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Absolute Quantification of Histone PTM Marks by MRM-Based LC-MS/MS

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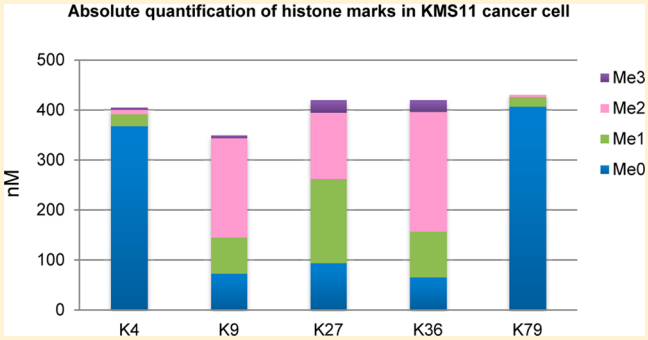
Supporting Information

ABSTRACT: The N-terminal tails of core histones harbor the sites of numerous post-translational modifications (PTMs) with important roles in the regulation of chromatin structure and function. Profiling histone PTM marks provides data that help understand the epigenetics events in cells and their connections with cancer and other diseases. Our previous study demonstrated that specific derivatization of histone peptides by NHS propionate significantly improved their chromatographic performance on reversed phase columns for LC/MS analysis. As a step forward, we recently developed a multiple reaction monitoring (MRM) based LC-MS/MS method to analyze 42 targeted histone peptides. By using stable isotopic labeled peptides as internal standards that are spiked into the reconstituted solutions, this method allows to measure absolute concentration of the tryptic peptides of H3 histone proteins extracted from cancer cell lines. The method was thoroughly validated for the accuracy and reproducibility through analyzing recombinant histone proteins and cellular samples. The linear dynamic range of the MRM assays was achieved in 3 orders of magnitude from 1 nM to 1 μM for all targeted peptides. Excellent intrabatch and interbatch reproducibility (<15% CV) was obtained. This method has been used to study translocated NSD2 (a histone lysine methyltransferase that catalyzes the histone lysine 36 methylation) function with its overexpression in KMS11 multiple myeloma cells. From the results we have successfully quantitated both individual and combinatorial histone marks in parental and NSD2 selective knockout KMS11 cells.



Dynamic post-translational modifications (PTMs) at multiple sites of histone N-terminal tails are known to be intricately involved in gene expression and cell replication, and are also participating in epigenetic crosstalk and feedback mechanism.[1−4] Quantitative analysis of prevalent histone protein PTMs can provide the insights into understanding of the mechanistic linkages between the modifications and disease pathways.[5,6] As a common practice in biology research, immunoassays, such as Western blotting and enzyme-linked immunosorbent assay (ELISA),[7,8] are traditionally used for detecting histone PTMs, such as methylation, acetylation, and phosphorylation. In general, these antibody-based assays are highly specific, thus typically only analyzing one specific PTM at a time (e.g., the antibody for detecting trimethylation on histone H3 lysine 27 is different from the one for H3 lysine 36). It is virtually impossible to profile multiple PTMs in a single experiment.

Mass spectrometry has become a popular technology in characterization of the combinatorial histone modifications to decipher the potential histone code or crosstalk in various cell lines or tissues samples.[9−15] For example, SILAC and i-TRAQ approaches are currently used for comparison of relative histone PTM levels through the analysis of histone peptides where the histone proteins are digested into tryptic peptides. In 2009, Garcia and colleagues reported a comprehensive study on the quantitative study of PTM modified histone peptides in knockout and wild-type samples. In this work, the percentage of the methylation levels were calculated based on the mass spectrometry responses by summing up the peak areas of all modification forms of the same peptide.[10] This method is based on an assumption that the signal response factors among these

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Table 1. List of Histone Peptides and Their Post-Translational Modifications/Derivatization[a]

