Enhancing protein quantification and sample throughput with TMTpro 32plex label reagents and extended supporting features from the Orbitrap Ascend MultiOmics mass spectrometer

Authors

Julia Kraegenbring[1], Jingjing Huang[2], Dustin Frost[3], William J. Comstock[2], Bernard Delanghe[1], Ryan D. Bomgarden[3], Rosa Viner[2], Tonya Pekar Hart[2], Rafael Melani[2], Amirmansoor Hakimi[2]

[1]Thermo Fisher Scientific, Bremen, Germany

[2]Thermo Fisher Scientific, San Jose, CA, USA

[3]Thermo Fisher Scientific, Rockford, IL, USA

Keywords

Orbitrap Ascend MultiOmics Tribrid mass spectrometer, Tandem Mass Tags, TMTpro 32plex, TMT, quantitative proteomics, real-time search, synchronous precursor selection, SPS MS3, TMTpro 16plex

Goal

This study evaluates the performance of the Thermo Scientific™ Orbitrap™ Ascend MultiOmics Tribrid™ mass spectrometer (MS) in combination with the Thermo Scientific™ TMTpro™ 32plex label reagents for high-throughput, multiplexed quantitative proteomics.

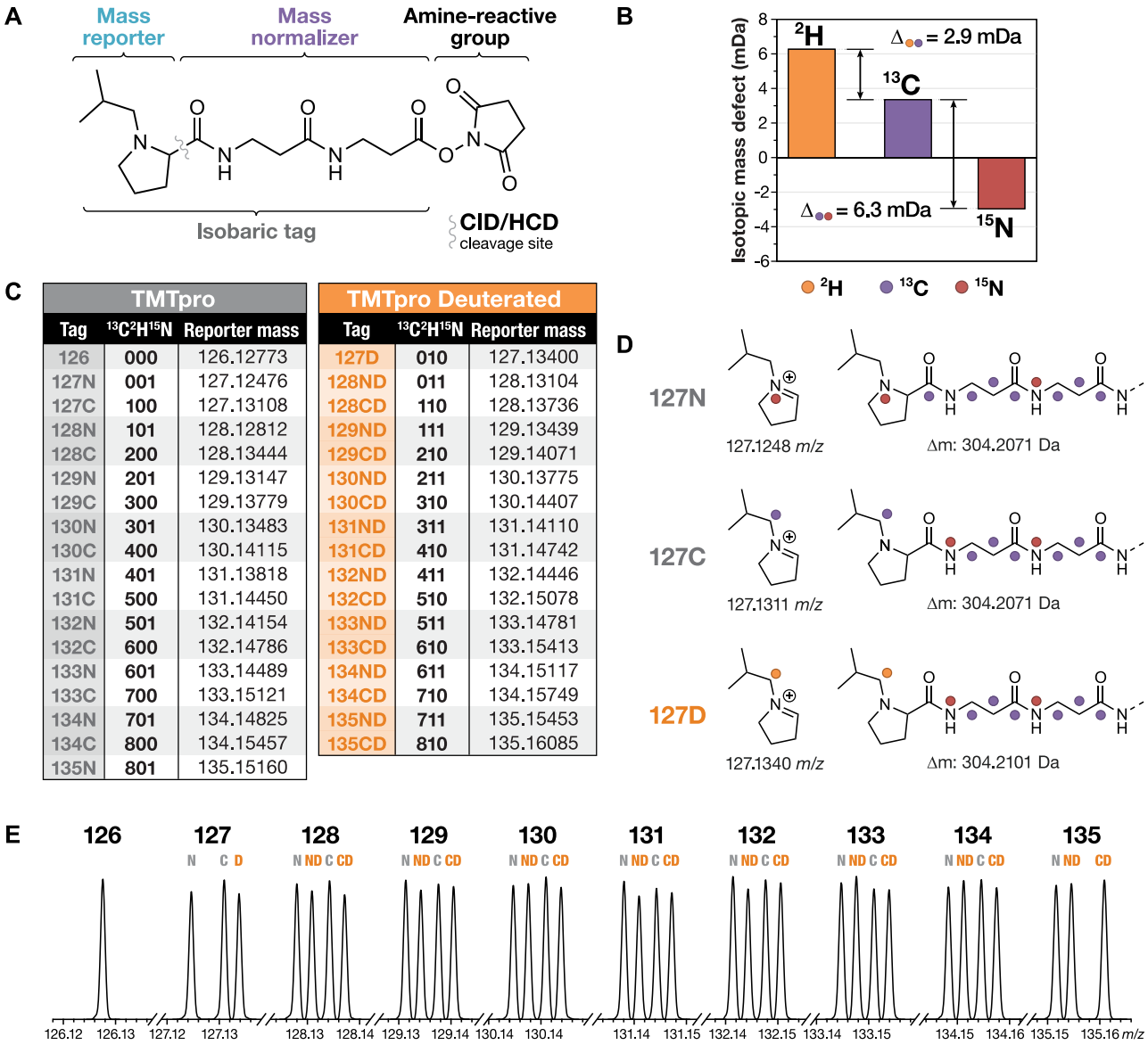
Introduction

Quantitative proteomics has become a cornerstone technique in molecular biology, facilitating in-depth analysis of protein expression patterns, post-translational modifications, and molecular interactions in complex biological systems. MS-based approaches have evolved from broad-spectrum protein profiling into highly precise tools capable of interrogating the proteome at scale, capturing dynamic signaling processes and regulatory mechanisms. Among these, Thermo Scientific™ Tandem Mass Tag™ (TMT) labeling reagents have had a transformative impact, enabling simultaneous protein identification and relative quantification across multiple biological samples within a single experiment.

