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Single-Shot LC-MS Workflow for Comprehensive Proteome Identification on an Orbitrap Astral Mass Spectrometer

Santosh Renuse[1], Tabiwang N. Arrey[2]; Anna Pashkova[2]; Maowei Dou[3]; Jeff Op De Beeck[4]; Ryan Bomgarden[3]; Li Wang[5]; Qiong Wang[5] ; Bernard Delanghe[2]; Xinyan Wu[5], Sally Webb[1] and Eugen Damoc[2]

[1]ThermoFisher Scientific, San Jose, CA; [2]Thermo Fisher Scientific (Bremen) GmbH, Bremen, Germany; [3]Thermo Fisher Scientific, Rockford, IL; [4]Thermo Fisher Scientific, Ghent, Belgium; [5]Mayo Clinic, Rochester, MN

GOAL

To develop an end-to-end single-shot LC-MS workflow for in-depth quantitative proteome characterization of eleven commonly used cell lines using a label-free data-independent acquisition (DIA) method on the new Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer interfaced with Vanquish Neo UHPLC system.

INTRODUCTION

Traditionally, deep proteomic profiling was achieved using prefractionation prior to LC-MS analysis of 24-48 fractions. approach wherein the complex peptides were pre-fractionated either using ion-exchange chromatography or more recently high-pH reversed-phase fractionation followed by analysis of 24-48 fractions by LC-MS/MS. Early studies using ultra-long 3 hrs gradients resulted in identification of 5,400 proteins [1]. Recent reports on single-shot proteomic analyses showed identification of up to ~8,200 proteins at 6-8 samples per day throughput [2, 3]. Such methods are not suitable for analysis of medium to large sample cohorts considerably adding sample to sample variation. Here, we show that ultra-high proteome depths can be achieved using novel Astral mass analyzer using one hour gradient thereby significantly increasing sample throughput and maximizing instrument time for analysis of more samples in less time. Using µPAC Neo 50cm column, deep proteome coverage of eleven commonly used cell lines was achieved on a new Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer interfaced with Thermo Scientific™ Vanquish™ Neo UHPLC system.

MATERIALS AND METHODS

Sample Preparation

A549, HCT116, HEK293T, Jurkat, K562, MCF10A, MCF7, MDA-MB-231, OV90, OVCAR8 and PEO1 cell lines (**Table 1**) were cultured in appropriate media followed by lysis using EasyPep lysis buffer. Protein estimation was carried out using BCA assay. 50 ug of protein lysates were processed using Thermo Scientific™ AccelerOme™ platform with label-free MS sample preparation kit for reduction, alkylation, in-solution trypsin digestion and peptide clean-up. The digested peptides were vacuum dried and reconstituted in 0.1% TFA followed by LC-MS/MS analysis. Thermo Scientific™ Pierce™ HeLa digest was used as a QC standard.

