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Using SELDI-TOF Mass Spectrometry on Amniotic Fluid and for Clinical Proteomics and Theranostics in Disorders of Pregnancy

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Abstract

Clinical proteomics encompasses a multitude of experimental approaches, tools and techniques based on proteomics technology that are directly aimed to accelerate and improve diagnosis and treatment of human diseases. Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry is a variant of matrix-enhanced laser desorption ionization (MALDI) that makes use of chemically-modified surfaces to reduce the complexity of biological samples prior to separation in the mass analyzer. Compared to other proteomic techniques, SELDI has several important advantages such as ability to analyze complex biological samples with minimal pre-processing, ease of handling and high throughput. Importantly, once the biomarker or combination of biomarkers with potential clinical value has been established, validation analyses can be conducted in close proximity to clinical settings which is important for establishing the utility of new diagnostics in clinical decision making and perhaps future theranostic interventions. This chapter provides protocols for experimental design and methodology aimed at **1) Undiscovering** biologically relevant biomarkers in amniotic fluid using SELDI-TOF; **2) validating** the clinical utility of the biomarkers as new diagnostics; **3) translating** the biomarker findings into pathophysiological phenomena to provide further insight and extend the current understanding of the disease process. Many of the principles described herein for amniotic fluid could be generalized to studies involving other types of biological samples and other clinical questions.

Keywords

Biomarkers; proteomics; SELDI; mass spectrometry; proteomics; profile; diagnostics

1. Introduction

1.1. SELDI-TOF as diagnostics platform for amniotic fluid analysis

A biomarker is defined as a physical or biochemical characteristic that can be objectively assessed or measured as an indicator of “diseased” or “normal” state. The theory of medical diagnosis relies on the physicians’ ability to recognize signs and symptoms of individual patients as biomarkers and corroborate them with their own prior experience or learned medical knowledge derived from the experience of their predecessors. The physician learns with practice to assign intuitively different certainty levels on each individual piece of information. In other words when establishing a diagnosis some disease signs are weighed more heavily than others. However, after a comfortable level of certainty has been achieved, the physician will generally deduce a diagnosis and decide on treatment strategies based on

the diagnosis. Difficulties arise because not all signs of disease are obviously detectable and therefore a certain degree of suspicion is required before deciding to perform a medical measurement test. Most diseases have a dynamic course and the skill is to corroborate a minimal number of signs and symptoms of early disease into a reasonable degree of suspicion to order a decisive diagnostic test. With respect to amniotic cavity this results in performance of an amniocentesis procedure and retrieval of amniotic fluid which is then sent for laboratory analysis.

Depending on gestational age, the composition of amniotic fluid is the result of amniotic membrane secretion, fetal urine and lung secretions. The fetus swallows amniotic fluid during most of pregnancy and thus participates directly to both the inflow and the outflow of substances in amniotic fluid. Therefore changes in fetal well-being are likely to result in earlier and more profound changes in composition of the amniotic fluid than of the maternal peripheral blood or urine. For this reason, the amniotic fluid is a preferred biological sample to start searching for proteomic biomarkers reflective of a fetal affection.

For amniotic fluid biomarker discovery, the principles of medical diagnosis described are the key in differentiating biomarkers of biological relevance (that should be weighed more upon in deciding final diagnosis) from redundancy (that could be discarded) and noise (that must be discarded). It is also important to understand that biological relevance does not overlap with clinical utility as the criteria for the latter depend on the disease that is studied, on the current state of clinical practice, and may change with time as more is learned about the disease and new drugs are discovered.

Within the last 50 years, advances in technology have facilitated measurements of various physical or biochemical characteristics and as a result the number of biologically relevant biomarkers has increased tremendously. Of these, however, only a handful has proven clinically useful. Therefore before embarking on proteomics experimentation to further identify proteins that are differentially expressed in a certain disease state, one needs to clearly set out the goals in terms of whether biomarkers are sought to mine further the disease process (biological relevance), to improve diagnosis in the current state of clinical practice (clinical relevance), or both.

Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry is a variant of matrix-enhanced laser desorption ionization (MALDI) that makes use of chemically-modified surfaces to reduce the complexity of biological samples prior to separation in the mass analyzer. SELDI has several important advantages such as the ability to analyze complex biological samples with minimal pre-processing, ease of handling, high throughput and requirement for small amounts of sample. Importantly, once the biomarker or combination of biomarkers with potential clinical value has been established, validation analyses can be conducted in close proximity to clinical settings, which is important to establish utility of new diagnostics for clinical decision making. Critics of SELDI-TOF often quote mass accuracy of the current technology as a limitation relative to other mass-spectrometry platforms as well as difficulty with biomarker identification for peaks of low intensity. Yet, an appropriate experimental design is generally able to overcome these alleged limitations. The ability to conduct experiments with larger numbers of clinical specimens thereby filtering through the biological variability is likely to result in more robust proteomic biomarkers for which identification will not be problematic.

1.2. General principles of experiment design

Just as bedside medical diagnosis is both art and science, so is proteomics-based biomarker discovery and certain choices have to be made *a priori* from experimentation. These choices

refer to: **i)** the disease, **ii)** the biological sample and **iii)** the proteomic techniques. In Table 1 we provide a few general guidelines which are derived from our experience as applied to disorders of pregnancy and to **SELDI-TOF** as the choice of initial proteomic technique, but these principles can be easily extrapolated to other clinical scenarios. In many instances, however, the choices are driven by combinations of less than ideal situations such as availability of a certain biological sample rather than its relevance for the disease of interest. As a result, the expectation for successful discovery of biomarkers with clinical utility should be lower. Unfortunately, many unsuccessful attempts in biomarker discovery are often blamed on the technology rather than the poor choices of experimental design. On the other hand, it is possible that the current clinical practice will benefit from even small improvements in diagnostics or that the newly discovered biomarkers will point to new means for therapeutical intervention, which could not have been envisioned by hypothesis-driven approaches. These possibilities certainly make some of the less than ideal experimental undertakings worthwhile, but also underscore the importance of an educated and carefully thought-out experimental design that can minimize disappointment and maximize the chance of success.

