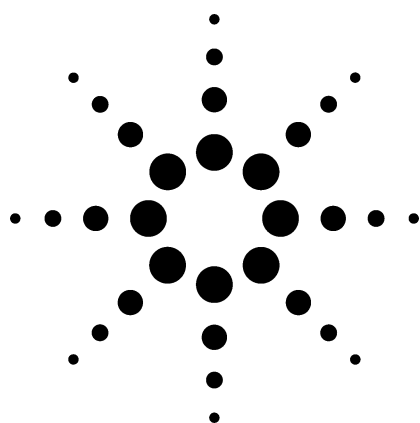# Removal of Multiple High-Abundant Proteins from Monkey Plasma for Proteomics Sample Preparation

Application

Proteomics

Author

Nelson Guerreiro and Stephane Charmont

Novartis Pharma AG

BioMarker Development

CH 4002 Basel, Switzerland

Abstract

The plasma proteome constitutes a highly complex array of circulating proteins, and is a rich source of potential diagnostic and prognostic biomarkers. The application of proteomic technologies for biomarker discovery has been limited by the presence of a few highly-abundant proteins, which constitute over 80% of the total plasma proteins. The ability to effectively remove high-abundant proteins in plasma is essential in order to increase the detection sensitivity of proteins present in low abundance. In this study we show the applicability of the Agilent Multiple Affinity Removal System for the immunodepletion of high-abundant target proteins from monkey plasma prior to separation by two-dimensional gel electrophoresis (2DGE). The removal of six highly-abundant plasma proteins enhanced the detection and resolution of less-abundant proteins by 2DGE. In conclusion, efficient and selective immunodepletion of plasma proteins prior to proteomic separation can increase the likelihood of detecting novel biomarkers

Introduction

Blood plasma is a rich source of circulating molecules derived from many tissues and processes that can act as indicators of a physiological condition.

Currently in the drug development and risk assessment process there is a growing interest in the discovery and use of biomarkers in plasma. The noninvasive collection of plasma is particularly amenable for monitoring treatment-related effects in a clinical setting. Many of the rapidly evolving technologies of proteomics are being used to profile the dynamic changes in the plasma proteome associated with treatment or disease. Plasma, however, is exceptionally difficult to analyze due to a dynamic range of more than 10 orders of magnitude in concentration that separate the highest- and lowest-abundant proteins. This is further compounded by the presence of a few high-abundant proteins such as albumin and immunoglobulins, which together may account for as much as 80% of the total plasma protein. A larger number of proteins, including potential biomarkers, are present at far lower concentrations. Removal of abundant serum proteins will help increase the relative amounts of less-abundant proteins to the detectable dynamic range of the various proteomic platforms.

The Multiple Affinity Removal System developed by Agilent has been shown to simultaneously remove multiple high-abundant proteins from human plasma/serum in a single step with high specificity. The Multiple Affinity Removal System is comprised of an affinity column packed with immobilized affinity-purified polyclonal antibodies designed for the removal of human albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin. In drug development and risk assessment, various

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animal models are used in preclinical safety to monitor the safety of compounds before going into man. The identification of circulating biomarkers from these animal models, which are indicative of an adverse effect, can be of high value to treatment monitoring of patients in clinical trials. In this application, we have investigated the applicability of the Multiple Affinity Removal System for the removal of high-abundant proteins in plasma samples collected from cynomolgus monkey (*macaca fascicularis*).

Experimental

Immunoaffinity Chromatography

High-abundant protein removal from crude monkey and human plasma was performed according to a standard liquid chromatography (LC) protocol provided with the Agilent Multiple Affinity Removal System. Briefly, crude plasma samples were diluted five times with Buffer A containing protease inhibitor PMSF and filtered through 0.22-µm spin filters by spinning at $16000 \times g$. A 200-µL sample of diluted plasma was used for injection in Buffer A (0% B) at a flow rate of 0.5 mL/min for 10 min. After the flow-through fraction was collected, the bound fraction was eluted with Buffer B (100% B) at a flow rate of 1 mL/min for 7 min. Collected flow-through fractions were stored at –20 °C until use.