Label-free quantification (LFQ), which depends on separate MS^1 intensity measurements for data-dependent acquisition (DDA) approaches and MS^1 or MS^2 intensity measurements for data-independent acquisition (DIA) approaches, is prone to run-to-run variability and data incompleteness. In contrast, isobaric tagging with chemically identical mass tags relies on post-digestion labeling of peptides from several samples that are measured in a multiplexed manner under the same conditions. Therefore, TMT-based quantification offers minimized inter-sample variation, more efficient use of instrument time, and enhanced statistical power—often detecting

abundance shifts below 10% in large-scale studies. As highlighted by Pappireddi et al.[1], increased multiplexing reduces both missing values and batch effects, two major limitations in traditional multi-run proteomics workflows.

The introduction of TMTpro 32plex label reagents represents the most significant advancement in isobaric multiplexing capacity to date, nearly doubling the sample capacity of the previous generation while retaining the same chemical structure (Figure 1A). This expanded set incorporates seventeen novel reagent isotopologues generated through targeted incorporation of a deuterium (^{2}H) atom into the reporter group (Figure 1C and 1D). The deuterated reporter ion channels differ from their non-deuterated counterparts by a precise 2.9 millidaltons (mDa) (Figure 1B), forming dense reporter ion peak clusters that require an MS/MS resolving power of ≥90,000 (at 200 *m/z*) to separate at full width at baseline in the Thermo Scientific™ Orbitrap™ mass analyzer (Figure 1E). This carefully engineered mass difference enables high-fidelity quantification across up to 35 distinct channels to greatly surpass previous throughput limitations while improving inter-sample normalization and reducing batch effects in complex study designs.[2] As a result, TMTpro 32plex label reagents are particularly well-suited for clinical and population-scale proteomics where robustness, scalability, and per-sample cost efficiency are critical for detecting subtle but biologically relevant proteomic signatures.



TMTpro			TMTpro Deuterated		
Tag	$^{13}C^2H^{15}N$	Reporter mass	Tag	$^{13}C^2H^{15}N$	Reporter mass
126	000	126.12773	127D	010	127.13400
127N	001	127.12476	128ND	011	128.13104
127C	100	127.13108	128CD	110	128.13736
128N	101	128.12812	129ND	111	129.13439
128C	200	128.13444	129CD	210	129.14071
129N	201	129.13147	130ND	211	130.13775
129C	300	129.13779	130CD	310	130.14407
130N	301	130.13483	131ND	311	131.14110
130C	400	130.14115	131CD	410	131.14742
131N	401	131.13818	132ND	411	132.14446
131C	500	131.14450	132CD	510	132.15078
132N	501	132.14154	133ND	511	133.14781
132C	600	132.14786	133CD	610	133.15413
133N	601	133.14489	134ND	611	134.15117
133C	700	133.15121	134CD	710	134.15749
134N	701	134.14825	135ND	711	135.15453
134C	800	134.15457	135CD	810	135.16085
135N	801	135.15160			

Figure 1. (A) Chemical structure of the TMTpro label reagent; (B) isotopic mass defects; (C) reporter ion masses; (D) heavy stable isotope configurations and tag Δm (127 channel examples); and (E) reporter ion peak clusters acquired in the Orbitrap mass analyzer at an MS/MS resolving power of 90k.

The Orbitrap Ascend MultiOmics MS is optimally suited for TMTpro 32plex label reagents applications, combining high-resolution, high-mass-accuracy detection with advanced acquisition strategies such as Real-Time Search with synchronous precursor selection (SPS) MS^3. By enabling SPS MS^3 and real-time peptide identification, real-time search with SPS MS^3 minimizes ratio distortion typically observed in MS^2-based methods and enhances quantitation accuracy—especially critical for complex samples and in densely multiplexed experiments. While MS^3 acquisition incurs slightly longer duty cycles, the increased selectivity and sensitivity allow for robust detection of low-abundance proteins and improved confidence in peptide sequence identification. These capabilities make the Orbitrap Ascend MultiOmics MS particularly advantageous for highly multiplexed quantitative proteomics in complex biological or clinical sample cohorts.

Experimental

Recommended consumables

- Fisher Chemical™ Optima™ LC/MS Grade Water with 0.1% Formic Acid (FA) (v/v) (Part No. **LS118-500**)
- Fisher Chemical™ Optima™ LC-MS Grade 80% Acetonitrile (ACN), 20% Water with 0.1% Formic Acid (Part No. **LS122500**)
- Fisher Chemical™ Optima™ LC-MS Grade Formic Acid (Part No. **A117-50**)
- Fisher Chemical™ Optima™ LC-MS Grade Water (Part No. **10505904**)
- Fisher Chemical™ Optima™ LC-MS Grade Acetonitrile (Part No. **A955-1**)
- Fisher Chemical™ Optima™ LC-MS Grade Isopropanol (Part No. **A461-212**)
- Thermo Scientific™ TMTpro™ 32plex and TMTpro Deuterated Label Reagents (Part No. **A40000839**)
- Thermo Scientific™ TMTpro™ 16plex Label Reagent Set (Part No. **A44520**)
- Thermo Scientific™ Pierce™ 6 Protein Digest, equimolar, LC-MS grade (Part No. **88342**)
- Thermo Scientific™ EasyPep™ MS Sample Prep Kits (Part No. **A45733**)

LC columns

- Thermo Scientific™ Easy-Spray™ PepMap™ Neo UHPLC Column, 2 µm C18 75 µm x 500 mm (Part No. **ES75500PN**)

HPLC system

- Thermo Scientific™ Vanquish™ Neo UHPLC System, including:
  — Thermo Scientific™ Vanquish™ Neo Pump/Autosampler (Part No. **VN-S10-A-01**)

Mass spectrometer

- Orbitrap Ascend MultiOmics MS
- Thermo Scientific™ EASY-Spray™ source

Data analysis software

- Thermo Scientific™ Proteome Discoverer™ Software version 3.3 (Part No. **PROTEOMEDISC3**)

