Microflow LC-Nanospray MS for Bottom-up Proteomics

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Key Words

M3 emitter, Multinozzle,
Microflow LC, Nanospray ESI,
Bottom-up Proteomics,
HeLa Digest, Plasma Digest,
Thermo Fisher EasyPrep™ Kit,
Q Exactive Plus

Goal

To develop a Microflow LC-Nanospray ESI-MS (MnESI-LC-MS) platform for bottom-up proteomics that achieves high-throughput, high-sensitivity, and robustness, by using Newomics® M3 Emitters and Thermo Fisher PepMap™ C18 columns on a Q Exactive™ Plus mass spectrometer.

Introduction

Liquid chromatography-mass spectrometry (LC-MS) is the enabling technology for global-scale analysis of proteins (proteomics). Nanoflow LC-MS (flow rate < 1 μL/min) is routinely used to achieve high sensitivity for identifying and quantifying thousands of peptides and proteins in small volumes of biological samples, but lacks robustness and throughput (i.e., speed). By contrast, microflow LC-MS (flow rate 1-50 μL/min) typically achieves higher throughput and robustness with relative standard deviation (RSD) < 20%, but lacks the sensitivity of nanoflow LC-MS. The Newomics award-winning silicon multinozzle emitters (M3 emitters) enable optimization of sensitivity, throughput, and robustness by splitting the microflow eluent evenly into multiple nanoflows at the emitter, thereby dramatically enhancing the ionization efficiency. In this Application Note, we demonstrate the application of our microflow LC-nanospray ESI-MS platform (MnESI-LC-MS) for bottom-up proteomics studies of HeLa whole cell lysates and human plasma samples.

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Methods

Sample Preparation

Pierce™ HeLa Protein Digest Standard (Thermo Fisher, USA, Cat.#: 88329) and human plasma digest, prepared by EasyPep™ Mini MS Sample Prep Kit (Thermo Fisher, USA, Cat.#: A40006), were used for bottom-up LC-MS analysis. During the experiments, standards for robustness and sensitivity tests were resuspended in Solvent A (3% acetonitrile in 97% water with 0.1% formic acid). HeLa protein digests were diluted into 25, 50, 100, 200, 500, 1000, and 2500 ng/mL with Solvent A. Standards were prepared in glass vials to reduce adsorption to plastic surfaces. All standards were kept at -20°C until analysis.

Instrumentation

A Thermo UltiMate 3000 nanoUPLC system was used as the LC system. Chromatographic separation was achieved with the conditions shown in Table 1. Data dependent analysis (DDA) was performed on a Thermo Q Exactive ™ Plus mass spectrometer with the parameters for full MS/dd-MS2 detection shown in Table 2. The position of M3 emitters relative to ion transfer tube was tuned based on the TIC signal and was optimized at about 3 to 4 mm.

Data Analysis

We performed analysis of dynamic range, sensitivity, and robustness using our microflow LC-nanospray MS platform and compared the results to those obtained from microflow LC-MS and nanoLC-MS, respectively. In the study of dynamic range and sensitivity, HeLa protein digests at each concentration were repeated 3 times to obtain an average. In the robustness test, the HeLa protein digest and plasma digest samples were run ~30 times to generate performance statistics, including RSD (relative standard deviation) values for protein groups, protein abundance, and peptide retention time. The MS raw data were loaded onto Proteome Discoverer 2.2.0.388 (Thermo Fisher, USA) and searched against the Swiss-Prot human sequence DBs using SEQUEST HT with 10 ppm of precursor mass tolerance, 0.02 Da of fragment mass tolerance. Percolator was employed to calculate the protein false discovery rate (FDR) by searching decoy database and the cutoff was FDR = 0.01 (for high-confidence search) and p-value < 0.05.

