SIRT1 FRET-Based Screening Assay Kit

Item No. 10010991

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TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
10011080	SIRT1 FRET Assay Buffer (10X)	1 vial
10011081	SIRT1 (human recombinant)	1 vial
10011082	SIRT1 FRET Peptide	2 vials
10011083	SIRT1 FRET NAD^+	1 vial
10011084	SIRT1 FRET Stop Solution	1 vial
10011085	SIRT1 FRET Developer	1 vial
10011086	SIRT1 FRET Fluorophore	1 vial
10011288	Half Volume 96-Well Plate (white)	1 plate
400012	96-Well Cover Sheet	1 cover

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.

Sold under license from CycLex® Co., Ltd (Patent Nos. EP 1243658, US 7033778, US 7256013, and JP 4267043).

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.

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 at -80°C 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 335-345 and emission wavelength of 440-465 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 post-translationally modified 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]

Sirtuins (SIRTs) 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] SIRT1, which is located in the nucleus, is the human sirtuin with the greatest homology to yeast Sir2 (Silent information regulator 2) and has been shown to regulate the activity of the p53 tumor suppressor and inhibit apoptosis.[8-10] These results have significant implications regarding an important role of SIRT1 in modulating the sensitivity of cells in p53-dependent apoptotic response and the possible effect in cancer therapy. Since the growth suppressive function of p53 is strongly enhanced by DNA damaging reagents, it is expected that inhibitors of SIRT1 may be effective anticancer drugs.[11,12]

Recent screens for modulators of SIRT1 activity yielded a number of small molecule activators. Several of these activating compounds extended yeast, *D. melanogaster*, and *C. elegan* lifespans in a way that mimicked caloric restriction.[13,14] Resveratrol, the most potent of these compounds, activated SIRT1 in human cells and enhanced the survival rate of cells stressed by irradiation.[13] However, this activation of SIRT1 is the subject of much debate. It was determined that the activation of SIRT1 and Sir2 by resveratrol was a substrate-specific event (i.e. the binding of resveratrol to SIRT1 promoted a conformational change that better accommodated the attached coumarin group in the peptide substrate.)[15,16] The Cayman FRET-based assay does not exhibit this phenomena and can be used to eliminate false SIRT1 activators found with the coumarin-based substrate. Additional research needs to be done to further elucidate the mechanism behind SIRT1 activation, as well as to establish the role of SIRT1 activation in aging, cancer, and neurodegenerative disease.[17,18]

About This Assay

Cayman's SIRT1 FRET-based Screening Assay Kit provides a convenient fluorescence-based method for screening SIRT1 inhibitors or activators. The procedure requires only two easy steps, both performed in the same microplate (Figure 1). In the first step, the substrate which is coupled to the fluorophore and quencher is incubated with human recombinant SIRT1 along with its cosubstrate NAD^+. Deacetylation sensitizes the substrate such that treatment with the Developer in the second step results in the separation of the quencher and fluorophore thus emitting fluorescence. The fluorescence can be easily analyzed using a fluorescence plate reader or a fluorometer with excitation wavelength of 335-345 nm and emission wavelength of 440-465 nm.

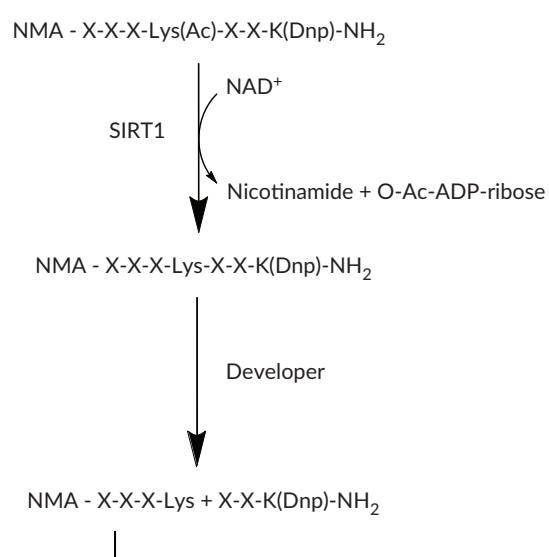

Figure 1.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **SIRT1 FRET Assay Buffer (10X) - (Item No. 10011080)**

   Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This final Buffer (50 mM Tris-HCl, pH 8.8, containing 4 mM $MgCl_2$ and 0.5 mM DTT) should be used in the assay and for diluting reagents. When stored at 4°C, this diluted buffer is stable for at least six months.

