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Abstract

Our objective is to develop and test proteomic methods for the prediction of breast cancer risk, an approach that has not been attempted previously. Our underlying hypothesis is that proteomic analysis of serum will identify proteins differentially expressed in women who do versus those who do not develop invasive breast cancer, and that these differences will be identifiable prior to the clinical presentation of breast cancer. Our work is being conducted in two phases, a training phase and a test phase. Both phases will be conducted as case-control studies nested in a population-based cohort of women who were members of Kaiser Permanente. These serum specimens were collected between 1986 and 1992. To date, we have finalized our cohort definition; finalized the definition of cases and controls; finalized the criteria for matching controls to cases; selected the cases and controls; pulled and aliquotted the serum specimens; and we have developed a detailed protocol for the proteomic analysis of the serum samples. Briefly, with respect to the latter, the serum sample is loaded onto an immunoaffinity column to deplete the twelve most abundant proteins, and the flow-through fraction is collected and subjected to tryptic digestion. Subsequently, the peptides are labeled with iTRAQ reagents and fractionated by strong cation exchange chromatography (SCX). Each SCX fraction is loaded onto a reverse phase column and spotted onto a MALDI target followed by MALDI-TOF/TOF (4700 Proteomic Analyzer) analyses. The data collected are automatically processed, combined, and searched against human protein databases. This procedure has been thoroughly tested for reproducibility, quantification, and complexity (dynamic range), and the collection of case-control data has been initiated. By applying a high-resolution proteomic approach to a prospective setting, this ongoing project should enhance our ability to identify those women at increased risk of breast cancer and intervene before they progress to cancer. Furthermore, it may provide insight into the biological processes underlying breast cancer development through the identification of protein markers of disease and the associated disease susceptibility genes.

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Introduction

New approaches to breast cancer risk prediction are needed given the modest discriminatory accuracy of existing risk prediction models at the individual level. Our objective is to develop and test proteomic methods for the prediction of breast cancer risk, an approach that has not been attempted previously. Our underlying hypothesis is that proteomic analysis of serum will identify proteins differentially expressed in women who do versus those who do not develop invasive breast cancer, and that these differences will be identifiable prior to the clinical presentation of breast cancer. Specifically: (1) using a training set of serum specimens sampled from a population-based cohort of women who were members of Kaiser Permanente, who had their blood samples taken at a multiphasic health examination (MHC) between 1986 and 1992**,** and who were followed up to determine subsequent breast cancer occurrence, we are applying highly sensitivity proteomic approaches in order to identify biomarkers that discriminate between women who developed invasive breast cancer within 5 years of having a serum sample collected (cases) and women who remained free of breast cancer for at least that long (controls); (2) using a validation set of serum samples collected from a separate group of cases and controls in the cohort, we plan to test the biomarkers identified using the training set. Nested case-control studies will be undertaken at the training (40 cases/40 controls) and validation stages (20 cases/20 controls). Cases will be women with no history of breast cancer at baseline who developed a subsequent incident, invasive breast cancer within 5 years of collection of a serum sample; controls (no breast cancer history) will be selected from the same cohort using risk-set sampling and matched individually to cases on calendar year of the MHC exam at which the serum sample of interest was collected, age at that MHC exam, and time since last meal. To reduce the possible impact of breast cancer heterogeneity on interpretation of the results, we have restricted inclusion to white, postmenopausal women; should the results prove informative, we plan to study other subgroups (e.g., African American, premenopausal, etc.) in the future. Data on breast cancer risk factors will come from questionnaire and physical exam data gathered at the MHC. All proteomic spectra will be analyzed by the MASCOT program to identify the protein sequences generating all peptide ions. After biomarker ions have been identified, validation will be carried out by the same analytical procedures following only the biomarker ions of interest. The laboratory work will be performed "blinded" to case-control status. Supervised clustering algorithms such as support vector machines will be used for the statistical analysis of the training dataset to identify candidate marker patterns that best discriminate between cases and controls. The most promising markers identified in the training set will be applied to subjects in the validation set and used to classify them as cases, controls, or neither. In contrast to the training set, where case-control status will be known to the data analysts, case-control status will not be known during analysis of the validation set. Therefore, we will determine how well the method developed at the training stage discriminates between cases and controls in the validation set.

Body

(i) Eligibility criteria

We have finalized eligibility for inclusion in the cohort. The cohort consists of white, postmenopausal women aged 55 to 80 years at the time of the blood draw who were recruited through Kaiser Permanente between 1986 and 1992. We chose to restrict attention to white women to reduce the possible impact of breast cancer heterogeneity on interpretation of the results.

(ii) Case/control definition and selection

The work for this project is being conducted as nested case-control studies, both at the training and at the validation stages. Cases are defined as white, postmenopausal women with no history of breast cancer at the time of recruitment. Inclusion is restricted to subjects between the ages of 55 and 80 years. We identified 68 potentially eligible cases by merging data from the multiphasic cohort/serum repository databases with data from the Kaiser Permanente tumor registry. The 60 cases selected for the study are a random sample of all eligible cases. Controls are matched 1:1 to the corresponding case. They are white, postmenopausal women with no history of breast cancer and who had not developed breast cancer by the date of diagnosis of the corresponding case. The controls were selected using risk-set sampling with replacement. They were matched to the corresponding case on age (within 1 year), date of serum collection (to within 1 month), and time since last meal (0-3 hours, 4-9 hours). Furthermore, cases and controls were matched with respect to membership of Kaiser Permanente, in the sense that as with the cases, controls were required to have been members from one year prior to serum collection and to have been a member at the time of diagnosis of the corresponding case (those whose membership lapsed for a period exceeding 3 months ceased to be eligible to be selected as a control).

(iii) Data file

Study identification numbers were assigned to the cases and controls. These numbers are linkable to the Kaiser IDs, which allowed extraction of corresponding covariate information from the cohort database. The data file containing the covariate information required for the analysis has been created.

(iv) Aliquoting of serum samples

The pulling, testing (for dessication), and aliquoting of the serum specimens was completed at the Orentreich Foundation. For each subject included in the study, 5 x 20 µl aliquots were made. Furthermore, a common serum pool was created by adding 50 µl from each study subject to the pool. The pool serves as a common standard that is included in each run. For each case-control pair, 5 sets of samples were prepared, each set consisting of 20 µl aliquots for the case, the matched control, and the common pool. The location of the case and control aliquot within each triplet was assigned randomly and the laboratory staff are blinded to the identity of the case and control samples. The specimens were shipped to and received at the Albert Einstein College of Medicine where they are held in storage at -80°C until they are needed for analysis.

(v) Development of methods for proteomic analysis of serum samples

In the previous report, the depletion, labeling, and digestion steps had been worked out, with the strong cation exchange (SCX) and reversed phase-MALDI target spotting routines remaining to be optimized. In addition, the protocol for automated data acquisition required fine-tuning. Despite several technical difficulties, we have now established detailed protocols and reaction conditions for each step of the proteomic analysis. Specifically, we have developed and validated an integrated, highly sensitive, proteomic analysis involving a fractionation by immunodepletion and multi-dimensional HPLC and analysis by MALDI-TOF/TOF-MS, to tackle the complexity and dynamic range of the serum proteome. The sequence of steps in this analysis are summarized in Figure 1 and the detailed description of the procedure is provided in the Proteomic Laboratory Operation Manual (Appendix 1) and in the checklists for each step (Appendix 2). Briefly, each serum sample is loaded onto an immunoaffinity column to deplete the twelve most abundant proteins, and the flow-through fraction is collected and subjected to tryptic digestion. Subsequently, the peptide digest is labeled

with iTRAQ reagent, mixed with two other digests (to combine differentially labeled case, control and pool), and subjected to strong cation exchange (SCX) chromatography. Each SCX fraction is then loaded onto the reverse phase column and pooled fractions spotted onto targets for MALDI-TOF/TOF (4700 Proteomic Analyzer) analyses. The data collected are automatically processed, combined, and searched against human protein databases. The raw spectral files are processed to provide appropriate bioinformatic data. All stages of this procedure have been developed and refined over the last year as described below.

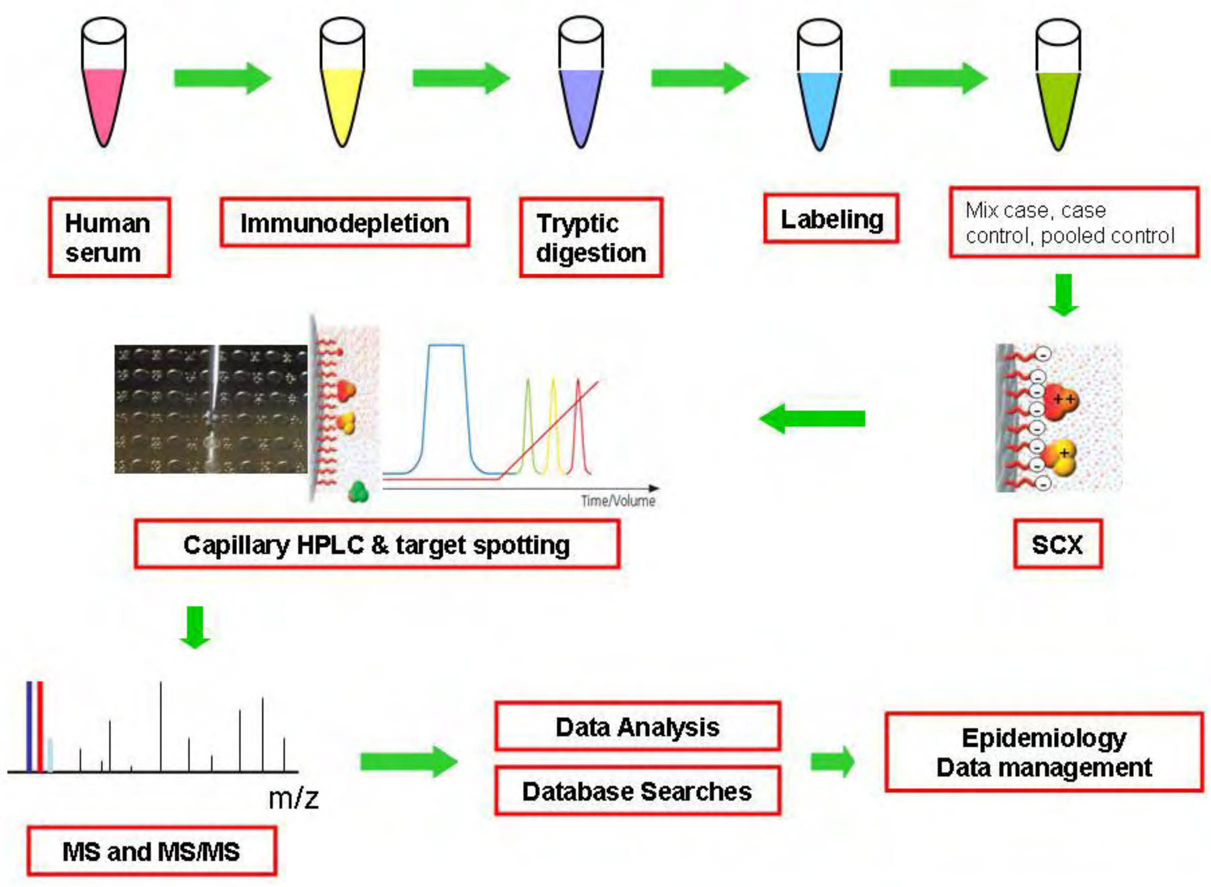


Figure 1. Steps in the proteomic analysis of breast cancer serum samples

(a) Sample preparation. The serum sample (20 µl) is loaded onto the immunodepletion column (6.4 × 63 mm, Genway Biotech Inc, San Diego, CA), the flow-through fraction is collected, its volume is reduced by ultrafiltration, and the concentrate digested overnight with trypsin. Each tryptic digest is then concentrated by SpeedVac and approximately 40% of the digest subjected to labeling with approximately 100 µg of iTRAQ reagent (114, 115 or 116) overnight at 37°C. Residual reagent is quenched by adding 300 µl of water and allowing excess reagent to completely hydrolyze over an additional 30 minutes. The three differently labeled samples are then mixed.

