Quantifying histone modifications using mass spectrometry (Prot 51)

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Introduction

A variety of different posttranslational modifications (PTMs) on histone proteins display an important layer of chromatin regulation. PTMs mostly occur on the N-terminal tails of histones that are protruding out of the nucleosome, with histones H3

and H4 featuring more described PTMs than H2A and H2B (Figure 1). Among the set of known histone PTMs, methylation and acetylation are the most abundant. In order to understand more about chromatin regulation, it is crucial to analyze histone modifications in a quantitative way.

Different technical approaches are available to determine the amount and location of these histone modifications, with antibody-based techniques and mass spectrometry being the most popular. We will describe a procedure to isolate histones, prepare them for MS analysis and interpret the results (Peters et al, 2003).

Due to their basicity, histones can be isolated from biological tissues by extracting with hydrochloric acid (HCl) (Villar-Garea et al, 2008). Further separation by SDS-PAGE gel and Coomassie staining will show a characteristic pattern of histones (Figure 2). The corresponding bands can be excised from the gel to undergo protease digestion prior to MS analysis. Commonly, trypsin is used to cleave proteins after lysine and arginine. As these amino acids are very abundant in histone tails, trypsin will generate peptides too short to be analyzed efficiently by MS. Therefore, a treatment with propionic anhydride is performed to block lysines from tryptic cleavage. By that means, longer peptides are generated after tryptic digestion and can be better analyzed in subsequent steps (Figure 3).

The resulting peptides are then separated by C18 reversed phase chromatography (RP-C18). The eluting peptides are directly ionized and infused into the mass spectrometer, which measures the mass-to-charge ratio (m/z) of these infused ions. Additionally, the most intense ions present at a certain time point are isolated and collided with an inert gas, generating fragment ions recorded in a so-called MS/MS spectrum. This spectrum contains additional information that together with the m/z value will be used to identify the peptides and the location and mass of PTMs present.

Procedure

Acid extraction of histones from a tissue

Cells are harvested and washed twice with PBS. The pellet is resuspended in 0.8 M HCl. For $2x10^6$ of mammalian cells 0.5 mL HCl are used. The sample is rotated over night at 4 °C to dissolve the basic proteins in the acid. On the next day, samples are centrifuged at 20.000 g for 30 minutes and the supernatant is dialyzed three times for one hour against 100 mM HOAc at 4°C. After dialysis, the sample is concentrated in a vacuum or freeze-dried by lyophilization. The pellet is dissolved in Laemmli buffer and boiled at 95 °C for 5 minutes (Note A). The samples can be stored after this step at -20 °C.

For separation of basic proteins, the sample is run on an 18% SDS protein gel. A protein amount according to 10^6 cells per lane is applied (Note B). Then, the gel is stained for 20 minutes with 0.25% Coomassie G-250/50% MeOH/10% HOAc (Note C) and destained with 10% HOAc until clear protein bands are visible enough to be cut (Note D) (Figure 2).

In order to avoid contamination with keratin from skin or hair, caution should be taken when cutting protein bands from the gel. Therefore, it is advisable to wear a lab coat and gloves to protect the samples and to cut in a fume hood. The gel is placed into a clean plastic tray and scalpel and spatula are cleaned thoroughly with 70% EtOH before and between cutting histone bands. Cutting should be performed tightly

around the stained band, so that the resulting band is not bigger than 1 cm x 1 mm (see comment 1). On the spatula, the band is cut into around 5-6 pieces to end up with pieces of approx. 2 mm x 1 mm x 1 mm. This gives a better accessibility for chemicals and enzymes in the subsequent steps. If the pieces are smaller than 1 mm, they can get lost more easily during sample preparation. The gel slices are put in a 0.2 mL plastic strip tube filled with 200 µL water (Note E) to prevent them from drying out. The gel pieces can be stored at 4 °C at this step.

Notes:

A) The pellet is quite acid, so the Laemmli buffer can turn yellow after dissolution. Add 1 M Tris base until it gets blue again to avoid problems with the SDS-PAGE running behavior.
B) For mammalian cells, 10^6 cells should give a Coomassie band that consists of a sufficient amount of histones for analysis. Smaller cells should be upscaled accordingly. For insect cells, like *Drosophila* cells, we recommend 10 times more cell material.
C) Coomassie G-250 is used instead of R-250 as it takes less time to destain.
D) For some cell lines, it is better to prepare nuclei for the acid extraction to obtain a more distinct histone pattern.
E) The reagents used for destaining of gel pieces, tryptic digestion and LC-MS analysis should be LC-MS grade.

