Effective Quenchers Are Required to Eliminate the Interference of Substrate: Cofactor Binding in the HAT Scintillation Proximity Assay

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ABSTRACT

Histone acetyltransferases (HATs) mediate the transfer of an acetyl group from the cofactor, acetyl-CoA, to the side chain amino group of specific lysines in diverse protein substrates, most notably nuclear histones. The deregulation of HATs is connected to a number of disease states. Reliable and rapid biochemical assays for HATs are critical for understanding biological functions of protein acetylation, as well as for screening small-molecule inhibitors of HAT enzymes. In this report, we present a scintillation proximity assay (SPA) for the measurement of HAT enzymatic activities. The acetyl donor was [³H]Ac-CoA, and a biotin-modified histone peptide served as the HAT substrate. After the HAT reaction, streptavidin-coated beads were added to induce proximity of acetylated substrate to the scintillant molecules. However, we observed strong nonspecific binding between the cofactor and the histone peptide substrates, which adversely complicated the SPA performance. To prevent this problem, a set of chemical agents were evaluated to eliminate the cofactor-substrate interaction, thus providing reliable SPA readings. With optimization, the SPA showed consistent and robust performance for HAT activity measurement and HAT inhibitor evaluation. Overall, this mix-and-measure assay does not require any washing procedure, can be utilized in the microplate format, and is well suited for high-throughput screening of HAT chemical modulators.

INTRODUCTION

Histone acetyltransferases (HATs) transfer the acetyl group from the cofactor, acetyl-coenzyme A (Acetyl-CoA, Ac-CoA), to the ε-amino group of specific lysines in histones and non-histone proteins. HATs are classified into several main families, which include GCN5/PCAF, MYST, and CBP/p300.¹ Studies have shown that these enzymes play critical roles in the regulation of chromatin restructuring, gene transcription, and signal transduction. Deregulation of HAT levels and activities can result in the development and progression of various diseases such as cancers and cardiac hypertrophy.^{2–4} Given that these diseases affect millions of lives, it is of great importance to discover inhibitors for these HAT enzymes. In this drug development journey, reliable and efficient biochemical methods that are able to screen numerous HAT inhibitor compounds are critically needed.

A few reported methods have been used to detect the acetyltransferase reaction mediated by HATs, which include radioactive filter binding assays, coupled fluorogenic assays, Flash plate assays, and fluorography assays.^{5–7} Among these assays, the radioactive filter binding method using negatively charged phosphocellulose paper discs is considered the gold standard, because it has greater sensitivity compared with the other methods and it is not as time consuming as the fluorography assays, which require mixture separation by gel electrophoresis and radiographic film development. Despite these amenable attributes, the major limitation for the filter binding assay method is the requirement of repeated washing steps, which not only are labor intensive but also cause a large accumulation of radioactive waste. This technical hindrance also poses a challenge for the filter binding method to be used for high-throughput screening (HTS) of HAT inhibitors. Due to these problems, it is of interest to design a biochemical assay that would provide comparable data to the filter binding method but would be more time efficient, environmentally

friendly, and, importantly, possess robustness and capability for HTS of HAT inhibitors.

Of the HTS assays available, scintillation proximity assay (SPA) is known for being robust, reliable, sensitive, and quick.⁸ A common SPA protocol utilizes the strong interaction between biotin and streptavidin ($K_d = 10^{-15}$ M, Richards⁹) to bring the radiolabeled ligand close to the streptavidin-coated polyvinyl toluene (PVT) microbeads to emit light.^{10,11} The emitted photons can be detected by the photomultiplier of a scintillation counter. Generally, tritium (^3H) is used due to its strong but short-path β -particle emission (1.5 μm) that ensures that only the bead-bound radiolabels can stimulate the polymer-immobilized scintillant molecules.^{8,11} The marked advantage of using SPA is the elimination of the laborious separation of product from unused radioactive cofactor, which greatly improves assay speed and allows for an efficient mix-and-measurement assay of the product. With these advantageous properties, the SPA method has been applied to study other post-translational enzymes such as protein methyltransferases and kinases.^{12,13}

We attempted to implement a biochemical assay for discovering HAT inhibitors based on the principle of SPA. In this strategy, biotin-labeled histone peptides were used as the HAT substrate and [^3H]Ac-CoA was used as the acetyl donor. After the homogeneous acetyltransferase reaction, the [^3H]-labeled product would bind to the streptavidin-coated SPA beads and produce detectable photon signals. We initially posited that such an SPA protocol should work straightforwardly based on the great success of previously using an analogous SPA method for the study of histone methyltransferase activities.^{12,14} Unexpectedly, when applying the SPA to detect the HAT reaction, we observed strong false-positive readings, especially in enzyme-negative controls. This acetylation-specific problem had to be illuminated and resolved to validate this SPA approach for HAT probe discovery. Here, we found out that the cofactor-substrate interaction interfered with acetyltransferase SPA readings. Further, we tested chemical reagents that could remove the interference, characterized this SPA approach for quantitative analysis of HAT inhibitor potencies, and proved robustness and high potential of this method for use as a high-throughput assay for HAT inhibitor screening.

