Proteomic Society–India (PSI)

PSI News Letter Vol 1 (No. 2) December 2011

Editor
Abhijit Chakrabarti

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Editor: Abhijit Chakrabarti

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From the Council

Message from the President

If we look at the progress in Proteomics research in India, the situation seems very promising. We started in the beginning of the last decade with just a handful of investigators trying their bit and today we have close to 20 functional centres with modern infrastructure and many groups actively working. From the stage of generating small datasets, we have moved to large scale proteomics and are even combining functional proteomics, we have made a mark in plant proteomics and have been using mass spectrometry based proteomics route to unravel the genome. While I write this, I know there are also efforts in the pipeline to integrate other omics approaches with proteomics and generate integrated data which is the trend of the day and also theme of the 3nd Annual Meeting of the Society. Antibody proteomics had so far remained outside Indian labs but last couple years see this area expanding with Protein array and large scale immunohistochemistry tissue microarray efforts in place. While we pursue our goals, our learning curve will keep extending and PSI will continue work hard with the spirit of education, cooperation and collaboration to support all the goals. We have already drawn the attention of the national bodies and the international forums. And I can tell you we are moving with a lot of good will and support from various corners. I have no doubt this enhancement and progress will continue to grow among the Indian Proteomics Community and I wish everyone the very best in their efforts.

Ravi Sirdeshmukh

From Editor's Desk

Welcome to Kolkata. The second News Letter of PSI has also come out in a hurry and could only feature few updates on past 7 months since the IPCON meet in April this year. I thank Suchismita, Shilpita and Avik again for making the design and lay-out of it. Like last number, Ravi has contributed the most also to this News Letter. I have bulldozed and worked for the fact that the 2nd number of the volume 1 comes out in the year 2011. I request all all PSI members to contribute generously for the good health of the PSI News Letters by providing with articles of different kind, news, views and anything and everything on this great adventure of Proteomics research in India.

Abhijit Chakrabarti

Message from the president of HUPO

Dear Ravi and members of the Proteomic Society of India:

I want to extend my greetings to all of you as you gather together for the meeting of the Society. Your group is young but already has an impressive record of activities, including hosting of the very successful 2010 Asia-Oceania HUPO Congress. I have read your January 2011 newsletter with great interest and am much impressed by the quality and range of your scientific progress, and especially by your substantial efforts to provide training to young scientists and to those who are coming to proteomics from adjacent fields. Your programs are models for similar societies around the world ! I am also pleased to see that women students and faculty are very much involved at all levels. I congratulate you on your past achievements, wish you well in your meeting this week, and look forward to following your future adventures.

Yours truly,

Catherine Costello, Ph.D.
Professor of Biochemistry, Biophysics and Chemistry
Director, Boston University Center for Biomedical Mass Spectrometry
President, Human Proteome Organization

Research Highlights

Nuclear Matrix Proteome Analysis of Drosophila melanogaster.

The nucleus is a highly structured organelle and contains many functional compartments. Although the structural basis for this complex spatial organization of compartments is unknown, a major component of this organization is likely to be the non-chromatin scaffolding called nuclear matrix (NuMat). Experimental evidence over the past decades indicates that most of the nuclear functions are at least transiently associated with the NuMat, although the components of NuMat itself are poorly known. Here, we report NuMat proteome analysis from Drosophila melanogaster embryos and discuss its links with nuclear architecture and functions. In the NuMat proteome, we found structural proteins, chaperones, DNA/RNA-binding proteins, chromatin remodeling and transcription factors. This complexity of NuMat proteome is an indicator of its structural and functional significance. Comparison of the two-dimensional profile of NuMat proteome from different developmental stages of Drosophila embryos showed that less than half of the NuMat proteome is constant, and the rest of the proteins are stage-specific dynamic components. These NuMat dynamics suggest a possible functional link between NuMat and embryonic development. Finally, we also showed that a subset of NuMat proteins remains associated with the mitotic chromosomes, implicating their role in mitosis and possibly the epigenetic cellular memory. NuMat proteome analysis provides tools and opens up ways to understand nuclear organization and function.

S Kallappagoudar, P Varma, R Upadhyay Pathak, R Senthilkumar and Rakesh K. Mishra (2010) Mol Cell Proteomics 9, 2005-'18.

Gamma radiation induced proteome of *Deinococcus radiodurans* primarily targets DNA repair and oxidative stress alleviation.

The extraordinary radioresistance of *D. radiodurans* primarily originates from its efficient DNA repair ability. The kinetics of proteomic changes induced by 6 kGy dose of gamma irradiation was mapped during the post-irradiation growth arrest phase by two dimensional protein electrophoresis coupled with mass spectrometry. The results revealed that at least 37 proteins displayed either enhanced or *de novo* expression in the first 1h of post irradiation recovery. All the radiation responsive proteins were identified and they belonged to the major functional categories of DNA repair, oxidative stress alleviation and protein translation/folding. The dynamics of radiation responsive protein levels throughout the growth arrest phase demonstrated (i) sequential upregulation and processing of DNA repair proteins such as single stranded DNA binding protein (Ssb), DNA damage response protein A (DdrA), DNA damage response protein B (DdrB), pleiotropic protein promoting DNA repair (PprA) and recombinase A (RecA) substantiating step wise genome restitution by different DNA repair pathways, and (ii) concurrent early upregulation of proteins involved in both DNA repair and oxidative stress alleviation. Among DNA repair proteins, Ssb was found to be the first and most abundant radiation induced protein only to be followed by alternate Ssb, DdrB, indicating aggressive protection of single strand DNA fragments as the first line of defense by *D. radiodurans* thereby preserving genetic information following radiation stress. The implications of both qualitative or quantitative and sequential or co-induction of radiation responsive proteins for envisaged DNA repair mechanism in *D. radiodurans* are discussed.

Bhakti Basu and Sri Kumar Apte (2011). Mol Cell Proteomics Oct 11. [Epub ahead of print]

Proteogenomic Analysis of Mycobacterium tuberculosis By High Resolution Mass Spectrometry.

The genome sequencing of H37Rv strain of Mycobacterium tuberculosis was completed in 1998 followed by the whole genome sequencing of a clinical isolate, CDC1551 in 2002. Since then, the genomic sequences of a number of other strains have become available making it one of the better studied pathogenic bacterial species at the genomic level. However, annotation of its genome remains challenging because of high GC content and dissimilarity to other model prokaryotes. To this end, we carried out an in-depth proteogenomic analysis of the M. tuberculosis H37Rv strain using Fourier transform mass spectrometry with high resolution at both MS and tandem MS levels. In all, we identified 3176 proteins from Mycobacterium tuberculosis representing ~80% of its total predicted gene count. In addition to protein database search, we carried out a genome database search, which led to identification of ~250 novel peptides. Based on these novel genome search-specific peptides, we discovered 41 novel protein coding genes in the H37Rv genome. Using peptide evidence and alternative gene prediction tools, we also corrected 79 gene models. Finally, mass spectrometric data from N terminus-derived peptides confirmed 727 existing annotations for translational start sites while correcting those for 33 proteins. We report creation of a high confidence set of protein coding regions in Mycobacterium tuberculosis genome obtained by high resolution tandem mass-spectrometry at both precursor and fragment detection steps for the first time. This proteogenomic approach should be generally applicable to other organisms whose genomes have already been sequenced for obtaining a more accurate catalogue of protein-coding genes.

Kelkar DS, Kumar D, Kumar P, Balakrishnan L, Muthusamy B, Yadav AK, Shrivastava P, Marimuthu A, Anand S, Sundaram H, Kingsbury R, Harsha HC, Nair B, Prasad TS, Chauhan DS, Katoch K, Katoch VM, Kumar P, Chaerkady R, Ramachandran S, Dash D, Pandey A. Mol Cell Proteomics. 2011 Dec 10 (12):M111.011445.

Few Important Publications :

1. Panga Jaipal Reddy, Rekha Jain, Young-Ki Paik, Robin Downey, Adam S. Ptolemy, Vural Ozdemir and Sanjeeva Srivastava (2011) Personalized Medicine in the Age of Pharmacoproteomics: A Close up on India and Need for Social Science Engagement for Responsible Innovation in Post-Proteomic Biology, Curr. Pharmacogenomics & Personalized Med 9, 159-167.

2. 1 .Chaerkady R, Kelkar DS, Muthusamy B, Kandasamy K, *et al.* (2011) A proteogenomic analysis of Anopheles gambiae using high-resolution Fourier transform mass spectrometry. Genome Res. 21: 1872-81.

