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Automated 2D Peptide Separation on a 1D Nano-LC-MS System

Paul Taylor,[†] Peter A. Nielsen,[‡] Morten B. Trelle,[‡] Ole B. Hørning,[‡] Michael B. Andersen,[‡] Ole Vorm,[‡] Michael F. Moran,[†,§,||] and Thomas Kislinger*[,⊥,▽]

Program in Molecular Structure and Function, Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada, Proxeon Biosystems, Odense, Denmark, Department of Molecular Genetics, University of Toronto, Toronto, Ontario, M5S 1A8, Canada, McLaughlin Centre for Molecular Medicine, Department of Medical Biophysics, University of Toronto, Toronto, Ontario, M5G 1L7, Canada, and Cancer Genomics & Proteomics, Ontario Cancer Institute, Toronto, Ontario, M5G 2M9, Canada

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Abstract: Given the complexity of the mammalian proteome, high-resolution separation technologies are required to achieve comprehensive proteome coverage and to enhance the detection of low-abundance proteins. Among several technologies, Multidimensional Protein Identification Technology (MudPIT) enables the on-line separation of highly complex peptide mixtures directly coupled with mass spectrometry-based identification. Here, we present a variation of the traditional MudPIT protocol, combining highly sensitive chromatography using a nanoflow liquid chromatography system (nano-LC) with a two-dimensional precolumn in a vented column setup. When compared to the traditional MudPIT approach, this nanoflow variation demonstrated better first-phase separation leading to more proteins being characterized while using rather simple instrumentation and a protocol that requires less time and very little technical expertise to perform.

Keywords: Proteomics • MudPIT • nano-LC • 2D peptide separation • vented column

Introduction

One of the goals of proteome research is the spatial and temporal detection of all proteins in a qualitative and quantitative manner.[1−3] Despite significant improvements in mass spectrometry technology, automated peptide identification algorithms and bioinformatics data mining strategies in recent years, the complexity of biological samples remains a daunting challenge and *whole proteome* analyses are currently not possible. A multitude of analytical platforms have been introduced to improve proteome coverage, all aimed at minimizing sample complexity and hence increasing the detection of lower-abundance proteins by MS-based proteomics. Commonly used technologies include (i) two-dimensional gel electrophoresis (2D-PAGE), in which proteins are separated by two consecutive electrophoretic dimensions, partitioning proteins based on their isoelectric point and molecular mass, respectively.[4,5] Separated spots are excised, in-gel digested and analyzed by MS; (ii) gel-enhanced LC-MS,[6] in which proteins are first separated by SDS-PAGE according to their molecular mass. Then, the entire gel is cut into defined sections, which are in-gel digested and analyzed by LC-MS; (iii) off-line 2D-LC, where traditional chromatographic separations (i.e., ion-exchange chromatography, reversed-phased chromatography, etc.) are used to separate proteins or peptides into a user defined number of fractions which are in turn analyzed by LC-MS;[7,8] (iv) online capillary LC-MS is used to separate peptide mixtures in narrow fused silica chromatography columns (∼75−100 μm) packed with reversed-phase resin. Peptides elute directly into a MS where they are identified by tandem mass spectrometry (hence on-line).[9] This technology was vastly improved by Yates and colleagues who developed a technology termed Multidimensional Protein Identification Technology (MudPIT).[10−12] In MudPIT, fused silica emitters are packed with two orthogonal chromatographic resins, a strong cation exchange (SCX) resin in the first dimension and a reversed-phase (RP) resin in the second dimension. Briefly, protein extracts of interest (i.e., cell homogenates, organelles, tissue lysates) are digested with trypsin and peptide mixtures are bound to the SCX resin. A step-sequence consisting of defined salt bumps elute a subset of the bound peptides from the SCX resin to the RP material from which they are eluted directly into the MS by a water/acetonitrile gradient. This separation reduces the sample complexity entering the mass spectrometer to a level with which the instrument can deal. Traditionally, MudPIT columns are packed and peptides are loaded off-line using a pressure vessel. Chromatographic separations are accomplished using quaternary HPLC systems with a precolumn split effectively reducing the flow-rate to ∼400 nL/min. Despite the tremendous success of MudPIT (in terms of providing data with superior information content relative to one-dimensional separation), relatively few laboratories are currently using this technology, which might be due to specific expertise required to optimally utilize this system. More recently, variations of the traditional MudPIT protocol, employing tetraphasic precolumns in a vented column setup in high flow-rate mode, have been presented.[13]

* To whom correspondence should be addressed. Thomas Kislinger, Toronto Medical Discovery Tower, 101 College Street, Room 9-807, Toronto, ON, M5G 1L7, Canada. Tel.: 416-581-7627. Fax: 416-581-7629. E-mail: thomas.kislinger@utoronto.ca.

[†] Program in Molecular Structure and Function, Hospital for Sick Children.
[‡] Proxeon Biosystems.
[§] Department of Molecular Genetics, University of Toronto.
[||] McLaughlin Centre for Molecular Medicine.
[⊥] Department of Medical Biophysics, University of Toronto.
[▽] Cancer Genomics & Proteomics, Ontario Cancer Institute.

