Protein Depletion for Plasma and Serum Proteomic Analysis

Human plasma and serum represents an important biological material for disease diagnosis. However, the wide dynamic range in protein concentration remains a major challenge in the development of diagnostic assays for the very low concentration of biomarker proteins in the presence of high abundance proteins. A practical and effective strategy is to remove 99% of the diagnostically uninformative proteins in order to enhance the detection of the low abundance proteins and penetrate deeper into the plasma proteome. Among a number of plasma protein depletion techniques, the ProteoPrep® 20 represents the most powerful enabling technology currently available.

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1. INTRODUCTION

1.1 Why blood plasma?

Blood plasma is not only the most studied among biological fluids, but also the primary material for disease diagnosis. Blood plasma contains a very high concentration of proteins, typically in the range of 60-80 mg of protein per ml. Estimates of the number of proteins in blood plasma start from 10,000, but the actual number of distinct proteins may be several orders of magnitude higher [1,2]. This is because each protein has a potential for a variety of post-translational and metabolic modifications [3-6], both in normal and diseased cells.

The global composition of proteins in the blood plasma represents the plasma proteome. Perfusion of blood through the different organs and tissues can result in the addition of new proteins, removal of some proteins, or modification of existing proteins, which may vary according to specific physiological or pathological conditions [7-14]. It is logical to expect correlation between the proteomic profiles of blood plasma with the specific physiological or pathological states. A recent extensive compilation of human plasma proteins indicated that most of the major categories of proteins in the human body were represented in the blood plasma [15]. Thus, the plasma proteome is an ideal source of diagnostic markers and therapeutic targets for many human diseases [10,11,15]. A protein, or most likely a set of proteins, that undergo changes in concentration or structural composition (e.g. PTM) as a result of disease or physiological state can potentially be used as diagnostic biomarkers. A biomarker is an identified protein or group of proteins, which change in concentration or structural composition due to a particular disease state.

When blood is coagulated and centrifuged, a translucent liquid called serum separates as a top layer. The coagulated portion is presumed to be mostly fibrin and other proteins involved in the coagulation process. The serum still contains a very high concentration of proteins. While both plasma and serum have been extensively used for diagnostic purposes, there is an increasing trend towards the use of blood plasma for proteomic profiling to ensure that important proteins are not trapped and lost into the coagulated portion.

As alternatives to blood plasma and serum, proteomic analyses of other biological fluids such as cerebral spinal fluid (CSF), urine [16-18], saliva [19-21], interstitial fluid [22], amniotic fluid [23-26], follicular fluid [27], and platelet-derived microparticles in blood [28] are also now being investigated for diagnostic biomarker discovery. In addition, proteomic profiles of human tissues like the brain, heart, liver, lung, muscle, pancreas, spleen, and testis are now being explored [29]. While the usefulness of these alternative biological fluids or tissues has not yet been clearly established, it is very conceivable that their profiles will complement or supplement those obtained from blood plasma or serum proteomics.

1.2 Why proteomics?

There are two important biomolecular disciplines used in identifying disease-associated biomarkers: genomics and proteomics. In the genomics approach, genes that are associated with specific diseases or physiological processes are identified and studied. The Human Genome Project (HGP) led to the successful sequencing of the human genome [30,31], which resulted in the identification of about 20,000 – 25,000 genes in the human body [32]. In various diseased states the expression of specific genes may either be enhanced (turned on) or suppressed (turned off). Thus, the levels of mRNA generated from the relative expression of these genes have been thought to correlate to specific diseased states.

However, there are still questions about the correlation between the expression levels of mRNAs and the corresponding changes in expression levels of proteins expressed, whether in human tissues [12,33,34] or in yeast cells [35,36]. In addition, one gene may express multiple proteins [35,37], with multiple biological functions. Finally, the proteins expressed from the genes may undergo a variety of post-translational modifications [4,5], as well as isoforms [38], some of which may be important in disease processes. For example, human plasma has been shown to contain 22 different forms of α-1-antitrypsin [39]. In many cases, the processes that regulate post-translational protein modifications are independent of gene function. Thus, despite the abundance of scientific data, diagnostic approaches based on genomic studies are still limited and are not always practical for clinical use.

