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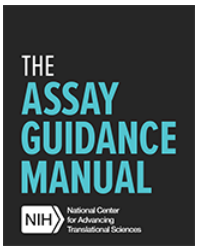

Histone Acetyltransferase Assays in Drug and Chemical Probe Discovery

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Abstract

Histone acetyltransferases (HATs) are a class of epigenetic enzymes involved in critical cellular processes like nucleosome assembly, DNA damage repair, and transcriptional regulation. HATs are implicated in many human pathologies including cancers. This chapter describes essential experimental considerations for performing high-throughput screening and follow-up assays, and offers practical strategies for assay optimization and validation. Illustrative walkthroughs for several HAT assay formats are provided. This content should be useful for those performing HTS assays, orthogonal assays, and counter-screens involving HATs in the context of drug discovery, chemical biology, and molecular pharmacology.

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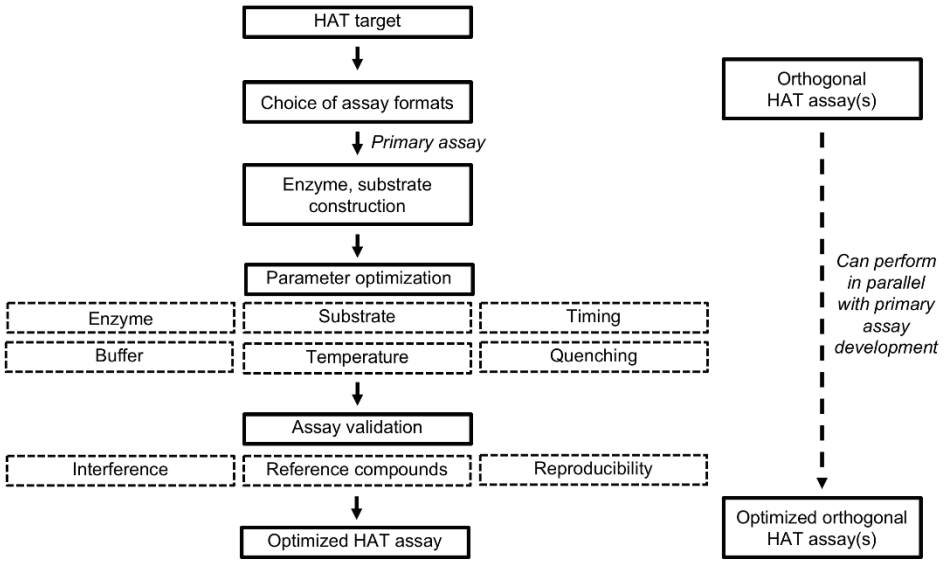
Flowchart



Abbreviations

Acetyl-CoA	acetyl coenzyme A
BSA	bovine serum albumin
CoA	coenzyme A
CPM	*N*-[4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl]maleimide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FRET	fluorescent/Förster resonance energy transfer
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEPES	hydroxyethyl piperazineethanesulfonic acid
HTS	high-throughput screening
IS	internal standard
KAT	lysine acetyltransferase
LoD	limits of detection
LoQ	limits of quantitation
MS	mass spectrometry
MSR	minimum significant ratio
NEM	N-ethylmaleimide
PAINS	pan assay interference compounds
PTM	post-translational modification

Table continued from previous page.

SIRT	sirtuin
SPE	solid-phase extraction
TCEP	*tris*(2-carboxyethyl)phosphine

Introduction and Background

Introduction

HATs are a class of epigenetic enzymes involved in many critical cellular processes. Aberrancies in HAT function have been implicated in human disease. Modulators of HAT activity may therefore be highly useful for chemical probes of epigenetic systems, or potential therapeutics in HAT-related pathologies.

This chapter describes essential experimental factors needed to develop, optimize, and validate assays for the purpose of assessing compound modulation of HAT activity. Several example protocols of representative HAT assays are provided. This information should be useful for novices as well as more seasoned researchers. While this chapter specifically focuses on HAT assays, the general methodologies should also be informative to a variety of epigenetic systems (e.g., methyltransferases, deacetylases) as well as non-epigenetic bi-substrate enzymatic systems.

Note: This chapter emphasizes biochemical HAT assays. Cell-based HAT assays are briefly discussed. However, many of the same principles discussed with respect to biochemical HAT assays should apply to cell-based HAT assays.

Background

Epigenetics is defined as heritable changes in gene expression that occur without altering the underlying genetic sequence. This process is regulated in part by a series of epigenetic enzymes acting *via* post-translational modifications (PTM) such as histone acetylation, methylation, phosphorylation, and ubiquitylation. Histone PTMs are regulated by histone reader, writer, and eraser enzymes.

Histone acetylation is critical for regulation of gene transcription, nucleosome assembly, and DNA repair (1,2). HATs have been implicated in a variety of human pathologies, including cancers (3). Consequently, there is great interest in developing potent and specific small-molecule modulators of HAT activity, either as chemical probes or potential therapeutics (4,5).

Human HATs can be divided into several large families: MYST (TIP60, MOZ/MYST3, MORF/MYST4, HBO1/MYST2, MOF/MYST1), p300/CBP (P300, CBP), and GNAT (GCN5, PCAF, ELP3). In addition, there are also transcription factors with HAT activity and nuclear receptor co-activators. HATs are also present in other eukaryotic species, including *Drosophila* and fungi, the latter of which includes opportunistic human pathogens such as *Aspergillus*, *Candida*, and *Pneumocystis*.

HATs catalyze the transfer of an acetyl moiety from acetyl-CoA to lysine side chains present on histone proteins (**Figure 1**). Catalysis can occur through a random-order ternary complex mechanism, a compulsory-order ternary complex, or a ping-pong mechanism. The final product neutralizes the positively-charged lysine ε-amino group and reduces steric bulk, which effectively "loosens" the interaction between histone proteins and negatively-charged DNA, leading to less compacted chromatin and increased transcription. Acetylated histone residues can be specifically recognized by histone reader domains called bromodomains. The biological counter-part to HATs are histone deacetylases (HDACs) and sirtuins (SIRTs), epigenetic enzymes which catalyze the removal of the acetyl moiety from acetylated histone lysines. HATs are complex bi-substrate systems, utilizing both an acetyl donor (acetyl-CoA) and an acetyl acceptor (histone protein).

HATs acetylate many histone lysine residues. (6) These occur on H2A (K5), H2B (K12, K15), H3 (K9, K14, K18, K36, K56), and H4 (K5, K8, K12, K16). Often a specific HAT can acetylate multiple histone lysine residues. HAT activity and substrate specificity can be regulated by multiple factors, including autoacetylation and chaperone proteins (7-9). Note that certain HATs can also acetylate non-histone substrates, and can also be referred more broadly as lysine acetyltransferases (KATs).

Introduction to Common HAT Assays

Multiple biochemical HAT assays have been described (**Table 1**). These assays measure HAT activity by detecting either the acetylated histone-based product ("direct") or the free CoA product ("indirect").

So-called indirect methods utilize fluorescent thiol-scavenging probes or enzyme-coupling reactions. In general, these assays are inexpensive and straightforward to establish, but are highly susceptible to compound-mediated assay interference. This can necessitate multiple counter-screens and orthogonal assays to rule-out interference and confirm true compound activity. While they are usually highly amenable to HTS, the time and resources required to perform interference counter-screens and orthogonal studies to triage assay artifacts and poorly tractable chemical matter has the potential to offset the initial up-front benefits of indirect assays.

Direct HAT assays can measure the acetylated histone-based product by one of multiple technologies. These include radiolabels (e.g., filter-binding, scintillation proximity), mass spectrometry (MS), microfluidic mobility, and antibody-based techniques such as traditional enzyme-linked immunosorbent assay (ELISA), Alpha-based technologies (e.g., AlphaScreen, AlphaLISA), and FRET (including time-resolved FRET). The advantage of direct assays is that they measure the actual protein product from the HAT reaction. Direct assays can require more technical expertise, specific instrumentation, and more customized and expensive reagents compared to the most common indirect methods. Furthermore, even direct HAT assays are susceptible to compound-mediated assay interference, and should be accompanied with appropriate readout interference counter-screens and orthogonal assays.

Each HAT assay technology has characteristic advantages and disadvantages (**Table 1**). When selecting an assay type for either a primary or orthogonal assay, one should consider multiple (and often competing) factors, including but not limited to: available instrumentation, resources, expertise, project timelines, the nature of the HAT system being assayed, possible technology-related interferences, and the number and type of test compounds.

The indirect assays and most of the direct assays can be suitable for HTS (**Table 1**), while the direct and indirect assays can all be adapted for orthogonal assays. Suggestions for specific primary assay-orthogonal assay pairings are discussed in the subsequent section "Use of Orthogonal Assays/Common Interferences".

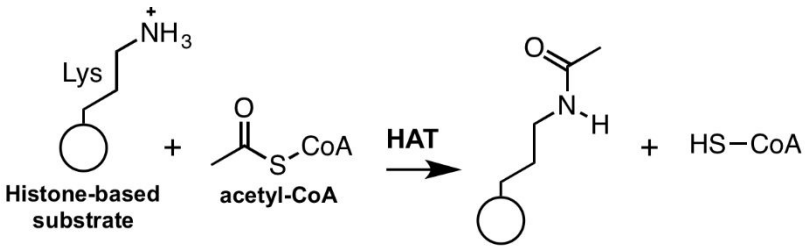


Figure 1. Schematic of HAT reaction. HATs catalyze the transfer of an acetyl moiety from acetyl-CoA to a histone substrate. The resulting products are free coenzyme A ("CoA-SH" or "CoA") and an acetylated histone lysine residue.

Table 1. Common HAT assay formats. Each HAT assay format has advantages and disadvantages, including susceptibilities to technology-related compound-mediated assay interferences.

Assay technology	Assay principle	Advantages	Disadvantages	Compound-mediated readout interferences	General suitability for HTS	Example references
Fluorometric (thiol-scavenging probe)	Free CoA forms fluorescent adduct with probe (indirect)	-Inexpensive -Easy to set-up -High-throughput -Label-free	-Significant potential for compound-mediated interference -Enrichment of thiol-reactive compounds -Can require large concentrations of product formation for detection	-Light-based interferences (absorbance, auto-fluorescence, quenching, light scattering) -Thiol-scavenging	+	(10-14)
Enzyme-coupling	Second enzyme requires CoA, secondary product measured (indirect)	-Inexpensive -Easy to set-up -High-throughput -Adaptable to continuous readout -Label-free	-Significant potential for compound-mediated interference -Enrichment of thiol-reactive compounds -Can require large concentrations of product formation for adequate signal	-Secondary enzyme system modulation -Light-based interferences (absorbance, auto-fluorescence, quenching, light scattering) -Thiol-scavenging	+	(15)
Radiolabeled substrate (filter-binding)	Radiolabeled acetate (*via* acetyl-CoA) incorporated onto histone product (direct)	-High S:N -Few compound-mediated interferences -Label-free	-Cost -Radiation -Lower-throughput in most settings	-Scintillation quenching	–	(10,16-18)
Alpha technology (AlphaScreen, AlphaLISA)	Amplified luminescent proximity (direct)	-High S:N -Customizable -No radiation -Amenable to miniaturization -Homogenous format	-Cost -Specialized instrumentation -Dependent on antibody quality -Light sensitivity	-Singlet oxygen quenching -Light-based interferences (absorbance, quenching, auto-fluorescence, light-scattering) -Capture reagent disruption (e.g., disruption of antibody/tag interaction)	+	(19)

Table 1. continued from previous page.

Assay technology	Assay principle	Advantages	Disadvantages	Compound-mediated readout interferences	General suitability for HTS	Example references
FRET	Fluorescent/ Förster resonance energy transfer (direct)	-Customizable -No radiation -Usually cheaper than Alpha -Can enhance with time-resolved FRET (TR-FRET) -Homogenous format	Dependent on antibody quality	-Light-based interferences (absorbance, quenching, auto-fluorescence, light-scattering) -Capture reagent disruption (e.g., disruption of antibody/tag interaction)	+	(20)
Fluidic mobility	Electrophoretic separation of histone-based products (direct)	-Monitor multiply-acetylated substrates -No radiation -Adaptable to continuous readouts	-Highly specialized instrumentation -Highly-charged substrates challenging -Large substrates challenging		+	(21,22)
Mass spectrometry	Histone-based products ionized and *m/z* measured (direct)	-Highly sensitive (less product formation required) -Label-free -Monitor multiply-acetylated products -No radiation	-Highly specialized instrumentation -Throughput	-Ion suppression -Compound-analyte adducts	+	(23)
Traditional antibody (ELISA, Western)	Antibody binds to acetylated histone product (direct)	-Straightforward to establish -Highly sensitive -No radiation	-Dependent on antibody quality -Lower-throughput (heterogeneous format)	-Antibody interference -Quenching of antibody reporter -Lower dynamic ranges	–	(10,11,17)
Scintillation	Radiolabeled product scintillates in proximity to capture matrix (direct)	-High S:N -Homogenous format	-Radiation -Specialized instrumentation	Scintillation quenching (24,25)	+	(20,26-28)

Section Summary

HATs are epigenetic enzymes involved in critical cellular processes, and have been linked to human disease. There are multiple robust, orthogonal assay platforms assessing modulation of HAT activity by test compounds.

