# ab133099 – Histone Acetyltransferase Inhibitor Screening

Instructions for Use

For the evaluation of pCAF HAT inhibitors

This product is for research use only and is not intended for diagnostic use.

Version 2 Last Updated 8 September 2014

# Table of Contents

## 1 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. 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. 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. The p53 tumor suppressor gene is the major target for genetic alteration or biochemical inactivation in human cancer(s). Numerous studies have demonstrated that p53 acetylation can greatly enhance its transactivation activity, increase its stability, and induce apoptosis. 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.

ab133099 provides a fast, fluorescence-based method for evaluating pCAF HAT inhibitors.

The Assay Reaction is shown below in Figure 1.

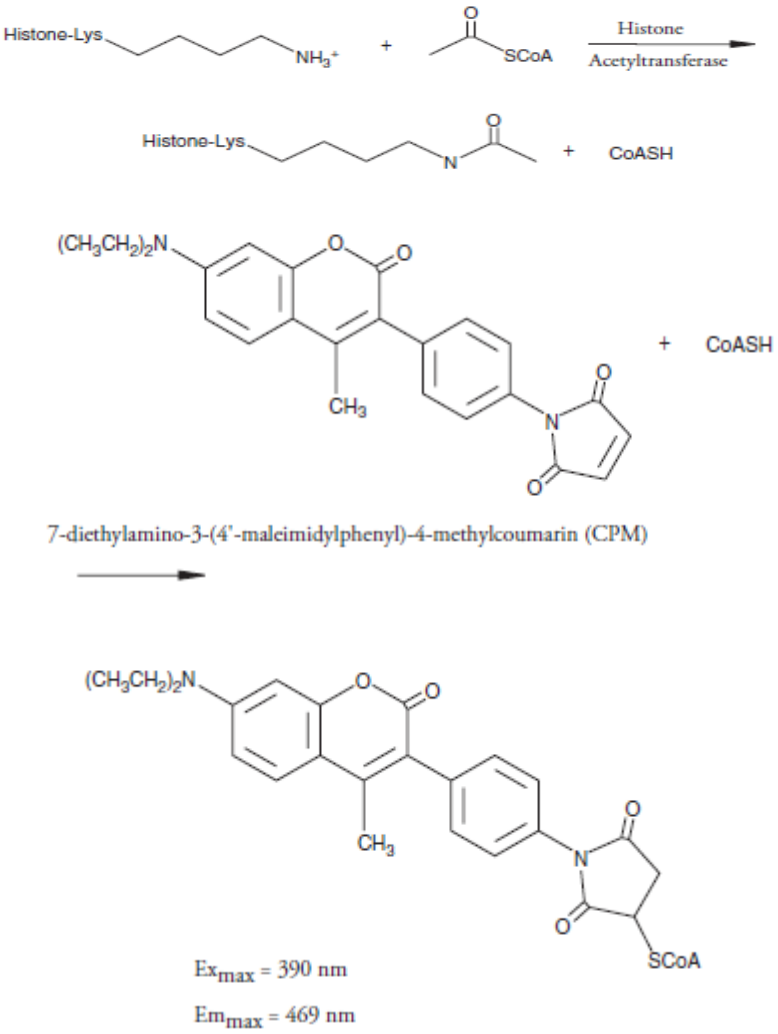



Figure 1. Reaction sequence for the HAT assay

## 2 ASSAY SUMMARY

HAT incubation with Acetyl CoA and Histone Peptide

Reaction Stopped with Isopropanol

CPM added and product read fluorescently

## 3 PRECAUTIONS

**Please read these instructions carefully prior to beginning the assay.**

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

## 4 STORAGE AND STABILITY

**Store kit as given in the table upon receipt and away from light.**

Observe the storage conditions for individual prepared components in sections 9 & 10. The components of the kit are stable for 6 months when stored properly.

For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

## 5 MATERIALS SUPPLIED

| Item | Quantity (96 tests) | Storage Condition (Before Preparation) |
|---|---|---|
| HAT Assay Buffer (5X) | 1 vial | -20°C |
| HAT Acetyl CoA | 1 vial | -20°C |
| Histone Acetyltransferase (pCAF) | 1 vial | -20°C |
| HAT Peptide | 1 vial | -20°C |
| HAT Stop Reagent | 1 vial | -20°C |
| HAT Developer | 1 vial | -20°C |
| 96-Well Plate (white) | 1 plate | -20°C |
| 96-Well Cover Sheet | 1 cover | -20°C |

## 6 MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 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.

## 7 LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

## 8 TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

## 9 REAGENT PREPARATION

Prepare fresh reagents immediately prior to use.

9.1 **HAT Assay Buffer**

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.

9.2 **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.

9.3 **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). 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.

9.4 **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. 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.*

9.5 **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.

