Chapter 6

Low-Molecular Weight Plasma Proteome Analysis Using Centrifugal Ultrafiltration

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Abstract

The low-molecular weight fraction (LMF) of the human plasma proteome is an invaluable source of biological information, especially in the context of identifying plasma-based biomarkers of disease. This protocol outlines a standardized procedure for the rapid/reproducible LMF profiling of human plasma samples using centrifugal ultrafiltration fractionation, followed by 1D-SDS-PAGE separation and nano-LC–MS/MS. Ultrafiltration is a convective process that uses anisotropic semipermeable membranes to separate macromolecular species on the basis of size. We have optimized centrifugal ultrafiltration for plasma fractionation with respect to buffer and solvent composition, centrifugal force, duration and temperature to facilitate >95% recovery, and enrichment of low-M_r components from human plasma. Using this protocol, >260 unique peptides can be identified from a single plasma profiling experiment using 100 μL of plasma (Greening and Simpson, J Proteomics 73:637–648, 2010). The efficacy of this method is demonstrated by the identification, for the first time, of several plasma proteins (e.g., protein KIAA0649 (*Q9Y4D3*), rheumatoid factor D5, serine protease inhibitor A3, and transmembrane adapter protein PAG) previously not reported in extant high-confidence Human Proteome Organization Plasma Proteome Project datasets.

Key words: Blood, Plasma, Low-molecular weight, LMF, LMW, Ultrafiltration, Proteomics, HUPO

1. Introduction

Human plasma is one of the most informative and important proteomes from a clinical perspective. For example, characteristic changes in protein levels in plasma are indicative of many clinical conditions, including severe liver disease, hemolytic anemia and Down's syndrome, schizophrenia, Alzheimer's disease, amyotrophic lateral sclerosis, and Creutzfeldt–Jakob disease (1). Hence, characterization of plasma proteins (both in qualitative and quantitative

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terms) should provide a foundation for the discovery of candidate markers for disease diagnosis and development of new therapeutics. However, human plasma is limited by its dynamic range of protein abundances [ten orders of magnitude between the least abundant (1–5 pg/mL, e.g., interleukins and cytokines) and most abundant ($35–70 \times 10^9$ pg/mL, e.g., albumin and IgG (2))]. For example, albumin and immunoglobulin G constitute approximately 51–71 and 8–26% of the total protein content in human plasma, respectively (3). This complexity creates extensive difficulties in the use of many proteomic separation tools (e.g., free-flow electrophoresis, 10^5 (4)) for the identification of low-abundance species directly in plasma (overview: (2)). The strategies that have been most frequently used to overcome this issue of dynamic range are to fractionate the plasma proteome into smaller subsets and/or to deplete one or more of the major proteins. Immunoaffinity is an established method that addresses the dynamic range of plasma by specific depletion of high-abundance proteins (5). However, although the efficiency of immunodepletion ranges from 96 to 99%, the remaining concentration of albumin, for example, would still be ~50–1,000 μg/mL – a value ~10^4-fold higher than blood CEA levels (~5 ng/mL) and 5×10^6-fold higher than blood IL-6 levels (~10 pg/mL). Hence, MS-based detection of most already known biomarkers in blood requires the use of additional separation/enrichment technologies.

In 2005, Human Proteome Organization (HUPO) Plasma Proteome Project (PPP) generated a high-confidence core set of 889 serum and plasma proteins (6). Interestingly, the low-molecular weight (low-M_r, ≤25K) component of the blood proteome (considered a rich source of plasma biomarkers) was significantly under-represented (2.9% coverage (6)) in these studies. Known plasma polypeptides such as the defensins, and bioactive peptides such as glucagon, insulin, growth hormone, and neuropeptides are involved in a variety of biological functions. The low-molecular weight fraction (LMF) also contains proteolytic peptide fragments of several abundant proteins such as albumin, transthyretin, and the apolipoproteins (7, 8). The plasma or serum proteome has been the focus of recent attempts to identify low-abundance and low-M_r endogenous peptides which hold diagnostic and prognostic potential in cancer biology (9–11) (reviewed in ref. 12).

