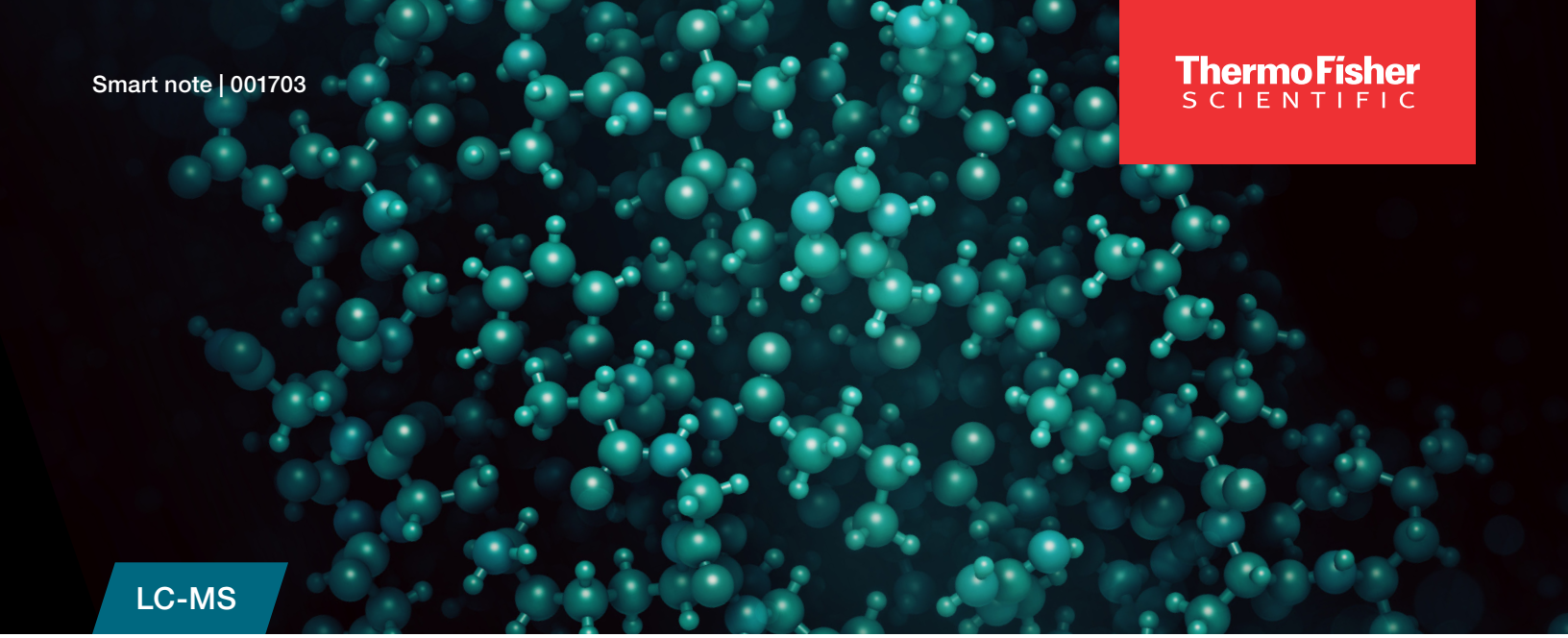

Smart note | 001703

LC-MS

Multiplexed sample analysis using TMT and Orbitrap Tribrid technology delivers proteome-wide biological assays

Introduction

Sample multiplexing using Thermo Scientific™ Tandem Mass Tag™ (TMT™) isobaric labeling has emerged as a powerful approach for liquid chromatography-mass spectrometry (LC-MS)-based proteomics. TMT labeling strategies allow scientists to perform proteome-wide biological assays that reveal novel insights about cellular processes and pathways. TMT-based quantitative proteomics has the potential to become the premier biological assay.

LC-MS sensitivity and throughput previously were a challenge, limiting full-proteome interrogation of biological systems. This smart note explains how TMT-based sample analysis using the Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer overcomes these challenges, enabling quantification of more samples in less time at lower concentrations while revealing protein abundance changes across whole proteomes with superior depth and coverage.

Why is sample multiplexing important?

