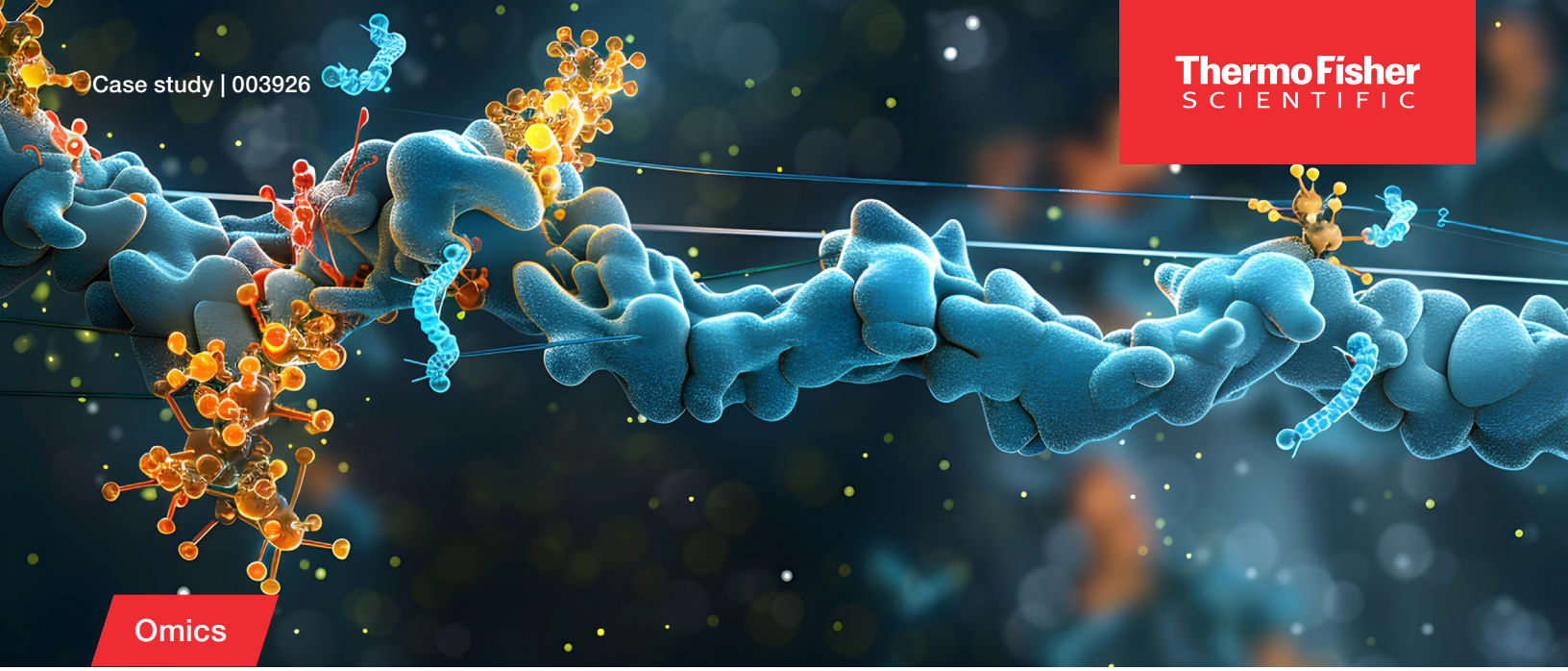

Post-translational modification characterization

Harnessing cutting-edge technology for unprecedented depth and accessibility to probe protein dynamics

Introduction

The analysis of post-translational modifications (PTMs) on proteins is essential to support the understanding of cellular and systemic functional dynamics of biology. Modification of proteins can significantly alter the function, activity, interactions, stability, and localization of proteins in cells or biological fluids. A single protein can perform many functions depending on its modification state or proteoform. PTMs also play an important role in signal transduction pathways. For example, phosphorylation is a common PTM that activates or deactivates signaling proteins. The study of PTMs can support understanding of disease mechanisms (as aberrant PTMs are often linked to diseases including cancer, neurogenerative disorders, and metabolic diseases), development of therapeutics that target modified proteins, or discovery of biomarkers indicative of disease or physiological state.

