Application Note

Proteomics

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Comprehensive Protein Quantification in Plasma Using the Agilent 6495D Triple Quadrupole LC/MS System

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Abstract

This application note highlights the ultrahigh performance of the novel Agilent 6495D triple quadrupole liquid chromatography/mass spectrometry (LC/TQ) system with Agilent MassHunter Workstation software version 12.1 for comprehensive multiplexed protein quantification in human plasma. Artificial intelligence (AI)-based SWARM autotune and enhanced system features provide seamless protein quantification method optimization, ultimate analytical sensitivity, and high analytical precision as well as user-friendly instrument care and maintenance. Learn how using a standardized LC/TQ configuration can save time so that users can quickly start generating biological insights.

Introduction

In proteomics research, multiplexed mass spectrometry using multiple reaction monitoring (MRM)-based liquid chromatography/mass spectrometry (LC/MS) methods has become increasingly important for quantification of protein biomarkers. To quantify protein biomarkers at biologically relevant levels, researchers often preselect signature peptides from protein biomarkers, then quantify those selected peptides in digested protein matrices with the use of stable isotope-labeled standard (SIS) peptides.[1,2] This requires LC/MS instruments that are highly sensitive, accurate, and reproducible. Furthermore, to investigate synergistic effects and expand study coverage, researchers often target signature peptides from many protein biomarkers and monitor expression changes across biological cohorts. This type of study often requires analytical platforms to have ultrahigh performance while simultaneously monitoring as many analytes as possible. Agilent has developed a novel 6495D triple quadrupole mass spectrometer with several enhanced features, which will benefit comprehensive peptide quantification in complex matrices.

This application note demonstrates the high-performance of the Agilent 6495D triple quadrupole LC/MS system (LC/TQ) system for peptide quantification in human plasma. MRM method development for multiplexed protein quantification panel assays is an iterative and time-consuming process. The integrated Skyline-Agilent automated MRM method development workflow can be enabled on the 6495D LC/TQ with MassHunter Workstation software version 12.1. The comparison of MS signal responses between the novel 6495D LC/TQ and its predecessor demonstrated significant MS signal gain. The standard curve analysis of stable isotope-labeled standard (SIS) peptides in plasma shows the ultimate analytical sensitivity on the 6495D LC/TQ. Excellent MS signal reproducibility with submillisecond acquisition dwell time dramatically improves the system capability to handle large panel multiplexed mass spectrometry methods. The novel 6495D LC/TQ provides state-of-the-art technology for comprehensive peptide quantification analysis in complex matrices.

Experimental

Instrumentation

Standardized omics LC configuration:

- Agilent 1290 Infinity II bio LC system including:
  - 1290 Infinity II bio high-speed pump (G7132A)
  - 1290 Infinity II bio multisampler with thermostat (G7137A)
  - 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6495D triple quadrupole LC/MS system (G6495D)

Materials

Raw human plasma was purchased from Bioreclamation (catalog number HMPLEDTA2). A PeptiQuant biomarker assessment kit (part number BAK-A6495-76, MRM Proteomics Inc.) was purchased from Cambridge Isotope Laboratories.

Sample preparation

Human plasma was diluted with 25 mM ammonium bicarbonate followed by denaturation with trifluoroethanol (TFE), reduction with dithiothreitol (DTT), and alkylation with iodoacetamide. The sample was then digested with trypsin overnight at 37 °C. The digested plasma was dried down, then reconstituted and spiked with the SIS peptide mixture at various concentrations yielding standard samples. The standard samples had a dynamic range of 1.25 amol/µL to 50 fmol/µL in 0.5 µg/µL of human plasma protein digest.

LC/MS analysis

For standard curve analysis, all standard samples were analyzed with replicates (n = 5) using the LC/MS/MS acquisition method provided by the PeptiQuant biomarker assessment kit. Agilent MassHunter Workstation software version 12.1 was used for data acquisition. The LC/TQ method parameters are listed in Table 1.

