HAT Assay Kit

Fluorescent

(version B2)

Catalog No. 56100

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Overview

Active Motif's fluorescent HAT Assay Kit is an easy and sensitive assay to either determine the activity of your own source of purified histone acetyltransferases (HATs) or to screen potential inhibitors of HAT activity.

The HAT Assay Kit contains a purified recombinant active p300 catalytic domain protein, histone H3 and H4 N-terminal substrate peptides, anacardic acid as a control inhibitor, and all the essential buffers for a full 96-well plate assay.

This fluorescent HAT Assay works in three easy steps: first, the HAT protein is incubated with acetyl-CoA and either a histone H3 or histone H4 substrate peptide. During this reaction, the HAT enzyme catalyzes the enzymatic transfer of acetyl groups from the acetyl-CoA to the histone peptide thereby generating the acetylated peptide and CoA-SH. The reaction is stopped by adding stop solution, and after adding the complete developing solution, the developer reacts with the free sulfhydryl groups on the CoA-SH to give a fluorescent reading of acetyltransferase activity1.

product	format	catalog no.
HAT Assay Kit (Fluorescent)	1 x 96 rxns	56100

Introduction

Histone Acetylation

The basic structural unit of chromatin is the nucleosome, which consists of 146 base pairs (bp) of DNA wrapped around a histone octamer. The histone octamer consists of two copies each of the core histone H2A-H2B dimers and H3-H4 dimers, and the N-terminal tails of the histones are free from the bound core of the nucleosome structure where they can be modified by protein interactions in the nucleus. Histones, especially the N-terminal tails, are highly conserved from yeast to humans, and the modifications are commonly acetylation, phosphorylation and methylation. The modifications that occur at specific amino acid residues on the histone tails are very important for downstream signaling events and are of much interest in recent studies[1].

Histone acetylation is a modification that effects the nucleosome structure and therefore the ability of transcription factors to access the DNA and regulate gene expression. Recent studies have found that hyperacetylation of histones can be associated with relaxed chromatin structure and active gene transcription, while hypoacetylated histones can lead to transcriptional repression by condensing the structure of chromatin and restricting the access of transcription factors to the DNA[2].

Histone acetylation is a reversible process. Two superfamilies of enzymes are involved in histone acetylation: histone acetyltransferases (HAT) and histone deacetylases (HDAC), which catalyze the addition and removal of acetyl groups on histones. Histone acetyltransferases (HAT) are enzymes that catalyze the acetylation of lysine residues in the N-terminal tails of histone proteins.

The first nuclear HATs discovered were the general control of nuclear-5 (GCN5), and its paralog p300/CBP accessory factor (PCAF). Both are conserved in organisms from yeast to humans, and GCN5/PCAF HATs have served as paradigms for the biochemical analysis of HAT structure, mechanism and function[3]. In addition to histones, GCN5/PCAF HATs target non-histone protein substrates, such as transcription factors (***i.e.***, p53) and structural proteins (***i.e.*** α-tubulin). More than 20 HATs have been identified and these have been classified into one of five families (GNAT1, MYST (MOZ, YBF2/Sas3, Sas2, Tip60), TAFII250, nuclear receptor co-activators, and p300/cAMP response element-binding protein (CBP)). To date, a small number of HAT inhibitors have been reported. A few natural small molecules such as anacardic acid, garcinol and curcumin have been described as potent p300 and PCAF inhibitors[4].

Kit Performance and Benefits

The HAT Assay Kit (Fluorescent) is for research use only. Not for use in diagnostic procedures.

Recombinant p300 catalytic domain protein, active

p300 catalytic domain is a purified recombinant human protein expressed in insect cells. p300 catalytic domain is supplied at 0.1 mg/ml. This recombinant protein corresponds to amino acids 965-1810 from the GenBank sequence accession number U01877.

Anacardic Acid

Anacardic acid is supplied at 28.7 mM in DMSO. Anacardic acid is a small molecule compound from cashew nut shell liquid. 15 µM anacardic acid has been shown to quench HAT activity more than 90%. The IC_{50} value of anacardic acid for p300 using histones as substrates was found to be 8.5 µM and the IC_{50} value of anacardic acid for p300 using H3 peptide as the substrate was found to be 500 nM[4].

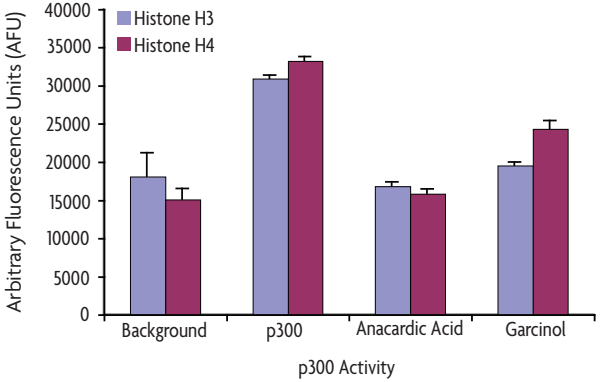

Figure 1: Effect of inhibitors on p300 HAT activity.

