Exploring the sensitivity differences for peptide quantification in the low flow rate regime

NanoLC™ 400 System for high performance nanoflow and microflow LC

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A commonly held view of the protein / biomarker research pipeline is diagrammed in Figure 1. Typically, high end mass spectrometers are used to perform non-targeted discovery experiments where the goal is to quantitatively profile large numbers of proteins across small numbers of samples to find protein targets (blue). In the next phase, this subset of proteins is analyzed across a larger set of samples. The outcome of this verification step (green) is hopefully a small subset of very promising protein markers that are then taken forward to perform large scale validation across a much larger sample size (orange), in hopes to find a small panel that provides the desired read-out.

While much focus has been on the mass spectrometry innovations, the importance of the separation component for getting the highest quality data cannot be underestimated. The sensitivity, robustness and throughput of the LC strategy must also evolve as research progresses through the pipeline. The nanoflow regime is used extensively for high sensitivity discovery experiments but more recently life science researchers are exploring the use of the microflow regime for increased throughput and robustness for quantification. The sensitivity differences between nanoflow and microflow rates are explored here with the goal of establishing general sensitivity guidelines.

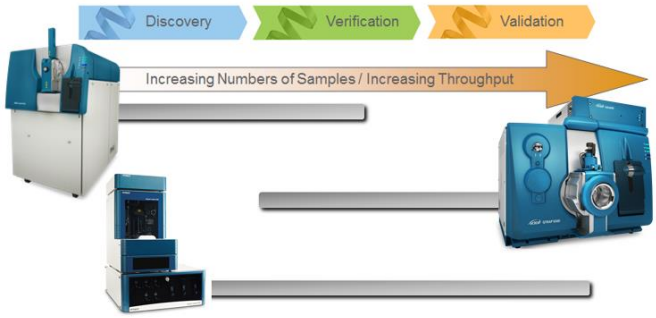


Figure 1. Protein / biomarker research pipeline. The NanoLC 400 System has the flexibility and reliability to support a broad range of LC-MS workflows, from high sensitivity global discovery to high throughput targeted quantitation.

Key features of the NanoLC 400 System for quantitative proteomics

- The NanoLC 400 system[1] has the flexibility and reliability to support a broad range of workflows, from global discovery to targeted quantitation
  - Easy to change flow module cartridges allow the user to rapidly switch between flow rate ranges[2]
  - MFCPlus™ Technology provides flow stability for highest 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.

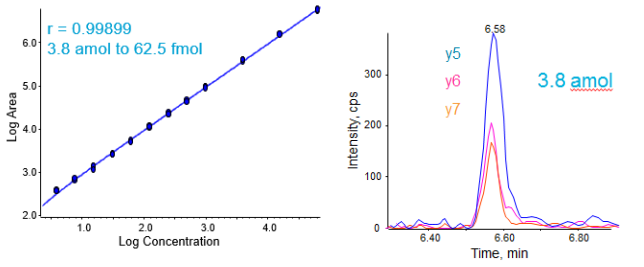
Methods

In this work, four different column diameters were assessed, all containing the ChromXP C18CL (120Å, 3 µm) reverse phase media and all 15 cm in length:

- 75 µm cHiPLC® column, 300 nL/min
- 200 µm cHiPLC® column, 1 µL/min
- 300 µm column, 4 µL/min
- 500 µm column, 10 µL/min

Experiments were done using MRM analysis on the QTRAP® 5500 system. Concentration curves were analyzed and the lower limits of quantitation (LLOQs) were measured for each peptide on each column to compare impact of flow rate and column diameter on sensitivity. LLOQ is defined as lowest point on concentration curve with <20 %CV precision and accuracy between 80 and 120% and S/N >10.

Ten unlabeled tryptic peptides (6 Protein Mix, Michrom Bioresources) were used for this sensitivity assessment in order to establish some general sensitivity guidelines (Figure 2). The matrix used was a simple matrix so the sensitivity differences could be measured with minimal impact from interferences or ion suppression. The matrix was a Serum Albumin tryptic digest (Michrom Bioresources) at 10 fmol on column, held constant across the concentration curve. Standard concentration curves



Row	Component Name	Actual Conc	Num. V	Mean	Standard Devi	Percent CV	Accuracy
1	CA.VGDANPALQK.2/y5	3.80	3 of 3	4.460e0	4.431e-2	0.99	117.37
2	CA.VGDANPALQK.2/y5	7.60	3 of 3	7.663e0	2.563e-1	3.34	100.82
3	CA.VGDANPALQK.2/y5	15.20	3 of 3	1.398e1	1.330e0	9.51	92.00
4	CA.VGDANPALQK.2/y5	30.50	3 of 3	2.825e1	7.548e-1	2.67	92.63
5	CA.VGDANPALQK.2/y5	61.00	3 of 3	5.632e1	2.142e0	3.80	92.33
6	CA.VGDANPALQK.2/y5	122.00	3 of 3	1.185e2	5.275e0	4.45	97.10
7	CA.VGDANPALQK.2/y5	244.00	3 of 3	2.379e2	1.197e1	5.03	97.52
8	CA.VGDANPALQK.2/y5	488.00	3 of 3	4.761e2	1.200e1	2.52	97.56
9	CA.VGDANPALQK.2/y5	976.00	3 of 3	9.972e2	2.869e1	2.88	102.18
10	CA.VGDANPALQK.2/y5	3900.00	3 of 3	4.191e3	1.523e2	3.63	107.45
11	CA.VGDANPALQK.2/y5	15630.00	2 of 2	1.672e4	4.673e2	2.79	106.98
12	CA.VGDANPALQK.2/y5	62500.00	3 of 3	6.149e4	2.346e3	3.82	98.38

Figure 3. Measuring the lower limits of quantitation for a set of tryptic peptides. Serial dilutions were done to generate linear concentration curves of the peptide mixture and the peptides were monitored by LC-MRM. Shown is the concentration curve for the peptide VGDANPALQK analyzed on the 300 µm ID column (top left). The statistics table (bottom) illustrates the very high reproducibility achieved across the concentration range. The MRM signal for this peptide at the LLOQ of 3.8 amol on column is shown in the top right.