histone residues	peptide sequence	modification	derivatization	abbreviation
H3(3−8)	TKQTAR	Me2(K4)	Pr(N-term)	K4(Me)2
H3(3−8)	TKQTAR	Me3(K4)	Pr(N-term)	K4(Me)3
H3(3−8)	TKQTAR	Me1(K4)	Pr(N-term, K4)	K4(Me)1
H3(3−8)	TKQTAR		Pr(N-term, K4)	K4
H3(9−17)	KSTGGKAPR	Ac (K9)	Pr(N-term, K14)	K9(Ac)
H3(9−17)	KSTGGKAPR	Ac (K9), PO3(S10)	Pr(N-term, K14)	K9(Ac)S10(PO3)
H3(9−17)	KSTGGKAPR	Me2(K9), Ac(K14)	Pr(N-term)	K9(Me)2K14(Ac)
H3(9−17)	KSTGGKAPR	Me2(K9)	Pr(N-term, K14)	K9(Me)2
H3(9−17)	KSTGGKAPR	Me3(K9), Ac(K14)	Pr(N-term)	K9(Me)3K14(Ac)
H3(9−17)	KSTGGKAPR	Me3(K9)	Pr(N-term, K14)	K9(Me)3
H3(9−17)	KSTGGKAPR	Me1(K9), Ac(K14)	Pr(N-term, K9)	K9(Me)K14(Ac)
H3(9−17)	KSTGGKAPR	Me1(K9)	Pr(N-term, K9, K14)	K9(Me)1
H3(9−17)	KSTGGKAPR	Ac(K14)	Pr(N-term, K9)	K14(Ac)
H3(9−17)	KSTGGKAPR	Ac(K9, K14)	Pr(N-term)	K9(Ac)K14(Ac)
H3(9−17)	KSTGGKAPR		Pr(N-term, K9, K14)	K9
H3(27−40)	KSAPA TGGVKKPHR	Ac(K27), Me2(K36)	Pr(N-term, K37)	K27(Ac)K36(Me)2
H3(27−40)	KSAPA TGGVKKPHR	Ac(K27), Me3(K36)	Pr(N-term, K37)	K27(Ac)K36(Me)3
H3(27−40)	KSAPATGGVKKPHR	Ac(K27), Me1(K36)	Pr(N-term, K36, K37)	K27(Ac)K36(Me)1
H3(27−40)	KSAPATGGVKKPHR	Ac(K27)	Pr(N-term, K36, K37)	K27(Ac)K36
H3(27−40)	KSAPATGGVKKPHR	Me2(K27, K36)	Pr(N-term, K37)	K27(Me)2K36(M)2
H3(27−40)	KSAPATGGVKKPHR	Me2(K27), Me3(K36)	Pr(N-term, K37)	K27(Me)2K36(M)3
H3(27−40)	KSAPATGGVKKPHR	Me2(K27), Me1(K36)	Pr(N-term, K36, K37)	K27(Me)2K36(M)1
H3(27−40)	KSAPATGGVKKPHR	Me2(K27)	Pr(N-term, K36, K37)	K27(Me)2K36
H3(27−40)	KSAPATGGVKKPHR	Me3(K27), Me2(K36)	Pr(N-term, K37)	K27(Me)3K36(M)2
H3(27−40)	KSAPATGGVKKPHR	Me3(K27, K36)	Pr(N-term, K37)	K27(Me)3K36(M)3
H3(27−40)	KSAPATGGVKKPHR	Me3(K27), Me1(K36)	Pr(N-term, K36, K37)	K27(Me)3K36(M)1
H3(27−40)	KSAPATGGVKKPHR	Me3(K27)	Pr(N-term, K36, K37)	K27(Me)3K36
H3(27−40)	KSAPATGGVKKPHR	Me1(K27), Me2(K36)	Pr(N-term, K27, K37)	K27(Me)1K36(M)2
H3(27−40)	KSAPATGGVKKPHR	Me1(K27), Me3(K36)	Pr(N-term, K27, K37)	K27(Me)1K36(M)3
H3(27−40)	KSAPATGGVKKPHR	Me1(K27, K36)	Pr(N-term, K27, K36, K37)	K27(Me)1K36(M)1
H3(27−40)	KSAPATGGVKKPHR	Me1(K27)	Pr(N-term, K27, K36, K37)	K27(Me)1K36
H3(27−40)	KSAPATGGVKKPHR	Me2(K36)	Pr(N-term, K27, K37)	K27K36(Me)2
H3(27−40)	KSAPATGGVKKPHR	Me3(K36)	Pr(N-term, K27, K37)	K27K36(Me)3
H3(27−40)	KSAPATGGVKKPHR	Me1(K36)	Pr(N-term, K27, K36, K37)	K27K36(Me)1
H3(27−40)	KSAPATGGVKKPHR		Pr(N-term, K27, K36, K37)	K27K36
H3(41−49)	YRPGTVALR		Pr(N-term)	H3(41−49)
H3(50−52)	EIR		Pr(N-term)	H3(50−52)
H3(70−72)	LVR		Pr(N-term)	H3(70−72)
H3(73−83)	EIAQDFKTDLR	Me2(K79)	Pr(N-term)	K79(Me)2
H3(73−83)	EIAQDFKTDLR	Me3(K79)	Pr(N-term)	K79(Me)3
H3(73−83)	EIAQDFKTDLR	Me1(K79)	Pr(N-term, K79)	K79(Me)1
H3(73−83)	EIAQDFKTDLR		Pr(N-term, K79)	K79

[a]PTM abbreviations: Ac, acetylation; PO3, phosphorylation; Me1, monomethylation; Me2, dimethylation; Me3, trimethylation; Pr, propionylation.

