Superior Proteome Depth with a Bruker Narrow-window dia-PASEF Method

Deep single-shot proteome analysis in minimal time is gaining increasing attention due to recent advances in hardware and software development, yet it remains a challenge.

Abstract

In this study we further optimized a dia-PASEF® acquisition mode by using narrow isolation windows of 5 Da combined with focused *m/z* and ion mobility window placement, resulting in the identification of 8850 protein groups and 134,728 peptides for a human cell line sample, 14,366 and 170,917 from a complex triple proteome sample and 707 protein groups and 4818 peptides from neat plasma with a 15-minute gradient.

Keywords:
Proteomics, high throughput, dia-PASEF, timsTOF HT, Spectronaut

Introduction

In proteomics studies, accurate and precise relative protein quantitation, together with high protein sequence coverage, is the key to unravel the complex secrets of biological processes. Scientists in the pharmaceutical and biopharmaceutical industry, as well as in the life sciences are committed to advancing knowledge, often relying on using cutting-edge technologies to achieve this goal. Data-independent acquisition (DIA) offers the advantage of comprehensive proteome coverage and reliable quantitation of proteins, while also reducing missing information, leading to more robust results. dia-PASEF is an advanced variant of DIA, capitalizing on the additional dimension of separation unlocked on the timsTOF platform by trapped ion mobility separation (TIMS). Due to the two-dimensional mass and mobility space in combination with varying TIMS scan/accumulation times, dia-PASEF enables method creation with tailored window schemes and duty cycles. When introduced in 2020 [1], the standard dia-PASEF method consisted of constant 25 Da *m/z* window widths covering the mass range of interest. Already with the introduction of py_diAID [2] variable window schemes became

Stephanie Kaspar-Schoenefeld, Andreas Schmidt, Torsten Mueller, Jonathan Krieger; Bruker Daltonics GmbH & Co. KG.

frequently used, showing a benefit of smaller isolation windows in the very dense ion cloud region. Recently a new flavor of dia-PASEF has been introduced, called thin-PASEF [3], which applies very small isolation windows of 10 Da focusing exclusively on regions of high ion density. The application of thin-PASEF resulted in the identification of nearly 11,000 protein groups from human cell line digests within a 100-minute active gradient. While identification of 11,000 proteins is impressive, the field is tending towards shorter gradients as high throughput is crucial in proteomics. Thus, we further optimized the approach for shorter gradients of 15-minutes, resulting in a narrow-window dia-PASEF method with 5 Da windows.

Material and Methods

Tryptic in-house digests of a human cell lysate, yeast, and *E. coli* were used to evaluate the performance of narrow-window dia-PASEF for short gradients on the timsTOF HT. For hybrid proteome measurements, samples were mixed in defined ratios of: Hela: 1:1, yeast: 3:2, *E. coli*: 2:3. Samples were diluted to 400 ng/µL and 2 µL were injected for LC-MS/MS analysis.

For plasma proteomics measurements, 54 individual plasma samples (18 from citrate, 36 from EDTA plasma from 20 different healthy donors) were digested with the PreOmics iST-BCT kit for biofluids. A pooled sample of all digestions was generated and diluted to 500 ng/µL for LC-MS/MS analysis.

Following parameters have been applied:

LC conditions	
nanoLC method parameters	
LC system	nanoElute
Mobile Phase A	0.1% formic acid (FA) in water
Mobile Phase B	0.1% FA in acetonitrile (ACN)
Flow rate	300 nL/min
Column	Aurora Ultimate 25 x 75 C18 UHPLC column (IonOpticks)
Column temperature	50°C
Gradient	0 min: 2% B 2 min: 5% B 13 min: 29% B 15 min: 35% B 15.5 min: 95% B 18 min: 95% B
MS conditions	
MS method parameters	
Source	Captive Spray ionization source
MS system	timsTOF HT
Acquisition mode	dia-PASEF
	5 Da window size IM Range: 0.8 to 1.1 1/K_0 *m/z* range: 350 to 900 112 windows a 44 frames plus 10 MS1 scans Accu/Ramp time: 30 ms Collision Energy: 20 (0.60 1/K_0) to 59eV (1.6 1/K_0)
Data processing	
Processing parameters	
Software	Spectronaut® v19 (Biognosys)
Workflow	1. File conversion via HTRMS converter Deselect „Sum multiple MS1 scans per Cycle“ 2. directDIA+ workflow Default settings Use MS1 level quant

