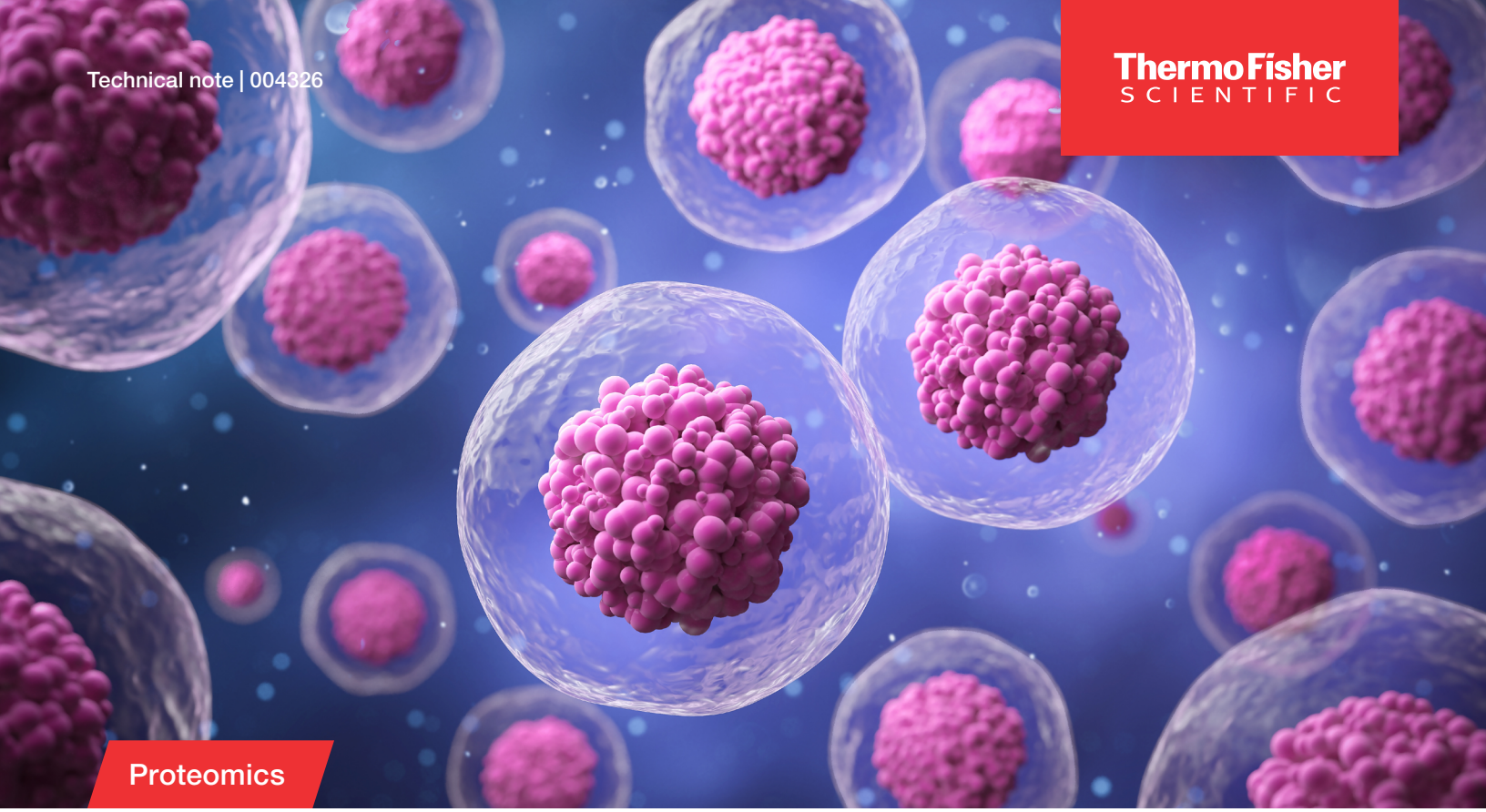
Technical note | 004326



Proteomics

Unlocking biological insights with the Stellar mass spectrometer for Adaptive RT-enhanced quantitative proteomics for plasma biomarker analysis

Authors

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Keywords

plasma, PQ500, Stellar mass spectrometer, blood, biomarker, multiplexed, targeted proteomics, Adaptive RT, clinical study, large-scale peptide panel, PRM

Goal

Develop a large-scale, multiplexed targeted proteomics assay with Adaptive Retention Time (Adaptive RT) to quantify potential biomarkers in human plasma.

