Quantitative test kit for histone deacetylase activity

CycLex HDACs Deacetylase Fluorometric Assay Kit

100 Assays

Cat# CY-1150

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Intended Use

The CycLex Research Product **CycLex HDACs Deacetylase Fluorometric Assay Kit** detects HDAC activity in lysates. Primarily, the CycLex Research Product **CycLex HDACs Deacetylase Fluorometric Assay Kit** is designed for the rapid and sensitive evaluation of HDAC inhibitors using crude HDAC fraction. Additionally, any cultured primary cell, cell line, or tissue homogenate can be assayed for HDAC activity with the CycLex Research Product **CycLex HDACs Deacetylase Fluorometric Assay Kit** if the appropriate dose of HDAC specific inhibitor e.g. Tricostatine A is used.

Applications for this kit include:

1) Monitoring the purification of HDACs including HDAC1, 2, 3 and 8.
2) Screening inhibitors or activators of HDACs.
3) Detecting the effects of pharmacological agents on HDACs.

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

Storage

- Upon receipt store Crude HDAC at -70°C and all other components below -20°C.
- Don't expose reagents to excessive light.

Introduction

Histone deacetylase (HDAC) is considered to play a crucial role in regulating gene expression by changing nucleosome structure. HDAC is also thought to participate in regulation of cell cycle and differentiation, and it has been reported that the failure of this regulation leads to some types of cancer. Inhibition of HDAC activity by HDAC inhibitors such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) induce differentiation and/or apoptosis of transformed cells *in vitro* and inhibit tumor growth in a mouse model. It has been reported that HDAC inhibitors are effective for the medical treatment of acute promyelocytic leukemia (APL) and various cancers. Thus, HDAC inhibitors are expected to function as new anti-tumor drugs and antibacterial reagents. It is thought that screening of histone deacetylase inhibitors is likely to be further carried out, as one way to discover additional substances with similar properties.

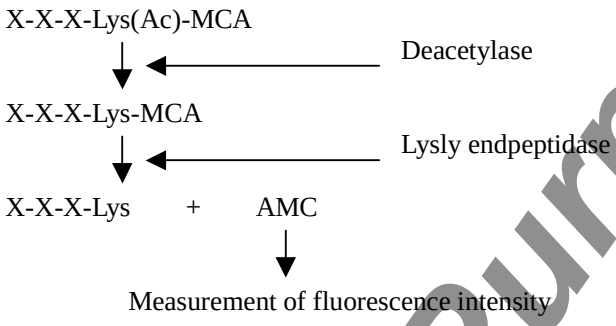
However, the conventional method for measuring HDAC activity is very complicated and laborious. In order to measure HDAC enzyme activity, it is necessary to prepare radioactive acetylated histone as a substrate. First, cells have to be labeled metabolically with radioactivity by adding radioactive acetic 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

CycLex HDACs Deacetylase Fluorometric Assay Kit measures the activity of HDAC by the basic principle of changing an HDAC reaction into the activity of the protease. Since it is very simple to measure common protease activity and it can be performed at a low price, the measurement of HDAC 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, HDAC activity measurement, which could not be made by the conventional method, is now possible with the CycLex HDACs 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 HDACs 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 Assay buffer	1ml x 2	Below -20°C
② 50X Fluoro-Substrate Peptide (1 mM)	100 µL x 1	Below -20°C
③ 50X Fluoro-Deacetylated Peptide (1 mM)	50 µL x 1	Below -20°C
④ Lysylendpeptidase (100 mAU/ml)	50 µL x 1	Below -20°C
⑤ 200X Trichostatin A (0.2 mM)	100 µL x 1	Below -20°C
⑥ Crude HDAC (crude nuclear extract from HeLa)	100 µL x 1	-70°C
⑦ 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 350-380 nm and detection of emitted light in the range 440-460 nm.
- **Pipettors:** 2-20 µL , 20-200 µL and 200-1000 µL 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 ②50X Fluoro-Substrate Peptide and ③50X 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 Crude HDAC in this kit. There is a possibility that the enzyme activity may be inactivated. Aliquot to 10-20 µL and store at -70℃
- Please avoid mixing of protease inhibitors such as PMSF, or alkyl amine in the sample that will be measured HDAC 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 pipette 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 HDACs Deacetylase Fluorometric Assay Kit can measure the enzyme activity of HDAC with two kinds of measuring methods, the 1 step method and the 2 steps method. In the 1 step method, the reaction is initiated and the fluorescence intensity is measured by mixing simultaneously fluorescence-labeled acetylated peptide, which is substrate, HDAC and lysly endpeptidase. 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 trichostatin A, an HDAC inhibitor, and to measure fluorescence intensity. Conversely, the 2 step method begins by initiating a reaction of fluorescence-labeled acetylated peptide and HDAC within a set time period to remove an acetyl group from substrate peptide; then in the second step adds lysly endpeptidase and trichostatin A as an HDAC enzyme inhibitor to stop the HDAC reaction, while simultaneously cleaving the resultant deacetylated fluorescence-labeled peptide by lysly endpeptidase.

