Automation of a Generic Fluorescence Methyltransferase Activity Assay

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Abstract

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 known to be a ubiquitous covalent modification involved in regulation of a diverse range of biomolecules. Histone methyltransferases (HMTs) are of particular interest as drug targets as histone methylation is linked to certain disease states, including a wide variety of cancer types. A high-throughput screening (HTS)-ready, universal methyltransferase activity assay was recently developed based on competitive fluorescent polarization immunodetection of AMP, formed from the methyltransferase (MT) reaction product S-adenosylhomocysteine in a dual enzyme coupling step. Here we demonstrate automation of the assay in a 384-well format suitable for HTS.

A combination 8-channel and single channel liquid handling instrument was used to automate titration of the histone methyltransferase (HMT) G9a and transfer of the titration series to the assay plate in quadruplicate. Automated serial dilution and transfer of the HMT inhibitor sinefungin was also performed using the 8-channel head of the liquid handling instrument. During determination of the EC_{80} of the HMT, addition of the cofactor, substrate, stop buffer and detection reagent was automated using a non-contact dispenser. Dispensing of the EC_{80} concentration of HMT enzyme as well as all assay components were also automated during determination of the IC_{50} of a HMT inhibitor. The EC_{80} of G9a was determined to be ~80 ng/mL and used for subsequent inhibition studies, while the IC_{50} value of sinefungin 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 both 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 (Esteller, 2006, Jirtel *et al*, 2007).

Histone modifications can 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, are 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 tail of histone H4 is associated with human tumors and represent a nearly universal marker for malignancy (Fraga *et al*, 2005).

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 (Seligson *et al*, 2005).

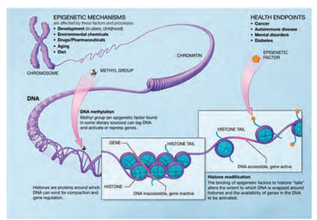

Figure 1 – *Histones play a central role in chromosomal structure affecting the regulation of gene expression. Epigenetic factors act as modulators of histones in turn controlling epigenetic mechanisms such as development and aging.*

Introduction *(continued)*

Here we demonstrate the combination of a fluorescence-based assay with liquid handling and dispensing instrumentation and a multi-mode reader which can be used to monitor the biological activity of the histone methyltransferase (HMT) G9a, a model system. A recently developed generic methyltransferase (MT) assay method uses fluorescent immunodetection of AMP, which is formed from the MT reaction product S-adenosylhomocysteine in a dual enzyme coupling step (figure 2). The Transcreener® EPIGENE 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.

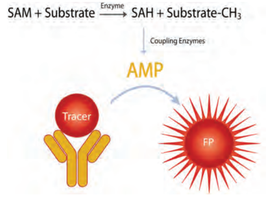

Figure 2 – *The Transcreener EPIGEN Methyltransferase Assay Principle. SAH produced by the target methyltransferase is converted to AMP by coupling enzymes which allows homogenous fluorescent detection using the Transcreener AMP²/GMP² Assay.*

BioTek Instrumentation

Figure 3 – *MultiFlo™ Microplate Dispenser offers up to four reagents dispensed in parallel with one compact instrument. The MultiFlo offers a choice of either peristaltic pump or microprocessor controlled syringe drive technologies. A wide array of plate types are accommodated from 6- to 1536-well formats as well as a broad volume range from 1 µL to 3 mL. The peristaltic pump cassettes consist of 8 individual tubes allowing for dispensing of a range of different reagents across the channels.*

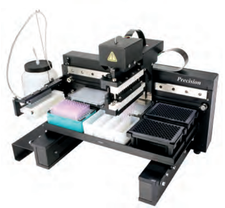

Figure 4 – *Precision™ Microplate Pipetting System is an affordable, innovative solution for automated 96-/384-well microplate liquid handling. The unique XY transport design provides effortless 96- to 384-well plate transfers with the same pipette mechanism. The instrument was used to serially titrate enzyme as well as inhibitor across a 96-well plate, as well as transfer the to the 384-well assay plates*

BioTek Instrumentation *(continued)*

Figure 5 – *Synergy™ H4 Microplate Reader with Hybrid Technology™ is a patent-pending multi-mode detector ideal for research and drug discovery applications when having to choose between flexibility and performance is not an option. The instrument contains both filter-based and monochromator-based fluorescence optical systems to ensure high-performance fluorescence detection needed for the Transcreener® EPIGEN assay.*

Materials

Assay Components

- Transcreener EPIGEN Methyltransferase Assay Kit (Cat. # 3017-1K, gift of BellBrook Labs, LLC, US)
- G9a, human, recombinant, HMT (Cat. # 51001, BPS Bioscience, San Diego, CA, US)
- Histone H3 (1-25), amide (Cat. # 83643A, ANASpec, Fremont, CA, US)
- Sinefungin (Cat. # A-9145, Enzo Life Sciences, Plymouth Meeting, PA, US)
- MT assay buffer (50 mM Tris, pH= 8.5, 5 mM $MgCl_2$, 1 % DMSO, 4 mM DTT)

