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Revolutionizing translational research: Large-scale targeted PRM proteomics assays enabled by the Stellar mass spectrometer

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Introduction

The primary developments made on Thermo Scientific™ Stellar™ MS is geared towards quantitative targeted MS^n experiments. The hardware enhancements enables faster data acquisition rates compared to previous generations of Thermo Scientific™ Linear Ion Traps (LITs). It offers capabilities to build biomarker verification workflows for translational and clinical research using biological matrices such as plasma.

Biomarker verification workflows on Stellar provide increased sensitivity, greater specificity, and extended quantitative accuracy at expanded scale and unrivaled productivity. Maximum injection times are dynamically adjusted based on assay concurrency, ensuring the longest possible injection times while preserving the necessary and optimal points-per-peak sampling rate. Chromatographic retention time shifts are managed with Adaptive RT real-time chromatogram alignment which provides real-time adjustment for each tMS2 acquisition cycle. Furthermore, software has been developed to expedite the creation of targeted assays by automating the GPF DIA or transition data to PRM strategy (Figure.1), which is implemented in a Skyline external tool called PRM Conductor.

Here, we show a thorough workflow from generating a Skyline file (and a spectral library file) using the transition list to select LC peaks, create PRM methods, and export results for further analysis within 3-4 days. The workflow was built using PQ500™ reference peptides, to profile plasma samples and quantify hundreds of proteins in a targeted manner. The PQ500 can be used to investigate novel biomarkers and study disease and possible treatment options in a research setting. A detailed tutorial along with raw data can be found on the Skyline website[1]. Linearity, LOD, LOQ, and precision (CV%) of PQ500 peptides were validated on Stellar. Overall, the Stellar MS provides a cost-effective platform for highly multiplexed targeted proteomics on a larger scale analysis compared to previous SRM and PRM technologies.

Experiment procedures

Sample preparation

For the PRM method development, PQ500 reference peptides were obtained from Biognosys AG. The samples were diluted following the manufacturer's instructions. Digested plasma were procured from Pierce™ (unreleased product) and used as a diluent at 300 ng/µl.

To verify the linearity, LOD, and LOQ, a 3x serial dilution was created with 11 steps, from 1x manufacturer's concentration = 100% to 0.005% with a 100% plasma blank for the final level.

Liquid chromatography-mass spectrometry parameters

The Thermo Scientific™ Vanquish™ Neo™ UHPLC was used with the EASY-Spray™ HPLC ES906A column with a trap-and-elute injection scheme set to 60 SPD and 100 SPD methods. Gradients were optimized and shown in Figure 2.A. Column temperature was kept at 45˚C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in 80% acetonitrile. The critical mass spectrometer parameters are shown in Figure 2.B. The scan range was *m/z* 200-1500 for the 60 SPD methods and was customized by PRM Conductor for the 100 SPD method. The eluted peptides were analyzed on a Stellar MS using both methods (Figure 2.C).

Figure 2. Liquid chromatography-mass spectrometry settings and TIC chromatogram for 60SPD and 100SPD methods

(A)

100SPD

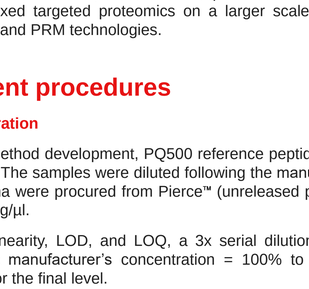
No	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
1	0.000			Run		
2	0.000	0.000	1.800	1.0	0.00	0.00
3	0.700	0.700	1.800	4.0	1.26	0.71
4	1.600	0.300	1.800	8.0	0.54	0.30
5	7.700	6.700	1.800	22.5	12.06	6.79
6	11.400	3.700	1.800	35.0	6.66	3.75
7	11.800	0.400	2.500	55.0	0.86	0.48
8	11.800			Column Wash		
9	12.300	0.500	2.500	99.0	1.25	0.70
10	13.000	0.700	2.500	99.0	1.75	0.99
11	13.000			Stop Run		
12	13.000			Column Equilibration		

