HPLC columns

Getting started with µPAC Neo HPLC columns

Goal

To provide a comprehensive guide for the installation of the Thermo Scientific™ µPAC™ Neo HPLC Columns on the Thermo Scientific™ Vanquish™ Neo UHPLC System. Complete installation instructions and best practices for direct injection and trap-and-elute LC bottom-up proteomic workflows are given.

Keywords

Thermo Scientific, µPAC Neo HPLC columns, Vanquish Neo UHPLC system, installation instructions, best practices, direct injection workflow, trap-and-elute workflow, gradient optimization, EASY-Spray bullet emitter, bottom-up proteomic, limited sample proteomic, high-throughput proteomic, deep proteome coverage

Improved performance

Complementary to the first-generation micro-pillar array HPLC columns (µPAC), the µPAC Neo columns have been developed to provide both novice and experienced proteomic scientists a highly robust and reproducible separation solution for nanoLC-MS workflows. The improved separation performance that is possible with the next generation microfluidic pillar array-based separation bed, in combination with the Thermo Scientific™ nanoViper™ connection capillaries aids in the generation of high-quality data. This results in considerable higher chromatographic resolution, offering deeper analysis of complex and often scarce biological samples (Figure 1).

The µPAC Neo column provides this increased separation performance at a much lower back pressure compared to packed-bed columns. They can be used over a wide range of nano to capillary flow rates: 100–2500 nL/min; while remaining below an operation pressure limit of 450 bar (6,500 psi).

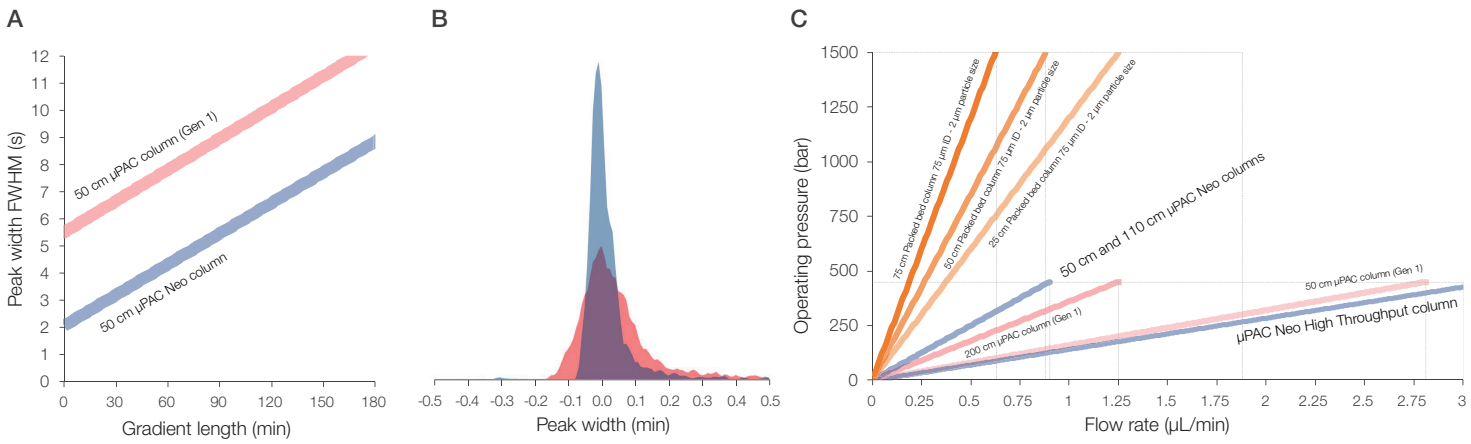


Figure 1. Improved separation performance observed with the µPAC Neo columns. A) Peak width (FWHM) as a function of gradient length for µPAC Neo column (blue) versus Generation 1 µPAC column (pink). B) Extracted ion chromatogram (XIC) of Thermo Scientific™ Pierce™ Retention Calibration Peptide 15 (LSSEAPALFQFDLK) on a 50 cm µPAC Neo column (blue) and a 50 cm Generation 1 µPAC column (pink), 60 min gradient at 300 nL/min. C) Pressure to flow rate characteristics for µPAC Neo columns (blue), µPAC Generation 1 nanoLC columns (pink), and a selection of packed-bed columns (orange) of differing lengths. The 50 cm and 110 cm µPAC Neo columns have similar pressure to flow rate characteristics, µPAC Neo high througput operates at much lower pressures.

Connections made simple

The microfluidic separation bed of the µPAC Neo columns is embedded in a protective aluminium case and critical fluidic connections are pre-assembled and thoroughly assessed via a rigorous quality control procedure at the manufacturing site. Correct connection is one of the key factors that affects the quality of the results from the nanoLC analyses. As a consequence of the low-flow rates applied, the quality of analyte separation can be completely compromised if these connections are poorly made. Although nanoLC consumables have become considerably more user-friendly, correctly assembling the column without introducing any dead volumes remains a challenge. When coupling µPAC HPLC columns to ESI-MS, this issue becomes even more challenging; the µPAC columns require an additional grounded connector proximal to the separation chip to prevent the leakage of current from the ion source back to the column. By integrating these critical fluidic connections into the protective casing and attaching low I.D. nanoViper capillaries on both ends (Figure 2), easy and reproducible connection with minimal introduction of dead volumes can be achieved.

The grounding clip on the outside of the metal casing is in direct contact with the two integrated 50 µm I.D. unions which subsequently permits the use of the column in either direction. Nevertheless, we would advise that the column is used with the long capillary (500 mm × 20 µm I.D.) on the outlet side. In this configuration it is possible to place the column in the heated LC column compartment and then directly couple the column to an Thermo Scientific™ EASY-Spray™ Emitter with an integrated high voltage liquid junction. If a column compartment is not available or a third-party portable column oven is used, then a direct connection between the short capillary (150 mm × 20 µm I.D.) and the emitter can be made, and the analyses are performed in the opposite direction. The bidirectionality of the columns simplifies column regeneration when gradual pressure increases take place, or when the column shows signs of a blockage (Figure 2).

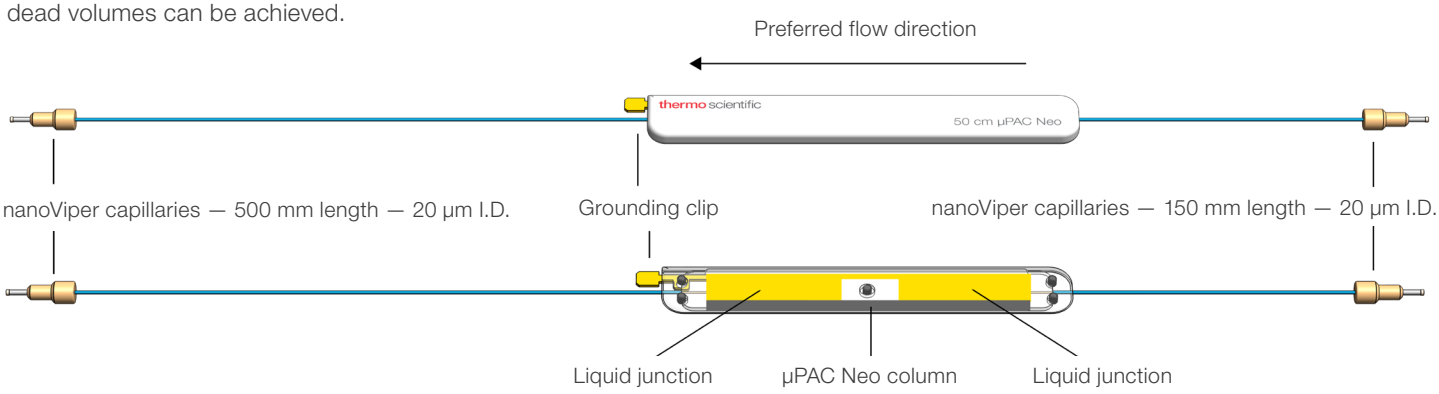


Figure 2. µPAC Neo column fluidic configuration. The µPAC separation chip is embedded in a protective aluminum case and connected to Thermo Scientific™ nanoViper™ capillaries via 50 µm internal bore stainless-steel unions.

Selecting the best µPAC Neo column and workflow for your application

Within the µPAC Neo column portfolio, four different columns, each with its own unique separation performance are available. To analyse very dilute single-cell samples and detect trace amounts of material, the non-porous 50 cm µPAC Neo Low Load column is recommended.[1] Medium-to-high throughput analyses of more conventional nanoLC sample quantities (0.01–0.5 µg) will benefit from the increased stationary phase surface area provided by the superficially-porous 50 cm µPAC Neo column. When the highest throughput is required, low capillary flow analyses are best performed using the µPAC Neo High Throughput column, which has a separation channel length of 5.5 cm and increased loadability due to the unique rectangular pillar design. And finally, even larger sample quantities and comprehensive 'single-shot' analyses will benefit from the unrivalled peak capacity offered by the superficially porous 110 cm µPAC Neo column.

The decision to analyze in either direct injection (1-column configuration) or in trap-and-elute mode (2-column configuration) should be based on the nature of the sample, the volume of the sample that will be injected, and the required sample throughput. Direct injection mode will always provide a higher absolute number of protein identifications; however, it is recommended that only very clean samples are directly injected or that additional off-line sample purification steps are implemented prior to injection. In trap-and-elute mode, sample impurities (debris, detergents, salts, etc.) can be removed and are prevented from entering the analytical column and ultimately the mass spectrometer. This practice significantly improves workflow robustness. The impact of sample loading and column equilibration on the total analysis time should also be taken into consideration when deciding between direct injection and trap-and-elute workflows. In direct injection mode, longer overhead times can be expected when large volumes are injected. For example, when applying an optimized direct injection protocol with the µPAC Neo columns, it will take 8 min to load 5 µL. Typically, sample volumes and loading volumes (that are added to the injection volume) below 2 µL are used in direct injection workflows. In contrast, loading samples onto a trap column can be carried out at higher flow rates, meaning that the proportion of total run time required to inject the sample can be drastically reduced. This is typically referred to as the MS utilization time or instrument productivity. Examples are given for the different columns and workflows in Table 1. The time required to draw the sample and load onto a trap column can be limited to approximately 2 min for volumes up to 15 µL. Additionally, re-equilibration of the analytical column will always have a detrimental impact on the total analysis time in direct injection mode whereas for optimized trap-and-elute methods, re-equilibration can occur in parallel to sample draw/sample loading.[2] If sample throughput is a priority, then the trap-and-elute mode is recommended.

Table 1. µPAC Neo column and system configuration selection

	50 cm µPAC Neo Low Load column		µPAC Neo High Throughput column		50 cm µPAC Neo column		110 cm µPAC Neo column	
	Direct injection mode	Trap-and-elute mode	Direct injection mode	Trap-and-elute mode	Direct injection mode	Trap-and-elute mode	Direct injection mode	Trap-and-elute mode
Sample load	0–5 ng	0–5 ng	50–1000 ng	50–1000 ng	5–500 ng	5–500 ng	500–2000 ng	500–2000 ng
Sample preparation	Offline SPE clean-up	Basic	Offline SPE clean-up	Basic	Offline SPE clean-up	Basic	Offline SPE clean-up	Basic
Injection volume	0.05–2 µL	1–10 µL	0.05–5 µL	1–20 µL	0.05–5 µL	1–10 µL	0.05–5 µL	1–20 µL
Flow rate range	0.1–0.75 µL/min		0.75-2.5 µL/min		0.1–0.75 µL/min		0.1–0.75 µL/min	
Gradient length	15–90 min	15–90 min	5–45 min	3–45 min	15–120 min	15–120 min	60–240 min	60–240 min
Throughput	20–60 samples/day	20–100 samples/day	60–180 samples/day	60–300 samples/day	20–60 samples/day	20–100 samples/day	6–16 samples/day	6–16 samples/day
MS utilization	60–90%	70–95%	57–90%	63–95%	60–90%	70–95%	70–90%	70–95%
Relative coverage	100%	80–90%	100%	95%	100%	80–90%	100%	80–90%

Configuring the Vanquish Neo HPLC system for µPAC Neo columns

Direct injection mode

Preparing the instrument

Prepare the instrument by following the scripts and actions listed in Table 2. Recommended solvents are listed in Table 3. A schematic overview of the fluidic configuration in the direct injection workflow is given in Figure 3. Before installing the column, ensure that the correct column specifications have been entered using script A04. Separation column specifications for µPAC Neo columns can be found in Table 4.

Table 2. Setting up the Vanquish Neo HPLC system for direct injection workflows in nano/cap fluidic configuration

Scripts for system set-up	
Script	**Direct injection workflow**
A01	Set pump solvent type
A02	Auto start up with diagnostics on
C02	Purge pump (pump & flow meter)
C04	Purge sampler
D01	Test system back pressure
A03	Set separation column type
A04	Set separation column specifications (see Table 4 for details)
	Install the analytical column (see Table 5)
	Check that the system is configured for the correct flow regime “nano/cap” and workflow “direct” y-under the information workflow tab. If necessary execute script A06 (see Table 14).

