

## 39.1

**Isolation of Intact Mitochondria from Tissue****P. Bell, R. Ignacio, and M. Benton**

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The isolation of mitochondria from tissues is typically time-consuming and labor-intensive. Pierce has developed a Mitochondria Isolation Kit for Tissue that circumvents these bottlenecks. Two methods were designed for the isolation of intact mitochondria from both soft and hard tissues. The first is a unique reagent-based procedure that enables multi-sample processing of up to six samples concurrently. The second is a Dounce homogenization procedure that processes one sample at a time, but requires 50% fewer strokes than traditional protocols. Following initial tissue homogenization, a series of proprietary buffers were used to promote cell lysis. The mitochondria were subsequently separated from the cytosolic fraction using differential centrifugation, maximizing the yield of mitochondrial protein while minimizing damage to the integrity of the organelle. Purity of the isolated mitochondria was evaluated by western blot analysis of LAMP2 and PMP70, protein markers used to monitor cross-contamination from lysosomes and peroxisomes, respectively. A 50% reduction in these common contaminants was found in a heavy versus a light mitochondrial fraction. Integrity of the mitochondria was assessed by assaying Cytochrome C in the cytosolic fraction. Release of cytochrome C was found to be minimal. Both the Dounce and reagent-based procedures could be completed in less than one hour. The isolated mitochondria were used in several downstream applications, including 1DE and 2DE western blotting and mass spectrometry analysis.

## 39.2

**Biotinylation and Isolation of Cell Surface Proteins for Western Blot Analysis****M. Benton, R. Ignacio, J. Loeb, and P. Bell**

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Cell fractionation reduces the complexity of samples and greatly simplifies proteomics research. Cell surface proteins represent a key subset of the cell, most notably due to a high concentration of integral membrane proteins. These proteins play major roles in signal transduction, cell adhesion, and ion transport and serve as common pharmacological targets. We have optimized a procedure for the isolation of this important group of proteins using cell surface biotinylation followed by affinity purification. This methodology was used for the identification of proteins using 1-D Western blot analysis. Cultured mammalian cells were first labeled with Sulfo-NHS-SS-Biotin, lysed with a mild detergent and biotinylated proteins affinity purified using NeutrAvidin™ resin. The captured proteins were subsequently eluted with nearly 100% efficiency using SDS-PAGE sample buffer containing 50 mM DTT. The protocol was optimized for diverse cell lines, including NIH 3T3, HeLa and C6, with minimal contamination by intracellular proteins. This procedure has utility in differential expression analysis between treated and untreated cells or between two or more cell types.

## 39.3

**Detection and Quantification of Proteins Using a Novel Fluorescent Dye****T. Berkelman**

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Protein detection and quantification are most commonly performed by dye binding, utilizing the ability of some dyes to bind to proteins non-specifically with an accompanying change in the spectral properties of the bound dye. Fluorescent dyes with this property are now widely used for protein analysis due to the high detection sensitivity and dynamic range achievable using fluorescence.

A novel dye (Flamingo Pink) has been identified that has properties well-suited for non-specific quantification of proteins. At low pH, it is essentially non-fluorescent in the absence of protein, but acquires strong fluorescence when protein-bound. The excitation maximum of the protein-bound dye is 510 nm, and is thus suitable for excitation with laser light. This dye also has significant UV absorbance, making it fully compatible with UV transillumination. To investigate the utility of Flamingo Pink for protein quantification in gels, fixed 1-D and 2-D PAGE gels were stained with a solution of the reagent and imaged with both UV-transilluminator-based and visible laser-based fluorescence imagers. Limit of sensitivity, linearity with respect to protein load, protein-to-protein variability and compatibility with subsequent MS analysis were all evaluated and compared with respect to other fluorescent staining methods. The utility of Flamingo Pink for solution quantification of protein was also investigated. When used as a gel stain, Flamingo Pink allows protein detection down to 0.25 ng and linear quantification from 0.5 to 500 ng. Its protein-induced fluorescence enhancement is only minimally affected by detergents or carrier ampholytes, rendering the dye particularly useful for the solution-phase quantification of samples that have been prepared for proteomic analysis, or for quantifying proteins that have been separated on 2-D gels. The staining technique is fully compatible with peptide mass fingerprinting by MALDI-MS.

39.4

### Discover Multiple Facets of the Innovative Phenyx Platform and Achieve Increased Confidence in Protein Identification

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Phenyx is a software platform for the identification and characterization of proteins and peptides from mass spectrometry data, specifically designed to meet the concurrent demands of high-throughput MS data analysis and dynamic high quality results assessment. Phenyx uses a client/server approach with modular architecture. The Phenyx MS/MS core calculation unit incorporates OLAV, a published family of true statistical scoring models. Each derived scoring scheme can be tailored through a learning phase for all kinds of instruments, instrumental set-ups and sample treatments. For PMF data, Phenyx includes Aldente, a scoring approach developed at the Swiss Institute of Bioinformatics. The collaboration with the SIB allows us take full advantage of the annotations (Post-translational modifications, amino acid mutations and protein splice variants) in Swiss-Prot and TrEMBL, thus allowing an even greater degree of pertinence to results. Phenyx can be handled via a Java interface (Phenyx Virtual Desktop), Web-based interfaces or even command lines. In order to manipulate Phenyx files and executables and to incorporate them in a proteomics workflow (e.g. LIMS), a battery of open source Perl modules is also available. Phenyx is available in several versions. The standalone version (Linux or Windows) allows user to install Phenyx locally within his own secure environment, with the possibility to search any .dat (Swiss-Prot native format) or .fasta formatted protein and nucleotide databases. The public version, accessible via secure login and password, is hosted on a server at the Vital-IT computing center. Phenyx accepts peak list data formats from major MS hardware and software providers, including the new mzData standard and mzXML format. Its interactive interfaces provide a number of innovative features, including the possibility to flexibly define your own protein cleavage rules and AA modifications, perform comparisons of identification runs, dynamically recalculate scores as a function of peptide validation status and easily export Phenyx tables to Excel. Scripts can be utilized to extract quantitative information (i.e. from iTRAQ labeling experiments). Phenyx also enables scientists to evaluate the pertinence of their results; A peptide match conflicts-resolution algorithm provides color-coded interpreted results to accelerate the validation. The software also gives the option to import results from other software such as Mascot and Sequest. It also offers statistical tools to graphically evaluate the calibration status of an instrument, the homogeneity of a complete run and the global pertinence of peptide and fragment ion assignments.

39.5

### High Recovery Liquid Chromatographic Methods for Protein Fractionation

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Protein sample prefractionation methods are emerging as useful approaches to reducing sample complexity prior to MS or integrated multi-dimensional LC/MS protein identification methods. We describe the use of LC methods that have been usefully applied to the fractionation of complex protein samples, prior to enzymatic fragmentation and MS analysis. Multiple affinity LC methods have been applied to the depletion of high abundance proteins from biological fluids (serum, plasma, CSF, etc.) obtained from a variety of species (human, rat and mouse), in a variety of circumstances, including human disease states, such as cancer, or induced genetic abnormalities (mouse knock-out disease models). Recently, this approach has been combined with downstream whole protein fractionation using reversed-phase LC methods designed to maximize protein recovery, thereby reducing the risk of biasing differential expression proteomic analysis. We present examples of the combination of these two LC modes to soluble protein fractionation, in such a way that the dynamic range of protein detection and identification is extended to the ng/mL level.

We have also found that appropriate reversed-phase HPLC methods can also be applied to the fractionation of samples that have previously been thought to be intractable - integral membrane proteins. Effective methods for the extraction, reversed-phase separation, recovery, fragmentation and MS-based identification of integral membrane proteins are exemplified by analysis of membrane raft protein preparations obtained from human and mouse neocortex and from HeLa cells.

39.6

### Unique Multivariate Approach to Biomarker Discovery Using New Technology for Analysis of Proteomics Data

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 and W. Dracup

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Traditional analysis of 2D gel electrophoresis (2DE) images relies on sophisticated spot detection algorithms to reduce the amount of information present on images to what is considered important to the researcher. Inevitably, this can and will result in the loss of faint discriminatory spots which fall below the detection limit. Moreover, the process of defining spot outlines and their subsequent matching across images can introduce bias and inconsistencies that can result in inaccurate comparisons and missing values at a later stage of the analysis.

We have developed a novel technology based on multivariate statistics that allows researchers to fully exploit all the information present in images and/or traces. This new technology has been applied to various types of proteomics data in order to achieve a statistically robust analysis. Results will be presented that show the power of this multivariate approach when applied to proteomics data in the identification of areas of interest. These areas are highly predictive of classification and serve as potential biomarkers. In addition, we will discuss how this technology can be applied to a wide variety of scientific data to provide a multiplexed cross technique analysis approach to biomarker localization and identification.

39.7

## An ISO9001 Industrialized Platform for Yeast Two-hybrid Screening

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In order to study protein-protein interactions on a large scale, Hybrigenics has developed efficient protocols and a proprietary Laboratory Integration Management System (LIMS) enabling the industrialization and semi-automation of yeast two-hybrid (Y2H) library screens. This platform (ISO9001) allows the screening of up to 2,500 Y2H screens per year in a cost-effective manner and retains the high quality standards that have been described originally (Rain *et al.*, 2001, *Nature* 409:211–215) and more recently with the *Drosophila* protein interaction map (Formstecher *et al.*, 2005, *Genome Research* 15:376–384). Typically,  $5.10^7$  interactions are tested for each screening of a bait with a highly complex random-primed cDNA fragments library of  $10^7$  independent clones. Using highly complex cDNA libraries to ensure a comprehensive representation of expressed mRNAs, exhaustively screening these cDNAs for interactions, and sequencing all prey clones are critical technical steps. These ensure detection of interactions with over 90% reproducibility and enable the computation of a level of confidence (Predicted Biological Score, PBS®) for each detected interaction. This score predicts the probability of an interaction to be specific. In addition, identification of the interacting domain on the prey proteins (SID®) may be useful for functional analysis.

39.8

## Biomarker Validation Using ProteusLIMS™; the Proteomics Lab Information and Scientific Data Management System

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The complexity of proteomic samples and experiments results in massive amounts of data generated that are collected at a faster pace than researchers have the ability to validate, interpret, and integrate them with other known data. Utilizing proteomics technologies for biomarker discovery and validation is very difficult without an integrated software platform to track lab information and sample genealogies, manage workflows and integrate data from instruments and bioinformatics tools.

Concerns regarding experiment traceability and long-term reproducibility are increasing, and researchers require a method of warehousing data for biomarker validation and data mining. ProteusLIMS will automate, manage, integrate and analyze lab and scientific data in academic and pharmaceutical research laboratories. Proteomics techniques, instrument data, laboratory management tools and software are integrated into a single, open informatics platform. Secure web access is facilitated to post scientific results for communication among collaborators.

