Towards Turnkey Targeted Proteomics Solutions Using Internal Standard Triggered Acquisitions on Modified Orbitrap MS

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ABSTRACT

Purpose: A novel internal standard triggered targeted quantification workflow, called 'SureQuant' workflow (Figure 1), has been developed and implemented in the native instrument control software of novel Orbitrap mass spectrometers: Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer and Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer. It aims at enabling large-scale target profiling with superior quantitative performance, robustness, and usability.

Methods: The SureQuant internal standard triggered-PRM (IS-PRM) acquisition scheme was developed and tested on Orbitrap Exploris 480 and Orbitrap Eclipse Tribrid mass spectrometers. Chromatographic separations were carried out using Thermo Scientific™ UltiMate™ 3000 RSCLC system and Thermo Scientific™ EASY-nLC™ 1200 system. Thermo Scientific™ Pierce™ SureQuant™ AKT Pathway Multiplex Panel kit and PQ500 kit from Biognosys were used to evaluate SureQuant workflows applied to the analyses of cancer cell line models and undepleted plasma samples.

Results: The novel SureQuant IS-PRM method significantly enhanced targeted data acquisition efficiency in comparison with conventional PRM method (*i.e.*, 80-90% of the instrument time dedicated to the collection of pertinent data), and exhibited strong robustness against chromatographic variations. Applied to the analyses of a variety of samples supplemented with different sets of stable isotopically labeled (SIL) peptides used as internal standards (IS) in various formats (30-800 IS), the method demonstrated the capability to quantify endogenous peptides in the low amol range at all scales. In addition, Embedding pre-set methods, associated with predefined kits of IS peptides, directly into the instrument control software turned out to be a decisive step towards the provision of turnkey targeted proteomics solutions.

INTRODUCTION

The advances in HRAM mass spectrometry instrumentation have enabled new approaches for targeted quantitative proteomics, such as the parallel reaction monitoring (PRM) technique. An extension of PRM uses spiked-in internal standards to dynamically control the acquisition process and to maximize its efficiency. The original implementation of this internal standard triggered-PRM method on a quadrupole Orbitrap instrument has enabled the systematic monitoring of larger sets of proteins with higher analytical performance. Here, the acquisition scheme of the approach has been revised, while keeping the same rationale, to enable a more generic implementation on latest generation Orbitrap-based instruments. Under this new implementation, the method has exhibited significantly enhanced usability and robustness without sacrificing analytical benefits.

MATERIALS AND METHODS

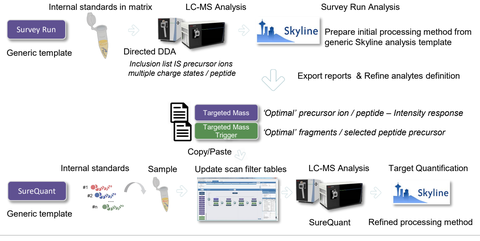
Sample Preparation

Cell Culture: HCT 116, MCF7, and A549 cells were grown in McCoy's 5A Media with 10% FBS/1xPenStrep to ~70-80% confluency. HCT116 cells were serum starved in 0.1% charcoal stripped FBS for 24 hours prior to stimulation (15 min hIGF-1 (100ng/mL; Cell Signaling Technology PN#8917SF)). Then cells were lysed with IP-Lysis buffer (Thermo Fisher Scientific PN#87788), and processed by reduction, alkylation and trypsin (Thermo Fisher Scientific PN#90057) digestion overnight at 37°C. The digested samples were acidified with TFA.
A commercial human plasma sample pooled from several individuals purchased from BioreclamationIVT was processed using Thermo Scientific™ EasyPrep™ Mini MS Sample Prep Kit.
LC-MS sample preparation: A set of 30 SIL peptides from SureQuant AKT Pathway Multiplex Panel (Thermo Fisher Scientific PN#A40080) was spiked at 50 fmol in 500 ng of HeLa digest (Thermo Fisher Scientific PN#88329), HCT116 digest, MCF7 digest, and A549 digest. One µL of the sample was injected for LC-MS/MS analyses.
A set of 804 SIL peptides from PQ500 kit (Biognosys PN#Ki-3019-96) was spiked at around 80 fmol (median value) in 1 µg of undepleted plasma sample. One µL of the sample was injected for LC-MS/MS analyses.

