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Histone Modification Screening using Liquid Chromatography, Trapped Ion Mobility Spectrometry, and Time-Of-Flight Mass Spectrometry

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Abstract

Histone proteins are highly abundant and conserved among eukaryotes and play a large role in gene regulation as a result of structures known as posttranslational modifications (PTMs). Identifying the position and nature of each PTM or pattern of PTMs in reference to external or genetic factors allows this information to be statistically correlated with biological responses such as DNA transcription, replication, or repair. In the present work, a high-throughput analytical protocol for the detection of histone PTMs from biological samples is described. The use of complementary liquid chromatography, trapped ion mobility spectrometry, and time-of-flight mass spectrometry (LC-TIMS-ToF MS/MS) enables the separation and PTM assignment of the most biologically relevant modifications in a single analysis. The described approach takes advantage of recent developments in dependent data acquisition (DDA) using parallel accumulation in the mobility trap, followed by sequential fragmentation and collision-induced dissociation. Histone PTMs are confidently assigned based on their retention time, mobility, and fragmentation pattern.

SUMMARY:

An analytical workflow based on liquid chromatography, trapped ion mobility spectrometry, and time-of-flight mass spectrometry (LC-TIMS-ToF MS/MS) for high confidence and highly reproducible “bottom-up” analysis of histone modifications and identification based on principal parameters (retention time [RT], collision cross section [CCS], and accurate mass-to-charge [*m/z*] ratio).

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DISCLOSURES:

Melvin A. Park and Matthew Willetts are employees of Bruker Daltonics Inc., the manufacturer of the timsTOF instrument.

INTRODUCTION:

In eukaryotic cells, DNA is packaged as chromatin into functional units called nucleosomes. These units are composed of an octamer of four core histones (two each of H2A, H2B, H3, and H4)[1–4]. Histones are amongst the most abundant and highly conserved proteins in eukaryotes, which are largely responsible for gene regulation[5]. Histone posttranslational modifications (PTMs) play a large role in the regulation of chromatin dynamics and rigger various biological processes such as DNA transcription, replication, and repair[6]. PTMs occur primarily on the accessible surface of the N-terminal regions of histones that are in contact with DNA[3,7]. However, tail and core modifications influence chromatin structure, altering inter-nucleosome interactions and recruiting specific proteins[3,8].

A current challenge during liquid chromatography-mass spectrometry (LC-MS)-based proteomics is the potential co-elution of analytes of interest. In the case of data-dependent analyses (DDA), this translates into the potential loss of several precursor ions during the MS/MS acquisition process[9]. Time-of-flight (ToF) instruments acquire spectra at very high frequency[9,10] (up to tens of kHz)[11]; this makes them capable of rapidly scanning the total precursor ions within a complex sample (MS1), thus promising optimal sensitivity and MS/MS sequencing rates (up to 100 Hz)[9] and making them ideal for biological sample analysis[10]. Nevertheless, the sensitivity available at these high scan rates is limited by the MS/MS rate[9]. The addition of trapped ion mobility spectrometry (TIMS) in combination with an orthogonal quadrupole time-of-flight (qToF) mass spectrometer was used to mitigate these limitations. In TIMS, all precursor ions are accumulated in tandem and eluted as a function of their mobility, rather than selecting single precursor masses with a quadrupole[9]. Parallel accumulation–serial fragmentation (PASEF) allows for hundreds of MS/MS events per second without any loss of sensitivity[9].

The principal aim of this work was to show the recent developments of DDA using parallel accumulation in the mobility trap followed by sequential fragmentation and collision-induced dissociation (CID). Histone PTMs were confidently assigned based on their retention times (RTs), mobilities, and fragmentation patterns.

PROTOCOL:

NOTE: Histone samples were extracted using a method adapted from Bhanu et al. (2020)[12].

1. Sample preparation

1.1. Harvesting cultured cells

1.1.1. When cells are 80% confluent, ensure they are viable using trypan blue exclusion.

NOTE: A HeLa S3 cell line was used for these experiments, but this method can be applied to any cultured cells.

1.1.2. Aspirate the media, then apply 5 mL of 1x phosphate-buffered saline (PBS) to each plate.

1.1.3. Swirl the plate(s) to rinse all residual media, then aspirate PBS and apply 5 mL of 1x PBS.

1.1.4. Gently separate the cells from the plate by scraping them with a disposable cell lifter.

1.1.5. Transfer each cell suspension to a 15 mL conical tube.

1.1.6. Pellet the cells by centrifuging at 800 x *g* for 5 min.

1.1.7. Aspirate the PBS from the cell pellet.

1.1.8. Proceed to histone extraction.

NOTE: Flash-freeze the cell pellet in liquid nitrogen if it cannot be processed immediately. Store the pellets at −80 °C until ready to proceed.

1.2. Histone extraction

1.2.1. Estimate the volume of each cell pellet and mark the meniscus with a permanent marker.

1.2.2. Prepare enough nuclear isolation buffer (NIB; 15 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 250 mM sucrose) for all the samples. Alternatively, if many samples need to be processed over time, make the buffer in bulk and store at 2–8 °C for up to 6 months, or aliquot and freeze at −15 °C to −25 °C indefinitely by thawing only the amount necessary for each extraction.

NOTE: The buffer should remain clear during storage. If the buffer takes on a cloudy or otherwise abnormal appearance at any time, discard and prepare fresh buffer.

1.2.3. Prepare 50 times the volume of the cell pellets of wash buffer and add inhibitors as follows (approximately 10 mL of wash buffer per 2 samples).

1.2.3.1. To prepare 10 mL of wash buffer, mix 10 mL of NIB, 30 µL of 200 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride [AEBSF], 10 µL of 1 M dithiothreitol [DTT], 20 µL of 5 µM microcystin, 20 µL of 5 M sodium butyrate.

1.2.4. Remove 1/5 of the wash buffer to prepare the lysis buffer (1/5 volume from wash buffer, 0.3% NP-40 or NP-40 alternative).

NOTE: Do not use Triton-X 100 instead of NP-40 or NP-40 alternative, as it may be too abrasive for certain cell types.

1.2.5. Wash the cell pellet thoroughly by suspending it in 5 columns of wash buffer and centrifuging at 800 x *g* for 5 min at 4 °C. Complete this step twice, aspirating and discarding the supernatant between washes.

1.2.6. Ensure the volume of the cell pellet is still marked with a permanent marker. Resuspend in 10 volumes of lysis buffer.

1.2.7. Pipette-mix each pellet thoroughly to resuspend, then incubate for 15 min on ice.

1.2.8. After 15 min, centrifuge at 800 x *g* for 5 min at 4 °C.

1.2.9. Aspirate and discard the supernatant.

NOTE: The pellet should reduce to ≤ $^1/_2$ the original pellet size (as indicated by the marker line). If the pellet has not reduced sufficiently, repeat the lysis procedure and include a gentle homogenization step using a pestle to break open the cells.

1.2.10. Once lysis is complete, resuspend the pellet in 500 µL of wash buffer, then centrifuge at 800 x *g* for 5 min at 4 °C. Aspirate and discard the supernatant, then repeat the wash step once more to remove all traces of NP-40.

NOTE: At this point, the pellet consists of chromatin, which contains histones.

1.2.11. Resuspend the pellet in 5 volumes (of the original cell pellet size) of 0.4 N H_2SO_4.

1.2.12. Incubate for 2 h in a cold room or refrigerator using an agitator.

1.2.13. After 2 h, centrifuge the sample(s) at 3400 x *g* for 5 min at 4 °C. Do not discard the supernatant.

1.2.14. Transfer the supernatant to new tubes, and spike 100% trichloroacetic acid (TCA) to 1/3 the volume of the contents (the final TCA concentration will be approximately 20%).

1.2.15. Gently invert the tube and observe that the clear, colorless solution turns white and/or cloudy, indicating protein precipitation.

NOTE: For solutions with low histone concentrations, the protein precipitation may not be immediately noticeable, but the precipitate should be visible after the overnight incubation.

1.2.16. Incubate without disturbance overnight (12–18 h) at 4 °C to completely precipitate the histone proteins.

1.2.17. The following day, centrifuge at 3400 x *g* for 5 min at 4 °C.

1.2.18. Aspirate the supernatant, being careful not to touch the sides of the tube with the pipette tip. At this stage, the histones are deposited primarily as a film around the sides of the tube(s).

1.2.19. Add 500 µL of ice-cold acetone + 0.1% HCl (acid acetone) to each tube, using a glass Pasteur pipette, then gently invert the tube(s) several times. Arrange the samples in order (1, 2, 3, etc.) while doing this, as any errant acetone may remove the markings on the tubes. Centrifuge at 3400 x *g* for 5 min at 4 °C and gently decant the supernatant.

1.2.20. Repeat this rinsing step with 500 µL of ice-cold 100% acetone, also with a glass Pasteur pipette. Centrifuge at 3400 x *g* for 5 min at 4 °C and gently decant the supernatant.

1.2.21. Leave the tubes open to dry at room temperature until the remaining acetone has evaporated.

1.2.22. When dry, add 100 μL of mass spectrometry (MS)-grade water to each tube. Use this droplet to swab all sides of the container to resuspend the entire histone film. Do this by pipetting the droplet onto the side of the tube and rotating it while pipetting up and down or dispensing half of the 100 μL and using the tip to stir it all around. A combination of both methods works best. Histones are readily soluble in water and will be in the solution.

1.2.23. After resuspending all samples, if there is any remaining white solid, sonicate in a bath at room temperature for 5 min.

1.2.24. Centrifuge at 800 x *g* for 5 min at 4 °C. Transfer the clear solution to fresh tubes. Discard any remaining insoluble pellet.

1.2.25. Run an SDS-PAGE under reducing conditions to verify that the extraction is clean.

NOTE: Gels can be run using any appropriate concentration of polyacrylamide as long as it can differentiate proteins in the range of 10–20 kDa. See the **Table of Materials** for the gels used in this protocol.

1.2.26. Perform a protein concentration assay (i.e., Bradford or BCA) to determine the total protein concentration.

1.3. Chemical derivatization (propionylation) of the lysine residues

1.3.1. Transfer 20 μg of histones (determined by the protein assay) to a clean tube. Dry this sample down to <5 μL using a vacuum concentrator, then resuspend using 20 μL of 100 mM ammonium bicarbonate (NH_4CO_3) (~1 μg/μL solution). Adjust the pH to ~8 using ammonium hydroxide if needed.

CAUTION: Do not use ammonium hydroxide (NH_4OH) to resuspend, only to adjust the pH if necessary. Otherwise, proteins will denature and precipitate.

NOTE: To check the pH with minimal sample loss, use a pipette tip to dip in the sample and dab onto a pH strip. This testing procedure will be useful throughout the remaining sample preparation steps.

1.3.2. Prepare the propionylation reagent by adding propionic anhydride to acetonitrile (ACN) in a 1:3 (v/v) ratio (i.e., to make 40 μL of reagent, combine 10 μL of propionic anhydride with 30 μL of ACN).

NOTE: Traditionally, methanol or isopropanol have been used in preparation of a propionylation reagent. As propionylation is an amide formation reaction, a non-protonic solvent, like acetonitrile, is required to prevent unwanted side products and reactions, such as methyl propionyl ester, which results from using methanol. Only prepare enough propionylation reagent for up to 4 samples at a time so the reagent remains fresh. Use the reagent within 1–2 min of preparation. As the reagent sits, the propionic anhydride will react with any ambient moisture, and acetic acid will begin to form, which can change the effectiveness of the reagent and will change the pH of the histone solution once the reagent is added.

1.3.3. Add propionylation reagent to each sample in a 1:4 (v/v) (i.e., for 20 μL of histones, add 5 μL of propionylation reagent).

1.3.4. Quickly add 1:5 (v/v) NH_4OH (i.e., add 4 μL for 20 μL of the histone solution) to re-establish the pH of the solution to ~8. If pH is still too low, add 1–2 μL of NH_4OH at a time until a pH of 8 is achieved. Typically, a 1:5 (v/v) ratio is adequate.

