

# Automated Compound Profiling Applications for the Assessment of Lead Compound Off-target Effects



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## Overview

Profiling lead compounds against other enzymes within the same target class has become increasingly important in the drug discovery process. Drugs must demonstrate specificity for the target enzyme, or run the risk of generating adverse health effects among patients. This process involves generating IC<sub>50</sub> or EC<sub>50</sub> values with a variety of enzymes in order to determine specificity.

Here we demonstrate profiling applications for two important drug target classes, kinases and histone deacetylases, using an 8-channel liquid handler and hybrid multi-mode microplate reader. Assay optimization and profiling data demonstrate the validity of each automated process.

## Introduction

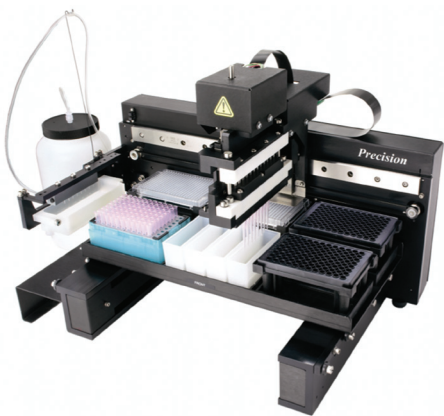
Lead compounds, identified during hit and hit to lead screening, are normally profiled to determine selectivity for the drug target. Compounds demonstrating inhibitory effects to other non-target enzymes may then be sent back for modification or be removed from further testing. The procedure typically involves performing the test assay with a dilution series of the compound in order to ascertain the IC<sub>50</sub> or EC<sub>50</sub> potency. Therefore, a separate set of instrumentation is required from that used for primary screening. Liquid handling must be able to serially titrate compounds, as well as dispense multiple assay reagents to high density plates.

Here we demonstrate the ability to perform automated compound profiling applications for two separate drug target families, known for selectivity issues. Kinases are one of the most diverse and highly studied enzyme families. It is becoming increasingly important for compounds to be profiled against other kinases due to the non-specificity of ATP competitive inhibitors. In this application, we used a luminescent assay chemistry which is able to detect small changes in ATP consumption from kinases having large ranges of ATP<sub>K<sub>m</sub> app</sub> values. A single set of reagents is used for all kinase/substrate combinations.

Histone deacetylases (HDACs) are an emerging drug target family. Histone hyperacetylation correlates with an open, decondensed chromatin structure and gene activation, while hypoacetylation correlates with chromatin condensation and transcriptional repression. Due to their importance in gene regulation, HDACs are becoming an increasingly accepted target for cancer therapy. Here we incorporated a green-emitting fluorescent assay which is capable of detecting the activity and inhibition of multiple HDAC enzymes using the same set of reagents.

Assay optimization and pharmacology data are demonstrated, and confirm the ability to generate compound profiling data in an automated fashion.

## 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.



**Figure 1 – B.** Synergy H4 with Hybrid Technology™ is a patent pending multi-mode detector system designed to combine flexibility and performance. 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.

## Assay Description

**Fluor de Lys®-Green HDAC 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.

**ADP-Glo™ Kinase Assay** – The ADP-Glo assay is based upon a single set of reagents capable of being used with any kinase/substrate combination. The assay is performed in two steps. After the kinase or ATPase reaction, ADP-Glo™ Reagent is added to terminate the kinase reaction and deplete the remaining ATP. The Kinase Detection Reagent is then added to convert ADP to ATP and allow the newly synthesized ATP to be measured using a luciferase/luciferin reaction. The light generated correlates to ADP present and kinase or ATPase activity.

