APPLICATION NOTE 65729

Real-Time Search enables a new gold standard for TMT quantitation accuracy on the Orbitrap Eclipse Tribrid mass spectrometer

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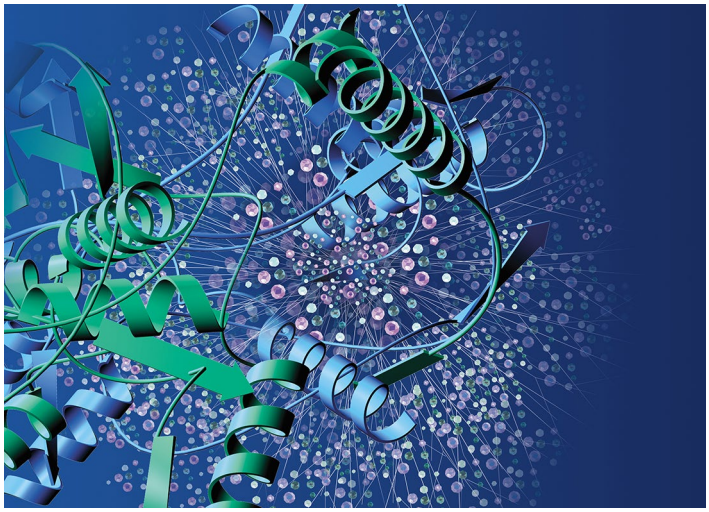

Introduction

Isobaric chemical tagging strategies using Thermo Scientific™ Tandem Mass Tags™ (TMT™) are powerful tools for studying global protein dynamics within a cell, tissue or an organism.[1] The primary advantage of the tags is their ability to simultaneously identify and quantify proteins in multiple sample sets in a single liquid chromatography coupled to mass spectrometry (LC-MS) analysis. The TMT reagent consists of an MS/MS reporter group, a spacer arm, and an amine-reactive group. The amine-reactive groups covalently bind to the peptide N-termini or to lysine residues. After labeling, the peptides are introduced into the mass spectrometer where each tag fragments during MS^2, producing unique reporter ions. Peptide quantitation is accomplished by comparing the intensities of the reporter ions. However, achieving quantitative accuracy is highly dependent on the purity of the precursor ion population selected for MS^2 analysis.[2] Innovations such as synchronous precursor selection (SPS) MS^3 technology are required to obtain accurate quantitation, and particularly important to measure subtle changes in low-abundance proteins.[3] The SPS MS^3-based methods provide higher accuracy compared to MS^2 methods for TMT quantitation. However, even with added selectivity of SPS MS^3, the accuracy can still suffer if non-specific fragments get selected for the MS^3 step. Furthermore, due to the requirement for an MS^3 scan, SPS MS^3 methods lower the scan rate of data acquisition. To improve upon the existing SPS MS^3 methods, we recently implemented a Real-Time Search step between the MS^2 and MS^3 scans on the Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer (Figure 1), which allows an MS^3 scan acquisition only if the MS^2 spectrum produces a positive peptide identification. In this application note we evaluate

