

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 multiple Class I and II HDAC enzymes in an automated fashion. Validation and pharmacology results demonstrate how the fluorescent assay technology and instrumentation 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 sub-units of eukaryotic chromatin. Histone N-terminal 'tails' are subject to a variety of post-translational 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).

Here we demonstrate the combination of a fluorescence-based assay with liquid handling and detection instrumentation to be used to examine the selectivity of lead compounds for a specific HDAC enzyme, in a profiling fashion. The assay is based upon a *green* pro-fluorescent substrate/developer combination. The substrate, which comprises an acetylated lysine side chain, is incubated with the HDAC enzyme. Deacetylation of the substrate sensitizes the substrate so that treatment with the developer produces a fluorophore. The liquid handler provides a simple way to perform compound titration, as well as dispense all assay reagents to the 384-well assay plate. Detection is provided by 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 instrumentation to be used together to detect activity and inhibition of this important emerging drug target.

BioTek Instrumentation



Figure 1 – A. The Precision™ Microplate Pipetting System combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate compounds across a 96-well polypropylene plate, transfer the compounds to the 384-well assay plates, as well as dispense all assay components to the plates.

B. Synergy™ H4 with Hybrid Technology™ is a patent pending Multi-Mode Detector System that includes both filters and monochromators. 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.

