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

Brad Larson and Peter Banks, Applications Department, BioTek Instruments, Inc., Winooski, VT
Nicolas Pierre, Thomas Roux, Suzanne Graham, and François Degorce, Cisbio US, Inc., Bedford MA

Key Words:

Epigenetics
Methyltransferase
Demetylase
SET7/9
JMJD2A
HTRF
TR-FRET
Automation

BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Phone: 888-451-5171
Outside the USA: 802-655-4740
Email: **customercare@biotek.com**
www.biotek.com

Introduction

Epigenetics is the study of modifications, which can affect the transcriptional state of DNA at the chromatin level. The epigenetics field has increased in recent years, and now includes research into alterations such as acetyl- and deacetylation, methyl- and demethylation, ubiquitylation, and phosphorylation. Modifications take place mainly at the histone protein N-terminus, or histone tail, and affect gene expression in that portion of the DNA sequence. These changes are a normal, essential part of a cell's embryonic differentiation from its original totipotent state, however, aberrant modifications are linked to autoimmune disease, diabetes and many human cancers[1].

Epigenetic-based drug development has focused on histone acetyltransferases (HATs) and histone deacetylases (HDACs). However, recent research shows that histone methylation is also dynamic. It is controlled on one side mainly by the lysine-specific SET-domain protein methyltransferase family, and on the other side by demethylases such as lysine-specific demethylase 1 (LSD1) and JmjC domain-containing histone demethylase (JHDM). These opposing processes serve as another important gene transcription regulator. For example, abnormal histone methylation patterns, such as hyper- and hypomethylation, are associated with human malignancies via multiple mechanisms including unscheduled gene silencing. Many drug discovery projects now focus on methyltransferase and demethylase enzyme classes, and a number of promising new inhibitors are currently in preclinical studies[2]. Therefore, it is essential for assay technologies to allow easy assessment of new potential histone methylation modulators in high-throughput format.

Here, we describe two homogeneous time-resolved fluorescence (HTRF®) assay formats from Cisbio Bioassays (Bedford, MA) to assess the small molecule inhibitor capabilities of methyltransferase and demethylase enzymes. The automated assay procedures were carried out using high-throughput liquid handling and detection instrumentation. Optimization, validation and screening data confirm that the automated process delivers accurate results in a simple yet robust manner.

HTRF® Biochemical Epigenetic Assay Principle

The HTRF assay principle relies on an europium cryptate-labeled antibody specific to the substrate's methylation state, and a streptavidin-acceptor molecule. In the HTRF assays used here, the methyltransferase assay (Figure 1A) detects the monomethylation of biotinylated histone H3 at the lysine 4 residue by the SET family's SET7/9 orphan. Inhibitor, SET7/9 enzyme, S-(5'-Adenosyl)-L-methionine chloride dihydrochloride (SAM) and biotinylated non-methylated H3(1-21) peptide substrate mixture are combined. The SET7/9 enzyme converts the H3(1-21)me0 peptide into H3K4me1 peptide.

The demethylase assay (Figure 1B) targets demethylation at the histone H3 lysine 36 residue by the JMJD2A enzyme. Inhibitor, JMJD2A enzyme, α-ketoglutarate and biotinylated tri-methylated H3(21-44) peptide substrate mixture are combined. The JMJD2A enzyme converts the H3K36(21-44)me3 peptide into H3K36me2 peptide.

When the substrate products are incubated with europium cryptate-labeled anti-histone H3 antibodies specific for the methylation site, binding occurs. Upon addition of streptavidin-XL665 (SA-XL) in the detection step, the close proximity allows energy to be transferred from the europium donor to the streptavidin acceptor, known as time-resolved fluorescence resonance energy transfer (TR-FRET).

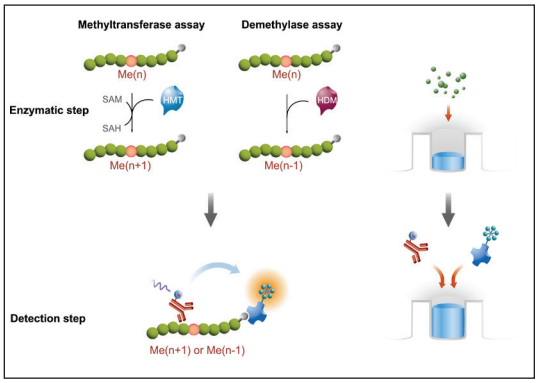


Figure 1. HTRF® biochemical epigenetic assay principle for (A) methyltransferase and (B) demethylase formats.

