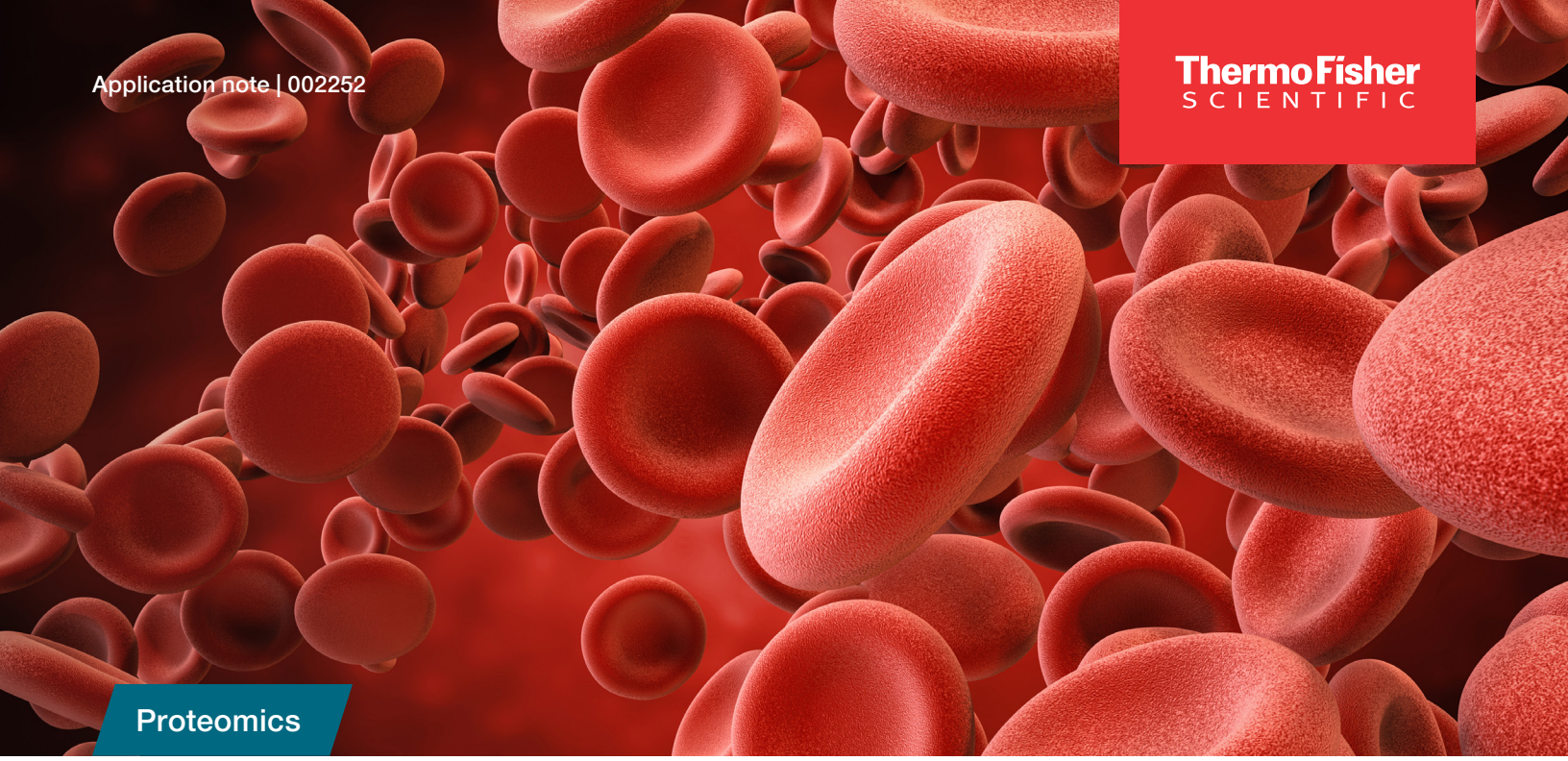

Application note | 002252

Proteomics

New standards for plasma proteomics—Balancing throughput for large sample cohorts and depth of analysis for biomarker discovery

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Keywords

Orbitrap Astral mass spectrometer, plasma proteomics, enrichment, fractionation, proteome coverage, throughput