Introduction

The development of targeted assays to monitor biomedically relevant proteins is crucial for translating discovery experiments into large-scale clinical studies. However, current targeted assays struggle to scale up to hundreds or thousands of targets. To address this challenge, the Thermo Scientific™ Stellar™ Mass Spectrometer, combined with Skyline Software, was employed to successfully generate large-scale assays.

With hyper-fast acquisition speeds, the Stellar mass spectrometer handles shifting retention times typically observed over the course of large longitudinal studies through a real-time retention time alignment mode called Adaptive RT. This feature automatically adjusts the retention time window for targeted analytes, so an eluting peak is never missed. This enables the acquisition of the target at the sensitivity and speed required while also allowing for the management of numerous concurrent targets that are typically possible without Adaptive RT. Without Adaptive RT, the acquisition window for a peak

is typically 2 minutes wide to reliably capture the peak despite day-to-day variations in chromatography. With Adaptive RT, this acquisition window can be reduced to 0.6 minutes or less, allowing more targets to be added to the method.

Another key feature of the Stellar mass spectrometer is the option to perform MS^3 fragmentation. This enables additional fragmentation of the MS^2 product ions of interest, which improves the signal-to-noise ratio (S/N) by reducing interferences. This results in improved sensitivity that is not achievable with typical triple-stage quadrupole mass spectrometers and is only possible with an ion trap mass analyzer made available on the Stellar mass spectrometer.

Leveraging the advanced features of the Stellar mass spectrometer, we developed a cutting-edge multiplex targeted proteomics method with Adaptive RT functionality in two to three days, using peptides from the Biognosys PQ500™ Reference Peptides Kit as heavy standards. This innovative method was then applied to the quantification of potential protein biomarkers in plasma samples from patients with lung cancer, Alzheimer's disease (AD), and colorectal cancer (CRC).

Experimental approach

Consumables and chemicals

- Fisher Chemical™ LC-MS grade water with 0.1% formic acid (Part No. LS118-500)
- Fisher Chemical™ Optima™ LC-MS grade 80% acetonitrile with 0.1% formic acid (Part No. LS122500)
- Thermo Scientific™ SureSTART™ 9 mm screw caps
- Thermo Scientific™ SureSTART™ 0.2 mL TPX screw top microvial with glass insert
- Thermo Scientific™ EASY-Spray™ HPLC Column, 2 µm C18, 150 µm × 15 cm (Part No. ES906)
- PQ500 Reference Peptide kit

Instrumentation

- Thermo Scientific™ Vanquish™ Neo™ UHPLC System
- Stellar mass spectrometer
- Thermo Scientific™ Easy-Spray™ Source

Sample preparation

Disease and healthy plasma were purchased from BioIVT and digested using Thermo Scientific™ AccelerOme™ Automated Sample Preparation Platform. A pooled plasma sample was used to develop a large-scale targeted MS^2 assay with Adaptive RT. PQ500 reference peptides standard was resuspended following the manufacturer's instructions.

LC-MS analysis

A Vanquish Neo UHPLC system coupled with the Stellar mass spectrometer setup was used. Mobile phase A was 0.1% formic acid (FA) in H_2O and mobile phase B was 0.1% formic acid in 80% acetonitrile. Liquid chromatography parameters and gradient settings are shown in Table 1A. EASY-Spray HPLC column temperature was set at 55 °C and autosampler temperature was 7 °C. Peptides were analyzed using a 30-minute gradient. MS parameters such as AGC values and maximum injection time were optimized and shown in Table 1B-D. Skyline software was utilized to generate scheduled retention time and a parallel reaction monitoring (PRM) panel for 804 peptides contained in the PQ500 reference peptides kit, with the complete workflow for the method setup described in Figure 1 and our previous method technical note.[1] An MS^3 method was created using PRM Conductor for low-abundant peptides with possible matrix interferences to improve overall S/N.