The obvious alternative is the proteomics approach since, as the final form of the gene product, proteins are most directly related with biological function. The proteome is also more responsive to physiological and diseased states, as well as external stimuli. The dynamic nature of the proteome, as opposed to the static nature of the genome, makes the proteome a "real time" indicator of physiological processes. The proteomes of normal and diseased states are quantitatively compared, and biomarker proteins are then identified based on their relative abundance or structural form (i.e. PTM state) [7,8,10,11,40-56]. Once identified, these biomarker proteins are utilized for developing diagnostic tools, and the processes that regulate their expression, processing and functions can be used as therapeutic targets for drug candidates. Proteomic analyses have been used to investigate potential biomarkers for such diseases as cancer [7,8,22,40-52,56-65], hemophilia [53], osteoarthritis [54], and cardiovascular diseases [55].

The major goal of plasma and serum proteomics is to obtain the most reliable information possible for diagnostic and therapeutic purposes. This requires the establishment of accurate and comprehensive baseline data of the serum proteome, including as many of the low abundance proteins as possible, against which subsequent data from a variety of serum samples can be compared. A baseline profile includes both the identification and quantitation of different proteins. Such a baseline would permit better detection of significant changes in biomarker levels as a result of specific physiological conditions or disease, as well as indicate whether the condition warrants further investigation. Highly sensitive and accurate biomarkers are very important in detecting the early onset of diseases, since these biomarker proteins are usually present at very low concentrations.

Although simple in principle, obtaining reliable baseline information is extremely difficult in practice [2,66]. Major issues include variability in sample collection and handling [66-69], a lack of standardized protocols and instrumentation [64,70-74], and differences in handling, processing and interpreting the data [68,75-79]. The recognition of the enormity of the problem and potential benefits of success has brought international cooperation and coordination within the research community, [e.g. Human Proteome Organisation (HUPO)]. HUPO was organized in an attempt to provide a comprehensive analysis of the proteins of human plasma and serum, annotate the entire human proteome, and make the data publicly accessible. An initial set of data generated from the Plasma Proteome Project (PPP) of HUPO identified 9504 proteins with one or more peptides, and 3020 proteins with two or more peptides and were taken to represent their Core Dataset [80]. A similar database has annotated gene products encoded by 3778 distinct genes [81]. Current data from HUPO and elsewhere have successfully mapped 6342 peptides to EnsEMBL 29.35b genome build [82]. As more sensitive procedures are developed, the number of proteins identified will likely increase. However, the present results indicate that the number of proteins identified is still below the predicted number of proteins present in the plasma or serum.

Proteomics is certainly a promising approach to revolutionize clinical diagnostics, improve prognosis, and lead to potentially life-saving medical treatments. However, it is very likely that genomics and proteomics will complement each other in establishing the most comprehensive approach to biomarker discovery and identification of therapeutic targets that will ultimately find clinical applications in the bedside.

1.3 The analytical challenge: Detecting the low abundance proteins

The presence of a large number of proteins in blood plasma makes human plasma an excellent material for discovering biomarkers for potential clinical diagnostics and therapeutics. However, it also represents a tremendous analytical challenge because the estimated dynamic range of protein concentrations in human serum may be up to 12 orders of magnitude [83-86]. Albumin, the most abundant protein, constitutes over half of the plasma proteins and is present at 30-50 mg/ml concentration. In contrast, most of the potential biomarkers are secreted into the blood stream at very low copy number [11,26,86-89], especially in the early onset of diseases [7,8,40,85,88]. For example, the cytokines and the prostate specific antigen (PSA) are present in the low pg/ml levels. Based on this wide dynamic range, quantitation of all proteins simultaneously in a single assay is enormously difficult. The more abundant proteins will certainly mask the detection of the very low abundance proteins.