Goal

To develop a high-throughput plasma proteomics analysis workflow without compromising the depth of analysis using a label-free data-independent acquisition (DIA) method on the new Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer

Introduction

Plasma is a rich source for protein biomarkers that can be used to reveal disease biology, measure responses to therapeutic treatments, or for diagnostic and prognostic purposes. A major advantage of plasma proteomics is the convenience of obtaining samples from routine blood draws and the availability of large cohorts of samples stored awaiting analysis such as in biobanks. However, identifying these biomarkers with bottom-up proteomics has substantial challenges due to the wide range of protein concentrations present in plasma. The analysis of low-abundant proteins can be analytically challenging since 99% of plasma consists of highly abundant proteins like albumin, globulins, and coagulants that essentially crowd out these low-abundant proteins from mass spectrometry-based detection and could potentially serve as biomarkers.[1,2]

There are multiple ways to perform sample preparation for plasma proteomics with no standardization. Some of the approaches are discussed below, highlighting their advantages and disadvantages.

1. Neat, undepleted plasma—This method offers the easiest sample preparation and highest throughput. This approach prevents the removal of non-specific proteins and minimizes technical variation. However, low abundant proteins may not be detected.
2. Immunodepletion—This approach removes 99% of the most abundant proteins and alleviates the dynamic range problem. The disadvantage is the removal of non-specific proteins. The sample preparation needs to be automated to keep up with sample throughput as well as the added reagent cost per sample.
3. Fractionation—Involves off-line methods to separate proteins into less complex fractions. This approach increases the time of preparation and analysis but enables the identification of more plasma proteins.
4. Multi-nanoparticle enrichment with the Seer Proteograph™ Product Suite (www.seerbio.com), where the use of a panel of diverse nanoparticles enables broad and deep coverage of the plasma proteome. Automation is necessary to keep up with throughput and there are additional reagent costs per sample.

Regardless of the approach undertaken for sample preparation, there is a need for a standardized liquid chromatography-mass spectrometry (LC-MS) workflow for plasma proteomics that balances the depth of coverage and quantitative accuracy and precision with the scalability to address large sample cohort sizes. The Orbitrap Astral mass spectrometer delivers a new set of performance standards for plasma proteomics with the flexibility for both deeper proteome coverage and fast throughput with accurate and precise quantitation due to its speed and high sensitivity.

In this application note, we evaluate the performance of the novel Orbitrap Astral mass spectrometer for plasma proteomics using four of the most common plasma sample preparation methods. The Orbitrap Astral mass spectrometer enables twice the proteome coverage compared to the current commercial mass spectrometers for the same gradient. In addition, with double the throughput, it provides higher proteome coverage.

An illustration of the workflows used in these experiments is shown in Figure 1.

Experimental

Recommended consumables

- Fisher Scientific™ LC-MS grade water with 0.1% formic acid (FA), (P/N LS118-500)
- Fisher Scientific™ LC-MS grade 80% acetonitrile (ACN with 0.1% formic acid (P/N LS122500)
- Fisher Scientific™ LC-MS grade formic acid (P/N A117-50)
- Thermo Scientific™ EasyPep™ MS sample prep kits (P/N A40006)
- Thermo Scientific™ High Select™ Depletion Spin Columns (P/N A36370)

Figure 1. At the heart of the plasma proteomics workflow, the Orbitrap Astral mass spectrometer delivers the analytical flexibility to enable your exact experimental goals. The mass spectrometer provides faster throughput for large cohorts and deeper coverage for extended biomarker insights with accurate and precise quantitation to increase statistical power.

LC columns

- Thermo Scientific™ EASY-Spray™ PepMap™ column, 2 µm C18 150 µm x 15 cm (P/N ES906)
- Thermo Scientific™ PepMap™ Neo trap cartridge, 5 µm C18 300 µm x 5 mm (P/N 174500)
- Thermo Scientific™ EASY-Spray™ PepMap™ Neo column, 2 µm C18 75 µm x 75 cm (P/N ES75750PN)
- Thermo Scientific™ µPAC™ Neo HPLC column, 2.5 µm x 16 µm, 110 cm (P/N COL-NANO110NEOB)

UHPLC system

Thermo Scientific™ Vanquish™ Neo UHPLC system including:

