Next-generation TMTpro reagents for increased sample multiplexing

Introduction

Quantitative proteomics workflows using tandem mass tags (TMTs) enable precise measurement of protein abundance from multiple samples from a single high-resolution liquid chromatography–mass spectrometry (LC-MS) experiment. In addition to significant increases in LC-MS sample throughput, the use of Thermo Scientific™ TMT™ reagents offers some unique advantages over label-free approaches, including fewer missing values between multiplexed samples, ability to add internal controls or replicates in a single analysis, and an increase in precursor signal from combined samples (Figure 1).

Notably, TMT technology has found utility for a broad range of advanced proteomic applications, including posttranslational modification profiling, thermal shift assays to evaluate drug binding, global protein half-life determination, subcellular localization studies, and single-cell analysis. The latest generation of tandem mass tags called Thermo Scientific™ TMTpro™ reagents was developed to increase sample multiplexing from 11-plex with the previous generation of products to 16-plex with the current reagent set (Figure 2). Compared to the TMT reagents, the latest TMTpro reagents are ~20% larger with a longer linker region between the amine-reactive group and reporter region, which can support more stable ^{13}C and ^{15}N isotopes (9 vs. 5 in TMT labels). Incorporation of the additional isotopes into the tag structure, combined with a proline-based reporter region, enabled generation of a set of 16 unique isobaric tags, with potential to expand to 18 tags for sample labeling and multiplexing. Remarkably, although the TMT and TMTpro reagents are structurally different, the atomic composition of the reporter ions is the same, resulting in the same reporter ion detection in the low-mass region of the tandem mass spectrum, which has lower interference from other sample ions.

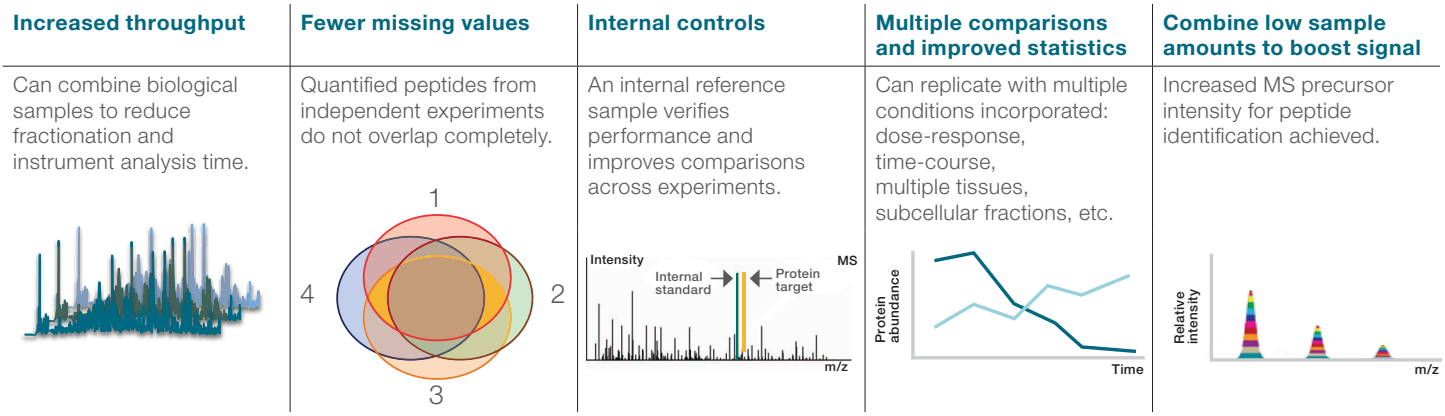

Figure 1. Benefits of using TMT reagents in proteomic analyses.

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Advantages of higher sample multiplexing using TMTpro reagents

One of the key advantages of higher sample multiplexing in proteomics experiments, apart from the obvious time and cost savings from increased sample throughput, is fewer missing values between samples. Missing values arise from being unable to identify and quantify proteins using the same unique peptides sequenced in a particular LC-MS analysis. Although this can be somewhat mitigated by software that matches proteins using different peptides between runs for protein abundance measurements, it can also result in null or missing values for quantification if no peptides are identified in a particular sample. This issue increases as more samples and LC-MS runs are used for large sample cohorts. Using the TMTpro reagents in higher sample multiplexing enables analysis of samples in fewer LC-MS runs, as well as identify and quantify more proteins across all samples. This is illustrated in Figure 3, which shows results from a study comparing analysis of 30 HeLa cell protein digests prepared using the Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit and labeled using either TMT reagents to generate three 11-plex samples or TMTpro reagents to generate two 16-plex samples. Despite containing the same total number of samples, performing the LC-MS analysis in two runs for the TMTpro reagent–labeled samples resulted in a 10% gain in the number of quantifiable proteins due to more overlap between runs.

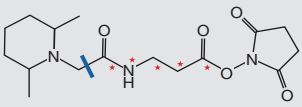
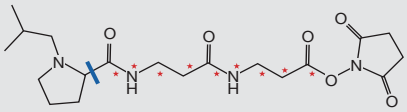
	TMT reagent	TMTpro reagent
Structure		
MW	339.39	410.46
Isotopes	5 total: $^{15}N_1$, $^{13}C_5$	9 total: $^{15}N_2$, $^{13}C_7$
Multiplexing	11-plex	16-plex
Reporter ion	Dimethyl piperidine	Isobutylproline
Reportor ion masses	126–131C	126–134N

Figure 2. Comparison of structural features of TMT and TMTpro reagents.

