A simplified orthogonal electrospray source setup for robust nanoflow or microflow proteomics analysis

Nanoflow and microflow flow rates can be run stably using the microflow probe on the OptiFlow Turbo V ion source on the ZenoTOF 7600 system

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This technical note describes the use of the OptiFlow Turbo V ion source on the ZenoTOF 7600 system at nanoflow rates. The source is configured using the microflow probe that allows for automated calibration between runs, increases throughput, maintains instrument robustness and provides comparable data to those acquired using a dedicated nanoflow ion source.

The OptiFlow Turbo V ion source is a dual-flow ion source designed for microliter-per-minute flow rates (microflow 1-10, 10-50, or 50-200 µL/min) or nanoflow (≤ 1 µL/min), depending on the configuration. It was determined that this source also works effectively when running nanoflow through the microflow probe/electrode assembly. This configuration does not require a specialized emitter and the spray is orthogonal to the orifice, which minimizes instrument contamination. Furthermore, the calibrant delivery system (CDS) inlet (when connected in-line to the horizontal probe on the source) acts as an E Lens, pushing to the ions into the mass spectrometer to improve sensitivity.

Data presented here demonstrate the equivalent performance of the microflow probe/electrode (Micro probe) and the nanospray probe/electrode (Nano probe) on the OptiFlow Turbo V ion source at nanoflow rates (Figures 1, 6, 7). The increased robustness, the E Lens effect from the CDS inlet and the ease of use make this an ideal option for nanoflow proteomics analysis on the ZenoTOF 7600 system.

Key features of using the Micro probe on the OptiFlow Turbo V ion source for nanoflow

- Results acquired using the OptiFlow Turbo V ion source configured to run with the Micro probe at nanoflow rates are equivalent to those obtained using the dedicated Nano probe
- Use of the Micro probe enables the use of the CDS for in-batch calibration and system tuning
- The spray through the Micro probe is directed orthogonal to the orifice of the ZenoTOF 7600 system, which reduces potential instrument contamination

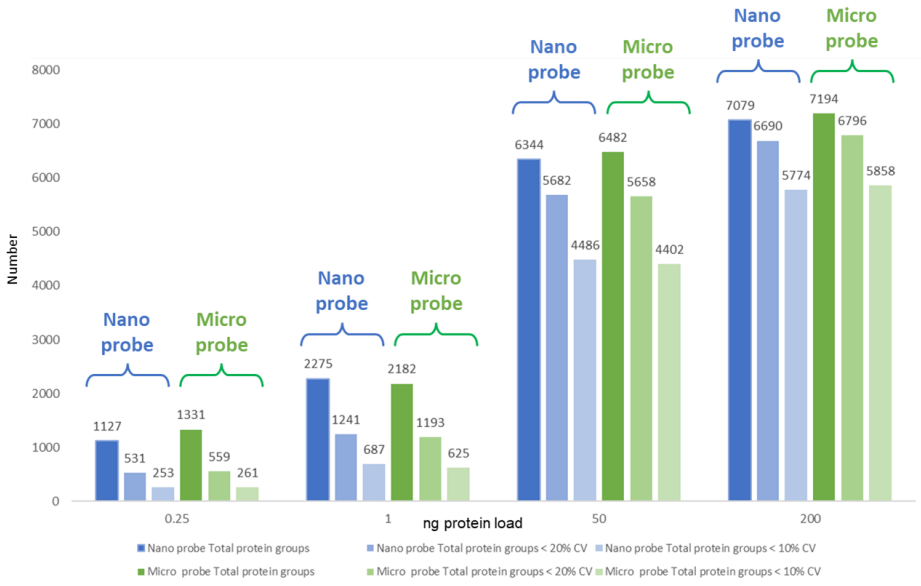


Figure 1. Comparison of protein groups identified using the OptiFlow Turbo V ion source with either the Nano probe or the Micro probe. Protein loadings of 0.25, 1, 50 and 200 ng on column were analyzed by Zeno SWATH DIA on the ZenoTOF 7600 system at a flow rate of 300 nL/min using either the Nano probe (blue) or at 500 nL/min using the Micro probe (green). Data shown indicate the total number of protein groups identified and those that had %CVs of less than 20 and 10, respectively. Results indicate equivalent performance between the 2 means of sample introduction.

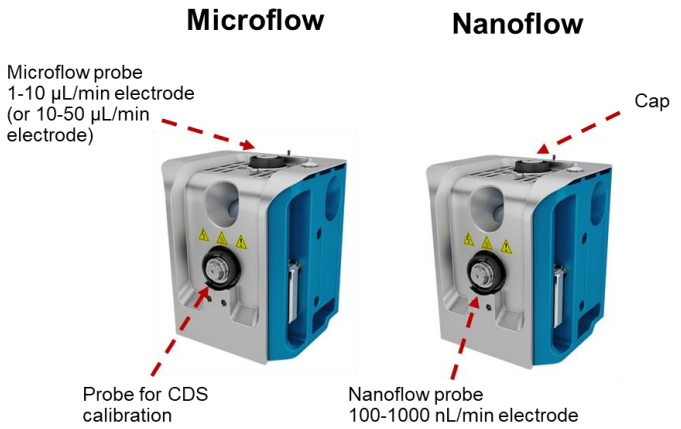


Figure 2. Standard configurations for the OptiFlow Turbo V ion source for dedicated microflow and nanoflow rates. When the microflow configuration is used, the output from the CDS can be attached to where the nanoflow probe is normally used. This enables automated calibration during analysis. The nanoflow configuration requires a nanoflow probe to be inserted into the horizontal position, permitting on-column system calibration to be achieved by injecting a standard during a batch run.

