Jet Stream Proteomics for Sensitive and Robust Standard Flow LC/MS

Technical Overview

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Introduction

Proteomics research has typically used nanospray LC/MS to achieve maximum sensitivity when the sample amount is limited. Nanoflow LC/MS takes advantage of the well-known sample concentration effect of electrospray ionization (ESI). It produces high ionization efficiency due to the small droplet size and efficient sampling of the ions as the nanospray tip is in close proximity to the MS inlet. The use of a small internal column diameter (id) for analysis results in the highest sample concentration on-column, and allows low flow rates to the mass spectrometer ion source. However, nanoflow LC/MS systems require more skill to use and maintain, and have limited nanocolumn capacity, which can result in less robust chromatography for complex samples.

The larger column id of standard ultra-high performance liquid chromatography (UHPLC) allows for much greater sample loading and does not have the limitations of nanoflow LC for routine and robust analysis of complex proteomics samples. However, standard flow chromatography sensitivity has been the limiting factor in wide spread use for LC/MS proteomics analysis. Jet Stream proteomics uses Agilent's high performance mass spectrometers incorporating iFunnel technology to change this paradigm by achieving new levels of sensitivity for peptides in complex matrixes using standard flow LC/MS.

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Agilent iFunnel Technology Enhances Sensitivity

Agilent iFunnel technology combines the efficient electrospray ion generation and focusing of Agilent Jet Stream technology with a hexabore capillary sampling array, which enables a much larger fraction of the ESI spray plume to enter the mass spectrometer ion optics[1]. A unique dual-stage ion funnel allows increased ion capture and transmission while removing the much higher gas load from the hexabore capillary. This innovative design greatly improves overall system signal-to-noise (S/N) by providing greater signal with lower system noise (Figure 1).

Agilent Jet Stream thermal gradient focusing technology was developed to significantly enhance sensitivity for ESI-MS by improving the desolvation and spatial focusing of ions[2]. Superheated nitrogen sheath gas confines the nebulizer spray to more effectively dry ions, and concentrate them in a thermal confinement zone. The improved desolvation also reduces noise, and the full confinement of the spray by the superheated nitrogen gas eliminates sample recirculation and reduces chromatographic peak tailing. Improved ion production results in higher MS and MS/MS signal intensities and improved S/N ratios. On average, a 5- to 10-fold improvement in MS and MS/MS sensitivity is realized by using Agilent Jet Stream technology (Figure 2).

The Jet Stream source has also been investigated with capillary flow LC/MS for targeted multiple reaction monitoring (MRM) assays for protein biomarker quantitation[3]. Results demonstrated that even at low flow rates (15 µL/min) the Jet Stream source provided greater than 3x signal enhancement versus standard ESI.

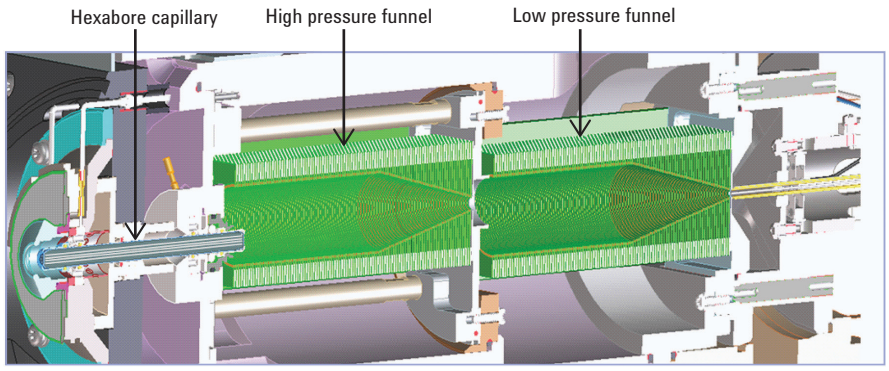

Figure 1. The Agilent iFunnel dual ion funnel assembly removes atmospheric gas and neutral species, and focuses and directs ions into the low pressure optics of the Agilent 6495 Triple Quadrupole LC/MS.

