High-throughput homogeneous epigenetics assays using HTRF® technology and the SpectraMax® Paradigm® microplate detection platform

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Overview

Histone proteins are dynamic proteins that form the scaffold for DNA packaging and also undergo a variety of post-translational modifications that help to determine which areas of the genome are active or inactive. Histone methylation is a post-translational modification that occurs on arginine and lysine residues and that represents one of the most studied post-translational modifications involved in epigenetic regulation. On histone H3, several lysines are potential targets for methyltransferases and demethylases, enzymes that convert these residues to mono-, di-, or tri-methylated marks. Over the past decade, these modifications have been shown to have a prominent role in a broad variety of diseases, and the related enzymes and chromatin interacting proteins are now considered as important druggable therapeutic targets in the drug discovery field. Cisbio Bioassays has generated a panel of reagent tools using the HTRF® technology based on time-resolved fluorescence energy transfer (TR-FRET) between europium cryptate (donor) and XL665 (acceptor). Cisbio Bioassays epigenetic reagent toolbox consist of specific anti-methylated histone H3 antibodies labeled with europium cryptate and streptavidin-XL665 (SA-XL665) that can be combined to form a pair of reagents able to detect a specific methylation state on a histone substrate biotinylated. Each anti-histone HTRF® antibody is specific for a single histone H3 lysine residue and methylation mark enabling development of assays with the highest levels of specificity and sensitivity. This poster features data showing the optimization of G9a (histone methyltransferase) and LSD1 (histone demethylase) assays on the SpectraMax® Paradigm® microplate detection platform. Enzyme titration and time course, cofactor and substrate K_m, and IC_{50} for known inhibitors are demonstrated. These results indicate that HTRF® G9a methyltransferase and LSD1 demethylase assays are suitable for high throughput screening of inhibitors of these disease-relevant enzymes.

HTRF Assay Principle

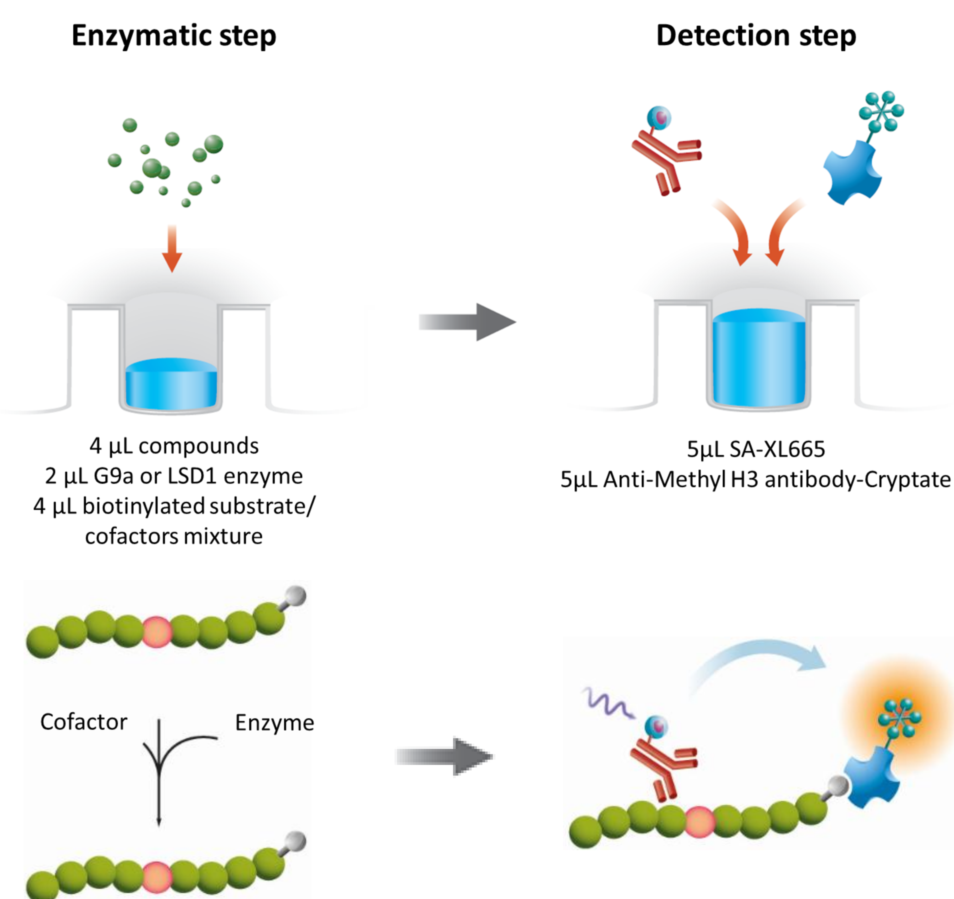
G9a Histone H3K27 di-methylation assay
The HTRF G9a Histone H3K9 dimethylation assay uses an H3(1-21) lysine 9 mono-methylated biotinylated peptide substrate, Eu3+-cryptate-labeled anti-H3K9 me2 detection antibody, and XL665-conjugated Streptavidin (SA-XL665).

