Quantitative test kit for histone deacetylase activity

CycLex HDACs Deacetylase Fluorometric Assay Kit Ver.2

100 Assays

Cat# CY-1150V2

Intended Use

The MBL Research Product **CycLex HDACs Deacetylase Fluorometric Assay Kit** detects HDAC activity in lysates. Primarily, the MBL 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 MBL Research Product **CycLex HDACs Deacetylase Fluorometric Assay Kit** if the appropriate dose of HDAC specific inhibitor e.g. Trichostatin 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 #5. Developer and #6. HDACs at -70°C and all other components below -20°C.
- Do not 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.

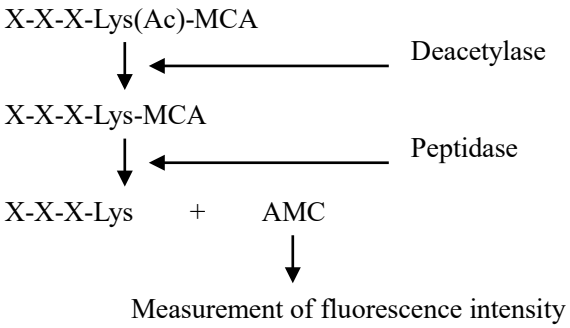
However, the conventional method for measuring HDAC activity is very complicated and laborious. In order to measure HDAC enzyme activity, it is necessary to prepare radioactive acetylated histone as a substrate. First, cells have to be labeled metabolically with radioactivity by adding radioactive acetic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

The 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 peptidase. Since it is very simple to measure common protease/peptidase 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



Materials Provided

All assays should be run in duplicate. The following components are supplied and are sufficient for one hundred assays.

Components of Kit

Components	Quantity	Storage
#1. HDAC 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. Trichostatin A (10 µM)	**500 µL x 1**	**Below -20°C**
#5. Developer	**500 µL x 1**	**-70°C**
#6. HDACs (crude nuclear extract from HeLa)	**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 350-380 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**

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. HDACs"**. There is a possibility that the enzyme activity may be inactivated. Aliquot to 10-20 µL and store at -70°C.

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

- If enzyme samples or test compounds themselves emit fluorescence at excitation wavelength: 350-380 nm and fluorescence wavelength: 440-460 nm, the assays cannot be evaluated correctly.

- 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.**

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.

Detailed Protocol

CycLex HDACs Deacetylase Fluorometric Assay Kit can measure the enzyme activity of HDAC with two kinds of measuring methods, one-step method and two-step method. In the one-step method, the reaction is initiated and the fluorescence intensity is measured by mixing simultaneously fluorescence-labeled acetylated peptide, which is a substrate, HDAC 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 the stop solution, and to measure fluorescence intensity.

Conversely, the two-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, and then in the second step adds the stop solution to stop the HDAC reaction, while simultaneously cleaving the resultant deacetylated fluorescence-labeled peptide by the developer.

I. Assay Procedures for Measurement of HDAC Activity

1. One-Step Method

1) Following Table.1 below, first, add **"Distilled water"**, **"#1. HDAC Assay Buffer"** and **"#2. Fluoro-Substrate Peptide"** to microtiter plate wells. Second, add **"#4. Trichostatin A"** and **"#5. Developer"** to each well of the microtiter plate and mix well.

Table.1: Reaction mixture of One-Step Method for measurement of HDACs activity

Assay reagents	Enzyme Sample Assay	No Enzyme Control Assay	Positive Control Assay	Inhibitor Control Assay
Distilled water	30 µL	30 µL	30 µL	25 µL
#1. HDAC Assay buffer	5 µL	5 µL	5 µL	5 µL
#2. Fluoro-Substrate Peptide	5 µL	5 µL	5 µL	5 µL
#4. Trichostatin A	-	-	-	5 µL
#5. Developer	5 µL	5 µL	5 µL	5 µL
Enzyme Sample	5 µL	-	-	5 µL
Buffer of Enzyme Sample	-	5 µL	-	-
#6. HDACs	-	-	5 µL	-
Total Volume of the mixture	50 µL	50 µL	50 µL	50 µL

2) Initiate reactions by adding **5 µL** of your **"Enzyme Sample"** or **"Buffer of Enzyme Sample"** or **"#6. HDACs"** 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 350-380 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 350-380 nm and detection of emitted light in the range 440-460 nm.