- Vanquish Neo UHPLC system, (comprising Binary Pump N, Split Sampler, NT, Solvent Rack, System base with drawer and Ship kit) (P/N VN-S10-A-01)
- Vanquish Display (P/N 6036.1180)
- Column Compartment N (P/N VN-C10-A-01)

Mass spectrometer

- Thermo Scientific Orbitrap Astral mass spectrometer
- Emitter, Fused Silica, ID 10 µm (P/N EV1111)
- EasySpray Adapter (P/N EV-1072)

Data analysis software

- Thermo Scientific™ Proteome Discoverer™ 3.1 software powered by Thermo Scientific™ Ardia™ with CHIMERYS™ intelligent search algorithm by MSAID™

Neat plasma preparation

Two LC-MS methods were utilized for neat plasma analysis. The 180 sample per day (SPD) method was used for maximum throughput, while the 36 SPD method aimed for maximum depth of proteome coverage (Figure 2).

The neat plasma samples used in these experiments were from a pooled sample collected from multiple donors. The samples were prepared using the EasyPep MS sample prep kit according to the manufacturer's protocol. After preparation, the dried peptides were resuspended in water with 0.1% FA to a final concentration of 200 ng/µL. Depending on the experiment, 200 ng to 2,000 ng of digested peptides were loaded on the column for LC-MS and data were acquired in triplicates. For all the LC-MS runs described here (depleted and enriched plasma), mobile phase A = 0.1% FA in H_2O, and mobile B = 0.1% FA in 80% ACN used, with a column temperature of 50 °C and an autosampler temperature of 7 °C.

The MS method parameters used for all the experiments (including depleted and enriched plasma) were identical, (Table 1), except for maximum injection time, which was adjusted per SPD method (Table 2). This parameter was set depending on the experiment and gradient length.

180 SPD method (trap/elute), 200 ng load
EASY-Spray PepMap 15 cm column

Time, min	Duration, min	%B	Flow rate, µL/min
0.0	0.0	4.0	2.5
0.2	0.2	8.0	2.5
4.0	3.8	20.0	2.5
5.8	1.8	35.0	2.5
Column wash			
6.2	0.4	99.0	2.5
6.7	0.5	99.0	2.5
Stop run			
Column equilibration			

24 SPD method (direct injection), 1 µg load
EASY-Spray PepMap Neo 75 cm column

Time, min	Duration, min	%B	Flow rate, µL/min
0.0	0.0	4.0	0.3
20.0	20.0	25.0	0.3
25.0	5.0	35.0	0.3
Column wash			
30.0	5.0	99.0	0.3
40.0	10.0	99.0	0.3
Stop run			
Column equilibration			

Figure 2. 180 SPD method for maximum throughput and 24 SPD method for maximum depth of coverage. These SPD methods include loading, gradient, wash, and equilibration times, so a 180 SPD method has a total run time of 8 min.

Table 1. MS method parameters used for all the experiments

Category	Property	Setting
Method setting	Application mode	Peptide
Ion source	Positive ion (V)	1,900
	Ion transfer tube temp (°C)	275
MS global settings	Advanced peak determination	TRUE
	Default charge state	2
Orbitrap analyzer full scan	Scan range (*m/z*)	380–980
	Orbitrap resolution	240,000
	Max IT (ms)	5
	RF lens (%)	40
	AGC target (%)	500
Astral analyzer DIA MS^2 scan	Scan range (*m/z*)	380–980
	Isolation window (*m/z*)	2
	Windows overlap (*m/z*)	0
	Window placement optimization	On
	Number of scan events	300
	HCD collision energies (%)	25
	Detector type	Astral
	Max IT (ms)	Experiment dependent
	AGC target (%)	500
	Loop control	Time
	Loop time (s)	0.6

Table 2. Maximum injection time used for each SPD method

SPD method	Maximum injection time
180 SPD	3.5 ms
60 SPD	5 ms
48 SPD	10 ms
24 SPD	7 ms
18 SPD	10 ms
14 SPD	10 ms
4 SPD	10 ms

Depleted plasma preparation

The top 14 abundant proteins in the human plasma samples were depleted using the High Select Depletion Spin Column. Samples were dried in a Thermo Scientific™ Savant™ SpeedVac™ concentrator, reconstituted with the EasyPep lysis buffer, and processed with an EasyPep MS Sample Prep Kit according to the manufacturer's protocol.

