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Plasma Biomarker Discovery Using 3D Protein Profiling Coupled with Label-Free Quantitation

Lynn A. Beer, Hsin-Yao Tang, Kurt T. Barnhart, and David W. Speicher

Abstract

In-depth quantitative profiling of human plasma samples for biomarker discovery remains quite challenging. One promising alternative to chemical derivatization with stable isotope labels for quantitative comparisons is direct, label-free, quantitative comparison of raw LC–MS data. But, in order to achieve high-sensitivity detection of low-abundance proteins, plasma proteins must be extensively pre-fractionated, and results from LC–MS runs of all fractions must be integrated efficiently in order to avoid misidentification of variations in fractionation from sample to sample as “apparent” biomarkers. This protocol describes a powerful 3D protein profiling method for comprehensive analysis of human serum or plasma proteomes, which combines abundant protein depletion and high-sensitivity GeLC–MS/MS with label-free quantitation of candidate biomarkers.

Keywords

Proteomics; Major protein depletion; Plasma biomarkers; Label-free quantitation

1. Introduction

The human plasma proteome is a major focus of proteomic studies directed toward disease biomarker discovery, because blood is a routinely collected biological fluid that is likely to contain multiple novel biomarkers for many physiological conditions and diseases (1). It is believed that most cells in the body secrete proteins into the blood and, therefore, plasma is very likely to contain information concerning the physiological status of all tissues and organs in the body – and is potentially the most informative sample to describe an individual's current health state (1–4). Although plasma is a potential source of disease biomarkers, systematic discovery of potential clinical biomarkers is greatly complicated by the wide dynamic range of known blood proteins, which spans 9–12 orders of magnitude (3, 5–8). The huge complexity and dynamic nature of the plasma proteome present great technological challenges for proteomic analyses, specifically when trying to identify and quantitate low-abundant (<100 ng/mL) proteins (9, 10).

Recent advances in proteomic technologies, specifically high-resolution, high-mass-accuracy, mass spectrometry (MS), have made quantitative comparisons of proteins in biospecimens more robust and reproducible. However, the wide dynamic concentration range of plasma proteins greatly exceeds the analytical capabilities of single LC–MS/MS runs and other 1D and 2D proteomic methods. One strategy to increase dynamic range of the plasma proteome is to use multi-dimensional separation strategies that involve orthogonal protein/peptide fractionation steps (5, 11–13).

The first step in our multi-dimensional plasma profiling scheme, outlined in Fig. 1, is major protein depletion. Until approximately 2003, the most effective technology available for depletion of abundant blood proteins was represented by kits that combined Cibacron® blue dye for removal of albumin, which constitutes ~65% of total plasma protein, with Protein A or G for removal of immunoglobulins, which constitute an additional 15% of plasma protein. Unfortunately, these columns incompletely removed albumin, which remained as one of the most abundant proteins. Furthermore, many lower abundance proteins were also removed by the dye columns due to nonspecific binding as visualized by 2D gels (14). The Multiple Affinity Removal System (Agilent Technologies, Santa Clara, CA) developed in 2003 was the first commercial product that simultaneously depleted several abundant proteins (15). Specifically, this technique effectively depleted six abundant proteins and reduced the protein content of human plasma or serum by ~85%. This substantially improved protein profiling capacities, but the next tier of abundant proteins continues to limit volumes of plasma that can be analyzed. Ideally, additional medium-abundant proteins should be depleted so that at least 98–99% of total protein content is initially removed (5, 14). Other manufacturers have recently produced antibody columns to target 7, 12, 14, or 20 abundant plasma proteins in the next phase of immunodepletion. In addition, the next generation of immunodepletion methods will likely target more medium-abundance proteins. For instance, the recently introduced Seppro® Supermix column (Sigma-Aldrich, St. Louis, MO) is an immunoaffinity column designed to be used in tandem with IgY columns and targets medium-abundance proteins. A recent report showed that the tandem IgY columns were reproducible and greatly increased detection of lower-abundance proteins by removing ~60 medium- to high-abundance proteins (16).

Following major protein depletion, there are multiple alternative protein and peptide separation techniques for further fractionation of the proteome. Our strategy uses GeLC–MS/MS, an efficient strategy to identify a broad dynamic range of protein abundances in complex proteomes. Samples are separated using 1D SDS-PAGE, and gel slices are excised and typically digested followed by nano-LC–MS/MS. The advantages of GeLC–MS/MS approaches are that the molecular weight (MW) of the proteins of interest are known, providing information on possible protein processing or posttranslational modifications with relative changes in MW distribution (17). In addition, the routine method combines reproducible protein separation methods with well-established sensitivity of in-gel digestions, followed by LC–MS/MS for protein identifications (18).

Finally, we have chosen to use comparative LC–MS pattern analysis for label-free quantification as an alternative approach to chemical derivatization with stable isotope labels for several reasons. First, most methods used to compare proteins quantitatively select either portions of the total proteome, e.g., cysteine-containing peptides or glycosylated peptides, or label proteins after trypsin digestion. Such strategies make separations at the protein level prior to labeling impractical due to potential misinterpretation of technical variations as biological differences. Second, the cost of isotopic labeling reagents is a disadvantage for large-scale experiments with high numbers of samples. Third, direct, label-free, quantitative comparison of raw LC–MS data is a promising alternative approach based on the observation that ion peak intensity in ESI-MS is usually proportional to the concentration of a peptide in a sample (9, 19). By comparing ion intensities between LC–MS runs of multiple samples, comprehensive quantitation of each peptide between samples is possible (9, 10). However, one limitation of the label-free method is that in order to achieve accurate, relative quantitation of peak intensities in multiple LC-MS datasets, it is necessary to have high-resolution and high-mass-accuracy mass spectrometers (LTQ-FT or LTQ-Orbitrap™ instruments). Furthermore, dedicated software for identification, matching, and alignment of peaks, as well as for integration of data across fractions is essential. High-performance MS has become more routinely available in the past few years, and it has been shown that

precise and comprehensive analysis of complex proteomes is best achieved using high-resolution proteomics technologies (20).

