ab156069

HDAC8 Activity Assay Kit (Fluorometric)

Instructions for Use

For the quantitative measurement of HDAC8 activity in cell lysates

This product is for research use only and is not intended for diagnostic use.

Version 3 Last Updated 3 March 2015

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1. Background

HDAC proteins are vital regulators of fundamental cellular events, including cell cycle progression, differentiation, and tumorigenesis. A small-molecule inhibitor of HDAC, trichostatin A (TSA), arrests mammalian cells in both G1 and G2, while overexpression of HDAC1 in mouse cells reduces their growth rate by lengthening the duration of G2 and M. 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. With this precedent, HDAC inhibitors are being actively explored as potential agents for the treatment of certain forms of cancer.

The human HDACs are organized into three different classes based on their similarity to yeast HDAC proteins. 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.

2. Overview

Abcam's HDAC8 Activity Assay Kit (Fluorometric) (ab156069) detects HDAC activity in lysates.

Primarily, the HDAC8 Activity Assay Kit (Fluorometric) 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 HDAC8 Activity Assay Kit (Fluorometric) after immunoprecipitation with an appropriate HDAC8 specific antibody.

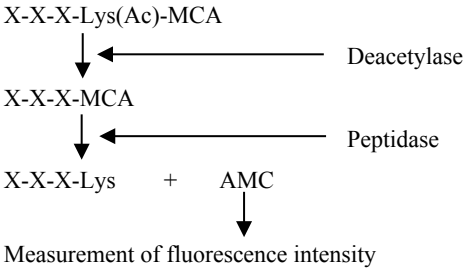
Applications for this kit include:

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.

3. Principle of the Assay

Abcam's HDAC8 Activity Assay Kit (Fluorometric) 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 HDAC8 Activity Assay Kit (Fluorometric) using the same equipment. This new method of measurement should dramatically raise the efficiency of inhibitor screening and biochemical analysis of these enzymes.

Assay Principle:



4. Protocol Summary

Prepare samples

Prepare reaction wells (sample and control)

Add inhibitor and developer to appropriate wells & mix thoroughly

Add HDAC8 enzyme (control enzyme or your sample) to each well.

(optional) Add Stop Solution to stop reaction

Measure Fluorescence Intensity at 1 – 2 min intervals

at Ex = 350 – 380 nm and Em = 440-460.

5. Materials Supplied

Item	Identifier	Quantity	Storage
HDAC Assay Buffer	#1	2 x 1 mL	-20°C
Fluoro-Substrate Peptide (0.2 mM)	#2	500 µL	-20°C
Fluoro-Deacetylated Peptide (0.2 mM)	#3	100 µL	-20°C
Trichostatin A (200 µM)	#4	500 µL	-20°C
Developer	#5	500 µL	-80°C
Recombinant HDAC8	#6	500 µL	-80°C
Stop Solution	#7	2 x 1 mL	-20°C

6. Storage and Stability

All reagents included in this kit have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, store the Developer and Recombinant HDAC8 at -80°C, all other kit reagents should be stored below -20°C.

- Thaw Fluoro-Substrate Peptide and Fluoro-Deacetylated Peptide at room temperature before use. Then, thaw the other reagents in ice and use after they are completely thawed.

- Avoid repeated freezing and thawing of Recombinant HDAC8. There is a possibility that the enzyme activity may be inactivated. Aliquot to 10-20 µL and store at -80°C.

- Avoid mixing of protease/peptidase inhibitors such as PMSF, or alkyl amine in samples that will be measured HDAC8 activity.

7. Materials Required, Not Supplied

- 96 well plate – black wells
- MilliQ water or other type of double distilled water (ddH_2O)
- Microcentrifuge
- Pipettes and pipette tips
- Microplate reader capable of measuring fluorescence at Ex/Em = 350-380/440-460 nm
- Orbital shaker

8. Assay Protocol

Abcam's HDAC8 Activity Assay Kit (Fluorometric) 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 for Quantification of HDAC8 Activity

1. Following the table below and **in duplicate,** add ddH_2O, #1. HDAC8 Assay Buffer, #2. Fluoro-Substrate Peptide and #4. Trichostatin A to microtiter plate wells.
2. Add #5. Developer to each well of the microtiter plate and mix well.