Preparation of Protein Fractions for 2DGE

Flow-through fractions from four injections were pooled into a spin concentrator with 5 kDa molecular weight cutoff. The samples were spun at $7500 \times g$ at 10 °C. Buffer exchange was performed by three rounds of spinning of 4 mL of buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and PMSF. Protein content was determined using Bradford assay (BioRad). Concentrated protein fractions with 250 µg of proteins were diluted to 350 µL in IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% IPG 3–10 pharmalytes) and applied to an IPG strip for overnight in-gel sample rehydration. IPG gel strips were focused for 67500 Vh at 20 °C using the Multiphor II unit (Amersham, Uppsala, Sweden). The second dimension (SDS-PAGE) was carried out on vertical

gradient slab gels (9%–18% T) using the ISO-DALT system. The gels were stained with SyproRuby® (Molecular Probes, Eugene OR) following standard procedures.

Results and Discussion

A representative chromatogram of protein depletion from monkey plasma on a 4.6 mm × 100-mm Agilent Multiple Affinity Removal column is shown in Figure 1A. The resulting chromatogram is almost identical to that obtained for human plasma (Figure 1B) under the same running conditions.
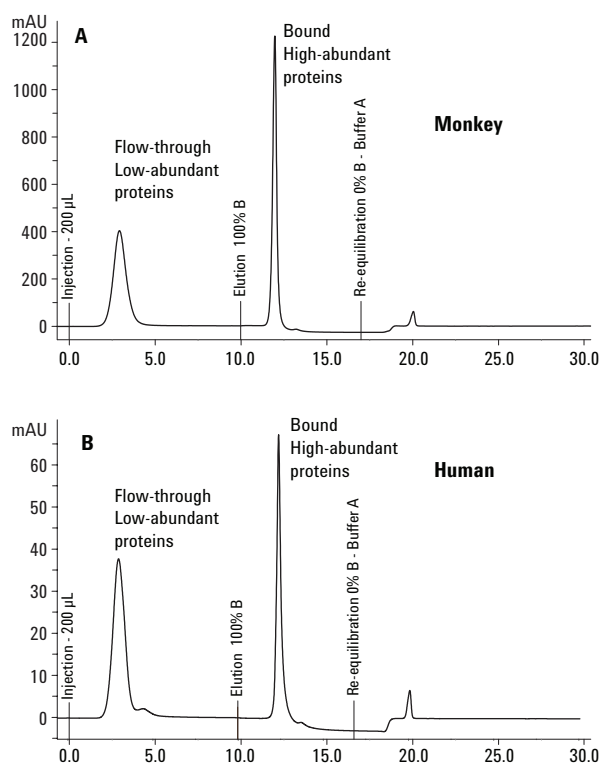

Figure 1. **Chromatogram of the affinity removal of high-abundant proteins from A) monkey plasma and B) Human plasma. A 200-µL sample of 5 × diluted plasma was used for injection in Buffer A (0% B) at a flow rate of 0.5 mL/min for 10 min on a 4.6 mm × 100-mm Multiple Affinity Removal Column. Depleted protein fractions (flow-through) were collected from 2–4 min. The bound fractions were eluted with Buffer B (100% B) at a flow rate of 1 mL/min for 7 min.**

The flow-through fraction and bound fraction of monkey plasma were collected and concentrated. A volume of sample containing 250 µg of protein was loaded for 2DGE. Figure 2 shows the 2D protein profile of the flow-through fraction and the bound fraction compared to crude serum. Circled areas indicate where the targeted high-abundant proteins are removed. Proteins that bind to the column are clearly absent from the flow-through fraction indicating a high degree of efficiency in the performance of the affinity column. However, spots possibly representing haptoglobin could still be seen in the flow-through fraction of monkey plasma albeit at much lower intensities when compared to the bound fraction. The identity of these spots has not been confirmed by mass spectrometry (MS). Furthermore, numerous additional spots were detected in the bound fraction and are likely to represent albumin fragments.