Data processing

Skyline software from MacCoss lab at University of Washington and Agilent MassHunter Workstation software version 12.1 were used for data analysis.

Table 1. LC/MS parameters.

Agilent 1290 Infinity II Bio LC System	
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (part number 959759-902)
Sampler Temperature	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 (min) B (%) 0.00 2 2.00 7 50.00 30 53 45 53.5 80 55.0 80
Stop Time	55.0 minutes
Post Time	5 minutes
Agilent 6495D 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	3,500 V
Nozzle Voltage	0 V
MS1/MS2 Resolution	Unit/unit
Autotune Mode	Large molecule mode

Results and discussion

Integrated Skyline-Agilent automation workflow

MassHunter acquisition software has been integrated with Skyline software on Agilent legacy LC/MS instruments for a long time.[3] This workflow allows users to start peptide method optimization from a customized Skyline file that automatically creates a series of LC/MS methods for retention time (RT) determination and collision energy optimization. The methods are then exported as a dynamic MRM (dMRM) method without user interference (Figure 1). This workflow is also integrated in the latest MassHunter Workstation software version 12.1 with improvements to accommodate enhancements in the 6495D LC/TQ system. The 6495D LC/TQ contains several features that are especially suitable for comprehensive peptide analysis, including:

- Intelligent SWAM autotune, scheduled tune, and early maintenance feedback (EMF) providing user-friendly instrument care and maintenance
- iFunnel enhancements provide analytical sensitivity gain
- Improved acquisition cycle time with submillisecond dwell time allowing 150% more concurrent dMRMs, enabling a larger panel of targeted peptides in a single method
- Intelligent reflex workflow for data-driven automation, saving instrument time and enhancing lab productivity