Preparation Method for Assay Reagents

Thaw ②50X Fluoro-Substrate Peptide and ③50X Fluoro-Deacetylated Peptide at room temperature. Stand other reagents in ice to thaw. Use them after they thaw completely.

#1. 1X Assay buffer (20 mM Tris-HCl, pH 8.0, 125 mM NaCl, 1 % glycerol)
Quantity Required: 100 µL/assay

・Dilute the ①10X Assay buffer 1: 10 with distilled water.
Since this is the base buffer for the assay, prepare 1 vial (1 ml) of 10X assay buffer mixed with 9 ml distilled water and store 10 ml of assay buffer at 4°C.

#2. 2X Fluoro-Substrate Peptide (40 µM Fluoro-Substrate Peptide)
Quantity required: 25 µL/assay

・Dilute the ②50X Fluoro-Substrate Peptide 1: 25 with #1. 1X Assay buffer.

#3. 2X Fluoro-Deacetylated Peptide (40 µM Fluoro-Deacetylated Peptide)
Quantity required: 25 µL/assay

・Dilute the ③50X Fluoro- Deacetylated Peptide 1:25 with #1. 1X Assay buffer.

#4. X20 diluted Lysylendpeptidase (5 mAU/ml)
Quantity required: 5 µL/assay

・Dilute the ④Lysylendpeptidase 1: 20 with #1. 1X Assay buffer.

#5. HDAC stop solution (1 mAU/ml Lysylendpeptidase and 2 µM Trichostatin A)
Quantity Required: 50 µL/assay

・Mix following reagents (200 µL/4 assays).

1.	④Lysylendpeptidase	2 µL
2.	⑤200X Trichostatin A	2 µL
3.	#1. 1X Assay buffer	196 µL

#6. One-step assay buffer (Final 0.25 mAU/ml Lysylendpeptidase and 20 µM Fluoro-Substrate Peptide in 50 µL of assay mixture)
Quantity Required: 30 µL/assay (in case of adding 10 µL of enzyme and 10 µL of inhibitor or equivalent.)
・Mix following reagents (30 µL/1 assay).

1.	①10X Assay buffer	5 µL
2.	②50X Fluoro-Substrate Peptide	1 µL
3.	#4. X20 diluted Lysylendpeptidase	2.5 µL
4.	Distilled water	21.5 µL

#7. 5X Inhibitor or equivalent (5X final concentration)
Quantity required: 10 µL/assay
・Dilute Inhibitor or equivalent to 5X final desired concentration with #1. 1X Assay buffer.

#8. 5X Trichostatin A (5 µM Trichostatin A)
Quantity Required: 10 µL/assay
・Dilute the ⑤200X Trichostatin A 1: 40 with #1. 1X Assay buffer.

#9. X10 diluted crude HDACs (1: 10 diluted crude nuclear extract from HeLa)
Quantity required: 10 µL/assay
・Dilute the ⑥Crude HDAC 1: 10 with #1. 1X Assay buffer.
(Note: Use "#9. X10 diluted crude HDACs within the same day they are prepared.)

Assay Procedures

1. One-step method

Assay reagents	Test sample	No enzyme control	No inhibitor control	Inhibitor control
#6. One-step assay buffer	**30 µL**	**30 µL**	**30 µL**	**30 µL**
#1. 1X Assay buffer	**-**	**20 µL**	**10 µL**	**-**
#7. 5X Inhibitor or equivalent	**10 µL**	**-**	**-**	**-**
#8. 5X TSA	**-**	**-**	**-**	**10 µL**
#9. X10 diluted crude HDACs (or Your enzyme sample)	**10 µL**	**-**	**10 µL**	**10 µL**

1) Following the above table, add Reagent #6, #1, #7, and #8 to each well of the microplate. Finally, initiate reaction by adding 10 µL of "#9. X10 diluted crude HDACs" or "your enzyme" to each well and mixing thoroughly at room temperature.