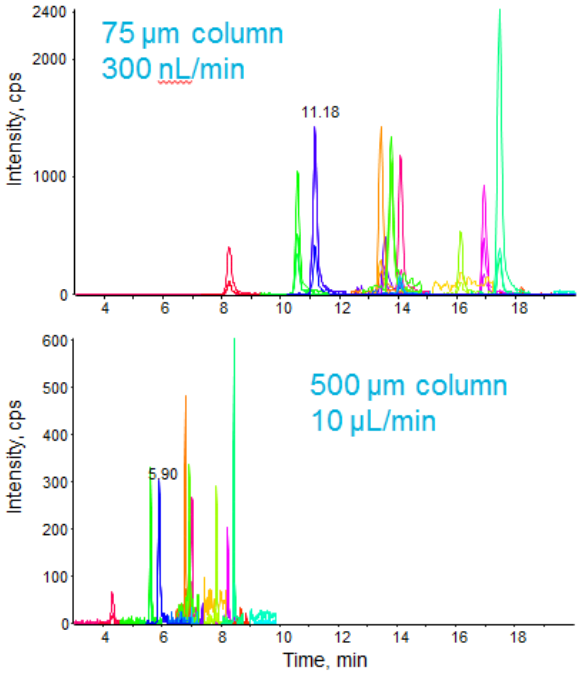


Figure 2. Comparison of the MRM traces at the lowest and highest flow rates. The MRM chromatogram for the 10 peptides from the 75 µm ID column was obtained using a 15 min linear gradient, peptides are observed from 8 – 18 mins (top). At the higher flow rates, faster gradients can be used. Analyzing the same peptides on a 500 µm ID column (bottom) shows a small decrease in sensitivity but an increase in throughput (7 minute linear gradient).

were analyzed using MultiQuant™ Software and LLOQs were computed for each peptide on each column to compare impact of flow rate and column diameter on sensitivity. The concentration range measured for each column diameter tested was 62.5 fmol down to 1.9 amoles of peptide standard.

Exploring sensitivity differences at different column diameters

This experiment was straight forward to do with the NanoLC 400 System as switching between flow regimes takes minutes due to the easily exchangeable flow modules. The Nano flow module was used for the 75 and 200 µm ID columns, along with the NanoSpray® Source. For the 300 and 500 µm ID columns, the Low Microflow module was used, along with the Turbo V™ Source with the 25 µm ID hybrid electrode.

The LLOQ for each peptide was measured on each of the different columns using MRM analysis on a QTRAP® 5500 system. A set of 10 different tryptic peptides was used such that an average sensitivity difference could be determined. An example of the data acquired is shown in Figure 3, for the peptide VGDANPALQK from carbonic anhydrase. A concentration curve was run across a wide range of concentrations, the curve was assessed for linearity and

reproducibility and the LLOQ was determined to be 3.8 amol on column for the 300 µm ID column.

Table 1 shows the average increase in LLOQ (or the decrease in sensitivity) relative to running a 75 µm ID column at 300 nL/min for each column diameter. For the 200 µm ID column running at 1 µL/min, a ~2.5 fold decrease in sensitivity or increase in LLOQ was observed. Moving to the 300 µm ID column at 4 µL/min on the Turbo V™ Source, a 3x difference in sensitivity was seen. Finally, 4x differences in sensitivity was observed when compared to a 500 µm ID column at 10 µL/min as compared to the same experiment on a 75 µm ID column. Therefore, as flow rate increases, increased robustness and throughput can be obtained with just a small decrease in sensitivity. Obviously with the larger column diameters, more sample can often be loaded if available to offset this sensitivity difference.

As these averages are for only 10 different tryptic peptides, these relative differences should be used as a guidance for deciding on the right column diameter for the planned experiment.

Conclusions

The flexibility and reproducibility of the NanoLC 400 System makes it an excellent LC system for labs performing proteomics workflows, from high sensitivity protein expression analysis to high throughput peptide quantitation.

- User exchangeable flow modules enables both nanoflow experiments and microflow experiments to be used as the workflows demand
- Microflow experiments can be performed with higher throughput and robustness with a small decrease (~4x) loss in sensitivity
- The fast, high precision autosampler provided accurate, reproducible sample injections (<1% CV) even in µL-pickup mode with speeds compatible with high throughput microflow experiments.

Table 1. Comparison of sensitivity differences between the column sizes. The LLOQ difference for each peptide at each column diameter was computed relative to the LLOQ for the 75 µm ID column, then this difference was averaged across the 10 peptides monitored.

Column ID	Flow Rate	Source	LLOQ* ↑ Relative to 75 µm
75 µm	300 nL/min	NanoSpray® Source	*1*
200 µm	1 µL/min	NanoSpray® Source	*2.5*
300 µm	4 µL/min	*Turbo V™ Source*	*3*
500 µm	10 µL/min	*Turbo V™ Source*	*4*

* LLOQ defined as lowest point on concentration curve with <20 %CV precision and accuracy between 80 and 120%; S/N >10

References

1. Achieving very high reproducibility for quantitative proteomics with nanoflow LC-MS - NanoLC™ 400 System. SCIEX technical note RUO-MKT-02-5755-A.

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