CHAPTER ONE

Identification and Quantification of Histone PTMs Using High-Resolution Mass Spectrometry

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Contents

Abstract

DNA is organized into nucleosomes, composed of 147 base pairs of DNA wrapped around an octamer of histone proteins including H2A, H2B, H3, and H4. Histones are

critical regulators of many nuclear processes, including transcription, DNA damage repair, and higher order chromatin structure. Much of their function is mediated through extensive and dynamic posttranslational modification (PTM) by nuclear enzymes. Histone PTMs are thought to form a code, where combinations of PTMs are responsible for specific biological functions. Here, we present protocols to identify and quantify histone PTMs using nanoflow liquid chromatography coupled to mass spectrometry (MS). We first describe how to purify histones and prepare them for MS. We then describe three MS platforms for histone PTM analysis, including bottom-up, middle-down, and top-down approaches, and explain the relative benefits and pitfalls of each approach. We also include tips to increase the throughput of large experiments.

1. INTRODUCTION

DNA must be highly organized and tightly regulated within the nucleus to maintain proper gene expression. The cell accomplishes this task by organizing DNA into a protein–DNA complex called chromatin. Within chromatin, DNA is contained in nucleosomes, which are composed of 147 base pairs of DNA wrapped around an octamer of histone proteins with two copies of each core histone—H2A, H2B, H3, and H4 (Luger, Mäder, Richmond, Sargent, & Richmond, 1997). Linker histone H1 can bind the free DNA that exists between nucleosomes. Due to their intimate association with DNA, histones are major regulators of chromatin structure and function. Histone proteins are extensively and dynamically posttranslationally modified on specific residues by a myriad of enzymes in the nucleus. These posttranslational modifications (PTMs) mediate histone function by directly altering the chemistry of the surrounding chromatin or through the action of other proteins that can bind these modifications. A growing body of research supports the hypothesis that PTMs form a "histone code" and can act in tandem to illicit a specific biological response (Jenuwein & Allis, 2001). Histone PTM profiles are critical to maintain nuclear stability, and aberrant regulation of histone PTMs is implicated in many diseases including cancer. As such, the ability to identify and quantify histone PTMs in biological systems is vital for understanding nuclear processes and how disease states may arise.

Histone PTM analysis had traditionally been accomplished using antibody–based approaches such as Western blots, chromatin immunoprecipitation, and deep sequencing. These methods have been instrumental in elucidating the roles of many histone PTMs but suffer from several critical

drawbacks (Britton, Gonzales-Cope, Zee, & Garcia, 2011). For example, many antibodies are not entirely site specific and can cross-react with similar modifications on different residues. A similar issue is epitope occlusion, where a PTM on a nearby residue can block interaction with an antibody. Perhaps the biggest drawback is that these methods require previous knowledge of the modification and are therefore unable to identify novel PTMs (Rothbart et al., 2015).

Mass spectrometry (MS) is an unbiased and quantitative method to comprehensively analyze histone PTM profiles. One of the greatest advantages of MS is that it can identify novel PTMs and can also measure the cooccurrence of PTMs on a single peptide. As such, MS has emerged as a critical tool for characterization of histone modifications. There are three major MS approaches, namely, bottom–up, middle–down, and top–down MS, each of which is useful for specific applications (Fig. 1). Bottom–up MS involves digestion of a protein sample into small peptides (5–15 amino acid residues) followed by online separation by reversed phase chromatography coupled to tandem MS. This method is very robust and sensitive. One major drawback, however, is that the cooccurrence between PTMs located on different peptides cannot be measured. Top–down MS, on the other hand, is performed by directly analyzing intact proteins and, as such, preserves complete connectivity between PTMs. However, this method is much less sensitive than bottom–up MS and thus has much larger sample
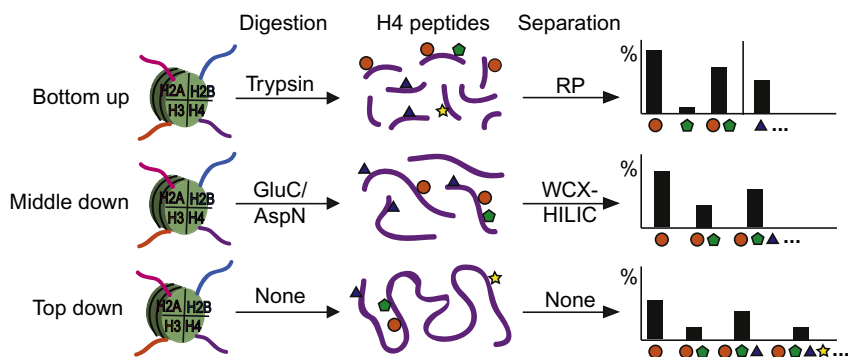
Figure 1 Workflows for bottom-up, middle-down, and top-down histone PTM analysis by high-resolution tandem MS. In bottom-up MS, the relative abundances and PTM cooccurrences can be monitored for PTMs contained within a single tryptic peptide. Longer peptides are generated in middle-down MS, allowing for better connectivity than bottom-up MS. In top-down MS, full connectivity is preserved, allowing for identification of complete protein isoforms.

requirement. Furthermore, the data analysis is much more challenging due to the large complexity of the tandem mass spectra, which sometimes results in the impossibility of discriminating proteoforms when cofragmented. Using chip-based infusion, rather than injection with a syringe, can reduce the sample requirement for top-down experiments that do not include online chromatographic separation. Middle-down MS offers a compromise between these two methods and is performed by digesting proteins into large peptides (about 30–60 amino acid residues). Analyzing large peptides allows for connectivity of many PTM locations, but offers better sensitivity and simpler data analysis than the top-down approach.

Bottom-up MS is the most commonly used MS approach for histone PTM analysis as it is technically more facile than the other approaches and does not require specialized equipment such as a 2D HPLC or chip-based electrospray ionization. Furthermore, small peptides generated in bottom-up MS are fragmented by collision-induced dissociation (CID) or high-energy collision dissociation (HCD), which is available in most commercial instruments. Large peptides or intact histone proteins, however, result in high charge state analytes when electrospray ionized and therefore do not fragment well with CID or HCD. Electron transfer dissociation (ETD) fragmentation is highly efficient for highly charged peptides or proteins and it is thus the fragmentation technique of choice for middle-down and top-down approaches.

In this chapter, we present the protocol to isolate histone proteins from cells and prepare the protein for MS analysis. We also outline how to perform bottom-up, middle-down, and top-down MS to identify and characterize histone PTMs. Tips to increase the throughput of experiments are also included.

2. HISTONE EXTRACTION FROM CELLS

Histones are among the most basic proteins in the cell, and as such, can be easily purified using an acid extraction. Here, we describe how to first isolate nuclei from cells and then perform an acid extraction to obtain purified histones. The whole protocol takes 1 day.

2.1 Materials and Buffer Recipes

1. 0.25% Trypsin
2. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$·2H$_2$O, 2 mM KH$_2$PO$_4$

3. Nuclei isolation buffer (NIB): 15 mM Tris–HCl (pH 7.5), 60 mM KCl, 15 mM NaCl, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 250 mM sucrose
4. 1 M DTT
5. 200 mM AEBSF
6. 2.5 µM Microcystin
7. 5 M Sodium butyrate

2.2 Cell Harvest

2.2.1 Cell Harvest: Tissue Samples

1. Rinse tissue sample with cold PBS (4°C). This can be stored at −80°C after flash freezing or used immediately.
2. Use a razor blade to cut the tissue into small pieces, as small as possible.
3. Record the approximate volume of the tissue sample.

