Gaining Advantage

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Proteomics Without Labels: Maximizing Label-Free Quantitation in MS

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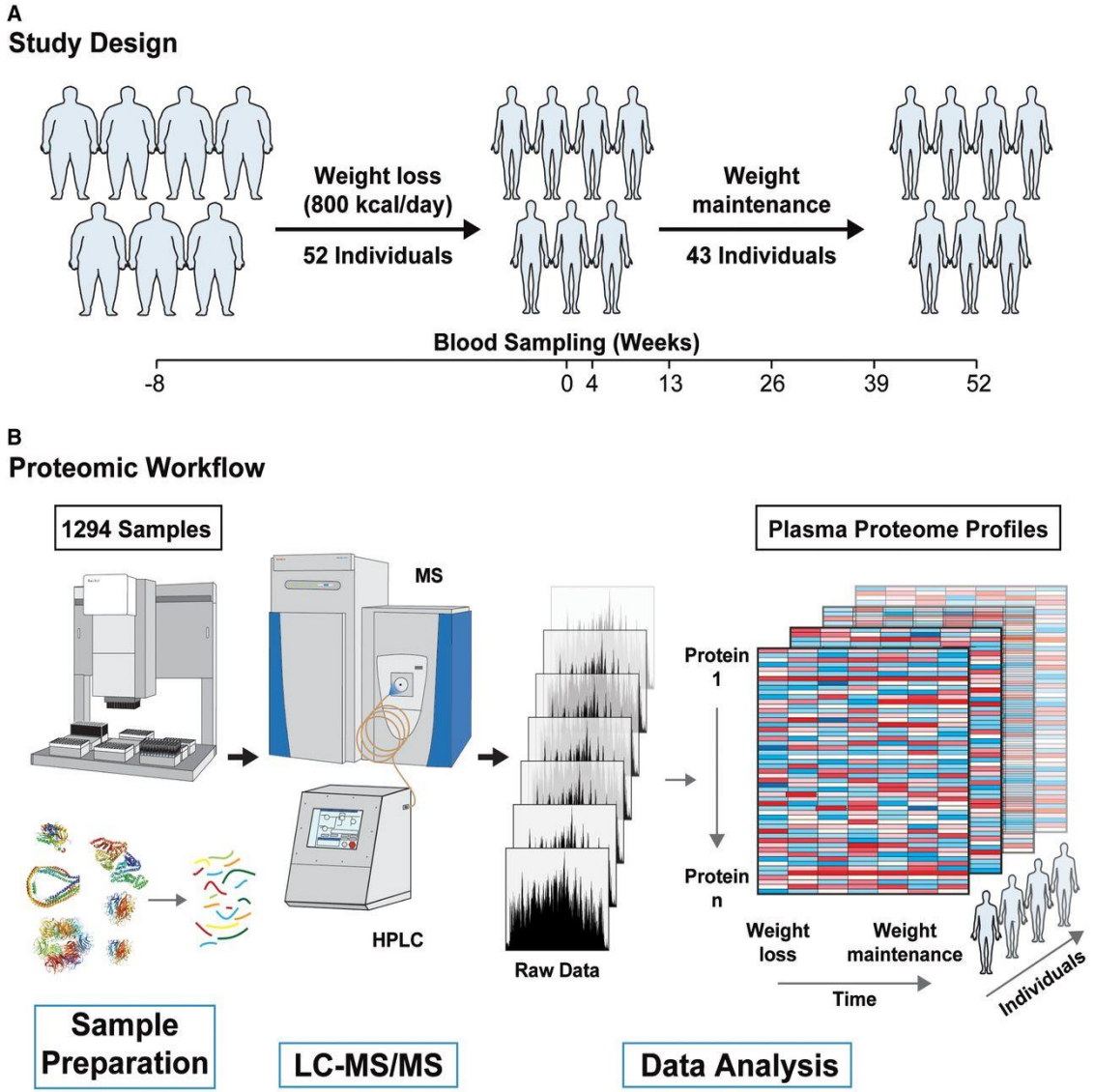
Product Marketing Manager – Obitrap Fusion Lumos

C&E News Webinar
May 15, 2019

What is Global Proteome Profiling

Large-scale study design with well-defined groups, timepoints, etc.



- Well-defined sample preparation protocols
- Incorporation of generic ISTD/control
- Reference groups or pooled response

- Set LC-MS method with qual and quant considerations built into the method
- Standard protocol for LC-MS robustness and reproducibility assessment (based on generic ISTD)
- Bioinformatics pipeline for automated data processing
- Processing output into a format for biological interpretation (KEGG, String, Ingenuity, etc.)

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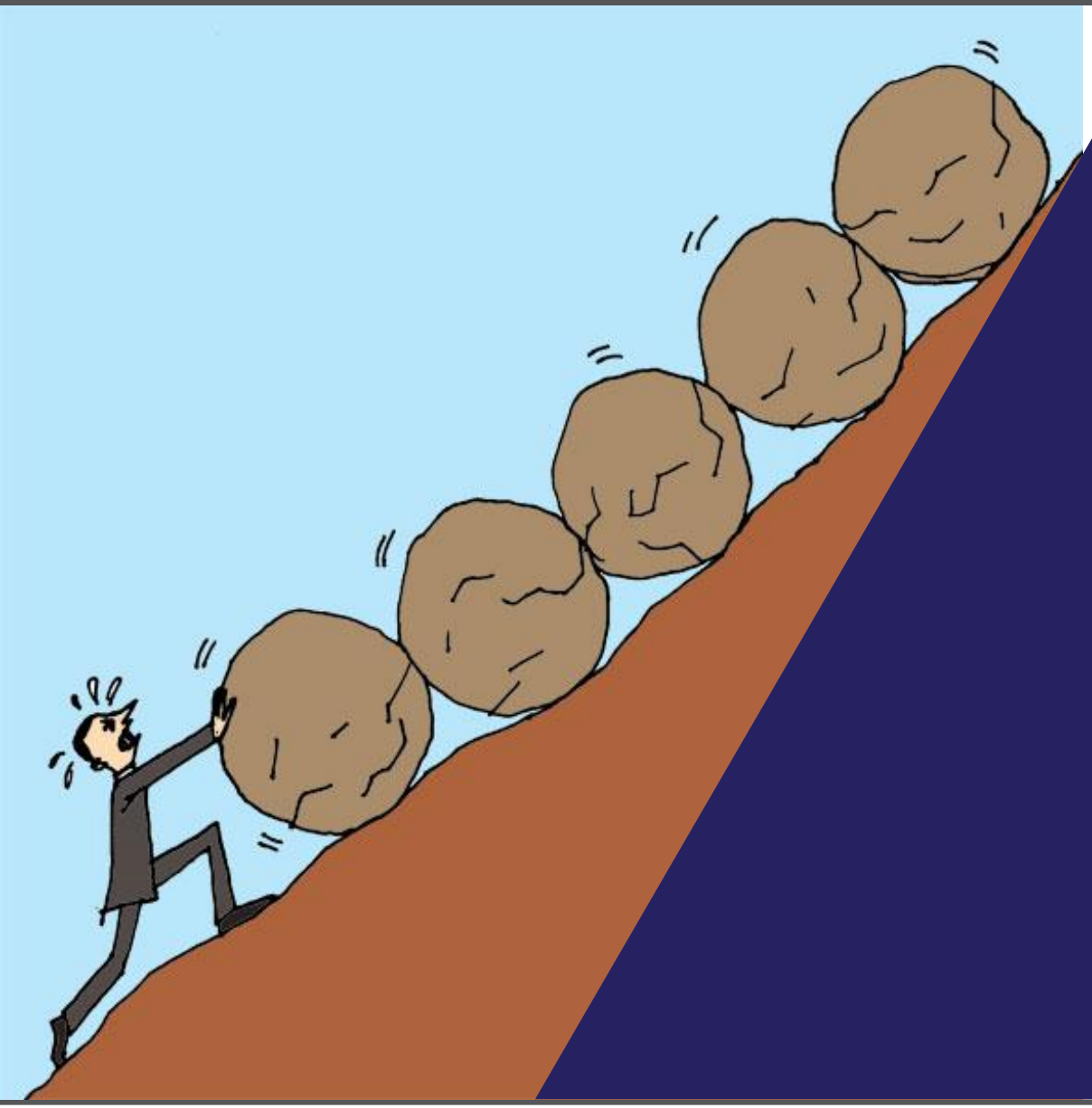
Pushing the Limits of LFQ

- Maximize breadth and depth of proteome coverage for each sample – peptide detection
- Maintain high throughput
- Robust and reproducible data acquisition across each sample for the entire study
- Leverage “match between runs”
- Employ automated data processing pipeline(s)

Challenges – LC-MS Perspective



- Speed of data acquisition (MS and MS/MS)
- Reproducibility for match-between-runs analysis
- Data quality for peptide identification – MS data quality plays as much of a role as MS/MS
- Data quality for peptide XIC, integration, and quantitation
- Platform stability across the study

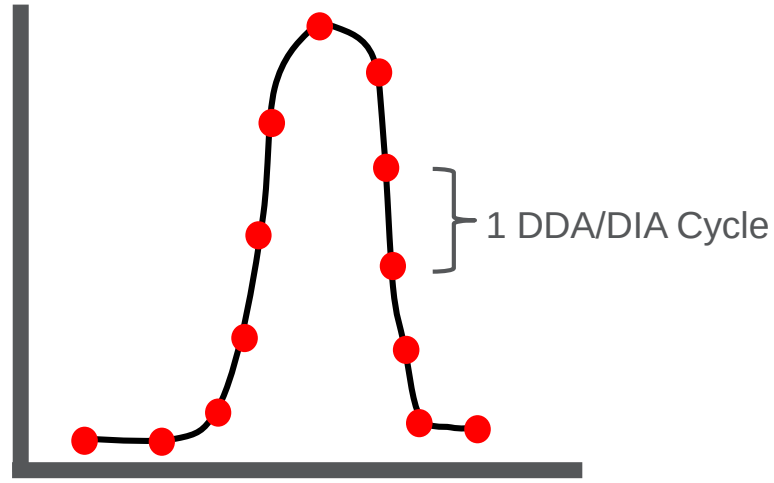
Things to Consider When Creating Maximum Data Acquisition Methods

Quantitative Data Processing

- Minimum data points per chromatographic peak
- Resolution to extract target m/z values
- Mass tolerance for data extraction
- Reproducibility across samples

Qualitative Data Processing

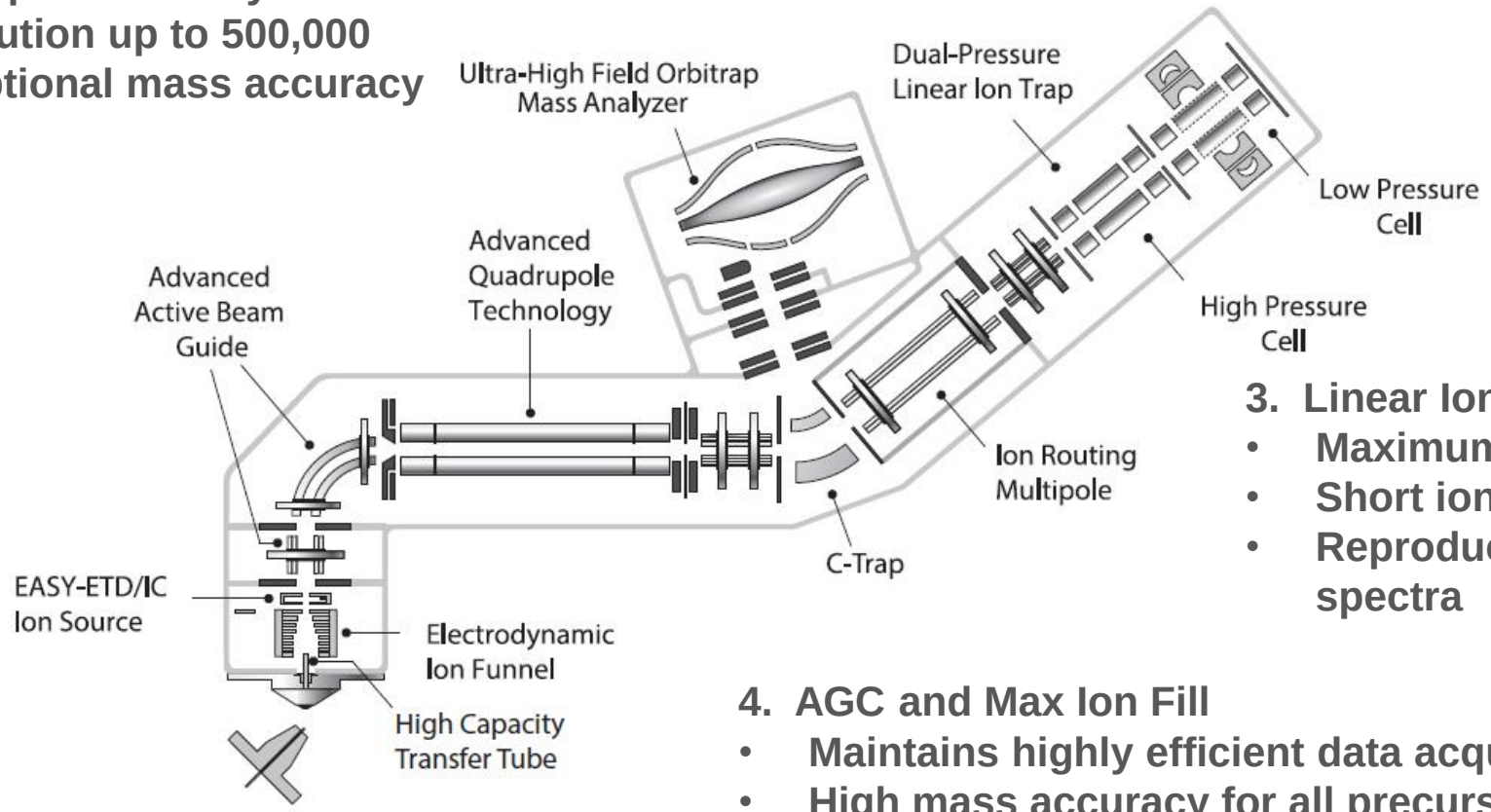
- Confident peptide sequencing (HRAM for DIA)
- Accurate mass analysis at MS stage
- Match between runs



Top 5 Attributes of the Orbitrap Fusion Lumos™ Tribrid™ Mass Spectrometer

2. Orbitrap mass analyzer
- Resolution up to 500,000
- Exceptional mass accuracy

1. Tribrid Architecture
- 3 mass analyzers
- 2 mass detectors
- Ion-routing multiple



3. Linear Ion Trap mass analyzer
- Maximum sensitivity
- Short ion fill times
- Reproducible product ion spectra

4. AGC and Max Ion Fill
- Maintains highly efficient data acquisition
- High mass accuracy for all precursors across the dynamic range
- Enables reliable quantitation between samples
-

5. FAIMS Pro Interface
- Enhances Tribrid performance
- Extends proteome coverage through CV fractionation
- Reproducible performance
- Extends robustness

LC-MS Experimental Design

Standard Sample

Pierce HeLa Protein Digest Standard
(P/N 88239)

Reversed phase LC-MS

Easy nLC coupled with Orbitrap Fusion Lumos
(300 nL/min)

Aurora UHPLC Column with **nanoZero**™ fitting

Product Specifications

• **Column Format:**	Analytical Column	• **Max Pressure:**	1200 bar
• **Column Type:**	Reversed Phase	• **Temp Limits:**	65°C (Low pH)
• **For use with:**	UHPLC	• **Particle Size:**	1.6µm
• **Length:**	250mm	• **pH Stability:**	1-8
• **Diameter**	75µm	• **Stationary Phase:**	C18
• **Pore Size:**	120Å		

IonOpticks (P/N AUR-25075C18A)

Data Processing

Proteome Discoverer

MS/MS were searched in the UniProt Human database with Sequest HT and only 3 standard Modifications:

-Carbamidomethylation (C)
-Oxidation (M)
-Acetylation (Protein Nter)

Unique Peptides were filtered with 1% FDR.

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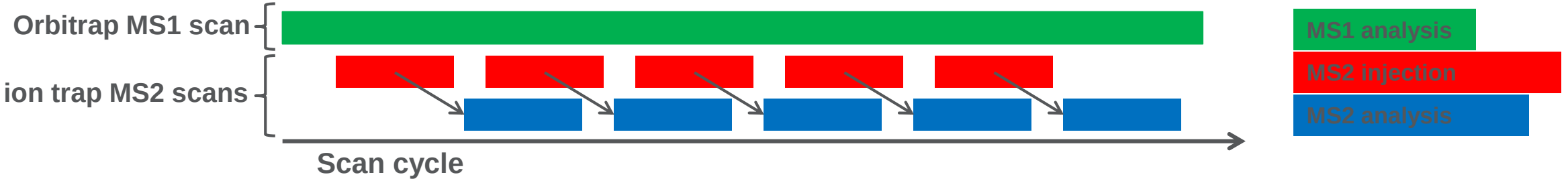
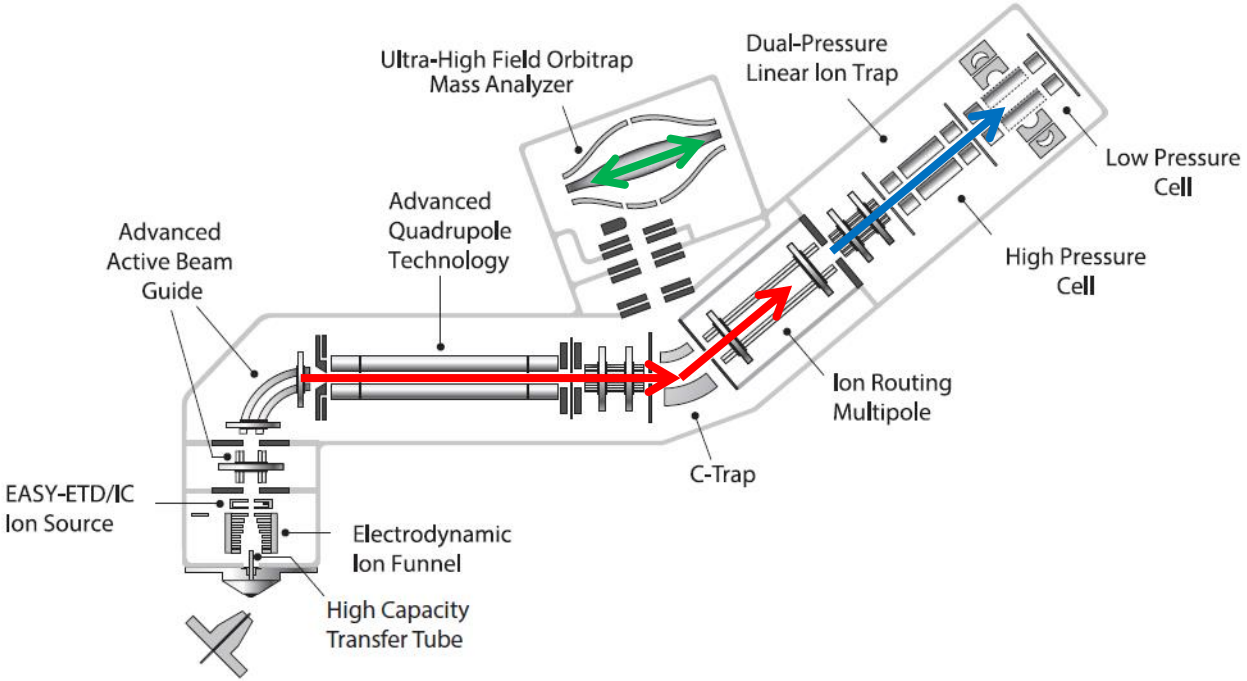
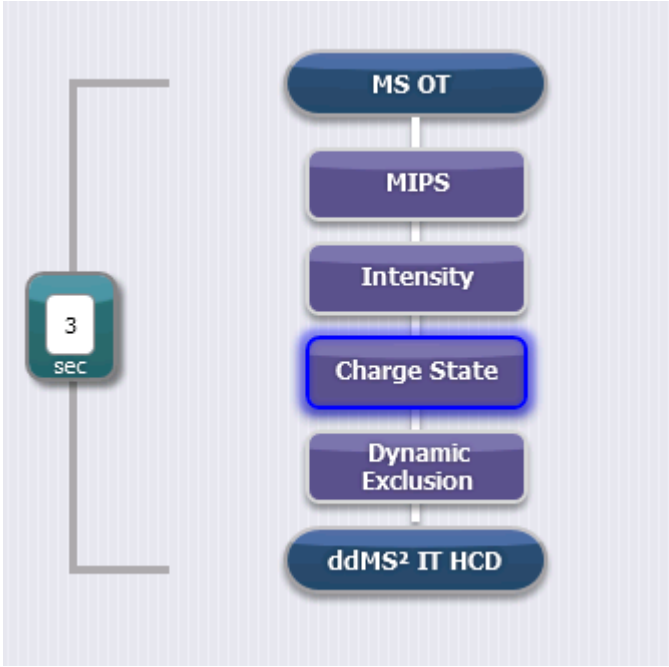
Parallel ion processing on the Tribrids in OTIT Mode – Fastest Data Acquisition

