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Intact-Protein Analysis System for Discovery of Serum-Based Disease Biomarkers

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Abstract

Profiling of serum and plasma proteins has substantial relevance to the discovery of circulating disease biomarkers. However, the extreme complexity and vast dynamic range of protein abundance in serum and plasma present a formidable challenge for protein analysis. Thus, integration of multiple technologies is required to achieve high-resolution and high-sensitivity proteomic analysis of serum or plasma. In this chapter, we describe an orthogonal multidimensional intact-protein analysis system (IPAS) (Wang et al., *Mol Cell Proteomics* 4:618–625, 2005) coupled with protein tagging (Faca et al., *J Proteome Res* 5:2009–2018, 2006) to profile the serum and plasma proteomes quantitatively, which we have applied in our biomarker discovery studies (Katayama et al., *Genome Med* 1:47, 2009; Faca et al., *PLoS Med* 5:e123, 2008; Zhang et al. *Genome Biol* 9:R93, 2008).

Keywords

Multidimensional protein fractionation; Serum; plasma; Quantitative proteomics; Disease biomarkers

1. Introduction

Comprehensive profiling of the serum and plasma proteomes has substantial relevance to the identification of circulating protein biomarkers that may have predictive value and utility for disease diagnosis and management. However, the complex spectrum of serum and plasma proteins spans a dynamic range of protein abundances of at least 12 orders of magnitude (1). Moreover, proteins often occur in multiple isoforms due to posttranslational modifications, cleavages, or splice variants. As a result, decomplexing biological fluids through fractionation or enrichment is beneficial for disease biomarker discovery. As an orthogonal separation technique, 2D-PAGE (2–4) was initially relied upon for quantitative separation of proteins. However, intrinsic limitations of 2D-PAGE, such as the dynamic range, inability to resolve small and large proteins, and the difficulty for online evaluation coupled with MS analysis, do not allow for sufficient capacity for analysis of low-abundance proteins in such complex proteomes as serum and plasma. Furthermore, extraction of proteins from gels for their identification following digestion or their recovery for other applications is difficult and associated with substantial losses. Therefore, alternative strategies using liquid-phase-based intact-protein fractionation approaches are increasingly relied upon (5–10). An advantage of separation of intact proteins prior to MS analysis is the ability to assess protein

variation associated with posttranslational modifications, cleaved forms, or splice variants, given the altered chromatographic properties of such variants.

In this chapter, we describe a liquid-phase-based orthogonal multidimensional intact-protein analysis system (IPAS) that we have developed for disease biomarker discovery. We incorporate intact-protein isotopic labeling into IPAS to profile protein differences quantitatively in samples being compared. The strength of the intact-protein-based multidimensional separation system is in its ability to resolve related proteins, such as differentially modified forms, and to reduce the complexity of samples through fractionation prior to MS analysis. The IPAS platform is illustrated in Fig. 1. First, serum/plasma samples from control and case are processed through immunodepletion chromatography to remove the high-abundance proteins. Then, the remaining protein mass in each of the samples to be compared is concentrated and isotopically labeled with acrylamide, i.e., the control sample is labeled with one isotope, e.g., ^{12}C -acrylamide (light), and the case sample is labeled with another ^{13}C -acrylamide (heavy). A mixture consisting of each of the intact-protein-labeled samples is subjected to an orthogonal 2D-HPLC separation with anion-exchange chromatography (AEX) as the first dimension that separates proteins based on charge, and reversed-phase (RP) chromatography as the second-dimension separation that further separates proteins based on hydrophobicity. Collected protein fractions from 2D-HPLC are lyophilized by freeze-drying, followed by in-solution tryptic digestion. The digests are subjected to LC-MS/MS analysis. Relative quantification of identified proteins is achieved by comparing the signal intensities of identified peptides from the control and case samples in MS scan events. We have applied our current liquid-phase-based orthogonal multidimensional IPAS, coupled with the intact-protein isotopic labeling approach and nano-LC ESI MS, to studies of serum/plasma from several cancer types (11–14). Thus, with the multidimensional intact-protein fractionation approach, sample complexity is greatly reduced prior to MS analysis. In general, we have identified ~1,500 proteins with high confidence and obtained relative quantitative data for some 40% of identified proteins that span across seven orders of magnitude in protein concentration in a given experiment.