The assay, performed in a single well, consists of two steps: enzymatic and detection. In the enzymatic step, G9a enzyme, inhibitor or enzymatic buffer, S-(5'-Adenosyl)-L-methionine chloride (SAM), and biotinylated mono-methylated H3(1-21) peptide substrate mixture are added to wells of a 384-well low-volume microplate. H3K9(1-21)me1 peptide is converted into H3K9me2 peptide with G9a. In the detection step, cryptate-labeled anti-dimethyl-histone H3K9 antibody (anti-H3K9me2-K) and streptavidin-XL665 (SA-XL665) are added to the reaction mixture. HTRF signal is proportional to the concentration of dimethylated H3(1-21) peptide.

LSD1 Histone H3K4 mono-demethylation assay
The HTRF LSD1 Histone H3K4 demethylation assay uses an H3(1-21) lysine 4 mono-methylated biotinylated peptide substrate, Eu3+-cryptate-labeled anti-H3K4 me0 detection antibody, and XL665-conjugated Streptavidin (SA-XL665).

The LSD1 demethylase assay also consists of enzymatic and detection steps. In the enzymatic step, LSD1 enzyme, inhibitor or buffer, and biotinylated mono-methylated H3(1-21) peptide substrate/FAD pre-mixture are added to wells. H3K4(1-21)me1 peptide is converted into H3K4me0 peptide with LSD1. In the detection step, cryptate-labeled anti-nonmethyl-histone H3K4 antibody (aH3K4me0-K) and streptavidin-XL665 (SA-XL) are added. HTRF signal is proportional to the concentration of demethylated H3(1-21) peptide.

Assay Setup



Results: Enzyme Titration and Time Course

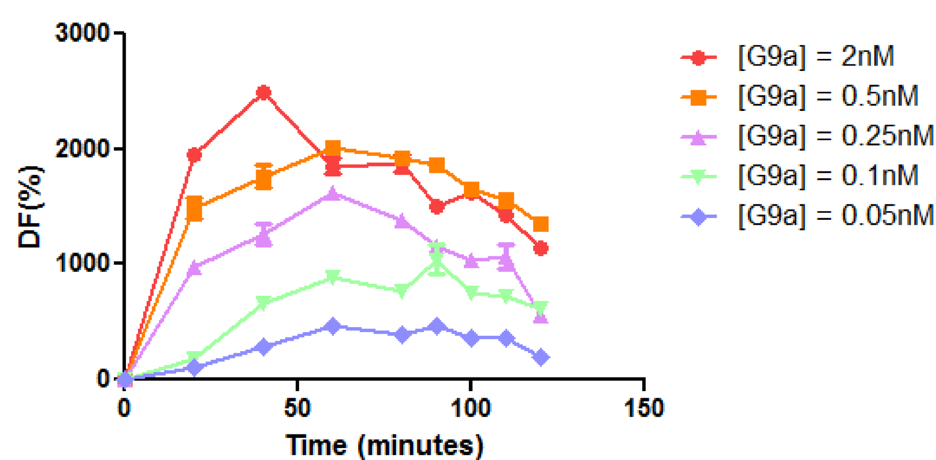


Figure 1. G9a enzyme titration and time course. This experiment allowed the optimal enzyme concentration and enzyme reaction time to be determined. For further experiments a reaction time of 1 hour and 0.25 nM enzyme were chosen.

