Poster Reprint

ASMS 2024
Poster number ThP 076

A Rapid and Economical Workflow for Protein Biomarker Discovery Using Agilent 6495D Triple Quadrupole LC/MS System

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Introduction

The advancement of triple quadrupole LC/MS systems and the publicly available peptide MS/MS spectral libraries have enabled researchers to develop comprehensive MRM-based assays without performing extensive proteome identifications in the first place.

In this study, we performed a proof-of-concept study in human plasma samples using Uniprot human reference proteome, PeptideAtlas plasma spectral libraries, Skyline software and 6495D LC/TQ system (Figure 1). A comprehensive peptide quantitative assay was developed quickly without using stable isotope-labeled standard peptides (SILs). This assay was applied to analyze plasma samples from 40 normal human subjects (20 males and 20 females) for group comparison analysis. The results demonstrated that this rapid and economical workflow can discover protein biomarkers in complex biological samples.

Key features on the Agilent 6495D LC/TQ system for peptide/protein quantification workflow include:

- Simple MRM-based LC/MS method development using Skyline software with Agilent Automation plugin and MassHunter software
- Faster MS acquisition time allowing up to 500 concurrent dMRMs
- Improved analytical sensitivity
- Excellent LC and MS signal reproducibility allowing label-free quantification in cohort samples

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)

Agilent AssayMAP Bravo platform (G5571AA)

Materials

Human plasma samples (20 males and 20 females) were purchased from BioIVT. Stable-isotope labeled standard peptides (SILs) were from MRM Proteomics Inc and Biognosys Inc.

Experimental

Sample preparation

All human plasma samples were subjected to overnight trypsin digestion, followed by peptide cleanup using AssayMAP Bravo. An aliquot from each digested sample was pooled together to make a QC sample which was also used for method development.

LC/MS analysis

LC/MS method shown in Table 1. Data acquisition and analysis were carried out using MassHunter software (v12.1) and Skyline-daily software (v23.1.1.353).

Table 1. LC/MS method.

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 vol.	15 µL	
Gradient program	Time (min)	B (%)
	0.00	2
	0.20	8
	20.00	28
	21.00	80
	23.00	80
	23.10	2
Stop time	24.00 min	
Post time	1 minute	

6495D Triple Quadrupole Mass Spectrometer	
Ion source	AJS
Polarity	Positive
Gas temperature	150 °C
Drying gas	16 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
Scan type	dMRM
Total MRMs	3604
Min./Max. concurrent MRMs	4 / 358
Cycle time	1050 ms

