Isobaric Mass Tagging Quantification Using Q Exactive Instruments – Approach and Expectations

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Overview

Purpose: Establish optimal parameters for relative quantification with Thermo Scientific™ Tandem Mass Tag™ (TMT™) Reagents on the Thermo Scientific™ Q Exactive™ Mass Spectrometer platforms.

Methods: Data dependent MS/MS bottom-up proteomics.

Results: Improved accuracy and precision of quantitation for TMT labeled proteins.

Introduction

Isobaric mass tagging (e.g., using TMT reagents) has become a common technique in mass spectrometry for relative quantification of proteins [1]. The tags are designed in a way that the same peptides from samples with different experimental conditions will have an identical precursor ion m/z. Upon fragmenting in mass spectrometer, however, diagnostic fragments - reporter ions - are generated. The intensities of these reporter ions are used to determine the relative amount of a particular peptide in individual samples. Nevertheless, there are a couple of factors that impede reliable quantification in complex proteomic samples. The co-isolation/co-fragmentation of peptides leading to the systematic compression of quantitative ratios presents a major problem [2]. Co-isolation interference has been addressed using various kinds of approaches, such as relying on MS^3 experiments [3] or employing a narrow isolation width.

The Q Exactive Plus and Q Exactive HF mass spectrometers are characterized by a fast acquisition speed and an efficient quadrupole isolation. In this study we demonstrate the influence of certain parameter settings, i.e. collision energy (NCE), isolation width, and MS^2 resolving power settings, on the protein quantification results from the point of protein quantification accuracy and precision.

Methods

Sample Preparation/Liquid Chromatography

Thermo Scientific™ Pierce™ HeLa Protein Digest Standard or MassPREP™ *E.coli* Digestion Standard (Waters Corporation) were labeled according to manufacturer's instructions with selected channels of the Thermo Scientific™ TMT-10plex™ reagents. Aliquots from 8 or all 10 labeled HeLa digest channels were mixed in equimolar ratios. The labeled E.coli channels (127C, 128N, 128C, 129N, 129C, 130N) were mixed in the ratio 20:10:1:1:10:20. Furthermore, for selected experiments 3 channels from the HeLa labeled sample (127N, 128N and 128C) mixed in equimolar portions were spiked into the equal amount of E.coli digest mixture (as above). The gradient is summarized in Table 1.

TABLE 1. Liquid Chromatography

Chromatography	Settings
LC	Thermo Scientific™ Ultimate™ 3000RSLCnano equipped with nano pump NCS-3000 and autosampler WPS-3000TPL
Mobile Phases	A: 0.1 % FA in water; B: 0.1 % FA in Acetonitrile (Fisher Chemicals)
Gradients*	10–25 % B in 120 min; 5min to 40 % B; 5 min to 90 % B; 8 min at 90 % B back to 5 % B in 2 min.
Flow Rate	250 nL/min
Trapping Column	Thermo Scientific™ Acclaim™ PepMap™100 µCartridge Column C18, 300 µm x 0.5 cm, 5 µm, 100 Å (back flush mode).
Separation Column	Acclaim PepMap C18, 75 µm x 50 cm, 2 µm, 100 Å

*The total run time (including washing and equilibration steps) was 146 min for the 120 min gradient.

Mass Spectrometry

Eluting peptides were analyzed on the Q Exactive Plus and Q Exactive HF mass spectrometers. The instruments were operated in the data-dependent acquisition mode selecting the top most intense 15 or 20 precursors from each scan. A summary of the MS parameters is shown in Table 2.

TABLE 2. Acquisition method parameters (Q Exactive Plus MS and Q Exactive HF MS)

Parameter	Q Exactive HF MS	Q Exactive Plus MS
Full MS parameters		
Resolution settings (FWHM at *m/z* 200)	120000	70000
Full MS mass range (*m/z*)	350-1400	350-1400
Target value	3e6	3e6
Max. injection time (ms)	50	50
MS2 parameters		
Resolution settings (FWHM at *m/z* 200)	30000; 60000	35000
Target value	1e5	1e5
Max. injection time (ms)	120; 200; 250	120; 200; 250
Isolation width	0.7; 1.0; 1.2; 2.0 Da	0.7, 1.2 Da
Collision energy (HCD)	30; 32; 35	32
Loop count	15; 20	15
Charge state recognition	2-6	2-6
Peptide match	Preferred	Preferred
Dynamic exclusion (s)	30 s	30 s
Intensity threshold	2 e4	2 e4