Mass spectrometry (MS) is an essential tool for the analysis of PTMs. MS offers the ability to comprehensively analyze a wide range of PTMs for thousands of proteins simultaneously over a wide dynamic range of abundance, including phosphorylation, ubiquitination, acetylation, methylation, succinylation, or glycosylation. It offers unbiased interrogation of biological systems with relative or absolute quantitative interrogation and frequently site localization of the modification to the amino acid residue. Some PTMs, such as phosphorylation, play a key role in cancer cell signaling and could be therapeutic drug targets.

The analysis of PTMs presents several challenges, as they are highly diverse, dynamic, and present over a wide dynamic range of abundance. PTMs often occur at low stoichiometry of abundance, where only a fraction of the protein population is modified.

PTMScan® HS is an enhanced PTMScan methodology with improved identification of post-translationally modified peptides. PTMScan technology employs a proprietary methodology from Cell Signaling Technology (CST) for peptide enrichment by immunoprecipitation using a specific bead-conjugated antibody in conjunction with liquid chromatography—tandem mass spectrometry (LC-MS/MS) for quantitative profiling of PTM sites in proteins. PTMs that can be analyzed by PTMScan technology include phosphorylation, ubiquitination, acetylation,

and methylation, among others. PTMScan technology enables researchers to isolate, identify, and quantitate large numbers of post-translationally modified peptides with a high degree of specificity and sensitivity (HS), providing a global overview of PTMs in cell and tissue samples to allow unbiased modification site determination. More information on PTMScan products and services can be found at cellsignal.com/applications/proteomics.

The Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer enables higher sensitivity and selectivity to provide deeper insights into the proteome, including the analysis of PTMs. In this study, we utilized the PTMScan enrichment platform coupled with a Thermo Scientific™ Vanquish™ Neo UHPLC system and an Orbitrap Astral mass spectrometer to selectively enrich classes of PTMs for analysis to achieve a depth of coverage not previously reported.

An interview with Dr. Mukesh Kumar, Cell Signaling Technology®

Dr. Mukesh Kumar is a principal scientist at Cell Signaling Technology in Danvers, Massachusetts.

Q: Can you describe your role at Cell Signaling Technology?

A: Cell Signaling Technology (CST®) is a different kind of life sciences company—one founded, owned, and run by active research scientists, with the highest standards of product and service quality, technological innovation, and scientific rigor for over 25 years. At CST, I am leading the mass spec laboratory, and my role involves providing mass spec support for antibody discovery, carrying out internal research that focuses on key areas like chemoproteomics, target protein degradation, and neurodegeneration as well as helping develop workflow solutions for proteins and PTM analysis. CST also provides a range of proteomics services including total proteome and PTM analysis for outside customers.

Q: Can you tell us about the goals of your workflow solutions to support investigation of post-translational modifications of proteins?

A: One of the core missions of CST is to provide fellow scientists around the globe with best-in-class products and services to fuel their quests for discovery. PTMScan technology is an example of one such tool which enables researchers to perform enrichment of thousands of PTM-containing peptides prior to analysis by liquid chromatography—tandem mass spectrometry (LC-MS/MS). PTMs are essential for many cellular functions such as protein activity, subcellular localization, degradation, and protein-protein interactions. PTM analysis aids in understanding of cellular functions, disease biology, mechanism of action of drugs/chemical modulators, biomarker discovery, and identification of therapeutic targets. There are many different PTMs that can be analyzed by PTMScan technology including phosphorylation, ubiquitination, acetylation, succinylation and methylation, among others (a complete list of PTMScan Kits can be found here: PTMScan Kits).

Q: What are the main challenges with your work?

A: The complexity, dynamic nature, and low abundance of PTMs compared to the unmodified counterpart makes the analysis very challenging. Biologically different sample types (cells/tissues/body fluids) have different types and varying degrees of modifications. The same proteins can be differently modified in different cell types and/or within same cell type under different cellular conditions. To add to the challenge, the same sites can be modified with different PTM types. Recent developments in analytical tools and software have helped the identification and characterization of certain types of PTMs. However, there are many other PTMs for which there is the need for improved identification and accurate site localization by currently available software, particularly for data generated by data independent acquisition (DIA).

Q: Can you share how newly developed analytical tools like PTMScan and the Orbitrap Astral mass spectrometer have helped to address these challenges?

