Isotopic Labeling and Quantitative Proteomics of Acetylation on Histones and Beyond

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Abstract

Lysine acetylation is an important post-translational modification (PTM) that regulates the function of proteins by affecting their localization, stability, binding, and enzymatic activity. Aberrant acetylation patterns have been observed in numerous diseases, most notably cancer, which has spurred the development of potential therapeutics that target acetylation pathways. Mass spectrometry (MS) has become the most adopted tool for not only the qualitative identification of acetylation sites, but also for their large-scale quantification. By using heavy isotope labeling in cell culture combined with MS, it is now possible to accurately quantify newly synthesized acetyl groups and other PTMs, allowing differentiation between dynamically regulated and steady-state modifications. Here, we describe MS-based protocols to identify acetylation sites and quantify acetylation rates on both proteins in general and in the special case of histones. In the experimental approach for the former, 13C-glucose and D3-acetate are used to metabolically label protein acetylation in cells with stable isotopes, thus allowing isotope incorporation to be tracked over time. After protein extraction and digestion, acetylated peptides are enriched via immunoprecipitation and then analyzed by MS. For histones, a similar metabolic labeling approach is performed, followed by acid extraction, derivatization with propionic anhydride, and trypsin digestion prior to MS analysis. The procedures presented may be adapted to investigate acetylation dynamics in a broad range of experimental contexts, including different cell types and stimulation conditions.

Keywords

histone acetylation; protein acetylation; epigenetics; mass spectrometry; acetylation dynamics; proteomics; isotopic labeling; post-translational modifications
1. Introduction

Post-translational modifications (PTMs), which can bestow different biophysical properties to otherwise identical sequences of amino acids, serve as a primary means by which protein function is regulated. While this paper focuses on methods dealing with the analysis of acetylation at lysine residues, especially the quantitative analysis of acetylation dynamics, acetylation represents just one of the many types of PTMs known to occur on proteins. Nonetheless, much is known about lysine acetylation and its effect on protein stability, localization, and function in the broader context of regulating cell signaling pathways and gene expression. Acetylation of lysine residues occurs through the catalytic activity of lysine acetyltransferases (KAT), which transfer the acetyl moiety from acetyl-CoA to the ε-amino group of a specific lysine residue \[1\]. Deacetylases (KDAC) mediate the removal of acetylation through a mechanism dependent on Zn\(^{2+}\) or NAD\(^{+}\)\[1,2\], making this PTM dynamic and reversible.

The first observation of protein acetylation occurred in the 1960s on histones \[3,4\], which provided a foundation for the modern field of epigenetics as it relates to histone modifications. In eukaryotic cells, histones associate closely with DNA to form the basic repeating unit of chromatin, the nucleosome, which consists of approximately 147 bp of DNA wrapped around a histone octamer composed of the core histones H2A, H2B, H3, and H4 \[5,6\]. In addition to providing a structural scaffold that permits packaging of DNA in cells, histone proteins have a major role in the regulation of genetic processes, such as DNA replication, DNA repair, and transcription \[7,8\]. In many cases, this regulation involves the N-terminal tails of histones, which protrude out from the nucleosome and are subject to a multitude of PTMs, including acetylation, methylation, phosphorylation, ubiquitination, and ADP ribosylation \[2,9–11\]. In the contemporary paradigm of histone modifications, these marks, which are thought to represent a combinatorial “histone code” \[12\], are placed by “writers,” recognized by “readers,” and removed by “erasers” \[13\]. Since the topic at hand is protein acetylation, further discussion is focused specifically on histone acetylation, which usually correlates with the transcriptional activation of a genomic locus. Histone acetylation is “written” by histone acetyltransferases (HATs), of which three main families exist: GNAT, MYST, and CBP/p300\[2,8,9\]. Compared to other histone modifying enzymes, histone acetyltransferases generally lack strict site selectivity, as exemplified by p300, which targets H3K14, H3K18, H4K5, and H4K8 \[8,11\]. Responding to the actions of histone acetyltransferases, “reader” proteins, such as PCAF and SMARCA4 \[14,15\], can then bind to the acetylated lysine residues via their bromodomains, thereby promoting additional modifications, transcription, or other biological processes. Finally, histone deacetylases (HDACs), which belong to one of four classes (I-IV), act as “erasers” of acetylation to return histones to an unmodified state \[2,9\]. Beyond serving as a mechanism for the recruitment of regulatory proteins, histone modifications are also thought to affect nucleosome structure. For example, compared to unmodified lysine residues, which carry a net positive charge at physiological pH, acetylated lysine residues are neutral. Thus, acetylation may weaken electrostatic interactions between histones and the DNA, possibly affording greater accessibility to the transcriptional machinery \[9,16\].
Beyond histones, acetylation has been detected on more than 80 transcription factors as well as many other nuclear, cytoplasmic, and mitochondrial proteins with significant effects on protein function [17–19]. For example, acetylation has been found to increase the stability of some proteins, such as the tumor suppressor p53, the transcriptional activator HNF6, and the ATP-dependent helicase WRN, but decrease the stability of others, such as the transcription factor GATA1. Specifically, treatment with the deacetylase inhibitor trichostatin A (TSA) not only increased the levels of acetylated p53, as expected, but also increased the half-life of p53, suggesting that acetylated p53 is more stable [20]. Similarly, for HNF6, increased levels of the acetyltransferase CBP led to increased acetylation of HNF6 with a concomitant increase in its half-life [21]. Acetylation of WRN at residues K366, K887, K1117, K1127, K1389, and K1413 by CBP was shown to suppress its ubiquitination, thus increasing its stability [22]. On the other hand, acetylation of GATA1 appears to signal its ubiquitination and subsequent degradation. TSA treatment resulted in decreased levels of GATA1, which was recovered via treatment with the proteasome inhibitor MG-132 [23]. Acetylation can also modulate, in either positive or negative fashion, the ability of a protein to interact with binding partners, as in the case of the transcriptional activator STAT3, the structural protein actin, and the molecular chaperone HSP90[24]. Acetylation can also affect the localization of proteins. For instance, acetylated PCAF and SRY localize to the nucleus, whereas the acetylated p300 localizes to the cytosol [24]. Autoacetylation of lysine residues within the nuclear localization signal (NLS) of acetyltransferase PCAF is required for PCAF nuclear localization, potentially by affecting its interaction with nuclear import factors or by driving conformational changes [25]. Acetylation of K136 on SRY, a transcriptional regulator involved in male development, increases the SRY-importin β interaction three-fold, resulting in nuclear localization of SRY [26]. Given that acetylation is important in regulating protein activity, abnormal acetylation can perturb the normal functioning of biological processes and become pathological. In particular, aberrant acetylation can lead to disease states such as tumorigenesis, cancer cell proliferation, persistence of DNA damage due to misregulation of repair machinery, dysregulation of the immune response, and neurodegeneration [1,24,27,28]. In fact, bioinformatics studies on cancers show that mutations may arise more frequently at acetylation sites compared to sequences of amino acids without PTMs [29]. Although numerous studies have focused on mapping acetylation sites across the proteome [19,30], it is also critical to consider the dynamics of acetylation at these sites. Studying acetylation dynamics can help improve understanding of the kinetics, usage, recycling, and regulation of this modification, while also providing insights about protein function. Specifically, the dynamics of acetylation indicate which sites are added or removed quickly, thereby providing information about the activity of the KATs or KDACs responsible for regulating these sites. In addition, knowledge of acetylation dynamics for a particular protein may give insight as to the role of the protein in its signaling pathway, such as whether the protein is part of a transient or more sustained signaling network. Since acetylation can also affect protein-protein interactions, knowledge of the dynamics of these sites may reveal the duration over which the protein interacts with its partners, perhaps to accomplish a given function. Acetylation on some proteins is also known to affect protein stability and localization, implying that characterization of the dynamics of the acetylation sites may provide hints about protein half-life or whether a protein spends more time in one compartment of the cell compared to another.
Protein-modifying enzymes rely on small molecule co-factors as donors of the chemical groups being transferred to proteins, and in some cases (e.g., some demethylases and deacetylases), as essential components in the reaction mechanism responsible for their removal [2,31]. Since the levels of these co-factors reflect the activity of various metabolic pathways, it is now appreciated that cell metabolism interfaces with cell signaling through PTMs [32,33]. As mentioned, protein acetylation originates from acetyl-CoA, which bridges both glycolysis and fatty acid oxidation to the tricarboxylic acid (TCA) cycle for oxidative phosphorylation and the generation of ATP [31,34]. Acetyl-CoA is produced primarily in the mitochondria by β-oxidation of fatty acids or by the action of the pyruvate dehydrogenase complex on pyruvate derived from glycolysis. This mitochondrial acetyl-CoA then enters the TCA cycle to produce citrate, which is subsequently exported from the mitochondria into the cytoplasm and nucleus, where it is converted back into acetyl-CoA for acetylating cytoplasmic and nuclear proteins (Figure 1) [35,36]. Thus, the metabolic state of a cell determines the relative levels of acetyl-CoA, a higher level of which might promote increased amounts of protein acetylation as the excess acetyl-CoA not entering the TCA cycle is available to KATs [34].

