

# Analysis of Human Plasma Protein Mixtures by Automated 2D HPLC Coupled With Ion Trap Tandem Mass Spectrometer

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## Overview

**Purpose:** Demonstrate a method for global identification of proteins in complex sample mixtures.

**Methods:** Proteins from neat and HSA-stripped human plasma were digested enzymatically and separated by a two dimensional HPLC, using strong cation exchange and reversed phase chromatography, then analyzed by on-line LC/MS/MS with an ion trap mass spectrometer. Proteins were identified using TurboSEQUENT™ software to search observed MS/MS spectra against a human protein database.

**Results:** 359 proteins were identified with high confidence.

## Introduction

Protein identification using peptide mapping has become an important technique for proteomic studies. In this application, proteins are digested with a site-specific enzyme and analyzed by tandem mass spectrometry to generate MS/MS spectra. Comparing measured peptide fragment ions with those predicted by protein sequence, one can use protein databases to derive the identities of proteins in a sample. Previously, analysis of proteins relied on 1D- or 2D-gel separations, followed by MS analysis. In this paper, we describe analysis of a complex protein mixture, human plasma, using an automated 2D-LC/MS/MS system. Human plasma was digested and loaded onto a 0.32mm diameter strong cation exchange column and then gradually released to a 0.18mm diameter C18 column by stepwise elution with salt steps of increasing molarity. After reversed phase HPLC separation, the peptides were analyzed by a Finnigan LCQ™ Deca XP Plus mass spectrometer with a microspray interface. Using TurboSEQUENT software, more than 350 proteins were identified in one experiment. Compared to current gel-based methods, this on-line 2D LC/MS/MS system has several advantages: more peak capacity, higher sensitivity, greater throughput and a higher degree of automation.

## Methods

### Sample preparation:

HSA was removed from samples with Cibacron Blue (Sigma). Proteins from human serum lysates were reconstituted with 6M guanidine to 1 mg/ml. After reduction, alkylation, and proteolytic digestion, the final sample concentration was approximately 5 µg/µL. 17µL of sample were analyzed by 1D or 2D LC/MS. About 85 µg of total protein was injected for each analysis.

### HPLC conditions:

**1D LC:** Using a Finnigan ProteomeX™ Workstation (Thermo Finnigan), a flow rate of 200 µL/min was split and maintained at 1.5 µL/min. An 11-step salt gradient was used for sample fractionation on strong cation column (SCX) (Thermo Hypersil-Keystone) with steps of 0, 5, 10, 15, 20, 40, 60, 100, 200, 400, 800mM ammonium chloride. After step elution from SCX, peptides were eluted from a BioBasic™ C18 reversed phase column (Thermo Hypersil-Keystone) with a 120 minute gradient from 5% to 65% ACN in water, 0.1% formic acid.

**1D LC:** If only the reversed phase column was used for analysis, the gradient profile was set from 5% to 65% ACN in 240 minutes.

### MS conditions (LCQ Deca XP):

The temperature of the ion transfer tube was set at 140°C. The spray voltage was set at 3.2 kV and Normalized Collision Energy was set at 35% for MS/MS. Dynamic Exclusion™ was used with exclusion duration for 3 minutes. The data was analyzed by Xcalibur® software and the proteins were identified by TurboSEQUENT in the BioWorks™ 3.0 software suite (Thermo Finnigan).

## Results

Figure 1 shows the separation of human serum proteins with and without removal of human serum albumin (HSA). HSA is by far the most abundant protein in plasma, representing almost 70% by weight. Without removal of HSA, low abundance proteins will be not detected due to ion suppression by more prevalent HSA peptides.

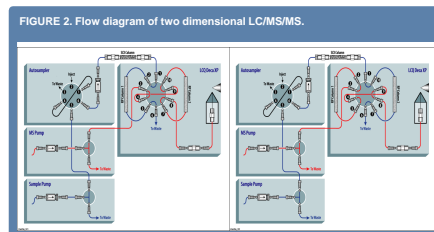
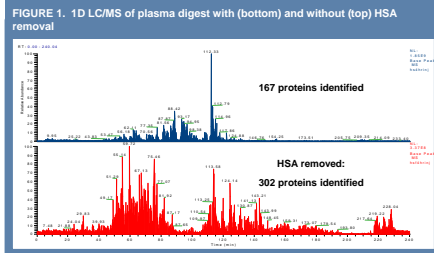


Figure 2 shows the flow diagram of an automated two dimensional LC/MS/MS system - ProteomeX. The system consists of two quaternary HPLC pumps, one autosampler and an ion trap mass spectrometer. One strong cation exchange column and two reversed phase C18 columns were used during analysis. The sample is loaded on the ion exchange column and eluted with a series of step gradients onto alternating C18 columns - while one C18 column is loaded and equilibrated, the second C18 column is eluted and analyzed by the mass spectrometer. This parallel process allows high-throughput analysis of complicated protein mixtures. The MS/MS spectra are analyzed by TurboSEQUENT, part of BioWorks 3.0 software. The peptide sequence is determined by comparison of experimental MS/MS spectra with predicted spectra from a relevant protein database.

Figure 3 shows the chromatogram of peptides from a digest of human serum. Eleven salt steps were used for the 2D separation. The 2D LC system increases the overall system peak capacity and greatly enhances the LC separation. Figure 4 shows a representative, high quality MS/MS spectrum upon which protein identifications are based.

