Complex Peptide Mixture Fractionation via Parallel Isoelectric Focusing on a digital ProteomeChip for Direct LC-MS/MS Analysis

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Overview

Purpose: To develop an LC-MS/MS compatible workflow for peptide analysis

Introduction

Protein Forest Inc. has developed the digital ProteomeChip (dPC) that traps peptides in acrylamide gel features based on charge, with a pH resolution of less than 0.1 units and run times less than one hour. The dPC is composed of 41 gel plugs each at a specific pH, creating a discrete 2.0 pH gradient across the dPC with a resolution of 0.05 pH units. This workflow employs buffers and conditions that have been designed to be MS compatible, eliminating the need for post-separation sample clean-up and dramatically reducing artifacts introduced by the separation process.

Methods

Sample preparation for dPC fractionation

Frozen Human A431 cell lysates were reduced with tributylphosphine 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. After proteolysis, peptides were reconstituted with dPC running buffer. Chips were run for a maximum of 45 min, briefly rinsed with water and gel plugs ejected into microcentrifuge tubes.

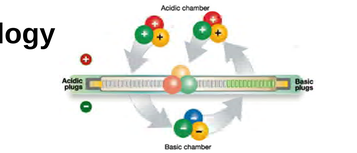
Sample preparation for LC-MS/MS analysis

Gel plugs were pooled in groups of 5, corresponding to 0.25 pH units, and extracted with 200 µL of 0.2% formic acid in 50% acetonitrile at 37°C for 1 hour. Extracts were collected in fresh microcentrifuge tubes and concentrated to a volume of 50 µL. Samples were then loaded onto a Thermo Scientific Micro AS, equipped with a 25 µL syringe, a 2.4 µL sample needle, a 200 µL buffer tubing and a 10 µL sample loop. The wash solvent used was 0.1 % formic acid in 30% acetonitrile. The pump was a Surveyor MS pump plus equipped with a static split at a ratio of 800:1 at 250 µL/min at 95% A (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 C_{18} media. The Thermo LTQ™ linear ion trap mass spectrometer was used in a "Top 8" configuration.

Data Analysis

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

Technology



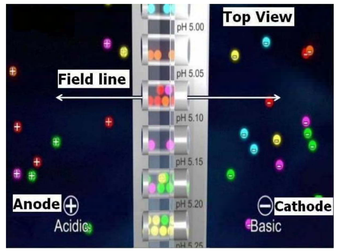


Figure 1. Top. Fractionation by parallel isoelectric focusing on the dPC. **Bottom.** Peptides and proteins are charged when in an environment that is either above or below their isoelectric point (pI) allowing them to migrate in an electric field until they encounter a gel that is at or near their pI and lose both charge and mobility.

Workflow

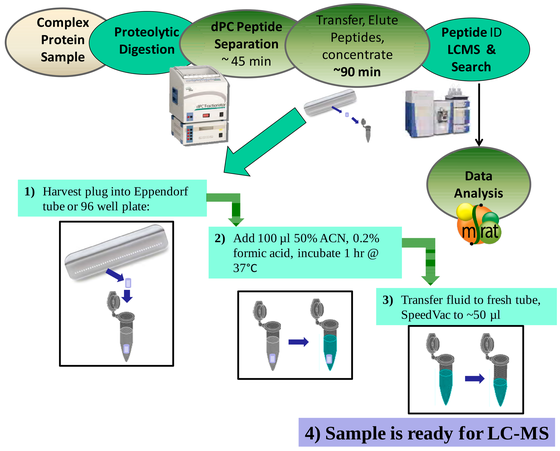


Figure 2. Overview of integrated workflow for maximum peptide recovery

Results

Figure 3. Comparison of two separate dPC runs on the 4.2-6.2 chip, each loaded with 50 µg aliquot of an A431 cell lysate, 25 µg of red and 10 µg of yellow flourescent peptides.

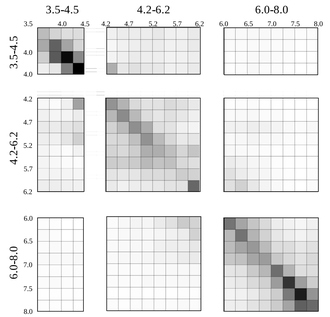


Figure 4. Focusing reproducibility between two separate dPC runs across the current working range. Each dPC was loaded with a 50 µg aliquot of an A431 tryptic digest. Grey to black boxes represent pools of plugs in which the same peptides were found in each of the two chips. The number of peptides observed is represented by the degree of intensity towards black.

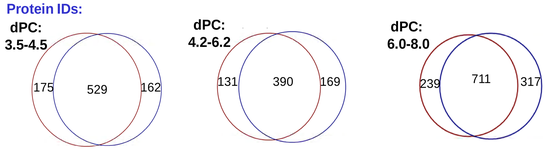


Figure 5. Number of proteins identified in each pH range and reproducibility observed between two technical replicates at each pH range.

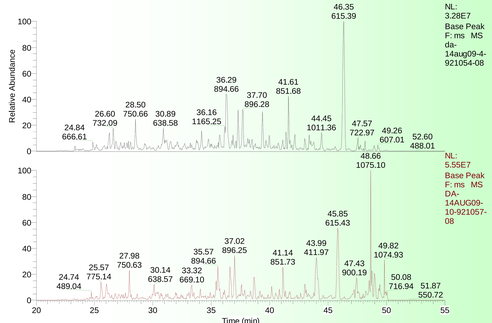


Figure 6. LC-MS/MS base peak chromatograms for 5-plug pH fraction 5.95-6.20 from two 4.20-6.20 dPC technical replicates each loaded with a 50 µg aliquot of an A431 tryptic digest. Illustrating the typical run to run degree of reproducibility between both dPC fractionation and LC-MS/MS analysis.

Advantages

- Minimized sample handling prior to LC-MS/MS analysis
- Fractionation to MS ready samples in 3 hours
- Only three simple steps to LC-MS/MS analysis
  - Harvest plug→ Elute peptides→ Concentrate
- Reproducible peptide focusing with overall RSD <20%

Conclusions

- More than 3,500 peptides were found using three dPCs with a combined pH range of 3.5-8.0
- Approximately 1,600 unique proteins were reproducibly identified across the entire working pH range
- Future work will further optimize the workflow for targeted quantitaive assays and extend pH range to pH= 9.0

References

(1) Immobilized pH Gradient Isoelectric Focusing as a First-Dimension Separation in Shotgun Proteomics; Journal of Biomolecular Techniques 2005,16, 181–189.

(2) G. Zilberstein et al, Parallel isoelectric focusing chip; Proteomics 2004, 4, 2533–2540.

Acknowledgements

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