2. **SIRT1 (human recombinant) - (Item No. 10011081)**

   Each vial contains 100 µl of human recombinant SIRT1. Thaw the enzyme on ice, dilute 20 µl of SIRT1 with 780 µl of diluted Assay Buffer, and vortex. The diluted enzyme is stable for four hours on ice.

3. **SIRT1 FRET Peptide - (Item No. 10011082)**

   Each vial contains 100 µl of a 1 mM peptide that is coupled to a fluorophore and a quencher. It is ready to use to make the Substrate Solution.

4. **SIRT1 FRET NAD^+ - (Item No. 10011083)**

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

5. **SIRT1 FRET Stop Solution - (Item No. 10011084)**

   The vial contains 2 ml of a 100 mM solution of nicotinamide, a sirtuin inhibitor. Dilute 1.5 ml of Stop Solution with 4.5 ml of HPLC-grade water. Unused diluted Stop Solution can be stored at -80°C for six months.

6. **SIRT1 FRET Developer - (Item No. 10011085)**

   The vial contains 50 µl of a lysyl endopeptidase solution. Thaw the Developer on ice, dilute 15 µl of Developer with 735 µl of diluted Assay Buffer, and vortex. The diluted Developer is stable for four hours on ice.

7. **SIRT1 FRET Fluorophore - (Item No. 10011086)**

   The vial contains 50 µl of 6 mM N-methylanthranilic acid (NMA). The Fluorophore can be used to assay for interference (see page 17).

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 background and three wells designated as 100% initial activity 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 22. A typical layout of samples and compounds to be measured in triplicate is given below 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	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 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.
- Use the diluted Assay Buffer in the assay.
- All reagents except SIRT1 and Developer 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 dilution 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 forty-six in duplicate.
- The assay temperature is 37°C.
- Monitor the fluorescence on a fluorometer with an excitation wavelength of 335-345 nm and an emission wavelength of 440-465 nm.

Performing the Assay

1. **Preparation of Substrate solution -** Add 150 µl of NAD^+ solution (Item No. 10011083) and 500 µl of diluted Assay Buffer to one of the thawed SIRT1 Peptide vials (Item No. 10011082). One vial of Peptide will make enough Substrate Solution for 50 wells. The Substrate Solution is stable for six hours. The addition of 15 µl to the assay yields a final concentration of 40 µM Peptide and 3 mM NAD^+. *NOTE: The K_m values for the Peptide and NAD^+ are 34 and 57 µM, respectively.*
2. **100% Initial Activity Wells -** add 20 µl of Assay Buffer, 5 µl of diluted SIRT1, 5 µl of Developer, and 5 µl of solvent (the same solvent used to dissolve the inhibitor/activator) to three wells.
3. **Background Wells -** add 25 µl of Assay Buffer, 5 µl of Developer, and 5 µl of solvent (the same solvent used to dissolve the inhibitor/activator) to three wells.
4. **Inhibitor/Activator Wells -** add 20 µl of Assay Buffer, 5 µl of diluted SIRT1, 5 µl of Developer, and 5 µl of inhibitor/activator* to three wells.
5. Initiate the reactions by adding 15 µl of Substrate Solution to all the wells being used.
6. Cover the plate with the plate cover and incubate on a shaker for 30 minutes at 37°C.
7. Remove the plate cover and add 50 µl of Stop Solution.
8. Read the plate in a fluorometer using an excitation wavelength of 335-345 nm and an emission wavelength of 440-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples. The fluorescence is stable for 20 minutes.

*Inhibitors/activators can be dissolved in Assay Buffer, methanol, or dimethylsulfoxide 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 SIRT1 inhibition or activation is completely unknown, we recommend that several dilutions of the inhibitor/activator be assayed.

ANALYSIS

Calculations

1. Determine the average fluorescence of each sample.
2. Subtract the fluorescence of the background wells from the fluorescence of the 100% initial activity and the inhibitor/activator wells.
3. Determine the percent inhibition/activation for each sample. To do this, subtract each inhibitor/activator sample value from the 100% initial activity sample value. Divide the result by the 100% initial activity value and then multiply by 100 to give the percent inhibition/activation.
4. Either graph the Percent Inhibition or Percent Initial 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 16.[19,20]