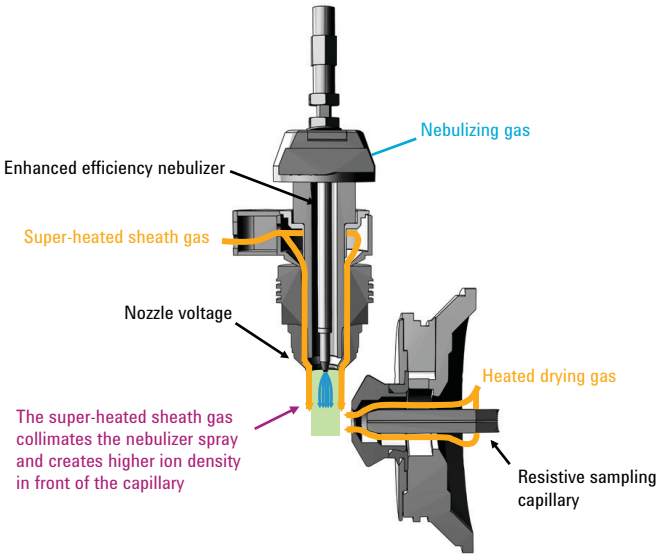

Figure 2. Agilent Jet Stream technology uses super-heated nitrogen to desolvate the spray and confine the electrospray plume, making more ions accessible to sampling by the mass spectrometer.

Sensitivity

A major benefit in lowering column id and flow rate is the increased sensitivity observed as the analyte elutes in a smaller volume of liquid. ESI typically shows significant concentration-dependence, however, the response can be mixed, as recently shown[4,5]. With Jet Stream technology, a mass-sensitive response (sensitivity proportional to amount of sample introduced into the source) was observed for the analytes measured. This is demonstrated by an experiment in which a constant linear velocity was maintained across columns with different id dimensions, thus keeping the same chromatographic peak width but different sample concentrations introduced into the ion source. The signal measured for the same amount of analyte injected on each column was nearly identical. This mass-dependence allows the use of the more robust chromatographic separations of 2.1 mm id columns without sacrifice in sensitivity (Figure 3).

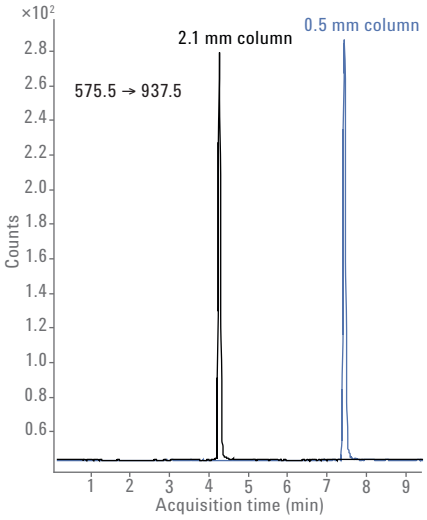

Figure 3. Capillary flow (blue) versus standard flow (black) with 1 fmol on-column.

Even with the increased sensitivity provided by Jet Stream compared to conventional ESI, nanoflow LC/MS still provides about 10x lower detection levels for material limited samples due to the high ionization efficiency and proximity of nanospray to the MS inlet. This sensitivity difference can be mitigated by injecting more where sample amount is not limited. As demonstrated in human plasma by Percy *et al.*[6], loading 10x more plasma on a standard flow LC/MS system with a Jet Stream source resulted in equal or better sensitivity for 72 of the 81 target peptides. This improvement in sensitivity results from a combination of the mass dependent response of the Jet Stream source, the greater loading capacity of the 2.1-mm column, and the narrower chromatographic peaks resulting in less interference. Figure 4 shows a comparison between response for a synthetic peptide spiked into mouse plasma and analyzed by both nanoflow and standard flow LC/MS. For standard flow, 10x more sample was injected than for the nanoflow analysis, and results demonstrate almost identical signal.