Table 1: LC-MS conditions for robustness test

	nanoLC-MS	MnESI-LC-MS
Flow Rate (µL/min)	0.3	1.5
Column	A standard C18 column, 75 µm ID x 150 mm L, 1.7 µm beads	Thermo Acclaim PepMap 100, C18, 150 µm ID x 150 mm L, 2 µm beads (Prototype)
Column Temperature	40°C (using a commercial column heater)	
Mobile Phase	A: 3% acetonitrile in water, 0.1% FA B: 3% water in acetonitrile, 0.1% FA	
LC pluming	Direct injection without trap column	
Injection Mode and Volume	Full loop, 1 µL loop	
Samples	1. Pierce™ HeLa Protein Digest Standard (Thermo Fisher) 2. Human plasma digest prepared by EasyPep™ Kit (Thermo Fisher)	
Run Time (min)	70	
LC Gradient	Time: 0 / 1 / 8 / 58 / 61 / 61.5 / 65 / 65.1 / 70 %B: 5 / 5 / 8 / 24 / 36 / 85 / 85 / 5 / 5	
Mass Spec	QE-Plus	
Acquisition Method	full MS/dd-MS^2	
Emitter/Sprayer	Stainless steel nano-bore emitter (ES542)	M3 emitter (10µm ID, 8-nozzle)
Spray Voltage (V)	2,500	3,500
Spray Angle	10°	30°
Gas Flow	0	1L/min

Table 2: Parameters of data dependent analysis (DDA)

Full MS		dd-MS^2	
Resolution	70,000	**Resolution**	17,500
AGC target	3.00E+06	**AGC target**	5.00E+04
Maximum IT	30 ms	**Maximum IT**	50 ms
Scan range	375-1,500 m/z	**Loop count**	20
		TopN	20
		Isolation window	2.4 m/z
		Fixed first mass	180.0 m/z
		(N)CE	27

Results and Discussion

1. MnESI-LC-MS for bottom-up proteomics of HeLa cell lysates

HeLa protein digest was serially diluted from 2,500 ng/mL to 25 ng/mL for the dynamic range study. The flow rate was 1.5 μL/min using the Thermo PepMap™ C18 LC column (2 μm particle size, 150 μm x 150 mm) and M3 Emitter (8 nozzles, 10 μm ID). The numbers of protein group (Figure 1a) and peptide group (Figure 1b) increase significantly from 25 to 500 ng injection amount. After 500 ng injection amount, the numbers reached plateaus at ~3,500 protein groups and ~18,000 peptide groups, respectively.

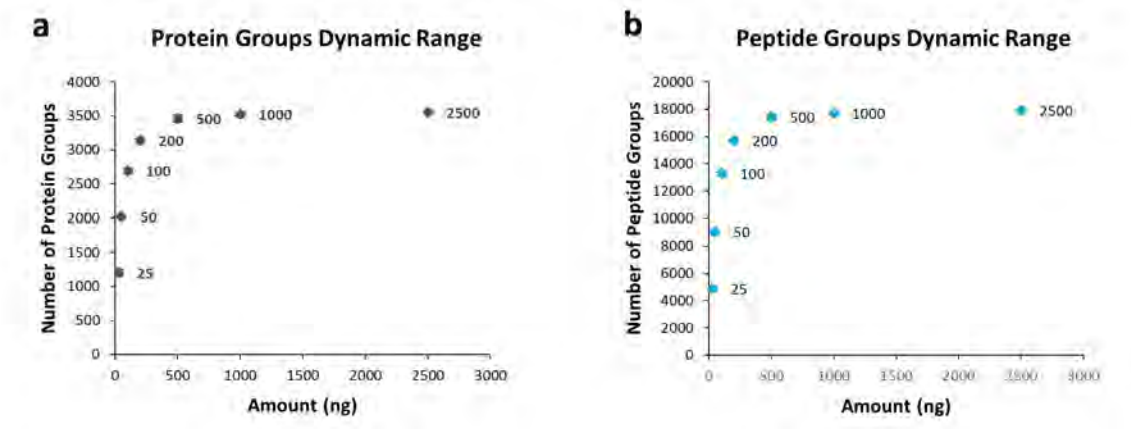

Figure 1: Dynamic ranges for protein groups (a) and peptide groups (b) for HeLa digests, ranging from 25 ng to 2,500 ng, at the flow rate of 1.5 μL/min. LC-MS was performed using our MnESI-LC-MS platform.

We also studied the dynamic range of 10 selected peptides across the 70 min LC-MS run, with their retention times ranging from 5 min to 60 min (Table 3). All peptides showed a good linearity in their peak area intensities from 25 to 2,500 ng injection of HeLa lysate (Figure 2).