The analytical challenge is further increased when we consider that the very low concentrations of potential biomarker proteins in raw samples are beyond the detection limit of most analytical instruments [90]. For example, while mass spectrometry (MS) represents the most sophisticated and sensitive analytical tool currently available, the current dynamic range of detection is only about 10^3 when analyzed in a single spectrum. Even when MS is combined with an on-line separation such as HPLC, enhancement of the dynamic range will only be in the 10^4 to 10^6 ranges.

Innovations in both sample preparation and protein analysis are therefore necessary to push the analytical capabilities towards the required 10^{12} dynamic range. In sample preparation, depletion of the abundant, mostly high molecular weight proteins is a necessity to enable loading of a much higher amount of the low copy and/or low molecular weight proteins for analysis. This strategy has been shown to effect a general enhancement of the intensity of the low abundance proteins, as discussed in greater detail in Section 2.

Innovations in protein analysis consist of a large group of multidimensional separation technologies that are applied orthogonally to fractionate the proteins and peptides prior to mass spectrometric analysis. These multidimensional technologies for protein and peptide separation vary in principle and instrumentation, and include such techniques as electrophoresis (1D-PAGE, 2D-PAGE, capillary, free-flow, etc.), chromatography (reversed-phase, ion exchange, size exclusion, affinity, etc), ultrafiltration, solvent precipitation, and other less common fractionation techniques. Traditionally, each orthogonal separation technique is a separate process step. However, a significant innovation was developed and termed Multi-Dimensional Protein Identification Technology (MuDPIT), where two separation techniques are achieved in a single column packed with two different separation matrices [91]. Typically MudPIT uses a strong cation exchange and a reversed phase resin in single column that can be interfaced directly with the mass spectrometer. This technology allows a higher level of automation in sample handling, analysis and data processing

Different combinations of these multidimensional separation technologies are used in both "top down" and "bottom up" proteomic analysis. In the "top down" approach [92] a mixture of proteins in a sample are separated into individual spots or fractions using different separation techniques, and the individual proteins are then analyzed by mass spectrometry to establish their identity. This is accomplished by determining the mass of the whole protein ion and then fragmenting the ionized protein to yield relatively large segments whose masses can then be deconvoluted and compared against known proteins in protein databases. On the other hand, the "bottom up" approach can be performed by using either of two strategies: In one strategy, samples containing a mixture of different proteins are subjected to multidimensional separation techniques and the individual protein spots or fractions are digested with trypsin to yield peptide fragments. With or without another separation step, the tryptic peptides are analyzed by mass spectrometry to establish their identify, either based on their peptide mass fingerprints or by further mass fragmentation to obtain sequence information. Recently, most "bottom up" proteomics employ the "shotgun" strategy [91,93-99] where, without prior separation, entire samples containing a mixture of a large number of different proteins, such as plasma or serum, are proteolytically digested into peptides. The peptides in the tryptic digest are then separated by multidimensional separation techniques and then analyzed by mass spectrometry to establish the identities of the proteins present in the sample. In other words, the "top down" approach utilizes the mass spectral information from the whole protein for identification, while in the "bottom up" approach the mass spectral data of the peptides are used to identify their source proteins. In both "top down" and "bottom up" proteomics, the combination of protein depletion and multidimensional separation technologies offer significant enhancement in sensitivity for low abundance proteins by removing the masking effect of the highly abundant proteins, thereby enabling deeper penetration into the plasma proteomes.

2. PROTEIN DEPLETION

Since protein depletion is becoming a common choice as the first dimension in orthogonal protein separation strategies, this subject will be emphasized in this review. Depletion of plasma proteins can be accomplished using different strategies, but the final goal is to separate the high abundance, non-diagnostic proteins from the low abundance proteins.

