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ab156064

Histone Deacetylase (HDAC) Activity Assay Kit (Fluorometric)

Instructions for Use

For the quantitative measurement of Histone Deacetylase activity in crude HDAC fraction (nuclear extracts).

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

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1. Background

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.

2. Overview

Abcam’s Histone Deacetylase (HDAC) Activity Assay Kit (Fluorometric) is designed for the rapid and sensitive evaluation of HDAC inhibitors using crude HDAC fraction (nuclear extracts). Additionally, any cultured primary cell, cell line, can be assayed for HDAC activity with the Histone Deacetylase (HDAC) Activity Assay Kit if the appropriate dose of HDAC specific inhibitor e.g. Trichostatin A is used.

Abcam’s Histone Deacetylase (HDAC) Activity Assay Kit (Fluorometric) has been shown to detect the activity of HDAC family, at least class I HDACs in Human or animal cell lysates or in column fractions. The assay shows good linearity of sample response. The assay may be used to follow the purification of HDACs or may be used to detect the presence of HDACs in cell lysates

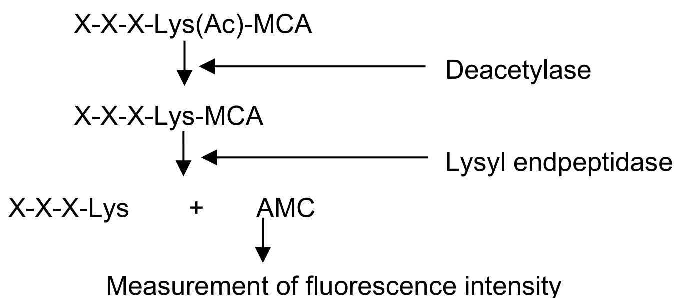
Applications for this kit include:

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.

3. Principle of the Assay

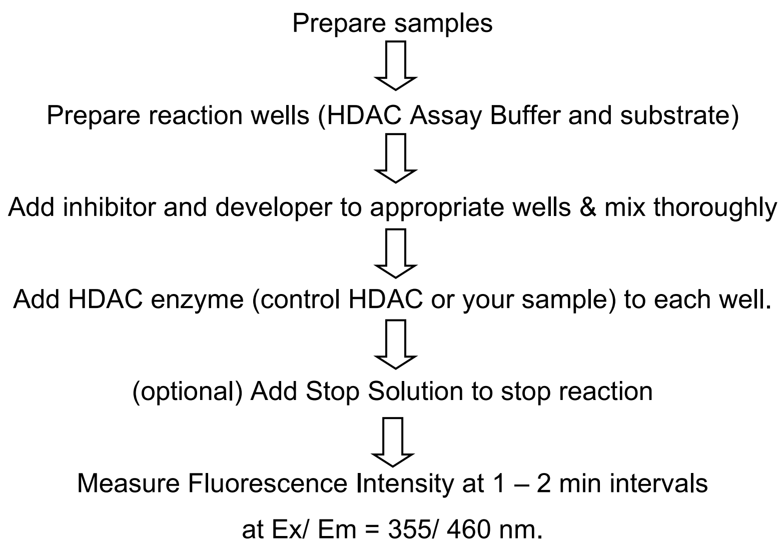
Abcam’s Histone Deacetylase (HDAC) Activity Assay Kit (Fluorometric) measures the activity of HDAC by the basic principle of changing an HDAC reaction into the activity of the peptidase. Since it is very simple to measure common protease activity and it can be performed at a low price, the measurement of HDAC activity in most laboratories is possible if they are equipped with a fluorescent reader for microtiter plates. This new method of measurement should dramatically raise the efficiency of inhibitor screening and biochemical analysis of these enzymes.

