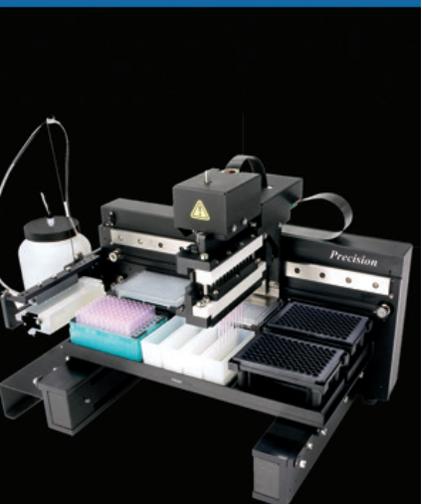




## Assessment of HDAC1 Inhibition Using an Automated, Bioluminescent Histone Deacetylase I/II Assay

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### Introduction

The DNA molecules in each cell are wrapped around histone proteins, and collectively referred to as chromatin. This serves to compact the DNA strand, and also controls gene expression. Changes to the chromatin structure are controlled by the addition or removal of epigenetic factors, such as methyl or acetyl groups, to histone tails. This is a normal process during cell differentiation. However, the loss of proper epigenetic control can lead to aberrant gene expression and the development of multiple diseases, including diabetes, cancer, or neurological disorders. This has led to much new research in this field, and has made epigenetics one of the fastest growing drug discovery markets.

One of the most popular set of epigenetic targets, due to their implication in many human diseases, are histone deacetylases (HDACs). This group of enzymes removes acetyl groups from  $\epsilon$ -N-acetyl lysine residues on histones and other proteins. Acetyl group removal serves to increase the positive charge of histone tails, thus encouraging binding between histones and the DNA backbone, preventing transcription, and silencing that part of the genetic code. HDAC inhibitors block this activity, therefore maintaining a relaxed chromatin structure allowing gene transcription to take place.

Here we demonstrate the capacity of a bioluminescent HDAC class I/II assay to be used in

a biochemical format to rapidly screen compounds for their ability to inhibit HDAC1 enzyme activity. Automated assay procedures were carried out in low volume 384-well (LV384) format using high throughput liquid handling and detection instrumentation. Experimental results confirm the automated cellular assay's ability to accurately detect the target-specific inhibitory characteristics of test compounds, with a low false positive rate, in a simple, yet robust manner for these important epigenetic targets.

### HDAC-Glo™ I/II Assay Principle

The HDAC-Glo™ I/II Assay from Promega Corporation (Madison, WI) is a single-reagent-addition, homogeneous, luminescent assay that measures the relative activity of histone deacetylase (HDAC) class I and II enzymes from cells, extracts or purified enzyme sources<sup>1</sup>. The assay is broadly useful for class I and II enzymes, and uses an acetylated, live cell-permeant, luminogenic peptide substrate that can be deacetylated by HDAC activities (Figure 1). Deacetylation of the peptide substrate is measured using a coupled enzymatic system in which a protease in the developer reagent cleaves the peptide from aminoluciferin, which is quantified in a reaction using Ultra-Glo™ Recombinant Luciferase. The HDAC-mediated luminescent signal is proportional to deacetylase activity.