2. Materials

2.1. FPLC Affinity Depletion of Plasma or Serum

1. Human plasma or serum.
2. HPLC or FPLC system capable of operating at low pressure (<30 psi).
3. ProteoPrep® 20 LC Plasma Immunodepletion Column kit (#PROT20LC-1EA, Sigma–Aldrich). Individual components are as follows:
 - (a) ProteoPrep® 20 LC Plasma Immunodepletion Column, 9.4 mL resin, 100 mL plasma (50–70 mg) capacity, storage: 2–8°C.
 - (b) ProteoPrep® 20 equilibration buffer, 10× concentrate, storage: 2–8°C.
 - (c) ProteoPrep® 20 elution solution, 10× concentrate, storage: 2–8°C.
 - (d) ProteoPrep® preservative concentrate, storage: 2–8°C.
4. Stericup® GP Express Plus membrane (Millipore, Billerica, MA), 0.22-µm filter units.
5. Amicon Ultrafree®-MC (Millipore), 0.22-µm microcentrifuge filters.
6. Milli-Q® (Millipore) water or equivalent.
7. 20% (v/v) Ethanol in water.

2.2. Ethanol Precipitation

1. Unbound fraction from ProteoPrep® 20 LC depletion.
2. Ethanol (e.g., molecular biology grade, 200 proof, Sigma–Aldrich), chilled to –20°C.
3. SpeedVac® (Thermo Scientific) centrifuge.

2.3. Reduction and Alkylation of Samples Prior to 1D SDS-PAGE

1. Depleted and ethanol-precipitated pellet of human plasma or serum.
2. SDS-resuspension buffer: 1.0–1.2% (w/v) SDS in 100 mM aqueous Tris–HCl, pH 8.6.
3. 1 M Aqueous dithiothreitol.
4. 0.5 M Iodoacetamide in 100 mM aqueous Tris–HCl, pH 8.6.
5. 37°C Thermostatically controlled incubator/shaker.

2.4. 1D SDS-PAGE

1. Reduced and alkylated ProteoPrep® 20 depleted sample.
2. 2× Protein solubilizing buffer: 0.4 M sucrose, 6% (w/v) SDS, 125 mM Tris–HCl, 4 mM Na₂EDTA, 2% (v/v) 2-mercaptoethanol, and 2% (v/v) saturated bromophenol blue solution.
3. NuPAGE® MES running buffer 20× (Invitrogen, Carlsbad, CA), storage: 2–8°C.
4. NuPAGE® 12% Bis–Tris gel, 1 mm, 10-well (Invitrogen), storage: 2–8°C.

5. XCell *SureLock*TM Mini-Cell (Invitrogen).
6. Heat block set to 90°C.
7. BenchMarkTM (Invitrogen) molecular weight marker, storage: −20°C.
8. 1 cc Insulin syringe.
9. India ink, several drops.
10. Novex® Colloidal blue staining kit (#LC6025, Invitrogen):
 - (a) Stainer A.
 - (b) Stainer B.
11. Fixing solution: 50% (v/v) methanol/10% (v/v) acetic acid in water.
12. Methanol (Optima® 0.2-µm filtered, Fisher Scientific, Pittsburgh, PA).
13. Glacial acetic acid.
14. Milli-Q water or equivalent.
15. Staining trays.

2.5. In-Gel Trypsin Digestion

1. PCR hood with built-in laminar flow and equipped with a HEPA filter and a lightbox.
2. 0.1% (v/v) trifluoroacetic acid/50% (v/v) methanol in water.
3. Methanol (Optima® 0.2-µm filtered, Fisher Scientific).
4. 96-well pierced V-bottomed plate (e.g., Thermo Fisher Scientific, Waltham, MA, or BioMachines, Research Triangle Park, NC).
5. V-bottomed, 96-well storage plates (e.g., Thermo Fisher Scientific).
6. Polystyrene plate covers (e.g., Thermo Fisher Scientific).
7. Glass plate (cut to the same size as a 96-well plate, 8 × 12 cm).
8. Gel-cutting device: e.g., MEG-1.5 Gel Cutter (The Gel Company, San Francisco, CA) or custom razor-blade array.
9. Stainless steel razor blades.
10. Ziploc bags.
11. Jewelers microforceps (e.g., Fisher Scientific).
12. SpeedVac® centrifuge equipped with 96-well plate centrifuge rotor.
13. Eight-channel pipette.
14. 37°C thermostatically controlled incubator/shaker.
15. Destain solution: 50% (v/v) acetonitrile in 0.2 M aqueous ammonium bicarbonate.
16. Sequencing grade-modified trypsin (Promega, Madison, WI).
17. Trypsin working solution: 0.02 µg/µL trypsin in 40 mM aqueous ammonium bicarbonate.
18. Trypsin wash buffer: 0.3% (v/v) formic acid in 40 mM aqueous ammonium bicarbonate.

19. Autosampler sample tubes.

2.6. LC-MS/MS

1. Solvent A: Milli-Q water containing 0.1% (v/v) formic acid (LC-MS grade, Sigma-Aldrich).
2. Solvent B: Acetonitrile (HPLC grade, Thomas Scientific, Swedesboro, NJ) containing 0.1% (v/v) formic acid (LC-MS grade, Sigma-Aldrich).
3. 180 μm i.d. $\times$ 2 cm UPLC symmetry trap column packed with 5 μm C18 resin (Waters, Milford, MA).
4. Reversed-phase 75 μm i.d. $\times$ 25 cm length analytical column packed with 1.7- μm C18 resin [e.g., nanoACQUITY UPLC BEH C18 Column (Waters, Milford, MA)].
5. UPLC equipped with a chilled microvolume autosampler, a 10- μL injection loop, and a column heater maintained at 40°C.
6. Hybrid linear ion trap-Fourier transform (LTQ FT ICR Ultra) or LTQ Orbitrap™ XL mass spectrometer.