2. Materials and Instrumentation

2.1. Samples

Samples (control or case) are from individual subjects or pooled donors. The protocol described here is designed for a total of 600 μL of serum/plasma, i.e., 300 μL of serum/plasma from control and 300 μL of serum/plasma from case samples.

2.2. Intact-Protein Fractionation

1D-HPLC system (Shimadzu Corporation, Columbia, MD). For immunodepletion chromatography, the system comprises the following:

2.2.1. Automated 1D-HPLC System

1. Pump system: Two LC-10Ai pumps.
2. Degasser: DGU-20A3 Prominence Degasser.

3. Column oven: CTO-20 Prominence Column Oven.
4. Detector: SPD-20 UV/VIS Prominence Detector.
5. Collector: FRC-10A Fraction Collector.
6. Controller: SCL-10A VP System Controller.
7. Workstation: EZStart 7.4 SP1.

2.2.2. Automated Online 2D-HPLC System—Automated online 2D-HPLC system (Shimadzu Corporation, Columbia, MD).

For isotopically labeled intact-protein 2D-HPLC fractionation (see Fig. 2), the system comprises the following:

First dimension: AEX.

Second dimension: RP chromatography.

1. Auto sampler: SIL-10AP.
2. Pump system: One LC-10Ai pump for the first-dimension HPLC, Two LC-20AD pumps for the second-dimension HPLC.
3. Degasser: DGU-20A5 Prominence degasser.
4. Column oven: CTO-20AC Prominence column oven.
5. Detector: SPD-20 UV/VIS Prominence Detector.
6. Collector: Four FRC-10A Fraction Collectors.
7. Controller: A SCL-10A VP system controller.
8. Workstation: Class-VP 7.4 (Shimadzu Client/Server).

2.2.3. Immunodepletion Chromatography

1. Hu-6 HC column (10 × 100 mm; Agilent Technologies, Wilmington, DE).
2. Ms-3 HC column (10 × 100 mm; Agilent Technologies, Wilmington, DE).
3. *Buffer A* (5185-5987), equilibration/loading/washing (Agilent Technologies, Wilmington, DE).
4. *Buffer B* (5185-5988), for elution (Agilent Technologies, Wilmington, DE).
5. 0.22-μm RC syringe filter (Fisher Scientific).

2.2.4. Anion-Exchange Chromatography

1. SAX/10 column, 7.5 mm ID × 150 mm L (Column Technology Inc., Fremont, CA).
2. *AEX Mobile Phase A*: 20 mM Tris-HCl, 6% isopropanol, 4 M urea, pH 8.5 (pH is adjusted by adding 1 N HCl).

3. *AEX Mobile Phase B*: 20 mM Tris-HCl, 6% isopropanol, 4 M urea, and 1 M NaCl, pH 8.5 (pH is adjusted by adding 1 N HCl).
4. Trizma base (T1503, Sigma, St. Louis, MO).
5. Hydrochloric acid, trace metal grade (A508-500, Fisher Scientific).
6. Sodium chloride (S271-3, Fisher Scientific).
7. Urea (U15-3, Fisher Scientific).
8. Isopropanol, HPLC grade (A451-1, Fisher Scientific).

2.2.5. Reversed-Phase Chromatography

1. RP/5D column, 4.6 mm ID × 150 mm L (Column Technology Inc., Fremont, CA).
2. *RP Mobile Phase A*: 95% HPLC-grade water, 5% acetonitrile + 0.1% trifluoroacetic acid (TFA).
3. *RP Mobile Phase B*: 90% acetonitrile, 10% HPLC-grade water + 0.1% TFA.
4. Water, HPLC grade (W5-1, Fisher Scientific).
5. Acetonitrile, HPLC grade (A998-1, Fisher Scientific).
6. TFA (3-3077, Supelco, Bellefonte, PA).