Fluor-de-Lys®-Green HDAC Assay

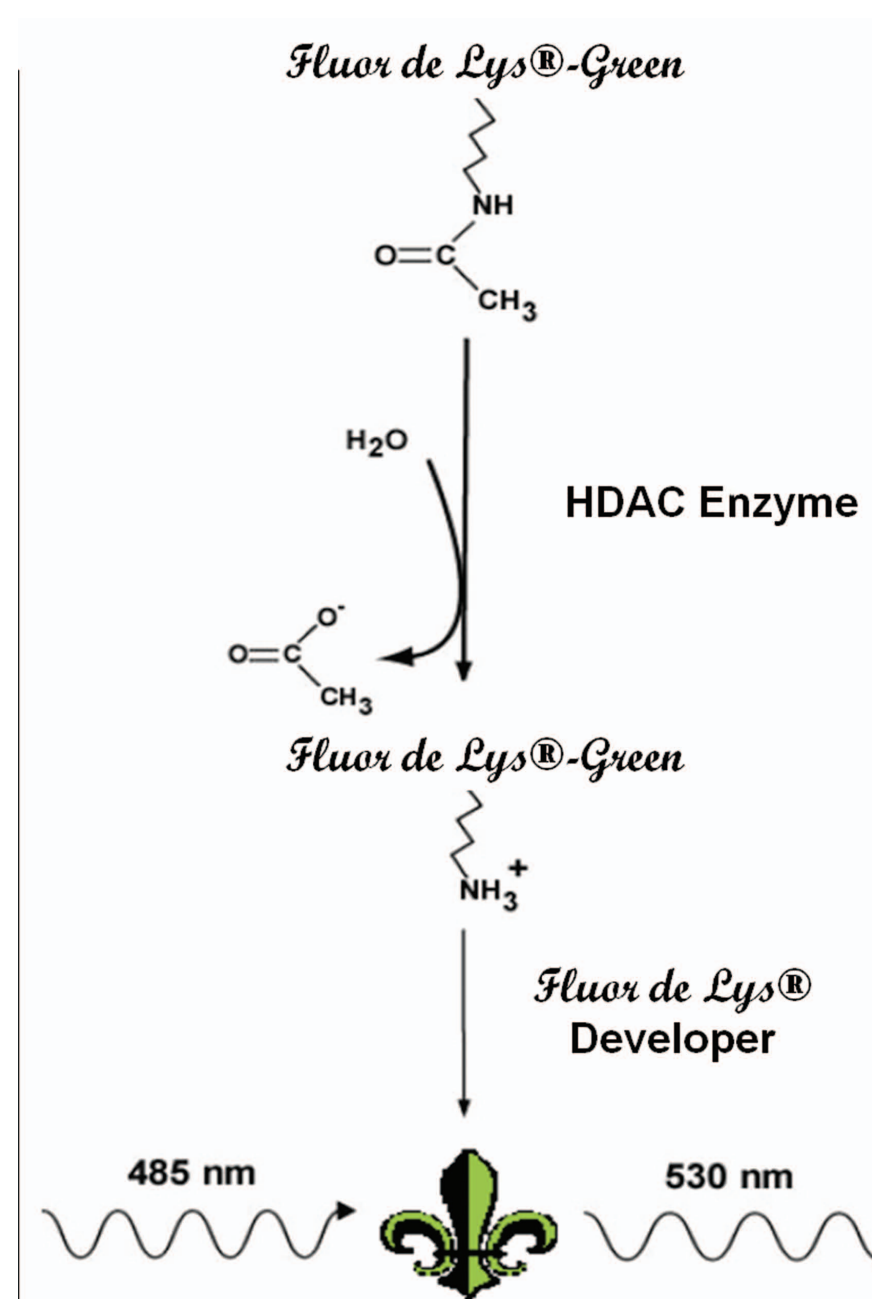


Figure 2 – Representation of Fluor de Lys®-Green Assay.

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.

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), Suberoyl bis-hydroxamic acid (BML-GR323), Tubacin (BML-GR362), Niltubacin (BML-GR363), 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.

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 Sample_{Enz} are assay wells containing enzyme, substrate, and developer, and Sample_{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.