9.6 **Plate Set Up**

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 at the end of the booklet. A typical layout of samples and inhibitors to be measured in triplicate is shown below.

| | 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

## 10 SAMPLE PREPARATION

**Inhibitors**

Sample (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.

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.

## 11 ASSAY PROCEDURE

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. The assay temperature is 22-25°C.

11.1 100% 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.
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.
Sample (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.

11.2 Initiate the reactions by adding 20 µL of HAT Peptide to all the wells being used except the background wells.

11.3 Cover the plate with the plate cover and incubate on a shaker for five minutes at room temperature.

11.4 Remove the plate cover and add 50 µL of HAT Stop Reagent to all the wells being used including the background wells.

11.5 Add 20 µL of HAT Peptide to the background wells only.

11.6 Add 100 µL of HAT Developer to all the wells being used including the background wells.

11.7 Cover the plate with the plate cover and incubate for 20 minutes at room temperature.

11.8 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.

# 12 ANALYSIS

## 12.1 Calculations

12.1.1 Calculate the average fluorescence of each sample.

12.1.2 Subtract the fluorescence of the background wells from the fluorescence of the 100% initial activity and the inhibitor wells.

12.1.3 Determine the percent inhibition for each sample.

% Inhibition = [(100% Initial Activity - Inhibitor Sample Activity)/ 100% Initial Activity] x 100

12.1.4 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).

## 12.2 Precision

When a series of 16 HAT samples 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%.

## 12.3 Interferences

12.3.1 Interference Protocol

12.3.1.1 100% 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.

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.

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.

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.

12.3.1.2 Initiate the reactions by adding 20 µL of HAT Peptide to all the wells being used except the background and interference wells.

12.3.1.3 Cover the plate with the plate cover and incubate on a shaker for five minutes at room temperature.

12.3.1.4 Remove the plate cover and add 50 µL of HAT Stop Reagent to all the wells being used including the background and interference wells.

12.3.1.5 Add 20 µL of HAT Peptide to the background and interference wells only.

12.3.1.6 Add 100 µL of HAT Developer to all the wells being used including the background and interference wells.

12.3.1.7 Cover the plate with the plate cover and incubate for 20 minutes at room temperature.

12.3.1.8 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.

12.3.2 Determining Interference

12.3.2.1 Determine the average fluorescence of each sample.

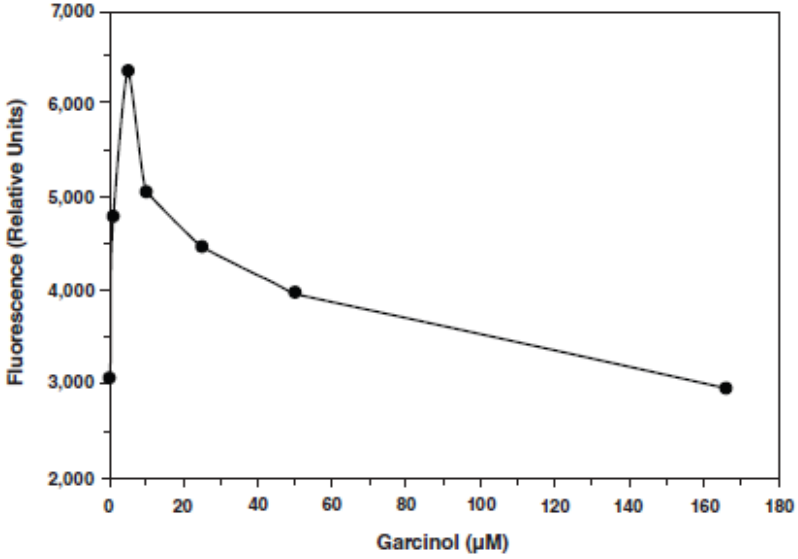
12.3.2.2 Subtract the fluorescence of the background wells from the fluorescence of the 100% initial activity wells.

12.3.2.3 Subtract the fluorescence of the interference test wells from the fluorescence of the inhibitor wells.

12.3.2.4 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.

12.4 **Typical Results.**

An example of a known pCAF HAT inhibitor, garcinol, interfering with the assay is shown.




Interference of garcinol in the assay

## 13 TROUBLESHOOTING

| Problem | Possible Causes | Recommended Solutions |
|---|---|---|
| Erratic values; dispersion of duplicates/ triplicates | A. Poor pipetting/technique<br>B. Bubble in the well(s) | A. Be careful not to splash the contents of the wells<br>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)<br>B. Inhibitor concentration is too high and inhibited all of the enzyme activity | A. Make sure to add all the components to the wells<br>B. Reduce the concentration of the inhibitor and re-assay |
| High background (NSB) | Incorrect dilution of antibody (too high) | Check antibody dilutions and use amounts outlined in the instructions |
| Fluorescence value was at the maximal level in the wells | A. The enzyme is too concentrated<br>B. The Gain setting is set too high | A. Set the gain to a lower setting and measure the fluorescence<br>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 |

## 14 NOTES

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