Table 3: Peptide sequence and average retention time (RT) of the 10 peptides from HeLa protein digest

	Peptide	Average RT (min)
Peptide 1	[K].IDEPLEGSEDR.[I]	5.54
Peptide 2	[K].EAYMGNVLQGGEGQAPTR. [Q]	13.06
Peptide 3	[K].DLLLTSSYLSDSGSTGEHTK.[S]	19.04
Peptide 4	[K].VFDAIMNFK.[K]	23.79
Peptide 5	[R].NQVEDLLATLEK.[S]	34.78
Peptide 6	[R].SILLSVPLLVVDNK.[Q]	41.42
Peptide 7	[K].DLLPSDMAVALLEAQAGTGHIIDPATSAR.[L]	48.39
Peptide 8	[-].mELITILEK.[T]	55.47
Peptide 9	[R].ALGLGVEQLPVVFEDVVLHQATILPK.[T]	56.84
Peptide 10	[K].DLLSDWLDSTLGcDVTDNSIFSK.[L]	61.86

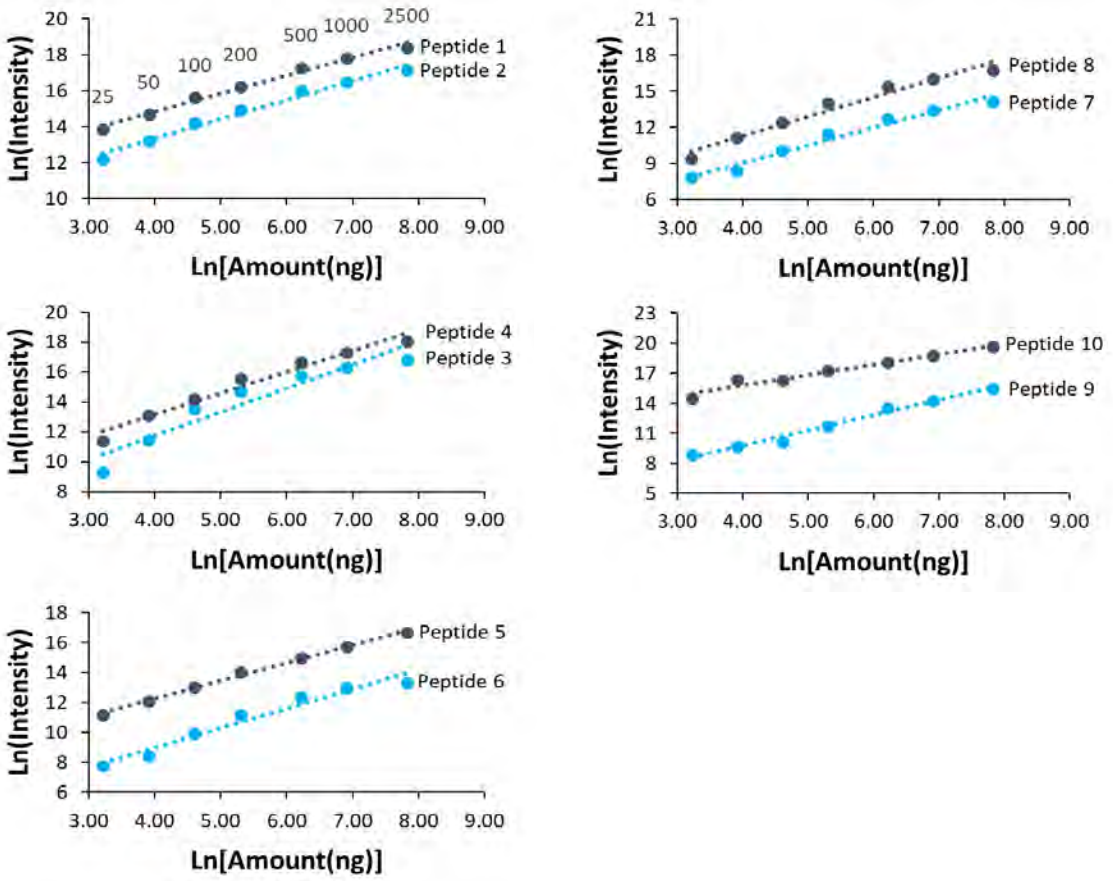

Figure 2: Dynamic range for 10 individual peptides (Peptide 1-10 in Table 3) with HeLa lysates ranging from 25 ng to 2,500 ng at the flow rate of 1.5 μL/min

