Quantitation of Protein Phosphorylation Using Multiple Reaction Monitoring

Application Note

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Abstract

This application note demonstrates quantitation of percentage of phosphorylation of two sites on a single peptide using the HPLC-Chip/QQQ system. ^{13}C-labeled peptides from the extracellular signal-regulated kinase (ERK) were synthesized and MRM assays were developed to quantify each peptide. The standard curve of each ^{13}C-labeled peptide was established. The amount of each phosphopeptide from active ERK spiked into human plasma was measured and calculated in reference to the ^{13}C-labeled standards.

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Introduction

Peptide quantitation using multiple-reaction monitoring (MRM) has emerged as an important methodology for biomarker validation. MRM on a triple quadruple mass spectrometer provides superior sensitivity and selectivity for targeted compounds in a complex sample. MRM also offers high precision in quantitation and fast scan speed, which makes it an ideal technology for validating biomarkers in a high-throughput fashion.

One area of great interest is the quantitation of protein phosphorylation. Reversible protein phosphorylation plays an important role in cell signaling pathways and the percentage of phosphorylation is often very important to signal transduction. The ERK pathway is essential to transmitting signals from many extracellular agents to regulate cellular processes such as proliferation, differentiation and cell cycle progression. Signaling via the ERK cascade is mediated by sequential phosphorylation and activation of protein kinases in the different tiers of the cascade. The recombinant human ERK1 containing an N-terminal GST tag was expressed in *E. coli* and activated with MEK1.

Up to 7 phosphorylation sites were identified from the activated ERK1. The two key regulatory phosphorylation sites have been identified as the neighboring t202 and y204.[1] Phosphorylation of these sites, located upstream of the conserved protein kinase subdomain VIII region implicated in substrate binding, might function by relieving inhibition of substrate access to the active sites of the kinase domain. The peptides that contain either no phosphorylation site, a single phosphorylation site or both phosphorylation sites have been identified. Hence, the degree of phosphorylation of these two sites is very important to the overall function of ERK1 in the signal transduction pathway. Therefore, in this study, we developed an MRM-based assay to quantify the percentage of phosphorylation by reference to ^{13}C-labeled peptides.

Figure 1. HPLC-Chip/QQQ system for protein quantitation.

Experimental

Sample Preparation

Four peptides labeled at the valine residue with ^{13}C were purchased. The 4 peptides were as follows: 1) without phosphorylation, 2) with single phosphorylation at t202 or 3) with single phosphorylation at y204 and 4) double phosphorylation at both t202 and y204.

Active MAP kinase ERK1 was purchased from Millipore (Temecula, CA). It was denatured, reduced, alkylated and digested using trypsin. ^{13}C-labeled peptide standards were purchased from Cell Signaling Technology (Danvers, MA). The peptides were labeled at the valine residue. Human plasma was purchased from Sigma (St. Louis, MO). The plasma was depleted of 6 highly abundant proteins using an Hu-6 immunoaffinity column (Agilent) following the standard protocol. After depletion, the sample was buffer-exchanged into an ammonium bicarbonate solution, then reduced, alkylated (IAA) and digested with trypsin under denaturing conditions. Internal calibration curves were acquired using different amounts of ^{13}C-labeled peptides spiked into 1 µg of human plasma digest. ERK digest (35 fmol/µg plasma protein) was spiked into the samples as the internal standard.

LC/MS Analysis

An Agilent 6410 Triple Quadrupole (QQQ) LC/MS was connected to the HPLC-Chip interface (see **Figure 1** above).

HPLC-Chip Conditions:
Protein ID chip with 150 x 0.075 mm analytical column and 160 nL enrichment column.

Sample load: 1 µg of human plasma digest spiked with different amounts of standard protein digests

Injection volume: 1 µL

Flow: 300 nL/min analytical pump, 3 µL/min loading pump

Mobile phases A: 0.1% formic acid (FA) in water, B: 90% acetonitrile, 0.1% FA

Gradients: 2% B to 42% B at 20 min, 90% B at 23–25 min, then 2% B at 25.1 min

Stop time: 35 min

Triple Quadrupole MS Conditions:
Drying gas: 6 L/min, 325°C

Fragmentor: 100 V

Collision energy: optimized for each transition

Dwell time: 70 ms

Delta EMV: 400 V

Results and Discussion

Previous work[2] demonstrated that peptide quantitation using the HPLC-Chip Agilent 6410 Triple Quadrupole LC/MS can be achieved down to 10 amol. In this study, we took on the challenge of quantitating the degree of phosphorylation of two sites on a peptide from ERK protein (amino acid residues 190-208).

The sequence and the product ions selected for monitoring the synthetic peptides in the MRM assay are illustrated in **Figure 2** and **Table 1**. The selection of the transitions was critical because of the similarity of the sequences.