#### Key Words:

Epigenetics

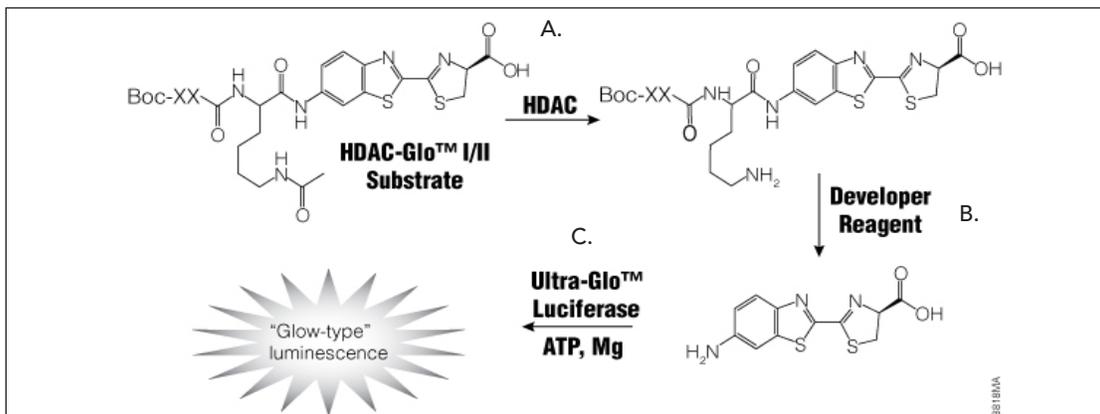
Histone Deacetylase

HDAC

HDAC-Glo

Luminescence

Automation



**Figure 1.** HDAC-Glo I/II Assay Principle. (A.) HDAC activity deacetylates the luminogenic HDAC-Glo™ I/II Substrate, making the peptide sensitive to a specific proteolytic cleavage event (B.) that is mediated by HDAC-Glo™ I/II Reagent and liberates aminoluciferin. (C.) Free aminoluciferin then can be measured using the Ultra-Glo™ firefly luciferase reaction to produce a stable, persistent emission of light. All three enzymatic modifications of the substrate occur in coupled, nearly simultaneous reactions upon addition of a single reagent.

## Materials and Methods

### Materials

#### Assay Components

HDAC-Glo™ I/II Assay (Catalog No. G6421), including HDAC-Glo I/II Buffer, Lyophilized HDAC-Glo I/II Substrate, Developer Reagent, and 10 mM Trichostatin A, was donated by Promega Corporation.

#### Compounds

The 43 compound Screen-Well® Epigenetics Library, Version 1.0 (Catalog No. BML-2836-0500) was generously donated by Enzo Life Sciences (Plymouth Meeting, PA). The known bromodomain inhibitor (+)-JQ1 (Catalog No. 92-1149) was donated by DiscoverX Corporation. Sinefungin (Catalog No. S8559) and 2,4-Pyridinedicarboxylic acid (2,4-PDCA) monohydrate (Catalog No. P63395) 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).

#### Instrumentation

##### Precision™ Microplate Pipetting System

The Precision™ Microplate Pipetting System (BioTek Instruments, Inc., Winooski, VT) combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. Precision was used to dilute the Epigenetics library and transfer the final 2X concentrations to the LV384 assay plates.

##### MultiFlo™ Microplate Pipetting System

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 2X enzyme and prepared detection reagent to the assay plates.

##### 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 dedicated, high-performance luminescence detection system was used to quantify the luminescent signal from each assay well using an integration time of 0.1 seconds and gain of 153.

### Methods

#### Enzyme Concentration Optimization

A serial titration of HDAC1 was created using a 1:4 dilution scheme starting at a 1X concentration of 500 ng/mL. Ten microliters of each concentration was transferred to the LV384 assay plate, followed by 10 µL of detection reagent. The luminescent signal was then quantified after a 45 minute incubation time at room temperature (RT) using the Synergy Neo and aforementioned luminescence settings.

#### Automated HDAC-Glo I/II LV384 Assay Workflow

Five microliters of 2X compound was transferred to the appropriate assay plate well using the Precision. 5 µL of 20 ng/mL (2X) HDAC1, optimized in the previous experiment, was then dispensed into each well using the MultiFlo and the plate was incubated at room temperature for 30 minutes. 10 µL of detection mixture was then added to each well, and the plate was incubated a final time for 45 minutes at room temperature. The luminescent signal was then quantified using the Synergy Neo and aforementioned settings.

#### Automated Assay Known Inhibitor IC<sub>50</sub> and Z'-factor Validation

An 8-point serial titration of the known HDAC inhibitor Trichostatin A was created using a 1:5 dilution scheme starting at a 2X concentration of 1,000 nM. Four 5 µL replicates of each concentration were transferred to an LV384 assay plate for the inhibitor IC<sub>50</sub> confirmation. Twenty-four 5 µL replicates were also dispensed of the 1,000 and 0 nM concentrations for the Z'-factor validation. The remaining steps of the assay procedure were then completed as previously described.

