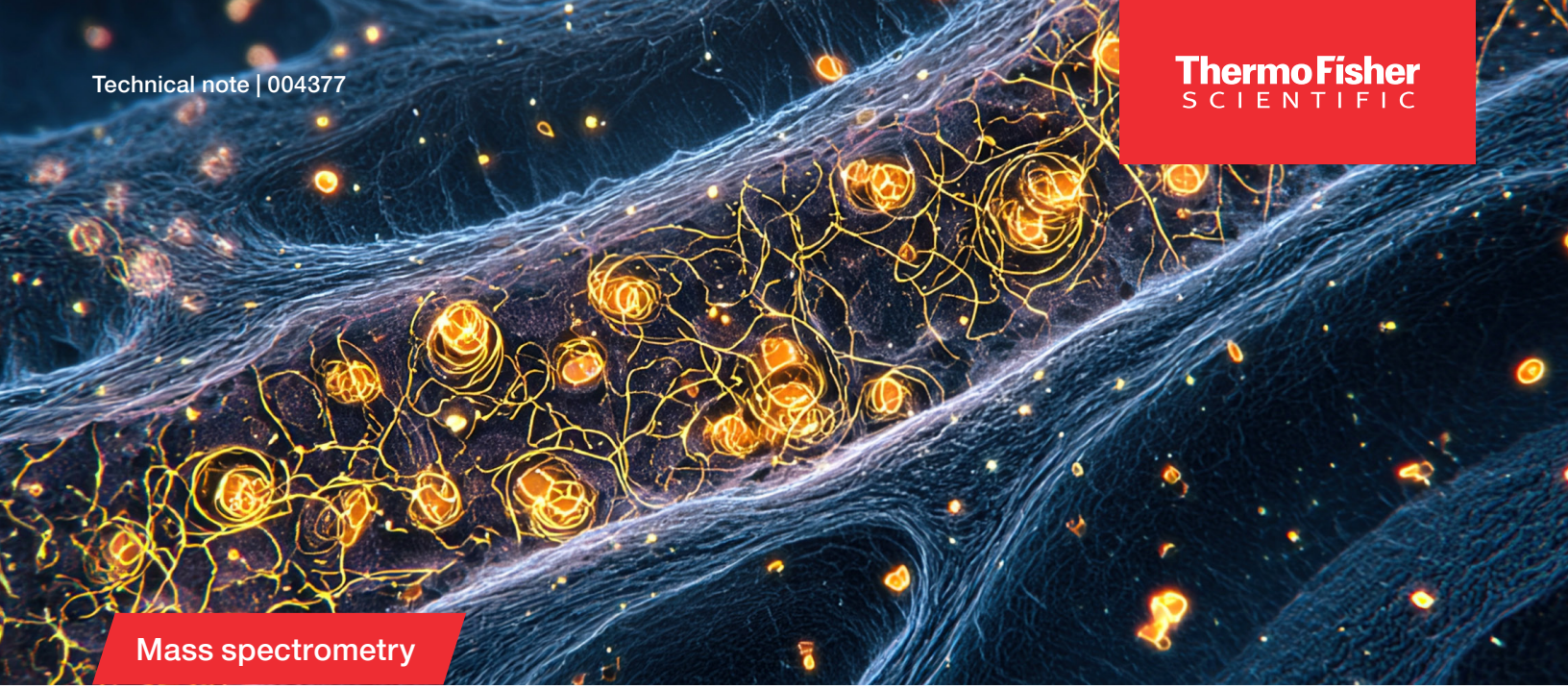

Technical note | 004377

Mass spectrometry

Advancing phosphorylation, ADMA, and O-GlcNAc PTM analysis with HCD and EThcD on the Orbitrap Excedion Pro mass spectrometer

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Keywords

Post-translational modifications, PTMs, glycosylation, phosphorylation, O-linked glycosylation, phosphopeptide, glycopeptide, asymmetric dimethylarginine, ADMA, EThcD, Orbitrap Excedion Pro, phosphotyrosine

Goal

Demonstrate fast and sensitive performance of the Thermo Scientific™ Orbitrap™ Excedion™ Pro hybrid mass spectrometer using higher-energy collisional dissociation (HCD) and electron transfer dissociation with supplemental higher-energy collisional (EThcD) for analyzing post-translationally modified peptides.

Introduction

There is increasing recognition of the critical role that protein post-translational modifications (PTMs) play in regulating protein structure, function, stability, and interactions. PTMs are essential for modulating a wide range of cellular processes, including signal transduction, cell growth and differentiation, and responses to environmental stimuli. Phosphorylation, for example, is a central regulator of cellular signaling networks, where the addition of a phosphate group can alter protein function, structure, localization, and stability. Other PTMs, such as asymmetric dimethylarginine (ADMA), can significantly influence protein activity and have been implicated in endothelial dysfunction as well as various cardiovascular and renal diseases. O-linked glycosylation also plays an important role in modulating protein–protein interactions, trafficking, and cellular recognition processes, but its structural diversity and lability make it one of the most analytically challenging PTMs to study. Collectively, such modifications contribute to the complexity of cellular regulation, and

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aberrant PTM patterns have been linked to numerous diseases, highlighting the importance of accurately identifying and characterizing these modifications in complex biological systems.

However, the detection and analysis of PTMs present significant analytical challenges. Many PTMs occur at low stoichiometry, are transient in nature, or are distributed across multiple potential modification sites, often within specific subcellular locations. These characteristics demand highly sensitive and precise analytical techniques capable of distinguishing modified from unmodified peptide species and accurately localizing modification sites.

Mass spectrometry (MS) has emerged as the gold standard for comprehensive proteome and PTM analysis due to its high sensitivity, mass accuracy, and ability to detect a wide dynamic range of peptide abundances. Among the various MS fragmentation techniques, electron transfer dissociation (ETD) has proven particularly powerful for PTM analysis, as it preserves labile modifications and minimizes neutral losses during peptide fragmentation, resulting in confident site localization. Furthermore, the addition of supplemental collisional energy to ETD—known as EThcD—enables secondary fragmentation of ETD product ions, thereby increasing sequence coverage and enhancing localization confidence of the modified residues.

In this study, we demonstrate the improved detection and characterization of PTM-enriched samples using HCD and EThcD fragmentation on the Orbitrap Excedion Pro mass spectrometer. The results highlight the benefits of employing multiple fragmentation methods to enhance spectral quality, expand PTM site coverage, and enable deeper insights into complex PTM landscapes across phosphorylation, ADMA, and O-glycosylation.

