Quantification of Proteins by TMT-labeling LC-MS/MS

Tandem mass tags (**TMT** or **TMTs**) are chemical labels used for mass spectrometry (MS)-based quantification and identification of biological macromolecules such as proteins, peptides and nucleic acids. TMT belongs to a family of reagents referred to as isobaric mass tags. They provide an alternative to gel- or antibody-based quantification but may also be used in combination with these and other methods.[1] There are currently four varieties of TMT available: TMTzero (TMT-0), a non-isotopically substituted core structure; TMTduplex (TMT-2), an isobaric pair of mass tags with a single isotopic substitution;[1] TMTsixplex (TMT-6), an isobaric set of six mass tags with five isotopic substitutions;[2] TMT 10-plex (TMT-10) - a set of 10 isotopic mass tags which use the TMT-6 reporter region, but use different elemental isotope (^{15}N and ^{13}C) to create a mass difference of 0.0063 Da.[3] [4]

The tags (**Figure 1**) contain four regions, namely a mass reporter region (M), a cleavable linker region (F), a mass normalization region (N) and a protein reactive group (R). The chemical structures of all the tags are identical but each contains isotopes substituted at various positions, such that the mass reporter and mass normalization regions have different molecular masses in each tag. The combined M-F-N-R regions of the tags have the same total molecular weights and structure so that during chromatographic or electrophoretic separation and in single MS mode, molecules labelled with different tags are indistinguishable. Upon fragmentation in MS/MS mode, sequence information is obtained from fragmentation of the peptide back bone and quantification data are simultaneously obtained from fragmentation of the tags, giving rise to mass reporter ions.

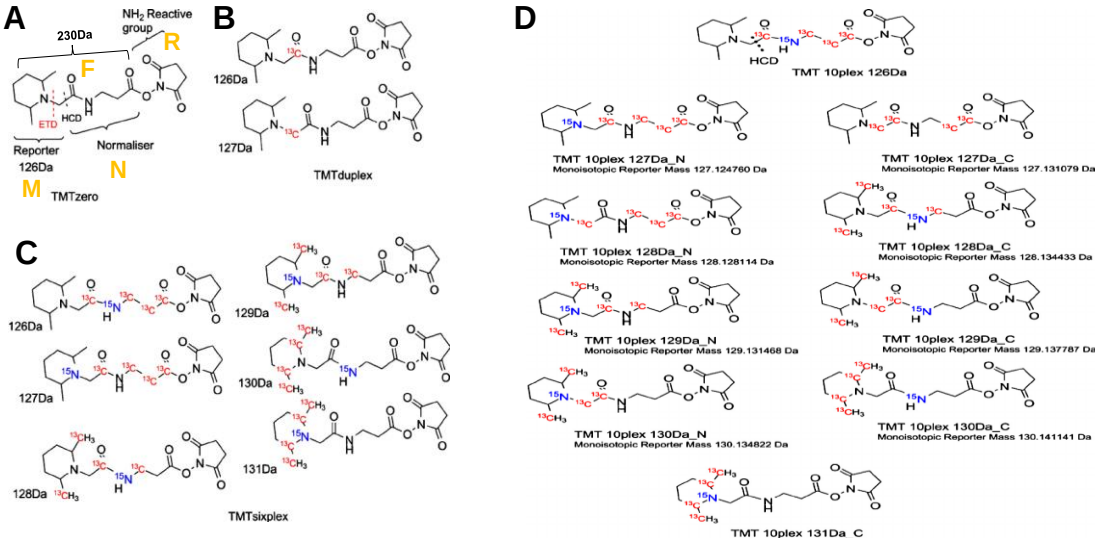

Figure 1. Chemical structures and marked stable-isotope labeled positions of TMT reagents

A. TMT-0 indicates the mass reporter region (**M**), the cleavable linker region (**F**), the mass normalization region (**N**), and the protein reactive group (**R**).
B. TMT-2
C. TMT-6
D. TMT-10

Rationale for quantification of proteins by TMT-labeling LC-MS/MS

Isobaric labeling-based quantification has many advantages such as its ability to perform high-throughput quantification due to sample multiplexing, the ability to combine and analyze several samples within one experiment without the need to compare multiple LC–MS/MS data sets, thereby reducing overall analytical time and run-to-run variation. The reason for being able to do this is that, via the isobaric labeling strategy, the mass spectrometer gives rise to the same precursor ions for any peptides (MS1) with the same amino sequence and modifications (if they have) from a mixture of multiple peptide samples subjected to LC/MS/MS analysis, regardless of the TMT tags used; however, after fragmentation, each peptide in one specific sample in the mixture produces not only the same sets of MS2 fragmentation “y” and “b” ions as the peptides with the same sequence in other samples based upon which a peptide sequence can be established and a protein accordingly identified from which that peptide is originally digested from, but also one unique reporter ion generated from a TMT chemical tag purposely used to label peptides in that sample at the beginning of sample preparation. The ratio between the two reporter ions generated by tandem mass spectrometry from the two peptide isomers with the same sequence, same MS1 ion, and same MS2 peptide fragmentation ions, but different TMT tag-linked, reflects the peptide or protein concentration ratio in the mixture of samples (**Figure 2**).