2. Two-Step Method

1) Following Table.2 below, first, add **"Distilled water"**, **"#1. HDAC Assay Buffer"** and **"#2. Fluoro-Substrate Peptide"** to microtiter plate wells. Second, add **"#4. Trichostatin A"** to each well of the microtiter plate and mix well

Table.2: Reaction mixture of Two-Step Method for measurement of HDACs activity

Assay reagents	Enzyme Sample Assay	No Enzyme Control Assay	Positive Control Assay	Inhibitor Control Assay
Distilled water	35 µL	35 µL	35 µL	30 µL
#1. HDAC Assay buffer	5 µL	5 µL	5 µL	5 µL
#2. Fluoro-Substrate Peptide	5 µL	5 µL	5 µL	5 µL
#4. Trichostatin A	-	-	-	5 µL
Enzyme Sample	5 µL	-	-	5 µL
Buffer of Enzyme Sample	-	5 µL	-	-
#6. HDACs	-	-	5 µL	-
Total Volume of the mixture	50 µL	50 µL	50 µL	50 µL

2) Initiate reactions by adding **5 µL** of your **"Enzyme Sample"** or **"Buffer of Enzyme Sample"** or **"#6. HDACs"** to each well and mixing thoroughly at room temperature.

3) Incubate for 20 minutes or desired length of time at room temperature.

4) Add **20 µL** of **"#7. Stop Solution"** to each well of the microtiter plate and mix thoroughly.

5) Add **5µL** of **"#5. Developer"** to each well of the microtiter plate and mix thoroughly.

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

7) Read fluorescence intensity using microtiter plate fluorometer with excitation at 350-380 nm and emission at 440-460 nm.

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

Note-2: Although the volume of addition of **"Enzyme Sample"** or **"Buffer of Enzyme Sample"** or **"#6. HDACs"** is set to 5 µL in the 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.

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 **"Inhibitor Control Assay"**, the HDAC activity in the samples cannot be evaluated correctly.

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

II. Assay Procedures for Inhibitor Screening

1. One-Step Method

1) Following Table.1 below, first, add **"Distilled water"**, **"#1. HDAC Assay Buffer"** and **"#2. Fluoro-Substrate Peptide"** or **"#3. Fluoro-Deacetylated Peptide"** to microtiter plate wells. Second, add **"Test Compound"** or **"Solvent of Test Compound"** or **"#4. Trichostatin A"**, and **"#5. Developer"** to each well of the microtiter plate and mix well.

Table.1: Reaction mixture of One-Step Method for inhibitor screening

Assay reagents	Test Compound Assay	Solvent Control Assay	Inhibitor Control Assay	No Enzyme Control Assay	Development Control Assay
Distilled water	25 µL	25 µL	25 µL	30 µL	30 µL
#1. HDAC Assay buffer	5 µL	5 µL	5 µL	5 µL	5 µL
#2. Fluoro-Substrate Peptide	5 µL	5 µL	5 µL	5 µL	-
#3. Fluoro-Deacetylated Peptide	-	-	-	-	5 µL
Test Compound	5 µL	-	-	-	5 µL
Solvent of Test Compound	-	5 µL	-	5 µL	-
#4. Trichostatin A	-	-	5 µL	-	-
#5. Developer	5 µL	5 µL	5 µL	5 µL	5 µL
#6. HDACs (or Enzyme Sample)	5 µL	5 µL	5 µL	-	-
Total Volume of the mixture	50 µL	50 µL	50 µL	50 µL	50 µL

2) Initiate reactions by adding **5 µL** of **"#6. HDACs"** (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 350-380 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 350-380 nm and detection of emitted light in the range 440-460 nm.

2. Two-Step Method

1) Following Table.2 below, first, add **"Distilled water"**, **"#1. HDAC Assay Buffer"** and **"#2. Fluoro-Substrate Peptide"** or **"#3. Fluoro-Deacetylated Peptide"** to microtiter plate wells. Second, add your **"Test Compound"** or **"Solvent of Test Compound"** or **"#4. Trichostatin A"** to each well of a microtiter plate and mix well.

