Proteomics

# Enhancing the resolving power on an Orbitrap Astral Zoom mass spectrometer for TMTPro 35plex applications

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## Abstract

**Purpose:** A novel data acquisition scheme was developed to allow dedicated MS2 scans for peptide identification with standard Astral resolution and TMT HR QUAN scans with extended Astral resolution.

**Methods:** A novel acquisition scheme (TMT HR mode with separate QUAN scans) was used for analysis of Thermo Scientific™ TMTpro™ 32plex labeled human cell line samples.

**Results:** Differential abundances in a human cell line TMTpro 32plex sample are measured with high accuracy and precision with a throughput of 440 samples per day.

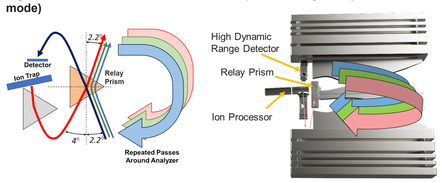
## Introduction

Thermo Scientific™ TMTpro™ reagents are isobaric tandem mass tags that enable multiplexed relative quantitation of samples via LC-MS/MS analysis with high precision and minimal missing values. The current TMTpro reagent tags have been expanded by incorporating 17 additional isotopologues, utilizing a $^{2}$H isotope on the reporter group to produce distinct reporter ion masses that differ from the existing set by 3 mDa[1]. This mass difference necessitates a resolving power of over 90,000 FWHM at *m/z* 130, which was achieved by allowing multiple passes of ions through the Astral mass analyzer on the novel Thermo Scientific™ Orbitrap™ Astral™ Zoom mass spectrometer.

### TMT High Resolution (TMT HR) Mode

The resolving power of Thermo Scientific™ Astral™ analyzer is closely linked to the length of its asymmetric track. In the TMT HR mode, the Relay Prism (a trans-axial deflector) can be set to reflect returning ions back into the analyzer for two additional passes, effectively tripling the length of the asymmetric track. While this mode restricts the *m/z* range, it significantly enhances resolution. With additional space-charge tolerance tuning, this setup is suitable for measuring TMTpro 32plex reporter ions, which require higher resolving power under substantial ion loads than the Astral analyzer can provide in regular operation.

**Figure 1. Schematic of TMT HR Mode (Astral analyzer operated in multi-pass mode)**




## Materials and methods

HeLa digest was labelled with Thermo Scientific™ TMTpro™ 18-plex and TMTPro-D 14plex and mixed in different ratios. The resulting sample was cleaned up using the Thermo Scientific™ EasyPep™ MS Sample Prep Kit. Method optimization was done using Thermo Scientific™ Pierce™ TMT11plex Yeast Digest Standard (TKO).

The samples were separated on an Ionopticks Aurora Frontier XT (75 µm x 60 cm) column coupled to a Thermo Scientific™ Vanquish™ Neo UHPLC system configured in direct injection using an 80 min gradient (see Figure 2). The data-dependent acquisition (DDA) method used a 120k full scan with *m/z* 400-1500 and 300% AGC target. The precursor fit threshold was set to 70%. The detailed parameters of ID and TMT HR QUAN MS2 scans are shown in Figure 2.

Data were analyzed using a beta version of Thermo Scientific™ Proteome Discoverer™ 3.3 software with new analysis options especially designed for TMT analysis: the new type of TMT HR QUAN MS2 scan can be specified in the analysis method and data were filtered for reporter ion signal-to-noise (S/N) as shown in Figure 3A. Data were searched using SEQUEST algorithm and precursor detector node, as well as INFERYS™ Rescoring node. All data were filtered for protein groups and peptides being quantified in all channels and quan spectra were considered as complete when filtered as shown in Figure 3B.

### Implementation of novel TMT HR acquisition scheme

In order to combine Astral MS2 scans for identification with high resolution multi-pass scans for quantitation, a novel acquisition scheme has been implemented. This novel scheme allows to set parameters for each scan that are best for identification and quantitation.

For TMT ID scans, a wider isolation window is employed to achieve high-quality spectra, along with higher transmission to minimize loss during quadrupole isolation. Optimal HCD collision energy is used to generate primary fragment ions, with b/y fragment ions still carrying the reporter tag. The scan range starts at *m/z* ≥ 150. In that way, there is a clear distinction between TMT ID scans and TMT HR QUAN scans, with higher RF applied for TMT ID scans resulting in more efficient fragment ion trapping.
For TMT HR QUAN scans, a narrower isolation window is used to reduce ratio compression, although this comes with some transmission loss. Higher collision energies are applied for efficient TMT tag cleavage.

