HDAC8 Inhibitor Screening Assay Kit

Item No. 700230

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Customer Service 800.364.9897
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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
700231	HDAC8 Assay Buffer (10X)	1 vial	-20°C
700232	HDAC8 (human recombinant)	2 vials	-80°C
700234	HDAC8 Substrate	2 vials	-20°C
700233	HDAC8 Trichostatin A	1 vial	-20°C
700235	HDAC8 Developer	1 vial	-20°C
700236	HDAC8 Fluorophore	1 vial	-20°C
10011288	Half Volume 96-Well Solid Plate (white)	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 specified in the **Materials Supplied** section, on page 3, 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 an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

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. The best studied of these modifications, acetylation of the ε-amino groups of specific histone lysine residues, are catalyzed by histone acetyltransferases (HATs). Histone deacetylases (HDACs) are responsible for hydrolytic removal of these acetyl groups.[2,3] Inhibition of HDAC can result in transcriptional activation through relaxation of DNA conformation. As a result, HDAC inhibitors are able to block angiogenesis and cell cycling, and promote apoptosis and differentiation. By targeting these key components of tumor proliferation, HDAC inhibitors are currently being explored as potential anticancer agents.[4-7] HDAC inhibitors modify the expression of many genes, and it is possible that inhibition of one isoform could cause epigenetic changes that are beneficial to the treatment of a disease, while inhibition of another isoform could cause contradictory changes. Selective HDAC inhibitors will be better able to avoid these types of situations than non-specific inhibitors, and may also be less toxic than pan-HDAC inhibitors. Many potent pan-HDAC inhibitors have already been developed, leaving the development of selective inhibitors at the forefront of HDAC drug development.

Eleven human class I and class II HDACs have been identified. They are all trichostatin A-sensitive and homologs of either the yeast RPD3 protein (class I) or the yeast HDA1 (class II).[8] Human HDAC8 is a class I HDAC and has been identified in a variety of human cancer tissues.[9-11] Earlier studies suggested that HDAC8 localized to the nucleus and was ubiquitously expressed. Recently, it was demonstrated that HDAC8 is a novel, prominently cytosolic marker of smooth muscle differentiation and may play an important role in neuroblastoma pathogenesis.[12,13]

About This Assay

Cayman's HDAC8 Inhibitor Screening Assay provides a convenient fluorescence-based method for screening HDAC8 inhibitors. The procedure requires only two easy steps, both performed in the same microplate (Figure 1). In the first step, the Substrate, which comprises the p53 sequence Arg-His-Lys-Lys(ε-acetyl)-AMC, is incubated with human recombinant HDAC8. Deacetylation sensitizes the Substrate such that treatment with the Developer in the second step releases a fluorescent product. Fluorescence is then analyzed with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. The acetylated p53 peptide substrate is also recognized by the other class I HDAC enzymes such as HDAC2 and can be used to assess their respective deacetylase activities.

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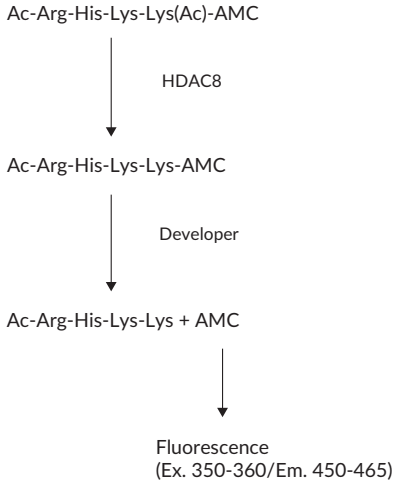

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **HDAC8 Assay Buffer (10X) - (Item No. 700231)**

   Dilute 5 ml of Assay Buffer concentrate with 45 ml of HPLC-grade water. This final Buffer (25 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$) should be used in the Assay and for diluting reagents. When stored at 4°C, this diluted Buffer is stable for at least six months.

2. **HDAC8 (human recombinant) - (Item No. 700232)**

   Each vial contains 150 µl of human recombinant HDAC8. Thaw the enzyme on ice, add 150 µl of diluted Assay Buffer to the vial, and vortex. The diluted enzyme is stable for four hours on ice. One vial of enzyme is enough HDAC8 to assay 60 wells. Use the additional vial if assaying the entire plate.

3. **HDAC8 Substrate - (Item No. 700234)**

   Each vial contains 100 µl of a 3 mM peptide solution comprising amino acids 379-382 of human p53 conjugated to aminomethylcoumarin (AMC). To one of the thawed vials, add 800 µl of diluted Assay Buffer and vortex. One vial of Substrate will make enough Substrate Solution for 60 wells. The Substrate Solution is stable for six hours at room temperature. The addition of 15 µl to the assay yields a final concentration of 100 µM peptide. *NOTE: The K_m value for the peptide is 235 µM.*

4. **HDAC8 Trichostatin A - (Item No. 700233)**

   The vial contains 100 µl of 3 mM Trichostatin A. Trichostatin A is a HDAC inhibitor and will be used to make the Stop/Developing Solution.