LC-MS/MS Analysis

Chromatographic separations were performed on UltiMate 3000 RSLC system equipped with C_{18} trap cartridge (5 µm, 0.3 x 5 mm) and analytical column (2 µm, 0.15 x 150 mm) and EASY-nLC 1200 system equipped with a C_{18} analytical column (2 µm, 0.15 x 150 mm) . The LC systems were coupled to Orbitrap Exploris 480 and Orbitrap Eclipse Tribrid mass spectrometers operated in conventional PRM and SureQuant IS-PRM mode. The acquisition parameters are detailed in the pertinent sections of the poster.

Figure 1. Overall SureQuant acquisition workflow



SureQuant IS-PRM Methodology

The original implementation of the IS-PRM method, using instrument application programming interface, toggled between i) a "watch mode", in which IS were continuously measured through fast PRM scans over their elution time monitoring windows (dynamically corrected), and ii) a "quantitative mode" that was triggered by the real-time detection of the IS (through dot-product spectral matching), wherein the corresponding pairs of IS and endogenous peptides were measured over their pre-defined elution profiles with optimized PRM acquisition parameters. In order to foster a broader adoption of the approach, we produced a generic implementation of the method (Figure 2), also benefiting from higher usability, in the native instrument control software of latest generation Orbitrap-based instruments. The acquisition scheme has been revised while keeping the same rationale of using IS to drive the acquisition in two modes. More specifically, the watch mode portion has been substantially modified and relies on a two-step process interrogating both MS1 and MS2 data to track the elution of IS. With such set-up, the acquisition fully avoid any dependency on time scheduling. In addition, substituting dot product spectral library matching with fragment matching only for the MS2-based confirmation of the IS improves the robustness and portability of the approach. The structure of the revised acquisition method, detailed in Figure 3, includes different scan events and filters operated with parameters optimized for the highest data quality and the sensitivity of triggering as first priority but also to retain sufficient triggering specificity (Figure 4).

Figure 2. SureQuant acquisition scheme.

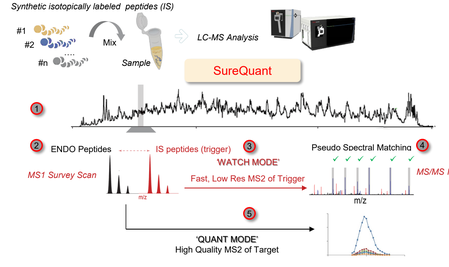
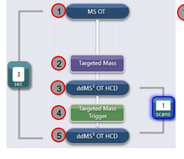


Figure 3. SureQuant method structure.



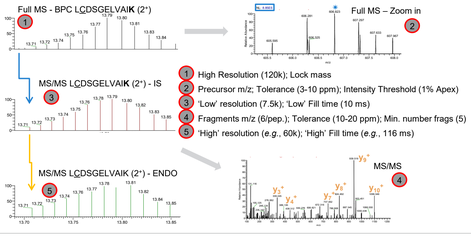
1 2 Predefined precursor ions and corresponding intensity threshold of IS are monitored in Full scan MS

3 Candidate precursor ions satisfying filtering criteria subjected to fast MS/MS (low resolution / low fill time)

4 Several "selective" fragment ions of IS are monitored in fast MS/MS

5 The detection of IS triggers the high-quality measurement of corresponding ENDO peptide (high resolution / fill time)

Figure 4. SureQuant acquisition process and critical parameters.