Labs can streamline workflow, ensure quality and increase throughput while cost effectively managing resources and tracking results. ProteusLIMS is a flexible platform that pulls together disparate data pools that exist within a lab into one common interface to ensure project data is saved in one platform, organized by project and sample, allowing researchers to search similar environmental, experimental or results data across a set of samples. Data from several separate laboratory locations can be merged and synchronized, allowing collaborators to share results and data instantly. A key objective of the ProteusLIMS platform is to allow researchers to work across other “omics” data sets, resulting in a systems biology approach. The software platform is more than a laboratory workflow solution. It provides a comprehensive integrated lab, instrument and scientific data management solution into a sophisticated analytical system to speed scientific discovery.

39.9

### IgY-Immunoaffinity Separation and Detection; Divide and Conquer the Proteome

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Avian IgY antibodies (Immunoglobulin isolated from egg Yolk) are the ideal immunoaffinity reagent alternative to IgG. IgY has unique and advantageous features, which include strong avidity, high specificity, low background, and efficient production. IgY antibodies have been demonstrated useful in various types of immunoassays, especially in screening for discovery and validation of drug targets or biomarkers. Polyclonal IgY antibodies covalently coupled to microbeads are specifically effective to remove highly abundant proteins from plasma, serum, CSF, urine, or other body fluid or cellular sources. IgY-12 is a composition of IgY microbeads designed for one-step removal of the 12 most abundant proteins in human serum or plasma: albumin, IgG, transferrin, fibrinogen,  $\alpha$ 1-antitrypsin, IgA, IgM,  $\alpha$ 2-macroglobulin, haptoglobin, apolipoproteins A-I and A-II, and orosomucoid ( $\alpha$ 1-acid glycoprotein). IgY-12 microbeads in spin or liquid chromatography (LC) columns were used to treat five types of plasma or serum samples. Sample preparation and protein separation were monitored using SDS-PAGE, 2-dimensional electrophoresis, multidimensional liquid chromatography, mass spectrometry (LC-MS/MS), and clinical protein assays. The results demonstrated high specificity of protein separation, with removal of 95–99.5% of the abundant human proteins. In addition, IgY microbeads can selectively remove orthologous proteins of other mammals, such as monkey, mouse and rat. A composition of IgY-R7 was designed to remove top 7 abundant proteins in rodent. Results also confirmed that IgY microbeads are useful in capture and detection of proteins for proteomic screening and profiling.

39.10

### CodeLink™ Polymer Slides for Multiplex Protein Array Applications

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GE Healthcare is developing a slide for multiplex protein array applications. The slide is based on the CodeLink™ 3D polymer matrix used for CodeLink expression bioarrays. Users will be able to generate their own unique protein arrays in 16-assay format with 144 spots per array. The slide allows arrays of consistent spot quality and minimal background for ELISA-based and 2-color differential protein expression profiling. An easy to follow guidebook with reference to appropriate reagents will aid users from sample preparation through analysis resulting in high quality and reproducible data. In house, we have demonstrated high reproducibility (CV < 20%), sensitivity down to 10 pg/mL and a dynamic range of 2–3 orders of magnitude. No post-labeling purification is required during sample preparation for 2-color differential experiments. In addition, assays are amenable to commonly used automation systems. Protein bioarrays represent an enabling tool with immediate applications in disease profiling and drug discovery with a potential in diagnostics. For researchers looking to exploit the utility, speed and multiplexing capabilities of protein bioarrays to look at protein profiles, GEHC has the solution with the slides and reagents. Use of the slide will become a valuable tool in identification of key signature sets from complex samples such as cell culture and serum, and lead to target model identification and validation.

39.11

### Rapid Screening of pl-separated Protein Samples from a MicroRotofor Using the Experion Automated Electrophoresis System

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The Experion Automated Electrophoresis System with the Pro260 Analysis Kit provides size and quantitative data on 10 protein samples in ~30 minutes, with sensitivity similar to Coomassie stained polyacrylamide gels. Here, we demonstrate the use of the Experion System for rapid analysis of protein samples obtained from the new Bio-Rad MicroRotofor. The MicroRotofor allows for separation and recovery of up to ~5mg of proteins by their pI. The use of the Experion System for electrophoresis of samples separated on the MicroRotofor can be used to generate a "virtual" 2-D gel from a small percentage of the sample. The remainder of the samples can then be further analyzed by mass spectrometry or other approaches.

An important consideration with use of the Experion System is the compatibility of the sample buffer with the electrophoresis. In the Experion System, the samples are electrokinetically injected and electrophoresed down a 10 micron deep microchannel which is 1 cm long and 50 microns across. Ideally, one would prefer to use a sample in its present buffer rather than having to exchange into a buffer more compatible with the Experion system. It is widely recognized that samples in buffers with too high of salt concentration will not be efficiently injected and electrophoresed by the Experion system, resulting in low sensitivity or other anomalous results. What is less recognized are pH effects of the buffer on the electrokinetic injection and separation process. Initial analysis of MicroRotofor samples on the Experion system which were in rotofor buffer (3.5 M urea, 1 M thiourea, 2% CHAPS, 2% Biolytes) indicated that proteins in the highly acidic pH fractions were not seen following electrophoresis, presumably due to lack of electrokinetic injection of the sample. Modifying the pH of the sample by simple addition of basic pH Tris buffer to the sample allows for proper injection and separation of the proteins, even in the presence of the high salt rotofor buffer.

39.12

### Target Profiling Aiding the Drug Discovery Process

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Small molecule inhibitors of protein kinases have gained considerable importance as a new class of ATP-competitive therapeutics within signal transduction therapy. Independent of whether a broad spectrum inhibitor or a compound selective for just one target is intended, the target profile provides valuable information during a drug development process. We have developed an efficient proteomics method to identify the cellular targets of protein kinase inhibitors. The method, known as KinaTorTM, is based on optimised affinity chromatography using immobilised protein kinase inhibitors and subsequent analysis by nano-LC-MS/MS. Target profiling can be applied to monitor selectivity at various stages throughout a lead optimisation program. This technology has proven useful in different stages of the drug-discovery process, beginning with hit-class evaluation, being furthermore useful to create a specific selectivity panel for lead optimisation, as well as being finally helpful to check pre-clinical candidates for potential toxicity problems. With a series of CDK9 inhibitor compounds, increased potency could be well correlated with a gain in target selectivity. For a set of quinazoline derivatives (two approved drugs emerged from this compound class, namely Iressa and Tarceva), minor changes in the substitution pattern of the molecular periphery yielded in a discriminating profile of kinase binding partners. In addition, by the detection of alternative targets, our method was successfully applied to re-direct drug discovery optimised for the treatment of one initial indication towards the development of anti-cancer drugs. Starting from such a privileged structure, an efficient shift to a new indication was possible, initiating a viable starting point for a new lead optimisation process.

39.13

### A Device to Ease Rapid In-gel Digestion

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We developed a device, to operate a three step rapid and simple in-gel digestion protocol. Mass spectra demonstrate that peptide yield and spectrum quality are increased. For operation, a thermo shaker and a laboratory micro centrifuge are required. The device features are carried out within 60 min working and 15 min handling time. Complete sample preparation is possible in one self-contained reaction tube whereby multiple transfer steps are omitted, and the risk of sample contamination and sample loss is minimized. We demonstrate that the novel protocol results in better signal intensities in mass spectrometric analysis of peptides. Utilizing the model protein GAPDH we show that sequence coverage is increased, small amounts of protein, and long and/or highly hydrophobic peptides are detected.

39.14

### Multidimensional Capillary Electrophoresis Separation Interfaced with Mass Spectrometry for Bioanalytical Drug Characterization

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Many therapeutic proteins, including products isolated from human blood, recombinant human cytokines, and recombinant growth factors, are exposed to structural changes or modifications when administered to patients. In some cases, the altered protein may lose efficacy and/or produce unwanted clinical conditions. Therefore, the development of safe and effective engineered and non-human protein therapeutics is crucial to the continued expansion of the biopharmaceuticals industry. Equally important is the development of better assays to quantify changes or modification of proteins used for therapeutic purposes.

This presentation describes the technological advances that have brought the application of capillary electrophoresis (CE) to the forefront of protein research, including the identification of molecular and structural changes of proteins, various degradation pathways, and the monitoring of co- and posttranslational modifications. The goal of CE is to overcome and improve the technological limitations of other methodologies that are still employed in many laboratories engaged in protein studies. The design and operation of a CE instrument that provides both multistep separation and assay of proteins and peptides are reported. The instrument contains a series of solid-phase, microextraction devices fabricated for use in on-line, affinity capillary electrophoresis. Different molecular-recognition affinity adsorbents were covalently bound to a solid support in order to capture specific proteins or peptides present in biological fluids. The captured protein or peptide is eluted with a small plug of a desorption buffer and capillary electrophoresis is performed on the eluted compound(s) within the separation capillary in a sequential mode, one capillary at the time. The separated compound(s) are monitored using one or an array of detectors, including mass spectrometry, as they exit the capillary. This instrument promises to be a powerful asset as an automated protein analyzer for the study of structural changes and modifications affecting the large majority of therapeutic proteins and peptides.

39.15

### Free Liquid IEF Pre-fractionation in the MicroRotofor™; Improved 2-D Resolution of Low Abundance Proteins in Microscale Sample Volumes

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The effective study of low abundance proteins by 2-D PAGE often requires a pre-fractionation step. Sample pre-fractionation reduces the overall complexity of the sample, and enriches low abundance proteins relative to the original sample. Proteins that may originally have been undetectable are thus rendered amenable to analysis. Removal of higher abundance proteins prevents them from obscuring the lower abundance proteins and allows a more effective 2-D separation by limiting the precipitation and smearing that are a consequence of higher protein loads. An efficient and reproducible micro-scale pre-fractionation technique is necessary for samples with limited availability.

Here we demonstrate that free liquid IEF pre-fractionation of mouse liver protein extract in the MicroRotofor system enhances resolution and increases the visibility of low abundance proteins in specific regions of a 2-D gel. Temperature control during separation results in improved run-to-run reproducibility. A 2.5 ml sample of mouse liver protein extract was pre-fractionated into 10 discrete samples using a customized pH gradient in the MicroRotofor system. Sample separation was conducted at a constant 20 °C and was completed in less than three hours. Individual fractions were separated by 2-D electrophoresis using narrow pH ranges for the first dimension. These separations were compared to identical separations of unfractionated sample. Analysis of the gels with PDQuest software showed improved resolution of low abundance proteins.

39.16

### Monitoring the Expression, Purification, and Processing of GST-tagged Proteins with Bio-Rad's Experion Automated Electrophoresis System

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Expression and purification of recombinant proteins is prerequisite for the structural and functional characterization of these proteins, including proteomic studies concerned with protein-protein interactions. Over the past 20 years, affinity chromatography has become the predominant approach to obtaining these target proteins in sufficiently high purity and quantity for subsequent analyses. Among the widely-used affinity tags, the glutathione S-transferase (GST) sequence has been demonstrated to be able to facilitate the overexpression of some recombinant proteins in soluble form. However, the 26-kDa GST tag may sometimes interfere with the target proteins' structure and/or function, and its removal thus becomes desirable. We have used Bio-Rad's recently launched Experion Automated Electrophoresis Station and the Experion Pro260 Analysis Kit to monitor the expression of GST-tagged proteins in *E. coli* cell cultures, and to analyze the protease digestion/removal process for optimizing the target protein purification scheme. Our results demonstrate that the Experion system offers high quality data comparable to the SDS-PAGE method, while it eliminates the tedious process associated with traditional gels. This microfluidic system integrates protein separation, detection, and data analysis within a single platform. It provides a convenient and accurate way to obtain qualitative and quantitative information on protein samples, which is valuable to downstream proteomic applications.

