



# Biological Reference Materials for Proteomics: Application of Tandem Mass Tag® labeled Plasma Reference for MS/MS-Quantification of Individual Samples

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## Overview

- Combination of Tandem Mass Tags (TMT) with the idea of a universal reference.
- Tagged reference materials improve reproducibility and comparability of proteomics studies.
- Relative quantification of hundreds of proteins in samples of interest achieved by referencing against specific reporter ion from added aliquot of TMT-labeled reference material.
- Sixplex-TMT-labeled peptides used to generate multipoint calibration mixture which is spiked into Sixplex-TMT-labeled samples 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.

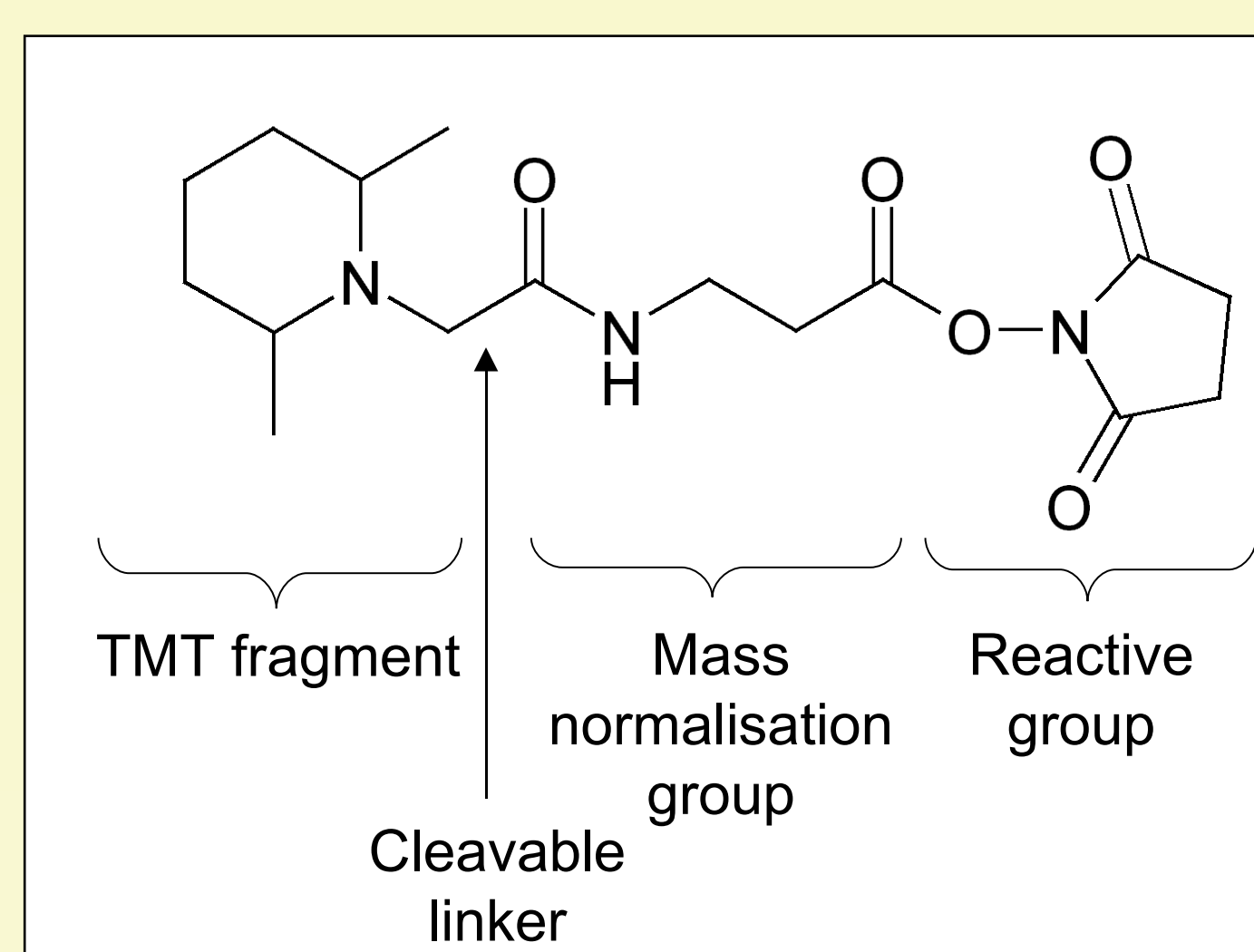


Figure 1: Design of the TMT label reagent

- TMT fragment: 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

By appropriate incorporation of <sup>13</sup>C and <sup>15</sup>N isotopes into the shown structure, different sets of TMT-reagents can be designed. We have developed Duplex-TMT (2TMT-126 + 2TMT-127) and Sixplex-TMT (6TMT-126 to 6TMT-131) sets for clinical and preclinical applications.