2.2.2 Cell Harvest: Cell Cultures

1. For adherent cells, detach the cells by covering the plate with a thin layer of trypsin 0.25% for 5 min at room temperature. Move cells to a centrifuge tube and spin at 300 rcf for 5 min. For cells grown in suspension, centrifuge cells at 300 rcf for 5 min. Aspirate the trypsin or pour it off into bleach (10% final concentration).
2. Wash with PBS (10 × volume). Centrifuge cells at 600 rcf for 5 min and remove PBS.
3. Repeat step 2. Pellets can be stored at −80°C or used immediately.

2.3 Nuclei Isolation

1. If cell pellets were frozen, thaw them on ice.
2. Estimate the amount of NIB that will be needed, which is approximately 50 × the total volume of cell pellets. Chill the buffer on ice.
3. Add inhibitors to the NIB to the final concentrations of 1 mM DTT, 500 µM AEBSF, 5 nM microcystin, and 10 mM sodium butyrate. For 50 mL buffer, add 50 µL of 1 M DTT, 125 µL of 200 mM AEBSF, 100 µL of 2.5 µM microcystin, and 100 µL of 5 M sodium butyrate. Inhibitors will degrade over time, so prepare NIB + inhibitors freshly for each experiment and store inhibitor stocks at −20°C.
4. Move 25% of the NIB + inhibitors to a new beaker and add 10% NP-40 alternative to a final concentration of 0.3%. For 50 mL, add 1.5 mL of 10% NP-40 alternative.
5. Resuspend cell pellets in 10 × volume of NIB with NP-40 and homogenize by gentle pipetting (cultured cells) or douncing (tissue samples).

6. Incubate on ice for 5 min to lyse outer cell membranes. Nuclei isolation efficiency can be approximated using Trypan Blue staining.
7. Centrifuge at 4°C for 5 min at 1000 rcf. The supernatant contains the cytoplasm and can be reserved if desired. Otherwise, discard the supernatant. The pellet contains nuclei and should be smaller than the original cell pellet.
8. Wash the pellet by resuspending it in NIB + inhibitors without NP-40 alternative (10 × volume of cell pellet).
9. Centrifuge cells at 4°C at 1000 rcf for 5 min.
10. Repeat steps 8 and 9 until no NP-40 remains. Usually, two washes are sufficient. NP-40 forms bubbles when resuspending and so lack of bubbles indicates successful removal of NP-40.
11. Nuclei can be stored in NIB + inhibitors + 5% glycerol at −80°C after freezing in liquid nitrogen or used immediately for acid extraction.

2.4 Acid Extraction

1. Gently vortex the nuclei pellet and slowly add 0.4 N H_2SO_4 (5 × volume of pellet, ie, add 5 mL H_2SO_4 to a 1 mL pellet).
2. Incubate at 4°C with intermittent mixing or on a rotator for 1 h up to overnight. We recommend incubation of 2 h for pellets larger than 500 μL or 4 h for pellets smaller than 500 μL. Longer incubation can result in extraction of other basic proteins besides histones.
3. Centrifuge extracts at 3400 rcf for 5 min. The supernatant contains the histone proteins and the pellet contains other proteins.
4. Transfer the supernatant to a new tube.
5. Repeat steps 3 and 4 to remove any traces of the pellet.
6. Gently add 100% TCA to the supernatant to a final concentration of 20% (ie, add 1/4 volume of the supernatant).
7. Incubate on ice for at least 1 h without agitation. For most samples, 1 h is sufficient. We recommend overnight incubation for small samples (<50 μL pellet).
8. Centrifuge at 3400 rcf for 5 min. The histones will form a film around the bottom of the tube. Other proteins and nonprotein material will form a white pellet at the bottom of the tube, which cannot be solubilized.
9. Carefully aspirate the supernatant, avoiding the protein film on the side of the tube.

10. Wash the protein with acetone + 0.1% HCl by pipetting gently down the side of the tube. Use a glass pipettor as acetone will dissolve plastic pipette tips.
11. Centrifuge at 3400 rcf for 5 min. Aspirate acetone.
12. Repeat steps 10 and 11 using 100% acetone two times.
13. Allow remaining acetone to evaporate by leaving the tubes open for 30 min up to overnight.
14. Resuspend the histone film in ddH_2O. The volume will depend on the size of the tube and pellet, but generally 100 μL is sufficient for pellets in a 1.5 mL microcentrifuge tube.
15. Centrifuge at 3400 rcf for 2 min.
16. Move supernatant to new tube, being careful to avoid the pellet, which can be discarded.
17. Measure the protein concentration using a Bradford assay or another method. Histones can be stored in ddH_2O at −80°C. If samples are dilute, concentrate them in a vacuum centrifuge.
18. If doing bottom-up MS, continue with Section 3. If doing middle-down or top-down MS, continue with Section 4 then 5 or 6, respectively.

3. BOTTOM-UP MASS SPECTROMETRY

Bottom-up MS is the most commonly used MS platform for proteomics. In bottom-up MS, proteins are digested into small peptides (5–15 amino acid residues) with trypsin, which are then separated with online reversed phase high-performance liquid chromatography (RP-HPLC) and analyzed via tandem MS (MS/MS). Histones are among the most basic proteins in the cell and contain a large number of lysine and arginine residues. Therefore, digestion with trypsin results in peptides that are too small to be retained by RP chromatography. To overcome this issue, histone proteins can be chemically derivatized on the ξ-amino groups of unmodified or monomethylated lysine residues (Garcia et al., 2007). This derivatization prevents trypsin cleavage after lysine residues, allowing the enzyme to cleave only after arginine residues thus generating longer peptides. We recommend using propionic anhydride as the derivatization reagent due to its high efficiency (Sidoli et al., 2015). After digestion with trypsin, a second round of derivatization is performed to modify the amino groups of the newly generated N-termini. This increases the hydrophobicity of the peptides and

allows for better interaction with RP columns. Propionylation and trypsinization can be performed either in microcentrifuge tubes or in 96-well plates to reduce sample preparation time if a large number of samples are being processed.

3.1 Materials and Buffer Recipes

1. 100 mM ammonium bicarbonate, pH 8.5
2. Ammonium hydroxide (NH$_4$OH)
3. Propionic anhydride
4. Isopropanol
5. pH paper
6. Trypsin
7. Glacial acetic acid
8. Vacuum centrifuge
9. C18 Disc (3M Empore)
10. Methanol (MeOH)
11. Wash buffer: 0.1% acetic acid in water
12. Elution buffer: 75% acetonitrile, 5% acetic acid, 20% water
13. Buffer A: 0.1% formic acid in water (all MS grade solvents)
14. Buffer B: 0.1% formic acid in 75% acetonitrile/25% water (all MS grade solvents)

3.2 Derivatization and Digestion

1. Dry samples down in a vacuum centrifuge and resuspend in 20 μL ammonium bicarbonate, pH 8.5. Ensure that the pH of the samples is between 7 and 9 using pH paper. If they are too basic, add some glacial acetic acid. If they are too acidic, add some powdered ammonium bicarbonate. If using the plate format, transfer samples to a 96-well plate.
2. Prepare the propionylation reagent by combining propionic anhydride and isopropanol in a 1:3 ratio. The propionic anhydride in the reagent will begin to dissociate into propionic acid so it is important to use it immediately after preparation. If processing samples on a plate, make new reagent for each plate. If processing samples in microcentrifuge tubes, make new reagent every three to five samples.
3. Add 10 μL of the propionylation reagent to the sample and vortex briefly. The pH will drop to 4–6 due to the propionic acid.