Centrifugal ultrafiltration has been the most widely used method to extract peptides and remove proteins with higher molecular weights from plasma/serum based on a size-exclusion filtration mechanism (13–17). Typically, membranes have a mean pore size between 10 and 500 Å (or 1.0 and 50 nm).

In the proteomic studies investigating the low-M_r region of plasma, issues with membrane selectivity, centrifugal conditions, buffers and solvents, filtrate heterogeneity, and contamination with abundant, high-M_r plasma proteins have limited the enrichment of

the LMF at present (13, 14, 18, 19). Here, we report optimized conditions for the use of Sartorius Vivaspin® tangential centrifugal ultrafiltration membranes that influence transmembrane pressure and permeability (20).

2. Materials

Throughout the protocol, Milli-Q deionized water (HPLC grade, ≥18 MΩ) should be used for making up all aqueous solutions. All washing, lysis, and HPLC buffers should be prepared using clean glassware on the day analysis is to be performed.

2.1. Blood Collection, Plasma Preparation, and Storage (See Notes 1–4)

1. EDTA blood collection tubes (e.g., BD Vacutainer® #366450).
2. Polypropylene tubes (1.5, 15 mL).
3. Freezer (−80°C or lower).
4. Gloves, gown, and eye protection.
5. Pipettes.
6. Disposal container for contaminated tubes.
7. Centrifugation unit (either/or bench-top/swing bucket rotor – compatible with 1.5/15-mL tubes, programmable temperature setting; range 4–25°C).
8. Labels for blood sample tubes.
9. Alcohol (70% (v/v) aqueous ethanol) and swabs for cleaning venipuncture site.
10. Micro BCA protein assay kit, sufficient reagent to perform 480 standard tube assays or 3,200 microplate assays (#23235, Pierce, Rockford, IL).
11. Water bath or incubator set at 37°C.

2.2. Centrifugal Ultrafiltration

1. Centrifugation unit (bench-top series – compatible with 1.5-mL tubes, programmable temperature setting; range 4–25°C).
2. Centrifugation unit (swing bucket rotor – compatible with 15-mL tubes, programmable temperature setting; range 4–25°C).
3. Centrifugal ultrafiltration membranes – Vivaspin-2® MWCO of 20,000 (#VS02X1, cellulose triacetate (CTA), Sartorius Stedim Biotech, Aubagne, France) (see Notes 5 and 6). For selecting the correct NMWL of the filtration membrane device, refer Notes 7 and 8.
4. Acetonitrile, HPLC grade (Fisher, A998-1 or equivalent) (see Note 9).
5. Water, HPLC grade (Fisher, W5-1 or equivalent).

2.3. SDS-PAGE

1. Laemmli nonreducing sample buffer (0.2 M Tris–HCl, 40% (v/v) aqueous glycerol, 4% SDS, and trace bromophenol blue).
2. Heat block – up to 95°C (compatible with 1.5-mL centrifuge tubes).
3. NuPAGE® LDS sample buffer (Invitrogen), store at 4°C.
4. 1D-Gel apparatus (Invitrogen Novex Mini-Cell).
5. Precast SDS polyacrylamide 12-well, 1.5-mm gel (4–12% Bis–Tris precast gel, Invitrogen).
6. 20 × NuPAGE® MES SDS running buffer (Invitrogen): 50 mM MES, pH 7.2, 50 mM Tris–NaOH, 0.1% SDS, and 1 mM EDTA, pH 7.3, stored at room temperature (RT). Add 25 mL of 20 × running buffer to 475 mL water for preparing 1 × SDS running buffer.
7. Benchmark or Mark 12 protein standard mix, store at 4°C.

2.4. Protein Visualization

1. SilverSNAP® Stain Kit II (#24612, Pierce, Rockford, IL) gel stain, sufficient reagents to stain up to 20 SDS-PAGE minigels.
2. Fixing solution, 30% (v/v) aqueous ethanol containing 10% (v/v) aqueous acetic acid (>99.7%, Sigma–Aldrich, Saint Louis, MO).
3. Personal Densitometer SI (Molecular Dynamics).
4. Coomassie R-250 (#24615, Imperial Protein Stain, Pierce Biotechnology), 1 L, sufficient reagent for staining up to 50 minigels (see Note 10).
5. ImageQuant™ software (Molecular Dynamics).

