Characterizing 32-plex TMTpro reagents for high-throughput quantitative proteomics on Orbitrap platforms

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Abstract

Thermo Scientific™ TMTpro™ Reagents enable researchers to simultaneously identify and quantify proteins and peptides from many samples in a single LC-MS/MS experiment. Current TMTpro isobaric mass tags incorporate ^{13}C & ^{15}N stable isotopes to allow quantitative analysis of up to 18 samples in parallel by high-resolution MS/MS analysis. To further increase multiplexing capability, we developed an additional isobaric set of 17 isotopologues that incorporate a single ^{2}H isotope on the reporter group to yield distinct reporter ion masses that differ from the existing set by 3 mDa. In combination with the traditional reagent set, the deuterated reagents enable multiplexed quantitative analysis of up to 35 samples on Thermo Scientific™ Orbitrap™ Mass Spectrometer Platform. Here, we characterize the novel TMTpro reagent variants and assess their performance for 32-plex quantification.

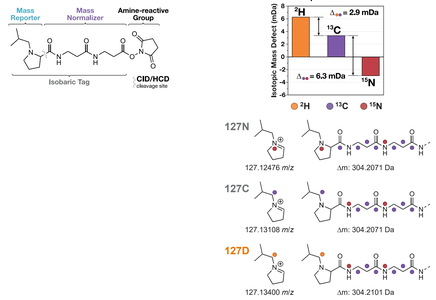
Introduction

Thermo Scientific TMTpro reagents are isobaric tandem mass tags that permit multiplexed relative quantification of samples by LC-MS/MS analysis. The reagent structure features a reporter group and a mass normalizer group, across which heavy stable isotopes are incorporated, and an amine-reactive group (Figure 1) for derivatization of peptide N-termini and lysine side chains. All reagents in the multiplex set are structurally identical and have the same nominal mass, but each incorporates nine ^{13}C & ^{15}N heavy stable isotopes in a distinct configuration between the reporter and mass normalizer. Samples are differentially labeled with each reagent in the multiplex set, the labeled samples are combined, and the pooled sample is subjected to LC-MS/MS analysis on an Orbitrap MS platform using high-resolution acquisition. Differentially labeled peptides incur the same mass shift, and single precursors are measured in MS spectra. Peptides across all samples are thus co-analyzed upon MS/MS acquisition, and fragmentation generates unique reporter ions in the low mass region of MS/MS spectra with intensities that reflect the relative protein abundance in each sample.

The TMTpro reagent structure supports a maximum of nine $^{13}C/^{15}N$ isotopes on the isobutylproline reporter group, permitting a total of ten nominal mass reporters between 126-135 Da. By exchanging a ^{13}C for a ^{15}N isotope on the reporter group, up to 18 distinct reporters with Δm: 6 mDa are possible that can be distinguished in Orbitrap MS/MS spectra acquired at resolving power (RP) ≥50K (at 200 *m/z*).

To increase multiplexing further without changing the tag structure, we incorporated a single ^{2}H isotope onto the reporter group to impart a 3 mDa mass difference and permit an additional 17 unique reporter ion masses to be created. To do this without requiring expensive custom starting reagents, adding synthetic steps, or reducing synthetic yield, we opted to incorporate the ^{2}H isotope into the isobutyl group via a deuterated reducing agent during synthesis. The Δm: 3 mDa reporters can be distinguished in MS/MS spectra acquired in the Orbitrap mass analyzer at RP ≥75K or TurboTMT 45K. Combining the deuterated tags with the existing tags permits multiplexed quantitative analysis of up to 35 samples.

Figure 1. TMTpro 35-plex isotopic configurations



Materials and methods

Sample preparation

HeLa S3 cells were grown in sMEM supplemented with 10% FBS, 1x Glutamax and 1% Pen/Strep. HeLa digest samples were prepared using the Thermo Scientific EasyPep™ MS sample prep kit protocol. Peptides were reconstituted in 100 mM TEAB pH 8.5, labeled for 1 hr with TMTpro reagents, and cleaned up using the EasyPep SPE protocol.

