

# On-line cHiPLC based digestion in nanoLC-MS for increased reproducibility

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## Introduction

We recently reported on a new microfluidic platform (cHiPLC-nanoflex) for nanoLC-MS applications. In addition to delivering easy-to-use, dead-volume-free connections to microfluidic devices, the platform's cHiPLC columns provide excellent column-to-column separation reproducibility.

In this poster we report on the addition of an immobilized trypsin digestion column to the cHiPLC workflow to increase overall proteomics workflow reproducibility. Completion of nanoLC analyses immediately after on-chip protein digestion has several advantages. Two of which are reduced sample handling and, perhaps more importantly, very little time lag between digestion and analysis. Both improvements reduce loss of peptides associated with adsorption to the sample vial - a problem that is enhanced for low quantity peptides when there is a long time between (in solution) digestion and analysis

## Methods

**LC Instrumentation and Method:** A direct flow nanoLC system (NanoLC-Ultra 2D, Eksigent Technologies) was used in combination with two cHiPLC nanoflex units (Eksigent); one containing a 2 x 1 x 0.2 mm (0.4  $\mu$ l) cHiPLC column packed with Poroszyme immobilized trypsin (Applied Biosystems), and one containing a 0.2 x 0.5 mm cHiPLC trap packed with 5  $\mu$ m 300  $\text{\AA}$  ChromXP C18 and a 15 cm x 75  $\mu$ m cHiPLC nanoLC column packed with 3  $\mu$ m 300  $\text{\AA}$  ChromXP C18. For the digestion 50 mM Tris; 10 mM CaCl<sub>2</sub>; pH 8.0 with 4% Acetonitrile was used. On-line digestion was performed at 1  $\mu$ l/min for 5 min at 37 C with the cHiPLC trap in-line to trap the tryptic peptides. Mobile phases for the separation were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A linear gradient from 3-35% B in either 15 or 30 min with a 15 min column wash at 90%B was used. Flow rate was 300 nl/min. During analysis of the tryptic peptides the digest column was washed with 50 mM Tris; 10 mM CaCl<sub>2</sub>; pH 8.0 with 50% Acetonitrile.

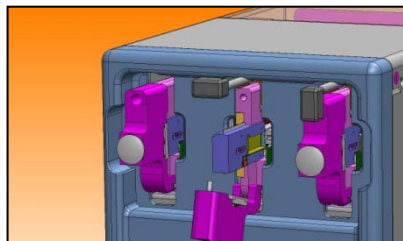
**Samples:** Cytochrome C from horse heart and BSA were obtained from Sigma Aldrich. BSA was reduced and alkylated with DTT and Iodoacetamide (Sigma Aldrich) and for comparison digested in solution overnight with sequence modified Trypsin from Invitrogen. Sample volume for all experiments was 1  $\mu$ l.

**MS Instrumentation:** An LTQ ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA) was used with a Picoview nanospray source with 20  $\mu$ m ID capillary /10  $\mu$ m ID tips (New Objective, Woburn, MA, USA). The MS method for peptide ID consisted of one MS scan followed by 4 MS/MS scans on the 4 most intense precursors. Dynamic exclusion was used (repeat count 2, 30 sec. duration, 15 sec. exclusion).

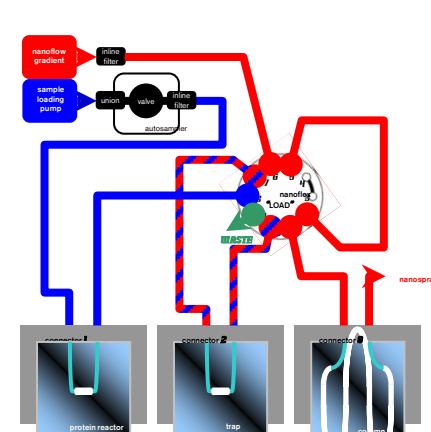
**Data analysis:** Database searching was performed using Mascot Distiller and MASCOT (Matrix Sciences, London, UK) using the NCBI nr database.

## cHiPLC nano columns

The cHiPLC columns are constructed from quartz using lithography to form the ideal circular channels for chromatography. Connections are made through the edge of the chip using the proprietary Eksport connection system for connecting chips together and to the outside world. The Eksport connector allows the digest reactor, trap and column chips to be quickly removed and replaced. The connectors are also temperature controlled for long-term retention time stability and selecting the ideal temperature for reproducible on-line digestion.

