Application Note

Clinical Research

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From Nanoflow to Standard Flow LC/MS for Routine Quantitative Plasma Proteomics in Diabetic Kidney Disease Research

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Abstract

This application note showcases the capability, reproducibility, and analytical sensitivity of MRM-based LC/MS analysis of human plasma proteins using the Agilent 1290 Infinity II LC coupled with the Agilent 6495 triple quadrupole (TQ) mass spectrometer. The results demonstrate equivalent data from eight peptides across 12 samples on the Agilent standard flow system compared to a nanoflow counterpart system. Successful and reproducible analysis of the eight peptides in whole plasma comparable to depleted plasma analysis highlights the analytical sensitivity and robustness offered by the 6495 LC/TQ for routine protein measurement in biomarker research.

Introduction

Diabetic kidney disease (DKD) is a significant complication of diabetes with one in three adult diabetics having some degree of DKD. The current clinical tests to monitor DKD are the urinary albumin:creatinine ratio (ACR) and the estimated glomerular filtration rate (eGFR), which have minimal power to predict early DKD.[1] Therefore, there is a need to identify new protein biomarkers and develop tests for early DKD prediction.

PromarkerD is a MS-based clinical research test method developed to predict chronic kidney disease (CKD) in a cohort validation of people with type 2 diabetes.[2,3] It was developed using a proteomics biomarker discovery and validation workflow by Proteomics International (PI). Initial development of PromarkerD involved the measurement of 12 proteins in immunodepleted plasma samples using a nanoflow LC/MS platform.[4] During method development the number of protein markers was narrowed down to four proteins (APOA4, CD5L, FHR2, and IBP3) with a total of eight peptides. However, a nanoflow LC/MS system is not suitable for routine protein biomarker measurements. As a future option for clinical assay development, a standard flow LC/MS system provides an alternative platform with excellent performance for routine protein measurement.[5]

This application note explores the ease of transferring the nanoflow LC/MS-based PromarkerD method to a standard flow LC/MS platform using the Agilent 1290 Infinity II LC coupled to the Agilent 6495 LC/TQ. The extent to which nanoflow LC/MS can be matched by standard flow LC/MS measurements is also explored. It is demonstrated that the four PromarkerD protein markers could be comparably measured from nanoflow to standard flow LC/TQ systems, and in whole plasma and depleted plasma samples. Since the standard flow LC/MS platform is a more robust and reproducible platform than nanoflow LC/MS, our study indicates that the Agilent 1290 Infinity II LC coupled to the Agilent 6495 LC/TQ is more suitable for routine measurement of PromarkerD protein markers.

Materials and methods

Plasma samples

All subjects' plasma samples were provided by the Fremantle Diabetes Study (FDS), a longitudinal observational cohort.[6] EDTA (Ethylenediaminetetraacetic acid) plasma was collected from all subjects after an overnight fast and stored at –80 °C until required. The FDS protocol was approved by the South Metropolitan Area Health Service Human Research Ethics Committee (07/397), Western Australia. All subjects gave informed consent before participation. A standard reference plasma was collected from three healthy volunteers and combined before aliquoting and storage at –80 °C.

Method transfer

To establish the PromarkerD method on the Agilent 6495 LC/TQ (G6495B), a synthetic peptide mix was generated and spiked into Atturos reference serum (ARS). Synthetic peptides were purchased from Thermo Fisher Scientific and resuspended in 50/50 acetonitrile (ACN)/H_2O (ddH_2O, 18.2 Ω) and a stock solution was prepared to give a final concentration of 0.1 pmol/µL. The synthetic peptide mix (1 pmol) was spiked into 4 µg of ARS for analysis of PromarkerD. An MRM method, which targets the 12 proteins including 21 endogenous peptides and 17 isotopically labeled peptides was generated.

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 (part number 959757-902). LC/MS data was acquired for PromarkerD on the Agilent 6495 LC/TQ using the parameters shown in Table 1. Buffer A consisted of 99.9% H_2O (ddH_2O, 18.2 Ω) with 0.1% formic acid and buffer B consisted of 99.9% acetonitrile with 0.1% formic acid. Once the MRM assay was set up and the peptide retention times determined, the data was acquired in dynamic MRM mode. Data analysis for targeted peptide quantification was carried out using Skyline software.

Table 1. Overview of LC/TQ acquisition parameters.

