Application Note

A Novel, Substrate-Agnostic Class III HDAC Activity Assay

Michael Sturges, Haizhen Liu, Lucas Armstrong, Merck Millipore
Basil Hubbard and David Sinclair, Paul F. Glenn Laboratories for the Biological Mechanisms of Aging, Harvard University, Boston, MA

Introduction

Class III histone deacetylases (HDACs), also known as sirtuins, are mechanistically distinct from class I and class II HDACs in that they couple deacetylation of the peptide/protein substrate to cleavage of NAD^+ to form nicotinamide and O-acetyl-ADP-ribose. The mammalian sirtuins consist of 7 members, termed Sirt1-7, which share the NAD^+-binding catalytic domain, but differ in N- and C-termini, subcellular localization, substrate preference, and biological function.

Lysine acetylation of histone proteins is controlled by the opposing activities of HATs (histone acetyltransferases) and HDACs. Given the diversity of lysine-acetylated proteins and the biological processes they regulate, modulators of HAT and HDAC enzymes represent potential candidates for drug discovery efforts. Consistent with this assertion, sirtuins became the focus of intense research when it was discovered that their activation led to reduced incidence of aging and age-related diseases such as diabetes[1]. Initial studies pointed to the small molecule, resveratrol, as a sirtuin activator and potential therapeutic. However, other results demonstrated that unlabeled peptides did not activate sirtuins in a similar manner. These data suggested that the activation observed was due to the use of fluorescently tagged substrates[2].

Despite these data, multiple lines of evidence are consistent with sirtuins having an impact on health and disease[3-6]. To better understand the biological roles of sirtuins, researchers would benefit from an alternative assay that uses untagged, native peptide substrates, enabling the study of sirtuins without the complication of fluorophore-mediated activation.

To avoid the pitfalls of fluorescent substrates, measure sirtuin activity in a more physiologically relevant manner and address the diversity of sirtuin isoforms and potential substrates, we developed an assay platform that enables the analysis of sirtuin activity using virtually any appropriate substrate. This new SIRTainty™ class III HDAC assay is a flexible, reliable, homogeneous, no-wash assay for quantifying sirtuin activity. Based upon novel, patent-pending technology, this easy-to-perform assay is coupled to nicotinamidase, which catalyzes breakdown of nicotinamide generated upon cleavage of NAD^+ during sirtuin-mediated deacetylation of a substrate. Thus, the SIRTainty™ assay provides a direct assessment of the activity of class III HDAC enzymes. In contrast to other sirtuin assays that use a fluorescently tagged, acetylated peptide substrate, the SIRTainty™ assay employs an untagged, acetylated peptide substrate.

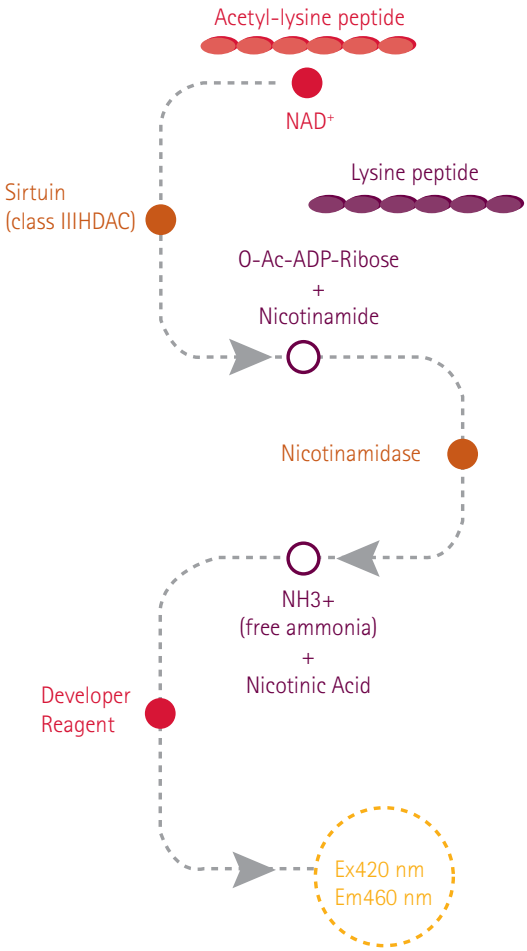

Figure 1. SIRTainty™ Assay Principle.

Sirtuin-mediated deacetylation of unlabeled peptide substrate generates nicotinamide as a product. The SIRTainty™ assay couples sirtuin enzyme activity to nicotinamidase, which cleaves nicotinamide into nicotinic acid and free ammonia. A developer reagent is added, which reacts with the free ammonia to generate a fluorophore. The resulting fluorescent signal is quantified with a conventional fluorometric plate reader.

In this study, we used the SIRTainty™ assay to compare enzymatic activity of three different sirtuins, assess their inhibition by suramin (a previously identified sirtuin-binding molecule[7]), compare their substrate preferences and, finally, compare the effects of resveratrol with the effects observed using a conventional HDAC assay.