differentially modified peptides with same amino acid sequence are the same. As studied by Pesavento et al., the mixtures of differentially modified forms of human histone peptides displayed large discrepancy between solution ratio and MS ratio.[11] This work actually represents a common concern on the use of MS signal directly for quantification without calibration by response factors. To achieve more accurate quantitative measurement, Philip Andrews et al. recently employed N15 metabolically labeled histone proteins as internal references for relative comparison of histone PTMs changes in wild-type and knockout strains.[15] In recent years, MRM-based LC-MS/MS has been increasingly employed as a label free approach in quantitative proteomics studies.[16−18] This technology offers some unique advantages over i-TRAQ or other semi- quantitative proteomics approaches in terms of extraordinary sensitivity and specificity in highly complex sample matrices.[16,19−22]

In this study, we aimed at developing an MRM based LC-MS/MS method to quantify selected histone peptides with methylation and acetylation on lysine residues of human histone H3 N-terminal tails based on the current literature knowledge (see Table 1).[1,5,6] The first part of our work is to set up MRM transitions and to optimize a procedure to measure the absolute concentrations of tryptic histone peptides by using stable isotope labeled peptides. Spiking a mixture of the labeled peptides with known concentrations into the biological sample solution allows us to accurately calculate the relative abundance of one single PTM on the same residue of histone H3 N-terminal tail. The second part of the work is to evaluate the recovery of the histone peptides through the sample treatment procedure including trypsin digestion and chemical derivatiza-

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tion. The feasibility of quantifying the global histone modifications at the protein level through bottom-up peptide analysis is discussed.

EXPERIMENTAL SECTION

Chemicals and Reagents. All chemicals were purchased from Sigma-Aldrich (St.Louis, MO) at the highest purity, unless otherwise specified. The double deionized water was produced by Milli-Q Gradient A10 system (Millipore, Bedford, MA). The isotope labeled histone peptides (with propionylation on the free and monomethylated amine) were purchased from New England Peptide LLC (MA, USA). The purity of isotope labeled peptides was determined by LC-MS and confirmed by amino acid analysis.

Standard Isotope Labeled Histone Peptides Preparation. For the 42 isotope labeled histone peptides stock solutions were prepared by dissolving accurate amounts in acetonitrile: water (3:7, v/v) at concentrations of 100 μM. A series of working standard solutions were made by further diluting the mixed stock solutions in water. Calibration standards were prepared by spiking the appropriate amounts of the standard solutions into 20 μL of biological samples to yield final concentrations of 1, 5, 10, 50, 100, 500, and 1000 nM. The quality control (QC) samples were similarly prepared at concentrations of 3.0, 100, and 850 nM for the low, medium and high concentration QC samples, respectively. A 100 nM working solution of 42 mixed labeled each peptides was also prepared in acetonitrile: water (3:7, v/v). All solutions and samples were stored at −80 °C and brought to room temperature before use.

Mammalian Cell Culture, Cellular ELISA, Histone Extraction, and Purification. KMS11-parental (PAR) and NSD2 selective knockout (TKO) cell lines were obtained from Horizon Discovery, Ltd. (Cambridge, UK) and were grown in RPMI-1640 (Gibco) with 12.5% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). TKO is a derivative line of the corresponding parental KMS-11 line (PAR) with selective disruption of the translocated NSD2 allele using somatic cell gene targeting.[23] To collect cells, the medium was removed from the plates. The cells were scratched off and centrifuged at 300 × *g* for 10 min. For cellular ELISA, 5×10^3 KMS11 PAR or TKO cells were seeded in PDL coated TC 384-well plate (PerkinElmer) per well. Histone H3 (dimethyl K36) antibody (Abcam) and Histone H3 Rabbit mAb (Cell Signaling Technology) were used as primary antibody. Secondary antibody (Peroxidase AffiniPure Goat Anti-Rabbit IgG, Jackson ImmunoResearch Laboratories) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) were utilized for detection with PerkinElmer Envison.

Histone proteins were extracted and purified as described by Shechter et al.[24] Briefly, the cells ($n = 5 \times 10^6$) were lysed using 500 μL hypotonic lysis buffer (10 mM Tris−HCl pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and cocktail of protease inhibitors) for 40 min at 4 °C and centrifuged at 10 000 × *g* for 10 min to isolate nuclei. The core histones were acid extracted from nuclei with 0.4 M H_2SO_4 and precipitated with trichloroacetic acid (TCA), followed by washing with ice-cold acetone twice. The core histones were further fractionated on a C8 column (150 mm × 4.6 mm, Agilent), using an Agilent series 1200 system (Waldbronn, Germany). Fractions of histone H3 were pooled together and evaporated to complete dryness with a SpeedVac for further derivatization.

