HAT Inhibitor Screening Assay Kit

Catalog Number KA1318

96 assays

Version: 02

Intended for research use only

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Introduction

Background

DNA is organized into a nucleoprotein complex termed chromatin, which not only is involved with the compaction of DNA within the nucleus but also serves as an important means to regulate genome function. The basic unit of chromatin is the nucleosome. Each nucleosome core contains 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 lysine residues, is catalyzed by histone acetyltransferases (HATs) producing a histone modification that 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] Functional defects of either of these enzymes can lead to several diseases, ranging from cancer to neurodenerative dieases. HATs and HDACs thus are potential therapeutic targets.

The p300/CBP-Associated Factor (pCAF) is an important HAT belonging to the GCN5-related N-acetyltransferase (GNAT) family. pCAF acetylates specific lysines on the N-terminal tails of histones H3 and H4. pCAF has also been shown to acetylate the tumor suppressor genes, p53 and PTEN.[4,5] The p53 tumor suppressor gene is the major target for genetic alteration or biochemical inactivation in human cancer(s).[5] Numerous studies have demonstrated that p53 acetylation can greatly enhance its transactivation activity, increase its stability, and induce apoptosis.[5] Acetylation of PTEN by pCAF, results in the inhibition of PTEN regulation of phosphatidylinositol 3-kinase signaling and inhibition of PTEN-regulated cell cycle arrest.[4]

Principle of the Assay

HAT Inhibitor Screening Assay Kit provides a fast, fluorescence-based method for evaluating pCAF HAT inhibitors. The procedure requires only three easy steps, all performed in the same microwell plate. In the first step of the protocol, HAT is incubated with acetyl-CoA and the histone H3 peptide. During this time, HAT catalyzes the enzymatic transfer of acetyl groups from acetyl-CoA to the H3 peptide producing an acetylated peptide and CoASH. Following addition of isopropanol to stop the enzymatic reaction (step 2), CPM is added to the wells of the plate (step 3). CPM reacts with the free thiol groups present on CoASH forming a highly fluorescent product that is detected using excitation and emission wavelengths of 360-390 nm and 450-470 nm, respectively. The scheme is shown below in Figure 1.