In our laboratory we embarked on discovery of biomarkers with both biological and clinical relevance for prediction of preterm birth using proteomics, on the premise that alterations at protein levels may relate closer to the disease process than alterations in genes or mRNA. Our choice of using SELDI-TOF as proteomics platform was based on the reasoning that minimal sample handling, high throughput and speed of analysis in a clinical setting were more important than mass accuracy or protein identification, especially for the discovery phase. The two diseases that have been at the center of our attention are spontaneous preterm delivery and preeclampsia, which are two important obstetrical syndromes, main determinants of preterm birth and of maternal and neonatal morbidity and mortality. Both diseases are especially difficult to tackle with respect to development of new diagnostics due to patient heterogeneity (i.e. different etiologies and pathogenic pathways converging into identical clinical scenarios), difficulty in obtaining relevant biological samples from the fetus and subjective gold standards for presence and absence of disease. Despite these difficulties, by using the experimental design described in the following section we were able to extract several proteomic profiles with both biological and clinical significance. One amniotic fluid proteomic profile, the MR (Mass Restricted) score composed of four biomarker peaks, appears best to diagnose intra-amniotic inflammation leading to preterm delivery and predict histological chorioamnionitis, funisitis and early onset neonatal sepsis (**1,2,3,4,5,6,7,8**). Another proteomic profile composed of 5 different peaks was found to occur with increased frequency in idiopathic spontaneous preterm birth which by our definition encompasses women who do not exhibit the proteomic profile characteristic for intra-amniotic inflammation or intra-amniotic bleeding yet still deliver preterm (**9**).

In addition to amniotic fluid we have applied the same principles to other biological sample such as cerebrospinal fluid and urine. One proteomic profile composed of 4 different peaks in cerebrospinal fluid identifies subclinical subarachnoidal bleeding such as that in the context of severe preeclampsia (PPB score) (**10**) while a urine proteomic profile (UPS score) composed of 13 biomarker peaks was demonstrated as having the highest accuracy to diagnose severe preeclampsia and predict an early indicated delivery (**11**). This chapter is centered on experimental details which we believe have been critical to our successful experimentation but have remained to the most part unpublished.

Each project performed in our laboratory can be dissected into three phases each with one goal and a number of steps as indicated in Table 2. The three phases do not necessarily need to be sequential. For efficiency purposes we prefer they overlap in time. A critical issue, however, is that patient enrollment and biological specimen collection is progressing

prospectively throughout the entire timeline of the study. The inclusion criteria relate to the physician's judgment to either perform a procedure or order tests to rule-out the disease of interest in the real clinical setting. In our case, patients with signs and symptoms of spontaneous preterm birth where the physician has a suspicion for intra-amniotic infection are managed with amniocentesis and a battery of five tests is performed on amniotic fluid. These patients make up the *rule-out inflammation-induced preterm birth cohort*. Of note is that once the exploratory phase has been completed, the challenge and translational phases can progress in a parallel timeline for enhanced productivity as schematically represented in Figure 1 (12).

2. Methods

2.1 Reagents

1. Centrifuge tubes (0.5 mL, 1.5 mL, 15 mL, 50 mL).
2. 1.5 mL centrifuge dark colored tubes (for matrix dilutions).
3. PapPen (hydrophobic pen).
4. Acetonitrile (ACN), HPLC grade diluted 20% and 50% with ultrapure water.
5. Trifluoroacetic acid (TFA) (1mL ampules, Pierce, Rockford, IL)
6. Protein assay (bicinchoninic acid/cupric sulfate [BCA] kit; Pierce).
7. Hydrochloric acid (HCl, 5M, for pH adjustment).
8. Acetic acid (glacial, for pH adjustment).
9. Sodium hydroxide (10N, for pH adjustment).
10. 100 mM sodium acetate (NaOAc) adjusted to pH 4 with acetic acid.
11. 100 mM sodium phosphate buffer pH 7
12. Phosphate buffer saline (PBS) (1X) pH 7.4
13. 100 mM Tris-HCl, pH 8
14. Sodium carbonate (NaRR_2CO_3) adjusted to pH 9.
15. 100 mM nickel (NiPP^{2-}) sulphate aqueous solution
16. 100 mM copper (Cu^{2-}) sulphate aqueous solution
17. 100 mM zinc (Zn^{2+}) sulphate aqueous solution
18. 100 mM gallium (Ga^{3+}) sulphate aqueous solution
19. ProteinChip™ arrays: reverse phase hydrophobic surfaces (H4 and H50), anion/cation exchangers (Q10 and CM10) or immobilized metal affinity coupling (IMAC30) from BioRad Laboratories, Hercules, CA USA.
20. Matrix solvent: 50% (v/v) acetonitrile (ACN) and 0.5% (v/v) trifluoroacetic acid (TFA).
21. Energy absorbing molecules (matrix): alpha-cyano-4-hydroxy cinnamic acid (CHCA), sinapinic acid (SPA) or EAM-1, 5 mg/tube (Bio-Rad) prepared as indicated in package insert to obtain a saturated matrix solution. Centrifuge briefly. Using the dark colored tubes further dilute with matrix solvent to obtain a 20% saturated CHCA solution, 50% saturated SPA or 50% saturated EAM-1. Diluted matrix samples are stable up to 1 week.

22. Biological samples (frozen at -80°C and access to clinical source of fresh samples).

2.2 Equipment

1. Refrigerated centrifuge.
2. Deep freezer (-80°C).
3. Spectrophotometer (for protein assay per kit instructions).
4. Medium vacuum for spot-washing the arrays.
5. Pipettes and tips (low retention).
6. SELDI TOF ProteinChip™ reader (PBSIIC or PCS4000) externally calibrated using the $[M + H]^+$ ion peaks of 1296.5 Da (angiotensin), 1570.6 Da ([Glu1]fibrinogen), 2147.5 Da (porcine dynorphin A [209-225]) and 5733.6 (bovine insulin) (BioRad SELDI mass standards).

3. Methods, Experiment Design and Data Analysis

3.1 Exploratory phase

The exploratory phase may begin when biological samples (of choice) from approximately 20 cases in the “diseased” category and 20 cases in the “non-diseased” category have been gathered (see Note 1). The “diseased” cases have to be intentionally selected as having at least 3 clinical criteria of disease presence and/or severity including poor outcome. This method is detailed in particular for amniotic fluid but can be applied to other low complexity fluids such as cerebrospinal fluid, urine, peritoneal fluid, tracheal aspirate.

3.1.1. Step 1: Optimization of SELDI conditions using wide SELDI profiling and pools of samples from “diseased” and “non-diseased” subjects.

1. Measure total protein concentration and test if there is a statistically significant difference between the diseased and non-diseased groups. If yes, then protein normalization is strongly encouraged.
2. Prepare 4 composite pools (2 diseased and 2 non-diseased) by mixing together different volumes of 10 samples per pool (either diseased or non-diseased) so that each individual sample contributes to its pool with an equal amount of total protein.
3. Measure total protein of the 4 pools using the same assay as before and equalize/dilute protein concentration for all pools at 2 mg/mL with water (see Note 2).
4. Prepare the 9 different types of binding/washing buffers listed in Table 3. For each energy absorbing molecule, 16 arrays are required. CHCA works best for low molecular weight biomarkers (<10 kDa) while SPA is preferred for desorption of proteins >9 kDa. Generally CHCA and SPA are sufficient in this step. EAM-1 is a proprietary matrix formulation recommended for proteins of 10-50 kDa and for glycosylated proteins and could be tested to improve resolution of peaks which appear broad on the initial SPA SELDI tracings.
5. Prepare working dilutions (8-fold) of normalized pools in each of the respective binding/washing buffer. The working dilutions should have about 0.25 mg/mL total

¹This starting number of exploratory phase samples is ideal but practically it cannot always be achieved. In these cases we prefer to start with smaller pools of severe cases rather than dilute the diseased group with milder forms.

