

TMT Workflow on the Q Exactive Series - Instrument Parameter Optimization and Data Analysis in Proteome Discoverer 2.1 Software

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Key Words

Q Exactive Plus, Q Exactive HF, TMT, Proteome Discoverer 2.1, identification, relative quantification, isobaric tagging, multiplexing

Goal

To develop an optimal acquisition method for relative quantification of proteins using Thermo Scientific™ TMTsixplex™ and TMT10plex™ workflows on the Thermo Scientific™ Q Exactive™ platforms. The topics of sample preparation, peptide separation and mass spectrometric analysis, and data processing using Thermo Scientific™ Proteome Discoverer™ software version 2.1 are covered in detail.

Introduction

Isobaric mass tagging (e.g., Tandem Mass Tag™ (TMT™)¹ or isobaric Tag for Relative and Absolute Quantification (iTRAQ®)²) has become a common technique in mass spectrometry for relative quantification of proteins.³⁻⁶ Some advantages of TMT-based multiplexed relative quantification include reduced overall experiment time and experimental variance, increased sample throughput, and fewer missing quantitative values among samples. Amine-reactive TMT10plex reagents share an identical chemical structure with TMTsixplex reagents but contain an increased number of combinations of ¹³C and ¹⁵N isotopes in the mass reporter region. Some of these combinations give rise to reporter ions that differ only by a single neutron mass, requiring instruments that deliver highly resolved tandem mass spectra for their analysis.

The most accurate TMT quantification on high dynamic range complex mixtures can be accomplished by employing the Synchronous Precursor Selection (SPS) MS3 method available on the Thermo Scientific™ Tribrid™ mass spectrometer family, including the Orbitrap™ Fusion™ MS and Orbitrap Fusion Lumos™ MS instruments.^{7,8} Another strong candidate for isobaric

quantification analyses is the Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer family, which combines the selectivity and specificity of quadrupole precursor selection with high-resolution, accurate-mass (HRAM) detection in the orbitrap analyzer. The Thermo Scientific™ Q Exactive™ Plus mass spectrometer is equipped with advanced quadrupole technology (AQT) to optimize the ion selection and transmission, improving the quantification of low abundance ions within narrow isolation windows.⁹ The Thermo Scientific™ Q Exactive™ HF mass spectrometer represents a further augmentation in performance with the incorporation of an ultra-high-field Orbitrap mass analyzer, delivering high resolution at twice the speed of the Q Exactive Plus MS.⁹

A successful TMT-labeling experiment on a Q Exactive mass spectrometer delivers maximal identifications for peptides and proteins, while simultaneously providing relative quantification of up to ten different samples at high quantitative accuracy. This requires well-separated chromatography, mass spectrometry resolved, abundant, and interference-free reporter ions, as well as high quality fragmentation patterns in MS2 spectra collected at high speed. This application note presents a step-by-step guide on TMT labeling and relative quantification, including sample preparation, instrument settings, and data processing of multiplexed data using Thermo Scientific™ Proteome Discoverer™ 2.1 software. The effect of key instrument parameters (resolving power, injection time, target value, collision energy, and isolation window) on protein and peptide identification and quantification is discussed in detail to assure the success of a TMT workflow on the Q Exactive Series of mass spectrometers.

Experimental

TMT Labeling

E. coli digest standard (Waters® Corp, Milford, MA) was labeled with Thermo Scientific TMT reagents as follows:

1. Equilibrate triethylammonium bicarbonate (TEAB) buffer, TMT tags, and dried protein samples from -20 °C to room temperature.
2. Make 100 mM TEAB by adding 500 µL of the Dissolution Buffer (1 M TEAB) to 4.5 mL of ultrapure water.
3. Dissolve each sample (up to 100 µg/TMT tag) in 100 mM TEAB buffer to obtain a concentration of 1 µg/uL, vortex well, wait for 10 minutes.
4. Add 41 µL of fresh LC-MS grade acetonitrile to each TMT reagent vial, vortex, and wait for 10 minutes.
5. Add up to 100 µL sample solution made in step 3 to each TMT reagent vial, vortex, and incubate for 1 hour.
6. After 1 hour, add 8 µL of 5% hydroxylamine (dilute 50% stock solution of hydroxylamine provided in the kit 10 times using water) to each vial to quench reaction. Incubate for 15 minutes.
7. After the labeling, immediately combine different samples at desired ratios. In the presented study, TMTsixplex reagents (channels 126–131) were mixed at 20:10:1:1:10:20. Six selected TMT10plex reagents (channels 127C, 128N, 128C, 129N, 129C, 130N) were mixed at the same ratios as well.
8. Aliquot prepared samples, dry, and store at -80 °C.
9. Resuspend prepared TMT sample in 0.1% TFA/5% ACN (v:v) just before LC-MS analysis; resuspended TMT-labeled samples remain stable for one week at 4 °C, avoid freeze-thaw.

To test quantification accuracy, Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (P/N 88328) was labeled using the above protocol, mixed at 10:10:10 ratio for TMTsixplex channels 129–131 and TMT10plex channels 127N, 127C, and 128N, and added into the *E. coli* sample in 1:1 ratio per channel (33 µg of HeLa digest + 67 µg of *E. coli* digest).

Liquid Chromatography

Details of the chromatography settings are listed in Table 1. A sample amount corresponding to 1 µg of the starting protein sample was injected.

Table 1. Detailed LC settings for TMT-labeled samples.

Parameter	Setting
LC	Thermo Scientific™ EASY-nLC™ 1000
Mobile phases	A: 0.1% formic acid in water; B: 0.1 % formic acid in acetonitrile (Fisher Chemical)
Gradients	7–25% B in 100 min; 25–60% B in 20 min; 1 min to 95% B, and 5 min hold at 95%
Flow rate	300 nL/min
Trapping column	Thermo Scientific™ Acclaim™ PepMap™ 100 C18 LC column, 75 µm x 2 cm, Thermo Scientific™ Dionex™ nanoViper™, C18, 3 µm, 100 Å (P/N 164705)
Separation column	Thermo Scientific™ EASY-Spray™ C18 LC column, 75 µm x 50 cm, 2 µm, 100 Å (P/N ES803)

Mass Spectrometry

Eluting peptides were analyzed on the Q Exactive Plus and Q Exactive HF mass spectrometers in the data-dependent acquisition mode. The instrument parameter settings are summarized in Table 2.

Table 2. Recommended instrument parameter settings for TMTsixplex- and TMT10plex-labeled samples for the Q Exactive Plus and the Q Exactive HF instruments, respectively.