Derivatization of Histones and Trypsin Digestion. The histone N-propionylation derivatization was performed according to the previously published method by Liao et al.[25] First, the histone H3 fraction pool was redissolved in 100 μL water solution. Ten microliters of H3 protein solution was derivatized in a final buffer containing 100 mM NHS acid, 25 mM NH_4HCO_3 and 50% acetonitrile for 30 min at 50 °C. Then the proteins were concentrated to dryness with a SpeedVac and digested with trypsin in 25 mM NH_4HCO_3 overnight. Finally, the digested peptides were concentrated to dryness again and derivatized with the same method used for proteins. Prior to LC-MS analysis, the derivatized peptides were redissolved in 50 μL of water and then combined with 50 μL of isotope labeled peptides mixture. A 5 μL aliquot of the resulting solution (100 μL) was injected onto the LC-MS/MS system for analysis.

LC-MS/MS Instrument. The high-performance liquid chromatography was performed on a Shimadzu Prominence UFLC system (Shimadzu Scientific Instruments, Pleasanton, CA) equipped with a binary pump, a degasser, an autosampler, and a column compartment. The UFLC system was coupled to a QTRAP 5500 mass spectrometer (Applied Biosystems, Singapore) via a TurboIonspray ionization (ESI).

The chromatographic separation was performed on an Agilent Zorbax 300 SB-C18 column (150 mm × 4.6 mm, 5 μm; Agilent, USA). Gradient elution was performed as follows: 2% B for 0−2.0 min; 2−30% B for 2.0−30 min; 30−90% B for 30−31 min, 90% B for 31−34 min followed by 4 min re-equilibration to 2% B at a flow rate of 0.6 mL/min. Solvent A was 0.1% formic acid in water, solvent B 0.1% formic acid in acetonitrile (v/v). The injection volume was 5 μL and the column temperature was maintained at 25 °C.

The ESI interface operated in the positive mode. The ion spray voltage was set to 5000 V, and the turbo spray temperature was kept at 550 °C. Nebulizer gas (gas 1) and heater gas (gas 2) was set at 60 and 65 arbitrary units, respectively. The curtain gas was kept at 40 arbitrary units and interface heater was on. MRM was employed, with two transitions for all endogenous and isotope labeled peptides of which the declustering potential (DP) and collision energy (CE) were optimized to obtain maximum MRM intensity. Relevant parameters are shown in Table S1 of Supporting Information. The endogenous and isotope labeled histone peptides (168 MRM transitions) were separated into six periods in mass spectrometry based on their retention times on the HPLC column, and the dwell times for each MRM transition were set as 0.02 s. All data was controlled and synchronized by Analyst software (versions 1.6.1) from AB Sciex.

RESULTS AND DISCUSSION

To develop the MRM-based LC-MS/MS methods, 42 isotope labeled histone peptides with propionylation on the free and monomethylated amines (Table 1) were used. As the C-terminal of each labeled peptide consist of an [13C6, 15N4] arginine, all corresponding singly charged *y* fragment ions have an *m*/*z* shift of 10 compared to the endogenous peptide. As the *y* fragment ions are usually the dominant species in CID fragmentation spectra of tryptic peptides, at least one of the *y* fragment ions was therefore included in the MRM transitions for each peptide for increased specificity. The MRM transitions of the endogenous histone peptides were calculated from the theoretical mass difference between the endogenous and the corresponding standard peptides. Identical MRM parameters

