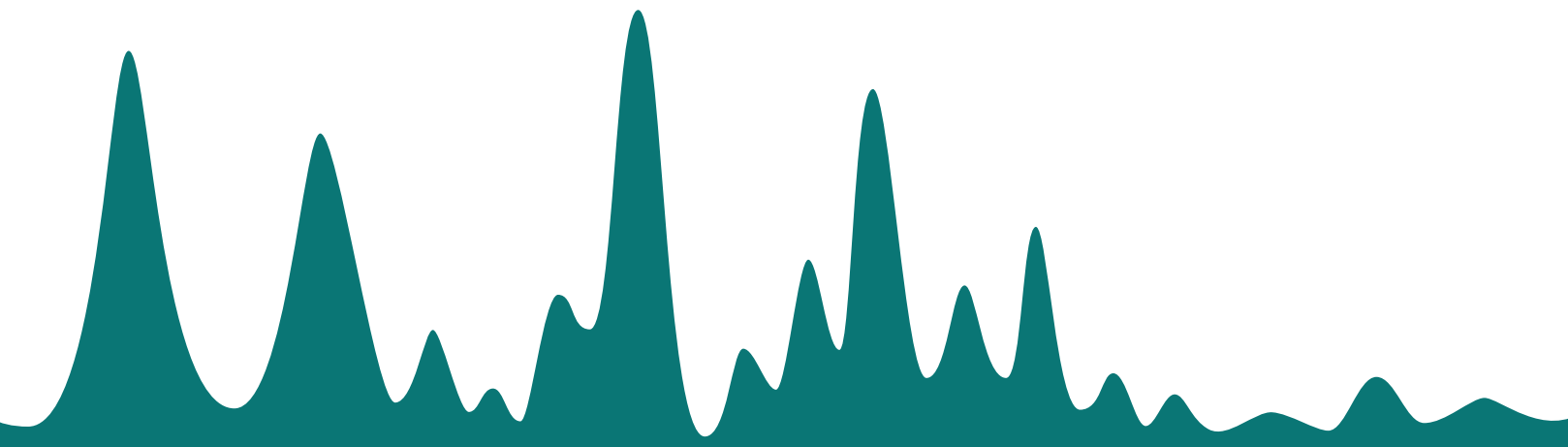
QPrEST+

Validated Isotope-Labeled Protein Standard for Absolute Quantification Using Mass Spectrometry



ATLAS ANTIBODIES

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Protein quantification

Proteins perform a vast array of functions within the human body and are therefore quantified in universal research from early discovery to the clinic. In mass spectrometry-based proteomics different label-free quantification strategies have been used providing mainly relative quantification. Often, however, there is a need to determine absolute concentrations, e.g. in clinical settings, where samples are compared to a normal reference interval. The preferred method for absolute protein quantification in targeted studies is to use stable isotope-labeled spike-in standards in combination with external calibration curves.

