

# Plasma Cleanup: A Prerequisite for Gel-based Biomarker Discovery

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## INTRODUCTION

There is currently widespread interest in government, academia and pharmaceutical companies in finding plasma or serum biomarkers for diseases and exposures. Because mass spectrometry provides the ability to identify and analyze femtomole levels of proteins, a great deal of effort is now focused on proteomic analysis of plasma and serum.

A key component in biomarker discovery is, of course, sample preparation. Our involvement with numerous groups pursuing biomarker discovery has led us to initiate experiments examining how different plasma and serum preparation protocols affect the ability to detect target plasma proteins, using 1-D and 2-D gel electrophoresis in combination with a variety of mass spectrometry techniques.

A major difficulty in uncovering serum or plasma biomarkers is presence of a small number of highly abundant proteins such as serum albumins and immunoglobulins. One solution to this problem is immunodepletion of particular proteins using one or more of the affinity matrices that are currently available.

## RESULTS AND DISCUSSION

Table 1. Serum purification kits used and their targets.

Kit	Target Protein(s)
Agilent Hu-6 Spin	HSA, IgG, IgA, Transferrin, Haptoglobin, $\alpha$ 1-Antitrypsin
Amersham	HSA, IgG
Genway Seppro-HSA	HAS
-Mix 6	HSA, IgG, Fibrinogen, Transferrin, IgA, IgM
-Mix 12	HSA, IgG, Fibrinogen, Transferrin, IgA, IgM, HDL (Apo A-I & Apo A-II) $\alpha$ 1-Antitrypsin, $\alpha$ 1-Acid Glycoprotein, IgY, $\alpha$ 2-Macroglobulin
Vivascience	HSA

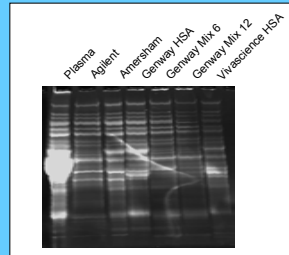


Figure 1. One-dimensional gel analysis of plasma cleanup. Following plasma processing samples 5% of the total protein was run on an SDS-polyacrylamide gel. An equivalent amount of unprocessed plasma was run for comparison. Note the differences observed between kits that nominally remove the same target protein (Amersham, Genway HSA and Vivascience HSA). With some kits non-targeted proteins may be being removed, potentially leading to loss of biomarkers.

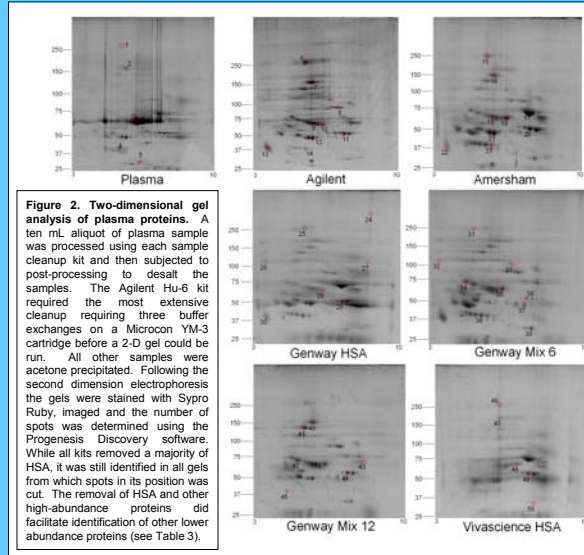


Figure 2. Two-dimensional gel analysis of plasma proteins. A ten mL aliquot of plasma sample was processed using each sample cleanup kit and then subjected to post-processing to desalt the samples. The Agilent Hu-6 kit required the most extensive cleanup requiring three buffer exchanges on a Microcon YM-3 cartridge before a 2-D gel could be run. All other samples were acetone precipitated. Following the second dimension electrophoresis the gels were stained with Sypro Ruby, imaged and the number of spots was determined using the Progenesis Discovery software. While all kits removed a majority of HSA, it was still identified in all gels from which spots in its position was cut. The removal of HSA and other high-abundance proteins did facilitate identification of other lower abundance proteins (see Table 3).

While the Agilent sample required the most extensive post-processing, it also gave the highest number of spots (see Table 3). This is likely due to the more effective removal of salts etc. that tend to reduce resolution in the first dimension. The Genway Mix 6 and Mix 12 gave the second highest numbers of spots. It should be noted that reduced yield with the Mix 12 may have contributed to the slightly lower number of spots detected. Several representative spots were then excised from the gels (numbered red circles) and subjected to in-gel digest and protein identification. While all kits removed a majority of HSA, it was still identified in all gels from which its position was cut. The removal of HSA and other high-abundance proteins did facilitate identification of other lower abundance proteins.