²This protein concentration is ideal but it cannot always be achieved as some biological samples are inherently more diluted in vivo (e.g. cerebrospinal fluid). In such cases adjust the concentration of the pools so that by diluting 3-fold with binding/washing buffers, the concentration on the spot will remain about 0.2 mg/mL.

protein. Spot volumes of 6 μL (containing 1.5 μg total protein) will be applied per spot. An example of the array layout for this experiment is shown in Table 4.

6. Prepare the arrays for on spot sample application. We prefer on-spot application and individual spot washings because of the small sample volumes required. Alternatively, a bioprocessor may be used and the array may be washed in-bulk. For our protocol, one spot application volume refers to 6 μL /spot. One wash volume refers to 120 μL partitioned into 6 applications (of about 20 μL each) which are rapidly applied on spot and then aspirated to create a flow of wash buffer over the spot. It generally takes about 2-3 min to wash sequentially all 8 spots of an array.
 - 6.a. H4 array preparation: circle each spot with PapPen and air dry. Precondition each spot by applying two 6 μL volumes of wash buffer and incubating for 5 minutes each.
 - 6.b. H50 array preconditioning: to each spot add 50% AcCN for 5 min., wash with the respective buffer then leave the last spot volume on each spot for 5 min. Aspirate and apply samples to respective spots.
 - 6.c. CM10 and Q10 arrays are pre-equilibrated with two 5 min. changes of the respective washing/binding buffer prior to sample application.
 - 6.e. IMAC30 array preconditioning: apply 6 μL of respective metal solution to each spot, wait for 15 min, aspirate and apply a second 6 μL volume for another 15 min. Wash with respective wash buffer and then apply the samples.
7. Incubate the arrays in a moist chamber for 1 hour at room temperature (20°C).
8. Wash each spot with one wash volume of binding/washing buffer. Air-dry the array for 10 min.
9. Apply matrix solution (one application of 1 μL 20% CHCA or 2 sequential applications of 1 μL 50% SPA, allowing for the spots to dry completely between applications). Air dry the array for 10 min.
10. Read each array under 3 laser intensities/detector sensitivities combinations (low laser, mid-laser and high laser). The array reading setting should be determined empirically by carefully examining signal-to-noise ratios for each reading as well as baseline elevations. The array reading protocols used in our laboratory are listed in Notes 3 and ⁴ and can be used as starting point when using a PBSIIC system.
11. Examine carefully each tracing and determine the optimal experimental combinations of binding buffer and array surfaces. First eliminate conditions where few peaks are observed. Examine the remaining conditions by comparing the tracings of the four pools within each condition with the auto-scale option disabled. Search for at least two conditions with highest signal-to-noise ratio which complement each other in resolved peaks. The best scenario is to find conditions that resolve peaks that are present in “diseased” state, while absent in “non-diseased” state. For example the tracings we illustrate in Figure 2 resulted from a wide profiling screen of one diseased pool (**D**) and one non-diseased (**ND**) pool under 4 different conditions represented by the combination of the array, binding buffer, matrix and reading conditions. Note that both the H4 and H50 conditions show unique peaks in D1 tracing compared to ND1. These peaks do not resolve at the same mass indicating that they may represent different biomarkers and that these two conditions complement each other. By contrast, IMAC-Ga3+ resolved the same markers as the H4 condition albeit at a lower signal-to-noise ratio. The

Q10 array condition was not able to resolve any peaks above the noise level for this sample and thus this condition was eliminated without hesitation.

3.1.2. Step 2: Data Collection, Spectrum Processing, and Peak Detection

1. Next, all samples that have been used to prepare the pools (n=40) are run in the 2 or 3 selected conditions established during Step 1. To estimate, there will be six arrays needed for each chosen condition. On each array, one spot is assigned as “blank” and is spotted with the respective binding buffer alone. The blank spots serve to determine the level of “background noise”. It is critical in this step for samples to be arranged on arrays at random and not to assign them to spots based on categories (“diseased”, “non-diseased” and buffer). This simple experimental layout can avoid much of the bias related to possible array inconsistencies or any unexpected change

³Standardized spot protocols used in our laboratory for low mass readings (CHCA spots).

a. Low Laser protocol (LL: 150-5)

Set high mass to 20000 Daltons, optimized from 2000 Daltons to 5000 Daltons.
 Set starting laser intensity to 150.
 Set starting detector sensitivity to 5.
 Focus by optimization center.
 Set Mass Deflector to 800 Daltons.
 Set data acquisition method to Seldi Quantitation
 Set Seldi acquisition parameters 22. delta to 5. transients per to 15 ending position to 82.
 Set warming positions with 2 shots at intensity 155 and Don't include warming shots.
 Process sample.

b. Mid Laser protocol (ML: 160-6)

Set high mass to 20000 Daltons, optimized from 2000 Daltons to 5000 Daltons.
 Set starting laser intensity to 160.
 Set starting detector sensitivity to 6.
 Focus by optimization center.
 Set Mass Deflector to 800 Daltons.
 Set data acquisition method to Seldi Quantitation
 Set Seldi acquisition parameters 20. delta to 5. transients per to 15 ending position to 80.
 Set warming positions with 2 shots at intensity 165 and Don't include warming shots.
 Process sample.

c. High Laser protocol (HL: 170-7)

Set high mass to 20000 Daltons, optimized from 5000 Daltons to 8000 Daltons.
 Set starting laser intensity to 170.
 Set starting detector sensitivity to 7.
 Focus by optimization center.
 Set Mass Deflector to 800 Daltons.
 Set data acquisition method to Seldi Quantitation
 Set Seldi acquisition parameters 24. delta to 5. transients per to 15 ending position to 84.
 Set warming positions with 2 shots at intensity 175 and Don't include warming shots.
 Process sample.

in instrument settings. Such issues have been at the center of several controversies that emerged in earlier stages of biomarkers discovery using SELDI (13).

2. Next, each array is read using 3 spot protocols with increasing level of laser intensity and/or detector sensitivity (see Notes 3 and ⁴ for reading settings used with our PBSIIC instrument). However, the exact reading settings will need to be determined empirically by critically examining the level of baseline elevation on tracings that received both samples and blank. As a guide, in Figure 3 we display SELDI tracings (before baseline subtraction) obtained from one sample spot. A tracing from the blank spots is shown for the mid-laser setting only. In the end, there will be about 135 tracings generated for each array/binding buffer combination chosen in Step 1. At least until now, we found that analyzing in this step just the tracings obtained with the mid-laser setting has been sufficient. Thus

⁴Standardized spot protocols for high mass readings (SPA or EAM covered spots).

a. Low Laser protocol (LL: 190-8)

Set high mass to 100000 Daltons, optimized from 8000 Daltons to 40000 Daltons.