Methods

OptiFlow Turbo V ion source set up: The OptiFlow Turbo V ion source is a dual flow source with 2 configurations: orthogonal microspray and on-axis nanospray. Figure 2 shows the standard configurations for the source, depending on the flow rate. The LC flows directly toward the orifice when using the standard configuration for nanoflow. Although the curtain gas (CUR) instrument parameter is used to minimize neutrals from entering and contaminating the instrument, orthogonal spray inherently contaminates less due to the active exhaust design of the source.

An alternative nanoflow configuration for the OptiFlow Turbo V ion source is presented here (Figures 3 and 4), in which the nanoflow column output is connected to the Micro probe instead of the Nano probe. This assembly requires specific fittings to accommodate this configuration. Figure 3 shows the different options for connections that enable the attachment of the column or capillary tubing to the Micro probe. By connecting the column output to the Micro probe, the Nano probe port can then be connected to the CDS using the calibration probe, which enables automated calibration throughout the batch in addition to instrument tuning and optimization.

Before connecting the nanoflow column outlet to the fittings, the fused silica should be freshly cut and polished using >2000 grit emery paper to ensure a clean, 90° angle on the tubing. These alternative configurations are robust and have minimal dead volumes to avoid adversely affecting peak shape.

Sample preparation: Lyophilized commercial K562 tryptic digest was reconstituted in LC-MS grade water containing 5% acetonitrile and 0.1% formic acid to a stock concentration of 0.5 µg/µL. Water with 0.1% formic acid or 5 fmol/µL bovine serum albumin (BSA) tryptic digest was used for further dilutions to achieve working concentrations of 200, 50, 5 and 0.5 ng/µL. SCIEX PepCalMix (P/N 5045759) was reconstituted to a final concentration of 20 fmol/µL with water containing 5% acetic acid and 2% acetonitrile.

Chromatography: A Waters ACQUITY UPLC M-class system with nanoflow tubing (20 µm ID line from µSample Manager valve to column) was used with the Evosep EV-1106 column (15 cm × 150 µm ID), Evosep EV-1112 column (15 cm × 75 µm ID) or Waters HSS T3 column (25 cm × 75 µm ID, Waters, P/N 186007474) with direct-inject mode. The nanoflow columns

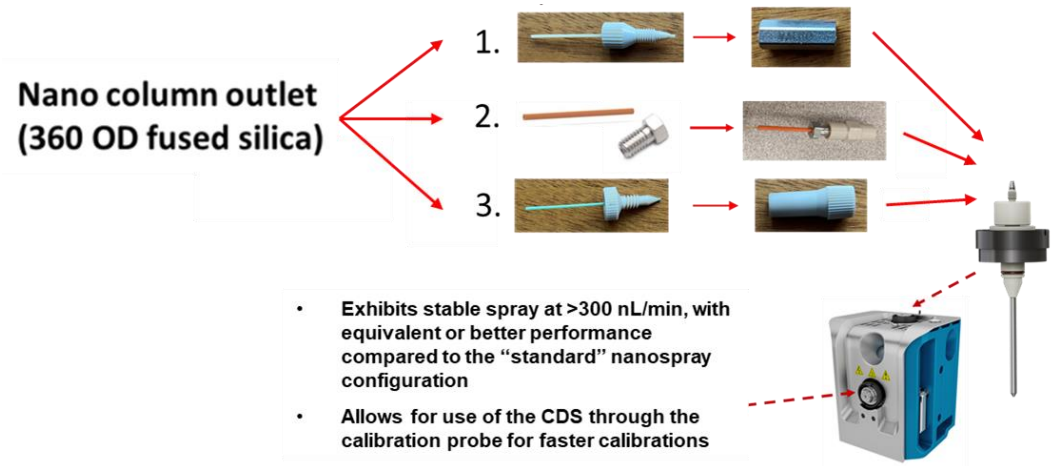


Figure 3. Alternative nanoflow connections to the OptiFlow Turbo V ion source. The nanoflow column outlet can be connected to the Micro probe using the following options: (1) a 1/32" PEEK nut with a tan sleeve for 360 OD fused silica and a VALCO 1/16"-to-1/32" reducing union (0.15 mm bore), (2) a high-pressure union body for NanoTight sleeves or 1/16" OD tubing, PEEK, 0.005" ID 10-32 (coned) and a 1/16" VICI nut or (3) a MicroTight nut (360 OD, 6-32 thread) with a green PEEK sleeve (0.0155" ID x 0.05" OD) and a PEEK ZDV MicroTight adapter.

