Ultrafast Histone Deacetylase Selectivity Screening Using the Agilent RapidFire High-Throughput Mass Spectrometry System

Application Note

Authors

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Abstract

Selectivity screening of a promising new compound requires testing against a host of enzymes related to the target of interest, resulting in the analysis of a large number of samples. Mass spectrometry offers robust, dependable data, and is less prone to artifacts than optical screening methods; however, the utility of MS can be limited by the comparatively low throughput necessitated by the slow, serial desalting step required. The Agilent RapidFire High-Throughput Mass Spectrometry System addresses this bottleneck by employing SPE upstream of MS detection, allowing analysis speeds of approximately 8–15 seconds per well. Multiplexed analysis of reactions can increase the speed of data acquisition even further. Here, full biological assays were developed for four histone deacetylases, which were then used in selectivity screening of two example inhibitors. Multiplexed data were acquired at an effective rate of approximately 3 seconds per sample, with no loss of data quality or modification of results as compared to singleton analysis. This increased effective analysis rate enables the acquisition of MS-quality selectivity screening data at speeds comparable to optical methods.

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Introduction

Selectivity screening is a vital step in the drug discovery and development process that weeds out compounds that have undesirable off-target effects. Eliminating these compounds from the pipeline as quickly as possible saves both time and money. Mass spectrometry (MS) delivers reliable data using label-free substrates, and is less susceptible to interfering artifacts (for example, autofluorescence) than optical screening techniques. However, the utility of MS can be limited by the comparatively low throughput necessitated by the slow, serial desalting step required. The Agilent RapidFire High-Throughput Mass Spectrometry System addresses this bottleneck by replacing traditional chromatography with an online solid phase extraction (SPE) step upstream of MS analysis, allowing analysis speeds of approximately 8–15 seconds per well, which approaches the timescale offered by optical methods. Multiplexed analysis of reactions can increase this data acquisition speed even further, in this example to an effective rate of approximately 3 seconds per sample, which is comparable to optical methods. This increased analysis rate opens up the possibility of generating approximately 10,000 MS data points in one 8 hour shift with no loss of data quality or alteration of results, and enables the acquisition of MS-quality selectivity screen data without sacrificing speed.

Here we demonstrate the application of this approach to selectivity screening of four histone deacetylases (SIRT1, SIRT3, HDAC1, and HDAC6), and find that the data acquired in multiplex (at four data points in approximately 12 seconds) are statistically indistinguishable from those acquired in singleton (at approximately 12 seconds per sample). The best-fit K_m and IC_{50} values acquired in multiplex agree well within 2-fold with the values derived from the individual data, and all multiplex values fall within the 95 % confidence interval calculated for the values acquired individually for all four enzymes.

Experimental

Chemicals and reagents

Enzymes were purchased from Enzo Life Sciences (Farmingdale, NY). The SIRT1, HDAC1, and HDAC6 peptide substrates were from Anaspec (Fremont, CA), while the SIRT3 peptide substrate was from EMD Millipore (Billerica, MA). All reference inhibitors, buffers, HPLC-grade solvents, and other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO).

Assay setup

Biochemical assays, including enzyme linearity, substrate K_m, and reference inhibition experiments, were developed for four histone deacetylases, SIRT1, SIRT3, HDAC1, and HDAC6. Acetylated peptide substrates representing a portion of p53 (SIRT1, HDAC1, and HDAC6) or histone 4 (SIRT3) were chosen as in Table 1. Reactions were carried out at room temperature in 50 mM Tris pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.05 % BSA, with 250 µM NAD^+ added for SIRT reactions, and quenched with a final concentration of 1 % formic acid. Reference inhibitors were suramin sodium (SIRT1), nicotinamide (SIRT3), valproic acid (HDAC1), and tubacin (HDAC6).

Analysis conditions

Samples were analyzed as is (individual) or post-reaction pooled (multiplex) on an Agilent RapidFire High-Throughput Mass Spectrometry System consisting of an Agilent RapidFire 365 coupled to an Agilent 6490 iFunnel Triple Quadrupole MS running Agilent MassHunter Acquisition Sofware (B.07.00). The RapidFire conditions are outlined in Table 2. Eight transitions were monitored in an MS method detailed in Tables 3 and 4. Data were analyzed using Agilent MassHunter Qualitative Analysis Sofware (B.05.00), Microsoft Excel 2010, RapidFire Integrator v4.0 and GraphPad Prism 5.0. All experiments were carried out in triplicate, and error bars represent one standard deviation.

