

# A Modified Orbitrap Tribrid Mass Spectrometer with Real Time Search and Advanced Spectral Processing Enhances Multiplexed Proteome Coverage and Quantification Accuracy

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## ABSTRACT

Isobaric tagging strategies using Thermo Scientific™ Tandem Mass Tags™ (TMT™) are powerful tools for studying how proteins interact and function in biological systems. Up to 11 samples can be multiplexed in a single high-resolution LC/MS experiment to enable state-of-the-art quantitative analysis of peptide and protein abundance. Here we evaluate the benefits of the Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer including real time search capabilities, advanced spectral processing algorithms, and modified hardware to enhance TMT quantification accuracy and proteome coverage.

## INTRODUCTION

To assess the accuracy, precision, and sensitivity of the Orbitrap Eclipse Tribrid mass spectrometer for TMT based quantitation, we utilized the Thermo Scientific™ 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. Quantitative proteomics strategies using Tandem Mass Tags (TMT) allow up to 11 samples to be multiplexed in a single high resolution LC/MS experiment while enabling precise measurement of protein abundance. However, co-isolated ion interference can suppress ratio quantification and thereby mask true differences in protein abundance. Multiple methods and hardware solutions help alleviate the negative influence of interfering ions including Synchronous Precursor Selection (SPS) and a Thermo Scientific™ FAIMS Pro™ interface. However, all of these solutions come with drawbacks. Furthermore, high resolution MS2 or MS3 scanning is necessary for accurate ratio determination in greater than TMT6plex experiments, which can reduce the frequency of acquisition. Here we evaluated the effects of Real Time Search on a modified Orbitrap Tribrid mass spectrometer to reduce the above stated limitations on TMT SPS MS3 quantitative experiments.

## MATERIALS AND METHODS

To assess the sensitivity of the modified Orbitrap™ Tribrid™ mass spectrometer for TMT based quantitation, we utilized the Pierce TMT11plex yeast digest standard. For liquid chromatography (LC) conditions, we used an analytical gradient from 8-32% acetonitrile (vol/vol) with 0.1% (vol/vol) formic acid in 50min with a column heater set to 45°C, unless otherwise indicated. Experiments were run with a Thermo Scientific™ EASY-nLC™ 1200 HPLC system in combination with a Thermo Scientific™ EASY-Spray™ C18 50cm column coupled to a Thermo Scientific™ EASY-Spray™ ion source. Samples were analyzed on a Orbitrap Eclipse Tribrid mass spectrometer. Settings for Orbitrap Tribrid Series Instrument Control Software Version 3.3 follow below (Table 1). Raw data files were processed using Thermo Scientific™ Proteome Discoverer™ 2.3 software using the SEQUEST® HT search engine with a 10ppm MS1 and 0.5 Da MS2 mass tolerance, TMT6plex (229.163 Da) set as a static modification, and a 1% false discovery rate.

Table 1. Mass spectrometer data acquisition settings.

