

# Characterization of HDAC Substrates and Inhibitors using a Microfluidic Mobility-Shift Assay

Laurel M. Provencher, Ph.D., Abbie L. Esterman, Ph.D., Seth P. Cohen Ph.D.,  
Caliper Life Sciences, Hopkinton, MA USA

## Abstract

Modulation of histone deacetylase (HDAC) activity has emerged as a viable strategy for the treatment of various conditions including neurodegenerative diseases and cancers. We present an assay for identification and characterization of HDAC substrates and inhibitors using a microfluidic mobility-shift format. Three fluorescent peptides representing sequences derived from Histone 3, Histone 4, or p53 were designed as HDAC substrates. High quality ratiometric data was generated in both endpoint and kinetic mode by electrophoretic separation of acetylated peptide substrates from deacetylated products. Enzymes from three HDAC classes were tested for substrate specificity by comparing real-time deacetylation rates for each enzyme against the different peptide targets. Using the preferred substrate for each HDAC,  $IC_{50}$  values for known HDAC inhibitors were determined. One enzyme/inhibitor pair, HDAC6 and Trichostatin A (TSA) was used to demonstrate the utility of the assay for mechanism of inhibition studies. Analysis of reaction progress curves showed time-dependent inhibition consistent with a single-step, reversible, non-competitive mode of action.

## Materials and Methods

Kinetic reactions were assembled in low volume microtiter plate wells by adding 1  $\mu$ L DMSO/31X compound, 15  $\mu$ L 2X Enzyme (and NAD+ for Sirtuins) in reaction buffer and 15  $\mu$ L 2X Peptide in reaction buffer. Plates were immediately placed into the EZ Reader where the wells were sampled periodically throughout the timecourse of the experiment.

Stopped reactions, for inhibitor titrations, were assembled in high volume microtiter plate wells by adding 1  $\mu$ L inhibitor in DMSO, 15  $\mu$ L 2X Enzyme (and NAD+ for Sirtuins) in reaction buffer and 15  $\mu$ L 2X Peptide in reaction buffer. Reactions were incubated at room temperature for 1 hour, 45  $\mu$ L Stop Solution was added, and plates were read in the EZ Reader.

Percent conversion and/or percent inhibition values were calculated by Caliper's Reviewer software. Curve fitting and non-linear regression analysis was done in GraphPad Prism 5.



Reaction Buffer	Stop Buffer
25 mM Tris/Cl, pH 8.0	100 mM HEPES, pH 7.5
137 mM NaCl	10 mM EDTA (disodium salt)
2.7 mM KCl	0.015% Brij-35
1 mM MgCl <sub>2</sub>	0.1% CR-3
0.1 mg/ml BSA (fatty acid free)	2.5 $\mu$ M Trichostatin A (HDAC3 and 6)
0.5 mM NAD <sup>+</sup> (SIRT1 only)	5 $\mu$ M Suramin (SIRT3)
1.25 mM NAD <sup>+</sup> (SIRT3 only)	
Separation Buffer	Separation Conditions
100 mM HEPES, pH 7.5	Upstream Voltage -500 V
10 mM EDTA (disodium salt)	Downstream Voltage -2000 V
0.015% Brij-35	Pressure -1.3 psi
0.1% CR-3	Sample Sip Time 0.2 sec
	Buffer Sip Time 30 sec

Enzyme	Source	Catalog #	Lot #
HDAC3	BPS	50003	1004
HDAC5	BPS	50005	1006
HDAC6	BPS	50006	1004
HDAC8	BPS	50008	1002
SIRT1	BioMol	SE-239	T5667
SIRT2	BioMol	SE-251	T5668
SIRT3	BioMol	SE-270	T5495

Inhibitor	Source	Catalog #
Trichostatin A	BioMol	GR-309
Oxamflatin	Sigma	O3139
Suramin	BioMol	G-430
Valproic Acid	Sigma	P4543

Peptide	Source	Catalog #
H212 (Histone 3)	Caliper	760511
H218 (p-53)	Caliper	760512
H219 (Histone 4)	Caliper	760513

## Results

### Caliper's Fluorescent HDAC Substrates

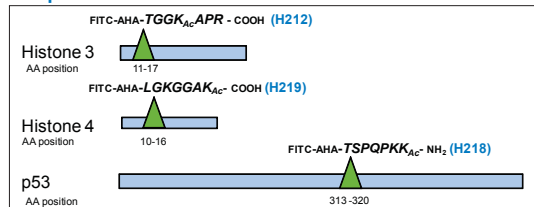


Figure 1. Peptide amino acid (AA) position on target protein and peptide sequence used for HDAC assay development.