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Here, we present an automatable variation of the traditional MudPIT technology combining highly reproducible and sensitive splitless nanoflow HPLC technology with a vented two-dimensional column setup. Comparison with the traditional MudPIT technology demonstrated a measurable performance improvement when using this modified two-dimensional peptide separation methodology. The combination of fast sample loading from a microplate autosampler in a vented column setup, with highly sensitive splitless nanoflow chromatography will allow investigators with lesser experience in LC-MS to utilize this technology and significantly increase the number of detected proteins. Using the same column for multiple samples allows for retention time reproducibility that is lacking in traditional approaches, allowing postacquisition software to better compare data sets. We also introduce a shorter modification of the full 2D experiment, which allows for 3 samples to be fully characterized in a 24 h period with little compromise, enabling projects consisting of larger sample sets to be entertained.

Materials and Methods

Materials. Ultrapure grade urea, ammonium acetate, calcium chloride, HEPES and TRIS were from BioShop Canada, Inc. (Burlington, ON, Canada). Ultrapure grade iodoacetamide, DTT and formic acid were obtained from Sigma. HPLC grade solvents (methanol, acetonitrile and water) were obtained from Fisher Scientific. Recombinant, proteomics grade trypsin was from Roche Diagnostics (Montreal, QC, Canada). OMIX solid phase extraction pipet tips were from Varian (Mississauga, ON, Canada).

Protein Extraction and Digestion. Primary mouse cardiomyocytes were prepared and cultured as previously described.[14] Cells were homogenized in lysis solution (250 mM Sucrose, 25 mM HEPES, pH 7.4, and 20 mM KCl) followed by centrifugation at 14 000 rpm for 15 min. The supernatant was used for analysis (see below). An aliquot of 150 μg of total protein (determined by Bradford assay) was precipitated overnight with 5-vol of ice-cold acetone followed by centrifugation at 14 000 rpm for 15 min. The protein pellet was solubilized in 8 M urea, 2 mM DTT, and 50 mM Tris-HCl, pH 8.5, at 37 °C for 1 h, followed by carboxyamidomethylation with 10 mM iodoacetamide for 1 h at 37 °C in the dark. Samples were diluted with 50 mM ammonium bicarbonate, pH 8.5, to ~1.5 M urea. Calcium chloride was added to a final concentration of 1 mM and the protein mixture was digested with a 1:30 molar ratio of recombinant, proteomics grade trypsin at 37 °C overnight. The resulting peptide mixtures were solid phase-extracted with Varian OMIX cartridges (Mississauga, ON, Canada) according to the manufacturer's instructions, and stored at −80 °C until further use.

The multiple myeloma cell line OPM2 (Gift from Suzanne Trudel) was grown in Iscove's Modified Dulbecco's Media (IMDM; Gibco, Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 μg/mL) at 37 °C and 5% CO_2. Proteins were extracted from harvested cells using 1% Triton X-100 (Sigma) lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM sodium fluoride, 100 mM sodium phosphate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM PMSF, and 1 mM sodium pervanadate). The cysteine reduction and alkylation, tryptic digestion and protein purification were as described above.

Chromatography. 1D Chromatography. Peptides were loaded on a 3 cm precolumn (150 μm i.d.) containing a Kasil frit packed with 5 μm Magic C18 100 Å reversed-phase material (Michrom Bioresources). The precolumn was connected via a titanium micro-tee splitter fitted with an electrode for voltage application (i.e., vented column system)[15] to a 10 cm fused silica microcapillary analytical column (75 μm i.d.) with a homemade laser-pulled spray tip packed with 5 μm Magic C18 100 Å reversed-phase resin.

Traditional MudPIT. A fully automated 9-cycle MudPIT procedure was set up similar as previously described.[11,16,17] A quaternary HPLC-pump was interfaced with a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a nanoelectrospray source (Proxeon Biosystems, Odense, Denmark). A 100 μm i.d. fused silica microcapillary column was packed with ~7 cm of 5 μm Magic C18 100 Å reversed-phase material (Michrom Bioresources), followed by ~5 cm of Luna 5 μm SCX 100 Å strong cation exchange resin (Phenomenex, Torrance, CA). Samples were loaded manually using an in-house pressure vessel. Columns were placed in-line with a Surveyor quaternary HPLC system (Thermo Fisher Scientific, San Jose, CA) using a precolumn split and a three buffer system (Buffer A, water, 0.1% formic acid; Buffer B, 100% acetonitrile and 0.1% formic acid; Buffer C, 500 mM ammonium acetate in buffer A). HPLC gradients were as previously described using salt bumps of 0%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, and 100% followed by a water/acetonitrile gradient. Peptides that eluted from the microcapillary columns were electrosprayed directly into the MS. A distal 2.3 kV spray voltage was applied to the microsplitter tee (Proxeon Biosystems). The MS operated in a cycle of one full-scan mass spectrum (400−1800 m/z), followed by 6 data-dependent MS/MS spectra at 35% normalized collision energy, which was continuously repeated throughout the entire MudPIT separation. The MS functions and the HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Fisher Scientific, San Jose, CA).

