Interrogation of SET7/9 Histone Lysine Methyltransferase Using a High Throughput Fluorescence Polarization Screening Assay

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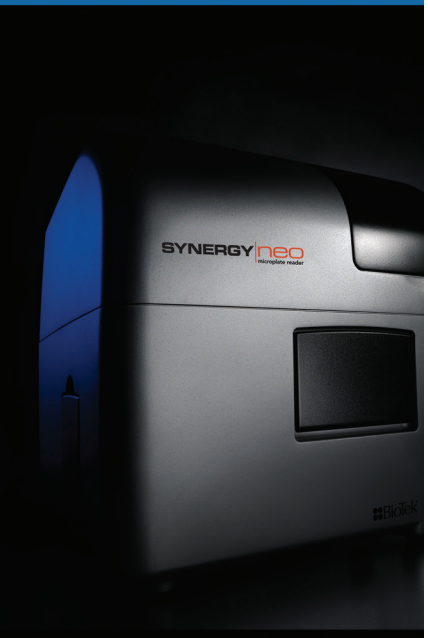

Regulation of chromatin structure provides a key regulatory mechanism for a variety of cellular processes involving DNA. Nucleosomes form the primary unit of chromatin and are comprised of DNA wrapped around a core of histone proteins. A variety of post-translational modifications are known to occur within histone N-terminal tails. The resulting means by which these modifications influence biological processes are currently the focus of several areas of study. A common posttranslational modification is methylation of lysine residues within the N-terminal tail of histones. Methylated lysines are the target of a wide range of recognition domains contained within chromatin-associated regulatory factors. Here we demonstrate the use of a fluorescent polarization assay using SET7/9, a SET domain-containing mono-methyltransferase, as a model system suitable for HTS applications in a 384-well microplate format using automated methods and a HTS microplate reader.

Key Words:

SET7/9

Methyltransferase

Epigenetics

Fluorescence Polarization

Introduction

There continues to be increasing interest in epigenetic mechanisms of control that alter chromatin structure and influence gene regulation. Posttranslational modifications (PTMs) of histones have been shown to provide a complex mechanism of influencing chromatin structure that play a primary role in many DNA regulatory processes[1]. Structural studies of nucleosomes have shown that highly basic histone amino (N)-terminal tails remain unstructured, allowing contacts to be made with neighboring nucleosomes. It has subsequently been shown that modifications to the N-terminal tails indeed influence global chromatin structure due to these inter-nucleosomal contacts. While structural changes are a result of histone modifications, recruitment of proteins and protein complexes have also been shown to correlate with specific histone modifications. Once recruited, these proteins perform a variety of enzymatic activities and regulate a range of DNA processes including remodeling, repair, replication and recombination, as well as transcriptional regulation[1].

Histone modifications include acetylation, phosphorylation, methylation, deamination, and ubiquitylation, to name a few. Histone methylation has been shown to primarily occur on the sidechains of lysine and arginine residues and has been widely studied as a potential druggable target due to the association of some histone methylation states with disease[1]. Thus, histone methyltransferases are divided into two groups, lysine or arginine, based on the target residue.

Histone lysine methyltransferases, or HKMTs, that target N-terminal lysine residues generally contain a SET domain responsible for the methyltransferase activity. While relatively specific for a particular N-terminal residue within the histone tail, some HKMTs can target additional non-histone proteins[2]. Methyl groups from the cofactor S-adenosylmethionine (SAM) are transferred by HKMTs to the ε-amino group of target lysine residues. The methylation state can vary from one to three groups per residue depending on the HKMT. Furthermore, it has been shown that the methylation state is influenced by adjacent residues of the N-terminal tail[1]. SET7/9 (KMT7) is a mono-methyltransferase targeting H3K4, as well as several non-histone targets such as TAF10, p53, and viral Tat, and can be used as a model to study SET domain histone methylation[2].

The Set7/9 SAM-Screener Assay is a fluorescence polarization assay designed around a small molecule fluorescent probe that binds to the SET7/9 SAM binding site (Figure 1). When bound to SET7/9, an increase in fluorescence polarization is seen when compared to free probe. The probe is displaced by the endogenous cofactor SAM as well as known SAM-binding site inhibitors (e.g. sinefungin). Probe binding is not altered by SET7/9 substrate peptides, suggesting a binding modality that is purely dependent upon the SAM-binding site. Here we demonstrate the use of automated methods to improve assay performance in a 384-well microplate format suitable for high-throughput screening (HTS) applications.