HDAC Enzyme Titration

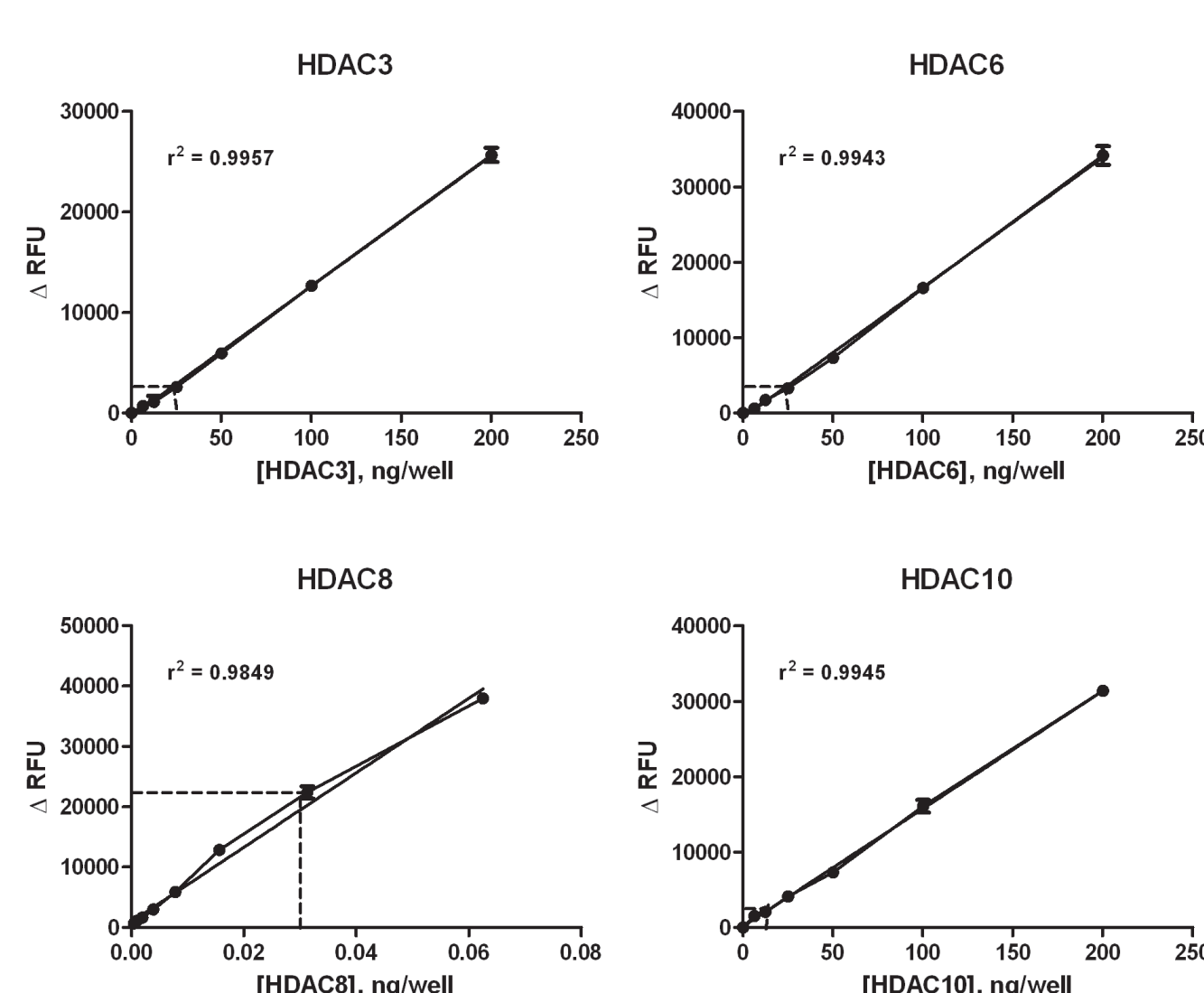


Figure 3 – HDAC Enzyme Titration Curves. The results demonstrate the linearity of the fluorescent response across a wide range of enzyme concentrations. Dashed lines show the concentration of enzyme chosen to perform the substrate K_m determination.

An enzyme titration was performed to demonstrate the ability of the HDAC enzymes to function correctly with the *Fluor de Lys®-Green* assay. Enzymes were serially titrated using 1:2 dilution scheme, and then added to the assay plate in triplicate. *Fluor de Lys®-Green* substrate was then added at a 2X concentration of 20 μM to create a substrate incubation time of 60 minutes. *Fluor de Lys®* Developer was then added to stop the reaction, and the plate was read after a 20 minute incubation time.