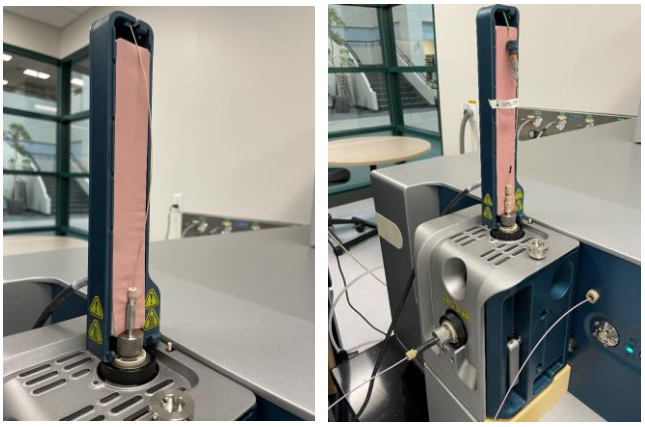


Figure 4. OptiFlow Turbo V ion source assembly. The images above show the OptiFlow Turbo V ion source configured with nanoflow columns attaching to the Micro probe, with a nanoflow column, such as the Waters HSS T3 column, having a distal fused silica ending (left) or a 1/16" fitting (right). In both cases, the calibration probe is connected to the horizontal probe assembly.

Evosep EV-1106 and Evosep EV-1112 were operated at either 300 nL/min or 500 nL/min using the Micro probe (orthogonal to nanoflow) and 300 nL/min with the OptiFlow interface using the standard Nano probe. A Waters HSS T3 column was operated at 400 nL/min with the Nano and Micro probes. PEEK Sil tubing for microflow (40 µm ID) was used with the Phenomenex Kinetex XB-C18 column (0.3 × 15 cm, 2.6 µm bead, P/N 000F-4496-AC) in direct-inject mode. The Phenomenex Kinetex XB-C18 microflow column was run at 5 µL/min flow rate with the Micro probe. The LC mobile phases used were water with 0.1% formic acid (buffer A) and acetonitrile with 0.1% formic acid (buffer B). Zeno SWATH data-independent acquisition (DIA) and Zeno data-dependent acquisition (DDA) methods were run with 45 min and 120 min gradients, respectively, with buffer B concentration increasing from 3% to 35%.

Evosep EV-1106 and EV-1112 columns with a 1/16" fitting were connected to the Nano probe via a 1/16"-to-1/32" reducing union (1/32" bore; VICI Valco, P/N ZRU1.5T), as shown in Figure 3, path 1. The reducing union was connected to a 1/32" PEEK nut (SCIEX, P/N 5019621) with a tan plastic sleeve for 360 µm OD fused silica (Eksigent P/N 910-00088) to the Nano probe using a 20 µm ID × 360 µm OD fused-silica tubing (4 cm long) and the supplied fitting for the Nano probe. The connection to the Micro probe (orthogonal to nanoflow) was established via a PEEK 1/16"-to-1/16" nano-tight union (IDEX, P/N P-779), as shown in Figure 3, path 2.

The Waters HSS T3 column with a 360 µm OD fused silica distal tubing was connected directly to a Nano probe (Figure 2). A connection to the Micro probe also utilized the 1/16"-to-1/32" reducing union (1/32" bore; VICI Valco, P/N ZRU1.5T) along with the 1/32" PEEK nut (SCIEX, P/N 5019621) and a tan sleeve (Eksigent P/N 910-00088), as shown in Figure 3, path 1.

Mass spectrometry: A ZenoTOF 7600 system was equipped with an OptiFlow Turbo V ion source that is compatible with the (a) Micro probe (with an electrode for 1-50 µL/min flow rates, P/N probe with an electrode for 0.1-1 µL/min flow rates. The ZenoTOF 7600 system was operated either in Zeno DDA or Zeno SWATH DIA mode.

The Zeno SWATH DIA method consisted of 85 variable-width windows enabling the selection of precursors from 400-900 m/z. A TOF MS scan covering 400-1500 m/z with an accumulation time of 50 ms and TOF MS/MS scans covering 140-1750 m/z with accumulation times of 18 ms were used. Source conditions for the Micro probe were Gas 1 = 12 psi, Gas 2 = 60 psi, CUR = 25 psi, voltage = 4500 V and temperature = 150°C. Source conditions for the Nano probe were Gas1 = 10 psi, CUR = 25 psi, voltage = 3200 V and temperature = 225°C.

The Zeno DDA method with Zeno trap pulsing activated (Zeno threshold = 100,000 cps) consisted of a TOF MS scan (400-1500 m/z) and TOF MS/MS scans with accumulation times of 100 ms and 20 ms, respectively. The top 45 precursor ions (intensity threshold = 100 cps) were selected for fragmentation by CID with an exclusion time of 15 sec, exclusion tolerance of 15 ppm and selection of charge states 2-5.