Assay Principle:

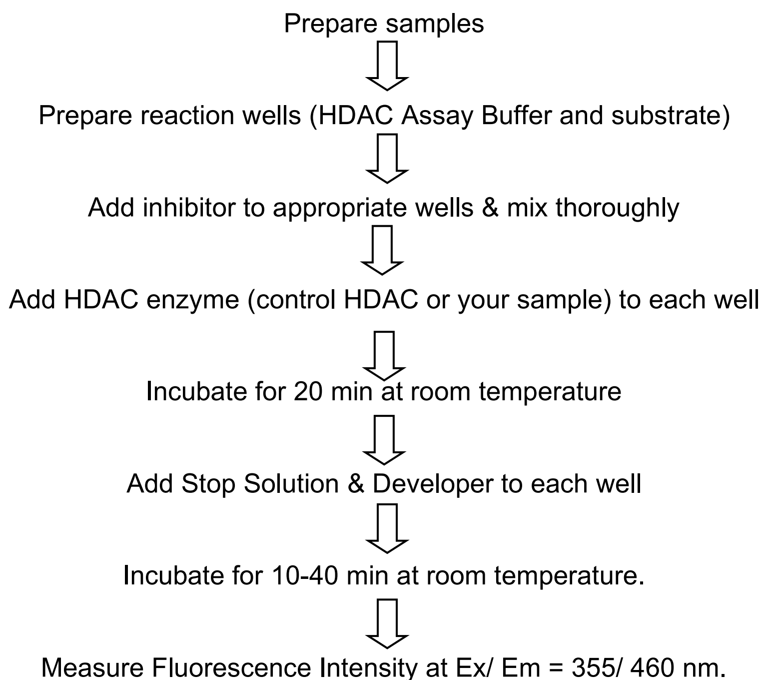


4. Protocol Summary

One-Step Method



Two-Step Method



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 (10 µM)	#4	500 µL	-20°C
Developer	#5	500 µL	-80°C
Crude HDAC (crude nuclear extract from HeLa)	#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. Upon receipt, store the Developer and Crude HDAC at -80°C, all other kit reagents should be stored below -20°C.

- Thaw Fluoro-Substrate Peptide and Fluoro -Deacetylated Peptide at room temperature (RT) before use. Then, thaw the other reagents in ice and use after they are completely thawed.
- Avoid repeated freezing and thawing of Developer and Crude HDAC as it may affect the enzyme stability. Aliquot to 10-20 µL and store at -80°C
- Avoid mixing of protease inhibitors such as PMSF, or alkyl amine in the sample that will be measured HDAC 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
- 500 or 100 mL graduated cylinder
- Reagent reservoirs

8. Assay Protocol

The Histone Deacetylase (HDAC) Activity Assay Kit (Fluorometric) (ab156064) can measure the enzymatic activity of HDAC with two kinds of measuring methods, one-step method and two-step methods.

In the **one-step method**, reaction is initiated and fluorescence intensity is measured by mixing simultaneously fluorescence-labeled acetylated peptide (which acts as substrate), HDAC (enzyme) and the developer. The reaction is not stopped, so it is necessary to measure fluorescence intensity at regular intervals after the reaction is initiated. Alternatively, when the reaction velocity reaches a constant rate, it is possible to stop the reaction by adding the stop solution and then measure fluorescence intensity.

Conversely, the **two-step method** begins by initiating a reaction of fluorescence-labeled acetylated peptide (substrate) and HDAC (enzyme) within a set time period to remove an acetyl group from the substrate peptide, while simultaneously cleaving the resultant deacetylated fluorescence-labeled peptide by the developer.

1. Assay for Quantification of HDAC Activity

One-Step Method

1. Following the table below and **in duplicate**, add ddH_2O, HDAC Assay Buffer (#1) and Substrate Peptide (#2) to microtiter plate wells.
2. Add Trichostatin A (#4) - HDAC inhibitor to the Inhibitor Control assay wells and mix.
3. Add Developer (#5) to each well of the microtiter plate and mix well.