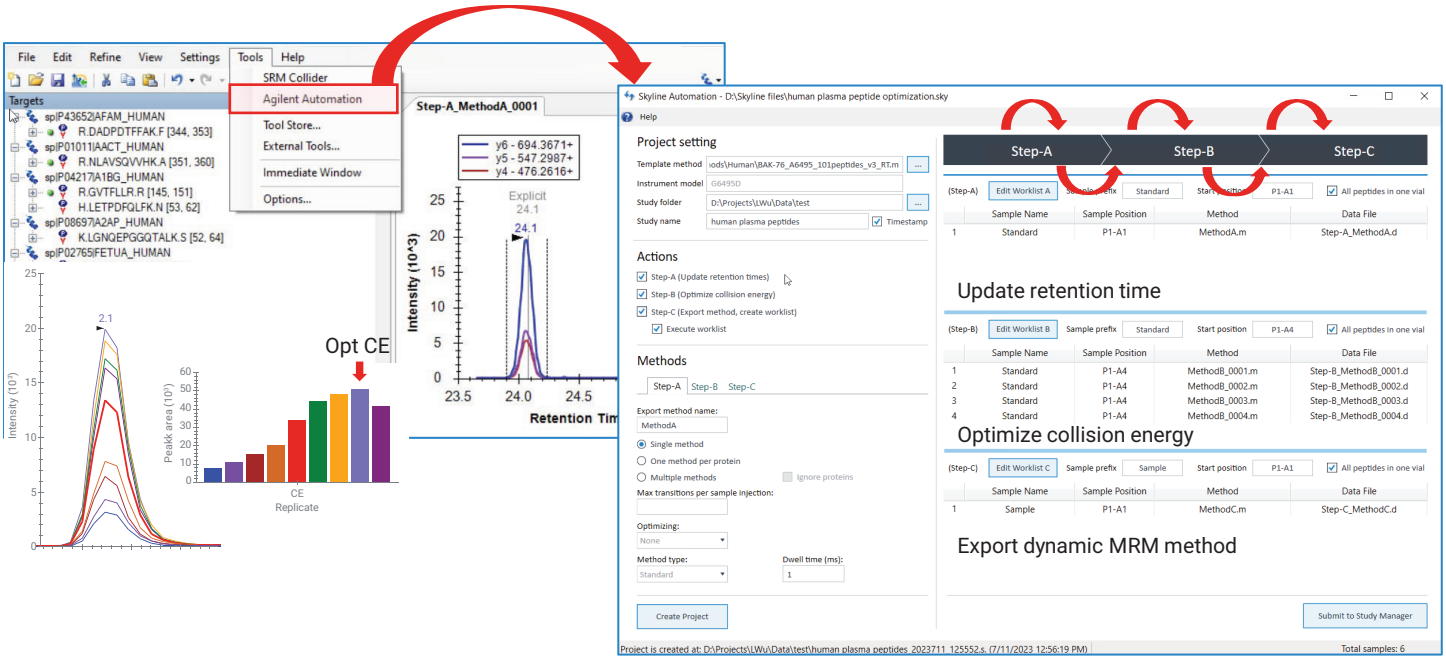

Figure 1. Schematic overview of Skyline-Agilent automation workflow for MRM-based LC/MS method optimization.

MS signal response gain

The 6495D triple quadrupole mass spectrometer is implemented with fourth-generation iFunnel technology, resulting in an improvement of signal response and detection limit compared to the predecessor. To evaluate the MS signal response of peptides in a complex matrix using the 6495D LC/TQ system, 101 peptide pairs (SIS peptides and their corresponding endogenous peptides) matching 76 human plasma protein biomarkers were analyzed. The analysis was performed in a human plasma protein digest spiked with SIS peptides. The same sample was analyzed using the same LC system on the 6495D LC/TQ and its predecessor for head-to-head comparison. The observations are summarized as follows:

- An increased MS signal response was observed for all of the targeted peptides (Figure 2).
- A median 2.4-fold increase in MRM signal response was observed using the 6495D LC/TQ without an obvious increase in background noise.
- MRM peak comparison of a selected SIS peptide (ATEHLSTLSEK), which was spiked in plasma, demonstrated a 2.2-fold increase in MS signal response and improvement of quantification precision at a low on-column amount (Figure 3).