Fluor de Lys®-Green Substrate Deacetylation Kinetics

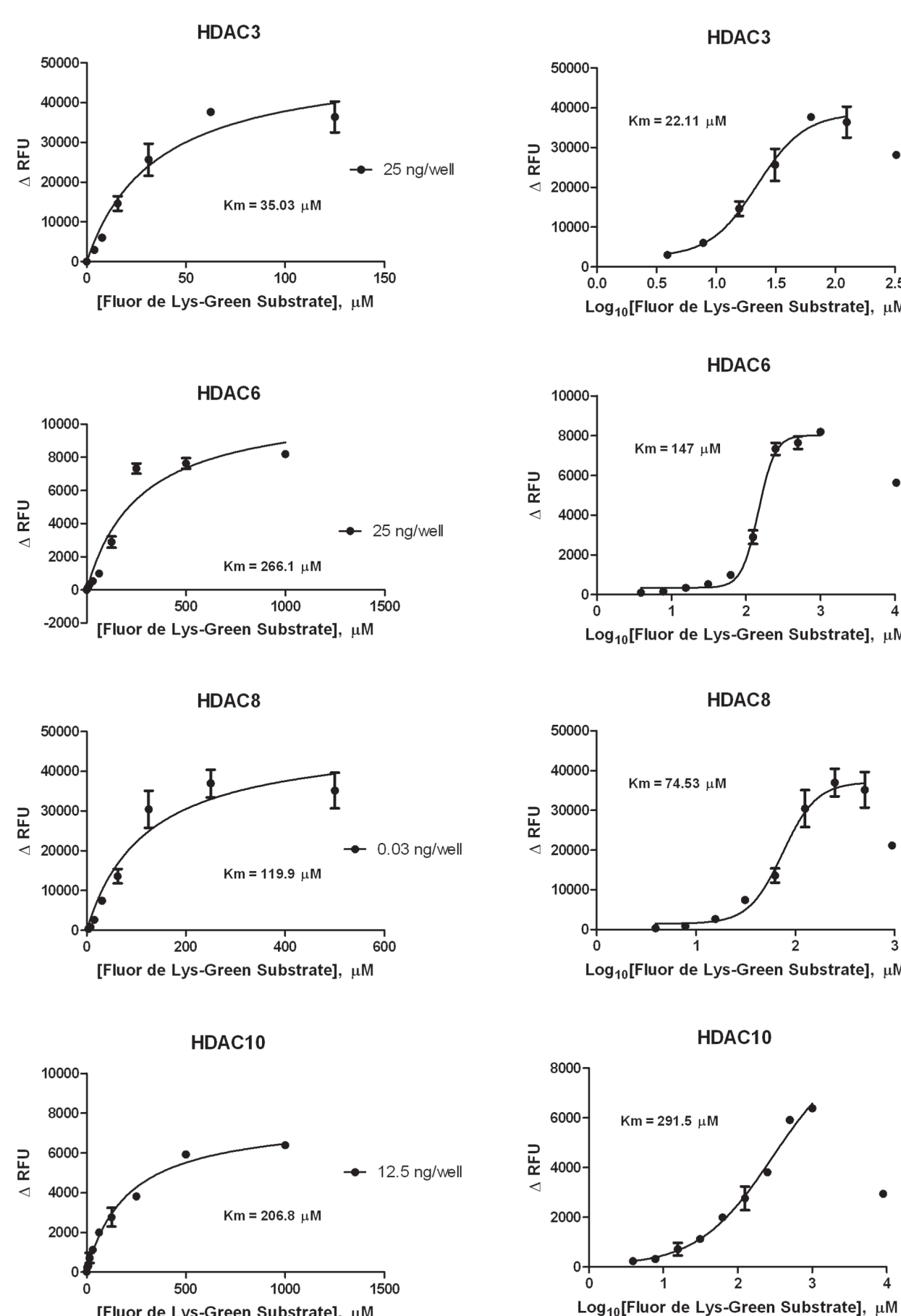


Figure 4 – The data show the increase in fluorescence in relation to changes in substrate concentration. A fit of the data to the Michaelis-Menten equation is shown on the left. The plot of ΔRFU vs. [Fluor de Lys®-Green] shows a slight sigmoidal appearance. Therefore the K_m value determined does not 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 ΔRFU vs. \log_{10} [Fluor de Lys®-Green] shows a more proper fit of the data, and yields a more true K_m value.

