



Quantitative test kit for NAD-dependent histone deacetylase activity

CycLex SIRT6 Deacetylase Fluorometric Assay Kit

100 Assays

Cat# CY-1156

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Intended Use

The CycLex Research Product **CycLex SIRT6 Deacetylase Fluorometric Assay Kit** detects deacetylase activity of recombinant SIRT6. Primarily, the CycLex Research Product **CycLex SIRT6 Deacetylase Fluorometric Assay Kit** is designed for the rapid and sensitive evaluation of SIRT6 inhibitors or activators using recombinant SIRT6 or purified SIRT6.

Applications for this kit include:

- 1) Screening inhibitors or activators of SIRT6.
- 2) Detecting the effects of pharmacological agents on SIRT6.

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

Storage

- Upon receipt store recombinant SIRT6 at -70°C and all other components below -20°C.
- Don't 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. 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 (1).

The distant mammalian Sir2 homolog SIRT6 is a broadly expressed, predominantly nuclear protein (2) like SIRT1. Inactivation of the Sirt6 gene in mice leads to dramatically shortened life span and a premature aging-like phenotype (3). It was shown that SIRT6 is a histone H3 lysine 9 deacetylase, and identified a role for SIRT6 activity at telomeric chromatin, where it prevents telomere dysfunction in human cells (4). In addition, SIRT6 also deacetylates histone H3 acetylated lysine 56 in vivo to promote genomic stability (5). In term of function, SIRT6 binds to the NF- κ B subunit RELA and attenuates NF- κ B signaling by modifying chromatin at NF- κ B target genes (6). Thus SIRT6-mediated control of NF- κ B prevents aging-associated, hyperactive NF- κ B-dependent gene expression, and inhibition of NF- κ B can rescue the early lethality of Sirt6 knockout mice. Overexpression of wild-type Sirt6, but not the catalytically inactive form, consistently resulted in increased tumor necrosis factor protein production relative to its mRNA levels (7).

The conventional method for measuring SIRT6 activity is very complicated and laborious. In order to measure SIRT6 enzyme activity, it is necessary to prepare radioactive acetylated histone H3 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.



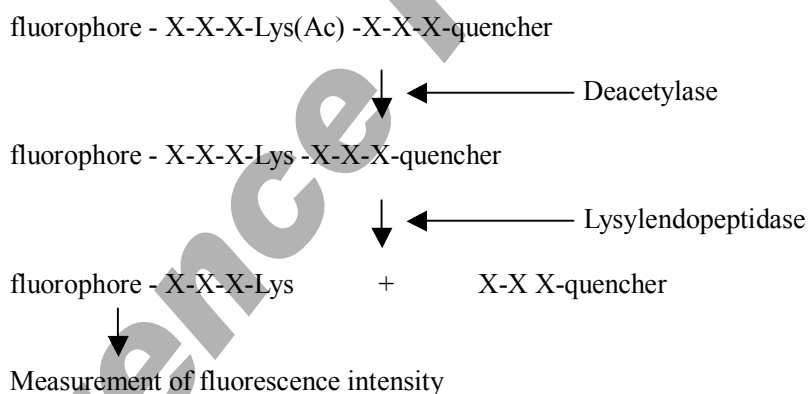
Principle of the Assay

CycLex SIRT6 Deacetylase Fluorometric Assay Kit measures the activity of SIRT6 by the basic principle of changing a SIRT6 reaction into the activity of the protease. In order to measure the enzyme activity of SIRT6, which is the NAD dependent histone deacetylase, 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 SIRT6 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 SIRT6 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, SIRT6 activity measurement, which could not be made by the conventional method, is now possible with the CycLex SIRT6 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 SIRT6 Deacetylase Fluorometric Assay Kit



Note: This measuring principle and kit are covered under CycLex's patents.

