

# TMT data acquisition on the LTQ-Orbitrap XL Mass Spectrometer

TR0070.0

### Introduction

Successful acquisition of quantitative tandem mass tag (TMT\*) data with the Thermo Scientific LTQ-Orbitrap XL Mass Spectrometer requires several considerations. As many peptides as possible should be analyzed, and reporter ions (found in the low mass region of MS/MS spectra) must be acquired with sufficient intensities for good quantitative analysis. Careful adjustment of instrument settings and acquisition parameters must be made to obtain these data. These settings are detailed in this document.

## Preparing for the experiment

#### A. Materials Required

- Binding buffer (0.1 to 1.0% TFA in water)
- Formic Acid for the mobile phase
- Bottled HPLC water
- Acetonitrile
- Capillary LC column ( $75\mu$ m × 20cm C18 column is recommended)
- Liquid Chromatograph Instrument capable of flow rates down to 250nL/min.

#### **B.** Procedure

- 1. Prepare fresh mobile phases.
- 2. Use a fresh column that has been well equilibrated. The column length should be at least 20cm.
- 3. Calibrate the mass spectrometer. It is especially important to calibrate the positive ion electron multipliers. Doing so will ensure that the instrument is operating with maximal sensitivity.
- 4. Dilute all samples with binding buffer. Target loading amounts to be in the 0.25 to 1.0µg range.

## Preparing the LC method

Proper setup of the LC method is critical to the success of the experiment. While LC methods vary widely, several common points have emerged:

- 1. Sample complexity determines the choice of gradient. Simple samples (e.g., from immunoprecipitation or enrichment experiments) require short 1-hour gradients (usually 4 to 40% B (acetonitrile/0.1% formic acid) while more complex samples require much longer gradients (up to 3 hours; 4 to 40% B over 3 hours).
- 2. Flow rates should be set to 250 to 600nL per minute.
- 3. Trap columns should be avoided as they tend to lose sample.
- 4. Always run a labeled positive control sample before analysis of the test sample. Be sure that the chromatographic peaks are less than 30 seconds wide (on average). If the peak widths are broad, change the LC gradient until your peaks sharpen.
- 5. Also, the more pre-fractionation done before hand (e.g., SCX fractionation), the more likely the experiment will be successful. This will help to ensure the under-sampling is minimized.



## Preparing a suitable Tune File

Proper setup of the tune file is essential for a successful experiment. All tune file parameters are entered in the Tune window.

1. First adjust the Scan Time Settings (Setup  $\rightarrow$  Injection Control).

Ion Trap	P   FT	OK		
				Cancel
	Scan Type	Microscans	Max. Inject Time (ms)	Apply
	Full MS	1	50.000	Reset
	SIM	1	25.000	
	MSn	3	150.000	Help
	Zoom	1	100.000	

**Figure 1.** Scan Time Settings for the Ion Trap. While the quantitation is done in the HCD cell and mass measurements are done in the Orbitrap, MS/MS spectra (CID) will be measured in the Ion-Trap.

Ion Trap	FT			OK
	Scan	Microscans	Max. Inject	Cancel
	Туре	North Control	Time (ms)	Apply
	Full MS	1	500.000	
	SIM	3	100.000	Reset
	MSn	1	300.000	Help

Figure 2. Scan Time Settings for the Orbitrap.

2. Next, enter the appropriate target values for both the Ion trap and the Orbitrap (FT) (Figures 3, 4).

	Cancel
3.00e+04	Apply
1.00e+04	Beset
1.00e+04	
3000	Help
	3.00e+04 1.00e+04 1.00e+04 3000

Figure 3.



on Trap FT	Reagent	OK
AGC Target Setti	Cance	
		Apply
Full M	S 5.00e+05	Devel
SI	M 1.00e+05	Heset
MS	n 1.00e+05	Help
✓ Enable Full Sca	an Injection Waveforms	

Figure 4.

