A Simplified Affinity Proteomics Workflow for Rapid, Sensitive, Quantitative Analysis of Proteins in Plasma

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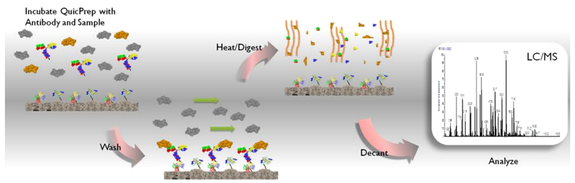
Overview

- Novel method for protein quantitation from plasma matrices
- Sample to results in <4 hours
- Potential for rapid method development
- LLOQ of 1ng/mL achieved on an analytical flow instrument through rapid affinity sample cleanup

Introduction

The improving efficacy of many biological therapeutics and identification of low level biomarkers is driving the analytical community to deal with extremely high levels of analyte and sample complexity. Many protein quantitation and biomarker validation procedures utilize an immunoaffinity enrichment step to purify the sample and maximize the sensitivity of the corresponding liquid chromatography tandem mass spectrometry measurements. In order to generate surrogate peptides with better mass spectrometric properties, protein enrichment is followed by a proteolytic cleavage step. This is often a time consuming, multistep, serial process. Presented here is a workflow which enables rapid protein enrichment and proteolytic cleavage to be performed in a single, easy to use reactor in under 4 hours.

Methods



Human Immunoglobulin G (IgG) and NHS-biotin were obtained from Sigma Aldrich (St Louis, MO). Anti-Human IgG (a-hIgG) was obtained from Pierce (Rockwood, IL). Mouse Plasma was obtained from Golden West Biologicals (Temecula, CA). Perfinity QuicPrep™ kits are made by Perfinity Biosciences, Inc (West Lafayette, IN).

Samples containing varying amounts of IgG in 500µL of mouse plasma, 3µg of a-hIgG, and QuicPrep – a co-immobilized streptavidin and heat activated temperate stable trypsin – were incubated together in 1.5 mL Eppendorf LoBind tubes at room temperature for 2 hours. Following incubation, the resin was washed 5 times with a wash buffer at room temperature which prevented trypsin activation. After washing, digestion buffer was added to the resin and the entire system was incubated at 70°C and 1400 rpm for 1 hour. Samples were then analyzed on a Shimadzu Nexera coupled to a SCIEX Triple Quad™ 5500 System.

A duplicate set of samples was processed using a magnetic form of the co-immobilized streptavidin and heat activated temperature stable trypsin. This was done to test the effectiveness of magnetization as a method of sample handling and to ensure that performance was equivalent on magnetic materials. These samples were analyzed using the same detection system.

Human IgG detection:
LC system: Shimadzu Nexera LC 30 Series
MS System: SCIEX Triple Quad™ 5500 System
Column: Kinetex XB-C18 100A column (2.1x50mm, 2.6µm)
Column conditions: 40°C with a flow rate of 500 µL/min
Solvent A: 0.1% formic acid in 98% water and 2% acetonitrile
Solvent B: 0.1% formic acid in 10% water and 90% acetonitrile
Gradient: 10%B kept for 1 minute then ramped to 70% B over 5 minutes
Injection volume: 20 µL digested purified protein solution
MS Settings: Positive Mode; CEM: 2100; CUR: 30; IS: 2500; Probe temp: 450°C; Sheath Gas: 40; Aux Gas: 40; CAD: 5; DP: 125; EP: 10; CXP:14
Monitored transitions:
Q1 Mass: 937.7; Q3 Mass: 836.5; Dwell: 100msec; CE: 40
Q1 Mass: 603.4; Q3 Mass: 805.4; Dwell: 100msec; CE: 40

Data was analyzed using Analyst 1.5.2 and tabulated using Excel.

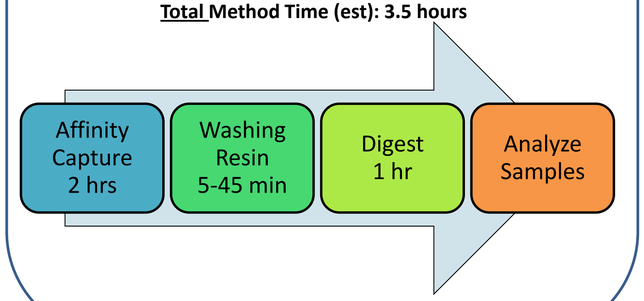
Results

Workflow Time Reduction

The co-immobilized streptavidin and heat activated temperature stable trypsin allow for a dramatically accelerated workflow with very high sensitivities. The ability to perform digestion on the same resin as the antigen capture removes the need for elution and buffer exchange. Additionally, the thermally stable trypsin allows for a rapid digestion that is significantly faster than the off-bead digestion protocols that may be found in the literature. Coupling these two advancements allows for a workflow with unprecedented speed and sensitivity from raw sample to purified digest.