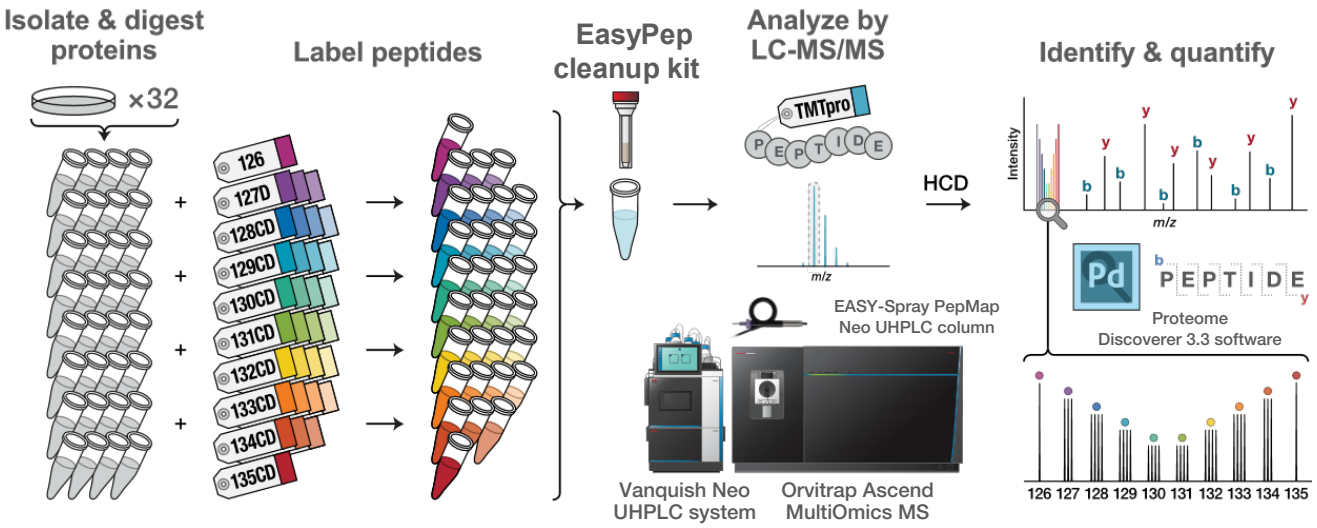


Figure 2. Workflow schematic for TMTpro 32plex label reagent sample labeling, cleanup, and LC-MS/MS analysis.

Sample preparation

TMTpro 32plex-labeled HeLa digest samples mixed at 1:4 and 1:10 ratios between deuterated and non-deuterated channels (Figure 3A) were used to evaluate instrument parameters necessary to resolve reporter ions on the Orbitrap Ascend MultiOmics MS. Additionally, a TMTpro 32plex-labeled HeLa digest with a 6 protein digest mix spiked in at different ratios across channels (Figure 3B) was used to assess quantitative metrics. The six proteins from different species were spiked into a background of HeLa cell lysate digest at 100, 200, 400, and 800 fmol/µg of HeLa digest. Samples were labeled with TMTpro 32plex label reagents, pooled, and cleaned by EasyPep MS sample prep kit prior to LC-MS/MS analysis.

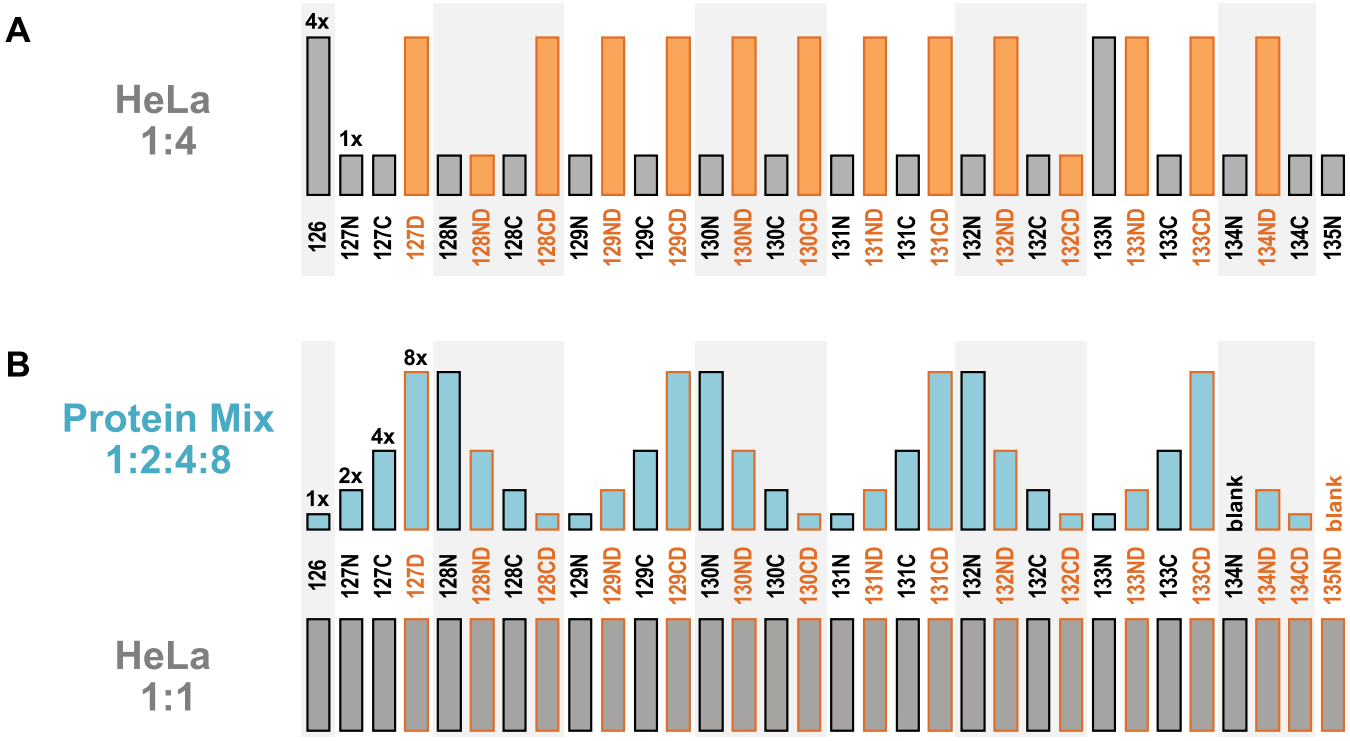


Figure 3. TMTpro 32plex-labeled digest sample designs. (A) HeLa digest mixed at 1:4 ratios between deuterated and non-deuterated channels and (B) 6-protein mix digest spiked at different concentrations into a static HeLa digest background.