Concomitant to the removal of the six abundant proteins there was a significant enhancement in the staining intensity of several protein spots. In addition, many less-abundant proteins that were obscured by comigration with the high-abundant target proteins are now visible. Figure 3 shows a 2D region in a gel displaying improved resolution and detection of proteins after immunodepletion of monkey and human plasma samples.

Results from this study demonstrate that the Agilent Multiple Affinity Removal System for human proteins can be applied for effective removal of high-abundant proteins from monkey plasma. The removal of these highly-abundant plasma proteins enhances the detection of less abundant proteins by 2DGE, and aids the display of proteins that comigrate and are otherwise obscured by the widely spread pattern of highly-abundant proteins. In addition, computer-aided image analysis of the 2D profiles is improved, particularly with gel-to-gel matching and quantitation.
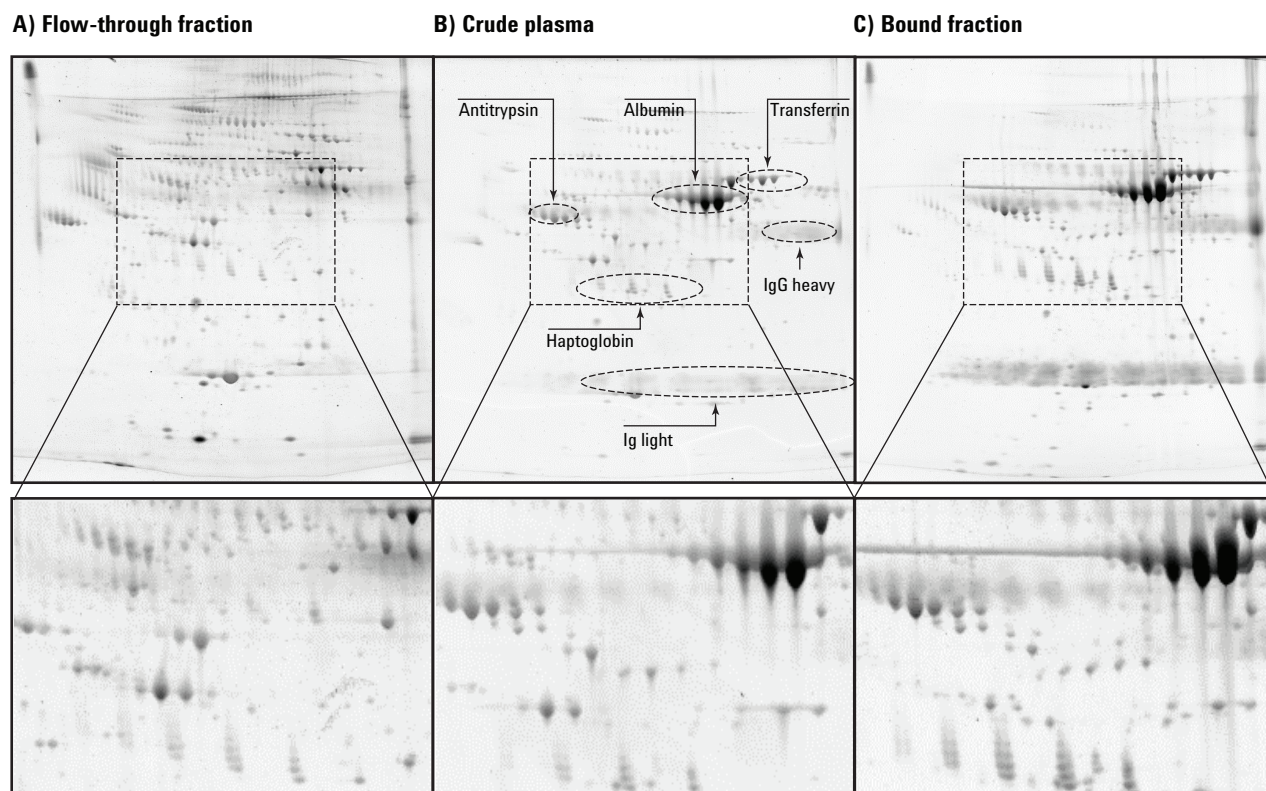
A) Flow-through fraction   **B) Crude plasma**   **C) Bound fraction**

