Quantitative test kit for histone deacetylase activity

CycLex HDAC8 Deacetylase Fluorometric Assay Kit

100 Assays

Cat# CY-1158

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Intended Use

The CycLex Research Product **CycLex HDAC8 Deacetylase Fluorometric Assay Kit** detects HDAC activity in lysates. Primarily, the CycLex Research Product **CycLex HDAC8 Deacetylase Fluorometric Assay Kit** is designed for the rapid and sensitive evaluation of HDAC inhibitors using recombinant HDAC8. Additionally, any cultured primary cell, cell line, or tissue homogenate can be assayed for HDAC8 activity with the CycLex Research Product **CycLex HDAC8 Deacetylase Fluorometric Assay Kit** after immunoprecipitation with an appropriate HDAC8 specific antibody.

Applications for this kit include:
1) Monitoring the purification of HDACs including HDAC1, 2, 3 and 8.
2) Screening inhibitors or activators of HDAC8.
3) Detecting the effects of pharmacological agents on HDAC8.

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

Storage

- Upon receipt store recombinant HDAC8 at -70°C and all other components below -20°C.
- Don't expose reagents to excessive light.

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Introduction

HDAC proteins are vital regulators of fundamental cellular events, including cell cycle progression, differentiation, and tumorigenesis (1, 2). A small-molecule inhibitor of HDAC, trichostatin A (TSA), arrests mammalian cells in both G1 and G2 (3, 4), while overexpression of HDAC1 in mouse cells reduces their growth rate by lengthening the duration of G2 and M (5). TSA induces terminal differentiation of mouse erythroleukemia cells and apoptosis of lymphoid and colorectal cancer cells. In addition, TSA treatment of cells expressing the PML zinc finger protein derepresses transcription and allows cells to differentiate normally (6). With this precedent, HDAC inhibitors are being actively explored as potential agents for the treatment of certain forms of cancer (7-9).

The human HDACs are organized into three different classes based on their similarity to yeast HDAC proteins (1, 2). Class I enzymes are ubiquitously expressed and include HDAC1, -2, -3, and -8, which are homologous to the yeast RPD3 protein. Class II includes HDAC4, -5, -6, -7, -9, and -10, which are similar to yeast HDA1 and are expressed in a tissue-specific manner. The Sir2-like class III HDACs, including SIRT1 to -7, require NAD(+) for enzymatic activity.

It has been reported that HDAC8 is important for the growth of human tumor cell lines and has a distinct inhibition pattern that differs from that of HDAC1 and -3, which both share 43% sequence identity with HDAC8. These findings lead to open the way to the development of selective inhibitors of this subtype as potential novel anticancer therapeutics.

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.

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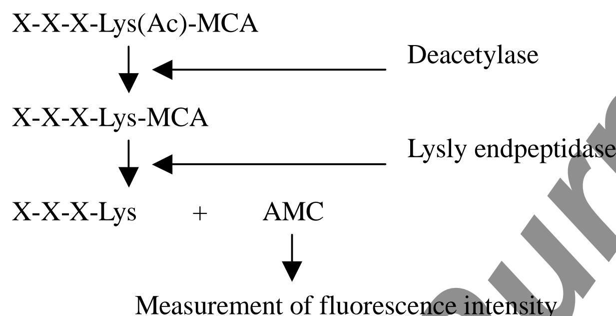
Principle of the Assay

CycLex HDAC8 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 HDAC8 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 HDAC8 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 50X Trichostatin A (1 mM)	20 µL x 1	Below -20°C
⑥ Recombinant HDAC8	200 µL x 1	-70°C
⑦ 100X Stop solution	100 µ l x 1	Below -20°C
⑧ Instruction manual	1	room temp.

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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 ⑥Recombinant HDAC8 in this kit. 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 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.**

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Detailed Protocol

Description of assay system

CycLex HDAC8 Deacetylase Fluorometric Assay Kit can measure the enzyme activity of HDAC8 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, HDAC8 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 the Stop solution, and to measure fluorescence intensity.