**Figure 2. Method details for final TMT HR MS method and LC gradient**

| No | Time | Duration [min] | Flow [µl/min] | %B | Volume [µl] | No. of Column Volumes |
|---|---|---|---|---|---|---|
| 1 | 0.000 | Run | | | | |
| 2 | 0.000 | 0.000 | 0.500 | 5.0 | 0.00 | 0.00 |
| 3 | 1.000 | 1.000 | 0.500 | 5.0 | 0.50 | 0.28 |
| 4 | 1.100 | 0.100 | 0.300 | 7.0 | 0.04 | 0.02 |
| 5 | 59.100 | 58.000 | 0.300 | 36.0 | 17.40 | 9.80 |
| 6 | 79.100 | 20.000 | 0.300 | 42.0 | 6.00 | 3.38 |
| 7 | 79.100 | Column Wash | | | | |
| 8 | 80.000 | 0.900 | 0.300 | 90.0 | 0.27 | 0.15 |
| 9 | 87.000 | 7.000 | 0.300 | 99.0 | 2.10 | 1.18 |
| 10 | 87.000 | Stop Run | | | | |
| 11 | 87.000 | Column Equilibration | | | | |

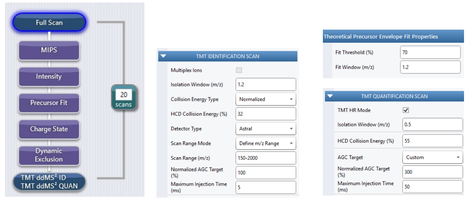
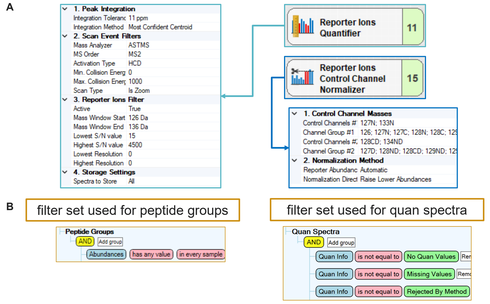



**Figure 3. Workflow and filter set in Proteome Discoverer 3.3 software for analyzing TMTpro 32plex data acquired using TMT HR mode**




## Results

### Optimization of Various Method Parameters with Triple-Knock Out Yeast Standard

TKO standard was used to optimize the parameters of TMT HR methods for runs with TMTpro 32plex-labelled samples. First, a baseline performance using traditional TMT MS2-based methods was established and benchmarked against TMT HR methods (Figure 4). Notably, numbers of quantified proteins and peptides are not highly affected by the TMT HR mode (Figure 4A). However, due to the differences in the two instrument methods, a decrease can be seen in the average S/N measured for all reporter ions where a loss of about 50% S/N is observed when using a TMT HR method compared to the MS2 method (Figure 4B). However, this can be partly mitigated by higher loading amounts and should be considered as good practice especially for running TMTPro 32plex samples where signal is split into more reporter ion channels. Using dedicated TMT HR QUAN scans results in good quantitative performance, as demonstrated by low interfering signal in the knocked-down channels is detected for the TMT HR method (Figure 4C).

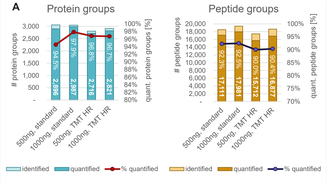



**Figure 4. Comparison of identified and quantified protein groups and quantification performance for TMT HR and conventional MS2 methods.** (A) Protein groups and peptides, (B) reporter ion S/N and % complete quan spectra, and (C) interfering abundances of knocked-out yeast proteins in respective channels from two different loads acquired with Astral MS2 and TMT HR MS2 methods.

Figure 5 illustrates the importance of sufficient sample loading amounts for TMT HR methods. The overall numbers can be increased significantly especially on peptide level where 20% more peptides were quantified for 1.5 µg as compared to 500 ng. This effect will be more pronounced for higher plex samples like 32plex, especially when dealing with very differential abundances that must be detected and quantified precisely in each sample.

**Figure 5. Total sample load influences percentage of quantifiable protein and peptide groups**

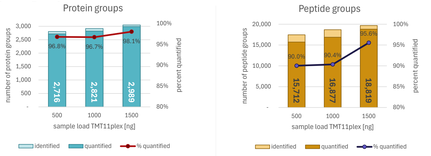
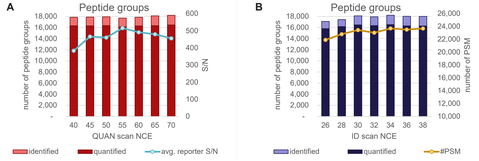
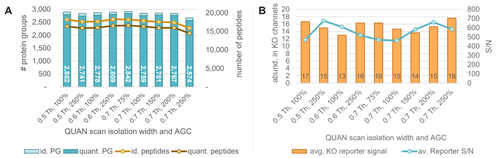



**Figure 6. Optimal normalized collision energy for TMT HR QUAN scan (A) and ID scan (B)**




The novel acquisition scheme for TMT HR methods allows for separate optimization of method parameters for identification and quantitation, like normalized collision energy (NCE). In Figure 6, different NCE values were used for both scan types while keeping the NCE for the other scan type constant at 32% for the ID scan and 55% for the QUAN scan. Overall, the numbers are not significantly influenced by the NCE. However, the reporter ion S/N is highest at 55%, decreasing for both lower and higher NCE values. Additionally, the number of PSMs does not increase beyond an NCE of 30%.