Set starting laser intensity to 190.

Set starting detector sensitivity to 8.

Focus by optimization center.

Set Mass Deflector to 500 Daltons.

Set data acquisition method to Seldi Quantitation

Set Seldi acquisition parameters 20. delta to 5. transients per to 15 ending position to 80.

Set warming positions with 2 shots at intensity 195 and Don't include warming shots.

Process sample.

b. Mid Laser protocol (LL: 200-8)

Set high mass to 100000 Daltons, optimized from 10000 Daltons to 40000 Daltons.

Set starting laser intensity to 200.

Set starting detector sensitivity to 8.

Focus by optimization center.

Set Mass Deflector to 500 Daltons.

Set data acquisition method to Seldi Quantitation

Set Seldi acquisition parameters 22. delta to 5. transients per to 15 ending position to 82.

Set warming positions with 2 shots at intensity 205 and Don't include warming shots.

Process sample.

c. High Laser protocol (LL: 210-10)

Set high mass to 100000 Daltons, optimized from 10000 Daltons to 40000 Daltons.

Set starting laser intensity to 210.

Set starting detector sensitivity to 10.

Focus by optimization center.

Set Mass Deflector to 500 Daltons.

Set data acquisition method to Seldi Quantitation

Set Seldi acquisition parameters 24. delta to 5. transients per to 15 ending position to 84.

Set warming positions with 2 shots at intensity 215 and Don't include warming shots.

Process sample.

only from this reading setting we will have to analyze 40 SELDI tracings resulting from sample analyses and at least 5 blank tracings. The spectra obtained from the low- and high-laser reading settings are saved separately and used at the end of the exploratory phase to verify that the reading condition are indeed optimal for the final discriminatory profile or to make minor adjustments to laser intensity and/or detector sensitivity before the challenge phase (see section 3.1.4.)

3. With all the tracings selected (using CRL-A command), subtract the baseline using the tool built into the ProteinChip™ software but do not use the normalization tool () or other filtering options in this step.
4. With all the tracings selected (CRL-A command), use the centroid peak tool built into the ProteinChip™ software to select all peaks that are visually detectable. Even when a peak is visually present in just one tracing, by selecting that peak in that tracing, the respective m/z value will be selected in all tracings given that the tool finds what it would automatically consider as peak even if practically no peptide was resolved at that mass (as in the case of the blank spots). The investigator selecting the peaks in this way will need to establish some rules for that he/she considers as peak. In our case, we would select a peak if it remains visible to the eye after covering the tracing above and below on the screen. Despite its tediousness, in this phase we prefer this rather simple manual method to the automated tool of all peak detection.
5. Next, save the analyzed file separately and export to a Microsoft Excel spreadsheet (as comma separated value [CVS] file) the following peak information: Spectrum tag, spectrum #, peak #, intensity, (m/z)-1 (substance mass) and signal-to-noise ratio (S/N).

3.1.3 Data Analysis

Mass Restriction (MR) analysis, definition of SELDI diagnostic profiles and scores:

Once the peak information has been obtained, you are ready to begin sorting out which peaks are informative and hence could classify as potential biomarkers. These have to be clearly filtered from peaks that carry redundant information that could be discarded, and peaks which belong to noise and thus must be discarded. In our laboratory, this procedure is performed by applying a sequential order of rules that together compose the algorithm of MR scoring, which is essentially an algorithm of additional complexity reduction optimized specifically for this purpose and to avoid data over-fitting. Although there have been several automated software tools proposed for this purpose, none of these were able to achieve, at least in our hands, the performance of MR scoring. One of the reasons might be that the MR scoring method bases the analysis of proteomics data on a few practical principles of medical diagnosis rather than statistical conventions which, at least in our opinion, do not have yet the ability to fully encompass the complexity of human diseases. One of these is that by definition, “diseased” is not a standalone principle but depends of what is considered “normal”. In other words “one has to stand out from the crowd” to be classified as abnormal.

1. To begin, each SELDI tracing is visually composed of sequences of “hypervariable” and “silent” areas or simply mass areas with peaks or just with noise, respectively. Mathematically however, SELDI tracings are functions in a Euclidian plane defined by two Cartesian coordinates. By convention, we define a SELDI peak by 3 attributes: mass (peak mass = (m/z)-1) which is the attribute of identity and the *x axis* coordinate, peak intensity (the attribute of relative abundance and the *y axis* coordinate) and peak presence (signal-to-noise ratio (S/N) greater than a cut-off of average S/N+2 standard deviations (SD) for each

corresponding mass value calculated from the tracings of the blank spots in the identical experimental condition including laser/detector settings). Peaks considered present are assigned a Boolean indicator of 1 as opposed to peaks below the cut-off which are assigned a Boolean indicator of 0.

2. The principles of MR scoring have been published earlier (**4,7,11**) and are listed together in Table 5. Next, the exported peak attributes are arranged in a spreadsheet and a Macro tool assigned to perform the necessary logical operations of the MR scoring algorithm. A representative section of a spreadsheet is shown in Figure 4 which displays criteria I-III applied to 3 SELDI peaks. Some of the criteria of the MR scoring method are disease and sample dependent and should be viewed as optional. For instance it is possible that various degrees of oxidation will produce valuable biomarkers which may relate to the disease process (**11**) (criterion IV). Alternatively, oxidation may be an *ex vivo* phenomenon and thus will appear inconsistently among the diseased tracings and thus one should be cautious when selecting both the parent and the oxidized form into the final profile. Criterion V assures that the final biomarkers are absent in non-diseased state. This is of utmost importance because the final diagnostic profile will not rely merely on quantitative differences in intensity of peaks which could be impacted nonspecifically for instance by small variations in concentration but rather on generation of new peptides. In the rare instance where there are no unique peaks characteristic of the disease state Criterion III will still assure a highly significant difference in S/N between diseased and non-diseased state. The final criterion (VI, optional) refers to restriction or parsimony. In other words, the profile should contain the minimal number of peaks required for maximum discrimination. Applying this criterion is dependent on the clinical context where improving the diagnosis is sought. For instance if the biological sample is the result of an invasive procedure, unlikely clinically to be repeated and a rapid result turnaround is necessary then restriction should be performed. This was the case both with the MR profile in amniotic fluid obtained at amniocentesis and the PPB profile in cerebrospinal fluid obtained by spinal tap. In contrast, if the purpose is to detect asymptomatic disease, the biological sample can be obtained non-invasively and thus the test can be easily repeated then the last criterion should not be applied. The reason for this is that a restricted profile will likely perform well in challenge phase at detecting severe and likely symptomatic disease but will not comprise a sufficient spectrum of biomarkers to perform adequately in the challenge phase where the profile will be tested to detect asymptomatic disease or disease in the presence of other co-morbidities. In our experience, in all clinical scenarios where we sought and then validated proteomic profiles we noted a sequential and ordered appearance of the biomarkers which ultimately comprised the final profile. The number of biomarkers and their intensity was in direct correlation with disease severity. Therefore to be able to detect a proteomic abnormality in asymptomatic patients one needs a large enough number of peaks to explore during the challenge phase. This was the case with the urine UPS profile of severe preeclampsia and the reason for the 13 markers required (Figure 5).
3. After the final composition of unique biomarkers (defined by unique average masses) that fulfill the rules of the Mass Restricted algorithm is determined, this proteomic information is transformed into a named operator (score) which characterizes each sample and can be manipulated with regular statistics. This can be done two ways. The simplest way is to sum the numbers of biomarkers of the final profile present in that particular sample. We think this applies best to restricted profiles such as the amniotic fluid MR score and cerebrospinal fluid PPB

