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An Effective Method for the Analysis of Human Plasma Proteome using Two-dimensional Gel Electrophoresis

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Abstract

Proteome analysis of plasma is increasingly leading to biomarker discovery of human diseases. However the high-abundant proteins, excess of salt and lipid in plasma makes the analysis very challenging. Therefore it is necessary to improve the sample preparation procedures before/after the two-dimensional gel electrophoresis analysis of plasma proteins. The objective of this study was to develop a reproducible method by examining the following parameters: (1) depletion of the high-abundant proteins (2) effect of different precipitation methods (3) comparing optimised rehydration buffer using modified Taguchi method with the standard rehydration buffer and (4) comparing the effects of different staining methods. Our results showed that the depletion of two high-abundant proteins improved the visualization of less abundant proteins present in human plasma and precipitation with TCA/acetone resulted in an efficient sample concentration and desalting. We found that using optimized rehydration buffer as compared to standard rehydration buffer increased protein solubility, improved resolution and reproducibility of 2D gels. We also found that visualization of 2D gel profiles by silver staining and fluorescent staining enhanced the detection of low abundant plasma proteins as compared to Coomassie staining. In conclusion, the optimized conditions in our study can be applied to produce a better reference 2-DE gel of plasma samples for the identification of novel disease markers.

Keywords: Human blood plasma; Two-dimensional gel electrophoresis (2-DE); Proteomics

Abbreviations: 2DE: Two-Dimensional Electrophoresis; CHAPS: 3-[(3- Cholamidopropyl) dimethylammoniol]-1-propanesulfonate; DTT: Dithioreitol; EDTA: Ethylenediaminete-traacetic Acid; TCA: Tricholoroacitic Acid; SDS-PAGE: Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis; IEF: Isoelectric Focusing

Introduction

Human plasma is considered to be one of the best studied largest and most diverse proteomes among body fluids and its value in clinical diagnostic is well known (Jacobs et al., 2005; Neddelkov et al., 2005; Thadikkaran et al., 2005). As compared to serum, plasma tends to be preferred for proteome studies as its constituents reflect more closely to the pathological status of a patient than do the serum constituents. Beside having classic components such as albumin, immunoglobulins, hepatoglobulins, antitrypsin and transferin, it contains a host of other proteins including cytokines, growth factors, receptors, leakage products, aberrant secretions and foreign proteins. Alternative splicing and post-translational modification such as proteolysis further increase this repertoire. These protein concentrations in plasma are controlled tightly to balance their physiological functions in areas including immunity, coagulation, small molecule transport, and inflammation and lipid metabolism (Anderson et al., 2002). Therefore, alterations in plasma protein concentrations if specific for a certain disease situation, may then serve as biomarkers for screening the disease in individuals. Alternatively, they may be used to design specific antibodies or fragments for disease treatment or applied in diagnostic screenings. Recently, the discovery of biomarkers from blood plasma has become the subject of the intensive attention, with the considerable advances that have thus far been made in proteomics research. Several studies have been done to identify new biomarkers for disease and toxicity processes via proteomic techniques (Li et al., 2005; Allard et al., 2005; Zimmermann-Ivol et al., 2004). However, it has been estimated that over 10000 different proteins commonly present in the plasma, most of which are at very low relative abundances (Adkins et al., 2002). The majority of potential disease biomarkers may be present at extremely low concentrations at low ng/ml to pg/ml levels within the plasma, and thus might be masked by the presence of more abundant proteins with similar characteristics (Jacobs et al., 2005; Anderson et al., 2002). However, to detect these lower abundance proteins in plasma at least two problems must be settled: (1) Efficient throughput depletion strategy of high-abundance proteins and (2) postdepletion fractionation. Depletion of albumin or IgG has been demonstrated to enable greater sensitivity for the remaining proteins in the complex mixture of blood fluids (Adkins et al., 2002; Lollo et al., 1999).