An 18 SPD method (80 minutes total) was employed to assess the depth of depleted plasma proteome coverage. The samples were analyzed in triplicate (Figure 3).

18 SPD method (direct injection), 1 µg load
µPAC Neo 110 cm column

Time, min	Duration, min	%B	Flow rate, µL/min
0.0	0.0	4.0	0.75
0.4	0.4	4.0	0.75
47.4	47.0	22.5	0.75
60.4	13.0	45.0	0.75
Column wash			
64.9	4.5	99.0	0.75
66.5	1.6	99.0	0.75
Stop run			
Column equilibration			

Figure 3. 18 SPD method details that include loading, gradient, wash, and equilibration times, showing a total run time of 80 minutes

Enriched and fractionated plasma sample preparation

The Seer Proteograph Product Suite was developed to address the dynamic range limitations in plasma by using a panel of five proprietary engineered nanoparticles (NPs) with distinct physicochemical properties. The Proteograph allows for sampling of plasma proteins across the wide dynamic range of the proteome, resulting in five plasma fractions for LC-MS analysis.

Human plasma samples were prepared with the Proteograph Assay kit according to the manufacturer's protocols using the Seer SP100 automation instrument. Several LC-MS methods were evaluated for high to moderate throughput and for maximum depth of analysis. The maximum depth for plasma analysis can be achieved via analyzing the five individual fractions generated by the Seer Proteograph Product Suite. For samples generated in this manner, we also evaluated throughput and depth of proteome coverage (Figure 4).

In addition to analyzing the five fractions individually, the fractions were also pooled for a single shot LC-MS analysis to further evaluate throughput and depth of proteome coverage (Figure 5).

180 SPD method (trap/elute), 500 ng load
EASY-Spray PepMap column

Time, min	Duration, min	%B	Flow rate, µL/min
0.0	0.0	4.0	2.5
0.2	0.2	8.0	2.5
4.0	3.8	20.0	2.5
5.8	1.8	35.0	2.5
Column wash			
6.2	0.4	99.0	2.5
6.7	0.5	99.0	2.5
Stop run			
Column equilibration			

48 SPD method (direct injection), 500 ng load
µPAC Neo column

Time, min	Duration, min	%B	Flow rate, µL/min
0.0	0.0	6.0	1.0
0.4	0.4	6.0	1.0
10.4	10.0	22.5	1.0
15.4	5.0	45.0	1.0
Column wash			
16.4	1.0	99.0	1.0
22.0	5.6	99.0	1.0
Stop run			
Column equilibration			

18 SPD method (direct injection), 1 µg load
µPAC Neo column

Time, min	Duration, min	%B	Flow rate, µL/min
0.0	0.0	4.0	0.75
0.4	0.4	4.0	0.75
47.4	47.0	22.5	0.75
60.4	13.0	45.0	0.75
Column wash			
64.9	4.5	99.0	0.75
66.5	1.6	99.0	0.75
Stop run			
Column equilibration			

Sample	Total time, min		
	180 SPD	48 SPD	18 SPD
Per fraction	8	30	80
Plasma (5 fractions)	40	150	400
Number of plasma samples over 24 hours	36	10	4

Figure 4. Several methods were used to evaluate the balance between high throughput and depth of coverage for Seer individual plasma fractions.

60 SPD method (trap/elute), 500 ng load
EASY-Spray PepMap 15 cm column

Time, min	Duration, min	%B	Flow rate, µL/min
0.0	0.0	4.0	1.3
0.5	0.5	4.0	1.3
0.6	0.1	8.0	0.8
13.9	13.3	22.5	0.8
20.8	6.9	35.0	0.8
21.2	0.4	55.0	2.0
Column wash			
21.9	0.7	99.0	2.0
22.6	0.7	99.0	2.0
Stop run			
Column equilibration			

14 SPD method (direct injection), 2 µg load
EASY-Spray PepMap Neo 75 cm column

Time, min	Duration, min	%B	Flow rate, µL/min
0.0	0.0	5.0	0.3
55.0	55.0	25.0	0.3
65.0	10.0	35.0	0.3
Column wash			
70.0	5.0	99.0	0.3
80.0	10.0	99.0	0.3
Stop run			
Column equilibration			

Figure 5. Two methods were used to evaluate the balance between moderate throughput and depth of coverage for Seer pooled plasma fractions.

