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Combinatorial Peptide Libraries Facilitate Development of Multiple Reaction Monitoring Assays for Low-Abundance Proteins

Andrei P. Drabovich[†] and Eleftherios P. Diamandis[*,†,¶,▽,§]

Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Department of Clinical Biochemistry, University Health Network, and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

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Low-abundance proteins present in biological fluids are often considered an attractive source of new disease biomarkers. Since such proteins are poorly observed in proteome-scale discovery experiments due to an overwhelming mass of high-abundance proteins, the development of quantitative multiple reaction monitoring (MRM) assays for low-abundance proteins is a challenging task. Here, we present a strategy that facilitates the development of MRM assays for large numbers of unpurified low-abundance proteins. Our discovery strategy is based on the reduction of the dynamic range of protein concentrations in biological fluids by means of one-bead one-compound combinatorial peptide libraries (CPL). Our 2D-LC-MS/MS approach allowed us to identify a total of 484 unique proteins in ovarian cancer ascites, and 216 proteins were assigned as low-abundance ones. Interestingly, 74 of those proteins have never been previously described in ascites fluid. Treatment with CPL allowed identification of a significantly higher number of unique peptides for low-abundance proteins and provided important empirical fragmentation information for development of MRM assays. Finally, we confirmed that MRM assays worked for 30 low-abundance proteins in the unfractionated ascites digest. Using a multiplexed MRM method, relative amounts of five proteins (kallikrein 6, metalloproteinase inhibitor 1, macrophage migration inhibitory factor, follistatin-related protein, and mesothelin) were determined in a set of ovarian cancer ascites. Multiplexed MRM assays targeting large numbers of proteins can be used to develop comprehensive panels of biomarkers with high sensitivity and selectivity, and to study complex protein networks.

Keywords: Low-abundance proteins • multiple reaction monitoring • multiplexed assays • mass spectrometry • ovarian cancer • ascites • combinatorial peptide libraries

Introduction

Biomarker discovery is a rapidly advancing field of biological and clinical sciences with challenging incentives to estimate the chance of developing a certain pathological state, to predict its progression, and to monitor the outcome of treatment. Protein biomarkers are of particular interest in clinical studies of cancer,[1] cardiovascular diseases,[2] neurological disorders,[3] and drug toxicity.[4] Ovarian cancer is one the most aggressive malignancies responsible for as many as 6% of all cancer mortalities.[5] The well-known ovarian cancer biomarker, CA-125, is not suitable for ovarian cancer screening due to its low sensitivity and specificity.[6,7] Thus, there is an urgent need for ovarian cancer biomarkers with better diagnostic potential.

Biological fluids, as opposed to serum, are considered a better source of biomarkers due to the lower complexity of their proteome and elevated levels of disease-relevant proteins. Low-abundance proteins (present in biological fluids at concentrations lower than 1−5 μg/mL)[8] are an excellent pool for novel biomarker discovery. Biological fluid present in the peritoneal cavity (ascites) is heavily accumulating due to cirrhosis, peritoneal carcinomatosis or other severe pathological processes.[9] The proteome of ascites is as complex as that of serum and contains high amounts of albumin, immunoglobulins, and other high-abundance serum proteins.[10,11] Ovarian cancer ascites is thought to contain many known and unknown ovarian cancer biomarkers.[10]

A promising technique to search for disease biomarkers is mass-spectrometry,[12] which enables global proteome profiling of hundreds to thousands of proteins. Discovery of low-abundance proteins with mass spectrometry is a challenging task due to the masking effect of abundant proteins. Quantification of low-abundance proteins with mass spectrometry is an even more challenging undertaking which still needs considerable instrumental and methodological improvements. Multiple reaction monitoring (MRM) assays were introduced

* To whom correspondence should be addressed. Eleftherios P. Diamandis, Ph.D. Tel, 416-586-8443; fax, 416- 619-5521; e-mail, ediamandis@mtsinai.on.ca.

† Samuel Lunenfeld Research Institute, Mount Sinai Hospital.

¶ Department of Pathology and Laboratory Medicine, Mount Sinai Hospital.

▽ Department of Clinical Biochemistry, University Health Network.

§ Department of Laboratory Medicine and Pathobiology, University of Toronto.

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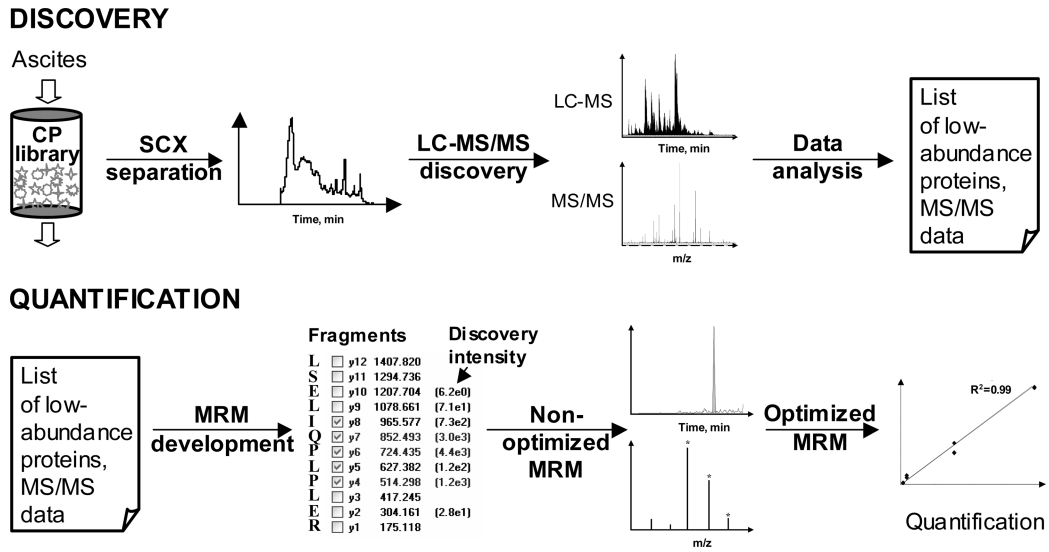


Figure 1. An integrated approach to discovery and MRM quantification of low-abundance proteins.

as an antibody-free means for quantitative proteomics in an attempt to compete with widely used antibody-based ELISAs. Development of MRM assays requires specific information about fragmentation of monitored peptides. This information cannot always be accurately predicted *a priori* but can be extracted from the MS discovery data. High-quality fragmentation spectra for low-abundance proteins, however, may not be available for all proteins of interest. Thus, additional purification and depletion procedures are usually required which significantly increase analysis time and may lead to loss of some low-abundance proteins.

Recently, one-bead one-compound combinatorial peptide libraries (CPL) were proposed as an efficient tool for reducing the wide dynamic range of protein concentrations in biological fluids and thus facilitating "deep" proteome analysis.[13] Such combinatorial libraries contain millions of polymer beads, each of which harbors many copies of a short unique peptide sequence. It has been hypothesized that the majority of proteins in a complex biological mixture may find their unique peptide binders in such library. Upon incubation of the protein mixture with the CP library, high-abundance proteins quickly saturate all their binders. Consequently, only a small proportion of such proteins binds to the beads while their bulk mass is washed away. On the other hand, low-abundance proteins are preferably concentrated on beads.[13] As a result, the range of protein concentrations after CPL treatment is becoming narrower, and low-abundance proteins are relatively enriched. However, since interaction of a random peptide library with a complex mixture of proteins may be facilitated by different mechanisms and with unknown affinity parameters, kinetics and stoichiometry, such fractionation is not fully quantitative. It has also been estimated that 3 to 7% of known proteins are not detected after treatment with random hexapeptide libraries.[14]

In this work, our primary motivation was to introduce an integrated approach for discovery of low-abundance proteins and development of MRM assays for such proteins. We used CPL to discover low-abundance proteins in ovarian cancer ascites. Since CPL treatment led to significantly higher number of identified peptides and fragmentation information of better quality, we used this advantage to develop MRM assays for a large number of low-abundance proteins in ascites fluid. Our integrated approach for discovery and quantification of low-abundance proteins is shown in Figure 1.

