# The need for speed: new strategies for label-free quantitation

## Thermo Scientific™ Velocity LFQ HR-DIA platform

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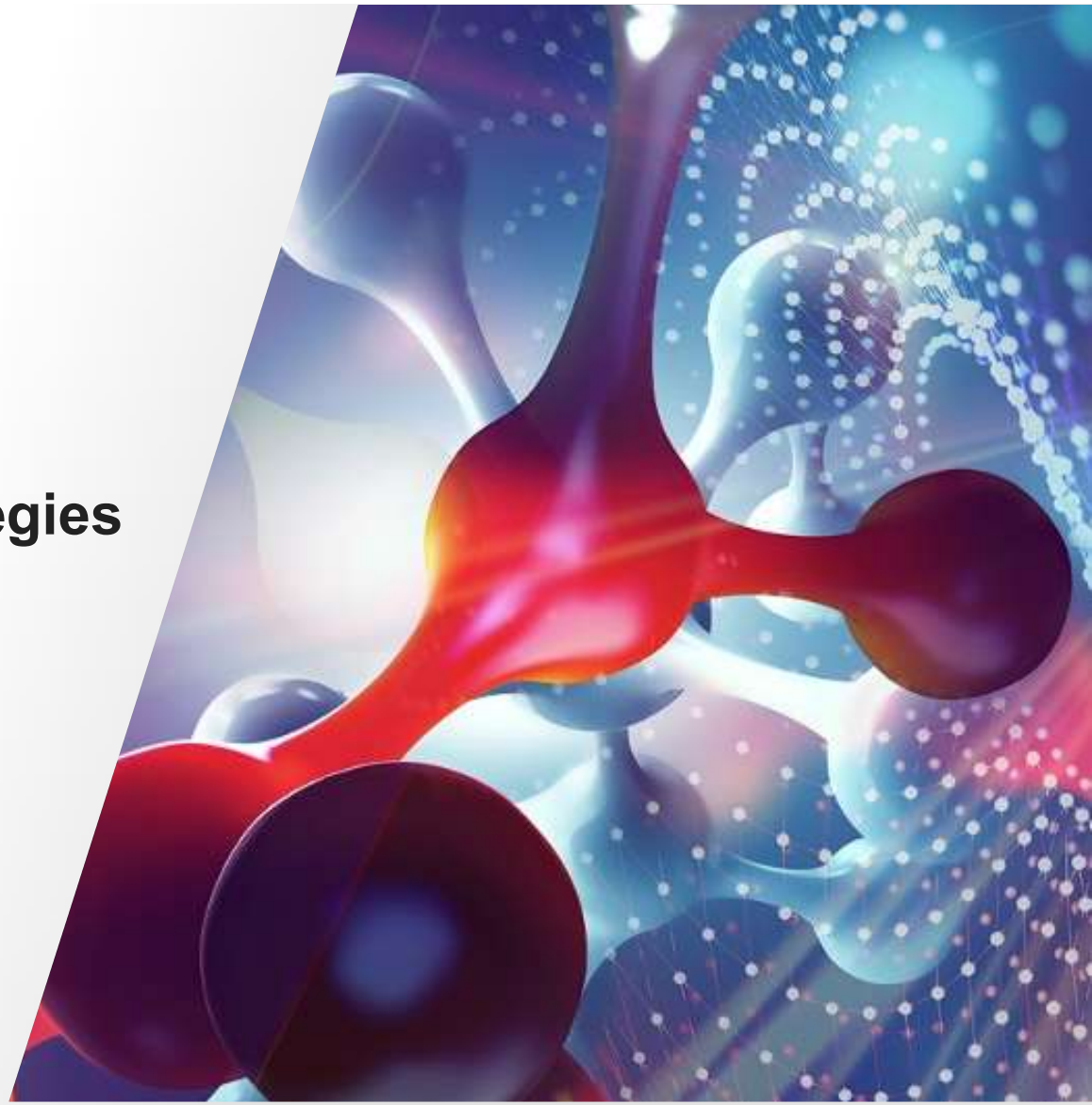
Director of Marketing, Mass Spectrometry



The world leader in serving science

# How do we quantify more proteins?

## Go beyond just IDs - adding quantitative information makes the difference

Confident characterization of differences in biological systems needs exact measurements for hypothesis testing of predictive theoretical models

Quantitative Proteomics

Translational Research

Biomarker Discovery

Single Cell Proteomics

**Accuracy**

determine correct protein abundance

**Precision**

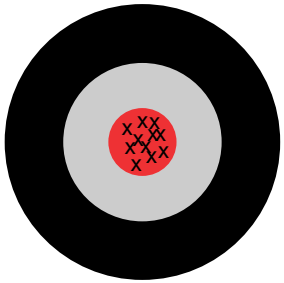
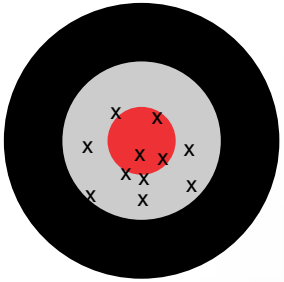
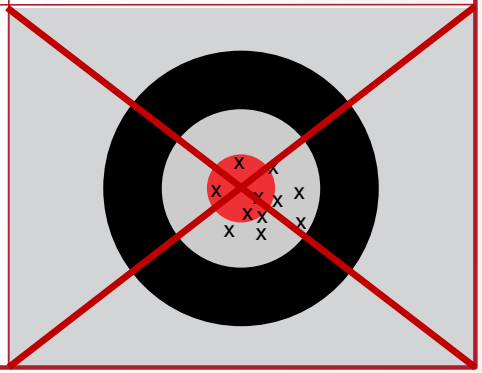
reduces number of measurements

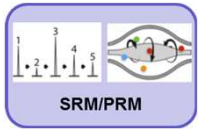
**Throughput**

yields statistical significance

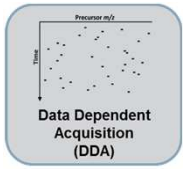
# Choices for Proteomic Quantitation

## Each method is a fit for purpose assay

| | ACCURATE | INACCURATE (SYSTEMATIC ERROR) |
|---|---|---|
| PRECISE (LIMITED TARGETS) | Targeted  | Multiplexing  |
| IMPRECISE (REPRODUCIBILITY ERROR) | LFQ  |  |

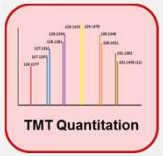








Topic of Presentation

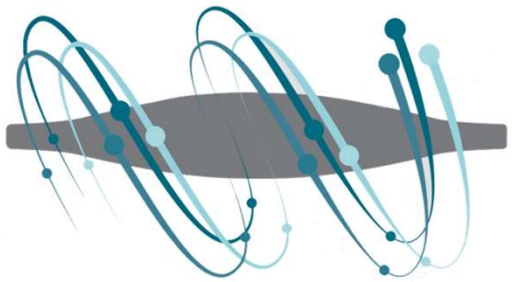



**Low resolution / Low mass accuracy** systems are less precise and accurate overall


ThermoFisher
SCIENTIFIC
1000
ORBITRAP DIA PUBLICATIONS

# Top 10 impactful Orbitrap DIA papers that came out in 2022

1. Changes in protein shapes as markers for Parkinson's disease
2. SARS-CoV-2 mimics a host protein to bypass defences
3. When spinal fluid from ALS patients was put into mice, the mice got weak. An unlikely protein could be the culprit
4. Old Drugs Could Reveal a New Way to Attack the Coronavirus
5. Noninvasive proteomic biomarkers for alcohol-related liver disease
6. Spatial region-resolved proteome map reveals mechanism of COVID-19-associated heart injury
7. The proteogenomic subtypes of acute myeloid leukemia
8. How are T-cells able to repeatedly kill virus and cancer infected cells
9. How the 'Alpha' Coronavirus Variant Became So Powerful
10. How a single phosphorylation site can affect protein function, cell signaling and drug response