score. Thus, these scores ignore any quantitative information related to peak intensity and rely solely on the presence of peaks that are otherwise absent in the respective biological compartment. This is specifically advantageous when a rapid diagnosis is sought as normalization for total protein is not required to achieve the same accuracy. Both the MR score and the PPB score can return a test result within 15 minutes and do not require a standard curve (*1, 10*). For profiles obtained after protein normalization, however, adding the semiquantitative information in the equation will result in a continuous rather than ordinal result. Because continuous tests have higher power it is expected this type of operator will perform better in the challenge phase (*14*). This was exactly the case of the UPS score where the ranked variant (UPSr, continuous operator ranging from 0 to infinity) displaced the Boolean variant (UP Sb, ordinal operator ranging from 0 to 13) when they were both entered into a multivariate model in the challenge phase (*11*).

3.1.4. Step 3: Verification of accuracy in the exploratory phase cohort

1. This step simply checks the accuracy of the new operators (SELDI scores) in segregating back the 40 samples used for their development based on the initial clinical criteria. In receiver operator curve analysis using clinical diagnosis as gold standard, 100% sensitivity and 100% specificity should be expected since you are fitting back the model to the samples used to create it.
2. If outliers are observed we found it useful to review the medical records of those cases carefully before embarking onto the challenge phase. Because in the exploratory phase the diseased cases have been intentionally selected as having at least 3 disease criteria, outcome outliers are not expected unless the possibility for misdiagnosis in the current clinical practice is high. During the exploratory phases of the three projects to which we refer throughout the article, the only instance of misclassification in the exploratory phase was a case that lacked the cerebrospinal fluid profile characteristic of severe preeclampsia, yet the patient was clinically categorized as having severe preeclampsia by pulmonary edema in the absence of increased blood pressure or proteinuria. Our post-proteomic review of the medical record revealed that, according to the obstetric care provider, the pulmonary edema likely resulted from iatrogenic fluid overload (*10*).
3. Accuracy calculations restricted to the exploratory phase sample will indicate a cut-off of maximum accuracy for this population. However, expect that this cut-off will likely need adjustment in the challenge phase given that the exploratory phase purposely omits cases with asymptomatic or mild disease.
4. These arrays are read with all 3 reading protocols (low-laser, mid-laser and high-laser) and scores are compared between the settings. The reading protocol displaying the largest level of significance between diseased and non-diseased samples will be used for the challenge phase.

3.2. Challenge Phase

3.2.1. Step 1: Challenge phase in cross-sectional cohort and calculation of diagnostic accuracy of SELDI proteomic scores

1. The key for a successful challenge phase is to test the SELDI scores in a setting that is as close as possible to the one where the new diagnostics are most needed. Thus, in our setting, as soon as the SELDI method and MR score model have been finalized, we begin the challenge phase by testing virtually every case that is enrolled in our prospective cohort and has a clinical work-up for either preterm birth or preeclampsia (“intent to diagnose”).

2. To increase the relevance of the challenge phase and to be able to judge the clinical utility of the SELDI scores, we perform the SELDI testing from fresh sample collection and generate the scores in blinded fashion in parallel with the clinical laboratory's testing and before a clinical diagnosis is confirmed or ruled-out. In this phase we also test samples from patients often encountered in the same clinical setting with related or un-related diagnoses to examine the specificity of the SELDI profile for our disease of interest.
3. Because sample testing in the challenge phase is done based on availability, there will not always be 8 patient samples to test at a time. We use an improvised modality to incubate samples on just few spots on the array as needed for each test while simultaneously keeping any unused spots dry. We also validated that the unused spots retain their surface-enhanced binding ability. In contrast, exposing unused spots to a humidifying environment diminish their reactivity and subsequently, cannot be used without a loss in surface affinity. In Figure 6 we illustrate how this improvised and reusable device allows testing of fresh patient samples individually and economically during the challenge phase. Every reading is compared to a positive control tracing (with all biomarkers present) to indicate the m/z location of each of the sought biomarkers. Thereafter UPSb and UPSr are calculated from the S/N ratios at each of the m/z values of interest.
4. If possible, the gold standard in the challenge phase should be independent of clinical diagnosis. In cancer studies histological classification offers an acceptable gold standard. In contrast, in preterm birth or preeclampsia the selection of an objective classification is hindered due to the fact that a diagnosis of disease will require the physician to interrupt the course of pregnancy per management protocols. However, by using an outcome variable (neonatal sepsis, histology of the placenta or indicated early delivery) the accuracy of SELDI scores can be compared using receiver operating curve (ROC) analysis with that of other laboratory tests accepted in clinical practice or research and development for the same purpose.
5. The number of cases needed for the challenge phase is a statistical matter and requires comment. Although there has been no statistical publication specifically targeting sample size calculation for proteomics operators derived from SELDI experimentation the belief remains that a sample size calculation prior to initiating the challenge phase is absolutely necessary especially if clinical utility relative to other diagnostic tools is sought. Several software routines can be used for sample size estimation of diagnostic accuracy based on the algorithms of Hanley and Mc Neil ¹⁵ and/or Obuchowski. ¹⁶ The PASS program by NCSS (Kayseville, UT; [www://www.ncss.com](http://www.ncss.com)) and Power and Precision by Biostat (Englewood NJ; <http://www.power-analysis.com>) run on Microsoft Windows, are both user friendly and can be evaluated by downloading free trial versions over the internet. To obtain a general estimate of how many cases are needed, the prevalence of the sought outcome in the challenge cohort needs to be known. For example, at our institution the prevalence of an indicated delivery for preeclampsia in cases with expressed intent to diagnose is about 40%. The accuracy of a clinically used test alternative (protein-to-creatinine ratio, measured as area under the curve) is less than 0.6 and that of a proposed alternative in research testing (ratio of urine angiogenic factors) is about 0.8. In order to detect a significant difference in area under the curve of the SELDI UPSr score versus ratio of angiogenic factors at a power level of 0.9 and an alpha of 0.5 we determined by using PASS 2008 software that a minimum of 66 cases are needed (25 positive and 41 negative for outcome). We decided, however, to stop the challenge phase only after enrolling at least double this calculated

