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A Novel Prefractionation Method Combining Protein and Peptide Isoelectric Focusing in Immobilized pH Gradient Strips

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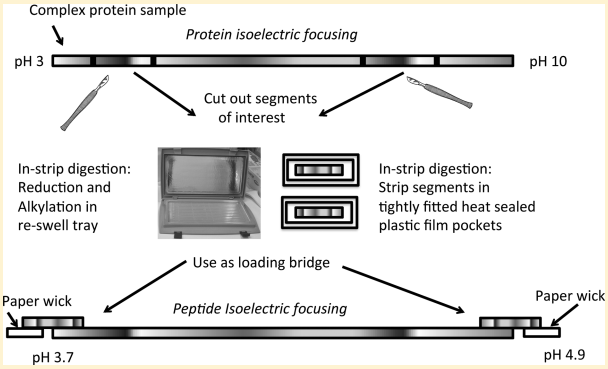
Supporting Information

ABSTRACT: To increase sensitivity and analytical depth in shotgun proteomics, prefractionation of complex samples is often used. Here we describe a novel prefractionation method, Sandwich high resolution isoelectric focusing, which combines both protein and peptide isoelectric focusing. In the first step, intact proteins are separated on the basis of isoelectric point (p*I*) using traditional immobilized pH gradient (IPG) strips. Segments in the IPG-strip containing proteins of interest are subsequently cut out and applied to in-strip digestion, without subsequent peptide elution. In the second *peptide* isoelectric focusing step, the strip segments are used as loading bridges. The peptides are thereby directly applied to the peptide isoelectric focusing, without an intermediate elution step, and separated on narrow range IPG strips to reduce the complexity on the peptide level. In the final step, the peptides are eluted into 96-well plates and analyzed with mass spectrometry. In a proof of principle experiment, using this method to zoom in on p*I* regions of interest in human plasma, we identify over 800 proteins, with concentrations spanning over 6 orders of magnitude.



KEYWORDS: *isoelectric focusing, prefractionation, plasma, depletion*

INTRODUCTION

Proteomics analysis is often characterized by high sample complexity. This high sample complexity is partly due to the high dynamic range of concentrations and partly due to the large number of protein species with a wide range of physiochemical properties. High sample complexity introduces challenges both for global shotgun analysis and targeted proteomics analysis, especially in organisms with complex proteomes such as the human proteome. To overcome these challenges, robust protein and peptide fractionation methods are required.

Isoelectric focusing (IEF) of proteins has long been used in protein research and proteomics as a robust protein separation method. Two dimensional gel electrophoresis combining isoelectric focusing (first dimension) and SDS-PAGE (second dimension) have up until recently been the preferred method in proteomics and have shown its applicability over numerous studies.[1] In shotgun proteomics, narrow range *peptide* isoelectric focusing has been applied as a fractionation method prior to mass spectrometry.[2−8] The rationale behind using narrow range peptide isoelectric focusing as a prefractionation method prior to MS/MS is that it reduces the complexity induced by tryptic digestion. This is done by selectively analyzing a subfraction of peptides with an acidic p*I* (pH 3.7−4.9). The pH range is chosen because 96% of human proteins have at least one tryptic peptide with a p*I* between pH 3.7−4.9.[3] This ensures high proteome coverage while reducing the number of peptides with two-thirds. In addition, the focusing precision is optimal in this range. The separation power and precision of isoelectric focusing has shown to be higher than the standard strong cation exchange performed in shotgun proteomics.[5] As the theoretical p*I* of peptides can be calculated, the p*I* of the identified peptides can be used to validate the peptide sequence or detect modified peptides.[9] Narrow range peptide isoelectric focusing is compatible with iTRAQ labeling, enabling quantitative analysis of the proteins.[10] In summary, by analyzing this subfraction of peptides in the pH range 3.7−4.9 the complexity of the sample can be reduced without significant loss of proteome coverage.