In the past, the fractions containing the most abundant proteins were presumed to be diagnostically unimportant and usually not analyzed. However, recent proteomic analyses indicate that other proteins may be concomitantly removed during depletion due to non-specific binding to the depleted proteins [26,70,73,74,100-111]. For example, comparative experiments between non-depleted serum and serum depleted of the six most abundant proteins have shown that while depletion significantly increased the number of proteins analyzed and identified, some of the proteins found in the non-depleted serum were not found in the depleted serum [70,109,112]. This is mostly attributed to the so-called "sponge effect," where small proteins and peptides may bind to large proteins that normally serve as their carriers [109,112]. In reality there is no quantitative data to show how much of the non-targeted proteins are non-specifically bound to the specifically depleted proteins, and how much are bound to the depletion matrices. Nevertheless, these observations raise concerns about the validity of the quantitative representation of the whole proteome when only the protein-depleted sample is analyzed. Therefore, for particular applications the specifically depleted bound fraction may also be analyzed to ensure that no important proteins are inadvertently omitted.

2.1 Depletion of albumin and the IgGs

Human serum albumin (HSA) and the various forms of immunoglobulins (IgGs) represent the most abundant proteins in the serum, constituting up to 80% of the total plasma proteins. The classical depletion strategy for albumin involves the use of the hydrophobic dye Cibacron blue, a chlorotriazine dye which has high affinity for albumin [104,105,113-115]. This strategy of removing albumin is still sometimes used in proteomic analyses because of it's relatively low cost [52,116-120]. Other small molecules have been designed (e.g. mimetic dyes) which demonstrate greater specificity than Cibacron Blue. Another classical affinity medium is the Protein A/G [121,122], which is used for the removal of the immunoglobulins [123,124]. As a group, the immunoglobulins represent the second most abundant proteins in the plasma or serum. A low cost depletion kit for simultaneous depletion of albumin and immunoglobulins (Cat. No. PROTBA) is available which includes both types of resins.

Comparative studies indicate that using antibody affinity ligands for HSA and IgG result in more specific depletion compared to the traditional

Cibacron blue/Protein A or G depletion methods [71,100,106]. Because of this demonstrated specificity, the trend is now towards the use of immunoaffinity media for most proteomic analyses. Affinity media are made up of matrices with covalently attached antibodies to the specific abundant proteins [15,124-126]. An immunoaffinity media for HSA and IgG depletion is available (Cat. No. PROTIA), conveniently packed as spin columns that are compatible with centrifugation.

Despite the efficiency of immunoaffinity media, depletion of more proteins besides HSA and the IgGs is necessary to enhance the detection of very low abundance proteins that are present at the low ng/ml to pg/ml levels. For example, it was estimated that even if 99.9% of albumin were removed, the remaining albumin concentration would be about 50 µg/ml, which is still 50,000-fold higher concentration compared to the tumor marker prostate-specific antigen [26,127,128]. In addition, there are still many other highly abundant proteins that can potentially mask the analysis of the low abundance proteins and should, therefore, be removed.

2.2 Depletion of six abundant proteins

While removing HSA and the IgGs has consistently shown improvement in the detection of some low abundance proteins, analytical efficiency is expected to improve even farther by increasing the number of proteins depleted. Depletion of 6 and 12 abundant proteins is expected to remove about 85% and 90%, respectively, of the total proteins [71,100]. For example, columns containing affinity ligands for the top six abundant proteins have been shown to improve the visualization, detection and identification of more low abundance proteins [38,70,73,74,99-101,106,109,112,129-133], when compared to depletion of only HSA and IgGs. In addition, data from the HUPO Plasma Proteome Project clearly showed that depletion of the most abundant proteins in serum, whether only albumin, albumin and IgGs, or the six most abundant proteins, improved detection of some of the low abundance proteins [80]. However, the same report also indicated "incomplete sampling of proteins is a dominant feature." Part of the reason is likely the limitation in the amount of sample that can be loaded for analysis, before the remaining high abundance proteins interfere with the analysis. An affinity column designed to remove the 12 most abundant proteins is also available, but experimental data on this product is yet to emerge.

2.3 Depletion of 20 abundant proteins

It has been suggested that removal of 18 to 22 of the most abundant proteins is desirable in order to effect an overall depletion of 98 to 99 percent of the total proteins [100,134]. A new affinity column with high binding capacity has been developed. The ProteoPrep® 20 Plasma Immunodepletion Kit (PROT20) is the only commercially available product that contains immunoaffinity ligands designed to remove 20 of the abundant proteins (Table 1) in human plasma or serum [128]. This novel technology is the most powerful tool currently available, and has demonstrated the ability to deplete more proteins to visualize low copy number proteins in plasma samples and subsequently identify them by mass spectrometry [135].