Table 1. The LC-MS/MS parameters in tMS^2 method with retention time alignment. (A) LC gradient of the assay. (B–D) The mass spectrometer parameters in the three experiments.

A

48SPD (EASY-Spray HPLC column)			
	Time	**%B**	**Flow (µL/min)**
Gradient	0	3	0.8
	22.5	30	0.8
	7.5	45	0.8
	0.2	99	0.8
	2.8	99	0.8
	Stop run		
	Column equilibration		

B

tMS^2 experiment parameters	
Isolation window (*m/z*)	1
Activation type	HCD
HCD collision energy (%)	30
AGC target	Standard
Maximum injection time mode	Dynamic
Points per peak	6
Loop control	All
Cycle time(s)	2
Dynamic time scheduling	Adaptive RT
Reference file	Generated when export method from PRM Conductor or when Acquire Reference is checked in "Adaptive RT DIA" experiment
Scan rate (kDa/s)	125
RF lens (%)	30
Scan range	200–1,500

C

MS experiment parameters	
Scan rate (kDa/s)	125
Scan range (*m/z*)	350–2,000
RF lens (%)	30
AGC target	Standard
Maximum injection time mode	Auto

D

Adaptive RT DIA parameters	
Precursor mass range (*m/z*)	400–1,000
Isolation window (*m/z*)	50
Scan rate (kDa/s)	200
Scan range (*m/z*)	200–1,000
HCD collision energy (%)	30
RF lens (%)	30
AGC target	Standard
Maximum injection time mode	Auto
Window placement optimization	Off
Acquire reference	Checked in scheduled PRM methods to generated reference file

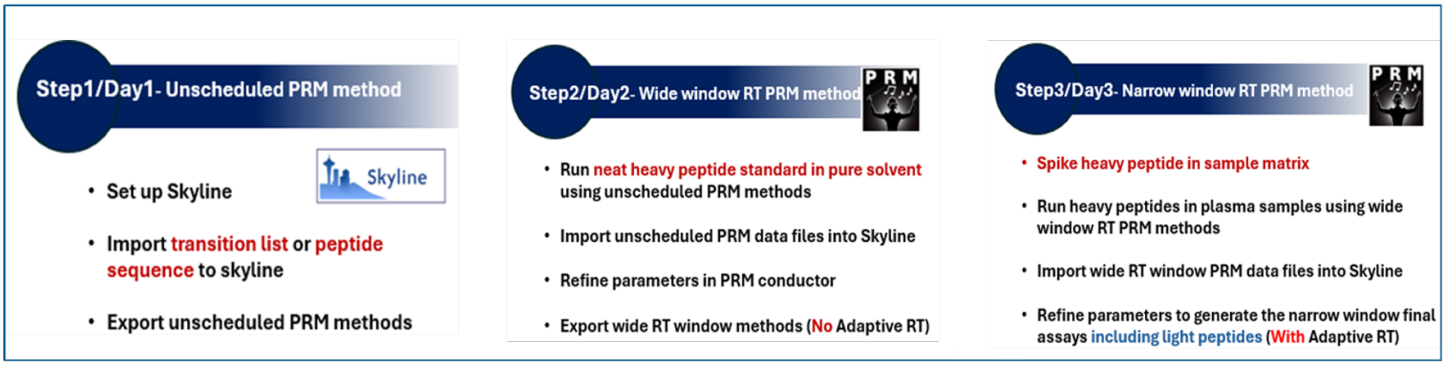

Figure 1. The workflow from the heavy peptide list to large panel of tMS2 assay using Stellar mass spectrometer.

