



Quantitative test kit for NAD(+)-dependent deacetylase SIRT1 activity

CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit Ver.2

100 Assays

Cat# CY-1151V2

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Intended Use

The MBL Research Product **CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit** detects SIRT1/Sir2 activity in lysates. Primarily, the MBL Research Product **CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit** is designed for the rapid and sensitive evaluation of SIRT1/Sir2 inhibitors or activators using crude SIRT1/Sir2 fraction or purified SIRT1/Sir2. Additionally, any cultured primary cell, cell line, or tissue homogenate can be assayed for SIRT1/Sir2 activity with the MBL Research Product **CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit** if the appropriate antibody directed against SIRT1 or Sir2 is used for immunoprecipitation.

Applications for this kit include:

- 1) Monitoring the purification of SIRT1/Sir2.
- 2) Screening inhibitors or activators of SIRT1/Sir2.
- 3) Detecting the effects of pharmacological agents on SIRT1/Sir2.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store #5. Developer and #6. Recombinant SIRT1 at -70°C and all other components below -20°C.
- Do not expose reagents to excessive light.



Introduction

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. 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/Sir2 activity is very complicated and laborious. In order to measure SIRT1/Sir2 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.



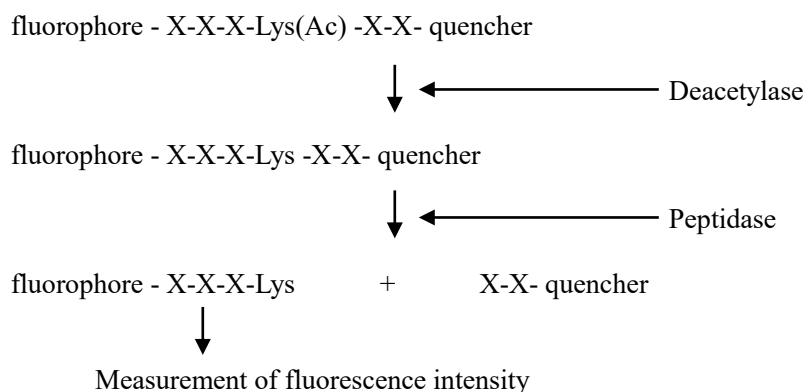
Principle of the Assay

The CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit measures the activity of SIRT1/Sir2 by the basic principle of changing a SIRT1/Sir2 reaction into the activity of the peptidase. In order to measure the enzyme activity of SIRT1/Sir2, 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/Sir2 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 SIRT1/Sir2 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/Sir2 activity measurement, which could not be made by the conventional method, is now possible with the CycLex SIRT1/Sir2 Deacetylase Fluorometric 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.

Measuring Principle of The CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit





Materials Provided

Components of Kit

Components	Quantity	Storage
#1. SIRT1 Assay Buffer	1 mL x 2	Below -20°C
#2. Fluoro-Substrate Peptide (0.2 mM)	500 µL x 1	Below -20°C
#3. Fluoro-Deacetylated Peptide (0.2 mM)	100 µL x 1	Below -20°C
#4. NAD (2 mM)	500 µL x 1	Below -20°C
#5. Developer	500 µL x 1	-70°C
#6. Recombinant SIRT1	500 µL x 1	-70°C
#7. Stop Solution	1 mL x 2	Below -20°C
Instruction manual	1	Room temp.

Materials Required but not Provided

- **Microplate for fluorometer**
- **Microplate reading fluorometer** capable of excitation at a wavelength in the range 340-360 nm and detection of emitted light in the range 440-460 nm.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Multi-channel pipette**
- **Microplate shaker**
- **Deionized water of the highest quality**
- **500 or 1,000 mL graduated cylinder**
- **Reagent reservoirs**
- **Control compound(s)**



Precautions

- Please thaw “#2. Fluoro-Substrate Peptide” and “#3. Fluoro-Deacetylated Peptide” at room temperature before use. Then, thaw the other reagents in ice and use after they are completely thawed.
- Please avoid repeated freezing and thawing of “#5. Developer” and “#6. Recombinant SIRT1”. There is a possibility that the enzyme activity may be inactivated. Aliquot to 10-20 μ L and store at -70°C .
- Please avoid mixing of protease/peptidase inhibitors such as PMSF, or alkyl amine in samples that will be measured SIRT1/Sir2 activity.
- Do not use kit components beyond the indicated kit expiration date.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- Do not mouth pipet or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**

NOTE: THE FOLLOWING PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER.