Table.2: Reaction mixture of Two-Step Method for inhibitor screening

Assay reagents	Test Compound Assay	Solvent Control Assay	Inhibitor Control Assay	No Enzyme Control Assay	Development Control Assay
Distilled water	30 µL	30 µL	30 µL	35 µL	35 µL
#1. HDAC Assay Buffer	5 µL	5 µL	5 µL	5 µL	5 µL
#2. Fluoro-Substrate Peptide	5 µL	5 µL	5 µL	5 µL	-
#3. Fluoro-Deacetylated Peptide	-	-	-	-	5 µL
Test Compound	5 µL	-	-	-	5 µL
Solvent of Test Compound	-	5 µL	-	5 µL	-
#4. Trichostatin A	-	-	5 µL	-	-
#6. HDACs (or Enzyme Sample)	5 µL	5 µL	5 µL	-	-
Total Volume of the mixture	50 µL	50 µL	50 µL	50 µL	50 µL

2) Initiate reactions by adding **5 µL** of **"#6. HDACs"** (or your **"Enzyme Sample"**) to each well and mixing thoroughly at room temperature.

3) Incubate for 20 minutes or desired length of time at room temperature.

4) Add **20 µL** of **"#7. Stop Solution"** to each well of the microtiter plate and mix thoroughly.

5) Add **5µL** of **"#5. Developer"** to each well of the microtiter plate and mix thoroughly.

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

7) Read fluorescence intensity using microtiter plate fluorometer with excitation at 350-380 nm and emission at 440-460 nm.

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

Note-2: In order to estimate the inhibitory effect on HDAC 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 **"Inhibitor Control Assay"** at least once for the first experiment, in addition to **"Test Compound Assay"** as indicated in the tables. When test compounds cause an inhibitory effect on HDAC activity, the level of increase of fluorescence intensity is weakened as compared with "**Solvent Control Assay**". The increase in fluorescence intensity is not observed in "**Inhibitor Control Assay**".

Note-3: The efficacy of the test compounds on the HDAC 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 HDAC 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 **"#4. Trichostatin A"** 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"**. 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 **"#6. HDACs"** 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 compounds that have an inhibitory effect on the peptidase in the development reaction are mixed in a crude HDACs fraction purified from various cells or the immunoprecipitate using a specific antibody against HDACs or other proteins, precise HDAC activity cannot be measured. Since protease/peptidase inhibitors used in the usual protein purification process strongly inhibit the peptidase in the development reaction, please avoid the use of any protease/peptidase inhibitors during the protein purification process.

2. Final fluorescence intensity will not increase, both when test compounds have an inhibitory effect on HDAC activity, and also when there is an inhibitory effect on the peptidase in the development reaction.

3. If enzyme samples or test compounds 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.

4. All samples and controls should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Poor duplicates indicate inaccurate dispensing. If all instructions in the **Detailed Protocol** were followed accurately, such results may indicate a need for multi-channel pipettor maintenance.

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. Incubation times or temperatures significantly different from those specified may give erroneous results.

Reagent Stability

All of the reagents included in the MBL Research Product **CycLex HDACs Deacetylase Fluorometric Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, store **"#5. Developer"** and **"#6. HDACs"** 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.

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.

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 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 µL of extraction buffer.
2. Sonic ate for 30 seconds.
3. Stand on ice for 30 minutes.
4. c.f.g. 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!!

Example of Test Results

Fig.1 Dose dependency of HDACs (Two-Step Method)

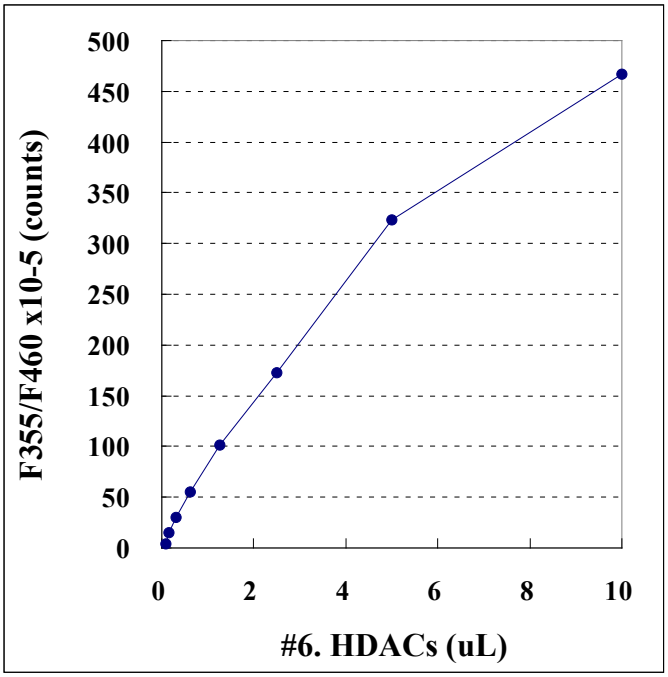


Fig.2 Time course of HDAC reaction (Two-Step Method)