Materials and Methods

Materials. The following materials and chemicals were used: one-bead one-compound combinatorial peptide library ("Proteominer", Bio-Rad Laboratories, Mississauga, ON, Canada), sequencing grade modified trypsin (Promega; Madison, WI), iodoacetamide, dithiothreitol (DTT), and glycine (Sigma-Aldrich; St. Louis, MO).

Patients and Specimens. Ascites fluid was obtained with informed consent and Institutional Review Board approved protocol from women with advanced stage ovarian cancer undergoing paracentesis. These patients had stage IV serous ovarian carcinoma and they have been previously treated with surgery plus carboplatin/paclitaxel chemotherapy. Ovarian cancer ascites fluids were aliquoted in 1 mL portions and centrifuged at 16 000*g* for 30 min at 4 °C three times, to separate the fluid from lipids and cellular components. Fluids were stored at − 80 °C until use.

CPL Treatment. Peptide library in a spin tube was washed three times with phosphate buffered saline by using a centrifuge at 1000*g*. One milliliter of ascites fluid was added to the library, incubated for 2 h at room temperature with very gentle shaking, and washed three times with phosphate buffered saline.

In the sequential elution procedure (CPL-SE), we used 2 × 100 μL of four different solutions: (i) 1 M NaCl, 20 mM HEPES at pH 7.5, (ii) 200 mM glycine-HCl at pH 2.4, (iii) 60% ethylene glycol, and (iv) organic solvent mixture in water (13.3% isopropyl alcohol, 7% ACN, 0.1% trifluoroacetic acid (TFA)). These solutions were supposed to disrupt (i) weak ionic interactions, (ii) strong ionic and affinity interactions, (iii) hydrophobic interactions, and (iv) very strong hydrophobic interactions, respectively. Acidic fractions were neutralized immediately after elution; all fractions were pooled together, dialyzed overnight against 50 mM ammonium bicarbonate at pH 7.4, total protein content was measured, and finally, the mixture was lyophilized to dryness and reconstituted in 100 μL of ammonium bicarbonate buffer.

In the on-bead digestion procedure (CPL-OB), beads were denatured with 6 M urea, disulfide bonds were reduced with 10 mM dithiothreitol and alkylated with 20 mM iodoacetamide. Finally, 140 μg of trypsin was added to the beads, and proteins were digested overnight at 37 °C with gentle shaking. Urea at

6 M concentration was added at the end of digestion to denature and elute tryptic peptides that could remain bound to the beads.

Total Protein Assay. Total protein was quantified using a Coomassie (Bradford) protein assay reagent (Pierce). Five microliters of sample and 5 μL of water were loaded in duplicate in a microtiter plate along with the reagent, and protein concentrations were estimated by reference to absorbance obtained for a series of bovine serum albumin standard protein dilutions.

Trypsin Digestion. Ascites (100 μL) or CPL-SE sample was denatured with 6 M urea, and the disulfide bonds were reduced with 10 mM dithiothreitol. Following reduction, the samples were alkylated with 20 mM iodoacetamide, diluted to 0.5 M urea final concentration, and then digested with trypsin (1:50) overnight at 37 °C.

Strong Cation Exchange Liquid Chromatography. Trypsin-digested samples were diluted to 500 μL with mobile phase I (0.26 M formic acid (FA) in 10% ACN). The samples were directly loaded onto a PolySULFOETHYL A column (The Nest Group, Inc.) containing a hydrophilic, anionic polymer (poly-2-sulfethyl aspartamide) with a pore size of 200-Å and a diameter of 5 μm. Prior to each run, a protein cation exchange standard (Bio-Rad) was analyzed to evaluate column performance. A 1-h fractionation run was performed using high-performance liquid chromatography (HPLC), with an Agilent 1100 system at a flow rate of 200 μL/min. A linear gradient of mobile phase II (0.26 M FA in 10% ACN and 1 M ammonium formate) was employed. The eluate was monitored at a wavelength of 280 nm. Three 4-min fractions, 800 μL each, were collected at the beginning and at the end of separation, and 20 2-min fractions, 400 μL each, were collected from 12 to 52 min. Fractions with 800 μL were lyophilized to dryness and reconstituted in 200 μL of buffer A (95% water, 0.1% FA, 5% ACN, and 0.02% TFA). Fractions with 400 μL were diluted with 100 μL of buffer A.

Protein Identification by Mass Spectrometry. All SCX fractions were extracted and desalted with 10 μL C18 OMIX pipet tips (Varian; Lake Forest, CA) and eluted into 4 μL of Buffer B (90% ACN, 0.1% FA, 10% water, and 0.02% TFA). Eighty microliters of acidic solution (0.1% FA and 0.02% TFA in water) was added to each sample and 40 μL was loaded on an Agilent 1100 HPLC system by the autosampler and injected onto a 2 cm C18 trap column (inner diameter, 150 μm). Peptides were eluted from the trap column onto a resolving-5-cm analytical C18 column (inner diameter, 75 μm) with an 15-μm tip (New Objective). Liquid chromatography setup was coupled online to a hybrid linear ion trap-Orbitrap (LTQ Orbitrap XL, Thermo Fisher Scientific, Inc.) mass spectrometer using a nanoelectrospray ionization source (ESI) in data-dependent mode. Each fraction was analyzed with a 90-min LC gradient and eluted peptides were subjected to tandem mass spectrometry (MS/MS). Each MS/MS cycle had a scan of precursor ions (double- and triple-charged, 450−1450 m/z range) in the orbitrap at resolution of 60 000, followed by data-dependent fragmentation of the six most intensive precurson ions (CID, 1 m/z isolation width, 35 V collision energy, 0.25 activation Q, and 30 ms activation time) and analysis of fragments in the ion trap.

Data Analysis. Data files (DATs) were created using the Mascot Daemon (version 2.2) and extract_msn. The parameters for DAT creation were minimum mass, 300 Da; maximum mass, 4000 Da; automatic precursor charge selection; minimum peaks, 10 per MS/MS scan for acquisition; and minimum scans per group, 1. The mass spectra for each fraction were analyzed using Mascot (Matrix Science, London, U.K.; version 2.2) and X!Tandem (Global Proteome Machine Manager, version 2.0.0.4) search engines on the nonredundant International Protein Index (IPI) human database (version 3.54, 20 January 2009),[15] which included the forward and reversed sequences for calculating false positive error rates. Up to one missed cleavage was allowed, and searches were performed with fixed carbamidomethylation of cysteines and variable oxidation of methionine residues. A fragment tolerance of 0.4 Da and a parent tolerance of 10 ppm were used for both search engines with trypsin as a digestion enzyme. The resulting DAT and XML files were loaded into Scaffold (version 2.0, Proteome Software Inc., Portland, OR). All samples were searched against the IPI database with MudPIT (multidimensional protein identification technology) option selected. Peptides identified with a single MS/MS spectrum with probability <95% were discarded since such identifications, as we realized, are the major source of false-positive identifications. Mascot and GPM scores in a "custom peptide identity" mode were adjusted in such way that the false positive identification rate for all protein entries would be ≤1%. Sample reports were exported from Scaffold and each protein entry was assigned a cellular localization based on information available from Genome Ontology (GO) and Swiss-Prot databases.