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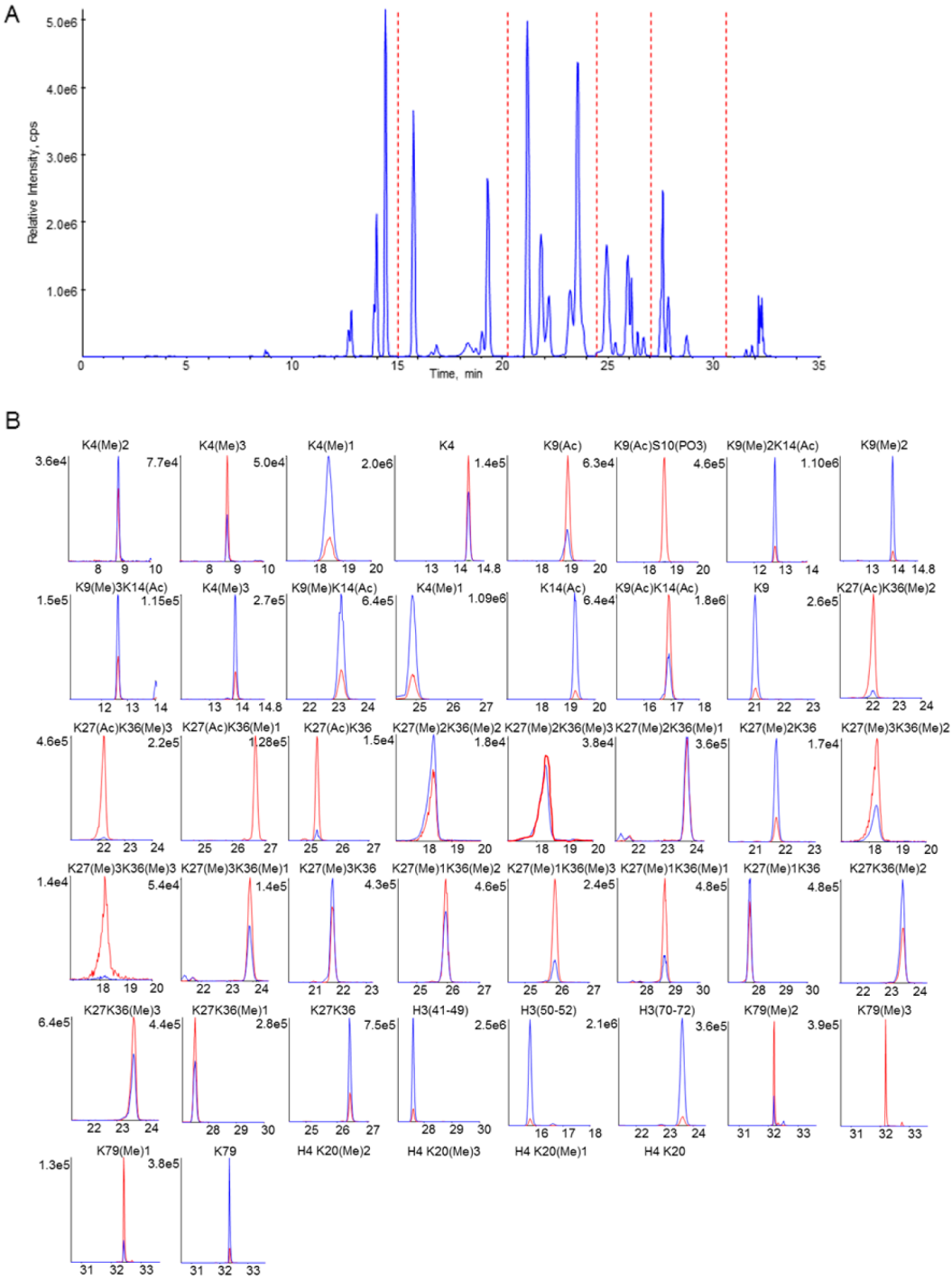

Figure 1. MRM quantitation of 42 peptides in a single LC-MS/MS analysis. (A) Representative chromatograms of 42 peptides in a single 38 min LC-MS/MS analysis of KMS11 cell spiked with a mixture of 42 isotope labeled peptides (red lines marked the time periods in LC). (B) XICs of MRM transitions for endogenous (blue) and labeled peptides (red).

were used for both endogenous and isotope forms of each peptide. As summarized in the Table S1 of Supporting Information, there are totally 168 MRM channels (2 MRM transitions for each peptide). These transitions are grouped into 6 data acquisition windows based on the elution order of the peptides, allowing sufficient dwell time for each peak.

Figure 1 shows the TIC (A) and XICs (B) of a single 38 min LC-MS/MS analysis of cellular histone peptides samples spiked with a mixture of 100 nM of 42 isotope labeled peptides. The concentration C of the targeted peptide was calculated as

$$C_{\text{peptide}} = C_{\text{labeledpeptide}} \times \text{peak area}_{\text{peptide}} / \text{peak area}_{\text{labeledpeptide}}$$

It is reasonable to assume that the MRM response factors are identical between the peptides and their respective labeled analogues, given that they are fully coeluted and all the MRM transitions and the CID parameters were set identically. However, there are still a number of peptides that are not well separated by LC or MS. For example, two peptides

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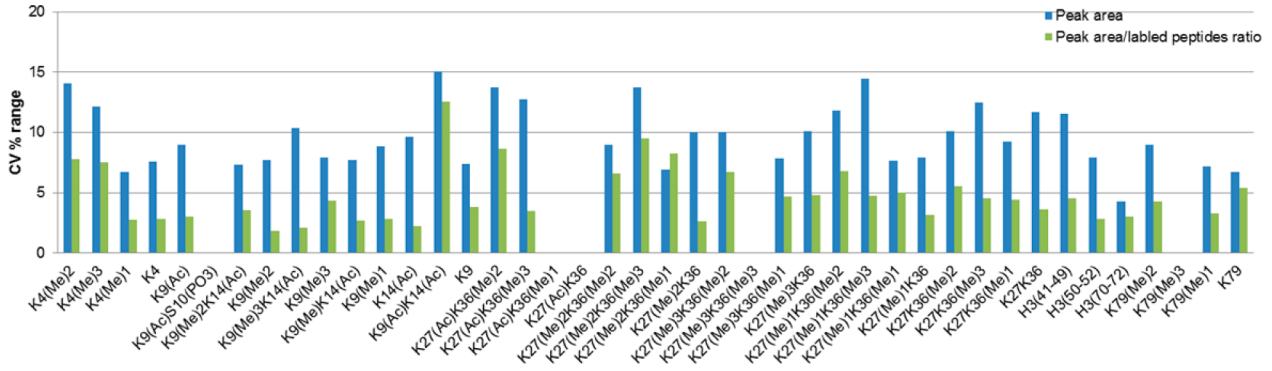

Figure 2. Analytical reproducibility of MRM-based quantitation without/with 42 labeled standards. CV% of 42 peptide assays using raw peak areas, peak area ratios normalized to labeled peptides mixture.