Several changes to the original procedure were necessary in optimization, including the elimination of reduction and alkylation of the immunodepleted flow through fraction under denaturing conditions and its subsequent concentration on a C4 column, due to the difficulty in removing guanidium hydrochloride which adversely affected the C4 column step. The end result was to streamline the procedure. The optimization of the biochemical steps was completed using standard proteins and the NIST serum standard, often used for proteomics methods development. The reduction in the amount of sample that is labeled, significantly improved labeling efficiency. Only 40% of the sample is labeled, the remaining 60% being saved. Of the 40%, a proportion corresponding to 33% of the total proceeds to 2D LC step. A proportion corresponding to 7% is saved for multi-lectin affinity chromatography to prepare glycopeptides that will be treated with PNGase-F to obtain the peptides that possessed N-linked sugars (Yang Z, Harris LE, Palmer-Toy DE, Hancock WS. Multilectin affinity chromatography for characterization of multiple glycoprotein biomarker candidates in serum from breast cancer patients. Clin Chem. 2006;52:1897-905. Plavina T, Wakshull E, Hancock WS, Hincapie M. Combination of abundant protein depletion and multi-lectin affinity chromatography (M-LAC) for plasma protein biomarker discovery. J Proteome Res. 2007;6:662-71). This glycopeptide analysis will not be performed as part of the ongoing study, but may, in future analyses, provide additional useful information. As can be seen in the detailed protocol (Appendix 1), other modifications to the sample preparation protocol were also made at this time.

(b) Off-line 2D LC coupled with MALDI-TOF/TOF analyses. In the first dimension, the combined peptide mixture is separated by strong cation exchange (SCX) chromatography on an AKTA purifier 10 system from GE Healthcare Bio-Sciences (Piscataway, NJ) using a PolySulfoethyl A ^{TM} column (2.1 × 100 mm, 5 µm, 300Å) from Poly LC Inc (Columbia, MD). The sample is diluted in 1 mL of SCX loading buffer (20% acetonitrile, 10 mM KH_2PO_4, pH 3), loaded and the column washed isocratically for 20 min at 0.1 mL/min to remove excess reagent. Peptides are eluted with a linear gradient of 0-700 mM KCl (20% acetonitrile, 10 mM KH_2PO_4, pH 3) over 15 minutes at a flow rate of 0.1 mL/minute, with fractions collected at 1-minute intervals (Fig. 2). The second dimension of the peptide separation was performed on an Ultimate^{TM} 3000 chromatography system equipped with a Bai Probot MALDI spotting device (Dionex Corp, Sunnyvale, CA). Six individual SCX fraction pools were injected and captured onto a trap column (1× 8mm) from Michrom Bioresources Inc (Auburn, CA) and then eluted onto an RP –C_{18} capillary column (300 µm ×150 mm) purchased from Dionex Corp (Sunnyvale, CA) with a gradient of buffer B (buffer A, 0.1% TFA, 5% acetonitrile, 95% H_2O; buffer B, 0.1% TFA, 95% acetonitrile, 5% H_2O). Column effluent was mixed automatically in a 2:1 ratio with MALDI matrix (saturated alpha-cyano-4-hydroxcinnamic acid) with a probot MALDI spotting device, and spotted directly on MALDI plate. MALDI plates were analyzed on an ABI 4700 Proteomic Analyzer from Applied Biosystems (Framingham, MA).

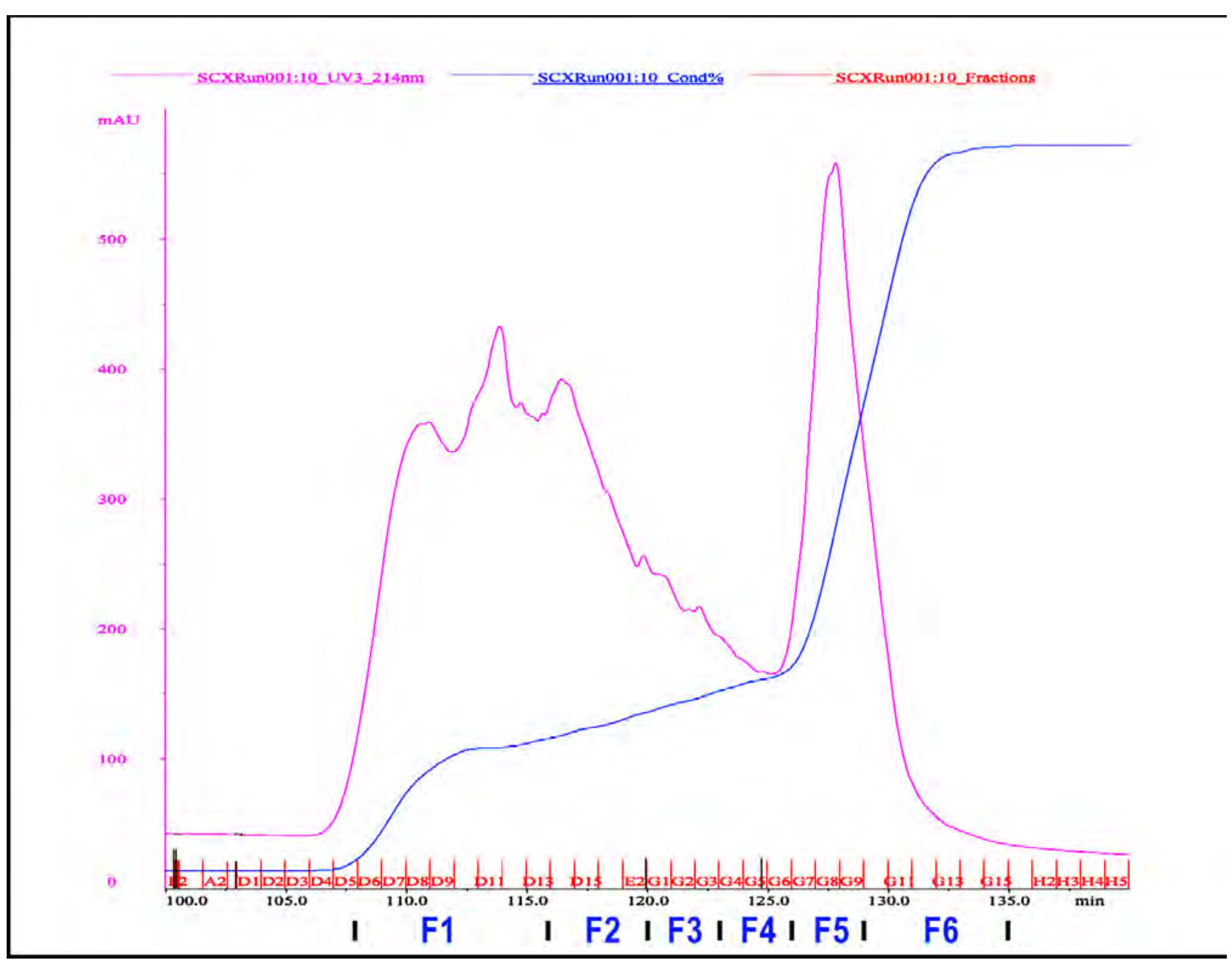
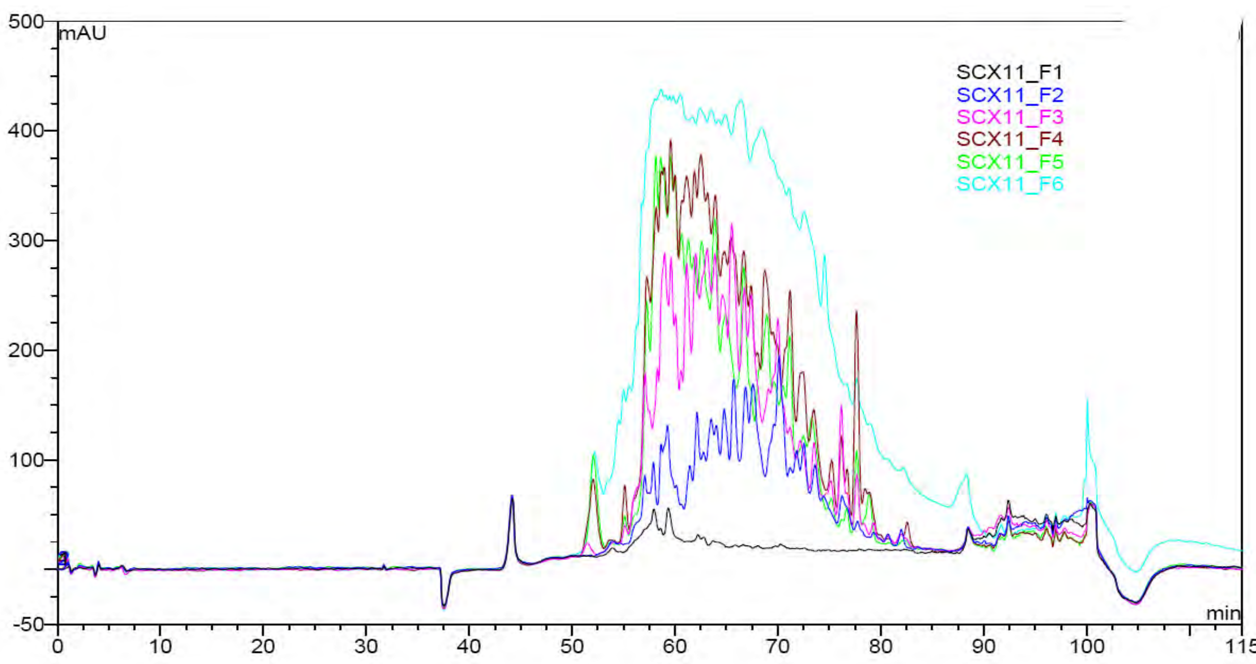


Figure 2. SCX chromatogram of iTRAQ-labeled digest of Stratum 01, showing absorbance at 214 nm (purple), the KCl gradient (blue, 0-700mM) and the 6 fraction pools (F1-F6) subjected to capillary HPLC and spotting onto 6 TOF/TOF target plates.

In the development of this step, the slope of the gradient was optimized so that approximately equivalent amounts of peptide ions would be detected in each of six fraction pools (Fig. 3). This process entailed testing of each gradient variation by capillary HPLC spotted onto the TOF/TOF targets, followed by data acquisition and comparison of the data sets. Each SCX fraction is deposited onto one target of 192 fractions. Optimization of the SCX chromatography was followed by optimization of the capillary HPLC gradient to distribute the peptides as evenly as possible across the TOF/TOF targets.



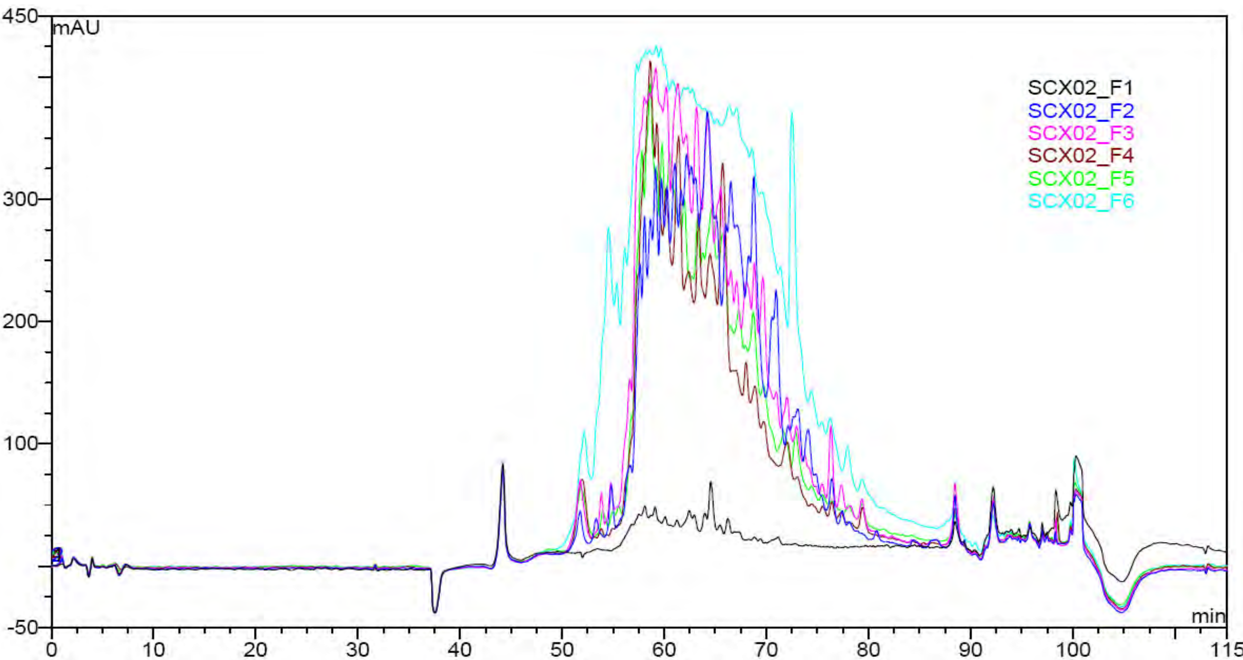


Figure 3. Improvement of sample distribution among fractions from capillary LC. (Upper panel) Uneven amounts and distribution of peptides from each of the 6 SCX fraction pools. (Lower panel) More uniform amounts and distribution of peptides from each of the 6 SCX fraction pools after optimization. Fractions eluted between 50-85 min from each LC run were spotted onto a single 192-well MALDI target plate (6 plates per stratum).

