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# An In-depth Plasma Proteomics Workflow Powered by Orbitrap Astral Mass Spectrometer

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## 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 using different varieties of gradients from maximum throughput methods to deepest coverage methods.

## 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 been very challenging with substantial analytical challenges due to the wide range of protein concentrations present in plasma. Here we present a label-free plasma proteomics workflow using an Orbitrap Astral Mass Spectrometer as a robust analytical setup for in-depth analysis of plasma proteins. Three different types of samples were used including 1) a neat plasma sample, 2) Depleted plasma and 3) Plasma sample enriched utilizing a multi-nanoparticle-based approach with the Seer Proteograph™ Product Suite.

## MATERIALS

**Materials:**

- Fisher Scientific™ LC-MS grade water with 0.1% formic acid
- Fisher Scientific™ LC-MS grade 80% acetonitrile with 0.1% formic acid
- Thermo Scientific™ EasyPep™ MS Sample Prep Kit
- Thermo Scientific™ High Select™ Depletion Spin Columns

**Analytical Column:**

- Thermo Scientific™ EASY-Spray™ Pepmap™ Column, 2 µm C18, 150 µm x 15 cm
- Thermo Scientific™ EASY-Spray™ Pepmap™ Neo Column, 2 µm C18, 75 µm x 75 cm
- Thermo Scientific™ uPAC™ Neo Column, Pillar 2.5 µm x 16 µm, Length 110 cm

**Trap Column**: PepMap Neo Trap Cartridge, 5 µm C18 300 µm x 5 mm

**Samples:**

1. Neat plasma sample (a pooled sample collected from multiple healthy donors*)
2. Depleted plasma sample (depleted using Thermo Scientific™ High Select™ Depletion Spin Columns*)
3. Enriched plasma sample (plasma sample proteins of which enriched using Proteograph™ Assay kit according to the manufacturer's protocols on the Seer SP100 automation instrument)

***** These samples were prepared using LFQ EasyPep-36 on a Thermo Scientific™ AccelerOme™ automated proteomics sample preparation system.

## METHODS

**Figure 2.** Multiple gradients were used to provide options based on the experiment needs from highest throughput to the deepest proteome coverage (**Figure 2, A**). The number of samples to analysis per day includes loading, gradient, wash and equilibration time. The MS method parameters used for all the experiments were identical, (**Figure 2, B**), except for maximum injection time, which was adjusted per SPD method. This parameter was set depending on the experiment and gradient length. Proteome Discoverer software, version 3.1, was used for data processing (**Figure 2, C**). The CHIMERYS node using Inferys prediction model version 3.0 was used for the search.

LC Gradients — A

| Time | Flow Rate | %B |
|---|---|---|
| 0.00 | 2.5 µl/min | 4.0 |
| 0.20 | 2.5 µl/min | 8.0 |
| 4.00 | 2.5 µl/min | 20.0 |
| 5.80 | 2.5 µl/min | 35.0 |
| 6.20 | 3.0 µl/min | 99.0 |
| 6.70 | 3.0 µl/min | 99.0 |

180 SPD

| Time | Flow Rate | %B |
|---|---|---|
| 0.0 | 1.0 µl/min | 6.0 |
| 0.4 | 1.0 µl/min | 6.0 |
| 10.4 | 1.0 µl/min | 22.5 |
| 15.4 | 1.0 µl/min | 45.0 |
| 16.4 | 1.0 µl/min | 99.0 |
| 22.0 | 1.0 µl/min | 99.0 |

48 SPD

| Time | Flow Rate | %B |
|---|---|---|
| 0.00 | 0.300 µl/min | 5.0 |
| 20.00 | 0.300 µl/min | 25.0 |
| 25.00 | 0.300 µl/min | 35.0 |
| 30.00 | 0.300 µl/min | 99.0 |
| 40.00 | 0.300 µl/min | 99.0 |

24 SPD

| Time | Flow Rate | %B |
|---|---|---|
| 0.0 | 0.75 µl/min | 4.0 |
| 0.4 | 0.75 µl/min | 4.0 |
| 47.4 | 0.75 µl/min | 22.5 |
| 60.4 | 0.75 µl/min | 45.0 |
| 64.9 | 0.75 µl/min | 99.0 |
| 66.5 | 0.75 µl/min | 99.0 |

18 SPD

| Time | Flow Rate | %B |
|---|---|---|
| 0.0 | 1.3 µl/min | 4.0 |
| 0.6 | 1.3 µl/min | 4.0 |
| 13.9 | 0.8 µl/min | 22.5 |
| 20.8 | 0.8 µl/min | 35.0 |
| 21.2 | 2.0 µl/min | 55.0 |
| 21.9 | 2.0 µl/min | 99.0 |
| 22.6 | 2.0 µl/min | 99.0 |