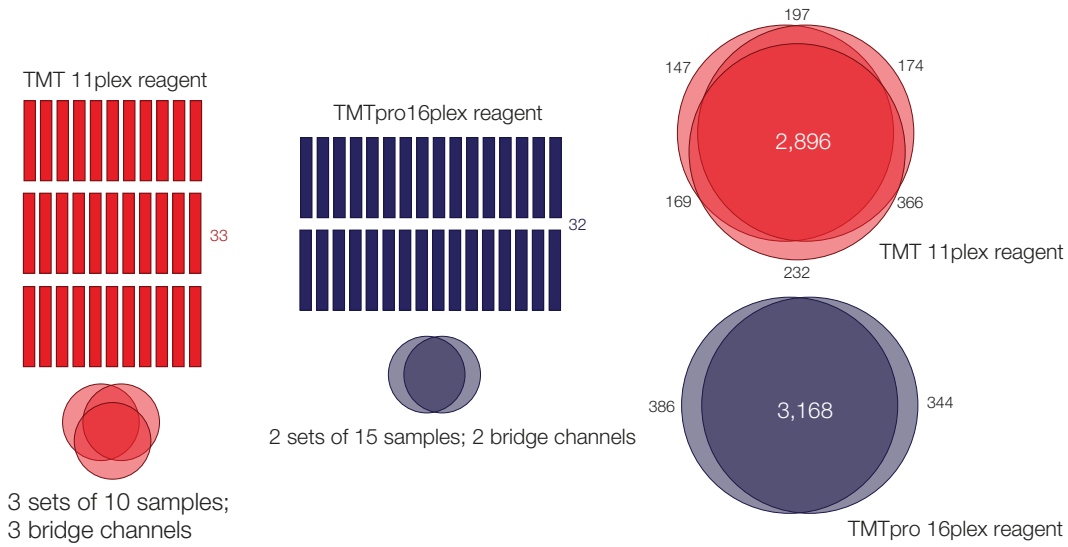


Figure 3. Reducing the missing values using TMTpro reagent–labeled sample sets.

High-pH fractionation of samples at the peptide level has become a popular technique in proteomics that enables deep analysis of complex samples. An advantage of using TMT labels in peptide fractionation is a reduction in the total number of fractions, to optimize comprehensive analysis of proteomes across many samples or replicates. To demonstrate this, HeLa cells were serum-starved and then subjected to four different treatments, including stimulation with epidermal growth factor (EGF) or insulin-like growth factor (IGF) in the presence or absence of the cancer therapeutic erlotnib (EGF receptor (EGFR) tyrosine kinase inhibitor, Figure 4). Duplicate or triplicate samples were labeled with Thermo Scientific™ TMT™ 11plex or TMTpro™ 16plex reagents, respectively, with a pooled sample as a reference for relative quantitation. After labeling individual samples equally mixed with the two tags, the samples were cleaned up using the Thermo Scientific™ EasyPep™ Maxi Sample Prep Kit. Labeled samples were also enriched for phosphopeptides using the Thermo Scientific™ High-Select™ Fe-NTA Phosphopeptide Enrichment Kit, and fractionated into eight fractions using the Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit before LC-MS analysis. Overall, there was no difference in total protein abundance for the replicate samples before or after labeling with TMT or TMTpro reagents (Figure 5A), as expected. However, an EGFR phosphotyrosine peptide, GSTAENEpYLR, was found to be significantly increased after EGF stimulation and decreased upon adding erlotinib. It is important to note that this phosphopeptide was observed only after both phosphopeptide enrichment and high-pH reversed-phase fractionation—reinforcing the importance of sample fractionation to achieve more comprehensive proteome coverage. Similar abundance-level profiles for the phosphopeptide were observed for the various treatments regardless of whether TMT or TMTpro reagents were used for sample multiplexing. However, it was possible to introduce more replicates for each condition using the TMTpro reagent set, enabling more statistical power for quantitative comparisons.