2) Read fluorescence intensity for 30 to 60 minutes at 1 to 2 minute intervals using microtiter plate fluorometer with excitation at 355 nm and emission at 460 nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Alternate procedure

1') Following the above table, add Reagent #6, #1, #7, and #8 to each well of the microplate. Finally, initiate reaction by adding 10 µL of "#9. X10 diluted crude HDACs" or "your enzyme" to each well and mixing thoroughly at room temperature.

2') While the reaction rate is kept constant, add 12 µL of "#8. 5X TSA " 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 350-380 nm and detection of emitted light in the range 440-460 nm.

3') The difference in fluorescence intensity between "No inhibitor control" and "No enzyme control" indicates the HDAC activity.

2. Two-step method

Assay reagents	Test sample	No enzyme control	No inhibitor control	Inhibitor control	Assay control
#1. 1X Assay buffer	**5 µL**	**25 µL**	**15 µL**	**5 µL**	**15 µL**
#2. 2X Fluoro-Substrate Peptide	**25 µL**	**25 µL**	**25 µL**	**25 µL**	**-**
#7. 5X Inhibitor or equivalent	**10 µL**	**-**	**-**	**-**	**-**
#8. 5X TSA	**-**	**-**	**-**	**10 µL**	**-**
#3. 2X Fluoro-Deacetylated Peptide	**-**	**-**	**-**	**-**	**25 µL**
#9. X10 diluted crude HDACs (or Your enzyme sample)	**10 µL**	**-**	**10 µL**	**10 µL**	**-**
Your enzyme sample					**10 µL**

1) Following the table above, add Reagent #1, #2, #7, and #3 to each well. Finally, add 10 µL of "#9. X10 diluted crude HDACs" or "your enzyme" to each well and mix thoroughly to initiate reaction.

2) Incubate for 20 min or desired length of time at room temperature.

3) Add 50 µL of "#5. HDAC stop solution" to each well.

4) Incubate for at least 10 min or desired length of time at room temperature (Measurement should be made between 10 minutes and 40 minutes).

5) Read fluorescence intensity using microtiter plate fluorometer with excitation at 355 nm and emission at 460 nm.

6) During the time in which HDAC reaction rate is maintained, the difference in fluorescence intensity between "No inhibitor control" and "No enzyme control" or "inhibitor control" indicates the HDAC activity.

Note-1: Although the above table indicates the volume of addition of "#7. 5X Inhibitor or equivalent" as 10 µL, the concentration and the volume of test reagents to add can be changed so that the concentration of test reagent becomes the setting concentration. For example, since the final volume of reaction is 50 µL here, it is also possible to add 5 µL of 10X test reagent. In this case, please add "#1. 1X Assay buffer" to set to the final reaction volume of 50 µL.

Note-2: Although the volume of addition of "Your enzyme sample" is set to 10 µL in this table, it may be changed to a volume up to 20 µL at your discretion. In that case, please add "#1. 1X Assay buffer" to set the final reaction volume of 50 µL.

Note-3: Duplicate measurement is recommended.

Cautions

1. In order to measure the activity of HDAC correctly, it is necessary to conduct the control experiments for "No enzyme control" and "Inhibitor control" at least once in addition to "**No inhibitor control**," as indicated in the above table. Although fluorescence intensity increases in "**No inhibitor control**" when HDAC enzyme activity is in the sample, the increase in fluorescence intensity is not observed in "No enzyme control" and "Inhibitor control".

2. In order to estimate the inhibitory effect on HDAC activity in the test chemicals correctly, it is necessary to conduct the control experiment of "No inhibitor control" at least once for every experiment and "Inhibitor control" at least once for the first experiment, in addition to "**Test sample**," as indicated in the above table. When test chemicals cause an inhibitory effect on HDAC activity, the level of increase of fluorescence intensity is weakened as compared with "No inhibitor control". The increase in fluorescence intensity is not observed in "Inhibitor control".