4. Immediately add 3–7 μL NH$_4$OH to bring the pH up to ∼8, checking with pH paper. Do not allow the sample to go above pH 10 as propionylation of other residues such as serine can occur at high pH.
5. Incubate the samples at 37°C for 15 min.
6. Dry down samples to less than 5 μL in a vacuum centrifuge.
7. Repeat steps 1–6 one more time.
8. Resuspend samples in 50–100 μL of ammonium bicarbonate.
9. Add trypsin in a 1:20 protease:histone ratio and incubate at 37°C for at least 6 h up to overnight.
10. Quench trypsin by freezing at −80°C or adding glacial acetic acid to lower pH to ∼4.
11. Dry samples to 20 μL or less. If dried to less than 20 μL, add more ammonium bicarbonate to a final volume of 20 μL.
12. Repeat steps 2–6 twice.

3.3 Desalting

Desalting is performed on home-made C$_{18}$ columns called stage-tips.
1. Cut off the last centimeter of a P1000 tip to make the opening bigger. Use this pipet tip to punch out a piece of C$_{18}$ material from the disc. If desalting more than 25 μg of protein, use two discs. Ensure that there is no space between the discs.
2. Use a piece of fused silica capillary (or any other long, thin item) to firmly push the C$_{18}$ material out of the P1000 pipet tip into a P200 pipet tip. Ensure that the C$_{18}$ disc is firmly positioned in the tip, but do not push hard enough to puncture the material.
3. Place the column in a microcentrifuge adaptor inside a 1.5- or 2-mL microcentrifuge tube.
4. Activate the resin by adding 50 μL methanol to the column. Push the methanol through using compressed air or by spinning in a centrifuge at 500 × g for 30–60 s.
5. Repeat step 4 once. After this point, keep the disc wet at all times.
6. Equilibrate the column by adding 200 μL washing buffer to the column and push the solution through as described in step 5. Note that the collection tube may need to be emptied.
7. Repeat step 6 once.
8. Add wash buffer to samples to a final volume of approximately 200 μL. The pH should be below 4.0 (check with pH paper). If needed, adjust the pH using glacial acetic acid.

9. Add the sample to the stage-tip and centrifuge for 2–5 min at $200 \times g$ until the sample passes through the column.
10. Wash the column by adding 50 μL wash buffer to the stage-tip and centrifuge at $500 \times g$ for 30–60 s until the buffer passes through.
11. Repeat step 10 once.
12. Remove the collection tube and replace with a clean 1.5-mL microcentrifuge tube.
13. Add 75 μL elution buffer to the column and centrifuge at $200 \times g$ for 2–5 min.
14. Repeat step 13 once.
15. Dry down sample in a vacuum centrifuge to less than 5 μL. Desalted samples can be stored at $-80°C$.
16. Resuspend samples to approximately 1 μg/μL in buffer A for MS analysis.

3.4 Online RP-HPLC and MS Acquisition

We present protocols to perform bottom-up MS using nanoelectrospray ionization (nano-ESI) on a hybrid LTQ-Orbitrap (ie, Thermo Orbitrap Elite).

MS data can be acquired in two different modes: data–dependent acquisition (DDA) or data-independent acquisition (DIA). DDA is the traditional method used in histone PTM analysis, where a high-resolution full MS scan is acquired followed by low-resolution MS/MS acquisition of the topN (eg, 10) most abundant peaks from the full MS scan. Peptide abundance is quantified by integrating the area of the extracted ion chromatogram (XIC) at the MS1 level. One issue with DDA is that it cannot accurately quantify coeluting isobaric peptides (different peptides that have the same mass) because they cannot be discriminated at the MS1 level. To overcome this limitation, coeluting isobaric peptides must be targeted across their elution profile and quantified based on the fragment ion intensities.

DIA is performed by acquiring a high-resolution full MS scan followed by a series of high-resolution MS/MS scans covering a larger m/z window (ie, 50) that step across the desired m/z range (Gillet et al., 2012). All ions present in the m/z window get fragmented and detected together, and so the size of the window and complexity of the sample will dictate the quality of the MS/MS spectra and consequently identification. DIA methodology has been optimized for histone PTM analysis also performing low-resolution MS/MS scans, and 50 m/z windows were found to be a good balance for

allowing sufficient identification and cycle speed (Sidoli, Simithy, Karch, Kulej, & Garcia, 2015). Peptide identifications should be obtained before the DIA experiment using a spectral library generated with DDA data. In other words, DIA is not the recommended acquisition method to identify unknown peptides. On the other hand, one major benefit of DIA is that MS/MS spectra are obtained for all peptides across the entire elution peak. Thus, DIA provides higher confidence in determining the correct chromatographic peak for a given peptide, as it allows for XIC for both precursor and fragment ions, which will appear as coeluting peak profiles. The abundance of the peptide is still quantified by calculating the area under the curve for the parent ion, as the precursor ion signal is always more intense than any fragment mass ion. Moreover, DIA eliminates the need for targeting of coeluting isobaric species, as all analytes are already fragmented at each MS scan cycle and allows for future data mining. DIA methods have been gaining popularity due to these advantages.

1. Fit the HPLC with a C_{18} column (75 μM inner diameter, 10–20 cm in length), either purchased commercially or packed in-house.
2. Load 1–2 µg histone peptides onto the column with an autosampler.
3. Program the HPLC gradient: 0–30% buffer B in 30 min, 30–100% buffer B in 5 min, 100% buffer B for 8 min. For HPLC systems that do not automatically equilibrate the column before sample loading, include equilibration steps in your gradient: 100–0% buffer B in 1 min and 0% buffer B for 10 min. Set the flow rate to 250–300 nL/min.
4. Program a method to acquire and record MS data.
   a. DDA: Composed of three segments. All full MS scans are obtained in the Orbitrap and all MS/MS scans are obtained in the ion trap.
      i. Segment 1: 14 min. One full MS scan followed by CID MS/MS of the top seven most abundant ions (in DDA mode).
      ii. Segment 2: 27 min. One full MS scan followed by CID fragmentation of isobaric species [for, eg, human and mouse H3 9–17aa 1 acetyl (528.296 m/z), H3 18–26aa 1 acetyl (570.841 m/z), H4 4–17 1 acetyl (768.947 m/z), H4 4–17 2 acetyls (761.939 m/z), H4 4–17aa 3 acetyls (754.930 m/z)] and DDA of the top five most abundant ions.
      iii. Segment 3: 19 min. One full MS scan followed by DDA of the top 10 most abundant ions.
      iv. *Note*: The time and length of each segment will vary depending on the exact elution times of the targeted species, which can vary between columns. For first time users, run one sample to

determine the elution time for the targeted species and modify the method, if needed, to allow for accurate targeting of the desired species.

  b. DIA: Set up method according to Table 1.

3.5 Data Analysis

Histone PTM analysis is a computationally challenging process. Given that histones are highly modified proteins, each peptide can contain several PTM

Table 1 Parameters for DIA MS Acquisition of Histone Samples

Scan #	Scan Type	Detector	Scan Range	MS2 Scan Range
1	MS1	Orbitrap	300–1100	N/A
2	MS2	Ion trap	300–350	120–1500
3	MS2	Ion trap	350–400	120–1500
4	MS2	Ion trap	400–450	120–1500
5	MS2	Ion trap	450–500	130–1500
6	MS2	Ion trap	500–550	140–1500
7	MS2	Ion trap	550–600	155–1500
8	MS2	Ion trap	600–650	170–1500
9	MS2	Ion trap	650–700	185–1500
10	MS1	Orbitrap	300–1100	N/A
11	MS2	Ion trap	700–750	195–1500
12	MS2	Ion trap	750–800	210–1500
13	MS2	Ion trap	800–850	225–1500
14	MS2	Ion trap	850–900	240–1500
15	MS2	Ion trap	900–950	250–1500
16	MS2	Ion trap	950–1000	265–1500
17	MS2	Ion trap	1000–1050	280–1500
18	MS2	Ion trap	1050–1100	295–1500

