Highly multiplex targeted proteomics assay in plasma using 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 System 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 °C and autosampler temperature was 7°C. Peptides were analyzed using a 30-minute gradients. Mass spectrometer parameters such as AGC values and maximum injection time were optimized. Skyline was utilized to generate scheduled retention time and PRM panel.

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. Figure 1. The workflow from the heavy peptide list to large panel of

targeted MS² assay using Stellar mass spectrometer



- Import transition list or peptide sequence to skyline
- Export unscheduled PRM methods

Step2/I	Day2- Wide window RT PRM method	
• Run	neat heavy peptide standard in pure solvent	t

- using unscheduled PRM methods Import unscheduled PRM data files into Skyline
- Refine parameters in PRM conductor
- Export wide RT window methods (No Adaptive RT)



Mode

Spike heavy peptide in sample matrix

Step3/Day3- Narrow window RT PRM metho

- Run heavy peptides in plasma samples using wide
- vindow RT PRM methods
- Import wide RT window PRM data files into Skyline
- Refine parameters to generate the narrow window final assays including light peptides (With Adaptive RT)

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1 2 3	





Figure 2. The three experiments in the adaptive RT method

5 .5 11 16.	5 22	27.5	33
Adaptive	RT DIA		
MS			
tMS	n		\supset

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

(EASY-Spray HPLC ES906A column)							
nt	Time	%В	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						

Vindow (m/z) 1
Туре НСD
sion Energy (%) 30
et Standard
Injection Time Mode Dynamic
peak 6
trol All
e(s) 2
Time Scheduling Adaptive RT
File Generated when export method from PRM conductor or When Acquire Reference is checked in "Adaptive RT DIA" experiment
e(kDa/s) 125
%) 30
ge 200-1500
(d)
meters Adaptive RT DIA parameters
Precursor Mass Range (m/z) 400-1000
000 Isolation Window (m/z) 50
Scan Rate (kDa/s) 200
ard Scan Range (m/z) 200-1000
HCD Collision Energy (%) 30
RF Lens (%) 30
AGC Target Standard
Maximum Injection Time Mode Auto
Window Placement Off Optimization

Acquire Reference

Checked in scheduled PRM methods to generated reference

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 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

		<u> </u>		Fragmentation Stage	Compound	m/z	z	t start (min)	t stop (min)	Scan Range (m/z)	Polarity
	PRECURSOR ION	PROPERTIES	1680 [837.4345,952.4614,1065.5455,1228.6088,1315.6408,1443.6	1			827-1454	
	Isolation Window (m/z)	1	1681		LSEGASYLDHTFPAEK	588.9528	3	14.27	14.87		Positive
	Activation Type	HCD	, 1682			829.4203,944.4472,1057.5313,1220.5946,1307.6266,1435.6	1			819-1446	Positive
	HCD Collicion Energy		1683		TVAAC[+57]NLPIVR[+'	612.3438	2	14.32	14.92		Positive
	Туре	Normalized	1684 L			272.1605,494.3325,721.4595,881.4901,1023.5643	1			262-1034	
Ħ	HCD Collision Energy/	25	1685		TVAAC[+57]NLPIVR	607.3397	2	14.32	14.92		Positive
_	Energies (%)		1686			272.1605,484.3242,711.4512,871.4818,1013.5561	1			262-1024	
	PRODUCTION P	ROPERTIES	1687		ALLAFQESK[+8]	507.7891	2	14.35	14.95		Positive
⊞	Activation Type	HCD .	1688 ^l			242.159,499.2602,646.3286,717.3657,830.4498	1			232-841	Positive
	HCD Collision Energy	Normalized	, 1689 ,		ALLAFQESK	503.782	2	14.35	14.95		Positive
	lype		1690			234.1448,491.246,638.3144,709.3515,822.4356	1			224-833	
⊞	Energies (%)	30	1691		GNLC[+57]VNLMR[+1	543.7747	2	14.35	14.95		Positive
-			1692 ¹			285.1557,316.1677,429.2518,543.2947,642.3631	1			275-653	
	Use Multi-Stage		1693		GNLC[+57]VNLMR	538.7706	2	14.35	14.95		Positive
	Fragmentation		1694			285.1557,306.1594,419.2435,533.2864,632.3548	1			275-643	
=	MS2 Scan Rate (kDa/s)	125	1695		IVIEYVDR[+10]	508.7862	2	14.42	15.02		Positive
Ħ	MS3 Scan Rate (kDa/s)	125	1696			562.2859,691.3285,804.4126,903.481	1			552-914	Positive
	Scan Bange (m/z) MS3		1697		IVIEYVDR	503.782	2	14.42	15.02		Positive
	spectra	Defined in Table	1698			552.2776,681.3202,794.4043,893.4727	1			542-904	
	RF Lens (%)	30	1699 [1	FLLYNR[+10]	418.2386	2	14.5	15.1		Positive

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 8(a)). 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 8(b)). A total of 292 endogenous proteins and 472 peptides were identified in disease and healthy plasma using the targeted MS² method (Figure 8(c)). 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
TIHY	Prealbumin	OVA1 test
CERU	Ceruloplasmin	Wilson disease
CYIC	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

Figure 4. CV% values of

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

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S C I E N T I F I C

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





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.
- . 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.

Reference

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

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

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