

# Global Plasma Proteome Quantification Using Internal Standard Triggered Targeted Analyses

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## ABSTRACT

**Purpose:** A novel global plasma proteome quantification workflow has been developed. It relies on a new high density targeted acquisition method, called 'SureQuant' method, implemented on next generation Orbitrap mass spectrometers: Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer and Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer. In addition, the workflow leverages a large set of stable isotopically labeled (SIL) peptides, used as internal standards, to drive the systematic screening of more than 500 plasma proteins (PQ500 kit from Biognosys).

**Methods:** The SureQuant PQ500 analyses of undepleted plasma samples were performed using Orbitrap Exploris 480 and Orbitrap Eclipse Tribrid mass spectrometers, coupled to Thermo Scientific™ UltiMate™ 3000 RSLC system or Thermo Scientific™ EASY-nLC™ 1200 system. An acquisition variant of the internal standard triggered targeted acquisition, dedicated to higher throughput analyses, was executed through an application programming interface (API) controlling a Thermo Scientific™ Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer coupled to a Evosep One LC system. PQ500 kit from Biognosys was spiked into the plasma samples to drive the targeted acquisition.

**Results:** The SureQuant method has been adapted from the internal standard triggered PRM (IS-PRM) method in order to improve its usability and robustness, while retaining its analytical performance, and has been implemented in the native instrument control software of next generation Orbitrap mass spectrometers. The application of the method to the analysis of plasma samples, leveraging spiked-in SIL peptides from PQ500 kit, allowed robust and precise quantification of around 560 endogenous peptides used as surrogate of 400 plasma proteins using a 70-min LC gradient. Therefore, the method combined data quality of targeted analyses with proteome coverage of state-of-the-art profiling experiments. In addition, the availability of pre-set PQ500 SureQuant methods embedded into the instrument control software enables a 'load and play' execution of such plasma proteomics experiments.

## INTRODUCTION

The success of plasma proteomics studies to deliver useful protein biomarkers has remained lower than initial expectations, prompting a redefinition of the plasma biomarker development pipeline. One proposed measure consisted in the implementation of a "rectangular" approach, relying exclusively on broad proteome profiling (through DDA or DIA analyses) across large cohorts for both the discovery and validation/verification stages. We describe here an alternative global quantification workflow, relying on a high density targeted acquisition method implemented on next generation Orbitrap mass spectrometers. This adaptation of the IS-PRM method leverages spiked-in stable isotopically labeled (SIL) peptides to drive the systematic screening of more than 500 plasma proteins per analysis across large sample sets.

## MATERIALS AND METHODS

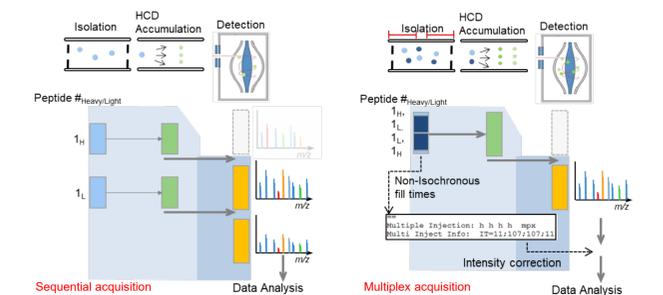
### Sample Preparation

A commercial human plasma sample pooled from several individuals purchased from Bioreclamation/VT was processed using Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit. A set of 804 SIL peptides from PQ500 kit (Biognosys PNJKI-3019-96) was spiked at around 80 fmol (median value) into 1 µg of undepleted plasma sample. One µL of the sample was injected for LC-MS/MS analyses.

### LC-MS/MS Analysis

For SureQuant analyses, chromatographic separations were performed on UltiMate 3000 RSLC system equipped with C<sub>18</sub> 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 C<sub>18</sub> analytical column (2 µm, 0.15 x 150 mm), using a 70-min gradient separation. The LC systems were coupled to Orbitrap Exploris 480 and Orbitrap Eclipse Tribrid mass spectrometers. In SureQuant analyses, Full MS scans were acquired at a resolution of 120k with a maximum fill time of 50 ms, while ddMS2 of IS and endogenous peptides were acquired sequentially at a resolution of 7.5k or 60k and maximum fill times of 10 ms and 116 ms, respectively. The analyses using the high throughput IS-PRM acquisition variant were performed on Evosep One LC system equipped with Evosep C<sub>18</sub> analytical column (3 µm, 0.1 x 80 mm), using a 21-min gradient separation. The LC system was coupled to a Q Exactive HF-X controlled by an API. In the analyses performed with the 'Single Scan Mx IS-PRM' method (Figure 1, right panel), PRM scans in watch mode were acquired at a resolution of 7.5k and a maximum fill time of 10 ms. In the quant. mode, the pairs of IS and endogenous peptides were measured simultaneously by 'multiplex' acquisition of their fragments in a single PRM scan generated at a resolution of 120k and non-isochronous maximum fill times of 20 or 124 ms for the IS and endogenous peptides, respectively.