3. Abhijit Chakrabarti, Dipankar Bhattacharya, Avik Basu, Sutapa Saha & Suchismita Halder. (2011) Differential expression of red cell proteins in hemoglobinopathy. Proteomics – Clinical applications. 5: 98-108.

4. Arivusundar Marimuthu, et al. (2011) A Comprehensive Map of the Human Urinary Proteome. J Proteome Res. 10, 2734-2743.

5. Polisetty RV, Gupta MK, Nair SC, Ramamoorthy K, Tiwary S, Shiras A, Chandak GR, Sirdeshmukh R. (2011). Glioblastoma cell secretome: analysis of three glioblastoma cell lines reveal 148 non-redundant proteins. J of Proteomics. 74 : 1918-25.

6. Saxena S, Singh SK, Lakshmi MG, Meghah V, Sundaram CS, Swamy CV, Idris MM. (2011). Proteome profile of zebrafish kidney. J of Proteomics. 74: 2937-47.

7. Saijyothi AV, Angayarkanni N, Syama C, Utpal T, Shweta A, Bhaskar S, Geetha IK, Vinay PS, Thennarasu M, Sivakumar RM, Prema P. (2010) Two dimensional electrophoretic analysis of human tears: collection method in dry eye syndrome. Electrophoresis 31: 3420-7.

8. Ananthi S, Santhosh RS, Nila MV, Prajna NV, Lalitha P, Dharmalingam K. (2011) Comparative proteomics of human male and female tears by two-dimensional electrophoresis. Exp Eye Res. 92: 454-63.

9. Chattopadhyay A, Subba P, Pandey A, Bhushan D, Kumar R, Datta A, Chakraborty S, Chakraborty N. (2011) Analysis of the grasspea proteome and identification of stress-responsive proteins upon exposure to high salinity, low temperature, and abscisic acid treatment. Phytochemistry. 72:1293-307.

10. Bhushan D, Jaiswal DK, Ray D, Basu D, Datta A, Chakraborty S, Chakraborty N. (2011) Dehydration-responsive reversible and irreversible changes in the extracellular matrix: comparative proteomics of chickpea genotypes with contrasting tolerance. J Proteome Res. 10:2027-46.

11. Chakraborty S, Chakraborty N, Agrawal L, Ghosh S, Narula K, Shekhar S, Naik PS, Pande PC, Chakrborti SK, Datta A. (2010) Next-generation protein-rich potato expressing the seed protein gene AmA1 is a result of proteome rebalancing in transgenic tuber. Proc Natl Acad Sci U S A. 107:17533-8.

12. Pandey A, Rajamani U, Verma J, Subba P, Chakraborty N, Datta A, Chakraborty S, Chakraborty N. (2010) Identification of extracellular matrix proteins of rice (Oryza sativa L.) involved in dehydration-responsive network: a proteomic approach. J Proteome Res. 9:3443-64.

The coverage is not exhaustive and there may be many other important Publications that are not listed.

Events and Reports

Report of the IInd Annual meeting of the Proteomics Society (India) IPCON 2011

The IInd annual meeting of the Proteomics Society (India)-IPCON2011 was organized by Jawaharlal Nehru University (JNU) with Dr. Dipankar Ghosh as the convenor and Organizing Secretary and Prof. Rakesh Bhatnagar as the Chairman. The meeting that was held from the 3rd to 5th April 2011 at "The Parkland Retreat" hotel, Delhi was attended by more than 275 delegates from all parts of the country and included prominent scientists working in the field of proteomics, besides wide-group of students and technicians.

The executive committee meeting of the Proteomics Society (India) was held on the 2nd of April while the general body meeting was held on the 3rd of April.

IPCON 2011 was introduced by Dr. Dipankar Ghosh and the symposium was inaugurated by Prof. Sudhir Sopory, Vice Chancellor, Jawaharlal Nehru University. Prof Avadesh Surolia, Director, National Institute of Immunology, New Delhi was the chief guest and addressed the gathering. Prof. Bhatnagar welcomed the delegates and Dr. Ravi Sirdeshmukh gave the Presidential address. Dr. Shantanu Sengupta, Vice President PS(I) proposed the vote of thanks which concluded the inaugural session.

There were 8 sessions in the meeting besides two plenary talks. Among the speakers five were from abroad. The first plenary talk was given by Prof. James Scrivens of Warwick University, UK. Besides this there were three sessions starting with infectious disease proteomics which was followed by proteomics of model systems and lipidomics and post translational modifications. The annual general body meeting of the Proteomics Society (India) was held after the session followed by dinner in the lawns of the hotel.

The second day began with a plenary talk given by Dr. Sanjoy Bhattacharya from Bascom Palmer Eye Institute, USA and was followed by sessions on proteomics of vision. The final session on the second day focused on proteomics of plant systems. After the sessions a cultural programme was organized in JNU followed by dinner.

There were three sessions on the final day which started with Secretomics and was followed by sessions in non-communicable disease proteomics. The final session of the day focused on novel approaches and techniques in proteomics.

One of the unique features of this meeting was presentation of the sponsors during lunch and tea/coffee breaks which was held in a separate hall where the sponsors also exhibited their products in the booths. Apart from this scientific posters were also displayed in this hall. There were about 85 posters displayed in various fields.

Shantanu Sengupta

PROTEOMICS – PRINCIPLES, METHODS AND APPLICATIONS
Two day seminar for researchers, April 15-16, 2011, Aravind Medical Research Foundation, Madurai

A regional two-day seminar entitled "Proteomics-Principles, Methods and Applications" was conducted during the 15th and 16th April, 2011 at the Aravind Medical Research Foundation, Madurai, Tamil Nadu under the auspices of Proteomics Society, India. Participants for this seminar were mainly researchers (Ph.D scholars and Principal Investigators) who are already involved in or interested in embarking on proteomics research.

The seminar was inaugurated by the chairman of Aravind Eye Care System, Dr. R. D. Ravindran followed by the presidential address by Dr. P. Namperumalsamy who emphasized the opportunities in clinical proteomics and also highlighted the importance given by AMRF for proteomics research. The inaugural session concluded with an

introductory lecture by Dr. V. R. Muthukaruppan (Aravind Medical Research Foundation, Madurai) in which he emphasized the importance of proteomics research in clinical medicine. Scientists invited from across the country with diverse research interests with proteomics gave lectures following the inauguration.

The lectures delivered during the two days could be broadly categorized under three headings.

1. Fundamentals of proteomics experiments.
2. Applications and technical issues in proteomics.
3. Application of proteomics in clinical research.

The participants were introduced to the basics of performing a proteomics experiment by Dr. K. Dharmalingam (Madurai Kamaraj University, MKU), Dr. Angayarkanni (Sankara Nethralaya, Chennai) and Dr. Masilamani (PALL Life Sciences, Bangalore). These lectures covered the various methodologies available for sample preparation, gel based and non-gel based proteomics approaches and mass spectrometry. The pros and cons of many of the methodologies were discussed along with their suitability for a wide range of purposes. While Dr. Matt Openshaw (Shimadzu Biotech, Manchester) discussed the applications of MALDI-ToF mass spectrometry, Dr. Ranjan Mogre (Dionex, Mumbai) dealt with the technical issues related to Nano LC MS/MS platform. Dr. Dipankar Chatterji's (IISc, Bangalore) talk revealed how mass spectrometric approaches can be utilized for a bottom-up approach to address even a single biological task. Dr. Venkat Manohar (IICMS, Chennai) discussed the requirements of effective biomarker discovery. The work presented by Dr. Abhijit Chakrabarti (SAHA institute, Kolkata), Dr. Sureka Zingde (ACTREC, Mumbai), Dr. Kumaravel Somasundaram (IISc, Bangalore), Dr. Sivaramaiah Nallapeta (Bruker Daltonics, India), Dr. Venkatesh Prajna (Aravind Medical Research Foundation, Madurai) emphasized on the utility of proteomics approaches for studying a wide range of clinical conditions such as fungal infection, cancer and hematological disorders. Each talk was for 45 minutes and the interaction of participants was excellent.

In addition to the lectures, the participants were presented with a tour of the proteomics facilities available at the Aravind Medical Research Foundation. Also, a number of posters were displayed on the applications of proteomics research in eye diseases and leprosy which are being carried out in Aravind Medical Research Foundation and Madurai Kamaraj University, respectively. A feedback session at the end of the two day seminar reflected the interest generated amongst the participants. It was felt unanimously that these activities should continue on regular intervals. Seminars and hands on sessions were the favored pattern from the point of view of the participants.