Given its capability for high-resolution and high-throughput measurements, MS provides a powerful platform for the unbiased analysis of proteins and PTMs. The latter are identified and localized by detecting mass adducts on individual amino acids in peptide fragmentation spectra (MS/MS). Because high resolution MS is remarkably accurate (<1–2 ppm mass error), it can easily resolve mass shifts caused by isotopes. Thus, it is frequently used in conjunction with labeling techniques based on metabolic incorporation of amino acids or small molecules bearing stable isotopes, such as deuterium, carbon-13, and nitrogen-15. Therefore, an informative way to track the dynamics of PTMs is to exploit the metabolic pathways that underlie their synthesis. Under normal cell culture conditions, acetyl-CoA is derived primarily from glucose, though acetate may also contribute in some circumstances [35,37]. Accordingly, protein acetylation can be labeled using 13C-labeled glucose and acetate, as demonstrated in a study of histone acetylation dynamics [38]. Other PTMs, including methylation and phosphorylation, have also been studied through stable isotope labeling with 13CD3-labeled S-adenosylmethionine and 18O4-labeled ATP, respectively [39,40]. In contrast to other metabolic labeling strategies, such as SILAC [41], in which heavy amino acids serve as the vehicle by which proteins acquire isotopic labels on the peptide backbone or sidechains but not on PTMs, supplying cells with isotopically labeled small molecule precursors to label PTMs provides additional information. For instance, treating cells with heavy glucose or acetate not only enables an assessment of acetylation dynamics, but also an assessment of which molecule contributes more efficiently to lysine acetylation on a protein by protein basis. However, different acetylation sites may exhibit unique dynamics, even when on the same protein, highlighting the necessity of using mass spectrometry to analyze acetylation sites in a broad and high-throughput manner. Mass spectrometry also has the added potential of identifying novel sites of acetylation.

In the following protocol, we describe methods for the quantitative analysis of protein acetylation dynamics (Figure 2) followed by a focus on histone proteins (Figure 5) by using MS and stable isotope labeling. Each protocol begins by metabolically labeling cells with stable isotopes (Section 3.1). In the case of non-histone proteins, cells are lysed to extract...
and digest proteins (Section 3.2.1) followed by enrichment for acetylated peptides by immunoprecipitation with anti-acetyl antibodies (Section 3.2.2) [42]. In the case of histone proteins, cell nuclei are isolated and treated with acid to extract histones, which are then derivatized and digested (Section 3.3). Finally, peptides are analyzed by MS to detect (Sections 3.2.3, 3.3.2) and quantify (3.3.3, 3.2.4) the relative ratio between light and isotope labeled (heavy) acetyl groups as outlined in section 3.3.3.

2. Materials

2.1 Solutions and Buffers

2.1.1 Solutions and buffers for general protein acetylation analysis

1. Joklik medium: Joklik Minimum Essential Medium, 10% newborn calf serum (NCS), 1X GlutaMax, 1X Penicillin/Streptomycin

2. 13C-glucose medium: Joklik Minimum Essential Medium (ingredients list is available online, add everything except light glucose), 2.5 g/L 13C6-glucose, 2 mM Alanine, filter using a 0.2 μm Nalgene disposable filter and then add 10% dialyzed fetal bovine serum (dFBS), 1X GlutaMax, 1X Penicillin/Streptomycin

3. D3 -acetate media: Joklik Minimum Essential Medium (ingredients list is available online, add everything including light glucose), 2 mM Alanine, 10 mM D3-acetate, filter using a 0.2 μm Nalgene disposable filter and then add 10% dialyzed fetal bovine serum (dFBS), 1X GlutaMax, 1X Penicillin/Streptomycin

4. Lysis buffer: 6 M urea, 2 M thiourea, 50 mM ammonium bicarbonate, pH 8.2, 1X Halt Protease Inhibitors Cocktail or equivalent, 10 mM sodium butyrate, 10 mM dithiothreitol (DTT)