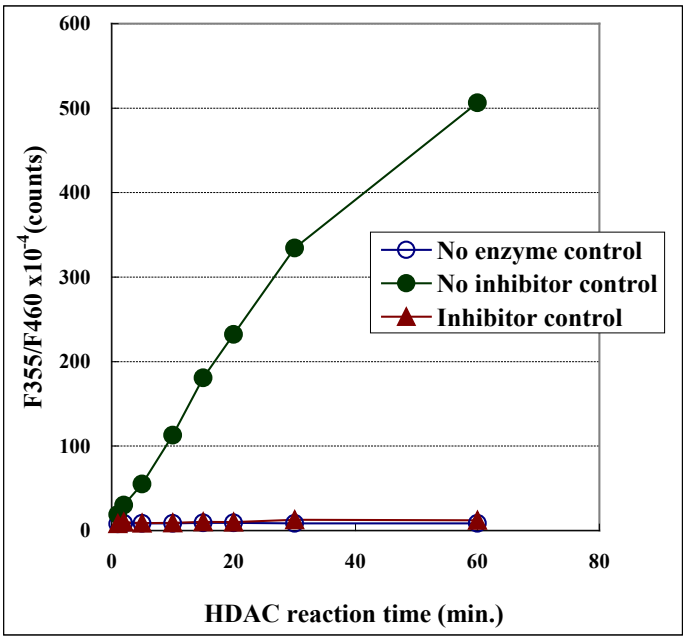


Fig.3 Effect of Trichostatin A on HDAC activity (One-Step Method)

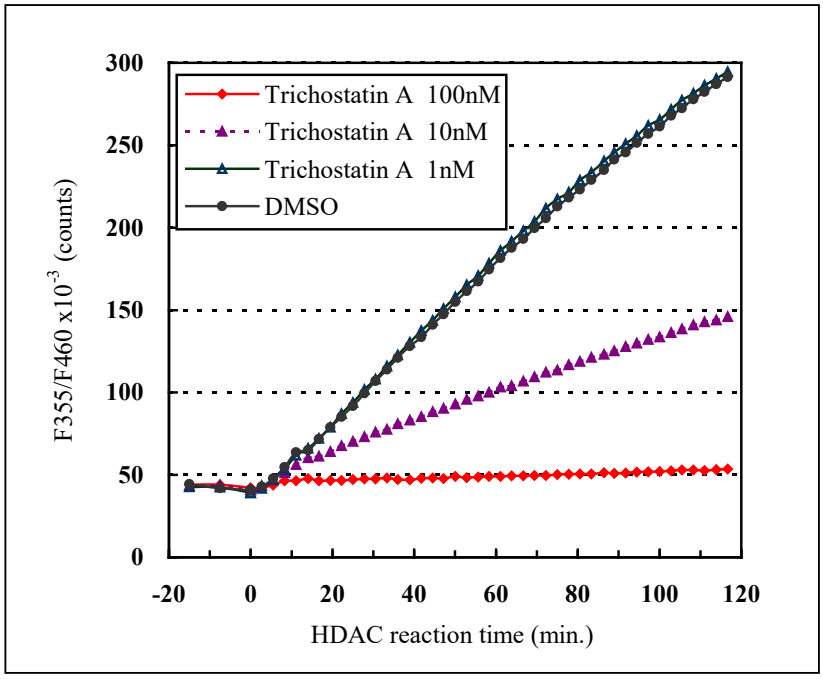


Fig.4 Substrate preference of HDAC and SIRT1 using CycLex HDACs Deacetylase Fluorometric Assay Kit

