Achieving robust, accurate TMT quantitation efficiency with Tribrid technology

Goal

To provide conclusive arguments to adopt the revolutionary Tribrid technology for a high throughput quantitative proteomics workflow.

Summary

Multichannel, isobaric tagging offers confident and reliable quantitation information for biological experiments. The multiplexing potential of the Thermo Scientific™ TMT10plex™ Isobaric Mass Tag Labelling Kit (Proteome Sciences,® commercially available from Thermo Fisher Scientific) is fully maximized when used with a SPS MS^3-based method exclusive to Thermo Scientific™ Tribrid™ mass spectrometers. Uniquely designed Tribrid MS systems are capable of performing synchronous precursor selection (SPS) multi-notch MS^3 quantitative analyses to improve experimental throughput and yet still providing the depth and coverage, eliminating issues encountered with TMT ratio distortions caused by interfering ions, without neglecting sensitivity that is often observed. Other commercially available mass analyzers have limitations to handle highly multiplexed isobaric tags as they lack the analytical performance features, i.e. high resolution and multiple analyzers for concurrent isolation and detection of ions, to accomplish a SPS MS^3-type technique for quantitation.

Introduction

Multiplexing protein quantitation has found broad adoption in biological research recently, with applications ranging from human disease and cell signaling studies to microbiology research. Mass spectrometry becomes a highly essential analytical tool in relative protein quantitation comparison studies to derive functional understanding of biological perturbations. Currently, there are a few methods available for relative quantitation and one such method involves the use of isobaric chemical tags. These multichannel isobaric tags offer greater parallelization potential in quantitative mass spectrometry experiments and have the key advantages as listed:

- *Increased throughput* is achieved with the concurrent MS analysis of multiple samples and would consume less sample amounts, and require less instrument time.
- *Fewer missing values* are guaranteed as identification and quantification are done in a single run, without worrying about irreproducibility.
- *Sample origin flexibility* increases experimental possibilities as samples can be derived from cells, tissues or biological fluids and are compatible with the isobaric tags.
- *Increased multiplexing* can be accomplished as more than three biological and/or experimental conditions can be compared (up to 10 samples with commercially available 10plex tags).
- *Multiple comparisons and improved statistics* by incorporating replicates with multiple conditions: dose-response, time-course, multiple tissues, subcellular fractions, etc.

Table 1. Depending on your research application, tandem mass tags (TMT) come in different formats.

Tag Chemistry	Available TMT Channels	Application Areas
Amine-reactive TMT	TMT0, TMT2plex, TMT6plex, TMT10plex	Total peptide quantitation, improved ETD fragmentation for glycopeptides
Sulfhydryl-reactive iodoTMT	Sixplex	Enrichment of cysteine-labeled peptides, cysteine PTM quantitation and general quantification, Biotin Switch assay
Carbonyl-reactive aminoxyTMT	Sixplex	Glycan, steroid and oxidized peptide quantitation

An analytical approach to multiplexed proteomics

The use of TMT-based labeling in combination with ultra-high resolution accurate mass (UHRAM) mass spectrometry provides better quantitative performance across more sample sets due to availability of multiplexing capabilities. More samples with differentiated conditions can be compared in a single MS experiment with the use of TMT-labeling. The high throughput TMT10plex isobaric mass tag labeling allows simultaneous comparison of up to 10 different samples in a single run. The combined use of TMT isobaric tags with SPS MS^3 scan method achieved on a Tribrid mass spectrometer provides greater selectivity, sensitivity, and the ability to probe low-abundance proteins to accurately measure the most subtle changes in protein expression.

MS application

Issue 1: Working with limited sample, limited instrument analysis time, and statistical variations.

Label free quantitation (LFQ) experiments are considered to be sample intensive and require biological and technical replicates. Multiple LC-MS runs are unavoidable, necessitating substantial instrument time to obtain meaningful statistics across the samples. Potential issues with this technique may include limited sample quantity and impact on reproducibility as run-to-run variations are introduced, possibly causing statistical errors and consequently inaccurate reporting of experimental results.

