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Examining Histone Posttranslational Modification Patterns by High Resolution Mass Spectrometry

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Abstract

Histone variants and posttranslational modifications (PTMs) are essential for epigenetic regulation of transcriptional expression. Single and/or combinatorial PTMs of histones play important roles in development and disease formation. Mass spectrometry (MS) has been a powerful tool to study histone variants and PTMs as it not only can identify novel PTMs but also can provide quantitative measurement of a spectrum of histone variants and PTMs in the same sample. In this chapter, we employ a combination of chemical derivation and high resolution mass spectrometry to identify and quantify multiple histone variants and PTMs. Histones are acid extracted and modified with propionyl groups, and subsequently produces suitable sizes of fragments for MS analysis by trypsin digestion. The newly generated N-termini of histone peptides can be differentially marked by stable isotope labeling in a second reaction of propionylation, which enables direct comparison between two different samples in the following MS analysis.

Keywords

epigenetics; histone posttranslational modifications; mass spectrometry; high performance liquid chromatography; propionylation; stable isotope labeling

1. Introduction

Epigenetics refers to the inheritable changes that affect gene expression and phenotypes without alterations in the DNA sequence. Epigenetic regulation of transcriptional expression is essential and important for cell type specificity as well as plasticity in development (Bird, 2007; Goldberg et al., 2007). Epigenetic variations often involve changes at the nucleosome level, such as DNA methylation, and in addition, the use of histone variants and posttranslational modifications (PTMs) on histone proteins. Histone PTMs can dictate the higher-order chromatin structure. For instance, tri-methylation of histone H3 lysine 9 (K9me3) and 27 (K27me3) are often important for heterochromatin formation and gene silencing (Kouzarides, 2007). Furthermore, many transcription factors, histone modifying enzymes, as well as the transcriptional machinery components can bind to different histone PTMs in an ordered manner and manipulate DNA. Misregulation of histone variants and

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PTMs are known to be associated with various human diseases including cancer (Egger et al., 2004).

Antibody-related techniques such as western blotting have been important tools for studying histone PTMs. However, there are intrinsic limitations in these techniques (Fuchs and Strahl, 2011): they rely on available antibodies; they cannot identify novel modifications or histone variants that are very similar to each other; and they are limited by the number of PTMs that can be analyzed simultaneously. In addition, antibodies may have cross-reaction to multiple PTMs (e.g. di- and tri-methylation of lysine residues, as seen in many commercially available antibodies) (Fuchs et al., 2011) and may have epitope masking effects when two PTMs are closely positioned (e.g. histone H3 serine 10 phosphorylation and H3K9me3) (Duan et al., 2008). Histones, H3 and H4 N-termini in particular, are highly enriched in the number of possible modifications (Young et al., 2010). Therefore, a different technique is required to identify single and combinatorial histone PTMs in an unbiased manner.

Mass spectrometry (MS) has been a powerful tool to study histone variants and PTMs as it can not only identify novel PTMs but also provide quantitative measurement of a spectrum of histone variants and PTMs in the same samples. In this chapter, we describe a protocol for analysis of histone PTMs by high resolution bottom-up MS (sequencing of digested small peptides and measuring PTMs). Briefly, histones are purified from biological samples of interest and are subjected to chemical derivation and trypsin digestion before MS analysis. For quantitative comparison between two biological states (including tissue culture and tissue samples), a cost effective stable isotope labeling approach is implemented in a second chemical reaction.

2. Materials

2.1. Reagents

Trypsin for tissue culture; Tissue culture medium; Glycerol; Acetone; Glycerol; Bradford protein assay reagent; Ammonium hydroxide (NH_4OH), 28% NH_3 in water; Propionic anhydride (D0 & D10); 2-propanol; 3M Empore^{TM} Solid Phase Extraction Disk C18 and C8; HPLC grade water; HPLC grade acetonitrile; Methanol; C18 phase 3 μm or 5 μm resin particles with 200Å pore size.

2.2. Buffers

1. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4 \cdot 2H_2O$, 2 mM KH_2PO_4
2. Nuclei Isolation Buffer (NIB-250): 15 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 250 mM Sucrose
3. Ammonium Bicarbonate (NH_4HCO_3): 100 mM NH_4HCO_3, pH 8.0
4. Offline HPLC buffer A: 5% Acetonitrile, 0.2% Trifluoroacetic Acid (TFA) in HPLC grade water
5. Offline HPLC buffer B: 95% Acetonitrile, 0.188% TFA in HPLC grade water

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6. Stage-tip sample and wash buffer: 0.1% Acetic acid
7. Stage-tip elution buffer 1: 50% Acetonitrile, 5% Acetic acid
8. Stage-tip elution buffer 2: 75% Acetonitrile, 5% Acetic acid
9. TiO_2 loading buffer: Saturated glutamic acid, 65% Acetonitrile, 2% TFA
10. TiO_2 wash buffer 1: 65% Acetonitrile, 0.5% TFA
11. TiO_2 wash buffer 2: 65% Acetonitrile, 0.1% TFA
12. TiO_2 elution buffer 1: 50% Acetonitrile, 300 mM NH_4OH
13. TiO_2 elution buffer 2: 60% Acetonitrile, 500 mM NH_4OH
14. Online HPLC buffer A: 0.1 M Acetic acid in HPLC grade water
15. Online HPLC buffer B: 0.1 M Acetic acid, 95% Acetonitrile, in HPLC grade water

2.3. Solutions

1. Protease inhibitors (add fresh to buffers prior to use) : 1 M Dithiothreitol (DTT) in ddH2O (1000X); 200 mM AEBSF in ddH2O (400X)
2. phosphatase inhibitor (add fresh to buffers prior to use): 2.5 µM Microcystin in 100% ethanol (500X)
3. HDAC inhibitor (add fresh to buffers prior to use): 5 M Sodium butyrate, made by titration of 5M butyric acid using NaOH to pH 7.0 (500X)
4. NP-40 Alternative: 10% v/v in ddH_2O
5. H_2SO4: 0.4 N/0.2 M in ddH_2O
6. Trichloroacetic acid (TCA): 100% w/v in ddH_2O
7. Acetone+0.1% Hydrochloric acid (HCl): 0.1% v/v HCl in acetone

2.4. Equipment

Razor blades; 1.5 mL and 2.0 mL microcentrifuge tubes; 15 and 50 mL conical tubes; Pipetmans and tips; −80 °C refrigerator; Tissue and cell homogenizers; Liquid nitrogen; Glass Pasteur pipets; pH indicator strips (pH 0–14); Heat blocks or water baths; Offline-HPLC; Fused silica tubing; Kim-wipes; Laser tip puller; Dissection microscope; Micro-stir Bars; Pressure cells; Compressed helium gas; Compressed argon gas; Online LC-MS/MS.

3. Nuclei isolation

3.1. Cell harvest from tissue

1. Dissect out desired tissue, rinse with ice-cold PBS.
2. Dissected tissue can be snap-frozen and kept at −80 °C, or continue with the following steps and nuclei isolation.
3. Mince fresh or frozen tissue with a razor blade into small pieces.

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4. Collect minced tissue in microcentrifuge tubes or 15 ml conical tubes, estimate the volume of tissue.
5. Continue with nuclei isolation.

3.2. Cell harvest from tissue culture

1. For suspension cells, centrifuge cells at 300 rcf for 5–10 min; for attached cells, trypsinize cells, stop the trypsinization and centrifuge at 300 rcf for 5–10 min.
2. Wash cell pellets with PBS and centrifuge at 300 rcf for 5–10 min.
3. Repeat Step 2.
4. After the second wash, remove PBS and estimate the volume of cell pellets.
5. Cell pellets can be frozen at −80 °C, or continue with nuclei isolation.

3.3. Nuclei isolation

1. Based on the number of samples and sample sizes, estimate the amount of NIB-250 buffer need. Add protease inhibitors and other inhibitors to NIB-250 buffer. Typically, for 1 mL of cell pellet, make 50 mL of NIB-250 buffer+inhibitors by adding 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.
2. Lyse the cell pellet with 10:1 (v/v) ratio of NIB-250+inhibitors+0.2% NP-40 Alternative. For instance, for 1 mL of cell pellet, take 10 ml of NIB-250+inhibitors, add 200 μL of 10% NP-40 Alternative.
3. Homogenize with appropriate instrument. For example, liver samples can be homogenized using pestles or dounce homogenizers. Tissue culture cells can be homogenized with gentle pipetting.
4. Incubate homogenized cells on ice for 5–10 min, the cells will lyse and release nuclei.
5. Centrifuge at 1000 rcf for 5–10 min at 4 °C. The supernatant contains mostly cytoplasmic components. The pellet contains mostly the nuclei. The size of the nuclei pellet should be smaller than the size of the original cell pellet.
6. Wash the nuclei pellet by gently resuspending with 10:1 (v/v) NIB-250+inhibitors (without NP-40 Alternative).
7. Centrifuge at 1000 rcf for 5 min at 4 °C.
8. Wash 2–4 more times until no NP-40 Alternative is left (Note: residual NP-40 Alternative forms bubbles during washes. Repeat the washes until no more bubbles form).
9. Isolated nuclei can be frozen in liquid nitrogen in NIB-250+inhibitors+5% glycerol or used promptly for acid extraction.