2.5. In-Gel Digestion and Peptide Extraction

1. Gel cutter – 40 slices (or could use scalpel for gel lane excision).
2. 96-Well polypropylene plates (#AB-1058, ABgene™ Thermo-Fast, Thermo Fisher Scientific).
3. 100 mM Ammonium bicarbonate. Dissolve 0.79 g of ammonium bicarbonate in 100 mL of Milli-Q water to make 100 mM ammonium bicarbonate. Prepare fresh for every digest.
4. Dehydration buffer, 100% acetonitrile (>99.7%, Sigma–Aldrich, Saint Louis, MO).
5. Reduction buffer. Dissolve 15.4 mg of dithiothreitol (DDT, Clelands reagent) in 10 mL of 100 mM ammonium bicarbonate to make 10 mM DDT. A volume of 1 mL is adequate for ten samples (prepare fresh for each digest). Preweighed DDT can be stored at –20°C.

6. Alkylation buffer. Dissolve 90 mg of iodoacetic acid (IAA) in 10 mL of 100 mM ammonium bicarbonate to make 50 mM IAA. Preweighed IAA can be stored at –20°C.
7. Promega trypsin Gold, mass spectrometry grade (#V5280), in 50 mM acetic acid, diluted to 6 ng/μL in 100 mM ammonium bicarbonate, stored covered at –20°C.
8. Extraction buffer, 1% (v/v) aqueous formic acid/2% (v/v) aqueous acetonitrile in Milli-Q water. Stock solutions can be stored. A volume of 150 μL of buffer is required for each well.
9. SpeedVac, centrifugal lyophilization (Savant AES1010, Savant, USA).
10. Adhesive plate seals or polypropylene plate covers.

2.6. Nano-LC Analysis

1. HPLC solvents. Solvent A: 0.1% (v/v) aqueous formic acid (HPLC/Spectrograde). Solvent B: 60% (v/v) aqueous acetonitrile (ChromAR grade; Mallinkrodt) containing 0.1% formic acid (v/v; HPLC/Spectrograde). For Solvent A, mix 1 mL of neat formic acid (pipette) in 1 L of Milli-Q water in a glass-stoppered measuring cylinder, into a clean HPLC reservoir bottle. For Solvent B, add 600 mL of acetonitrile to 1-L glass-stoppered measuring cylinder, adjust the volume to 1 L with HPLC-grade water, add 0.9 mL of neat formic acid [final concentration 0.09% (v/v)], and mix thoroughly.
2. RP-capillary column, nanoACQUITY™ (C18) 150×1.0 mm I.D. (nanoACQUITY™-C18, 1.8 μm, Waters Corp, MA, USA).
3. Software: Chemstation 1200 series (Agilent Technologies).

2.7. MS/MS Analysis

1. Mass spectrometer with fast scan rate [e.g., Electrospray-Ion Trap (ESI-IT) tandem mass spectrometry (MS/MS) (LTQ-Orbitrap, Thermo Fisher Scientific, MA, USA)].

2.8. Data Processing and Analysis

1. Mass spectrometry program; *extract-msn* version 3, Bioworks 3.2 (Thermo Finnigan, USA).
2. MS/MS data analysis software (e.g., Mascot or Sequest). Mascot search algorithm (http://www.matrixscience.com/).
3. Mascot Daemon (http://www.matrixscience.com/daemon.html).
4. Java™ spectrum applet.
5. IPI human database (IPI.-HUMAN. current version; i.e., v.3.38, the number of entries were 70,757) from the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/).

3. Methods

These methods assume the use of proper venipuncture technique for obtaining blood samples. For blood collection, standard protocols recommended by well-established organizations must be utilized (see Appendix A) (21). During phlebotomy, hemolysis can be caused by several factors including needle insertion for blood withdrawal (see Note 2). For routine venipuncture procedures, a 21-gauge needle is recommended to minimize hemolysis (see Note 1).