Data processing parameters

Proteome Discoverer software, version 3.1, was used for data processing (Figure 6). The CHIMERYS node was used with Human FASTA files selected as the protein database of choice (downloaded from uniport Tx ID 9606) and Inferys prediction model version 3.0 was used for the CHIMERYS search. Oxidation of methionine and carbamidomethylation of cysteine were selected as dynamic and static modifications respectively. Trypsin was the enzyme of choice with a maximum number of 2 missed cleavage sites per peptide.

Results and discussion

Neat plasma

Neat plasma offers the highest throughput given there is no depletion or enrichment steps involved in sample preparation. The presence of abundant proteins limits the depth of proteome coverage compared to the other sample preparation methods. However, due to the sensitivity and speed of the Orbitrap Astral mass spectrometer, the proteome coverage for neat plasma for both short and long LC gradients surpasses the current published data to date[3-5] (Figure 7).

Depleted plasma

The depleted plasma sample preparation method offers moderate throughput taking the depletion step into account. For the plasma sample prepared in this manner, we employed a long gradient method (24 SPD method) to evaluate the depth of proteome coverage.

The proteome coverage increased by ~2x compared to neat plasma when using methods designed for greater depth of coverage than the method for high throughput. The sensitivity and the speed of the Orbitrap Astral mass spectrometer provided proteome coverage for depleted plasma that surpassed the current published data to date[6] (Figure 8).

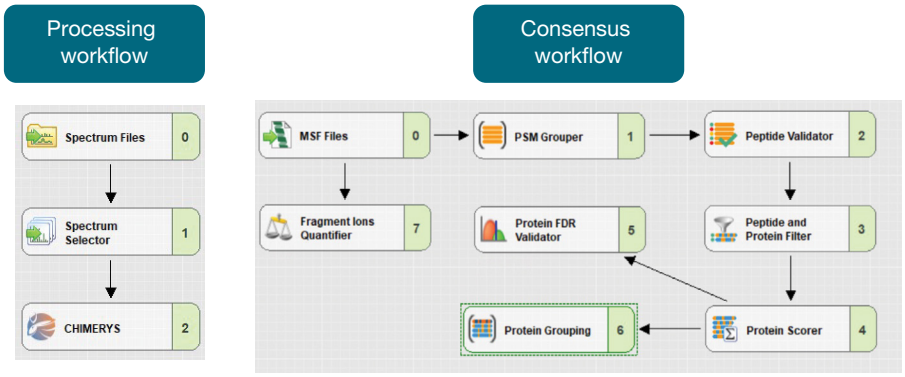


Figure 6. Proteome Discoverer 3.1 software powered by Ardia with CHIMERYS intelligent search algorithm by MSAID

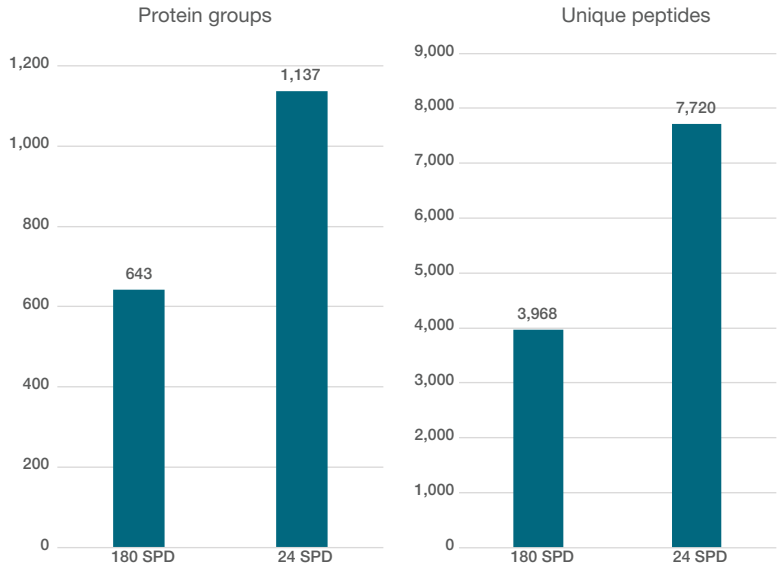


Figure 7. The high throughput method of 180 SPD delivered a depth of coverage of 643 protein groups, substantially higher than what is currently reported in the literature[3] under similar conditions. By running a longer gradient using the 24 SPD method, 1137 protein groups were identified, also, higher than what is reported in the literature to date[4,5].