#### 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 HDAC-Glo I/II Buffer from their original 10 mM concentration to final 2X concentrations of 40 µM, 4 µM and 400 nM. Five microliters of each compound concentration was transferred to an LV384 assay plate. The remaining steps of the automated assay procedure were then completed as previously described.

#### Inhibitor Validation

Dose response tests were performed on compounds exhibiting greater than 50% inhibition of enzyme activity at any of the three concentrations tested, including known HDAC inhibitors, and the SIRT activators Resveratrol, Piceatannol, and Amino-resveratrol Sulfate. 12-point titrations were created for each test using a 1:4 dilution scheme starting at a 1X concentration of 100 µM. Two different tests were completed. The first determined the specific inhibitory characteristics of each lead compound.

The assay was performed as previously described and  $IC_{50}$  values were calculated from each inhibition curve. The second test determined the inhibitory potential of each compound on the components used in the detection step. A nonacetylated HDAC-Glo I/II control substrate was added to the detection reagent. The control substrate does not require deacetylation to produce luminescence, and therefore is not affected by HDAC inhibitors. The assay was then performed as previously described. assay procedure were then completed as previously described.

## Results

### Enzyme Concentration Optimization

Optimization of enzyme concentration is critical to maximize the assay window, or signal-to-background ratio (S:B), while at the same time minimizing cost per well. S:B ratios were calculated for each enzyme concentration tested, ranging from 500 to 0.12 ng/mL, by comparing Relative Luminescent Units (RLU) for wells containing enzyme to negative control wells containing HDAC-Glo I/II Buffer only. S:B values were calculated as follows:

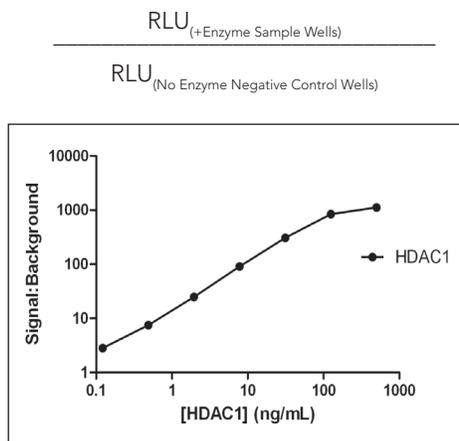


Figure 2. HDAC1 concentration optimization data.

Calculated S:B ratios were then plotted (Figure 2). A linear relationship between [enzyme] and S:B ratio is seen up to an HDAC1 concentration of 125 ng/mL. For subsequent experiments a concentration of 10 ng/mL was chosen due to the fact that a high S:B ratio is generated, and this value is well within the linear range for the enzyme.

### Automated Assay Known Inhibitor $IC_{50}$ and Z'-factor Validation

Using the optimized enzyme concentration from the previous experiment, two validation experiments were performed to determine the accuracy and robustness of the automated assay procedure. Trichostatin A was chosen because of its known inhibitory effects on HDAC1. S:B ratios were once again plotted for each inhibitor concentration tested (Figure 3).

An  $IC_{50}$  value of 1.04 nM was calculated from the curve generated using a variable slope (four parameter) non-linear regression curve fit. This value agrees with previously published  $IC_{50}$  values ranging from 1.5-2.9 nM<sup>2</sup>.

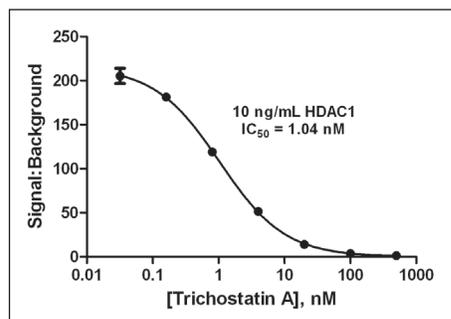


Figure 3. Dose response curve showing inhibition of HDAC1 enzyme activity by Trichostatin A.

The Z'-factor value takes into account the difference in signal between a positive and negative control, as well as the signal variation amongst replicates<sup>3</sup>. A scale of 0-1 is used, with values greater than or equal to 0.5 being indicative of an excellent assay.

