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A quantitative multiplexed mass spectrometry assay for studying the kinetic of residue-specific histone acetylation

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Abstract

Histone acetylation is involved in gene regulation and, most importantly, aberrant regulation of histone acetylation is correlated with major human diseases. Although many lysine acetyltransferases (KATs) have been characterized as being capable of acetylating multiple lysine residues on histones, how different factors such as enzyme complexes or external stimuli (e.g. KAT activators or inhibitors) alter KAT specificity remains elusive. In order to comprehensively understand how the homeostasis of histone acetylation is maintained, a method that can quantitate acetylation levels of individual lysines on histones is needed. Here we demonstrate that our mass spectrometry (MS)-based method accomplishes this goal. In addition, the high throughput, high sensitivity, and high dynamic range of this method allows for effectively and accurately studying steady-state kinetics. Based on the kinetic parameters from *in vitro* enzymatic assays, we can determine the specificity and selectivity of a KAT and use this information to understand what factors influence histone acetylation. These approaches can be used to study the enzymatic mechanisms of histone acetylation as well as be adapted to other histone modifications. Understanding the post-translational modification of individual residues within the histones will provide a better picture of chromatin regulation in the cell.

Keywords

histone; acetylation; post-translational modification; enzyme kinetics; mass spectrometry

1. INTRODUCTION

Histones are highly basic proteins that organize DNA in eukaryotic cells. This compact DNA-histone conformation limits accessibility to the DNA. Post-translational modification (PTM) of histones modulates DNA accessibility, which is one of the mechanisms that regulates gene transcription and DNA repair [1–3]. However, different modifications, or even the same modification found on a different site, can lead to different functions in cells. For example, acetylation on lysine 5 of H4 (H4K5) is related to histone deposit in many eukaryotes [4]. H3K56 acetylation is involved in DNA damage repair [3], while H3K14 acetylation is important for gene transcription *in vivo* [5]. In addition, aberrant regulation of lysine acetylation not only alters gene activation but also has been shown to correlate with

human diseases [6–9]. Thus, determining both the location and quantity of acetylation on histones is important to characterize how genes are regulated in response to DNA damage.

Lysine acetyltransferases (KATs) catalyze histone acetylation, which is the transfer of an acetyl group from acetyl-CoA to the lysine residues of a histone [10]. While histones usually have a positive charge, the addition of an acetyl group to a lysine residue results in neutralization of this charge, which in turn contributes to a decreased histone-DNA or nucleosome-nucleosome interaction. This increases the accessibility of DNA to enzymes, allowing for initiation of transcription, DNA replication, and DNA damage repair [1–5]. However, many KATs, such as p300 and Gcn5, are able to acetylate multiple lysine residues on histones and different acetylation sites can lead to different downstream effects [11–13]. Regarding this multiplexing ability, the acetylation specificity and selectivity of a KAT becomes adjustable by different factors such as chaperone complex or the addition of KAT activators/inhibitors. Note that specificity is the ability of a KAT to acetylate a specific residue on histones, while selectivity is the efficiency of a KAT to acetylate one site relative to another. Therefore, in order to understand the contribution to the histone acetylation by a particular KAT with or without the corresponding factors, we require a multiplexed technique to detect each potential site of histone acetylation simultaneously.

Although under ideal conditions conventional site-specific antibody methods can provide high specificity for detection of histone modifications, the drawback to this technique is that one antibody can only measure one modification of one location at a time and could be difficult to quantitate. In addition, varying quality of antibodies and the potential for epitope occlusion when utilizing antibodies may cause errors for quantitative measurements. These problems make it less feasible to have accurate quantification via antibody assays, not to mention how time consuming and arduous such a process would make the measurement of multiple residues and multiple samples from kinetic assays. While the use of radioactive or fluorescence methods can meet the criterion of being high throughput [14,15], it is only capable of measuring the total amount of acetylation, not site-specific amounts and are not capable of measuring histone modifications in cells. The approach we present herein has the advantage of being able to quantitate histone acetylation at multiple sites on multiple proteins at the same time and the label free nature of this approach allows for the ability to also quantitate modifications on histones extracted from cells.

