# Automated sample preparation via rapid on-line trypsin digestion prior to mass spectrometry

Dagley LF*, Infusini G*, Hewetson J#, and Webb AI*
*Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia
#Shimadzu Scientific Instruments (Oceania), Rydalmere, NSW 2116, Australia

## Introduction

Sample preparation for mass spectrometry analysis often represents the main bottleneck in proteomics experiments. Manual protein digestion is a laborious, multi-step process that is commonly used and often requires up to 18 hours to achieve sufficient proteolysis. Recent advances in this field have led to the routine use of the filter-aided sample preparation (FASP) method, which has improved the efficiency and coverage of protein identification by mass spectrometry[1]. However this technique remains cumbersome, time-consuming and somewhat variable and is therefore unsuitable for high-throughput, quantitative studies. Moreover, benchtop desalting and buffer exchange procedures can result in significant sample losses. To this end, the Perfinity Workstation (Shimadzu™) was used as a front-end tool for performing rapid trypsin digestion and on-line desalting prior to high resolution LC-MS/MS on the hybrid quadrupole-Orbitrap instrument (Q-Exactive, Thermo Fisher Scientific™). This system (Figure 1) allows for affinity selection, buffer exchange, trypsin digestion, desalting and reverse phase separation. The UV detection enables the visualisation of peptide absorbance at 214 nm subsequent to trypsin digestion.

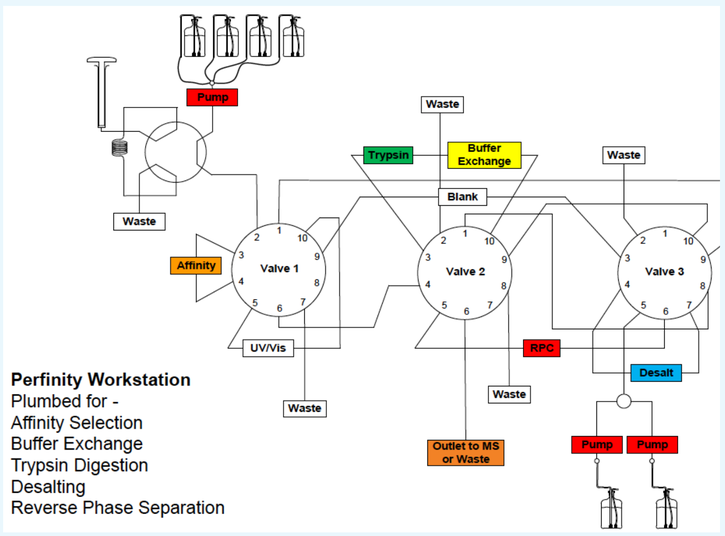


**Figure 1:** Schematics of the LC configuration for the Perfinity workstation based on the Shimadzu™ modular HPLC system.

## Methods

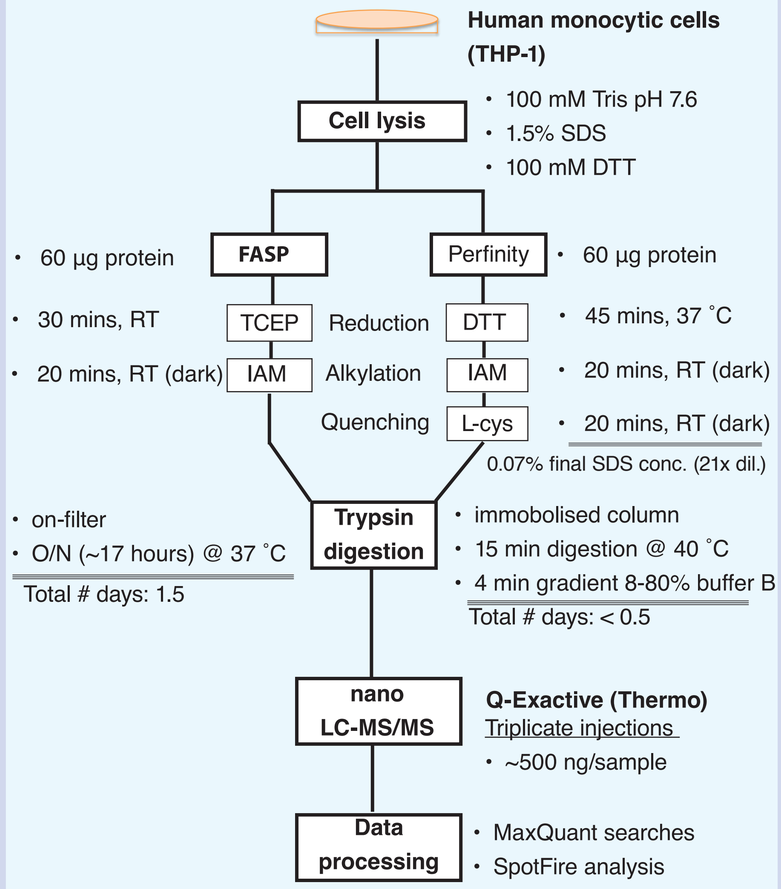


**Figure 2:** Workflow illustrating the methodology used in this study to compare the FASP protocol with the online trypsin digestion procedure via the Perfinity LC system. In this study human monocytic cells (THP-1) were first solubilised in a buffer containing 1.5% SDS and digested at 0.07% SDS final concentration[2] prior to performing on-column trypsin digestion.