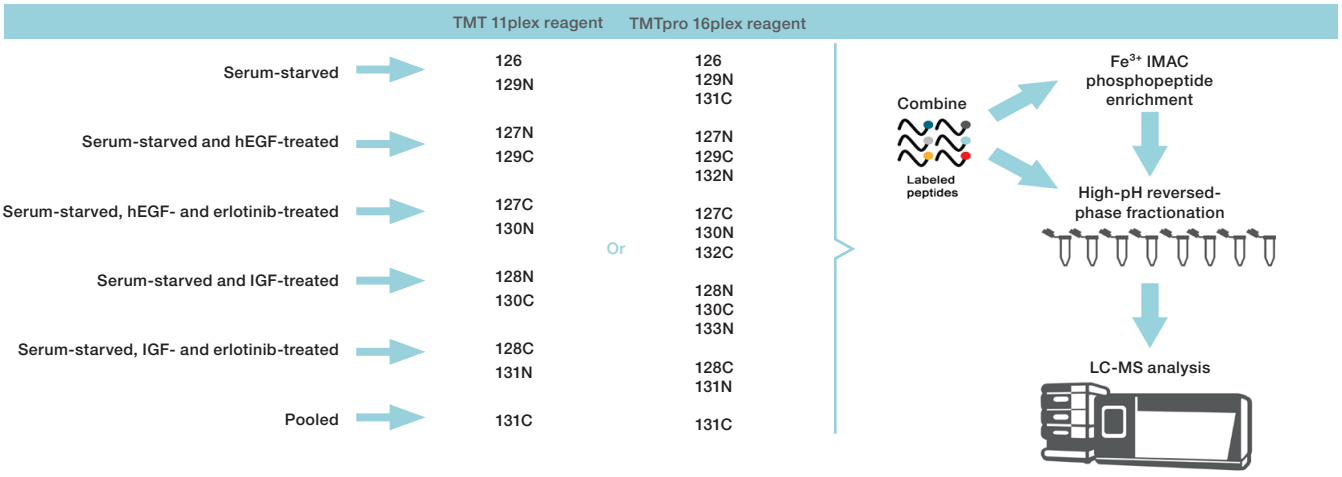


Figure 4. Schematic workflow of a sample phosphoproteomics study utilizing TMT and TMTpro reagents.

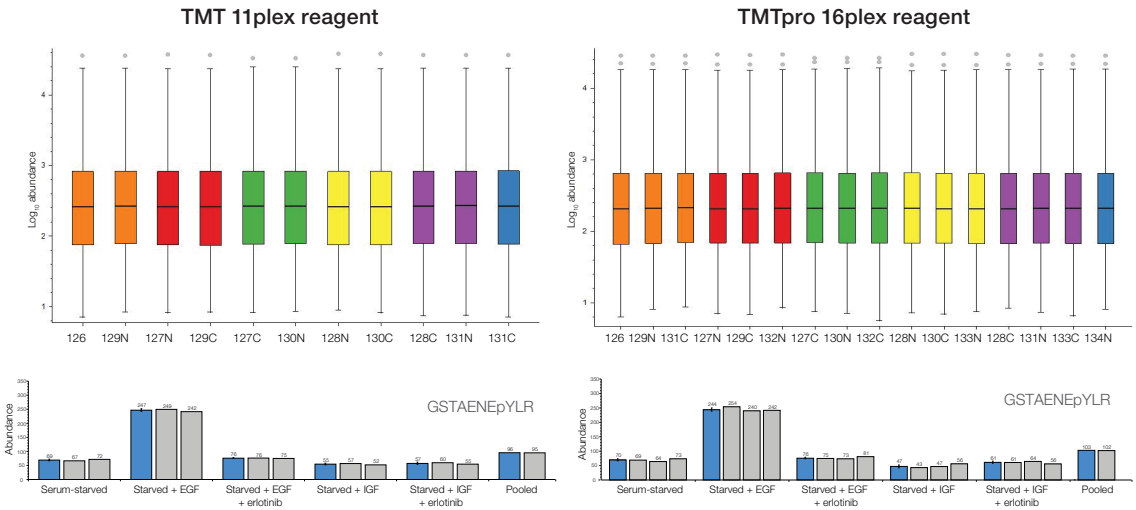


Figure 5. Accurate relative quantitation of phosphopeptides with high precision using TMT and TMTpro reagents.

Impacts of tag structure on chromatography

Compared to unlabeled (i.e., label-free) peptide samples, isobaric tag–labeled peptides are significantly more hydrophobic. Since the TMTpro tag is larger in size compared to the first-generation TMT tag, TMTpro reagent–labeled peptides are also slightly more hydrophobic than TMT reagent–labeled peptides. LC-MS gradients should be optimized for high protein identification numbers. Figure 6A shows representative total ion count (TIC) chromatograms for label-free (top), TMT reagent–labeled (middle), and TMTpro reagent–labeled (bottom) HeLa cell protein digests separated under LC conditions optimized for label-free samples. For both of the labeled samples, higher TIC signals are observed for the first part of the chromatogram compared to label-free samples.

However, as shown in Figure 6B, this increased TIC signal does not result in increased peptide identifications. In fact, more peptide identifications are found through the middle and later half of the gradient, consistent with increased hydrophobicity of TMT reagent–labeled peptides. Although excess free TMT label is routinely observed at the beginning of the LC gradients if samples are not fully cleaned up, the increased TIC signal for these samples was actually due to small peptides (1–3 amino acids in length) being above the low mass cutoff in the MS scan range upon introduction of the tag. With the increase in tag size, there is also a proportional increase in peptide charge states—from +2 to +3 (Figure 6C).