MATERIALS AND METHODS

Protein expression and purification

The expression of p300 HAT domain (1287–1666) was done following the method developed by Cole's lab.¹⁵ Transformation was done with BL21(DE3)-RIL competent cells using heat-shock, spreading on plates containing both ampicillin and chloramphenicol antibiotics. Colonies were harvested, grown at 37°C in 8 mL, and inoculated to 1 L culture of 2XYT media

containing both ampicillin and chloramphenicol. Protein expression was induced with isopropyl β -D-1-thiogalactopyranoside (IPTG) and shaken for 16 h at 16°C. The cells were collected by centrifugation at 4,000 rpm for 25 min and were resuspended in lysis buffer (25 mM Na-HEPES [pH 8], 500 mM NaCl, 1 mM MgSO_4 , 10% glycerol, and 2 mM phenylmethanesulfonyl fluoride [PMSF]).

The cells were lysed by passing them through a microfluidizer (Microfluidics) at 17,000 psi, and the supernatant was purified on chitin resins that were equilibrated with column buffer (25 mM Na-HEPES [pH 8], 250 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 1 mM PMSF). The protein-loaded beads were thoroughly washed with the column buffer and wash buffer (25 mM Na-HEPES [pH 8], 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 1 mM PMSF). Next, the CT14 peptide (CMLVELHTQSQDRF) was dissolved in the cleavage buffer (25 mM Na-HEPES [pH 8], 250 mM NaCl, 1 mM EDTA, and 200 mM 2-Mercaptoethanesulfonic acid [MESNA]) and added to the column. The column was set out at room temperature for 16 h before the protein was eluted from the column, and several volumes of cleavage buffer were added to ensure the complete protein elution. Further purification was done using the Bio-Rad NGC fast protein liquid chromatography system. p300 HAT domain protein band was verified with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Millipore centrifugal filter and Bradford assay were, respectively, used to concentrate and determine the protein's concentration. Lastly, the protein was aliquoted and stored at -80°C .

Peptide synthesis

The biotinylated peptide containing the N-terminal 20-amino-acid sequence of histone H4, that is, Ac-SGRGKGKG LGKGGAKRHRK(Biotin)- NH_2 [abbreviated as H4(1–20)-BTN], and histone H3, that is, Biotin-ARTKQTARKSTGGKAPRKQL-OH [abbreviated as H3(1–20)-BTN], were synthesized using Fmoc-based solid-phase peptide synthesis protocol according to Wu *et al.*¹² The CT14 peptide for chemical ligation contained the residues 1653–1666 of p300 catalytic domain (CT14, *i.e.*, CMLVELHTQSQDRF).

Measurement of histone acetylation with SPA

As a standard enzymology practice, we monitored the HAT-catalyzed reactions for a range of time and at different enzyme concentrations. Typically, the reaction time and enzyme concentration were controlled such that the reaction yield was maintained under the linear, initial condition (*Supplementary Figs. S1 and S2*; Supplementary Data are available online at www.liebertpub.com/adt). The time of 6-min and enzyme concentration of 25-nM were chosen based on the fact that

both these points were in the linear range of rate–time and rate–[E] relationship. By keeping the reactions within the linear range, we ensure that the enzymatic reaction occurred under a constant and maximal rate, and the product formation can be accurately quantitated using a linear equation. When the reaction time becomes overly long or the enzyme concentration becomes too high, the reaction goes out of the linear range, and the reaction rate starts decreasing due to a number of factors such as substrate consumption, product feedback inhibition, and enzyme denaturing.

The SPA experiments were conducted in a 96-well plate (Isolate-96; Perkin Elmer) at 30°C using a reaction buffer containing 50 mM HEPES (pH 8), 1 mM EDTA, and 0.5 mM dithiothreitol (DTT). The cofactor used as an acetyl donor was [³H]Ac-CoA (PerkinElmer), and the substrate was either H4(1–20)-BTN or H3(1–20)-BTN. The 30 μL reaction volume typically consisted of 2.5 μM of substrate, followed by 1 μM [³H]Ac-CoA. After 5–10 min of incubation, 0.025 μM of p300 (final concentration) was added and samples were re-incubated for 6–10 min. The reaction was quenched with 30 μL of the tested quenchers or with varying volumes of quenchers. Lastly, 10 μL of suspended 20 mg/mL streptavidin-coated SPA beads (Perkin Elmer) in the reaction buffer were added to each well and thoroughly mixed. The plate was placed in the MicroBeta2 scintillation counter (Perkin Elmer) in total darkness for 1 min before each of the wells containing samples was scanned. Samples were performed in duplicate and were typically within 20% of each other. The typical SPA protocol specifics are shown in *Table 1*.