1.3.5. Incubate the samples at room temperature for 15 min without disturbance.

1.3.6. Repeat the propionylation reaction for no more than 3–4 samples per batch of propionylation reagent to ensure minimal acid formation.

1.3.7. Repeat the propionylation procedure steps 1.3.2–1.3.5. A second round of propionylation ensures that >95% of available lysines are derivatized.

1.3.8. Dry the samples down to <5 μL using a vacuum concentrator. This will evaporate any unreacted propionylation reagent, acid products, and ammonia gas released from the NH_4OH. If the samples dry out completely, this is fine, as no significant sample losses occur.

NOTE: Displace air in the propionic anhydride bottle with argon gas prior to storage to prevent the formation of acetic acid due to contact with ambient moisture remaining in the bottle.

1.4. Proteolytic digestion with trypsin

1.4.1. Resuspend histones in 100 mM NH_4HCO_3 to achieve a volume of 20 μL, achieving an optimal concentration of 1 μg/μL.

NOTE: Sample solutions with concentrations lower than 1 μg/μL will result in decreased Trypsin efficiency.

1.4.2. Add trypsin to histone samples at a 1:10 ratio (wt/wt) (i.e., add 2 μL of 1 μg/μL solution of trypsin to 20 μg of histones).

1.4.3. Incubate reactions at 37 °C for 6–8 h. Alternatively, incubate overnight (12–18 h) at room temperature.

1.4.4. Stop the digestion by freezing at −80 °C for at least 1 h.

NOTE: Do not use acid to quench the digestion reaction, as this will cause an unwanted drop in pH at this point in the procedure. The sample can be stored at −80 °C until ready to proceed (Interim stopping point).

1.5. Chemical derivatization (propionylation) of peptide N-terminals

1.5.1. Dry the samples to <5 μL using a vacuum concentrator.

1.5.2. Resuspend the samples up to 20 μL (1 μg/μL) using 100 mM NH_4HCO_3.

1.5.3. Repeat propionylation as before (step 1.3).

NOTE: It is normal that the samples take longer to dry at this step due to a higher aqueous: organic phase ratio.

1.6. Sample desalting with stage-tips

1.6.1. Resuspend or dilute samples with 50 µL of MS-grade water + 0.1% TFA.

1.6.2. Using an 11-G sample corer, punch 5 disks of C_{18} material from a solid phase extraction disk (punch all 5 disks before transferring to the pipette tip). Insert and ensure the disks are securely and evenly wedged at the bottom of a 200 µL pipette tip (Figure 1).

NOTE: Use 15-G corer if desalting over 25 µg of sample through a single stage-tip.

1.6.3. Use a centrifuge adapter to hold the stage-tips in place in 1.5 mL or 2 mL microcentrifuge tubes.

NOTE: For the following centrifugation steps, use slow (400–500 x *g*) revolution at 4 °C, for 1–2 min at a time; the solvents normally pass through the resin in less than 1 min, depending on how tightly the C_{18} material is packed into the tips.

1.6.4. Rinse the resin by centrifuging with 50 µL of 100% acetonitrile to activate the C_{18} material and remove potential contaminants.

NOTE: It may be easier to load solutions onto the stage-tips using gel-loading pipette tips. Once the C_{18} material has been activated, it is important not to allow the resin to dry out for the duration of the desalting procedure.

1.6.5. Equilibrate the disk material with 80 µL of MS-grade water + 0.1% TFA by centrifugation.

1.6.6. Acidify the sample to pH 4 or lower using glacial acetic acid. Check the pH with pH strips as before to minimize sample loss.

1.6.7. Load the entirety of the sample onto the resin disk by slow centrifugation.

1.6.8. Wash the sample with 80 µL of MS-grade water + 0.1% TFA by centrifugation.

1.6.9. Elute the sample into a clean 1.5 mL tube by flushing 70 µL of 75% acetonitrile and 0.5% acetic acid by slow centrifugation. Additional centrifugation time may be used to ensure the full sample volume is eluted from the stage-tip. It is okay if the resin dries out with the additional centrifugation time, as it is no longer needed past the elution of the sample.

1.6.10. Dry each sample completely in a vacuum concentrator.

NOTE: Sample(s) can be stored at −80 °C until ready to proceed (Interim stopping point).

1.6.11. For LC-MS/MS analysis, reconstitute the samples in a volume of Solvent A (0.1% formic acid) from the liquid chromatography (LC) protocol that gives the final concentration of 0.4 µg/µL (i.e., dissolve 20 µg of histones in 50 µL of Solvent A).

2. TIMS software interface

2.1. Select the **Instrument** tab and switch to **Operate** (verify that the instrument name becomes highlighted in green) (Figure 2).

2.2. Verify TIMS parameters (Figure 2).

2.3. Verify MS Settings (scan begin, scan end, ion polarity, scan mode) (Figure 2).

2.4. Verify TIMS settings (mode, mobility start, mobility end, ramp time, accumulation time, duty cycle, ramp rate, MS rate, MS averaging, and autocalibration) (Figure 2).

2.5. Go to the **Source** tab and activate the syringe option (**Hamilton 500 µL**) only for the **TuneMix** calibration step (Figure 3).[13]

2.6. Go to the **Calibration** tab, click **m/z**, select **Calibration Mode**, and choose the mode (**Enhanced Q**, generally), zoom (+0.01%), and click **Calibrate**; when a score of 100% is achieved, accept (Figure 4).

2.7. Go to the mobility tab and repeat the calibration process (**Linear Mode**, generally), detection range (+5%), width (0.1 Da), then click calibrate; when you get a score ≥ 98.5%, accept (Figure 5).

2.8. Go to the method and select the method to be used; for this example, **Proteomic_/2023–01-19-CF/20230119-Hela Control histone_prep_pasefDIA_1–24_1_451.d/451.m-TimsControl** was selected (Figure 5).

3. LC-TIMS-PASEF-ToF MS/MS

3.1. Use the typical nESI operating conditions: 4500 V capillary voltage, 800 V endplate offset, 3.0 bar nebulizer pressure, 10.0 L/min dry gas, 200 °C dry heater, and 50 µL/min injection flow rate.

3.2. Use the typical MS settings: 6 eV collision energy, 1200 Vpp collision RF, 75 µs transfer time, 5 µs prepulse storage.

3.3. Determine the drift gas flow using the pressure difference from the entrance funnel P1 and the exit funnel P2. Parallel accumulation-serial fragmentation (PASEF) occurs in the TIMS cell, accumulating all precursor ions simultaneously rather than individually. Precursor ions are then released in narrow ion peaks versus the normally much wider peaks (about 50 times shorter), increasing the signal-to-noise ratio while still separating co-eluting peptides via mobility[14].

3.4. Develop an LC-TIMS-ToF MS/MS method for analyzing proteolytic histone peptides. Couple a high-performance liquid chromatograph (HPLC) fitted with a C_{18} (300 Å, 5 µm, 4.6 mm x 250 mm) column with a commercial TIMS-TOF MS instrument with proprietary PASEF technology.

NOTE: This column size was determined to provide good separation at both high and low pH for peptide mixtures, based on previously published works[15–17].

3.4.1. Set the injection volume to 20 µL (8 µg) of sample and a 0.4 mL/min flow rate.

3.4.2. Run a 60-min, non-linear LC gradient using water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). Set the gradient: 10% B for 2.7 min, then to 20% B in 5.3 min, 28% B in 4 min, 35% B in another 18 min,

to 40% B in 13 min, and 100% B in another 2 min. After holding 100% B for 5 min, lower the concentration to 10% B in 5 min and hold for the final 5 min.

3.5. Verify sample elution from the HPLC into the TIMS-TOF via nano-electrospray ionization (nESI) in positive ionization mode.

4. Data analysis

4.1. Identify the peptide sequences and modification sites.

4.1.1. Prepare a theoretical list of peptides using ProteinProspector [https://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest] under the MS-digest tool.

4.1.1.1. Perform a theoretical digest while taking into consideration the conditions of the digest (enzyme used), types of PTMs being searched for (e.g., mono-, di-, or trimethylation), the size range of peptides being searched for, as well as mass detection range and the potential number of missed cleavages.

4.2. Manually analyze the acquired data based on theoretical peptides (Figure 6)[12].

4.2.1. Search for the masses at several charge states (+1 to +4) for each theoretical peptide are searched for.

4.2.2. Following the initial identification of each *m/z,* select the peak and confirm the MS/MS using a theoretical list of fragmentation ions based on the peptide sequence, including PTMs.

NOTE: If the mobility of the identified peptide was known previously, this is also confirmed.

4.3. Calculate the relative abundances of various PTMs and report each modification as a percentage of the specified peptide sequence.

4.3.1. The relative abundance of each detected PTM is calculated using the following equation: Relative abundance = Area of PTM/Total area of unmodified and PTMs for a given peptide

REPRESENTATIVE RESULTS:

A bottom-up proteomic workflow (Figure 7) typically involves the following: extraction of the target protein(s) from a crude sample, followed by quantifying the concentration of the protein(s), and then fractionation, usually by gel electrophoresis or liquid chromatography. After fractionation, the proteins are digested using a proteolytic enzyme (often trypsin), and finally, mass spectrometric analysis of the resulting peptides and protein identification using an established database[18]. Sequence information is derived from precursor ions within the mass-to-charge (*m/z*) range indicated, which are subjected to collision-induced dissociation (CID), producing fragmentation patterns to be identified and sequenced using a database[19] (Figure 8).

For this work, the principal goal was to develop and apply an LC-TIMS-PASEF-ToF MS/MS DDA method following the steps described previously in the protocol section.

Determining the positionality of posttranslational modifications on isomeric and isobaric peptides has presented a particular challenge regarding identification and spectrum interpretation. In this study, recombinant human histone standards and HeLa S3 cells were used as samples.

Histone PTM analysis of human histone standards via ESI-TIMS-PASEF-ToF MS/MS yielded mid- to large-sized peptides (3–30 amino acids in length) detected with as many as 5 charges per peptide. The propionylation procedure was successful in producing longer, more informative peptides than those commonly produced by Tryptic digestion. Upon data analysis, peptides were identified in variously modified states. As an advantage, the TIMS-based method differentiated some positional isomeric peptides carrying the same PTMs. For example, two isomeric species may overlap in retention time and *m/z*; however, the two signals could be separated in the mobility domain (Figure 9).

The corresponding fragmentation spectra for the peptides shown in Figure 9 were annotated by proteomic analysis software using the appropriate FASTA files. In Figure 9A, the unmodified peptide is seen with three propionyl (+56.03) groups (on the N-terminal, lysine 18, and lysine 23). In Figure 9B, the peptide is observed with an acetyl group (+42.02) on lysine 18 and two propionyl groups (one at the N-terminal and one on lysine 23). Finally, in Figure 9C the peptide is seen with an acetylation observed on lysine 23 and two propionyl groups (on the N-terminal and lysine 18). As published previously, the PASEF advantage could be used for increasing sequencing speed and sensitivity by targeting the same feature repeatedly[9]. This allows the user to obtain more structural information from biological samples. In this case, this is applied to the type and position of PTMs occurring on each histone.

Posttranslational modification analysis can also be represented visually as a sequence coverage plot, as seen in Figure 10. In Figure 10A, the histone H3 standard which has been propionylated prior to digestion, presents with longer peptides than would have otherwise resulted, denoted by the blue lines. Histones extracted from HeLa S3 cells were processed in the same fashion, as represented by Figure 10B. Several PTMs were indicated, including many different patterns at the same amino acid positions. This is to be expected from biological samples. Of note, the few gray lines in Figure 10B denote peptides that were identified ambiguously due to the lack of an MS^2 resulting from the low intensity.

DISCUSSION:

Histones are basic proteins that regulate chromatin structure by interacting with DNA in the form of octamers consisting of the four core histones (two each of H2A, H2B, H3, and H4)[20]. Histones contain numerous lysine and arginine residues, which are readily modified, leading to extensive PTMs that alter the chromatin chemistry by influencing histone function or by binding to other cellular proteins[21]. PTMs can elicit biological responses by working in tandem, with specific groups of PTMs having been reported in several diseases, most notably, several types of cancer[22].