number on the empirical basis that we were expecting a high rate of misclassification, as currently there is no clinical means of discriminating severe preeclampsia from other proteinuric or hypertensive conditions complicating pregnancy. In the challenge phase such cases were all targeted for enrollment. In Figure 6 we present the distribution of proteomic scores UPSb and UPSr in the cases analyzed as part of the exploratory versus the challenge phase in our most recent study. Of note, although in the exploratory phase there is no overlap of scores between diseased (preeclamptic with indicated early delivery) and non-diseased cases (non-preeclamptic with normal delivery at term), a significant overlap is noted in the challenge phase despite all graphs statistically differing at $P < 0.0001$. Yet, the fact that the proteomic score performs in the same population significantly better ($P < 0.001$) than the other tests used or proposed for the same purpose indicates that the limitation lies not in our test but in the available gold standard. However, given its highly significant clinical relevance these results underscore the importance of providing clinicians with additional markers to aid with clinical decisions with significant impact. Urine SELDI scoring can find thus immediate application in current clinical practice with selecting the cases that need immediate intervention and from those that can be management expectantly at least until repeat analysis.

3.2.2. Step 2: Challenge phase in longitudinal (serial) cohort—This step is important to perform when the goal is to test accuracy in detecting asymptomatic disease. As detailed earlier, an outcome variable should be used as gold standard. Otherwise, a test that truly detects subclinical disease will appear falsely positive if the clinical diagnosis at the time of sample collection is used for classification.

3.2.3. Step 3: Reliability and stability of biomarkers—When analyzing reliability (inter- and intra-rater variability) as well as stability of biomarkers it is important to use agreement and not correlation analysis. Statistical algorithms such as Bland and Altman agreement and kappa coefficients are especially useful in evaluating robustness of the newly developed test (*II*). For inter and intra-rater variability, 8 samples are tested in triplicate by three independent observers from chip preparation to score calculation. For biomarker stability, scores from fresh samples are compared to those obtained after samples have been stored at either -80°C , -20°C , 4°C or at room temperature for different periods of time (*II*).

3.3. Translational Phase—The translational phase requires identification of the proteins responsible for the peaks included in the final profile. In all instances where we employed the above described algorithm of discovery and validation, the identity of the proteins was highly informative and found to closely relate to the pathogenic process of the disease. The methods for identification of SELDI markers are beyond the focus of this chapter. Importantly, however, in all our studies the identification of the markers led us to pathogenic pathways that could not have been envisioned or detected solely through hypothesis-driven approaches.

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Buhimschi IA. Figure 1

Figure 1. Timeline of proteomics workflow in our laboratory

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Figure 2. Representative SELDI tracings obtained in Step 1 of the Exploratory Phase (wide profiling)

D: pool of diseased samples (urine from women with severe preeclampsia). **ND:** pool of non-diseased samples (urine from normal pregnant women). CHCA: (alpha-cyano-4-hydroxy cinnamic acid). Other annotations refer to binding/washing conditions (ACN: acetonitrile, TFA: trifluoroacetic acid) and instrument reading settings such as laser intensity (160) and detector sensitivity (6). The red asterisks note different biomarkers resolved on two complementing conditions (ideal situation and retained in final profile). The green asterisks mark non-identical peaks, but disease marker resolves in close proximity to a peak present in nondiseased sample (eliminated based of Criterion V). The blue asterisks mark

peaks representing the same peptide (i.e resolved at the same m/z), which is not a disease marker (eliminated based on criterion III). After baseline subtraction the tracings were normalized for total ion current (at 1,500 Da) as indicated by the blue mark to the left of each tracing.

Figure 3. Unprocessed amniotic fluid SELDI tracings obtained in Step 2 of the Exploratory Phase

These tracings are shown without baseline subtraction and without normalization. The baseline is marked by the red line and allows subjective judgment of reading conditions such as laser intensity and detector sensitivity which are the two variables we vary in our standardized array reading protocols. Panel A shows low molecular weight tracings of spots covered with alpha-cyano-4-hydroxy cinnamic acid (CHCA). Note that the matrix peaks also appear in the tracing from the blank spot. Panel B is a partial display of high molecular weight tracings from spots covered with sinapinic acid (SPA). The SPA matrix peaks are not outside the displayed part of the spectrum. The instrument reading settings are listed in Notes 3 and 4.