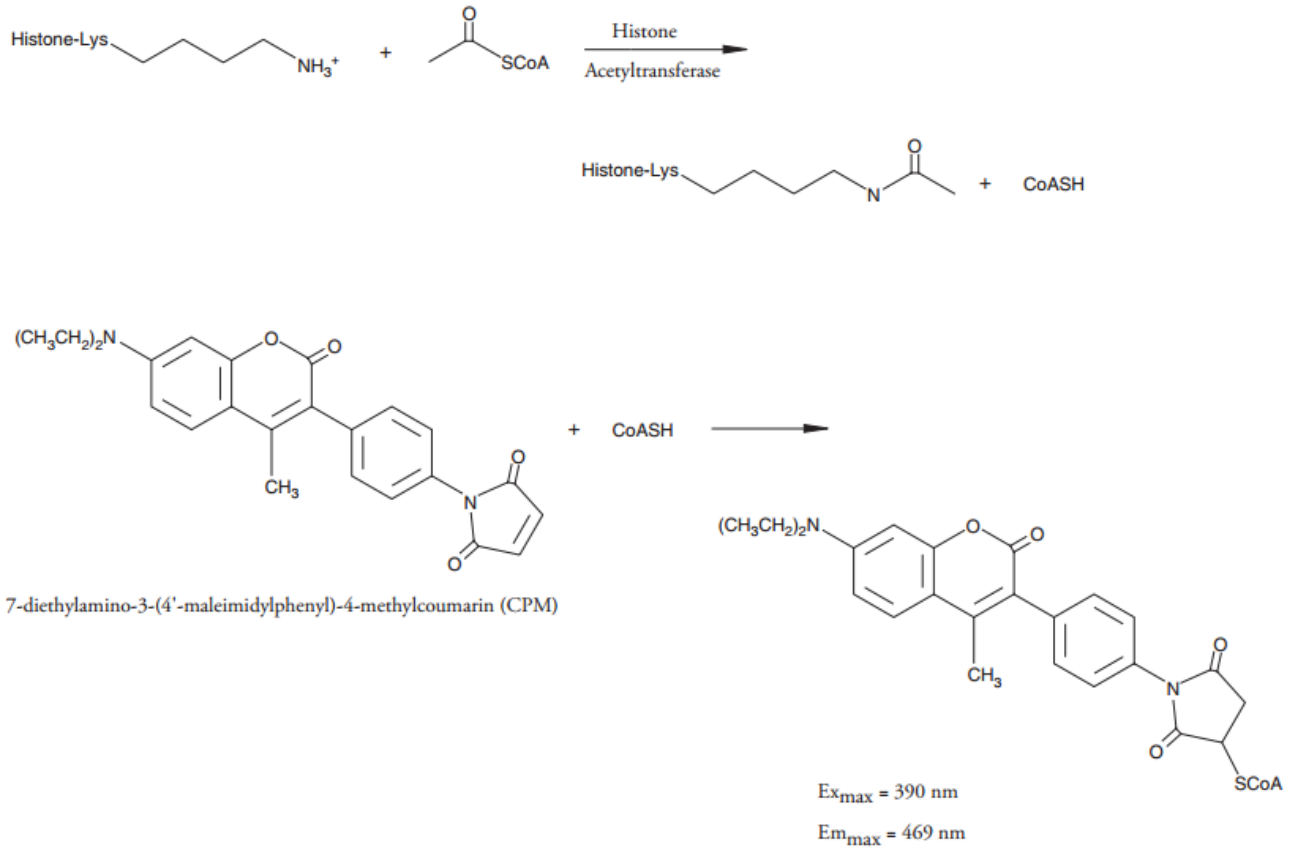


Figure 1. Schematic of the HAT Inhibitor Screening Assay

General Information

Materials Supplied

List of component

Item	Amount
HAT Assay Buffer (5X)	1 vial
HAT Acetyl CoA	1 vial
Histone Acetyltransferase (pCAF)	1 vial
HAT Peptide	1 vial
HAT Stop Reagent	1 vial
HAT Developer	1 vial
96-Well Plate (white)	1 plate
96-Well Cover Sheet	1 cover

Storage Instruction

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Required but Not Supplied

- ✓ A fluorometer capable of measuring fluorescence at an excitation wavelength of 360-390 nm and an emission wavelength of 450-470 nm
- ✓ Adjustable pipettes and a repeat pipettor
- ✓ A source of UltraPure water (*i.e.*, Milli-Q or HPLC-grade water)

Precautions for Use

- ✓ WARNING

  This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

- ✓ 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 200 µl in all the wells.
- Use the diluted assay buffer in the assay.
- All reagents except HAT (pCAF) 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.
- If the appropriate effective inhibitor concentration is not known, it may be necessary to assay at several dilutions.
- It is recommended that the samples be assayed at least in triplicate, but it is the user's discretion to do so.
- 30 inhibitor samples can be assayed in triplicate or 46 in duplicate.
- The assay temperature is 22-25°C.
- Monitor the fluorescence using an excitation wavelength of 360-390 nm and an emission wavelength of 450-470 nm.

✓ Plate Set Up

There is no specific pattern for using the wells on the plate. We suggest that there be at lease 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 trmplate sheet provided in Plate Layout.