2D Vented Column Setup. A fully automated 9-cycle two-dimensional chromatography sequence was set up identical as described above. Peptides were loaded on a 7 cm precolumn (150 μm i.d.) containing a Kasil frit packed with 3.5 cm 5 μm Magic C18 100 Å reversed-phase material (Michrom Bioresources) followed by 3.5 cm Luna 5 μm SCX 100 Å strong cation exchange resin (Phenomenex, Torrance, CA). Samples were automatically loaded from a 96-well microplate autosampler using the EASY-nLC system (Proxeon Biosystems, Odense, Denmark) at 3 μL/min. The precolumn was connected to an 8 cm fused silica analytical column (75 μm i.d.) via a microsplitter tee (Proxeon Biosystems) to which a distal 2.3 kV spray voltage was applied. The analytical column was pulled to a fine electrospray emitter using a laser puller. For the peptide separation on the analytical column, a water/acetonitrile gradient was applied at an effective flow rate of 400 nL/min, controlled by the EASY-nLC. Ammonium acetate salt bumps (8 μL) were applied at the identical concentrations as above, using the 96-well microplate autosampler at a flow-rate of 3 mL/min in a vented-column setup. Samples were analyzed on the LTQ-Orbitrap XL under the identical instrument setup as described above. A shorter variant of the 2D vented column experiment was performed essentially as described with the following exceptions; data collection was for only 60 min during the sample loading phase and only 3 salt elution steps were used at 25, 100, and 500 mM ammonium acetate.

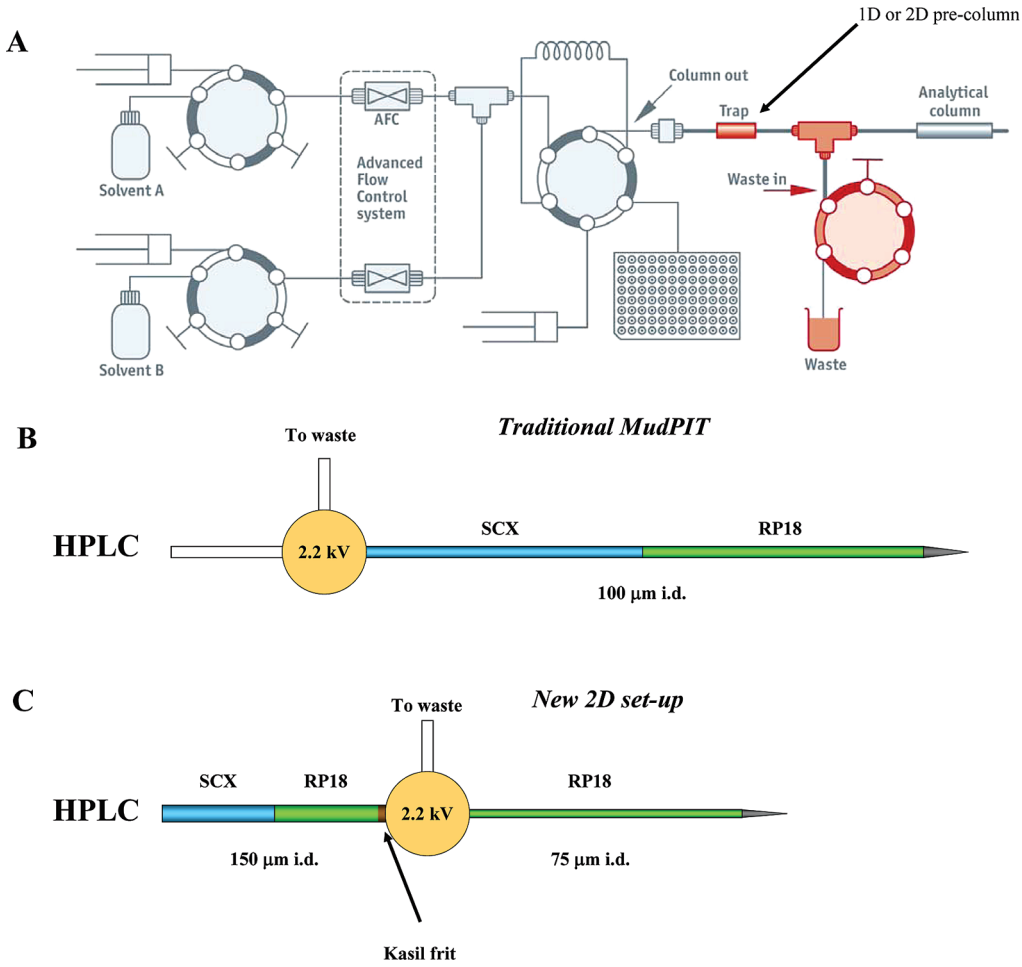

Figure 1. Instrumental setup. (A) Shown is the very simple and user-friendly instrumental setup of the Easy-LC operation. (B) Schematic of the traditional MudPIT procedure. (C) Schematic of the peptide separation using a vented two-dimensional precolumn setup.