5. Liquid chromatography coupled to MS (LC-MS) Buffer A: 0.1% formic acid (FA) in MS grade water

6. LC-MS Buffer B: 80% HPLC-grade acetonitrile (ACN), 0.1% FA in MS grade water

7. 50 mM triethylammonium bicarbonate (TEAB)

8. Column pre-equilibration buffer for the desalting column procedure: 75% ACN

9. Equilibration and sample wash buffer for the desalting column procedure: 0.1% Trifluoroacetic acid (TFA)

10. Elution buffer for desalting procedure: 60% ACN, 0.1% TFA

11. Immunoaffinity purification buffer (IAP) buffer 1X: 50 mM MOPS/NaOH pH 7.2, 10 mM Na2HPO4, 50 mM NaCl

12. Immunoaffinity purification elution buffer: 0.15% TFA

13. Stage-tip elution buffer: 75% ACN, 0.1% TFA

14. 30 mM iodoacetamide (IAA)
2.1.2. Solutions and Buffers for analysis of histone protein acetylation

1. Growth medium: DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 10% fetal bovine serum and 1X penicillin/streptomycin.

2. Labeling medium: DMEM with L-glutamine without glucose or pyruvate, supplemented with 10% dialyzed fetal bovine serum, 1X penicillin/streptomycin, 2 mM alanine, and desired concentration of unlabeled or labeled substrate e.g. 25 mM unlabeled glucose or $^{13}$C$_6$-glucose.

3. Trypsin-EDTA solution (0.25%).

4. Nuclear isolation buffer (NIB): 15 mM Tris pH 7.5, 15 mM NaCl, 60 mM KCl, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 250 mM sucrose, stored at 4°C and supplemented with 1 mM dithiothreitol (DTT), 500 μM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 5 nM microcystin, and 10 mM sodium butyrate prior to use.

5. 10% NP-40 Alternative.

6. 0.4 N Sulphuric acid H$_2$SO$_4$, stored at 4°C.

7. 100% trichloroacetic acid (1 g/ml in water), stored at 4°C.

8. 0.1% HCl in acetone, stored at −20°C.

9. 0.1M Ammonium bicarbonate NH$_4$HCO$_3$.

10. Trifluoroacetic acid, 1% and 10%.

11. Bradford assay reagent, cuvettes, and spectrophotometer.

12. Reversed-phase (RP) LC buffer A (0.1% formic acid in water)

13. RPLC buffer B (80% acetonitrile with 0.1% formic acid)

2.2 Equipment

2.2.1 Equipment for general protein acetylation analysis

1. Humidified 37 °C incubator with 5% CO$_2$

2. 1.5 ml microcentrifuge tubes

3. 15 and 50 mL conical tubes

4. 1 L spinner flasks

5. 500 mL centrifuge bottles

6. 5 mL Eppendorf tubes

7. Pipettes (P10, P20, P200, P1000) and tips

8. −80°C freezer

9. 4°C cold room
10. Centrifuge
11. Table-top centrifuge
12. 0.2 μm Nalgene disposable filter
13. Cell culture hood
14. Cell counter
15. Vortex mixer
16. Sonicator probe
17. Spectrophotometer
18. Rotator
19. Speed-vac
20. Sep-Pak C18 Vac columns or equivalent, 50 mg sorbent and 55–105 μm particle size
21. Vacuum manifold
22. C8 filter paper
23. Repro-Sil Pur C18-AQ 3 μm resin for analytical column for nanoLC
24. Appropriate nanoflow (nanoLC-MS) setup: HPLC with at least two channels (one for buffer A/loading buffer and one for buffer B) and high-resolution MS
25. Autosampler vials

2.2.2 Equipment for histone acetylation analysis
1. Humidified 37°C incubator with 5% CO2 atmosphere
2. Tissue culture flasks and dishes (10 cm)
3. 15 ml conical tubes
4. Centrifuge
5. Liquid nitrogen
6. Freezer (−80°C and −20°C)
7. 1.5 ml tubes
8. Rotator
9. 4°C cold room
10. Pasteur pipettes
11. C18 stage tips and buffers (0.1% trifluoroacetic acid [TFA], 0.5% acetic acid, and 50% acetonitrile with 0.1% formic acid)
12. C18 columns (75 μm i.d. × 15–20 cm fused silica packed with 3 μm ReproSil-Pur 120 C18-AQ from Dr. Maisch GmbH)
13. Pressure bomb set-up
14. Appropriate nanoflow (nanoLC-MS) setup: HPLC with at least two channels (one for buffer A/loading buffer and one for buffer B) and high-resolution MS

3. Methods

3.1 Isotope labeling of cell cultures

3.1.1 Isotope labeling of suspension cell cultures for general acetylation analysis

1. From a single established culture, sub-culture HeLa suspension cells into 4 spinner flasks using Joklik medium and grow the cultures until they reach 1 liter in volume at \(4 \times 10^5\) cells/mL (see Note 1 for recommendations on number of replicates and information on growing HeLa cells).

2. After reaching 1 liter in volume, collect 120 mL of each of the four cultures at a cell confluence of about \(4 \times 10^5\) cells/mL for a baseline unlabeled control (time point 0) for each replicate culture.

3. Pellet cells at 140 \(\times\) g for 5 minutes in a centrifuge at room temperature. Discard the supernatant.

4. Wash cells by resuspending in 6 mL PBS and centrifuge as in step 3. Discard the supernatant.

5. Repeat step 4 (2 washes total) and then freeze the cell pellet at - 80°C until ready to begin sample processing (Section 3.2.1, step 1).

6. Pellet the remaining volume of each culture at 140 \(\times\) g for 5 minutes at room temperature, discard the supernatant, and wash twice with 10 mL PBS. Resuspend two of the four pellets in 1 L of 2.5 g/L\(^{13}\)C-glucose medium. Resuspend the other two pellets in 1 L of 10 mM D\(_3\) acetate medium. In this manner, there are two biological replicates for each labeling method. Note: 2 mM alanine is added to the labeling media to minimize incorporation of \(^{13}\)C\(_3\) - labeled alanine derived from pyruvate [38].

7. Incubate cells at 37°C. Following the procedures in steps 3–5, collect cells from each culture at 0.5, 1, 4, 8, 12, 16, and 24 hours after transfer into isotopically labeled medium (see Note 2 for explanation on why specific time points were chosen). Assess cell density and aim to collect approximately \(4.8 \times 10^7\) cells at each time point, which will be around 120 mL for the earlier time points (0–8 hours) and 100 mL for the later time points (12–24 hours).