2.7. Label-Free Quantitation

1. Label-free analysis software (e.g., Rosetta Elucidator® System, Rosetta Biosoftware, Cambridge, MA).
2. Database search engine (e.g., BioWorks v. 3.3.1, Thermo Scientific).

3. Methods

3.1. FPLC Affinity Depletion of Plasma or Serum

The following protocol describes HPLC/FPLC immunoaffinity depletion using the ProteoPrep® 20 column (21), which currently depletes the largest number of proteins and removes approximately 95% of total plasma protein (see Note 1). The ProteoPrep® 20 LC column packing has a rated pressure maximum of 30 psi, which is much lower than the pressure system requirements of most HPLCs (typically, at pressures of 100 psi or greater). Hence, an FPLC-type system, capable of operating at low pressures, is strongly preferred. If the available HPLC requires higher operating pressures, the necessary backpressure can be generated in front of the column with a flow restrictor, such as an appropriate length of small internal diameter PEEK tubing (e.g., 1 m of 0.005 or 0.007 in. i.d.), and after the column with a larger ID PEEK tubing (0.030 or 0.040 in. i.d.) to avoid exposing the column

¹The current commercially available antibody columns deplete 6, 7, 12, 14, or 20 abundant plasma proteins in both HPLC/FPLC and spin column formats. In our experience, most commercial multi-protein immunodepletion columns perform similarly, in that they efficiently remove >98–99.5% of most targeted abundant proteins, while not removing significant amounts of untargeted minor proteins (14). However, if serum albumin is present at 40–50 mg/mL and 99% is removed, the residual 0.4–0.5 mg/mL of albumin is still a predominantly abundant remaining major component. Therefore, for studies directed toward low-abundant protein analyses, it is often necessary to remove greater than 99%, at least for the most abundant proteins.

There are several factors to consider when choosing a depletion column. First, whether to use an FPLC or spin column format. FPLC columns require relatively expensive instrumentation not available in every laboratory, and antibody LC columns are usually larger and more expensive than spin columns. However, the FPLC columns are highly robust, more completely remove the targeted proteins, and require less of the operator's time because they usually remove larger volumes of plasma or serum in a single pass.

Other factors that may influence decisions concerning the type of column to use include (1) column size and capacity, i.e., spin column capacities typically range from 8 to 20 μL of serum or plasma, while LC columns can be purchased with standard capacities ranging from 20–80 μL or 100–300 μL for high capacity columns, (2) price-to-capacity differences, (3) compositions of buffers and whether they are defined or proprietary, and, most importantly, (4) the amount of the total plasma protein removed by the column. In general, it is most beneficial to deplete as many high- to medium-abundant proteins as possible with the aim of improving the identification of proteins of lower abundance.

to high pressures. The maximum pressure limit must be set only slightly above the operating pressure to reduce the risk of compressing the column bed:

1. Prepare Buffer A (1× equilibration buffer): Dilute one part of ProteoPrep® 20 equilibration buffer, 10× concentrate with nine parts of room temperature ultrapure water. Filter the diluted buffer through a 0.22-µm filter unit and degas for 5 min at room temperature using vacuum filtration with stirring. The final volume needed for depletion is ~55 mL; buffers should be prepared fresh each day.
2. Prepare Buffer B (1× elution solution): Dilute one part of ProteoPrep® 20 elution solution, 10× concentrate with nine parts of room temperature ultrapure water. Filter the diluted buffer through a 0.22-µm filter unit and degas for 5 min at room temperature using vacuum filtration with stirring. The final volume needed for depletion is ~30 mL; buffers should be prepared fresh each day.
3. Before connecting the column, flush Buffer A through the system to the column inlet line for 15 min at 1–3 mL/min. Transfer the ProteoPrep® 20 LC plasma immunodepletion column from 2–8°C to room temperature at this time.
4. Connecting the column: To prevent air from being introduced into the column, after flushing the system as described in step 3, stop flow, remove the plug from the top of the column only, start the pump at 0.5 mL/min, and while holding the column inlet tubing over the top end of the column, fill the column end fitting with Buffer A. Stop flow and connect the tubing to the column inlet.
5. Remove the screw plug from the bottom of the column, connect the detector inlet solvent line to the column bottom, and equilibrate with Buffer A for 5 min at 1 mL/min (see Note 2).
6. Filter undiluted plasma (generally 100 µL) with a pre-rinsed 0.22-µm microcentrifuge filter (see Note 3). Keep the filtered sample on ice until ready for use (see Note 4).

²The ProteoPrep® LC column comes packed in a storage solution containing glycerol. To remove the glycerol solution prior to the first use of the column, attach the top of the column to the tubing, as described in Subheading 3.1, step 4. Do not connect the column outlet line to the bottom of the column. Instead, place the column over a beaker to collect the glycerol solution. Allow the column to equilibrate with 20–25 mL of 1× equilibration buffer at no higher than 0.5 mL/min. It is important to monitor pressure and decrease the flow rate carefully as to not exceed 30 psi. Increased pressures at this step may cause bed compression and a void at the top of the resin bed, which can be readily observed because the column is made of glass. When the resin has changed from opaque to white and the buffer runs clear, the column outlet line may be connected. The column should be conditioned with two complete blank cycles prior to injecting a sample. It is also preferable to run several noncritical control plasma samples before depleting plasma samples that will be used in comparative studies in case there is minor irreversible absorption of low-abundance proteins or antibody bleed during the first several runs.