All Tribrid mass spectrometers optimize the duty cycle by parallelizing ion injection with *m/z* analysis







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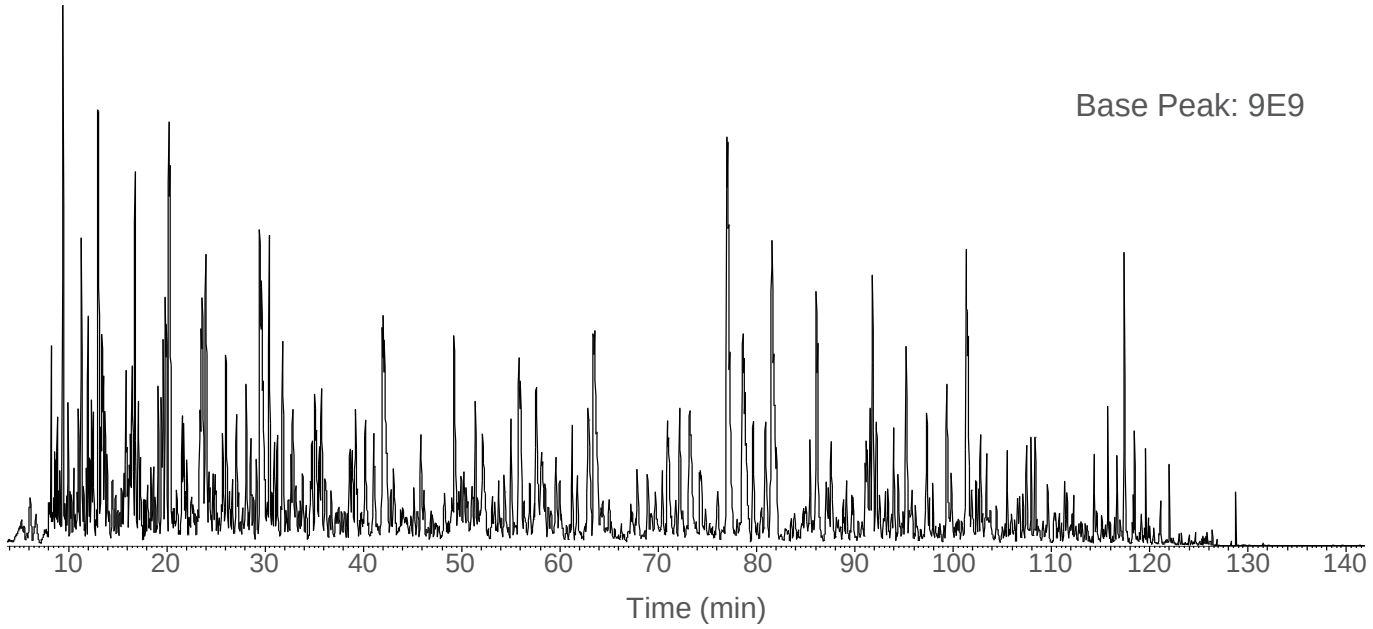
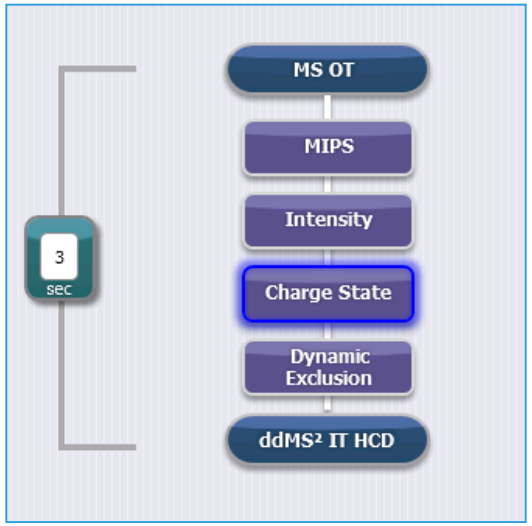
Maximizing Proteome Coverage on the Orbitrap Fusion Lumos Tribrid





New Depths of Analysis of Complex Samples

- To maximize the sampling available precursors, we configured the ion trap to collect MS/MS spectra at ≥40 Hz

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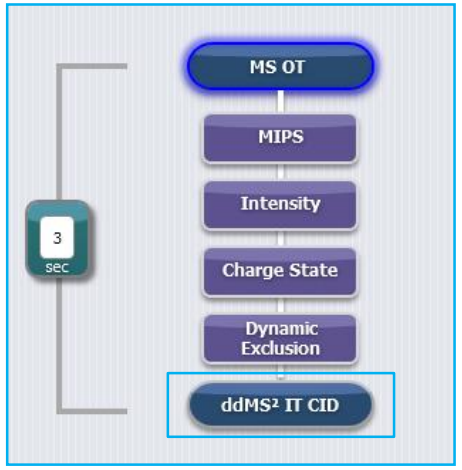
Maximizing Proteome Coverage on the Orbitrap Fusion Lumos Tribrid

Resolution = 240,000



Data-Dependent MSⁿ Scan Properties

Multiplex Ions	☐
Isolation Mode	Quadrupole
Isolation Window (m/z)	1.6
Isolation Offset	Off
Activation Type	CID
Collision Energy Mode	Fixed
CID Collision Energy (%)	35
CID Activation Time (ms)	10
Activation Q	0.25
Multistage Activation	☐
Detector Type	Ion Trap
Scan Range Mode	Auto: m/z Normal
Ion Trap Scan Rate	Rapid
AGC Target	2.0e3
Inject Ions for All Available Parallelizable Time	☑
Maximum Injection Time (ms)	300
Microscans	1
Data Type	Centroid

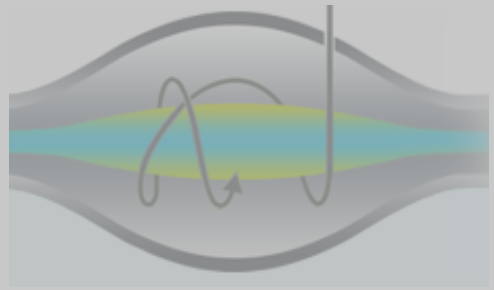
Data-Dependent MSⁿ Scan Properties

Multiplex Ions	☐
Isolation Mode	Quadrupole
Isolation Window (m/z)	**0.7**
Isolation Offset	Off
Activation Type	**HCD**
Collision Energy Mode	Fixed
HCD Collision Energy (%)	35
Detector Type	Ion Trap
Scan Range Mode	**Define m/z range**
Ion Trap Scan Rate	**Turbo**
Scan Range (m/z)	**200-1400**
AGC Target	**3.0e4**
Inject Ions for All Available Parallelizable Time	☐
Maximum Injection Time (ms)	**10**
Microscans	1
Data Type	Centroid
Scan Description	

- To maximize the sampling available precursors, we configured the ion trap to collect MS/MS spectra at ≥40 Hz

Method template also available on:

http://planetorbitrap.com/orbitrap-fusion tips/templates



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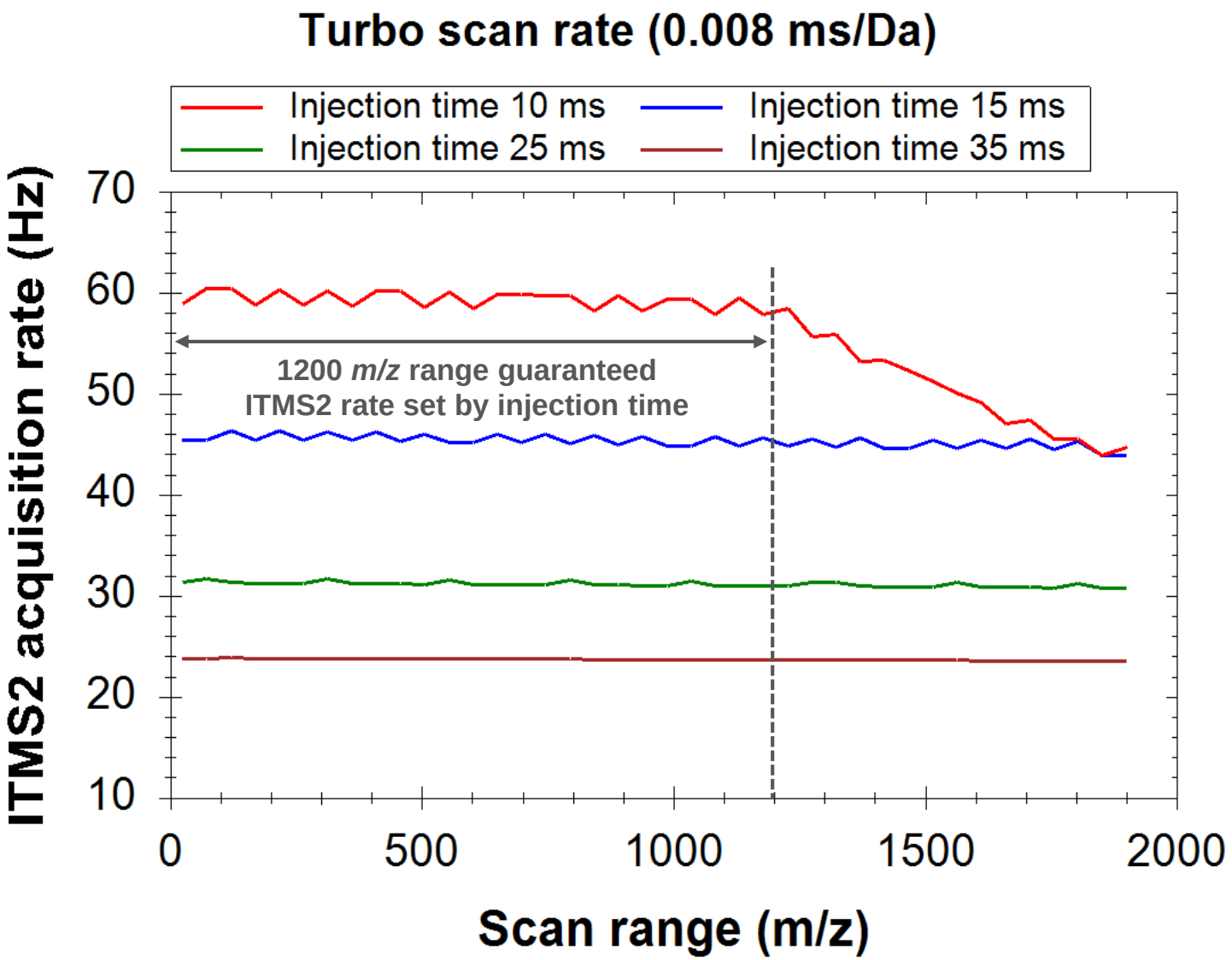
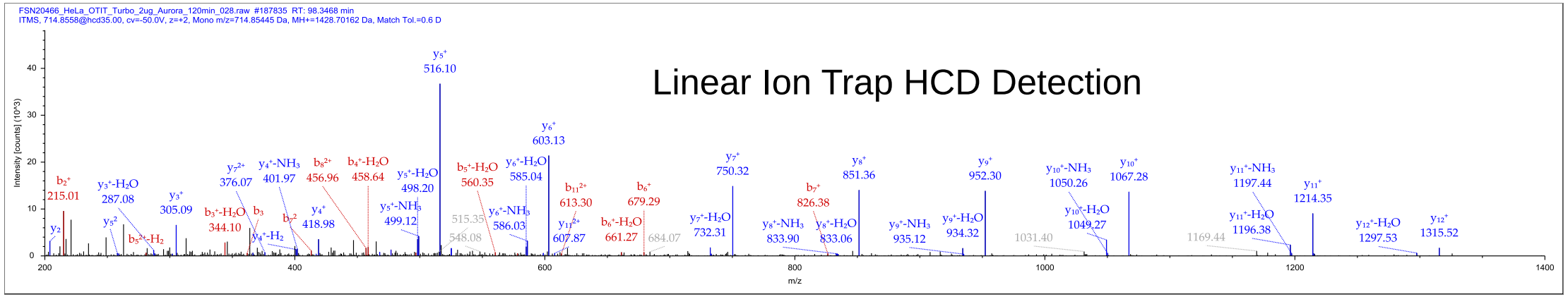
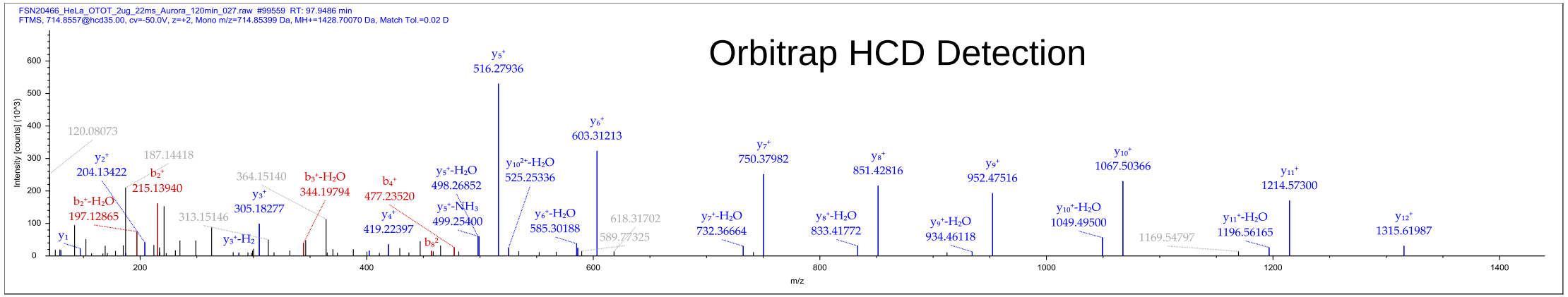
Either ion injection or ion trap *m/z* analysis can limit the acquisition rate



- The ion trap analysis time is proportional to the size of the *m/z* range analyzed
- The MS2 acquisition rate will be limited by the *m/z* analysis step if the time needed to scan ions out of the trap is longer than the injection time
- In order to interrogate the largest population of precursors possible, we need to control the acquisition rate with the MS2 Maximum injection time

Comparative HCD Data - Measured in Both Detectors





HCD method is consistent between the two experiments – the ion-routing multiple moves the resulting product ions into the defined mass analyzer for HCD spectral analysis

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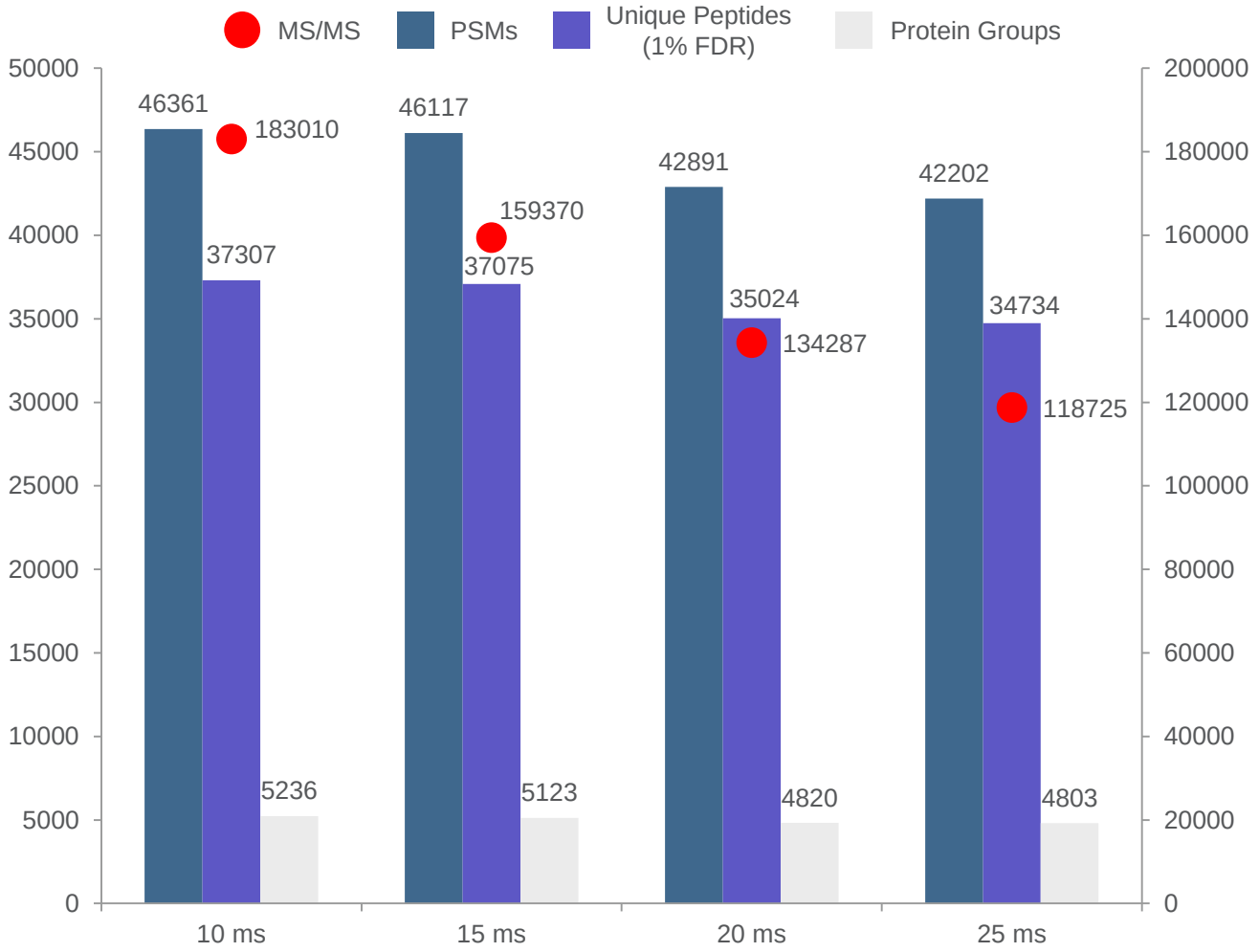
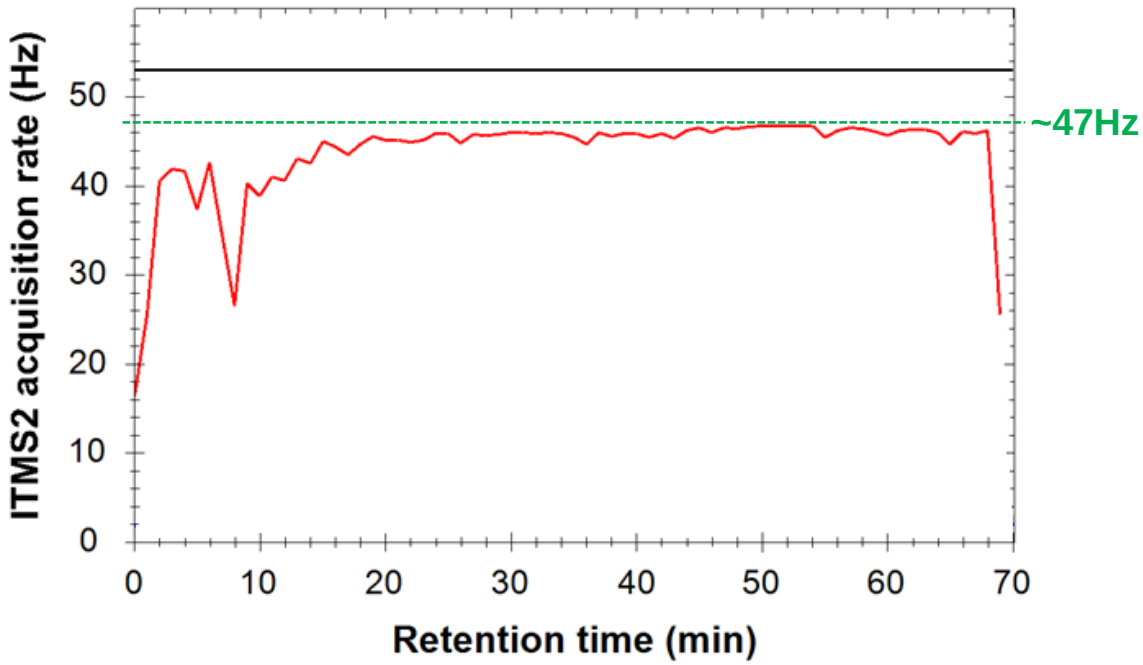
Optimal ITMS2 settings are sample and method dependent