U.S. Patent No. 7,033,778 and No. 7256013

European Patent No. 1243658

Japanese Patent No. 4267043

Canadian Patent No. 2392711



Materials Provided

Each kit contains

Materials	Quantity	Storage
① 10X SIRT6 assay buffer	1 ml x 2	Below -20°C
② 50X Fluoro-Substrate Peptide (0.5 mM)	100 μ l x 1	Below -20°C
③ 10X Fluoro-Deacetylated Peptide (0.1 mM)	125 μ l x 1	Below -20°C
④ Lysylendopeptidase (1 AU/ml)	50 μ l x 1	Below -20°C
⑤ 100X NAD (80 mM)	100 μ l x 1	Below -20°C
⑥ 200X Trichostatin A (0.2 mM)	100 μ l x 1	Below -20°C
⑦ Recombinant SIRT6	200 μ l x 1	-70°C
⑧ 100X Stop solution	100 μ l x 1	Below -20°C
⑨ 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 490 ± 10 nm and detection of emitted light in the range 530 ± 10 nm.
- Pipettors: 2-20 μ L, 20-200 μ L and 200-1000 μ L precision pipettors with disposable tips.
- multi-channel pipette
- Microplate shaker
- Deionized water of the highest quality
- 500 or 1000 mL graduated cylinder
- Reagent reservoirs



Precautions

- Please thaw ② 50X Fluoro-Substrate Peptide and ③ 10X 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 the ⑦ Recombinant SIRT6 in this kit. 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 inhibitors such as PMSF, or alkyl amine in the sample that will be measured SIRT6 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.**



Detailed Protocol

Description of assay system

CycLex SIRT6 Deacetylase Fluorometric Assay Kit can measure the enzyme activity of SIRT6 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, SIRT6, 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.

Preparation Method for Assay Reagents

Thaw ②50X Fluoro-Substrate Peptide and ③10X Fluoro-Deacetylated Peptide at room temperature. Stand other reagents in ice to thaw. Use them after they thaw completely.

#1. 1X SIRT6 assay buffer (50 mM Tris-HCl (pH 8.8), 0.5 mM DTT)

Quantity Required: 50 μ L/assay

- Dilute the ①10X SIRT6 assay buffer 1:10 with distilled water.

Since this is the base buffer for the assay, prepare 1 vial (1 ml) of ①10X SIRT6 assay buffer mixed with 9 ml of dH₂O and store SIRT6 assay buffer at 4°C.

#2. X20 diluted Lysylendopeptidase (50 mAU/ml Lysylendopeptidase)

Quantity required: 2.5 μ L/assay

- Dilute the ④Lysylendopeptidase 1:20 with #1. 1X SIRT6 assay buffer.

#3. 10X NAD (8 mM β -NAD)

Quantity required: 5 μ L/assay

- Dilute the ⑤100X NAD 1:10 with #1. 1X SIRT6 assay buffer.

#4. 10X TSA (10 μ M)

Quantity required: 5 μ L/assay

- Dilute the ⑥200X Trichostatin A 1:20 with #1. 1X SIRT6 assay buffer.

#5. 10X Test sample (10X final concentration, e.g. a candidate of inhibitor or activator)

Quantity Required: 5 μ L/assay

- Dilute Test sample to 10X final desired concentration with #1. 1X SIRT6 assay buffer.

#6. X5 diluted recombinant SIRT6

Quantity Required: 10 μ L/assay

- Dilute the ⑦Recombinant SIRT6 1:5 with #1. 1X SIRT6 assay buffer.

(Note! Use “#6. 5X diluted recombinant SIRT6” within the same day they are prepared.)

#7. 2X Stop solution

Quantity required: 50 μ L/assay

- Dilute the ⑧100X Stop solution 1:50 with dH₂O.