Assay reagents	No enzyme Control Assay	Positive Control Assay	Inhibitor Control Assay	Enzyme Sample Assay
ddH$_2$O	30 µL	30 µL	25 µL	30 µL
HDAC Assay buffer (#1)	5 µL	5 µL	5 µL	5 µL
Fluoro-Substrate Peptide (#2)	5 µL	5 µL	5 µL	5 µL
Trichostatin A (#4)	-	-	5 µL	-
Developer (#5)	5 µL	5 µL	5 µL	5 µL
Enzyme Sample/ Extract[(1)]	-	-	5 µL	5 µL
Buffer of Enzyme Sample	5 µL	-	-	-
HDACs (#6)	-	5 µL	-	-
Total volume	50 µL	50 µL	50 µL	50 µL

(1)More information on Sample preparation in Section 10

4. Initiate reactions by adding 5 µL Buffer of Enzyme Sample (for no enzyme control assay) or 5 µL of HDACs (#6) (positive control assay) to each well and mix thoroughly at RT.

NOTE: *Although the volume of your Enzyme sample of Extract is set to 5 μL in the table, it may be changed to a volume up to 20 μL if necessary. In that case, reduce the volume of dd*H_2O *accordingly to set a final volume of 50 μL.*

5. Read fluorescence intensity for 30 – 60 minutes at 1 – 2 minute intervals using a microplate fluorescence reader at Ex/ Em = 350 – 380 nm/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 Stop Solution (#7) to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader at Ex/ Em = 350 – 380 nm / 440 – 460 nm.

Two-Step Method

1. Following the table below and **in duplicate**, add ddH_2O, HDAC Assay Buffer (#1) and Substrate Peptide (#2) to microtiter plate wells.
2. Add Trichostatin A (HDAC inhibitor) (#4) to the Inhibitor Control assay wells and mix.

Assay reagents	No enzyme Control Assay	Positive Control Assay	Inhibitor Control Assay	Enzyme Sample Assay
ddH_2O **HDAC Assay buffer (#1)** **Fluoro-Substrate Peptide (#2)**	35 µL 5 µL 5 µL	35 µL 5 µL 5 µL	30 µL 5 µL 5 µL	35 µL 5 µL 5 µL
Trichostatin A (#4)	-	-	5 µL	-
Enzyme Sample/ Extract[(1)] **Buffer of Enzyme Sample** **HDACs (#6)**	- 5 µL -	- - 5 µL	5 µL - -	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 Buffer of Enzyme sample (for No enzyme control assay) or 5 µL of HDACs (#6) (positive control assay) to each well and mix thoroughly at RT.

   NOTE: A*lthough the volume of your Enzyme sample of Extract is set to 5 µL in the table, it may be changed to a volume up to 20 µL*

if necessary. In that case, reduce the volume of ddH_2O accordingly to set a final volume of 50 µL.

4. Incubate for 20 min or desired length of time at RT.
5. Add 20 µL of Stop Solution (#7) to each well of the microtiter plate and mix thoroughly.
6. Add 5 µL of Developer (#5) to each well of the microtiter plate and mix thoroughly.
7. Incubate for at least 10 min or desired length of time at RT – measurement should be done between 10 – 40 minutes.
8. Read fluorescence intensity using microplate fluorescence reader at Ex/Em = 350 – 380 nm / 440 – 460 nm.

2. Assay for HDAC Inhibitor Screening

One-Step Method

1. Following the table on the next page and **in duplicate**, add ddH_2O, HDAC Assay Buffer (#1) and Substrate Peptide (#2) or Deacetylated Peptide (#3) to microtiter plate wells.
2. Add Test Compound (inhibitor compound to test) or just the Solvent in which compound is dissolved (control) or Trichostatin A (HDAC inhibitor) (#4) to the Inhibitor Control assay wells and mix.
3. Add Developer (#5) to each well of the microtiter plate and mix well.

4. Initiate reactions by adding 5 µL of your Enzyme sample or 5 µL of HDACs (#6) (positive control assay) to each well and mix thoroughly at RT.

