

SIRT6 Direct Fluorescent Screening Assay Kit

Item No. 700290



Customer Service 800.364.9897 * **Technical Support** 888.526.5351
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TABLE OF CONTENTS

GENERAL INFORMATION

INTRODUCTION

PRE-ASSAY PREPARATION

ASSAY PROTOCOL

ANALYSIS

RESOURCES

3

4

4

4

4

5

6

7

8

9

11

12

13

15

16

17

18

19

20

Materials Supplied

Precautions

If You Have Problems

Storage and Stability

Materials Needed but Not Supplied

Background

About This Assay

Reagent Preparation

Plate Set Up

Performing the Assay

Calculations

Performance Characteristics

Interferences

Troubleshooting

References

Related Products

Warranty and Limitation of Remedy

Plate Template

Notes

GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity	Storage
700291	SIRT6 Direct Assay Buffer (10X)	1 vial	-20°C
700292	SIRT6 (human recombinant)	2 vials	-80°C
700293	SIRT6 Direct Peptide	2 vials	-20°C
700294	SIRT6 Direct NAD ⁺	1 vial	-20°C
700295	SIRT6 Direct Nicotinamide	1 vial	-20°C
700296	SIRT6 Direct Developer	1 vial	-20°C
700297	SIRT6 Direct Fluorophore	1 vial	-20°C
10011288	Half-Volume 96-Well Plate (white)	1 plate	Room temperature
400012	96-Well Cover Sheets	1 cover	Room temperature

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.

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WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

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 described in the **Materials Supplied** section on 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 350-360 nm and an emission wavelength of 450-465 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Nucleosomes, which fold chromosomal DNA, contain two molecules each of the core histones H2A, H2B, H3, and H4. Almost two turns of DNA are wrapped around this octameric core, which represses transcription.¹ The histone amino termini extend from the core, where they can be modified post-translationally by acetylation, phosphorylation, ubiquitination, and methylation, affecting their charge and function. Acetylation of the ϵ -amino groups of specific histone lysines is catalyzed by histone acetyltransferases (HATs) and correlates with an open chromatin structure and gene activation. Histone deacetylases (HDACs) catalyze the hydrolytic removal of these acetyl groups from histone lysine residues and correlates with chromatin condensation and transcriptional repression.^{2,3}

The sirtuins represent a distinct class of trichostatin A-insensitive lysyl-deacetylases (class III HDACs) and have been shown to catalyze a reaction that couples lysine deacetylation to the formation of nicotinamide and O-acetyl-ADP-ribose from NAD⁺ and the abstracted acetyl group.⁴⁻⁶ There are seven human sirtuins, which have been designated SIRT1-SIRT7.⁷ SIRT6 is a nuclear protein that has both ADP-ribosyl transferase and deacetylase activities.⁸ SIRT6 associates specifically with telomeres and functions at chromatin to decrease NF- κ B signaling.^{9,10} Mammalian cells depleted of SIRT6 display abnormal telomere structures similar to defects found in Werner syndrome, a premature aging disorder, and have a shortened life span.^{9,10} Since SIRT6 binds and attenuates NF- κ B signaling, it is proposed that activators of SIRT6 may be effective anti-cancer and anti-inflammatory drugs and may increase longevity.⁹

About This Assay

Cayman's SIRT6 Direct Fluorescent Screening Assay provides a convenient fluorescence-based method for screening SIRT6 inhibitors or activators. The procedure requires only two easy steps, both performed in the same microplate (see Figure 1). In the first step, the substrate, which comprises the p53 sequence Arg-His-Lys-Lys(ϵ -acetyl)-AMC, is incubated with human recombinant SIRT6 along with its cosubstrate NAD^+ . 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 emission wavelength of 450-465 nm.