When DNA damage is recognized at the cellular level, it is instantly followed by the action of a complex signaling cascade where lesions are marked, followed by the coordination of cell cycle progression and activation of the required repair pathways. In addition, DNA damage induces various modifications, such as acetyl and methyl adducts, which facilitate protein recruitment[23]. The great variety of PTMs that are involved in DNA lesions leads to the question of how these molecular mechanisms regulate their coexistence and what the functional importance of defending the integrity of the genome through an extremely complex integrated network is. For example, lysine 9 trimethylation of histone H3 (H3K9me3) has been linked to different pathologies in various diseases[24]. For reasons such as this, it is necessary to develop instrumental analytical methodologies that allow the complete characterization of these modifications at the cellular level[23].

Analysis of the HeLa S3 histone extractions using manual data analysis software and proteomic analysis software revealed PTMs, including acetylation (+42.01 Da), methyl-propionylation (+70.04 Da), dimethylation (+28.03 Da), and trimethylation (+42.05) for several histone proteins. Additionally, the PASEF-based MS/MS method was able to differentiate some positional isomeric peptides carrying the same PTMs.

In the introduction, the advantages of coupling LC-TIMS-ToF MS/MS in the study of PTMs to show the recent developments of DDA using parallel accumulation in the mobility trap followed by sequential fragmentation and collision-induced dissociation are briefly described. The main idea is to establish a methodology that allows for the resolution of signals coming from different peptides and that, up until now, classical techniques have not been able to resolve. The derivatization process using propionic anhydride prevents the cleavage of lysine C-terminals by Trypsin, generating longer, more informative peptides. Peptides with the same *m/z* and retention time were able to be identified by their fragmentation patterns, but it was also seen that some of these species could be separated in the mobility domain using this LC-TIMS-PASEF-ToF MS/MS method.

To better understand this, Figure 8 represents three main characteristics of any molecule, thus allowing the identification of a compound, whether they are intact proteins, lipids, or peptides (in this case, histone H3 18–26), to name a few examples. These characteristics include the retention time (min) of a compound in the chromatographic column, the mass-charge ratio (*m/z*) of each compound, and the mobility ($1/K_o$) that these compounds present when they interact with the drift gas. In Figure 8A, the unmodified H3 peptide 18–26 is shown to have an RT of 28.15 min and that it presents two bands in its mobility spectrum, indicating that it has at least two conformations, a result that is suspected to be a result of the two lysines (18 and 23) that have been propionylated following the previously described protocol. The following spectra (Figure 8B,C) show the same peptide (H3 18–26) but varying the position of the acetylation group (42.02) between B, K18Ac and C, K23Ac. These two isomers (K18Ac and K23Ac) have been identified through the mobilogram, as they present with different spatial distributions, which results in different interactions with the gas in the TIMS cell. The importance of this method lies in the possibility of identifying and studying in more detail the different PTMs that have been associated with different diseases through, for example, DNA damage.

When fragmentation data are sparse, identifying a modification at a specific residue is challenging because two or more dissimilar modifications could occur simultaneously at (or near) the same residues and may be understood as a single modification[25]. This could be resolved by ensuring that the unmodified peptide has been identified, especially by using a standard to confirm or deny the presence of a single modification rather than multiple modifications (Table 1).

To avoid excessive contamination or extractions of impure histones, it is important to check the quality of the reagents before use. For example, if the NIB buffer solution is stored and used in bulk, ensure that the solution is clear with no outward appearance of turbidity or abnormal presentation. Turbidity may be the result of bacterial growth, which would contaminate samples and could result in a mixture of histones and bacterial proteins. In addition, it is recommended to prepare fresh calibration curves for assays, such as the BCA or Bradford assay used to determine protein concentration, ensuring that the protein used for the calibration curve is not expired or degraded.

This method can be extended to other types of cells or organisms, for example, mosquitoes. In the case of whole or partial organisms, selecting an appropriate number of organisms is especially important to ensure that the final histone concentration is suitable for analysis.

Also, as a general guideline for mass spectrometer maintenance, the front end should be cleaned periodically to prevent buildup on the instrument and contamination between runs. This cleaning should include the curtain, orifice plate, and quadrupole, as required.

Generally, when an LC is used, it is necessary to take into consideration preparing fresh mobile phase(s) each week using MS-grade solvents. It is good practice to keep dedicated pipettes and glassware for mobile phase preparation and to purge the LC lines whenever new solutions are placed on the system. Guard and separation columns should usually be replaced every 100–200 injections and 500–1500 injections, respectively[26]. Be sure to inject blanks before and after running a batch of samples. If there are a large number of samples within a given batch, one may also consider running a blank at various intervals within the batch.

The protocol provides a PASEF-based DDA workflow for detecting histone PTMs and differentiation of isobaric and isomeric species based on ion mobility.

This protocol requires extensive sample preparation, and overall experimental sample preparation time should be accounted for. On average, the sample preparation protocol requires 2–3 business days to complete. Additionally, differences between laboratories and instrument versions can affect the overall sensitivity of the analysis.

Very few proteomic data analysis software have been deemed adequate for use in analyzing histones *via* bottom-up methods without manual adjustment or correction[27–29]. Results should (at least at first) be confirmed using manual analysis, which is also time-consuming. If analytical software is used, it should have MS/MS annotation capabilities, which are generally easy to confirm or reject.

It is also worth mentioning that it is impossible to separate isomers through mass spectrometry unless a TIMS cell is inserted and mobility values are used; for example, the positions of histone modifications can be determined using fragmentation patterns (PASEF).

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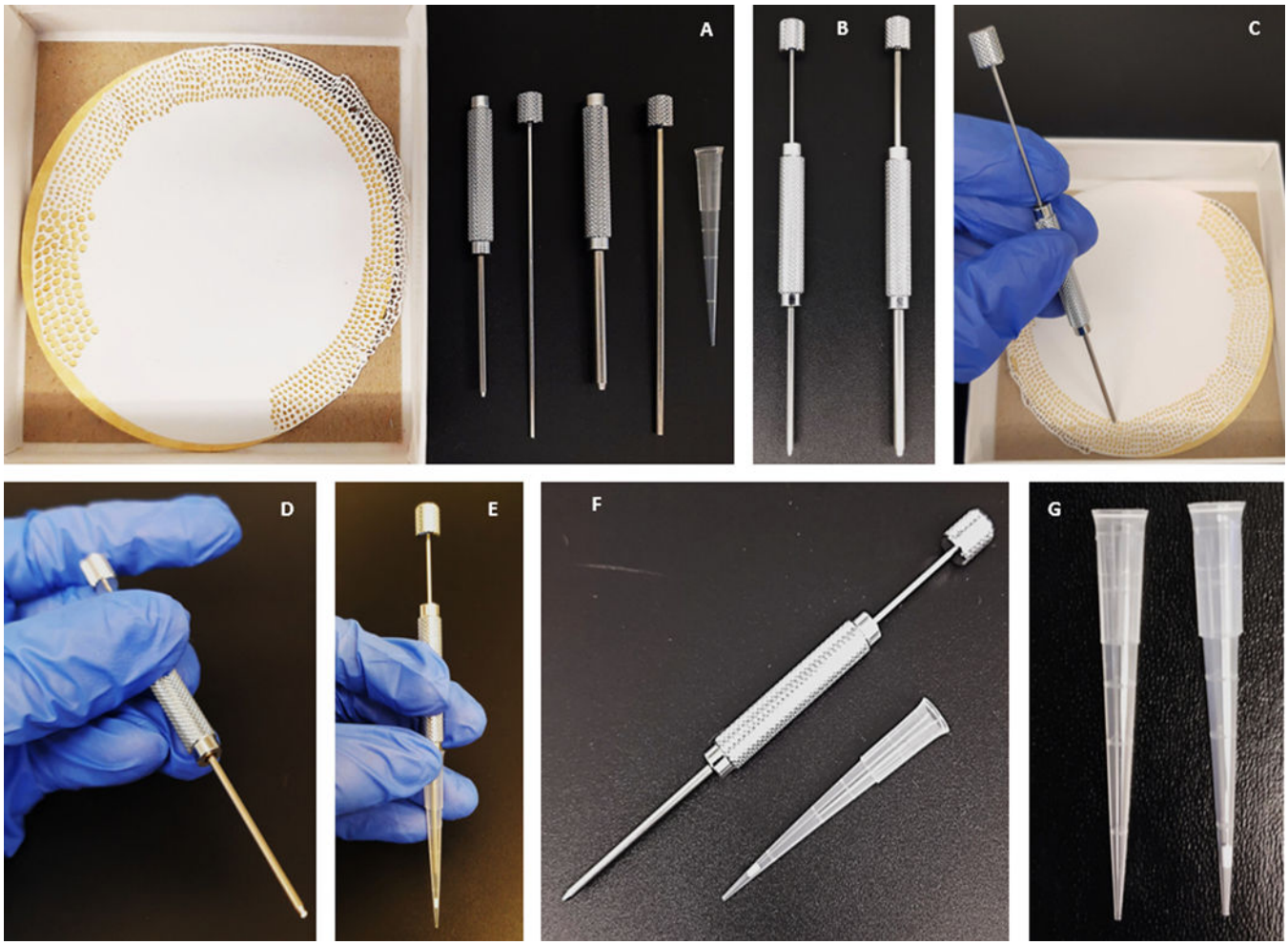

Figure 1: Schematic representation and production of stage tips.
(**A–G**) Step-by-step guide on the manufacture of a C18-silica disk stage tip.

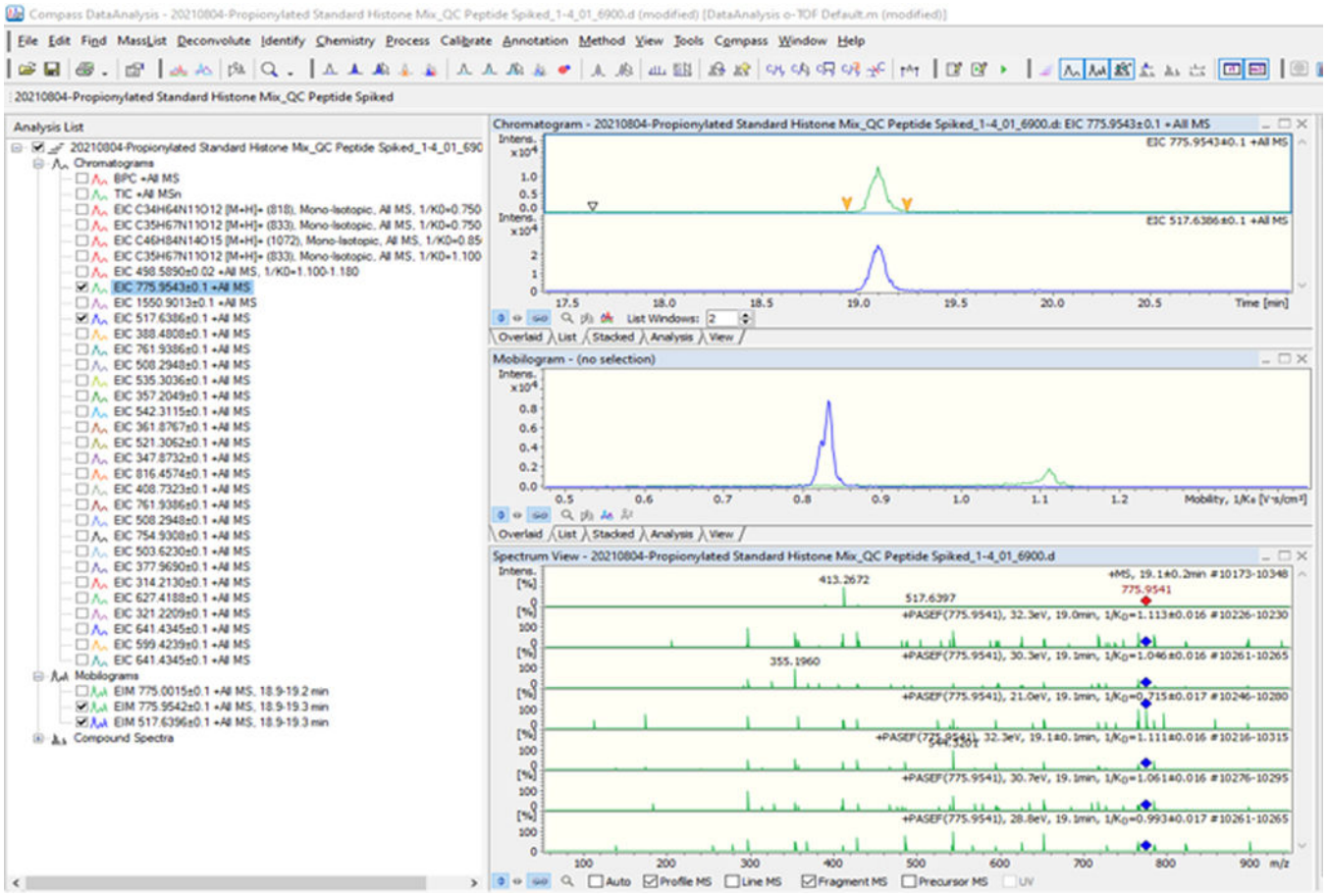

Figure 2: timsControl protocol steps 1–4.