Proteomics samples vary in complexity from individual proteins to entire tissues, organisms, and even communities. The increasing levels of sample complexity make identification and quantification of proteins more challenging. One important reason to use sample multiplexing is that it facilitates the complex experimental designs that better reflect and capture the nuances of complex and dynamic biological systems. Multiplexing streamlines use of multiple experimental models to increase biological insight such as biological replicates, positive and negative controls, dose-response, time series data, and rescue experiments. Because quantitation occurs for each quantifiable protein for each sample and in each experimental condition, there are essentially no missing values in the data sets obtained. In other words, because every measurement is made across every sample and treatment, TMT multiplexing experiments are closed systems with unique statistical properties that allow for more confidence in comparative results. Of course, an obvious advantage of multiplexing is increased sample throughput. Combining up to 18 biological samples into one permits experiments to be completed in a fraction of the instrument time. And finally, quantitation based on the principle of stable isotope dilution theory provides unmatched precision.

Workflow overview: TMT quantitative proteomics

TMTs are isobaric chemical tags that provide multiplexing capabilities for quantitative proteomics. The ability to perform concurrent LC-MS analysis of multiple samples using TMT reagents increases throughput and enables relative quantitation of up to 18 different samples derived from cells, tissues, or biological fluids.

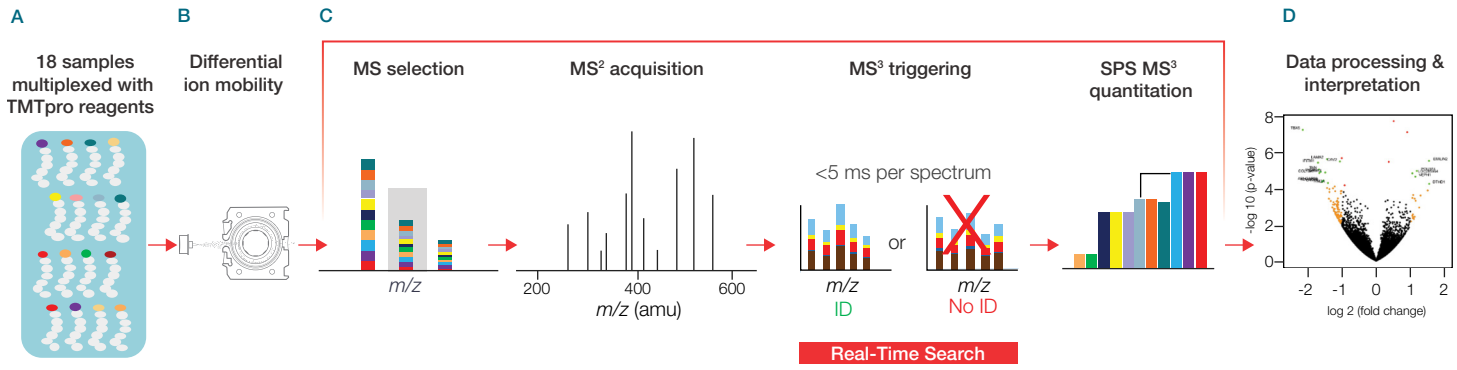

Figure 1. Overview of the TMTpro 18-plex proteomics workflow on Orbitrap Ascend Tribrid MS. (A) Peptides from up to 18 different samples are labeled with TMTpro 18plex reagents and pooled together. (B) Differential ion mobility with FAIMS Pro interface provides gas-phase fractionation. (C) High resolution, accurate mass method using SPS-MS³ with Real-Time Search is utilized for precursor selection, fragmentation, and TMTpro reporter ion quantitation. (D) Data processing, interpretation, and visualization is done in Thermo Scientific™ Proteome Discoverer™ software.

Figure 1 presents a generalized overview of the Thermo Scientific™ TMTpro™ 18-plex experimental workflow. In this example, 18 samples containing proteomes that have been proteolyzed to peptides are chemically labeled using TMTpro reagent. TMT labeling produces 18 different peptide populations in which every peptide is labeled. The samples are then combined into one and analyzed by LC-MS. Because the isobaric tags possess the same chemical properties, all the peptides from the different TMT-labeled samples co-elute during LC separation. Once the peptides enter the mass spectrometer, they are simultaneously detected in the MS¹ scan as a single precursor ion peak. Application of higher-energy dissociation (HCD) in the MS² or MS³ scan cleaves off the TMT reporter ions at the position shown in Figure 2, resolving the labeled peptides to produce a spectrum that represents the abundance of each in the original mixture. Peptide identification is achieved by matching the resulting MS² peaks to those in fragment databases. Peptide quantitation is accomplished by comparing the intensities of the reporter ions.

