Automated, High Throughput, HTRF®-Based Detection of Histone Methyltransferase and Demethylase Enzyme Activity

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Introduction

The study of modifications which can affect the transcriptional state of DNA at the chromatin level, otherwise known as epigenetics, has seen increased emphasis in recent years. The alterations that have currently been described include, but are not limited to, acetyl- and deacetylation, methyl- and demethylation, ubiquitylation, and phosphorylation. These modifications take place mainly at the N-terminus of histone proteins, or histone tails, and affect gene expression in that portion of the DNA sequence. While epigenetic changes are normal, and essential, during the embryonic differentiation of cells from their original totipotent state, aberrant modifications have been linked to autoimmune disease, diabetes, and many human cancers.

Initial drug development in this area has concentrated on histone acetyltransferases (HATs) and histone deacetylases (HDACs). However, recent investigations have revealed that the process of histone methylation is also a dynamic process, controlled on one side mainly by the SET-domain protein methyltransferase family, and on the other by demethylases such as lysine-specific-demethylase-1 (LSD1) and JmjC domain-containing histone demethylase (JHDM). These two opposing processes are another important regulator of gene transcription. Abnormal histone methylation patterns, such as hypermethylation and hypomethylation, have been associated with human malignancies via multiple mechanisms including unscheduled gene silencing. As a result, the number of drug discovery projects focused on these two enzyme classes has increased in the last five years and resulted in a number of promising new inhibitors currently in preclinical studies (Wagner, et al., 2012). Because of this recent trend, it has also become essential to have access to assay technologies that allow for easy assessment of new potential modulators of histone methylation in a high throughput format.

Here we describe two new HTRF®-based assay formats for the assessment of small molecule inhibitor capabilities of methylase and demethylase enzymes. The first focuses on activity leading to monomethylation of histone H3 at the lysine 4 residue by the SET7/9 orphan member of the SET family. The second targets demethylation activity at the lysine 36 residue of histone H3 by the JMJD2A enzyme. Both assays are biochemical in nature and rely on the addition of a europium cryptate labeled antibody specific for the methylation state of the substrate, as well as a streptavidin-acceptor molecule, in a TR-FRET format. Enzyme activity in the well leads to the creation of the appropriate number of methyl groups on the substrate, allowing for antibody binding. Energy can then be transferred from donor to acceptor molecule, creating FRET. Inhibition of enzyme activity leads to the opposite effect, and decreasing FRET. The assay procedures were carried out using high throughput liquid handling and detection instrumentation. Optimization, validation, and screening data confirm the ability of the automated process to deliver accurate results in a simple, yet robust manner.

BioTek Instrumentation

MultiFlo™ Microplate Dispenser. The dispenser offers fast, accurate plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 0.5-3000 µL. The instrument was used to dispense all assay components including enzyme, substrate mixtures, cofactors, and antibody mixes in volumes as low as 2 µL.

Synergy™ NEO Multi-Mode Microplate Reader. The reader combines a filter-based and monochromator-based detection system in one unit. The HTRF certified reader uses a filter-based system, high performance Xenon flash lamp, as well as dual matched PMTs to simultaneously detect the 665 nm and 620 nm fluorescent emissions from the assay chemistry with the following settings: Delay after plate movement: 0 msec; Delay after excitation: 150 µsec; Integration time: 500 µsec; Read height: 8.5 mm.

HTRF Biochemical Epigenetic Assays

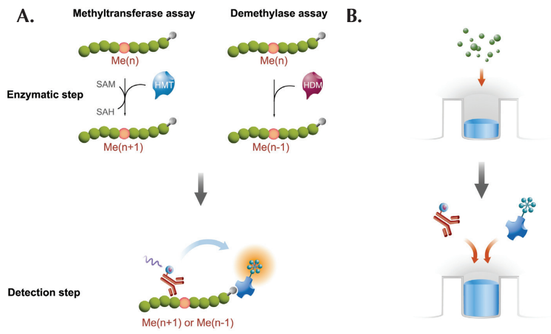

Figure 1 – *Each assay consists of enzymatic and detection steps. The enzymatic step is carried out in the appropriate enzymatic buffer. In the following order,* ***(A.)*** *Inhibitor, SET7/9 enzyme, SAM (S-(5'-adenosyl)-L-methionine) and biotinylated non-methylated H3(1-21) peptide substrate mixture are added. H3(1-21)me0 peptide is converted into H3K4me1 peptide with SET7/9.* ***(B.)*** *Inhibitor, JMJD2A enzyme, alpha-ketoglutarate and biotinylated tri-methylated H3(21-44) peptide substrate mixture are added to the well. H3K36(21-44)me3 peptide is converted into H3K36me2 peptide with JMJD2A. When the substrate products are incubated with cryptate-labeled anti-Histone H3 antibodies specific for the methylation site, and streptavidin-XL665 (SA-XL) in the detection step, time-resolved fluorescence resonance energy transfer (TR-FRET) between europium cryptate (donor) and XL665 (acceptor) is observed.*