LC parameters

The Vanquish Neo UHPLC system was configured in direct-injection setup and coupled to an EASY-Spray PepMap Neo UHPLC column (75 µm × 500 mm) which was heated using the EASY-Spray source to 40 °C. The details of the UHPLC method are shown in Tables 1 and 2.

Table 1. Vanquish Neo UHPLC system parameters.

Column setup	
Inner diameter	75 µm
Length	50 cm
Void volume	1.479 µL
Maximum pressure	1,500 bar
Maximum flow	100 µL/min
Max. pressure change up	500 bar/min
Max. pressure change down	500 bar/min
Sampler settings	
Sampler temperature	7.0 °C
Wash mode	After draw
Strong wash solvent	0.1% FA in 80% ACN
Weak wash solvent	0.1% FA in water

Loading settings	
Fast loading	True
Mode	Combined control
Pressure	1,500 bar
Loading volume	Automatic
Loading flow	100 µL/min
Wash and equilibration settings	
Fast equilibration	True
Equilibration mode	Combined control
Pressure Equilibration factor Equilibration flow	1,500 bar 6.0 100 µL/min

Table 2. LC gradient for 15 samples per day (SPD). Mobile phase A: H_2O with 0.1% FA. Mobile phase B: 80/20 ACN/H_2O with 0.1% FA.

Time [min]	Duration [min]	Flow rate [µL/min]	Mobile phase B [%]
0.0	Run		
0.0	0.0	0.25	4.0
120.0	120.0	0.25	25.0
140.0	20.0	0.25	40.0
140.0	Column wash		
141.0	1.0	0.35	99.0
150.0	9.0	0.35	99.0
150.0	Stop run		
150.0	Column equilibration		

MS parameters

For clarity, resolution values such as 45k, 60k, 75k, and 90k refer to standard eFT spectral processing (at 200 *m/z*) unless otherwise stated. In contrast, resolution values obtained using ΦSDM (TurboTMT) spectral processing are explicitly denoted as, e.g., 45k TurboTMT.

TMTpro 32plex-labeled samples were analyzed by DDA methods using an Orbitrap Ascend MultiOmics MS. In Table 3, the real-time search with SPS MS^3 method with an Orbitrap resolution setting of 90,000 is summarized. The MS^2-based method uses the same parameters for full scans and quantification scans (MS^3 scans in the table), except: HCD energy of 34% and a first mass of 110 *m/z*.

Table 3. MS acquisition parameters for TMTpro 16plex and TMTpro 32plex samples using real-time search and SPS MS^3.

Global parameters			
Settings		**Ion source properties**	
Application mode	Peptide	Spray voltage (V)	2,250
Advanced peak determination	True	Ion transfer tube temperature (°C)	305
Scan parameters			
Orbitrap full MS scan (MS-OT)		**Data-dependent properties**	
Orbitrap resolution	120,000	Data-dependent mode	Cycle time
Scan range (*m/z*)	400–1,600	Time between master scans (s)	3
Max. injection time (ms)	50	**Data-dependent scans**	
Normalized AGC target (%)	100	**Ion trap ID scan (ddMS² IT CID)**	
RF lens (%)	60	Isolation window (*m/z*)	0.7
Filters for data-dependent MS^2 triggers		Ion trap scan rate	Turbo
MIPS		Scan range (m/z)	400–1,600
Monoisotopic peak determination	Peptide	Normalized AGC target (%)	100
Isolation window center	Most abundant peak	Max. injection time (ms)	23
Intensity		**Filters for SPS MS^3 scans**	
Filter type	Intensity threshold	**Real-time search**	
Intensity threshold	5e3	Static modification	Carboxymethyl (C), TMTpro 16plex (Kn)
Precursor fit		Variable modification	Oxidation (M)
Fit threshold (%)	50	Max. missed cleavages	1
Fit window (*m/z*)	1.2	Max. modification/peptide	2
Charge state		TMT SPS MS^3 mode	True
Include charge state(s)	2-6	Scoring thresholds: precursor PPM	20
Include undetermined charge states	False	**Orbitrap QUAN scan (ddMS³ OT HCD)**	
Dynamic exclusion		Number of SPS precursors	10
Exclusion duration (s)	60	Isolation window (*m/z*)	1.2
Mass tolerance high/low (ppm)	10	Normalized HCD collision energy (%)	55
Exclude isotopes	True	Orbitrap resolution	90,000*
Perform dependent scan on single charge state per precursor only	True	Scan range (*m/z*)	100–500
Filter type	Intensity threshold	Normalized AGC target (%)	300
		Max. injection time (ms)	187*

*Maximum injection times for the tested resolution settings (45k TurboTMT, 60k TurboTMT, 75k eFT, 90k eFT) were selected to favor spectral acquisition rates with maximum duty cycle parallelization: 91 ms, 123 ms, 155 ms, 187 ms.

Data processing parameters

Data were processed in Proteome Discoverer software version 3.3 using the Processing Workflow nodes shown in Figure 4. In the Reporter Ions Quantifier node, an integration tolerance of 11 ppm and integration method of Most Confident Centroid is used to extract reporter ion abundances for the TMTpro 32plex Δm: 3 mDa reporter ions, and MS order is selected (MS^2 or MS^3) based on the MS/MS parameters used for data acquisition. Proteome Discoverer software version 3.3 includes the Reporter Ions Control Channel normalizer node to re-scale abundances between the non-deuterated (Channel Group #1) and deuterated (Channel Group #2) sub-plexes based on control channels (one channel per group, or the average of multiple channels per group) using the parameters shown. Dynamic modifications were specified as shown with static modification of TMTpro (+304.207 Da) on N-termini and Lysine (K) for both TMTpro 16plex and 32plex experiments.

