A simplified approach to fast and accurate, high throughput targeted MS^2 quantitation using internal standard

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ABSTRACT

Purpose: To develop a simplified, retention time (RT) independent approach for fast and accurate, high-throughput targeted MS^2 quantitation.

Methods: A QuanDirect™ method was developed for the analysis of complex, peptide-containing samples, wherein the sample is spiked with heavy labeled versions of the endogenous peptide of interest.

Results: An RT independent method has been developed that obtains improved LOQs, and is able to target a large number of precursors in complex mixture, all while being easy to set up and execute.

INTRODUCTION

Quantifying peptides in a proteomics sample is often performed using selected/multiple reaction monitoring (SRM/MRM) on a triple quadrupole MS and parallel reaction monitoring (PRM, or targeted MS/MS) on an Orbitrap-based MS. These techniques are far more sensitive and reproducible than standard data-dependent analysis; however, the breadth of these targeted approaches are limited and the methods themselves can be hard to develop and maintain. This is particularly true for methods requiring complex retention time scheduling, which is highly reliant on reproducible chromatography and often requires constant optimization.

As an alternative to these traditional approaches, two labs have published workflows that attempt to realize the depth and reproducibility of the targeted workflow, while still leveraging the ease of use of the data-dependent approach (1,2). These workflows begin with the addition of heavy peptide standards to the analytical sample, with sequences that are analogous to endogenous peptides of interest. By incorporating specific heavy isotopes, these standards differ in mass from the endogenous forms of the peptides; however, their retention times match the endogenous peptides exactly. In the published workflows, the mass spectrometer method includes low-quality targeted scans on the spiked-in standards. Following acquisition of the targeted MS/MS transitions/spectra, the data is analyzed, and if certain conditions are met, such as specific peaks are detected or a library match is achieved, the instrument triggers a high-quality MS/MS analysis on the expected location of the endogenous form of the peptide.

These workflows have successfully addressed some of the limitations and disadvantages of the established data-dependent and targeted methods; however, both workflows rely on retention time scheduling to achieve acceptable duty cycle and selectivity. This scheduling increases method setup complexity and can compromise robustness, particularly where run-to-run variation of chromatographic separation exits.

Here we present a novel and simplified approach to fast and accurate targeted MS/MS quantitation using internal standards on an Orbitrap Fusion Lumos MS system.

MATERIALS AND METHODS

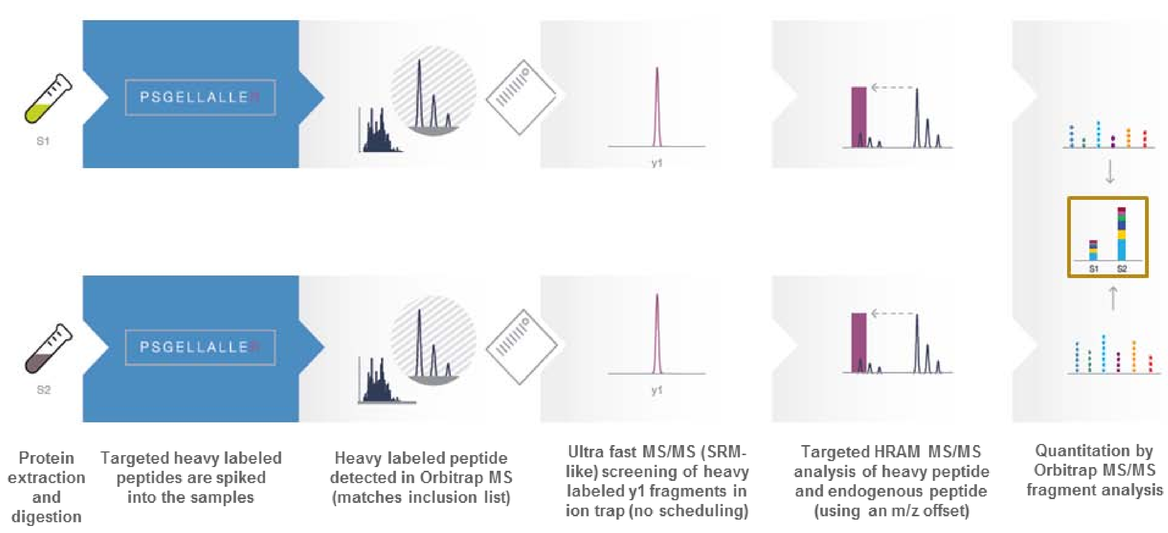

Figure 1. The QuanDirect experimental overview

Sample Preparation

All of our experiments were based around hybrid peptide samples which consisted of mixtures of unlabeled endogenous peptides combined with heavy internal standards (labeled at the C-termini using heavy forms of the K and R amino acids).