Preparation for MS analysis

For a more detailed description of the histone preparation prior to MS analysis, see Villar-Garea et al (Villar-Garea et al, 2008).

Destaining

If the Coomassie or other stains are not removed, they might give a signal in the following analysis. As the proteins are trapped in the gel before digestion, there is no danger of mixing the samples while using the same pipet tips. However, after destaining, one has to pay attention while handling the gel pieces: They might stick to the pipet tips and are not clearly visible.

1. The gel pieces are washed with 200 µL water shaking 1 minute at 37 °C.
2. Remove supernatant.
3. To neutralize the slices after the acid destaining solution, they are incubated with 200 µL of 50 mM ammonium bicarbonate (NH_4HCO_3) for 1 minute at 37 °C with shaking.
4. Remove supernatant.
5. For destaining gel pieces, 200 µL of 50% acetonitrile (ACN)/50 mM NH_4HCO_3 are added. Shake at 37 °C until the pieces are destained. This should take around 60 minutes. The supernatant will turn blue while the gel pieces destain.
6. Remove supernatant. If the gel pieces are not destained yet, repeat step 5.
7. Wash with 200 µL of water. The gel pieces can be stored at 4 °C over night.
8. Remove supernatant.

Acylation

As described before, lysines should be blocked chemically before trypsin digestion. Here we describe the method using propionic anhydride, although other organic anhydrides are also possible. This treatment will add a propionyl group (C_3H_4O) to the unmodified or monomethylated ε-amino group of the lysine residue and the unmodified amino group of the protein N-terminus. Lysines carrying di- or trimethylations as well as acetylations are not modified but are as well protected from trypsin digestion. By this means, trypsin only cuts after arginines.

1. Add 1 µL of propionic anhydride and 10 µL of 0.1 M NH_4HCO_3 of a master mix to the gel pieces (Note F). As the bicarbonate ions neutralize the protons created during the acylation, there is a buildup of CO_2. Open and close the tubes several times so that the gas can vaporize and an explosion of the tubes that might lead to a loss of gel pieces is avoided.
2. Add 39 µL of 1 M NH_4HCO_3. 5 minutes after start of the reaction, check the pH and add 1 M NH_4HCO_3 until the pH is in neutral (pH 7-8) to prevent modification of other functional groups. Incubate 30 to 60 minutes at room temperature.
3. Remove supernatant (see comment 2).
4. Wash with 100 µL of 0.1M NH_4HCO_3 for 10 minutes at 37 °C with shaking.
5. Remove supernatant.
6. Repeat step 4 and 5 two times.
7. Wash with 100 µL of water for 1 minute shaking at 37 °C.
8. Remove supernatant.
9. Wash with 100 µL of 50% ACN for 15 minutes shaking at 37 °C.
10. Remove supernatant.
11. Dehydrate gel pieces by adding 50 µL of ACN.
12. Remove supernatant. The volume is bigger than 50 µL as the gel pieces get dehydrated.
13. Repeat steps 11 and 12 until the gel pieces are white. Usually, one or two repetitions should be enough.
14. Put the tubes on ice.

Note

F) Propionic anhydride should be handled under a fume hood.

Digestion

If trypsin is not cooled during handling, it will digest itself. To avoid this, we are preparing the master mix on ice.

1. The lyophilised trypsin (20 µg) is dissolved in 100 µL of the resuspension buffer (50mM acetic acid) to generate a concentration of 200 ng/µL. This solution can be aliquoted and stored at -20 °C. Avoid multiple freeze-and-thaw cycles.
2. A master mix is prepared on ice containing 10 µL of 50 mM NH_4HCO_3 and 1 µL of trypsin per sample.
3. 11 µL of the master mix are added immediately to the dehydrated gel pieces and incubated on ice for 5 minutes. The gel pieces absorb the trypsin solution and turn transparent again.
4. Add 30 µL of ice-cold 50 mM NH_4HCO_3 to each sample.
5. Incubate the samples over night, at least 3 hours, shaking at 37 °C.

Acid extraction

After digestion of the proteins, the peptides are released from the gel pieces to the supernatant. Thus, it is important after digestion to use separate pipet tips and to not mix up different samples. In order to release a higher amount of peptides from the gel slices, we extract them from the gel piece with different chemicals and pool the supernatants.