TMT labeling

As shown in Figure 2, a standard TMT consists of an MS² reporter group, a spacer arm (also called a balancer or mass normalizer) and an amine reactive group. During labeling, the amine reactive group binds to the N-terminus of a peptide or a lysine residue. It is the reporter ions that are measured during quantitation. The TMT contains a specific bond where cleavage occurs during HCD fragmentation. The asterisks on the TMT structures represent the different atoms containing heavy isotopes, which are either Nitrogen-15 or Carbon-13. Because the location of the heavy isotopes is different on each reporter ion cleaved during HCD, the mass differences are distinguished by the high-resolution (HR) MS² or MS³ scan in a Thermo Scientific™ Orbitrap™ mass analyzer.

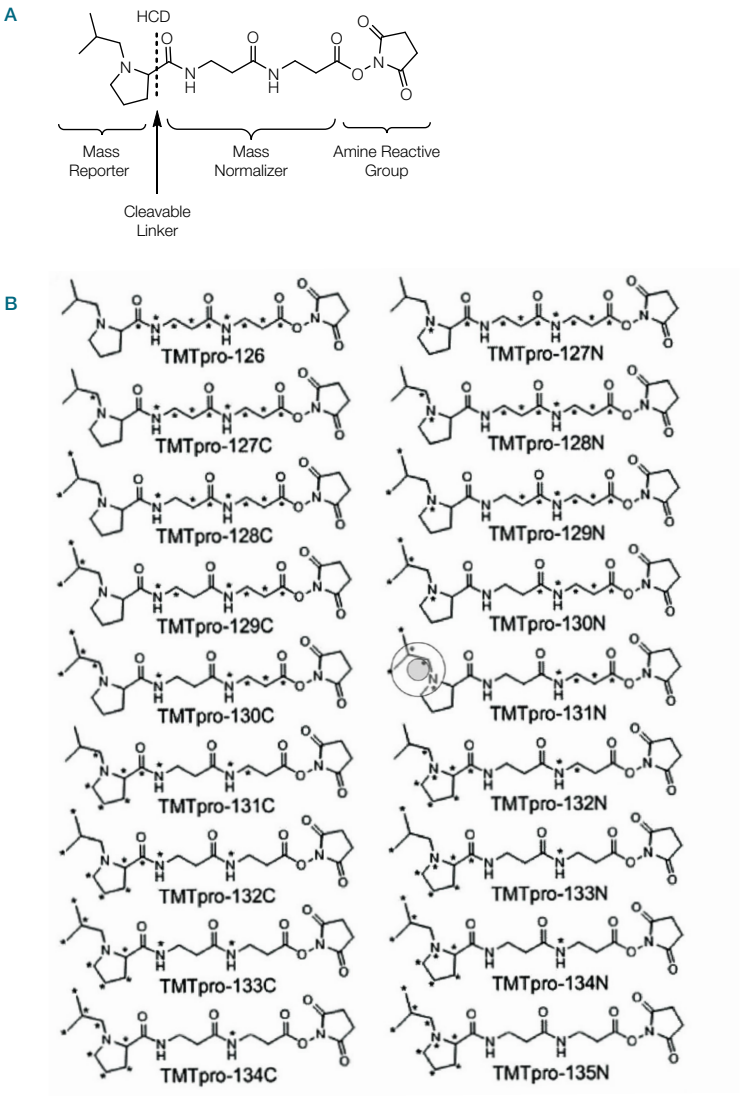

Figure 2. TMTpro 18-plex labeling. (A) Chemical structure of a standard TMT. (B) The asterisks on the structures represent the atoms containing heavy isotopes, either Nitrogen-15 or Carbon-13. Because the location of the heavy isotopes differs on each reporter ion cleaved during HCD, the mass differences are resolved in the MS² or MS³ scan.