The method for monitoring PepCalMix consisted of a TOF MS scan of 400-1250 m/z with an accumulation time of 250 ms and a dedicated TOF MS/MS scan for the 758.91 m/z precursor, scanning 100-1500 m/z with an accumulation time of 500 ms and a CE of 40. The LC gradient length was 15 min with a flow rate of 300 nL/min.

Data processing: DDA data were searched with the ProteinPilot app within the OneOmics suite against a canonical + isoform FASTA database to identify peptides and protein groups at 1% global false discovery rate (FDR).

Triplicate injections of K562 Zeno SWATH DIA data were processed in DIA-NN software and Spectronaut software, version 16.2 (Biognosys) using a HeLa and K562 spectral library. A spectral library was previously generated from high-pH fractionation of HeLa and K562 cell lines, followed by IDA analysis on a Zeno TOF 7600 system and subsequent database searching[1]. Results from DIA-NN were reported at 1% global FDR using the *.pr_matrix and *.pg_matrix output files for precursors and protein groups, respectively.

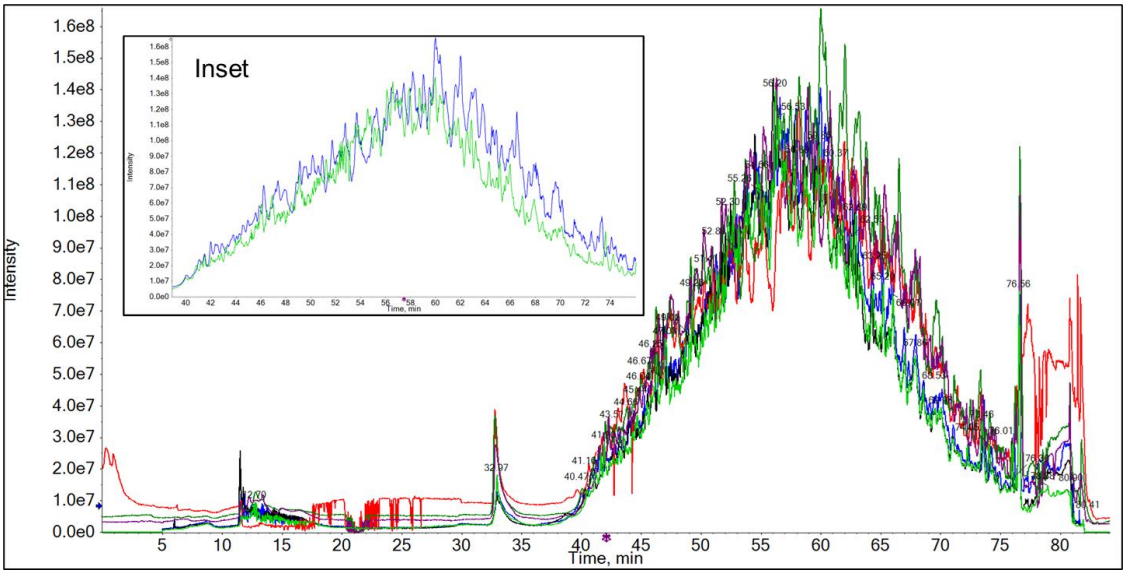


Figure 5. TIC comparison of Zeno SWATH DIA data acquired on the ZenoTOF 7600 system at 300 nL/min using the Nano probe or the Micro probe on the OptiFlow Turbo V ion source with an Evosep EV-1106 column. The 50 ng K562 cell digest was analyzed under 2 conditions in triplicate. Overlaid results show that the OptiFlow Turbo V ion source configured for nanoflow rates using the Micro probe provides comparable results to the dedicated Nano probe. The inset region of the graph shows an expanded view of the chromatogram between 40 and 75 minutes of LC time for a single injection, each using the Micro probe (green) or the Nano probe (blue).

Effects of using the Micro probe at nanoflow rates on protein group identifications and peptide detection

Most bottom-up proteomics analysis workflows are based on nanoflow chromatography. This flow regime typically increases the sensitivity of the assay compared to microflow and analytical flow rates. However, there are significant challenges with nanoflow that impact productivity. For example, nanoflow spray emitters are fragile and susceptible to clogging, plumbing connections can leak and most nanoflow sources spray directly into the orifice of the instrument, which can lead to eventual instrument contamination.

An effective bottom-up proteomics experiment combines broad coverage of the peptides eluting from the chromatographic column with good spectral quality and sensitivity to ensure spectral matching with the proteomic database. The initial experiments performed here were designed to assess the effectiveness of the OptiFlow Turbo V ion source configured with nanoflow connected to the Micro probe and compare it to an equivalent flow using the dedicated Nano probe.

Figure 1 and Figure 6 shows a histogram comparing the respective numbers of protein groups and peptides identified using either the dedicated Nano probe or nanoflow through the Micro probe with Zeno SWATH DIA or IDA. In these experiments, protein loadings of 0.25, 1, 50 and 200 ng on column were analyzed with Zeno SWATH DIA or 50 and 200 ng loadings with IDA. For the standard Nano probe data, the flow rate was set to 300 nL/min, whereas the flow rate of the Micro probe setup was 500 nL/min. Three replicate injections were performed for each condition for Zeno SWATH DIA and the data

Table 1. Protein group identifications with 50 ng K562 cell extract using either the Nano probe (300 nL/min) or the Micro probe (500 nL/min) on the OptiFlow Turbo V ion source with an EV-1106 column and at 5000 nL/min with the Phenomenex Kinetex XB-C18 column. Data were acquired by Zeno SWATH DIA on the ZenoTOF 7600 system and processed as described above.