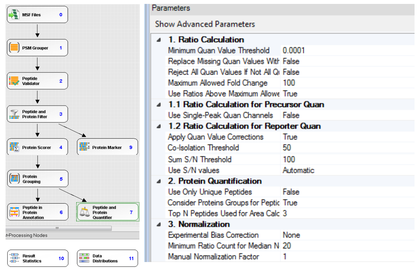
Data Analysis

Thermo Scientific™ Proteome Discoverer™ software version 2.1 was used to search MS/MS spectra against the IPI-human database or Swiss-Prot® E. coli using Sequest HT™ search engine. Static modifications included carbamidomethylation (C), and Thermo Scientific™ TMT-6plex™ reagents (peptide N-terminus; K). Dynamic modifications included methionine oxidation and deamidation (N; Q).

Peptide groups were filtered for maximum 1% FDR using Percolator with Qvality [4]. Protein groups were filtered to 1% FDR based on the number of hits obtained for searches against the forward and decoy database.

The TMT reporter ion quantification method within Proteome Discoverer software was used to calculate the reporter ratios with mass tolerance ±10 ppm. Isotopic correction factors were applied according to the pertaining CoA. Only confidently identified peptides containing all reporter ions were designated as "quantifiable spectra". Protein ratio was expressed as a median value of the ratios for all quantifiable spectra of the unique peptides pertaining to that protein group. The Consensus workflow and the parameter settings for "Peptide and Protein Quantifier " node are shown in Figure 1.

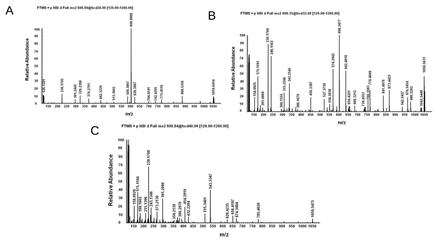
FIGURE 1. Consensus workflow and parameter settings for the quantifier node used in the study.



Results

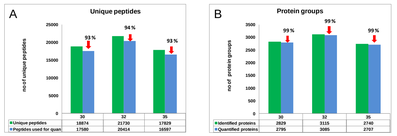
One of the several factors that affect relative quantification using TMT reagents is the applied collision energy. HCD fragmentation of TMT labeled peptides requires higher NCE as shown in Figure 2. With lower collision energies (Fig.2 A) the precursor ion dominates the spectrum. Increasing NCE to 32 (B) significantly improves fragmentation. Such a well-balanced spectrum serves well for both identification and quantification. Further increasing NCE to 40 (C) improves quantification but decreases identification score.

FIGURE 2. Spectra of an *E.coli* peptide acquired at different normalized collision energy settings. (A) NCE 28; (B) NCE 32; (C) NCE 35.



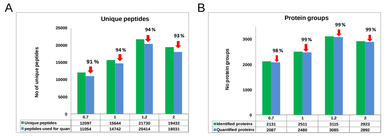
In the second set of experiments, we further optimized the NCE values. Figure 3 shows the results from TMT6plex HeLa digest (ratio of 1:1:1:1:1:1) for NCE 30, 32, and 35. Slightly more unique peptide and protein groups were identified and quantified using NCE of 32% compared to the other two settings.

FIGURE 2. Effects of different NCE on the number of unique peptide and protein groups identified (green) and quantified (blue) from TMT6plex HeLa digest. The results represent an average of triplicate experiments.



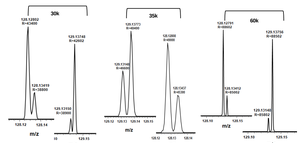
The isolation width is another important setting that directly influences the TMT-based quantification accuracy due to possible precursor co-isolation[3]. However, the extent of co-isolation depends to a large degree on sample complexity (the extent of pre-separation). Narrowing the isolation width results in a lower co-isolation interference. However, this requires longer ion fill times to accumulate sufficient number of ions. Depending on the sample complexity and other parameters of the acquisition method, there would be an optimum representing an acceptable compromise between the quantitative accuracy and identification success.
Figure 4 shows the results from TMT-6plex HeLa digest (in the ratio of 1:1:1:1:1:1) obtained for different isolation width settings. The number of identified and quantified peptide and protein groups increased with increasing isolation width. However, using the isolation width of 2 Da, the number of identified and quantified protein groups starts to drop. It is worth mentioning though, that larger isolation width might improve the identification should appropiate software algorithms be used, conducting, for example, a further round of a search for unassigned fragments. Nevertheless, an adverse effect on quantification would be expected. The best overall outcome was achieved with isolation width of 1.2 Da on the Q Exactive HF MS.