Table 3. Solvents used for instrument operation

Module	Property	Setting
Binary pump N	Mobile phase A	H_2O with 0.1% FA
	Mobile phase B	80/20 (v/v) ACN/H_2O with 0.1% FA
Metering device	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	80/20 (v/v) ACN/H_2O with 0.1% FA
Wash port	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	80/20 (v/v) ACN/H_2O with 0.1% FA
Binary pump N and metering device	Rear seal wash buffer	25/75 (v/v) H_2O/isopropanol with 0.1% FA

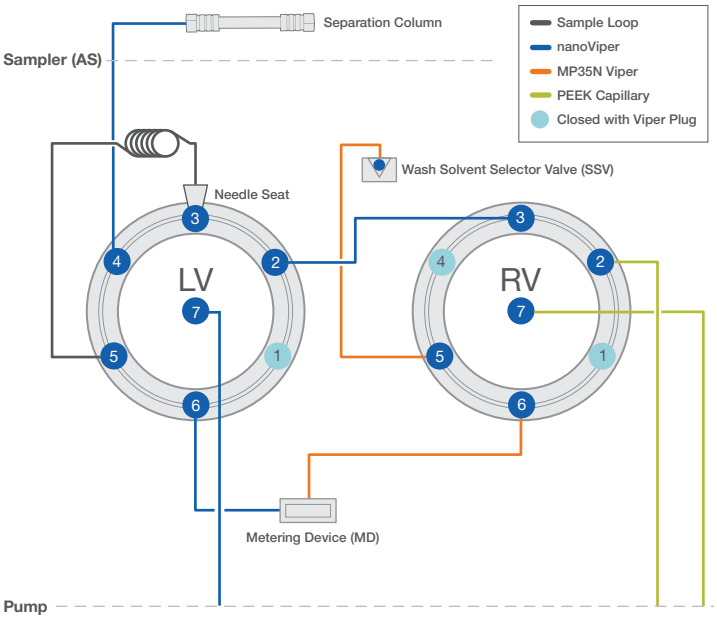


Figure 3. Vanquish Neo HPLC system direct injection workflow schematic.

Table 4. Separation column specifications for µPAC Neo columns

	Separation column specifications			
	50 cm µPAC Neo Low Load column	**µPAC Neo High Throughput column**	**50 cm µPAC Neo column**	**110 cm µPAC Neo column**
Inner diameter	75 µm	75 µm	75 µm	75 µm
Length	50 cm	5.5 cm	50 cm	150 cm
Void volume	1.480 µL	1.480 µL	1.480 µL	4.440 µL
Maxiumum pressure	450 bar	450 bar	450 bar	450 bar
Maximum flow	0.8 µL/min	3 µL/min	0.8 µL/min	0.8 µL/min
Maximum temperature	60°C	60°C	60°C	60°C
Maximum pressure change up	1,000 bar/min	1,000 bar/min	1,000 bar/min	1,000 bar/min
Maximum pressure change down	1,000 bar/min	1,000 bar/min	1,000 bar/min	1,000 bar/min

Installing the analytical column

Please consult the installation instructions provided with the µPAC Neo HPLC column. An overview of the required procedure to install the column is given in Table 5. The correct positioning of the µPAC Neo column in the Vanquish Neo HPLC system column compartment is shown in Figure 4. The grounding attachment points on the EASY-Spray source and the Vanquish Neo UHPLC system; plus connection of the µPAC Neo column nanoViper outlet capillary to the female ESI bullet emitter with an integrated liquid junction or to a reducing union and conductive emitter are given in Figure 5.

Table 5. Mounting a µPAC Neo column on the Vanquish Neo UHPLC system

µPAC Neo column installation steps	
Action	**Description**
1	Install µPATCH holder
2	Position µPAC column in µPATCH holder
3	Connect viper union to column inlet
4	Connect nanoViper transfer line from autosampler to column inlet
5	**For 50 and 110 cm µPAC Neo columns:** Apply a flow rate of 300 nL and equilibrate column with desired solvent starting conditions (1%B) - make sure pressure readback is within specifications (90 - 160 bar). **For µPAC Neo High Throughput columns:** Apply a flow rate of 750 nL and equilibrate column with desired solvent starting conditions (4%B) - make sure pressure readback is within specifications (60 - 100 bar).
6	Connect grounding cable to grounding point LC or MS
7	Connect grounding cable to grounding clip µPAC column
8	Connect column outlet directly to 1/16" female emitter with integrated liquid junction or to 1/16" receiving reducing uinion equipped with conductive ESI emitter
9	Make sure column pressure does not increase more than 10% after connecting ESI emitter
10	Apply high voltage (1.7–2.5 kV) and start acquiring

A

B

Figure 4. µPAC Neo column positioned inside the column compartment of the Vanquish Neo UHPLC instrument. (A) Column orientation with the MS positioned on the left side of the UHPLC system. (B) Column orientation with MS positioned on the right side of the UHPLC system.

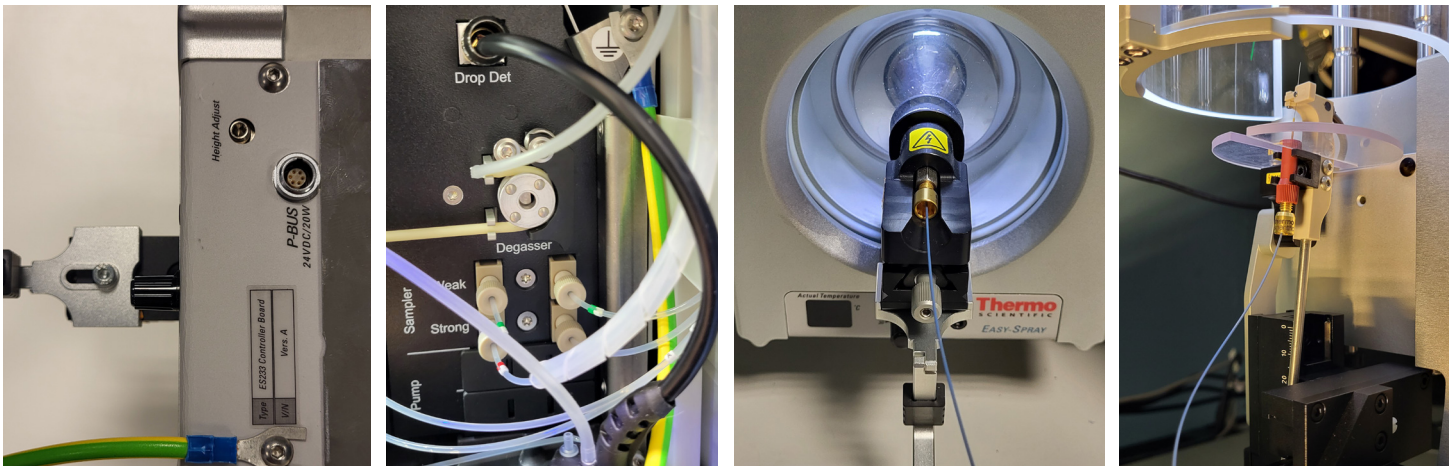


Figure 5. (A) Grounding attachment points on the EASY-Spray source, and (B) the Vanquish Neo UHPLC system. (C) The connection of the µPAC Neo nanoViper outlet capillary to the female ESI bullet emitter, (D) The connection of the µPAC Neo nanoViper outlet capillary to a stainless steel emitter.

Conditioning and installation analyses

50 cm µPAC Neo Low Load column

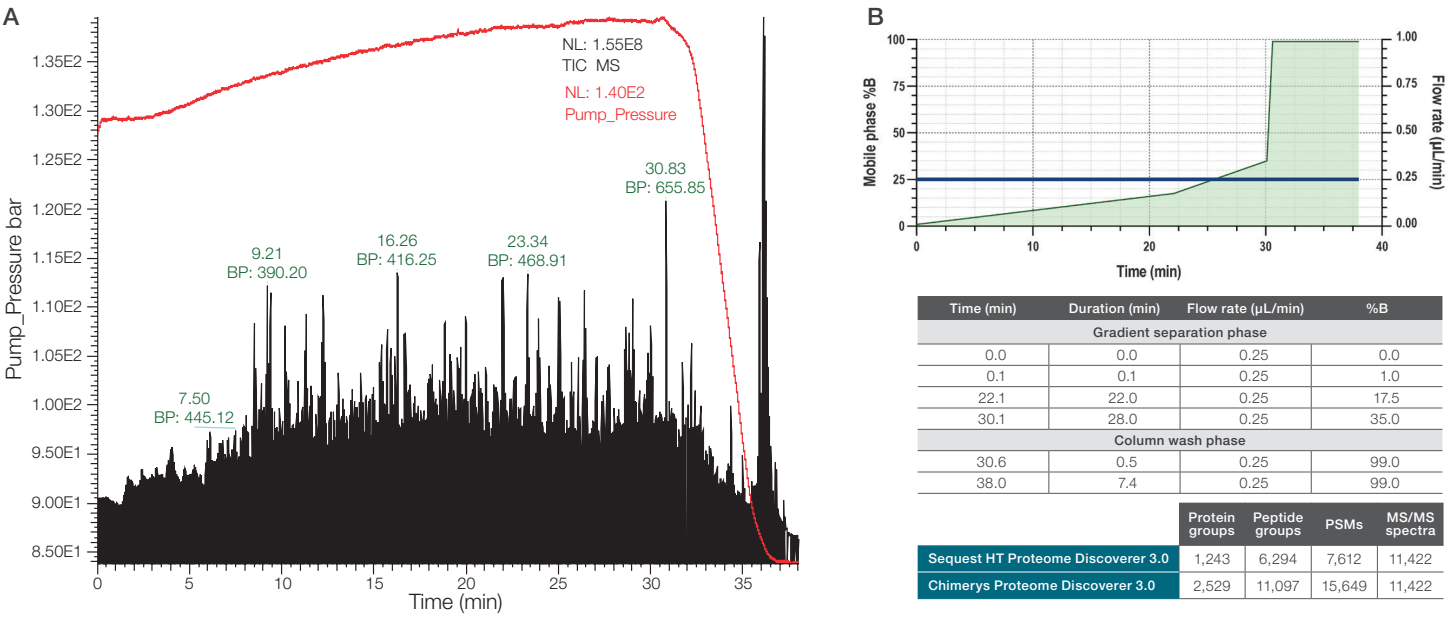
After the column has been installed and the pump back pressure is within specifications, proceed with conditioning the column (Table 5), injecting and analyzing a blank, and performing some installation assessment injections/analyses. All these runs can be performed using the operating conditions listed below in Table 7. For the 50 cm µPAC Low Load column, the recommendation is to condition the column with 10 ng of a protein tryptic digest (Thermo Scientific™ Pierce™ HeLa Digest Standard - P/N 88328) before beginning the installation assessment. All consumables required are listed in Table 6. The generic parameters for sample loading and column equilibration are provided in Table 7. The separation gradient is described in Figure 6.

Table 7. LC method parameters and sample conditions

50 cm µPAC Neo Low Load column direct injection workflow		
Category	**Parameter**	
Sample specifications	Thermo Scientific Pierce HeLa digest standard	P/N 88328
	Concentration	1 ng/µL
	Injection volume	2 µL
	Solvent	H_2O + 0.1% TFA
Sample loading	Fast loading	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Loading volume	1.5 µL
Column equilibration	Fast equilibration	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Equilibration factor	1.5
Temperature	Column compartment temperature	40°C
	Autosampler temperature	7°C

Table 6. Fluidics and accessories

50 cm µPAC Neo Low Load direct injection workflow		
Part number	**Description**	**Quantity**
6PK1655	Vial and cap screw with septa kit, 100/pack	**1**
	• Vial 0.2 mL amber TPX screw 9 mm short thread conical glass insert	**1**
	• Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	**1**
ES993	EASY-Spray Nano Emitter, bullet type without transfer line (10 µm I.D.)	**1**
COL-LOLO050NEOB	50 cm µPAC Neo Low Load HPLC column	**1**
6040.2304	Union for Viper and nanoViper tubing	**1**
6250.5260	20 µm I.D. × 550 mm nanoViper capillary	**1**



Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.25	0.0
0.1	0.1	0.25	1.0
22.1	22.0	0.25	17.5
30.1	28.0	0.25	35.0
Column wash phase			
30.6	0.5	0.25	99.0
38.0	7.4	0.25	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	1,243	6,294	7,612	11,422
Chimerys Proteome Discoverer 3.0	2,529	11,097	15,649	11,422

Figure 6. (A) Typical TIC trace obtained for the installation method on the 50 cm µPAC Neo Low Load column in direct injection mode. 2 ng HeLa cell digest was loaded on column. Pressure profile is overlaid in red. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Thermo Scientific™ Proteome Discoverer™ Software, version 3.0 (1% FDR).