The tryptic digests of HeLa cells (1 μg/run) were injected consecutively for 25 runs to evaluate the robustness of MnESI-LC-MS. Figure 3a shows a representative TIC (Total Ion Chromatogram) from a LC-MS run of HeLa protein digest. Among the 25 replicates, the RSDs of the top 100 most abundant proteins were calculated and shown in Figure 3b. More than 90% of the RSDs were below 5% with an average value of 2.7%. Out of the total 27,587 peptides identified, the average RSD of abundance was 19.1%, and 1,297 peptides had an RSD below 5%.

The number of protein groups discovered from each replicate is plotted in Figure 3c. The average number of protein groups is 3,114 with an RSD of 1.1%, which shows good reproducibility. The retention time of 10 peptides from Table 3 was also studied with their RSDs shown in Figure 3D. All RSDs were below 1%, with an average of 0.30%.

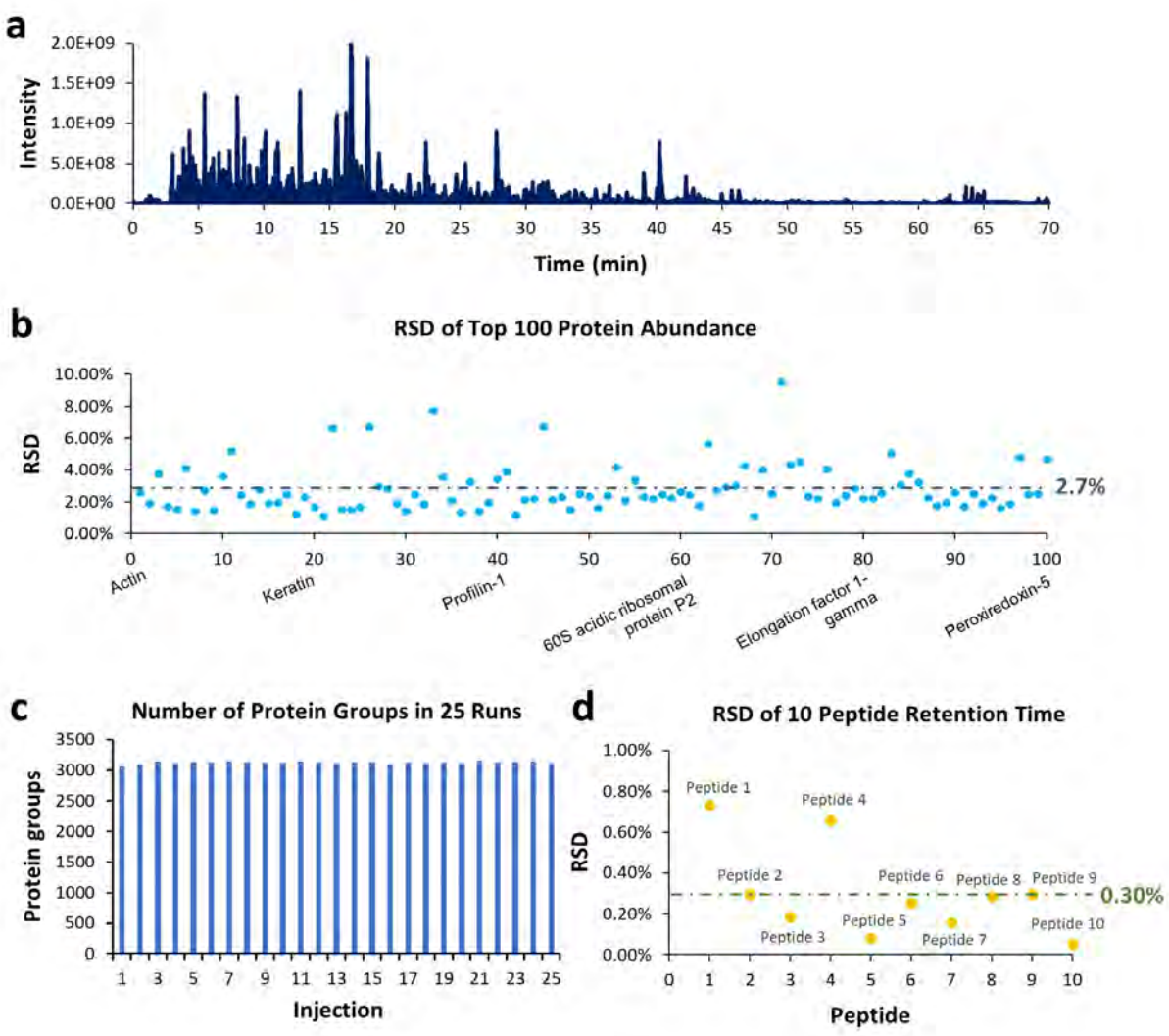

Figure 3: (a) Representative TIC for a 70 min LC-MS run (with 60 min gradient) of 1 μg HeLa protein digest using a M3 Emitter interfaced with a Thermo C18 column. (b) RSD of the top 100 most abundant proteins in 25 replicates; average value was 2.7%. (c) Number of the protein groups in 25 replicates; average value was 3,114. (d) RSD of retention times of the 10 selected peptides; average value was 0.30%

2. MnESI-LC-MS for bottom-up proteomics of human plasma samples

We next performed discovery proteomics studies of human plasma samples for a 1.5 μL/min flow rate. Plasma digests were injected for 30 consecutive runs with the same setup and parameters as those for HeLa digests described above. Figure 4a shows a representative TIC for a 70 min LC-MS run of 1 μg plasma digest at 1.5 μL/min using a M3 Emitter interfaced with a C18 column (150 μm x 150 mm). RSDs of top 25 most abundant proteins were below 10%, with an average value of 2.7% (Figure 4b). The average number of protein groups discovered from 30 injections was 244, with an RSD of 2.5% (Figure 4c). Ten peptides (Table 4) were selected to characterize the reproducibility of their retention times. The RSDs of their retention times were plotted in Figure 4d and the average value was 0.48%.