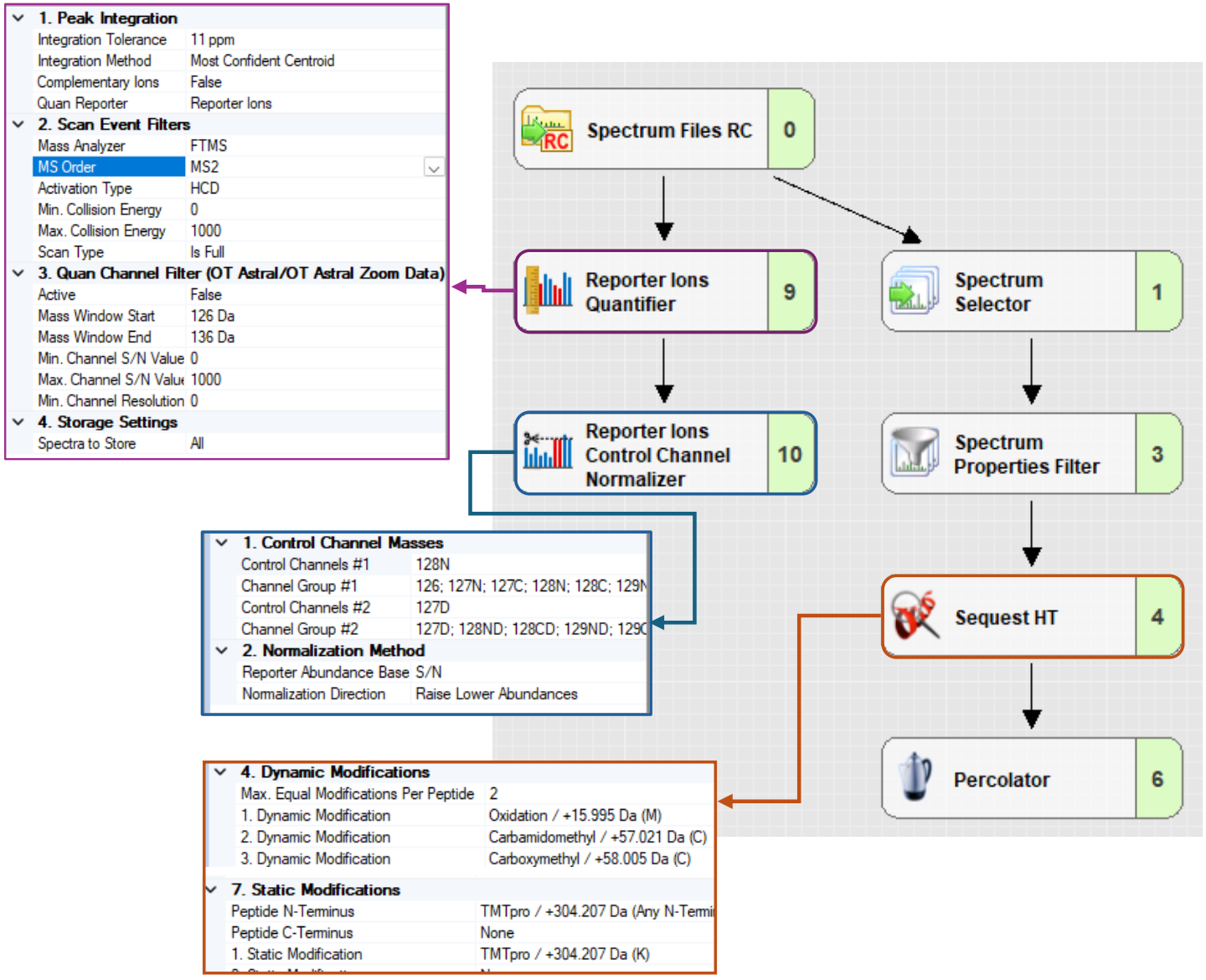


Figure 4. Parameters for processing workflow for TMTpro 32plex data in Proteome Discoverer version 3.3 software.

Results

Orbitrap resolving power requirements for TMTpro 32plex experiments

TMTpro 32plex label reagents 128N, 128ND, 128C, and 128CD were mixed in 1:1, 10:1, and 1:10 amounts and acquired by direct infusion MS/MS to determine the resolving power required to distinguish Δm: 3 mDa reporter ions in HCD FT-MS^2 spectra (Figure 5). Reporter ion peaks are distinguishable but not well-resolved from each other at 60k, whereas they are resolved to 5% of baseline at 75k and baseline resolved at 90k and higher.

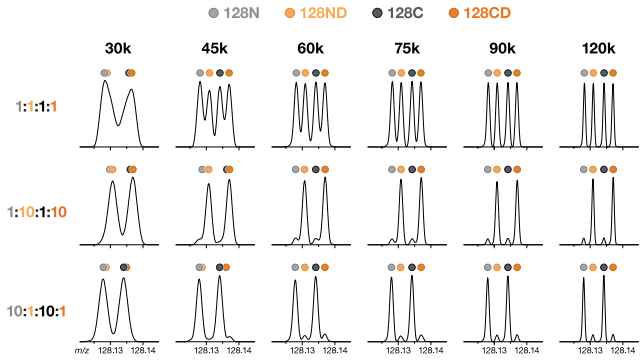


Figure 5. Resolving Δm: 3mDa TMTpro reporter ions in the Orbitrap mass analyzer.