For research use only, not for use in diagnostic or therapeutic procedures



Detailed Protocol

The CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit can measure the enzyme activity of SIRT1/Sir2 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, SIRT1/Sir2, 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 Method for Measurement of SIRT1/Sir2 Activity

- Following Table.1 below, first, add “**Distilled water**”, “**#1. SIRT1 Assay Buffer**”, “**#2. Fluoro-Substrate Peptide**” and “**#4. NAD**” to microtiter plate wells. Second “**#5. Developer**” to each well of the microtiter plate and mix well.

Table.1: Reaction mixture for measurement of SIRT1/Sir2 activity

Assay reagents	Enzyme Sample Assay	No Enzyme Control Assay	Positive Control Assay	No NAD Control Assay
Distilled water	25 μ L	25 μ L	25 μ L	30 μ L
#1. SIRT1 Assay Buffer	5 μ L	5 μ L	5 μ L	5 μ L
#2. Fluoro-Substrate Peptide	5 μ L	5 μ L	5 μ L	5 μ L
#4. NAD	5 μ L	5 μ L	5 μ L	-
#5. Developer	5 μ L	5 μ L	5 μ L	5 μ L
Enzyme Sample	5 μ L	-	-	5 μ L
Buffer of Enzyme Sample	-	5 μ L	-	-
#6. Recombinant SIRT1	-	-	5 μ L	-
Total Volume of the mixture	50 μ L	50 μ L	50 μ L	50 μ L

- Initiate reactions by adding 5 μ L of your “**Enzyme Sample**” or “**Buffer of Enzyme Sample**” or “**#6. Recombinant SIRT1**” to each well and mixing thoroughly at room temperature.
- 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.

Alternate procedure

- While the reaction rate is kept constant, add 20 μ L 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 340-360 nm and detection of emitted light in the range 440-460 nm.

Note-1: During the time in which SIRT1/Sir2 reaction rate is maintained, the difference in fluorescence intensity between “**Enzyme Sample Assay**” and “**No Enzyme Control Assay**” indicates the SIRT1/Sir2 activity of your “**Enzyme Sample**”.



Note-2: Although the volume of addition of “**Enzyme Sample**” or “**Buffer of Enzyme Sample**” or “**#6. Recombinant SIRT1**” is set to 5 μ L in Table.1, it may be changed to a volume up to 20 μ L at your discretion. In that case, please reduce the volume of “**Distilled water**” to set the final reaction volume of 50 μ L.

Note-3: 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 SIRT1/Sir2 activity in the samples cannot be evaluated correctly.

Note-4: If enzyme samples contain inhibitors for protease/peptidase, precise SIRT1/Sir2 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.

Note-5: 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 Procedures for Inhibitor/Activator Screening

1) Following Table.2 below, first, add “**Distilled water**”, “**#1. SIRT1 Assay Buffer**”, “**#2. Fluoro-Substrate Peptide**” or “**#3. Fluoro-Deacetylated Peptide**” and “**#4. NAD**” to microtiter plate wells. Second, add “**Test Compound**” or “**Solvent of Test Compound**” or “**Control Compound (not provided)**”, and “**#5. Developer**” to each well of the microtiter plate and mix well.

Table.2: Reaction mixture for inhibitor/activator screening

Assay reagents	Test Compound Assay	Solvent Control Assay	Control Compound Assay	No Enzyme Control Assay	Development Control Assay
Distilled water	20 μ L	20 μ L	20 μ L	25 μ L	30 μ L
#1. SIRT1 Assay Buffer	5 μ L	5 μ L	5 μ L	5 μ L	5 μ L
#2. Fluoro-Substrate Peptide	5 μ L	5 μ L	5 μ L	5 μ L	-
#3. Fluoro-Deacetylated Peptide	-	-	-	-	5 μ L
#4. NAD	5 μ L	5 μ L	5 μ L	5 μ L	-
Test Compound	5 μ L	-	-	-	5 μ L
Solvent of Test Compound	-	5 μ L	-	5 μ L	-
Control Compound (not provided)	-	-	5 μ L	-	-
#5. Developer	5 μ L	5 μ L	5 μ L	5 μ L	5 μ L
#6. Recombinant SIRT1 (or Enzyme Sample)	5 μ L	5 μ L	5 μ L	-	-
Total Volume of the mixture	50 μL	50 μL	50 μL	50 μL	50 μL

2) Initiate reactions by adding 5 μ L of “**#6. Recombinant SIRT1**” (or your “**Enzyme Sample**”) to each well and mixing thoroughly at room temperature.

