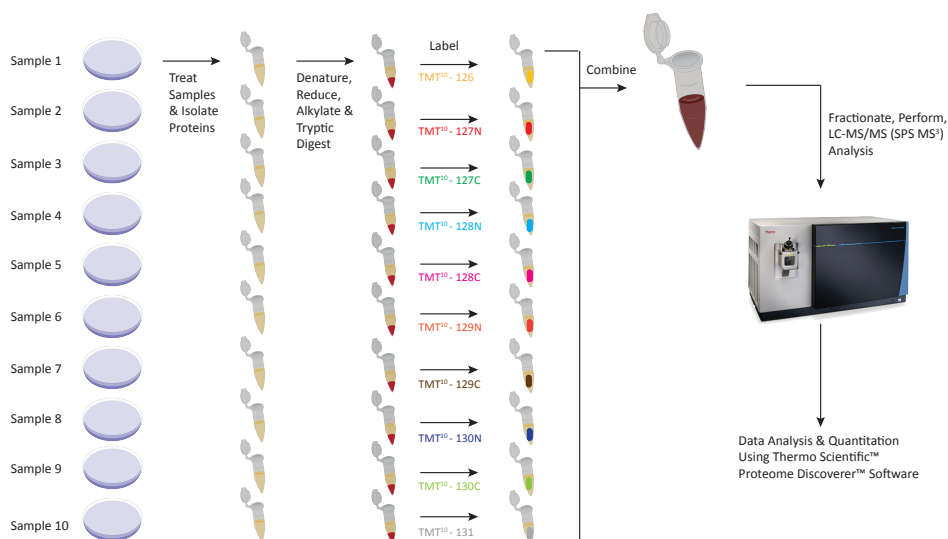


# HIGH THROUGHPUT QUANTITATIVE PROTEOMICS USING ISOBARIC TAGS

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Proteomics techniques are rapidly evolving to become a highly sensitive, quantitative, and high-throughput approach to analyzing global protein dynamics within a cell, tissue or an organism.

The ability to perform accurate protein quantification at low levels lets scientists unravel the complexity of protein interactions and track protein abundance changes in a wide variety of samples. When combined with multiplexing capabilities that measure increasing numbers of samples across varying conditions in a single experiment, quantitative proteomics can provide a deep and comprehensive understanding of the molecular mechanisms underlying biological processes and disease states.



Isobaric chemical tags, such as TMT tags, are used to quantify relative protein abundance changes in complex protein samples across multiple experimental conditions.

Critical advances in analytical technology are driving this revolution in proteomic analysis. Indeed, in the last decade, the innovation across the proteomic workflows has been transformative. Novel Tandem Mass Tag (TMT)-based labeling combined with ultra-high resolution accurate mass (UHRAM) mass spectrometry (MS) enable greater multiplexing capacity, which results in increased depth of quantitative proteomic analysis across larger numbers of samples. Specifically, Thermo Scientific™ TMT10plex™ isobaric mass tag labeling makes it possible to compare up to 10 samples in a single run.

The improved sensitivity and accuracy are achieved with UHRAM MS and synchronous precursor selection (SPS)-based MS<sup>3</sup> methods on the Thermo Scientific™ Orbitrap Tribrid™ systems, which provide the capability to probe low-abundance proteins and to accurately measure the most subtle changes in protein levels. This whitepaper describes the state-of-the-art technologies and methods for multiplexed quantitative proteomics and presents real-world applications from leading research laboratories.

### **TMT ISOBARIC TAGS**

Understanding complex biological phenomena at the proteome level requires a fast, unbiased, and sensitive method to comprehensively quantify proteins in a biological sample. Isobaric chemical tags are ideal for this approach, mainly because they can be used to identify and quantify relative changes in complex protein samples across several experimental conditions in a single analysis. They are compatible with various sample types, including those derived from cells, tissues, and biological fluids.