Condition	Total protein groups	Rep 1	Rep 2	Rep 3	Total protein groups < 20% CV	Total protein groups < 10% CV	% Protein groups at <20% CV	% Protein groups at <10% CV
Nano probe (300 nL/min)	*6344*	*6247*	*6246*	*6243*	*5682*	*4486*	*89.6*	*70.9*
Micro probe (500 nL/min)	*6482*	*6337*	*6377*	*6328*	*5658*	*4402*	*87.3*	*67.9*
Micro probe (5000 nL/min)	*5573*	*5372*	*5372*	*5359*	*3930*	*2541*	*70.5*	*45.6*

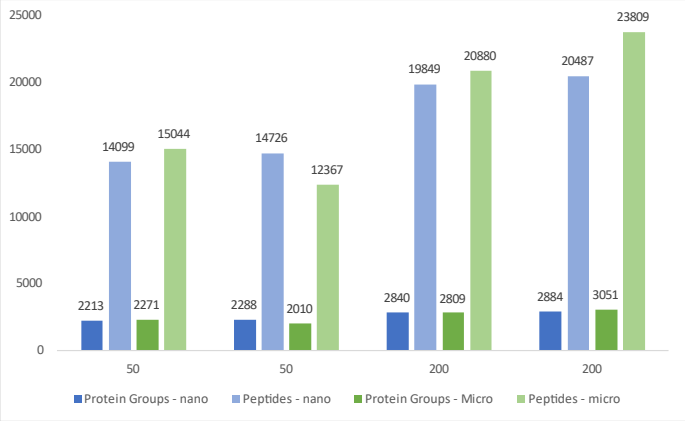
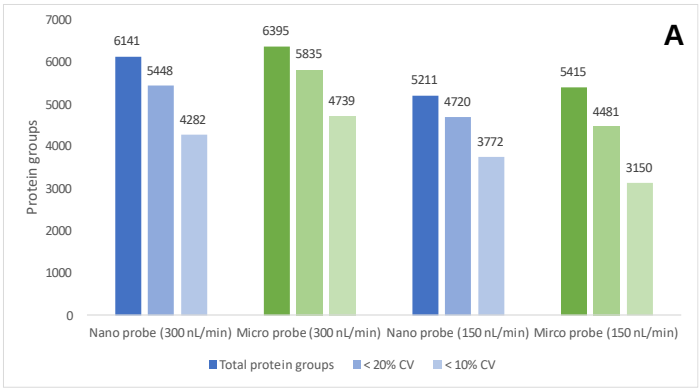


Figure 6.* Protein and peptide identification *comparisons for Zeno IDA data acquired on the ZenoTOF 7600 system at 300 nL/min using the Nano probe (blue) or 500 nL/min using the Micro probe (green) with the OptiFlow Turbo V ion source. *On-column K562 loadings of 50 ng or 200 ng were analyzed in duplicate with both regimes as indicated and data were processed to show total numbers of protein groups and peptides identified.*

were acquired and processed as described above to identify peptides and protein groups. Although the flow to the Micro probe was higher than that for the Nano probe (500 vs. 300 nL/min, respectively), as shown in Figures 1 and 6, the data show equivalent performance, as measured by the total numbers of both protein identifications and peptides characterized in both DIA and IDA approaches. When the same sample (50 ng) was run on the Micro probe at relatively higher flow rates (5 µL/min), the numbers of peptides and protein groups decreased, as expected (Table 1). This experiment was reproduced with 2 different Micro probes (data not shown).

Figure 5 shows the overlaid total ion chromatograms (TICs) of similar intensities acquired from 2 experimental runs of 50 ng of K562 protein digest run in triplicate on an Evosep EV-1106 column. This experiment was performed with the Nano probe and the Micro probe using a flow rate of 300 nL/min. The inset of Figure 5 expands the time period in which most peptides elute and shows a single TIC each from the Nano probe (blue) and from the Micro probe (green) elution.

In another experiment, a 50 ng K562 cell extract analyzed with a 150 nL/min flow rate using the Nano probe or the Micro probe was also evaluated. Using the EV-1106 column, 50 ng of K562 cell digest showed a comparable number of protein groups (Figure 7A) and precursors (Figure 7B) identified between the 2 configurations for 300 nL/min and 150 nL/min flow rates. Source conditions were maintained the same for all flow rates. Thus, the Micro probe can effectively run nanoflow at rates as low as 150 nL/min. These results indicate that the lower flow rate does not offer any significant advantage using this column.