- 3. Note that some of the parameters are adjustable, especially the MSn parameters. For example, a scan time of 300ms for the FT-MSn is generally adequate. However, if the sample amount is low (less than 250ng on column), then increasing the scan time by 50 to 100ms is advisable.
- 4. Save the tune file since you will need to point to this exact file in the acquisition method.
- 5. Calibration prior to analysis is essential, especially the electron multipliers, in order to obtain maximal sensitivity. Failure to calibrate will lead to large CV's of technical replicates. If you are unfamiliar with the calibration process, consult the instrument documentation. Successful calibration will result in a green check mark next to the parameter in question (Figure 5).

Calibrate			
Mass Ra	nge: 💽 N	lormal 🔿 High (I	lon Trap)
Automatic Semi-Automatic Check FT Manu	Jal		
What to Calibrate	Besult	Last Cal. Date	~
	Troodic	2000000.0000	
			=
	2	12/15/2009	
- Main BE Frequency	-	12/15/2009	
- Positive Ions Electron Multiplier	1	5/17/2010	
- Negative Ions Electron Multipli		5/10/2010	
- Mass Calibration		0/10/2010	
- Normal Scan Bate Tunes	-	5/10/2010	
- Enhanced Scan Bate Tunes	-	12/15/2009	
- Zoom Scan Bate Types	-	12/15/2009	
	_	4/0/2010	×
			>
- Status			
13:07:18: (diff: -0.160205) with FWHM: 1.6	17391		^
13:07:21: m/z 1522.340700 found at m/z 15 13:07:21: (diff: -0.122072) with FWHM: 1.6	22.21862	в	
13:07:24: m/z 1822.388610 found at m/z 18	22.85656	7	
13:07:24: (diff: 0.467957) with FWHM: 1.94	5770		
13:07:26: AGC Prescan Calibration Check P/ 13:07:26:	ASSED		
13:07:26: Calibration is OK!			
			-
<u></u>			
-			
Set Instrument to Standby when Finished			
Start Cancel		Print I	Help

Figure 5.



## Constructing an acquisition method

The following method has been developed to maximize protein identification with quantitation. The method is a top  $3 \times 2$ , which consists of three HCD events followed by 3 CID events on the same ions selected for the first 3 HCD events. Use the following procedure to systematically set up the method.

- 1. Go to the Xcalibur software program and click on the Instrument Setup Icon (Figure 6).
- 2. Right click on the mouse button and select Go to.
- 3. A new page will appear (Figure 7).



Figure 6.



Figure 7.



- 4. Set up the LC method according to the manufacturer's instructions.
- 5. Select the MS/MS instrument icon and then click on the icon called Data dependent MS/MS.
- 6. A new screen will appear. Enter the parameters as they appear in Figure 8 for Scan event 1. Select the Tune method that was saved in the last section and be sure to enter an Acquire Time that is the same length as the LC gradient (can be shorter, but not longer). In this method we are implementing seven scan events. Scan event 1 is the precursor selection step, scan events 2-4 are the HCD steps and 5-7 are the CID steps. In this example, we have set the resolution to 15000. However, higher resolutions in scan event 1 are recommended for highly complex samples so that a maximal number of peptides can be selected.

lun settings							
Acquire time (min): 1	50.00		<u>S</u> egme	nts: 1			Start delay (min): 0.00
	To display	a chromatogram he	re, use LTQ (	)rbitrap XL/Open	raw file		
<			Segment	1			> 1
0 10 20	30 40	50 60	70	90 90	100 1	10 120	130 140 150
			Retention	time (min)			
egment 1 settings		-					
egment time (min): 15	0.00 S	can <u>e</u> vents:  7	- Tu	ne method: C:V	Xcalibur\T	une\MMR\20	110\TuningForTMT.LT
Scan Event 1	Scan Event 2	Scan Event 3	Scan Even	t 4 Scan Ev	ent 5	Scan Event 6	Scan Event 7 > 1
can event 1 settings							
Scan Description		MSn Settings					Scan Ranges
Analyzer: FT	MS 💌	Parant	Act	o. Normalized	to A	Act.	First Mass
Mass Range: No	ormal 💌	n Mass (m/z)	Type (n	lath Collision	Q.	Time (ms)	# (m/z) (m/z)
Besolution: 15	000 -	2	CID 1	0 35.0	0.250	30.000	1 380.00 1800.00
Scan Type: Fu							
Polarity: Po	sitive 👻						
Data type: Pro	ofile 💌						
Source Fragmentation -							
□ Dn Energy (V)	35.0 -						
🗖 Dependent scan	Settings						
		☐ <u>W</u> ideband Act	ivation	🗖 Supp	lemental Ad	tivation	
FAIMS		HCD Charge State	1 -	SA Charg	e State:	2	Input: From/To 💌
C <u>V</u> (V)	0.00	Use MS/MS M	lass List 🔽	APCI Corona O	n 🔽 AF	PI Lamp On	<b>B</b>
			1	-		1	
		New method	Tun	e Pius	Help		