Assay Protocol

Reagent Preparation

- ✓ HAT Assay Buffer (5X) - Dilute 10 ml of Assay Buffer concentrate with 40 ml of UltraPure water. This final Assay Buffer (100 mM HEPES, pH 7.5, containing 0.8% Triton X-100) must be used in the assay and for diluting HAT Acetyl CoA, HAT, and the HAT Developer. When stored at 4°C, this diluted Assay Buffer is stable for at least three months.
- ✓ HAT Acetyl CoA - The vial contains 200 µl of an acetyl CoA solution. Prior to use in the assay, dilute 100 µl of Acetyl CoA with 500 µl Assay Buffer. The diluted Acetyl CoA solution is stable for one week at -20°C.
- ✓ Histone Acetyltransferase (pCAF) - The vial contains 200 µl of human recombinant pCAF histone acetyltransferase. The enzyme is the catalytic domain of pCAF (p300/CREB-binding protein Associated Factor).[7] Prior to use in the assay, thaw the enzyme on ice and dilute 40 µl of pCAF with 960 µl of Assay Buffer. Store the diluted enzyme on ice. The diluted enzyme will be stable for four hours.
- ✓ HAT Peptide - The vial contains 2.5 ml of 250 µM Histone H3 peptide. The peptide comprises residues 5-23 of the human histone H3 N-terminal tail and is centered on Lys-14, the preferred acetylation site for the GCN5/pCAF family of HATs.[8] The solution is ready to use as supplied. *NOTE: The final concentration of peptide in the assay as described below is 100 µM. This concentration may be reduced with diluted Assay Buffer at the user's discretion.*
- ✓ HAT Stop Reagent - The vial contains 10 ml of isopropanol. It is ready to use as supplied.
- ✓ HAT Developer - The vial contains 500 µl of 7-diethylamino-3-(4'-maleimidylphenyl)-4-methyl-coumarin (CPM) in dimethylsulfoxide. Prior to use in the assay, dilute 100 µl of CPM with 11.9 ml of Assay Buffer. Cover the vial with tin foil. The diluted developer is stable for six hours.

Assay Procedure

1. 100% Initial Activity Wells - add 15 µl of Assay Buffer, 5 µl of Acetyl CoA, 10 µl of diluted pCAF, and 5 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
2. Background Wells - add 15 µl of Assay Buffer, 5 µl of Acetyl CoA, 10 µl of diluted pCAF, and 5 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
3. Inhibitor Wells - add 15 µl of Assay Buffer, 5 µl of Acetyl CoA, 10 µl of diluted pCAF, and 5 µl of inhibitor* to three wells.
4. Initiate the reactions by adding 20 µl of HAT Peptide to all the wells being used **except** the background wells.
5. Cover the plate with the plate cover and incubate on a shaker for five minutes at room temperature.
6. Remove the plate cover and add 50 µl of HAT Stop Reagent to all the wells being used including the background wells.
7. Add 20 µl of HAT Peptide to the background wells only.
8. Add 100 µl of HAT Developer to all the wells being used including the background wells.
9. Cover the plate with the plate cover and incubate for 20 minutes at room temperature.
10. Remove the plate cover and read the plate using an excitation wavelength of 360-390 nm and an emission wavelength of 450-470 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples. The fluorescence is stable for 30 minutes.

Table 1. Assay protocol

Steps	Reagent	100% Initial Activity	Background Wells	Inhibitor Wells*
1. Pipette reagents	HAT Assay Buffer	15 µl	15 µl	15 µl
	HAT Acetyl CoA	5 µl	5 µl	5 µl
	pCAF	10 µl	10 µl	10 µl
	Solvent	5 µl	5 µl	---
	Inhibitor	---	---	5 µl
2. Initiate Reaction	HAT Peptide	20 µl	---	20 µl
3. Incubate	5 minutes at room temperature			
4. Stop Reaction	HAT Stop Reagent	50 µl	50 µl	50 µl
	HAT Peptide	---	20 µl	---
5. Develop	HAT Developer	100 µl	100 µl	100 µl
6. Incubate	20 minutes at room temperature			
7. Read	Excitation 360-390 nm; Emission 450-470 nm			

*Inhibitors can be dissolved in Assay Buffer, ethanol, 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 HAT inhibition is completely unknown, we recommend that several concentrations of the inhibitor be assayed.

Data Analysis

Calculation of Results

Note:Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used.

1. Calculate the average fluorescence of each sample.
2. Subtract the fluorescence of the background wells from the fluorescence of the 100% initial activity and the inhibitor wells.
3. Determine the percent inhibition for each sample.

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

4. 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 is 50% inhibition).

Performance Characteristics

✓ Pricision

When a series of 16 HAT sample were performed on the same day, the intra-assay coefficient of variation was 3.2%. When a series of 16 HAT samples were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 4.4%.

✓ Interferences

There is a possibility that potential HAT inhibitors may interfere with the assay. If you are experiencing erratic fluorescence alues in the inhibitor wells, test the inhibitor for interference using the protocol outlined below.

