High Throughput Quantitation of 46 Histone PTMs through Unscheduled SRM-based Method Development on a Nano-HPLC Triple Quadrupole Platform

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Overview

Histone posttranslational modifications (PTM) was analyzed on a high throughput, unscheduled quantification platform using nano HPLC coupled with a Thermo Scientific™ TSQ™ Quantiva™ mass spectrometer. Forty-six modified histone peptides were selected for quantitation using 186 transitions in 35 and 60 minute program. The sensitivity and reproducibility of these research methods are evaluated. Results here establish a baseline to guide MS-based quantification method development for research.

Introduction

Histone posttranslational modifications (PTM) are important aspects of epigenetic gene regulation, and are linked to many diseases. The core histones (H2A, H2B, H3, and H4) are densely populated with numerous PTMs. Most histone PTM analysis has involved antibody-based techniques, but mass spectrometry (MS) has emerged as an attractive alternative, overcoming limitations of antibodies such as poor specificity and epitope occlusion by nearby PTMs. The most common MS strategy is to analyze enzymatically-digested histones, with chemical modification of lysine residues for better detection. The complexity of histone peptide digests, with many closely related peptides and large dynamic ranges make fast and accurate quantitation challenging. We therefore developed a workflow for high throughput quantitation of histone peptides using nano-HPLC triple quadrupole instrumentation.

Methods

Sample Preparation

Core histones were acid-extracted from cultured HEK293T cells, column-purified by ion exchange, and desalted by perchloric acid precipitation. The purified histones were prepared for MS using a hybrid chemically derivatization strategy, whereby an initial conversion of free lysines to their propionylated forms under mild aqueous conditions was followed by trypsin digestion and labeling of new peptide N-termini with phenyl isocyanate. Labeled histone samples were desalted using C18-stage-tips. Approximately 100 ng of peptides were loaded onto a Thermo Scientific™ PepMap™ C18, 75µm x15cm column and separated by a linear gradient of 35 or 60 minutes. MS data were acquired in selected reaction monitoring (SRM) mode using "Scheduled" and "Unscheduled" SRM lists on TSQ Quantiva triple quadrupole MS.

Mass Spectrometry

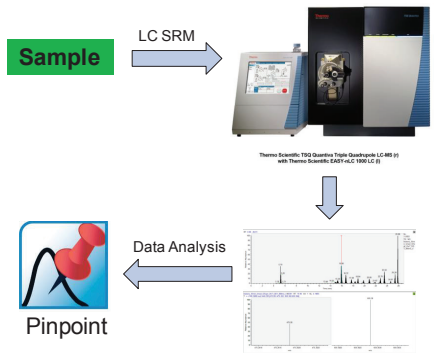
A mixture of peptides from human histones H3 and H4 was quantified on a Thermo Scientific™ Easy1000™ nano™ HPLC coupled with TSQ Quantiva triple quadrupole MS. These samples had been previously analyzed by data-dependent tandem mass spectrometry on a Thermo Scientific™ Orbitrap Elite™ mass spectrometer where a 90-minute HPLC program was required to resolve closely related and isobaric peptides. As a more rapid and targeted alternative for quantitation, 186 SRM transitions for “light” and “heavy” isotopically labeled forms of 46 peptides were established, first in "Unscheduled", and then "Scheduled" modes. In " Unscheduled " mode, all SRM transitions were performed in each duty cycle throughout the experiment, using 0.7 unit mass resolution in both Q1 and Q3, and chrome filter at 7 sec. Cycle times and collision gas pressures in the 60 minute program were investigated. All 186 SRMs were quantified by using targeted SRM methods at both 35 and 60 minute gradients.

A "Scheduled" mode was then implemented to improve sensitivity. Eight time segments in a 60-minute program were created. An even higher throughput was investigated using a 35min program in "Scheduled" mode.

Data Analysis

Data was acquired using Thermo Scientific™ Xcalibur™ software version 2.2. Thermo Scientific™ Pinpoint™ software version 1.4 was used for simultaneous quantitative data processing.

