

ab156065

SIRT1 Activity Assay Kit

(Fluorometric)

Instructions for Use

For the quantitative measurement of SIRT1 activity in cell lysates

[View kit datasheet: www.abcam.com/ab156065](http://www.abcam.com/ab156065)

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

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1. Background

SIRT1 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 SIRT1 protein is a NAD(+)-dependent histone deacetylase that plays a critical role in transcriptional silencing, genome stability and longevity. A Human homologue of Sir2, SIRT1, also functions as a NAD(+)-dependent-p53 deacetylase as well as a NAD(+)-dependent histone deacetylase. SIRT1 was shown to regulate the activity of the p53 tumor suppressor and inhibits apoptosis. These results have significant implications regarding an important role for SIRT1 in modulating the sensitivity of cells in p53-dependent apoptotic response and the possible effect in cancer therapy. Since the function of p53 is made to strengthen powerfully by using together with DNA damaging reagent, it is expected that inhibitor of SIRT1 becomes an effective anticancer drug.

However, the conventional method for measuring SIRT1 activity is very complicated and laborious. In order to measure SIRT1 enzyme activity, it is necessary to prepare radioactive acetylated histone 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 SIRT1 Activity Assay Kit (Fluorometric) (ab156065) detects SIRT1 activity in lysates. Primarily, the SIRT1 Activity Assay Kit is designed for the rapid and sensitive evaluation of SIRT1 inhibitors or activators using crude SIRT1 fraction or purified SIRT1. Additionally, any cultured primary cell, cell line, or tissue homogenate can be assayed for SIRT1 activity with the SIRT1 Activity Assay Kit if the appropriate antibody directed against SIRT1 is used for immunoprecipitation.

Abcam's SIRT1 Activity Assay Kit (Fluorometric) has been shown to detect the activity of Sirtuins, at least SIRT1 in Human or animal cell lysates or in column fractions. The assay shows good linearity of sample response. The assay may be used to follow the purification of Sirtuins or may be used to detect the presence of Sirtuins in cell lysates.

Applications for this kit include:

- 1) Monitoring the purification of SIRT1.
- 2) Screening inhibitors or activators of SIRT1.
- 3) Detecting the effects of pharmacological agents on SIRT1.

3. Principle of the Assay

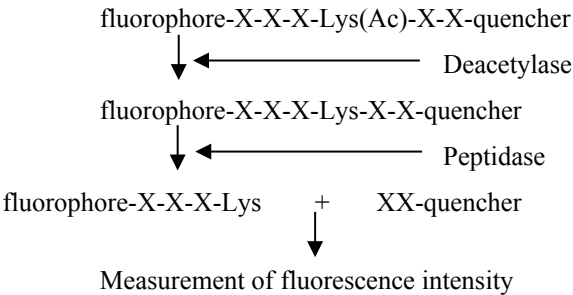
Abcam's SIRT1 Activity Assay Kit (Fluorometric) measures the activity of SIRT1 by the basic principle of changing a SIRT1 reaction into the activity of the protease. In order to measure the enzyme activity of SIRT1, which is the NAD dependent Histone deacetylase, and its homolog, this kit is designed 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 SIRT1 performs deacetylation, substrate peptide will

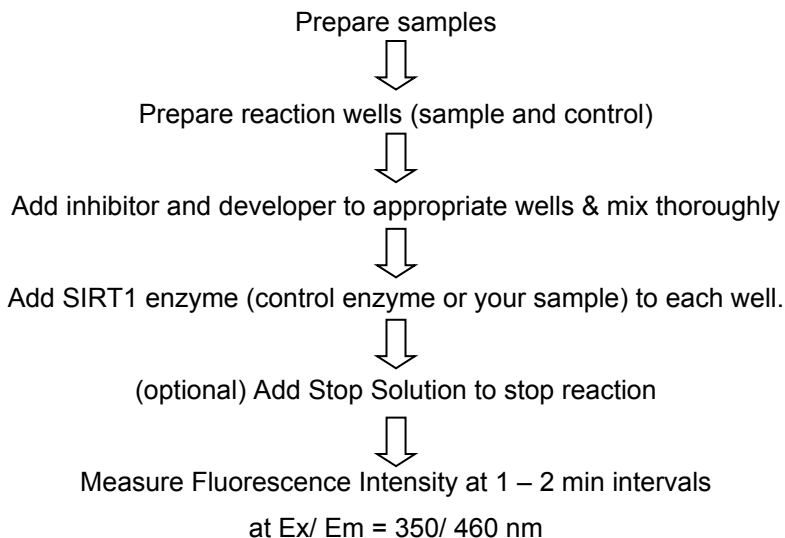
become cut by the action of protease added simultaneously, quencher will separate from fluorophore, and fluorescence will be emitted. Deacetylase enzyme activity is measured by measuring this fluorescence intensity.