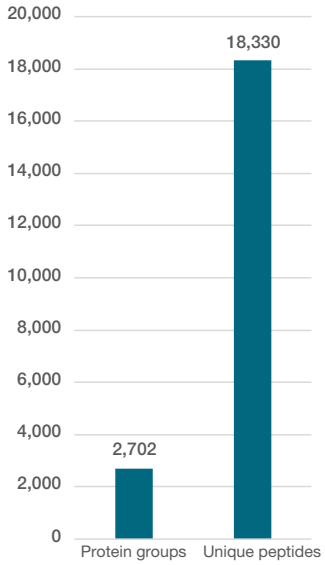


Figure 8. For depleted plasma, a longer gradient using a 18 SPD method was employed for greater depth of coverage, resulting in 2,702 protein groups being identified, 1.5 times more than currently reported under similar conditions[6].

Seer enriched plasma fractions

Individual fractions generated by Proteograph were evaluated for both throughput and depth of coverage. The fractions were also pooled to maximize throughput and evaluate the depth of coverage. For the individual fractions, the SPD method/time refers to each individual fraction, so the 180 SPD method means 36 plasma samples with each plasma sample consisting of 5 fractions.

The methodology for plasma profiling varies among users. Some undertake capillary flow to tap into the robustness the setup affords, while others prefer to work in the nanoflow space to take advantage of the sensitivity. In our capillary flow experiments, we were able to identify 3,104 protein groups (individual fraction analysis) at 36 SPD throughput, while 3,285 protein groups (pooled fractions, single shot) were identified with nanoflow at 60 SPD throughput. Comparing the throughput and depth of coverage of the individual fractions and the pooled fractions, the numbers of proteins identified surpassed those reported in the literature (Figure 9).