FIGURE 1. Peptide Quantitation Workflow using nano-HPLC coupled with Quantiva MS



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Histone Peptide Information

A total of 46 histone peptides from 5 backbone peptides (the list below) were quantified with their isotopic –labeled heavy pairs using 186 SRMs. The peptide backbones are:

TKQTAR
KSTGGKAPR
KQLATKAAR
KSAPATGGVKKPHR
GKGGKGLGKGGAKR

Results

Quantitation Optimization using "Unscheduled" Mode

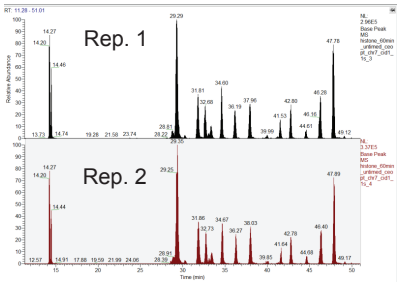
The quantitation of histone peptides are optimized using the heavy histone peptides in "unscheduled" mode. All the heavy peptide transitions were subjected to detection through the 60min LC run. Optimization of the cycle time and collision gas pressure found best signal for most peptides at a cycle time of 800ms with 1mTorr of collision gas. For each peptide, its collision energy was optimized based on its predicted collision energy by Pinpoint. In addition, with 500ms cycle time and 1ms interscan delay, all 186 SRMs were detected.

Table 1. Target List for "Unscheduled" SRM at 60min Run Time

SRM Table

	Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)
1	K(Me2)SAPATGGVK(Me2)K(un)PHR light	5	55	Positive	416.996	434.24	24
2	K(Me2)SAPATGGVK(Me2)K(un)PHR light	5	55	Positive	416.996	532.322	24
3	K(Me2)SAPATGGVK(Me2)K(un)PHR heavy	5	55	Positive	418.493	440.231	24
4	K(Me2)SAPATGGVK(Me2)K(un)PHR heavy	5	55	Positive	418.493	532.322	24
5	K(Me2)SAPATGGVK(Me3)K(un)PHR light	5	55	Positive	420.499	434.24	22
6	K(Me2)SAPATGGVK(Me3)K(un)PHR light	5	55	Positive	420.499	539.33	22
7	K(Me3)SAPATGGVK(Me2)K(un)PHR light	5	55	Positive	420.501	448.256	22
8	K(Me3)SAPATGGVK(Me2)K(un)PHR light	5	55	Positive	420.501	532.322	22
9	K(Me2)SAPATGGVK(Me3)K(un)PHR heavy	5	55	Positive	421.997	440.231	22
10	K(Me2)SAPATGGVK(Me3)K(un)PHR heavy	5	55	Positive	421.997	539.33	22
11	K(Me3)SAPATGGVK(Me2)K(un)PHR heavy	5	55	Positive	421.999	454.247	22
12	K(Me3)SAPATGGVK(Me2)K(un)PHR heavy	5	55	Positive	421.999	532.322	22
13	TK(Me2)QTAR light	5	55	Positive	426.241	475.262	28
14	TK(Me2)QTAR light	5	55	Positive	426.241	631.388	28
15	TK(Me2)QTAR heavy	5	55	Positive	429.236	475.262	28
16	TK(Me2)QTAR heavy	5	55	Positive	429.236	631.388	28
17	TK(Ac)QTAR light	5	55	Positive	433.23	475.262	23
18	TK(Ac)QTAR light	5	55	Positive	433.23	645.367	23
19	TK(Me3)QTAR light	5	55	Positive	433.249	373.729	28
20	TK(Me3)QTAR light	5	55	Positive	433.249	620.341	28
21	TK(Ac)QTAR heavy	5	55	Positive	436.226	475.262	23
22	TK(Ac)QTAR heavy	5	55	Positive	436.226	645.367	23
23	TK(Me3)QTAR heavy	5	55	Positive	436.244	373.73	28
24	TK(Me3)QTAR heavy	5	55	Positive	436.244	626.36	28

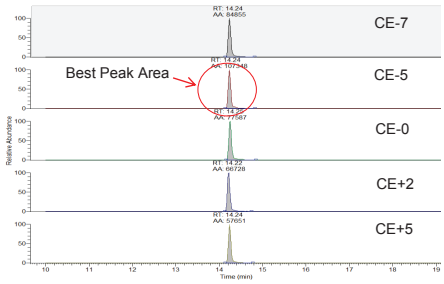
Figure 2. Base Peak Chromatogram of 60 min "Unscheduled" Analysis of 186 SRMs



LC Gradient

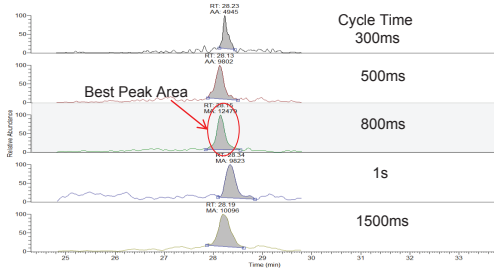
Time (min)	Flow (nl/min)	B%
0	700	0
10	700	0
11	400	0
12	400	4
47	400	25
48	400	50
49	700	80
55	700	80
56	700	0
60	700	0