Full workflow for protein biomarker discovery

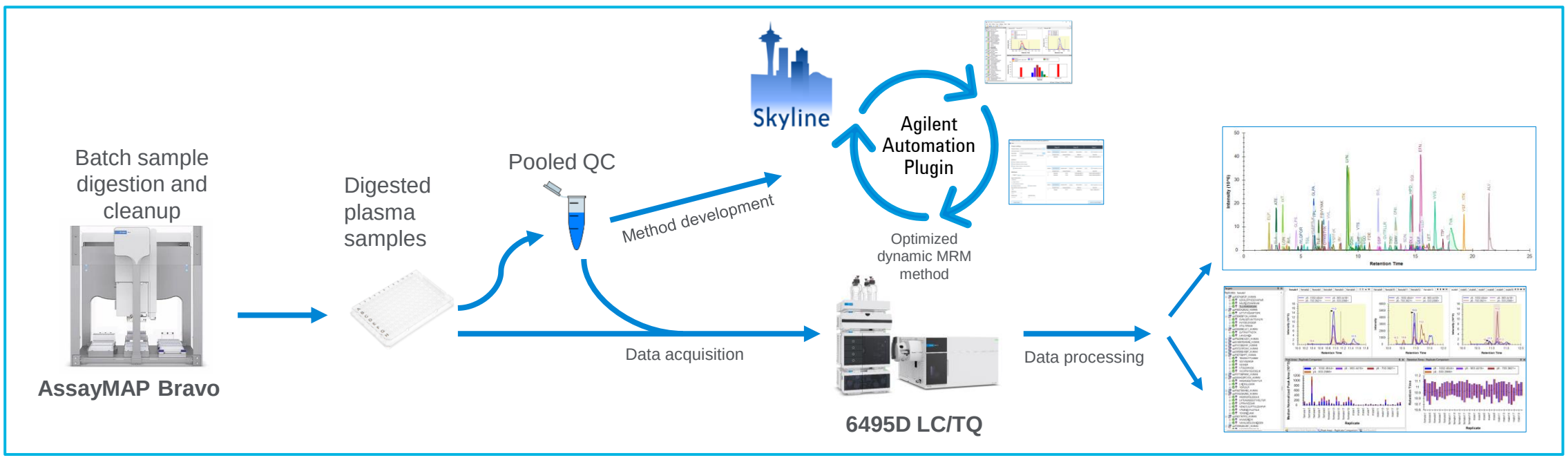

Figure 1. Diagram of study workflow for protein biomarker discovery using AssayMAP Bravo platform, Skyline software and 6495D LC/TQ system with MassHunter 12.1.

Method development

The dMRM method was developed and optimized using Skyline software with Agilent automation plugin on the 6495D LC/TQ (Figure 2). A list of 581 target proteins was initially imported. The whole method development was completed within 2 days.

