On-line, Chip-Based Strategy for Increased Depth of Coverage in Proteomic Samples

Two Dimensional Reverse Phase / Reverse Phase Fractionation with cHiPLC® System

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On-line two-dimensional (2D) liquid chromatography is widely used for protein identification and quantification because of the advantage of increased peak capacity. A common workflow is the combination of strong cation exchange (SCX) as an orthogonal first dimension to reversed-phase (RP) chromatography as the second dimension. A more recently developed strategy has emerged where the first dimension is a high pH RP separation which has less orthogonality, but provides higher peak capacity compared to SCX. These 2D workflows are essential for measuring lower abundant proteins in complex proteomes, however can be complex to implement. Eksigent's cHiPLC® system is a microfluidic chip based “docking station” with a flexible design that allows for easy switching between different types of LC workflows.

In this application note, we describe a simplified chip based 2D-LCMS workflow using a high pH/RP first dimensional separation and a low pH/RP secondary dimension coupled directly to mass spectrometer for proteomic analysis.

Key Features of the ekspert™ nanoLC 400 and cHiPLC® System

- The ekspert™ nanoLC 400 system with the cHiPLC® system supports a broad range of workflows, from global discovery to targeted quantitation
- Flexible cHiPLC® System easily enables many workflows:
  - Direct injection
  - Trap and Elute – dirty samples
  - Dual column multiplexing – increasing throughput
  - Serial 2-column – increasing peak capacity and depth of proteome coverage
- On-line 2 dimension LC fractionation
- Easy to change flow module cartridges allow the user to rapidly switch between flow rate ranges
- MFCPlus™ Technology provides flow stability for high retention time reproducibility, with retention time variability below 0.35% RSD at 500 nL/min
- Ultra high pressure (10 000 psi) for high resolution separations
- High precision autosampler enables excellent injection reproducibility with little or no sample waste.
Experimental

**Sample Preparation:** A standard beta-galactosidase digest (25 fmol/µL) was used for method development. For proteomic analysis, E. coli cell lysates were denatured, reduced and alkylated with iodoacetamide, before being subjected to tryptic digestion. The final concentration was 1 µg/µL.

**2D-LC-MS/MS:** Online 2D-LC workflow was performed using the ekspert™ nanoLC 425 system with two cHiPLC® system (Eksigent, part of AB SCIEX). First dimension mobile phases were A: 50 mM ammonium formate in water (pH 9.8) and B: pure ACN. The second dimension mobile phases were A: water with 0.1% formic acid, pH 2.5 and B: acetonitrile with 0.1% formic acid. The loading pump was water with 0.1% formic acid, pH 2.5. Digested peptides were first loaded onto a 200 µm x 15 cm C18 cHiPLC® column under basic conditions with a flow rate of 1 µL/min. A step gradient of acetonitrile was used to sequentially elute peptide fractions, which were then diluted to acidic conditions before being captured by a 200 µm x 6 mm C18 cHiPLC® trap. Each fraction was then separated with a 75 µm x 15 cm C18 cHiPLC® column at 300 nL/min, a detailed workflow diagram is shown in Figure 1.

**Mass Spectrometry:** The fractionated samples were analyzed using the TripleTOF® 5600+ System (AB SCIEX). Data was processed with ProteinPilot™ Software.

**Figure 2. On-line 2D RP-RP for Beta-Galactosidase Digest Separation.** Method development was performed using a standard Beta-Galactosidase digest. The %ACN indicates the step gradient condition in the first dimension to elute the corresponding peptides.

**Figure 3. Peptide Distribution across Ten 2D Fractions.** (Top) The fractionation in the first dimension provided a good distribution of peptides across individual fractions, with the most peptides eluting in the 20%-26% ACN fractions. (Bottom) Analysis of the fractionated peptides as seen in the second dimension TOF MS TICs show the peptide hydrophobicity does vary with the different first dimension fractions. Further optimization of the second dimension analytical gradient could further improve the depth of coverage and the total experimental time.

**On-line 2D RP-RP for Peptide Fractionation**

Peptides normally contain ionic acidic and basic functional groups and their retention time can be affected by changing the pH of the mobile phases. The peak capacity of 2D strategy involving high pH RP followed by low pH RP has been reported to be better than several other 2D-LC methods, such as those using strong cation exchange in the first dimension. Here, a chip based LC system was used to configure and simplify the on-line 2D RP-RP workflow (Figure 1). The method development was performed using a standard beta-galactosidase digest. The peptides were sequentially eluted from the first cHiPLC column, captured and enriched on the trap column, then eluted to second RP column for MS analysis. As shown in Figure 2, some acidic and basic peptides showed their retention time shifted at high pH.
demonstrating orthogonality to provide increased depth of coverage for peptide identification.