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

Performance Characteristics

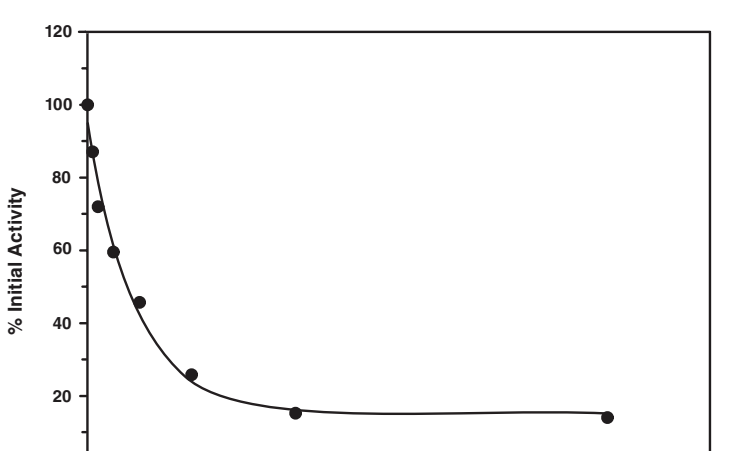

Figure 3. Inhibition of SIRT1 by Sirtinol (IC_{50} = 38 µM)

Precision:

When a series of sixteen SIRT1 measurements were performed on the same day, the intra-assay coefficient of variation was 5.2%. 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 5.7%.

Interferences

It is possible that a compound tested for SIRT1 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 SIRT1 FRET Fluorophore. A procedure is outlined below.

Testing for Fluorophore Interference

1. Dilute 10 µl of Fluorophore (Item No. 10011086) with 990 µl of diluted Assay Buffer. The diluted Fluorophore is stable for four hours.
2. **Fluorophore wells -** add 10 µl of diluted Fluorophore, 5 µl of solvent (the same solvent used to dissolve the compound), and 85 µl of diluted Assay Buffer to three wells.
3. **Compound wells -** add 10 µl of diluted Fluorophore, 5 µl of compound, and 85 µl of diluted Assay Buffer to three wells.
4. Cover the plate with the plate cover and incubate for 10 minutes at room temperature.
5. Remove the plate cover and read the plate in a fluorometer using an excitation wavelength of 335-345 nm and an emission wavelength of 440-465 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. **SIRT1 wells -** add 20 µl of Assay Buffer and 5 µl of diluted SIRT1 to three wells.
2. **Compound wells -** add 20 µl of Assay Buffer and 5 µl of diluted SIRT1 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 30 minutes at 37°C.
5. Remove the plate cover and add 50 µl of Stop Solution and 5 µl of Developer to the SIRT1 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 SIRT1 wells.
7. Cover the plate with the plate cover and incubate for 30 minutes at 37°C.
8. Remove the plate cover and read the plate in a fluorometer using an excitation wavelength of 335-345 nm and an emission wavelength of 440-465 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 SIRT1 value. Divide the result by the SIRT1 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 SIRT1 or Developer 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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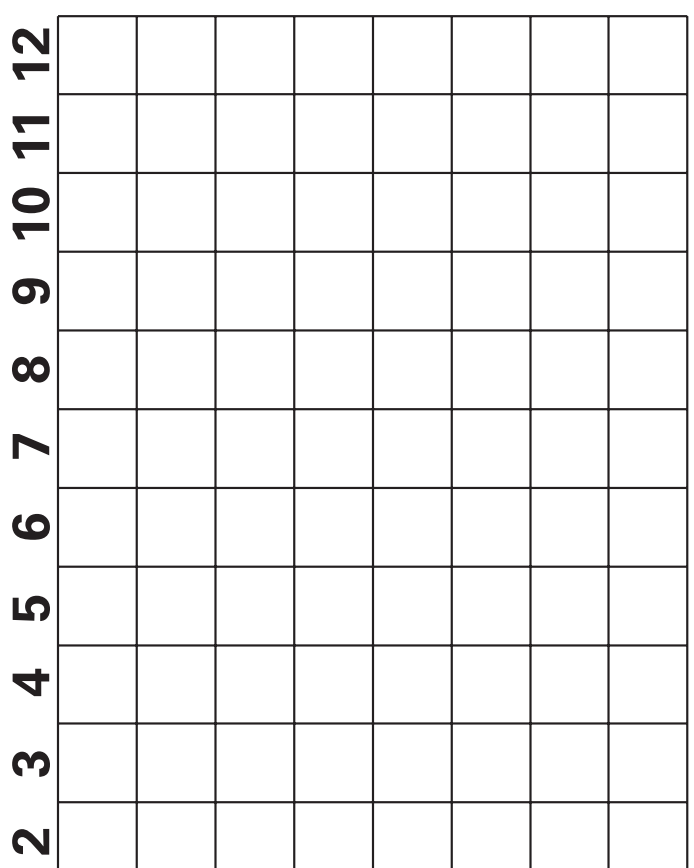

NOTES

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.