Highly multiplex targeted proteomics assay in plasma using the Stellar mass spectrometer with adaptive RT

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Abstract

Purpose: Develop a large-scale of multiplexed targeted proteomics assay with adaptive RT to quantify potential biomarkers in patient plasma.

Method: The targeted method with an adaptive RT function was created following the steps in Figure 1. The established assay includes three experiments, as shown in Figure 2. The detailed LC-MS/MS parameters are included in Table 1 (a)-(d).

Results: More than 1600 peptide precursors were analyzed using MS2 and MS3 assays with a 30-minute gradient. The MS3 assay improved the signal-to-noise ratio for low abundant peptides. Certain proteins were found to be significantly changed in the plasma of disease patients compared to that of healthy donors. Distinct protein changes were detected in the plasma of colorectal cancer patients.

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 to hundreds or thousands of targets. To overcome the challenge, Thermo Scientific™ Stellar™ mass spectrometer combined with Skyline™ software were utilized to generate large-scale assays. With hyper-fast acquisition speeds, Stellar MS handles shifting retention times through real-time alignment mode called adaptive RT and maintains the sensitivity and speed required to manage numerous concurrent targets. We developed a multiplex targeted proteomics method with adaptive RT function within 3 days using PQ500 peptides as heavy standards. This method was further applied to the quantitation of potential protein biomarkers in plasma from lung cancer, Alzheimer's disease and colorectal cancer patients.

Materials and methods

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 MS2 assay. PQ500 peptides were obtained from Biognosys AG. The peptide standard was resuspended following the manufacturer's instructions.

Methods: A Thermo Scientific™ Vanquish™ Neo UHPLC coupled with Stellar mass spectrometer scheme was used. Mobile phase A was 0.1% formic acid(FA) in H2O and mobile phase B was 0.1% FA in 80% ACN. Thermo Scientific™ EASY-Spray™ ES906 column temperature was set at 55 ºC and autosampler temperature was 7ºC. Peptides were analyzed using a 30-minute gradients. Mass spectrometer parameters such as AGC values and maximum injection time were optimized. Skyline software was utilized to generate scheduled retention time and PRM panel.

Data Analysis

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

Figure 1. The workflow from the heavy peptide list to large panel of targeted MS2 assay using Stellar mass spectrometer

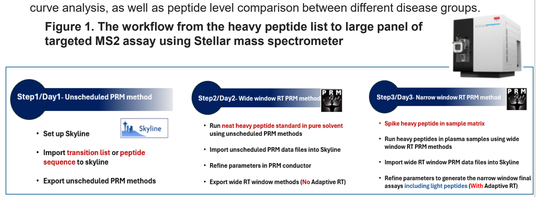


Figure 2. The three experiments in the adaptive RT method

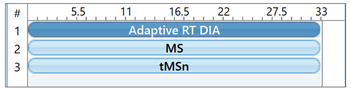


Table1. The LC-MS/MS parameters in tMS2 method with retention time alignment: (a) LC gradient of the assay; (b)-(d) the mass spectrometer parameters in the three experiments

(a)

48SPD (EASY-Spray HPLC ES906A column)			
Gradient	Time	%B	Flow (ul/min)
	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)

tMS2 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	200-1500

(c)

MS experiment parameters	
Scan rate (kDa/s)	125
Scan range (*m/z*)	350-2000
RF lens (%)	30
AGC target	Standard
Maximum injection time mode	Auto

(d)

Adaptive RT DIA parameters	
Precursor mass range (*m/z*)	400-1000
Isolation window (*m/z*)	50
Scan rate (kDa/s)	200
Scan range (*m/z*)	200-1000
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

Results

1. Peptides showed good reproducibility, linearity and sensitivity using Stellar mass spectrometer.

A large panel targeted PRM method was developed on Stellar MS with good reproducibility, linearity, and sensitivity (Figures 3-5). More than 94% of peptides had CV values less than 25% in disease plasma samples. About 90% of peptides had R2 values greater than 0.9. The Stellar MS achieved ultrasensitive detection of peptides in plasma samples at the low amol level.

Figure 3. Chromatogram of 804 peptides

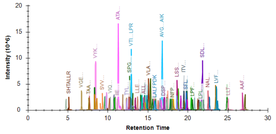


Figure 4. CV% values of peptides in plasma (n=7)

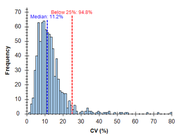
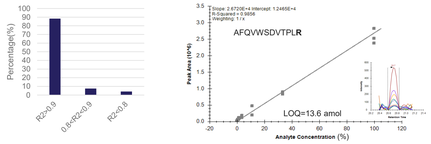


Figure 5. The linearity and limit of quantitation of an example peptide



2. MS3 analysis improved signal to noise ratio (S/N) for low abundant peptides

MS3 assay, which was easily created using PRM conductor, improved S/N for low abundant peptides or peptides with interference (Figure 7). Utilizing tMS3 capabilities increased the number of identified peptides.