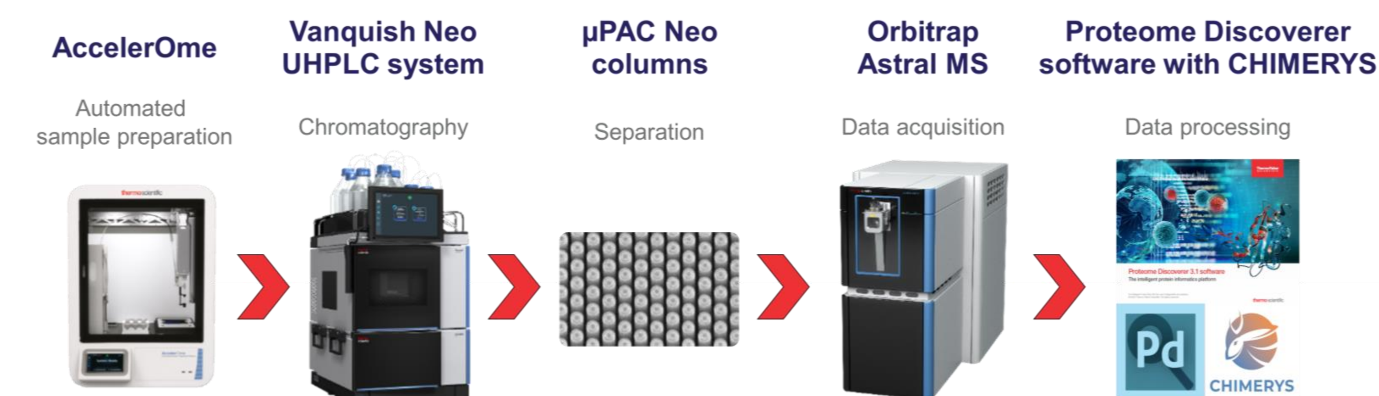


Figure 1. An end-to-end single-shot LC-MS workflow for deep proteome characterization

Figure 2. Schematic representation of the Orbitrap Astral mass spectrometer indicating various components. Ion-path in Astral analyzer shown in red lines.

Cell line	Description	Origin
A549	Adenocarcinoma; human alveolar basal epithelial cells	Squamous
HCT116	Carcinoma; Colorectal; Large intestine; Colon	Epithelial
HEK293T	Kidney, embryo	Epithelial
Jurkat	T lymphocyte cells	Lymphocytes
K562	Chronic Myeloid Leukemia;	Bone marrow
MCF10A	Fibrocystic disease; human breast, mammary gland cells	Epithelial
MCF7	Adenocarcinoma; human breast, mammary gland cells	Epithelial
MDA-MB-231	Adenocarcinoma; human breast, mammary gland cells	Epithelial
OV90	HGSOC;	Epithelial
OVCAR8	Adenocarcinoma; ovary	Epithelial
PEO1	Adenocarcinoma; ovary	Epithelial

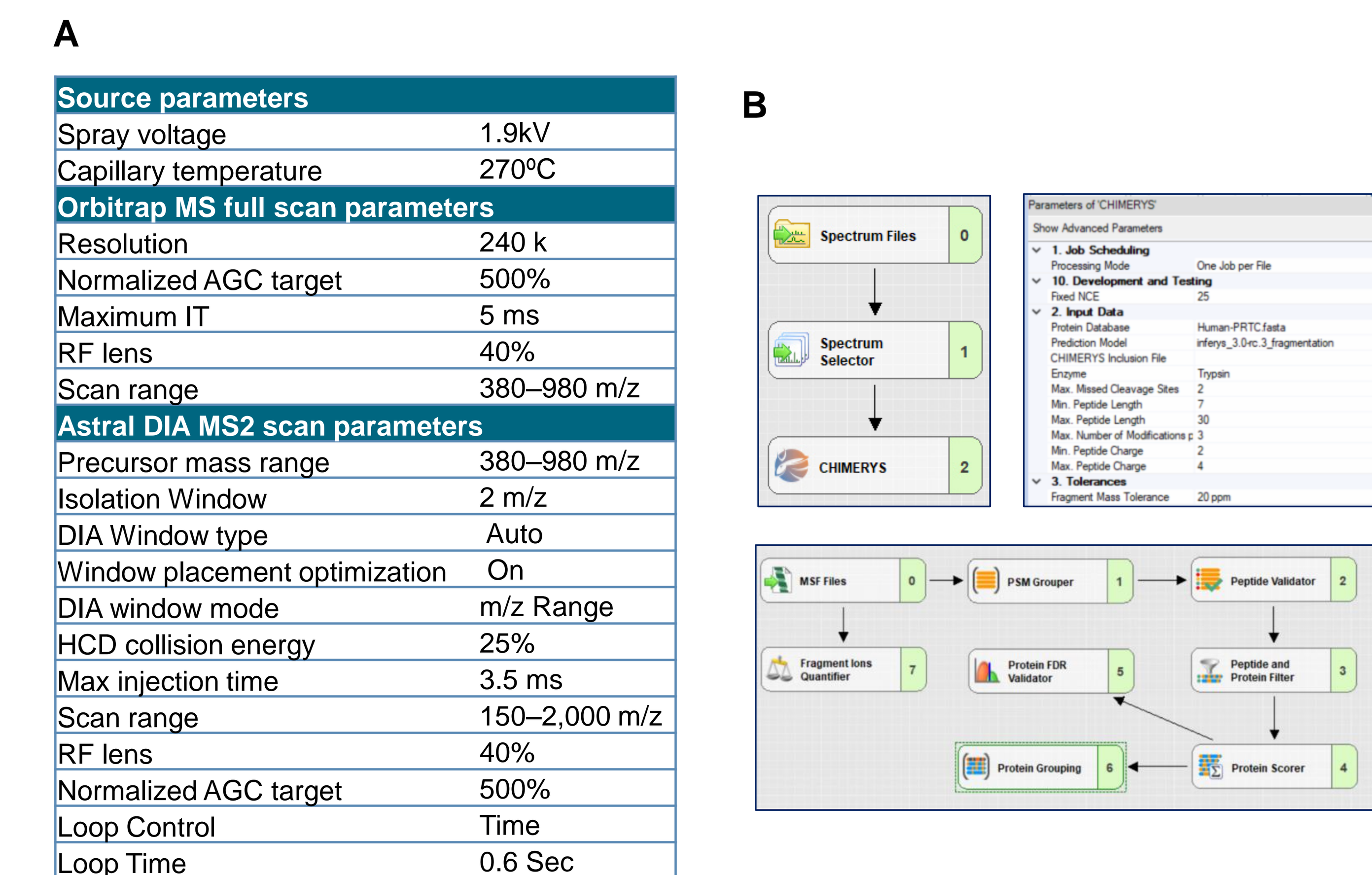
Table 1. Details of the cell lines used in this study.