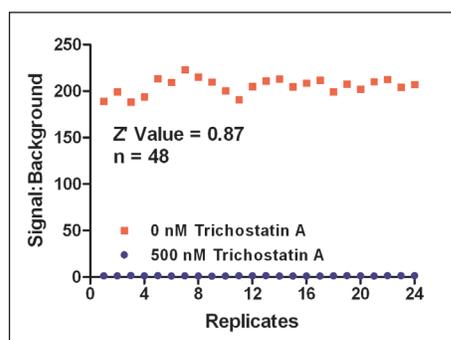


Figure 4. Z'-factor results from 24 replicate measurements each at [Trichostatin A] of 500 nM and vehicle.

Per Figure 4 data, the Z'-factor calculation of 0.87 is indicative of a robust assay performance. The automated assay method was then used to perform a compound library screen to look for potential HDAC1 enzyme inhibitors.

### Compound Library Screen

The Screen-Well Epigenetics Library and 5 known inhibitors were screened using the validated, automated HDAC1 assay. Percent inhibition (Figure 5) was determined for each of the three compound concentrations tested by comparing the luminescent signal from compound-containing wells to uninhibited positive control wells using the following formula.

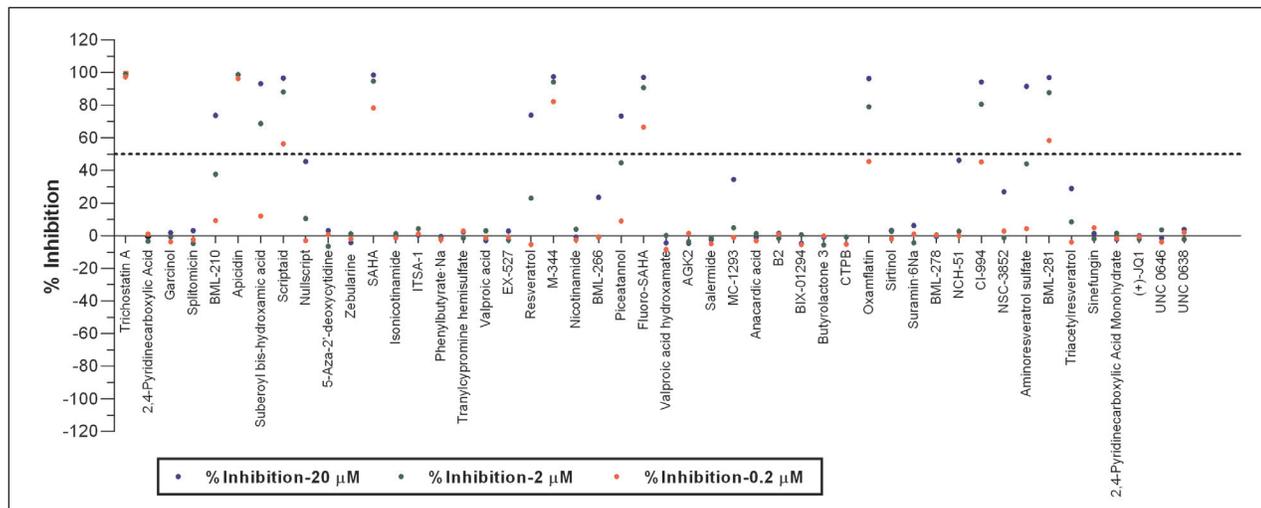


Figure 5. % Inhibition results from HDAC1 assay screen using Screen-Well Epigenetics Library.

Compounds exhibiting greater than 50% inhibition of enzyme activity at any of the three concentrations tested, including known HDAC inhibitors, and the SIRT activators Resveratrol, Piceatannol, and Aminoeresveratrol Sulfate, were labeled as hits and carried forward for dose response testing to discern their inhibitory profile.