**Figure 7. Influence of TMT HR QUAN scan isolation width and AGC target on quantified peptides and protein groups (A) and quantification performance (B)**




The importance of sufficient reporter ion signal intensity was demonstrated in previous figures and can be primarily influenced by isolation width and the AGC target of the QUAN scan. A smaller isolation width decreases reporter ion signal intensity but increases its selectivity and reduces interfering signals. Therefore, these two parameters need to be balanced carefully, as demonstrated in Figure 7. The numbers of quantified and identified protein groups and peptides do not change significantly with different parameters, except for 0.7 Th and 250% AGC (Figure 7A). However, the reporter ion S/N is optimal for 0.5 Th, 250% AGC, and 0.7 Th, 200% AGC. Similarly, the lowest measured interfering abundances in the knock-out channels are observed for these parameters, but also for a slightly lower AGC target of 100% (Figure 7B). For a TMTpro 32plex sample, AGC targets must be adjusted to accommodate more TMT channels; a rough estimation would be a factor of 2 to 3.

**Figure 8. Design of TMTpro 32plex HeLa sample with 1:4 expected ratio**
For normalization to accommodate for the retention time shift of the deuterated (D) vs. the non-deuterated (non-D) TMT sets, 4 different channels were used (red squares) that carried a 4x amount of HeLa in both D and non-D channels.

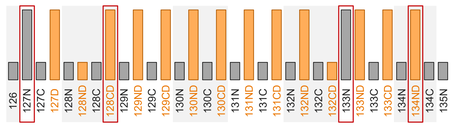
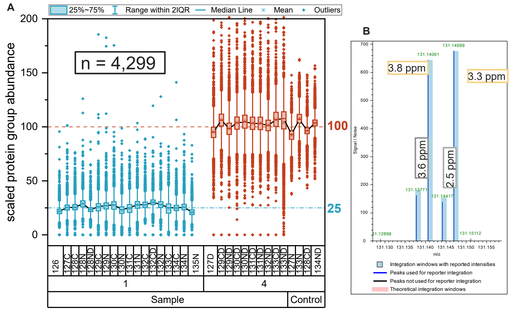



**Figure 9. Measured protein abundances in a TMTpro 32plex HeLa sample with 1:4 ratio**




### Results of TMT HR methods used for analysis of TMTpro 32plex sample

From TKO measurements, an optimized method for TMTpro 32plex samples was developed (Figure 2) and used to analyze a 1:4 HeLa digest, with a sample design shown in Figure 8.
In Figure 9A, the measured abundance ratios of all 32 samples (18 non-D and 14 D) are presented. Overall, the expected ratios (dotted line) are well reflected by the median measured abundances of all channels. The reported abundances were measured in all 32 samples, covering a total of 4,299 protein groups and 22,146 peptides. These account for 94% and 78% of all identified protein groups and peptides, respectively, and 78% of all quan spectra are fully quantified (see Figure 3B).
In Figure 9B, a spectrum of the TMT quadruplets for mass 131 is shown. All four reporter ion peaks are well resolved and detected with their respective 1 to 4 ratios, with a minimum resolution of over 100,000 for all peaks.

## Conclusions

- Novel acquisition scheme on Orbitrap Astral Zoom mass spectrometer enables the analysis of TMTpro 32plex-labelled samples
- In TMT HR mode, Astral resolving power is sufficient to resolve 3 mDa mass difference of deuterated and non-deuterated reporter ions
- Differential abundances in a TMTpro 32plex sample are measured accurately and precisely for >94% of all detected protein groups
- With a throughput of 440 samples per day, a proteome coverage of 4,300 quantified protein groups and 22,300 peptides was achieved.

## References

1. Zuniga *et al.*, **Achieving a 35-Plex Tandem Mass Tag Reagent Set through Deuterium Incorporation**; Journal of Proteome Research 2024 23 (11), 5153-5165; DOI:10.1021/acs.jproteome.4c00668

## Conflict of interest

All authors are employees of Thermo Fisher Scientific, the manufacturer of instrumentation used in this study.

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