A: Tools like PTMScan are invaluable for the enrichment of low abundant PTM-containing peptides. The Orbitrap Astral mass spectrometer has been a game changer for us and has enabled the identification and quantification of thousands of PTM sites across multiple different PTMs including ubiquitination, phosphorylation, acetylation, succinylation, and methylation. Analysis of PTMScan-enriched peptides on the Orbitrap Astral MS has enabled us to detect thousands of sites that we were

not able to detect earlier. Compared to previous generation instruments, DIA analysis on the Orbitrap Astral MS provides 2-3x more PTM peptides in half the analysis time. On one hand, the sensitivity of the Orbitrap Astral MS has greatly increased the identification and quantification rate for low abundant PTMs like phosphotyrosine, and on the other hand, the speed of the Orbitrap Astral MS has greatly increased the identification and quantification rate for high complexity PTMs like ubiquitination.

Q: Could you share any opinions you have on future directions for PTM analysis that will have an impact on research or development in the field?

A: Technical advancements like the Orbitrap Astral MS and PTMScan enable researchers to measure both the unmodified and modified proteome at scale. Studies combining total proteome and PTM analysis will greatly advance our understanding of complex biological systems including the role of PTMs in protein-protein interactions, regulation of protein activity, localization, degradation, as well as functional relationships of proteoforms. Also, significant progress has been made to improve the speed and robustness of LC-MS/MS analysis, enabling hundreds of analyses per day. This also creates an opportunity for immunoprecipitation (IP)-LC-MS/MS applications to be used for highly multiplexed assays for clinical diagnostics. At CST, we continue to expand the PTMScan Kit to include additional PTMs, which are otherwise difficult to detect, and work on expanding future applications such as the use of PTMScan in single cell analysis.

Cell Signaling TECHNOLOGY®

Rethink what is possible

The Orbitrap Astral MS provides faster throughput, deeper coverage, higher sensitivity, and accurate and precise quantitation for the analysis of proteomics samples, including PTM analysis. The Orbitrap Astral MS combines three mass analyzers: a quadrupole mass analyzer for high selectivity and high ion transmission, an Orbitrap mass analyzer for high dynamic range and high-resolution measurements, and a novel Astral analyzer for fast and sensitive measurements with complete synchronization of ion transfer and processing throughout the instrument.

This synchronization and parallel handling of five separate ion packets simultaneously enables each mass analyzer to be used at once to optimize performance. The Orbitrap Astral MS expands the scale and scope of experiments by offering up to four times faster throughput, up to two times deeper proteome coverage, and higher sensitivity with accurate and precise quantitation.

The Orbitrap Astral MS offers the ability to do narrow window DIA (nDIA). nDIA enables the separation of modified and unmodified peptides into distinct MS/MS fragmentation spectra. For example, a methylation results in a mass shift of +14 Da, but peptides are multiply charged, resulting in precursor *m/z* shifts of 7 for a +2 charge state or 4.6 for a +3 charge state. Thus, an unmodified peptide and its methylated counterpart can be co-isolated with DIA windows larger than 4 Th, creating chimeric spectra where some fragment ions are shared between the modified and unmodified species. This leads to significant bioinformatic deconvolution challenges and can mask the detection of low stoichiometry PTMs with standard DIA methods. nDIA provides added specificity to DIA data with smaller isolation windows resulting in fewer co-fragmented peptides in each nDIA spectrum. The reduced spectral complexity and a narrower precursor mass range improves sensitivity in PTM site assignment.

Exemplary results

Sample preparation

Total proteins were extracted from untreated, MG132 and pervanadate treated human colorectal carcinoma (HCT116), and T-cell acute lymphoblastic leukemia (Jurkat) cell lines, respectively, and from untreated mouse liver tissue. Extracted proteins were reduced, alkylated, and digested using trypsin and Lys-C enzymes into peptides. Digested peptides were purified using a Sep-Pak™ C18 cartridge. Purified peptides were subjected to IMAC for the enrichment of phosphopeptides. PTMScan HS Kits were used to enrich target classes of modified peptides, including lysine-ubiquitination (lysyl-diGly), lysine-acetylation, tyrosine-phosphorylation, lysine-succinylation, and arginine-monomethylation. Prior to LC-MS/MS analysis, the enriched peptides were cleaned by C18 stage-tips. PTMScan HS enrichments can optionally be automated by leveraging the Thermo Scientific™ KingFisher™ Flex Purification System for automated sample preparation with magnetic beads.