TMT reagents (Proteome Sciences®, commercially available from Thermo Fisher Scientific) are isobaric chemical tags consisting of a signature reporter group, a spacer arm, and an amine reactive group. The reagents covalently bind to the N-terminus of a peptide or to lysine residues. Upon MS/MS fragmentation, each version of the tag fragments and produces a unique reporter ion. In an experiment that compares several experimental conditions in a single analysis, the protein digest from each experimental condition is labeled with one of the isobaric versions of the TMT reagent. Afterwards, all the samples are pooled together and analyzed with LC-MS/MS. During the first dimension separation (RPLC), the same peptide labeled with different versions of the tag will elute together as they have the same chemical properties. On subsequent MS<sup>1</sup> analysis, these peptides will be detected simultaneously as a single indistinguishable precursor ion peak. Following MS/MS fragmentation of the precursor, the relative levels of each version of the tag used to label the peptide can be quantified by comparing the intensities of the unique reporter ion generated from each tag. Additionally, protein identification is achieved by matching the peptide fragment ions in the MS/MS spectrum to sequences in the appropriate database.

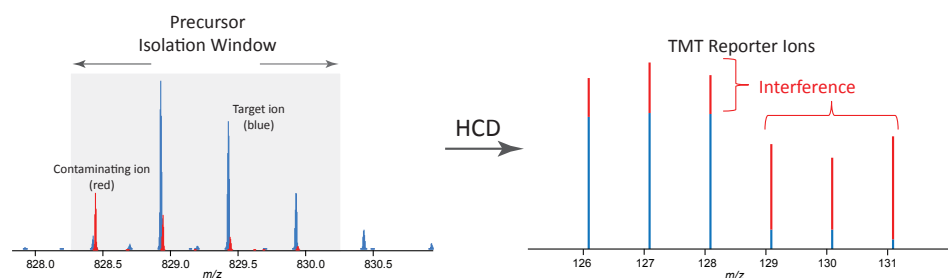
With the introduction of the TMT10plex Isobaric Mass Tag Labeling Kit, Thermo Fisher Scientific greatly increased the multiplexing potential of this experiment. The use of TMT10plex tags allows for simultaneous analysis of up to 10 different samples. This leads to increased throughput and more comprehensive quantitative analysis and results in fewer missing values.

**QUANTITATIVE ACCURACY OF THIS APPROACH IS HIGHLY DEPENDENT ON THE PURITY OF THE PRECURSOR ION POPULATION THAT IS SELECTED FOR MS/MS ANALYSIS.**

### MAXIMIZING QUANTITATIVE ACCURACY AND PRECISION

Traditional TMT experiments involved the isolation of precursor ions and generation of peptide fragment and TMT reporter ions in a single spectrum, producing MS/MS data that enabled both peptide identification and relative quantitation.

The quantitative accuracy of this approach is highly dependent on the purity of the precursor ion population that is selected for MS/MS analysis. However, the long-underestimated reality is that when dealing with complex digests, even rigorous pre-fractionation and subsequent reversed-phase liquid chromatography (RPLC) separation steps fail to remove all co-eluting isobaric species. This results in these interfering species being co-isolated and co-fragmented with the ions of interest. The reporter ions generated from fragmentation of these species are indistinguishable from the reporter ions generated from the parent ions of interest and contribute to a significant loss of quantitative accuracy and precision. A comprehensive account of this phenomenon appeared in the literature several years ago. Ting and co-workers from Harvard University used a two-proteome model to show that nearly all the quantitative measurements they obtained with a standard MS/MS approach were largely distorted by interfering ions, leading to significant compression of the reporter ion ratios and loss of quantitative accuracy and precision.<sup>1</sup>



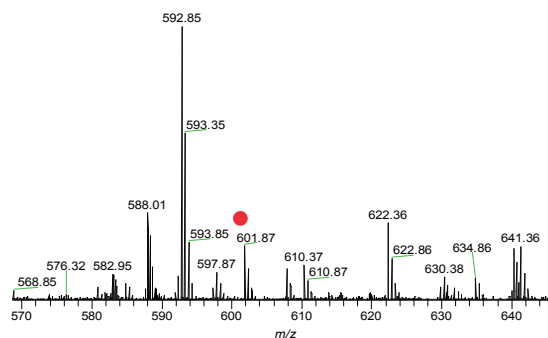
Interference at the MS level leads to distorted reporter ion ratios in the MS/MS spectra and, as a result, significantly affects quantitative accuracy, precision and dynamic range of MS/MS-based TMT quantitation.