2.3. Acrylamide Isotope Labeling

1. Centricon YM-3 devices (Millipore).
2. *Labeling Buffer*: 8 M urea, 100 mM Tris-HCl, and 0.5% octyl-beta-D-glucopyranoside (OG), pH 8.5 (pH is adjusted by adding 1 N HCl).
3. Dithiothreitol (DTT, ultrapure, USB).
4. Light acrylamide: 1,2,3-¹²C₃-acrylamide (>99.5% purity, Fluka).
5. Heavy acrylamide: 1,2,3-¹³C₃-acrylamide (>98% purity, Cambridge Isotope Laboratories, Andover, MA).

2.4. Protein Fractionation Processing

FreeZone Plus 12 Liter Cascade Console Freeze Dry Systems (Labconco Corporation, Kansas City, MO).

2.5. Nano-LC-ESI MS/MS Analysis of 2D-HPLC Fractionated Samples

2.5.1. In-Solution Protein Digestion

1. Sequence-grade modified trypsin (Porcine, V511A/20 µg, Promega, Madison, WI).
2. *Digestion buffer*: aqueous 50 mM ammonium bicarbonate containing 4% acetonitrile in HPLC-grade water (v/v).
3. Ammonium bicarbonate (A643-500, Fisher Scientific).
4. Acetonitrile, Optima LC/MS grade (A955-1, Fisher Scientific).

5. Urea (U15-3, Fisher Scientific).
6. Formic acid, 99+% (28905, Pierce).

2.5.2. Nano-LC–ESI Mass Spectrometry Analysis

1. Nano-ESI LTQ-FT or Orbitrap nano-ESI mass spectrometer (Thermo Scientific).
2. Eksigent nano-LC-1D plus (Dublin, CA).
3. Capillary column: 25-cm Pico frit 75 μ m ID (New Objectives) in-house packed with Magic C18 (100 Å pore size/5- μ m particle size, Michrom Bioresources, Inc.).
4. Trap column: Symmetry C₁₈ 180 μ m $\times$ 20 mm, particle size 5 μ m (Waters Corporation).
5. *Nano-LC Mobile phase A*: 0.1% formic acid in HPLC-grade water (v/v).
6. *Nano-LC Mobile phase B*: 0.1% formic acid in acetonitrile (v/v).
7. Water, HPLC grade (W5-1, Fisher Scientific).
8. Acetonitrile, Optima LC/MS grade (A955-1, Fisher Scientific).
9. Formic acid, 99+% (28905, Pierce).

2.5.3. Data Analysis

1. All the LC–MS/MS data management are performed using the Computational Proteomics Analysis System (15). <https://proteomics.fhcrc.org/CPAS/project/home/begin.view?>
2. Search Algorithm: X!Tandem (16).
3. Scoring: PeptideProphet (16) and ProteinProphet (17).
4. Quantitation: Q3 (18).

3. Methods

3.1. Immunodepletion Chromatography (Performed at Room Temperature)

1. Filter serum/plasma samples with a 0.22- μ m RC syringe filter.
2. Equilibrate the Hu-6 HC immunodepletion column with *Buffer A* (Agilent) at 3 mL/min for 10 min.
3. Wash the 2-mL sample loop (PEEK) with 5 mL *Buffer A* (Agilent).
4. Load 300 μ L of the serum/plasma sample onto 2-mL sample loop. Start the step elution at 0.5 mL/min (the step-elution program is listed in Table 1).
5. Collect the flow-through fraction (i.e., the low-abundance protein fraction) for 20 min and immediately store the fraction at -80°C until use.
6. Elute the bound fraction (i.e., the high-abundance protein fraction) and regenerate the column with *Buffer B* at 3 mL/min for 8 min (collect if wanted).