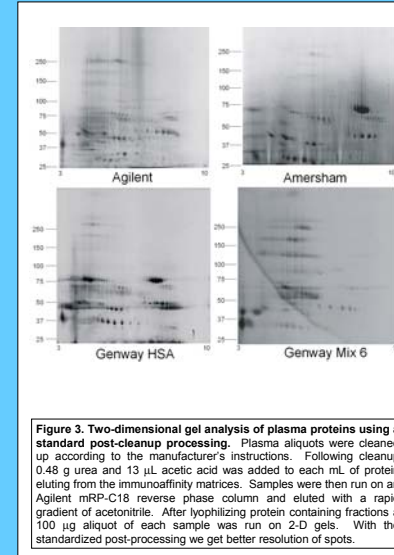


Figure 3. Two-dimensional gel analysis of plasma proteins using a standard post-cleanup processing. Plasma aliquots were cleaned up according to the manufacturer's instructions. Following cleanup 0.48 g urea and 13  $\mu$ L acetic acid was added to each mL of protein eluting from the immunoaffinity matrices. Samples were then run on an Agilent MRP-C18 reverse phase column and eluted with a rapid gradient of acetonitrile. After lyophilizing protein containing fractions a 100  $\mu$ g aliquot of each sample was run on 2-D gels. With the standardized post-processing we get better resolution of spots.

Spot	Protein	Spot	Protein	Spot	Protein	Spot	Protein	Spot	Protein
1	A*	11	Fibrinogen $\beta$ -chain precursor	21	Fibrinogen	31	B*	41	Fibrinogen $\alpha$ -chain precursor
2	A*	12	Fibrinogen	22	Cholesterol 2	32	B*	42	Actin
3	HSA	13	Cholesterol 1 or 2	23	Haptoglobin precursor	33	A*	43	Fibrinogen $\beta$ -chain precursor
4	HSA	14	Myosin heavy chain	24	B*	34	B*	44	Fibrinogen $\beta$ -chain precursor
5	HSA	15	B*	25	B*	35	HSA	45	A*
6	Fibrinectin	16	Complement factor H	26	A*	36	Fibrinogen $\beta$ -chain precursor	46	A*
7	Complement factor H	17	Transferrin	27	A*	37	A*	47	A*
8	Complement factor B	18	Hydroxyacid	28	HSA	38	A*	48	HSA
9	Hemoglobin	19	HSA	29	Fibrinogen $\beta$ -chain precursor	39	Fibrinogen $\beta$ -chain precursor	49	Fibrinogen $\beta$ -chain precursor
10	HSA	20	Fibrinogen $\beta$ -chain precursor	30	Cholesterol 1 or 2	40	Complement factor H	50	A*

Table 1. Protein identifications from the spots indicated in Figure 2.

A\* Non-trypsin peptides detected, but no significant hits from the database  
B\* Only trypsin peptides detected

Protein listed in blue was just below the level of significance

Table 2. A comparison of processing time and spot detection in 2-D gel analysis for the various cleanup kits.

Kit	Processing Time	Post-Cleanup Sample Prep. (time)	No. of Spots
Agilent	25 min	Microconcentrator (4+ hrs)	1308
Amersham	40 min	Acetone Precip. (2 hrs)	512
Genway HSA	30 min	Acetone Precip. (2 hrs)	545
Genway Mix 6	30 min	Acetone Precip. (2 hrs)	789
Genway Mix 12	30 min	Acetone Precip. (2 hrs)	745
Vivascience HSA	20 min	Acetone Precip. (2 hrs)	141

## CONCLUSIONS

- Highly abundant serum proteins must be removed from plasma samples to effectively identify proteins that may be biomarkers of disease.
- While more extensive post-cleanup processing is time consuming it generates cleaner samples with higher resolution in 2-D gel electrophoresis and more protein identifications.
- Given that protein biomarkers will likely be low abundance proteins, methods which increase the numbers of spots detected have the clear advantage. However a standardized post-cleanup protocol is required for effective side-by-side comparisons.

## ACKNOWLEDGEMENTS

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## METHODS

Six commercial depletion kits from four manufacturers were compared with respect to the efficiency of their depletion of plasma proteins, and the specificity of this removal. Plasma was collected from a single patient using a collection vial containing protease inhibitors. The plasma was stored at -80 °C. Aliquots of plasma were then processed according to each of the manufacturers protocols for the specific cleanup procedure.

For 1-dimensional gel analysis equivalent percentages of the processed plasma was loaded on the gel. For 2-dimensional gel analysis 100 mg of sample was analyzed (except Mix-12, 60 mg). For protein identification the indicated spots were excised from the gels and subjected to in-gel trypsin digest. Peptides were then analyzed on an ABI 4700 Proteomics Analyzer in positive ion mode and spectra were searched against the human protein MSDB database using Mascot.