3.1.2 Isotope labeling in cell culture for histone acetylation analysis

1. Plate cells in growth medium and incubate at 37°C overnight such that they will reach roughly 50% confluency the next day (see Note 3). Prepare one plate for each replicate or treatment. As a control, include at least one plate for untreated cells or, preferably, cells treated with unlabeled substrate (See Note 4).
2. Remove growth medium and rinse cell monolayer with 10 ml PBS.
3. Add 10 ml of labeling medium containing isotope-labeled substrate and incubate at 37°C for the desired amount of time (See Note 5).
4. When ready to harvest cells, transfer the conditioned medium from the cell monolayer to a 15 ml tube, rinse with 10 ml of PBS, add 2 ml of trypsin-EDTA, and swirl briefly to distribute evenly. Incubate for approximately 5 mins at room temperature until cells detach. As an alternative to detachment with trypsin, cells may be harvested with a scraper. For cells grown in suspension, collect cells in 15 ml tube and skip to step 6.
5. When cells appear rounded and detached, add the conditioned medium (or alternatively, fresh growth medium) back to the 10 cm dish to inactivate the trypsin. Pipette up and down briefly to create a homogenous single cell suspension and transfer to the 15 ml tube.
6. Pellet cells at 1000 rpm (218 × g) for 5 mins at room temperature.
7. Aspirate the supernatant and gently resuspend the cell pellet in 10 ml of PBS to wash.
8. Pellet cells at 1000 rpm (218 × g) for 5 mins at room temperature.
9. Aspirate the supernatant.
10. Flash freeze the cell pellet by carefully immersing in liquid N₂ for 5–10 seconds.
11. Store cell pellets at −80°C until ready to proceed with sample preparation.

3.2 Identification of acetylation sites and quantification of protein acetylation rates

3.2.1 Cell lysis and trypsin digestion

1. Lyse the cell pellets in 2.5 times the pellet volume of cold lysis buffer (for example: to a 300 ul pellet add 750 ul lysis buffer) by vortexing followed by sonication on ice in the cold room (See Note 6 for suggestions on sonication).
2. Incubate the samples for 30 minutes at room temperature.
3. Alkylate reduced cysteine residues with 30 mM iodoacetamide (ΓAA) for 30 minutes at room temperature in the dark.
4. A Bradford assay may be conducted to determine protein concentration. Proceed with an equal amount of protein for each time point sample for further processing (see Note 7 for suggestions on protein amount).
5. Digest proteins first with endopeptidase Lys-C for 3 hours to enhance digestion efficiency. Use 100 μg (0.38 AU) of Lys-C per 10 mg of protein sample.
6. Dilute the solution with five volumes of 50 mM triethylammonium bicarbonate (TEAB) to dilute out the urea from the lysis buffer, as it will interfere with trypsin digestion.
7. Add sequencing grade trypsin at an enzyme-to-substrate mass ratio of approximately 1:50 and incubate for 12 h at room temperature.

8. Desalt the peptide digest using Sep-Pak C_{18} Vac cartridges, 50 mg sorbent and 55–105 micron particle size and a vacuum manifold. To do so, pre-equilibrate the columns with 3 mL (3X column volume) of 100% ACN and then 2 mL of 75% ACN. Equilibrate with 2 mL 0.1% TFA. Acidify the sample with TFA to pH 3. Load peptide samples and run through column, and then re-load the flow-through on the column for a total of two loads. Wash the loaded sample on the column with 1 mL 0.1% TFA. Elute the sample with 500 ul of 60% ACN + 0.1% TFA into a clean collection tube and repeat once for a total of two elutions.

9. Dry samples using a speed-vac to complete dryness.

3.2.2 Enrichment for acetylated peptides—Enrich for acetylated peptides by immunoaffinity purification using the PTMScan Acetyl-Lysine Motif Kit (Cell Signaling Technologies) following the manufacturer’s protocol. The protocol is briefly described below.

1. Resuspend the peptide samples in 1X immunoaffinity purification buffer (IAP) (Cell Signaling Technologies). (See Note 8 about saving an input fraction)

2. Wash the antibody-bead slurry four times with 1mL 1X PBS, centrifuging at 2,000 × g in a table top centrifuge for 30 seconds to pellet the beads after each wash, and remove previous PBS before adding new PBS for next wash. After the last wash, resuspend the beads as a 50% slurry.

3. Incubate the peptide solutions with the beads overnight at 4C while rotating.

4. After incubation, wash the beads with IAP buffer two times, followed by three washes with MS grade water. Complete the wash steps on ice. Between each wash step, centrifuge at 2,000 × g in a table top centrifuge for 30 seconds to pellet the beads so that the supernatant can be carefully removed.

5. Elute acetylated peptides from the beads using 75 μl of 0.15% TFA, and let stand at room temperature for 10 minutes, mixing gently by tapping the bottom of the tube several times every 2–3 minutes. Centrifuge for 30 seconds at 2,000 × g in a table top centrifuge to collect the elution. Repeat the elution/centrifugation steps for a second elution using 150 ul of 0.15% TFA. After collecting the second elution, combine both eluates in a single tube.

6. Remove any beads that may be present in the eluate by stage-tipping, as described in other studies [43], but with C_{8} filter paper to act as a plug. Most of the peptides will flow through the C_{8} paper while the beads are retained, so make sure to collect the flow-through. Elute any other possible binding peptides from the stage-tips in 75% ACN+0.1% TFA

7. Completely dry the elutions in a speed-vac.
3.2.3 NanoLC-MS/MS analysis of acetylated peptides

1. Prepare a picofrit 18 cm long fused silica capillary column (75 μm inner diameter, 360 μm outer diameter) by packing with reversed-phase Repro-Sil Pur C<sub>18</sub>-AQ 3 μm resin for peptide loading. Preparing a nanoLC column has been described previously [44]. Briefly, cut a 75 μm inner diameter fused silica capillary column at about 30 cm. Pull a tip at one side of the capillary with a laser tip puller. Add Repro-Sil Pur C<sub>18</sub>-AQ 3 μm resin to a clean HPLC glass vial and resuspend in 100% methanol or another organic solvent for packing the column. Add a miniature stir bar to the glass vial. Place the glass vial with the resin slurry in a pressure (gas) bomb and turn on the magnetic stirrer. Place the pulled silica column in the pressure bomb and turn on the pressure to pack the column. Make sure that the pressure does not exceed the maximum pressure on the gas bomb. After the column is packed, cut the column to a final length of 15–18 cm.