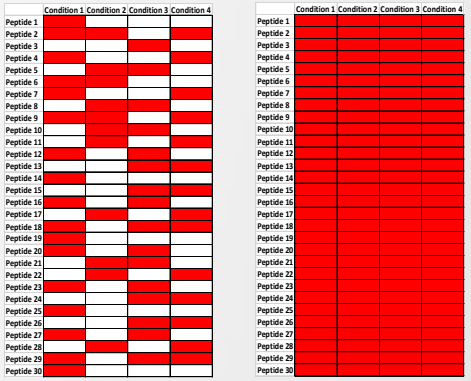
# High throughput DIA platform

## Data Independent Acquisition (DIA) strategies for proteomics

### Why have customers moved to data independent analysis:

- **Easy to adopt**
  - Especially for large sample studies (>30 samples)
- **Greater proteome coverage for short gradients <30min**
  - Fear of missing out on key protein biomarkers of interest
- **Easy-to-use advanced software options**
  - Less expertise needed to succeed with computational performance gains
- **Demonstrated LOD/LOQ potential**
  - Minimize sample cost while increasing data confidence in relative quantitation
- **Fewer missing values for LFQ**
  - Direct queries (and p-values) for peptides of interest: Hypothesis Driven > Stochastic Sampling *(from Michael MacCoss)*

**DDA** **DIA**




Modified from Michael MacCoss Lab's

- ***Pain Point:*** Missing values for quantitation across large cohorts

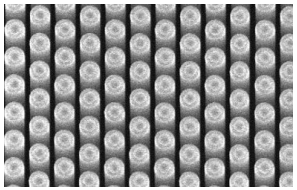
| Ideal Method | Sample Size |
|---|---|
| LFQ (DDA) | 1-6 samples per experiments |
| TMT(DDA) | 6-90 samples across multiple plexes |
| LFQ (DIA) | 10-1000 samples in cohorts |

# Velocity LFQ HR-DIA platform

## Workflow for high-throughput label-free quantitation and proteome

Data Independent Acquisition (DIA)

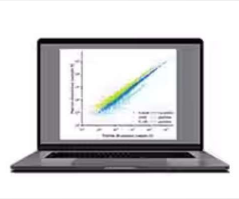
Thermo Scientific™ Vanquish™ NEO UHPLC



Thermo Scientific™ µPAC™ NEO 50cm

Thermo Scientific™ EASY-Spray™ Nano Source

Thermo Scientific™ Orbitrap Exploris™ 240 MS



Software of choice

**Quantitation**

Accuracy | Precision

robust
reproducible
consistent

**Throughput**

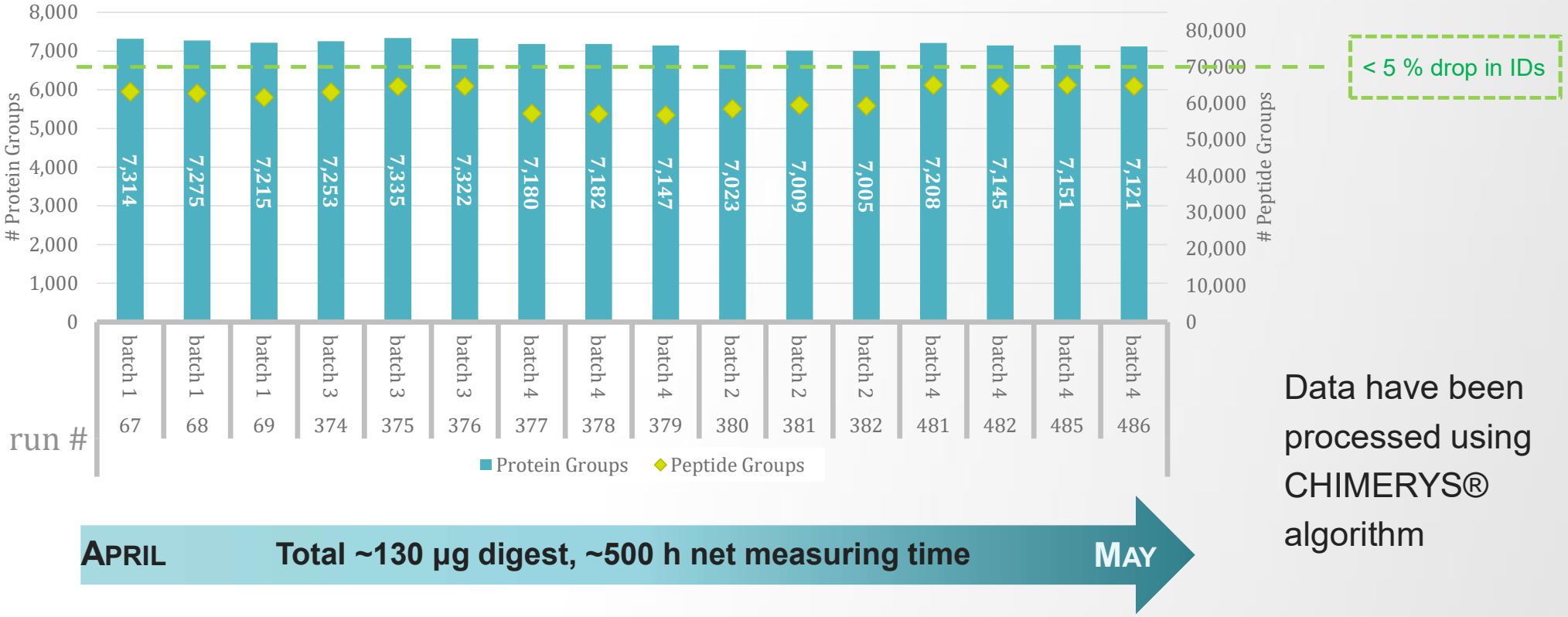
(sample per day)

**Identifications**

# peptides/proteins

# Workflow robustness including µPAC Neo column

## QC runs (200ng HeLa, DDA, 67min gradient) interspersed to quantitative study



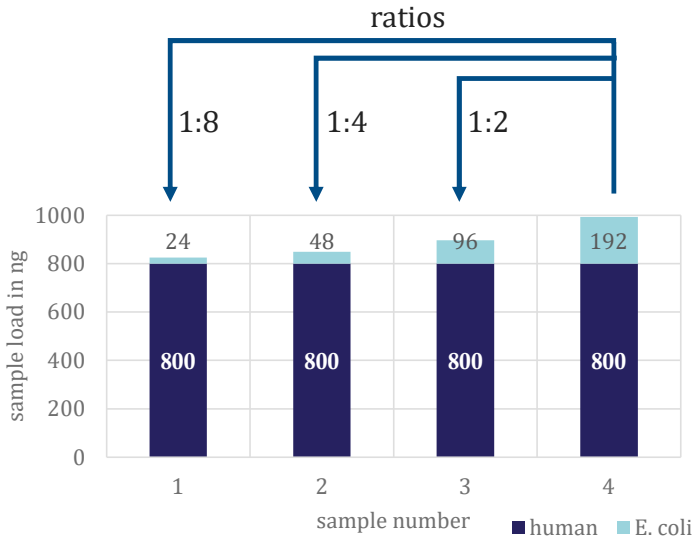

APRIL — Total ~130 µg digest, ~500 h net measuring time — MAY

Data have been processed using CHIMERYS® algorithm

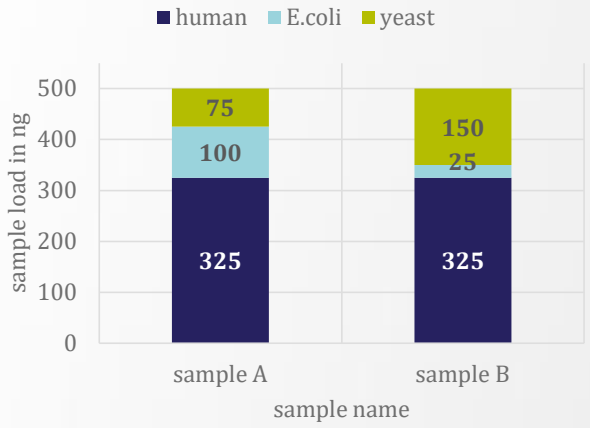
CHIMERYS is a registered trademark of MSAID

