Complex Peptide Mixture Fractionation via Parallel Isoelectric Focusing for Direct LC-MS/MS Analysis

P. Fung, D. Argoti, W. Kuhlman, J. Dasch, S. Haralampu
Cell Biosciences, Inc., Santa Clara, CA, USA.

Introduction
Charge-based separation of peptides prior to LC-MS/MS analysis has been performed by long run times and the presence of components such as detergents and carrier ampholytes that interfere with MS (1). Cell Biosciences has developed a workflow using the digital ProteomeChip® (dPC®), that captures peptides in acrylamide gel plugs according to charge, with run times less than one hour (2,3) (figure 1). This workflow employs buffers and conditions that have been designed to be "MS-friendly," eliminating the need for post-separation sample clean-up and dramatically reducing artifacts introduced by the separation processes. Cell Biosciences ProteomeChips are available in three pH ranges: 3.5-4.5, 4.2-6.2 and 6.0-8.0, providing an overall pH range of 3.5-8.0.

Processes. Cell Biosciences ProteomeChips are reducing artifacts introduced by the separation buffers and conditions that have been designed to improve reproducibility and speed of the ProteomeChip for "bottom-up" proteomic screening is accomplished utilizing Cell Biosciences digital ProteomeChip technology. Encompassing mass spectrometry friendly solvents, complex mixtures of peptides are separated in less than 1 hour, with LC-MS ready peptide solutions processed in less than 3 hours. These findings demonstrate the reproducibility and speed of the ProteomeChip for peptide fractionation and identification. The technology easily integrates into standard mass spec workflow and establishes a foundation for improved reproducibility and more rapid throughput in areas such as discovery proteomics and biomarker screening.

Discussion
Novel separation and pre-fractionation of samples for "bottom-up" proteomic screening is accomplished utilizing Cell Biosciences digital ProteomeChip technology. Encompassing mass spectrometry friendly solvents, complex mixtures of peptides are separated in less than 1 hour, with LC-MS ready peptide solutions processed in less than 3 hours. These findings demonstrate the reproducibility and speed of the ProteomeChip for peptide fractionation and identification. The technology easily integrates into standard mass spec workflow and establishes a foundation for improved reproducibility and more rapid throughput in areas such as discovery proteomics and biomarker screening.

Conclusions

• More than 3,800 unique peptides (17,066 total) were found using three dPCs with a combined pH range of 3.5-8.0.
• 1,132 unique proteins (1,189 total) identified on at least two dPCs.
• Targeted analysis results of specific peptides identified on the LTQ MS showed high reproducibility.

Workflow

Figure 1. Overview of integrated peptide separation workflow using the dPC.

Methods

Sample Preparation and dPC Fractionation
Frozen human A431 cell lysate were generated from trityl/phosphine and alkylated with iodoacetamide. Proteins were precipitated in methanol/chloroform and dissolved in 50 mM ammonium bicarbonate. Trypsin was added and the sample was allowed to digest overnight at 37°C. Following digestion, the samples were diluted with running buffer and run on ProteomeChips in each of the three available pH ranges: 3.5-4.5, 4.2-6.2 and 6.0-8.0. ProteomeChips were run for a maximum of 45 minutes. After the run, each chip was briefly rinsed with water and gel plugs were harvested into microcentrifuge tubes.

LC-MS/MS Analysis
Gel plugs were pooled into groups of 5, corresponding to 0.25 pH units, and extracted with 200 µL of 0.2% formic acid in acetonitrile at 37°C for 1 hour. Extracts were collected in fresh microcentrifuge tubes and concentrated to a volume of 50 µL via SpeedVac. Samples were then loaded onto a Thermo Scientific Micro AS, equipped with a 25 µl syringe, 2.4 µL sample needle, 200 µL buffer tubing and 10 µL sample loop. The wash solvent used was 0.1% formic acid in water, 5% B (0.1% formic acid in acetonitrile) with a 75 µm x 15 cm column packed with Michrom Bioresources Magic 5 µm C18 media. A Thermo LTQ linear ion trap mass spectrometer was used in a "Top 8" configuration.

Data Analysis
Raw files from the LTQ mass spectrometer were collated using SEQUEST® through Thermo Scientific Bioworks 3.3.1 SR1 software package against the Rebase Human database, with static carbamidomethyl modified cysteines and differentially modified oxidized methionines. SEQUEST data was exported and analyzed further by the Cell Biosciences Mass Spectrometry Research Analysis Tool (MSRAT®).

Results

Figure 3 demonstrates dPC-based fractionation using 2 fluorescently tagged peptides mixed with A431 trypic digested cell lysate on two pH 4.2-6.2 ProteomeChips. Studies performed using this A431 cell lysate, identified 3,876 unique (17,066 total) peptides using 3 ProteomeChips with a combined pH range of 3.5-8.0. Reproducibility was evaluated by comparing identity and position of specific peptides in multiple dPC runs (figure 4), leading to strong agreement in peptide IDs between 2 ProteomeChips (figure 5). On the basis of these peptide IDs, 1,873 unique proteins (2,025 total) were identified across the entire pH range (figure 6), with 1,132 unique proteins (1,189 total) identified on at least two ProteomeChips.

References
(1) B. Gouge et al. Unravelled pH Gradient Isotopic Focusing as a First-Dimension Separation in Shotgun Proteomics; Journal of Proteome Research 2007, 6, 3350-3358.
(2) G. Zilberstein et al. Parallel isoelectric focusing chip; Proteomics 2004, 4, 2533–2540.

Figure 4. Reproducibility between two dPC runs across different working range. Each ProteomeChip was loaded with a 50 µg aliquot of an A431 trypic digest. Each box represents pools of plugs in which the same peptide were found in each of the two ProteomeChips. The number of unique peptides observed is represented by the degree of intensity towards black (scale shown below).

Figure 5. Number of proteins identified in each pH range and reproducibility observed between two technical replicates.