Issue 2: Analyses limited by number of observable experimental conditions, compatibility of labeling technique with sample types.

SILAC quantitation experiments improve quantitation throughput and lowers samples variation but limits sample comparison of up to only three experimental conditions. MS^1 spectral complexity increases and proteins have to be metabolically labelled in cell culture, thus restricting the various sample types that can utilize this technique.

To resolve the aforementioned two issues, TMT isobaric mass tagging introduces the multiplexing potential in an experiment allowing simultaneous analysis of up to ten different samples. This increases MS analysis throughput and gives a more comprehensive quantitative analysis allowing for improved statistical analysis and fewer missing values. Attaining quantitative accuracy however, is highly dependent on the purity of the precursor ion population selected for MS/MS analysis.

Even with the most rigorous pre-fractionation followed by reversed-phase liquid chromatography separation, the issue of co-elution of isobaric species cannot completely be eliminated. The issue of co-isolation and co-fragmentation of interfering ions with ions of interest, further impact quantitation accuracy and precision (Figure 1). There have been reported phenomena of reporter ion ratio compression associated with isobaric tagging. This distortion in TMT ratios influences the final reporter ion population and results in the underreporting of true fold changes and true reporter ion intensities, leading to unpredictable losses of quantitative values.

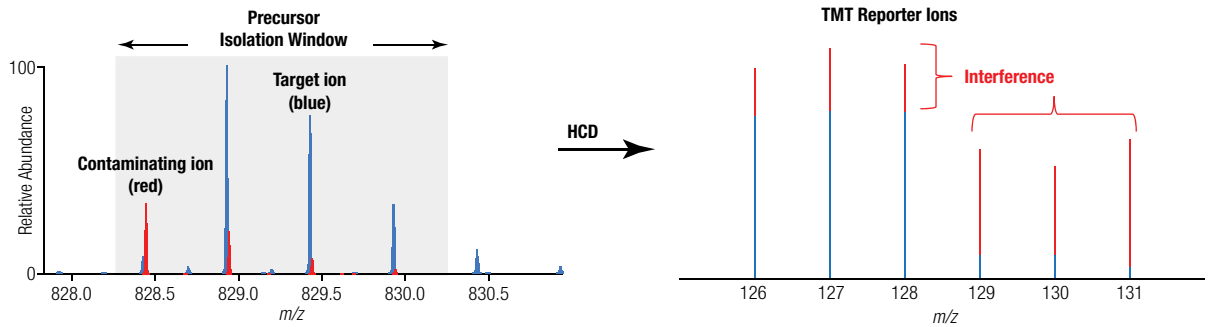

Figure 1. Interference at MS level leads to reporter ion ratios distortion in the MSMS spectra, therefore affecting quantitative accuracy, precision, and dynamic range of MSMS-based TMT quantitation significantly.[1]

An MS^3-based experiment has been demonstrated to mitigate this problem and restore the accuracy and precision of TMT quantitation.[2] This novel solution resolves the issue with interference ions but causes an overall loss in sensitivity and the number of quantifiable peptides.

Solution and benefits

To overcome the isobaric ion contamination problem and still maintain quantitation accuracy and precision without the resultant loss in sensitivity, a synchronous precursor selection (SPS) for MS^3 method was designed and implemented. SPS is based on the concept of using isolation waveforms with multiple frequency notches.

How SPS MS^3 works

The SPS method begins with parent ion selection in the MS scan, followed by its isolation in the quadrupole and fragmentation by collisional induced dissociation (CID) in the ion trap. After fragmentation, SPS facilitates simultaneous isolation of up to 20 MS^2 fragment ions. A selected group of MS^2 fragment ions gets transferred back into the ion routing multipole (IRM) and are subjected to high energy collisional dissociation (HCD) fragmentation. The resultant MS^3 fragments undergo detection in the Orbitrap analyzer (Figure 2).