Figure 8.

7. Click on Scan Event 2 and enter the parameters as shown in Figure 9.



Acquire time (min): 150.00	Segments: 1	Start delay (min): 0.00
To displa	y a chromatogram here, use LTQ Orbitrap XL/Open raw file	
	Segment 1	> 1
0 10 20 30 4	50 60 70 80 90 100 110 120 Retention time (min)	130 140 150
egment 1 settings egment time (min): 150.00 Scan Event 1 Scan Event 2	ican events: 7 Tune method: C:\\Calibur\Tune\MMR\201	0\TuningForTMT.LT
an event 2 settings		
Ccan Description Analyzer: FTMS ▼ Mass Range: Normal ▼ Resolution: 7500 ▼	MSn Settings n Parent Act. Iso. Normalized Act. Act. Time Mass (m/z) Type (m/z) Energy Q (ms)	Scan Ranges           #           First Mass (m/z)           Last Mass (m/z)
Scan Iype: Full 👻 Polarity: Positive 👻 Data type: Centroid 💌		
Gource Fragmentation           Image: Display the second s		
Dependent scan Settings		
AIMS		Input: From/To
C⊻ (M):  0.00 ≟	Use MS/MS Mass List 🔽 APCI Corona On 🔽 APPI Lamp On	<b>Pb</b>

Figure 9.

8. Continue to enter the parameters for the other scan events described here.

Table 1. Settings for Scan Events 3 to 7. All other settings are the same as for Scan Event 2 (Figure 9					
Scan Events 3 & 4 (same as 2)		Scan Events 5 to 7			
Analyzer	FMTS	Analyzer	Ion Trap		
Mass Range	Normal	Mass Range	Normal		
Resolution	7500	Scan Rate	Normal		
Scan Type	(off)	Scan Type	(off)		
Polarity	(off)	Polarity	(off)		
Data Type	Centroid	Data Type	Centroid		
Dependent Scan	Checked (on)	Dependent Scan	Checked (on)		



9. Click on Scan Event 2. Select the Dependent Scan setting box and then click on the Settings tab. A box will appear (Figure 10).

Global     Global     Global     Scan Widths     Dynamic Exclusion     Mass Tags	Exclusion mass width     By mass     Low: 10.0     High: 10.0
Analog     Analog     Neutral Loss     Product     Segment	Parent mass width ○ By mass ● Relative to mass (ppm) Conversion Low: 10.00 → High: 10.00 → High: 10.00 →
Current Segment Chromatography Parent Mass List Reject Mass List Charge State Neutral Loss	Reject mass width         ○ By mass         ③ Relative to mass (ppm)    Low: 10.00
Product Mass List	OK Cancel Help

10. Enter the parameters as shown in Figures 11 to 12.

Global     Mass Widths     Scan Widths     Dynamic Exclusion     Mass Tags     Isotopic Data Depenc     Analog     Neutral Loss     Product     Current Segment     Chromatography     Parent Mass List     Reject Mass List     Charge State     Neutral Loss	Repeat count: 2 Repeat duration (s): 30.0 Exclusion list size: 500 Exclusion duration (s): 20.0 Exclusion mass width C By mass  Relative to reference mass (ppm) Low: 20.0 Early expiration Early expiration Enabled S/N threshold 20
Product Mass List	

Figure 11.