#8. SIRT6 reaction buffer (Final 50 mM Tris-HCl (pH 8.8), 0.5 mM DTT, 2.5 mAU/ml



SIRT6 Deacetylase Fluorometric Assay Kit

User's Manual

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Lysylendopeptidase, 1 μ M Trichostatin A and 10 μ M Fluoro-Substrate Peptide in 50 μ L of reaction mixture)

Quantity Required: 30 μ L/assay (in case of adding 10 μ L of “#6. X5 diluted recombinant SIRT6” and 5 μ L of “#5. 10X Test sample”)

- Mix following reagents (30 μ L/1 assay)

1.	①10X SIRT6 assay buffer	5 μ L
2.	②50X Fluoro-Substrate Peptide	1 μ L
3.	#2. X20 diluted Lysylendopeptidase	2.5 μ L
4.	#4. 10X TSA	5 μ L
5.	dH ₂ O	16.5 μ L
Total		30 μ L



SIRT6 Assay Procedures

1. Assay method

Assay reagents	Test sample	No enzyme control	No Test sample control	No NAD control
#8. SIRT6 reaction buffer	30 μ L	30 μ L	30 μ L	30 μ L
#3. 10X NAD	5 μ L	5 μ L	5 μ L	-
#5. 10X Test sample	5 μ L	-	-	-
dH ₂ O	-	15 μ L	5 μ L	10 μ L
#6. X5 diluted recombinant SIRT6 (or Your enzyme sample)	10 μ L	-	10 μ L	10 μ L

- 1) Following the above table, add Reagent #3, #5 and #8 or dH₂O to each well of the microplate. Finally, initiate reaction by adding 10 μ L of “#6. X5 diluted recombinant SIRT6” or “your enzyme sample” to each well and mixing thoroughly. Incubate at room temperature (Ca.25°C).
- 2) Read fluorescence intensity for 60 to 120 minutes at 5 to 10 minute intervals using microtiter plate fluorometer with excitation at 490 \pm 10 nm and emission at 530 \pm 10 nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Alternate procedure

- 1') Following the above table, add Reagent #3, #5 and #8 or dH₂O to each well of the microplate. Finally, initiate reaction by adding 10 μ L of “#6. X5 diluted recombinant SIRT6” or “your enzyme” to each well and mixing thoroughly. Incubate at room temperature (Ca.25°C).
- 2') While the reaction rate is kept constant, add 50 μ L of “#7. 2X Stop solution “ to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader with excitation at 490 \pm 10 nm and emission at 530 \pm 10 nm.
- 3') The difference in fluorescence intensity between “No Test sample control” and “No enzyme control” indicates the SIRT6 activity.

Note-1: It is possible to change the volume of assay reagents and sample as far as it sets up the final concentration of each reagents in a reaction mixture as indicated as below.

Note-2: Duplicate measurement is strongly recommended.



2. Assay control

1. When the chemicals that have an inhibitory effect on lysylendopeptidase come to be mixed in SIRT6 assay mixture, precise SIRT6 enzyme activity cannot be measured. Since the protease inhibitors used in the usual protein purification process strongly inhibit lysylendopeptidase activity, please avoid using any protease inhibitors during the process of recombinant SIRT6 purification.

If there is such a possibility, please carry out the experiment of “**Positive control**” and “**Assay control-1**” in the following table, using Fluoro-Deacetylated Peptide to reference. When Fluoro-Deacetylated Peptide is used, fluorescence intensity should increase whenever there is no SIRT6 activity in your enzyme sample. When there is an inhibitory effect on lysylendopeptidase activity, even if there is SIRT6 activity in a sample, fluorescence intensity should not increase.

2. Not only when an inhibitory effect on SIRT6 is in test chemicals, but also when there is an inhibitory effect on lysylendopeptidase, final fluorescence intensity will not increase. Please use Fluoro-Deacetylated Peptide instead of Fluoro-Substrate Peptide, and please carry out the experiment of “**Positive control**” and “**Assay control-2**” that does not add SIRT6 in the following Table. Although fluorescence intensity increases when Fluoro-Deacetylated Peptide is used, when an inhibitory effect on lysylendopeptidase activity occurs in a test chemicals, fluorescence intensity does not increase.