2. Set up a 120 minute gradient following the conditions outlined in Table 1. Use a flow rate of 300 nl/min.

3. Set up the MS method to perform data-dependent acquisition (DDA). Here, we describe the method with an Orbitrap Fusion Tribrid mass spectrometer; however, the DDA can be performed with any high resolution (>20,000 FWHM) MS instrument using an optimized shotgun proteomics method for medium-complex peptide mixtures. If using an Orbitrap Fusion, perform the full MS scan in the Orbitrap at 120,000 resolution (FWHM at 200 m/z) with a mass range of 350–1200 m/z and an AGC target value of 5×10<sup>5</sup>. Perform DDA MS/MS using TopSpeed mode with the Quadrupole as the isolation mode, and the Orbitrap as the detector. Dynamic exclusion should be set for 60 seconds. Set HCD collision energy to 27, AGC target to 5e4, and maximum injection time to 200 msec.

4. Resuspend dried samples in 12 μL of LC-MS buffer A (0.1% formic acid), transfer 11 μl to autosampler vials, and inject 5 ul onto an Easy-nLC system (Thermo) coupled online with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Other HPLC and MS systems may be used instead, depending on to which instruments the lab has access.

3.2.4 Database searching and quantification of protein acetylation

1. Perform database searching with MS raw files using software of choice, such as pFind [45,46], MaxQuant [47], or vendor associated software. In this specific study we illustrate data processing and analysis by using pFind, setting the search parameters as listed in Table 2.

2. Filter the output file for a list of all of the unique acetylated peptides that were identified along with the precursor m/z, charge state, retention time, search score, peptide sequence, modifications, and the respective protein accession numbers.

3. Calculate the fraction of heavy labeled acetylation over total acetylation (heavy acetyl<sup>13</sup>C/ (heavy acetyl<sup>13</sup>C+ light acetyl<sup>12</sup>C) for each acetylated peptide. To
calculate the fraction of heavy labeled acetylation over total acetylation, extract the area under the curve (AUC) of the chromatographic peaks corresponding to the monoisotopic peak (M0), the M+2 peak, and the M+4 peak (+2 and +4 Da, respectively; see Figure 3 for representative mass spectra). An unlabeled reference for each peptide should be used to calculate the natural abundance of the M+2 isotope (due to natural occurrence of $^{13}$C). This abundance, extracted by integrating the AUC, should be subtracted when estimating the intensity of the labeled acetylation M+2 peak. In presence of doubly acetylated peptides, this calculation should be repeated also for the M+4 signal. The sum of M+2 AUC and M+4 AUC is divided by the sum of M0, M+2, M+4 AUC to calculate $^{13}$C/$^{13}$C+$^{12}$C, the fraction of heavy labeled acetylation.

4. To visualize the heavy acetylation incorporation, it may be useful to graph the ratio of heavy acetylation incorporation calculated in step 3 $^{13}$C/($^{13}$C+$^{12}$C) plotted against the time as shown in Figure 4.

5. The half-time of heavy acetyl incorporation can be calculated by fitting the ratios of $^{13}$C/($^{13}$C+$^{12}$C) acetylated peptides at each time point to the best fit equation. This can be done using Matlab. Previously, it has been determined by Matlab that the best fit equation is $y=a*(1- e^{-x/b})$. Here, y and x are the input data: y is the ratio $^{13}$C/($^{13}$C+$^{12}$C), and x is the time point (hours). The constants a and b are the output of the fitting curve function, and vary for each peptide based on the data. A Matlab script for calculating the half-life for each peptide given their heavy acetylation ratio at each time point can be distributed upon request.

6. If after the latest time point (i.e., 24 hours) the amount of $^{13}$C labeled acetylation does not reach 0.5, the linear polynomial equation $y=ax+b$ can be used to calculate the heavy acetylation incorporation half-life.

7. Reproducibility between the two biological replicates across all quantified acetylated peptides can be assessed using Pearson correlation to plot the correlation between time points, and correlation significance (t conversion). We recommend a calculated p-value < 0.05 for significance.

8. After converting the quantification values into ratios of heavy form / total heavy + light form, the significance of the monotonic increase of the ratio across the time points can be assessed using the Mann-Kendall test on the average of the two biological replicates (p-value < 0.05).

9. Once data are filtered for significantly regulated acetyl sites, further analysis of the data can include, but not be restricted to, hierarchical clustering of trends using e.g. Perseus [48], Gene Ontology enrichment analysis of acetylated proteins with e.g. Gorilla [49], visualization of known protein-protein interactions with e.g. STRING [50], and further network visualizations with e.g. Cytoscape [51].

3.3 Quantitative analysis of histone acetylation rates

3.3.1 Histone extraction, derivatization, and proteolytic digestion—The following procedure is similar to previously published protocols [43,44,53,54].
1. Supplement NIB with 1 mM dithiothreitol (DTT), 500 μM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 5 nM microcystin, and 10 mM sodium butyrate prior to use. Prepare 3 ml buffer for every 100 μl of packed cell volume (e.g. 30 ml for 10 tubes of 100 μl cells) plus a few ml extra to account for pipetting errors. Remove 1/3 of prepared NIB to another tube and to this aliquot, add NP-40 Alternative to a final concentration of 0.2%.

2. Resuspend cell pellet in NIB containing 0.2% NP-40 Alternative. Incubate 10 mins on ice to lyse cells. Use approximately 1 ml buffer per 100 μl of packed cells.

3. Pellet lysed cells at 500 x g for 5 mins at 4°C.

4. Remove the supernatant (cytosolic contents) and resuspend the pellet (nuclei) in NIB without NP-40 alternative to wash. Use the same volume of NIB here as used in step 12.

5. Pellet nuclei at 500 x g for 5 mins at 4°C.

6. Repeat steps 4–5.

7. Remove the supernatant of the second wash and resuspend the nuclear pellet in 0.4 N (0.2 M) H₂SO₄ to extract histones. Use a volume of H₂SO₄ equal to one half of the volume of NIB used in step 1.

8. Rotate at 4°C for 2–4 hrs.

9. Pellet insoluble debris by centrifugation at 3400 x g for 5 mins at 4°C.

10. Transfer the supernatant, which contains histones, to a 1.5 ml tube. Precipitate proteins by adding 1 volume of 100% trichloroacetic acid to 3 volumes of supernatant (e.g. 167 μl trichloroacetic acid to 500 μl acid extract).

11. Mix well and incubate overnight on ice in a 4°C cold room.

12. Pellet precipitated protein by centrifugation at 3400 x g for 5 mins at 4°C.

13. Remove supernatant and wash pellet by adding approximately 500 μl cold 0.1% HCl in acetone with a glass Pasteur pipette.