3.1. Blood Collection (See Notes 1–4)

1. It is important to obtain the required volume of blood using specific blood collection tubes. This is essential to ensure that the blood to anticoagulant ratio is not exceeded. Blood collection should be completed within 5 ± 2 min from the starting time.
2. After blood collection, gently mix the unit by inverting the tube eight to ten times.
3. Label the donor collection tube(s). If storage is required, do so immediately at –20°C.
4. Thawing of the plasma sample on the day of use should be performed at 37°C (not at RT or on ice) (see Notes 11 and 12). This is to prevent the formation of cryoprecipitate.
5. Protein concentration of the thawed plasma sample should be determined. For consistency, the bicinchoninic (BCA) protein assay, using bovine serum albumin (BSA) as a standard, should be used (22).

3.2. Centrifugal Ultrafiltration

1. Prepare centrifugal filter membranes according to the manufacturer's instructions by rinsing in 15 mL of HPLC-grade water at $2{,}000 \times g$ for 10 min (see Note 13). Set the centrifugal temperature to 20°C. Twist off the lock cap and remove the inner tube (filtrate collector). Make sure not to touch or bend the membrane. If the device is not to be used immediately, store it at 4°C with Milli-Q water covering the membrane surface.
2. Dilute 100 µL of thawed plasma with 900 µL of 10% (v/v) aqueous acetonitrile and allow to stand at RT for 2 min (see Note 9). Centrifuge each plasma sample (with a counterbalance) at $14{,}000 \times g$ for 2 min at RT to precipitate any insoluble material that may clog the filters.
3. Apply the supernatant to the prepared centrifugal filter(s) and place the samples in an M4 swing bucket rotor and centrifuge (with a counterbalance) at $4{,}000 \times g$ for 35 min at 20°C (see Note 6). A small aliquot (50 µL) of the sample is set aside to assess LMF recovery. This sample is stored at –80°C.

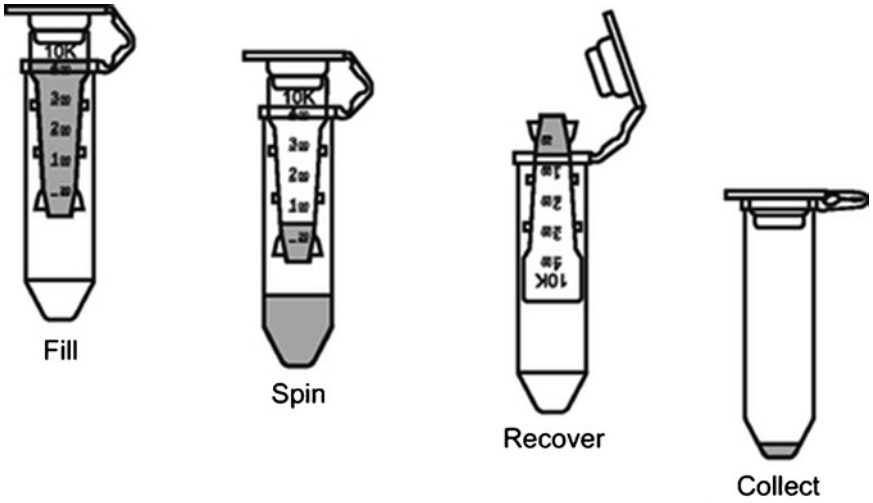

Fig. 1. Centrifugal filtration device. The assembly and operation of the centrifugal ultrafiltration device are shown, with retained volume (retentate, *upper*) and filtrated volume (filtrate, *lower*) indicated. Obtained with permission from the Millipore product catalog for protein purification and concentration (Reproduced with permission from http://www.millipore.com/catalogue/module/c82301).

4. The retentate (retained fraction, ~5% initial volume) should be removed and stored separately. The filtrate (flow-through fraction, ~90–95% initial volume) volume can be removed using a pipette or the filtrate is recovered by inverting the tube and centrifuging at 2,000 × *g* for 1 min (see Fig. 1).
5. The LMF recoveries of the filter membrane can be analyzed by BCA protein assay (22), comparing the initial plasma concentration to the concentration and volume of both the retained (retentate) and filtered (filtrate) samples. Typical recoveries for this experiment should be in the range of 94–97% (three experimental replicates). Retentate samples are stored at –80°C.
6. The plasma LMF filtrates are lyophilized to dryness by centrifugal lyophilization and resuspended in Laemmli nonreducing sample buffer.