1 High Resolution (120k); Lock mass
2 Precursor m/z; Tolerance (3-10 ppm); Intensity Threshold (1% Apex)
3 'Low' resolution (7.5k); 'Low' Fill time (10 ms)
4 Fragments m/z (6/pep.); Tolerance (10-20 ppm); Min. number frags (5)
5 'High' resolution (*e.g.*, 60k); 'High' Fill time (*e.g.*, 116 ms)

Figures of Merit of SureQuant Method

The native implementation of SureQuant method in the instrument control software facilitates method building, especially for large scale experiments, through the use of the new 'Group ID' feature (Figure 5). This feature offers a way to streamline the upload of the necessary information associated with the precursor and fragment ions of the targeted peptides, only requiring a few branches to be included in the method. In order to illustrate the enhanced productivity of SureQuant acquisition, it has been compared with time-scheduled PRM measurement of a pair of IS and endogenous peptides, using a 2.5-min monitoring window (Figure 6). A significantly higher proportion of productive scans was observed in SureQuant analysis (*i.e.*, 80-90% against 10-15% in PRM analysis). In addition, SureQuant acquisition exhibited a strong robustness against chromatographic variations (Figure 7). The introduction of an artificial elution time drift (offset gradient mimicking chromatographic dysfunction) compromised PRM measurements while the capture of the targeted peptides in SureQuant analysis was not affected.

Figure 5. Improved method building usability through 'Group ID' feature implementation

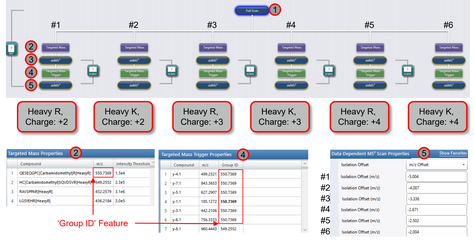


Figure 6. Acquisition efficiency of conventional PRM and SureQuant methods

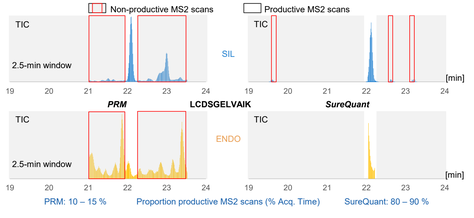
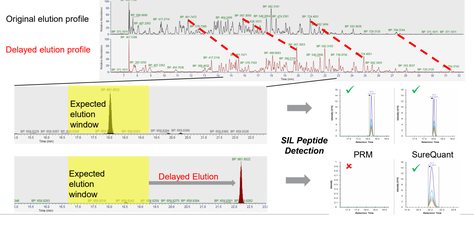


Figure 7. Robustness of SureQuant acquisition against chromatographic variations



Application to Signaling Pathway Monitoring

SureQuant method was applied to the monitoring of AKT/mTOR signaling pathway using SureQuant AKT Pathway Multiplex Panel kit. The kit includes 30 SIL peptides associated to the 12 main protein components of the pathway. After initial assay development, such as described in the upper part of Figure 1, PRM and SureQuant methods have been optimized in order to maximize the sensitivity/selectivity of endogenous peptides measurements in cell line digests while maintaining a chromatographic sampling rate of at least 8 data points over the LC separation. It turned out that with the 30-min gradient used, MS/MS of endogenous peptides can be acquired with 6 times more fill times in SureQuant analyses (Figure 8, upper panel), translating into significantly improved sensitivity of measurements. This higher sensitivity enabled 26 of the 30 targeted endogenous peptides to be detected / quantified by SureQuant, against only 11 by PRM (Figure 8, lower panel). The higher acquisition time (resolution and fill time) dedicated to endogenous peptides measurements in SureQuant analyses significantly improved signal to noise ratio of their extracted fragment ion traces. Applied to other cancer line digests stimulated with IGF-1, SureQuant method delivered near comprehensive quantification of the entire set of targets over a quantification range between 10 amol and 1 fmol, and with exquisite precision, reflected by 60% of the endogenous peptides quantified with CV<5% (Figure 9).