Since it is very simple to measure and it can be performed at a low price, the measurement of SIRT1 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, SIRT1 activity measurement, which could not be made by the conventional method, is now possible with the SIRT1 Activity Assay Kit 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
SIRT1 Assay Buffer	#1	2 x 1 mL	-20°C
Fluoro-Substrate Peptide (0.2 mM)	#2	500 µL	-20°C
Fluoro-Deacetylated Peptide (0.2 mM)	#3	100 µL	-20°C
NAD (2 mM)	#4	500 µL	-20°C
Developer	#5	500 µL	-80°C
Recombinant SIRT1	#6	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 SIRT1 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 Recombinant SIRT1. 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 SIRT1 activity.
- Do not expose reagents to excessive light.

7. Materials Required, Not Supplied

- 96 well plate – black wells
- MilliQ water or other type of double distilled water (ddH₂O)
- Microcentrifuge
- Pipettes and pipette tips
- Microplate reader capable of measuring fluorescence at Ex/Em = 340-360/ 440-460 nm
- Orbital shaker
- 500 or 1000 mL graduated cylinder
- Reagent reservoirs

For SIRT1 Activity in an Immunoprecipitate:

- Cell Lysis Buffer (1X): 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT.
- 50 mM Tris-HCl (pH 8.8), 0.5 mM DTT
- Protein A Agarose Beads

8. Assay Protocol

Abcam's SIRT1 Activity Assay Kit (Fluorometric) can measure the enzyme activity of SIRT1 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 substrate, SIRT1, trichostatin A, NAD and lysylendopeptidase. 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 2X stop solution and to measure fluorescence intensity

1. Assay for Quantification of SIRT1 Activity

1. Following the table below and **in duplicate**, add ddH₂O, SIRT1 Assay Buffer (#1), Fluoro-Substrate Peptide (#2) and NAD (#4) to microtiter plate wells.
2. Add Developer (#5) to each well of the microtiter plate and mix well.

Assay reagents	Test sample	No enzyme control	Positive Control	No NAD control
ddH ₂ O	25 µL	25 µL	25 µL	30 µL
SIRT1 Assay Buffer (#1)	5 µL	5 µL	5 µL	5 µL
Fluoro-Substrate Peptide (#2)	5 µL	5 µL	5 µL	5 µL
NAD (#4)	5 µL	5 µL	5 µL	-
Developer (#5)	5 µL	5 µL	5 µL	5 µL
Enzyme Sample	5 µL	-	-	5 µL
Buffer of Enzyme Sample ⁽¹⁾	-	5 µL	-	-
Recombinant SIRT1 (#6)	-	-	5 µL	-
Total volume	50 µL	50 µL	50 µL	50 µL

(1) More information on Sample preparation in Section 10

- Initiate reactions by adding 5 µL of your Enzyme Sample or Buffer of Enzyme Sample or Recombinant SIRT1 (#6) to each well and mixing thoroughly at room temperature.

NOTE: Although the volume of addition of Enzyme Sample or Buffer of Enzyme Sample or Recombinant SIRT1 (#6) 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 340-360 nm and emission at 440-460 nm. 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 μ L of Stop Solution (#7) 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 340 – 360 nm and detection of emitted light in the range 440 – 460 nm.