K Dharmalingam

Technical notes

Improving Proteomics Reproducibility using Chip-Based Nanoflow Chromatography

Bryce Young[1] , Christie Hunter[2] , Nicole Hebert[1] , Annu Uppal[3] , Manoj Pillai[3]
[1]*Eksigent Technologies, USA,* [2]*AB SCIEX,USA,* [3]*AB SCIEX, India*

The continuous advancements in mass spectrometric technologies have made mass spectrometry based proteomics as an indispensible tool in biomarker and drug discovery programs (1,2). Mass spectrometry based protein identification generally involves 1-D or 2-D gel electrophoresis of complex protein mixtures followed by digestion with trypsin (or other proteases) to peptides and analyzed by tandem mass spectrometry. In order to detect the low abundant proteins in a complex mixture for biomarker discovery workflows, peptides are pre-fractionated before MS analysis by liquid chromatography (3,4). Thus having a robust, reproducible and high resolution liquid chromtographic separation is a prerequisite for indepth profiling of proteome analysis (5).

Proteomics Researchers prefer nanoelectrospray liquid chromatography over conventional LC techniques due to its improved resolution, lower sample injection requirements, and better ionization efficiency leading to improved sensitivity in proteomics analysis (3,5,6). However, to obtain reproducible results on tryptic peptides at low flow rates, maintaining separation efficiency, the measurement of the *m/z* ratio is not always easy due to the complexity of protein mixtures. Nanoflow LC has a number of challenges that has limited its adoption in higher throughput proteomics experiments.

One of the major challenges in the field of proteomics is to generate the consistent proteomics results across several samples. For example, primary focus in the biomarker discovery workflows is to identify the differences between the healthy and diseased sample groups. In this scenario it is critical to minimize the variation between technical replicates, i.e. repeated analysis of the same sample and to move the focus onto biological variations to allow for the sensitive detection of biologically relevant differences between the groups (3). Thus, the measurement of reproducible retention time is important to define in terms of reliable proteomics results. The nano columns do not typically exhibit very good reproducibility and are known for column to column variability, additionally, a high skill level is required to use capillary columns and make the required low-dead volume intricate connections. Therefore, reproducibility considerations become critical and the system ease-of-use is essential for routine proteomics operations (3,5,6).

High throughput analysis is required in order to screen the maximum possible number of samples to provide sufficient statistical power needed to address biological variability in most of the proteomics experiments (7). This has become important especially with the advent of mass spectrometers capable of doing high speed acquisitions. The current LCMS proteomics platforms typically involve LC separations with gradients of 80 min or longer depending upon the sample complexity which limits the throughput. In a classical Nano LC run low solvent flow rate means that loading sample and re-equilibrating the columns takes a long time further limiting the throughput. In addition, switching

between the in depth protein id experiments to validation experiments is challenging and time consuming especially in an multi user lab.

In order to provide solutions to these technical challenges in proteomics research, utilization of microfabricated systems such as chip based microfluidic devices is gaining importance (8, 9). The chip based systems have the potential to improve the development, automation, reproducibility of peptide separation and throughput of various proteomic approaches. Thus, in a proof- of- principle experiment, we investigated the chip based nano HPLC technology and the unique two column switching multiplexed workflow enabled by cHiPLC®-nanoflex system coupled to Eksigent nanoLC-Ultra® 2Dplus System. The Eksigent cHiPLC®-nanoflex is a microchip platform that allows columns and traps to be easily installed for plug-and play simplicity, without any sacrifice in chromatographic fidelity. This system allows easy switching between multiple workflows like direct injection, trap-elute, two column switching and serial two-column nano LC-MS workflows.

Methods

For the sample analysis yeast cell lysate was isolated and digested. The Bovine Serum Albumin (BSA) & Betagalactosidase (BGal) tryptic digests, and other peptide standards used in this study were procured either from ABSCIEX, Sigma or from elsewhere. The chromatographic separation has been accomplished by using Eksigent nanoLC-Ultra® 2Dplus System combined with the cHiPLC®- system in Two-Column mode with two nano chip columns (75 µm x 15 cm ChromXP C18-CL 3 µm 300 Å). The elution gradients consisted of 10-30% acetonitrile (0.1% formic acid) of varying gradient lengths. For the two column switching workflow, a workflow jumper chip and two column chips are loaded into the Nanoflex. The columns are used in alternating fashion: while one column is online with the LC-MS, the other is offline being washed, equilibrated and loaded with the next sample. Figure 1 illustrates these events with a three-run snapshot taken from a typical multi-run sequence. Two traces, top and bottom, represent how the Nanoflex switches between the two chip columns:

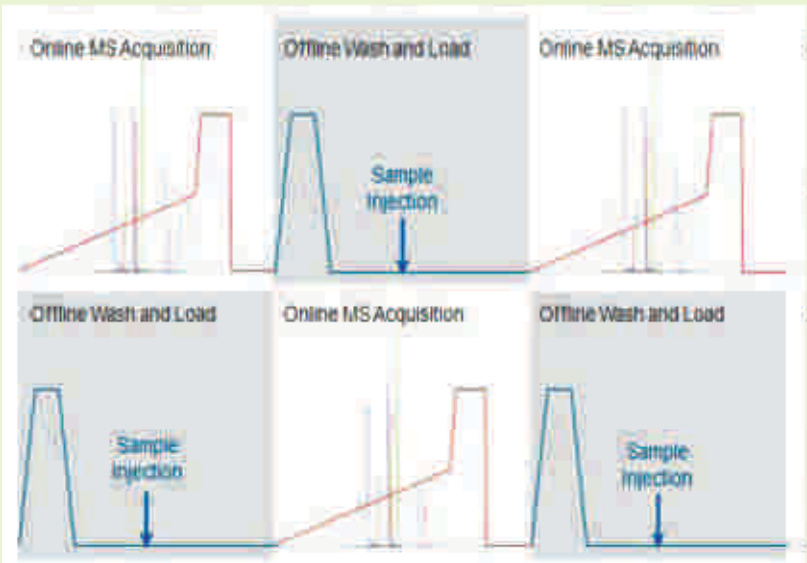


Figure 1: Two-Column Switching Workflow using cHiPLC®-nanoflex. This easy-to setup workflow provides up to a 2-fold increase in

- Column 1 (top trace): gradient elution, followed by column equilibration and loading, then another gradient elution (online-offline-online).

- Column 2 (bottom trace): this channel is undergoing the opposite cycle of events (offline- online-offline).

Essentially, the Nanoflex switches between the two columns as the sample queue advances; by the time the first LC-MS run is complete, the second column is ready for the next LC-MS analysis. Mass Spectrometric

Analysis was done using a ABSCIEX QTRAP® 5500 systems equipped with a NanoSpray® III source. Data was acquired in either full scan EMS mode or using MRM analysis. Data processing was done using MultiQuant™ Software.

Results

Reproducibility in retention time

It is important to have the reproducible chromatography for the correct identification of proteins especially which are present at relatively low levels. With the help of reproducible retention times, coeluting peptides or the peptides having similar m/z can be easily assigned to the correct protein identification. The difference deduced from the retention time can then be verified with their respective MS/MS spectra.

The results of the reproducibility check in retention time across the different nanoflex chip columns using BSA tryptic digest in two-Column mode showed correctly overlapped super imposable retention times (Figure 2). Further, reproducibility in the peptide retention time was tested by injecting the [LEU]-enkephalin peptide on 100 separate chip columns. The column to column reproducibility was found to be <2% RSD (Figure 2).

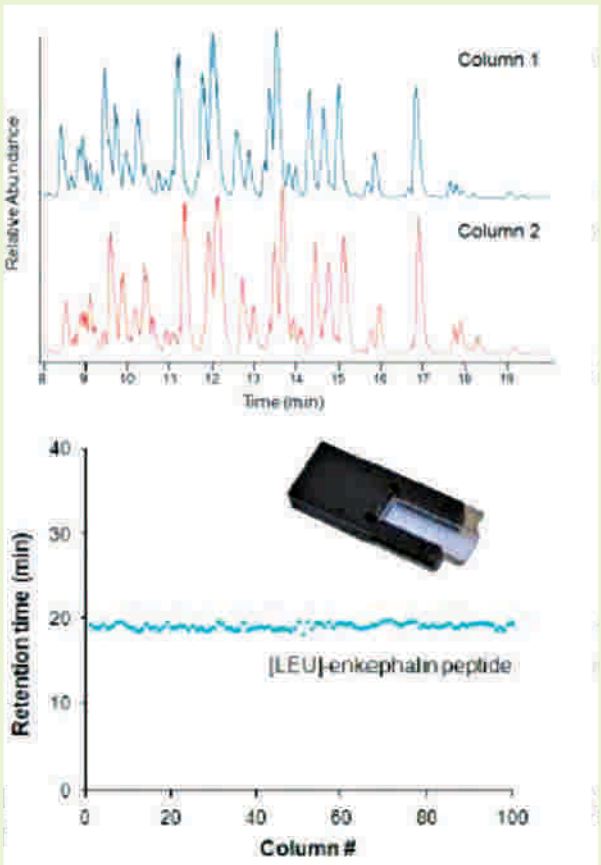


Figure 2: Matched Columns. (Top) BSA reproducibility between columns (Bottom). Testing peptide RT on 100 separate chip columns (<2% RSD).

