HDAC Assay Kit
(Fluorescent)

(version B2)

Catalog No. 56200

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Overview

Active Motif's fluorescent HDAC Assay Kit is an easy and sensitive assay to determine histone deacetylase activity or to screen potential inhibitor compounds in your cell or nuclear extracts, column fractions, immunoprecipitates or purified enzymes.

The HDAC Assay Kit (Fluorescent) contains positive control HeLa Nuclear Extracts, deacetylated HDAC assay standard, fluorescent HDAC substrate, trichostatin A as a control inhibitor, and all the essential buffers for a full 96-well plate assay.

This fluorescent HDAC Assay utilizes a short peptide substrate that contains an acetylated lysine residue that can be deacetylated by certain HDAC enzymes. Once the substrate is deacetylated, the lysine residue reacts with the Developing Solution and releases the fluorophore from the substrate resulting in a fluorescent product that can be easily measured using a fluorescent plate reader with an excitation wavelength of 340-360 nm and emission wavelength of 440-465 nm.

The HDAC Assay Kit (Fluorescent) is not recommended for every HDAC enzyme, please see the chart below for guidance. Class I, IIB and IV HDAC enzymes are recommended for use in the assay. Class III HDAC enzymes, Sirt1, 2, 3, 4, 5, 6 and 7, require the addition of the NAD+ cofactor in the assay. We do not recommend using the HDAC Assay Kits for Class IIA enzymes which include HDAC 4, 5, 7 & 9.

HDAC enzyme	Guidelines
HDAC 1, 2, 3, 6, 8, 10, 11	Are validated to work in the HDAC fluorescent assay kit (Cat. No. 56200)
HDAC 4, 5, 7, 9	Are not recommended for use in the HDAC assay kit
Sirtuins	Require NAD+ cofactor in the assay

product	format	catalog no.
HDAC Assay Kit (Fluorescent)	1 x 96 rxns	56200

Introduction

The reversible acetylation of lysine residues contained in proteins, originally discovered about 40 years ago, is necessary for appropriate regulatory control of gene expression in eukaryotes. However, only recently has the mechanistic nature of acetylation of the epsilon amino group of specific lysine residues contained in the N-terminal tail domains of core histones become better appreciated. In general, acetylation of lysine residues in the tail regions of histones leads to a more relaxed local chromatin structure that favors transcriptional activation. In contrast, deacetylation of these lysine residues leads to a more condensed chromatin structure which is associated with transcription repression. Two distinct classes of enzymes, histone acetyltransferases (HAT) and histone deacetylases (HDAC) are involved in creating the appropriate contextual acetylation environment appropriate for transcriptional states at specific gene loci[1].

Human histone deacetylases (HDAC) are divided into four classes based on homology. Class I HDACs include HDAC1, 2, 3, and 8, all of which show some homology with the yeast Rpd3 HDAC[1]. Class II enzymes are comprised of Class IIA HDAC4, 5, 7, & 9 and Class IIB with HDAC 6 & 10. Class II enzymes share homology with the yeast enzyme Hda1. Class III HDACs differ from other classes in that they require the co-factor NAD+ for activity. Class III HDACs include Sirt1, 2, 3, 4, 5, 6, and 7, all are homologues of the yeast Sir2 deacetylase[2]. Finally, class IV HDAC includes only HDAC11. Class I, II, and IV share some sequence homology, and share an enzymatic requirement for zinc. Class III share very little sequence homology with members of classes I, II, or IV[2].

The HDAC Assay Kit (Fluorescent) utilizes a short peptide substrate that contains an acetylated lysine residue that can be deacetylated by most HDAC enzymes. However, class III enzymes require the addition of the NAD+ co-factor for a functional assay. Once the substrate, BOC-(Ac) Lys-AMC is deacetylated, a fluorescent product results with an excitation wavelength of 353 nm and emission wavelength of 448 nm. To enable the calculation of enzymatic activity, a fluorescent assay standard is also provided. This standard is the non-acetylated version of the substrate peptide, BOC-Lys-AMC and can be used to generate a standard curve for calculation of enzymatic activity.