As plasma contains such a variety of proteins in a wide and dynamic concentration range, two–dimensional gel electrophoresis (2-DE) has proven to be a valuable method for the separation and comparison of complex protein mixtures. It has major advantages in discovery of the overall alterations of protein expression from metabolic disorders and in identification of specific protein responding to a certain effect in uncharacterized

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crude samples (Watarai et al., 2000). The comparison of proteome expression between normal and abnormal states of biological samples such as tissue and body fluids provides information regarding the variation, relative quantities, and structures of the intact proteins. Development of immobilized pH gradients (IPG) coupled with pre-cast gradients polyacrylamide gels and introduction of new sensitive staining methods have considerably simplified and greatly improved the capacity, sensitivity and reproducibility of 2D gels. These recent technologies advances do not however eliminate a number of difficulties associated with the separation of 2DE. One major problem is the solubilisation of protein mixtures during Isoelectric focusing (IEF) (Rabilloud et al., 2002). As a consequence, conventional approaches for protein solubilsation and modification do not reliably provide the best samples for electrophoresis. However, 2-DE is in the face of an obstacle in the sample preparation and solubilisation of biological fluids. Especially, plasma is known to be difficult to resolve by 2-DE for the abundance of lipid and salt.

To find the optimal and most robust condition for 2DE, we applied depletion method, different precipitation methods and modified Taguchi method for the formulation of rehydration buffer used to solubilise human plasma protein samples. We also applied different staining methods in order to visualize the low abundance proteins present in the plasma. The resulting protocol, substantially improved the solubility and resolution of human plasma protein for 2-DE.

Materials and Methods

Sample collection and processing

Human blood sample from six healthy individuals were used. To prepare plasma, anticoagulants either EDTA, heparin or sodium citrate, were added to the blood specimens immediately after the blood was drawn to prevent clotting (EDTA plasma: 10 mL containing approximately 1.7 mg potassium EDTA; heparin plasma: 5 mL containing heparin 1 vial ; sodium citrate plasma: 10 mL containing 1 mL of 0.118 mol/L (3.2%) citrate solution). The specimens were then centrifuged at 1500 g for 10 mins /4 ^{0}C to avoid hemolysis, decanted and transferred into Eppendorf tubes as aliquots. To each 1.0 mL plasma aliquots, 10 µl of protease inhibitor were added to obtain the reproducible results by 2DE analysis (Hulmes et al., 2004). Proteases used are less active at lower temperature; therefore it is recommended that the plasma be prepared at as low a temperature as possible.

Depletion of high-abundance plasma proteins

Because albumin and immunoglobulin IgG collectively account for ~ 70 % of the total plasma protein content (Anderson et al., 2002), we selectively removed these proteins to enrich for proteins of lower abundance. A dye-based Proteoprep blue albumin and IgG depletion kit (Sigma Aldrich, Germany) was used according to the manufacturer ´s instructions. Briefly, the provided suspended slurry medium were added to the spin columns, centrifuged and equilibrated at 8000 x g for 10 seconds. The spin columns were collected in fresh collection tube. To each spin column 0.1 mL of plasma sample were added to the packed medium bed, incubated for 10 minutes, centrifuged at 8000 x g for 60 seconds, repeated the same step twice to remove the additional albumin. The two times depleted plasma were remained in the collection tube and pooled for optimal protein recovery. The albumin/IgG depleted plasma samples were stored at -80°C for long-term storage.

Acetone/TCA precipitation

A 100 µl of depleted plasma sample was diluted with 900 µl of 10 %TCA in acetone. The mixture was incubated overnight at -20^{0}C and centrifuged at 15 000g, 4^{0}C for 10 min. The supernatant was removed and 1000 µl of 90 % ice-cold acetone were added to wash the pellet. The sample was incubated at -20^{0}C for 10 min and centrifuged as above. The acetone containing supernatant was removed and the pellet was air dried. For 2D gel electrophoresis, the protein pellet was suspended in 100 µl of lysis buffer containing 8 M urea , 2.5 M thiourea, 40 Mm Tris-HCl , 3% w/v CHAPS and 0.5 % v/v Igepal CA-630. The protein sample was stored frozen at -80^{0}C until analysis.

TCA Precipitation

A 100ul of depleted plasma was diluted to 25 µl of 100 % TCA with vortexing. The diluted plasma solution was incubated for 10 min at 4^{0}C and centrifuged at 14 000 rpm for 5 min, the supernatant was removed and 200 µl of 90 % ice-cold acetone were added to wash the pellet. The pellet was dried by placing the tube in 95^{0}C heat block for 10 min to drive off acetone. For 2D gel electrophoresis, the pellet was suspended in 100 µl of lysis buffer as above.