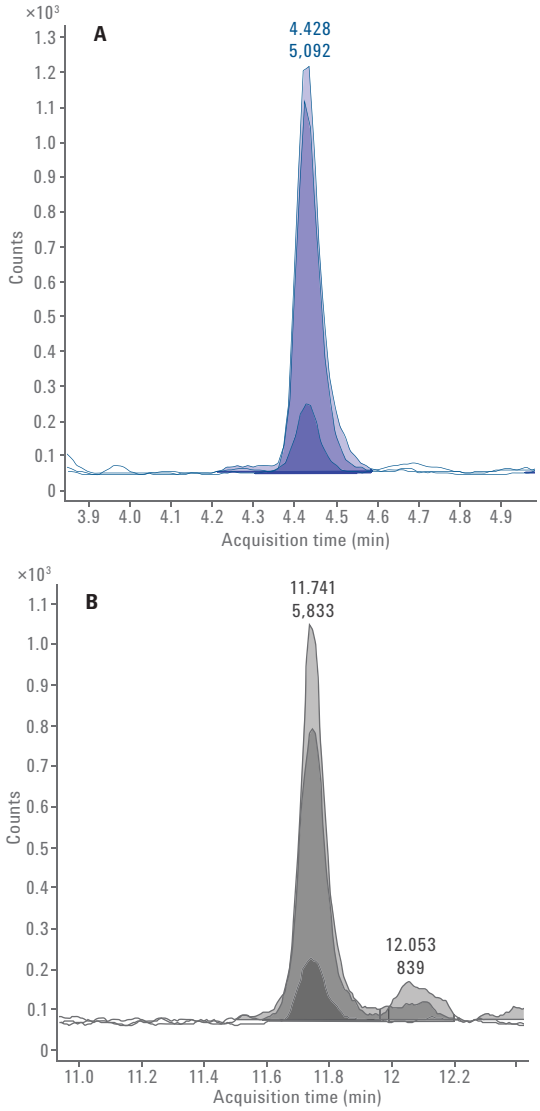

Figure 4. Agilent Jet Stream (A) versus nanoflow (B) for the peptide INDISHTQSVSSK.

Quantitative Proteomics on Triple Quadrupole

For complex biological matrixes such as plasma, the improved chromatographic performance with standard flow LC/MS provides significant improvement in the overall analysis where there is a strong need for short analysis time, high-throughput, and robust assays[7,8]. Agilent triple quadrupole mass spectrometers interfaced to the Agilent 1290 Infinity LC system through the Jet Steam source enables high sensitivity and robustness as well as outstanding retention time stability. For assays involving many peptides and transitions, creating a dynamic MRM method with scheduled retention time windows provides the best performance. When retention times are very stable, narrower windows can be set, which allows for more transitions in an assay (Figure 5). Stable retention times also allow large sample sets to be analyzed over several weeks without method adjustment. After 3.5 weeks of continual operation with these plasma digests, retention times showed an average of less than 1.5 % RSD for 40 peptides monitored in a QC kit (MRM Proteomics, Inc.).

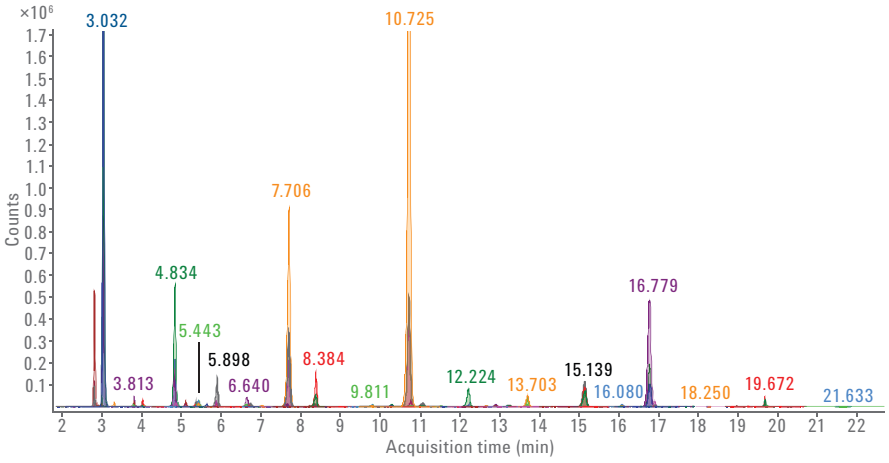

Figure 5. Overlay of 238 transitions from 40 peptides in QC kit from MRM Proteomics, Inc.