60SPD

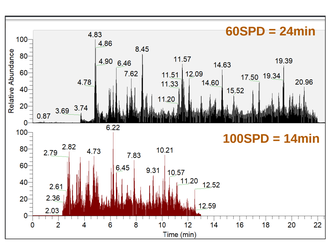
No	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
1	0.000			Run		
2	0.000	0.000	3.000	4.0	0.00	0.00
3	0.500	0.500	1.200	4.0	1.08	0.61
4	0.600	0.100	0.800	8.0	0.11	0.06
5	0.900	0.300	0.800		0.24	0.14
6	13.900	13.000	0.800	22.5	10.40	5.86
7	20.800	6.900	0.800	35.0	5.52	3.11
8	21.200	0.400	2.000	55.0	0.56	0.32
9	21.200			Column Wash		
10	21.700	0.500	3.000	99.0	1.25	0.70
11	24.000	2.300	3.000	99.0	6.90	3.89
12	24.000			Stop Run		
13	24.000			Column Equilibration		

(B)

60SPD 100SPD

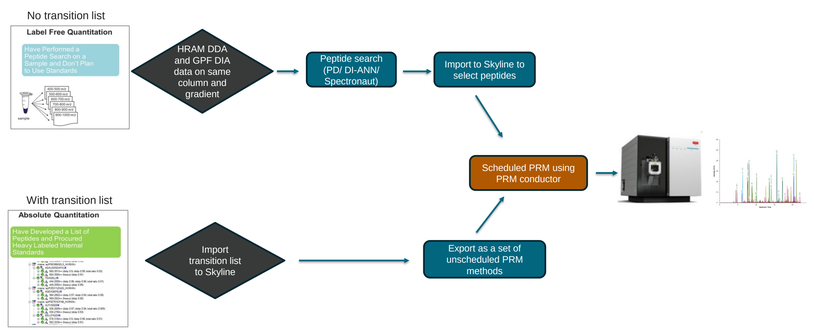


(C)



(A) Separation column gradient for 60SPD and 100SPD methods; (B) The targeted MS_2 parameters for the 100 SPD and 60 SPD methods; (C) TIC chromatogram of eluted peptides from 60SPD and 100SPD gradients.

Figure 1. Targeted assay development workflow using Stellar MS and Skyline streamline tMSn method creation and data acquisition management.



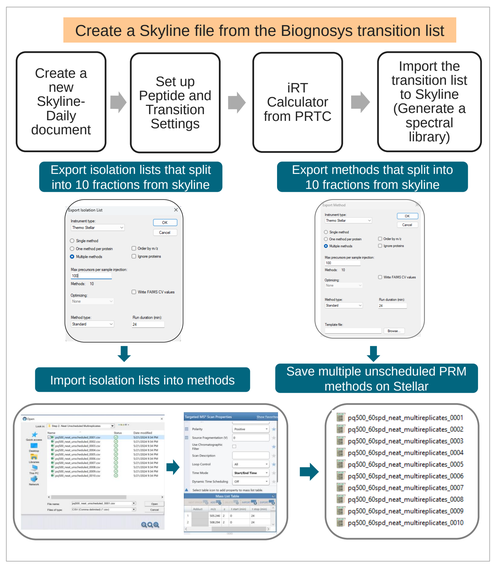
There are two main routes to create the scheduled PRM methods depending on whether the data comes from the transition list, high resolution accurate mass instrument or whether the discovery data is generated using gas phase fractionation(GPF) DIA with Stellar mass spectrometer.

PRM conductor to build multiplex targeted panel for PQ500 analysis in plasma

Step 1 (Day 1): Create unscheduled PRM methods using a transition list for neat heavy PQ500 peptides

In the first step, the PQ500 peptide transition list (provided by Biognosys) was imported into Skyline to generate a Skyline file and spectral library ((804 PQ500 peptides plus 14 PRTC)/11088 transitions). The Indexed Retention Time (iRT) calculator was created using PRTC as a reference peptide list. The unscheduled isolation lists or methods were exported from Skyline to create a set of unscheduled PRM methods. From this step, the unscheduled PRM methods were created for both 60 and 100 SPD gradients that analyzed the 804 PQ500 peptides split into 10 fractions (Figure 3).

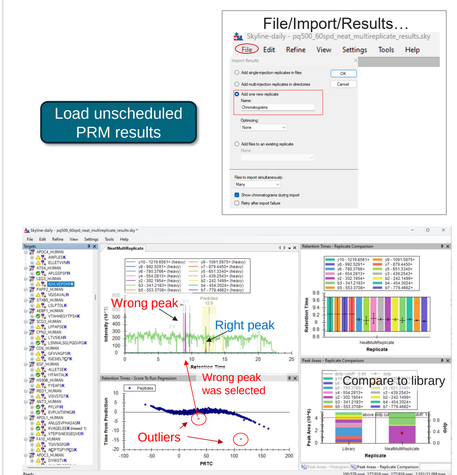
Figure 3. Build a set of unscheduled PRM methods from Biognosys PQ500 peptide transition list.