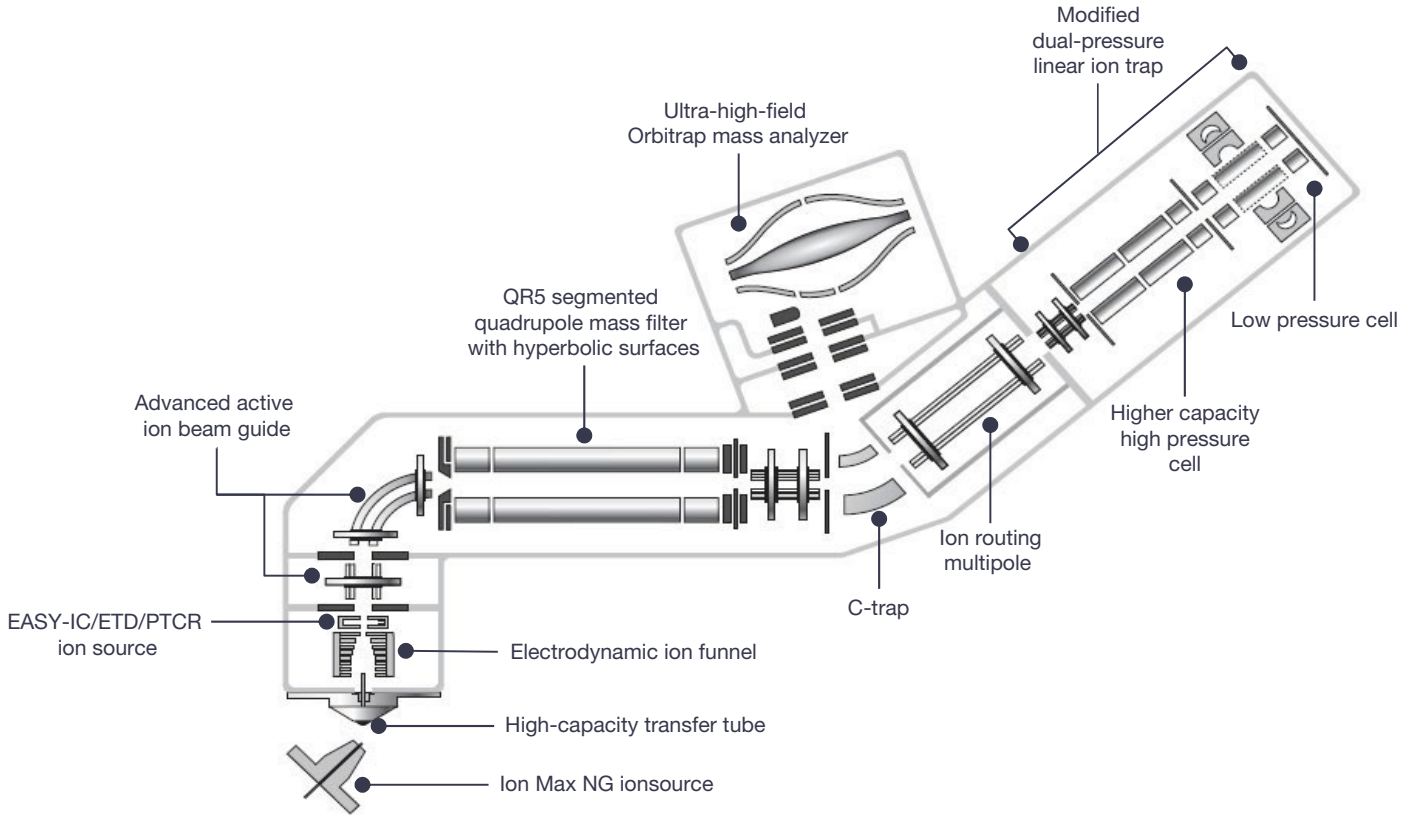

Figure 1. Schematic representation of the Orbitrap Eclipse Tribrid mass spectrometer that enables Real-Time Search

the benefits of Real-Time Search for SPS MS^3 implemented on the Orbitrap Eclipse Tribrid mass spectrometer to enhance TMT quantification accuracy and proteome coverage. We also apply this approach to the new Thermo Scientific™ TMTpro 16plex Label Reagent, which enable multiplexing of up to 16 samples.[4] Overall, we demonstrate that Real-Time Search sets a new gold standard for quantitative performance and throughput for isobaric tagging experiments.

Experimental methods and materials

Materials used

- Thermo Scientific™ Pierce™ TMT11plex Yeast Digest Standard, P/N A40939
- TMTpro 16plex Label Reagent Set, P/N A44520
- Thermo Scientific™ EASY-Spray™ LC columns, 50 cm × 75 µm diameter, P/N ES803A
- Thermo Scientific™ EASY-Spray™ ion source
- Thermo Scientific™ EASY-nLC™ 1200 system
- Orbitrap Eclipse Tribrid mass spectrometer
- Thermo Scientific™ Proteome Discoverer™ 2.3 software

Glossary of key terms

- **Tandem Mass Tags (TMT):** isobaric tags used for the precise quantitation of multiplexed samples
- **Liquid chromatography (LC)-Mass Spectrometry (MS):** combines physical separation and mass measurement
- **Automatic Gain Control (AGC) target:** specifies the maximum number of charges to accumulate for a given analysis
- **Peptide Spectrum Matches (PSM):** search engine match between theoretical and observed peptide spectra
- **Synchronous Precursor Selection (SPS):** MS^3 based method that increases TMT quantification accuracy
- **Phase-Constrained Spectrum Deconvolution (ΦSDM):** an advanced processing method for Fourier transform MS
- **Quality Control (QC):** standardized sample, LC/MS method, and data analysis workflow to assess system readiness
- **Triple Gene Knockout (TKO):** met6Δ, his4Δ, or ura2Δ yeast strains used to access ratio distortion for TMT quantitation
- **Interference Free Index (IFI):** calculated as average scaled protein abundance of (BY4741 - met6Δ, his4Δ, or ura2Δ)

Instrumentation settings and experimental setup

To assess sensitivity, accuracy, and precision of the Orbitrap Eclipse Tribrid mass spectrometer for TMT-based quantitation, we utilized the Pierce TMT11plex Yeast Digest Standard. This standardized sample provides users with a tool to measure the accuracy, precision, and proteome depth of TMT methods across different instrumentation. For a detailed user guide, please refer to "A quality control standard for Tandem Mass Tags (TMT) proteomic workflows" Technical Note 72968.[5] In brief, for liquid chromatography (LC) conditions, we used an analytical gradient from 8% to 32% acetonitrile (vol/vol) with 0.1% (vol/vol) formic acid in 50 min with a column heater set to 45 °C, unless otherwise indicated. Experiments were run with an EASY-nLC 1200 system in combination with an EASY-Spray C18 50 cm long column coupled to an EASY-Spray ion source. Samples were analyzed on an Orbitrap Eclipse Tribrid mass spectrometer. Settings for Thermo Scientific™ Orbitrap™ Tribrid™ Series Instrument Control Software Version 3.3 are found in Table 1. Raw data files were processed using Proteome Discoverer 2.3 software using the SEQUEST® HT search engine with a 10 ppm MS^1 and 0.5 Da MS^2 mass tolerance, TMT6plex (229.163 Da) or TMTpro16plex (304.207) set as a static modification, and a 1% false discovery rate (FDR).