NOTE:

- *During the time in which SIRT1 reaction rate is maintained, the difference in fluorescence intensity between Enzyme Sample Assay and No Enzyme Control Assay indicates the SIRT1 activity of your Enzyme Sample.*
- *If enzyme samples contain some protease/peptidase able to break down Fluoro-Substrate Peptide (#2), resulting in an increase of fluorescence intensity in No NAD Control Assay, the SIRT1 activity in the samples cannot be evaluated correctly.*

- *If enzyme samples contain inhibitors for protease/peptidase, precise SIRT1 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 Fluoro-Deacetylated Peptide (#3) instead of Fluoro-Substrate Peptide (#2), and conduct a control experiment.*

2. Assay for SIRT1 Inhibitor/Activator Screening

1. Following the table on the next page and **in duplicate**, add ddH₂O, SIRT1 Assay Buffer (#1), Fluoro-Substrate Peptide (#2) or Fluoro-Deacetylated Peptide (#3) and NAD (#4) 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 Developer (#5) to each well of the microtiter plate and mix well.

4. Initiate reactions by adding 5 μL of your of Recombinant SIRT1 (#6) or your Enzyme Sample to each well and mix thoroughly at RT.

NOTE: *Although the volume of addition of Recombinant SIRT1 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 ddH₂O 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 340 – 360 nm and emission at 440 – 460 nm. 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
ddH₂O	20 µL	20 µL	20 µL	25 µL	30 µL
SIRT1 Assay Buffer (#1)	5 µL	5 µL	5 µL	5 µL	5 µL
Fluoro-Substrate Peptide (#2)	5 µL	5 µL	5 µL	5 µL	-
Fluoro-Deacetylated Peptide (#3)	-	-	-	-	5 µL
NAD (#4)	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	-	-
Developer (#5)	5 µL	5 µL	5 µL	5 µL	5 µL
Recombinant SIRT1 (#6) (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 Stop Solution (#7) to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader at Ex/ Em = 350 – 380 nm/440 – 460 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 SIRT1 reaction rate is maintained, the difference in fluorescence intensity between Solvent Control Assay and No Enzyme Control Assay indicates the SIRT1 activity.*

- *In order to estimate the active or inhibitory effect on SIRT1 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 SIRT1 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 SIRT1 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 SIRT1 activity cannot be evaluated correctly.*

3. SIRT1 Activity In An Immunoprecipitate

Reagents Required (not supplied):

- Cell Lysis Buffer (1X): 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT.
- Protein A Agarose Beads: Add 5 mL of 1X PBS to 1.5 g of Protein A Agarose Beads. Shake 2 hours at 4°C; spin down. Wash the pellet twice with PBS. Re-suspend beads in 1 volume of PBS (can be stored for 2 weeks at 4°C).
- 50 mM Tris-HCl (pH 8.8), 0.5 mM DTT

Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing test compound for desired time.
2. To harvest cells under non-denaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 mL 1X ice-cold Cell Lysis Buffer to each plate (10 cm dish) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate 4 times for 5 seconds each on ice.
6. Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at -80°C.

Immunoprecipitation

1. Take 200 μ L cell lysate and incubate with a suitable anti-SIRT1 antibody according to the manufacturer's instructions.
2. Add Protein A agarose beads (20 μ L of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
3. Microcentrifuge for 30 seconds at 4°C.
4. Wash pellet 3 times with 500 μ L of 1X Cell Lysis Buffer (20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT) and once with 500 μ L of cold 50 mM Tris-HCl (pH 8.8), 0.5 mM DTT. Keep on ice during washes.
5. After immunoprecipitation, add reaction mixture containing Fluoro-Substrate peptide solution to Protein A agarose beads as an "Enzyme Sample" and measure NAD dependent deacetylase activity according to the procedure in Section 8.1 1. Assay for Quantification of SIRT1 Activity.

