Targeted Quantitative Proteomics Using Selected Reaction Monitoring (SRM)-Mass Spectrometry Coupled with an ^{18}O-labeled Reference as Internal Standards

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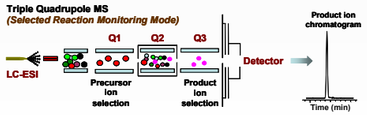
Overview

- To address the need of stable isotope labeled internal standards for accurate quantification, we introduce the use of an ^{18}O-labeled reference as comprehensive internal standards for accurate SRM-MS-based quantification instead of using labeled synthetic peptides.
- ^{18}O-labeling efficiency for most peptides is sufficiently high to provide a residue of $^{16}O/^{18}O$ ratio of <1%.
- A linear dynamic range of quantification ~10^4 in relative concentration was obtained for most of the peptides being monitored based on the observed peak area ratios ($^{16}O/^{18}O$) versus concentration ratios for each peptide.
- $^{16}O/^{18}O$ based quantification also provided significantly better reproducibility in terms of the coefficient of variances among the triplicate analyses compared to label-free quantification.

Introduction

- Selected reaction monitoring (SRM)-MS coupled with stable isotope dilution is becoming a powerful tool for targeted quantitative proteomics, e.g., biomarker verification.
- Selective and sensitive detection of specific peptides are achieved by selecting a specific parent ion and monitoring specific fragment ions (See Fig.1).



- To achieve accurate quantification, isotope labeled synthetic peptides are often used as internal standards, which limits the feasibility to monitor a large number of candidates due to expense.
- The ^{18}O-labeled "universal" reference will serve as ideal internal standards for quantitative monitoring for any candidates of interest.

Methods

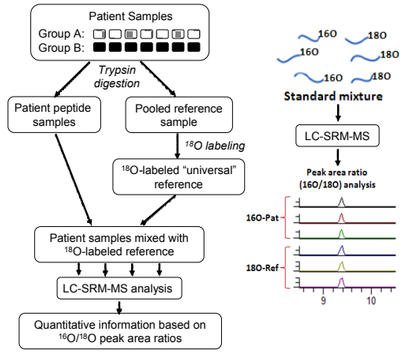


Fig. 1 SRM quantification strategy using ^{18}O-labeled "universal" reference.[1]

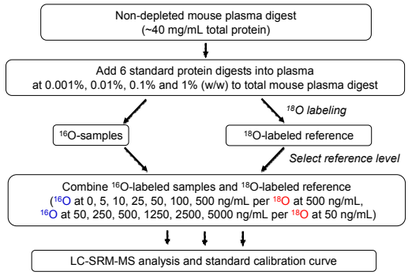


Fig. 2 Flow chart for preparing the non-depleted mouse plasma samples with 6 spiked standard protein digests for calibration curve.

- Bovine carbonic anhydrase, beta-lactoglobulin, cytochrome *c*, chicken ovalbumin, equine skeletal muscle myoglobin and E.coli beta-galactosidase were used as 6 standard proteins.
- C-reactive protein (CRP) and prostate specific antigen (PSA) were spiked in human female plasma (non-depleted) for absolute quantification using standard addition.
- ^{18}O labeling was performed with the recently published protocol to completely prevent ^{18}O back-exchange.[2]
- LC: Agilent 1100; 75 μm x 25 cm column (3 μm; C18); 60-min gradient; 400 nL/min.
- MS: Thermo Scientific TSQ vantage.
- SRM: 10 ms dwell time; 0.002 Da scan width; unit resolution in Q1 and Q3 (0.7 FWHM).
- Pinpoint software (Thermo Fisher Scientific) was used for data analysis.

Results

^{18}O labeling efficiency

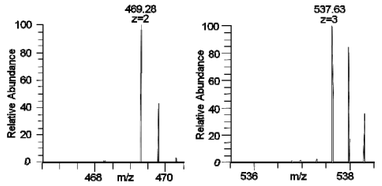


Fig. 3 LC-LTQ-Orbitrap MS analysis of ^{18}O labeled peptides.

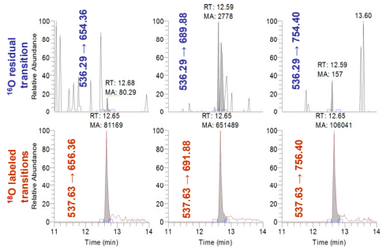


Fig. 4 LC-TSQ-SRM chromatogram showing ^{16}O residual (top) and ^{18}O labeled (bottom) transitions of VDEDQPFPAVPK(3+) peptide after ^{18}O labeling.