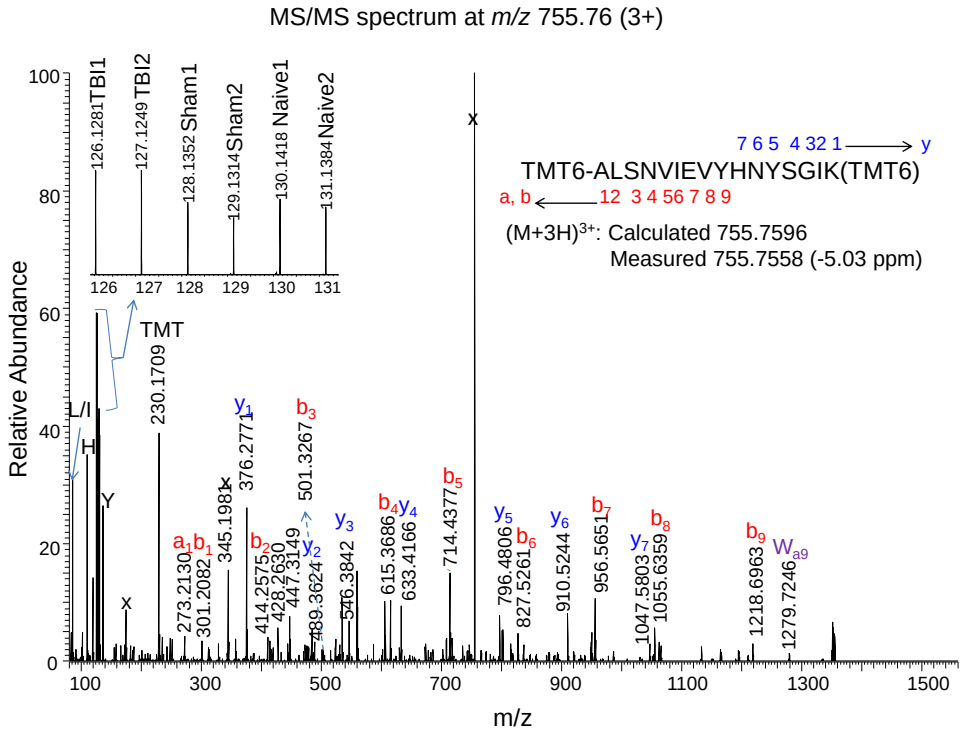

Figure 2. HCD MS/MS spectrum of peptide at m/z at *m/z* 755.76 (3+)

The spectrum corresponds to the highlighted (in red color) peptide sequence of S100A8:
(M)ATELEKALSNVIEVYHNYSGIKGNHHALYRDDFRKMVTTECPQFVQNKNTESLFK ELDVNSDNAINFEE FLVLVIRVGVAAHKDSHKE.

Trauma-Brain-Injury (TBI) samples are labeled with TMT-126 and -127, Sham samples with TMT-128, and -129, and Naïve samples with TMT-130 and -131 using TMT-6. From this analysis, protein S100A was found upregulated in rat TBI samples based on the reporter ion ratio TBI-(I_{126}+I_{127})/Sham-(I_{128}+I_{129}).

Advantages:

1. Quantification of multiple samples, for example, up to six samples (if each sample is only labeled with only one TMT tag) can be analyzed by one single LC-MS/MS analysis with multiple technical repeats. Quantification is relatively more accurate than label free because variation between separated runs of multiple samples is eliminated.
2. Other separation methods, such as cation-exchanging chromatography, can be applied to reduce sample complexity, enrich peptide concentration, in order to increase the amount of proteins for identification and quantification.

Disadvantage:

1. Extra cost of the TMT reagents.
2. Extra steps of sample preparations.

Precautions:

1. Accuracy of quantification is affected by co-eluted peptides or co-existed impurities that generate the same reporter ions.
2. Accuracy of quantification is also significantly affected by insufficient or uneven labeling of TMT and incomplete removal of un-reacted TMT reagents before mixing samples.

Useful solutions to the precautions:

1. Refer to our on-line TMT sample preparation protocol. The major attention is to make sure to have “absolutely” equal amount of proteins in each sample before labeling of samples and mixing them after labeling.
2. Increase the time of HPLC gradient or use other better chromatographic conditions (such as column size, length, and particle bead dimension) to enhance separation and decrease co-elution.
3. Use the new mass spectrometric acquisition methods, such as the MultiNotch method that pools the high-intensity MS2 CID fragmentation ions (b and y) together as the new precursor ion cluster which is dissociated again under HCD to produce new MS2 (namely MS3) ions. [5]

References

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