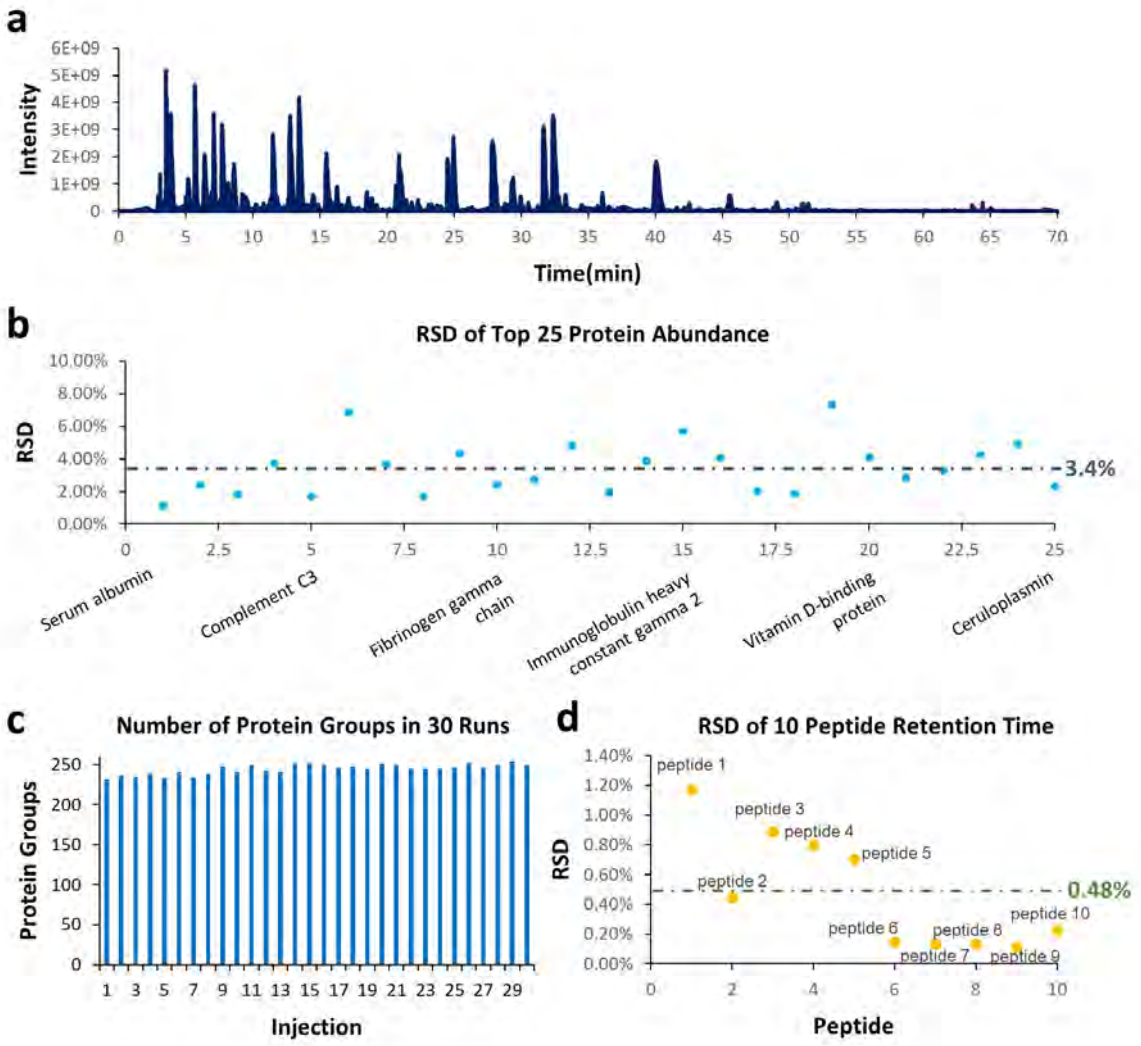

Figure 4: (a) Representative TIC for a 70 min LC-MS run of 1 µg human plasma digest using a M3 Emitter with a Thermo C18 column. (b) RSD of top 100 most abundant proteins in 30 replicates; average value was 3.42%. (c) Number of protein groups in 30 replicates; average value was 244. (d) RSD of retention times of the 10 selected peptides; average value was 0.48%.

Table 4: Peptide sequence and average retention time (RT) of the 10 peptides from the human plasma digest across the 60 min LC gradient

	Peptide	Average RT (min)
Peptide 1	[K].AVGDKLPEcEAVcGKPK.[N]	4.09
Peptide 2	[K].YAATSQVLLPSK.[D]	12.27
Peptide 3	[K].AADDTWEPFASGK.[T]	17.57
Peptide 4	[-].RTVAAPSVFIFPPSDEQLK.[S]	27.28
Peptide 5	[K].GDTFScMVGHEALPLAFTQK.[T]	30.31
Peptide 6	[K].VEGTAFVIFGIQDGEQR.[I]	33.66
Peptide 7	[R].AGFGNQESEFWLGNENLHQLTLQGNWELR.[V]	42.44
Peptide 8	[K].AALTELSLGSAYQAMILGVDSK.[N]	46.62
Peptide 9	[R].VFAIPPSFASIFLTK.[S]	50.64
Peptide 10	[R].AFQPFFVELTMPYSVIRGEAFTLK.[A]	53.61

3. Comparison between MnESI-LC-MS and nanoLC-MS for bottom-up proteomics

To compare the robustness of MnESI-LC-MS with that of nanoLC-MS, HeLa and plasma digests were tested on a QE Plus mass spectrometer with M3 Emitter at 1.5 µL/min flow rate, and Thermo stainless steel nano-bore emitter at 0.3 µL/min flow rate. The comparison is shown in Figure 5 and Figure 6. For the RSDs of peak area intensity, values obtained by MnESI-LC-MS had an average of 7.97% for HeLa digest and 10.83% for plasma digest for the peptides listed in Table 3 and Table 4, respectively, which was about 50% smaller than the values obtained by nanoLC-MS (Figure 5). The RSDs of peptide retention time showed a similar trend (Figure 6). Compared to nanoLC-MS, the average RSDs achieved by MnESI-LC-MS were approximately 50% smaller. Especially for more hydrophilic peptides eluted at low organic mobile phase (retention time < 20 min), MnESI-LC-MS has achieved much lower RSD values than nanoLC-MS.