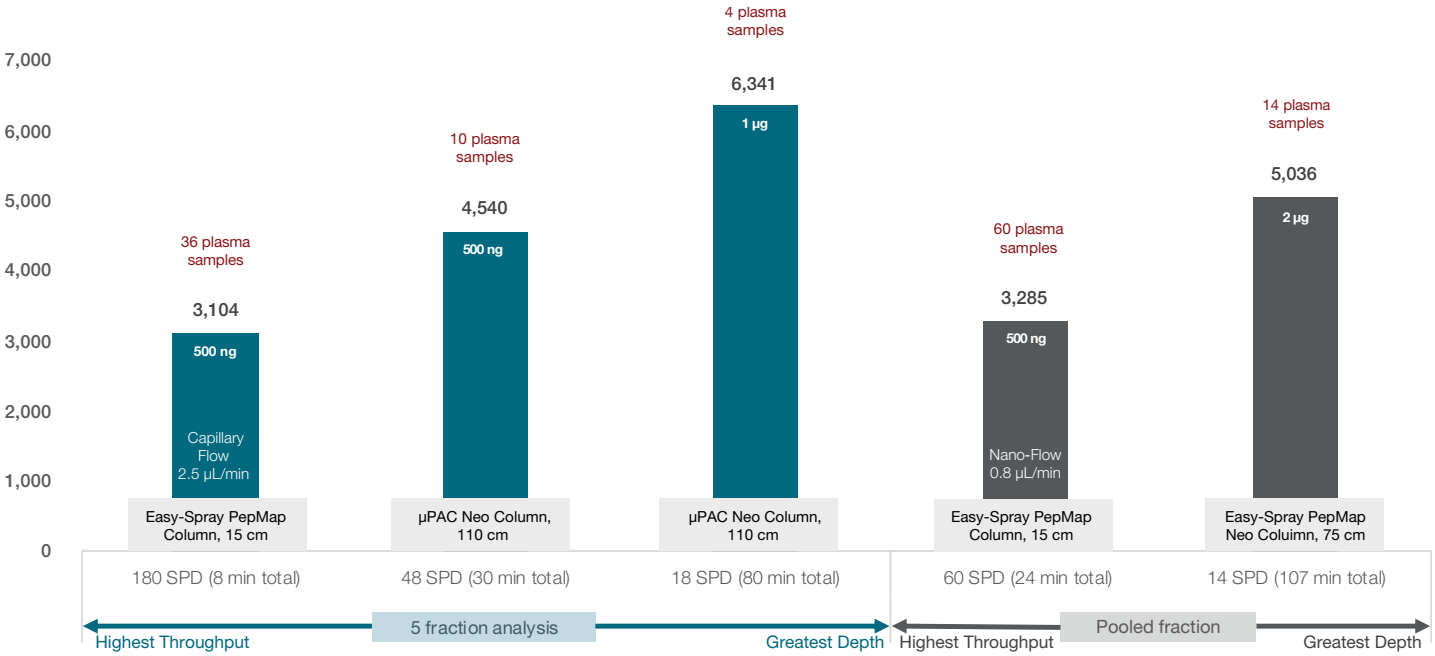
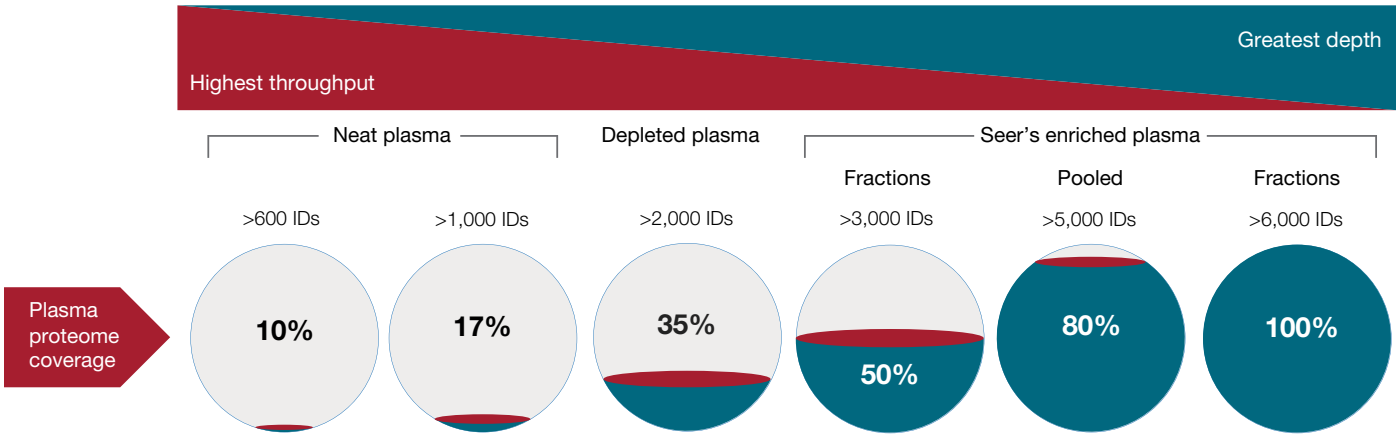


Figure 9. Seer enriched plasma sample results on the Orbitrap Astral mass spectrometer. Both individual fractions and pooled samples were evaluated for proteome coverage and throughput.

Summary

Neat plasma suffers from dynamic range issues resulting in lower coverage compared to depleted plasma. Enrichment/ fractionation with the Seer methodology offers more depth of analysis while balancing the need for high throughput.

Regardless of the sample preparation method chosen for plasma proteomics, the Orbitrap Astral mass spectrometer delivers the highest proteome coverage and throughput compared to current mass spectrometers[3-6] (Figure 10).



	PepMap column 15 cm	PepMap Neo column 75 cm	µPAC Neo column 110 cm	PepMap column 15 cm	PepMap Neo column 75 cm	µPAC Neo column 110 cm
Plasma samples/day	180	24	18	36	14	4
Sample load	0.5 µg	0.5 µg	0.5 µg	0.5 µg	2 µg	1 µg

Figure 10. Summary of plasma results on the Orbitrap Astral mass spectrometer

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