	Q Exactive Plus	Q Exactive HF
Full MS Parameters		
Resolving power (FWHM at <i>m/z</i> 200)	70,000	120,000
AGC target	3e6	3e6
Max. injection time (ms)	50	50
Scan range (<i>m/z</i>)	375–1400	375–1400
MS2 Parameters		
Resolving power (FWHM at <i>m/z</i> 200)	35,000	30,000 (TMTsixplex), 60,000 (TMT10plex)
AGC target	1e5	1e5
Maximum injection time (ms)	100	100
Isolation width (Th)	0.7 or 1.2	0.7 or 1.2
Normalized collision energy (NCE)	32	32
Loop count	10 or 15	10 or 15
First fixed mass	100	100
Underfill ratio	2%	2%
Charge state recognition	2–7	2–7
Peptide match	Preferred	Preferred
Dynamic exclusion (s)	30	30

Data Processing

Data analysis was performed using Proteome Discoverer software version 2.1 (PD 2.1). It features a new and improved TMT quantification workflow, which includes: 1) a new user interface for adding reporter ion isotopic distributions; 2) the ability to account for reporter ion isotopic impurities for TMT10plex reagents; 3) the implementation of TMT quantification based on S/N values¹⁰; 4) protein and peptide quantification based on the sum of S/N values; and 5) new methods for normalization and scaling for peptide and protein abundance values.

The critical steps for setting up the data processing in Proteome Discoverer 2.1 software are described in detail below.

1. Create a Study-Specific Quantification Method

Before setting up a new study, the quantification method needs to be updated. To edit the quantification method:

1. Go to *Administration*, select *Maintain Quantification Methods*. The common quantification templates are provided.
2. Select *Add* and choose from existing method. Each existing TMT quantification template includes the modifications on the residues/peptide N-terminal, and the Reporter Ion Isotope Distribution.
3. The isotope distribution values need to be added as provided in the certificate of analysis (CoA) of the specific TMT lot used in the study. This information can be found at <http://www.thermofisher.com/order/catalog/product/90110?CID=search-product> using the lot number displayed on the reagents vials.

4. Select/unselect reporter ion channels according to the experiment using the “active” column.

5. Save the newly created Quantification Method.

A new study-specific quantification method is shown in Figure 1.

2. Import FASTA Database

1. Open *Maintain FASTA Files* under the *Administration*.
2. Select *Add* to import the existing .FASTA file from the local drive.
3. Alternatively, select *Download*, type in the taxonomy ID, and obtain the database from ProteinCenter.

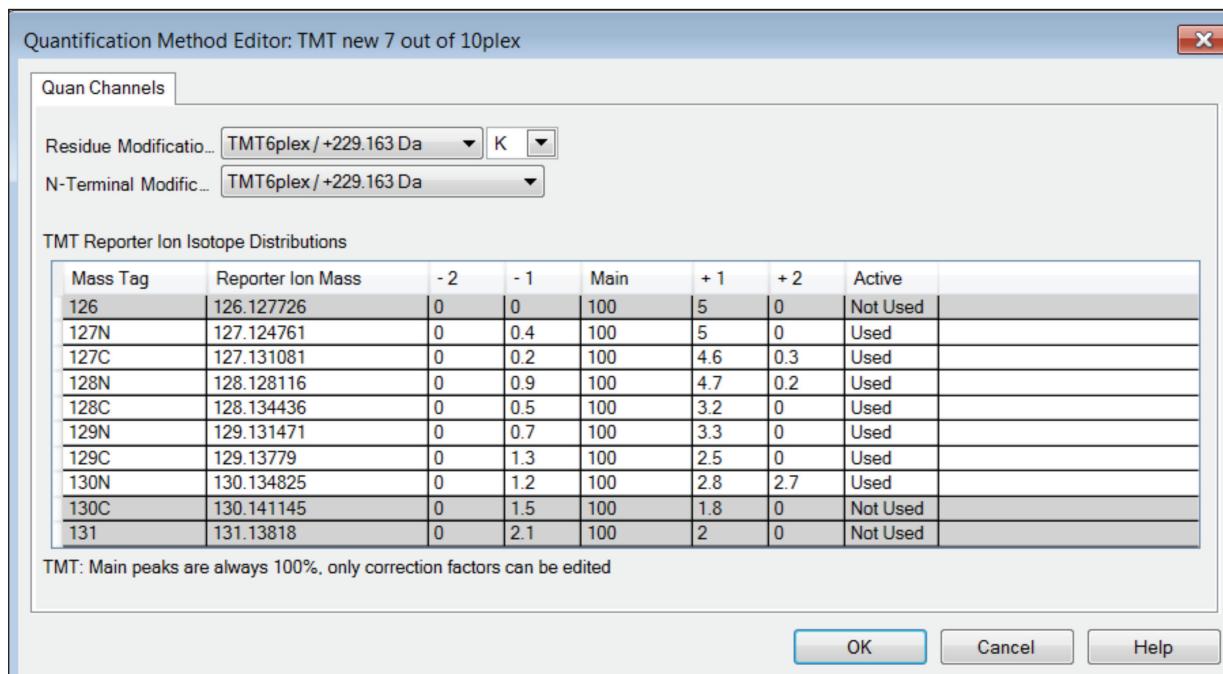


Figure 1. Example of a user-defined study-specific TMT10plex quantification method.

3. Create New Study

1. Select *New Study and Analysis* from the Proteome Discoverer software start page (Figure 2).
2. Define a *Study Name* and assign it a working *Study Directory* to save files to a specific folder.
3. For the Processing Workflow, select the down triangle to the templates available, select “ProcessingWF_QE active\PFW_QE_Reported_Based_Quan_Sequ tHT_Percolator.pdProcessingWF”.
4. For Consensus workflow, select “ConsensusWF CWF_Comprehensive_Enhanced_Annotation _Quan pdConsensusWF”.
5. Specify the *Quantification Method* just created in section 1, followed by the *Control Channel Selection*.
6. Import .raw files to be processed. In an example shown in Figure 2, four raw files representing replicate analyses employing two different isolation width settings are being imported.
7. Select *OK* and the study will open in a new window.
8. For complex studies with replicates, *Study Factors* management is recommended for proper results grouping, normalization, and display. To add *Study Factors*, go to *Study Definition tab* in the current study window, select *Add Categorical or Numeric Factors*. Type in factor name and all related factors (Figure 3). If no Study Factor is needed, continue to workflow setup in section 4.
9. Under the *Input Files tab*, the imported raw files should be displayed. Select the triangle left of each file to expand the details. Specify the *Sample Type* and *Study Factors* per reporter ions and files accordingly to experiment settings (Figure 4).
10. When completed, check the *Samples tab* - all study factors should be updated automatically.

