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Evaluation of Comprehensive Quantification Assays for Plasma Protein Analysis Using a Novel Triple Quadrupole LC/MS

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Introduction

Introduction

Targeted multiplexing assays using triple quadrupole LC/MS system have been increasingly used for proteome-wide quantitation analysis. Plasma from human or model animals is one of the most used sample types for academic and clinical research. However, it is challenging to detect and quantify proteins in plasma due to the sample complexity and huge dynamic range.

In this study, we evaluated large panel peptide quantification assays for protein biomarker analysis in human and mouse plasma using a novel intelligent 6495 triple quadrupole LC/MS system (G6495D) which is implemented with the 4th generation iFunnel technology and MassHunter software 12.1 (Figure 1).

Key enhanced features on the 6495 LC/TQ (G6495D) for improving peptide quantification workflow include:

- Intelligent SWAM autotune and maintenance automation
- Streamlined LC/MS/MS method optimization with integrated Agilent Automation tool in Skyline software and MassHunter software 12.1
- iFunnel enhancements providing analytical sensitivity gain
- Improved acquisition cycle time (including sub milli sec dwell times) allowing more concurrent MRMs and total MRMs in a single method

Experimental

Instrumentation

Agilent 1290 Infinity II Bio LC system coupled to a 6495 Triple Quadrupole LC/MS system (G6495D) with Jet Stream Technology Ion Source (AJS).

Materials

Raw human plasma was purchased from Bioreclamation. PeptiQuant kits for quantitative analysis of human plasma protein and mouse plasma protein were from MRM Proteomics Inc.

LC/MS analysis

All samples were separated using the Agilent ZORBAX Eclipse Plus Rapid Resolution C18 analytical column: 50 × 2.1 mm, 1.8 µm in size (p/n 959757-902).

LC/MS method shown in Table 1. Data acquisition and analysis were carried out using MassHunter software 12.1 and Skyline software.

Experimental

Instrumentation

- 1290 Bio High-Speed Pump (G7132A)
- 1290 Bio Multisampler with Cooler (G7137A)
- 1290 Multicolumn Thermostat (G7116B)
- 6495 Triple Quadrupole LC/MS (G6495D) with AJS

1290 Infinity II Bio LC System		
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (p/n 959759-902)	
Sampler temp.	4 °C	
Mobile phase	A) H_2O, 0.1% formic acid B) Acetonitrile, 0.1% formic acid	
Flow rate	0.4 mL/min	
Injection volume	10 µL	
Gradient program	Time	B (%)
	0.00	2
	2.00	7
	50.00	30
	53	45
	53.5	80
	55.5	80
	56.0	2
Post time	4 minutes	
6495 Triple Quadrupole Mass Spectrometer		
Ion source	AJS	
Polarity	Positive	
Gas temperature	150 °C	
Drying gas	17 L/min	
Nebulizer gas	30 psi	
Sheath gas	250 °C	
Sheath gas flow	12 L/min	
Capillary voltage	3500 V	
Nozzle voltage	0 V	
MS1/MS2 resolution	Unit/Unit	
Autotune mode	Large molecule mode	

Table 1. LC/MS Method.

Figure 1. 1290 Infinity II LC and the novel 6495 LC/TQ.

Enhanced MRM Response

Stable heavy-isotope labeled standard (SIS) peptide mixture for 101 peptide sequences was spiked at 2.5 amol/ µL in human plasma protein digest. Both heavy and matched endogenous peptides were monitored with a dMRM method. The same sample was analyzed on both the novel 6495 LC/TQ (G6495D) and its predecessor.

Figure 2 shows the overlaid comparison of total MRM response performed on the 6495 LC/TQ (G6495D) and on the predecessor.

An ~2-3 -fold increase in MS signal response was observed on the 6495 LC/TQ (G6495D).