Results

Deeper proteome coverage with the highest depth and quantitative accuracy for TMT-based quantitation

To improve upon existing SPS methods, we implemented a Real-Time Search filter between the MS^2 and MS^3 scans. Real-Time Search compares the raw data collected by the mass spectrometer to the information in the user provided FASTA database and identifies peptides from the mass spectra using the open source Comet search engine.[6] Real-Time Search benefits TMT SPS MS^3 methods in two distinct ways.[7,8] First, MS^3 scans are only triggered if a peptide-spectrum match (PSM) is identified from the preceding MS^2 spectrum. This increases the number of peptides identified with SPS MS^3. Secondly, Real-Time Search identifies precursor fragments ions that are specific to the identified peptide sequence, and then passes their masses to be selected for SPS MS^3 event. This adds extra specificity to the SPS MS^3 analysis, as it eliminates the possibility of a non-specific fragment to be included in the SPS MS^3 event. To assess the accuracy and sensitivity of TMT quantification methods, we utilized the Pierce TMT11plex Yeast Digest Standard (Figure 2)[5,9] analyzed with Proteome Discoverer software. TMT SPS MS^3 quantitation with Real-Time Search improves the quantitation accuracy, enabling up to 95% interference

Table 1. Mass spectrometer data acquisition settings

Acquisition settings	MS^2	SPS MS^3 (with or without Real-Time Search)
Top speed (s)	2.5	2.5
RF lens	30%	30%
Orbitrap MS^1 resolution	120,000	120,000
Scan range (*m/z*)	400–1400	400–1400
Standardized MS^1 AGC target	100%	100%
MS^1 max IT (mode)	Auto	Auto
Charge state	2-5	2-5
Dynamic exclusion (s)	45	45
MS^2 resolution	45,000	Turbo
MS^2 scan range (*m/z*)	First mass 110	200–1200
MS^2 isolation window	0.7 *m/z*	0.7 *m/z*
Standardized MS^2 AGC target	500%	100%
MS^2 max IT (mode)	Auto	Auto
MS^2 HCD NCE%	36	36
SPS MS^3 resolution	-	50,000
MS^2 scan range (*m/z*)	-	100–500
SPS MS^3 isolation window	-	0.7 *m/z*
Standardized SPS MS3 AGC target	-	500%
SPS MS^3 max IT (mode)	-	Auto
SPS MS^3 HCD NCE%	-	55
SPS MS^3 notches	-	10

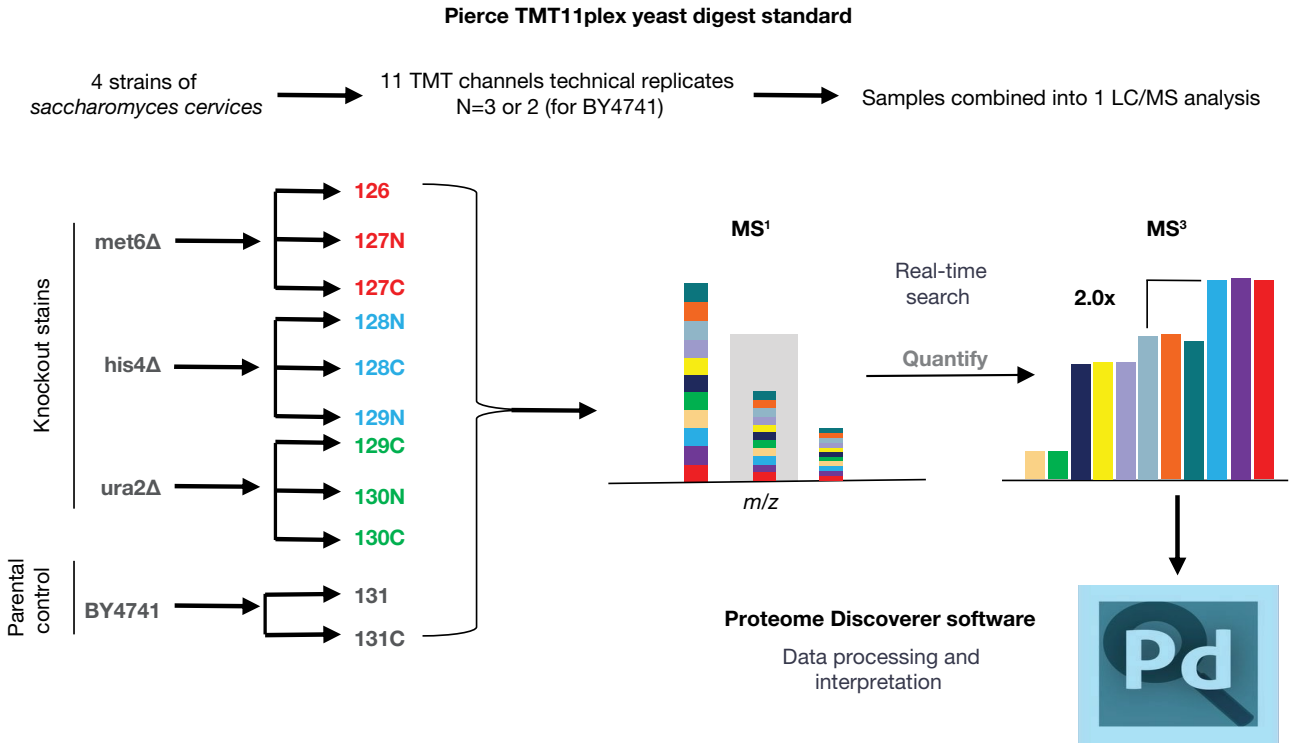

Figure 2. Schematic representation of Pierce TMT11plex Yeast Digest Standard method workflow. The standard is composed of four *Saccharomyces cerevisiae* strains with the three lines respectively lacking the non-essential proteins Met6, His4, or Ura2, and the parental strain BY4741 for reference channels.