Although powerful, the narrow range peptide isoelectric focusing is not enough as single fractionation method to reach high proteome coverage in samples with an extreme dynamic range of concentrations such as human plasma. In a recent study combining high abundance protein depletion and narrow range isoelectric focusing, we identified and quantified around 300 proteins from human plasma,[2] which is not enough to cover low abundance tissue leakage proteins.

In this study, we wanted to combine protein isoelectric focusing with narrow range peptide isoelectric focusing. The

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aim of this approach is to increase the sensitivity and specificity in highly complex proteomics samples by zooming in on certain protein p*I* areas of interest and then applying the already established narrow range peptide IEF to reduce sample complexity. By combining these two steps directly, without an intermediate elution step, our aim is to create a robust, sensitive and versatile prefractionation approach for mass spectrometry based proteomics.

MATERIALS AND METHODS

Plasma Preparation

Patients assigned for bronchoscopy, regardless of diagnosis, were asked to participate in the study. All donors signed informed consent forms, and the study was approved by the ethics committee at the Karolinska University Hospital, Stockholm (2003-413 amendment 2005-71-32). Sample collection and storage was performed in line with guidelines presented in ref 11. Peripheral venous blood was collected in EDTA tubes (BD Vacutainer K2E 7.2 mg, BD Diagnostics) and kept at 4 °C until preparation to prevent coagulation and minimize protein degradation. EDTA tubes were centrifuged at 1500*g* at 4 °C for 10 min. Supernatant was transferred to a new tube and centrifuged at 3000*g* at 4 °C for 10 min. Supernatant was aliquoted and kept in −80 °C until analysis. All samples used in this study were prepared within 1 h of sample collection and showed no signs of hemolysis.

First Dimension Protein Isoelectric Focusing (IEF)

Twenty microliters of EDTA plasma was loaded on linear 24 cm pH 3−10 IPG strips from GE Healthcare by reswelling overnight in rehydration buffer containing 0.5% IPG buffer according to manufacturer's instructions. The strips were then focused in an Ettan IPGphor 3 (GE Healthcare) overnight as recommended by the manufacturer.

Comassie Staining

Strips were stained using the Novex Colloidal Blue Staining kit (Invitrogen) according to manufacturers instructions.

In-Strip Digestion

In-strip digestion was performed on the basis of an already established in-gel digestion protocol.[12] The following modifications were done to adapt the protocol to in-strip digestion. After the protein IEF strip was cut in either 2- or 4-cm segments using a clean scalpel and the distance was measured from the anode end of the gel with a ruler, the segments were transferred to a conventional IPG box with insert and reswell tray (GE Healthcare). The in-strip protocol was then performed as described in ref 12, using sufficient volumes to cover the strip segments, usually 1.5 mL, and performing the incubation steps in a IPG box with insert and reswell tray. For the trypsin rehydration step, 100 μL of trypsin in 10 mM ammonium bicarbonate, 10% acetonitrile, and 2 M urea was placed in a IPG box with insert and reswell tray, and the pieces were allowed to absorb as much trypsin buffer as possible for 1 h at 4 °C. Once rehydrated with trypsin solution, the pieces were transferred to small tightly fitted heat-sealed plastic film pockets to reduce the evaporation from the gel and digested overnight at 37 °C.

Elution of Peptides

In the elution experiments, the elution of the peptides was performed from the intact segment in 2 mL Eppendorf tubes using same acetonitrile buffers as described in ref 12. The eluted peptides were then dried in a speedvac and stored at −20 °C until they were applied to the second peptide separation as described in ref 3.

Direct Loading and Second Dimension Narrow Range Peptide IEF

Following overnight digestion at 37 °C, the cut segments (still in the plastic pockets) were allowed to reach room temperature (RT) and incubated at RT for approximately 1 h to allow the reabsorption of any fluid that had vaporized before removing the plastic pockets. The segments were then removed from the plastic pockets and applied to IPG strips by acting as two gel bridges (gel sides facing each other), one at the cathode end and one at the anode end. Narrow range isoelectric focusing was performed essentially as described in ref 3. Briefly, linear 3.7−4.9 IPG strips provided from GE Healthcare were reswollen overnight. Samples were focused until 100 kVhs was reached. The peptides were passively eluted in 72 fractions of 150 μL Milli-Q water and transferred to 96-well plates using a robot kindly provided by GE Healthcare. Eluted peptide samples were then dried in a speed vac system.