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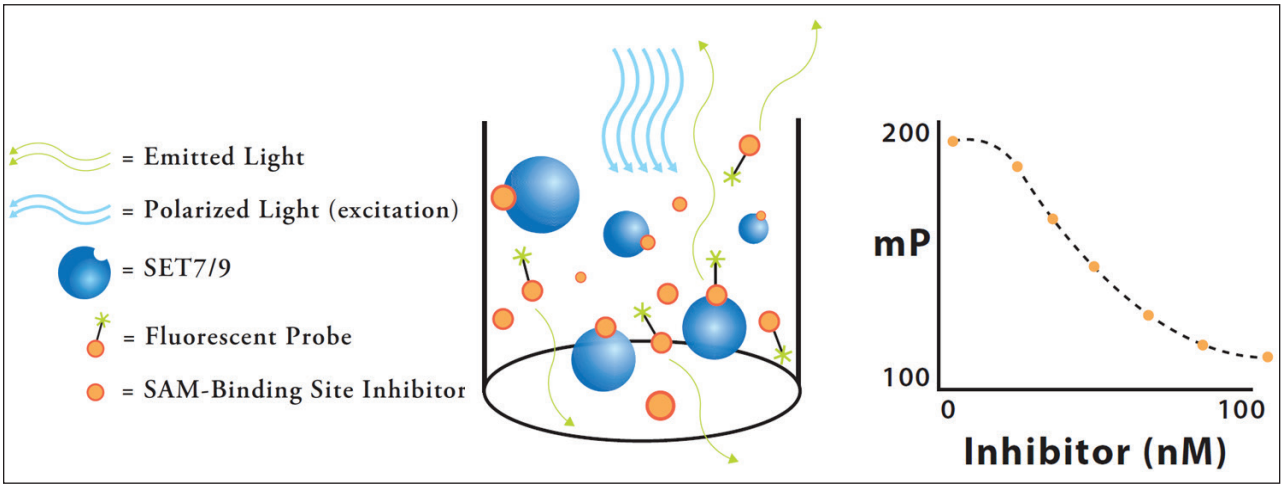

Figure 1. **Assay Principle.** The Set7/9 SAM-Screener Assay relies on an in increase in FP signal upon binding of the SAM-binding Site Probe to SET7/9. Loss of FP is directly proportional to the displacement of the fluorescently-labeled SAM-Binding Site Probe in the presence of competing molecules.

Materials and Methods

Materials

The SET7/9 SAM-Screener Assay Kit was a gift of Cayman Chemical Company (Ann Arbor, MI, USA) and the assay was performed per the manufacturer's protocol. Sinefungin was purchased from Enzo Life Sciences (Plymouth Meeting, Pennsylvania, USA). S-adenosyl-L-methionine (SAM or AdoMet) was a gift of BellBrook Labs, LLC (Madison, Wisconsin, USA). The workflow is summarized in Figure 2. Briefly, SAM-binding site assay buffer (assay buffer) was prepared fresh from a 10X stock with MilliQ™ water. Human recombinant SET7/9 was thawed on ice and 20 µL was diluted in a final volume of 4 mL in 1X SAM-binding site assay buffer for each 384-well plate. The SAM-binding site probe was reconstituted by addition of 2 mL of 1X SAM-binding site assay buffer to one vial per 384-well plate. The SAM-binding site inhibitor was reconstituted by addition of 250 µL of 1X SAM-binding site assay buffer into one tube containing the control. Samples and standards were prepared in DMSO and diluted to 4X the desired final concentration prior to use. DMSO concentration was normalized, not to exceed a final concentration of 2% in the assay.

Methods

Inhibitor Titration

For each inhibitor, an 11 point 1:2 serial dilution was performed in a 96-well microplate using the Precision automated pipettor. Following the titration, 5 µL of each concentration was transferred, in quadruplicate, to the 384-well assay plate also using the Precision automated pipettor. SET7/9 assay enzyme, 10 µL, was added to each assay well using the MultiFlo™ Dispenser.