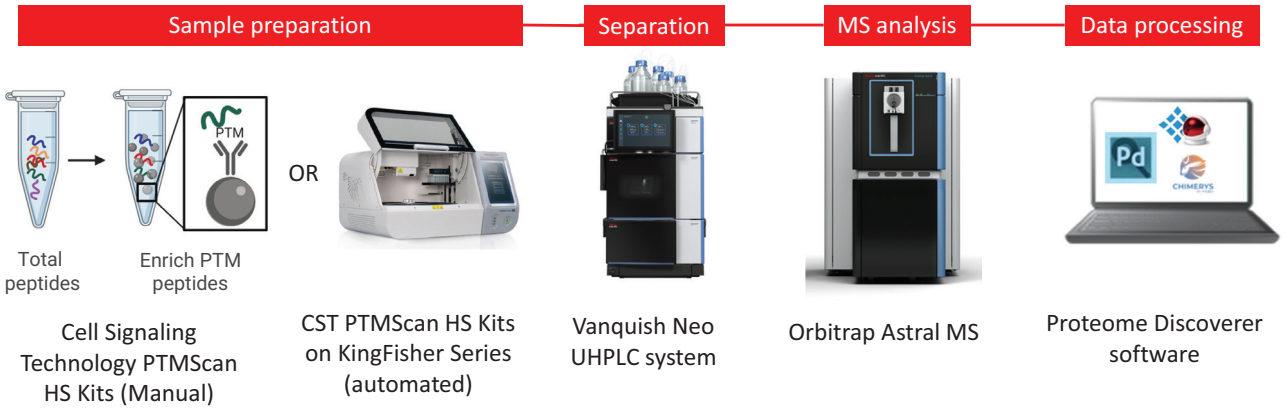

Figure 1. Overview of the workflow for PTMScan enrichment of selected classes of PTMs peptides following protein extraction and proteolytic digestion. A detailed protocol for protein extraction, digestion, peptide cleanup and PTMScan enrichment can be found **here**.

Test method and data analysis

Samples were loaded onto a reverse phase C18 column and separated chromatographically at 350 nL/min flow rate over either a 45-minute or 90-minute gradient with the Vanquish Neo UHPLC system. Mass spectrometry analysis was performed on the Orbitrap Astral MS. Data was acquired using data-dependent acquisition (DDA) and DIA methods employing unique narrow-window DIA (nDIA) isolations of 2 amu made possible by the acquisition speed and sensitivity of the Orbitrap Astral MS. nDIA methods enable the enhanced separation of peptides into different MS^2 acquisition isolation windows, increasing the sensitivity and selectivity of PTM measurements while also allowing discrete PTM site localization. DIA data were analyzed using Spectronaut™ 19 software with either a library free search (LFS) or hybrid search (HBS), and Thermo Scientific™ Proteome Discoverer™ 3.2 software with the CHIMERYS™ intelligent search algorithm by MSAID for spectral matching and INFERYS™ rescoring algorithm for PTM site localization validation. DDA data were analyzed using MSFragger and Proteome Discoverer 3.2 software.

Results

The analysis of PTMScan-enriched peptides by the Orbitrap Astral MS yielded identification and quantification of thousands of post-translationally modified peptides. The PTMScan HS Ubiquitin/SUMO Remnant Motif (K-ε-GG) Kit was used for the enrichment of ubiquitin remnant peptides (K-ε-GG) from different amounts of untreated and MG132 (a proteasome inhibitor) treated human colorectal carcinoma cell line (HCT 116). From the lowest input amount of 100 µg, the Orbitrap Astral MS analysis using DDA, nDIA with library free search, and nDIA with hybrid search resulted in 1,379, 1,716, and 5,724 unique ubiquitinated quantified peptides, respectively (Figure 2A). When 3 mg of total peptide was used for enrichment and analyzed by the Orbitrap Astral MS DIA HBS, a total of up to >41K and >72K unique ubiquitinated peptide were quantified from untreated and MG132 treated cells, respectively (Figure 2A). The identified ubiquitinated peptides mapped to >9,200 protein groups.

Similar to ubiquitinated peptides enrichment, PTMScan Kits detailed in Table 1 were used for the enrichment of specified PTMs and subsequently analyzed by the Orbitrap Astral MS. nDIA analysis on the Orbitrap Astral MS resulted in identification and quantification of up to >13K, >6K, and >1.4K acetylated, succinylated, and methylated peptides, respectively (Figures 2B, 2C, and 2D).