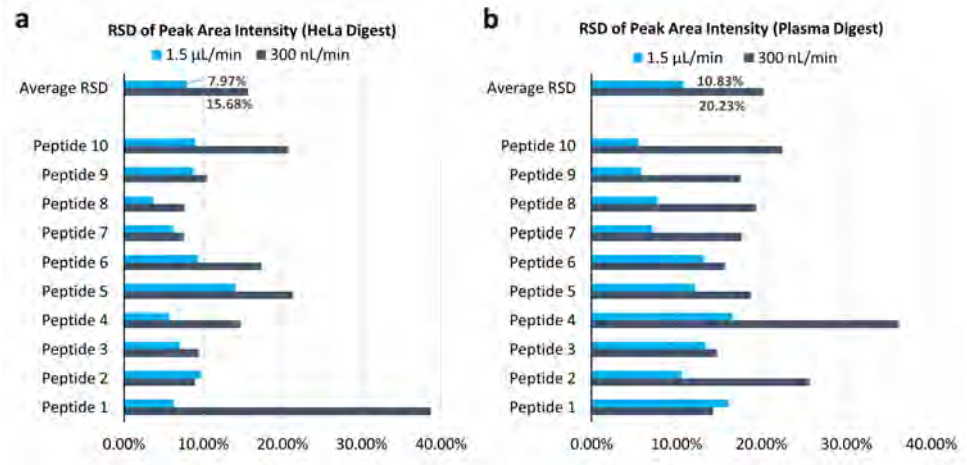

Figure 5: Comparison between nanoLC-MS (300 nL/min) and MnESI-LC-MS (1.5 µL/min) in terms of RSD of peptide peak area intensity using (a) HeLa digest and (b) human plasma digest.

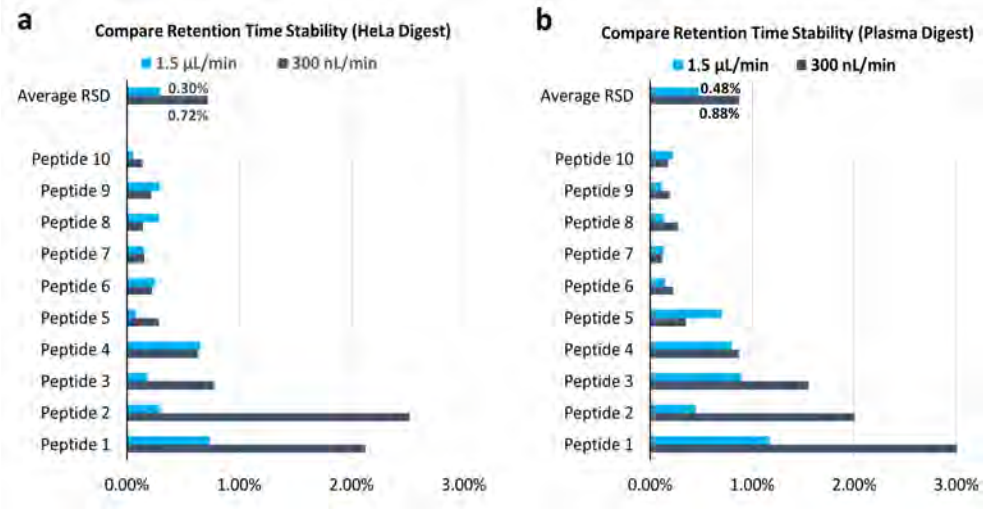

Figure 6: Comparison between nanoLC-MS (300 nL/min) and MnESI-LC-MS (1.5 µL/min) in terms of RSD of peptide retention time using (a) HeLa digest and (b) human plasma digest.

Finally, we evaluated the detection sensitivity of our MnESI-LC-MS platform in comparison to nanoLC-MS for profiling HeLa cell lysate digests. The LC-MS conditions are shown in Table 5.