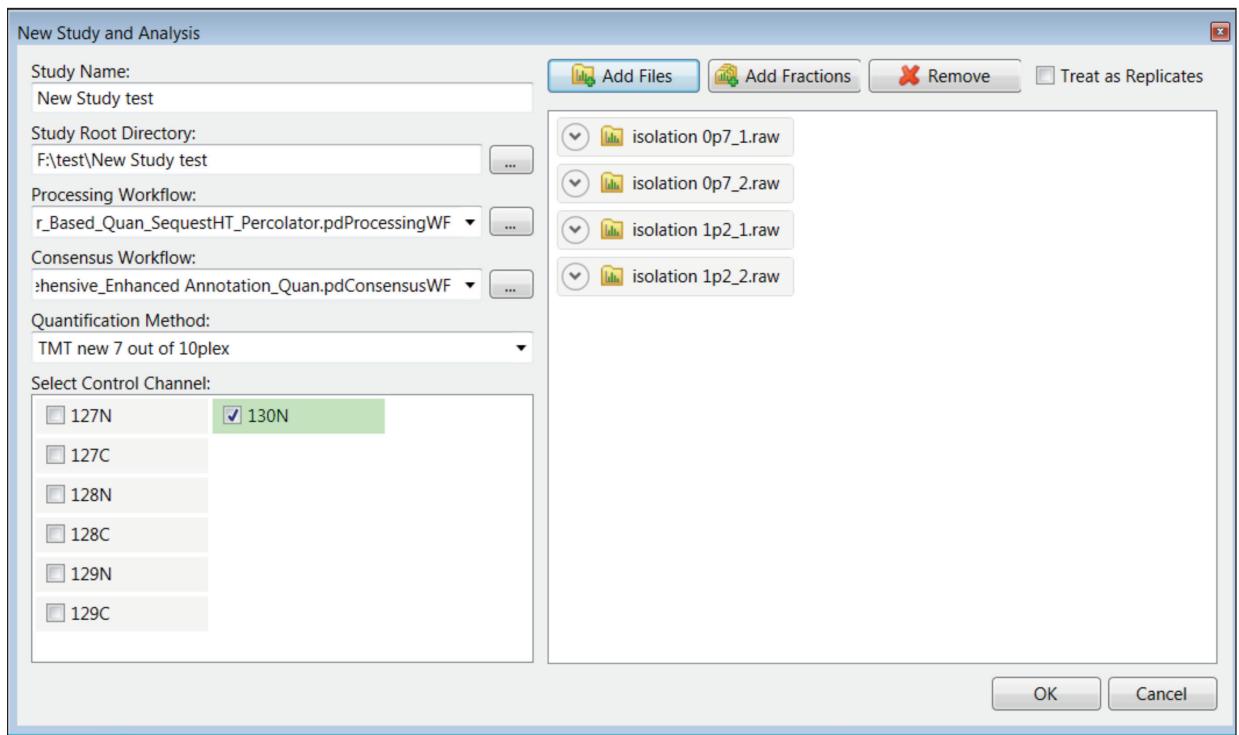


Figure 2. The New Study and Analysis setup dialogue screen.

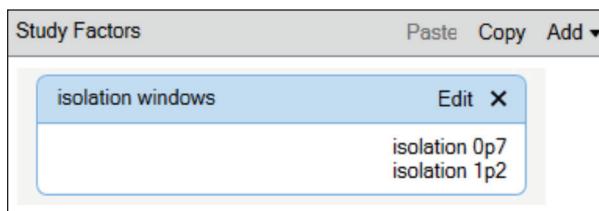


Figure 3. Adding new Study Factors within the Study Factor management screen.

EN English (United States)

Study Definition		Input Files		Samples	Analysis Results		Workflows	Grouping & Quantification									
ID	Name	File Type	Quan Method	Sample Information													
F1	isolation 0p7_1	.raw	TMT new 7 out of 10plex	Sample Type: [Sample, Control], isolation windows: [isolation 0p7]													
F2	isolation 0p7_2	.raw	TMT new 7 out of 10plex	Sample Type: [Sample, Control], isolation windows: [isolation 0p7]													
				Sample	Sample Identifier	Sample Type	Quan Channel	isolation windows									
	2	isolation 0p7_2 - [127N]	Sample	127N	isolation 0p7												
	11	isolation 0p7_2 - [127C]	Sample	127C	isolation 0p7												
	12	isolation 0p7_2 - [128N]	Sample	128N	isolation 0p7												
	13	isolation 0p7_2 - [128C]	Sample	128C	isolation 0p7												
	14	isolation 0p7_2 - [129N]	Sample	129N	isolation 0p7												
	15	isolation 0p7_2 - [129C]	Sample	129C	isolation 0p7												
	16	isolation 0p7_2 - [130N]	Control	130N	isolation 0p7												
File:																	
<table border="1"> <thead> <tr> <th>ID</th> <th>Name</th> <th>Date Modified</th> <th>Size</th> </tr> </thead> <tbody> <tr> <td>F2.1</td> <td>G:\test\isolation 0p7_2.raw</td> <td>4/3/2015 4:38:26 AM</td> <td>1022746265 [Byte]</td> </tr> </tbody> </table>										ID	Name	Date Modified	Size	F2.1	G:\test\isolation 0p7_2.raw	4/3/2015 4:38:26 AM	1022746265 [Byte]
ID	Name	Date Modified	Size														
F2.1	G:\test\isolation 0p7_2.raw	4/3/2015 4:38:26 AM	1022746265 [Byte]														
F3	isolation 1p2_1	.raw	TMT new 7 out of 10plex	Sample Type: [Sample, Control], isolation windows: [isolation 1p2]													
	Sample	Sample Identifier	Sample Type	Quan Channel	isolation windows												
	3	isolation 1p2_1 - [127N]	Sample	127N	isolation 1p2												
	17	isolation 1p2_1 - [127C]	Sample	127C	isolation 1p2												
	18	isolation 1p2_1 - [128N]	Sample	128N	isolation 1p2												
	19	isolation 1p2_1 - [128C]	Sample	128C	isolation 1p2												
	20	isolation 1p2_1 - [129N]	Sample	129N	isolation 1p2												
	21	isolation 1p2_1 - [129C]	Sample	129C	isolation 1p2												
	22	isolation 1p2_1 - [130N]	Control	130N	isolation 1p2												
File:																	
<table border="1"> <thead> <tr> <th>ID</th> <th>Name</th> <th>Date Modified</th> <th>Size</th> </tr> </thead> <tbody> <tr> <td>F3.1</td> <td>G:\test\isolation 1p2_1.raw</td> <td>4/3/2015 12:52:22 AM</td> <td>1023110068 [Byte]</td> </tr> </tbody> </table>										ID	Name	Date Modified	Size	F3.1	G:\test\isolation 1p2_1.raw	4/3/2015 12:52:22 AM	1023110068 [Byte]
ID	Name	Date Modified	Size														
F3.1	G:\test\isolation 1p2_1.raw	4/3/2015 12:52:22 AM	1023110068 [Byte]														
F4	isolation 1p2_2	.raw	TMT new 7 out of 10plex	Sample Type: [Sample, Control], isolation windows: [isolation 1p2]													

Figure 4. Study Factors and Quantification Channels defined for individual sample quantification.