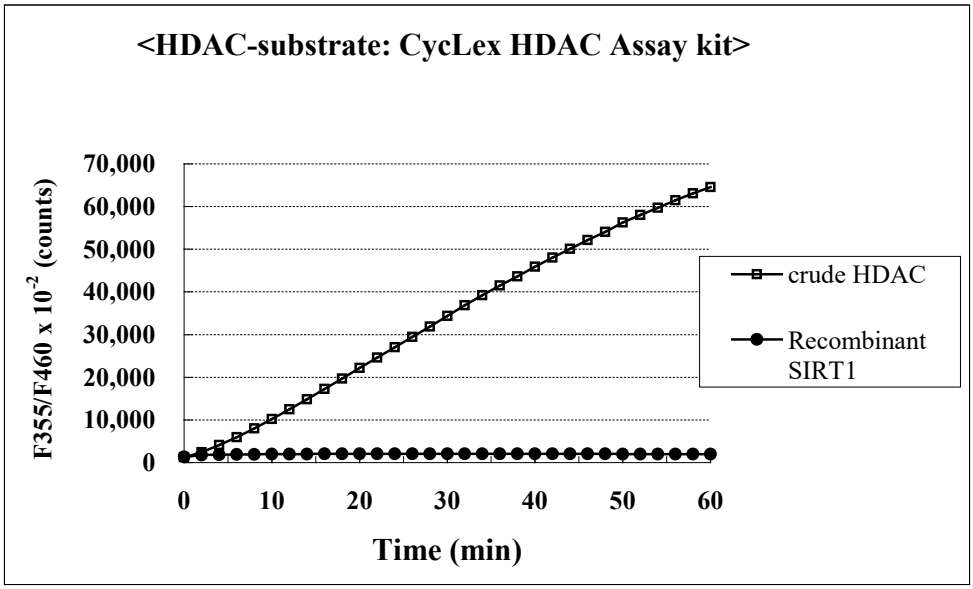


Fig.5 Substrate preference of HDAC and SIRT1 using CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (Cat# CY-1151)

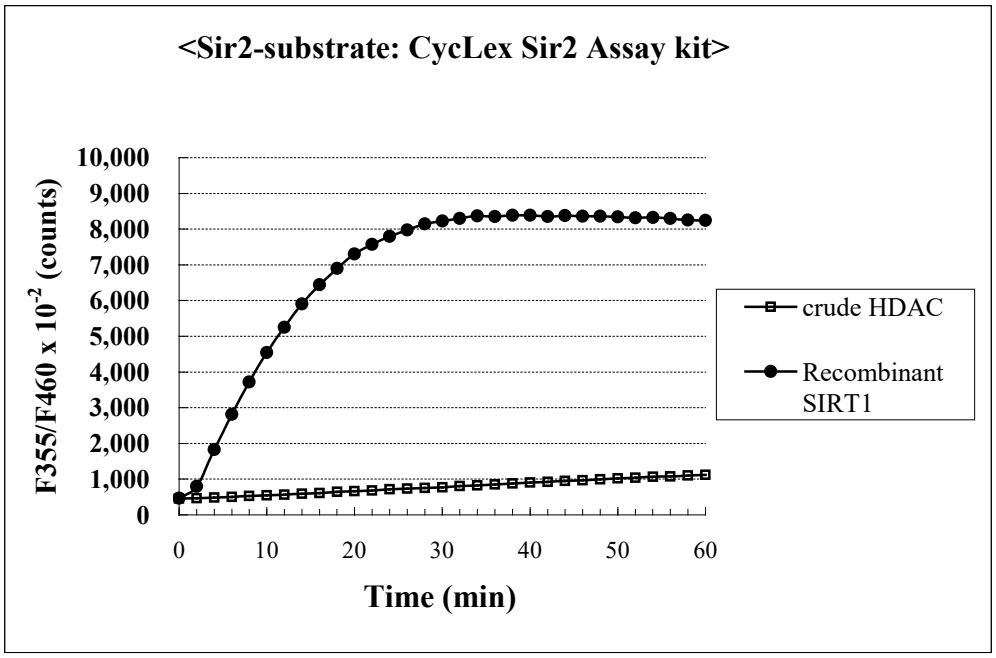


Fig.6 Time course of 2nd reaction in Two-Step Method (development reaction)