   NOTE: A*lthough the volume of your Enzyme sample of Extract is set to 5 µL in the table, it may be changed to a volume up to 20 µL if necessary. In that case, reduce the volume of ddH*$_2$*O accordingly to set a final volume of 50 µL.*

5. Read fluorescence intensity for 30 – 60 minutes at 1 – 2 minute intervals using a microplate fluorescence reader at Ex/ Em = 350 – 380 nm/440 – 460 nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Assay Reagents	No enzyme Control Assay	Develop control assay	Inhibitor Control Assay	Solvent control assay	Test compound assay
ddH_2O	30 µL	30 µL	25 µL	25 µL	25 µL
HDAC Assay buffer (#1)	5 µL	5 µL	5 µL	5 µL	5 µL
Fluoro-Substrate Peptide (#2)	5 µL	-	5 µL	5 µL	5 µL
Fluoro-deacetylated Peptide (#3)	-	5 µL	-	-	-
Test Compound	-	5 µL	-	-	5 µL
Solvent of Test Compound	5 µL	-	-	5 µL	-
Trichostatin A (#4)	-	-	5 µL	-	-
Developer (#5)	5 µL	5 µL	5 µL	5 µL	5 µL
HDACs (#6) / Enzyme Sample[(1)]	-	-	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 Stop Solution (#7) to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader at Ex/ Em = 350 – 380 nm / 440 – 460 nm.

   NOTE: *Although the volume of Test Compound is set to 5 µL in the table, the concentration and the volumes of the reagents may be changed so that the concentration of the compounds becomes the setting concentration. In that case, reduce the volume of ddH$_2$O accordingly to set a final volume of 50 µL.*

Two-Step Method

1. Following the table on the next page and **in duplicate**, add ddH$_2$O, HDAC Assay Buffer (#1) and Substrate Peptide (#2) or Deacetylated Peptide (#3) to microtiter plate wells.
2. Add Test Compound (inhibitor compound to test) or just the Solvent in which compound is dissolved (control) or Trichostatin A (HDAC inhibitor) (#4) to the Inhibitor Control assay wells and mix.

   NOTE: *Although the volume of Test Compound is set to 5 µL in the table, the concentration and the volumes of the reagents may be changed so that the concentration of the compounds becomes the setting concentration. In that case, reduce the volume of ddH$_2$O accordingly to set a final volume of 50 µL.*

3. Initiate reactions by adding 5 µL of your Enzyme sample or 5 µL of HDACs (#6) (positive control assay) to each well and mix thoroughly at RT.

   NOTE: *Although the volume of your Enzyme sample of Extract is set to 5 µL in the table, it may be changed to a volume up to 20 µL if necessary. In that case, reduce the volume of ddH$_2$O accordingly to set a final volume of 50 µL.*
4. Incubate for 20 min or desired length of time at RT.
5. Add 20 µL of Stop Solution (#7) to each well of the microtiter plate and mix thoroughly.
6. Add 5 µL of Developer (#5) to each well of the microtiter plate and mix thoroughly.
7. Incubate for at least 10 min or desired length of time at RT – measurement should be done between 10 – 40 minutes.
8. Read fluorescence intensity using microplate fluorescence reader at Ex/Em = 350 – 380 nm / 440 – 460 nm.

Assay reagents	No enzyme Control Assay	Develop control assay	Inhibitor Control Assay	Solvent control assay	Test compound assay
ddH$_2$O	35 µL	35 µL	30 µL	30 µL	30 µL
HDAC Assay buffer (#1)	5 µL	5 µL	5 µL	5 µL	5 µL
Fluoro-Substrate Peptide (#2)	5 µL	-	5 µL	5 µL	5 µL
Fluoro-deacetylated Peptide (#3)	-	5 µL	-	-	-
Test Compound	-	5 µL	-	-	5 µL
Solvent of Test Compound	5 µL	-	-	5 µL	-
Trichostatin A (#4)	-	-	5 µL	-	-
HDACs (#6) / Enzyme Sample(1)	-	-	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

9. Data Analysis

1. Quantification of HDAC activity

- Determine the average fluorescence of each data point based on the duplicates
- During the time in which HDAC reaction rate is maintained, the HDAC activity of the Enzyme sample can be calculated as follows:

HDAC activity = Enzyme Sample Assay – No Enzyme Control Assay

Assay considerations:

1. If enzyme samples contain some protease /peptidase able to break down Fluoro-substrate Peptide (#2), this can result in an increase of fluorescence intensity in the **Inhibitor Control Assay** and therefore the HDAC activity in the samples cannot be evaluated correctly.
2. 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 purification process.
3. If enzyme samples have an inhibitory effect on the peptidase in the development reaction, the final fluorescence intensity will not

increase. Please use Fluoro-deacetylated Peptide (#3) instead of Fluoro-substrate Peptide (#2), and proceed with experiment as described.

2. Assay for HDAC Inhibitor Screening

- Determine the average fluorescence of each data point based on the duplicates
- During the time in which HDAC reaction rate is maintained, the HDAC activity of the Enzyme sample can be calculated as follows:

HDAC activity = Solvent Control Assay – No Enzyme Control Assay

- The efficacy of inhibition of the test compounds on the HDAC activity can be calculated as follows:

Inhibition effect =

[Test compound Assay – No enzyme control] – [HDAC activity]

Assay considerations:

1. 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 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**.

2. If test compounds have an inhibitory effect on protease/ peptidase, there is little or no increase seen in the **Development Control Assay** and therefore the inhibitory effect on HDAC activity cannot be evaluated correctly.

3. Typical Results

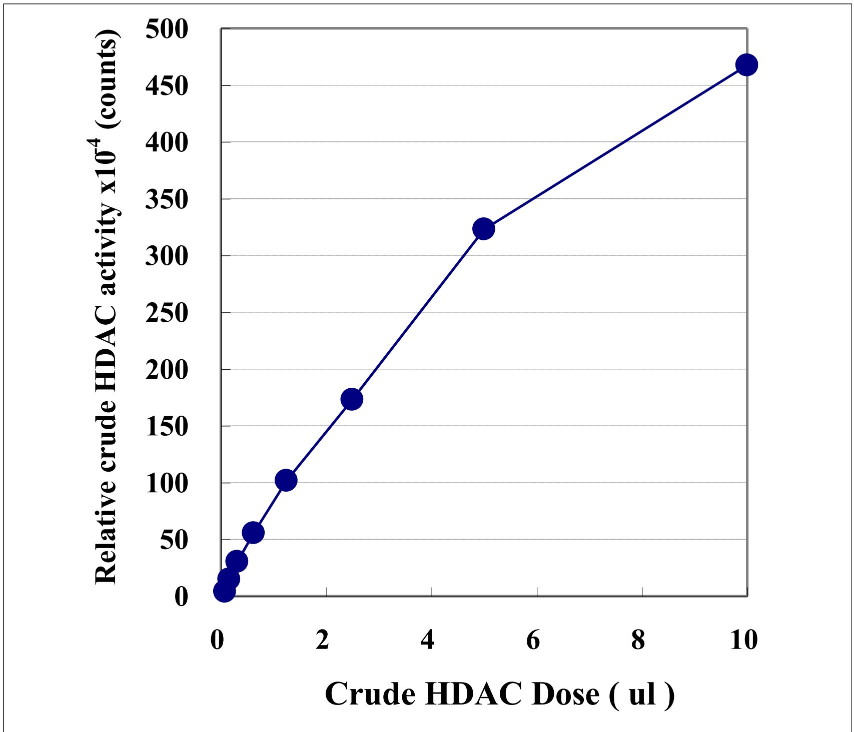


Figure 1: Dose dependency of HDAC (Two-Step Method)