Mass Spectrometry. All samples were analyzed on a LTQ-Orbitrap XL. The instrument method consisted of one MS full scan (400−1800 m/z) in the Orbitrap mass analyzer, an AGC target of 500 000 with a maximum ion injection of 500 ms, 1 μscan and a resolution of 60 000 and using the preview scan option. Six data-dependent MS/MS scans were performed in the linear ion trap using the six most intense ions at 35% normalized collision energy. The MS and MS/MS scans were obtained in parallel. AGC targets were 10 000 with a maximum ion injection time of 100 ms. A minimum ion intensity of 1000 was required to trigger a MS/MS spectrum. The dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count with a repeat duration of 30 s and exclusion duration of 45 s.

Protein Identification and Data Analysis. Tandem mass spectra were extracted by BioWorks version 3.3.1 (ThermoFisher, San Jose, CA). All MS/MS samples were analyzed using Sequest (ThermoFisher, San Jose, CA) and X! Tandem (www.thegpm.org; version 2007.01.01.2). The databases used were the Human IPI database (version 3.41, 72 155 entries) for the OPM2cells and the Mouse IPI database (version 3.41, 53 204 entries) for the murine cardiomyocytes cytoplasm preparations. Sequest and X! Tandem were searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 0.010 Da. Complete tryptic digestion was assumed. The iodoacetamide derivative of cysteine was specified in Sequest and X! Tandem as a fixed modification. The oxidation of methionine was specified in Sequest as a variable modification. The oxidation of methionine and the phosphorylation of serine, threonine and tyrosine were specified in X! Tandem as variable modifications.

Scaffold (version Scaffold 2.1.1, Proteome Software, Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.[18] Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[19] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Results and Discussion

Experimental Setup. As mentioned above, the MudPIT technology was a ground breaking discovery for the proteomics community, both in its traditional[11] and vented column setup.[13] Here, we extended on these concepts by both implementing new and improved HPLC technology (i.e., splitless nanoflow system) and most importantly by simplifying the experimental setup to a *plug-and-play* system. We demonstrate a slightly improved performance of this highly automatable method. In Figure 1, the experimental overview of our 2D

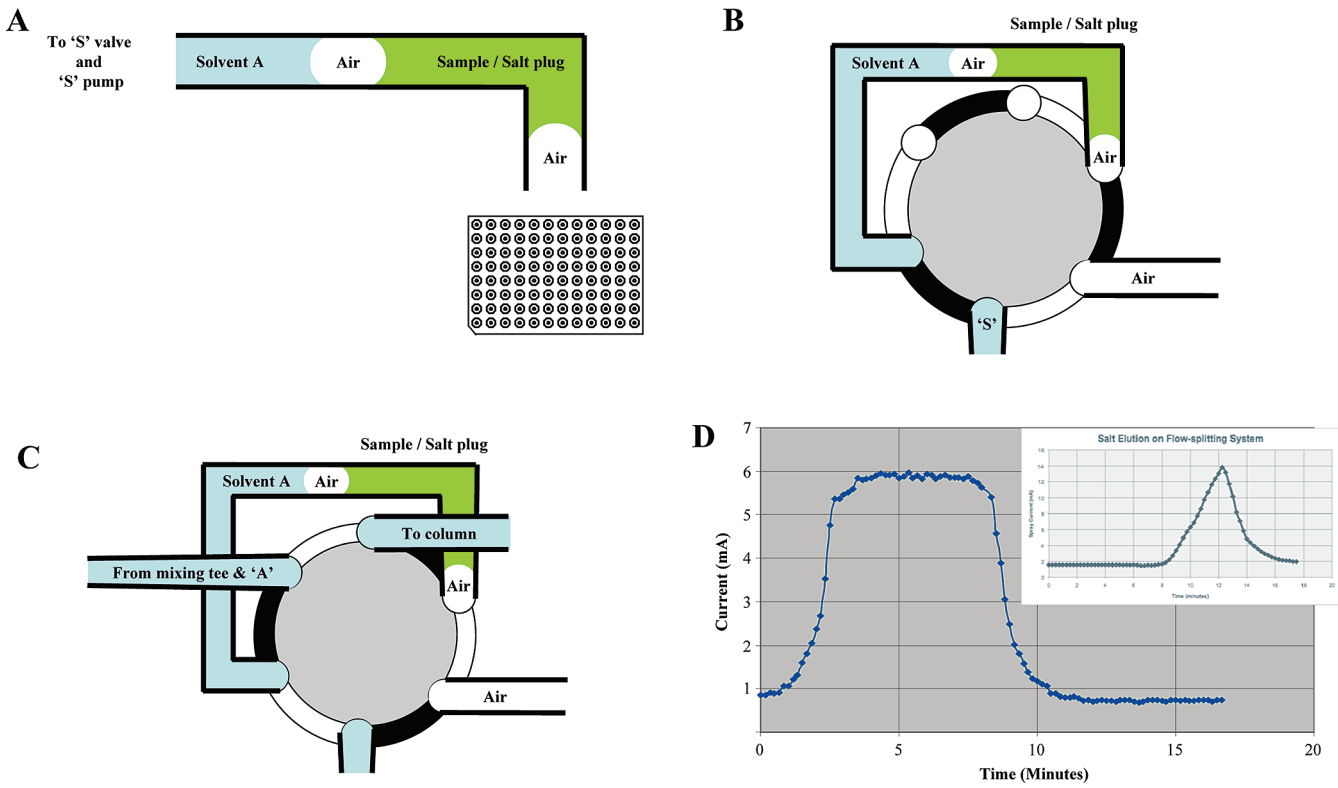

Figure 2. Nano-LC autosampler schematics. (A) The sample or salt plug is sandwiched between small air aliquots to prevent dilution by the carrier liquid. (B) Sample valve shown in the load position. (C) Sample valve shown in the inject position, placing the sample or salt plug into the high-pressure solvent stream. (D) The spray current at a column tip from the split-free system, the spray current is proportional to the salt concentration at the tip. The inset in panel D shows a similar spray current experiment measure using the traditional MudPIT protocol.