3.3. SDS-PAGE Analyses

1. A plasma LMF protein sample (50 µg) is mixed with pre-warmed NuPAGE® LDS sample buffer (in a 2:1 ratio of sample: buffer).
2. The sample mixture is heated for 5 min on a heat block at 95°C and cooled (2 min) prior to sample loading.
3. Separation is performed using a precast 12-well SDS polyacrylamide gel (4–12% Bis–Tris precast gel).
4. 1 × MES SDS running buffer (500 mL) is prepared – approximately 200 mL in the upper (inner) buffer compartment and 300 mL in the lower (outer) buffer compartment.

5. Samples are loaded into defined gel lanes. Benchmark protein standards (5 μL) are used for molecular weight comparison.
6. Protein separation is performed at 150 V (constant voltage) until tracking dye reaches the bottom of the gel (approximately 75 min).
7. Immediately following electrophoresis, the gel should be washed with water and stained with colloidal Coomassie R-250, as described elsewhere (23) (Fig. 2) (see Note 14). Destain the background with water.

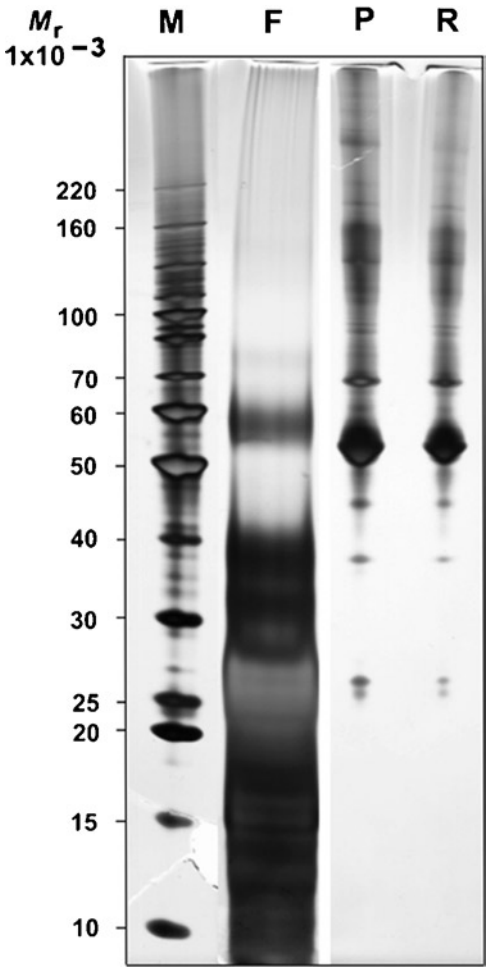

Fig. 2. 1-DE analysis of human plasma fractionated with centrifugal ultrafiltration. A volume of 100 μL of plasma was diluted 1:9 with 900 μL of 10% ACN, pH 8.5 v/v, as per Subheading 3.2. This sample was fractionated using Vivaspin-2 20K MWCO membrane filter at 4,000 × *g* until 95% of the input plasma had passed through the 20K filter. Aliquots of whole (*lane P*) or ultrafiltered plasma (filtrate, *lane F*, and retentate, *lane R*) were subjected to 1-DE and stained using silver staining. *Lane M*, benchmark molecular weight marker. Reproduced with permission from *Journal of Proteomics.*