The PhiSDM spectral processing algorithm can be applied to shorter Orbitrap FT transients to increase effective resolution within the reporter ion mass range (termed TurboTMT) to adequately separate Δm: 3 mDa reporter ions and permit TMTpro 32plex quantification at a higher acquisition rate. TMTpro 32plex-labeled HeLa digest samples were mixed at 1:4 or 1:10 ratios between deuterated and non-deuterated channels and acquired by LC-MS/MS at MS^2 resolving power settings of 30k TurboTMT, 45k TurboTMT, 60k TurboTMT, 75k eFT, 90k eFT, and 120k eFT. Figure 6 shows sufficiently resolved reporter ion peaks of channels 130N, 130ND, 130C, and 130CD acquired at 45k TurboTMT, 60k TurboTMT, 75k eFT, 90k eFT for an example peptide from each of the two samples.

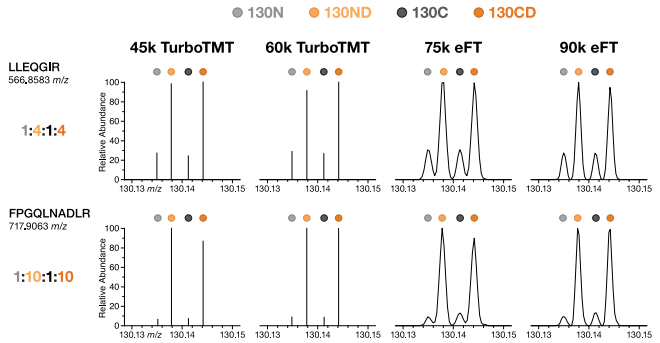


Figure 6. Reporter ion spectra acquired at Orbitrap resolving power settings suitable for distinguishing and quantifying peptides.

Figure 7 shows a comparison of quantitative ratio boxplots for the 1:10 sample (8 of the 32 channels shown; reference channels 134N and 128ND for non-deuterated and deuterated channels, respectively; isotopic interference correction not applied). The 30k TurboTMT acquisition did not reliably resolve the Δm: 3 mDa reporter ions, resulting in poor quantitative accuracy and precision, whereas 45k TurboTMT and 75k eFT achieved a good balance of acquisition speed and high quantitative performance. The average medians and coefficients of variation for the non-deuterated and deuterated channels are also summarized in Figure 7 for each resolving power setting.

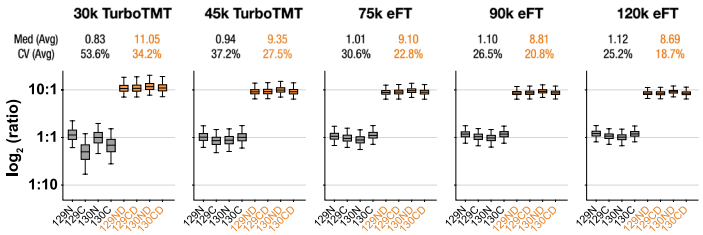


Figure 7. Comparison of TMTpro 32plex quantitative performance (PSMs) at different MS^2 resolving powers; 1:10 mixture, 8 out of 32 channels shown; box plots demarcate the median (line), the 25th and 75th percentile (box), and the 5th and 95th percentile (whiskers).

Quantitative performance at different resolving powers for HeLa + 6 protein mix sample

The 16plex and 32plex TMTpro-labeled HeLa digest with 6 protein digest mix samples were analyzed under various MS/MS acquisition settings using the same on-column load and the number of protein and peptide groups identified and quantified across all reporter ion channels is summarized in Figure 8. For a 16plex experiment, a resolving power of 45k is recommended which serves as the baseline for comparison against the 32plex experiments acquired at 45k TurboTMT, 75k eFT, and 90k eFT. The best performance for the 32plex is achieved using real-time search with SPS MS^3 acquisition with 45k TurboTMT for the SPS-MS^3 scan, resulting in approximately an equivalent number of quantified proteins between the two reagent sets with a difference of <5%, while increasing the resolution to 75k or 90k results in a difference of ~10%. Likewise, the 32plex performs equivalently to the 16plex with MS^2 acquisition using the 45k TurboTMT scan, whereas 75k and 90k result in fewer quantified proteins by 21% and 28%, respectively.