Figure 1. Sequential and multiplex acquisition modes



## SureQuant Methodology

The developed SureQuant acquisition method includes different scan events and filters, with parameters optimized for the highest data quality and sensitivity of triggering, but also to retain sufficient triggering specificity (Figure 2). During SureQuant analyses, a high resolution (120k) full scan MS (1) is acquired, using lock mass re-calibration, to monitor the predefined optimal precursor ions of the IS, based on the list of associated m/z values and intensity thresholds (defined at 1% of the expected MS1 intensity at the chromatographic peak apex) included in the 'Targeted Mass' filter (2). In case of the detection of a candidate precursor ion satisfying the filtering criteria (MS peak with an m/z value within 3-10 ppm tolerance and intensity exceeding the threshold), it is subjected to fast MS/MS acquisition (3) performed at low resolution and short fill times (typically 7.5k/10 ms), which is used as a second layer of confirmation of the detection of the IS. Several fragment ions, predefined as associated with the IS (6 optimal frag.) are included in the 'Targeted Mass Trigger' filter (4), and the detection of a subset of them (at least 5 frag., 10-20 ppm tolerance) confirms the actual elution of the IS. This two-step process interrogating MS1 and MS2 data to track IS elution is the 'watch' mode of the SureQuant method. The detection of an IS in watch mode triggers the 'quant' mode, and therefore MS/MS acquisition of the corresponding endogenous pept. (5) with parameters favoring data quality, i.e., high resolution and long fill times (typically 60k/116 ms). The process is repeated over the entire LC separation with a systematic cycle-to-cycle assessment of the elution of the IS. The development of a SureQuant assay to be associated with a custom peptide panels requires the preliminary determination of the optimal precursor ion of each peptide target together with their intensity response and optimal associated fragment ions. This information is typically extracted from an initial directed DDA analysis of the corresponding IS peptide mixture spiked into a representative matrix. 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 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 a 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 responses of predefined IS precursor ions need to be re-adjusted though an additional survey run performed under the modified experimental conditions. Therefore, the ability of embedding pre-set methods associated with predefined kits of IS peptides expedites SureQuant assay development at the user sites. Such validated method templates have already been included in the instrument control software for the PQ500 related methods (Figure 3).

Figure 2. SureQuant acquisition Scheme and method structure

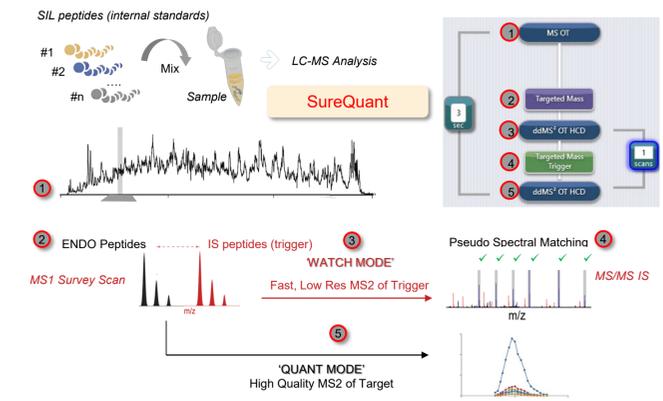
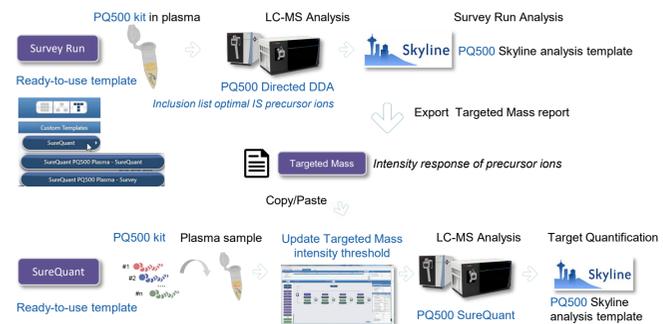


Figure 3. Overall PQ500 SureQuant acquisition workflow.