³The manufacturer originally recommended diluting the plasma sample 5× with Buffer A prior to filtering. This dilution step has subsequently been omitted to enhance depletion of apolipoproteins and recovery of unbound proteins. We have not observed any major changes in apolipoprotein removal, and a fivefold dilution of the plasma did not significantly increase the final volume of the unbound pool. Also, diluting the sample makes it more convenient to add protease inhibitors to the sample before injection, if desired.

⁴Several considerations to note during sample preparation are (1) the number of depletions and/or (2) different plasma samples that will be run in a single day. If multiple runs of the same sample are being depleted, it is best to thaw and filter the entire sample at once and store it on ice until all depletions are complete for optimal reproducibility. If multiple samples from different donors are being depleted, it is recommended that a single sample is thawed and filtered shortly before its injection. To minimize proteolysis, thaw and prepare the next sample while the previous sample is running. Alternatively, another option is to add protease inhibitors to the plasma sample prior to injection; however, the value of adding protease inhibitors is unclear. Instead of adding protease inhibitors to plasma or serum, we prefer to minimize exposure of samples to conditions that favor proteolysis. However, the one step where it is not practical to avoid protease-favorable conditions is during binding to the antibody resin. This is because in order to optimize binding affinity, conditions close to physiological pH without denaturants must be used, and in order to achieve reasonable binding kinetics, low temperatures should be avoided. However, it should be noted that plasma and serum are far more stable to proteolysis than cell lysates and intrinsically contain many proteases and protease inhibitors. Moderate-length exposure to physiological conditions at room temperature appears to have only very minor effects on plasma proteomes (22). Regardless of the exact sample preparation and handling methods to be used, it is critical to ensure that all plasma samples to be compared are processed in a consistent manner.

7. Prior to injection of the first sample of the day, run a blank run (no sample injection) and re-equilibrate the column using Buffer A (see Note 5).
8. Inject 100 μ L of filtered plasma at 0.3 mL/min (see Note 6).
9. Elute the depleted plasma (unbound fraction) from the column with Buffer A at 0.3 mL/min. Collect the flow-through fractions and pool the unbound fractions containing protein as it comes off the column (see Note 7). Keep the pooled sample on ice.
10. Elute bound proteins with Buffer B at 3 mL/min. Collect and pool bound proteins and store on ice (see Note 8).
11. Re-equilibrate column with Buffer A for 10 min at 3 mL/min.
12. Inject the next sample and repeat steps 8–11 until all samples are separated or a sufficient volume of a single sample is depleted.
13. Disconnect and store the column at 4°C in Buffer A (see Note 9).
14. Replace Buffers A and B with ultrapure water and flush the system, including detector, for 30 min at 1 mL/min followed by 20% (v/v) ethanol in water for 30 min at 1 mL/min (see Note 10).

3.2. Ethanol Precipitation

Precipitation of proteins with ethanol is an advantageous alternative to ultra-filtration for concentrating unbound and bound fractions. Ethanol precipitation requires less operator time and more effectively removes salts and detergent. The ethanol should be of high quality, chilled to -20°C , and added quickly for efficient protein precipitation. If precipitating the bound fraction, the pooled sample should be neutralized by adding 1 M NaOH to bring the pH to ~ 8.0 prior to precipitation:

⁵Running an initial “quick blank gradient” at 3 mL/min for the entire gradient with no sample injection is useful for several reasons. First, it will ensure that the column is equilibrated to room temperature prior to the plasma sample being injected. Also, as the column warms up, solvent out-gassing may occur; but the increased flow rate forces any small air bubbles out of the column before large pockets can form and disrupt the column media packing. Finally, a blank cycle run as the first run of the day removes any residual protein that may be bound to the column from previous depletions. Any protein peaks observed during the blank run should be run on 1D SDS-PAGE and analyzed with silver stain.

⁶The manufacturer describes this column's capacity as 100 μ L of plasma with a concentration of 40–50 mg/mL. However, this is a very low estimate for protein concentration. For example, the range for total protein in normal human serum is quite broad, ~ 60 –85 mg/mL, and plasma samples will have similar or slightly higher values. Furthermore, in our experience, plasma concentration ranges in patients with diseases are often even more variable. Total protein concentration may also be affected by the use of different anticoagulants and collection tubes. Therefore, it is recommended to determine the protein concentration of each sample prior to depletion and base injection loads upon total protein, not volume.

⁷To monitor depletions and analyze individual fractions, it is useful to reserve a small aliquot of each fraction before pooling the unbound and bound samples for analysis on 1D SDS-PAGE.

⁸Bound proteins should ideally be collected and stored at -20°C for potential future analysis. Prior to freezing, the bound pool should be neutralized by adding 1 M NaOH to bring the pH to approximately 8.0.

⁹It is essential to store and monitor antibody LC columns properly to maintain optimal column performance. Storage in equilibration buffer at 2 – 8°C is suitable for short-term storage (less than 1 week). Long-term column storage (1 week or more) requires the addition of a preservative solution, included with the ProteoPrep® 20 LC columns. Otherwise, flushing the column with equilibration buffer containing 0.01% sodium azide should prevent any microbial growth in the column. It is important to keep track of the total number of blank and depletion cycles run on each column. Periodically, and especially when the number of recommended uses has been reached, column performance should be critically evaluated. If column capacity has inevitably declined, it is usually adequate to reduce protein loads slightly to achieve a degree of depletion similar to that observed with a new column. To track antibody bleed and/or proteolysis of the coupled antibodies from the column, bound fractions from plasma depletions and bound fractions from blank cycles after extended column storage can be monitored by digesting an aliquot of the bound pool with trypsin, followed by LC–MS/MS. The resulting data ideally should be searched against an all-species database. Alternatively, a human sequence database supplemented with all immunoglobulin sequences from the species of origin for the antibodies on the column should be used.

¹⁰After the final run of each day, the FPLC or HPLC system should be thoroughly flushed with water, followed by 20% ethanol. This will prevent corrosion due to halogen salt-containing buffers or clogging of lines due to bacterial growth.