39.17

### Biomarker Discovery and Validation in One Step; Combination of High Throughput Monoclonal Antibody (MAB) and Mass Spectrometry Technologies

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It is an important principle that the discovery of novel, disease specific protein biomarkers can speed the complex process of drug discovery and validation, as well as facilitate the identification of the optimum patient population for subsequent clinical trials. To date these efforts have been hampered by the lack of an efficient biomarker discovery platform coupled with an effective validation process. Although two popular approaches of biomarker discovery mass spectrometry based protein profiling and systems biology exploration of multiple biomarkers can generate disease relevant candidates, these markers are generally based on abundant proteins and lack true disease specificity. Thus, such activities are rarely translated into a bedside clinical assay. The bottleneck is in the validation/qualification of relevant candidates with sensitive, reproducible and easily applicable clinical assays.

The centerpiece of clinical protein biomarker assays is the availability of high quality antibodies, as these can be applied both in conventional ELISAs, or in antibody array formats for disease specific pattern analysis.

We describe a proprietary platform that combines high throughput monoclonal antibody-based global disease specific analyte profiling technology with mass spectrometry-based determination of antibody specificity. In a single step, differential epitope profiling of the protein analyte is obtained using our 10K mAb library or custom made disease/condition specific libraries in terms of the immunogenic space of a given complex sample, in conjunction with protein IDs. Large-scale mAb profiling has the sensitivity and efficiency of the ELISA assay. The mABs derived from discovery programs can be readily incorporated into the biomarker development process of pharmaceutical industry partners in the short time necessary for today's rapid product development cycles.

39.18

### Mild Tissue Dissociation and Subcellular Fractionation Sample Preparation Tools, Facilitating Functional Investigations of Proteins

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Increased awareness of cellular proteins as potential drug targets, or biological markers, necessitates the development of standardized sample preparation procedures. Apart from the dynamic expression level, post-translational modifications of proteins and the subcellular localization are features that are extremely informative for elucidating protein function. Besides investigations using tissue culture cells as model systems, the analysis of tissue samples with medical relevance is of high interest. Such samples are more complex, and contain structural material that may not be the target of the analysis. For such analysis, standardized tissue dissociation and cellular extraction and separation procedures are still a bottleneck.

The objective of this study was to develop robust sample preparation methods that facilitate protein analysis at the subcellular level for both tissue culture cells as well as tissues. To selectively isolate membrane proteins in their native state from both sample types, a scalable 2-step extraction procedure could be set up. Furthermore, a convenient subcellular extraction scheme was developed for extraction of cells, yielding four subproteomes enriched in (a) cytosolic, (b) membrane and membrane organelle-localized, (c) soluble and DNA-associated nuclear and d) cytoskeletal proteins. Robustness and versatility of the procedures, that do not require ultra-centrifugation, are demonstrated by independent methods, including immunological and enzyme assays. In addition, a reproducible and convenient method to disintegrate fresh tissues was developed and shown to work in concert with the subcellular protein extraction schemes. The applicability to study spatial changes of signaling molecules demonstrates the suitability of these sample preparation tools for functional protein investigations. The methods have recently become available under Calbiochem's ProteoExtract™ product line.

39.19

### First Functional Protein Microarrays for Multiplexed Investigation of Cancer Mechanisms

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One of the most significant challenges facing cancer biologists and drug developers today is the need to investigate the function of large numbers of proteins in a multiplexed format. Sigma-Aldrich Inc., has partnered with Procognia Ltd., to introduce the first functional Human protein microarrays specifically for the elucidation of cancer pathways. Fundamental to this technology is a proprietary BCCP fusion tag that ensures the arrayed proteins are folded correctly and remain functional on the array surface. This new technology enables the functional characterisation of many proteins in a parallel, miniaturised and multiplexed manner.

Two protein function arrays have currently been developed using this technology, p53 SNP variant array and cancer protein array. The p53 microarray (Part No, HPFM1) contains wild type human p53 alongside 48 germline SNP variants commonly identified in tumours. In many cancers, mutations in p53 result in reduction or loss of the normal tumour suppression function (1).

The cancer microarray contains a set of 129 Human proteins known to be implicated in cancer (2). These genes and their corresponding proteins have roles in cell proliferation, differentiation, death and DNA-repair processes.

These arrays have been demonstrated to be extremely powerful tools in conducting multiplex assays for protein interactions with; proteins, DNA and nucleic acids, together with enzyme assays. These arrays have significant implications in accelerating new discovery in cancer through the identification new pathways and lead compounds in drug discovery.

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39.20

### Protein Prefractionation, Passing Phase or Forward Looking Approach?

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The challenge in proteomics is not the efficiency in mass spectrometry but rather the associated chemistry and sample preparation approaches. Shotgun proteomics, starting with total protein digestion, does not only dramatically increase the level of sample complexity but also the correlation to the proteins from which the peptides are derived is lost. Fractionation at protein level reduces the complexity and offers the advantage to correlate identified peptides with “parent” proteins. Furthermore, efficient protein prefractionation with high loadability is an essential tool to tackle the dynamic range issue. The subdivision of samples into more manageable fractions results in improved protein identification rate and sequence coverage. In order to utilize the full separation power of LC techniques, combinations which are both orthogonal and compatible with each other, must be selected. Direct compatibility, without any intermediate reconditioning steps, is a key prerequisite for on-line coupling of the different dimensions.

We present results allowing automated and unattended prefractionation of proteomics samples at the protein level. Examples of protein prefractionation by combination of orthogonal chromatography methods as affinity, ion-exchange and reversed-phase chromatography were performed. The main reason for choosing an on-line strategy is the demand to maintain a high recovery and to gain reproducibility. The sample is kept in a closed system under well defined conditions which provide stability and minimizes sample losses. Collected protein fractions were digested and further analysed using peptide MDLC (Multidimensional liquid chromatography) MS/MS analysis for identification and characterisation of proteins. A comparison of comprehensive MDLC on the protein and peptide level to the MudPIT shotgun approach will be presented and discussed.

39.21

## Developments in TOF/TOF Technology Applied to Proteomics

**M. Hornshaw**

Applied Biosystems, Warrington, United Kingdom

The aim of this presentation will be to present an overview of a novel MALDI TOF/TOF technology and its application to proteomics. A MALDI TOF/TOF instrument was used for this work that provides substantially higher overall ionization and transfer efficiency than was available previously. Coupled with this improved mass spectrometric performance a number of software enhancements have been made to allow intelligent use of the major feature of LC-MALDI MS/MS workflows, the decoupling of LC from MS and MS/MS. Several examples of the use of this novel TOF/TOF technology in proteomics workflows will be described briefly. For example, amine-specific, isobaric stable isotope labelling of digested proteins coupled with multidimensional liquid chromatography followed by MS/MS by MALDI TOF/TOF was used to compare the protein content of Alzheimer disease tissue, with Parkinson's disease, Lewy body dementia and control tissue. A significant number of protein expression differences were observed which will hopefully serve to better elucidate the mechanisms of action of these dementias. Other examples will be described.

39.23

## EpiTags; a Scaleable Approach for Building Binders and Assays to Study the Proteome

**J. Tonkinson, C. Williams, T. Nadler, and N. Gordon**

Epitome Biosystems, Waltham, Massachusetts, USA

Multiplex immunological methods (antibody arrays) provide great potential for proteome analysis. Unlike other proteomic methods, antibody arrays allow simultaneous, quantitative analysis of multiple known gene products at a high level of sensitivity and specificity. However, the promise of antibody arrays has been slow to develop due to the lack of unique antibodies and robust assay systems. Currently available tools are insufficient for proteome analysis due to a lack of menu choice complexity and due to difficulties in taking measurements on complex protein samples. We have developed a robust and comprehensive approach for multiplex protein analysis that combines the ability to generate antibodies to all proteins in a predictable and scaleable manner with a quantitative peptide-based assay format. Antibody content generation begins with a computational approach to identify unique, antigenic peptide sequences (EpiTags™) within proteins that can serve as immunogens (7–10 amino acids). The antibodies produced to the tags are then used for multiplex measurements on planar or bead based arrays. The test sample is digested enzymatically to produce a relatively homogenous peptide mixture where fragments containing the EpiTags™ are easily accessible to bind to their respective antibodies. Quantification occurs by generating a standard curve with the synthetic peptide. Precise targeting of antibody reagents to different portions of the protein sequence enables measurement of total protein as well as post-translational modifications. Using this system we have developed arrays to quantitatively assess phosphorylation of intracellular signaling proteins and biomarker levels in serum.

39.22

## Cryptomics; Identification of Bioactive Fragments from Intact Proteins

**L. Ilag**

Cryptome Pharmaceuticals, Melbourne, Australia

Most proteomic technologies are focused on cataloguing different proteins and protein interactions. However, most of the activities are buried within intact proteins. Novel activities buried in the proteome, which is of limited size, leads to significant increases in the numbers of regulators of biological function, and thus, therapeutic opportunities. It is now clear that a number of biological activities that regulate the cardiovascular system, the coagulation process, the complement cascade and endocrine signalling reside within (cryptic) fragments or *crypteins* of other proteins. A variety of naturally occurring crypteins have been discovered by detection of novel bioactivities.

Thus, it is apparent that evolution has perfected the use of crypteins as a mechanism to regulate protein function through a limited number of genes. It is also very likely that the human cryptome contains a plethora of undiscovered crypteins involved in regulating disease processes yet to be discovered and representing potential human therapeutics. It is also possible that additional crypteins in search of function or have not yet been subjected to the forces of natural selection reside in the cryptome.

A systematic approach of finding crypteins in vitro will be described. It involves a reiterative process of systematic fragmentation, screening and identification of functional crypteins focussing on (but not limited to) the human cryptome. Novel crypteins with anti-coagulation and anti-proliferative properties were identified using the aforementioned approach.

39.24

### Integration of Databases, TRANSFAC®, TRANSPATH®, and Proteome, Is the Comprehensive Resource to Study Protein Functions

A. Kel, N. Voss, M. Tillberg, P. Stegmaier, O. Kel-Margoulis, F. Schacherer, and E. Wingender

Biobase GmbH, Wolfenbuettel, Germany

The databases: TRANSFAC®, TRANSPATH® and Proteome Databases, containing various protein functional and structural information have been developed and are maintained by accurate and high-throughput curation of the scientific literature. TRANSFAC® is a database containing detailed information on transcription factors and their binding sites in the target genes; TRANSPATH® gives information about various functional properties of a large amount of known proteins involved in signal transduction pathways in the cell; Proteome Databases consider all known proteins in several eukaryotic organisms and give extensive information about molecular functions of the proteins, protein-protein interactions, gene ontology assignment, disease information, mutant phenotypes, tumor and cell type expression. All the databases contain manually curated protein classifications according to their structural and functional characteristics. Such classifications are indispensable resources for creation of the computational methods for automatic functional characterization of new proteins found in various organisms. Based on the manual annotation of transcription factors stored in the TRANSFAC® database, we developed a library of hidden Markov models (HMM) to represent their DNA-binding domains and use it for a comprehensive classification. An automatic classification tool based on a genetic algorithm has been developed to obtain the most discriminative library of HMM models. We also implemented the ArrayAnalyzer™ tool to enable causal interpretation of microarray gene expression data using integrated protein functional annotation. Integration of TRANSFAC® and TRANSPATH® with the Proteome databases empowers possibilities to use disease-related information and drug target and other functional information in the context of signal transduction and regulatory pathways.