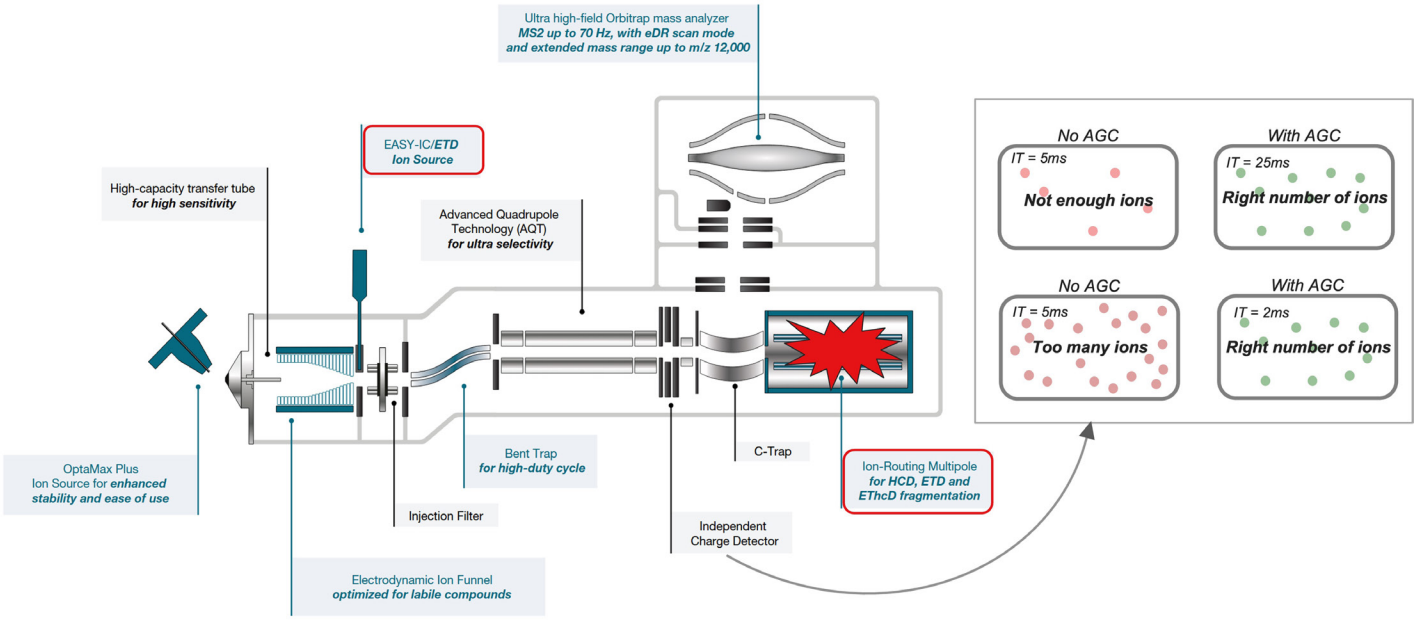

Figure 1. The Orbitrap Excedion Pro mass spectrometer with fast and sensitive HCD and EThcD fragmentation for analysis of PTM peptides and easy-to-use turnkey methods. Both HCD and EThcD fragmentation parameters are automatically adjusted for each peptide, leading to confident site assignments. Automatic Gain Control (AGC) uses the independent charge detector to dynamically control the ion charge density for each spectrum and plays an important role ensuring the best quality data for every single peptide, enabling confident detection of hundreds and thousands of PTMs.

Experimental

Recommended consumables

- Fisher Chemical™ Optima™ LC-MS Grade Water with 0.1% Formic Acid (FA) (v/v) (Part No. LS118-500)
- Fisher Chemical™ Optima™ LC-MS 80% Acetonitrile (ACN), 20% Water with 0.1% Formic Acid (Part No. LS122500)
- Fisher Chemical™ Optima™ LC-MS Grade Formic Acid, 99.0+% (Part No. A117-50)

Sample enrichment

- Cell Signaling Technology™ PTMScan™ HS Phospho-Tyrosine (P-Tyr-1000) Kit (Part No. 38572)
- Cell Signaling Technology™ PTMScan™ Phospho-Enrichment IMAC Fe-NTA Magnetic Beads (Part No. 20432)
- Cell Signaling Technology™ PTMScan™ O-GlcNAc [GlcNAc-S/T] Motif Kit (Part No. 95220)
- Cell Signaling Technology™ PTMScan™ Asymmetric D-Methyl Arginine Motif [adme-R] Kit (Part No. 13474)

LC columns

- Ion Opticks Aurora™ Ultimate™ 25×75 XT C18 UHPLC Column (Part No. AUR3-25075C18-XT)

Instrumentation

- Thermo Scientific™ Vanquish™ Neo UHPLC System (Part No. VN-S10-A-01)
- Orbitrap Excedion Pro hybrid mass spectrometer (Part No. BRE725572)
- Thermo Scientific™ EASY-Spray™ Source (Part No. ES081)

Data analysis software

- Thermo Scientific™ Proteome Discoverer™ Software, version 3.2
- Protein Metrics™ Byonic™ Software, version 5.2.5
- MSFragger via FragPipe, version 22.0
- O-Pair via MetaMorpheous, version 1.1.4

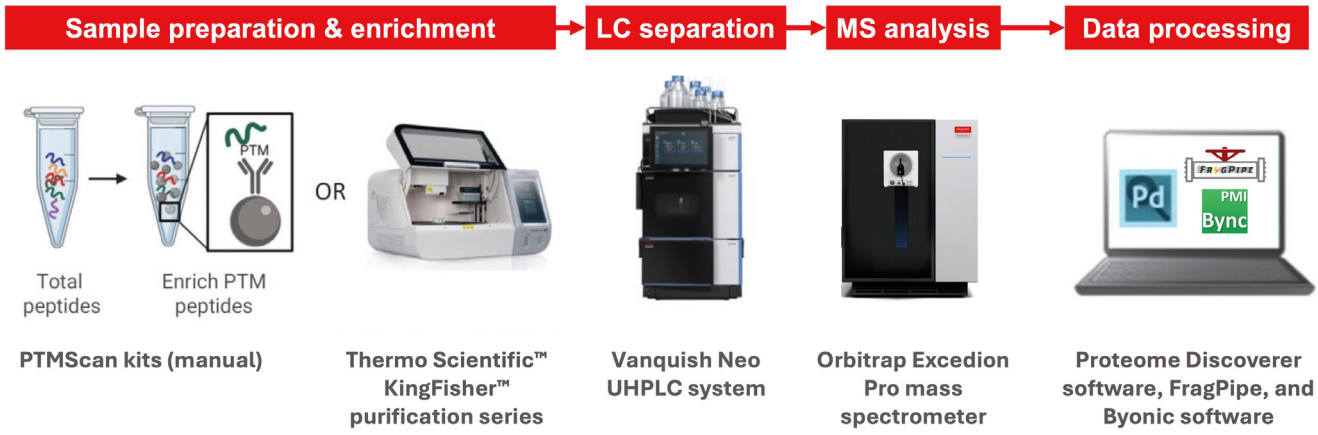

Figure 2. Analysis workflow: PTMScan enrichment of peptide samples, LC-MS analysis and PTM search analysis.

