

# HTRF® human SIRT1 Assay: A new format for SIRT1 modulator screening

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## Introduction

SIRT1, a NAD<sup>+</sup> dependent enzyme, belongs to class III histone deacetylase family known as sirtuins. It modulates gene expression through the deacetylation of acetylated lysine residues of histones as well as non histones proteins (such as transcription factors). SIRT1 is involved in a variety of biological processes such as glucose homeostasis, mitochondrial biogenesis, adipogenesis, apoptosis, senescence and metabolism, making it an interesting target for modulator screening. Furthermore, the beneficial role of SIRT1 inhibitors as anti-tumor agents has been demonstrated making them an attractive target for the development of new anti-cancer drugs. Finally, SIRT1 overexpression by activator compounds has been described as a potential solution for treating diabetes, aging, neurodegenerative disease, and cardiovascular disease.

Cisbio has developed a two step deacetylation assay using the HTRF technology. It consists of the recognition of an acetylated d2 labeled substrate by a Eu-cryptate labeled antibody. The maximum signal is obtained in the absence of enzyme, and it disappears when the substrate's acetyl group is removed by SIRT1 enzyme. Signal extinction is thus directly correlated to the quantity of deacetylated substrate.

The assay is a homogeneous method for high throughput screening of SIRT1 inhibitors and activators. This solution allows highly sensitive, robust detection with the advantage of very low enzyme and substrate consumption.

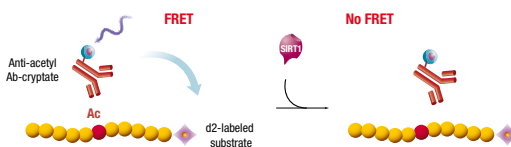
## Assay principle

The HTRF deacetylation assay uses substrate-d2 containing a single acetylated lysine. It is recognized by an Eu-cryptate anti-acetyl lysine antibody.

The test is performed in two steps: an enzymatic step in presence of enzyme and acetylated substrate, and a detection step in which the deacetylation event is quantified.

The maximum signal obtained in the absence of enzyme decreases when the deacetylation reaction occurs. The quantity of deacetylated peptides is then directly correlated to the signal extinction.

Deacetylation assay is carried out in low volume 384 well plates. Enzymatic reaction is performed in 10 µl. The reagents are distributed in the following order : 4 µl of substrate-d2, 2µl of compound (or enzymatic buffer) and 2µl of human SIRT1 enzyme. The reaction is started by adding 2µl NAD<sup>+</sup>, and stopped by adding 10 µl of the Eu-cryptate conjugated Ab diluted in a detection buffer containing 5 mM nicotinamide. The final assay volume is 20µl.

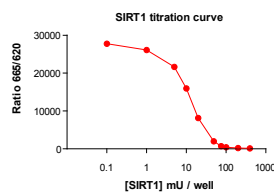


## SIRT1 Investigation

### 1. Enzyme titration curve

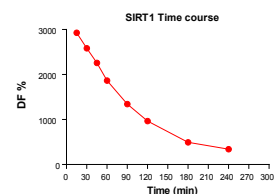
SIRT1 titration assay was run with enzyme concentrations ranging from 0.1 to 400 mU/well, 6 nM of d2-substrate and 500 µM of NAD<sup>+</sup> in the enzymatic step. Enzymatic reaction time is 1H.

The titration curve indicates an EC50 of 12 mU/well of SIRT1.



### 2. Time course

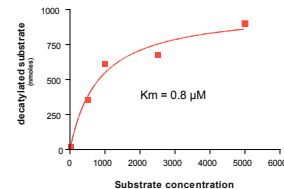
The assay was performed with an enzyme concentration of 10mU/well, 6 nM of substrate-d2 and 500 µM of NAD<sup>+</sup> in the enzymatic step. The reaction was stopped at the indicated time.