Step 2 (Day 2): Schedule a wide window PRM using neat plasma

Step 2.1 After data acquisition, the unscheduled PRM results were imported into Skyline as multi-injection replicate to assess the retention time and peptide intensities. iRT calculator and spectral library were used to assist peak picking (Figure 4).

Figure 4. Unscheduled PRM files were imported into Skyline to check the peak retention times



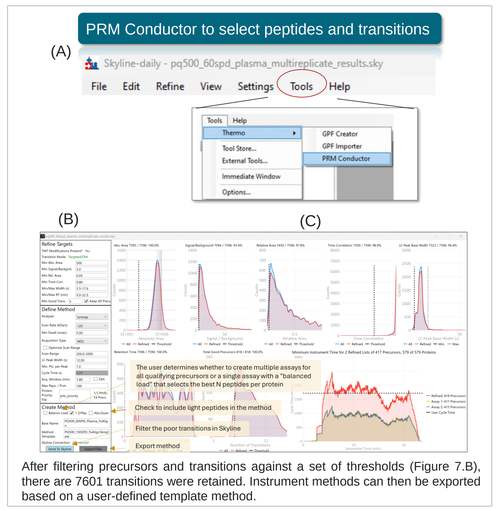
We can use Score-to-Run Regression to check RT Outliers in Skyline. These outliers can be potential missed peaks. One can click on these dots and inspect them. If wrong peak is picked, the right peak should be selected manually.

Step 2.2: Schedule PRM methods for heavy PQ500 peptides in plasma digest with wide acquisition windows

In this step (Figure 5), the PRM conductor was applied to filter precursor ions and transitions based on parameters including signal to noise, peak area, retention time and charge state (Figure 7.B). The method was refined based on the parameters including points across the peak, peak width, scan rate, acquisition window width and scan range. Without checking the Balance Load box, precursors were split into 2 assays for 60SPD method and 3 assays for 100SPD methods. The wide-window PRM methods were then exported to verify the retention time (RT) of the PQ500 heavy peptides in plasma.

After the method was ready, PQ500 peptides were spiked into 300ng of digested plasma matrix and analyzed with wide acquisition window PRM methods to confirm retention times and retention time drift in plasma matrix. After data acquisition, the result files were imported into Skyline. The retention times were compared between neat PQ500 peptides and PQ500 spiked in plasma (Figure 6).

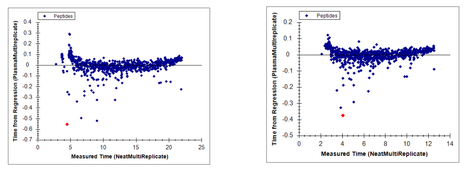
Figure 5. PRM conductor to select precursors and transitions and generate wide window acquisition PRM methods



After filtering precursors and transitions against a set of thresholds (Figure 7.B), there are 7601 transitions were retained. Instrument methods can then be exported based on a user-defined template method.

(A) Open PRM conductor; (B) Skyline metadata was utilized to filter transitions against a set of thresholds; (C) Visualization area that illustrates the concurrency of the assay.

Figure 6. The retention times from wide acquisition window PRM methods were compared



The retention times were compared between neat PQ500 peptides and spiked PQ500 in plasma. Only a very small portion of peptides RT shifted in plasma compared to neat reference peptides.

The final step (Day 3): Schedule a narrow window PRM method in plasma to analyze both heavy and light peptides

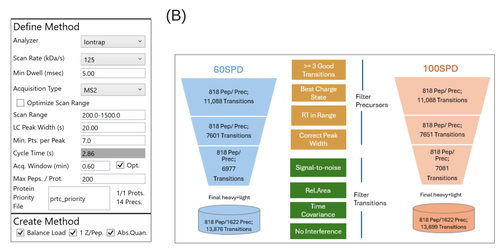
To obtain the final method, the wide window results were imported into Skyline. The acquisition window in PRM conductor was narrowed down to 0.6 min for 60SPD (Figure 7.A) and 0.35 min for 100SPD gradient. The Opt. box, Balance Load, 1 Z/prec and Abs. Quan boxes were checked. The Abs Quan option instructed the Export command to include light targets for each of the heavy targets. CV values can be used as a threshold to filter transitions with bad precisions(Refine\Advanced…\Consistency).