iTRAQ Labeling

Each of the 72 dried fractions in the 96-well plates were dissolved in 15 μL 0.5 M triethylammonium bicarbonate (TEAB) using a multichannel pipet. Four 8-plex iTRAQ labels were then dissolved in 1200 μL isopropanol, and 15 μL was added to each fraction. After 2 h incubation, the fractions were pooled into one 96 well plate and dried in a speedvac. The dried fractions were applied directly to LC−MS/MS analysis without further cleanup.

High Abundance Protein Depletion

Agilent Plasma 7 Multiple Removal System 4.6 × 100 (Agilent Technologies) was set up on a ÄKTA system (GE Healthcare) and run four times according to the manufacturer's instructions. The flow-through was concentrated on 5 kD molecular weight cut off (MWCO) filter (Agilent Technologies) followed by buffer exchange >100 times to 50 mM TEAB.

In-Solution Digestion

One hundred micrograms of depleted and crude plasma was digested using standard trypsin digestion protocol. Briefly, DL-dithiothreitol (DTT) was added to all samples to an end concentration of 5 mM, and the samples were incubated at 60 °C for 30 min. After reduction, the samples were alkylated in end concentration of 15 mM iodoacetamide (IAA) for 30 min in room temperature in the dark. Trypsin (Mass Spec Gold Promega) was then added 1:20 w/w, and the samples were incubated at 37 °C overnight. The samples were subsequently dried in a speedvac and stored at −20 °C until analysis.

LC−MS/MS

Prior to mass spectrometry analysis, the samples/fractions were dissolved in 10 μL of 3% ACN, 0.1% formic acid. Two micrograms of sample in triplicate were loaded of the crude plasma/depleted plasma. Each sample/fraction was injected into online HPLC−MS performed on a hybrid LTQ-Orbitrap Velos mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). An Agilent HPLC 1200 system (Agilent) was used to provide the 70 min gradient (4 h for crude plasma and depleted plasma) for online reversed-phase nano-LC at a flow of 0.4 μL/min. The sample was injected into a C18 guard desalting column (Agilent) prior to a 15 cm long C18 picofrit column (100 μm internal diameter, 5 μm bead size, Nikkyo

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Technos Co., Tokyo, Japan) installed on to the nano electrospray ionization (NSI) source. Precursors were isolated with a 2 m/z width, and dynamic exclusion was used with 60 s duration. We enabled "preview mode" for FTMS master scans, which proceeded at 30 000 resolution (profile mode). Data-dependent MS/MS (centroid mode) followed in two stages: first, the top 5 ions from the master scan were selected for collision induced dissociation (CID, at 35% energy) with detection in the ion trap (ITMS), and afterward, the same 5 ions underwent higher energy collision dissociation (HCD, at 45% energy) with detection in the orbitrap (FTMS). The entire duty cycle lasted ~3.5s. In the full analysis of the 12 segments from the 3−10 strip, only the CID branch was used.

The data was searched by Sequest under the software platform Proteome Discoverer 1.3.0.339 (Thermo) against the Swissprot_human_20120202_canonical_n_isoform_seq.fasta protein sequence database using a one peptide, 95% confidence cut off limit. A precursor mass tolerance of 10 ppm, and product mass tolerances of 0.02 Da for HCD-FTMS and 0.8 Da for CID-ITMS were used. Further settings used were as follows: trypsin with 1 missed cleavage; IAA on cysteine as fixed modification and iTRAQ 8-plex on lysine and N-terminal (if applicable) and oxidation of methionine as variable modification. Quantitation of iTRAQ 8-plex reporter ions was done by Proteome Discoverer on HCD-FTMS tandem mass spectra using an integration window tolerance of 20 ppm, and false discovery rate was estimated using percolator version 1.17 (2010/11/30) for false discovery rate cut off.