## Optimized Automated Fluor de Lys®-Green 384-Well Assay Procedure

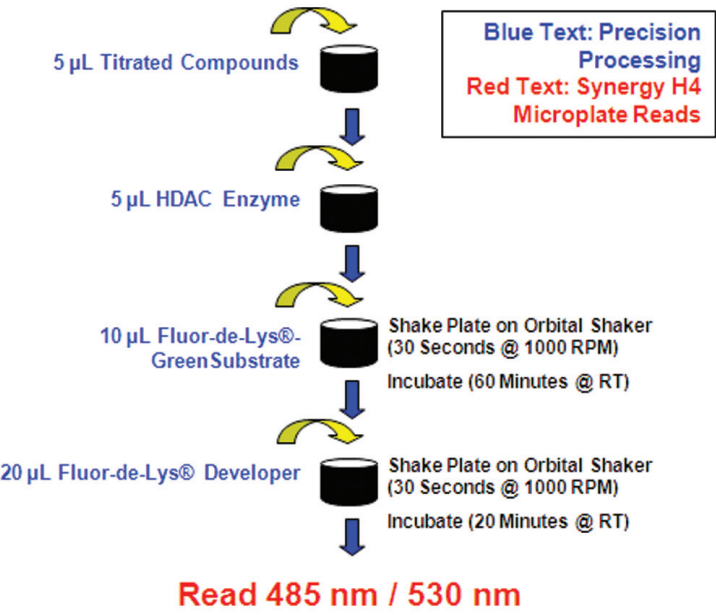


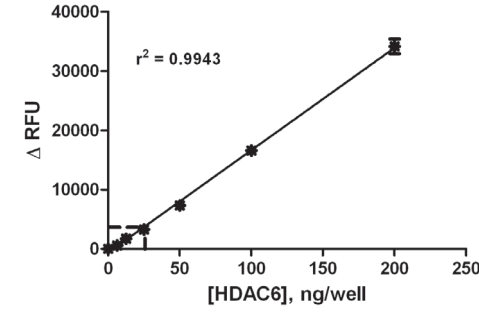
Figure 2 – Fluor de Lys®-Green HDAC 384-well Assay Workflow.

## Fluor de Lys®-Green Assay Optimization

Assay optimization was carried out before compound profiling took place. HDAC3, 6, 8, and 10 assays were optimized. Results for HDAC6 shown here.

### Enzyme Titration

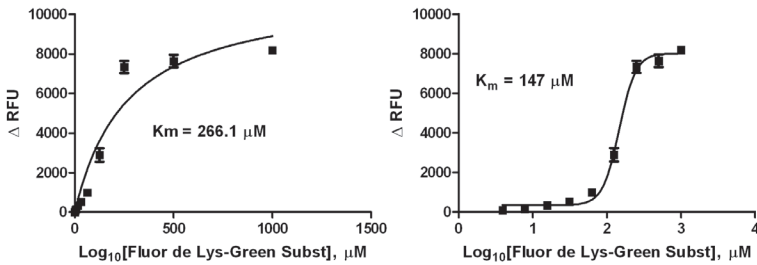
Enzyme titrations were performed to demonstrate the ability of the Precision to properly dilute the HDAC enzymes. Enzymes were serially titrated using a 1:2 dilution scheme, and then added to the assay plate in triplicate. Fluor de Lys®-Green substrate was then added at a 2X concentration of 20 µM to 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.



**Figure 3 – Representative HDAC Enzyme Titration Curve.** The results demonstrate the titration ability of the Precision, as well as 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.

### Substrate Titration

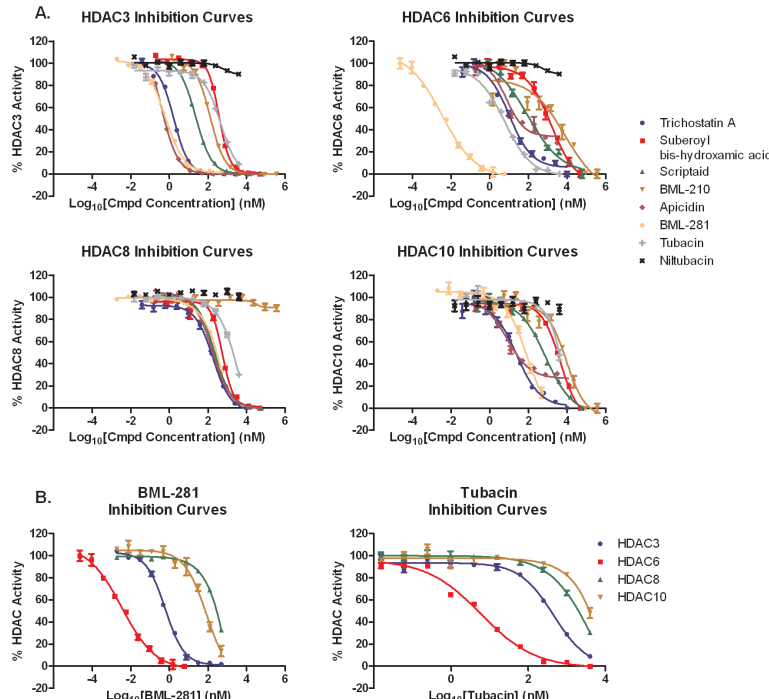
Fluor de Lys®-Green substrate was titrated to determine the substrate  $K_m$  with each HDAC enzyme. Substrate was serially titrated using a 1:2 dilution scheme creating final 1X concentrations ranging from 1000-0 µM. Enzyme was added to the assay plate at a concentration of 25 ng/well for HDAC6. The titrated substrate was then added to the assay plate in triplicate. A 60 minute substrate incubation time was used, in addition to a 20 minute incubation following the Fluor de Lys® Developer addition.