To overcome these limitations, we have developed a label-free quantitative mass spectrometry (MS)-based method that is able to quantitate acetylation at all known sites of histone H3 and H4 in a single run [16,17]. Because we use a tandem MS, we can utilize the mode of selected reaction monitoring (SRM) to gain sensitivity and selectivity for peptide analysis. Briefly, SRM is used to detect the decomposition reactions (product ions) of the selected ions that are characteristic of individual peptides (parent ions). Thus, we are able to monitor specific parent-ion-to-product-ion transitions that are both unique to the peptides of interest and to the sites of modification. Here we describe the workflow for performing the kinetic analysis of a KAT, sample preparation for MS detection, and data analysis (Fig. 1). While our work allows examining the histone acetylation patterns of KATs on the histone monomer and tetramer, in a broader sense, this MS-based method can be applied to studying PTMs of different histone conformations (e.g. nucleosome) by those multi-targeting

enzymes, and can provide a rapid and accurate workflow for the determination of kinetic parameters of such enzymes.

2. MATERIAL AND METHODS

2.1 Steady-state experimental setup for histone acetylation

All Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher (Pittsburgh, PA) and the purity at least meets LC/MS grade. Ultrapure water was generated from a Millipore Direct-Q 5 ultrapure water system (Bedford, MA). Recombinant histone H3 and H4 were purified and provided from the Protein Purification Core at Colorado State University. H3/H4 was refolded from purified H3 and H4 using previously published methods [18,19]. KATs (e.g. p300, CBP, and Rtt109) were also prepared and purified following the reported procedures [16,20,21]. Protein molecular weight and purity was confirmed through SDS-PAGE with Coomassie stains. The concentrations of purified KATs and histones were determined by UV absorbance and calculated from the extinction coefficients [22,23].

To conduct steady-state kinetics with histone titration (0.15–10.3 μM), our enzyme concentrations need to be much less than substrate (histones) concentrations while using saturating acetyl-CoA concentration (200 μM). On the other hand, to conduct steady-state kinetics where we titrate acetyl-CoA (0.1–20 μM), we make substrate (acetyl-CoA) concentrations much larger than enzyme concentrations while saturating histones (10 μM). All kinetic assays were conducted under the identical buffer condition (100 mM HEPES buffer (pH 6.8) and 0.08% Triton X-100 at 37°C). Note that we need to adjust the enzyme amount (2–18 nM) and/or sampling time to ensure that the collected samples analyze the initial acetylation rates for each individual. To fulfill steady-state assumptions (i.e. that we are measuring acetylation events that occurs before more than 10% of the total substrate is acetylated), 5–8 different time points of each substrate concentration should be collected. In addition, substrate concentrations ranging from 0.25–5-fold of the Michaelis constant (K_m) should be used to sufficiently analyze steady-state kinetics [24].

2.2 Quench steps for enzyme kinetics

An efficient quenching reagent should immediately stop the acetylation at each time point and is key to achieving the accuracy of a kinetic assay. However, considering the incompatibility of numerous surfactants with MS detection, they cannot be used as a quench reagent. Thus, we examined the quenching efficiency of three different reagents (trichloroacetic acid (TCA), isopropanol, and acetone), which are compatible with MS detection. We found that 4 volumes of 100% TCA for 30-min incubation (on ice) was the most efficient quench procedure [17], which had no observable acetylation detected. However, there was maximum 2% and 5% acetylation found with isopropanol and acetone quench, respectively, for over-night 4°C incubation. Therefore, at varying time points, the collected samples were quenched with at least 4 volumes of 4°C TCA and cooled on ice for 30 min. Each precipitate was then washed twice with 150 μL acetone (–20°C). By doing so, excess salts and acetyl-CoA are removed, and individual samples can easily dry for either further processes or storage at –80°C.

