

# Simplifying the Hunt for Optimal SRM Transitions: Utilizing Discovery Data to Expedite Targeted Protein Quantitation Methods

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## Overview

**Purpose:** To demonstrate integrated workflows between discovery/differential expression experiments and targeted protein quantitation utilizing SRM assays on a triple quadrupole mass spectrometer.

**Methods:** Enzymatically digested plasma samples were analyzed using an LTQ Orbitrap XL™ mass spectrometer obtaining peptide identification/sequences using both linear ion trap CID and orbitrap HCD detection. A set of peptides were selected to be analyzed on a triple quadrupole MS using a large number of SRM transitions per peptide. Over 120 peptides were analyzed by each method and separated into groups based on peptide length (+2 charge state precursors) and missed cleavage peptides and +3 charge state. The effectiveness of each experimental and theoretical approach for identifying the optimum SRM transition was based on comparative relative product ion abundances between CID, HCD, SRM assays, as well as rules-based determination.

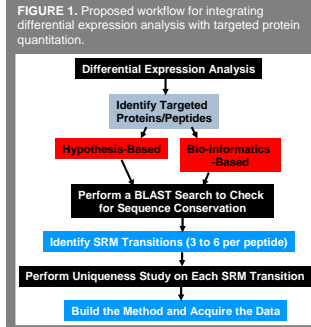
**Results:** The most abundant product ion from the SRM assays matched the most abundant product ion obtained from orbitrap HCD and linear ion trap CID for 70% of the peptides under study compared to only 48% using a rules-based approach. The agreement between experimental methods showed over an 85% agreement when considering the top 2 most abundant product ions.

## Introduction

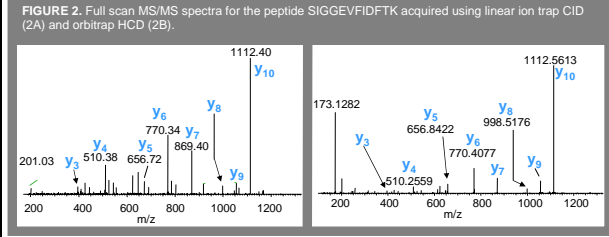
Greater emphasis has been placed on advancing proteomics studies from discovery and/or relative quantitation to validated quantitative methods in an effort to establish clinical assays. The typical workflow involves first performing discovery-based experiments to identify differentially expressed proteins that are reproducibly changing between biological samples. In doing so, product ion information is generated and used to sequence the precursor peptide. The difficulty arises in transferring discovery-based methods directly to validated quantitative methods since each is generally performed on separate mass spectrometry platforms. Low confidence has been placed on relating relative product ion abundance obtained from ion trap CID to that observed using a triple quadrupole mass spectrometer due to the difference in ion activation mechanisms and the timescale of the excitation. This study compares the product ion relative abundance from discovery-based instrumental approaches to SRM transitions acquired on a triple quadrupole mass spectrometer to determine the frequency of matching the top two most abundant product ions measured per peptide via SRM assays with that obtained by HCD. Showing a strong agreement between the two instrumental platforms underscores the benefits of spectral library-based determination for selecting not only the targeted proteins and peptides for a subsequent SRM assay, but also the most sensitive product ions. To incorporate a means of evaluating the selectivity of each individual SRM transition, prototype software is presented that compares the frequency of precursor/product ion pairs that are isobaric with the selected pair and directs the user for additional SRM transitions needed to increase uniqueness based on the specific biological matrix.

## Methods

Discovery-based experiments were performed using an LTQ Orbitrap XL (Thermo Scientific, Bremen, Germany) while targeted peptide quantitation experiments were performed using a TSQ Quantum Access™ (Thermo Scientific, San Jose, CA). Full scan MS data was acquired using 30k resolution. For one experiment, the Data Dependent™ product ion spectra was acquired in the linear ion trap (CID) while the second experiment utilized HCD acquisition in the orbitrap. The resolution setting for the HCD data was 7500. All resulting data was searched using BioWorks™ 3.3.1. A total of 120 peptides identified by both detection schemes were transferred to the TSQ Quantum Access for SRM analysis. Each peptide was divided into a separate category based on peptide length; most were doubly-charged. An additional set of peptides was selected that were identified as either having a missed cleavage site or having a +3 precursor charge state. Each peptide was monitored on the triple quadrupole using at least five SRM transitions. The selected product ions contained the two rules-based transitions as well as the most abundant product ions as determined by the HCD and CID product ion spectra. A five msec dwell time setting was used for all triple quadrupole experiments.



**FIGURE 1.** Proposed workflow for integrating differential expression analysis with targeted protein quantitation.

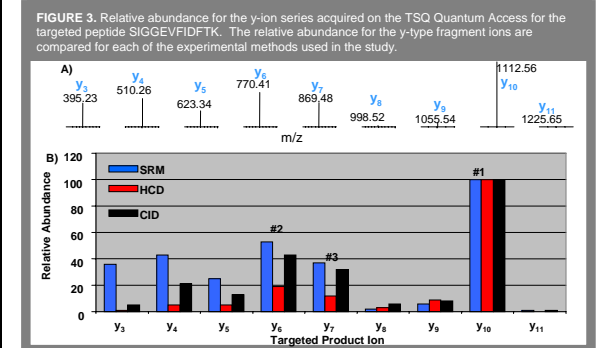


**FIGURE 2.** Full scan MS/MS spectra for the peptide SIGGEVDFDTK acquired using linear ion trap CID (2A) and orbitrap HCD (2B).

