

# Exploring the use of cICAT for the Discovery of Biomarkers in Plasma

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## INTRODUCTION

1. We have assessed the cleavable Isotope-Coded Affinity Tag (cICAT) for the identification of disease markers in plasma. The abundance of albumin and immunoglobulin within serum/plasma presents a huge analytical challenge, as biomarkers of interest are expected to circulate at concentrations several orders of magnitude below this level. To address this issue we have used test plasma from stroke patients that has been depleted of albumin and IgG. We report identification and quantitation of two proteins.

2. Prefractionation allows increased detection of low abundant proteins in a highly dynamic and complex sample such as serum/plasma. Therefore, in conjunction with cICAT, we investigated different depletion and separation methods. One of the advantages of depletion is that the removal of highly abundant proteins allows more serum/plasma to be used for analysis. Samples were depleted using the BioRad Aurum kit or the Agilent affinity column and the results were compared to data obtained from undepleted samples.

## 1. ANALYSIS OF THE STROKE SAMPLES

A pool of 37 plasma samples from stroke patients and a control pool of 20 plasma samples were depleted of albumin and IgG using the BioRad kit, quantified by LC/MS and proteins identified by directed-LC/MS/MS. The aim was to identify potential biomarkers for stroke.

**Method:** The stroke and control plasma pools were treated following the cICAT protocol. After tryptic digestion and labelling of the stroke and control plasma pools with light and heavy cICAT reagents, respectively, the two pools were mixed. Ten strong cation exchange fractions including the flow-through were obtained, followed by enrichment for cysteine-containing peptides on an avidin affinity column. The fractions were acid-cleaved and lyophilised prior to MS analysis.

The raw MS data for all 10 fractions was manually interrogated to determine peptides, which were differentially expressed. The data were normalised to light and heavy cICAT-labelled ion pairs generated by Mascot Distiller (Matrix Science) based on mass difference and m/z ratio present at a 1:1 ratio. A correction factor of 1.25 was calculated for normalisation of the data (Fig.1). The m/z of the most intense ion of each differential pair was added to an include list for directed MS/MS to obtain protein identification. Oxidised cICAT heavy and light labels were included as a variable modification within the search parameters of Mascot (Matrix Science), we deduced that this was a common side reaction in the process.

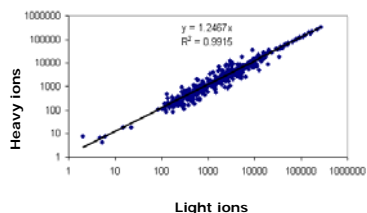


Figure 1. Normalisation value obtained using Mascot Distiller

**Results:** This preliminary study identified 58 proteins in plasma samples. Two proteins were found to be differentially expressed. MS survey data in Figure 2 illustrates an ion pair, identified as fibrinogen, as an example of a quantitative change in stroke (light) and control (heavy). Proteins found to be up-regulated in stroke are:

- Fibrinogen** – regulates soft clot formation and platelet aggregation.
- Antithrombin-III** – regulates the blood coagulation cascade.

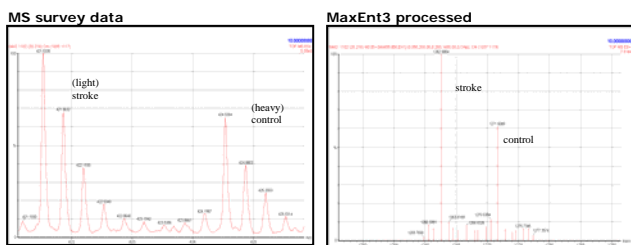


Figure 2. MS survey data showing the differential changes of fibrinogen beta chain

**Discussion:** The cICAT, LC/MS and directed LC/MS/MS strategy has enabled the identification of proteins that are differentially expressed in stroke. It is known that both antithrombin and fibrinogen are up-regulated following tissue damage (Fig. 3). The data shows that there is a 1.6- and 2.1-fold increase in abundance respectively, in the stroke compared to control.