method is presented. The heart of the system is two syringe pumps enabling a reproducible flow-rate in the low nanoliter per minute (nL/min) range and a 96-well microplate autosampler for high sample throughput. Importantly, a single column out line connects the HPLC to the 2D precolumn which in turn is connected to the analytical column through a titanium tee for high voltage application. A single waste line enables high-flow sample loading (up to 7 μL/min). This methodology eliminates the sophisticated setup of earlier vented column MudPIT approaches,[13] while enabling true nanoflow HPLC separations and significantly simplifying this methodology to earlier applications.

Validation of the Salt Delivery Using a 1D Chromatography System. The total spray ion current delivered to the mass spectrometer during the salt elution phase was measured to determine the characteristics of the salt plug produced. The ion current at the spray tip is proportional to the salt concentration of the solution, higher salt concentrations producing larger currents. Figure 2D shows the ion current plotted as a function of time. The elution profile demonstrated approximates the ideal square form, which would occur if no mixing occurred. The inset in Figure 2D shows a similar experiment using the flow-split quaternary pump system used for the traditional on-line 2D experiments in this study. The square spray current pattern created by the split-free method represents a known salt concentration delivered with a predictable flow rate, whereas the v-shaped salt elution pattern in the split-flow system is indicative of the mixing which occurs in the high flow rates of the splitting process. Moreover, the salt concentration at the apex of the v-shape curve will be difficult

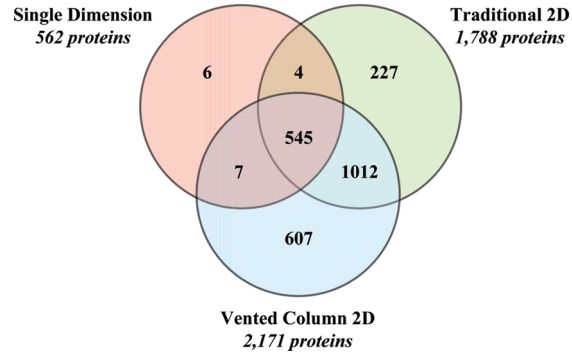

Figure 3. Venn diagram of the number of proteins identified in each experiment. The total number of proteins identified using each approach is listed.

to control and reproduce since it is a function of flow-rates, back pressures and viscosity (which increases for each step as one aims to increase the salt concentration). Because of the well-controlled delivery of the salt steps in the split-free system, a more complete delivery of peptides from the SCX resin to the RP resin is achieved and should result in less tailing of peptides between SCX fractionation steps leading to less repeat MS/MS spectra for the high-abundance ions. The split-free system is set to deliver a constant flow rate and will adjust pump pressure to do so; the traditional quaternary pump split-flow systems also operates at a constant flow rate (100 μL/min), although, due to the precolumn split, the effective column flow rate will change in response to pressure changes in a way that cannot be effectively controlled. Since retention times are a function of solvent composition and volume delivered across

Table 1. The 15 Most Abundant Proteins Identified in Lysates from a Human Multiple Myeloma Cell Line[a]

hit no.	identified proteins	accession no.	unique peptides		total spectra	
			split-free	split-flow	split-free	split-flow
1	GAPDH Glyceraldehyde- 3-phosphate ehydrogenase	IPI00219018	18	17	600	1860
2	ENO1 Isoform alpha-enolase of Alpha-enolase	IPI00465248	28	28	338	1287
3	ACTB Actin, cytoplasmic 1	IPI00021439	16	16	437	1083
4	TUBB2C Tubulin beta-2C chain	IPI00007752	23	19	229	1079
5	VIM Vimentin	IPI00418471	50	48	490	787
6	HSPD1 60 kDa heat shock protein, precursor	IPI00784154	38	39	431	822
7	HIST2H2BE Histone H2B type 2-E	IPI00003935	8	6	374	875
8	HIST1H4E	IPI00453473	12	10	234	987
9	FASN Fatty acid synthase	IPI00026781	76	72	433	605
10	ALDOA Fructose-biphosphate aldolase A	IPI00465439	22	18	133	989
11	HSP90AA1 heat shock protein 90 kDa alpha (cytosolic)	IPI00382470	43	47	367	551
12	LDHA Isofrom 1 of L-lactate dehydrogenase A chain	IPI00217966	18	17	109	774
13	TUBA4A Tubulin alpha-4A chain	IPI00007750	21	16	241	599
14	TPI1 Isofomr 1 of Triosephosphate isomerase	IPI00465028	19	16	91	783
15	HSP90B1 Endoplasmin precursor	IPI00027230	45	43	233	456

[a] The unique peptides identified in the split-free (vented column) or split-flow (traditional MudPIT) methods are shown, as well as the total number of MS/MS spectra which could be associated with each protein.