7. Re-equilibrate the system between sample injections.
8. The typical immunodepletion chromatogram is shown in Fig. 3.

3.2. Intact-Protein Isotopic Labeling (Performed at Room Temperature)

1. Concentrate the control and case immunodepleted serum/plasma samples separately (only the flow-through low-abundance protein fraction, see Fig. 3) with the Amicon YM-3 device until volume of <100 μ L is reached.
2. Dilute each of the concentrated samples up to 1 mL with *Labeling buffer*: 8 M urea, 100 mM Tris-HCl, and 0.5% OG, pH 8.5.
3. Measure protein concentration using Bradford method (19) for case and control samples, respectively (see Note 1).
4. Reduce protein disulfide bonds for 2 h at room temperature by adding 0.66 mg DTT per milligram of protein (see Note 2).
5. Alkylate cysteines with acrylamide immediately after the reduction step by adding 7.1 mg/mg of total protein of ^{12}C 3-acrylamide (*light*) to the control sample and 7.4 mg/mg of total protein of ^{13}C 3-acrylamide (*heavy*) to the case sample. The reaction is carried out for 1 h in the dark at room temperature (see Note 3).
6. Mix the acrylamide-labeled control and case samples, dilute the mixture to 9 mL with AEX mobile phase A, and submit it immediately to 2D-HPLC intact-protein separation (see Note 4).

3.3. Protein 2D-HPLC Fractionation (Performed at Room Temperature)

3.3.1. The First-Dimensional AEX—Step-elution program is listed in Table 2:

1. Equilibrate the anion-exchange column with AEX mobile phase A for 60 min at 1.0 mL/min.
2. Wash the 10-mL sample loop (PEEK) with 20 mL AEX mobile phase A.
3. Filter the mixed isotope-labeled sample (diluted with AEX mobile phase A) with a 0.22- μ m RC syringe filter.
4. Load the filtered sample onto the 10-mL sample loop through the injector.
5. Start the run (the online 2D-HPLC system diagram is shown in Fig. 2).

¹As a result of immunodepletion, the total protein concentration is reduced by 90%, from approximately 70 to 7 mg/mL. These calculations are useful in evaluating the efficiency/performance of the immunodepletion column. The life time of the immunodepletion column is 200 injections, and efficiency of capture is between 90 and 95%.

²Since the samples are in the *Labeling Buffer* that contains 8 M urea, the protein reduction reaction should be done at room temperature to avoid protein carbamylation due to the formation of isocyanic acid from urea at higher temperatures.

³We suggest performing the labeling of control and case samples with ^{12}C 3-acrylamide (*light*, control) and ^{13}C 3-acrylamide (*heavy*, case). Also, the labeling reaction should be at room temperature as explained in Note 2.

⁴When the labeling reaction is finished, add 1 mL of the AEX *Mobile phase A* to the control sample and the case sample, respectively. Then, mix these two samples and further dilute the mixed samples to 9 mL with AEX *Mobile phase A*. The size of the sample loop (PEEK) is 10 mL for the first-dimensional AEX.

3.3.2. The Second-Dimensional RP Chromatography—Gradient-elution program is listed in Table 3:

1. Equilibrate the two RP columns with 95% of RP *Mobile Phase A* (95% HPLC-grade water, 5% (v/v) acetonitrile + 0.1% TFA) for 15 min at 2.4 mL/min.
2. Protein eluted from the first-dimensional anion exchange column is loaded onto one of the two RP columns (the online 2D-HPLC diagram is shown in Fig. 2).
3. There is a total of eight-step elution's from the first-dimensional AEX (Table 2), and each of the eight eluted fractions is alternatively online loaded onto one of the two RP columns for sequential second-dimensional RP chromatography separation (see Fig. 2).
4. Desalt the RP column with 95% RP *Mobile Phase A* for 10 min before starting the gradient elution. The gradient-elution program for second-dimension RP chromatography is listed in Table 3 (see Note 5).
5. Apply RP separation to each of the eight anion-exchange step elutions. Typical online 2D-HPLC chromatograms are shown in Fig. 4.
6. Collect the protein fractions from the RP column (3 fractions/min, 800 μ L/per fraction) and keep the protein fractions at -80°C until use.
7. Lyophilize the protein fractions using the Freeze-Dry Systems. It usually takes 48 h to dry the protein fractions completely.