Data analysis

Skyline-daily (64-bit) 24.1.1.398 was used for peptide quantitation, calibration curve analysis, as well as peptide level comparison between different disease groups.

Results and discussion

The results were assessed for reproducibility, linearity, sensitivity, and S/N. The large panel of 804 peptides detected by targeted PRM method were chromatographically separated within 30 minutes as observed in Figure 2. Of the 804 peptide targets, more than 94% of the peptides had CV% values less than 25% in disease plasma samples and nearly 90% of peptides had calibration curve R2 values greater than 0.9 as seen in Figures 3 and 4. The Stellar mass spectrometer enabled ultrasensitive detection of plasma peptides down to the low attomole range, with one example peptide showing a limit of quantitation (LOQ) of 13.6 amol (Figure 5). The sensitivity results are consistent with our previous method validation.[1] For low-abundant peptides, the MS^3 assay created with PRM Conductor improved the S/N when there were observed interferences as shown in Figure 6.

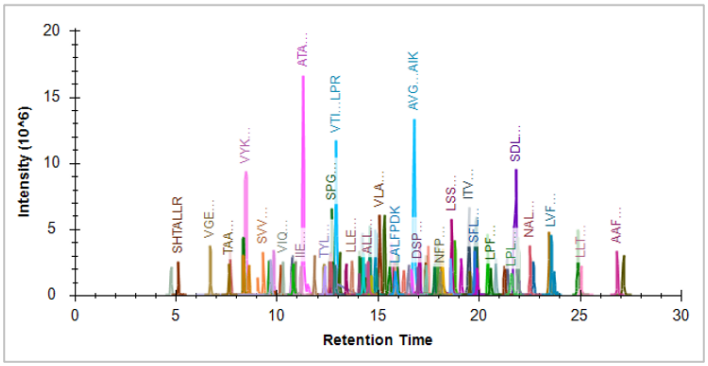

Figure 2. Chromatogram of 804 peptides.

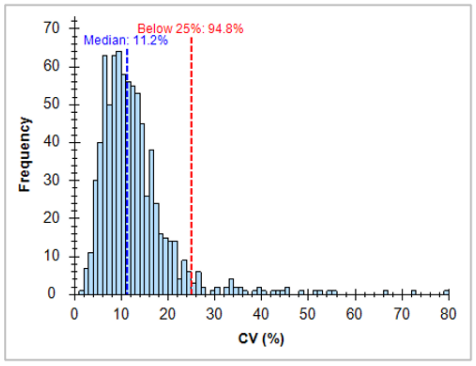

Figure 3. CV% values of peptides in plasma (n=7).

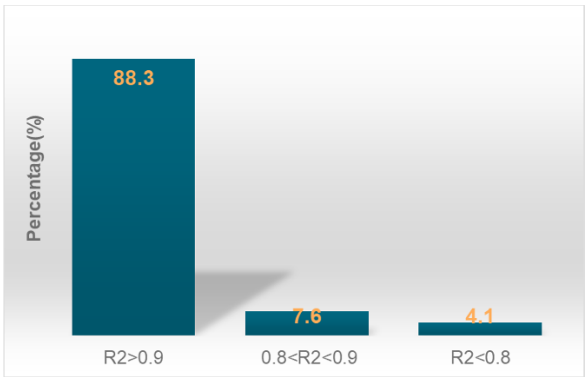

Figure 4. Percent distribution of peptides with R2 of greater than 0.9, 0.8, or less than 0.8.

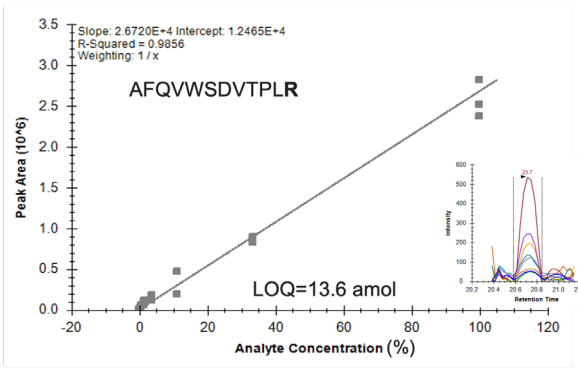

Figure 5. Calibration curve for AFQVWSDVTPLR with a LOQ of 13.6 amol on column.