RESULTS AND DISCUSSION

In this study, we describe a novel fractionation method combining protein and peptide isoelectric focusing by in-strip digestion and direct gel bridge loading of the peptides onto the second peptide isoelectric focusing strip.

In-strip digestion has to our knowledge previously not been described in the literature. Therefore, to test the efficiency of the novel in-strip digestion approach as well as the subsequent direct gel loading of the peptides, we evaluated two different peptide loading approaches for the narrow range peptide isoelectric focusing step.

In the first *direct loading* approach, we separated crude plasma on a conventional 3−10 strip. Once the sample was focused, we cut out a 4-cm long segment of the strip (11.6−15.6 cm from the anode end, pH 6.5−7.7). We then performed in-strip digestion essentially as described by Shevchenko et al.[12] for in-gel digestion, but in a minimal volume and in a heat sealed plastic pocket to keep the peptides confined within the strip. To facilitate the direct loading and to increase the compatibility with the downstream peptide IEF, the in-strip digestion was performed in low salt concentrations (10 mM ammonium bicarbonate) and 2 M urea. Following overnight digestion, the strip was directly applied as a loading bridge onto the second narrow range peptide IPG strip (Figure 1). The aim of the direct loading bridge approach was to reduce the loss of peptides that could occur during an elution process, as well as minimize the additional time and variability that an intermediate step could introduce.

In the second *elution* approach, we separated the proteins on an identical 3−10 IPG strip, cut out the same 4-cm long section from the strip as above, performed in-strip digestion as described above, but then *eluted* the peptides from the segment using acetonitrile, similarly as done when performing in-gel digestion. The eluted peptides were then dried and applied to narrow range peptide isoelectric focusing as previously described.[2,3]

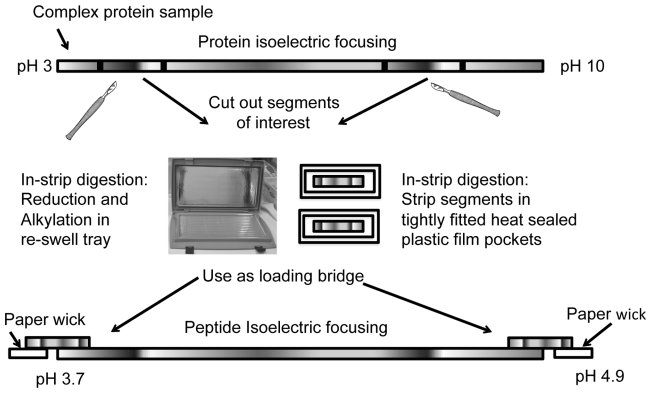

Figure 1. Schematic overview of the workflow. The protein sample is first separated using conventional isoelectric focusing. Segments of interest are cut out and in-strip digested. The cut strip segments containing tryptic peptides are then applied as loading bridges, and the peptides are separated using narrow range peptide isoelectric focusing. Once focused, the peptides are eluted into 72 fractions, iTRAQ labeled (if applicable) and analyzed with LC−MS.

The narrow range peptide strips from the two approaches were then focused, and the peptides were eluted in 72 fractions using passive diffusion in water and transferred using a robot into a 96-well plate. Twenty-one fractions from each plate were then analyzed with LC−MS/MS.

To ensure that all the proteins were digested, we stained both the two protein strips and the cut out sections using Comassie to check for presence of intact protein remnants in the cut out sections. No remnant proteins were visible in either of the cut out sections of the strips, indicating that the in-strip digestion worked well (Figure 2).

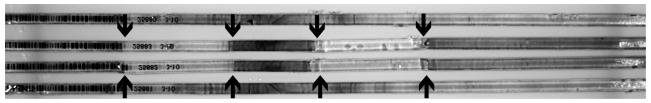

Figure 2. Efficiency of digestion. Photo illustrating in-strip digestion where two 4-cm segments have been cut out and in-strip digested, and the peptides have been sequentially eluted from the strip (indicated by black arrows). The top strip and bottom strip are not cut and can be used as reference of normal staining in the corresponding section. The cut segments and undigested strips are both stained using Comassie staining.

