

# Incorporation of an Internal Trypsin Cleavage Site into Protein-AQUA™ Standards for Absolute Quantification

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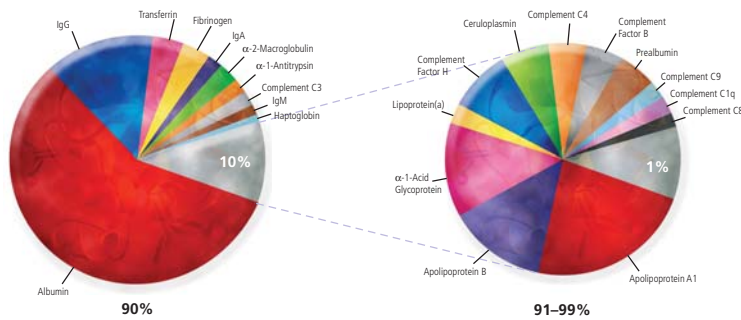
## Overview

Protein-AQUA has been utilized to absolutely quantify targeted proteins from a variety of sources. Initially, the method was employed for the quantification of a single protein in a complex mixture. In an extension of the technology, Protein-AQUA has enabled the absolute quantification of protein phosphorylation and ubiquitination sites, expanding the capabilities of post-translation modification profiling. Typically, in Protein-AQUA workflows, software is utilized to predict tryptic fragments in silico, enabling the selection of a suitable peptide for monitoring. An isotopically labeled version of this peptide is synthesized, and spiked, in known amounts, into a tryptic digestion of the test sample. However, tryptic digestion of a target protein frequently results in the generation of many additional peptides not predicted using in silico digestion, in part due to missed and/or partial cleavages. In the present work, we have employed the use of isotopically labeled peptides that incorporate a trypsin cleavage site into the sequence to monitor variance in the digestion procedure. Our results indicate that proper selection of a suitable peptide sequence for Protein-AQUA analysis can eliminate variability in the method, providing reliable quantitative data.

## Introduction

The quantification of protein(s) in a complex mixture is one of the greatest challenges facing proteomics researchers today. While recent advances in differential protein analysis have provided a means for comparing relative protein expression between populations, there exists a need for the absolute quantification of a single protein in a complex population. Methods for the absolute quantification of proteins have been previously described, which employ the use of an isotopically labeled internal standard corresponding to a tryptic peptide of the protein being quantified. However, the accuracy of this method inherently relies on the efficiency of tryptic digestion. Although an internal standard is employed, variations in the digestion efficacy hamper the accuracy of this method. In the present work, we have employed the use of isotopically labeled peptides that incorporate a trypsin cleavage site into the sequence to monitor variance in the digestion procedure.

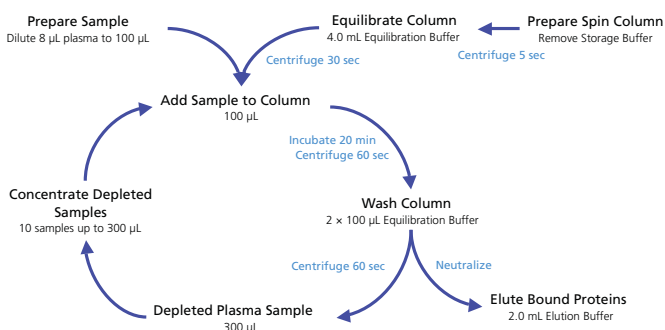
## Methods



- The 10 most abundant proteins represent approximately 90% of the total protein mass in human plasma.
- The 22 most abundant proteins are said to represent approximately 99% of the total protein mass in human plasma.
- The ProteoPrep 20 plasma immunodepletion column removes the 20 high-abundance plasma/serum proteins listed below. These 20 proteins represent approximately 97% of the total human plasma protein mass

Albumin	α-2-Macroglobulin	Apolipoprotein A1	Complement C4
IgGs	IgMs	Apolipoprotein A2	Complement C1q
Transferrin	α-1-Antitrypsin	Apolipoprotein B	IgDs
Fibrinogen	Complement C3	Acid-1-Glycoprotein	Prealbumin
IgAs	Haptoglobin	Ceruloplasmin	Plasminogen

## ProteoPrep® 20 Workflow



## Selection of Protein-AQUA Peptide Sequences

The amino acid sequence of human serum albumin was analyzed in-silico to select a peptide sequence suitable for monitoring using the Protein-AQUA approach. Isotopically labeled versions of the predicted tryptic fragment, FQNALLVR, and two potential missed cleavage products, FQNALLVRYTK and FQNALLVRYTKK, were synthesized by Sigma-Genosys (The Woodlands, TX).