free measurements.[10] Real-Time Search enables on-the-fly MS² data processing, matching the MS² spectrum acquired in the linear ion trap to the user-defined FASTA database. Advantageously, this results in near immediate determination of PSM, taking less than 5 ms per MS² spectrum when searching the UniProt human reference proteome, containing 74,449 proteins, with one variable modification and one missed cleavage. The search process is conducted concomitantly with the acquisition of the next MS² spectrum, in this way it does not impact the cycle times of the MS experiment. If a peptide spectrum match is made, the peptide is selected for subsequent SPS MS³ based quantitation. With Real-Time Search, MS³ scans are only triggered if a PSM is identified from the preceding MS² spectrum. This increases the number of peptides identified with Real-Time Search for SPS MS³ to nearly the level that could be obtained in MS²-only experiments while maintaining a significantly higher interference free index (IFI).

A schematic representation of Real-Time Search for TMT SPS MS³ is shown in Figure 3. We initially evaluated how Real-Time Search on the Orbitrap Eclipse Tribrid mass spectrometer influenced TMT peptide and protein identification rates. A 500 ng sample of Pierce TMT 11plex Yeast Digest Standard was analyzed on a 50 min gradient using MS², SPS MS³, or Real-Time Search for SPS MS³ methods. With Real-Time Search for SPS MS³, 53% more peptides and 55% more proteins were identified than in the classic SPS MS³ experiment, approaching the results of the MS² experiment. Additionally, Erickson *et al.* demonstrated that Real-Time Search for SPS-MS³ could achieve the same proteome coverage as classical SPS-MS³, but in half the analysis time, thus further increasing sample throughput.[7] Importantly, Real-Time Search for SPS MS³ results produced the highest IFI, i.e., the most accurate results, far surpassing what can be achieved in an MS² experiment. The IFI is calculated based upon the Pierce TMT11plex Yeast Digest Standard. This standard contains three yeast strains with gene deletions for met6Δ, his4Δ, or ura2Δ. In the channels where the genes have been deleted, any signal observed for the three proteins is due to co-isolation interference which can mask true differences in biological changes. An IFI of 0.75 indicates that twenty five percent of the observed signal came from co-isolated ions, incorrectly contributing to the reporter ion signal. This negatively affects quantitation and can produce a significant alteration if the interfering ions are expressed at higher abundance than the selected precursor. Thus, the IFI provides a method to measure if new approaches can reduce co-isolation interference and improve TMT quantitation accuracy.[9]