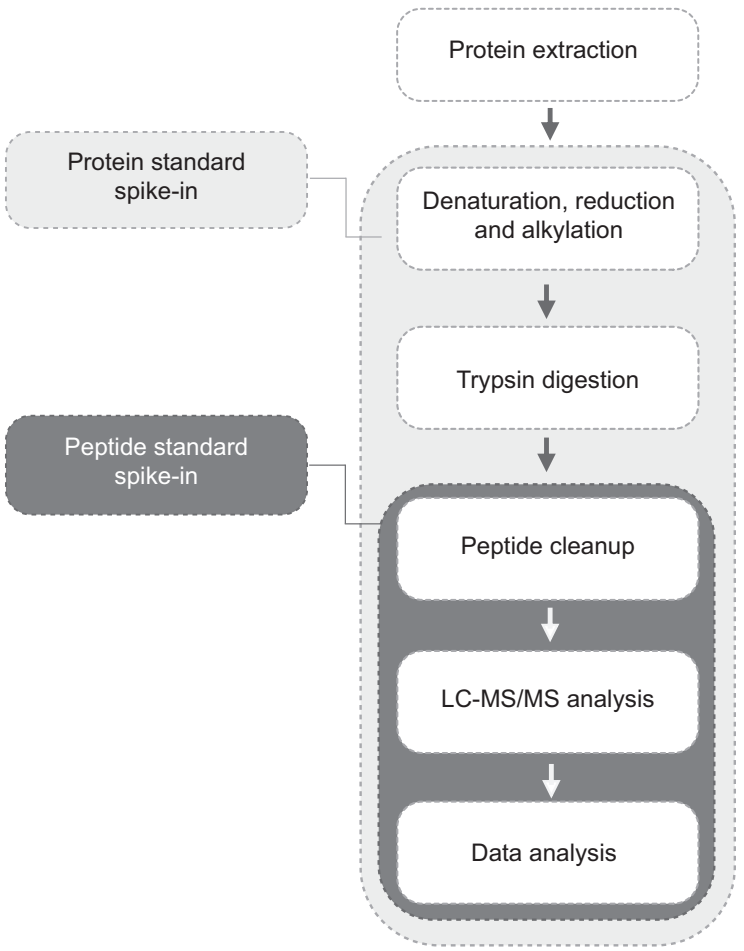

Figure 1. Proteomic workflow
Stable isotope-labeled standards used as internal standards for protein quantification are spiked in at different stages of the sample preparation. The stable isotope-labeled peptide standards are added to the sample after digestion and only normalize for variation occurring thereafter (*dark gray*). Stable isotope-labeled protein standards are spiked in early in the preparation, before the trypsin digestion and the peptides are formed simultaneously as for the protein to be quantified (*light gray*).

Stable isotope-labeled standard approaches

The most common stable isotope-labeled standards used for protein quantification are synthesized peptides that are added after the sample has been reduced, alkylated and subsequently digested with a protease such as trypsin (**Fig 1**).

These labeled peptides can therefore normalize for variation in the subsequent steps such as peptide cleanup, sample injection and LC-MS/MS analysis, but they cannot account for variation introduced during the digestion process.

A more reliable option is the stable isotope-labeled full-length proteins. These are added before the sample preparation and digestion, which results in a similar digestion efficiency as the protein to be quantified. Also, since the isotope-labeled peptides are released together with the endogenous peptides during the digestion process, the requirements on peptide stability are lower and more peptides can be considered for quantification. However, few stable isotope-labeled full-length proteins are available for purchase and they are often expensive, while labeled peptides are widely available and often synthesized on demand.

QPrEST

Quantitative Protein Epitope Signature Tags (QPrESTs) are stable isotope-labeled protein standards developed for absolute quantification.

The QPrEST sequence is identical to a part of a human protein and is designed to have low sequence similarity to other proteins and theoretically form several tryptic peptides. The QPrESTs are accurately quantified by LC-MS/MS using an amino acid analyzed reference protein.

As the stable isotope-labeled full-length proteins, the QPrEST standards are added to the sample of interest before the sample preparation and trypsin digestion (**Fig 2**).

Due to identical amino acids surrounding the cleavage sites, peptides are subsequently formed and released similarly for both the QPrEST and the endogenous protein.

The QPrESTs are available for the majority of all human proteins and are generally produced on demand.