K27(Me)2K36 (Me)3 and K27(Me)3K36(Me)2 are isobaric and difficult to be resolved by HPLC or precursor peptide ions in MS. To solve this problem, the b^{3+} fragment ions can be selected in the MRM transitions to distinguish both peptides.

Method Evaluation and Validation. *Specificity.* To assess the specificity for the 42 histone peptides we measured two MRM transitions per peptides in neat solution as well in biological samples and calculated their ratios. The data are summarized in Table S2 of Supporting Information. The values are rather consistent (RE< 25%) indicating negligible interference from the sample matrix to the labeled peptides. Furthermore, we measured the ratios of two MRM transition signals for endogenous peptides (as shown in column 3 in Supporting Information Table S2). The ratios for all the peptides (except those not detected) are in a good agreement with the labeled peptides. It is worthwhile pointing out that the absolute MRM signals of the labeled peptides, as spiked into the biological samples, decreased to different extents as compared with their neat solution. This observation reflected the fact that there was matrix suppression effect. Owing to the use of labeled peptides as internal standard, this effect should have negligible impact on the accuracy of the quantification in this assay.

Linearity and Reproducibility. The quantification of the endogenous histone peptides is based on a one point calibration using labeled peptides. In order to use that calculation, the MRM responses for all measured peptides from unknown samples should be within the linear range and the assays must be validated over a wider concentration range. In our study, we spiked isotope labeled peptides into the biological samples to evaluate the linearity and the assay yielded dynamic responses over a 10^3-fold concentration range. The correlation coefficients of the weighted calibration curves ($1/x^2$ as weighting factor for curve fitting) ranged from 0.9902 to 0.9982 (Supporting Information Table S3). The lower limit of quantifications (LLOQ) was determined to be 1 nM for all 42 isotope labeled peptides with the signal-to-noise ratios greater than 10. To assess the reproducibility of the method in biological sample, we analyzed the cellular histone peptide samples spiked with the 42 labeled peptides at three different concentrations (low, medium and high) to create QC pools (see Table S3 of Supporting Information).

In the analysis of biological samples, one of the major concerns has been the reproducibility of the histone marks, especially for those with low abundances. To assess the intra and inter batch reproducibility, we analyzed three samples from independently collected cancer cells in triplicates prepared on different days. The overall CVs (coefficient of variation) are less than <15% even at low concentrations (~1 nM). The interbatch CVs of 42 endogenous peptides of either the raw peak area or of the peak area ratio against labeled standard are shown in Figure 2. These results clearly demonstrate the better reproducibility of the assay using internal standards.

Quantification of Histone Marks at Protein Level through Analysis of Digested Peptides. In bottom-up proteomics studies, the peptides from enzymatic digestion are often used to quantify the precursor protein levels. The question being always asked is whether peptides concentrations as determined are a truly reflective of the protein level in samples. In our studies, two rounds of chemical derivatization were carried out at the precursor protein level and the tryptic peptides level. So it inevitably raises a big concern on the correlation on the modification levels between histone proteins and measured peptides. Although the method reported here, as discussed earlier, is mainly used to determine the absolute concentrations of the histone peptides in the final solution, an assessment of the recovery of histone peptides would provide useful information on how the peptide quantification results can used to assess the precursor protein amount. Due to technical limitation to produce a perfect histone H3 standard protein carrying various cellular PTMs, we attempted to use a recombinant histone H3 protein without any modifications as a first step toward the assessment. Basically we measured the concentrations of the tryptic peptides prepared from 2.6 uM recombinant histone H3.1 proteins from *Escherichia coli* expression. The data are shown in Figure 3, the K4, K9,

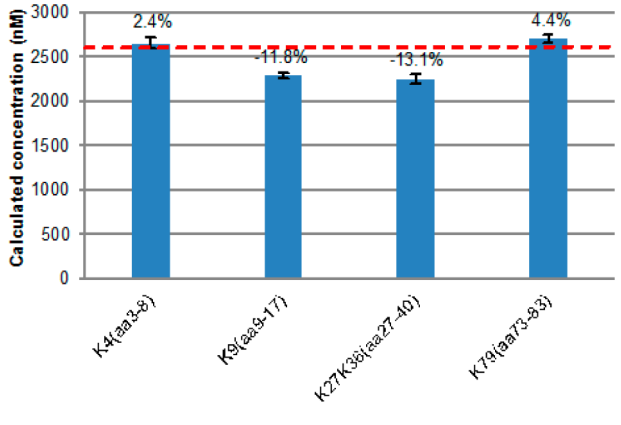

Figure 3. Recombinant H3.1 protein concentration determined by peptide MRM method (n = 3).