Long Term Retention Time Stability in Complex Matrix

Generally the peptide quantitation studies involve low femtomol level quantitation from complex mixtures which requires long term retention time stability and reproducibility in chromatography. In two-column switching workflows, it is of the utmost importance that the columns exhibit optimal column-to-column reproducibility. The Two-Column Switching workflow for multiplexing and long term reproducibility was tested in a stability study using a simple BSA protein digest. Very high retention time reproducibility was observed when 320 total injections (including 64 blanks) were run, using a 30 min linear gradient and offline column regeneration (Figure 3).

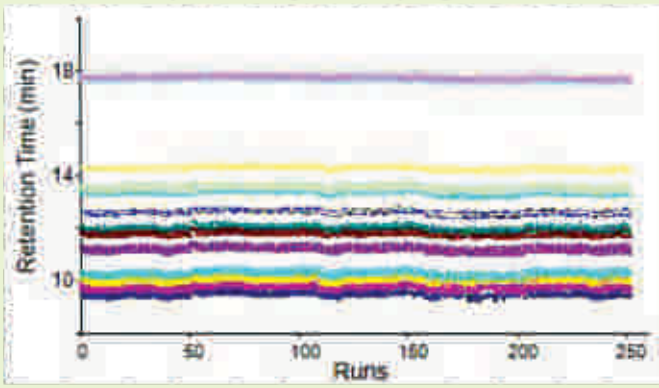
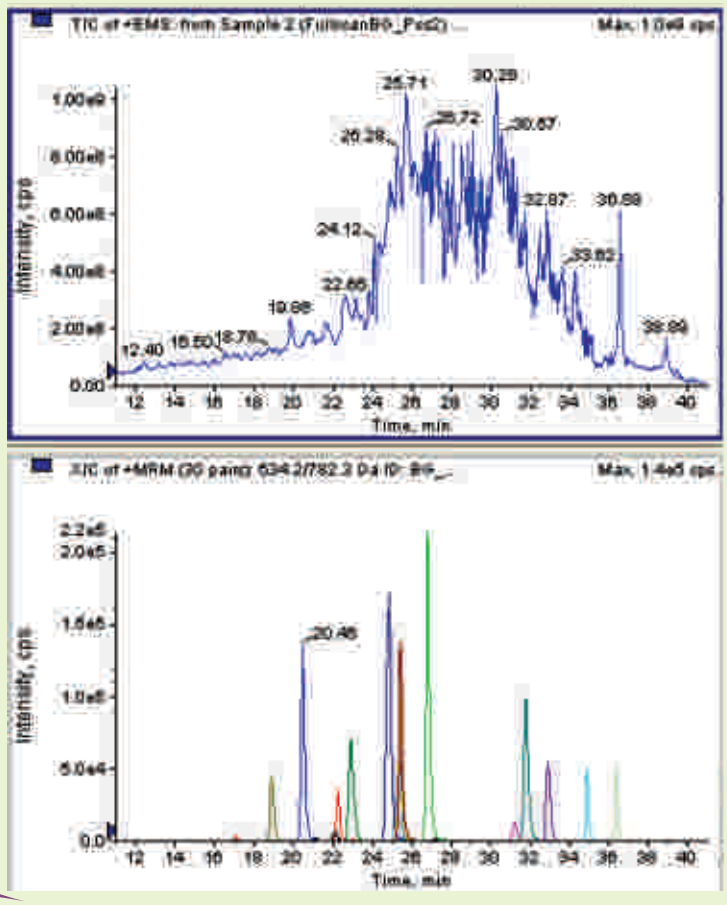


Figure 3: Retention Time Reproducibility of Simple BSA Mixture. The stability in retention time was assessed using replicate injections of a BSA digest. Shown is the measured retention time on both columns across 320 consecutive runs (64 were blanks).

As majority of the proteomics workflows involve analyzing proteins from complex tryptic digests, we tested the retention time reproducibility of this workflow in a complex matrix. A sample containing 100 ng/ul of a digest of yeast cell lysate was spiked with 100 fmol/ul beta-galactosidase (BGal) digest and analyzed; the separation was carried out using the two-column switching workflow, and the BGal peptides were monitored by MRM. Using a 45 min gradient program, and offline column regeneration, the run-to-run cycle time was 45 minutes. A total of 100 injections were run, for a total acquisition time of 3 days and 3 hours.



The complexity of the sample is represented in Figure 4, which shows the Total Ion Chromatogram (TIC) of a yeast extract digest followed by the extracted ion chromatogram of the MRMs for BGal digest from the LC-MS analysis of the same sample. Despite the complexity of the sample matrix and the short gradient, the BGal peptides have very good peak shapes in terms of peak width and symmetry.

Figure 4: Two-Column Switching Workflow in Complex Matrices. Protein digest (BGal) was spiked into a digested yeast cell lysate. (Top) Total ion chromatogram of the EMS scan shows the complexity of the sample on column. (Bottom) MRM data on the spiked protein shows the quality of the chromatogram within this complex mixture.

Figure 5 plots the retention times of selected MRM transitions of the yeast and BGal peptides over the course of 100 runs using the Two-Column Switching workflow. The yeast / BGal sample is showed excellent retention time reproducibility, suggesting that increased sample complexity has little or no effect on the effectiveness of separations on the Eksigent cHiPLC®-nanoflex. The RSD for the peptide retention times for 100 sample run data set was found to be less than 2% further confirming the excellent reproducibility in the two-column switching workflow.

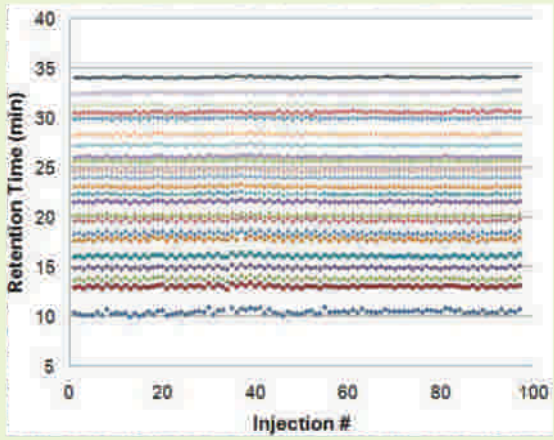


Figure 5: Retention Time Reproducibility in Complex Matrices. The retention times of yeast and Beta galactosidase peptides over the course of 100 nanoLC runs using the Two-Column Switching workflow are plotted.

The Throughput Advantage

Increasing throughput in LC-MS experiments by alternating between two columns where one column is online with the MS while the other column is switched off-line, and available to be washed, re-equilibrated and loaded with sample, is highly time-saving. This innovative approach can achieve up to a twofold increase in throughput and also significantly reduce sample carryover between runs. By programming gradients into the offline equilibration/sample loading method, the column can be very thoroughly washed, reducing the number of blanks that are required for low level quantitative work.

Conclusion

In summary, this study suggests that, with the use of cHiPLC®-nanoflex system, it has become possible to easily switch between various workflows, to generate more reproducible results with increased throughput and to obtain in depth coverage of the proteome, along with excellent retention time reproducibility, which are of primary requirements to couple high-through put protein expression and identification platforms. In addition, the simple plug and play connector system for precise low dead volume connections eliminates the intricate fittings and connections typically required in classical nano flow LCMS systems.

Thus this approach greatly facilitates the ease of use of nano- LCMS for less experienced proteomics users with high sensitivity, robustness and reproducibility and will be of great significance for mass spectrometry based proteomics in biomarker and drug discovery programs.

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electrophoresis

tech note 6136

PROTEAN® i12™ IEF System: Independent Voltage and Current Control Enables Optimization of First-Dimension IEF Conditions

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Introduction

Two-dimensional (2-D) electrophoresis is a powerful method for the analysis of complex protein samples. It is unparalleled among protein separation techniques in its ability to generate an array of several hundred or more individual separated protein species. The first dimension consists of isoelectric focusing (IEF), most commonly on immobilized pH gradient (IPG) strips, which separate proteins based on their isoelectric point (pI). Following the first dimension, the IPG strip is applied to a slab gel for the second-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation, which separates proteins on the basis of size. The first dimension separation is often the most critical with respect to its influence on the quality and resolution of the 2-D separation.