µPAC Neo High Throughput column

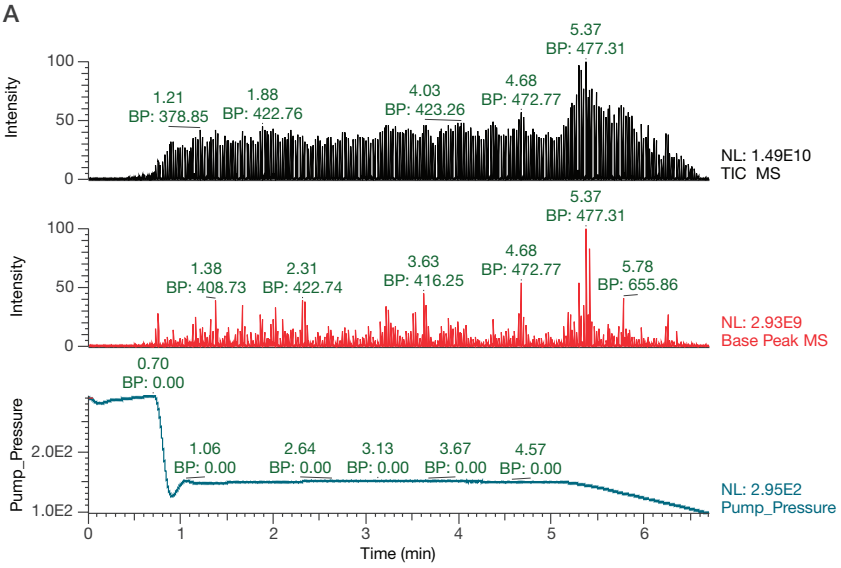
After the column has been installed and the pump back pressure is within specifications , proceed with conditioning the column (Table 5), injecting and analyzing a blank, and performing some installation assessment injections/analyses. All these runs can be performed using the operating conditions listed in Table 8. For the µPAC Neo High Throughput column, the recommendation is to condition the column with 500 ng of a protein tryptic digest (Pierce HeLa digest standard - P/N 88328) before beginning the installation assessment. All consumables required are listed in Table 9. The generic parameters for sample loading and column equilibration are provided in Table 8. The optimal separation gradients at respectively 180 and 100 samples per day throughput are described in Figure 7 and 8.

Table 8. LC method parameters and sample conditions

µPAC Neo High Throughput column		
Category	**Parameter**	
Sample specifications	Thermo Scientific Pierce HeLa digest standard	P/N 88328
	Concentration	200 ng/µL
	Injection volume	1 µL
	Solvent	H_2O + 0.1% TFA
Sample loading	Fast loading	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Loading volume	1.5 µL
Column equilibration	Fast equilibration	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Equilibration factor	1
Temperature	Column compartment temperature	50°C
	Autosampler temperature	7°C

Table 9. Fluidics and accessories

µPAC Neo High Throughput column		
Part number	**Description**	**Quantity**
6PK1655	Vial and cap screw with septa kit, 100/pack	1
	• Vial 0.2 mL amber TPX screw 9 mm short thread conical glass insert	1
	• Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	1
P-881	MicroTight Adapter for 1/16" OD tubing to 1/32" OD tubing	1
ES542	Thermo Scientific Nano Bore Emitter stainless steel 40mm OD 1/32	1
COL-CAPHTNEOB	µPAC Neo High Throughput HPLC column	1
6040.2304	Union for Viper and nanoViper tubing	1
6250.5260	20 µm I.D. × 550 mm nanoViper capillary	1



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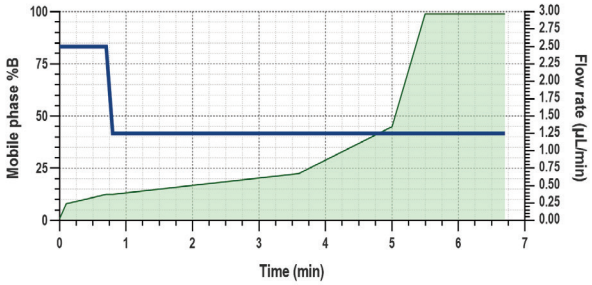
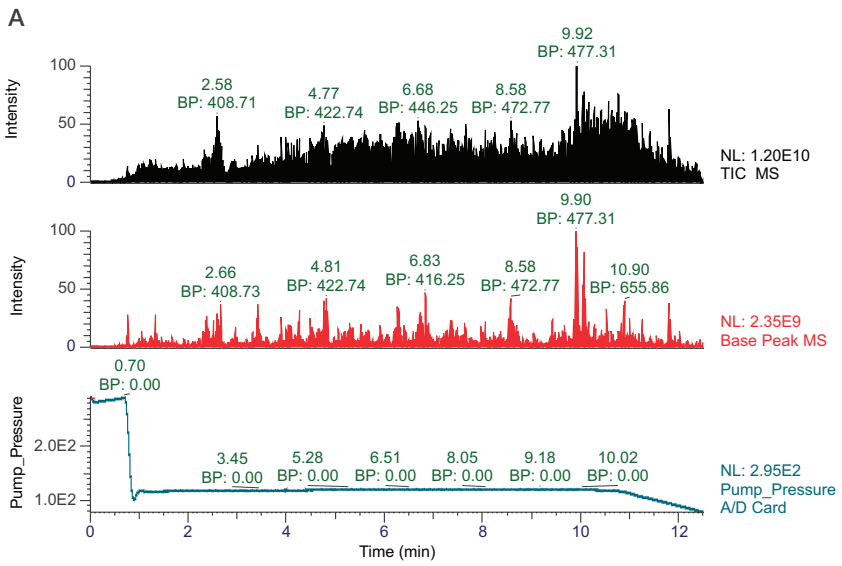
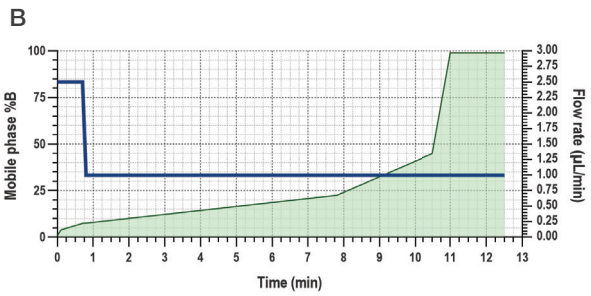


Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	2.5	1.0
0.1	0.1	2.5	8.0
0.7	0.6	2.5	12.5
0.8	0.1	1.25	12.6
3.6	2.8	1.25	22.5
5.0	1.4	1.25	45.0
Column wash phase			
5.5	0.5	0.25	99.0
6.7	1.2	0.25	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	793	2,588	2,979	9,488
Chimerys Proteome Discoverer 3.0	3,676	14,596	19,440	9,488

Figure 7. (A) Typical TIC trace obtained for the 5.5 min gradient installation method on the µPAC Neo High Throughput column in direct injection mode. 200 ng HeLa cell digest was loaded on column. BPC and Pressure profile are shown below the TIC trace. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Proteome Discoverer software, version 3.0 (1% FDR).





Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	2.5	1.0
0.1	0.1	2.5	4.0
0.7	0.6	2.5	7.5
0.8	0.1	1.0	7.6
7.8	7.0	1.0	22.5
10.8	3.0	1.0	45.0
Column wash phase			
11.3	0.5	0.25	99.0
12.5	1.2	0.25	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	1,514	6,715	8,323	17,901
Chimerys Proteome Discoverer 3.0	4,547	23,835	36,169	17,901

Figure 8. (A) Typical TIC trace obtained for the 11min gradient installation method on the µPAC Neo High Throughput column in direct injection mode. 200 ng HeLa cell digest was loaded on column. BPC and Pressure profile are shown below the TIC trace. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Proteome Discoverer software, version 3.0 (1% FDR).

50 cm µPAC Neo column

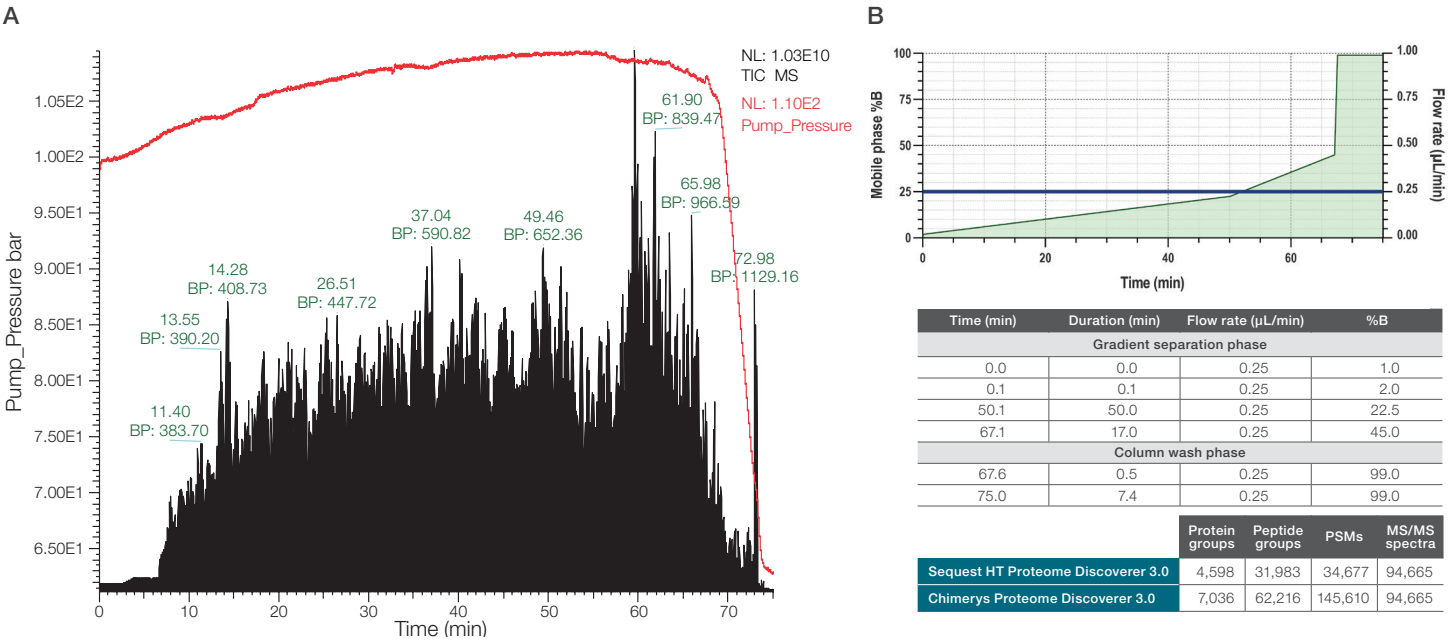
After the column has been installed and the pump back pressure is within specifications , proceed with conditioning the column (Table 5), injecting and analyzing a blank, and performing some installation assessment injections/analyses. All these runs can be performed using the operating conditions listed in Table 10. For the 50 cm µPAC Neo column, the recommendation is to condition the column with 500 ng of a protein tryptic digest (Pierce HeLa digest standard - P/N 88328) before beginning the installation assessment. All consumables required are listed in Table 11. The generic parameters for sample loading and column equilibration are provided in Table 10. The separation gradient is described in Figure 9.

Table 10. LC method parameters and sample conditions

50 cm µPAC Neo column direct injection workflow		
Category	**Parameter**	
Sample specifications	Thermo Scientific Pierce HeLa digest standard	P/N 88328
	Concentration	200 ng/µL
	Injection volume	1 µL
	Solvent	H_2O + 0.1% TFA
Sample loading	Fast loading	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Loading volume	1.5 µL
Column equilibration	Fast equilibration	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Equilibration factor	1.5
Temperature	Column compartment temperature	50°C
	Autosampler temperature	7°C

Table 11. Fluidics and accessories

50 cm µPAC Neo column direct injection workflow		
Part number	**Description**	**Quantity**
6PK1655	Vial and cap screw with septa kit, 100/pack	**1**
	• Vial 0.2 mL amber TPX screw 9 mm short thread conical glass insert	**1**
	• Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	**1**
ES993	EASY-Spray Nano Emitter, bullet type without transfer line (10 µm I.D.)	**1**
COL-NANO050NEOB	50 cm µPAC Neo HPLC column	**1**
6040.2304	Union for Viper and nanoViper tubing	**1**
6250.5260	20 µm I.D. × 550 mm nanoViper capillary	**1**



Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.25	1.0
0.1	0.1	0.25	2.0
50.1	50.0	0.25	22.5
67.1	17.0	0.25	45.0
Column wash phase			
67.6	0.5	0.25	99.0
75.0	7.4	0.25	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	4,598	31,983	34,677	94,665
Chimerys Proteome Discoverer 3.0	7,036	62,216	145,610	94,665

Figure 9. (A) Typical TIC trace obtained for the installation method on the 50 cm µPAC Neo column in direct injection mode. 200 ng HeLa cell digest was loaded on column. Pressure profile is overlaid in red. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Proteome Discoverer software, version 3.0 (1% FDR).