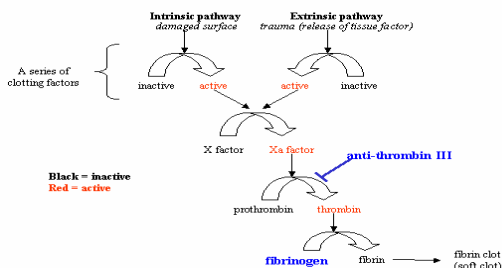


Figure 3. A simplified diagram of the blood clotting cascade

## 2. COMPARISON OF METHODS

The proteins identified, including those that vary in abundance between stroke patients and controls, are all abundant proteins in plasma. We have investigated methods to improve the detection of lower abundance proteins prior to labelling using cICAT. Here, we report comparisons of the number of proteins identified in undepleted and depleted serum using two different depletion methods, the BioRad kit (removal of albumin and IgG) and the Agilent column (removal of albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin). Sample preparation for each approach is shown in Figure 4.

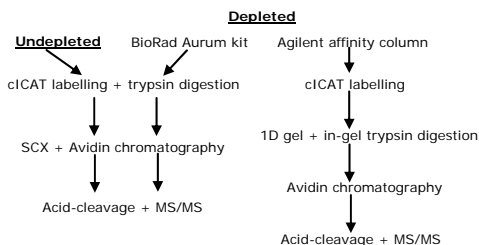


Figure 4. A flow-chart to illustrate the methods used for each sample

There was no significant difference between the total number of proteins found in crude serum and the BioRad kit depleted samples (Table 1). However, the total number of ions for albumin was significantly reduced and increased for the other plasma proteins. Whereas, the Agilent column depletion increased the number of proteins identified, with a reduction of the number of ions for the depleted proteins (albumin <0.1% and IgG 1.3%) and an increase for other plasma proteins including low abundance tissue leakage proteins. The pie-charts in Figure 5 illustrate the comparisons of the total number of ions identified for each protein group and gives an indication of the concentration of the proteins in each group.

Proteins	No. found in crude serum	No. found after BioRad kit	No. found after Agilent column
Serum albumin	1	1	1
Immunoglobulin G	4	4	3
Other immunoglobulin	8	8	6
Classical plasma proteins	50	44	77
Others	0	2	9
<b>Total</b>	<b>63</b>	<b>59</b>	<b>96</b>

Table 1. Number of proteins identified from crude serum and two depleted samples (BioRad kit and Agilent column-1D gel)

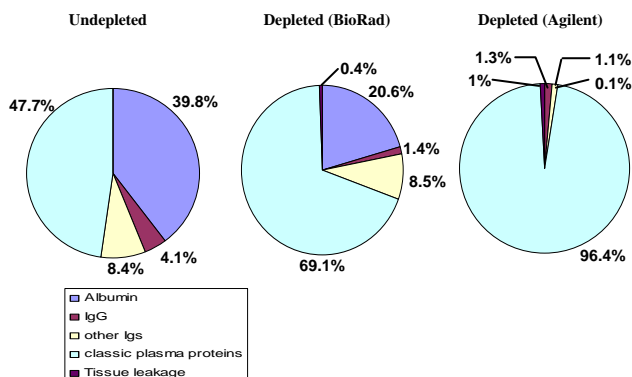


Figure 5. Pie-chart representation of the total number of ions found in each of the five groups of proteins as a percentage of the total ions

**Conclusions:** Depletion did not eliminate all albumin and IgG but the reduction has allowed detection of increased number of ions and proteins for the other groups. The total number of proteins identified in crude serum and following depletion using the BioRad kit were similar. A significant improvement in the number of proteins identified was obtained using the Agilent column followed by 1D gel separation. Levels of albumin and IgG were negligible.