A detailed analysis of the mass spectrometry data obtained from the HPLC of 1 pooled SCX fraction revealed that 82% (1,071) of the 1,313 precursor ions that were selected for MS/MS contained the iTRAQ reporter ions. This clearly shows that the improvements in our protocol have been optimized.

Table 1. Percentage of precursor ions selected for MS/MS containing iTRAQ reporter ions (m/z 114, 115, 116). Data represents 1 pooled SCX fraction (combination of F3 & F4) obtained from the HPLC on-target spotting of samples from stratum 2. The majority of peptides elute between 55-80 minutes (see Figure 3) where 82% (1,071) of the 1,313 precursor ions that were selected for MS/MS contained the iTRAQ reporter ions.

		HPLC Retention Time (min)	**# precursor ions selected for MS/MS**	**# precursor ions selected for MS/MS containing iTRAQ reporter ions**	**%**
SCX F4	**A1-A24**	50-55	115	4	3.48
SCX F3	**B1-B24**	55-60	270	228	84.44
SCX F3	**C1-C24**	60-65	362	303	83.70
SCX F4	**D1-D24**	65-70	341	289	84.75
SCX F4	**E1-E24**	70-75	110	107	97.27
SCX F4	**F1-F24**	75-80	230	144	62.61
SCX F4	**G1-G24**	80-85	172	81	47.09
SCX F4	**H1-H24**	85-90	128	33	25.78
		Total	1728	1189	68.81

(c) Data processing. Data analysis was performed using in-house software (Du P, Angeletti RH. Automatic deconvolution of isotope-resolved mass spectra using variable selection and quantized peptide mass distribution. Anal Chem., 78:3385-92, 2006; P Du, R Sudha, MB Prystowsky, R Hogue Angeletti (2007) Data reduction of isotope resolved LC-MS spectra. Bioinformatics, in press) to build arrays with SCX fraction number, retention time, precursor monoisotopic mass of all ions containing the iTRAQ reporter ions (114, 115 and 116), along with their signal intensities. The retention time drift is corrected for by using the retention time of the internal standard peptides. Peptides having similar mass (± 0.1 Da) and retention time (±60 sec) were considered to be the same peptide. Intensity levels of ions at *m/z* 117 were used as indicators of noise level. In analyses of replicate samples of the pool, a high level of reproducibility was observed for all iTRAQ reporter ions having intensities greater than 500 counts (Fig. 4).

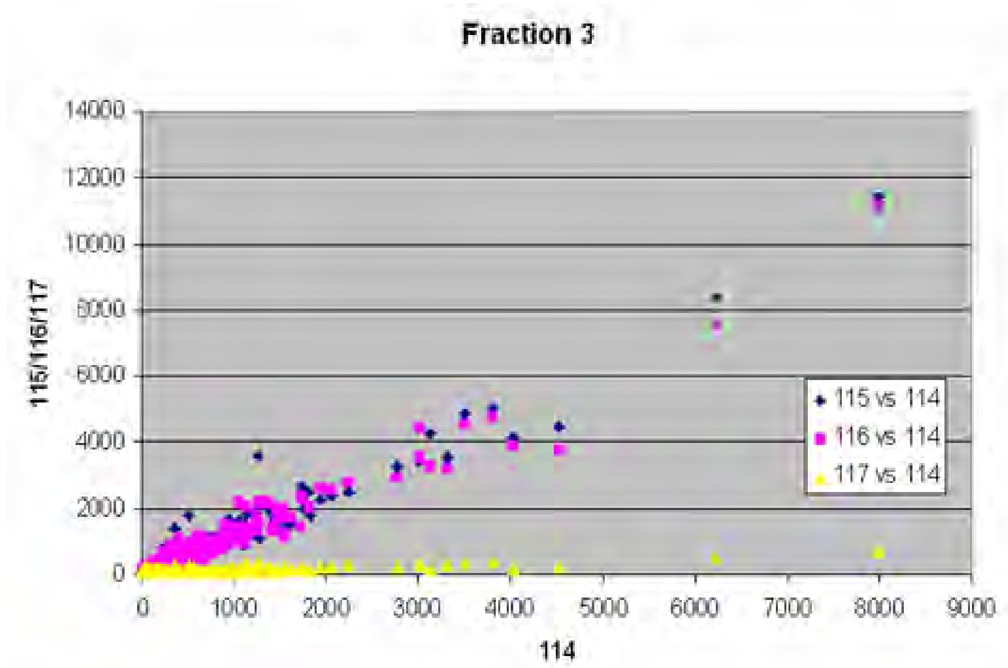


Figure 4. Plot of the iTRAQ reporter ion intensities (*m/z* 114, 115 & 116) of replicate control serum samples. The ion intensity value plot of *m/z* 117 depicts our baseline noise level. Ideally, the ion intensity values from *m/z* 114, 115 & 116 should all be equal to each other. The calculated correlation coefficients are: 114:115, 0.9701; 114:116, 0.9719; and 115:116, 0.9811.

In addition, the data are searched through the TOF/TOF GPS server to identify the sequenced peptides and their parent proteins. As an example from stratum 02 immunodepleted serum, more than 1,098 proteins can be identified with a Confidence Interval (C.I.%) of 95% or greater. It should be noted that in quantitative analysis of serum, most peptides will be unchanged. In the present experiments, that means that in most cases the 114 and 115 marker ions, representing blinded case and case control samples, should be indistinguishable from the 116 marker ion, the pooled control (Figs. 5 and 6). Of the 1,098 proteins identified, 305 proteins are highly relevant to pathways and disease processes and are not in the upper tier of abundant proteins (Appendix 3).

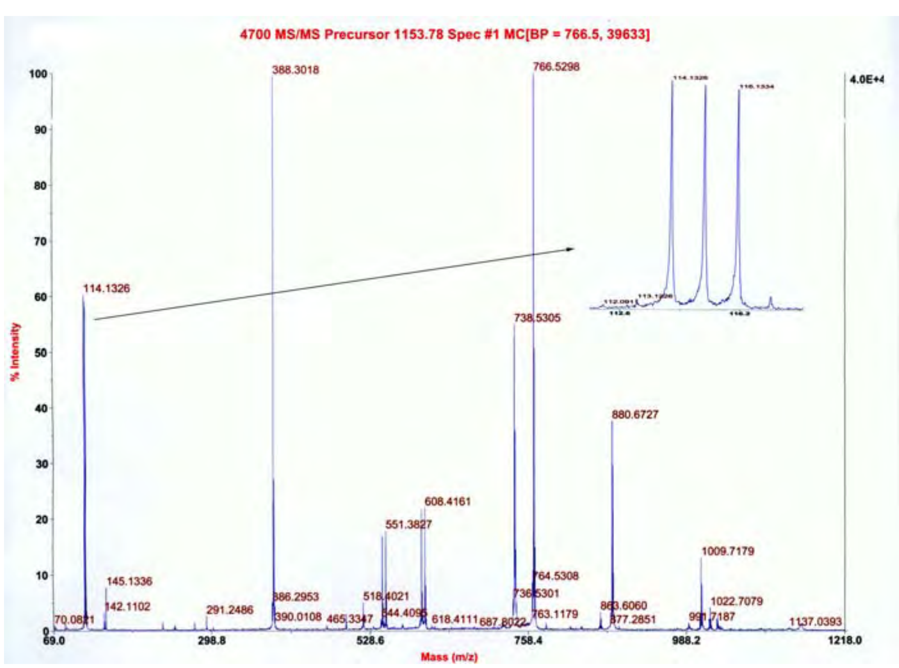


Figure 5. MS/MS spectrum of a peptide ion with approximately equal intensities of the reporter ions for case, case control, and pooled control. The inset is a magnification of the reporter ions.

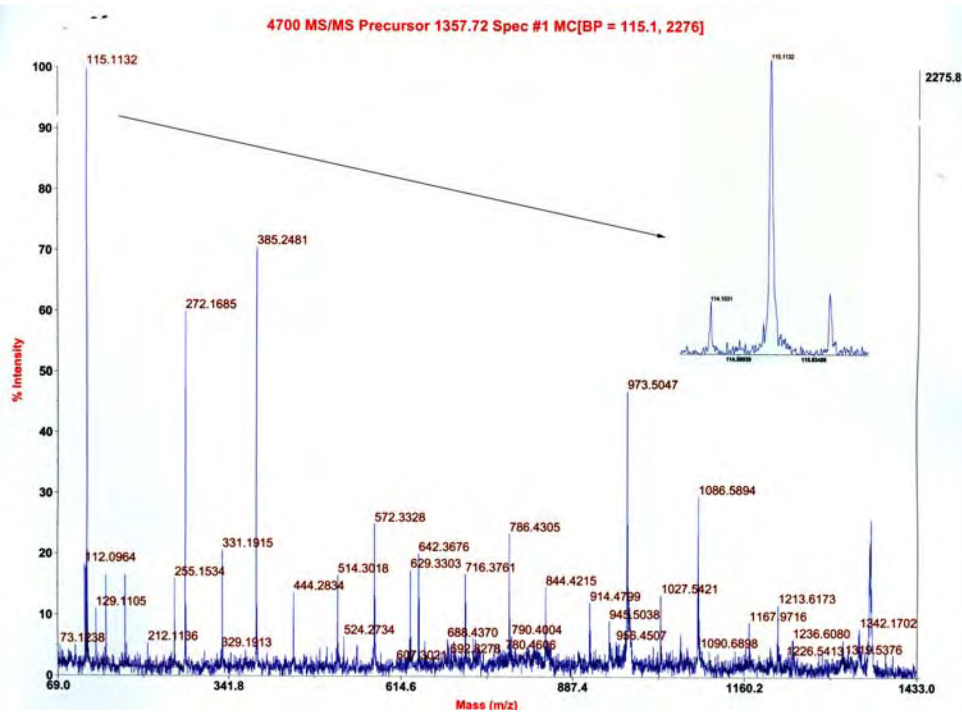


Figure 6. MS/MS spectrum of a peptide ion with an intense 115 reporter ion (inset), indicating the increased expression of this peptide in either the case or case control serum.

(vi) Data and operations management
The process of tracking samples and data throughout the complicated process from the initial immunodepletion phase through the MSMS phase is crucial in this study. Each of the procedures in this process has been documented in an operations manual and checklists have been devised for each step of the process (Appendices 1 and 2) to ensure that all procedures are in place and are adhered to strictly. In order to track the many different test tubes that are produced at each juncture, cryo-labels have been created that clearly identify the current procedure and the sample ID number.

Data are stored on three different computers, each of which is connected to the equipment necessary for each step of the process. Directories have been created in each of these machines to clearly identify project data and these directories are backed up weekly onto a portable hard drive. After analysis of each case-control stratum is completed, the hard drive is copied to the data management servers and data on the hard drive are deleted so that data resulting from the analysis of the next stratum can be backed up.

During the MSMS phase, data files for each of the six fractions are created. These files are then converted into separate T2D files in order to prepare analysis files. An analysis program is run on each of these files and space delimited text files are outputted which contain one line of data for each spectrum in the given fraction. Data included in this file include the spot number (spectrum), M/Z, mascot score, protein ascension number, and the intensity for each sample by peptide. Quality assurance procedures include steps to ensure that the file is created correctly before raw data files are moved. After these interim analysis files are created, all data for each stratum are merged into one file which will be used to create the final analysis file for the study.