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. X20 diluted Lysylendpeptidase (5 mAU/ml)
Quantity required: 2.5 µL/assay
・Dilute the ④Lysylendpeptidase 1:20 with #1. 1X Assay buffer.

#3. 10X TSA (200 µM)
Quantity required: 5 µL/assay
・Dilute the ⑤50X Trichostatin A 1:5 with #1. 1X Assay buffer.

#4. 10X Inhibitor or equivalent (10X final concentration)
Quantity Required: 5 µL/assay
・Dilute Inhibitor or equivalent to 10X final desired concentration with #1. 1X Assay buffer.

#5. X5 diluted recombinant HDAC8
Quantity Required: 10 µL/assay
・Dilute the ⑥Recombinant HDAC8 1:5 with #1. 1X Assay buffer.
(Note! Use "#5. X5 diluted recombinant HDAC8" within the same day they are prepared.)

#6. 2X Stop solution
Quantity required: 50 µL/assay
・Dilute the ⑦100X Stop solution 1:50 with dH_2O.

#7. HDAC8 reaction buffer (Final 0.25 mAU/ml Lysylendpeptidase and 20 µM Fluoro-Substrate Peptide in 50 µL of assay mixture)
Quantity Required: 35 µL/assay (in case of adding 10 µL of enzyme and 5 µL of inhibitor or equivalent)
・Mix following reagents (35 µL/1 assay)

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	Component	1 assay	10 assays	50 assays	100 assays
1.	①10X Assay buffer	5 µL	50 µL	250 µL	500 µL
2.	②50X Fluoro-Substrate Peptide	1 µL	10 µL	50 µL	100 µL
3.	#2. X20 diluted Lysylendpeptidase	2.5 µL	25 µL	125 µL	250 µL
4.	dH_2O	26.5 µL	265 µL	1325 µL	2650 µL
	Total	35 µL	350 µL	1750 µL	3500 µL

HDAC8 Assay Procedures

1. Assay method

Assay reagents	Test sample	Vehicle control	No enzyme control	Inhibitor control
#7. HDAC8 reaction buffer **#4. 10X Inhibitor or equivalent** **Vehicle for Inhibitor** **#3. 10X TSA**	**35 µL** **5 µL** **-**	**35 µL** **-** **5 µL**	**35 µL** **-** **5 µL**	**35 µL** **-** **-** **5 µL**
Buffer for your enzyme sample	**-**	**-**	**10 µL**	**-**
#5. X5 diluted recombinant HDAC8 or Your enzyme sample	**10 µL**	**10 µL**	**-**	**10 µL**

1) Following the above table, add Reagent #7 and #3 or #4, to each well of the microplate. Finally, initiate reaction by adding 10 µL of "#5. X5 diluted recombinant HDAC8" or "your enzyme sample" to each well and mixing thoroughly. Incubate at room temperature (Ca.25°C).

2) Read fluorescence intensity for 30 to 60 minutes at 1 to 2 minute intervals using microtiter plate fluorometer with excitation at 340 nm and emission at 440 nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Alternate procedure

1') Following the above table, add Reagent #7 and #3 or #4 to each well of the microplate. Finally, initiate reaction by adding 10 µL of "#5. X5 diluted recombinant HDAC8" 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 µL of "#6. 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 350-380 nm and emission at 440-460 nm

3') The difference in fluorescence intensity between "Vehicle control" and "No enzyme control" indicates the HDAC8 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 recommended.

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2. Assay control

1. When the chemicals that have an inhibitory effect on lysylendpeptidase come to be mixed in HDAC8 fraction purified from various cells or the immunoprecipitate using the specific antibody against HDAC8 or other proteins, precise HDAC8 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 **"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 HDAC8 activity in your enzyme sample. When there is an inhibitory effect on lysylendpeptidase activity, even if there is HDAC8 activity in a sample, fluorescence intensity should not increase.

