HAT Inhibitor Screening Assay Kit

Catalog No. 10006515

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CONTENTS OF THE KIT

Number	Item	Quantity
1	HAT Assay Buffer (5X)	1 vial
2	HAT Acetyl CoA	1 vial
3	Histone Acetyltransferase (pCAF)	1 vial
4	HAT Peptide	1 vial
5	HAT Stop Reagent	1 vial
6	HAT Developer	1 vial
7	96 Well White Plate	1 plate
8	Plate Cover	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.

PRECAUTIONS

WARNING: THIS PRODUCT IS NOT INTENDED OR APPROVED FOR DIAGNOSTIC USE IN HUMANS OR VETERINARY ANIMALS. RELIANCE ON THIS PRODUCT FOR ANALYTE MEASUREMENTS IN A THERAPEUTIC SETTING IS HAZARDOUS AND MAY RESULT IN ILLNESS OR INJURY.

- Please read these instructions carefully before beginning this assay.
- For research use only. Not for human or diagnostic use.

WARRANTY AND LIMITATION OF REMEDY

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery.

Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence.

This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.

IF YOU HAVE PROBLEMS

Our technical service staff may be reached by phone (888-526-5351, 734-975-3888), fax (734-971-3641), or E-Mail (techserv@caymanchem.com) Monday through Friday 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 at -20°C and used before the expiration date indicated on the outside of the box.

ADDITIONAL ITEMS REQUIRED

1. A fluorometer with the capacity to measure fluorescence using excitation wavelengths of 360-390 nm and emission wavelengths of 450-470 nm.
2. An adjustable pipettor and a repeat pipettor.
3. A source of UltraPure water (i.e., Milli-Q or HPLC-grade water).

ABOUT THIS ASSAY

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 phosphatidyliniositol 3-kinase signalling and inhibition of PTEN-regulated cell cycle arrest.[4]

Cayman's 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.