1. Transfer the supernatant (~40µL) to a 0.5 mL low binding tube (Note G).
2. Add 50 µL of 50mM NH_4HCO_3 to the gel pieces. Shake 15 minutes at 37 °C.
3. Add supernatant to the tubes.
4. Apply 50 µL of 25 mM NH_4HCO_3/50% ACN from a master mix on the gel pieces. Shake 15 minutes at 37 °C.
5. Add supernatant to the tubes.
6. Apply 50 µL of 5% TFA on the gel pieces. Shake 15 minutes at 37 °C.
7. Add supernatant to the tubes.
8. Apply 50 µL of 2.5% TFA/50% ACN from a master mix to the gel pieces. Shake 15 minutes at 37 °C.
9. Add supernatant to the tubes.
10. Add 30 µL ACN to the gel pieces (see comment 3).
11. Add supernatant to the tubes.
12. Dry to a pellet in a vacuum.
13. The pellet is resuspended in 15 µL of 0.1% TFA. About 20 to 50% of this solution, depending on the intensity of the Coomassie gel band and the peptide mass fingerprint (see below), is then injected into the LC-MS system.

Note

G) At this step, it is possible to perform a peptide mass fingerprint to check for a successful digestion of the samples. This procedure is described below (*peptide mass fingerprint*).

Peptide mass fingerprint

After digestion and before performing the acid extraction of the gel pieces, a quick check of the sample quality and intensity can be performed using MALDI-TOF mass spectrometry. For this purpose, 2 µL out of the 40 µL of the supernatant after digestion (see point 1 from “Acid extraction”) are taken, mixed with 8 µL of 0.1% TFA, desalted by using ZipTips (µC18) and spotted on a hydrophobic target plate with α-cyano-4-hydroxycinnamic acid matrix (saturated solution of the matrix in 0.3% TFA/50% ACN) (Note H).

After the spot has dried, the sample is measured by MALDI-TOF. The theoretical peak mass-to-charge of H3 or H4 (see Table 1, column MH^+) can be compared to the peaks detected by MALDI-TOF.

For a closer description of MALDI-TOF mass spectrometry for histone modifications, refer to Villar-Garea et al. (Villar-Garea et al, 2008).

Note

H) The solution is prepared, matrix is added in excess and the two components are mixed for 1 minute to dissolve the matrix. For a homogeneous mixture, the dissolved matrix is kept for 1 hour on ice and centrifuged before use.

MS/MS analysis

For MS/MS analysis, the digest dissolved in 0.1% TFA (see point 13 from "Acid extraction") is injected in an Ultimate 3000 HPLC system (LC Packings Dionex). Samples are desalted on-line in a C18 micro column (300 µm i.d. x 5 mm, packed with C18 PepMap™, 5 µm, 100 Å by LC Packings), and peptides are separated with a gradient from 5 to 60% ACN in 0.1% formic acid (FA) over 40 minutes at 300 nl/min on a C18 analytical column (75 µm i.d. x 15 cm, packed with C18 PepMap™, 3 µm, 100 Å by LC Packings). The effluent from the HPLC is directly electrosprayed into an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). The MS instrument is operated in the data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 300 – 2000) are acquired in the Orbitrap with resolution R = 60,000 at m/z 400. The six most intense peptide ions with charge states between 2 and 5 are sequentially isolated to a target value of 10,000 and fragmented in the linear ion trap by collision induced dissociation (CID) (see comment 4).

Fragment ion spectra are recorded in the ion trap part of the instrument. For all measurements with the Orbitrap detector, 3 lock-mass ions from ambient air (m/z = 371.10123, 445.12002, 519.13882) are used for internal calibration as described (Olsen et al, 2005). Typical mass spectrometric conditions are: spray voltage, 1.4 kV; no sheath and auxiliary gas flow; heated capillary temperature, 200 °C; normalized collision energy, 35% for CID in LTQ. The ion selection threshold is 10,000 counts for MS2. An activation q = 0.25 and activation time of 30 ms are used.

Analysis of raw files

For figures 4 and 5 and table 1, we are focusing on two histone peptides carrying methylation (peptide 27-40 on H3) and acetylation (peptide 4-17 on H4).