PTM enrichment

All enrichments were performed in collaboration with Cell Signaling Technology. Mouse liver or mouse brain samples were homogenized and protein extracts digested with Trypsin or LysC proteases. PTMScan HS kits were used to enrich ADMA or O-GlcNAc modified peptides. Human colorectal carcinoma cell line (HCT 116) and human gastric adenocarcinoma cell line (MKN-45) were lysed, and protein extracts digested with Trypsin. Phosphorylated peptides were enriched globally using IMAC and with a phospho-tyrosine PTMScan HS kits.

LC-MS analysis

Samples were analyzed by data-dependent acquisition (DDA) using a Vanquish Neo UHPLC system coupled to an Orbitrap Excedion Pro mass spectrometer with either HCD or EThcD to fragment precursor peptides in an automated manner. Peptides were separated on the Vanquish Neo UHPLC system using an Aurora Ultimate 25×75 XT C18 UHPLC column, and chromatographic gradients were formed using 0.1% formic acid in water for mobile phase A and 0.1% formic acid in 80% acetonitrile for mobile phase B. Liquid chromatography parameters and gradient settings can be found in Table 1. Mass spectrometer source parameters are available in Table 2, and scan parameters can be found in Tables 3–6. Default settings were used for the instrument to automatically select optimal fragmentation settings for each peptide during DDA acquisition.

Table 1. HPLC parameters.

High-throughput HPLC parameters					
	Time (min)	% Mobile phase B			Flow (µL/min)
		Phospho-tyrosine	IMAC	O-GlcNAc & ADMA	
Gradient	0.0	1	1	4	0.25 (0.85 O-GlcNAc & ADMA)
	0.5	8	8	8	0.25
	40.5	35	27	27	0.25
	43.0	50	50	25	0.25
	45.5	99	99	99	0.25
	48	99	99	99	0.25
LC parameters	LC configuration	Direct inject			
	Fast loading/Equilibration mode	Pressure control			
	Loading/Equilibration	1,400 bar			
	Equilibration factor	2			
	Sampler temperature	7 °C			
	Mobile phase A/Weak wash	0.1% formic acid in water			
	Mobile phase B/Strong wash	0.1% formic acid in 80% acetonitrile			
	Analytical column cartridge temperature	50 °C			
Column specifications	Analytical column	Aurora Ultimate 25×75 XT C18 UHPLC column			

Table 2. Orbitrap Excedion Pro MS global source and mass spectrometer parameters.

Global parameters (source & MS)	
Positive ion voltage	1,900 Volts
Ion transfer tube temperature	290 °C
Gas mode	Static
Expected peak width	10 seconds
Advanced peak determination	True
Pre-accumulation for MS^2 scans	True
Default charge state	2
Lock mass correction	EASY-IC™ (RunStart)

Table 3. Orbitrap Excedion Pro MS MKN45/HCT116 phosphotyrosine DDA experiment parameters.

MS^1 full scan parameters		
	HCD method	**EThcD method**
Orbitrap resolution	60K	60K
Scan range (*m/z*)	375–1,500	375–1,500
RF lens (%)	50	50
AGC target	200% (2.00e6)	100% (1.00e6)
Maximum injection time	120	Auto
Microscans	1	1
Filters		
	HCD method	**EThcD method**
MIPS	Peptide	Peptide
	Relax restrictions: False	Relax restrictions: False
Dynamic exclusion	30s, mass tolerance +/– 10 ppm	20s, mass tolerance +/– 10 ppm
	Exclude isotopes: True	Exclude isotopes: True
	Dependent scan on single charge start only: True	Dependent scan on single charge start only: True
Charge state	2–6	2–6
	Include undetermined charge states: False	Include undetermined charge states: False
MS^2 DDA scan parameters		
	HCD method	**EThcD method**
Data dependent mode	Number of scans, 40	60K
Orbitrap resolution	15K	15K
Scan range	Define first mass, 120 *m/z*	Define first mass, 120 *m/z*
Isolation window (*m/z*)	1.6	0.8
AGC target	Standard	50% (5.00e4)
Maximum injection time	Auto	27
Activation type	HCD	ETD
HCD collision energy (%)	28	–
Use calibrated charge-dependent ETD parameters	–	True
Supplemental activation energy (%)	–	30

Table 4. Orbitrap Excedion Pro MS HTC116 IMAC DDA experiment parameters.

MS¹ full scan parameters		
	HCD method	EThcD method
Orbitrap resolution	60K	60K
Scan range (*m/z*)	375–1,500	375–1,450
RF lens (%)	50	50
AGC target	200% (2.00e6)	100% (1.00e6)
Maximum injection time	120	Auto
Microscans	1	1
Filters		
	HCD method	EThcD method
MIPS	Peptide	Peptide
	Relax restrictions: False	Relax restrictions: False
Dynamic exclusion	30s, mass tolerance +/– 10 ppm	30s, mass tolerance +/– 10 ppm
	Exclude isotopes: True	Exclude isotopes: True
	Dependent scan on single charge start only: True	Dependent scan on single charge start only: True
Charge state	2–6	2–6
	Include undetermined charge states: False	Include undetermined charge states: False
MS² DDA scan parameters		
	HCD method	EThcD method
Data dependent mode	Number of scans, 40	Cycle time, 1 sec
Orbitrap resolution	30K	15K
Scan range	Define first mass, 120 *m/z*	150–1350 *m/z*
Isolation window (*m/z*)	1.6	0.8
AGC target	Standard	50% (5.00e4)
Maximum injection time	Auto	27
Activation type	HCD	ETD
HCD collision energy (%)	28	–
Use calibrated charge-dependent ETD parameters	–	True
Supplemental activation energy (%)	–	30

Table 5. Orbitrap Excedion Pro MS mouse tissue ADMA DDA experiment parameters.