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4. Acid extraction

1. Slowly add 0.4 N H_2SO_4 to the nuclei pellet at a 5:1 (v/v) ratio, resuspend the pellet by gentle pipetting to break up the pellet.
2. Incubate the sample with constant rotation or gentle shaking for 2–4 hr at 4 °C. For sample beginning with more than 500 μL cell pellet, a 2 hr extraction is enough incubation time. Longer extraction is not recommended because basic proteins other than histones will be also extracted. For small sample size such as 100 μL cell pellet, 4 hr extraction will give a better yield.
3. Centrifuge at 3,400 rcf for 5 min.
4. Transfer the supernatant to a new 1.5 mL or 15 mL tube based on the volume.
5. Repeat Steps 3–4.
6. Estimate the volume of the supernatant; add 1/4 volume of 100% TCA and mix.
7. Let this mixture precipitate on ice for at least 1 hr. Do not disturb the precipitation. For samples that start with small cell numbers, overnight precipitation is recommended.
8. Centrifuge at 3,400 rcf for 5 min. Histones form a “film” like layer around the bottom of the tube, and the white pellet on the bottom contains mostly other proteins or non-protein material. Carefully remove the supernatant by aspiration without touching the precipitated proteins.
9. Rinse the tube with acetone+0.1% HCl to cover the precipitated proteins. (Note: Use glass Pasteur pipettes to transfer acetone).
10. Centrifuge at 3,400 rcf for 2 min and discard supernatant.
11. Rinse the tube with 100% acetone and repeat Step 10. (Note: Use glass Pasteur pipettes to transfer acetone).
12. Air dry pellet for 30 min to overnight until completely dry.
13. Dissolve the histones with ddH_2O, make sure the “film” like layer is completely dissolved. Typically, for pellets in a 1.5 mL microcentrifuge tube, 100 to 250 μL ddH_2O is used.
14. Centrifuge the tube containing reconstituted histones at 3,400 rcf or higher for 2 min and transfer the supernatant to a new tube. Discard the pellet which contains mostly non-histone proteins and other material.
15. Measure the protein concentration by Bradford protein assay or other methods.
16. Purified reconstituted histones can be stored at −80 °C or continue with the following steps. If the protein concentration is too low for the next steps, histones can be concentrated using a SpeedVac concentrator.

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5. Offline reverse phase-high performance liquid chromatography for histone variant purification (Optional)

If analysis of specific histone variants are desired, offline reverse phase-high performance liquid chromatography (RP-HPLC) may be performed.

1. For a 4.6 mm Vydac C18 5 μm column (4.6 mm internal diameter × 250 mm, Vydac), Set up the flow rate to be 0.8 mL/min.
2. Use a buffer gradient with offline HPLC buffer A and B as shown in Table 1.
3. Set up automatic sample collector to collect samples for 1-min intervals between 15–80 min.
4. Load 100–200 μg of histones, run the buffer gradient. A typical histone run is shown in Fig. 1.
5. Transfer desired histones to microcentrifuge tubes and dry down in a SpeedVac concentrator to dryness to remove organic solvent and TFA.
6. Dry histones can be stored at room temperature or histones that are reconstituted can be stored at −80 °C or you can continue with the chemical derivatization.

6. Chemical derivatization of histones

Histone proteins are highly enriched in basic residues, especially on the N-termini of histone H3 and H4 where most PTMs reside. This is problematic for proteomic analysis because the most commonly used proteases usually cleave on either basic or acidic residues. For example, trypsin cleaves at the C-termini of arginine and lysine residues when neither is followed by proline. Trypsin digestion of histones results in small peptides that are difficult to retain on RP-HPLC columns and analyzed by MS. On the other hand, Glu-C cuts at the C-termini of glutamic acid and aspartic acid residues, and Glu-C digestion of histones generates large multiply charged peptides whose MS/MS spectra are very difficult to interpret if fragmented with standard collisionally activated dissociation (CAD) methods. To resolve this problem, we developed a method with a combination of chemical derivatization of histones and trypsin digestion (Garcia et al., 2007). By treating histones with propionic anhydride before trypsin digestion, any free amine groups including the N-termini of peptides and ε-amino groups of unmodified and mono-methylated lysine residues are converted to propionyl amides. Therefore, trypsin digestion only induces proteolysis at the C-termini of arginine residues. In addition, propionylation also reduces charges on treated peptides, which makes the histone peptides less hydrophilic. The resulting histone peptides can also be easily resolved by standard RP-HPLC. Furthermore, these peptides generate doubly and triply charged ions in electrospray ionization MS, resulting in CAD MS/MS spectra that are easy to interpret. The work flow for chemical derivatization of histones is shown in Fig. 2. (Note: Histones H1, H2A and H2B have fewer lysine residues than H3 and H4. Therefore, for H1, H2A and H2B, the propionylation before trypsin digestion should be skipped due to the arrangement of the lysine and arginine residues in their sequences.)

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6.1. Propionylation before trypsin digestion

1. Take 60–125 μg histone sample dissolved in ddH_2O, add 15 μL 100 mM NH_4HCO_3, pH 8.0. Dilute or dry the sample to achieve a desired volume of ~20–30 μL. (Note: smaller amount of histones can also be used; however the relative yields maybe poorer. NH_4HCO_3 provides buffering of pH for future reactions.)

2. Use a pH indicator strip to monitor the pH of samples. Add 0.5–2 μL of NH_4OH (28% NH_3 in water) to get pH 7–9 if necessary. Use glacial acetic acid to balance the pH if needed. (Note: NH_4OH and glacial acetic acid should be handled in a fume hood)

   Step 3–8 must be performed in a streamlined manner without any interruptions for maximum reaction efficiency. These steps should be performed in a fume hood.

3. Prepare propionylation reagent by adding 1 volume of propionic anhydride to 3 volumes of 2-propanol. Vortex briefly to mix. This reagent must be made freshly for every three samples (For three samples that have the volume of 20–30 μL, mix 10 μL of propionic anhydride and 30 μL of 2-propanol).

4. Immediately add 1/3 to 1/2 volume of propionylation reaction to a histone sample (i.e. 10 μL propionylation reaction for 30 μL histone sample).

5. For one volume of the propionylation reaction used in step 4, immediately add 1/4 to 1/2 volume of NH_4OH to the histone sample (2.5–5 μL NH_4OH for 10 μL propionylation reaction). (Note: This step is to keep the pH to be around 8.0. The reaction between propionic anhydride and amino groups produces propionic acid, leading the pH to drop. Low pH environment facilitates the hydrolysis of propionic anhydride, which further releases propionic acid. On the other hand, when pH is larger than 10.0, labeling of other residues with higher pK_a is possible. To achieve the best efficiency of amino groups labeling, the pH needs be kept at around 8.0.)

6. Mix immediately by vortex.

7. Test pH by pipetting a small amount (<0.5 μL) on a pH indicator strip, make sure the pH is between 7–9. If the pH is acidic, add more NH_4OH to achieve ~ pH 8.0. If the pH is >10.0, add glacial acetic acid.

8. For each propionylation reaction prepared, repeat Steps 3–7 for two more samples. If any steps are interrupted, make new reagents for additional samples.

9. Briefly centrifuge and incubate samples at 37 °C on a heat block or in a water bath for 15 min.

10. Dry samples down to 5–10 μL in a SpeedVac concentrator at room temperature. This step usually takes 20–30 min. The unreacted propionic anhydride and 2-propanol, as well as remaining acetic acid or ammonia gas released from NH_4OH evaporate during the drying step and therefore do not affect later reactions.

11. Dilute sample to 30 μL by adding ddH_2O.

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12. Adjust pH to be around 8.0 by NH_4OH and/or possibly glacial acetic acid.
13. Repeat Steps 3–10. Two runs of propionylation ensure maximum conversion (>95%) of amino groups to propionyl amides.
14. Histone samples can be stored at −80 °C or continue with trypsin digestion. 2-propanol and propionic anhydride containers need to be filled with argon gas to keep moisture from the air away from the surface of the reagent.