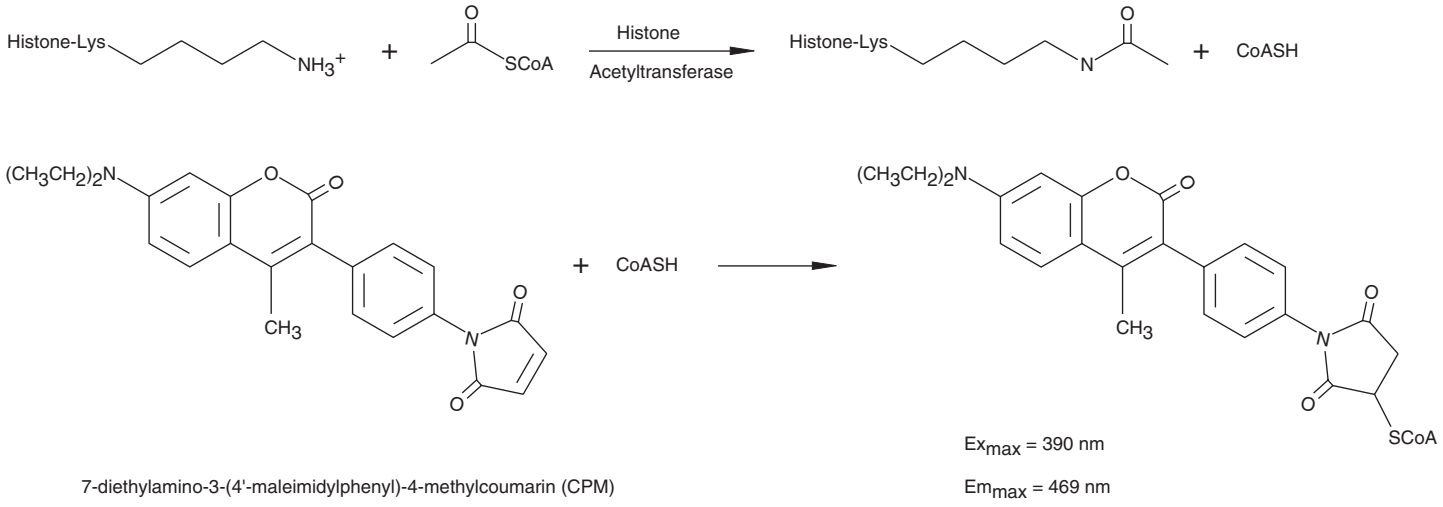


Figure 1. Reaction sequence for the HAT assay

PRECISION

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

PRE-ASSAY PREPARATION

Preparation of reagents

1. **HAT Assay Buffer (5X) – (vial #1)**

   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 acetyl CoA, HAT, and the HAT developer. When stored at 4°C, this diluted Assay Buffer is stable for at least three months.

2. **HAT Acetyl CoA – (vial #2)**

   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.

3. **Histone Acetyltransferase (pCAF) - (vial #3)**

   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.

4. **HAT Peptide - (vial #4)**

   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.}*

5. **HAT Stop Reagent - (vial #5)**

   The vial contains 10 ml of isopropanol. It is ready to use as supplied.

6. **HAT Developer - (vial #6)**

   The vial contains 500 µl of 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (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.

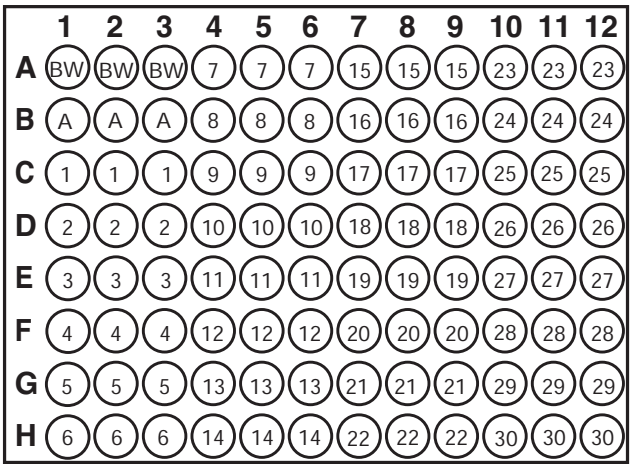
PIPETTING HINTS

- **It is recommended that a repeat pipettor be used to deliver reagents to the wells. This saves time and helps to maintain more precise incubation times.**
- **Before pipetting each reagent, equilibrate the pipet tip (i.e., fill the tip and expel the contents several times).**
- **Do not expose the pipet tip to the reagent(s) already in the well.**

PERFORMING THE ASSAY

Plate configuration

There is no specific pattern for using the wells on the plate. We suggest that there be at least 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 on page 9. A typical layout of samples and inhibitors to be measured in triplicate is shown in Figure 2 (see page 5).



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

Figure 2. Sample Plate Format

- *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.*

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 in a fluorometer 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.

	Reagent	100% Initial Activity	Background Wells	Inhibitor Wells*
Pipet Reagents	Assay Buffer (µl)	15	15	15
	Acetyl CoA (µl)	5	5	5
	pCAF (µl)	10	10	10
	Solvent (µl)	5	5	---
	Inhibitor	---	---	5
Initiate Reaction	HAT Peptide (µl)	20	---	20
Incubate	5 minutes at room temperature			
Stop Reaction	HAT Stop Reagent (µl)	50	50	50
	HAT Peptide (µl)	---	20	---
Develop	HAT Developer (µl)	100	100	100
Incubate	20 minutes at room temperature			
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.

CALCULATING THE PERCENT INHIBITION

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.

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

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).