4. Workflow Setup and Quantification Ratio Definition

Go to *Workflows tab* and open the processing workflow first. The template already includes the mass tolerance, reporter ion quantification specifications, and common modifications. Several additional parameters need to be specified.

- Under the SequestHT node of the processing workflow, select *Protein Database*. More than one protein database can be selected.
- Under the SequestHT node of the processing workflow, specify TMT as a *static modification* on peptide N-terminus and lysine residues. (Note: the TMTsixplex and TMT10plex both correspond to the addition of 229.163 Da).
- Check the *MS order* in the Reporter Ion Quantifier node is *MS2*.
- Open the consensus workflow, select on Protein Marker node. Select the proteins to mark in the result. As the sample is a mixture of *E. coli* and human proteins, it is convenient to define two markers (*Ecoli* and *Human*) (Figure 5).
- Go to Peptide and Protein Quantifier node. Set *Apply Quan Value Corrections to True, Co-Isolation Threshold* to 50, and *Average Reporter S/N Threshold at least 10* (Figure 6). Select *On Channels Average*

(*Per File*) for *Scaling Mode* so that the abundance values of each channel are scaled for each replicate. For more details on how to set up these parameters, please check Reference 11.

- Drag the .raw files from under the Input Files tab into the designated area on the right, under Analysis.
- Go to the Grouping & Quantification tab and specify study variables. This setting determines how the quantification results will be grouped and displayed. In this case, the Quan Channel and isolation windows are selected so that the results for the replicate analyses of each isolation width are grouped and averaged and thus can be easily compared in the final report. To achieve this, check these two variables and adjust the isolation windows to the top of Quan Channel by selecting the up and down triangles at the left end of each variable. Note how the order of the variables could affect the grouping in Bulk Ratio Generation. Select the control 130N Quan Channel as the Denominator for two sets of isolation windows and select *Add Ratios*. The Generated Ratios are shown in Figure 7. For more discussion on how to set study variables, see Reference 11.
- Select *Run* on the right upper corner of the *Analysis* to start the Processing and Consensus workflow searches in the Job Queue.

1. Contaminant Database	
Protein Database	
2. Additional Marker Database	
Column Name	Ecoli
Protein Database	Escherichia coli K-12 (SwissProt TaxID=83333) (v2015-09-16)
3. Additional Marker Database	
Column Name	Human
Protein Database	Homo sapiens (SwissProt TaxID=9606) (v2015-09-16)

Figure 5. Settings of the Protein Markers node, part of the consensus workflow, used in the study.

1. Quantification - General	
Peptides to Use	Unique + Razor
Consider Protein Groups for Peptide Uniqueness	True
Replace Missing Values with Minimum Value	False
Reject Quan Results with Missing Channels	False
Maximum Allowed Fold Change	100
Top N Peptides Used for Area Calculation	3
2. Reporter Quantification	
Reporter Abundance Based On	Automatic
Apply Quan Value Corrections	True
Co-Isolation Threshold	50
Average Reporter S/N Threshold	10
3. Precursor Quantification	
Use Single-Peak Quan Channels	False
4. Normalization and Scaling	
Normalization Mode	None
Proteins For Normalization	
Scaling Mode	On Channels Average (Per File)
5. Display Options	
Show Standard Errors	True
Show Quan Value Counts	False
Show Quan Ratios As	Normal Space Values
6. Quan Ratio Distributions	
1st Fold Change Threshold	2
2nd Fold Change Threshold	4
3rd Fold Change Threshold	6
4th Fold Change Threshold	8
5th Fold Change Threshold	10

Figure 6. Parameter settings for the Peptide and Protein Quantifier node, part of the consensus workflow.

The screenshot shows the 'Grouping & Quantification' tab of a software application. It includes several panels:

- Sample Group and Quan Ratio Specification:** Contains sections for 'Study Variables' (File, isolation windows, Quan Channel, Sample Type), 'Manual Ratio Generation' (Numerator and Denominator dropdowns, Add Ratio button), and 'Bulk Ratio Generation' (Denominators to be used: Quan Channel: 129C, Quan Channel: 130N, isolation windows: isolation 0p7, Quan Channel: 127N, Quan Channel: 127C, Quan Channel: 128N, Quan Channel: 128C, Quan Channel: 129N, Quan Channel: 129C, Quan Channel: 130N).
- Generated Sample Groups:** Lists sample groups and their components:
 - isolation 1p2 | 127N: 127N Sample isolation 1p2, F3: isolation 1p2_1, 127N Sample isolation 1p2, F4: isolation 1p2_2
 - isolation 0p7 | 127N: 127N Sample isolation 0p7, F1: isolation 0p7_1, 127N Sample isolation 0p7, F2: isolation 0p7_2
 - isolation 1p2 | 127C: 127C Sample isolation 1p2, F3: isolation 1p2_1, 127C Sample isolation 1p2, F4: isolation 1p2_2
 - isolation 0p7 | 127C: 127C Sample isolation 0p7, F1: isolation 0p7_1, 127C Sample isolation 0p7, F2: isolation 0p7_2
- Generated Ratios:** Lists generated ratios:
 - X isolation 1p2 | 127N / isolation 1p2 | 130N
 - X isolation 0p7 | 127N / isolation 0p7 | 130N
 - X isolation 1p2 | 127C / isolation 1p2 | 130N
 - X isolation 0p7 | 127C / isolation 0p7 | 130N
 - X isolation 1p2 | 128N / isolation 1p2 | 130N
 - X isolation 0p7 | 128N / isolation 0p7 | 130N
 - X isolation 1p2 | 128C / isolation 1p2 | 130N
 - X isolation 0p7 | 128C / isolation 0p7 | 130N
 - X isolation 1p2 | 129N / isolation 1p2 | 130N
 - X isolation 0p7 | 129N / isolation 0p7 | 130N

Figure 7. Grouping and Quantification tab for ratio generation.