6.2. Trypsin digestion of histone samples

1. Start with 5–10 μL propionylated histones. Add 15 μL 100 mM NH_4HCO_3 and ddH2O to 50–100 μL.
2. Check and adjust pH to be around 8.0.
3. Add trypsin to histone samples at a 1:20 ratio (e.g. 5 μg of trypsin for 100 μg of histones).
4. Incubate at 37 °C for 6 hr.
5. Stop the digestion by adding 2–5 μL (or more) of glacial acetic acid to reach pH 3.0, which prevents trypsin from further digestion.
6. Freeze the sample at −80 °C to fully deactivate trypsin.

6.3. Propionylation of histone peptides after trypsin digestion

1. Dry down the sample to 5–10 μL in a SpeedVac concentrator. This step takes 45 min – 1.5 hr.
2. Add NH_4OH and/or glacial acetic acid to reach pH 8.0.
3. Repeat Steps 3–13 in 6.1. These steps are to convert the newly trypsin-generated N-termini to propionyl amides. Because there are accumulative salts generated from propionylation reactions and pH adjustments, the drying steps at this point could take much longer time (1–2 hr) than previous drying steps.

   For relative quantification of histone peptides between two types of cells or tissues, one sample is modified by the D0 propionic anhydride $(CH_3CH_2CO)_2O$, while the other sample is modified by D10 propionic anhydride $(CD_3CD_2CO)_2O$ at this step. A propionyl group with five hydrogen (D0) or deuterium (D5) atoms is added to each newly synthesized N-terminus. In the following steps, these two samples are mixed together (Step 7.10 or Step 9.2.1).
4. Propionylated peptides can be stored at −80 °C and need to be desalted before running MS. 2-propanol and propionic anhydride containers need to be filled with argon gas to keep away air that contains water.

7. Stage-tip clean-up of peptide samples prior to MS analysis

Peptide samples treated with propionylation contain salts that ionize much more efficiently than peptides and can lead to signal suppression in the MS. Therefore, peptides samples need to be desalted prior to MS analysis.

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1. Drill holes on top of one 2 mL and one 1.5 mL microcentrifuge tube using a suitable size screw driver for each peptide sample.
2. Reside a P200 tip on each 2 mL microcentrifuge tube with a hole on the lid. The 2 mL microcentrifuge tubes are used as flow-through collectors in the following steps. Discard the flow-through when needed.
3. Measure and cut out 1.3 cm from a P1000 pipette tip (USA Sci. Catalogue number 11112721) using a razor blade.
4. Use this pipette tip to punch out three mini disks from a C18 extraction disk. The mini disks stick in the pipette tip and can be transferred into the P200 tip.
5. Push the mini disks out of the P1000 tip using a piece of fused silica or tubing fitting in the inside of the P1000 tip. Ensure that the discs are securely wedged in the bottom of the P200 tip and there is not space in between the disks (Fig. 3A).
6. Remove the fused silica and P1000 tip.
7. To activate the C18 mini disks, add 50 μL methanol to each P200 tip, centrifuge at 500 g for 2 min.
8. Repeat Step 7. Once activated, the C18 mini disks need to be kept wet in the following steps. Do not let the stage-tips sit without buffer for excessive amount of time.
9. Wash the stage-tips by adding 200 μL of 0.1% acetic acid and centrifuge at 500 g for 2 min or longer, until most wash buffer pass through the C18 mini disks.
10. Dilute the peptide samples to 200 μL with 0.1% acetic acid. The pH should be acidic (pH 4.0 or below). Adjust pH using glacial acetic acid if needed.
11. Transfer diluted samples to the stage-tips and centrifuge at 200 g for 5–10min until most samples pass through the C18 mini disks.
12. Wash with 200 μL of 0.1% acetic acid and centrifuge at 500 g for 2–5 min.
13. Repeat Step 12 and make sure no wash buffer is retained on the stage-tip.
14. Switch the P200 tips to the 1.5 mL microcentrifuge tubes with holes on the lid.
15. Elute with 75 μL of Elution buffer 1, centrifuge at 200 g for 5 min.
16. Elute again with 75 μL of Elution buffer 2, centrifuge at 200 g until all elution buffer pass through the stage-tips.
17. Transfer eluted samples to new 1.5 mL microcentrifuge tubes.
18. Dry down in a SpeedVac concentrator to 5–25 μL. The purified peptides can be stored at −80 °C or continue with Section 8 or directly subjected to RP-HPLC and MS analysis.

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8. Enrichment for phosphorylated peptides using titanium dioxide (TiO_2) beads (optional)

Protein phosphorylation on serine, threonine or tyrosine residues is well-known to be important for numerous cell processes such as signal transduction pathways. Histone phosphorylation on histones H1, H2A.X, H2B, H3 and H4 have been reported over the past few decades. Unlike methylation and acetylation, phosphorylation on histones is usually at a low abundance and is challenging for proteomic analyses. In order to concentrate phosphorylated peptides, we employed techniques established by others (Larsen et al., 2005; Li et al., 2009) using TiO_2 beads. We use the same set up as the stage-tips to make TiO_2 micro-columns. We typically start with 100–500 μg peptide samples. For sample size larger than 500 μg, TiO_2 enrichment can be done in microcentrifuge tubes with rotating incubation and centrifuge.

1. Weigh TiO_2 beads in a microcentrifuge tube. For each peptide sample, use a minimum of 1.5 mg or 4 fold of the sample amount (Li et al., 2009), whichever is the smaller.
2. Resuspend the TiO_2 beads in TiO_2 loading buffer, shake or rotate at room temperature for 15 min or longer. Continue with Steps 3–8 while waiting for this step.
3. Drill holes on top of one 2 mL and two 1.5 mL centrifuge tubes using a suitable size screw driver for each peptide sample.
4. Reside a P200 tip on each 2 mL microcentrifuge tube with a hole on the lid. The 2 mL microcentrifuge tubes are used as flow-through collectors in the following steps. Empty the 2 mL tubes in the following steps when needed.
5. Measure and cut out 0.3 cm from a P1000 pipette tip (USA Sci. Catalogue number 11112721) using a razor blade.
6. Use this pipette tip to stamp out one mini disk from a C8 extraction disk for each sample. Transfer the C8 mini disk to the P200 tip.
7. Make a C8 plug by pushing the C8 mini disk to the bottom of the P200 tip, as in Step 7.5.
8. Activate the C8 plug by adding 50 μL methanol and centrifuge at 500 g for 2 min.
9. Transfer TiO_2 beads in loading buffer from step 8 to the P200 tips with C8 plugs. Right before each transfer, vortex the beads briefly to make sure the TiO_2 beads are well suspended.
10. Centrifuge at 300 g for 2–5 min. The TiO_2 beads form a micro-column on top of the C8 plug (Fig. 3B). Keep the TiO_2 micro-column wet during the following steps. Do not let the micro-column sit without buffer for excessive amount of time.
11. Wash the TiO_2 micro-column with 200 μL of loading buffer and centrifuge at 500 g for 2–5 min. Make sure all buffer go through the column.
12. Switch the P200 tip to a 1.5 mL microcentrifuge tube with a hole on the lid.

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13. Dilute the peptide sample at least 1:2 with loading buffer and transfer to the TiO_2 micro-column. Centrifuge at 200 g for 10 to 20 min. If the sample volume is more than 200 μL, load multiple times.
14. (Optional) To maximize the binding of phosphorylated peptides, reapply the flow-through back to the TiO_2 micro-column, centrifuge at 300 g for 5–10 min.
15. Switch the TiO_2 micro-columns back to the 2 mL microcentrifuge tubes and continue with Step 16. Save the flow-through from Step 14 and dry down in a SpeedVac concentrator. Make sure no acetonitrile is left in the flow-through sample before proceeding to step 23.
16. Wash the TiO_2 micro-column with 200 μL of loading buffer and centrifuge at 500 g for 2–5 min.
17. Wash the micro-column with 200 μL of TiO_2 wash buffer 1 and centrifuge at 500 g for 2–5 min.
18. Wash the micro-column with 200 μL of TiO_2 wash buffer 2 and centrifuge at 500 g for 2–5 min. Make sure all wash buffer pass through the column.
19. Switch the P200 tip to the second 1.5 mL tube with a hole on the lid.
20. Elute with 200 μL TiO_2 elution buffer 1, centrifuge at 200 g for 5–10 min.
21. Elute again with 200 μL TiO_2 elution buffer 2, centrifuge at 200 g for 5–10 min until all elution buffer pass through the TiO_2 micro-column.
22. Discard the TiO_2 micro-columns. Dry the eluted peptides down to less than 50 μL in a SpeedVac concentrator.
23. Stage-tip both flow-through samples and TiO_2-enriched samples following instruction in Section 7. The flow-through samples contain TFA and therefore the pH should be acidic; whereas the TiO_2-enriched samples are basic because the elution buffers contain NH_4OH. Use glacial acetic acid to adjust the pH to be below 4.0 before performing stage-tip clean-up.
24. The samples are now ready to be submitted to online RP-HPLC and MS analysis, or be stored at −80 °C.