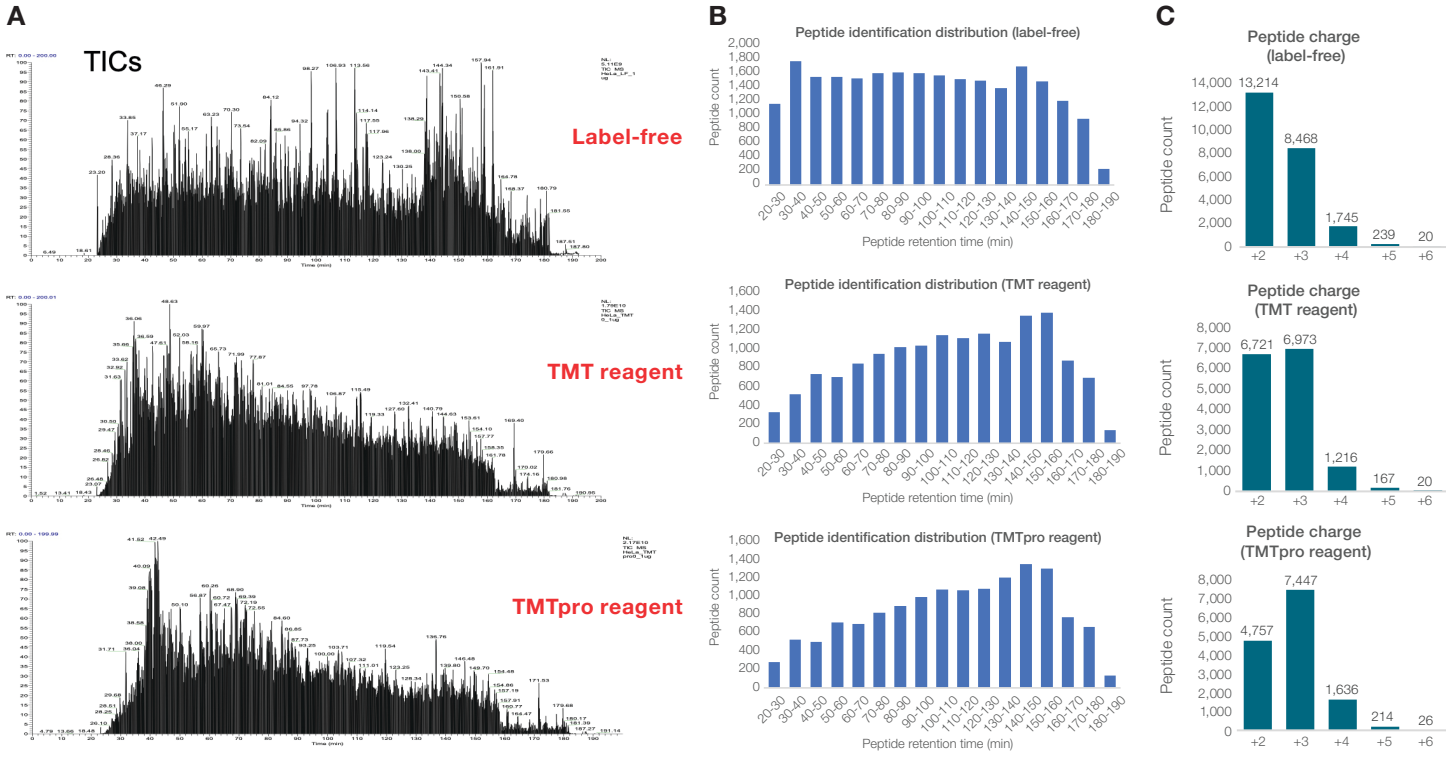


Figure 6. Chromatographic and ionization features of tandem mass tag–labeled complex peptide samples. (A) Representative TIC chromatograms. **(B)** Peptide identification distribution. **(C)** Peptide charge states.

Analysis of over 1,600 tryptic peptides from HeLa cells digested with trypsin, labeled using TMT and TMTpro reagents and separated using a 3 hr LC gradient, revealed that there are average shifts of 7 min and 5 min in the retention times of the lysine- and arginine-containing peptides, respectively (Figure 7). This correlates with the overall increase in hydrophobicity for peptides that have two labels per tryptic peptide containing lysine residues, and only one label for tryptic peptides containing arginine residues. However, the retention time difference was not consistent across the gradient, with the largest shift observed at the beginning of the gradient and virtually no significant differences observed at the end of the gradient. This necessitates optimization of the low-pH reversed-phase gradients for specific tag-labeled peptide samples to maximize identification rates for unique peptides. In contrast, there are no differences in fractionation of TMT reagent–labeled and TMTpro reagent–labeled peptides using standard high-pH reversed-phase gradients (data not shown).

