Utility of a Sensitive, Fluorescence-Based Assay for the Detection of Histone Deacetylase 6 (HDAC6) Activity

Brad Larson[1], Peter Banks[1], Diana Hulboy[2], Kara Cannon[2], Wayne Patton[2]

[1]BioTek Instruments, Inc., Winooski, Vermont, USA • [2]Enzo Life Sciences, Farmingdale, New York, USA

Overview

Histone deacetylases (HDACs) are a class of enzymes that remove acetyl groups from ε-N-acetyl lysine amino acids on histones and other proteins. The removal of acetyl groups serves to increase the positive charge of histone tails, encouraging binding between histones and the DNA backbone, and preventing transcription. Because of this role, HDACs have been implicated in a variety of human diseases, including cancer; making them an increasingly popular target for drug discovery research. Here we demonstrate an application to monitor the deacetylation activity of the HDAC Class II enzyme, HDAC6. Validation and pharmacology results demonstrate how the fluorescent assay technology and microplate reader can be used together to provide a relevant system to assess enzyme kinetics and inhibition.

Introduction

Histones form the core of nucleosomes, the DNA/protein complexes that are the subunits of eukaryotic chromatin. Histones' N-terminal "tails" are subject to a variety of posttranslational modifications, including phosphorylation, methylation, ubiquitination, ADP-ribosylation and acetylation. These modifications have been proposed to constitute a 'histone code' with profound regulatory functions in gene transcription (Strahl *et al.*, 2000). The best studied of these modifications, ε-amino acetylations of specific histone lysine residues, are catalyzed by histone acetyltransferases (HATs). Histone deacetylases (HDACs) are responsible for the removal of these acetyl groups (Grunstein *et al.*, 1997; Ng *et al.*, 2000; Cheung *et al.*, 2000). Histone hyperacetylation correlates with an open, decondensed chromatin structure and gene activation, while hypoacetylation correlates with chromatin condensation and transcriptional repression (Kadosh *et al.*, 1998; Rundlett *et al.*, 1998; Zhang *et al.*, 1998). Due to their importance in gene regulation, HDACs are becoming an increasingly accepted target for cancer therapy (Marks *et al.*, 2003; Marcus et al., 2005; Saji *et al.*, 2005; Carew *et al.*, 2008).

HDAC6 in particular has seen increasing interest as a drug target due to its role in cancer-causing cell transformation. Overexpression of HDAC6 has been shown to correlate with tumorigenesis and improved survival of cancerous cells. It has also been shown to contribute to breast cancer metastasis, due to the fact that its upregulation increases motility in breast cancer MCF-7 cells (Aldana-Masangkay *et al.*, 2011).

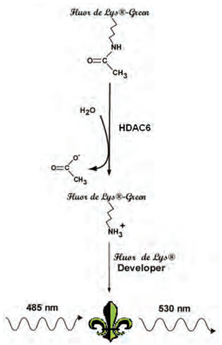
Here we demonstrate the combination of a fluorescence-based assay and multi-mode reader to be used to screen HDAC6 inhibitors. The assay is based upon a green pro-fluorescent substrate/developer combination. The substrate, which comprises an acetylated lysine side chain, is incubated with HDAC6. Deacetylation of the substrate sensitizes the substrate so that treatment with the developer produces a fluorophore. The instrument is a hybrid multi-mode microplate reader, which combines a monochromator-based as well as a filter-based detection system. The filter-based system, used here, is completely independent of the monochromator system, and provides increased sensitivity and speed during detection. Validation and pharmacology data demonstrate the ability of assay and reader to be used together to detect activity and inhibition of this important emerging drug target.

BioTek Instrumentation

The Synergy H4 combines a filter-based and monochromator-based detection system. The filter-based system and Xenon flash lamp was used to detect the 530 nm fluorescent emission using Ex: 485/20 and Em: 528/20 filters, along with a 510 nm cut-off mirror.

Figure 1 – *Synergy™ H4 Hybrid Multi-Mode Microplate Reader.*

Fluor de Lys®-Green HDAC6 Assay



The ***Fluor de Lys®-Green*** assay is based upon the ***Fluor de Lys-Green*** substrate and ***Fluor de Lys*** Developer combination. The assay procedure has two steps. First, the ***Fluor de Lys-Green*** substrate, which comprises an acetylated lysine side chain, is incubated with the HDAC enzyme. Deacetylation of the substrate sensitizes the substrate so that, in the second step, treatment with the ***Fluor de Lys*** Developer produces a fluorophore. The fluorophore is excited with 485 nm light (470-500) and emits at approximately 530 nm.