Agilent 6495B Triple Quadrupole Mass Spectrometer			
Ion Mode	AJS ESI, positive mode		
Gas Temperature	150 °C		
Drying Gas Flow	15 L/min		
Capillary Voltage	4,000 V		
Nozzle Voltage	300 V		
High/Low Pressure RF Voltage	150/60 V		
Delta EMV	300 V		
MS1/MS2 Resolution	Unit/Unit		
Cycle Time	500 ms		
Agilent 1290 Infinity II LC			
Time (minutes)	**A (%)**	**B (%)**	**Flow (mL/min)**
2	92	8	0.400
18	70	30	0.400
22	10	90	0.400
25	10	90	0.400
25.10	97	3	0.400
26	97	3	0.400

Results and discussion

Nanoflow to standard flow comparison

To assess the measurement comparability of the PromarkerD biomarkers on nanoflow and standard flow LC/TQ platforms, 12 plasma samples (PI-4205 A-L) were processed by Proteomics International (PI) and then sent to Atturos. PI analyzed the 12 samples on a non-Agilent nanoflow LC/TQ platform. At Atturos, the samples were separated using a 26-minute LC-dMRM method, using the Agilent standard flow 6495 LC/TQ. The MRM peak area of eight selected peptides matching four protein biomarkers (APOA4, CD5L, FHR2, and IBP3) were extracted for relative peak area comparison to the average peak area of each peptide (n = 12, in % terms). This brought the two sets of MS data to scale with each other and allowed observation of any trends across the 12 samples for each peptide. Comparison results for the four proteins and eight peptides are shown in Figure 1. Overall, a consistent trend was observed between results obtained on the nanoflow and standard flow LC/TQ platforms. The data obtained on the Agilent system are largely equivalent to that of PI.

Occasionally deviation between the two data sets was observed, e.g. peptides from APOA4 in PI-4205D. Examination of the targeted MS data of the LEPYADQLR peptide from APOA4 on the 6495 LC/TQ proved good chromatographic peaks with high dot-product (dotp) values between the peak areas and the matching MS/MS peak intensities (Figure 2). In addition, both LEPYADQLR and ISASAEELR peptides of APOA4 showed the same trend in the plasma sample of PI-4205D. This indicates the high quality of targeted MS data on the 6495 LC/TQ for this peptide in PI-4205D. Therefore, the observed deviation might be due to some unknown reason in the sample handling.

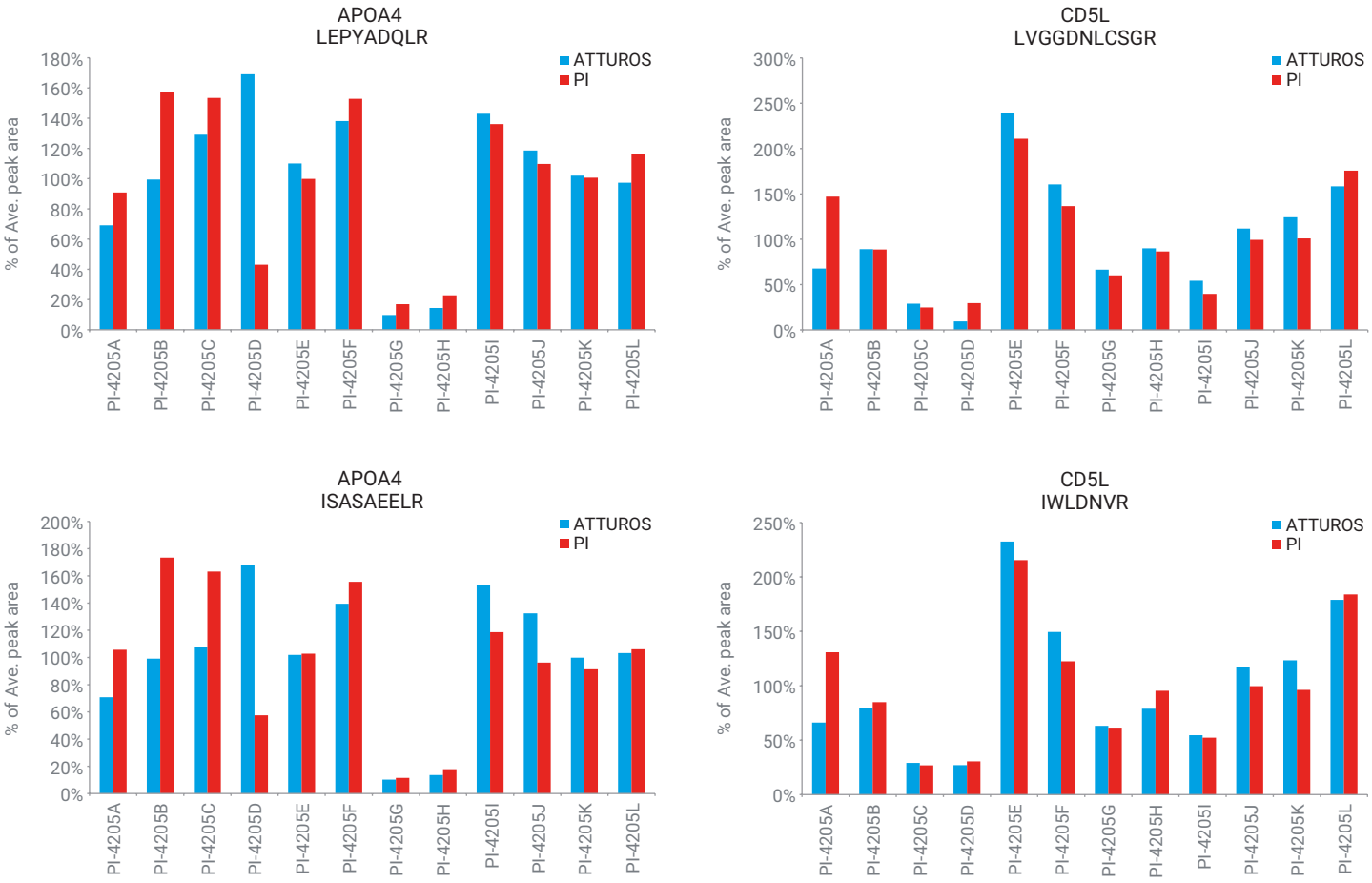
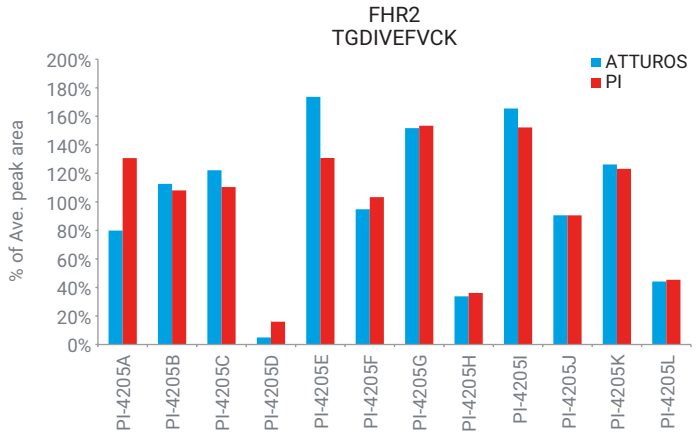