3) 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.

*Alternate procedure*

3') 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 340-360 nm and detection of emitted light in the range 440-460 nm.

Note-1: During the time in which SIRT1/Sir2 reaction rate is maintained, the difference in fluorescence intensity between “**Solvent Control Assay**” and “**No Enzyme Control Assay**” indicates the SIRT1/Sir2 activity.

Note-2: In order to estimate the active or inhibitory effect on SIRT1/Sir2 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/Sir2 activity, the level of increase of fluorescence intensity is strengthened or weakened as compared with “**Solvent Control Assay**”.

Note-3: The efficacy of the test compounds on the SIRT1/Sir2 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**”].

Note-4: 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/Sir2 activity cannot be evaluated correctly.

Note-5: 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.

Note-6: 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 “**Distilled water**” to set the final reaction volume of 50 µL.



Troubleshooting

1. When chemicals that have an inhibitory effect on the peptidase are mixed in a crude SIRT1/Sir2 fraction purified from various cells or the immunoprecipitate using a specific antibody against SIRT1/Sir2 or other proteins, precise SIRT1/Sir2 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.
2. Final fluorescence intensity will not increase, both when test chemicals have an inhibitory effect on SIRT1/Sir2, and also when there is an inhibitory effect on the peptidase.
3. 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.
4. All samples and controls should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.
5. 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.
6. Poor duplicates indicate inaccurate dispensing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for multi-channel pipettor maintenance.

Reagent Stability

All of the reagents included in the MBL Research Product **CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, store the “**#5. Developer**” and “**#6. Recombinant SIRT1**” at -70°C, all other kit reagents should be stored below -20°C.



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 -70°C to prevent loss of enzymatic activity.

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Buffers

***Lysis Buffer:**

10 mM Tris-HCl (pH7.5)
10 mM NaCl
15 mM MgCl₂
250 mM Sucrose
0.5 % NP-40
0.1 mM EGTA

***Sucrose cushion:**

30 % Sucrose
10 mM Tris-HCl (pH7.5)
10 mM NaCl
3 mM MgCl₂

***Extraction buffer:**

50 mM Hepes-KOH (pH 7.5)
420 mM NaCl
0.5 mM EDTA Na₂
0.1 mM EGTA
10 % glycerol

Procedure

Isolation of Nuclei

1. Suspend 1×10^7 cells (100 mm dish sub-confluent) into 1ml of Lysis buffer.
2. Vortex for 10 seconds.
3. Keep on ice for 15 minutes.
4. Spin the cells through 4 ml of sucrose cushion at 1,300 x g for 10 minutes at 4 C.
5. Discard the supernatant.
6. Wash the nuclei pellet once with cold 10 mM Tris-HCl (pH7.5), 10 mM NaCl.

Extraction of Nuclei

1. Suspend the isolated nuclei in 50-100 μ L of extraction buffer.
2. Sonicate for 30 seconds.
3. Stand on ice for 30 minutes.
4. Microcentrifuge at 20,000 x g for 10 minutes.
5. Take supernatant (the crude nuclear extract).
6. Determine protein conc. by Bradford method or equivalent.
7. Store the crude nuclear extract at -70°C until use.

Note: Do not use any kind of protease/peptidase inhibitor!!



SIRT1 Activity in an Immunoprecipitate

Immunoprecipitation Followed by Measuring SIRT1 Activity Protocol

NOTE: THE FOLLOWING PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER.

Solutions and Reagents

Note: Prepare solutions with Milli-Q or equivalently purified water.

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. Resuspend beads in 1 volume of PBS. (Can be stored for 2 weeks at 4°C)

Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing test compound for desired time.
2. To harvest cells under nondenaturing 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 -70°C.