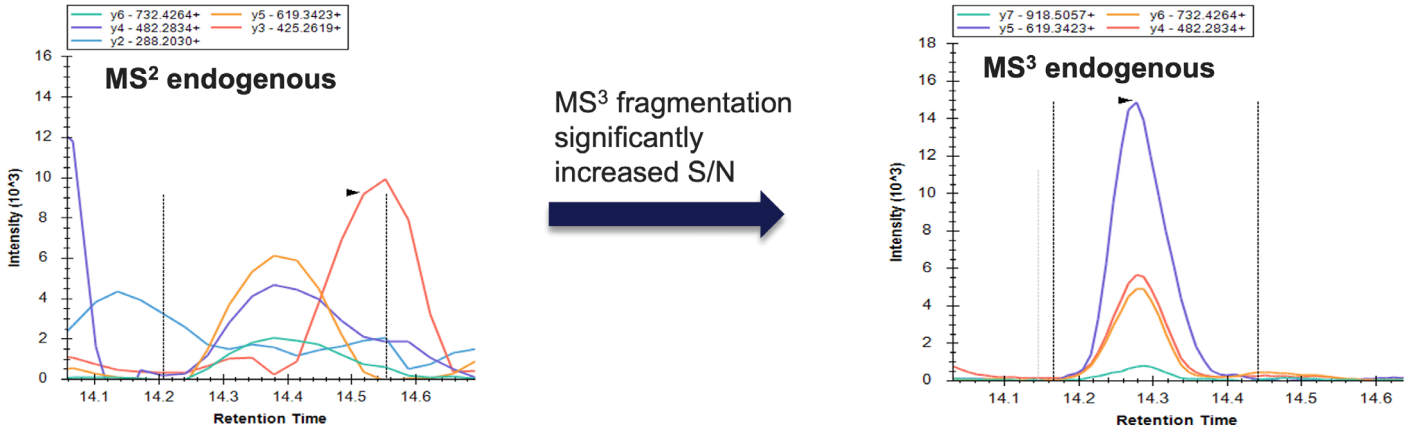

Figure 6. Peptide YLDWIHGHIR from the same AD plasma sample using MS² and MS³ methods.

The retention times of all 804 peptides during disease sample analysis were stable, with an average retention time CV of 0.36% and a median CV value of 0.35%. The excellent time stability on the Stellar mass spectrometer was also reported in a recent large-scale study, in which 311 SST runs were completed during the acquisition of the entire 1,002-sample inflammatory bowel disease (IBD) cohort dataset. The retention times of the 83 spiked SIL peptides demonstrated good stability, with a median CV of only 3%.[2]

Endogenous proteins and peptides were identified using PQ500 from disease plasma and compared to the list of FDA-approved biomarkers. These peptides were analyzed from 25 ng to 1 µg plasma digest (Figure 7A). The LC-MS/MS response was linear in the plasma matrix with 2x serial dilutions. The number of detected proteins increased by 73% using 1 µg compared to 25 ng plasma digest on column (Figure 7B). Using the targeted MS^2 method, a total of 292 endogenous proteins and 472 peptides were identified in disease and healthy plasma. Meanwhile, the combined use of MS^2 and MS^3 methods identified a total of 322 endogenous proteins and 507 peptides. (Figure 7C). About 10.3% more proteins and 7.3% more peptides were identified using the MS^3 assay. Of the endogenous proteins in plasma, 57 were FDA biomarkers for various known diseases. Examples of detected FDA biomarkers are shown in Table 2.

Table 2. Detected FDA biomarkers using PQ500 heavy peptides as reference standards.