Protein-AQUA Peptide 1 417 ELFEQLGEYK**FQNALLVRYTKK**VPQVST 444

Protein-AQUA Peptide 2 417 ELFEQLGEYK**FQNALLVRYTK**VPQVST 444

Protein-AQUA Peptide 3 417 ELFEQLGEYK**FQNALLVRYTK**VPQVST 444

## High Abundance Protein Depletion

Twenty high abundance proteins were depleted from plasma using the ProteoPrep® 20 Immunodepletion Kit. A large 3.7-mL prototype spin column for depleting 100 μL of human plasma was used. The protein concentration of whole and depleted plasma was determined using Bradford assay (B6916), with BSA as the standard.

## Tryptic Digestion

Samples of both whole and depleted plasma were prepared in 100 μg aliquots. Each sample was diluted to 0.25 mg/ml in ammonium bicarbonate buffer. Acetonitrile was added to a final concentration of 9% (v/v), and each sample was reduced and alkylated using the ProteoPrep Reduction/Alkylation Kit (PROTRA). Proteomics Grade Trypsin (T6567) was added at a concentration of 1% (w/w) and the sample incubated for 3 hours at 37 °C. An additional aliquot of trypsin at 1% (w/w) was added and then the sample further incubated at 37 °C overnight. The digests were dried to completion in a SpeedVac™.

## LC-MS/MS Analysis

An injection of a tryptically digested ProteoPrep 20 depleted plasma sample (prepared as described in Methods) was made using an Agilent capillary 1100 HPLC. The reverse phase separation was performed on a 15 cm × 2.1 mm Discovery C18 column, employing a peptide trap column for peptide pre-concentration and desalting. The mobile phase consisted of formic acidified water and acetonitrile. The separation system was coupled to a Thermo Finnigan LTQ linear ion trap mass spectrometer set to perform tandem MS on the ten most abundant ions in each full scan spectrum. After obtaining tandem MS results on an ion of interest, it was added to an exclusion list to facilitate the interrogation of lower abundance ions. An LC-SRM method for each of the isotopically labeled peptides was developed, as outlined in the Results section.

## AQUA Analysis

An AQUA Peptide stock solution was prepared by dissolving isotopically labeled versions of the three target peptides (FQNALLVR\*, FQNALLVRYTK\*, and FQNALLVRYTKK\*) in 0.1% formic acid. Each digest sample (whole and depleted plasma) was dissolved in 20 μL of the AQUA Peptide stock solution. Samples were analyzed using an Agilent Capillary 1100 HPLC coupled to a Thermo Finnigan LTQ linear ion trap mass spectrometer. Using the LC-SRM methods previously developed, the absolute quantity of each peptide fragment was determined.

## The Protein-AQUA Method

### Step 1:

#### AQUA Peptide Selection

VPQVSTPTLVEVSR  

 Select an optimal tryptic peptide and stable isotope amino acid from the sequence of your protein of interest

VPQVSTPTLVEVSR\*

Order synthetic AQUA Peptide from Sigma-Genosys

### Step 2: Implementation

Optimize LC-MS/MS separation protocol for quantitation

Extract protein from biological samples and add known quantity of AQUA Peptide

Digest

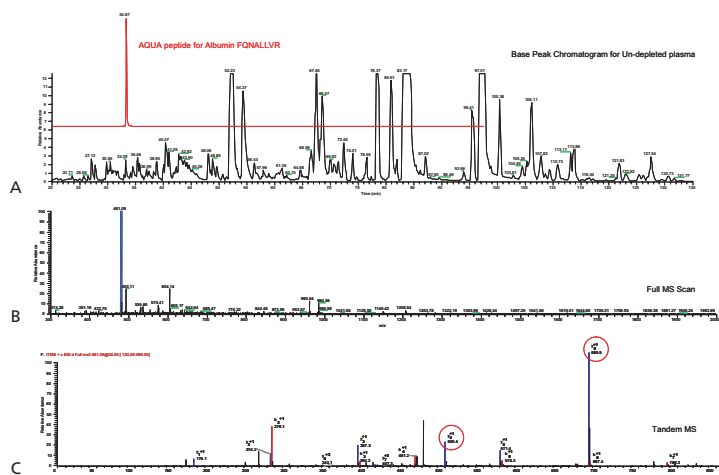
Analyze by LC-MS/MS or MALDI to quantitate protein of interest