Peptide Selection for MRM. A final list of proteins from three samples included 134 secreted and membrane-bound low-abundance proteins. The MRM candidate peptides that had clear, intense and unambiguous y-ion fragments (especially at proline residue) were selected. Peptides that had modifications and/or cysteine, methionine and tryptophan amino acids were avoided, when possible. To confirm the choice of empirical peptides, *in silico* digestion and fragmentation were done using "Pinpoint" software prototype (Thermo Fisher Scientific, Inc.), which was also used to generate analytical methods for candidate peptides.

Ascites Digestion for MRM Assay. All digests were performed using 5 μL of ascites without prior purification or removal of high-abundance proteins. Ascites samples containing 10−1912 ng/mL of KLK6 (as determined by ELISA) were denatured with 6 M urea, and the disulfide bonds were reduced with 10 mM dithiothreitol. Following reduction, the samples were alkylated with 20 mM iodoacetamide. They were then diluted to 200 μL and digested with trypsin overnight at 37 °C. Ten microliters of digest was extracted with 10 μL OMIX C18 tips with 2 μg of total peptide binding capacity (Varian; Lake Forest, CA) and injected into the TSQ Quantum triple-quadrupole mass spectrometer. Three replicates were analyzed per each ascites sample.

MRM Conditions. Tryptic peptides were separated on a 2 cm C18 trap column with an inner diameter of 150 μm. The peptides were eluted from the trap column onto a resolving 5 cm analytical C18 column (inner diameter 75 μm) with a 15 μm tip. The LC setup was coupled online to a triple-quadrupole mass spectrometer (TSQ Quantum, Thermo Fisher Scientific, Inc.) using a nanoelectrospray ionization source (nano-ESI). Buffer A contained 0.1% FA, 5% ACN, and 0.02% TFA in an aqueous solution, and buffer B contained 90% ACN, 0.1% FA, and 0.02% TFA in water. The eluted peptides were analyzed by MRM methods in positive-ion mode. Preliminary screening methods were set up to monitor two different peptides with five unique MRM transitions per peptide and had the following parameters: collision energy values predicted with Pinpoint

software, 0.002 m/z scan width, 0.05 s scan time, 0.2 Q1, 0.7 Q3, 1.5 mTorr Q2 pressure, tuned tube lens values, 7 V skimmer offset. A three-step gradient was used with an injection volume of 40 μL, which was loaded onto the column via an Agilent 1100 Cap-LC series autosampler. A 30 min method with 15 min gradient was used in all experiments. Three best transitions for each peptide were left in the final MRM method. To enhance sensitivity and reproducibility, scan times (80 ms) were tuned to provide 20−25 scans per chromatographic peak (~12 s).

Confirmation of Peptide Identity with MRM-Triggered Data Dependent Full Scan MS/MS. The identities of some peptides observed with MRM assays were confirmed with an MRM-triggered data dependent full scan MS/MS using TSQ Quantum. Such methods were set up to monitor a single peptide and included two events. First event had MRM scans for two transitions with 0.010 m/z scan width, 0.05 s scan time, 0.7 Q1, 0.7 Q3, 1.5 mTorr Q2 pressure, previously used collision energy values, tuned tube lens values, and 7 V skimmer offset. Second event was a data-dependent full MS/MS scan with the following parameters: most intense ion from scan 1, minimal signal threshold 10 000, 1 s scan time, charge state +2, 0.7 Q1, 0.7 Q3, no dynamic exclusion, full scan mass range 200−1200 m/z, previously used collision energy values, and 7 V skimmer offset. Individual MS/MS scans in the resulting .RAW files were searched with Mascot against IPI human database version 3.54 as described above. The search confirmed the identity of peptides and corresponding proteins.

Data Analysis. The peak area responses recorded for each sample digest were analyzed using LCquan (version 2.5.6). The peak areas of fragment ions were examined manually for verification and used for quantification.

Quantification of KLK6 by ELISA. The concentration of KLK6 was quantified with a KLK6-specific immunoassay as described previously.[16,17]

Multiplexed MRM Assay of Five Proteins. Multiplexed MRM assay was targeting one unique peptide for each of five low-abundance proteins (kallikrein 6, metalloproteinase inhibitor 1, macrophage migration inhibitory factor, follistatin-related protein, and mesothelin) and thus included 15 precursor-to-fragment ion transitions (5 peptides × 3 transitions). Multiplexed MRM method had the following instrumental parameters: collision energy values predicted with Pinpoint software, 0.010 m/z scan width, 0.06 s scan time, 0.2 Q1, 0.7 Q3, 1.5 mTorr Q2 pressure, tuned tube lens values, 7 V skimmer offset. Each ascites digest sample was run 3 times. Area for each protein was normalized to total protein concentration (normalized abundance = MRM area × total protein concentration). Such normalization was required because different ascites fluids contained significantly different amounts of total proteins. OMIX tips used for micro extraction bound a maximum of 2 μg of total peptides, and 10 μL of ascites digests were used per MRM analysis. Thus, we exceeded the binding capacity of tips by several fold (2.3−6.5) and needed to account for that with normalization.

Results

Depletion of High-Abundance Proteins. To evaluate the efficiency of CP libraries in reducing the wide range of protein concentrations in complex biological fluids, we treated ascites with a CP library and compared the identified proteins to those found in the direct digest (DD) of ascites. Two different sample preparation approaches were used with the CP library: (i) a four-step sequential elution (CPL-SE) of proteins from the beads with NaCl, glycine-HCl, ethylene glycol and organic solvent mixture; and (ii) on-bead digestion (CPL-OB) with trypsin.

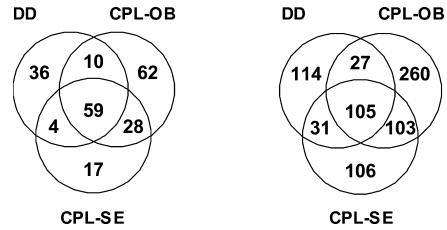


Figure 2. Low-abundance proteins (left) and their unique peptides (right) discovered in the direct digest of ascites (DD), CPL-treated ascites followed by sequential elution (CPL-SE), and CPL-treated ascites followed by on-bead digestion (CPL-OB).

A distinct advantage of CPL treatment was the use of a much higher volume of ascites for proteome discovery. With the direct digestion protocol, we were limited to 100 μL of ascites, or 3.4 mg of total proteins. With the CPL-SE protocol, we were able to treat 1 mL of ascites, or 34.4 mg of total proteins, which resulted in only 0.83 mg of total proteins eluted from the beads. Thus, 98% of total proteins were depleted with the CPL-SE protocol. Similar result would be expected for the CPL-OB protocol. On-bead digestion also allowed us to avoid additional sample preparation steps such as dialysis and lyophilization which could lead to the loss of some proteins.

Mass Spectrometry and Protein Identification. Digested proteins were separated with SCX chromatography into 26 fractions for each sample. Microextraction with C18 OMIX pipet tips was followed by reverse-phase LC-MS/MS. Such 2D-LC-MS/MS approach allowed us to identify in all samples a total of 484 unique proteins with 1% false positive rate, and 251 proteins were identified with two or more peptides (Table S1 in Supporting Information). The number of proteins discovered in the direct digest, CPL-SE and CPL-OB samples was 333, 272, and 314, respectively. The total number of proteins, however, was not a relevant parameter to compare, since the overlap between, for example, the direct digestion protocol (no enrichment) and the on-bead digestion protocol was only 42%. In the context of our experiment, it would be more relevant to compare the number of low-abundance proteins and, especially, the number of unique peptides identified per each low-abundance protein.