- The Orbitrap Fusion Lumos collects > 150,000 ion trap MS/MS spectra during a 1 h data-dependent LC-MS/MS analysis of 1 µg of HeLa tryptic digest



ITMS2 acquisition rate with Turbo Scan and 10 ms Injection Time during a 60 min LC-MS analysis



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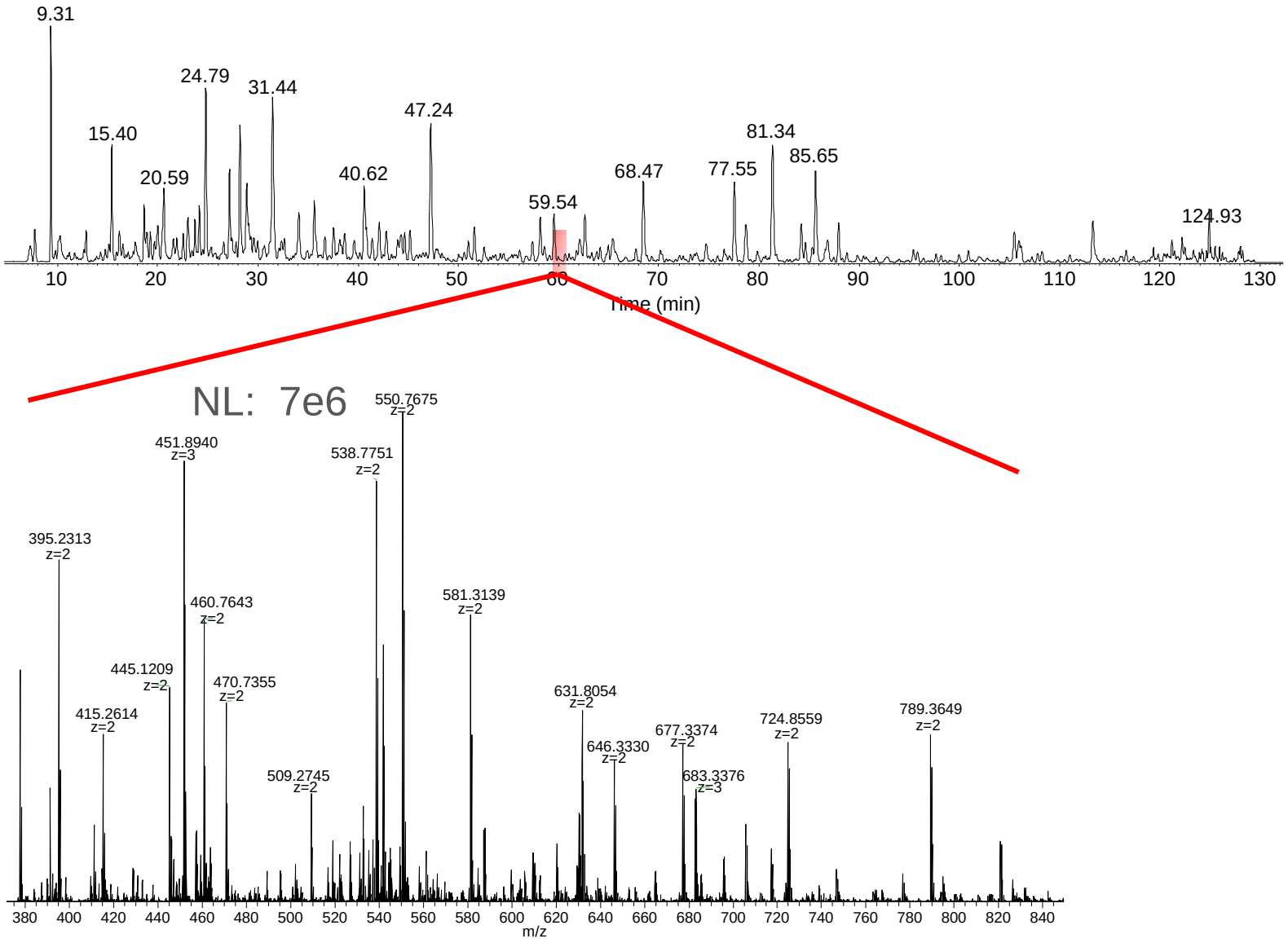
Spectral Complexity Presents Unknown Challenges



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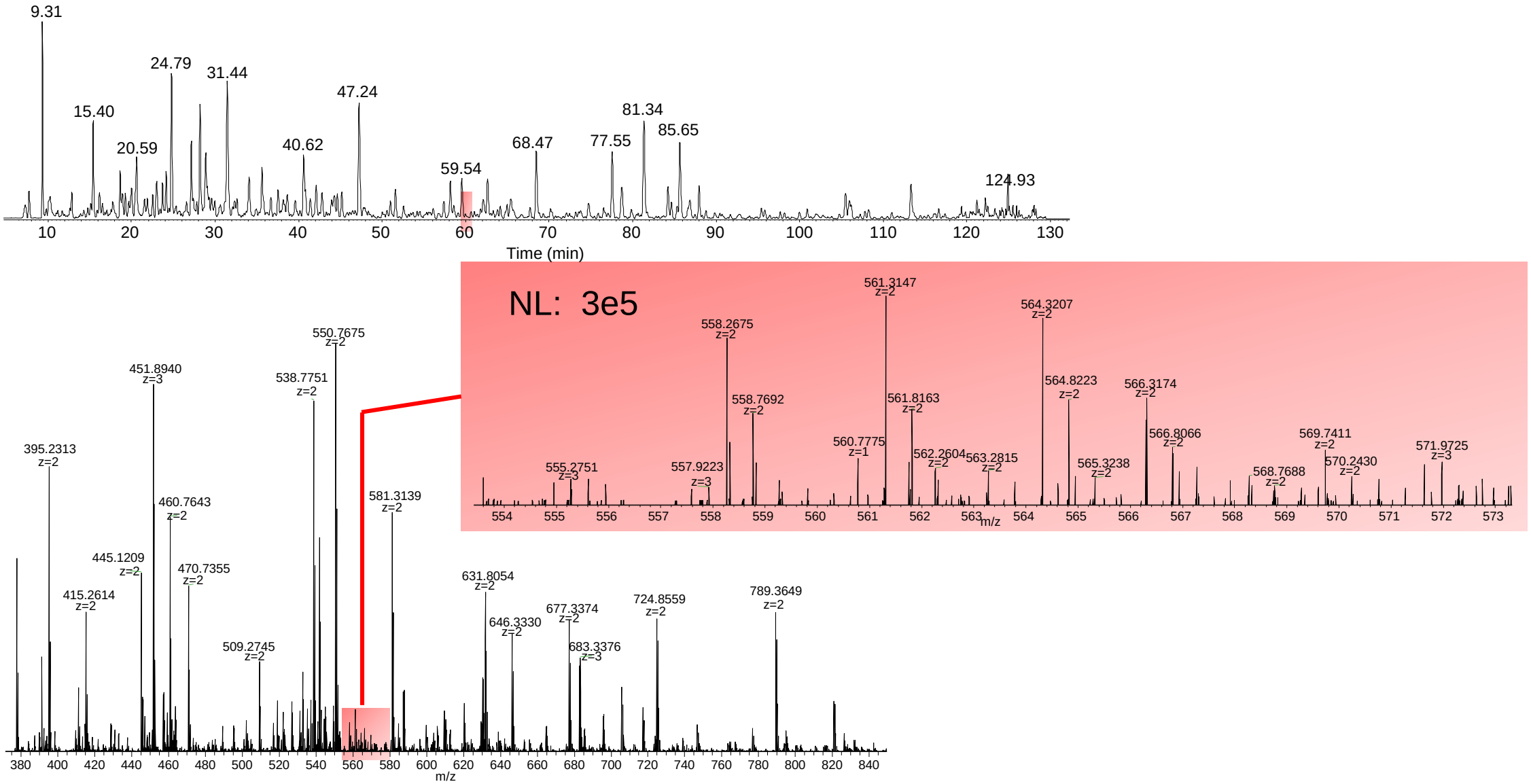
Spectral Complexity Presents Unknown Challenges



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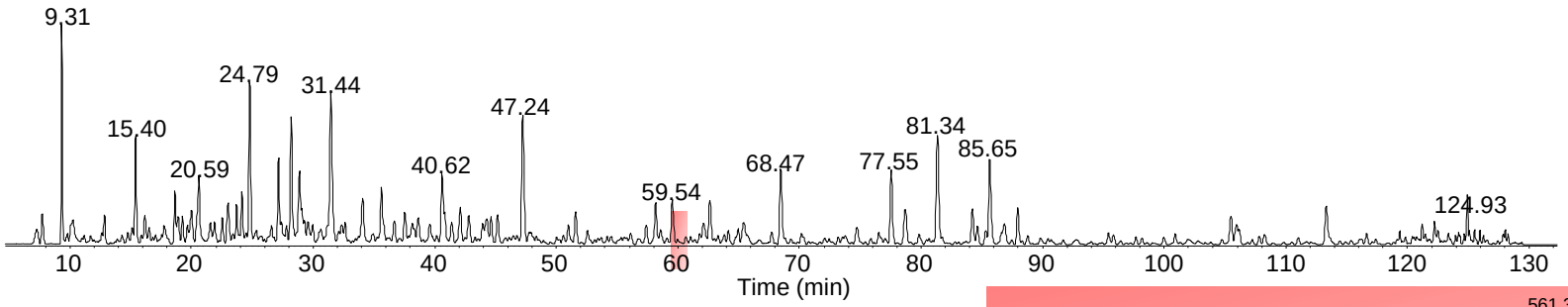
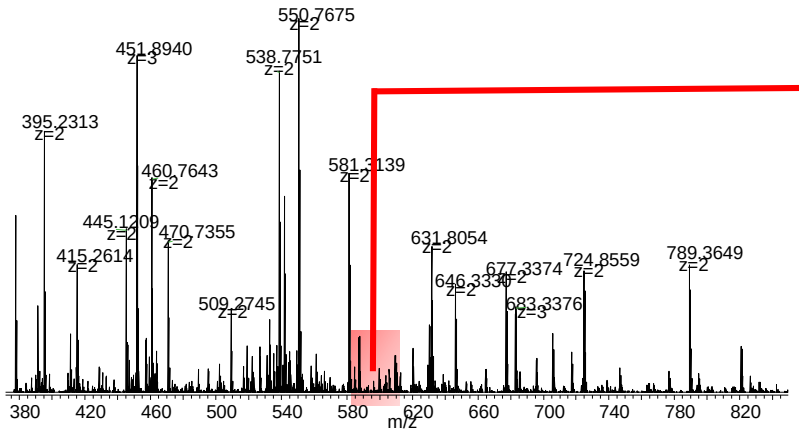
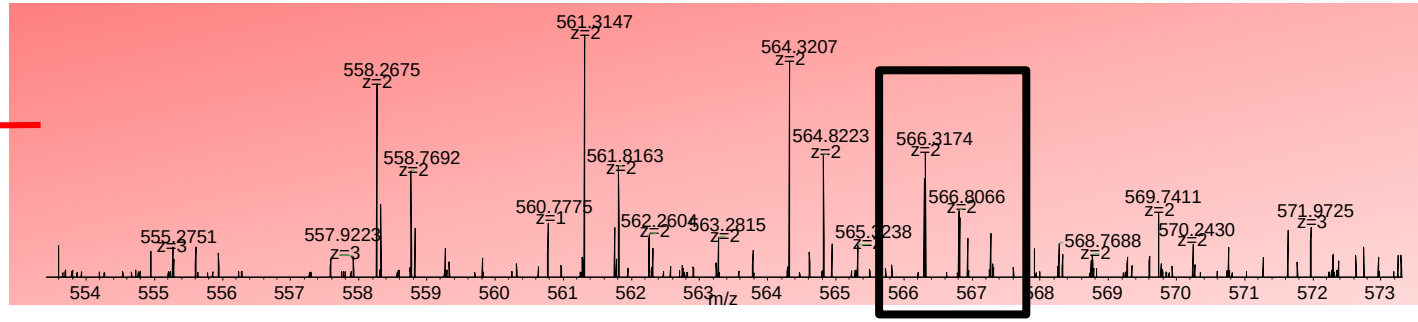
Spectral Complexity Presents Unknown Challenges







IVAIGTSDLDK

566.3175
R=157841
z=2

566.3050
R=155205
z=2

NL: 7e4

NTEAVDLITR

566.2870
R=159781
z=2

Δ13 mmu

566.8192
R=157310
z=2

566.8066
R=157310
z=2

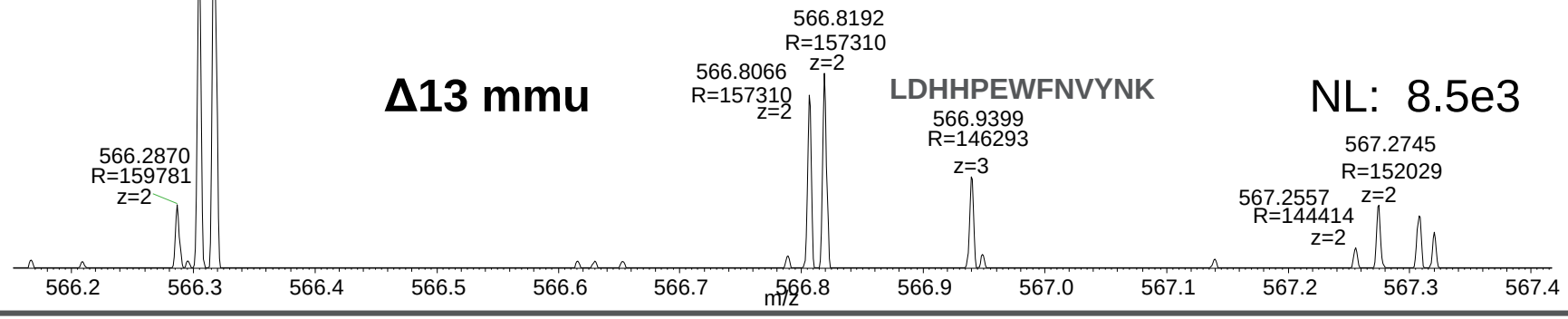
LDHHPEWFNVYNK

566.9399
R=146293
z=3

NL: 8.5e3

567.2745
R=152029
z=2

567.2557
R=144414
z=2



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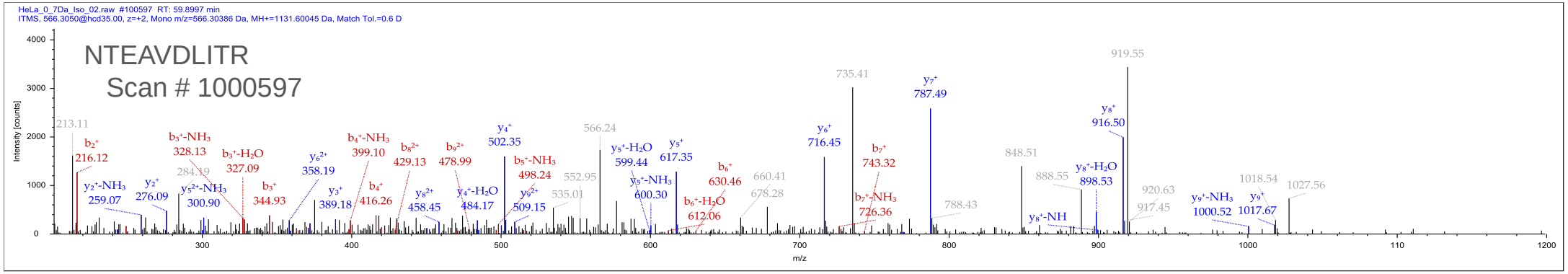
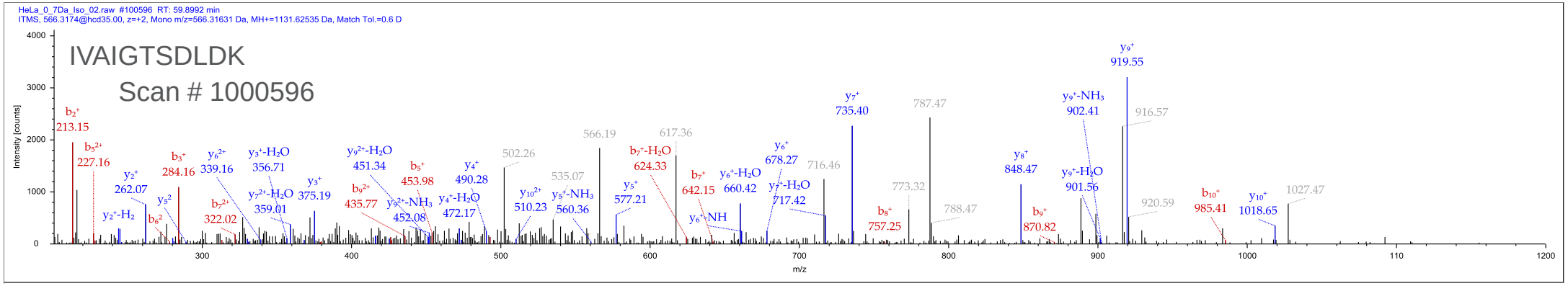
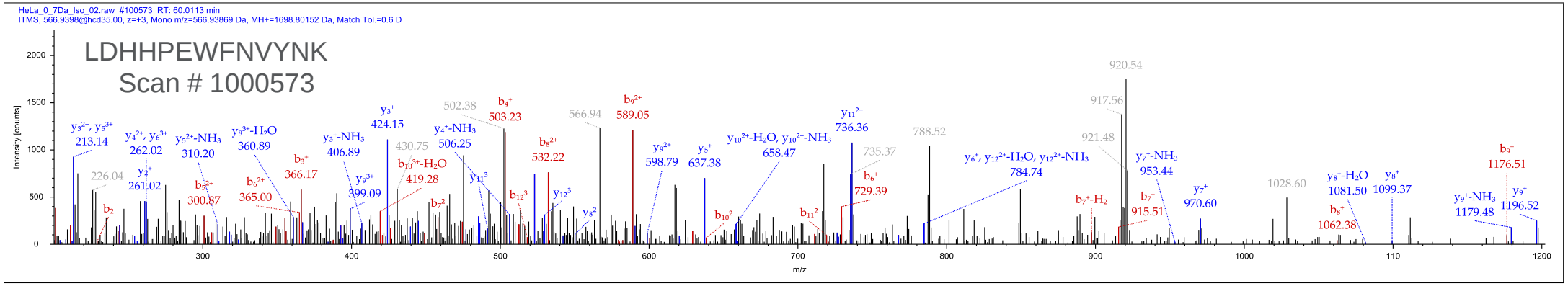
Comparative Product Ion Spectra