To determine LOQ, we analyzed a tryptic HeLa digest (200 ng, Pierce, Rockford, IL) spiked with 250 fmol *Heavy*PRTC kit trigger peptides (Pierce, Rockford, IL). *Light* PRTC peptides were spiked at 10 amol – 100 fmol

To test the method with a large number of targets in a complex mixture, we analyzed a non-small-cell lung cancer cell line digest (1ug, provided by Bruno Domon) spiked with 404 synthesized heavy peptides (each peptide with a fixed concentration between 200 and 400 fmol).

Liquid Chromatography

We separated the mixture with an Easy-nLC 1000 ultra-high pressure LC on a Thermo Scientific™ EASY-Spray™ C18 column (75 µm x 50 cm). Solvent A consisted of 0.1% formic acid in H_2O, and B consisted of 0.1% formic acid in 100% acetonitrile. Peptides were eluted over 40 minutes or 80 minutes at a flow rate of 300 nL/min. The linear gradient for 40 minutes was 5% to 25% B in 20 minutes, followed by 25% to 40% in 8 minutes, and 40% to 95% in another 1 minute. We finished by holding at 95% B for 8 minutes. The linear gradient for 80 minutes was 5% to 35% B in 69 minutes, followed by 35% to 95% in 5 minutes, and holding at 95% B for 11 minutes.

Mass Spectrometry

The peptides were interrogated with an Orbitrap Fusion™ Lumos mass spectrometer. We collected sets of 3 replicates on three separate MS systems. The inclusion list-driven, hybrid data-dependent/targeted LC-MS/MS method (QuanDirect) was developed in the Orbitrap Fusion Series Instrument Control Software (ver. 2.1) (Fig. 2 and 3). The PRM method used for LOQ comparison entailed Orbitrap MS/MS with the same settings as the QuanDirect method. The PRM method also used retention time windows that were 1 minute wide. The Orbitrap MS Full scan was collected with a scan range of 325-1600 *m/z*, 120K resolution, AGC target 2E5, maximum injection time (max IT) of 50 ms, and RF Lens set to 30%. Targeted precursors were interrogated for a maximum 3 second cycle. The target list consists of the *m/z* ratios and charge states of the heavy peptides (Table 1) only (*no retention time info is required)*. To trigger a data-dependent scan, the precursor must match the expected charge state and the *m/z* within 20 ppm. These precursors were interrogated with a short SRM scan (MS^2 IT HCD) of the predicted y1 fragment ion region for the expected heavy R (180-190 *m/z*) or heavy K (150-160 *m/z*) peptides. These ultra fast SRM scans were performed using a 10 *m/z* mass range, rapid ion trap scan rate, HCD NCE 40%, 0.7 *m/z* isolation window, AGC target 1E4, and a max IT of 10 ms. The y1 ion in the SRM scan must be above an intensity threshold of 5e2 and within 1 Da of the expected *m/z*. If the expected heavy y1 fragment ion is detected in the SRM scan, 185.1 (heavy R) or 155.1 (heavy K), then full HRAM HCD MS/MS scans are triggered on the spiked-in heavy peptide as well as the endogenous form (Table 1). These scans use the following parameters: scan range 150-1500 *m/z*, 60K resolution, 35% NCE HCD, AGC target 2e5, and max IT of 118 ms. To trigger on the endogenous peptide, an *m/z* Offset of -5 or -4 u was used for the R and K peptides, respectively.