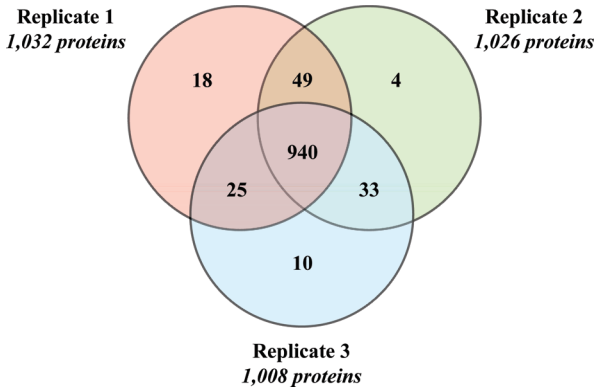

Figure 4. Venn diagram derived from 3 technical replicates of the vented precolumn 2D analysis. The total number of proteins identified in each replicate is listed.

the analytical column, pressure increases due to sample impurities will cause the split-flow systems to show increased apparent retention times compared to constant volume systems. This can complicate the alignment of peaks when running sample comparison analysis such as the label-free quantitation approaches.

Comparison with the Traditional On-Line MudPIT Approach. A tryptic digest of proteins derived from a human multiple myeloma cell line was used to determine the efficacy of single versus multiple dimension peptide separation as well as evaluating the relative performance of the two multidimensional protocols under discussion. The number of proteins identified using a given amount of sample is larger using either of the 2D protocols when compared to a single 1D reverse-phase separation. When the database and acceptance criteria outlined in Materials and Methods was used, 562 proteins were identified using only one dimension, and 1788 proteins and 2171 proteins were identified using the traditional split-flow system and the split-free nano LC systems, respectively. Figure 3 is a Venn diagram indicating the identification overlaps. The proteins identified by the 1D approach were essentially a subset of the proteins identified by either 2D approach, although repeat analyses of the 1D separation to achieve the same MS analysis time as in the 2D separation would likely increase the number of identified proteins by ~20−30% due to the random sampling effect of data-dependent MS analysis.[16,20]

The number of times any given ion is selected for MS/MS indicates how often that peptide is present to a significant level in the reverse-phase column eluent. Table 1 shows the 15 most abundant proteins in this study with respect to the number of unique peptides found, as well as the total MS/MS spectra assigned to that protein. It is clear that more mass spectrometer time is being spent fragmenting the most abundant ions using the traditional MudPIT system. In total, 2.85 times more sequencing events are spent on identifying the top-15 proteins using the traditional split-flow system relative to the split-free nanoflow system. This will result in less time available for the fragmentation of peptides of lower-abundance proteins. Note that the number of unique peptides for each protein is generally greater using the split-free nano-LC system (e.g., 6% greater when averaged over the 15 most abundant proteins). This observation would be expected if there were a more complete delivery of peptides from the SCX column to the reverse-phase resin, that is, less tailing in the SCX separation.

Reproducibility of Replicate Analyses. To ascertain the reproducibility of technological replicates using the split-free 2D approach, three identical 2D analyses were performed on a tryptic digest of a cytosolic preparation isolated from mouse cardiomyocytes. Figure 4 shows a Venn diagram of the proteins identified in this experiment. Of the 1079 proteins identified in total, 87% of the proteins were detected in all three replicates with 97% of the proteins accounted for in at least 2 of the 3 analyses. Figure 5 shows the reverse-phase total ion chromatograms (TICs) for the 25 mM ammonium acetate elution salt "bump" from the three technical replicates of the myocardial cytosolic samples.

Reduced Step Variant of the Split-free Experiment. Mass spectrometer time is often a major consideration in experimental design. It is a common occurrence to have larger sample sets than can be reasonably analyzed within the given projects budget. Quite often, a single dimension approach is employed as a compromise, but this can result in the characterization of only the 500−600 most abundant proteins in the sample. We modified the full 9-step protocol presented in this study to address such sample sets. If the reverse-phase elution (i.e., the gradient) following sample loading step is reduced to 60 min and a simple 3 salt step approach is used (acidic, neutral and basic peptides), we are able to analyze 3 samples per 24 h