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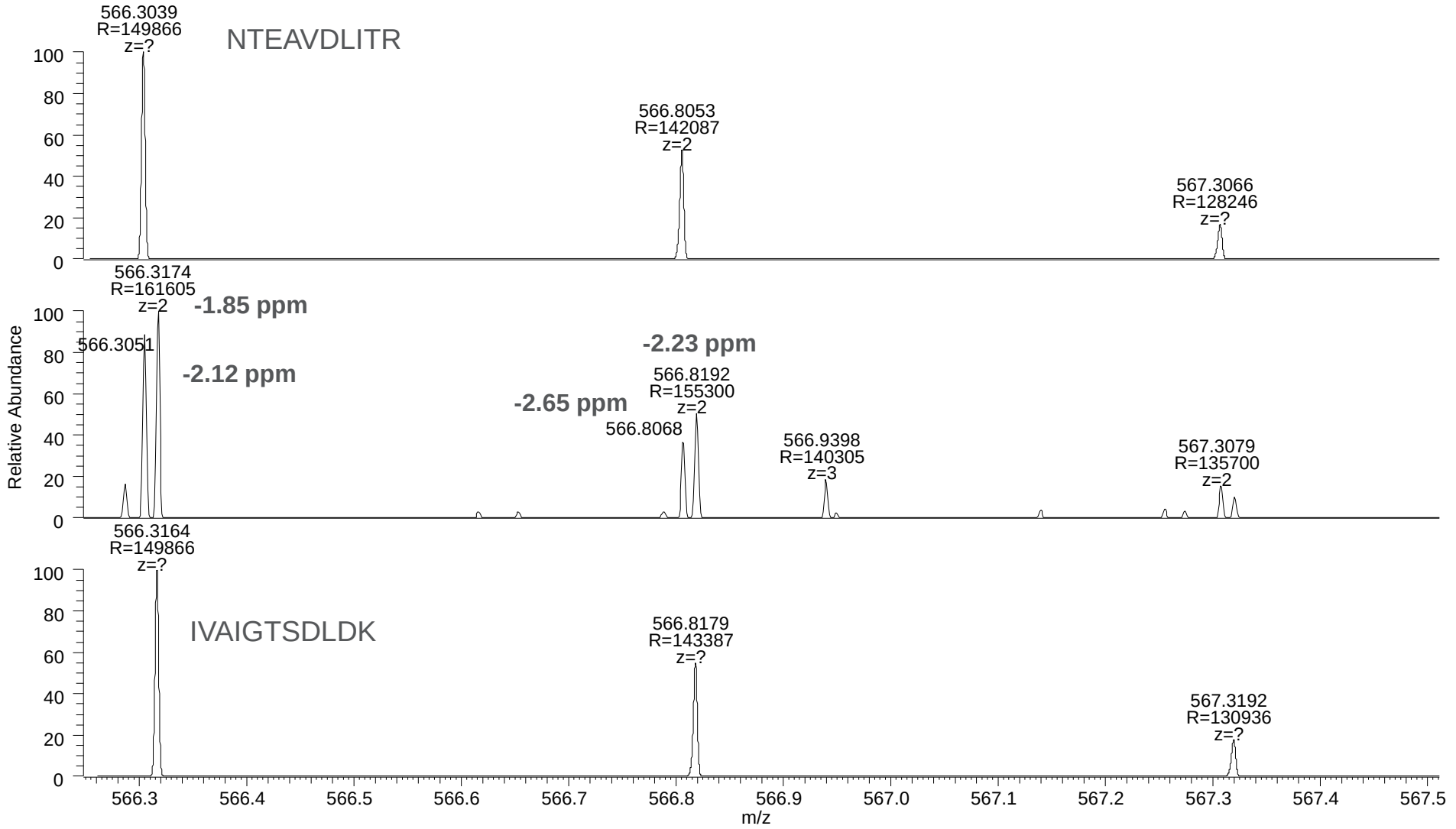
Theoretical Isotopic Analysis



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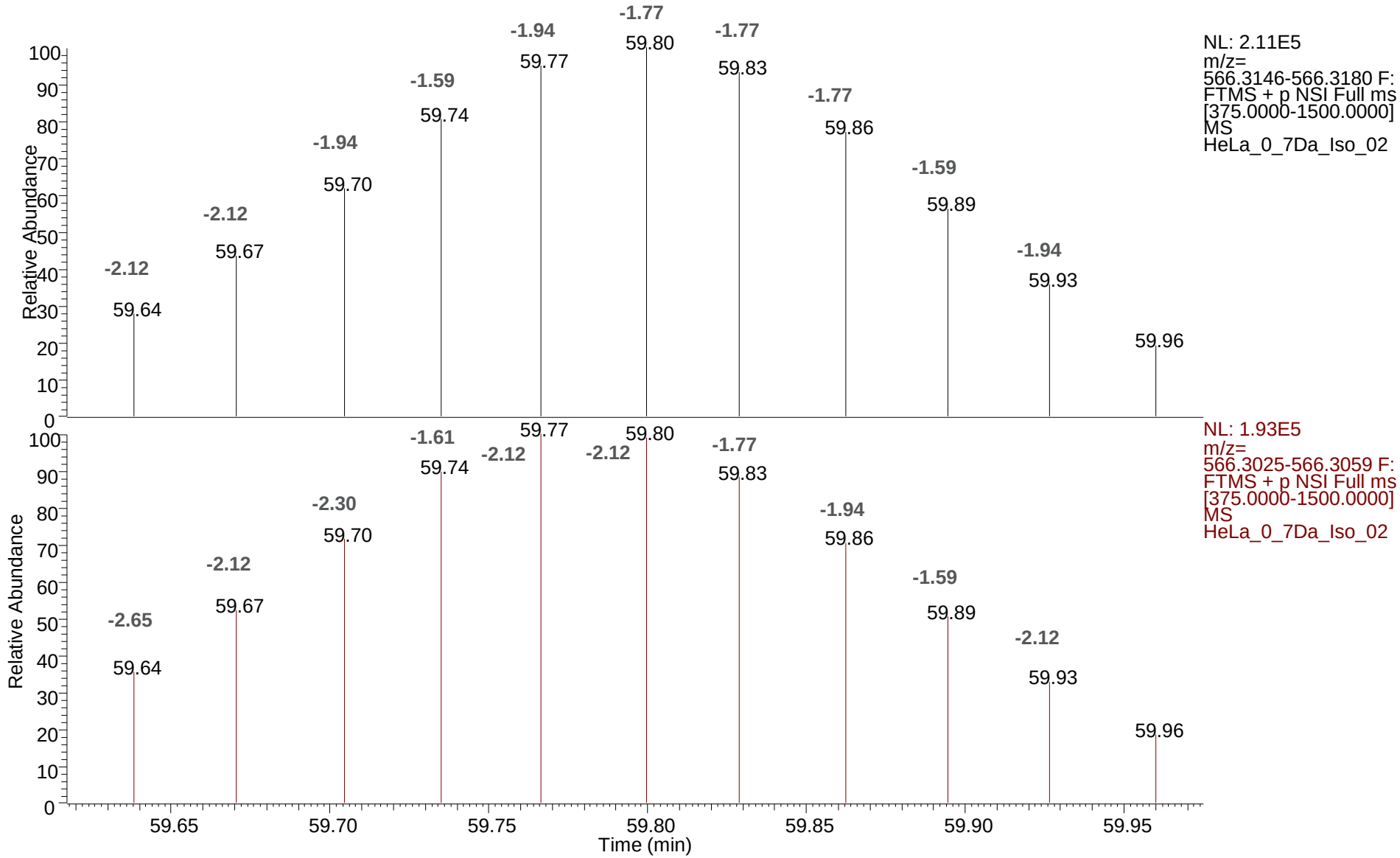
Benefits of Mass Measurement Accuracy for XIC Analysis



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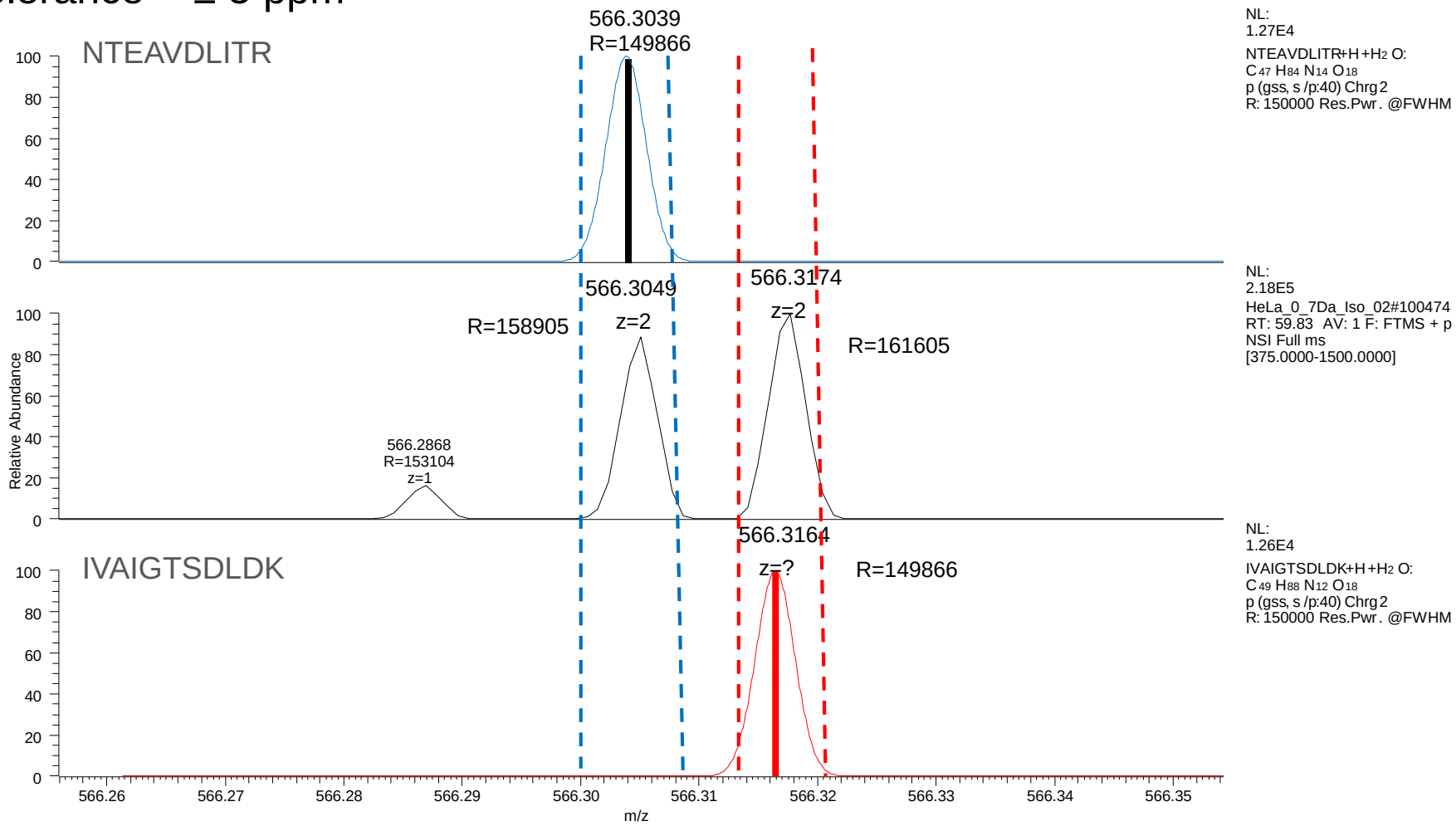
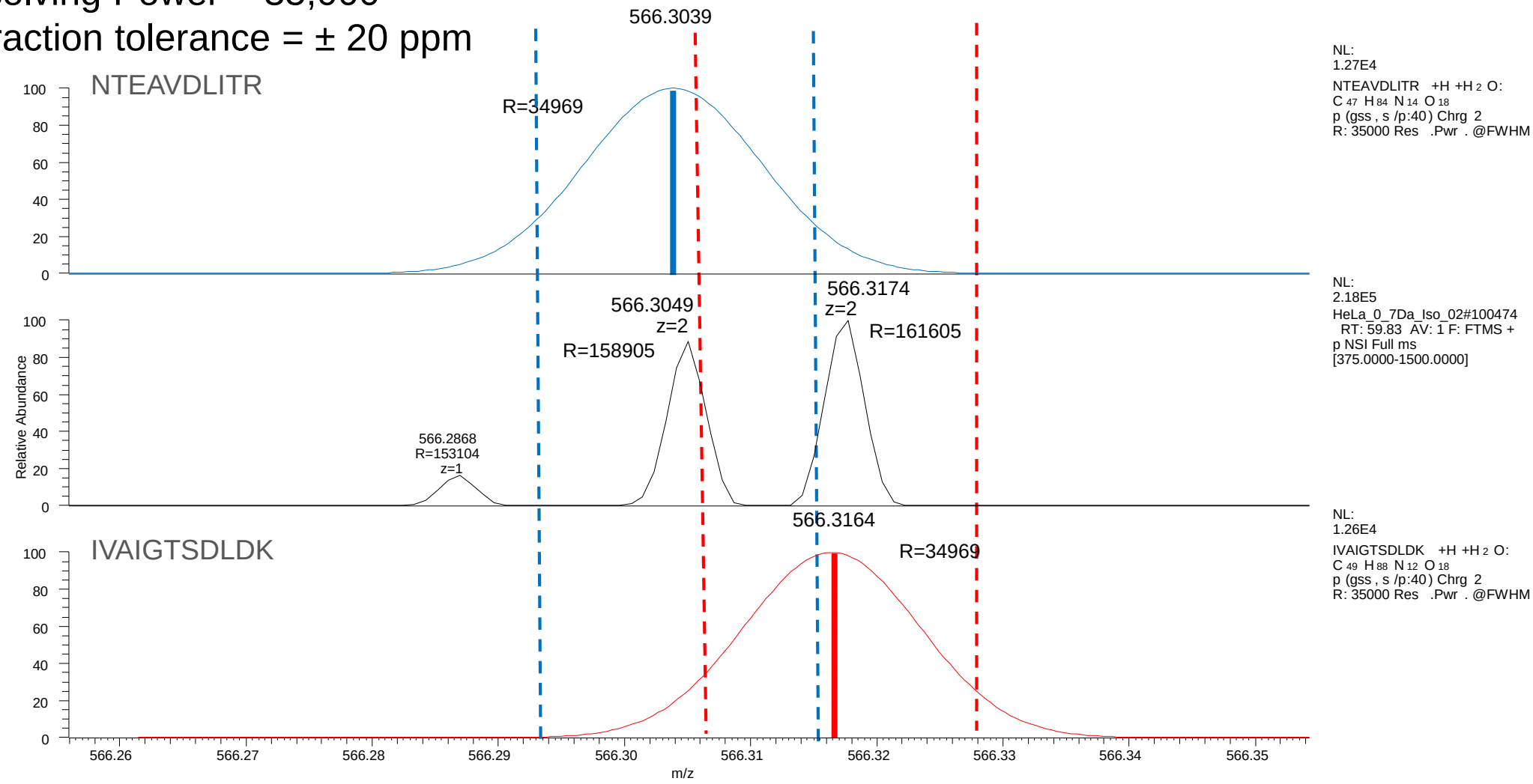
OT Resolution and Precursor Extraction Tolerance

Resolving Power = 150,000
Extraction tolerance = ± 5 ppm



Lower Resolution with Wide Mass Extraction Parameters

Resolving Power = 35,000
Extraction tolerance = ± 20 ppm



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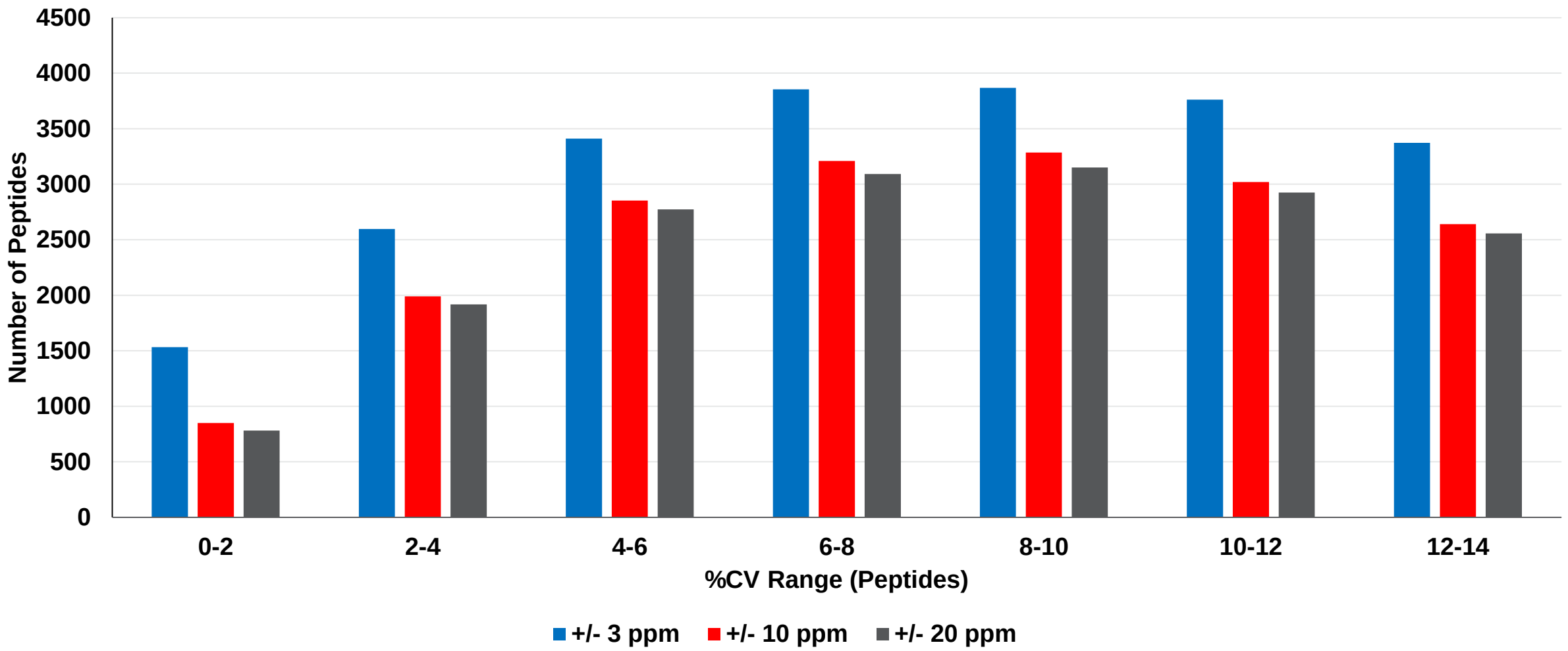
Distribution of Peptide Variance as a Function of Precursor Mass Tolerance