### Mass spectrometry

Peptides were separated by nanoflow reversed-phase LC on a Waters nanoAcquity C18 150 mm x 75 µm I.D. column with a 60 min gradient set at a flow rate of 400 nl/min from 100% buffer A (0.1% formic acid, 3% acetonitrile, 97% water) to 55% B (80% acetonitrile, 0.1% formic acid, 20% water). The nano HPLC was coupled on-line to a Q-Exactive mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher Scientific™) for automated MS/MS. The Q-Exactive was run in a data-dependent acquisition mode with resolution set at 70K and a top 10 method dynamically choosing the most abundant precursor ions from the survey scan (350–1850 Th) for HCD fragmentation with the resolution set at 17.5K. Dynamic exclusion was enabled for 90 secs.

## Results

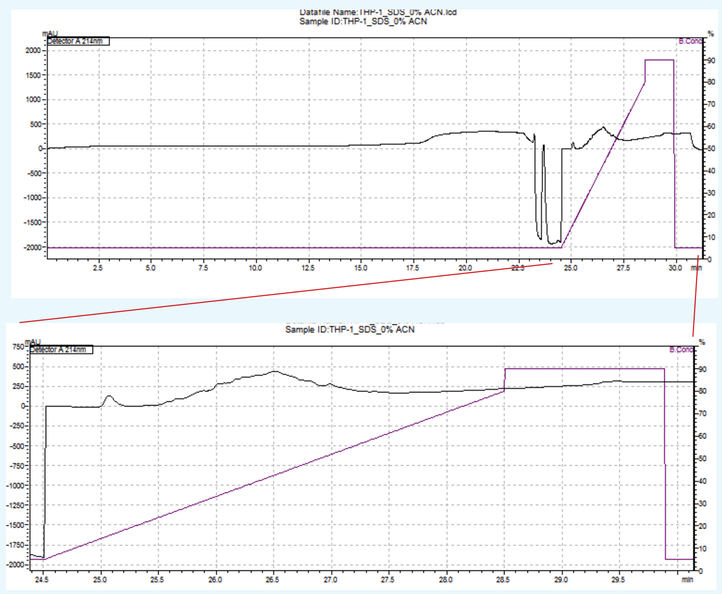


**Figure 3:** Chromatographic UV absorbance traces of the THP-1 cell lysate subjected to trypsin digestion on the immobilised column using the Perfinity LC workstation. Inset: a zoomed in version of the trace illustrating the increase in UV absorbance detected between 25-28 mins representing the peptides that were eluted off the RPC column (in line with the trypsin column) and collected for subsequent MS analysis.

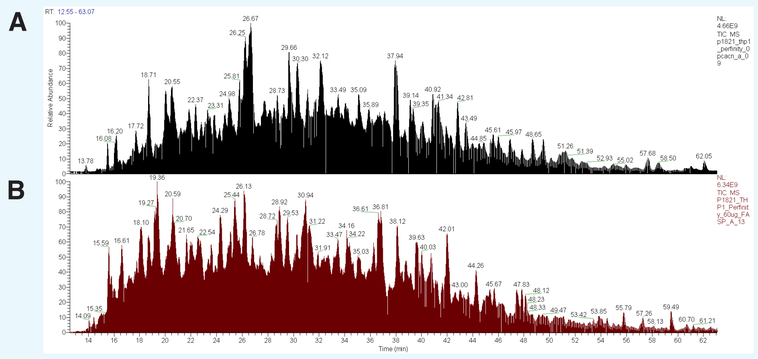


**Figure 4:** TIC traces (12-63 mins) during the analysis of peptides derived from (A) the Perfinity workstation or (B) FASP digestion.

The number of proteins identified from the automated digestion of THP-1 cells on the Perfinity were found to be comparable to that of an overnight FASP digestion. A total of 2150 unique proteins were identified overall with 1557 proteins shared between the two preparative methods whilst 113 and 389 proteins were exclusively identified in the Perfinity and FASP methods, respectively (Figure 5A). These results indicate that over 90% of proteins (1738) can be identified from a 15 minute digestion on an immobolized trypsin column compared with an overnight on-filter digestion with the FASP method (1969 proteins). However, the FASP technique recovered a larger number of unique integral membrane proteins (111 versus 11), which can likely be attributed to the multiple steps of urea exchange used in the FASP workflow which facilitate detergent removal via dissociating detergent micelles (Figure 5B). Triplicate analyses of peptides on the Q-Exactive instrument resulted in a similar coefficient of variability (CV) of 5.5% for the automated digests and 5.2% for the FASP digests.