3. When the chemicals that have an inhibitory effect on lysylendpeptidase come to be mixed in crude HDAC fraction purified from various cells or the immunoprecipitate using the specific antibody against HDAC or other proteins, precise HDAC enzyme activity cannot be measured. Since the protease inhibitors used in the usual protein purification process strongly inhibit lysylendpeptidase activity, please avoid using any protease inhibitors during the process of protein purification.
   If there is such a possibility, please carry out the experiment of **"Assay control"** using Fluoro-Deacetylated Peptide to reference. When Fluoro-Deacetylated Peptide is used, fluorescence intensity should increase whenever there is no HDAC activity in a sample. When there is an inhibitory effect on lysylendpeptidase activity, even if there is HDAC activity in a sample, fluorescence intensity should not increase.

4. Not only when an inhibitory effect on HDAC is in test chemicals, but also when there is an inhibitory effect on lysylendpeptidase, final fluorescence intensity will not increase. Please use Fluoro-Deacetylated Peptide instead of Fluoro-Substrate Peptide, and conduct a control experiment that does not add HDAC. Although fluorescence intensity increases even if HDAC is not added when Fluoro-Deacetylated Peptide is used, when an inhibitory effect on lysly endpeptidase activity occurs in a test sample, fluorescence intensity does not increase.

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Troubleshooting

1. Although trichostatin A is added in the 2 steps method in order to stop a HDAC reaction, the activities of Sir2 and it's human homologue, SIRT1 cannot be measured correctly even if NAD is added, since they do not have susceptibility in trichostatin A. (Please use CycLex's Sir2 assay kit.)

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

3. Final fluorescence intensity will not increase, both when test chemicals have an inhibitory effect on HDAC, and also when there is an inhibitory effect on lysylendpeptidase.

4. If the test reagents themselves emit fluorescence at excitation wavelength: 360-380 nm and fluorescence wavelength: 440-460 nm, the inhibitory effect of the test assay cannot be evaluated correctly.

5. The Crude HDAC 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.

6. 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.

7. 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 **HDAC Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, **store the ⑥ Crude HDAC 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 HDACs. 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 HDACs 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.

Buffers

*Lysis Buffer:	*Sucrose cushion:	*Extraction buffer:
10 mM Tris HCl pH7.5 10 mM NaCl 15 mM $MgCl_2$ 250 mM Sucrose 0.5 % NP-40 0.1 mM EGTA	30 % Sucrose 10 mM Tris HCl pH7.5 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.

Procedure

Isolation of Nuclei

1. Suspend 1x 10^7 cells (100 mm dish sub-confluent) into 1ml 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.

Extraction of Nuclei

1. Suspend the isolated nuclei in 50-100 µL of extraction buffer.
2. Sonic ate for 30 seconds.
3. Stand on ice for 30 min.
4. c.f.g. at 20,000 x g for 10 min.
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 inhibitor!!

Example of Test Results

Fig.1 Dose dependency of crude HDAC (Two-step method)

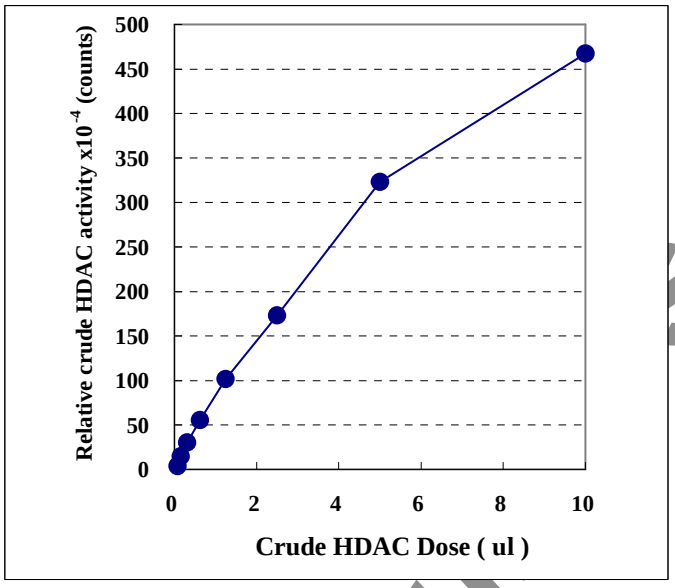


Fig.2 Time course of HDAC reaction (Two-step method)