General Considerations for HAT Assays

Introduction

Regardless of technology choice, HAT assays require optimization of many experimental parameters. This section describes essential experimental considerations for performing HAT assays, including optimization of enzyme and substrates, other reaction components, reaction timing, and reaction quenching.

Enzyme/Substrate Source

HATs and histone-based substrates can be purchased commercially or produced in-house. Commercially-sourced HATs and histones have the advantage of being readily available, but often they are prohibitively expensive in amounts needed for an HTS. Proteins produced in-house can allow for greater customization and control over the purification process, but production can be time-consuming, and can be difficult to achieve at large scales and acceptable purities.

Commercially-sourced enzymes and substrates are often "one size fits all" entities, and may not have the specific features optimal for a particular HAT assay. Users may need to customize: (a) a specific affinity or purification tag, (b) the construct of the HAT enzyme or histone (e.g., truncations), (c) the introduction of PTMs such as fluorophores, and (d) expression and purification conditions to optimize protein folding and stability. For some applications, full-length HAT or histone substrate may be necessary to capture the essential features of the native system being probed by the HTS. In other cases, users may only need the catalytic HAT domain or specific histone peptides. In some cases, supposed non-catalytic domains may have profound effects on catalysis, system stability, and substrate specificity.

Depending on the research question and HTS method, users may need to produce their own HAT or histone substrate (29). While the details of producing recombinant enzymes are beyond the scope of this chapter, we recommend first consulting any original literature on the specific HAT or histone substrate to be assayed for details about potential production and purification strategies.

The identity and purity of HAT enzymes and protein substrates should be rigorously validated, especially when performing experiments on the HTS scale. For details, refer to Validating Identity, Mass Purity and Enzymatic Purity of Enzyme Preparations.

Acetyl-CoA

There are several important considerations with respect to the acetyl-CoA substrate:

- Acetyl-CoA is often sold in salt/hydrate preparations with variable stoichiometry. Most commercial forms are sold as a sodium or lithium salt.
- To ensure accurate K_M determination and to enhance assay precision, the concentration of acetyl-CoA should be determined spectrophotometrically. For example, acetyl-CoA (tri-lithium salt) can be measured by monitoring light absorbance at 260 nm (ε = 16000 M^{-1} cm^{-1} in H_2O) or at 232 nm (ε = 8700 M^{-1} cm^{-1} in H_2O) (30).
- In solid form, acetyl-CoA should be stored in desiccated conditions and in -20°C.
- In solution, acetyl-CoA stocks should be stored at -20°C. Acetyl-CoA is unstable in alkaline conditions and in highly acidic conditions. A recommended storage buffer is 50 mM sodium acetate, pH 5.0. Minimize freeze-thaw cycles.
- Commercial acetyl-CoA can contain minor amounts of free CoA. This may interfere with some types of HAT assay readouts or inhibit certain HATs by product inhibition (22). Free CoA can be treated with acetic anhydride to mitigate this effect (13).

Histone-Based Substrates

Many choices of substrates are available for HAT assays (**Table 2**). These include (in order of complexity): histone peptides, full-length histones, histone dimers, core histones, histone-chaperone complexes, and nucleosomes.

For any given HAT assay, the optimal histone-based substrate is multifactorial. One factor is the nature of the specific HAT system being assayed, as HAT specificity and catalytic efficiency can depend on whether the

histone is presented in monomeric or multimeric form(s), or whether histone chaperones are present. Users should also consider the physiologic context of the HAT target. As with the choice of enzyme constructs, using full-length substrates and/or include chaperone proteins can potentially better approximate physiological catalysis, HAT stability, and substrate specificity. Generally speaking, it is best to approximate physiologic conditions in any given assay, but such considerations must be weighed against practical and competing factors such as compatibility with assay technology, overall system complexity, and reagent costs.

In general, histone peptides are practical and relatively easy to customize (with respect to length, sequence, labels, affinity tags), and can reduce system complexity. Depending on the particular epigenetic system, histone peptides can have a higher K_M than their full-length counterparts (31). Furthermore, a potential downside to peptide substrates is that they may not sufficiently model the physiologic context of a given HAT system.

Full-length histone products may therefore be required in this regard. Potential downsides to full-length protein use are their more challenging production procedures and the increased assay complexity due to many HATs that can acetylate multiple lysine residues. Fortunately, full-length histone monomers and multimers can be produced using recombinant technology in large scales with sufficient technique. For additional details regarding histone product production, we refer the reader to several published protocols (32-35). For full-length histone products, users should pay particular attention to protein precipitation at higher concentrations. This potential problem can often be mitigated by carefully optimizing pH and salt conditions.

Users should also consider the species of the histone-based substrate. While histones are highly conserved species in eukaryotes, subtle inter-species sequence variations may have dramatic consequences. For some applications, it may be acceptable to use histones derived from *Drosophila*, yeast, chicken, or calf thymus. In other cases, human-based histones may be required.

Table 2. Common histone-based substrates in HAT assays. Each substrate type has advantages and disadvantages. The optimal substrate will depend on the nature of a given HAT system, the choice of assay, and other factors.

Substrate type	Potential advantages	Potential disadvantages
Histone peptide	-Soluble -Customizable -Can simplify complex multi-substrate histones	-Non-native substrate -Potentially higher K_M
Full-length histone protein	Potential physiologic substrate	-Precipitation risk -PTM heterogeneity (e.g., non-recombinant source)
Histone dimer/multimer/core	Potential physiologic substrate	-Precipitation risk -Increased assay complexity -PTM heterogeneity (e.g., non-recombinant source)
Histone-chaperone complex	Potential physiologic substrate	-More laborious to prepare in bulk quantities -Precipitation risk -Increased assay complexity -PTM heterogeneity (e.g., non-recombinant source)
Nucleosome	Potential physiologic substrate	-More laborious to prepare in bulk quantities -Precipitation risk -Increased assay complexity -PTM heterogeneity

Reaction Buffer and Additives

The essential components of a biochemical HAT reaction include buffer, salts, chelating agents, reducing agents, detergents, and carrier proteins.

- Buffer

    - Buffers are used to maintain the reaction solution within a fixed pH range optimal for HAT activity.
    - Common buffering agents include Tris HCl and HEPES, typically at 50-100 mM final concentrations.
    - Most HAT reactions are performed in slightly alkaline conditions (usually pH 7.5-8.0).
    - A common buffering agent is 50 mM Tris, pH 8.0.
- Salts
    - Most biochemical HAT assays utilize NaCl, though KCl can often be used in its place.
    - The most common ionic strength is 50-100 mM NaCl.
    - Full-length histone substrates (± chaperones) can be highly sensitive to salt concentrations, especially at higher concentrations.
- Chelating agents
    - Chelating agents can be added to prevent metal-catalyzed proteolysis and oxidation.
    - The most common chelating agent used in HAT reactions is ethylenediaminetetraacetic acid (EDTA), often at 0.1 mM final concentrations.
    - Be aware that certain HAT systems may show metal-dependent activity. Therefore, it is reasonable to assess the performance of a HAT assay in the presence and absence of chelating agents like EDTA, as well as using similar methods to investigate compounds for chelation-related bioactivity. (36)
- Reducing agents
    - Reducing agents are added to prevent oxidation of protein side chains, most notably cysteine thiols. Reducing agents can also mitigate the effect of thiol-reactive screening compounds by acting as scavenging reagents (17).
    - Strong reducing agents have the potential to fuel redox-cycling, a phenomenon in which compounds produce H_2O_2 *in situ* (37,38).
    - Examples of commonly used reducing agents include dithiothreitol (DTT), β-mercaptoethanol (BME), and *tris*(2-carboxyethyl)phosphine (TCEP).
    - The most common reducing agent is DTT, often at 1-5 mM final concentrations.
    - Note that DTT is unstable in aqueous conditions. Freshly prepare solutions containing DTT, and keep on ice.
- Detergents
    - Detergents are added for two reasons: (a) to prevent nonspecific protein adsorption to container walls, and (b) to mitigate aggregation formation by test compounds, which can nonspecifically modulate HAT activity.
    - Examples detergents include Triton X-100, Tween 20, Pleuronic F-68, and Brij 35.
    - The authors have had good experience with 0.01% Triton X-100 (v/v). Triton X-100 can produce H_2O_2 *in situ*, which should be considered in systems highly sensitive to oxidation. This effect can be mitigated by preparing fresh detergent-containing buffers.
    - Many detergents are highly viscous. Handling can be enhanced by preparing as 10% (v/v) solutions and pipetting slowly. Allow time (often several hours with gentle mixing) for detergents to fully dissolve in water.
- Carrier proteins
    - Carrier proteins are included in many biochemical reactions to enhance enzymatic stability, as many enzymes are unstable in dilute concentrations. Carrier proteins can prevent nonspecific adsorption of assay proteins to container walls.
    - Carrier proteins (also known as "decoy proteins" in the context of preventing aggregation) can also mitigate compound aggregation (39), and can also serve as low-level thiol-scavenging reagents to mitigate the effect of thiol-reactive screening compounds.
    - The most common carrier protein used in biochemical HAT assays is bovine serum albumin (BSA). Common concentrations of BSA range from 5 to 100 μg/mL.

- Protease, HDAC inhibitors
  - Protease inhibitors are typically added to certain biochemical solutions to prevent proteolysis. Examples include phenylmethylsulfonyl fluoride ("PMSF") and 4-(2-aminoethyl)benzenesulfonyl fluoride ("Pefabloc"). Commercial cocktails are also widely available.
  - While useful in protein purification, protease inhibitors are generally not required for most biochemical HAT assay applications.
  - Inhibitors are often used at relatively high concentrations (high micromolar/low millimolar) and have the potential to cause assay interference.
  - Consider adding protease inhibitors if there is evidence of significant protein degradation. Extended incubation or reaction times may also warrant trials of protease inhibitors.
  - Nonspecific HDAC inhibitors such as sodium butyrate are occasionally added to prevent deacylatation. They are typically used at low millimolar concentrations. In purified systems, such inhibitors are probably unnecessary. When analyzing reactions containing cellular extracts, appropriate control experiments should be performed to determine whether HDAC inhibitors are necessary and whether they interfere with HAT activity or assay readout.
- Example reaction buffer
  - The following is a useful initial reaction buffer: 50 mM Tris, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% Triton X-100 (v/v), 50 µg/mL BSA.
  - It is often useful to prepare reaction buffers in concentrated form. Consider making a 5X solution (250 mM Tris, pH 8.0, 250 mM NaCl, 0.5 mM EDTA, 5 mM DTT, 0.05% Triton X-100 (v/v), 250 µg/mL BSA).
  - Consider performing simple titration experiments with each reagent to determine optimal reaction conditions and components.

Timing/Enzyme Concentration

Assay timing and enzyme concentration can usually be optimized in one experiment. Monitor HAT reactions over time at multiple enzyme concentrations at multiple time points (**Figure 2**). Optimal timing in HAT assays is during the linear reaction phase, at times feasible for the desired throughput, instrumentation, and system stability, among other factors. Sampling too soon after reaction initiation (e.g., seconds to minutes) may be impractical in many HTS settings, and may lead to imprecise results if the reaction proceeds quickly. Later sampling times are more susceptible to system stability effects (e.g., enzyme instability). For most compounds, enzyme modulation is best apparent at earlier reaction times. For inhibitors, the continued product accumulation by uninhibited enzyme can eventually confound the final readout (40). Reaction times can range from approximately five minutes to several hours, depending on the nature of the HAT system and assay specifics. Linearity can change with subtle perturbations in reaction conditions, so it is advised to periodically reassess linearity when changing assay conditions.

Optimal enzyme concentration should produce a readout intensity sufficient for robust sampling above background. Too much enzyme, and the reaction can be completed too quickly to practically measure in the linear range. Too little enzyme, and the readout is not strong enough to reliably measure relative to background signal. For reference, high picomolar to lower nanomolar concentrations of HAT enzyme are most often reported in the literature.

For additional details, refer to Basics of Enzymatic Assays for HTS.

Substrate Concentrations

The choice of substrate concentrations is an important consideration when screening for compound modulators of enzymatic activity. The choice of substrate concentrations can favor the selection of various modes of compound modulation. For instance, screening at high substrate concentrations relative to K_M will disfavor

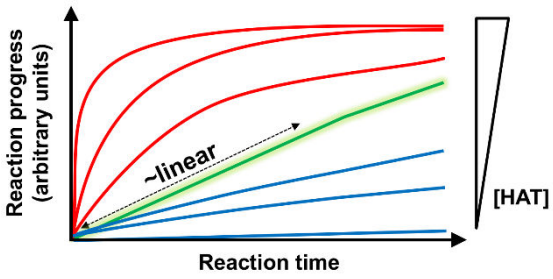


Figure 2. Optimization of enzyme concentration and timing in HAT assays. Optimal enzyme concentration and assay timing can be determined by a straightforward time-course experiment combined with enzyme titrations. In an ideal assay, HAT activity should be measured during the linear phase of the reaction. In this cartoon, the reaction progress curves in blue, performed with lower enzyme concentrations, are sufficiently linear but signal strength would be insufficient. The red progress curves, performed with higher enzyme concentrations, produce sufficient amounts of product but linearity is relatively transient. In this example, the green curve denotes an optimal enzyme concentration, as it is linear for sufficient duration to be assayed, and has a sufficient amount of product to measure. Assay timing should also consider other factors, including system stability, instrumentation, and workflow.

substrate-competitive inhibition, as competitive inhibitors will have more competition by the natural substrate for target binding to the substrate site. Sometimes, screening conditions are chosen to enrich certain mechanisms of target modulation. Most often, screens are performed around the K_M (so-called "balanced screening conditions") so as not to favor any particular mechanism. In either case, the K_M for each HAT substrate must be experimentally determined under the final assay conditions.