Table 1. Percentage of ^{16}O residual (unlabeled) peptide in an ^{18}O-labeled reference samples determined by SRM mode

Protein	Sequence	% of ^{16}O residual peptides*	% CV (triplicate analyses)
Carbonic anhydrase	DGPLTGTYR	0.35	10.7
Carbonic anhydrase	DFPIANGER	0.50	27.8
Carbonic anhydrase	EPISVSSQQMLK	0.73	33.5
Carbonic anhydrase	AVVQDPALKPLALVYGEATSR	0.15	10.1
β-Lactoglobulin	LIVTQTMK	0.29	35.8
β-Lactoglobulin	VLVLDTDYKK	0.06	4.2
β-Lactoglobulin	TPEVDDEALEK	2.27	13.2
β-Lactoglobulin	VYVEELKPTPEGDLEILLQK	0.20	13.0
Cytochrome *c*	EDLIAYLK	0.09	37.7
Cytochrome *c*	TGPNLHGLFGR	0.93	17.6
Myoglobin	LFTGHPETLEK	0.57	14.0
Myoglobin	VEADIAGHGQEVLIR	0.48	3.0
Ovalbumin	AFKDEDTQAMPFR	0.05	21.6
Ovalbumin	GGLEPINFQTAADQAR	0.25	34.5
β-Galactosidase	VDEDQPFPAVPK	0.35	32.8
β-Galactosidase	LWSAEIPNLYR	0.11	6.9

* An average of ~0.5% $^{16}O/^{18}O$ ratio indicates the high percentage of heavy label incorporation, a necessity for accurate quantification.

Effect of Q1 resolution

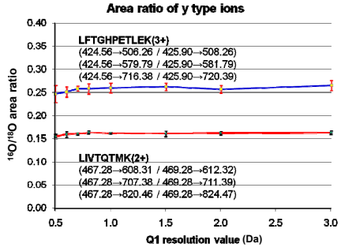


Fig. 5 Effect of Q1 window width on y type ions. ^{16}O / ^{18}O y ion pair without interference under wide Q1 window.

Linear dynamic range of ^{18}O labeled reference–based quantification

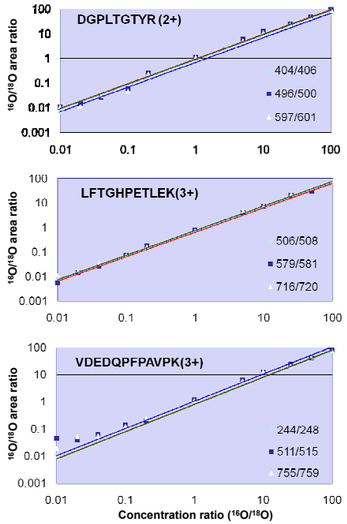
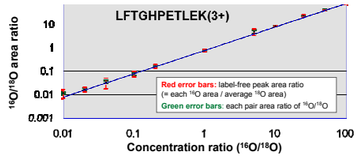


Fig. 6 Calibration curve showing linear dynamic range of quantification ~10^4 in relative concentration.

Reproducibility



Concen. Ratio	0.01	0.02	0.04	0.1	0.2	1	5	10	25	50	100
$^{16}O/^{18}O$ pairs	6.1	12.5	13.7	13.8	5.5	7.7	16.8	2.8	4.6	4.2	1.6
Label free (%)	39.2	23.4	47.5	26.5	19.3	10.6	31.2	3.2	10.7	4.8	3.5

Fig. 7 Comparison of reproducibility (% CV in table) between $^{16}O/^{18}O$ pair and label free ($^{16}O/^{18}O_{avg}$) approach.

Absolute quantification by standard addition

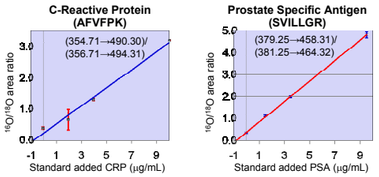


Fig. 8 Standard addition calibration curve for absolute quantification of endogenous CRP and spiked PSA in non-depleted human female plasma.

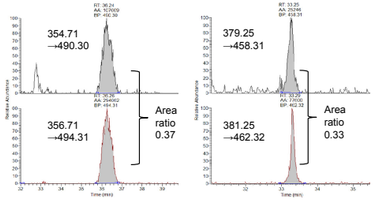


Fig. 9 XIC of $^{16}O/^{18}O$ transition pair for endogenous CRP (left) and spiked PSA (right) at 500 ng/mL in non-depleted human female plasma.

Table 2. SRM Quantification results of endogenous CRP and spiked PSA (500 ng/mL) in non-depleted human female plasma by standard addition method.

	(354.71→ 490.30) / (356.71→494.31)	
CRP (ng/mL) (endogenous)	*concentration*	*CV*
	790 ng/mL	4.1%
	(379.25→458.31) / (381.25→464.32)	
PSA (ng/mL) (spiked)	*concentration*	*CV*
	800 ng/mL	0.8%

Conclusions

- The utility of ^{18}O-labeled "universal" reference as internal standards for targeted quantitative proteomics has been successfully demonstrated
  - A linear dynamic range of quantification ~ 10^4 in relative concentration
  - Better reproducibility than label-free approach
  - Sufficient effectiveness for monitoring ^{16}O and ^{18}O y-fragments at unit or even higher resolution
- Endogenous concentration of CRP and spiked PSA in human plasma successfully determined by "standard protein addition/^{18}O-labeled reference"

Acknowledgements

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References

1. Qian W-J, et al. Large-Scale Multiplexed Quantitative Discovery Proteomics Enabled by the Use of an ^{18}O-Labeled "Universal" Reference Sample. *J Proteome Res* 8:290-299 (2009).
2. Petritis BO et al. A Simple Procedure for Effective Quenching of Trypsin Activity and Prevention of ^{18}O-Labeling Back-Exchange. *J Proteome Res* 8:2157-2163 (2009).

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