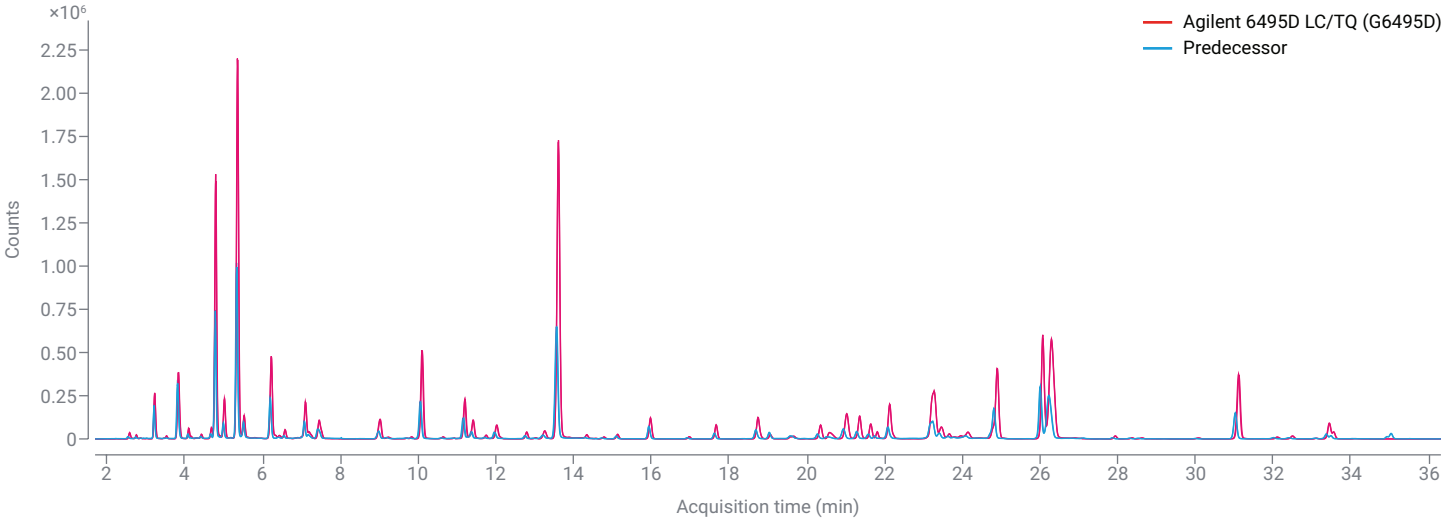

Figure 2. Overlaid total MRM chromatograms from the Agilent 6495D LC/TQ (G6495D) and predecessor system.

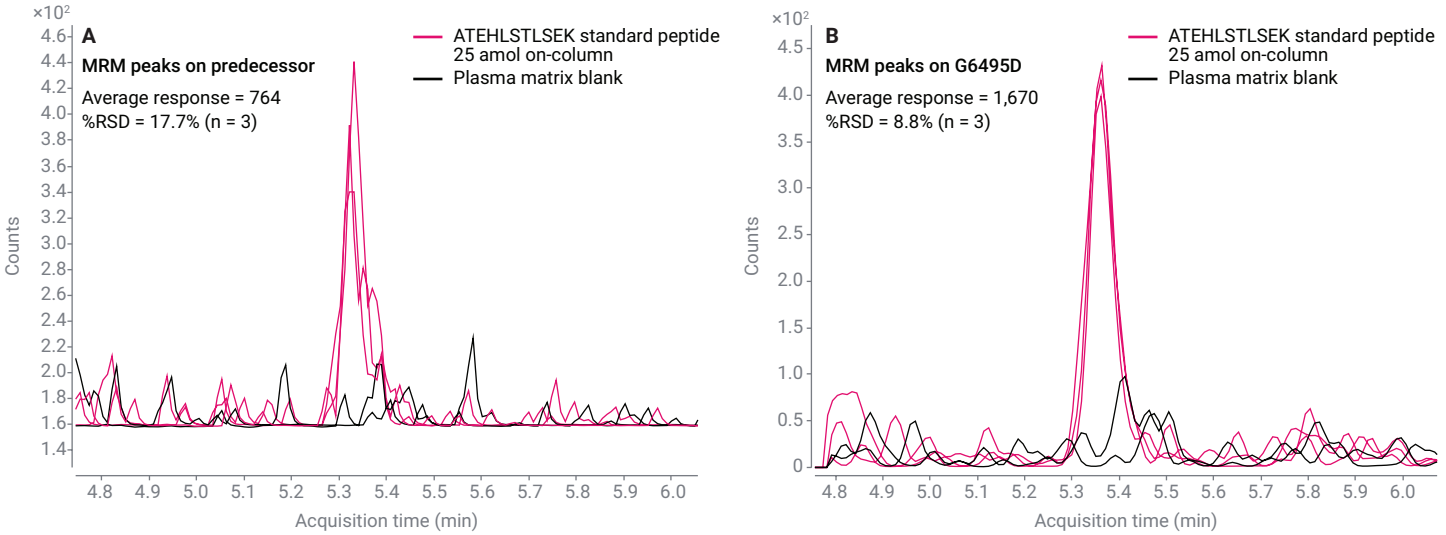

Figure 3. Comparison of replicate MRM peaks of selected SIS peptide (ATEHLSTLSEK) in plasma from the Agilent 6495D LC/TQ (G6495D) and the predecessor system. Improvements on signal response and quantification precision with low on-column sample loading were achieved with the novel Agilent 6495D LC/TQ.

Excellent quantification sensitivity

To evaluate quantification sensitivity of peptides in a complex matrix, the SIS peptide mixture was spiked in 0.5 µg/µL human plasma protein digest at different concentrations ranging from 1.25 amol/µL to 50 fmol/µL. Replicate (n = 5) injections were analyzed for all concentration levels to produce standard curves using the 6495D LC/TQ.