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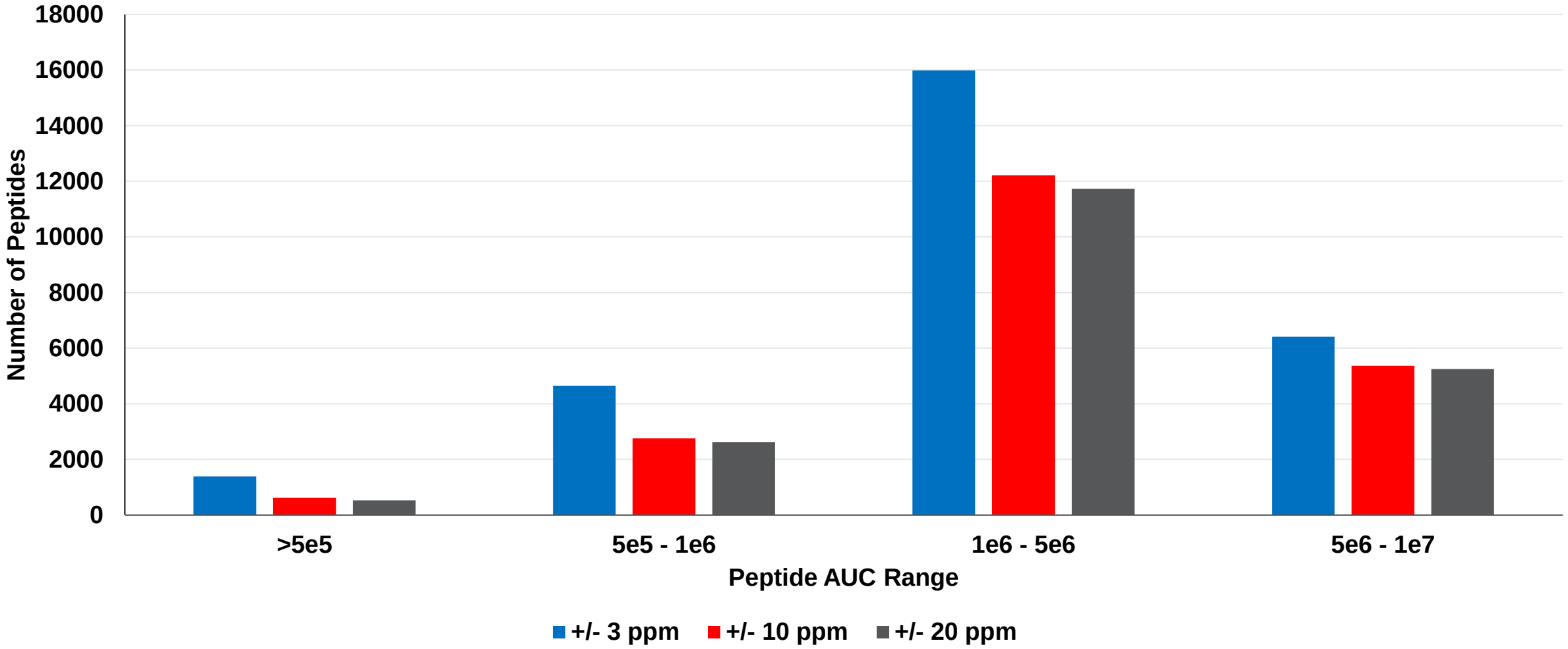
Integrated Peak Area Analysis



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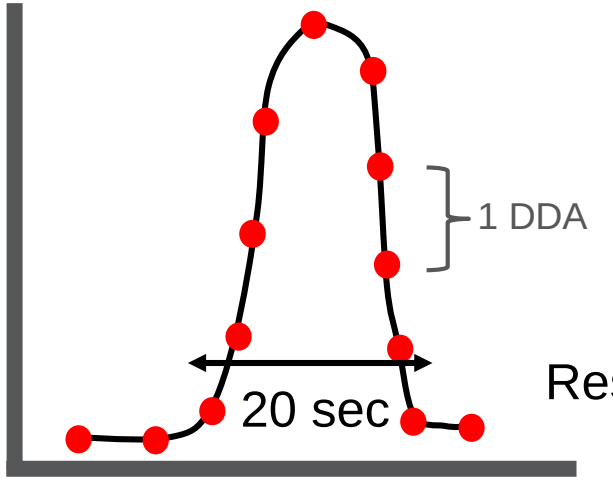
Chromatographic Fit-For-Purpose Methods

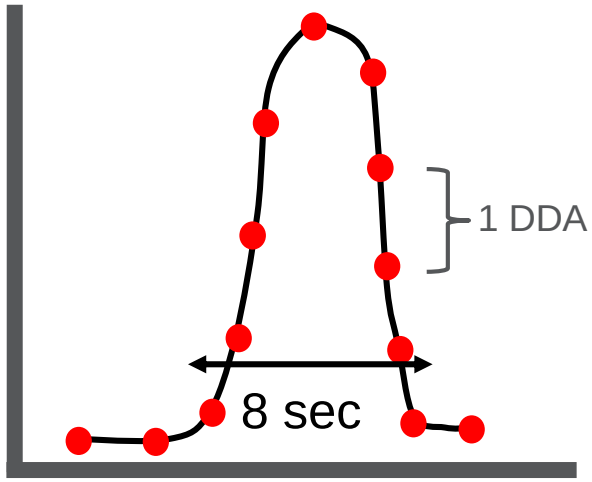


1 second

RP = 240,000 (@m/z 200)

IT MS/MS (26 msec each) 44 per cycle

Results in ~ 20 HRAM Full Scan MS and ~ 440 IT MS/MS



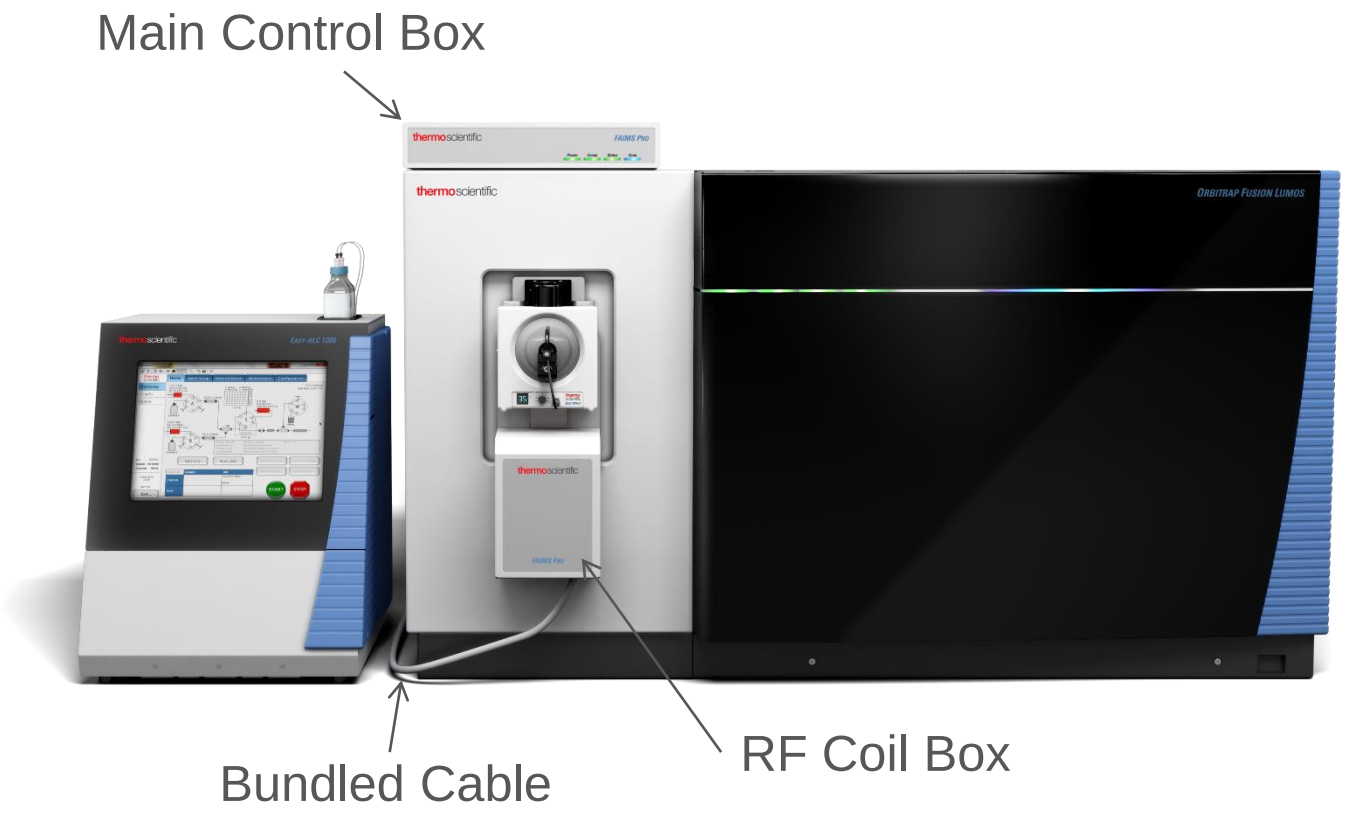
0.7 second

120,000 (@m/z 200)

IT MS/MS ~ 30 per cycle

Results in ~ 10 HRAM Full Scan MS and ~ 300

New High-Field Asymmetric Waveform Ion Mobility Spectrometry – FAIMS Pro



Dr. Satendra Prasad
R&D

Dr. Michael Belford
R&D

Thermo Scientific™ FAIMS Pro™ Details

- FAIMS Pro hardware comprises the main control box (MCB), the RF coil box and electrode assembly mounted to a collar flange, and a bundled cable connecting the two.
- Hardware and software ease-of-use makes it simple to attach and use in <2 minutes
- Method templates help a customer to hit the ground running for DDA proteomics experiments
- No Instrument Configuration necessary, FAIMS Pro interface is recognized by the software when mounted and powered up

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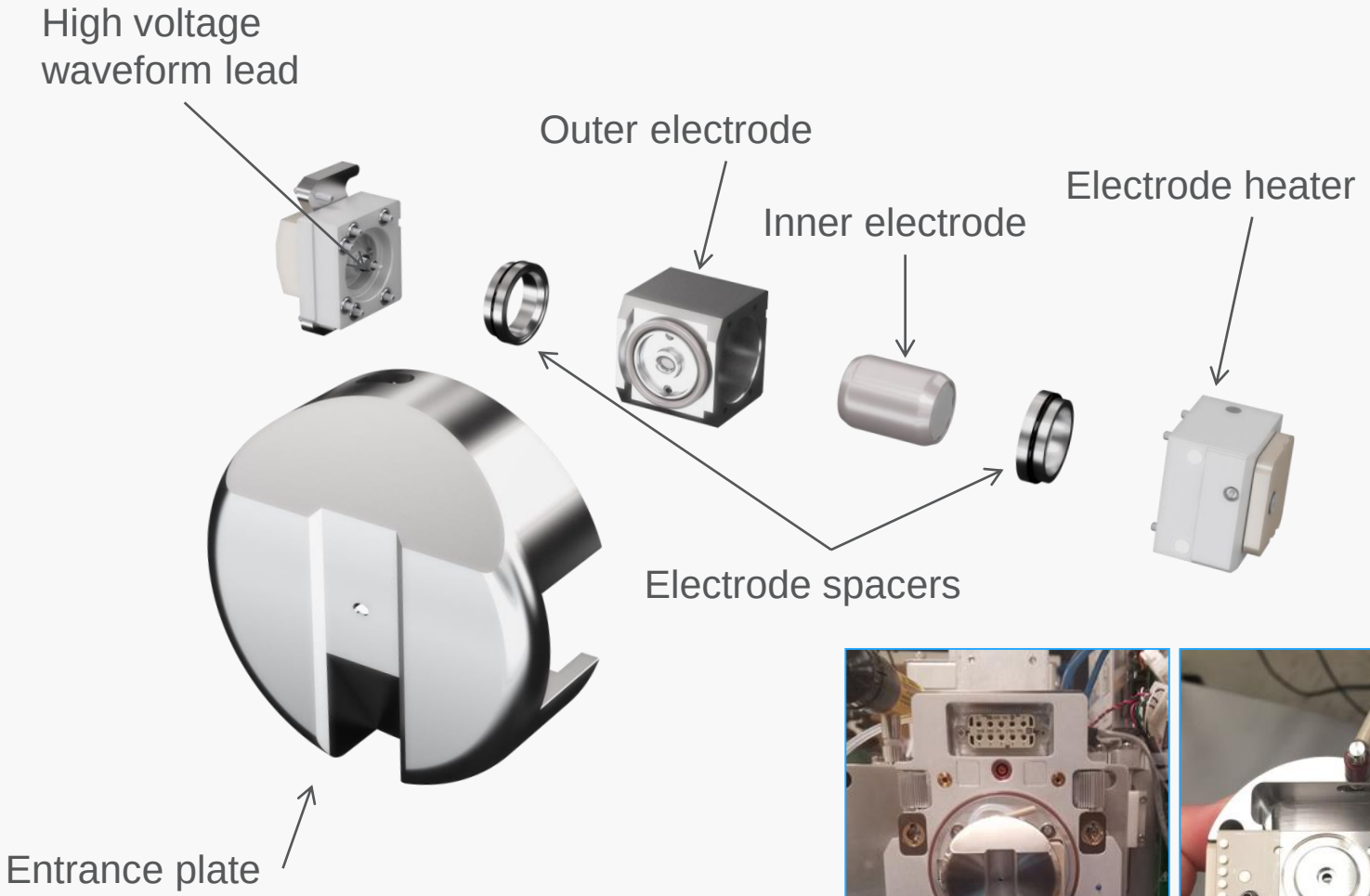
FAIMS Pro Hardware is Tool-Free and Easy to Maintain



www.thermofisher.com/faimspro

User-Friendly Implementation

- One-way, tool free assembly and disassembly
- No venting required for maintenance
- Inner electrode blocks line-of-sight, which improves MS robustness
- Maintenance is simple and electrodes can be cleaned by sonication, at needed frequency (≥ 1 week)
- The only parts requiring cleaning are stainless steel

FAIMS Pro CV Fractionation of Human K562 Cell Line Tryptic Digest

analytical chemistry

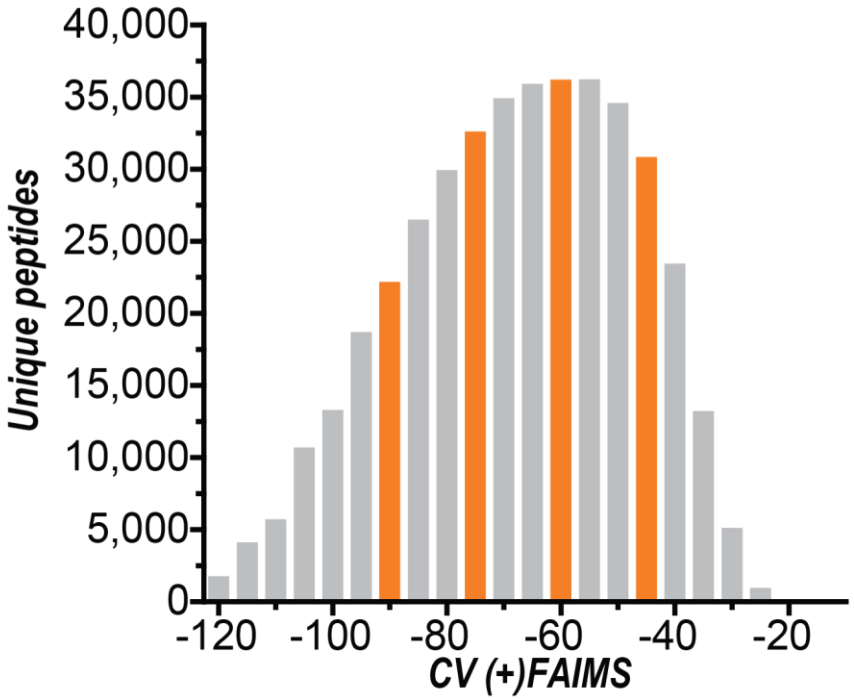
WISCONSIN
UNIVERSITY OF WISCONSIN–MADISON

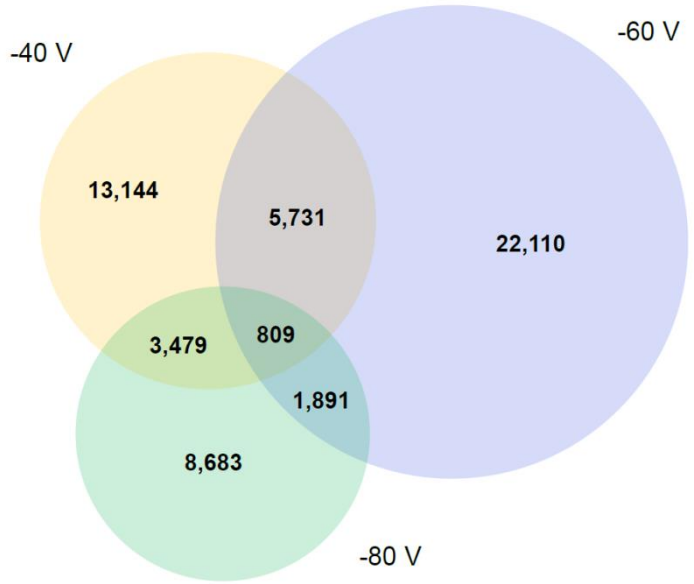
Comprehensive Single-Shot Proteomics with Liquid Chromatography–High-Field Asymmetric Waveform Ion Mobility Spectrometry–Tandem Mass Spectrometry

Alexander S. Hebert,[†] Satendra Prasad,[∥] Michael W. Belford,[∥] Derek J. Bailey,[∥] Graeme C. McAlister,[∥] Susan Abbatiello,[⊥] Romain Huguet,[∥] Eloy R. Wouters,[∥] Jean-Jacques Dunyach,[∥] Dain R. Brademan,[‡] Michael S. Westphall,[†] and Joshua J. Coon*[,†,‡,§,#]

Pr. Josh Coon

Dr. Alex Hebert





Unique Sequence Coverage

- LC-MS analysis of K562 Human cell line
- Individual runs at single CVs show a distribution of Unique Peptide identifications
- When select CVs are combined in 1 LC-MS/MS run, orthogonal selection results in increased peptide IDs (Here with HeLa)