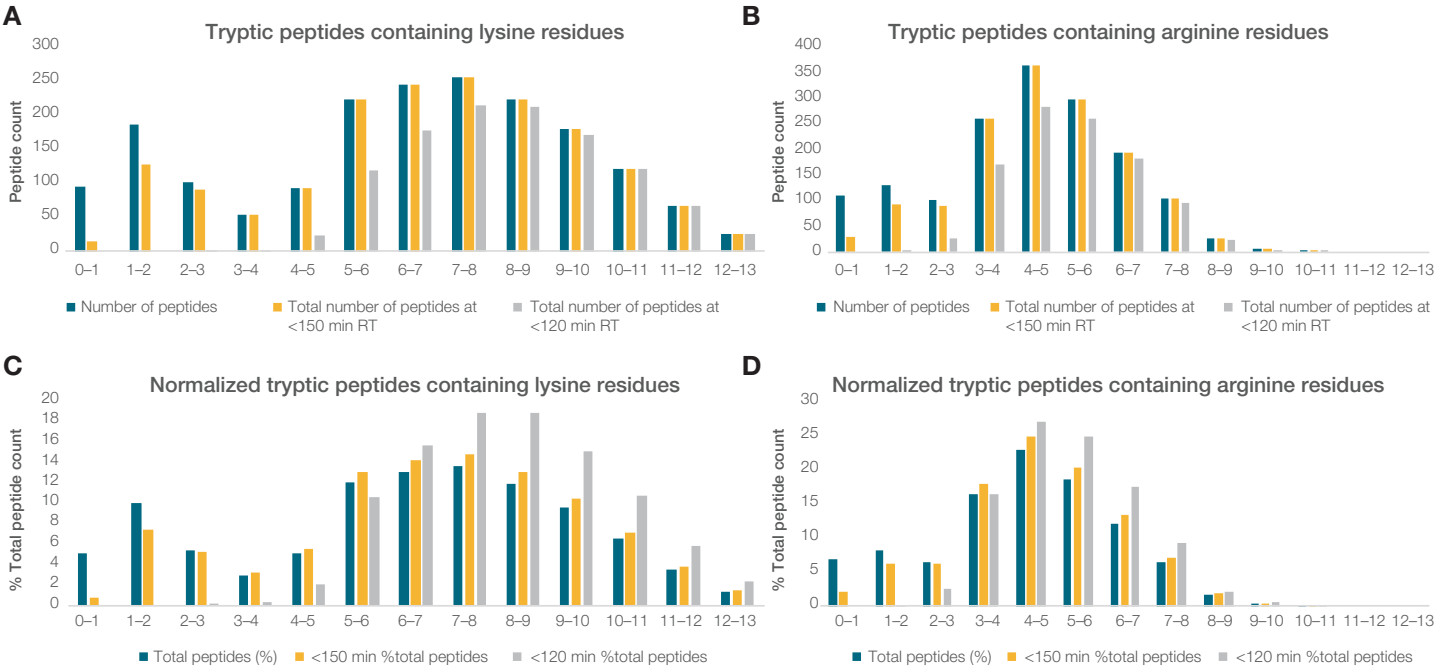


Figure 7. Differences in reversed-phase retention time (RT) of peptides labeled with TMT reagent and TMTpro reagents.

Optimizing instrument settings and data analysis in Proteome Discoverer software

In addition to the LC gradient, parameters in the mass spectrometer should also be optimized to maximize protein identification and quantitation of multiplexed samples. Predefined acquisition methods for different types of experiments are available in the latest versions of the instrument control software for Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ and Orbitrap Exploris™ mass spectrometers (Figure 8). These methods include optimal settings for MS and MS/MS resolution, normalized collision energy, injection time, automatic gain control (AGC) targets, and intensity thresholds.

For data analysis, Thermo Scientific™ Proteome Discoverer™ software 2.4 and 2.5 have quantitative method templates for application of isotopic correction factors and analysis of TMT and TMTpro reagent–labeled samples. Information from the Certificate of Analysis on isotopic purity of lot-specific reagents can be added to the Proteome Discoverer quantification method to create a lot-specific quantification method. For TMTpro sample data analysis using Proteome Discoverer software 2.3, please refer to our **application note**.