5. Interpretation of Protein and Peptide Identifications and Quantification Results

- When the searches finish, highlight the Consensus search and select *Open Results*. Or go to the *Analysis Results tab and Open Results* by highlighting the analysis row.
- The numbers of Protein Groups and Peptide Groups are summarized at the bottom of the screen.
- The species assignment according to the settings of the Protein Marker node is shown for each protein/peptide group (Figure 8).
- Go to Toolbar *View* and select *Display Filter*. Filter for total identifications of protein groups and peptide groups for *E. coli* from all files (Figure 9).
- To obtain numbers of identifications for each of the two study factor groups, apply filters shown in Figure 10.
- To get the numbers of quantifiable peptides, go to *View*, select *Distribution Charts*. Choose *Peptide Groups-Quan Info* in Bar Charts to display peptide groups with and without Quan Values (Figure 11).
- As designed in the Grouping&Quantification tab, quantification ratios for two replicates of each condition are grouped and averaged, as shown in *Abundance Ratios* column (Figure 12). In addition, the *Grouped* and *Scaled Abundances*, *Standard Error* for replicates, and the *Scaled Abundances* for individual replicate dataset are displayed as well (Figure 13). This allows easy comparison of quantification results for different conditions.

Proteins	Protein Groups	Peptide Groups	PSMs	MS/MS Spectrum Info	Quan Spectra	Result Statistics	Ecoli	Human	Coverage	# Peptides	# PSMs
1	<input type="checkbox"/>	<input checked="" type="checkbox"/>	P0A6Y8	Chaperone protein DnaK [OS=Escherichia coli K-12]			X		66%	38	496
2	<input type="checkbox"/>	<input checked="" type="checkbox"/>	P0A853	tryptophanase [OS=Escherichia coli K-12]			X		58%	27	614
3	<input type="checkbox"/>	<input checked="" type="checkbox"/>	P14618	Pyruvate kinase PKM [OS=Homo sapiens]				X	58%	27	508
4	<input type="checkbox"/>	<input checked="" type="checkbox"/>	P06733-1	alpha-enolase [OS=Homo sapiens]				X	72%	24	537
5	<input type="checkbox"/>	<input checked="" type="checkbox"/>	P14618-3	Isoform 3 of Pyruvate kinase PKM [OS=Homo sapiens]				X	53%	24	467
6	<input type="checkbox"/>	<input checked="" type="checkbox"/>	P0AFH8	osmotically-inducible protein Y [OS=Escherichia coli K-12]			X		52%	8	460
7	<input type="checkbox"/>	<input checked="" type="checkbox"/>	P0A6F5	60 kDa chaperonin [OS=Escherichia coli K-12]			X		56%	26	502
8	<input type="checkbox"/>	<input checked="" type="checkbox"/>	Q09666-1	Neuroblast differentiation-associated protein AHNAK [OS=Homo sapiens]			X		51%	146	653
9	<input type="checkbox"/>	<input checked="" type="checkbox"/>	P14618-2	Isoform M1 of Pyruvate kinase PKM [OS=Homo sapiens]			X		51%	25	432

Figure 8. Proteins marked according to their species of origin.

The screenshot shows the 'Display Filter' interface with two main sections: 'Proteins' and 'Peptide Groups'. Both sections have a toolbar with 'Load', 'Save', 'Clear', 'Clear All', and 'Apply' buttons. The 'Proteins' section has a filter for 'Proteins' (selected) and 'Ecoli' (selected). The 'Peptide Groups' section has a filter for 'Peptide Groups' (selected) and 'Ecoli' (selected). Below the toolbar are lists of other filterable categories: Protein Groups, PSMs, MS/MS Spectrum Info, Quan Spectra, and Result Statistics.

Figure 9. Filters for identifications of proteins (top) and peptide groups (bottom) for *E.coli*.

This screenshot shows a more detailed view of the filter interface for identified proteins and peptide groups. It includes complex logic like AND and OR operators. For proteins, it filters by 'Master' being equal to 'Master' and 'Ecoli' being true. For peptide groups, it filters by 'Ecoli' being true. Both sections include additional filters for 'Found in Files' with 'High' confidence in specific files (F3 and F4).

Figure 10. Filters for identified proteins (top) and peptide groups (bottom) from each study factor used in this study.

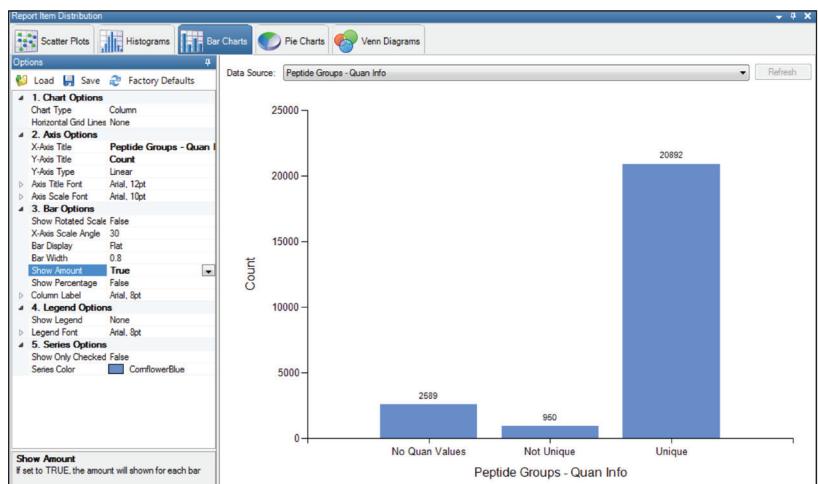


Figure 11. Quantification overview for filtered peptide groups.

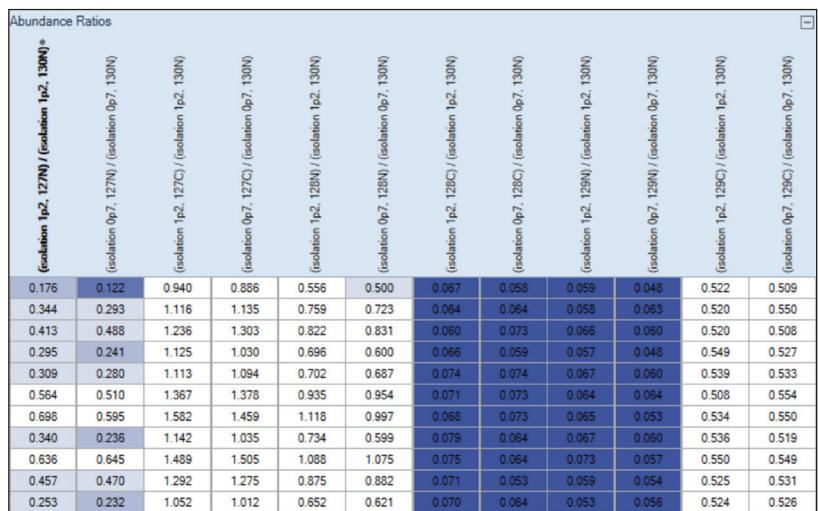


Figure 12. Abundance Ratios columns in the result. The ratios are sorted by Isolation Windows and Quan Channels. The ratio changes for up or down regulation are highlighted by colors. The darker colors are for more significant changes compared to the lighter colors.