Automated Assay Procedure

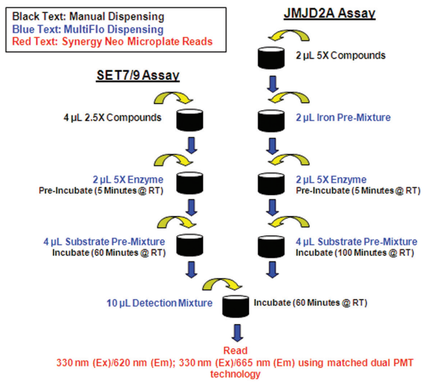

Figure 2 – *Automated SET7/9 and JMJD2A assay workflows.*

Assay Optimization and Validation

Enzyme Concentration and Reaction Time Optimization

Optimization of enzyme concentration and reaction time were performed in order to determine the proper amount of enzyme and incubation period to use to maximize the assay window, while still minimizing customer reagent costs. For SET7/9, four different concentrations of enzyme were used in the reaction (5, 10, 20, and 40 nM), and three for JMJD2A (5, 10, and 20 nM). Remaining reaction components were in excess. Enzyme reactions were stopped by adding detection reagents after each time point, ranging from 0 to 120 minutes.

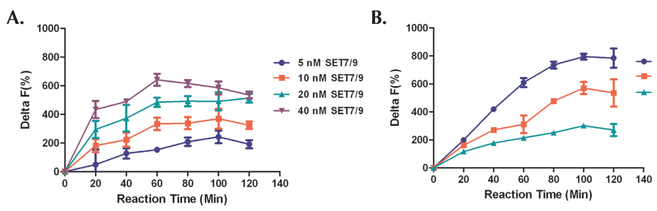

Figure 3 – Enzyme Titration and Reaction Time Results. *Delta F(%) calculated from results generated for each enzyme concentration and time point by the following formula: ((HTRF Value$_{(Time\ X)}$ – HTRF Value$_{(Time\ 0)}$)/HTRF Value$_{(Time\ 0)}$).*

The results shown in Figure 3A demonstrate that a SET7/9 enzyme concentration of 40 nM generates the highest Delta F(%), or assay window. Furthermore, the reaction plateaus after 60 minutes, with no increase being seen after that timepoint. Figure 3B also illustrates that the largest concentration of JMJD2A enzyme tested, 10 nM, yields the largest Delta F(%) values. This enzyme reaction, however, appeared to take a longer time to reach a steady state, and did not plateau until 100 minutes. The enzyme concentrations and reaction incubation times optimized here, SET7/9: 40 nM/60 minutes; and JMJD2A: 10 nM/100 minutes were used for all subsequent experiments performed.

Substrate K_m Determinations

Incorporating the correct substrate concentration is essential to ensure proper enzyme reaction kinetics. Typically a concentration that is at or around the K_m value is accepted. Use of a concentration that is higher than the K_m value can cause a right-shift in the results from test compounds, causing them to appear less potent than what may be seen *in-vivo*. The H3(1-21)me0 peptide and α-ketoglutaric acid represent the substrate for the SET7/9 and JMJD2A enzymes, respectively. Titrations of each molecule were tested with the enzyme concentrations previously optimized, and varying amounts of the methyl donors SAM and H3K36(21-44)me3 peptide.