UniProt entry name	Protein name	Disease
FIBG	Fibrinogen	COPD
IC1	Complement C1 Inhibitor	Hereditary angioedema (HAE)
KLK3	Prostatic Specific Antigen (PSA)	Prostate cancer
TFR1	Transferrin Receptor (TFR)	Iron deficiency anemia
THBG	Thyroxine Binding Globulin (TBG)	Thyroid diseases
TTHY	Prealbumin	OVA1 test
CERU	Ceruloplasmin	Wilson disease
CYTC	Cystatin C	Drug-induced kidney injury
CRP	C-Reactive Protein (CRP)	Inflammatory disorders and cardiovascular risk

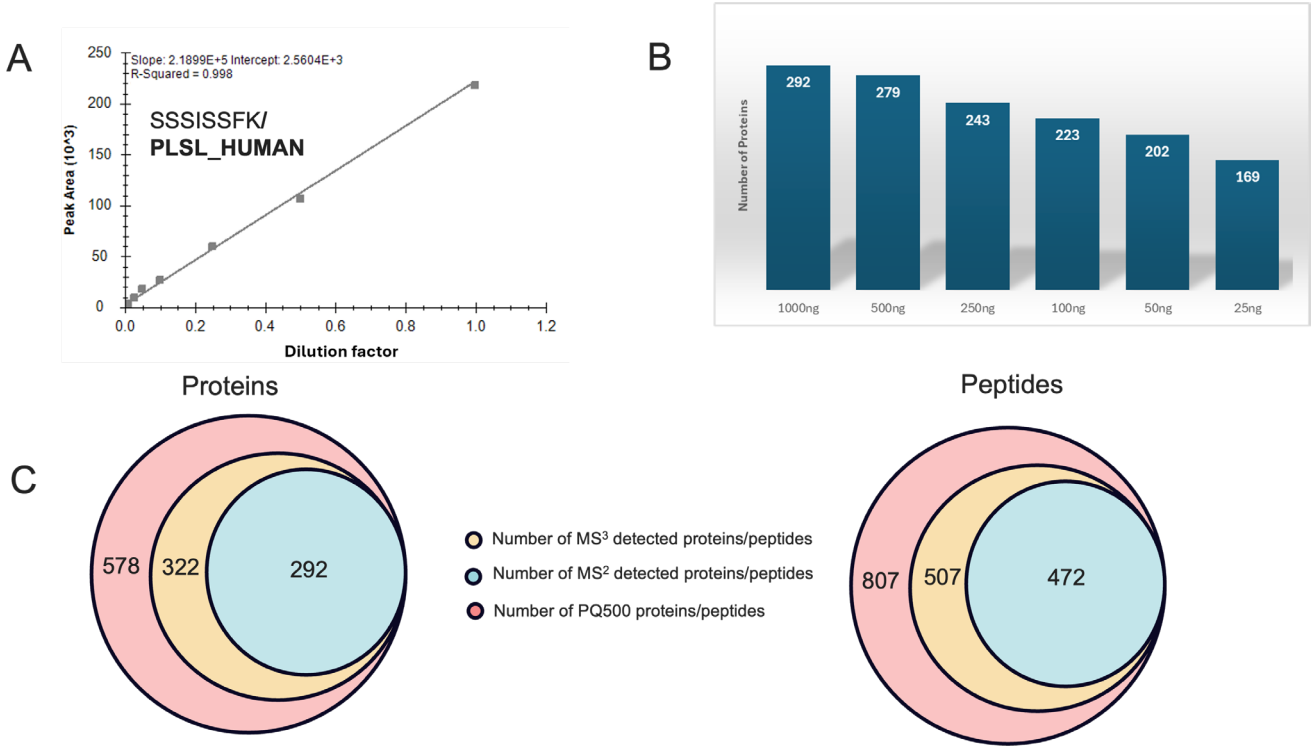

Figure 7. Detected endogenous proteins and peptides using PQ500 heavy peptides as reference standards. (A) The peak area of an example peptide from 25 ng (dilute 40x) to 1 µg (dilute 1x) plasma digest. (B) The number of detected proteins increased by 73% using 1 µg compared to 25 ng plasma digest on column. (C) Total number of endogenous proteins and peptides using MS^2 and MS^3 methods.