Materials and Methods

Materials

Reagents and Compounds

H3K4me1-Eu(K) antibody (Catalog No. 61KA1KAE), H3K36me2-Eu(K) antibody (Catalog No. 61KD2KAE), streptavidin XL-665 (Catalog No. 610SAXLA) and Detection Buffer (Catalog No. 62SDBRDF) were procured from Cisbio Bioassays. The 43 compound Screen-Well® Epigenetics Library, Version 1.0 (Catalog No. BML-2836) was generously donated by Enzo Life Sciences (Plymouth Meeting, PA). The known inhibitor (+)-JQ1 (Catalog No. 92-1149) was donated by DiscoveRx Corporation (Fremont, CA). Sinefungin (Catalog No. S8559), 2,4-Pyridinedicarboxylic acid (2,4-PDCA) monohydrate (Catalog No. P63395), ammonium iron(II) sulfate hexahydrate (Catalog No. F3754), L-ascorbic acid (Catalog No. A5960), α-ketoglutaric acid disodium salt hydrate and S-(5'-Adenosyl)-L-methionine chloride dihydrochloride were purchased from Sigma-Aldrich Co. (St. Louis, MO). UNC 0646 (Catalog No. 4342) and UNC 0638 (Catalog No. 4343) were purchased from R&D Systems (Minneapolis, MN). H3K36(21-44)me3 substrate and H3(1-21)me0 substrate were purchased from AnaSpec, Inc., (Fremont, CA). JMJD2A (Catalog No. 50123) and SET7/9 (Catalog No. 51010) were procured from BPS Bioscience, Inc., (San Diego, CA).

Instrumentation

MultiFlo™ Microplate Dispenser

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

Synergy™ Neo HTS Multi-Mode Microplate Reader

Synergy™ Neo HTS Multi-Mode Microplate Reader (BioTek Instruments, Inc.) with patented Hybrid Technology™ combines filter-based and monochromator-based detection systems in one unit. The reader is HTRF certified and uses a filter-based system, high performance xenon flash lamp and dual-matched photomultiplier tubes (PMT) 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: 8.5mm.

Methods

Enzyme Concentration and Reaction Time Optimization

SET7/9 was titrated to 5, 10, 20 and 40 nM, and JMJD2A was titrated to 5, 10 and 20 nM, and all concentrations were subject to the assay's enzyme reaction. Remaining reaction components were in excess. The reactions were stopped by adding detection reagents after each time point, ranging from 0 to 120 minutes.

Substrate K_m Determinations

The H3(1-21)me0 peptide was used to represent the substrate for the SET7/9 enzyme. Serial 1:2 dilutions of H3(1-21)me0 were performed, starting at 500 nM, and the reaction was run using SAM concentrations ranging from 200-0.01 µM. The α -ketoglutaric acid was used to represent the substrate for the JMJD2A enzyme. Serial 1:5 dilutions of α -ketoglutaric acid were performed, starting at 1600 µM, and the reaction was run using H3K36(21-44) me3 peptide concentrations ranging from 1200-0 nM.

Automated Assay Workflow

Per the SET7/9 assay, 4 µL of 2.5X compound was added to each well of a microplate followed by 2 µL of 5X enzyme. The plate was pre-incubated for 5 minutes at room temperature. Following incubation, 4 µL of substrate pre-mixture was added to each well, and the plate was incubated for 60 minutes at room temperature. 10 µL of detection mixture was then added to each well, and the plate was incubated a final time for 60 minutes at room temperature, then read on the Synergy Neo using 330 nm excitation/620 nm emission and 330 nm excitation/665 nm emission using matched dual PMT technology.

Per the JMJD2A assay, 2 µL of 5X compound was added to each well of a microplate followed by 2 µL of iron pre-mixture and 2 µL of 5X enzyme. The plate was pre-incubated for 5 minutes at room temperature. Following incubation, 4 µL of substrate pre-mixture was added to each well, and the plate was incubated for 100 minutes at room temperature. 10 µL of detection mixture was then added to each well, and the plate was incubated a final time for 60 minutes at room temperature, then read on the Synergy Neo using the aforementioned excitation and emission settings.