The consequent effect of using SPS with MS^3-based methods is a dramatic increase in reporter ion signal intensity compared to a single notch MS^3 experiment and improvement in reporter ion ratio accuracy. These improvements were enabled by improved ion counting statistics that leads to an apparent and significant increase in the number of peptides quantified.

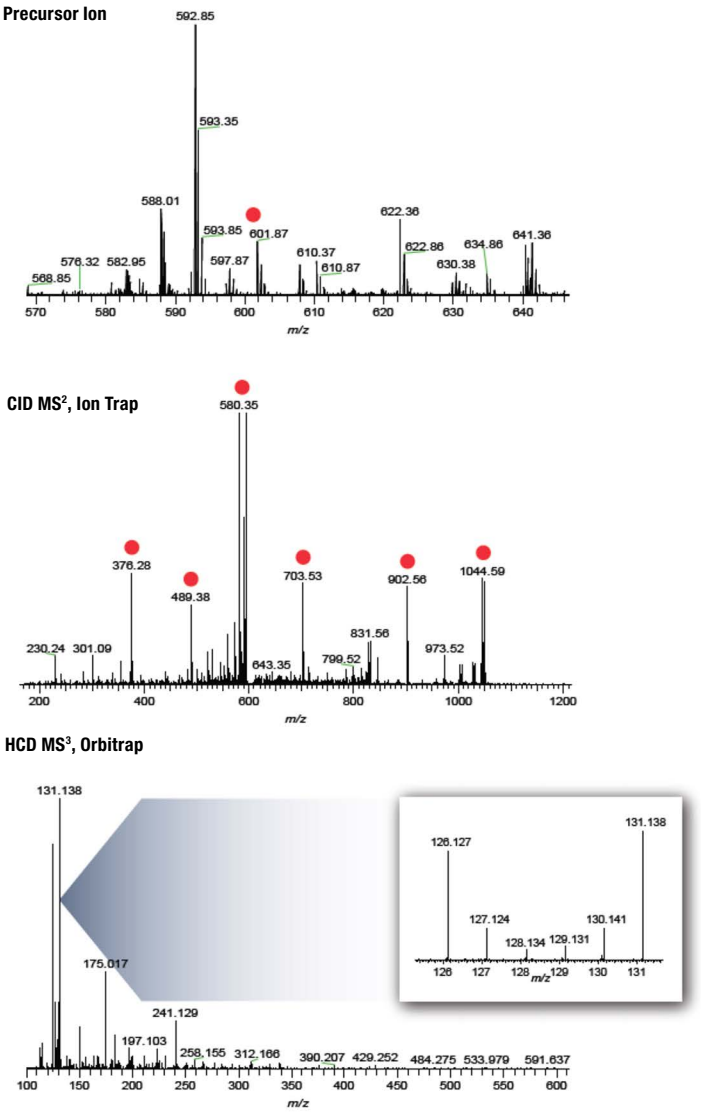

Figure 2. SPS facilitates the isolation of several MS^2 fragments simultaneously, resulting in increased reporter ion signal in the MS^3 spectrum and improving the depth of quantitation significantly.

Differentiating our technology

The use of multichannel isobaric tagging revolutionizes quantitative proteomics, given its multiplexing potential to tackle issues with throughput and missing values that impact coverage and spectral complexity over other labeling quantitation techniques. Problems with reporter ion ratio distortion due to co-isolation and co-fragmentation of interference species are however, inherent with this technique.

An initial study published by Lily Ting and colleagues from Harvard Medical School[2] demonstrated that performing MS^3 on one of the TMT-labeled MS^2 fragment largely reduced the negative effects of interfering signals. Although this method reduces the contribution of interfering signals dramatically, it had an adverse effect on overall sensitivity.