110 cm µPAC Neo column

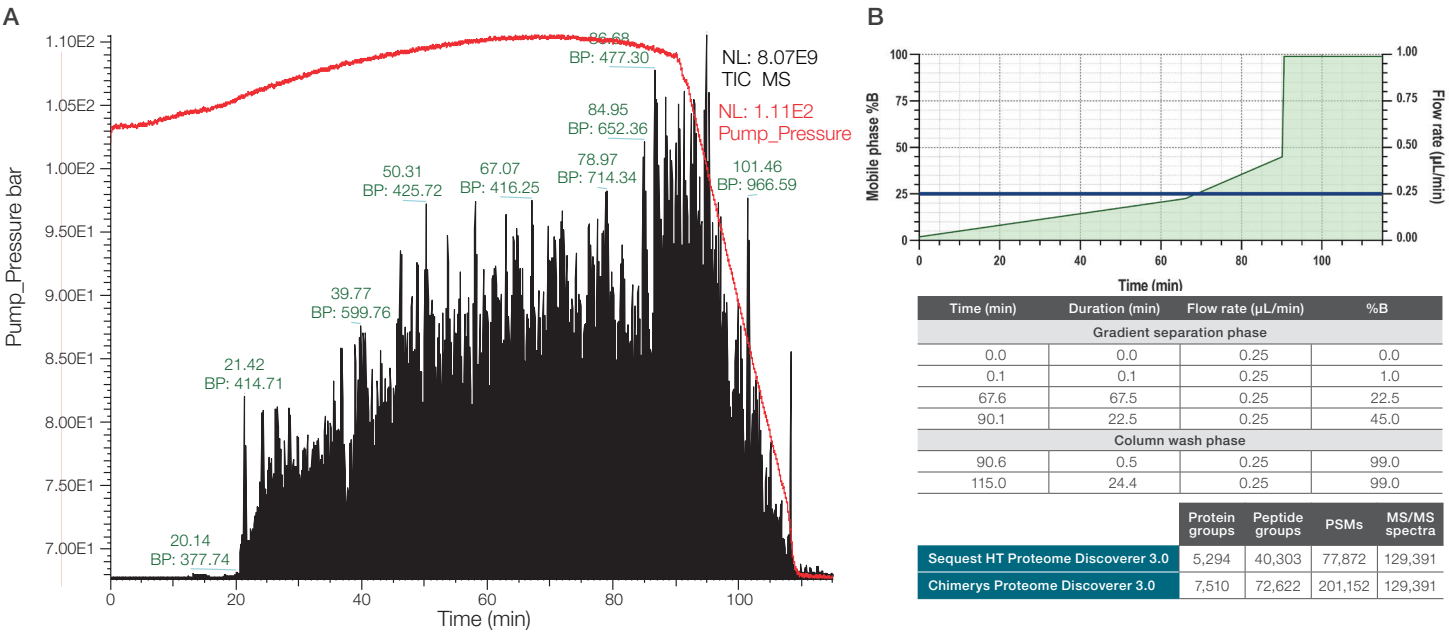
After the column has been installed and the pump back pressure is within specifications, proceed with conditioning the column (Table 5), injecting and analyzing a blank, and performing some installation assessment injections/analyses. All these runs can be performed using the operating conditions listed in Table 13. For the 110 cm µPAC column, the recommendation is to condition the column with 500 ng of a protein tryptic digest (Pierce HeLa digest standard - P/N 88328) before beginning the actual installation assessment. All consumables required are listed in Table 12. The generic parameters for sample loading and column equilibration are provided in Table 13. The separation gradient is described in Figure 10.

Table 13. LC method parameters and sample conditions

110 cm µPAC Neo column direct injection workflow		
Category	**Parameter**	
Sample specifications	Thermo Scientific Pierce HeLa digest standard	P/N 88328
	Concentration	200 ng/µL
	Injection volume	2.5 µL
	Solvent	H_2O + 0.1% TFA
Sample loading	Fast loading	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Loading volume	1.5 µL
Column equilibration	Fast equilibration	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Equilibration factor	1.5
Temperature	Column compartment temperature	50°C
	Autosampler temperature	7°C

Table 12. Fluidics and accessories

110 cm µPAC Neo column direct injection workflow		
Part number	**Description**	**Quantity**
6PK1655	Vial and cap screw with septa kit, 100/pack	**1**
	• Vial 0.2 mL amber TPX screw 9 mm short thread conical glass insert	**1**
	• Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	**1**
ES993	EASY-Spray Nano Emitter, bullet type without transfer line (10 µm I.D.)	**1**
COL-NANO110NEOB	110 cm µPAC Neo HPLC column	**1**
6040.2304	Union for Viper and nanoViper tubing	**1**
6250.5260	20 µm I.D. × 550 mm nanoViper capillary	**1**



Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.25	0.0
0.1	0.1	0.25	1.0
67.6	67.5	0.25	22.5
90.1	22.5	0.25	45.0
Column wash phase			
90.6	0.5	0.25	99.0
115.0	24.4	0.25	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	5,294	40,303	77,872	129,391
Chimerys Proteome Discoverer 3.0	7,510	72,622	201,152	129,391

Figure 10. (A) Typical TIC trace obtained for the installation method on the 110 cm µPAC Neo column in direct injection mode. 500 ng HeLa cell digest was loaded on column. Pressure profile is overlaid in red. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Proteome Discoverer software, version 3.0 (1% FDR).

Sample loading and injection volume

When using the Vanquish Neo HPLC system in direct injection mode, a predefined sample volume is isocratically loaded (solvent conditions are defined through the selection of the Weak Wash liquid) onto the µPAC Neo column and once this task has been completed, the separation gradient is executed. Instrument overhead time can be minimized by loading the sample at high pressure and/or flow rate. Conditions should, however, be carefully selected to obtain the optimal performance for the µPAC Neo columns. In contrast to columns that have a fully porous stationary phase, µPAC Neo columns are either non-porous or superficially-porous and this impacts the ability of these columns to retain hydrophilic analytes during an isocratic separation. To ensure that the entire sample volume is correctly loaded onto the analytical column, the Vanquish Neo UHPLC system direct injection protocol uses an extra loading volume in addition to the actual sample volume. When this value is set to Automatic, an additional volume of 5 µL is loaded onto the column which can cause hydrophilic peptide 'breakthrough' before the analytical gradient reaches the column. This will also cause extensive dispersion in the early part of the chromatogram and results in poor reproducibility and reduced identification of hydrophilic species. The effect of loading volume on peptide elution is illustrated in Figure 11. This can be prevented by manually setting the loading volume to a value of ≤1.5 µL. Reconstituting samples in 0.1% trifluoroacetic acid (TFA) rather than in 0.1% formic acid (FA) will also reduce peptide breakthrough and will result in a higher resolution for hydrophilic peptides (Figure 12).

Figure 11. Base peak chromatograms obtained from the separation of 200 ng HeLa tryptic digest (1 µL injected) using different loading volumes. 50 cm µPAC Neo column, 67 min gradient. The shaded light blue section is expanded to the left.

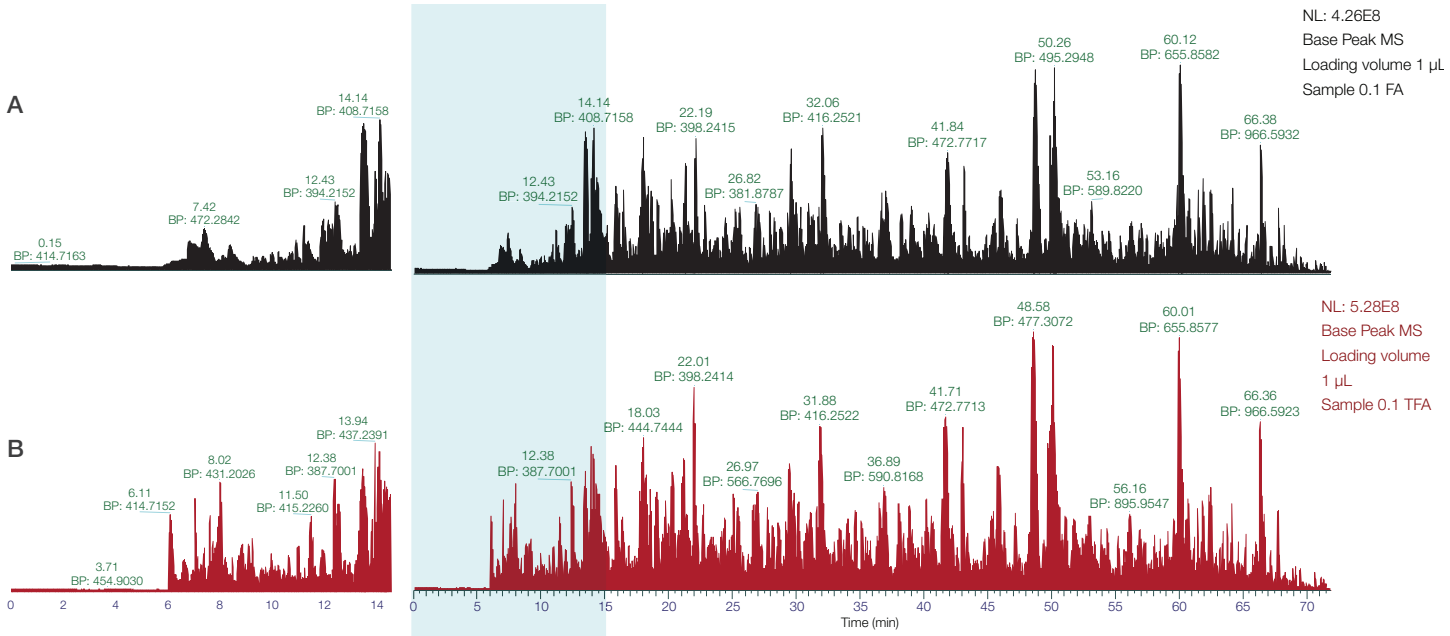


Figure 12. Base peak chromatograms obtained from the separation of 200 ng HeLa tryptic digest (1 µL injected) using different solvents to reconstitute the sample. (A) 0.1% formic acid (FA). (B) 0.1% trifluoroacetic acid (TFA). 50 cm µPAC Neo column, 67 min gradient. The shaded light blue section is expanded to the left.

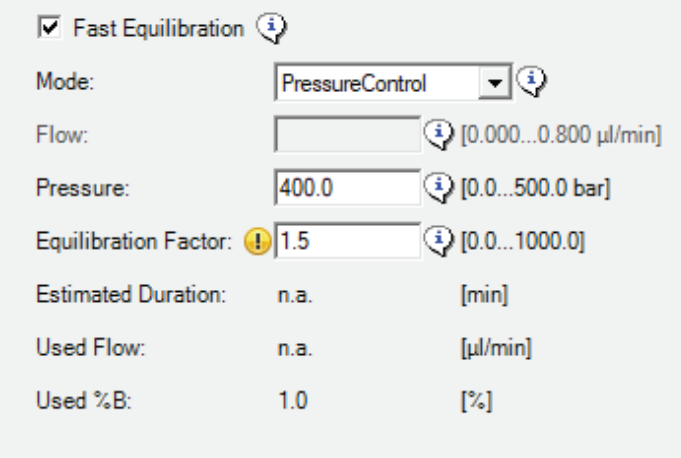
Column equilibration

Column equilibration is carried out with fast column equilibration activated to maximize sample throughput and MS utilization. In PressureControl mode, a pressure of 400 bar will minimize the impact of column equilibration on instrument overhead time. As a result of the reduced interaction surface, µPAC Neo columns require less equilibration and have reduced column-related sample carry-over compared to conventional fully-porous stationary phases.[3] Equilibrating the column with 1.5 column volumes (1% B) is sufficient to regenerate the column for a subsequent analysis. This takes approximately 0.5, 2.5 and 9 min for the High Throughput 50 cm, and 110 cm µPAC Neo columns (Figure 13).

Flow rate optimization

The fast sample loading and equilibration options for the Vanquish Neo UHPLC system are innovative features to increase instrument productivity. When the Vanquish Neo UHPLC system is combined with a µPAC Neo column, an additional gain in instrument productivity can be obtained by increasing the flow rate employed during gradient separation. At a flow rate of 750 nL/min, sample elution can be accelerated resulting in void times of 2 and 6 min for the 50 cm, and 110 cm µPAC Neo columns. For the µPAC Neo High Throughput column, the void time can even be reduced to 0.5 min by applying an initial flow rate of 2.5 µL/min. Depending on the required sample throughput and sensitivity, the entire gradient separation can be run at an elevated flow rate or; alternatively, a method that utilizes variable flow rates can be employed. To enhance the sensitivity of the separation, the flow rate is reduced during the portion of the run where the analytes are eluted. Decreasing the analytical flow rate to 300 nL/min or lower during analyte elution significantly increases ionization efficiency and therefore MS sensitivity. This enables the combination of high sample throughput with increased sensitivity for low quantities of sample. An example of such a method employing flow rate ramping is given for the 50 cm µPAC Neo column in Figure 14. Significantly, higher proteome coverage was obtained for 50 ng of material when the flow rate was decreased to 250 nL/min after 2 min (shown below the TIC trace). When developing a method with variable flow rate, it is crucial that the increase in %B is kept constant as a function of the volume delivered by the pumps. This will ensure that there is even distribution of peptides across the elution window (shown below the TIC trace). Such a strategy can also be used to decrease instrument overhead time with the other column types within the µPAC Neo column family. For the 110 cm µPAC Neo column, peptide elution can be reduced from 20 min to approximately 6 min, resulting in a gain in instrument productivity of approximately 14 min.