Excellent quantification performance was achieved using the 6495D LC/TQ. The results show a wide dynamic range linearity in plasma (1.25 amol/µL to 50 fmol/µL) for most peptides. A log scale view of linear standard curves at low concentrations was created for two selected SIS peptides ATEHLSTLSEK and SGIPIVTSPYQIHFTK to show quantification precision and accuracy at low abundance. Both peptides were successfully quantified at 1.25 amol/µL with excellent precision and accuracy (Figure 4 and Table 2). These results demonstrate the great analytical sensitivity in heavy matrix using the 6495D LC/TQ.

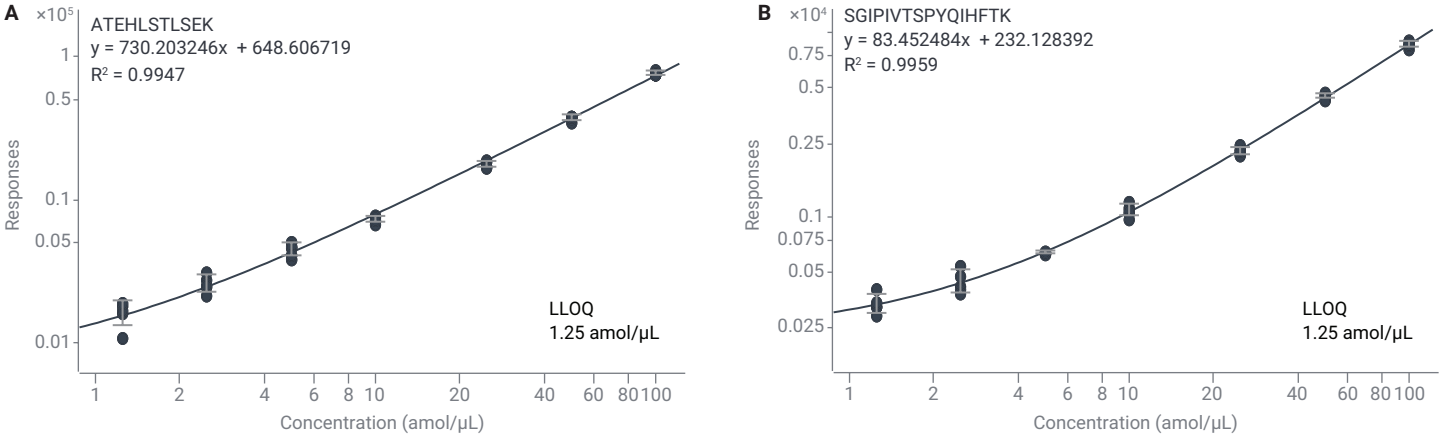

Figure 4. Log scale view of linear standard curves of selected SIS peptides in plasma. (A) ATEHLSTLSEK peptide from Apolipoprotein A-I. (B) SGIPIVTSPYQIHFTK peptide from Complement C3. Lower limit of quantification (LLOQ) of 1.25 amol/µL was achieved for both peptides using quantification criteria of %RSD <20% and accuracy within 80 to 120%. These results demonstrate the excellent quantification sensitivity in heavy matrix on the 6495D LC/TQ.

Table 2. Precision and accuracy for the selected SIS peptides in plasma.

Concentration (amol/µL)	ATEHLSTLSEK (n = 5)			SGIPIVTSPYQIHFTK (n = 5)		
	Average Response	RSD (%)	Accuracy (%)	Response	RSD (%)	Accuracy (%)
1.25	1,617	19.4	106.1	337	12.1	100.3
2.5	2,585	14.3	106.1	446	14.3	102.4
5	4,436	10.7	103.7	638	1.4	97.2
10	7,217	4.7	90.0	1,078	7.9	101.3
25	17,413	4.9	91.8	2,261	4.7	97.2
50	36,903	3.6	99.3	4,477	3.3	101.7
100	75,846	3.4	103.0	8,558	3.5	99.8

High precision with submillisecond dwell time

The 6495D LC/TQ continues the excellent MS signal reproducibility with submillisecond dwell time of Agilent legacy LC/TQs. In addition, it is implemented with enhanced acquisition cycle time, which enables analysis of up to 500 concurrent static MRM or dMRMs.

