SIRT1 Direct Fluorescent Screening Assay Kit

Item No. 10010401

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Customer Service 800.364.9897
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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	Quantity	Storage
10010993	SIRT Assay Buffer (10X)	1 vial	-20°C
10010994	SIRT1 (human, recombinant)	2 vials	-80°C
10010995	SIRT1 Peptide	2 vials	-20°C
10010996	SIRT NAD^+	1 vial	-20°C
10010997	SIRT Nicotinamide	1 vial	-20°C
10010998	SIRT Developer	1 vial	-20°C
10010999	AMC Fluorophore	1 vial	-20°C
10011288	Half Volume 96-Well Plate (white)	1 plate	RT
400012	96-Well Cover Sheet	1 cover	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometer with the capacity to measure fluorescence using excitation wavelength of 350-360 and emission wavelength of 450-465 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or deionized water is acceptable
4. An orbital microplate shaker

INTRODUCTION

Background

Sirtuins (SIRTs) comprise a family of seven NAD^+-dependent class III histone deacetylases (HDACs) with roles in aging, inflammation, oxidative stress, tumorigenesis, and DNA repair.[1,2] SIRTs have different biological functions based on their subcellular localization and variation in their substrate-binding sites. Though originally characterized as deacetylases, SIRTs are multifunctional enzymes involved in additional post-translational modifications of proteins, including poly ADP-ribosylation, demalonylation, lipoamidation, phosphorylation, and ubiquitination.[1-3] SIRT1 is an NAD^+-dependent HDAC localized to both the nucleus and the cytoplasm and has roles in the regulation of inflammatory signaling, mitochondrial biology, and oxidative stress.[1,3] Dysregulation of SIRT1 is implicated in several diseases, including psoriasis, atherosclerosis, and kidney disease.[1,2,4,5]

About This Assay

Cayman's SIRT1 Direct Screening Assay Kit provides a convenient fluorimetric method for screening SIRT1 modulators in a two-step reaction (Figure 1). In the first step, the SIRT1 peptide and NAD^+ are incubated with human recombinant SIRT1. Deacetylation sensitizes the substrate such that treatment with the developer in the second step releases a fluorescent product. The fluorophore can be easily analyzed using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm.

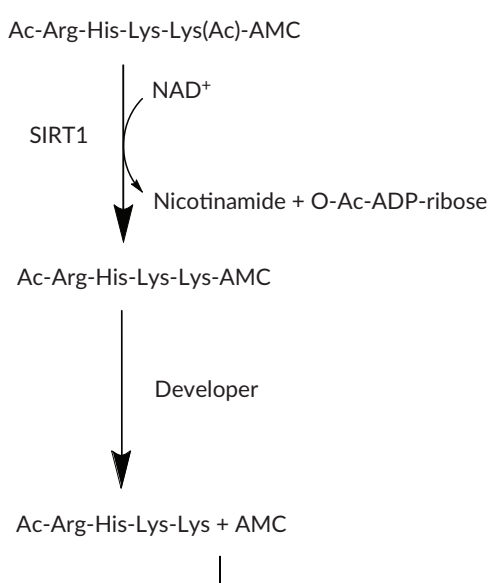

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **SIRT Assay Buffer (10X) - (Item No. 10010993)**

   Dilute 3 ml of the assay buffer concentrate with 27 ml of pure water. When stored at 4°C, this diluted buffer is stable for at least six months.

2. **SIRT1 (human, recombinant) - (Item No. 10010994)**

   Each vial contains 100 µl of human recombinant SIRT1. Thaw the enzyme on ice, add 300 µl of Assay Buffer (1X) to the vial, and mix gently. The diluted enzyme is stable for four hours on ice. One vial of enzyme is sufficient to assay 50 wells.

3. **SIRT1 Peptide - (Item No. 10010995)**

   Each vial contains 100 µl of a 5 mM peptide solution comprising amino acids 379-382 of human p53 conjugated to aminomethylcoumarin (AMC). It is ready to use to make the substrate solution.

4. **SIRT NAD^+ - (Item No. 10010996)**

   The vial contains 500 µl of a 50 mM solution of NAD^+. It is ready to use to make the substrate solution.

5. **SIRT Nicotinamide - (Item No. 10010997)**

   The vial contains 500 µl of a 50 mM solution of nicotinamide, a sirtuin inhibitor. It is ready to use to make the stop/developing solution.