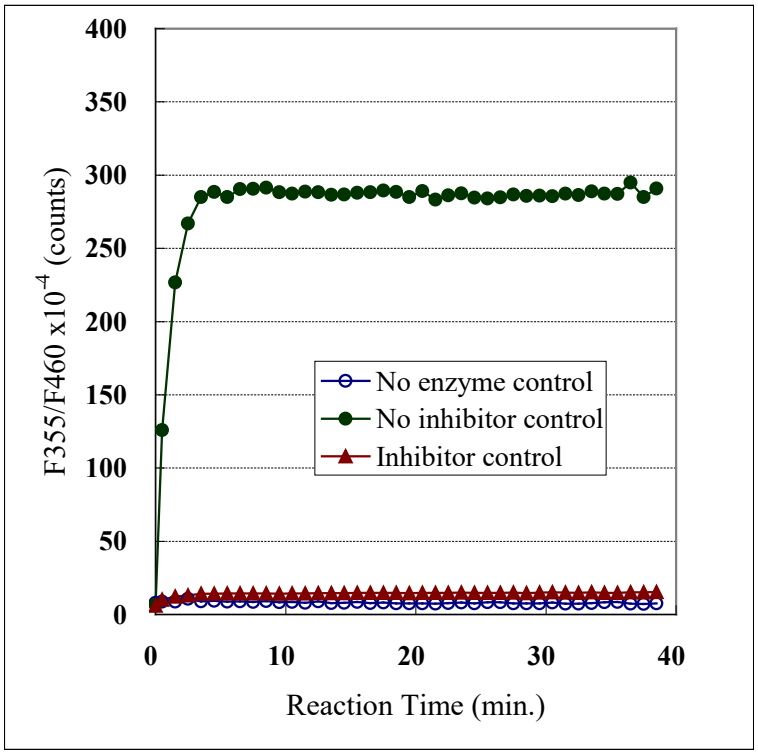


Fig.7 Measurement of HeLa cell endogenous HDAC1 in an immunoprecipitate using anti-HDAC1 antibody (Cat# CY-P1011) by means of CycLex HDACs Deacetylase Fluorometric Assay Kit

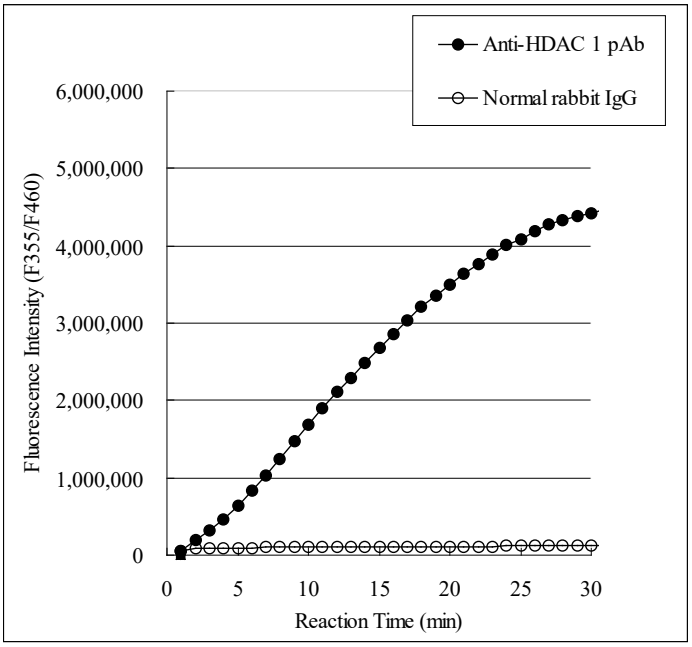
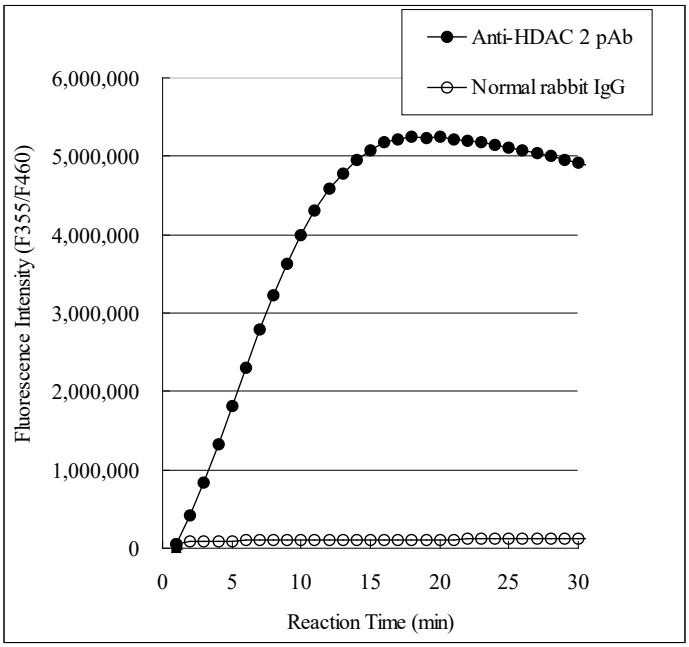


Fig.8 Measurement of HeLa cell endogenous HDAC2 in an immunoprecipitate using anti-HDAC2 antibody (Cat# CY-P1012) by means of CycLex HDACs Deacetylase Fluorometric Assay Kit



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