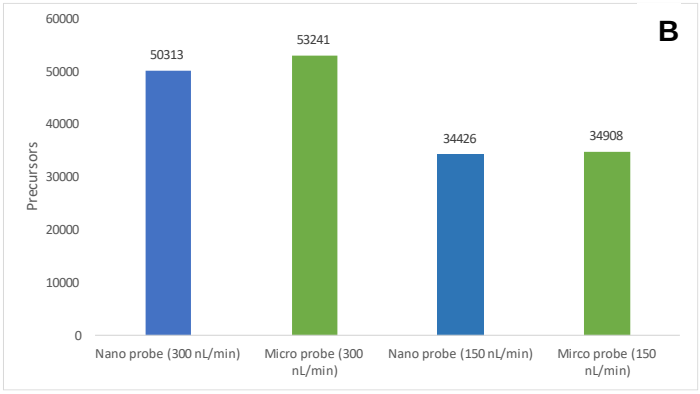


Figure 7. Total protein groups (A) and precursor peptides (B) identified (50 ng load, 300 nL/min and 150 nL/min) using the OptiFlow Turbo V source configured using with either the Nano probe or the Micro probe. Data from Zeno SWATH DIA analysis indicate equivalent performance between the 2 probes, with the added benefits of being able to do automated calibration and the improved robustness of the orthogonal spray configuration.

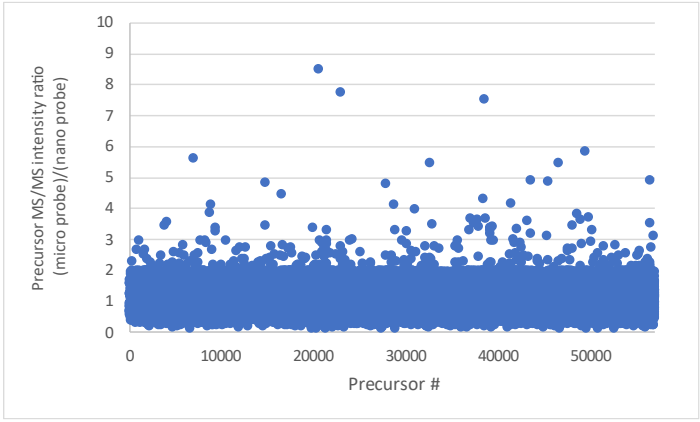


Figure 8. Precursor (peptide) intensity ratios from MS/MS data acquired using the Micro probe and those from the Nano probe assembly at 300 nL/min using the EV-1106 column. These ratios correspond to the data shown in Figure 7. The average and median precursor ratios ((Micro probe)/(Nano probe)) were 1.06 and 0.97, respectively, which demonstrate equivalent signal using either OptiFlow Turbo V ion source configuration.

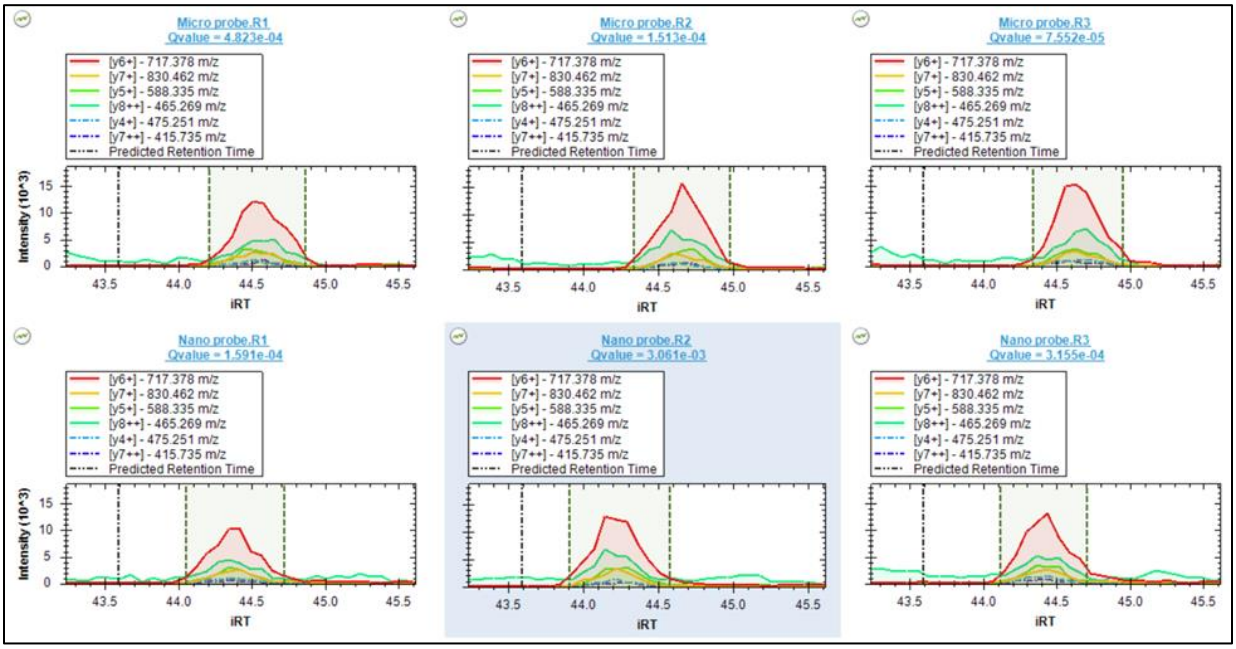


Figure 9. Effects of the OptiFlow Turbo V ion source configuration on the intensity of MS/MS peptide fragments. The data shown in Figure 7 were processed using Spectronaut software to extract chromatographic peaks for the top 7 peptide fragments. Shown here are the results from the peptide VLELDNVK. Data for the Micro probe are presented on the top and data acquired using the Nano probe are presented on the bottom. Note the equivalent peak intensities.