The peptide H3 27-40 contains two lysines: K27 that can be either mono-, di-, trimethylated or acetylated, and K36 that is also known to be methylated (see (Bhaumik et al, 2007) for an overview of histone modifications). The presence of methylation on K27 or K36 in the monomethylated peptide can not be distinguished in the described LC system. On the other hand, higher degrees of methylation in either K27 or K37 show different retention times (RT) in the RP-C18 and therefore are easily distinguished with this method.

For peptides that are isobaric, the LC separation prior to MS is crucial to distinguish them. For instance, the peptide K27me1/K36me2 elutes later than K27me2/K36me1, giving two different peaks at different RT. In the case of almost isobaric peptides like those containing a trimethylated or acetylated lysines (mass difference of 0.0364 amu), the first one is more hydrophilic, elutes earlier and the two different modified peptides can be distinguished. Alternatively, the mass difference can also be resolved using an Orbitrap mass spectrometer at the resolution (R=60,000) as described here.

As an example of acetylation, we chose a peptide of histone H4 that contains 4 lysines that can be acetylated: Lysine 5, 8, 12 or 16 (see (Selvi & Kundu, 2009) for an overview of histone acetylation). Acetylations can be on either one of the residues or also occur in different combinations. The peptide can thus be acetylated from zero to four times. Depending on the number of acetyl groups, peptides elute at different RT in the RP-C18. MS/MS analysis helps to distinguish the modified residue.

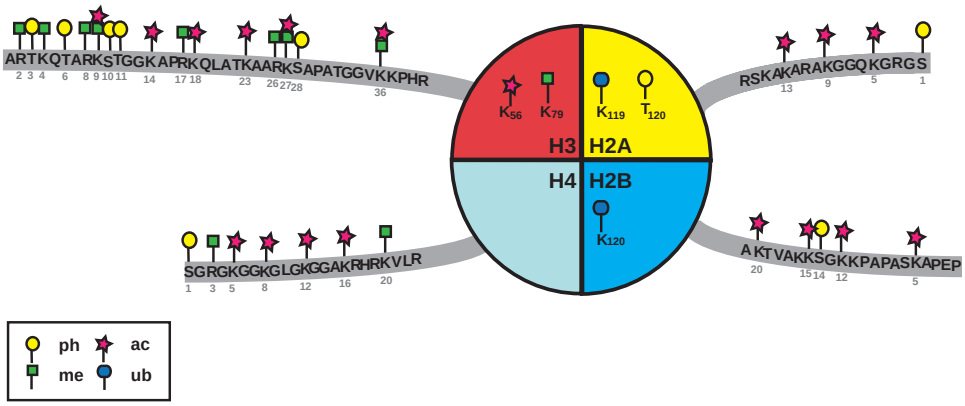
Masses of the peptides can be calculated using the GPMAW software where *in silico* digestion of the protein of interest containing the different modifications can be performed. ArgC digestion mimicks the tryptic peptides after propionylation. The output is a table of peptide masses with the monoisotopic peptide mass and different charges of the peptides (see Table 1).

To quantify the different peptides, the corresponding m/z is searched in the acquired MS file using XCalibur Qual Browser software. For our example peptides (Table 1), the m/z values for the doubly and triply charged peptides are used to obtain the extracted ion chromatogram (XIC). This XIC is defined by the intensity of the ion of interest at different retention times, resulting in a chromatographic peak whose area will reflect the abundance of the considered peptides. General parameters are: user-defined mass tolerance = 10ppm, display masses with 4 decimals, ICIS algorithm for peak detection. Qual Browser will display the retention time of the searched m/z and automatically integrate the area under the peak (Figure 4). The areas of the XICs are exported to an Excel file where quantification can be conducted (Figure 5).

The identity of the ions quantified using XIC can be confirmed by analyzing the MS/MS spectra of these ions (see Figure 4 for MS/MS display). Several softwares are available for this purpose (Eng et al, 2011; Kapp & Schutz, 2007). In the case of histone modifications, we use Protein Discoverer based on the Sequest algorithm (MacCoss et al, 2002; Yates et al, 1995) to correlate MS/MS spectra in a protein database. It is convenient to design a database in FASTA format containing only histones to decrease the time of search. Propionylation as well as the PTMs of interest (i.e. methylation and acetylation) are taken into account in the search parameters. The result will show a list of the identified peptides, the residue carrying the modifications of interest and a score to determine the confidence of identification, amongst other parameters.