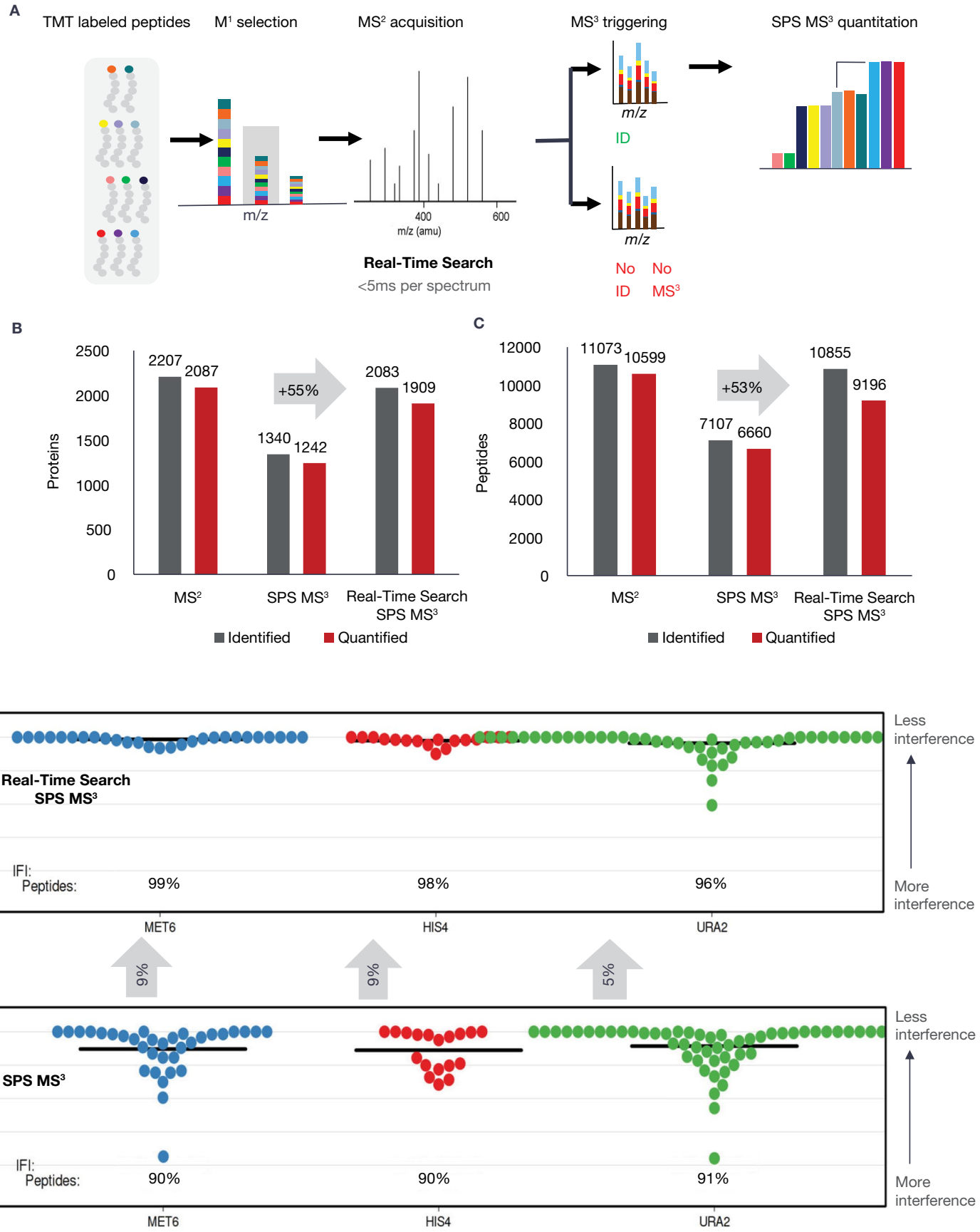

Figure 3. Achieving improved proteome depth and accuracy for SPS MS³ quantitation. (A) Schematic representation of TMT workflow on the Orbitrap Eclipse Tribrid mass spectrometer. If an MS² spectrum results in a PSM, a corresponding MS³ scan is triggered for quantitation. We evaluated how Real-Time Search influenced TMT identification rates for (B) proteins and (C) peptides. (D), the IFI was measured at the peptide level and visualized by TKOmics.com. 500 ng of TMT11plex Yeast Digest Standard was measured using a 50 min gradient using MS², SPS MS³, or Real-Time Search SPS MS³ methods.

Real-Time Search improves quantitative accuracy

Real-Time Search utilizes the open source search engine Comet to identify PSM in real time.[6] Importantly, post-acquisition data processing is required to accurately control the FDR. The results of searching the same dataset in an offline version of Comet with the online Real-Time Search implementation of Comet demonstrate a near perfect correlation. However, due to subtle differences in how search algorithms function, the choice of post-acquisition search engine other than Comet will influence both the identification and the assigned Xcorr (Figure 4A-B). An optional LC-MS survey experiment can be run with wide tolerances for Xcorr and dCn to further refine the MS^3 trigger thresholds. In addition to the doubled rate of peptide identification and quantitation, the quantitative accuracy is improved with Real-Time Search. In the traditional SPS MS^3 method, where fragment ions are selected based on the intensity, an n-notch experiment would select the n most abundant fragment ions from an MS^2 spectrum. In contrast, the Real-Time Search filter would select up to a maximum of n notches, which correspond to fragment ions from the identified PSM (Figure 4C-D), as well as specific b- and y-type ions that also contain the TMT label. For example, in the case of a peptide that is only N-terminally labeled, the y-type ions will not contain a TMT label; therefore, these fragments will not contribute to the overall TMT reporter ion signal and will be excluded from SPS by Real-Time Search. A portion of the SPS MS^3 signal is derived from the ions that do not belong to the peptide of interest (Figure 4E), since the fragment ions are selected based on intensity, and therefore lowered the accuracy of the experiment. When Real-Time Search is employed, 91% of the PSM quantified used only fragment ions from the correct peptide of interest (Figure 4F). The remainder of PSMs with non-matching fragment ions were likely due to subtle differences in the Real-Time Search engine (Comet) and the post-acquisition database search engine (SEQUEST HD). Overall, the added selectivity afforded by the Real-Time Search significantly improves quantitative accuracy of the experiment.

Real-Time Search can also be used with custom amino acid modifications and supports the analysis of commonly found post-translation modifications (PTMs) such as phosphorylation. The Orbitrap Eclipse Tribrid MS enables additional functionalities that improve the results of the quantitative experiment. These include Precursor Fit Filter, which allows for selecting ions based on precursor ion specificity of the isolation window, TurboTMT powered by the ΦSDM algorithm that increases scan acquisition rates,[11,12] and a QR5 segmented quadrupole mass filter with its improved precursor ion selectivity and transmission, making it possible to use narrower isolation widths to further improve the precursor ion specificity and with that, the TMT quantitation accuracy.

TMTpro 16plex isobaric label reagent enables up to 16 samples to be quantified simultaneously

Increasingly, biological experiments are requiring higher multiplexing capability and sample throughput. Applications such as thermal shift assays, time courses, drug dose curves, large scale translational cohort studies, hyperLOPIT subcellular localization studies, or whole cell interactomics approaches such as QMIX[13] require an extensive number of comparisons to be able to measure statistically meaningful differences. TMTpro 16plex isobaric label reagents are next generation reagents that increase the level of sample multiplexing up to sixteen without compromising identification or quantitation. The TMTpro tag is isobaric and amine reactive, similar to the classic TMT tags, but differ by the incorporation of a longer spacer region and isobutyl proline mass reporter region. After fragmentation within the mass spectrometer, the TMTpro reagents generate unique reporter ions having masses in the range of 126 to 134 Da that are used for relative quantitation of the isobaric precursor ions. The TMTpro 16plex isobaric label reagent enables increased sample throughput and results in fewer missing values for quantitation when analyzing up to 16 samples in a single LC-MS analysis or across multiplex experiments.