Both systems utilized a Surveyor™ MS pump and Micro AS autosampler. All separations were done on a Hypersil™ 50 x 1 mm Gold ac column (Thermo Fisher, Runcorn, UK). For each sample, a 1 µg/mL amount of human ascite was digested. A 2 µL aliquot was injected for each analysis. For quantitation, a protein digest standard of yeast enolase was spiked into the plasma digest at a concentration range of 10 to 1000 fmol.

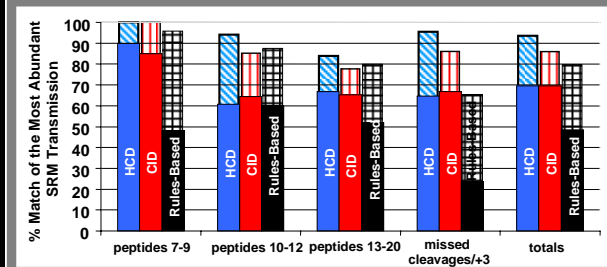
## Results

Figure 1 shows a decision tree for the total workflow for determining targeted peptide quantitation used to characterize protein expression levels in biological systems. The first step of the process is to identify the targeted proteins and/or peptides. The best approach used to generate an unbiased view of the biological sample is to utilize a linear ion trap-based platform operated in data dependent/dynamic exclusion mode. From these initial studies, protein and peptide information are obtained that can be processed using networking software such as Ingenuity Pathway Analysis™ (Ingenuity Systems, Redwood City, CA) to determine biological relationships. In addition to protein information, peptide information such as the most abundant precursor charge state and product ion abundance ratios are measured. An example of linear ion trap and orbitrap HCD are shown in Figure 2A and 2B for the targeted peptide SIGGEVDFDTK. Although the method of ion activation and detection are different, the resulting base peaks are consistent. In addition, all of the higher mass y-type fragment ions are detected for increased confidence in sequence assignment. The resulting SRM assays were built based on the Data Dependent product ion spectra acquired on the linear ion trap based platform. For the targeted peptide shown in Figure 2, a total of 9 product ions were incorporated into the triple quad method and the relative product ion abundance measurements are shown in Figure 3. Note the most abundant transition was the y<sub>10</sub>, which is consistent with both the CID and HCD results in Figure 2. The comparative product ion abundance measurements across the three experimental methods are presented in Figure 3B.



**FIGURE 3.** Relative abundance for the y-ion series acquired on the TSQ Quantum Access for the targeted peptide SIGGEVDFDTK. The relative abundance for the y-type fragment ions are compared for each of the experimental methods used in the study.

**FIGURE 4.** Comparison analysis for identifying the most abundant SRM transition for 120 targeted plasma peptides. Two experimental methods (linear ion trap CID and orbitrap HCD) and rules-based selection were used. The solid bars identify the success of matching the most abundant SRM transition, while the lined bars indicate matching the top two SRM transitions.



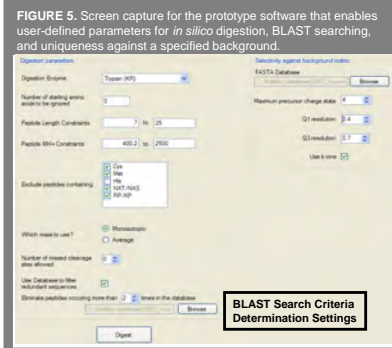
The results obtained using SRM assays clearly match with those from the orbitrap. The two product ions that would be selected using common rules-based determination are the y<sub>6</sub> and y<sub>7</sub> product ions but are not the most abundant product ions. The rules-based selection criteria assumes that the most abundant product ions are directly greater in m/z value than that for the precursor. Each method used in this study was compared to determine the most robust means of determining optimal SRM transitions to be included in the resulting targeted experiment.

Figure 4 shows the frequency of matching the most abundant SRM transition for the peptides in each method. Both experimental results are shown to match at a greater frequency than that for the rules-based approximation by almost 20% for each peptide category. Clearly the shorter the peptide length the more likely the fragmentation patterns match between instrument platforms and activation/detection methods. Extending the search from the most abundant to the top two most abundant product ions dramatically increases the likelihood that library-based determination for resulting SRM transitions will result in detection limits for targeted peptides with the overall frequency of matching at 92% for HCD and 85% for CID compared to 80% for rules-based determination. Selectivity can be increased by utilizing more than 1 SRM transition per peptide and the results from Figure 4 show the utility of a library-based determination at finding the most abundant product ions.

