HDAC Fluorometric Activity Assay Kit

Item No. 10011563

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity	Storage
10006389	HDAC Assay Buffer (10X)	1 vial	-20°C
10011617	HDAC1 Positive Control	1 vial	-80°C
10006391	HDAC Trichostatin A	1 vial	-20°C
10006392	HDAC Substrate	1 vial	-20°C
10006393	HDAC Deacetylated Standard	1 vial	-20°C
10006394	HDAC Developer	2 vials	-20°C
400017	96-Well Plate (Black)	1 plate	RT
400012	96-Well Cover Sheet	1 cover	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

Sold under license from CycLex® Co., Ltd (Patent Nos. EP 1243658, US 7033778, US 7256013, and JP 4267043).

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -80°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometer capable of measuring fluorescence using excitation wavelengths of 340-360 nm and emission wavelengths of 440-465 nm.
2. Adjustable pipettes and a repeating pipettor.
3. A source of UltraPure water (Milli-Q or HPLC-grade water).

INTRODUCTION

Background

Nucleosomes, which fold chromosomal DNA, contain two molecules each of the core histones H2A, H2B, H3, and H4. Almost two turns of DNA are wrapped around this octameric core, which represses transcription.[1] The histone amino termini extend from the core, where they can be modified post-translationally by acetylation, phosphorylation, ubiquitination, and methylation, affecting their charge and function.

Acetylation of the ε-amino groups of specific histone lysines is catalyzed by histone acetyltransferases (HATs) and correlates with an open chromatin structure and gene activation. Histone deacetylases (HDACs) catalyze the hydrolytic removal of acetyl groups from histone lysine residues and correlates with chromatin condensation and transcriptional repression.[2,3] Therefore, HDAC inhibition results in transcriptional activation through the conformational relaxation of DNA. The human class I HDACs include HDAC1, 2, 3, and 8. Class II HDACs are divided into class IIa (HDAC4, 5, 7, 9) and class IIb (HDAC6 and 10) based on structure. The human class III HDACs include the sirtuin family (SIRTs 1-7) of NAD^+-dependent protein deacetylases. SIRTs can be distinguished from other HDACs based on their insensitivity to inhibition by trichostatin A. The novel HDAC11 has a distinct structure and is a class IV HDAC. Changes in the transcription of key genes has linked HDAC inhibitors to blocking angiogenesis and cell cycling, and promoting apoptosis and differentiation. By targeting these key components of tumor proliferation, HDAC inhibitors are currently being explored as potential anticancer agents.[4-6]

About This Assay

Cayman's HDAC Fluorometric Activity Assay Kit provides a fast, fluorescent-based method for measuring HDAC activity that eliminates radioactivity, extraction, or chromatography. The procedure requires only two easy steps, both performed in the same microplate. In the first step, an acetylated lysine substrate is incubated with samples containing HDAC activity. Deacetylation sensitizes the substrate such that treatment with the HDAC developer in the second step releases a fluorescent product. The fluorophore can be easily analyzed using a fluorescence plate reader or a fluorometer with excitation wavelengths of 340-360 nm and emission wavelengths of 440-465 nm. The assay can be used for quantifying Class I and II HDAC activity from various sources.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **HDAC Assay Buffer (10X) - (Item No. 10006389)**

   Dilute 5 ml of Assay Buffer concentrate with 45 ml of UltraPure water. This final Assay Buffer (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$) should be used in the assay. It is used for diluting the HDAC1 Positive Control, samples, deacetylated standard, and for dissolving the HDAC Developer. The diluted buffer is stable for six months at 4˚C.

2. **HDAC1 Positve Control - (Item No. 10011617)**

   The vial contains 50 µl of human recombinant HDAC1. To avoid repeated freezing and thawing, the control should be aliquoted into several small vials and refrozen at -80˚C. Dilute 10 µl of the control with 190 µl of diluted Assay Buffer. The diluted HDAC1 is stable for four hours when stored on ice.

3. **HDAC Trichostatin A - (Item No. 10006391)**

   The vial contains 250 µl of 0.21 mM Trichostatin A. Trichostatin A is a HDAC inhibitor. Dilute 50 µl of Trichostatin A stock with 450 µl of diluted Assay Buffer. A 10 µl aliquot in the assay results in a final concentration of 1 µM. At this concentration, HDAC activity will be completely inhibited.

