Development of a Robust, Routine, and Highly Multiplexed Plasma Profiling Method Using UHPLC-SRM Assays

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ABSTRACT

Purpose: To demonstrate monitoring of TMT0 and TMT6 labeled peptides on the Thermo Scientific™ TSQ Altis™ MS.

Methods: Using digested peptides in plasma labeling them with TMT™ reagents on the Thermo Scientific™ Vanquish™ HPLC/TSQ Altis™ MS.

Results: Monitored a large panel of peptides to monitor TMT labeled species.

INTRODUCTION

Clinical research methods strive to maximize the number of proteins routinely quantified to better characterize individual donor samples and identify putative biomarkers. This trend, however, becomes challenging for triple quadrupole mass spectrometers as generally the analytical performance of SRM assays is inversely proportional to the number of SRM transitions monitored per assay. To increase the protein panel size, we have leveraged chromatographic peak capacities via UHPLC separations and chromatographic stability to maintain quantitative confidence for over 200 plasma proteins using 30-minute gradients. A quantitative strategy using tandem mass tag™ (TMT) reagents is used to introduce a global standard reducing the complexity of sample preparation.

MATERIALS AND METHODS

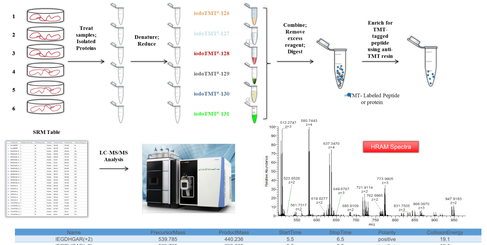
Sample Preparation

LC-MS Parameters

All experiments were performed on a TSQ Altis triple quadrupole mass spectrometer. A total of 3010 scheduled SRM transitions were monitored to quantitate 215 proteins and 430 peptides using variable dwell times depending on the targeted peptide abundance based average peak widths of 7 seconds. A Vanquish Horizon UHPLC system was used for chromatographic separations. Chromatographic separation was performed using a standard binary solvent system and 30-minute gradient resulting in 8-10 second wide peaks. A stock plasma digest sample was obtained from a single source and divided into samples labeled with Thermo Scientific™ TMTzero and Thermo Scientific™ TMTsixplex reagents and spiked at known TMTzero:TMTsixplex ratios from 1:1 to 30:1.

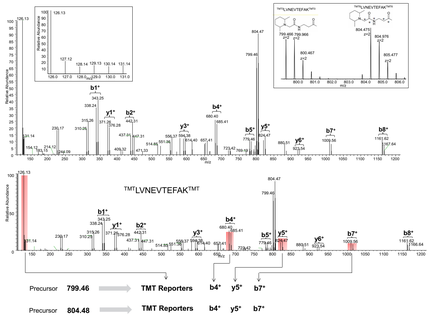
Quantitation data was processed in Thermo Scientific™ TraceFinder™ software. Peptide optimization was performed in Skyline 3.1 (University of Washington).

Figure 1. Workflow of TMT labeling in LC/MS analysis.



Peptide	Precursor m/z	Product m/z	Start (min)	End (min)	Polarity	Collision Energy
IEGDHGAR(+2)	539.785	440.236	5.5	6.5	positive	19.1
IEGDHGAR(+2)	539.785	539.325	5.5	6.5	positive	19.1
IEGDHGAR(+2)	542.29	440.236	5.5	6.5	positive	19.2
IEGDHGAR(+2)	542.29	644.345	5.5	6.5	positive	19.2
IEGDHGAR(+2)	542.29	741.377	5.5	6.5	positive	19.2
VTSGSTTTTR(+2)	617.835	757.409	6	7	positive	21.4
TMT 0 VTSGSTTTTR(+2)	617.835	810.395	6	7	positive	21.4
VTSGSTTTTR(+2)	617.835	911.443	6	7	positive	21.4
VTSGSTTTTR(+2)	620.34	762.42	6	7	positive	21.5
TMT 6 VTSGSTTTTR(+2)	620.34	810.395	6	7	positive	21.5
VTSGSTTTTR(+2)	620.34	911.443	6	7	positive	21.5
NFANPVQR(+2)	560.317	499.299	7	8	positive	19.7
NFANPVQR(+2)	560.317	621.335	7	8	positive	19.7
NFANPVQR(+2)	560.317	781.432	7	8	positive	19.7
NFANPVQR(+2)	562.822	499.299	7	8	positive	19.8
NFANPVQR(+2)	562.822	626.346	7	8	positive	19.8
NFANPVQR(+2)	562.822	781.432	7	8	positive	19.8