A



B

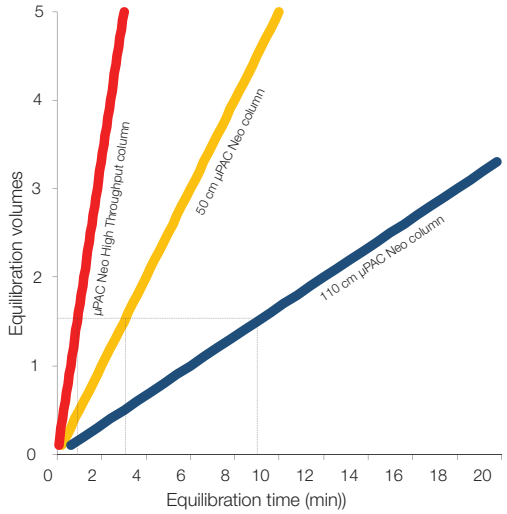
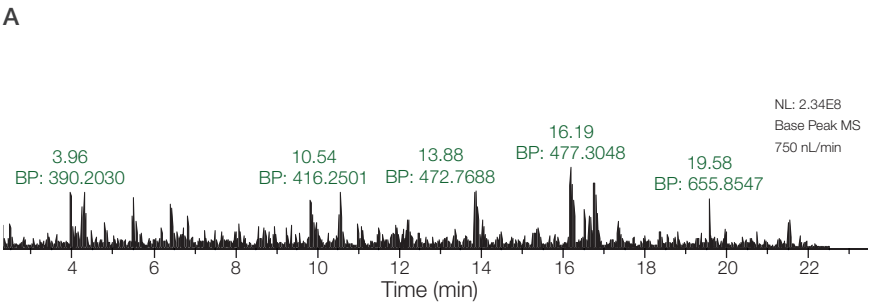
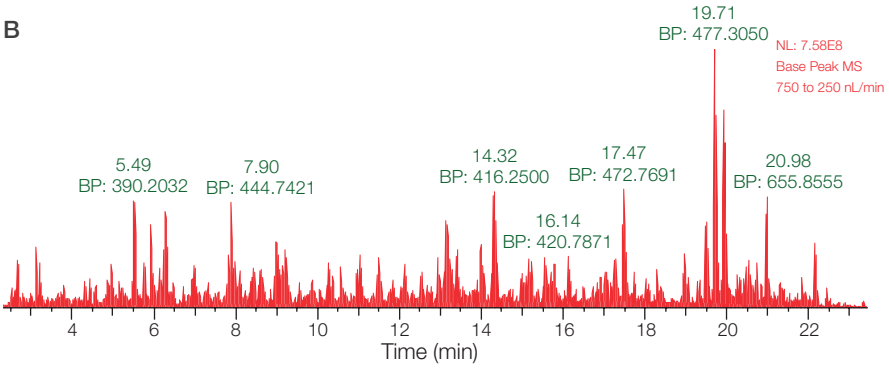
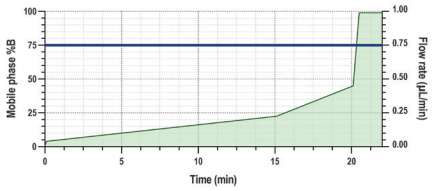


Figure 13. (A) Tab for column equilibration settings in the workflow editor. (B) Time required to perform column equilibration at 400 bar. µPAC Neo High througput column (red), 50 cm µPAC Neo Low Load column and 50 cm µPAC Neo column (yellow), 110 cm µPAC Neo column (blue). Note: the yellow exclamation mark next to the equilibration factor value is a warning suggesting that the user employs a larger number of column volumes for sufficient column equilibration (≥2). For the µPAC Neo columns, 1.5 column volumes are sufficient for the reasons given above.



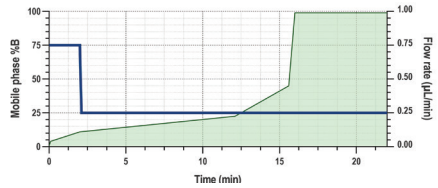


C High flow rate - 20 min gradient



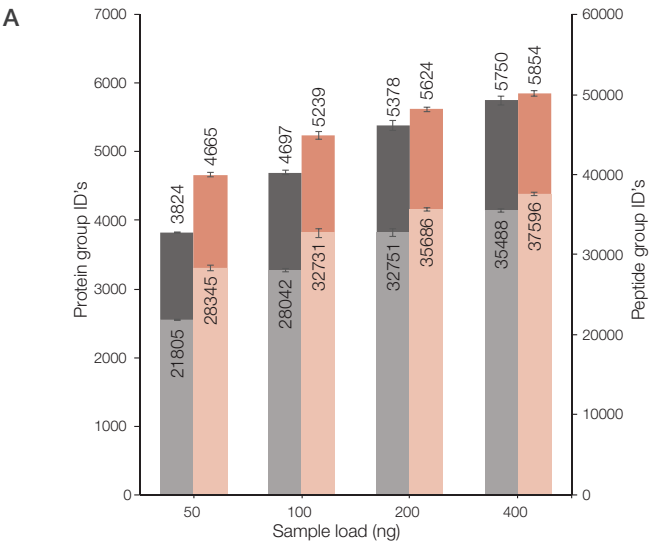
Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.75	1.0
0.1	0.1	0.75	4.0
15.1	15.0	0.75	22.5
20.1	5.0	0.75	45.0
Column wash phase			
20.5	0.4	0.75	99.0
22.0	1.5	0.75	99.0

Flow rate optimization - 20 min gradient



Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.75	1.0
0.1	0.1	0.75	4.0
2.0	1.9	0.75	11.0
2.1	0.1	0.25	11.1
12.1	12.0	0.25	22.5
15.6	3.5	0.25	45.0
Column wash phase			
16.0	0.5	0.25	99.0
22.0	6.0	0.25	99.0

Figure 14. Left: Base peak chromatograms obtained from separating 50 ng HeLa tryptic digest (0.25 µL injected). (A) The method at a constant flow rate of 750 nL/min. and (B) optimized method with a reduced flow rate of 250 nL/min after 2 min. Elution window = 20 min. (C) Solvent gradient and flow rate profiles.



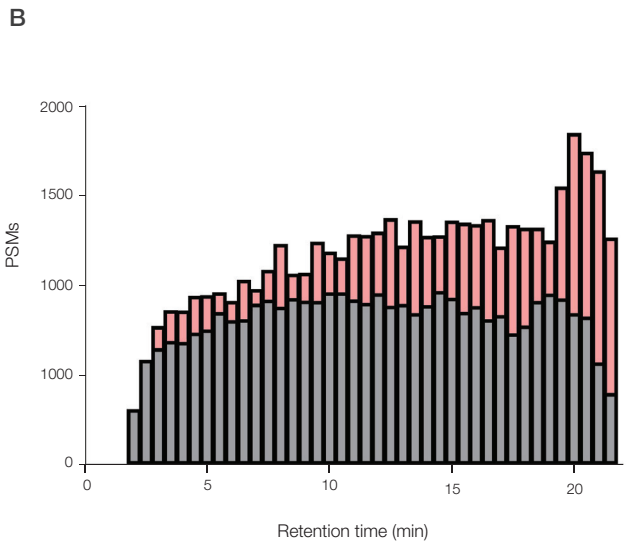


Figure 15. (A) Protein and peptide group identifications obtained with a constant flow method at a high flow rate of 750 nL/min (grey) vs an optimized method with a reduced flow rate of 250 nL/min after 2 min (red). Sample is HeLa cell digest. Elution window = 20 min. (B) Peptide spectral matches (PSMs) as a function of retention time, binned in 0.5 min windows.

Trap-and-elute mode

Preparing the instrument

Prepare the instrument by following the scripts and actions listed in Table 14. Recommended solvents are listed in Table 15. A schematic overview of the fluidic configuration in the (non-heated) trap-and-elute workflow is given in Figure 16. Before installing the columns, make sure the correct column specifications are entered using scripts A04 and A05. Specifications for the µPAC Neo columns can be found in Table 4, specifications for the trapping columns can be found in Table 17.

Selecting the appropriate trapping column and Vanquish Neo UHPLC system specifications

µPAC Neo analytical columns have different surface morphologies and sample loading capacities. To obtain an optimal separation, it is crucial that the analytical and trapping columns are carefully paired (Table 16). The pillars in the µPAC Neo trapping column have a non-porous surface that has been functionalized with C8 and subsequently, the trap has a maximum loading capacity of only 10 ng. The trapping column can be paired with the 50 cm µPAC Low Load analytical column; or, if low quantities of sample (≤10 ng) are analyzed, with the 50 cm µPAC Neo column. For sample quantities >10 to ≤500 ng, the Generation 1 µPAC trapping column (pillars have a superficially-porous surface functionalized with C18) is recommended for the 50 cm, 110 cm, and µPAC Neo High Throughput columns. For sample quantities >500 ng, the Thermo Scientific™ PepMap™ Neo Trap Column (which is included with every Vanquish Neo UHPLC system ship kit) is recommended for the 110 cm µPAC Neo column (Figure 17).

Table 14. Vanquish Neo UHPLC system configuration for trap-and-elute workflows in nano/cap fluidic configuration

Scripts for system set-up	
Script	**Direct injection workflow**
A01	Set pump solvent type
A02	Auto start up with diagnostics on
C02	Purge pump (Pump & Flow Meter)
C04	Purge sampler
D01	Test system back pressure
A03	Set separation column type
A04	Set separation column specification
A05	Set trap column specification
A06	Change workflow/fluidics (includes the installation of trap and analytical columns)

Table 15. Solvents used for instrument operation

Module	Property	Setting
Binary pump N	Mobile phase A	H_2O with 0.1% FA
	Mobile phase B	80/20 (v/v) ACN/H_2O with 0.1% FA
Metering device	Weak wash liquid	H_2O with 0.1% FA (or 0.1%TFA)
	Strong wash liquid	80/20 (v/v) ACN/H_2O with 0.1% FA
Wash port	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	80/20 (v/v) ACN/H_2O with 0.1% FA
Binary pump N and metering device	Rear seal wash buffer	25/75 (v/v) H_2O/isopropanol with 0.1% FA

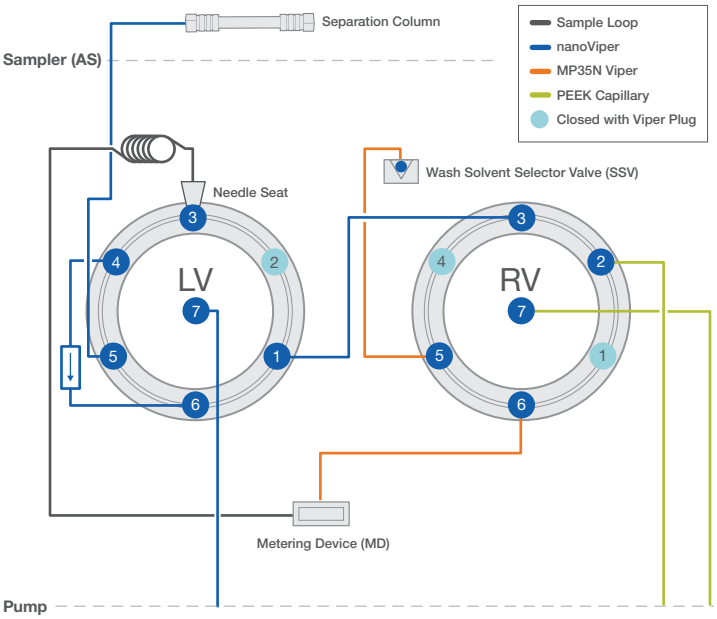


Figure 16. Vanquish Neo UHPLC system trap-and-elute schematic

Table 16. Trapping column specifications and compatibility

	µPAC Neo Low Load trapping column	µPAC trapping column	PepMap trapping column
Loading capacity (ng)	0–10	0–500	0–3000
Maximum pressure (bar)	400	400	1500
Loading flow rate (µL/min)	20	20	60
Washing/equilibration flow rate (µL/min)	40	40	200
50 cm µPAC Neo Low Load column	Compatible	Not compatible	Not compatible
µPAC Neo High Throughput column	Compatible	Compatible	Compatible
50 cm µPAC Neo column	Compatible	Compatible	Not compatible
110 cm µPAC Neo column	Not compatible	Compatible	Compatible