6. **SIRT Developer - (Item No. 10010998)**

   The vial contains 100 mg of SIRT developer.

7. **AMC Fluorophore - (Item No. 10010999)**

   The vial contains 50 µl of 10 mM 7-amino-4-methylcoumarin (AMC) in DMSO. The fluorophore can be used to assay for interference (see page 16).

8. **SIRT1 Substrate Solution**

   Prepare the SIRT1 substrate solution according to the table below. It will be stable for 6 hours. *NOTE: The addition of 15 µl of substrate solution to the assay yields a final concentration of 125 µM peptide and 3 mM NAD^+. The K_M values for the peptide and NAD^+ are 200 and 435 µM, respectively.*

Reagent	50 Wells	100 Wells
SIRT1 Peptide	100 µl (1 vial)	200 µl (2 vials)
Assay Buffer (1X)	850 µl	1,700 µl
SIRT NAD^+	240 µl	480 µl

9. **SIRT Stop/Developing Solution**

   Prepare the SIRT stop/developing solution according to the table below. Vortex as necessary to ensure that the developer is completely in solution. Store on ice where it will be stable for four hours.

Reagent	50 Wells	100 Wells
SIRT Developer	15 mg	30 mg
Assay Buffer (1X)	2,400 µl	4,800 µl
SIRT Nicotinamide	100 µl	200 µl

Sample Preparation

All test compounds, be they small molecules, natural products, or proteins, should be prepared in Assay Buffer (1X) at a concentration 10X the desired final assay concentration (*e.g.*, for 5 µM final assay concentration, a 50 µM stock should be made). This solution may contain up to 25% DMSO, dimethyl formamide (DMF), or short-chain alcohols (*e.g.*, MeOH, EtOH). The final concentration of organic solvents in the assay will then be ≤2.5%.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as vehicle control and three wells designated as background wells. It is recommended that each test compound be assayed in triplicate and that the contents of each well are recorded on the template sheet provided on page 21. A typical layout of samples and compounds to be measured in triplicate is given in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	V	V	V	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW = Background Wells
V = Vehicle Control Wells
1-30 = Test Compound Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 100 µl in all the wells.
- All reagents except SIRT1 and stop/developing solution must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- If the appropriate dilution of the test compound is not known, it may be necessary to assay at several dilutions.
- Thirty test compounds can be assayed in triplicate or forty-six in duplicate.
- The assay temperature is 22-25°C.
- Monitor the fluorescence on a fluorometer with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm.

Performing the Assay

1. Add the appropriate amount of prepared reagent(s) to the corresponding wells according to the table below. If test compounds prepared in different solvents are to be assayed at the same time, separate sets of background and vehicle control wells should be run for each solvent.

Reagent	Background Wells	Vehicle Control Wells	Test Compound Wells
Assay Buffer (1X)	30 µl	25 µl	25 µl
Diluted SIRT1	--	5 µl	5 µl
Solvent	5 µl	5 µl	--
Test Compound	--	--	5 µl

2. Initiate the reactions by adding 15 µl of SIRT1 substrate solution to all the wells being used.
3. Cover the plate with the 96-Well Cover Sheet (Item No. 400012) and incubate on a shaker for 45 minutes at room temperature.
4. Remove the plate cover and add 50 µl of the SIRT stop/developing solution to each well.
5. Cover the plate and incubate on a shaker for 30 minutes at room temperature.
6. Remove the cover and read the plate with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting to allow for the measurement of all samples. The signal is stable for 30 minutes.

ANALYSIS

Calculations

1. Determine the average fluorescence of each sample.
2. Subtract the fluorescence of the background wells from the fluorescence of the vehicle control and the test compound wells.
3. Using the corrected values, determine the percent inhibition or percent activity for each test compound using the following equations:

$$\% \text{ inhibition} = \left[1 - \frac{\text{corrected value of test compound}}{\text{corrected value of vehicle}}\right] \times 100$$

$$\% \text{ activity} = \frac{\text{corrected value of test compound}}{\text{corrected value of vehicle}} \times 100$$

4. Either graph the Percent Inhibition or Percent Activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). An example of SIRT1 inhibition by Sirtinol, a sirtuin-specific inhibitor, is shown in Figure 3 on page 15.[6]

Performance Characteristics

Precision:

When a series of sixteen SIRT1 measurements were performed on the same day, the intra-assay coefficient of variation was 2.5%. When a series of sixteen SIRT1 measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 4.3%.

Sample Data:

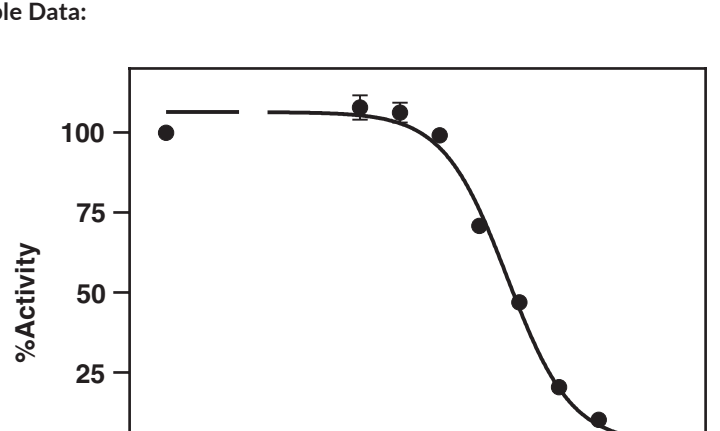

Figure 3. Inhibition of human recombinant SIRT1 by sirtinol. Data are plotted as the mean of duplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% activity. The IC_{50} value of sirtinol is 34 µM.

Effects of Solvents:

Compounds may be prepared in organic solvents such as DMSO, DMF, or short-chain alcohols (*e.g.* MeOH, EtOH). A titration of organic solvents showed that signal decreases with increasing solvent concentration, so the proper vehicle control should be included in the assay.

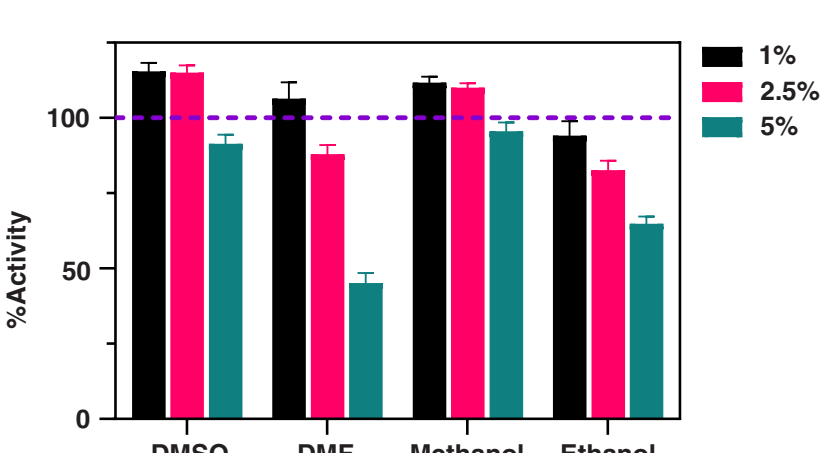

Figure 4. The effect of solvent on the readout of SIRT1 activity. The data are shown as the mean ± standard deviation for triplicate reactions containing the indicated concentration of solvents. The dotted line represents SIRT1 activity in the absence of additional solvent.

Interferences

It is possible that a compound tested for SIRT1 modulation will interfere with the development of the assay or interfere with the fluorophore. Potential fluorophore interference can be tested by assaying the compound in question with the included AMC fluorophore. A procedure is outlined below.

Testing for Fluorophore Interference

1. Dilute 20 µl of AMC Fluorophore (Item No. 10010999) with 480 µl of Assay Buffer (1X).
2. Add the appropriate amounts of prepared reagents to the corresponding wells according to the table below.
3. Cover the plate with the plate cover and incubate for 10 minutes at room temperature.
4. Remove the plate cover and read the plate with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm.

Reagent	Fluorophore Wells	Test Compound Wells
Assay Buffer (1X)	90 µl	90 µl
Diluted Fluorophore	5 µl	5 µl
Solvent	5 µl	--
Test Compound	--	5 µl

Testing for Developer Interference

Follow the procedure outlined in the table below to test for developer interference:

Procedure	SIRT1 Wells	Test Compound Wells
Add Assay Buffer (1X)	25 µl	25 µl
Add Diluted SIRT1	5 µl	5 µl
Initiate reaction with 15 µl SIRT1 substrate solution Cover and shake for 45 minutes at room temperature		
Remove cover		
Add SIRT Stop/Developing Solution	50 µl	50 µl
Add solvent	5 µl	--
Add Test Compound	--	5 µl
Cover and incubate for 30 minutes at room temperature Read at excitation λ=350-360 nm and emission λ=450-465 nm		

Calculating the Percent Interference

1. Determine the average fluorescence of each sample.
2. Determine the percent interference for the test compounds using the equation below. The percent interference should be less than 10% for the compound to be not affecting the fluorophore or the developer.

$$\% \text{ interference} = \left[1 - \frac{\text{compound fluorescence value}}{\text{fluorophore or SIRT1 fluorescence value}}\right] \times 100$$

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence detected above background in any of the wells	Either SIRT1 or developer solution was not added to the wells	Make sure to add all the components to the wells and re-assay
The fluorometer exhibited 'MAX' values for the wells	The *gain* setting is too high	Reduce the *gain* and re-read
No inhibition/activation seen with compound	A. The compound concentration is not high enough B. The compound is not an inhibitor/activator of the enzyme	Increase the compound concentration and re-assay

References

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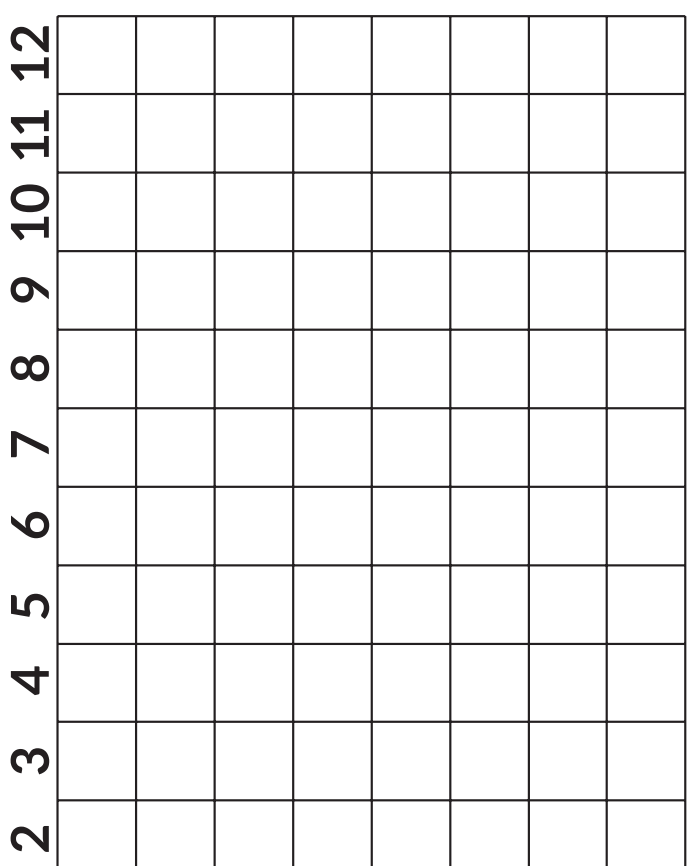
2. Jin, Q., Ma, F., Liu, T., *et al.* Sirtuins in kidney diseases: potential mechanism and therapeutic targets. *Cell Commun. Signal.* **22(1)**, 114 (2024).
3. Wątroba, M., Dudek, I., Skoda, M., *et al.* Sirtuins, epigenetics and longevity. *Ageing Res. Rev.* **40**, 11-19 (2017).
4. Lee, H. and Yoon, H. Mitochondrial sirtuins: Energy dynamics and cancer metabolism. *Mol. Cells* **47(2)**, 100029 (2024).
5. Xu, C., Liang, Z., Zhong, Y. *et al.* Sirtuins in macrophage immune metabolism: A novel target for cardiovascular disorders. *Int. J. Biol. Macromol.* **256**, 12870 (2024).
6. Grozinger, C.M., Chao, E.D., Blackwell, H.E., *et al.* Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. *J. Biol. Chem.* **276(42)**, 38837-38843 (2001).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.