Assay reagents	Assay control-1	Assay control-2	Positive control
① 10X SIRT6 assay buffer	5 μ L	5 μ L	5 μ L
③ 10X Fluoro-Deacetylated Peptide	5 μ L	5 μ L	5 μ L
#3. 10X NAD	5 μ L	5 μ L	5 μ L
#4. 10X TSA	5 μ L	5 μ L	5 μ L
#5. 10X Test sample	-	5 μ L	-
#6. X5 diluted recombinant SIRT6 (or Your enzyme sample)	5 μ L	-	-
dH ₂ O	22.5 μ L	22.5 μ L	27.5 μ L
#2. X20 diluted Lysylendopeptidase	2.5 μ L	2.5 μ L	2.5 μ L

1) Following the table above, add Reagent ①, ③, #3, #4, #5 or #6 and dH₂O to each well. Finally, add 2.5 μ L of “#2. X20 diluted Lysylendopeptidase” to each well and mix thoroughly to initiate reaction.

2) Incubate for 60 min or desired length of time at room temperature (Ca.25°C).

3) Add 50 μ L of “#7. 2X Stop solution” to each well.

4) Read fluorescence intensity using microtiter plate fluorometer with excitation at 490 ± 10 nm and emission at 530 ± 10 nm.



Evaluation of Results

Analysis of Kinetics

Time course curve

1. Run reactions as described in the Detailed Protocol.
2. Subtract fluorescence intensity at the 0 time from all reaction time points.
3. Plot fluorescence intensity at 530 ± 10 nm versus reaction time.
4. Determine the reaction time range in which the increase in fluorescence intensity at 530 ± 10 nm is linear.
5. Calculate activity:

$$\text{Activity (reaction velocity)} = \frac{\text{Fluorescence Intensity of Test Sample}}{\text{Reaction time (min.)}}$$

Note: Usually, the linear range is from 10 to 40 min. This value is variable depending on reaction conditions and storage/handling of the recombinant SIRT6. Decreasing the amount of recombinant SIRT6 in the assay may help to lengthen the time range.

Cautions

1. In order to measure the activity of SIRT6 correctly, it is necessary to conduct the control experiments for “**No enzyme control**” and “**No NAD control**” at least once in addition to “**No Test sample control**” as indicated in the above table of Assay method. Although fluorescence intensity increases in “**No Test sample control**” when SIRT6 enzyme activity is in the sample, the increase in fluorescence intensity is not observed in “**No enzyme control**” and “**No NAD control**”.
2. In order to estimate the inhibitory effect on SIRT6 activity in the Test sample correctly, it is necessary to conduct the control experiment of “**No Test sample control**” at least once for every experiment and “**No NAD control**” at least once for the first experiment, in addition to “**Test sample**” as indicated in the above table of Assay method. When test chemicals cause an inhibitory effect on SIRT6 activity, the level of increase of fluorescence intensity is weakened as compared with “**No Test sample control**”. The increase in fluorescence intensity is not observed in “**No NAD control**”.

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



Troubleshooting

1. When chemicals that have an inhibitory effect on lysylendopeptidase are mixed in recombinant SIRT6 or the immunoprecipitate using a specific antibody against SIRT6 or other proteins, precise SIRT6 enzyme activity cannot be measured. Since the protease inhibitors used in the cell lysate buffer inhibit lysylendopeptidase activity strongly, please avoid the use of any protease inhibitors during the immunoprecipitation process.
2. Final fluorescence intensity will not increase, both when test chemicals have an inhibitory effect on SIRT6, and also when there is an inhibitory effect on lysylendopeptidase.
3. 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.
4. The recombinant SIRT6 should be run 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 CycLex Research Product **CycLex SIRT6 Assay kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, **store the ⑦ Recombinant SIRT6 at -70°C**, all other kit reagents should be stored below -20°C.