### 3. Determination of the Km for SIRT1 and substrate-d2

The Km of the enzyme for the substrate-d2 was determined using a range of substrate concentrations from 0 to 5 µM. The assay was performed with an excess of NAD<sup>+</sup> (2 mM), and a SIRT1 concentration of 20 mU/well. Enzymatic reaction was stopped after 120 min with the detection buffer containing the Eu-cryptate antibody. The test was carried out at the initial velocity of SIRT1. For high substrate concentrations (more than 20 nM), dilutions were done after reaction arrest in order to have an optimal detection and to quantify the deacetylation event correctly. Data analysis of the graph shows a Km of 0.8 µM. A very similar Km of 1.2 µM was obtained (data not shown) for the same experiment done with a non fluorogenic substrate (biotin substrate).

Several SIRT1 characteristics allowing complete and easy assay development have been determined with HTRF SIRT1 assay.

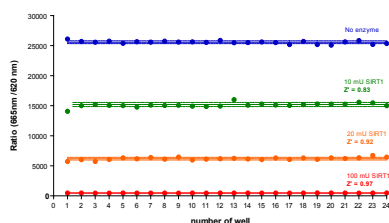


## HTS suitability

### 1. Screening robustness

#### Z' values for various SIRT1 concentration

Twenty-four replicates were run using 4 selected SIRT1 concentrations: 0mU of enzyme (no deacetylation), 100 mU of enzyme (100% deacetylation), 10 mU and 20 mU corresponding to the EC50 and EC80 respectively. The assay was carried out as previously described.



#### Assay characteristics

| Condition   | Average signal | SD  | Delta F | % deacetylation |
|-------------|----------------|-----|---------|-----------------|
| Negative    | 384            |     |         |                 |
| 0 mU SIRT1  | 25558          | 236 | 6557%   |                 |
| 20 mU SIRT1 | 6186           | 224 | 1151%   | 77%             |

S/B Z'

54 0.9

The above results show the assay's ability to detect low amounts of enzyme (10 mU) with excellent assay robustness (Z>0.8) and a reliable signal.

## Conclusion

This poster presents the new HTRF SIRT1 assay and examples of applications for enzyme characterization and compound screening are described. Generated data shown:

- Low enzyme (mU range) and substrate consumption (nM range).
- Ability to detect 10 mU of enzyme with excellent robustness (Z>0.8)
- Signal to background of 54
- Suitability for inhibitor and activator screening with polyphenolic compound discrimination.

### 2. Compound screening

The assay was performed using 3 classes of compounds: polyphenolic compounds (resveratrol and piceatannol), non-polyphenolic compounds (compound 1 and compound 2), and one inhibitor of reference, nicotinamide.

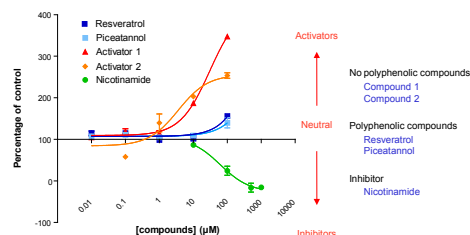
Concentrations tested ranged from 0.1 to 100 µM for polyphenolic and non-polyphenolic compounds, and from 10 to 500 µM for nicotinamide.

In the enzymatic step reagent concentrations were the following: 6 nM of substrate-d2, 150 µM NAD<sup>+</sup> and 10mU/well of SIRT1. DMSO concentration is 1% in all the wells. An enzymatic buffer containing 1% DMSO was added for control wells (with no compound addition).

Results were expressed in % of the control =  $(1 - (\%DF_{sample} / \%DF_{control})) \times 100$

The IC50 values determined were 30 µM, 3µM and 62 µM for compound 1, compound 2 and nicotinamide respectively. No EC50 could be calculated for resveratrol and piceatannol due to the lack of modulation.

Additional experiments carried out with resveratrol at 200µM failed to demonstrate any effect on SIRT1 modulation (data not shown).



The assay is able to detect different types of compound modulators