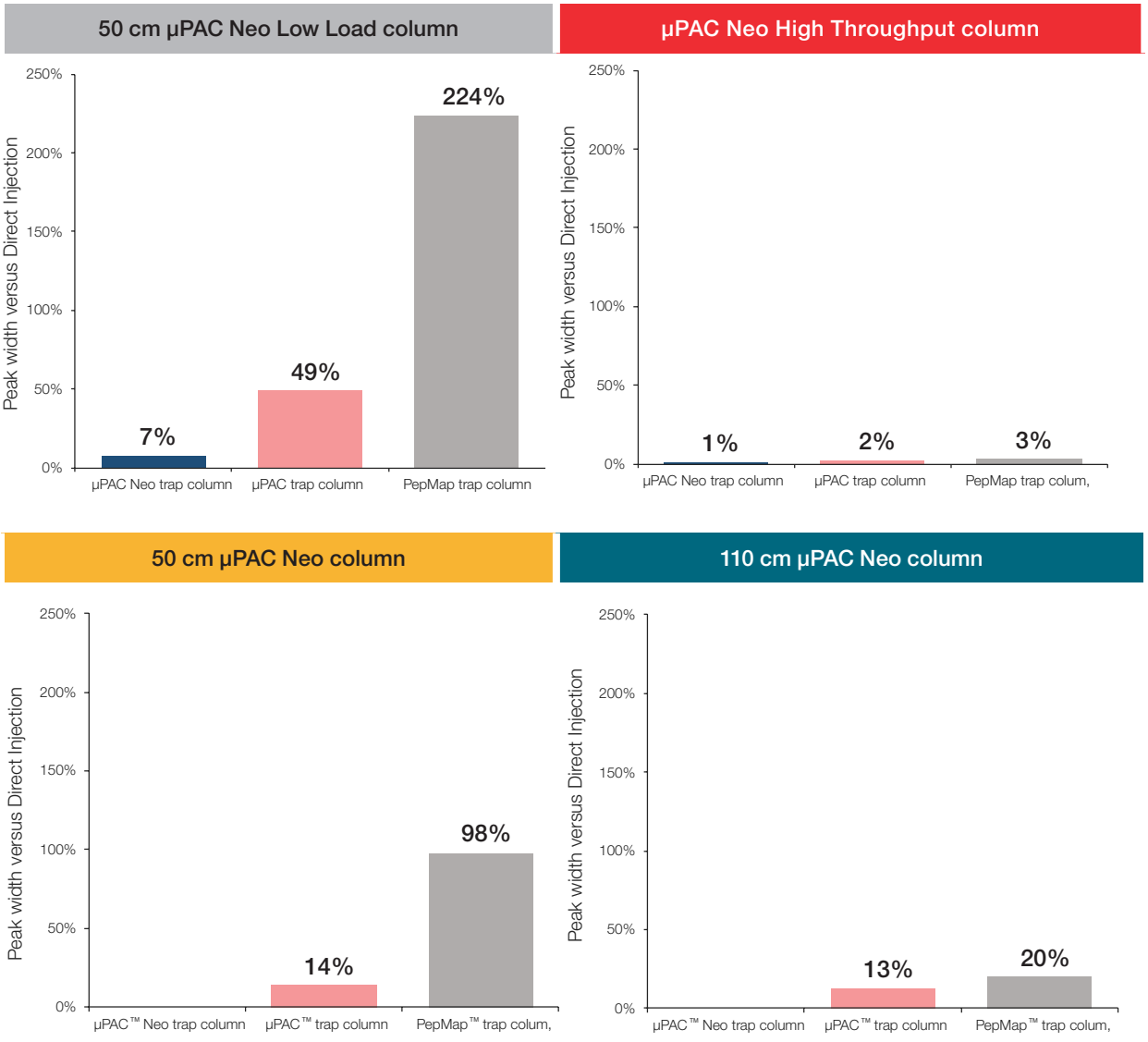


Figure 17. Trapping column compatibility. Bar chart representing the relative increase in peak width for trap-and-elute versus direct injection workflows using the non-porous µPAC Neo trapping column (blue), the µPAC trapping column (red), and the PepMap trapping column (grey). Sample = 10 fmol of Thermo Scientific™ Pierce™ Peptide Retention Time Calibration Mixture (P/N 88320) spiked into a background of HeLa cell digest.

Table 17. Trapping column specifications

	Trapping column specifications		
	µPAC Neo trapping column	µPAC trapping column	PepMap trapping column
Inner diameter	300 µm	300 µm	300 µm
Length	1 cm	1 cm	0.5 cm
Void volume	0.474 µL	0.474 µL	0.237 µL
Maxiumum pressure	400 bar	350 bar	1,500 bar
Maximum flow	60 µL/min	60 µL/min	200 µL/min
Maximum temperature	60°C	60°C	60°C
Maximum pressure change up	1,000 bar/min	1,000 bar/min	1,000 bar/min
Maximum pressure change down	1,000 bar/min	1,000 bar/min	1,000 bar/min
Supports backward flush	Yes	Yes	Yes

Installing the trap column

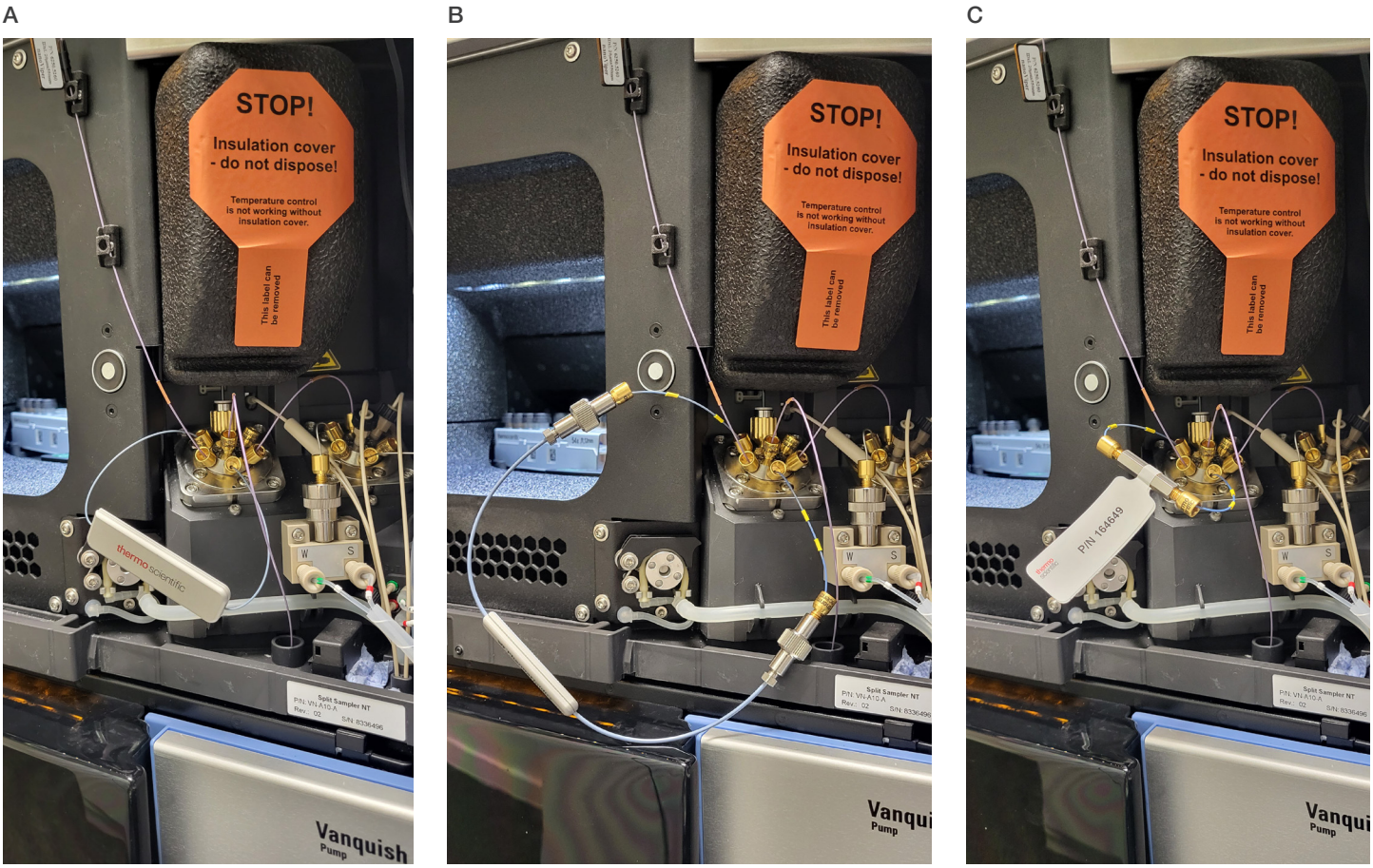


Figure 18. Trapping column position in the autosampler of a Vanquish Neo UHPLC instrument. (A) µPAC Neo column, (B) µPAC column, (C) PepMap column.

Table 18. Mounting µPAC Neo column and trapping column on a Vanquish Neo UHPLC instrument.

µPAC Neo column and trapping column installation steps	
Action	**Description**
1	Install trapping column in ports 4 and 6 of the Vanquish Neo autosampler
2	Install µPATCH holder
3	Position µPAC column in µPATCH holder
4	Connect viper union to column inlet
5	Connect nanoViper transfer line from autosampler (port 5) to column inlet
6	**For 50 and 110 cm µPAC Neo columns:** Apply a flow rate of 300 nL and equilibrate column with desired solvent starting conditions (1%B) - make sure pressure readback is within specifications (90 - 160 bar). **For µPAC Neo High Throughput columns:** Apply a flow rate of 750 nL and equilibrate column with desired solvent starting conditions (4%B) - make sure pressure readback is within specifications (60 - 100 bar).
7	Connect grounding cable to grounding point LC or MS
8	Connect grounding cable to grounding clip µPAC column
9	Connect column outlet directly to 1/16" female emitter with integrated liquid junction or to 1/16" receiving reducing uinion equipped with conductive ESI emitter
10	Make sure column pressure does not increase more than 10% after connecting ESI emitter
11	Apply high voltage (1.7-2.5 kV) and start acquiring

Conditioning and installation analyses

µPAC Neo trapping column and 50 cm µPAC Neo Low Load column

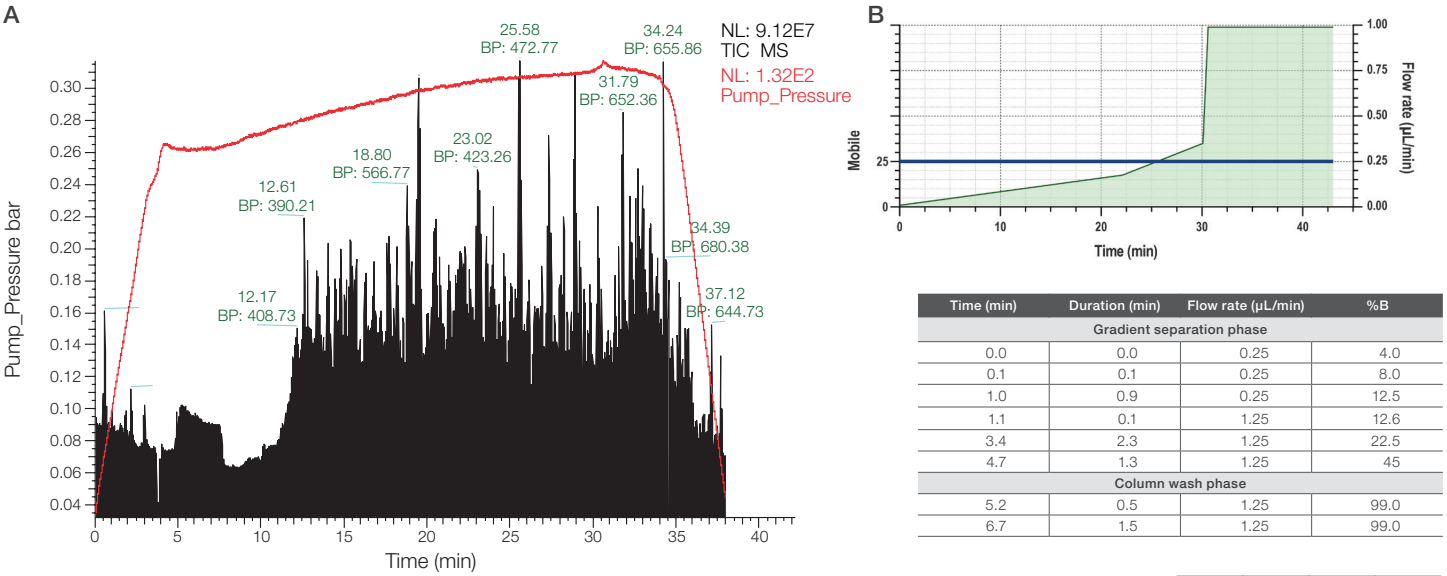
After the column has been installed and the pump back pressure is within specifications (Table 18), proceed with conditioning the column, injecting and analyzing a blank, and performing some installation assessment injections/analyses. All these runs can be performed using the operating conditions listed below in Table 19. For the 50 cm µPAC Low Load column, the recommendation is to condition the column with 10 ng of a protein tryptic digest (Pierce HeLa digest standard - P/N 88328) before beginning the actual installation assessment. All consumables required are listed in Table 20. The generic parameters for sample loading and column equilibration are provided in Table 19. The separation gradient is described in Figure 19.