Filter binding assay of HAT reaction

Filter binding assay was done in the same buffer mentioned earlier. Radio-labeled Ac-CoA was used to donate the acetyl group to either H4(1–20)-BTN or H3(1–20)-BTN. The total reaction volume was 30 μL. Peptide and cofactor were incubated for 5 min, followed by the addition of the enzyme. After another 6–10 min of incubation, the reaction was then quenched by spreading 20 μL of the reaction mixture over a Whatman P81 phosphocellulose filter disc (GE Healthcare Life Sciences). Once the filter discs were dried in air, they were washed thrice with 50 mM NaHCO₃ (pH 9.0) and re-dried. Acetylated products were quantified with the addition of scintillation cocktail, which was scanned using the MicroBeta2 (Perkin Elmer). Assays were performed in duplicate with a standard deviation within 20% of each other.

RESULTS

Design of the SPA for HATs

To design a fast and sensitive assay for HATs, we investigated the practicability of the SPA for HAT activity measurement. The

Table 1. The SPA Protocol Table			
Step	Parameter	Value	Description
1	Inhibitor to wells	6 μL	100 μM for screening
2	Mixture	18 μL	Mixture: H3(1–20)-BTN and [³ H]Ac-CoA
3	Incubation time	5 min	30°C
4	Add enzyme	6 μL	0.025 μM
5	Reaction time	6 min	30°C
6	Quencher	30 μL	0.5 M (final) guanidine HCl
7	SPA beads	10 μL	20 mg/mL
8	Assay readout	1 min	MicroBeta2
Step Notes			
1. Ninety-six-well plate (Isolate-96; Perkin Elmer); fixed 100 μM of inhibitor for quick screening or various inhibitor concentrations were added to determine IC ₅₀ ; Inhibitor was absent in controls.			
2. Mixture contained reaction buffer (50 mM HEPES [pH 8], 1 mM EDTA, and 0.5 mM dithiothreitol [DTT]), [³ H]Ac-CoA, and biotin-labeled peptide.			
3. Plate was placed in a mini incubator with the temperature set at 30°C.			
4. 0.025 μM p300.			
5. Plate was placed in a mini incubator with the temperature set at 30°C.			
6. 30 μL of 0.5M (final) guanidine HCl was added to every sample to quench the reaction.			
7. 10 μL of 20 mg/mL SPA beads was added and mixed well with every sample.			
8. Each well was scanned for 1 min using the MicroBeta2 scintillation counter (Perkin Elmer).			
SPA, scintillation proximity assay.			

SPA approach is built on the interaction between streptavidin-coated scintillation bead and biotin-labeled HAT substrate¹⁶ (*Fig. 1A*). In this protocol, an HAT enzyme catalyzes the transfer of [³H]acetyl group from [³H]Ac-CoA to a biotin-labeled substrate (*e.g.*, H3 or H4 peptide). The HAT enzyme used in the assay was p300, which can effectively acetylate the N-terminal tails of both histone H3 and H4. The substrate was a biotinylated peptide containing the N-terminal 20-aa sequence of H3 or H4, namely H3(1–20)-BTN or H4(1–20)-BTN. After incubation of the enzyme, [³H]Ac-CoA, and biotinylated substrate, the acetyltransferase reaction was stopped by adding a quenching reagent, such as isopropanol. Lastly, SPA scintillation beads were added and the acetylated product was counted. Through a specific biotin-streptavidin interaction, the [³H]-radiolabeled acetylated peptide product is brought into close proximity to the SPA bead. The beta-particle emission of [³H] excites the scintillants immobilized in the SPA bead, generating luminescence that is detected by a microplate-compatible scintillation counter, for example, the MicroBeta or TopCount. Any unbound [³H]Ac-CoA is out of the scintillation proximity distance; thus, it will not generate SPA signals.

Fig. 1. Scheme of the scintillation proximity assay (SPA) for detecting histone acetylation. **(A)** The enzyme transfers the radioactive acetyl group to the biotinylated peptide, which is brought into proximity of the streptavidin-coated scintillant microsphere beads. **(B)** Pathway 1 produces signals as a result of the acetyltransferase reaction. Pathway 2 shows that the electrostatic interaction of acetyl-CoA and histone substrate leads to undesired SPA signals even in the absence of HAT enzymes. Pathway 2 must be removed in a reliable HAT SPA measurement.