Figure 1. Comparison of eight peptides from four selected proteins (APOA4, CD5L, FHR2, and IBP3) across 12 plasma samples analyzed by nanoflow LC/TQ in PI (red bars) and standard flow LC/TQ in Atturos (blue bars). The individual subject's peak area for a given peptide is expressed as a percentage of the average peak area for that peptide across the 12 plasma samples (continued on next page).


FHR2
TGDIVEFVCK
ATTUROS
PI
% of Ave. peak area
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
PI-4205A
PI-4205B
PI-4205C
PI-4205D
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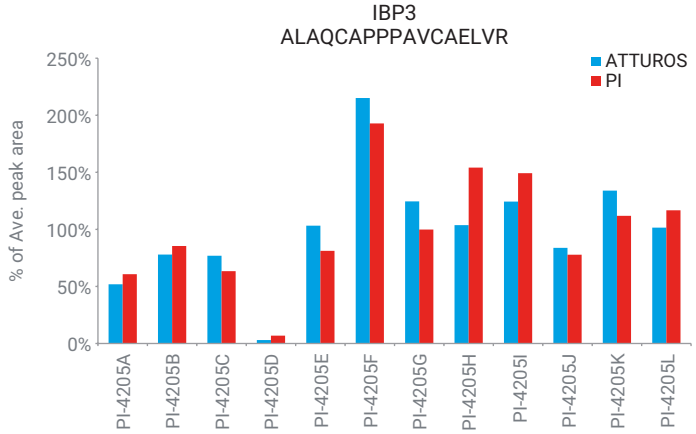
PI-4205J
PI-4205K
PI-4205L


IBP3
ALAQCAPPPAVCAELVR
ATTUROS
PI
% of Ave. peak area
250%
200%
150%
100%
50%
0%
PI-4205A
PI-4205B
PI-4205C
PI-4205D
PI-4205E
PI-4205F
PI-4205G
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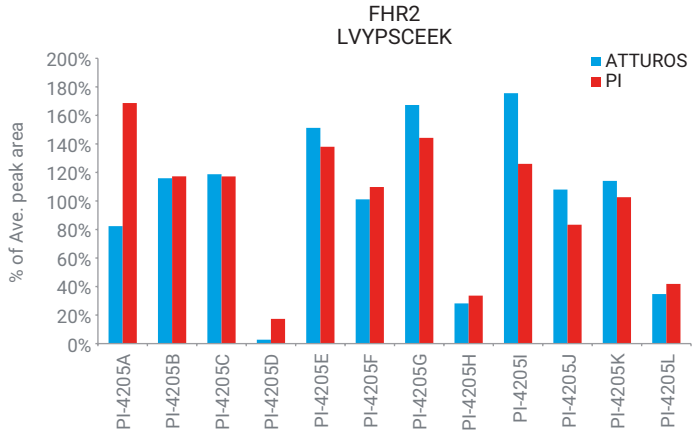
PI-4205J
PI-4205K
PI-4205L


