# Increasing Depth of Coverage using Serial Two- Column Workflows

***Powerful Proteomics Workflows Enabled by the cHiPLC® System***

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Nanoflow liquid chromatography coupled with high performance mass spectrometry has proven to be an invaluable tool for sensitive peptide and protein analysis. High resolution peptide separations are a prerequisite for diving deeper into complex proteomes. Advances in high resolution mass spectrometry can capitalize on increases in chromatographic peak capacity, and yield dividends in proteome coverage. The emergence of microfluidic chip technology for nanoflow chromatography has greatly simplified the execution of LC-MS analyses in proteomics. However, the great diversity of applications in proteomics demands a microfluidic device that offers great workflow flexibility, in addition to powerful separations.

The cHiPLC® system is a unique chip platform that enables many different workflows. For the deepest proteome coverage, the cHiPLC system supports a Serial Two-Column workflow that maximizes the peak capacity available on chip. The advantages of the Serial Two-Column workflow will be demonstrated in this work.

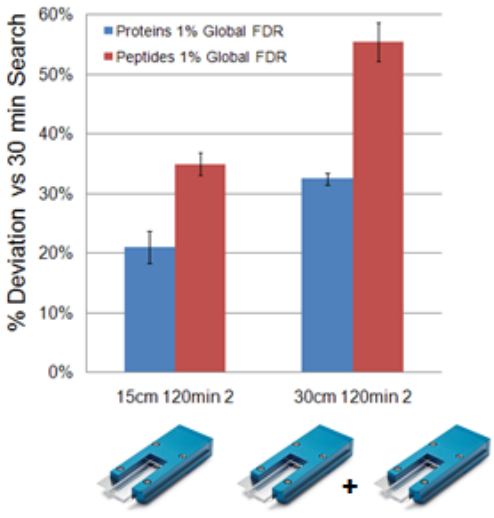


**Figure 1. Serial Two-Column Workflow for Better Depth of Coverage.** With the cHiPLC system, a serial two-column workflow is easily established with a simple connection change. Instead of running one 75 µm x 15 cm column, two columns can be put in serial to double the length of column and enhance peak capacity.

## Key Features of the cHiPLC® Systems

- The cHiPLC system is a flexible platform for using chip-based nanoLC traps and columns with a SCIEX nanoflow LC system
- Flexible platform and modular components allow for many different workflows to be performed on a single system
  - Direct injection
  - Trap-elute
  - Two-Column switching[1]
  - Serial Two-column
- Unique connection system makes changing columns and traps, and therefore workflows, very easy.
- Excellent column-to-column reproducibility (<2%)
- Serial Two-Column workflow enables two chip columns to be run in serial, doubling the length of the column and increasing peak capacity. The first column can also be used as a trap column, loading and washing to waste, then switching the second column in line for elution

## Methods

***Sample Preparation:*** The cytosolic fraction of a human cell lysate was isolated and digested.

***Chromatography:*** The sample was analyzed using the nanoLC Ultra® 2Dplus System combined with the cHiPLC® system in Serial Two-Column mode with two nano cHiPLC columns (75 µm x 15 cm ChromXP™ C18-CL 3 µm 300 Å). The sample was loaded using either the 15 or 30 cm column configuration. Then, elution gradients of 10-30% acetonitrile (0.1% formic acid) were used with either a 30, 60 or 120 min gradient (Figure 2). All conditions were tested in triplicate.

***Mass Spectrometry:*** The TripleTOF® 5600 System equipped with a NanoSpray® III source (SCIEX) was used for high speed data acquisition. The IDA method was constructed to acquire a TOF MS survey scan at >30 000 resolution, followed by 20 MS/MS in a second at >15 000 resolution.

***Data Processing:*** All data was processed using ProteinPilot™ Software 4.0 with integrated false discovery rate (FDR) analysis. Further data analysis was performed using the accompanying ProteinPilot Descriptive Statistics Template[3].

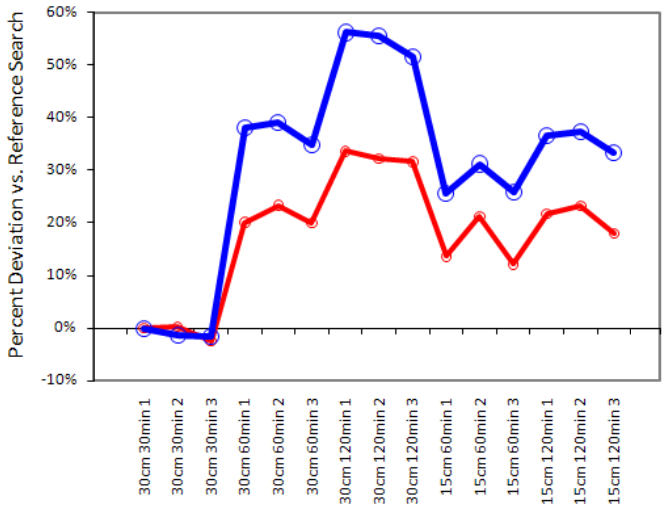


**Figure 3. Comparison of Proteins and Peptides Found under Various Chromatographic Conditions.** Using the 1% Global FDR as the key number, the numbers of proteins (red) and peptides (blue) found under the various chromatographic conditions were compared. Extending the gradient to 120 mins on the 30 cm column provided the highest number of identifications.