FIGURE 4. Number of peptide and protein groups identified (green) and quantified (blue) using different isolation width settings. The results represent an average of triplicate experiments.



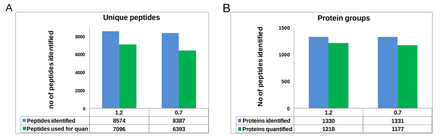
With recent advances in multiplexed quantitative approaches based on isobaric tagging, high resolving mass spectrometers are indispensable. For instance, in the case of TMT10plex, the difference between ^{13}C and ^{15}N isotopologues is only 6.32 mDa. Figure 5 shows the reporter ion region of a peptide labeled with 6 channels (127C, 128N, 128C, 129N, 129C, 130N) in different ratios. The MS^2 spectra were acquired with resolving power setting 30,000, 35000 and 60,000 at m/z 200. All other parameters were kept constant. With increasing fold-change in concentration of the samples, resolving the reporter ions employing *"just"* the 30,000 resolving power setting, becomes a challenge; quantitative precision will be affected. Using 35000 or 60,000 resolving power setting, both *N* and *C* isotopologues are fully resolved.

FIGURE 5. Detail of the reporter ion region for an *E.coli* peptide acquired with resolving power setting 30,000 (30k), 35000 (35k) and 60,000 (60k). The isobaric channels are only partially resolved when 30,000 resolving power setting was used.



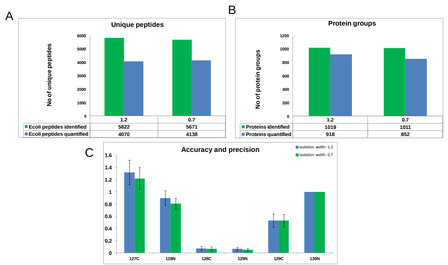
The applicability of the optimized parameters for TMT quantitation was evaluated using both a neat *E.coli* sample (6plex mixed in the ratio 20:10:1:1:10:20) and a more complex mixture where the neat *E.coli* sample was mixed with the same amount of a HeLa digest (channels 127N, 127C and 128N), total peptide load on column was 1 mg. Since the Q Exactive HF, operating with an MS2 resolution setting of 60000 performs the same as the Q Exactive Plus MS, operating at 35000 and to demonstrate the versatility of our method, both samples were analyzed on the Q Exactive Plus MS using 2 isolation widths 0.7 and 1.2 amu.
The results for the neat *E.coli* sample are shown in figure 6. Generally, there are only slight difference in the number of identified/quantified *E.coli* protein groups for both isolation widths. In both cases more than 90 % of the identified proteins were quantified. The standard deviation is, however, larger for 0.7 amu isolation width compared to 1.2 amu for the neat sample, however, quantification precision of more complex sample (HeLa+*E.coli*) as expected is better with 0.7 amu isolation width (Figure 7, C).

FIGURE 6. Number of identified (blue) and quantified (green) peptides (A) and protein (B) groups obtained from the analysis of *E.coli* digest employing isolation with settings 0.7 and 1.2 Da.



Results from the *E.coli* and HeLa mix is summarized in Figure 7. The goal of the this experiment was to determine approximately the number of *E.coli* proteins that can be quantified in the complex background and the effect HeLa peptides may have on the precision and accuracy of quantification. In total, approximately 4000 protein groups (HeLa + *E.coli*) were identified, about a third of these were *E.coli* proteins. This represents about 86 % of the number of proteins that were quantified in the neat *E.coli* sample (Figure 6). However, both precision and accuracy were significantly affected by presence of the HeLa peptides. Both accuracy and precision were improved by using narrow isolation width without penalty for identified or quantified proteins and peptides (Figure 7)

FIGURE 7. Number of identified (green) and quantified (blue) peptide (A) and protein (B) groups obtained from the analysis of *E.coli* digest employing isolation with settings 0.7 and 1.2 Da. The results represent an average of duplicate experiments.



Conclusion

- Small isolation width can be used to address the issue of co-isolation; however, the isolation width should be set in relation with the ion accumulation time.
- NCE plays an important role in accuracy of quantitation. The best compromised between identification and quantification was achieved at NCE of 32. However, the use of step collision energy, where the lower energy is used for identification and higher energy for quantification is another approach to improve quantitation.
- High resolution > 30000 is a necessity for multiplexed quantitation, especially with TMT-10 plex reagents.
- Up to 86% of the number of *E.coli* proteins identified/quantified in a neat sample was identified/quantified

References

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