The figure shows the first four steps of the timsControl procedure. On the upper left-hand side, click the **Instrument** button to turn on and off the connection between the instrument and the software. Before executing any task, one must ensure that the software is in the operating mode. Finally, verify that the TIMS parameters are correct.

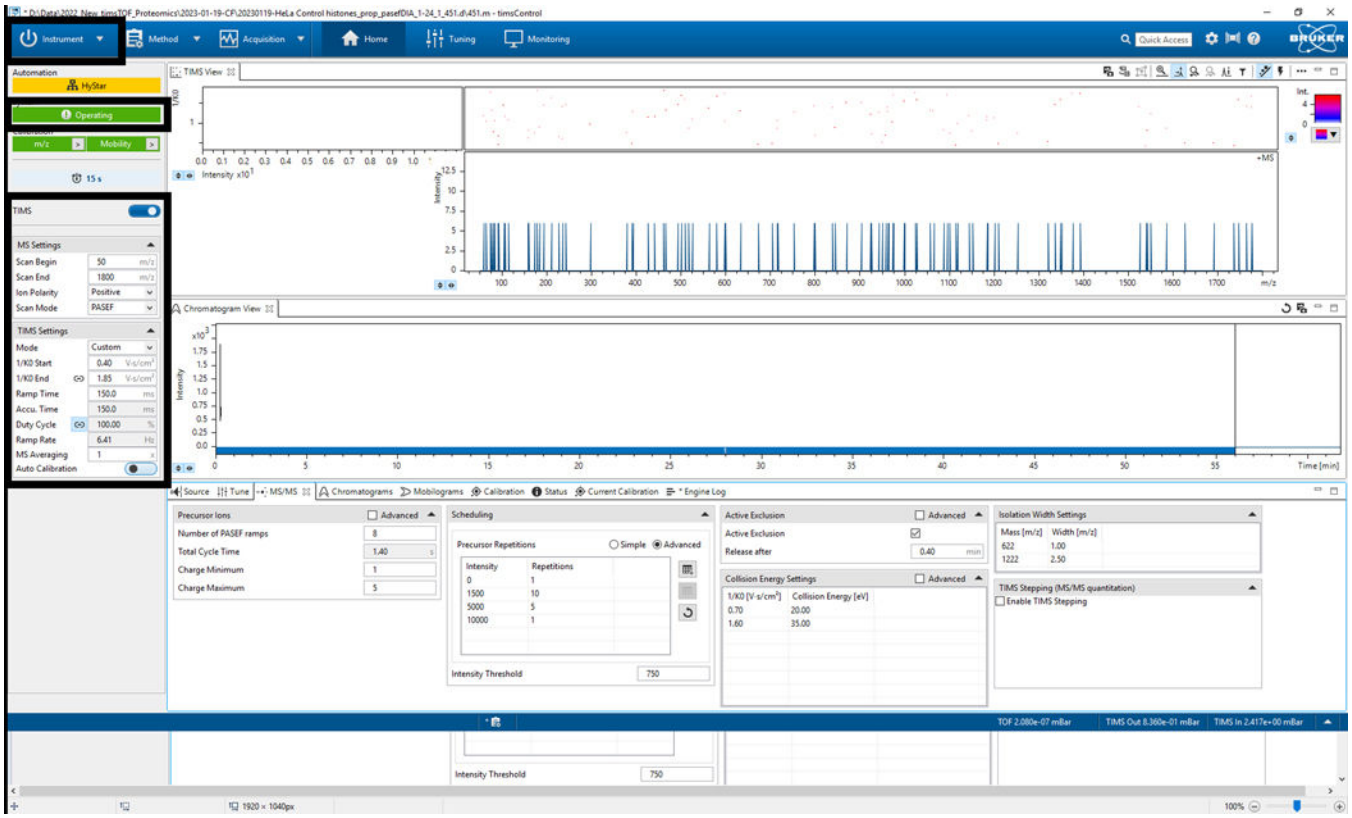

Figure 3: Source parameters.

In this case, Syringe Hamilton 500 µL was used only for **TuneMix**. Verify that the other parameters remain correct.

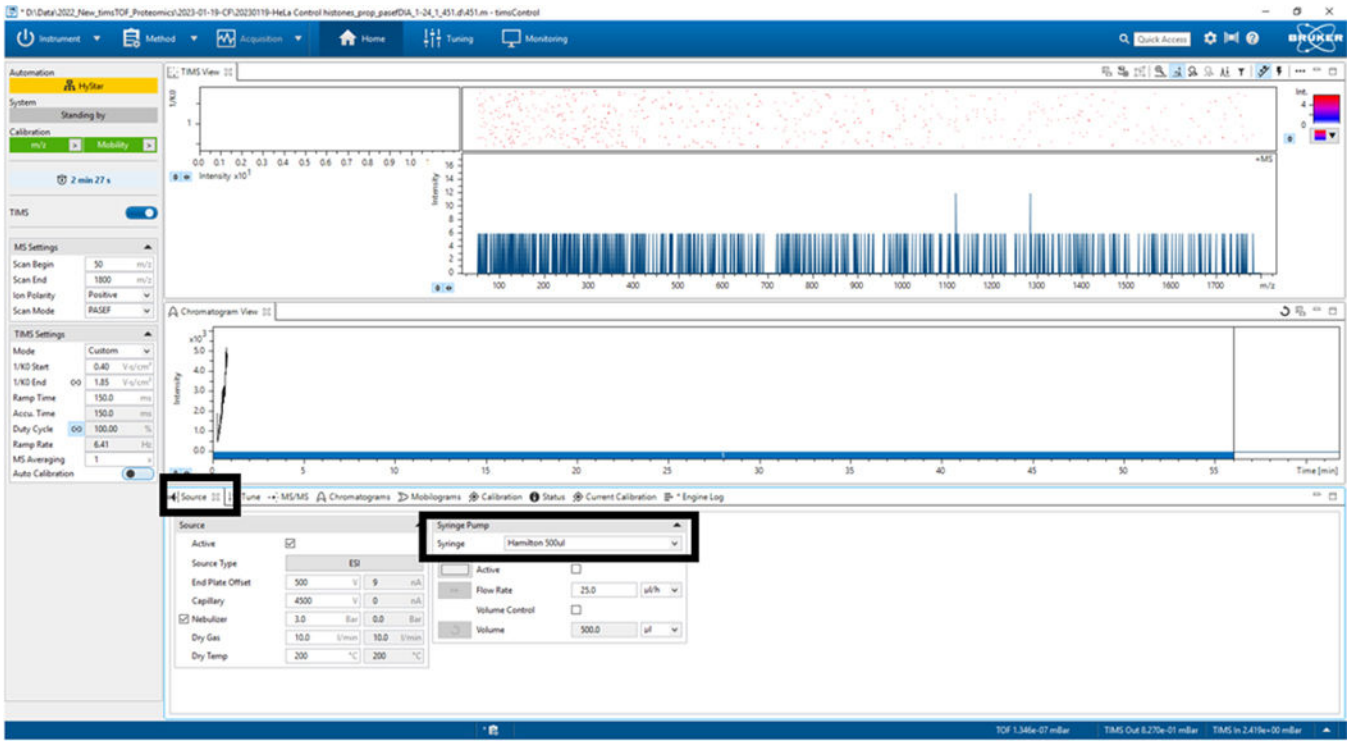

Figure 4: Mass-to-charge (*m/z*) calibration.

Select **Calibrate** until a score of 100% has been obtained in the bottom left panel **Calibration Mode**.

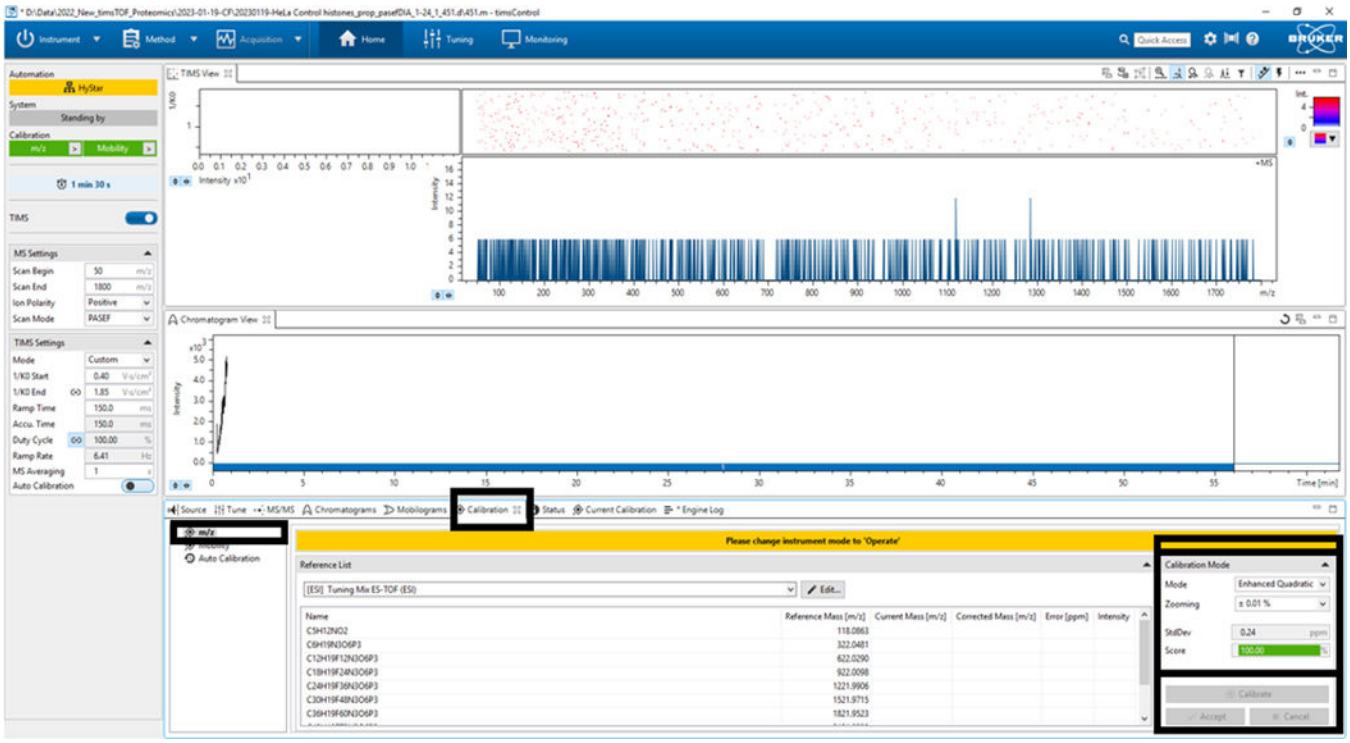

Figure 5: Mobility calibration.

