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Combining a new hybrid nominal mass platform and intelligent data acquisition to enable highly multiplexed targeted proteomics

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Abstract

Purpose: Characterization of the Thermo Scientific™ Stellar™ mass spectrometer, a hybrid quadrupole, linear ion trap mass spectrometer with improved capabilities for targeted MSn experiments.

Methods: Quantitative performance is assessed for >700 peptides compared to a triple quad. Larger scale assays are created for the PQ500™ Reference Peptides kit diluted in plasma and a *E. coli* digest standard dilution in HeLa.

Results: Stellar MS outperformed the triple quad by more than 10x in terms of limit of quantitation due to the inherent advantage of PRM over SRM (or MRM). Large scale assays are easily created with PRM Conductor, and the new Adaptive RT algorithm improves assay throughput and reproducibility.

Introduction

Modern discovery mass spectrometers continue to improve in sensitivity and coverage, to the point that the one-hour proteome is within reach[1]. As researchers move their findings from discovery towards the clinic, the technology switch from global assays to targeted triple quad assays is a major stumbling block, due to lengthy optimizations, limited throughput, serial transition acquisition and the need to choose transitions before acquiring data. We demonstrate how Stellar MS with fast, full scan MS^n, parallel transition accumulation, new software for method development with retrospective transition selection, and the Adaptive RT real-time chromatogram alignment can help surmount the traditional, targeted technology switch challenges.

Materials and methods

Samples and liquid chromatography

Comparison to the Thermo Scientific™ TSQ Altis™ Plus mass spectrometer was performed with 333 ng matrix-matched dilutions of digested human into digested chicken plasma. Absolute quantitation experiments were performed using the PQ500 kit spiked into 300 ng of human plasma. *E. coli* experiments used a base level of 200 ng of *E. coli* spiked into 200 ng of HeLa.

LC separation was achieved using the Thermo Scientific™ Vanquish Neo™ UHPLC system with various gradients, ranging from 30 minutes and 0.45 µl/min for the human/chicken experiment, to 60 and 100 SPD methods with 0.8 and 1.8 µl/min flow rates for the PQ500 and *E. coli* experiments.

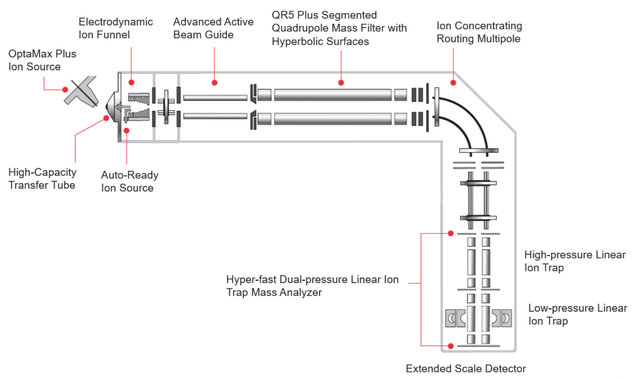
Data acquisition

Methods were created using a new program called PRM Conductor[2] that plugs into the Skyline SW ecosystem. This program automates the process of selecting high quality precursors for targeting and exporting to instrument method files, based on any kind of data that can be imported into Skyline.

For absolute quantitation, heavy standards are detected using Skyline's integrated RT and spectra prediction using Prosit™. For the plasma and *E. coli* experiments, discovery data were generated using gas-phase-fractionation (GPF) data independent acquisition (DIA) using Stellar MS with peptide detection performed with CHIMERYS in Proteome Discoverer.

Stellar MS (Figure 1) performs a standard targeted MS2 experiment by isolating precursors in the Q1, fragmenting and accumulating multiple product ions in parallel in the Q2 and mass analyzing them in the dual-cell linear ion trap (LIT). The LIT can also be used to perform MS3 experiments by isolating multiple product ions with synchronous precursor selection and utilizing resonance CID at either the 1st or 2nd activation stage.

Figure 1. Thermo Scientific Stellar MS diagram.



Adaptive RT

Adaptive RT is a new strategy for acquiring targeted MS^n data with real-time chromatogram alignment[3]. It performs a comparison of spectra during targeted acquisition with a reference data set, such as a previous targeted or discovery run (Figure 2). Adaptive RT enables narrow scheduled windows as low as 0.35 minutes for 100 SPD and 0.6 minutes for 60 SPD.

Figure 2a. Adaptive RT methodology summary.

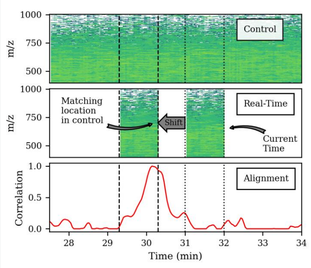
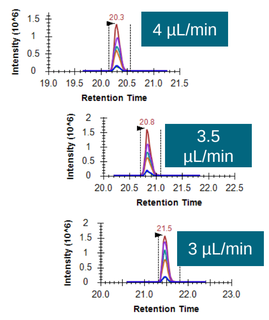


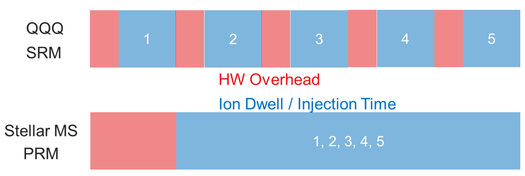
Figure 2b. Adaptive RT test case changing flow rate by 15% increments.