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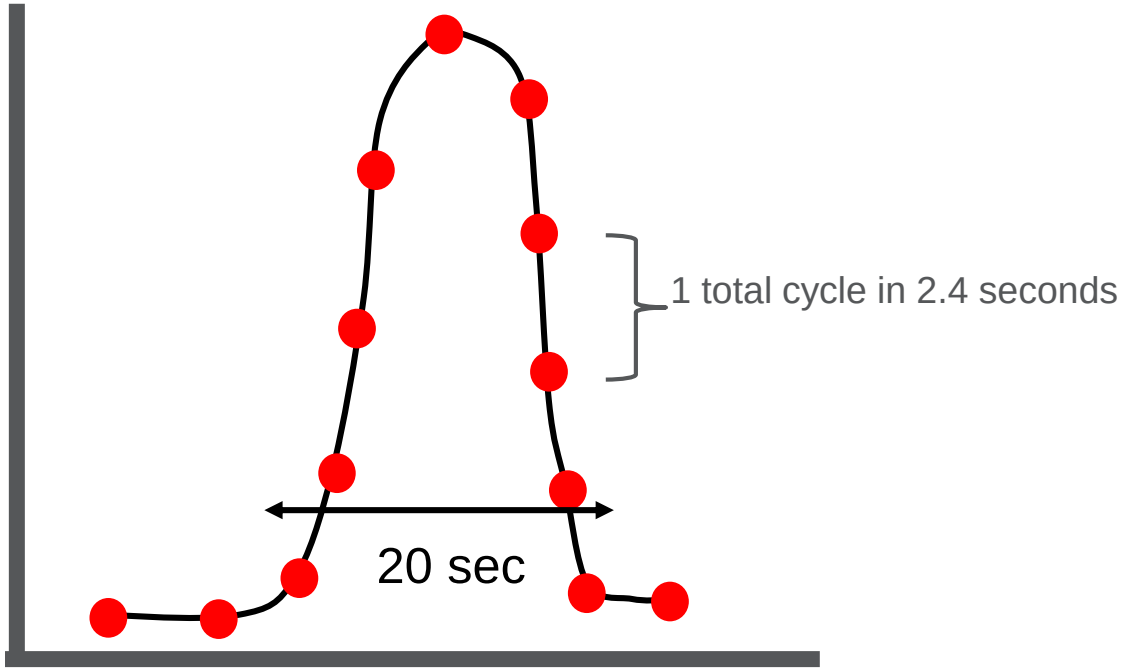
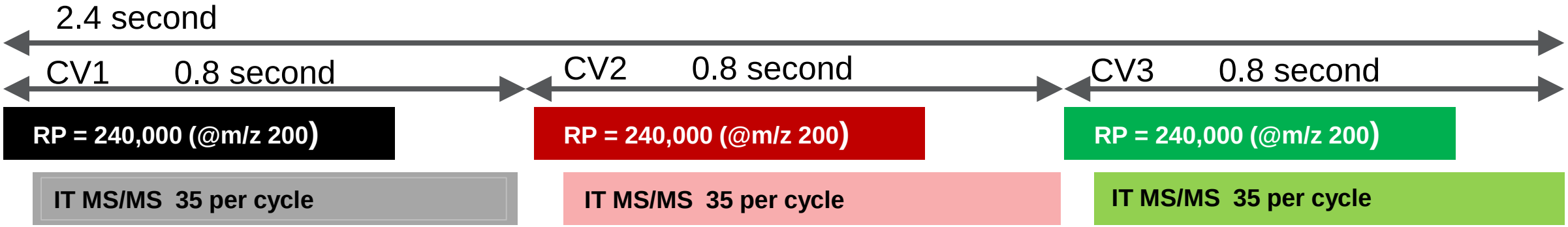
Applying FAIMS Pro Interface – Leveraging Parallel Data Acquisition





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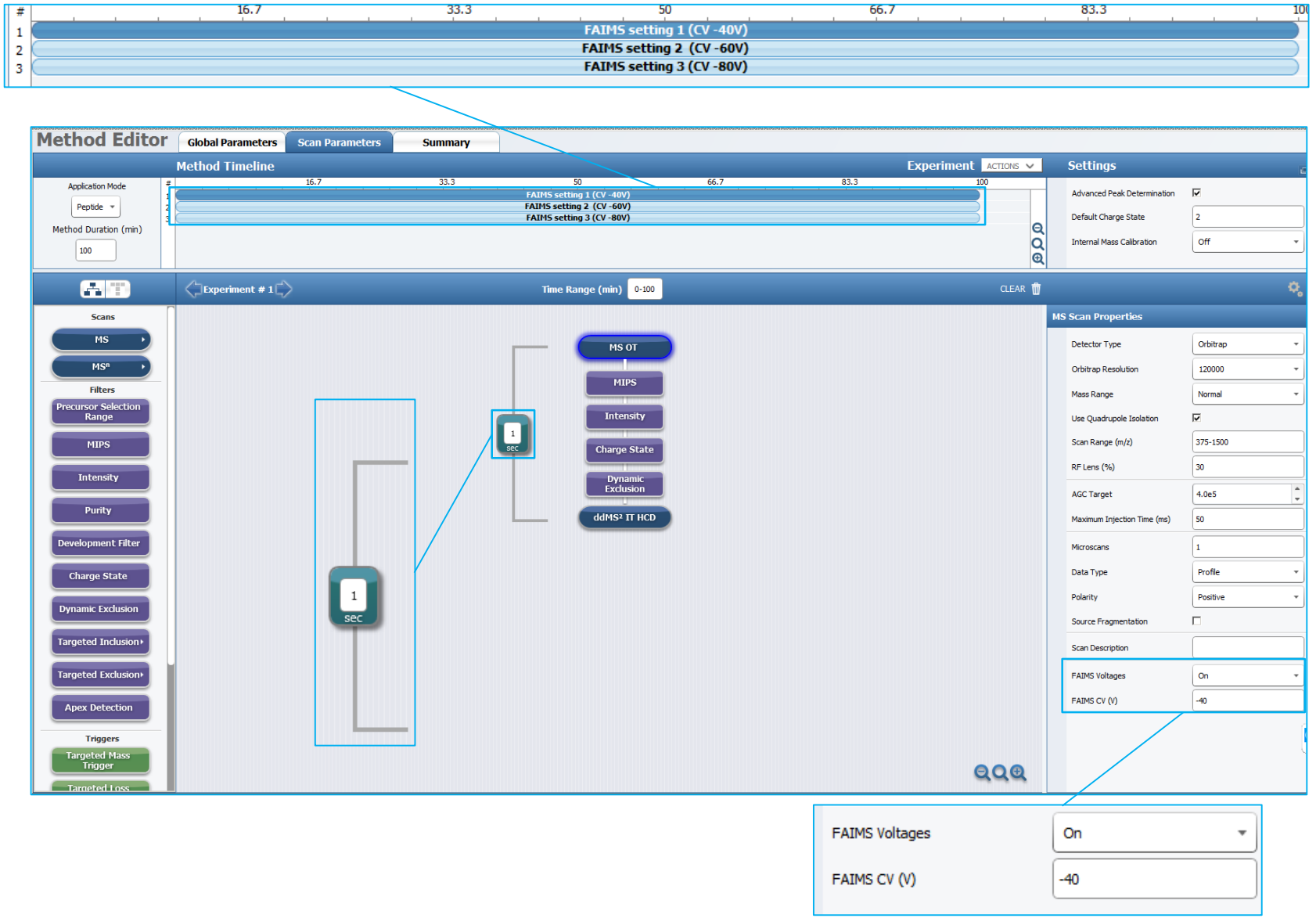
Maximizing Proteome Coverage on the Orbitrap Fusion Lumos Tribrid + FAIMS Pro



Full OTMS scan at CV1

DDA MS2 acquired at CV1

Full OTMS scan at CV2

DDA MS2 acquired at CV2, etc

- CVs are pre-selected based on tryptic peptide transmission
- Gas-phase separation at different FAIMS CVs sends different populations of ions to the MS, increasing number of precursors available for MS/MS
- The result is improved peptide and protein IDs in one DDA run compared to not using FAIMS Pro technology

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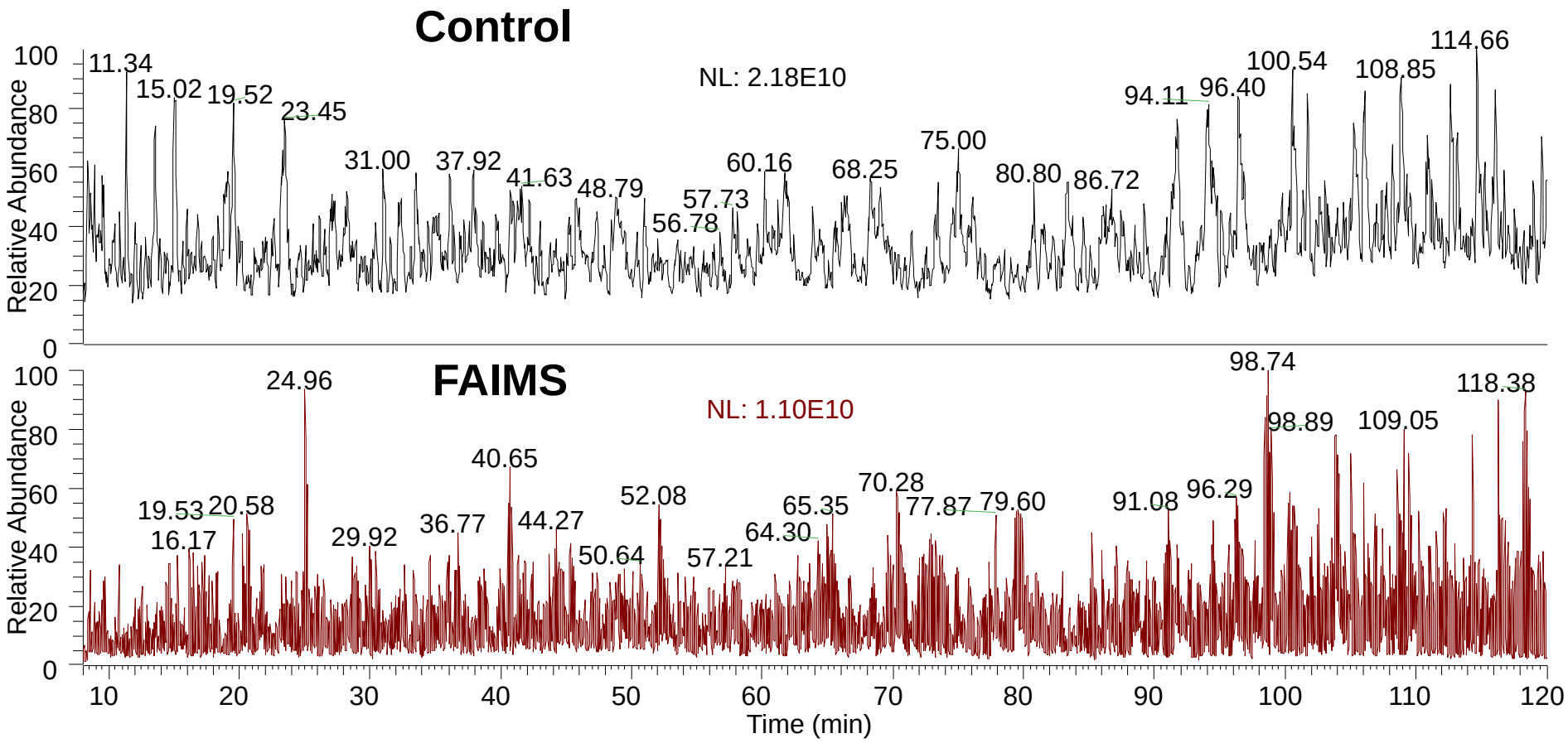
Evaluating the TIC Comparison



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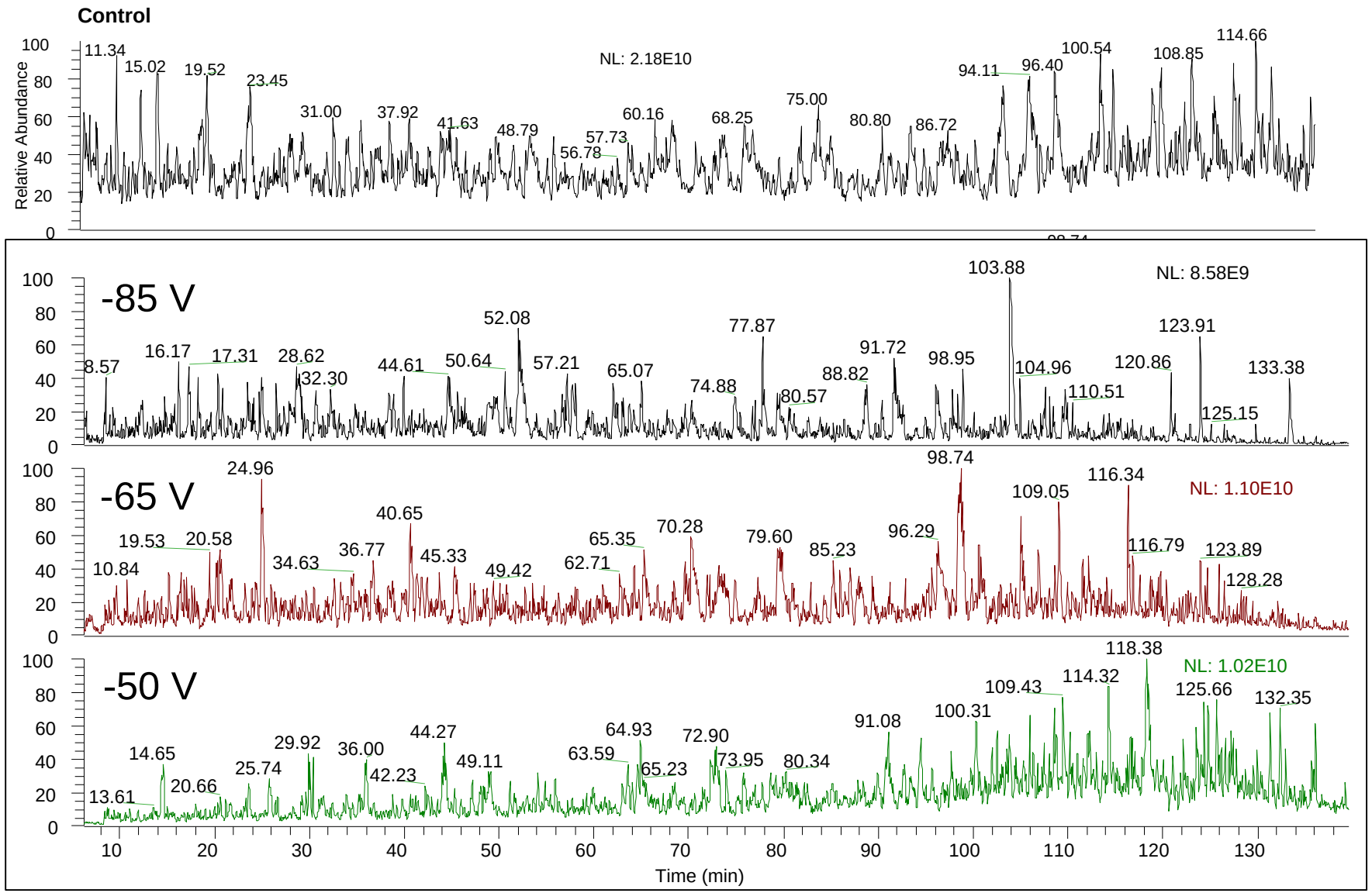
Extracting out the CV Fractions – 3 Experiments in 1 Injection

FAIMS



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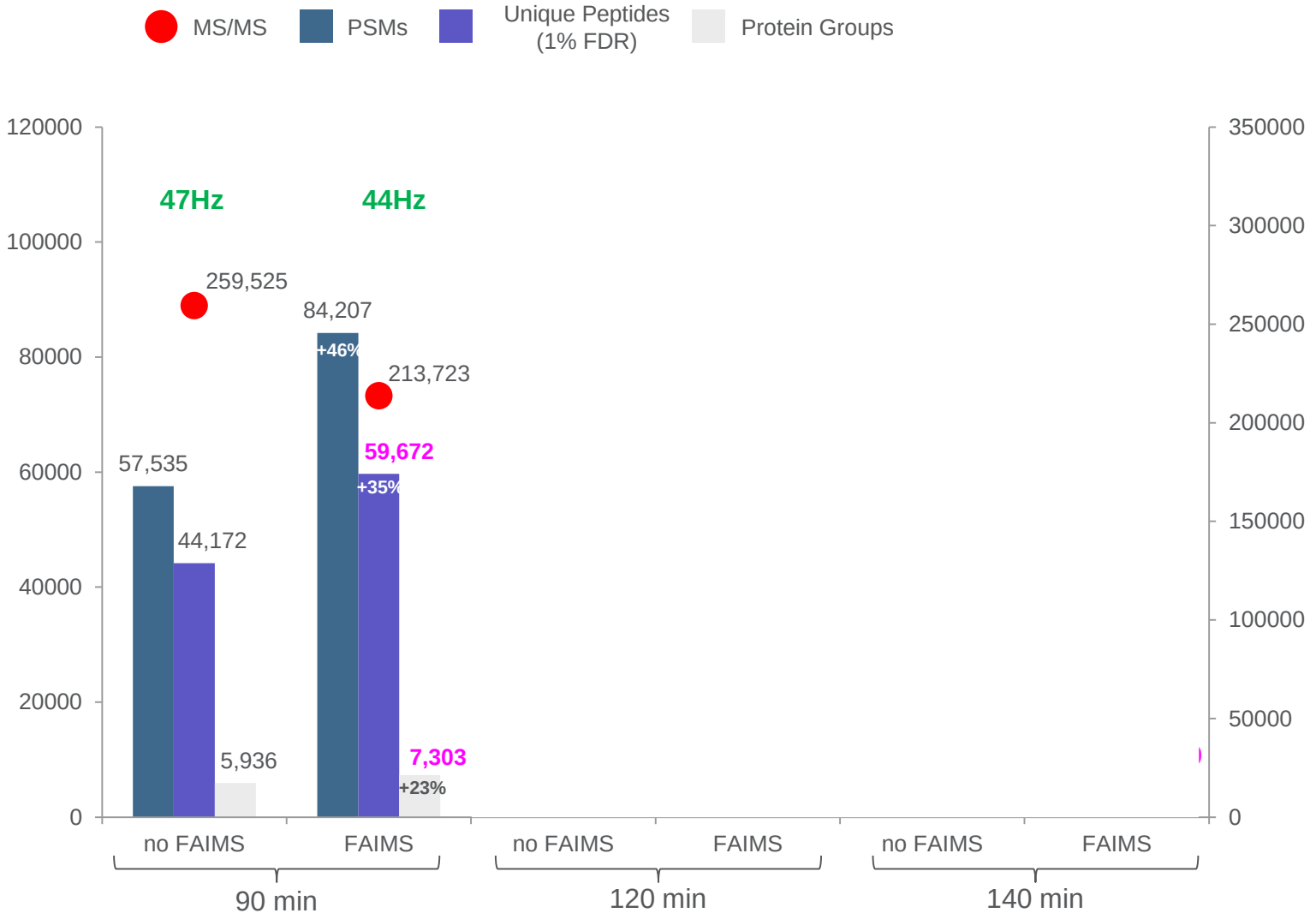
FAIMS Pro Technology Improves Peptide and Protein Coverage



- 1 ug of HeLa lysate digest analysis with 90, 120 and 140 min gradients, detected with OTIT and FAIMS Pro (-50/-65/-85V in every single run).
- Data were searched against the Uniprot human database using Sequest (3 standard modifications, acetylation, oxidation and carbamidomethylation) in Proteome Discoverer 2.3 SW with 1% FDR using Percolator.