The bi-substrate nature of HATs requires determining the K_M of each substrate: acetyl-CoA and the histone-based substrate (**Figure 3**). While holding the concentration of acetyl-CoA constant and in excess, the reaction rates can be determined with titrations of histone-based substrate, and plotted to determine the K_M of the histone-based substrate. Next, while holding the concentration of histone-based substrate constant and in excess, the reaction rates can be determined with titrations of acetyl-CoA, and plotted to determine the K_M of acetyl-CoA.

A few technical reminders on K_M determination:

- Reaction rates should be based on initial reaction velocity (linear range).
- A general guideline for excess substrate is > 100:1 substrate:enzyme.
- A guideline for substrate titrations is between 0.2-5.0 K_M. Use at least eight substrate concentrations to determine K_M.
- Examine literature for reports on K_M to help guide initial conditions.

For additional details on optimizing substrate concentrations, refer to Basics of Enzymatic Assays for HTS.

Determining balanced screening conditions for bi-substrate reactions can be complex, especially in cases with significant substrate cooperativity (i.e., the concentration of one substrate significantly alters the binding behavior of another substrate). Cooperativity can be assessed by determining the K_M for each substrate at several different fixed concentrations of its partner substrate. In cases where significant cooperativity is suspected, additional mechanistic details about the HAT system should be characterized. For this undertaking, and for subsequently selecting optimal screening conditions, we recommend collaborating with an experienced enzymologist (40).

Quenching

For end-point assays, the HAT reaction should be quenched. This prevents additional substrate consumption and guards against time-dependent enzyme modulation. This is especially important in HTS applications, as

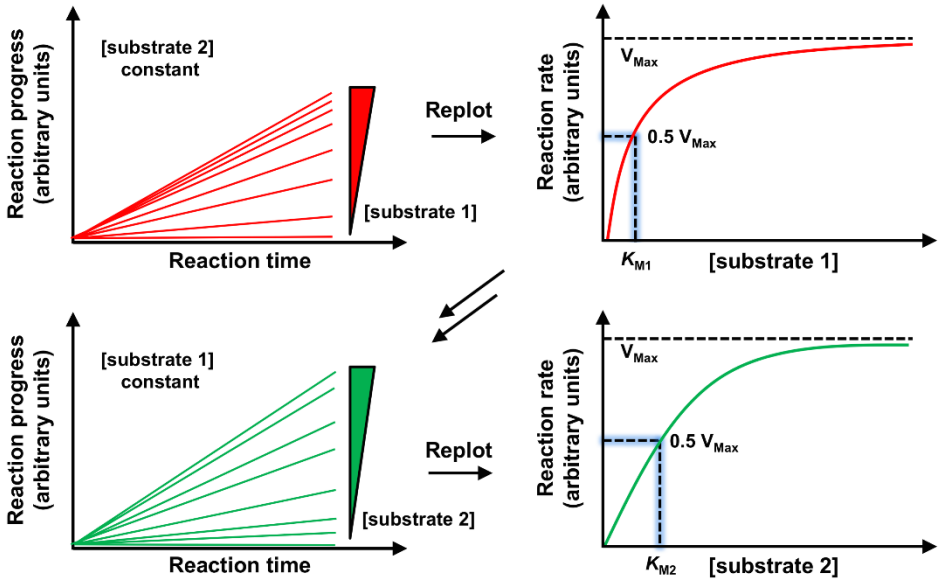


Figure 3. Optimization of substrate concentrations in HAT assays. Optimal substrate concentrations should be guided by the K_M for both HAT substrates. These can be determined experimentally. (Top) While keeping the concentration of substrate **2** constant (in excess), measure the enzymatic rates while titrating substrate **1**, and calculate K_{M1}. (Bottom) Next, while keeping concentration of substrate **1** constant (in excess), measure the enzymatic rates while titrating substrate **2**, and calculate K_{M2}.

there can be a significant time lag between reading the initial and subsequent microplates. Quenching can also have the effect of increasing assay precision by ensuring reproducible reaction and sampling times.

There are several strategies for efficiently quenching biochemical HAT assays:

- Chaotropic agents: ethanol, isopropanol, urea, guanidine HCl (often comparable in volume to reaction sample).
- Detergents such as SDS.
- Strongly basic or acidic solutions.
- Rapid freezing or heating.
- Directly spotting of the reaction solution onto filter paper.
- High concentrations of a HAT inhibitor at compound concentrations sufficient to ensure complete quenching (>> IC_{50}) (20,41).
- Hydroxylamine (21).

Consider several factors when picking quenching conditions.

- Depending on the specifics of the assay, quenching can be accomplished by adding the quencher to the reaction, or transferring the reaction mixture to a known quantity of quencher.
- With any of these approaches, verify that the HAT reaction is in fact quenched. This can be done by attempting to quench the reaction, and taking serial measurements to ensure a stable signal. Changing readouts usually indicate a non-quenched reaction.
- The type of quenching should be compatible with the assay readout. For example, certain quenching reagents may interfere with a given fluorescence readout, while general protein denaturants may prevent protein capture.

Compound Solvent

Determine the optimal percentage of DMSO (or other compound solvent) in the assay. This can be done by straightforward titration of the particular compound solvent (which is usually neat DMSO in most compound libraries). This percentage is often less than 2.5% total volume, though some assays may tolerate less organic solvent. The amount of compound solvent should be kept constant regardless of compound concentration.

Temperature

During the assay optimization process, determine the effect of temperature on the reaction system. Most biochemical HAT assays perform reactions at 30°C. This is a reasonable starting temperature point for most HAT assays. Users should experimentally determine the optimal reaction temperature that balances between convenience, reaction rate, and system stability for their particular assay application.

For HTS and even smaller scale follow-up experiments, it may be possible to conduct assays at room temperature. This convenience can circumvent the need to perform reactions in a heating oven and can reduce temperature gradient effects.

Higher temperatures can accelerate the reaction rate, which may be desirable depending on the assay timetable (this is true to a point, as too high of a temperature will eventually result in reagent or protein denaturation). Lower assay temperatures can slow the reaction rate and better stabilize the assay system, which again may be desirable for some applications.

Regardless of temperature, one should ensure assay temperature equilibration, especially if certain assay components are being kept on ice. Strategies include short pre-incubation steps and concentrated stock solutions.

Controls/Reference Compounds

It is often useful to test the effect of positive controls or reference compounds when validating an assay. Their use can help in proof-of-concept, can monitor assay performance over time, and can help compare results from different experiments.

There are many small-molecule HAT inhibitors reported in the scientific literature, many of which are sold commercially (**Table 3**) (5). Unfortunately, to-date most are insufficiently validated with respect to target selectivity in biochemical assays, and most have not been demonstrated to show specific target engagement in cells (42,43). Many of these reported compounds contain thiol-reactive moieties, while others form aggregates in common assay conditions. Both of these properties are associated with assay interference and poor target selectivity (39,44,45). Many of these compounds have the potential to interfere with certain light-based readouts, and several appear to be chemically unstable in biological buffer (36).

Using most of these small-molecules in HAT assay development should be done with full knowledge of their considerable off-target liabilities. For example, many of their reported activities may become significantly attenuated if detergents and/or reducing agents are included in the reaction buffer. Furthermore, their reactive or aggregating tendencies may interfere with some assay readouts. Until more promising HAT inhibitors are developed (especially for HATs other than p300/CBP) that demonstrate better potency and target selectivity in biochemical and cell-based assays (among other criteria), we recommend that most currently reported small-molecule HAT inhibitors be restricted to monitoring assay performance (such as intra- and inter-run precision). Their use in more complex cell-based HAT assays should be used with extreme caution or in many cases avoided, as evidence of direct, selective target engagement in cells have not been demonstrated. While many of the reported HAT inhibitors produce decreases in histone acetylation in cells at low-to-mid micromolar compound concentrations, such readouts could also be produced by well-characterized promiscuous

compounds, suggesting some observed decreases in histone acetylation may be due to a variety of nonspecific target engagements at these relatively high compound concentrations (36).

Recently, the discovery of A-485, a potent, selective indane spirooxazolidinedione inhibitor of p300 was reported by Lasko and colleagues (43). While external validation of this compound is pending, it appears promising as a useful chemical probe given strong supporting evidence for potent and selective target engagement, including: (a) low nanomolar IC_{50} values versus human p300/CBP, (b) approximately 1000-fold biochemical selectivity versus other HATs and greater than 100 unrelated biological targets, (c) biophysical evidence of target engagement (SPR, x-ray crystallography), (d) robust chemical characterization and SAR including the description of an inactive analog A-486, (e) nanomolar potencies in multiple cellular assays for histone acetylation, and (f) multiple lines of evidence consistent with target engagement in cells and *in vivo*. If using A-485 in HAT assays, we strongly encourage inclusion of its inactive analog A-486 as a negative control.

It is also worth mentioning Lys-CoA, a bi-substrate inhibitor (46). This compound, not a small-molecule *per se*, may be useful as a control compound in biochemical HAT assays involving p300. As it is cell-impermeable, this specific compound is not recommended in cell-based HAT assays, though cell-permeable analogs such as Tat-CoA have been reported (47).

Table 3. Reported HAT inhibitors. Most of the reported HAT inhibitors have structural alerts or poor physicochemical properties, and most inhibit HATs by nonspecific reactivity or aggregation. Most should be used in validating HAT assays with caution and with knowledge of their potential liabilities (36).

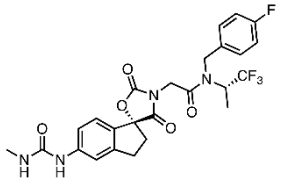
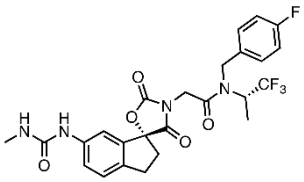
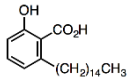
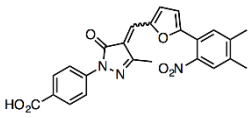
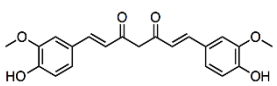
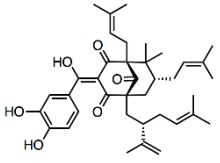
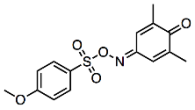
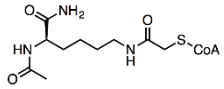
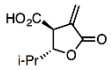
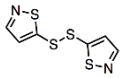
Compound	Chemical structure	Reported target, *in vitro* IC_{50} (μM)	Comments	Reference
A-485		p300-BHC, 0.010 CBP-BHC, 0.003	-External validation on-going, though promising potential chemical probe -Nanomolar cellular activities -Paired with A-486 inactive analog control	(43)
A-486		p300, > 50	External characterization on-going; use with active analog A-485	(43)
Anacardic acid		p300, 8.5	Aggregator	(41)
C646		p300, 1.6	Thiol-reactive	(48)
Curcumin		p300, 25	Thiol-reactive, aggregator, unstable	(49)
Garcinol		p300, 5	Aggregator	(50)

Table 3. continued from previous page.

Compound	Chemical structure	Reported target, *in vitro* IC_{50} (μM)	Comments	Reference
L002		p300, 1.98	Thiol-reactive	(51)
Lys-CoA		p300, 0.5	Cell-impermeable	(46)
MB-3		Gcn5, 100	Thiol-reactive	(52)
NU-9056		Tip60, 2	Thiol-reactive	(53)

Use of Orthogonal Assays/Common Interferences

Assay technologies are subject to various modes of compound-mediated interference (**Table 1**). Specific modes of interference for several assay technologies are also described in further detail in the subsequent section "BIOCHEMICAL HAT ASSAYS". It is best practice to perform at least one orthogonal assay method to help confirm actual modulation of HAT activity (11,17,54). Depending on project and test compound specifics, a second orthogonal assay may also be useful to provide additional mechanistic confidence and further de-risk interference.

In the authors' opinion, the gold standard of confirmatory biochemical HAT assays is some variation of the radiolabeled acetyl-CoA filter-binding method because: (a) it offers excellent signal:noise ratio, (b) it is a direct measurement of substrate acetylation, (c) it does not require much specialized instrumentation aside from a scintillation counter, (d) and is not prone to many of the common interference modalities seen in other assay types because test compounds are removed by filtration. While it requires specialized instrumentation, chromatographic separation coupled to mass spectrometry (i.e., LC-MS) is another high-quality HAT assay format.