Assay reagents	Test sample	No enzyme control	No Test Sample control	No NAD control
ddH_2O	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(1)	-	5 µL	-	-
#6. Recombinant HDAC8	-	-	5 µL	-
Total volume	50 µL	50 µL	50 µL	50 µL

(1)More information on Sample preparation in section 10

3. 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.

NOTE: Although the volume of addition of Enzyme Sample or Buffer of Enzyme Sample or #6. Recombinant HDAC8 is set to 5 µL in the previous table, 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.

4. 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.

Alternative procedure

1. Follow procedure described above till step 4.
2. 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 for HDAC8 Inhibitor/Activator Screening

1. Following the table on the next page and **in duplicate**, add ddH_2O, #1. HDAC Assay Buffer and #2. Fluoro-Substrate Peptide or #3. Fluoro-Deacetylated Peptide to microtiter plate wells.
2. Add Test Compound (inhibitor compound to test) or just the Solvent in which compound is dissolved (control) or #4. Trichostatin A to each well of the microtiter plate and mix.
3. Add #5. Developer to each well of the microtiter plate and mix well.
4. Initiate reactions by adding 5 µL of #6. Recombinant HDAC8 or your Enzyme Sample to each well and mix thoroughly at RT.
   NOTE: Although the volume of addition of 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 ddH_2O to set the final reaction volume of 50 µL.
5. 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.

Assay reagents	Test Compound Assay	Solvent Control Assay	Control Compound Assay	No Enzyme Control Assay	Development Control Assay
ddH_2O	25 µL	25 µL	25 µL	30 µL	30 µL
#1. HDAC8 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	50 µL	50 µL	50 µL	50 µL	50 µL

(1)More information on Sample preparation in section 10

Alternative procedure

1. Follow procedure described above till step 4.
2. 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:

- Although the above tables indicate the volume of addition of Test Compound or Solvent of Test Compound or Control Compound (not provided) 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 or Control Compound (not provided). In this case, please reduce the volume of Distilled water to set the final reaction volume of 50 µL.

- During the time in which HDAC8 reaction rate is maintained, the difference in fluorescence intensity between Solvent Control Assay and No Enzyme Control Assay indicates the HDAC8 activity.

- In order to estimate the active or inhibitory effect on HDAC8 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 Control Compound Assay at least once for the first experiment, in addition to Test Compound Assay as indicated in the Table.2. When test compounds cause an active or inhibitory effect on HDAC8 activity, the level of increase of fluorescence intensity is strengthened or weakened as compared with Solvent Control Assay.

- The efficacy of the test compounds on the HDAC8 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.

- 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 HDAC8 activity cannot be evaluated correctly.

9. Data Analysis

1. Typical Results

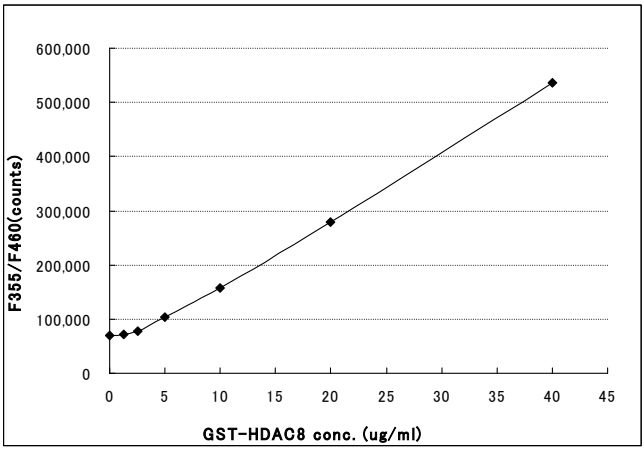


Figure 1: Dose dependency of recombinant HDAC8 (30min.)