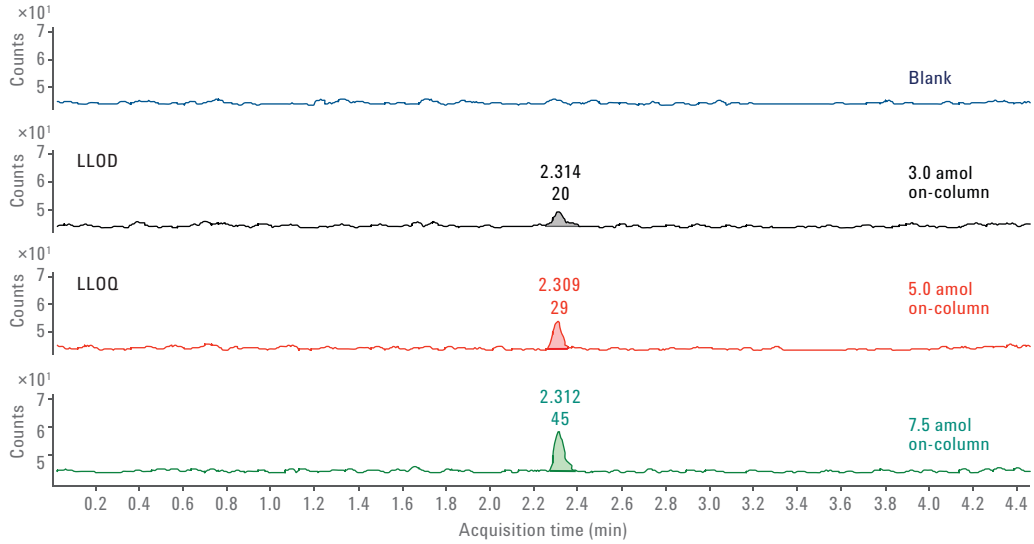

Figure 6. The outstanding sensitivity of the Agilent 6495 Triple Quadrupole Mass Spectrometer with standard flow chromatography is demonstrated using a synthetic peptide standard spiked into an enolase tryptic digest. At the lower limit of quantitation (LLOQ), the reproducibility was 14 % (n = 10) and accuracy was 109.8 %.

With the latest Agilent 6495 iFunnel Triple Quadrupole and Jet Stream source, low attomole limits of detection (LOD) can be achieved for peptides. For LVNEVTEFAK from human serum albumin, a lower limit of quantitation (LLOQ) of 5 amol on-column was reached along with six orders of linear dynamic range (Figure 6). This performance, combined with the robustness of UHPLC, provides an excellent platform for routine, high-sensitivity peptide quantitation.

Discovery Proteomics on Q-TOF

In discovery proteomics, data-dependent acquisition using nanoflow LC/MS has long been the preferred technique. Previously, with the wide dynamic range and sensitivity requirements for proteomics samples, there were not many options. To improve depth of coverage, it has not been uncommon to significantly overload nanocolumns, leading to shifting chromatography and displacement effects. With the advent of Jet Stream technology and the Agilent 6550 iFunnel Q-TOF, it is now feasible to use standard flow UHPLC for protein identification workflows where sample amount is not a limitation. A preliminary study performed using an *Escherichia coli* (*E. coli*) tryptic digest demonstrated the effect of gradient length and sample loading on the number of proteins identified.

As shown in Figure 7, longer gradient lengths increase the number of protein identifications, however, the benefit is not as dramatic as typically seen with nanoflow chromatography. In addition, the excellent chromatographic separation achieved allowed high validation rates of the acquired MS/MS spectra (average > 60 % validated), which supports shorter methods and higher throughput. As expected, increased sample loading is another critical factor in maximizing protein identification, as illustrated in Figure 8.

This high-throughput UHPLC/MS analysis strategy has also been applied to the proteome of more complex samples. Triplicate analysis of a human breast cancer cell line (Figure 9) demonstrates the performance of the Jet Stream enabled protein discovery workflow. A total of 32,446 unique peptides and 5,905 unique proteins were identified across the triplicate 100 minute LC/MS analyses.

With 2-dimensional analysis, the number of identifications can be significantly greater, however, this substantially increases the amount of LC/MS time. With Jet Stream proteomics, shorter gradients and rapid column re-equilibration result in faster analysis times. For the 2-dimensional experiment shown in Table 1, a high pH fractionation was performed prior to LC/MS analysis as described by Wang *et al.*[9]. A total of 96 high pH fractions were collected for each cell line and concatenated to 30 fractions for each of two cell lines. With a 30 minute gradient and 10 minute re-equilibration time, a combined total of 13,650 unique human proteins were identified in the two cell lines in approximately 40 hours of LC/MS time. These results demonstrate that Jet Stream proteomics generates excellent results for discovery proteomics workflows while providing the ease-of-use and higher throughput of UHPLC.

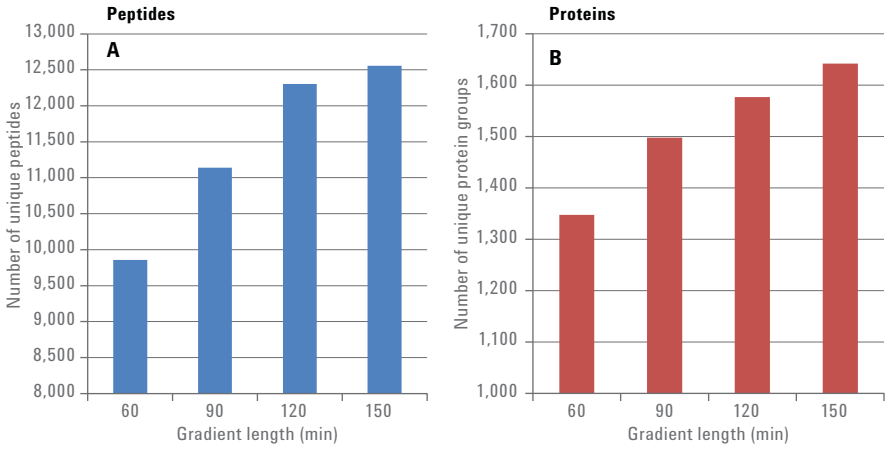

Figure 7. Protein identification results from an *E. coli* digest (15 µg on-column) for different gradient lengths using a 2.1 × 250 mm Agilent AdvanceBio Peptide Mapping column. Protein database searching was done using Agilent Spectrum Mill Software, and peptide spectral matches were validated using a 1.2 % false discovery rate (FDR) filter.