Select **Calibrate** until a score of at least 98.5% has been obtained in the bottom left panel **Calibration Mode**.

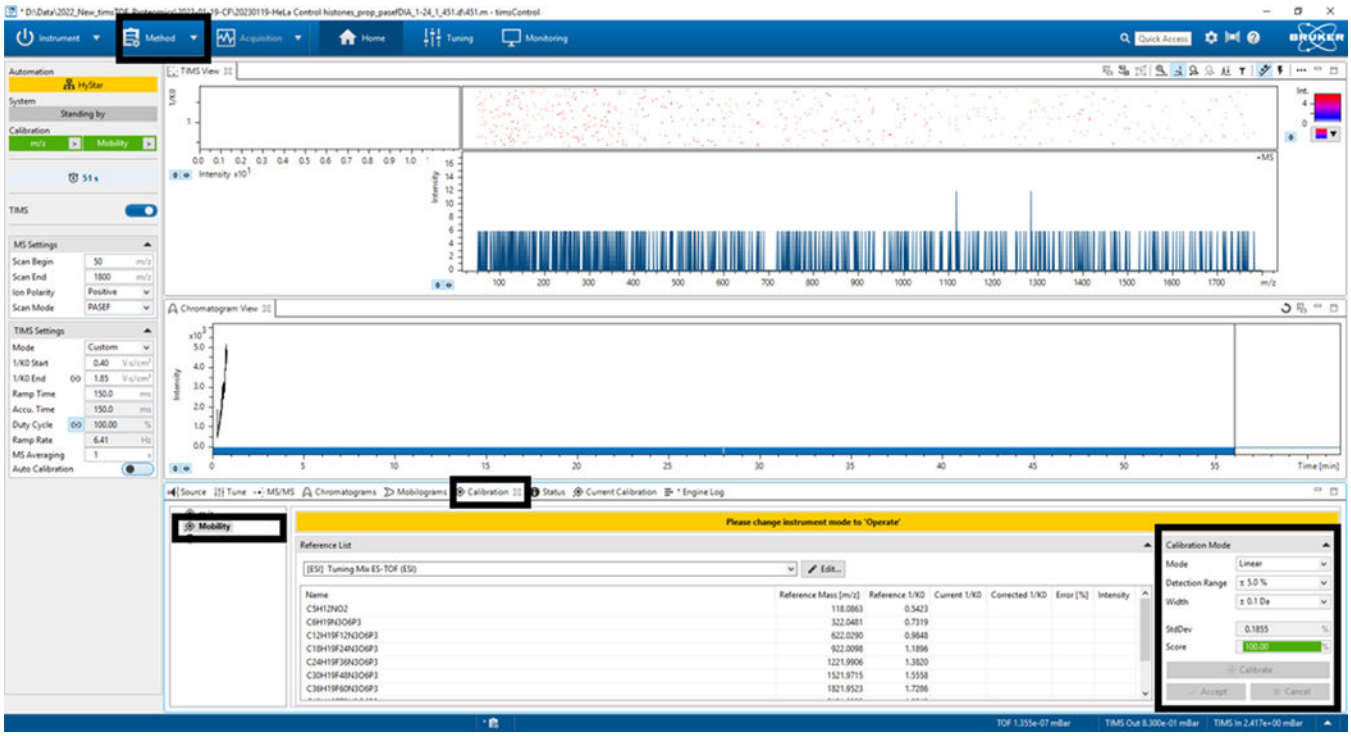

Figure 6: Data processing: 20210804 Propionylated Standard Histones Mix_QC Peptide.

Before starting to process the data, make sure to prepare the theoretical list of possible charge states and their fragmentations (1550.9013; 775.9543; 517.6386; etc.) to extract those values from the base peak chromatogram (BPC +All MS). After extracting each peptide, make sure it looks like the analysis list shown in the figure. The peak 775.9543 was selected as an example. On the right side of the figure, three graphs are shown: the first corresponds to the chromatogram (intensity vs. time graph), the second to the mobilogram, and the third to the mass spectrum with PASEF fragmentation included.

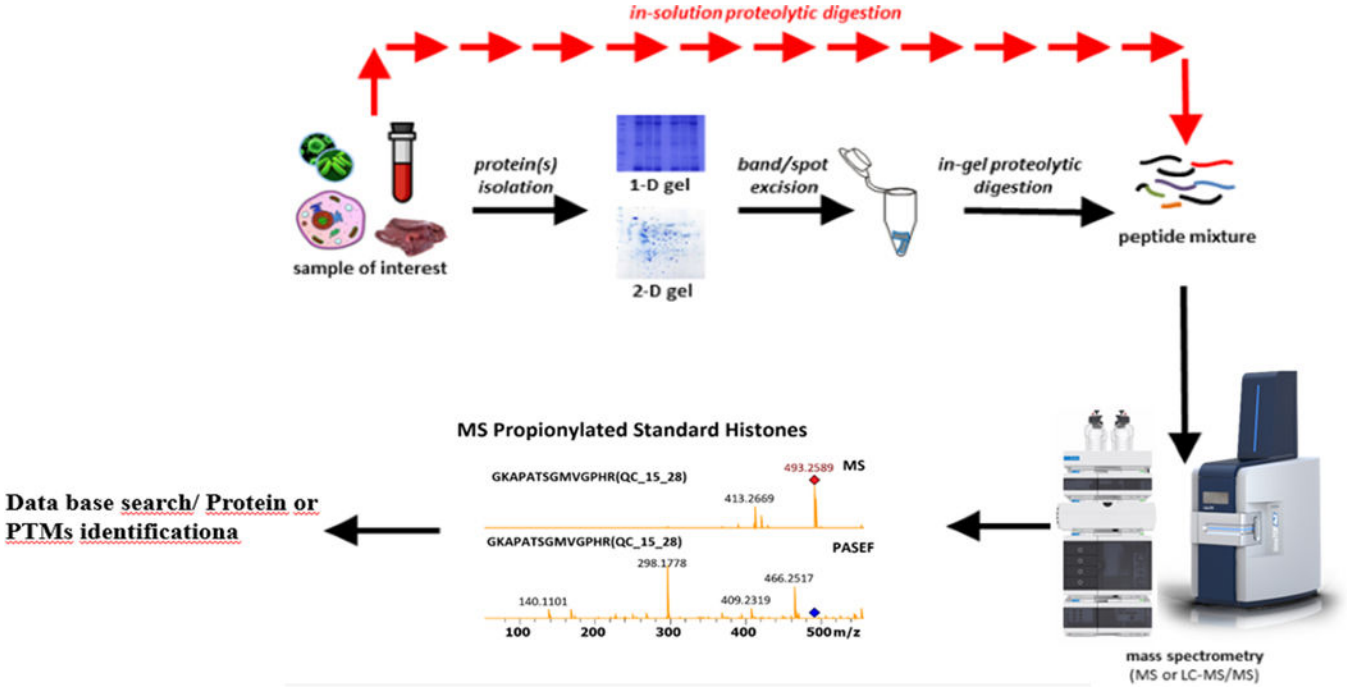

Figure 7: Typical bottom-up proteomics workflow.

Step-by-step of the bottom-up procedures from sample preparation to identification[9].

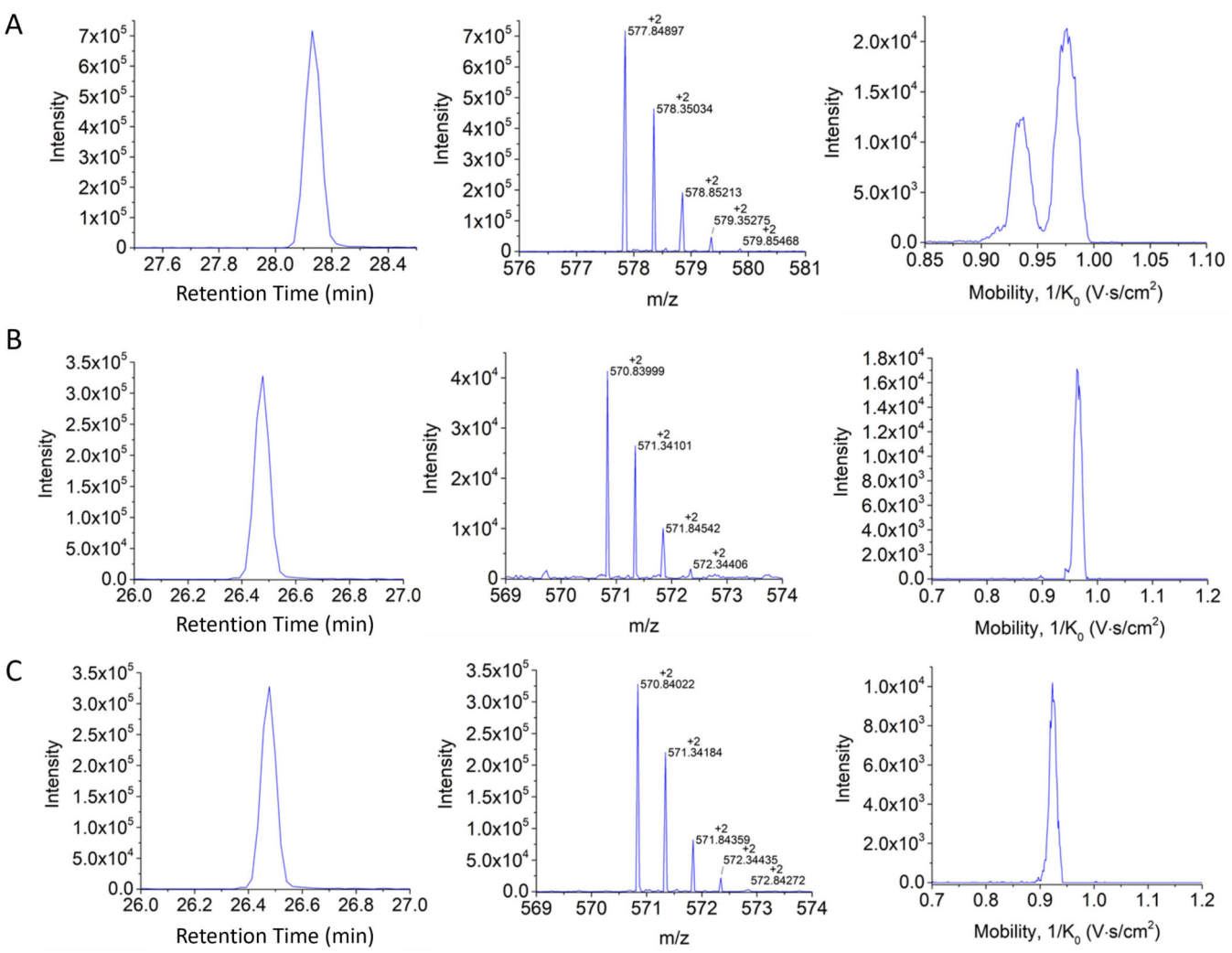

Figure 8: Retention time, isotopic pattern, and H3 18–26 mobility profiles.

(**A**) Unmodified propionylated, (**B**) K23Ac peptide propionylated in the other two positions, and (**C**) K18Ac propionylated in the other two positions. Notice the advantages of the mobility separation for the case of the structural isomers shown in panels B and C.