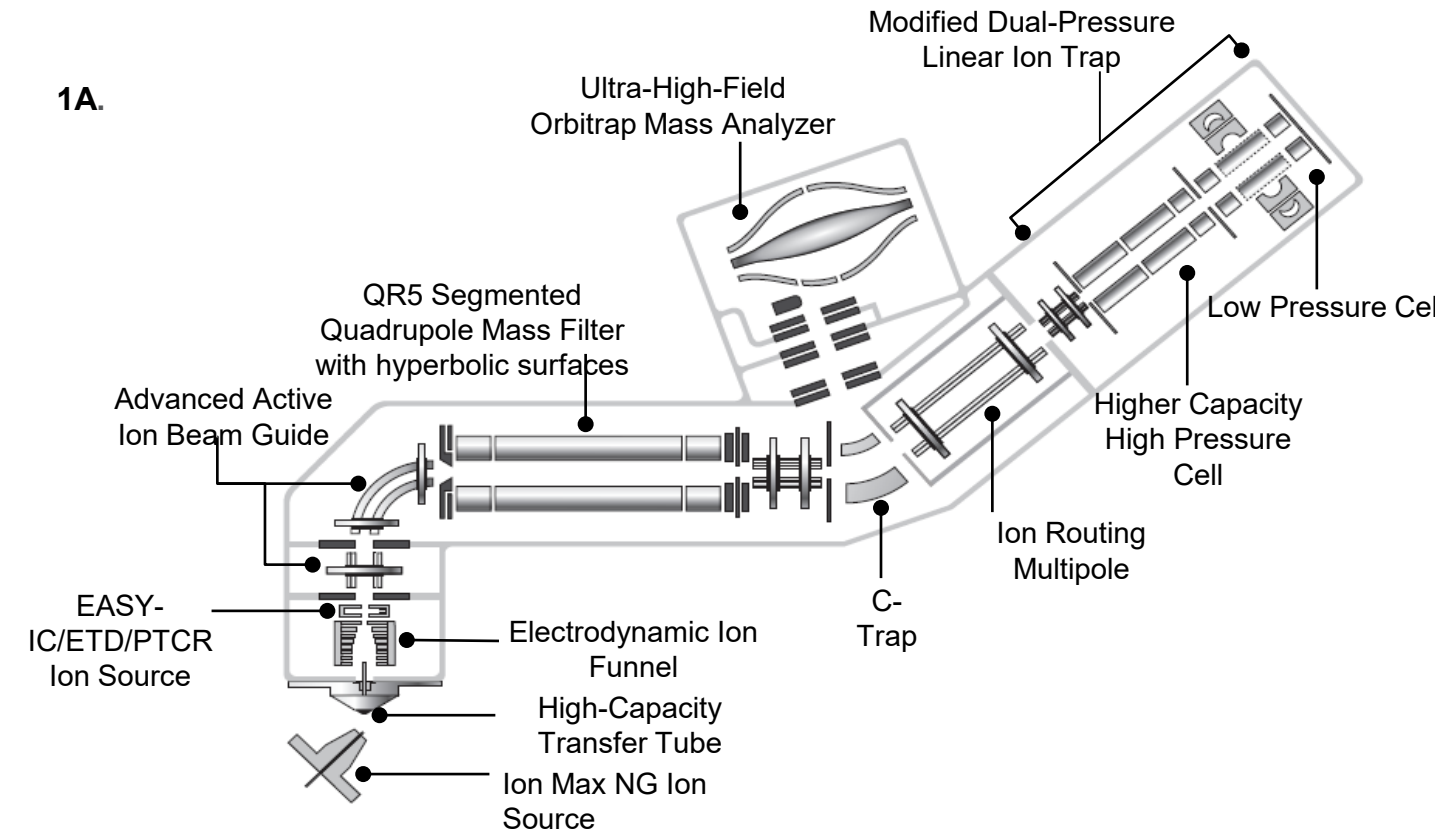
Acquisition Settings	MS2	SPS MS3	RTS SPS MS3
Top Speed (sec)	2.5	2.5	2.5
RF lens	30%	30%	30%
Orbitrap MS1 resolution	120000	120000	120000
Scan range (m/z)	400-1400	400-1400	400-1400
Standardized MS1 AGC target	100%	100%	100%
MS1 max IT (mode)	Auto	Auto	Auto
Charge state	2-5	2-5	2-5
Dynamic Exclusion (sec)	45	45	45
MS2 resolution	45000	Turbo	Turbo
MS2 Scan range (m/z)	First mass 110	200-1200	200-1200
MS2 Isolation Window	0.7 m/z	0.7 m/z	0.7 m/z
Standardized MS2 AGC target	500%	100%	100%
MS2 max IT (mode)	Auto	Auto	Auto
MS2 HCD NCE%	36	36	36
SPS MS3 Resolution	-	50000	60000
MS2 Scan range (m/z)	-	100-500	100-500
SPS MS3 Isolation Window	-	0.7 m/z	0.7 m/z
Standardized SPS MS3 AGC target	-	500%	500%
SPS MS3 max IT (mode)	-	Auto	Auto
SPS MS3 HCD NCE%	-	55	55
SPS MS3 Notches	-	10	10