FHR2
LVYPSCEEK
ATTUROS
PI
% of Ave. peak area
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
PI-4205A
PI-4205B
PI-4205C
PI-4205D
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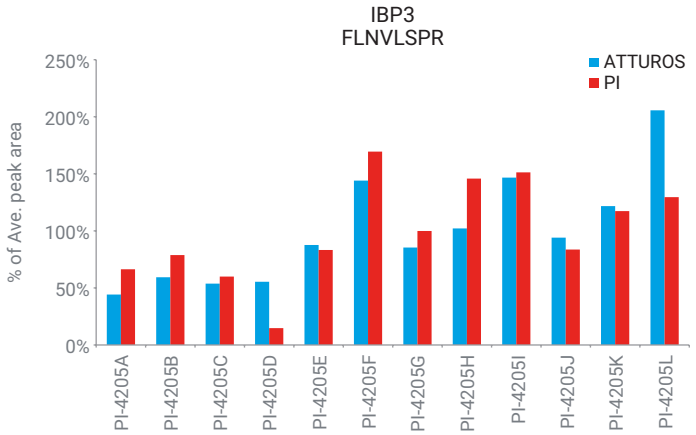
PI-4205J
PI-4205K
PI-4205L


IBP3
FLNVLSPR
ATTUROS
PI
% of Ave. peak area
250%
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PI-4205A
PI-4205B
PI-4205C
PI-4205D
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PI-4205L