1. Quickly add nine volumes of 200-proof ethanol (-20°C) to the pooled unbound fraction.
2. Vortex the sample thoroughly.
3. Incubate overnight at 0°C .
4. Centrifuge the sample for 30 min at $1,500 \times g$, 4°C .
5. Carefully remove the supernatant and carefully air-dry pellet under a gentle stream of argon (see Note 11).
6. Freeze pellets and store the samples at -20°C for short-term (less than 1 week) or -80°C for long-term storage.

3.3. Reduction and Alkylation of Sample Prior to 1D SDS-PAGE

Reducing and alkylating the unbound sample prior to running gels save time in the downstream in-gel digestion protocol, especially for large-scale experiments with many fractions and/or samples. This protocol assumes that 100 μL of human plasma has been depleted, precipitated with ethanol, and will be re-suspended in 100 μL (original plasma volume) of SDS re-suspension buffer. Reduction, alkylation, and quenching buffer volumes should be calculated and subtracted from the final sample volume (in this case, 100 μL) to determine the volume of re-suspension buffer to be used:

1. Thaw ethanol-precipitated plasma pellet from -20°C .
2. Re-suspend pellet in 81 μL of 1.2% SDS and 100 mM Tris-HCl, pH 8.0 (final SDS concentration: $\sim 1\%$).
3. Reduce samples by adding 2 μL of 1 M DTT (final concentration: 20 mM). Incubate for 1 h at 37°C with shaking.
4. Alkylate proteins by adding 12 μL of 0.5 M iodoacetamide/100 mM Tris-HCl, pH 8.6 (final concentration: 60 mM). Incubate for 1 h at 37°C in the dark, with shaking.
5. Quench the reaction by adding an additional 5 μL of 1 M DTT (final concentration: 50 mM) to the sample and incubate for 15 min at 37°C with shaking.

3.4. 1D SDS-PAGE

The following instructions are for SDS-PAGE with precast NuPAGE® gels, buffers, and electrophoresis equipment, followed by gel staining with colloidal Coomassie® blue. To prevent keratin contamination, prior to electrophoresis all gel units and staining containers should be thoroughly washed with a 1% solution of mild laboratory detergent, followed by rinses with neat methanol and ultrapure water. It is also important to wear gloves that have been rinsed with ultrapure water, and staining solutions should be removed with vacuum aspiration to avoid any contact with the gel:

1. Prepare reduced and alkylated plasma samples in protein solubilizing buffer (mix 1:1 v/v with 2 $\times$ solubilizing buffer).

¹¹It is preferable to dry the pellet completely under a gentle stream of argon (this may be done at room temperature) or, alternatively, in a SpeedVac® for a few minutes to prevent airborne contamination or sample oxidation. The pellet is then resuspended in the desired resolubilization buffer, or the dried pellet may be stored at -20 to -80°C until it is needed. To check for potential sample loss during the ethanol precipitation, dry 10% of the ethanol supernatant in a SpeedVac®, and run this sample in parallel with proportional volumes of sample before and after precipitation on 1D SDS-PAGE.

2. Mark a precast 1-mm, 12% Bis–Tris, 10-well NuPAGE® gel cassette at a point 2–4 cm from the bottom of the sample wells (see Note 12).
3. Prepare 1× MES running buffer: Add 50 mL of 20× NuPAGE® MES buffer to 950 mL of Milli-Q water.
4. Heat the samples at 90°C for 2 min.
5. Assemble the gel in the XCell *SureLock*™ Mini-Cell unit and add the running buffer to the upper and lower chambers. Load samples into each lane, with a MW standard in the first lane (see Note 13).
6. Run gels at 200 V using constant voltage.
7. Stop the gels when the dye front has reached the desired separation distance.
8. Disassemble the gel unit and remove the gel from plastic plates. Cut the left, bottom corner of the gel for proper orientation and, using a syringe, carefully mark the outer edges of the dye front with India ink (see Note 14).
9. Prepare fixing solution (100 mL per gel): 50% (v/v) methanol/10% (v/v) acetic acid in water.
10. Place the gel in fixing solution for 10 min, with gentle agitation on a platform shaker.
11. Prepare staining solution (95 mL per gel): 20% (v/v) methanol/20% (v/v) Stainer A in water.
12. Remove the fixing solution with vacuum aspiration and add the staining solution to the gel container. Shake the gel in the staining solution (without Stainer B) for 10 min.
13. Add the colloidal Coomassie® Stainer B (5 mL per gel container) to the existing staining solution. Shake the gel in the staining solution between 3 and 12 h.
14. Remove the staining solution with vacuum aspiration and replace with 200 mL of ultrapure water to destain. The gel will have a clear background after approximately 7 h, but the water can be changed several times to expedite the destaining process.

3.5. In-Gel Trypsin Digestion

To minimize airborne keratin contamination, we generally perform the in-gel digestion in a PCR hood equipped with laminar flow and a HEPA filter. For higher throughput, large-scale

¹²12% Bis–Tris gels combined with NuPAGE® MES buffer effectively separate complex serum proteins and provide high resolution of small- and medium-sized proteins. Ten-well gels allow larger volumes to be loaded, and the wider gel lane is advantageous for in-gel digestions, as any protein smearing at the lane edges can be removed with a razor blade while keeping an optimal lane width of ~4 mm. Gel length can differ per experiment based on sample complexity, but in general, running plasma samples for a short distance (2 or 3 cm) will effectively separate the proteins while reducing the total number of fractions to be analyzed by LC–MS/MS.

