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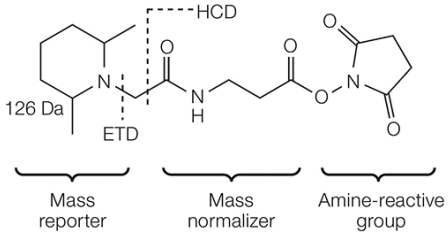
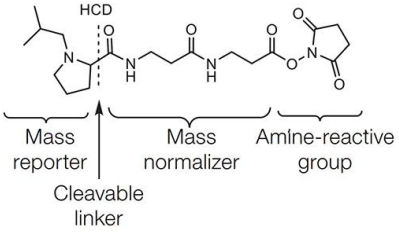
TMT and TMTpro Mass Tag Labelling

Protein extracts isolated from cells or tissues are reduced, alkylated, and digested. Samples are labeled with TMT or TMTpro Reagents and then mixed before sample fractionation and clean-up. Labeled samples are analyzed by high resolution Orbitrap LC-MS/MS before data analysis to identify peptides and quantify reporter ion relative abundances.

TMT reagents: TMT 6plex to 11 plex	*TMTpro reagents: TMT 16 plex and 18 plex*
	
Mass tag: 229.162932	*Mass tag: 304.207146*

TMTpro reagents are ~20% heavier than TMT reagents, so a ratio range of 1:5-1:10, sample to tag, w:w is recommended compared to standard TMT reagent labeling ratio range of 1:4-1:8, w:w.

Note: The TMT and TMTpro reagents are highly moisture-sensitive. To avoid moisture condensation onto the product, the reagents must be equilibrated to room temperature before opening.

Peptide Labeling of 20-50 µg digested proteins

Note: All samples must be digested, desalted and lyophilized prior to labeling.

Sample dissolution

- Add 20-50 µl HEPES Buffer *(see right panel)* to the lyophilized samples.

Note! Peptide concentration, use between 0.5 and 2 µg peptides/µl.

- Vortex 30s at 1500 rpm) following 30s in a ultrasonication bath.
- Centrifuge at 13000 rpm for 1 min, measure peptide conc. on Nanodrop, and normalize the amounts for all samples.
- Keep at 4 deg prior to TMT labeling.

100ml 100 mM HEPES buffer pH 8.5

Dissolve **2.3831g HEPES** *(2-[4-(2 hydroxyethyl)piperazin-1-yl] ethanesulfonic acid, MW=238.31)* in 90 ml water. Adjust pH to 8.5 with 6M NaOH solution. Adjust volume to 100 ml with water.

TMT and TMT pro reagent preparation

- Immediately before use, equilibrate the TMT/TMTpro Label Reagents to room temperature in the foil pouch.
- Dissolve each TMT label in anhydrous acetonitrile (ACN).
- Vortex, and place in ultrasonication bath for 30s every 2 minute for 10 minutes.

Vial size (mg)	TMT reagent	Molecular weight	Volume anhydrous acetonitrile (µl)	Concentration (mM)	Concentration (µg label/µl)
0.8	6,10,11 plex	342.4	41	57.0	19.5
5			256	57.0	19,5
0.5	Pro (16,18 plex)	419.4	20	59.6	25
5			200	59.6	25

Sample labeling

- Add 5-12.5 µl TMT/TMTpro label to each sample (5x dilution, final TMT concentration above 10 mM)
- Vortex 30s at 1500 rpm, and spin, incubate for 60 min at 25 °C and 400 rpm.
- Add 5% hydroxylamine *(see right panel)* to 0.4 % final concentration (12.5x dilution) and incubate for 15min at 25 °C and 400 rpm.
- Combine samples, acidify with 10% FA in 10% ACN, and make 100 µg aliquots. Freeze at -80 °C.
- Lyophilize one aliquot, add 500 µl 0.1% TFA, an desalt using 10mg OASIS plate, freeze at -80 °C and lyophilize.
- Proceed with fractionation and LC-MSMS

5% Hydroxylamine

Add 50 µL of the 50% hydroxylamine to 450 µL of 50 mM HEPES buffer.

Combining experiments

When #samples exceed #labels, more than one experiments is needed. To be able to compare results from two or more experiments, they need to be linked. This is done by using identical reference samples, usually made from an even mix of all samples in the study, usually performed by taking a small aliquot of all samples after digestion and mix. It is recommended to use 2 reference samples per experiment, labeled with the first and last reporter tag for each experiment. It is also important to randomize samples for each experiment, and take care to have conditions evenly distributed in each experiment.

Proteomic Unit at University of Bergen

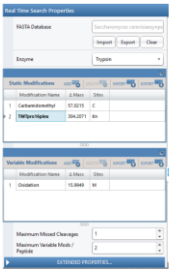
Instrument Acquisition Settings

Orbitrap Eclipse (Tune 3.3)

Fit Filter: 70+, APD on, FAIMS: CV -50/-70, 1.5 sec/CV

Properties	SPS 120 min TMT 11plex	SPS 120 min TMTpro 16 plex	MS2 120 min TMT 11plex	MS2 120 min TMTpro 16 plex
Resolution Full MS	120000	120000	120000	120000
AGC target Full MS	100%(4e5)	100%(4e5)	100%(4e5)	100%(4e5)
MS max IT, ms	50	50	50	50
Scan range, m/z	400-1400	400-1400	400-1400	400-1400
Top Speed, s	3	3	3	3
MS2 max IT, ms	50	50 (custom)	105	120
MS2 Isolation window, Th	1.2(2)-0.7(3)-0.5 (4+)	0.7	0.7(2-3)-0.5 (4+)	0.7
MS2 NCE, %	35	30	38-40	35
MS2 Intensity threshold	5e3	5e3	5e4	5e4
Dynamic exclusion, s	60, single charge	60, single charge	60, single charge	60, single charge
MS2 Resolution	turbo	rapid	50000	50000
MS2 AGC target	100%(1e4)	100%(1e4)	200%(1e5)	200%(1e5)
MS3 AGC target	200%(1e5)	200%(1e5)		
SPS Isolation window, Th	1.3(2)-0.7(3)-0.5 (4+)	0.7		
SPS NCE, %	65	55		
SPS max IT, ms	105 (custom)	120 (custom)		
SPS settings: # notches, mass range, Tag Exclusion	5-10-10 m/z 110-500, TMT	10 m/z 110-500, TMTpro	m/z 110	m/z 110

Synchronos Precursor Selection (SPS) MS3 with Real Time Search (RTS)



Orbitrap Exploris 480 (Tune 1.1)

Fit Filter: 70+, APD on, FAIMS: CV -50/-70, 1.5 sec/CV

Properties	MS2 120 min TMT 11plex	MS2 120 min TMTpro 16 plex
Resolution Full MS	120000	120000
AGC target Full MS	100%(4e5)	100%(4e5)
MS max IT, ms	50	50
Scan range, m/z	400-1400	400-1400
Top Speed, s	3	3
MS2 max IT, ms	105	120
MS2 Isolation window, Th	0.7(2-3)-0.5 (4+)	0.7
MS2 NCE, %	38-40	32
MS2 Intensity threshold	5e4	5e4
Dynamic exclusion, s	60, single charge	60, single charge
MS2 Resolution	50000	50000
MS2 AGC target	200%(1e5)	200%(1e5)
First mass	m/z 110	m/z 110
SPS Isolation window, Th		
SPS NCE, %		
SPS max IT, ms		

For 120 min LC-gradient setting and 2 CVs