Stellar MS vs Altis Plus MS

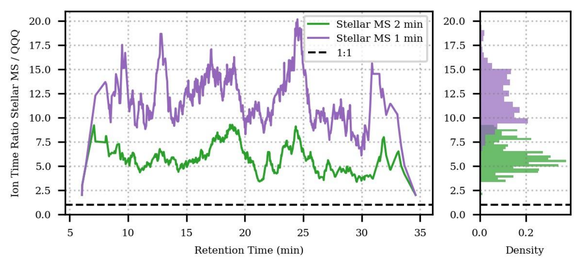
Fundamentals of PRM vs SRM/MRM

Targeted analysis of peptides often uses 3 or more transitions for data analysis to ensure the uniqueness of the data signature and to protect against new interferences induced by LC drift. Accumulation of multiple transitions happens in serial for triple quads, while full scan instruments like Stellar accumulate these products in parallel. This leads to N-fold higher sensitivity or even higher when the dwell times approach the hardware switching times (Figure 3).



The PRM advantage means that not only are multiple products accumulated at the same time, but the total accumulation time per transition is higher, as shown in Figure 4. This first study used a 2-minute window for compatibility with the triple quad, but 1-minute or smaller windows are more typical with Adaptive RT.

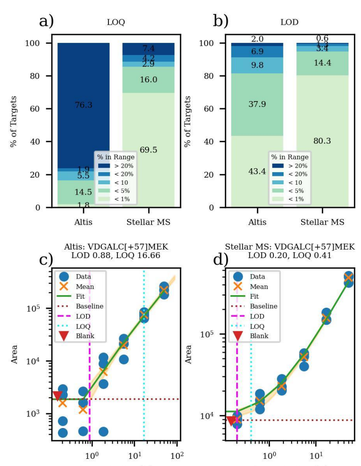
Figure 4. Ratio of accumulation times for Stellar MS versus a Triple quad for 786 peptides with ~5 transitions each. Green represents a 2-minute scheduling window and purple a 1-minute window.



Dilution curve results summary

The increased ion accumulation times for Stellar MS resulted in better precision and an average of 15x better limits of quantification (LOQ) than the Altis Plus. The limits of detection (LOD), which measure the concentration where the signal disappears in the baseline, are about 2x better on Stellar MS. Of note is that the Altis Plus achieves similar LOQs to Stellar MS for smaller scale MS2 assays.

Figure 5. Stellar MS vs Altis dilution curve results for 786 peptides in a 30-minute gradient. a) summary of limits of quantification. b) summary of limits of detection. c) representative Altis dilution curve. d) representative Stellar MS dilution curve

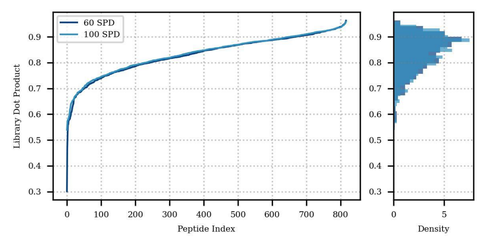


Absolute quantitation for 804 PQ500 reference peptides

Method creation and characterization

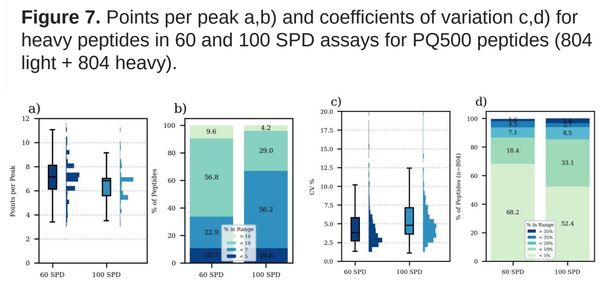
Creating large scale targeted assays with absolute quantitation is facilitated with Skyline's integrated retention time and spectral library prediction. Heavy standards are detected via comparison with the predicted library. Normalized collision energy is used instead of optimizing for each transition, such that once the LC system is set up, an assay can be created in a few injections. The match between Prosit and experimental spectra at 30% NCE is excellent, despite Prosit having been trained on spectra from other Thermo Scientific MS platforms.

Figure 6. Library dot products of experimental and Prosit predicted MS2 HCD spectra for 804 PQ500 heavy peptides.



Assays were created for 60 and 100 SPD (24 and 14 minute) total experiment lengths that included all 804 light and 804 heavy peptides. Analysis of replicate results at the nominal heavy peptide concentration yielded more than 6 points per peak and high precision for nearly all peptides for both assays.