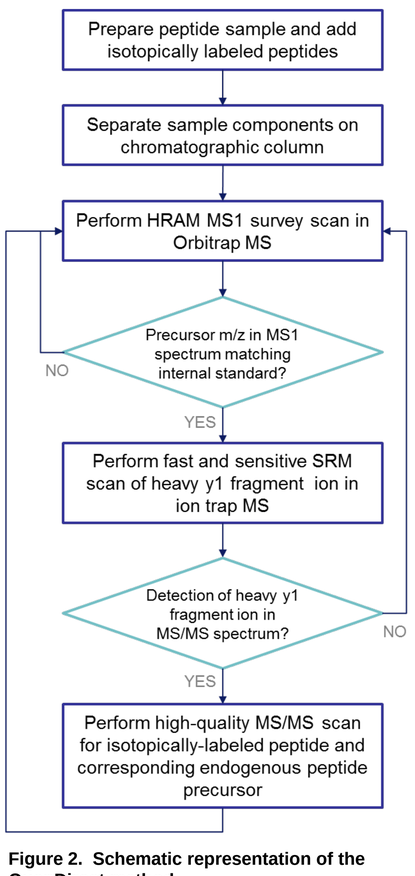

Figure 2. Schematic representation of the QuanDirect method

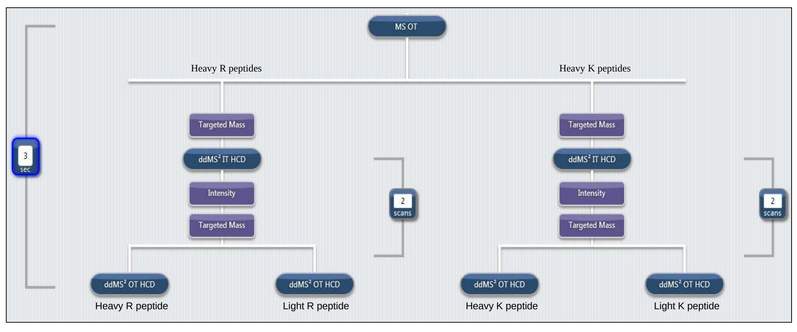

Figure 3. Experiment workflow representation of the QuanDirect method in the Method Editor software.

Table 1. PRTC targeted mass list with expected y1 fragment ion trigger *m/z*

Peptide #	Sequence	Charge	Precursor m/z	Product (y1) m/z
1	SSAAPPPPPR*	+2	493.7683	185.1 (heavy arginine)
2	GISNEGQNASIK*	+2	613.3167	155.1 (heavy lysine)
3	HVLTSIGEK*	+2	499.2867	155.1 (heavy lysine)
4	DIPVPKPK*	+2	451.2834	155.1 (heavy lysine)
5	IGDYAGIK*	+2	422.7363	155.1 (heavy lysine)
6	TASEFDSAIAQDK*	+2	695.8324	155.1 (heavy lysine)
7	SAAGAFGPELSR*	+2	586.8003	185.1 (heavy arginine)
8	ELGQSGVDTYLQTK*	+2	773.8955	155.1 (heavy lysine)
9	GLILVGGYGTR*	+2	558.3259	185.1 (heavy arginine)
10	GILFVGSGVSGGEEGAR*	+2	801.4115	185.1 (heavy arginine)
11	SFANQPLEVVYSK*	+2	745.3924	155.1 (heavy lysine)
12	LTILEELR*	+2	498.8018	185.1 (heavy arginine)
13	NGFILDGFPR*	+2	573.3025	185.1 (heavy arginine)
14	ELASGLSFPVGFK*	+2	680.3735	155.1 (heavy lysine)
15	LSSEAPALFQFDLK*	+2	787.4212	155.1 (heavy lysine)

Data Analysis

All data were analyzed with Skyline (MacCoss lab, University of Washington), and with an integrated data analysis environment that is based in Lua and that was written in-house.

RESULTS

QuanDirect Method is easy to set up and execute

The QuanDirect Method is easy to set up and execute (Figure 1). There is no dependence on reproducible chromatography, because there is no retention time scheduling or correction. As the *m/z* of the heavy y1 fragment ion is known, there are no libraries to create and maintain and no fragment ion spectra to optimize. As a result, the QuanDirect method is system or lab independent and does not require constant optimization/modification.

High selectivity

The QuanDirect method uses an extremely fast and sensitive, data-dependent ion trap SRM scan of a characteristic product ion (heavy y1) – which is derived from the dissociation of a spiked-in, isotopically-labeled, peptide ion – to trigger high resolution, accurate mass (HRAM) MS/MS analysis of the corresponding endogenous peptide. The observation of the heavy y1 ion, paired with the HRAM measurement of the precursor, confidently identifies the presence of the isotopically-labeled peptide (Figure 4). The following HRAM MS/MS spectra are used for highly selective and sensitive MS/MS-based quantitation of the targeted peptides in complex mixtures.