2.3 Chemical derivatization and tryptic digestion of histones

There is a dilemma when selecting a protease to digest histones for MS analysis; that is, not all proteolytic enzymes are suitable for fragmenting histones. For example, a proteolytic peptide with over 20 amino acids may be too long to be detected by triple quadrupole MS. Some other proteases (e.g. Arg-C) need salts and/or surfactants to stimulate their activities and, more importantly, to ensure their reproducibility. All of those additives could hamper the precision and accuracy of MS analysis due to ion suppression. While trypsin can provide high reproducibility of digestion under minimum salts, the large numbers of lysines and arginines present on histones can result in fragmented peptides that are too small, losing backbone structural information. To overcome this drawback caused by tryptic digestion, we decided to chemically modify the lysines on histones prior to the addition of trypsin. When lysines are modified (either by enzymes or by chemicals), only arginines can be digested by trypsin. Thus, the sizes of fragmented peptides are appropriate for MS analysis to study histone acetylation. To differentiate chemical derivatization from the acetylation catalyzed by KATs, we chose to propionylate the unmodified lysines on histones [17,25,26]. With this derivatization, either unacetylated (propionylated) or acetylated lysines will be found on the identical peptide sequence. This not only avoids loss of detection for very short peptides generated by trypsin alone but also increases the hydrophobicity, as well as neutralizes the charge at the unacetylated lysine residuals, providing greater separation on the C-18 column. Thus, propionylation reduces the number of experimental steps, contributes to higher reproducibility of analysis, and simplifies data processing. This protocol has been successfully used to identify and quantify histone PTMs for several different research groups [27–29].

To propionylate the samples, we took the dried samples from section 2.2, sequentially added in 5 μ L water and 1.5 μ L propionic anhydride, and quickly titrated ammonium hydroxide to adjust the pH to ~ 8 [17,25]. Samples were then incubated at 51°C for 1 h. After the 1 h incubation, 30 μ L of 50 mM ammonium bicarbonate was added in, as the buffer for tryptic digestion. The amount of added trypsin depends on the amount of the proteins in each tube. A optimal trypsin:protein ratio is recommended ranging from 1:100 to 1:20 (w/w). Before incubation for tryptic digestion (overnight at 37°C), we ensure the final pH is ~ 8 by the titration of ammonium hydroxide. The addition of 1 μ L ammonium hydroxide is a good starting point. After tryptic digestion, the solution was transferred to a proper sample plate or an autosampler vial for MS analysis.

2.4 Chromatography and mass spectrometry analysis

An ultra-high performance liquid chromatography (UPLC Acquity H-class, Waters, Milford, MA) coupled to a triple quadrupole mass spectrometer (TSQ Quantum Access, Thermo, Waltham, MA) was used to quantify acetylated H3 and H4 peptides. The trypsin digested H3 and H4 peptides were injected into an Acquity BEH C18 column (2.1 $\times$ 50 mm; particle size 1.7 μ m) with 0.2 % formic acid (FA) aqueous solution (solution A) and 0.2 % FA in acetonitrile (solution B). Peptides were eluted over 11 min at 0.6 mL/min and 60°C, and the gradient was programmed from 95 % solution A and 5 % solution B and down to 80% solution A and 20% solution B in 11 min. The resolution power provided by UPLC can be equivalent to HPLC separation. However, UPLC provides the advantage being high

throughput, which is harder to achieve by HPLC and even nanoflow LC. The mass spectrometric conditions were: electrospray voltage: +4 kV; sheath gas pressure: 45 psi; auxiliary gas pressure: 20 psi; ion sweep gas pressure: 2 psi; collision gas pressure: 1.5 mTorr; and capillary temperature: 380°C. SRM is used to monitor the elution of the acetylated and propionylated H3 and H4 peptides in one sample run (i.e. a multiplexed assay). For SRM, doubly or triply charges were monitored for parent ions, whereas the product ions were detected under the singly charged state. The detailed mass transitions are shown in Table 1.

3. DATA ANALYSIS AND RESULTS

3.1 Validation of quantitative calculations

Each acetylated and propionylated peak was identified by retention time and specific mass transitions. The identification and integration of the resolved peaks were done using Xcalibur software (version 2.1, Thermo), and data was fit using Prism (version 5.0d).

$$F_s = I_s / I_p \quad \text{eq.1}$$

The fraction of a specific peptide (F_s) is calculated by eq. 1, where I_s is the intensity (integrated area) of a specific peptide state and I_p is the total intensity of any state of that peptide [30,31]. This relative quantitation is based on the assumption that within one specific tryptic peptide, there is no difference of ionization efficiency observed between acetylation and propionylation. To validate this assumption, we mixed two different states of modifications (i.e. $K_a\text{STGGK}_a\text{APR}$ and $K_p\text{STGGK}_a\text{APR}$) on individual synthetic peptides with different molar ratios and then analyzed by UPLC-MS/MS. A good linear regression, with the slope equal to one, indicates the same ionization efficiency between acetylation and propionylation within a peptide (Fig. 2).