Trichostatin A is included as a potent inhibitor of Class I and Class II HDAC activity[3]. Trichostatin A is the byproduct of microbial metabolites, and is thought to inhibit HDAC enzymatic activity by chelating zinc ions and blocking the active-site pocket of the enzyme[3]. Class III HDACs are mechanistically different by being NAD-dependent enzymes whose activity can be inhibited by their metabolite, nicotinamide.

Kit Performance and Benefits

The HDAC Assay Kit (Fluorescent) is for research use only. Not for use in diagnostic procedures.

Range of detection: The HDAC Assay Kit (Fluorescent) works with nuclear extract samples in the range of 2-25 µg per well. For purified recombinant HDAC enzyme or enzymes isolated by immunoprecipitation (IP) we recommend trying a range of concentrations to determine the amount of enzyme needed.

Reactivity: The HDAC Assay Kit (Fluorescent) utilizes a short peptide substrate that contains an acetylated lysine residue that can be deacetylated by Class I, IIB and IV HDAC enzymes. Class III HDAC enzymes, (Sirt1, 2, 3, 4, 5, 6 and 7), require the addition of the NAD+ cofactor in the assay. Class IIA HDAC enzymes do not work in the assay.

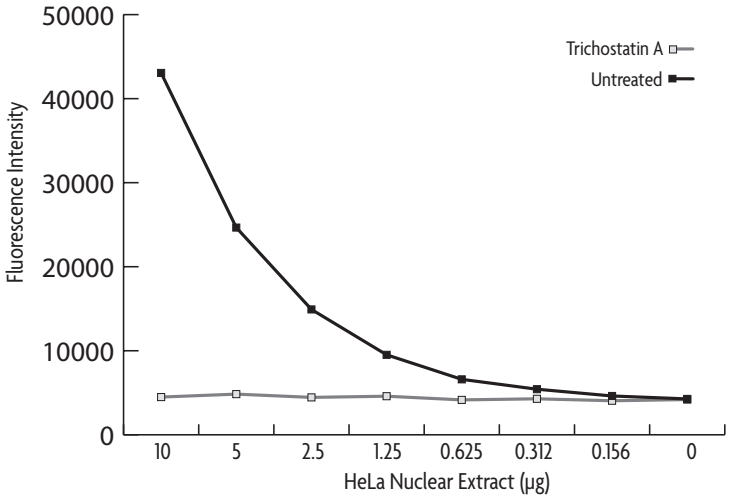


Figure 1: Fluorescent HDAC Assay results.

HeLa Nuclear Extracts were assayed from 0 to 10 µg per well in duplicate. The black line represents activity from untreated HeLa Nuclear Extracts and the gray line represents results from HeLa Nuclear Extracts treated with 1 µM Trichostatin A inhibitor.

Kit Components and Storage

The HDAC Assay Kit (Fluorescent) is for research use only. Not for use in diagnostic procedures. Except for the nuclear extract that must be kept at -80°C, kit components can be stored at -20°C prior to the first use. Then, we recommend storing each component at the temperature indicated in the table below. All components are guaranteed stable for six months when stored properly.

Reagents	Quantity	Storage / Stability
HDAC Assay Buffer	10 ml	-20°C to room temp.
HDAC Substrate (Fluoro), 10 mM	70 µl	-20°C
HDAC Assay Standard (Fluoro), 1 mM	10 µl	-20°C
Trichostatin A, 0.2 mM	50 µl	-20ºC
HDAC Developer Solution	5 ml	-20°C
HeLa Nuclear Extract (2.5 mg/ml)	100 µg	-80°C
96-well half-volume black plate	1	-20°C to room temp.