There are numerous combinations of primary assays and orthogonal assays. The choice of primary and orthogonal assays will depend on a variety of factors (discussed in prior sections). In general, indirect primary assays should be paired with direct orthogonal assays, while direct primary assays can be paired with direct and/or indirect orthogonal assays (**Table 4**). Note that compound activity in both a primary and an orthogonal assay does not completely rule out compound-mediated interference. For example, many of the assays discussed in this chapter are (in principal) susceptible to light-based interferences across multiple assay formats, which can be analyzed with appropriate interference counter-screens.

HAT systems will almost inevitably show some degree of susceptibility to generalized compound-mediated assay interferences (so-called because they will often modulate biological targets regardless of technology), such as nonspecific reactivity or aggregators. Additional studies should also confirm tractable mechanisms of target modulation by test compounds, as HATs sensitive to nonspecific modes of target modulation by thiol reactivity, redox activity, chelation, or aggregation may still show activity, albeit poorly tractable, in orthogonal assays.

Table 4. Potential primary assay-orthogonal assay pairings.

Primary assay	Potential orthogonal assays	Comments
Fluorometric, enzyme-coupling (indirect)	-Filter-binding radiolabel -Antibody-based (Western, ELISA, Alpha) -Scintillation proximity -MS* -Fluidic mobility*	-Pair indirect primary assay with direct orthogonal assay -Caution when pairing with light-based orthogonal assay readouts without separation steps
Alpha technology (AlphaScreen, AlphaLISA)	-Filter-binding radiolabel -MS* -Fluidic mobility* -Scintillation proximity -Fluorometric, -Enzyme-coupling	-Pair direct primary assay with direct and/or indirect orthogonal assays -Prioritize a non-antibody-based orthogonal assay -Caution when pairing with light-based orthogonal assay readouts without separation steps
TR-FRET	-Filter-binding radiolabel -MS* -Scintillation proximity -Fluidic mobility* -Enzyme-coupling	-Pair direct primary assay with direct and/or indirect orthogonal assays -Prioritize a non-antibody-based orthogonal assay -Caution when pairing with light-based orthogonal assay readouts without separation steps
Fluidic mobility	-Filter-binding radiolabel -Scintillation proximity -MS* -Fluorometric -Enzyme-coupling	Pair direct primary assay with direct and/or indirect orthogonal assays
MS	-Filter-binding radiolabel -Scintillation proximity -Fluidic mobility*	-Pair direct primary assay with direct and/or indirect orthogonal assays -Can also monitor Ac-CoA depletion, CoA production by MS; non-MS-based orthogonal assay still recommended
Scintillation proximity	-Antibody-based (Western, ELISA, Alpha) -MS* -Fluidic mobility* -Fluorometric -Enzyme-coupling -Filter-binding radiolabel	-Pair direct primary assay with direct and/or indirect orthogonal assays -Caution when pairing with light-based orthogonal assay readouts without separation steps

* MS and fluidic mobility orthogonal assays useful to minimize light-based compound interferences, but their implementation may be limited by available instrumentation and resources.

Assay Validation

Assay validation represents a critical last step in assay development and optimization, but often overlooked or rushed. Best practices for HTS assay validation should include:

- Characterization of reagent stability.
- Characterization and correction of plate effects.
- Characterization and optimization of signal variability (e.g., intra-plate, inter-plate, day-to-day).
- Characterization of assay performance using reference compounds (e.g., intra-plate, inter-plate, day-to-day).
- Characterization of HTS performance using mini-compound libraries such as LOPAC (e.g., inter-plate, day-to-day).

Assay performance can be assessed with several statistical methods during both the validation and production phases:

- *Z'* factor (55). This calculation can monitor assay signal dynamic range and data variation on each microplate, as well as assay performance over time when examining multiple microplates.
- Minimum significant ratio (MSR) (56). In a robust assay, a given compound should have similar bioactivity across independent experiments. This calculation utilizes reference compound activity to monitor assay performance and variability. For an excellent discussion, the reader is referred to Minimum Significant Ratio – A Statistic to Assess Assay Variability.

For more detailed discussion on assay validation, the reader is referred to HTS Assay Validation and *In Vivo* Assay Guidelines.

Section Summary

Constructing and validating a robust assay capable of identifying modulators of HAT activity requires careful thought and experimental optimization of multiple, often competing parameters.

Biochemical HAT Assays

Introduction

This section describes special considerations for multiple types of biochemical HAT assays. For several assay platforms, generic HAT assay protocols are provided.

Filter-Binding Radiolabeled HAT Assays

Radiolabeled substrate HAT assays were some of the first HAT assays to be reported. Benefits include directly measuring acetylated histones, intrinsic high signal:noise ratios which can facilitate lower amounts of enzyme and substrate, absence of required protein labels, and resistance to certain forms of compound-mediated assay interference such as compound fluorescence. The main drawback is the use of radioisotopes, which necessitates additional safety disposal protocols and regulatory compliance.

The assay principle is relatively simple: acetyl-CoA substrate is labeled with radioactive tritium ([^{3}H]) or carbon ([^{14}C]) on the acetyl moiety. HATs catalyze the transfer of the labeled acetate to histone-based substrates, and the amount of radiation can be quantified by capturing the radiolabeled histone-based product and quantifying radioactivity, usually though a scintillation counter. HAT activity is therefore proportional to radioactivity (**Figure 4**).

Even though filter-based radiolabeled HAT assays are generally less susceptible to light-based forms of compound-mediated interference relative to homogenous light-based assay formats (because in filter-based assays most test compounds are removed by filtration prior to scintillation counting), orthogonal assay(s) should still be performed to rule out compound-mediated interference. For example, scintillation quenchers can still interfere with the assay readout if they are not separated from the radiolabeled analyte by filtration. Another source of potential interference are compounds that prevent the binding of acetylated product onto capture matrix.

In parallel with liquid scintillation counting, reaction aliquots can be separated by SDS-PAGE and analyzed by autoradiography to verify radiolabeled acetate incorporation onto the desired substrate.

The following is a sample protocol for a generic radioisotope-based HAT assay. Specific parameters may vary for a particular assay. Specific reagents, concentrations, volumes, dispensing procedures, and time intervals would need to be optimized for a particular application.

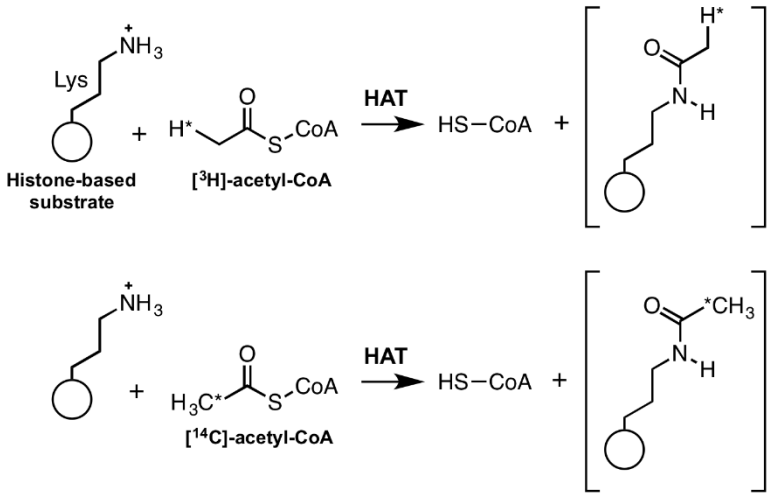


Figure 4. General schematic of cell-free radioisotope HAT assays. Radiolabeled acetyl-CoA is used to directly quantify HAT activity, either in the form of (top) [^{3}H]-acetyl-CoA or (bottom) [^{14}C]-acetyl-CoA. After the HAT reaction, histones are bound to a capture matrix (e.g., filter), and unreacted radiolabeled acetyl-CoA is removed by washing steps. The amount of radiolabeled (*) acetate incorporated onto histone-based substrates (brackets) is proportional to HAT activity.

Consumable specifications

- Microplates: standard-volume polystyrene 384-well microplates (final reaction volume = 15 µL).
- For lower-throughput applications, reactions can be performed in Eppendorf-style tubes instead of microplates.

Instrumentation specifications

- Compound dispenser: capable of dispensing in nL increments; usually acoustic droplet or pintool transfer.
  - Alternatively, serial dilutions can be made and subsequently transferred by manual pipetting. In such cases, accurate and precise compound dispensation may require higher reaction volumes.
- Fluid dispenser: capable of dispensing in 0.5 µL increments.
  - Alternatively, solution can be dispensed by serial pipetter or multichannel pipette.
- Liquid scintillation counter.

Solutions (for total reaction volume, 15 µL)

- 5X reaction buffer: 250 mM Tris, pH 8.0, 250 mM NaCl, 0.5 mM EDTA, 0.05% Triton X-100 (v/v), 250 µg/mL BSA, 5 mM DTT.
- 1X reaction buffer: 50 mM Tris, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 0.01% Triton X-100 (v/v), 50 µg/mL BSA, 1 mM DTT.
- Reaction solution: 375 nM histones (H3H4 tetramers), 15 nM HAT enzyme in 1X reaction buffer (dilute concentrated histones and HAT enzyme with appropriate volumes of H_2O and 5X reaction buffer).
- Control solution: 375 nM histones (H3H4 tetramers), 0 nM HAT enzyme in 1X reaction buffer (dilute concentrated histones with appropriate volumes of H_2O and 5X reaction buffer).
- Acetyl-CoA solution: 7.5 µM [^{3}H]-acetyl-CoA in 1X concentration (dilute concentrated enzyme stock solution with appropriate volumes of H_2O and 5X reaction buffer).
- Compounds: 10 mM DMSO stocks.

Safety and disposal considerations

- Radioactive waste should be disposed of in concordance with institutional radioactive safety protocols.

- Always wear personal protective equipment when handling radioactive material, including lab coat, gloves, and eye protection.

Sample protocol

1. Dispense compounds and vehicle controls in microplates.
    - Ensure equal organic solvent in each reaction.
    - Add 15 nL DMSO in control wells with fluid dispenser; 15 nL test compound in test wells (to assay at 10 µM final compound concentration) with compound dispenser.
2. Dispense reaction buffer into appropriate wells.
    - Add 10 µL reaction solution into test wells and positive control wells with fluid dispenser.
    - Add 10 µL control buffer into negative control wells with fluid dispenser.
3. Allow compounds to pre-incubate for 10 min at 30°C.
    - Perform incubation in temperature-controlled microplate oven.
4. Initiate HAT reaction by adding acetyl-CoA solution.
    - Add 5 µL acetyl-CoA solution into all wells with fluid dispenser.
    - Seal microplates.
5. Allow system to equilibrate.
    - Mix reaction contents with microplate shaker for 1 min.
    - Centrifuge microplates for 1 min.
6. Allow HAT reaction to proceed for 10 min at 30°C.
    - Perform incubation in temperature-controlled microplate oven.
7. Quench reaction.
    - Transfer 12.5 µL aliquot of reaction mixture into 12.5 µL neat isopropanol with pipette. Gently mix.
8. Transfer reaction aliquots onto filter paper.
    - Transfer 20 µL aliquot of quenched reaction solution from Step 7 to Whatman P-81 phosphocellulose paper. Allow to air dry completely (approximately 30 min).
9. Wash filter paper.
    - Wash filter paper disks with 50 mM $NaHCO_3$, pH 9.0 for 5 min with gentle agitation. Repeat 3 times.
    - Wash filter paper disks with acetone for 5 min with gentle agitation. Allow to air dry completely.
10. Read samples.
    - Transfer washed filter paper disks from Step 9 to 20 mL liquid scintillation vials containing 5 mL liquid scintillation cocktail and briefly vortex.
    - Read radioactivity of samples using liquid scintillation counter.
11. Analyze data.
    - Calculate the average readout for the negative controls. This value constitutes the background signal.
    - Subtract the background signal from the remaining reactions.
    - Calculate the average readout for the positive (vehicle) controls. This value constitutes the uninhibited reaction.
    - Calculate the percent HAT modulation for each reaction. Percent HAT inhibition (%) = (1 - (test solution/positive control)) x 100.

Miscellaneous notes

- Phosphocellulose filter disks are often used as a capture matrix (57).
- DTT and other reducing agents can be used in this assay.

- Labelled acetyl-CoA can often be mixed with unlabeled acetyl-CoA. This reduces the amount of total assay radioactivity, and expensive radiolabeled substrate. The exact ratio labelled:unlabelled acetyl-CoA will depend on the specific radioactivity of labelled acetyl-CoA, instrument settings, assay parameters, and amount of radioisotope transferred to capture matrix.
- Ensure scintillation counter is properly calibrated.
- Filter-binding assays are end point assays, but they can be adapted for continuous reaction monitoring by sampling reaction aliquots.
- Always keep solutions containing proteins and peptides on ice when not in use.

Additional examples/protocols: For additional examples of radioisotope-based HAT assays, we refer the reader to several studies (11,16,17,30,57).