### Representative Data Tracings

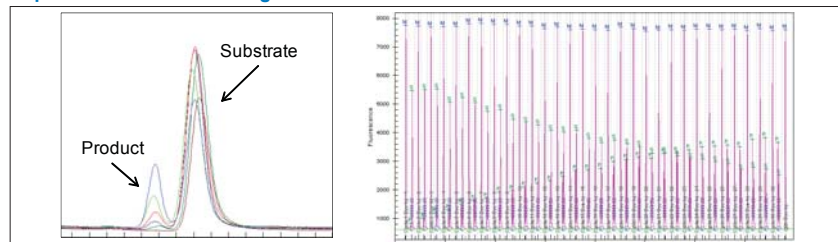


Figure 2. Removal of acetyl groups from peptide substrates produces distinct mobility shifts. Reactions containing 1  $\mu$ M peptide H219 and increasing concentrations of HDAC6 were assembled and read in the EZ Reader in kinetic mode. A. Tracings from 6 independent channels showing enzyme dependent appearance of deacetylated product peaks. Each color represents data from a different reaction well. B. Sequential slips from a single reaction well showing the consumption of acetylated peptide substrate (S) and the accumulation of deacetylated peptide product (P) over time.

### Substrate Selectivity of HDACs from Different Classes

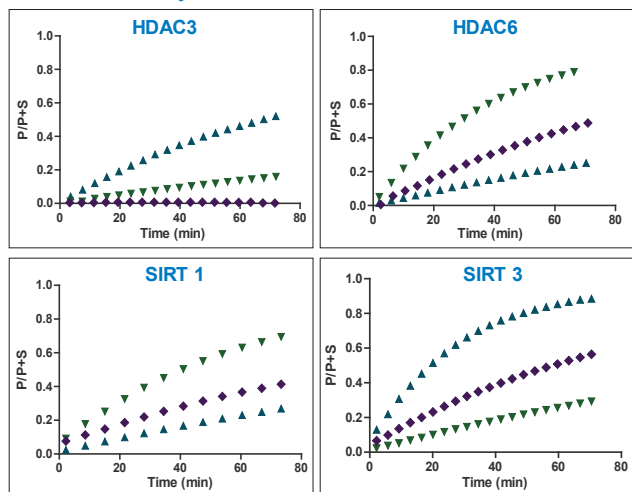


Figure 3. Activity of HDACs representing class I, II, and III were tested against a panel of three different acetylated substrates, representing acetylated regions of P53, Histone H3 and Histone H4. Reactions were assembled in duplicate and read in the EZ Reader in kinetic mode. Differences in activity were noted in each case, as summarized in Table 1.

### HDAC Substrate Selectivity

Enzyme	Peptide		
	H218	H212	H219
Class I	HDAC3	2.51	0.63
	HDAC8	0.025	0.047
Class II	HDAC5	0.009	0.26
	HDAC6	0.052	0.10
Class III (Sirtuins)	SIRT1	0.59	2.12
	SIRT2	1.09	0.41
	SIRT3	10.3	1.74

Table 1. Values represent enzyme activity in nmol product formed/min/mg enzyme. Yellow shading highlights enzyme-substrate pairs chosen for assay development.

## Results (cont)

### Effect of HDAC Specific Inhibitors

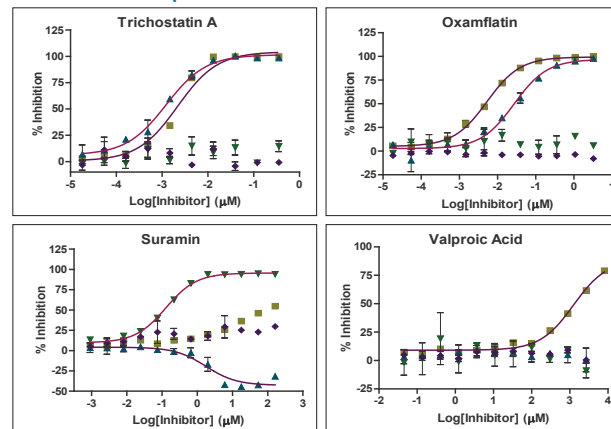


Figure 4. Stopped reactions were assembled in microtiter plate wells by adding 1  $\mu$ L inhibitor in DMSO, 15  $\mu$ L 2X Enzyme, and 15  $\mu$ L 2X Peptide. Reactions were incubated for 1 hour at RT, stopped with the addition of 45  $\mu$ L Stop Solution, and read on the EZ Reader. The curves were fit with sigmoidal dose-response algorithm using GraphPad Prism, producing the  $IC_{50}$  values shown in Table 2.

### EC<sub>50</sub> Values ( $\mu$ M) for HDAC Inhibitors

Enzyme	TrichostatinA	Oxamflatin	Suramin	Valproic Acid
HDAC3	0.0022	0.0053	--	>1mM
HDAC6	0.0012	0.027	1.75**	--
SIRT1	--	--	0.128	--
SIRT3	--	--	--	--

Table 2. Relative potency of HDAC inhibitors with enzymes from 3 different classes. Two independent inhibitor titrations were performed in duplicate, as described in figure 6. \*\*Suramin showed inhibition of SIRT1 and reproducibly activated HDAC6.