Identifying effective quenchers and optimizing their concentrations for SPA measurements

To start, we first measured the SPA reading of $[^3\text{H}]\text{Ac-CoA}$ in the reaction buffer. As expected, the sample showed only a background-level count (~ 100 cpm) (Fig. 2A), supporting that the tritium atoms in the bulk Ac-CoA solution are not being brought into close enough proximity to the microsphere-coated scintillant molecules. Nevertheless, when the biotinylated histone H3 or H4 peptide was added, we observed strong SPA signals, which increased proportionally with the increasing amount of the added peptide (Fig. 2A). This result was quite unexpected because no HAT enzyme was present, and the 10-min incubation time could not have allowed for non-enzymatic acetylation to occur. Instead, the undesirable SPA signals were likely caused by nonspecific electrostatic interactions between the positively charged histone peptide and the negatively charged $[^3\text{H}]\text{Ac-CoA}$. Nevertheless, this interaction needed to be eliminated; otherwise, it would interfere with the signals from the HAT reaction.

In end-point enzymatic assays, typically a quenching reagent is added to stop the enzymatic reaction so that the amount of products can be quantified at precise time points. In the previously reported HAT assays, the oft-used quencher is a water-miscible organic solvent, such as isopropanol and DMSO, which can stop the HAT reaction by disrupting the active enzyme folding structures.¹⁷ In our previous SPA experiments for histone methylation, dilution of the reaction mixture with a fivefold buffer was also found to be effective in diminishing enzymatic activity.¹²

We reasoned that a different chemical quencher should be selected to remove the undesirable cofactor-substrate

Fig. 2. Interaction of histone H3 and H4 substrates with $[^3\text{H}]\text{Ac-CoA}$ measured by SPA (A) and the effects of various quenchers on the interaction between substrate and cofactor (B). Assays containing peptide [0–10 μM H3(1–20)-BTN or H4(1–20)-BTN peptide (A), or 2.5 μM H3(1–20)-BTN or H4(1–20)-BTN (B)] and 1 μM $[^3\text{H}]\text{Ac-CoA}$ were incubated at 30°C for 6 min, before adding 30 μL of isopropanol (A) or various quenchers (B) and 10 μL of 20 mg/mL SPA beads. Quenchers in figure (B) are as follows: a, negative; b, positive; c, 50% isopropanol; d, 1% SDS; e, 1% Triton X-100; f, 4 M guan; g, 2 M guan; h, 50 mM NaHCO_3 ; i, 50% DMSO; j, 50 mM AcOH. In the positive control, water was added as the quencher. Negative controls did not contain any peptide.

interaction in an HAT reaction. In this regard, several chemical quenching agents were tested, which included isopropanol, SDS, Triton X-100, guanidine HCl, NaHCO_3 , DMSO, and acetic acid. In the first experiment, a reaction mixture containing biotinylated histone peptide and $[^3\text{H}]\text{Ac-CoA}$ was incubated for 6 min. Then, an equal volume of individual quenchers was added to each reaction mixture. As seen in Figure 2B, several quenchers, including 1% SDS, 50 mM NaHCO_3 , 2 and 4 M guanidine HCl, gave low SPA signals that were comparable to the negative control, which was indicative of their effects on disrupting the interaction between the histone peptide (both biotinylated H3 and H4 peptides) and $[^3\text{H}]\text{Ac-CoA}$. Although 1% SDS showed promising

results in this experiment, it was harder to work with, because it is prone to generate air bubbles during the mixing process.

We are particularly interested in using guanidine HCl as the quenching reagent, because it is a cheap agent and has been widely used in protein denaturing studies. The minimum guanidine HCl concentration that would produce reliable and accurate SPA readings by inhibiting the histone–Ac-CoA interaction was determined. Various concentrations of guanidine HCl were tested with samples containing 2.5 μM of H3(1–20)-BTN and 1 μM $[^3\text{H}]\text{Ac-CoA}$. In the absence of enzyme, at least 0.125 M guanidine HCl was needed to keep the reading signal as low as the negative control and presumably blocking any interaction between H3 peptide and Ac-CoA (Fig. 3A). Ideally, the quenching agent should also stop the

Fig. 3. The dose response of SPA signals at different concentrations of guanidine HCl (A) and NaHCO_3 (B). All samples were incubated for 6 min at 30°C. Samples not containing enzyme had 2.5 μM H3(1–20)-BTN, 1 μM $[^3\text{H}]\text{Ac-CoA}$, and they were quenched with 30 μL of guanidine HCl (A) or NaHCO_3 (B) at various concentrations. Samples with enzyme contained 6 μL of guanidine HCl (A) or NaHCO_3 (B) at various concentrations in the reaction mixture of 2.5 μM H3(1–20)-BTN, 1 μM $[^3\text{H}]\text{Ac-CoA}$, and 0.025 μM p300. After the reaction, 30 μL of isopropanol was added. Positive control did not contain any guanidine HCl or NaHCO_3 . H3(1–20)-BTN was absent in the negative control samples.

enzymatic reaction as well, which is required for end-point enzymatic assays. So, a similar assay was performed with the presence of HAT enzyme, p300, and readings showed that a guanidine HCl concentration at 0.25 M was needed to keep the readings at the background level (Fig. 3A). This suggests that under these experimental conditions, denaturation of p300 required a higher concentration of guanidine HCl than to break down histone–Ac–CoA interaction. Together, 0.25 M of guanidine HCl was sufficient to both stop the HAT reaction and meanwhile eliminate histone–Ac–CoA interaction.