\*Labeled amino acid

## Figure 1: Overview of the Protein-AQUA Method.

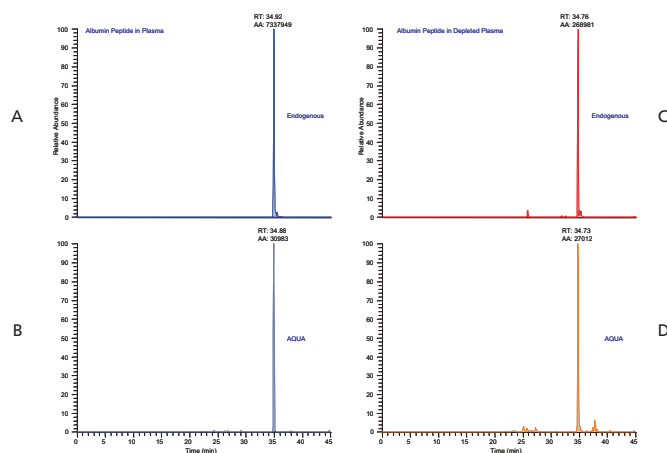
This method was developed by Dr. Steve Gygi and colleagues at Harvard Medical School [Stemmann O., Zou H., Gerber S. A., Gygi S. P., Kirschner M. W.; Dual inhibition of sister chromatid separation at metaphase, Cell 2001, Dec 14, 107: 715-726]. Limited use of this method is permitted under a licensing arrangement with Harvard Medical School.

## Results

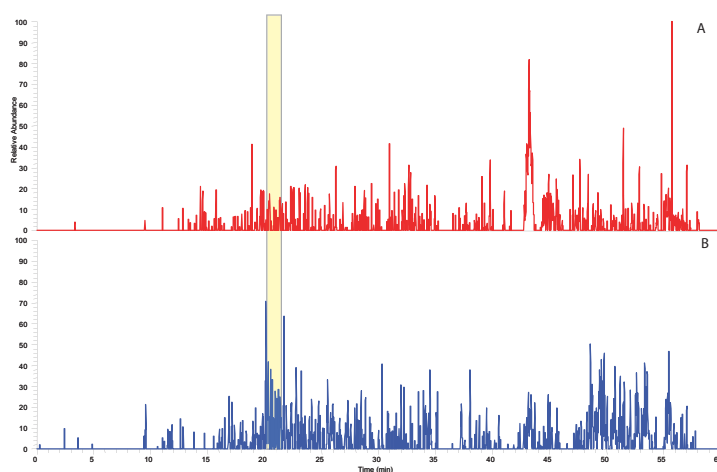


**Figure 2: Development of a Protein-AQUA LC-SRM for Absolute Protein Quantification.**

An injection of a typically digested ProteoPrep 20 depleted plasma sample (prepared as described in Methods) was made using an Agilent capillary 1100 HPLC coupled to a Thermo Finnigan linear ion trap mass spectrometer. The total ion current (Figure 2A) shows a complex mixture of species that may or may not have been chromatographically resolved. By harnessing the power of tandem MS, more than one hundred proteins were positively identified, with confidence, from the depleted plasma sample. One of these proteins (albumin) was chosen for further study. The full scan MS shown in (Figure 2B) demonstrates the high abundance of the 481 Da doubly charged ion from one of the albumin peptides identified (FQNALLVR). This sequence is then confirmed in (Figure 2C) with an abundance of y- and b-ions. This experimental approach lays the groundwork for choosing an appropriate AQUA peptide in addition to providing the necessary information for designing the SRM experiment. As shown in (Figure 2B and C), the doubly charged parent ion of this specific albumin peptide was selected for fragmentation and the two most abundant y-ions present in the tandem MS were selected for detection.