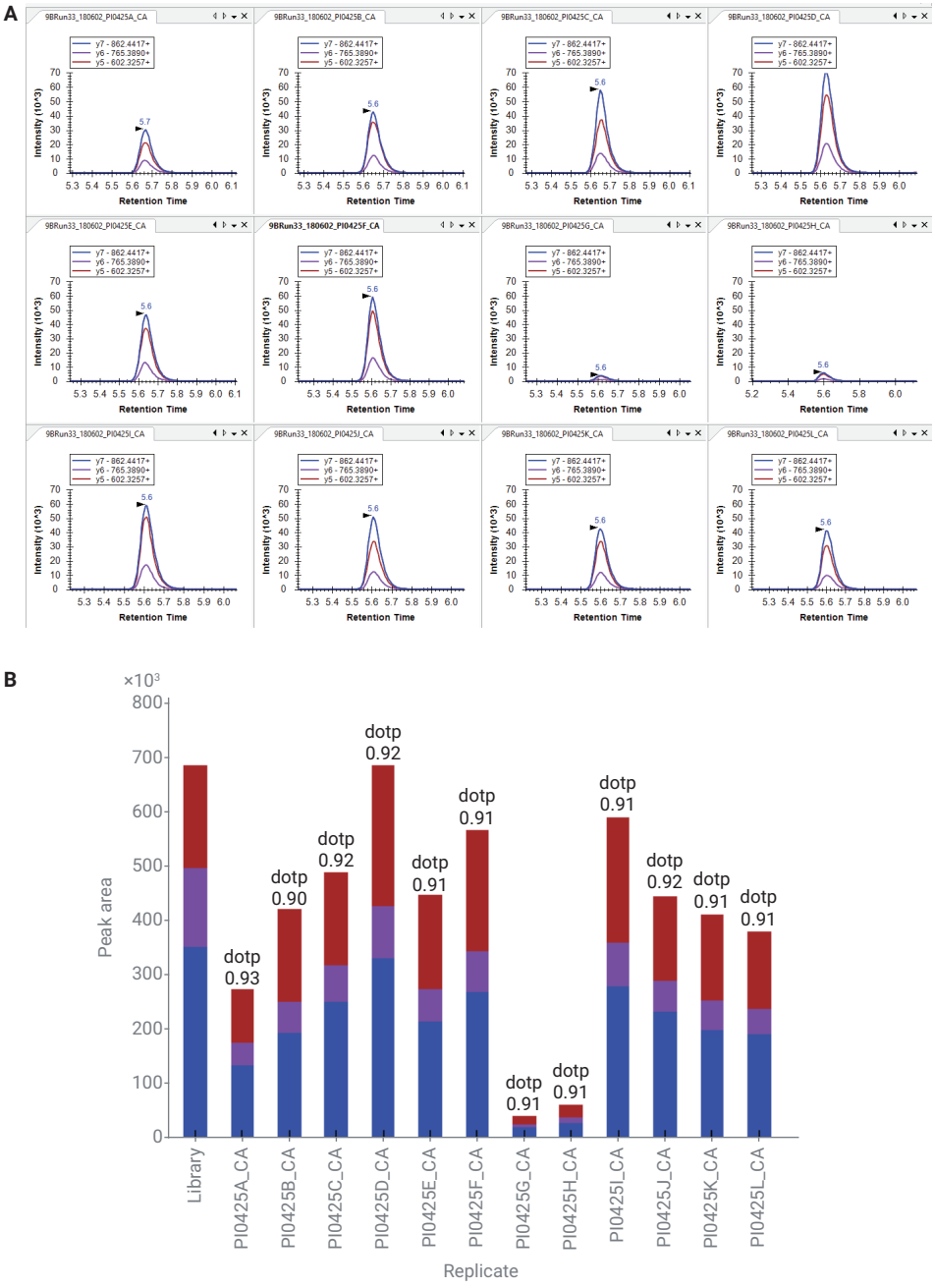


Figure 2. Peak area of the LEPYADQLR peptide from APOA4 across the 12 plasma samples. (A) MRM chromatograms of LEPYADQLR peptide in 12 plasma samples. (B) Stacked bar chart of peak areas of the targeted transitions displayed with dot-product (dotp) value between the peak areas and the matching MS/MS peak intensities.

Depleted plasma versus whole plasma on the 6495 LC/TQ

The PromarkerD biomarkers were measured on the Agilent 6495 LC/TQ in whole reference plasma and reference plasma depleted of the top 14 serum proteins using the Agilent Multiple Affinity Removal Column Human 14. The samples were analyzed using the PromarkerD method parameters described previously in technical replicate injections (n = 6). Replicates 1 to 3 were run at the beginning of the worklist, 40 injections were then run to analyze the 12 plasma samples described previously and finally replicates 4 to 6 of the depleted and whole reference plasma were analyzed; 17-hours after replicates 1 to 3. Figure 3 shows the comparison of MRM chromatograms of peptides from the eight peptides matching four selected proteins (APOA4, CD5L, FHR2, and IBP3) in whole and in depleted reference plasma. In whole reference plasma, the PromarkerD biomarkers produced good MRM chromatograms for all peptides from APOA4 and CD5L, and good MRM chromatograms for at least one peptide from each of FHR2 and IBP3. APOA4, CD5L, and IBP3 have since been independently validated on a separate, larger cohort of people with type 2 diabetes and proved alongside standard clinical features to be an accurate prognostic test for future renal decline.[3] The PromarkerD test may be useful for risk stratification in future clinical trials.

Similar reproducibility was observed in depleted and whole reference plasma with all peptides having a relative standard deviation (RSD) below 20% across the six replicates (Table 2). This result demonstrated that the standard flow LC/MS on the 6495 LC/TQ could be used to measure the PromarkerD biomarkers in both whole plasma and depleted plasma.