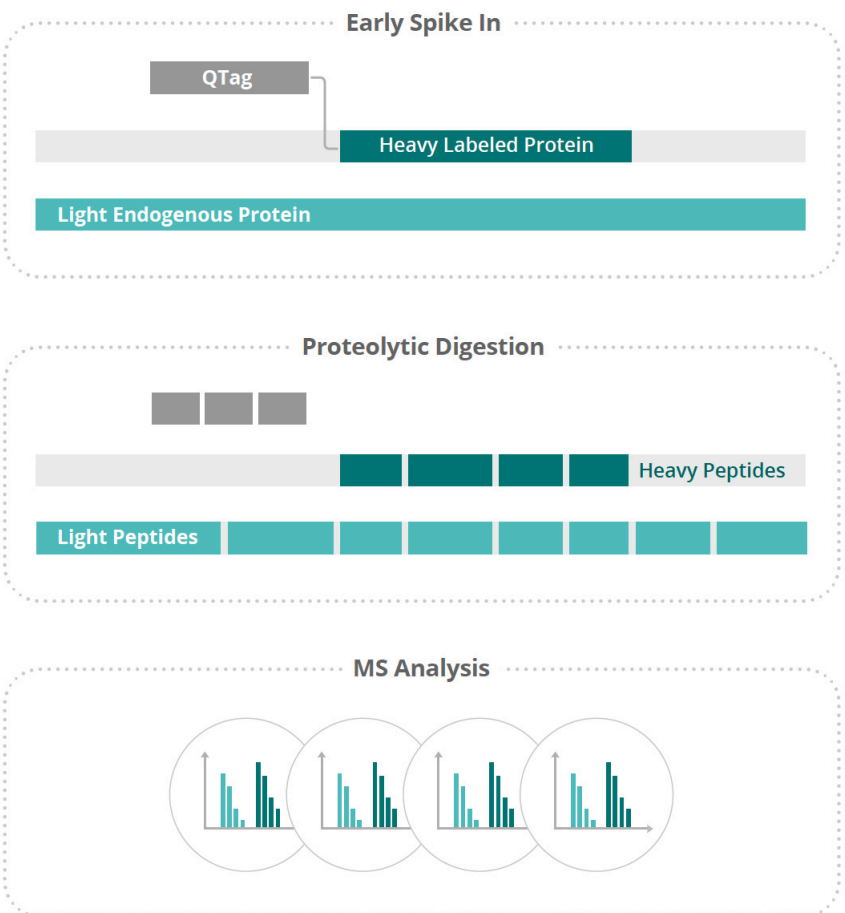

Figure 2. Suggested workflow for quantification using QPrEST
Accurately quantified lyophilized QPrEST is reconstituted and spiked into the sample prior reduction, alkylation, and trypsin digestion. After peptide cleanup, the sample is analyzed using LC-MS/MS and the data is analyzed with a quantification software using single-point calibration.

Quantotypic peptides

For a peptide to be suitable for quantification, several criteria must be fulfilled. Firstly, a peptide must ionize well to give a high detectable signal in the mass spectrometer, and secondly, it must be proteotypic, i.e. having a sequence unique to the protein of interest. However, a proteotypic peptide that gives a good signal needs to be further evaluated to establish if it could also function as a quantotypic peptide. For instance, amino acids and motifs of established or predicted post translational modifications should be avoided in quantotypic peptides.

The definition of a quantotypic peptide depends on the type of stable isotope-labeled standard used. For synthesized peptides, some amino acids are prone to be modified, such as N-terminal glutamine residues that can undergo cyclization and form pyroglutamate. Since modifications before addition to the sample would compromise the quantitative precision and accuracy, these amino acids should be avoided.

Proteins are in general more persistent in solution than peptides, although modification sites in the quantotypic peptides should preferably be avoided also for stable isotope-labeled full-length protein and QPrEST standards. Modifications occurring after addition of the protein standards to the sample are theoretically formed similarly in both the standard and protein of interest and would therefore not affect the accuracy in quantification.

Peptides with surrounding cleavage sites that contain multiple arginines or lysines tend to be only partially digested, hence these should be avoided for the peptide standards. However, since the tryptic peptides are formed in the same way for the protein to be quantified and for the protein standards, even miscleaved peptides could be considered as quantotypic peptides in this strategy.

In conclusion, stable isotope-labeled protein standards, like QPrESTs, are more stable and since the peptides are released during trypsin digestion, more peptides can be considered for quantification.