With this in mind, Graeme McAlister and colleagues at Harvard Medical School went on to apply the SPS MS^3 method and described the technique's benefits in a study of differential protein expression across cancer cell line proteomes.[3] "Using isolation waveforms with multiple frequency notches, we co-isolated and co-fragmented multiple MS^2 fragment ions, thereby increasing the number of reporter ions in the MS^3 spectrum 10-fold over the standard MS^3 method." Furthermore, "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." This research group "reproducibly quantified 172,704 protein abundance changes between individual cell lines." This technique is highly advantageous over others when it comes to achieving similar breadth of quantitative

analysis with multiple samples and biological replicates, albeit in lesser number of analytical experiments on the mass spectrometer, thus providing greater throughput and coverage. Following these successful reports, the SPS MS^3 technique was developed and implemented on the Orbitrap Fusion and Orbitrap Fusion Lumos Tribrid mass spectrometers. The use of SPS MS^3 undoubtedly combines multiplexing capacity with quantitative sensitivity and accuracy, providing greater informational value available from proteomic experiments.

Hardware Benefits

Key hardware improvements have been made on the Tribrid MS systems that contribute to greater sensitivity, multiplexing, multi-stage fragmentation capabilities and faster acquisition scan rates up to 20 Hz. A brighter ion source was introduced for increased sensitivity, which includes a combination of a high capacity transfer tube (HCTT) that increases ion flux into the MS system and the electrodynamic ion funnel (EDIF) that focuses ions after the HCTT (exclusively available on Orbitrap Fusion Lumos instruments). The ion source optics provide a 2–5 fold increase in ion transmission, resulting in sensitivity improvement over previous generations of ion optics found on other ion trap Orbitrap hybrid platforms. Selectivity and ion transmission are positively impacted with the segmented quadrupole. Improved transmissions at higher resolutions are also observed across the *m/z* range and within narrow isolation windows (Figure 3). This is particularly advantageous when working with complex peptide mixtures as tighter precursor ion isolation can be applied without loss in peptide identifications and would benefit TMT experiments which are highly reliant on precursor ion purity (Figure 4).

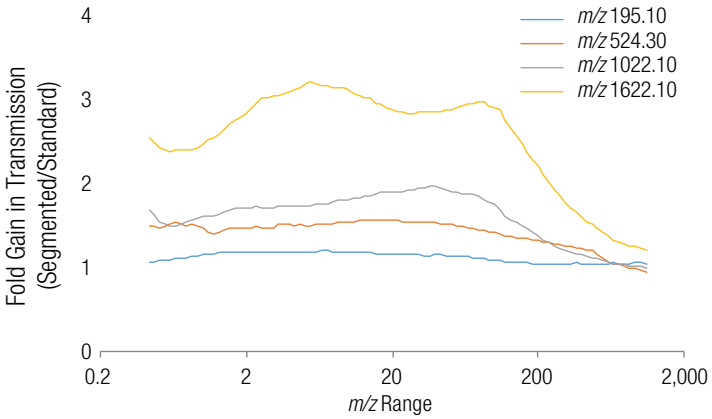

Figure 3. Improved selectivity of precursors with adoption of segmented quadrupole.

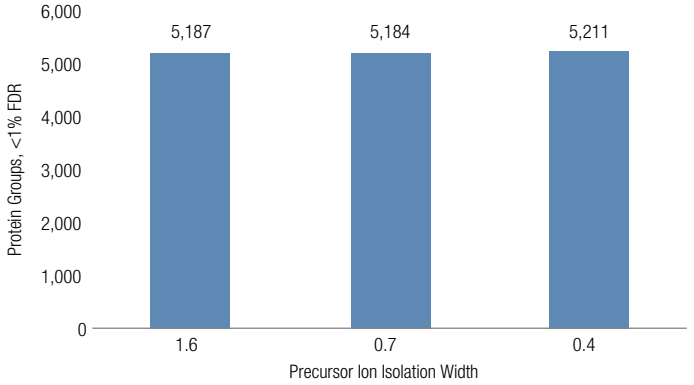

Figure 4. Use of brighter ion source and segmented quadrupole allows use of 0.4 µ isolation without loss of IDs for a 1 µg HeLa digest standard on an Orbitrap Fusion Lumos MS.