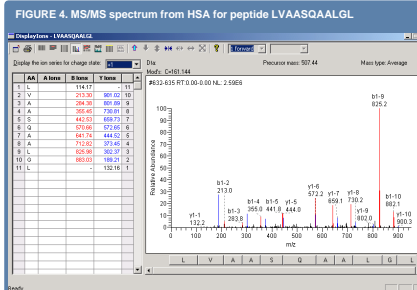
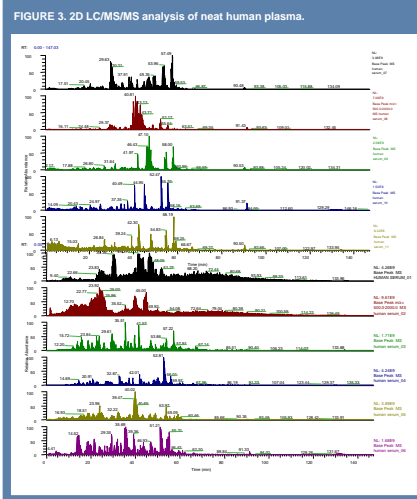


Table 1. The top 20 proteins identified by 2D LC/MS/MS analysis of human plasma

Neat Plasma	HSA removed
#1 ALBU_HUMAN SERUM ALBUMIN PRECURSOR	A1AT_HUMAN Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor) (A1AT-1)
#2 KAC_HUMAN KAPPA CHAIN C REGION	(NM_000566) complement component 3 precursor (Protein sapiens)
#3 A1AT_HUMAN Alpha-1-antitrypsin precursor (Alpha-1 pro	(NM_000566) complement component 3 precursor (Protein sapiens)
#4 TRIF_HUMAN TRICHOPTERIN PRECURSOR (ISODIOPHYLLIN IB	ITPA5 protein - human
#5 GC2_HUMAN IG G2MB1A CHAIN C REGION HEAVY CHAIN IDEK	(NM_005322) similar to Alpha-1-antitrypsin precursor (ACT-1) (Gene)
#6 GC2_HUMAN IG G2MB1A CHAIN C REGION	A Chain A, Transferrin (Also Called Prealbumin) Complex 199k, Mitron
#7 GC1_HUMAN IG G1MB1A CHAIN C REGION	(NM_000505) conalbumin (Hemostatic), Conalbumin (Protein sapiens)
#8 HPR_HUMAN HPTGLOBLIN-RELATED PROTEIN PRECURSOR	(NM_000143) heptoglobin (Protein sapiens)
#9 DMM_HUMAN DNA (cytosine-5)-methyltransferase 1 (DNM	A Chain A, Crystal Structure Of Human Apolipoprotein A-I-1
#10 PCCA_HUMAN PROPIONYL-CoA CARBOXYLASE ALPHA CHAIN, MT	03375 alpha-2-macroglobulin - human (Fragment)
#11 ALC1_HUMAN IG ALFA1 CHAIN C REGION	CRF4 complement C4A precursor - human
#12 APAA_HUMAN APOLIPOPROTEIN A1 PRECURSOR (APO-A)	Human Serum Albumin In A Complex With Malic Acid And Triiodothyronine Acid
#13 C03_HUMAN COMPLEMENT C3 PRECURSOR (CONTANG, C3A ANAPH	(ITP2_HUMAN) Inter-alpha-lysin inhibitor heavy chain H2 precursor (IT2 heavy ch)
#14 A2M3_HUMAN ALPHA-2-MACROGLOBULIN PRECURSOR (ALPHA-2-M	SC2101 complement component C2b - human (Fragment)
#15 V10B_HUMAN VITAMIN BINDING PROTEIN PRECURSOR (VBP)	SP4301 Inter-alpha-lysin inhibitor heavy chain H1 precursor - human
#16 A1AH_HUMAN ALPHA-1-ACID GLYCOPROTEIN 2 PRECURSOR (AGP	SP4301 Inter-alpha-lysin inhibitor heavy chain H1 precursor - human
#17 DAB2_HUMAN DISABLED HOMOLOG 2 (DIFFERENTIALLY EXPRESS	AFM_HUMAN Apolipoprotein A1V precursor (Apo-A1V)
#18 IF2P_HUMAN Translation initiation factor IF-2	T09H_HUMAN Trochalydin
#19 MCA2_HUMAN MITOCHONDRIAL CARNITINE/ACETLYCARNITINE CAR	(NM_000177) gelatin (gelatins), Porcine type I; Gelatin (Protein sapiens)

Table 1 shows the top 20 proteins identified in both the neat and HSA-stripped plasma samples. Proteins were selected based on charge state and Xcorr: +1, Xcorr>1.7; +2, Xcorr>2.0; +3, Xcorr>2.5. HSA is the most abundant protein in plasma and is the #1 protein identified in the neat sample. After treatment with Cibacron Blue, all the human albumin was removed and the A1AT\_HUMAN Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor) became the top choice in the protein search. It appears that removal of high abundance, known proteins may help the identification of lesser-abundant proteins. The total number of proteins identified increased from 245 to 359.

## Conclusions

1. Removal of HSA appears to aid in the identification of lesser-abundant proteins in human plasma.
2. 2D LC separation greatly increases peak capacity and allows the automated analysis of highly complex protein mixtures such as plasma.
3. TurboSEQUENT database searching provides confident protein identifications based on MS/MS spectral information.
4. ProteomeX, an integrated 2D LC/MS/MS workstation, combining separation, MS/MS analysis and TurboSEQUENT software to allow for automated analysis of complex biological samples.

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