Note: The full MS1 scan is performed twice within the same duty cycle to allow for a more resolved definition of the precursor peak profile. This is not necessary for MS2 ions, as these are commonly not used for quantification, but only for increasing the confidence of the selected chromatographic peak and, in specific cases, to discriminate isobaric forms. The differences in MS2 scan range, in particular for the low mass range, are due to the intrinsic limitations of the ion trap, which cannot hold for scanning fragment ions smaller than ∼1/3 of the isolated precursor mass.

acceptor sites, resulting in a large number of possible modified forms for a given peptide. For example, the H3 9–17 peptide can contain mono-, di-, or trimethylation (me1/me2/me3) on K9 and K14 as well as acetylation (ac) on K9, for a total of 10 possible forms of the peptide. Some isobaric peptides are also generated by the derivatization process, further complicating analysis. For example, the H3 9–17 peptide has two sets of isobaric peptides: unmodified and K9me1K14ac ($[M + 2H]^{2+} = 535.304$ m/z) and K9me3K14ac and K9me2 ($[M + 2H]^{2+} = 521.306$ m/z. Many of these isobaric species arise because the mass of a propionyl group is the same as the mass of an acetyl group and monomethyl group (56.026 Da). Fortunately, many of these peptides elute at different times and therefore do not require MS/MS targeting across their elution profiles in DDA experiments (ie, K9me3K14ac and K9acK14ac). This is because the modifications impart different hydrophobicities, causing the peptides to elute at different times (Fig. 2).

Some isobaric species, however, coelute and cannot be discriminated based on retention time (RT). A prime example is the H4 4–17 peptide that contains four lysine residues that can be acetylated, resulting in many isobaric species. The diacetylated peptide is the most complicated example, as there are six isobaric forms that coelute. These peptides can be differentiated by collecting MS/MS spectra across their elution and using the elution profiles of unique fragment ions to define the ion chromatogram (Fig. 3A).

In both DDA and DIA experiments, the relative abundances of histone peptides must be calculated by extracting the raw peak area of all modified and unmodified forms for a given peptide sequence. The relative abundance is obtained by dividing the total area of a single peptide isoform (including all charge states) by the total area of that peptide sequence in all of its modified forms. We developed a software tool to automate this type of data analysis for both DDA and DIA experiments (Yuan et al., 2015).

3.5.1 Software-Based Peak Area Extraction and Abundance Calculation

EpiProfile is a freely available Matlab-based automated tool to quantify histone PTM profiles in DDA experiments (Yuan et al., 2015). The software reads raw data and provides a table of quantified histone peptides, layouts (MS1 elution profiles), and annotated MS/MS spectra used for identification. EpiProfile can quantify coeluting isobaric peptides (if they were targeted in the MS method) as well as isotopically labeled peptides (ie, labeled by SILAC).
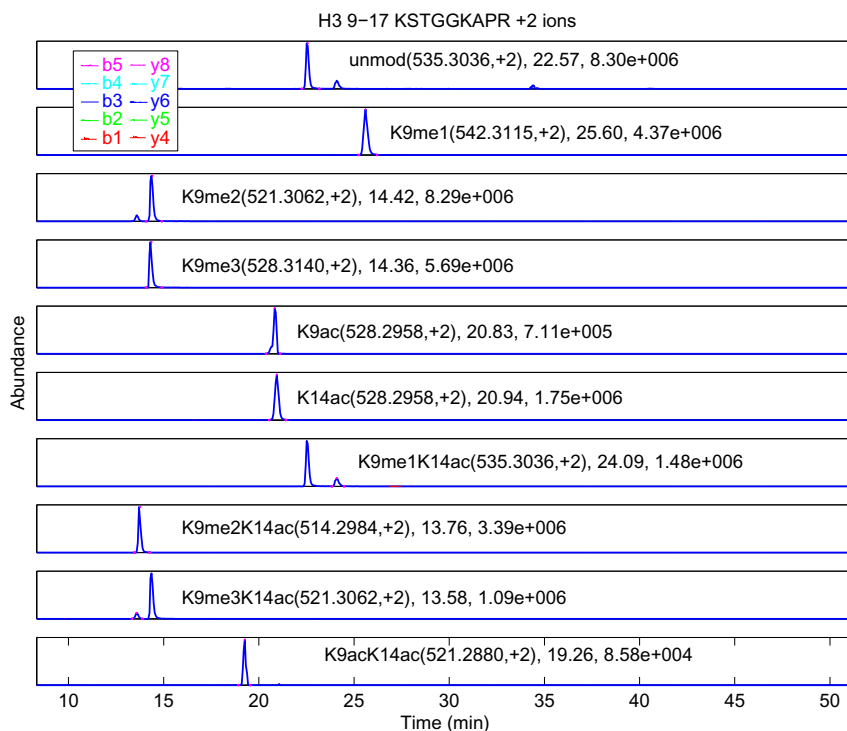
Figure 2 Example layout for H3 9–17 peptide provided by EpiProfile. Each row represents an extracted ion chromatogram (XIC) for a specific modified form of the peptide. The script next to each peak provides the modification state, *m/z* value, charge state, rentention time, and intensity, respectively. The XIC of fragment ions are also illustrated in colors, however, they cannot be easily visualized because they overlap with the XIC trace. Note that some isobaric peptides (ie, K9meK14ac and K9acK14ac) have nearly the same mass but elute at different times, while others (ie, K9ac and K14ac) have overlapping XICs.

EpiProfile uses previous knowledge about relative elution times to aid in quantification and identification of histone peptides. It uses this information, as well as mass information, to perform XIC of each unique peptide. Epi-Profile then calculates the area under the curve of the XIC, which is then used to calculate the relative abundance of each peptide.

1. Open the "params.txt" file in EpiProfile and specify the file path containing your raw files. Other options can be specified as well, such as isotopic labeling and mass tolerance.
2. Open Matlab and specify the file path containing EpiProfile.
3. Enter "EpiProfile" in the command window to start the program.

A   K8acK12acK16ac/K5acK12acK16ac/K5acK8ac
K16ac/K5acK8acK12ac:43%:17%:15%:25%

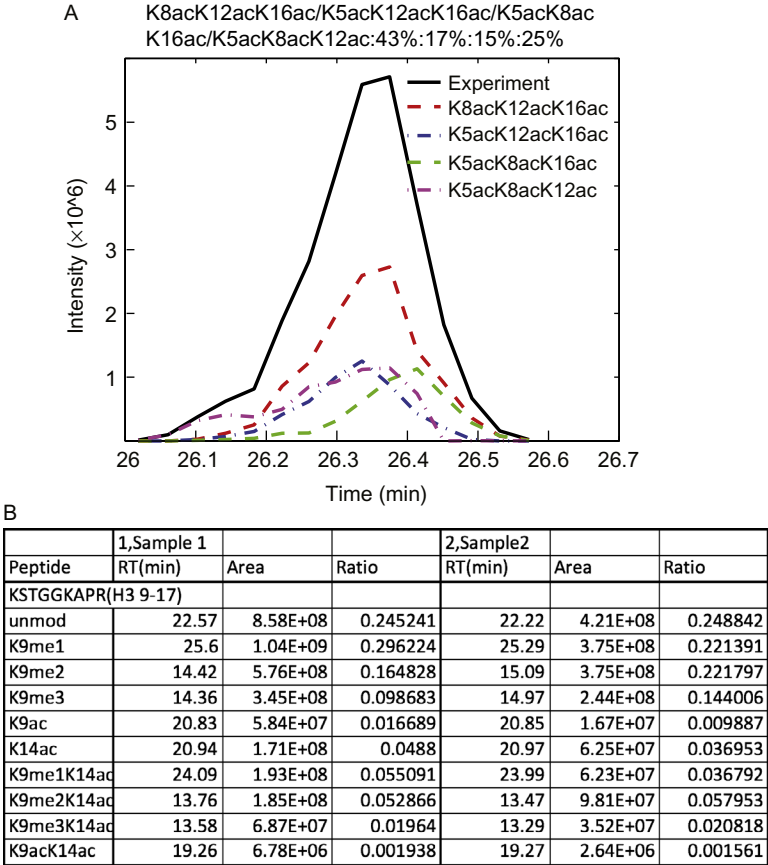| Peptide	1,Sample 1			2,Sample2		
	RT(min)	Area	Ratio	RT(min)	Area	Ratio
KSTGGKAPR(H3 9-17)						
unmod	22.57	8.58E+08	0.245241	22.22	4.21E+08	0.248842
K9me1	25.6	1.04E+09	0.296224	25.29	3.75E+08	0.221391
K9me2	14.42	5.76E+08	0.164828	15.09	3.75E+08	0.221797
K9me3	14.36	3.45E+08	0.098683	14.97	2.44E+08	0.144006
K9ac	20.83	5.84E+07	0.016689	20.85	1.67E+07	0.009887
K14ac	20.94	1.71E+08	0.0488	20.97	6.25E+07	0.036953
K9me1K14ac	24.09	1.93E+08	0.055091	23.99	6.23E+07	0.036792
K9me2K14ac	13.76	1.85E+08	0.052866	13.47	9.81E+07	0.057953
K9me3K14ac	13.58	6.87E+07	0.01964	13.29	3.52E+07	0.020818
K9acK14ac	19.26	6.78E+06	0.001938	19.27	2.64E+06	0.001561