### HDAC6 Substrate $K_m$ Determination

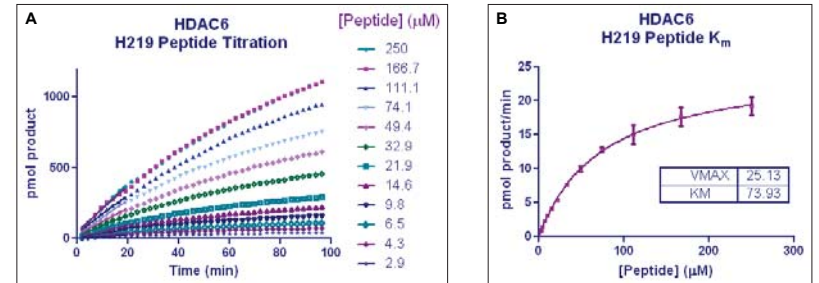


Figure 5. Kinetic reactions were run with 10 nM HDAC6 and varying concentrations of H219 peptide substrate. Initial rates were determined from the linear portions of reaction progress curves (A) and plotted against peptide concentration for determination of substrate  $K_m$  (B).

### Mechanism of Action for TSA Binding to HDAC6

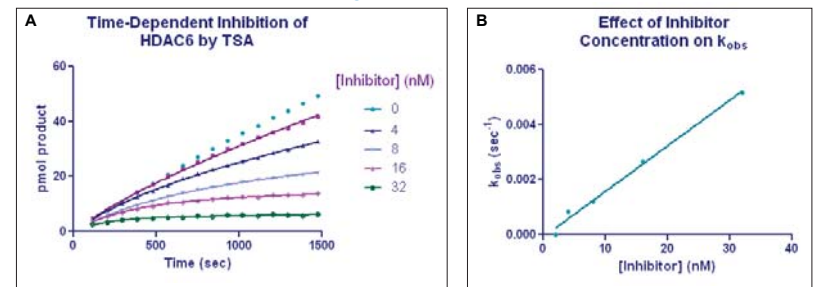


Figure 6. Characterization of slow-binding inhibition of HDAC6 by Trichostatin A. Progress curves from reactions initiated by the addition of 15  $\mu$ L 2X enzyme to wells containing pre-mixed 2X inhibitor and substrate (A). Values for  $k_{obs}$  were determined by non-linear regression analysis in GraphPad Prism using the equation shown (C). Plotting  $k_{obs}$  vs. inhibitor concentration shows a linear relationship (B), consistent with a single-step, reversible, slow-binding inhibition mechanism.

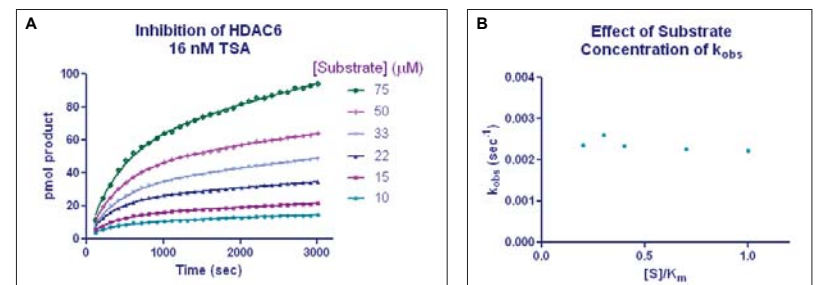


Figure 7. Non-Competitive Inhibition of HDAC6 by Trichostatin A. Progress curves from reactions initiated by the addition of 15  $\mu$ L 2X enzyme to wells containing pre-mixed 2X inhibitor and substrate (A). Values for  $k_{obs}$  were determined by non-linear regression analysis in GraphPad Prism using the equation describing slow-binding inhibition (Figure 6 C). At a fixed concentration of inhibitor (16 nM) and enzyme (10 nM), increasing substrate concentration did not change the value for  $k_{obs}$  (B). This data is consistent with a non-competitive mode of inhibition.

## Summary

- Peptides representing different biologically relevant HDAC targets (histone 3, histone 4 and p53) were designed and tested on Caliper's microfluidic mobility-shift platform.
- HDAC substrate selectivity, based on relative conversion rates, was determined for HDACs representing class I, II, and III.
- Four known HDAC inhibitors with different ranges of potency (trichostatin A, oxamflatin, suramin and valproic acid) were tested and showed  $IC_{50}$ s similar to published values.
- Real-time kinetics capability enabled rapid assay development and characterization of the mechanism of trichostatin A inhibition of HDAC6 activity.
- Caliper's platform provides a flexible assay format that can be adapted for peptide substrate profiling and inhibitor characterization with multiple classes of HDACs.