Example of Test Results

Fig.1 Dose dependent curve of recombinant SIRT6 activity

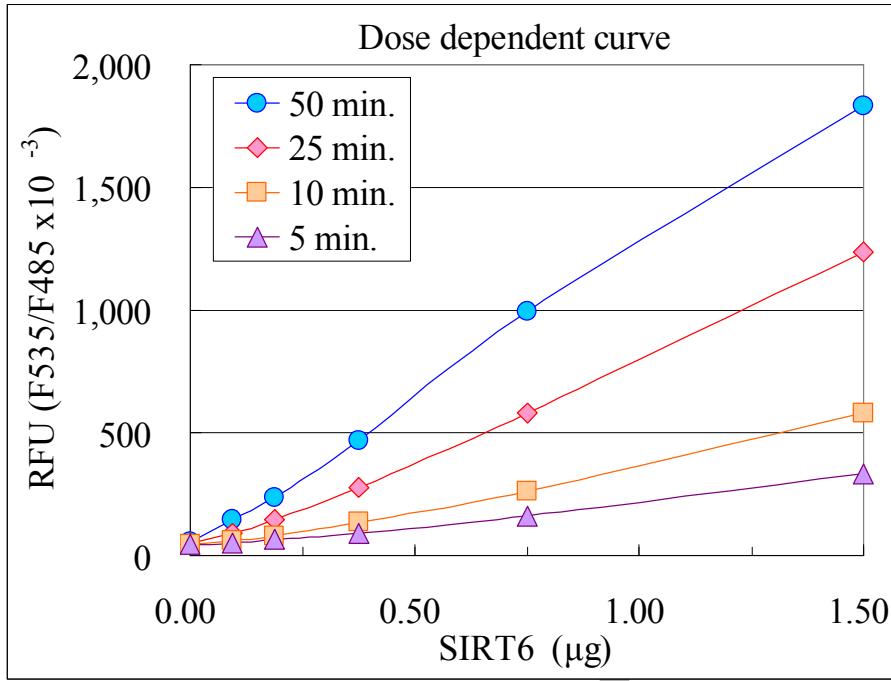


Fig.2 Time course of SIRT6-substrate deacetylation by recombinant SIRT6

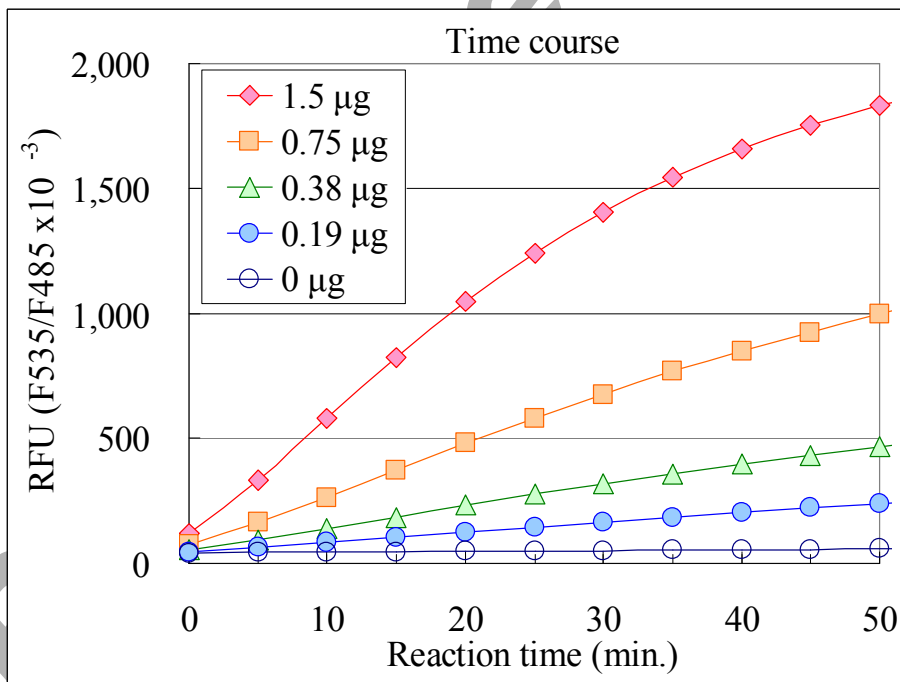