A number of factors can affect IEF separations. The solution in which the sample is prepared and in which IEF is conducted can strongly influence the quality of the separation. In general, one wishes individual protein species to separate cleanly and resolve well from one another. This requires solution conditions in which the proteins are maximally soluble, which vary to some degree from sample to sample. The duration of the focusing protocol and the rate at which voltage increases during the run also influence the outcome. For these reasons, first-dimension IEF separations often require optimization with respect to both the separation solution components and the instrument settings used. Failure to optimize these parameters can result in a poor-quality 2-D separation, with horizontal streaking and poorly resolved protein spots being the most likely undesirable outcomes.

Optimization is, however, often forgone in favor of using a standard IEF solution and a standard focusing protocol. The primary reason for this is that standard IEF equipment allows only one set of conditions to be run at a time so that conditions can be compared only among sequential runs. Optimization is therefore very time-consuming if one has access to only a single IEF instrument. Although commercial IEF instruments allow 12 or more IPG strips to be run simultaneously, they rely on a single anode and cathode to apply voltage across all of them. As a result, the current applied to each IPG strip can vary depending on the conductivity of the sample solution. Samples of varying conductivity run simultaneously can lead to inconsistent results, so the general recommendation is to use a consistent sample solution within a single run. A single anode and cathode also means that only a single focusing protocol can be applied at a time.

The PROTEAN i12 IEF system is unique in that the voltage and current are controlled separately and independently for up to 12 individual IPG strips. An individual focusing protocol can be applied to each IPG strip and the current and voltage may be reproducibly controlled regardless of whether sample conductivity is consistent within a run. This allows multiple conditions to be tested simultaneously. As a result, experiments to optimize the sample solution or focusing protocol are much simpler and quicker to carry out.

To demonstrate first-dimension IEF optimization using the PROTEAN i12 IEF system, a sample was first prepared in six different sample solutions, systematically testing the effect of the chaotrope mixture (8 M urea vs. 7 M urea plus 2 M thiourea), the detergent (4% CHAPS vs. 2% C7BzO), and the carrier ampholyte concentration (0.2% vs. 0.6% Bio-Lyte® 3/10). Each of the solutions tested was expected to differ from the others in its characteristic conductivity, and this experiment would not have been advisable using an instrument in which the current and voltage applied to each IPG strip could not be independently controlled. A second experiment tested the effect of varying the duration of IEF and the rate at which voltage was increased. Six different focusing protocols were applied to the sample in the optimal IEF solution selected from the first experiment.

Materials and Methods

Urea, Bio-Lyte 3/10, dithiothreitol (DTT), bromophenol blue, ReadyStrip™ IPG strips, SDS, electrophoresis buffers, SDS-PAGE gels, and Oriole™ fluorescent gel stain were supplied by Bio-Rad laboratories, Inc. Thiourea was supplied by Sigma-Aldrich, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) was supplied by Amresco, and 3-(4-heptyl)phenyl-3-hydroxypropyl) dimethylammoniopropanesulfonate (C7BzO) was supplied by EMD.

A crude preparation of yeast mitochondrial membranes was prepared as described previously (Arnold et al. 1998) from a log-phase culture of *Saccharomyces cerevisiae* strain BJ2168. The protein content was estimated using the *DC*™ protein assay (Bio-Rad) with BSA as a standard.

The mitochondrial membrane pellet was suspended to a protein concentration of 1 mg/ml into various sample solutions described below. Each sample was sonicated briefly and clarified by centrifugation (5 min, 14,000 × g at room temperature). Samples (200 µl) were loaded by overnight rehydration onto 11 cm pH 4–7 IPG strips. The first dimension was run on the PROTEAN i12 IEF cell as described below. Equilibration and second-dimension SDS-PAGE were performed as recommended in Bio-Rad bulletin #2651, using Criterion™ 8–16% Tris-HCl gels. A lane of Precision Plus Protein™ unstained standards was run on each second dimension. Gels were stained with Oriole fluorescent gel stain, imaged using the VersaDoc™ MP 4000 system, and analyzed using PDQuest™ software.

Experiment 1 — Variation of Sample Solution

The sample was prepared in each of six different sample solutions (Table 1). Each solution was loaded in duplicate. All 12 IPG strips were run simultaneously in the PROTEAN i12 IEF cell using the preprogrammed Rapid protocol for the IPG strip type (8,000 V, 50 µA, 26,000 Vh followed by a hold step at 750 V). The IPG strips were allowed to remain at the hold voltage until all 12 had completed the run, at which time they were transferred to –80°C.

Table 1. Sample solutions tested in experiment 1.

Solution	Composition*
1	8 M urea, 4% [w/v] CHAPS, 0.2% [w/v] Bio-Lyte 3/10
2	8 M urea, 4% [w/v] CHAPS, 0.6% [w/v] Bio-Lyte 3/10
3	7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 0.2% [w/v] Bio-Lyte 3/10
4	7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 0.6% [w/v] Bio-Lyte 3/10
5	7 M urea, 2 M thiourea, 2% [w/v] C7BzO, 0.2% [w/v] Bio-Lyte 3/10
6	7 M urea, 2 M thiourea, 2% [w/v] C7BzO, 0.6% [w/v] Bio-Lyte 3/10

* All solutions also contained 40 mM DTT and 0.001% bromophenol blue.

Experiment 2 — Variation of Focusing Protocol

The sample was prepared in a 7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 0.6% [w/v] Bio-Lyte 3/10 solution (solution 4 from Table 1). Twelve IPG strips were loaded with this sample and six different focusing protocols were applied, each to a pair of IPG strips. The focusing protocols are shown in Table 2. Each IPG strip was removed from the instrument and stored at –80°C within 30 minutes of entering the final Hold step.

Table 2. Focusing protocols tested in experiment 2.

	Step Sequence*			
Protocol	Step 1	Step 2	Step 3	Step 4
1	8,000 V, 22,000 Vh	Hold at 750 V		
2	8,000 V, 26,000 Vh	Hold at 750 V		
3	8,000 V, 30,000 Vh	Hold at 750 V		
4	250 V, 30 min	Gradual ramp to 8,000 V, 1 hr	8,000 V, 22,000 Vh	Hold at 750 V
5	250 V, 30 min	Gradual ramp to 8,000 V, 1 hr	8,000 V, 26,000 Vh	Hold at 750 V
6	250 V, 30 min	Gradual ramp to 8,000 V, 1 hr	8,000 V, 30,000 Vh	Hold at 750 V

* A 50 µA current limit was maintained throughout.

Results

Effect of Sample Solution Composition

Each sample solution (Table 1) showed a distinct voltage vs. time profile during IEF for 26,000 Vh (Figure 1). All of the sample solutions tested resulted in qualitatively similar 2-D patterns (Figure 2), but with varying spot counts (Table 3).

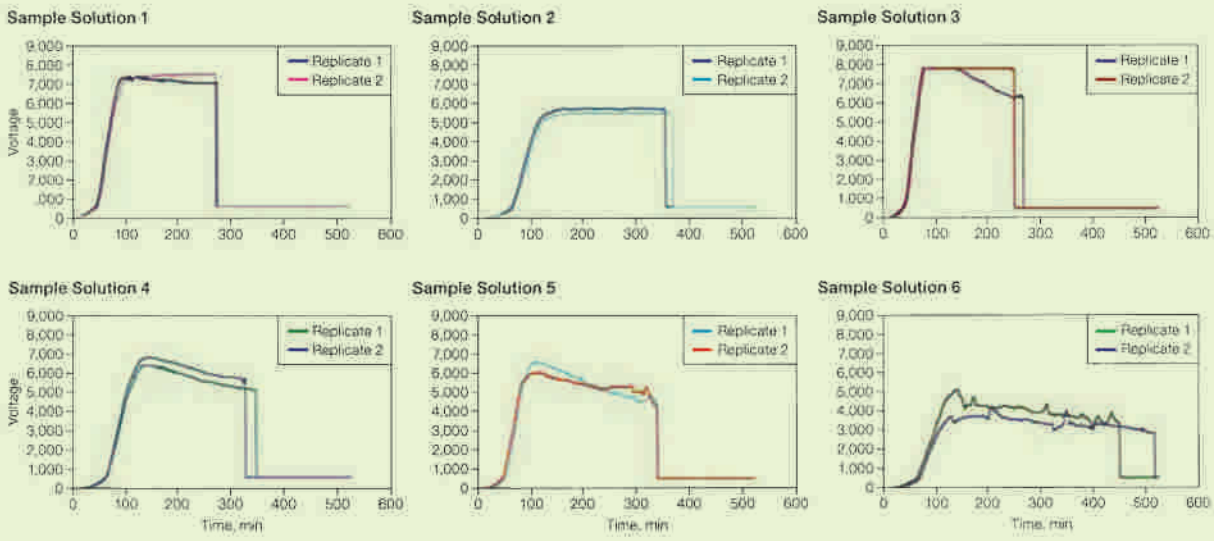

Fig 1. Effect of sample solution on voltage vs. time profile. Sample solutions were as described in Table 1. IEF was conducted for 26,000 Vh with a single-phase protocol with voltage and current limits of 8,000 V and 50 µA, respectively. Run data were downloaded from the PROTEAN i12 IEF cell and analyzed using Microsoft Excel.