Figure 3 EpiProfile allows for quantification of histone PTMs, including isobaric species. (A) Fragment ion XICs for H4 4–17 peptide containing three acetyl groups. These fragment ion XICs are used for quantification because the parent ion XICs overlap. (B) Example of EpiProfile quantification output from "histone_ratios.xls" file. The retention time, area under the XIC and relative abundance (ratio) for each peptide in each sample are listed.

4. The program will run and the results will be stored in the same file path that was specified in the "params.txt" file. Several result files and folders will be provided:

   a. An excel file called "histone_ratios.xls." This file contains the RT, area, and relative abundance of each peptide for all raw files (Fig. 3B).

   b. A folder called "histone_layouts." This contains all of the XICs used for area calculations, including all of the XICs for fragment ions (ie,

Fig. 2). The "details" folder in this directory contains XICs for coeluting isobaric species (ie, Fig. 3A).

5. The layout files can be used to manually validate that the program chose the correct peak to quantify.

   Note: EpiProfile, although it is the software we recommend for histone analysis, is not the only one available. Manual quantification can be performed using the Xcalibur QualBrowser (for Thermo instruments), which is mostly used to visualize raw files. The mass of the monoisotopic peak can be entered to obtain the XIC, and the area under the curve function automatically calculates the peak area. Moreover, the free software Skyline can be adopted for the purpose (MacLean et al., 2010). Skyline is optimized to extract precursor and fragment XIC upon a precompiled list of peptides, and thus it is definitely more automated than the manual quantification procedure. However, it does not include unique features of EpiProfile such as RT-based peak calling and automatic calculation of ratios for coeluting isobaric peptides.

4. OFFLINE FRACTIONATION OF HISTONE SPECIES

4.1 Materials and Buffer Recipes

1. Offline buffer A: 5% acetonitrile, 0.2% trifluoroacetic acid (TFA) in water (all HPLC-grade)
2. Offline buffer B: 95% acetonitrile, 0.188% TFA in water (all HPLC-grade)
3. 5 μm C_{18} column (size depending on application)

4.2 Histone Variant Purification

1. Attach a C_{18} column to the offline HPLC and set the flow rate according to the size of the column. Generally, a flow rate of 0.2 mL/min is used for a 2.1 mm column, 0.8 mL/min for a 4.6 mm column, and 2.5 mL/min for a 10 mm column. Allow the column to equilibrate in buffer A for $10\times$ column volumes.
2. Load the sample. Generally, 100–200 μg histone should be loaded for a 4.6 mm column.
3. Run the following gradient: 30–60% buffer B over 100 min, 60–100% buffer B over 20 min, 100–30% buffer B over 10 min. The elution profile is shown in Fig. 4.
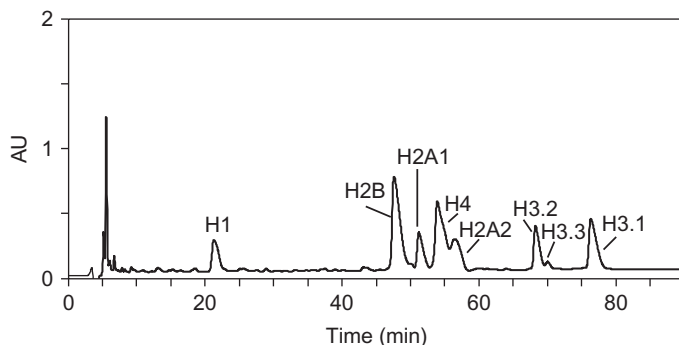
Figure 4 Chromatogram for histone variant purification. This chromatogram was obtained by injected 200 μg of acid-extracted histone from HeLa cells on a 4.6 mm C_{18} column. Note that other cell types may contain varying abundances of histone variants, but the elution times should remain the same. The identity of each peak is provided.

4. Set up a fraction collector to collect samples in 1 min intervals from 15 to 80 min.
5. Transfer the desired fractions to centrifuge tubes and dry to completion in a vacuum centrifuge to remove organic solvent and TFA. Fractions corresponding to the same histone can be pooled before drying. Dried histones can be stored at room temperature. If reconstituted, store at $-80°C$.

5. MIDDLE-DOWN MASS SPECTROMETRY

Middle-down MS is a different proteomics strategy valuable for quantifying combinatorial histone PTMs. In middle-down MS, proteases that generate long peptides are employed to allow for greater connectivity between PTM sites than bottom-up MS. It is important to use a protease that does not cleave in the tail domain as this is where most PTMs are catalyzed. Endoproteinase GluC (in bicarbonate buffers) is recommended for histone H3, H4, and canonical H2A, as it generates N-terminal peptides of 50, 53, and 41 amino acid residues, respectively. Endoproteinase AspN is a valid alternative, as it cleaves all canonical histones providing middle-down sized N-terminal peptides; specifically, 71 amino acid residues for H2A, 76 for H3, 24 for H4, and 51 for H2B. Because the peptides are longer and trypsin is not being used, derivatization of the protein is unnecessary. The high charge states occupied by histone tail peptides are incompatible

with CID fragmentation. ETD fragmentation, on the other hand, generally results in high coverage of the histone tail peptides.

Middle-down histone tail peptides do not separate well by RP chromatography. Weak cation exchange hydrophilic interaction liquid chromatography (WCX-HILIC) is currently the stationary phase of choice for this application, as it employs a hydrophilic stationary phase containing negatively charged residues (ie, glutamic acid), which is the ideal binding pocket for basic hydrophilic polypeptides (Jung et al., 2013; Sidoli et al., 2014; Young et al., 2009). The HILIC separation occurs by decreasing the amount of organic buffer in the solvent so that more hydrophobic species elute first. The WCX separation is accomplished by incorporating a pH gradient into the mobile phase. As the pH decreases during the gradient, the resin becomes increasingly protonated, which removes the charge on the resin and abolishes the cation exchange interaction.