Another quencher tested was NaHCO₃. This chemical is commonly used as the washing reagent in the filter-binding assay for HATs. The dose-dependent effect of NaHCO₃ on the histone–Ac–CoA interaction and p300 HAT activity was determined in the same way as the guanidine HCl experiment. As displayed in Figure 3B, 50 mM of NaHCO₃ was sufficient enough to break down the cofactor–substrate interaction, and to show low readings close to the background level. However, in the presence of p300, 100 mM of NaHCO₃ was needed to bring down the SPA readings in parallel to the negative control. This difference, again, suggests that inhibition of p300 activity needs a higher strength of quencher solution than does blocking the histone–Ac–CoA interaction.

Further comparison of guanidine quenching with the other quenching methods

We further evaluated and compared the quenching properties of different quenching reagents on a substrate–cofactor interaction, and on the p300 enzymatic activity in the SPA measurement. As shown in Figure 4A (white column), the addition of isopropanol was unable to break the histone–Ac–CoA interaction; even with 150 μ L isopropanol (83% v/v), the SPA reading was still as high as 1,251 cpm. Another often used quenching method is diluting the reaction mixture with buffer. By dilution, the enzyme will have a less access to its substrates, resulting in a dramatic decrease of product formation. The results showed that even with a fivefold dilution using the reaction buffer, the SPA readings were not able to stay as low as the negative control (Fig. 4A, square checked column). This clearly suggests that the dilution method is insufficient to break the histone–Ac–CoA interaction. The addition of 90 μ L DMSO (75% v/v) to the reaction mixture was able to bring the readings down close to the background level. Guanidine HCl and NaHCO₃ were the only quenchers that were able to keep readings close to the background level with only one-volume addition (Fig. 4A, gray and slanted line column).

Fig. 4. Quenching effects of 1xRB, isopropanol, DMSO, NaHCO₃, and guanidine HCl on samples (A) without HAT enzyme, and (B) with p300 enzyme. (A) Samples containing 2.5 μ M H3(1–20)-BTN, and 1 μ M [³H]Ac-CoA was incubated for 6 min at 30°C before various volumes of the different quenchers were added. Lastly, 10 μ L of 20 mg/mL SPA beads was added. The background did not contain any biotinylated peptide. (B) These samples followed the same conditions with the addition of 0.025 μ M p300.

This quenching effect was retested under the same conditions except that p300 enzyme was added to the reaction mixture (Fig. 4B). Before the addition of any quenching agents, the reaction mixture was incubated for 6 min to allow histone acetylation to occur. Therefore, unlike the results in Figure 4A, we expected to observe a high SPA reading in every test due to the radiolabeling of the H3(1–20)-BTN substrate. Indeed, as displayed in Figure 4B, the SPA readings remained at high levels (>900 cpm) for guanidine HCl, NaHCO₃, isopropanol, and 1xRB, independent of volume addition (Fig. 4B). Comparing the data in Figure 4A and B, we can conclude that (1) quenching with isopropanol and extra volumes of buffer solution is not sufficient enough to block a nonspecific

histone–Ac-CoA interaction and thus cannot produce reliable data in SPA HAT assays. (2) Guanidine HCl and NaHCO_3 own the ability of inhibiting both HAT activity and the histone–Ac-CoA interaction, thus providing reliable results with just a one-volume addition. (3) Surprisingly, as DMSO volumes increased, the readings gradually decreased close to the background level in both *Figure 4A* and *B*. The observation of this trend for DMSO is very important for this assay; the decrease of SPA signals in *Figure 4A* was because the histone–Ac-CoA interaction was inhibited by DMSO, whereas the diminished SPA readings in *Figure 4B* strongly indicate that high volumes of DMSO interfered with the interaction between biotin and streptavidin, which is detrimental to SPA efficiency.