The assay plate was incubated at room temperature (~ 23 °C) for 15 minutes to allow equilibration of compounds with the SET7/9 enzyme. The SAM-binding site probe, 5 µL, was added to all assay wells using the MultiFlo dispenser and Liquid Handling Control (LHC) software (BioTek Instruments, Inc., Winooski, VT). The plate was sealed using an adhesive aluminum foil seal and incubated at room temperature, with shaking, for 30 minutes.

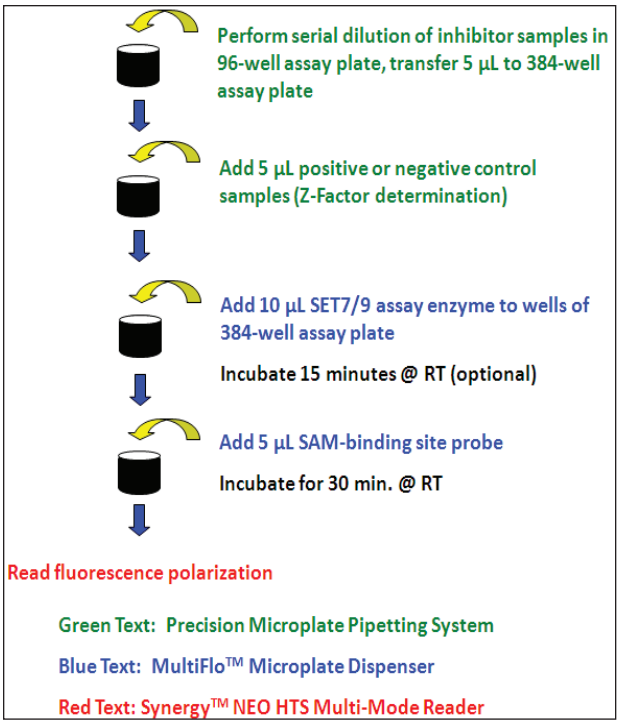

Figure 2. **SET7/9 SAM-Screener Assay Workflow.** Automated workflow for high-throughput screening applications. Serial dilution of inhibitor samples and transfer to 384-well assay plate were performed with the Precision™ Microplate Pipetting System. Enzyme and reagent dispensing was performed with the MultiFlo™ Microplate Dispenser. Fluorescence polarization was read using the Synergy™ Neo HTS Multi-Mode Reader using 590/20 nm excitation filter and 635/32 nm emission filter, along with a 595 nm cutoff dichroic mirror with matched dual PMT technology.

Z'-Factor Determination

For the determination of the Z'-factor, 48 replicates of both positive and negative control, 10 µM sinefungin and buffer, respectively, were assayed as described above and the Z'-factor calculated as previous described[1]. One outlier was removed from the negative control data.

Reader Settings

The plate was read in a Synergy™ Neo HTS Multi-Mode microplate reader as summarized in Table 1. Briefly, the plate was read with the following excitation and emission wavelengths and bandpass: 590/20 nm and 635/32 nm, respectively, with a 595 nm dichroic filter. Data acquisition and reader control was performed using Gen5™ Data Analysis software (BioTek Instruments, Inc., Winooski, VT) using the parameters described below. Automatic gain adjustment was used for reader optimization with scaling to the negative control well to a parallel scale value of 50,000 RFU and requested polarization value of 20 mP. Read Speed was set to normal with no delay after plate movement, 50 measurements per data point and a read height of 11 mm. The light source selected was the Xenon Flash with lamp energy set to High. Data reduction was performed using Microsoft Excel (Redmond, WA) and GraphPad Prism (La Jolla, CA) software.

Synergy Neo Read Parameters	
Filter Sets	
Mode	FP/Dual PMT
Excitation	590/20 nm
Emission	630/32 nm
Dichroic mirror	595 nm
Gain (Side/Top PMTs)	Auto
FP Parameters	
Parallel scale value	50,000 RFU
Req. Polarization value	20 mP
Read Speed	
Read speed	Normal
Delay after plate movement	0
Measurement per data point	50
Read Height	
Auto-Adjust determined	11 mm
Light Source	
Souce	Xenon Flash
Lamp energy	high

Table 1. **Synergy™ Neo HTS Multi-Mode Microplate Reader Parameters.** The assay made use of a 590/630 nm fluorescent polarization filter cube and dual-matched PMTs for rapid measurements in a 384-well density microplate.