## SureQuant PQ500 Plasma Quantification

The PQ500 SureQuant acquisition workflow has been applied to the analyses of 1 µg of undepleted plasma sample supplemented with PQ500 kit (median spiked-in amount of SIL peptides of 80 fmol) using a 70-min LC gradient on UltiMate 3000 RSLC system. The survey run revealed that 802 of 804 SIL peptides were compatible (intensity response and hydrophobicity) with the LC-MS setup used (Figure 4), and were retained for subsequent SureQuant analyses. The high overall intensity response of the SIL peptides (median MS1 intensity at chromatographic peak apex of 1e8) insured high acquisition efficiency (>90%) in SureQuant analyses, benefiting from the median MS1 triggering intensity threshold at 1e6. This translated into a good overall sampling rate over the LC separation, exceeding 8 data points collected over the elution profile of the vast majority of the peptides, which also remained acceptable in the most crowded time ranges, with 6 to 8 data points collected per peak (Figure 5). The 802 retained internal standards were systematically detected in the triplicated SureQuant analyses of plasma samples, and triggered high quality measurement of the corresponding endogenous peptides, translating into a broad coverage of the undepleted plasma proteome (Figure 6). Around 400 proteins were detected in each replicate (based on around 560 surrogate endogenous peptides), while 90% of the total sets were systematically quantified across triplicates.

Figure 4. Characterization of PQ500 kit through survey run analysis.

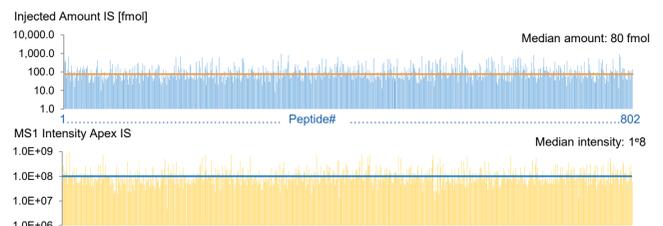


Figure 5. Sampling rate in SureQuant PQ500 global plasma analysis

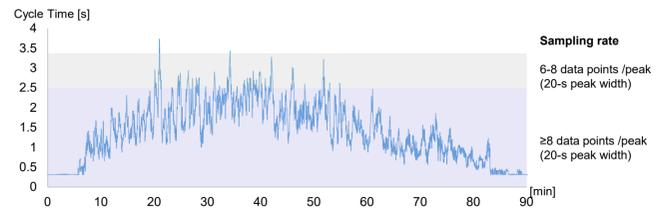


Figure 6. Peptide detection/quantification in SureQuant PQ500 analyses of undepleted plasma



In addition to delivering a broad plasma proteome coverage, rivaling that of state-of-the-art profiling methods, SureQuant PQ500 analyses still provide targeted quantification performance (Figure 7). The amounts of endogenous peptides were estimated based on the known amounts of IS injected and single-point quantification strategy. This revealed a quantification range between 4 amol and 15 pmol, spanning more than 6 orders of magnitude, which is in theory sufficient to cover the vast majority of current FDA-approved plasma biomarkers. The fragment ion signals of the lowest abundant quantified endogenous peptide (PFLVFIR, 4 amol/µg of plasma) have been extracted (Figure 7, lower panel), illustrating the acceptable data quality even obtained for this challenging analyte. The quantification precision obtained on the entire dataset was excellent, with 75% of the endogenous peptides quantified with a CV below 10% (median CV value of 6%). The experiments were reproduced at a second site by another operator, using a different LC set-up (EASY-nLC 1200 system operated in a one-column setup instead of UltiMate 3000 RSLC system operated in a two-column setup) (Figure 8). After initial survey analysis, enabling the adjustment of internal standards MS1 triggering intensity threshold, SureQuant PQ500 analyses of undepleted plasma sample were repeated. It turned out that the quantification of the endogenous peptides between sites / systems / operators was very consistent, as reflected by the quantitative values tightly aligned along the theoretical 'perfect' correlation line, and the absence of bias revealed by the equation of the regression. The acquisition scheme of SureQuant method, fully avoiding any dependency on time-scheduling, enables efficient method portability and strong robustness.

Figure 7. Quantification performance of SureQuant PQ500 analyses of undepleted plasma

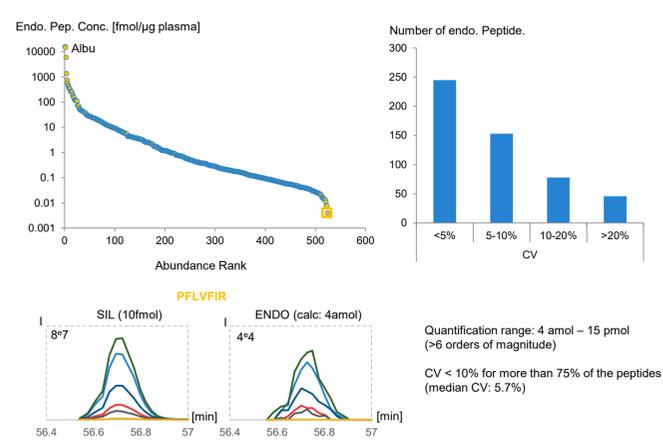
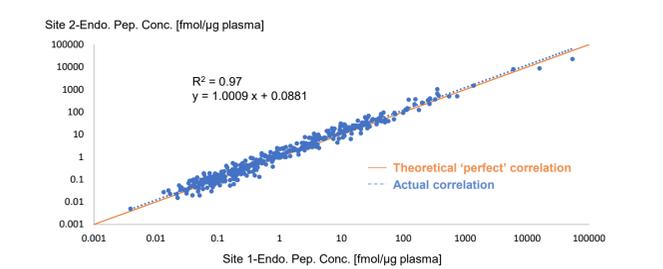