## RESULTS

Synchronous precursor selection (SPS) based methods provided higher accuracy compared to MS2 methods for TMT quantitation. However, depending on precursor isolation specificity and which fragments are selected for MS3 fragmentation, quantitative accuracy can still be distorted. To improve upon existing SPS methods, we implemented a Real Time Search (RTS) filter between the MS2 and MS3 scans. This feature benefits TMT SPS MS3 methods in two distinct ways. First, MS3 scans are only triggered if a peptide-spectrum match (PSM) is identified from the preceding MS2. This increased the number of peptides identified with SPS MS3. Secondly, Real Time Search identifies precursor fragments that are generated from the identified peptide on the fly, and then selects those fragment ions for MS3 quantitation. Thus, TMT SPS MS3 quantitation with Real Time Search can be improved to be 95% interference free. Next, we evaluated a new feature call TurboTMT, powered by the ΦSDM algorithm. ΦSDM is an advanced spectra processing algorithm that increases resolution within a range of the spectrum without requiring a longer transient. Applying ΦSDM specifically to the TMT reporter ions increased the resolution sufficient to baseline resolve TMT isotopologues even when using transients that produce a 30,000 or 15,000 resolving power MS2 scan. ΦSDM increased both the spectral acquisition rate for TMT11plex experiments and the number of identifications for SPS-MS3. Additionally, the modified Orbitrap Tribrid mass spectrometer has an optimized quadrupole that improves ion transmission. Thus it is possible to use narrower isolation widths to improve TMT quantitation accuracy further. Overall, the Orbitrap Eclipse Tribrid mass spectrometer includes unique features such as Real Time Search for TMT SPS MS3 based quantitation, Precursor Fit filter, and TurboTMT, which together allow for intelligent acquisition methods that improve quantitation accuracy, precision, and proteome coverage.