TMTpro 18-plex label reagents are next-generation TMT solutions designed to increase sample multiplexing without compromising protein identification and quantitation. These label reagents are similar in design to the standard 6- to 11-plex TMT label reagents in that they are isobaric and amine-reactive, but the TMTpro reagents have longer balancer and isobutyl proline mass reporter regions. After MS^2 fragmentation, each TMTpro tag generates a unique reporter mass ranging from 126 to 134 Da. Thermo Scientific™ TMTpro 134C and TMTpro 135N label reagents increase sample multiplexing from 16-plex to 18-plex, enabling concurrent comparison of a larger number of proteomes.

TMT-labeled sample analysis workflow

A typical TMT-based quantitative proteomics study is shown in Figure 3. It begins with up to 18 samples, containing up to 18 different "treatments" including replicates and positive and negative controls. After the samples are denatured, reduced, alkylated, and enzymatically digested, and the peptides are labeled with the TMT reagent, the samples are combined to create one sample containing up to 18 proteomes. This sample can be fractionated to acquire more mass spectrometer data in separate runs for improved coverage and quantitation. Starting with 10 to 25 µg of material per sample channel, it's possible to consistently quantify over 8,000 proteins across 18 sample channels in 18 hours (12 fractions, each with 90 min runtimes) of LC-MS instrument time.

LC-MS quantitation methods for TMT experiments

Quantitative proteomics strategies using TMT reagents enable sample multiplexing and precise measurement of protein abundance. However, co-isolated ion interference can suppress accurate ratio quantification and thereby mask true differences in protein abundance across biological systems. Thus the method choice for quantifying TMT reporter ions must balance proteome depth of coverage with quantitation accuracy. The method chosen depends on sample complexity, the quantitation depth and accuracy needed, and the instrumentation used (Table 1). TMT experiments can be carried out using an Orbitrap instrument to collect HR MS^2 data with excellent depth, but this approach is subject to interferences that reduce quantitation accuracy for highly complex samples. The Thermo Scientific™ FAIMS Pro interface enables gas-phase fractionation to reduce chemical noise and matrix interferences, providing better accuracy for MS^2 quantification. Adding the FAIMS Pro interface to an MS^2 method therefore provides broad coverage with good quantitation accuracy.

Table 1. Comparison of TMT quantitation methods

	Depth	Accuracy	Instrumentation
HR-MS^2	●●●	●	Orbitrap MS
FAIMS-MS^2	●●●	●●	Orbitrap MS + FAIMS Pro interface
SPS-MS^3	●	●●	Orbitrap Tribrid MS
FAIMS-SPS-MS^3	●	●●	Orbitrap Tribrid MS + FAIMS Pro interface
RTS-SPS-MS^3	●●●	●●●	Orbitrap Tribrid MS + Real-Time Search
FAIMS-RTS-SPS-MS^3	●●●	●●●●	Orbitrap Tribrid MS + FAIMS Pro interface + Real-Time Search