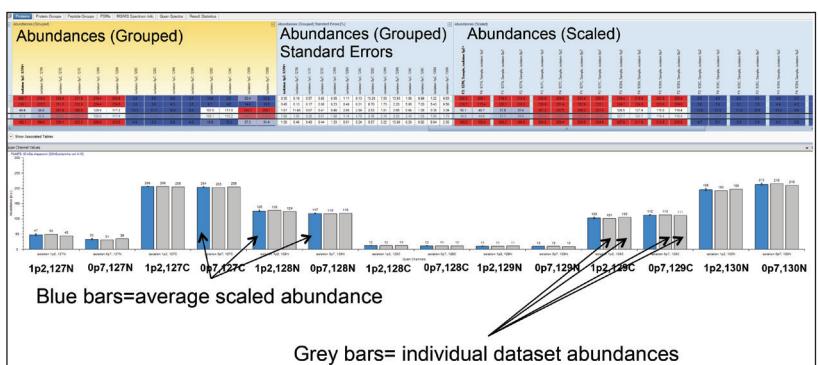


Figure 13. Abundances (Grouped), Standard Errors, and scaled Abundances for each dataset are illustrated for proteins.

Results and Discussion

The success of a TMT-labeling experiment for achieving absolute sensitivity and quantification accuracy is determined by the careful setting of each analytical parameter. A longer gradient provides more chromatographic resolution and less ion suppression effects as well as less potential interference from co-elutants. Highly resolved MS1 and MS2 spectra contribute to the accurate relative quantification of peptides and proteins. MS2 max injection time, AGC target, NCE, and MS2 isolation width instrument parameter setup all influence the quality of MS/MS spectra as well as the quantification. All of these parameters should be carefully chosen while setting the instrument acquisition rate as high as possible.

Liquid Chromatography

Chemical modification of a peptide can affect its hydrophobicity and thus change its chromatographic behavior. Elution of TMT-labeled samples typically requires a higher percentage of organic solvent. Tables 3 and 4 contain the recommended gradient profiles used for non-labeled and labeled cell lysate for a two hour total gradient length.

Table 3. LC gradient for non-labeled samples.

Time	Duration	Flow	%B
0	N/A	300	2
5	5	300	2
105	100	300	20
125	20	300	32
126	1	300	95
134	8	300	95

Table 4. LC gradient for TMT-labeled samples.

Time	Duration	Flow	%B
0	N/A	300	5
3	3	300	5
5	2	300	7
105	100	300	25
125	20	300	60
126	1	300	95
134	8	300	95

With increasing sample complexity, the gradient should be lengthened (e.g., 4 hours) to enable better chromatographic separation. Extending the gradient, however, is bound to result in a widening of LC peaks and a decrease in precursor S/N. It is therefore advisable to increase sample injection amount or the maximum injection time limit. The final metric used for the evaluation of such experiments is the number of quantifiable peptides defined as peptides whose fragmentation spectra contain all of the expected reporter ions. As shown in Figure 14A, for a 1 µg sample injection, increasing the gradient from 2 hours to 4 hours results in an increase in the number of identified peptides from 3,241 to 3,810. The identification results were clearly benefiting from better separation, indicating increased sensitivity for lower abundant peptides leading to improved protein coverage. On the other hand, the number of the quantifiable peptides did not improve as max. injection time setting was the same as for 2-hour gradient (Table 2). Injecting 2 µg of sample while at the same time using a longer gradient resulted in additional 400 quantifiable peptides, raising the ratio of quantifiable/identified peptides to 85%. Similarly, the number of quantified protein groups increased with improvement in both chromatography and sample loading amount (Figure 14B). The results highlight the importance of optimally matched chromatographic separation conditions with appropriately chosen sample injection amounts and injection time settings to obtain reliable quantitative results for samples with high dynamic range (20:1).

MS1 Parameters

As the TMT label is truly isobaric for precursor ions, the resolving power settings used for MS1 are identical to those used for the unlabeled samples: 70,000 and 120,000 for the Q Exactive Plus MS and the Q Exactive HF MS, respectively. This is sufficient to resolve most of the co-eluting species selected for data-dependent scans without substantially impacting the overall cycle time. The AGC target of 3e6 is recommended to improve the dynamic range of the full scan.

MS2 Resolving Power

Insufficient MS2 resolving power results in the convergence of the signal from background or contaminant ions with that of the reporter ions, interfering with S/N estimation. For TMTsixplex-labeled samples, a resolving power setting of 30,000 ensures the reporter ions get baseline resolved from the isotopes of adjacent reporter ions.¹²

In the case of TMT10plex reagents, the difference between ¹³C and ¹⁵N isotopologues is only 6 mDa. Figure 15 shows the reporter ion region of a peptide labeled with 4 channels (128N, 128C, 129N, 129C) at different ratios. A resolution setting of 30,000 is inadequate to baseline resolve the N and C isotopologues. Suitable resolving power settings for TMT10plex-labeled sample would be 35,000 and 60,000 for the Q Exactive Plus MS and the Q Exactive HF MS, respectively.