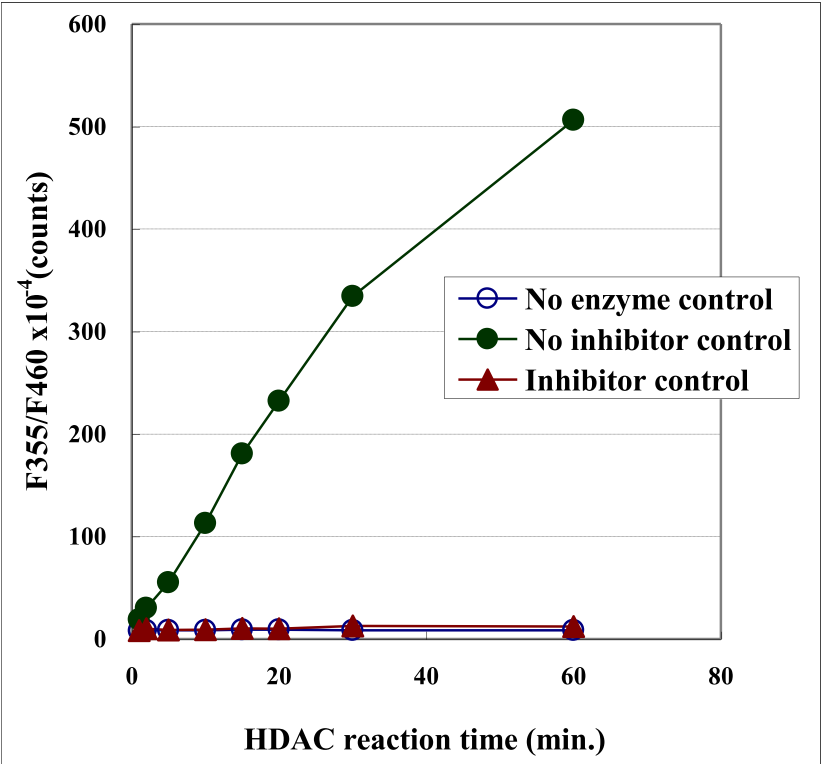


Figure 2: Time course of HDAC reaction (Two-step method)

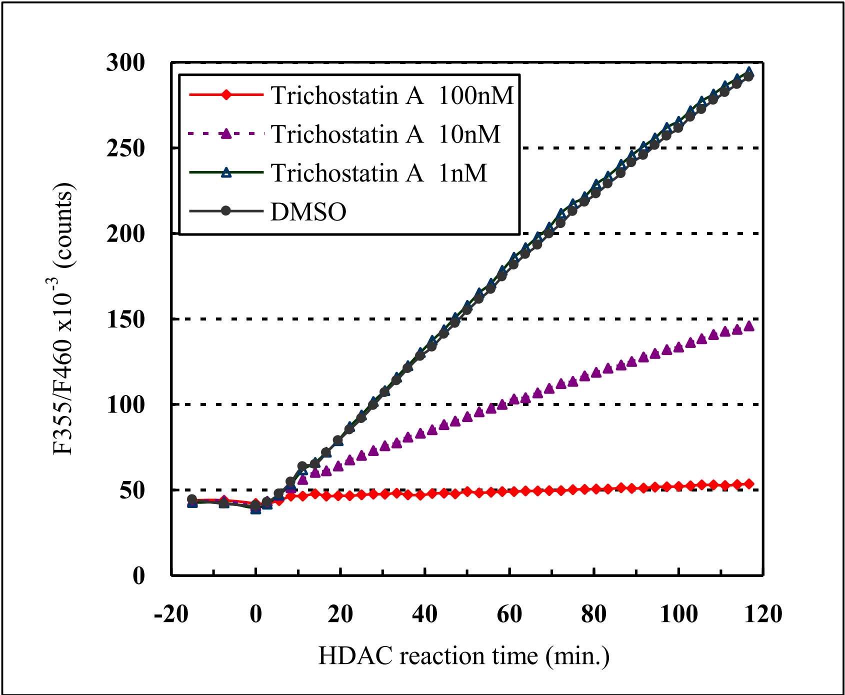


Figure 3: Effect of Trichostatin A on HDAC activity (One-step method)