Fig.3 NAD dependency of recombinant SIRT6 activity

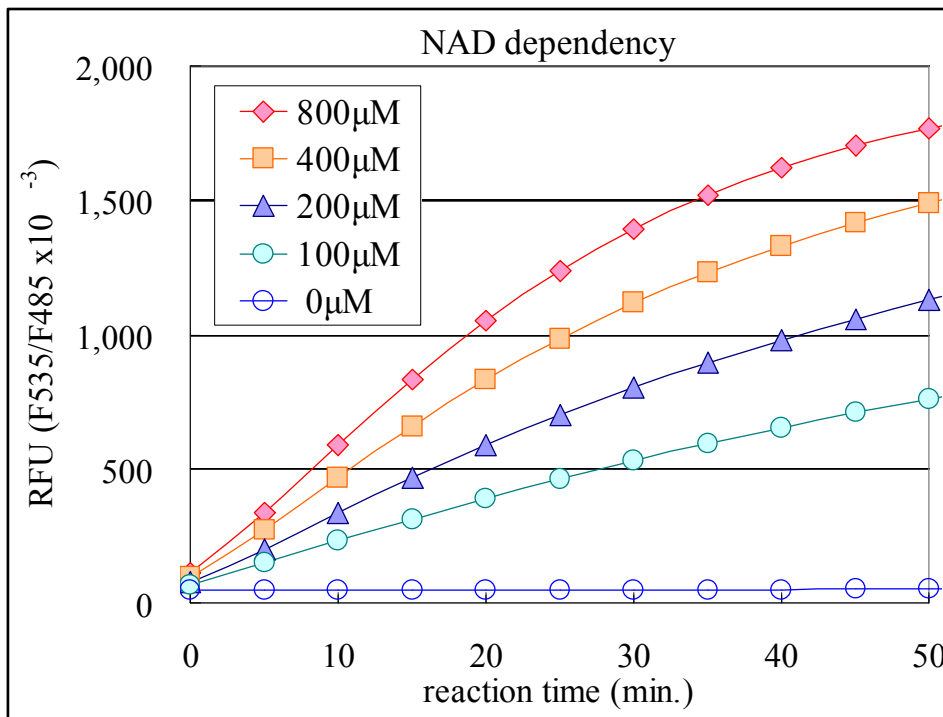


Fig.4 Effect of Trichostatin A and NAD on recombinant SIRT6 activity

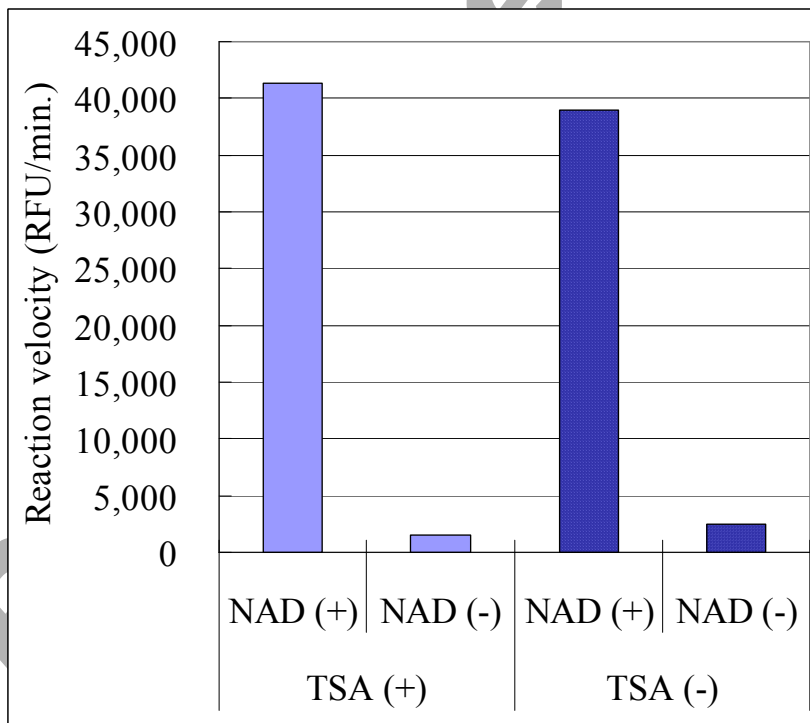
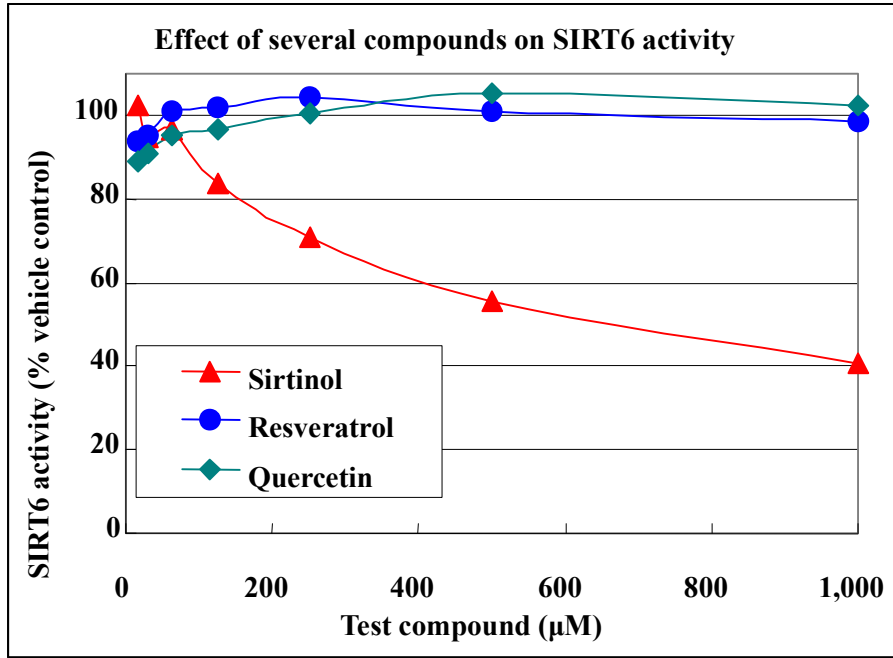