60 SPD

| Time | Flow Rate | %B |
|---|---|---|
| 0.0 | 0.3 µl/min | 5.0 |
| 55.0 | 0.3 µl/min | 25.0 |
| 65.0 | 0.3 µl/min | |
| 70.0 | 0.3 µl/min | 99.0 |
| 80.0 | 0.3 µl/min | 99.0 |

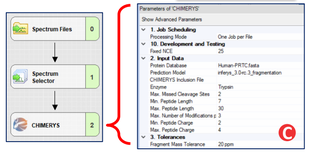
14 SPD

MS Method — B

| Property | Setting |
|---|---|
| Positive Ion (V) | 2000 |
| Ion Transfer Tube Temp (˚ C) | 275 |
| Advanced Peak Determination | TRUE |
| Default Charge State | 2 |
| Scan Range (m/z) | 380-980 |
| Detector Type | **Orbitrap** |
| Orbitrap Resolution | 240000 |
| Max IT (ms) | 5 |
| RF Lens (%) | 40 |
| AGC Target (%) | 500 |
| Scan Range (m/z) | 380-980 |
| Isolation Window (m/z) | 2 |
| Windows Overlap (m/z) | 0 |
| Window Placement Optimization | On |
| Number of Scan Events | 299 |
| HCD Collision Energies (%) | 25 |
| Detector Type | **Astral** |
| Max IT (ms) | Experiment Dependent |
| AGC Target (%) | 500 |
| Loop Control | Time |
| Loop Time (sec) | 0.6 |

| Method | 180 SPD | 60 SPD | 48 SPD | 24 SPD | 18 SPD | 14 SPD | 4 SPD |
|---|---|---|---|---|---|---|---|
| Max-IT | 3.5 ms | 5 ms | 10 ms | 7 ms | 10 ms | 10 ms | 10 ms |

Data Processing



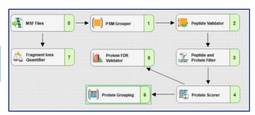
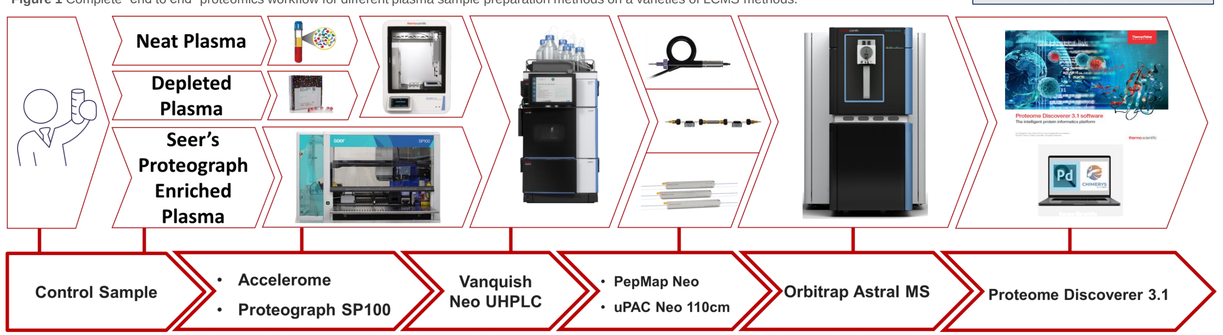
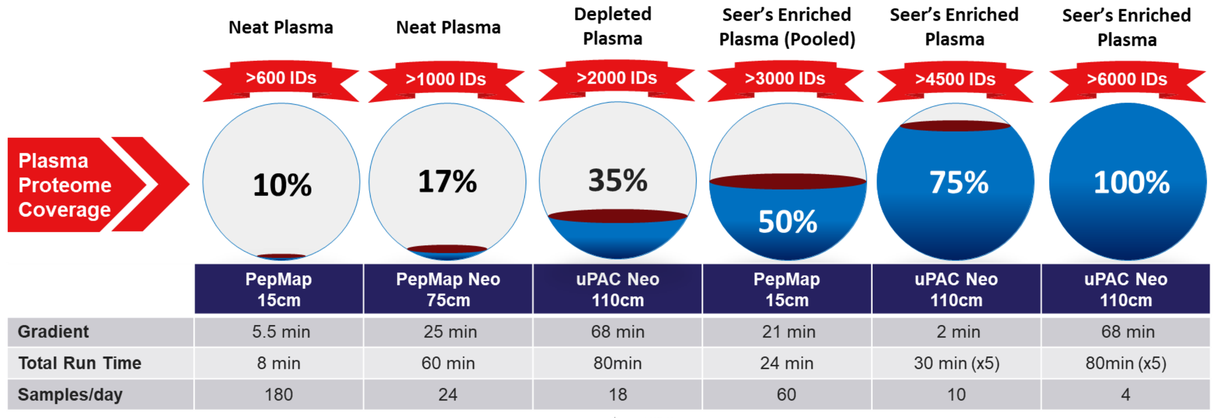