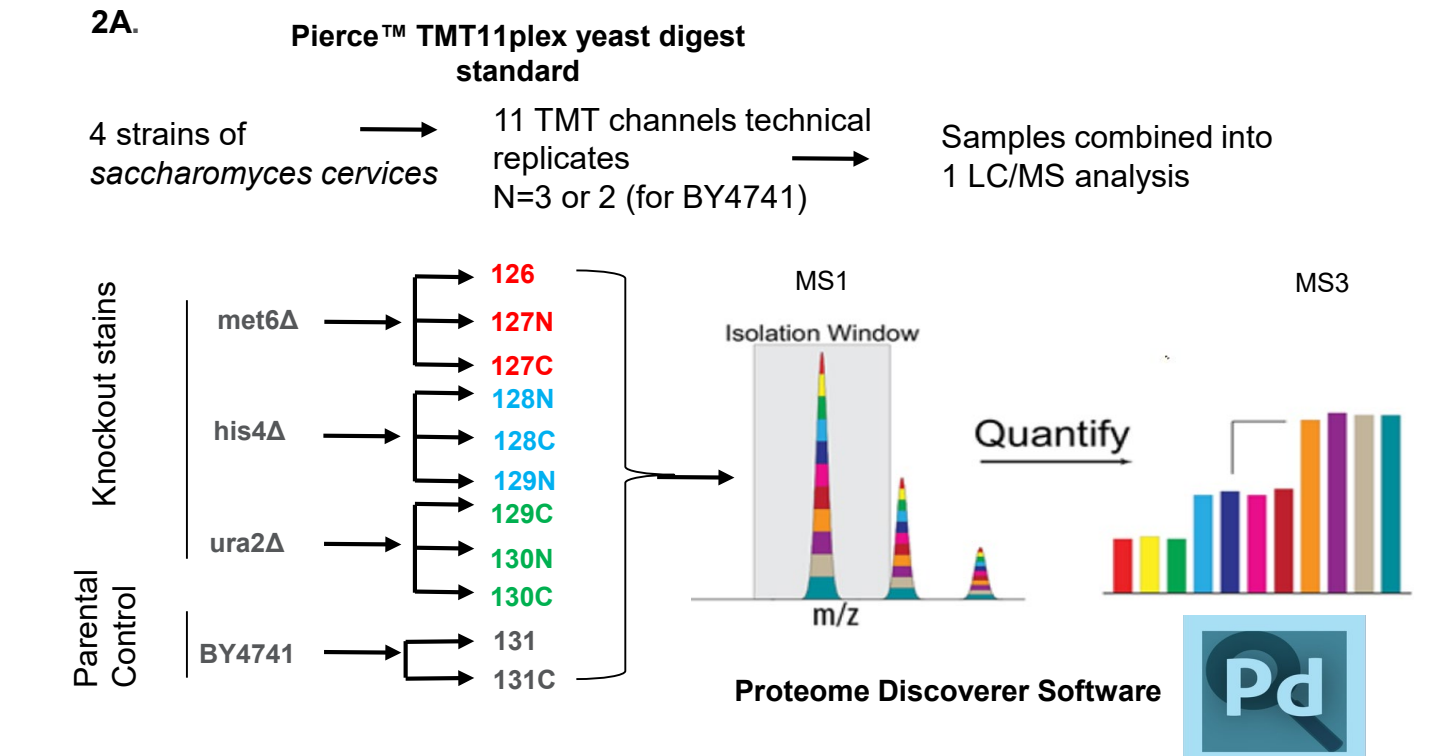
## A MODIFIED ORBITRAP TRIBRID MASS SPECTROMETER

Figure 1. (1A.) Schematic representation of Orbitrap Eclipse Tribrid mass spectrometer.



