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Enhancing protein quantification and sample throughput with TMTpro 32-plex reagents and extended supporting features from the Thermo Scientific Orbitrap Ascend MultiOmics Tribrid Mass Spectrometer

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Abstract

Purpose: To assess the performance of the Thermo Scientific™ Orbitrap™ Ascend MultiOmics Tribrid™ mass spectrometer (MS) with the novel Thermo Scientific™ TMTpro™ 32-plex reagent set.

Methods: LC-MS/MS experiments employing either MS2 or Real-Time Search (RTS) Synchronous Precursor Selection (SPS)-MS3 acquisition were performed using newly-enabled Orbitrap resolving power settings of TurboTMT 45k, TurboTMT 60k, eFT 75k, and eFT 90k. We compared a TMTpro 32-plex labeled sample to a TMTpro 16plex labeled counterpart to evaluate identification efficiency and quantification accuracy.

Results: Increased sample throughput with TMTpro 32-plex reagents and improved quantification accuracy with RTS SPS-MS3 acquisition.

Introduction

Multiplexed quantitative proteomics has gained significant attention in various research domains, including chemoproteomics, drug discovery, single-cell analysis, and plasma profiling. To address the demand for higher multiplexing capabilities and improved sample throughput, a novel TMTpro 32-plex reagent was developed that permits quantification of proteins from twice the number of samples in a single LC-MS/MS experiment without sacrificing quantitative accuracy and precision.

To fully leverage the potential of the TMTpro 32-plex reagent set[1], it is crucial to employ mass spectrometers capable of resolving Δm at 3 mDa reporter ion peaks with maximum duty cycle efficiency. Consequently, the Thermo Scientific Orbitrap Ascend MultiOmics Tribrid mass spectrometer now offers new resolving power (RP) settings at TurboTMT 45k, TurboTMT 60k, eFT 75k, and eFT 90k to ensure optimal resolution and acquisition speed for accurate quantification of proteins and peptides in highly multiplexed experiments.

One of the key features that enhances data acquisition efficiency while maintaining quantitative accuracy for complex sample mixtures is the Real-Time Search feature that is implemented on Orbitrap Tribrid MS systems. RTS enables on-the-fly peptide sequence matching for MS2 spectra, triggering SPS-MS3 acquisition only upon confident peptide sequence identification.

Materials and methods

Sample preparation

TMTpro 32-plex-labeled HeLa digest samples mixed at 1:4 and 1:10 ratios between deuterated and non-deuterated channels were used to evaluate instrument parameters necessary to resolve reporter ions. Furthermore, a TMTpro 32-plex-labeled HeLa digest with a Thermo Scientific™ Pierce™ 6 protein digest mix spiked in at different ratios across channels was also used (Figure 2).

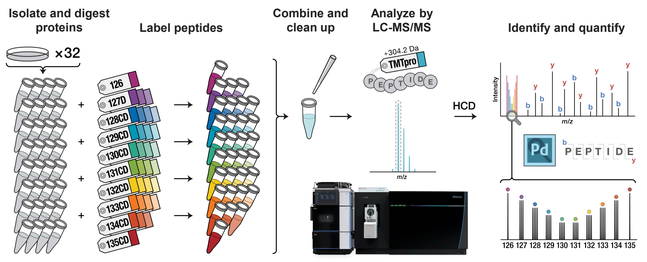


Figure 1. TMTpro 32-plex workflow. TMTpro 32-plex labeled cell lysate digest samples are analyzed by nanoflow LC-MS/MS using an Orbitrap Ascend MultiOmics Tribrid mass spectrometer (MS) coupled with a Thermo Scientific™ Vanquish™ Neo UHPLC system using a Thermo Scientific™ 50cm C18 Easy-Spray™ PepMap™ Neo UHPLC column (#ES75500PN). Data are processed by Thermo Scientific™ Proteome Discoverer™ 3.2 software.