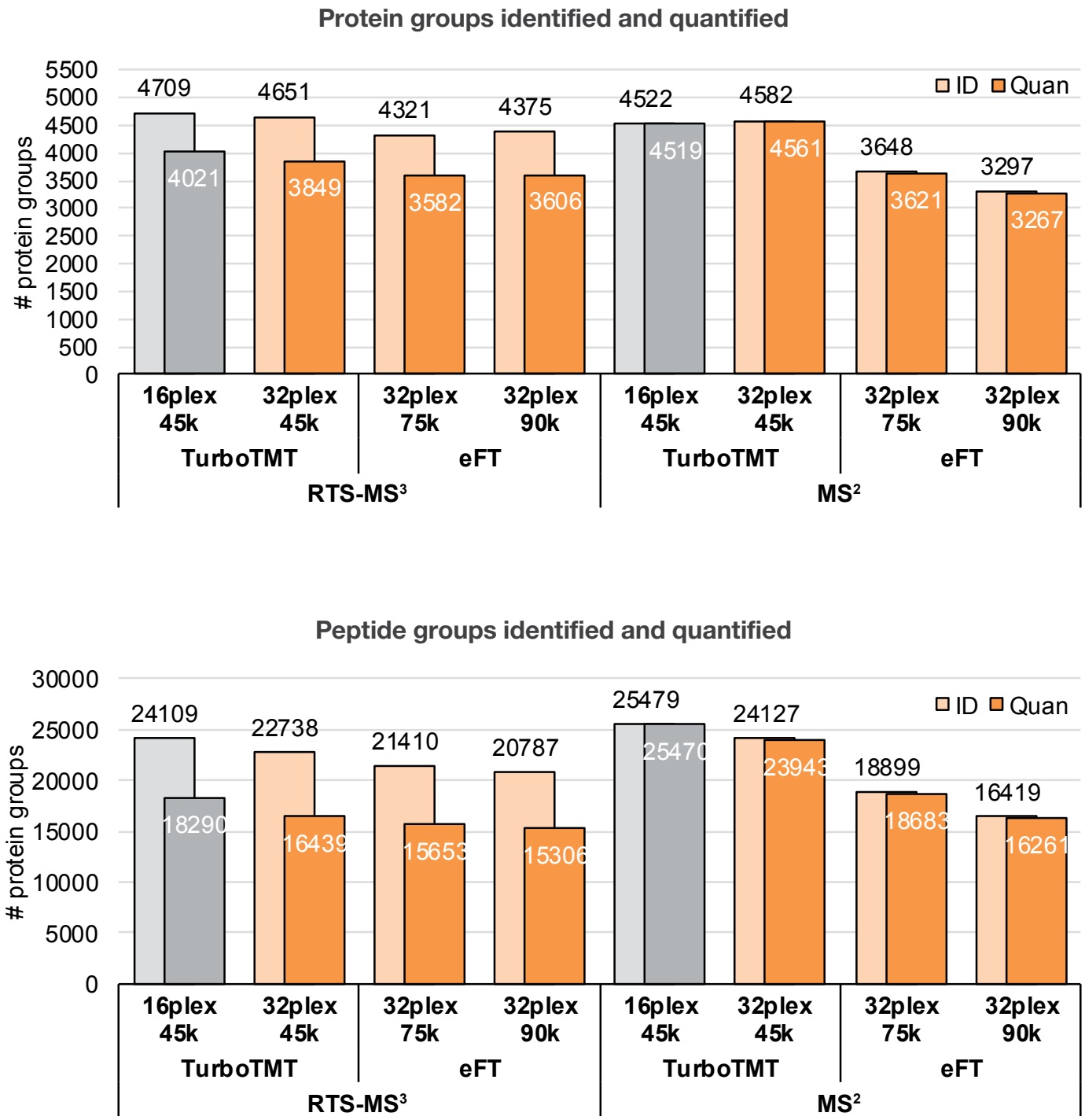


Figure 8. Comparison of identified and quantified protein and peptide groups for TMTpro 32plex for different MS/MS acquisition methods vs. TMTpro 16plex acquired at 45k TurboTMT.

Figure 9 shows a comparison of quantitative boxplots for the scaled and normalized abundances (%) of PSMs from the 6-protein mix digest spiked into a HeLa digest background. Co-isolation interference from the HeLa background results in additional reporter ion signals being measured for the 6 protein mix peptides, distorting the resulting abundances. The MS^2 acquisitions are impacted more significantly by co-isolation, resulting in less accurate abundances being measured. The real-time search with SPS MS^3 acquisitions partially mitigates the effect of co-isolation interference to yield better quantitative accuracy and precision.

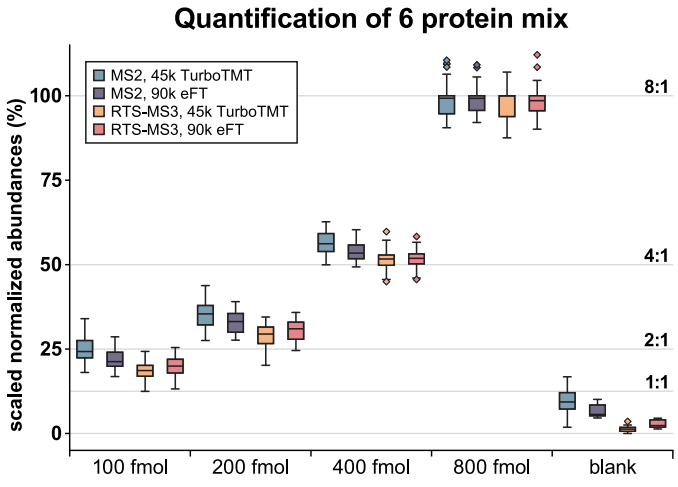


Figure 9. Comparison of TMTpro 32plex quantitative performance (PSMs) of 6 protein mix digest spiked into HeLa digest for different MS/MS acquisition methods. Box plots demarcate the median (line), the 25th and 75th percentile (box), the range within 1.5 × IQR (whiskers), and outliers (diamonds).

Increased sample throughput and greater numbers of quantified proteins and peptides with TMTpro 32plex label reagents

Analyzing samples across two TMTpro 16plex label reagents LC-MS experiments results in missing values between runs, yielding fewer fully quantified proteins and peptides compared to a single LC-MS run. A TMTpro 32plex label reagents experiment acquired using a real-time search with SPS MS^3 method using a resolving power of either 45k TurboTMT or 90K eFT for the MS^3 scan achieves greater numbers of quantified proteins (8.8% and 7.8%, respectively) and peptides (21.9% and 13.4%, respectively) compared to two 16plex runs (Figure 10). Similarly, an MS^2 method using a resolving power of 45k TurboTMT achieves greater numbers of quantified proteins and peptides (12.2% and 15.1%, respectively) for the TMTpro 32plex label reagents experiment.

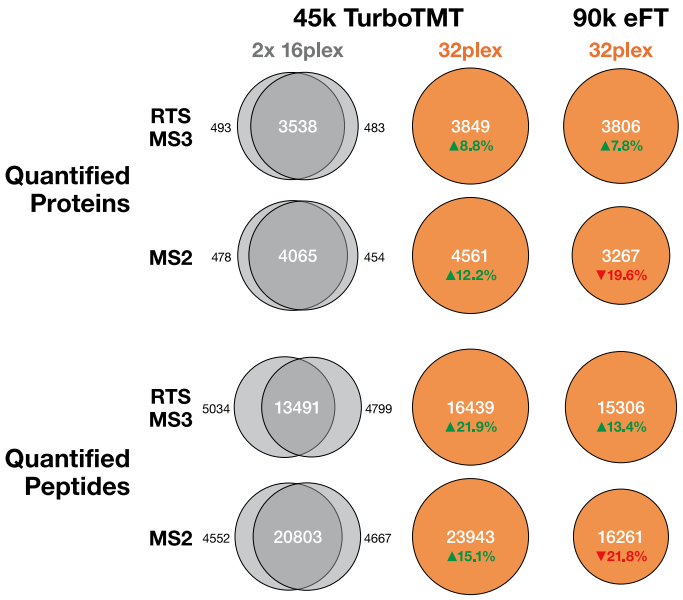


Figure 10. Increased sample throughput and greater numbers of quantified proteins and peptides in one TMTpro 32plex experiment compared to two TMTpro 16plex experiments with 45k TurboTMT and 90k eFT resolving power settings for real-time search with SPS MS^3 and MS^2 acquisition methods.