HDAC Enzyme Activity Confirmation

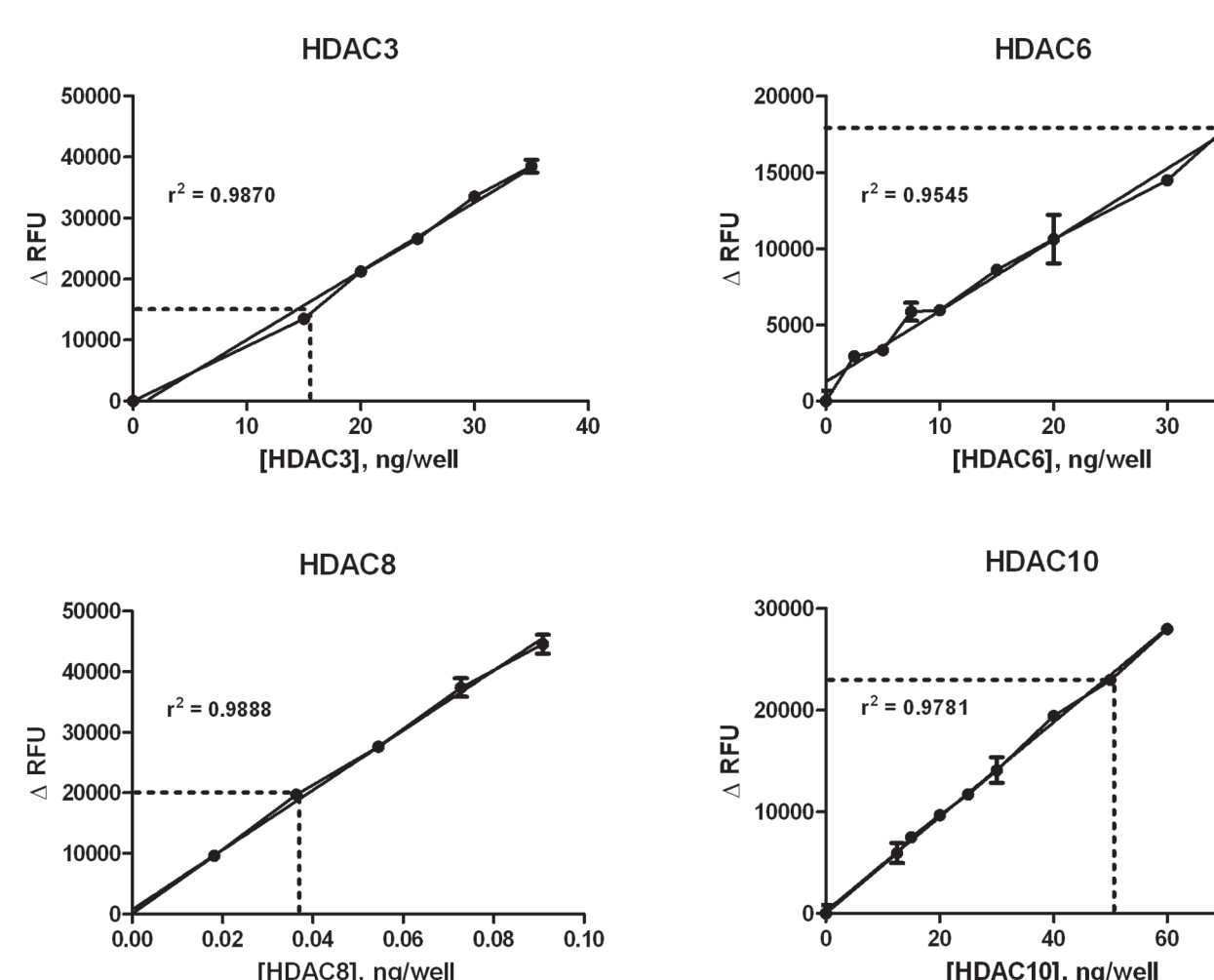


Figure 5 – The results show that a concentration of 15, 35, 0.03, and 50 ng/well yield a significant change in fluorescence for HDAC3, 6, 8, and 10, while still well within the linear range of the curve. This was further tested using a Z'-factor test to determine the robustness of the assay using the optimized conditions.