A closer investigation into CRC patient plasma was conducted and revealed 29 proteins to be significantly changed with an adjusted p value of <0.05 and more than twofold concentration changes when comparing CRC patient plasma samples to healthy controls. Proteins such as SAA2, A2GL, and CO9[2] were found to be significantly increased in CRC patient plasma (Figure 8). These proteins were also reported as potential biomarkers for CRC disease and listed in Table 3. These results show alterations in concentration levels for proteins that play critical roles in the development and progression of CRC (Figure 9) as cited in the literature.[3,4,9]

Table 3. Significantly changed proteins in CRC patient plasma.

Potential protein markers in CRC patients	
HBA	LYSC
A2GL	CXCL7
CO9	THBG
HBB	SAA2
IGHM	CAP1
FGL1	BLVRB
B2MG	COF1
SEPP1	C163A
CRP	ITB3

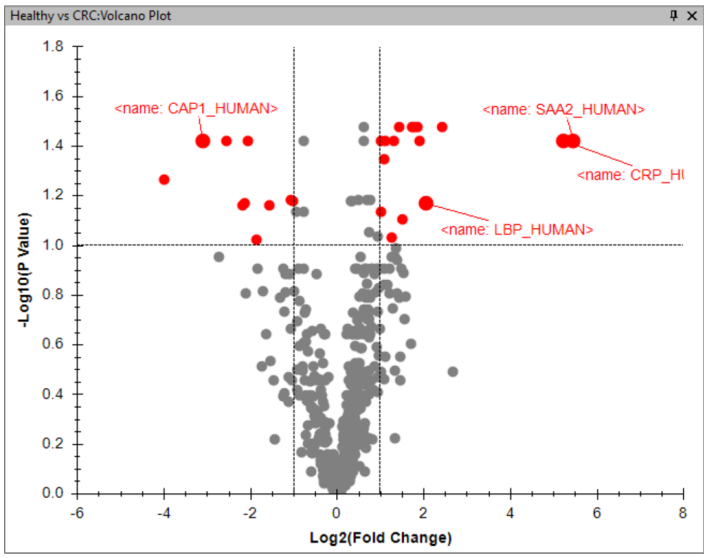

Figure 8. Volcano plot of the protein comparison in plasma from healthy donors and CRC patients.