**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 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 more proper fit of the data, and yields a more true  $K_m$  value.**

## HDAC Profiling

Inhibitor dose response curves were generated using Trichostatin A, Scriptaid, Apicidin, bis-hydroxamic acid, and BML-210. Also included were the HDAC6 specific inhibitors BML-281 and Tubacin, with Niltubacin as the inactive control. All inhibitors were run with each enzyme to determine the specificity of the inhibitors for each enzyme.



**Figure 5 – 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.

## HDAC Inhibitor Pharmacology Validation

	BML-281		Tubacin	
	Literature IC <sub>50</sub> Value	Fluor de Lys®-Green HDAC Assay	Literature IC <sub>50</sub> Value	Fluor de Lys®-Gr HDAC Assay
HDAC3	0.42 nM <sup>1</sup>	0.61 nM	~4000 nM <sup>3</sup>	450.1 nM
HDAC6	0.002 nM <sup>2</sup>	0.003 nM	4 nM <sup>1</sup>	5.9 nM
HDAC8	5-10 µM <sup>1</sup>	>500 nM	~4000 nM <sup>3</sup>	>4000 µM
HDAC10	90.7 nM <sup>1</sup>	86.94 nM	~4000 nM <sup>3</sup>	>4000 nM

<sup>1</sup>Enzo Life Sciences Internal Unpublished Results; <sup>2</sup>Kozlowski et al., 2008

<sup>3</sup>Butler et al., 2010; <sup>4</sup>Wong et al., 2003

Table 1 – Inhibitor IC<sub>50</sub> Values.

The IC<sub>50</sub> values for BML-281 and Tubacin with HDAC6 compare favorably to literature IC<sub>50</sub> values or internal IC<sub>50</sub> values generated by Enzo Life Sciences for this enzyme.

## Optimized Automated ADP-Glo 384-Well Assay Procedure

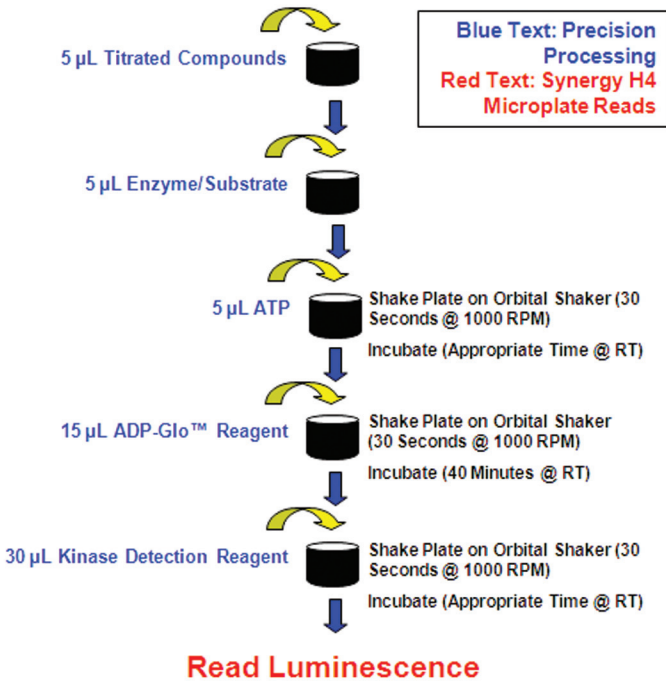


Figure 6 – ADP-Glo 384-well Kinase Assay Workflow.

## ADP-Glo Assay Optimization

Assay optimization was carried out for multiple kinases before profiling took place. Results for Src kinase shown here.

### ATP Titration

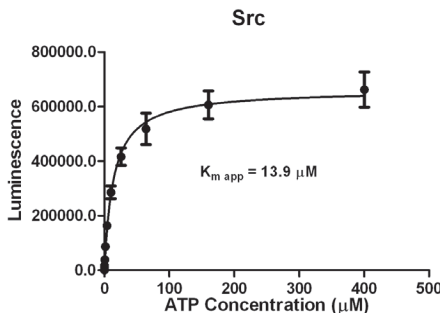


Figure 7 – ATP titration for Src kinase.