Finally, precursor intensities at the MS/MS level (extracted ion chromatogram, XIC) corresponding to the results presented in Figure 5 and Figure 7 were compared to evaluate the 2 probes. Using the Evosep EV-1106 column operating at 300 nL/min, the average and median ratios ((Micro probe)/(Nano probe)) were calculated to be 1.06 and 0.97, respectively (Figure 8). This observation correlates well with the comparable number of identifications obtained in Figure 7 and it demonstrates that the absolute signal obtained from either configuration of the OptiFlow Turbo V ion source remains unchanged.

Spectral evidence demonstrating the equivalent quality of the data obtained from these experiments is shown in Figure 9. The acquired data were processed by Spectronaut software and the XICs for the top 7 fragments of a representative peptide identified in the K562 cell extract (VLELDNVK) are shown for triplicate injections. Despite slight shifts in the retention times, the chromatographic peak shapes and intensities of the peptide fragments were similar between the 2 different OptiFlow Turbo V ion source configurations.

The overall results from these experiments indicate that using the Micro probe at <1 µL/min flow rates provides comparable data to those acquired using the dedicated Nano probe. Additionally, there are significant advantages to this approach: 1) being able to use the CDS to auto-calibrate during batch analysis with a short calibration procedure (2 min infusion) compared to calibrations from dedicated LC injections of a calibration standard, 2) the ability to fully optimize and tune the ZenoTOF 7600 system without changing the probe and 3) increased instrument robustness resulting from the spray being orthogonal to the orifice.

Increased sensitivity of the Micro probe configuration due to the E Lens effect

The OptiFlow Turbo V ion source has an added benefit to those listed above when run at nanoflow rates using the Micro probe. As mentioned, in this configuration, the calibration probe can be used simultaneously, enabling automated calibration via the CDS. An unintended consequence of using this configuration is that the calibration probe also acts as an E Lens that pushes ions toward the orifice as they emit from the Micro probe. The concept of an E Lens effect was introduced on the Triple Quad version of the SCIEX 7500 system[2]. This system is configured with the OptiFlow Pro ion source, which is distinctly different than the OptiFlow Turbo V ion source described here, and is equipped with a stainless-steel rod (the E Lens) that sits on-axis, directly in front of the instrument orifice. During data acquisition the E Lens is held at potential and creates an electrical field of the same polarity as the ions being generated in the source. This field essentially pushes ions into the mass spectrometer, resulting in a 2- to 3-fold increase in overall sensitivity.

Figure 10 shows the increased sensitivity that resulted from the E Lens effect caused by using the calibration probe with the OptiFlow Turbo V ion source. In this experiment, SCIEX PepCalMix was run both with and without the calibration probe assembly. When the calibration probe was not used, a probe port plug was utilized to cap the port entry. The flow rate for both runs was 300 nL/min on an Evosep EV-1106 column. Representative data from 6 peptides from the PepCalMix are shown for both experiments in an overlay. The ratios of the TOF MS peptide areas [(with Cal probe)/(without Cal probe)] are shown in Table 2. The data from Table 2 demonstrate that there are average and median increases in sensitivity of 1.37-fold and 1.49-fold, respectively, when the calibration probe is installed compared to when the calibration probe was not installed.

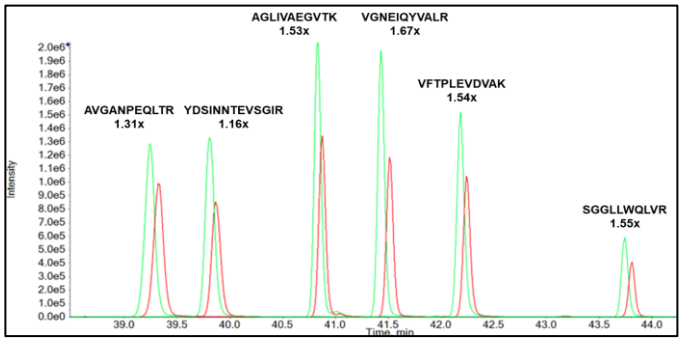


Figure 10. E Lens effect due to calibration probe on sensitivity when using the Micro probe. The 40 fmol SCIEX PepCalMix was analyzed using the ZenoTOF 7600 system with the OptiFlow Turbo V ion source configured to run nanoflow through the Micro probe with (green) and without (red) the calibration probe attached.