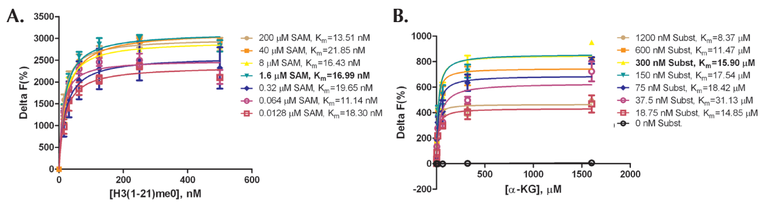

Figure 4 – Substrate K_m Curves. A. *H3(1-21)me0 analysis. Serial 1:2 dilutions of substrate performed starting at 500 nM. Reaction run using SAM concentrations ranging from 200-0.01 µM SAM. B. α-ketoglutaric acid analysis. Serial 1:5 dilutions of substrate performed starting at 1600 µM. Reaction run using H3K36(21-44)me3 concentrations ranging from 1200-0 nM peptide. K_m values determined from data plotted and analyzed using a Michaelis-Menten curve fit.*

The results in Figure 4a illustrate that K_m values ranged from 11-22 nM H3(1-21)me0 peptide across the different concentrations of SAM tested in the enzyme reaction. The graph also shows that a concentration of approximately 1 µM SAM gives the largest Delta F(%) at the K_m value. In addition, the graph in Figure 4b shows Km values for α-ketoglutaric acid ranging from 8-31 µM, as well as a value of 300 nM H3K36(21-44)me3 peptide yielding the largest Delta F(%) at the K_m concentration. Therefore, further experiments will include concentrations of 20 nM H3(1-21)me0 peptide and 1 µM SAM for the SET7/9 enzyme reaction, and 20 µM α-ketoglutaric acid and 300 nM H3K36(21-44)me3 peptide for the JMJD2A enzyme reaction.

Automated Assay Z'-factor Validation

The automated SET7/9 and JMJD2A assay procedures were then validated using the optimized reaction concentrations previously mentioned. 0 and 100 µM concentrations of either Sinefungin (SET7/9) or 2,4-PDCA (JMJD2A) were used as the positive and negative controls in a Z'-factor experiment to measure assay robustness. The Z'-factor[1] value takes into account the difference in signal between a positive and negative control, as well as the variation in the signal amongst replicates. A scale of 0-1 is incorporated, with values ≥0.5 being indicative of an excellent assay.

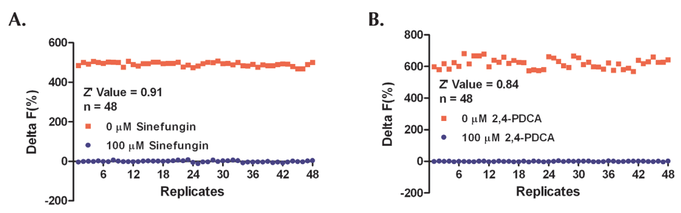

Figure 5 – Z'-factor results. *Forty-eight replicates of 0 µM and 100 µM Sinefungin (A.) or 2,4-PDCA (B.) used as positive and negative controls, respectively. Enzyme reactions stopped by addition of detection mixture after the appropriate optimized incubation time mentioned previously. 620 nm and 665 nm signals detected after an additional 60 minute room temperature incubation.*

The Z'-factor values of 0.91 and 0.84 which were generated, as explained previously, are indicative of an excellent, robust assay. The instrument methods for the MultiFlo, and detection settings on the Synergy NEO used for this experiment would then be used to perform a compound library screen to look for potential inhibitors of the SET7/9 and JMJD2A enzymes.

Screen-Well® Epigenetics Compound Library Screen

The 43 compound Screen-Well® Epigenetics Library V. 1.0 (BML-2836), generously donated by Enzo Life Sciences, was then screened using the validated, automated SET7/9 and JMJD2A assays. Five other known inhibitors for various epigenetic targets were also screened, including Sinefungin, 2,4-PDCA, (+)-JQ1, UNC 0646, and UNC 0638. All compounds were diluted in the appropriate assay buffer from their original 10 mM concentration. The final 1X compound concentrations used in the screen were 20 µM, 2 µM, and 200 nM.