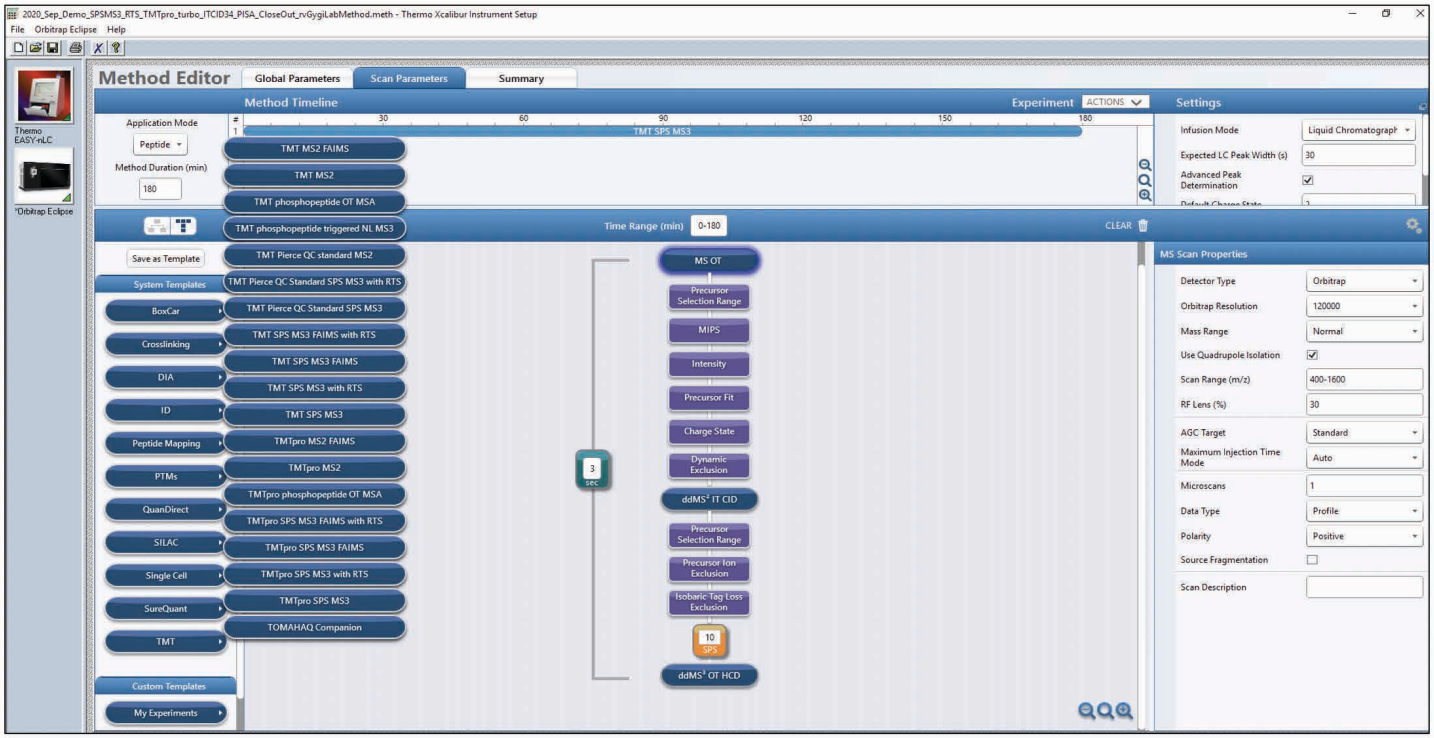


Figure 8. Method templates option for TMT reagent workflow on the Orbitrap Eclipse Tribrid mass spectrometer.

Going deeper into the proteome with gas-phase fractionation

The Thermo Scientific™ FAIMS Pro™ interface for differential ion mobility coupled to the Orbitrap Eclipse Tribrid mass spectrometer is the foremost platform for comprehensive analysis of complex labeled proteomics samples. The FAIMS Pro interface enables efficient online gas-phase fractionation of peptides at different charge states and sizes by the ion source. By varying compensation voltage (CV) settings for a single sample or over several sequential runs, more proteins from complex samples can be identified and quantified without any additional off-line fractionation.

To demonstrate the advantages of using the FAIMS Pro interface coupled to the latest Orbitrap Eclipse Tribrid mass spectrometer, HeLa and *E. coli* cell lysates labeled with TMTpro 16plex reagents were mixed at fixed ratios as shown in Figure 9. Different sample ratios were chosen to assess the quantitation accuracy of the MS with a constant complex background. The samples were subjected to online gas-phase fractionation as described previously [1]. Figure 10 shows the gas-phase fractional distribution of unique peptides and protein groups observed at different CVs ranging from –30 to –90. Overall, each run at the different CVs resulted in nearly 4,000 proteins identified, except for the lowest CV at –90, which only resulted in 2,100 protein identifications.

Replicate	Sample	Labeling reagent	HeLa	*E. coli*
Replicate 1	Sample 1	TMTpro-126	40	0
	Sample 2	TMTpro-127N	40	1
	Sample 3	TMTpro-127C	40	2
	Sample 4	TMTpro-128N	40	4
	Sample 5	TMTpro-128C	40	8
Replicate 2	Sample 1	TMTpro-129N	40	0
	Sample 2	TMTpro-129C	40	1
	Sample 3	TMTpro-130N	40	2
	Sample 4	TMTpro-130C	40	4
	Sample 5	TMTpro-131N	40	8
Replicate 3	Sample 1	TMTpro-131C	40	0
	Sample 2	TMTpro-132N	40	1
	Sample 3	TMTpro-132C	40	2
	Sample 4	TMTpro-133N	40	4
	Sample 5	TMTpro-133C	40	8
	Sample 1	TMTpro-134N	40	0

Figure 9. Multiplexed sample design of mixed proteomes from HeLa and *E. coli* cells.

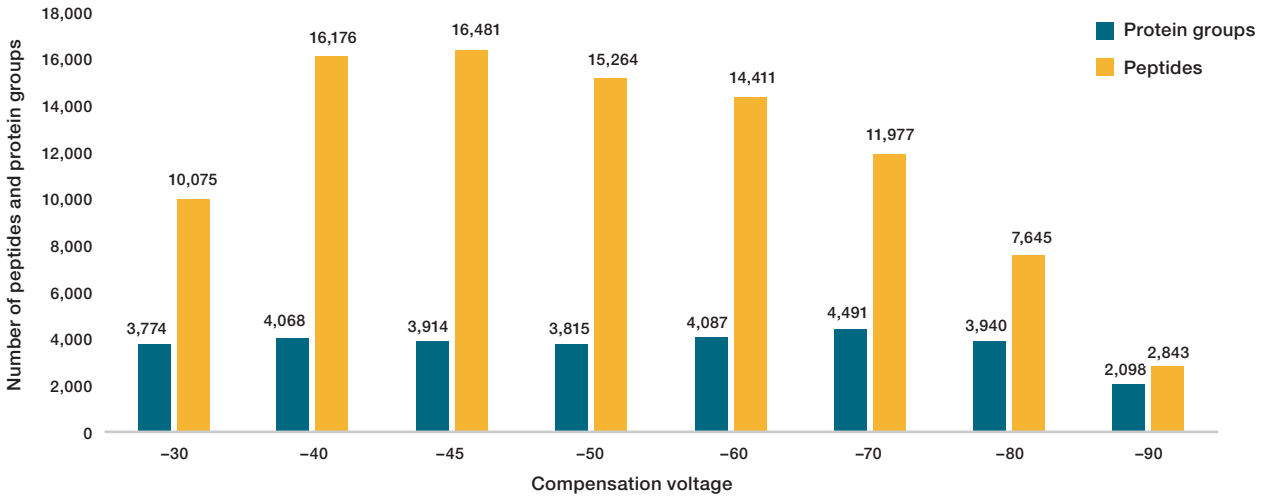


Figure 10. Gas-phase fractional peptide and protein identification profiles.