3.4. In-Gel Digestion and Peptide Extraction

1. After staining, gel sections are excised (using either scalpel or gel-excision tool with slices ~1.0–1.5-mm thick) from a single lane.
2. The excised gel sections (23 sections in this study) are placed in a 96-well flat-bottom tissue culture plate (polypropylene, BD Biosciences) and digested with trypsin (0.05 μg).
3. The samples are first washed in 50 μL of 100 mM ammonium bicarbonate, followed by 5 μL of acetonitrile at 37°C for 20 min. This is repeated twice.
4. Each gel piece is subsequently treated with 50 μL of reduction buffer (10 mM DTT) at 37°C for 30 min and alkylated by incubation with 50 μL of alkylation buffer at 37°C for 30 min. After removal of excess buffer, the gel pieces are washed with 50 μL of 100 mM ammonium bicarbonate buffer at 37°C for 5 min, followed by 50 μL of acetonitrile at 37°C for 5 min. This washing step is repeated with 50 μL of 50 mM ammonium bicarbonate for 5 min. The wash is then removed as waste.
5. The gel pieces are dehydrated by the addition of 50 μL of acetonitrile and then dried for 10 min using centrifugal lyophilization.
6. Each gel section is rehydrated with 25 μL of the diluted trypsin stock solution (see Subheading 2.5). The plate should be sealed properly with adhesive plate seals or polypropylene plate covers.
7. Digestion is performed by incubating the plate at 37°C for 16 h.
8. To the gel pieces, add 60 μL of extraction buffer and incubate them at RT for 30 min. Carefully remove the extraction buffer containing generated tryptic peptides and place it into separate 100-μL glass autosampler vials. This is repeated twice with extraction/digestion buffer retained in the autosampler vial.
9. Extraction/tryptic digests are concentrated to 10 μL by centrifugal lyophilization in preparation for nano-LC–mass spectrometry (LC–MS).

3.5. Nano-LC Analysis

1. Peptide fractionation is achieved by capillary reversed-phase HPLC using the nano ACQUITY™ (C18) 150×1.0 mm I.D. RP-capillary column (nano ACQUITY™-C18, 1.8 μm) as detailed. The column is developed with a linear 60-min gradient from 0 to 100% B with a flow rate of 0.8 μL/min. The samples (~7 μL) are loaded onto the column via the autosampler.
2. The column temperature is maintained at 45°C and the eluent monitored for UV absorption at 215 and 280 nm.

3. The capillary HPLC is coupled online to the ESI-IT mass spectrometer for automated MS/MS analysis.
4. Tune and calibrate the LTQ Orbitrap according to the manufacturer's instructions (see Note 15).
5. Positive ion mode was used for data-dependent acquisition. Survey MS scans were acquired with the resolution set to a value of 30,000. Each scan was recalibrated in real time by co-injecting an internal standard from ambient air into the C-trap (24) (see Note 15). Up to five of the most intense ions per cycle were fragmented and analyzed in the linear trap. Target ions already selected for MS/MS were dynamically excluded for 180 s to optimize peptide coverage.

3.6. Data Processing and Analysis

1. The parameters used to generate the peak lists using *extract-msn* were as follows: minimum mass 700; maximum mass 5,000; grouping tolerance 0.01 Da; intermediate scans 200; minimum group count 1; 10 peaks minimum, and TIC of 100.
2. Peak lists for each LC–MS/MS run were merged into a single MGF file for Mascot searches using Mascot Daemon. Charge state of the selected ions was automatically determined from the survey scan.
3. Acquired MS/MS spectra were searched against the IPI human database (IPI.-HUMAN. current version; i.e., v.3.38, the number of entries were 70,757) from the EBI (http://www.ebi.ac.uk/).
4. Database search parameters were as follows: fixed modification, carboxymethylation of cysteine (+58 Da), variable modifications, NH_2-terminal acetylation (+42 Da), and methionine oxidation (+16 Da). Peptide mass tolerance was ±20 ppm, and #13C is defined as 1 with allowance for up to three missed tryptic cleavage sites.
5. Acceptance criteria based on ProteinScore, IonScore > Homology Score, and a <1% false discovery rate (FDR), in addition to manual verification of the spectra (Java™ spectrum applet), were determined in accordance with previously established guidelines for inclusion of true peptide identifications (25).
6. Proteins were submitted to BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) with the criteria of >95% to remove redundancy.
7. Proteins were correlated with prediction of nonclassical protein secretion (SecretomeP) (http://www.cbs.dtu.dk/services/SecretomeP/) and also the Secreted Protein Database (http://spd.cbi.pku.edu.cn/spd_search.php).

8. Other resources to classify identified proteins based on several predictive algorithms included the SignalP (http://www.cbs.dtu.dk/services/SignalP/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), Gene Ontology (GO) (http://www.geneontology.org/index.shtml?all/), UniProt (http://www.uniprot.org/) and Bioinformatic Harvester (http://harvester.fzk.de/harvester/) databases.

