Biological Reference Materials for Proteomics: Multipoint Calibration of Plasma Proteins by Mass Spectrometry using Tandem Mass Tag-labeled Reference Materials

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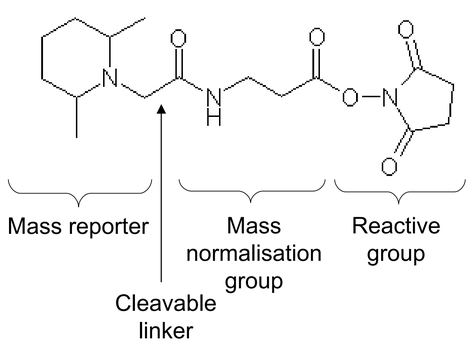
Overview

- **Combination of four different Tandem Mass Tags (TMT) to label biological samples to generate a universal reference with 4-point calibration.**
- **These reference materials improve quantification, reproducibility and comparability and allow for harmonization of proteomics studies.**
- **Relative quantification of hundreds of proteins in samples of interest is achieved by referencing against specific reporter ion calibration curves from reference material labeled with TMT reagents.**
- **TMT-labeled peptides can be used to generate a multipoint calibration mixture which is suitable for absolute quantification.**

Tandem Mass Tags

We have developed isobaric Tandem Mass Tags (TMT) [1], which can be applied for the MS/MS-based relative or absolute quantification of peptides and proteins in proteomics studies.



- Mass reporter: reports the abundance of a peptide upon MS/MS in individual samples being mixed
- Cleavable linker: enables the release of the TMT fragment from the whole tag upon MS/MS
- Mass normalisation group: balances mass differences from individual TMT fragments to ensure the same overall mass of the label reagents from a set
- Reactive group: allows for the labeling of amino groups

Figure 1: Design of the TMT label reagent

By appropriate incorporation of ^{13}C and ^{15}N isotopes, different sets of TMT-reagents can be designed. We have developed TMT-Duplex (TMT^2-126 + TMT^2-127) and TMT-Sixplex (TMT^6-126 to TMT^6-131) sets for clinical and preclinical applications.

Use of reference materials and 4-point calibration

Lack of precision and reproducibility of workflows has a negative impact on proteomics. A key development to overcome these limitations was the introduction of isobaric Tandem Mass Tags which allow the specific labeling of proteins and peptides from a sample via tagging of amino functions. We have recently extended this concept by developing isobarically labeled reference materials for standardisation of proteomics studies.

Under this approach an identical amount of one TMT-labeled biological reference is added to each individual sample allowing relative quantification of a large number of proteins across all study samples. Since the biological reference can be used by multiple laboratories, cross-study and cross-laboratory comparisons are now possible.

To extend this concept, we here present a reference material which delivers 4-point calibration curves for all proteins contained in the reference material. Human plasma was labeled with TMT^6-128 to TMT^6-131 according to figure 2. Four different quantities of the labeled materials were combined to generate a calibration mixture (1:2:4:8).