14. Centrifuge as in step 12.

15. Remove supernatant and wash pellet by adding approximately 500 μl cold acetone with a glass Pasteur pipette.

16. Centrifuge as in step 12.

17. Remove supernatant, leave the tube uncapped, and allow the pellet to air dry for 5–10 mins on the bench.

18. Resuspend the pellet in 0.1 M NH₄HCO₃. Use approximately 20–40 μl per 100 μl of original cell pellet, which should result in a protein concentration of roughly 2–4 μg/μl. Check pH with pH paper and, if necessary, add additional NH₄HCO₃ salt or concentrated (e.g. 1 M) NH₄HCO₃ so that the pH is 7–8.
19. Pellet insoluble debris by centrifugation at 17,000 x g for 5 mins at room temperature.

20. Measure protein concentration of supernatant by Bradford assay.

21. For each sample, place 20 μg of protein in a 1.5 ml tube. Add 0.1 M NH₄ HCO₃ to a final volume of 20 μl. See Note 9.

22. Add 10 μl of 25% propionic anhydride in 2-propanol (e.g. 10 μl propionic anhydride to 30 μl 2-propanol) to each 20 μl sample (scale accordingly if using different volumes). Add 2–3 scoops of NH₄HCO₃ salt using a beveled P1000 pipette tip to counteract acidification caused by the reaction as the derivatization is less efficient at acidic pH. Ammonium hydroxide may also be used [43,44,53,54]. The pH of the reaction should be approximately 8. Incubate for 15 mins in a 37°C water bath. See Note 10.

23. Dry samples in a speed-vac. See Note 11.

24. Resuspend samples in 20 μl of 0.1 M NH₄HCO₃ and repeat steps 22–23.

25. Resuspend samples in 20 μl of 0.1 M NH₄HCO₃, ensure the pH is 7–8 with pH paper, and add 1 μg trypsin for every 20 μg of protein.

26. Incubate overnight at room temperature.

27. Repeat steps 22–23 twice to derivatize the newly generated N-termini by propionylation.

28. Resuspend samples in 1% trifluoroacetic acid (TFA) and add additional concentrated 10% TFA as necessary so that the pH is 2–3. Pellet insoluble debris by centrifugation at 17,000 x g for 5 mins at room temperature.

29. Desalt supernatant with C18 stage tips. See Note 12.

30. Dry samples in speed-vac and resuspend in 0.1% formic acid at approximately 1 μg/μl. Pellet insoluble debris by centrifugation at 17,000 x g for 5 mins at room temperature, and then transfer the supernatant to an autosampler vial and proceed to analysis by LC-MS/MS. See Note 13.

3.3.2 Nano LC-MS/MS analysis of acetylated histone peptides

1. Pack a column with C₁₈ material and connect it to a nano liquid chromatography system coupled to a mass spectrometer.

2. Set the chromatography gradient as listed in Table 3. Use a flow rate of 300 nl/min.

3. Program the mass spectrometer for data-independent acquisition (DIA), which results in fragmentation of all peptide ions at a given retention time to facilitate quantitation of isobaric peptides bearing the same marks at different positions. To cover a mass range from 300 to 1100 m/z, each instrument cycle consists of 16 DIA MS/MS scans of 50 m/z isolation width with two full MS scans, one at the beginning and one after the eighth MS/MS scan ([54],[55]). Full scans are
performed with the orbitrap in positive profile mode from 300 to 1100 m/z at a resolution of 120,000. DIA MS/MS scans are performed in the ion trap in centroid mode using CID fragmentation with a normalized collision energy of 35%, activation Q of 0.25, activation time of 10 ms, and maximum injection time of 50 ms. The mass range of each MS/MS scan is varied slightly depending on the isolation window. Table 4 outlines the scan settings.

4. Inject approximately 1 μg of histone peptides and collect data. See Note 14.

3.3.3 MS data analysis for quantification of histone acetylation analysis

1. We analyze MS data with a custom Matlab script called EpiProfile [56], which reports the chromatographic peak areas of acetylated histone peptides bearing either unlabeled or $^{13}$C$_2$-labeled acetyl groups and then calculates the relative abundance of each. For a given acetylation event (e.g. H4K12ac), the percent of $^{13}$C-labeling is obtained by dividing the relative abundance of the $^{13}$C-labeled form by the sum of the relative abundances of the $^{13}$C- labeled and unlabeled forms. The script is freely available from the Garcia Lab upon request (see http://hosting.med.upenn.edu/garcialab/resources/). Any labeling due to naturally occurring $^{13}$C can be subtracted out based on the percent $^{13}$C-labeled peptide found in the unlabeled control cells. Peak areas may also be manually extracted, using software such as Xcalibur, based on the expected m/z value of a peptide of interest, keeping in mind to add the mass of a propionyl group (+56.0262 Da) for the N-terminus and every unmodified or mono- methylated lysine residue, the mass of a methyl group (+14.0157 Da) for every methylation event (e.g. multiply by 3 for tri-methylation), the mass of an acetyl group for every acetylation event (+42.0106), and the mass of a proton (+1.0073) based on the charge state z (usually +2 but occasionally +3). The $^{13}$C-labeled form of an acetylated peptide should be (+2n)/z units greater than the unlabeled form, where n equals the number of labeled acetyl groups. As before, the relative abundance of isotopic labeling is calculated by dividing the peak area of the $^{13}$C-labeled peptide by the sum of the peak areas of the unlabeled and $^{13}$C-labeled forms. See Notes 15 and 16. Representative mass spectra data are shown in Figure 6. An example of a plot showing acetylation kinetics for peptides from H3 is depicted in Figure 7.

4. Notes

1. At least two replicates are strongly suggested for each isotopically labeled substrate, which is why this protocol calls for splitting the HeLa cultures into four separate cultures. However, the number of replicates can be varied by splitting the HeLa cultures accordingly. The optimal concentration for HeLa cell growth is 2 × 10$^5$ cells/mL. Culture the cells by diluting them to 2 × 10$^5$ cells/mL with Joklik medium every day. We recommend starting this labeling experiment at 4 × 10$^5$ cells/mL so that there will be a larger yield of protein for each time point without the cells becoming overly dense at the later time points. Variation between 2 – 4 × 10$^5$ cells/mL is fine, but avoid starting with a culture that is denser than 4 × 10$^5$ cells/mL because the culture will be overgrown by the later
time points, which may affect cell viability. Growing cells to a volume of 1 L will allow 120 ml of cells (at an approximate density of $4 \times 10^5$ cells/ml for a total of $4.8 \times 10^7$) to be collected across 8 time points. If larger amounts of starting material are desired, it is possible to grow the HeLa cultures to a volume larger than 1 L so that a larger volume may be collected at each time point.