For convenience, the ProteoPrep 20 Plasma Immunodepletion Kit (PROT20) is supplied as a complete kit containing 3 spin columns and the necessary reagents and consumable supplies. The kit also includes protocols that have been optimized for specific applications. Carefully controlled tests [135] indicated that each spin column removed the 20 high abundance proteins with an average depletion of 99.6% when 10 x 8 µl plasma depletions were concentrated and depleted twice. This depletion enabled a 38-fold and a 3-fold increase, respectively, in the load of low abundance proteins compared to the sample without depletion and depletion of just 6 proteins. This enrichment consequently enabled the identification of several low abundance proteins that could not be detected either in the non-depleted serum nor the 6-protein depleted serum. Finally, the spin columns have high economic value because they are re-usable for at least 100 times. Ordering information for PROT20 and companion reagents/consumables is shown in Table 2.

As indicated previously, protein depletion can be considered an initial dimension in orthogonal protein separation, the purpose of which is to separate the highly abundant proteins from the low abundance proteins. Since the flow through from ProteoPrep 20 spin column (low abundance proteins) and the fraction derived from the proteins bound to the affinity media (high abundance proteins) are both in solution phase, they are amenable to subsequent protein separation steps. A variety of possible combinations of orthogonal protein separation techniques are shown in the workflow (Figure 1), depending on the application and instrumentation available to the researcher. Finally, the different fractions from the different multi-dimensional separation techniques are subjected to trypsin digestion and analyzed by LC-mass spectrometry. Multi-dimensional analysis and mass spectrometry will be discussed separately elsewhere.

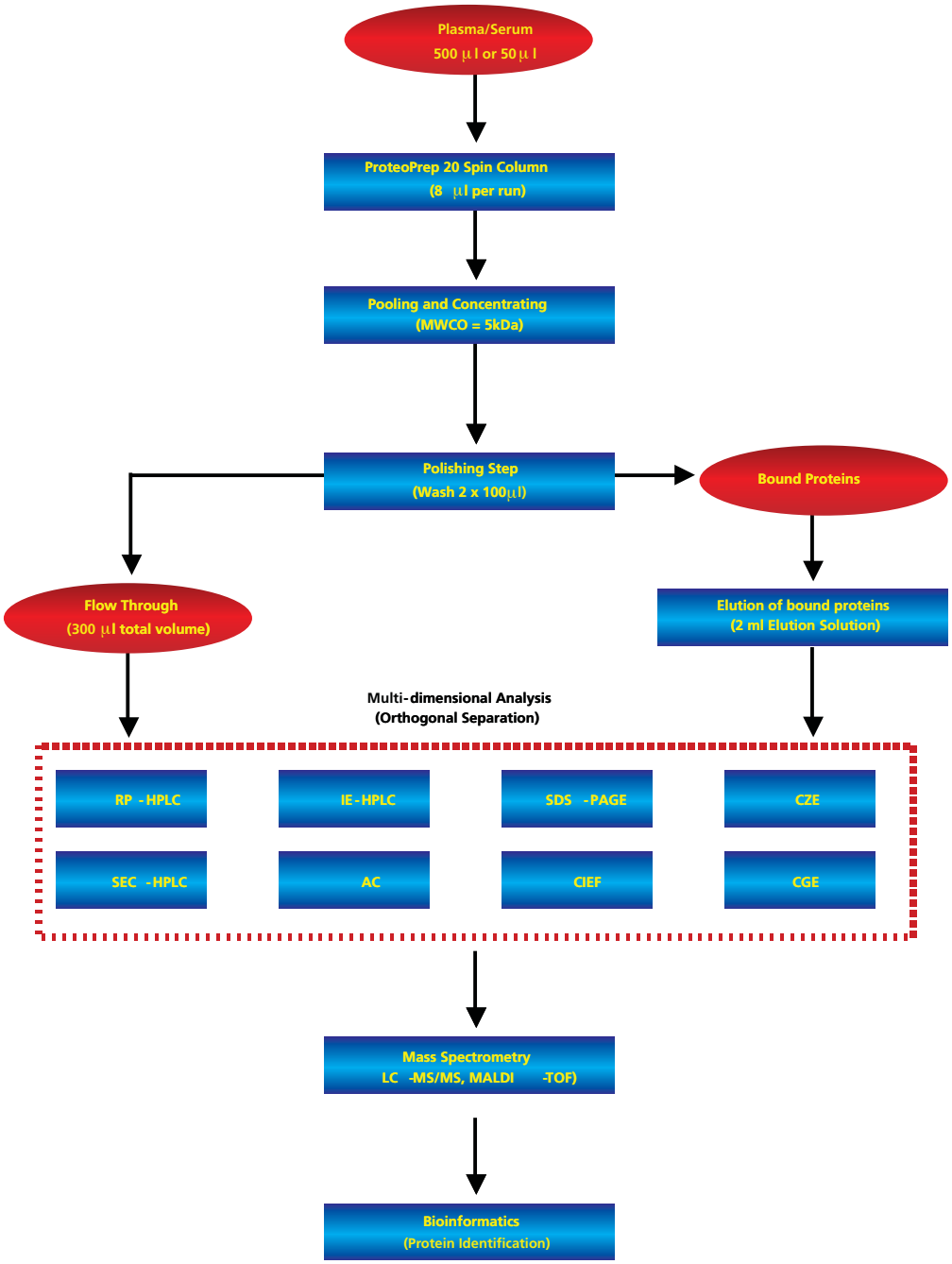
Table 1. The 20 abundant proteins in human plasma depleted by the PROT20 technology.