MS¹ full scan parameters		
	HCD method	EThcD method
Orbitrap resolution	60K	60K
Scan range (*m/z*)	350–1,800	350–1,800
RF lens (%)	50	50
AGC target	200% (2.00e6)	100% (1.00e6)
Maximum injection time	120	Auto
Microscans	1	1
Filters		
	HCD method	EThcD method
MIPS	Peptide	Peptide
	Relax restrictions: False	Relax restrictions: False
Dynamic exclusion	20s, mass tolerance +/– 10 ppm	20s, mass tolerance +/– 10 ppm
	Exclude isotopes: True	Exclude isotopes: True
	Dependent scan on single charge start only: True	Dependent scan on single charge start only: True
Charge state	3–7	3–7
	Include undetermined charge states: False	Include undetermined charge states: False
MS² DDA scan parameters		
	HCD method	EThcD method
Data dependent mode	Number of scans, 40	Cycle time, 1 sec
Orbitrap resolution	30K	15K
Scan range	Define first mass, 130 *m/z*	Define first mass, 130 *m/z*
Isolation window (*m/z*)	1.6	2
AGC target	Standard	200% (2.00e5)
Maximum injection time	Auto	25
Activation type	HCD	ETD
HCD collision energy (%)	28	–
Use calibrated charge-dependent ETD parameters	–	True
Supplemental activation energy (%)	–	30

Table 6. Orbitrap Excedion Pro MS mouse tissue O-GlcNAc DDA experiment parameters.

MS¹ full scan parameters				
	Stepped HCD method	HCD-pd-EThcD method		
Orbitrap resolution	60K	60K		
Scan range (*m/z*)	350–1,800	350–1,800		
RF lens (%)	50	50		
AGC target	200% (2.00e6)	200% (2.00e6)		
Maximum injection time	120	Auto		
Microscans	1	1		
Filters				
	Stepped HCD method	**HCD-pd-EThcD method**		
MIPS	Peptide	Peptide		
	Relax restrictions: False	Relax restrictions: False		
Dynamic exclusion	20s, mass tolerance +/– 10ppm	10s, mass tolerance +/– 10 ppm		
	Exclude isotopes: True	Exclude isotopes: True		
	Dependent scan on single charge start only: True	Dependent scan on single charge start only: True		
Charge state	2–8	2–8		
	Include undetermined charge states: False	Include undetermined charge states: False		
MS² DDA scan parameters				
	Stepped HCD method	**HCD**	**Targeted mass trigger**	**EThcD**
Data dependent mode	Number of scans, 40	Cycle time, 2 sec	Time mode: Unscheduled Mass list: 138.0545 274.0921 204.0876 366.1396 Mass tolerance: +/– 10 ppm	Number of scans, 3
Orbitrap resolution	15K	15K		15K
Scan range	Define first mass, 130 *m/z*	Define first mass, 130 *m/z*		Define first mass, 130 *m/z*
Isolation window (*m/z*)	1.6	1.6		1.6
AGC target	Standard	100% (1.00e5)		100% (1.00e5)
Maximum injection time	Auto	35		100
Activation type	HCD	ETD		ETD
HCD collision energy (%)	28, 35	–		–
Use calibrated charge-dependent ETD parameters	–	True		True
Supplemental activation energy (%)	–	35		35

LC-MS data processing and analysis

All phosphopeptides and ADMA-enriched LC-MS data were processed using Proteome Discoverer software or FragPipe, while O-GlcNAc data were processed with Byonic and O-Pair software. All sample preparation methods were searched separately. A human UniProt™ database (downloaded 2025-02-05, 20,435 entries) was used for all phosphopeptide samples, and a mouse UniProt database (downloaded 2025-04-04, 17,858 entries) was used for all ADMA and O-GlcNAc samples. The Byonic "O-glycan 6 most common" modification list was used with all modifications set to variable-common 1. All results were processed and filtered with a 1% precursor and 1% protein group false discovery rate (FDR). Downstream data visualization was completed in RStudio™ (2025.05.1 Build 513) using R (v4.4.2).

Results

Enriched PTM samples present significant analytical challenges due to their wide dynamic range and the low abundance of many modified peptides, necessitating high instrument sensitivity, speed, and precision. The Orbitrap Excedion Pro mass spectrometer equipped with fast and sensitive ETD provides a flexible and robust platform for addressing these complexities.

PTM analysis: Phosphorylation

IMAC and phosphotyrosine enrichment

Peptides modified by phosphorylation on serine, threonine, and tyrosine sites were enriched from digested lysate with IMAC and analyzed by DDA using HCD and EThcD fragmentation. Figure 3A shows 5,892 unique phosphosites were identified, with 92% phosphoserine sites and only 16 phosphotyrosine sites detected. Many signaling pathways controlling cell growth and differentiation involve phosphotyrosine, making it a PTM of interest for potential disease implications. After lysate enrichment with the PTMScan™ HS kit for phosphotyrosine, 2,941 unique phosphotyrosine sites were detected. Both HCD and EThcD fragmentation resulted in many confident peptide detections and site assignments (Figure 3B), with some phosphosites detected uniquely with one fragmentation method.

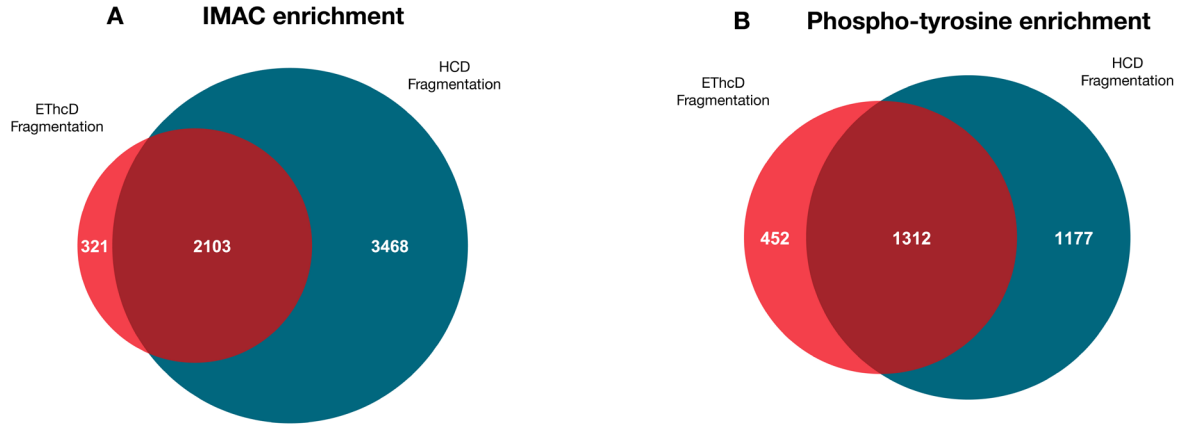

Figure 3. (A) IMAC enriched samples analyzed by DDA resulted in 5,892 unique phosphosites identified from EThcD and HCD fragmentation methods. (B) Distribution of the 2941 unique phosphotyrosine sites detected from EThcD fragmentation and HCD fragmentation—15% of sites are only detected in the EThcD data and 40% of the sites were only detected in the HCD data.