3.2 Example - Gcn5 kinetic assays

Gcn5, the first identified KAT, is directly related to gene transcription in *Tetrahymena thermophila* [32], and the function of Gcn5 is highly conserved in eukaryotes [33–35]. Thus, we chose Gcn5 to study its specificity and selectivity for histone H3 acetylation. First, we conducted a time course assay to monitor H3 acetylation progress by Gcn5. By analyzing the peak intensities of individual peptides, the fraction of each peptide can be obtained. For example, the fraction of $K_a\text{QLATK}_a\text{AAR}$ (Histone H3 K18 to R26) can be calculated by the intensity of $K_a\text{QLATK}_a\text{AAR}$ divided by the summed intensities of $K_a\text{QLATK}_a\text{AAR}$, $K_p\text{QLATK}_a\text{AAR}$, $K_a\text{QLATK}_p\text{AAR}$, and $K_p\text{QLATK}_p\text{AAR}$, which are all possible states of this peptide (subscript a and p are acetylation and propionylation, respectively) (Fig. 3A and B). By monitoring multiple lysine residues on a single peptide, we can detect the site-specific acetylation order of a KAT, if that happens within this one tryptic peptide. Here we observed that Gcn5 preferentially acetylate H3K23 prior to H3K18 acetylation, because the appearance of H3K23 acetylation ($K_p\text{QLATK}_a\text{AAR}$) was followed by both K18 and K23 being acetylated ($K_a\text{QLATK}_a\text{AAR}$), and K18 acetylation by itself ($K_a\text{QLATK}_p\text{AAR}$) was only modestly quantitated (< 2%). However, we cannot detect acetylation order of a KAT if the site-specific acetylation of a KAT occurs on two or more different tryptic peptides,

because the intact acetylation pattern from individual histone proteins was lost after proteolytic cleavage. In addition, the fraction for a specific lysine acetylated can be calculated as the sum of all F_s that contain that acetylated lysine. For instance, the fraction of K23 acetylation can be obtained by summation of the fractions of K_aQLATK_aAAR and K_pQLATK_aAAR (Fig. 3C). Thus, we can quantitate the acetylation fraction on a specific residue and calculate the concentration of acetylation at specific lysine residues by simply multiplying the fraction by the initial concentration of histone.

$$\frac{v}{[E]} = k_{cat} \frac{[S]^{nH}}{([S]^{nH} + K_{(app)}^{nH})} \quad \text{eq.2}$$

The initial rates (v) of acetylation are calculated from the linear increase in either acetylation ratio or acetylation concentration as a function of time. To ensure all those data points obey the assumption of steady-state kinetic conditions and can be fit by Michaelis-Menten equation, we only use the data prior to the acetylation fractions reaching a total 10% of the substrates (Fig. 4). In addition, to measure steady-state parameters for acetyl-CoA titrations, the initial rates are calculated based on time where less than 10% of the acetyl-CoA is consumed. For the acetylation of specific lysines, the steady-state parameters k_{cat} , $K_{(app)}$ (i.e. $K_{m(app)}$ or $K_{1/2}$), and Hill coefficient (nH) can be determined by fitting eq. 2, where $[S]$ is the concentration of substrate (histone or acetyl-CoA), and $[E]$ is the concentration of KAT (Fig. 4). The Hill coefficient (nH) is only used when the appearance of sigmoidal kinetics is observed in the enzyme velocity curves (e.g. positive cooperativity) [36]. In classical terms, positive cooperativity represents multiple substrate binding sites on an enzyme where the first binding event increases the affinity of the second [37]. Thus, a sigmoidal curve occurs in the plot of velocity vs. substrate concentration. However, the appearance of a sigmoidal substrate dependence can also be found in a monomeric, single-site enzyme that has a slow transient enzyme conformation change that is stabilized by substrate binding [38].

Specificity of a KAT for each lysine is defined by the specificity constant, $k_{cat}/K_{m(app)}$ [39] (or $k_{cat}/K_{1/2}^{nH}$ if the sigmoidal curve is present [40]), which is a quantitative parameter to indicate the capability of a KAT to catalyze a site/substrate. In addition, we can compare the differences of these specificity constants between different lysines to understand how a KAT selectively acetylates one site over the other. When a KAT can catalyze multiple lysine acetylations, we need to have a complicated model to describe this competitive situation. However, from our previous study [17], we have demonstrated that if all these competitive sites either have no cooperativity or the same nH , the specificity constant of individual sites derived from eq. 2 (a one-site model) is conserved. Thus, we can use this advantage to characterize the acetylation specificity of a KAT for either single site or multiple sites with the same nH .