Results

We optimized a dia-PASEF method to cover the high ion density region of interest combined with high specificity by using narrow windows of 5 Da, resulting in reduced complexity of MS2 spectra. In detail, we developed a method covering a significantly smaller *m/z* range from 350 to 900 *m/z* combined with a focused mobility range from 0.8 to 1.1 $1/K_0$ (Figure 1). 110 MS/MS windows with 5 Da window width were covered in 44 TIMS frames with 10 MS1 frames in between to ensure good peak coverage on MS1 level for quantitation (on average 8 data points per peak). The multiplexing capabilities of dia-PASEF allowing measurements of more than one window per frame combined with the ability to use very short accumulation and ramp times of 30 ms in the TIMS cartridge resulted in an overall cycle time of 1.76 s on MS/MS level and 0.45 s on MS level.

When applying the optimized narrow-window dia-PASEF method to a human cell line digest, we were able to identify 8850 protein groups and 134,728 peptides from triplicate injections with a 15-minute active gradient (Figure 2) using library-free directDIA+ in Spectronaut (v.19, Biognosys). These results relate to an increase in number of identifications of 12% on protein group level and 30% on peptide level, compared to a previously published study using the same setup [4]. This significant improvement can be accounted to the combination of a focused dia-PASEF window placement combined with narrow 5 Da windows, very short accumulation and ramp times in the TIMS dimension of 30 ms as well as an improved data processing by using the latest Spectronaut version (v19). Impressively, 88% of the protein groups were quantified