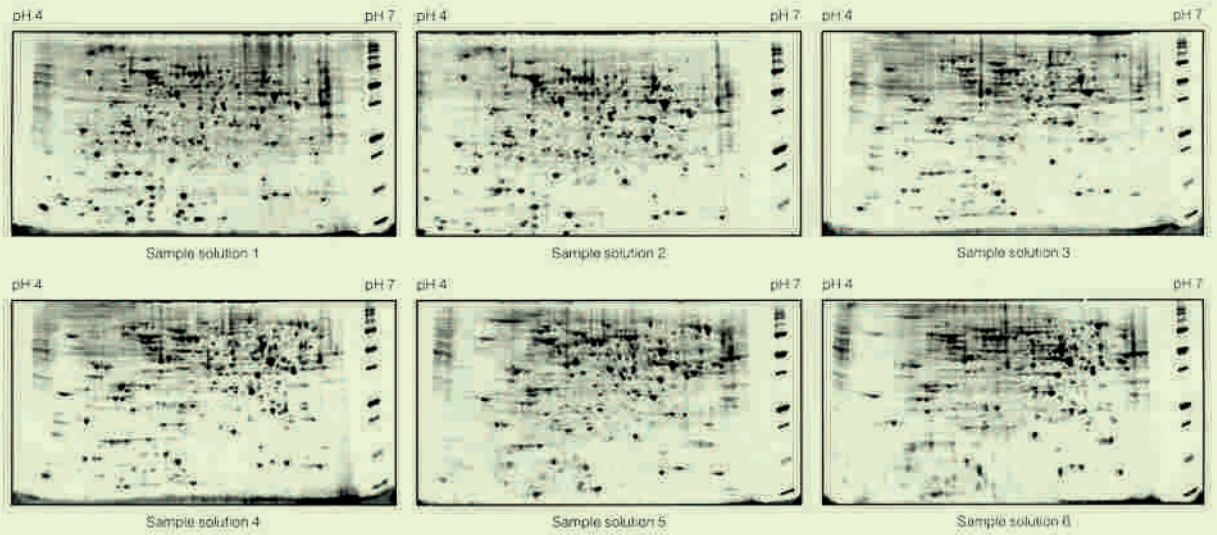

Fig 2. Effect of sample solution composition on 2-D electrophoresis of yeast mitochondrial membrane proteins. Sample solutions are as described in Table 1. First dimensions were run on 11 cm pH 4–7 IPG strips. Second dimensions were run on Criterion 8–16% Tris-HCl gels. A representative Oriole-stained gel from each replicate pair is shown.

Table 3. Effect of sample solution composition on spot counts.
Spot counts for the gels shown in Figure 2 were generated with PDQuest software using identical spot detection parameters for each gel.

Sample Solution	Spot Count
1	327
2	364
3	473
4	506
5	416
6	439

Focusing in 7 M urea, 2 M thiourea resulted in noticeably more spots, particularly among higher molecular weight proteins and those in the higher pH region of the gradient (Figure 3).

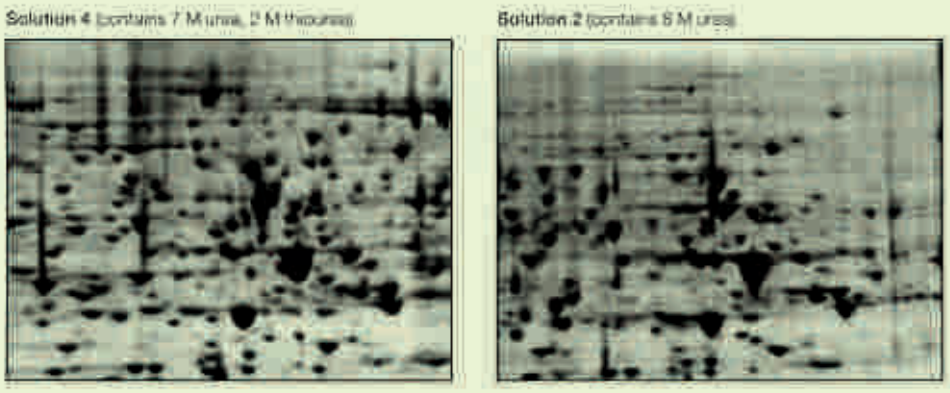

Fig 3. Effect of chaotrope mixture. Equivalent regions from two of the gels in Figure 2 are expanded to show increased protein representation, definition, and resolution as a consequence of the use of a urea/thiourea mixture (left) as opposed to urea alone (right).

The use of CHAPS as the detergent resulted in sharper, more distinct spots than C7BzO in the lower molecular weight, more acidic region of the 2-D gel (Figure 4).

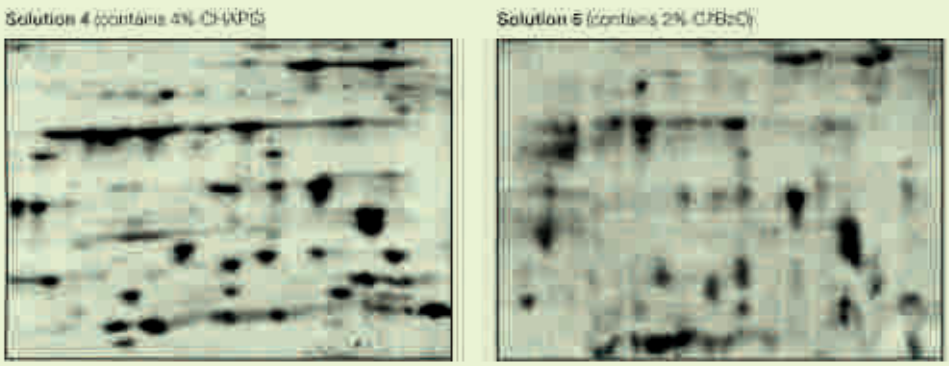

Fig 4. Effect of CHAPS and C7BzO in the sample solution. Equivalent regions from two of the gels in Figure 1 are expanded to show better separation resolution as a consequence of the use of CHAPS (left) as opposed to C7BzO (right).

The use of a higher carrier ampholyte concentration (0.6% Bio-Lyte 3/10) resulted in sharper, more distinct spots than did a lower concentration (0.2% Bio-Lyte 3/10) (Figure 5).

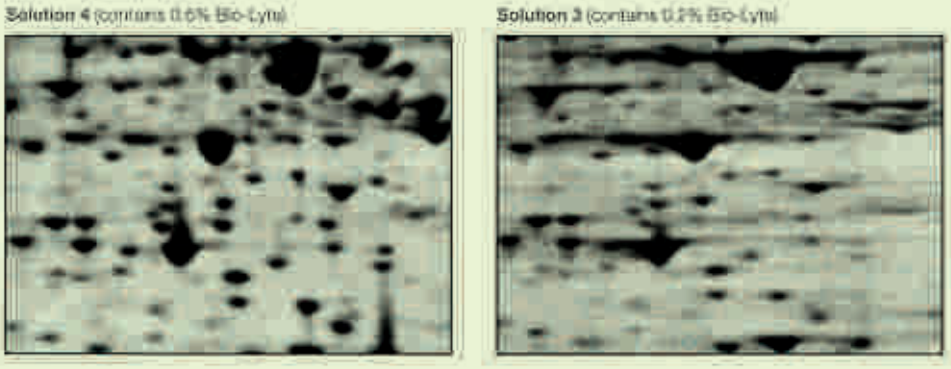

Fig 5. Effect of Bio-Lyte concentration. Equivalent regions from two of the gels in Figure 1 are expanded to show better separation resolution as a consequence of the use of 0.6% Bio-Lyte 3/10 (left) as opposed to 0.2% Bio-Lyte 3/10 (right).