4. **HDAC Substrate - (Item No. 10006392)**

   The vial contains 1.2 ml of 3.4 mM acetylated fluorometric substrate in dimethylsulfoxide (DMSO). The solution is ready to use as supplied. *NOTE: The final concentration of HDAC substrate in the assay is 200 µM.*

5. **HDAC Deacetylated Standard - (Item No. 10006393)**

   The vial contains 400 µl of 2.1 mM Deacetylated Standard in DMSO. The Deacetylated Standard is used to prepare a standard curve for quantitative determination of HDAC activity.

6. **HDAC Developer - (Item No. 10006394)**

   The vial contains the HDAC Developer. Dissolve the contents of the vial in 4 ml of diluted Assay Buffer and store on ice. Add 100 µl of undiluted HDAC Trichostatin A (Item No. 10006391) to the reconstituted Developer. One vial of Developer will develop the entire plate. The reconstituted Developer is stable for two hours.

Sample Preparation

Isolation of Nuclei

1. Suspend 1 x 10^7 cells (100 mm dish sub-confluent) in 1 ml of cold Lysis Buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 15 mM $MgCl_2$, 250 mM sucrose, 0.5% NP-40, and 0.1 mM EGTA).
2. Vortex for 10 seconds and keep on ice for 15 minutes.
3. Spin the cells through 4 ml of cold sucrose cushion (30% sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 3 mM $MgCl_2$) at 1,300 x g for 10 minutes at 4°C.
4. Discard the supernatant.
5. Resuspend the nuclei pellet in 1 ml of cold 10 mM Tris-HCl, pH 7.5, containing 10 mM NaCl.
6. Centrifuge at 1,300 x g for 10 minutes at 4°C.
7. Discard the supernatant.

Extraction of Nuclear components

1. Suspend the isolated nuclei in 100-200 µl of Extraction Buffer (50 mM HEPES, pH 7.5, containing 420 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, and 10% glycerol).
2. Sonicate for 30 seconds and incubate on ice for 30 minutes.
3. Centrifuge at 10,000 x g for 10 minutes at 4°C in a microcentrifuge.
4. The supernatant contains the crude nuclear extract.
5. Store the crude nuclear extract at -80°C until use.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of HDAC standards and samples to be measured in duplicate is given below in Figure 1. We suggest you record the contents of each well on the template sheet provided (see page 18).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	1	1	5	5	9	9	13	13	17	17
B	B	B	1T	1T	5T	5T	9T	9T	13T	13T	17T	17T
C	C	C	2	2	6	6	10	10	14	14	18	18
D	D	D	2T	2T	6T	6T	10T	10T	14T	14T	18T	18T
E	E	E	3	3	7	7	11	11	15	15	19	19
F	F	F	3T	3T	7T	7T	11T	11T	15T	15T	19T	19T
G	H	H	4	4	8	8	12	12	16	16	20	20
H	HT	HT	4T	4T	8T	8T	12T	12T	16T	16T	20T	20T

A-F - Standards A-F
H - HDAC1 Positive Control
HT - Positive Control + Trichostatin A
1-20 - Samples 1-20
1T-20T - Samples 1-20 + Trichostatin A

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 210 µl in all the wells.
- Use the diluted Assay Buffer in the assay.
- All reagents except Positive Control, samples, and Developer must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time, however a standard curve should be run every time.
- If the HDAC activity of the sample is not known or if it is expected to be beyond the range of the standard curve, it is prudent to assay the sample at several dilutions.
- It is recommended that the samples and deacetylated standards be assayed at least in duplicate (triplicate recommended).
- Twelve samples in triplicate or twenty samples in duplicate can be run in the assay.
- Assay temperature is 37°C.
- Monitor the fluorescence with an excitation wavelength of 340-360 nm and an emission wavelength of 440-465 nm.

Standard Preparation

Dilute 200 µl of standard with 1.8 ml of diluted Assay Buffer to obtain the stock solution. Take six clean glass test tubes and mark them A-F. Add the amount of standard stock and Assay Buffer to each tube as described in Table 1. Diluted standards are stable for four hours at room temperature.