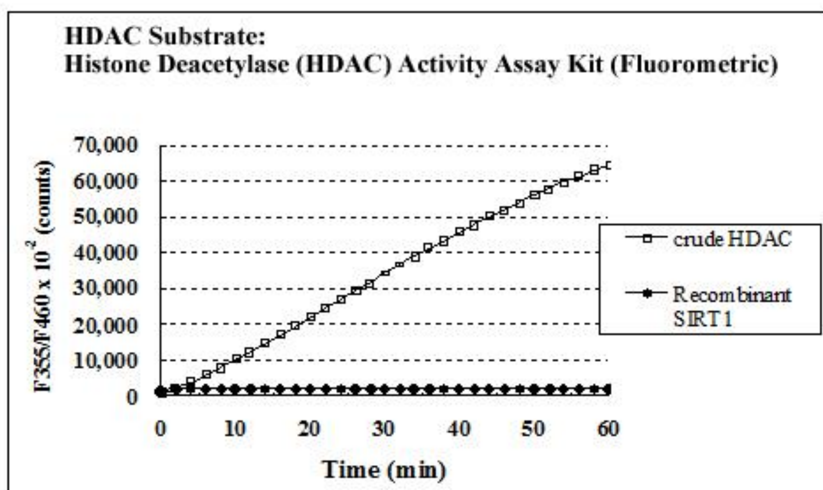


Figure 4: Substrate preference of HDAC and SIRT1 using ab156064

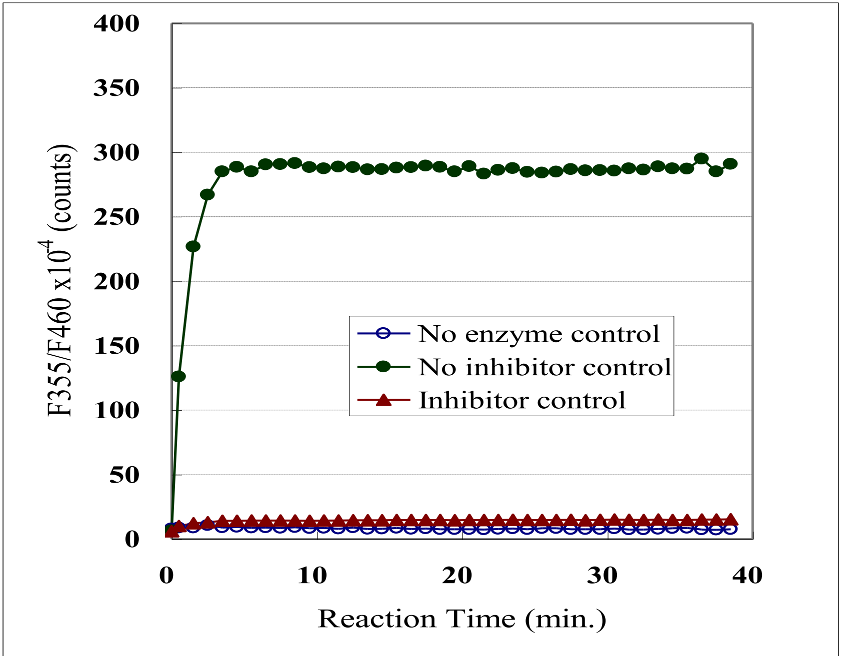


Figure 5: Time course of the second reaction in a Two-Step method (Lysylendpeptidase reaction)

10. Sample Preparation

Different extraction and purification methods can be used to isolate HDACs. The following protocol has been shown to work with a number of different cells and is provided as an example of a suitable method. 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, purified protein 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

- 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 nicotinamide adenine dinucleotide (NAD) is added, since they do not have susceptibility in trichostatin A (Please use SIRT2 Assay Kit (ab156066)).

- When test compounds that have any inhibitory effect on the peptidase in the development reaction 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 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.

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

- If enzyme samples or test compounds themselves emit fluorescence at the same range as peptide (Ex/ EM = 360 – 380/ 440 – 460 nm), the inhibitory effect of the test assay cannot be evaluated correctly.

- The assays should be run in duplicate as described in the protocols in Section 8. 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.

- 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.

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

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