Automated Assay Z'-factor Validation

The automated SET7/9 and JMJD2A assay workflows were run using 0 and 100 µM concentrations of sinefungin (SET7/9) or 2,4-PDCA (JMJD2A) as positive and negative controls, respectively in a Z'-factor experiment to measure assay robustness. Forty-eight replicates of each compound concentration were included for each test, and the enzyme reactions were stopped by addition of detection mixture at the end of the incubation period. The plates were read using the aforementioned settings following a 60-minute room temperature incubation.

Compound Library Screen

Forty-eight compounds, including the 43 compound Screen-Well Epigenetics Library, and inhibitors sinefungin, 2,4 PDCA, (+)-JQ1, UNC 0646 and UNC 0638, were each diluted in the appropriate assay buffer from their original 10 mM concentration to final 1X concentrations of 20 µM, 2 µM and 200 nM. Each compound concentration was tested using the SET7/9 and JMJD2A assays following the aforementioned automated workflow.

Dose Response

Dose response tests were performed by using serial 1:4 titrations on each hit compound from the compound library screen starting at a 100 µM 1X concentration. Each hit compound was tested using the SET7/9 and JMJD2A assays following the aforementioned automated workflow.

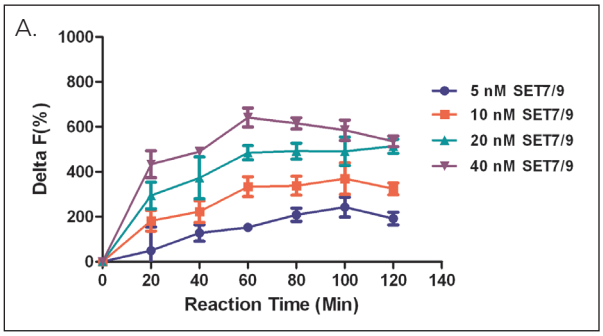
Results

Enzyme Concentration and Reaction Time Optimization

Optimization of assay parameters is essential to Optimal enzyme concentration and reaction time were calculated to maximize the assay window while still minimizing reagent costs. The Delta F(%), or assay window, was calculated from results generated for each enzyme concentration and time point using the following formula:

$$\frac{(\text{HTRF Value}_{(\text{Time X})} - \text{HTRF Value}_{(\text{Time 0})})}{\text{HTRF Value}_{(\text{Time 0})}}$$

Results shown in Figure 2A demonstrate that 40 nM of SET7/9 enzyme generates the highest Delta F(%). Furthermore, the enzyme reaction plateaus after 60 minutes. The SET7/9 40 nM concentration and 60-minute reaction time were used for all subsequent experiments performed. Figure 2B data shows that 10 nM of JMJD2A enzyme generates the highest Delta F(%) values. This enzyme reaction took longer than the SET7/9 reaction to reach a steady state, and did not plateau until 100 minutes. The JMJD2A 10 nM concentration and 100-minute reaction time were used for all subsequent experiments performed.



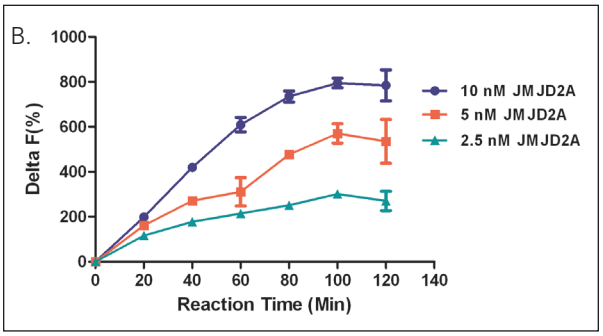
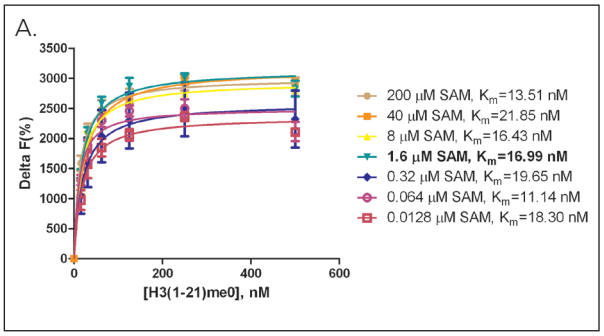


Figure 2. Enzyme titration and reaction time optimization data for (A.) SET7/9 and (B.) JMJD2A.