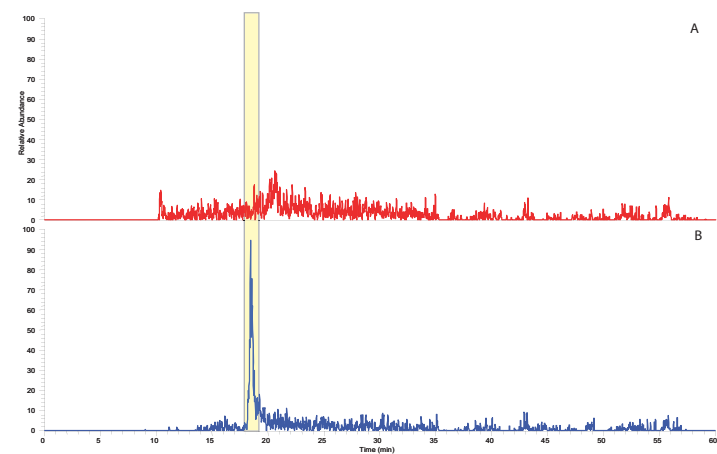


**Figure 3: Protein-AQUA Analysis of Albumin in Whole Plasma and ProteoPrep 20 Depleted Samples (using Protein-AQUA Peptide 1).** Selected reaction monitoring (SRM) specifically targets MS detection of the selected parent that produces specific daughter ions. (Figures 3A) (whole plasma) and (3C) (depleted) depict the endogenous albumin peptide, FQNALLVR, as the doubly charged ions fragment to produce two abundant y-ions. These same y-ions are monitored in the AQUA peptide standard shown in (Figures 3B) (whole plasma) and (3D) (depleted); however the absolute mass-to-charge of both parent and daughter ions in the AQUA spike are of higher mass due to the incorporation of a stable isotope amino acid. Quantitation is performed by integrating the co-eluting peak areas shown here at 34.8 minutes. Protein-AQUA analysis of the samples indicates 99.8% depletion of albumin using the ProteoPrep 20 Plasma Immunodepletion Kit, which is in excellent concordance with previously obtained ELISA data.



**Figure 4: Protein-AQUA Analysis of Albumin in ProteoPrep 20 Depleted Plasma (using Protein-AQUA Peptide 2).**

LC-SRM analysis of the predicted missed cleavage peptide, FQNALLVRYTK, results in a complex chromatogram with low signal-to-noise (Figure 4A), suggesting the endogenous peptide was not present in the depleted plasma digestion. However, a similar result is observed when the isotopically labeled version of the peptide is monitored (Figure 4B). Due to the fact the Protein-AQUA peptide was not observed, this indicates a suppression effect of the peptide target, indicating that the sequence is not suitable for quantitation of albumin.



**Figure 5: Protein-AQUA Analysis of Albumin in ProteoPrep 20 Depleted Plasma (using Protein-AQUA Peptide 3).**

LC-SRM analysis of the potential missed cleavage peptide, FQNALLVRYTKK, shows only background noise (Figure 5B) at the expected retention time (shown in yellow). However, analysis of the isotopically labeled version of this peptide shows a strong signal (Figure 5C). The lack of signal for the endogenous peptide indicates the potential missed cleavage peptide, FQNALLVRYTKK, is not present in the depleted plasma digestion.

## Conclusions

- Protein-AQUA is a powerful, precise quantitative proteomics tool
- Not all tryptic peptides are suitable sequences for Protein-AQUA Analysis
- The efficiency of trypsin digestion can be monitored by employing isotopically-labeled peptides corresponding to missed and/or partial cleavages of the protein being studied

## Acknowledgments

The authors wish to thank Jeffrey Porter for providing *E. coli* cell paste and Rick Mehigh for aiding in the selection of AQUA peptides.

## References

1. Anderson, N. L.; Anderson, N. G. The Human Plasma Proteome. *Mol. Cell. Proteomics* **2002**, *1*, 845.
2. Gerber, S.A., Rush, J., Stemman, O., Kirschner, M.W., and Gygi, S.P. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 6940-6945.