Fluorescent HAT Assay Kit results using 50 ng p300 catalytic domain per well with 50 µM Histone H3 peptide, 50 µM Acetyl-CoA, 15 µM Anacardic Acid, 25 µM Garcinol and 30 minute incubation at room temperature. The background fluorescence was subtracted from the data.

Kit Components and Storage

The HAT Assay Kit (Fluorescent) is for research use only. Not for use in diagnostic procedures. Store components at the temperatures indicated below. All components are guaranteed stable for six months when stored at the indicated temperature.

Reagent	Quantity	Storage / Stability
5X Assay Buffer AM1	1.8 ml	-20°C to room temp.
Acetyl-CoA	105 µl	-80°C
Recombinant p300 catalytic domain	50 µl (0.1 mg/ml)	-80°C
Histone H3 Peptide (MW=2068 Da)	0.275 µmoles (568 µg)	-20ºC
Histone H4 Peptide (MW=1905 Da)	0.275 µmoles (524 µg)	-20°C
Stop Solution (Fluor)	5 ml	room temp.
Developer (Fluor), 120X	84 µl	-20°C
Developing Solution (Fluor)	10 ml	-20°C to room temp.
96-well plate	1 plate	room temp.
Anacardic Acid	4 µl (10 mg/ml)	-80°C to -20°C
Plate sealer	1	room temp.

Additional materials required

- β-mercaptoethanol or Coenzyme A
- DMSO
- multi-channel pipette
- multi-channel pipette reservoir
- Fluorescent plate reader capable of exciting at 360-390 nm and reading emission at 460-490 nm

Reagent Preparation

5X Assay Buffer AM1

Prior to the assay, dilute to 1X with sterile water. Allow solution to warm to room temperature before use. This assay buffer is used in the assay and for diluting the Acetyl-CoA, Histone peptide substrates, and inhibitors. Prepare the quantity required for your assay including what you need to dilute the components. The diluted 1X Assay Buffer AM1 is stable for 3 months.

Acetyl-CoA

Acetyl-CoA is provided as a 6 mM stock solution. Prior to the assay, dilute 1:12 with the 1X Assay Buffer to a final concentration of 0.5 mM. 5 µl are used per assay well. Use freshly diluted Acetyl-CoA for each assay.

Recombinant p300 catalytic domain

Avoid multiple freeze-thaws of the protein. Please aliquot and store frozen at -80°C if only a portion of the enzyme is needed for your assays.

Histone H3 Peptide and Histone H4 Peptide

Peptides are supplied lyophilized. Rehydrate each peptide with 2.2 ml of the 1X Assay Buffer to obtain a 125 µM stock solution (125 pmol/µl). The peptide is supplied in a tube with a 1.5 ml volume capacity. Start by adding a portion of the 2.2 ml 1X Assay Buffer to transfer the peptide to a new tube to resuspend in a 2.2 ml final volume. After the peptide is in solution, store at -20°C.

Use 20 µl per assay well for a 50 µM final concentration in the reaction. This concentration may be reduced or increased with the 1X Assay Buffer if another final concentration is preferable.

Anacardic Acid

Anacardic Acid is supplied at 28.7 mM in DMSO. Dilute in 1X Assay Buffer AM1 to make a 150 µM solution. For example, dilute 1 µl of Anacardic Acid stock solution in 190 µl 1X Assay Buffer. For a 15 µM Anacardic Acid treatment, add 5 µl of the 150 µM solution per assay well.

DMSO

Prepare a DMSO dilution equivalent to the Anacardic Acid dilution to serve as a background control within the assay. Add 1 µl of DMSO to 190 µl of 1X Assay Buffer. Use 5 µl of diluted DMSO per assay well.

Stop Solution (Fluor) Ready to use as supplied.

Developer (Fluor)

Developer stock is 3 mM in DMSO. Prior to use, dilute the developer 1:120 with Developing Solution to obtain a final developing solution concentration of 25 µM. Protect the Developer and final Developing Solution from light. The final diluted developing solution is stable for 6 hours at room temperature.

Note: The assay will not work if the developer is diluted in assay buffer instead of developing solution.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Minimize the use of sample buffers containing DTT or β-mercaptoethanol for your HAT source. Buffers containing these reducing agents or other sources of reactive thiol groups will result in high background.

This protocol has been optimized for use with recombinant proteins. If you wish to calculate enzymatic activity, a standard curve must be prepared.

It is recommended to run test samples, controls, standards and background wells at least in duplicate.

Preparation of Standard Curve (Optional)

If you want to calculated enzymatic activity, you will need to run a standard curve alongside your samples in the assay. A standard curve may be prepared with Coenzyme A or β-mercaptoethanol.

For example, to prepare a standard curve with β-mercaptoethanol, make dilutions in duplicate ranging from 0 to 14 µM in 2 µM increments in a 50 µl volume to result in a 0 to 500 pmol range for the X-axis (Figure 1). The volumes below are enough for duplicate wells.