Figure 2 – *Representation of* ***Fluor de Lys-Green*** *Assay.*

Materials

Assay Components

Fluor de Lys-Green Substrate (BML-KI572), ***Fluor de Lys*** Developer Concentrate (20X) (BML-KI105), Trichostatin A (BML-GR309), HDAC Assay Buffer II (BML-KI422) are a part of the ***Fluor de Lys*** HDAC2 Fluorimetric Drug Discovery Kit (BML-AK512).

Compounds

Trichostatin A (BML-GR309), BML-210 BML-GR330), Apicidin (BML-GR340), Scriptaid (BML-GR326), and BML-281 (BML-GR361) were attained from Enzo Life Sciences. Compounds were resuspended in 100% DMSO to the correct 200X concentration for use in the pharmacology validation.

Instrument Components

A 485/20 nm excitation filter (7082221), 528/20 nm emission filter (7082247), and 510 nm cut-off mirror (7138510) from BioTek Instruments, Inc. were used for all reads on the Synergy H4.

Assay Plates

384-well, flat bottom, black, non-treated plates (3710) from Corning Life Sciences were used in all experiments.

Optimized 384-Well Assay Procedure

Figure 3 – *Fluor de Lys-Green HDAC6 384-well Assay Workflow.*

Data Reduction

The delta RFU value for each well was calculated using the following formula:

$$\Delta \text{ RFU} = (\text{Sample}_{\text{Enz}} - \text{Sample}_{\text{NoEnz}})$$

Where $\text{Sample}_{\text{Enz}}$ are assay wells containing enzyme, substrate, and developer, and $\text{Sample}_{\text{NoEnz}}$ are control wells containing substrate, developer and no enzyme. The measure of a sample's deacetylation is the difference between its fluorescence and the fluorescence of the no enzyme control.

HDAC6 Enzyme Titration

An enzyme titration was performed to demonstrate the ability of the HDAC6 enzyme to function correctly with the ***Fluor de Lys-Green*** assay. Enzyme was serially titrated from 200-0 ng/well using 1:2 dilutions, and then added to the assay plate in triplicate. ***Fluor de Lys-Green*** substrate was then added at a 2X concentration of 20 µM at various timepoints to create substrate incubations of 60, 30, 15, and 5 minutes. ***Fluor de Lys*** Developer was then added to stop the reaction, and the plate was read after a 25 minute incubation time.

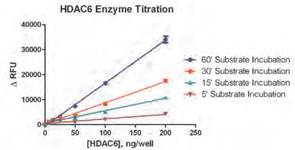


Figure 4 – *HDAC6 Enzyme Titration Data.*

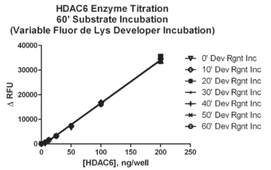


Figure 5 – *HDAC6 enzyme titration. Variable* ***Fluor de Lys*** *Developer incubation time with 60' substrate incubation.*

The results shown in Figure 4 demonstrate the linearity of the fluorescent response across a wide range of enzyme concentrations. It can also be seen that the largest change in fluorescence is seen using a 60' substrate incubation. Figure 5 shows that the change in fluorescence seen from the 60' substrate incubation data is consistent across multiple developer incubation times.

Fluor de Lys-Green Substrate Deacetylation Kinetics by HDAC6

Fluor de Lys-Green substrate was titrated to determine the substrate K_m with HDAC6. Substrate was serially titrated using a 1:2 dilution scheme creating final 1X concentrations ranging from 1000-0 µM. HDAC6 enzyme was added at a concentration of 25 ng/well and then added to the assay plate in triplicate. A 60 minute substrate incubation time was used, in addition to a 25 minute incubation following the ***Fluor de Lys*** Developer addition.