¹³Protein loads on gels should be maximized to increase the total sample digested and analyzed by LC–MS/MS. We typically load the equivalent of 10 µL of original serum per lane and run triplicate lanes per sample, for a total of 30 µL of original serum analyzed. When running more than one gel for quantitation, it is critical to measure the separation distance precisely to eliminate gel-to-gel migration differences. Therefore, we usually only run one gel per chamber because two gels in the same electrophoresis unit can migrate slightly differently. While some gel-to-gel variability is inevitable, tracking protein migration with MW standards will help to point out any major gel separation discrepancies. Figure 2 shows a typical 2-cm gel used for a serum biomarker discovery study; specifically, detection of protein changes between ectopic (EP) and normal intrauterine (IUP) pregnancy.

¹⁴Marking the tracking dye with India ink is helpful for measuring the exact gel running distance. This is important because the low MW region of plasma may be sparse with few stained proteins, and it can be difficult to determine where to cut the bottom of the gel to ensure that these low-abundance proteins are included in the subsequent in-gel digestion. Also, some gel swelling may occur after staining and can increase gel volume slightly, thereby artifactually shifting gel migration. MW standards should be carefully examined to correct for any migration differences.

digestions are done in 96-well plates with laser-cut holes allowing for centrifuge-aided removal of liquids. Prior to cutting gels, all 96-well plates and autosampler tubes and caps should be washed twice with 0.1% (v/v) trifluoroacetic acid/50% (v/v) methanol in water, followed by two rinses with 100% methanol, and allowed to air-dry in the PCR hood. All reagents are added with an eight-channel pipette and removed with centrifugation for 1 min in a bench-top centrifuge equipped with a 96-well plate rotor. A clean polystyrene plate cover should be used to cover the 96-well plate any time it is removed from the PCR hood for centrifugation or incubations:

1. Turn on the fan in the PCR hood approximately 15 min prior to cutting gels to achieve optimal air flow and to remove any dust particles that may be inside the hood.
2. Slice lanes of each gel into uniform 1×4 mm slices using a gel-cutting device (see Note 15). Several adjacent lanes (2–3) of the same sample may be combined to maximize protein load (see Note 16).
3. Using microforceps, transfer the excised gel slices into separate wells of a pre-cleaned, pierced, 96-well plate that has been stacked on top of a clean, V-bottomed, 96-well collection plate.
4. Destain the gel slices for 30 min with 100 μ L of 200 mM ammonium bicarbonate/50% acetonitrile at 37°C, with gentle shaking. Centrifuge the plates for 1 min to remove the buffer into the collecting plates. Discard the destaining extract after centrifugation.
5. Dry gel slices for approximately 20–30 min using a SpeedVac® evaporator.
6. Re-hydrate gel bands by adding either 20 μ L (one to two $1 \times 1 \times 4$ mm bands per well) or 40 μ L (three bands per well) of trypsin working solution (see Note 17). Incubate for 16–18 h at 37°C in a thermostatically controlled incubator (see Note 18).
7. Collect the digested protein extract into a clean, 96-well collecting plate by centrifuging for 1 min.
8. Add 15 μ L (for one to two bands per well) or 30 μ L (for three bands per well) of wash buffer and incubate for 30 min at 37°C.

¹⁵If using the gel cutter tool, which is currently the only commercial gel cutting device for 1D SDS-PAGE, gel lanes will have to be sliced individually. This tool is convenient, but some mashing of the gel occurs. Hence, a custom razor blade array constructed of stainless steel razor blades separated by 1-mm spacers is preferred. The custom array is beneficial because the width of the blades (4 cm) allows up to three lanes to be sliced simultaneously, preventing sample mix-up that can occur when lanes are sliced separately. Also, the sharpness of the razor blades, compared to the thin grid-like design of the gel cutter, prevents mashing of the gel, which can cause small particles of gel to contaminate other protein fractions.

¹⁶To combine three lanes of a sample, the entire lanes of the gel can be cut with a stainless steel razor, removing any portion with no protein, i.e., loading wells, outer edges, gel region between lanes, or the bottom of the gel. We typically cut along the inner edges of the gel lane to eliminate any edge effects on the sides of the gel lane. Adjacent lanes can be aligned by matching up major proteins horizontally prior to cutting the gel.

¹⁷A 96-well pierced plate will easily hold up to three $1 \times 1 \times 4$ mm gel slices. However, increasing the number of gel slices, and consequently gel volume, will absorb more trypsin solution as the gels re-hydrate during the overnight incubation. To prevent significant buffer losses, we increase the trypsin extract volumes in Subheading 3.5, steps 5 and 7 to account for the increased gel volume if three slices are combined. Using more than three $1 \times 1 \times 4$ mm gel slices is not recommended due to the need for excessive reagent volumes and increased trapping of peptides in the large gel volume.

¹⁸To prevent sample evaporation during overnight incubation, place the pierced plate (top) and collection plate (middle) on top of a third 96-well plate with ~80 mL of Milli-Q® water in each well. Place all stacked plates into a Ziploc® bag. The humidity created in the bag will prevent sample evaporation. To prevent sample leakage from the pierced plate, cover the top (pierced) plate with a glass plate placed under the polystyrene plate cover. The glass plate acts as a seal to prevent leakage of trypsin solution into the collecting plate. Shaking the samples during overnight incubation should be avoided, as the agitation may promote sample leakage.

9. Collect the second extract into the 96-well collecting plate containing extract 1 by centrifuging for 1 min.
10. Transfer the samples into pre-cleaned autosampler sample vials. If desired, the samples may be pooled at this point (see Note 19).