3.4. Nano-LC–ESI Mass Spectrometry Analysis

3.4.1. In-Solution Protein Digestion

1. Individual lyophilized protein fraction is suspended with 200 ng of trypsin in the *Digestion Buffer*. The protein digestion is carried out at 37°C overnight.
2. The digestion is quenched by adding 5 μ L of 1.0% formic acid solution.
3. Based on the RP chromatography pattern, individual fractions (see Fig. 4) are combined into 12 pooled fractions for nano-LC–MS/MS analysis (the pooling sequence is listed in Table 4).

3.4.2. Nano-Flow Rate LC ESI Mass Spectrometry Analysis—Eksigent nano-LC-1D plus RP capillary HPLC system directly delivers nano-flow without using A splitter. This system is coupled with a nano-ESI LTQ-FT or LTQ-Orbitrap. The gradient elution program is listed in Table 5:

1. Load 20 μ L of each pooled digested protein fraction into the 20- μ L sample loop.
2. After injection, the sample is trapped onto the trap column and desalted with 3% of nano-LC mobile phase B (0.1% formic acid in acetonitrile) for 10 min at 5 μ L/min.

⁵Protein fractions eluted from the first-dimensional AEX are trapped onto the RP column with the anion-exchange buffer. It is important to desalt the protein fraction before initializing the second-dimensional RP chromatography to achieve higher resolution separation and prevent salt precipitation within the RP column in the presence of acetonitrile.

3. Start the nano-RPLC gradient elution (Table 5) and acquire spectra in a data-dependent mode in m/z range of 400–1,800. Select the five most abundant +2 or +3 ions in each MS spectrum for MS/MS analysis.
4. Nano-ESI LTQ-FT mass spectrometer parameters are capillary voltage of 2.0 kV, capillary temperature of 200°C, resolution of 100,000, FT target value of 800,000, and ion trap target value of 10,000.
5. Nano-ESI LTQ-Orbitrap mass spectrometer parameters are capillary voltage of 2.0 kV, capillary temperature of 200°C, resolution of 60,000, FT target value of 1,000,000, and ion trap target value of 10,000.

3.4.3. Data Analysis—The acquired LC–MS/MS data from nano-RPLC LTQ-FT or nano-RPLC LTQ-Orbitrap are automatically processed by the Computational Proteomics Analysis System (CPAS; see Subheading 2.5.3) (15, 16), <https://proteomics.fhcrc.org/CPAS/project/home/begin.view?>

1. For protein database search, $^{12}\text{C}_3$ -acrylamide-labeled cysteine-containing peptide (light) is considered as the fixed modification and $^{13}\text{C}_3$ -acrylamide isotope-labeled cysteine-containing peptide (heavy) is detected using a delta mass of 3.01884 Da. The relative quantitation (ratio = heavy/light) information of the paired cysteine-containing peptides is obtained by a script called “Q3” that was developed in-house to obtain the relative quantification for each pair of cysteine-containing peptides sequenced by MS/MS (18).
2. Apply the criteria for peptide sequence matching with a PeptideProphet score equal or greater than 0.2 (20). Then, the matched peptides are further grouped to protein queue using the ProteinProphet algorithm at an error rate of <5% (17). Furthermore, the sequenced peptides with PeptideProphet scores equal or greater than 0.75 are selected for quantitation (ratio). Based on the sequenced cysteine-containing peptides, their corresponding theoretical mono-isotopic mass and mass charge are obtained. The intensity of peaks in each MS scan is computed and each light peak (labeled with $^{12}\text{C}_3$ -acrylamide) is paired with the corresponding heavy peak (labeled by $^{13}\text{C}_3$ -acrylamide). For the final quantitation, a total of 20 MS scans before and after the MS/MS identification are summed. Proteins containing more than one pair of peptides that yield quantitation have all their peptide ratios averaged in that particular 2D-HPLC fraction (see Note 6).