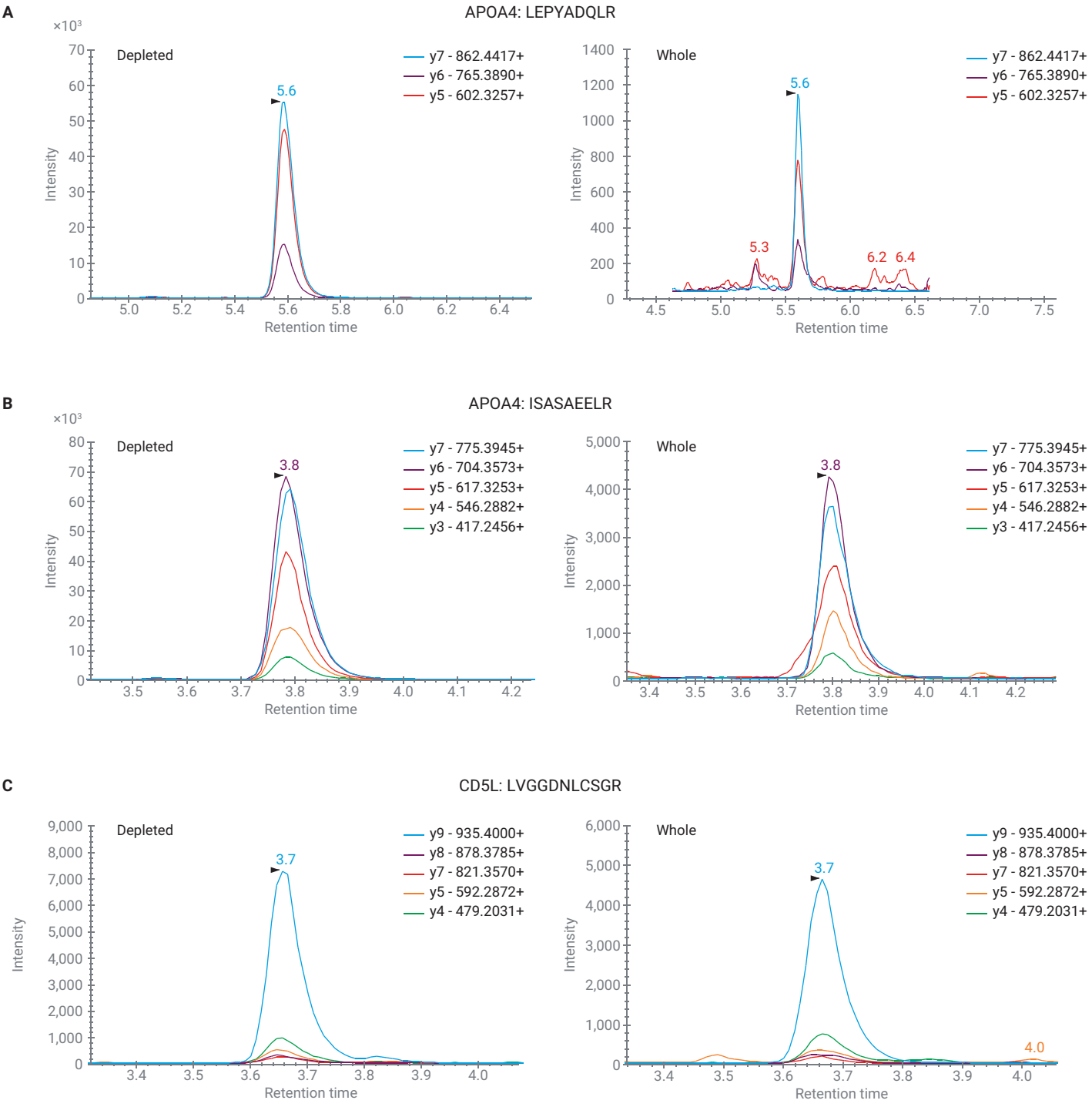
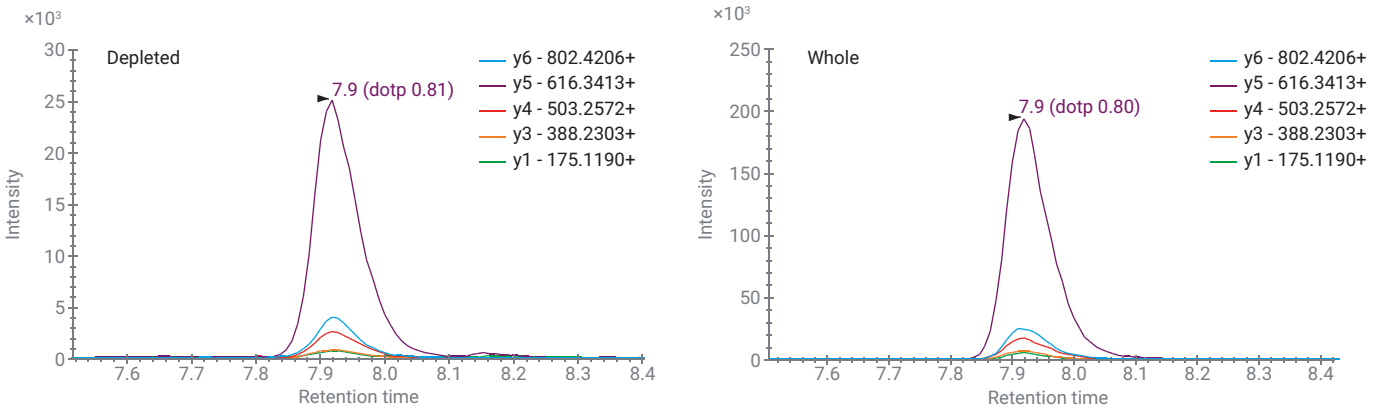


Figure 3. MRM chromatograms for all eight peptides (A-H) targeting four proteins (APOA4, CD5L, FHR2, and IBP3) in depleted and whole reference plasma (continued on next page).