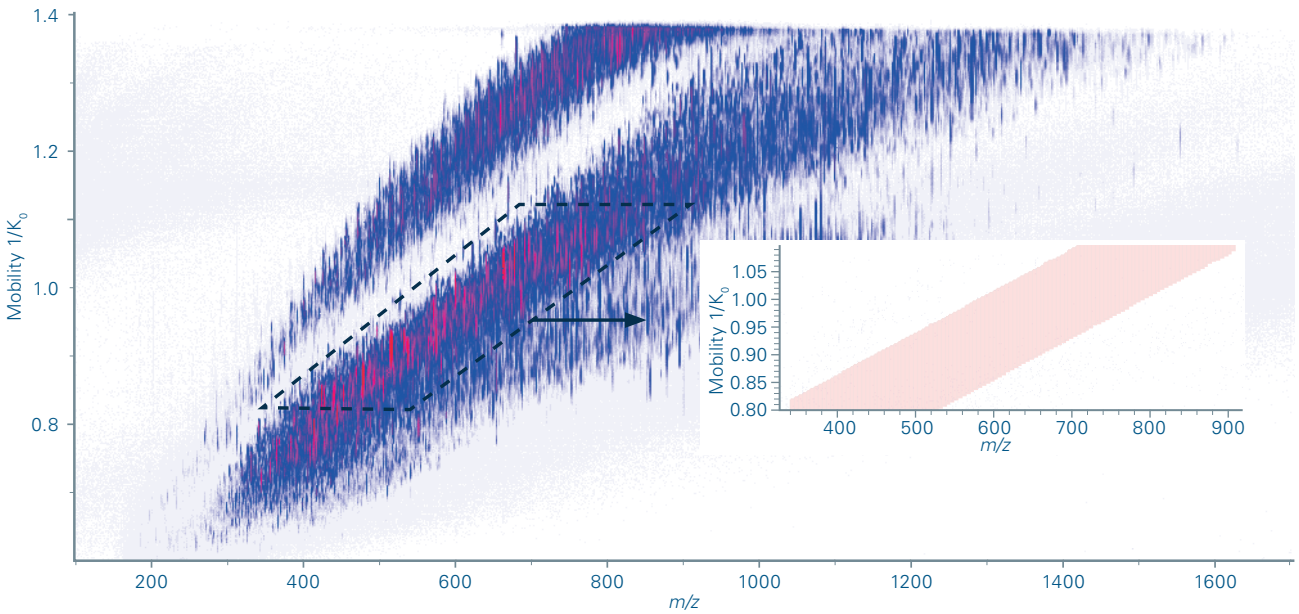


Figure 1

Overview narrow-window dia-PASEF method.

To optimize deep proteome coverage a method has been generated with focus on most intense ion region regarding *m/z* and ion mobility pane.

with a coefficient of variation (CV) below 10%. Even though mass and mobility ranges were dramatically reduced compared to typical dia-PASEF default methods (standard range: 400 to 1200 *m/z* and 0.6 to 1.3 $1/K_0$), we were still able to identify more than 134,720 peptides from triplicate injections. Applying the narrow-window dia-PASEF method to a less complex proteome, namely yeast, resulted in the identification of 4677 protein groups. This is an increase of 6% on protein group level and more than 60% on peptide level compared to our previous study running a 15-minute active gradient [4].

For both samples, median CV values were 3% on protein group level and 8% on peptide level (Figure 2).