Figure 8. Comparison of the analytical performance of PRM and SureQuant analyses

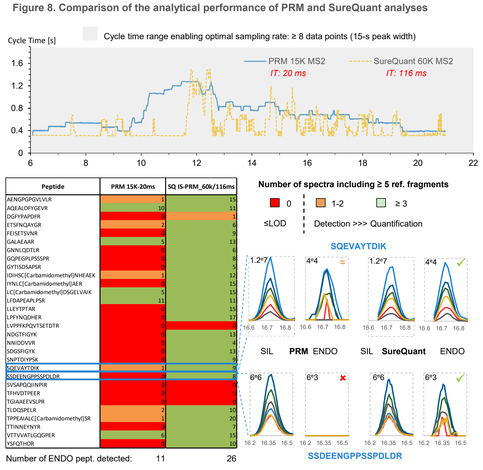
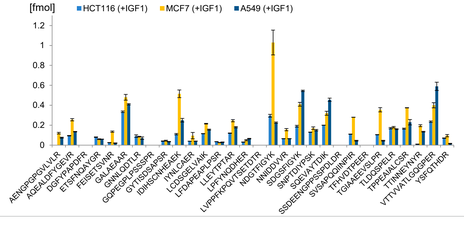


Figure 9. Sensitive and precise quantification of AKT/mTOR peptides by SureQuant analyses.



Towards Turnkey Targeted Proteomics Solutions

The development of a SureQuant multi-peptides assay requires the preliminary determination of the optimal precursor ion of each peptide target together with their intensity response and optimal associated fragment ions (Figure 1). This information is typically obtained from a directed DDA analysis of the corresponding IS peptide mixture. The target list of multiple theoretical precursor ions of each IS peptide included in such survey runs enables comprehensive data collection, and therefore the refinement of analytes definition for subsequent SureQuant analyses. The process is supported by Skyline software and facilitated by the generic data acquisition and processing templates provided. The transfer of the generated information can be achieved through simple copy/paste from Skyline reports to 'Targeted Mass' and 'Targeted Mass Trigger' tables of SureQuant method. The survey analysis of a SureQuant assay needs to be run one time as the onset of the project without further adjustment over time, unless a major change in the experimental design is applied (*e.g.*, LC set up, spiked amount of SIL peptides). In the latter case, only the intensity response of predefined IS precursor ions needs to be re-adjusted though an additional survey run performed under the modified experimental conditions (Figure 10). Therefore, the ability of embedding pre-set methods associated with predefined kits of IS peptides expedites SureQuant assay development at the user sites. Several validated method templates of this type have already been included in the instrument control software, such as the PQ500 related methods (Figure 11), prepared for the analyses of plasma samples supplemented with PQ500 kit (which includes 804 SIL peptides associated with more than 500 plasma proteins). The applications of the SureQuant PQ500 method to the analyses of undepleted plasma samples enabled the robust and precise quantification of around 560 endogenous peptides, corresponding to around 400 proteins in a 70-min LC gradient.

Figure 10. Overall SureQuant acquisition worklow of embedded application specific kits.

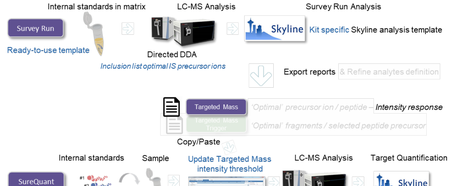
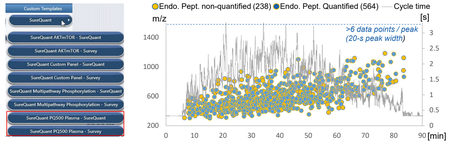


Figure 11. SureQuant analysis of plasma enabled by embedded preset methods



CONCLUSIONS

- A novel IS triggered targeted quantification workflow, called 'SureQuant' workflow has been successfully implemented in the native instrument control software of Orbitrap Exploris 480 and Orbitrap Eclipse Tribrid MS.
- The SureQuant method demonstrated exquisite acquisition efficiency and robustness, translating into systematic and highly sensitive quantification of large sets of endogenous peptides (up to 800) in a variety of samples. Its 'load-and-play' execution, especially for embedded application specific kits, significantly improves user experience.

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TRADEMARKS/LICENSING

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