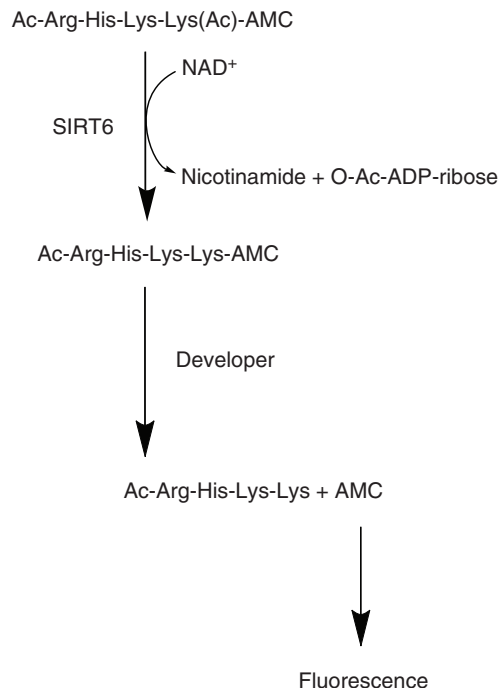


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. SIRT6 Direct Assay Buffer (10X) - (Item No. 700291)

Dilute 3 ml of Assay Buffer (10X) with 27 ml of HPLC-grade water. The final Assay Buffer (50 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl_2) should be used in the Assay and for diluting reagents. When stored at 4°C, this diluted Assay Buffer is stable for at least six months.

2. SIRT6 (human recombinant) - (Item No. 700292)

Each vial contains 100 μl of human recombinant SIRT6. Thaw the enzyme on ice, add 200 μl of diluted Assay Buffer to the vial, and vortex. The diluted enzyme is stable for four hours on ice. One vial of enzyme is enough SIRT6 to assay 60 wells. Use the additional vial if assaying the entire plate.

3. SIRT6 Direct Peptide - (Item No. 700293)

Each vial contains 80 μl of a 15 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. One vial of peptide will make enough Substrate to assay 60 wells. Use the additional vial if assaying the entire plate.

4. SIRT6 Direct NAD^+ - (Item No. 700294)

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

5. SIRT6 Direct Nicotinamide - (Item No. 700295)

The vial contains 2 ml of a 1 M solution of nicotinamide, a sirtuin inhibitor. It is ready to use to make the Stop/Developing Solution.

6. SIRT6 Direct Developer - (Item No. 700296)

The vial contains 100 mg of the SIRT6 Developer.

7. SIRT6 Direct Fluorophore - (Item No. 700297)

The vial contains 50 μl of 5 mM 7-amino-4-methylcoumarin in DMSO. The Fluorophore can be used to assay for interference (see page 13).

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 100% initial activity and three wells designated as background wells. We suggest that each Inhibitor/Activator sample be assayed in triplicate and that you record the contents of each well on the template sheet provided on page 19. A typical layout of samples and compounds to be measured in triplicate is given below (see 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	A	A	A	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

A - 100% Initial Activity Wells

1-30 - Inhibitor/Activator Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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 μ l in all the wells.
- Use the diluted Assay Buffer in the Assay.
- All reagents except SIRT6 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 Inhibitor/Activator concentration is not known, it may be necessary to assay at several dilutions.
- We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- Thirty Inhibitor/Activator samples can be assayed in triplicate or 46 in duplicate.
- The assay temperature is 37°C.
- Monitor the fluorescence with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm.

Performing the Assay

- Preparation of Substrate Solution** - To one of the thawed SIRT6 Direct Peptide vials add 190 μ l of NAD⁺ Solution and 630 μ l of diluted Assay Buffer. One vial of peptide will make enough Substrate Solution for 60 wells. The Substrate Solution is stable for six hours. The addition of 15 μ l to the assay yields a final concentration of 400 μ M peptide and 3 mM NAD⁺. *NOTE: The K_m values for the peptide and NAD⁺ are 600 and 310 μ M, respectively.*
- 100% Initial Activity Wells** - add 25 μ l of diluted Assay Buffer, 5 μ l of diluted SIRT6 (human recombinant), and 5 μ l of solvent (the same solvent used to dissolve the inhibitor) to three wells.