Optimized Automated 384-Well Assay Procedure

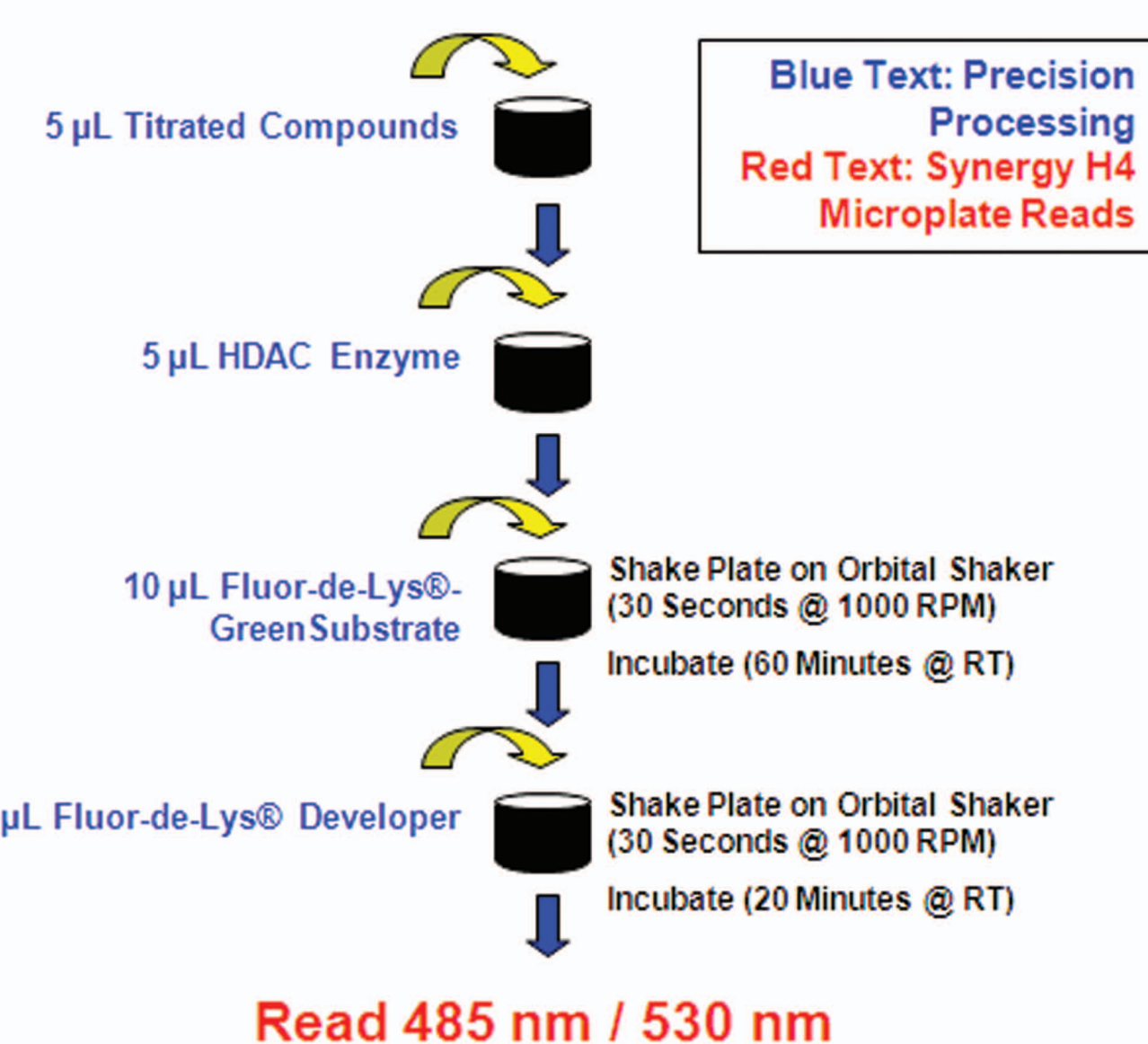


Figure 6 – Fluor de Lys®-Green HDAC 384-well Assay Workflow.