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Peak Attributes

Assigns Boolean indicator of 1 if S/N > dynamic cut-off value at that mass

Enters mass value only for present peaks

Code	Type	14.5 kDa Mass	14.5 kDa Real mass	14.5 kDa Intensity	14.5 kDa S/N	14.5 kDa present?	15.1 kDa Mass	15.1 kDa Real mass	15.1 kDa Intensity	15.1 kDa S/N	15.1 kDa present?	15.3 kDa Mass	15.3 kDa Real mass	15.3 kDa Intensity	15.3 kDa S/N	15.3 kDa present?
Sample1	ND	14554.25	14554.25	0.13	3.91	1	15138.69		1.19	0.04	0	15306.50		0.04	1.47	0
Sample2	ND	14536.54	14536.54	0.20	6.08	1	15078.13		1.29	0.04	0	15343.02		0.04	1.24	0
Sample3	ND	14542.37	14542.37	0.35	10.85	1	15124.95		1.82	0.06	0	15283.11		0.05	1.63	0
Sample4	D	14542.97	14542.97	0.40	11.17	1	15120.37	15120.37	4.77	0.16	1	15334.57	15334.57	0.08	2.49	1
Sample5	ND	14543.09	14543.09	0.30	8.59	1	15078.13		1.44	0.05	0	15320.67		0.04	1.42	0
Sample6	D	14538.49	14538.49	0.37	10.01	1	15096.62	15096.62	3.94	0.14	1	15325.59		0.04	1.29	0
Sample7	D	14530.58	14530.58	0.35	9.10	1	15110.94	15110.94	23.90	0.84	1	15312.88	15312.88	0.28	8.28	1
Sample8	D	14540.84	14540.84	0.37	10.25	1	15097.56	15097.56	15.33	0.52	1	15310.94	15310.94	0.13	4.09	1
Sample9	ND	14545.59	14545.59	0.38	10.29	1	15166.98		1.96	0.07	0	15304.28		0.06	1.70	0
Sample10	ND	14547.46	14547.46	0.35	10.73	1	15157.92		2.21	0.07	0	15352.79		0.05	1.58	0
Sample11	ND	14546.59	14546.59	0.28	8.00	1	15147.30	15147.30	3.11	0.10	1	15358.60		0.04	1.34	0
Sample12	ND	14552.85	14552.85	0.52	12.59	1	15085.38	15085.38	1.83	0.07	1	15293.10		0.06	1.68	0
Sample13	D	14550.15	14550.15	0.59	15.05	1	15110.72	15110.72	11.54	0.42	1	15317.45	15317.45	0.12	3.28	1
Sample14	ND	14546.46	14546.46	0.24	7.63	1	15084.03		1.49	0.04	0	15267.36		0.05	1.63	0
Sample15	ND	14548.01	14548.01	0.30	9.75	1	15176.49		1.41	0.04	0	15290.58		0.05	1.60	0
Sample16	ND	14544.50	14544.50	0.46	12.91	1	15111.42	15111.42	2.26	0.08	1	15281.73		0.06	1.97	0
Sample17	ND	14548.07	14548.07	0.20	6.17	1	15106.36		1.53	0.05	0	15303.13		0.04	1.20	0
Sample18	D	14550.02	14550.02	0.20	6.43	1	15110.62	15110.62	5.88	0.17	1	15316.17	15316.17	0.07	2.40	1
Sample19	ND	14557.22	14557.22	0.14	5.15	1	15099.40		1.32	0.03	0	15299.18		0.02	0.82	0
Sample20	ND	14554.55	14554.55	0.22	7.52	1	15157.85		1.67	0.05	0	15356.54	15356.54	0.05	1.77	1
Sample21	ND	14548.85	14548.85	0.20	6.64	1	15081.09		1.01	0.03	0	15309.73		0.03	1.29	0
Sample22	ND	14544.76	14544.76	0.15	5.18	1	15147.39		1.41	0.04	0	15318.74		0.03	1.05	0
Sample23	ND	14544.06	14544.06	0.16	5.98	1	15135.73		1.41	0.04	0	15294.12		0.03	1.18	0
Blank		14536.72		0.03	1.36		15164.29		2.90	0.06		15344.02		0.03	1.38	
Blank		14552.00		0.05	1.84		15174.10		1.93	0.05		15333.48		0.06	1.72	
Blank		14514.06		0.02	0.89		15086.66		2.19	0.05		15330.15		0.07	1.76	
average S/N Blank					1.30					0.05					1.62	
SD S/N Blank					0.38					0.01					0.21	
dynamic cut-off (average + 2SD)					2.05					0.07					2.04	
average real mass		14546.01	0.30	8.69			15110.10	4.08	0.14			15324.76	0.06	2.02		
SD real mass		6.09	0.12	2.84			17.47	5.57	0.20			17.69	0.05	1.55		
number tracings		23.00	23.00	23.00			17.00	23.00	23.00			23.00	23.00	23.00		
number tracings with peak present					23					9					6	
average Diseased			0.38	10.34				10.89	0.37				0.12	3.64		
average Nondiseased			0.27	8.12				1.67	0.05				0.04	1.45		
F-test			0.67641	0.79123				0.00000	0.00000				0.00000	0.00000		
P value < 0.0001			no	no				yes	yes				yes	yes		

Criterion III

Not a biomarker but adequate as reference peak

Biomarker

Biomarker

Average mass of only present peaks

Figure 4. Representative spreadsheet calculations for the Mass Restricted (MR) scoring method
 The MR method is an algorithm to extract peaks of biological relevance from SELDI tracings and consists of a set of rules which are sequentially applied to keep or eliminate peaks from the final profile.

Figure 5. Biomarker components of the urine proteomic score (UPS) characteristic of severe preeclampsia in need of indicated early delivery

Representative SELDI profile from a patient with severe preeclampsia at 30 weeks of pregnancy illustrating the 13 biomarker components (P1-P13, marked with red bullets) of the urinary proteomic scores (Boolean UPSb =13 and Ranked UPSr =47) presented in comparison with a SELDI profile of urine from a healthy pregnant control patient (both UPSb and UPSr of 0) at the same gestational age. kDa: kilodaltons. Republished from Am. J. Obstet. Gynecol. 2008 (reference 11) with permission from Elsevier.

Figure 6. Economical modality to use partial SELDI arrays in the Challenge Phase

The tube opening is covered with Parafilm. A central slit is cut into the Parafilm and the array carefully inserted inside the tube. After incubation the blue cap is placed on the tube over the parafilm and the entire device reused in the next experiment.

Figure 7.

Clustering of cases and receiver operating curve (ROC) analysis in the Exploratory versus Challenge Phase. Cases in the Exploratory (A & C) and Challenge Phases (B & D) are grouped by their urine proteomic scores [UPSb (ordinal variable) and UPSr (continuous variable)] and by the occurrence of poor outcome (MID: medically indicated delivery for preeclampsia). The figure illustrates that while there is no overlap between the two groups in the Exploratory Phase we expect a significant overlap in the Challenge Phase where enrollment is consecutive based on “decision to diagnose” and the outcome is not natural but dictated by the physician’s decision to intervene and interrupt the course of pregnancy. Despite the overlap, the SELDI score is superior to any other equivalent tests from the same sample such as protein-to-creatinine ration (P/C ratio) or ratio in angiogenic factors (uFP) (E & F). Panel F is republished from *Am. J. Obstet. Gynecol.* 2008 (reference 11) with permission from Elsevier.

Table 1

Decisions to be made prior to experimentation.

Choice	Question	Answer most likely to yield greatest success	Answer likely to yield lower success rates
Choice of disease	1. Is the disease important?	1. Disease is highly prevalent and/or outcome is unacceptable (severe disability or death).	1. Disease is rare and/or outcome is acceptable.
	2. Is there a current need for better diagnostics?	2. Current diagnostics predictor outcome poorly and/or have long turnaround time and/or a wrong diagnosis leads to treatment decisions with consequences that are final and cannot be reversed.	2. No, several diagnostic test(s) are implemented in practice and/or are used to guide treatment and/or the adverse effects of treatment are minimal.
	3. Is there a clear distinction between patients who have severe disease and those who do not?	3. Yes, but only in advanced stages where treatment options are limited.	3. No, current diagnosis is clinical (subjective) and/or treatment interrupts disease progress before disease can be clearly distinguished.
	4. Is there considerable patient heterogeneity?	4. None or minimal.	4. Yes, the disease is likely a syndrome with multiple etiologies and pathogenic pathways converging in a single clinical scenario.
	5. If diagnostics is perfected (higher accuracy, earlier detection) would it change clinical management?	5. Yes, doctors need to decide on diagnosis quickly and/or accurately and/or the decision impacts treatment options and outcome.	5. No, there is no treatment available and/or adverse effects and cost of treatment are minimal.
Choice of biological sample	1. Is the sample relevant for the disease process?	1. Yes, the biological sample is in special and temporal proximity of an affected organ which is typically affected part of the disease.	1. No, the biological sample is remote from affected organs, or the onset of disease is after the sample is available.
	2. Is the sample likely to be changed in response to unrelated diseases in the same population?	2. Likely in a different way.	2. Yes, disease states of other systems or organs are likely to change the composition on the sample in a similar way.
	3. Is the sample easily available?	3. Yes, sample can be obtained safely and non-invasively (i.e. urine, saliva) or minimally invasive (i.e. blood, tear fluid).	3. No, invasive surgical procedures are required to obtain the sample and/or specialized training (i.e. internal organ tissue or fluid).
	4. Is the sample available repeatedly?	4. Yes, sample can be obtained repeatedly without discomfort for the patient.	4. No, invasive procedure cannot be repeated without substantial risk or discomfort for the patient.
	5. Is the sample likely to degrade rapidly ex vivo?	5. No, sample is a fluid that accumulates in vivo inside a body cavity. Proteolysis likely has occurred already occurred in vivo before sample is obtained.	5. Yes. Sample is a tissue rich in proteases.