One drawback of middle-down MS compared to bottom-up MS is reduced sensitivity. Larger peptides are electrospray ionized in multiple charge states, which reduces the signal for any given charge state compared to smaller peptides. Similarly, larger peptides can have a larger number of modified forms, which dilutes the signal for any single form. This limitation can be partially alleviated by using larger amounts of starting material. Fractionation of histones before MS analysis is the most effective method to increase sensitivity because it reduces the complexity of the sample, and thus allows for loading more material of a single histone variant. Another caveat of middle-down MS is that the data analysis for middle-down experiments is more complicated and less automated compared to bottom-up MS, although few software tools have been developed for the purpose (DiMaggio, Young, Baliban, Garcia, & Floudas, 2009; Sidoli et al., 2014).

5.1 Materials and Buffer Recipes

1. 100 mM ammonium acetate, pH 4.0
2. 10 mM Tris–HCl, pH 7.5
3. GluC
4. AspN
5. Offline buffer A: 5% acetonitrile, 0.2% TFA in water (all HPLC-grade)
6. Offline buffer B: 95% acetonitrile, 0.188% TFA in water (all HPLC-grade)
7. Online buffer A: 75% acetonitrile, 20 mM propionic acid, pH 6.0 (generated using ammonium hydroxide)
8. Online buffer B: 15% acetonitrile, 0.2% formic acid

9. WCX–HILIC column: 75 μm ID, 15 cm length, 1.7 μm (diameter) 1000 Å (porosity) particle size is recommended. The 3 μm (diameter) 1500 Å (porosity) particle size can also be used

5.2 Digestion

5.2.1 GluC
1. Resuspend sample in 100 mM ammonium acetate to a final concentration of 0.5 μg/μL.
2. Dilute the GluC enzyme to 0.2 μg/μL in the same buffer.
3. Add GluC to a final concentration of 1:10 GluC:histone.
4. Incubate for 6 h at room temperature.
5. Block digestion by adding 1% formic acid.

5.2.2 AspN (Alternative to GluC)
1. Resuspend enzyme to 40 ng/μL in 10 mM Tris–HCl, pH 7.5.
2. Resuspend sample in the same buffer and add enzyme at 1:100 enzyme: histone by weight.
3. Digest at 37°C for 6 h.
4. Block digestion by adding 1% formic acid.

5.3 WCX-HILIC and MS

Middle–down has been optimized by different research laboratories (Jung et al., 2013; Sidoli et al., 2014; Young et al., 2009). Currently, the most automated platform includes an RP trap column for sample loading and a WCX-HILIC analytical column for the gradient (Fig. 5). This setup allows
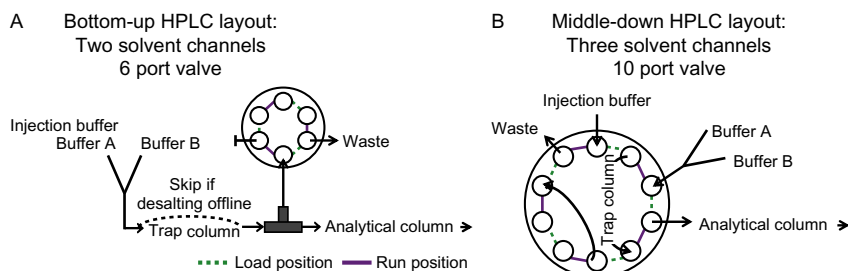

Figure 5 Valve layouts for bottom-up and middle-down HPLC-MS/MS analysis. (A) Bottom-up HPLC layout. The trap column can be used to desalt online. (B) Middle-down HPLC layout. Three solvent channels are needed: two are used to deliver the WCX-HILIC gradient, and one is used to load and desalt the sample on the trap column.

for sample loading in aqueous conditions and separation using WCX–HILIC (Sidoli et al., 2014).

1. Program the HPLC method as follows: from 0% to 55% buffer B in 1 min, from 55% to 85% B in 160 min, and from 85% to 100% in 5 min. If the HPLC is not programmed for automated column equilibration before sample loading then include this part in the method: switch the valve in position load (Fig. 5B), from 100% to 0% B in 1 min and isocratic flow at 0% B for 10 min. The flow rate of the analysis should be 250–300 nL/min.

2. Program the MS acquisition method to perform MS/MS DDA of the 5–8 most abundant precursor masses. Do not apply dynamic exclusion, as this increases the number of isobaric forms quantified. The full MS scan range should be 450–750 m/z, as this is the region of the most intense charge states for histone polypeptides. If only histone H3 is analyzed, the window can be narrowed to 660–720 m/z, in order to include only charge state 8^+.

3. Program the MS/MS acquisition to be performed with ETD at a resolution of ~30,000. The reaction time should be around 20 ms for polypeptides with 8–10 charges. Include three microscans to improve the quality of the MS/MS spectra acquired, as ETD spectra are overall less reproducible than CID.

4. Load ~2 µg of sample onto the HPLC trap column. The sample can be loaded as is from the digestion step (Section 5.2.1 or 5.2.2).

5. Run the HPLC-MS/MS method as programmed. Since the sample is loaded onto a trap column it does not require prior desalting, as the salts will be eluted during the trap loading and thus they will not be sprayed into the MS.

5.4 Data Analysis

Identification and quantification of middle-down peptides is a more challenging process than bottom-up. This is because each precursor mass might correspond to hundreds of isobarically modified peptides. Currently, the most comprehensive algorithm to quantify histone peptides employs mixed integer linear optimization (DiMaggio et al., 2009). However, this software is currently not in a user-friendly format and it is not flexible through unexpected modification sites. Thus, we developed a workflow that includes database searching and quantification based on virtual histograms. Briefly, the obtained LC-MS/MS result file is searched using Mascot (Matrix

Science), which provides identification and spectrum intensities, and then filtered with the freely available isoScale slim (http://middle-down. github.io/Software) (Sidoli et al., 2014).

1. Collect all raw files and submit them to a deconvolution tool. MS/MS spectra ions should be all singly charged before Mascot database searching. We recommend Xtract as deconvolution algorithm if Thermo Scientific instrument is used (eg, LTQ-Orbitrap), although it is important to highlight that utilizing Thermo instruments is not mandatory.

2. Perform database searching using a sequence database containing only histones; large databases increase dramatically searching time. Search parameters should be as following: MS mass tolerance: 2.2 Da, to include possible errors of the deconvolution algorithm in isotopic recognition. MS/MS mass tolerance: 0.01 Da. Enzyme: GluC (or AspN) with no missed cleavages. Sample preparation does not generate static modifications on the peptide, while variable modifications can be chosen as desired.

   Note: Recommended variable modifications are mono- and dimethylation (KR), trimethylation (K), acetylation (K) and, optionally, phosphorylation (ST).

3. Export Mascot results in .csv, including the following information to the file: all Query level information, all the default information (these last are already selected by default).

4. Import the .csv file in isoScale slim. Set the tolerance for the search (recommended: 30 ppm) and the type of fragmentation adopted (in this case ETD). The result table contains only confidently assigned combinatorial PTMs. A peptide is defined as confident if all the modifications are uniquely validated by site determining ions, which are ions that unambiguously confirm the localization of a modification site. For instance, in order to verify that a methylation is on H3K27 the software requires at least one ion proving that the modification is not either on H3R26 or H3K36. The output table contains the list of peptides that passed the site determining ions validation and their MS/MS ion intensity. isoScale also quantifies cofragmented isobaric species, as soon as all these species pass the confidence threshold, in a similar manner as EpiProfile performs for bottom-up peptides (Section 3.5.1).

5. The output table contains duplicates (peptides with the same sequence and PTM combination). Remove them by using the "Remove duplicates" option in Excel.