- Background Wells** - add 30 µl of diluted Assay Buffer and 5 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
- Inhibitor/Activator Wells** - add 25 µl of diluted Assay Buffer, 5 µl of diluted SIRT6 (human recombinant), and 5 µl of Inhibitor/Activator* to three wells.

*Inhibitors/Activators can be dissolved in Assay Buffer, ethanol, methanol, or DMSO and should be added to the assay in a final volume of 5 µl. In the event that the appropriate concentration of Inhibitor/Activator needed for SIRT6 inhibition or activation is completely unknown, we recommend that several dilutions of the Inhibitor/Activator be assayed.

	Assay Buffer (µl)	Solvent (µl)	SIRT6 (µl)	Test Compound (µl)
100% Initial Activity	25	5	5	-
Background	30	5	-	-
Inhibitor/Activator	25	-	5	5

Table 1. Pipetting summary

- Initiate the reactions by adding 15 µl of Substrate Solution to all the wells being used.
- Cover the plate with the plate cover and incubate on a shaker for 45 minutes at 37°C.
- Preparation of Stop/Developing Solution** - Weigh 30 mg of Developer into a vial that will hold 5 ml then add 200 µl of Nicotinamide and 4.8 ml of diluted Assay Buffer. Vortex until the Developer is into solution. This is enough Stop/Developing Solution for the entire plate. The Stop/Developing Solution is stable for four hours on ice.
- Remove the plate cover and add 50 µl of Stop/Developing Solution. Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
- Remove the plate cover and read the plate using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples. The development is stable for 30 minutes.

ANALYSIS

Calculations

- Determine the average fluorescence of each sample.
- Subtract the fluorescence of the Background wells from the fluorescence of the 100% Initial Activity and the Inhibitor/Activator wells.
- Determine the percent Inhibition or percent Initial Activity for each compound using one of the following equations.

$$\% \text{ Inhibition/Activation} = \left[\frac{\text{Initial Activity} - \text{Sample}}{\text{Initial Activity}} \right] \times 100$$

$$\% \text{ Initial Activity} = \left[\frac{\text{Sample Activity}}{\text{Initial Activity}} \right] \times 100$$

- Either graph the Percent Inhibition or Percent Initial Activity as a function of the inhibitor concentration to determine the IC₅₀ value (concentration at which there was 50% inhibition). An example of SIRT6 inhibition by Nicotinamide, a sirtuin-specific inhibitor, is shown in Figure 3, on page 12.

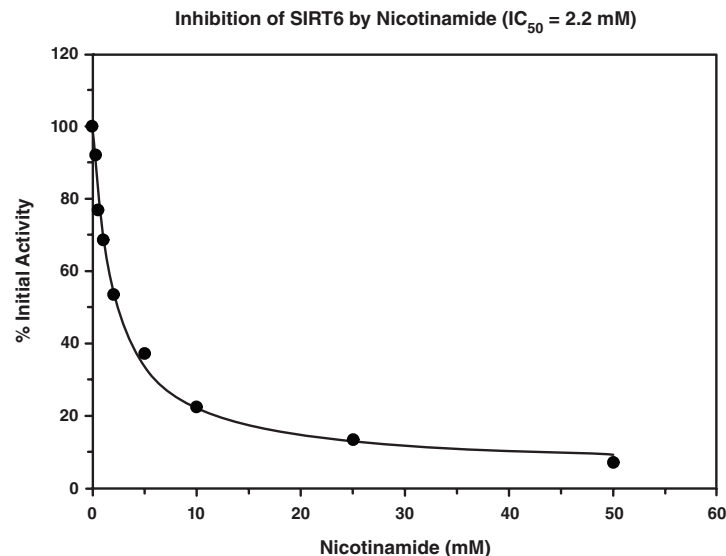


Figure 3. Inhibition of SIRT6 by nicotinamide ($IC_{50} = 2.2 \text{ mM}$)

Performance Characteristics

Precision:

When a series of 16 SIRT6 measurements were performed on the same day, the intra-assay coefficient of variation was 2.7%. When a series of 16 SIRT6 measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 3.3%.