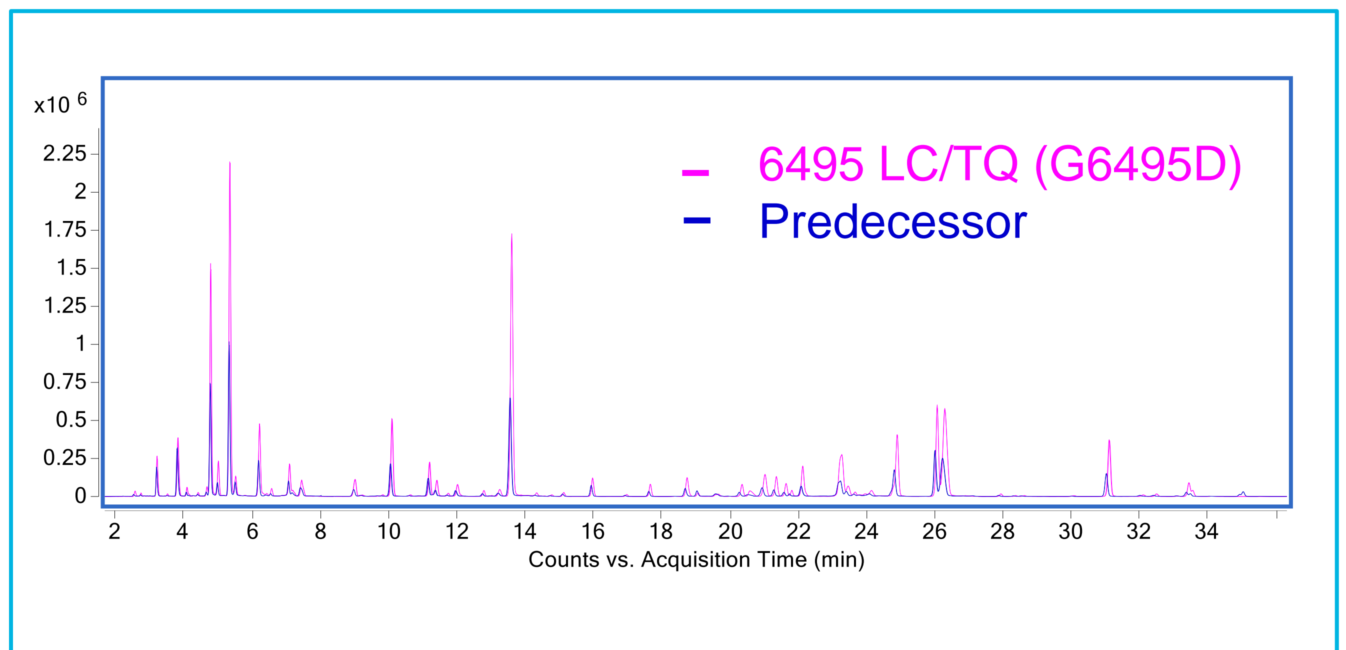

Figure 2. Overlaid total MRM chromatograms from 6495 LC/TQ (G6495D) and predecessor system

Quantification Sensitivity for Peptide Analysis

To evaluate quantification sensitivity of peptides in complex matrix, the SIS mixture was spiked into 0.5 µg/µL human plasma protein digest at various concentrations. Replicate (n=5) injections were made for all sample levels to produce linear standard curves for targeted SIS peptides using the 6495 LC/TQ (G6495D).

Quantification results of a selected heavy peptide ATEHLSTLSEK in plasma matrix :

- Outstanding linearity with R^2 =0.9996 for a wide dynamic range of 1 amol/µL to 50 fmol/µL (Figure 3A)
- Excellent precision and accuracy at low levels ranging from 1 amol/µL to 50 amol/µL (Figure 3B)
- Very good chromatograms, S/N, precision, and accuracy were achieved in complex matrix with low on-column amount (Figure 4)