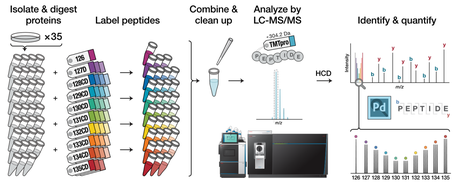
LC-MS/MS analysis

Samples were analyzed by nanoflow LC-MS/MS using a Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ Mass Spectrometer interfaced with a Thermo Scientific™ Vanquish™ Neo UHPLC System using a 50cm C18 Thermo Scientific™ EASY-Spray™ Column with a gradient elution of 2-32% ACN with 0.1% formic acid over 120 min at a flow rate of 300 nL/min. FT-MS scans (RP 120K at 200 *m/z*) and HCD FT-MS^2 scans (RP ≥75K or TurboTMT 45K) were acquired using a 3 sec DDA scan cycle with 60 sec dynamic exclusion. RTS SPS-MS^3 acquisition was also performed.

Data analysis

Raw data was processed by Thermo Scientific™ Proteome Discoverer™ 3.2 Software using the SEQUEST® HT search algorithm with an UniProt human protein database. Peptide modifications consisted of static carbamidomethylation (C), dynamic oxidation (M), and either static or dynamic TMTpro tags (N-terminus, K; Δm: 304.2071 Da). Protein and peptide identifications were filtered to a 1% FDR threshold using Percolator. Reporter ions were integrated within an 11 ppm window.

Figure 2. TMTpro 35-plex reagent workflow



Results

Figure 3. Resolving 3mDa TMTpro reporter ions in the Orbitrap mass analyzer
TMTpro 128N, 128ND, 128C, 128CD reagents were mixed at 1:1, 1:10, and 10:1 and analyzed by direct infusion MS to determine resolving power required to distinguish TMTpro Δm: 3 mDa reporter ions in HCD FT-MS^2 spectra. Reporter ion peaks are resolved to 5% of baseline at RP 75K (at 200 *m/z*) and baseline resolved at RP ≥90K.

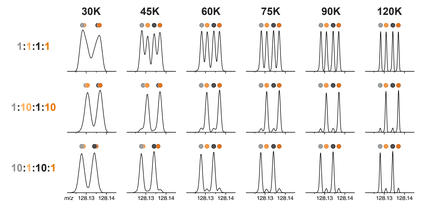


Figure 4. TMTpro 35-plex reporter ions acquired at RP 90K in the Orbitrap

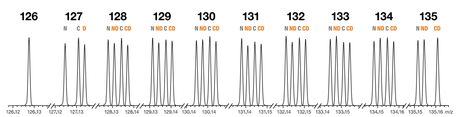


Figure 5. Characterization of RPLC retention time shift
Peptides labeled with the TMTpro deuterated reagent set are shifted ahead slightly in retention time during reversed-phase chromatography. Peptides labeled with non-deuterated reagents co-elute exactly, and peptides labeled with deuterated reagents co-elute exactly. To achieve accurate & precise quantification, reference non-deuterated channels to a non-deuterated control channel and reference deuterated channels to a deuterated control channel.

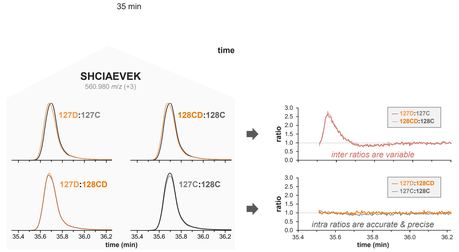


Figure 6. TMTpro 32-plex vs. 16-plex reagents quantification – HCD FT-MS^2, PSMs
HeLa digest samples labeled with TMTpro 16-plex, 16-plex deuterated, and 32-plex reagents show that the three multiplex sets achieve equivalent quantitative performance for MS^2 acquisition at RP 50K for 16-plex samples and 75K for the 32-plex sample. The % abundance was calculated separately for the deuterated and non-deuterated sets by dividing the abundance of each channel by the sum of abundances for that set. Isotopic interference correction not applied.

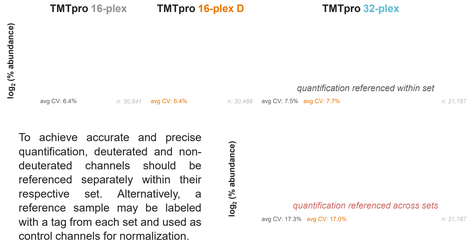


Figure 7. Quantitative performance at different MS^2 resolving powers – MS^2, PSMs
HeLa digest samples labeled with TMTpro 32-plex reagents were combined at 1:10 ratio between non-deuterated and deuterated channels to compare quantitative performance at different MS^2 resolving powers. TurboTMT RP 45K and eFT RP 75K provide a good balance of quantitative accuracy & precision and acquisition speed. 8 of 32 channels shown; reference channels 134N & 128ND; isotopic interference correction not applied.