2. Not only when an inhibitory effect on HDAC8 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 please carry out the experiment of **"Positive control"** and **"Assay control-2"** that does not add HDAC8 in the following Table. Although fluorescence intensity increases when Fluoro-Deacetylated Peptide is used, when an inhibitory effect on lysly endpeptidase activity occurs in a test chemicals, fluorescence intensity does not increase.

Assay reagents	Assay control-1	Assay control-2	Positive control
① 10X Assay buffer	**5 µL**	**5 µL**	**5 µL**
③ 50X Fluoro-Deacetylated Peptide	**1 µL**	**1 µL**	**1 µL**
#4. 10X Inhibitor or equivalent	**-**	**5 µL**	**-**
#6. Your enzyme sample	**5 µL**	**-**	**-**
dH_2O	**26.5 µL**	**26.5 µL**	**31.5 µL**
#2. X20 diluted Lysylendpeptidase	**2.5 µL**	**2.5 µL**	**2.5 µL**

1) Following the table above, add Reagent ①, ③, #4 or #6 and dH_2O to each well. Finally, add 2.5 µL of "#2. X20 diluted Lysylendpeptidase" to each well and mix thoroughly to initiate reaction.

2) Incubate for 30 min or desired length of time at room temperature (Ca.25°C).

3) Add 50 µL of "#6. 2X Stop solution" to each well.

4) Read fluorescence intensity using microtiter plate fluorometer with excitation at 360 nm and emission at 460 nm.

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Cautions

1. In order to measure the activity of HDAC8 correctly, it is necessary to conduct the control experiments for "**No enzyme control**" and "**Inhibitor control**" at least once in addition to "**Vehicle control**," as indicated in the above table. Although fluorescence intensity increases in "**Vehicle 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 HDAC8 activity in the test chemicals correctly, it is necessary to conduct the control experiment of "**Vehicle 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 "**Vehicle 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 HDAC8 fraction purified from various cells or the immunoprecipitate using the specific antibody against HDAC8 or other proteins, precise HDAC8 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 HDAC8 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 HDAC8. 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. When chemicals that have an inhibitory effect on lysylendpeptidase are mixed in a HDAC8 fraction purified from various cells or the immunoprecipitate using a specific antibody against HDAC8 or other proteins, precise HDAC8 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.

2. Final fluorescence intensity will not increase, both when test chemicals have an inhibitory effect on HDAC8, and also when there is an inhibitory effect on lysylendpeptidase.

3. If the test reagents themselves emit fluorescence at excitation wavelength: 350-380 nm and fluorescence wavelength: 440-460 nm, the inhibitory effect of the test assay cannot be evaluated correctly.

4. The recombinant HDAC8 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 **HDAC Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, **store the ⑥ Recombinant HDAC8 at -70°C**, all other kit reagents should be stored below -20°C.

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Example of Test Results

Fig.1 Dose dependency of recombinant HDAC8 (30min.)