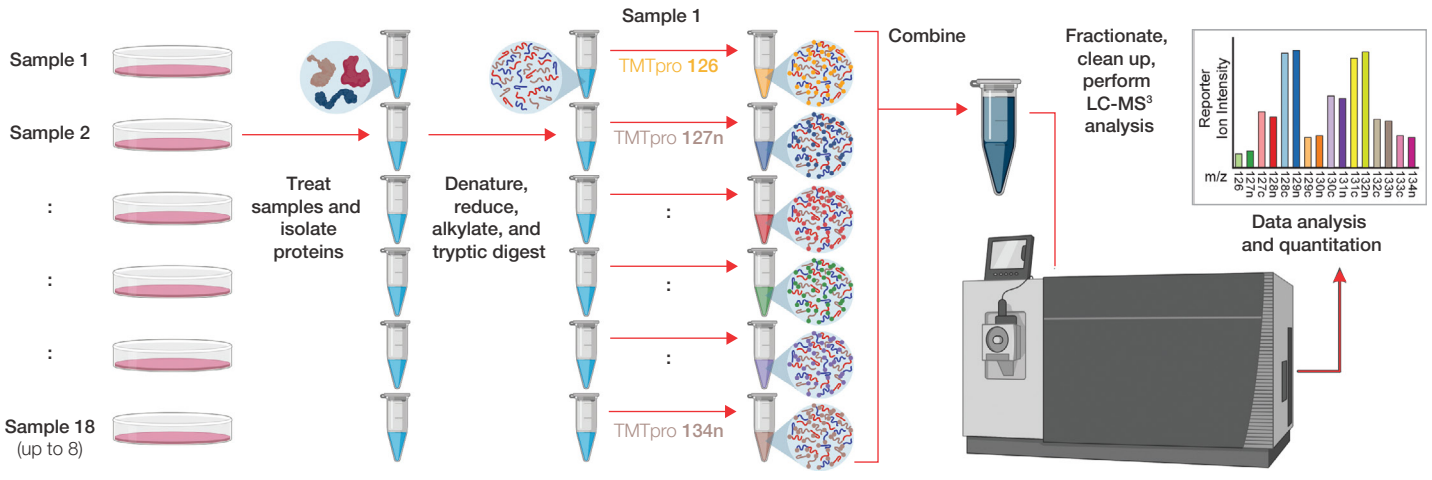

Figure 3. Standard TMT-based experimental workflow from sample to data analysis and quantitation.

When the highest quantitation accuracy is required, such as analysis of PTMs, then an Orbitrap Tribrid mass spectrometer using synchronous precursor selection (SPS) with an MS^3 scan for quantitation provides the higher quantitation accuracy. Synchronous precursor selection removes interferences prior to the MS^3 scan, increasing quantitation accuracy. The FAIMS Pro interface offers additional robustness to SPS-MS^3 workflows. However, greater quantitation depth and accuracy, as well as experimental speed, can be achieved using Real-Time Search (RTS) in combination with SPS-MS^3. During data acquisition, RTS uses the MS^2 scan to generate peptide spectrum matches from a FASTA database to trigger an MS^3 scan only when there is a positive peptide identification. This improves data acquisition efficiency while maintaining quantitation precision and accuracy. Adding the FAIMS Pro interface to the RTS-SPS-MS^3 workflow maximizes all aspects of workflow performance for complex samples and experimental designs.

Performance: TMT multiplexing with the Orbitrap Ascend Tribrid mass spectrometer

Since its commercialization in 2008, TMT isobaric labeling has co-evolved with Thermo Scientific Orbitrap mass spectrometer technology to increase experimental performance. While TMT multiplexing capacity has increased, enhancements like SPS-MS^3 and Real-Time Search have similarly increased TMT quantitation accuracy and speed. Introduced in 2022, the Orbitrap Ascend Tribrid mass spectrometer features new architecture that adds even more capabilities to enhance multiplexed quantitative proteomics.

Table 2 presents the increase in protein and peptide identification and quantitation achieved using the Orbitrap Ascend Tribrid mass spectrometer. In addition to identifying more unique peptides (4015) under the same experimental conditions, the Orbitrap Ascend Tribrid instrument increased the number of quantifiable peptides by more than 14,600 (about 21%). Protein identifications and quantifiable proteins were likewise increased by more than 200 and 490 (about 6%), respectively. The 6% increase is a substantial improvement in the depth of analysis achieved because it reflects quantification of very low-abundance proteins that were not observed by the Thermo Scientific™ Orbitrap Eclipse™ Tribrid mass spectrometer.

New Orbitrap Tribrid instrumentation design enhances TMT method performance

In SPS-MS^3 and RTS-SPS-MS^3 TMT quantitation methods, Orbitrap mass analyzer MS^1 scans detect, ion trap MS^2 scans identify, and Orbitrap mass analyzer MS^3 scans quantify ions. Previous Tribrid mass spectrometer designs used a single ion routing multipole (IRM), where ions are shuttled back and forth and accumulated by the c-trap. In this scheme, every ion must traverse the c-trap, limiting ion transmission and transfer efficiency, parallelizability, scan speed and cycle time, and therefore proteome depth. To advance TMT-based quantitative proteomics, the Orbitrap Ascend Tribrid mass spectrometer incorporates a dual-IRM design. Illustrated in Figure 4, the additional IRM is positioned in front of the c-trap, allowing more efficient and parallelizable Orbitrap mass analyzer scans. Ions can pass back and forth through the c-trap without delays because the additional IRM (IRM #1) can accumulate ions, freeing the c-trap from that step. For TMT-based experiments, the improved ion transfer and acquisition rate substantially boosts quantifiable peptides and quantitation of lower-abundance proteins, providing the greater proteomic depth and coverage that is essential to characterizing biological systems. Alternatively, the performance gains created by the dual-IRM design can be applied to shorter LC gradients to save time and increase sample throughput.