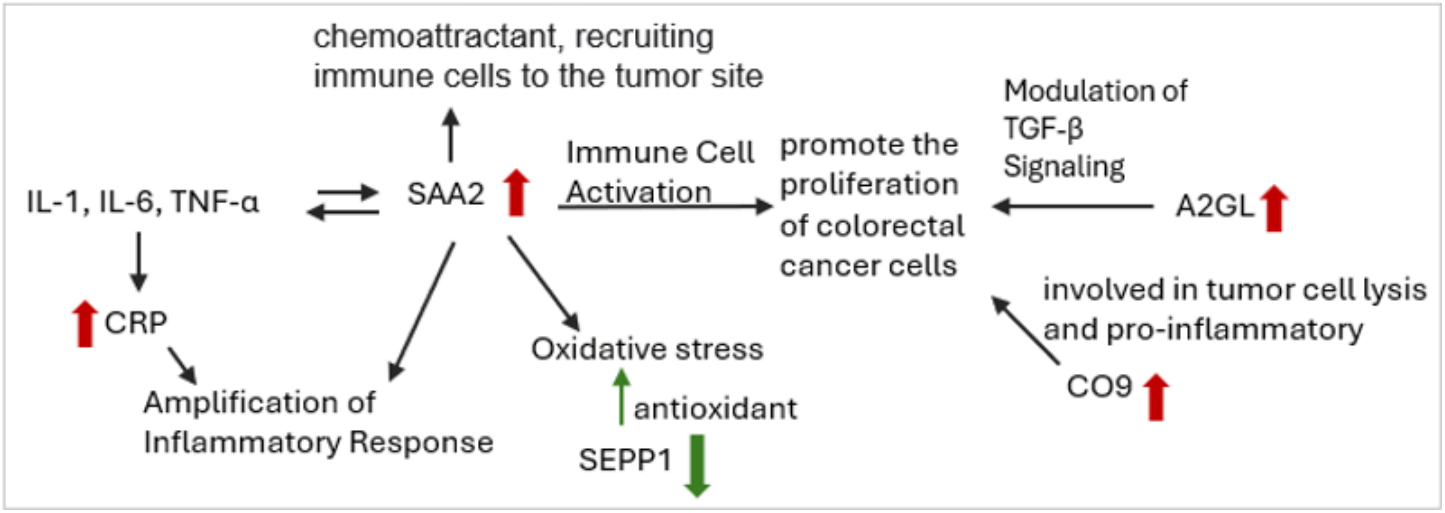

Figure 9. Significantly changed proteins play crucial roles in the development and progression of CRC.

Conclusions

The Stellar mass spectrometer platform represents a significant advancement in mass spectrometry-based proteomics, offering exceptional precision, linearity, and sensitivity. This technology facilitates the analysis of over 1,600 peptide precursors using MS^2 and MS^3 assays within a 30-minute gradient, enabling the identification of 322 endogenous proteins and 507 peptides in total in both disease and healthy plasma, including 57 FDA-approved biomarkers. The MS^3 assay significantly enhances the S/N for low-abundant peptides or those with interference, resulting in the identification of 10.3% more proteins. The Adaptive RT function and a 0.65-minute scheduled RT window ensure the successful capture of all 804 peptides without the need for rescheduling retention time windows during analysis.

The Stellar mass spectrometer platform's advanced linear ion trap analyzer allows for extremely rapid and sensitive PRM and MS^3 targeting, achieving high reproducibility and low coefficients of variation. This capability is crucial for clinical applications, as it enables the accurate quantification of multiple biomarkers, from hundreds of micrograms per milliliter to picograms per milliliter in a single run. The platform's high sensitivity and specificity make it ideal for longitudinal studies and real-time monitoring of disease progression, bridging the gap between proteomics discovery and routine clinical testing.

Significantly altered proteins influencing CRC progression were identified, with proteins such as CO9 and A2GL significantly increased in the plasma of CRC patients compared to healthy controls. This underscores the platform's potential in identifying clinically relevant biomarkers. The Stellar mass spectrometer platform's advanced capabilities make it an indispensable tool for clinical laboratories aiming to enhance their diagnostic and prognostic capabilities, ultimately driving the future of precision medicine.

Key takeaways

Innovative PRM methods on the Stellar mass spectrometer: Developed large-scale targeted PRM methods on the Stellar mass spectrometer, showcasing exceptional precision, linearity, and sensitivity.

Comprehensive peptide analysis: Analyzed over 1,600 peptide precursors using MS^2 and MS^3 assays within a swift 30-minute gradient.

Extensive protein identification: Identified 322 endogenous proteins and 507 peptides in both disease and healthy plasma using MS^2 and MS^3 methods, including 57 FDA-approved biomarkers.

Enhanced detection with MS^3 assay: The MS^3 assay significantly improved the S/N for low-abundant peptides or those with interference, resulting in the identification of 10.3% more proteins.

Efficient peptide capture: Successfully captured all 804 peptides using an Adaptive RT function and a 0.65-minute scheduled RT window, eliminating the need for rescheduling retention time windows during analysis.

CRC biomarker discovery: Identified significantly altered proteins influencing CRC progression, with notable increases in proteins such as CO9 and A2GL in the plasma of CRC patients compared to healthy controls, highlighting their potential as biomarkers for CRC.

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