Quantotypic peptide definition for stable isotope-labeled QPrEST+ protein standard

- Formed during protease digestion
- Unique sequence for protein of interest
- Cross-validated by another peptide or method

Further more

- For QPrEST-products miscleaved peptides can also be used

CPTAC assay

The Clinical Proteomic Tumor Analysis Consortium (CPTAC) is a public repository of well-characterized, MS-based targeted proteomics assays, which are specific and precise and could be standardized and reproduced to be distributed across laboratories. An assay is defined as the quantification of a peptide in a complex mixture of peptides derived after proteolytic digestion of a relevant sample matrix.

On the CPTAC portal, the research community can upload and share information of an assay, i.e the analytical performance of the developed quantification method.

Two experiments are required for upload:

- Lower limit of quantification and linearity of a response curve
- Repeatability of the assay

QPrEST+ peptide validation

QPrEST+ is a QPrEST protein standard with a minimum of one peptide validated according to the CPTAC assay criteria.

To identify the linear range (define slope and intercept), the limit of detection (LOD) and the lower limit of quantification (LLOQ), samples are prepared in the relevant matrix in a multipoint response reversed curve (at least six points). Three transitions per peptide are included in the analysis and their results should be within ±30 %.

Based on the first data, three concentrations, low, medium and high, are used to access the repeatability (intra- and inter-assay variability) for three replicates at five different occasions. Approved intra- and inter-assay variation (CV%) should be lower than 20 %.

To further validate the suitability of QPrEST+ peptides as quantotypic peptides, in-house developed strategies are applied. As a first option, generated peptide result can be cross-validated by another peptide. Using this strategy, generated ratios are required to be confirmed (±20 %) by at least one other QPrEST peptide (peptide could originate from the same or another QPrEST) in the same sample. If this is not applicable, alternative strategies include using an orthogonal method for validation of the concentration, e.g. an immunoassay or comparing the generated result to a well-defined reference value.

The QPrEST+ products are validated in at least one sample type, e.g. human plasma or a tissue lysate, and come with a recommended spike-in level. However, since protein concentrations can vary considerably between samples, depending on many factors such as disease state and sample handling, this value should only be used as a starting point.

Benefits of using QPrESTs

- Sequence covers at least two theoretical tryptic peptides
- Accurately quantified using an amino acid determined reference protein
- Shipped in lyophilized form
- Digested together with the endogenous protein

Extra benefits of QPrEST+

- Internal peptide(s) validated according to CPTAC
- Peptide cross-validated by another peptide or orthogonal method
- Recommended spike-in level for single-point calibration in specified matrix
- Skyline file with CPTAC validation data

Single-point calibration

The classical approach for absolute quantification using mass spectrometry relies on the creation of an external protein standard curve and spike-in of the same amount of a stable Isotope-labeled standard to the standard curves and the samples. In this approach, the stable Isotope-labeled standard is used only for normalization purposes and does not need to be accurately quantified.

However, the use of an external standard curve requires several additional LC-MS analyses to be run and the accuracy of this kind of assay highly depends on the quality of the protein used for the standard curve. Firstly, the concentration of the standard has to be accurately determined using a highly quantitative assay. Secondly, the standard protein needs to be well characterized regarding structure and modifications and preferably be identical to the endogenous protein of interest.

A more common and high-throughput approach for absolute quantification is the addition of stable isotope-labeled standards with accurately determined concentration to the samples. The endogenous protein is then quantified by direct comparison of the labeled: unlabeled peptide ratio.

The potential use of a single-point calibration would require both the unlabeled and the labeled peptides to be at concentrations within the linear response range and assumes that slope and intercept of a properly prepared standard curve should be one and zero, respectively.

QPrEST+ standards are well suited for use in single-point calibration experiments since they are pre-quantified with high accuracy and contain one or more validated peptide(s).