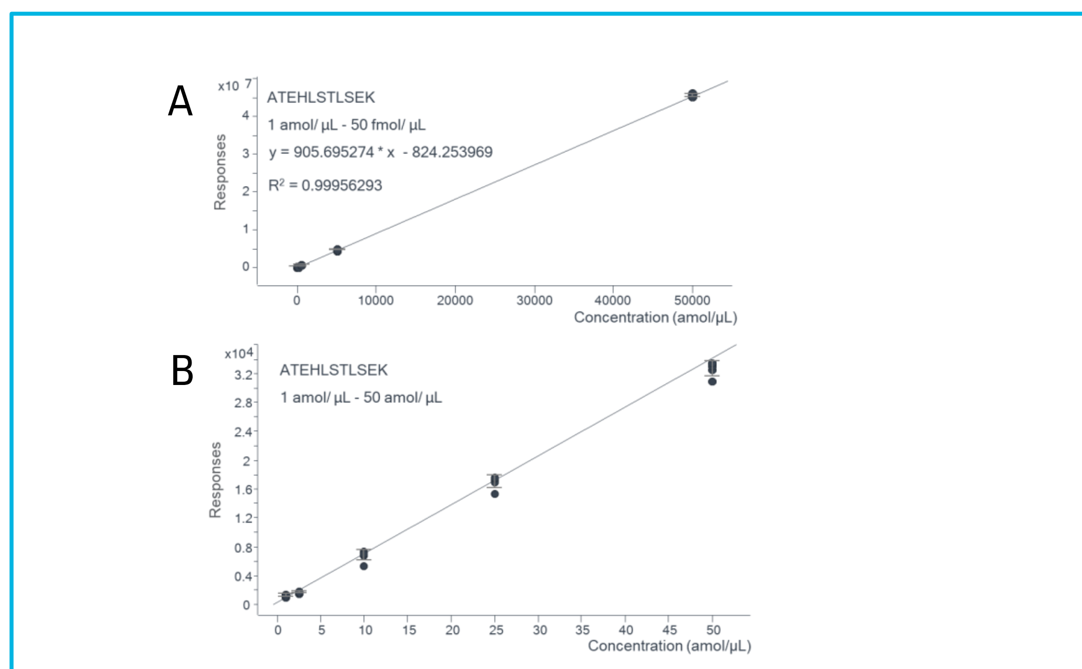

Figure 3. Standard curve of SIS peptide ATEHLSTLSEK in 0.5 µg/µL human plasma protein digest ranging from 1 amol/µL to 50 amol/µL.