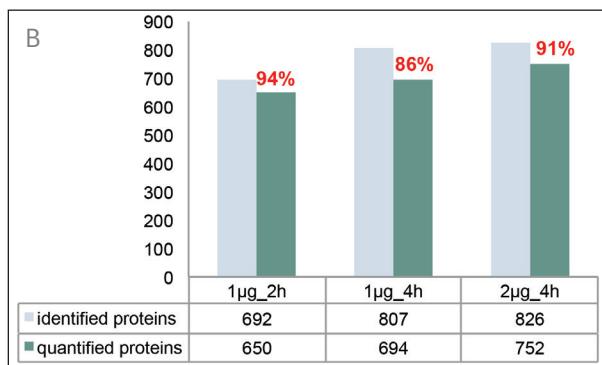
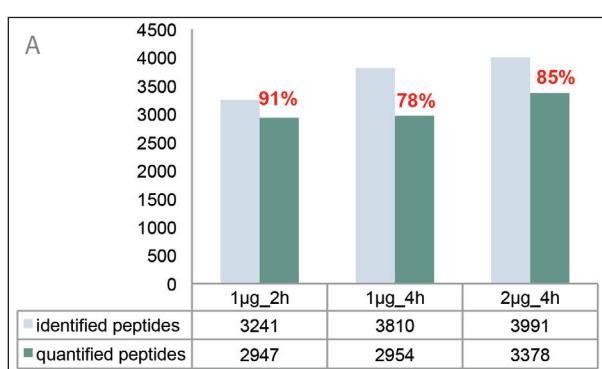


Figure 14. The numbers of identified and quantified peptide and protein groups for *E. coli* when analyzing TMTsixplex-labeled *E. coli* and Hela mixture samples at increasing sample injection amounts and gradient lengths.

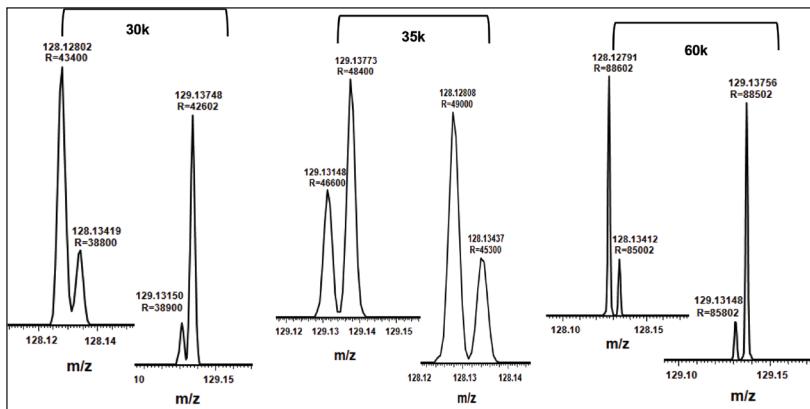


Figure 15. Details of the reporter ion region of a TMT10plex-labeled peptide analyzed at resolving power settings 30,000, 35,000 and 60,000. Note that the isobaric channels are only partially resolved when just 30,000 resolving power setting is used.

MS2 Maximum Injection Time

MS2 maximum injection time needs to be adjusted based on the injected sample amount, its complexity, as well as the length of gradient. Generally, a longer injection time allows the accumulation of more precursor ions to generate higher reporter ion intensity, thus facilitating the identification and quantification of medium and low abundance peptides. In Figure 16, the increase of injection time from 64 ms to 100 ms boosted the number of proteins and peptides quantified, leading to a much higher quantification rate.

As mentioned above, longer gradients require longer injection times. On the other hand, the injection time should not significantly impact the overall speed of the instrument, especially for complex lysate samples. An ideal method will be to match the allowed maximum injection time with the FT transient detection time, which is directly correlated with the resolution settings. For the Q Exactive Plus MS and the Q Exactive HF MS, the resolving power settings 35,000 and 60,000, respectively, correspond to the Orbitrap analyzer detection time of 128 ms. Employing a 100 ms injection time thus matches well the detection time, effectively allowing the maximum parallelization. Longer injection times also benefits quantitative accuracy, as shown in Figure 17.

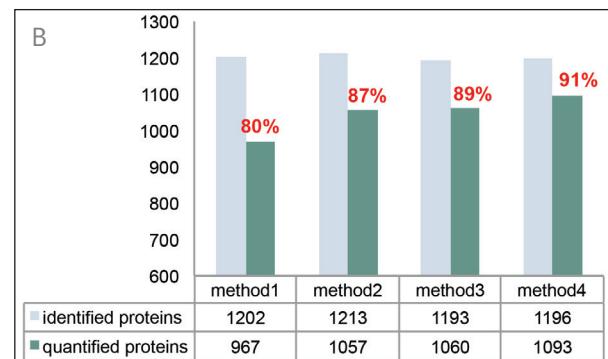
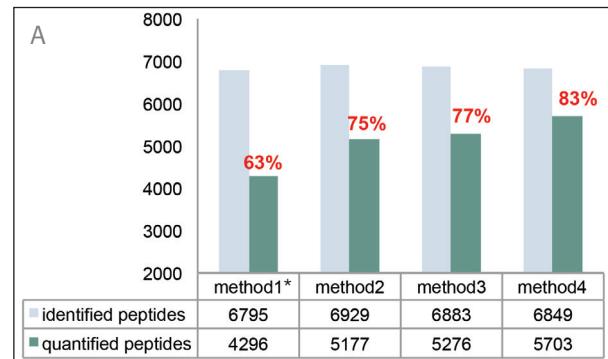


Figure 16. Number of identified and quantified peptide and protein groups from neat *E. coli* sample, when using different instrument settings. *The detailed method settings can be found in Table 5.

Table 5. Detailed parameters for each method compared in Figure 16 in the study.

	MS2 IT	MS2 AGC	MS2 NCE
Method 1	64 ms	5e4	30
Method 2	100 ms	5e4	30
Method 3	100 ms	1e5	30
Method 4	100 ms	1e5	32

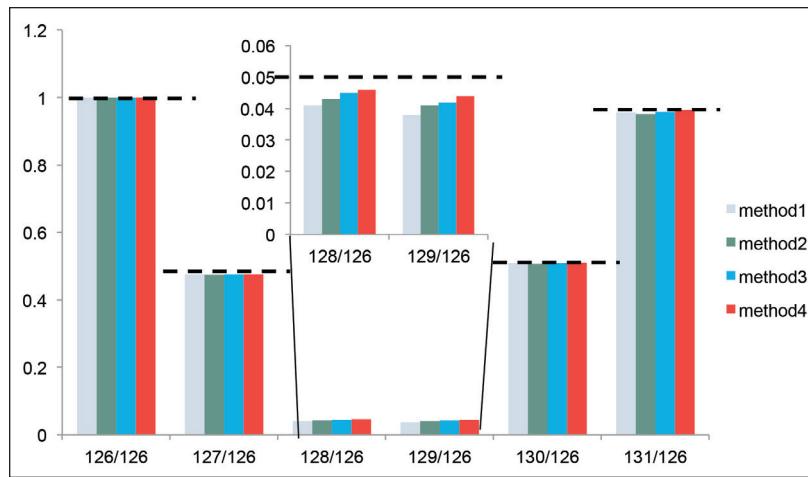


Figure 17. Ratios for TMT-labeled neat *E. coli*. The expected ratio is marked in dashed lines. Note that for the 128/126 and 129/126 ratios, the method 4 provides the best quantification accuracy.