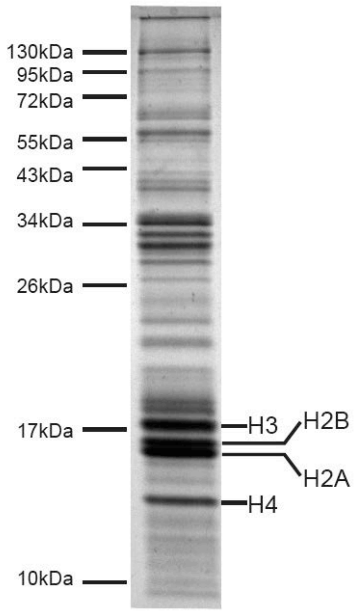
Figures

Figure 1:



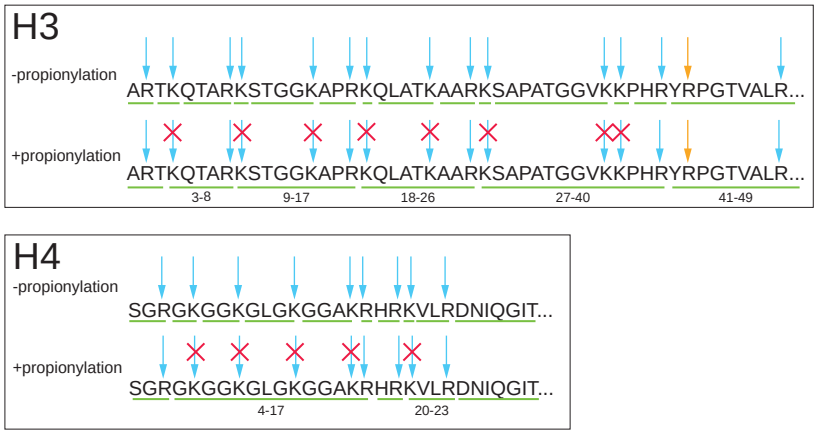
Overview of different histone posttranslational modifications. Scheme of the four different core histones (H2A, H2B, H3 and H4) with their protruding N-terminal tails. Displayed are examples of different modifications that are known from literature. Some residues, like lysine 27 on histone H3, can carry either methylation or acetylation as a modification.

Figure 2



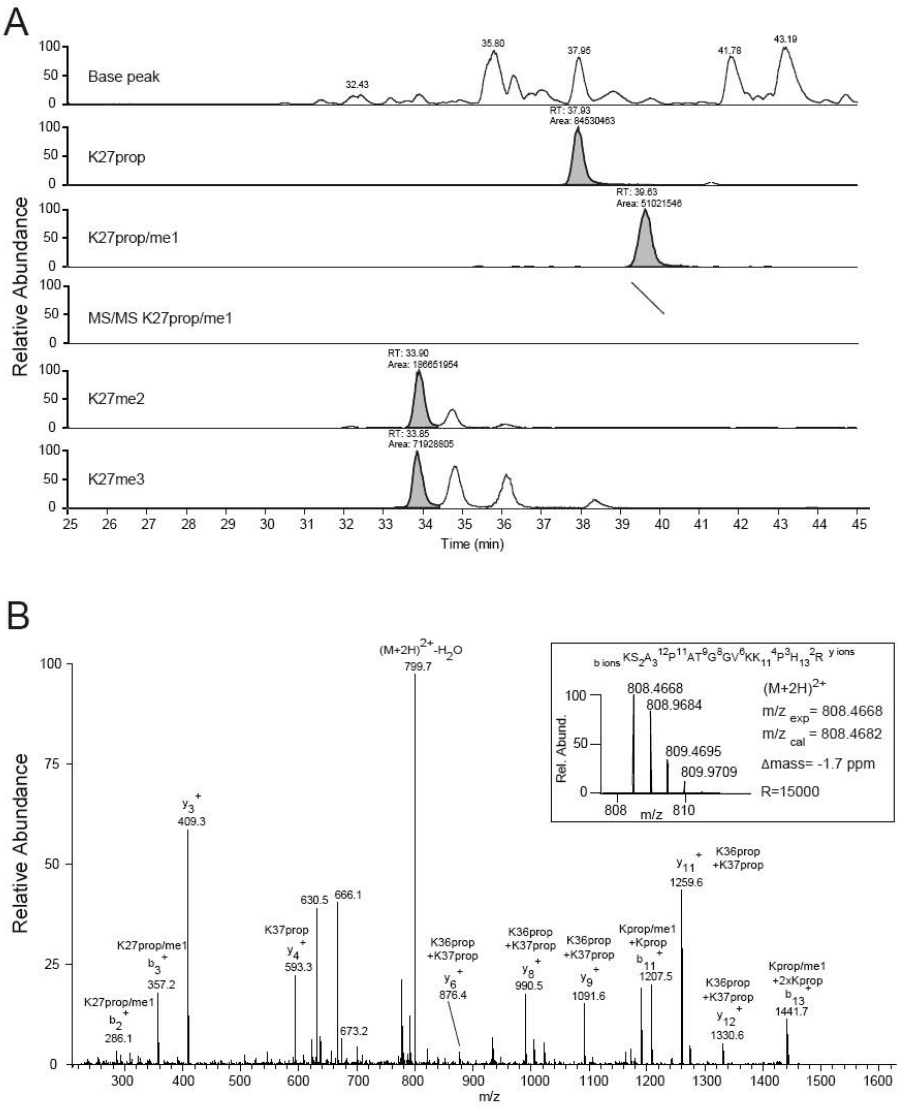
Example gel for an acid extraction. The supernatant of 10^6 HeLa cells is applied per lane. The histone pattern is indicated. The proteins are separated according to size on an 18% SDS-PAGE gel.