Project management is facilitated by a portal web site which has been created for this study and allows all members of the study to collaborate in a shared environment. The portal site hosts a project calendar and a tracking system which allows each member to update the status of each sample as it is completed and document issues that occur. The data manager is automatically notified when this system is updated to ensure that the project is tracked efficiently and monitored appropriately.

Key research accomplishments

We have developed and validated an integrated, highly sensitive, proteomic analysis involving fractionation by immunodepletion and multi-dimensional HPLC, analysis by MALDI-TOF/TOF-MS and sophisticated computational analysis of the data, to tackle the complexity and dynamic range of the serum proteome. We have shown that this procedure identifies proteins that are highly relevant to pathways and disease processes and that are not in the upper tier of abundant proteins. We have established a sophisticated procedure for operations management and quality control.

Reportable Outcomes

So far our publications have been on the development of algorithms for signal processing:
1. Du P, Angeletti RH. Automatic deconvolution of isotope-resolved mass spectra using variable selection and quantized peptide mass distribution. Anal Chem., 78:3385-92, 2006,
2. P Du, R Sudha, MB Prystowsky, R Hogue Angeletti (2007) Data reduction of isotope resolved LC-MS spectra. Bioinformatics, in press)

Conclusions

Using the procedures outlined herein, we are now analyzing the serum samples from the 4th case-control stratum from the training set, and are analyzing the strata at the rate of about one per week (the samples in each stratum consist of serum from a case, the matched control, and the pooled sample). While we are behind schedule, we believe that we have developed procedures that provide the complexity required to uncover new markers of disease. Furthermore, we have initiated the analysis of the actual study specimens at a reasonable rate. The personnel involved are highly trained and familiar with all aspects of the project. We are now in the intense data acquisition phase and they are highly motivated to complete the project. Due to the fact that we are behind schedule, there are two strategies that we could adopt to reduce the time required to complete the project:

(1) we could reduce the sample size for the training phase of the study to 32 case-control pairs. A sample size of 32 matched pairs will still offer over 90% power to detect the 70% sensitivity/95% specificity specified in the original grant, corresponding to a conditional odds ratio of over 11, and over 80% power to detect a conditional odds ratio of 5, which would result from 63% sensitivity and 88% specificity. Thus, if we were to reduce the sample size, the study would still provide adequate statistical power to detect reasonably large differences between cases and controls. Furthermore, reducing the size of the training set to 32 case-control pairs would save approximately 2 months; and

(2) we could abandon the development of immunoassays (Task 5). We would propose instead to analyze the test set of 20 case-control pairs using the same protocol used to analyze the training set. This would not compromise our ability to test the proteomic patterns identified in the training phase to discriminate between cases and controls in the test phase. Once the marker proteins are known, the development of the immunoassays could be performed at a later date, using funding that we would obtain from other sources. We estimate that this would save us at least 6 months.

Previous proteomic studies of breast cancer have used lower resolution proteomic approaches in cross-sectional settings only. By using high-resolution proteomic approaches in a prospective setting, this ongoing project may enhance our ability to both identify those women at increased risk of breast cancer and to intervene before they progress to cancer. Furthermore, it may provide insight into the biological processes underlying breast cancer development, for example, by identifying the protein markers underlying case-control proteomic differences and by leading to identification of the associated candidate genes that might be responsible for differences in susceptibility.

Proteomic Lab Operation Manual

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I. Test tube preparation

A. For the first phase of this study, we will be analyzing 40 case control pairs. Each case control pair has been assigned a stratum number (01-40). Each sample has a unique ID number (1-80). The lab will receive 5 aliquots for each one of the samples. For each case-control pair (i.e. stratum), the lab will also receive a pooled sample. The labeling schema developed for this study identifies each test tube as: STRATUM# _ ID # _ ALIQUOT #
Example: For the first stratum (01), the lab will receive 3 test tubes.
- The first test tube will be labeled 01_1_1. 01 represents the stratum number. The 1 in the middle represents the ID for this sample. The last 1 represents the 1st aliquot for this sample.
- The second test tube will be labeled 01_2_1. 01 represents the stratum number. The 2 represents the ID for this sample. The last 1 represents the 1st aliquot for this sample.
- The third test tube will be the pooled sample for the 01 stratum and will be labeled 01_PL_1. 01 represents the stratum number. The PL represents POOL for this sample. The last 1 represents the 1st aliquot for this pooled sample.

B. A label sheet will be prepared by the Department of Epidemiology and Population Health (DEPH) for each stratum. The sheet will contain all of the labels that are needed for the process. Each label contains the procedure step name (CONC, DIGEST, iTRAQ etc) and the numbers used to identify the sample. The first 2 columns of the label sheet contain the labels for the first aliquot of each stratum. The other 4 columns contain extra labels for the other aliquots in case they are needed. As a rule, only the first 2 columns of the label sheet will be used unless a 2nd aliquot is needed for a particular sample.

II. Immunodepletion
(Removal of major abundant serum proteins by immunodepletion chromatography, 3 serum samples consecutively at a time)

Buffers **Buffer A1**: Phosphate Buffered Saline, pH 7.4, from Sigma (P3813-10PAK, contains 0.01 M phosphate, 0.138 M NaCl, 0.0027M KCl). Freshly prepare before use for the day.
Buffer B1: Stripping Buffer: 0.1 M glycine-HCl, pH 2.5, containing 0.02% sodium azide.
Neutralization Buffer A2: 0.1M Na_2HPO_4-NaH_2PO_4, pH 8.0, containing 0.02% sodium azide.
Column Storage Buffer: PBS containing 0.02% sodium azide.
Filter all buffers through a 0.2 µm sterile membrane filter and store at 4°C, except buffer A1 which need to be prepared freshly each time.

Column 6.4 × 63 mm, affinity column from Genway Biotech Inc (San Diego, CA) Cat. Number : MIXED12-LC12. Now it is sold as 'ProteomeLab-IgY column kits' by Beckman. **The column must be stored at 4°C in column storage buffer. The column must be washed with 3 column volumes of room temperature degassed dilution buffer IMMEDIATELY after it is taken from 4°C storage**. This procedure will prevent air bubble formation inside the column.

Instrumentation
AKTA purifier
Injection loop size: 250 µl
Flow cell (Cat. 18-1147-25): 3 mm pathlength, 0.7 µl
(Check the synchronization of UV tracing and sample collection of the fraction collector)
Syringe: 250 µl syringe from Hamilton

Procedure
1. Bring PBS, Stripping buffer, neutralization buffer and column storage buffer to room temperature and re-dissolve precipitates. (Phosphate precipitates when stored at 4°C). Degas all buffers by purging the buffers with helium for 10 minutes.
2. Turn on AKTA work-station and computer. Wait for system synchronization to complete.
3. Connect the 250 µl loop to the right position.
4. Connect UV light, pH meter, and Conductivity meter to the right position.
5. Run the *equilibrate-system* program under Method, to bring all solutions to running-condition.

6. Switch valve to "inject" position to wash and purge the loop with 2 ml of Buffer A1. Then switch valve to "load" position to wash and purge the loading syringe adaptor and the loop with 2 ml of Buffer A1 from a syringe fitted with the loading needle.
7. Connect the depletion column immediately after brought out from 4°C, and purge with 10 ml of Buffer A1 at 1ml/min. Make sure that no air is introduced to the column.
8. Test the fraction collector tubing to make sure it's not clogged.
9. Dilute 20 µl serum sample with 80 µl PBS and spin at 13,000 rpm for 5 min to pellet insoluble debris.
10. End any run program.
11. Insert 10 1.5 ml microfuge tubes to position A1 to A10 on the 12mm collector plate.
12. Draw up the 100 µl supernatant with 100 µl syringe and inject the sample into the loop. Do not remove the syringe from the injection port until the current run is finished.
13. Using the *Rohan\DepletionRunMethod* program under depletion method saved on the AKTA purifier, start the run.
14. After finishing all runs, wash the immunodepletion column with 3 column volumes of column storage buffer and store the column at 4°C.
15. Pool the flow-through fraction A3 to A8, with absorption at 280 nm into a 15 ml round-bottomed polyethylene tube (with cap), about 2.5 ml. Place the appropriate CONC label on the tube. Put on ice.
16. Save the 3 depletion files for this triplet (case, control and pool) in the Rohan directory. The naming convention for these files should be:
    a. 'DP'_<Stratum#>_<ID#>_<Aliquot#>

       Example: DP_01_1_1
       DP_01_2_1
       DP_01_PL_1
17. When the process is finished, back up the 3 deletion files onto the Rohan portable hard drive in the DEPLETION directory.

III. Trypsin Digestion

(concentration, buffer exchange and trypsin digestion; process & digest the 3 samples from section 1 above simultanously)

Buffers & reagents

5% Triton X-100 containing 0.02% sodium azide, store at room temperature.
50 mM TEAB (triethylammonium bicarbonate), freshly prepared from 1M stock.
TPCK treated trypsin, 20 µg /vial (Promega, Cat. # : V5111), store at -20°C.

Procedure

18. Pre-treat the 5 kDa cut-off Ultra-4 centrifugal concentrator (Millipore, Bedford, MA 01730. Size: 5,000 MWCO. Cat. No. UFC800596, 96pk) with 4 ml 5%

Triton X-100 for 30 min at room temperature, followed by extensive washing with distilled water to remove the Triton X-100.

19. Transfer the pooled fractions from step 15 to the concentrator prepared in step 18.

20. Spin at 4,000 x g for 40 min at 8°C using a swinging bucket centrifuge to reduce the volume to 80 µl or less.

21. Dilute the concentrate with 3 ml of 50 mM TEAB (triethylammonium bicarbonate buffer, Fluka, lot and filling code: 1192744 34005180) in the concentrator to change buffer to trypsin digestion solution (50 mM TEAB).

22. Concentrate as in step 20 to 80 µl or less.

23. Transfer and measure the volume of the final concentrate from step 22 to a 1.5 ml microfuge tube. Make up the sample volume to 80 µl with 50 mM TEAB if needed. Add and mix 20 µl acetonitrile to make the sample containing 20% acetonitrile.

24. Dissolve 1 vial of trypsin in 20 µl of 50 mM TEAB. Each vial contains 20 ug TPCK treated trypsin (Promega, Cat: V5111).

25. Add 5 µl of trypsin from step 24 to the sample from step 23. The total digestion volume is 105 µl.

26. Place the appropriate DIGEST label on each tube.

27. Incubate at 37°C overnight at 400 rpm on a thermomixer.

IV. iTRAQ labeling

(Label the peptides with iTRAQ reagents (label the 3 samples from section 2 above simultaneously, each with a unique iTRAQ reagents)

Buffers & reagents:

100% (absolute, HPLC grade) ethanol

iTRAQ reagents 114, 115, 116 from the "iTRAQ Reagent Multi-Plex Kit" from Applied Biosystem. Store at -80°C.

Procedure

28. ~~Speed-Vac the digest from 27 briefly to reduce the volume to 30 ul. The digest should not allow to dry completely. Add water to make the volume to 30ul if necessary~~. Spin down the digest.

29. Add 105 ul 100% (absolute, HPLC grade, from iTRAQ kit) ethanol to each vial of iTRAQ reagent. Votex to dissolve the reagent completely. Spin briefly to collect all solution to the bottom. Transfer 40 ul of each digest to each corresponding iTRAQ solution vial, vortex each tube to mix, and then spin to collect all solution to the bottom. Incubate the tube at room temperature overnight. Save the rest 60% of digest at -80C.

30. Stop the labeling by adding 420 µl of SCXA1 and allowing excess reagent to completely hydrolyze over an additional 30 min at room temperature. Spin briefly to collect all solution to the bottom.

31. Combine 466 ul (out of 560) of each to a 50 ml tube. Add 4,300 ul buffer A from SCX-cation exchange in the subsequent procedure below. Save the rest 94 (1/6 of the labeled sample) at -80C.
32. Place the iTRAQ label for the current stratum on the tube.
33. The combined and diluted sample (4.5 ml) should be at pH 3.0 and can be stored at 4°C overnight. (It will contain 1 mM TEAB, 4.7% ethanol, 6.7mM potassium phosphate, 16.6% acetonitrile.)