Figure 3. Optimization of Collision Energy (CE) for Heavy Peptide K(Me3)SAPATGGVK(Me2)K(un)PHR



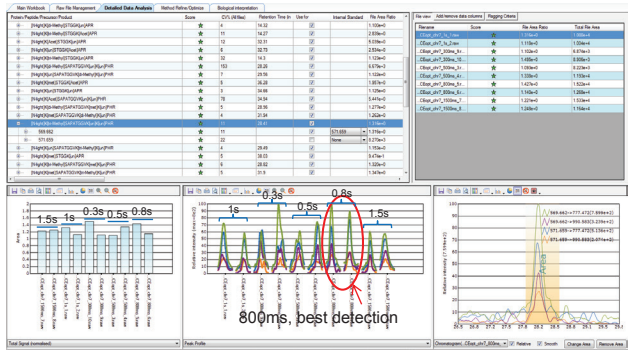
421.999->454.247
421.999->532.322
$CE_{predicted}$= 27
$Ce_{opt.}$= 22

Figure 4. Cycle Time Optimization of Low Abundant Peptide [N-light]K[tri-Methyl]SAPATGGVK[un]K[un]PHR using Best Peak Area



569.662->777.472
569.662->990.583
Cycle Time Opt.=800ms

Figure 5. Pinpoint Comparison of Histone Peptide Detection at Different Cycle Time



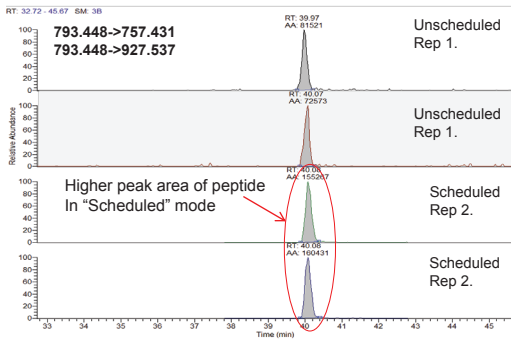
Quantitation using "Scheduled" Mode

A "Scheduled" mode was then developed to improve sensitivity. Eight time segments in a 60-minute program were created, with less than 30 SRMs in each time segment. Quantitation of the 46 histone peptides via the Pinpoint program found high reproducibility, with CV <15% (n=4). To achieve higher throughput , a 35min program in "Scheduled" mode was developed.

Table 3. Example of SRM List in "Scheduled" Mode

	Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)
1	TK(Me3)QTAR light	11.7	16.7	Positive	433.249	373.729	28
2	TK(Me3)QTAR light	11.7	16.7	Positive	433.249	620.341	28
3	TK(Me3)QTAR heavy	11.7	16.7	Positive	436.244	373.73	28
4	TK(Me3)QTAR heavy	11.7	16.7	Positive	436.244	626.36	28
5	K(Me2)SAPATGGVK(Me2)K(un)PHR light	11.8	16.8	Positive	416.996	434.24	24
6	K(Me2)SAPATGGVK(Me2)K(un)PHR light	11.8	16.8	Positive	416.996	532.322	24
7	K(Me2)SAPATGGVK(Me2)K(un)PHR heavy	11.8	16.8	Positive	418.493	440.231	24
8	K(Me2)SAPATGGVK(Me2)K(un)PHR heavy	11.8	16.8	Positive	418.493	532.322	24
9	K(Me2)SAPATGGVK(Me3)K(un)PHR light	11.8	16.8	Positive	420.499	434.24	22
10	K(Me2)SAPATGGVK(Me3)K(un)PHR light	11.8	16.8	Positive	420.499	539.33	22
11	K(Me3)SAPATGGVK(Me2)K(un)PHR light	11.8	16.8	Positive	420.501	448.256	22
12	K(Me3)SAPATGGVK(Me2)K(un)PHR light	11.8	16.8	Positive	420.501	532.322	22
13	K(Me2)SAPATGGVK(Me3)K(un)PHR heavy	11.8	16.8	Positive	421.997	440.231	22
14	K(Me2)SAPATGGVK(Me3)K(un)PHR heavy	11.8	16.8	Positive	421.997	539.33	22
15	K(Me3)SAPATGGVK(Me2)K(un)PHR heavy	11.8	16.8	Positive	421.999	454.247	22
16	K(Me3)SAPATGGVK(Me2)K(un)PHR heavy	11.8	16.8	Positive	421.999	532.322	22