9. Online RP-HPLC and MS

9.1. Packing HPLC capillary column (this step can be omitted if using commercially available columns)

1. Cut 30–40 cm of 75 μm internal diameter & 360 μm external diameter fused silica tubing.
2. Burn off about 5 cm of the coating in the middle of the tubing. Wet a Kim-wipe with methanol and wipe off the remaining of the coating.
3. Pull the tubing using a laser tip puller aiming the middle of the tubing where the coating is burned off. Each tubing gives two columns at this step.

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4. Check under a dissection microscope. The tips of the columns should be very sharp.

5. In a clean HPLC glass vial, mix ~50 μL of dry C18 reversed phase 3 μm or 5 μm particles with 200Å pore size (Magic C18, Michrom BioResources Inc.) in 1 mL of 1:1 2-propanol and methanol. Put in a micro-stir bar.

6. Pack the column in an available pressure cell (e.g. Brechbuehler) using helium gas at around 1000 psi. The pressure cell should be placed on a magnetic plate. Turn on the stirring to prevent the C18 material from settling down during packing. (Note: The tip of the column may need to be tapped to create an opening.) This step takes around 2–5 hr.

7. Stop the packing when the column is packed around 10–15 cm. Turn off the pressure slowly and remove the column once the pressure is down to 1 psi.

8. Block the column by running peptide mixtures (we use a mixture of 5–10 pM of angiotensin and bradykinin in 0.1% acetic acid) through once or twice. This step can be done either in a pressure cell or on HPLC.

9.2. Running online RP-HPLC and MS

1. Load peptides on the column. For propionylated and desalted histone peptides, 5–20 μg equivalent of the total starting protein amount (measured in 4.15) should be used. For TiO_2 enriched samples, depending on the cell and tissue type, use 50–100 μg equivalent of the starting protein amount; Use 5–20 μg for the flow-through samples.

   For relative quantification of histone peptides between two types of cells or tissues, mix equal amount of peptides modified using D0 and D10 propionic anhydride. If different amount of the two samples are mixed, very different ionization efficiency of the same peptide may occur in the MS run and therefore cause inaccurate quantification. It may be necessary to run each sample individually to estimate the relative amount before mixing.

2. Run nanoflow LC using a buffer gradient with online HPLC buffer A and B as shown in Table 2.

3. The peptides are separated by the online RP-HPLC and are directly eluted into an electrospray ionization high-resolution tandem mass spectrometer (i.e. an Orbitrap) operated in data-dependent MS/MS mode.

9.3. Data analysis

The example data shown below was collected by Nanoflow LC tandem MS (LC-MS/MS), on a hybrid linear quadrupole ion trap-orbitrap mass spectrometer (Thermo Electron) coupled to an Agilent 1200 Series binary HPLC pump (Agilent Technologies) and an Eksigent AS2 autosampler (Eksigent Technologies). The mass spectra (MS1) was collected in the orbitrap at 30000 resolution, followed by 8 data-dependent tandem mass spectra (MS2) collected in the linear quadrupole ion trap. Data is visualized and analyzed in Thermo Xcalibur Qual Browser.

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9.3.1. Identification of histone PTMs—Because histones are highly modified and multiple combinatorial forms of modifications exist, it is challenging to separate all different forms of histone peptides. The propionylation treatment not only prevents trypsin digestion of lysine residues and removes charges from digested peptides, but also allows better chromatography in RP-HPLC. Taking the histone H3 9–17 peptide KSTGGKAPR as an example, we will explain how to identify different forms of this peptide.

The most frequent observed modifications on this peptide are acetylation of K9 and/or K14, mono-, di- and tri-methylation of K9. Taking into account of unmodified K9 and K14, there are at least 5×2=10 different forms of this peptide. Our system allows us to identify and further quantify all different forms of this H3 9–17 peptide. First, they can be distinguished according to their difference in mass over charge (m/z) (Table 3 and Fig. 4).

Second, we can additionally identify peptides based on their retention time on the RP-HPLC column. For the propionylated histone peptides that only differ at one PTM, the order of hydrophobicity is: di- and tri-methylated < acetylated < unmodified (propionylated) < mono-methylated (propionylated) peptides. As shown in Fig. 4C and D, K9me3K14ac and K9me2 peptides have the same m/z. Because K9me3K14ac and K9me3 only differ at K14ac, therefore the first peak in Fig. 4C should be K9me3K14ac, which has an earlier retention time as compared with K9me3; whereas K9me2 has a very similar retention time as K9me3. However, in Fig. 4A, in the spectra of m/z 535.304, two peaks were detected, which should be from the unmodified histone H3 9–17 peptide and the K9me1K14ac peptide. Both the mono-methylation and acetylation should add hydrophobicity to the peptide; however, the acetylation also prevents the peptide from being propionylated, which causes the peptide to be more hydrophilic relatively. In addition, both peaks in Fig. 4A were released from the RP-HPLC in between the peaks in Fig. 4B (K9me1) and Fig. 4E (K9ac and/or K14ac), suggesting they are both more hydrophilic than a mono-methylated peptide, and are more hydrophobic than an acetylated peptide. To determine the identity of these peptides, a third layer of information, the tandem MS, is required. Fig. 6 shows the MS/MS spectrum of two precursor ions with m/z around 535.304. Using this information we determined that the first peak in Fig. 4A came from an unmodified peptide, whereas the second peak came from the K9me1K14ac peptide. Similarly, we determined the two peptides in Fig. 4C to be K9me3K14ac and K9me2, respectively.

9.3.2. Relative quantification of histone PTMs—To quantify the abundance of histone PTMs, we measure the area of each peak in the MS chromatogram (Fig. 4). Usually $[M+H]^{+}$, $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions of the same peptide, if detectable, are measured (Fig. 7). The sum of all different modified forms of a histone peptide is designed as 100%; and the relative quantity of each PTM is calculated by dividing the area of the particular PTM MS signal peaks by the total area. For K9ac and K14ac, because they have the identical m/z and retention time, we also need to use the MS/MS spectrum to find the ratio of b and/or y ions that were different between the two peptides (Fig. 8). The averages of all these ratios are used to estimate the relative amount of K9ac and K14ac.

9.3.3. Relative quantification of histone peptides between two types of cells or tissues by stable isotope labeling—Very often in research we need to compare

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relative abundance of histone PTMs between two different cell/tissue types. There are unavoidable run to run system variations in RP-HPLC and MS. In order to avoid these variations, histone peptides from two origins can be labeled by different stable isotopes and analyzed together. To achieve best quantitative results, equal amount of histone peptides from two origins need to be used to accomplish similar ionization efficiency.

The histone peptides from two different cell/tissue types are labeled by D0 and D10 propionic anhydride in step 6.3, respectively. Equal amount of peptides are mixed together before stage-tip in Step 7.10 or after stage-tip in Step 9.2.1. It may be necessary to run each sample separately and adjust the amount according to the signals from the individual runs before mixing. An example is given in Fig. 9. Samples 1 and 2 are from two different cell types and are differentially labeled. The histone H3 41–49 peptide YRPGTVALR is used for loading control because it is not modified and only one form exists. Fig. 9A and B show this peptide had roughly similar signal strength between the two samples, demonstrating approximately equal loading. Fig. 9C and D show the histone H3 9–17 peptide with K9 mono-methylated, and sample 2 has around 30% enrichment of this modification compared with sample 1. A more precise measurement needs to take into account of all different forms of the H3 9–17 peptides as well as multiple charge states of these peptides, as discussed in 9.3.2.