To test the performance of the new tags, we labeled the Thermo Scientific™ Pierce™ 6 Protein Digest Standard with the TMTpro 16plex isobaric label reagent. The sample was then spiked into the Thermo Scientific™ Pierce™ HeLa Protein Digest Standard at a ratio of 1:8 as shown in Figure 5A. The combined mixture was then analyzed on the Orbitrap Eclipse Tribrid MS using the SPS MS^3 method with and without utilizing Real-Time Search. We observed that Real-Time Search increased the numbers of quantified proteins by 26%, while also improving both the precision and accuracy of quantitation (Figure 5D). This improvement in quantitation accuracy for large ratios is particularly useful for single cell proteomics approaches that utilize TMT-based quantitation.[14]

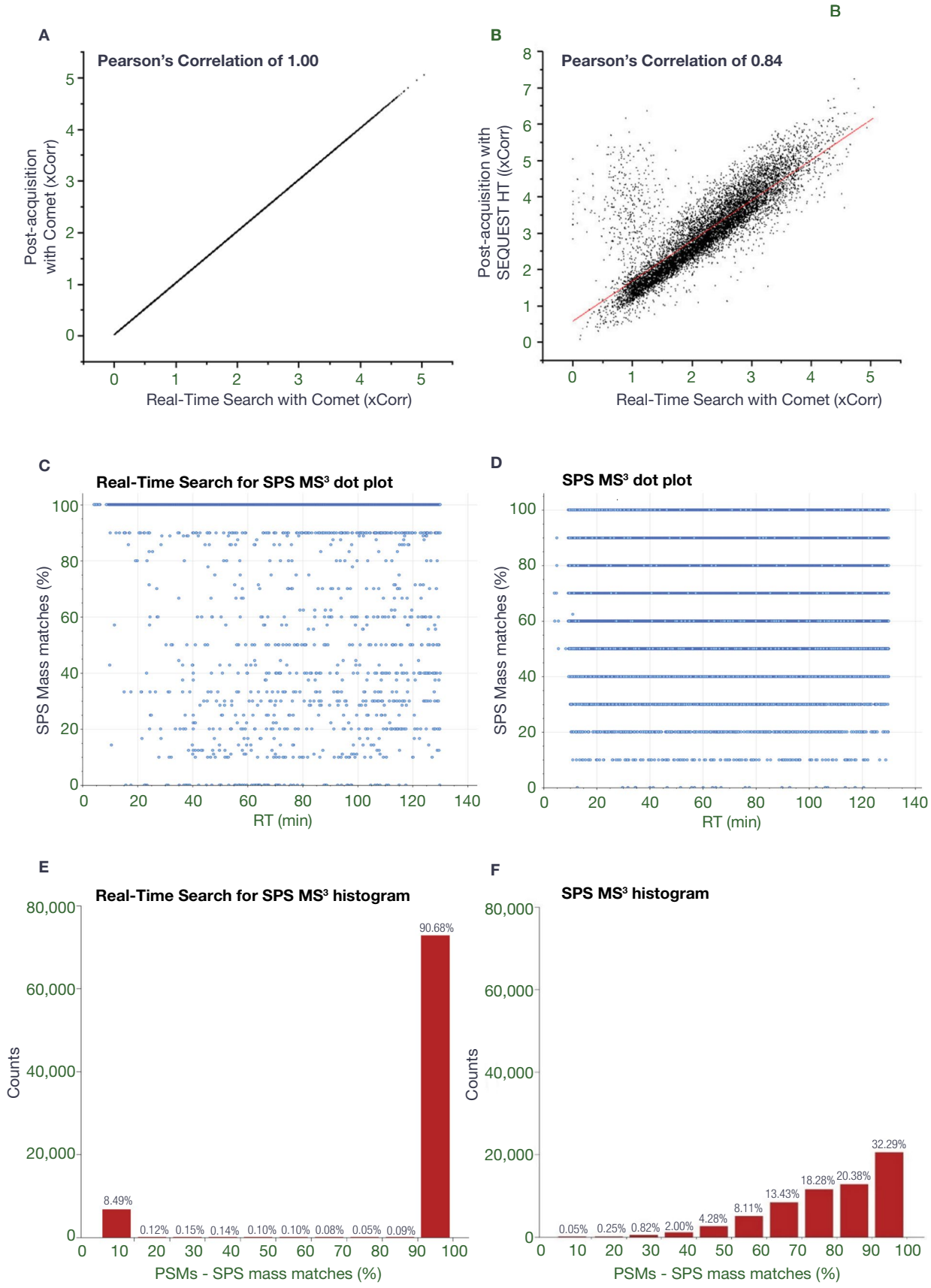

Figure 4. Real-Time peptide identification for TMT quantitation. Real-Time Search utilizes the open source search engine Comet. We evaluated the correlation of Xcorr scores between (A) Real Time Search with Comet and post-acquisition search with Comet, or (B) Proteome Discoverer software using SEQUEST HD. Secondly, we evaluated the number of fragment ions selected for a 10-notch SPS MS^3 experiment that correctly matched the post-acquisition identification in Real-Time Search for SPS MS^3 (C and E) or SPS MS^3 (D and F). Selection of a fragment ion that does not correspond to the correct peptide negatively impacts quantitation in classical SPS-MS^3.

