ab156066
SIRT2 Activity Assay Kit (Fluorometric)

Instructions for Use

For the quantitative measurement of SIRT2 activity in cell lysates

This product is for research use only and is not intended for diagnostic use.

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1. Background

Sir2 is a conserved protein and was recently shown to regulate lifespan extension both in budding yeast and nematode. In 2000, it was reported that the yeast Sir2 protein is a NAD(+)-dependent histone deacetylase that plays a critical role in transcriptional silencing, genome stability and longevity. In mammals, the homologs of Sir2 have been named sirtuins (SIRT), with seven members in a family termed SIRT1 through SIRT7. They share a conserved central deacetylase domain but have different N- and C termini and display distinct subcellular localization, suggesting different biological functions.

In contrast to SIRT1, mammalian SIRT2 is localized mainly in the cytoplasm. SIRT2 colocalizes with the microtubule network and deacetylates Lys40 of alpha-tubulin. The same residue of alpha-tubulin is also deacetylated by HDAC6, a class II HDAC, and deacetylation by HDAC6 leads to changes in cellular motility.

A role for SIRT2 in cancer pathogenesis was demonstrated using a proteomic approach. The SIRT2 gene, which is located at chromosome 19q13.2, lies within a region that is frequently deleted in human gliomas, and levels of SIRT2 mRNA and protein expression are severely reduced in a large fraction of human glioma cell lines. Ectopic expression of SIRT2 in these cell lines suppressed colony formation and modified the microtubule network. These

results indicate that SIRT2 may act as a tumor suppressor and may function to control the cell cycle by acetylation of alpha-tubulin. It was reported that SIRT2 inhibitor rescued alpha-synuclein toxicity and modified inclusion morphology in a cellular model of Parkinson’s disease, however the exact mechanism remains uncertain.

However, the conventional method for measuring SIRT2 activity is very complicated and laborious. In order to measure SIRT2 enzyme activity, it is necessary to prepare radioactive acetylated histone H4 as a substrate. First, cells have to be labeled metabolically with radioactivity by adding radioactive acetic acid to the culture medium. Second, radioactive acetylated histone has to be purified from the cells. Following the reaction, it is necessary to extract and separate the radioactive acetyl group, which has been released from acetylated histone, using ethyl acetate to measure the activity of the enzyme based on the radioactivity.

Although a method for measuring the activity of deacetylase without the use of radioactive substances was reported in recent years, owing to the use of fluorescent-labeled acetylated lysine as a substrate, the reaction product must be separated from the intact substrate and the fluorescent intensity measured by reverse phase HPLC. As mentioned above, these measurement systems are difficult to adapt for processing many samples under a variety of conditions, because of their complicated operation. Thus a simple

system for biochemical analysis as well as for inhibitor screening without the use of radioactive substances is preferred.

2. Overview

Abcam's SIRT2 Activity Assay Kit (Fluorometric) (ab156066) detects deacetylase activity of recombinant SIRT2.

Primarily, Abcam's SIRT2 Activity Assay Kit (Fluorometric) is designed for the rapid and sensitive evaluation of SIRT2 inhibitors or activators using recombinant SIRT2 or purified SIRT2.

This Kit has been optimized for assaying SIRT2 from nuclear extracts (see Section 10) but if required a cytoplasmic extraction can be carried out using the following kits: Cell Fractionation Kit – Standard (ab109719) and Cell Fractionation Kit - High Throughput (HT) (ab109718).

Applications for this kit include:

1) Screening inhibitors or activators of SIRT2.
2) Detecting the effects of pharmacological agents on SIRT2.