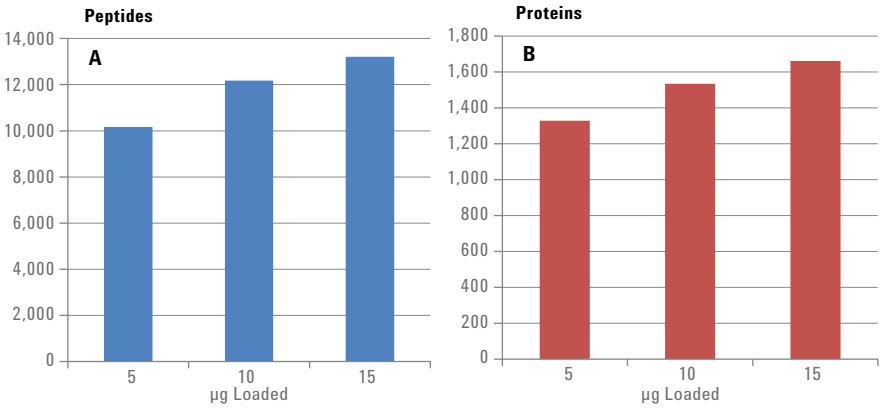

Figure 8. Protein identification results from loading different amounts of an *E. coli* digest for the 120-minute gradient length using a 2.1 × 250 mm Agilent AdvanceBio Peptide Mapping column. Protein database searching was done using Agilent Spectrum Mill Software, and peptide spectral matches were validated using a 1.2 % false discovery rate (FDR) filter.

Table 1. Protein identification results for the 2-dimensional analysis of two cell lines. Samples were fractionated by high pH RPLC and concatenated (n = 30) before LC/MS analysis without phosphopeptide enrichment. Total analysis time was 40 hours.

	Phosphoproteins		All proteins	
Cell line	No. of unique peptides	No. of unique proteins	No. of unique peptides	No. of unique proteins
Embryonic stem cells	4,514	3,230	120,564	12,613
Neural progenitor cells	4,369	3,207	92,904	11,945
Total		5,113	151,838	13,650

Conclusions

Jet Stream proteomics uses iFunnel technology to enable the use of standard flow LC/MS. Combining the mass-flow dependent detection and enhanced sensitivity of the Agilent Jet Stream source with the improved sensitivity of ion funnel mass spectrometers, it is now feasible to use UHPLC for high-throughput, robust, and reproducible LC/MS analysis of complex proteomics samples for both discovery and targeted workflows.

Acknowledgements

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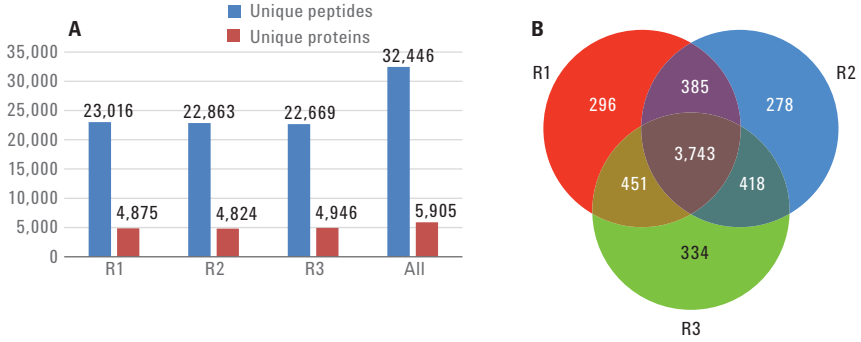

Figure 9. Triplicate analysis of MDA-MB-231 cell lysate digest (25 µg on-column) analyzed on an Agilent AdvanceBio Peptide Mapping 2.1 × 250 mm column with a 100 minute gradient. A) The number of unique proteins and peptides identified (1 % FDR) in each analysis as well as the total number for all three runs demonstrating excellent reproducibility. B) Venn diagram of unique proteins identified in the triplicate analyses.

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