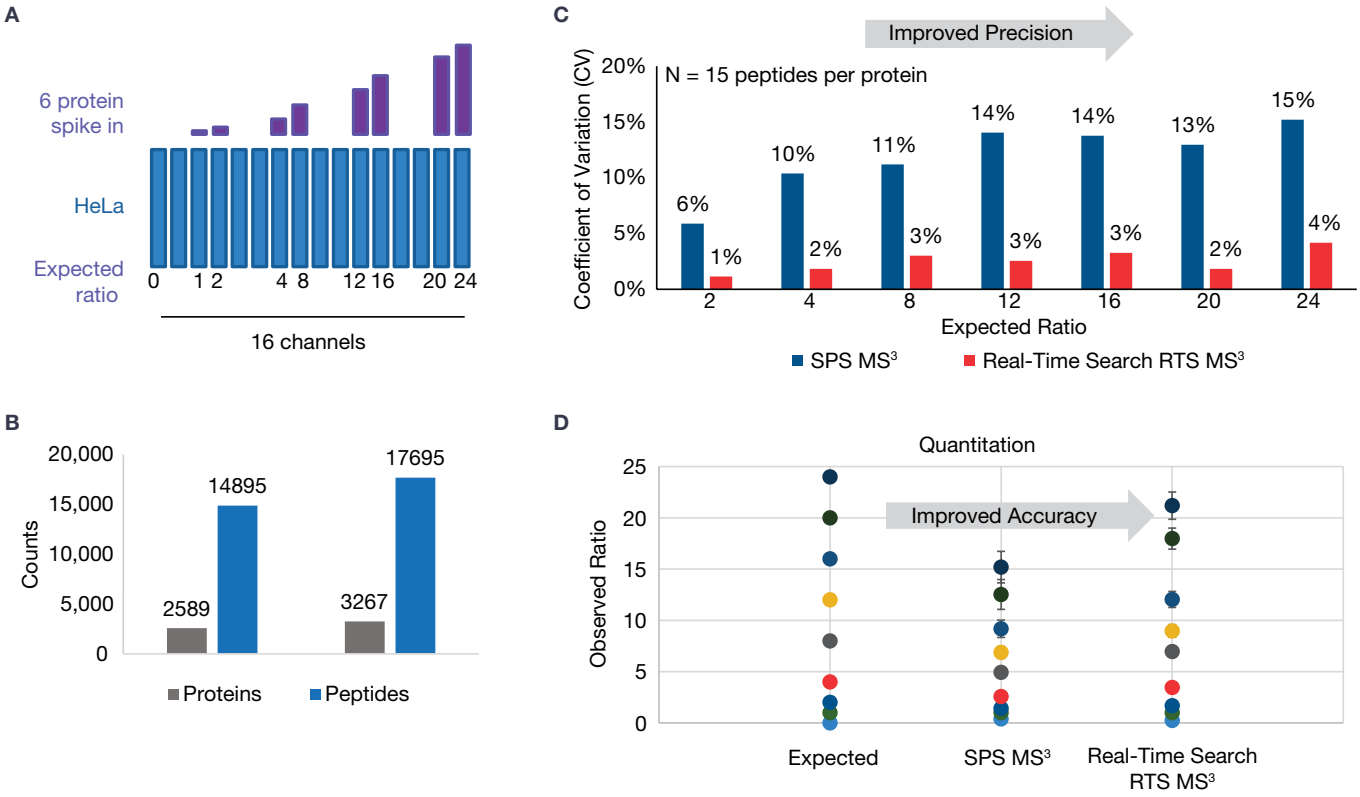

Figure 5. Next generation TMTpro isobaric tags increases sample multiplexing. We evaluated the potential for Real-Time Search to use custom modifications using next generation TMTpro isobaric tags (A). An equimolar mixture of Pierce 6 Protein Digest (red) was mixed in various ratios into the Pierce HeLa Protein Digest Standard and labeled (B). 1 µg of sample was then analyzed on a 120 min gradient using Real-Time Search for SPS MS³ or SPS MS³ on the Orbitrap Eclipse Tribrid MS. Data was analyzed using Proteome Discover 2.3 software. We analyzed how Real-Time Search effected TMT SPS MS³ quantitation precision (C) and accuracy (D). This work is for research purposes only.

Conclusion

We evaluated the utility of Real-Time Search for TMT SPS MS³-based quantitation, which, in addition to other new features including Precursor Fit Filter and TurboTMT, allow for intelligent acquisition methods that improve quantitation accuracy, precision, and proteome coverage. The combination of isobaric tag reagents, high resolution accurate mass instrumentation, and advanced software enable the identification of thousands of proteins multiplexed from up to 16 samples in a single run while achieving accurate and precise quantitation including of low level precursors and/or small ratios. Together, the TMT and TMTpro multiplexing works enables the investigation of novel biological questions, such as the heterogeneity of single cell protein expression across a tissue.