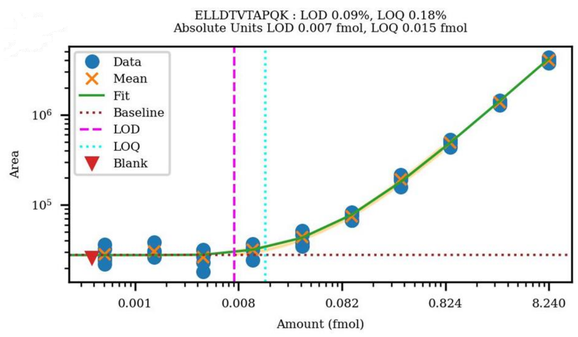
Figure 7. Points per peak a,b) and coefficients of variation c,d) for heavy peptides in 60 and 100 SPD assays for PQ500 peptides (804 light + 804 heavy).



Analysis of PQ500 dilution curves

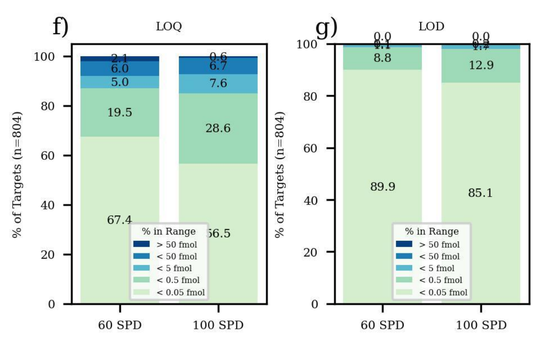
The heavy standards were diluted in 300 ng of plasma at various dilution levels. LOQ was determined as the first level with coefficient of variation < 20%, using Pinot-style[4] bootstrapping to interpolate between levels and including a constraint that LOQ > 2 x LOD. LOD was determined as the intersection of the bilinear fit to the data.

Figure 8. Example dilution curve result for ELLDTVTAPQK heavy peptide.



The LOQ and LOD values were determined for each peptide and converted to absolute concentration using the Biognosys-supplied standard amounts. The results in Figure 9 demonstrate that most peptides had LOQ and LOD below 50 attomoles. The 100 SPD results were about 40% worse on average than the 60 SPD results, for the 2x gain in throughput. The difference is likely due to the combination of higher LC flow rates and ~50% lower ion injection times at 100 SPD.

Figure 9. Summary of PQ500 dilution curve analysis.

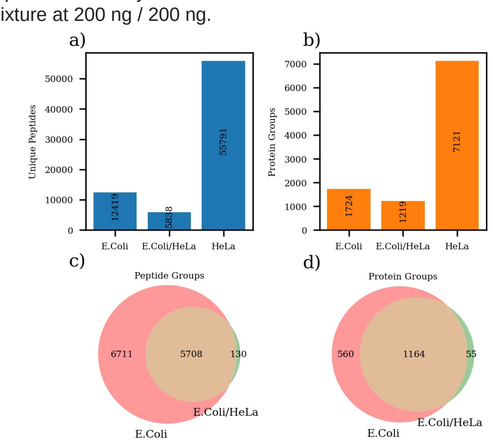


*E. Coli d*iscovery and targeted quantitation

Gas-Phase-Fractionation results

One of the primary advantages of Stellar MS relative to a triple quadrupole is that it can be used for qualitative as well as quantitative experiments. For example, a reasonable number of *E. coli* and HeLa peptides can be detected with GPF DIA experiments (Figure 10).

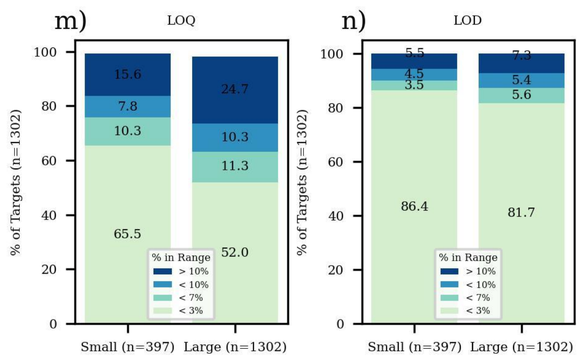
Figure 10. Peptide detections from Stellar MS GPF DIA experiment. Assays were created based on the E. Coli/HeLa mixture at 200 ng / 200 ng.



Analysis of E. Coli dilution curves

Two assays were created for 60 SPD methods based on the GPF DIA results, one with 397 targets and another with 1302 targets. Dilutions of E. Coli into a constant amount of 200 ng HeLa showed excellent limits of quantitation for both assays, which were limited somewhat at the low end by not diluting past 1%. Like with the PQ500 assay, a modest difference in LOQ and LOD about 20% and 5%, respectively, is observed when increasing the throughput by 3x, likely due to the reduction in ion accumulation time of ~30%.

Figure 10. Summary of dilution curve analysis for large and small E. Coli assays.



Conclusions

- Stellar MS had a 15x and 2x advantage in LOQ and LOD compared to a high-end triple quadrupole for a high-throughput plasma peptide assay.
- PRM Conductor software[2] in Skyline allows for rapid assay development of absolute and label-free methods.
- Stellar MS can detect respectable numbers of peptides using GPF DIA experiments that serve as the input for targeted assays.
- See our pre-print paper with data and processing tools for more information[5].

References

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