## Use of reference materials

Lack of precision and reproducibility of workflows have had 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 extended this concept by developing isobarically labeled reference materials for standardisation of proteomics studies.

Under this approach an identical amount of 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 illustrate the concept aliquots of human plasma (100µg each, ~1.5µl plasma) were processed and labeled with 2TMT-126 according to figure 2. In parallel, a larger amount of human plasma was processed and labeled with 2TMT-127. Each 2TMT-126 labeled individual plasma samples was spiked with a 100 µg aliquot of the 2TMT-127 labelled plasma reference.

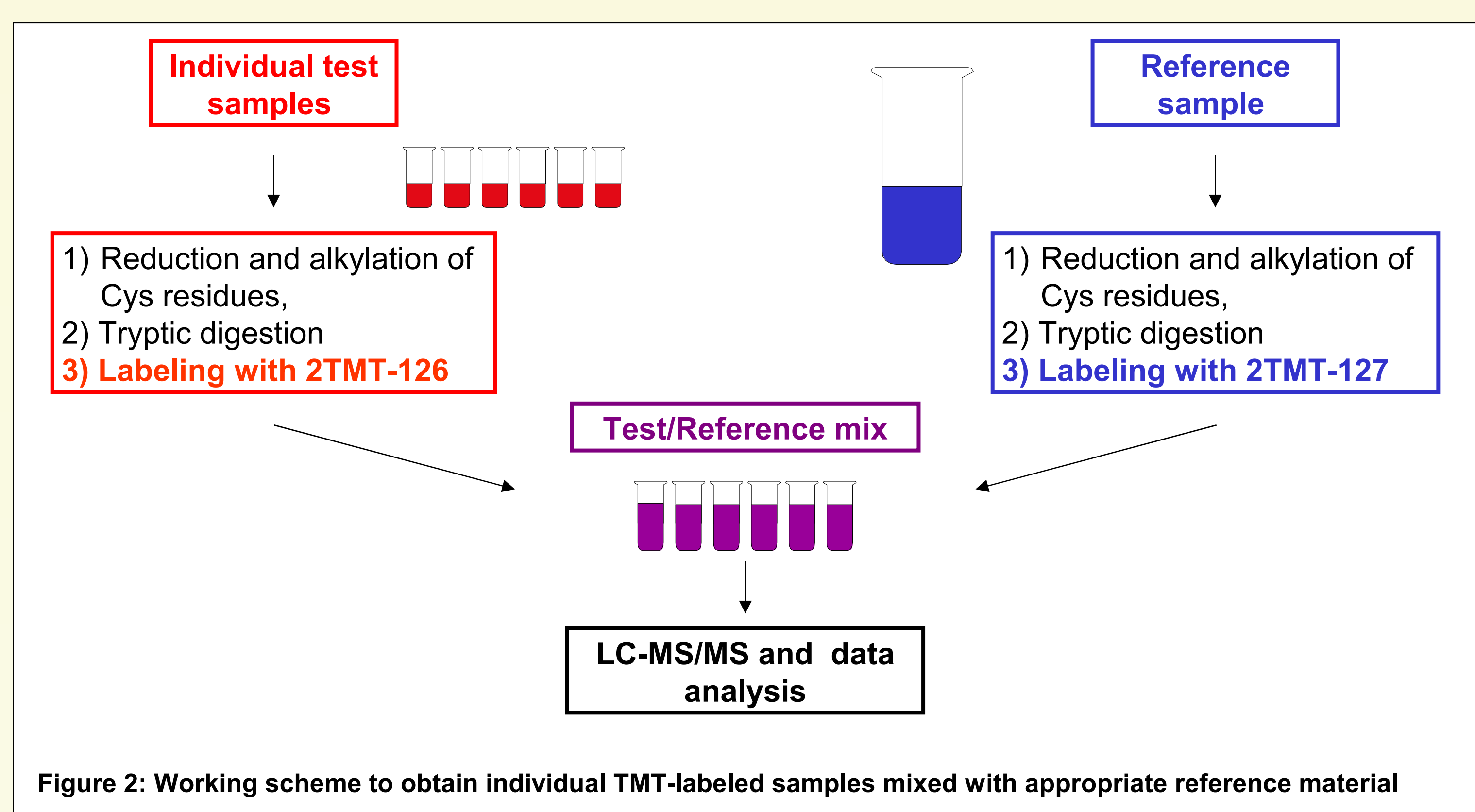


Figure 2: Working scheme to obtain individual TMT-labeled samples mixed with appropriate reference material