Overall, the fractional peptide resolution for four sample runs at CVs ranging from –40 to –70 was sufficient to identify 38,628 unique peptides and 6,136 protein groups (Figure 11).

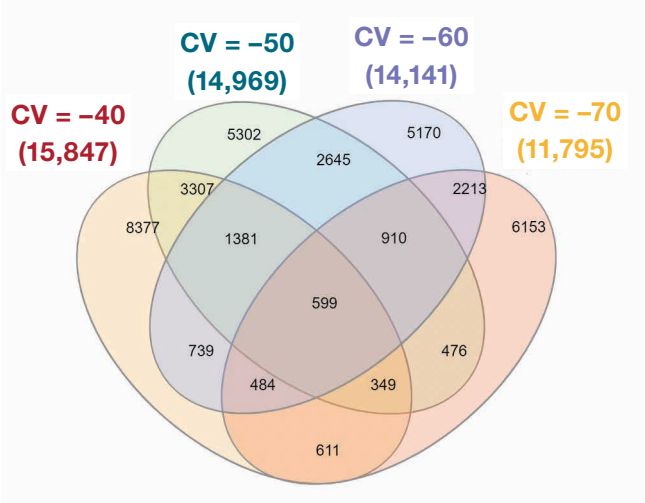


Figure 11. Combined analysis results of selected gas-phase fractions.
Single CV, 2 hr gradient, SPSMS^3 method with real-time search (RTS).
SPS: synchronous precursor selection.

In addition, when normalized to the equimolar human proteome background, the *E. coli* proteins mixed at different fixed ratios were quantified with a high degree of accuracy and precision (Figure 12). Using a median log_2-fold change for *E. coli* ratios of 1:2, 1:4, and 1:8, we observed fold changes of 1.002 (expected log_2-fold change = 1), 1.873 (expected log_2-fold change = 2), and 2.850(expected log_2-fold change = 3), respectively.

Run 1: CV = –40
Run 2: CV = –50
Run 3: CV = –60
Run 4: CV = –70

Protein groups	Peptides
6,136	38,628

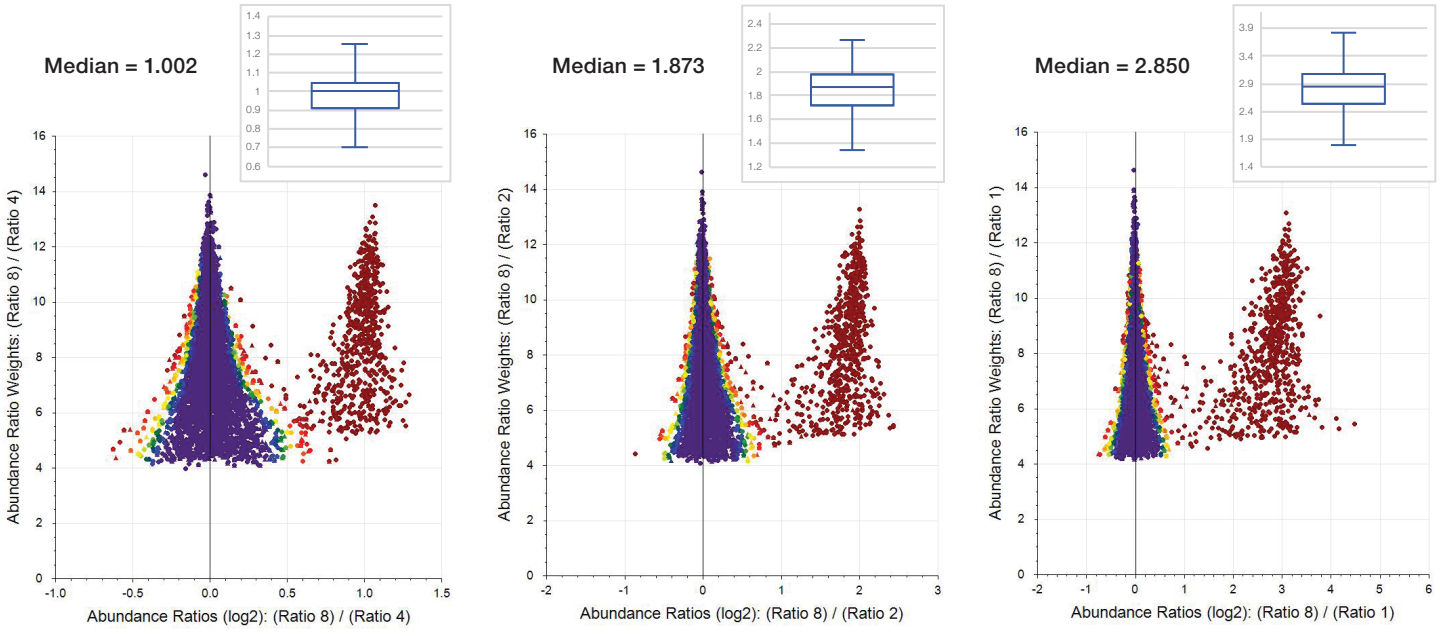


Figure 12. Quantitative accuracy of *E. coli* proteome analysis in the mixed-proteome sample.