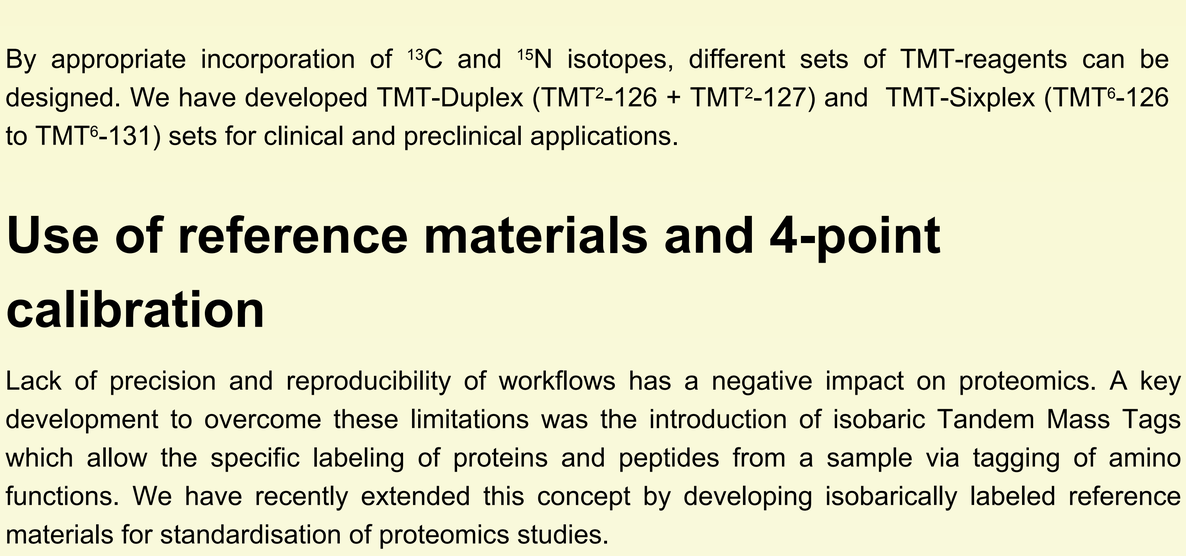


Figure 2: Workflow to generate a 4-point calibration mix of a reference sample

Subsequently, combined samples were separated by strong cation exchange chromatography and fractions were analysed by LC-MS/MS on an ESI-QTOF2 or MALDI-TOFTOF instrument. Peptide and protein identification was performed with SEQUEST. For each identified peptide, the reporter signals in MS/MS spectra at m/z = 128-131 were evaluated to assess their quantitative behaviour.

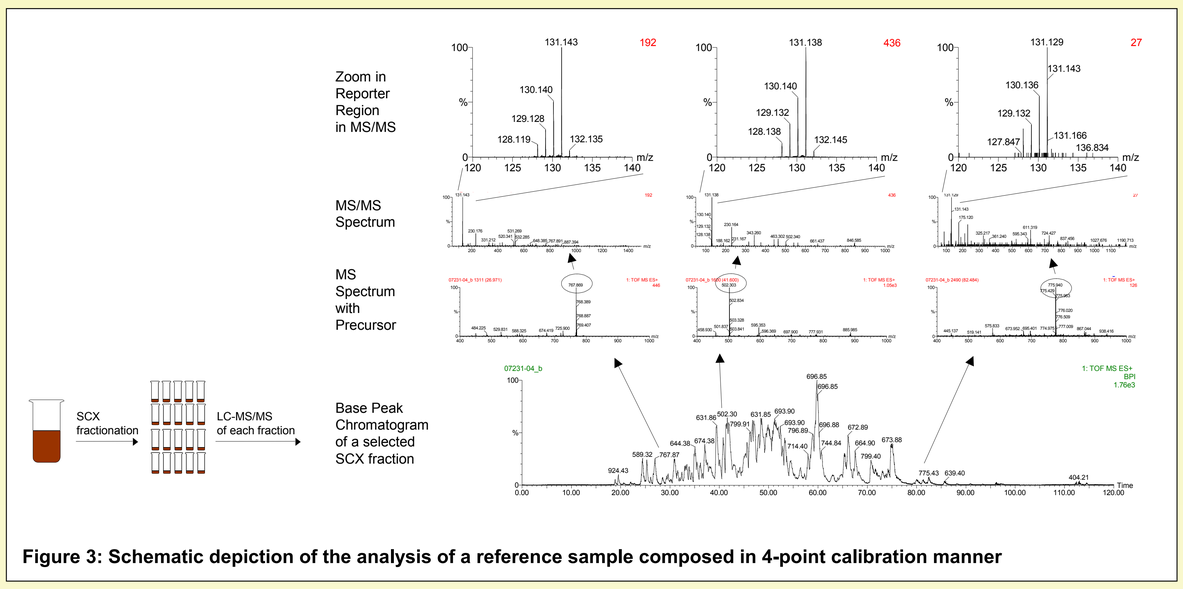


Figure 3: Schematic depiction of the analysis of a reference sample composed in 4-point calibration manner

In the experiment shown here about 4000 MS/MS spectra were acquired in data dependent acquisition mode. Over 1,000 peptides were identified which showed reporter ions suitable to generate 4-point calibration curves, typically with R-square values of >0.99 which make them suitable for quantification purposes. Sensitivity is only limited by the individual experimental setup, e.g. sample load, degree of prefractionation, and MS/MS conditions employed.