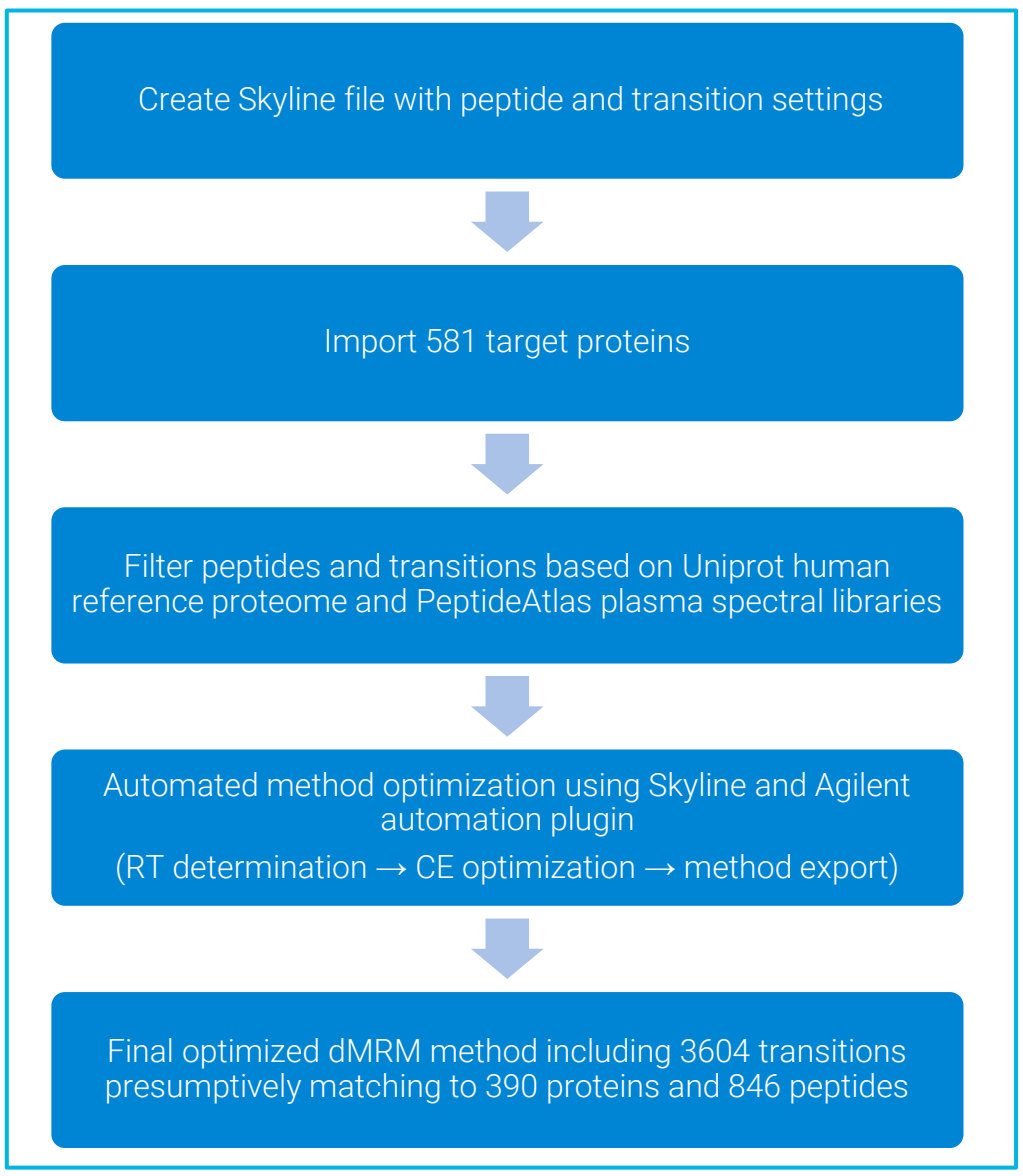

Figure 2. dMRM method development steps with Skyline and Agilent automation plugin using plasma QC sample without SILs.

Accuracy of peptide RT determination

For dMRM method development, endogenous peptide peaks were automatically selected by Skyline during RT determination. We also used other Skyline filters to facilitate peak picking and remove poor peptide peaks, e.g., customized retention time predictor, peptide peak found ratio > 0.75, shape correlation > 0.9, etc.

In a separate experiment, we measured the true RTs of 364 peptides matching to 278 plasma proteins using spiked SIL peptide analogs as gold standards. The real RTs were compared to the RTs determined by the Skyline workflow using endogenous plasma sample without SILs (Figure 3). 68% of peptides yielded correct RTs using the Skyline workflow without SILs.

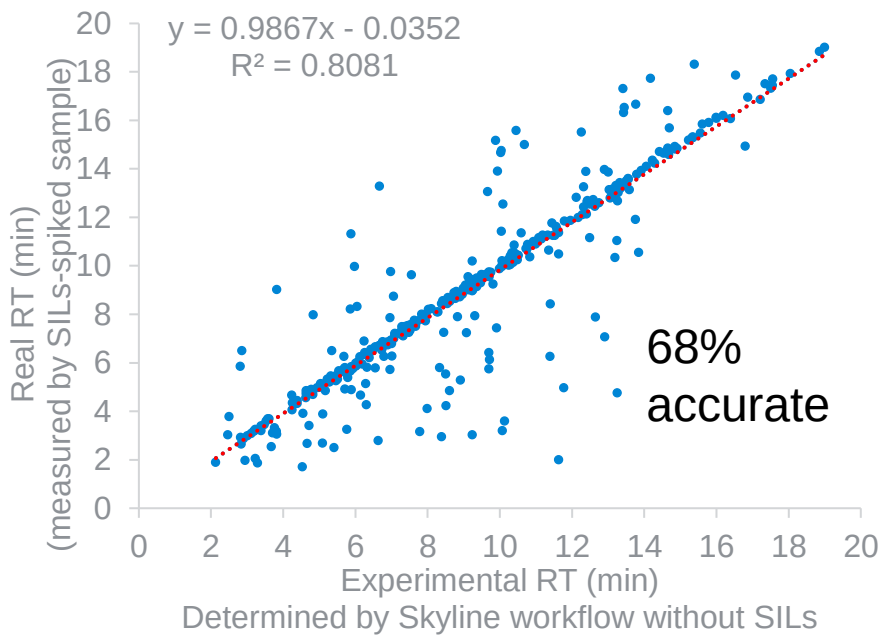

Figure 3. Accuracy assessment of peptide RTs determined by Skyline workflow.