Table 1. Peptide substrates used for each enzymatic assay.

Enzyme	Peptide substrate	Molecular weight
SIRT1	Ac-GQSTSRHK-K(Ac)-LMFKTEG-NH2	1,918
HDAC1	Biotin-LC-KKGQSTSRHK-K(Ac)-LMFKTEG-NH2	2,472
HDAC6	KKGQSTSRHK-K(Ac)-LMFKTEG	2,133.5
SIRT3	SGRGKGGKGLGKGGA-K(Ac)-RHRC	2,009.5

Table 2. Agilent RapidFire method parameters.

Agilent RapidFire conditions	
Cartridge	A (C4, G9203A)
Buffer A (pump 1)	ddH_2O + 0.1 % formic acid
Buffer B (pumps 2 and 3)	70 % acetonitrile + 0.1 % formic acid
State timings	1) 600 2) 3,000 3) 8,000 4) 500
Pump flow rates	1) 1.5 mL/min 2) 1.25 mL/min 3) 0.6 mL/min

Table 3. Agilent 6490 iFunnel Triple Quadrupole parameters.

Agilent 6490 iFunnel Triple Quadrupole conditions, electrospray, positive ionization	
Drying gas	17 L/min, 290 °C
Sheath gas	12 L/min, 400 °C
Nebulizer	45 V
Capillary	4,000 V
Nozzle	2,000 V

Table 4. MRM transitions.

Compound ID	Q1	Res	Q3	Res	Dwell	CE	CAV	Polarity
HDAC6 sub	427.6	unit	424	unit	20	9	3	pos
HDAC6 prod	419.1	unit	415.5	unit	20	9	3	pos
SIRT1 sub	480.4	unit	564.4	unit	20	12	2	pos
SIRT1 prod	469.9	unit	550.3	unit	20	12	2	pos
HDAC1 sub	495.3	unit	226.9	unit	20	25	3	pos
HDAC1 prod	608.4	unit	226.9	unit	20	25	3	pos
SIRT3 sub	340.0	unit	84	unit	20	42	3	pos
SIRT3 prod	333	unit	84	unit	20	42	3	pos

Results and Discussion

Substrate K_m curves

Timecourses through 2 hours (SIRTs) or 4 hours (HDACs) at various substrate concentrations were analyzed individually or in multiplex and found to be linear (data not shown). The initial velocities were plotted against substrate concentration (Figure 1) to allow for the calculation of a K_m value. All best-fit K_m value pairs matched well within 2-fold (Table 5), and all multiplex values fell within the 95 % confidence interval generated from the individual values, indicating that they are statistically indistinguishable.