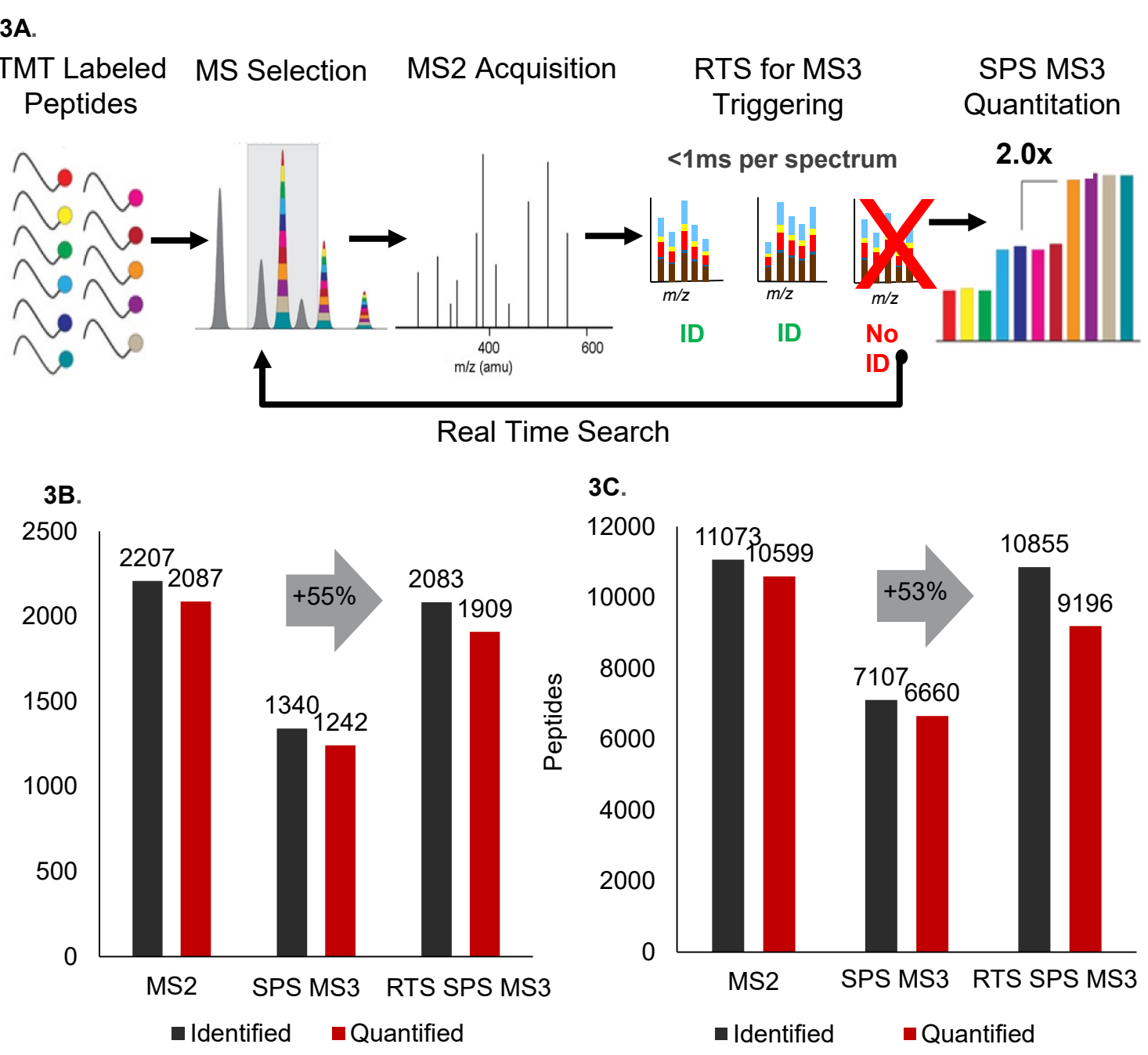
## A TMT11PLEX YEAST DIGEST STANDARD

Figure 2. (2A.) Schematic representation of Pierce TMT11plex Yeast Digest Standard. The standard is composed of four *Saccharomyces cerevisiae* strains: three lines respectively lacking the non-essential proteins Met6, His4, or Ura2, and the parental strain BY4741 for reference. Modified from Schweppe et al. 2019.