In order to understand the kinetics of secondary sites catalyzed by a KAT, one would need to prepare the histones with the primary site(s) already acetylated. For example, in our past study [17], Gcn5 primarily acetylates H3K14, so we prepared histone H3 protein with K14 acetylation (H3K14ac) by the previously published method [41,42] and followed the same

experimental procedures of aforementioned steady-state kinetic assays, sample processing, and data analysis. The specificity of Gcn5 for secondary sites can be quantitated; however, we should note that these constants may not directly compare with the specificity constant of primary site but could represent the specificity of a KAT only after the primary site(s) are completely acetylated.

4. CONCLUDING REMARKS

This multiplexed MS-based technique demonstrates a high throughput (47 peptides in 12 min) and sensitive analysis of the histone acetylation kinetics of a KAT *in vitro*. Most KATs are capable of catalyzing multiple lysines, and many factors (e.g. subunit proteins, histone chaperones, or external stimuli) could alter their specificity. Therefore, to comprehensively understand how acetylation is regulated by a KAT and how this acetylation is altered by other cellular factors, we used this multiplexed MS-based method that can simultaneously monitor all possible acetylated lysines on H3 and H4. This method provides the high sensitivity necessary to effectively detect the small fraction of acetylation (<10%) required for steady-state kinetic analysis. Using this MS-based method, we have characterized Gcn5-mediated histone H3 acetylation and have quantitated the Gcn5 specificity by calculating the specificity constants for individual lysine residues [17]. Through our studies, we have measured the specificity and selectivity of Gcn5 between the primary and secondary lysines on histone H3. We have also distinguished the different acetylation specificities of p300 and CBP, suggesting although they are highly conserved structural homologs, they each possess unique acetylation activities [16]. In addition, we have found that both the histone conformation (H3 vs. H3/H4) and/or the amount of acetyl-CoA available can alter the acetylation specificity of p300 and CBP.

In addition to these steady-state experiments, another strength of this MS-based method is that it can be applied to any histone H3 and/or H4 time course assay, catalyzed by any KAT. That is, in addition to steady-state kinetics, we can carry out single turnover assays or determine the kinetics of progress curves by this method. Furthermore, the histone substrate is not limited to histone H3 monomer or H3/H4. It can be expanded to any conformation of histones that incorporates H3 or H4; for example, tetrasome or nucleosome. In addition, we can examine the effect of pre-existing modification(s) on histones on the specificity of a KAT. This versatility will facilitate studies investigating such topics as histone crosstalk and the role of histone acetylation in chromatin dynamics.

Finally, it is important to note that the method presented here is a label-free assay. This fact has eliminated the labor associated with fluorescence labeling or the use of radioactive isotopes and has also reduced the generation of labeling contamination. Importantly, this fact means that this method is not only suitable for *in vitro* assays but also compatible with *in vivo* samples. Thus, this MS-based method can aid in monitoring the histone H3 and H4 acetylation profiles of a cell, which would be invaluable to further facilitating the pharmacodynamic studies of epigenetic therapy.

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Figure 1.
Experimental flow chart of the multiplexed MS-based assay.

Figure 2.

Comparison of the expected and detected molar fractions of K_aSTGGK_aAPR when mixing with different levels of synthetic peptide, K_pSTGGK_aAPR (the subscript a and p are acetylation and propionylation, respectively). The linear regression yields a $y=0.994x-2.26$ ($R^2=0.998$). This shows that the ionization efficiency has no significant difference between acetyl and propionyl modifications on a single peptide.

Figure 3.

Acetylation profiles of K(18)QLATK(23)AAR (the subscript a and p on lysines are acetylation and propionylation, respectively) obtained from *in vitro* KAT kinetic assay, where [acetyl-CoA] = 200 μ M, [H3] = 12 μ M, and [Gcn5] = 180 nM. (A) Chromatograms of all states of 18 KQLATKAAR 26 at $t=0$, 80 s, 10 min, and 60 min of KAT assay. The signals of individual peptides were normalized to the highest peak of each time point. (B) The percentage of each state of a peptide can be calculated for individual time points by eq. 1. This figure demonstrates that the acetylated peptides increase with time. (C) The acetylation percentage of a specific lysine can be calculated by summation of the percentage of all peptides that contain this acetylated lysine.