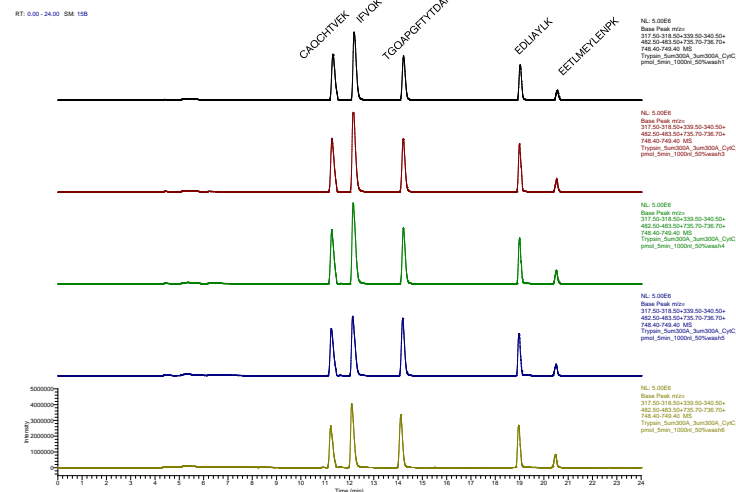


## cHiPLC nanoflex set-up



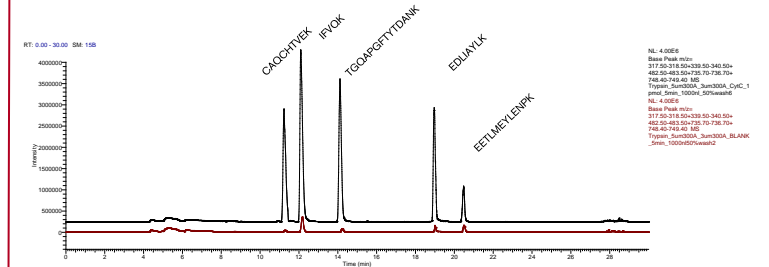
The built-in valve in the cHiPLC Nanoflex is used to switch the trap-column on-line with the immobilized trypsin column after injection of the sample. After the sample has passed through the digest column and trap column, the valve is switched and the analysis started. During the analysis the digest column is washed with high organic.

## Reproducibility



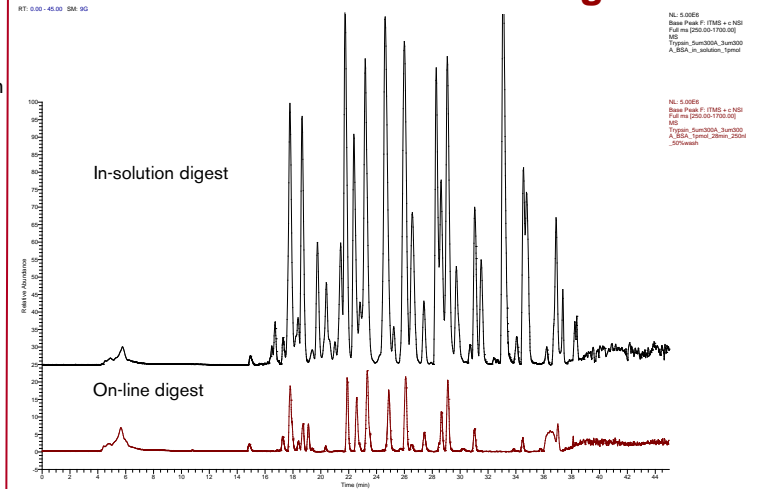
Reproducibility of the on-line digestion was measured with 1 pmol Cytochrome C injections. In the figure above selected ion chromatograms for five tryptic fragments for 5 consecutive injections are shown. RSD's on peak area (n=8) where 9.4 to 22%

## Carry-over



Carry-over for the studied tryptic fragments of Cytochrome C is in the range of 1.4 % for the earliest eluting fragment to 22 % for the last eluting in the first blank, and reduces to 0.7 to 9.3% for the second blank. By taking the immobilized trypsin chip off-line before running a blank, it was determined that the majority of the carry-over came from the trypsin chip.

## BSA on-line vs. in-solution digestion



A comparison between 1 pmol of on-line vs. in solution digested BSA was made. Intensity of the tryptic peptides in the in-solution digest were 4-10x higher, and also coverage was much higher (66% vs. 26%). The conversion possibly can be improved by increasing the residence time of the protein in the digest chip by reducing the flowrate and/or increasing the digest temperature.

## Conclusions

The experiments show differences between the in-solution and on-line digestion; further optimization of the on-line digestion may be still possible. Although run-to-run reproducibility is good, carryover from the immobilized trypsin reactor is substantial. Different wash strategies of the immobilized trypsin reactor will be tested for their effect on carryover. The performance of the cHiPLC digestion column for lower concentrations sample also needs to be studied

Overall, on-line digestion in the easy to use cHiPLC format shows good potential as a replacement for batch processed in-solution digestion, especially when processing larger numbers of samples and when complete digestion is not required, i.e. for protein identification.