Table 1. PTMScan Kits used for IP enrichment of specified PTMs

PTMScan Kit	CST part number
HS Acetyl-Lysine Motif (Ac-K) Kit	Cat# 46784
HS Succinyl-Lysine Motif (Succ-K) Kit	Cat# 60724
HS Mono-Methyl Arginine Motif (mme-RG) Kit	Cat# 98567
HS Ubiquitin/SUMO Remnant Motif (K-ε-GG) Kit	Cat# 59322
HS Phospho-Tyrosine (P-Tyr-1000) Kit	Cat# 38572

For the enrichment of phosphorylated peptides from HCT 116 cells, immobilized metal affinity chromatography (IMAC) was used and resulted in identification and quantification of up to >24K, >22K, and >32K phosphorylated peptides by DDA, nDIA-LFS, and nDIA-HBS, respectively (Figure 3A). Compared to DDA, nDIA analysis resulted in up to >2.6x more peptides quantified per minute. Among all phosphorylated peptides enriched by IMAC, the majority of them are phosphoserine (87%), followed by phosphothreonine (11%), and least phosphotyrosine (2%) (Figure 3E).

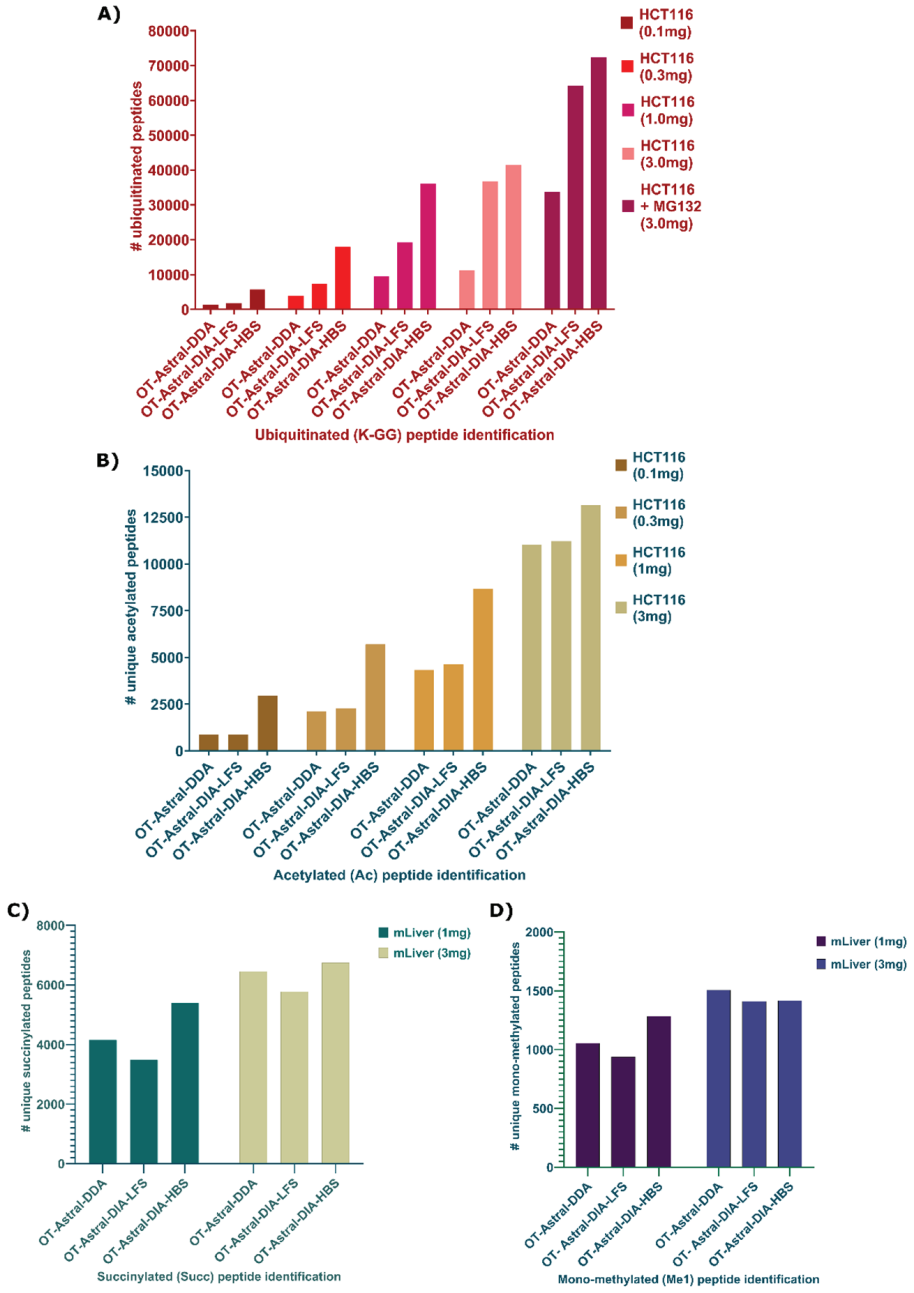

Figure 2. PTMScan HS enrichment and analysis of ubiquitinated, acetylated, succinylated, and mono-methylated peptides on the Orbitrap Astral MS. (A) Number of unique ubiquitinated peptides quantified with the Orbitrap Astral MS using DDA, nDIA-LFS, or nDIA-HBS methods from different amounts of peptides