The combination of several peptides derived from the same protein assists in very robust quantification behaviour. Preferentially peptides are selected which are proteotypic for one protein (i.e. are not present in other proteins of that species). By defining m/z and elution time windows, directed quantification at improved sensitivity and signal to noise ratio is facilitated.

Figure 4 shows one example of the 4-point reference plasma on different MS instruments. The left MS/MS spectrum was acquired on an ESI-TOF MS/MS type instrument (QTOF2), the right MS/MS spectrum is from a MALDI-TOFTOF instrument (Proteomics Analyser 4800). The inserted zoom spectra of the reporter regions nicely show the reporters in 1:2:4:8 manner.

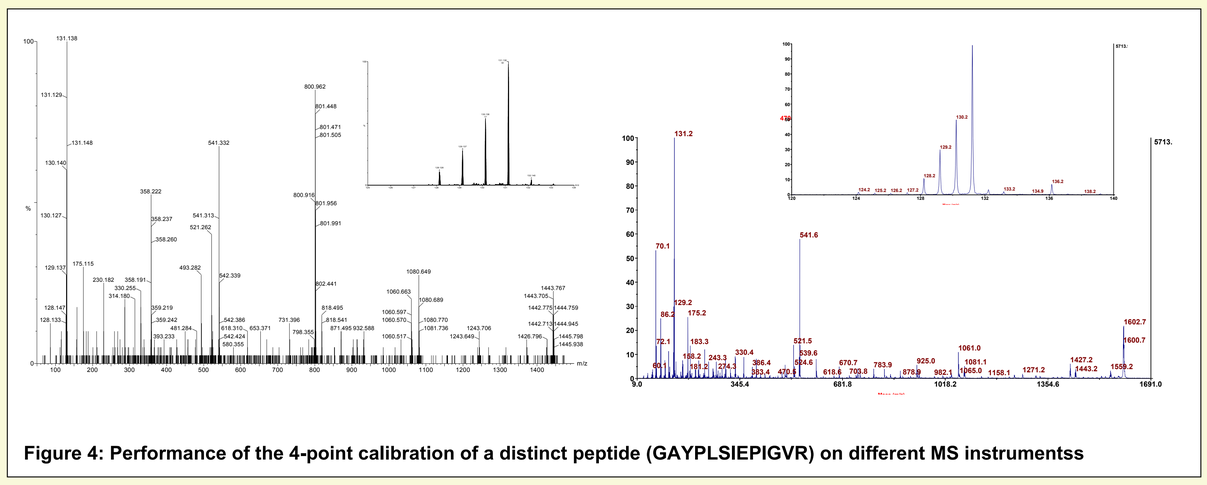


Figure 4: Performance of the 4-point calibration of a distinct peptide (GAYPLSIEPIGVR) on different MS instrumentss

Absolute Quantification

In addition to the use of the “full proteome reference”, absolute quantification of regulated peptides can be obtained. Here, known amounts of regulated peptides are labeled with several TMTs. Deliberate combinations of tagged peptides are then used to establish individual calibration curves.

Based on calibration curves from the spiked TMT-labeled peptides, the absolute amount of the sample peptide of interest can be determined accurately. By making use of this 4-point calibration concept, multiplex assays with absolute quantification of selected proteins can be developed. Simultaneously, relative quantification of other proteins is achieved using the reference material.

Summary

- **The concept of a universal biological reference material based on labeling with Tandem Mass Tags Is extended to generate multipoint calibration references.**
- **Validity is demonstrated in human plasma samples showing dramatic improvement in precision and CV’s.**
- **Proof-of-concept for accurate absolute quantification for biomarker validation and routine measurement using 4-point calibration standards is demonstrated.**

Literature:

[1] Thompson A, Schäfer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Hamon C: “Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS“, *Anal. Chem.* **2003**, *75*, 1895-1904.