In total 175 proteins were identified from the 21 fractions from eluted section and 235 proteins were identified from the 21 fractions from direct loading bridge approach, showing the benefit of the direct loading approach in terms of proteome coverage and analytic depth. When analyzing all 72 fractions from the loading bridge approach, 384 proteins were detected.

In summary, this experiment proved that trypsin digestion can be performed in IPG strips with high efficiency, as well as the benefit of direct loading of peptides compared to performing an intermediate elution.

Human plasma is characterized by high dynamic range of concentrations, making it very challenging for global shotgun proteomics analysis. We have previously shown the benefit of high abundance protein depletion on the number of identified proteins, as well as high abundance protein depletion in combination with downstream narrow range peptide IEF,

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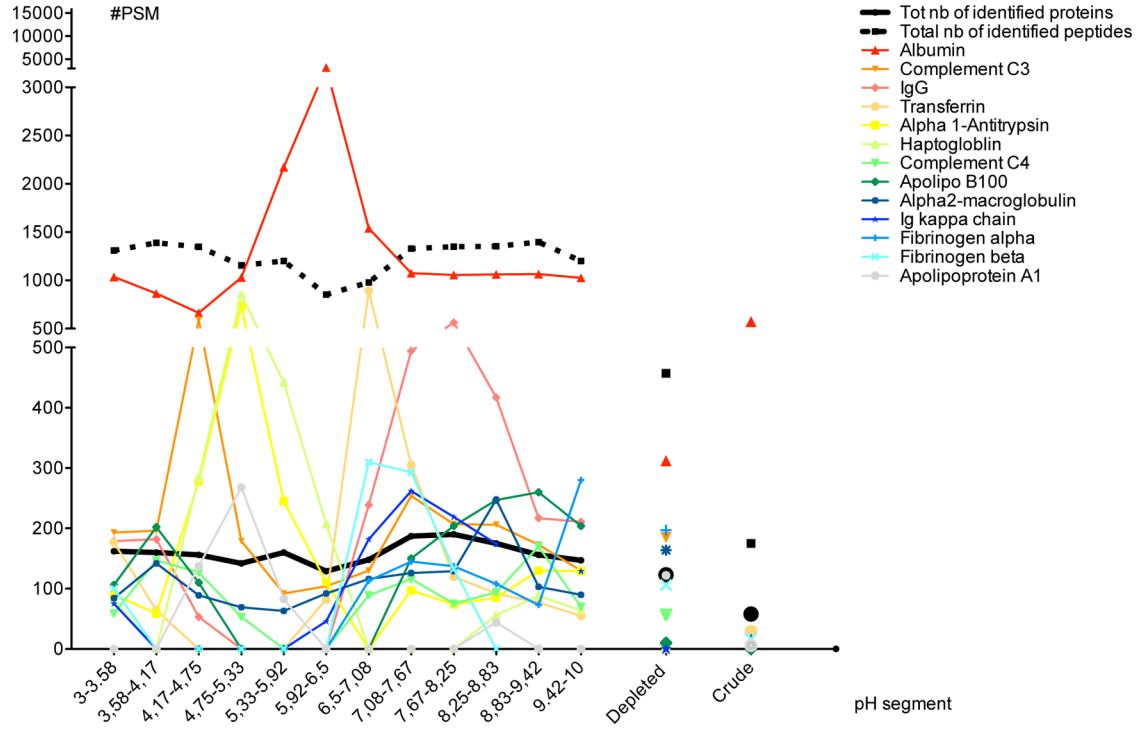

Figure 3. High abundance protein content. The plot is showing the number of peptide-spectrum matches (PSMs) of the 14 high abundance proteins from each pH segment of the 3−10 strip as well as the corresponding information for depleted plasma and crude plasma. The total number of proteins and peptides identified in each segment is illustrated by bold black line with circles and dashed black line with squares respectively.

identifying 138 and 282 proteins, respectively, from human plasma.[2,13] However, improvement in terms of analytical depth and proteome coverage is still needed. One possible application of the developed method would be to perform a negative selection or "depletion step" of high abundance plasma proteins in the first protein isoelectric focusing dimension. By cutting out and analyzing only segments of the 3−10 strip that should not contain high abundance plasma proteins, one could theoretically zoom in on pH areas of interest and increase the analytical depth and proteome coverage.

