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# Intra-sample three-point quantitative calibration using multiple stable isotope labelled QconCATs

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## Introduction

Quantitative concatamers (QconCATs) have been used since almost 20 years as quantification standards for multi-protein panels in targeted and untargeted proteomics assays. Classical approaches use heavy stable isotope labelled QconCATs for single-point internal calibration. The most common labelling strategy uses labelled arginine and lysine residues, a technique known as SILAC, which is also often used for peptide standards.

Peptides are quantified by adding the labelled standards in known amounts to the samples and by comparing the signal intensity of unlabelled, native peptides with their labelled counterparts.

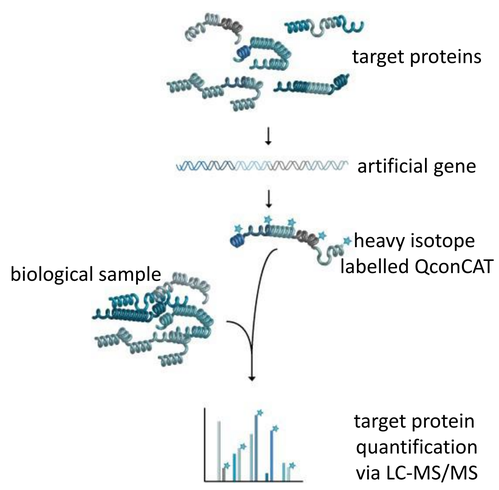
Here, we show the advantages and drawbacks of metabolic QconCAT labelling using $^{15}N$ salt and/or $^{13}C$ glucose compared to classical SILAC labelling. By spiking multiple differently labelled QconCATs at increasing concentrations into the same sample, we establish an intra-sample three-point calibration curve.

## Concept

### QconCAT technology

QconCATs are artificial concatamers of quantitative peptides for several target proteins. They are typically used as internal standards in LC-MS/MS based proteomics, but can also serve as external standards.

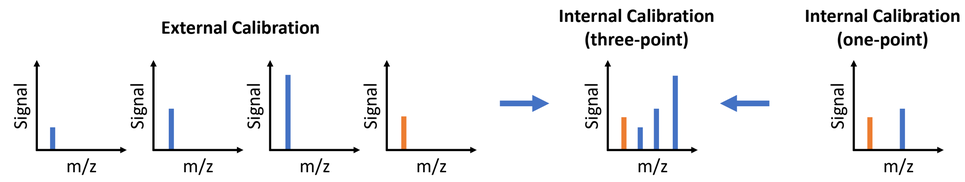
QconCATs can be produced as recom-binant proteins in *E.coli*. For heavy iso-tope labelling, bacterial cultures are provided with either heavy labelled amino acids (SILAC), $^{15}N$ salt and/or $^{13}C$ glucose.




### Three-point internal calibration

MS signal intensities show a linear positive correlation with protein / peptide abundance. However, the slope of the of the line often differs from 1, i.e. a doubling of the peptide's intensity does not lead to a doubling of the observed signal intensity. This phenomenon reduces the accuracy of one-point calibrations, especially when the signal intensities of standard and unknown differ strongly.

Therefore, quantitative clinical MS assays most often use external calibration curves. A drawback of external calibration is lacking intra-sample controls that can correct e.g. for matrix effects or ion suppression. Also, external calibration curves need additional measurements and thus increase the needed machine time.




## Results

### Analysis of labelling strategies

A common problem in quantitative proteomics is incomplete labelling of the standards during production. We discriminate two sources of error that may disturb exact quantification:

### 1) Contamination with unlabeled peptides

Standards are often contaminated by fully unlabelled peptides that have the same mass than native proteins. The resulting signals in the light trace cannot be distinguished from the native protein and thus limit the linearity and sensitivity of the method. Unlabelled peptides occur in relevant amounts only in SILAC and $^{13}C$ labelling, the signal detected in $^{15}N$ and $^{13}C^{15}N$ labelling is neglegible.

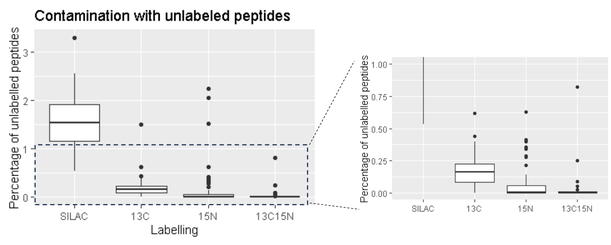



**Figure 1: Percentage of unlabelled peptide signal of total peptide signal.** SILAC peptides show a median unlabelled signal of 1.54 %, 13C labelled peptides a median of 0.16 %. The unlabelled signal in 15N and 13C15N peptides is less than 0.01% and can be mostly attributed to noise.

### 2) Partial labelling

A different problem arises when not all $^{12}C$ or $^{14}N$ atoms of a given peptide are replaced by heavy isotopes. Incomplete labelling results in the observation of peaks that are lighter than the expected monoisotopic peak.

While 15N and SILAC labelling show an isotope pattern comparable to unlabelled peptides, 13C and 13C15N labelling show a shift towards lighter isotopes.