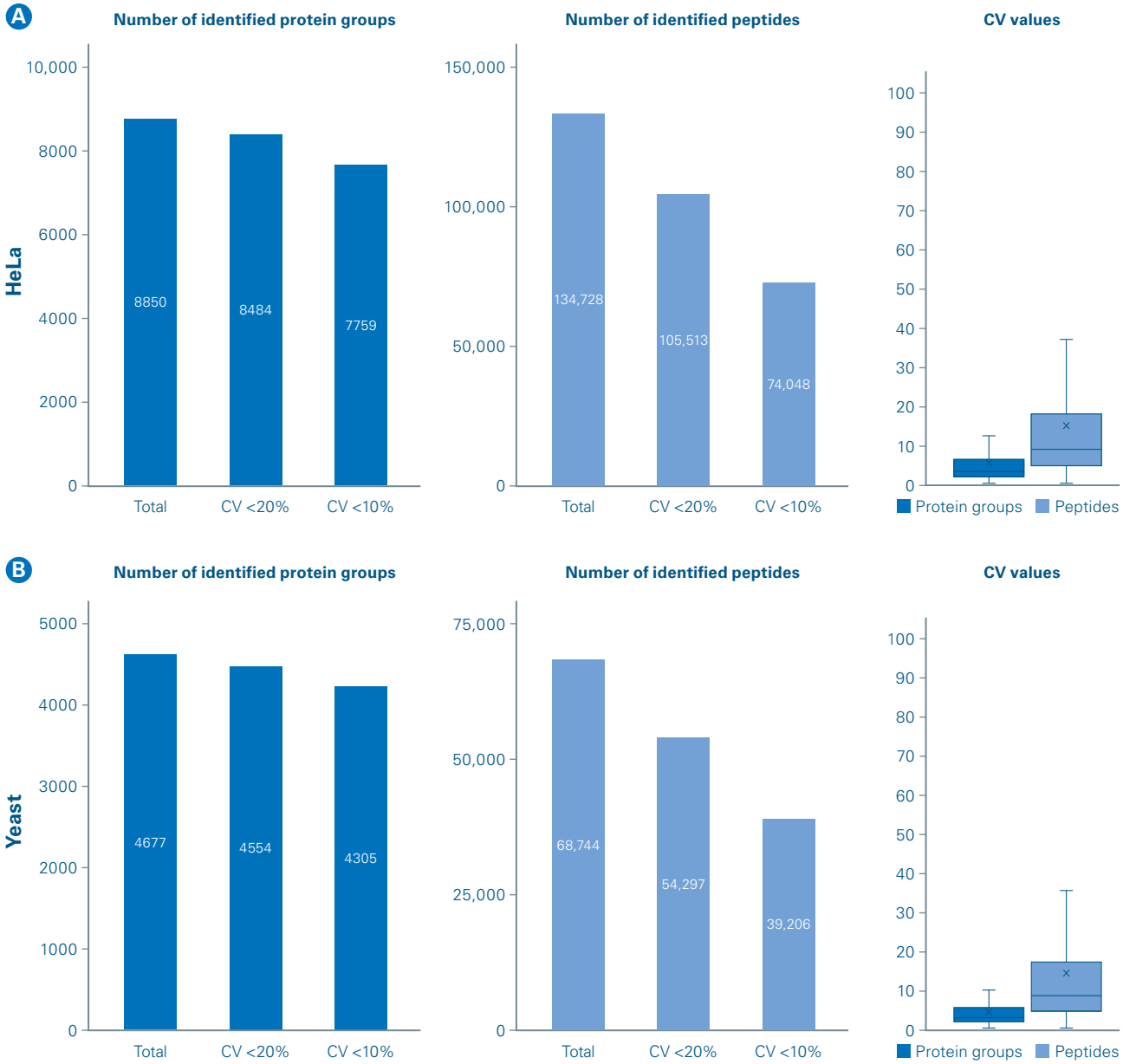


Figure 2

Reproducible and in-depth peptide and protein identification from human (A) and yeast (B) protein digest with total protein group and peptide numbers shown together with number of quantified with CV values below 20% and 10% and box plot visualisation of CV distribution on protein groups and peptide level.

In the next step we analyzed a complex hybrid proteome sample consisting of HeLa, yeast, and *E. coli* digests combined in defined ratios (HeLa: 1:1, Yeast: 3:2, *E. coli*: 2:3). The narrow-window dia-PASEF method resulted in an on average identification of 14,366 (7978 HeLa, 3885 yeast, 2473 *E. coli*) protein groups and 170,917 peptides from six injections per sample within a 15-minute gradient (Figure 3). The median coefficient of variation for six runs per sample was around 6% on protein group level and 12% on peptide level.

Confidence in the applied proteomics approach is determined by the reproducibility of the identification and quantitation and by the accuracy of the quantitative outcome. The chosen experimental design enabled the evaluation of the quantitative accuracy of the narrow-window dia-PASEF method in complex proteomics mixtures with pre-determined theoretical ratios. Background human proteins were spiked in equal amounts resulting in a theoretical ratio of 1:1. We found the relative quantitation of the corresponding human proteins to be centered at the expected ratio across the complete dynamic range (Figure 3B, median $\log_2$ ratio of –0.01, measured ratio = 0.99). The measured ratios obtained for yeast (1.4) and *E. coli* (0.8) were close to the expected ratios with low levels of standard deviation making the developed narrow-window dia-PASEF method perfectly suited for investigation of proteome-wide changes in protein abundance in different biological contexts.