Other Radiolabeled HAT Assay Methods

Several other radiolabeled methods have been reported for assaying HAT activity:

Scintillation proximity assays (SPA) have been applied to HATs (26,43,58). SPA is a solid-phase homogenous technique that couples a scintillation matrix (beads; coated plate, "FlashPlate") to a capture system (e.g., anti-histone antibody, or biotin-tagged histones and streptavidin-coated beads/plates). Histone-based products can be captured in close proximity to the matrix surface. In certain cases, histone-based products can also be immobilized to the matrix (i.e., microplate) without special tags through nonspecific adsorption. When a radiolabeled product is in close proximity to the scintillator, the signal from radioactive decay of the radioisotope is amplified. Unbound radiolabeled acetyl-CoA in solution is not in close enough proximity to the scintillation matrix, and therefore does not generate signal. An advantage of SPA is that it does not require a separation event such as filter-binding. Consequently, SPA is an attractive assay technique when the detection sensitivity of radiolabels is needed in a high-throughput setting. Development and optimization of SPA-based HAT assays utilizes the same general assay development principles described in this chapter. For additional information on SPA assay development, several reviews may be useful (24,59).

Some SPA-specific comments include:

- Reducing agents such as DTT should not interfere with SPA-based technologies.
- A potential source of compound-mediated interference in SPA are compounds that disrupt scintillation (24). For example, such inner-filter effects can be quite significant in certain scintillation proximity assays where photon emission occurs in the blue region (which can by quenched by yellow-colored compounds) (25). Orthogonal assays, including filter-binding assays that remove interfering compounds, can help identify this type of assay interference.
- Excessive radiolabels can lead to high background signals, causing nonproximity effects. This can be countered by reducing the concentration of radiolabels, or aspirating reaction solution before measuring.
- Nonspecific protein binding to either beads or surfaces can also cause high background. This effect can often be mitigated with blocking agents, change of matrix, or detergent.
- Interactions between anionic acetyl-CoA and cationic histone interactions can also lead to nonspecific background signal, though these can be disrupted with appropriate selection of quenching agent to disrupt this interaction (e.g., guanidine HCl) (58). This effect can be identified by high background signal in the presence of acetyl-CoA and histone-based substrates and the absence of HAT enzyme.
- High-throughput plate-based SPA requires specialized instrumentation, such as PMT-based microplate-compatible scintillation counters or CCD-based readers (25).

Autoradiography of HAT reactions is well-described in the literature (60). Autoradiography methods are typically employed for lower-throughput confirmatory applications. If reaction aliquots are separated by gel chromatography, they can be useful for confirming acetylation of specific substrates in multi-protein reaction

mixtures (61). Reactions can also simply be applied to a capture matrix (e.g., filter paper, nitrocellulose membrane) and assayed for total radioactivity (60,62). Radiation can be quantified by radiodensitometry. The main drawback is lower-throughput. For example, gel separation requires a separate gel lane for each reaction. Furthermore, signal acquisition depends on the specific radioisotope and the amount of radioisotope per reaction. Development can require hours to days of product exposure to film.

Antibody-Based HAT Assays

Another common class of HAT assays probes for histone acetylation using antibodies targeting either specific acetylated histone residues (e.g., H3K27ac) or nonspecific histone acetylation (e.g., H3ac). These assays directly probe for the acetylated histone-based product. Antibody-based assays are versatile and come in several varieties, including Western blot, slot blot (a modified Western blot), and ELISA. For examples of immunoassays, we refer the reader to several studies (10,11,17).

For details on developing immunoassays, we refer readers to Immunoassay Methods. Some considerations for antibody-based HAT assays include:

- Certain chemotypes, such as acetimides, have been reported to interfere with acetylated histone antibodies (19). Useful counter-screens include a second, independent antibody, as well as orthogonal assays.
- Immunoassays are limited by the quality and specificity characteristics of the antibody. Verify antibody performance. Antibodies designed for specific acetylated histones have potential for significant cross-reactivity (63,64).

Fluorometric HAT Assays

Cell-free fluorometric HAT assays are relatively inexpensive, are straightforward to establish, and require only standard HTS instrumentation. The assay principle is relatively simple: free CoA produced by the HAT reaction reacts with a sulfhydryl-scavenging probe such as *N*-[4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl]maleimide (CPM), to form a highly fluorescent CoA-CPM adduct quantified by fluorescence intensity (**Figure 5**).

The actual acetylated histone product is not directly measured in this assay platform. Therefore, a significant drawback to this method is the significant burden of compound-mediated assay interference (17). The method also has a lower signal:noise ratio compared to radioisotopic methods. In principle, the detection of the CoA reaction product can serve as a convenient orthogonal assay to direct HAT assays. The decision to utilize this assay format, and whether to implement as a primary screen or orthogonal assay, will depend on multiple factors including: available resources, the availabilities of other orthogonal assays, the desired throughput and timelines at each project stage, and the expected test compound chemotypes. For instance, if many of the test compounds are colored or contain potential reactive groups, this assay format may not be an ideal choice due to the high likelihood of readout interference.

If choosing this method for assaying HAT activity, assay-specific counter-screens should be included to characterize any compound-mediated fluorescence interference (both quenching and auto-fluorescence) and CoA-scavenging reactions by test compounds. For details on performing these counter-screens, we refer the reader to several worked-out examples (14,16,17).

Furthermore, at least one orthogonal assay that directly quantifies the protein product (acetylated histone) is all but required when utilizing this assay method.

The following is a sample protocol for a generic CPM-based HAT assay. Specific parameters may vary for a particular assay. Specific reagents, concentrations, volumes, dispensing procedures, and time intervals would need to be optimized for a particular application.

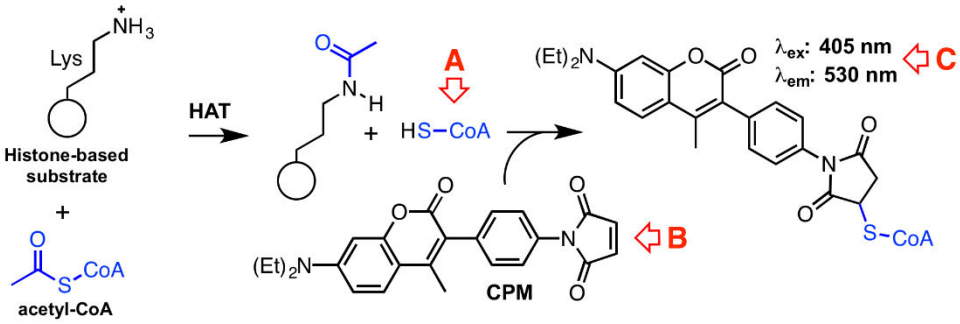


Figure 5. General schematic of cell-free fluorometric HAT assays. HATs produce acetylated histone-based products and free CoA (CoA-SH). After quenching the reaction, HAT activity is assayed by adding a sulfhydryl-scavenging probe, CPM, which reacts with CoA to produce highly fluorescent adducts. In absence of interference, the amount of fluorescence is proportional to HAT activity. There are three main sources of compound-mediated readout interference: (A), thiol-scavenging of CoA-SH by electrophilic test compounds; (B), formation of compound-CPM adduct by nucleophilic test compounds; and (C), fluorescence interference by test compounds (auto-fluorescence, quenching, inner-filter effects, light scattering).

Consumable specifications

- Microplates: low-volume black polystyrene 384-well microplates (final reaction volume = 20 µL)
- Note: this assay can be miniaturized to 1536-well format with reaction volumes less than 5 µL (11).

Instrumentation specifications

- Compound dispenser: capable of dispensing in nL increments; usually acoustic droplet or pintool transfer.
  - Alternatively, serial dilutions can be made and subsequently transferred by manual pipetting. In such cases, accurate and precise compound dispensation may require higher reaction volumes.
- Fluid dispenser: capable of dispensing in 0.5 µL increments.
  - Alternatively, solution can be dispensed by serial pipetter or multichannel pipette.
- Microplate reader: capable of reading fluorescence intensity.

Solutions (for total reaction volume, 15 µL)

- 5X reaction buffer: 250 mM Tris, pH 8.0, 250 mM NaCl, 0.5 mM EDTA, 0.05% Triton X-100 (v/v), 250 µg/mL thiol-deactivated BSA.
- 1X reaction buffer: 50 mM Tris, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 0.01% Triton X-100 (v/v), 50 µg/mL thiol-deactivated BSA.
- Substrate solution: 150 µM histone peptide, 30 µM acetyl-CoA in 1X reaction buffer (dilute concentrated peptide and acetyl-CoA stock solutions with appropriate volumes of H_2O and 5X reaction buffer).
- Enzyme solution: 60 nM HAT enzyme; add appropriate amounts of 5X reaction buffer in 1X concentration (dilute concentrated enzyme stock solution with appropriate volumes of H_2O and 5X reaction buffer)
- Quenching/probe solution (concentrated): 80 µM CPM in 1:1 H_2O:EtOH, 1% DMSO solution.
- Test compounds: 10 mM DMSO stocks.

Sample protocol

1. Dispense compounds and vehicle controls in microplates.
   - Ensure equal organic solvent in each reaction.
   - Add 15 nL DMSO in control wells with fluid dispenser; 15 nL test compound in test wells (to assay at 10 µM final compound concentration) with compound dispenser.
2. Dispense reaction buffer into appropriate wells.

    - Add 5 µL reaction buffer (1X) into control wells, 2.5 µL reaction buffer into test wells with fluid dispenser.
3. Dispense concentrated enzyme solution into appropriate wells.
    - Add 2.5 µL enzyme in reaction buffer into test wells with fluid dispenser.
4. Allow compounds to pre-incubate for 5-10 min at 30°C.
    - Perform incubation in temperature-controlled microplate oven.
5. Initiate HAT reaction by adding concentrated acetyl-CoA solution.
    - 10 µL substrate solution in reaction buffer into all wells with fluid dispenser.
    - Seal microplates.
6. Allow system to equilibrate.
    - Mix reaction contents with microplate shaker for 1 min.
    - Centrifuge microplates for 1 min.
7. Allow HAT reaction to proceed for 1 h at 30°C.
    - Perform incubation in temperature-controlled microplate oven.
8. Quench reaction and initiate CoA-CPM adduct formation by adding quenching/probe solution.
    - Add 5 µL quenching/probe solution into all wells with fluid dispenser.
9. Allow thiol-probe reaction to proceed for 15 min at room temperature
    - Mix reaction contents with microplate shaker for 1 min.
    - Centrifuge microplates for 1 min.
10. Read fluorescence intensity of microplate wells.
    - Settings: excitation 405 nm; emission 535 nm.
11. Analyze data.
    - Calculate the average readout for the negative controls. This value constitutes the background signal.
    - Subtract the background signal from the remaining reactions.
    - Calculate the average readout for the positive (vehicle) controls. This value constitutes the uninhibited reaction.
    - Calculate the percent HAT modulation for each reaction. Percent HAT inhibition (%) = (1 - (test solution/positive control)) x 100.

Miscellaneous notes

- DTT and other reducing agents are omitted from this assay, as they will react with the thiol-scavenging probe. This includes TCEP, whose phosphine lone pair of electrons can also react with maleimides (65).
- Besides CPM, there are several other maleimide-based fluorogenic probes available. We refer the reader to an excellent comparative study of such probes (12).
- Note some HAT enzymes may also contain free cysteine residues that may contribute to background.
- BSA can be highly useful for stabilizing HAT reactions, especially in the prolonged reaction times often necessitated in large-scale HTS settings. As BSA contains multiple cysteine residues (greater than 30 in mature BSA), free thiols on BSA must be deactivated ("capped") to prevent interference with sulfhydryl-scavenging probes. A facile workaround involves treating BSA with excess N-ethylmaleimide (NEM) (taking into account BSA contains multiple cysteine residues), and multiple rounds of dialysis to remove excess, unreacted NEM (11,13). Ensure thiol inactivation by performing a test reaction with CPM and measuring the fluorescence intensity under assay-like conditions.
- Substrates may contain free cysteine residues. To reduce background from probe-peptide/protein adducts, it may be necessary to replace cysteine residues with alternative residues (e.g., peptide synthesis or site-directed mutagenesis for recombinant proteins), or alternatively treated with NEM or similar reagent.
- CPM and other probes can usually be prepared as 10 or 50 mM stock solutions in DMSO. Store in -20°C, protect from light.

- Protect probe solutions from light. Prepare working probe solutions fresh to prevent degradation/non-specific hydrolysis in alkaline, aqueous conditions (66).
- The reaction between free CoA and maleimide probes is relatively fast, usually on the order of minutes (12). However, during assay optimization verify the reaction is complete by monitoring fluorescence intensity over time. Look for a signal plateau to signify reaction completion. Continued increases in fluorescence intensity may indicate inadequate mixing, insufficient reaction quenching, or CPM-protein adduct formation.
- Ensure microplate reader is properly calibrated.
- Fluorometric assays are end point assays given the ability of thiol-scavenging probes to modulate HAT activity. Like filter-binding assays, they can be adapted for continuous reaction monitoring by sampling reaction aliquots.
- Always prepare solutions containing proteins and peptides on ice. Keep on ice when not in use.
- An advantage of this assay is that the protein product may also be assayed by an orthogonal method such as Western blot which may be useful for examining compound-mediated assay interference (10,17).

Additional examples/protocols: For additional examples of fluorometric HAT assays, we refer the reader to several studies (10-14).