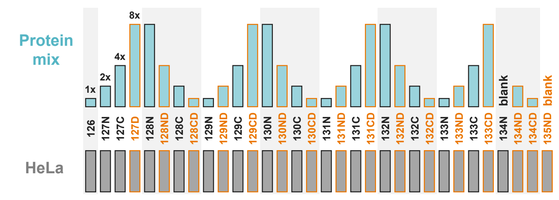
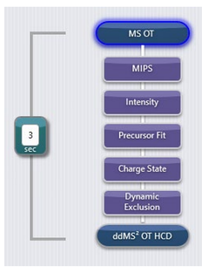


Figure 2. TMTpro HeLa + protein digest mix. Pierce 6 protein digest standard (equimolar) was spiked into a background of HeLa cell lysate digest at 100, 200, 400, and 800 fmol per µg of HeLa digest. Samples were labeled with TMTpro 32-plex reagents, pooled in equal amounts, and cleaned by EasyPep SPE prior to LC-MS/MS analysis.

Pierce 6 protein digest standard
Serum albumin, *Bovine*
Cytochrome C, *Bovine*
Lysozyme, *Chicken*
β-galactosidase, *E. coli*
Apo-Transferrin, *Bovine*
Alcohol dehydrogenase, *S. cerevisiae*

Instrument methods

Our experiments utilized the Orbitrap Ascend MultiOmics Tribrid MS and Vanquish Neo UHPLC systems with a PepMap 50cm C18 column, employing a 140-minute LC gradient length with a gradient elution from 4% to 40% of 80% Acetonitrile with 0.1% formic acid at LC flowrate of 0.250 µL/min. About 1ug sample was loaded on column for each injection. Both MS2 and RTS SPS-MS3 experiments were performed using resolving powers of TurboTMT 45k, TurboTMT 60k, eFT 75k, and eFT 90k with optimized maximum injection time for each resolving power. For SPS-MS3 experiments with Real-Time Search filter, the following parameters were specified: Xcorr = 1.4, dCn = 0.1, and precursor mass tolerance = 20 ppm.



MS Parameter	
Instrument Control Software	Orbitrap Ascend Tune Application 4.2
MS¹	
RF lens (%)	60
Orbitrap Resolution	120,000
Normalized AGC Target (%)	100
Maximum Injection Time (ms)	50
Precursor Fit Filter (%, *m/z*)	50/1.2
Dynamic Exlusion (s)	60
MS²	
Isolation Window (*m/z*)	0.7
HCD Collision Energy (%)	34
Orbitrap Resolution	45,000/Turbo TMT 60,000/Turbo TMT 75,000 90,000
First Mass (*m/z*)	110
Normalized AGC Target (%)	300
Maximum Injection Time (ms)* ** Note that the parameters we present here are selected to optimize the total number of quantified proteins for this sample and this LC-MS method. In this case we opted to use maximum injection times that favored spectral acquisition rates over spectral quality. For other samples, LC gradients, etc., an instrument operator may see better absolute performance using longer injection times.*	91 (for 45,000 resolution/ Turbo TMT) 123 (for 60,000 resolution/Turbo TMT) 155 (for 75,000 resolution) 187 (for 90,000 resolution)