3. Principle of the Assay

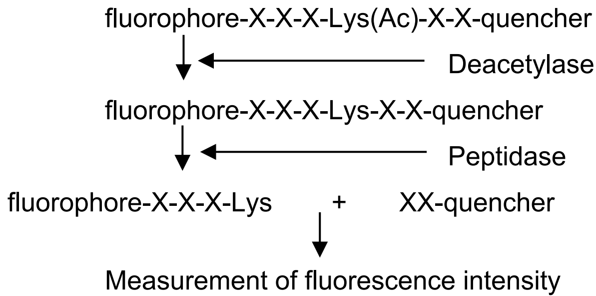
Abcam's SIRT2 Activity Assay Kit (Fluorometric) measures the activity of SIRT2 by the basic principle of changing a SIRT2 reaction into the activity of the peptidase. In order to measure the enzyme activity of SIRT2, which is the NAD dependent Histone deacetylase, and its homolog, this kit is designed so that the activity of NAD dependent Histone deacetylase can be measured under existence of Trichostatin A, which is the powerful inhibitor of HDACs.

In this kit, fluorophore and quencher are coupled to amino terminal and carboxyl terminal of substrate peptide, respectively, and before reaction of deacetylase, the fluorescence cannot be emitted. However, if SIRT2 performs deacetylation, substrate peptide will become cut by the action of peptidase added simultaneously, quencher will separate from fluorophore, and fluorescence will be emitted. Deacetylase enzyme activity is measured by measuring this fluorescence intensity.

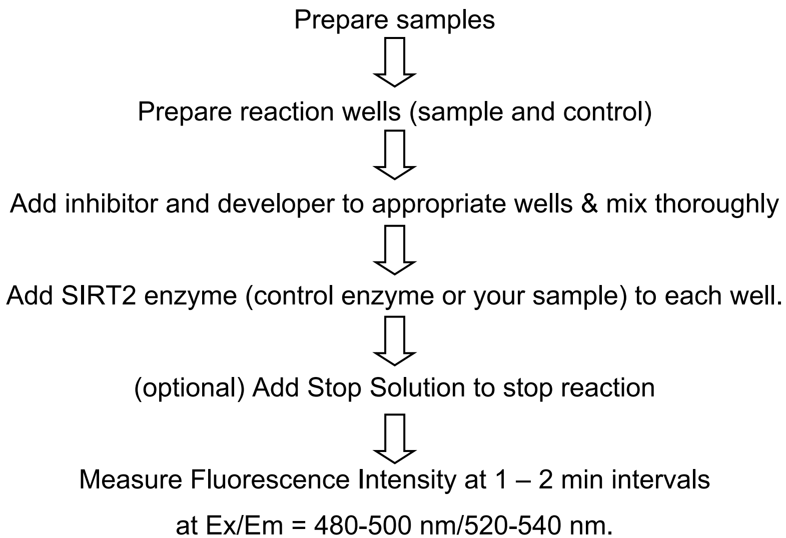
Since it is very simple to measure and it can be performed at a low price, the measurement of SIRT2 activity in most laboratories is possible if they are equipped with a fluorescent reader for microtiter plates. Considering that the use of fully automatic apparatus to measure fluorescence intensity has become widespread, SIRT2 activity measurement, which could not be made by the conventional method, is now possible with the SIRT2 Activity Assay Kit

(Fluorometric) using the same equipment. This new method of measurement should dramatically raise the efficiency of inhibitor screening and biochemical analysis of these enzymes.

Assay Principle:



4. Protocol Summary



5. Materials Supplied

Item	Identifier	Quantity	Storage
SIRT2 Assay Buffer	#1	2 x 1 mL	-20°C
Fluoro-Substrate Peptide (0.08 mM)	#2	1 x 500 µL	-20°C
Fluoro-Deacetylated Peptide (0.08 mM)	#3	1 x 100 µL	-20°C
NAD (8 mM)	#4	1 x 500 µL	-20°C
Developer	#5	1 x 500 µL	-80°C
Recombinant SIRT2	#6	1 x 500 µL	-80°C
Stop Solution	#7	2 x 1 mL	-20°C

6. Storage and Stability

All reagents included in this kit have been tested for stability. Upon receipt, store the Developer and Recombinant SIRT2 at -80°C, all other kit reagents should be stored below -20°C.

- Thaw Fluoro-Substrate Peptide and Fluoro-Deacetylated Peptide at room temperature before use. Then, thaw the other reagents in ice and use after they are completely thawed.

- Avoid repeated freezing and thawing of ‘Developer’ and ‘Recombinant SIRT2’. There is a possibility that the enzyme activity may be inactivated. Aliquot to 10-20 µL and store at -80°C.

- Avoid mixing of protease/peptidase inhibitors such as PMSF, or alkyl amine in samples that will be measured SIRT2 activity.

7. Materials Required, Not Supplied

- 96 well plate – black wells
- MilliQ water or other type of double distilled water (ddH_2O)
- Microcentrifuge
- Pipettes and pipette tips
- Microplate reader capable of measuring fluorescence at 480-500 nm (ex) and 520-540 nm (em).
- Orbital shaker
- Deionized water of the highest quality (ddH_2O)
- 500 or 1000 mL graduated cylinder
- Reagent reservoirs
- Control compound(s)

8. Assay Protocol

Abcam's SIRT2 Activity Assay Kit (Fluorometric) can measure the enzyme activity of SIRT2 with a homogeneous method. In this method, the reaction is initiated and the fluorescence intensity is measured by mixing simultaneously fluorescence-labeled acetylated peptide, which is a substrate, SIRT2, NAD and the developer. Since the reaction is not stopped, it is necessary to measure fluorescence intensity at regular intervals after the reaction is initiated, and to determine reaction velocity. Alternatively, within a time in which the reaction velocity is kept constant, it is also possible to stop the reaction by adding stop solution and to measure fluorescence intensity.

1. Assay for Quantification of SIRT2 Activity

1. Following the table below and **in duplicate,** add ddH_2O, #1. SIRT2 Assay Buffer, #2. Fluoro-Substrate Peptide and #4. NAD to microtiter plate wells.
2. Add #5. Developer to each well of the microtiter plate and mix well.

Assay reagents	Test sample	No enzyme control	No Test Sample control	No NAD control
ddH_2O	25 µL	25 µL	25 µL	30 µL
#1. SIRT2 Assay Buffer	5 µL	5 µL	5 µL	5 µL
#2. Fluoro-Substrate Peptide	5 µL	5 µL	5 µL	5 µL
#4. NAD	5 µL	5 µL	5 µL	-
#5. Developer	5 µL	5 µL	5 µL	5 µL
Enzyme Sample	5 µL	-	-	5 µL
Buffer of Enzyme Sample(1)	-	5 µL	-	-
#6. Recombinant SIRT2	-	-	5 µL	-
Total volume	50 µL	50 µL	50 µL	50 µL

(1)More information on Sample preparation in section 10

3. Initiate reactions by adding 5 µL of your Enzyme Sample or Buffer of Enzyme Sample or #6. Recombinant SIRT2 to each well and mixing thoroughly at room temperature.
   NOTE: Although the volume of addition of Enzyme Sample or Buffer of Enzyme Sample or #6. Recombinant SIRT2 is set to 5 µL in the previous table, it may be changed to a volume up to 20 µL at your discretion. In that case, please reduce the volume of Distilled water to set the final reaction volume of 50 µL.

4. Read fluorescence intensity for 30 to 60 minutes at 1 to 2 minute intervals using microtiter plate fluorometer with excitation at 480-500 nm and emission at 520-540nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Alternative procedure

1. Follow procedure described above till step 4.
2. While the reaction rate is kept constant, add 20 μL of #7. Stop Solution to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader capable of excitation at a wavelength in the range 480-500 nm and detection of emitted light in the range 520-540 nm.

NOTE:

- During the time in which SIRT2 reaction rate is maintained, the difference in fluorescence intensity between Enzyme Sample Assay and No Enzyme Control Assay indicates the SIRT2 activity of your Enzyme Sample.

- If enzyme samples contain some protease/peptidase able to break down #2. Fluoro-Substrate Peptide, resulting in an increase of fluorescence intensity in No NAD Control Assay, the SIRT2 activity in the samples cannot be evaluated correctly.

- If enzyme samples contain inhibitors for protease/peptidase, precise SIRT2 enzyme activity cannot be measured. Since protease/peptidase inhibitors used in the usual protein purification process strongly inhibit the peptidase activity in the development reaction, please avoid using any protease/peptidase inhibitors during the process of protein purification.

- If enzyme samples have an inhibitory effect on the peptidase in the development reaction, the final fluorescence intensity will not increase. Please use #3. Fluoro-Deacetylated Peptide instead of #2. Fluoro-Substrate Peptide, and conduct a control experiment.

2. Assay for SIRT2 Inhibitor/Activator Screening

1. Following the table on the next page and **in duplicate**, add ddH$_2$O, #1. SIRT2 Assay Buffer, #2. Fluoro-Substrate Peptide or #3. Fluoro-Deacetylated Peptide and #4. NAD to microtiter plate wells.
2. Add Test Compound (inhibitor compound to test) or just the Solvent in which compound is dissolved (control) or Control Compound (not provided) to each well of the microtiter plate and mix.
3. Add #5. Developer to each well of the microtiter plate and mix well.
4. Initiate reactions by adding 5 μL of your of #6. Recombinant SIRT2 or your Enzyme Sample to each well and mix thoroughly at RT.

   NOTE: Although the volume of addition of Recombinant SIRT2 or your Enzyme Sample is set to 5 μL in above tables, it may be changed to a volume up to 20 μL at your discretion. In that case, please reduce the volume of ddH$_2$O to set the final reaction volume of 50 μL.
5. Read fluorescence intensity for 30 to 60 minutes at 1 to 2 minute intervals using microtiter plate fluorometer with excitation at 480-500 nm and emission at 520-540nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Assay reagents	Test Compound Assay	Solvent Control Assay	Control Compound Assay	No Enzyme Control Assay	Development Control Assay
ddH_2O	20 µL	20 µL	20 µL	25 µL	30 µL
#1. SIRT2 Assay Buffer	5 µL	5 µL	5 µL	5 µL	5 µL
#2. Fluoro-Substrate Peptide	5 µL	5 µL	5 µL	5 µL	-
#3. Fluoro-Deacetylated Peptide	-	-	-	-	5 µL
#4. NAD	5 µL	5 µL	5 µL	5 µL	-
Test Compound	5 µL	-	-	-	5 µL
Solvent of Test Compound	-	5 µL	-	5 µL	-
Control Compound (not provided)	-	-	5 µL	-	-
#5. Developer	5 µL	5 µL	5 µL	5 µL	5 µL
#6. Recombinant SIRT2 (or Enzyme Sample)	5 µL	5 µL	5 µL	-	-
Total volume	50 µL	50 µL	50 µL	50 µL	50 µL

(1)More information on Sample preparation in section 10

Alternative procedure

1. Follow procedure described above till step 4.
2. While the reaction rate is kept constant, add 20 μL of #7. Stop Solution to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader capable of excitation at a wavelength in the range 480-500 nm and detection of emitted light in the range 520-540 nm.

NOTE:

- Although the above tables indicate the volume of addition of Test Compound or Solvent of Test Compound or Control Compound (not provided) as 5 μL, the concentration and the volume of the reagents to add can be changed so that the concentration of test compounds becomes the setting concentration. For example, since the final volume of reaction is 50 μL here, it is also possible to add 10 μL of Test Compound or Solvent of Test Compound or Control Compound (not provided). In this case, please reduce the volume of Distilled water to set the final reaction volume of 50 μL.

- During the time in which SIRT2 reaction rate is maintained, the difference in fluorescence intensity between Solvent Control Assay and No Enzyme Control Assay indicates the SIRT2 activity.

- In order to estimate the active or inhibitory effect on SIRT2 activity by the test compounds correctly, it is necessary to conduct the control experiment of Solvent Control Assay at least once for every experiment and Control Compound Assay at least once for the first experiment, in addition to Test Compound Assay as indicated in the Table.2. When test compounds cause an active or inhibitory effect on SIRT2 activity, the level of increase of fluorescence intensity is strengthened or weakened as compared with Solvent Control Assay.

- The efficacy of the test compounds on the SIRT2 activity is the difference in fluorescence intensity between Test Compound Assay minus No Enzyme Control Assay and Solvent Control Assay minus No Enzyme Control Assay.

- If test compounds have an inhibitory effect on protease/peptidase, resulting that the increase in fluorescence intensity is not or a little observed in Development Control Assay, the effect on SIRT2 activity cannot be evaluated correctly.

9. Data Analysis

1. Typical Results

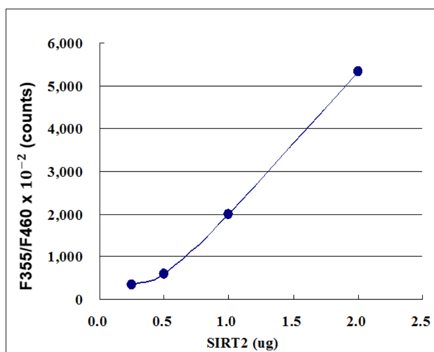


Figure 1: Dose dependency curve of recombinant SIRT2 activity

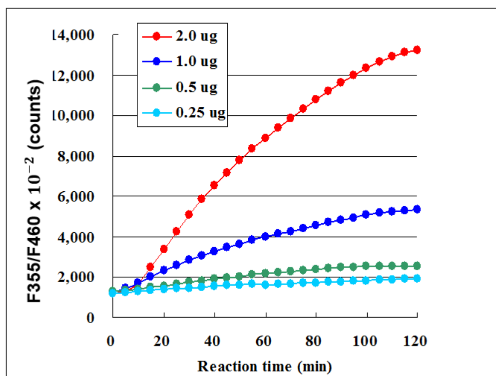


Figure 2: Time course of SIRT2-substrate deacetylation by recombinant SIRT2

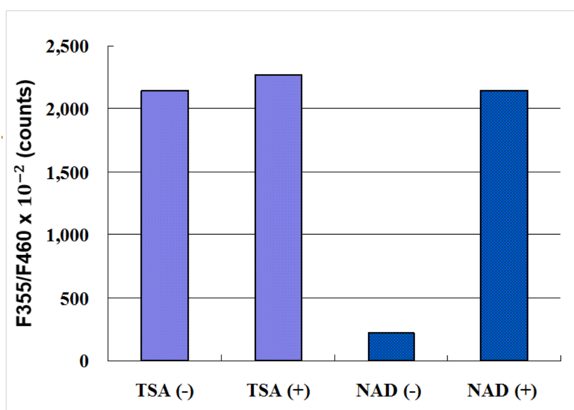


Figure 3: Effect of Trichostatin A and NAD on recombinant SIRT2 activity

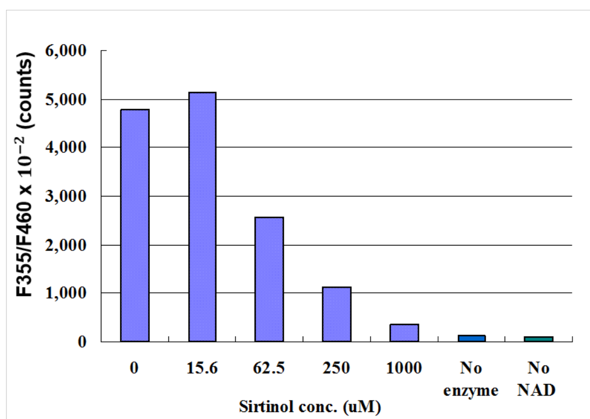


Figure 4: Effect of Sirtinol on recombinant SIRT2 activity

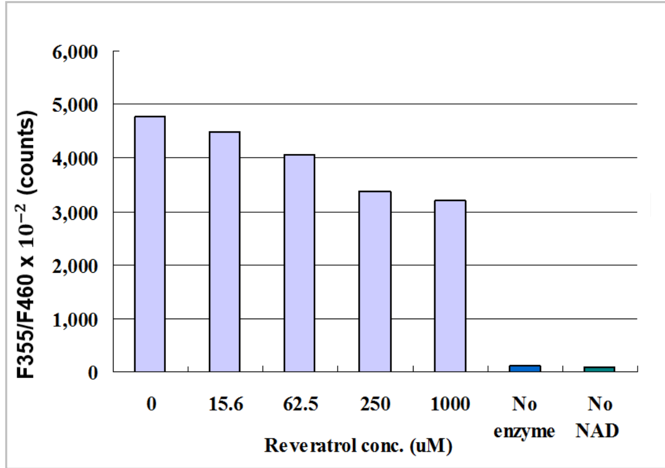


Figure 5: Effect of Resveratrol on recombinant SIRT2 activity

10. Sample Preparation

Numerous extraction and purification methods can be used to isolate SIRT2. The following protocols have been shown to work with a number of different cells and enzyme sources and are provided as examples of suitable methods. Crude samples can frequently be used without dilution while more concentrated or highly purified SIRT2 should be diluted.

It is strongly advised that the user always perform an initial experiment to determine the proper dilution to be used in subsequent experiments. This need not be any more than a single time point assay using serial dilutions of the crude extract, cell lysate or sample fraction taken prior to a purification step. All sample preparation should be performed at 4°C and recovered fractions should be kept at -80°C to prevent loss of enzymatic activity.

A. Buffer Preparation

Lysis Buffer	**Sucrose Cushion**	**Extraction Buffer**
10 mM Tris HCl (pH 7.5) 10 mM NaCl 15 mM $MgCl_2$ 250 mM Sucrose 0.5 % NP-40 0.1 mM EGTA	30 % Sucrose 10 mM Tris HCl (pH 7.5) 10 mM NaCl 3 mM $MgCl_2$	50 mM Hepes KOH (pH 7.5) 420 mM NaCl 0.5 mM EDTA Na_2 0.1 mM EGTA 10 % glycerol

B. Isolation of Nuclei

1. Resuspend 1x 10^7 cells into 1 mL of lysis buffer.