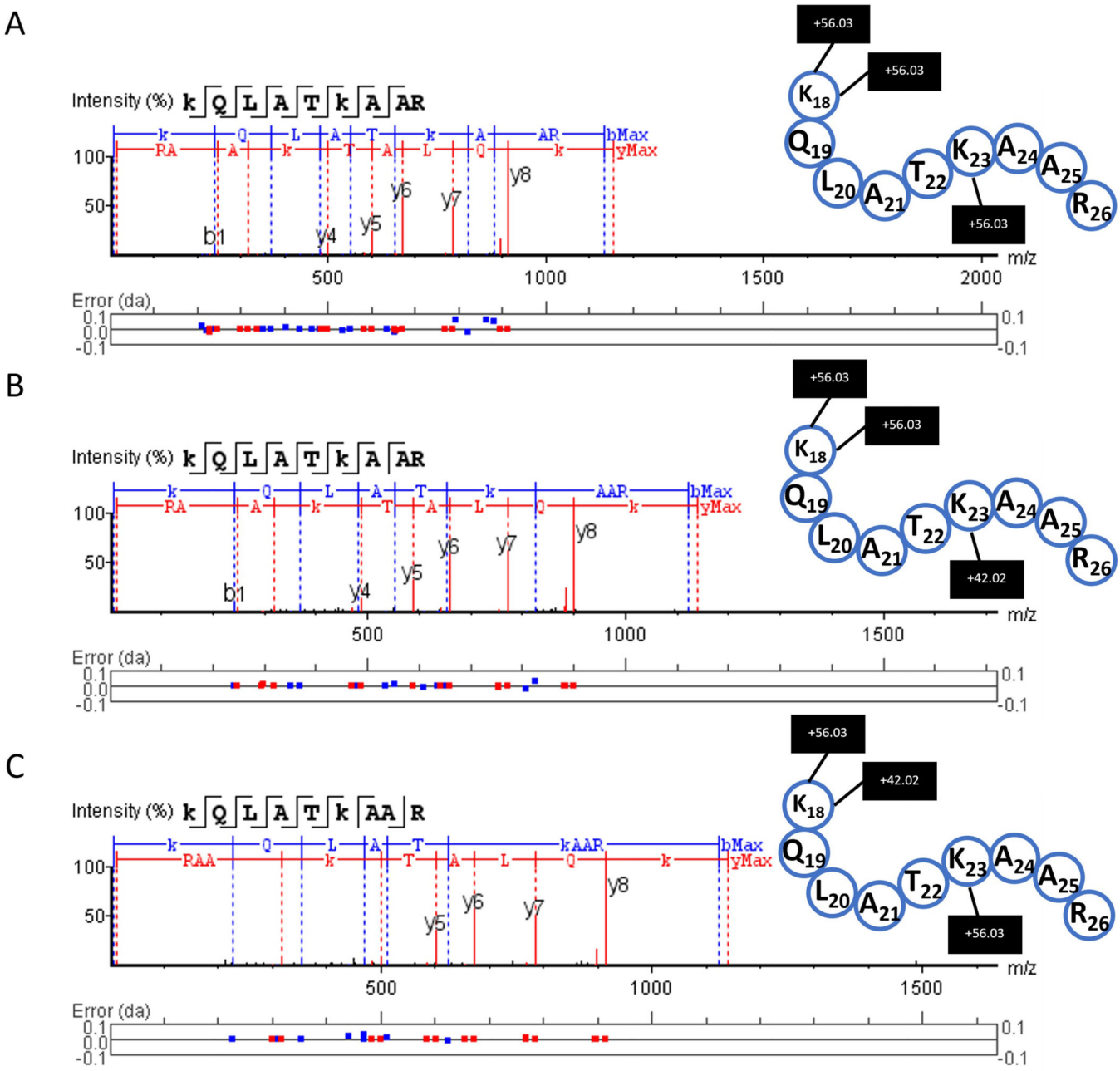

Figure 9: Example of MS/MS fragmentation peptide sequencing using PASEF.

Fragment spectra were obtained from proteomic analysis software for the H3 peptide with amino acid positions 18–26. (**A**) unmodified propionylated, (**B**) K23Ac peptide propionylated in the other two positions, and (**C**) K18Ac propionylated in the other two positions.

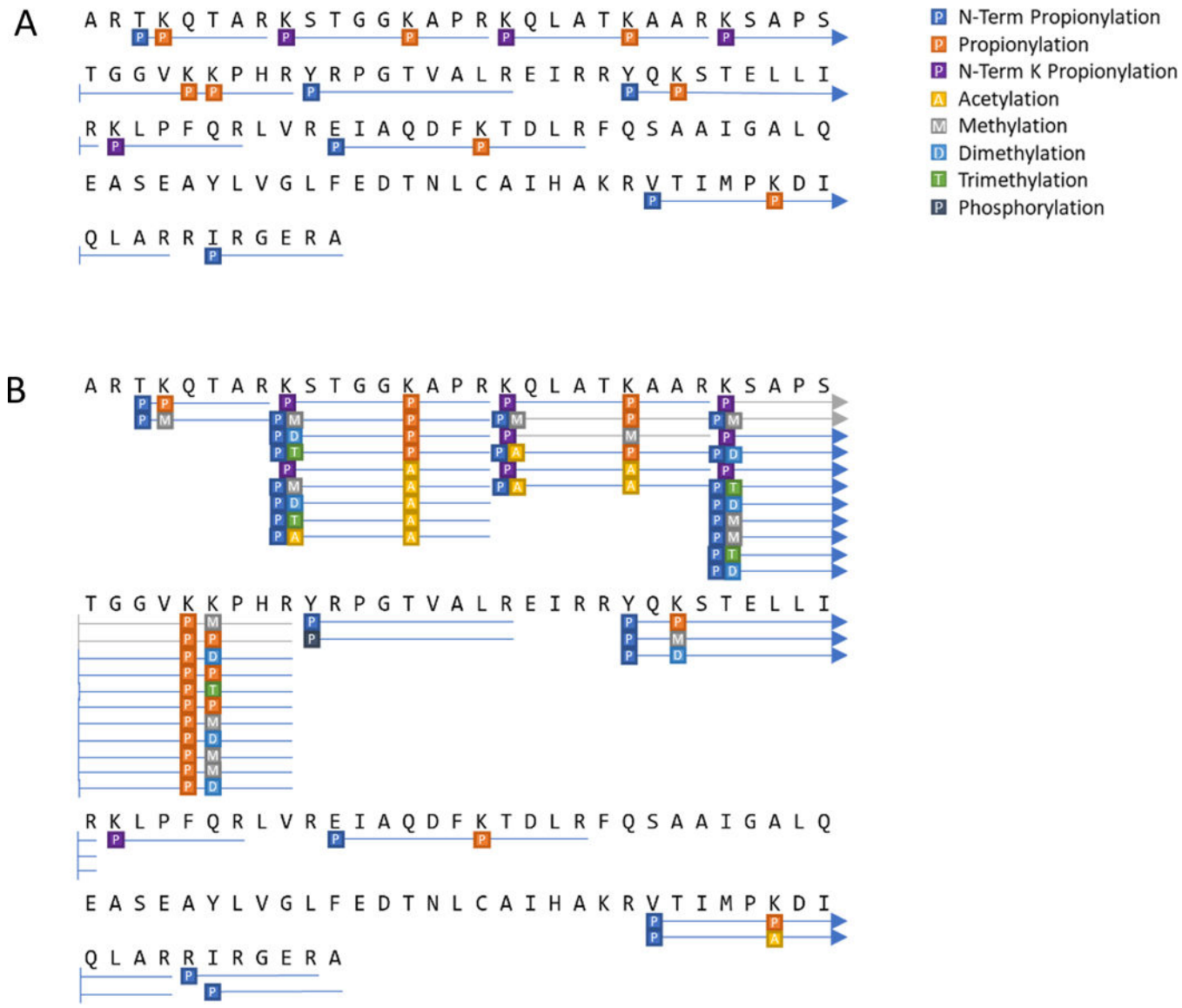

Figure 10: Example of a visual histone PTM analysis summary.

Results of observed peptides and PTMs from (**A**) an H3 standard and (**B**) H3 from HeLa S3 cells.

Table 1:

Standard and HeLa S3 peptide LC-TIMS-ToF MS/MS characteristics.

Target and observed peptide list, including experimental properties (i.e., retention time, *m/z*, $1/K_o$, and LC peak areas).

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
H3_01_3_8	TKQTAR											
	unmod	1	816.4574		14.3	816.4552	1.314	92098	14.3	816.4555	1.338	14192
		2	408.7323	659.38, 342.20		408.7326	0.746, .769			408.7325	.771, .792	
	K4me1	1	830.473									
		2	415.7402									
	K4me2	1	788.4625									
		2	394.7349									
	K4me3	1	802.4781									
		2	401.7427									
	K4ac	1	802.4417									
		2	401.7245									
H3_02_9_17	KSTGGKAPR											
	unmod	1	1069.6		16.7	1069.5965	1.585, 1.607, 1.616	171405	16.5			5083
		2	535.3036	829.45, 742.42, 641.37, 584.35, 527.33, 328.19, 429.23, 486.26, 543.28		535.3028	0.919			535.3033	0.921	
		3	357.2049									
	K9me1	1	1083.6157						17.9			3056
		2	542.3115							542.3113	0.91	
		3	361.8767									
	K9me2	1	1041.6051						13.2			8285

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		2	521.3062	829.45, 742.42, 641.37, 584.35, 527.33, 300.19, 401.24, 458.26, 515.28						521.3065	0.847	
		3	347.8732									
	K9me3	1	1055.6207						13.1			3468
		2	528.314							528.3144	.856, .876	
		3	352.5451									
	K9ac	1	1055.5844									
		2	528.2958	829.45, 742.42, 641.37, 584.35, 527.33, 314.17, 415.22, 472.24, 529.26								
		3	352.533									
	K14ac	1	1055.5844									
		2	528.2958	815.44, 728.41, 627.36, 570.34, 513.31, 328.19, 429.23, 486.26, 543.28								
		3	352.533									
	K9me1K14ac	1	1069.6									
		2	535.3036	815.44, 728.41, 627.36, 570.34, 513.31, 342.21, 443.25, 500.28, 557.30								
		3	357.2049									
	K9me2K14ac	1	1027.5894									
		2	514.2984									
		3	343.2013									
	K9me3K14ac	1	1041.6051									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		2	521.3062	815.44, 728.41, 627.36, 570.345, 513.31, 314.21, 415.26, 472.28, 529.30								
		3	347.8732									
	K9acK14ac	1	1041.5687									
		2	521.288									
		3	347.8611									
H3_03_18_26	KQLATKAAR											
	unmod	1	1154.6892		21.1	1154.6895	1.728	1749137	21	1154.6826	1.725	737276
		2	577.8482	914.54, 786.48, 673.40, 602.36, 501.31, 369.21, 482.30, 553.33, 654.38		577.8482	.918, .966			577.8478	.919, .967	
		3	385.5679									
	K23me1	1	1168.7048									
		2	584.856									
		3	390.2398									
	K18me1	1	1168.7048									
		2	584.856									
		3	390.2398									
	K18me1K23me1	1	1182.7205									
		2	591.8639									
		3	394.9117									
	K18ac	1	1140.6735									
		2	570.8404	914.54, 786.48, 673.40, 602.36, 501.31, 355.20, 468.28, 539.32, 640.37								
		3	380.896									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
	K23ac	1	1140.6735						20.3	1140.672	1.709	204353
		2	570.8404	900.53, 772.47, 659.38, 588.35, 487.30, 369.21, 482.30, 553.33, 654.38						570.8405	.910, .957	
		3	380.896									
	K18acK23ac	1	1126.6579									
		2	563.8326									
		3	376.2241									
H3_04_27_40	KSAPATGGVKKPHR											
	unmod	2	829.4728		17.7	829.4698	1.118	247198				
		3	553.3176	1417.79, 1330.76, 1259.72, 1162.67, 1091.63, 990.58, 933.56, 876.54, 777.47, 593.35, 328.19, 399.22, 496.28, 567.31, 668.36, 725.38, 782.40, 881.47		553.3176	0.891					
		4	415.2401									
	K36me1	2	836.4807									
		3	557.9895	1431.81, 1344.78, 1273.74, 1176.69, 1105.65, 1004.60, 947.58, 890.56, 791.49, 328.19, 399.22, 496.28, 567.31, 668.36, 725.38, 782.40, 881.47								
		4	418.744									
	K27me1	2	836.4807						18.5	836.478	1.123, 1.141	7173
		3	557.9895	1417.79, 1330.76, 1259.72, 1162.67, 1091.63, 990.58, 933.56, 876.54,						557.9894	.890, .900, .909	