Based on both spot count (Table 3) and general gel appearance (Figures 3–5), solution 4 (7 M urea, 2 M thiourea, 4% CHAPS, 0.6% Bio-Lyte 3/10) was selected as the optimal sample solution.

Effect of IEF protocol

Within the ranges tested, the IEF protocol had no discernable effect on the quality of the resulting 2-D pattern (Figure 6).

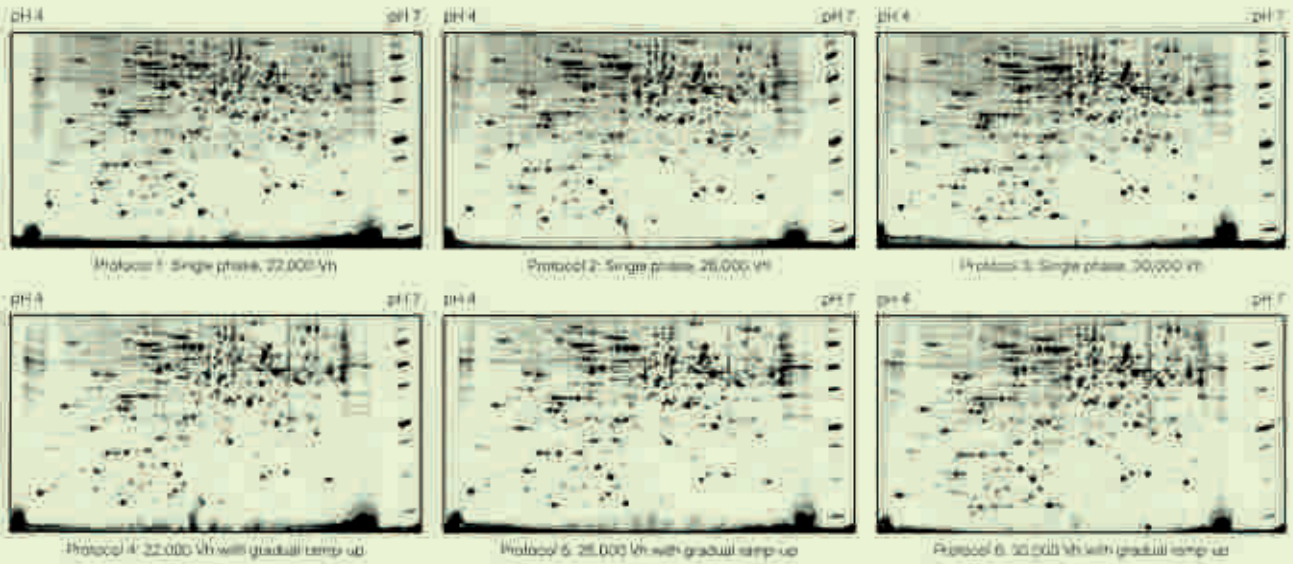

Fig 6. Effect of IEF protocol on 2-D electrophoresis of yeast mitochondrial membrane proteins. IEF protocols described in Table 2 were applied. First dimensions were run on 11 cm pH 4–7 IPG strips. Second dimensions were run on Criterion 8–16% Tris-HCl gels. A representative Oriole-stained gel from each replicate pair is shown.

Discussion and Conclusions

In most cases, the success of a 2-D electrophoresis experiment depends on the ability to resolve as many proteins as possible and to reproducibly detect changes in position or abundance. Optimization of the parameters affecting resolution and reproducibility is therefore often an essential step toward generating a useful experimental result.

The sample tested was an insoluble membrane protein fraction prepared from yeast cells by differential centrifugation. It was enriched in mitochondrial membranes and was therefore expected to contain membrane protein complexes that can be difficult to analyze by electrophoretic methods. The rationale for the variables tested in the composition of the rehydration solution came from literature reports on the ability of various additives to increase the resolution and representation of membrane proteins on 2-D gels. The use of urea-thiourea mixtures, as opposed to urea alone, is reported to result in an increased yield of distinguishable protein spots visible on 2-D gels, particularly when applied to membrane preparations (Rabilloud et al. 1997). Selection of the detergent present during IEF is also reported to influence representation of proteins on 2-D gels. CHAPS is the standard detergent used in 2-D electrophoresis (Görg et al. 2000, Friedman et al. 2009), however C7BzO is a representative of a class of zwitterionic detergents that is reported to provide increased solubilizing power for certain membrane proteins (Rabilloud et al. 1999). Carrier ampholytes have been reported to enhance protein solubility, particularly of membrane proteins, when used during IPG-IEF (Rimpilainen and Righetti 1985), so the effect of an elevated carrier ampholyte concentration was tested as well.

The PROTEAN i12 was invaluable in allowing rapid determination of optimal focusing conditions for this sample. It allowed effective optimization of the components of the sample solution in a single run. It was determined that the standard composition of 7 M urea, 2 M thiourea, and 4% CHAPS gave the best resolution and spot count. Additionally, it was determined that elevating the concentration of carrier ampholyte used from the standard 0.2% to 0.8% resulted in enhanced resolution and spot count. It also allowed the verification, in a single separate run, of the optimal focusing protocol for this particular sample and sample solution. Optimal results were achieved independent of total volt-hours or whether a phased voltage ramp was applied, and the preprogrammed protocol for the IPG strip length and pH range used was determined to be adequate. In comparison, conducting these experiments using one of the previous generation of commercial first-dimension IEF systems would have required several sequential experiments. As well as requiring considerably more time, this approach can introduce variation through different handling across sequential runs.

References

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Görg A et al. (2000). The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21, 1037–1053.

Rabilloud T et al. (1997). Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 18, 307–316.

Rabilloud T et al. (1999). Analysis of membrane proteins by two-dimensional electrophoresis: Comparison of the proteins extracted from normal or *Plasmodium falciparum* – infected erythrocyte ghosts. Electrophoresis 20, 3603–3610.

Rimpilainen MA and Righetti PG (1988). Membrane protein analysis by isoelectric focusing in immobilized pH gradients. Electrophoresis 8, 419–432.

High-throughput screening for antibodies and histidine-tagged proteins using Mag Sepharose™ magnetic beads

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Mag Sepharose magnetic beads have been developed to simplify handling in protein sample preparation. The beads are an excellent choice for enrichment, small-scale purification, and screening of target proteins. In this study, automated screening methods for purification of monoclonal antibodies and histidine-tagged proteins are described. A combination of Mag Sepharose magnetic beads in a 96-well microplate, MagnaBot™ 96 Magnetic Separation Device and Tecan Freedom EVO™ liquid handling workstation was used.

Introduction

The Mag Sepharose platform combines well-established enrichment and purification methods with the magnetic bead format to provide high quality and reproducible results. The magnetic beads are scalable and provide simple capture of target protein in small or large sample volumes, from low microliter to high milliliter scale. Mag Sepharose magnetic beads are suitable for enrichment, small-scale purification and screening of target proteins such as in screening for optimal purification conditions.

Reproducible purification

Combining Tecan Freedom EVO, MagnaBot 96 Magnetic Separation Device, and Protein G Mag Sepharose Xtra ensures high reproducibility. To demonstrate this, 96 replicate runs on a 96-well microplate filled with Protein G Mag Sepharose Xtra beads were performed to purify a monoclonal human IgG expressed in CHO cells. The load was 60% of the total binding capacity and the yield of the eluted fractions was determined via absorbance measurements.

Figure 1 shows the yield of the monoclonal human IgG. The results show good well-to-well reproducibility with low relative standard deviation (RSD) of 4.6%. All 12 samples from wells H 1–12 in the 96-well microplate had a lower amount of eluted monoclonal human IgG. This reduced amount of eluted human IgG Indicates a systematic error but no further investigation was made. The relative standard deviation for 96 samples was only 3.0% when omitting the 12 samples from wells H 1-12.

Flexible purification

Adapting the purification scale to different sample volumes is one advantage with the magnetic bead format. In this study, different volumes of magnetic beads were scaled down from 5 µl magnetic beads to 1 µl. Human IgG was purified using Tecan Freedom EVO, MagnaBot 96 Magnetic Separation Device, and Protein G Mag Sepharose Xtra filled in 96-well plates. The yield and recovery of the eluted fractions was determined by absorbance measurements. The purification showed good well-to-well variation with relative standard deviations (Fig 2).