To facilitate the building of SRM assays, a prototype software package was created. The software enables entire protein databases to be loaded and processed via user-defined *in silico* parameters to create a list of proteotypic peptides that can be incorporated into a final SRM table. To aid in identifying proteotypic peptides,

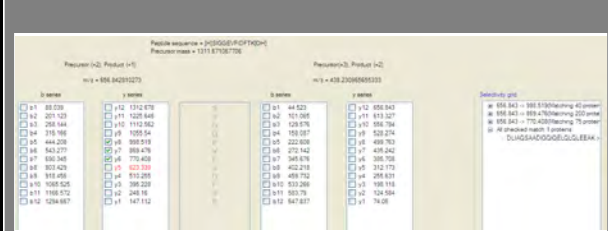
performing a BLAST search as shown in the screen capture at the bottom of Figure 5. In addition to performing a BLAST search, the user can also check for uniqueness of each SRM transition by specifying experimental conditions such as the background matrix, the Q1 and Q3 resolution settings, and the charge state for the background peptides.

To assess the effects of the user-defined parameters, the previously described targeted peptide is used. Figure 6 shows a screen capture of the software to predict the potential background interference for the targeted peptide. In this example, 3 transitions were selected for inclusion in the SRM table.

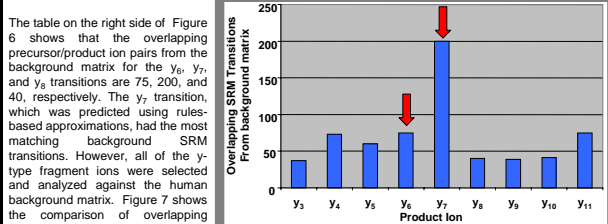


**FIGURE 5.** Screen capture for the prototype software that enables user-defined parameters for *in silico* digestion, BLAST searching, and uniqueness against a specified background.

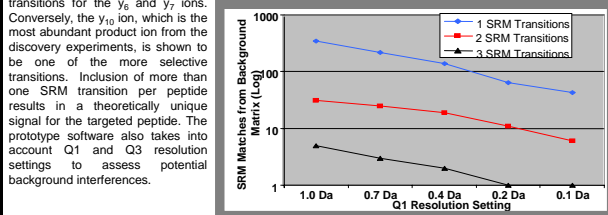
**FIGURE 6.** Screen capture from the prototype software that shows the uniqueness for each SRM transition against the user-defined back



**FIGURE 7.** Frequency of matrix precursor-product ion pairs with each y-type transition for the targeted peptide sequence SIGGEVDFDTK.

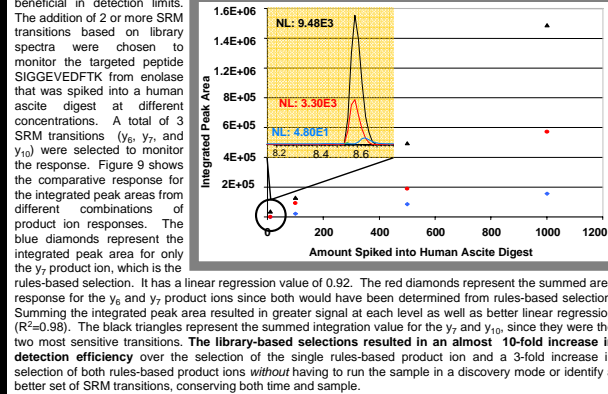


**FIGURE 8.** Q1 resolution effect on generating unique sets of SRM transitions for targeted peptide assays.



By increasing the resolution setting of Q1 from 0.7 Da (FWHM) to 0.1 Da, the number of overlapping background precursors should be reduced and therefore dramatically decrease the number of product ions that could potentially interfere with the targeted SRM transitions. To illustrate this, the peptide SIGGEVDFDTK was assessed for overlapping background interference as a function of Q1 resolution setting. Figure 8 shows the number of SRM transitions for the targeted peptide to achieve a unique set of product ions against the human protein database. Clearly, the increase in Q1 resolution reduces the overlapping background interference as well as the need for additional SRM transitions.

**FIGURE 9.** Effects of sensitivity for targeted peptide detection using the rules-based selection (y<sub>6</sub> and y<sub>7</sub> product ions) vs. library-based selection of SRM transitions (y<sub>7</sub> and y<sub>10</sub>).



## Conclusions

- Most proteomics labs utilize an LTQ-based platform for initial screening of proteins, peptides, and expression levels, rendering a list of protein biomarker candidates that must be further validated.
- From these discovery-based experiments, a wealth of knowledge and thousands of MS and MS/MS spectra have been accumulated and can be used to direct more selective SRM-based analyses.
- The targeted quantitation workflow presented here incorporates software and libraries that identify optimal peptide and product ions to be used in a subsequent SRM assay.
- Utilizing product ion abundances from LTQ-based experiments provides a higher probability of matching the most abundant SRM transition than relying on a rules-based approach.
- The prototype software can be used to evaluate overlapping precursor/product ion pairs from the user-defined matrix and determine if more than 1 SRM transition is needed.
- The addition of the top two or three product ions not only increases the selectivity for identifying the targeted peptide, but can dramatically increase the detection and quantification capabilities of the resulting method.
- Future work will include modifying the prototype software to automatically read in discovery-based data and generate spectral libraries that can be used to automatically build SRM tables for a specified protein.

## Acknowledgements

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