Compression of the ratios is especially pernicious as it underreports true fold-change and leads to the unpredictable loss of quantitative values. The same group suggested using an MS<sup>3</sup>-based experiment as a novel solution to this problem, i.e., to restore the accuracy and precision of TMT quantitation. The addition of a MS<sup>3</sup> stage, triggered on one of the TMT-labeled MS<sup>2</sup> fragment ions, largely eliminated the negative effects of the interfering signals. However, it resulted in an overall decrease in sensitivity, leading to a significant loss in overall numbers of quantifiable peptides.

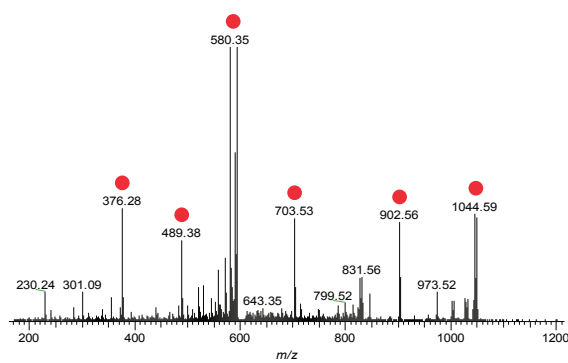
Synchronous precursor selection (SPS) for MS<sup>3</sup> is a novel method designed to overcome the problem of isobaric ion contamination and to restore quantitative accuracy and precision while mitigating sensitivity loss of the MS<sup>3</sup> experiment. The SPS method begins with selection of the parent ion in the MS scan, followed by its isolation in the quadrupole and fragmentation by collisionally induced dissociation (CID) in the ion trap. Following fragmentation, SPS enables simultaneous isolation of up to 20 MS<sup>2</sup> fragment ions. A select group of MS<sup>2</sup> fragment ions are

then transferred back into the ion routing multipole (IRM) where they undergo higher energy collisional dissociation (HCD) fragmentation, with the resulting MS<sup>3</sup> fragments subsequently detected in the Orbitrap analyzer. The use of SPS dramatically increases the reporter ion signal intensity and improves the ratio accuracy, due to improved ion counting statistics, leading to a significant increase in the number of peptides quantified.

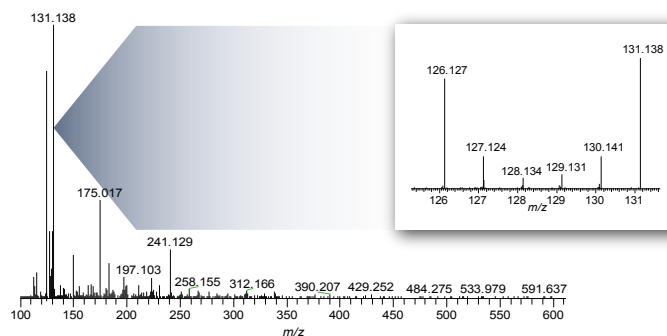
Precursor Ion



CID MS<sup>2</sup>, Ion Trap



HCD MS<sup>3</sup>, Orbitrap



**FOLLOWING THESE SUCCESSFUL REPORTS, THE SPS MS<sup>3</sup> TECHNIQUE HAS BEEN DEVELOPED AND IMPLEMENTED ON ORBITRAP FUSION AND ORBITRAP FUSION LUMOS TRIBRID MASS SPECTROMETERS.**

SPS allows for isolating several MS<sup>2</sup> precursors at the same time, increasing reporter ion signal in the MS<sup>3</sup> spectrum and leading to significantly improved quantitation.