2. The time points 0.5, 1, 4, 8, 12, 16, and 24 hours, as listed in this study, are recommended in order to get a better analysis of heavy acetylation incorporation trends. The 0 time point is the control sample that is harvested from the unlabeled media, as described in the protocol. The specific time points chosen and the number of time points may be varied, but it is recommended that at least 6 time points are used, as 6 degrees of freedom has good statistical power in correlation analysis. Additionally, the initial time points after switching to heavy media should be closer together (0, 0.5, 1, 4) compared to the later time points in order to get a better record of the initial incorporation. Time points up to 24 hours are recommended because it has been previously shown in HeLa cells that the average turnover rate of proteins is ~20 hours [52].

3. For instance, add $3 \times 10^6$ HEK293 cells to 10 ml of growth medium in a 10 cm dish. Scale appropriately for different sizes of culture vessels. This number of HEK293 cells should yield approximately 50–100 μg of histone extract, which is well above the target of 20 μg.

4. To monitor kinetics, a time course may be performed by adding substrate and then harvesting cells at various times after addition. Alternatively, a reverse time course may be performed in which substrate is added at various times ahead of a set time for harvesting all treatments at once (e.g. add substrate at t - 24 hrs and t - 2 hrs and harvest at time t).

5. The substrate and its concentration are specified by the user based on the desire to know how much a given carbon source contributes to acetylation. The only requirement is that the substrate provides carbon for acetyl-CoA production. We have specified $^{13}$C$_6$-labeled glucose as an example as mentioned in section 2.1.2 in the labeling medium composition. In the case of isotopic labeling with $^{13}$C$_6$-glucose, exogenous unlabeled alanine may be added to suppress the appearance of the isotope label on amino acids instead of the acetyl group due to conversion of glucose to alanine via pyruvate [38].

6. Sonication of cells in lysis buffer was performed at power setting 4 on a Sonic Dismembrator Model 100 for 3 cycles of 10 seconds with 30 second breaks in between. The duration or number of sonication cycles may be varied as necessary to reduce sample viscosity. Remember to keep the tubes in ice in the cold room, as sonication generates heating, which could result in chemical derivatization of the proteins by urea.

7. For large scale analysis, a total amount of 10 mg of protein per sample is suggested to use as input for the digestion. However, the amount of protein used for digestion can be varied, depending on the amount of total protein extracted.
from the cells at each time point. It is important that an equal amount of protein is used for each time point for digestion and further processing.

8. If you would like to calculate the percent enrichment achieved using the anti-acetyl lysine antibodies, save a fraction of the input from each of your time point samples before adding them to the anti-acetyl lysine beads. Analyze these input fractions by MS (with the same MS conditions as for the time point samples) in order to compare the number of acetylated peptide identifications in the enriched sample versus the input sample. In addition, saving an input fraction is useful to account for any changes in protein expression levels that may appear as changes in acetylation levels.

9. Alternative amounts and volumes may be used, though starting with less than 10–20 μg may impact the quality of results. Excessively large volumes will also require additional drying time. Any remaining histones may be stored at −80°C.

10. In this step, N-termini and unmodified and mono-methylated lysine residues are derivatized by propionylation to block trypsin cleavage at lysine residues, which, given the high lysine content of histones, would otherwise produce smaller peptides that are not suitable for LC-MS/MS analysis. Additionally, the propionyl group increases peptide hydrophobicity, resulting in better retention by the C18 resin. Prepare fresh propionylation reagent every 3–5 samples and complete this step rapidly by performing the whole process (up to the incubation) on no more than 3–5 samples at a time. Be aware that adding NH4HCO3 salt to the reaction will cause some bubbling as gas is released, which could lead to a build-up of pressure in the tube and make the cap prone to popping, though this rarely happens in our experience. To extend its shelf life, propionic anhydride can be stored under a thin layer of argon gas.

11. Drying times may vary greatly, and sometimes, a translucent pellet remains. In our experience, adding more ammonium bicarbonate salt seems to result in faster drying times.

12. Briefly, we create C18 stage tips by cutting out a circular piece (1–2 mm diameter) of C18 material (C18 Empore, 3M) with a shortened P1000 pipette tip (cut a few mm off the end with a razor blade to obtain a larger diameter bore) and packing it into a P200 pipette tip with a piece of capillary. The stage tip is mounted with an adaptor on 1.5 ml tubes for centrifugation at 1000 x g for 1 min (or more time or higher speed as necessary) to force liquid through the resin. The resin is activated with 30 μl methanol, equilibrated twice with 30 μl 0.1% TFA, loaded with sample, and washed twice with 30 μl 0.1% TFA and once with 30 μl of 0.5% acetic acid with the collected liquid being discarded as necessary such that the tip does not contact the waste liquid. The desalted peptides are then eluted with 30 μl 50% ACN/0.1% formic acid into a clean 1.5 ml tube.

13. We assume 50% sample loss throughout the procedure. We typically resuspend the dried peptides in 12 μl, pellet the insoluble debris, and then remove 10 μl to the sample vial to avoid transferring any particulates into the sample vial, which
could clog the LC column. We also centrifuge the sample vials briefly (1–2 mins at 3400 x g) before placing in the autosampler for the same reason and to eliminate any air bubbles. If peptides will not be analyzed immediately, they can be stored at −20°C.

14. We typically inject 2 μl of sample. If the protein yield was low in step 31, a larger volume can be injected.

15. Beyond the incorporation of $^{13}$C$_2$-labeled acetyl groups, an additional cause of a shift in the isotopic distribution, which is not accounted for here, may be the incorporation of $^{13}$C$_3$-labeled alanine derived from pyruvate [38]. Alanine is added to the labeling medium to minimize the contribution of this pathway. Fragmentation spectra can be inspected to confirm that the isotopic shift is due to labeling of acetylated lysines.

16. Statistical analysis of $^{13}$C isotope incorporation into histone peptides may be performed in the same manner as in the case of non-histone proteins (section 3.2.4).