Magnetic separation was very rapid and required less washing due to the ability to decant larger volumes without disturbing the resin. Coupled with an automated system, washing should be easily completed in 5 minutes or less.

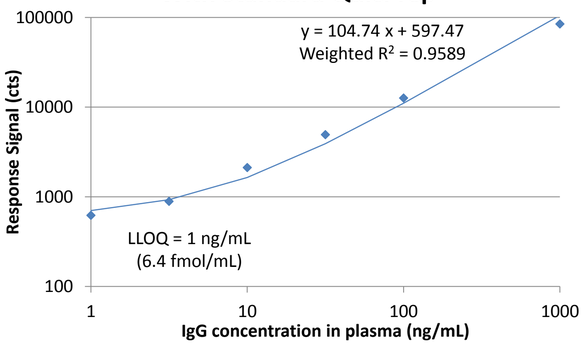
Total Method Time (est): 3.5 hours



Detection of human IgG in plasma samples

Using the workflow specified below we analyzed our samples employing a previously investigated tryptic peptide from the Fc region of human IgG [1]. Using this peptide we were able to detect single-ng/mL levels of human IgG in mouse plasma consistently and without the use of an internal standard, still maintaining CVs below 14% for all points, including our LLOQ.

Calibration Curve of Human IgG in Mouse Plasma With Standard QuicPrep

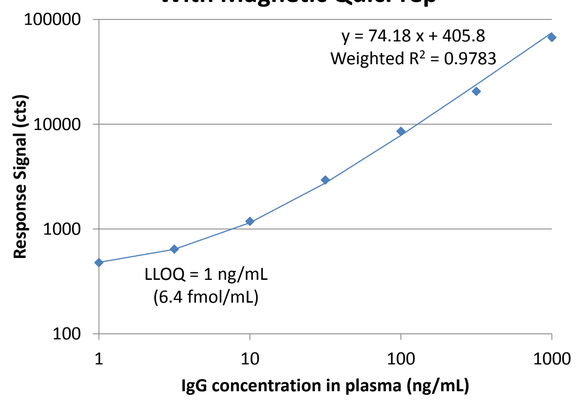


Regression: Linear, weighted to 1/x where x = analyte concentration

Using Magnetic Materials For Ease of Automation

Using the magnetic materials a similar calibration curve was developed. The signal was only slightly lower, and was still within the range of run-to-run variance for samples without an internal standard. All CVs were below 12%, including the LLOQ.

Calibration Curve of Human IgG in Mouse Plasma With Magnetic QuicPrep



Regression: Linear, weighted to 1/x where x = analyte concentration

Conclusions

Workflow time

A critical part of protein analysis in pharmaceuticals, food, agriculture, and many other industries is developing detection methods with good sensitivities. Our ability to purify protein from plasma and perform a complete digestion in under 4 hours dramatically improves upon what is currently available. The capture protocols found in the literature typically require overnight binding of the biotinylated antibody before sample addition as well as a multistep process to prepare purified samples for digestion [2].

IgG Detection Capabilities

Previously reported sensitivities for IgG in plasma with the application of affinity or other sample cleanup steps is limited to LLOQs at the 20-100 ng/mL level [2,3]. Here we have shown that by performing rapid immunoaffinity, minimizing sample handling and losses, and achieving a complete digestion without the peak decay caused by autolysis we are able to quantitatively detect human IgG in mouse plasma down to levels of 1 ng/mL, more than an order of magnitude lower than previously reported.

As pharmaceuticals grow more efficacious, reporting regulations on food and agriculture grow more rigorous, and more low abundance proteins are shown to be key biomarkers, effective digestion and detection strategies are becoming increasingly critical to effective work flows in proteomics analysis.

The work here shows that a well designed co-immobilized streptavidin and heat-activated temperature stable trypsin such as the QuicPrep kits is capable of significantly increasing assay sensitivity. This should also hold true with a wide array of immunoaffinity targets and matrices.

References

1. Kaur S, Saad O, Xu K (2012) US Patent No. 2012155019. Washington, DC: U.S. Patent and Trademark Office.
2. Li H, Oritz R, Tran L, Hall M, Spahr C, Walker K, Laudemann J, Miller S, Salimi-Moosavi H, Lee JW. (2012) General LC-MS/MS method approach to quantify therapeutic monoclonal antibodies using a common whole antibody internal standard with application to preclinical studies. Analytical Chemistry 84:1267-1273.
3. Becher F, Dubois M, Fenaille F, Ezan E. (2013) Mass spectrometry protocol for the absolute quantification of a monoclonal antibody in serum with immunopurification. Methods in Molecular Biology 988:345-352.