Subsequently, the combined samples were analysed by LC-MS/MS on a QTOF-2 instrument (injected amount per LC-MS/MS run corresponds to ~0.01µl of the individual plasma sample). Peptide and protein identification was performed with SEQUEST. For each identified peptide, the resulting reporter signals m/z = 126 and m/z = 127 were normalised and their ratio was calculated. The peptide identification and relative quantification results were grouped per protein and their means and standard deviation calculated.

Figure 3 shows the quantitative ratios and the corresponding standard deviation of proteins measured over 6 individual experiments using human plasma. Based on the experimental design quantitative ratios of 1 were expected. LC-MS/MS was run in data dependent acquisition mode with 1.4 sec. scantime. The top 50 proteins from all experiments were analysed for their quantitative behaviour.

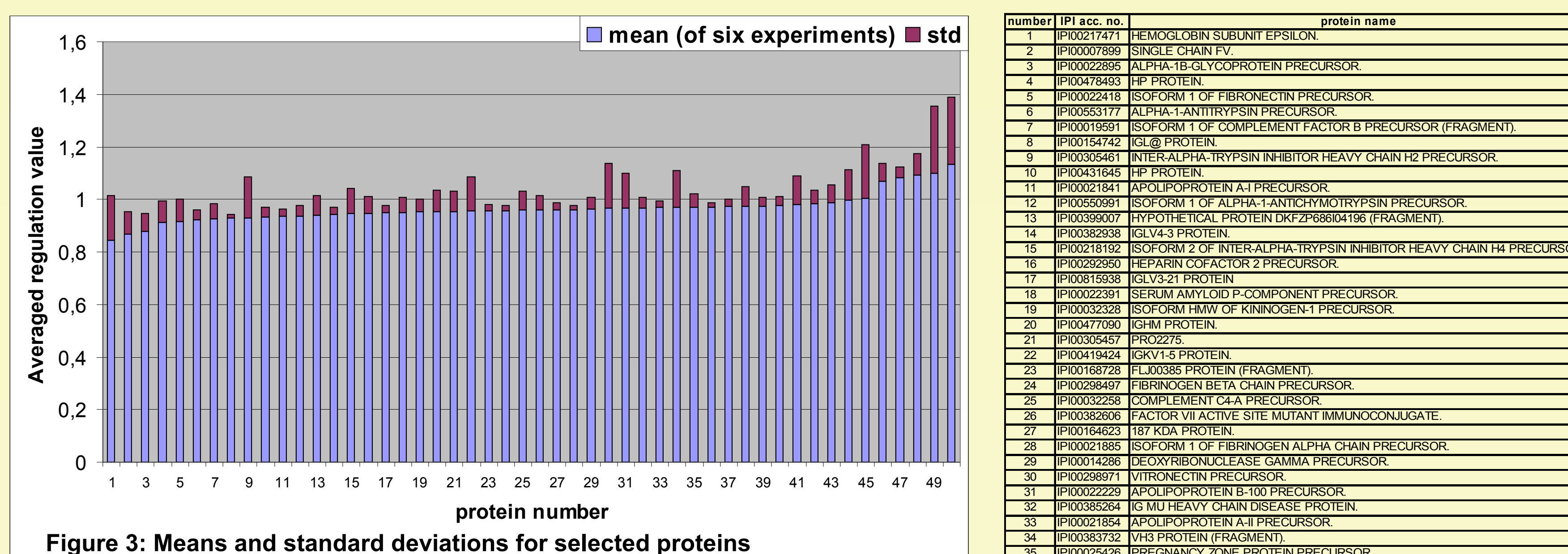


Figure 3: Means and standard deviations for selected proteins

The averaged ratios of the proteins over 6 experiments range from 0.84 to 1.13. 46 out of 50 (92%) exhibit a precision of > 90%. 44 out of 50 (88%) show a coefficient of variation of less than 15% over the 6 experiments.

The experimental results demonstrate the validity of the underlying concept.

Further optimisation of LC-MS/MS parameters such as inclusion lists and/or the use of longer MS/MS scan times lead to improved precision and reduced coefficients of variation. Routinely, precision better 10% and coefficients of variation of less than 10% can be achieved.