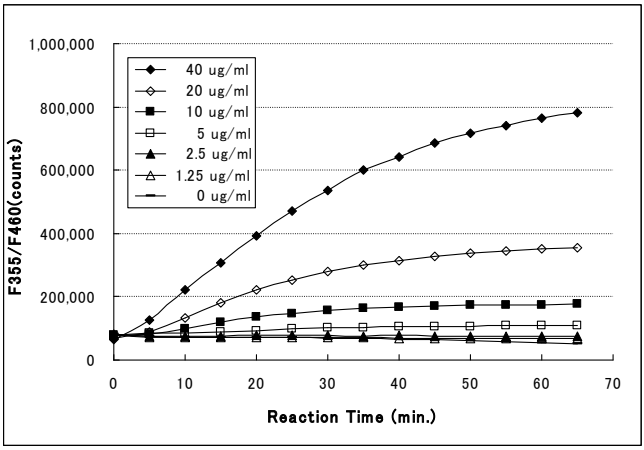


Figure 2: Time course of HDAC8 reaction

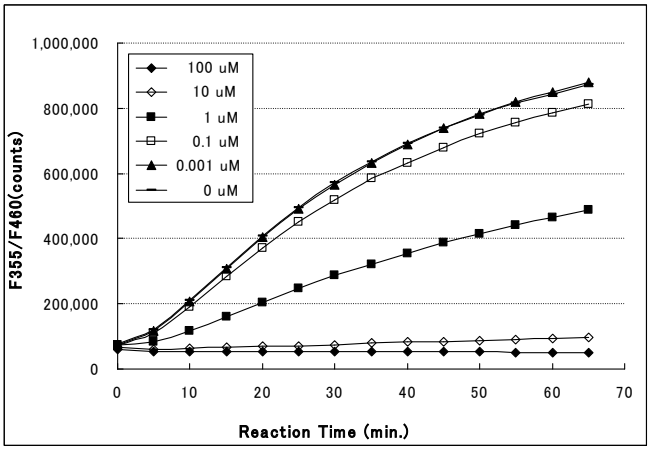


Figure 3: Effect of Trichostatin A on HDAC8 activity

10. Sample Preparation

Numerous extraction and purification methods can be used to isolate HDAC8. 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 HDAC8 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 -80°C to prevent loss of enzymatic activity.

A. Buffer Preparation

Lysis Buffer	**Sucrose Cushion**	**Extraction Buffer**
10 mM Tris HCl (pH 7.5) 10 mM NaCl 15 mM $MgCl_2$ 250 mM Sucrose 0.5 % NP-40 0.1 mM EGTA	30 % Sucrose 10 mM Tris HCl (pH 7.5) 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

B. Isolation of Nuclei

1. Resuspend 1x 10^7 cells into 1 mL 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.

C. Extraction of Nuclei

1. Resuspend the isolated nuclei in 50-100 µL of extraction buffer.
2. Sonicate for 30 seconds.
3. Stand on ice for 30 min.
4. Centrifuge at 20,000 x *g* for 10 min.
5. Take supernatant (the crude nuclear extract).
6. Determine protein concentration by Bradford method or equivalent.
7. Store the crude nuclear extract at -80°C until use.

11. Troubleshooting

- When chemicals that have an inhibitory effect on the peptidase are mixed in a crude 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/peptidase inhibitors used in the usual protein purification process inhibit the peptidase activity strongly, please avoid the use of any protease/peptidase inhibitors during the protein purification process.

- 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 the peptidase.

- 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.

- The recombinant HDAC8 should be run in duplicate, using the protocol described in the Assay Protocol. Incubation times or temperatures significantly different from those specified may give erroneous results.

- 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.

- Poor duplicates indicate inaccurate dispensing. If all instructions in the Assay Protocol were followed accurately, such results indicate a need for multi-channel pipettor maintenance.

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