V. SCX-strong cation exchange chromatography

Column

PolySulfoethyl A column (2.1x 100 mm, 5 µm, 300Å, volume≈0.3ml) from Poly LC Inc. Cat. No. 102 se0503
Guard column for the above

Buffers:

Buffer A: 25% acetontrile, 10 mM potassium phosphate, pH 3 (Made by titrating phosphoric acid with 5M KOH to pH 3.0)
Buffer B: 25% acetontrile, 10 mM potassium phosphate, pH 3, 700 mM KCl.

Instrumentation

AKTA purifier
Injection loop (8 ml)
Flow cell (Cat. 18-1147-25) (3 mm path length, 0.7 µl hold up volume)
(check the synchronization of UV tracing and sample collection of the fraction collector).
Syringe: syringe from Hamilton (10 ml), cat. No.: 7650-01; or 10 ml PP/PE lubricants free syringe (10 ml).

Procudure

34. Get ready all solutions
35. Turn on AKTA work-station and computer. Wait for system synchronization to complete.
36. Connect the 8 ml loop to the right position.
37. Connect UV light, pH meter, and Conductivity meter to the right position.
38. Run the *SCX equilibrate-system* program under Method, to bring all solutions to running-condition.
39. Switch valve to "inject" position to wash and purge the loop with 20 ml of Buffer A. Then switch valve to "load" position to wash and purge the loading syringe adaptor and the loop with 20 ml of Buffer A from a syringe fitted with the loading needle.
40. Connect the PolySulfoethyl A column (connect the guard column first) to the right position of the flow path.
41. Wash the column with 2 ml of buffer A at 0.1 ml/min.
42. Test the fraction collector tubing to make sure it's not clogged.
43. End any run program.

44. Insert 1.5 ml tubes (or the LC vials) to collector rows A and B.
45. Draw up the sample from 31 with syringe and load the sample into the loop. Do not remove the syringe from the injection port until the current run is finished.
46. Using the *Rohan\SCXRunMethod* method saved on the AKTA purifier, start the run. Collect the eluted peptide. Combine fractions D6-D14 as F1, D15 to E12 as F2, G1 to G3 as F3, G4to G6 as F4, G7 to G9 as F5, G10 to G15 as F6. Store at 4C if not using immediately.
47. After the run, regenerate the column by running the column with a blank gradient followed by 6 ml of buffer A. Finally, wash the column and the system with at least 20 ml of 20% ethanol and store the column in 20% ethanol at room temperature.
48. ~~Save the fractions that have UV reading at 214nm, about 15-20 fractions in all.~~
49. Save the computer file generated by this process in the Rohan directory and on the portable hard drive in the ITRAC subdirectory. The naming convention for these files should be:
    b. 'iTRAQ'_<Stratum#>_<Aliquot#>
        Example: iTRAQ_01_1

50. ~~Label the 10 tubes containing the fractions with the appropriate SCX labels.~~
51. ~~Store at 4C.~~

VI. RPLC-reversed phase chromatography (Interfaced with Probot automatic spotting)

Columns:

Trap column
    Dionex P/N 6720.0012
Loop
    250 µl
RP C18 column
    Dionex, P/N 160295, 300 um I.D.x, 15cm, static phase: C18, PepMap100, 3um, 100A

Solutions:

Solvent A
    5% acetronitrile, 0.1% TFA
Solvent B
    95% acetontrile, 0.1% TFA

Procedure

52. Speed-Vac each SCX fraction to 100 ul. Adjust to 0.1% TFA by adding 1 ul of 10% TFA.
53. Prespot the 192-well MALDI plates with a-cyno matrix dissolved in 50% acetonitrile, 0.1% TFA at 7mg/ml. Use only the perfectly pre-spotted plates for the subsequent LC over-spotting.
54. Set the Dionex ultimate 3000 and the Probot at working condition

55. Place 6 prespotted 192-well MALDI plate on the Probot.
56. Put the LC sample vials in Autosampler positions RA1 to RA6
57. Start the Sequence program SCX_0X for LC and spotting, each run takes 115 min.
58. When each step is complete, save the computer files generated by this process in the Rohan directory and a second copy on the portable hard drive in the SCX directory. The naming convention for these files should be:

c. 'SCX'_<Stratum#>_<Aliquot#>_'FR'<Fraction#>

Example: SCX_01_1_F1
SCX_01_1_F2
.
.
SCX_01_1_F

59. After all runs, regenerate the column by running the column with a blank gradient followed by 1 ml of buffer A. Finally, wash the column and the system thoroughly with 70% isopropanol and store the column in 70% isopropanol at room temperature.

VII. MS/MS
(ABI 4700 MALDI-TOF/TOF for MS and MS/MS of iTRAQ labeled peptides)

60. On the MSMS computer, create a sub-directory in the Rohan directory for the stratum about to be processed.   The naming convention for this sub-directory should be:

d. 'MS'_<Stratum#>_<Aliquot#>

Example: MS_01_1
MS_02_1
.
.
MS_40_1

61. Begin the MSMS program. The computer will create a sub-directory for each plate (fraction). The naming convention for these directories should be:

- 'MS_S'<Stratum #>_'F'<Fraction #>

Example: MS_S01_F1
MS_S01_F2
.
.
MS_S01_F6

62. Open the ABI4700 software
63. Load the plates in the autoloader, write the slot number
64. Create new spot set for each plate (MS_0X_1_F1,…) using the correct LC/MALDI spot set template

65. For MS calibration, in the Spot Set Manager, select the Calibration spots for Job-Using Run_Specific Methods, choose the Acq Method for MS calibration, choose the corresponding Proc Method.
66. For MS/MS calibration, in the Spot Set Manager, select the Calibration spots for Job-Using Run_Specific Methods, choose the MS/MS Acq Method for MS/MS at 1570.60 m/z, choose the corresponding Proc Method for 1570.60 m/z.
67. For sample run, in the Spot Set Manager, select the sample spot rows fro Job-Using Run _Specific Methods, choose the Acq Method for MS service runs, choose the Proc Method of internal calibration (903 and 2465 m/z).
68. Select the Run job-wide interpretation function. In Cal Types Updates, select None. In the interpretation method, choose the correct methods for MS/MS acquisition and processing (these methods need to be tested and updated every before every batch run). Select Peak Width Number as 2. Parent ion selection from 900 to 3000 m/z.
69. Save each spot set.
70. Correlate spot set to the correct plate number and slot number
71. Load each plate to align the plate position
72. Test the acquisition method, processing method, and interpretation method with the calibration standard peptide
73. Calibrate the mass accuracy.
74. Re-select the newly updated methods for each spot set.
75. Save the spot set again.
76. Submit the spot set job to queue run, observe the validation process
77. Switch the acquisition mode from interactive to batch mode
78. Start the queue run
79. Observe the beginning of the automatic run, pay attention to the signals.

VIII. Data analysis (Protein ID and label ions comparison)

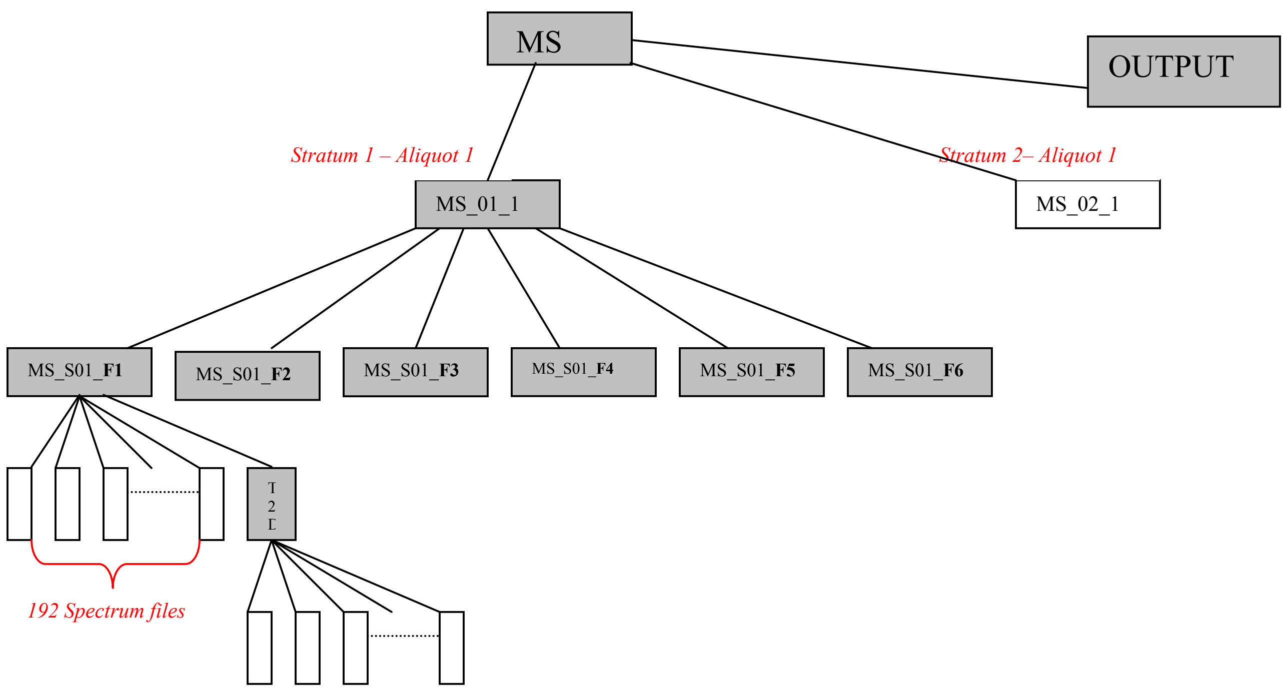
In this step, the spectrum will be analyzed and the volume of the ion peaks of the labels will be compared. At the conclusion of the MSMS run for each stratum, the following procedures will be run.

Procedure

80. Connect the portable hard drive to the MSMS computer, open Windows Explorer, highlight the MS directory name for the stratum(e.g. MS_01_1) and copy the entire MS directory for the current stratum into the MS directory on the portable hard drive. Open the spotset, display MS and MS/MS runs, highlight all rows from A1 to H2, right mouse click on the highlighted rows, select export to folder, select or create the application folder, export as T2D files.
81. Connect the portable hard drive to the Unix machine.
82. On the portable hard drive, create a sub-directory in each fraction directory called T2D. Convert all files in each of the 'Fraction' directories for the stratum T2D files format using the web-based program and place them in the T2D directory for

that stratum. A figure representing the directory structure for each stratum appears below*: (Directories are shaded)*

.



83. A program will be run on all .t2d in a given fraction directory and will create a space delimited text file in the MS/Output directory on the portable hard drive. This file will contain 192 lines (one line for each spectrum in the given fraction). The name of the file will be 'S'<Stratum#>_'F'<Fraction#>pepdata.txt (e.g. S01_F1pepdata.txt for Stratum1/Fraction1) and it will contain the following data fields:

not normalized (Intensity for 114, Intensity for 115, Intensity for 116)

MS2 spectrum data (last four columns)

Fraction #	Spot #	M/Z (peptide mass data)	Intensity for 114	Intensity for 115	Intensity for 116	AA Sequence	Mascot Score	Protein Asc #							
F1	A1														
F1	A2														
F1	A3														
F1	A4														
F1	A5														
F1	.														
F1	.														
F1	B1														
F1	B2														

F1	.														
F1	.														
F1	C1														
F1	C2														
F1	.														
F1	.														
F1	.														
F1	.														
F1	.														
F1	.														
F1	.														
.	.														
.	.														
F1	H26														

84. After the program is run, the .txt file should be opened to confirm that it was created properly.

85. Proceed to run the program on the remaining fraction directories for the given stratum.

IX. Data Management:

A. Data are backed up onto the portable hard drive after each stage of the process (see sections III.17, V.51, VI.58, VIII.64 above). After a complete run on a stratum is complete (approximately once every week to 10 days) , the DEPH will copy the hard drive onto their servers, delete all data on the hard drive and return the hard drive to the lab so that analysis on the next stratum can begin.