## Absolute Quantification

Absolute quantification of regulated peptides identified with the reference material approach can be obtained by labeling known amounts of these peptides with four of the six reagents of the Sixplex-TMT set (6TMT-128 to 6TMT-131) and mix them in certain ratios. The TMT-reporter ions of these labeled peptides are used to establish a calibration curve. This curve can be used to determine the absolute amount of this peptide in the individual sample which itself was labeled with 6TMT-126.

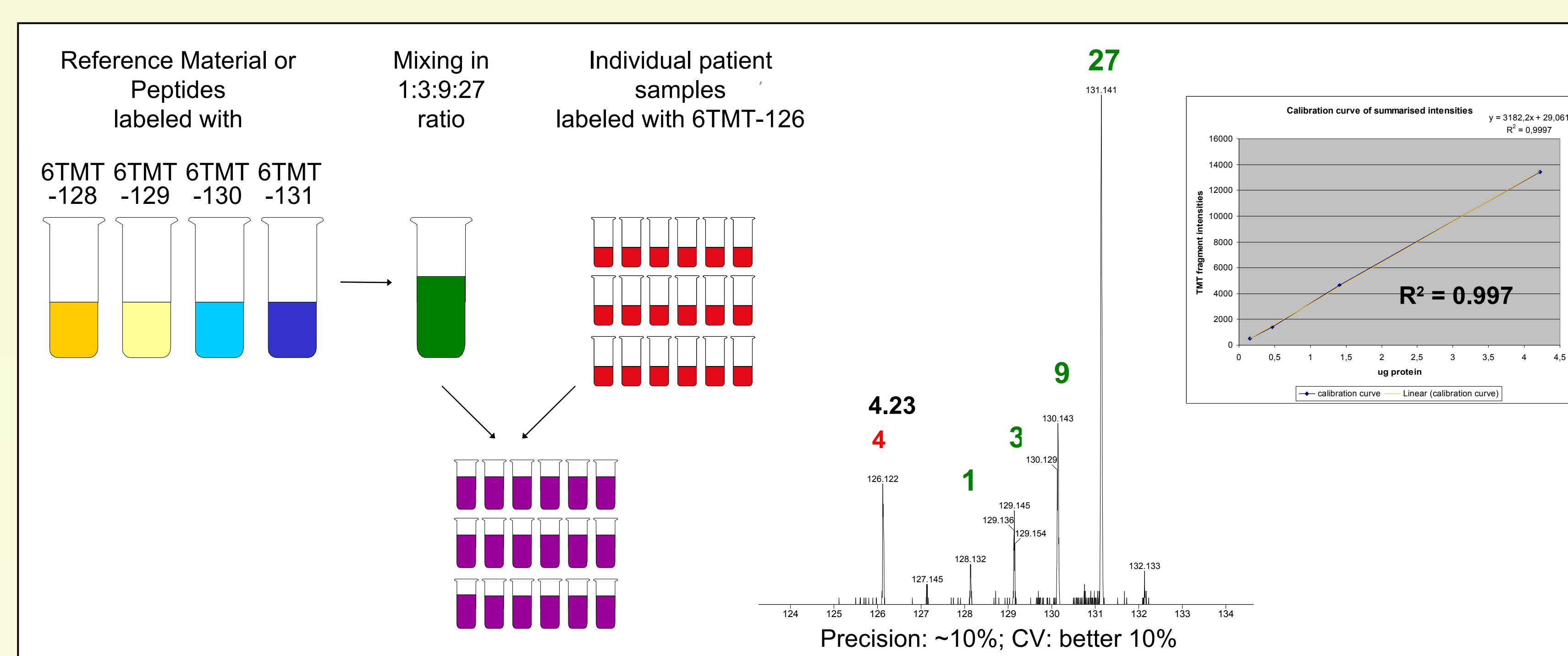


Figure 4: Schematic depiction of the absolute quantification approach using a fourpoint calibration

The four data points from the known calibrants show an excellent correlation between signal intensity and peptide content. Based on this fourpoint calibration, the absolute amount of the peptide of interest being labeled with 6TMT-126 can be determined in a very exact and reliable manner. By either using a 4-point biological reference label or using selected proteins, multiplex assays can be developed with absolute quantification.

## Summary

- Development of a universal reference material based on labeling with Tandem Mass Tags.
- Validity demonstrated with human plasma samples showing dramatic improvement in precision and CV's.
- Proof-of-concept for exact and reliable absolute quantification for biomarker validation and routine measurement using fourpoint calibration standard 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.