39.25

### Quantitative Immunodetection Using Infrared Technology

M. Krings

Li-Cor Biosciences GmbH, Bad Homburg, Germany

A new technology for sensitive and quantitative 2-colour immunodetection - the Odyssey® Infrared Imaging System - has been developed by LI-COR® Biosciences. The system combines unparalleled sensitivity in fluorescence detection with precise quantification and provides new perspectives for quantitative analysis of immuno-stained samples. The Odyssey® is unique in that it combines two independent infrared (IR) laser/detection systems for simultaneous detection of two targets: e.g. in signal transduction experiments both total protein and phosphorylated protein can be simultaneously detected and quantified.

In-Cell Western – a cell-based assay - provides a new tool for drug discovery and signal transduction research. This application enables antibody mediated protein detection and quantification in the whole cell environment. Again two targets can be simultaneously detected in a microplate format and normalised for cell numbers in each well. Consequently this technique is well suited for high throughput quantitative analysis of protein modifications in fixed cells. Compared to traditional Western Blots, experiments are carried out in a 96 or 384 well format, saving time while gaining accuracy: Time consuming and error-prone steps such as lysate preparation, gel loading, electrophoresis and membrane transfer are eliminated with In-Cell Westerns.

This flexible and powerful imaging system can also be used for analyzing EMSAs, Protein Arrays, Tissue Section & Small Animal Imaging as well as for documentation of 1D/2D-Coomassie Gels.

39.26

### Protein Biomarkers for Inflammatory Disease Dissection

C. Labeur, K. De Cremer, L. Vanneste, F. D'hondt, G. Thomas, K. Kas, and L. Krols

Peakadilly, Zwijnaarde, Belgium

We describe the use of our clinical biomarker discovery engine in a proteome-wide quantitative analysis of the inflammatory status in mouse serum. Inflammation was induced by IV injection of 800 µg Lipopolysaccharide (LPS)/mouse. Sera were collected 15h post-injection, pooled and depleted for abundant proteins. Samples were prepared for N-terminal differential COFRADIC analysis. The LPS sample was labeled with the O18 isotope during trypsin digestion, the control serum (PBS injected mice) was kept in non-isotopically labeled water. Samples were mixed in a 1:1 (w:w) ratio and analyzed both in MS and MS/MS modus. The ratio of the peptide intensity of the O18/O16 mono-isotopic mass reflects the relative abundance of each protein in the original sample. Our proteome analysis identifies close to 1200 unique mouse proteins, 157 of which are differentially regulated: 37 proteins were identified as O18 singles (only present in the LPS sample), 50 proteins were highly up-regulated, 20 proteins were unique to the PBS sample (O16 singles) and 50 were down-regulated. We confirmed differential regulation using experiments where the O18/O16 label was swapped.

Besides the expected markers (acute phase proteins, LPS- and cytokine receptors, anti-oxidant proteins, lipocalins, . . . ) we discover and validate novel processing in known proteins (belonging to the complement cascade). On top, we characterize a number of novel candidate protein biomarkers. Strikingly, this analysis unequivocally proves that blood mirrors disease processes and that identification of novel diagnostic and therapeutic biomarkers in blood is now reality. An extended survey of changing proteome profiles in this inflammation model will contribute significantly to our understanding of systemic inflammation, sepsis and septic shock.

39.27

### MPEP MALDI Chips for High Sensitivity and Throughput Peptide Analysis by MALDI-TOF MS

M. Lutz and K. Reihls

Sunyx, Cologne, Germany

Over the last decade MALDI-TOF MS (matrix assisted laser desorption and ionization mass spectrometry) has been evolved into a primary tool in modern biochemical analysis. In addition to improvements of MS instruments, MALDI-TOF MS performance can be largely increased “upstream” in the proteomics workflow. This can be predominantly achieved by optimizing preparation techniques using novel plate surfaces and spot deposition techniques. Sunyx recently developed a novel sample plate for MALDI MS peptide analysis (MPep Chip) featuring pre-deposited matrix spots of CHCA ( $\alpha$ -cyano-4-hydroxycinnamic acid).

With these ready-made and easy-to-use plates, high sensitivity analysis of peptides by MALDI-TOF MS down to the low attomolar range can be reproducibly obtained. The MPep Chip also provides superior reproducibility within a spot and from spot to spot due to the homogeneous structure of the matrix spots. The single-use, disposable targets reduce cross contamination and allow archiving and revisiting of samples. This technology ideally supports offline LC-MALDI-TOF MS.

39.28

### Reproducible Sample Complexity Reduction for 2D Gel and MALDI-based Biomarker Discovery

C. Lynch<sup>1</sup>, M. Lopez<sup>1</sup>, E. Golenko<sup>1</sup>, A. Mikulskis<sup>1</sup>, S. Kuzdzal<sup>1</sup>, D. Bennett<sup>2</sup>, and W. Patton<sup>1</sup>

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Objective: Reproducible sample complexity reduction is an essential first step in biomarker discovery experiments. Analysis of low molecular weight peptide biomarkers in serum and plasma has been extremely difficult due to the vast number of contaminating species present. These contaminants pose problems for separations methods as well as MS detection systems. High concentrations of lipids and salts can suppress the ionization of peptides biomarkers in MALDI-TOF MS analysis. 2-D gels cannot effectively separate proteins below 10 kilodaltons.

Methods: Human brain tissue samples were fractionated using ProXPRESSION™ Acidic and Basic Kits (PerkinElmer, MA) and separated on 2-D gels. Serum samples were processed using ProXPRESSION™ Biomarker Enrichment Kits (PerkinElmer, MA) and analyzed by MALDI O-TOF MS (ProTOF 2000™, PerkinElmer SCIEX).

Results: Efficient, reproducible, microscale fractionation for complex protein mixtures is demonstrated using patented membrane absorber chromatography technology (Vivascience AG) combined with PerkinElmer buffers and elution chemistries. Vivascience’s MA technology, in contrast to traditional resins and beads, features a rigid format that makes it highly reproducible and robust.

Fractionation of acidic and basic proteins resulted in significant complexity reduction of protein patterns and better resolution of individual protein spots as compared to the protein patterns for non-fractionated proteins [Lopez *et al.*, *Electrophoresis*, 2004 Aug 25, 15, 2557–63].

New biomarker enrichment kits based on carrier protein-bound serum proteomic profiling [Mehta *et al.*, *Dis Markers*, 2003–2004, 19, 1–10] enable reproducible, high-fidelity serum proteomic profiling of the low molecular weight proteome (*i.e.* “fragmentome”).

39.29

### Protein Production in a Wheat Germ Cell-free Translation System

**K. Madin, M. Watzela, T. Metzler, and B. Buchberger**  
Roche Diagnostics GmbH, Penzberg, Germany

The vast amount of information revealed by numerous whole genome-sequencing projects has dramatically increased number of genes with unknown functions and structures and generated a new emphasis on studies that will lead to information regarding biochemical function of proteins. Pharmaceutical companies and structural genomics consortia are dedicating massive amounts of time and effort to solve protein structures for a variety of uses, most notably for use in health care. This information is to be used to achieve a better understanding of biology and in diagnosis and treatment of disease including rational design of new drugs. But those efforts are hampered by problems associated with sufficient protein expression and inability to produce enough proteins with high purity.

Cell-free protein synthesis has received renewed attention as a verification tool for fast and parallel expression of functional proteins with high productivity since availability of sufficient amount of protein of interest is a requirement for proteome analysis.

Here we report about a product line for efficient and optimized protein expression based on Wheat Germ extract. It combines several new technologies and enables high success rates for expression and solubility, particularly for eukaryotic target proteins. Convenience is greatly improved due to ability to use PCR-generated linear templates which in combination with a newly developed CECF device allows utilization in robotic systems for processing of many genes under HT-conditions. Larger reaction format enables synthesis of up to 1 mg of expressed protein in a 1 ml of transcription-translation cell-free reaction.

39.30

### ECL Plex; Fluorescent Multiplex Western Blotting

**E. Malmport, Å. Hagner-McWhirter, M. Seddighzadeh, A. Hurynowicz, A. Edman-Örlefors, I. Gottschalk, and S. Edlund**

Amersham Biosciences AB, Uppsala, Sweden

A new competitive multiplexed fluorescent Western blotting system, with optimized CyDye™ conjugated secondary antibodies and low fluorescent membranes, has been developed. The aim was to improve the results that could be obtained in Western blotting by combining absolute sensitivity, linear dynamic range and minimal cross-reactivity. The ability to multiplex would reduce the need for stripping and re-probing and the stable dyes should make re-scanning possible after many months.

Proteins separated by electrophoresis and transferred to low-fluorescent membrane were detected by specific primary antibodies followed by cross-reactivity validated CyDye conjugated secondary antibodies; direct fluorescence was detected using Typhoon™.

The experiment resulted in very high sensitivity (0.6 pg), good linearity ( $R^2$ -value of 0.998) and wide dynamic range (more than 3-fold). This made it possible to perform accurate relative quantification of low abundant endogenous proteins.

The low fluorescent membrane improves the sensitivity and minimizes background noise.

The optimized CyDye conjugated antibodies are stable, have lower self-quenching and are compatible with many scanners and CCD cameras on the market.

39.31

### Selective Labeling of Cell Surface Proteins

**R. Marouga, S. Bourin, M. Rafiyan Nejad, and Å. Hagner-McWhirter**

GE Healthcare, Uppsala, Sweden

Cell surface proteins are relevant to almost all protein-protein interactions and are often involved in disease pathogenesis. Therefore, they are of great interest to the pharmaceutical industry as they form potential drug targets. 2-D gel electrophoresis is used extensively for detection of protein biomarkers and targets. However, cell surface proteins can be difficult to detect in 2-D gels, partly due to their low abundance, without fractionation or some other type of enrichment. They are also often poorly represented due to their hydrophobic nature and high molecular weight.

In this study, we present a new protocol using CyDye™ DIGE Fluor minimal dyes to visually enrich and detect this important group of proteins. This protocol is rapid and simple to use. Intact CHO-K1 cells were first labeled with CyDye DIGE Fluor minimal dyes, then lysed and fractionated into hydrophilic and hydrophobic fraction before multiplexing and separation according to the 2-D DIGE technology. The membrane fraction contains labeled proteins, whereas the cytosolic fraction is devoid of labeled proteins. DeCyder™ 2-D Differential Analysis Software was used to compare protein spot maps where small changes in abundance can be detected with high accuracy. Over 80 new proteins spots were found present only in the cell surface fraction at a ratio of more than 10. The proteins were further identified as cell surface proteins by MALDI-ToF analysis.