ATP titrations were performed to determine the ATP  $K_{m app}$ . ATP was serially titrated using a 1:2.5 dilution scheme, and then transferred to the plate. Enzyme was then added at a concentration of 10 ng/rxn in saturating substrate. The  $K_{m app}$  was determined to be 13.9 µM using the Michaelis-Menten equation.

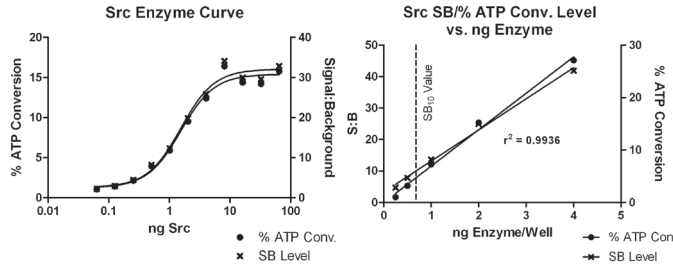


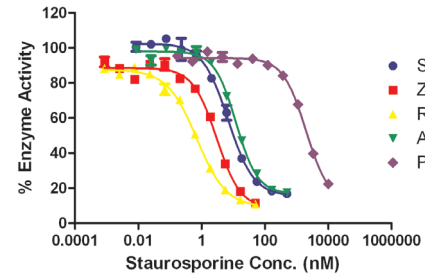
Figure 8 – Enzyme titration and confirmation graphs for Src kinase.

Once  $K_{m app}$  values are established, appropriate enzyme concentrations need to be determined. This is typically a balance of minimizing enzyme consumption and obtaining sufficient signal relative to background (SB) for adequate performance during profiling experiments. Enzyme titrations were performed in order to determine enzyme concentrations yielding SB levels between 5 and 20. Standard curves were also included to determine % ATP conversion. Confirmation of SB level at various individual enzyme concentrations was then performed, and the SB<sub>10</sub> concentration determined. The SB<sub>10</sub> enzyme concentration was determined to be 0.68 ng/rxn, which represents 4.71% ATP conversion.

## Kinase Profiling

### Universal Kinase Inhibitor

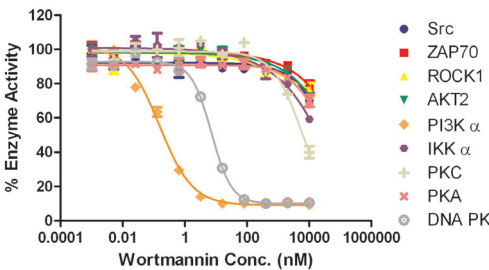
Staurosporine is a potent, but non-selective kinase inhibitor due to the fact that it directly binds to ATP-binding sites which are largely conserved through the kinase. Thus it is a useful tool to perform an initial assessment of pharmacology for kinase assays.



**Figure 9 – Results were as expected, as IC<sub>50</sub> values for Src, ZAP70, ROCK1, and AKT2 were all below 15 nM. PI3 Kinase Alpha, being a lipid kinase, reacts differently with staurosporine and will possess IC<sub>50</sub> values generally orders of magnitude higher than for kinases with non-lipid substrates.**

### PI3 Kinase Specific Inhibitor

The optimized kinase assays were profiled using Wortmannin as the model lead compound of interest. This compound, being a specific inhibitor for PI3 Kinase, generates IC<sub>50</sub> values typically in the low nM range for this kinase. At higher concentrations, this compound has also shown inhibitory effects on DNA PK.



**Figure 10 – Results once again agreed with expected results, with IC<sub>50</sub>s for PI3 Kinase Alpha and DNA PK being 0.15 and 7.1 nM, respectively. Wortmannin showed little or no effect on the remaining protein Tyrosine or Serine/Threonine Kinases.**

## Conclusions

1. Compound titration, transfer, and reagent dispense with the Precision Microplate Pipetting System is suitable for the automation of dose-response curves
2. The Synergy H4 is able to quantify the luminescent and fluorescent signals from each assay chemistry
3. Each assay chemistry is able to be run with multiple enzyme/substrate combinations with a single set of reagents
4. The combination of assay and automation create simple, yet robust solutions for performing profiling applications of lead compounds