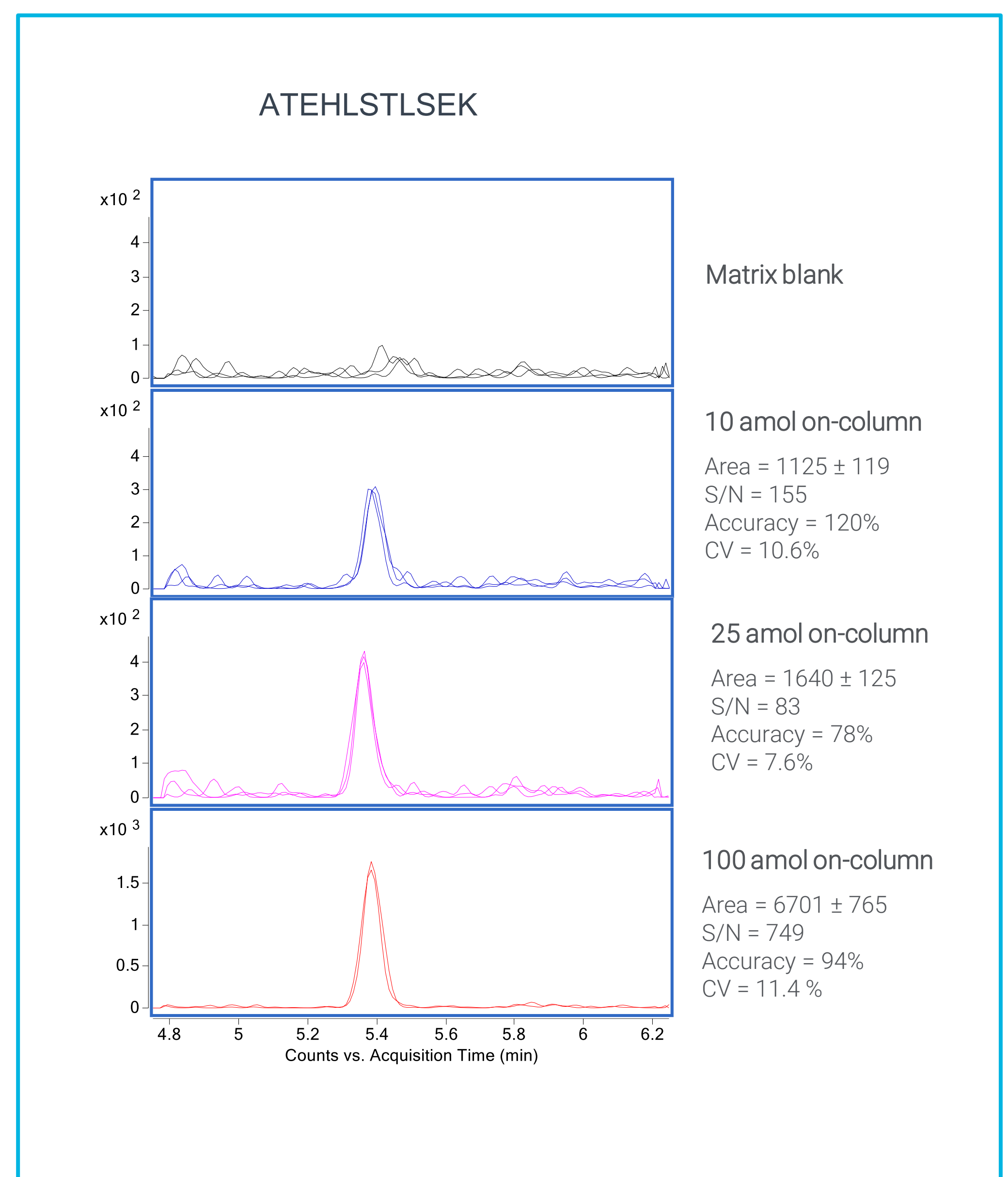

Figure 4. MRM chromatograms of SIS peptide ATEHLSTLSEK in human plasma protein digest with low on-column loading.

Results and Discussion

Large Panel Quantification Assay

Quantitating large numbers of analytes reproducibly in a single sample can be challenging. The 6495 LC/TQ (G6495D) with enhanced acquisition cycle time (sub milli second dwell times) and comprehensive dMRM/MRM methods (more total MRMs and concurrent MRMs in a single method) enables high precision sampling of complex samples.

To evaluate this feature for peptide analysis, a large panel dMRM method from PeptiQuant Plus kits which covers 375 mouse plasma proteins was applied to analyze a batch of 56 mouse plasma samples. A pooled sample was prepared and used as a sample QC, which was analyzed nine times and evenly distributed across the 3-days instrument acquisition time. The results are summarized below:

- The dMRM acquisition method with a total of 2250 transitions, 204 maximum concurrent MRMs, and 650 millisecond (ms) cycle time was successfully applied (Figure 5)
- Most peptides (80.8%) have an average acquisition dwell time < 4 ms in this one-hour method (Figure 6)
- The peak areas of all the MRMs from targeted SIS peptides span over 6-orders of dynamic range
- The distribution of normalized peak area CVs of detected SIS peptides shows a median value of 4.9% and 100% having a CV < 20% (Figure 7)

The above results demonstrated that the enhanced 6495 LC/TQ (G6495D) is suitable for large panel multiplexing peptide quantitation analysis.