# Experimental design

## Two sample mixtures with different ratios and background levels




***Two-Proteome Mix***



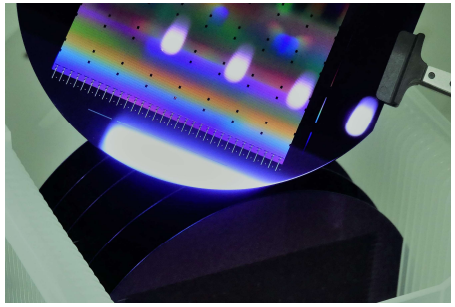

***Three-Proteome Mix***

| species | ratio A:B |
|---|---|
| human | 1:1 |
| yeast | 1:0.5 |
| E.coli | 1:4 |

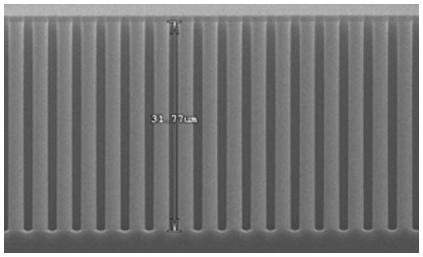
- High human background levels (up to 30x more than the spiked species)
- 30 min gradients for two and three proteome mixes

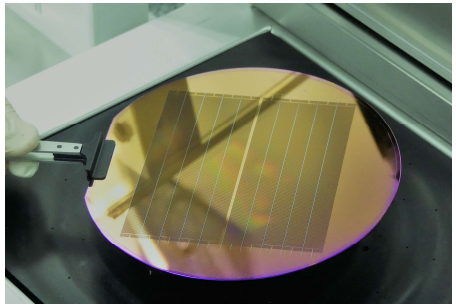
# µPAC HPLC Columns

From silicon wafer to highest resolution separation channel

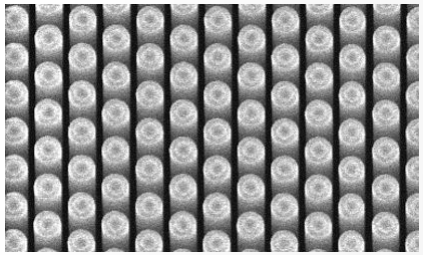


Micromachined µ-pillars





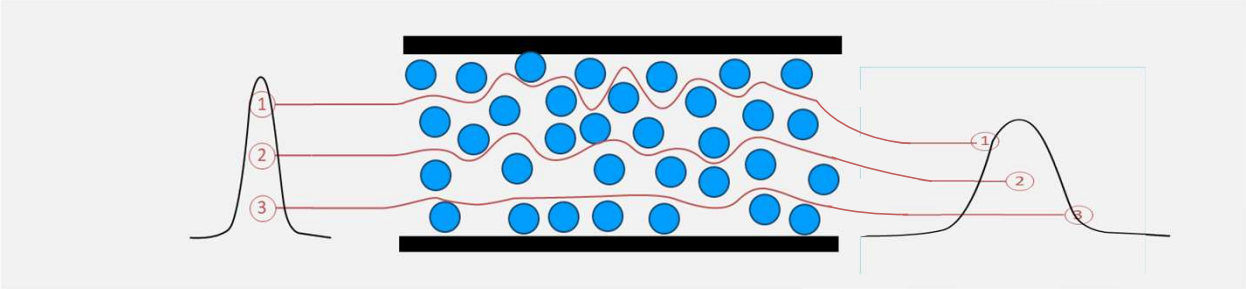
µ-Pillars separation channel

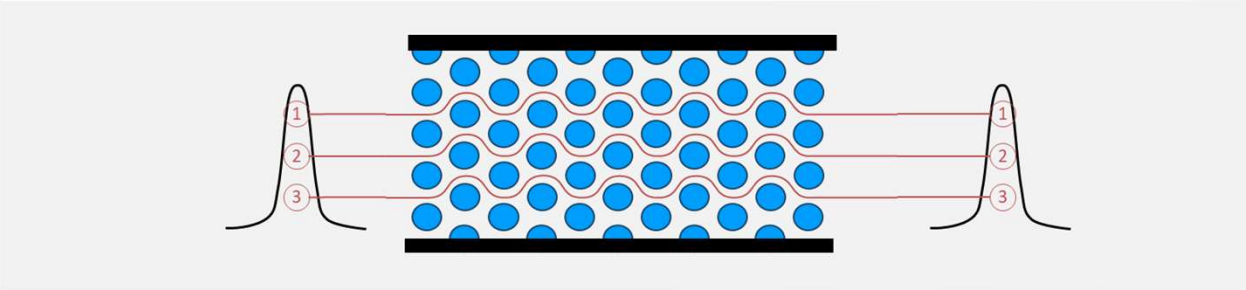


- µPAC overcomes the boundaries limiting packed-bed columns
  - Efficiency – no effect of eddy diffusion
  - Peak capacity – long columns at moderate pressure
  - Reproducibility – lithography and microtechnology
  - Robustness – no potentially moving particles/no frits

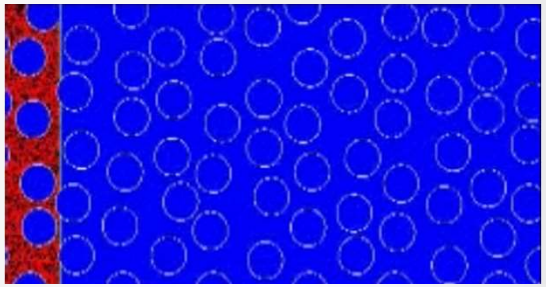
# Characteristic of the µPAC HPLC columns

**Perfect order in chromatography with micropillar array-based technology**

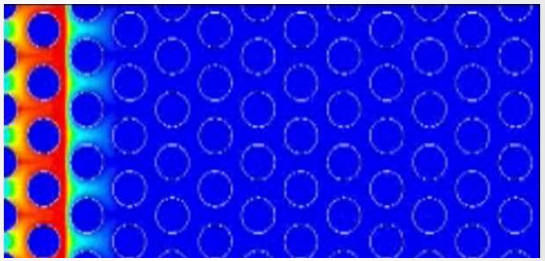



Disorder – Packed bed



Order – Pillar Array

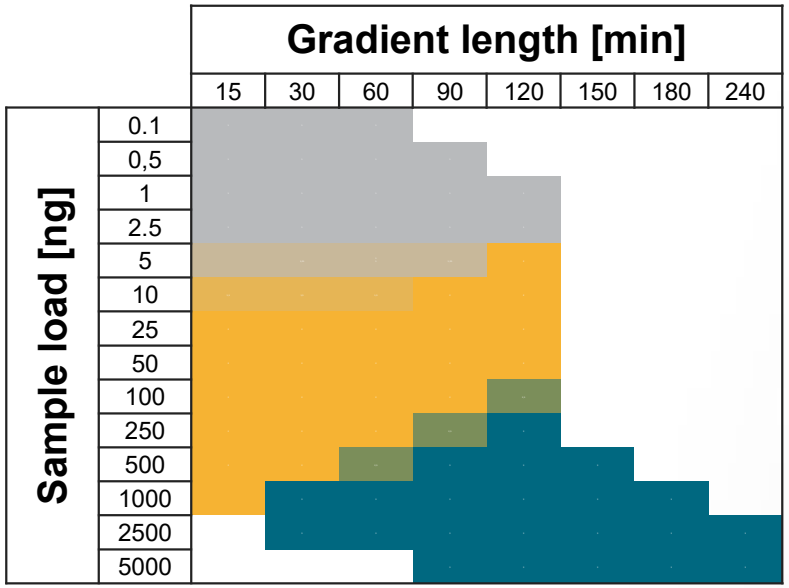


Reduced eddy-dispersion results in sharper peaks – higher intensity

# µPAC Neo column selection

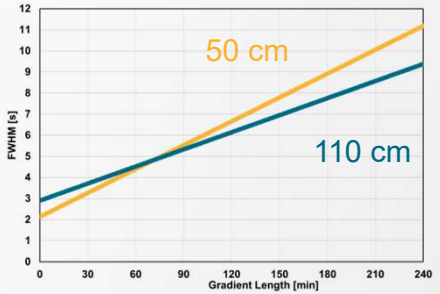
## Ideal sample and gradient conditions

| 50 cm µPAC Neo low-load column | 50 cm µPAC Neo column | 110 cm µPAC Neo column |
|---|---|---|
| Highest sensitivity<br>Lowest carry-over | Higher throughput<br>Shorter gradients | Highest #ID's<br>Longer gradients |