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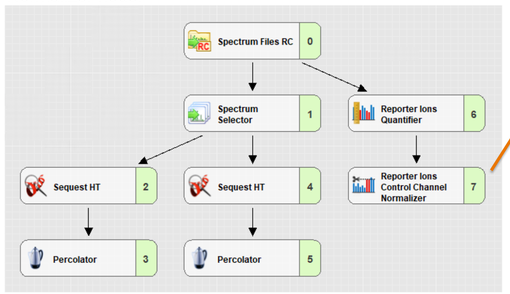
Precursor Fit Filter (%, *m/z*)	50/1.2
Dynamic Exlusion (s)	60
MS²	
Isolation Window (*m/z*)	0.7
CID Collision Energy (%)	34
IT Scan Rate	Turbo
Scan Range (m/z)	400-1600
Normalized AGC Target (%)	100
Maximum Injection Time (ms)	23
MS³	
Isolation Window (*m/z*)	1.2
HCD Energy (%)	55
Orbitrap Resolution	45,000(Turbo) 60,000(Turbo) 75,000 90,000
Scan Range (*m/z*)	100-500
Normalized AGC Target (%)	300
Maximum Injection Time (ms)** *** Note: settings are selected to favor spectral acquisition rate to optimize total number of quantified proteins for this sample and LC-MS method. For other samples, LC gradients, etc., an instrument operator may see better absolute performance using longer injection times.*	91 (for 45,000 resolution/ Turbo TMT) 123 (for 60,000 resolution/Turbo TMT) 155 (for 75,000 resolution) 187 (for 90,000 resolution)

Figure 3. Orbitrap Tribrid acquisition methods for MS2 and SPS-MS3 with Real-Time Search utilizing new resolving power options. Orbitrap Tribrid Series Tune Application 4.2 includes new Orbitrap mass analyzer resolving powers options, TurboTMT 45k/60k and eFT 75k/90k, that permit distinguishing TMTpro 32-plex reporter ions (Δm at 3 mDa) while maximizing acquisition speed.

Data analysis

Data analysis was performed using Proteome Discoverer 3.2 software and the SEQUEST® HT search algorithm. Peptide modifications included dynamic modifications of carbamidomethylation (C) for HeLa, carboxymethylation (C) for the protein mix, TMTpro tags (N-terminus, K), and Met oxidation. FDR threshold was set to 1% in the Percolator node for identifying PSMs at high confidence for peptide and protein identification. A reporter ion peak integration tolerance of 11 ppm was specified in the Reporter Ions Quantifier node, and normalization of the deuterated and non-deuterated channel groups was performed using deuterated and non-deuterated control channels via the newly integrated Reporter Ions Control Channel Normalizer node.



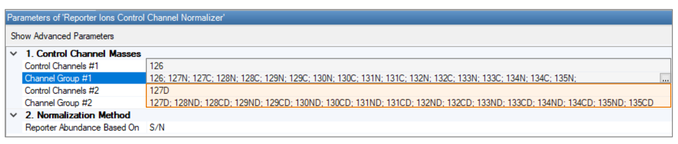
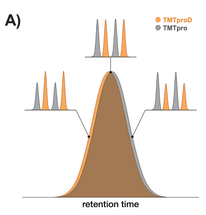


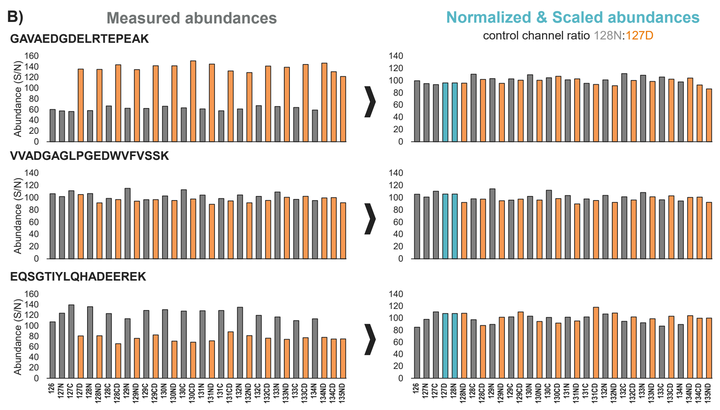
Figure 4. Proteome Discoverer 3.2 software supports TMT 32-plex data processing. The new Reporter Ion Control Channel Normalizer node permits referencing deuterated and non-deuterated channels separately and using control channels to normalize abundances between deuterated and non-deuterated sub-plexes to achieve accurate quantification.

Figure 5. Control channel normalization of TMTpro 32-plex data.