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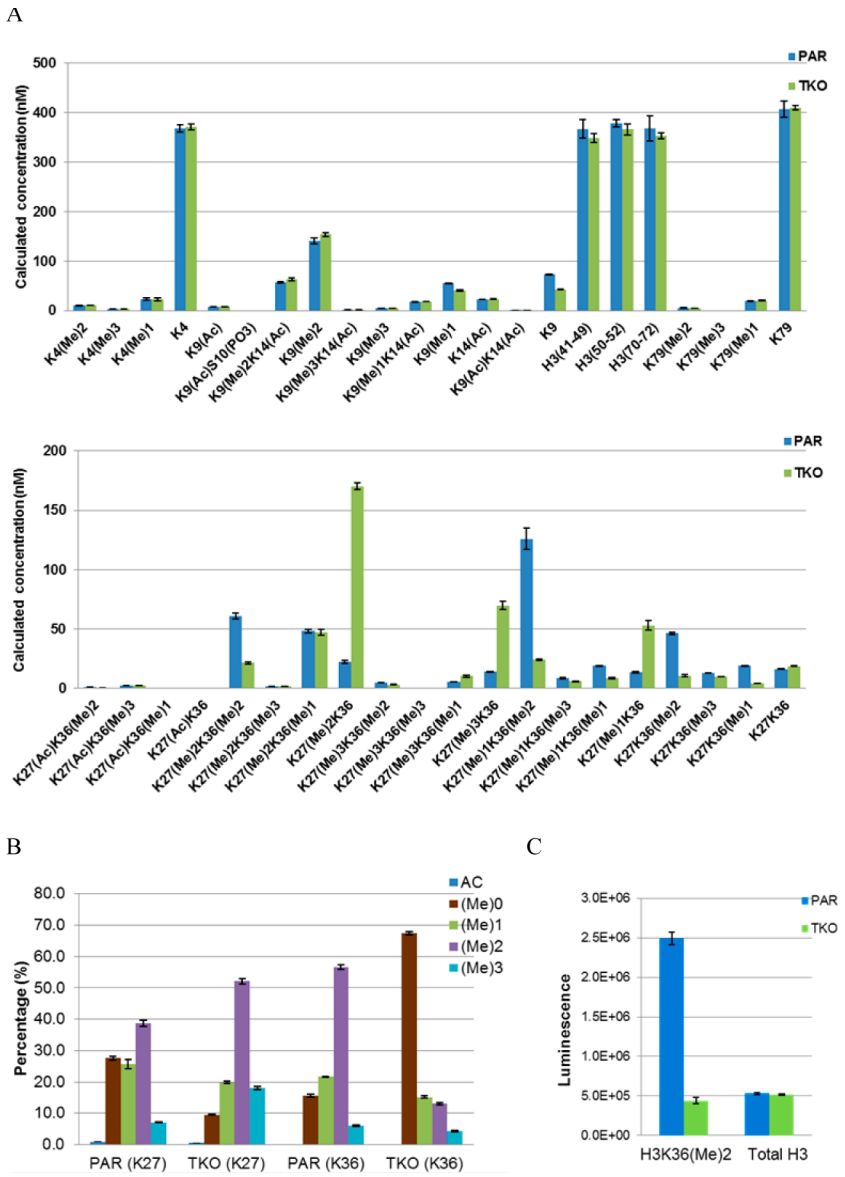

Figure 4. Calculated concentration of histone PTMs in KMS11 PAR and TKO cells. (A) Calculated concentration of each histone PTMs on K4, K9, K14, K27K36, and K79 in PAR and TKO cells (n = 3). (B) Individual K27 and K36 methylation distributions were further calculated by summing the appropriate data corresponding to each particular site in PAR and TKO cells. (C) K36 dimethylation level measured by ELISA assay in PAR and TKO cells.

K27K36, and K79 peptides concentrations are ranging from 2.0 to 2.7 μM based on MRM calculation, which is in a fairly agreement with the known original protein level. Therefore, this result gives us confidence that we can use certain stably reproducible peptides as "house-keeping" peptides to infer the parental protein concentration even after multiple preparation steps. Meanwhile, selection of these "house-keeping" peptides from a particular protein will serve as a quality control to track the experimental bias between biological replicates. For complex cellular histones, we may need to select those tryptic peptides without any known modifications as our "house-keeping" peptides simply because they are the only forms of complete digestion products. Three "house-keeping" histone peptides (H3:41−49; 50−52; 70−72) were selected for evaluating sample true ratios and extraction/digestion efficiency as they carry no endogenous modifications and locate at different regions of histones to avoid digestion efficiency bias. However, there are still many possible caveats in the selection of house-keeping peptides. Different stability and solubility may lead to different sample loss rates of house-keeping peptides during digestion and derivatization. Meanwhile it is very hard to know whether the digestion efficiency of the selected peptides remains constant in case that the conformation of precursor protein changes dramatically due to the dynamic modifications in different treatments/cells.