Instrument Components

- Parallel and perpendicular signals were read on the Synergy H4 using 620/40nm and 680/30nm excitation and emission filters, along with a 660nm cutoff dichroic mirror
- An optional red-shifted, increased sensitivity PMT was installed in the Synergy H4

Assay Plates

- 384-well, black, low-volume, non-binding plate (Cat. # 3676, Corning Life Sciences, Lowell, MA, USA) were used in all experiments

Data Reduction

- ΔmP values are reported as the difference between high (tracer + antibody) and sample polarization values
- Data was analyzed using GraphPad Prism (GraphPad Software, LaJolla, CA, USA)

Transcreener EPIGEN MT Assay Procedure

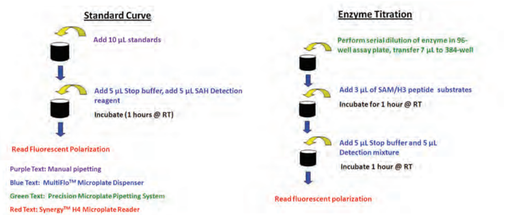

Figure 6 – *Transcreener EPIGEN Assay workflow*

Transcreener EPIGEN MT Assay Procedure *(continued)*

Figure 6 (continued) – *Transcreener EPIGEN Assay workflow.*

Optimization of Assay Window

- Two 11-point SAM/SAH standard curves were prepared plus zero substrate points at 1 µM and 10 µM and 10 µL were transferred in quadruplicate to a 384-well assay plate
- 5 µL of stop buffer and detect reagent were added either manually or using the MultiFlo
- Following a 1 hour incubation the plate was read on the Synergy H4 using Automatic Gain Adjustment and Xenon flash lamp with variable flash counts from 1 to 15 flashes

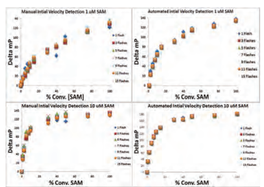

Figure 7 – *Initial Velocity detection of 1 µM and 10 µM SAM. Comparison of manual vs. automated reagent addition and affect of flash count on assay window.*

% SAM Conversion	1 uM		10 uM		
	Manual	Automated	Manual	Automated	
1	-8.51	-0.22	-1.30	0.27	Z'Factor
2.5	-4.05	0.33	-0.57	0.55	
5	-1.89	0.42	0.07	0.71	
7.5	-1.60	0.52	-0.07	0.75	
10	-0.92	0.64	0.09	0.81	
15	-0.51	0.67	-0.28	0.82	
30	-0.41	0.72	0.34	0.84	
40	0.12	0.74	0.34	0.80	
50	0.16	0.77	0.43	0.87	
75	0.05	0.79	0.40	0.85	
100	0.32	0.82	0.41	0.84	

Table 1 – *Representative Z' values at percent SAM conversion showing the comparison between manual and automated methods of lower limit of detection (yellow) and initial values that are ≥0.5 (green) at 5 flashes. It was determined that 2 µM SAM would provide sufficient limit of detection and Z'-factor for inhibition studies. It was determined that 5 flashes resulted in data with sufficient Z' values while minimizing the time required to read the assay plate.*

G9a Enzyme 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 G9a of 5 µg/mL
- Eight replicates, 7 µL, of each dilution were transferred to a 384-well assay plate
- Four replicates received 3 µL of either SAM alone or SAM/H3 peptide substrates
- 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

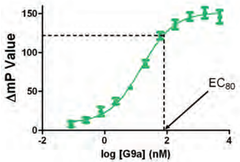

Figure 8 – *EC_{80} concentration for use in inhibition experiments was determined from enzyme titration curve.*

- The G9a EC_{80} was determine to be ~80 ng/mL and was used for inhibition studies

G9a Inhibition 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 at 250 µM
- Eight replicates, 5 µL, 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 alone or SAM/H3 peptide substrates
- Background correction was performed as described above

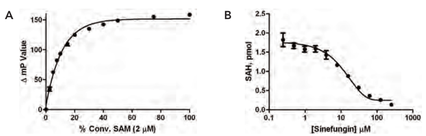

Figure 9 – *Pharmacology. **A)** A standard curve was generated mimicking the MT reaction. The curve was used to convert polarization values to product formation for quantitative data analysis. **B)** A dose response curve was generated for G9a methyltransferase sensitivity with the known inhibitor sinefungin. The IC_{50} value was 14.5 µM. The G9a enzyme reaction progressed to ~10 % conversion.*

- The IC_{50} value of 14.5 µM shows excellent correlation with published values (Reactions Biology Corp., Malvern, PA, USA)

Conclusions

1. The Transcreener EPIGEN Assay can be performed using simple, inexpensive automated methods
2. Higher sensitivity and lower background were achieved when using automated methods resulting in higher throughput and ease-of-use
3. Pharmacology of known compounds generated using the MultiFlo Microplate Dispenser for enzyme and Transcreener EPIGEN Methyltransferase reagent dispensing and Precision Microplate Pipetting System for serial dilution and compound transfer agree with published values (Reactions Biology Corp., Malvern, PA, USA)
4. An increased Z'-factor determination when compared to manual methods provides for an increase in assay performance as well as the additional benefit of increased throughput