Note: From the relative abundance of the combinatorial marks, it is possible to extract the relative abundance of single marks simply by summing all relative abundances of peptides that contain the given PTM. To estimate which histone marks tend to coexist with each other with high or low frequency, it is possible to calculate the interplay score (Jung et al., 2013; Schwämmle, Aspalter, Sidoli, & Jensen, 2014). This score is calculated as:

$$I_{xy} = \log_2\big(F_{xy}/\big(F_x \times F_y\big)\big)$$

where I_{xy} is the interplay score between the marks X and Y, F_{xy} is the coexistence frequency (or relative abundance) of the two marks, and F_x or F_y are the frequencies of the single marks in the dataset. Basically, F_{xy} is the observed coexistence frequency, while $(F_x \star F_y)$ is the theoretical coexistence frequency, calculated based on the relative abundance of single PTMs. This calculation provides a score of how much two marks tend to coexist or be mutually exclusive on the same histone protein. Positive values indicate tendency to coexist higher than if the two marks were completely independent from each other, while negative values indicate the opposite. This score has been used to predict crosstalk between histone marks (Schwämmle et al., 2014).

6. TOP-DOWN MASS SPECTROMETRY

Top-down MS is performed by fragmenting intact protein using ETD fragmentation. The biggest advantage of top-down MS over the other methods is that it provides a global view of the intact protein sequence. However, this advantage is accompanied by the caveat that it is also the least sensitive method because the histone proteins will occupy the maximal number of charge states and modified forms as compared to bottom–up and middle–down strategies. Furthermore, the data analysis is not only more difficult, but also currently prohibitive in specific cases. For instance, while the bottom-up sample preparation can produce peptides with up to six isobaric forms, an intact histone has potentially hundreds of thousands of combinatorial forms with the same identical precursor mass. The exponential number can be explained considering the many modifiable sites (eg, K and R) and the permutations of isobaric modified forms (eg, me3 is equivalent to me1me2, which is equivalent to me1me1me1, which is equivalent to the same combination on other sites, etc.). Therefore, a single MS/MS

spectrum might contain an impressive number of isobaric proteoforms, of which at the moment we can only scrape its surface.

Generally, fractionation is performed before conducting a top-down experiment to reduce the complexity of the sample. Histone species can be fractioned as described in Section 4. Usually, histone proteins are directly infused into the MS in top-down analysis, but online RP separation can also be conducted if desired (Contrepois, Ezan, Mann, & Fenaille, 2010; Eliuk, Maltby, Panning, & Burlingame, 2010; Tian et al., 2010). Direct infusion using a syringe requires a large amount of sample (about 20–30 µg for 10 min injection), so using a chip-based infusion system, such as an Advion Nanomate, is strongly recommended, as the amount of sample required can be as low as 5 µg for a 30 min injection. In these systems, sample is picked up by a small tip and delivered to a chip containing nano-ESI nozzles. Voltage is applied to the chip to enable ionization into the MS. Very small sample volumes can be stably sprayed for long periods of time using this system (10 µL of sample lasts for about 30–40 min).

6.1 Materials and Buffer Recipes

1. Sample buffer: 75% acetonitrile/25% of 0.8% formic acid adjusted to pH 2.5 with TFA
2. Chip-based nano-ESI autosampler such as Advion Nanomate

6.2 Top-Down MS Using Direct Infusion

The acquisition method in top-down MS will vary depending on the exact application. Most top-down experiments aim to characterize a specific modified form of histone protein. In this case,

1. If sample contains salt, desalt according to Section 3.3 using C_8 resin in place of C_{18} resin. One disc of C_8 resin can bind approximately 10 µg of intact histone protein. Use multiple discs if the sample contains more than 10 µg of protein. If offline fractionation was used, there is no need to desalt the sample.
2. Resuspend histone protein in sample buffer to a concentration of approximately 1 µg/µL.
3. Infuse sample in a chip-based direct infusion system and adjust parameters to achieve stable spray. Generally, stable spray can be achieved using a capillary temperature of 150°C, voltage between 1.7 and 2.5 kV, and a gas pressure between 0.3 and 0.4 psi. Capillary temperatures above 170°C can damage the chip and are not recommended. These

parameters will need to be adjusted for each run before data acquisition and may also need adjustment as data is being acquired. A sudden complete loss of signal usually indicates that the nozzle has become clogged, in which case another nozzle should be selected.

4. Run an MS acquisition method to collect data. The method will depend largely on the application. To analyze a specific modified form of a histone protein, set up an MS1 scan that spans the m/z range of that form but excludes other species (ie, Fig. 6A). Perform a data-dependent MS/MS on the top isotope of the distribution with a large number of microscans (10–20 should provide a clean spectra depending on the quality of signal and spray). Generally, a 3–5 min acquisition time is adequate to obtain a high-quality MS/MS spectrum that can be identified during data analysis. If characterization of more than one species is desired, set up the method to take MS1 scans corresponding to the other species followed by data-dependent MS2 scans.

6.3 Data Analysis

Top–down data require deconvolution before analysis, where each multiply charged ion is reduced to its singly charged monoisotopic mass. This process facilitates data analysis as most programs cannot analyze MS/MS spectra containing highly charged fragment ions. The deconvoluted data can then be searched using Mascot (Matrix Science) software.

1. Convert files into mzxml format using any of a number of available programs for this purpose.
2. Deconvolute data using any of a number of available programs for this purpose. Examples include the Xtract module from Thermo Xcalibur software and MS–Deconv (freely available at http://bix.ucsd.edu/projects/msdeconv/).
3. Use proper software to search the deconvoluted data. The traditional Mascot license can analyze intact molecules up to 16 kDa, making it suitable for intact histone proteins. Other software tools can be used, such as ProSightPC (Thermo) or the freely available MS–Align+ (Liu et al., 2012). Use a database containing all of the histone protein sequences, including variants. Select the modifications of interest as variable PTMs.

   Note: Manual validation of the results will be necessary, as all softwares are not optimized for heavily modified proteins such as histones. In order to achieve a confident localization for a given PTM, it is necessary to have specific fragment ions between two possible modifiable
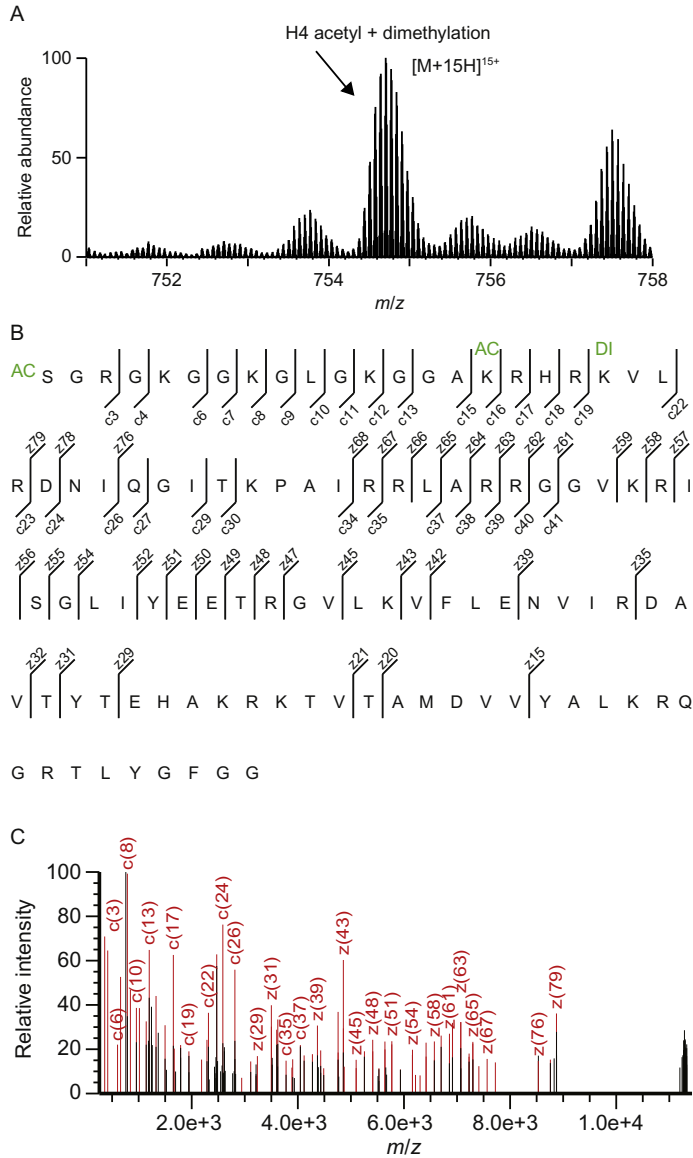
Figure 6 Example data obtained in a top-down experiment for intact H4 containing 1 dimethyl and 2 acetyl groups. (A) Full MS scan prior to isolation and fragmentation of intact H4 containing an acetyl and dimethyl group. The isolation window is set so that the species of interest is the most abundant ion in the scan so that it will be selected for data-dependent MS2 acquisition. (B) Fragment ion coverage of the H4 protein containing N-terminal acetylation (AC), K16ac (AC), and K20me2 (DI). There are fragment ions flanking each modification, allowing for confident identification of the PTMs. (C) Deconvoluted MS/MS spectra. Each identified fragment ion is indicated in gray and the most abundant fragment ions are labeled.