Results and Discussion

Cohort study

Plasma samples from 40 normal human subjects (20 males vs. 20 females) were analyzed on an Agilent 1290 Infinity II Bio LC and 6495D LC/TQ system using the dMRM method developed by the Skyline workflow. Sample injection order was randomized. Replicate injection of the pooled QC was performed every 5 injections throughout this study. The results were summarized below:

- The median CV of peptide signal responses based on 10 replicates of pooled QC is 5.8%, and 91.7% of targeted peptides had a CV < 20% (Figure 4)
- An excellent RT reproducibility was observed throughout the whole study with a median CV of 0.12% (Figure 5)
- Volcano plot of group comparison showed four proteins have at least 2 peptides with statistical differences (Figure 6), two of which have been published in the literature[1]
- The peak areas of 3 peptides from pregnancy zone protein (PZP_HUMAN) show synchronized expression level among subject samples, demonstrating quantification reliability (Figure 7)
- Single peptides with statistical differences need follow-up study to re-confirm peptide sequence

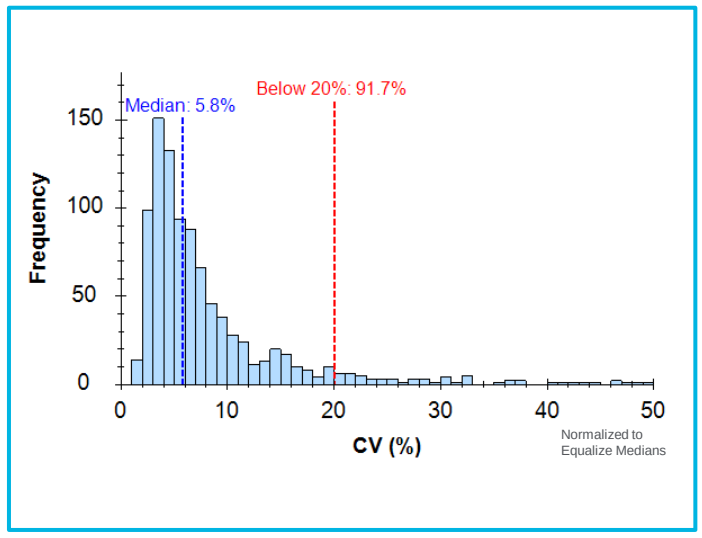

Figure 4. Distribution of peak area CV (%) for all the 846 targeted peptides based on QC injections (n=10).

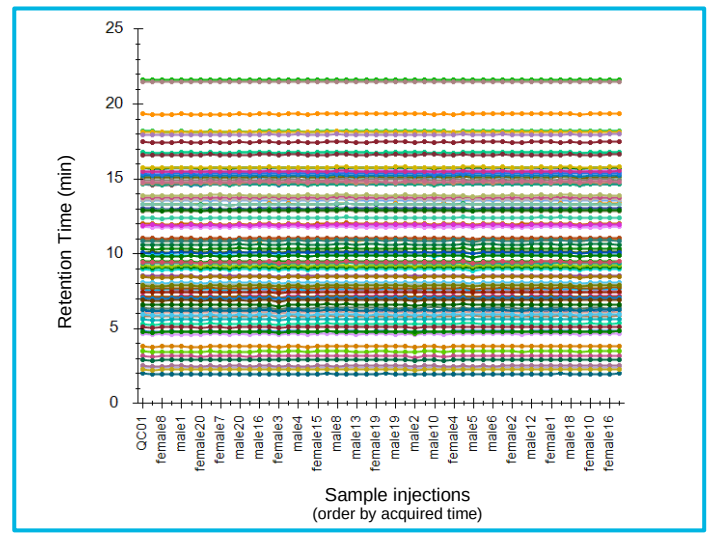

Figure 5. Distribution of all the peptides' retention times measured in all plasma samples and pooled QC.