INTERFERENCES

There is a possibility that potential HAT inhibitors may interfere with the assay. If you are experiencing erratic fluorescence values 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 of 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 in a fluorometer 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 activity wells.
3. Subtract the fluorescence 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, below.[9]

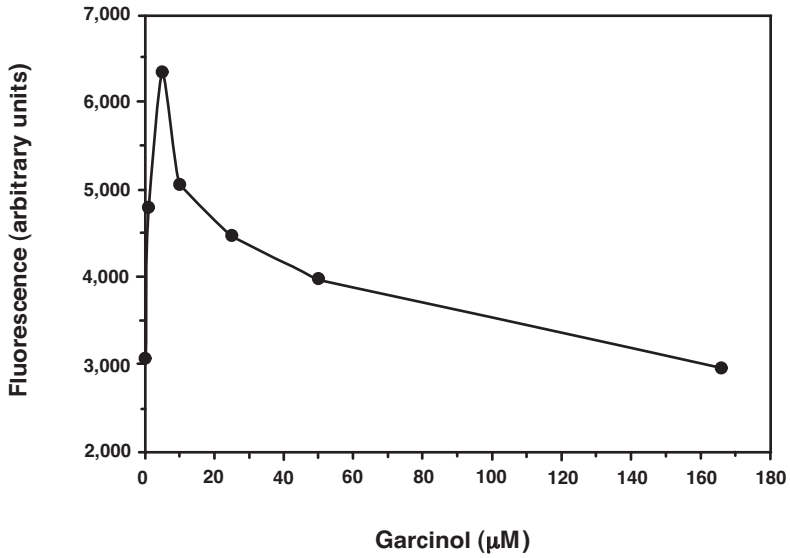


Figure 3. Inhibition of human recombinant pCAF histone acetyltransferase by garcinol

TROUBLESHOOTING

Problem: Erratic values; dispersion of duplicates/triplicates.
Cause: Poor pipetting/technique *-or-* Bubble in the well(s).
Solution: Carefully tap the side of the plate with your finger to remove bubbles. Be careful not to splash the contents of the wells.

Problem: No fluorescence above background is seen in the Inhibitor wells.
Cause: Enzyme, acetyl CoA, or HAT peptide was not added to the well(s). *-or-* Inhibitor concentration is too high and inhibited all of the enzyme activity.
Solution: Make sure to add all the components to the wells. Reduce the concentration of the inhibitor and re-assay.

Problem: Fluorescence value was at the maximal level in the wells.
Cause: The enzyme is too concentrated or the Gain setting is set too high.
Solution: Set the gain to a lower setting and measure the fluorescence. Make sure that you diluted the enzyme before assaying.

Problem: The inhibitor did not inhibit the enzyme.
Cause: Either the inhibitor concentration is not high enough or the compound is not an inhibitor.
Solution: Increase the inhibitor concentration and re-assay.

Problem: The fluorescence of the inhibitor wells is higher than the 100% Initial activity wells.
Cause: The inhibitor may be interfering with the assay.
Solution: See Interference section for guidance.

REFERENCES

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8. Lau, O.D., Courtney, A.D., Vassilev, A., *et al.* p300/CBP-associated factor histone acetyltransferase processing of a peptide substrate. Kinetic analysis of the catalytic mechanism. *J. Biol. Chem.* **275(29)**, 21953-21959 (2000).
9. Balasubramanyam, K., Altaf, M., Varier, R.A., *et al.* Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. *J. Biol. Chem.* **279(6)**, 33716-33726 (2004).

RELATED PRODUCTS

HDAC Activity/Inhibitor Screening Assay Kit - Cat. No. 789701 • p53 Monoclonal Antibody (Clone BP53-12) - Cat. No. 10004806 • p53 (Phospho-Ser^{392}) Polyclonal Antibody - Cat. No. 10004807 • PTEN Polyclonal Antibody - Cat. No. 10005059 • pCAF Histone Acetyltransferase - Cat. No. 10009115 • CREB (Phospho-Ser^{133}) Polyclonal Antibody - Cat. No. 10009181

PLATE TEMPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

NOTES