Figure 8. Consistency of SureQuant PQ500 Quantification across sites/systems



## High Throughput IS Triggered Plasma Analyses

A variant of SureQuant acquisition, dedicated to higher throughput analyses, has been developed using API-based IS-PRM application, which is only compatible with QE MS platforms. This method has not been natively implemented in the instrument control software of orbitrap instruments and does not benefit from the revised acquisition strategy of SureQuant method, but relies on the acquisition scheme of the original IS-PRM method. More specifically, in this 'Single Scan Mx IS-PRM' acquisition variant, the detection of an IS peptide in watch mode triggers the concomitant measurement of the corresponding pair of IS and endogenous peptides in a single PRM scan in quant. mode close to their elution profile apex, following their 'multiplex' isolation (Figure 1). This contrast with the regular sequential acquisition, in which data points are collected over the full elution profiles of the peptides. With this acquisition variant, quantification is performed based on the fragment ion intensities extracted from single high quality PRM scan, and not on the area under the curve of the corresponding extracted fragment traces. This quantification strategy alleviates sampling rate constraint.

The analyses of plasma sample supplemented with PQ500 kit were reproduced using the 'Single Scan Mx IS-PRM' acquisition method on QE HF-X MS coupled to Evosep One LC system operated at a 60 samples/day analytical throughput (21-min LC gradient). The sampling rate under these conditions remained significantly higher than the predefined (relaxed) limit of 4-5 data points collected per peptide chromatographic peak (Figure 9, upper panel). In comparison with SureQuant analyses performed with longer LC gradient, a substantial number of IS peptides (81) were not detected, corresponding to the most hydrophilic peptides, which are not compatible with the LC setup used (Figure 9, lower panel). The remaining 721 IS peptides were properly detected in the watch mode of the method and triggered multiplex PRM measurement in the quant. mode, enabling the detection of 421 endogenous peptides, surrogate of 301 plasma proteins. In Figure 10, the data generated by SureQuant and Single Scan Mx IS-PRM analyses are displayed for one endogenous peptide quantified by both methods. The quantification results were very consistent across the methods.

Figure 9. High throughput plasma analyses using Single Scan Mx IS-PRM method.

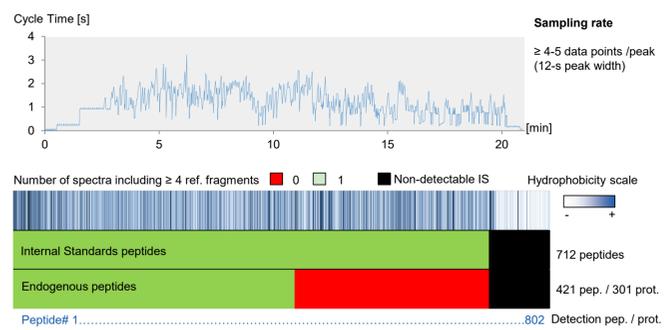
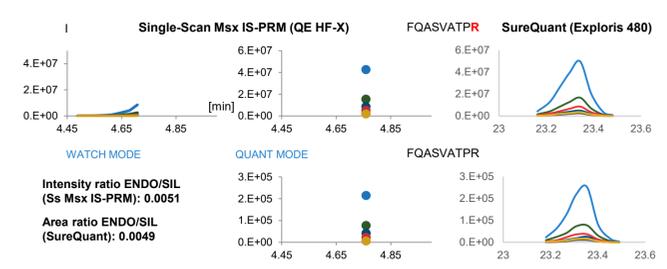


Figure 10. Endogenous peptide quantification from SureQuant and Single Scan Mx IS-PRM



## CONCLUSIONS

- A novel global plasma IS triggered targeted quantification workflow, based on 'SureQuant' method has been successfully implemented in the native instrument control software of Orbitrap Exploris 480 and Orbitrap Eclipse Tribrid MS.
- The global plasma quantification workflow combines the figures of merit of targeted acquisition method with the coverage capabilities of profiling methods for plasma proteomics. In addition, it exhibits an efficient portability and a high usability, benefiting from embedded pre-set PQ500 related methods.
- The developed IS-PRM acquisition variant was explored to carry out high throughput analyses. It exhibited slightly lower performance, but also the capability for a 5-fold increase in throughput/experiment scale. Further investigation is needed to evaluate the pertinence of its native implementation under the form of a SureQuant method variant.

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