4. Notes

1. *Safety*. All blood and biological specimens and materials should be considered to be biohazards. Hence, it is important to use gloves, gowns, eye protection, other personal protective equipment, and controls to protect from blood splatter, blood leakage, and potential exposure to bloodborne pathogens. Use aseptic techniques at all times and sterile disposables (tubes, pipettes, etc.) throughout to prevent blood contamination. Risk factors for possible transfusion transmissible infections should be rigorously screened *prior* to blood collection. Handle the specimens as if they are capable of transmitting infection and dispose of with proper precautions in accordance with federal, state, and local regulations. Refer to your institutional regulations regarding the screening of blood for specific infectious disease markers (i.e., HIV, hepatitis B, hepatitis C, etc.). Discard all blood collection materials in biohazard containers approved for their disposal.
2. *Sample hemolysis*. The release of cellular material due to hemolysis into serum/plasma may introduce additional confounding factors. We recommend that if hemolysis (pink to red tinge in serum/plasma sample) is observed following centrifugation, this information should be recorded. It is recommended that hemolyzed samples should not be used for proteomic/peptidomic analyses.
3. *Monitoring pre/post-analytical variation*. In 2005, the HUPO PPP report detailed an extensive analysis of the variables that affect the stability of plasma (26). These included (a) the anticoagulant used in collection tube types (e.g., EDTA and ascorbate), (b) sample processing times, (c) temperatures at which blood specimens were processed and stored, (d) sample storage parameters, and (e) thaw/refreeze cycles, associated with obtaining human plasma and serum samples for proteomic analyses directed toward clinical research. It is of upmost importance that for diagnostic use, these variables are controlled and monitored at all times, from blood collection

as an anticoagulated or coagulated source, processing, handling, to storage (27–29). Recently, it has been shown that biomarker validation studies should use standardized collection conditions, and multiple control groups to detect and correct for potential biases associated with sample collection (30).

4. *Data points.* For blood handling, it is important to note also (a) the date and time of blood collection, (b) the number and volume of samples/aliquots prepared, (c) the date and time the samples are placed at −80°C, (d) the date and time of shipping, (e) any freeze–thaw cycles that occur, and (f) variations or deviations from the standard-operating protocol, and problems or issues that arise.
5. *Centrifugal ultrafiltration membrane devices.* A wide range of centrifugal filters are commercially available for concentrating and filtering protein solutions, removing small solutes, and/or buffer exchanging. These devices consist (mostly) of two chambers separated by a semipermeable membrane. These membranes can be composed of different chemistries and different orientations depending on their application (see Note 8). Under centrifugal force, solvent and solute molecules smaller than the NMWL readily pass through the membrane (filtrate) (see Note 7). Vertical or angular membrane configuration reduces concentration polarization (membrane fouling) and allows high flow rates for optimal solvent passage even with high proteinaceous solutions. The direction of the centrifugal force and flow rate of solute differ between the membrane devices used. Additional information can be obtained from http://www.millipore.com/ and http://www.sartorius.com/.
6. *Optimized centrifugal ultrafiltration.* Conditions for each plasma sample should be optimized. Conditions provided in this protocol are the combined effect of analyzing multiple filter membrane units, with conditions optimized with respect to plasma buffer and solvent compositions, centrifugal force, duration, and temperature (Fig. 2). Typically, plasma LMF should represent 95% of the initial supernatant applied to the filtration devices. The amount of protein recovered in the filtrate and retentate can be calculated as a percentage of the initial plasma protein concentration loaded.
7. *Appropriate membranes – selecting the NMWL.* Ultrafiltration membranes are not absolute in their pore size (NMWL) ratings. Separation occurs as a result of differences in the filtration rate of different components across the membrane in response to a given pressure. Unlike UF membranes, microporous membranes have a precisely controlled pore size that ensures quantitative retention of particles and biomolecules greater than the pore size of the membrane. In selecting the most effective membrane for filtration applications, a rule has

been developed to calculate the appropriate membrane pore size (NMWL) rapidly. It is a simple calculation based on the molecular weight of the desired protein to be concentrated or removed in the retentate unit (upper level of the membrane apparatus). The "rule of 1.5–2" requires a membrane cut-off approximately two times smaller than the desired protein's molecular weight. For example, to remove proteins of ~65,000 MW and greater, use a 30,000 NMWL-regenerated cellulose membrane. Typically, this results in >90–95% recovery of the filtrate, containing proteins/peptides <65,000 MW. Other factors to consider when determining an optimal membrane include flow rate, also known as flux, solute concentration, solute composition, and temperature.