Acknowledgments

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Reference

Bird A. Perceptions of epigenetics. Nature. 2007; 447:396–398. [PubMed: 17522671]

Duan Q, Chen H, Costa M, Dai W. Phosphorylation of H3S10 blocks the access of H3K9 by specific antibodies and histone methyltransferase. Implication in regulating chromatin dynamics and epigenetic inheritance during mitosis. J. Biol. Chem. 2008; 283:33585–33590. [PubMed: 18835819]

Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature. 2004; 429:457–463. [PubMed: 15164071]

Fuchs SM, Krajewski K, Baker RW, Miller VL, Strahl BD. Influence of combinatorial histone modifications on antibody and effector protein recognition. Curr. Biol. 2011; 21:53–58. [PubMed: 21167713]

Fuchs SM, Strahl BD. Antibody recognition of histone post-translational modifications: emerging issues and future prospects. Epigenomics. 2011; 3:247–249. [PubMed: 22122332]

Garcia BA, Mollah S, Ueberheide BM, Busby SA, Muratore TL, Shabanowitz J, Hunt DF. Chemical derivatization of histones for facilitated analysis by mass spectrometry. Nat. Protoc. 2007; 2:933–938. [PubMed: 17446892]

Goldberg AD, Allis CD, Bernstein E. Epigenetics: a landscape takes shape. Cell. 2007; 128:635–638. [PubMed: 17320500]

Kouzarides T. Chromatin modifications and their function. Cell. 2007; 128:693–705. [PubMed: 17320507]

Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jørgensen TJD. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. Mol. Cell Proteomics. 2005; 4:873–886. [PubMed: 15858219]

Li, Q-run; Ning, Z-bin; Tang, J-shu; Nie, S.; Zeng, R. Effect of peptide-to-TiO2 beads ratio on phosphopeptide enrichment selectivity. J. Proteome. Res. 2009; 8:5375–5381. [PubMed: 19761217]

Young NL, Dimaggio PA, Garcia BA. The significance, development and progress of high-throughput combinatorial histone code analysis. Cell. Mol. Life Sci. 2010; 67:3983–4000. [PubMed: 20683756]

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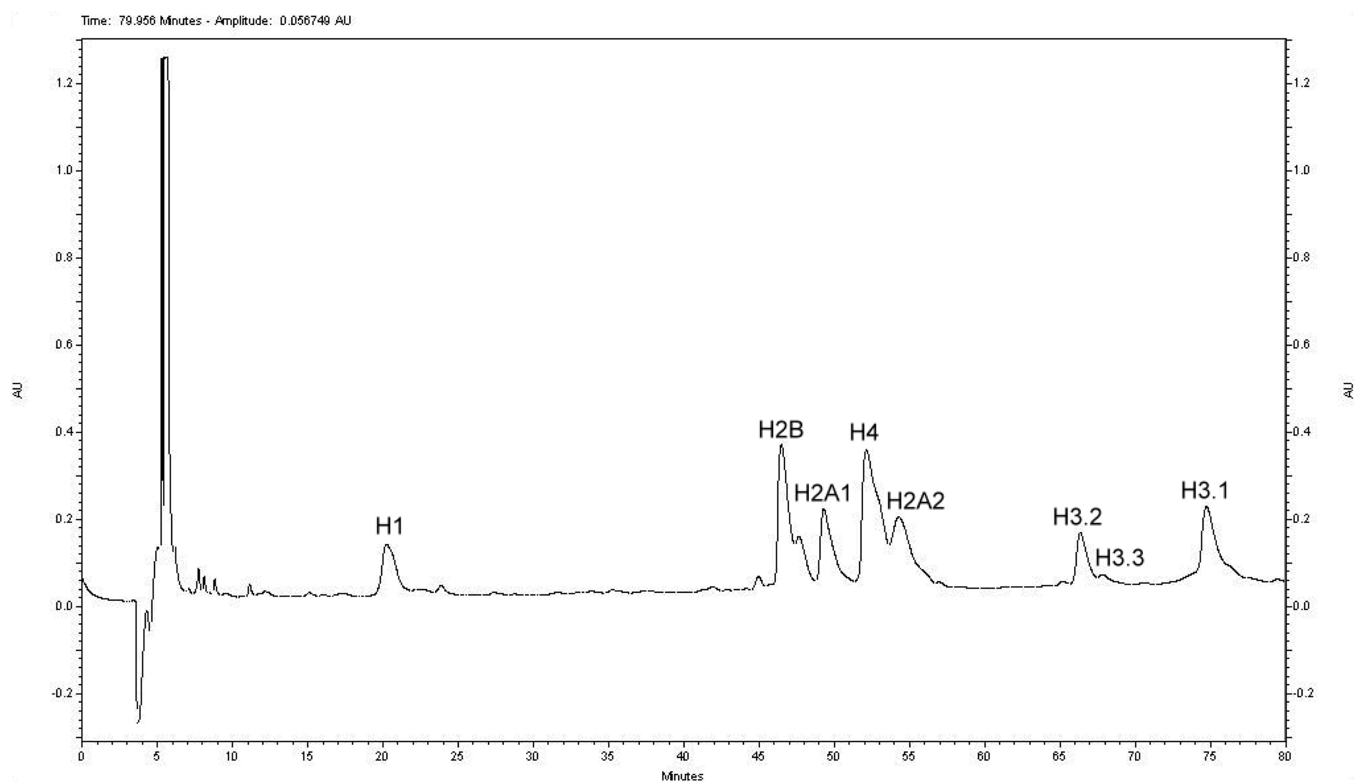

Figure 1.

Offline RP-HPLC separation of histones. H1, H2B, H2A1, H4, H2A2, H3.2, H3.3 and H3.1 are eluted in a time-wise manner.

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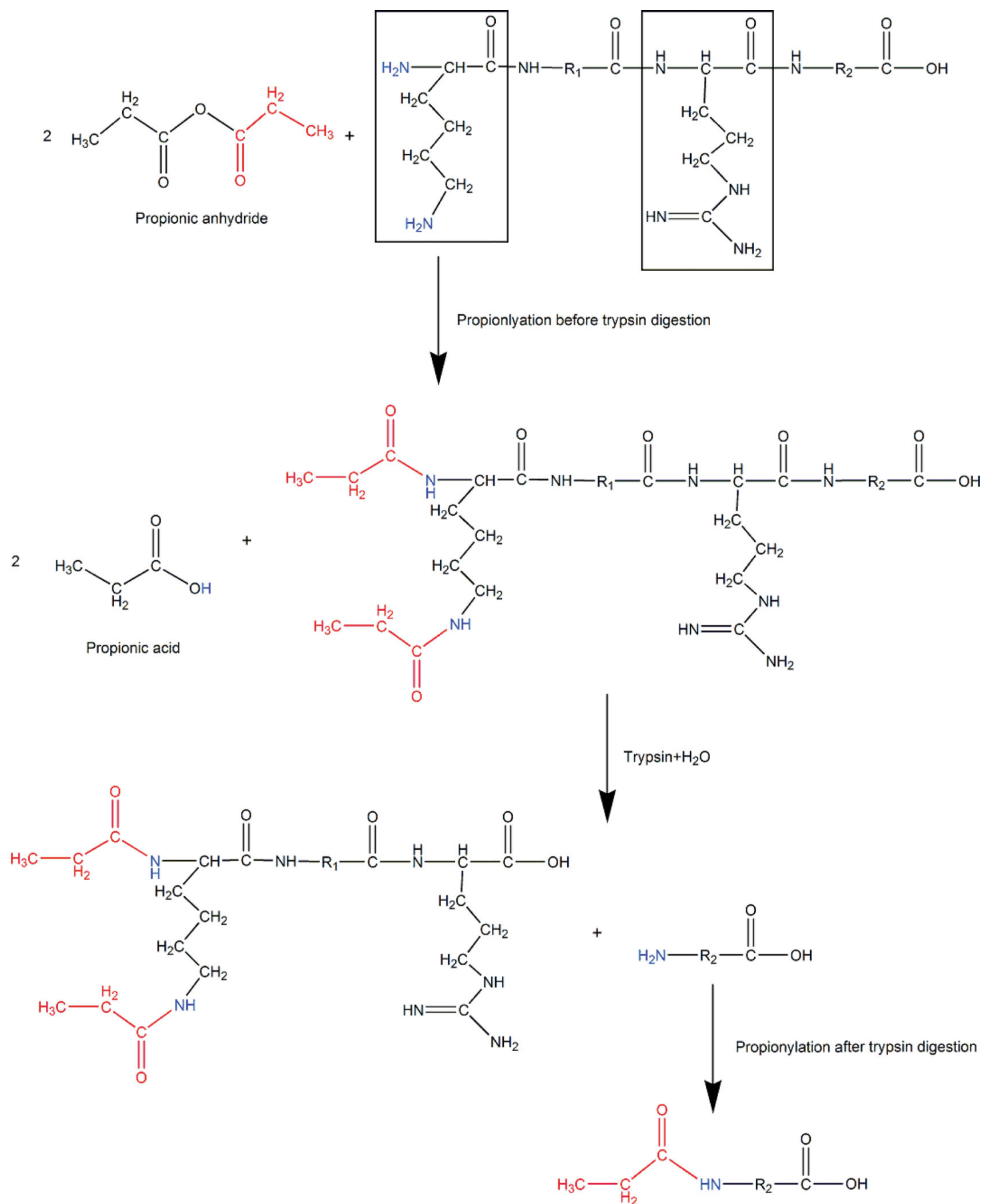

Figure 2.

Chemical derivation of histones. An example peptide with an N-terminal lysine residue (boxed) and an intermediated arginine residue (boxed) is shown. R1 and R2 represent other amino acid residues. After the propionylation treatments before trypsin digestion, the N-terminal amine group (in blue) and the free amine group (in blue) on the lysine residue are both modified with a propionyl group (in red). Propionic acids are the side products of this reaction. Trypsin cleavages at the C-terminal of the arginine residue, which produces two

shorter peptides. Propionylation after the trypsin digestion adds propionyl group (in red) to the newly generated N-terminus (in blue).