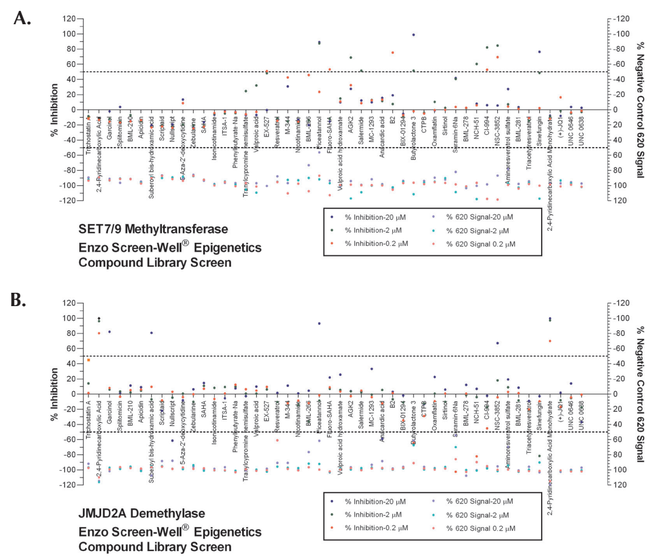

Figure 6 – Results from Epigenetics Library compound screen. *Percent inhibition for each compound concentration tested with either SET7/9 assay* ***(A.)*** *or JMJD2A Assay* ***(B.)*** *shown on left Y-axis. Percent of no compound negative control 620 signal shown on right Y-axis. Black dotted lines represent 50% inhibition of enzyme activity or 620 signal.*

Percent inhibition and percent no compound negative control 620 signal were plotted to determine the effect that the compounds had on enzyme activity and proper assay function. The second measurement can be used to detect filter effect or autofluorescence effect of compounds which could lead to false positives or false negatives. Compounds showing >50% inhibition at two separate concentrations tested, or a high degree of inhibition at a single concentration, with no appreciable effect on the donor fluorophore 620 signal were labeled as "hits" and potential true inhibitors of enzyme activity.

Dose Response Confirmation

"Hit" compounds were then included in a dose response test to discern their inhibitory profile. Serial 1:4 titrations were performed with each compound starting at a concentration of 100 µM. All compounds included in the experiment were tested with the SET7/9 and JMJD2A assays in order to determine the selectivity of the small molecule. Percent inhibition was determined by comparing the Delta F(%) value for wells containing compound to the value from wells containing no compound.

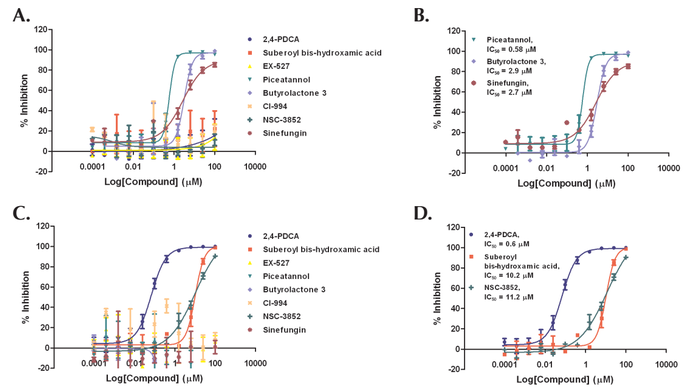

Figure 7 – Dose Response Test Results. *Percent inhibition for all compounds, across all concentrations tested with the SET7/9* ***(A.)*** *and JMJD2A* ***(B.)*** *assay. Inhibition curves and IC_{50} values listed for true inhibitory compounds of the SET7/9 methyltransferase* ***(C.)*** *and JMJD2A demethylase* ***(D.)*** *enzymes.*

Results of the screen and dose response test were validated by the fact that the known SET7/9 inhibitor, Sinefungin, and JMJD2A inhibitor, 2,4-PDCA, demonstrated the expected inhibitory profiles and IC_{50} values. Inhibition of SET7/9 was also seen by the SIRT activator Piceatannol, and the histone acetyl transferase inhibitor Butyrolactone 3, while the histone deacetylase inhibitors Suberoyl bis-hydroxamic acid and NSC-3852 demonstrated clear inhibition of JMJD2A. Similarity in target enzyme function may explain the inhibitory effects seen. However further investigation would be necessary to completely uncover the mechanism of action for these compounds.

Conclusions

1. The HTRF SET7/9 Methyltransferase and JMJD2A Demethylase assays afford sensitive, precise biochemical formats for the assessment of enzyme activity and inhibition.
2. Each assay procedure can be easily automated in low-volume 384-well format using the non-contact dispensing capabilities of the MultiFlo, using volumes as low as 2 µL.
3. The Xenon-based excitation and filter-based detection system of the Synergy NEO microplate reader is able to simultaneously quantify the emitted signals from the donor and acceptor fluorophores.
4. The combination of assay chemistry, as well as liquid handling and detection instrumentation, create a rapid, easy-to-use, robust solution for analysis of modulation of these important epigenetic targets.

[1]Zhang. *et al.*: A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* 1999 4(2): 67-73.

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