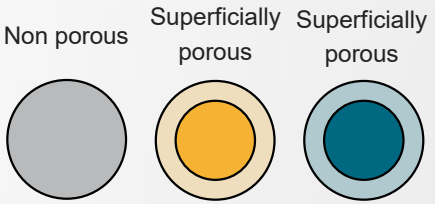



## Main differentiators

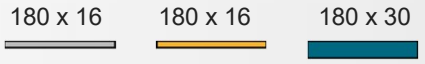
### 1) Column length




### 2) Surface morphology




### 3) Separation channel cross-section

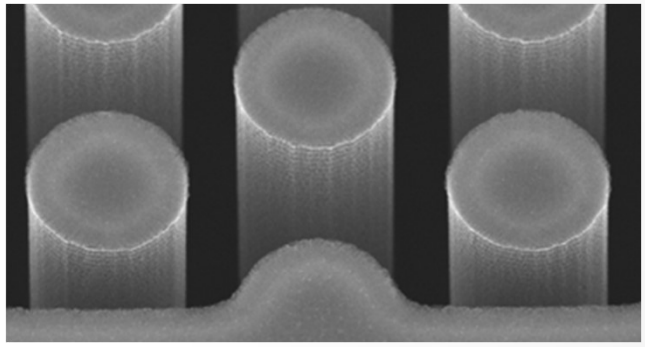


# µPAC Neo HPLC column

Comprehensive sample coverage injection after injection, column after column

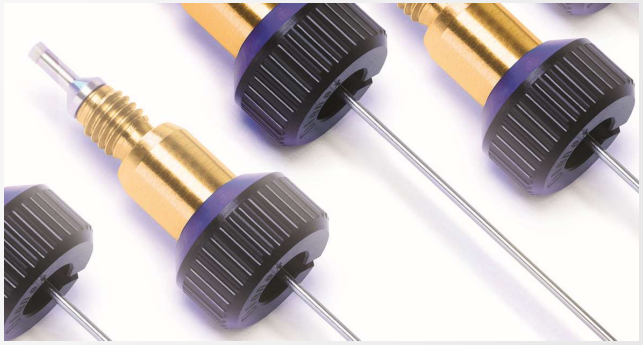
**A column to match separation needs**

- 50 cm: Routine LFQ DIA and data dependent acquisition (DDA) analysis
- 110 cm: Comprehensive proteomics with extended gradients >120 min
- 50 cm low-load: single-cell and few-cell proteomics



**Perfect order for consistent separations**

- Micro pillars etched into a silicon wafer provide virtually identical columns for consistent performance
- Optimized micro pillar design for highest separation performance and sensitivity

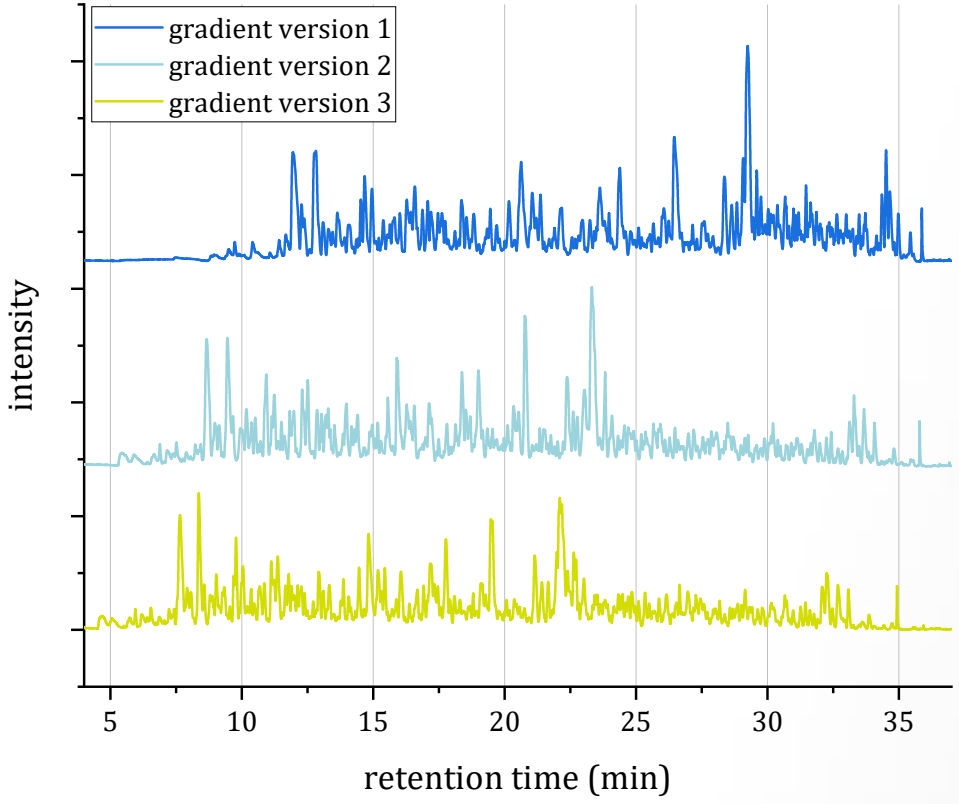


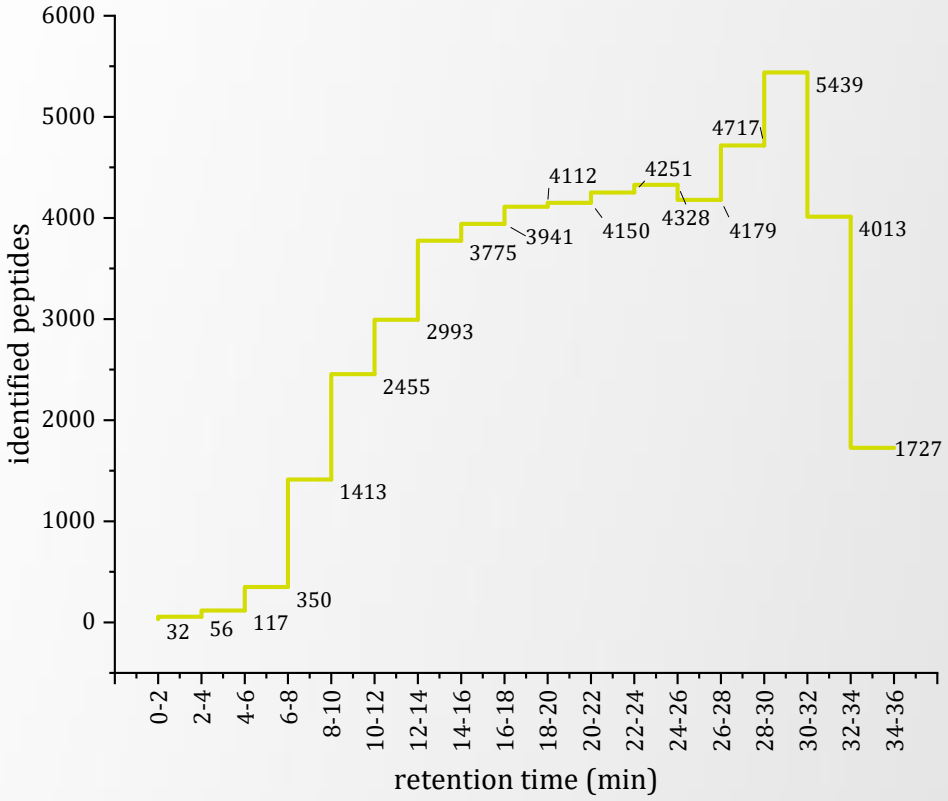
**Easy-to-use and robustness**

- Reduced back pressure for extended lengths and increased column lifetimes
- Thermo Scientific™ Double nanoViper™ Fitting for near zero-dead-volume connection every time