The initial successful application of the SPS MS<sup>3</sup> method in proteomics was published by Graeme McAlister and colleagues from Harvard Medical School. They described the benefits of this technique in a study of differential protein expression across cancer cell line proteomes.<sup>2</sup> “Using isolation waveforms with multiple frequency notches we co-isolated and cofragmented multiple MS<sup>2</sup> fragment ions, thereby increasing the number of reporter ions in the MS<sup>3</sup> spectrum 10-fold over the standard MS<sup>3</sup> method.” The same scientists report that, “By increasing the reporter ion signals, this method improves the dynamic range of reporter ion signal variance, and ultimately produces more high-quality quantitative measurements.” Furthermore, “we reproducibly quantified

172,704 protein abundance changes between individual cell lines. To achieve similar breadth of quantitative analysis, including eight samples and three biological replicates, would require 24 separate experiments using a simple duplex quantitative method (e.g. SILAC). In contrast, we only need three separate experiments using the TMT 8plex workflow.” Following these successful reports, the SPS MS<sup>3</sup> technique has been developed and implemented on Orbitrap Fusion and Orbitrap Fusion Lumos Tribrid mass spectrometers.

### **THE POWER OF ACCURATE TMT SPS MS<sup>3</sup>-BASED QUANTITATIVE PROTEOMICS**

The studies that follow, and many others in the literature, reflect the broad scope and diversity of quantitative proteomics applications in drug discovery and basic biological research. Newly developed methods based on TMT SPS MS<sup>3</sup> technology enable accurate, sensitive, and reproducible analyses with much higher throughput, allowing researchers to rapidly explore the proteome to greater depths by enabling the measurement of changes in protein localization and quantification of minute changes in low abundance proteins.

### **QUANTITATION OF RNA-INTERACTING PROTEINS**

Jeannie Lee, Ph.D, Wilhelm Haas, Ph.D., and colleagues at Massachusetts General Hospital and Harvard Medical School are working together to characterize the Xist interactome.<sup>3</sup> Xist is a long noncoding RNA that oversees the silencing of one of the two X chromosomes—the inactive X (Xi)—in female mammals. The team developed an RNA-centric proteomic method called identification of direct RNA-interacting proteins (iDRiP). One component of iDRiP is TMT-based multiplexed quantitative analysis using an Orbitrap Fusion mass spectrometer.

Utilizing the iDRiP methodology the researchers not only identified proteins already known to interact with Xist, but also identified proteins enriched on the Xi that had not previously been shown to interact directly with Xist. They reported that more than 80 proteins were at least 3-fold enriched over background and more than 200 proteins were at least 2-fold enriched. These proteins, comprising the Xist interactome, fall into several functional categories. The researchers performed targeted inhibition studies that demonstrated the ability to destabilize Xi silencing by disrupting multiple components of the interactome.

As Xi is “a reservoir of functional genes that could be tapped to replace expression of a disease allele on the active X,” discovering a mechanism to un-silence one or more genes on Xi in a targeted and controlled manner would be of great interest, according to the authors.

The iDRiP method and quantitative MS-based proteomic technology made it possible to carry out experiments “to identify the actual proteins that interact with Xist, and to distinguish small quantitative differences in protein levels,” says Prof. Lee. “We identified 100-200 new proteins that had not been shown to interact with the Xist before.”

Prof. Haas highlights the advantages that multiplexing using 10plex TMT labeling provides in helping to close the gap between proteomics and high-throughput genomic methods. The ability to test more samples and to analyze duplicates in one experiment “increases the statistical power,” says Prof. Haas. “We can select biological conditions where we expect to see changes in a complex, can run samples in duplicate, and examine the differences under varying conditions.”