Table 19. LC method parameters and sample conditions

50 cm µPAC Neo Low Load column trap-and-elute workflow		
Category	**Parameter**	
Sample specifications	Thermo Scientific Pierce HeLa digest standard	P/N 88328
	Concentration	1 ng/µL
	Injection volume	2 µL
	Solvent	H_2O + 0.1% TFA
Sample loading	Mode	FlowControl
	Flow	20 µL/min
	Loading volume	1.5 µL
Column equilibration	Fast equilibration	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Equilibration factor	1.5
Trap column	Fast wash and equilibration	Enabled
	Wash factor	Automatic
	Equilibration factor	Automatic
	Mode	CombinedControl
	Flow	40 µL/min
	Pressure	300 bar
	Trap flush direction	Backward
Temperature	Column compartment temperature	40°C
	Autosampler temperature	7°C

Table 20. Fluidics and accessories

50 cm µPAC Neo Low Load column trap-and-elute workflow		
Part number	**Description**	**Quantity**
6PK1655	Vial and cap screw with septa kit, 100/pack	**1**
	• Vial 0.2 mL amber TPX screw 9 mm short thread conical glass insert	**1**
	• Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	**1**
ES993	EASY-Spray Nano Emitter, bullet type without transfer line (10 µm I.D.)	**1**
COL-LOLO050NEOB	50 cm µPAC Neo HPLC Low Load column	**1**
COL-TRPLOLONEOB2	µPAC Neo trap column	**1**
6040.2304	Union for Viper and nanoViper tubing	**1**
6250.5260	20 µm I.D. × 550 mm nanoViper capillary	**1**



Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.25	4.0
0.1	0.1	0.25	8.0
1.0	0.9	0.25	12.5
1.1	0.1	1.25	12.6
3.4	2.3	1.25	22.5
4.7	1.3	1.25	45
Column wash phase			
5.2	0.5	1.25	99.0
6.7	1.5	1.25	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	723	2,351	2,680	8,778
Chimerys Proteome Discoverer 3.0	3,499	13,669	18,400	8,778

Figure 19. (A) Typical TIC trace obtained for the installation gradient on the 50 cm µPAC Neo Low Load column in trap-and-elute mode. 2 ng HeLa cell digest was loaded on column. Pressure profile is overlaid in red. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Proteome Discoverer software, version 3.0 (1% FDR).

PepMap trapping column and µPAC Neo High Throughput column

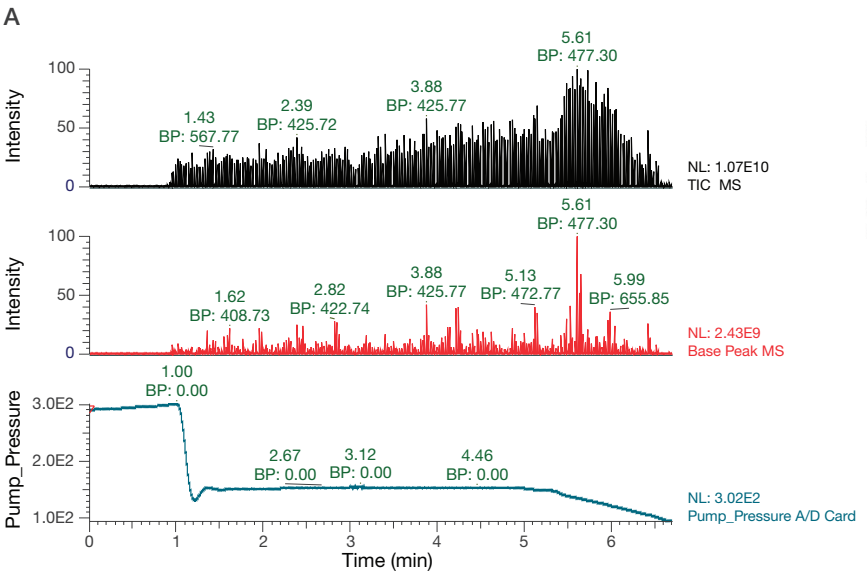
After the column has been installed and the pump back pressure is within specifications (Table 18), proceed with conditioning the column, injecting and analyzing a blank, and performing some installation assessment injections/analyses. All these runs can be performed using the operating conditions listed below in Table 22. For the µPAC Neo High Throughput column, the recommendation is to condition the column with 500 ng of a protein tryptic digest (Pierce HeLa digest standard – P/N 88328) before beginning the actual installation assessment. All consumables required are listed in Table 21. The generic parameters for sample loading and column equilibration are provided in Table 22. The optimal separation gradients at respectively 180 and 100 samples per day throughput are described in Figure 20 and 21.

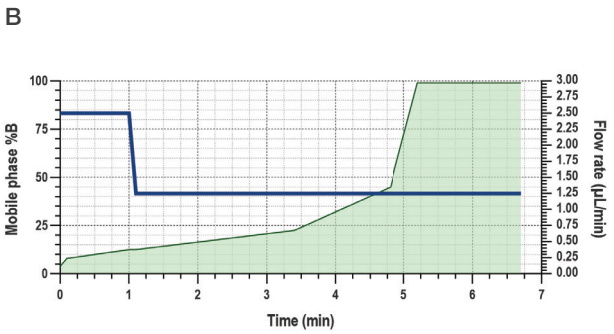
Table 22. LC method parameters and sample conditions

µPAC Neo High Throughput HPLC column		
Category	**Parameter**	
Sample specifications	Concentration	200 ng/µL
	Injection volume	1 µL
	Solvent	H_2O + 0.1% TFA
Sample loading	Fast loading	Enabled
	Mode	PressureControl
	Flow	/
	Loading volume	1.5 (µL)
Column equilibration	Fast equilibration	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Equilibration factor	0
Temperature	Column compartment temperature	50°C
	Autosampler temperature	7°C

Table 21. Fluidics and accessories

µPAC Neo High Throughput HPLC column		
Part number	**Description**	**Quantity**
6PK1655	Vial and cap screw with septa kit, 100/pack	**1**
	• Vial 0.2 mL amber TPX screw 9 mm short thread conical glass insert	**1**
	• Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	**1**
P-881	MicroTight Adapter for 1/16" OD tubing to 1/32" OD tubing	**1**
ES542	Nano Bore Emitter Stainl. Steel 40mm OD 1/32	**1**
COL-CAPHTNEOB	µPAC Neo High Throughput HPLC column	**1**
COL-TRPNANO16G1B2	µPAC trapping column	**1**
174500	PepMap Neo trap column 300 µm × 5 mm, 5 µm, 1500 bar	**1**
174502	Trap column holder and nanoViper Fitting System, 1500 bar	**1**
6040.2304	Union for Viper and nanoViper tubing	**1**
6250.5260	20 µm I.D. × 550 mm nanoViper capillary	**1**

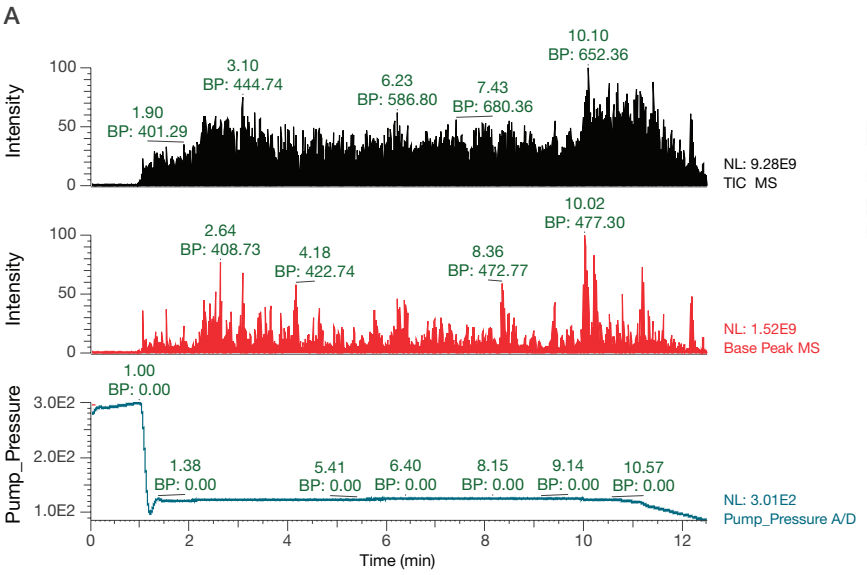


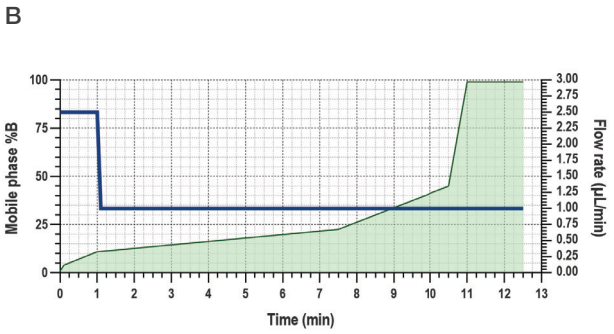


Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.25	0.0
0.1	0.1	0.25	1.0
22.1	22.0	0.25	17.5
30.1	8.0	0.25	35.0
Column wash phase			
30.6	0.5	0.25	99.0
43.0	12.4	0.25	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	1,108	5,446	7,276	11,552
Chimerys Proteome Discoverer 3.0	2,210	9,304	14,143	11,552

Figure 20. (A) Typical TIC trace obtained for the 5.5 min gradient installation method on the µPAC Neo High Throughput column in trap-and-elute mode. 200 ng HeLa cell digest was loaded on column. BPC and Pressure profile are shown below the TIC trace. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Proteome Discoverer software, version 3.0 (1% FDR).





Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.25	1.0
0.1	0.1	0.25	4.0
1.0	0.9	0.25	11.0
1.1	0.1	1.0	11.1
7.5	6.4	1.0	22.5
10.5	3.0	1.0	45.0
Column wash phase			
11.0	0.5	1.0	99.0
12.5	1.5	1.0	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	1,108	5,446	7,276	11,552
Chimerys Proteome Discoverer 3.0	2,210	9,304	14,143	11,552

Figure 21. (A) Typical TIC trace obtained for the 11 min gradient installation method on the µPAC Neo High Throughput column in trap-and-elute mode. 200 ng HeLa cell digest was loaded on column. BPC and Pressure profile are shown below the TIC trace. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Proteome Discoverer software, version 3.0 (1% FDR).

µPAC trapping column and 50 cm µPAC Neo analytical column

After the column has been installed and the pump back pressure is within specifications (Table 18), proceed with conditioning the column, injecting and analyzing a blank, and performing some installation assessment injections/analyses. All these runs can be performed using the operating conditions listed below in Table 24. For the 50 cm µPAC column, the recommendation is to condition the column with 500 ng of a protein tryptic digest (Pierce HeLa digest standard – P/N 88328) before beginning the actual installation assessment. All consumables required are listed in Table 23. The generic parameters for sample loading and column equilibration are provided in Table 24. The separation gradient is described in Figure 22.

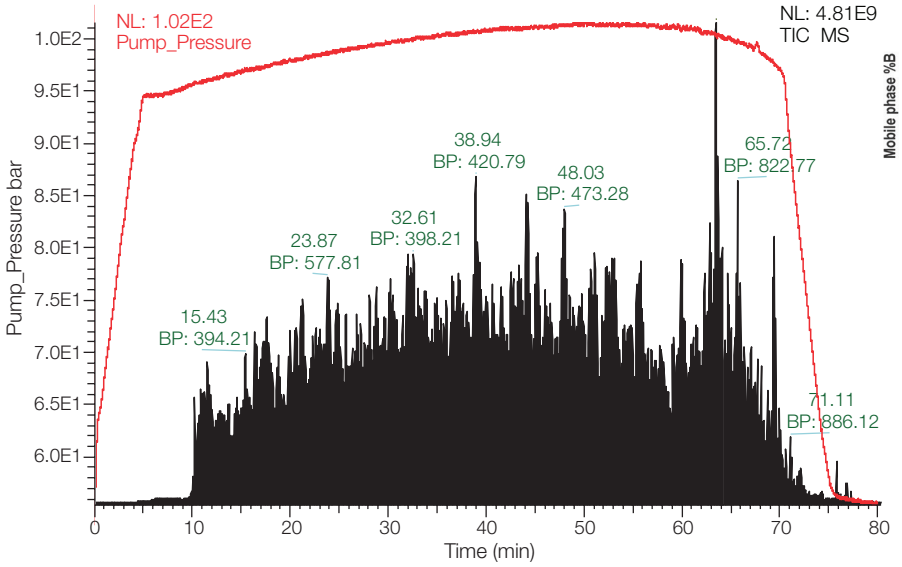
Table 24. LC method parameters and sample conditions

50 cm µPAC Neo trap-and-elute workflow		
Category	**Parameter**	
Sample specifications	Thermo Scientific Pierce HeLa digest standard	P/N 88328
	Concentration	50 ng/µL
	Injection volume	4 µL
	Solvent	H_2O + 0.1% TFA
Sample loading	Mode	FlowControl
	Flow	20 µL/min
	Loading volume	1.5 (µL)
Column equilibration	Fast equilibration	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Equilibration factor	1.5
Trap column	Fast wash and equilibration	Enabled
	Wash factor	Automatic
	Equilibration factor	Automatic
	Mode	CombinedControl
	Flow	40 µL/min
	Pressure	300 bar
	Trap flush direction	Backward
Temperature	Column compartment temperature	50°C
	Autosampler temperature	7°C
	Trap cartridge	Room temperature (ca. 23°C)

Table 23. Fluidics and accessories

50 cm µPAC Neo trap-and-elute workflow		
Part number	**Description**	**Quantity**
6PK1655	Vial and cap screw with septa kit, 100/pack	**1**
	• Vial 0.2 mL amber TPX screw 9 mm short thread conical glass insert	**1**
	• Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	**1**
ES993	EASY-Spray Nano Emitter, bullet type without transfer line (10 µm I.D.)	**1**
COL-NANO050NEOB	50 cm µPAC Neo HPLC column	**1**
COL-TRPNANO16G1B2	µPAC trapping column	**1**
174502	Trap column holder and Thermo Scientific™ nanoViper™ Fitting System, 1500 bar	**1**
6040.2304	Union for Viper and nanoViper tubing	**3**
6250.5260	20 µm I.D. × 550 mm nanoViper capillary	**1**

A



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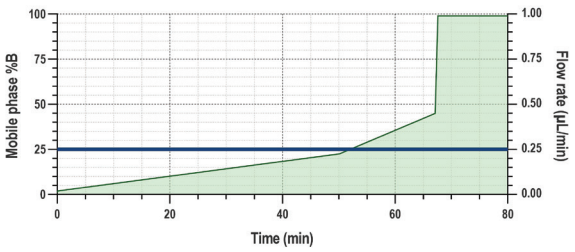


Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.25	1.0
0.1	0.1	0.25	2.0
50.1	50.0	0.25	22.5
67.1	17.0	0.25	45.0
Column wash phase			
67.6	0.5	0.25	99.0
80.0	12.4	0.25	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	4,376	31,382	59,697	93,229
Chimerys Proteome Discoverer 3.0	6,685	50,280	136,237	93,229

Figure 22. (A) Typical TIC trace obtained for the installation gradient on the 50 cm µPAC Neo columns in trap-and-elute mode. 200 ng HeLa cell digest was loaded on column. Pressure profile is overlaid in red. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Proteome Discoverer software, version (1% FDR).