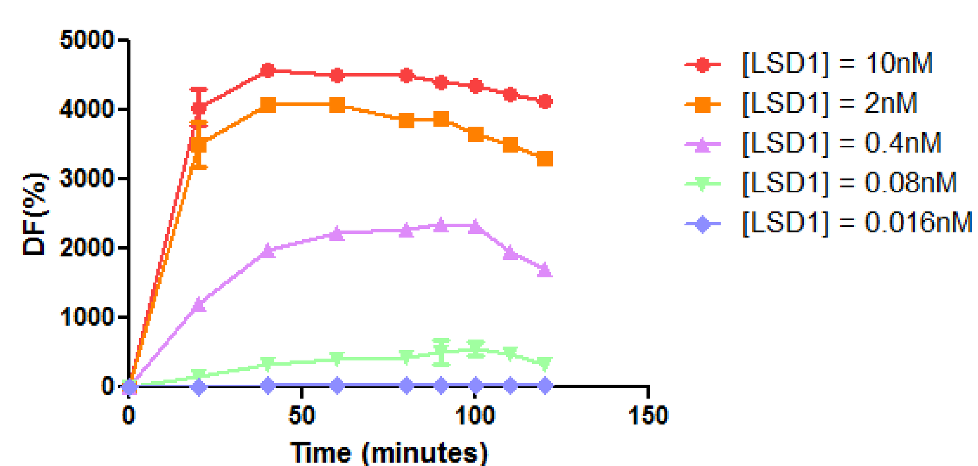


Figure 2. LSD1 enzyme titration and time course. Optimal reaction time was determined to be 1 hour, and an enzyme concentration of 0.4 nM was chosen for further experiments.

Results: K_m determination

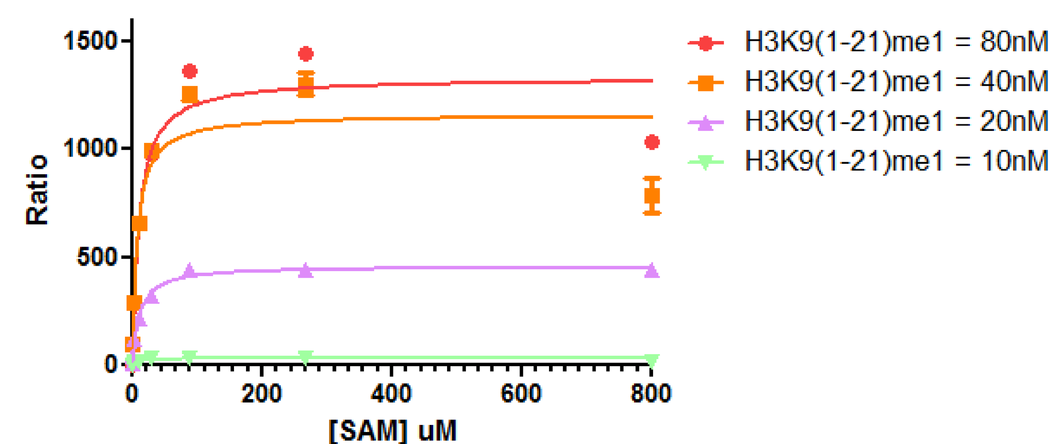


Figure 3. SAM titration. This experiment allowed the determination of K_m for SAM. The K_m value was determined using 0.2 nM G9a, and a range of substrate concentrations were tested. The K_m for SAM at 40 nM substrate was determined to be 7.2 µM, which is in agreement with published values.

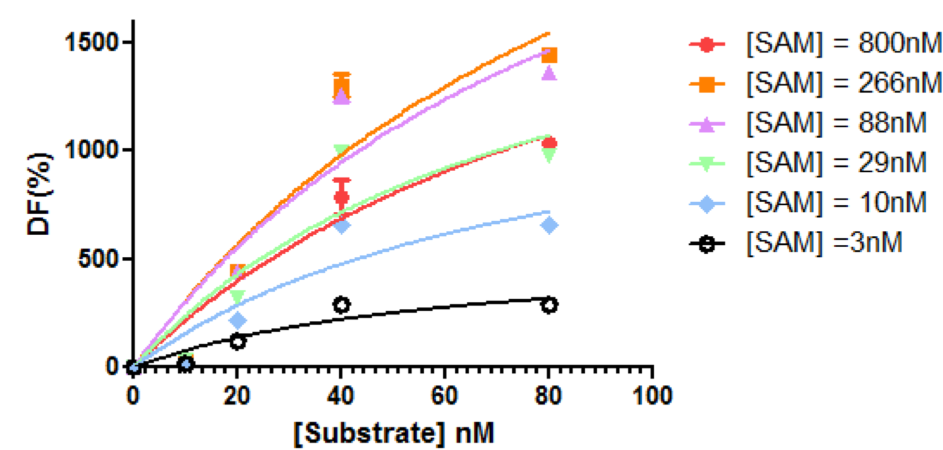


Figure 4. Substrate titration at different SAM concentrations. K_m values ranging from 80 to 100 nM SAM were calculated.