Mass Widths Scan Widths Dynamic Exclusion Mass Tags Isotopic Data Depenc Analog Neutral Loss Product Segment Current Segment Chromatography Parent Mass List Reject Mass List Charge State Neutral Loss Product Mass List	<ul> <li>Most intense if no Parent Masses found</li> <li>Exclude parent mass from MSn selection</li> <li>Use separate positive and negative polarity mass lists</li> <li>Enable preview mode for FTMS master scans</li> <li>Additional microscans event: 0</li> </ul>
---	---

Figure 12.

11. Figure 13 (Reject Mass List), is not required. But, if you have some background ions that you would prefer to ignore, it is recommended to include this step.

Global A	T Use Reject i	e global mass list masses:	\$
- Scan Widths	#	MS m/z	Name
- Dynamic Exclusion	1	389.11	
- Mass Tags	2	389.11	
Isotopic Data Depenc	3	389.11	
Analog	4	391.28	
Neutral Loss	5	391.28	
Product	6	391.28	
Segment	7	391.28	
- Current Segment	8	391.29	
- Chromatography	9	391.29	
- Parent Mass List	10	392.29	
Reject Mass List	11	392.29	
Charge State	12	392.29	10
Neutral Loss	12	303.30	
- Product Mass List 🛛 💌		Impo	rt
			K Cancel Help

12. Set up the charge state screening parameter as shown in Figure 14. The use of monoisotopic precursor selection and non-peptide monoisotopic recognition is optional.



Product     Segment     Current Segment     Chromatography     Parent Mass List     Reject Mass List     Charge State     Neutral Loss     Product Mass List     Add/Sub     Scan Event     Current Scan Event     Activation     FT HCD     FT ETD     Base Peak Ejection     Mass Tags     V	<ul> <li>Enable charge state screening</li> <li>Enable monoisotopic precursor selection</li> <li>Use non-peptide monoisotopic recognition</li> <li>Enable charge state dependent ETD time</li> </ul> Charge state rejection Enabled Reject charge states: <ul> <li>I</li> <li>I</li></ul>

Figure 14.

13. While still in the Data Dependent Settings box, click on the Current Scan Event tab. Enter the values as shown in Figures 15 to 17. These values apply to Scan event 2.

- Lurent Scan Event	Multistage activation
Activation FT HCD FT ETD Base Peak Ejection Mass Tags	
	Use procedure Procedures

Figure 15.



- Neutral Loss			
Product			
Segment			
- Current Segment	Activation type:	HCD	-
- Chromatography		2	- 1
- Parent Mass List	Default charge state:	12	-
- Reject Mass List	Isolation width (m/z);	3.0	
<ul> <li>Charge State</li> </ul>		1000	
- Neutral Loss	Normalized collision energy:	50.0	
- Product Mass List	Activation G	0.250	-
- Add/Sub			
Scan Event	Activation time (ms):	30.000	-
Achuation			
ET HCD			
- FT FTD	C Supplement	stal Activa	Hole
Base Peak Election	<ul> <li>pupplemental Activation</li> </ul>		
Mass Tags			



<ul> <li>Neutral Loss</li> <li>Product</li> </ul>	<u> </u>	
<ul> <li>Product</li> <li>Segment</li> <li>Current Segment</li> <li>Chromatography</li> <li>Parent Mass List</li> <li>Reject Mass List</li> <li>Charge State</li> <li>Neutral Loss</li> <li>Product Mass List</li> <li>Add/Sub</li> </ul>		First mass         Image: Fixed at m/z:         Image: Fixed at m/z:
- Scan Event - Current Scan Event - Activation - FT HCD - FT ETD - Base Peak Ejection - Mass Tags		
		OK Cancel Help

14. For Scan events 3 and 4, you will want to keep the same Activation and FT HCD values. Apply the values shown in Figure 18 for Scan event 3 and Figure 19 for Scan event 4.