Table 2. Comparison of protein and peptide identification and quantitation using Orbitrap Ascend Tribrid mass spectrometer and Orbitrap Eclipse Tribrid mass spectrometer under identical experimental conditions, using RTS-SPS-MS^3

Instrument	Unique peptides	Quantifiable peptides (Sum SN > 160)	Protein IDs	Quantifiable proteins (Sum SN > 160)
Orbitrap Eclipse Tribrid mass spectrometer	91,080	68,755	8,682	8,294
Orbitrap Ascend Tribrid mass spectrometer	95,095	83,386	8,889	8,788

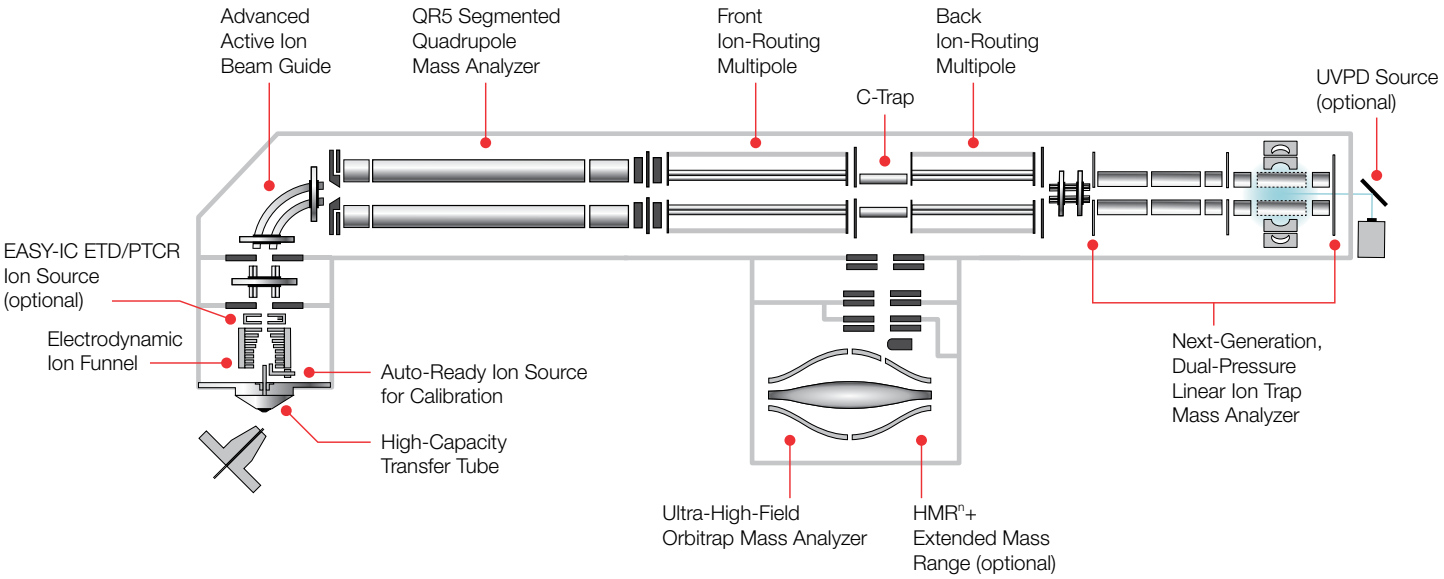

Figure 4. The Orbitrap Ascend Tribrid mass spectrometer with key hardware innovations shown on the schematic. Dual IRMs enable the simultaneous manipulation of up to three ion populations in parallel, increasing scan rate for methods like TMT that require long ion injection times.

Conclusion

Sample multiplexing using TMT isobaric labeling is a powerful strategy for performing proteome-wide biological assays which provide novel insights into cellular processes and pathways. TMTpro reagents allow simultaneous quantification of 9,000 proteins or more in up to 18 samples simultaneously, including positive and negative controls, time series, and replicates in a closed system with no missing values. When carried out with such rigor, TMT-based quantitative proteomics is becoming a premier biological assay. With added sensitivity, speed, and ease of use, the Orbitrap Ascend Tribrid mass spectrometer minimizes many of the challenges associated with TMT-based sample analysis, enabling quantification of samples with increased efficiency, depth, and coverage.

Learn more at **thermofisher.com/OrbitrapAscend**