To validate whether DMSO was, indeed, disrupting the biotin–streptavidin interaction in the SPA procedure, an experiment using histone methylation was designed (*Fig. 5*). In this methylation experiment, we used protein arginine methyltransferase 1 (PRMT1) enzyme and $[^3\text{H}]\text{SAM}$ as the methyl donor and H4(1–20)-BTN as the substrate. We had predetermined that there was no electrostatic interaction between $[^3\text{H}]\text{SAM}$ and the histone substrate (data not shown). The histone methylation was first carried out before the addition of any quenching agent. Therefore, we would expect to observe strong SPA readings regardless of the type of added quenchers. Indeed, the samples showed high and relatively stable SPA signals in the presence of guanidine, NaHCO_3 ,

isopropanol, and 150 μL reaction buffer (*Fig. 5*). The reactions that were quenched with less than three-volume 1xRB had greater reading values, likely because 1xRB did not denature the enzyme. Under a lower dilution of the reaction mixture, the enzyme activity was still measurable by the continual substrate methylation. Importantly, again, it was observed that increasing DMSO volumes greatly reduced the SPA signals, which was similar to that observed in *Figure 4B*. From these experiments, it is clear that high volumes of DMSO interfered with the biotin–streptavidin interaction. Thus, using DMSO as a quencher in SPA experiments should be cautioned or avoided.

Comparison between the SPA and the filter binding assay

The filter binding assay is considered the gold standard for measuring activities of HATs, both for enzyme mechanism studies and for inhibitor characterization studies. However, this method is laborious, creates a large amount of radioactive waste, and hinders HTS application. The development of a more efficient and environmentally friendly method with results comparable to the filter binding assay would be highly valuable. We measured and compared the time-dependent reaction progression using both the SPA and the filter binding assay under identical reaction conditions. As shown in *Figure 6*, an excellent linear relationship (correlation coefficient $[R]$ is 0.996) was observed between the data obtained from the

Fig. 5. Effects of 1xRB, isopropanol, DMSO, NaHCO_3 , and guanidine HCl on PRMT1-mediated H4 methylation. Samples containing 0.02 μM PRMT1, 1 μM H4(1–20)-BTN, and 0.5 μM $[^3\text{H}]\text{SAM}$ were incubated for 7 min at room temperature. Reaction was quenched with 30–150 μL of the different quenchers. Lastly, 10 μL of 20 mg/mL SPA beads was added. The background did not contain any biotinylated peptide.

Fig. 6. Linear correlation of the SPA with the filter binding assay. A time-dependent assay was carried out using identical conditions for both assays. Samples containing 2.5 μM H3(1–20)-BTN, 1 μM $[^3\text{H}]\text{Ac-CoA}$, and 0.025 μM p300 were incubated at 30°C before ending the reaction with 30 μL of 0.5 M (final) guanidine HCl or by spreading 30 μL of the reaction mixture on P81 paper. Reaction times were 0, 1, 3, 5, 7, 10, 15, 30, and 60 min.

SPA and the filter binding assay, demonstrating that these two methods are consistent and reliable.

We also compared the two assay formats for HAT inhibitor characterization (Fig. 7). C646 is a known inhibitor of p300 with submicromolar potency.¹⁸ We measured the p300 inhibition by C646 at varying concentrations using both the filter binding assay and the established SPA. Both assays contained 2.5 μM H3(1–20)-BTN and 1 μM [^3H]Ac-CoA and were reacted for a reaction time of 6 min at 30°C. The readings for the SPA measurements quenched with guanidine HCl followed the same trend as the readings for the filter binding. The IC_{50} value of the SPA measurement with guanidine HCl as quenching agent was calculated to be $3.4 \pm 0.1 \mu\text{M}$, which was very close to the value obtained from the filter binding assay ($2.3 \pm 0.01 \mu\text{M}$). Therefore, the SPA showed the same quality performance as the filter binding method for quantifying HAT activities in both the absence and presence of inhibitors. The SPA would be preferred, because it is faster and does not require washing procedures. For comparison purpose, we also

Fig. 7. Dose-dependent inhibition of p300 activity by C646 measured using SPA and filter binding assay. Both the SPA and filter binding assays included 0.025 μM p300, 2.5 μM H3(1–20)-BTN, 1 μM [^3H]Ac-CoA, and an incubation time of 6 min at 30°C. To quench the reaction, 30 μL of each reaction mixture was spread out on a Whatman filter binding paper or 30 μL of 100% isopropanol or 1 M guanidine HCl was added to the sample. IC_{50} was determined by fitting the data to the equation $\frac{y - y_{\min}}{y_{\max} - y_{\min}} = \frac{1}{(1 + [I]/\text{IC}_{50})^h}$, where $y_{\max}$, $y_{\min}$, h , and $[I]$ are maximum and minimum SPA signals on the y-axis, hill coefficient, and inhibitor concentration, respectively.²⁶

did the IC_{50} measurement using the SPA measurement with isopropanol as a quencher. An IC_{50} of $4.1 \pm 0.6 \mu\text{M}$ was obtained from this assay. Although the value was similar to that of the filter binding assay and the guanidine-quenched SPA, it is clearly seen from Figure 7 that even at 100 μM of the inhibitor where the p300 enzymatic activity was completely inhibited, the scintillation readings were still much higher than the background. This phenomenon of partial inhibition supports the fact that the interaction between the substrate and cofactor led to false SPA readings and isopropanol was not able to disrupt the interaction between the substrate and cofactor. Therefore, the high SPA readings caused by the substrate–cofactor interaction reduced the signal dynamic window, making it more difficult to distinguish between positive and negative hits.