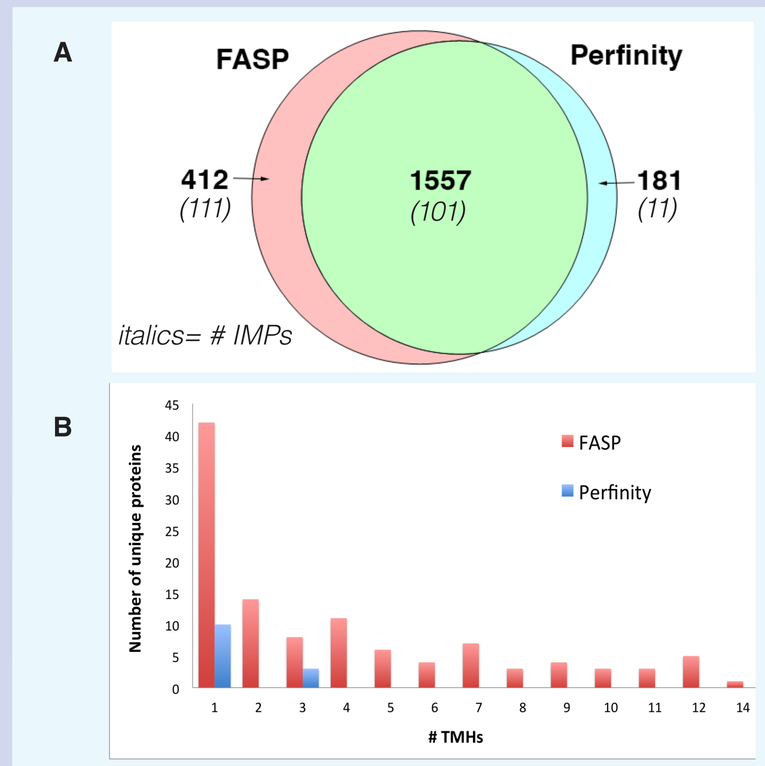


**Figure 5:** (**A**) Venn diagram illustrating the overlap in the number of unique protein identifications made in SDS-solubilised THP-1 cell lysate subjected to trypsin digestion either by FASP or Perfinity analysis. FASP resulted in a higher number of unique proteins, including integral membrane proteins (IMPs, numbers in italics) compared with the Perfinity workstation. (**B**) Plot illustrating the number of IMPs containing one or more transmembrane helix (TMH) exclusively identified in the FASP analysis compared with Perfinity. A total of 111 unique IMPs were identified from FASP compared with only 11 from the Perfinity workstation.

## Conclusions and Future work

The high-efficiency trypsin column on the Perfinity workstation provided fast on-line trypsin digestion in 15 minutes. Subsequent mass spectrometry analysis of these peptides resulted in the identification of 90% of the unique proteins detected using FASP, a procedure which requires overnight digestion plus a day of preparative benchtop work. However, a significant proportion of integral membrane proteins were not detected in the THP-1 samples subjected to the Perfinity LC workstation. Therefore, careful consideration must be made prior to embarking on peptide mapping studies involving the Perfinity system in terms of the suitability of membrane-protein enriched samples. Future work will be focused on establishing the degree of carryover observed after various amounts of protein is loaded onto the immobolised trypsin column, as well as determining the optimum amount of time requred for sufficient trypsin digestion.

The Perfinity workstation also allows for the affinity-based capture of target proteins and online digestion and separation of peptides for immunochromatographic analyses. In this way, user-specific affinity columns (Perfinity A, G or A/G) can be made by cross-linking with a desired antibody thus replacing the step for a second antibody in conventional assays.

Other potential future applications of the Perfinity workstation include its use in the clinic where it could be interfaced to a triple quadrupole instrument to streamline biomarker-based assays which are compatible with an urgent care setting e.g. Cardiac ischemia, stroke. This would allow for a remarkable improvements in assay speed as well as gains in reproducibility given the full automation of steps, ultimately translating into an increased ability to accurately identify quantitative differences in protein abundance. We will next test the Perfinity workstation using human plasma samples subjected to dimethyl labeling.

## References

1. Wisniewski, J. R., A. Zougman, N. Nagaraj and M. Mann (2009). "Universal sample preparation method for proteome analysis." Nat Methods 6(5): 359-362.

2. Zhou, Jian-Ying, et al. "Simple sodium dodecyl sulfate-assisted sample preparation method for LC-MS-based proteomics applications." Analytical chemistry 84.6 (2012): 2862-2867.