B. The DEPH will merge the output files for all fractions for each stratum (i.e. S01_F1pepdata.txt , S01_F2pepdata.txt etc.) into one output file for each stratum (S01final.txt, S02final.txt etc.). A program created by the proteomics lab will be run on these stratum files to create the final analysis file which will contain the following data:

Peptide Mass	Retention Time (Spot #)	Stratum 01 (114) Intensity After Normalization	Stratum 01 (115) Intensity After Normalization	Stratum 01 (116) Intensity After Normalization	Stratum 02 (114) Intensity After Normalization	Stratum 02 (115) Intensity After Normalization	Stratum 02 114 Intensity After Normalization		Stratum 40 116 Intensity After Normalization

Grand Check List

1. Depletion Run Check List-----p2
2. SCX Run Check List------------p5
3. U3000 LC Run Check List-----p6
4. MS/MS Run Check List--------p7
5. Data Processing Check List--p8

Depletion Run Check List (page 1)

Sample________
Date___________
Time___________

- [ ] 1. Prepare the following solutions freshly
  - Buffer A1: PBS. Dissolve 1 bag of sigma PBS powder in 1000 ml of H2O
  - Buffer B1: Stripping buffer: Dilute 20 ml of 10X stripping buffer in 180 ml of H2O
  - Buffer A2: Neutralization buffer: Dilute 20 ml of 10X neutralization buffer in 180 ml of H2O
- [ ] 2. Wash the pump heads with 20% ethanol
- [ ] 3. Turn on AKTA system and computer
- [ ] 4. Connect UV cell, conductivity meter to the correct position
- [ ] 5. Wash the pH meter tip with H2O, connect to the pH cell
- [ ] 6. Connect the correct loop (250 ul) to the correct position, wash the injection hole and check the hole size for compatibility with syringe needle
- [ ] 7. Connect the collection tubing directly to the collection tip, bypassing the accumulator.
- [ ] 8. Make sure that the collection path is not clogged. Wipe the Syncdrop with H2O to clean up any stain on its walls (if the walls are dirty, the SyncDrop message show up and collection will not start). Make sure that the tip is in the middle and is tightly screwed in.
- [ ] 9. Equilibrate the tubing and pumps with 10 ml of H_2O for each of the 4 pumps at 2 ml/min
- [ ] 10. Wash pumps A and B with H_2O, using the built in protocol (PumpWashBasic)
- [ ] 11. Wash the loop with 4 ml of H_2O at 4 ml/min
- [ ] 12. Stop flow if it is on
- [ ] 13. Replace Inlet A1 liquid with Buffer A1, B1 liquid with BufferB1, and A2 liquid with Buffer A2, degas for 5 min
- [ ] 14. Start a DepletionEquiSystem method, to bring solutions up to Outlets
- [ ] 15. Wash the loop with 4 ml of Buffer A1 at 1 ml/min, switch from Load to Injection position twice to remove any trapped air bubble
- [ ] 16. Set flow rate to 0.2 ml/min
- [ ] 17. Immediately after moving the depletion column from 4°C to room temperature, connect the column to the correct position following the correct flow direction.
- [ ] 18. Swiftly purge the column with Buffer A1 at 1 ml/min, watch the pressure (normal pressure=0.1)
- [ ] 19. Connect the column outlet to the system, watch the pressure (normal pressure=0.1)
- [ ] 20. Run 10 ml of Buffer A1 through the column, watch the pressure (0), UV(50 at 214), pH(7.4), Conductivity(?)
- [ ] 21. Add 1.5 ml tubes to the 12mm collector rack at A1 through A9
- [ ] 22. Thaw the serum samples on ice
- [ ] 23. Wash the 100 ul syringe with Buffer A1 3 times, 100 ul each, wipe clean the needle with Kimwipe
- [ ] 24. Draw 100 ul of Buffer A1 to the syringe, purge the air bubbles
- [ ] 25. Assure that the valve is at Load position
- [ ] 26. Inject the syringe content to the loop slowly. Make sure there is no leak at the needle tip and observe the liquid drop in the waste tank. Leave the syringe there
- [ ] 27. Double-check the above items
- [ ] 28. Make a blank depletion run with Buffer A1 that is injected
- [ ] 29. Go to File\Run\Rohan\DepletionRunMethod, click Start, observe the whole run especially at every check point.
- [ ] 30. Most importantly, the collector is on and the collection starts at the right time and in the right tubes. Observe the spectrum
- [ ] 31. After run, examine that each fraction contains 500 ul of run thru
- [ ] 32. Discard the collections from the blank run. Change to new tubes
- [ ] 33. Spin down the sera samples, vortex swiftly
- [ ] 34. Draw 20 ul of serum (Stratum0X-1-1) with 20 ul Pippetman, deliver to a new 500 ul microfuge tube
- [ ] 35. Add 80 ul of Buffer A1, mix and spin at full speed (13,000 rpm) in a microfuge for 5 min

- [ ] 36. with the 100 ul syringe, take 95 ul, avoid disturbing and drawing the top lipid layer. Wipe the needle
- [ ] 37. inject the 95 ul of serum to the loop
- [ ] 38. leave the syringe at inject position
- [ ] 39. Go to File\Run\Rohan\DepletionMethodRun, click Start, observe the whole run especially at every check point.
- [ ] 40. Most importantly, the collector is on and the collection starts at the right time and in the right tubes. Observe the spectrum
- [ ] 41. After run, examine that each fraction contains 500 ul of run thru
- [ ] 42. collect the fractions from A3 to A8, including A3 and A8 (total is 2.5ml)
- [ ] 43. designate the tube as Dp 0X-1-1
- [ ] 44. Label three spin ultrafiltration concentrators as conc0X-1-1, Conc-X-2-1 and Conc0X-pool-1, separately.
- [ ] 45. Discard the H_2O and add 4 ml of 5% Triton-X100 to each tube. Leave them on bench for more than 30 min
- [ ] 46. Draw 20 ul of serum (Stratum 0X-2-1) with 20 ul peppetman, move to a new 500 ul tube
- [ ] 47. Add 80 ul of Buffer A1, mix and spin at full speed for 5 min
- [ ] 48. with the 100 ul syringe, take 95 ul, avoid disturbing and drawing the top lipid layer. Wipe the needle
- [ ] 49. inject the 95 ul of serum to the loop
- [ ] 50. leave the syringe the at inject position
- [ ] 51. Go to File\Run\Rohan\DepletionMethodRun, click Start, observe the whole run especially at every check point.
- [ ] 52. Most importantly, the collector is on and the collection starts at the right time and in the right tubes. Observe the spectrum
- [ ] 53. After run, examine that each fraction contains 500 ul of run thru
- [ ] 54. Collect the fractions from A3 to A8, including A3 and A8 (total is 2.5ml)
- [ ] 55. Designate the tube as Dep 0X-2-1
- [ ] 56. Repeat steps 34 to 42 for sample 01-pool-1
- [ ] 57. Designate the tube as Dep 01-pool-1
- [ ] 58. Spin down the three collection tubes
- [ ] 59. Rinse the three spin concentrator from step (45) thoroughly with H_2O
- [ ] 60. Transfer the 3 Dep samples to the corresponding concentrators
- [ ] 61. Spin the concentrators (+samples) at 4000g for 40 min first (vol. reduce to 80 ul); mix in 3 ml of 50 mM TEAB buffer.
- [ ] 62. Spin again at 4000g for 40 min. Volume must reduce to less than 80ul. Spin once more if needed.
- [ ] 63. Transfer the concentrates individually to three 500-ul tubes labeled iTRAQ0X-1-1, 0X-2-1, and 0X-pool-1.
- [ ] 64. If the volume in the any tube is less than 80, add 50 mM TEAB buffer to make up the volume to 80 ul
- [ ] 65. Add 20 ul of 100% ACN to each tube
- [ ] 66. Dissolve one vial of trypsin in 20 ul of H_2O
- [ ] 67. Add 5 ul of the trypsin solution from step (66) to each sample
- [ ] 68. Vortex and spin down
- [ ] 69. Incubate at 37°C overnight, shaking at 300rpm.
- [ ] 70. Store the digest at 4°C if not labeling directly.

....

Day 2 continues on next page

- ☐ 71. Move iTRAQ reagent 114, 115 and 116, and 100% EtOH to room tesmprature, leave on bench for 10 min, spin briefly (10 sec in a microfuge) to collect the content to the bottom of the tubes.
- ☐ 72. Spin the digests briefly (10 sec. in a microfuge)
- ☐ 73. Quickly add 105 ul of EtOH to 114 vial, vortex briefly (1 min)
- ☐ 74. Quickly pipette 40 ul (40% of the original serum) of digest 0X-1-1 and add to the 114 vial, vortex briefly (20 sec.)
- ☐ 75. Quickly add 105 ul of EtOH to 115 vial, vortex briefly (1min)
- ☐ 76. Quickly pipette 40 ul (40% of the original serum) of digest 0X-2-1 and add to the 115 vial, vortex briefly (20sec.)
- ☐ 77. Quickly add 105 ul of EtOH to 116 vial, vortex briefly (1 min)
- ☐ 78. Quickly pipette 40 ul of digest 0X-pool-1 and add to the 116 vial, vortex briefly (10 sec.)
- ☐ 79. Spin briefly the vials and cover the vials with aluminum foil for overnight incubation at room temperature
- ☐ 80. Store the rest 60% of the digests at - 80°C (Box C3.1., Positions XXX)
- ☐ 81. Store the EtOH at -80°C with the Kit
- ☐ 82. Stop the reaction by add 420 ul of H_2O to each tube (total 560 ul in each tube), spin to pellet the precipitation
- ☐ 83. Take 466 ul of each to a 1.5 ml tube
- ☐ 84. Add 4,300 ul of SCXA1 solution , mix gently, text pH with paper. pH should be at 3.0
- ☐ 85. Save the rest of 94 ul at -80°C freezer Box C3.1. at position XXX.
- ☐ 86. Subject the diluted mixture to SCX fractionation.

SCX Run Check List

Sample_______________
Date_________________
Time_________________

- ☐ 1. Purge the pump heads with 25% ethanol using a 50 ml syringe to remove air bubble
- ☐ 2. Degas the H_2O for more than 5 min
- ☐ 3. Switch inlets to H_2O
- ☐ 4. Connect UV cell, conductivity meter to the correct position
- ☐ 5. Wash the pH meter tip with H2O, connect to the pH cell
- ☐ 6. Connect the correct loop to the correct position, wash the injection hole and check the hole size for compatibility with syringe needle
- ☐ 7. Connect the collection tubing directly to the collection tip, bypassing the accumulator.
- ☐ 8. Make sure that the collection path is not clogged. Wipe the Syncdrop with H2O to clean up any stain on its walls (if the walls are dirty, the SyncDrop message show up and collection will not start). Make sure that the tip is in the middle and is tightly screwed in.
- ☐ 9. Equilibrate the tubing and pumps with 10 ml of H_2O for each of the 4 pumps at 2 ml/min
- ☐ 10. Wash pumps A and B with H_2O, using the built in protocol (PumpWashBasic)
- ☐ 11. Wash the loop with 4- folds loop size (8 ml) of H_2O at 4 ml/min
- ☐ 12. Stop flow if it is on
- ☐ 13. Replace Inlet A1 liquid with SCXA1 and B1 liquid with SCXB1, degas for 5 min
- ☐ 14. Start a SCXEquiSystem method, to bring solutions up to Outlets
- ☐ 15. Wash the loop with 40 ml of SCXA1 at 4 ml/min, switch from Load to Injection position twice to remove any trapped air bubble
- ☐ 16. Set flow rate to 0.1ml/min
- ☐ 17. Connect the SCX Precolumn, watch the pump pressure (0.9 normal pressure)
- ☐ 18. Connect the SCX polyA column to the precolumn, watch the pressure (3.2 to 3.5 normal pressure)
- ☐ 19. Connect the column outlet to the system, watch the pressure (3.2 to 3.5 normal pressure)
- ☐ 20. Run 2 ml of SCX A1 through the columns, watch the pressure, UV, pH, Conductivity
- ☐ 21. Add 1.5 ml tubes to the following position on the 12mm collector rack: A1, A2, A3, D1 to D15, E1, E2, G1 to G15, H1 to H9
- ☐ 22. Bring the iTRAQ labeled digest mixture to room temperature, vortex, spin 1 min at full speed
- ☐ 23. Collect the 450 ul supernatant to 4 ml of SCXA1 in a 50ml tube, mix well gently
- ☐ 24. Wash the 10 ml syringe with SCX A1 3 times, 6 ml each, wipe clean the needle with Kimwipe
- ☐ 25. Draw all 4.50ml sample to the syringe, purge the air bubbles
- ☐ 26. Assure that the valve is at Load position
- ☐ 27. Inject the sample to the loop, slowly but firmly. Make sure there is no leak at the needle tip and observe the liquid drop in the waste tank. Leave the syringe there
- ☐ 28. Double-check the above items
- ☐ 29. Go to File\Run\SCX, click Start, observe the whole run especially at every check point.
- ☐ 30. Most importantly, the collector is on and the collection starts at the right time and in the right tubes. Observe the conductivity change when elution begins
- ☐ 31. After run, examine that each fraction contains 100 ul of elute
- ☐ 32. Pool fractions D6 to D14 into the D6 tube. Label the vial as SCX##F1
- ☐ 33. Pool fractions D15 and E2 into the D15 tube. Label the vial as SCX##F2
- ☐ 34. Pool fractions G1 and G3 into the G1 tube. Label the vial as SCX##F3
- ☐ 35. Pool fractions G4 and G6 into the G4 tube. Label the vial as SCX##F4
- ☐ 36. Pool fractions G7 and G9 into the G7 tube. Label the vial as SCX##F5
- ☐ 37. Pool fractions G10 to H1 into the G1 tube. Label the vial as SCX##F6
- ☐ 38. Store at 4C for next day's SpeedVac processing.