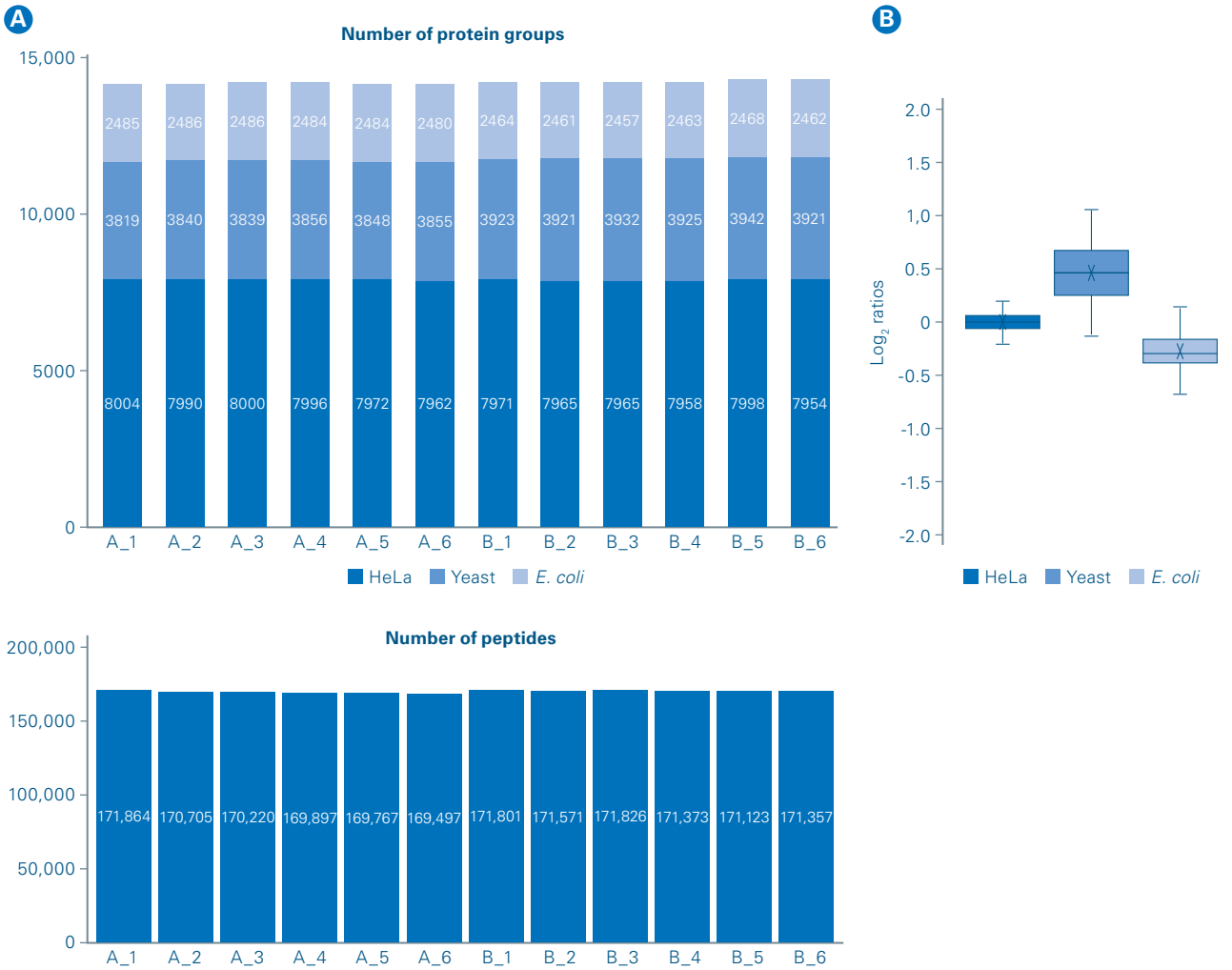


Figure 3

In-depth protein identification and quantitation from a hybrid proteome sample using library-free data processing (directDIA+, Spectronaut). More than 14,000 protein groups have been reproducibly identified and quantified in each of the 12 samples. (A) Total numbers of identified protein groups and peptides from the mixed proteome samples (sample "A": H50Y20E30, Sample "B": H50Y30E20) for each of the three species (HeLa, yeast, *E.coli*). (B) Box and Whisker plot of obtained log10 ratios for all three species (HeLa, yeast and *E.coli*).

Neat plasma is one of the most challenging samples in proteomics due to its high dynamic range. When applying the generated narrow-window dia-PASEF method to a plasma sample, we were able to identify on average 707 protein groups from 4818 peptides with a 15-minute gradient. 661 protein groups were identified in all three replicates displaying the high recovery rate of the method (Figure 4 A). Nearly 6 orders of magnitude can be covered by the used setup (Figure 4 B).