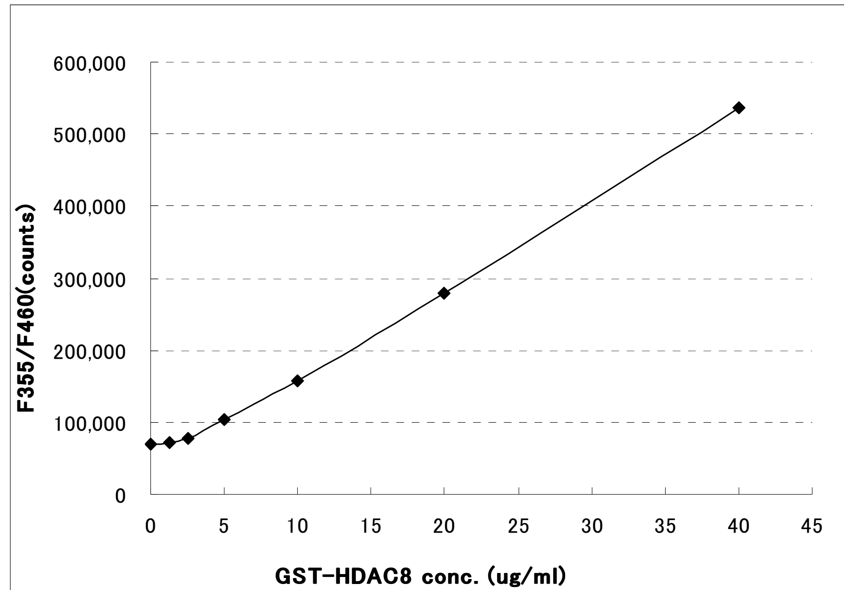

Fig.2 Time course of HDAC reaction

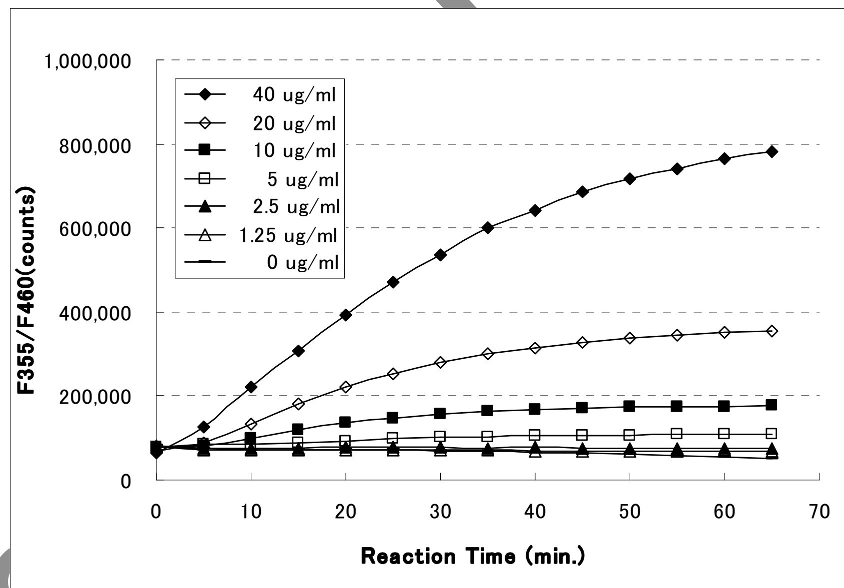

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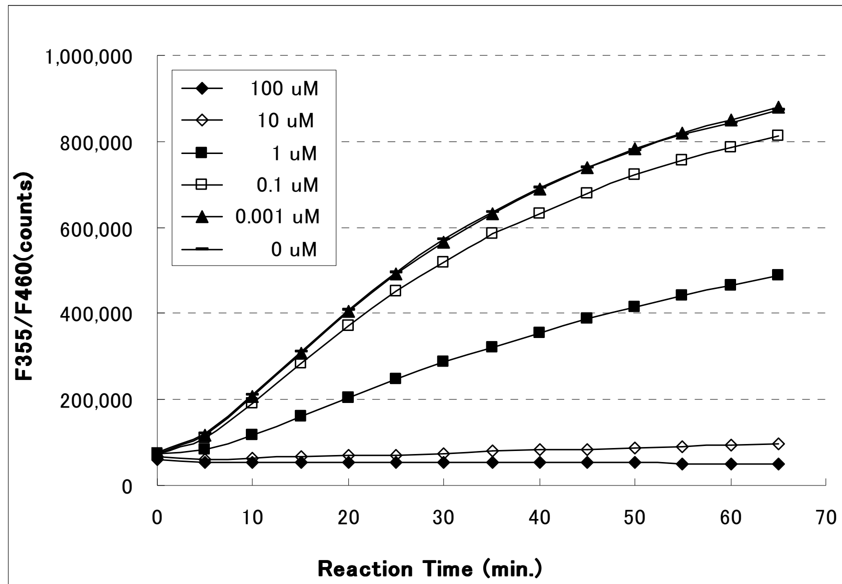
Fig.3 Effect of Trichostatin A on HDAC activity (One-step method)



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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

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