In conclusion, applying the three CyDye DIGE Fluor minimal dyes in this new protocol, and in combination with DeCyder 2-D software, provides a unique tool for studying cell surface proteins specifically. Detection of several new proteins was improved when compared to the standard Ettan™ DIGE protocol.

39.32

### Human Histone Isoform Profiling Using the ZOOM® Benchtop Proteomics System

**G. Meredith, A. Lindsey, and R. Rooney**

Invitrogen Corp., Carlsbad, California, USA

Histones, proteins of extremely basic pI that are essential for packaging genomic DNA into functional chromatin, are becoming increasingly recognized as central elements in the regulation of gene expression as well as in other key processes such as genome replication and repair. A "histone code" based on a diverse set of possible post-translational modifications of histones has been suggested to underlie much of this regulation; however, the tools to map these modifications and correlate them with cell-state are in need of further development. We present results showing that starting with human cells grown in tissue culture, simple pre-fractionation followed by 2DE separation with pH 9–12 ZOOM® IPG strips and ZOOM® gels using the ZOOM® IPGRunner™ System, permits in-gel visualization of histone isoforms. Further, blotting the gels permits visualization with blot stains and probing with isoform-specific antibodies. Within a day, using this mini-gel-based 2DE system, a significant degree of histone H3 isoform complexity is revealed that is undetectable with conventional 1D SDS-PAGE separation. In particular, several resolved Ser-10 phosphorylated H3 isoforms, with pIs ranging from ~10.5 to ~11.8, can be discerned on western blots. Furthermore, treatment of cells with colcemid to block mitosis leads to demonstrable changes in the distribution of Ser10 and Ser28 phosphorylated H3 isoforms.

39.33

### Genomic Antibodies™; Directly from DNA to Antibodies

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Strategic Biosolutions, Newark, Delaware, USA

The Genomic Antibodies process is a high throughput antibody reagent production service designed to enable rapid discovery and commercialization of products for customers in the proteomics, pharmaceutical, diagnostics and biomedical research industries. This initiative is based on genetic immunization and associated processing technologies for the high throughput production of high purity, high reactivity antibodies.

Traditional methods of making antibodies to proteins require that the protein of interest be produced and purified in relatively large quantities prior to injection for *in vivo* production of a specific antibody. Protein production and purification methods are time-consuming, laborious, technically challenging, and expensive. The Genomic Antibodies process eliminates the need to produce and purify protein. Instead, synthetic DNA, representing a genetic sequence of interest is introduced directly, via a proprietary process that produces antibodies to the protein encoded by the DNA. The Genomic Antibodies process results in antibodies that are easier to produce in large numbers thus facilitating large-scale research involved in drug development and academic research. Immunization can begin before purified protein antigen is available. Immunization is not delayed when the protein antigen is difficult to express in the laboratory.

This high throughput, high efficiency process is aimed at enabling more rapid discovery by providing faster, cost effective access to very large numbers of high sensitivity, and specific antibodies, or families of antibodies, that will collectively advance the investigator's research, development and commercialization efforts

Genomic Antibodies enables researchers to increase their success rate of identifying potential therapeutic or diagnostic protein targets by providing large numbers of unique antibody reagents generated from customer specified genetic sequences. The exponential increase in genetic information available from all types of living organisms has created a need for large numbers of antibody reagents to detect the proteins produced by these genes.

39.34

### Maximising Reproducibility and Improving the Resolution of Target Proteins with Automated Gradient Gel Casting for 2D Electrophoresis

R. Mount

NextGen Sciences Ltd., Cambridgeshire, United Kingdom

The use of gradient gels for 2D electrophoresis has been shown to enhance the resolution of target proteins, improving quantification, limit of detection, and identification. However, pre-cast gradient gels are expensive, with short shelf lives, and are only available in a limited choice of gradients. An alternative is to pour the gradient gels manually, but this can be time-consuming and generates considerable gel-to-gel variability. The a2DEoptimizer is the first system to automatically cast gels to user-defined gradients, with the performance of a pre-cast gel.

The a2DEoptimizer can pour homogeneous, linear, non-linear, and stepped gradients, allowing the user to optimise their gel for specific projects or protein samples. This presentation will show the effect of different gradient gels on protein separation and spot resolution. Protein spot mobility, over five separate casts, had an average reproducibility (as measured by %CV) over 32 gels of 3.3%.

The a2DEoptimizer is fully PC controlled. Through a graphical interface, the user may design any gel gradient profile. Once the gradient has been selected and stored on the computer, the gels are then cast automatically by the system, giving the same result each time that particular profile is recalled. In addition to gel casting capabilities, the a2DEoptimizer is available with internal power supplies. These are supported by the system's user-friendly software, which enables drag-and-drop programming of the power supplies and gel casting equipment, allowing the user to control and monitor the progress of each step and access current/voltage feedback in real-time.

39.35

### Software Solutions for the Analysis of Heterogeneity by Analytical Ultracentrifugation

S. Overkamp<sup>1</sup>, M. Chien<sup>2</sup>, J. Philo<sup>3</sup>, and A. Furst<sup>2</sup>

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Significant advances have taken place in the past few years in the analysis of sedimentation velocity data. A number of different algorithms and software packages have been developed that can be used to reveal sample heterogeneity under different circumstances. With these approaches, sedimentation velocity experiments can provide resolution that rivals or exceeds that available from other techniques. Although there are now many methods of analysis that may be used to reveal heterogeneity, such as the Van Holde-Weischet, the time derivative ( $\delta c/\delta t$ ), and the continuous distribution *c(s)* methods, each is applicable under particular circumstances and sample conditions. In this presentation, we review several of these algorithms and software packages and we define the conditions required for their use.

39.36

### Cost-efficient and High Capacity Depletion of Abundant Serum Proteins

M. Palm and T. Bergman

Affibody AB, Bromma, Sweden

The robust Affibody® molecules are well suited as affinity ligands for removing high-abundance proteins in serum samples. Affibody® molecules can be immobilized onto a variety of solid supports and the unique C-terminal cysteine residue allows oriented coupling, granting a flexible, user customized format.

With Affibody® lyophilized proteins; scientists are presented with an opportunity to design their bioseparation matrix in a cost-efficient manner, while other commercially available depletion products exclusively offer fixed formats of the ligands such as pre-packed LC columns, disposable spin columns and microbeads.

Here, we present the use of the new Anti-HSA Affibody® molecule for depletion of human serum albumin (HSA) in a cost efficient manner with superior capacity.

The capacity of the Anti-HSA Affibody® molecule immobilized column was corresponding to 160 µl serum per ml matrix. The reproducibility and stability of the column was tested, the procedure of injection, washing, elution and re-equilibration was repeated for 300 cycles with no decrease in column performance. Analysis of flow-through fractions up to 300 cycles showed that more than 99% of HSA was removed consistently proving supreme column stability.

Affibody's participation in the MolPAGE initiative, a European consortium of academic institutions and biotechnology companies, with the aim to identify biomarkers, assures further development of high capacity Affibody® ligands for future proteome studies of biological fluids. Additional Affibody® molecules to be utilized for sample preparation are currently under development and will continuously be added to the catalog of Affibody® products.

39.37

### Fluorescence-based Phosphopeptide Mapping; Interfacing Thin-layer Electrophoresis with Analytical Imaging and MALDI-TOF Mass Spectrometry

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Understanding how phosphorylation regulates specific signaling pathways typically requires knowledge of which amino acid residues are phosphorylated in a particular protein. Notwithstanding the recent dramatic increase in the use of mass spectrometry-based methods for phosphorylation site determination, cellulose-based thin-layer electrophoresis (TLE) of proteolytically-cleaved <sup>32</sup>P-labeled proteins remains an important technology for generating phosphopeptide maps. This mapping technique allows the analysis of temporal and positional changes in protein phosphorylation patterns under different physiological conditions. Experimental design considerations, economic issues and safety concerns related to disposal of radioactive waste have represented a significant barrier to the widespread adoption of TLE for phosphopeptide analysis. To overcome this hurdle, a fluorescent phosphorylation-sensing dye was used in combination with a xenon-arc lamp-based charge-coupled device (CCD) camera system to conduct phosphopeptide mapping without the use of radioisotopes. Multiplexed imaging of phosphorylated and nonphosphorylated peptides was also accomplished using two spectrally distinct fluorophores. The feasibility of subsequently analyzing particular peptides directly from TLE plates using orthogonal MALDI-TOF mass spectrometry was also explored. With this instrument, ionization and mass analysis are decoupled. Consequently, mass analysis is not affected by spatial variations and sample topography, allowing the TLE surface to serve as the sample target. The newly developed fluorescence-based phosphopeptide mapping technique should allow for the routine identification of phosphorylated residues in proteins without the use of radioisotopes.

39.38

### Biomarker Proteins in Diabetic and Normal Serum Samples Using Both Chromatographic and Preparative Electrophoretic Fractionation with 2D Gels

A. Paulus, S. Freeby, T. Wehr, K. Academia, N. Liu, and A. Posch

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The human serum proteome project underway with international contributions aims to catalogue, characterize and identify every protein found in human serum. Whole blood, serum and plasma are preferred samples for monitoring human health state both in a clinical environment and in pharmaceutical research. These samples are also widely used in the search for biomarkers in clinical studies. However, the search for biomarkers in serum, e.g. quantitative differential protein expression is difficult because the twelve most abundant proteins account for over 99% of the protein mass in the sample. Therefore, sample preparation strategies aimed at removing the most abundant protein and further fractionation is necessary. Here we demonstrate this strategy in a biomarker discovery study involving diabetic and normal control serums. As an alternative to the immuno depletion chromatography to remove the high abundant serum proteins, we used a less selective, but inexpensive and in spin columns easy-to-use approach to remove the majority of albumin and IgGs with an affi-blue and protein A stationary phase. The albumin and IgG depleted serum samples were further fractionated via preparative isoelectric focusing with the Rotofor, resulting in 20 fractions each for the diabetic and normal sample. Separation of these fractions in narrow-range IPG strips followed by second dimension SDS-PAGE analysis on Criterion 11-cm gels allowed for the identification of differentially expressed spots. We picked 50 differentially expressed spots for identification with an electrospray ion-trap instrument and the poster will be discussing the proteins and their relations

39.39

### Fourier Transform Mass Spectrometry-based Comparative Proteomics for PLAS, MA, and Profiling

C. Paweletz, F. Meng, E. Deyanova, M. Mazur, X. Zhao, N. Yates, and R. Hendrickson

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There exists an urgent need for gel-free proteomic technologies that can not only identify peptides by data-dependent analysis but also detect, quantify, and identify peptide changes within complex matrices, such as plasma, cerebral spinal fluid, or lavage in a high throughput manner. We have used a Fourier Transform Mass Spectrometry based plasma profiling platform to identify dose dependent protein changes in a large scale profiling experiment that involved the analysis of 200 individual murine samples treated with disease modifying agents at different concentrations in an animal model of disease. Plasma samples were processed using a glyco-peptide isolation procedure developed by Zhang *et al.*, (*Nat Biotechnol.* 2003, 21, 660–666), and analyzed using an LC-FTMS platform consisting of an Agilent microcapillary HPLC pump, a Famos autosampler, a Proxeon nano-electrospray source and a Thermo LTQ-FT Mass Spectrometer. Micro-capillary LC separations were performed on a New Objective peptide trap coupled to a 5 cm, 190 X 100 um column with a flame pulled tip. To detect differences in these large study set LCMS data were subjected to differential mass spectrometry (dMS) in which statistical tests are performed at each  $m/z$  value over a given time interval. This produces a single p value for each binned (time,  $m/z$ ) grid (Wiener *et al.*, *Anal Chem* 2004, 20, 6085–6096). A lower p value indicate that the difference is more statistically significant. Here, we describe key experimental parameters that can influence dMS plasma analysis, such as platform reproducibility, biological variability, sample handling, changes in chromatographic behavior, and sensitivity and specificity of dMS.