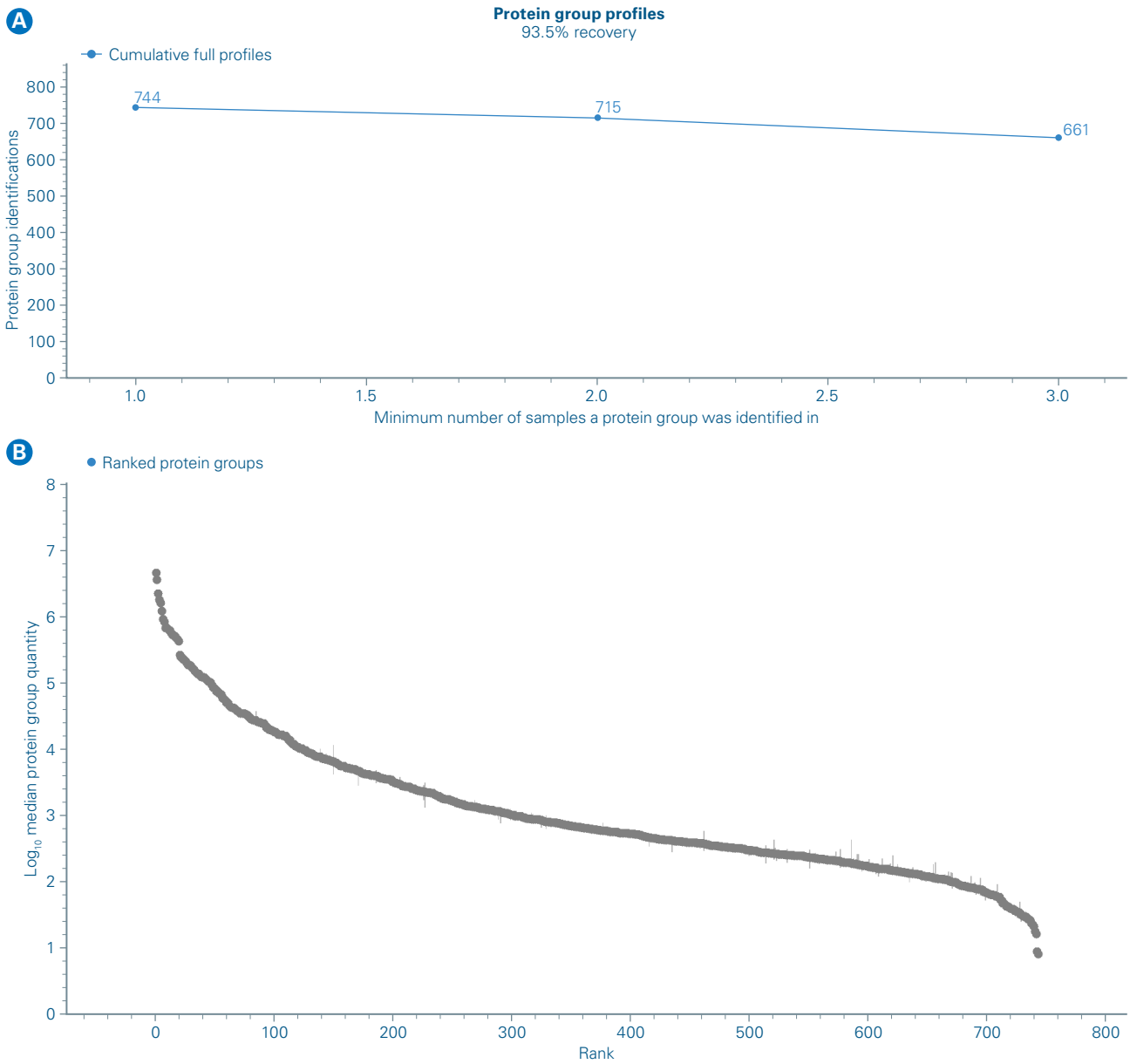


Figure 4

In-depth protein identification from a neat plasma proteome sample using library-free data processing (directDIA+, Spectronaut). More than 700 protein groups have been reproducibly identified and quantified in triplicate runs. (A) Protein group identifications from plasma proteome sample. Shown are the number of protein groups identified in all three runs (661) and the cumulative number of protein groups identified in at least one run (744). (B) Ranked protein group plot visualizing log10 median protein group quantity across all quantified protein groups.