Automated HDAC Assay Z'-factor Validation

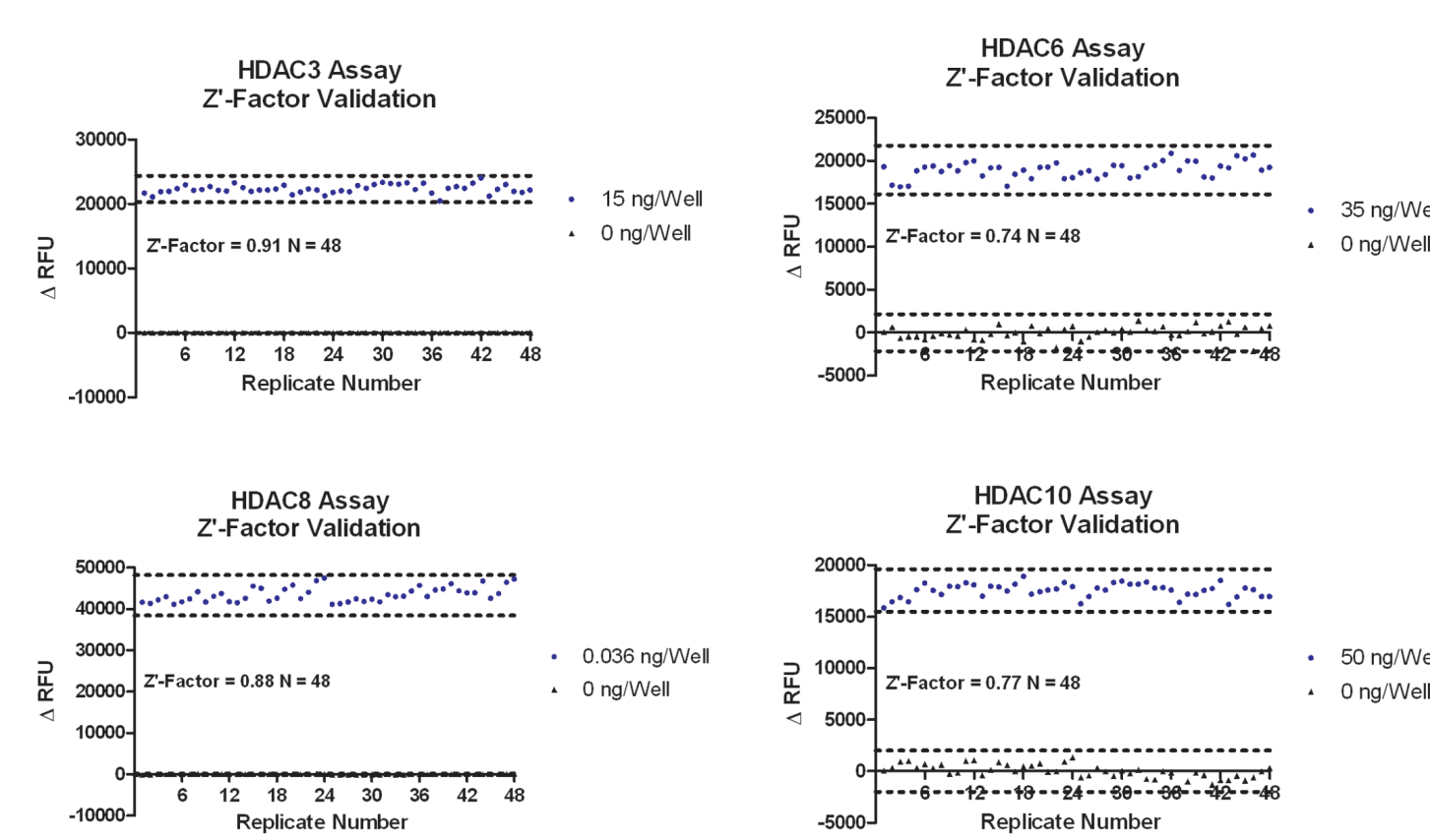


Figure 7 – Z'-factor validation results. Data shown for all replicates. Dashed lines represent the average * 3 standard deviations for each condition. A Z' value ≥ 0.5 is indicative of an excellent assay according to Zhang et al., 1999.