Figure 4.

Determination of steady-state kinetic parameters, fit by the Michaelis-Menten equation, for Gcn5-mediated K14 acetylation. Kinetic experiments were conducted where $[\text{acetyl-CoA}] = 200 \mu\text{M}$ with $0.15\text{--}10.3 \mu\text{M}$ H3 concentrations. The inset shows the determination of initial acetylation rate of $5 \mu\text{M}$ of histone H3 under steady-state condition.

Table 1

MS detection parameters of tryptic peptides from histone H3 and H4

Modification on lysines ^a	Peptide sequence	Parent ion (m/z)	Product ions (m/z)	Collision energy (eV)
H3 K4ac	³ TK _a QTAR ⁸	373.711	475.262, 645.367	16
H3 K4un	³ TK _p QTAR ⁸	380.706	475.262, 659.387	16
H3 K9ac-K14ac	⁹ K _a STGGK _a APR ¹⁷	493.275	570.335, 728.404, 815.437	20
H3 K9ac-K14un	⁹ K _a STGGK _p APR ¹⁷	500.270	584.355, 742.424, 829.456	20
H3 K9un-K14ac	⁹ K _p STGGK _a APR ¹⁷	500.272	570.335, 728.404, 815.437	20
H3 K9un-K14un	⁹ K _p STGGK _p APR ¹⁷	507.264	584.355, 742.424, 829.456	21
H3 K18ac-K23ac	¹⁸ K _a QLATK _a AAR ²⁶	535.819	659.383, 772.467	22
H3 K18ac-K23un	¹⁸ K _a QLATK _p AAR ²⁶	542.814	673.402, 786.486	22
H3 K18un-K23ac	¹⁸ K _p QLATK _a AAR ²⁶	542.816	659.383, 772.467	22
H3 K18un-K23un	¹⁸ K _p QLATK _p AAR ²⁶	549.809	673.402, 786.486	22
H3 K27ac-K36ac-K37ac	²⁷ K _a SAPATGGVK _a K _a PHR ⁴⁰	520.627	579.336, 905.531, 1231.690	26
H3 K27un-K36ac-K37ac	²⁷ K _p SAPATGGVK _a K _a PHR ⁴⁰	525.290	579.336, 905.531, 1231.690	26
H3 K27ac-K36ac-K37un	²⁷ K _a SAPATGGVK _a K _p PHR ⁴⁰	525.292	593.355, 919.550, 1245.709	26
H3 K27ac-K36un-K37ac	²⁷ K _a SAPATGGVK _p K _a PHR ⁴⁰	525.294	579.336, 919.550, 1245.709	26
H3 K27un-K36un-K37ac	²⁷ K _p SAPATGGVK _p K _a PHR ⁴⁰	529.953	579.336, 919.550, 1245.709	27
H3 K27un-K36ac-K37un	²⁷ K _p SAPATGGVK _a K _p PHR ⁴⁰	529.955	593.355, 919.550, 1245.709	27
H3 K27ac-K36un-K37un	²⁷ K _a SAPATGGVK _p K _p PHR ⁴⁰	529.957	593.355, 933.570, 1259.729	27
H3 K27un-K36un-K37un	²⁷ K _p SAPATGGVK _p K _p PHR ⁴⁰	534.616	593.355, 933.570, 1259.729	27
H3 K56ac	⁵⁴ YQK _a STELLIR ⁶³	646.864	744.461, 831.493, 1001.598	25
H3 K56un	⁵⁴ YQK _p STELLIR ⁶³	653.859	744.461, 831.493, 1015.588	26
H3 K64ac	⁶⁴ K _a LPFQR ⁶⁹	415.748	450.245, 547.298	17
H3 K64un	⁶⁴ K _p LPFQR ⁶⁹	422.742	450.245, 547.298	18
H3 K79ac	⁷³ EIAQDFK _a TDLR ⁸³	689.354	288.203, 936.478	27
H3 K79un	⁷³ EIAQDFK _p TDLR ⁸³	696.349	288.203, 950.497	27
H3 K122ac	¹¹⁷ VTIMPK _a DIQLAR ¹²⁸	476.274	600.382, 715.409, 885.515, 982.567	24
H3 K122un	¹¹⁷ VTIMPK _p DIQLAR ¹²⁸	480.938	600.382, 715.409, 899.534, 996.587	24
H4 K5ac-K8ac-K12ac-K16ac	⁴ GK _a GGK _a GLGK _a GGAK _a R ¹⁷	719.910	530.305, 757.432, 1211.685	25
H4 K5un-K8ac-K12ac-K16ac	⁴ GK _p GGK _a GLGK _a GGAK _a R ¹⁷	726.914	530.305, 757.432, 1211.685	25
H4 K5ac-K8un-K12ac-K16ac	⁴ GK _a GGK _p GLGK _a GGAK _a R ¹⁷	726.916	530.305, 757.432, 1225.701	25
H4 K5ac-K8ac-K12un-K16ac	⁴ GK _a GGK _a GLGK _p GGAK _a R ¹⁷	726.920	530.305, 771.447, 1225.701	25
H4 K5ac-K8ac-K12ac-K16un	⁴ GK _a GGK _a GLGK _a GGAK _p R ¹⁷	726.918	544.320, 771.447, 1225.701	25
H4 any 2 ac at K5-K8-K12-K16	⁴ GKGGKGLGKGGAKR ^{17, b}	733.926	530.305, 544.320, 757.432, 771.447, 785.463, 1225.701, 1239.717	25