**Figure 1** Complete "end to end" proteomics workflow for different plasma sample preparation methods on a varieties of LCMS methods.




| | PepMap 15cm | PepMap Neo 75cm | uPAC Neo 110cm | PepMap 15cm | uPAC Neo 110cm | uPAC Neo 110cm |
|---|---|---|---|---|---|---|
| **Gradient** | 5.5 min | 25 min | 68 min | 21 min | 2 min | 68 min |
| **Total Run Time** | 8 min | 60 min | 80min | 24 min | 30 min (x5) | 80min (x5) |
| **Samples/day** | 180 | 24 | 18 | 60 | 10 | 4 |

**Figure 3. Summary of plasma results on the Orbitrap Astral mass spectrometer**

## RESULTS

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, great proteome coverage for both short and long LC gradients was observed. 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. Superior proteome coverage was observed when samples enriched by Proteograph nanoparticles were evaluated for both throughput and depth of coverage either through individual NP injections or pooled sample (Figure 4).

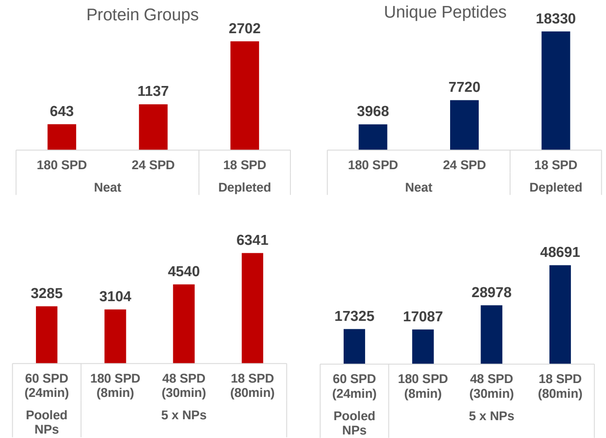
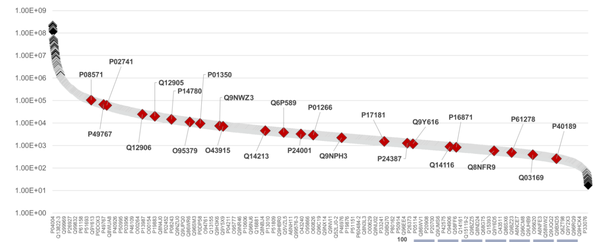



**Figure 4.** Protein and peptide identification for neat, depleted, and Seer's Proteograph Nanoparticles enriched plasma samples




| | | | |
|---|---|---|---|
| P40189 | Interleukin-6 receptor subunit beta | P24001 | Interleukin-32 |
| Q03169 | Tumor necrosis factor alpha-induced protein 2 | Q14213 | Interleukin-27 subunit beta |
| P61278 | **Somatostatin** | O43915 | Vascular endothelial growth factor D |
| Q8NFR9 | Interleukin-17 receptor E | Q9NWZ3 | Interleukin-1 receptor-associated kinase 4 |
| P16871 | Interleukin-7 receptor subunit alpha | P01350 | Gastrin |
| Q14116 | Interleukin-18 | O95379 | Tumor necrosis factor alpha-induced protein 8 |
| Q9Y616 | Interleukin-1 receptor-associated kinase 3 | P14780 | Matrix metalloproteinase-9 |
| P24387 | Corticotropin-releasing factor-binding protein | Q12905 | Interleukin enhancer-binding factor 2 |
| P17181 | **Interferon alpha/beta receptor 1** | Q12906 | Interleukin enhancer-binding factor 3 |
| Q9NPH3 | Interleukin-1 receptor accessory protein | P02741 | C-reactive protein |
| P01266 | Thyroglobulin | P49767 | Vascular endothelial growth factor C |
| Q6P589 | Tumor necrosis factor alpha-induced protein 8 | P08571 | Monocyte differentiation antigen CD14 |

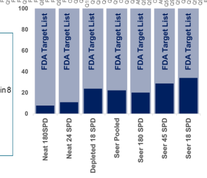



**Figure 5. Top:** great dynamic range coverage of more than 7 orders of magnitudes was observed. **Bottom left:** 24 of the most cited clinical biomarkers of plasma were found with 2 or more unique peptides per proteins when the Max-ID method was used. **Bottom right:** Between 5 to 255 proteins from the FDA drug target list were identified using different methods.

## CONCLUSIONS

- Regardless of the plasma proteomics workflow of choice, the Orbitrap Astral mass spectrometer delivers the highest coverage and throughput compared to current standards.

## TRADEMARKS/LICENSING



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