HDAC Inhibitor Pharmacology Validation

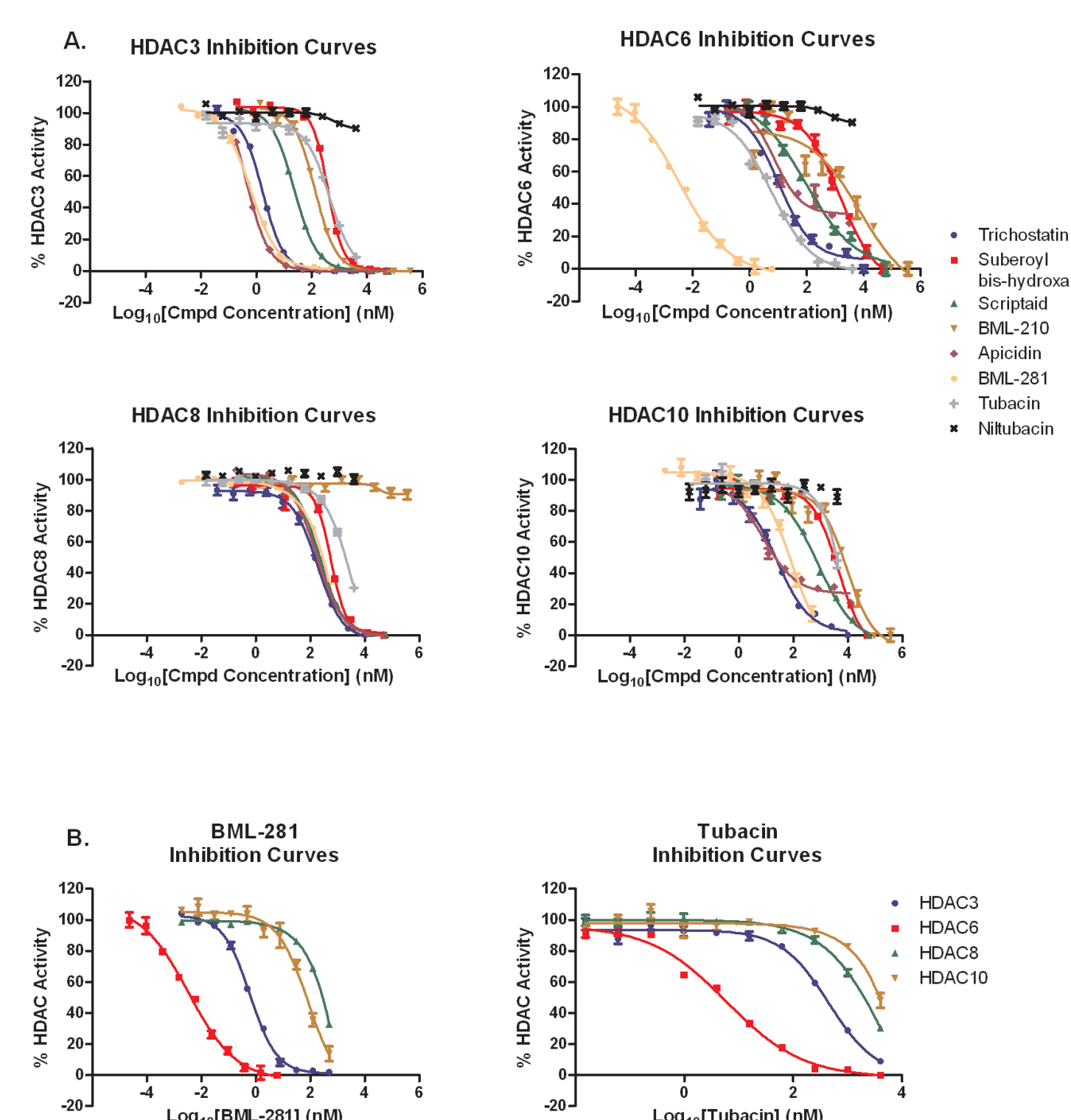


Figure 8 – Inhibitor validation data. A. Inhibition curves for all compounds with HDAC3, 6, 8, and 10. **B.** BML-281 and Tubacin inhibition curves demonstrating specificity for HDAC6.

	BML-281		Tubacin	
	Literature IC ₅₀ Value	Fluor de Lys®-Green HDAC Assay	Literature IC ₅₀ Value	Fluor de Lys®-Green HDAC Assay
HDAC3	73 nM ¹	0.61 nM	~4000 nM ³	450.1 nM
HDAC6	0.002 nM ²	0.003 nM	4 nM ⁴	5.9 nM
HDAC8	5-10 μM ¹	>500 nM	~4000 nM ³	>4000 μM
HDAC10	0.002 nM ¹	86.94 nM	~4000 nM ³	>4000 nM

¹Enzo Life Sciences Internal Unpublished Results; ²Kozikowski et al., 2008

³Butler et al., 2010; ⁴Wong et al., 2003

Table 1 – Inhibitor IC₅₀ Values.

The IC₅₀ values for BML-281 and Tubacin with HDAC6 compare favorably to literature IC₅₀ values or internal IC₅₀ values generated by Enzo Life Sciences for this enzyme. Discrepancies in IC₅₀ values with HDAC3 can be explained by the concentration and type of substrate used in the experiment. Substrate concentrations above the K_m value can lead to right shifted IC₅₀ values.

Conclusions

- The sensitivity of the Synergy H4 and *Fluor de Lys®-Green* assay allow for assay miniaturization to 384-well format
- Emission at the *green* end of the spectrum, as seen with the assay demonstrated here, avoids interference at shorter wavelengths often exhibited by screening compounds, and by substances commonly found in biological samples and tissue culture medium
- Compound titration, transfer, and reagent dispense is easily accomplished using the Precision Microplate Pipetting System
- Excellent assay robustness, using low concentrations of enzyme and substrate, is demonstrated with Z'-factor values >0.74
- Profiling of lead compounds against multiple HDAC enzymes can be easily carried out using the automated *Fluor de Lys®-Green* assay