Methods

Measuring deacetylation activity of three sirtuin family enzymes

Sirt1 (Merck Millipore Catalogue No. 524743), Sirt2 (Merck Millipore Catalogue No. 524744), and Sirt3 (Merck Millipore Catalogue No. 524745) at a range of concentrations were incubated with 25 µM acetylated peptide (H3K9) substrate and 0.2 mM β-NAD using the SIRTainty™ assay. The K_m values were determined by non-linear curve fit of Michaelis-Menten.

Analysis of peptide substrate preference of sirtuin family enzymes

Peptide substrates derived from p53, histone H3 and histone H4 (available from Merck Millipore) were selected. Peptides were either unacetylated (H4, H3 and p53), acetylated at one site (e.g. H4K8), or acetylated at more than one site (e.g. H3K9,14). Recombinant Sirt1 (5 Units), Sirt2 (0.7 Unit) and Sirt3 (3 Units) were incubated with indicated acetylated peptide substrates (25 µM) and 1mM β-NAD with the SIRTainty™ assay.

Effectiveness of suramin in inhibiting sirtuin family enzymes

Recombinant Sirt1 (5 Units), Sirt2 (0.7 Units) and Sirt3 (1.5 Units) were incubated with 25 µM substrate (H3K9), 0.2 mM β-NAD and suramin and analyzed using the SIRTainty™ assay. Sirt1 exhibited greatest sensitivity to inhibition by suramin.

Effectiveness of resveratrol in activating sirtuin family enzymes

Sirt1 was incubated with resveratrol at a range of concentrations, and activity assessed with a conventional commercially available kit employing a fluorophore-tagged acetylated peptide substrate, or an untagged acetylated peptide substrate (H3K9) with the SIRTainty™ assay. Resveratrol potentiated Sirt1 activity only with tagged substrate.

Results

Sirtuin isoform activity

The SIRTainty™ assay was effective in measuring activity of all three sirtuin isoforms tested (Figure 2). SIRT2 displayed the highest affinity (K_m = 0.14 units) for the acetylated H3K9 substrate used.

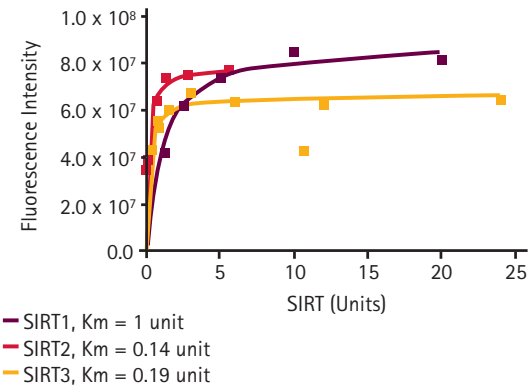

Figure 2.

The SIRTainty™ Sirtuin Assay is effective for multiple sirtuin family members.

Substrate preference

H3K9, H3K9/14, H4K8, and H4K5/8/12/16 displayed high efficiency for deacetylation as compared to the corresponding non-acetylated peptides for all three sirtuins, as shown in Figure 3. Sirt1 and 2, but not Sirt3, demonstrated higher deacetylation activity with a human p53 peptide acetylated at K382 compared to the non-acetylated peptide.

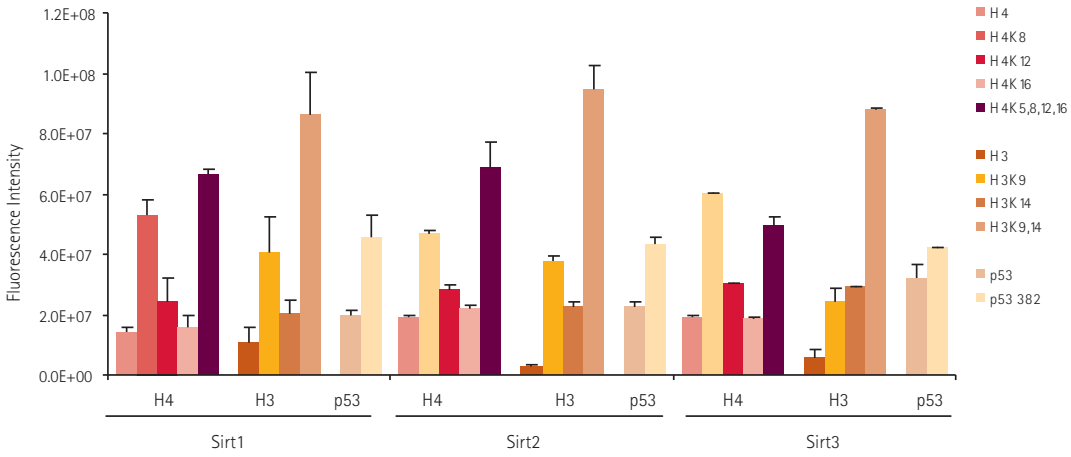

Figure 3.