Additional materials required

- Nuclear extract or HDAC enzyme source
- Incubator, ice and timer
- Multi-channel pipette, variable-volume pipettes
- Multi-channel pipette reservoir
- 96-well plate reader capable of reading fluorescence (excitation wavelength 340-360 nm and emission wavelength 440-465 nm)

Preparing HDAC samples

HDAC samples can be prepared using several techniques:

1. Recombinant HDAC enzyme sources can be used directly in the HDAC assay.
2. Nuclear extracts can be prepared using Active Motif's Nuclear Extract Kit (Catalog No. 40010) and used directly for analysis in the HDAC Assay Kit (Fluorescent). We recommend using nuclear extracts in the range of 2-25 µg per well.
3. HDAC proteins can be immunoprecipitated from whole-cell or nuclear extracts. It is important to find an antibody that will bind to the specific HDAC but does not interfere with the activity of the HDAC enzyme.

Buffer Preparation and Recommendations

HDAC Assay Buffer

The HDAC Assay Buffer should be thawed completely before use.

5X HDAC Substrate (Fluoro)

The fluorescent HDAC substrate is supplied at 10 mM in DMSO. Prior to the assay, dilute to 500 µM in HDAC Assay Buffer. 10 µl are used per well. For each milliliter required, dilute 50 µl of the provided 10 mM stock in 950 µl HDAC Assay Buffer. The final substrate concentration per assay well is 100 µM.

5X Trichostatin A, HDAC Inhibitor

Trichostatin A is a potent inhibitor of histone deacetylase class I and II activity at nanomolar concentrations. Trichostatin A is supplied at 0.2 mM in DMSO. Dilute Trichostatin A to 5 µM in HDAC Assay Buffer. 10 µl are used per well. For each milliliter required, dilute 25 µl of the inhibitor in 975 µl of HDAC Assay Buffer. The final Trichostatin A concentration per assay well is 1 µM.

HDAC Assay Standard (Fluoro)

The standard is supplied at 1 mM. Dilute the HDAC Assay Standard (Fluoro) to 20 µM using HDAC Assay Buffer. For each curve required, dilute 5 µl of the standard in 245 µl HDAC Assay Buffer.

HeLa Nuclear Extract

HeLa Nuclear Extract is supplied at 2.5 mg/ml. For duplicate wells add 5 µl of the provided HeLa nuclear extract to 70 µl Assay Buffer. Vortex to mix. Use 30 µl of diluted extract per well in the assay (5 µg). The HeLa Nuclear Extract should be kept on wet ice while preparing the assay.

Test Sample

Dilute your source of HDAC to the desired concentration. Each assay point can be diluted to as much as a total volume of 30 µl. If stability problems are an issue, the test sample can be diluted in a smaller volume and the total assay volume brought to 50 µl using HDAC Assay Buffer. The Test Sample containing active HDAC enzymes should be kept on wet ice until added to the assay plate. Nuclear extracts produced by Active Motif's Nuclear Extract Kit (Catalog No. 40010) can be used directly for analysis with the HDAC Assay Kit (Fluorescent) in a range of 2-25 µg per well.

HDAC Developer Solution

The working HDAC Developing Solution should be prepared during the incubation step. Allow the HDAC Developer Solution to warm to room temperature before use. For each milliliter required, add 10 µl of the provided 0.2 mM Trichostatin A stock into 0.990 ml HDAC Developer Solution to make a 2 µM final concentration of Trichostatin A. The final reaction concentration of Trichostatin A after addition to the reaction wells is 1 µM.

HDAC (Fluorescent) Protocol

Read the entire protocol before use.

Please read the Reagent Preparation section on page 5 before starting. If you wish to calculate enzymatic activity, a standard curve must be prepared.

It is recommended to run test samples, controls, standards and background wells in duplicate.

1. Thaw components and store on ice. Prepare reagents as described in the Buffer Preparation and Recommendation section on page 5.
2. Use the following table to determine the reagents required for each assay condition. Add components in the order listed below, adding the extract last.