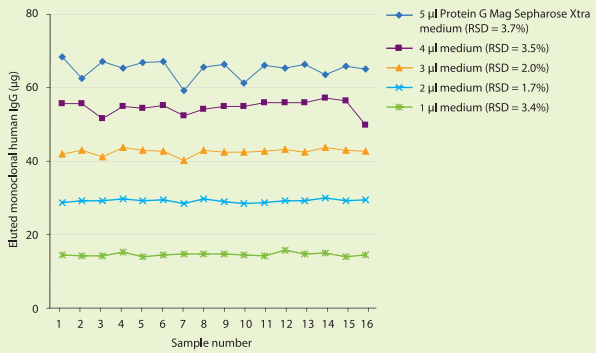

Fig 2. Human IgG was loaded with 50% of the total binding capacity for each magnetic bead volume. The yield and recovery of the eluted fractions was determined by absorbance measurements. The results showed good well-to-well variation with low relative standard deviations.

Screening for optimal IMAC purification conditions

Purification of histidine-tagged proteins by immobilized metal ion adsorption chromatography (IMAC) is a balance between yield and purity, modulated by the imidazole concentration in the sample and binding/wash buffer. The optimal imidazole concentration is protein dependent and can be determined for each histidine-tagged protein.

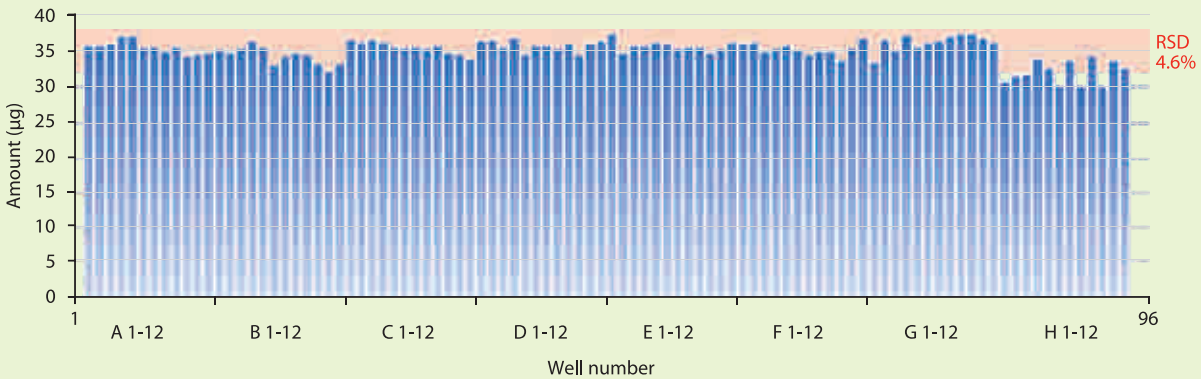

Fig 1. Elution reproducibility over 96 runs of monoclonal human IgG purified from CHO cells using Protein G Mag Sepharose Xtra magnetic beads.

A screening study for optimal sample loading and wash conditions was performed with eight different imidazole concentrations and four different sample loads, varying from 25% to 100% of the total binding capacity of His Mag Sepharose Ni. Histidine-tagged green fluorescent protein, GFP-$(His)_6$, was purified from Escherichia coli lysate using Tecan Freedom EVO, MagnaBot 96 Magnetic Separation Device, and His Mag Sepharose Ni filled in 96-well plates. The yield and purity of the eluted fractions was determined by absorbance measurements and SDS-PAGE analysis, respectively.

The results showed that during sample application and wash, a good balance between yield and purity was obtained with 40 mM imidazole (Fig 3) and a sample load of 50% to 100% of the total binding capacity (Fig 4).

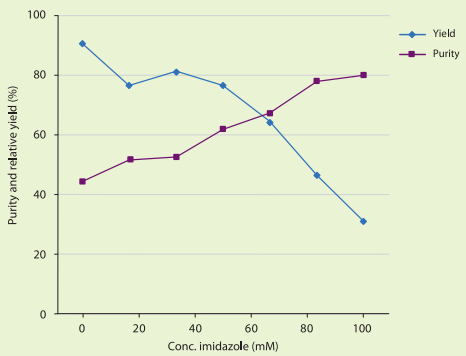

Fig 3. Purity and relative yield of GFP-$(His)_6$ with a 50% sample load relative to the total binding capacity of His Mag Sepharose Ni.

Conclusions

Mag Sepharose magnetic beads filled in 96-well plates and combined with robotics provide an excellent platform for screening of protein constructs and conditions that can be tested in drug development and structural studies.

Using screening methodology with Mag Sepharose beads combined with robotics enables highly reproducible results, as well as a fast and cost-effective screening with limited effort.

To see an animation demonstrating the practical use of His Mag Sepharose Ni beads, visit http://www.gelifesciences.com/aptrix/upp01077.nsf/content/sample_preparation~news~mag_sepharose?OpenDocument&intcmp=ibc000005

Ordering information

Product	Code number
Protein G Mag Sepharose Xtra, 2 × 1 ml	28-9670-66
Protein G Mag Sepharose Xtra, 5 × 1 ml	28-9670-70
His Mag Sepharose Ni, 2 × 1 ml	28-9673-88
His Mag Sepharose Ni, 5 × 1 ml	28-9673-90
His Mag Sepharose Ni, 10 × 1 ml	28-9799-17
MagRack 6	28-9489-64

For more information on the Mag Sepharose platform, visit www.gelifesciences.com/sampleprep

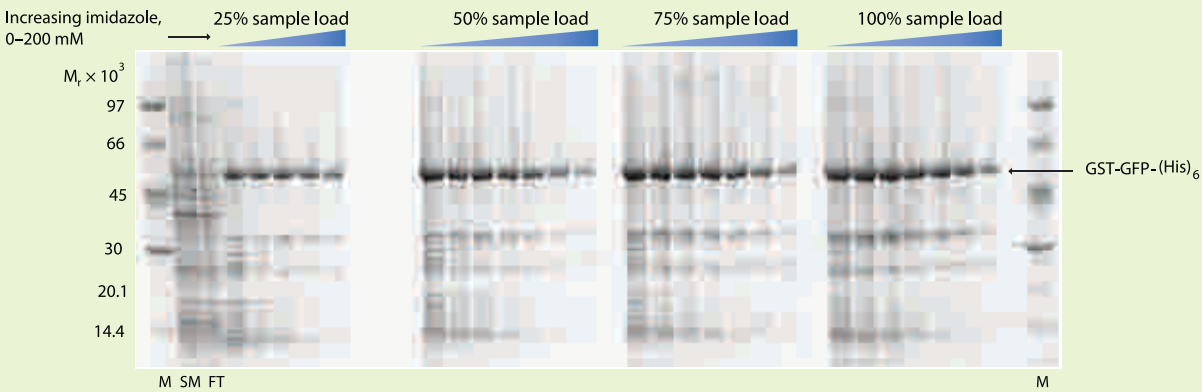

Fig 4. SDS-PAGE of GFP-$(His)_6$ enriched from a background of E. coli protein using His Mag Sepharose Ni beads. GFP-$(His)_6$ was detected using Deep Purple™ Total Protein Stain and Ettan™ DIGE Imager. The gel image was analyzed with ImageQuant™ TL software. M = molecular weight markers, SM = start material, FT = flowthrough from a sample load of 75% of the total binding capacity, 40 mM imidazole.

International Associations / Talks

Dr Niranjan Chakraborty gave an invited talk on at the 10th HUPO Congress held at Geneva, Switzerland, Sept 2011

Dr Shubhra Chakraborty, nominated to the Council of Asia Oceana Agricultural Proteomics Organization (AOAPO)

Dr Ravi Sirdeshmukh was elected to the Board of Central and Eastern European Proteomics Conference (CEEPC) forum.

Dr Ravi Sirdeshmukh gave an invited talk on Advances in Differential Proteomics of Gliomas – New Technologies and Approaches, at the 5th Central and Eastern European Proteomics Conference held in Prague, Czech Republic, September 2011.

Upcoming Events

- Workshop on Proteomics and the Translational Front (Shankar Netralaya, Chennai)
- Proteomics Methods in Cancer Research (Institute of Bioinformatics,Bangalore)

Tentative timing (Jan - June 2012). Exact dates will be announced later.

AOHUPO 6th Congress on Proteomics :

Better for life Beijing, China, May 5-7, 2012.
Registration is on.*

The congress aims to showcase progress within the field of proteomics and related disciplines, to promote international collaboration within Asia and the Pacific, and also to welcome scholars from all over the world to join the pageant and share their expertise. In keeping with this theme, a wide range of topics will be covered in various formats including workshops, speaker sessions and poster sessions. Over one thousand scientists are expected to attend this conference. You can plan enjoy the science, the warm friendship and the rich cultural activities in Beijing.