To evaluate analytical precision with a minimal dwell time in a real biological sample, a single large-panel MRM method was created. This method covers the 101 endogenous peptides from 76 human plasma protein biomarkers and was used to analyze replicate injections (n = 20) of the human plasma protein digest sample. The final method contains 303 concurrent static MRMs and 0.5 ms dwell time for each ion transition, resulting in a cycle time of 832 ms. The results were summarized as follows:

- With 303 concurrent MRMs and 0.5 ms dwell time per MRM, the system collected enough data points (17 points) across a narrow peptide peak (0.25 minutes peak width), providing high quantification precision (Figure 5).
- Excellent reproducibility of signal responses was observed for most targeted endogenous human plasma peptides, resulting in 79 peptides with %RSD <20% and 60 peptides with %RSD <10%. Note that some of the targeted endogenous peptides have low abundance in human plasma leading to the high %RSD observed in this test.
- Figure 6 shows the distribution of signal responses for four selected peptides covering a wide signal dynamic range (3.45E+04 to ~1.22E+07) and various retention times (5.88, 11.25, 13.55, and 20.23 minutes, respectively).

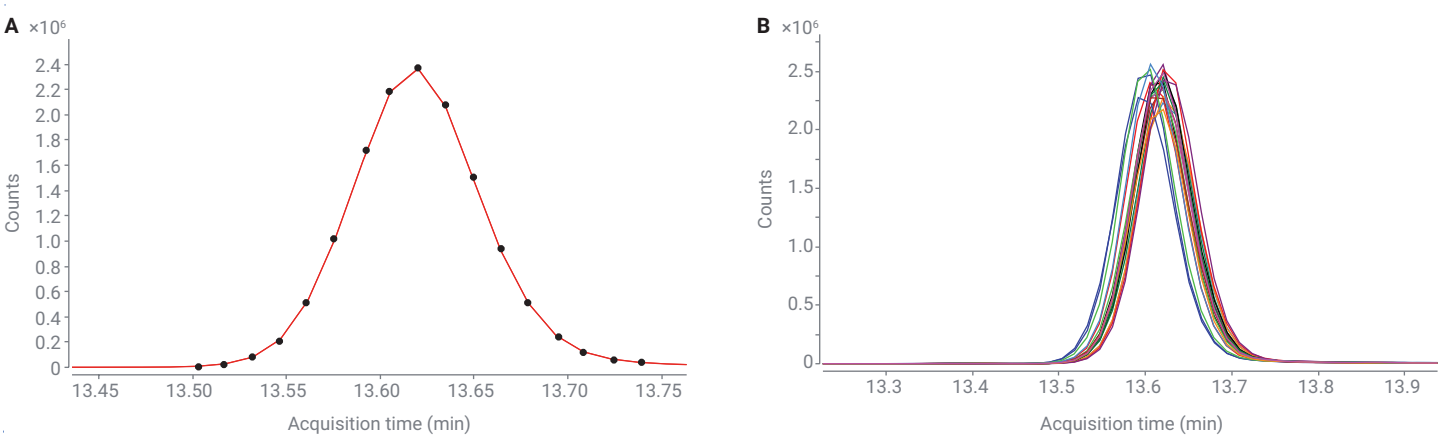

Figure 5. Enhanced acquisition cycle time enables a large panel acquisition method with 303 concurrent MRMs and small dwell time. (A) 17 data points were acquired across a narrow peptide peak with 303 concurrent MRMs. (B) Overlaid MRM chromatograms of replicate (n = 20) analyses of a representative transition acquired with 0.5 ms dwell time, showing excellent analytical reproducibility.