Substrate K_m Determinations

Incorporating the correct substrate concentration is essential to ensure proper enzyme reaction kinetics. Typically, a concentration at or around the K_m value is accepted, as higher values can cause a right-shift in test compound results, making them appear less potent than in vivo behavior. K_m values were determined from data plotted and analyzed using a Michaelis-Menten curve fit. Results in Figure 3A illustrate that H3(1-21)me0 peptide K_m values range from 11-22 nM across the different SAM concentrations tested. Additionally, 1 µM SAM provides the highest Delta F(%) at the K_m value. The H3(1-21) me0 20 nM concentration and 1 µM SAM concentration were used for all subsequent SET7/9 experiments performed. Figure 3B data show α-ketoglutaric acid K_m values range from 8-31 µM across the H3K36(21-44)me3 concentrations, and 300 nM of H3K36(21-44)me3 yielding the highest Delta F(%) at the K_m value. The α-ketoglutaric acid 20 µM concentration and 300 nM H3K36(21-44)me3 concentration were used for all subsequent JMJD2A experiments.



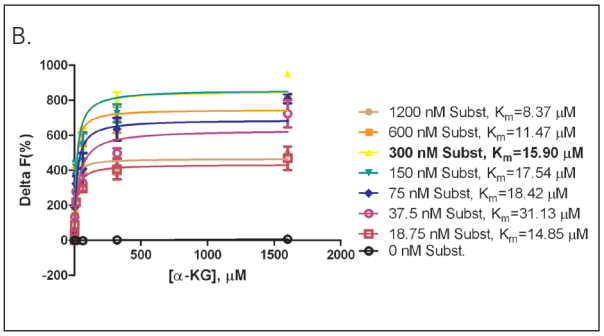


Figure 3. Substrate K_m curves for (A.) H3(1-21)me0 and (B.) α-ketoglutaric acid.

Automated Assay Z′-factor Validation

Using the optimized reaction conditions previously calculated, the automated SET7/9 and JMJD2A assay procedures were validated in a Z′-factor[1] experiment. The Z′-factor is a measure of assay robustness, and takes into account the difference in signal between positive and negative controls as well as the signal variation amongst replicates. A scale of 0-1 is used, with values greater than or equal to 0.5 indicative of an excellent assay. Per Figure 4 data, the Z′-factor calculations of 0.91 for sinefungin and 0.84 for 2,4-PDCA are indicative of robust assay performances. The automated assay method was then used to perform a compound library screen to look for potential SET7/9 and JMJD2A enzyme inhibitors.

Compound Library Screen

The 43-compound Screen-Well Epigenetics Library and 5 known inhibitors were tested using the SET7/9 (Figure 5A) or JMJD2A (Figure 5B) assay to determine the effect that each compound had on enzyme activity and proper assay function. Each compound's percent inhibition was plotted on the left Y-axes, while the percent no compound negative control 620 nm signal, used to detect filter effect or compound autofluorescence leading to false positives or false negatives, was plotted on the right Y-axes of each scatter plot. Compounds showing greater than 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 nm signal, were labeled as hits, and thus potential true enzyme activity inhibitors. These hits were then included in a dose response test to discern their inhibitory profile.

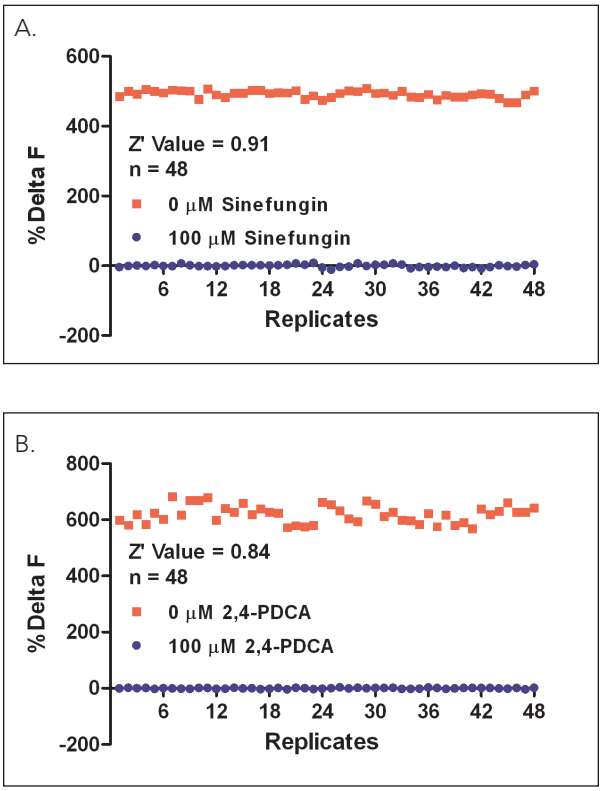


Figure 4. Z′-factor results for automated (A.) SET7/9 and (B.) JMJD2A assay methods, indicating excellent performance.