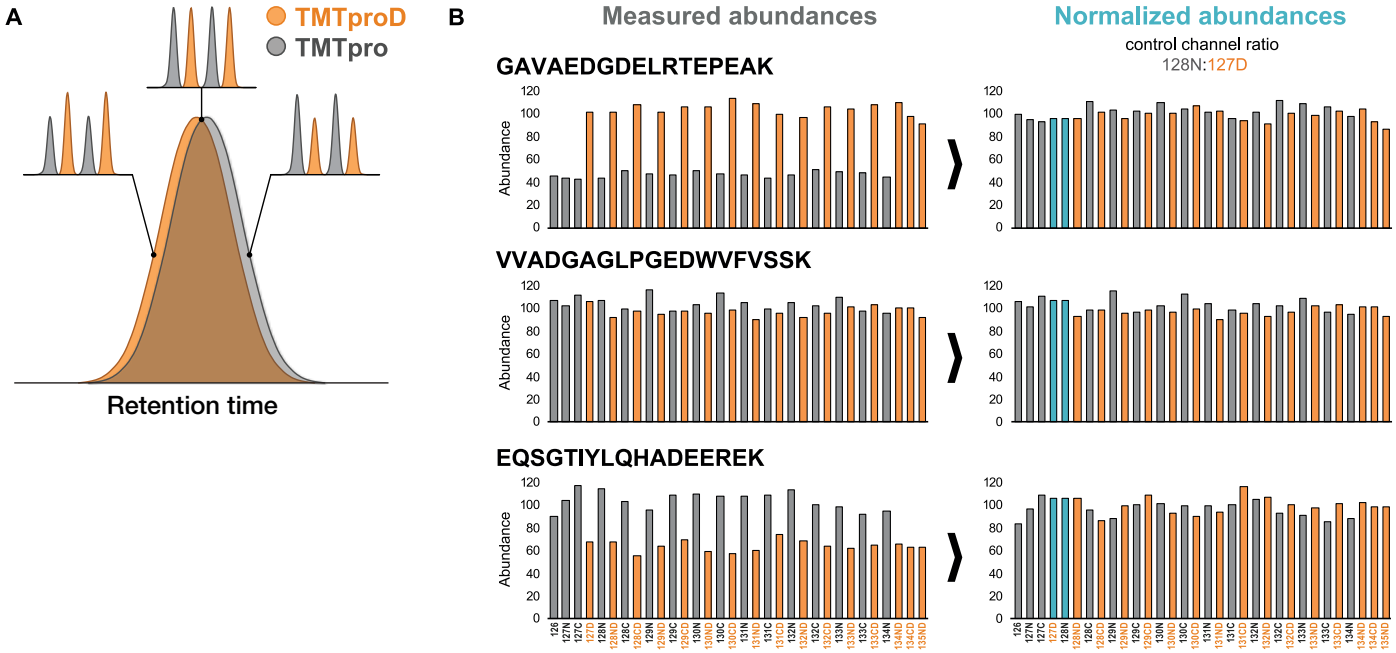


Figure 11. Normalization of TMTpro 32plex reporter ion abundances using the Reporter Ions Control Channel Normalizer node in Proteome Discoverer version 3.3 software.

Normalization of TMTpro 32plex data in Proteome Discoverer version 3.3 software

During reversed-phase chromatography separation of a TMTpro 32plex-labeled sample, a retention time difference of 0.5-1 second is observed between peptides labeled with deuterated and non-deuterated TMTpro label reagents of the 32plex set, resulting 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 (Figure 11A). A reference sample may be labeled with a tag from each set to be used as control channels to normalize abundances of the two sub-plexes in Proteome Discoverer version 3.3 software using the Reporter Ions Control Channel Normalizer node. After normalization, the abundances are scaled to correct for the effect of the retention time shift and achieve accurate quantification (Figure 11B).

Conclusion

- Newly-enabled Orbitrap resolving power options of 45k TurboTMT, 60k TurboTMT, and 75k sufficiently resolve the Δm: 3 mDa reporter ions of the novel TMTpro 32plex label reagent set for reliable quantification
- TMTpro 32plex label reagents double sample throughput, enabling relative quantification of >4,500 proteins across >30 samples in a single LC-MS acquisition
- A single TMTpro 32plex real-time search with SPS MS^3 acquisition yields 8% more quantified proteins compared to two TMTpro 16plex acquisitions due to missing values between runs
- Real-time search with SPS MS^3 achieves better quantification accuracy vs. MS^2 acquisition
- Proteome Discoverer version 3.3 software supports normalization and scaling of deuterated and non-deuterated sub-plexes to ensure accurate quantification

References

1. Pappireddi N, Martin L, Wühr M. A Review on Quantitative Multiplexed Proteomics. Chembiochem. 2019 May 15;20(10):1210-1224. doi: 10.1002/cbic. 201800650. Epub 2019 Apr 18. PMID: 30609196; PMCID: PMC6520187.
2. Zuniga NR, Frost DC, Kuhn K, Shin M, Whitehouse RL, Wei TY, He Y, Dawson SL, Pike I, Bomgarden RD, Gygi SP, Paulo JA. Achieving a 35-Plex Tandem Mass Tag Reagent Set through Deuterium Incorporation. J Proteome Res. 2024 Nov 1;23(11):5153-5165. doi: 10.1021/acs.jproteome.4c00668. Epub 2024 Oct 9. PMID: 39380184.

Learn more at **thermofisher.com/ascend**