# Optimization of LC gradient and flow rate

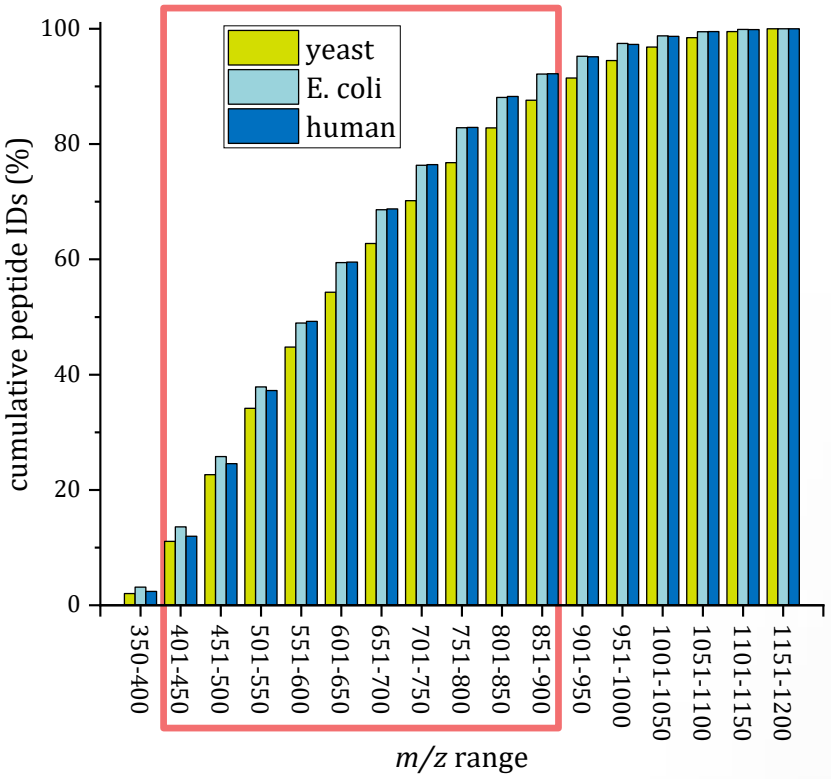
## Optimal conditions for the evenly distributed elution and IDs should be determined



# Optimization of an LC gradient and DIA method

## How large does the mass range have to be?




- Mass range and window size determine the duty cycle time
- Three-proteome mix:
  - mass range 350 – 1,200 Th
  - Total identified peptides >200,000 across three replicates

➢ **>90% of identified peptides in the mass range 400-900 Th**

# Optimized MS method and LC gradient

## LC method

- Direct injection setup
- Gradient optimized for µPAC Neo column

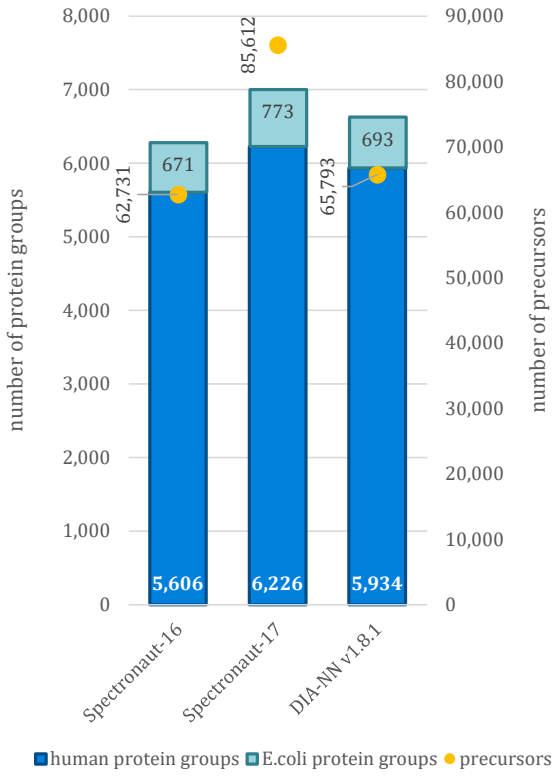
| No | Time | Duration [min] | Flow [µl/min] | %B | Volume [µl] | No. of Column Volumes |
|---|---|---|---|---|---|---|
| 1 | 0.000 | Run | | | | |
| 2 | 0.000 | 0.000 | 0.350 | 4.0 | 0.00 | 0.00 |
| 3 | 22.500 | 22.500 | 0.350 | 30.0 | 7.88 | 5.32 |
| 4 | 30.000 | 7.500 | 0.350 | 45.0 | 2.63 | 1.77 |
| 5 | 30.000 | Column Wash | | | | |
| 6 | 30.100 | 0.100 | 0.350 | 97.5 | 0.04 | 0.02 |
| 7 | 33.000 | 2.900 | 0.350 | 97.5 | 1.02 | 0.69 |
| 8 | 33.100 | 0.100 | 0.350 | 4.0 | 0.04 | 0.02 |
| 9 | 39.000 | 5.900 | 0.350 | 4.0 | 2.07 | 1.40 |
| 10 | 39.000 | Stop Run | | | | |
| 11 | 39.000 | Column Equilibration | | | | |

## MS DIA method:

- MS1 resolution: 60k
- MS1 AGC target: 300%
- MS2 (DIA) resolution: 15k
- MS1 mass range (*m/z*): 400-900
- Isolation width: 12 Th
- DIA scan range (*m/z*): 145 - 1,450
- MS2 AGC target: 800%
- HCD NCE: 30