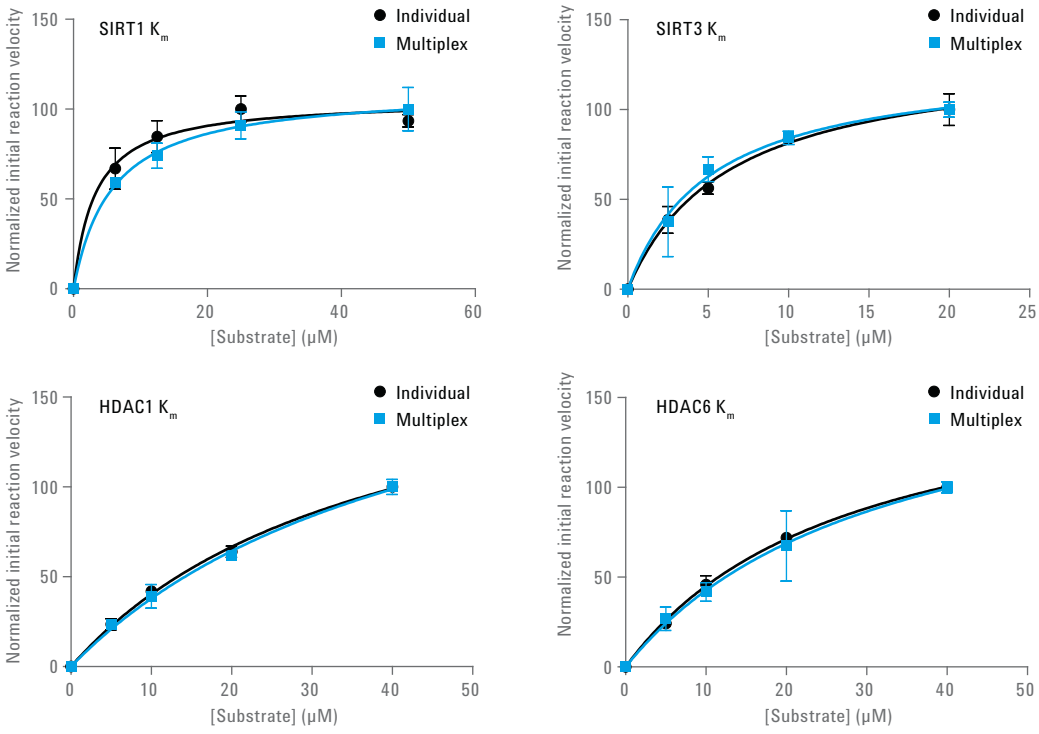

Figure 1. K_m curves analyzed individually or in multiplex are almost superimposable.

Table 5. K_m values fit from the individual and multiplex analyses agree to well within 2-fold and all multiplex values lie within the 95 % confidence interval calculated for the individual reads.

K_m (µM)	SIRT1	SIRT3	HDAC1	HDAC6
Individual	3.3	6.3	38.0	27.7
Multiplex	5.8	5.1	46.2	32.0

Reference inhibition curves

Inhibition curves using a different inhibitor for each enzyme and substrate concentrations of 5 µM (SIRTs), or 10 µM (HDACs) were analyzed individually or in multiplex (Figure 2). All best-fit IC_{50} value pairs matched well within 2-fold (Table 6), and all multiplex values fell within the 95 % confidence interval generated from the individual values, indicating that they are statistically indistinguishable.

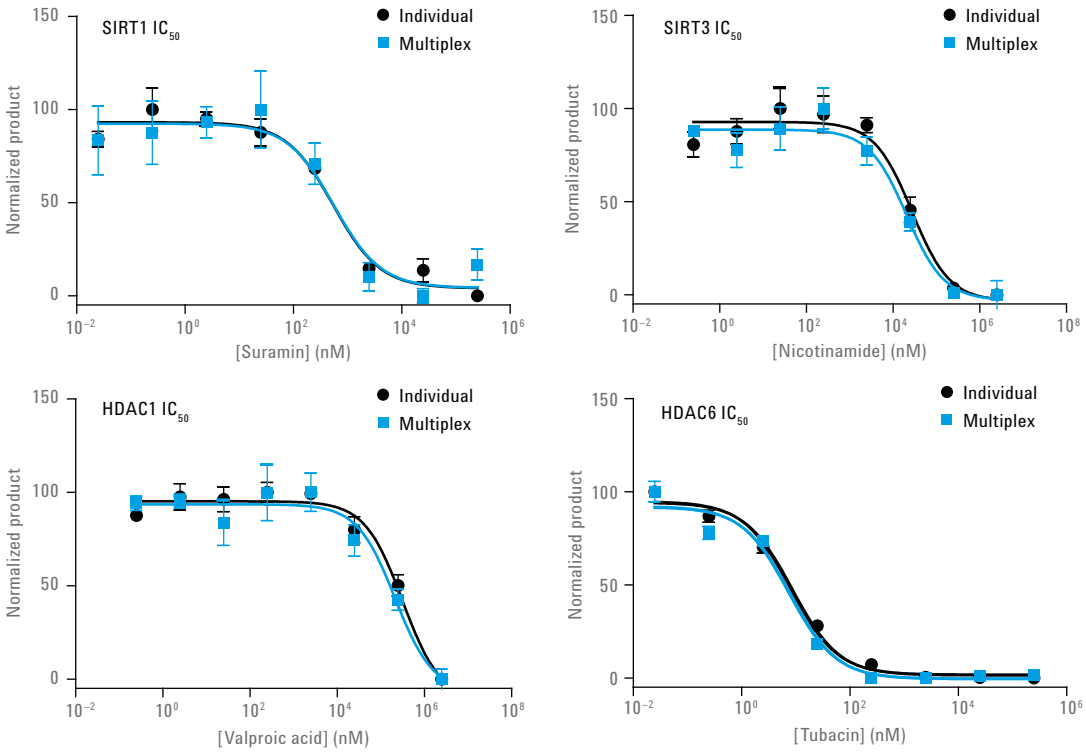

Figure 2. Inhibition curves analyzed individually and in multiplex are almost superimposable.

Table 6. IC_{50} values fit from the individual and multiplex analyses agree to well within 2-fold and all multiplex values lie within the 95 % confidence interval calculated for the individual reads.