Figure 2.   **2DGE profile of monkey plasma before and after immunodepletion with the Multiple Affinity Removal System. Gel images represent A) flow-through fraction B) crude plasma and C) bound fraction. Regions corresponding to albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin are circled. Enlarged areas show increased resolution. In the first dimension, proteins were focused on IPG strips (pH 4–7 linear, 18 cm) for 67500 Vh. In the second dimension, proteins were separated on vertical gradient slab gels (9%–18%). The gels were stained with SyproRuby .**
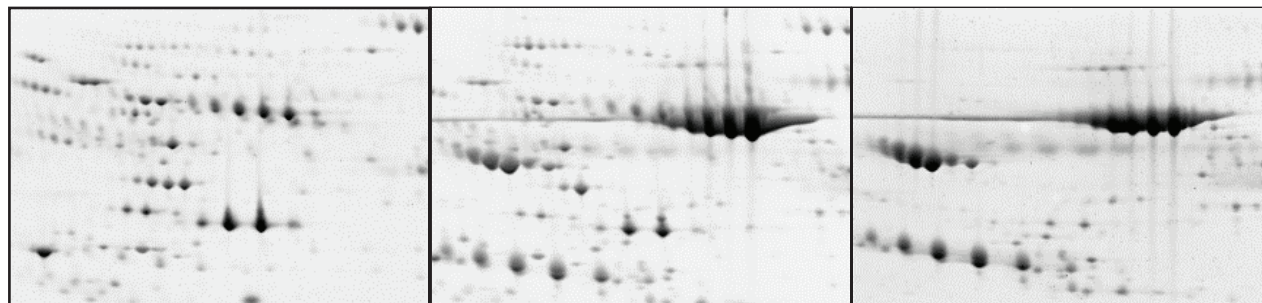
A

Human

Flow-through fraction | Crude plasma | Bound fraction
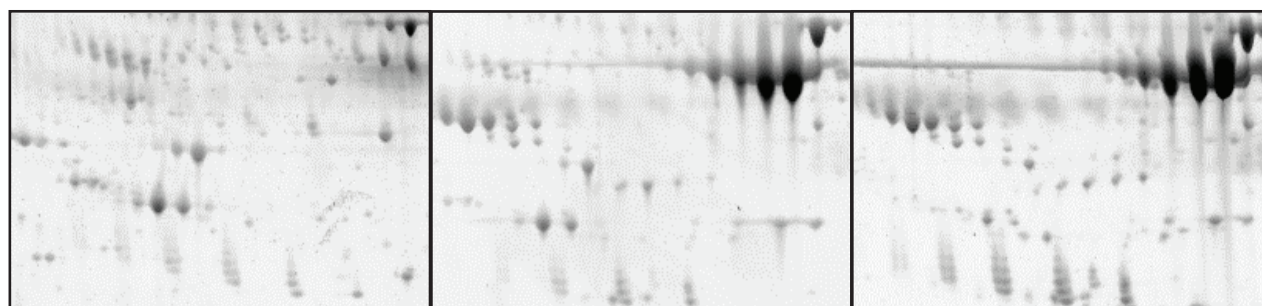

B

Monkey

Figure 3. Improved resolution and detection of proteins after immunodepletion. Corresponding regions of the 2D gel from A) human and B) monkey plasma samples after immunodepletion.

Additional data to the above mentioned will be released in a future journal publication

Nelson Guerreiro and Stephane Charmont are Principal Scientists at Novartis Pharma AG, BioMarker Development in Basel Switzerland.

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