Microfluidic-Based HAT Assays

The development of a microfluidic mobility shift platform for cell-free, fluorescence-based HAT assays provides a useful approach for both HTS and mechanistic analysis of HAT modulators (21,22,67). The assay principle is straightforward: fluorescent HAT substrates (peptides of histones H3 and H4 tails) are acetylated by a given HAT system, altering the charge-to-mass ratio on the peptide. Subsequently, acetylated peptides are separated from non-acetylated peptides *via* electrophoretic separation, enabling distinguishable fluorescent detection of each peptide. The ratiometric measurement of substrate/product fluorescence peak heights gives the percent conversion that can be directly related to HAT enzyme activity once correcting for non-enzymatic acetylation (**Figure 6**).

An advantage of this assay platform is its versatility in facilitating compound discovery by HTS and also compound mechanism of action studies. A hydroxylamine-based reaction quench allows for uniform HAT assay conditions amenable to HTS of large compound libraries in 384-well plates. Small sample aliquots and relatively fast electrophoretic separations allow for multiple end-point measurements of a single reaction. Therefore, when the hydroxylamine-based quench is not used, consecutive measurements of HAT activity can be obtained to determine time-dependent HAT modulation. An additional advantage of this assay platform is the direct measurement of the acetylated product. Therefore, this method can be an effective counter screen to triage hits from high-throughput primary screens, such as the CPM assay, that may yield several compound-mediated assay interference molecules. Lastly, the microfluidic aspect of this assay allows for following enzymatic reactions in small reaction volumes with a minimum amount of sample, increasing its feasible applications from an economic perspective.

As this assay technique directly measures fluorescently-labeled, electrophoretic-separated acetylated histone products, the assay readout is not subject to certain types of compound-mediated interference. For instance, light-based interference compounds are usually separated by the electrophoresis. However, like the other methods described in this chapter, the enzymatic system itself will be susceptible to nonspecific sources of bioassay interference, such as redox cycling compounds, aggregators, and nonspecific reactivity. The magnitude is dependent on the experimental conditions (target, concentrations, detergent, scavenging reagents, etc.).

A critical factor in experimental design is substrate choice. Users should verify that the acetylated and non-acetylated histone products can be adequately resolved. This is often achievable for peptide-based histone substrates, but may be considerably more difficult for larger (> 30-mer), multiply-charged histone-based

substrates, as electrophoretic separation is based upon mass/charge ratios. The net charge ideal for commercial microfluidic capillary electrophoresis instruments is from +3 to -3, and the substrate histone peptides used for HAT assays typically have net charges ranging from +3 (for the H4 peptide) and +4 (for the H3 peptide). Therefore, this system presents challenges for HAT assays requiring larger histone-based substrates (e.g., full-length histones, chaperones). The choice of fluorophore for microfluidic assays will depend on the light source and detector, as well as the spectral properties and other behaviors of the labelled analyte in the assay. Common fluorophores for the EZ-Reader include FITC, 5-FAM, and Alexa 488.

The following is a sample protocol for a generic microfluidic mobility shift HAT assay. Specific reagents, concentrations, volumes, dispensing procedures, and time intervals should be optimized for a particular application.

Consumable specifications

- 384-well microplates from E&K Scientific (EK2253): 58 µL well capacity volume with a U-bottom, clear, natural polypropylene (final reaction volume = 30 µL).
- PerkinElmer ProfilerPro^{TM} Separation Buffer (Cat# 760367): Prior to the first use of the separation buffer, add 2 mL of coating reagent 8 (CR8) that comes with the purchase of the separation buffer. CR8 is a positively-charged additive for coating the Lab-Chip sipper channels. Store separation buffer at 4°C but warm to room temperature before use.

Instrumentation specifications

- PerkinElmer Lab-Chip EZ-Reader instrument (obtains fluorescence measurements following microfluidic electrophoresis).
    - Suggested initial electrophoresis separation conditions: downstream voltage of -500 V, upstream voltage of -2500 V, and a pressure of -1.5 psi for both FITC H3/H4 peptides
- Lab-Chip EZ Reader 12-sipper chip: capable of sipping as little as 10 nL reaction mixture while simultaneously monitoring 12 reactions (proper treatment and troubleshooting points are listed under *Miscellaneous notes*).

Substrate peptide sequences

- FITC-H4 (3-14, FITC-Ahx-RGKGGKGLGKGG [Ahx = 6-amino-hexanoic acid])
- FITC-H3 (5-20, FITC-Ahx-TARKSTGGKAPRKQL)
- These peptides were synthesized by automated peptide synthesis on Rink amide resin, using Fmoc-based chemistry. Peptides can be synthesized in-house with appropriate instrumentation. Alternatively, custom peptides can be synthesized by commercial vendors.

Solutions (for total reaction volume, 30 µL)

- 10X reaction buffer: 500 mM HEPES, pH 7.5, 500 mM NaCl, 20 mM EDTA.
- Miscellaneous assay components (prepare new each experiment): 0.5% Triton X-100 (v/v) in PBS (10X), 20 mM DTT in PBS (10X), 0.5 M hydroxylamine in PBS (for reaction quench).
- Master mix solution: 1X reaction buffer, 0.05% Triton X-100 (v/v), 2 mM DTT, 2 µM FITC-H3/H4 peptide, 150 nM p300.
- Quenching solution: 0.5 M hydroxylamine in PBS, pH 7.5.
- Test compounds: 10 mM DMSO stocks.

Sample protocol

1. Dispense 0.5 µL of compounds (dissolved in DMSO) and vehicle controls in screening microplates.

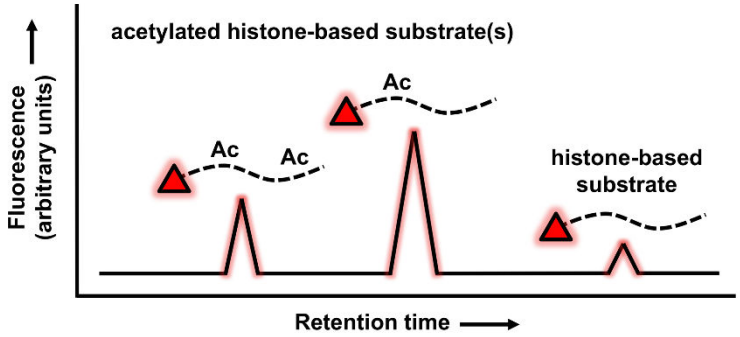


Figure 6. General schematic of the microfluidic mobility shift HAT assay. Acetyl-CoA and fluorescent-labeled histone peptides are the HAT substrates. Reaction aliquots are analyzed by microfluidic electrophoresis. Lysine acetylation alters the retention time of the histone peptide *via* decreasing product net charge and increasing product mass. HAT activity is proportional to the amount of acetylated product produced.

   a. When screening large compound libraries for inhibitor discovery, include samples having no enzyme as this will represent the percentage of non-enzymatic acetylation.
   b. DMSO percentage for the assay is 1.7%. Higher DMSO amounts will decrease enzyme activity.
2. Dispense 26.5 µL master mix into appropriate wells.
3. Allow compounds to pre-incubate with master mix solution for 10 min at room temperature.
4. Initiate HAT reaction by adding 3.0 µL of 10 µM acetyl-CoA (total reaction volume – 30 µL).
5. Allow HAT reaction to proceed for 10-15 min at room temperature.
   a. When performing an end-point analysis, keep product accumulation between 10-15%.
   b. For kinetic measurements, transfer the microplate to the PerkinElmer Lab-Chip EZ-Reader immediately following the addition of acetyl-CoA to begin obtaining fluorescence measurements.
6. If quenching the reaction, add 5 µL of 0.5 M hydroxylamine to each well.
7. Read fluorescence intensity of microplate wells.
   a Light source: LED.
8. Analyze data.
   a. Calculate averages and standard deviations of percent conversion for negative (no inhibitor) controls and positive (vehicle) controls.
   b. Calculate averages and standard deviations of percent conversion for inhibitor samples.
   c. Calculate the HAT percent activity: normalize the percent conversions by setting the negative controls to 0% and the positive control to 100% conversion.

Miscellaneous notes

- The separation resolution (SR) is calculated using the formula $SR = \Delta L/2(\sigma 1 + \sigma 2)$, where ΔL, $\sigma 1$, and $\sigma 2$ are the distance between peaks, standard deviation of the first peak, and standard deviation of the second peak, respectively.
- Protect fluorescent peptide solutions from light to prevent photo-catalyzed degradation.
- Always prepare solutions containing proteins and peptides on ice. Keep on ice when not in use.
- Prior to use in an experiment, proper treatment for the Lab-Chip EZ Reader 12-sipper chip includes:
  - Washing entire chip with distilled water thoroughly, followed by a complete drying *via* aspiration.
  - Washing the chip wells using separation buffer (2 x 250 µL), followed by filling the smaller wells with 250 µL and the two larger wells with 500 µL of separation buffer.
- Following completion of an experiment, proper treatment for the Lab-Chip EZ Reader 12-sipper chip and EZ Reader instrument includes:

    - Filling 12 wells within a screening microplate with 5% DMSO in water and running the Lab-Chip EZ reader instrument at 15 cycles with a final delay time of 300 s. while running at a pressure of -4.0 psi. This will rinse the chip sipper channels with the 5% DMSO followed by a 300 s rinse with separation buffer.
    - For the EZ Reader instrument, switch out separation buffer for distilled water and wash the lines for 10 min prior to either starting a new experiment or shutting the instrument down. Carryover may occur from one experiment to another if lines are not washed.
- Due to evaporation of reaction solution, the maximum kinetic runtime of a single experiment is 40 h when screening microplates utilizing 500 µL reactions. When less than 500 µL is used, wells will dry out quicker and runtimes should be reduced. Additionally, chip wells should be refreshed by removing old separation buffer and adding fresh separation buffer every 12 h.
- It is important to be vigilant for declining chip performance, which can occur due to blockage of a sipper, presence of air bubbles, or standard wear and tear. Signs a chip may need to be refreshed or retired include a lack of reproducibility among replicate samples, broadening of peak shape, or delayed elution of peptide when compared to a properly functioning chip.
- In cases when a chip shows signs of failing (potentially due to sipper blockage or heavy usage), we have found the following protocol can be useful to refresh and restore chip performance:
    - Run the Lab-Chip EZ-Reader instrument at a positive pressure of 4.0 psi for 1 h with no microplate loaded. This can aid the clearance of any blockages from the channel. Follow this with an additional wash step, by filling 12 wells within a microplate with 5% DMSO in water and running the EZ-Reader instrument at 50 cycles with a final delay time of 300 s and pressure of -4.0 psi. This wash should be performed for approximately 1 h.
    - Purchase a fresh bottle of separation buffer. Since reaction solutions are circulated through a bottle of separation buffer, poorly performing chip sipper channels can often be resolved by simply using a fresh bottle of separation buffer.

MS-Based HAT Assays

Mass spectrometry (MS) is an analytical technique that ionizes analytes and separates them based on their mass-to-charge ratios (m/z). Components of HAT reactions can be ionized through one of several technologies (e.g., electrospray, MALDI), then passed through an electric field where the ionized analytes are measured based on their m/z ratios. Prior to ionization, HAT reaction components can be separated by liquid chromatography and/or subjected to various extraction/enrichment strategies such as solid-phase extraction (SPE).

MS-based detection allows for the monitoring of multiple analytes in a single injection, which facilitates the detection of substrate, product, an internal standard (IS), and/or an orthogonal reaction. For instance, the net loss of histone lysine methylation catalyzed by an α-ketoglutarate-dependent recombinant demethylase can be simultaneously quantified by monitoring the production of modified histone and also succinate, with the conversion of α-ketoglutarate to succinate being an orthogonal reaction **(Figure 7)**. Similarly, in addition to monitoring histone-based substrates and products themselves, the production of histone acetylation states can also be validated by monitoring the deacetylation of acetyl-CoA and the production of free CoA (23). Measuring orthogonal reaction products can enable real-time identification of signal enhancement or suppression.

As a representative example of available HTS-compatible MS technology, the Agilent RapidFire (RF) MS system is a micro-SPE system enabling rapid sample analysis (7-10 s/sample) from 96- and 384-well assay plates. Aspirated sample is loaded onto a micro-SPE cartridge, washed (to remove MS-incompatible components such as salts), and immediately reverse-eluted to the mass spectrometer. The RF-MS system is amenable to wide range of SPE packing materials (e.g., C4, C18, HILIC). The SPE packing method is chosen based on the desired levels of chromatographic separation and sample clean-up, and analyte properties (e.g., polarity, solubility). Combined with the ability of pairing various mass spectrometers such as QQQ (triple quadrupole), ToF (Time of Flight),

and QToF (quadrupole ToF), RapidFire analysis can offer a versatile system capable of measuring HAT-relevant analytes. Note specific instrumentation and monitoring conditions (e.g., multiple reaction monitoring with triple quadrupoles, full-scan monitoring with ToF) will depend on multiple factors, including analyte, throughput, and mass accuracy specifications.