Conclusions

Tandem mass tag reagents enable high-throughput, quantitative proteomic analyses to identify changes in protein abundance and posttranslational modification states. The introduction of the latest TMTpro reagents has enabled even more efficient sample throughput for up to 16 samples in a single LC-MS run. Although TMTpro regents have structures different from previous-generation isobaric mass tags, only minor LC-MS method changes are required to enable enhanced multiplexing capabilities, which also results in fewer missing values between samples. Combining TMTpro reagent multiplexing with the latest-generation Orbitrap Eclipse Tribrid mass spectrometer equipped with the FAIMS Pro interface enables deep proteomic analysis of samples with the highest accuracy and precision for protein quantitation.

Materials and methods

LC method

For the TMTpro tag–labeled HeLa/*E. coli* samples, 1 µg of material was injected directly onto a 75 µm x 50 cm, 2 µm C18 Thermo Scientific™ EasySpray™ column (Thermo Fisher Scientific, Cat. No. ES903). The column was kept at 50°C. Peptides were separated over 140 min at 300 nL/min using a Thermo Scientific™ Easy-nLC™ 1200 System (Thermo Fisher Scientific, Cat. No. LC140) over a gradient from 5% B (A: 99.9% H_2O, 0.1% formic acid; B: 80% acetonitrile, 0.1% formic acid) to 25% B in 110 min, followed by a gradient ramp to 40% B in 10 min. The column was washed at 95% B for 10 min.

MS method

Data were acquired on the Orbitrap Eclipse Tribrid mass spectrometer (Thermo Fisher Scientific, Cat. No. FSN04-10000) equipped with the FAIMS Pro interface. The instrument was operated at "Top Speed", data-dependent MS/MS mode using multiple FAIMS CV values of –30 to –90. First, a full scan was acquired from m/z 400–1,400 at 120,000 resolving power with the ion funnel radio frequency (RF) lens value of 30% and an AGC target of 4×10^5. Data-dependent MS/MS were acquired with a 3 sec cycle using the following filters: monoisotopic precursor selection mode set to "peptide"; charge state filter set at 2–8; 60 sec dynamic exclusion with isotopes excluded; intensity threshold set at 5×10^3. The quadrupole was set to 0.7 Da for precursor isolation. MS^2 spectra were generated by collision-induced dissociation (CID) with a normalized collision energy of 30% and were acquired in the ion trap using a "rapid" scan rate, an AGC target of 1×10^4 ions, and a maximum inject time of 50 ms. The "Realtime Search" feature was used with *E. coli* and human FASTA file combined (downloaded from UniProt database), trypsin was used as the enzyme for cleavage, and the following peptide scoring thresholds were used: Xcorr of 1, dCn of 0, and precursor PPM of 15. MS^3 spectra were generated by high-energy collision dissociation (HCD) with a normalized collision energy of 50%. MS^3 spectra were acquired in the Orbitrap Eclipse Tribid mass spectrometer using 50,000 resolution, an AGC target of 1×10^5 ions, and a maximum inject time of 115 ms.

Data analysis

Data were processed in Proteome Discoverer software 2.4 for TMTpro reagent–labeled samples using a single-stage search. The spectra were analyzed by the SEQUEST HT engine, searched against human and *E. coli* databases considering dynamic methionine oxidation, N-terminal carbamylation, and N-terminal acetylation with and without methionine loss. For the static modifications, carbamidomethylation on cysteine and TMTpro labels on N-terminus and lysine were used. All peptide-spectrum matches (PSMs) were filtered to 1% false discovery rate (FDR). Normalization was performed against total human proteins. Protein abundances were calculated from the summed unique and razor peptide abundances, and protein ratios were calculated as the median of all possible pairwise ratios between replicates. *P* values were calculated using a background-based *t*-test.

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Related product information

- TMTpro 16plex Label Reagent Set (Cat. No. A44520)
- TMTpro Label Reagent Set (Cat. No. A44522)
- EasyPep Mini MS Sample Prep Kit (Cat. No. 40006)
- Thermo Scientific™ EasyPep™ 96 MS Sample Prep Kit (Cat. No. A45733)
- EasyPep Maxi Sample Prep Kit (Cat. No. A45734)
- Pierce High pH Reversed-Phase Peptide Fractionation Kit (Cat. No. 84868)
- Thermo Scientific™ Pierce™ Rapid Gold BCA Protein Assay Kit (Cat. No. A53226)
- Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay (Cat. No. 23290)
- Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay (Cat. No. 23275)
- Thermo Scientific™ High-Select™ TiO_2 Phosphopeptide Enrichment Kit (Cat. No. A32993)
- High-Select Fe-NTA Phosphopeptide Enrichment Kit (Cat. No. A32992)

Related publications

1. **Application note: FAIMS Pro interface application note**
2. **Using TMTpro reagents with Proteome Discoverer software 2.3**
3. **Application note: EasyPep sample preparation (COL33874)**

Reference

1. Liu X, Gygi SP, Paulo JA (2021) Isobaric tag-based protein profiling across eight human cell lines using high-field asymmetric ion mobility spectrometry and real-time database searching. *Proteomics.* 21(1):e2000218. doi: 10.1002/pmic.202000218. Epub 2020 Oct 26.

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