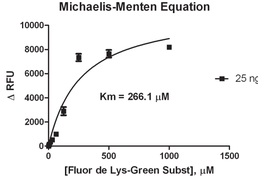


Figure 6 – *Substrate K_m Determination – (Michaelis-Menten Curve Fit).*

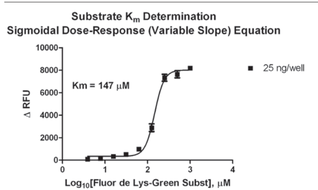


Figure 7 – *Substrate K_m Determination - (Sigmoidal Dose-Response Curve Fit).*

The data in Figure 6 show the increase in fluorescence in relation to changes in substrate concentration. A fit of the data to the Michaelis-Menten equation is shown, yielding a K_m value of 266 µM. The plot of Δ RFU vs. [***Fluor de Lys-Green***] shows a slight sigmoidal appearance. Therefore the K_m value determined does not completely fit the data and is right shifted. Due to this fact, the data were fitted to a sigmoidal dose-response curve. The plot of Δ RFU vs. $\log_{10}$[***Fluor de Lys-Green***] shows a much better fit of the data, and yields a more true K_m value.

HDAC6 Enzyme Activity Confirmation

A confirmation of HDAC6 activity was performed using enzyme concentrations of 30, 20, 15, 10, 7.5, 5, and 2.5 ng/well. A ***Fluor de Lys-Green*** substrate concentration of 35 µM was chosen to be below the determined substrate K_m. A 60 minute substrate incubation, as well as a 25 minute developer incubation was used for the confirmation.

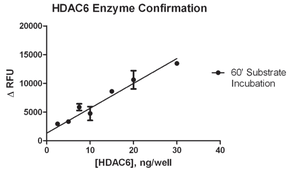


Figure 8 – *HDAC6 Confirmation of Δ RFU vs. [HDAC6].*

The results show that a concentration of 15 ng/well yields a significant change in fluorescence, while still well within the linear range of the curve. This was further tested using a Z'-factor test to determine the robustness of the assay using the optimized conditions.

HDAC6 Assay Z'-factor Validation

A Z'-factor assay was performed to validate the HDAC6 assay using the optimized enzyme and substrate conditions. 15 and 0 ng/well enzyme concentrations were used as the positive and negative controls, respectively. Forty-eight replicates of each condition were tested.

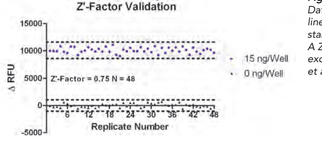
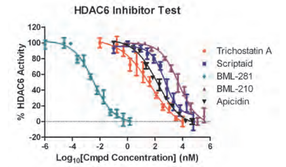


Figure 9 – *Z'-factor validation results. Data shown for all replicates. Dashed lines represent the average ± 3 standard deviations for each condition. A Z' value ≥ 0.5 is indicative of an excellent assay according to Zhang et al., 1999.*

HDAC6 Inhibitor Pharmacology Validation

Inhibitor dose response curves were generated using Trichostatin A, Scriptaid, Apicidin, and the Enzo Life Sciences inhibitors BML-210 and BML-281 (specific HDAC6 inhibitor). Concentrations tested ranged from 10 – 0 µM for Trichostatin A, 60 – 0 µM for Scriptaid, 50 – 0 µM for Apicidin, 365 – 0 µM for BML-210, and 1.5 – 0 nM for BML-281.

Figure 10 – *Inhibitor Validation Data.*



	Literature IC_{50} Value	Fluor de Lys®-Green HDAC6 Assay
Trichostatin A	73 nM[1]	46 nM
Scriptaid	6-8 µM[2]	517 nM
Apicidin	100 nM[3]	147 nM
BML-210	5-10 µM[4]	5.3 µM
BML-281	0.002 nM[5]	0.003 nM

[1]Takahashi *et al.*, 1996; [2]Su *et al.*, 2000; [3]Kwon *et al.*, 2002

[4]Herman *et al.*, 2006; [5]Kozikowski *et al.*, 2008

Table 1 – *Inhibitor IC_{50} Values.*

The IC_{50} values compare favorably to literature IC_{50} values or internal IC_{50} values generated by Enzo Life Sciences. The discrepancy seen between the literature IC_{50} value for Scriptaid, and that generated with the ***Fluor de Lys-Green*** HDAC6 assay can be explained by the fact that 8 µM is an optimum concentration used within a cell-based system, whereas the compound may be more potent in a purified enzyme system.

Conclusions

1. HDAC6 enzyme kinetics and pharmacology can be easily and accurately characterized using the ***Fluor de Lys-Green*** assay.
2. The sensitivity of the Synergy H4 allows for assay miniaturization to 384-well format.
3. Excellent robustness of the assay, using low concentrations of enzyme and substrate, is demonstrated with a Z'-factor of 0.75.
4. BML-281 demonstrates high potency for HDAC6, relative to other non-selective HDAC inhibitors.