39.40

### Array-based Systems for High Information Protein Interaction Analysis in Proteomics

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Protein interaction data from yeast two hybrid (Y2H) and mass spectrometry (MS), together with developments in bioinformatics, are expanding our understanding of protein function and enable predictions on how proteins are linked in functional interaction pathways. Although Y2H and MS enable rapid data throughput, these techniques suffer from a relatively high frequency of false positive results and can also result in some interactions being overlooked.

Optical detection techniques based on the phenomenon of surface plasmon resonance (SPR) permit label-free analysis of protein interactions and can be used to confirm Y2H and MS interaction data and most importantly, provide kinetic and affinity data on these interactions. Knowledge of these fundamental biophysical parameters greatly enhances the understanding of individual interactions and their roles in larger biological pathways. At the same time, affinity and kinetic data in combination with mutational approaches is key for understanding how protein function can be related to structure.

Two new systems are now available to provide interaction analysis data at a throughput suitable for efficient processing of the large number of interactions handled in proteomic studies. Biacore® A100 offers a protein array format for high information interaction analysis through multiplex screening of protein panels. Flexchip enables the study of the interaction of a single protein sample with up to 400 different targets spotted onto a sensor surface. The potential of these systems to improve the understanding of interactions is illustrated using transcription factor analysis with DNA arrays, protein binding to peptide arrays (Flexchip), screening of small molecules over a panel of drug targets and kinetic screening of antibodies (Biacore A100).

39.41

### Identification of Low Copy Number Human Plasma Proteins following Removal of 20 High Abundance Proteins

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The wide concentration range of individual human plasma proteins has been a significant challenge for identification and characterization of potential lower copy number biomarkers. Many technologies for serum pre-fractionation and removal of the high abundant proteins to potentially unmask the low abundant proteins have become commercially available over the past few years. To date removal of the top 6 or 12 abundant proteins has been employed to help identify low level proteins in serum. Removal of an additional set of high abundant proteins can potentially unmask many more proteins separated by 2DE and/or peptides separated by liquid chromatography. A new depletion technology, which removes 20 of the highest abundance serum proteins, has been tested for its ability to allow the identification of many more proteins following its use. The performance of this "Top 20" depletor is compared and contrasted against alternative plasma/serum depletion methodologies, including those that deplete "single or discrete" highly abundant proteins.

39.42

## 2D-PAGE-based Proteomics and Protein Analysis

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A wealth of information is encoded in the genome and converted to create biological structures - the perfectly organized interaction of macromolecules is of extreme complexity. Several lines of development of protein analytical technology have evolved as today's prime tools towards dissection of protein interaction. Though mass spectrometry, liquid chromatography, protein chips and other techniques have gained widespread acceptance as powerful sources of information, 2DE-PAGE, the electrophoretic separation of complex protein mixtures in two different dimensions, retains its extraordinary position. The unequalled power of separation of thousands of individual proteins underlines the importance of this method. We specialize in the 2DE-PAGE variant named NEPHGE (non-equilibrium pH-gradient electrophoresis, Klose & Kobalz) for more than a decade. We have developed proprietary formulations of ampholytes and components for gel preparation and processing of samples. Along with the improvement of the chemistry we are continuously working on the introduction of newly designed equipment. With our current state of the art technology we prepare up to 40 by 30 cm giant gels with more than 10,000 resolved protein spots. NEPHGE's intrinsic advantages encompass aspects like high sample load capacity, the superior ability to resolve basic and hydrophobic proteins and its versatility in view of subsequent analytical and micro-preparative options. Such features merge into an ideal platform for many of today's proteomics objectives, e.g. in glycobiology, membrane protein analysis etc. Embedded in a wide range of adjunct protein analytical techniques, like electrophoretic methods, MALDI-MS as well as nanoLC-ESI-MS<sup>n</sup> characterization of proteins, automated Edman micro sequencing and reference peptide synthesis, we are ready to accept the challenge of biological complexity. Advanced methods of protein analysis have often proved to be indispensable in order to shed light on biological interactions.

39.43

## Isoelectric Focusing in Serial Immobilized pH Gradient Gels to Improve Protein Separation in Proteomic Analysis

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In collaboration with the Sinha group we previously demonstrated the separation of proteins by IEF with pH 4–8 IPGs over 54 cm (Poland *et al.*, 2003, *Electrophoresis* 24:1271–5). In the present study we demonstrate that comparable results can be opportunely achieved using commercial IPGs of suitable pH ranges placed end-on-end in series during electrophoresis. We term this new mode of iso-electric focussing "Daisy Chain IEF." We show that proteins efficiently migrate between IPGs during electrophoresis by moving through porous bridges between the serial IPGs that contain suitable buffer. Several materials function well as bridges, that include paper, polyacrylamide gels, or actual IPGs. The 2-D protein patterns are not apparently worse in quality than those produced by conventional IEF employing the same IPGs. A great benefit of this process is that protein samples are consumed efficiently, without the need for prior preparatory fractionation, such as e.g. chamber isoelectric focusing. This advantage is considerable when working with very scarce samples, such as clinical biopsies or biochemical subfractions. The present study employed the most suitable commercially available pH gradients. The proteomics community would be well served if other suitable pH ranges were commercially available, e.g. to perform high resolution IEF separation of proteins in one electrophoretic step over the pH range 2–12 over 100 cm. The only technological limit thereto appears to be the convenient availability of IPGs with suitable pH ranges.

39.44

## An Automated Approach to Top Down Biomarker Analysis

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The use of top down mass spectrometry (MS) to dissect biological diversity in human proteins is now possible. Utilizing Fourier transform mass spectrometry, accurate and precise intact mass data combined with MS/MS fragmentation spectra can be used to facilitate the characterization of cSNPs, alternative splice events, post-translational modifications (PTMs), and other protein processing events. This is the only technique that can be used for comprehensive characterization of protein isoforms.

ProSight software (Taylor *et al.*, *Anal. Chem.* 2003, 75, 4018–4086) was developed to allow researchers to apply database search technology to top down proteomics through a technique called shotgun annotation. In shotgun annotation, the database of intact proteins is overpopulated with possible protein forms and multiple fragment ions are used to explain the measured intact mass.

Biomarkers may be intact proteins, modified proteins, fragments of proteins, or combinations thereof. Top down sequencing is an ideal method to identify all of these potential protein isoforms. In this study, ProSight software has been extended to allow searches for peptide biomarkers. Top down data is used to search an entire database for all possible sub-sequences of every protein form in the database that matches the observed exact intact peptide mass. The observed fragmentation spectra are matched with the predicted spectra of each theoretical subsequence and a probability score is assigned to the peptide match. Variations of protein primary structures, including post-translational modifications and cSNPs, will be simultaneously identified. This method offers a fast, automated tool to complement conventional biomarker analysis.

39.45

## Differential Protein Binding to the Enchant Albumin Depletion Kit Column as a Result of Altering Buffer Composition

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The study of low abundant plasma/serum proteins is of notable interest particularly for the research and discovery of biomarkers for disease. A significant challenge in this research is the reduction of plasma/serum complexity, which results in increased resolution and visualization of low abundant proteins by 2D gel analysis or mass spectrometry. The Enchant Albumin Depletion kit uses a Cibacron Blue based support in conjunction with a buffered salt solution, to reduce the concentration of albumin in plasma/serum containing samples. One complication with this method lies in the degree of non-specific binding of additional sample proteins to the column, potentially resulting in the loss of the protein of interest. In this study, we demonstrate that by altering the binding and/or wash buffer composition, such as by changing the pH or ionic strength, it is possible to modify the binding profile without affecting albumin removal properties.

39.46

## New Fluorescence Dyes for Protein Gel Stains

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Advanced proteomic techniques require highly sophisticated means for the detection and analysis of biomolecules.

3 new fluorescence dyes for the staining of proteins in polyacrylamide gels have been developed: LUCY-506, LUCY-565 and LUCY-569. These dyes were tested in different gel-systems, and applications for 1D- and 2D-Electrophoresis have been worked out. The following parameters were elaborated: sensitivity, detection-limits, linear dynamic range, background, specificity, photostability, pre- and post-electrophoretic staining protocols, electrophoresis conditions, time-optimization, ease of use, detection and imaging, and compatibility with subsequent MS-analyses.

Comparisons to current state-of-the-art dyes were drawn under different experimental conditions. The detection limits for the 3 dyes ranged between 3–10 ng/band. Linearity was given up to 1000–6000 ng/band and is therefore larger than for most silver stains, Coomassie blue or other fluorescence dyes. The standard procedure is a post-electrophoretic stain without fixation, which is completed after 60 min. Using a modified staining protocol, it is also possible to stain a gel and perform a western-blot afterwards. Native gels can be visualized by rinsing the gel in SDS immediately after the run, but before staining it.

Several devices can be used for the detection, e.g. illuminating the gel on a transilluminator (Dark-Reader, UV-Screen) and imaging the gel using a CCD- or Polaroid-Camera. Alternatively a laser scanner can be employed, using the corresponding excitation and filter settings. Photostability of the dyes was determined by continuous UV-illumination of the gels and imaging on a laser scanner in defined time intervals.

The new dyes offer a suitable alternative to current silver staining techniques or existing fluorescent staining methods for the detection of minute amounts of protein.

39.47

## Automated Electrophoresis by Slotgels

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Gel electrophoresis is one of the eldest and powerful methods to analyze biomolecules [Tiselius and Svendberg 1926]. Though capillary electrophoresis opens the field to automation, most separations are still performed in slabgels manually [Mikkers *et al.*, 1979].

To overcome the obstacles to automate slabgel electrophoresis, we propose a chip comprising an array of slots each filled with a polymer matrix. The chip design enables automated electrophoretic separation of samples in their individual slots without any loss of flexibility or performance. The matrix is permanently fixed by two walls of the slots via adhesive and chemical forces, however full-length flat side access to the matrix from opposite directions is ensured throughout the entire analysis process.