used for PTMScan enrichment from untreated and MG132 treated HCT116 cells. The number of unique (B) acetylated, (C) succinylated, and (D) monomethylated peptides quantified by DDA, nDIA-LFS, and nDIA-HBS methods from different amounts of peptides used for PTMScan HS enrichment.

To enrich the least abundant phosphotyrosine peptide, the PTMScan HS Phospho-Tyrosine (P-Tyr-1000) Kit (Cat# 38572) was used, and nDIA analysis on the Orbitrap Astral MS resulted in quantification of up to >1.8K unique phosphotyrosine peptides (Figure 3B). Among the enriched phosphopeptides by PTMScan, >96% were phosphotyrosine, approximately 3% were phosphoserine, and 1% phosphothreonine (Figure 3E).

To further test the enrichment efficiency and benchmark Orbitrap Astral MS nDIA performance for phosphotyrosine analysis, samples from untreated and pervanadate (a protein tyrosine phosphatase inhibitor that induces tyrosine phosphorylation in cells) treated Jurkat cells (an immortalized human T lymphocyte cell line) were used. PTMScan enrichment and nDIA analysis of pervanadate (PV) treated samples resulted in identification and quantification of up to 10K unique phosphotyrosine peptides (Figure 3C) with good analytical reproducibility (Figure 3D).