Tube	Deacetylated standard stock (µl)	Assay Buffer (µl)	Deacetylated standard concentration (µM)
A	0	1,000	0
B	50	950	10.5
C	100	900	21
D	200	800	42
E	400	600	84
F	800	200	168

Table 1. Preparation of deacetylated standards

Performing the Assay

1. **Deacetylated Standard Wells** - add 150 µl of diluted Assay Buffer and 10 µl of standard (tubes A-F) per well in the designated wells on the plate (see suggested plate configuration, Figure 1, on page 9).
2. **HDAC1 (positive control) Wells** - add 140 µl of diluted Assay Buffer and 10 µl of diluted HDAC1 to four wells.
3. **Sample Wells** - add 140 µl of diluted Assay Buffer and 10 µl of sample to four wells. To obtain reproducible results, HDAC activity must fall within the standard curve. When necessary, samples can be diluted with diluted Assay Buffer to bring the activity to this level.
4. Add 10 µl of diluted Trichostatin A to two of the positive control wells and to two of each of the sample wells. Trichostatin A will eliminate all HDAC activity and is used as a control for generating the sample background values. Add 10 µl of diluted Assay Buffer to the Positive Control and samples wells that were not treated with Trichostatin A.
5. Initiate the reactions by adding 10 µl of HDAC Substrate to all the wells being used including the standard wells. The final concentration of substrate is 200 µM in the wells.
6. Cover the plate with the plate cover and incubate on a shaker for 30 minutes at 37°C.
7. Remove the plate cover and add 40 µl of Developer. Cover the plate with the plate cover and incubate for 15 minutes at room temperature.
8. Remove the plate cover and read fluorescence using an excitation wavelength of 340-360 nm and an emission wavelength of 440-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples. The development is stable for 30 minutes.

ANALYSIS

Calculations

1. Calculate the average fluorescence of each standard, sample, and Trichostatin-treated sample.
2. Subtract the average fluorescence of Standard A from itself and all other standards.
3. Plot the corrected fluorescence of the standards (from step 2 above) as a function of the final concentration of deacetylated standard from Table 1, on page 11. See Figure 2 for a typical standard curve.

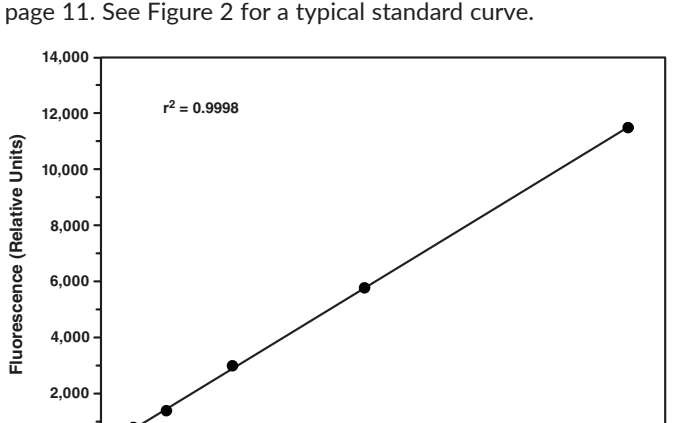


Figure 2. Deacetylated standard curve

4. Subtract the average fluorescence of the Trichostatin-treated samples from the average fluorescence of its corresponding samples to yield the corrected sample fluorescence (CSF).
5. Calculate the deacetylated concentration of the samples using the equation obtained from the linear regression of the standard curve substituting corrected sample fluorescence (CSF) values for each sample.

Deacetylated compound (µM) = [(CSF – y-intercept)/Slope]

6. Calculate the HDAC activity using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of deacetylated compound per minute at 37°C.

HDAC Activity (nmol/min/ml) = [µM/30 minutes] x Sample dilution

Performance Characteristics

Precision:

When a series of eight HDAC1 measurements were performed on the same day, the intra-assay coefficient of variation was 2.2%. When a series of eight HDAC1 measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 2.4%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0-118 nmol/min/ml of HDAC activity.