Furthermore, the QPrEST+ standards are provided with a recommended spike-in levels that give labeled: unlabeled ratios close to one in a standard sample. To achieve a good accuracy and repeatability for single point calibration a ratio close to one is preferred. However, since QPrEST+ peptides have a validated linear response, they generally provide good quantitative results also when measuring at rather large off-ratios (as long as both the heavy and light signals are not too close to the lower limit of quantification).

QPrEST validation example

A QPrEST (QPrEST22551) for Leucine-rich alpha-2-glycoprotein-1 (LRG1/A2GL) (**Fig 3**) was serially diluted in steps of three and spiked into pooled human plasma at eleven different concentrations. The samples were reduced, alkylated and digested with trypsin and subsequently analyzed by LC-MS/MS in parallel reaction monitoring (PRM) mode on a Thermo Q Exactive Plus MS instrument.

Two tryptic peptides (DLLLPQPDLR and VAAGAFQGLR) with three transitions each were evaluated using Skyline with an external tool from CPTAC to generate plots.

Linear response curves were produced from 2-13,300 fmol/µL plasma with similar slopes close to one and intercepts close to zero for both peptides (**Fig 4**). The peptides showed similar linear responses, hence validating each other. The LLOQ was determined to 2 fmol/µL. The plasma level of LRG1/A2GL was determined to 300 fmol/µL in a healthy plasma pool.

Repeatability for LRG1/A2GL peptides DLLLPQPDLR and VAAGAFQGLR was investigated in samples with high, medium and low spike-in levels. They were prepared and analyzed five times on five separate days (**Fig 5**). The three different spike-in levels gave similar results and the repeatability is well within the CPTAC criterium of variation less than 20 %.

MSSWSRQRPKSPGGIQPHVSRTLFLLLLLAASAWGVTLSPKDCQVFRSDHGSSISCQPPAEIPGYLPADTVHLAVEFFNLTHLPANLLQGASKLQELH
LSSNGLESLSPEFLRPVPQLRVLDLTRNALTGLPPGLFQASATLDTLVLKENQLEVLEVSWLHGLKALGHLDLSGNRLRKLPPGLLANFTLLRTLDLG
ENQLETLPPDLLRGPLQLERLHLEGNKLQVLGKDLLLPQPDLRYLFLNGNKLARVAAGAFQGLRQLDMLDLSNNSLASVPEGLWASLGQPNWDMRDGF
DISGNPWICDQNLSDLYRWLQAQKDKMFSQNDTRCAGPEAVKGQTLLAVAKSQ

Figure 3. Amino acids sequence for LRG1/A2GL
The Leucine-rich alpha-2-glycoprotein-1 (LRG1/A2GL) consists of 347 amino acids and the sequence is outlined above. The QPrEST22551 (*green font*) cover amino acids 193-326. The QPrEST22551 tryptic peptides DLLLPQPDLR and VAAGAFQGLR (*underlined*) were included in the CPTAC validation.