Results and Discussion

Inhibitor Titration

The SAM positive control wells representing the basal polarization for the SAM-Binding Site Probe were averaged and subtracted from all assay wells. The resulting data represents baseline-corrected FP as a percentage of the negative control and can be plotted versus inhibitor concentration. A 12-point 1:2 serial dilution of sinefungin and SAM were run in parallel resulting in typical sigmoidal dose response curves for the displacement of SAM-Binding Site Probe when plotted on a semi-Log axis fit to a 4-parameter logistic equation (Figure 3). IC_{50} values determined from the dose-response experiments were 2.5 and 0.86 µM for sinefungin and SAM. The IC_{50} determination for sinefungin correlates well with previously determined values[1].

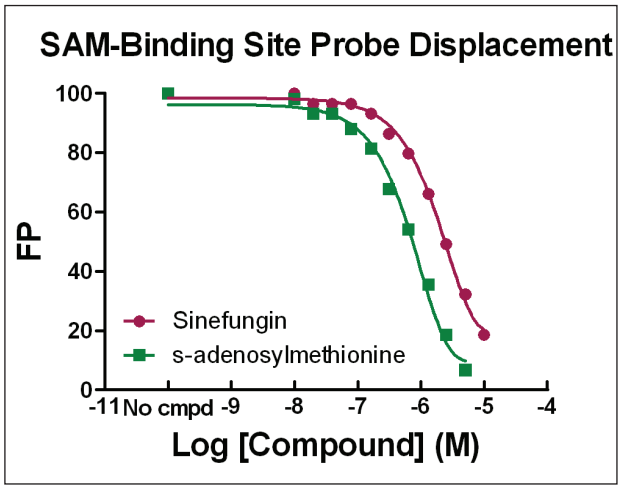

Figure 3. **Inhibition Curves.** A 12-point 1:2 serial dilution of sinefungin and SAM were used to generate inhibition profiles using automated methods. All FP measurements were corrected for mP measurements of unbound probe (i.e. probe in buffer).

Z'-Factor Determination

The Z'-factor is used to describe the robustness of an assay with a theoretical upper limit of 1.0. An assay is considered robust when it has a Z'-factor > 0.5. Automated methods have been shown to maintain or improve upon assay robustness while allowing increased throughput. Forty-eight replicates of both positive and negative controls were assayed for the Z'-factor determination. One data point was determined to be an outlier and subsequently removed from the positive control replicate data. The Z'-factor for the SET7/9 SAM-Screener Assay was determined to be 0.73 using automated methods (Figure 4). This correlates well with manual methods showing a slight improvement in assay performance when using automated methods[1].

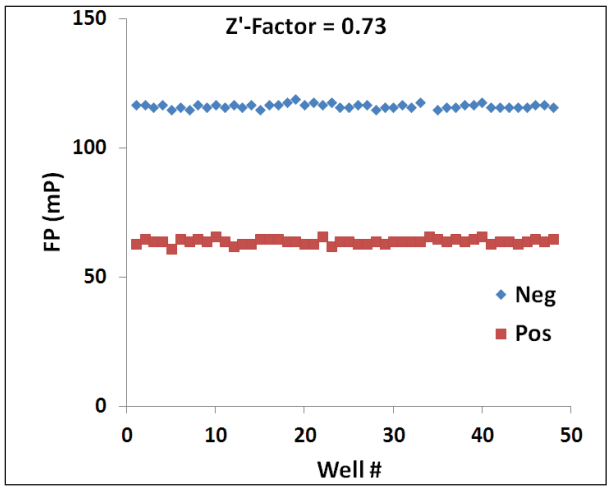

Figure 4. **Z'-Factor Determination.** Data is representative of 48 replicate wells of both positive and negative controls. The calculated Z' from this experiment was 0.73.

Conclusion

Posttranslational modifications to histones have been shown to play a significant role in regulation of chromatin structure and are being investigated as a potential druggable target. Robust assay technologies are required for HTS of potentially active compounds. The SET7/9 SAM-Screener is a simple biochemical assay suitable for HTS using a 384-well microplate format and automated methods. The use of the Synergy™ Neo HTS microplate reader, along with Precision™ Pipetting System and MultiFlo™ Dispenser, greatly increases throughput capabilities. Using this combination of instrumentation and chemistry resulted in robust assay performance.

References

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