### **COMPARATIVE PROTEOMICS IDENTIFIES A NOVEL SUBSTRATE**

Christopher Overall and colleagues from University of British Columbia, BC Children’s Hospital, Novartis Institutes for BioMedical Research, and Thermo Fisher Scientific used TMT10plex TAILS N-terminal Peptide proteomics to identify new

substrates for the paracaspase known as MALT1, an important drug target in lymphoma.<sup>4</sup> Mutated MALT1 amplifies lymphocyte responses during activation of the NF- $\kappa$ B pathway and in subtypes of B-cell lymphoma. The researchers performed comparative proteomic analysis using B cells from the only known living patient who is homozygous for MALT1 mutation (MALT1<sup>mut/mut</sup>) with those from healthy (MALT1<sup>+ /mut</sup>) family members.

Terminal amine isotopic labeling of substrates (N-TAILS) “enriches and identifies protease substrates simultaneously with their cleavage sites” using tandem MS. The 10plex TMT labeling made it possible to do the experiment “in a single mass spectrometric analysis to reduce experimental variability.” High-accuracy analysis of TMT reporter ion ratios using the SPS MS<sup>3</sup> method to reduce peak interferences led to the identification with high confidence of HOIL1 as a new MALT1 substrate.

“For the first time we used proteomics alone to work out the phenotype of a patient with an autoimmune disorder and to identify the defect,” says Dr. Overall. He emphasizes the risks of under-sampling when comparing two biological samples using conventional proteomics approaches, which may lead scientists to jump to wrong conclusions. Achieving less than full coverage of the proteome, not being able to analyze replicates, and relying on the results of MS1 spectra can all increase the risk of under-sampling. Dr. Overall adds that, with 10plex TMT labeling, “you have 10 chances to amplify the signal (in MS1) and detect low abundance proteins,” which might otherwise be missed.

“10plex was a huge advantage,” says Overall. “With only three or four channels, the data would not have generated a curve. Ten channels yield a curve, and a smooth curve.”

#### SPATIAL PROTEOMICS AT SUBCELLULAR SCALE

Kathryn Lilley, University of Cambridge, U.K., is using high-throughput quantitative proteomics to generate high-resolution subcellular protein maps. By designing experiments that examine the subcellular localization of proteins at a global level, rather than focusing on protein abundance, Dr. Lilley demonstrates that changes in protein localization when a cell is perturbed may be as important as changes in protein levels when it comes to understanding cell function and dysfunction and the underlying cause of many diseases.

Quantitative proteomics can be used to study protein localization at the level of the whole cell or of subcellular organelles. One way to sample the spatial proteome is to first purify a subcellular organelle of interest, such as the mitochondria, prior to analysis. Another approach is to fractionate the entire cell and examine how the proteins are distributed across different fractions, an approach known as protein correlation profiling (PCP). There are multiple cellular fractionation techniques, including centrifugation coupled with differential detergent solubility. Alternatively, a method that gives better resolution of subcellular niches involves separating the organelles based on their buoyant density using equilibrium density centrifugation, followed by quantitative proteomic methods to correlate the protein distributions. Dr. Lilley’s lab is using this method to determine where a particular protein is localized, whether it is present in multiple locations, what other proteins it interacts with, and how these findings vary for different protein isoforms. She uses quantitative proteomic analysis to detect dynamic changes on a proteome-wide scale, a cell-wide scale, and with sub-organelle resolution. As this work progresses, future questions will focus on how these things change when a cell is perturbed, for example, by disease or as a result of a drug.

Dr. Lilley applies this method known as LOPIT—localization of proteins using isobaric tagging—to identify proteins, and uses organelle markers to define clusters of proteins that co-localize. This provides a steady-state distribution of proteins across organelles and facilitates the identification of novel organelle proteins. Combining this method with TMT10plex labeling, analyzing samples with accurate SPS MS<sup>3</sup> analysis, and developing the informatics tools to manage the complex data produced allowed Dr. Lilley to “hyper-plex LOPIT.” She is using this higher multiplexed method to develop a comprehensive cellular map of a self-renewing mouse embryonic stem cell.<sup>5</sup>