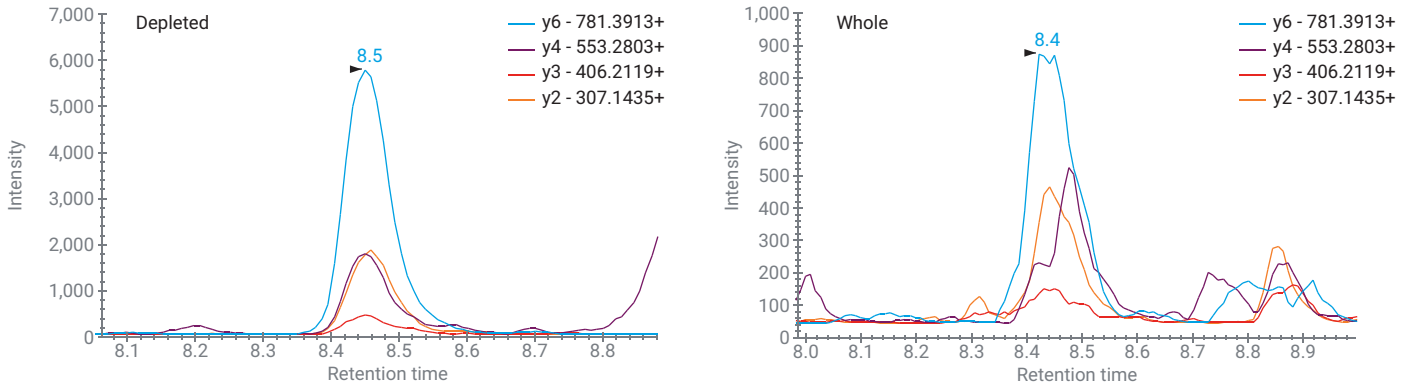
D

CD5L:IWLDNVR



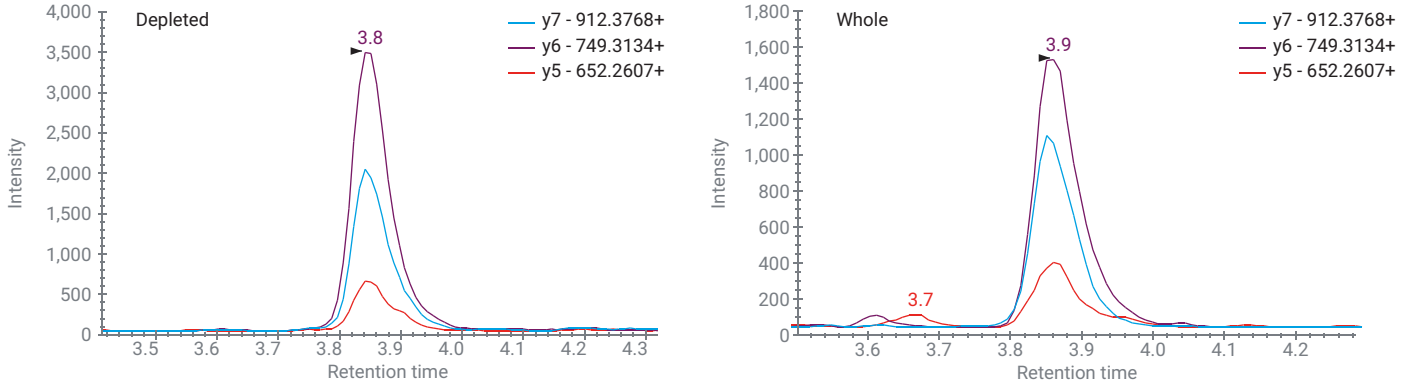
E

FHR2: TGDIVEFVCK



F

FHR2:LVYPSCEEK



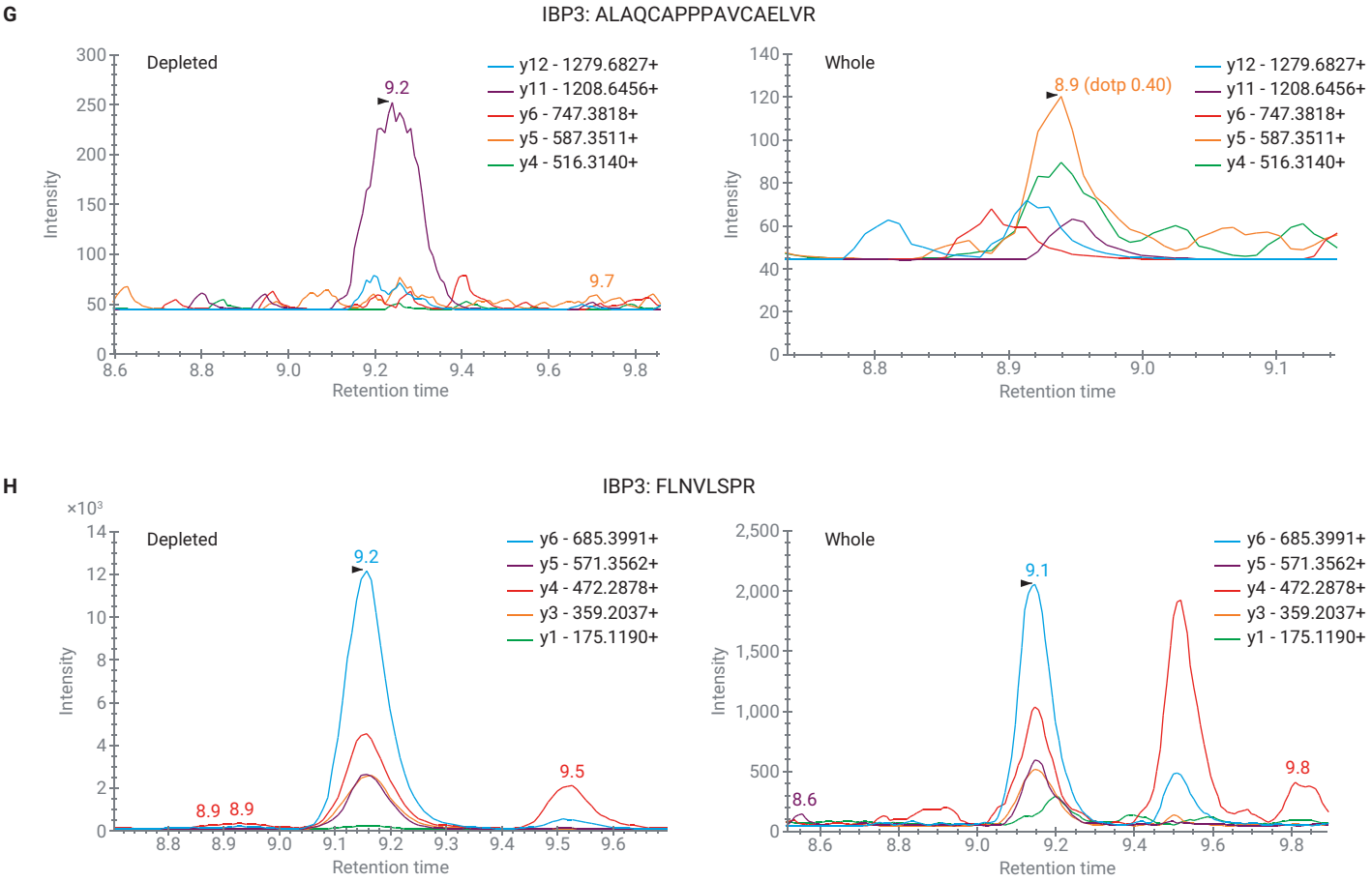


Table 2. Peak areas for all eight peptides in depleted and whole reference plasma. Reproducibility is shown in terms of percentage relative standard deviation (RSD) across six nonconsecutive- technical replicate injections of each sample measured over a 22-hour worklist.

			Depleted Reference Plasma (n = 6)		Whole Reference Plasma (n = 6)	
Accession	Protein	Peptide	Peak area	Peak area RSD	Peak area	Peak area RSD
P06727	APOA4	LEPYADQLR	257,570	9.97%	3,944	14.52%
P06727	APOA4	ISASAEELR	303,069	10.99%	18,759	14.11%
O43866	CD5L	LVGGDNLCSGR	31,643	10.50%	19,117	12.86%
O43866	CD5L	IWLDNVR	136,872	7.05%	947,395	6.12%
P36980	FHR2	TGDIVEFVCK	26,877	10.58%	7,392	17.39%
P36980	FHR2	LVYPSCEEK	15,943	6.91%	6,898	6.60%
P17936	IBP3	ALAQCAPPPAVCAELVR	1,894	16.67%	127	18.54%
P17936	IBP3	FLNVLSPR	68,437	10.00%	16,725	13.00%

Conclusion

The reproducibility, robustness, and analytical sensitivity of MRM-based LC/MS methods for peptide quantification in biological fluids are important considerations for high-throughput protein biomarker quantitation studies. This application note discusses the transfer of an MRM method from nanoflow to standard flow LC/TQ platforms and the analytical sensitivity of the Agilent 6495 LC/TQ in whole and depleted human plasma. For 12 subjects, equivalent results were obtained on the standard flow system compared to nanoflow. Standard flow LC/TQ measurement of protein biomarkers shown here does not negatively affect the data output. This application note also shows the analytical sensitivity of standard flow; when coupled with the 6495 LC/TQ it is capable of reproducibly detecting the four PromarkerD biomarker proteins in whole plasma without depletion. These results demonstrated the suitability of this high-performance LC/MS system on routine protein measurement in complex matrices for potential clinical research assay development in the future.

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