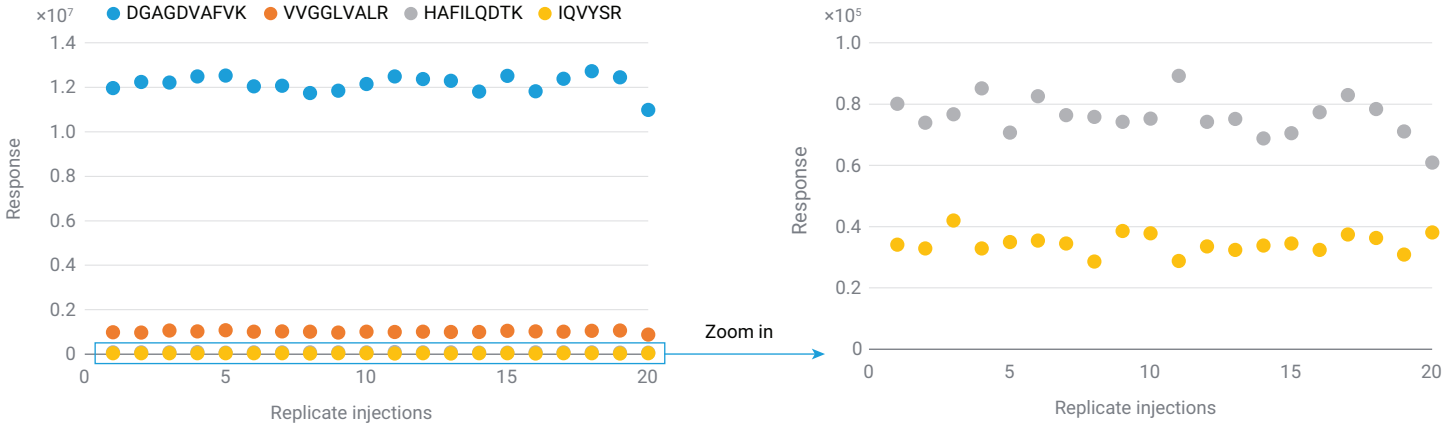

Figure 6. Distribution of MS responses for four selected peptides from 20 replicate injections that were acquired with a 0.5 ms dwell time per transition.

– The %RSD of MS response and retention time for the four selected peptides demonstrated excellent reproducibility with submillisecond dwell time for diverse peptides (Figure 6 and Table 3).

Table 3. Summary of MS response and retention time for the four selected endogenous peptides in human plasma.

Peptide Sequence	Protein Name	RT (min)	%RSD of RT (n = 20)	Average Response	%RSD of Response (n = 20)
DGAGDVAFVK	TRFE_HUMAN	13.55	0.06%	1.22E+07	3.20%
VVGGLVALR	FA12_HUMAN	20.23	0.05%	1.01E+06	4.30%
HAFILQDTK	CO2_HUMAN	11.25	0.10%	7.59E+04	8.30%
IQVYSR	B2MG_HUMAN	5.88	0.30%	3.45E+04	9.60%

Conclusion

The analytical sensitivity, precision, and accuracy of MRM-based LC/MS methods are important considerations for high-throughput protein biomarker analysis. Using commercially available proteomics quantitation kits from a third-party vendor (MRM Proteomics, Inc.), the ultrahigh performance of the Agilent 6495D LC/TQ coupled with the Agilent 1290 Infinity II bio LC system and Agilent MassHunter Workstation software version 12.1 was demonstrated for comprehensive peptide quantification in human plasma. The standardized acquisition LC/MS/MS method provided in the proteomics kit for human plasma (MRM Proteomics Inc.) allows users to directly analyze up to 270 human plasma protein biomarkers without any method development.[1,2] For researchers who want to develop an MRM-based LC/MS method from scratch, the integrated Skyline-Agilent automated workflow enables intelligent MRM method development without user intervention and in a user-friendly way. The improved features on the 6495D LC/TQ system enable researchers to monitor more protein biomarkers simultaneously, facilitating investigation of potential synergetic effects in biological states.

References

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