Acknowledgements

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References

1. Thompson, A.; Schafer, J.; Kuhn, K.; Kienle, S.; Schwarz, J.; Schmidt, G.; Neumann, T.; Johnstone, R.; Mohammed, A. K.; Hamon, C. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS., *Anal. Chem.* **2003,** *75*(8), 1895–1904.
2. Bantscheff, M.; Boesche, M.; Eberhard, D.; Matthieson, T.; Sweetman, G.; Kuster, B. Robust and sensitive iTRAQ quantification on an LTQ orbitrap mass spectrometer. *Mol. Cell Proteomics* **2008,** *7*(9), 1702–1713.
3. McAlister, G.C.; Nusinow, D.P.; Jedrychowski, M.P.; Wühr, M.; Huttlin, E.L.; Erickson, B.K.; Rad, R.; Haas, W.; Gygi, S.P. MultiNotch MS^3 enables accurate, aensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal. Chem.* **2014,** *86*(14), 7150–7158.
4. Li, J.; Van Vranken, J.G.; Pontano Vaites, L. et al. TMTpro reagents: a set of isobaric labeling mass tags enables simultaneous proteome-wide measurements across 16 samples. *Nat. Methods* **2020,** *17*(4), 399–404.
5. Robitaille, A.M.; Choi, J.; Viner, R.; Huhmer, A.; Opperman, K.; Rogers, J.C. A quality control standard for Tandem Mass Tags (TMT) proteomic workflows. Thermo Fisher Scientific, Technical Note 72968, **2019.** https://planetorbitrap.com/library?t=QTIzMzBlODg0OWE1NWYzYw%3D%3D&keywords=A2330#tab:keywords
6. Eng, J.K.; Jahan, T.A.; Hoopmann, M.R. Comet: an open source tandem mass spectrometry sequence database search tool. *Proteomics.* **2013**. *13*(1), 22–24.
7. Erickson, B.K.; Mintseris, J.; Schweppe, DK.; Navarrete-Perea, J.; Erickson, A.R.; Nusinow, D.P.; Paulo, J.A.; Gygi, S.P. Active instrument engagement combined with a real-time database search for improved performance of sample multiplexing workflows. *J Proteome Res.* **2019,** *18*(3), 1299–1306.
8. Schweppe, D.K.; Eng, J.K.; Bailey, D.; Rad, R.; Yu, Q.; Navarrete-Perea, J.; Huttlin, E.L.; Erickson, B.K.; Paulo, J.A.; Gygi, S.P. Full-featured, real-time database searching platform enables fast and accurate multiplexed quantitative proteomics. *J. Proteome Res.* **2020.**
9. Paulo, J.A.; O'Connell, J.D.; Gygi, S.P. A triple knockout (TKO) proteomics standard for diagnosing ion interference in isobaric labeling experiments. *Journal of the American Society for Mass Spectrometry* **2016,** *27*(10), 1620–1625.
10. Gygi, J.P.; Yu, Q.; Navarrete-Perea, J.; Rad, R.; Gygi, S.P.; Paulo, J.A. Web-based search tool for visualizing instrument performance using the triple knockout (TKO) proteome standard. *J. Proteome Res.* **2019,** *18*(2), 687–693.
11. Kelstrup, C.D.; Aizikov, K.; Batth, T.S.; Kreutzman, A.; Grinfeld, D.; Lange, O.; Mourad, D.; Makarov, A.; Olsen J.V. Limits for resolving isobaric tandem mass tag reporter ions using phase-constrained spectrum deconvolution. *Proteome Res.* **2018,** *17*(11), 4008–4016.
12. Grinfeld, D.; Aizikov, K.; Kreutzmann, A.; Damoc, E.; Makarov, A. Phase-constrained spectrum deconvolution for fourier transform mass spectrometry. *Analytical Chemistry* **2017,** *89*(2), 1202–1211.
13. Yu, C.; Huszagh, A.; Viner, R.; Novitsky, E.J.; Rychnovsky, S.D.; Huang, L. Developing a multiplexed quantitative cross-linking mass spectrometry platform for comparative structural analysis of protein complexes. *Anal. Chem.* **2016,** *88*(20), 10301–10308.
14. Dou, M.; Clair, G.; Tsai, C.F.; Xu, K.; Chrisler, W.B.; Sontag, R.L.; Zhao, R.; Moore, R.J.; Liu, T.; Pasa-Tolic, L.; Smith, R.D.; Shi, T.; Adkins, J.N.; Qian, W.J.; Kelly, R.T; Ansong, C.; Zhu, Y. High-throughput single cell proteomics enabled by multiplex isobaric labeling in a nanodroplet sample preparation platform. *Anal. Chem.* **2019,** *91*(20), 13119–13127.

Ordering information	
Pierce TMT11plex Yeast Digest Standard, 20 µg	Product Number: A40938
Pierce TMT11plex Yeast Digest Standard, 5 × 20 µg	Product Number: A40939
EASY-Spray LC Columns, 50 cm × 75 µm diameter	Product Number: ES803A
TMTpro 16plex Label Reagent Set, 1 × 5 mg	Product Number: A44520

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