Low-Abundance Proteins. Upon removing 268 high-abundance proteins according to the previously published list of serum proteins with concentration higher than 1−5 μg/mL,[18] 216 proteins were assigned as low-abundance (Table S2 in Supporting Information). Overall, 277 unique peptides in the direct digest versus 632 peptides in both CPL-treated samples were found (Figure 2). Significantly more unique peptides were identified in the CPL-OB sample (Figure 3).

Since secreted and membrane-bound proteins are thought to be a better source of biomarkers,[10] we further reduced our list of low-abundance proteins to 134 entries by removing intracellular proteins (Table S3 in Supporting Information). Interestingly, 74 secreted or membrane-bound low-abundance proteins identified in the current study (Table S4 in Supporting Information) have not been previously described in the mass spectrometry-based proteomic analysis of ascites fluid.[10,11] However, there is still a chance that some of these new proteins

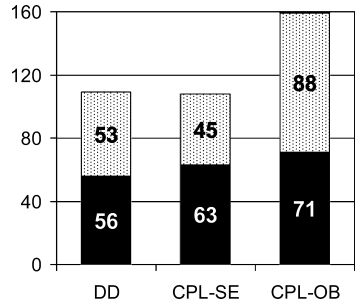


Figure 3. Unique peptides representing low-abundance proteins discovered in the direct digest of ascites (DD), CPL-treated ascites followed by sequential elution (CPL-SE), and CPL-treated ascites followed by on-bead digestion (CPL-OB). Proteins were discovered with one peptide (black area) or two and more peptides (dotted area).

were discovered as a result of the stochastic nature of shotgun proteomic methods, rather than as a result of our CPL-based approach.

Development of MRM Assays. We developed MRM assays based on the empirical fragmentation information obtained at the discovery step. We used "Scaffold" and "Pinpoint" software to facilitate the translation of discovery data into MRM methods. Development of MRM for large numbers of proteins (dozens to hundreds) would be quite a challenging task if such translation were done manually for each individual MRM transition. Thus, our target list of proteins consisted of 134 secreted and membrane-bound, low-abundance proteins.

Since y-ion peptide fragments obtained with ion traps match the fragments obtained with triple quadrupole mass spectrometers,[19] we thoroughly analyzed proteins of interest in the Scaffold file in order to select "proteotypic" peptides. Those peptides that were 7−15 amino acids long, had clear y-ion fragments with high intensities, and contained proline, but not methionine or cysteine, were considered as first priority candidates. Five transitions were chosen per peptide based on y-ion fragment intensities. Besides, uniqueness of each transition in the IPI human proteome database v3.54 was estimated at 0.2 Da fwhm Q1 resolution. Transitions with potential interference were omitted, if possible. One or two peptides were chosen per protein, and TSQ Quantum methods were generated with five transitions for each peptide.

Analysis with preliminary MRM assays was done with the unfractionated digest of ascites. Thirty-one methods were run on TSQ Quantum that targeted 62 unique peptides. MRM methods provided clear peaks with each individual precursor-to-fragment transition for 35 peptides that represented 30 low-abundance proteins (Table 1). Such moderate success rate with preliminary analysis can be explained by the low abundance of monitored peptides. On the other hand, all MRM methods that we have developed to analyze high-abundance proteins in the ascites and serum digests were successful. For example, MRM methods for transferrin (serum concentration 18 mg/mL), clusterin (0.14 mg/mL), and afamin (0.04 mg/mL[20]) had signal-to-noise ratios of 30×10^5, 3×10^5, and 0.9×10^5, respectively. This supports recently published data that high-

Table 1. A List of Low-Abundance Proteins for Which MRM Assays Were Developed

protein	biological process	proteotypic peptide
AMBP Protein AMBP	Transport	AFIQLWAFDAVK
BCAM Lutheran blood group glycoprotein	Cell adhesion	VAYLDPLELSEGK
BTD biotinidase	Metabolic process	VDLITFDTPFAGR
CD109 Isoform 1 of CD109 antigen	Immune response	GDVTLTFLPLSFWGK
CD14 Monocyte differentiation antigen CD14	Immune response	FPAIQNLALR
COMP Cartilage oligomeric matrix protein	Cell adhesion	ELQETNAALQDVR
CPN2 Carboxypeptidase N subunit 2	Proteolysis	LSNNALSGLPQGVFGK
ECM1 Extracellular matrix protein 1	Signal transduction	ELLALIQLER
FSTL1 Follistatin-related protein 1	Signal transduction	IIQWLEAEIIPDGWFSK
HABP2 Hyaluronan-binding protein 2	Cell adhesion	DEIPHNDIALLK
HSPG2 Basement membrane-specific heparan sulfate proteoglycan core protein	Cell adhesion	LEGDTLIIPR
IGFBP3 Insulin-like growth factor-binding protein 3	Cell growth	FLNVLSPR
KLK6 Isoform 1 of Kallikrein-6	Proteolysis	LSELIQPLPLER
LBP Lipopolysaccharide-binding protein	Immune response	ITLPDFTGDLR
LGALS3BP Galectin-3-binding protein	Cell adhesion	SDLAVPSELALLK
MIF Macrophage migration inhibitory factor	Inflammation	LLCGLLAER
MSLN Isoform 2 of Mesothelin	Cell adhesion	TDAVLPLTVAEVQK
MSN Moesin	Cell motion	IGFPWSEIR
MXRA5 Matrix-remodeling-associated protein 5	Cell adhesion	FSILSSGWLR
PON1 Serum paraoxonase/arylesterase 1	Catabolic process	IFFYDSENPPASEVLR
PRG4 Isoform A of Proteoglycan 4	Cell proliferation	ITEVWGIPSPIDTVFTR
SERPINA10 Protein Z-dependent protease inhibitor	Proteolysis	NLELGLTQGSFAFIHK
SERPINC1 SERPINC1 protein	Proteolysis	TSDQIHFFFAK
SERPIND1 Serpin peptidase inhibitor, clade D (Heparin cofactor), member 1	Proteolysis	FAFNLYR
SERPINF1 Pigment epithelium-derived factor	Proteolysis	TVQAVLTVPK
SHBG Isoform 1 of Sex hormone-binding globulin	Hormone transport	IALGGLLFPASNLR
TGFBI Transforming growth factor-beta-induced protein ig-h3	Cell adhesion	EGVYTVFAPTNEAFR
THBS1 Thrombospondin-1	Cell adhesion	FVFGTTPEDILR
TIMP1 Metalloproteinase inhibitor 1	Cell proliferation	GFQALGDAADIR
TNC Isoform 1 of Tenascin	Cell adhesion	LIPGVEYLVSIIAMK