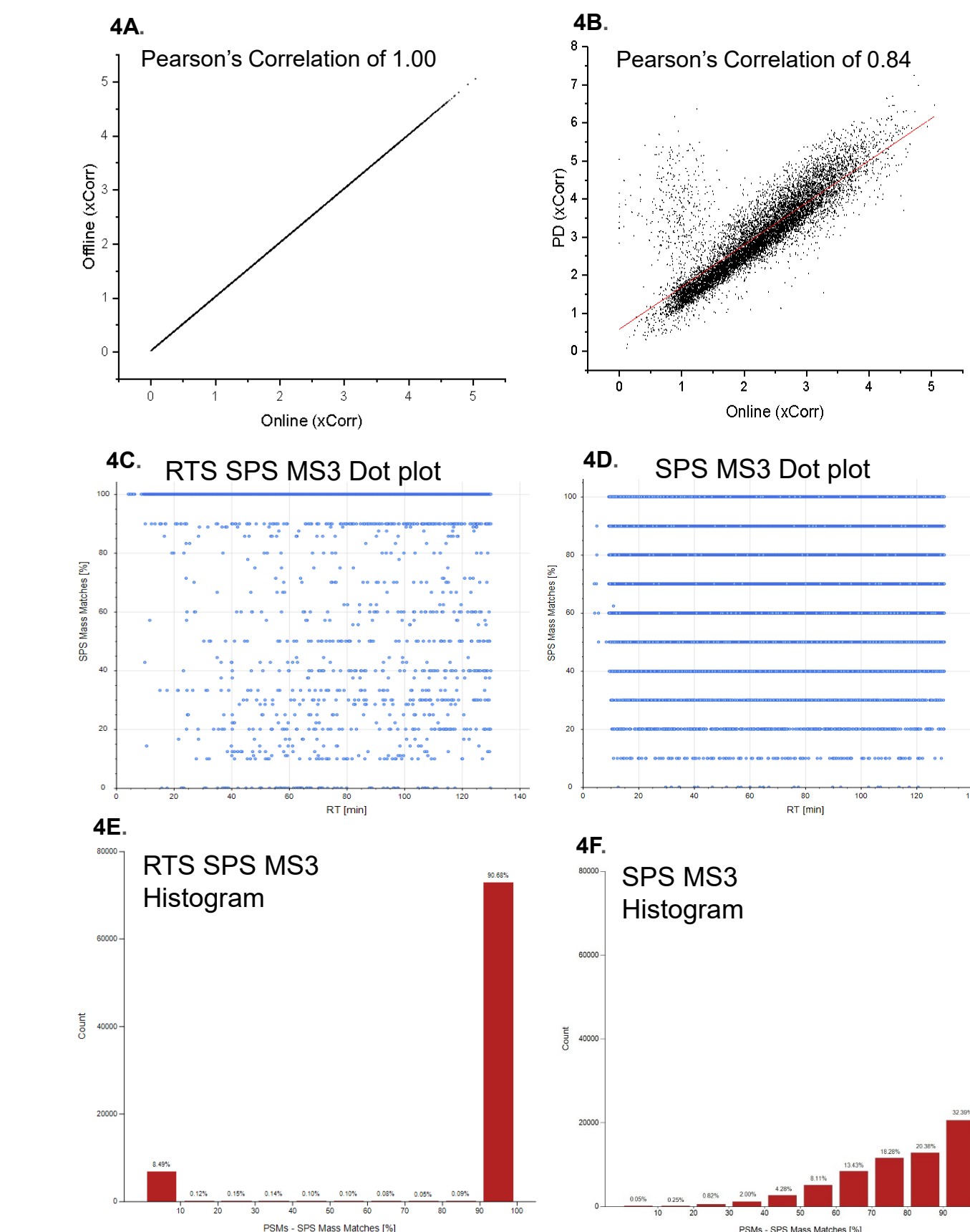
## ENHANCED TMT SPS MS3 PERFORMANCE

Figure 3. Achieving improved proteome depth and accuracy for SPS MS3 quantitation (3A.) Schematic representation of TMT workflow on Orbitrap Eclipse Tribrid mass spectrometer. We evaluated how Real Time Search on the modified Orbitrap Tribrid mass spectrometer influenced TMT identification rates for (3B.) proteins and (3C.) peptides, (3D.) as well as the interference free index at the peptide level as visualized in TKOomics.com. 500ng of TMT11plex Yeast Digest Standard was measured on a 50min gradient using MS2, SPS MS3, or RTS SPS MS3 methods.