Absolute Quantification of Histone PTMs in KMS11 Multiple Myeloma Cells. The nuclear receptor SET domain-containing protein 2 (NSD2, also named MMSET and WHSC1) is a histone lysine methyl-transferase that mediates the mono- and dimethylation on histone H3 Lys 36 (H3K36).[26,27] KMS11 cell line is a model t(4;14)+ multiple myeloma cell line with the overexpression of NSD2.[24] To investigate the biological consequence of NSD2 translocation on histone modification levels in vivo, we utilized our absolute MRM-based LC-MS/MS method to characterize and compare the histone modification levels from KMS11 cells with selective

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knock out of the translocated NSD2 allele (TKO) to the parental KMS11 cells (PAR). The absolute quantification results are shown in Figure 4A. The combinations of K27(Me)2K36 and K27(Me)1K36(Me)2 are the most abundant "histone codes" in TKO and PAR cells respectively and this data has not been reported in any other studies. The sum concentration was calculated at ∼400 nM for all K4, K9, K27K36, and K79 peptides, which suggest the experimental variation between different peptides from the same histone H3 protein can be well controlled in our method (data listed on Supporting Information Table S4). Meanwhile, the result from three additional "house-keeping" histone peptides (H3:41−49; 50−52; 70−72) showed very good consistency in calculated concentration (348−378 nM) and acceptable CV variation (Figure 4A). On the basis of our protocol and calculation, 0.5% of the extracted histone H3 proteins were digested and submitted for each MS run. Therefore, the MRM-calculated amount of extracted histone H3 proteins from 5×10^6 cells is roughly about 6.1 μg based on the peptide sum concentration result (∼400 nM). The original sample amount of extracted histone H3 proteins was also measured at about 15.2 μg by comparing the HPLC UV peak absorbance from the sample H3 fraction versus the pure recombination H3 protein standard. This difference can be explained by sample preparation loss and impurities in the sample H3 UV peak absorbance. And it would be more meaningful to measure the relative percentage of each individual modification on a specific residue rather than the absolute total protein amount. The relative level of a specific single-site modification is calculated based on the concentration according to the following formula using K36(Me)2 as an example

$$\begin{aligned}&\text{relative bundance of K36(Me)2}\\&= \sum \text{K36(Me)2}_{\text{containingpeptides}}\\&/[\sum \text{K36(Me)0}_{\text{containingpeptides}}\\&+ \sum \text{K36(Me)1}_{\text{containingpeptides}}\\&+ \sum \text{K36(Me)2}_{\text{containingpeptides}}\\&+ \sum \text{K36(Me)3}_{\text{containing peptides}}] \times 100\%\end{aligned}$$

According to the above calculation method, K36 dimethylation is the most abundant in PAR cells, whereas NSD2 knockout resulted in nearly 4.4 fold decrease of K36 dimethylation in TKO cells (Figure 4B). As the crosstalk evidence, it was observed that K27 trimethylation level more than doubled in response to K36 dimethylation reduction in TKO cells. Particularly, K27 dimethylation is the dominant modification form in TKO cells. We also compared the MRM results with ELISA assay, which showed 4.7 fold decreases of the total K36 dimethylation (Figure 4C). This data supports that our peptide MRM quantitation result is consistent with the independent data from other orthogonal assay in determining the relative changes of individual histone mark in cells.

CONCLUSIONS

The integration of mass spectrometry in chromatin biology has become more popular as it provides quantitative comparison between different histone marks. Absolute quantification of histone PTMs in biological samples is critical for answering various questions regarding cellular enzymatic activities, drug efficacy and toxicity, PTMs abundance and linkage between histone PTM combinations/crosstalk and downstream regulation. We have described a mixture of 42 isotope labeled peptides that we used as internal standards to develop rapid, specific, and sensitive MRM assays for quantitative profiling either individual histone marks or combinatorial "histone codes". Through the multiple sample preparation steps, these MRM assays were reproducible and linear across a broad range of concentrations. The absolute quantification approach was demonstrated to be highly reproducible and specific through the analysis of both analytical and biological replicates. This panel of isotope labeled peptides should be easily expandable to include more newly discovered histone PTMs. This would allow the assay become versatile to decipher specific "histone codes" in regulating chromatin structure and function during various epigenetic events. In future studies, the sensitivity of our method can be further improved by coupling with nano-LC system in the front end, therefore reducing the biological sample consumption. Also the histone extraction/separation from tissues/organs can be coupled with our method to have better understanding of epigenetic regulation and molecular mechanism at different stages of disease progression and tissue differentiation. However, due to the different recovery rates between histone peptides after preparation, it is still a big challenge to deduce the original cellular histone abundance solely relying on the measurement on the tryptic peptides.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text.This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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