The Tribrid-based mass spectrometers are equipped with three analyzers:

- Ultra-high-field Orbitrap mass analyzer: Offers resolution exceeding 500,000 resolving power at *m/z* 200 and scan speeds up to 20 Hz at 15,000 FWHM.
- Ion Routing Multipole: Facilitates parallel analysis and performance of HCD at any MS^n stage.
- Dual-Pressure Linear Ion Trap: Performs MS^n and sensitive mass analysis of four fragmentation types (CID, HCD, ETD HD and EThcD HD)

Tribrid architecture enables full parallelization of complex modes of analysis, allowing the concurrent isolation of ions with one analyzer and detection with the other two analyzers. The use of the SPS MS^3 technique requires the analyzers on the Tribrid MS system to work in sync. This includes implementation of multi-notch frequency isolation waveforms in the ion routing multipole to isolate ions of interest, collisional induced dissociation (CID) fragmentation of precursor, followed by high energy collisional dissociation (HCD) fragmentation of reporter ions to obtain MS^3 spectra. The Tribrid systems demonstrate clear advantages in the expanded flexibility offered through different fragmentation techniques and MS^n whereas other Orbitrap MS platforms are limited in the available fragmentation techniques and may not come with a linear ion trap analyzer to perform MS^n fragmentation. In the case for TMT analysis, these other Orbitrap systems do not have the built-in SPS MS^3 feature and would prove challenging when trying to overcome the known sensitivity issue associated with isobaric tagging co-isolation of contaminant ions. Previous generation Orbitrap hybrids are sufficient in performing single notch MS^3 experiments, but cannot perform multiple precursor isolation (SPS) as well.

Table 2. TMT quantitation capability of various sample types on different Orbitrap based instrument platforms.

Experiment	Orbitrap Tribrid Series		Q Exactive Series		Orbitrap Elite	
Complex Mixture	MultiNotch MS^3	+++++	MS^2	+	Singlenotch MS^3	++
Fractionated Mixture	MultiNotch MS^3	+++++	MS^2	++	Singlenotch MS^3	+++
TMT-Ab Enriched Sample	MS^2	+++++	MS^2	++++	MS^2	++++
Simple Mixture	MS^2	+++++	MS^2	++++	MS^2	++++

High resolution plays a crucial role for TMT analyses as the mass differences between TMT10plex reporter ion isotopologues are extremely small. High resolution accurate mass analyzers would be necessary to resolve these reporter ions (at least 50K at *m/z* 200). Due to the nature of quadrupole time-of-flight detection mechanism, other competitive MS technologies may not be optimal in striking the balance between achieving superior resolving power and good detection sensitivity.

The power of accurate TMT SPS MS^3-based quantative proteomics

Many other studies 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^3 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.

Specifications

Product specifications for the Orbitrap Fusion Tribrid MS and Orbitrap Fusion Lumos Tribrid MS systems can be found by using the links below:

Thermo Scientific Orbitrap Fusion Tribrid Mass Spectrometer

Thermo Scientific Orbitrap Fusion Lumos Tribrid Mass Spectrometer

For sole source specifications, contact your local sales representative or contact us at:
www.thermofisher.com/grantcentral

Why choose Orbitrap?