Conclusions

- The two-dimensional mass and mobility space of dia-PASEF enables generation of various methods tailored to sample complexities and throughput demands.
- The presented method using 5 Da isolation windows over a condensed ion mobility and *m/z* region resulted in high proteome coverage and accurate quantitation in short gradients of 15 minutes.
- Nearly full yeast proteome coverage can be achieved with on average 4677 protein groups identified.
- Analysis of complex mixed proteomes resulted in identification of 14,366 protein groups using library-free data processing.

References

[1] Meier et al. (2020). Nat Methods. **17**(12):1229-1236

[2] Skowronek et al. (2022). Molecular & Cellular Proteomics. **21**(9):100279

[3] Konno et al. (2024). bioXriv. https://doi.org/10.1101/2024.04.26.591246

[4] Bruker application note LCMS 218. https://www.bruker.com/en/products-and-solutions/mass-spectrometry/timstof/timstof-ht/_jcr_content/root/sections/more_information/sectionpar/search.download-asset.pdf/4922715e-6af9-4abd-a16c-070b6cb70ed4/1905946-lcms-218-timstof-ht-and-hela-ebook.pdf

Further reading

Setting the standard for Protein Analysis with PreOmics.
www.preomics.com/

Immediately dive into results of your experiment.
www.bruker.com/en/products-and-solutions/mass-spectrometry/ms-software/proteoscape.html

Immerse into the capabilities of 4D-Proteomics™.
www.bruker.com/en/applications/academia-life-science/proteomics/4d-proteomics.html

Everything you need. Maximum Sensitivity, Speed, and Robustness.
www.bruker.com/en/products-and-solutions/mass-spectrometry/lc-ms/pepsep-columns.html

Learn more about the timsTOF HT.
www.bruker.com/en/products-and-solutions/mass-spectrometry/timstof/timstof-ht.html

The gold standard for DIA proteomics analysis: Spectronaut®.
biognosys.com/software/spectronaut/