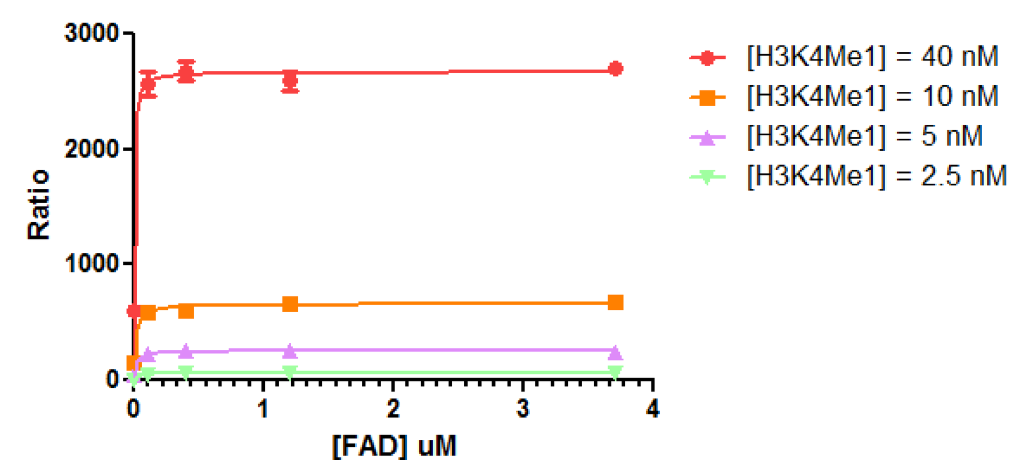


Figure 5. FAD titration with LSD1 and different concentrations of substrate. A K_m value of 0.013 µM was calculated for all but the highest substrate concentration.

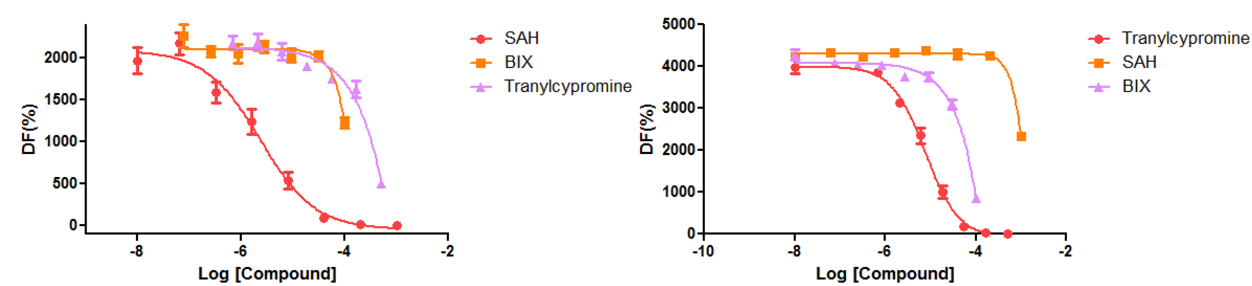


Figure 6. G9a (left) and LSD1 (right) inhibition curves. In this experiment, an IC_{50} value of 2.3 µM was calculated for SAH, in close agreement with published values. An IC_{50} value of 7.8 µM was obtained for tranylcypromine, similar to published values.

Conclusions

- The assay is shown to be highly sensitive, accurate, and robust, with no requirement for transfer or separation steps.
- HTRF® G9a methyltransferase and LSD1 demethylase assays are suitable for high throughput screening of inhibitors of G9a.
- HTRF epigenetic toolbox reagents give researchers the flexibility to create highly specific, custom histone modification assays.

SpectraMax Paradigm Microplate Reader

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- SpectraMax M5e Multi-Mode Microplate Reader
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