Sirt1, Sirt2, and Sirt3 exhibit preference for acetylated vs. nonacetylated peptides.

Suramin inhibition

Suramin and suramin analogues typically target purinergic binding sites, which is why they show activity against sirtuins and not other histone deacetylases[7]. We measured the IC_{50} of suramin inhibition of three sirtuin isoforms and showed that Sirt1 was most effectively inhibited by suramin (Figure 4).

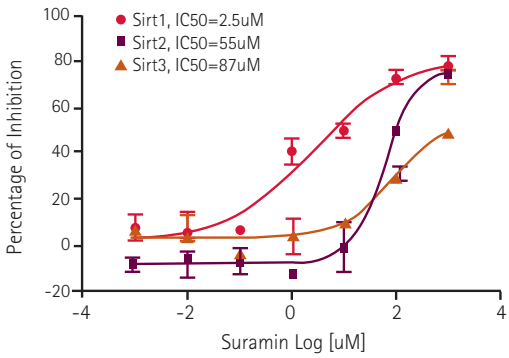

Figure 4.

Sirt1 shows the greatest sensitivity to inhibition by suramin compared to Sirt2 and Sirt3.

Effects of resveratrol

Conventional HDAC activity assays, which use fluorescently labeled substrates, have been shown to yield misleading results and fluorophore-mediated potentiation of sirtuin activity by resveratrol. We showed that, in the SIRTainty™ assay, resveratrol had no effect on Sirt1 activity (Figure 5). In contrast, resveratrol strongly activated Sirt1 activity measured using a conventional assay.

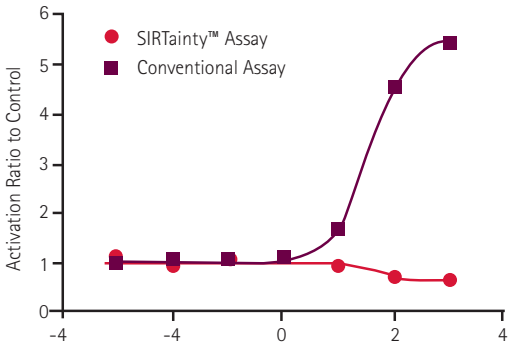

Figure 5.

Unlike a conventional HDAC activity assay with fluorescently tagged substrate, the SIRTainty™ Assay does not permit fluorophore-mediated Sirt1 enzyme activation by resveratrol.

Discussion

Our results indicate that SIRTainty™ Class III HDAC Assay offers a convenient, no-wash solution for quantifying sirtuin activity with any untagged acetylated substrate and for easily performing highly sensitive inhibitor assays with multiple sirtuin family members.

The SIRTainty™ assay not only enables unparalleled flexibility in choosing sirtuin isoforms and peptide substrates, but also helps avoid fluorophore-mediated activation observed using other commercial assay formats. Labeled substrates may affect enzyme activity via steric hindrance, electrostatic, or other mechanisms. Although there are other methods, such as mass spectrometry, for measuring acetylation status of native, unlabeled substrates, the SIRTainty™ assay is far simpler and enables higher throughput, making it amenable to lead discovery programs and systems-level analyses.

The flexibility and high throughput nature of the SIRTainty™ assay make it ideal for multiple applications, including assaying of peptide substrate panels to determine substrate specificity, screening for activators and inhibitors of sirtuins, comparison of sirtuin family members for profiling and selectivity studies, enzyme kinetics studies, and potency assessment for sirtuin modulators for rank-ordering purposes.

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References

1. Finkel T. et al. Recent progress in the biology and physiology of sirtuins. Nature. 2009 Jul 30;460(7255):587-91.
2. Pacholec M et al. SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. J Biol Chem. 2010 Mar 12;285(11):8340-51.
3. K. T. Howitz et al. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature. 2003 Sep 11; 425:191.4.
4. G. Boily et al. SirT1-null mice develop tumors at normal rates but are poorly protected by resveratrol. Oncogene. 2009 Aug 13; 28:2882.
5. J. A. Baur et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature. 2006 Nov 16; 444:337.
6. R. K. Minor et al. SRT1720 improves survival and healthspan of obese mice. Sci. Rep. 2011; 1.
7. Schuetz A et al. Structural basis of inhibition of the human NAD^+-dependent deacetylase SIRT5 by suramin. Structure. 2007 Mar;15(3):377-89.

Featured Products

Description	Qty/Pk	Catalogue No.
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SIRT1/2 Inhibitor VII		566327
SIRT1/2 Inhibitor VIII, Salermide		566330
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SIRT2 Inhibitor, AGK2		566324
SIRT2 Inhibitor II, AK-1		566331
Sirtinol		566320
InSolution™ Sirtinol		566321

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