# Deep proteome profiling with Velocity LFQ HR-DIA

## High proteome depth is achieved library free with multiple softwares of choice



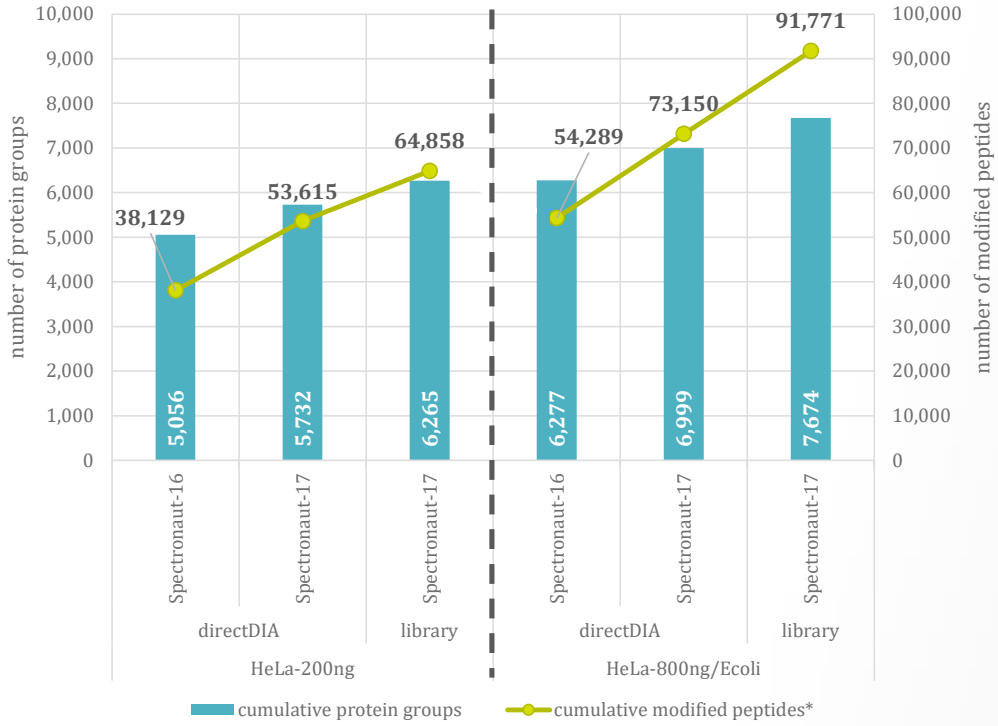

- Velocity LFQ HR-DIA provides deep coverage for complex two proteome mixture
- Optimized 30min DIA conditions provide similar depth of coverage as 67min DDA
- directDIA approuch: no library used
- protein groups were filtered for 1 % FDR

Orbitrap Exploris 240 MS, 50cm µPAC Neo column, 30min gradient

Spectronaut is a registered trademark of Biognosys

# Deep proteome profiling with Velocity LFQ HR-DIA

## Deeper proteome depth is achieved using a library

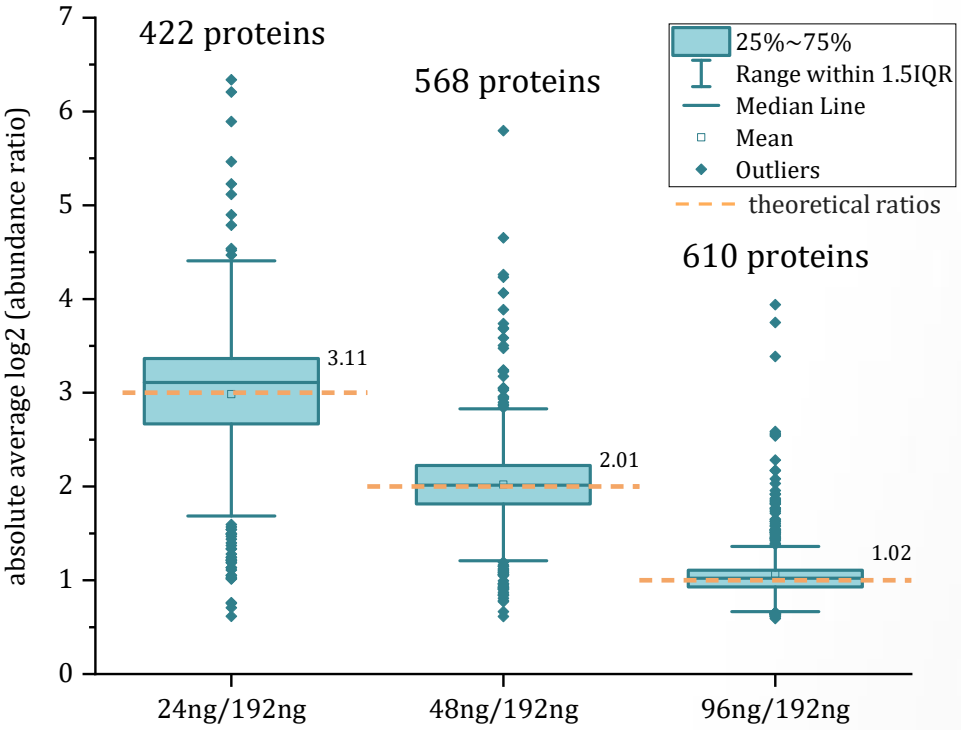



Orbitrap Exploris 240 MS, 50cm µPAC Neo column, 30min gradient

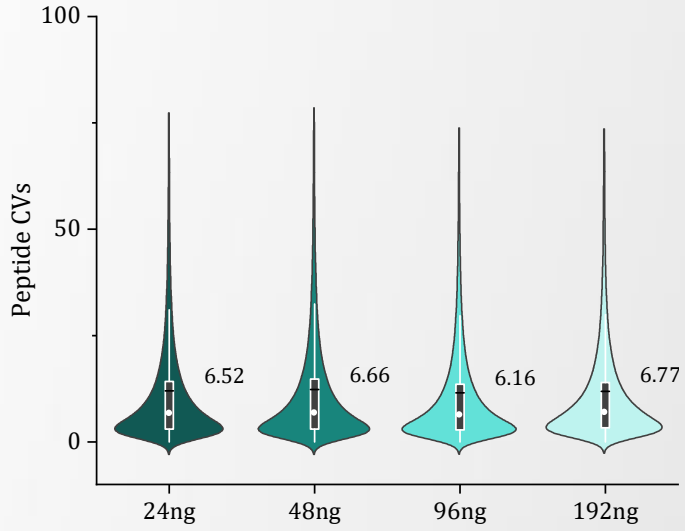
- Comparison directDIA vs. library
  - directDIA performance is significantly improved in Spectronaut 17
- Library approach yields higher proteome coverage:
  - ~ 20% more precursors
  - ~ 10% more protein groups
- Library generated from three 120min DDA runs
- Additional coverage can be expected by using a suitable library generated from fractionated samples under same conditions

# Quantitation accuracy & precision in two-proteome mix

## Bacterial proteome (E.coli) spiked in human cell digest (HeLa)




- great quantitation accuracy across all ratios
- low CVs of peptide quantities across triplicates