IC_{50} (nM)	SIRT1	SIRT3	HDAC1	HDAC6
Individual	547	27354	316978	8.6
Multiplex	564	20534	212729	7.4

Isoform-specific inhibition

Inhibition curves using tubacin, an HDAC6-specific inhibitor[1], at eight concentrations in triplicate against all four enzymes were analyzed in multiplex (Figure 3). Error bars represent standard deviations of triplicate points, and, as expected, orders of magnitude differences in inhibition potency are observed among the four enzymes. The entire 96-point data set was acquired in less than 5 minutes.

Additionally, nicotinamide was tested at eight points in triplicate against all four enzymes, and the 96 samples were analyzed in multiplex in less than 5 minutes (Figure 4). As expected, inhibition curves could be fit to the data for the NAD-dependent SIRT1 and SIRT3, but not to the data for HDAC1 and HDAC6, which do not use NAD in their reaction mechanism.

Conclusions

Robust biochemical assays have been developed for four histone deacetylase enzymes acting on four different peptide substrates. Analysis of these samples by the Agilent RapidFire High-Throughput Mass Spectrometry System solves the time bottleneck associated with MS detection, allowing an analysis rate of approximately 12 seconds per well. Multiplexed analysis of deacetylation reactions by SPE/MS/MS allows an even further increase in data acquisition to an effective rate of 3 seconds per sample. This faster analysis does not affect the quality of the data, as statistically indistinguishable K_m and IC_{50} values were achieved in the multiplexed analysis as compared to the individual analysis. Additionally, isoform-selective inhibitors are easily detected, suggesting great utility of the method in selectivity screening. Multiplexed analysis of biochemical reactions using the RapidFire system allows approximately 10,000 data points to be generated in 8 hours, which is on par with the speed of optical methods, and allows rapid selectivity screening based on MS-quality data.

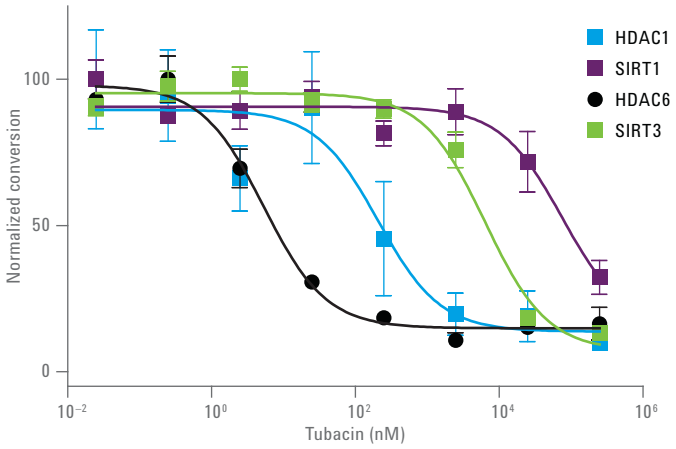

Figure 3. Isoform-selective inhibition by the HDAC6-specific inhibitor tubacin.

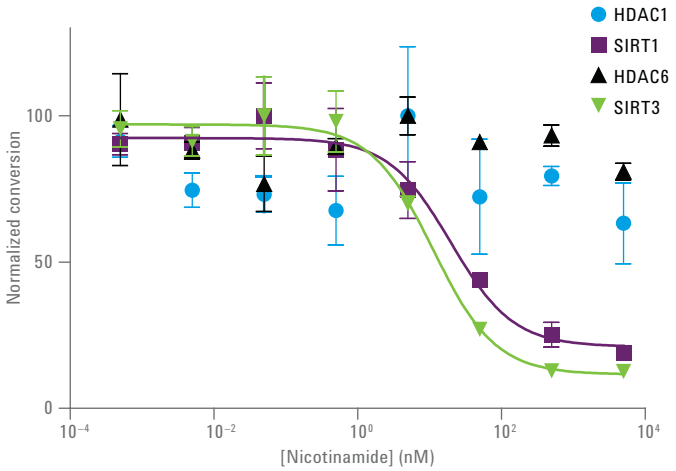

Figure 4. Inhibition of the NAD-dependent deacetylases SIRT1 and SIRT3, but not the NAD-independent deacetylases HDAC1 and HDAC6 by the NAD-competitive inhibitor nicotinamide.

Reference

1. Regna, N. L; Reilly, C. M. Isoform-Selective HDAC Inhibition in Autoimmune Disease. *J. Clin. Cell. Immunol*. **2014**, *5*:207.

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