Fig.5 Effect of several compounds on recombinant SIRT6 activity





References

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4. Michishita E et al.; SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature.* **452**: 492, 2008
5. Yang B et al.; The sirtuin SIRT6 deacetylates H3 K56Ac in vivo to promote genomic stability. *Cell Cycle.* **8**: 2662, 2009
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Related Products

- * CycLex Cellular Histone Acetylation Assay Kit: Cat# CY-1140
- * CycLex HDACs Deacetylase Fluorometric Assay Kit: Cat# CY-1150
- * CycLex HDAC8 Deacetylase Fluorometric Assay Kit: Cat# CY-1158
- * CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit: Cat# CY-1151
- * CycLex SIRT2 Deacetylase Fluorometric Assay Kit: Cat# CY-1152
- * CycLex SIRT3 Deacetylase Fluorometric Assay Kit: Cat# CY-1153
- * CycLex SIRT6 Deacetylase Fluorometric Assay Kit: Cat# CY-1156
- * Anti-Acetylated Histone/p53-K382 Mouse Monoclonal Antibody: Cat# CY-M1029
- * Anti-Histone Deacetylase 1 (HDAC1) Rabbit Polyclonal Antibody: Cat# CY-P1011
- * Anti-Histone Deacetylase 2 (HDAC2) Rabbit Polyclonal Antibody: Cat# CY-P1012
- * Anti-Human SIRT1 Rabbit Polyclonal Antibody: Cat# CY-P1016
- * NAD(+)-Dependent Deacetylase SIRT1: Cat# CY-E1151
- * NAD(+)-Dependent Deacetylase SIRT2: Cat# CY-E1152
- * NAD(+)-Dependent Deacetylase SIRT3: Cat# CY-E1153
- * NAMPT (Nicotinamide Phosphoribosyltransferase): Cat# CY-E1251
- * NMNAT1 (Nicotinamide Mononucleotide Adenylyltransferase 1): Cat# CY-E1252

Note:

This product is covered under CycLex's patents.
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European Patent No. 1243658
Japanese Patent No. 4267043
Canadian Patent No. 2392711

PRODUCED BY

CycLex Co., Ltd.
1063-103 Terasawaoka
Ina, Nagano 396-0002
Japan
Fax: +81-265-76-7618
e-mail: info@cyclex.co.jp
URL: <http://www.cyclex.co.jp>

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