Figure 3



Peptides generated by trypsin on the N-terminal tail of histone H3 and H4 in absence of presence of propionylated lysines. Blue arrow: trypsin cutting sites (lysines and arginines). Orange arrow: Trypsin cutting is blocked on this arginine because of the following proline. Red cross: trypsin cutting sites blocked by treatment with propionic anhydride.

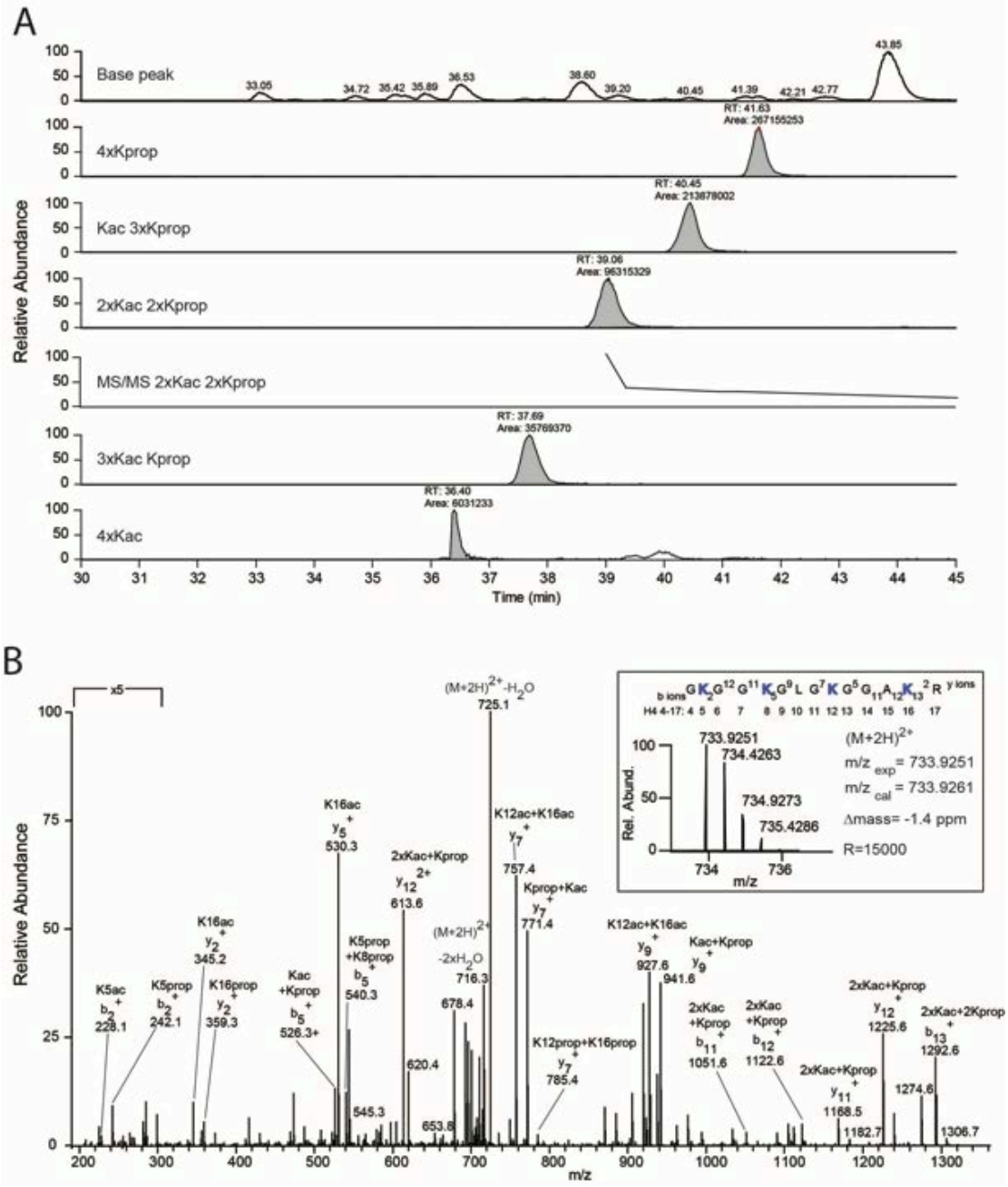
Figure 4.1



Quantification and identification of the example peptide H3 27-40 by mass spectrometry. Panel A: Base peak overview of the spectrum and XICs of unmodified, mono-, di- and trimethylated K27. The XICs are obtained in Xcalibur QualBrowser software by extracting the m/z value over time for doubly and triply charged peptides. The areas of peptides with different modification states are highlighted in grey. The presence of fragmentation spectra for the monomethylated peptide is also displayed. RT: retention time.

Panel B: MS/MS spectrum of the doubly-charged peptide 27-40 with monomethylated/propionylated K27. Assigning the fragment ions of these MS/MS spectra will confirm the identity of the peptide. Additionally, it points out the modified residue. In this case, fragment ions b_2^+ and b_3^+ clearly demonstrate methylation in K27 while y_6^+-y_9^+ show only propionylation in positions K36 and K37. The inlay shows the doubly charged ion [$(m+2H)^{2+}$] that is fragmented to obtain the MS/MS spectrum, the b and y ions that are identified on the sequence as well as the error of the measured mass. exp: expected, cal: calculated, R: resolution.

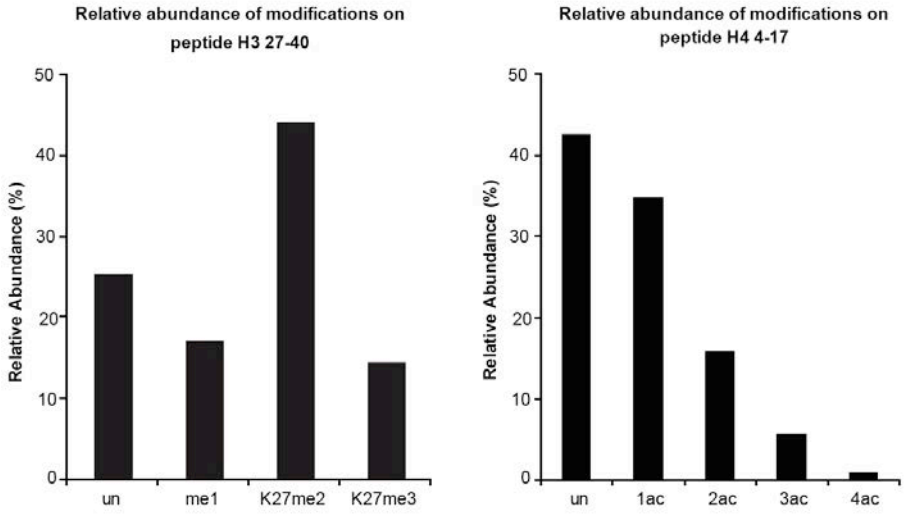
Figure 4.2



Quantification and identification of the example peptide H4 4-17 by mass spectrometry. Panel A: Base peak overview of the spectrum and XICs of zero to four times acetylated peptide. The presence of fragmentation spectra for the diacetylated peptide is also displayed. For description, refer to figure 4.1.

Panel B: MS/MS spectrum of doubly-charged diacetylated peptide 4-17. In this fragmentation spectrum, different combinations of acetylated residues are present. For instance, y_7^+ shows that there is acetylation present in K12 and K16 whereas b2+ demonstrates a fraction of acetylation also present on lysine 5. Automatic assignation by Sequest suggests the possibility of at least four acetylation combinations: K12/K16, K8/16, K5/K16 and K8/K12. For description of the inlay, refer to figure 4.1.