A) A retention time difference of 0.5-1 seconds is observed between peptides labeled with deuterated and non-deuterated TMTpro reagents during reversed-phase chromatography separation which results in the two sub-plexes of reporter ions measuring at different abundances depending on when MS/MS is triggered over the peptide's elution profile.



B) Accurate quantification may be achieved by referencing the two sub-plexes in isolation from each other by taking ratios of deuterated channels to a deterated denominator channel and doing the same of non-deuterated channels. Alternatively, a reference sample may be labeled with a tag from each set to be used as control channels to normalize abundance for the two sub-plexes in Proteome Discoverer 3.2. After normalization, the abundances of the two sub-plexes are scaled to correct for the effect of the retention time shift.



Results and discussions

TMTpro 32-plex resolving power requirement

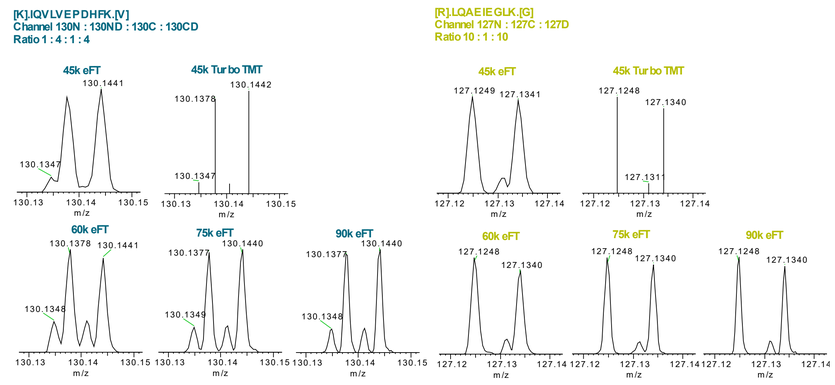


Figure 6. Characterizing resolving power on Orbitrap Ascend MultiOmics Tribrid MS. A TMTpro 32-plex-labeled HeLa digest mixed at 1:4 or 1:10 ratios between deuterated and non-deuterated channels was acquired at eFT resolving powers of 45k, 60k, 75k, and 90k to distinguish reporter ions. A resolving power of eFT 90k resolves Δm at 3 mDa reporter ions at baseline while eFT 75k resolves within ~5% of baseline.

Though eFT 45k and eFT 60k resolving power are not sufficient to adequately resolve Δm at 3 mDa reporter ions for quantification, PhiSDM spectral processing feature can be applied when using these shorter transients to increase effective resolution. By enabling the TurboTMT setting, the spectral processing is applied specifically to the reporter ion mass region to permit resolution of the TMTpro 32-plex reporter ions at a higher acquisition rate.

Quantification performance at different MS2/MS3 resolving powers for HeLa + 6 protein mix sample

Figure 7. Characterizing resolving power on Orbitrap Ascend MultiOmics Tribrid MS with a HeLa + 6 protein mix sample utilizing both MS2 and RTS MS3 methods. For MS2 analysis, 45k resolving power with the TurboTMT option enabled outperformed the rest of the available OT acquisition settings both in terms of identification and quantification of protein groups and peptide groups. For SPS-MS3 with Real-Time Search analysis, the performance differences are subtle amongst all MS3 OT acquisition settings.

Though RTS SPS-MS3 analyses produced a comparable number of protein groups/peptide groups identifications to the best MS2 analysis with TurboTMT 45k setting, the percentage of quantified groups/peptide groups in RTS SPS-MS3 analyses are not has high as the percentage from MS2 analyses in this case, as we opted to use maximum injection time that favored spectral acquisition rate over spectral quality for this sample and this LC-MS method. For other samples, LC gradients, etc., an instrument operator may see better absolute performance using longer injection times.

Both the 32-plex sample (in blue) and 16-plex non-deuterated sample (in green) show the trend described above.