Evaluation of method for the use in HTS

Screening a large number of compounds represents a task that can become very laborious, so it is very valuable to have an HTS method that works efficiently and robustly and shows superior signal-to-background ratios. The Z' and Z factors are excellent parameters that are used to evaluate effectiveness and robustness of a biochemical assay for HTS application.¹⁹ The theoretical Z' and Z values can range from 0 to 1, and a value greater than or equal to 0.5 is considered a preferable parameter for a stable HTS assay. We measured the Z' and Z factors of this HAT SPA. Three sets of samples, each with 24 data points, were tested in a 96-well plate format. The first set was the enzyme-negative control (containing 2.5 μM H3(1–20)-BTN and 1 μM [^3H]Ac-CoA), the second set was the enzyme-positive control (containing 2.5 μM H3(1–20)-BTN, 1 μM [^3H]Ac-CoA, and 0.025 nM p300), and the third set included an HAT inhibitor in the enzymatic reaction (containing 2.5 μM H3(1–20)-BTN, 1 μM [^3H]Ac-CoA, 0.025 μM p300, and 10 μM C646). From the sampling data shown in Figure 8, the calculated Z and Z' factors for this HTS assay were found to be 0.68 and 0.69, respectively, therefore supporting the robustness and effectiveness of this SPA protocol to screening HAT inhibitors in HTS format.

DISCUSSION

Lysine acetyltransferases have major physiological roles at the cellular level; however, in an unregulated state, they contribute to various detrimental diseases. To develop new HAT-targeting therapies, it is crucial to have a reliable HTS method to quickly obtain chemical hits from a large library of compounds. With this task in mind, we set out to implement an SPA for HATs. Using the SPA for biochemical assays of enzyme activities and protein–protein interactions is an

Fig. 8. The scatter plot of the SPA signals comparing enzyme-positive, enzyme-negative, and inhibitor-containing samples. *Squares*, the enzyme-positive reaction samples contain 0.025 μ M p300, 2.5 μ M H3(1–20)-BTN, and 1 μ M [3 H]Ac-CoA. *Circles*, the enzyme-negative samples contain 2.5 μ M H3(1–20)-BTN, and 1 μ M [3 H]Ac-CoA. *Diamonds*, the inhibitor samples contain 0.025 μ M p300, 2.5 μ M H3(1–20)-BTN, 1 μ M [3 H]Ac-CoA, and 10 μ M C646. All samples were incubated for 7 min at 30°C. Lastly, all samples were quenched with 30 μ L of 0.5 M (final) guanidine HCl, and 10 μ L of 20 mg/mL SPA beads was added before scintillation counting in MicroBeta2.

appealing approach. SPA methods have been adopted to analyze several PTM enzyme activities, such as protein methyltransferases and kinases.^{12,13} We reasoned that the SPA protocol could be analogously set up for HAT enzymes using [3 H]Ac-CoA as the acetyl donor and biotinylated histone peptide as the acetyl acceptor (Fig. 1A). Once the radioactive labeled acetyl group is transferred to the biotinylated peptide, it is brought to close proximity of streptavidin-coated scintillation beads by the specific binding of biotin with streptavidin. We want to mention that Ait-Si-Ali *et al.*²⁰ previously also performed radiometric HAT assays using biotinylated histone peptide. In their assay, the acetylated product was captured on streptavidin-modified agarose beads. Since it was not an SPA method, a washing step was still needed to remove unreacted Ac-CoA before scintillation counting, which is similar to the standard filter binding assay. In our strategy, we attempt to devise the HAT assay in the SPA format, so that scintillation readings can be directly measured after the HAT reaction without any washing, which would greatly improve the high-throughput capacity.

When implementing the SPA method for HAT activity measurement, however, we observed that the enzyme-negative samples showed unexpectedly high SPA signals (e.g., Fig.

2A). Using this assay to measure potency of HAT inhibitors also yielded a pattern of incomplete inhibition (Fig. 7). Through a series of comparative experiments, the issue was nailed down to the nonspecific electrostatic interaction between the cationic histone peptides and the anionic [3 H]Ac-CoA. The adverse histone-cofactor binding brought the tritium radiolabel in proximity to the SPA scintillation beads even in the absence of HAT enzyme, thus producing false readouts (Fig. 1B). Apparently, this extra intermolecular interaction interfered with the true readings of HAT enzymatic assay and needed to be avoided to set up a reliable HAT SPA measurement.