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Figure 1. Metabolic labeling pathway.
Acetyl-CoA is produced in the mitochondria from glucose and fatty acids. Mitochondrial
acetyl-CoA then enters the tricarboxylic acid (TCA) cycle to produce citrate, which is
exported out of the mitochondria into the cytoplasm and nucleus. Citrate is converted back
into acetyl-CoA for use in acetylating cytoplasmic and nuclear proteins. Acetate also
contributes to both the cytoplasmic and nuclear pool of acetyl-CoA, and is incorporated into
acetylation of proteins. The cartoon cell matter were slightly modified from Servier Medical
Art [57].
Figure 2. Workflow for tracking acetylation of non-histone proteins.
The basic workflow for studying acetylation dynamics of non-histone proteins involves collection of samples at specified time points, followed by cell lysis and peptide digestion, enrichment of acetylated peptides, and nano-LC MS/MS analysis of the acetylated peptides on any high-resolution mass spectrometer that is suitable for proteomics. The cartoon lab equipment were slightly modified from Servier Medical Art [57].
Figure 3. Representative spectra of acetylated peptides with heavy isotope incorporation from $^{13}$C$_6$-glucose and D$_3$-acetate over time. Isotopic patterns indicate heavy acetylation incorporation over time. The red dashed rectangle highlights the monoisotopic peak, and the green dashed rectangle highlights the heavy labeled peak. The isotopic patterns at 0, 8, and 24 hours are shown. The L- lactate dehydrogenase peptide by glucose labeling is an example of a peptide that shows minimal incorporation of heavy labeling by 24 h, whereas the ATRX peptide by glucose labeling exemplifies a substantial increase in heavy labeling incorporation by 24 h. The remodeling and spacing factor 1 peptide is an example of a peptide that does not show an increase in heavy labeling incorporation from 0 to 24 h, whereas the mediator of RNA polymerase II transcription subunit 1 peptide does show a mild increase in heavy labeling from 0 to 24 h.
**Figure 4. Analysis of acetylation rates on non-histone proteins.**

Graph demonstrates the ratio of heavy acetylation incorporation $^{13}$C/($^{13}$C+$^{12}$C) plotted against time. The half-time of heavy acetyl incorporation was calculated by fitting the ratios of $^{13}$C/($^{13}$C+$^{12}$C) acetylated peptides at each time point to the equation $y=a*(1-e^{-x/b})$. If after 24 hours the amount of $^{13}$C labeled acetylation did not reach 0.5, as shown by the lactate dehydrogenase peptide, we used the linear polynomial equation $y=ax+b$. The red dashed line demonstrates the half-life for the ATRX K898ac site.
Figure 5. Workflow for tracking acetylation of histone proteins.
To analyze acetylation dynamics on histone proteins, cells are incubated with isotopically labeled glucose (Section 3.1.2). After harvesting the cells, nuclei are isolated and then treated with acid to extract histones, which are derivatized with propionic anhydride and digested with trypsin (Section 3.3.1). Digested peptides are propionylated once more to derivatize newly generated N-termini and then analyzed by LC-MS/MS (Section 3.3.2 and 3.3.3).
Figure 6. Representative spectra of acetylated histone peptides.
The mass spectrum of the doubly charged H3 peptide^{18}KQLATKAAR^{26} bearing acetylation at K18 is shown for HEK293 cells treated with unlabeled glucose (Control) or with 25 mM^{13}C_{6}-glucose for various amounts of time. The relative abundance of the +2 Da peak at 571.84 m/z increases over time, indicating the presence of an acetyl group with two carbon-13 atoms.
Figure 7. Kinetic analysis of histone acetylation.
The relative abundance of the isotope-labeled species is plotted over time for several distinctly modified H3 peptides from HEK293 cells treated with 25 mM $^{13}$C$_6$-glucose (mean ± standard deviation of technical replicates). The residue bearing the labeled acetyl group is indicated in the legend with an asterisk.
Table 1. Chromatography gradient for analysis of non-histone peptides.

Non-histone peptides may be separated by online nanoLC using the gradient listed.

| Time (mins) | Buffer B (%) |
|------------|--------------|
| 0          | 3            |
| 100        | 38           |
| 115        | 98           |
| 120        | 98           |
Table 2.
Parameters for database searches using pFind.

Peptide identifications may be obtained by running MS raw files through the pFind database search tool using the parameters listed above.

| Parameter               | Value                                                                 |
|-------------------------|----------------------------------------------------------------------|
| Sequence Database       | Human UniProt FASTA database [tax id: 9606]                         |
| Precursor tolerance for Orbitrap | 10 ppm                                                               |
| Fragment tolerance for Orbitrap | 0.02 Da                                                              |
| FDR                     | 0.01                                                                |
| Enzyme specificity      | Trypsin                                                             |
| Fixed modifications     | Cysteine carbamidomethylation (+57.021 Da)                          |
| Modifications           | Lysine acetylation (+42.026 Da)                                      |
|                         | Methionine oxidation (+15.995 Da)                                    |
Table 3. Chromatography gradient for analysis of histone peptides.

Histone peptides may be separated by online nanoLC using the gradient listed above.

| Time (mins) | Buffer B (%) |
|------------|--------------|
| 0          | 5            |
| 45         | 33           |
| 50         | 98           |
| 60         | 98           |
Table 4.
Mass spectrometry scan settings for the analysis of histone peptides by data-independent acquisition (DIA).

Histone peptides may be analyzed by tandem mass spectrometry using the instrument cycle shown.

| Scan # | Type      | Isolation Window Center (m/z) | Mass Range (m/z) |
|--------|-----------|-------------------------------|------------------|
| 1      | Full MS   | -                             | 300–1100         |
| 2      | DIA MS/MS | 325                           | 120–1500         |
| 3      | DIA MS/MS | 375                           | 120–1500         |
| 4      | DIA MS/MS | 425                           | 120–1500         |
| 5      | DIA MS/MS | 475                           | 130–1500         |
| 6      | DIA MS/MS | 525                           | 140–1500         |
| 7      | DIA MS/MS | 575                           | 155–1500         |
| 8      | DIA MS/MS | 625                           | 170–1500         |
| 9      | DIA MS/MS | 675                           | 185–1500         |
| 10     | Full MS   | -                             | 300–1100         |
| 11     | DIA MS/MS | 725                           | 195–1500         |
| 12     | DIA MS/MS | 775                           | 210–1500         |
| 13     | DIA MS/MS | 825                           | 225–1500         |
| 14     | DIA MS/MS | 875                           | 240–1500         |
| 15     | DIA MS/MS | 925                           | 250–1500         |
| 16     | DIA MS/MS | 975                           | 265–1500         |
| 17     | DIA MS/MS | 1025                          | 280–1500         |
| 18     | DIA MS/MS | 1075                          | 295–1500         |