Table 7. DDA results for phosphotyrosine-enriched samples.

	Proteins	Peptides	PSMs	Phosphopeptides
HCD	3,008	12,879	16,855	4,293
EThcD	2,493	11,130	14,257	3,615
Default charge state	2			
Lock mass correction	EASY-IC™ (RunStart)			

Spectra examples (Figures 4–6) show high sequence coverage across different charge states for both HCD and EThcD. With some peptide sequences, fragment ion series detection is insufficient for correct site assignment (Figure 7). Having additional fragmentation options can provide clarifying results.

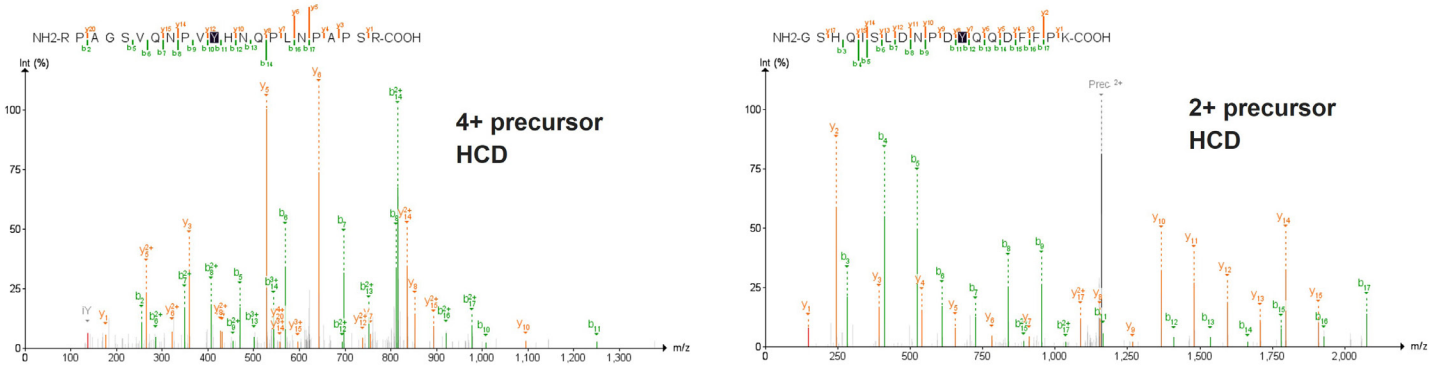

Figure 4. EGFR phosphotyrosine sites identified only in the HCD dataset.

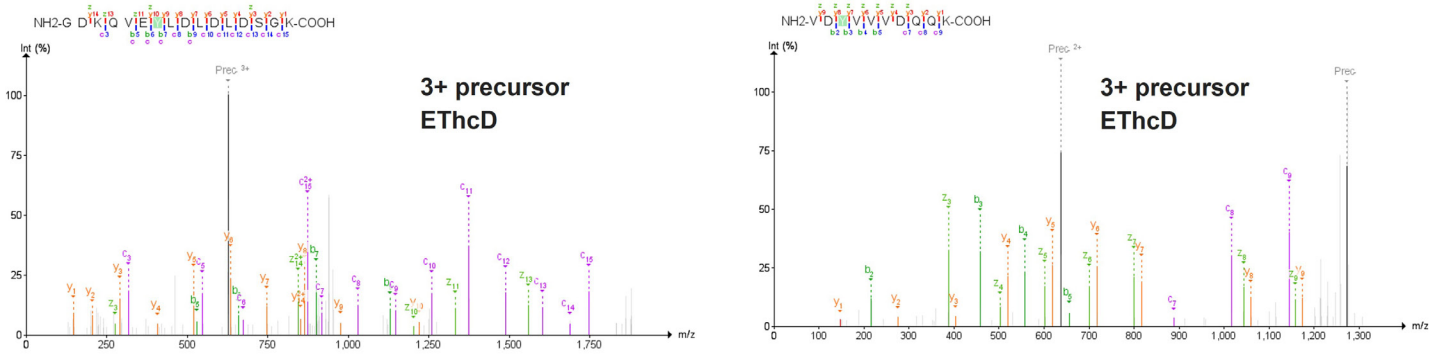

Figure 5. GAB1 phosphotyrosine sites identified only in the EThcD dataset.

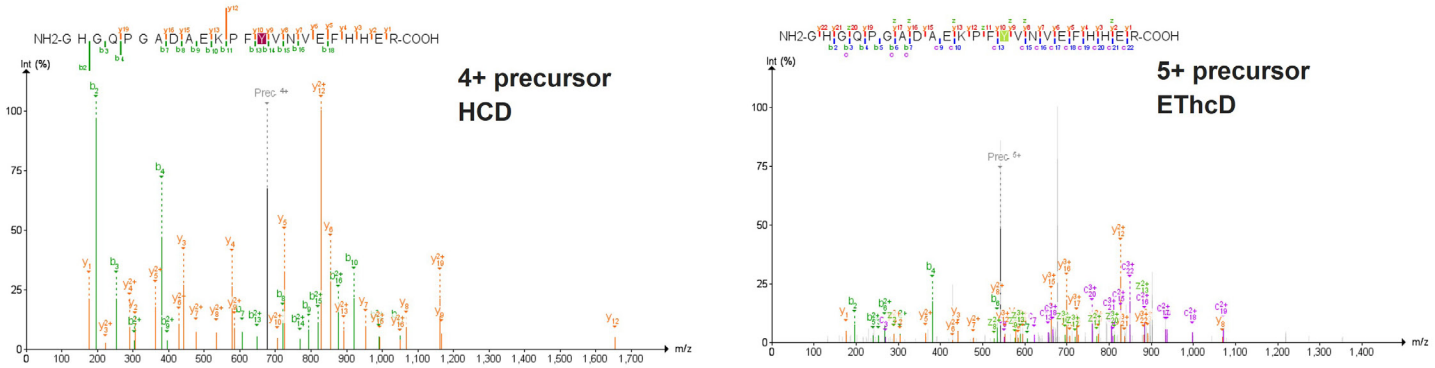

Figure 6. BCR phosphotyrosine site identified with both HCD and EThcD.