Selection of Transitions for MRM Assay

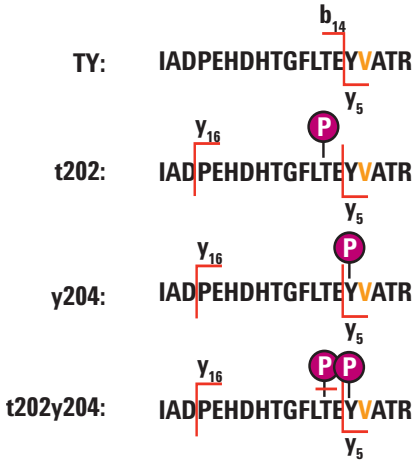

Figure 2. Sequence and product ions selected for monitoring the four ^{13}C-labeled peptides. Each peptide was named by the phosphorylation state at amino acids 202 and 204. Phosphotyrosine was labeled as lower case y and phosphothreonine was labeled as lower case t. V represents ^{13}C-labeled Valine.

Chromatographic Separation of the 4 Peptide Standards

The transition from 753.3 –> 979.9 is the same for both the t202 and y204 peptide, thus it was important to have complete chromatographic separation. The four ^{13}C-labeled peptide standards were mixed at equal molar ratios and 100 fmol was injected on the HPLC-Chip. A gradient method was developed to separate the 4 peptides (**Figure 3**).

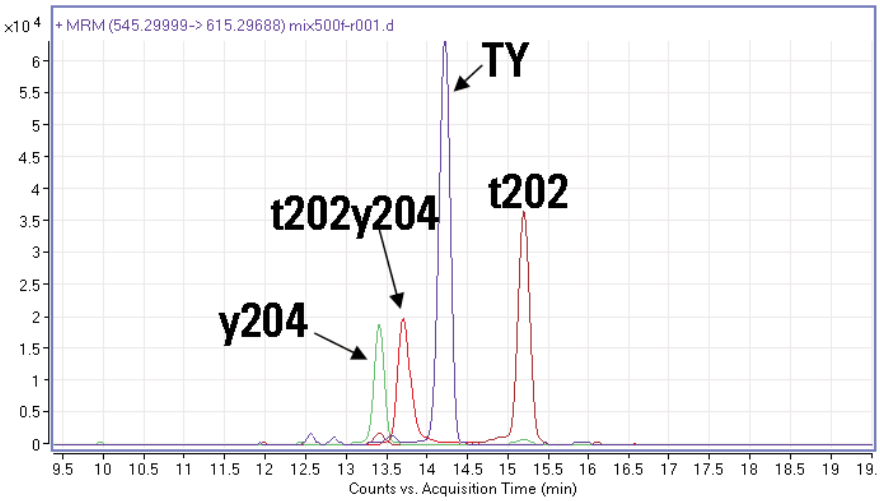

Figure 3. MRM chromatogram of four ^{13}C-labeled peptides showed complete chromatographic separation.

	Precursor ion	Product ions	
TY	545.3 $[M+3H]^{3+}$	615.3 y_5	782.5 b_{14}^{2+}
t202	**753.3** $[M+2H]^{2+}$	615.3 y_5	**979.9** y_{16}^{2+}
y204	**753.3** $[M+2H]^{2+}$	**979.9** y_{16}^{2+}	695.3 y_5
t202y204	780.0 $[M+2H]^{2+}$	647.6 y_{16}^{3+}-H_3PO_4	695.3 y_5

Table 1. The charge states, type of fragment and m/z values of the precursor ions and product ions monitored for each ^{13}C-labeled peptide standard. Bold indicates transition shared between two peptides.

QC of incoming phosphopeptide standards

We also performed quality control of the incoming ^{13}C-labeled peptides using the MRM assays developed. The four ^{13}C-labeled peptide standards were analyzed individually (500 fmol on-column). The non-phosphorylated peptide (TY) was pure and does not contain phosphorylation residues (**Figure 4**). The two singly phosphorylated peptides (t202 and y204) contained a small percentage of non-phosphorylated peptide (TY). The doubly phosphorylated peptide (t202y204) contained both the singly phosphorylated peptide (t202) and the non-phosphorylated peptide (TY). However, overall the percentage of impurities was relatively low (**Table 2**). The purity of each synthesized peptide was above 97.6%.

Quantitation Curves for the Four ^{13}C-labeled Peptide Standards

For each LC/MS analysis, 1 µg of human plasma digest was injected. The four calibration curves all had excellent linearity with correlation coefficients better than 0.995 (Figure 3). Calibration curves were acquired using human plasma digest spiked with both ^{13}C-labeled peptides and ERK1 tryptic digest. Limit of quantitation (LOQ) was determined to be 500 amol and limit of detection (LOD) was 50 amol.