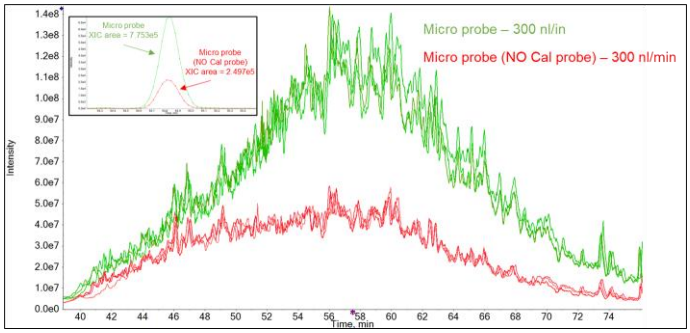


Figure 11. Increase in the TIC of 50 ng K562 cell digest analyzed by Zeno SWATH DIA using the Micro probe with (green) and without (red) the calibration probe installed (300 nL/min). The increase in the TIC is reflected by a commensurate increase in precursor peptide peak areas. Inset shows the XIC for a peptide (P35637-AAIDWFDGK, y6 - 767.336 m/z) run with and without the calibration probe. The average peak area increased 3.08-fold when the calibration probe was used.

The same approach was used to evaluate the E Lens effect on a more complex sample (Figures 11 and 12). In this experiment, 50 ng K562 cell digest was analyzed by Zeno SWATH DIA using the OptiFlow Turbo V ion source configured with the Micro probe with or without the calibration probe. In experiments without the

Table 2. Fold-increases in peak area from representative PepCalMix peptides from the experimental described in Figure 10. Ratios of peak areas of for peptides using the Micro probe with or without the calibration probe are shown.

Peptide	With Cal Probe	No Cal Probe	Ratio
AETSELHTSLK	4.90E+06	4.49E+06	**1.09**
GAYVEVTAK	4.76E+06	4.41E+06	**1.08**
IGNEQGVSR	4.27E+06	2.68E+06	**1.60**
LDSTSIPVAK	8.45E+06	6.30E+06	**1.59**
AGLIVEGVTK	9.47E+06	6.18E+06	**1.53**
LGLDFDSFR	4.19E+06	3.91E+06	**1.07**
GFTAYYIPR	5.14E+06	3.54E+06	**1.45**
SGGLLWQLVR	2.74E+06	1.77E+06	**1.55**
AVGANPEQLTR	8.76E+06	6.71E+06	**1.31**
SAEGLDASASLR	2.92E+06	2.03E+06	**1.44**
VFTPLEVDVAK	7.71E+06	5.02E+06	**1.54**
VGNEIQYVALR	9.18E+06	5.49E+06	**1.67**
YIELAPGVDNSK	6.06E+06	3.97E+06	**1.53**
DGTFAVDGPGVIAK	2.05E+06	1.27E+06	**1.61**
SPYVITGPGVVEYK	4.46E+06	2.89E+06	**1.54**
ALENDIGVPSDATVK	1.81E+07	1.09E+07	**1.65**
TVESLFPEEAETPGSAVR	2.22E+07	1.67E+07	**1.33**

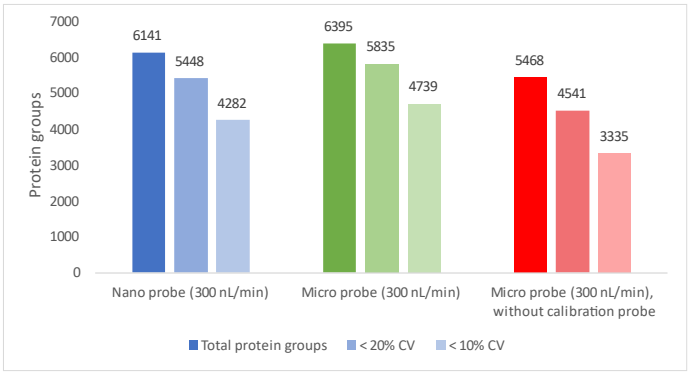


Figure 12. The E Lens effect on the total numbers of protein identifications and peptides detected in a Zeno SWATH DIA analysis of K562 cell digest. There were 17% more protein groups and 32% more precursor peptides identified when the calibration probe was used. These data were derived from the experiment detailed in Figure 11.

calibration probe, the port was capped. Figure 11 shows overlays of the TIC from triplicate runs either with or without the CDS probe. The TIC intensity at the chromatographic apex was nearly 2.5-fold higher when the calibration probe was used due to the E Lens effect. This increase was also seen at the XIC level, with average and median precursor peak area ratios (for 53,781 precursors) 3.08- and 2.97-fold higher, respectively, when the CDS probe was utilized (data not shown). The data presented in Figure 12 show an 17% increase in protein groups and 33% increase in precursors identified with the use of the calibration probe.

Conclusions

- The OptiFlow Turbo V ion source using the Micro probe on the ZenoTOF 7600 system is ideal for proteomics analysis at low-flow regimes
- Using the Micro probe at flow rates <1µL/min (nanoflow) provides robust performance, increases throughput and enables automatic calibration
- Data generated using the Micro probe show equivalent or better numbers of protein groups and precursor peptides identified and similar precursor XIC intensities

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