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, acetylation, deacetylation, methyl- and demethylation, ubiquitylation, and phosphorylation. These modifications take place mainly at the N-terminals 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 somatic state, aberrant modifications have been linked with a variety of diseases, 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 demethylases (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; multiple mechanisms including unbalanced and gene silencing. As a result, the number of drug discovery projects focused on these two enzyme classes has increased in the last few 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 methylases and demethylases 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 JUMPDAC enzyme. Both sets of assays are biochemical in nature and rely on the addition of europium cryptate labeled antibody specific for the methylation state of the substrate, as well as a streptavidin-XL665 molecule, in a TR-FRET format. Enzyme activity in the well leads to the creation 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 JUMPDAC enzyme.

Materials and Methods

Each assay consists of enzymatic and detection steps. The enzymatic step is carried out in the 150 µL reaction volume. The reaction conditions were as follows: 1X Reaction buffer, 1X Buffer for the fluorescent reagents, and 1X Buffer for the antibody mixes in volumes as low as 2 µL. Sodium NED 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 Xanop plate black and quartz microplates. The reader was used to detect fluorescence from 340 to 620 nm, with 5 nm increments; and absorbance from the assay chemistry with the following settings: Delay after plate movement 0; Delay after excitation 150; Integration time 300; Read height 8.5 mm.

Automated Assay Procedure

The automated SET7/9 and JMJD2A assay procedures were then validated using the optimized reaction conditions 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 2 factor experiment to measure assay robustness. The 2-factor value takes into account the difference in signal between a positive and 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.

Conclusions

1. The HTRF SET7/9 Methyltransferase and JMJD2A Demethylase assay formats afford sensitive, precise biochemical for the assessment of enzyme activity and inhibition.

2. Each assay procedure can be easily adapted in low-volume 384-well format using the non-contact dispensing capabilities of the MultiM, using volumes as low as 2 µL.

3. The Xenon-based excitation and filter-based detection system of the MultiM NED microscope 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 technology, create a rapid, easy-to-use, robust solution for analysis of modulation of these important epigenetic targets.