Table 2

General scheme of biomarker discovery using SELDI-TOF in our laboratory.

Experimental Phases	Goal	Steps
Exploratory phase (i.e. biomarker discovery)	Extract “best” combination of SELDI biomarkers from a disease relevant biological sample	Step 1: Optimization of SELDI conditions using wide SELDI profiling and pools of samples from “diseased” and “non-diseased” subjects.
		Step 2: Mass Restriction (MR) analysis, definition of SELDI diagnostic profiles & scores.
		Step 3: Accuracy calculation of SELDI scores in the exploratory phase cohort (individual samples).
Challenge phase (i.e. biomarker validation)	Determine clinical utility of proteomic scores as diagnostic and predictive tools in the context of current clinical practice	Step 1: Challenge phase in cross-sectional cohort and calculation of diagnostic accuracy of SELDI proteomic scores.
		Step 2: Challenge phase in serial cohort and calculation of predictive ability of SELDI proteomic scores.
		Step 3: Determination of reliability (intra- and inter-assay variations) and stability of proteomic scores.
Translational phase (i.e. biomarker pathophysiological relevance)	Extend knowledge on the disease process and discover potential therapeutical targets for a future theranostics trial.	Step 1: Biomarker identification.
		Step 2: Hierarchical cohort clustering based on proteomic scores.
		Step 3: Identification-centered proteomic techniques on samples selected based on SELDI proteomic scores.
		Step 4: Hypothesis driven experiments based on biomarker identity.

Table 3

Optimized combination of array surfaces and binding/washing buffers for wide profiling.

Arrays	Binding / washing buffer
H4	20% ACN
H4	50% ACN
H50	0.1% TFA
H50	15% ACN + 0.1% TFA
CM10	pH4 (100mM NaOAc)
CM10	pH8 (100mM Tris-HCl)
Q10	pH6 (100mM NaH ₂ PO ₄)
Q10	pH9 (100mM Na ₂ CO ₃)
IMAC30-metal	pH4 (100mM NaOAc)
IMAC30-metal	pH7.4 (PBS)

Table 4

Array layout for wide profiling using pools of biological samples in Step 1 of Exploratory Phase.

Spot	H4 arrays	H50 arrays	CM10 arrays	Q10 arrays
	1: CHCA & 2: SPA	3: CHCA & 4: SPA	3: CHCA & 4: SPA	3: CHCA & 4: SPA
A	20% ACN Pool D1	0.1% TFA Pool D1	pH4 Pool D1	pH6 Pool D1
B	20% ACN Pool D1	0.1% TFA Pool D2	pH4 Pool D2	pH6 Pool D2
C	20% ACN Pool ND1	0.1% TFA Pool ND1	pH4 Pool ND1	pH6 Pool ND1
D	20% ACN Pool ND2	0.1% TFA Pool ND2	pH4 Pool ND2	pH6 Pool ND2
E	50% ACN Pool D1	15% ACN+0.1% TFA Pool D1	pH8 Pool D1	pH9 Pool D1
F	50% ACN Pool D2	15% ACN+0.1% TFA Pool D2	pH8 Pool D2	pH9 Pool D2
G	50% ACN Pool ND1	15% ACN+0.1% TFA Pool ND1	pH8 Pool ND1	pH9 Pool ND1
H	50% ACN Pool ND2	15% ACN+0.1% TFA Pool ND2	pH8 Pool ND2	pH9 Pool ND2
Spot	IMAC-Zn ²⁺ arrays	IMAC-Cu ²⁺ arrays	IMAC-Ni ²⁺ arrays	IMAC-Ga ²⁺ arrays
	1: CHCA & 2: SPA	1: CHCA & 2: SPA	1: CHCA & 2: SPA	1: CHCA & 2: SPA
A	pH4 Pool D1	pH4 Pool D1	pH4 Pool D1	pH4 Pool D1
B	pH4 Pool D2	pH4 Pool D2	pH4 Pool D2	pH4 Pool D2
C	pH4 Pool ND1	pH4 Pool ND1	pH4 Pool ND1	pH4 Pool ND1
D	pH4 Pool ND2	pH4 Pool ND2	pH4 Pool ND2	pH4 Pool ND2
E	pH7.4 Pool D1	pH7.4 Pool D1	pH7.4 Pool D1	pH7.4 Pool D1
F	pH7.4 Pool D2	pH7.4 Pool D2	pH7.4 Pool D2	pH7.4 Pool D2
G	pH7.4 Pool ND1	pH7.4 Pool ND1	pH7.4 Pool ND1	pH7.4 Pool ND1
H	pH7.4 Pool ND2	pH7.4 Pool ND2	pH7.4 Pool ND2	pH7.4 Pool ND2

Table 5

Principles of the Mass Restricted (MR) scoring algorithm. Republished from Am. J. Obstet. Gynecol. 2008 (reference 11) with permission from Elsevier.

Criterion	Principle
Criterion I	All biomarker peaks should be present in the “diseased” state (i.e. the search is for peaks that appear rather than disappear in disease state)
Criterion II	The final profile should include peaks from at least two different experimental conditions
Criterion III	All peaks in the final profile should be significantly different at a level of <0.0001 between the “diseased” and “normal” state (i.e. for each remaining biomarker there should be a highly significant difference in S/N ratios between diseased and non-diseased tracings).
Criterion IV (optional)	Only parent peaks should be considered (singly ionized, least oxidized)
Criterion V	Peaks should not occur in areas where significant peaks appear in “normal” individuals as they could potentially interfere with informative peaks in “diseased” patients.
Criterion VI (optional)	The final diagnostic proteomic profile should be minimal (restricted).