Data acquisition

Standard HeLa digest (200 ng) samples were analyzed using 30, 60, 100, 180 and 300 samples per day (SPD) with trap-elute setup using PepMap 15 cm analytical column (150 µm i.d., 2 µm PepMap C_{18}) with 5 mm trap column (300 µm i.d., 5 µm PepMap C_{18}). For cell line samples, ~500 ng peptides were separated with 60-minute gradient method using a µPAC™ Neo™ column (Silicon Chip C_{18}, length 50 cm with pillar diameter of 180 µm and interpillar distance of 16 µm) on an Orbitrap Astral mass spectrometer interfaced with Vanquish Neo UHPLC system. The Orbitrap Astral MS was operated with 240,000 FWHM full MS in the Orbitrap Analyzer with normalized AGC target of 500%, and DIA m/z scan range of 380-980, isolation window width of 2 Th in the Astral analyzer with normalized AGC target of 500%, 3.5 ms max injection time, MS/MS m/z range of 150-2000 and 40% source-RF.

Data processing

The acquired DIA data were processed with Beta version of Proteome Discoverer 3.1 with CHIMERYS node (Version 2.5.15, DIA Stage 2) in a library-free approach against Human UniProt protein database including isoforms (49,004 FASTA entries, downloaded on Jan 19th, 2023) plus common contaminants. Identified peptides and proteins were filtered at 1% FDR. The protein group CVs (%) was calculated based on three replicates. DATAtab software was used for generation of violin plots.