Research trends and analytical needs have driven mass spectrometry innovation, especially in the last decade. Mass spectrometers of today must be equipped with superior performance features such as high resolution, mass accuracy, dynamic range and fast scanning capabilities in order to fulfil rigorous experimental demands and handle extremely complex samples. In today's research, these same instruments have to provide the flexibility to carry out a variety of analytical techniques including multiplexing, multi-stage fragmentation and multiple dissociation techniques, in addition to being highly robust and giving consistent performance for high throughput analysis. Since its introduction in 2005, Orbitrap technology has revolutionized mass spectrometry based research to meet these various challenges across multiple application and fields of interest. The exceptional value of Orbitrap based MS systems in delivering uncompromised analytical performance and achieving greater experimental possibilities have been well-recognized by the scientific community. Adoption of Orbitrap technology over the years has grown exponentially with the proven increase in numbers of Nature and Science family publications (Figure 5). Furthermore, with the implementation of SPS MS^3 functionality for isobaric labeling experiments, we see an increase in recognition for the analytical benefits this method brings to various research studies in the field (Figure 6).

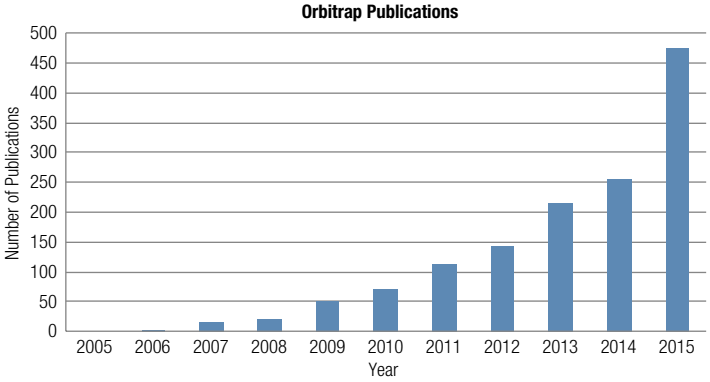

Figure 5. Rising trend in number of Orbitrap MS based research publications in Nature and Science journals over the past years since introduction in 2005.

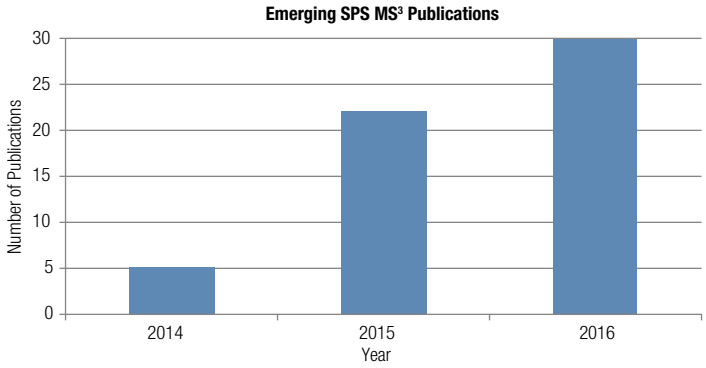

Figure 6. Emerging trend in increased number of publications focusing on SPS MS³ application since its implementation on Orbitrap Tribrid mass spectrometers.

Table 3. Which Orbitrap system is right for my research?