We changed LC Peak Width to 20 to get all targets exported in one assay in PRM conductor (Figure 7.A), and then changed the LC peak width back to 11 after the instrument method was created. In the end, there were 1622 precursors/13,876 transitions for the 60SPD method and 1622 precursors/13,699 transitions for the 100SPD method (Figure 7.B).

The final refined method was then exported from PRM conductor and 10 replicate PQ500 spiked plasma samples were analyzed using the final PRM method. The Adaptive RT real-time chromatogram alignment was also included in the exported method file.

Figure 7: The summary of precursors and transitions selection

(A) (B)



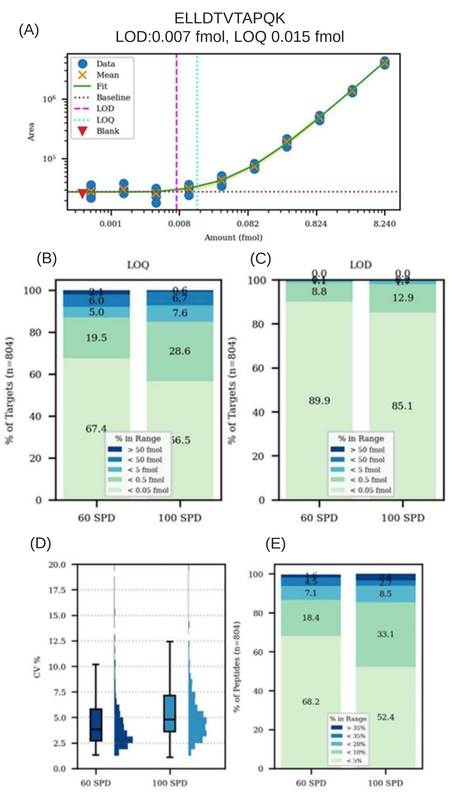
(A) The defined method parameters for the final method for 60SPD; (B) The summary of precursors and transitions selection.

PQ500 Results: Reproducibility, peptide linearity, LOD and LOQ in plasma

Dilution curves of the PQ500 into 300 ng of human plasma were analyzed using developed 60 and 100 SPD methods to assess limits of detection and quantitation. Limits of quantification and detection (LOQ and LOD) were determined offline with a similar script as was used previously which picks the set of ≥ 3 transitions that give the lowest LOQ[2]. The majority of peptides had an LOQ less than 50 attomoles, with about 85% of peptides having LOQ < 500 attomoles (Figure 8.B). The LOD's were nearly all < 50 attomoles (Figure 8.C). An example calibration curve for a well-performing peptide ELLDTVTAPQK was shown in Figure 8.A. The CV values (n=10) were < 20% for 94% peptides using 60 and 100SPD methods (Figure 8.D and E)

Results also showed that 60SPD is slightly better compared to 100 SPD method [2]. LOQs and LODs were 1.6x lower for 60SPD.

Figure 8. Reproducibility, peptide linearity, LOD and LOQ in plasma



(A) Calibration curve of peptide. ELLDTVTAPQK as an example; (B) and (C) LOD and LOQ of PQ500 peptides using 60SPD and 100SPD methods; (D) and (E) CVs of peptides using 60SPD and 100SPD methods. Data was processed in Python. Scripts are available in the Panorama repository.

Conclusions

- A targeted proteomics method was created rapidly for PQ500 reference and paired endogenous peptide measurements in plasma, showing that the Stellar mass spectrometer and Skyline combination is an ideal platform for targeted quantitative proteomics studies requiring analysis of the large-scale peptide panels.
- 94% of the PQ500 reference peptides had CV values < 20% (n=10). The majority of peptides had an LOQ < 50 attomoles, with about 85% of peptides having LOQ < 500 attomoles. The LOD's were nearly all < 50 attomoles.
- This developed and validated method will be used to quantify endogenous protein and peptide biomarkers from healthy donor and patient donor plasma samples as a next step.

References

[1] https://panoramaweb.org/prm_conductor.url

[2] Philip M. Remes, Cristina C. Jacob, etc. bioRxiv. Preprint. 2024 Jun 1. doi: 10.1101/2024.05.31.596848

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