Figure 2. Comprehensive HSRAM spectra from discovery experiments which are used to obtain the initial information for peptide fragmentation.



Results

The experimental method utilized demonstrated robust, accurate, and reproducible quantitation for over 200 medium- to high-level plasma proteins listed in prior publications. Incorporation of UHPLC separation methods maximized peak capacities (6-8 second wide peaks) and stable retention times (ca. 0.1% variance) enabling much shorter SRM scheduling windows facilitating highly multiplexed assays. In addition, the TSQ Altis triple quadrupole mass spectrometer performs exceptionally well at very short (<3 msec) dwell times which is key for 30-minute chromatographic gradients. High-resolution data of what the spectra look like with different labels is found in Figure 4.

The targeted peptide list was created using a novel automated selection routine in Pinnacle software based on the user-defined protein list and a set of experimental parameters. The routine required only two rounds of method refinement prior to routine data collection. Utilization of the TMTzero sample as the global standard further reduced method development time and cost as all targeted peptides were present at expected biological levels and confirmed using the spiking levels.

Over 87% of the targeted peptides had a coefficient of variance <20% despite some of the transitions being monitored using 3 msec dwell times. Evaluation of the measured AUC ratios between the TMTzero and TMTsixplex peptides showed excellent agreement for 201 proteins whereas the remaining 14 had AUC ratios outside of the allotted thresholds. While the presented method used only one of the TMTsixplex tags to perform relative AUC determination, extensions will be made to mix multiple TMTsixplex tags for a multiplexed sample analysis relative to the TMTzero standard.

Figure 3 demonstrates the various area responses for the peptides being monitored. The retention time reproducibility.

Figure 3. SRM chromatogram for all peptides being monitored and retention time for various peptides across the different samples.

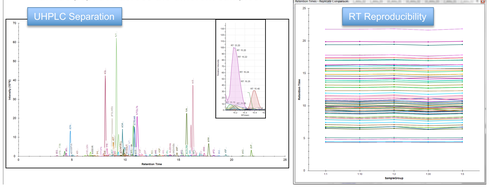
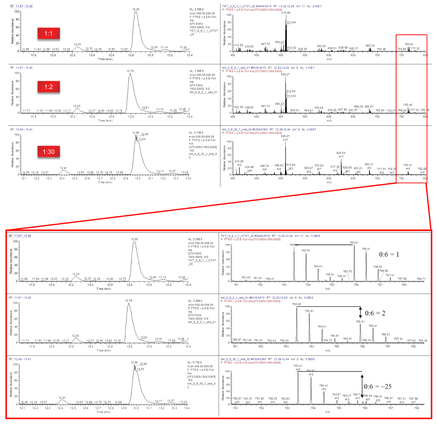


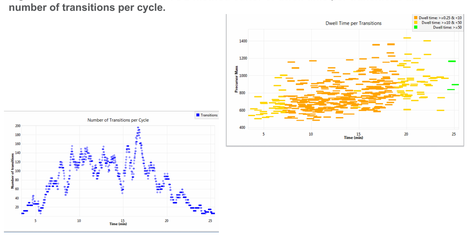
Figure 4. Extracted chromatograms and their corresponding spectra for different ratios of TMT6:TMT0.