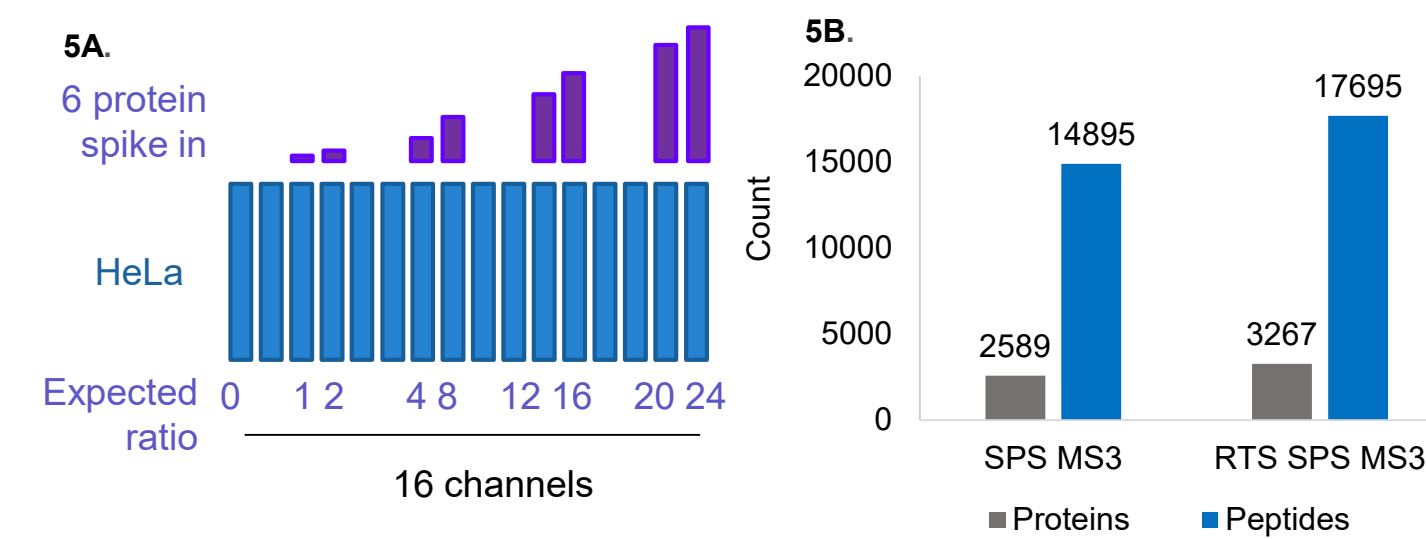
## REAL TIME SEARCH FOR SPS MS3 TRIGGERING

Figure 4. On the fly peptide identification for TMT quantitation. Real Time Search utilizes the open source search engine Comet. We evaluated the correlation of Xcorr scores between (4A.) Real Time Search (Online) and Comet post acquisition (offline) or (4B.) Proteome Discoverer (PD). Secondly, we evaluated the number of fragment ions selected for a 10 notch SPS MS3 that correctly matched the post acquisition identification in RTS SPS MS3 (4C., 4E.) or SPS MS3 (4D., 4F.).



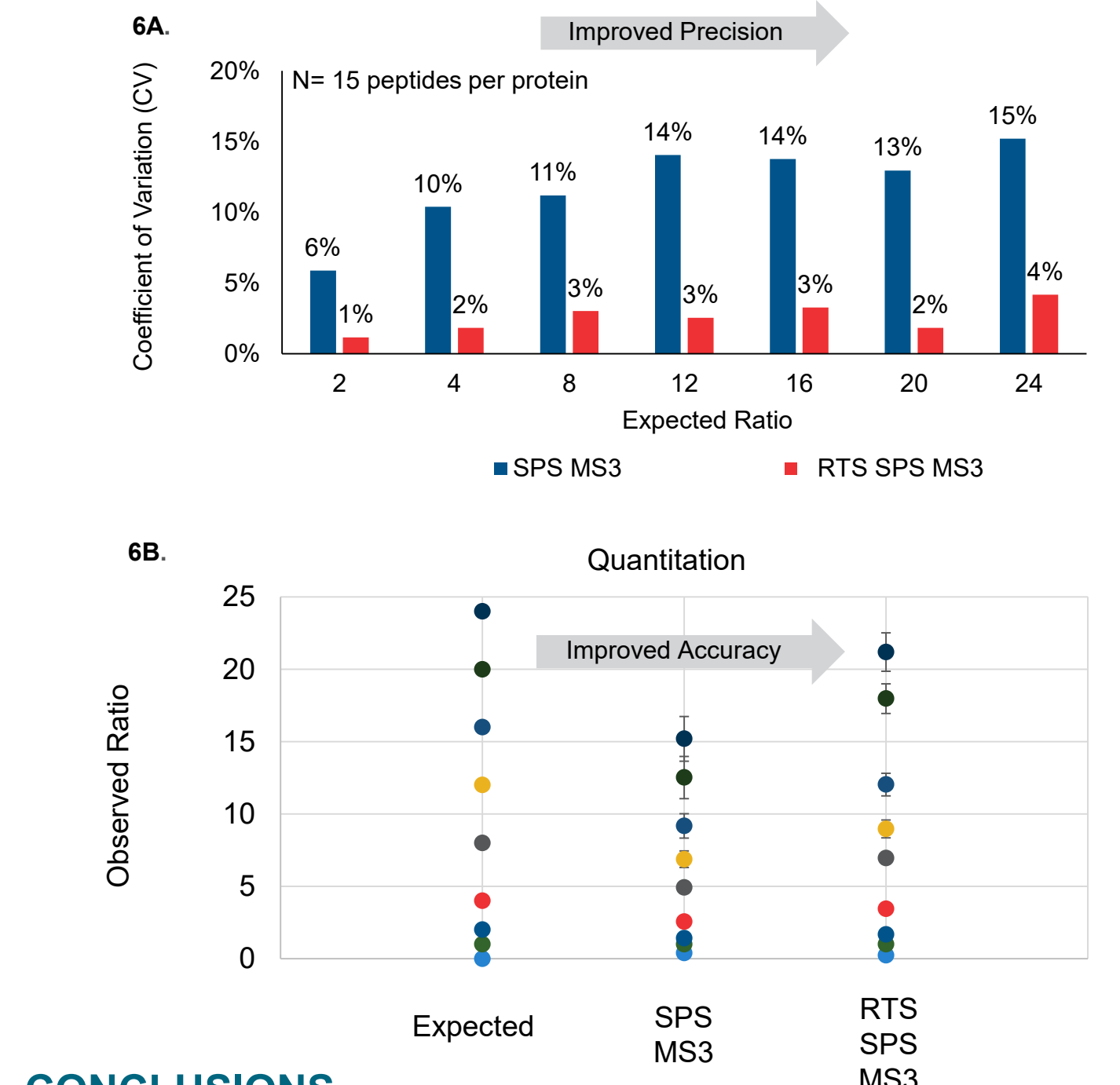
## REAL TIME SEARCH ENABLES CUSTOM MODIFICATIONS