Instrument Attributes	Q Exactive Focus MS	Q Exactive MS	Q Exactive Plus MS	Q Exactive HF MS	Orbitrap Elite MS	Orbitrap Fusion MS	Orbitrap Fusion Lumos MS
Analyzer	Orbitrap	Orbitrap	Orbitrap	Ultra High Field Orbitrap	Hybrid: Linear ion trap, Orbitrap	Tribrid: Quadrupole with dual pressure linear ion trap, Orbitrap D20	Tribrid: Quadrupole with dual pressure linear ion trap, Orbitrap D20
Mass Range	*m/z* 50–2000	*m/z* 50–6000	*m/z* 50–6000	*m/z* 50–6000	*m/z* 50–2000; *m/z* 200–4000	*m/z* 50–6000	*m/z* 50–6000
Maximum Resolution @ *m/z* 200	70,000	140,000	140,000	240,000	240,000	500,000	500,000
Scan Speed	12 Hz	12 Hz	12 Hz	18 Hz	4 Hz	20 Hz	15 Hz
Top N/MS^n	Top 2 $ddMS^2$	Top 2 $ddMS^2$	Top 2 $ddMS^2$	Top 2 $ddMS^2$	MS^n, n = 1 to 10	MS^n, n = 1 to 10	MS^n, n = 1 to 10
Mass Accuracy - Internal Calibration	< 1ppm	< 1ppm	< 1ppm	< 1ppm	< 1ppm	< 1ppm	< 1ppm
Polarity switching	<1 sec	<1 sec	<1 sec	<1 sec	No	<1 sec	<1 sec
Multiplex	No	Yes, up to 10 precursors	Yes, up to 10 precursors	Yes, up to 10 precursors	No	Yes, up to 10 precursors	Yes, up to 10 precursors
Intact Protein Mode	No	No	Yes	Yes	Yes	Yes	Yes
Enhanced Resolution	No	No	280,000 (Option)	N/A	N/A	N/A	N/A
Collision Energy	CE only	Normalized CE	Normalized CE	Normalized CE			
Dissociation	HCD	HCD	HCD	HCD	CID, ECD	CID, HCD, ETD, EThCD	CID, HCD, ETD HD, EThCD HD
ETD Option	No	No	No	No	Yes, efficiency > 15%	Yes, efficiency > 15%	Yes, efficiency > 15%

References

1. Ow, SY.; Salim, M.; Noirel, J.; Evans, C.; Rehman, I.; Wright, P.C. iTRAQ Underestimation in Simple and Complex Mixtures: "The Good, the Bad and the Ugly". *J. Proteome Res.,* **2009,** *8(11)*, pp 5347–5355.
   Description: Assessment of the accuracy and precision of iTRAQ quantification in a controlled experimental setup using low- and high-complexity protein mixtures.

2. Ting, L.; Rad, R.; Gygi, S.P.; Haas, W. MS^3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. *Nature Methods* **2011,** *8,* 937–940.
   Description: Application of MS^3 stage fragmentation to overcome reporter ion ratio distortion phenomena resulting from coisolation and cofragmentation of interfering ions.

3. McAlister, G.; Nusinow, D.; Jedrychowski, M.; Wuhr, M.; Huttlin, E.L.; Erickson, B.K.; Rad, R.; Haas, W.; Gygi, S.P. MultiNotch MS^3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal. Chem.* **2014,** *86,* 7150–7158.
   Description: Implementation of MultiNotch MS^3 technology to complement multiplexing quantitation while improving quantitation accuracy and precision without loss in sensitivity.

Supporting Resources

4. Saba, J.; Viner, R. High Throughput Quantitative Proteomics Using Isobaric Tags. C&EN whitepaper, American Chemical Society.
   Description: An overview of using TMT isobaric tags in parallel with SPS-MS^3 method to gain throughput and coverage through multiplexing in quantitative proteomics.

The following references demonstrate the application of TMT SPS MS^3 methods in multiple areas of scientific research:

5 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.*
   Description: Quantitation of RNA-Interacting Proteins; The advantages of multiplexing with 10plex TMT labeling is highlighted, where increased throughput, improved statistical power and observing differences in different biological conditions can all be achieved.

6 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-kB signalling. *Nat. Commun.* **2015,** *6.*
   Description: Comparative Proteomics Identifies a Novel Protease Substrate; Use of 10plex TMT enabled discovery of new protein *N*-termini in vivo and novel regulation of
   NF-kB pathway.

7. 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.*
   Description: Spatial proteomics at subcelluar scale: taking advantage of high throughput quantitative proteomics to generate high-resolution subcellular protein maps.

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8. 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.
Description: CRISPR-CAS9 Genomic Editing Characterizes the Prion Protein Knockout Proteome; Quantitative global proteome analysis to characterize molecular function of PrP through relative protein abundance.

9. 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.
Description: The Importance of pRb for Mitochondrial Function; Application of SPS MS^3 method to screen for biological effects in proteins, providing insight to new strategies for cancer treatment.

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