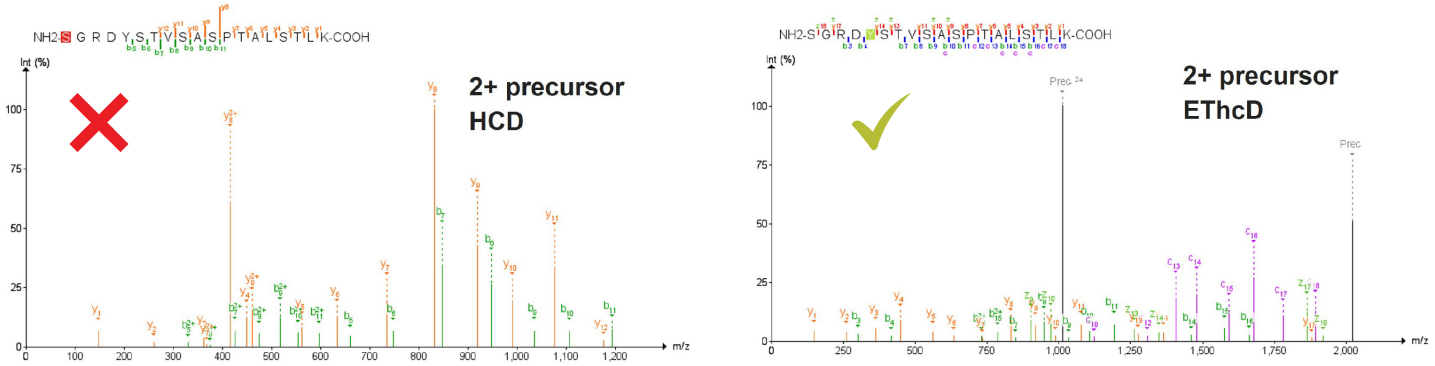

Figure 7. ARHGEF5 phosphotyrosine peptide fragmented in both HCD and EThcD data sets. The HCD spectra does not have enough fragment ion coverage at the N-term region to confirm S1 or Y5 as the modification site. The EThcD spectra has good fragment ion coverage across the peptide sequence resulting in confident site identification.

PTM analysis: ADMA

The PTMScan HS kit specifically enriches peptides with the asymmetric modification, two methyl groups on one of the terminal nitrogen atoms of the guanidine group of arginine. The enrichment resulted in approximately 10% of total identified peptides as ADMA-modified peptides and almost 300 ADMA sites using both HCD and EThcD (Figure 8). Even though more total peptides were identified with HCD fragmentation, more PSMs and unique ADMA sites were detected from EThcD fragmentation.

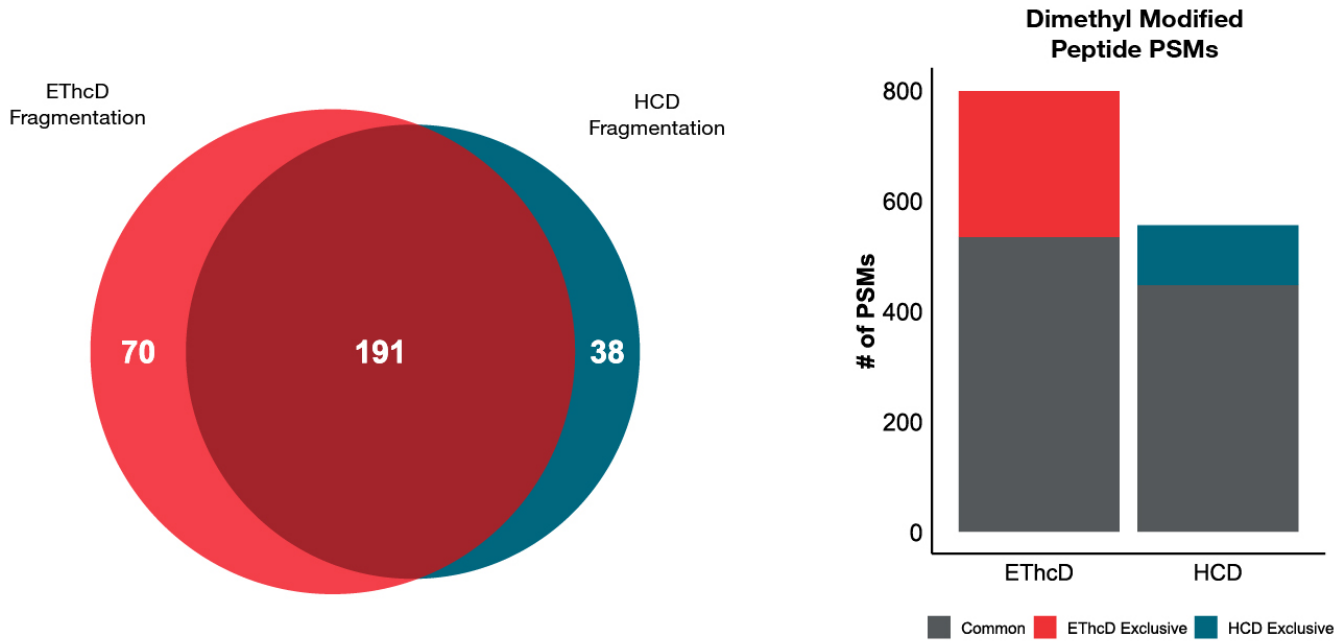

Figure 8. Venn diagram shows the distribution of 299 unique peptide sequences detected with 1 or more ADMA modifications. Stacked bar graph shows the data at PSM level.

Table 8. Peptide PSMs detected in ADMA-enriched samples.

	EThcD	HCD
Total PSMs	5,714	7,850
ADMA PSMs	798	555

PTM analysis: O-linked glycosylation

Protein glycosylation commonly occurs as either N-linked or O-linked modifications. However, glycosylation is labile under collisional activation (HCD or CID), which leads to loss of the glycan during MS/MS fragmentation and makes site localization difficult. O-linked glycosylation is particularly challenging because serine and threonine residues are widely distributed throughout a protein and there is no consensus sequence governing O-glycosylation. Furthermore, multiple sites on a single peptide can be modified. As a result, glycan loss from the peptide backbone during CID or HCD fragmentation prevents reliable assignment of modification sites.

For O-linked glycopeptides, alternative fragmentation approaches such as ETD or EThcD are preferred due to their non-ergodic dissociation behavior. These fragmentations generate extensive backbone fragmentation while preserving the glycans on the peptide backbone, enabling confident peptide sequencing and unambiguous localization of glycosylation sites.