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Modification on lysines ^a	Peptide sequence	Parent ion (m/z)	Product ions (m/z)	Collision energy (eV)
H4 K5un-K8un-K12un-K16ac	⁴ GK _p GGK _p GLGK _p GGAK _a R ¹⁷	740.929	530.305, 771.447, 1239.717	25
H4 K5un-K8un-K12ac-K16un	⁴ GK _p GGK _p GLGK _a GGAK _p R ¹⁷	740.931	544.320, 771.447, 1239.717	25
H4 K5un-K8ac-K12un-K16un	⁴ GK _p GGK _a GLGK _p GGAK _p R ¹⁷	740.935	544.320, 785.463, 1239.717	25
H4 K5ac-K8un-K12un-K16un	⁴ GK _a GGK _p GLGK _p GGAK _p R ¹⁷	740.933	544.320, 785.463, 1253.732	25
H4 K5un-K8un-K12un-K16un	⁴ GK _p GGK _p GLGK _p GGAK _p R ¹⁷	747.941	544.320, 785.463, 1253.732	25
H4 K31ac	²⁴ DNIQGITK _a PAIR ³⁵	684.386	727.446, 840.530, 897.552	23
H4 K31un	²⁴ DNIQGITK _p PAIR ³⁵	691.394	741.462, 854.546, 911.567	24
H4 K59ac	⁵⁶ GVLK _a VFLENVIR ⁶⁷	714.932	743.441, 890.509, 989.578	24
H4 K59un	⁵⁶ GVLK _p VFLENVIR ⁶⁷	721.940	743.441, 890.509, 989.578	25
H4 K77ac	⁶⁸ DAVTYTEHAK _a R ⁷⁸	666.831	553.321, 651.298, 946.474	23
H4 K77un	⁶⁸ DAVTYTEHAK _p R ⁷⁸	673.839	567.336, 651.298, 960.490	23
H4 K79ac-K91ac	⁷⁹ K _a TVTAMDVVYALK _a R ⁹²	839.963	371.229, 890.546, 1136.613	28
H4 K79un-K91ac	⁷⁹ K _p TVTAMDVVYALK _a R ⁹²	846.971	385.245, 890.546, 1136.613	28
H4 K79ac-K91un	⁷⁹ K _a TVTAMDVVYALK _p R ⁹²	846.973	371.229, 904.561, 1150.629	28
H4 K79un-K91un	⁷⁹ K _p TVTAMDVVYALK _p R ⁹²	853.979	385.245, 904.561, 1150.629	29

^a Acetylation and no acetylation on lysine are indicated as ac and un, respectively.

^b All mass transitions of di-acetylated ⁴GKGGKGLGKGGAKR¹⁷ are collected under one parent ion (m/z=733.926). We used the product ions to deconvolute 6 different states of double acetylation [43]. For example, the mass transitions 733.926→757.432 and 733.926→785.463 can only contribute from the peptides, ⁴GK_pGGK_pGLGK_aGGAK_aR⁹¹⁷ and ⁴GK_aGGK_aGLGK_pGGAK_pR¹⁷, respectively.