9. Data Analysis

Typical Results

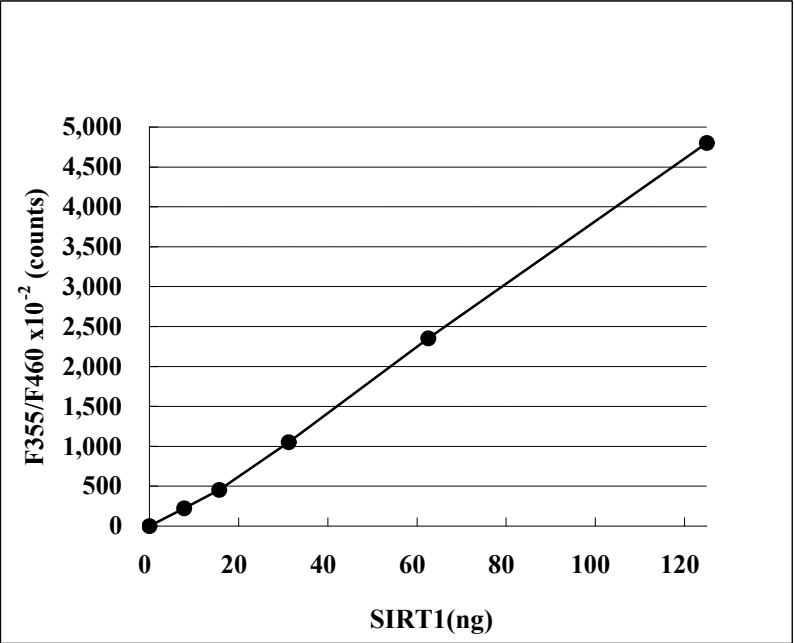


Figure 1. Dose dependency curve of recombinant SIRT1 activity

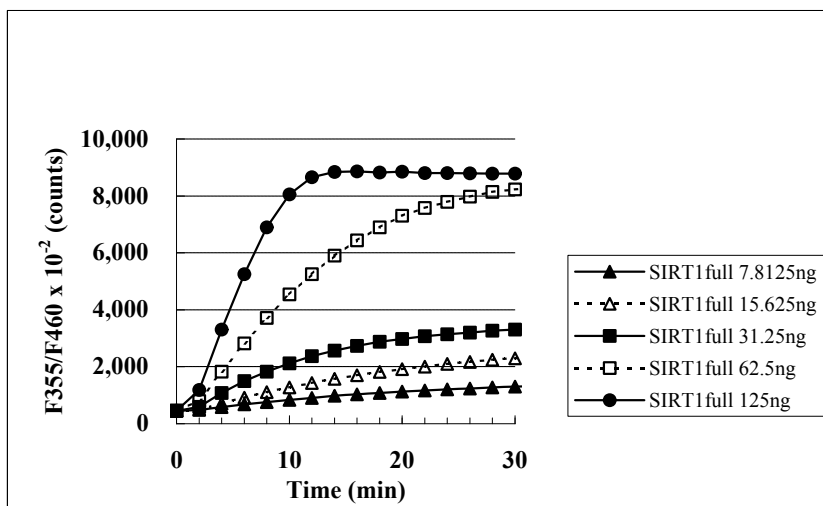


Figure 2. Time course of SIRT1-substrate deacetylation by recombinant SIRT1

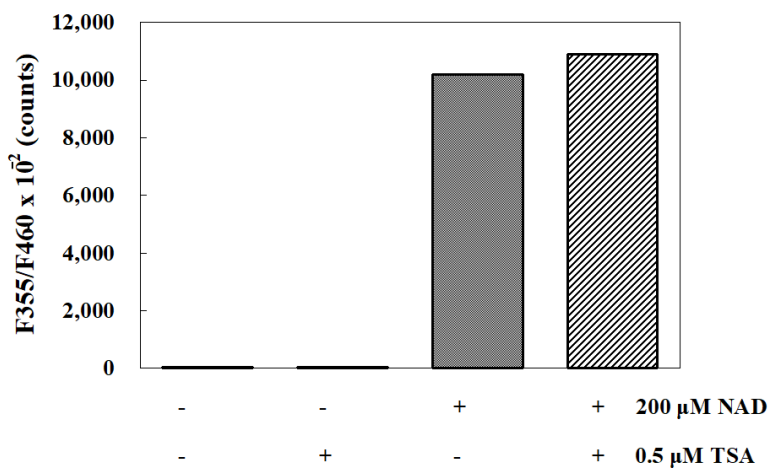


Figure 3. Effect of Trichostatin A and NAD on recombinant SIRT1 activity

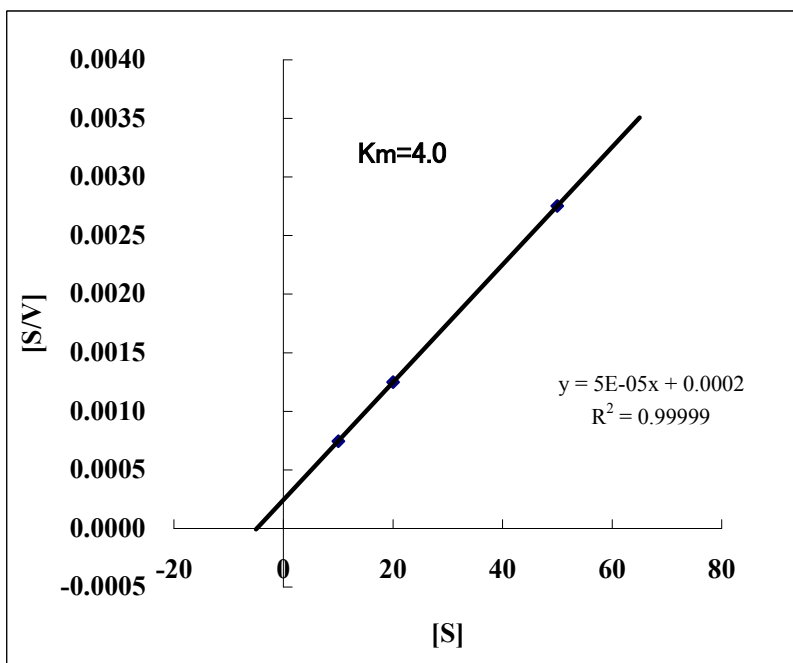


Figure 4. K_m value of recombinant SIRT1 for Fluoro-Substrate Peptide

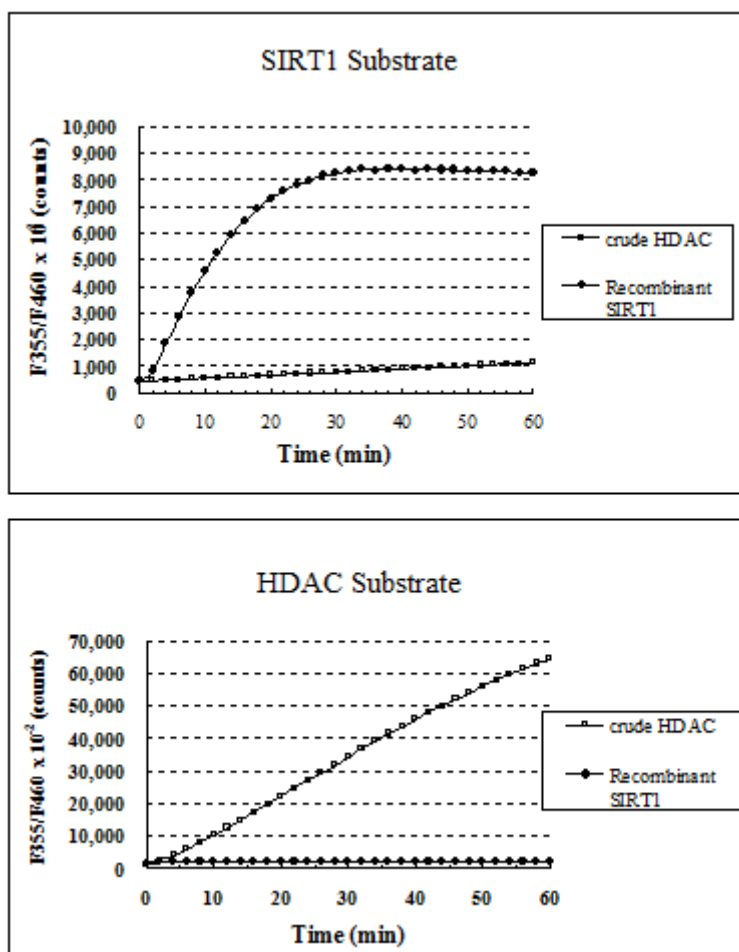


Fig.5 Substrate Preference of HDAC and SIRT1

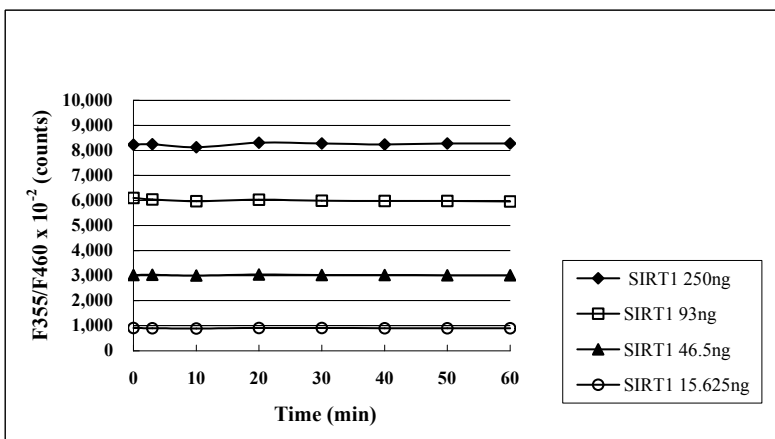