5. **HDAC8 Developer - (Item No. 700235)**

   The vial contains 40 mg of the HDAC8 developer. It will be used to make the Stop/Developing Solution.

6. **HDAC8 Fluorophore - (Item No. 700236)**

   The vial contains 50 µl of 10 mM 7-amino-4-methylcoumarin. The Fluorophore can be used to assay for interference (see page 17).

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background wells. We suggest that each inhibitor sample be assayed in triplicate and that you record the contents of each well on the template sheet provided (see page 22). A typical layout of samples and inhibitors to be measured in triplicate is given in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells
A - 100% Initial Activity Wells
1-30 - Inhibitor Wells

Figure 2. 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 100 µl in all the wells.
- All reagents except enzyme 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.
- It may be necessary to assay the inhibitor at several concentrations to determine an effective concentration.
- We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- 30 inhibitor samples can be assayed in triplicate or 45 in duplicate.
- The Assay temperature is 37°C.
- Monitor the fluorescence with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm.

Performing the Assay

1. **100% Initial Activity Wells -** add 25 µl of Assay Buffer, 5 µl of diluted HDAC8, and 5 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
2. **Background Wells -** add 30 µl of Assay Buffer and 5 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
3. **Inhibitor Wells -** add 25 µl of Assay Buffer, 5 µl of diluted HDAC8, and 5 µl of inhibitor* to three wells.
4. Initiate the reactions by adding 15 µl of Substrate Solution to all the wells being used.
5. Cover the plate with the plate cover and incubate for 30 minutes at 37°C.
6. **Preparation of Stop/Developing Solution -** Weigh 18 mg of Developer (Item No. 700235) into a vial that will hold 5 ml then add 3 ml of diluted Assay Buffer and 10 µl of Trichostatin A (Item No. 700233). Vortex until the Developer is into solution. This is enough Stop/Developing Solution for 60 wells. The Stop/Developing Solution is stable for two hours on ice.
7. Remove the plate cover and add 50 µl of Stop/Developing Solution. Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
8. Remove the plate cover and read the fluorescence using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 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.

*Inhibitors can be dissolved in Assay Buffer, methanol, or dimethylsulfoxide and should be added to the Assay in a final volume of 5 µl. In the event that the appropriate concentration of inhibitor needed for HDAC8 inhibition is completely unknown, we recommend that several concentrations of the compound be assayed.

Well	Assay Buffer	HDAC8	Solvent	Inhibitor	Substrate Solution	Stop Solution
100% Initial Activity	25 µl	5 µl	5 µl	-	15 µl	50 µl
Background	30 µl	-	5 µl	-	15 µl	50 µl
Inhibitor	25 µl	5 µl	-	5 µl	15 µl	50 µl

Table 1. Pipetting summary

ANALYSIS

Calculations

1. Determine the average fluorescence of 100% Initial Activity, Background, and inhibitor wells.
2. Subtract the fluorescence of the background wells from the fluorescence of the 100% initial activity and inhibitor wells.
3. Determine the percent inhibition for each compound. To do this, subtract each inhibitor sample value from the 100% initial activity sample value. Divide the result by the 100% initial activity value and then multiply by 100 to give the percent inhibition.
4. When multiple concentrations of inhibitor have been assayed it is possible to either graph the Percent Inhibition or Percent Initial Activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). An example of HDAC8 inhibition by Trichostatin A is shown in Figure 3, on page 15.

$$\% \text{ Inhibition} = \left[\frac{\text{Initial Activity - Inhibitor}}{\text{Initial Activity}} \right] \times 100$$

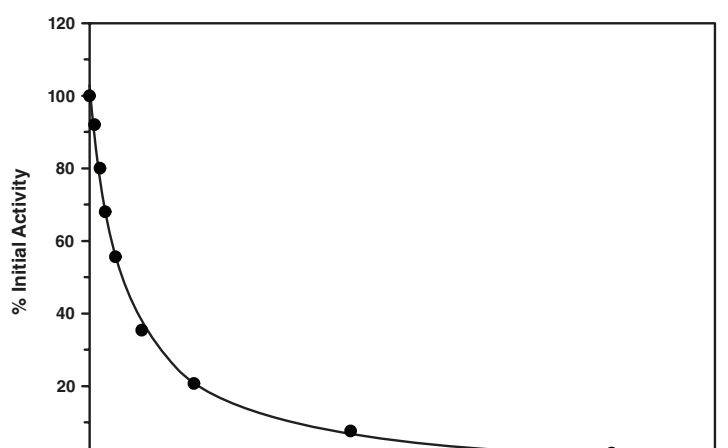

Figure 3. Inhibition of HDAC8 by Trichostatin A (IC_{50} = 0.6 µM)

Performance Characteristics

Precision:

When a series of sixteen HDAC8 measurements were performed on the same day, the intra-assay coefficient of variation was 3.3%. When a series of sixteen HDAC8 measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 3.6%.