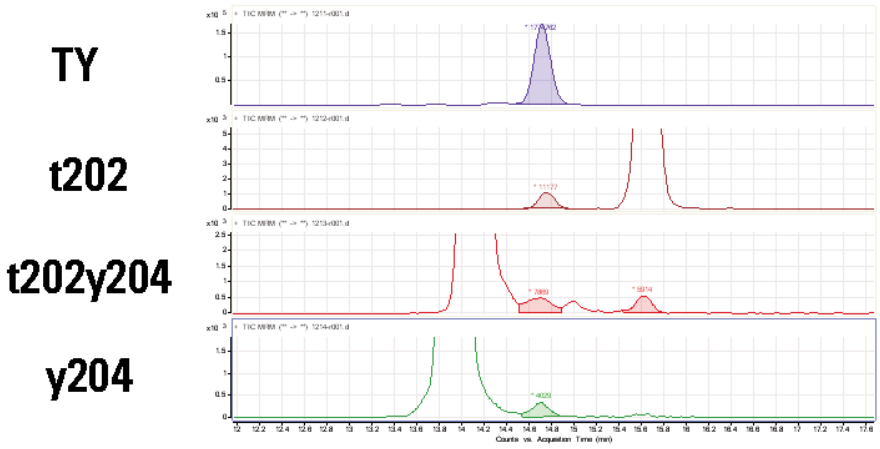

Figure 4. The four ^{13}C-labeled peptide standards were injected individually at 500 fmol on-column. Trace amounts of incomplete phosphorylation were observed. The percentage of impurities for each synthetic peptide was analyzed by MRM and calculated in Table 2 below.

Peptides	Percentage of components			
	TY	t202	y204	t202y204
TY	100%	–	–	–
t202	0.6%	99.4%	–	–
y204	0.2%	–	99.8%	–
t202y204	0.4%	2%	–	97.6%

Table 2. The percentage of each component in the synthetic peptides was calculated by the ratio of MRM peak area. All four peptides are greater than 97% pure.

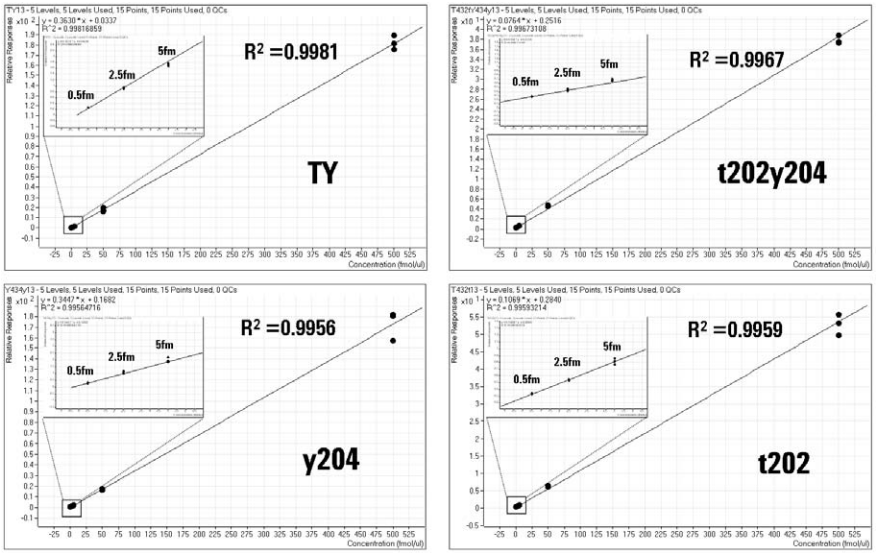

Figure 5. The calibration curves of the four ^{13}C-labeled peptides spiked into human plasma.

The Degree of Phosphorylation at t202 and y204 in Active ERK1 Protein

The degree of phosphorylation can be measured by the relative percentage of each peptide as shown in Figure 2. The ratio between the abundance of unlabeled peptide from ERK tryptic digest and the labeled peptide in each sample provided the absolute amount of unlabeled peptide. The percentage for each phosphorylation form was derived from these results and listed in Table 3 below. The relative standard deviation was below 15%.

	% Molar ratio	RSD (n=9)
TY	20%	0.13
t202	25%	0.15
y204	21%	0.12
t202y204	34%	0.08

Table 3. Relative amount of four different peptides detected in active ERK1. RSDs of all measurements are below 15%.

Conclusions

- An MRM-based method to quantitate the different phosphorylation states on a single peptide was developed. A critical part of the method development was the chromatographic separation of the target peptides.
- Because of the sequence similarity between these peptides, the selection of transitions was critical. Chromatographic separation of the peptides allowed the same transition to be used for different peptides.
- The microfluidic-based HPLC-Chip has been demonstrated to be sensitive, reproducible and capable of fast separation, especially in combination with a triple quadrupole MS/MS.

References

1. D. L. Charest, G. Mordret, K. W. Harder, F. Jirik, S. L. Pelech, "Molecular cloning, expression, and characterization of the human mitogen-activated protein kinase p44erk1," *Mol. Cell. Biol.* 13:4679-4690, **1993**.
2. N. Tang, C. Miller, K. Waddell, "Approaches for the Confirmation of Putative Biomarkers Using Multiple Reaction Monitoring," HUPO Conference Proceedings, **2007**.

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