(Items 32–37: This needs to be adjusted each preparation)

U3000 LC Run Check List

Sample_____________
Date________________
Time_____________

- ☐ 1. Move SCX fractions from 4°C to room temperature
- ☐ 2. Spin down drops on tube side
- ☐ 3. Prepare the SpeedVac, wait until the vacume reach the proper level (0.1 atm)
- ☐ 4. Place the tubes balanced in the centrifuge
- ☐ 5. Start Drying process. Stop and check level of sample every 10 min until the sample volume is less than 100 ul
- ☐ 6. Reconstitute the sample volume to 110 with H_2O using a 200 ul pipetman
- ☐ 7. Add to each fraction 1 ul of LC standard peptides (peptide 1900, middle, and peptide 1826, late) which are reconstituted in 20 ul of 50% CAN and 0.1%TFA
- ☐ 8. Add to each fraction 1 ul of 10% TFA, mix and spin
- ☐ 9. Label 6 U3000 autosampler 250ul vials as SCX##F1 to SCX##F6
- ☐ 10. Transfer the fractions to corresponding vial, tape the vial gently to remove trapped air bubbles in vials
- ☐ 11. Place the vials to designated positions in autosampler rack
- ☐ 12. Take 1 vial of matrix (CHCA, 25 mg/vial) from 4°C
- ☐ 13. Dissolve the matrix in 1 ml acetonitrile. Move it to the matrix bottle of the uCarrier dosage unit
- ☐ 14. Add another 1 ml of acetontrile, 2 ml of H_2O, mix by gentle pipetting, add 4 ul TFA
- ☐ 15. Add 10 ul of the MS internal standard peptides (bradykinin and ACTH), mix by pipeting
- ☐ 16. Place the bottle in the bottle holder in the dosage unit, insert the fill tubing to the bottle but keep a 5 mm distance between the bottle bottom and the tubing tip to avoid any precipitation from enterring the tubing
- ☐ 17. Manually purge the syringe, the valves and the spotting needle with the matrix by 5 strokes, be sure no air bubble in the syringe and be sure to switch the valve position prior to each operation
- ☐ 18. Wash 6 readable plates throughly and polish them completely
- ☐ 19. Place the plates in the uCarrier plate holder, write down the plate bar code number in correct order
- ☐ 20. Align the well positions
- ☐ 21. Place a shallow cup to collect the waste from the spotting needle (sample will be save in case of uCarrier failure)
- ☐ 22. Open the application file, and start application
- ☐ 23. Notice the message at the bottom of the application file as "waiting for the signal from the closure"
- ☐ 24. Prepare fresh solutions A and B for HPLC
- ☐ 25. Purge the pumps
- ☐ 26. Change a new trap column
- ☐ 27. Fill the autosampler wash liquid with solution A, be sure no air bubble in the autosampler syringe
- ☐ 28. Fill the R1 bottle with solution A (used for blank runs)
- ☐ 29. Fill the valve washing liquid bottle with H_2O
- ☐ 30. Turn on UV 214 and 254 nm, turn on data acquisition, watch the UV reading (650 mAu at 214 nm)
- ☐ 31. Set flow rat at 40 ul/min for loading pump and 2.0 ul/min for micropump
- ☐ 32. Observe the pressures (90 Value)
- ☐ 33. Start a blank run with R1 solution, 95ul ulPickup injection mode, observe the background
- ☐ 34. Estimate the flow rate at the outlet of UV meter (650 mAu at 214 nm)
- ☐ 35. Connect the outlet of UV to the uCarrier tubing, be sure that liquid comes out of the spotting needle and the pump pressures are not changed
- ☐ 36. Open the sequence editing page, set up a blank run before each sample run
- ☐ 37. Name the blank runs as blankR1, and the sample runs as LC-SCX##F1 to LC_SCX##F6
- ☐ 38. Make sure the correct vial positions for blank and samples are selected
- ☐ 39. Make sure set injection volume to 95ul
- ☐ 40. Make sure the correct program files are chosen for blank and samples
- ☐ 41. Save the sequence in a new folder under Rohan folder. Name the new folder as LC-SCX##. Name the sequence file as LC-SCX##, too
- ☐ 42. Final check: no liquid leak, LC-Spotting tubing connection, nothing block the uCarrier platform from moving, uCarrier power on, syringe filled with matrix, plates are in position
- ☐ 43. Start batch, watch any warning message, OK to start
- ☐ 44. Check the run periodically

MS/MS analysis ckeck list

Stratum________
Date_________
Time__________

- [ ] 1. Load the plates in the autoloader, write down the corresponding slot number.
- [ ] 2. Create a spot set for each plate in LC/MALDI mode
- [ ] 3. Correlate the spot set to the correct plate number and slot number.
- [ ] 4. Load each plate to align the position of the plate
- [ ] 5. Test the acquisition method, processing method, and interpretation method with the calibration standard peptide.
- [ ] 6. Calibrate the mass accuracy.
- [ ] 7. Choose the methods for the automatic runs.
- [ ] 8. Make sure the Chromatogram Peak Width is 2 and mass range in the job interpretation method is from 900 to 3000 m/z
- [ ] 9. Save the spot sets.
- [ ] 10. Submit the spot set job to queue run, observe the validation process.
- [ ] 11. Switch the acquisition mode from interactive to queue run mode.
- [ ] 12. Start the queue run.
- [ ] 13. Observe the beginning of the automatic run, pay attention to the signals.

Data Processing Check List

1. Export the ms/ms data files to the external hard drive as T2D file
2. Open the text conversion program
3. Convert the T2D files into text files
4. Backup the data files to another hard drive for data storage

Appendix 3

Proteins Relevant to Disease Pathways Identified in Stratum (set 2) Sera

1. Sarcometic mitochondrial creatine kinase isoform
2. serpin peptidase inhibitor, clade G (C1 inhibitor), member1
3. ZNF2 protein
4. myosin heavy chain 6
5. asp (abnormal spindle)-like, microcephaly associated
6. Cohen syndrome 1 protein splice variant 1
7. microtubule- associated protein 1a
8. phosphatidylinositol 4-kinase, catalytic, alpna polypeptide isoform 2
9. kinesin-like protein KIF13B (Kinsesin-like protein GAKIN)
10. retinoblastoma-binding protein 6 isoform 2
11. MDN1, midasin homolog
12. protein kinase C-binding protein RACK8
13. nuclear receptor binding SET domain protein 1 isoformb
14. protein kinase SAD1B
15. bromodomain PHD finger transcription factor
16. cdk2
17. zinc finger, CCHC domain containing 11 isoform a
18. Hu-Claspin
19. groth arrest-specific 2 like 2
20. nebulin
21. zinc finger protein 451 isoform 1
22. SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a1 isoform b
23. cyclin-dependent kinase 2 interacting protein
24. serine (or cysteine) proteinase inhibitor, clade (alpha-1 antiproteinase, antitrypsin), member 4
25. insulin-like growth factor binding protein, acid labile subunit
26. fragile X mental retardation-related protein 1 isoform a
27. PTPRF interacting protein alpha 2
28. guanine nucleotide-binding regulatory protein alpha-inhibitory subunit
29. Signal-induced proliferation-associated 1-like protein 1 (High-risk human papilloma viruses E6 onco
30. IQ motif containing GTPase activation protein 1
31. kininogen
32. olfactory receptor, family 5, subfamily P, member 2
33. caspase 1 isoform beta precursor
34. ADAM metallopeptidase with thrombospondin type 1 motif, 9 preproprotein
35. inter-alpha-trypsin inhibitor heavy chain H2 precursor
36. guanylate binding protein1, interferon-inducible, 67kDa
37. JAW1-related protein MRVI1B short isoform
38. retinoic acid inducible in neuroblastoma cells RAINB1d
39. MINDBOMB; ubiquitin E3 ligase

40. eukaryotic translation initiation factor 4 gamma, 3
41. doublesex and mab-3 relatd transcription factor2
42. X2 box repressor
43. laminin alpha 4 chain
44. utrophin
45. ovarian cnacer related tumor marker
46. JNK-associated leucine-zipper protein
47. transcriptional repressor NF-X1 (Nuclear transcription factor, X box-binding, 1)
48. glycerate kinase 2
49. angiotensinogen
50. RNA binding motif protein 19
51. hepatocyte growth factor isoform 3 precursor
52. phosphalipase C, gamma 2
53. down-regulated in metastasis
54. huntingtin interacting protein
55. programmed cell death 6 interacting protein
56. Nesprin-2 (Nuclear envelope spectrin repeat protein 2) (Syne-2) (Synaptin nuclear envelope protein)
57. disrupted in schizophrenia 1 isoform S
58. G-protein coupled purinergic receptor P2Y10
59. protein Tro alpha1 H, myeloma
60. heat shock 105kD
61. GTPase activating Rap/RanGAP domain-like 1 isoform 1
62. serine/threomine kinase 10
63. dopamine receptor interacting protein
64. splicing coactivator subun it SRm300
65. receptor tyrosine phosphatase gamma
66. PDZ and LIM domain 7 isoform
67. GRINL1A combined protein isoform 1
68. vaciolar protein sortin 13c protein isoform 1B
69. zinc finger protein 318
70. ankyrin repeat and IBR fomain-containing protein 1
71. serpin peptidase inhibitor , clade G (C1 inhibitor), member 1, (angioedema, hereditary)
72. TGF beta-induced transcription factor 2-like protein
73. dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3 isoform b
74. BRCA1 interacting protein C-terminal helicase 1
75. kinectin
76. mitogen-activated protein kinase kinase kinase 3 isoform1
77. brease cancer-associated antigen BRCAA1
78. HSPC322, Activator of HSP90 ATPase [Posttranslational modification, protein turnover
79. AHNAK nucleoprotein isoform 1. AHNAK mediates activation of phospholipase C-gamma1 through protein kinase C
80. FLJ00175 protein, protein kinase C conserved region 2 (CalB);
81. RNA binding motif protein 12B,