A

Source parameters	
Spray voltage	1.9kV
Capillary temperature	270ºC
Orbitrap MS full scan parameters	
Resolution	240 k
Normalized AGC target	500%
Maximum IT	5 ms
RF lens	40%
Scan range	380–980 m/z
Astral DIA MS2 scan parameters	
Precursor mass range	380–980 m/z
Isolation Window	2 m/z
DIA Window type	Auto
Window placement optimization	On
DIA window mode	m/z Range
HCD collision energy	25%
Max injection time	3.5 ms
Scan range	150–2,000 m/z
RF lens	40%
Normalized AGC target	500%
Loop Control	Time
Loop Time	0.6 Sec

B

Figure 3. The MS method parameters used for all the experiments (**Figure 3A**), Proteome Discoverer software, version 3.1, was used for data processing (**Figure 3B**). The CHIMERYS node using INFERYS prediction model version 3.0 was used for the search.

RESULTS

High-throughput methods with deep proteome coverage

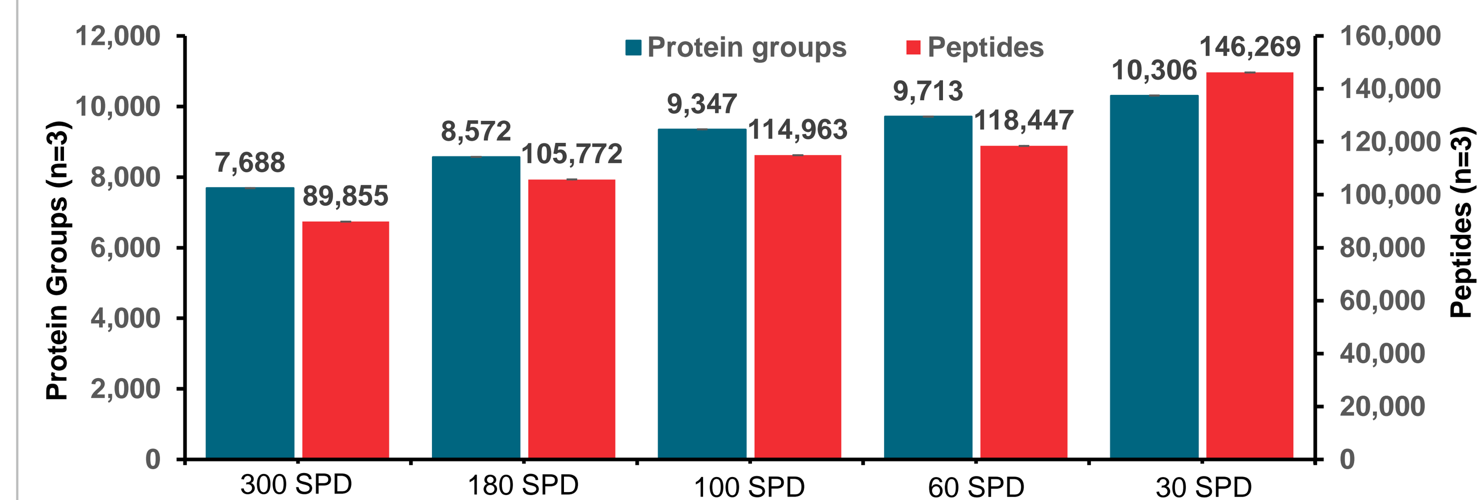


Figure 4. Protein groups and peptides identified in standard HeLa digest analyzed using throughput of 300, 180, 100, 60 and 30 samples per day (SPD).

In-depth proteome identification of common cell lines processed with AccelerOme platform