RESOURCES

Interferences

The following reagents were tested in the assay for interference in the assay:

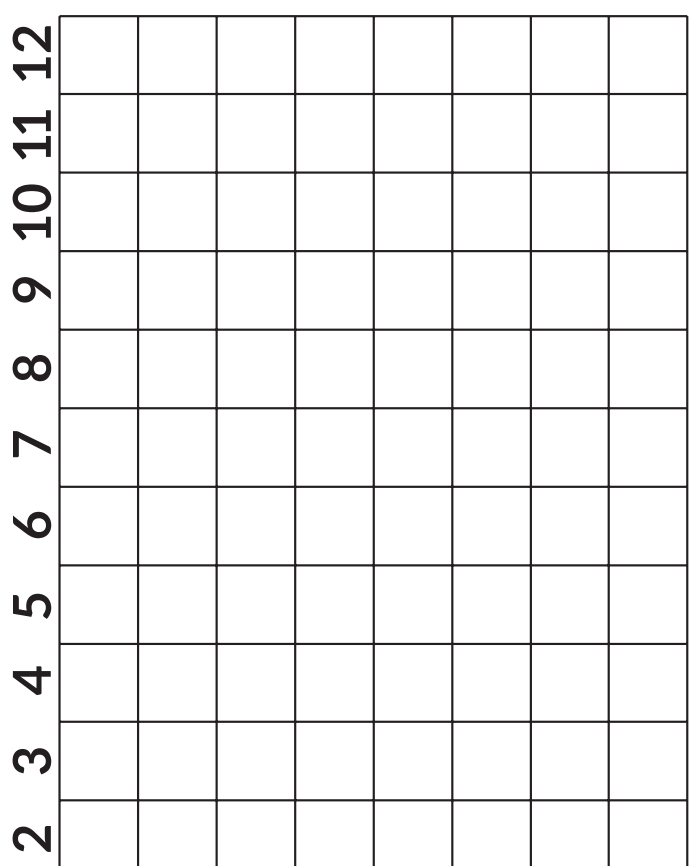
	Reagent	Will Interfere (Yes or No)
Buffers	Tris	No
	Borate	No
	HEPES	No
	Phosphate	No
Protease Inhibitors/ Chelators	≤200 µM PMSF	No
	≤10 µg/ml Leupeptin	No
	≤10 µg/ml Pepstatin	No
	≤10 µg/ml Chymostatin	No
	≤1 mM EGTA	No
	≤1 mM EDTA	No
Solvents	10 µl Ethanol	No
	10 µl Methanol	No
	10 µl Dimethylsulfoxide	No
Others	≤10% Glycerol	No
	≤1% BSA	No
	≤5 mM β-Mercaptoethanol	No

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
HDAC activity was not detected in the sample	Sample was too dilute	Re-assay the sample using a lower dilution
Fluorescence value was at the maximal level in the sample wells	A. The sample is too concentrated B. The Gain setting is set too high	Set the gain to a lower setting and measure the fluorescence. If the fluorescence is still too high, dilute your sample with diluted assay buffer and re-assay
The deacetylated standard curve did not work	A. The deacetylated standards were not diluted properly B. The deacetylated standard has deteriorated	Set-up the standards according to Table 1 and re-assay

References

1. Grunstein, M. Histone acetylation in chromatin structure and transcription. *Nature* **389,** 349-352 (1997).
2. Strahl, B.D. and Allis, D. The language of covalent histone modifications. *Nature* **403,** 41-45 (2000).
3. Cheung, W.L., Briggs, D.B., and Allis, C.D. Acetylation and chromosomal functions. *Curr. Opin. Cell Biol.* **12,** 326-333 (2000).
4. Kwon, H.J., Kim, M.S., Kim, M.J., *et al.* Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. *Int. J. Cancer* **97,** 290-296 (2002).
5. Suenaga, M., Soda, H., Oka, M., *et al.* Histone deacetylase inhibitors suppress telomerase reverse tarnscriptase mRNA expression in prostate cancer cells. *Int. J. Cancer* **97,** 621-625 (2002).
6. Johnstone, R.W. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nature Reviews Drug Discover* **1**, 287-299 (2002).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.