3.6. LC–MS/MS

Label-free quantitation through comparative LC–MS requires data acquisition with high-resolution and high-mass-accuracy mass spectrometers. Analysis of complex peptide mixtures on lower-resolution instruments is not sufficient to resolve co-eluting peptides with similar m/z ratios (20). MS-based proteomics has seen great improvements in the past 5–6 years, and extremely high-performance MS is now routinely available. The evolution of hybrid linear ion trap-FT and Orbitrap™ instruments has improved not only resolution, but also ion capacity, scan speed, and mass resolving power to reduce false-positive peptide identifications dramatically (20). Our analysis was performed using an LTQ-Orbitrap™ XL instrument:

1. Connect a 75- μ m i.d. $\times$ 25 cm column packed with BEH C18 resin, 1.7 μ m particle size, to the mass spectrometer. To avoid temperature fluctuations, the column should be contained in a thermostat-controlled compartment maintained at 40°C.
2. Set the flow rate at 200 nL/min and equilibrate the column with 3% Solvent B.
3. Inject 4–8 μ L (see Note 20) of each trypsin digest at 5 μ L/min over a 4-min period with 100% Solvent A. The HPLC gradient is as follows (see Note 21):
 - 3–28% Solvent B over 42 min.
 - 28–50% Solvent B over 25.5 min.
 - 50–80% Solvent B over 5 min.
 - Hold at 80% Solvent B for 5 min.
 - Return to 3% Solvent B over 1 min.
4. The following parameters are used for MS/MS data acquisition and have been optimized for downstream analysis with the Elucidator® system (see Note 22):
 - (a) Scan ranges: 400–2,000 m/z .
 - (b) Full MS scan: FT at 60,000 resolution in profile mode.

¹⁹The effect of pooling samples in biomarker discovery remains unclear. Pooling samples reduces cost and analysis time associated with multi-dimensional experiments, which may include tens to hundreds of patient samples. However, pooling averages the concentration of individual proteins across all pooled samples and, in some cases, outliers skew the results and overall intensities of some low-abundance proteins may fall below the detection threshold in pooled samples (23). Alternatively, pooling may help to reduce sample variability by minimizing individual variation. This is suitable for analyses where the focus is not specific to the individual, but rather on global changes in expression patterns (24). Pooling can be done in two ways: complete pools or sub-pools. Complete pooling of all samples in a treatment group reduces biological variation, and any replicates analyzed are technical rather than biological. Sub-pooling randomly combines several individual samples into multiple pools per experimental group, thus providing biological replicates and still allowing identification and removal of anomalous samples, if necessary (24, 25). We typically create sub-pools of samples after proteins are digested with trypsin. Tryptic digests of individual samples are then archived at –20°C for future use. Specifically, from nine serum samples from patients with ectopic pregnancy and nine gestational-stage matched controls, we pooled corresponding fractions from three patients each, for a total of six sub-pools. The sub-pools provide biological replicates for our analyses, yet reduce the total number of fractions to be analyzed by LC–MS/MS. In future validation stages of biomarker discovery, the individual samples can be easily thawed and analyzed in a highly reproducible manner, because the same samples used for discovery are being used for initial validation.

²⁰The injection volume can be adjusted based on the intensity of protein staining in individual digests to prevent overloading of the column.

²¹The sample run time is 78 min. To help minimize carryover between samples, a blank should be run between samples. A reasonable blank cycle time is 20 min, giving a total analysis time per sample of 98 min.

- (c) Number of MS/MS: three most abundant with data-dependant scans.
- (d) Charge state screening enabled.
- (e) Monoisotopic precursor selection enabled.
- (f) Charge state rejection enabled to reject $z = 1$.

3.7. Label-Free Quantitation in the Elucidator® System

There are a number of label-free quantitation software programs available as open-source or commercial packages. While the freely available packages are in various stages of development, the commercial packages seem to have more advanced data-processing algorithms and user-friendly interfaces (9). A critical distinguishing feature is whether the software can reliably combine quantities of a given peptide if it is distributed across fractions. This protocol describes label-free quantitation with the Rosetta Elucidator® System. The basic principles and typical workflow of the Elucidator® System are common to most comparative LC-MS packages, and this protocol can be used as a guideline for most available label-free quantitation programs:

1. Import RAW MS spectra into the Elucidator® System.
2. Define each experiment's treatment groups and corresponding technical and/or biological replicates. Multi-fraction experiments should be analyzed in a multidimensional work-flow (see Note 23). If the dataset is too large for the software program to handle in a single analysis, the proteome may be divided into subsets based on gel migration for quantitative comparisons (see Note 24).
3. Perform retention time (RT) alignment and extract features, i.e., unique time and m/z boundaries for peaks in individual runs, over the entire chromatographic time window. In the Rosetta Elucidator® System, this step is executed using the Peak Teller algorithm (see Note 25).

²²To facilitate the label-free pattern analysis in the Elucidator® system, it is essential that rigorous controls be maintained to ensure that HPLC column performance (resolution and peak widths), peak RTs, and mass spectrometer performance are maintained within restricted tolerances. Specifically, the same column (ideally temperature controlled) and gradients should be used, RTs of trypsin autolytic peptides should be monitored via extracted ion chromatograms and remain within a ± 1.0 -min window, and injection volumes – measured by weighing the sample vials before and after injection – should remain within $\pm 10\%$.

²³One of the benefits of using the Elucidator® system is the ability to utilize a multi-dimensional workflow. While there are a number of LC-MS and LC-MS/MS pattern analysis software programs available, most approaches and current commercial software can only handle data where an entire proteome is represented by a single LC-MS/MS run. Unfortunately, without some fractionation of the proteome, only a modest number of plasma proteins can be identified, which will include few, if any, low-abundance proteins. Alternatively, the Elucidator® system effectively integrates multiple LC-MS/MS datasets such as those resulting from separating a single proteome into 20 fractions (gel slices). The system initially aligns RT and m/z patterns from corresponding slices, e.g., all slice 1 samples or all slice 2 samples. Therefore, the initial comparison is within corresponding gel slices; but importantly, the software adjusts for variable distributions of some proteins between adjacent slices. This is critical because such complex samples represent a continuum of proteins on a 1D gel (or any other protein or peptide fractionation method), and there will always be some proteins that are variably distributed across adjacent slice boundaries in different gel lanes. After initial comparisons within gel slices are completed, the entire dataset is integrated by matching corresponding m/z values with the same RT between slices. Intensities for matching m/z values in adjacent slices are summed if they are identified as matching the same peptide, or if the m/z in one slice was identified and the m/z with matching mass and RT did not trigger an MS/MS. Because matching peptide intensities are summed even if they do not result in a high-quality MS/MS spectra, the major source of variation often encountered due to undersampling in MS/MS analyses of complex mixtures is avoided.