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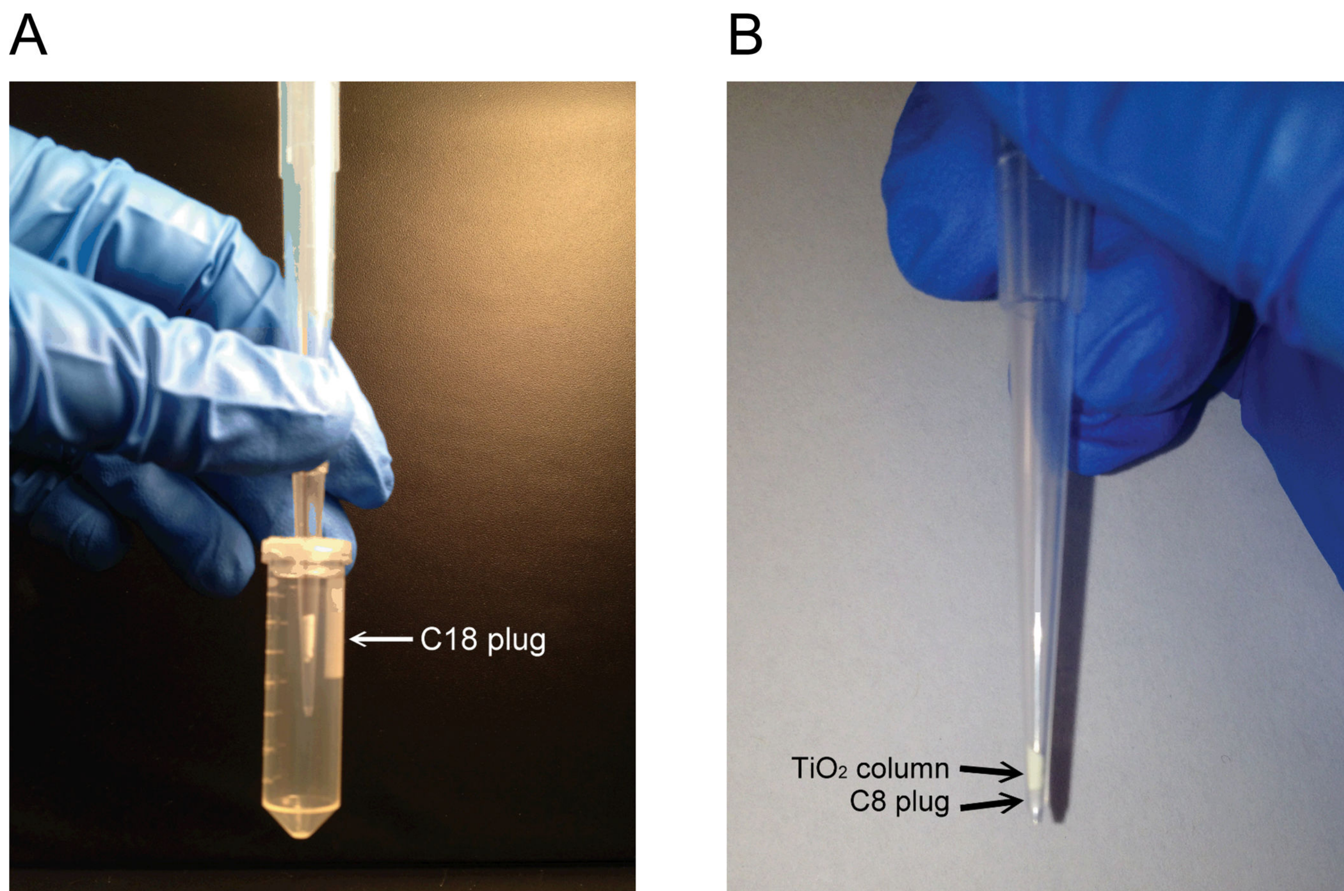

Figure 3.

Set up of stage-tip and TiO_2 micro-column. A. Stage-tip: the C18 mini disks are transferred to a P200 tip using a P1000 tip. A fused silica was used to push the C18 mini disks down to the bottom of the p200 tip. The arrow shows the C18 plug after all C18 mini disks are securely wedged. B. TiO_2 micro-column: the bottom layer of C8 plug turned clear after activation by methanol and the top TiO_2-bead layer looked white. 1.5 mg of TiO_2 was used to make this micro-column.

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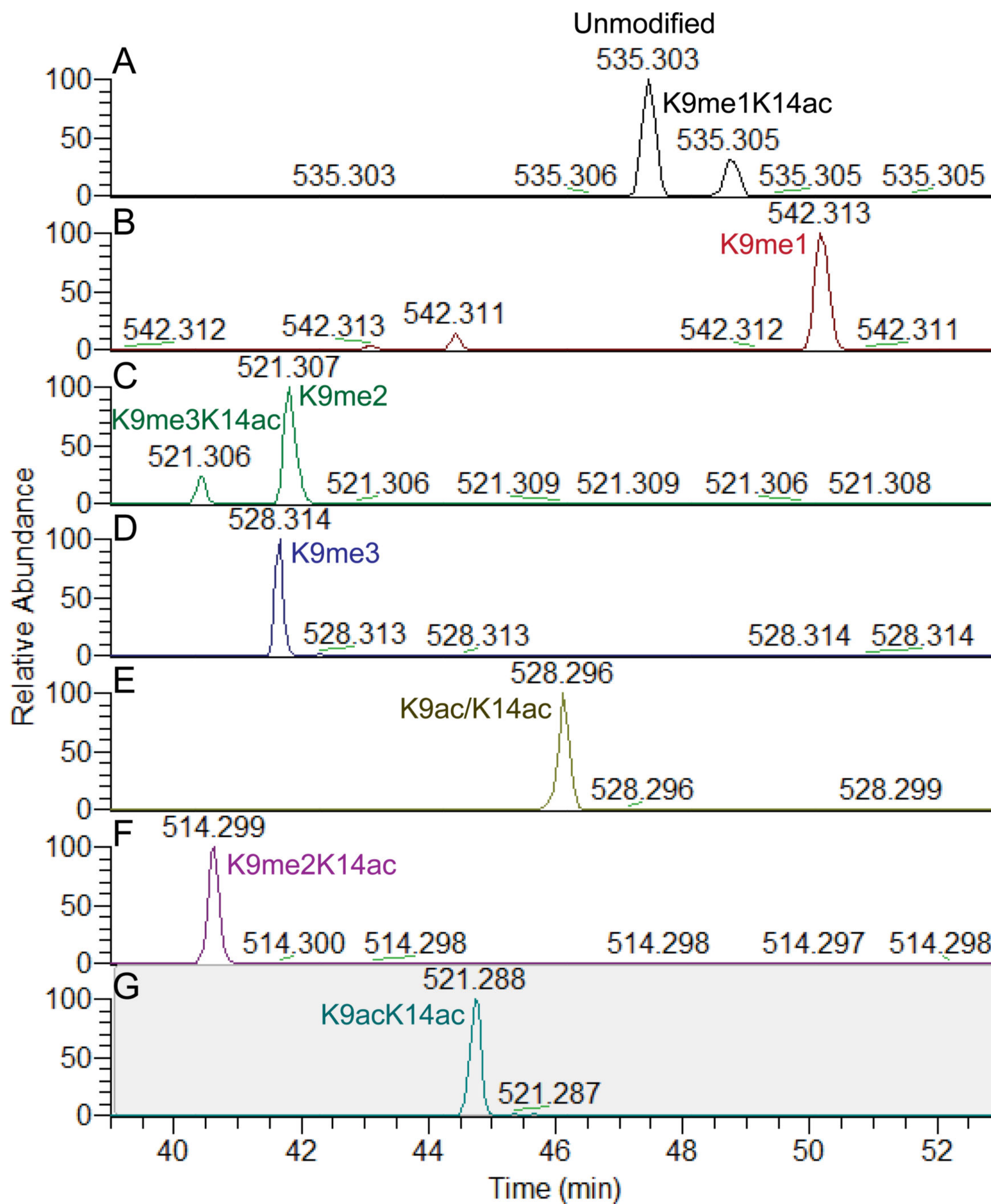

Figure 4.
MS chromatogram of the $[M+2H]^{2+}$ ions of histone H3 9–17 peptide. The peptides are propionylated and digested by trypsin. The modifications and m/z values are indicated. The peptides are identified by their m/z and MS/MS spectrum. The area under each peak represents the abundance of each ion and can be measured in the Qual Browser.