## CRISPR-CAS9 GENOMIC EDITING CHARACTERIZES THE PRION PROTEIN KNOCKOUT PROTEOME

Mohadeseh Mehrabian and co-workers at University of Toronto combined CRISPR Cas9-based gene editing tools to create knockout clones that lack prion protein (PrP), which is implicated in the neurotoxicity of prion disease and Alzheimer's disease. Using quantitative global proteome analysis, they determined the relative abundance of more than 3,000 proteins in an effort to characterize the molecular function of PrP.<sup>6</sup> The researchers compared the results obtained from total proteome analysis of the PrP-deficient cells and of the original cells. They identified more than 120 proteins whose levels changed on PrP knockout in this cell model. The proteins that exhibited the greatest change in abundance were associated with the organization of extracellular matrix, cell-cell junctions, and the cytoskeleton, shedding insight into biological pathways critical for PrP-related neurotoxicity.

## THE IMPORTANCE OF pRb FOR MITOCHONDRIAL FUNCTION

In a different application of the SPS MS<sup>3</sup> method, research groups from Massachusetts General Hospital and Harvard Medical School showed that loss of function of the retinoblastoma tumor suppressor (pRb) – a frequent event in cancer – leads to mitochondrial defects in mouse tissue.<sup>7</sup> Intriguingly, it was shown that this effect is reflected in concentration changes of mitochondrial proteins, but not in the concentrations of the corresponding mRNA molecules. This shows that this quantitative approach provides unique possibilities to screen for biological effects that are inaccessible when using other technologies. The mitochondrial defect caused by the loss of function of pRb has the potential to lead to new treatment strategies for specific cancers.

## References

1. Ting, L.; Rad, R.; Gygi, S.P.; Haas, W. [MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics](#) *Nature Methods* **2011**, *8*, 937-940.
2. McAlister, G.; Nusinow, D.; Jedrychowski, M.; Wuhr, M.; Huttlin, E.L.; Erickson, B.K.; Rad, R.; Haas, W.; Gygi, S.P. [MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes](#). *Anal. Chem.* **2014**, *86*, 7150-7158.
3. Minajigi, A.; Froberg, J.E., Wei, C.; Sunwoo, H.; Kesner, B.; Colognori, D.; Lessing, D.; Payer, B.; Boukhali, M.; Haas, W. [A comprehensive Xist interactome reveals cohesin repulsion and an RNA-directed chromosome conformation](#). *Science* **2015**, *349*.
4. Klein, T.; Fung, S.; Renner, F.; Blank, M.A.; Dufour, A.; Kang, S.; Bolger-Munro, M.; Scurll, J.M.; Priatel, J.J.; Schweigler, P.; Melkko, S.; Gold, M.R.; Viner, R.I.; Regnier, C.H.; Overall, C.M. [The paracaspase MALT1 cleaves HOIL1 reducing linear ubiquitination by LUBAC to dampen lymphocyte NF- \$\kappa\$ B signalling](#). *Nat. Commun.* **2015**, *6*.
5. Christoforou, A.; Mulvey, C.; Breckels, L.; Geladaki, A.; Hurrell, T.; Hayward, P.C.; Naake, T.; Gatto, L.; Viner, R.; Arias, A.M.; Lilley, K.S. [A draft map of the mouse pluripotent stem cell spatial proteome](#). *Nat. Commun.* **2016**, *7*.
6. Mehrabian, M.; Brethour, D.; MacIsaac, S.; Kim, J.K.; Gunawardana, C.G.; Schmitt-Ulms, G. [CRISPR-Cas9-based knockout of the prion protein and its effect on the proteome](#). *PLOS ONE* **2014** *9(12)*, e114594.
7. Nicolay, B.; Danielian, P.; Kottakis, F.; Lapek Jr., J.D.; Sanidas, I.; Miles, W.O.; Dehnad, M.; Tschop, K.; Gierut, J.J.; Manning, A.L.; Morris, R.; Haigis, K.; Bardeesy, N.; Lees, J.A.; Haas, W.; Dyson, N.J. [Proteomic analysis of pRb loss highlights a signature of decreased mitochondrial oxidative phosphorylation](#). *Genes Dev.* **2015**, *29*, 1875-1889.