Albumin	Apolipoprotein A1
IgGs	Apolipoprotein A2
Transferrin	Apolipoprotein B
Fibrinogen	Acid-1-Glycoprotein
IgAs	Ceruloplasmin
α-2-Macroglobulin	Complement C4
IgMs	Complement C1q
α-1-Antitrypsin	IgDs
Complement C3	Prealbumin
Haptoglobulin	Plasminogen

Table 2. Ordering information for ProteoPrep 20 plasma immunodepletion kit and related reagents.

Product No.	Description	Pack Size(s)
PROT20	ProteoPrep® 20 Plasma Immunodepletion Kit	1 kit
C0356	Chaotropic Membrane Extraction Reagent 4	1 bottle, 4 bottles
PROTRA	ProteoPrep® Reduction and Alkylation Kit	1 kit
I3531	IPG Strips, 11 cm, pH 4-7	12 each
I7281	IPG Equilibration Buffer	1 bottle
M4038	SigmaMarker™ Wide Range	1 vial, 10 vials
G1041	EZBlue™ Gel Staining Reagent	500 ml, 3.8 L
M0286	5,000 NMWL Filter	25 each
PROTPR	ProteoPrep® Protein Precipitation Kit	1 kit
C3041	Carbonate Bicarbonate Buffer	50 capsules, 100 capsules
T0440	3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA	100 ml, 1 L
QPBCA	QuantiPro BCA Assay Kit	1 kit
PP0100	Trypsin Profile IGD Kit	1 kit
PROTSIL2	ProteoSilver™ plus Silver Stain Kit	1 kit

Figure 1. Typical workflow for protein depletion using ProteoPrep® 20 Plasma Immunodepletion Kit (PROT20), leading to multidimensional separation, mass spectrometry, and protein identification. The different separation techniques are enclosed in a dotted box to indicate that any combination of these techniques can be used in an orthogonal manner. Abbreviations used: HPLC, High Performance Liquid Chromatography; RP, Reversed Phase; IE, Ion Exchange; SEC, Size Exclusion; AC, Affinity Chromatography; SDS-PAGE, SDS-Polyacrylamide Electrophoresis; CZE, Capillary Zone Electrophoresis; CIEF, Capillary Isoelectric Focusing; CGE, Capillary Gel Electrophoresis.



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