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
				777.47, 342.21, 413.24, 510.30, 581.33, 682.38, 739.40, 796.42, 895.49								
		4	418.744									
	K27me2	2	815.4754									
		3	543.986	1417.79, 1330.76, 1259.72, 1162.67, 1091.63, 990.58, 933.56, 876.54, 777.47, 300.19, 371.23, 468.28, 539.32, 640.37, 697.39, 754.41, 853.48								
		4	408.2413									
	K36me2	2	815.4754									
		3	543.986	1389.80, 1302.76, 1231.73, 1134.67, 962.59, 905.57, 848.55, 749.48, 328.19, 399.22, 496.28, 496.28, 567.31, 668.36, 725.38, 782.40, 881.47								
		4	408.2413									
	K27me3	2	822.4832						15.7			4310
		3	548.6579	1417.79, 1330.76, 1259.72, 1162.67, 1091.63, 990.58, 933.56, 876.54, 777.47, 314.21, 385.24, 482.30, 553.33, 654.38, 711.40, 768.43, 867.49						548.6568	0.896	
		4	411.7452									
	K36me3	2	822.4832									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		3	548.6579	1403.81, 1316.78, 1245.74, 1148.69, 1077.65, 976.61, 919.56, 862.56, 763.35, 328.19, 399.22, 496.28, 567.31, 668.36, 715.38, 782.40, 881.47								
		4	411.7452									
	K27me2K36me1	2	822.4832									
		3	548.6579	1431.81, 1344.78, 1273.74, 1179.69, 1105.65, 1004.60, 947.58, 890.56, 791.49, 300.19, 371.23, 468.28, 539.32, 640.37, 697.39, 754.41, 853.62								
		4	411.7452									
	K27me1K36me2	2	822.4832									
		3	548.6579	1389.80, 1302.76, 1231.73, 1134.67, 1063.64, 962.59, 905.55, 848.55, 749.48, 342.21, 413.24, 510.30, 581.33, 682.38, 739.40, 895.49								
		4	411.7452									
	K27me1K36me1	2	843.4885									
		3	562.6614									
		4	422.2479									
	K27me3K36me1	2	829.491									
		3	553.3298	1431.81, 1344.78, 1273.74, 1176.69, 1105.65, 1004.60, 947.58, 890.56, 791.49, 314.21,								

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
				385.24, 482.30, 553.33, 654.38, 711.40, 768.43, 867.49								
		4	415.2491									
	K27me1K36me3	2	829.491									
		3	553.3298	1409.81, 1316.78, 1245.74, 1148.69, 1077.65, 976.61, 976.61, 919.58, 862.56, 763.49, 342.21, 413.24, 510.30, 581.33, 682.38, 739.40, 796.42, 896.49								
		4	415.2491									
	K27me2K36me2	2	801.4779									
		3	534.6544									
		4	401.2426									
	K27me3K36me2	2	808.4857									
		3	539.3263									
		4	404.7465									
	K27me2K36me3	2	808.4857									
		3	539.3263									
		4	404.7465									
	K27me3K36me3	2	815.4936									
		3	543.9981									
		4	408.2504									
	K27ac	2	822.465									
		3	548.6458									
		4	411.7361									
H3_04v3_27_40	KSAPSTGGVKKPHR											

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
	unmod	2	837.4703									
		3	558.6493									
		4	419.2388									
	K36me1	2	844.4781									
		3	563.3212									
		4	422.7427									
	K27me1	2	844.4781									
		3	563.3212									
		4	422.7427									
	K27me2	2	823.4728									
		3	549.3176	1433.79, 1346.75, 1275.72, 1178.66, 1091.63, 990.58, 933.56, 876.54, 777.47, 593.35, 300.19, 371.23, 468.28, 555.28, 656.36, 713.38, 770.40, 869.47, 1053.59								
		4	412.2401									
	K36me2	2	823.4728									
		3	549.3176	1405.79, 1318.76, 1247.72, 1150.67, 1063.64, 962.59, 905.57, 848.55, 749.48, 565.36, 328.19, 399.22, 496.28, 583.31, 684.36, 741.38, 798.40, 897.47, 1081.59								
		4	412.2401									
	K27me3	2	830.4807									
		3	553.9895									
		4	415.744									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
	K27me3K36me3	2	823.491									
		3	549.3298									
		4	412.2491									
	K27ac	2	830.4625									
		3	553.9774									
		4	415.7349									
H3_05_41_49	YRPGTVALR											
	unmod	1	1088.6211		18.9			2910608	18.8			232847
		2	544.8142			544.8136	0.868			544.8148	0.87	
		3	363.5452									
	Y41ph	1	1168.5874									
		2	584.7973									
		3	390.2007									
H3_06_54_63	YQKSTELLIR											
	unmod	1	1362.7627		25.5			93690	25.4			63192
		2	681.885			681.884	1.012			681.8846	1.013	
		3	454.9258									
		4	341.4461									
	K56me1	1	1376.7784									
		2	688.8928									
		3	459.5976									
		4	344.9501									
	K56me2	1	1334.7678									
		2	667.8875									
		3	445.5941									
		4	334.4474									
	K56me3	1	1348.7835									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		2	674.8954									
		3	450.266									
		4	337.9513									
	K56ac	1	1348.7471									
		2	674.8772									
		3	450.2539									
		4	337.9422									
H3_07_73_83	EIAQDFKTDLR											
	unmod	1	1447.7427		29.5			65430	29.3			119424
		2	724.375			724.3749	1.022			724.3744	1.022	
		3	483.2524									
		4	362.6911									
	K79me1	1	1461.7584									
		2	731.3828									
		3	487.9243									
		4	366.195									
	K79me2	1	1419.7478									
		2	710.3775									
		3	473.9208									
		4	355.6924									
	K79me3	1	1433.7634									
		2	717.3854									
		3	478.5927									
		4	359.1963									
	K79ac	1	1433.7271									
		2	717.3672									
		3	478.5805									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		4	359.1872									
H3_08_117_128	VTIMPKDIQLAR											
	unmod	1	1496.8505		30.5			100245	30.3			55421
		2	748.9289			748.9275	1.021, 1.077			748.9276	1.021, 1.077	
		3	499.6217									
		4	374.9681									
	K122ac	1	1482.8348									
		2	741.9211									
		3	494.9498									
		4	371.4642									
H3_09u_64_135	unmod											
	KLPFQR	1	900.5301		23.7	900.528	1.406, 1.428	1024986	23.6	900.5261	1.404, 1.429	239181
		2	450.7687	660.38, 547.30, 450.25, 354.24, 451.29, 598.36		450.7693	0.808			450.7688	0.807	
		3	300.8482									
	RIRGER	1	842.4955		16.2			2722				
		2	421.7514			421.7505	.779, .794					
		3	281.5033									
	IRGERA	1	757.4315									
		2	379.2194									
		3	253.1487									
H4_01_4_17	GKGGKGLGKGGAKR											
	unmod	1	1550.9013		19.6			419558	19.5			250690

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		2	775.9543			775.9539	1.122, 1.140			775.9536	1.121, 1.140	
		3	517.6386			517.6376	.799, .829			517.6387	.815, .832	
		4	388.4808									
	K5ac	1	1536.8856									
		2	768.9464	1253.73, 1196.71, 1139.69, 284.16, 341.18, 398.20								
		3	512.9667									
		4	384.9769									
	K8ac	1	1536.8856									
		2	768.9464	955.57, 898.46, 785.46, 728.44, 298.17, 355.20, 412.22, 582.32, 639.35, 752.43, 809.45								
		3	512.9667									
		4	384.9769									
	K12ac	1	1536.8856									
		2	768.9464	941.55, 884.53, 771.45, 714.43, 544.32, 487.30, 430.28, 359.24, 596.34.653.36, 766.45, 823.47								
		3	512.9667									
		4	384.9769									
	K16ac	1	1536.8856						19			32497
		2	768.9464	530.30, 473.28, 416.26, 345.22, 1007.59, 1121.63, 1192.67						768.9464	1.112, 1.128	

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		3	512.9667							512.9662	.805, .824, .837	
		4	384.9769									
	K5acK8ac	1	1522.87									
		2	761.9386	1239.72, 1182.70, 1125.67, 955.57, 989.55, 785.46, 728.44, 544.32, 487.30, 359.24, 284.16, 341.18, 398.20, 568.31, 625.33, 738.41, 795.44, 979.56, 1036.58, 1093.6, 1164.64								
		3	508.2948									
		4	381.4729									
	K5acK12ac	1	1522.87									
		2	761.9386	1239.72, 1182.70, 1125.67, 941.55, 884.53, 771.45, 714.43, 544.32, 487.30, 430.28, 359.24, 284.16, 341.18, 398.20, 582.32, 639.35, 752.43, 809.45, 979.56, 1036.48, 1093.6, 1164.64								
		3	508.2948									
		4	381.4729									
	K5acK16ac	1	1522.87									
		2	761.9386	1239.72, 1182.70, 1125.67, 941.55, 884.53, 771.45, 714.43, 530.30, 473.28, 416.26, 345.22, 284.16, 341.18, 398.20, 582.32, 639.35,								

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
				752.43, 809.45, 993.57, 1050.59, 1107.62, 1178.65								
		3	508.2948									
		4	381.4729									
	K8acK12ac	1	1522.87									
		2	761.9386	941.55, 884.53, 771.45, 714.43, 544.32, 487.30, 430.28, 359.24, 298.18, 355.20, 412.22, 582.32, 639.35, 752.43, 809.45, 979.56, 1036.58, 1093.6, 1164.64								
		3	508.2948									
		4	381.4729									
	K8acK16ac	1	1522.87									
		2	761.9386	941.55, 884.53, 771.45, 714.43, 530.30, 473.28, 416.26, 345.22, 298.18, 355.20, 412.22, 582.32, 639.35, 752.43, 809.45, 993.57, 1050.59, 1107.62, 1178.65								
		3	508.2948									
		4	381.4729									
	K12acK16ac	1	1522.87									
		2	761.9386	927.54, 870.52, 757.43, 700.41, 530.30, 473.28, 416.26, 345.22, 298.18, 355.20, 412.22, 596.34, 653.36, 766.45, 823.47, 993.57,								

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
				1050.59, 1107.62, 1178.65								
		3	508.2948									
		4	381.4729									
	K5acK8acK12ac	1	1508.8543									
		2	754.9308	941.55, 884.53, 771.45, 714.43, 544.32, 487.30, 430.28, 359.24, 341.18, 568.31, 625.33, 738.41, 965.54, 1022.56, 1079.58, 1150.62								
		3	503.623									
		4	377.969									
	K5acK8acK16ac	1	1508.8543									
		2	754.9308	941.55, 884.53, 771.44, 714.43, 568.31, 625.33, 738.41, 795.44								
		3	503.623									
		4	377.969									
	K5acK12acK16ac	1	1508.8543									
		2	754.9308	927.54, 870.52, 757.43, 700.41, 582.32, 639.35, 752.43, 809.45								
		3	503.623									
		4	377.969									
	K8acK12acK16ac	1	1508.8543									
		2	754.9308	1211.69, 1154.66, 1097.64, 927.54, 870.52, 757.43, 700.41, 298.18, 355.20, 412.22, 582.32, 639.35, 752.43, 809.45								

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		3	503.623									
		4	377.969									
	K5acK8acK12acK16ac	1	1494.8387									
		2	747.923									
		3	498.9511									
		4	374.4651									
H4_02_20_23	KVLR											
	unmod	1	627.4188		20.8	627.4178	1.215	835125	20.8	627.4204	1.213	2079
		2	314.213			314.2138	.720, .735					
	K20me1	1	641.4345						22.3	641.4339	1.23	35976
		2	321.2209									
	K20me2	1	599.4239						16.5	599.4245	1.172	283059
		2	300.2156							300.2157	0.727	
	K20me3	1	613.4395						16.5			3838
		2	307.2234							307.223	.692, .707, .723	
	K20ac	1	613.4032									
		2	307.2052									
H4_03_24_35	DNIQGITKPAIR											
	unmod	1	1437.806		21.3			548144	21.2			337235
		2	719.4066			719.4054	.992, 1.013, 1.026			719.4055	.994, 1.018, 1.031	
		3	479.9402			479.9387	0.818			479.9401	.778, .797, .816	
		4	360.207									
H4_04_40_45	RGGVKR											