Figure 5



Modification on H3 27-40	Abundance (XIC area)	Relative Abundance (%)
un	6.2E+08	25.0
me1	4.2E+08	16.8
K27me2	1.1E+09	44.0
K27me3	3.5E+08	14.2

Modification on H4 4-17	Abundance (XIC area)	Relative Abundance (%)
un	2.7E+08	42.6
1ac	2.2E+08	34.8
2ac	9.9E+07	15.9
3ac	3.6E+07	5.7
4ac	6.0E+06	1.0

Quantifying histone modifications from XIC values. The percentages are calculated by adding up the XIC areas from all different modification states and setting them to 100%.

Table 1:

Peptide masses and mass-to-charge values for two example peptides. Upper panel: peptide 27-40 of histone H3. Lower panel: peptide 4-17 of histone H4. The lysines that are methylated or acetylated, respectively, are displayed in blue. In this protocol, the methylation example is focused on K27 although K36 is also known to be methylated. The table shows the masses of the peptides generated after treatment with propionic anhydride and trypsin for different modifications. m: peptide mass (amu); m/z: mass-to-charge ratio; (m+H)+: one times protonated peptide; $(m+2H)^{2+}$: two times protonated peptide; $(m+3H)^{3+}$: three times protonated peptide.

KSAPATGGVKKPHR				
Modification on H3 27-40	**m**	**$(m+H)^{+}$ m/z**	**$(m+2H)^{2+}$ m/z**	**$(m+3H)^{3+}$ m/z**
un	1600.9049	1601.9128	801.4603	534.6429
me1	1614.9206	1615.9285	808.4682	539.3147
me2	1572.9100	1573.9179	787.4629	525.3112
me3	1586.9257	1587.9335	794.4707	529.9831
ac	1586.8893	1587.8971	794.4525	529.971

GKGGKGLGKGGAKR				
Modification on H4 4-17	**m**	**$(m+H)^{+}$ m/z**	**$(m+2H)^{2+}$ m/z**	**$(m+3H)^{3+}$ m/z**
un	1493.8678	1494.8757	747.9418	498.9638
1ac	1479.8522	1480.8600	740.9340	494.2919
2ac	1465.8365	1466.8444	733.9261	489.62
3ac	1451.8209	1452.8287	726.9183	484.9482
4ac	1437.8052	1438.8131	719.9105	480.2763

List of Reagents

0.2mL tubes	PRC Strip tubes Axygen PCR-0208-C
Acetic acid	Roth 3738.1
Acetonitrile	Roth HN40.1
Ammonium bicarbonate	Roth T871.1
Coomassie Brilliant Blu G250	Serva 17524.01
Ethanol	Roth 9065.1
HPLC-grade water	Merck 1.15333.2500
Hydrochloric acid	Merck 100319.2500
Low binding tubes	Protein LoBind Tube 0.5 ml Eppendorf 022431064
Methanol	Roth AE71.1
Propionic anhydride	Merck 8.00608.0100
Trifluoroacetic acid	Roth P088.1
Trypsin	Sequencing Modified Grade Promega V5111
α-cyano-4-hydroxycinnamic acid matrix	Sigma C2020
µC18 ZipTips P10	Millipore ZTC18M960

Software

GPMAW	Lighthouse data
Protein discoverer	Thermo Scientific
XCalibur Qual Browser	Thermo Scientific

Abbreviations

FA	formic acid
ACN	Acetonitrile
CID	Collision-induced dissociation
EtOH	Ethanol
HOAc	Acetic acid
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LTQ	Linear trap quadrupole
m/z	mass-to-charge ratio
MeOH	Methanol
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PBS	Phosphate buffered saline
ppm	Parts per million
PTMs	Posttranslational modifications
RP-C18	Reversed phase chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
amu	Atomic mass unit
XIC	Extracted ion chromatogram

Reviewer comments:

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CeMM - Research Center for Molecular Medicine of the Austrian Academy of Sciences

(1) We cut all histones together, so that the original gel piece is much larger and can later be cut into 1X1 mm pieces.

(2) To ensure nearly 100% propionylation, we repeat steps 1-2 a second time.

(3) We extract twice with 50 µl 5% formic acid.

(4) We have defined an inclusion list of expected masses for known modified histone peptides to fragment these peptides preferentially

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Keywords

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Mass spectrometry

Acetylation

Methylation