Quantification accuracy evaluation at different MS2/MS3 resolving powers for HeLa + 6 protein mix sample

Figure 8. Evaluating quantification accuracy with HeLa + 6 protein mix sample utilizing both MS2 and RTS MS3 methods. Co-isolated ion interference can often result in distorted TMT quantitative values. Synchronous precursor selection (SPS)-MS3-based methods provide higher TMT quantitative accuracy compared to MS2 methods, but some degree of abundance distortion is still observed if precursor isolation specificity is suboptimal.

With Real-Time search for SPS-MS3, on-the-fly search and identification of MS2 fragment ions enables more efficient triggering of MS3 scans and can reduce interference to lower levels by ensuring that all selected MS2 fragment ions are associated with the target peptide.

The plots here show the TMTpro channel abundances for the 32-plex sample (in blue) and 16-plex sample (non-deuterated, in green), with MS2 experiments in the darker shade and Real-Time Search SPS-MS3 experiments in the lighter shade. Four resolving powers (TurboTMT 45k, TurboTMT 60k, eFT 75k, and eFT 90k) were evaluated for both MS2 and RTS SPS-MS3 methods. While MS2 acquisition yielded clearly distorted abundances, RTS SPS-MS3 achieved greater quantitative accuracy across different resolving power settings and different samples.

Increased sample throughput with TMTpro 32-plex

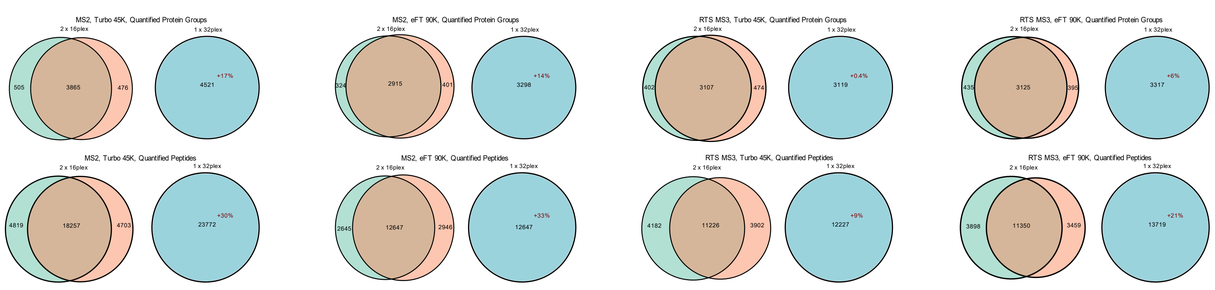


Figure 9. Increased sample throughput and greater numbers quantified proteins & peptides in one TMTpro 32-plex experiment compared to two TMTpro 16-plex experiments with TurboTMT 45k and eFT 90k resolving power settings. In large data sets, only the proteins which are identified in every analysis can be confidently used for quantification. Each TMTpro 32-plex experiment yielded equal or greater numbers of quantified proteins and peptides compared to two TMTpro 16-plex experiments, for both MS and RTS SPS-MS3 acquisition methods, due to missing values between two LC-MS/MS runs.

Conclusions

- Newly-available Orbitrap resolving power options of 75k and 90k resolve the Δm at 3 mDa reporter ions from the novel TMTpro 35-plex reagent set.
- Resolving power settings at TurboTMT 45k/60k and eFT 75k/90k are all suitable for TMTpro 32-plex experiments.
- Real-Time Search with SPS-MS3 achieves better quantification accuracy vs. MS2 acquisition for both 32-plex and 16-plex samples.
- Single TMTpro 32-plex experiment yields equal or better quantitative performance compared to replicates of TMTpro 16-plex experiments
- Proteome Discoverer 3.2 software supports normalization & scaling of deuterated and non-deuterated sub-plexes to ensure accurate quantification .

References

1. Dustin Frost, Joao A. Paulo, Steven P. Gygi, Karsten Kuhn, Ian Pike, Ryan Bomgarden, Expanding TMTpro reagents to 32-plex for high throughput quantitative proteomics on Orbitrap platforms, ASMS poster 2024.

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