⁶Acquired MS/MS data from LC-ESI mass spectrometry were automatically processed by the in-house built software (<https://proteomics.fhcrc.org/CPAS/project/home/begin.view>). In brief, LC–MS and MS/MS data were first converted to mzXML format using ReAdW software (version 1.2) to generate the peak list for protein database search. X!Tandem search engine (version 2005.12.01) was configured with Cys labeled with $^{12}\text{C}_3$ -acrylamide (71.03657@C) as the fixed modification and Cys labeled with $^{13}\text{C}_3$ -acrylamide (74.04663@C) and Methionine (Met) oxidation (15.9949@M) as the variable modifications. Protein database used for this search is the International Protein Index (IPI) Human protein knowledgebase (version v3.68), and the number of protein entries in the database is 87,061. The minimum criterion for peptide matching was PeptideProphet score > 0.2. Peptides that met with these criteria were grouped to protein sequences using the ProteinProphet algorithm at an error rate of < 5%. In a given experiment, ~1,500 proteins are identified, with 40% of identified proteins yielding relative quantitation information. Figure 6 shows the distribution of identified proteins using the number of MS/MS events as a measure of protein abundance in a given study. From Fig. 6, it is clear that the majority (70%) of identified proteins occur at low abundance, with < 10 MS/MS events per protein. This further illustrates the efficiency of intact protein multidimensional fractionation in reaching low-abundance proteins in serum/plasma.

3. Figure 5 shows a representative nano-flow rate LC–MS/MS analysis obtained by nano-ESI LTQ-FT, including the TIC chromatogram, MS spectrum, MS/MS sequence spectrum, and $^{13}\text{C}_3/^{12}\text{C}_3$ -acrylamide-labeled paired precursor ions for relative quantitation (ratio = heavy/light).
4. Figure 7 demonstrates the advantage of intact-protein-based multidimensional HPLC fractionation in studying serum/plasma samples in the pre-diagnosis of WHI-colon cancer (see Note 7).

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⁷Peroxisiredoxin 6 (PRDX6) is a cytoplasmic protein involved in redox regulation of the cell, detoxifying hydrogen peroxide and various organic peroxides (21). Two different forms of PRDX6 are detected by using IPAS analysis in a study of pre-diagnosis of WHI-colon cancer. Quantitative data are obtained for the two different forms of PRDX6 (Fig. 7). Form (A) is the intact-protein form containing the AhpC/TSA domain and C-terminal domain. Form (A) is upregulated in cancer with a ratio of 1.60 (cancer vs. control). Form (B) is the cleavage form containing the AhpC/TSA domain only. There is no significant difference in levels between cancer and control with a ratio of 1.14 (cancer/control). It was reported that overexpression of PRDX6 leads to a more invasive phenotype and metastatic potential in human breast cancer, at least in part, through the regulation of the levels of uPAR, Ets-1, MMP-9, RhoC, and TIMP-2 expression (22). It will be more interesting to investigate the PRDX6 serum level in other types of cancer, such as colon cancer.

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Fig. 1. Platform of intact-protein multidimensional HPLC fractionation, coupled with isotope labeling and nano-LC-ESI MS/MSMS for quantitative serum/plasma proteome analysis.

Fig. 2. Work-flow diagram of automatic online 2D-HPLC system

(a) The first-dimension pump loads the sample through the 10-mL sample loop onto the anion-exchange column with step elution Step 1. (b) Through the 10-port 2-position valve, the flow-through proteins (unable to be absorbed within anion-exchange column) will be trapped within one of the two RP columns, here the RP-2 column. (c) The second-dimension pump starts the gradient elution within the other RP column. Here is the RP-1 column that is directed to the Fraction Collector through the 7-port 6-position valve. (d) At the end of the RP-1 gradient elution, the 10-port 2-position valve will switch to another position. (e) The first-dimension pump will start the step elution Step 2. (f) Through the 10-port 2-position valve, proteins eluted from the anion-exchange column will be trapped within the RP-1 column. (g) The second-dimension pump starts the gradient elution within the RP-2 that is directed to the Fraction Collector through the 7-port 6-position valve and starts to collect protein fractions. (h) The 10-port 2-position valve automatically switches the position at the end of RP gradient elution, and the first-dimension pump will start the next step elution. The 2D-HPLC system will repeat the online two-dimension separation until it finishes the last step elution of anion-exchange chromatography.