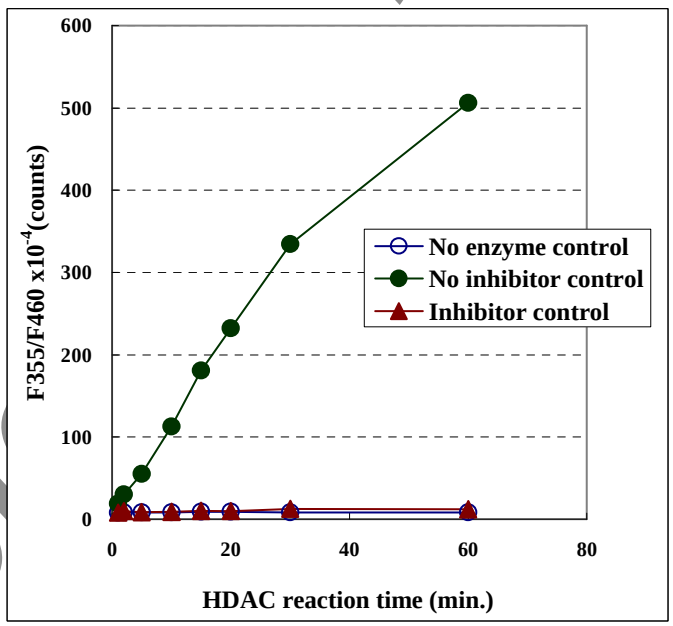


Fig.3 Effect of Trichostatin A on HDAC activity (One-step method)