Acetone precipitation

Four volumes of ice-cold acetone (400µl) were added to depleted plasma sample and kept overnight on ice. The sample solution was centrifuged at 15 000 g for 10 min at 4^{0}C, the supernatant was removed and the pellet was air dried. The pellet was suspended in 100 µl of lysis buffer as above.

Chloroform/Methanol Precipitation

Four volumes of methanol (400µl) and one volume of chloroform (100µl) were added to 100 µl depleted plasma sample and vortex well. Three volumes of milli-Q water was added to the mixture and centrifuged at 15000 g for 2 min. The aqueous layer was removed and four volumes of methanol were added and centrifuged 15000 g for 2 min. The aqueous layer was removed without disturbing the pellet. The pellet was dried and suspended in 100 µl of lysis buffer for 2D gel electrophoresis.

SDS-PAGE analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out with the Tris/glycine buffer system according to Laemmli, (1970). Two microlitres (20µg) albumin and IgG depleted proteins were separated under reducing conditions on 12% SDS-PAGE mini gels (10 x 10.5 cm) at 250 V, 40 mA, and constant currents for 2h and visualized by colloidal Comassie Blue G-250 or Silver staining according to standard protocols. The gels were scanned in Ultra Lum Omega 16Vs system.

One-dimensional IEF using the protean IEF cell

Total protein content in plasma samples was determined by Bradford assay and employed bovine albumin standards. Immobilized linear pH gradient strips (17 cm, pH 3-10, Bio-Rad) were rehydrated with the individual plasma samples, 500 µg of protein, in 300 µl of a improved rehydration buffer solution con-

taining 7 M urea , 2 M thiourea , 1.2%, w/v CHAPS, 0.4 % w/v ABS-14, 20 mM dithiothreitol (DTT) , 0.25%, v/v, pH 3-10 ampholytes and 0.005 % w/v bromophenol blue (BPB), for 18 h without current (in-gel passive rehydration). After rehydration, the focusing tray was renewed to remove any proteins not absorbed into the strip. IEF was conducted using a Protean IEF Cell (Bio-Rad) according to one of the following IEF parameters. (1) Standard procedure: 250 V for 20 min, linear ramping to 10 000 V for 2 h, 10,000 V for 45 kVh; (2) optimized procedure: 250 V for 1 h (slow ramping), changing the wicks every 30 min (to assist removal of ionic contaminants), 1000V for 1 h, linear ramping 10 000 V to over 3 h and a constant of 10 000V until approximately 60 kVh was reached. Strips were removed and stored at – 80^{0}C until run on the second dimension.

Two-dimensional SDS-PAGE

For 2DE analysis, individual samples (n=6) were repeated at minimum in triplicates. Prior to SDS-PAGE, the IPG strips were equilibrated twice for 15 min with gentle shaking. The first equilibration solution contained 50 mM of Tris-HCl, pH 8.8, 6 M urea, 30 % v/v glycerol, 2 % w/v SDS, 1 % w/v DTT and 0.01 % w/v BPB. In the second equilibration solution, DTT was replaced with 2.5% (w/v) iodoacetamide (Son WK et al, 2003). The equilibrated IPG strips were slightly rinsed with milli-Q water, blotted to remove excess equilibration buffer and then applied to SDS-PAGE gels (20 cm × 20cm × 1mm 8-19% polyacrylamide (30% (w/v)acrylamide:0.8% (w/v) bis-acrylamide, 37.5:1 stock) using a PROTEAN II XL system (Bio Rad) at 10mA per gel for 30 min followed by 35 mA per gel for 12 h until the dye front had run off the edge of the 2-D gel.