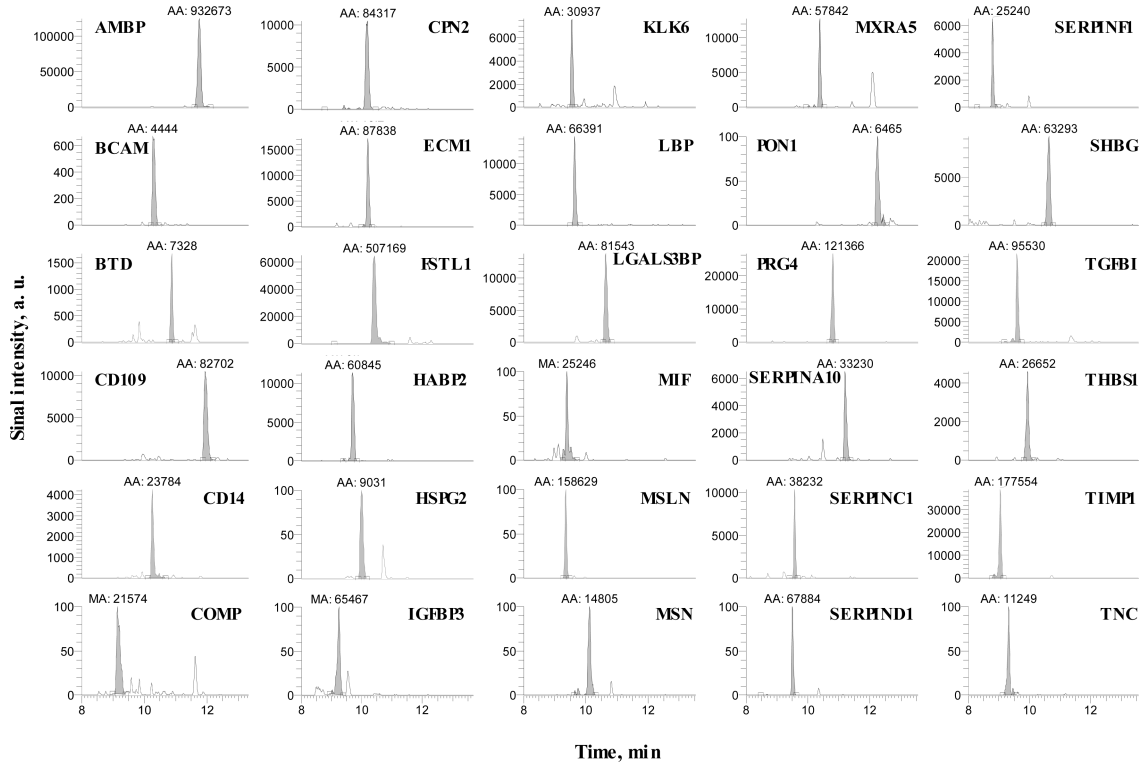


Figure 4. MRM assays for 30 low-abundance proteins listed in Table 1. AA: area under the peak.

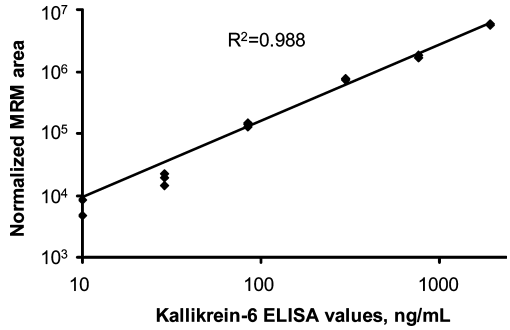


Figure 5. Analysis of LSELIQPLPLER peptide of kallikrein-6 in the unfractionated digest of ovarian cancer ascites from six patients. Details are presented in the Supporting Information, Table S8.

abundance proteins can be readily quantified by LC-MRM even in unfractionated serum at concentrations higher than 20 μg/mL.[20]

Finally, the three most intense and selective transitions from the preliminary analysis were chosen to improve sensitivity and reproducibility of MRM assays (Table S5 in Supporting Information). For example, y6, y7 and y8-ion fragments were used in the final MRM assay of the proteotypic peptide of kallikrein 6 (Figure 4). The identities of some peptides monitored with MRM assays were confirmed with an MRM-triggered, data-dependent full scan MS/MS using TSQ Quantum followed by Mascot search against IPI human database. Thus, we developed sensitive and selective MRM assays for 30 low-abundance proteins that can be analyzed in the unfractionated ascites (Figure 5).

Multiplexed MRM Assay of a Panel of Low-Abundance Proteins in a Set of Ovarian Cancer Ascites. To facilitate high-throughput analysis of proteins, we developed a multiplexed MRM assay to monitor simultaneously a panel of five proteins (kallikrein 6, metalloproteinase inhibitor 1, macrophage migration inhibitory factor, follistatin-related protein, and mesothelin). These proteins are involved in proteolysis, signal transduction, inflammatory response, cell adhesion and cell proliferation and were previously found to be overexpressed in ovarian and many other cancers.[21,22]

We have chosen ovarian cancer ascites from six different patients based on increasing concentrations of kallikrein 6, which is a widely studied ascites-based marker of ovarian cancer.[23] Concentration of kallikrein 6 in these samples was measured with ELISA as described earlier[17] and varied from 10 to 1,912 ng/mL. Multiplexed MRM assay simultaneously targeting five proteins provided their relative amounts. Kallikrein-6 MRM values matched well to ELISA values with a linear regression coefficient $R^2 = 0.988$ (Figure 5) and provided reasonable sensitivity (LOQ 29 ng/mL) and reproducibility (CV $\leq$ 20%, which included between-digest variability, between-micro extraction variability and between-injection variability).

Interestingly, relative amounts of five proteins varied significantly from sample to sample (Supporting Information, Table S8 and Figure S1). Even though there was no noticeable cross-correlation between relative amounts of proteins in present experiment, such multiplexed MRM assays armored with comprehensive mathematical models[24−26] may be used to correlate concentrations of dozens of proteins in order to achieve the maximum sensitivity and specificity of biomarker panels.

Discussion

Discovery of Low-Abundance Proteins. High-abundance proteins present in serum and biological fluids significantly compromise the MS detection of low-abundance proteins. Existing methods for "deep" analysis of the low-abundance proteome include immunoaffinity depletion of high-abundance proteins, immunoaffinity enrichment of proteins of interest,

and multidimensional fractionation methods. Even though these approaches are quite efficient in identifying low-abundance proteins,[27] they are usually time and resource consuming. For example, antibody-based columns[28] for the depletion of several high-abundance proteins are capable to handle only a small volume of sample (up to 250 μL), are not very affordable (cost up to tens of thousands of dollars, such as Agilent MARS-14 column), and may deplete some low-abundance proteins due to the "sponge" effect.[29,30]

In our work, we used commercially available synthetic one-bead one-compound combinatorial peptide libraries that were recently introduced as a tool to reduce the wide range of protein concentrations in complex mixtures of proteins.[13] Such libraries present a fast and affordable way to identify low-abundance proteins.

The potential of CP libraries for quantification, however, seems to be low due to the nature of enrichment, even though there were few successful trials to quantify proteins spiked into serum or cell lysates.[31,32] It is still not known what proteins are preferentially purified on CPL beads. Since weakly charged and hydrophilic proteins may be lost during washing with PBS, we can speculate that mainly highly charged and hydrophobic proteins are enriched upon CPL treatment.

Properties and Performance of CP Libraries. Each bead in the CP library contains around 10 pmol of a unique hexapeptide sequence. The library is synthesized using 17 amino acids (all natural aminoacids excluding cysteine, methionine and leucine) so that the theoretical diversity of the library is $17^6 \approx 2.4 \times 10^7$ unique sequences. The nominal amount of dry beads per 1 mL of sample is 20 mg or $\sim 2 \times 10^5$ beads. Since the latter number represents less than 1% of the theoretical diversity, each bead in the library should be unique. Thus, CPL enrichment is a random process in its nature since different batches of beads will have different pools of peptides and may enrich different proteins. However, stochastic processes, such as shotgun proteomics, are widely used for discovery because the present dynamic range of mass spectrometry instrumentation does not allow for reproducible identification of low-abundance proteins.

Taking into account instrumentation limit of detection and the range of protein concentrations in ascites fluid (similar to that of serum), a simple calculation shows that upon CPL depletion the limit of detection with LC-MS/MS in the identification mode may be decreased from 10 μg/mL to 10 ng/mL.

Ascites Proteome. Previous studies were able to identify 229[11] and 445[10] proteins in the soluble ascites proteome. In our experiment, we were able to identify 484 proteins with a false positive rate of 1%, and 267 proteins were identified with two or more peptides. The overlap between this study and the recently published ascites proteome[10] was 52%.