Figure 5. Next generation isobaric tags increases sample multiplexing. We evaluated the potential for Real Time Search to use custom modifications using a next generation isobaric tags. (5A.) Pierce 6 Protein Digest Standard mixed in various ratios into the Pierce HeLa Protein Digest Standard and labeled. (5B.) 1ug of sample was then analyzed on a 120min gradient using RTS SPS MS3 or SPS MS3.



## REAL TIME SEARCH IMPROVES TMT QUANTIFICATION

Figure 6. Evaluation of TMT quantification performance. We evaluated how Real Time Search affects TMT SPS MS3 quantitation precision (6A.) and accuracy (6B.) using Pierce 6 Protein Digest Standard mixed in various ratios into the Pierce HeLa Protein Digest Standard. 1ug of peptides was analyzed on a 120min gradient.



## CONCLUSIONS

We present a modified Orbitrap Tribrid mass spectrometer utilizing Real Time Search that improved identification rates and accuracy for TMT SPS MS3 based quantitation.

## REFERENCES

- Paulo et al. A Triple Knockout (TKO) Proteomics Standard for Diagnosing Ion Interference in Isobaric Labeling Experiments. J. Am. Soc. Mass Spectrom. 2016, 27: 1620
- Erickson et al. Active Instrument Engagement Combined with a Real-Time Database Search for Improved Performance of Sample Multiplexing Workflows. J. Proteome Res. 2019 1;18(3):1299-1306.
- Schweppe et al. Characterization and Optimization of Multiplexed Quantitative Analyses Using High-Field Asymmetric-Waveform Ion Mobility Mass Spectrometry. Anal. Chem. 2019, 91, 6, 4010-4016

## ACKNOWLEDGEMENTS

We would like to thank Ryan Bomgardner, Derek Bailey now at Google, Jimmy Eng from the University of Washington, and Steven Gygi, Devin Schweppe, and Qing Yu from the Department of Cell Biology at Harvard Medical School in Boston, USA for their collaboration and input.

## TRADEMARKS/LICENSING

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PO65536-EN0519S

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