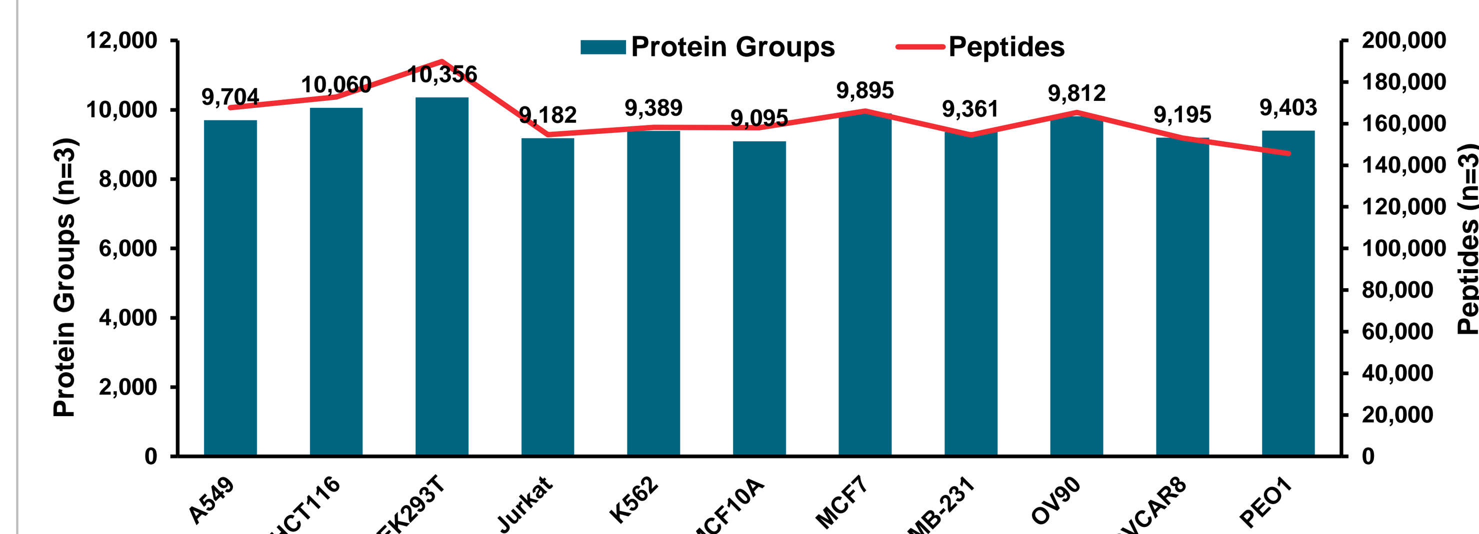


Figure 5. Proteins and peptides identified in cell lines using a 60-min gradient with library-free directDIA

High quantitation accuracy with high-throughput analysis

Standard HeLa digest analyzed using 60, 100 and 180 samples per day methods showed 4, 4.3 and 5.4% median CV at protein group level [**Figure 6A**] while peptide median %CVs were observed at 11, 11.3 and 13.2 for 60, 100 and 180 SPD method, respectively [**Figure 6B**].

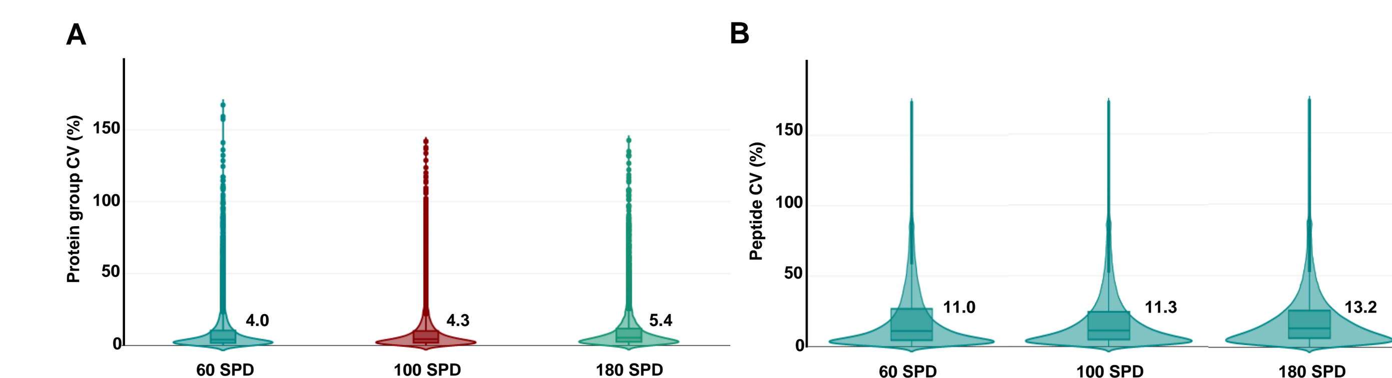


Figure 6. Protein group and peptide CVs (%) distribution for all protein groups identified in standard HeLa digest triplicate runs analyzed using 60, 100 and 180 samples per day (SPD) method. Median CV (%) indicated.