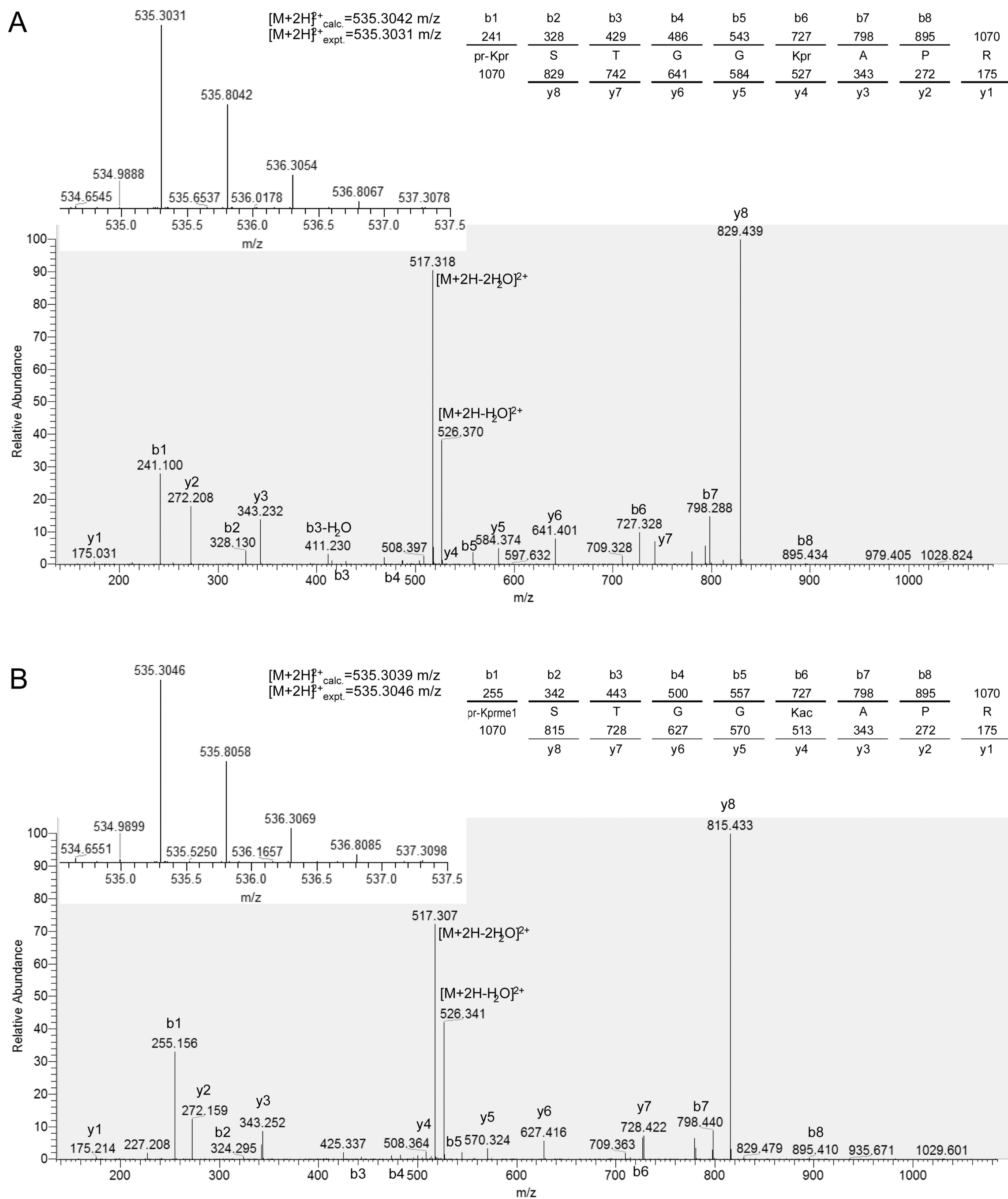

Figure 5.

MS/MS spectrum of two $[M+2H]^{2+}$ precursor ions from the digest of propionylated histone. Both peptides were determined to span histone H3 residues 9 to 17. A. the peptide is unmodified; B. the peptide is mono-methylated on K9 and acetylated on K14. All b and y ions are found and labeled. Inset: mass spectrum of the precursor parent ions. Calc. calculated m/z; expt. experimental m/z.

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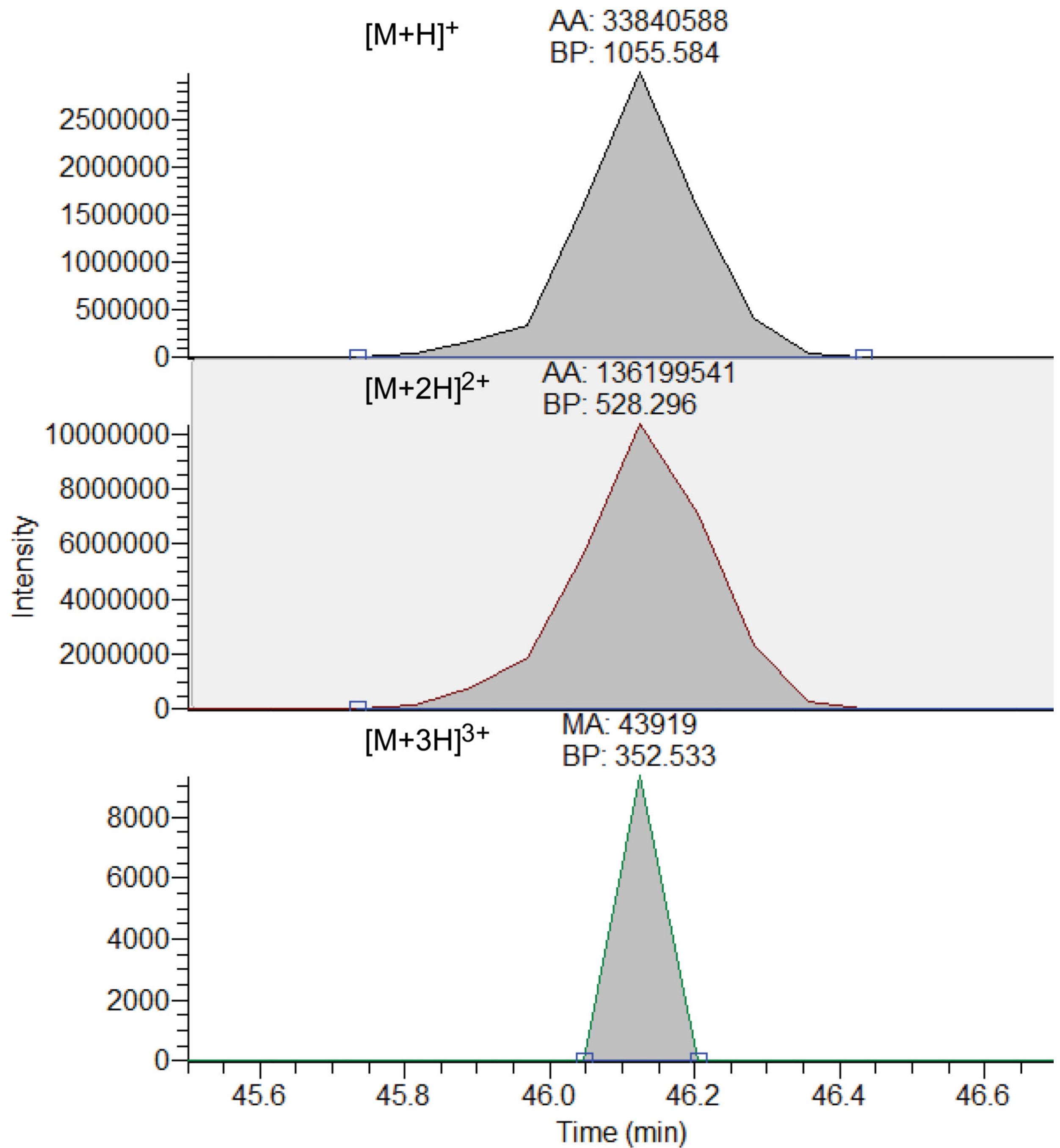

Figure 6.

MS chromatogram of three charge states of a histone H3 9–17 peptide with K9 or K14 acetylated. The area underneath each peak is measured either by automatic area (AA) or manual area (MA) measurement. BP represents the base peak m/z.