Protein visualization

Proteins were visualized using either Coomassie Blue staining, modified silver stain procedure compatible with MS (Yan et al., 2000) or SYPRO Ruby (Bio Rad). In Coomassie Blue staining method, after protein fixation for 2h with 50 % v/v methanol, containing 5% v/v phosphoric acid, the gels were stained with CBBG-250 for 12 h, destained with milli-Q water. The gels were scanned in Investigator ™ ProPic II Genomics Solutions. In sliver staining method, the gels were fixed in 50 % v/v methanol, 12 % v/v acetic acid and 0.05 % v/v formaldehyde for at least 2 h. The fixed gels were rinsed with 50% v/v ethanol three times for 20 min each, then again sensitized with 0.02% w/v sodium thiosulfate followed by three washings with milli-Q water each for 20 s. The gels were immersed in 0.1 % w/v silver nitrate and 0.075% v/v formaldehyde for 20 min and rinsed with milli-Q water twice for 20 s each. It was developed with 6 % sodium carbonate and 0.05 % v/v formaldehyde. Finally, the reaction was terminated by fixing with 50 % v/v methanol and 12 % v/v acetic acid. The stained gels were imaged using an Investigator ™ ProPic II Genomics Solutions. In SYPRO Ruby staining method, gels were fixed in 40 % v/v methanol and 10 % v/v acetic acid in milli-Q water for 1 h at room temperature then incubated in SYPRO Ruby for 16 h at room temperature on a rocking platform. Gels were destained for 1 h in 10 % v/v methanol and 7 % v/v acetic acid in milli-Q water and imaged using Investigator™ ProPic II Genomics Solutions.

Results

Our goal was to develop an effective method that had sufficient reproducibility to allow the accurate quantitative plasma proteomic analysis. In this study, an attempt is made to find out the various experimental steps (preparations) that improve the separation and identification of low-abundance proteins in human plasma.

SDS-PAGE analysis of depleted human plasma samples

In human plasma, highly abundant proteins such as albumin accounts for 57-71% (w/w) and IgG for 8- 26 % (w/w) of the total protein and they are therefore present at concentration of 35-50 mg/mL and 5-18 mg/mL, respectively (Anderson et al., 2002; Putnam, 1975; Putnam, 1983). Consequently, these proteins tend to prove overwhelming in 2-DE separation and detection assay. Therefore, the removal of these proteins facilitates the characterization of plasma proteomes. We applied Proteoprep blue albumin and IgG depletion kit from Sigma and removed the most abundant human plasma proteins, albumin and immunoglobulin chains. By the SDS-PAGE analysis of human plasma, we were able to confirm that the albumin band, at approximately 64 kDa and the IgG bands, at approximately 50 kDa and 25 kDa corresponding to heavy and light chains of IgG, respectively, had been effectively removed (Figure1). This dye-based depletion of human plasma (Figure1) clearly shows that the removal of two high abundance proteins lead to the appearance of several proteins that were not detectable in the non-depleted plasma samples.

Optimization of protein precipitation methods for sample preparation

Non protein contaminants also interfere with 2-DE, proving particularly detrimental to IEF (Shaw et al., 2003). Protein precipitation has been suggested for the removal of protease activity and biological contaminants and enrichment of proteins (Gorg et al., 2000; Jiang et al., 2004). However, precipitation is prone to protein loss due to poor precipitation or incomplete resolubilisation (Jiang et al., 2004; Bak-Jensen et al., 2004). To determine the effect of precipitation on depleted human plasma proteins, various procedures were carried out. Conventional acetone, TCA and chloroform/methanol precipitation resulted in

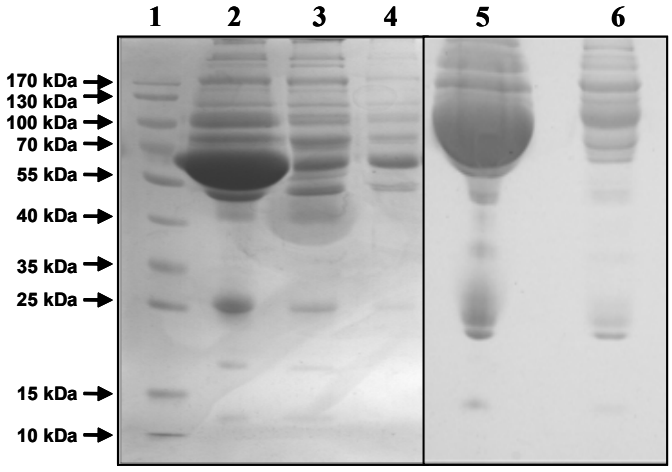

Figure 1: Effect of depletion of albumin and IgG on detection of low abundance plasma proteins. Lane1, Molecular weight marker; Lane 2, Undepleted human plasma, Lane 3 and Lane 4, depleted and eluted human plasma protein fraction detected by silver staining; Lane 5 and 6, Undepleted and depleted human plasma detected by coomassie staining.