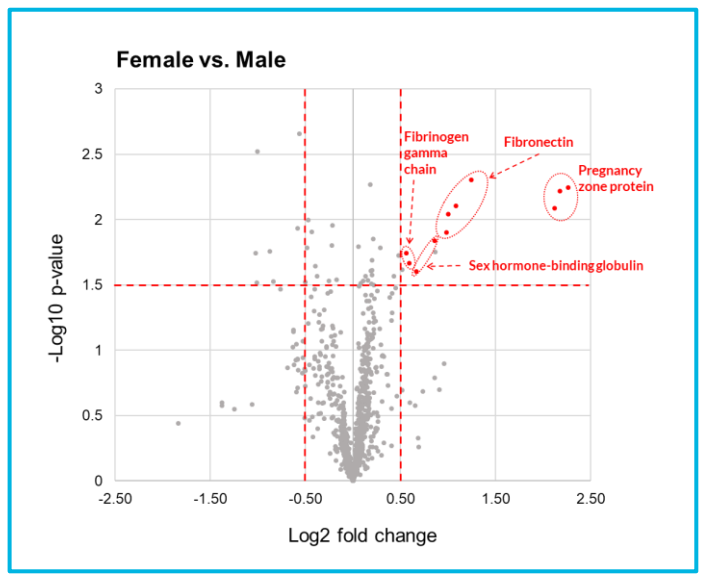

Figure 6. Volcano plot of group comparison (female vs. male). Each dot represents a targeted peptide. Four proteins with multiple peptides showing increased level in female were labeled.

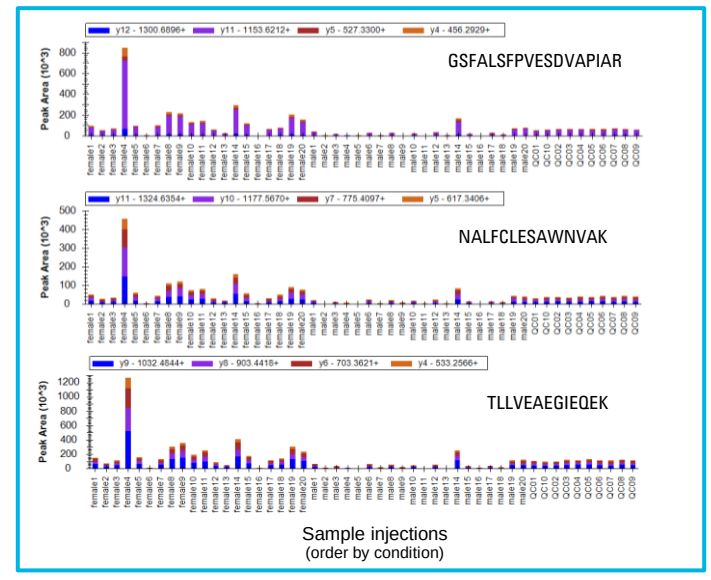

Figure 7. Peak areas for the 3 peptides of PZP_HUMAN measured in all plasma samples and pooled QC show synchronous level from the same subject.

Conclusions

This proof-of-concept study demonstrated that using publicly available libraries, 6495D LC/TQ and Skyline software with Agilent automation plugin, protein biomarkers can be quickly discovered in cohort samples:

- This platform provides a simple and fast solution for comprehensive assay development (< 2 days)
- The whole workflow is economical in practice as SIL peptides were not required
- 846 peptide peaks and 3604 transitions were precisely quantified in a 25-min LC/MS/MS method using the Agilent 1290 Infinity II Bio LC and 6495D LC/TQ system, which was successfully applied for protein biomarker discovery

References

[1]Determination of the concentration range for 267 proteins from 21 lots of commercial human plasma using highly multiplexed multiple reaction monitoring mass spectrometry. Gaither C, et al. *Analyst*. 2020

https://www.agilent.com/en/promotions/asms