To determine which sections of the IPG strip that would yield the highest number of identified proteins, crude plasma was run on a broad range 3−10 strip. The strip was cut in 12 2-cm pieces and digested and analyzed separately without running the second peptide IEF dimension. In parallel crude plasma and depleted (MARS-7) plasma were also analyzed as reference samples. The result from this experiment can be found in Figure 3.

The results clearly demonstrate the negative effect of high albumin concentration on total number of identified peptides and proteins in the corresponding pH segment (dashed black line with squares and black line with circles, respectively). The results also show that there is no segment without any high abundance proteins present. However, the major negative effect on number of identified proteins and peptides is clearly caused by high albumin content. A summary of the 15 most abundant proteins (based on number of PSM) in each pH segment as well as number of proteins and peptides identified per segment can be found in Supporting Information Table S1. Comparing the performance of the isoelectric focusing with the high abundance protein depletion, all pH segments yielded a higher number of protein identifications than the commonly used depletion.

The data from the depletion experiment also highlight differences in the efficiency of the individual antibodies in the depletion column. We have previously shown that the depletion of fibrinogen is poor using the MARS-7 column, and this was confirmed in the current experiment, with a higher number of PSMs of fibrinogen detected in depleted plasma than in crude plasma.[13]

Although the isoelectric focusing identified more proteins than high abundance protein depletion overall, the results indicate that not one pH segment alone is optimal for avoiding all high abundance proteins and in addition give a high number of identified proteins. We therefore wanted to test an approach of using two loading bridges in the second peptide dimension: one in the anode end and one in the cathode end.

We chose two 4-cm segments from the 3−10 strip to apply to the second dimension narrow range peptide IPG strip: 5−9 cm from the anode end of the 3−10 strip (corresponding to pH range 4.5−5.7) and 11.6−15.6 cm from the anode end (corresponding to pH range 6.5−7.7). These two segments were digested in-strip and then applied to the same narrow range peptide IPG strip, applying the 4.5−5.7 segment on the anode end and the 6.5−7.7 segment on the cathode end. The two segments were chosen to be complementary and to avoid the large segment of albumin around pH 6. In addition, the acidic segment had previously been suggested as an interesting pH range for plasma proteomics studies using free flow separation.[14] The experiment was performed in triplicates to further evaluate the technical reproducibility in terms of peptide and protein overlap between runs. In total 516, 533, and 477 proteins were found in the three replicates, respectively (95% confidence), which is far more than the 384 proteins that were detected analyzing the pH 6.5−7.7 segment alone in the first experiment. This clearly shows the benefit of using two loading bridges to increase the proteome coverage. Out of the 516, 533,

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and 477 proteins, respectively, a set of 270 proteins was found in all three pools. Searching the three data sets together resulted in a total number of 826 significantly identified proteins (Supporting Information File S1). As anticipated from the full 3−10 strip analysis, albumin and several other high abundance proteins could still be detected in this analysis, but we also identified several low abundance proteins exemplified in red in Figure 4. In Figure 4 we have plotted high confidence

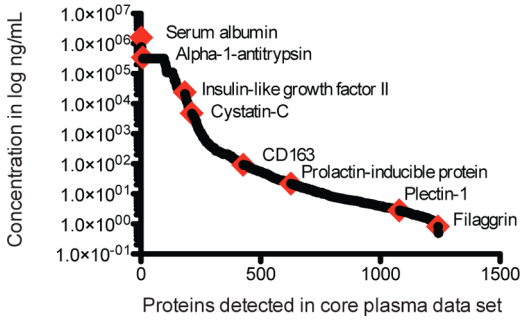

Figure 4. Protein concentrations in human plasma. Concentration plot of the proteins with concentration estimates from core plasma set (n = 1929) from Farrah et al.[15] Highlighted in red are examples of proteins at various concentrations selected from the 826 proteins.

proteins from a plasma core data set with concentration estimates from a recent publication by Farrah and co-workers.[15] The proteins highlighted in red are examples of proteins detected in our data set at various concentrations, spanning over 6 orders of magnitude.