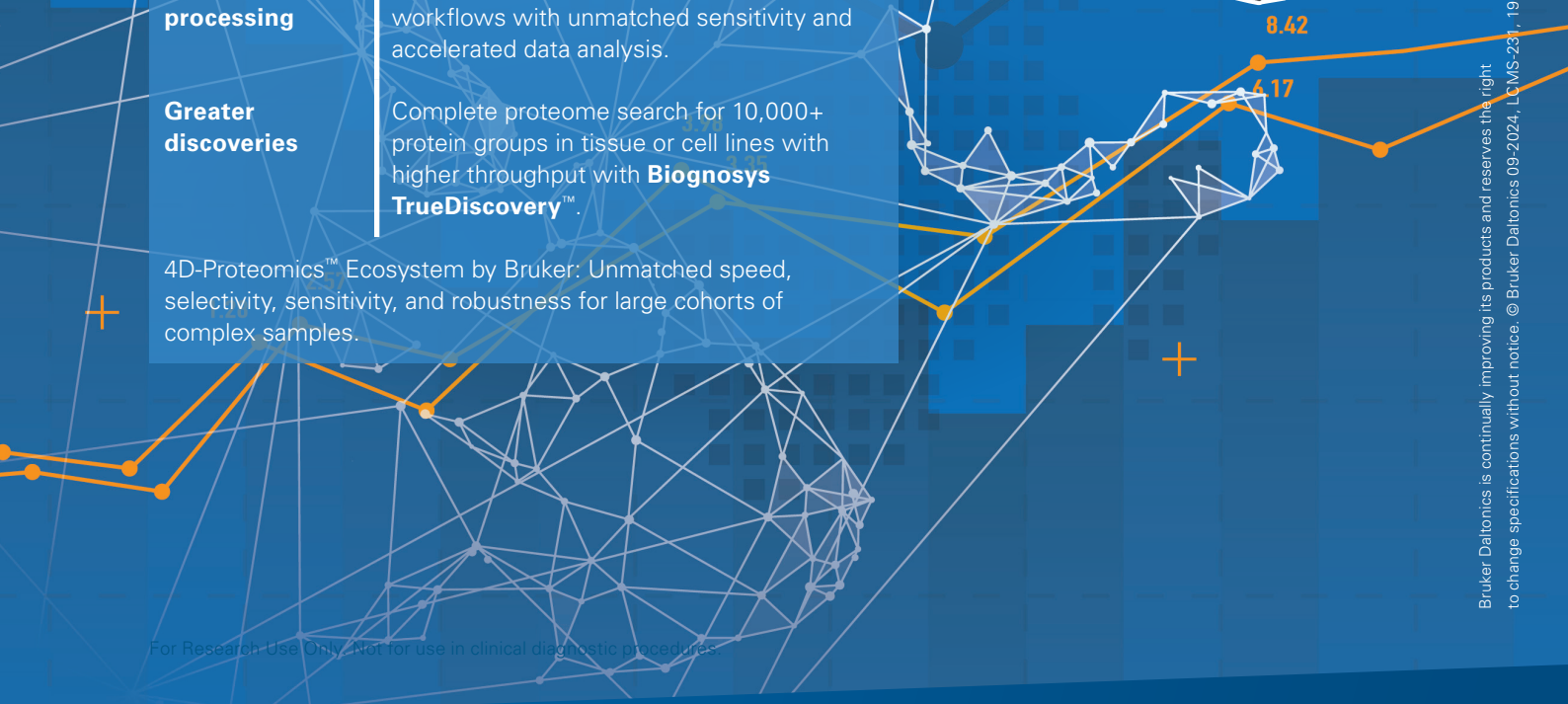
Expanding capabilities, see the timsTOF HT in action.
www.youtube.com/watch?v=_C8enJv2k1I

Experience PASEF
www.bruker.com/en/products-and-solutions/mass-spectrometry/timstof/pasef.html

Bruker 4D-Proteomics™ Ecosystem

Benefit	Feature
Maximum separation	**PepSep® MAX and PepSep® MAX HT:** High-throughput proteomics with reproducible, high-quality results at PASEF speeds.
Ultimate performance	**CaptiveSpray 2** delivers nano-flow sensitivity with simplicity.
Highest quality, sample to data	PreOmics protein and peptide **Sample Preparation** kits: Reliable results, faster than ever.
Higher throughput, deeper profiles	**TIMS** spreads sample complexity across the mobility dimension for fast turnaround, without compromise.
Enhanced confidence with TIMS	**Mobility offset, mass aligned** (MOMA) for confident identification and quantification of isobaric or nearly isobaric peptides.
Powerful insights	**Bruker ProteoScape™**: Real-time data processing for large experiments.
Limitless processing	**Spectronaut®** simplifies complex DIA workflows with unmatched sensitivity and accelerated data analysis.
Greater discoveries	Complete proteome search for 10,000+ protein groups in tissue or cell lines with higher throughput with **Biognosys TrueDiscovery™**.

4D-Proteomics™ Ecosystem by Bruker: Unmatched speed, selectivity, sensitivity, and robustness for large cohorts of complex samples.



For Research Use Only. Not for use in clinical diagnostic procedures.

Bruker Switzerland AG

Fällanden · Switzerland
Phone +41 44 825 91 11

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com

Learn more at **www.bruker.com/timsTOF-HT**