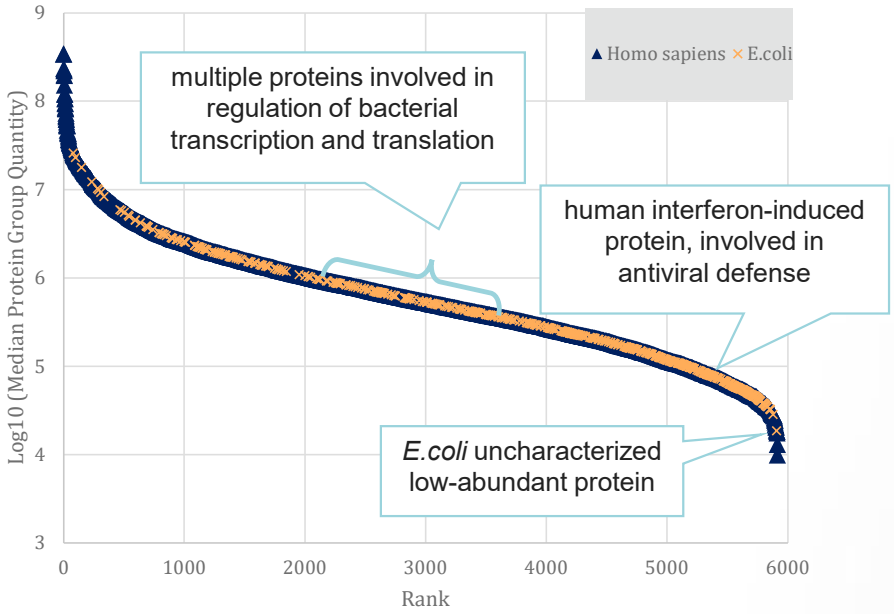


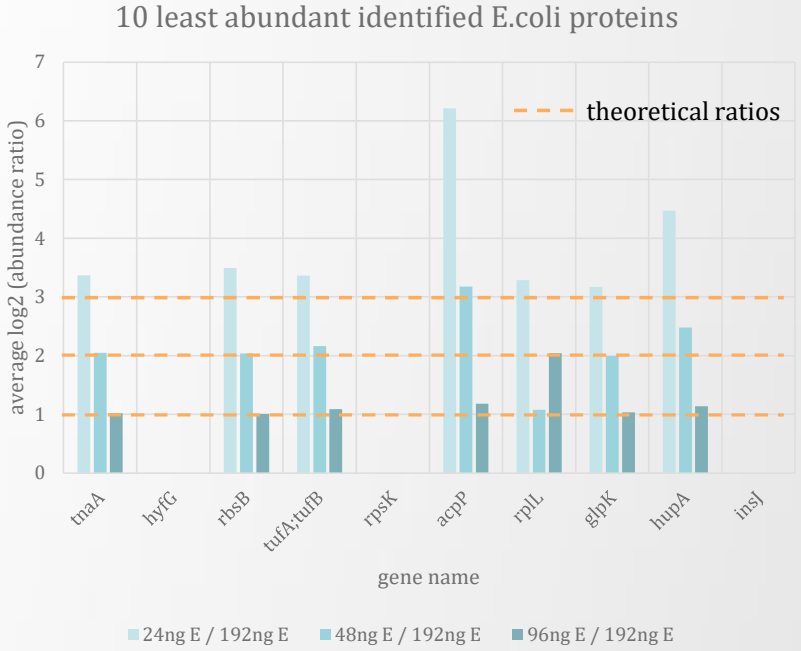

Accuracy

Precision

# Dynamic range: covering 4 orders of magnitude

## Discover differentially expressed low-abundant proteins of interest

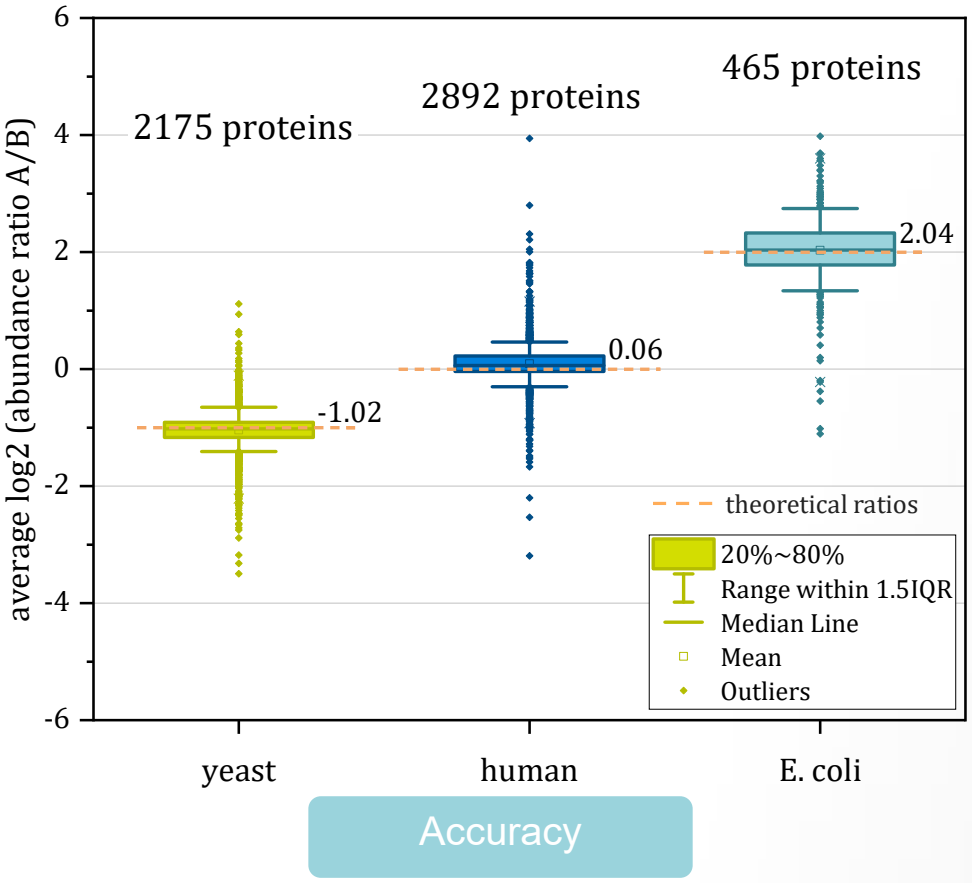


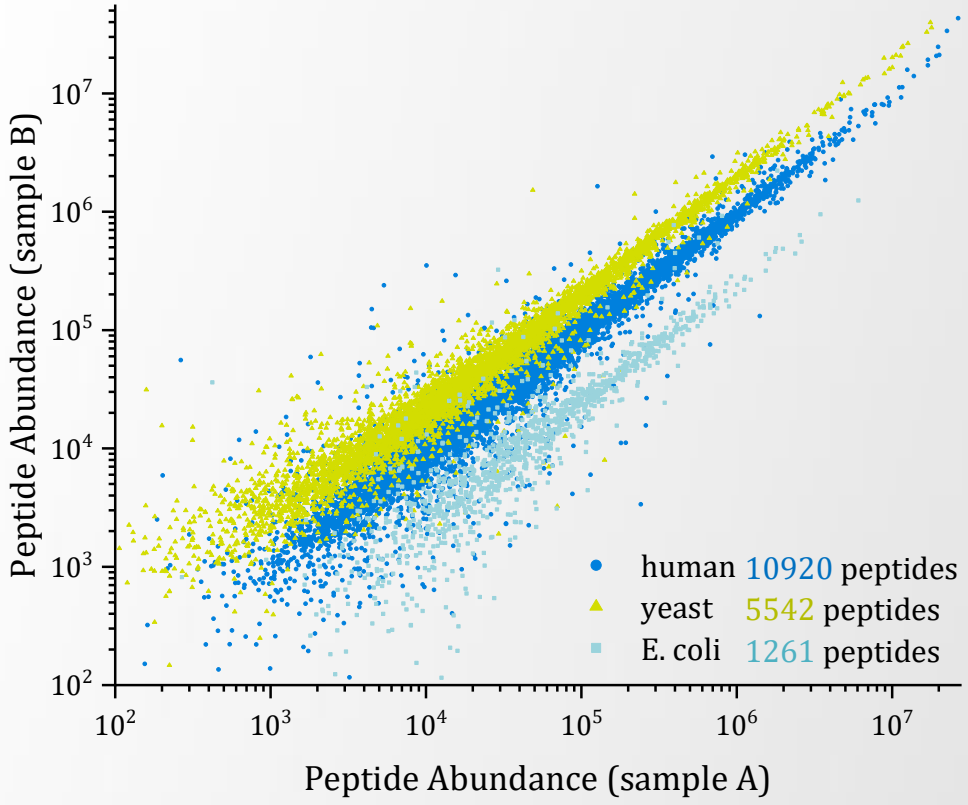

- 2 proteome mix, *E.coli* spiked in high human background, separated over 30 min LC gradient
- 7 out of 10 low-abundant *E.coli* proteins were quantified accurately

# Quantitation accuracy in three-proteome mixtures

## LFQ benchmark: a measure for quantitation accuracy




Accuracy



# Velocity LFQ HR-DIA platform

The new standard of quantitative accuracy, precision and data completeness at deep proteome coverage

## High-throughput high-resolution data-independent acquisition workflow for accurate label-free quantitation

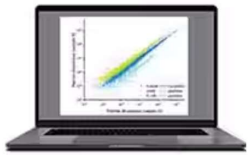
Thermo Scientific™
Vanquish™ NEO
UHPLC system

Thermo Scientific™
µPAC™ Neo UHPLC
column (50 cm)

Thermo Scientific™
EASY-Spray™
nano source

Thermo Scientific™
Orbitrap Exploris™ 240
mass spectrometer

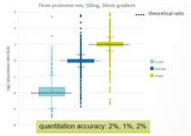
Thermo Scientific™
Orbitrap Exploris™ 480
mass spectrometer