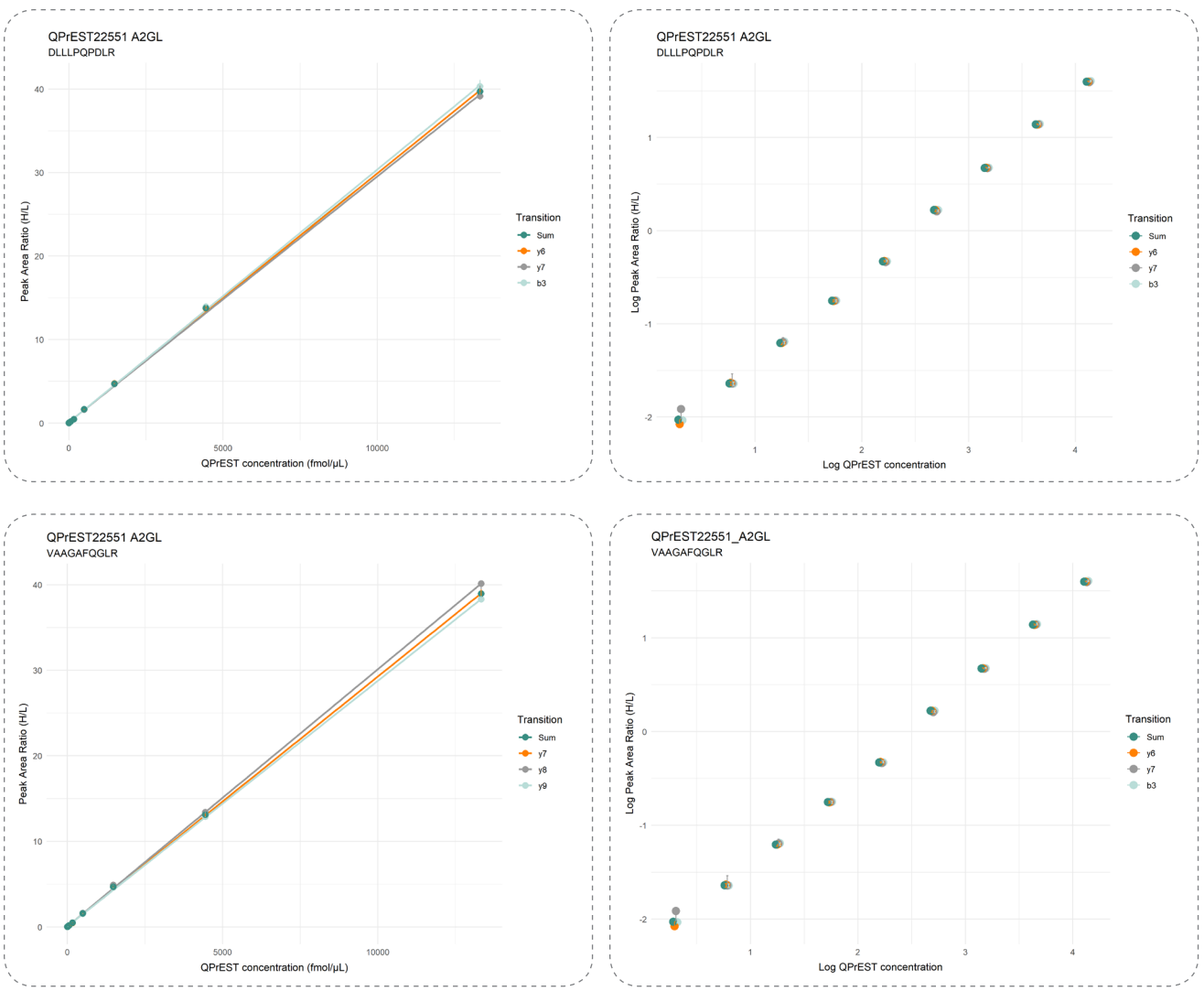

Figure 4. Response curves for LRG1/A2GL
Linear response curves for Leucine-rich alpha-2-glycoprotein-1 (LRG1/A2GL) peptides DLLLPQPDLR (*upper images*) and VAAGAFQGLR (*bottom images*) derived after protease digestion of QPrEST22551.