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
	unmod	1	784.4788		14			83319	13.7			12188
		2	392.743			392.7429	0.758			392.7437	0.749	
	K44ac	1	770.4631									
		2	385.7352									
H4_05_79_92	KTVTAMDVVYALKR											
	unmod	1	1762.9771		43.9			27760				
		2	881.9922			881.9916	1.234					
		3	588.3306									
		4	441.4997									
H4_06u_46_102	unmod											
	ISGLIYEETR	1	1236.647		30.4	1236.648	1.723	630923	30.2	1236.6429	1.715	101001
		2	618.8272			618.8266	0.951			618.826	0.952	
		3	412.8872									
		4	309.9172									
	GVLKVFLENVIR	1	1498.8991									
		2	749.9532									
		3	500.3046									
		4	375.4802									
	DAVTYTEHAKR	1	1402.6961		18.4			39543	18.3			1119
		2	701.8517			701.8503	1.008			701.8525	1.01	
		3	468.2369			468.2373	0.758					
		4	351.4295									
	TLYGFGG	1	770.3719		27.4	770.3708	1.283, 1.348	276638	27.1	770.3721	1.284, 1.303	17029
		2	385.6896									
		3	257.4622									
		4	193.3484									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
HH2A_01m1_36_42	KGNYAER											
	unmod	1	949.4738		17.1	949.4704	1.453	543640	17	949.4721	1.45	7694
		2	475.2405			475.2401	.812, .841			475.241	0.84	
	K36ac	1	935.4581									
		2	468.2327									
HH2A_01m3_36_42	KGNYSER											
	unmod	1	965.4687									
		2	483.238									
	K36ac	1	951.453									
		2	476.2302									
HH2A_01oX_36_42	KGHYAER											
	unmod	1	972.4897									
		2	486.7485									
	K36ac	1	958.4741									
		2	479.7407									
HH2A_02m1_4_11	GKQGGKAR											
	unmod	1	969.5476		15.5	969.5441	1.486	565276	15.4	969.5492	1.475, 1.486	51680
		2	485.2774	672.38, 544.32, 487.30, 430.28, 298.18, 426.23, 483.26, 540.28		485.2762	0.875			485.2779	0.873	
	K5ac	1	955.5319									
		2	478.2696	284.16, 412.22, 469.24, 526.26, 672.28, 544.32, 487.30, 430.28								
	K9ac	1	955.5319									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		2	478.2696	658.36, 530.30, 473.28, 416.26, 298.18, 426.23, 483.26, 540.28								
	K5acK9ac	1	941.5163									
		2	471.2618									
	K9me1	1	983.5632									
		2	492.2853	686.40, 558.34, 501.32, 444.30, 298.18, 426.23, 483.26, 543.28								
	K5me1	1	983.5632									
		2	492.2853	672.38, 544.32, 487.30, 430.28, 312.20, 440.25, 497.28, 554.30								
HH2A_02oJ_4_11	GKQGGKVR											
	unmod	1	997.5789									
		2	499.2931									
	K5ac	1	983.5632									
		2	492.2853	700.41, 572.35, 515.33, 458.31, 284.16, 412.22, 469.24, 526.26								
	K9ac	1	983.5632									
		2	492.2853	686.39, 558.34, 501.31, 444.29, 298.18, 426.23, 483.26, 543.28								
	K5acK9ac	1	969.5476									
		2	485.2774	686.39, 558.34, 501.31, 444.29, 284.16, 412.22, 469.24, 526.26								
	K9me1	1	1011.5945									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		2	506.3009	714.43, 586.37, 529.35, 472.33, 298.18, 426.23, 483.26, 543.28								
	K5me1	1	1011.5945									
		2	506.3009	700.41, 572.35, 515.33, 458.31, 312.20, 440.25, 497.28, 554.30								
HH2A_02oX_4_11	GKTGGKAR											
	unmod	1	942.5367									
		2	471.772									
	K5ac	1	928.521									
		2	464.7642	645.37, 544.32, 487.30, 430.28, 284.16, 385.21, 442.23, 499.25								
	K9ac	1	928.521									
		2	464.7642	631.35, 530.30, 473.28, 416.26, 298.18, 399.22, 456.25, 513.27								
	K5acK9ac	1	914.5054									
		2	457.7563									
	K9me1	1	956.5523						16.6			11778
		2	478.7798	659.39, 558.34, 501.32, 444.30, 298.18, 399.22, 456.25, 513.27						478.779	.843, .859	
	K5me1	1	956.5523									
		2	478.7798	645.37, 544.32, 487.30, 430.28, 312.20, 413.24, 470.27, 527.29								
HH2A_03m1_1_11	SGRGKQGGKAR											

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
	unmod	1	1269.7022		14			514				
		2	635.3547			635.3556	0.95					
		3	423.9056									
	S1ac	1	1311.7127									
		2	656.36									
		3	437.9091									
	K5ac	1	1255.6865									
		2	628.3469									
		3	419.2337									
HH2A_03oV_1_19	AGGKAGKDSGKAKAKAVSR											
	unmod	2	1062.0946									
		3	708.3988									
		4	531.5509									
		5	425.4422									
	K4ac	2	1055.0867									
		3	703.7269									
		4	528.047									
		5	422.6391									
	K7ac	2	1055.0867									
		3	703.7269									
		4	528.047									
		5	422.6391									
	K11ac	2	1055.0867									
		3	703.7269									
		4	528.047									
		5	422.6391									
	K15ac	2	1055.0867									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
	K7acK11acK15ac	2	1041.0711									
		3	694.3831									
		4	521.0392									
		5	417.0328									
	K4acK11acK15ac	2	1041.0711									
		3	694.3831									
		4	521.0392									
		5	417.0328									
	K4acK7acK15ac	2	1041.0711									
		3	694.3831									
		4	521.0392									
		5	417.0328									
	K4acK7acK11ac	2	1041.0711									
		3	694.3831									
		4	521.0392									
		5	417.0328									
	K4acK7acK11acK15ac	2	1034.0633									
		3	689.7113									
		4	517.5353									
		5	414.2297									
HH2A_03oZ_1_19	AGGKAGKDSGKAKTKAVSR											
	unmod	2	1077.0998									
		3	718.4023									
		4	539.0536									
		5	431.4443									
	K4ac	2	1070.092									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		5	423.0349									
	K4acK7acK11acK15ac	2	1049.0685									
		3	699.7148									
		4	525.0379									
		5	420.2318									
HH2A_04m1_12_17	AKAKTR											
	unmod	1	842.5094						17	842.5092	1.428	60315
		2	421.7583	531.33, 460.29, 312.20, 383.23, 276.17						421.7597	.772, .793	
	K13ac	1	828.4938									
		2	414.7505									
	K15ac	1	828.4938									
		2	414.7505									
	K13acK15ac	1	814.4781									
		2	407.7427									
	K15me1	1	856.5251									
		2	428.7662	531.33, 460.29, 326.21, 397.25								
	K13me1	1	856.5251									
		2	428.7662	545.34, 474.31, 312.20, 383.23								
HH2A_04m3_12_17	AKAKSR											
	unmod	1	828.4938		16.9	828.49	1.411	1140381	16.8	828.4922	1.409	44577
		2	414.7505			414.7502	0.788			414.7502	0.787	
	K13ac	1	814.4781									
		2	407.7427	517.31, 446.27, 298.18, 369.21								

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
	K15ac	1	814.4781									
		2	407.7427	503.29, 432.26, 312.20, 383.23								
	K13acK15ac	1	800.4625									
		2	400.7349									
	K15me1	1	842.5094									
		2	421.7583	531.33, 460.29, 312.20, 383.23, 262.15								
	K13me1	1	842.5094									
		2	421.7583	517.31, 446.27, 326.21, 397.25								
HH2A_05m1_72_77	DNKKTR											
	unmod	1	929.5051		16.2	929.5022	1.448	162881	16.1	929.5048	1.433, 1.446	18624
		2	465.2562			465.2559	0.816			465.2567	.805, .817	
	K74ac	1	915.4894									
		2	458.2483									
HH2A_06_82_88	HLQLAIR											
	unmod	1	906.5519		20.9			16685936	20.8			725428
		2	453.7796			453.7796	0.823			453.7799	0.825	
		3	302.8555									
HH2A_07v_1_88	unmod											
	H2AZ.AGGKAGKDSGKAKTKAVSR	2	1077.0998									
		3	718.4023									
	H2AY.SAKAGVIFPVGR	1	1313.7576									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		2	657.3824									
		3	438.5907									
	H2AX.GKTGGKAR	1	942.5367									
		2	471.772									
		3	314.8504									
HH2A_08u_4_99	unmod											
	HLQLAVR	1	892.5363						19.8			19140
		2	446.7718							446.7721	0.815	
		3	298.1836									
	GGKKKSTKTSR	1	1457.8322									
		2	729.4197									
		3	486.6156									
	SGKKKMSKLSR	1	1529.872									
		2	765.4396									
		3	510.6288									
	IHRHLKTR	1	1172.7011									
		2	586.8542									
		3	391.5719									
	IHRHLKSR	1	1158.6854									
		2	579.8463									
		3	386.9									
	NDEELNKLLGR	1	1412.738						27.5			12177
		2	706.8726							706.8716	0.996	
		3	471.5842									
	AGLQFPVGR	1	1000.5574		23.4	1000.5555	1.444, 1.478, 1.510	14000051	23.2	1000.5538	1.475, 1.506	737499

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
		2	500.7823			500.7814	.836, .855			500.7828	.837, .855	
		3	334.1907									
	VHR	1	467.2725		11.1	467.2726	1.009	68980				
		2	234.1362									
		3	156.4242									
	LLR	1	457.2832									
		2	229.1416									
		3	153.0944									
	IHPELLAKKR	1	1372.8311									
		2	686.9192									
		3	458.2819									
	YIKKGHPKYR	1	1513.8525									
		2	757.4299									
		3	505.289									
HH2B_01v_1_29	unmod											
	1C/ 1K.PEPAKSAPAPKK GSKKAVTKAQKKD GKKR	3	1226.0397									
		4	919.7816									
	1H.PDPAKSAPAPKK GSKKAVTKAQKKD GKKR	3	1221.3679									
		4	916.2777									
	2F.PDPAKSAPAPKK GSKKAVTKVQKKD GKKR	3	1230.7116									
		4	923.2855									

Target Peptides					Histone Standard Mix				HeLa S3			
Amino acid position	**Peptide/mod**	**Charge State**	**m/z**	**Reporter Ions**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**	**RT (min)**	**m/z experimental**	**1/k0**	**LC peak area**
	1B.PEPSKSAPAPKKGSKKAITKAQKKDGKKR	3	1236.0433									
		4	927.2843									
	1N.PEPSKSAPAPKKGSKKAVTKAQKKDGKKR	3	1231.3714									
		4	923.7804									
	1D.PEPTKSAPAPKKGSKKAVTKAQKKDGKKR	3	1236.0433									
		4	927.2843									
	1M.PEPVKSAPVPKKGSKKAINKAQKKDGKKR	3	1253.7309									
		4	940.55									
	1L.PELAKSAPAPKKGSKKAVTKAQKKDGKKR	3	1231.3835									
		4	923.7895									
HH2B_02u_1_100	unmod											
	LAHYNKR											
	unmod	1	1013.5527		19			815519	18.9			103031
		2	507.28			507.2788	0.883			507.2803	0.883	
		3	338.5224									
	LPHYNKR	1	1039.5683									
		2	520.2878									
		3	347.1943									
	EIQTAVR	1	872.4836		18.6	872.4829	1.386	754301				
		2	436.7454			436.7443	0.797					
		3	291.4994									