8. *Centrifugal ultrafiltration membrane chemistries.* For a detailed overview, refer (31).

   Polyethersulfone – General purpose membrane, providing excellent performance with most solutions when retentate recovery is of primary importance. Polyethersulfone membranes exhibit no hydrophobic or hydrophilic interactions and are usually preferred for their low fouling characteristics, exceptional flux, and broad pH range.

   CTA – High hydrophilicity and very low nonspecific binding characterize this membrane. These membranes are preferred for sample cleaning and protein removal and when high recovery of the filtrate solution is of primary importance.

   Regenerated cellulose/Hydrosart – These membranes demonstrate the same properties as regenerated cellulose, but with the added benefit of enhanced performance characteristics and extremely low protein binding, making it the membrane of choice for applications such as concentration and desalting of immunoglobulin fractions.

9. *Disrupting protein–protein interactions.* A low concentration of organic solvent (typically, 5–10% acetonitrile) is added to buffers to disrupt high-M_r protein–protein interactions. For chemical compatibility of membranes, be careful to read each company's manual prior to operation (based on 2-h membrane contact time). Normally, small uncomplexed proteins and peptides (i.e., less than 30K) are rapidly cleared from the circulation through enzymatic degradation, uptake by the reticuloendothelial system, or by glomerular filtration, which discriminates on the basis of molecular size and charge (32). It is believed that the circulation half-life of the LMF is directly related to its binding affinity to large high-abundance carrier proteins (8, 11).

10. *Coomassie dye staining.* The Coomassie dyes (R-250 and G-250) bind to proteins through ionic interactions between

dye sulfonic acid groups and positive protein amine groups. Coomassie R-250, the more commonly used of the two dyes, can detect protein levels down to 0.1 µg. Additionally, Coomassie R-250 does not require methanol/acetic acid fixation and destaining.

11. *Cryoprecipitate formation.* A cryoprecipitate is often formed if the fresh-frozen plasma unit is slowly thawed at temperatures just above freezing (1–6°C), typically in a water bath or a refrigerator. The product is then centrifuged at low speed (typically 5,000 × *g*) to remove the majority of the precipitate. Formation of the cryoprecipitate can be avoided by thawing the plasma at 37°C.
12. *Plasma thaw process.* Thawing of plasma can be achieved in various ways, the most common of which uses a recirculating water bath. This carries a risk of bacterial contamination and must be maintained in a controlled sterile environment. Denaturation of plasma proteins can be avoided by using a dry heating apparatus.
13. *Prerinsing membranes.* Most ultrafiltration membrane devices contain trace amounts of glycerine/sodium azide. If this interferes with subsequent sample analysis, prerinse the device extensively with buffer or Milli-Q water through the concentrator. If interference still persists, rinse the membrane with 0.1 M NaOH, followed by repeated centrifugation with buffer or Milli-Q water.
14. *Silver staining protocol.* For protein visualization, we used the sensitive SilverSNAP® Stain Kit II gel stain, as described earlier (20). Briefly, the gels were washed (2×) for 5 min in deionized water, fixed (2×) [30% (v/v) aqueous ethanol containing 10% (v/v) acetic acid] for 15 min, and washed for 10 min first in 10% ethanol and then in deionized water for 10 min. The gels were incubated in SilverSNAP® Sensitizer solution for 1 min, stained with SilverSNAP® silver solution for 30 min, washed (2×) in deionized water for 1 min, developed in SilverSNAP® develop solution for approximately 3–5 min, and fixed in 5% (v/v) aqueous acetic acid. SilverSNAP® stained gels were imaged with a Personal Densitometer SI (Molecular Dynamics) with 100-µm pixel size.
15. *Mass accuracy and tuning.* For Orbitrap mass analyzers to maintain the ability to measure peptide masses accurately with less than 5 ppm error requires constant calibration every 2–3 days. The "Lock Mass" capability of the LTQ Orbitrap, which allows for real-time recalibration using polydimethylcyclosiloxane ions present in ambient air (24), is recommended.

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