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	b1	b2	b3	b4	b5	b6	b7	b8	
	227	314	415	472	529	713	784	881	1056
K9ac	pr-Kac	S	T	G	G	Kpr	A	P	R
	1056	829	742	641	584	527	343	272	175
		y8	y7	y6	y5	y4	y3	y2	y1
	b1	b2	b3	b4	b5	b6	b7	b8	
	241	328	429	486	543	713	784	881	1056
K14ac	pr-Kpr	S	T	G	G	Kac	A	P	R
	1056	815	728	627	570	513	343	272	175
		y8	y7	y6	y5	y4	y3	y2	y1

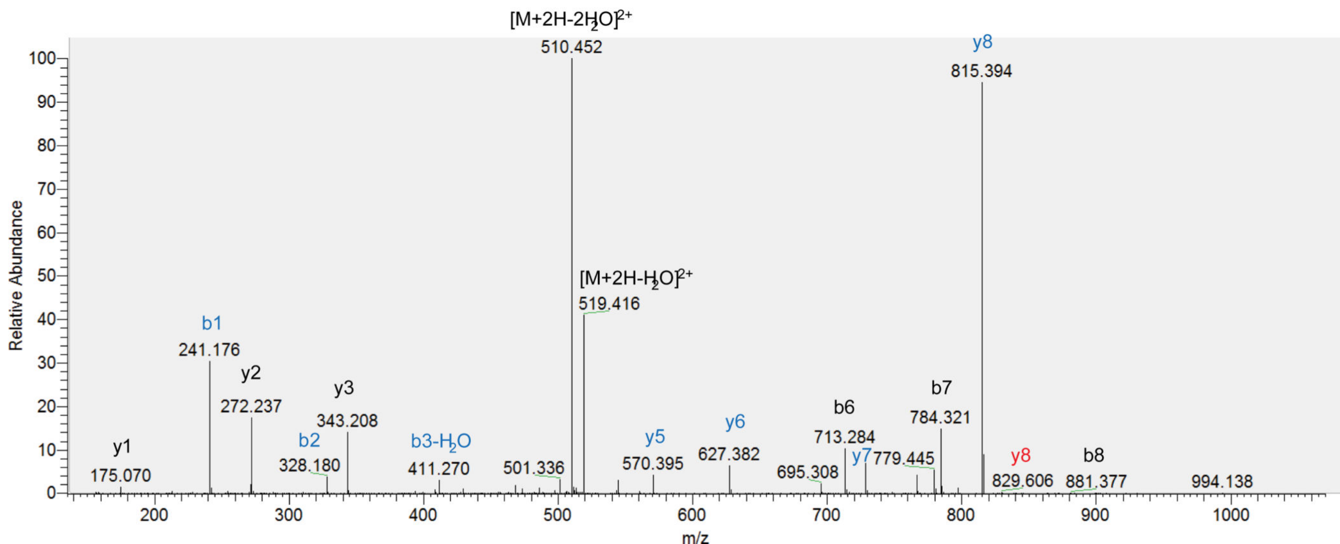

Figure 7.

MS/MS spectrum of the $[M+2H]^{2+}$ precursor ions in Fig. 4E, representing two histone H3 9–17 peptides with one lysine residue (K9 or K14) to be acetylated. Most b and y ions are found and the most abundant ones are labeled. The ions from the K9ac peptide are in red; and the ions from the K14ac peptide are in blue; the ions shared between the two peptides are in black. In this particular experiment, the majority of signal comes from the K14ac peptide.

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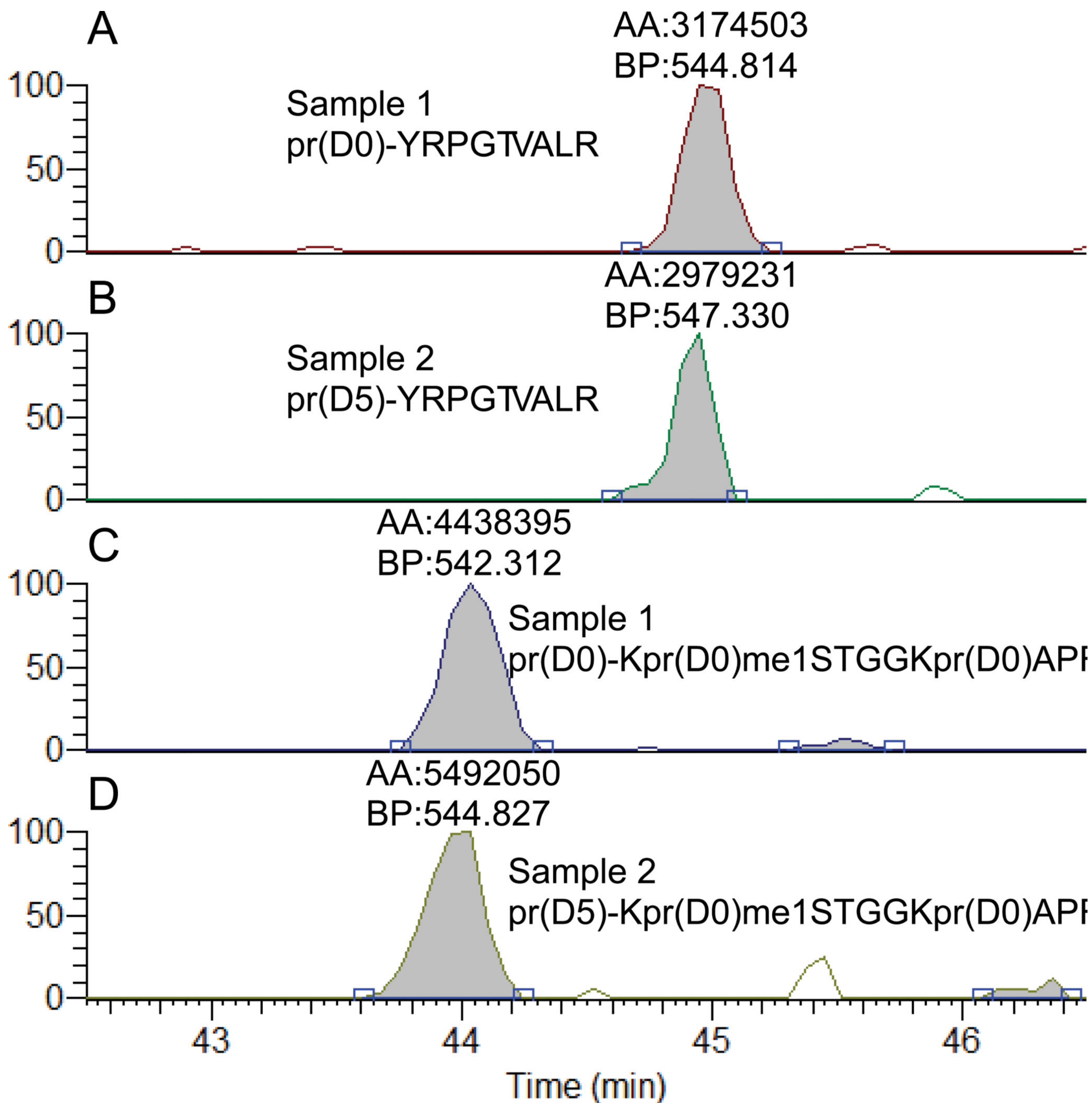

Figure 8.

Differential expression MS analysis of histone PTMs. A and B. MS chromatogram of the histone H3 41–49 peptides from sample 1 and 2, which were labeled with D0 and D10 propionic anhydride in Step 6.3, respectively. The propionyl groups that were added to the N-termini of peptides contain 5 hydrogen (D0) or 5 deuterium (D5) atoms (Fig. 2). C and D. MS chromatogram of the histone H3 9–17 peptides with K9me1 from sample 1 (D0) and 2 (D5), respectively. AA, automatic area measurement; BP: base peak m/z, z=2.

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Table 1

Offline HPLC solvent gradient

Time interval (min)	Gradient (%B)
0–100	30–60
100–120	60–100
120–130	100–30

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Table 2

Online HPLC solvent gradient

Time interval (min)	Gradient (%B)
0–5	0.7
5–40	0.7–30
40–70	30–98
70–72	98
72–77	98–0.7
77–110	0.7

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Table 3

Different modified forms of the histone H3 9–17 peptide

Modification	Peptide	$[M+2H]^{2+}$ m/z
Unmodified*	pr-KprSTGGKprAPR	535.304
K9me1	pr-Kpr-me1STGGKprAPR	542.312
K9me2$	pr-Kme2STGGKprAPR	521.307
K9me3	pr-Kme3STGGKprAPR	528.314
K9ac#	pr-KacSTGGKprAPR	528.296
K14ac#	pr-KprSTGGKacAPR	528.296
K9me1K14ac*	pr-Kpr-me1STGGKacAPR	535.304
K9me2K14ac	pr-Kme2STGGKacAPR	514.299
K9me3K14ac$	pr-Kme3STGGKacAPR	521.306
K9acK14ac	pr-KacSTGGKacAPR	521.288

*, # and $ indicate three pairs of peptides that have similar or same m/z. pr: propionylation; me1,2 and 3: mono- di- and tri-methylation; ac: acetylation