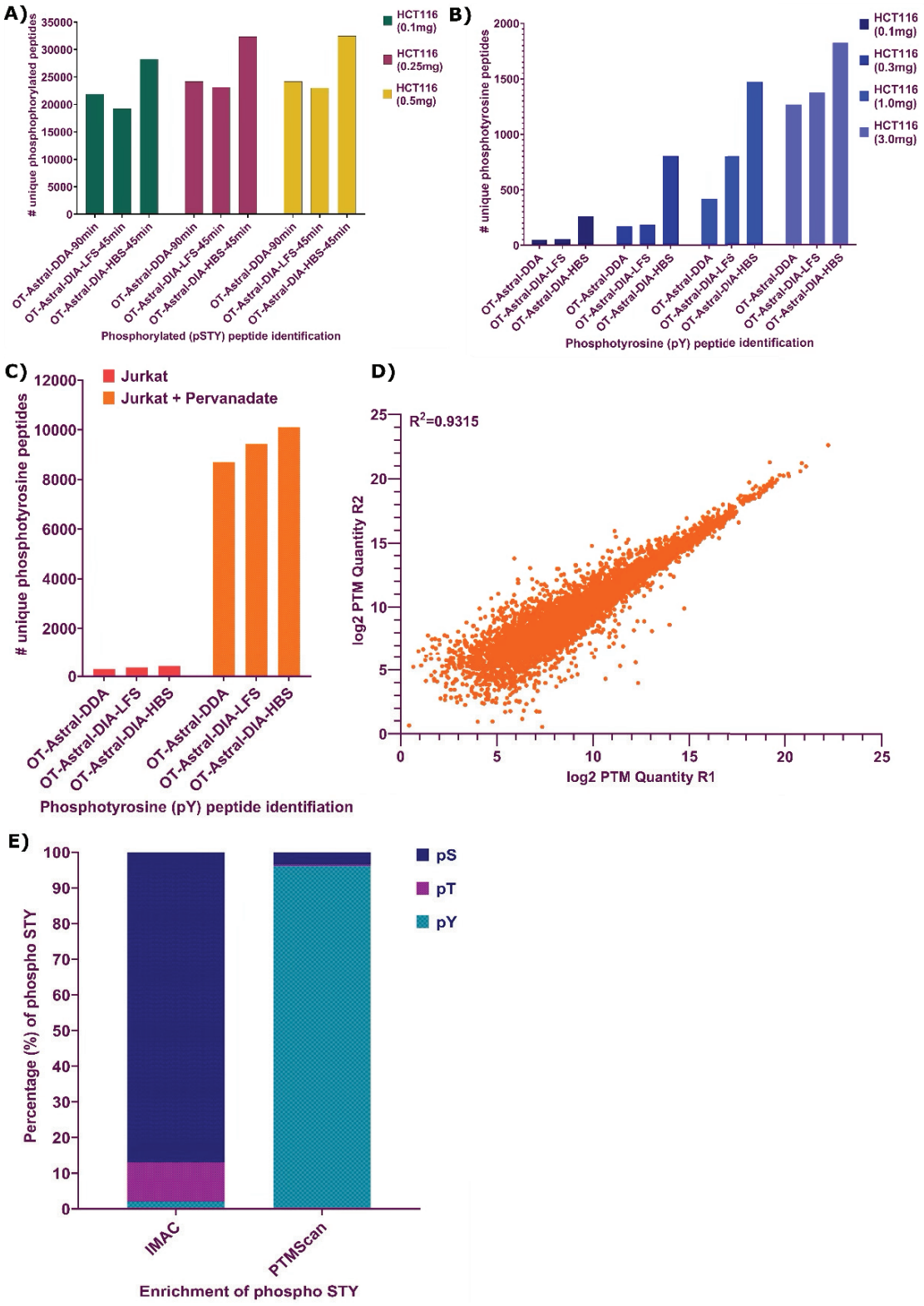

Figure 3. Enrichment and analysis of phospho peptides on the Orbitrap Astral MS. (A) Number of unique phosphorylated peptides quantified by DDA, nDIA-LFS, and nDIA-HBS methods from different amounts of peptides used for IMAC enrichment. (B) Number of unique phosphotyrosine peptides quantified by DDA, nDIA-LFS, and nDIA-HBS methods from different input amounts of peptides used for PTMScan HS pY enrichment. (C) Number of unique phosphotyrosine peptides enriched by PTMScan HS pY and quantified by DDA, nDIA-LFS, and nDIA-HBS from untreated and pervanadate treated Jurkat cells. (D) Correlation of phosphotyrosine peptide quantified in two technical nDIA runs. (E) Percentage of phosphoserine, phosphothreonine, and phosphotyrosine peptides identified by IMAC and PTMScan HS enrichment.