sites, confirming on which amino acid residue the modification is localized (Fig. 6B and C). Software will provide the "most probable" localization of the PTMs, but they rarely provide a score describing whether another localization for a given modification is equally probable. isoScale slim is applicable for this purpose, as previously illustrated for middle-down (Section 5.4). However, it demands result files from Mascot.

REFERENCES

Britton, L.-M. P., Gonzales-Cope, M., Zee, B. M., & Garcia, B. A. (2011). Breaking the histone code with quantitative mass spectrometry. *Expert Review of Proteomics*, *8*(5), 631–643. http://doi.org/10.1586/epr.11.47.

Contrepois, K., Ezan, E., Mann, C., & Fenaille, F. (2010). Ultra-high performance liquid chromatography–mass spectrometry for the fast profiling of histone post-translational modifications. *Journal of Proteome Research*, *9*(10), 5501–5509. http://doi.org/10.1021/pr100497a.

DiMaggio, P. A., Young, N. L., Baliban, R. C., Garcia, B. A., & Floudas, C. A. (2009). A mixed integer linear optimization framework for the identification and quantification of targeted post-translational modifications of highly modified proteins using multiplexed electron transfer dissociation tandem mass spectrometry. *Molecular & Cellular Proteomics*, *8*(11), 2527–2543. http://doi.org/10.1074/mcp.M900144-MCP200.

Eliuk, S. M., Maltby, D., Panning, B., & Burlingame, A. L. (2010). High resolution electron transfer dissociation studies of unfractionated intact histones from murine embryonic stem cells using on-line capillary LC separation. *Molecular & Cellular Proteomics*, *9*(5), 824–837. http://doi.org/10.1074/mcp.M900569-MCP200.

Garcia, B. A., Mollah, S., Ueberheide, B. M., Busby, S. A., Muratore, T. L., Shabanowitz, J., & Hunt, D. F. (2007). Chemical derivatization of histones for facilitated analysis by mass spectrometry. *Nature Protocols*, *2*(4), 933–938. http://doi.org/10.1038/nprot.2007.106.

Gillet, L. C., Navarro, P., Tate, S., Röst, H., Selevsek, N., Reiter, L., … Aebersold, R. (2012). Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: A new concept for consistent and accurate proteome analysis. *Molecular & Cellular Proteomics*, *11*(6). http://doi.org/10.1074/mcp.O111.016717. O111.016717.

Jenuwein, T., & Allis, C. D. (2001). Translating the histone code. *Science*, *293*(5532), 1074–1080. http://doi.org/10.1126/science.1063127.

Jung, H. R., Sidoli, S., Haldbo, S., Sprenger, R. R., Schwämmle, V., Pasini, D., … Jensen, O. N. (2013). Precision mapping of coexisting modifications in histone H3 tails from embryonic stem cells by ETD-MS/MS. *Analytical Chemistry*, *85*(17), 8232–8239. http://doi.org/10.1021/ac401299w.

Liu, X., Sirotkin, Y., Shen, Y., Anderson, G., Tsai, Y. S., Ting, Y. S., … Pevzner, P. A. (2012). Protein identification using top-down spectra. *Molecular & Cellular Proteomics*, *11*(6). M111.008524. http://doi.org/10.1074/mcp.M111.008524.

Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F., & Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature*, *389*(6648), 251–260. http://doi.org/10.1038/38444.

MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., … MacCoss, M. J. (2010). Skyline: An open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*, *26*(7), 966–968. http://doi.org/10.1093/bioinformatics/btq054.

Rothbart, S. B., Dickson, B. M., Raab, J. R., Grzybowski, A. T., Krajewski, K., Guo, A. H., … Strahl, B. D. (2015). An interactive database for the assessment of histone

antibody specificity. *Molecular Cell*, *59*(3), 502–511. http://doi.org/10.1016/j.molcel.2015.06.022.

Schwämmle, V., Aspalter, C.-M., Sidoli, S., & Jensen, O. N. (2014). Large scale analysis of co-existing post-translational modifications in histone tails reveals global fine structure of cross-talk. *Molecular & Cellular Proteomics*, *13*(7), 1855–1865. http://doi.org/10.1074/mcp.O113.036335.

Sidoli, S., Schwämmle, V., Ruminowicz, C., Hansen, T. A., Wu, X., Helin, K., & Jensen, O. N. (2014). Middle-down hybrid chromatography/tandem mass spectrometry workflow for characterization of combinatorial post-translational modifications in histones. *Proteomics*, *14*(19), 2200–2211. http://doi.org/10.1002/pmic.201400084.

Sidoli, S., Simithy, J., Karch, K. R., Kulej, K., & Garcia, B. A. (2015). Low resolution data-independent acquisition in an LTQ-Orbitrap allows for simplified and fully untargeted analysis of histone modifications. *Analytical Chemistry*, *87*(22), 11448–11454. http://doi.org/10.1021/acs.analchem.5b03009.

Sidoli, S., Yuan, Z.-F., Lin, S., Karch, K., Wang, X., Bhanu, N., … Garcia, B. A. (2015). Drawbacks in the use of unconventional hydrophobic anhydrides for histone derivatization in bottom-up proteomics PTM analysis. *Proteomics*, *15*(9), 1459–1469. http://doi.org/10.1002/pmic.201400483.

Tian, Z., Zhao, R., Tolić, N., Moore, R. J., Stenoien, D. L., Robinson, E. W., … Paša-Tolić, L. (2010). Two-dimensional liquid chromatography system for online top-down mass spectrometry. *Proteomics*, *10*(20), 3610–3620. http://doi.org/10.1002/pmic.201000367.

Young, N. L., DiMaggio, P. A., Plazas-Mayorca, M. D., Baliban, R. C., Floudas, C. A., & Garcia, B. A. (2009). High throughput characterization of combinatorial histone codes. *Molecular & Cellular Proteomics*, *8*(10), 2266–2284. http://doi.org/10.1074/mcp.M900238-MCP200.

Yuan, Z.-F., Lin, S., Molden, R. C., Cao, X.-J., Bhanu, N. V., Wang, X., … Garcia, B. A. (2015). EpiProfile quantifies histone peptides with modifications by extracting retention time and intensity in high-resolution mass spectra. *Molecular & Cellular Proteomics*, *14*(6), 1696–1707. http://doi.org/10.1074/mcp.M114.046011.