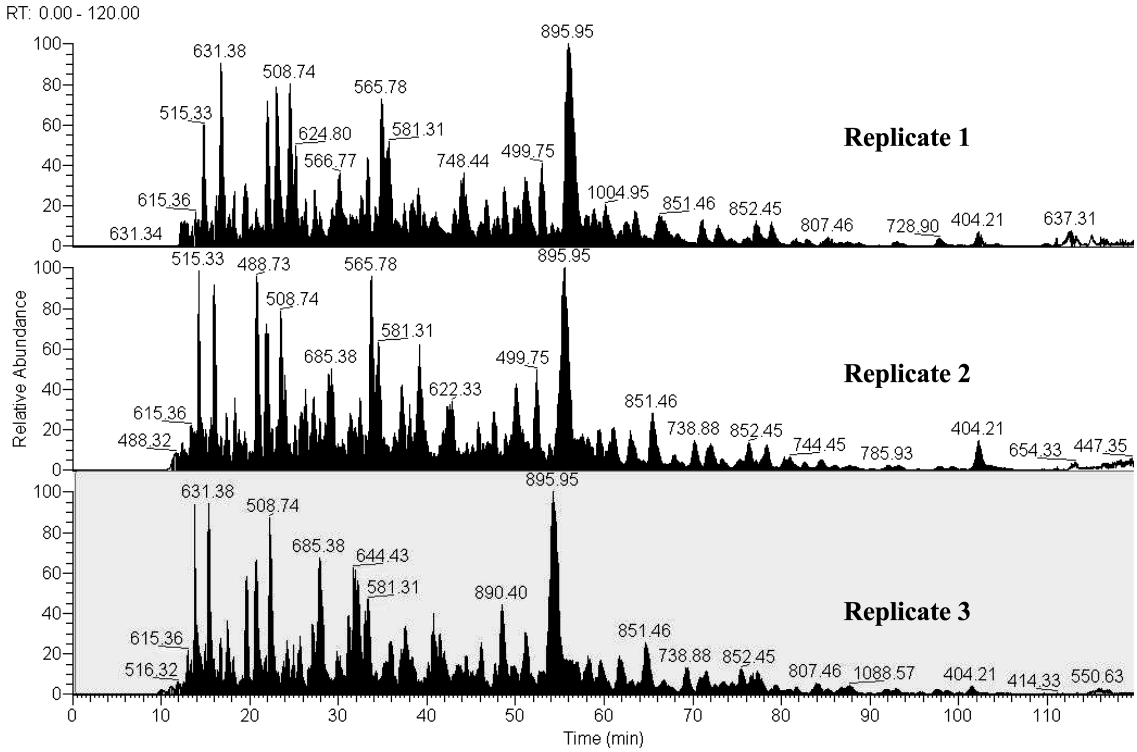

Figure 5. The reverse-phase second-dimension base peak total ion current (TIC) chromatograms from 3 technical replicates of peptides from mouse myocardial cytoplasm. The peaks are labeled with the *m*/*z* value of the most prominent ion in the peak.

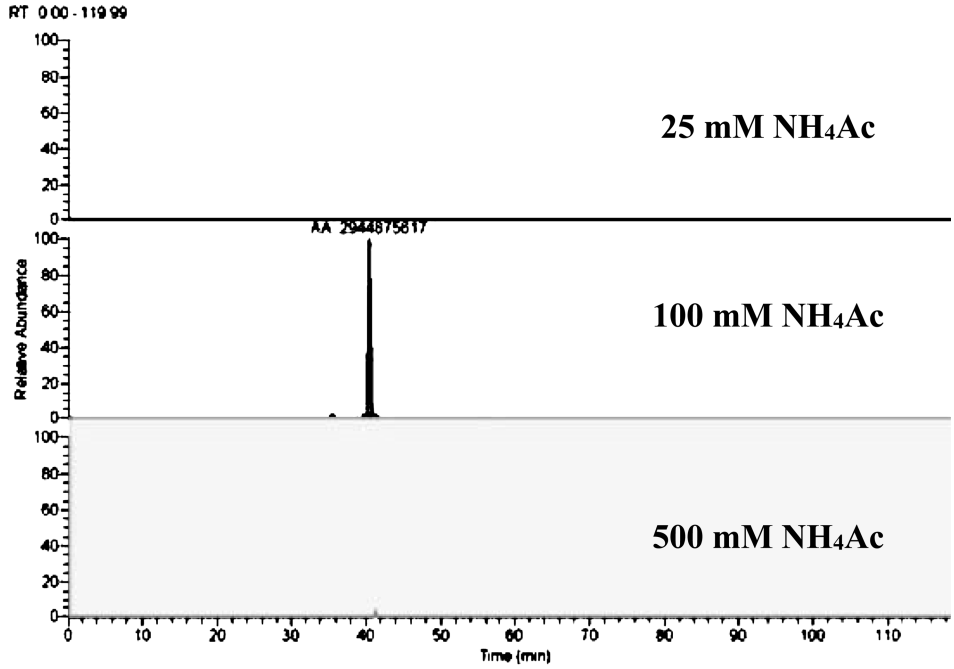

Figure 6. An extracted ion chromatogram (XIC) of a single ion (the doubly charged form of the peptide AEGPEVDVNLPK) from Neuroblast differentiation-associated protein AHNAK in each of the 3 salt elution steps from the three step modification of the vented 2D-precolumn experiment. The peak is labeled with the relative area.

period in a 2D fashion compared to one sample per 20 h period with either the 9-step split-free 2D or traditional MudPIT methods. The same sample of OPM2 lysate was processed using this modified protocol and 1533 proteins were identified using the acceptance criteria outlined. Figure 6 shows an extracted ion current (XIC) chromatogram of a single peptide ion in the 3-step experiment. The ion is almost completely eluted in the second step. These XIC chromatograms can be obtained for any ion (whether it has been identified or not) and used for relative quantitative purposes, either manually one at a time or by using any of the label-free quantitative software packages.