With our approach, 24 out of 33 previously studied ovarian cancer biomarker candidates were identified (Table S6, Supporting Information). Interestingly, 74 secreted and membrane-bound low-abundance proteins identified in our study have not been previously described in the soluble ascites fraction; 28 of those proteins were identified with 2 or more peptides.

To summarize up-to-date knowledge of the ascites fluid proteome, we combined in a single list all unique proteins identified in the current study and previously published by Kuk et al.[10] and Gortzak-Uzan et al.[11] (Table S7, Supporting Information). This is the longest possible list of soluble ascites fluid proteins (626 entries) ever published.

Biological Function of Proteins Chosen for MRM Assays. Secreted and membrane-bound proteins are often involved in key steps of tumor growth and metastasis and considered excellent candidate biomarkers. To develop MRM assays, we have thus chosen low-abundance proteins involved in signal transduction, cell growth, cell adhesion, immune and inflammatory responses, and regulation of proteolytic cascades (Table 1).

Developing MRM Assays for Unpurified Proteins. To facilitate the development of quantitative MRM assays for multiple low-abundance proteins in an unfractionated digest of a biological fluid, a set of requirements should be satisfied. First, the protein of interest should have at least one peptide easily detectable with mass spectrometry ("proteotypic" peptide). Second, bioinformatics software should be available to translate discovery data into MRM methods. Third, instruments used for discovery and MRM quantification should produce similar fragmentation patterns and intensities of fragments. Fourth, the MS instruments used for MRM quantification should have relatively high resolution and fast cycle times to provide high selectivity in the complex matrix and multiplexing capabilities, respectively.

To satisfy the first requirement, as many as possible peptides should be identified per protein at the discovery step. Each assigned spectrum at this step provides ample empirical information about MS/MS fragmentation, elution times and possible modifications. When matched to *in silico* simulations, this information is invaluable for the fast development of MRM assays.

The second requirement can be satisfied with a set of software tools that are capable of (i) processing raw files at the discovery step; (ii) filtering data with required confidence of identification to obtain the list of proteins with easily accessible sequence information, MS and MS/MS data; (iii) *in silico* digesting proteins of interest and predicting MS/MS fragmen-

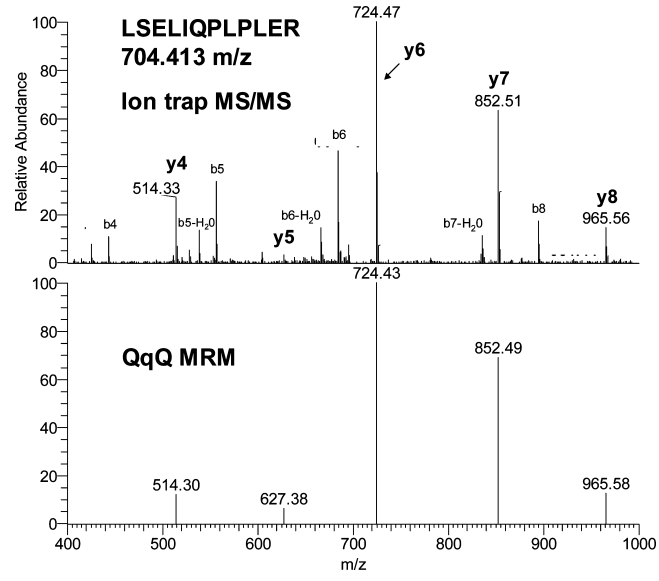
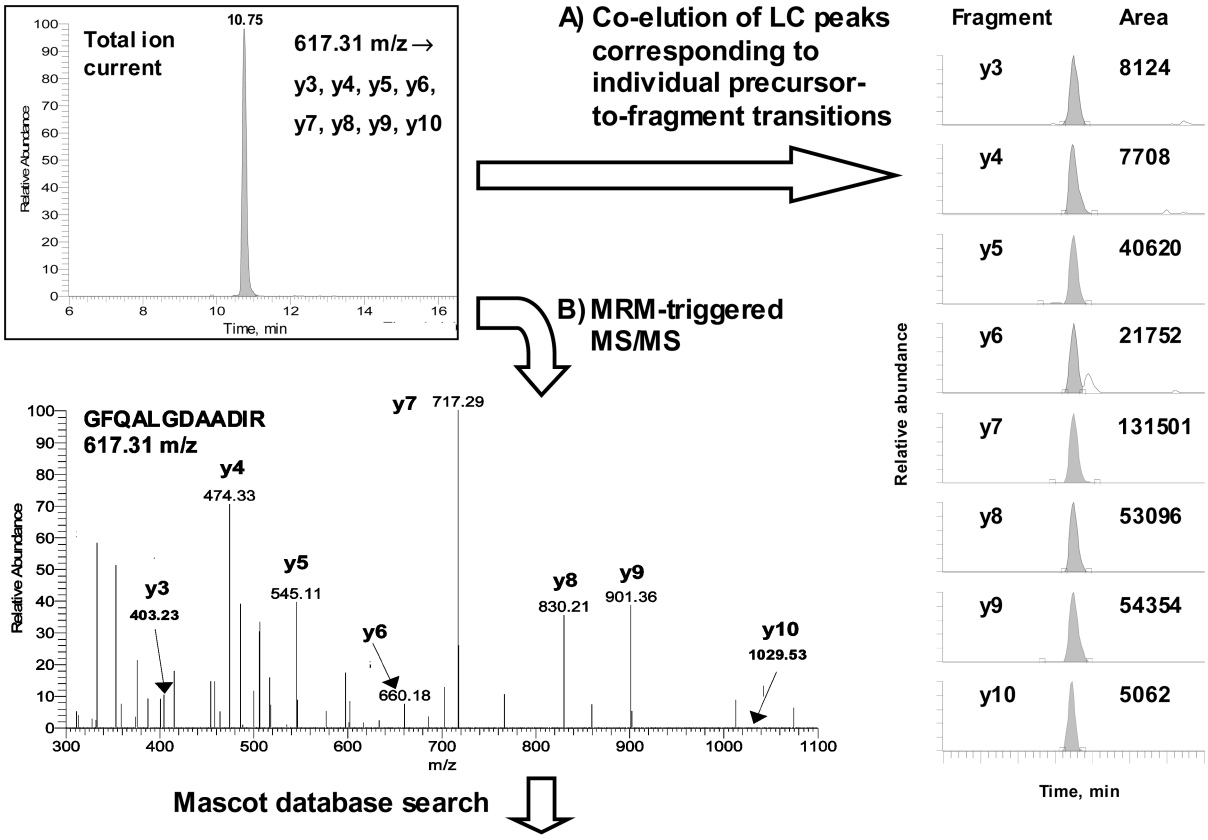


Figure 6. Fragmentation of proteotypic peptide LSELIQPLPLER of kallikrein 6 in the ion trap of LTQ Orbitrap (upper spectrum), and relative intensities of five y-ion fragments of this peptide in the triple-quadrupole (QqQ) mass spectrometer (lower spectrum). The three most abundant y-ion fragments (y6, y7 and y8) were chosen for the final MRM method.



IPI00032292 Mass: 23156 Score: 50 Queries matched: 1
Tax_Id=9606 Gene_Symbol=TIMP1 Metalloproteinase inhibitor 1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
1	617.3141	1232.6136	1232.6149	-0.0013	0	50	0.0021	1	K.GFQALGDAADIR.F

Figure 7. Confirmation of the identity of peptide GFQALGDAADIR of TIMP1 protein in the unfractionated ascites digest. Peak observed at 10.75 min is confirmed by (A) coelution of LC peaks corresponding to 8 individual precursor-to-fragment transitions; (B) MRM-triggered, data-dependent full scan MS/MS followed by Mascot database search.

tation of peptides; (iv) comparing empirical fragmentation to *in silico* simulations; and (v) generating instrumental methods for hundreds of proteins. Even though no single software program can fulfill all mentioned requirements, a combination of commercially available software packages such as Mascot (Matrix Science, Inc.), GPM (Global Proteome Machine Organization), Scaffold (Proteome Software, Inc.) and Pinpoint (Thermo Fisher Scientific, Inc.) can be used.