Table 5: LC-MS conditions for sensitivity test

	nanoLC-MS	MnESI-LC-MS	Microflow LC-MS
Flow Rate (µL/min)	0.3	1.5	
Emitter/Sprayer	EASY-Spray™ ES803 (7 µm emitter ID)	Newomics M3 emitter (10 µm ID, 8-nozzle)	EASY-Spray™ ES791 (7 µm emitter ID)
Column	75 µm ID x 50cm L	150 µm ID x 15cm L	
Run Time (min)	60 min		
Sample	Pierce™ HeLa protein digest standard (1 µg/injection)		
Mass Spec	Thermo Orbitrap Fusion Lumos		

Figure 7a shows the average sensitivity gain obtained by M3 Emitter and ES803 using ES791 as a baseline. M3 emitter achieved an average gain of 3.8 in peak intensity relative to a single nozzle emitter (ES791) operating at the same flow rate (1.5 µL/min). More interestingly, M3 Emitter at 1.5 µL/min achieved the same high-level sensitivity as nanoflow ES803 at 0.3 µL/min (Figure 7b). Because of its high sensitivity, our MnESI-LC-MS was able to identify a similar number of protein groups as nanoLC-MS with the same loading amount of 1 µg HeLa digests (Figure 7c). In summary, MnESI-LC-MS at capillary flow delivers the same sensitivity as nanoflow LC-MS, while achieving higher robustness with better stabilities of retention time and peak intensity.

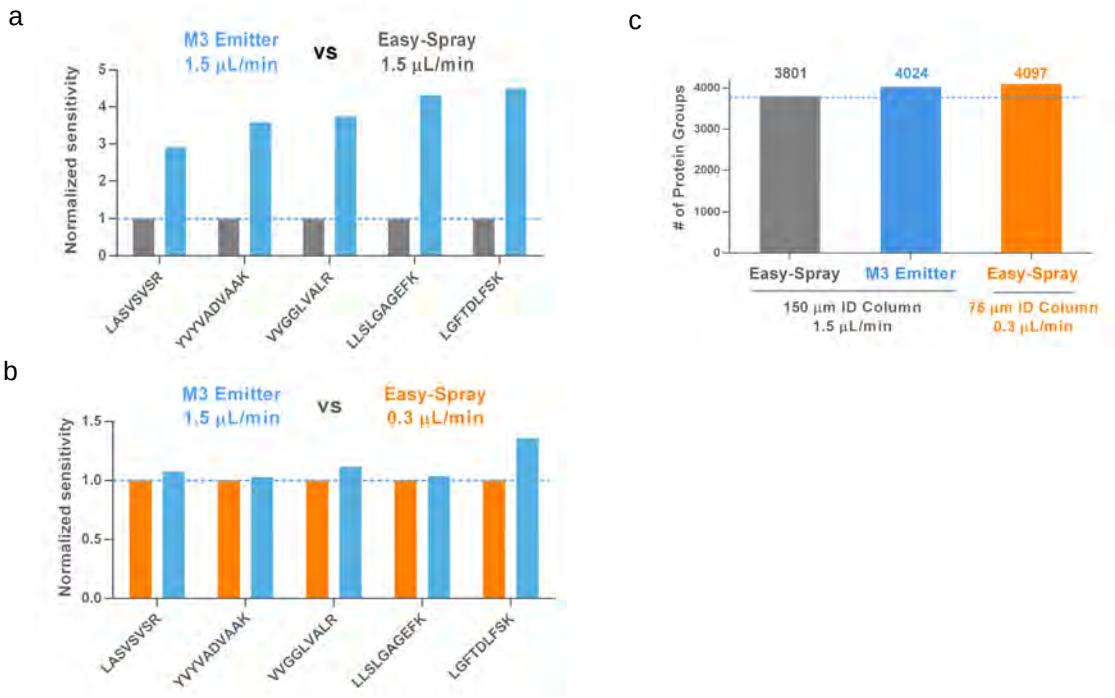

Figure 7: Comparison of sensitivity (a, b) and protein identification (c) between nanoLC-MS (ES803), MnESI-LC-MS (M3 Emitter), and microflow LC-MS (ES791).

Conclusions

The Newomics-Thermo MnESI-LC-MS platform, using Newomics® M3 Emitters and Thermo Fisher PepMap™ C18 columns on a Q Exactive™ Plus mass spectrometer, has achieved high-throughput, high-sensitivity, and robustness for bottom-up proteomics studies of complex biological samples including whole cell lysates and human plasma samples.

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4. ASMS 2018 poster: po-65308-lc-ms-capillary-flow-sensitivity-proteomics-asms2018-po65308-en

Ordering Information

Catalog #	Product
SKIT-T01	Newomics® Starter Kit for Thermo Fisher Nanospray Flex Ion Sources
E8N10MU01	Newomics® M3 Emitters, 10 µm I.D. – 8 nozzles

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