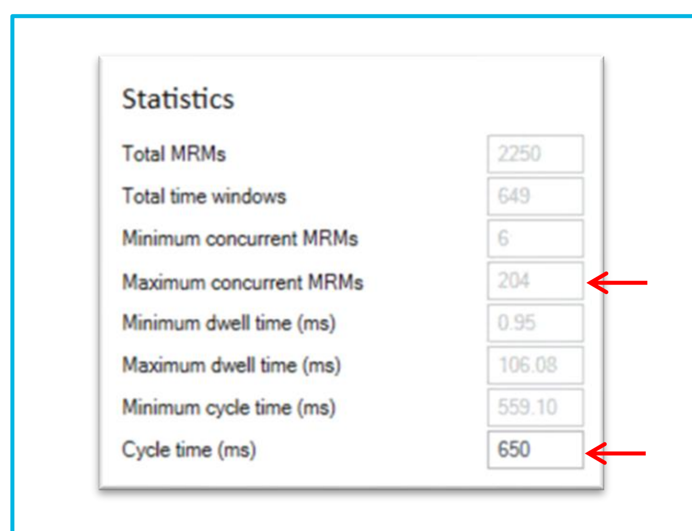

Figure 5. Screen capture of statistics of a large panel dMRM acquisition method on the 6495 LC/TQ (G6495D)

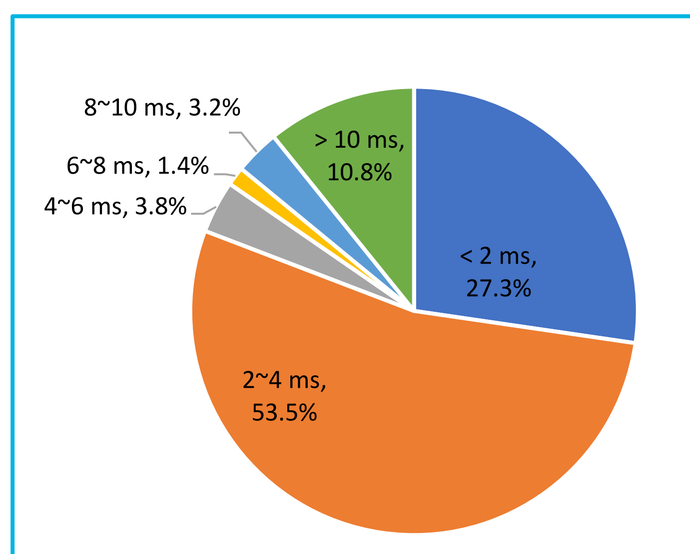

Figure 6. Distribution of acquisition time for all the targeted peptides

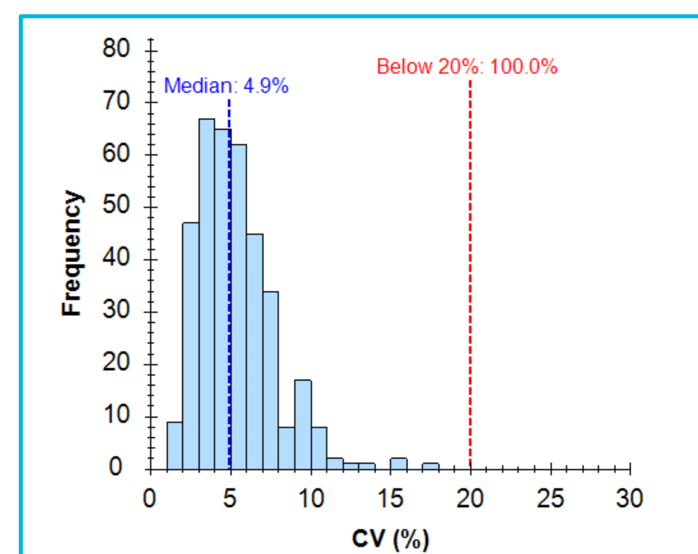

Figure 7. Distribution of normalized peak area CV (%) for all the detected SIS peptides based on QC injections (n=9).

Conclusions

This study demonstrated the 6495 LC/TQ (G6495D) is suitable for comprehensive protein quantification assays in complex matrix:

- The hardware enhancements provide an ~2-3 -fold increase in MRM signal responses
- Improved analytical sensitivity shows excellent quantification in complex matrix
- Reproducible peak areas were achieved with a large panel dMRM method during a 3-days study on mouse plasma samples

Acknowledgement

We thank MRM Proteomics Inc. for providing PeptiQuant Plus kits for mouse plasma protein analysis.

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