According to the third requirement, mass spectrometry instruments used for discovery and quantification phases should have comparable fragmentation patterns. An example of such successful combination of instruments is an ion trap and a triple quadrupole mass spectrometers. Even though peptide fragmentation in the triple quadrupole is deficient in b-ion fragments, y-ion fragments and their intensities match very well to those obtained with an ion trap (Figure 6).

And fourth, high resolution of Q1 of modern triple quadrupole instruments (up to 10 000 fwhm at 700 m/z vs 1500 fwhm for previous generations of triple quadrupoles) provides high selectivity of MRM assays and facilitates the analysis of proteins in the unfractionated digests of complex biological fluids. Besides, fast cycle times of triple quadrupole instruments allow for multiplexing, which is an important advantage for high-throughput quantification of large panels of biomarkers.[33,34]

If a biological fluid is the only source of a given protein, and if LC retention times of target peptides are not known, confirmation of the identity of peaks observed in MRM is required. Co-elution of LC peaks corresponding to multiple precursor-to-fragment transitions (Figure 7A) is the simplest way to confirm the identity. Five and more y-ion transitions with observed MRM areas correlated to known MS/MS intensities usually unambiguously identify a target peptide.[19] Besides, observation of multiple individual transitions helps to choose the best transitions with maximum intensity and minimum interference from contaminants to provide high selectivity of the assay in a given biological fluid. Another way to confirm the identity of peak is an MRM-triggered, data-dependent full scan MS/MS followed by Mascot search (Figure 7B).

In general, to target low-abundance proteins with LC-MS/MS data-dependent discovery methods, extensive multidimensional separation methods are required. On the other hand, MRM assay has two "intrinsic" dimensions of separation of precursor peptides in Q1 quadrupole and their fragments in Q3 quadrupole. Thus, LC-MRM with its three-dimensional separation allows bypassing the labor-intensive sample preparation steps. Interestingly, new hybrid mass spectrometry instruments, such as Q-IMS-TOF, may add another dimension of separation to MRM assays.[35] In perspective, when mass spectrometry instrumentation is capable of complete ionization of peptides, minimal loss of precursor and fragment ions in the ion optics, high-resolution analysis and ultrafast scanning,

high-throughput multiplexed MS^n-based MRM assays will become the methods of choice for quantitative proteomics.

Conclusion

Our work was motivated by the insight that all essential requirements to develop MRM assays for multiple unpurified low-abundance proteins can be satisfied in our integrated approach.

Finally, we would like to emphasize the advantages of CP libraries. First, CPL treatment facilitates discovery of low-abundance proteins. Second, CPL treatment increases the number of unique peptides and improves the quality of MS/MS fragmentation information which helps to choose the best MRM transitions. CPL treatment may also be used as a tool in the preliminary experiments to quickly develop MRM assays and evaluate their performance for uncharacterized proteins in complex biological fluids prior to time-consuming protein quantification by techniques that require specialized reagents (such as immuno-MS and ELISAs). Finally, our work paves the road to the fast development of multiplexed MRM assays for large numbers of proteins. Such assays will be invaluable tools for clinical analyses of panels of biomarkers and for studies of complex protein networks complemented with systems biology approaches.[36−38] Further validation of some proteins identified in our study may lead to identification of novel ovarian cancer biomarkers.

Abbreviations: BSA, bovine serum albumin; CA125, carbohydrate antigen 125; CE, collision energy; CV, coefficient of variation; Da, dalton; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; fwhh, full width at half height; GO, gene ontology; IPI, international protein index; KLK6, kallikrein 6; LC, liquid chromatography; LOQ, limit of quantification; MRM, multiple reaction monitoring; MS, mass spectrometry/spectrometer; MS/MS, tandem mass spectrometry; Q1, first quadrupole; Q3, third quadrupole; SCX, strong cation exchange chromatography.