For example, peptide VDEDQPFPAVPK (Figure 2) eluted first, at 15% ACN, in the first dimension, and the other two major peptides YSQQLMETSHR and RDWENPGVTQLNR eluted at 20% ACN (Figure 2). However in low pH condition, their elution order is opposite.

The optimized workflow was then applied to the analysis of E. coli cell lysates and a variety of comparisons were performed to assess impact on proteomic workflows. The peptide distribution across the ten individual fractions in the 2D 10 fraction experiment is plotted in Figure 3 (top). As shown, the first dimension fractions that contain the higher peptide numbers were the fractions between 20%-26% ACN. This peptide distribution information could be used for further optimization of the number of identification by increasing the number of fractions further. In addition, further improvements could also be achieved by optimizing the second dimension gradient for each different fraction, adjusting for the changing hydrophobicity of the eluting peptides (Figure 3, bottom).

**Experiments performed:**
1. 1 dimension with 1 µg total protein load
2. 2 dimensions of 6 fractions (10%, 15%, 20%, 25%, 30%, 50% ACN) with 1 µg protein load
3. 2 dimensions of 6 fractions (10%, 15%, 20%, 25%, 30%, 50% ACN) with 10 µg protein load
4. 2 dimensions of 10 fractions (10%, 14%, 17%, 20%, 22%, 24%, 26%, 28%, 30%, 50% ACN) with 1 µg protein load
5. 2 dimensions of 10 fractions (10%, 14%, 17%, 20%, 22%, 24%, 26%, 28%, 30%, 50% ACN) with 10 µg protein load

Figure 4 provides a summary of the impact on protein and peptide identification rates across the 5 different experiments performed. As expected, there is a steady increase in coverage as the fractionation increases. At the same protein load, there is a 1.8x and 2.1x increase in the identification numbers for the 2D-6 and 2D-10 fractions respectively, versus the 1D experiment at the peptide level (5% local FDR). The other advantage of 2D workflow is the larger sample loading capacities of the first dimension because of the larger diameter chiPLC® column.

When the loading amount was increased by 10x, the number of detected peptides increased by 3.3x and 4x for the 2D 6 and 10 fraction workflows, respectively, over the 1D workflow (Figure 4).

**Figure 5. Peptide Alignment Comparison for 1D and 2D-10 Fraction Experiments.** An alignment between the peptides found in the 1D 1 µg experiment and the 2D 10 fraction 10 µg experiment was performed. (Left) The measured retention time of peptides detected in both the 1D 1 µg and 2D 10 µg experiments were plotted and showed very good correlation (r² 0.99, slope 1.0). (Right) The intensity of the peptides also showed roughly 10x higher signal intensity as expected from the 10x higher load enabled by the 2D workflow.
A detailed comparison of peptide intersection was performed between the 1D – 1 µg load experiment and the 2D – 10 fraction – 10 µg load experiment (Figure 5). All of the shared peptides between the two experiments were found and the retention times and MS peak areas were compared. The retention time correlation for the peptides was very good. Also, the peak intensities of the peptides were about 10x higher with the 10x higher load as expected, highlighting another advantage of this workflow.

In a typical IDA experiment, multiple replicates normally give increased proteome coverage due to the stochastic nature of data dependent acquisition. Figure 6 compares the impact of the number of injections performed vs the identification rates, to highlight the economical nature of the 2D workflow. The number of peptides and proteins identified from the same number of injections is significantly better with the 2D workflow.

**Conclusions**

Multi-dimensional chromatography is a key technology for working on complex proteomes and obtaining deeper depth of coverage of proteins. Using high pH reverse phase as the first dimension has been increasing in popularity because of its higher resolution and reduced need for salt. Here, the workflow was implemented using two cHiPLC® systems for ease of use and fast switching between workflows. While further optimization can still be performed on the demonstrated workflow, substantial improvements in numbers of peptides identified was shown using the on-line 2D-LC RP-RP workflow compared to 1D separations.

**References**

1. Increasing Throughput of NanoLC using Two-Column Switching Workflows. AB SCIEX Technical Note 1870411-01
2. Increasing Depth of Coverage using Serial Two-Column Workflows. AB SCIEX Technical Note 1870211-02

**Figure 6. Comparison of Combined 1D vs 2D Fractions.** The 1D replicates (1 µg) were searched individually or in combination (blue circles of increasing darkness to indicate # of combined replicates), injections were reproducible and combined to provide a small increase in peptide identifications. When using the 2D workflow, more sample can be loaded and multiple fractions can be used giving many more identifications. X axis is expressed by number of injections to indicate rough time differences between the different workflows.