## Replicate Analysis

For each different chromatographic condition explored, three replicates were performed (Figure 3). This ensures that the results obtained are reproducible at each condition and the conclusions drawn are more robust. In addition, at the end of the set of experiments, repeats were done of earlier experiments to ensure stability across the whole dataset.

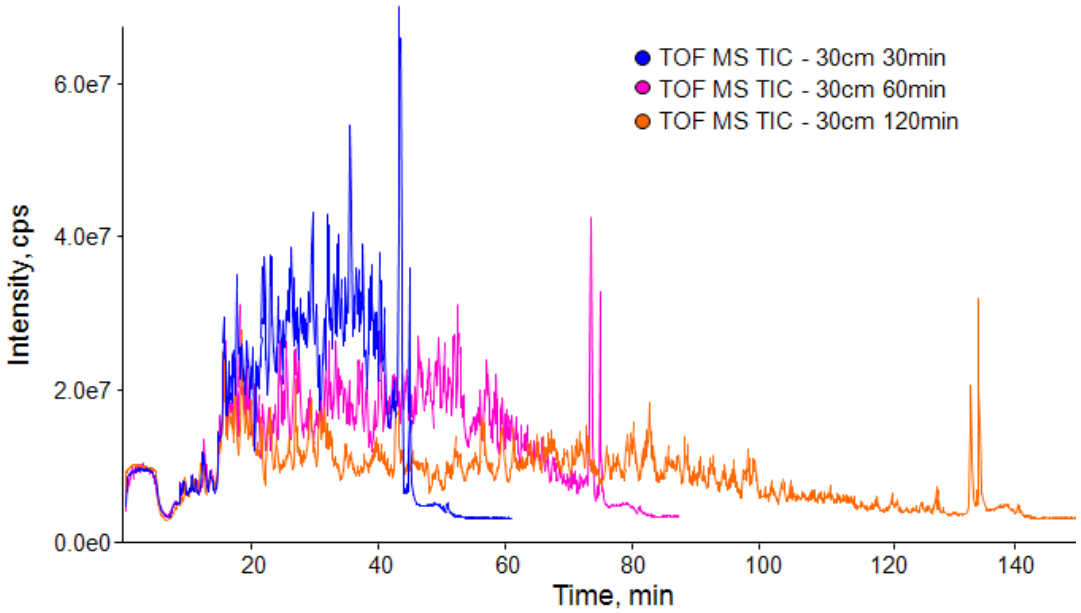


**Figure 2. Separation Achieved at Increasing Gradient Lengths in the Serial Two-Column Configuration.** As the gradient time is extended on the 30 cm column, the peptide separation time is optimized, as illustrated in the overlapping chromatograms of the 30 min (blue), 60 min (pink) and 120 min (orange) experiments.

## Increasing Peak Capacity in Separations

An important factor in maximizing the number of peptides identified in a LC-MS run is the quality of the LC separation. The peak capacity of the separation is a measure of the number of peaks that can be resolved during the course of the gradient elution. A number of variables are involved in determining peak capacity, but the ones that are most often used during method development include gradient length, column length and particle size.

The theoretical peak capacity can be determined as a function of column length and gradient rate. The dependence of peak capacity nc' on column length L and plate height H can be calculated with $nc' = \sqrt{(L/H)} / (1 + a\, L/tg)$ where $a = S\Delta\varphi/u$, and S is solvent strength, $\Delta\varphi$ is change in gradient composition, u is mobile phase velocity and tg is gradient time3. A simulation of the effect of column length on peak capacity at various gradient lengths is shown (Figure 4).

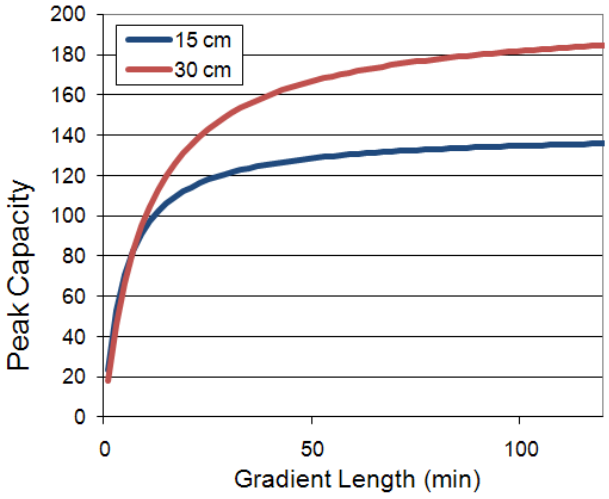


**Figure 4. Maximizing Peak Capacity to Maximize Peptide Identification.** A simulation of peak capacity shows that increasing gradient length should be accompanied by an increase in column length to obtain the maximal peak capacity.