Immunoprecipitation

1. Take 200 μ L cell lysate and add anti-SIRT1 antibody (CY-P1016; 1-2 μ g; incubate with gentle rocking for 2 hours or overnight at 4°C.
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. Wash pellet 3 times with 500 μ L of 1X Cell Lysis Buffer and once with 500 μ L of cold 50 mM Tris-HCl (pH 8.8), 0.5 mM DTT. Keep on ice during washes.
4. After immunoprecipitation, according to the procedure of "1. Assay Method for Measurement of SIRT1/Sir2 Activity" in the "Detailed Protocol", add reaction mixture containing Fluoro-Substrate peptide solution to protein A agarose beads as an "Enzyme Sample" and measure NAD dependent deacetylase activity.

Note: Do not use any kind of protease/peptidase inhibitor!!

Example of Test Results

Fig.1 Dose dependency curve of recombinant SIRT1 activity

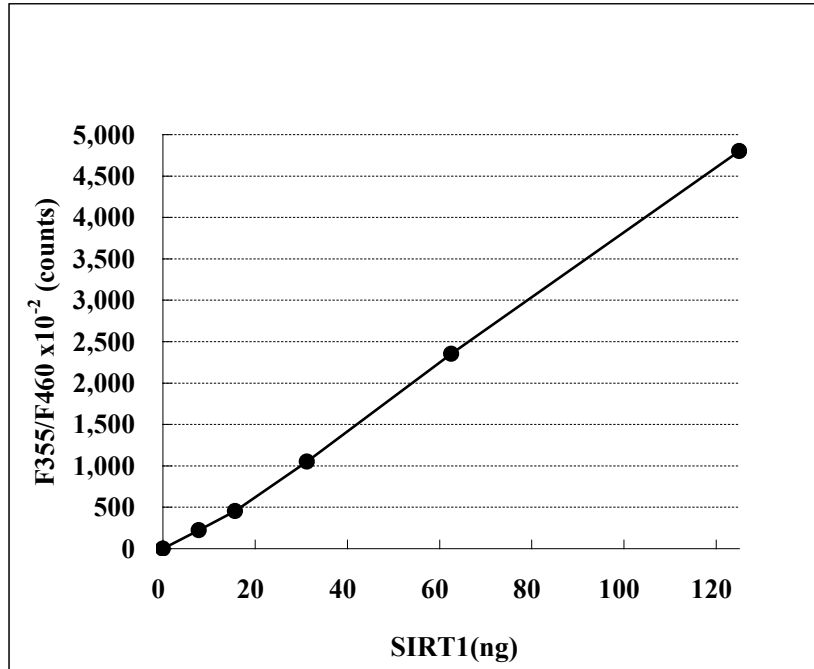


Fig.2 Time course of SIRT1-substrate deacetylation by recombinant SIRT1

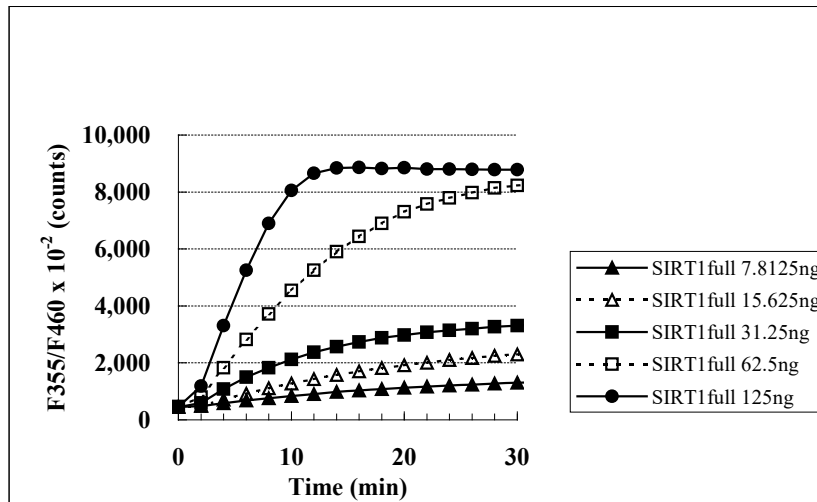


Fig.3 Effect of Trichostatin A and NAD on recombinant SIRT1 activity

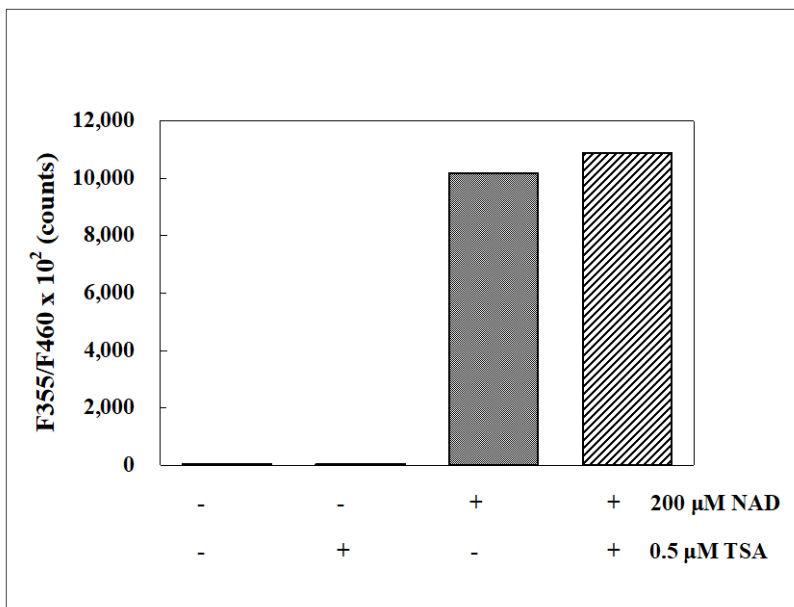