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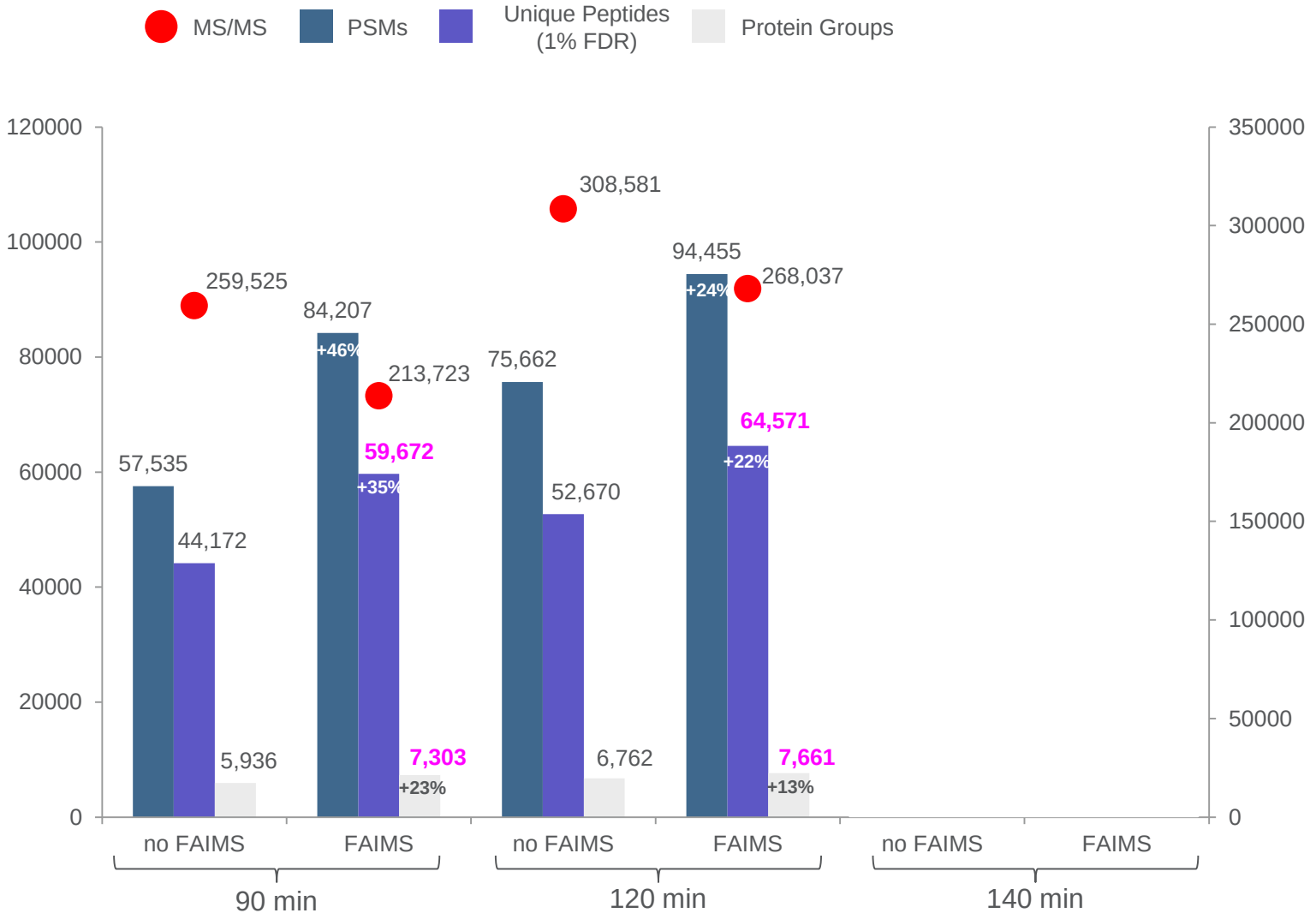

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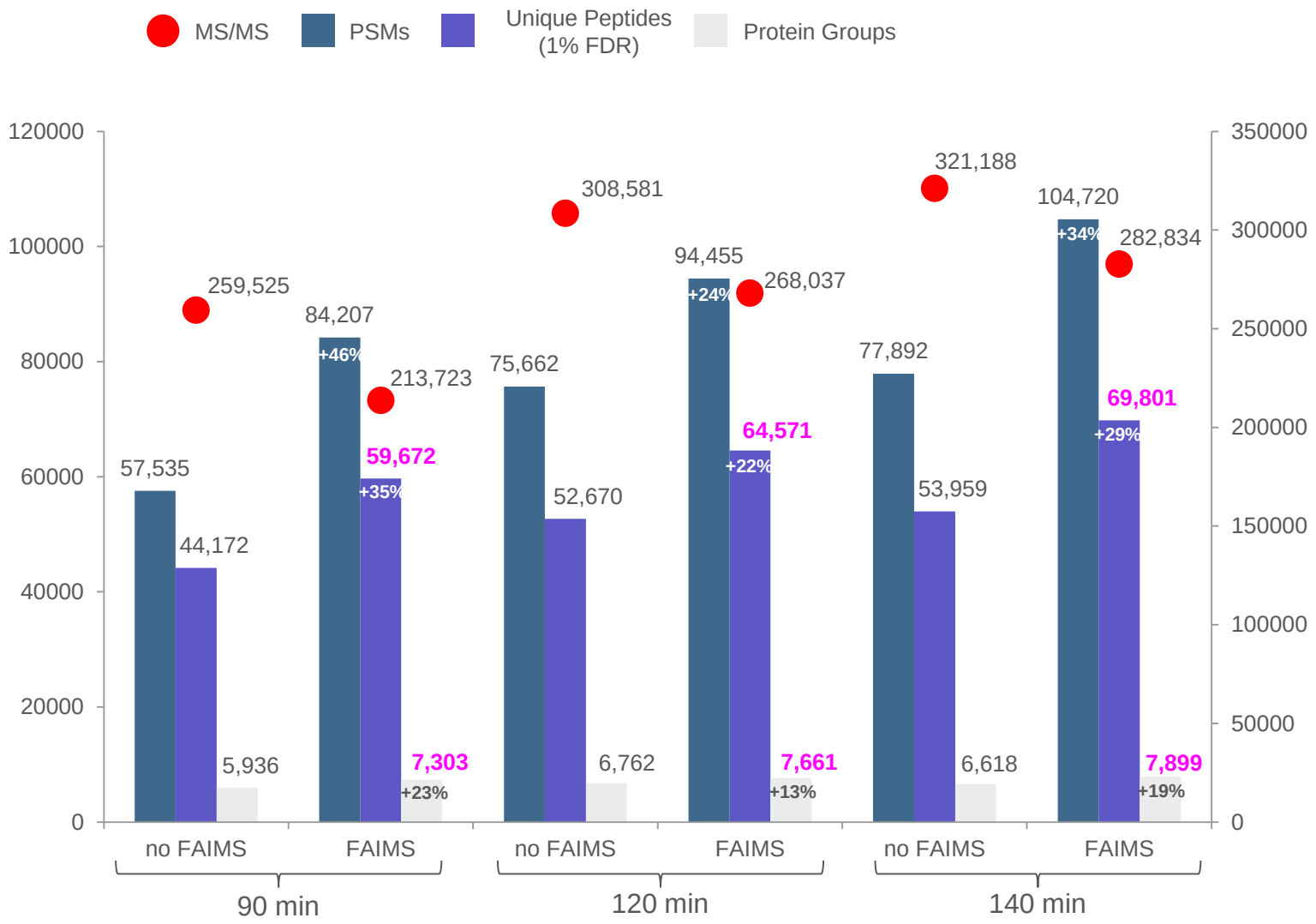

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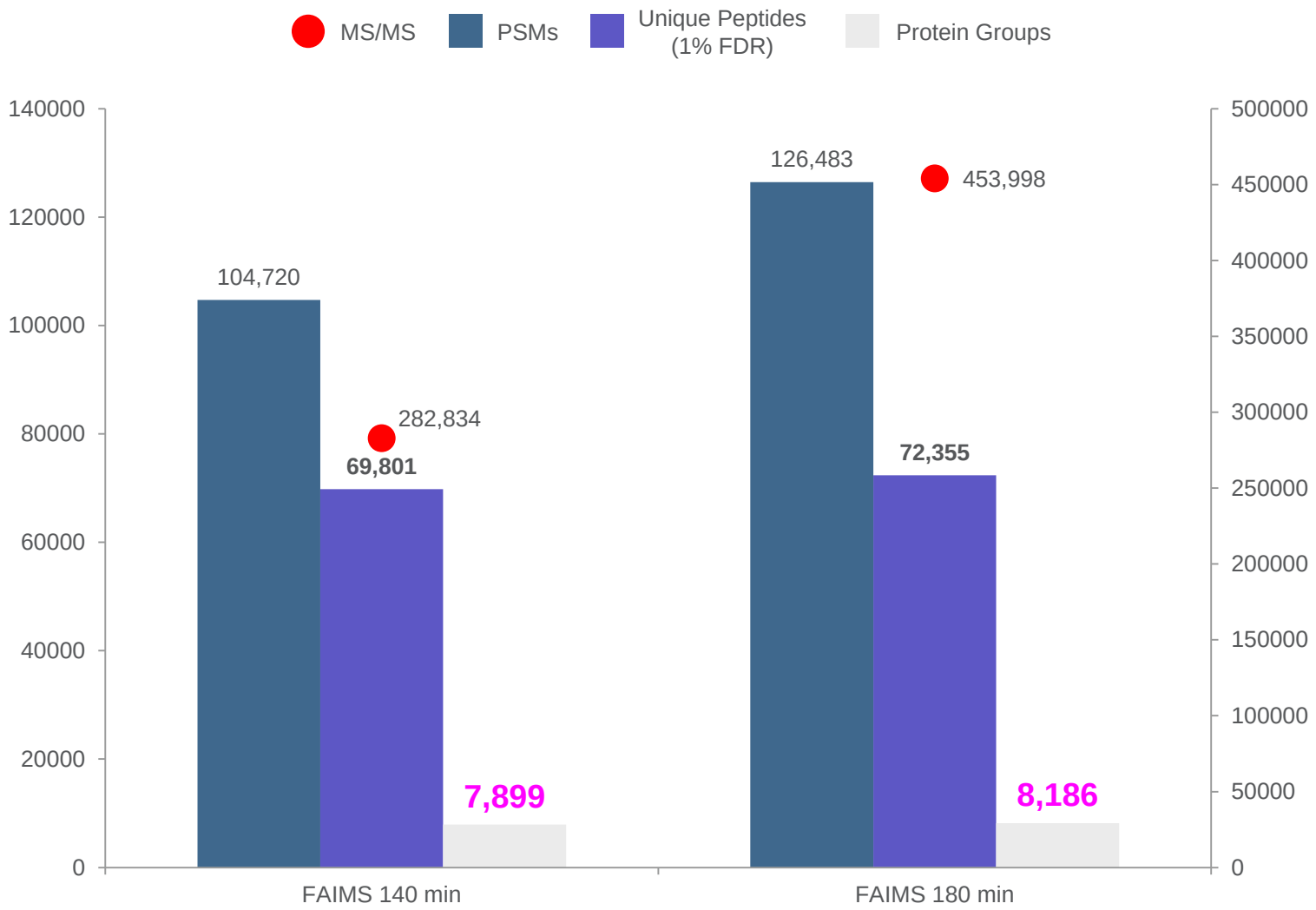
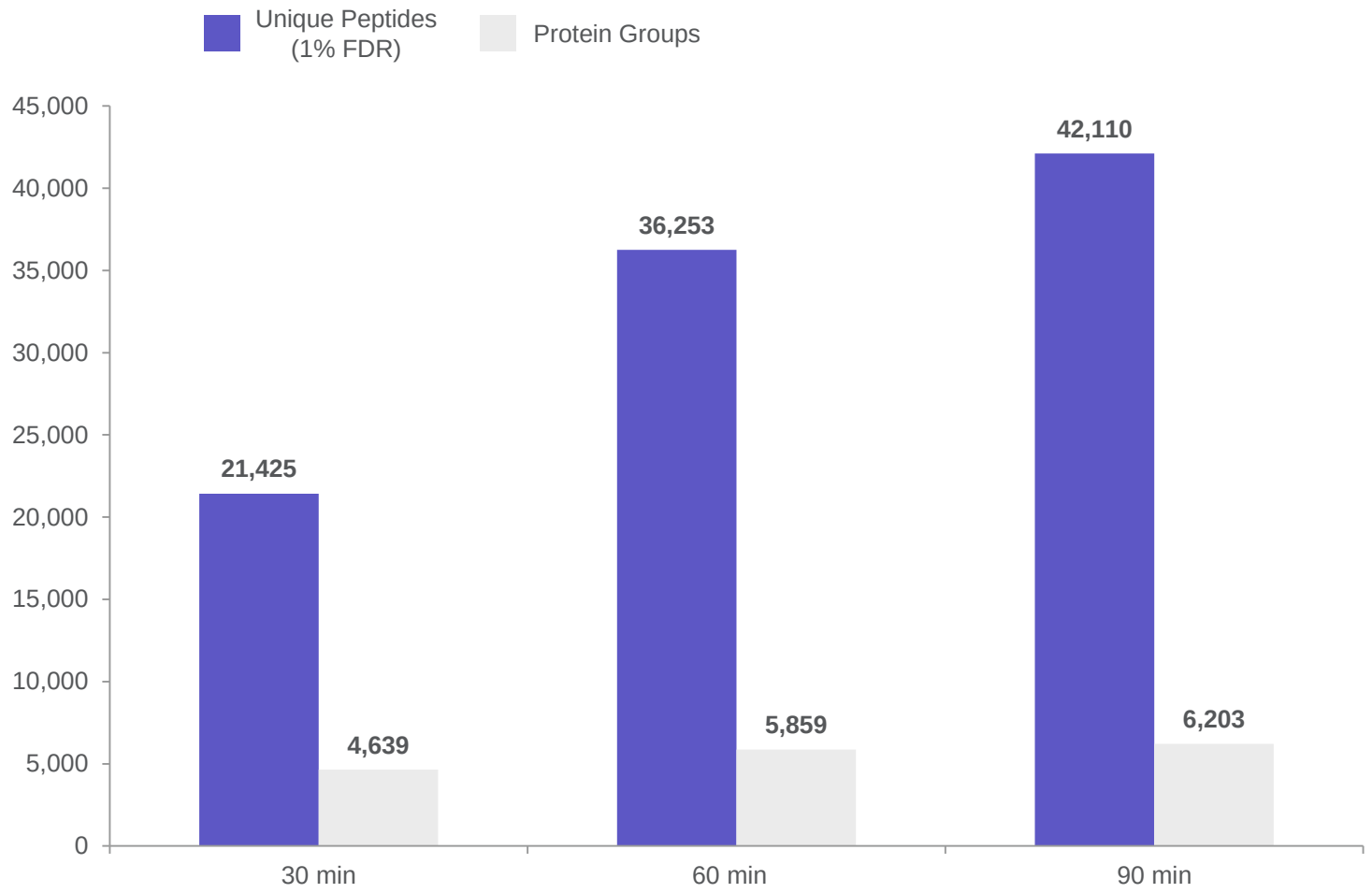


- 1 ug of HeLa lysate digest analysis with 90, 120 and 140 min gradients, detected with OTIT and FAIMS Pro (-50/-65/-85V in every single run).
- More optimization is needed for 180 min gradient
- Data were searched against the Uniprot human database using Sequest (3 standard modifications, acetylation, oxidation and carbamidomethylation) in Proteome Discoverer 2.3 SW with 1% FDR using Percolator.

FAIMS Pro Technology Improves Peptide and Protein Coverage



- **100 ng** of HeLa lysate digest analysis with 30, 60 and 90 min gradients, detected with OTIT and FAIMS Pro (-50/-65/-85V in every single run).
- Data were searched against the Uniprot human database using Sequest (3 standard modifications, acetylation, oxidation and carbamidomethylation) in Proteome Discoverer 2.3 SW with 1% FDR using Percolator.

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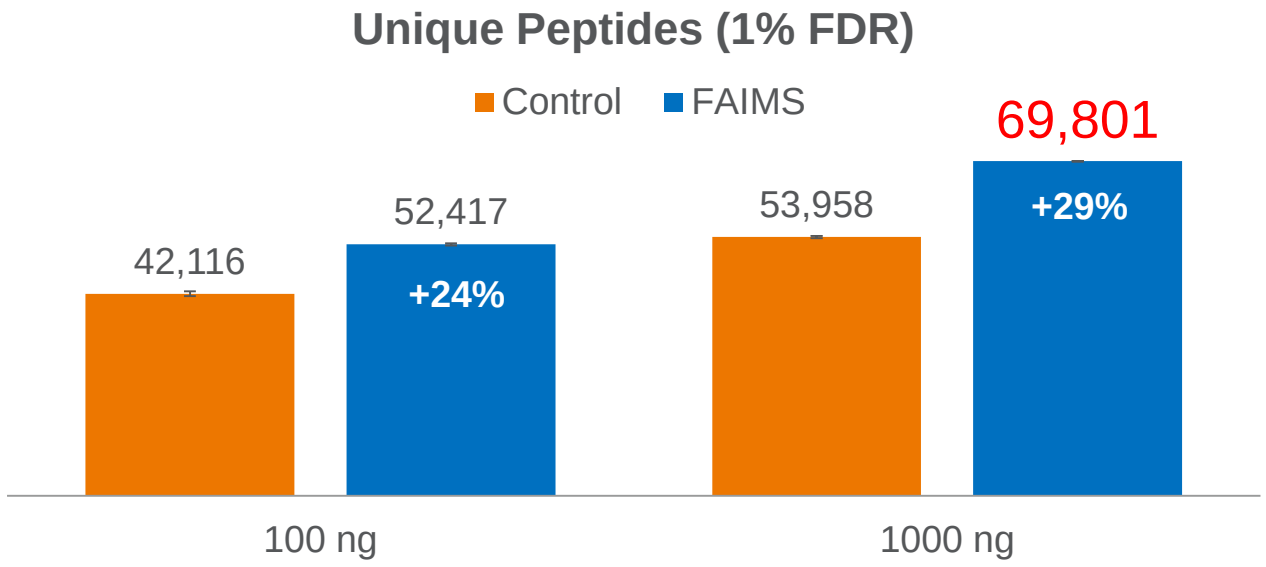
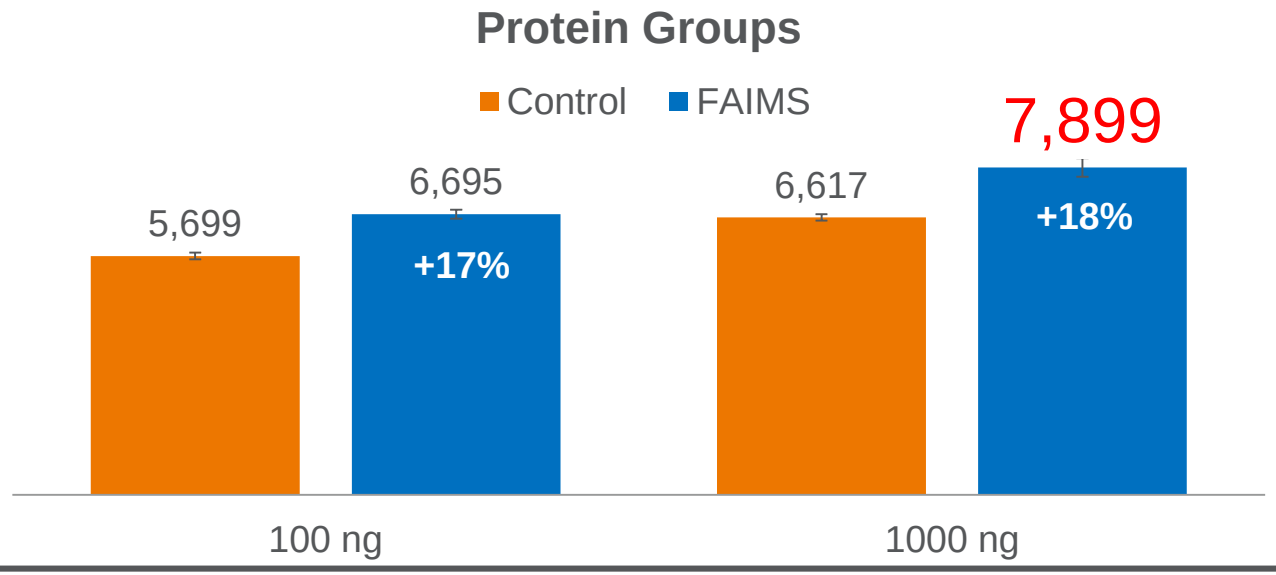
FAIMS Pro Technology Improves Peptide and Protein Coverage