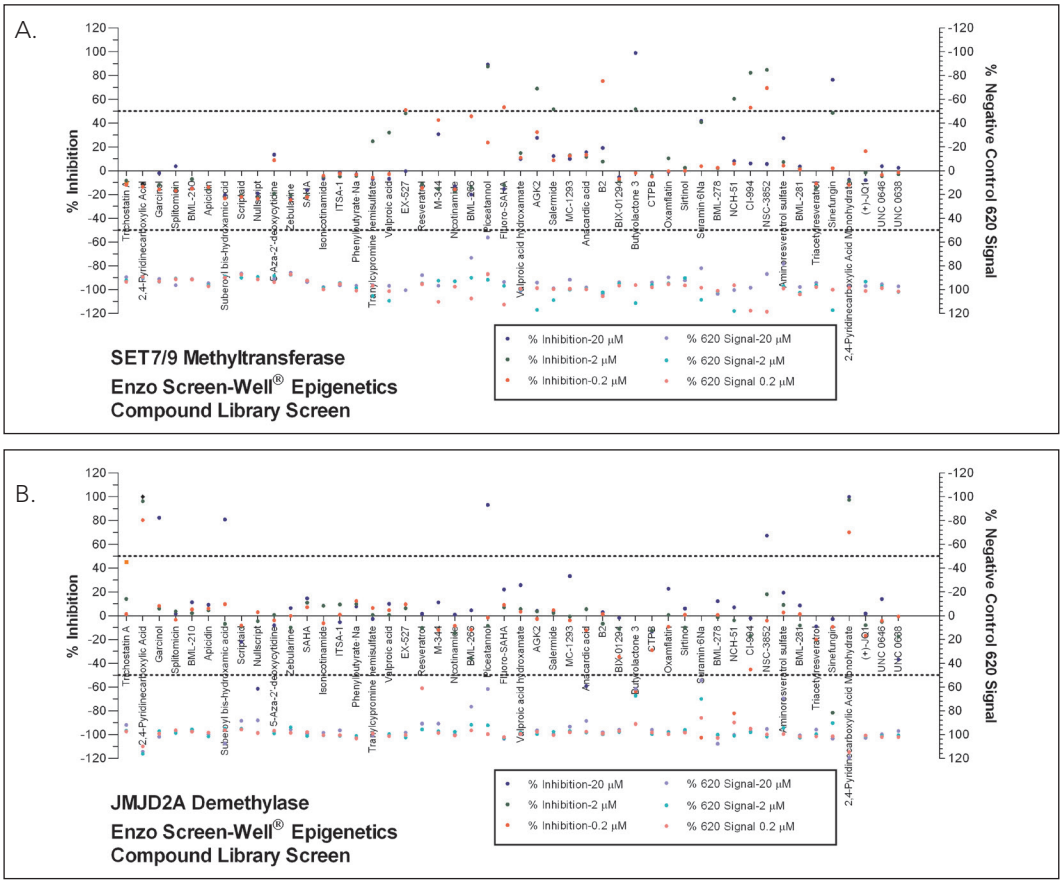


Figure 5. Screen-Well Epigenetics Library compound screen results. Black dotted lines represent 50% inhibition of the enzyme activity or of the 620 nm signal.

Dose Response

Hit compounds from the compound library screen were tested with the SET7/9 and JMJD2A assays to determine small molecule selectivity. Percent inhibition was graphed for all compounds across all concentrations tested using the SET7/9 assay (Figure 6A) and the JMJD2A assay (Figure 6C). Additionally, inhibition curves and IC_{50} values were calculated for true SET7/9 methyltransferase (Figure 6B) and JMJD2A demethylase (Figure 6D) enzyme inhibitors. SET7/9 inhibition was seen by the SIRT activator Piceatannol and the histone acetyl transferase inhibitor Butyrolactone3, while the histone deacetylase inhibitors Suberoyl bis-hydroxamic acid and NSC-3852 demonstrated clear JMJD2A inhibition. The similarity in target enzyme function may explain the inhibitory effects seen, and further investigation could uncover the mechanism of action for these compounds. Sinefungin and 2,4-PDCA, known SET7/9 and JMJD2A inhibitors, respectively, demonstrated expected inhibition curves and IC_{50} values, thus validating the dose response test.