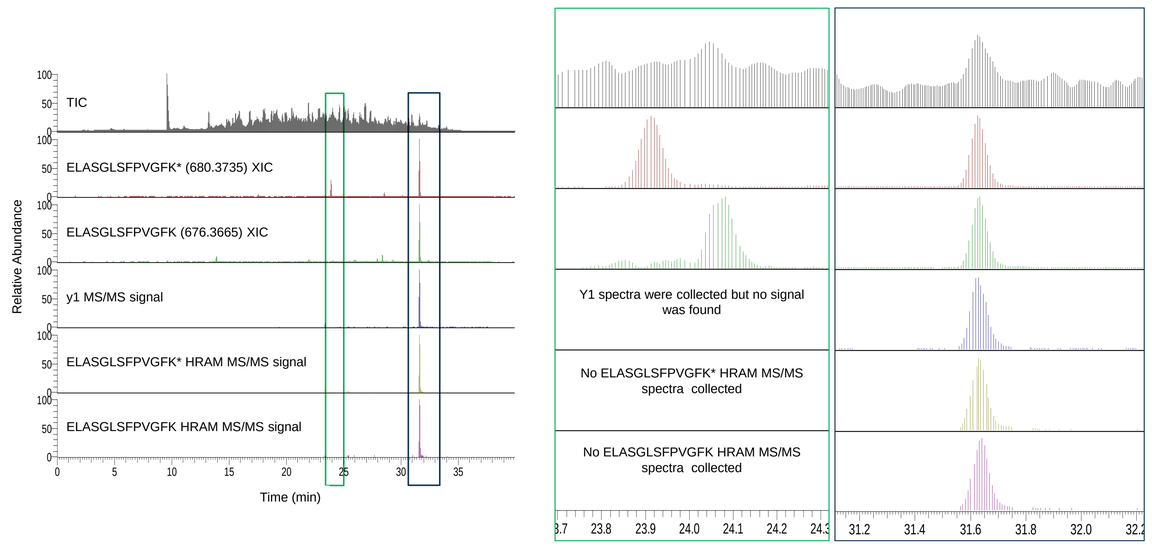

Figure 4. The presence of the heavy y1 ion paired with the accurate mass of the precursor indicates, with a high degree of confidence, the presence of the isotopically-labeled peptide (here an example for ELASGLSFPVGFK*, *m/z* 680.3735 and ELASGLSFPVGFK, *m/z* 676.3665).

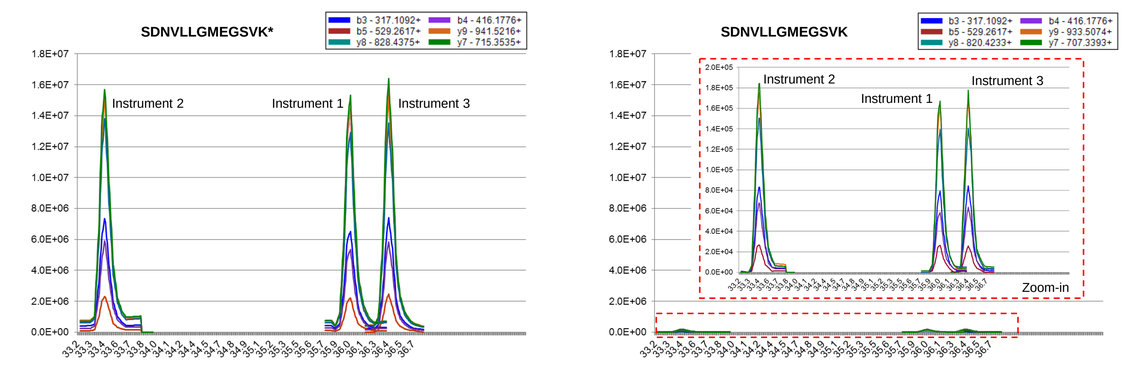

Figure 5. Retention time shifts do not affect quantitation. QuanDirect performance is independent of chromatography changes and no retention time scheduling or correction is required (here an example for SDNVLLGMEGSVK*, *m/z* 678.8496 and SDNVLLGMEGSVK, *m/z* 674.8425, +2).