Interferences

It is possible that a compound tested for HDAC8 inhibition will interfere with the development of the Assay or interfere with the HDAC8 Fluorophore. Potential Fluorophore interference can be tested by assaying the compound in question with the Fluorophore. A procedure is outlined below.

Testing for Fluorophore Interference

1. Dilute 10 µl of the HDAC8 Fluorophore (Item No. 700236) with 2.99 ml of diluted Assay Buffer.
2. **Fluorophore wells -** add 5 µl of diluted Fluorophore, 5 µl of solvent (the same solvent used to dissolve the compound), and 90 µl of diluted Assay Buffer to three wells.
3. **Compound wells -** add 5 µl of diluted Fluorophore, 5 µl of compound, and 90 µl of diluted Assay Buffer to three wells.
4. Cover the plate with the plate cover and incubate for 10 minutes at room temperature.
5. Remove the plate cover and read fluorescence using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Calculating the percent Fluorophore interference

1. Determine the average fluorescence of the HDAC8 Fluorophore and compound wells.
2. Determine the percent interference for the compound. To do this, subtract each compound value from the Fluorophore value. Divide the result by the Fluorophore value and then multiply by 100 to give the percent interference. The percent interference should be less than 10% for the compound to be not affecting the Fluorophore.

Testing for Developer interference

1. **HDAC8 wells -** add 25 µl of Assay Buffer and 5 µl of diluted HDAC8 to three wells.
2. **Compound wells -** add 25 µl of Assay Buffer and 5 µl of diluted HDAC8 to three wells.
3. Initiate the reactions by adding 15 µl of Substrate Solution to all the wells being used.
4. Cover the plate with the plate cover and incubate for 30 minutes at 37°C.
5. Remove the plate cover and add 50 µl of Stop/Developing Solution to the HDAC8 and compound wells.
6. Add 5 µl of compound to the compound wells and 5 µl of solvent (the same solvent used to dissolve the compound) to the HDAC8 wells.
7. Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
8. Remove the plate cover and read fluorescence using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Calculating the percent Developer interference

1. Determine the average fluorescence of the HDAC8 and compound wells.
2. Determine the percent interference for the compound. To do this, subtract each compound value from the HDAC8 value. Divide the result by the HDAC8 value and then multiply by 100 to give the percent interference. The percent interference should be less than 10% for the compound to be not affecting the Developer.

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
No fluorescence above background is seen in the Inhibitor wells	Inhibitor concentration is too high and inhibited all of the enzyme activity	Reduce the concentration of the inhibitor and re-assay
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
No inhibition was seen with the inhibitor	A. The inhibitor concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

References

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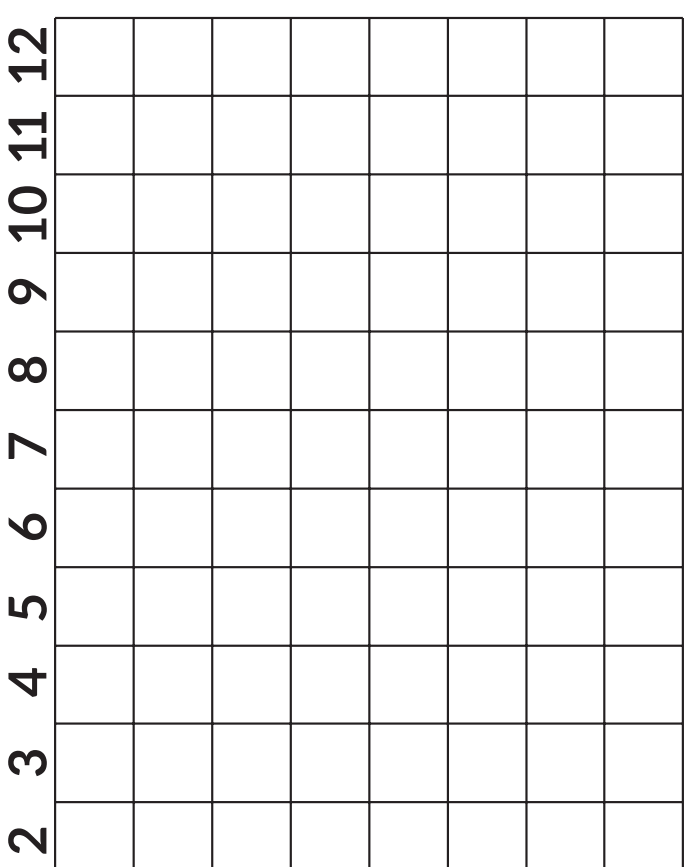

NOTES

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.