- Neutral Loss	
Product	Minimum signal threshold (counts): 50000.0
E Segment	
Current Segment	Mass determined from scan evenc
Chromatography	
Parent Mass List	Same MS order as referenced scan event
- Reject Mass List	• Nth most intense ion
- Charge State	C Mits must interest from East 2
- Neutral Loss	<ul> <li>With most intense from list</li> </ul>
- Product Mass List	
- Add/Sub	
Scan Event	
Current Scan Event	I Multistage activation
Activation	
- FT HCD	
FIETD	
Base Peak Ejection	
- Mass Lags	
	Use procedure Procedures
	OK Cawaal Hala



- Neutral Loss 🔥	1
- Product	Minimum signal threshold (counts): 50000.0
Segment	Mass determined from some quent
- Current Segment	Mass determined nom scan event.
<ul> <li>Chromatography</li> </ul>	
- Parent Mass List	I Same MS order as referenced scan event
<ul> <li>Reject Mass List</li> </ul>	Nth most intense ion 3 ÷
<ul> <li>Charge State</li> </ul>	
- Neutral Loss	C Nth most intense from list
<ul> <li>Product Mass List</li> </ul>	
Add/Sub	
🖃 Scan Event	
- Current Scan Event	Multistage activation
Activation	
- FT HCD	
- FT ETD	
Base Peak Ejection	
Mass Tags	
	Use procedure     Procedures

Figure 19.

15. Figures 20 to 22 apply to Scan Events 5, 6 and 7. These are the CID steps in the duty cycle.



Neutral Loss     Product Mass List     Add/Sub     Scan Event     Current Scan Event     Activation     Base Peak Ejection	Multistage activation
Mass Tags	



<ul> <li>The set of the test set of the set of the</li></ul>	
- Analog	Minimum signal threshold (counts): 10000.0
- Neutral Loss	
- Product	Mass determined from scan event:
Segment	
- Current Segment	Same MS order as referenced scan event
<ul> <li>Chromatography</li> </ul>	• Nth most intense ion 2
- Parent Mass List	C 101 - 11 - 1 - 12 - 11
- Reject Mass List	Nth most intense from list
- Charge State	
- Neutral Loss	
<ul> <li>Product Mass List</li> </ul>	
- Add/Sub	Multistage activation
Scan Event	
<ul> <li>Current Scan Event</li> </ul>	
- Activation	
Base Peak Ejection	
— Mass Tags 🛛 🗹	
	Use procedure Procedures

Figure 21.



- Isotopic Data Depenc 🔨	
- Analog	Minimum signal threshold (counts): 10000.0
- Neutral Loss	
- Product	Mass determined from scan event: 1
Segment	
- Current Segment	Same MS order as referenced scan event
- Chromatography	Nth most intense ion     3
- Parent Mass List	
- Reject Mass List	C Nth most intense from list
- Charge State	
- Neutral Loss	
- Product Mass List	
Add/Sub	Multistage activation
Scan Event	
- Current Scan Event	
- Activation	
- Base Peak Ejection	
Mass Tags 🛛 🖌	
	Use procedure Procedures

Figure 22.

16. For each of the scan events 5 to 7, the activation tab will require the values shown in Figure 23.

- Isotopic Data Depenc 🔨		
Analog		
- Neutral Loss		
- Product	Activation type: CID	*
B Segment		
- Current Segment	Default charge state: 6	-
- Chromatography	lealation width (m/a) 30	
- Parent Mass List		
- Reject Mass List	Normalized collision energy: 35.0	늰
- Charge State	0.050	
- Neutral Loss	Activation Q: U.250	-
- Product Mass List	Activation time (ms) 30.000	
Add/Sub	Mentagon and first Terrere	
∃-Scan Event		
- Current Scan Event		
Activation	Supplemental Activ.	ation
Base Peak Ejection		
Mass Tags 🛛 🖌		

Figure 23.

17. Enable the stepped collion energy feature. Go to the LTQ Orbitrap XL → Stepped Collision energy tab. Check the Enabled box and enter a Collision Energy Width of 10 % and for Number of steps a value of 2.

Stepped Normalized Collision E	nergy 🔀
✓ Enabled	
Collision energy width (%): Number of steps:	2
OK Cancel	Help

Figure 24.