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Supporting Information Available: Tables listing proteins detected in soluble ascites fluid; MRM transitions; list of previously studied ovarian cancer biomarkers; the longest possible list of soluble ascites fluid proteins ever published. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hanash, S. M.; Pitteri, S. J.; Faca, V. M. Mining the plasma proteome for cancer biomarkers. *Nature* **2008**, *452* (7187), 571–9.
(2) Edwards, A. V.; White, M. Y.; Cordwell, S. J. The role of proteomics in clinical cardiovascular biomarker discovery. *Mol. Cell. Proteomics* **2008**, *7* (10), 1824–37.
(3) Ekegren, T.; Hanrieder, J.; Bergquist, J. Clinical perspectives of high-resolution mass spectrometry-based proteomics in neuroscience: exemplified in amyotrophic lateral sclerosis biomarker discovery research. *J. Mass Spectrom.* **2008**, *43* (5), 559–71.
(4) Collins, B. C.; Clarke, A.; Kitteringham, N. R.; Gallagher, W. M.; Pennington, S. R. Use of proteomics for the discovery of early markers of drug toxicity. *Expert Opin. Drug Metab. Toxicol.* **2007**, *3* (5), 689–704.
(5) Jemal, A.; Siegel, R.; Ward, E.; Hao, Y.; Xu, J.; Murray, T.; Thun, M. J. Cancer statistics, 2008. *CA Cancer J. Clin.* **2008**, *58* (2), 71–96.
(6) Jacobs, I.; Bast, R. C., Jr. The CA 125 tumour-associated antigen: a review of the literature. *Hum. Reprod.* **1989**, *4* (1), 1–12.
(7) Gargano, G.; Correale, M.; Abbate, I.; Falco, G.; De Frenza, N.; Lorusso, V.; De Lena, M.; De Leonardis, A. The role of tumour markers in ovarian cancer. *Clin. Exp. Obstet. Gynecol.* **1990**, *17* (1), 23–9.
(8) Keshishian, H.; Addona, T.; Burgess, M.; Kuhn, E.; Carr, S. A. Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* **2007**, *6* (12), 2212–29.
(9) Tamsma, J. T.; Keizer, H. J.; Meinders, A. E. Pathogenesis of malignant ascites: Starling's law of capillary hemodynamics revisited. *Ann. Oncol.* **2001**, *12* (10), 1353–7.
(10) Kuk, C.; Kulasingam, V.; Gunawardana, C. G.; Smith, C. R.; Batruch, I.; Diamandis, E. P. Mining the ovarian cancer ascites proteome for potential ovarian cancer biomarkers. *Mol. Cell. Proteomics* **2009**, *8* (4), 661–9.
(11) Gortzak-Uzan, L.; Ignatchenko, A.; Evangelou, A. I.; Agochiya, M.; Brown, K. A.; St Onge, P.; Kireeva, I.; Schmitt-Ulms, G.; Brown, T. J.; Murphy, J.; Rosen, B.; Shaw, P.; Jurisica, I.; Kislinger, T. A proteome resource of ovarian cancer ascites: integrated proteomic and bioinformatic analyses to identify putative biomarkers. *J. Proteome Res.* **2008**, *7* (1), 339–51.
(12) Kulasingam, V.; Diamandis, E. P. Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. *Nat. Clin. Pract. Oncol.* **2008**, *5* (10), 588–99.
(13) Boschetti, E.; Righetti, P. G. The art of observing rare protein species in proteomes with peptide ligand libraries. *Proteomics* **2009**, *9* (6), 1492–510.
(14) Boschetti, E.; Lomas, L.; Citterio, A.; Righetti, P. G. Romancing the "hidden proteome", Anno Domini two zero zero seven. *J. Chromatogr., A* **2007**, *1153* (1−2), 277–90.
(15) Kersey, P. J.; Duarte, J.; Williams, A.; Karavidopoulou, Y.; Birney, E.; Apweiler, R. The International Protein Index: an integrated database for proteomics experiments. *Proteomics* **2004**, *4* (7), 1985–8.
(16) Diamandis, E. P.; Yousef, G. M.; Soosaipillai, A. R.; Grass, L.; Porter, A.; Little, S.; Sotiropoulou, G. Immunofluorometric assay of human kallikrein 6 (zyme/protease M/neurosin) and preliminary clinical applications. *Clin. Biochem.* **2000**, *33* (5), 369–75.
(17) Luo, L. Y.; Soosaipillai, A.; Grass, L.; Diamandis, E. P. Characterization of human kallikreins 6 and 10 in ascites fluid from ovarian cancer patients. *Tumor Biol.* **2006**, *27* (5), 227–34.
(18) Hortin, G. L.; Sviridov, D.; Anderson, N. L. High-abundance polypeptides of the human plasma proteome comprising the top 4 logs of polypeptide abundance. *Clin. Chem.* **2008**, *54* (10), 1608–16.
(19) Prakash, A.; Tomazela, D. M.; Frewen, B.; Maclean, B.; Merrihew, G.; Peterman, S.; Maccoss, M. J. Expediting the development of targeted SRM assays: using data from shotgun proteomics to automate method development. *J. Proteome Res.* **2009**, *8* (6), 2733–9.
(20) Kuzyk, M. A.; Smith, D.; Yang, J.; Cross, T. J.; Jackson, A. M.; Hardie, D. B.; Anderson, N. L.; Borchers, C. H. MRM-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol. Cell. Proteomics* **2009**, *8* (8), 1860–77.
(21) Polanski, M.; Anderson, L. N. A List of Candidate Cancer Biomarkers for Targeted Proteomics. *Biomarker Insights* **2006**, *2*, 1–48.
(22) Kobel, M.; Kalloger, S. E.; Boyd, N.; McKinney, S.; Mehl, E.; Palmer, C.; Leung, S.; Bowen, N. J.; Ionescu, D. N.; Rajput, A.; Prentice, L. M.; Miller, D.; Santos, J.; Swenerton, K.; Gilks, C. B.; Huntsman, D. Ovarian carcinoma subtypes are different diseases: implications for biomarker studies. *PLoS Med.* **2008**, *5* (12), e232.
(23) Diamandis, E. P.; Scorilas, A.; Fracchioli, S.; Van Gramberen, M.; De Bruijn, H.; Henrik, A.; Soosaipillai, A.; Grass, L.; Yousef, G. M.; Stenman, U. H.; Massobrio, M.; Van Der Zee, A. G.; Vergote, I.; Katsaros, D. Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma. *J. Clin. Oncol.* **2003**, *21* (6), 1035–43.
(24) Dudley, J. T.; Butte, A. J. Identification of discriminating biomarkers for human disease using integrative network biology. *Pac. Symp. Biocomput.* **2009**, 27–38.
(25) Zhang, S.; Zhang, X. S.; Chen, L. Biomolecular network querying: a promising approach in systems biology. *BMC Syst. Biol.* **2008**, *2*, 5.
(26) Adourian, A.; Jennings, E.; Balasubramanian, R.; Hines, W. M.; Damian, D.; Plasterer, T. N.; Clish, C. B.; Stroobant, P.; McBurney, R.; Verheij, E. R.; Bobeldijk, I.; van der Greef, J.; Lindberg, J.; Kenne,

K.; Andersson, U.; Hellmold, H.; Nilsson, K.; Salter, H.; Schuppe-Koistinen, I. Correlation network analysis for data integration and biomarker selection. *Mol. BioSyst.* **2008**, *4* (3), 249–59.
(27) Qian, W. J.; Kaleta, D. T.; Petritis, B. O.; Jiang, H.; Liu, T.; Zhang, X.; Mottaz, H. M.; Varnum, S. M.; Camp, D. G., 2nd; Huang, L.; Fang, X.; Zhang, W. W.; Smith, R. D. Enhanced detection of low abundance human plasma proteins using a tandem IgY12-SuperMix immunoaffinity separation strategy. *Mol. Cell. Proteomics* **2008**, *7* (10), 1963–73.
(28) Pernemalm, M.; Lewensohn, R.; Lehtio, J. Affinity prefractionation for MS-based plasma proteomics. *Proteomics* **2009**, *9* (6), 1420–7.
(29) Zolotarjova, N.; Martosella, J.; Nicol, G.; Bailey, J.; Boyes, B. E.; Barrett, W. C. Differences among techniques for high-abundant protein depletion. *Proteomics* **2005**, *5* (13), 3304–13.
(30) Ichibangase, T.; Moriya, K.; Koike, K.; Imai, K. Limitation of immunoaffinity column for the removal of abundant proteins from plasma in quantitative plasma proteomics. *Biomed. Chromatogr.* **2009**, *23* (5), 480–7.
(31) Hammond, D. J.; Lathrop, J. T., Identification and characterization of analytes from whole blood. US Patent Application 11/454,801, 2007.
(32) Roux-Dalvai, F.; Gonzalez de Peredo, A.; Simo, C.; Guerrier, L.; Bouyssie, D.; Zanella, A.; Citterio, A.; Burlet-Schiltz, O.; Boschetti, E.; Righetti, P. G.; Monsarrat, B. Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry. *Mol. Cell. Proteomics* **2008**, *7* (11), 2254–69.
(33) Kuhn, E.; Addona, T.; Keshishian, H.; Burgess, M.; Mani, D. R.; Lee, R. T.; Sabatine, M. S.; Gerszten, R. E.; Carr, S. A. Developing multiplexed assays for troponin I and interleukin-33 in plasma by peptide immunoaffinity enrichment and targeted mass spectrometry. *Clin. Chem.* **2009**, *55* (6), 1108–17.
(34) Wang, K. Y.; Chuang, S. A.; Lin, P. C.; Huang, L. S.; Chen, S. H.; Ouarda, S.; Pan, W. H.; Lee, P. Y.; Lin, C. C.; Chen, Y. J. Multiplexed immunoassay: quantitation and profiling of serum biomarkers using magnetic nanoprobes and MALDI-TOF MS. *Anal. Chem.* **2008**, *80* (16), 6159–67.
(35) Pringle, S. D.; Giles, K.; Wildgoose, J. L.; Williams, J. P.; Slade, S. E.; Thalassinos, K.; Bateman, R. H.; Bowers, M. T.; Scrivens, J. H. An investigation of the mobility separation of some peptide and protein ions using a new hybrid quadrupole/travelling wave IMS/oa-ToF instrument. *Int. J. Mass Spectrom.* **2007**, *261* (1), 1–12.
(36) Bachi, A.; Bonaldi, T. Quantitative proteomics as a new piece of the systems biology puzzle. *J. Proteomics* **2008**, *71* (3), 357–67.
(37) Feng, X.; Liu, X.; Luo, Q.; Liu, B. F. Mass spectrometry in systems biology: an overview. *Mass Spectrom. Rev.* **2008**, *27* (6), 635–60.
(38) Rho, S.; You, S.; Kim, Y.; Hwang, D. From proteomics toward systems biology: integration of different types of proteomics data into network models. *BMB Rep.* **2008**, *41* (3), 184–93.

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