Thus any staining technique, blotting, eluting or picking can be carried out. Dimensions of the chip similar to a conventional slabgel and a slot width of 1 mm were chosen to ensure high separation capacity, high sensitivity and, due to the large surface of each slot, short separation times. The chip format enables processing in an automated system designed as a production line.

Some aspects of slotgels which are not relevant under conditions of a standard slabgel format become serious technical issues by the geometry of a slot: Requirements for a reasonable electric field within the slots and the condition of the walls with respect to polymerization, adhesion and homogeneity of the matrix are taken into account. Shrinking and expansion of the matrix while applying chemicals is compensated.

The automated slotgel technology can adequately fulfill the requirements of high throughput analysis.

39.48

## Integrated Resource for Functional Proteomics Data

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**Abstract:** The German human brain protein project is a NGFN-II project embracing eleven consortial partners. A broad spectrum of proteomics techniques is employed within the consortium in order to acquire molecular data from primate and mice brains. These data include information on RNA-transcript levels, protein expression levels, protein identification, protein interaction and protein localization. A dedicated database system is developed and implemented by MicroDiscovery and will represent normalized and controlled data on a high quality level. Data is imported by experts following a number of protocols (SOPs). These protocols are designed to assess statistical properties of the data and are adapted to the specific requirements of the imported data types. In order to make the results accessible to the community a comprehensible interface is being developed by MicroDiscovery in order to provide easy access to the data. We present our concepts for an integrated database system, data import and web interface providing high throughput data generated in our consortium.

39.49

### Extending the Dynamic Range of Gel Based Proteomics For Biomarker Discovery

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For some years, continuous method improvements were implemented in our proteomics line to achieve utmost proteomics coverage using 2DGE and MALDI-TOF as a lot more protein spots can be detected in the gel images than can possibly be analysed by MALDI protein mass fingerprints (PMFs). As these lower intensity spots bear the greatest potential to unravel interesting protein candidates, our primary goal is matching the spot detection and protein identification sensitivity. We have explored the behaviour of Prespotted AnchorChip targets (PACs) –a disposable plastic MALDI target equipped with 384 HCCA matrix “anchor” spots- in our high throughput environment and evaluated performance characteristics such as the ID rate, robustness of sample preparation and suitability for protein quantification due to high signal reproducibility. We used several technical repeats of 2D gels of mouse brain cytosolic extracts. Identical spots were excised, digested under identical conditions of full automation and submitted to different target preparation protocols. MALDI-TOF mass spectra were acquired under suitable conditions, and protein identifications derived using home built PMF identification software. Basic findings: The Prespotted AnchorChip is compatible with relatively high concentrations of electrolytes in the digest buffer without additional purification devices, allows robust data acquisition at high speed due to the absence of crystallization hot-spots and provides a higher dynamic range of suitable digest concentrations avoiding memory effects. Using the newly adapted sample preparation methods we routinely achieve the analysis of 1200 spots per gel at good success rate, which appears promising enough to reconsider our “gridding approach” and cut the gel in arrayed pieces without any staining rather than picking just the stained spots.

39.50

### Quantitation of Amyloid Beta Peptides with MALDI-TOF-MS

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Amyloid beta peptides are found in an aggregated, poorly soluble form in senile plaques deposited in the brain of individuals affected by Alzheimer’s disease (AD). In addition, soluble amyloid beta peptides are identified normally circulating in human body fluids. Quantitation of these peptides is of major interest, as changing serum levels are considered to influence the onset of AD.

The objective of this study was developing a qualitative and quantitative assay for a potential AD marker using MALDI-TOF MS data in combination with an appropriate internal standard. Therefore, different dilutions series of two amyloid peptide fragments –amyloid beta peptide 1–38 and 1–42 –were prepared and amyloid peptide fragment 1–43 applied as internal standard. Firstly, different sample preparations and instrument conditions were tested to achieve highest sensitivity. Alpha-Cyano-4-hydroxycinnamic acid only and in combination with a nitrocellulose thin layer preparation was less sensitive as compared to dihydroxybenzoic acid (DHB) or sinapinic acid (SA). The method of choice was found to be a specific SA preparation, despite the fact that DHB offered a slightly better sensitivity. Measuring in linear mode offered higher sensitivity at the cost of mass accuracy and resolution. Secondly, a reasonable linearity between 10 to 200 nM amyloid peptide 1–42 and between 1 and 10 nM amyloid beta peptide 1–38 was observed. Whereas the amyloid peptides 1–42 and 1–43 had comparable detection limits, peptide fragment 1–38 showed an approximately ten times lower detection limit. Thirdly, current research is aiming for an improvement in throughput, versatility and sensitivity.

39.51

## Functional Validation of RNAi Knockdown at the Protein Level, via Sequential Quantitative Proteomics Techniques

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RNA-mediated interference (RNAi) offers a mechanism for the analysis of gene function as well as target validation, but currently available methodologies to interrogate and measure protein levels following gene knockdown are inadequate and imprecise. Indeed, there is an urgent and demonstrable need to functionally validate RNAi knockdown by rapid and accurate quantitative surveillance of affected protein(s).

Differential isotopic labeling of tryptic peptides using  $^{18}\text{O}$  water is a well-established technique for measuring relative amounts of protein in two related but discrete samples. Through incorporation of the isotopic label, a mass shift is generated in the test sample, thereby allowing it to be easily discriminated from the control sample - via mass spectrometric analysis. Most importantly,  $^{18}\text{O}$  labeling is a global strategy in that essentially all peptides in a sample are labeled - so any changes between control and test samples are revealed. Absolute Quantification (AQUA)<sup>1</sup> represents a complementary quantitative proteomics technique to  $^{18}\text{O}$  labeling. PROTEIN-AQUA experiments may be performed by employing isotopically labeled internal peptide standards, corresponding to analogous native tryptic peptides of the protein(s) being interrogated. This method is a targeted strategy that displays robust efficacy and is being increasingly utilized for a wide variety of quantitative proteomics studies.

A study has been carried out to demonstrate that the  $^{18}\text{O}$  labeling and PROTEIN-AQUA strategies can be sequentially coupled to RNAi technology in order to perform true quantitative functional validation of gene knockdown. Following gene knockdown,  $^{18}\text{O}$  labeling is undertaken to observe both the target protein and all other proteins involved in the knockdown cascade. Subsequent to this comprehensive survey of the proteome, directed AQUA experiments are executed to elucidate the absolute concentrations of several affected proteins.

This method was developed by Dr. Steve Gygi and colleagues at Harvard Medical School [Stemann, O., Zou, H., Gerber, S. A., Gygi, S. P., Kirschner, M. W.; Dual inhibition of sister chromatid separation at metaphase, *Cell* 2001, Dec. 14, 107: 715–726] and is the subject of both U.S. and PCT Patent Applications. Limited use of this method is permitted under a licensing arrangement with Harvard Medical School.

39.52

## Simultaneous Qualitative and Quantitative Analysis of the *E. coli* Proteome; a Sweet Tale

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Profiling of microbes has diverse applications ranging from the response of bacteria to external stimuli to the bacterial and fungal response to pharmaceuticals and disinfectants. A novel, label-free methodology for monitoring protein expression is presented, which is capable of identifying and quantifying proteins/peptides in a complex matrix such as *E. coli*.

The results obtained from this LCMS method provide the high level of mass accuracy and chromatographic reproducibility which affords the specificity to cluster peptide components from the entire study for subsequent quantitative analysis. Approximately 70% of the translation machinery was identified and found to exhibit a relative decrease in abundance when comparing proteins identified from either the lactose or glucose condition to proteins from the acetate condition. These findings are consistent with the literature regarding growth rate and protein production. Essential proteins for acetate metabolism (*aceA*, *aceB*, *mdh*, *eno*, *acs*) were identified and shown to be differentially expressed between acetate and the other two carbon sources. Other proteins involved in the Glyoxylate and Citric Acid Cycle, Glycolysis and Acetyl CoA production were identified and exhibited varying levels of abundance among the various conditions. The protein coverage of identified proteins ranged from 10–60% and appeared to be a function of the relative abundance and size of the corresponding protein. The inherent redundancy of this label-free method enables one to determine confidence intervals associated with the relative fold-changes. The reproducibility and robustness of this analytical methodology is evidenced by the high degree of Venn intersection from replicate analyses (60 to 80%).

39.53

### Top-down Versus Bottom-up Proteomics Approaches of Human Platelet Proteins Using Monolithic PS-DVB Capillary Columns in a Multidimensional LC/MS Set-up

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Platelets, circulating in the body, play a critical role in haemostasis and contribute to essential processes in wound repair. The biology of platelets has been studied for several decades using 2D gel electrophoresis, more recently in combination with tandem mass spectrometry (MS/MS). This "classical" proteomics technique applied to platelet samples has generated over 2000 protein features of which a much smaller number of proteins has been identified so far. While 2D gel electrophoresis still provides an unsurpassed resolution for protein separations, several drawbacks of this technique have stimulated the development of alternative techniques for large-scale proteomics research.

Two-dimensional liquid chromatography (2D LC) is a valuable, complementary separation technique to 2D gel electrophoresis. The key issue in multidimensional LC methods for protein identification is whether the separations are performed at the peptide or protein level, *i.e.* bottom-up or top-down proteomics, respectively. Both approaches have pros and cons but share the time consuming RP chromatography step that needs to be repeated for a large numbers of liquid fractions.

In this study we have employed monolithic capillary columns to increase the speed of analysis of multidimensional LC separations. A bottom-up as well as a top-down proteomic analysis approach is applied to human platelet samples. LC/MS/MS analysis of peptides is performed on micro column switching set-up employing 200  $\mu\text{M}$  ID monolithic columns. Separation of intact proteins is performed on 500  $\mu\text{M}$  ID monolithic columns followed by micro-preparative fractionation and proteolytic digestion. The two approaches are compared for system reproducibility, sensitivity, the number of identified proteins and usability.

39.54

### A New Chip Technology for Identification of Biomarkers

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We have developed a new chip technology by which biomarkers can be efficiently identified followed by differential display analysis. It could improve the sensitivity and reproducibility of peak intensity and/or peak area needed for profiling and differential display analysis. Moreover the new technology enables to identify biomarkers by MS/MS technique after digestion of them on the chip. It does not require any time-consuming purification processes. The new chip technology will be expected to comprehensively discover and identify disease-modifying biomarkers.

**Materials and Methods:** The different tissue extracts of mouse were subjected to electrophoresis on SDS-polyacrylamide gel (PAGE). The proteins separated in the gel by PAGE were electrically transferred onto the surface of the chip. Thus arrayed proteins on the chip are analyzed by MALDI type mass spectrometry for profiling and differential display. On the other hand, proteins transferred to the chip were directly digested by trypsin and the digested peptides on the chip were analyzed by MS/MS technique in order to determine amino acid sequences.

**Results:** The new methodology allowed completing the entire differential display procedure less than 4 hours. Several tissue specific proteins were found and identified by this technique. The new chip enables proteomic analysis for example: purification, detection, quantification and identification in a high-throughput and comprehensive manner by directly transferring proteins which are separated electrophoretically to a chip which use a mass spectrometry for identification.