Figure 6. Stability of Fluorescence Intensity after Stop the Reaction

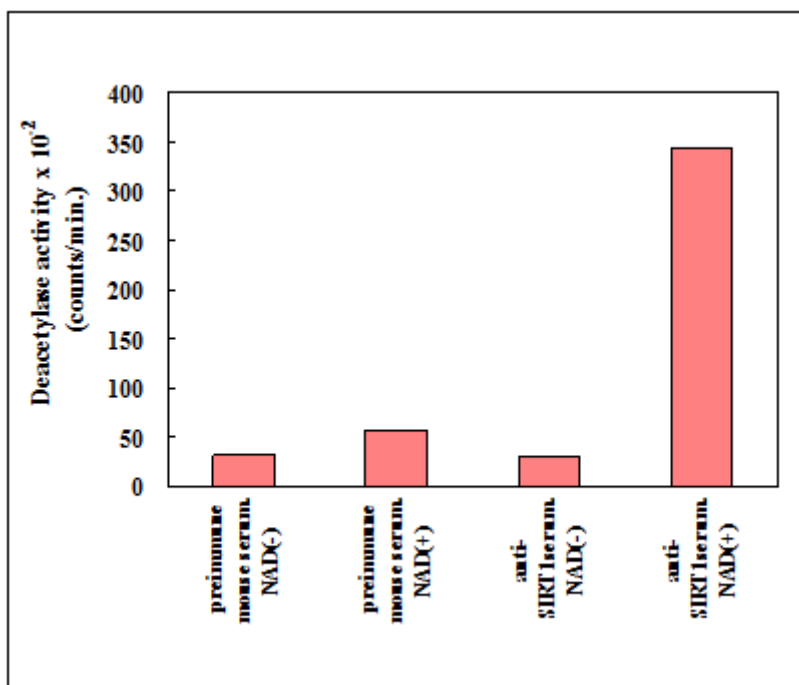


Figure 7. Measurement of 293T cell endogenous SIRT1 activity in an immunoprecipitate using antibody

10. Sample Preparation

Numerous extraction and purification methods can be used to isolate SIRT1. 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 SIRT1 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 1×10^7 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 $1,300 \times g$ for 10 min at $+4^\circ\text{C}$.
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 $20,000 \times g$ 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 SIRT1 fraction purified from various cells or the immunoprecipitate using a specific antibody against SIRT1 or other proteins, precise SIRT1 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 SIRT1, and also when there is an inhibitory effect on the peptidase.
- If the test reagents themselves emit fluorescence at excitation wavelength: 340 – 360 nm and fluorescence wavelength: 440 – 460 nm, the inhibitory effect of the test assay cannot be evaluated correctly.
- The recombinant SIRT1 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.

Technical Support

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