Quantitative test kit for histone deacetylase 8 activity

CycLex HDAC8 Deacetylase Fluorometric Assay Kit Ver.2

100 Assays

Cat# CY-1158V2

Intended Use.....1
Storage.....1
Introduction.....2
Principle of the Assay.....3
Materials Provided.....3
Materials Required but not Provided.....4
Precautions.....4
Detailed Protocol.....5-7
Cautions.....8
Troubleshooting.....8
Reagent Stability.....8
Example of Test Results.....9-10
References.....11

Intended Use

The MBL Research Product **CycLex HDAC8 Deacetylase Fluorometric Assay Kit** detects HDAC activity in lysates. Primarily, the MBL 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 MBL 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 #5. Developer and #6. Recombinant HDAC8 at -70°C and all other components below -20°C.
- Do not expose reagents to excessive light.

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.

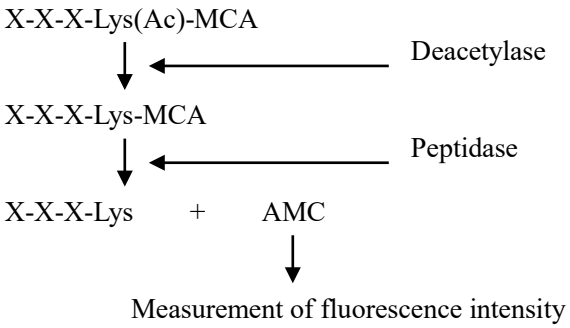
However, the conventional method for measuring HDAC activity is very complicated and laborious. In order to measure HDAC enzyme activity, it is necessary to prepare radioactive acetylated histone as a substrate. First, cells have to be labeled metabolically with radioactivity by adding radioactive acetic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

Principle of the Assay

The CycLex HDAC8 Deacetylase Fluorometric Assay Kit measures the activity of HDAC by the basic principle of changing an HDAC reaction into the activity of protease/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 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



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 (200 µM)	**500 µL x 1**	**Below -20°C**
#5. Developer	**500 µL x 1**	**-70°C**
#6. Recombinant HDAC8	**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. Recombinant HDAC8"**. 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.

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

Detailed Protocol

The 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 a substrate, HDAC8 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.

1. Assay Procedures for Measurement of HDAC Activity

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 HDAC activity

Assay reagents	Enzyme Sample Assay	No Enzyme Control Assay	Positive Control Assay	Inhibitor Control Assay
Distilled water #1. HDAC Assay buffer #2. Fluoro-Substrate Peptide	30 µL 5 µL 5 µL	30 µL 5 µL 5 µL	30 µL 5 µL 5 µL	25 µL 5 µL 5 µL
#4. Trichostatin A	-	-	-	5 µL
#5. Developer	5 µL	5 µL	5 µL	5 µL
Enzyme Sample Buffer of Enzyme Sample #6. Recombinant HDAC8	5 µL - -	- 5 µL -	- - 5 µL	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. Recombinant HDAC8"** 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. Assay Procedures for Inhibitor Screening

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. Recombinant HDAC8 (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. Recombinant HDAC8"** (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.

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. Recombinant HDAC8"** 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 HDAC8 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. Recombinant HDAC8"** at -70°C, all other kit reagents should be stored below -20°C.