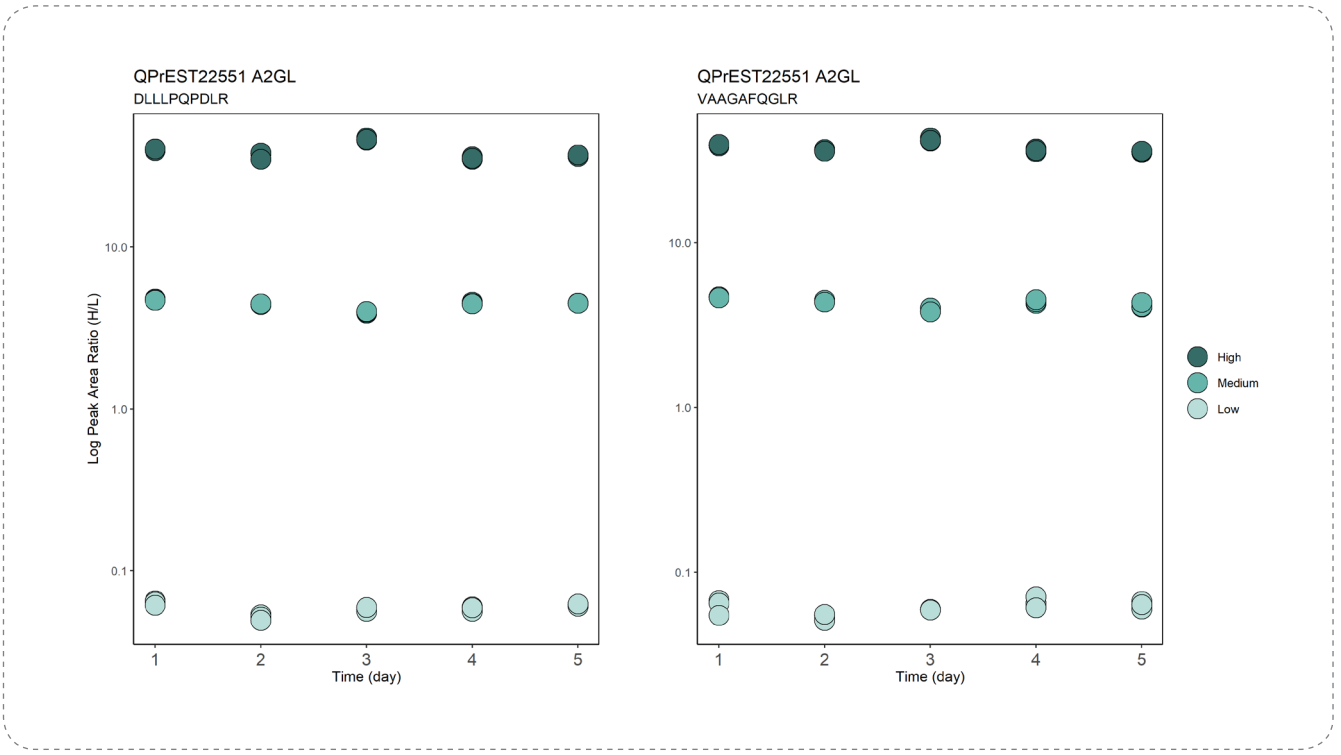

Figure 5. Repeatability study for LRG1/A2GL
Repeatability study for LRG1/A2GL peptides DLLLPQPDLR (*left*) and VAAGAFQGLR (*right*). Samples with high (13 pmol/µL plasma), medium (1.5 pmol/µL plasma) and low (0.02 pmol/µL plasma) spike-in levels were prepared five separate days and analyzed in triplicate injections.

References

Bantscheff M, Lemeer S, Savitski MM, Kuster B. (2012) Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present. *Anal Bioanal Chem* 404, 939-965

Zeiler M, Straube WL, Lundberg E, Uhlén M, Mann M. (2012) A Protein Epitope Signature Tag (PrEST) library allows SILIAC-based absolute quantification and multiplexed determination of protein copy numbers in cell lines. *Mol Cell Proteomics* 11, O11 009613

MacCoss MJ. Mass Spectrometry Signal Calibration for Protein Quantitation. Technical note from Cambridge Isotope Laboratories.

Summary

- QPrESTs are stable isotope-labeled protein standards for absolute quantification using mass spectrometry
- The QPrEST+ protein standards are readily available and contain peptides validated according to the CPTAC assay criteria
- The QPrEST+ protein standard provides quantotypic peptides comparable to stable isotope-labeled full-length proteins
- QPrEST+ shows good linear responses for quantotypic peptides and are suitable for single-point calibration

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