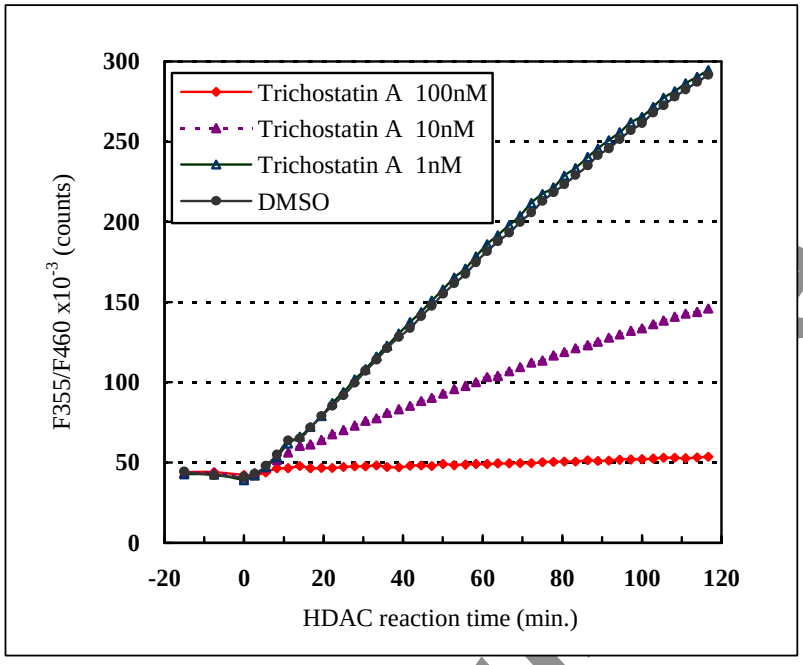


Fig.4 Substrate Preference of HDAC and SIRT1 using CycLex HDAC kit

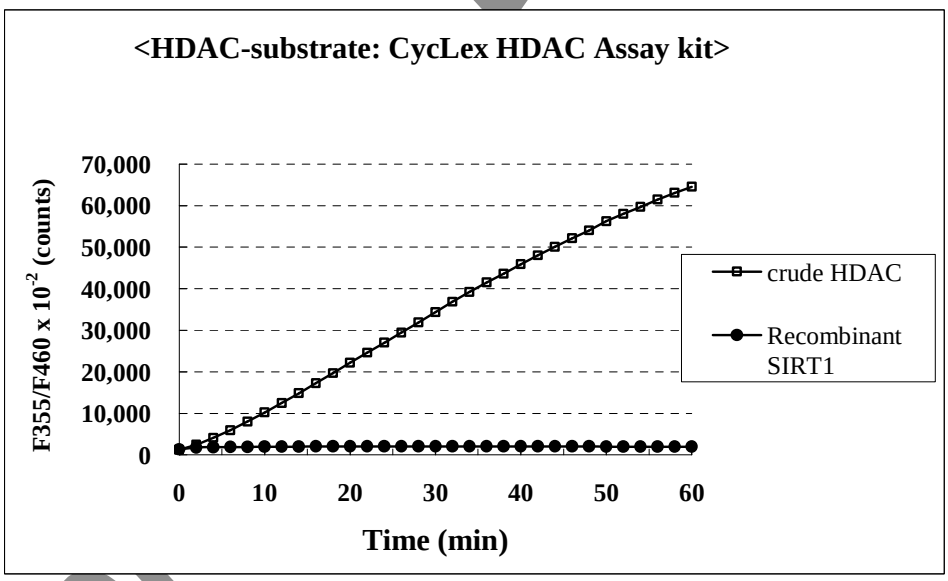


Fig.5 Substrate Preference of HDAC and SIRT1 using CycLex Sir2 kit

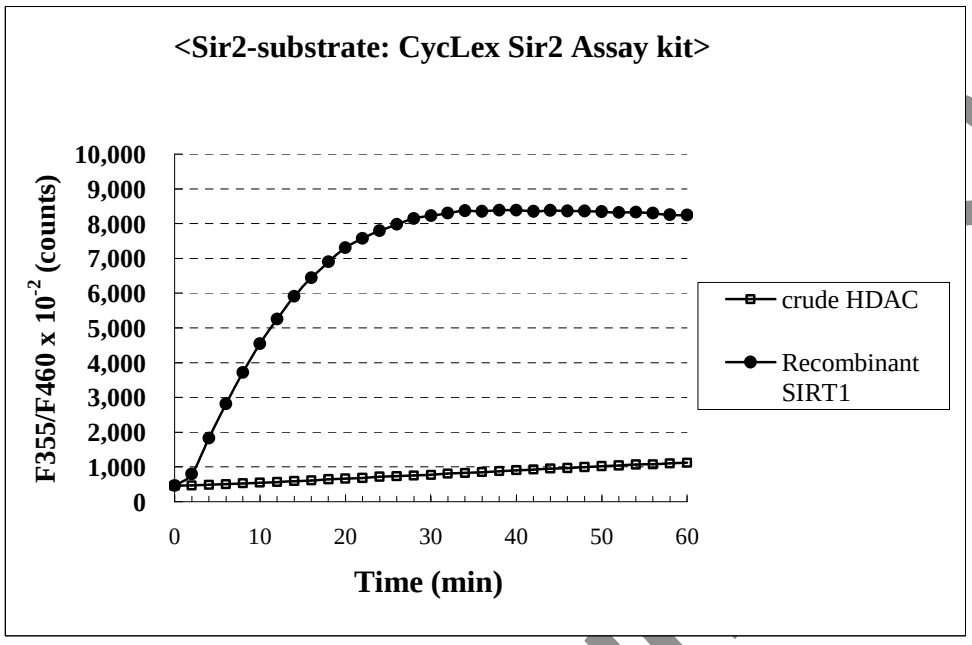


Fig.6 Time course of 2nd reaction in a Two-step method (Lysylendpeptidase reaction)