On the Orbitrap Excedion Pro mass spectrometer, we have implemented an intelligent acquisition strategy called HCD product-dependent EThcD (HCD-pd-EThcD) to accelerate O-glycopeptide identification and improve analytical productivity. In this approach, the Orbitrap Excedion Pro mass spectrometer acquires HRAM HCD spectra in a data-dependent manner, detects oxonium ions in real time in the spectrum, and triggers EThcD scans only for glycopeptide precursors that exhibit these ions (Figure 9). This selective triggering enhances dynamic range and duty cycle by avoiding unnecessary EThcD acquisitions.

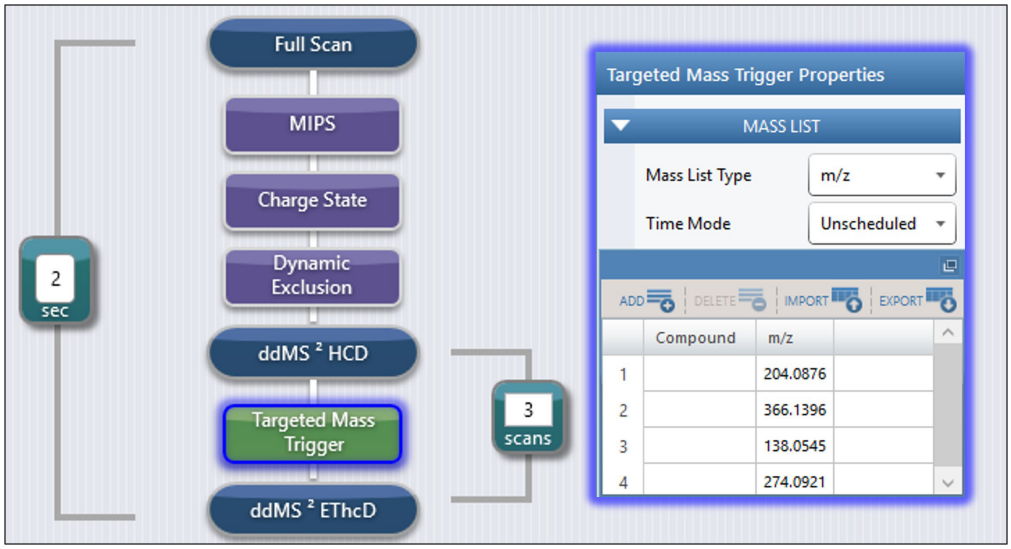

Figure 9. Method structure for HCD-pd-EThcD data acquisition.

To support downstream analysis and further increase confidence in sequencing and site localization, this workflow integrates effectively with software such as the O-Pair platform, a dedicated O-glycoproteomics platform designed to identify glycopeptides and assign glycosylation sites by combining HCD and ETD/EThcD data. O-Pair software first leverages HCD spectra to determine peptide sequence and glycan composition, then incorporates EThcD-derived backbone fragments that retain the intact glycan, enabling precise site localization, even when peptides carry multiple O-glycan modifications. Its probabilistic scoring framework merges spectral evidence, glycan composition, and site-level fragmentation patterns to provide confidence-ranked assignments, streamlining characterization of complex O-glycopeptides.

Overall, the combination of on-the-fly precursor selection using HCD-pd-EThcD on the Orbitrap Excedion Pro mass spectrometer and advanced data interpretation with O-Pair software enables efficient, confident, and high-throughput O-glycopeptide analysis.

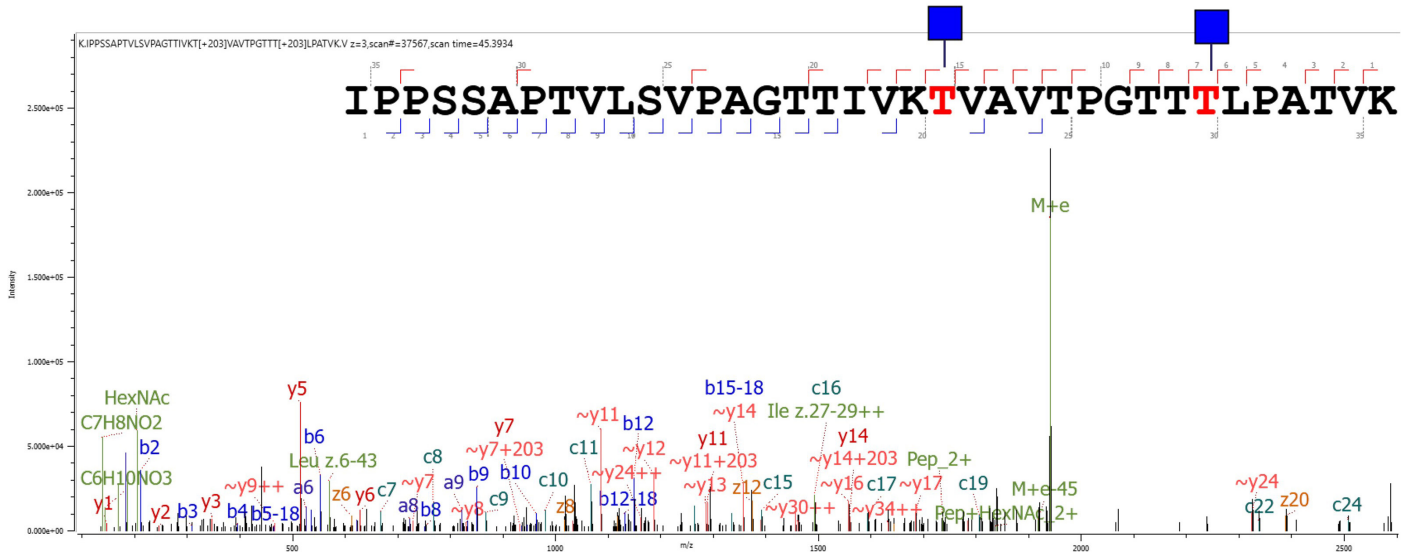

Figure 10. HCFC1 (Q61191) peptide with 12 potential O-glycosylation modification sites. EThcD fragmentation results in high sequence coverage and 2 confident glycosylation site assignments.