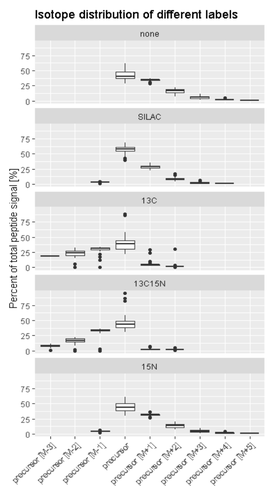



**Figure 2: Isotope distribution.** Isotopic peaks were identified and quantified using Skyline. All peaks with at least 1% signal compared to the mono-isotopic precursor were included.

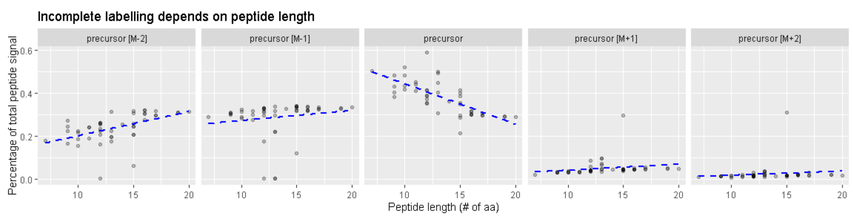



**Figure 3: Precursor isotopes observed in 13C labelling.** The percentage of not fully labelled standard increases with the peptide length, while the percentage of the monoisotopic precursor drops.

### Skyline XICs

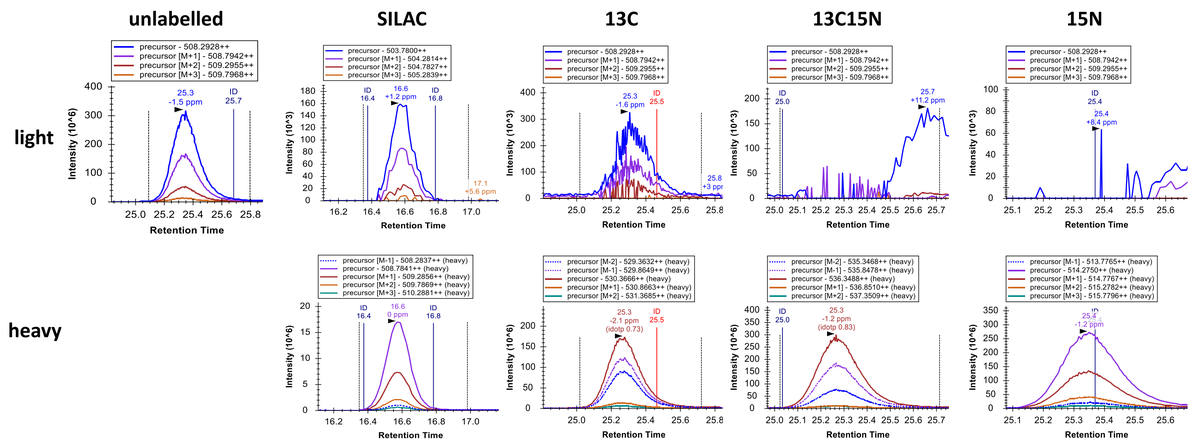
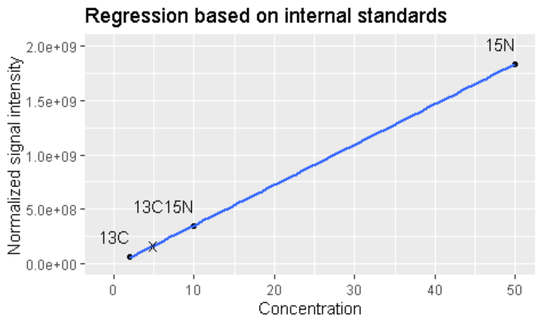



**Figure 4: Extracted ion chromatograms from labelled QconCATs.** The same QconCAT was expressed five times with different labelling: unlabelled, SILAC-$^{13}C^{15}N$ @ Lys & Arg, metabolic $^{13}C$, metabolic $^{13}C^{15}N$ and metabolic $^{15}N$. Each QconCAT was measured separately and the XICs for the light and heavy peptide LISEVIGER was extracted using the Skyline software.

### Proof of principle

Differentially labelled QconCATs were spiked into the same sample in increasing amounts, to calculate an intra-sample calibration curve. QconCATs with $^{13}C$, $^{13}C^{15}N$ and $^{15}N$ were spiked in a 2 : 10 : 50 ratio. Unlabelled QconCAT served as an "unknown" and was spiked at a ratio of 5. All signals were corrected for the observed differences in the isotope distribution and for slight differences in the peak integration.




| Label | Signal | True conc | Calculated |
|---|---|---|---|
| 13C | $6.6x\ 10^7$ | 2 | - |
| 13C15N | $3.5x\ 10^8$ | 10 | - |
| 15N | $1.8x\ 10^9$ | 50 | - |
| unlabelled | $1.7x\ 10^8$ | 5 | 4.997 |

**Figure 5: Intra-sample linear regression.** A linear fit was calculated from the different heavy signals. The unlabelled QconCAT was used as an unknown. Its concentration was back-calculated based on its signal from the linear regression.

## Conflict of interest

Florian Christoph Sigloch is employed by PolyQuant GmbH

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