18. Once the method setup is complete, click on the Summary tab, located at the top right portion of the screen. The summary of the method you have just created will be similar to that which is shown in Figures 25 and 26.

```
MS Detector Setup | Mass Lists | Syringe Pump | Divert Valve | Contact Closure | Summary
  Method summary.
   Additional Microscans:
                MS2
                                                 0
                                0
                MS3
                                0
                                                 0
                 MS4
                                                 Ō
                                0
                MS5
                                0
                                                 n
                MS6
                                0
                                                 0
                                0
                                                 Û
                 MS7
                 MS8
                                0
                                                 0
                 MS9
                                0
                                                 0
                MS10
                                0
                                                 0
   Segment 1 Information
                                               150.00
   Duration (min):
Number of Scan Events:
Tune Method:
                                               TuningForTMT
   Scan Event Details:
    1: FTMS + p norm res=15000 o(380.0-1800.0)
CV = 0.0V
     2:
            FTMS + c norm res=7500 Dep MS/MS Most intense ion from (1)
                Activation Type:
Min. Signal Required:
Isolation Width:
Normalized Coll. Energy:
Default Charge State:
Activation Time:
                                                                HCD
50000.0
                                                                 3.00
                                                                50.0
                                                                30.000
                Activation Time:
                FT first mass mode: fixed at m/z
FT first mass value: 100.00
CV = 0.0V
    CV = 0.0V

3: FTMS + c norm res=7500 Dep MS/MS 2nd most intense ion from (1)

Activation Type: HCD

Min. Signal Required: 50000.0

Isolation Width: 3.00

Normalized Coll. Energy: 50.0

Default Charge State: 3

Activation Time: 30.000

ET first proceeder first at proceeder
    FT first mass mode: fixed at m/z
FT first mass value: 100.00
CV = 0.0V
4: FTMS + c norm res=7500 Dep MS/MS 3rd most intense ion from (1)
                Activation Type: HCD
Min. Signal Required: 5000
Isolation Width: 3.00
Normalized Coll. Energy: 50.0
                                                                 50000.0
                 Default Charge State:
                                                                30.000
                 Activation Time:
                FT first mass mode: fixed at m/z
FT first mass value: 100.00
CV = 0.0V
```

Figure 25.



```
MS Detector Setup Mass Lists Syringe Pump Divert Valve Contact Closure Summary
  Method summary:
             Isolation Width: 3.00
Normalized Coll. Energy: 50.0
             Default Charge State:
                                                    3
             Activation Time:
                                                    30.000
             FT first mass mode: fixed at m/z
FT first mass value: 100.00
CV = 0.0V
    5: ITMS + c norm Dep MS/MS Most intense ion from (1)
             Activation Type:
Min. Signal Required:
Isolation Width:
Normalized Coll. Energy
                                                    CID
                                                    10000.0
                                                    3.00
                                       Energy:
                                                    35.0
             Default Charge State:
                                                    6
                                                    0.250
             Activation Q
   Activation Time: 30.000
CV = 0.0V
6: ITMS + c norm Dep MS/MS 2nd most intense ion from (1)
             Activation Type:
Min. Signal Required:
Isolation Width:
Normalized Coll. Energy:
                                                  CID
                                                    10000.0
                                                    3 00
                                                    35.0
             Default Charge State:
                                                    6
                                                    0.250
             Activation Q
   Activation Time: 30.000
CV = 0.0V
7: ITMS + c norm Dep MS/MS 3rd most intense ion from (1)
             Activation Type:
Min. Signal Required:
Isolation Width:
Normalized Coll. Energy
                                                  CID
                                                    10000.0
                                                    3.00
35.0
                                       Energy:
             Default Charge State:
                                                    6
             Activation Q
                                                    0.250
             Activation Time:
CV = 0.0V
                                                    30.000
  Lock Masses:
                                                          N/A
API Source
       Pos List Name:
                  Source
       Mass List:
Neg List Name:
                                                          (none)
N⁄A
                                                          API Source
                Source
             Mass List:
                                                          (none)
  Data Dependent Settings:
             Parent Mass List: (none)
                                                          (none)

389.11 389.11 389.11 391.28 391.28

391.28 391.28 391.29 391.29 392.29

392.29 392.29 392.29 393.08 413.26

413.27 413.27 419.31 419.31 419.32
             Reject Mass List:
```

Figure 26.

\*TMT is a registered trademark of Proteome Sciences plc.

Current versions of product instructions are available at www.thermoscientific.com/pierce.. For a faxed copy, call 800-874-3723 or your local distributor.

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