Protein groups for cell lines showed ~10% median CV for biological triplicate samples processed using the AccelerOme platform and analyzed on separate days on the Orbitrap Astral mass spectrometer [**Figure 7**],

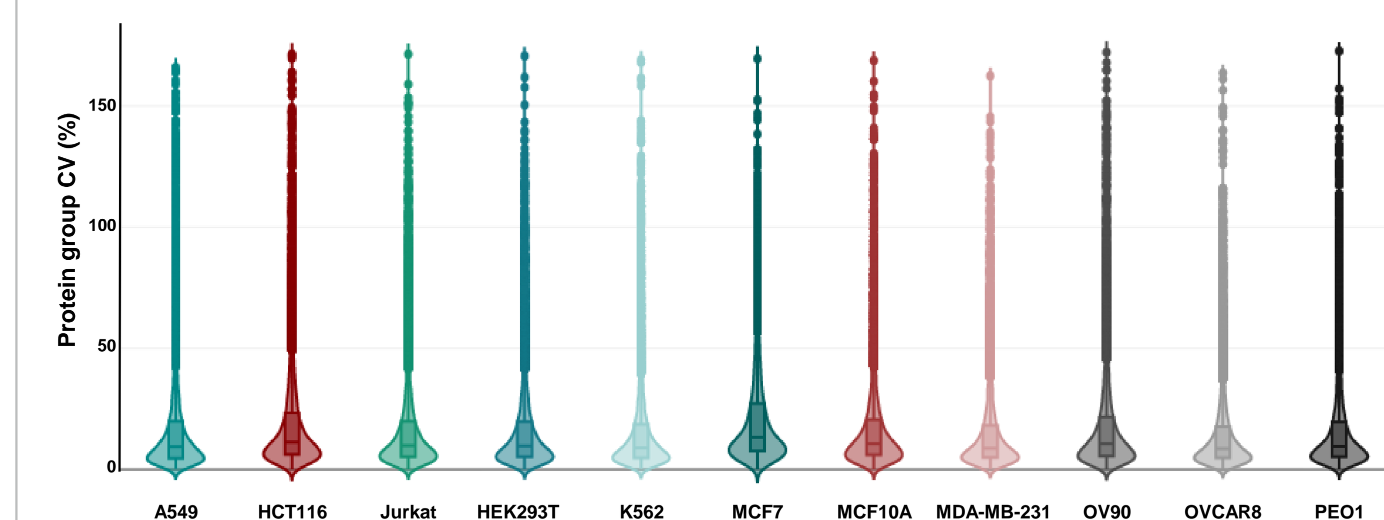


Figure 7. Protein group CVs (%) distribution for all protein groups identified in cell line data. Median CVs (%) indicated.

CONCLUSIONS

- A single-shot LC-MS workflow with sample preparation using AccelerOme followed by high-throughput analysis on a new Orbitrap Astral mass spectrometer
- The novel combines an Orbitrap mass analyzer and a novel Astral mass analyzer to enable robust, reproducible and deep proteome coverage data-independent acquisition
- Deep proteome coverage was achieved using 180 samples per day throughput enabling analysis of large sample cohort of >5,000 sampler per month per MS system
- High quantitation accuracy and precision was achieved maintaining deep proteome coverage at high sample throughput

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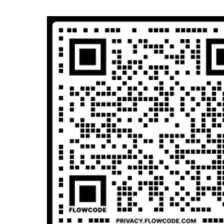

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