Reagent	Blank wells	Sample no inhibitor	Sample with inhibitor	Positive Control nuclear extract
HDAC Assay Buffer	40 µl	10 µl	–	10 µl
Trichostatin A (5 µM)	–	–	10 µl	_
HDAC Substrate (500 µM)	10 µl	10 µl	10 µl	10 µl
Test Sample	–	30 µl	30 µl	–
HeLa nuclear extract	–	–	–	30 µl
Total Volume	**50 µl**	**50 µl**	**50 µl**	**50 µl**

3. Incubate the plate at 37°C for 30-60 minutes. Longer incubations can be performed for samples with low levels of HDAC activity.
4. Prepare HDAC Assay Developing Solution as stated in the Buffer Preparation and Recommendation section during the incubation period.
5. Stop the HDAC reactions at the desired time point by adding 50 µl of the working HDAC Developing Solution per well. Mix thoroughly.
6. Incubate the plate at room temperature for 10-15 minutes.
7. Read the fluorescence of the plate in a plate reader with excitation wavelength at 360 nm and emission wavelength at 460 nm.

Standard Curve Preparation for HDAC Assay Standard (Fluoro)

Use this plate set-up example to prepare a standard curve for the HDAC Assay Standard (Fluoro) in duplicate. The standard curve is prepared at a range of 0-20 µM.

		1	2	3	4	5	6	7	8	9	10	11	12
50 µl	A	20 µM	20 µM	–	–	–	–	–	–	–	–	–	–
50 µl	B	10 µM	10 µM	–	–	–	–	–	–	–	–	–	–
50 µl	C	5 µM	5 µM	–	–	–	–	–	–	–	–	–	–
50 µl	D	2.5 µM	2.5 µM	–	–	–	–	–	–	–	–	–	–
50 µl	E	1.25 µM	1.25 µM	–	–	–	–	–	–	–	–	–	–
50 µl	F	0.62 µM	0.62 µM	–	–	–	–	–	–	–	–	–	–
	G	0.31 µM	0.31 µM	–	–	–	–	–	–	–	–	–	–
	H	0 µM	0 µM	–	–	–	–	–	–	–	–	–	–

1. Pipette 50 µl of assay buffer to wells B1 through H2.
2. Pipette 100 µl of the diluted HDAC Assay Standard to wells A1 and A2.
3. Perform a serial two-fold dilution of the standard by transferring 50 µl of the 20 µM HDAC Assay Standard in row A to the wells in row B.
4. Mix the contents of row B by pipetting up and down 3-5 times. Do not change pipette tips between well transfers.
5. Transfer 50 µl of the contents of row B to row C and mix, as previously described.
6. Continue this process until row G is reached.
7. When row G is reached, discard 50 µl of the well contents so that the final volume is 50 µl.
8. Row H will serve as the blank wells.
9. The standards may be incubated on the plate with the test samples and HeLa positive control extract. Incubate the plate at 37°C for 30-60 minutes.
10. Prepare HDAC Assay Developing Solution as stated in the Buffer Preparation and Recommendation section during the incubation period.
11. Add 50 µl of the working HDAC Developing Solution to all wells. Mix thoroughly.
12. Incubate the plate at room temperature for 10-15 minutes.
13. Read fluorescence in a plate reader with excitation wavelength at 360 nm and emission wavelength at 460 nm.

Example standard curve:

The following standard curve is provided for demonstration only. A standard curve should be made fresh every time you wish to calculate enzymatic activity. Generate a linear trendline for the plotted standard. The standard curve should have an R^2 greater than 0.95 in order to use the standard curve to determine enzymatic activity.

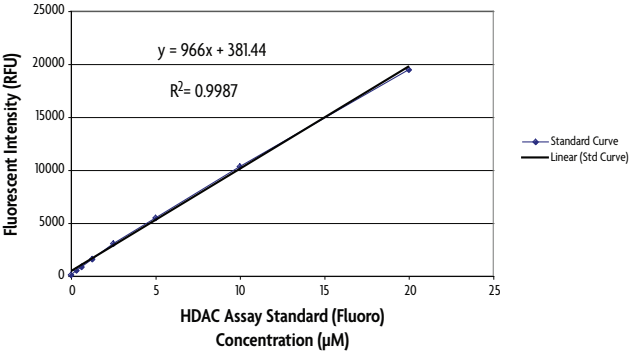


Figure 2. HDAC Assay Kit (Fluorescent) Example Standard Curve.