#### Inhibitor Validation

Hit compounds from the compound library screen were tested to determine their inhibitory effects on HDAC1 activity and proper detection reagent function. For the HDAC1 inhibition test, percent inhibition was

graphed for all compounds across all concentrations tested (Figure 6A).  $IC_{50}$  values were once again calculated from the generated curves. For the analysis of effect on detection reagent function (Figure 6B), % Negative Control Luminescence was calculated by comparing RLU values from wells containing compound to uninhibited reaction positive control wells using the following formula.

$$\left( \frac{RLU_{(\text{Compound Containing Wells})}}{RLU_{(\text{No Compound Positive Control Wells})}} \right)$$

Each value was then plotted to generate curves signifying inhibition of the HDAC-Glo I/II Reagent.

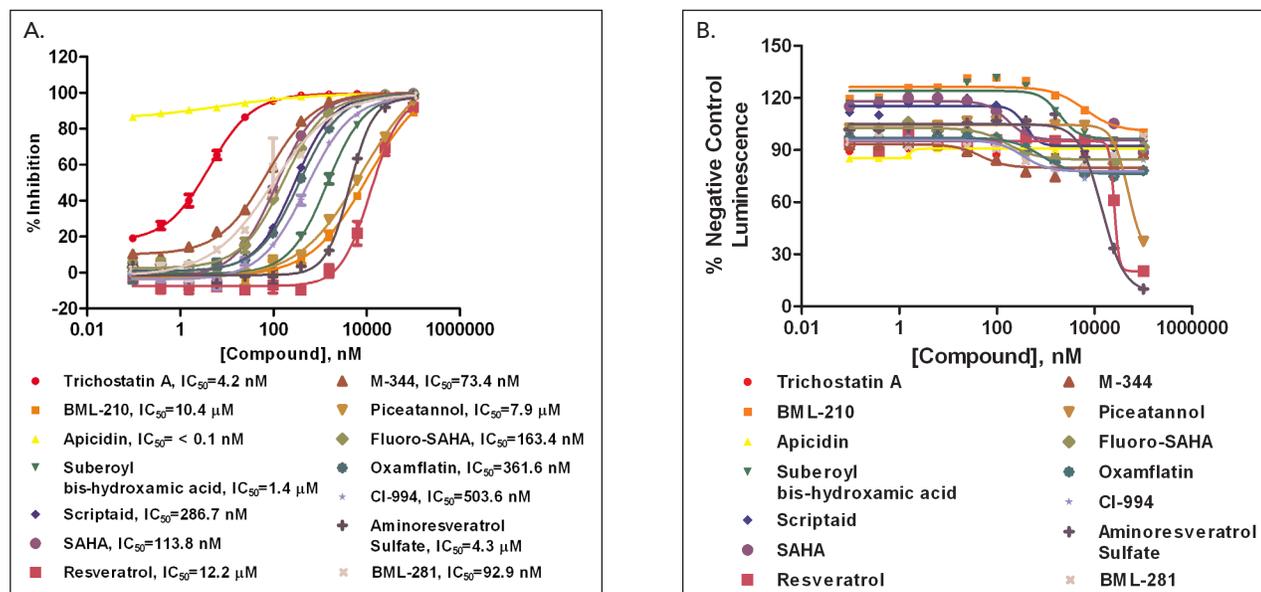


Figure 6. Dose response curves generated for "hit" compounds using (A) standard detection reagent components, or (B) detection reagent plus control substrate.

The dose response tests confirmed that each known HDAC inhibitor demonstrated true HDAC1 activity inhibition, as no significant negative change in luminescent signal was seen when each compound was tested with the control substrate. This was in contrast to the three SIRT activator compounds, which showed evidence of a negative effect on the detection reagent components at high compound concentration. Therefore these compounds should not be included with the others as actual inhibitors of HDAC1 activity.

## Conclusions

The HDAC-Glo I/II assay provides a sensitive, precise, easy to use format to screen for potential inhibitors of class I and II histone deacetylases. The assay procedure is easily automated in LV384 format using Precision™ and MultiFlo™. Additionally, Synergy™ Neo's high-performance luminescence detection affords rapid, sensitive bioluminescent signal detection, allowing use of minimal enzyme concentrations and integration times. Screening, counter-screening, and dose response data show that the automated assay delivers accurate information for unknown compounds. Finally, the combination of assay chemistry and liquid handling and detection microplate instrumentation create a simple, robust and definitive solution for inhibitory compound identification of these important epigenetic targets.

## References

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