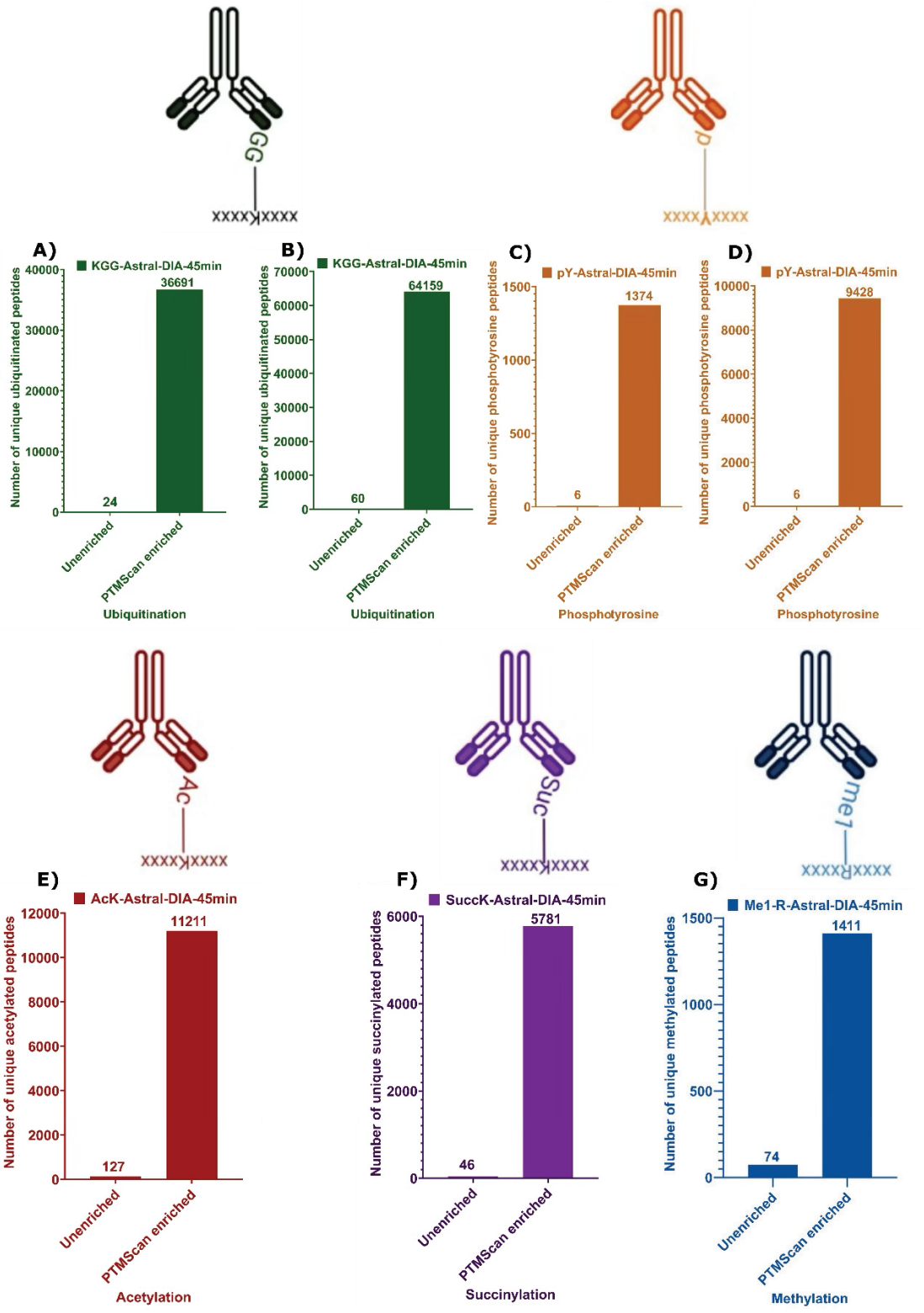

Figure 4. Comparison of PTM site identification with and without PTMScan HS enrichment. Panels A-G show the number of quantified PTM sites detected in a single experiment with nDIA analysis on the Orbitrap Astral MS coupled with the Vanquish Neo UHPLC system.

Owing to low abundance compared to its unmodified counterpart, the analysis of PTM peptides without enrichment is challenging even when using the most advanced analytical tools like nDIA analysis on the Orbitrap Astral MS. In a single-shot, 45-minute nDIA analysis on the Orbitrap Astral MS, the total number of ubiquitinated, phosphotyrosine, acetylated, succinylated, and methylated PTM peptides without enrichment and upon PTMScan HS enrichment are 60 vs. 64,159; 43 vs. 9,428; 127 vs. 11,211; 46 vs. 5,781; and 74 vs. 1,411 (Figures 4A/B, 4C/D, 4E, 4F, and 4G), respectively.

In conclusion, this case study highlights the importance of sample preparation with PTMScan HS enrichment, and the power of the Orbitrap Astral MS combined with the Vanquish Neo UHPLC system for in-depth characterization of thousands of PTM peptides across multiple types of PTMs.

Summary

- PTMScan HS enrichment of target modifications provides increased sensitivity of detection for target PTMs from 10 to >1,000 fold.
- Enhanced performance enabled by advanced technology platforms used in this study allows for detection and quantification of:
  - PTMs from low amounts of starting material (100 µg).
  - Novel unique PTM sites not yet reported in existing databases.
- Improved sensitivity and dynamic range of detection for PTM analysis with the Vanquish Neo UHPLC system coupled to the Orbitrap Astral mass spectrometer enables deeper coverage and higher sensitivity to study the functional dynamics of protein modifications, including the discovery of previously unreported novel PTM sites.

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[1] Cell Signaling Technology, Danvers, MA, USA

[2] Thermo Fisher Scientific, Mississauga, ON, Canada

[3] Thermo Fisher Scientific, San Jose, CA, USA

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