2. Vortex for 10 second.
3. Keep on ice for 15 min.
4. Spin the cells through 4 ml of sucrose cushion at 1,300 x *g* for 10 min at +4°C.
5. Discard the supernatant.
6. Wash the nuclei pellet once with cold 10 mM Tris HCl (pH7.5), 10 mM NaCl.

C. Extraction of Nuclei

1. Resuspend the isolated nuclei in 50-100 μL of extraction buffer.
2. Sonicate for 30 seconds.
3. Stand on ice for 30 min.
4. Centrifuge at 20,000 x *g* for 10 min.
5. Take supernatant (the crude nuclear extract).
6. Determine protein concentration by Bradford method or equivalent.
7. Store the crude nuclear extract at -80°C until use.

11. Troubleshooting

- When chemicals that have an inhibitory effect on the peptidase are mixed in a crude SIRT2 fraction purified from various cells or the immunoprecipitate using a specific antibody against SIRT2 or other proteins, precise SIRT2 enzyme activity cannot be measured. Since the protease/peptidase inhibitors used in the usual protein purification process inhibit the peptidase activity strongly, please avoid the use of any protease/peptidase inhibitors during the protein purification process.

- Final fluorescence intensity will not increase, both when test chemicals have an inhibitory effect on SIRT2, and also when there is an inhibitory effect on the peptidase.

- If the test reagents themselves emit fluorescence at excitation wavelength: 480-500 nm and fluorescence wavelength: 520-540 nm, the inhibitory effect of the test assay cannot be evaluated correctly.

- The recombinant SIRT2 should be run in duplicate, using the protocol described in the Assay Protocol. Incubation times or temperatures significantly different from those specified may give erroneous results.

- The reaction curve is nearly a straight line if the kinetics of the assay is of the first order. Variations in the protocol can lead to non-linearity of the curve, as can assay kinetics that are other than first order. For a non-linear curve, point to point or quadratic curve fit methods should be used.

- Poor duplicates indicate inaccurate dispensing. If all instructions in the Assay Protocol were followed accurately, such results indicate a need for multi-channel pipettor maintenance.

12. Notes

Technical Support

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