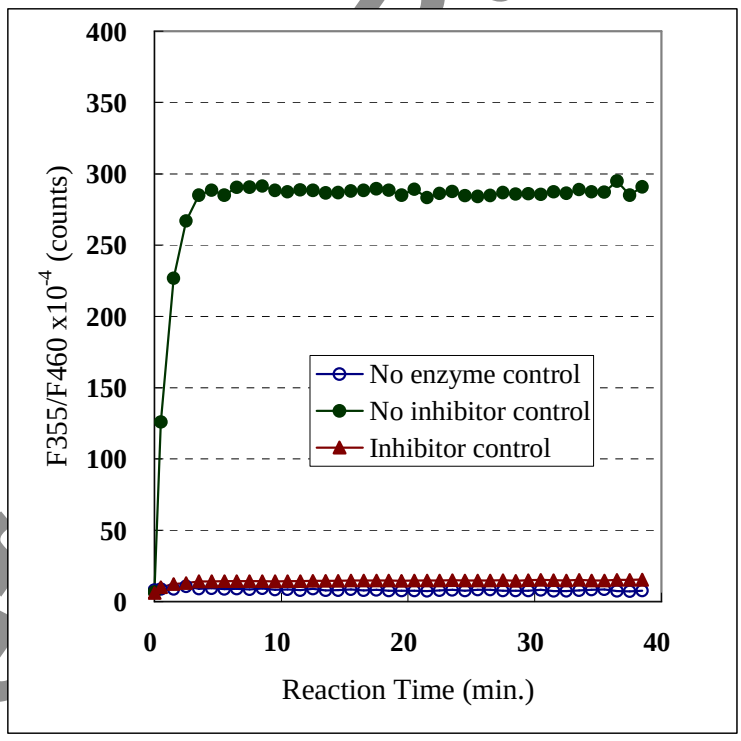


Fig.7 Measurement of HeLa cell endogenous HDAC1 in an immunoprecipitate using anti-HDAC1 antibody (Cat# CY-P1011) by means of CycLex HDACs Deacetylase Fluorometric Assay Kit

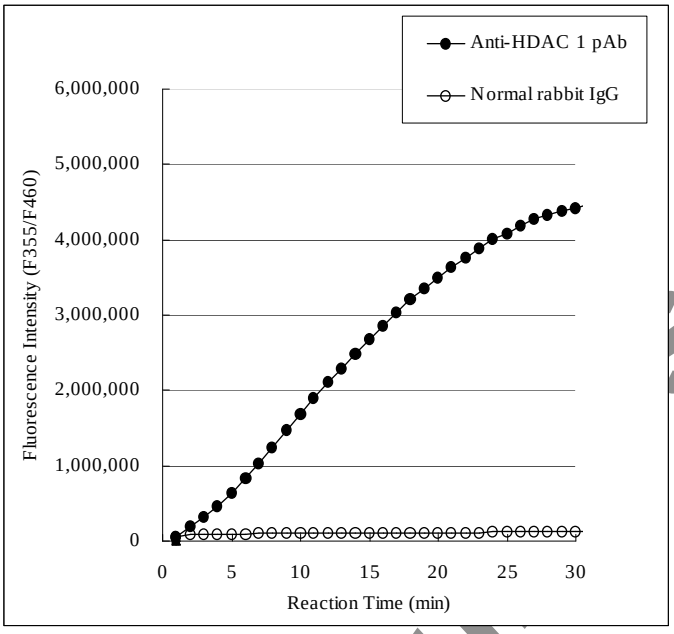
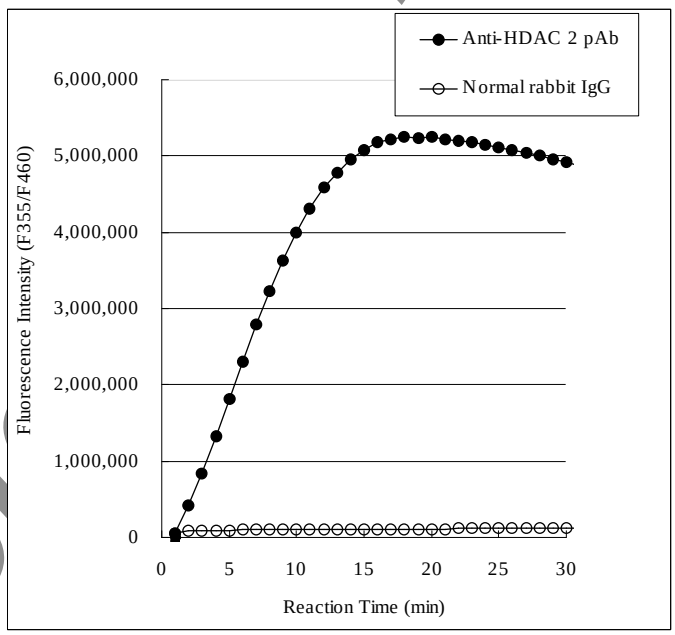


Fig.8 Measurement of HeLa cell endogenous HDAC2 in an immunoprecipitate using anti-HDAC2 antibody (Cat# CY-P1012) by means of CycLex HDACs Deacetylase Fluorometric Assay Kit



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