When performing a HTS run for small molecules or biological samples, minimizing cross contamination and run times is critical. Including blank injection(s) after each sample injection reduces cross contamination due to sample carryover, although this increases run times by at least two-fold. Hence, it is vital to ensure complete elution of the sample from the SPE cartridge by altering the RF method (flow and/or duration during each step) or by using appropriate mobile phases. The optimal solvent system will depend on the specific analytes being tested. Factors to consider include the solubility, chemical stability, and chromatographic properties of the specific analyte(s) in a given solvent system.

Compound-mediated assay interference in MS-based assays can manifest as ion suppression, but this phenomena is usually mitigated through sample purification, sample chromatography, the use of internal standards, and the aforementioned monitoring of orthogonal reactions (68). Chemical modification or adduct formation of the analyte by test compounds can also interfere with accurate analyte measurement by perturbing (a) analyte chromatographic and extraction behavior, or (b) measurement of parent ions due to mass shifts, especially when utilizing single or multiple reaction monitoring (69,70).

For additional details on MS-based assay development and optimization, refer to HPLC-MS/MS for hit generation.

The following is a sample protocol for a generic MS HAT assay. Specific reagents, concentrations, volumes, dispensing procedures, and time intervals should be optimized for a particular application.

Sample protocol

1. Dispense 1-2 µL enzyme mix (2X) into microplate using a liquid handling system such as Bioraptr. Centrifuge microplate briefly at 1000 rpm.
2. Dispense test compounds using a 384-head pin-tool or an acoustic dispenser
3. Dispense 1-2 µL substrate mix (2X) using a liquid handling system to initiate the reaction and centrifuge the plate at 1000 rpm for 1 min.
4. Incubate for desired time duration.
5. Add stop solution to quench the reaction using a large volume liquid dispenser; final volume should be at least 50 µL/well.
6. Briefly centrifuge and heat seal the plate(s) prior to RF-MS analysis. (Note: avoid using an adhesive sealer since the adhesive will clog the sipper tube in RF as well as leach on to the mass spectrometer, causing loss of signal).

In a representative RF method for a ~20-residue histone peptide, sample can be loaded on a C4 cartridge in 0.6 s, washed for 3 s with 100% dH_2O containing 0.1% (v/v) formic acid, eluted for 3 s with 80% acetonitrile containing 0.1% formic acid, and re-equilibrated for 5 s. The system can be programmed to perform a blank injection to minimize cross-contamination of subsequent samples.

Data processing

The Agilent RF mass spectrometry system is closely integrated with a proprietary data analysis software package (MassHunter Qualitative and Quantative Analysis) for measuring area under the curve (AUC) for each peak as well as converting the large data files into data for individual wells. Alternatively, RapidFire Integrator (Agilent) can be used for processing data from various mass spectrometer platforms (such as Sciex, Waters, and Agilent). These data can be processed further to calculate the amount of product (**Equation 1**).

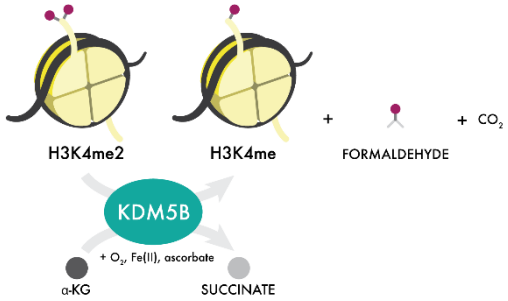


Figure 7. Measuring histone modifications by MS. Shown is a biochemical reaction demonstrating the KDM5B-catalyzed demethylation of H3K4 and the concurrent production of succinate. Similarly, HAT reactions can be monitored by measuring the production of acetylated histone products as well as the concurrent production of CoA/loss of acetyl-CoA (see **Figure 1**).

Equation 1. $\%\ \text{Product conversion} = \dfrac{\text{Product}_{\text{AUC}}}{\text{Product}_{\text{AUC}} + \text{Substrate}_{\text{AUC}}}$

Miscellaneous notes

- Due to the technical requirements of MS (e.g., instrumentation, chromatography/separation, troubleshooting), one may wish to consult with experienced mass spectrometrists when designing and optimizing an MS-based assay.
- The high sensitivity of many MS methods requires less product formation for detection relative to other technologies, potentially decreasing the amounts of required enzyme and substrate to complete a screening campaign.
- Polymeric detergents (e.g., Tween-20, Tween-80, Triton X-100, NP-40) are generally incompatible with MS assays, as they can broadly elude and saturate the assay signal. Monomeric detergents (e.g., CHAPS, octyl β-D-glucopyranoside, dodecyl β-D-glucopyranoside) are preferred, but can still interfere with MS assays.
- Multiple histone-based substrates have been reported for MS-based assays, including peptides and whole histone proteins (23).
- Like other detection methods, it is imperative to determine the limits of detection (LoD) and limits of quantitation (LoQ) for all analytes of interest, in presence and absence of IS. Determining the LoD and LoQ prevents detector saturation as well as determine the concentration of sample to be injected.
- Unlike LC-MS, RF-MS does not involve an elution gradient or prolonged retention times, which allows for cross contamination and carryover of samples. It is critical to establish optimal chromatography conditions for the analyte(s) of interest – appropriate SPE cartridge, alterations of load, wash and/or elute steps of RF method, along with the mass spectrometer detection method (positive or negative mode). If these manipulations fail to reduce carryover, it is advisable to incorporate blank injection(s) after each sample.
- Inclusion of an isotopically-labeled analyte (in stop solution) as an internal standard should also be considered to identify cases of incomplete sample injection, ion suppression, and matrix effects.
- Higher-throughput RF-MS can be achieved by minimizing the sample retention time on the system. This requires tubing with extremely small internal diameter combined with robust injection valves and LC-pumps. Thus, monitoring pump pressures facilitates the identification of clogs (if any), which might occur during longer runs using biological samples.
- Use of reverse-phase chromatography involving C4 or C18 cartridges requires sample preparation in 100% aqueous phase to prevent sample precipitation. In contrast, samples should be prepared in high organic (90 – 95% acetonitrile) when using HILIC cartridges (*see HPLC-MS/MS for hit generation for more details*).

- Arguably, RF-MS has lower throughput in comparison to a 1536-well biochemical assay read on a ViewLux. Multiplexing samples involving analytes that do not interfere in detection increases RF-MS throughput (71).

Non-Radiolabeled Proximity HAT Assays

There are several non-radioisotope, proximity-based technologies available to assay HAT activity, such as AlphaScreen (Amplified Luminescent Proximity Homogeneous Assay; **Figure 8**) and FRET (Fluorescent/Förster Resonance Electronic Transfer; **Figure 9**). Each of these technologies involves the excitation of a donor system by a light source, followed by signal transmission to an acceptor system that requires proximity for efficient transmission. The acceptor system, upon excitation by the donor system, then emits a signal which can be quantified. Alpha-based technologies (AlphaScreen, AlphaLISA) utilize singlet oxygen transfer, while FRET utilizes fluorescence resonance electronic transfer. Note that AlphaScreen acceptor beads contain a thioxene/anthracene/rubrene mixture, while AlphaLISA acceptor beads contain a thioxene/europium chelate mixture. The latter configuration creates a narrower emission band that is optimized for certain biological matrices. Time-resolved FRET (TR-FRET) adaptations using lanthanide fluorophore donors are common, and can enhance signal-to-noise by coupling an excitation pulse with delayed acquisition to allow for background fluorescence decay. For a well-designed example of a TR-FRET and AlphaLISA assay versus p300, we refer readers to a recent report by Lasko and colleagues (43).

Alpha- and FRET-based HAT assays share many advantages. Compared to conventional ELISA and Western blots, Alpha and FRET technologies are homogenous assays. This eliminates many assay steps such as washing, reducing assay time and simplifying the assay workflow. Both Alpha and FRET technologies require separate capture systems for the donor and acceptor components. Typically, this involves a combination of capture and affinity tags, and an acetylated histone recognition system (i.e., antibody or bromodomain). Both technologies are versatile, and allow for extensive customization of tags, substrates, and labeling strategies. For either technology, many reagent combinations are readily commercially available (so-called "off-the-shelf" assays), or are amenable to customization by relatively straightforward techniques or commercial services.

Alpha-based HAT assays have several potential advantages, including high sensitivity, low background, good dynamic range, and miniaturization (to 1536-well plates) (72). Additionally, singlet oxygen transfer can generally occur over longer distances compared to FRET. However, a disadvantage of Alpha technology can be reagent and instrumentation costs as well as light sensitivity. By contrast, TR-FRET assays may be less sensitive to DMSO (73). Both assay technologies are susceptible to specific modes of compound-mediated interference, including disruption of capture reagent systems and signal quenching (74,75).

Miscellaneous notes

- Optimization of the capture and affinity tags is advised for either technology. This includes experimenting with different tag combinations (e.g., 6xHis-Nickel, streptavidin-biotin, GST-glutathione) as well as the location of each tag on histone-based substrate.
- These technologies are dependent on the quality of the histone recognition motif. Characterize antibody specificity and optimize antibody titers. Specificity can also be assessed by titrating purified histone-based products.
- There have been reports of compounds interfering with antibody recognition of acetylated histone substrate (19). Consider a follow-up counter-screen with a second, independent antibody.
- As with fluorescence-based assays, avoid background light contamination when acquiring FRET and Alpha readouts.
- Perform titration experiments with the various assay components. Depending on the assay format and relative concentrations of each assay component, "hook effects" may be present (76). This can occur when a capture system is saturated by excess tagged substrate/product leading to an apparent signal plateau,

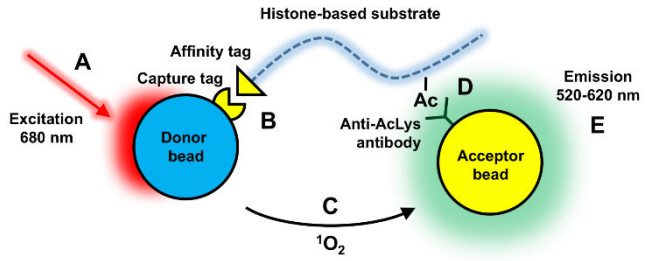


Figure 8. Example of an AlphaScreen-based HAT assay. A donor bead with a capture tag binds to a histone-based substrate with an affinity tag. An acceptor bead with an anti-acetyl lysine antibody binds to acetylated lysine on the histone-based substrate. Phthalocyanine from the donor bead is excited by a high-wavelength laser, which produces singlet oxygen ($^1O_2$). If in close proximity to the donor bead, thioxene, anthracene, and rubrene within the acceptor bead are excited by singlet oxygen to produce an emission at a lower wavelength. In the absence of interference, HAT activity is proportional to the emission intensity at this lower wavelength. There are five main potential areas of compound-mediated readout interference: (A) interference of excitation light source by test compound (quenching/absorbance/light-scattering); (B) disruption of donor bead-substrate interaction by test compound; (C) disruption of singlet oxygen transmission by test compound (quenching, chemical reaction); (D) disruption of acceptor fluorophore construct-product interaction by test compound; and (E) acceptor bead emission interference by test compounds (auto-fluorescence, quenching/absorbance/light-scattering).

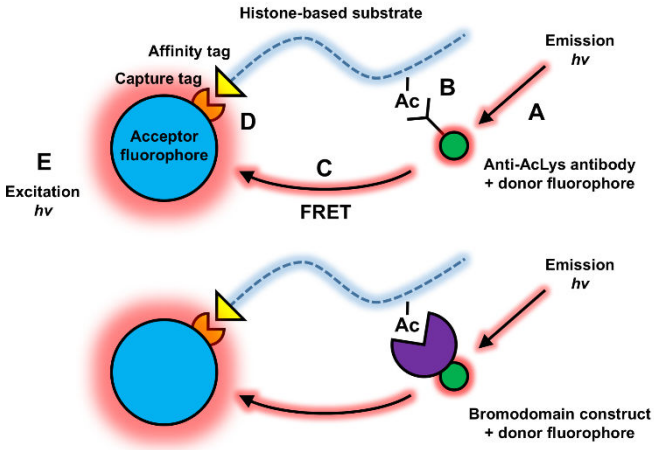


Figure 9. General schematic of (TR)-FRET HAT assays. A donor fluorophore conjugated to a recognition motif (top panel, antibody; bottom panel, bromodomain) binds to an acetylated lysine on the histone-based substrate. An acceptor fluorophore with a capture tag binds to the histone-based substrate with an affinity tag. The donor fluorophore is excited by a laser, which induces fluorescence. If in close proximity to the donor fluorophore, the acceptor fluorophore is excited by FRET. In the absence of interference, HAT activity is proportional to the emission intensity at this higher wavelength. There are five main potential areas of compound-mediated readout interference: (A) interference of excitation light source by test compound (quenching/absorbance/light-scattering); (B) disruption of donor fluorophore construct-substrate interaction by test compound; (C) disruption of FRET by test compound (quenching/absorbance/light-scattering); (D) disruption of acceptor fluorophore construct-product interaction by test compound; and (E) acceptor fluorophore emission interference by test compounds (auto-fluorescence, quenching/absorbance/light-scattering).

followed by signal attenuation with further substrate/product titrations (e.g., excess biotinylated histone-based substrate/products for available streptavidin beads).