Software of choice

# Optimized Velocity LFQ HR-DIA on Orbitrap Exploris 240 MS is the right combination for your lab

**Vanquish Neo UHPLC | µPAC Neo column | Orbitrap Exploris 240 MS**

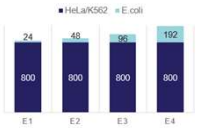


**Quantitation**
Accuracy |Precision

**Robustness**
reproducible performance

robust
reproducible
consistent

**Throughput**
samples per day



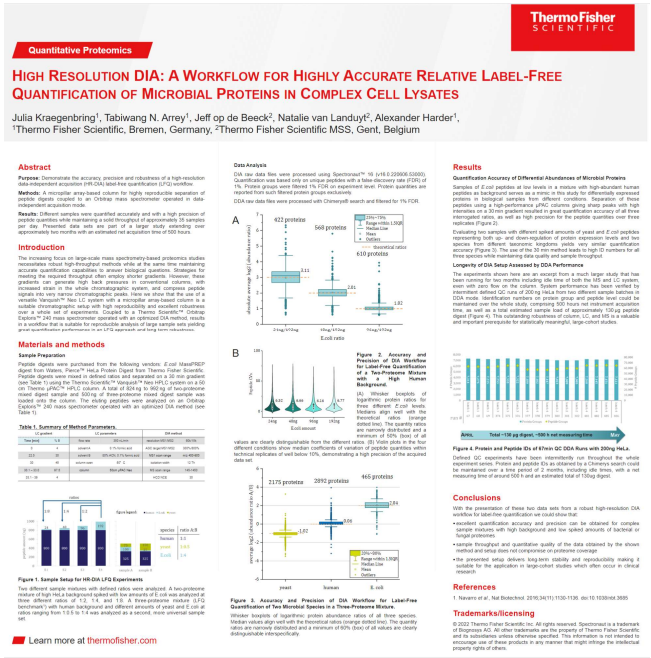

**Identifications**
# peptides/proteins

| | IDs |
|---|---|
| Peptides | > 94,000 |
| Proteins | > 12,500 |
| Protein Groups | > 9,500 |

# Learn more: www.thermofisher.com/VelocityDIA

Including details methods & templates

## Poster



Quantitative Proteomics

HIGH RESOLUTION DIA: A WORKFLOW FOR HIGHLY ACCURATE RELATIVE LABEL-FREE QUANTIFICATION OF MICROBIAL PROTEINS IN COMPLEX CELL LYSATES

Julia Kraegenbring[1], Tabiwang N. Arrey[1], Jeff op de Beeck[2], Natalie van Landuyt[2], Alexander Harder[1],
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Abstract

Introduction

Materials and methods

Results

Conclusions

References

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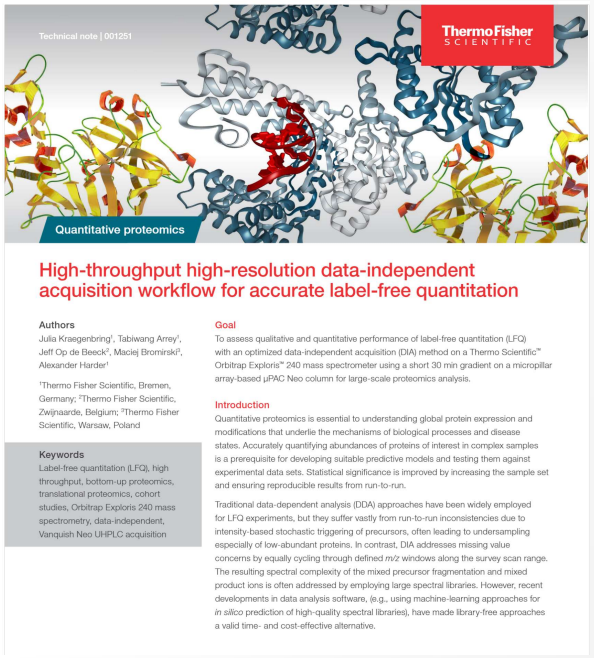
## Application note



Technical note | 001251

Quantitative proteomics

High-throughput high-resolution data-independent acquisition workflow for accurate label-free quantitation

Authors

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Keywords

Label-free quantitation (LFQ), high throughput, bottom-up proteomics, translational proteomics, cohort studies, Orbitrap Exploris 240 mass spectrometry, data-independent, Vanquish Neo UHPLC acquisition

Goal

To assess qualitative and quantitative performance of label-free quantitation (LFQ) with an optimized data-independent acquisition (DIA) method on a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer using a short 30 min gradient on a micropillar array-based µPAC Neo column for large-scale proteomics analysis.

Introduction

Quantitative proteomics is essential to understanding global protein expression and modifications that underlie the mechanisms of biological processes and disease states. Accurately quantifying abundances of proteins of interest in complex samples is a prerequisite for developing suitable predictive models and testing them against experimental data sets. Statistical significance is improved by increasing the sample set and ensuring reproducible results from run-to-run.

Traditional data-dependent analysis (DDA) approaches have been widely employed for LFQ experiments, but they suffer vastly from run-to-run inconsistencies due to intensity-based stochastic triggering of precursors, often leading to undersampling especially of low-abundant proteins. In contrast, DIA addresses missing value concerns by equally cycling through defined *m/z* windows along the survey scan range. The resulting spectral complexity of the mixed precursor fragmentation and mixed product ions is often addressed by employing large spectral libraries. However, recent developments in data analysis software, (e.g., using machine-learning approaches for *in silico* prediction of high-quality spectral libraries), have made library-free approaches a valid time- and cost-effective alternative.

## On-demand webinar

Webinars
Live and on-demand

Summary

High Throughput Label-Free Quantitation with Orbitrap HR-DIA Workflow

In this webinar we will be presenting a high throughput LF-Quan HR-DIA workflow based on Thermo Scientific™ Orbitrap™ technology for reliable protein identification and quantitation. The HR-DIA Orbitrap workflow with Thermo Scientific™ Vanquish™ NEO UHPLC and µPAC columns are easy to adopt especially for short gradients, its ease of use with advanced software options, and has fewer missing values for LF-Quan. This presentation will benefit all proteomics labs, especially those that focus on large cohort studies.

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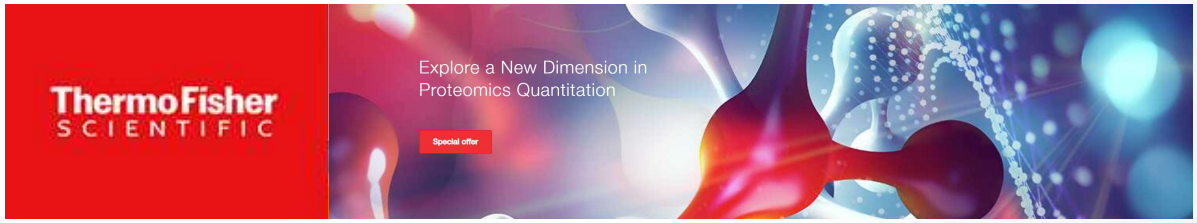


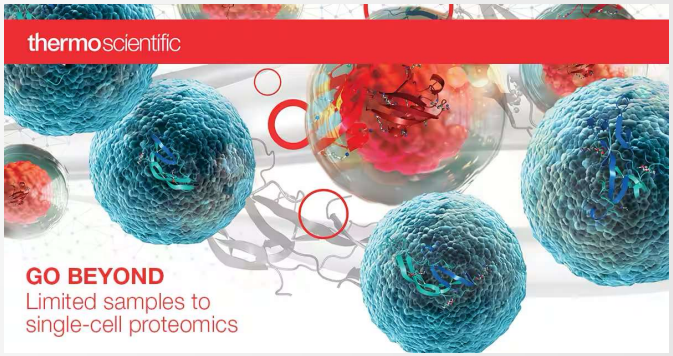


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