Conclusion

This study describes a simple automated approach to multidimensional peptide separation using strong cation ex-

change as the first dimension and reversed-phase chromatography as the second dimension. We have compared this setup to the traditional on-line MudPIT experiment and found that it characterizes more peptides and is fully automated while using simpler instrumentation and hence much easier technically to perform. Importantly, due to the automated sample loading via the microplate autosampler used by the Easy-LC, the system can be operated at a 24/7 mode, increasing the sample throughput as compared to the manual sample loading process used for the traditional MudPIT procedure, which is practically limited to one sample per day (at least for a ≥ 9-step setup). Many samples can be analyzed using a single precolumn/column setup compared with a new column used more frequently in the traditional approach (T.K. personal experience). Samples, which have been run on the same column, have chromatograms that are more easily aligned and make postacquisition analysis easier. We also presented an abbreviated variant of this experiment which allows for 3 times as many samples being analyzed without a huge compromise is data quality.

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References

(1) Aebersold, R.; Mann, M. Mass spectrometry-based proteomics. *Nature* **2003**, *422* (6928), 198–207.
(2) Domon, B.; Aebersold, R. Mass spectrometry and protein analysis. *Science* **2006**, *312* (5771), 212–7.
(3) Yates, J. R., III; Gilchrist, A.; Howell, K. E.; Bergeron, J. J. Proteomics of organelles and large cellular structures. *Nat. Rev. Mol. Cell Biol.* **2005**, *6* (9), 702–14.
(4) Klose, J. Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. *Humangenetik* **1975**, *26* (3), 231–43.
(5) O'Farrell, P. H. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **1975**, *250* (10), 4007–21.
(6) Wilm, M.; Shevchenko, A.; Houthaeve, T.; Breit, S.; Schweigerer, L.; Fotsis, T.; Mann, M. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **1996**, *379* (6564), 466–9.
(7) Faca, V.; Pitteri, S. J.; Newcomb, L.; Glukhova, V.; Phanstiel, D.; Krasnoselsky, A.; Zhang, Q.; Struthers, J.; Wang, H.; Eng, J.; Fitzgibbon, M.; McIntosh, M.; Hanash, S. Contribution of protein fractionation to depth of analysis of the serum and plasma proteomes. *J. Proteome Res.* **2007**, *6* (9), 3558–65.
(8) Matt, P.; Fu, Z.; Fu, Q.; Van Eyk, J. E. Biomarker discovery: proteome fractionation and separation in biological samples. *Physiol. Genomics* **2008**, *33* (1), 12–7.
(9) McCormack, A. L.; Schieltz, D. M.; Goode, B.; Yang, S.; Barnes, G.; Drubin, D.; Yates, J. R. 3rd, Direct analysis and identification of proteins in mixtures by LC/MS/MS and database searching at the low-femtomole level. *Anal. Chem.* **1997**, *69* (4), 767–76.
(10) Link, A. J.; Eng, J.; Schieltz, D. M.; Carmack, E.; Mize, G. J.; Morris, D. R.; Garvik, B. M.; Yates, J. R. 3rd, Direct analysis of protein complexes using mass spectrometry. *Nat. Biotechnol.* **1999**, *17* (7), 676–82.
(11) Washburn, M. P.; Wolters, D.; Yates, J. R., III. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **2001**, *19* (3), 242–7.
(12) Wolters, D. A.; Washburn, M. P.; Yates, J. R., III. An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem.* **2001**, *73* (23), 5683–90.
(13) Guzzetta, A. W.; Chien, A. S. A double-vented tetraphasic continuous column approach to MuDPIT analysis on long capillary columns demonstrates superior proteomic coverage. *J. Proteome Res.* **2005**, *4* (6), 2412–9.
(14) Simpson, P.; McGrath, A.; Savion, S. Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and by catecholamines. *Circ. Res.* **1982**, *51* (6), 787–801.
(15) Licklider, L. J.; Thoreen, C. C.; Peng, J.; Gygi, S. P. Automation of nanoscale microcapillary liquid chromatography-tandem mass spectrometry with a vented column. *Anal. Chem.* **2002**, *74* (13), 3076–83.
(16) Kislinger, T.; Cox, B.; Kannan, A.; Chung, C.; Hu, P.; Ignatchenko, A.; Scott, M. S.; Gramolini, A. O.; Morris, Q.; Hallett, M. T.; Rossant, J.; Hughes, T. R.; Frey, B.; Emili, A. Global survey of organ and organelle protein expression in mouse: combined proteomic and transcriptomic profiling. *Cell* **2006**, *125* (1), 173–86.
(17) Kislinger, T.; Rahman, K.; Radulovic, D.; Cox, B.; Rossant, J.; Emili, A. PRISM, a generic large scale proteomic investigation strategy for mammals. *Mol. Cell. Proteomics* **2003**, *2* (2), 96–106.
(18) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **2002**, *74* (20), 5383–92.
(19) Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **2003**, *75* (17), 4646–58.
(20) Liu, H.; Sadygov, R. G.; Yates, J. R., III. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **2004**, *76* (14), 4193–201.

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