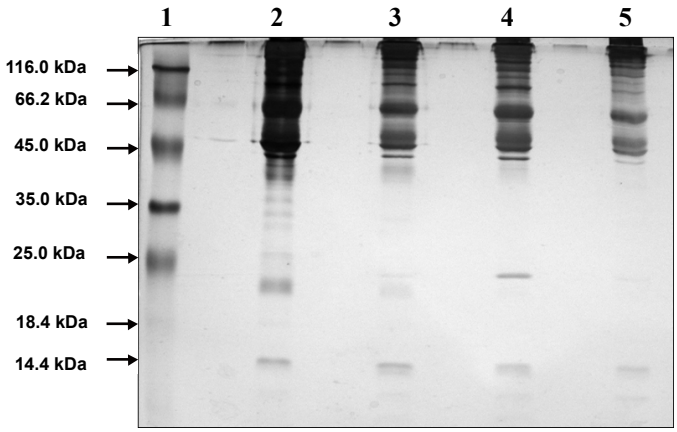

Figure 2: Effect of different precipitation protocol on detection of low abundance human plasma proteins. Lane1; Molecular weight marker; Lane 2; TCA/Acetone precipitation; Lane 3; Acetone precipitation; Lane 4; TCA precipitation; Lane-5 Chloroform/methanol precipitation.

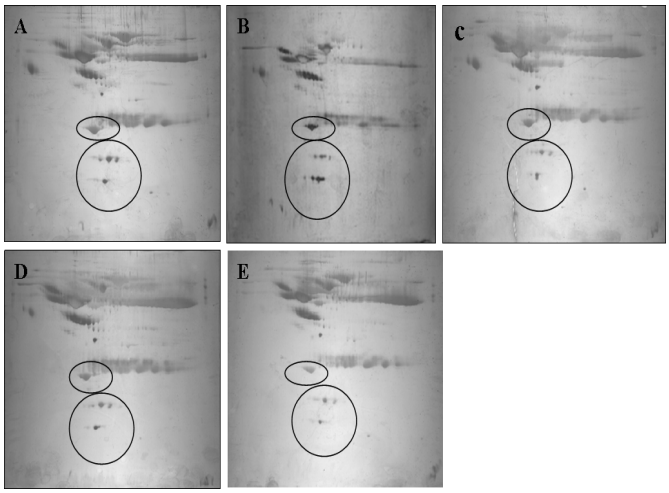

Figure 3: Effect of different precipitation protocol on detection of low abundance human plasma proteins by 2DGE. **A:** Depleted plasma. **B:** Depleted plasma after TCA/Acetone precipitation. **C:** Depleted plasma after Acetone precipitation. **D:** Depleted plasma TCA precipitation. **E:** Depleted plasma after Chloroform/methanol precipitation.

loss of protein as assessed by 1-D SDS-PAGE (Figure 2). In contrast, protein recovery was essentially better and satisfactory with TCA/acetone precipitation procedure. Figure 3 shows the results of the 2D electrophoresis analysis of four precipitation methods. No significant differences were observed between untreated plasma (Figure 3A) and treated samples (Figure 3B-E). On comparison it was found that the TCA/acetone precipitation method delivered a higher protein recovery. From the practical point of view, the easiest method to perform is precipitation with TCA/acetone, although it usually requires two steps, precipitation with TCA and removal of TCA trace with acetone. Precipitation with acetone requires larger organic solvent volumes (at least three fold of sample volume) and it is inconvenient to perform if the original sample volume is larger than 300 µl. Thus, the method of choice is precipitation, where time and cost are important factors.

Optimization of rehydration buffer for the protein solubilisation of human plasma

To achieve the good solubilisation of plasma proteins without disaggregation, we applied a modified Taguchi method (Khoudoli et al., 2004) for the formulation of rehydration buffer and compared with the standard formulation of rehydration buffer contains 8M urea (PROTEAN IEF Cell, 2000). However the combination of chaotropes, 7 M urea and 2 M thiourea was reported to produce better 2D images with an immobilized pH gradient (IPG) compared to 8 M urea alone (Molly, 2000) and this mix was chose as the basis for all subsequent rehydration solutions. It was also found that the combination of CHAPS with another surfactant (ABS14) improves solubilisation potential of plasma proteins for 2DE. The addition of carrier ampholytes enhances the solubility of individual proteins as they approach their isoelectric points. They also produce an approximately uniform conductivity across a pH gradient without affecting its shape. We observed at least a 50 % increase in detected spot number in Taguchi rehydration buffer compared to the standard buffer composition as shown in Figure 4B.