Summed signal intensity over the entire gel lane is obtained for every identified protein in the proteome by combining the intensities for all peptides contained within that protein. Average intensities and standard deviations for each protein are calculated for the control and experimental samples. The variance in these data points reflects a combination of experimental and biological variation. An advantage of this analysis is that signals arising from very low-level peptides that produce a detectable m/z peak, but are too low to trigger MS/MS analyses consistently in all samples, can still be quantitatively compared across all samples.

²⁴It was our experience with an earlier version of the Elucidator® system that software limitations prevented the analysis of entire proteomes from six pools with a total of 252 RAW files in a single quantitative comparison. Hence, we divided the data into four subsets based on gel migration (slices 1–4, 5–10, 11–15, and 16–21).

4. Acquire high-quality features: filter out any features that have $z \leq 1$ or $z \geq 5$, or peak time score or peak m/z score less than 0.7 and 0.8, respectively (see Note 26).
5. Create DTA files for all features that pass the above quality filters. DTAs should be created for the monoisotopic peak using the scan closest to the center of the chromatographic peak only.
6. Search MS/MS data from features of interest using SEQUEST or other search engines to determine peptide sequences and associated proteins.
7. Assign peptide and protein information to features using SEQUEST Annotation. For high-quality peptide and protein identifications, search results should be filtered at mass error ≤ 10 ppm and dCN ≥ 0.05 prior to annotation.
8. Find peptides or proteins of interest whose expression levels change significantly across treatment groups (see Note 27).

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²⁵The Peak Teller algorithm is a complex processing step that corrects for local shifts in RT across all runs in the experiment and removes noise and background. In addition, the algorithm creates peak regions, or features, to match peaks across runs and treatment groups correctly. The algorithm summarizes features into isotope groups, i.e., groups of features originating from the same peptide and an ion of one charge state, and isotope groups are associated into charge groups representing all detected charge states from a given peptide. Finally, the algorithm generates expression profiles, which include peak intensities and associated error model statistics. Figure 3 shows peak intensities at the isotope group level for three peptides of a candidate biomarker.

²⁶During RT alignment and feature ($RT \times m/z$ surface) extraction, the Peak Teller algorithm performs background subtraction and smoothing across the alignment window (both in RT and m/z dimensions) and uses a maximum alignment time correction of ± 4 min. Each feature is then scored for quality, where a score of 0 indicates uncorrelated intensities and a score of 1 represents ideal peak shape (26). In these experiments, only features with scores greater than 0.7 and 0.8 were included.

²⁷The Elucidator® system has a number of system tools to filter full sets of peaks and return a subset relevant to the specific experiment. Differentially expressed peaks across treatment groups can be detected using significance testing methods, such as ANOVA, as well as by applying fold-change criteria. We find that there are some advantages in using the trend analysis tool to detect increases or decreases in intensities at the peptide level between experimental groups. Trends were created for each segment of the gel proteome (see Note 24) and intensities for each protein were then manually combined across the four subsets. As expected, individual peptides were spread across several gel slices, and minor variations occurred from sample to sample. These gel migration differences caused medium- to high-abundant proteins within one region of the gel to look artifactually interesting in a flanking segment of the gel. When peptide intensities for these multi-fraction proteins were combined, the net fold change was often not significant. Variable intensity distributions across fractions may be due to a number of factors, as illustrated in Fig. 4. These include possible extensive aggregation/glycosylation (*Panel A*), and proteolysis (*Panel B*). These MW differences may also reflect an isoform shift or a posttranslational modification that could distinguish between normal and ectopic pregnancy. GeLC-MS/MS provides the advantage of identifying these potential MW shifts, which would be missed in most other fraction schemes.

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3-D Serum Profiling Method

Fig. 1.

Scheme for comprehensive protein profiling using major protein depletion and GeLC-MS/MS prior to label-free quantitation. To identify a broad range of proteins in plasma in a clinically relevant setting (ectopic pregnancy compared with intrauterine pregnancy), we utilize “Top 20” depletion and fractionation on short SDS-PAGE gels to improve detection of low-abundance proteins.

Fig. 2. Representative 1D SDS-PAGE gel used for GeLC-MS/MS. Depleted and concentrated unbound serum from patients with ectopic pregnancy (EP) and normal intrauterine pregnancy (IUP) was run for a short distance (2 cm) on SDS-PAGE. Each lane was sliced into 20 fractions and each fraction was digested with trypsin for further analysis on LC-MS/MS.

Fig. 3.

Differential peak intensity. Differential peak intensities quantitated using the Rosetta Elucidator® system. Peak intensities shown are summarized as isotope groups, i.e., groups of isotopic mass peaks determined to originate from the same charge state of one peptide. The isotope groups represent three different peptides of a candidate biomarker, which is decreased in ectopic pregnancy across all pooled samples ($n = 3$ in each pooled group).

Fig. 4.

Edge effects caused by dividing the proteome into multiple groups due to *software* capacity limitations. Individual peptides are spread across multiple gel slices as expected, and minor variations occur from sample to sample. These gel migration differences cause proteins within one region of the gel (*vertical dashed lines*) to exhibit an apparently significant fold change (values indicated above intensity bars). When peptide intensities for these multi-fraction proteins were combined across all gel fractions, the net fold change was not significant (values indicated below *panel title*). Variable intensity distributions across fractions are due to a number of factors, including extensive aggregation or glycosylation (**a**) and proteolysis (**b**). Arrows indicate expected MW (*gel slice*) of protein based on amino acid sequence.