HDAC Assay Standard (Fluoro) was diluted to 20 µM and diluted 2-fold as described in the protocol. The HDAC Assay
Standard (Fluoro) was incubated at 37°C for 10 minutes with the working Developing Solution and the fluorescence
intensity (ex. 360/em. 460 nm) measured on a plate reader. Data shown are the results from wells assayed in duplicate.

References

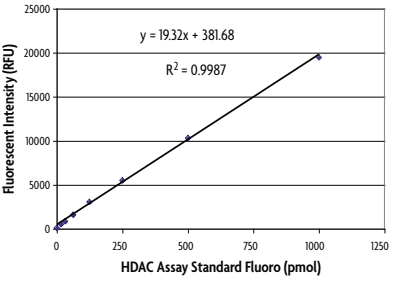
1. Ouaissi, M and Ouaissi, A. (2006) J Biomed and Biotech. 13474: 1-10.
2. Yang, X-G and Gregoire, S. (2005) Mol and Cell Biol. 25(8): 2873-2884.
3. Furumai, R, *et al.*, (2001) PNAS 98(1): 87-92.

Appendix

Section A. Calculating Activity from the Standard Curve

1. Calculate the average value for each duplicate data point in the standard curve.
2. To determine specific activity, the HDAC concentration (µM) is converted into mass (pmol). The average fluorescent intensity per point is plotted on the y-axis versus the pmol of HDAC on the x-axis. Generate a linear trendline for the plotted standard. The standard curve should have an R^2 greater than 0.95 in order to use the standard curve to determine enzymatic activity. The mass values for the provided HDAC Standard (Fluoro) in a 50 µl reaction volume and an example graph are provided below:

Concentration (µM)	Product (pmol)
20	1000
10	500
5	250
2.5	125
1.25	62.5
0.62	31.2
0.31	15.6



3. From the curve plotted in Step 2, extrapolate the pmoles of product formed based on the fluorescent intensity values obtained from the Test Sample.
4. Calculate specific activity by dividing the pmoles of product formed by the incubation time (minutes) and the mass of HDAC (milligrams) used to yield activity in pmoles/min/mg.

Section B. Troubleshooting Guide

Problem/question	Recommendation
Failure to obtain signal	Run standard curve as outlined in protocol to ensure plate reader acquisition parameters are set correctly and that the assay is in the linear range. A filter suitable for AMC is recommended: the excitation maximum is 353 nm and the emission maximum is 448 nm.
	Test buffers and compounds containing HDAC source for potential inhibitory compounds with developer reaction. Test for compounds that may inhibit the developer reaction by incubating the test samples with 250 µM of the HDAC Assay Standard and developing as outlined in the protocol. If the standard fails to yield increased fluorescence, then the samples contain interfering compounds that will have to be removed by dialysis, gel-filtration or similar technique.
	The HDAC Assay Kit (Fluorescent) is designed for use with HDAC Class I, IIB and IV enzymes. The kit is not recommended for use with Class IIA enzymes. To adapt the kit for use with HDAC Class III enzymes see the recommendations for NAD-dependent HDAC enzymes below.
Test sample is known to have low HDAC activity	The typical assay can be run successfully with a 37°C incubation for 30 to 60 minutes. However, if dilute samples are being assayed, the HDAC reaction time may be increased to maximize signal. Sample incubations of 2 hours to overnight have been successfully run. If the HDAC source is a recombinant protein, including a low amount of surfactant such as Brij-35 can aid enzymatic stability when prolonged incubations are necessary.
How can I adapt this assay for NAD-dependent HDAC enzymes?	Add 250 µM NAD (such as Sigma Cat. No. N1636) to assay buffers. 1-5 µM nicotinamide (such as Sigma Cat. No. 72340) can be used as an inhibitor.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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