Figure 6. MS3 method setup and fraction of a mass list table(more than 1600 precursors were included) in the tMS3 experiment

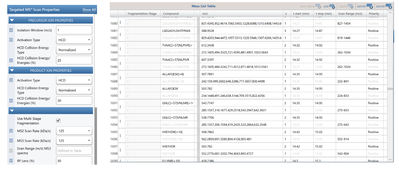
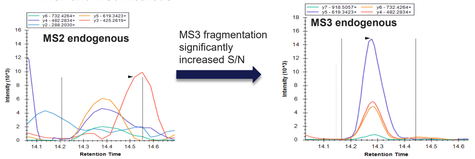


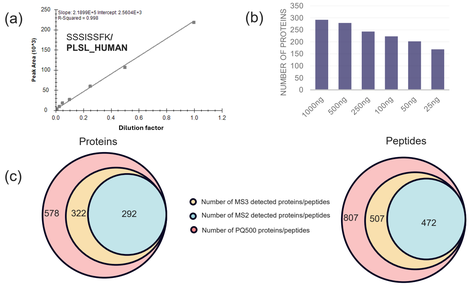
Figure 7. Peptide YLDWIHGHIR from the same AD plasma sample using MS2 and MS3 methods



3. Endogenous proteins and peptides identified using PQ500 standards from disease plasma

Peptides were analyzed from 25 ng to 1 µg plasma digest (Figure 8a). 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 8b). A total of 292 endogenous proteins and 472 peptides were identified in disease and healthy plasma using the targeted MS2 method (Figure 8c). About 10.3% more proteins and 7.3% more peptides were identified using the MS3 assay.

Figure 8. Detected endogenous proteins using PQ500 heavy peptides as reference standards



4. Certain detected endogenous proteins in plasma are FDA approved biomarkers

Among detected proteins, about 57 proteins are FDA approved biomarkers for different diseases.

Figure 9. 57 of detected proteins were FDA biomarkers

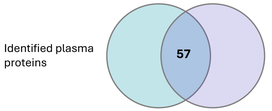


Table 2. Detected FDA biomarkers using PQ500 heavy peptides as reference standards

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.

5. Potential protein biomarkers were found significantly increased in colorectal cancer (CRC) patients' plasma

29 proteins were found significantly changed (adj.p<0.05) with more than 2-fold concentration changes in CRC patient plasma compared to healthy controls. Proteins such as SAA2, A2GL and CO9 were found to be significantly increased in CRC patient plasma. These proteins were also reported as potential biomarkers for CRC disease [1,2].

Figure 10. Volcano plot of the protein comparison in plasma from healthy donors and colorectal cancer patients

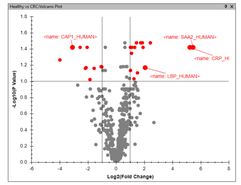
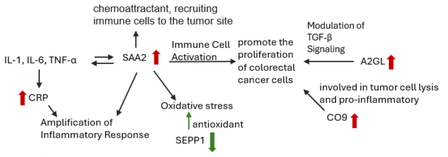


Table 3. Significantly changed proteins in CRC patient plasma

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 11. Significantly changed proteins play crucial roles in the development and progression of colorectal cancer



Conclusions

1. Large-scale targeted PRM methods were developed on Stellar MS, demonstrating good precision, linearity, and sensitivity. Over 1600 peptide precursors were analyzed using MS2 and MS3 assays with a 30-minute gradient.
2. In total, 292 endogenous proteins and 472 peptides were identified in disease and healthy plasma, including 57 FDA-approved biomarkers.
3. The MS3 assay enhanced the signal-to-noise ratio for low-abundant peptides or those with interference, leading to the identification of 10.3% more proteins.
4. Using an adaptive RT function and a 0.65-minute scheduled RT window, all 804 peptides were successfully captured without the need for rescheduling retention time windows during analysis.
5. Significantly changed proteins were found to influence colorectal cancer progression. Specifically, proteins such as CO9 and A2GL were significantly increased in the plasma of colorectal cancer patients compared to healthy controls, suggesting their potential as biomarkers for colorectal cancer.

References

[1] Juthamard Chantaraamporn, et.al. Proteomes 2020, 8(3), 26.

[2] Bethany Geary. Et.al. Cancers 2021, 13(10), 2443.

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