Optimization of different staining methods

The depletion of highly abundant proteins is not sufficient by itself to detect low abundance peptide and protein biomarkers but that highly sensitive visualization detection technique have to be employed as well. The majority of general staining protocols require a 1 to 8 h staining step, followed by an equally long destaining step as in case of Coomassie brilliant blue (CBB) method. For this reason, we have chosen silver staining and fluorescent staining of the gels rather than Coomassie as the later produced fewer overall spots, thus fewer data points to evaluate as shown in Figure 5.

Discussion

In order to use the information rich proteomic analysis of

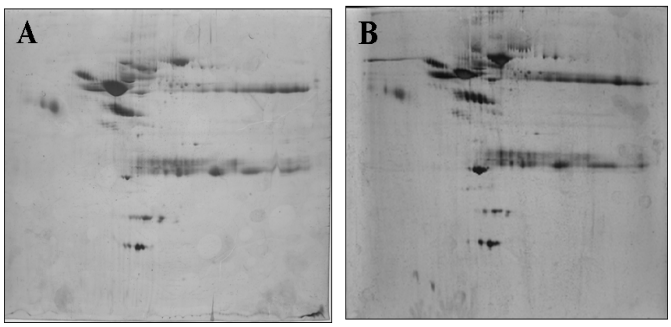

Figure 4: Effect of standard rehydration buffer and modified Taguchi rehydration buffer on 2DGE protein profiling of human plasma samples. **A:** Standard buffer composition, **B:** Modified Taguchi rehydration buffer composition.

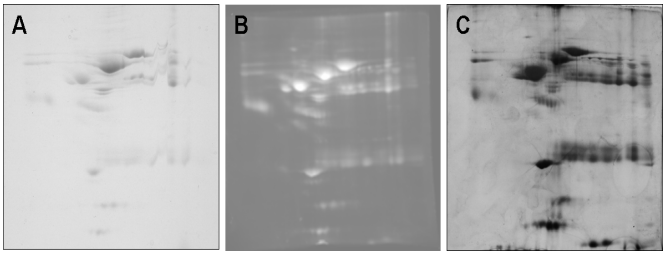

Figure 5: Comparison of sensitivity of different staining methods on 2DGE protein profiling of human plasma samples. **A:** Coomassie stained, **B:** Sypro Ruby stained, **C:** Silver Stained.

plasma in a diagnostic manner, it is essential that the method used to prepare the sample provide reproducible results. Although a variety of proteomic techniques have been attempted so far, no generally applicable technique has yet been developed for the identification of biomarker that can replace 2-DE with regard to its ability to separate and display several thousand plasma proteins simultaneously. The selection of an appropriate blood plasma preparation method is important for confident 2-DE results. The goal of the present study was to find the optimized method for a high throughout sample analysis of human plasma by 2-DE. The use of plasma as a protein sample because a large number of plasma samples are usually analyzed for diagnostic purposes and marker detection. This work describes that the selection and use of anticoagulants and protease inhibitors during blood collection increases the chances for consistent results. The removal of highly abundant proteins using an albumin and IgG removal kit results in 4- to 6- fold increase in relative protein concentration of medium- and low- abundance proteins . As a result, the detection, identification and quantification of medium- and low- abundance human plasma proteins by proteomic methods should easily achieved and aid in the characterization of the important human plasma proteome. TCA/acetone precipitation improves the pattern generated during 2-DE. The contribution of different detergents in the rehydration solution improves the solubility and resolution of proteins on 2D gels. The staining profile of proteins with the most sensitive method improves the detection of low abundance proteins in plasma. In conclusion, our study suggests that by using these procedures/steps for sample preparation before and after 2-DE analysis one can increase the likelihood of discovery of biomarkers of high sensitivity and specificity that can be used in early disease detection, as well as to monitor disease progression.

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