Example of Test Results

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

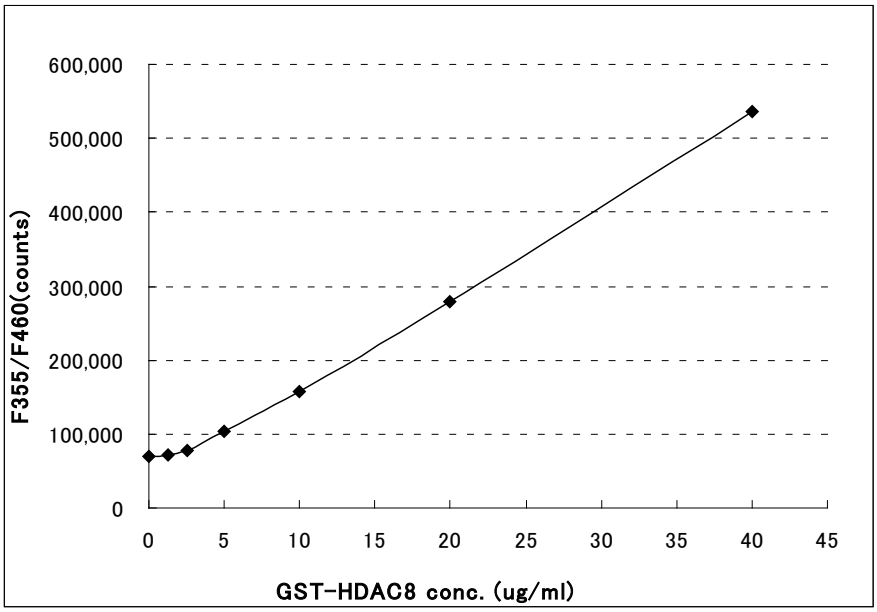


Fig.2 Time course of HDAC8 reaction

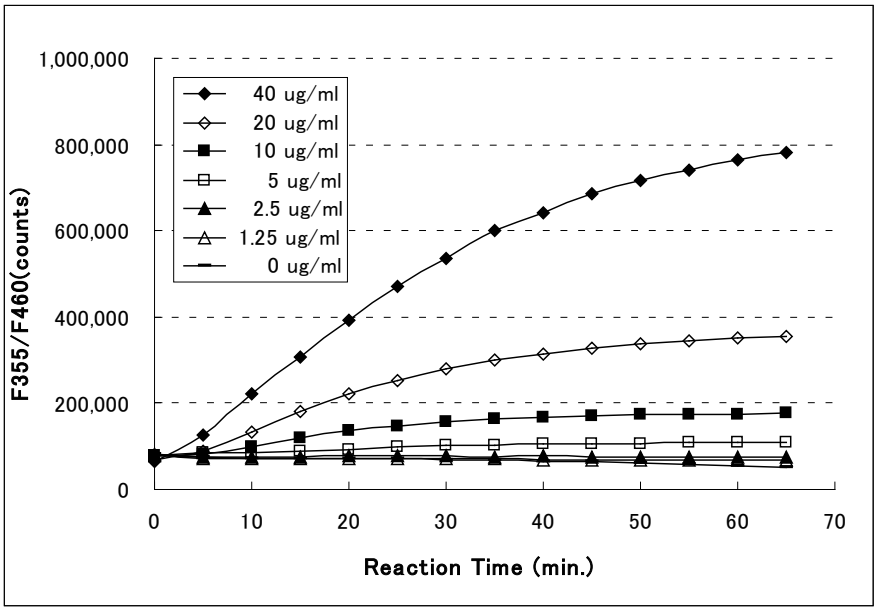
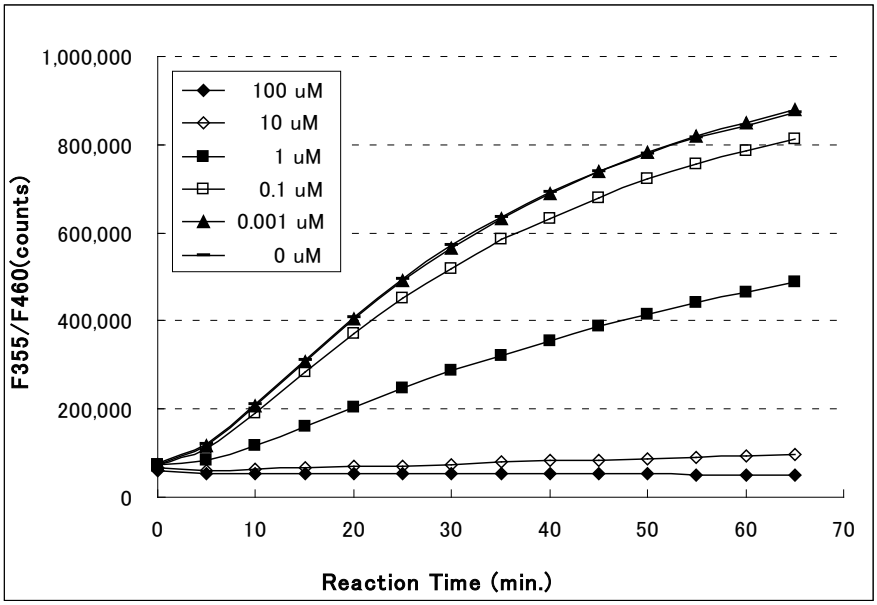


Fig.3 Effect of Trichostatin A on HDAC8 activity (One-step method)



References

1. Thiagalingam, S., K. H. Cheng, H. J. Lee, N. Mineva, A. Thiagalingam, and J. F. Ponte. 2003. Histone deacetylases: unique players in shaping the epigenetic histone code. Ann. N. Y. Acad. Sci. 983:84-100

2. Yang, X. J., and E. Seto. 2003. Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. Curr. Opin. Genet. Dev. 13:143-153

3. Ogryzko, V. V., T. H. Hirai, V. R. Russanova, D. A. Barbie, and B. H. Howard. 1996. Human fibroblast commitment to a senescence-like state in response to histone deacetylase inhibitors is cell cycle dependent. Mol. Cell. Biol. 16:5210-5218.

4. Wharton, W., J. Savell, W. D. Cress, E. Seto, and W. J. Pledger. 2000. Inhibition of mitogenesis in Balb/c-3T3 cells by trichostatin A. Multiple alterations in the induction and activation of cyclin-cyclin-dependent kinase complexes. J. Biol. Chem. 275:33981-33987

5. Bartl, S., J. Taplick, G. Lagger, H. Khier, K. Kuchler, and C. Seiser. 1997. Identification of mouse histone deacetylase 1 as a growth factor-inducible gene. Mol. Cell. Biol. 17:5033-5043

6. He, L. Z., F. Guidez, C. Tribioli, D. Peruzzi, M. Ruthardt, A. Zelent, and P. P. Pandolfi. 1998. Distinct interactions of PML-RAR{alpha} and PLZF-RAR{alpha} with co-repressors determine differential responses to RA in APL. Nat. Genet. 18:126-135

7. Johnstone, R. W. 2002. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat. Rev. Drug Discov. 1:287-299.[CrossRef][Medline]

8. Kelly, W. K., O. A. O'Connor, and P. A. Marks. 2002. Histone deacetylase inhibitors: from target to clinical trials. Expert Opin. Investig. Drugs 11:1695-1713

9. Melnick, A., and J. D. Licht. 2002. Histone deacetylases as therapeutic targets in hematologic malignancies. Curr. Opin. Hematol. 9:322-332

10. Gao, L., M. A. Cueto, F. Asselbergs, and P. Atadja. 2002. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J. Biol. Chem. 277:25748-25755

For more information, please visit our web site.
https://ruo.mbl.co.jp/

MANUFACTURED BY

A JSR Life Sciences Company
MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.
URL: https://ruo.mbl.co.jp
E-mail: support@mbl.co.jp