MS2 AGC Target

AGC target value is another key parameter that determines the total number of charges allowed to be accumulated in the C trap and sent to the Orbitrap analyzer for detection. While more ions may improve S/N and increase identification rate, an inappropriately high AGC target value can cause coalescence and other space-charge related effects, resulting in decreased mass accuracy.¹³ We compared the results obtained with AGC target value settings 5e4 and 1e5 for a 1 µg sample injection in Figures 16 and 17. The setting of 1e5 generated a higher number of quantifiable peptides and resulted in an overall better quantitative accuracy and precision without causing coalescence.

MS2 Collision Energy

NCE is a dimensionless number approximately equivalent to the collision energy (in eV) for a reference ion of mass 500 and charge 1. The actual energy to be applied is automatically calculated on the fly based on the mass and charge of the selected precursor ion. The commonly-used NCE setting (e.g., NCE 27–28) is sufficient to generate fragments for peptide identification, but it proves inadequate for dissociating the TMT reporter ions to ensure accurate quantification. Applying too much collision energy on the other hand leads to over-fragmentation of the peptide itself, resulting in a reduced identification rate.¹⁴ We found that increasing the collision energy from NCE 30 to NCE 32 significantly increased the number of quantifiable proteins without negatively impacting the identification rate (Figure 16). Moreover, using higher collision energy improved the quantification accuracy (Figure 17). We suggest using 4–6 NCE higher for TMT-labeled samples than for unlabeled peptides.

MS2 Isolation Width

An isolation width of 2 Th is usually recommended for Q Exactive-type instruments, ensuring sufficient ion transmission and sensitivity. However, a wider isolation width allows the interference ions to be co-isolated with the precursor ions, reducing reporter ion quantification accuracy.

Fragments from interfering ions can also potentially complicate the MS2 spectra and lead to lowered identification scores. The Q Exactive Plus MS and Q Exactive HF MS are equipped with AQT, which allows an efficient ion transmission even at very narrow isolation width settings. We analyzed neat *E. coli* samples with 0.7 and 1.2 Th isolation width settings and obtained practically similar results (Figure 18).

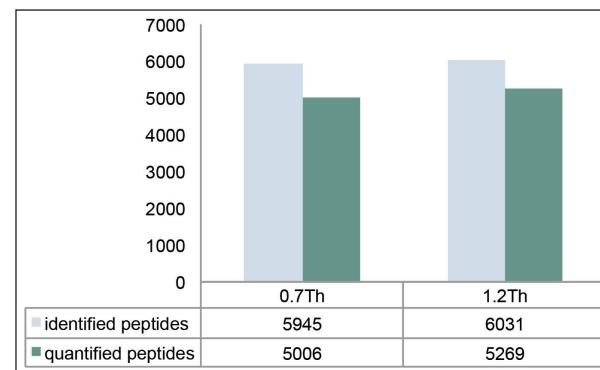


Figure 18. Numbers of identified and quantified peptide groups for different isolation width settings. The reported numbers are the combined results for two replicates for a neat *E. coli* sample.

To evaluate the appropriate isolation width for complex samples, neat *E. coli* sample (127C:128N:128C:129N:129C:130N mixed in the ratio 20:10:1:1:10:20) was mixed with the same amount of HeLa digest (channels 127N, 127C, and 128N mixed in 1:1:1 ratio) as background. The results showed for *E. coli* channels 127C and 128N, which were interfered by HeLa digest, displayed significantly distorted ratios compared to the theoretical values and other interference-free channels (Figure 19). A smaller isolation width (0.7 Th) provides slightly better quantitative accuracy compared to a wider one (1.2 Th) by reducing co-isolation interference. For samples with high complexity, we recommend the use of the SPS MS3 method available on the Orbitrap Fusion MS and Orbitrap Fusion Lumos MS instruments to achieve accurate quantification with high dynamic range. Alternatively, pre-fractionation (e.g., Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit, P/N 84868) could be employed to reduce the complexity of the sample.

Potentially, an even smaller isolation width (0.4 Th) could be used on the Q Exactive Plus MS or Q Exactive HF MS to gain quantification accuracy, but with a compromised sensitivity.

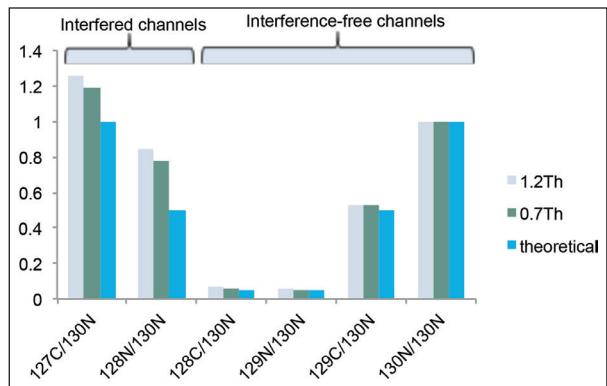


Figure 19. Quantification accuracy of different isolation windows as represented by median ratios of quantifiable peptide groups.

In summary, to perform a TMT experiment on any Q Exactive series mass spectrometer, all the parameters should be carefully chosen based on the nature of samples. High sensitivity and quantification accuracy can be achieved with:

- 1) Longer gradients, longer columns, and higher sample loads.
- 2) Correct MS2 resolution settings for different TMT tags.
- 3) Maximum injection time to match to resolving power for most optimal duty cycle.
- 4) Increased AGC MS2 targets based on number of tags and up to 1e5 for TMT10plex.
- 5) Higher normalized collision energy settings without over-fragmenting the peptides.
- 6) Narrower precursor isolation width of the quadrupole compared to the traditional method optimized for shotgun sequencing application.

Conclusion

TMT labeling increases sample throughput and enables relative quantification of up to ten different samples derived from cells, tissues, or biological fluids. The performances of the Q Exactive Plus MS and the Q Exactive HF MS instruments were evaluated with respect to protein/peptide identification rate and quantification accuracy using a TMTsixplex and TMT10plex labeled *E. coli* cell lysate sample. The Q Exactive Plus MS and Q Exactive HF MS are well suited for the analysis of low and medium complexity samples. A pre-fractionation is recommended, especially for high complexity samples to improve quantification accuracy and precision. Several different parameter settings were compared and optimized, including MS2 resolving power, injection time, AGC target, and isolation width, to achieve the highest rate of identification and the most accurate quantification. Best practices for sample preparation and liquid chromatography settings have been addressed in detail, as well as the aspects of data processing with Proteome Discoverer 2.1 software.

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