In total 311 proteins from the three replicates were overlapping with the core data set, and 59 of them at concentration estimates below 10 ng/mL, showing the power of this method to reach deep into complex proteomes such as human plasma. In addition, this experiment showed the benefit of using two loading bridges.

In clinical proteomics, quantitative analysis of the proteins is often a necessity. To investigate if the method would be compatible with peptide labeling for relative quantification, we performed a small pilot experiment using iTRAQ labels. After the elution from the narrow range peptide IEF the peptides were dried in a speedvac and labeled with iTRAQ labels directly in the 96 well plates. After the labeling the plates were pooled and dried, and 14 fractions were subsequently analyzed with LC−MS/MS without any additional sample cleanup.

In total 685 peptides with 95% confidence were identified from the 14 fractions in the iTRAQ experiment. Out of these, 63% had labeling in two or more of the iTRAQ channels. Calculating quantitative reproducibility on peptides present in all four channels yielded an average ratio of 0.95 and standard deviation of 0.39. This experiment shows that iTRAQ labeling can be performed post fractionation and easily could be incorporated in the fractionation workflow, enabling global quantitative proteomics analysis.

In this study, we have used human plasma to test the feasibility of the Sandwich high-resolution isoelectric focusing method in global shotgun analysis, but the method is not limited to plasma or global analysis separations per se. Examples of other possible applications could be the study of isoforms or post-translational modifications that shift the pI of the protein of interest, which would then be separated on the protein level and studied in depth on a peptide level.

When performing targeted analysis such as single reaction monitoring (SRM) on human plasma samples, increased sensitivity and specificity is often needed.[16,17,18] For SRM, the protein of interest would be cut out in the first protein IEF dimension, digested, and then applied to the second peptide IEF. Only the fractions containing the peptides of interest would then be analyzed in the triple quadropole mass spectrometer. This would theoretically greatly improve the sensitivity and specificity of the SRM analysis without being dependent on protein or peptide antibodies.

The compatibility with iTRAQ labeling and the increased analytical depth as well as the high dynamic range compatibility shows that this method is applicable also in more classical global clinical proteomics studies. In order to reduce the number of strips and increase the sample throughput, one could also consider performing protein isobaric labeling prior to the first protein IEF separation. The data from the replicates shows that proteins in low ng/mL concentrations easily could be detected even in a global analysis from only 20 μL of crude plasma sample, and the comparison with the high abundance protein depletion column illustrates the gain in number of identified proteins.

The large number of combinations of protein and peptide pI regions to analyze makes this approach highly versatile and opens up for a large number of applications. Here we have only used the wide rage 3−10 strip for the protein separation, but narrow range strips are also commercially available. One could also experiment with the pH gradient (linear/nonlinear) of the first protein IEF separation as well as the length of the cut segment. However, extremely acidic or basic proteins are known to precipitate and focus poorly using protein IPG IEF, so proteins with pI outside the pH range 2.5−12 will not be optimal for this method.[1]

In our hands, the narrow range *peptide* isoelectric focusing is highly reproducible and precise (approximately 90% of all peptides end up in only one fraction[3]), and in comparison with protein pI prediction, theoretical peptide pI prediction is highly accurate, enabling easy and versatile experimental optimization. As all samples and projects are different, applying one method that solves all challenges in proteomics is not likely. Modern proteomics laboratories need to be versatile and be able to provide custom-made solutions based on the question at hand.

In this paper, we describe a novel prefractionation method, Sandwich high resolution isoelectric focusing, for mass spectrometry based proteomics. The method is robust and versatile and provides increased analytical depth and sensitivity in complex clinical samples.

■ ASSOCIATED CONTENT

Supporting Information

Supporting tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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