82. Cullin-7 (CUL-7), Defects in CUL7 are the cause of 3M syndrome
83. epidermal growth factor receptor substrate
84. IQ motif containing GTPase activating protein 1
85. CENP-E protein, CENP-E is a putative kinetochore motor that accumulates just before mitosis
86. cell proliferation antigen Ki-67, short form – human, a new kind of cell cycle-maintaining proteins
87. NF-IL6
88. dedicator of cytokinesis 7
89. kelch-like 17
90. hepatocyte growth factor isoform 3 precursor
91. RNABP2-Like and GRIP domain-containing protein 8 (Ran-binding protein 2-like 1) (RanBP2L1), Sperm membrane protein BS-63
92. Zinc finger and BTB domain-containing protein 24 (Zinc finger protein 450). Alternative splicing; DNA-binding; Metal-binding; Nuclear protein; Repeat; Transcription; Transcription regulation
93. SNF2 histone linker PHD RING helicase
94. Snf2-related CBP activator protein
95. telomerase catalytic subunit
96. ubiquitin specific protease 42
97. Bardet-Biedl syndrome 7 protein isoform a
98. katanin p60 subunit A-like 2, AAA-superfamily of ATPases associated with a wide variety of cellular activities, including membrane fusion, proteolysis, and DNA replication
99. leucin rich neuronal protein
100. vacuolar protein sorting 13C protein isoform 1B
101. T-cell lymphoma invasion and metastasis 1
102. adlican
103. erythroblastosis retrovirus oncogene homologue 2
104. linker for activation of T cells isoform b
105. Kringle domains are believed to
106. HGFL(S) protein, play a role in binding mediators, such as peptides, other proteins, membranes, or phospholipids vacuolar protein sorting 13C protein isoform 1B
107. T-cell receptor beta chain
108. Inositol 1,4,5-trisphosphate receptor type 2 (Type 2 inositol 1,4,5-trisphosphate receptor) (Type 2 InsP3 receptor) (IP3 receptor isoform 2) (InsP3R2).
109. DNA topoisomerase I
110. Haptoglobin-related protein precursor
111. polymerase (RNA) I polypeptide D isoform 1
112. reverse transcriptase related protein
113. Conserved oligomeric Golgi complex component 3 (Vesicle docking protein SEC34 homolog) (p94)
114. Human Notch2, a novel member of cell-fate determining NOTCH family
115. Vertebrate centrosome proteins that share homology with yeast exit proteins are required for cytokinesis and cell cycle progression

116. c-Cbl-associated protein SH3P12
117. human immunodeficiency virus type I enhancer binding protein 2
118. DLG5, play a role in the regulation of enzymes by intramolecular interactions, changing the subcellular localization of signal pathway components and mediate multiprotein complex
119. VprBP protein, HIV-1 Vpr-binding protein
120. latrophilin 3 precursor, adhesion family of G-protein-coupled receptors
121. PI-3-kinase ATX
122. plectin 1 isoform 8
123. ADAMTS-16 precursor (A disintegrin and metalloproteinase withthrombospondin motifs 16) (ADAM-TS 16) (ADAM-TS16).
124. nucler protein GRB1
125. Cytoskeleton-associated protein 5 (Colonic and hepatic tumor over-expressed protein) (Ch-TOG protein)
126. membrane-spanning 4-domains, subfamily A, member 10
127. ovarian cancer related tumor marker CA125
128. voltage gated potassium channels, beta subunit
129. glucosaminyl (N-acetyl) transferase 2, I-branching enzyme isoform B
130. Na/Ca exchanger
131. mitosin
132. trace amine associated receptor 1
133. SMC4 structural maintenance of chromosomes 4-like 1
134. alpha 3 type VI collagen isoform 4 precursor
135. sulfate/anion exchanger
136. CLL-associated antigen KW-10, Identification of novel tumor antigens in CLL by SEREX: Assessment of their potential as targets for immunotherapeutic approaches
137. NUCB2 protein, Serological identification and expression analysis of gastric cancer-associated genes
138. LIM domain only 1, contributes to leukemogenesis in concert with LMO1
139. sug1 [Saccharomyces cerevisiae].
140. voltage-gated calcium channel beta 2 subunit splice variant CavB2cN1
141. DNA polymerase epsilon catalytic subunit
142. membrane associated guanylate kinase, WW and PDZ domain containing 3
143. regulator of G-protein signalling 9
144. dJ1175B15.2 (protein tyrosine phosphatase, non-receptor type 21)
145. matrix metalloproteinase 12 preproprotein
146. programmed cell death 6 interacting protein
147. retinoblastoma protein-binding zinc finger protein isoform a
148. protein phosphatase 1, regulatory (inhibitor) subunit 12B isoform b
149. vacuolar protein sorting 13D isoform 1
150. Trip230, A thyroid hormone receptor coactivator negatively regulated by the retinoblastoma protein
151. tissue specific transplantation antigen P35B
152. histidyl-tRNA synthetase
153. ribosomal protein S6 kinase, 90kDa, polypeptide 5 isoform a

154. dopamine receptor interacting protein
155. tubby related protein 1 TULP1, members of the novel tubby gene family and their possible relation to ocular
156. dihydrouridine synthase 2-like, SMM1 homolog,
157. novel human tRNA-dihydrouridine synthase involved in pulmonary carcinogenesis
158. Inter-alpha-trypsin inhibitor heavy chain H2 precursor,
159. glioma-associated oncogene homolog 1
160. fizzy-related protein homolog
161. intersectin 2
162. interleukin-1 receptor-associated kinase 4
163. phospholipase C, zeta 1
164. Gag-Pro-Pol protein
165. ribonuclease P (30kD) (RPP30)
166. MutS homolog 4, MutS protein initiates DNA mismatch repair by recognizing mispaired and unpaired bases embedded in duplex DNA and activating endo- and exonucleases to remove the mismatch;
167. nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta isoform a
168. gp200-MR6, The gp200-MR6 molecule which is functionally associated with the IL-4 receptor modulates B cell phenotype and is a novel member of the human macrophage mannose receptor family
169. beta-spectrin 2 isoform 2, A novel isoform of beta-spectrin II localizes to cerebellar Purkinje-cell bodies and interacts with neurofibromatosis type 2 gene product schwannomin
170. Rho-associated, coiled-coil containing protein kinase 1
171. receptor tyrosine phosphatase gamma
172. SALL4, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice
173. adenylate kinase 6
174. golgi apparatus protein 1
175. beta adrenergic receptor kinase 1
176. cell proliferation antigen Ki-67, short form
177. Kinesin-like protein KIF13B (Kinesin-like protein GAKIN)
178. huntingtin interacting protein 1
179. DNA topoisomerase I
180. CENP-E , a putative kinetochore motor that accumulates just before mitosis
181. caspase 1 isoform beta precursor
182. Na/Ca exchanger
183. nucleolar protein 8
184. dynein, cytoplasmic, heavy polypeptide 1
185. A-kinase anchor protein 9 isoform 3
186. DNA directed RNA polymerase II polypeptide A
187. MINDBOMB; ubiquitin E3 ligase
188. myosin IG
189. spermatid-specific linker histone H1-like protein

190. src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites
191. RBAP2, a novel retinoblastoma-protein-associated protein similar to the transcription regulator TFII-I
192. alpha1A-voltage-dependent calcium channel
193. Protein kinase N1
194. differentiation antigen
195. Fas-binding protein Daxx
196. brain-specific angiogenesis inhibitor 3
197. membrane metallo-endopeptidase
198. suppressor of cytokine signaling 5
199. SOX2=SRY DNA-binding domain
200. AHNAK nucleoprotein isoform 1
201. voltage gated potassium channels, beta subunit
202. ATP binding cassette, sub-family A (ABC1), member 13
203. B3GALT2, encompassing the hereditary prostate cancer (HPC1) locus
204. lung adenoma susceptibility 1-like protein
205. DEK oncogene
206. testis expressed sequence 10
207. 5' nucleotidase, ecto
208. epidermal growth factor receptor substrate
209. CPRP1, a novel gene related to the proliferation of tumor cells
210. T-cell lymphoma invasion and metastasis 1
211. X2 box repressor
212. gigaxonin, Gigaxonin interacts with tubulin folding cofactor B and controls its degradation through the ubiquitin-proteasome pathway
213. synovial sarcoma, X breakpoint 1
214. Pituitary tumor-transforming 2
215. calbindin 1
216. kinesin family member 18A
217. Heommbor asnaep-ieansss]ociated guanylate kinase-related 3 (MAGI-3)
218. ITF-2 DNA binding protein
219. calcium/calmodulin-dependent serine protein kinase (MAGUK family)
220. growth arrest-specific 2 like 2
221. Inhibitor of nuclear factor kappa-B kinase subunit alpha (I kappa-B kinase alpha)
222. Transcriptional repressor NF-X1 (Nuclear transcription factor, X box-binding, 1)
223. RAB6-interacting protein 2 isoform delta
224. psaroptieeinns k]inase, DNA-activated, catalytic polypeptide
225. ATP-binding cassette, sub-family A (ABC1), member 1
226. Bloom syndrome protein
227. angiomotin like 2, a protein that binds angiostatin, a circulating inhibitor of the formation of new blood vessels (angiogenesis).
228. Tax1 (human T-cell leukemia virus type I) binding protein 1
229. nuclear receptor co-repressor N-CoR. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex

230. Mig-6=mitogen-inducible gene mig-6 product
231. familial Alzheimer's disease protein 1
232. suppressor of cytokine signaling 5
233. disrupted in schizophrenia 1 isoform S
234. Extracellular matrix protein FRAS1 precursor
235. A kinase (PRKA) anchor protein 2
236. Germ cell associated 2 (haspin)
237. CD2 surface antigen
238. phospholipase C, gamma 2
239. fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
240. phosphatidylinositol-3-phosphate/phosphatidylinositol 5-kinase, type III isoform 2
241. pigment epithelial-differentiating factor
242. cardiomyopathy associated 4 isoform 1
243. anchor protein
244. Polycystic kidney and hepatic disease 1 precursor (Fibrocystin)
245. cyclin E1 isoform 1
246. Iron-responsive element-binding protein 2 (IRE-BP 2) (Iron regulatory protein 2) (IRP2).
247. suppressor of potassium transport defect 3
248. von Willebrand factor precursor (vWF) [Contains: von Willebrand antigen 2 (von Willebrand antigen II)]
249. breast cancer-associated antigen SGA-72M
250. breast cancer antigen NY-BR-1.1
251. dystrophin
252. Ran-binding protein 2-like 3 (RanBP2L3)
253. death associated protein 5
254. Mad2B protein, mitotic spindle checkpoint
255. PTPL1-associated RhoGAP 1 (PARG1)
256. Sec23-interacting protein p125
257. EGF-like, fibronectin type III and laminin G domains isoform 2
258. skeletal muscle ryanodine receptor
259. thyroid hormone receptor interactor 8
260. chorea-acanthocytosis
261. NMDA receptor regulated 1
262. apoptosis related protein 3 isoform b
263. potassium inwardly-rectifying channel J8
264. ryanodine receptor 2 (cardiac)
265. spermatogenesis-related protein 7
266. ksianpaiseen-interacting substance of 220 kDa
267. killer cell lectin-like receptor subfamily A, member 1
268. CD14 antigen precursor
269. huntingtin
270. Transmembrane protein 60

271. Ectonucleotide pyrophosphatase/phosphodiesterase 1 (E-NPP 1) (Phosphodiesterase I/nucleotide pyrophosphatase 1) (Plasma-cell membrane glycoprotein PC-1) [Includes: Alkaline phosphodiesterase I ; Nucleotide pyrophosphatase (NPPase)]
272. endothelial cell apoptosis protein E-CE1
273. calcium/calmodulin-dependent protein kinase IV
274. ATP/GTP-binding protein
275. myotubularin-related protein 1
276. Traf2 and NCK interacting kinase, splice variant 2
277. N-myc
278. sarcoma antigen NY-SAR-22
279. Rho-interacting protein 3
280. DNA-binding protein 5
281. Rap1 guanine-nucleotide exchange factor; Epac
282. squamous cell carcinoma antigen 2
283. myeloid/lymphoid or mixed-lineage leukemia 2
284. metastasis-associated protein 2
285. PTH reponsive osteosarcoma B1 protein
286. retinoblastoma protein-binding zinc finger protein isoform a
287. Lymphoid-restricted membrane protein (Protein Jaw1)
288. MAP/microtubule affinity-regulating kinase 4
289. proliferating cell nuclear protein P120
290. metallothionein-like 5, testis-specific isoform a
291. PTK2B protein tyrosine kinase 2 beta
292. CTCL tumor antigen L14-2
293. telomeric repeat factor 2
294. guanylate cyclase 2F
295. cancer-associated gene protein
296. Growth arrest-specific 1 [
297. tumor protein D52-like 2 isoform d
298. lung cancer-related protein 8
299. apoptotic protease activating factor 1
300. B-cell CLL/lymphoma 6 (BCL6) protein
301. CDC27 protein
302. fetal and adult testis expressed transcript protein
303. GTP-binding protein alpha q subunit
304. RAB3 GTPase-activating protein
305. CD22 protein