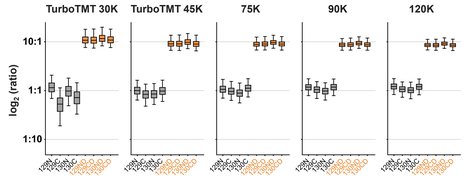


Figure 8. TMTpro 32-plex HeLa quantified proteins & peptides – HCD FT-MS^2
HeLa digest samples labeled with TMTpro 32-plex reagents were analyzed using MS^2 acquisition methods at increasing resolving powers to assess the impact on the number of quantified proteins & peptides and % of quantified peptide spectral matches (PSMs).

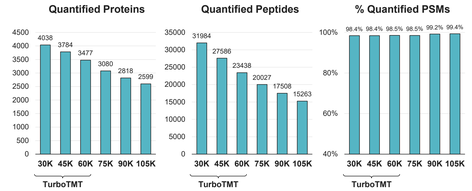


Figure 9. TMTpro 32-plex vs. 2x 18-plex reagent LC-MS experiments – RTS SPS-MS^3
HeLa digest samples labeled with TMTpro 32-plex & 18-plex reagents were analyzed using RTS SPS-MS^3 acquisition methods at RP 90K and TurboTMT 30K, respectively. The 32-plex experiment yields 15% more quantified proteins and 39% more quantified peptides than two 18-plex bridged experiments due to missing values between two LC-MS runs.

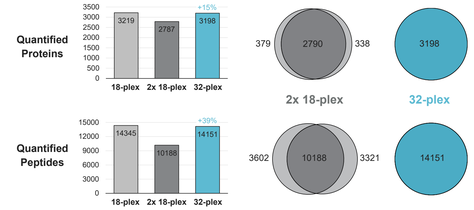


Figure 10. Normalization of TMTpro 32-plex reagent data in Proteome Discoverer 3.2 software
A) During reversed-phase chromatography separation, a retention time difference of 0.5-1 seconds is observed between peptides labeled with deuterated and non-deuterated TMTpro reagents, resulting in the two sub-plexes of reporter ions measuring at different abundances depending on when MS/MS is triggered over the peptide's elution profile.
B) 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 3.2 software. The Reporter Ion Control Channel Normalizer node permits defining control channels for the sub-plexes. After normalization, the abundances are scaled to correct for the effect of the retention time shift and achieve accurate quantification.

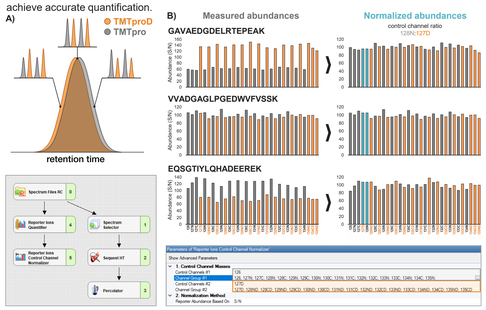
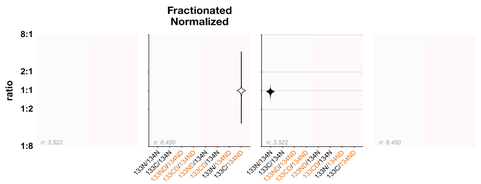


Figure 11. Offline high pH reversed-phase fractionation – HCD FT-MS^2, proteins
HeLa digest samples labeled with TMTpro 32-plex reagents were fractionated into 8 fractions using the Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit. Quantitative metrics of intra ratios (in gray and orange) are equivalent or better for the fractionated sample (6,450 proteins) compared to the unfractionated sample (3,522 proteins). Control channel normalization improves accuracy and precision of inter ratios (in red) between deuterated and non-deuterated channels. 4 of 32 channels shown; control channels 126 & 127D; ratio reference channels 134N & 134ND.



Conclusions

- Isobaric TMTpro deuterated reagents enable multiplexed quantification of up to 35 samples in a single LC-MS/MS experiment on Orbitrap MS platforms
- TMTpro reagent reporters with Δm: 3 mDa are resolved in MS/MS spectra at RP 75K & TurboTMT 45K
- TMTpro 32-plex reagents achieve equivalent quantitative performance and number of quantified proteins & peptides compared to TMTpro 18-plex reagents

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