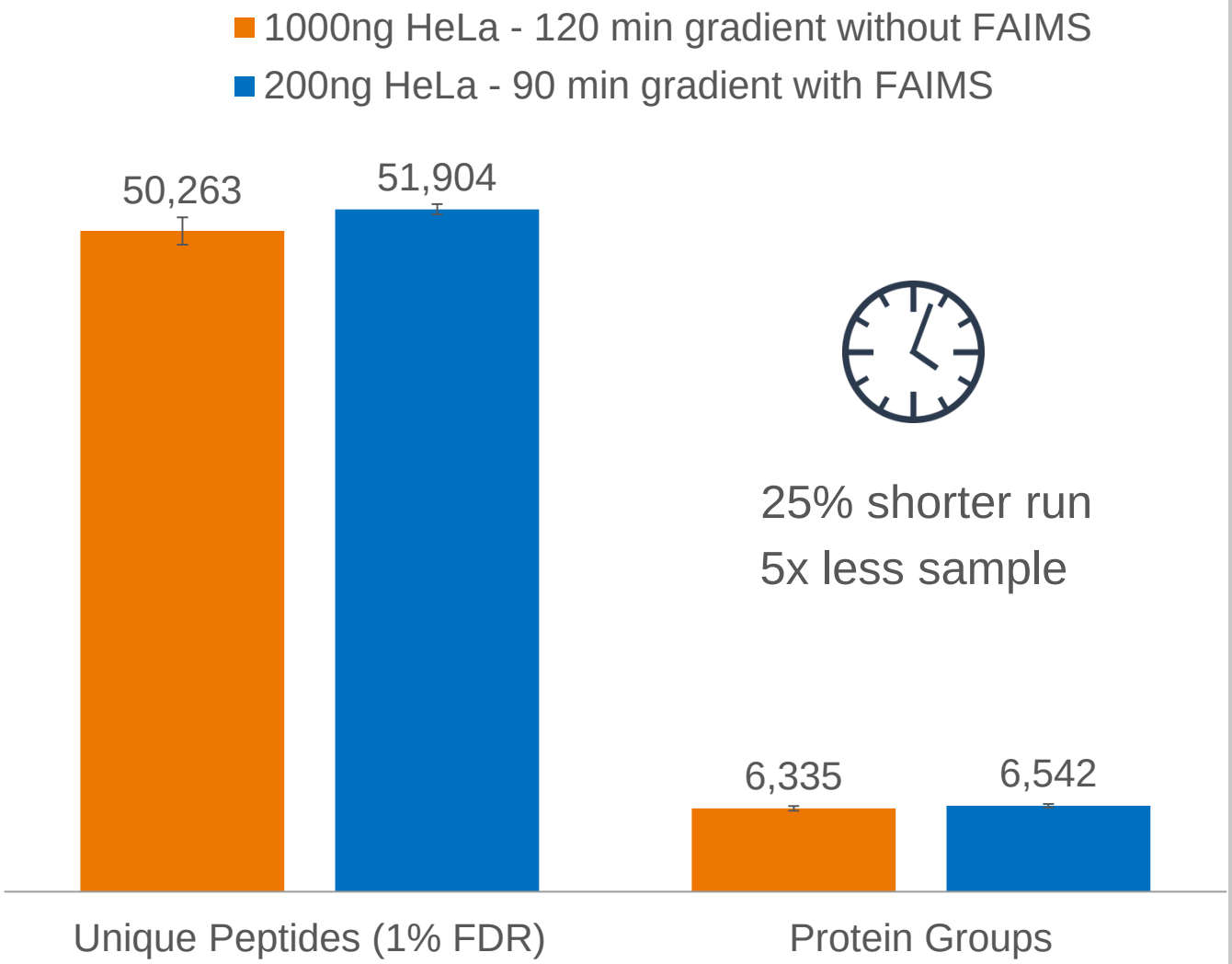




Improved Peptide Identifications

- HeLa lysate digest analysis with 140 min gradient, detected with OTIT and 3 FAIMS CVs in one run
- Peptide improvements exceeded 20% for both 100 ng and 1000 ng loads compared to no FAIMS
- Data were searched against the Uniprot human database using Sequest in Proteome Discoverer 2.3 SW with 1% FDR using Percolator
- Protein ID improvements with FAIMS Pro technology were nearly as high, approaching 8000 protein group IDs at the 1000 ng load

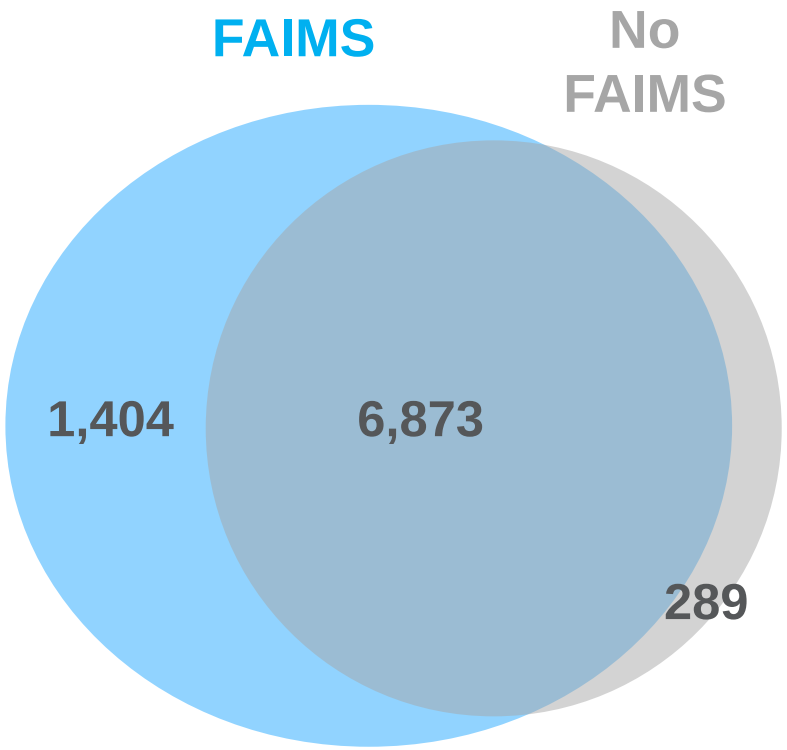
FAIMS provides same IDs with 5x less sample and shorter gradient



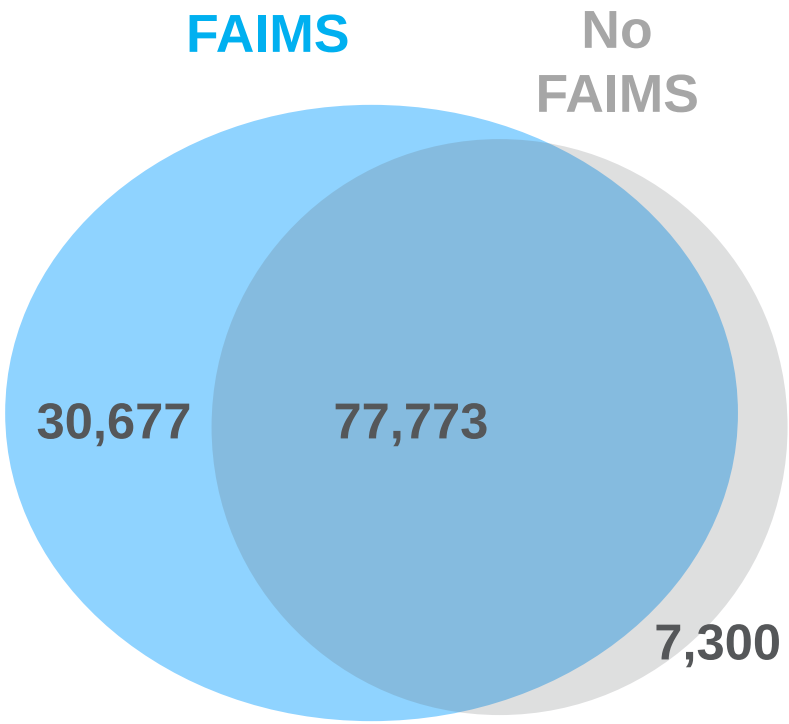
Increased Sensitivity and Throughput

- FAIMS can save sample throughput time by providing similar IDs in shorter chromatographic runs
- Loading only 200 ng and analyzing on a 90 min gradient with FAIMS achieves >1000 more peptide IDs and >200 protein IDs than a 1000 ng load and a 120 min gradient without FAIMS
- Over time, this could lead to savings in time and samples

FAIMS Pro Technology Improves Peptide and Protein Identifications



Protein groups (8,566)

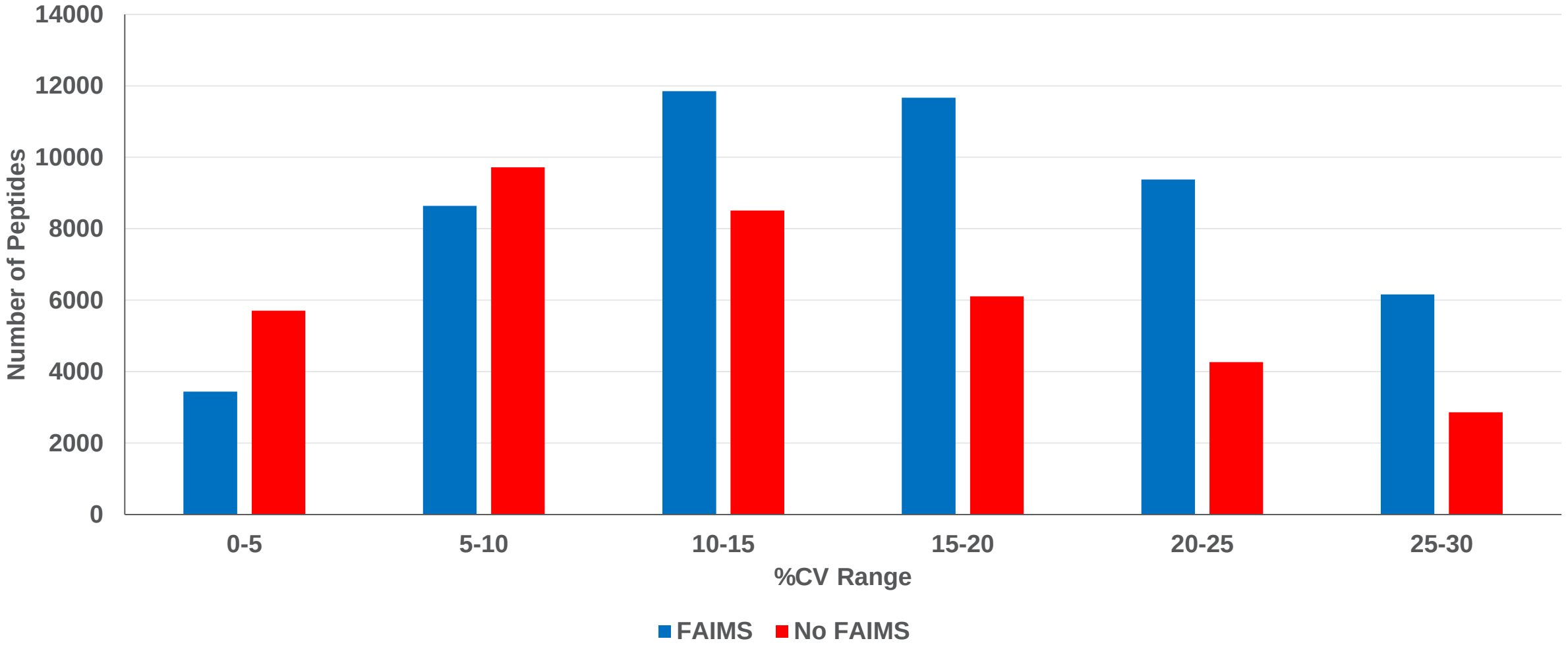


Unique Peptides (115,750 at 1% FDR)

Deeper Protein coverage

- 13 No FAIMS HeLa LC-MS runs and 13 FAIMS HeLa LC-MS runs were searched together, and the two global ID results were compared to identify unique peptides and protein groups that can only be observed with FAIMS.
- Over 25% of the unique peptides were identified only with FAIMS.
- Over 15% of protein groups were identified only with FAIMS, only 3% of proteins were identified without FAIMS
- Data was searched against the Uniprot human database using Sequest in Proteome Discoverer 2.3 SW with 1% FDR using Percolator.

Comparative Reproducibility as Defined by AUC Values per Peptide



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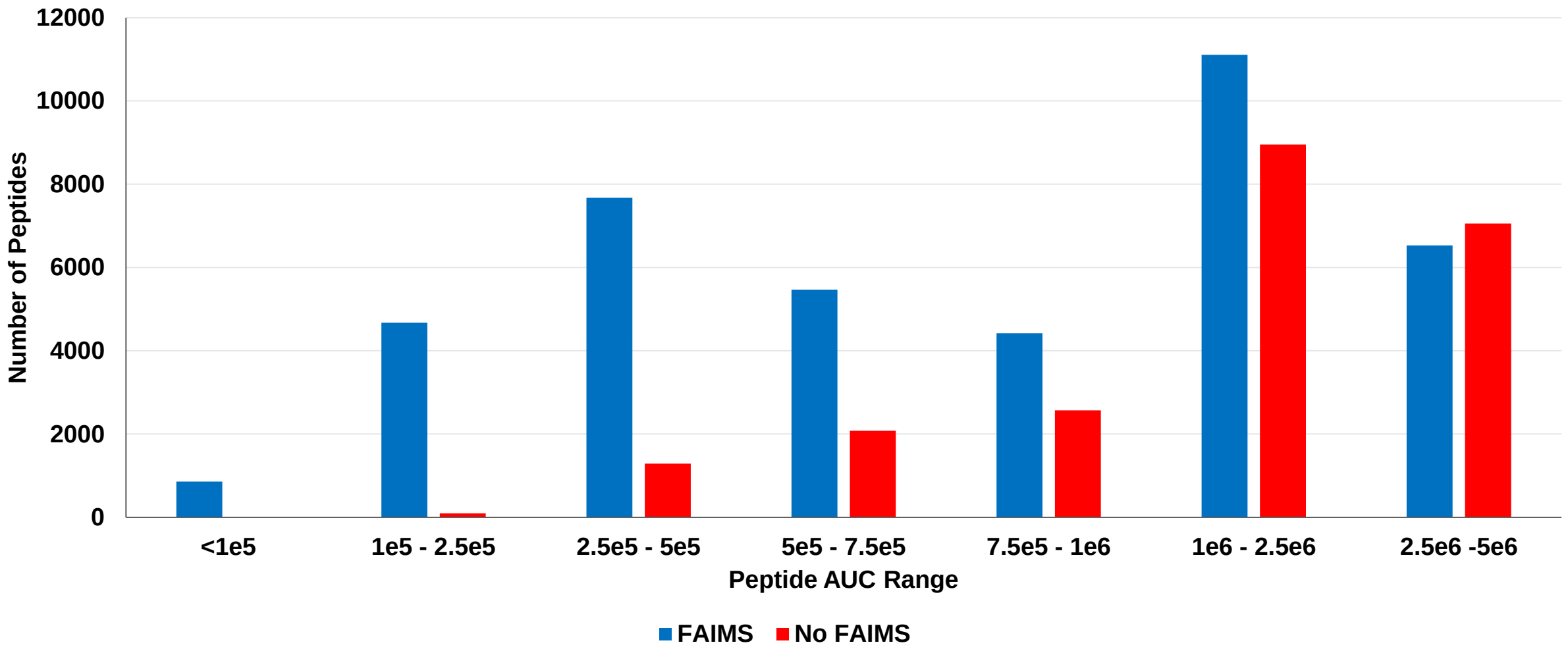
AUC Distribution Analysis – Peptides Measured with %CV ≤ 35%



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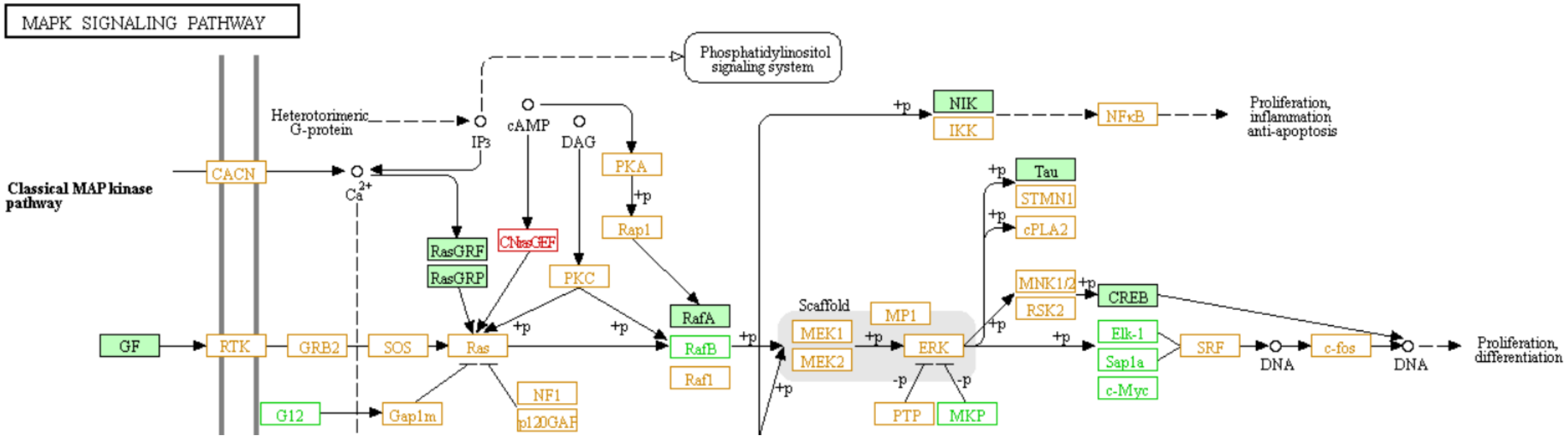
FAIMS Provides Higher Protein Coverage in Many Signaling Pathways

Pathway	# of proteins in No FAIMS	# of proteins in FAIMS	
Cytokine-cytokine receptor interaction	12	24	+100%
Phospholipase D signaling pathway	46	63	+73%
NF-kappa B signaling pathway	34	46	+74%
Jak-STAT signaling pathway	31	41	+76%
Lipoic acid metabolism	0	2	Only IDed with FAIMS
mTOR signaling pathway	76	90	+80%
ErbB signaling pathway	44	53	+83%

FAIMS unveils the MAPK Signaling Pathway

Unmatched protein detection

- Proteins shown in orange were identified in both No FAIMS and FAIMS LC-MS runs.
- Proteins in light green were identified only in the FAIMS LC-MS runs, in red , only in no FAIMS runs
- Proteins in black and green were not identified. FAIMS allows the identification of all the proteins involved in the MAPK Signaling Pathway.



Summary

- Tribrid architecture provides significant advantages for LFQ
- Quadrupole mass filter is extremely beneficial for OTIT acquisition schemes
- HRAM MS in OT is significantly better for routine XIC analysis
- FAIMS Pro can increase the proteome coverage while maintaining reproducible measurements

Acknowledgements

Michael Belford
Satendra Prasad
Ann Yadlowsky
Ryan Hermezian
Hoa Tran
Derek Bailey
David Minkler
Susan Abbatiello
Romain Huguet
Ray Chen
John MacAskill
Qingyu Song
Qiming Wan
Bennet Kalafut
Rae Ana Snyder
Zijuan Lai
Cornelia Boeser
Wei Wei
Jean-Jacques Dunyach
Mary Blackburn
Iain Mylchreest
August Specht
Vlad Zabrouskov
Eloy Wouters

Andreas Huhmer
Aaron Robitalle
Rajesh Raina
Daniel Ferrer-Lopez
Josh Silviera
Josh Nicklay
Fred Ayres
Ed Gonzalez
David Horn
Bernard Delanghe
Torsten Ueckert
Christoph Henrich
Carmen Paschke
Graeme McAlister
Scott Peterman
Stefan Houel
David Sarracino
Beth Anderson
Kien Le
Fishaye Gebrehiwet
Thomas Moehring
Ken Miller

Harvard Medical School
Steve Gygi
Devin K Schweppe

University of Wisconsin
Josh Coon
Alex Hebert
Michael Westphall

Université de Montreal
Pierre Thibault
Eric Bonneil
Sibylle Pfammater

University of Birmingham, UK
Helen Cooper
Rian Griffiths

Northwestern University
Neil Kelleher
R. Vince Gerbasi

The Broad Institute
Steve Carr
Hasmik Keshishian

University of Washington
Mike MacCoss
Brendan MacLean
Lindsay Pino