Fig.4 Km value of recombinant SIRT1 for Fluoro-Substrate Peptide

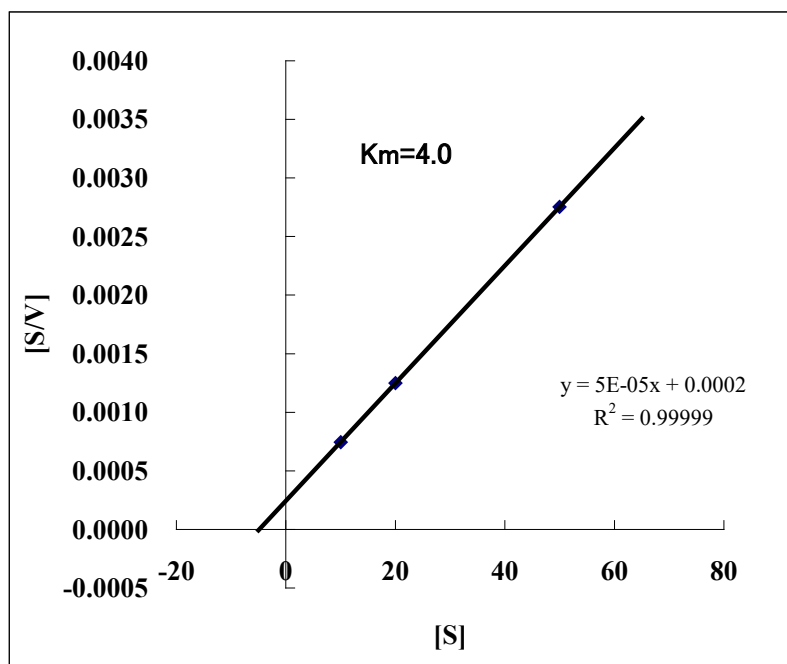


Fig.5 Substrate Preference of HDAC and SIRT1

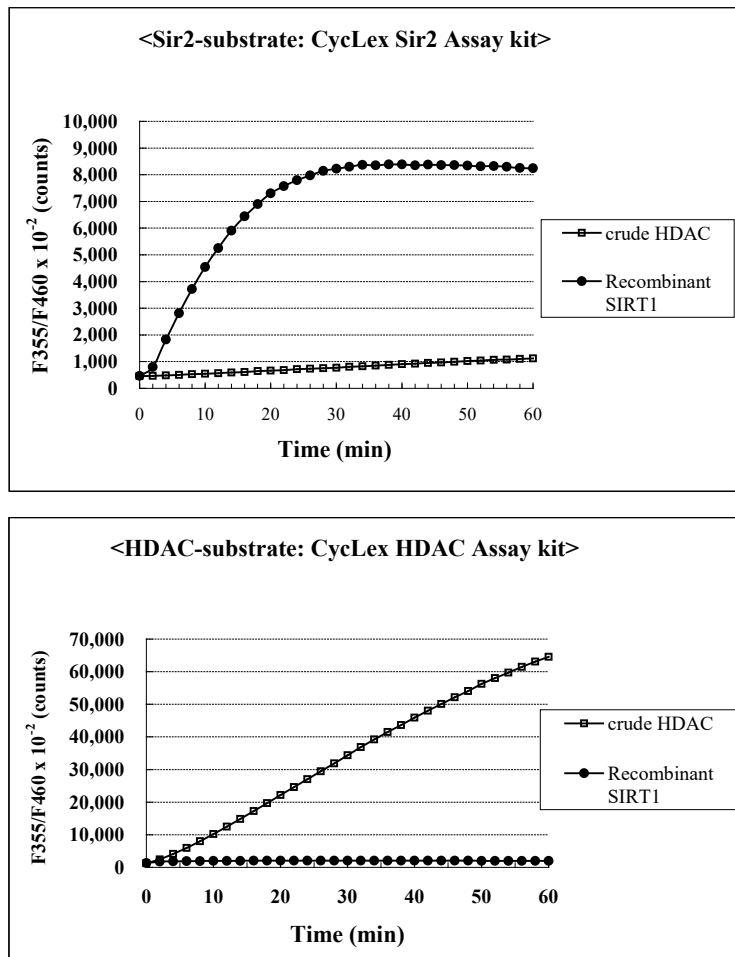


Fig.6 Stability of Fluorescence Intensity after Stop the Reaction

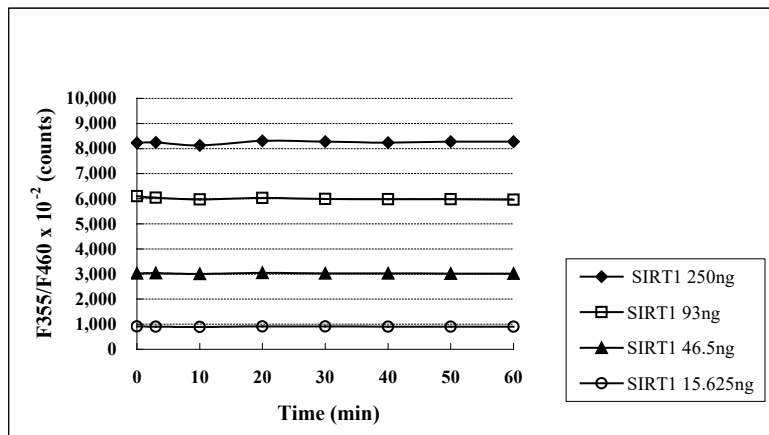
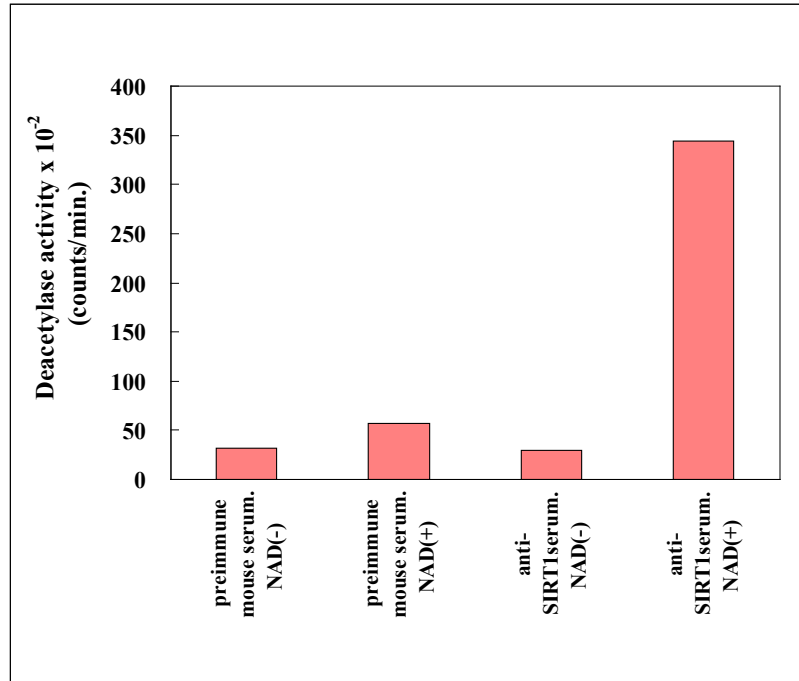


Fig.7 Measurement of 293T cell endogenous SIRT1 activity in an immunoprecipitate using anti-SIRT1 antibody (CY-P1016).





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