To provide unbiased results for HAT activity measurement using the SPA, several chemical agents were studied that could eliminate the undesirable substrate-cofactor interaction. In selecting a quenching agent, we reasoned that the chemical quenchers must satisfy three basic requirements. First, the quencher must disrupt the electrostatic interaction between histone peptide substrate and [3 H]Ac-CoA. Second, it has to inhibit the

HAT enzyme activity so that no further acetylation reaction proceeds after the quenching. Third, the quencher must not interfere with the biotin and streptavidin interaction. We found that guanidine HCl and NaHCO₃ were excellent quenchers, because they were able to satisfy all the three requirements. Isopropanol, 1% Triton X-100, and 50 mM AcOH were not strong enough to prevent a cofactor-histone interaction. Excess DMSO disrupted biotin-streptavidin binding, so we do not recommend it to be used as a quencher in the HAT SPA experiments.

Previously, Turlais *et al.*⁶ developed a radiometric assay utilizing the scintillating microplate (i.e., FlashPlate) to detect HAT activity. In their protocol, histone H3, [3 H]Ac-CoA, and HAT enzyme PCAF were incubated directly on the FlashPlate. In the HAT reaction, the tritium-labeled histone was captured by the walls of FlashPlate that contained immobilized scintillant molecules, generating a scintillation signal. That method apparently was also based on the principle of scintillation proximity. Interestingly, the authors did not show any interference from the Ac-CoA-histone interaction. It was possible that 150 mM of NaCl present in their assay buffer somehow diminished the undesirable cofactor-histone interaction. Compared with the FlashPlate assay, our assay

possesses additional advantageous features. The previous FlashPlate assay was heterogeneous in nature, because the histone substrate was adsorbed on the microplate surface throughout the enzymatic reaction. The solid surface may partially impact HAT-mediated histone acetylation. Further, the adsorption of histone H3 on FlashPlate was a nonspecific electrostatic interaction, so the efficiency of histone adsorption could be easily interfered with by buffer compositions.²¹ It was also difficult to assess the molecular stoichiometry of histone binding to the FlashPlate. The presence of a high salt concentration could be necessary to prevent cofactor–histone binding; however, it may also affect the efficiency of histone adsorption to the plate surface, which is needed for SPA detection. Furthermore, high salt concentration has the pitfall of inhibiting enzymatic activity of HATs, and it has been recommended that strong ionic strength be avoided in HAT reactions.^{22,23} In this study, we described a more clear SPA for HATs: The HAT reaction was conducted homogeneously in a buffer solution in a regular 96-well plate. After the HAT reaction, SPA beads were added so that the radiolabeled product was brought into proximity of the scintillant by the specific and potent biotin–streptavidin interaction. Importantly, we showed that using chemical agents such as guanidine HCl and NaHCO₃ effectively quenched the HAT reaction and meanwhile removed any nonspecific interaction between the acetyl-CoA and histone substrate. Using guanidine HCl or NaHCO₃ as a quencher is beneficial because of their intrinsic ionic properties as well as due to their being readily available and cheap in price. Guanidine HCl is a strong electrolyte that is known to be a strong protein denaturant.^{24,25} The chaotropic property of guanidine is very useful in this SPA for it assures that the HAT reaction ended once this reagent was added. The ability of guanidine HCl to break down the electrostatic substrate–cofactor interaction is critical to avoid false readings. At this point, we are not sure why NaHCO₃ can stop the HAT reaction. Possibly, NaHCO₃ may interact with the positively charged histone peptide, which prevents the substrate from entering into the active site of the HAT. Nevertheless, NaHCO₃ is an effective quencher-like guanidine HCl for this SPA experiment. Furthermore, in our studies, we found that 150 mM of sodium chloride or 30 mM of sodium phosphate (pH 8.0) fully eliminated the unwanted [³H]Ac-CoA–histone interaction (Supplementary Figs. S3 and S4). However, at those concentrations, neither sodium chloride nor sodium phosphate was capable of completely quenching the HAT activity (Supplementary Figs. S5 and S6).

Taken together, we have developed and validated an SPA to measure the enzymatic activity of HATs. This mix-and-measure assay does not require any washing procedures and can

be implemented in the microplate format. This SPA method is well suited for quantitative potency evaluation of HAT inhibitors and has the high-throughput capability to screen for new HAT chemical probes.

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DISCLOSURE STATEMENT

No competing financial interests exist.

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Abbreviations Used

HATs = Histone acetyltransferases

SPA = scintillation proximity assay

HTS = high-throughput screening

PMSF = phenylmethanesulfonyl fluoride

SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis