SIRT3 Direct Fluorescent Screening Assay Kit

Item No. 10011566

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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
10012541	SIRT3 (human, recombinant)	2 vials	-80°C
700283	SIRT2/SIRT3 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 ea	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 an excitation wavelength of 360 nm (350-360) and an emission wavelength of 465 nm (450-460)
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] SIRT3 is a mitochondrial NAD^+-dependent HDAC that is ubiquitously expressed, with high expression in metabolically active tissues.[4,5] It has roles in fatty acid oxidation, protein and carbohydrate metabolism, ATP and redox homeostasis, mitochondrial function, and thermogenesis.[6-9] Dysregulation of SIRT3 is implicated in several diseases, including cancer, cardiovascular diseases, and neurodegenerative diseases, as well as aging.[7,5,10-12]

About This Assay

Cayman's SIRT3 Direct Fluorescent Screening Assay Kit provides a convenient fluorometric method for screening SIRT3 modulators in a two-step reaction (Figure 1, on page 7). In the first step, the SIRT2/SIRT3 peptide and NAD^+ are incubated with human recombinant SIRT3. 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 360 nm and an emission wavelength of 465 nm.

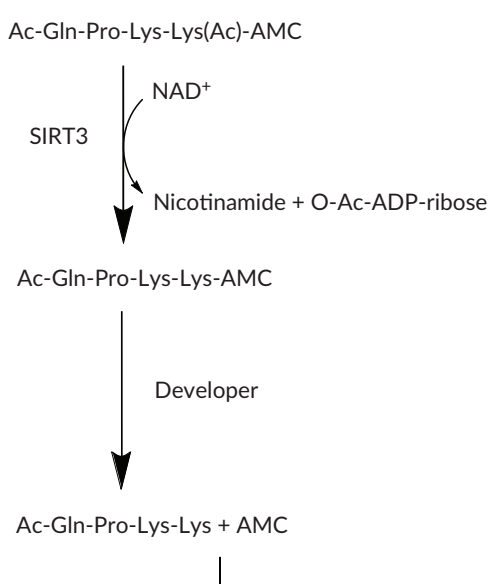

Figure 1. Assay scheme

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 to make 30 ml of Assay Buffer (1X). When stored at 4°C, this diluted buffer will be stable for at least six months.

2. **SIRT3 (human, recombinant) - (Item No. 10012541)**

   Each vial contains 50 µl of recombinant human SIRT3. Thaw the enzyme on ice, add 200 µl of Assay Buffer (1X) to the vial, and mix gently. The diluted enzyme will be stable for four hours on ice. One vial of enzyme is sufficient to assay half of a 96-well plate.

3. **SIRT2/SIRT3 Peptide - (Item No. 700283)**

   Each vial contains 100 µl of a 5 mM peptide solution comprising amino acids 317-320 of human p53 conjugated to 7-amino-4-methylcoumarin (AMC). It is ready to use to make the SIRT3 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 SIRT substrate solution.

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

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

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

   The vial contains 100 mg of SIRT developer. It is ready to use to make the SIRT stop/developing solution.

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

   The vial contains 50 µl of 10 mM AMC in DMSO. The fluorophore can be used to assay for interference (see page 17).

8. **SIRT3 Substrate Solution**

   Prepare the SIRT3 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 320 and 240 µM, respectively.*

Reagent	50 Wells	100 Wells
SIRT2/SIRT3 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

Test Compounds

Test compounds can be dissolved in DMSO, dimethyl formamide (DMF), methanol, or ethanol. Additional solvents have not been tested. The concentrated compound stock solutions must be further diluted in Assay Buffer (1X) to a concentration 10X the desired final assay (*i.e.* in-well) concentration. To minimize potential interference by solvents in the assay, refer to the **Effects of Solvents** section (page 16) to determine the maximum solvent concentration tolerated by the assay. Appropriate vehicle control wells containing the same concentration of solvent used for the test compounds must be included in each assay.

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 be 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 SIRT3 (human, recombinant) and SIRT 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 37°C.
- Monitor the fluorescence on a fluorometer with an excitation wavelength of 360 nm and an emission wavelength of 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 SIRT3	--	5 µl	5 µl
Solvent	5 µl	5 µl	--
Test Compound	--	--	5 µl

2. Initiate the reactions by adding 15 µl of SIRT3 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 37°C.
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 360 nm and an emission wavelength of 465 nm. It may be necessary to adjust the gain setting to allow for the measurement of all samples. The signal will be stable for 30 minutes.

ANALYSIS

Calculations

1. Determine the average fluorescence of each set of replicates.
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 SIRT3 inhibition by nicotinamide is shown in Figure 3, on page 15.[13]

Performance Characteristics

Precision:

When a series of sixteen SIRT3 measurements were performed on the same day, the intra-assay coefficient of variation was 2.5%. When a series of sixteen SIRT3 measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 2.7%.

Sample Data:

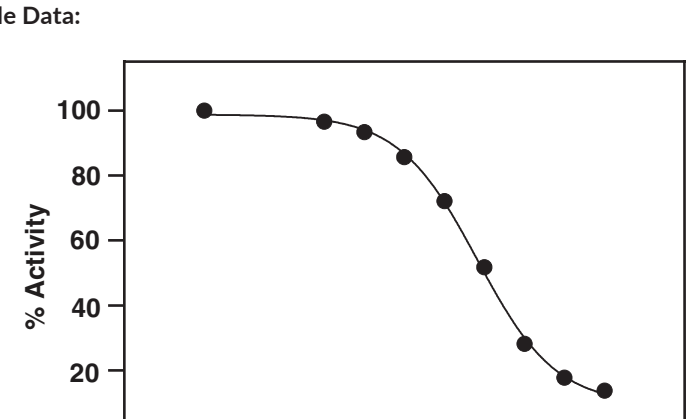

Figure 3. Inhibition of recombinant human SIRT3 by nicotinamide. Data are plotted as the mean of duplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% activity. The IC_{50} value of nicotinamide is 260 µM.

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 activity may decrease with increasing solvent concentration, so the proper vehicle control should be included in the assay.

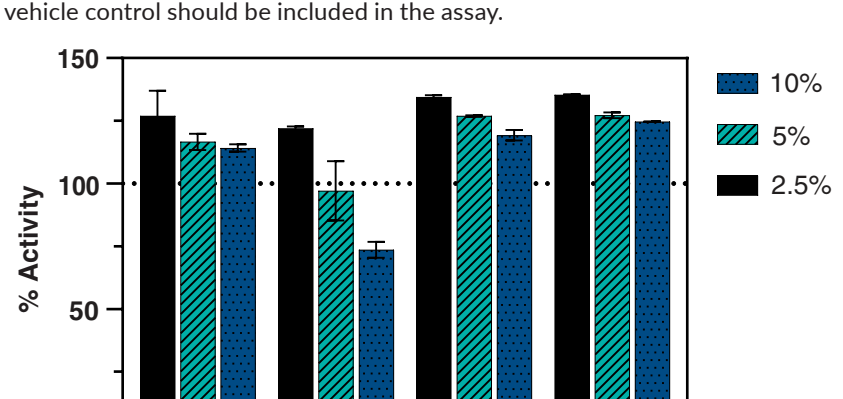

Figure 4. The effect of solvent on the readout of SIRT3 activity. The data are shown as the mean ± standard deviation for triplicate reactions containing the indicated concentration of solvents. The dotted line represents SIRT3 activity in the absence of additional solvent.

Interferences

It is possible that a compound tested for SIRT3 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 360 nm and an emission wavelength of 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	SIRT3 Wells	Test Compound Wells
Add Assay Buffer (1X)	25 µl	25 µl
Add Diluted SIRT3	5 µl	5 µl
Initiate reaction with 15 µl SIRT3 substrate solution Cover and shake for 45 minutes at 37°C		
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=360 nm and emission λ=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 not affect the fluorophore or the developer.

$$\% \text{ interference} = \left[1 - \frac{\text{compound fluorescence value}}{\text{fluorophore or SIRT3 fluorescence value}}\right] \times 100$$

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	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 SIRT3 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

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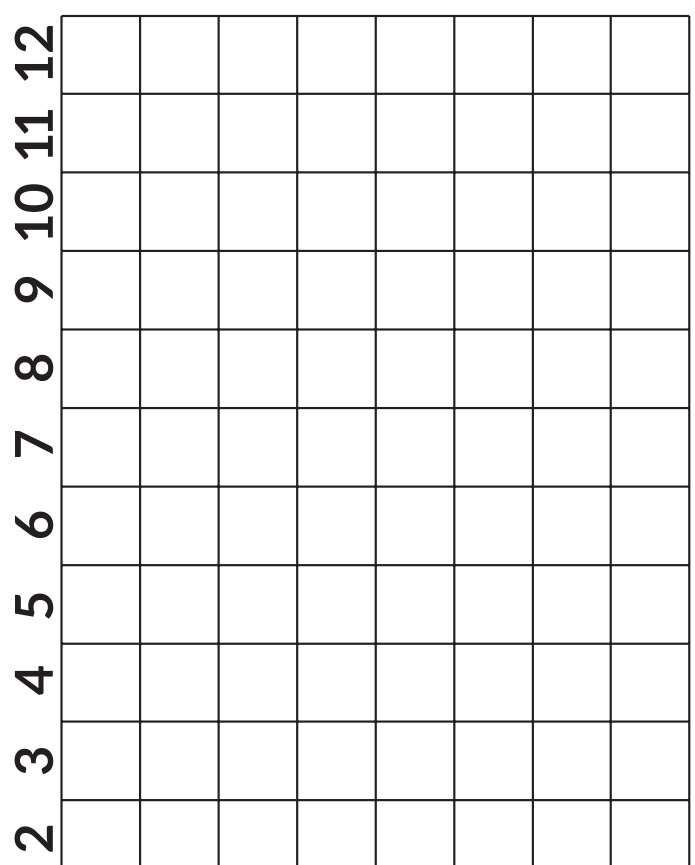

NOTES

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.

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