Figure 6. Detection Comparison of Peptide "GK[un]GGK[un]GLGK[Acet]GGAK[Acet]R Using "Scheduled" vs "Unscheduled" Mode

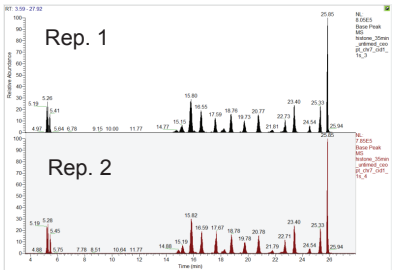


The cycle time for “unscheduled" mode was 800ms for all 186 transitions. The cycle time for “Scheduled” mode was also 800ms for less than 30 transitions in each of the eight time segments. The detection sensitivity of “scheduled” mode was higher, because MS now spent longer time on each transition. In addition, quantitation of the 46 histone peptides by “scheduled” mode found higher reproducibility with tighter CV (CV<15%, n=4, Figure 7.). However, “Unscheduled” mode still achieved reasonable sensitivity performance for all transitions and served as a quick and straightforward starting point for method optimization.

Histone Peptide Quantitation using 35 min HPLC run

A even higher throughput was achieved using a 35min program in "Scheduled" mode, maintaining sensitivity and quantitative performance. Figure 7. shows the base peak chromatogram of a 35min run for “Scheduled” quantitation of 46 histone peptides using 186 SRMs using also eight time segments. With shorter gradient, all the 186 transitions were still detected with good sensitivity and reproducibility (CV<15%, n=3). For example, Pinpoint analysis found that in 35min runs (Figure 9.) the histone peptide “ [N-light]K[di-methyl]STGGK[un]APR was well detected compared to 60min runs (Figure 8.).

Figure 7. Base Peak Chromatogram of 35 min “Scheduled” Analysis of 186 SRMs



LC Gradient

Time (min)	Flow (nl/min)	B%
0	700	0
1	700	0
2	400	0
3	400	4
23	400	25
24	400	50
25	700	80
30	700	80
31	700	0
35	700	0

Figure 8. Pinpoint Analysis of Histone Peptide Detection at “Scheduled” Mode at 60min Run Found Higher Reproducibility with Tighter CV

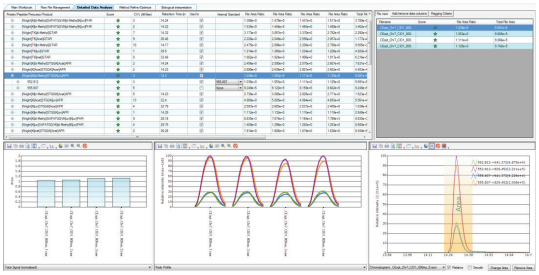
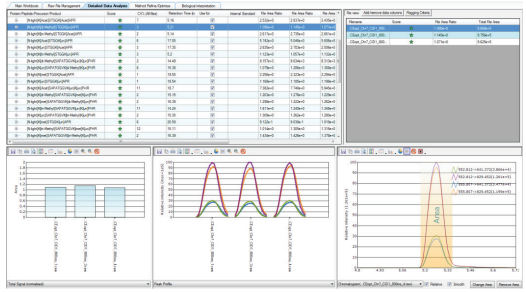


Figure 9. Pinpoint Analysis of Histone Peptide Detection at “Scheduled” Mode at 35min Run



Conclusion

We developed a workflow for sensitive and high throughput quantitation of 46 modified histone peptides through 186 transitions in 60min runs using nano-HPLC couple with triple quadrupole MS. The high scanning speed of TSQ Quantiva triple quadrupole MS allows MS detection sensitivity optimization through “Unscheduled” mode. Further improvement of detection sensitivity was achieved using “Scheduled” time segments. In addition, we demonstrated quantitation of the 46 histone peptides using 35min runs with better throughput.

The nano-HPLC and TSQ Quantiva MS platform allowed histone peptides with abundances spanning three orders of magnitude to be quantified in a single experiment, separating closely related peptides with identical precursor masses. The simplicity and convenience of this workflow on the TSQ Quantiva MS allows triple quadrupole mass spectrometry to contribute powerfully to epigenetic research programs.

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