Fig. 3. Immunodepletion Chromatogram. The control and case samples are immunodepleted, respectively. The low-abundance protein fraction is collected and processed for further studies. A total of 300 μ L of plasma/or serum from either control sample or case sample was loaded onto the immunodepletion column for immunodepletion chromatography.

Fig. 4. 2D-HPLC Chromatogram (second-dimension RP chromatography). Protein fractions were collected between 10 and 40 min. A total of 84 fractions are collected. There are eight RP-HPLC chromatograms corresponding to the eight anion-exchange step elution (from 0 to 1,000 mM NaCl).

Fig. 5.

Relative quantitation analysis of isotopic labeling of plasma protein by nano-LC-ESI LTQ-FT mass spectrometer. Two different plasma samples (control and cancer) were labeled with $^{12}\text{C}_3$ -acrylamide (*light*) and $^{13}\text{C}_3$ -acrylamide (*heavy*), respectively, after immunodepletion. The mixture of these samples is further fractionated using 2D-HPLC, followed by in-solution tryptic digestion for LC-MS/MS analysis. **(a)** The reconstructed total ion current (TIC) chromatogram corresponds to approximately 2.5 μg of protein on-column loading. **(b)** The MS/MS sequence spectrum of the precursor (*m/z* 550.92) and the quantifiable paired precursor ions from the MS scan.

Fig. 6. Distribution of identified proteins using the number of MS/MS events as an indication of the relative abundance. This distribution illustrates that the majority (70%) of identified proteins are of low abundance, with 10 MS/MS events per protein. This indicates the efficiency of intact-protein multidimensional fractionation to identify low-abundance proteins in plasma.

Fig. 7. Peroxiredoxin 6 (*PRDX6*) identified with different isoforms by 2D-HPLC and quantitatively measured by LC-MS. **(a)** Intact-protein isoform that is upregulated in cancer with a ratio of 1.60 (cancer/control). **(b)** Cleavage form resulted from C-terminal domain cleavage, where there is no significant change between cancer and control since the ratio is 1.14 (cancer/control).

Table 1

Immunodepletion chromatography program (UV = 280 nm)

Time (min)	Buffer B (%)	Total flow rate (mL/min)	Fraction collector
0.00	0	0.5	
6.30	0	0.5	Start to collect the flow-through fraction
25.00	0	0.5	
25.10	100	0.5	
26.30	100	0.5	Stop to collect the flow-through fraction
26.50	100	0.5	
27.00	100	3.0	
29.00	100	3.0	Start to collect the bound fraction
33.00	100	3.0	Stop collecting bound fraction
35.00	100	3.0	
35.10	0	3.0	
45.00	0	3.0	

Table 2

Anion-exchange chromatography, flow rate 0.8 mL/min

Step elution	AEX mobile phase B (%)
1 (AEX01)	0
2 (AEX02)	5
3 (AEX03)	10
4 (AEX04)	15
5 (AEX05)	20
6 (AEX06)	30
7 (AEX07)	50
8 (AEX08)	100

Table 3

Reversed-phase chromatography, flow rate: 2.4 mL/min, UV = 280 nm

Time (min)	Solvent B (%)	Fraction collector
0	5	
10	5	Start to collect the fraction, 3 fractions/min, 800 µL/fraction
12	20	
30	65	
32	65	
33	90	
35	90	
37	5	
40	5	Stop collecting fractions
46	Stop	

Table 4

Digested protein fraction pooling sequence for LC–MS/MS analysis

Pool no.	Fraction no.
1	From fraction RP #04 to #22
2	From fraction RP #23 to #24
3	From fraction RP #25 to #26
4	From fraction RP #27 to #28
5	From fraction RP #29 to #30
6	From fraction RP #31 to #32
7	From fraction RP #33 to #34
8	From fraction RP #35 to #36
9	From fraction RP #37 to #38
10	From fraction RP #39 to #40
11	From fraction RP #41 to #42
12	From fraction RP #43 to #72

Table 5

Nano-flow rate RPLC gradient elution, flow rate: 300 nL/min

Time (min)	Nano-LC mobile phase B (%)
0	3
2	7
92	35
93	50
102	50
103	95
108	95
109	3
130	3