- The absolute concentration and ratio of donor:acceptor beads is critical for optimal assay performance. Start with manufacturer recommendations and slowly titrate.
- Because many of the Alpha- and FRET-based HAT assay formats rely on several binding events, it is recommended to determine the optimal order of reagent addition (e.g., binding of capture tag to affinity tag may interfere with the binding of another the antibody-histone pair, but not vice-versa).

- For Alpha-based technologies, the manufacturer typically recommends Tween 20 (0.01-0.1% v/v), Triton X-100 (0.01-0.1% v/v), CHAPS (0.1% v/v), and/or BSA (0.1% w/v) to mitigate nonspecific interactions.
- Certain assay components may interfere with Alpha-based technologies:
  - Residual azide (a common antimicrobial used for reagent storage) may interfere with singlet oxygen transmission.
  - Certain transition metals (Al^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}) may quench singlet oxygen at low millimolar concentrations.
- To assess for assay interference:
  - Allow the reaction to go to completion, spike reaction with test compound, and then measure assay readout. If available, an alternative is to spike purified product and compound, then measure the assay readout.
  - For Alpha-based technologies, TruHit^{TM} beads can also be used to identify compound-mediated readout interference.
  - To assess interference with affinity tag systems, one can also test the effect of compounds on generic bead readouts that contain the affinity tag system of interest.

Enzyme-Coupled HAT Assays

Enzyme-coupled methods "indirectly" quantify HAT activity by measuring the product of a second, coupled enzymatic reaction **(Figure 10)**. This second enzyme system requires CoA substrate, which is produced from the primary HAT reaction. Additional substrates for this secondary enzyme are present in excess, so that the rate-limiting component of the final readout is the HAT reaction. Both α-ketoglutarate dehydrogenase or pyruvate dehydrogenase have been successfully used as the secondary enzyme (15,30). In both cases, α-ketoglutarate or pyruvate is oxidized, while NAD^+ is reduced to produce NADH. This latter product can be continuously monitored by spectrophotometry at 340 nm.

Enzyme-coupled assays have characteristic advantages and disadvantages. They are relatively inexpensive and amenable to higher-throughput set-ups. In the case of NADH readouts, these assays typically require larger amounts of product (low micromolar) for robust detection due to their absorbance-based readout. Like thiol-scavenging HAT assays, enzyme-coupled HAT assays are susceptible to multiple modes of compound-mediated assay interference. Compounds can react with free CoA, depleting substrate for the coupled reaction and producing either a false-negative or false-positive readout depending on the absorbance properties of the compound-CoA adduct. Compounds can also modulate the secondary enzyme. Finally, test compounds can interference with the absorbance readout.

The following are some considerations for enzyme-coupled HAT assays:

- The secondary enzyme reaction should not be rate-limiting, which can be verified by titrating each component of the secondary enzyme system. In many systems, the coupled enzyme is present in much higher concentrations than the primary (target) enzyme.
- Unlike thiol-scavenging HAT assays, DTT and other reducing agents do not typically interfere with the assay readout.
- Ensure each enzyme system components are compatible with one another, as certain HAT substrates (e.g., calf thymus histones) can interfere with previously described secondary reactions (15).
- Both α-ketoglutarate dehydrogenase or pyruvate dehydrogenase are available commercially.
- Reaction buffer should contain all of the necessary substrates and co-factors for the secondary enzyme (e.g., metals, NAD^+). Reaction buffer should be optimized for both the HAT activity and secondary enzyme activity.
- Cloudy solutions or precipitation may interfere with absorbance-based readouts.

- Background controls should be included. For instance, excluding HAT enzyme allows subtraction of non-enzymatic production of CoA. Additional controls can exclude secondary enzyme or various substrates to better characterize background.

Section Summary

There are a variety of technology platforms, each with signature advantages and disadvantages that must be carefully considered when choosing and subsequently optimizing a new HAT assay. Consideration should be given to the nature of the given HAT system being assayed, operator experience, available instrumentation, budget, project timeframe, as well as other factors. Regardless of the primary assay method, at least one orthogonal assay should be performed to confirm true compound-mediated HAT modulation.

Cell-Based HAT Assays

Introduction

Several cell-based HAT assays have been reported, most of which quantify histone acetylation through antibodies targeted acetylated histone lysine products. This section briefly describes common approaches to assaying HAT activity in cellular systems, as well as recommendations for their interpretation.

Cell-Based HAT Assays

Cell-based HAT assays assess the effect of test compounds on HAT activity in the cellular context. Most cellular HAT assays involve treating cells with test compound, followed by either (a) fixing treated cells and measuring histone acetylation by immunohistochemistry, or (b) lysing treated cells and measuring histone acetylation by an antibody-based method (e.g., ELISA, Western blot, and now Alpha-based technology) (**Figure 11**). Histone acetylation is usually normalized to total histone content or cell number. More recent approaches involve live-cell imaging that links an acetylated histone recognition system (e.g., bromodomain) with a reporter-linked histone substrate (**Figure 11**). These latter approaches require transfection of engineered reporter constructs. In principle, analysis of cellular histone isolates can also be performed by MS, though such methods are generally not readily adaptable to quantitative or high-throughput analyses.

Unlike most biochemical HAT assays, histone acetylation in cellular systems is confounded by multiple variables, including HAT expression, histone deacetylase activity, histone chaperones, cell cycle effects, and rates of histone synthesis and degradation. Due to the complexities of these higher-order systems and the inherent properties of current detection technologies, many cell-based assays have smaller dynamic ranges and decreased precision than simpler biochemical assays. For these reasons, those performing cell-based HAT assays should carefully evaluate intra- and inter-assay precision and accuracy.

Interpretation of Cell-Based HAT Assays

Many well-characterized promiscuous compounds (including known thiol-reactive, redox-active, and/or aggregators) can disrupt cell proliferation and histone acetylation in cells at low-to-mid micromolar compound concentrations, raising the strong possibility that certain histone acetylation readouts are also susceptible to off-targets (36). Therefore, caution is advised when interpreting such cell-based readouts, especially with chemical matter not sufficiently optimized or characterized for potency and selectivity. With nonspecific compounds, it is unclear whether changes in histone acetylation and other phenotypes are due to specific HAT engagement or off-target effects. It is likely that cellular HAT readouts are more interpretable with compounds possessing sufficient potency and selectivity (43).

The following are some cautions when interpreting cell-based HAT assay readouts:

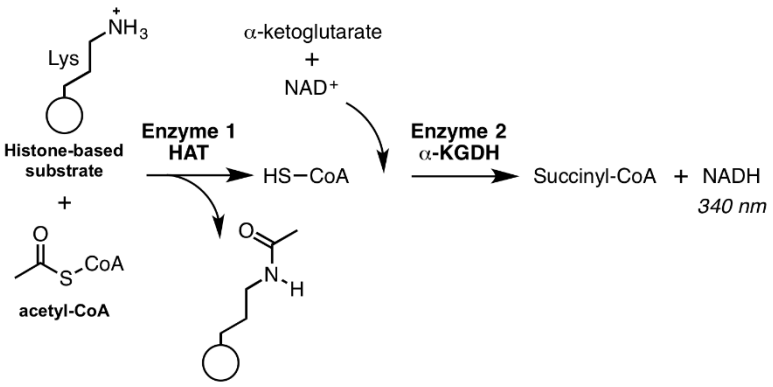


Figure 10. General schematic of enzyme-coupled HAT assays. Free coenzyme A produced by the HAT reaction serves as one of the substrates for a second enzymatic reaction. In the presence of excess co-substrate, the second enzymatic reaction produces a product that can be quantified. In absence of interference, HAT activity is proportional to the secondary enzyme product readout. In this example, the secondary enzyme α-ketoglutarate dehydrogenase (α-KGDH) catalyzes the conversion of free CoA and α-ketoglutarate to succinyl-CoA. The NAD^+ cofactor is reduced to NADH, which is monitored by absorbance at 340 nm. There are several potential areas of compound-mediated readout interference: (A) thiol-scavenging of CoA-SH by electrophilic test compounds; (B), modulation of secondary enzyme activity by test compounds; and (C), light-based interference by test compounds.

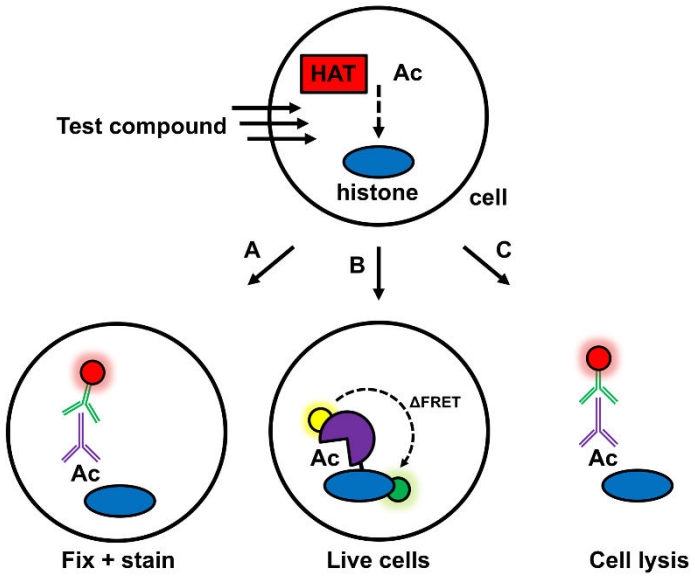


Figure 11. Schematic of representative cell-based HAT assays. Cells are incubated with test compound. (A) Cells can be fixed in preservative and analyzed by immunohistochemistry and related techniques. (B) HAT activity can be monitored in real-time with the introduction of special constructs, such as FRET-based systems. (C) Cells can be lysed, and histones can be probed for acetylation by antibody-based approaches, as well as mass spectrometry. Cell-based readouts should be interpreted with certain cautions (see main text).

- *HAT/substrate overlap*. HATs often have multiple substrates, and conversely, many histone modifications are catalyzed by more than one HAT. Changes in a particular histone acetylation after compound treatment may be due to simple engagement of the intended HAT. However, histone acetylation may be due to engagement of other HATs with overlapping substrate specificities.
- *Deacetylase activity*. Cellular histone acetylation exists in dynamic equilibrium between acetyltransferases and deacetylases. Histone acetylation can be reversed by HDACs and sitruins with deacetylase activity. One should carefully consider the effects of test compounds on HDAC/sirtuin activity in cellular systems.

- *Controlling for HDAC activity*. To control for HDAC activity, some researchers treat cells with HDAC inhibitors such as suberanilohydroxamic acid (SAHA) or trichostatin A (TSA). If employing this approach, one must consider the effects of HDAC inhibition in the context of the assay readout.
- *Sensitivity/acetylated histone content.* Depending on the experimental system including the specific histone acetylation, the concentration of analyte can be very low. One approach to increase measureable acetylated histones is to treat cells with HDAC inhibitors. As above, if employing this approach, again one must consider the effects of HDAC inhibition when interpreting the assay readout.
- *Cell cycle dependence*. Certain HATs and histone PTMs are cell-cycle dependent in their expression and activity. Furthermore, overall nucleosome content is cell-cycle dependent. Depending on the context, cell synchronization or cell cycle analyses may be necessary.
- *Cell proliferation*. Modulation of certain HATs can decrease cell proliferation and induce apoptosis. Decreases in cell proliferation or induction of apoptosis are not necessarily direct evidence of actual HAT engagement, and may be due to general compound toxicity.
- *Indirect readouts*. Due to complex cellular dynamics, histone acetylation is still an indirect marker of HAT engagement by a compound. Demonstrating direct target engagement in the cellular context generally requires sophisticated methodologies such as CETSA, radiolabeled compound, or chemical proteomics.

Section Summary

Compound modulation of HAT activity should be verified in a cell-based system. Due to the complex regulation of histone acetylation, changes in bulk as well as specific cellular histone acetylation may not always be attributable to a direct compound-HAT interaction. Subsequent efforts should be made to characterize direct target engagement and specificity in the cellular context using orthogonal methods (e.g. CETSA, compound-labeling).

Miscellaneous Considerations

The following are several miscellaneous considerations that may be especially useful in establishing and validating HAT assays:

- The relative order of compound and reagent addition may have significant effects on assay performance. It can affect enzymatic activity by altering HAT or substrate stability, for instance (40). Experiment with several configurations during assay development and even later mechanistic studies with promising lead compounds.
- For assays utilizing affinity tags, we recommend experimenting with several different tags, tag locations (i.e., *N*-terminal or *C*-terminal), and linkages. Often the optimal tag set-up is difficult to predict, and is best determined experimentally.
- Whenever possible use the highest quality reagents possible.

Conclusions

HATs are an emerging class of epigenetic targets in drug discovery and chemical probe development. Modulation of HAT activity by small molecules can be assayed by multiple orthogonal platforms, many of which are amenable to high-throughput formats. Designing robust and cost-effective HAT assays requires careful optimization of multiple experimental parameters. Identifying chemical matter capable of modulating HATs by tractable, useful mechanisms also requires well-designed orthogonal assays and counter-screens. Scientists investigating the effect of small molecules on HAT activity should incorporate the aforementioned strategies and principles into their assay design.

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Conflict of Interest Statement

The authors declare no financial conflicts of interest.

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