	0 µM	2 µM	4 µM	6 µM	8 µM	10 µM	12 µM	14 µM
1X Assay Buffer	120 µl	117.6 µl	115.2 µl	112.8 µl	110.4 µl	108 µl	105.6 µl	103.2 µl
β-mercaptoethanol	0 µl	2.4 µl	4.8 µl	7.2 µl	9.6 µl	12 µl	14.4 µl	16.8 µl

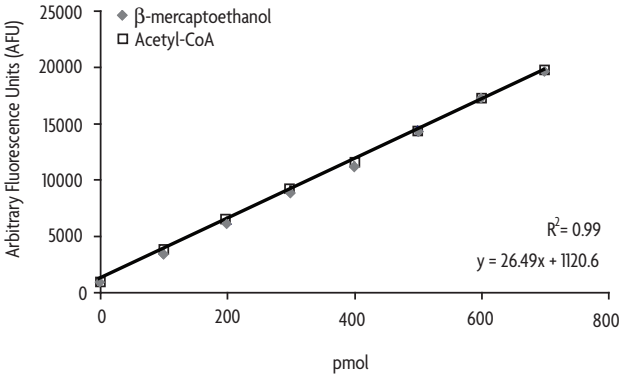

Figure 1: Standard Curve Data.

Example standard curve data of both acetyl-CoA and β-mercaptoethanol diluted from 0 to 14 µM. pmol of β-mercaptoethanol = µM of β-mercaptoethanol X 50 µl per well

Run standard curve wells by adding 50 µl of each dilute per well in duplicate and continue with step 4 in the following protocol.

Assay Protocol

1. Design your assay to test all samples in duplicate. Prepare the quantity required of 1X Assay Buffer, diluted Acetyl-CoA and desired inhibitors. If using the Anacardic acid inhibitor provided in the kit, prepare a DMSO dilution for background comparison. Prepare the amount of final developing solution required and keep protected from light. The HAT enzyme and Acetyl-CoA should be kept on ice.
2. Using any desired microcentrifuge tubes for samples including background, positive control and inhibition wells, gently mix the following components together in the quantities indicated. 30 µl of each mix is needed per assay well. Keep the reactions at room temperature. The volumes listed in the following table are enough for duplicate wells.

Reagent	Background Wells	Positive Control Wells	Inhibitor Wells	Standard Curve Wells
1X Assay Buffer	48.75 µl	48.75 µl	48.75 µl	
0.5 mM Acetyl-CoA	12.5 µl	12.5 µl	12.5 µl	
150 µM Anacardic Acid			12.5 µl	
Diluted DMSO	12.5 µl	12.5 µl		
p300 catalytic domain (0.1 mg/ml)	1.25 µl	1.25 µl	1.25 µl	
β-mercaptoethanol standard				120 µl
Total volume per well	**30 µl**	**30 µl**	**30 µl**	**50 µl**

3. Add 20 µl of Histone H3 or Histone H4 peptide to each assay well. Do not add peptide substrate to background wells so that autoacetylation activity of the acetyltransferase is included in the background measurement. Mix solution by pipetting contents up-and-down with multichannel pipettor. Cover plate with plate sealer and incubate for 10 to 30 minutes at room temperature.

   Note: If you are determining the activity of a new HAT protein, we recommend running a time course to establish the correct incubation time for your protein.

4. Add 50 µl of Stop Solution per well. To background wells, add 20 µl peptide substrate after adding the stop solution.
5. Add 100 µl final Developing Solution to each well and incubate 15 minutes in the dark at room temperature.
6. Read fluorescence with excitation at 360-390 nm and emission at 450 to 470 nm.

Data Analysis

Plot the standard curve data with fluorescence units on the Y-axis and pmol of β-mercaptoethanol on the X-axis.

Determine slope of the line through the linear part of the curve as arbitrary fluorescence units per pmol. Subtract the fluorescence of background from that of the assay samples and divide the difference by the incubation time (AFU/minute). Divide the AFU/minute by the slope of the standard curve (AFU/pmol) to obtain the rate in pmol/minute.

References

1. Trievel, R. C., et al., (2000) Analytical Biochemistry 287: 319-328.
2. Wang, L., et al., (1997) Mol. and Cell. Biol. 17(1): 519-527.
3. Marmorstein, R. (2001) Cell. Mol. Life Sci. 58: 693-703.
4. Balasubramanyam K, et al., (2003) J. Biol. Chem. 278(21):19134-19140.

Appendix

Section A. Troubleshooting Guide

Problem/question	Recommendation
Must the assay be incubated at room temperature?	The assay may be performed at 30˚C or room temperature without effecting results.
Is it possible to use diluted Acetyl-CoA?	No. Fresh Acetyl-CoA should be prepared for each assay.
Is it necessary to run a standard curve?	No. The standard curve is only necessary if the specific activity needs to be calculated (pmol/min/µg).
Why was no HAT activity detected?	The recombinant p300 supplied in this kit may lose activity after 3 freeze-thaws. Please aliquot at first use to store and reduce the number of freeze-thaws.
	Check that the developer has been diluted into developing solution. Do not use Assay Buffer AM1 to dilute!
Why is there high background?	The background is due to the free sulfhydryl groups in the formulation buffer. Some background may be due to the protein's auto-acetylation activity. Run controls with and without acetyl-CoA to determine background and auto-acetylation levels.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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