**Discussion:** It is obvious that many disease-modifying biomarkers can be found and identified by the methodology described here.

39.55

### A Compliant Solution for Monitoring Proteins Using a Lab-on-a-Chip Instrument

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Lab-on-a-Chip (LoaC) technology has had a major impact on the automatization of protein analysis. Traditionally, SDS-PAGE gels are run for sizing and quantitation of proteins. Microfluidic protein analysis is now beginning to replace this traditional method. In August 2002, the Food and Drug Administration (FDA) announced a significant new initiative, Pharmaceutical Current Good Manufacturing Practices (CGMPs) for the 21st Century, to enhance and modernize the regulation of pharmaceutical manufacturing and product quality. With the increasing focus on proteins as pharmaceutical drugs, there is a strong demand for standardized and reproducible protein analysis methods that comply with the GLP, GMP and 21CFR Part 11 requirements.

Here, LoaC technology can offer a benefit since for protein analysis it integrates sample handling, separation, staining, destaining, detection and digital data analysis. In addition, due to the integration of several individual procedures an increase in throughput and reproducibility can be achieved. We have compared chip-based protein analysis with regard to sensitivity, sizing accuracy and reproducibility to SDS-PAGE. Data were comparable to that obtained from Coomassie-stained PAGE gels. The benefits of working on the microfluidic scale include speed of analysis, sample size and fully automated data evaluation. Ten samples can be run in thirty minutes. Electropherograms plotting fluorescence intensity against separation time are generated for each sample. Data for individual constituents of a complex mixture are shown along with calculations of concentration and percent total for each protein in the trace.

Analysis of 10 samples took thirty minutes using only four microliters of sample. The data analysis is automatically performed in real-time and is stored and archived in digital format. IQ & OQ/PV tools and services as well as 21CFR Part 11 compliant software tailor the instrument for use in regulated environments.

39.56

### Reversible Covalent Binding of the Fluorophore Epicocconone to Proteins Provides a Novel Approach to the Sensitive Quantification of Proteins Across a Wide Variety of Different Platforms

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Epicocconone, a heterocyclic organic fluorophore, spontaneously conjugates to lysine residues in proteins yielding a red fluorescent product. This change in fluorescence provides a novel approach for the sensitive quantification of proteins. Covalent binding of epicocconone to proteins is only stable at low pH (pH 2.5) and the fluor can be removed by washing at a higher pH. This pH dependent reversible binding renders proteins amenable to subsequent analysis by MS and Edman-based sequencing. Deep Purple Total Protein Stain™ (DP) is a formulation of epicocconone used for staining electro-blots, SDS-PAGE, and IEF gels. DP provides at least an order of magnitude greater sensitivity than SYPRO Ruby for gel and blot staining. Staining is in water and procedures take between 3 hours to 1 day for gels and < 30 minutes for blots. DP does not show the high background or speckling typical of other stains and is biodegradable making disposal simple. DP staining does not affect enzymes activity or antibody binding. Epicocconone has also been formulated into a simple to use, sensitive (ng/mL) rapid protein quantification kit (FluoroProfile) that can be used in solution or on PVDF membranes. FluoroProfile is particularly suitable: for situations where the protein sample is scarce or contains high concentrations of salts and detergents, for rapid determination of loading levels for 2D-gels or where simplicity and speed are required. The method is robust and accurate over a wide range of protein concentrations, and is suitable for automation and high throughput screening. Deep Purple is a trademark of GE Healthcare. FluoroProfile is a trademark of Sigma Aldrich.

39.57

### Protein Assays by a High Performance Blotless Nano Western System

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Western blotting remains the primary tool of protein research, little has changed since its first reported development (*Proc. Nat. Acad. Sci. USA* 76:4350–4, 1979) We have developed a nano-volume, high performance immunoassay functionally equivalent to Western blotting while providing orders of magnitude better sensitivity, potentially down to the level of single cells. The system is inherently automatable, completely avoiding the electroblotting step of the conventional Western method. The system automatically separates, immobilizes, probes and detects proteins in less than one hour. All operations are performed in capillary chambers and simultaneous processing of multiple capillaries is readily achieved. Due to the small amount of sample used per capillary and ability to run multiple capillaries in parallel, this technique holds the potential of rapidly measuring all nodes in a signaling pathway from a limited, homogeneous cell population; something that is not currently possible. Thus, this approach can provide researchers a systems biology view of cell response to pathogens and therapies with a more complete understanding of cellular pathways.

39.58

### Protein Quantification by the SELDI-TOF-MS-based Protein Chip® System

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The ProteinChip System enables the direct and fast analysis of virtually any type of protein containing solutions like serum, cerebrospinal fluid, urine or cell lysates. Samples are applied on ProteinChip Arrays carrying spots with chromatographic surfaces for Expression Difference Mapping™ (EDM) studies or alternatively presenting pre-activated groups for coupling biomolecules of choice to run Interaction Discovery Mapping™ (IDM) experiments with antibody-antigen or receptor-ligand complexes. Before analysis in the ProteinChip Reader, non-bound proteins and contaminants are removed by selective wash buffers. Consequently, each analysis is linked to a fractionation of the original sample thereby increasing the resolution and the specificity for the target proteins.

The successful combination of chromatography and mass spectrometry is named Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS)-process. In contrast to the traditional Matrix Assisted Laser Desorption/Ionization (MALDI)-technology, SELDI enables the direct analysis of complex biological samples in a reproducible and quantitative manner.

The quantification capability of the ProteinChip System is essential in all proteomic applications for which this technology is used. In the following study we describe methods and results of quantification of myoglobin spiked into 1% human serum on chromatographic ProteinChip Arrays, quantification of a mixture of cytokines, captured on antibody coupled ProteinChip Arrays and monitoring of phosphatase activity on chromatographic ProteinChip Arrays. These three experiments mimic realistic analytical challenges to provide practical examples of quantification.

39.59

### TILLING®; High Throughput Functional Genomics

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An effective approach to do genome-wide functional genomics can be provided by the reverse genetics technique TILLING®. Targeting induced local lesions in genomes is a general technique to identify chemically induced point mutations which can be applied to any organism.

With this technique not only knock-out but also partial loss-of-function genes can be created/detected. No time-consuming transgenic steps are required and the individuals are not considered as genetically modified organisms.

A recent publication (Comai *et al.*, 2004) also proves this technique to be ideal for the detection of natural polymorphisms: "Ecotilling" allows the rapid and cost effective detection of genetic variation in many individuals. Not only single nucleotide polymorphisms (SNPs) are identified but also insertions and deletions (1–30 bases).

The instrument which is uniquely suited for TILLING® and Ecotilling is the DNA Analysis System from LI-COR® Biosciences (Colbert *et al.*, 2001; Till *et al.*, 2003; Wienholds *et al.*, 2003). This is due to the use of two-colour infrared fluorescence detection which provides high sensitivity, a wide dynamic range and two true gel images during electrophoresis. This system has demonstrated an outstanding throughput performance of up to 2000 samples and up to 2 million bases screened per day in the Arabidopsis Genome.

39.60

### Dynamic Analysis of Nucleolar Proteome by i-PROT Technology

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The nucleolus represents a large, highly dynamic multifunctional nuclear organelle. It plays a key role in ribosome biogenesis and participates directly or indirectly in cell-cycle regulation, senescence, proliferation, differentiation and maturation states. Recently, several studies have dramatically increased the scientific communities knowledge of nucleolar proteome regulation using mass-spectrometry-based quantitative analysis (J. S. Andersen *et al.*, *Nature*, 2005, 433, 77–83; A. Scherl *et al.*, *Mol Biol Cell* 2002, 11:4100–9). Here, we present a dynamic analysis on the nucleolar proteome using i-PROT technology, a novel quantitative system based on a class of isobaric reagents and tandem mass spectrometry (MS). The properties of this technology allow analytes modified by the i-PROT reagents, to be separated as a group from other molecules and distinguished from each other through tandem MS. Seven nucleolar protein extracts, isolated from HeLa cells that were treated with the metabolic inhibitor actinomycin D for different time periods, were modified with a unique i-PROT reagent on the free cysteine residues. Based on the MS results, a total of 614 proteins were qualitatively identified, and 506 proteins were unambiguously quantitated. In comparison to Manns nucleolar database, 70% of the proteins were matched, while additional novel proteins were identified and quantitated. The results demonstrate that the nucleolar proteome significantly changes over time in response to differences in growth conditions, which is consistent with the observation from Mann group. The i-PROT approach should provide a widely applicable multiplexing tool in the quantitative proteomics field.

39.61

### Analysis of Human Serum after Albumin and IGG Depletion with Vivapure Anti-HSA/IGG Kit

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In the proteomics era proteins from human body fluids are investigated due to the identification of possible markers for diseases. A main challenge of e.g. serum is the high dynamic range of the concentrations of the proteins which can be up to 12 orders of magnitude. Whereas serum albumin and IgG represent the most abundant proteins with ca. 60–80% of total. On two dimensional (2-D) gels many proteins are normally masked by albumin and IgG and cannot be detected. Specific removal of albumin and IgG yields in the enhanced resolution and detection of remaining proteins. Furthermore low abundant proteins can be loaded in higher concentration onto 2-D gels after depletion of albumin and IgG. Here, we focus on the typical Proteomic workflow for detection and identification of new biomarkers and potential drug targets. We present data for albumin and IgG depletion from human serum in order to increase the amount of low abundant proteins loaded onto 2D gels. The albumin and IgG depletion method based on a highly specific HSA-antibody and a Protein G ligand was applied on human serum samples prior to 2D-PAGE. The data presented show that more than 95% of the serum albumin and high rates of IgG could be depleted fast and conveniently with negligible non-specific removal of other sample proteins. This resulted in a much better resolution and hence in the detection of low abundance proteins.

39.62

### Rapid and Accurate Protein Sizing, Quantitation, and Analysis Using the Experion Automated Electrophoresis System and the Experion PRO260 Analysis Kit

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SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is commonly used to analyze protein samples to determine the molecular weight, concentration, and relative purity of a target protein. This method involves multiple steps that include gel running, staining, destaining, imaging and analysis, and it usually takes at least 2–3 hours to get the desired results. The newly introduced Experion automated electrophoresis system offers an innovative method for performing electrophoresis by providing a compact lab-on-a-chip platform for rapid and automated analysis of protein samples. This microfluidic-based system integrates separation, detection, and data analysis within a single platform. The Experion Pro260 analysis kit is used for separation and analysis of proteins ranging from 10 kD to approximately 260 kD, and requires approximately 30 minutes to complete the analysis of 10 samples. Here we describe the performance comparison of the Experion Pro260 analysis kit to traditional SDS-PAGE (4–20% Tris-HCl gels). We demonstrate that the performance of the Experion Pro260 analysis kit in areas of separation range, sensitivity, linear dynamic range, sizing precision and accuracy, quantitation precision, and resolution is comparable or superior to the traditional gel-based analysis. In addition, the Experion Pro260 analysis provides sample information not readily delivered by SDS-PAGE, such as the relative sample concentration and the purity of the sample.