High flow, UHPLC chromatography facilitates large numbers of targeted proteins/peptides while maximizing sample throughout. UHPLC separation results in narrow chromatographic peak widths (6-12 second wide peaks) and stable retention time performance (less than 0.02% retention time variance) providing similar peak capacities using a 30-minute gradient as that for 2 hour gradients using nanoflow rates.

Figure 5 demonstrates the extent of mass ranges being monitored and their corresponding dwell times, and the numbers of transitions being acquired throughout the LC run.

Figure 5. Visualization tool from TSQ method editor of dwell time per transitions and number of transitions per cycle.



215 Proteins; 430 Peptides; >2,500 Transitions

Figure 6. SRM transition of different ratios of TMT0 and TMT6.

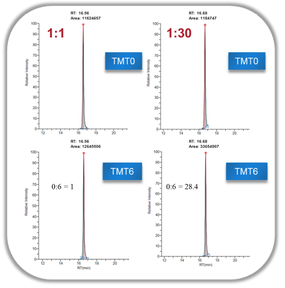
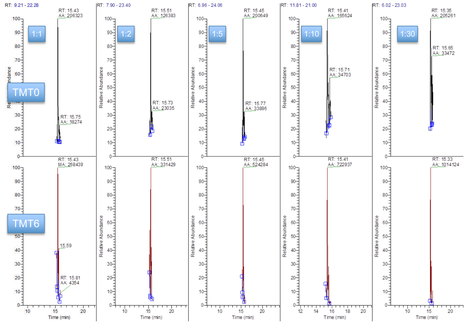


Figure 7. SRM transitions of different ratios of TMT0 and TMT6. Note TMT0 was not spiked in each concentrations for each of the different ratios.



Evaluation of the automated SRM method creation was performed based on conservation of retention time and product ion distribution. The retention time variance was less than 0.5% for all targeted peptides between instruments as well as within replicates on each mass spectrometer resulting in the creation of highly multiplexed, scheduled SRM transition methods. The highly multiplexed method facilitated both large numbers of peptides as well as an increased number of SRM transitions for the light and heavy version of each peptide to perform global targeted profiling. Second, the overlap between ion ratios observed between the light and heavy peptide versions were consistent between the discovery (DDA) and SRM quantitation enabled highly confident automated SRM data processing to determine data quality based on qualitative and quantitative measurements. Over 85% of the targeted peptides showed accurate AUC ratio determination for the TMT0/6 spiking experiment as defined by variance measurements and AUC ratios within 15%. In addition, the ion ratios showed over 87% of the targets with dot-product correlation coefficients in excess of 0.65. Various examples of different peptides can be found in Figure 6 through 8 demonstrating a difference in peak area.

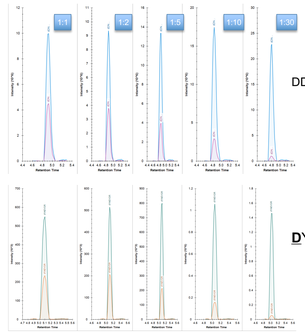


Figure 8. Overlaid chromatograms of TMT0 and TMT6 at different spiking levels. Pink chromatogram is represent of TMT6.

CONCLUSIONS

- The presented workflow leveraged high peak capacities due to UHPLC separations and unique triple quadrupole mass spectrometer capabilities to acquire highly multiplexed plasma profiling experiments. The results presented demonstrated to ability to screen a large amount of peptide transitions.
- Narrow chromatographic peak widths and stable retention times enabled much tighter scheduled acquisition windows per peptide.
- Variable dwell time settings ranging from 3 to 15 msec based on a unique method building process.
- The ability to perform relative peptide quantitation based on either sequence ions or the low-mass reporter ions from which the number of samples quantitated per injection could be increased to six.

FUTURE WORK

1. To label peptides with various TMTsix labels and have reporter ions be in the transition list.
2. Obtain clinical samples and do correlation studies to assays being run in these laboratories.

ACKNOWLEDGMENTS

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TRADEMARKS/LICENSING

Thermo Fisher SCIENTIFIC

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