Improved detection limit

The QuanDirect method achieves very good selectivity while retaining low LOQ, and avoids the need for retention-time scheduling and/or other techniques that complicate method development and render the method vulnerable to problems arising from run-to-run variations (Figures 5 and 6)

The method leverages the unique chemical properties of the heavy peptides and the high-resolution and mass accuracy of the Orbitrap analyzer to perform *on-line*- identification of the spiked-in standards without the need of a spectral library. We have compared the performance of this method to traditional PRM analysis. The QuanDirect method quantified all 15 PRTC peptides at 10 amol LOQ with CVs<10%, when spiked into a 200 ng HeLa digest. Only 12 PRTC peptides were quantified by the standard PRM method due to retention time shift (Figures 6 and 7). On average, the method achieved 11.9 points across the LC peak and ~75% of targets had 8 points across the peak or more.

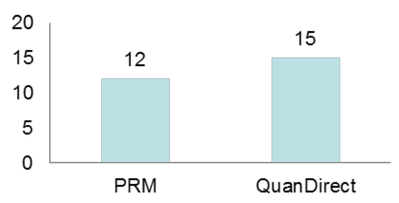

Figure 6. The QuanDirect method can achieve a lower LOQ than standard PRM. The QuanDirect method quantified all 15 PRTC peptides at 10 amol LOQ with CVs,10%, when spiked into a 200 ng HeLa digest. Only 12 PRTC peptides were quantified by the standard PRM method due to retention time shift. The experiments had n=3 and repeated on 3 instruments.

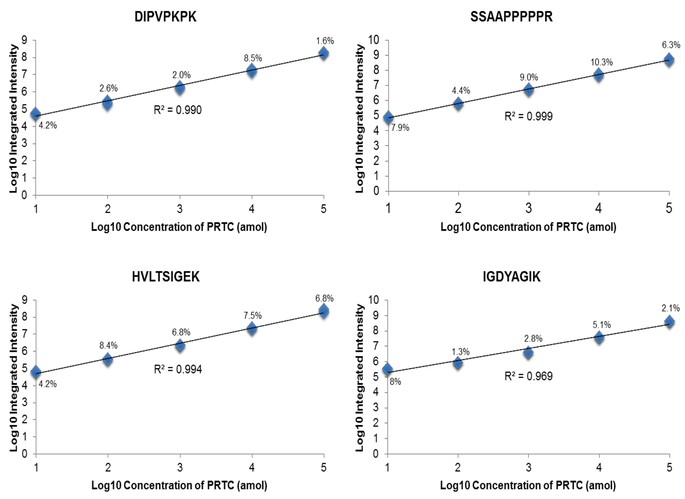

Figure 7. The QuanDirect method achieves high quantitative linearity.

Targeting a Large Number of Peptides in Complex Mixture

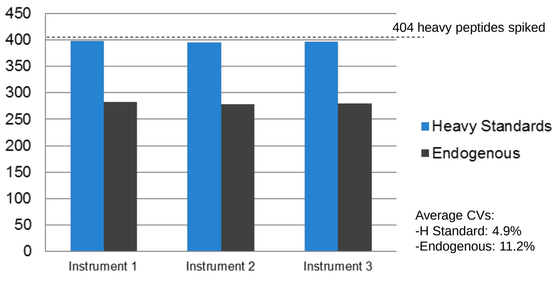

Figure 8. The QuanDirect method provides high-throughput quantitation of endogenous peptides in complex matrices (All peptides quantified at <20% CV, 3 instruments x 3 replicates).

Using a single QuanDirect method, greater than 98% of the 404 heavy peptide standards were quantified in each replicate on three separate systems and greater than 70% of the endogenous peptides were detected and quantified with CVs less than 20% (Figure 8).

CONCLUSIONS

The main advantages of the QuanDirect method are its low LOQs, high selectivity and its ability to target a large number of precursors in an extremely complex mixture, all while being completely independent of LC performance, making the method easy to set up without any pre-optimizations.

REFERENCES

1. Yan, W., Luo J., Robinson M., Eng J., Aebersold R., and Ranish J. (2011) Index-ion triggered MS2 ion quantification: A novel proteomics approach for reproducible detection and quantification of targeted proteins in complex mixtures. Mol. Cell. Proteomics 10
2. Gallien S., Yoon K. S., and Domon B. (2015) Large-scale targeted proteomics using internal standard triggered-parallel reaction monitoring (IS-PRM). Mol. Cell. Proteomics 14, 1630-1644.

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TRADEMARKS/LICENSING