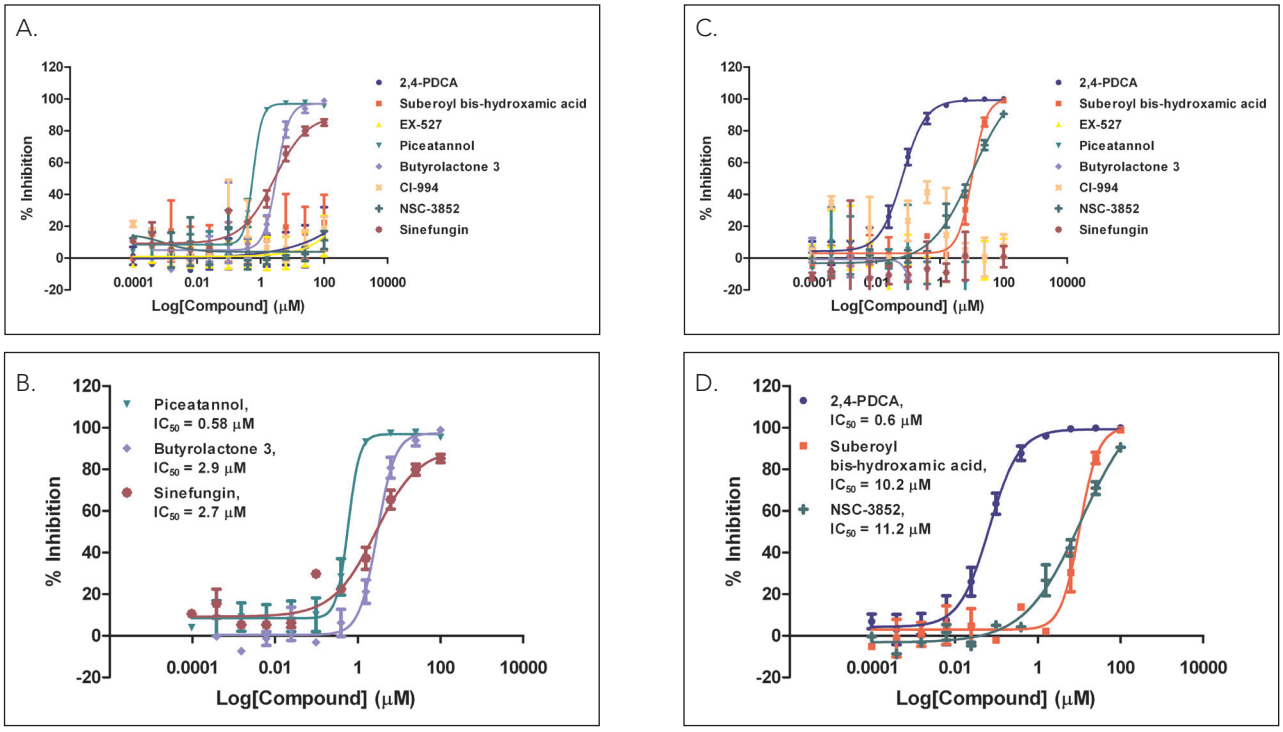


Figure 6. Dose response tests showing percent inhibition for all compounds and concentrations tested with (A) SET7/9 and (C) JMJD2A assays as well as inhibition curves and IC_{50} values for (B) SET7/9 methyltransferase and (D) JMJD2A enzyme inhibitors.

Conclusions

The HTRF SET7/9 Methyltransferase and JMJD2A Demethylase assays afford sensitive, precise biochemical formats for the assessment of enzyme activity and inhibition. 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. In addition, 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. Screening and dose response data illustrate the ease of use, and accuracy of each automated assay. Finally, the combination of assay chemistry, as well as liquid handling and detection instrumentation, create a rapid, robust solution for analysis of modulation of these important epigenetic targets.

References

1. Santos-Rosa, H.; Caldas, C. Chromatin modifier enzymes, the histone code and cancer. *Eur J Cancer*. **2005 Nov**, *41*(16), 2381-2402.

2. Wagner, T.; Jung, M. New lysine methyltransferase drug targets in cancer. *Nat Biotechnol*. **2012 Jul 10**, *30*(7), 622-623.

3. Zhang, J.; Chung, T.; Oldenburg, K. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen*. **1999**, *4(2)*, 67-73.

Acknowledgements

The authors would like to thank Enzo Life Sciences for the generous donation of Screen-Well Epigenetics Library.