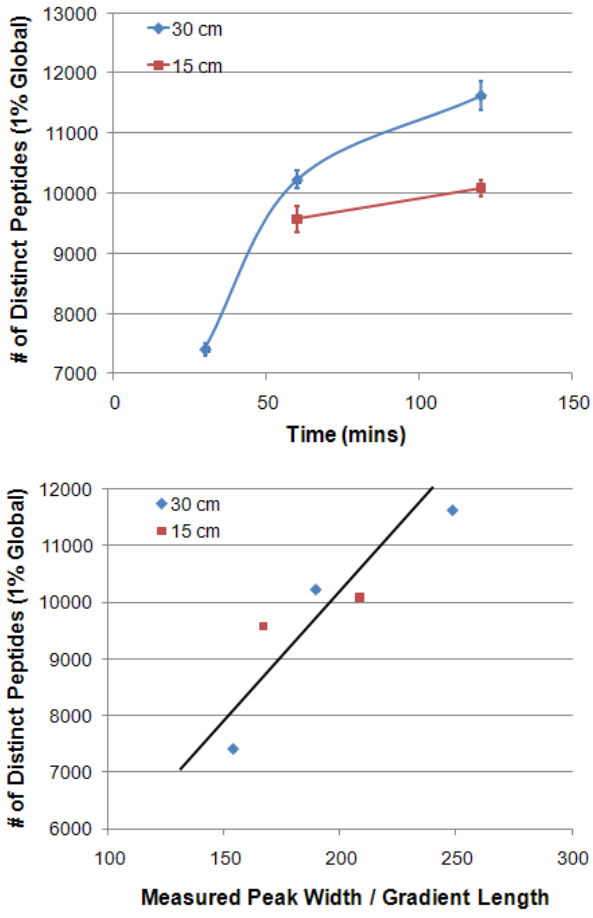


**Figure 5. Exploring the Effects of Gradient Length and Column Length on Peptide Identification.** (Top) The average number of peptides identified for each time point on both columns clearly shows the advantage of using the longer column for the longer length gradients. (Bottom) A rough estimate of peak capacity (peak width / gradient length) correlates well with average # of peptides identified, demonstrating that optimizing chromatography can greatly enhance protein ID experiments.

There are a number of ways to increase peak capacity with gradient chromatography. The gradient slope can be reduced, which increases the total gradient length. Another technique is to use columns with smaller particle size, thereby decreasing plate height (H). Finally, the column length (L) can be increased. The combination of longer columns and longer gradient times is explored in this study.

## Maximizing Peptide Identifications

A rough estimate of the peak capacity can be obtained by dividing the total separation time by the average peak width at half height obtained in the LC run (Figure 5, bottom). Good correlation was observed between this estimate and the number of distinct confident peptide identifications. Optimizing peak capacity can therefore help in maximizing the information content obtained from an LC-MS protein ID run.

On any fixed column length, the number of peptide IDs increases with increasing gradient length, dramatically at first, but then with diminishing returns. This is because peak widths also increase with increasing gradient length, and is a result of the contribution of longitudinal diffusion to peak width as the peaks spend more time on-column. This is represented by the flattening of the slope of the line (Figure 5, top), especially for the 15 cm column (red) as the gradient lengths are extended. This decrease in slope was not as pronounced for the 30 cm column (blue) and shows that an extension of gradient length should also be accompanied by an extension in column length, for maximal information return.

## Conclusions

- The flexibility of the cHiPLC® system allows users to explore multiple workflows and determine the best workflow for maximal throughput and maximal protein/peptide identifications.
- Switching between the 15 cm and 30 cm column configuration on the cHiPLC system when in Serial Two-Column configuration is as easy as switching the valve from Load to Inject.
- Increasing gradient length from 60 to 120 minutes on the 15 cm column configuration only produced about a 5% increase in peptide identifications.
- Numbers of peptides identified between 120 mins on a 15 cm column and 60 mins on a 30 cm column were quite similar; therefore throughput can be maximized by using the 30 cm column configuration.
- When using a 120 min gradient, maximal peptide and protein identifications were obtained using the 30 cm column configuration.

## References


1. Increasing Throughput of nanoLC using Two Column Switching Workflows. SCIEX Technical Note, Publication RUO-MKT-02-2892-A.
2. Improving Resolution in nanoLC Separations for Proteomics using Ultra High Pressures. ASMS 2008 Poster - R van Soest, DW Neyer; JE Siow, P Paul.
3. The ProteinPilot™ Descriptive Statistics Template is now part of ProteinPilot Report in ProteinPilot™ Software 5.0.





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