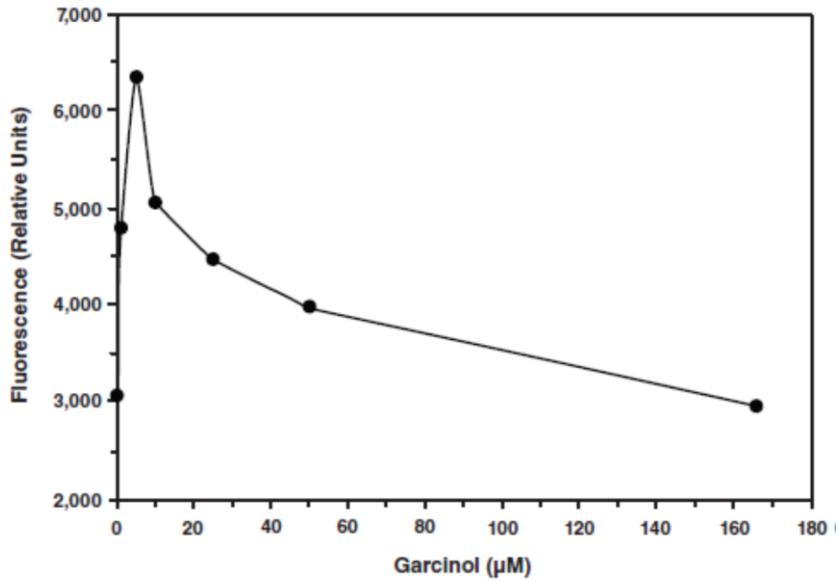
• Interference Protocol:

1. 100% Initial Activity Wells-add 15 µl of Assay Buffer, 5 µl of Acetyl CoA, 10 µl of diluted pCAF, and 5 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
2. Background wells- add 15 µl of Assay Buffer, 5 µl of Acetyl CoA, 10 µl of diluted pCAF, and 5 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
3. Inhibitor wells- add 15 µl of Assay Buffer, 5 µl of Acetyl CoA, 10 µl of diluted pCAF, and 5 µl of inhibitor to three wells.
4. Interference wells- add 15 µl of Assay Buffer, 5 µl of Acetyl CoA, 10 µl diluted pCAF, and 5 µl of inhibitor to three wells.
5. Initiate the reactions by adding 20 µl of HAT Peptide to all the wells being used except the background and interference wells.

6. Cover the plate with the plate cover and incubate on a shaker for five minutes at room temperature.
7. Remove the plate cover and add 50 µl of HAT Stop Reagent to all the wells being used including the background and interference wells.
8. Add 20 µl of HAT Peptide to the background and interference wells only.
9. Add 100 µl of HAT Developer to all the wells being used including the background and interference wells.
10. Cover the plate with the plate cover and incubate for 20 minutes at room temperature.
11. Remove the plate cover and read the plate using an excitation wavelength of 360-390 nm and an emission wavelength of 450-470 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.

- Determining Interference:

1. Determine the average fluorescence of each sample.
2. Subtract the fluorescence of the background wells from the fluorescence of the 100% initial acrivity wells.
3. Subtract the fuorescence of the interference test wells from the fluorescence of the inhibitor wells.
4. If the fluorescence seen in the inhibitor wells is greater than the 100% Initial Activity wells, then the compound is interfering in the assay and should not be used.
5. An example of a known pCAF HAT inhibitor garcinol, interfering with the assay is shown in Figure 3.[9]



Resources

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	A. Enzyme, acetyl CoA, or HAT peptide was not added to the well(s) B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all the components to the wells B. Reduce the concentration of the inhibitor and re-assay
Fluorescence value was at the maximal level in the wells	A. The enzyme is too concentrated B. The Gain setting is set too high	A. Set the gain to a lower setting and measure the fluorescence B. Make sure that you diluted the enzyme before assaying
The inhibitor did not inhibit the enzyme	Either the inhibitor concentration is not high enough or the compound is not an inhibitor	Increase the inhibitor concentration and re-assay
The fluorescence of the inhibitor wells is higher than the 100% Initial activity wells	The inhibitor may be interfering with the assay	See Interference section for guidance

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Plate Layout

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

BW-Background Wells

A-100% Initial Activity Wells

1-30-Inhibitor Wells