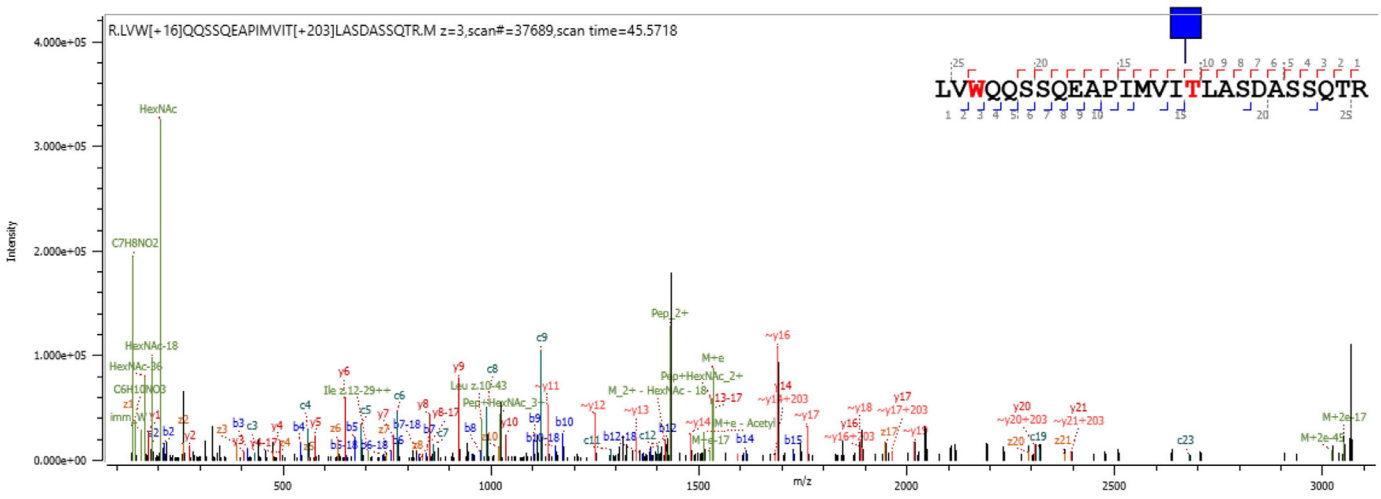

Figure 11. BSN (O88737) peptide with 7 potential sites of O-glycosylation shows good EThcD fragment ion coverage and confident modification site assignment.

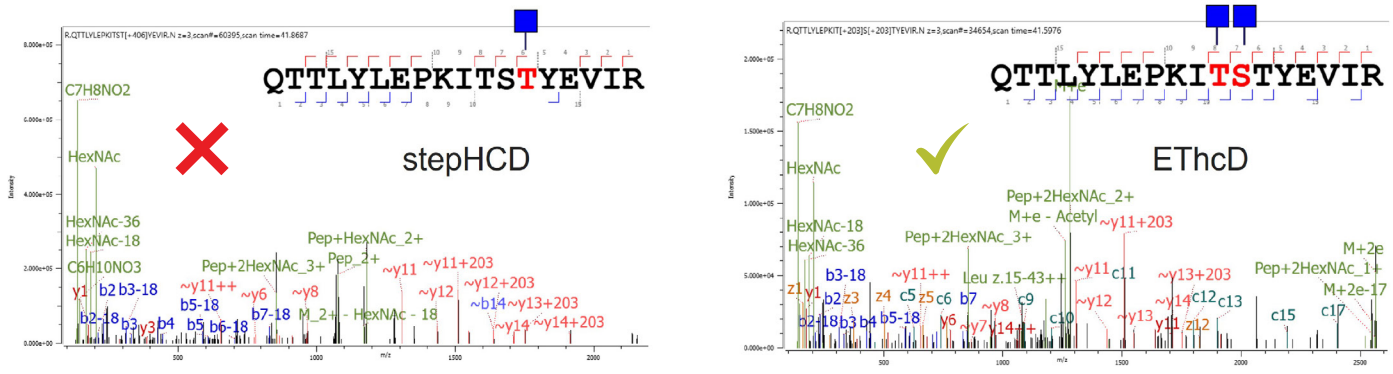

Figure 12. PCLO (Q9QYX7) peptide with 5 potential O-glycosylation sites. Stepped-HCD fragmentation data shows good peptide sequence coverage, but search results incorrectly indicate 2 HexNAc modifications on the wrong amino acid. EThcD fragmentation spectra has enhanced fragment ion coverage that gives confident site assignment at 2 sites.

Conclusion

Expand PTM coverage across multiple modification classes—the Orbitrap Excedion Pro mass spectrometer identified 5,892 unique phosphorylation sites from IMAC enrichment and 2,941 phosphotyrosine sites, showcasing deep coverage of both abundant and low-level PTMs.

Confidently localize challenging sites using EThcD fragmentation, which uniquely detected 15% of phosphotyrosine sites not observed with HCD and enabled clear site discrimination where HCD alone produced ambiguous assignments (e.g., ARHGEF5 peptide).

Increase sequence and site confidence in ADMA analysis with EThcD identifying more unique ADMA-modified peptides and more PSMs than HCD despite HCD producing a higher total peptide count—resulting in ~300 confidently assigned ADMA sites.

Improve O-glycopeptide characterization with HCD-pd-EThcD, achieving confident localization of modifications on highly complex peptide, such as 2 localized O-glycosylation sites across a peptide containing 12 potential sites (HCFC1) and accurate site assignments for multi-glycosylated peptides where stepped-HCD mislocalized modifications (PCLO example).

Enhance productivity and duty cycle through intelligent triggering—EThcD scans are acquired only for precursors with oxonium-ion signatures, increasing dynamic range and avoiding unnecessary fragmentation overhead.

Deliver high-quality spectra across PTM chemistries and charge states with both HCD and EThcD producing complementary identifications—40% of phosphotyrosine sites were unique to HCD and 15% unique to EThcD, demonstrating the power of combined fragmentation strategies.

The Orbitrap Excedion Pro mass spectrometer demonstrates exceptional capability for comprehensive PTM analysis through the integration of flexible fragmentation options. The combination of HCD and EThcD enables complementary peptide fragmentation, resulting in broader sequence coverage and more confident PTM site localization. EThcD fragmentation, in particular, provided enhanced detection and characterization of labile PTMs such as phosphorylation, asymmetric dimethylation, and O-linked glycosylation, improving localization accuracy and sequence information compared to traditional methods. Thousands of unique modification sites were identified across multiple enrichment strategies, illustrating the system's sensitivity and performance across diverse PTM classes.

The combination of speed, sensitivity, and versatility supports confident PTM identification in complex biological samples. Overall, the Orbitrap Excedion Pro mass spectrometer empowers researchers to achieve deeper proteome coverage and unlock new biological insights into cellular regulation, signaling, and disease mechanisms through advanced PTM analysis.

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