Interferences

It is possible that a compound tested for SIRT6 Inhibition/Activation 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 Fluorophore. A procedure is outlined below.

Testing for Fluorophore interference

1. Label two test tubes F1 and F2. Add 10 μl of the Fluorophore and 90 μl of the diluted Assay Buffer to the F1 tube and vortex.
2. To the F2 tube, add 990 μl of diluted Assay Buffer and 10 μl from the F1 tube and vortex.
3. **Fluorophore wells** - add 5 μl of diluted Fluorophore (F2), 5 μl of solvent (the same solvent used to dissolve the compound), and 90 μl of diluted Assay Buffer to three wells.
4. **Compound wells** - add 5 μl of diluted Fluorophore (F2), 5 μl of compound, and 90 μl of diluted Assay Buffer to three wells.
5. Cover the plate with the plate cover and incubate for 10 minutes at room temperature.
6. Remove the plate cover and read the plate using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Calculating the percent Fluorophore interference

1. Determine the average fluorescence of each sample.
2. Determine the percent interference for the compound. To do this, subtract each compound value from the Fluorophore value. Divide the result by the Fluorophore value and then multiply by 100 to give the percent interference. The percent interference should be less than 10% for the compound to be not affecting the Fluorophore.

Testing for Developer interference

1. **SIRT6 wells** - add 25 µl of Assay Buffer and 5 µl of diluted SIRT6 to three wells.
2. **Compound wells** - add 25 µl of Assay Buffer and 5 µl of diluted SIRT6 to three wells.
3. Initiate the reactions by adding 15 µl of Substrate Solution to all the wells being used.
4. Cover the plate with the plate cover and incubate on a shaker for 45 minutes at 37°C.
5. Remove the plate cover and add 50 µl of Stop/Developing Solution to the SIRT6 and Compound wells.
6. Add 5 µl of compound to the Compound wells and 5 µl of solvent (the same solvent used to dissolve the compound) to the SIRT6 wells.
7. Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
8. Remove the plate cover and read the plate using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Calculating the percent Developer interference

1. Determine the average fluorescence of each sample.
2. Determine the percent interference for the compound. To do this, subtract each compound value from the SIRT6 value. Divide the result by the SIRT6 value and then multiply by 100 to give the percent interference. The percent interference should be less than 10% for the compound to be not affecting the Developer.

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 SIRT6 or Stop 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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Related Products

HAT Inhibitor Screening Assay Kit - Item No. 10006515
HDAC Fluorometric Activity Assay Kit - Item No. 10011563
HDAC1 Inhibitor Screening Assay Kit - Item No. 10011564
HDAC8 Inhibitor Screening Assay Kit - Item No. 700230
LSD1 Inhibitor Screening Assay Kit - Item No. 700120
Methyltransferase Colorimetric Assay Kit - Item No. 700140
Methyltransferase Fluorometric Assay Kit - Item No. 700150
PAD4 Inhibitor Screening Assay Kit - Item No. 700560
SET7/9 Methyltransferase Inhibitor Screening Assay Kit - Item No. 700270
SET8 Methyltransferase Inhibitor Screening Assay Kit - Item No. 700350
SIRT1 Direct Fluorescent Screening Assay Kit - Item No. 10010401
SIRT1 FRET-Based Screening Assay Kit - Item No. 10010991
SIRT2 Direct Fluorescent Screening Assay Kit - Item No. 700280
SIRT3 Direct Fluorescent Screening Assay Kit - Item No. 10011566
SIRT6 (human recombinant) - Item No. 10315

Warranty and Limitation of Remedy

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Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.

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NOTES

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