PepMap trapping column and 110 cm µPAC Neo column

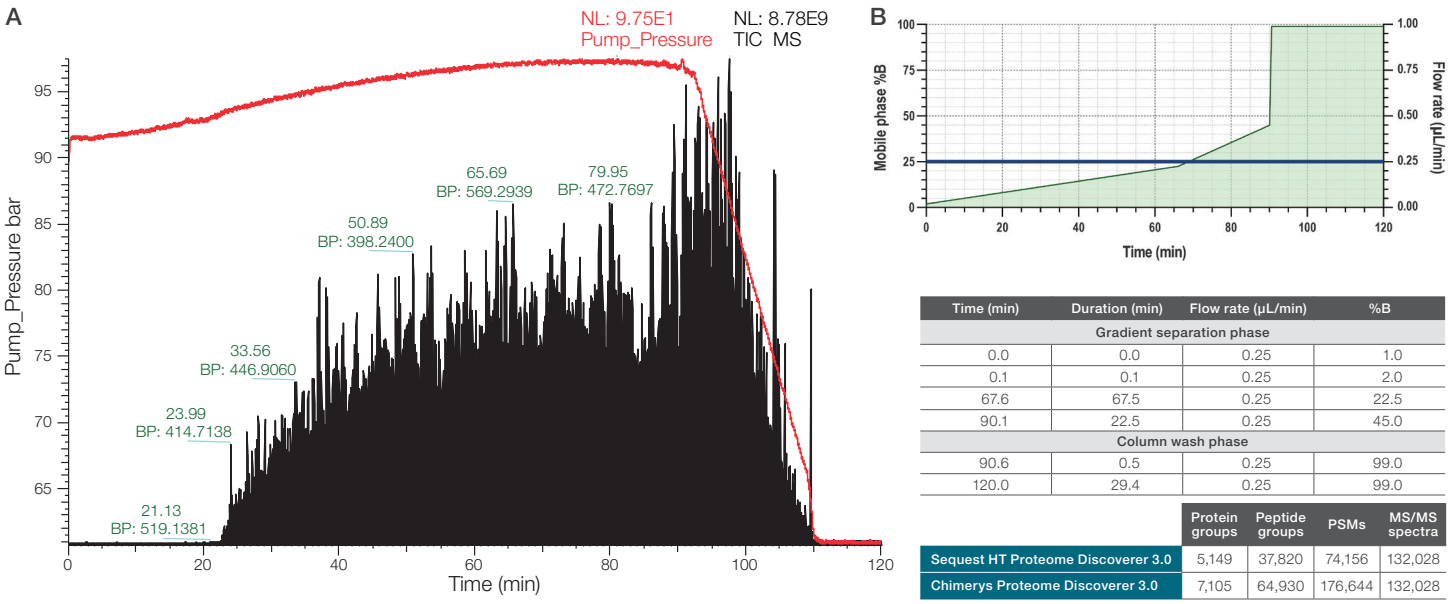
After the column has been installed and the pump back pressure is within specifications (Table 18), proceed with conditioning the column, injecting and analyzing a blank, and performing some installation assessment injections/analyses. All these runs can be performed using the operating conditions listed below in Table 25. For the 110 cm µPAC column, the recommendation is to condition the column with 500 ng of a protein tryptic digest (Pierce HeLa digest standard - P/N 88328) before beginning the actual installation assessment. All consumables required are listed in Table 26. The generic parameters for sample loading and column equilibration are provided in Table 25. The separation gradient is described in Figure 23.

Table 25. LC method parameters and sample conditions

110 cm µPAC Neo trap-and-elute workflow		
Category	**Parameter**	
Sample specifications	Thermo Scientific Pierce HeLa digest standard	P/N 88328
	Concentration	100 ng/µL
	Injection volume	5 µL
	Solvent	H_2O + 0.1% TFA
Sample loading	Mode	FlowControl
	Flow	20 µL/min
	Loading volume	1.5 µL
Column equilibration	Fast equilibration	Enabled
	Mode	PressureControl
	Flow	/
	Pressure	400 bar
	Equilibration factor	1.5
Trap column	Fast wash and equilibration	Enabled
	Wash factor	50
	Equilibration factor	Automatic
	Mode	CombinedControl
	Flow	200 µL/min
	Pressure	800 bar
	Trap flush direction	Backward
Temperature	Column compartment temperature	50°C
	Autosampler temperature	7°C
	Trap cartridge	Room temperature (ca. 23°C)

Table 26. Fluidics and accessories

110 cm µPAC Neo trap-and-elute workflow		
Part number	**Description**	**Quantity**
6PK1655	Vial and cap screw with septa kit, 100/pack	**1**
	• Vial 0.2 mL amber TPX screw 9 mm short thread conical glass insert	**1**
	• Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	**1**
ES993	EASY-Spray Nano Emitter, bullet type without transfer line (10 µm I.D.)	**1**
COL-NANO110NEOB	110 cm µPAC Neo HPLC column	**1**
174500	PepMap Neo trap column 300 µm × 5 mm, 5 µm, 1500 bar	**1**
174502	Trap column holder and Thermo Scientific nanoViper Fitting System, 1500 bar	**1**
6040.2304	Union for Viper and nanoViper tubing	**1**
6250.5260	20 µm I.D. × 550 mm nanoViper capillary	**1**



Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.25	1.0
0.1	0.1	0.25	2.0
67.6	67.5	0.25	22.5
90.1	22.5	0.25	45.0
Column wash phase			
90.6	0.5	0.25	99.0
120.0	29.4	0.25	99.0

	Protein groups	Peptide groups	PSMs	MS/MS spectra
Sequest HT Proteome Discoverer 3.0	5,149	37,820	74,156	132,028
Chimerys Proteome Discoverer 3.0	7,105	64,930	176,644	132,028

Figure 23. (A) Typical TIC trace obtained for the installation gradient on the 110 cm µPAC Neo Low Load columns in trap-and-elute mode. 500 ng HeLa cell digest was loaded on column. Pressure profile is overlaid in red. (B) LC solvent gradient conditions and profile. Number of protein groups, peptide groups, PSMs and MS/MS spectra obtained. Raw file processed with either Sequest HT or Chimerys in Proteome Discoverer software, version 3.0 (1% FDR).

Loading and injection volume

For the trap-and-elute configuration, sample volumes of ≤15 µL can be loaded in 2 min. The amount of solvent that is drawn in addition to the sample is determined by the loading volume and can be defined in the loading parameters section of the method editor. Increasing the loading volume will wash more impurities to waste, but inevitably, there will be some loss of hydrophilic peptides. When 0.1% formic acid (FA) in water is the weak wash solvent, optimal proteome coverage will be achieved with loading volumes of 1 to 2 µL. Changing the weak wash to 0.1% trifluoroacetic acid (TFA) in water will significantly increase trapping capacity and lead to higher peptide recovery and improved peak shapes for peptides with shorter retention times.

Figure 24. Base peak chromatograms obtained for the separation of 200 ng HeLa tryptic digest (1 µL injected) using different loading volumes. 50 cm µPAC Neo column, 67 min gradient. The loss of hydrophilic peptides at lower retention times is indicated by the arrows.

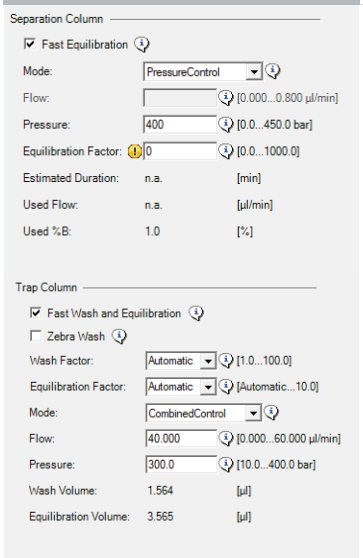
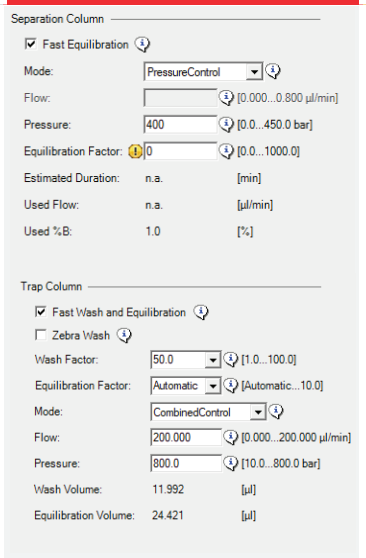
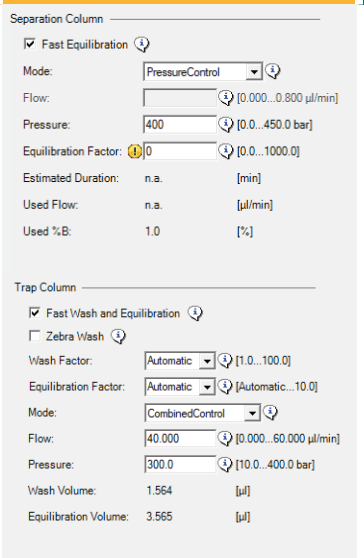
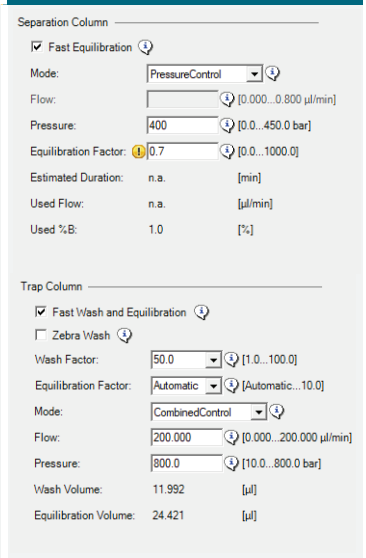
Column equilibration

Analogous to the procedure for direct injection, column equilibration is achieved off-line and is typically performed at high pressure and/or flow rate. At conventional nanoLC flow rates (≤500 nL/min), the equilibration volume must be defined in the method editor. Equilibrating the column with 1.5 column volumes (1% B) is sufficient to regenerate the column for the next analysis. This will take approximately 0.5, 2.5 and 9 min for the µPAC Neo High Throughput, 50 cm, and 110 cm µPAC Neo columns, respectively. Alternatively, the trap-and-elute configuration enables parallel sample loading and column equilibration for columns with a low internal volume. For the 50 cm µPAC Neo Low Load and the 50 cm µPAC Neo columns, the equilibration volume can be removed from the method. If the initial flow rate in the method is at 2500 or 750 nL/min, the columns (bed volume of 1.5 µL) are fully equilibrated during sample draw and loading. For the 110 cm columns, this approach cannot be used because of the larger column volume (4.5 µL). Even if a higher flow rate is used in the first section of the analytical method, a minimum of 0.7 column volumes should always be maintained to ensure adequate equilibration of the 110 cm µPAC Neo column (Table 27).

Flow rate optimization

Compared to the direct injection mode, the introduction of a trapping column will slightly increase the void volume and the peptides will elute later. The additional volume (including the nanoViper capillaries) will be approximately 1–1.25 µL and 0.5–0.75 µL for the µPAC and the PepMap trapping columns, respectively. These volumes should be added to the analytical column volume when the gradient and flow rate are optimized to accelerate peptide elution (Figure 25). Significant increases in sample throughput can be obtained by optimizing the flow rate and solvent composition of the gradient.

Table 27. Column equilibration settings for increased throughput (analytical flow rate should be at 2,500 or 750 nL/min at the beginning of the next analysis, depending on the µPAC Neo column used).

µPAC Neo trapping column 50 cm uPAC Neo Low Load column	PepMap trapping column µPAC Neo High Throughput column	µPAC trapping column 50 cm µPAC Neo column	PepMap trapping column 110 cm µPAC Neo column
			

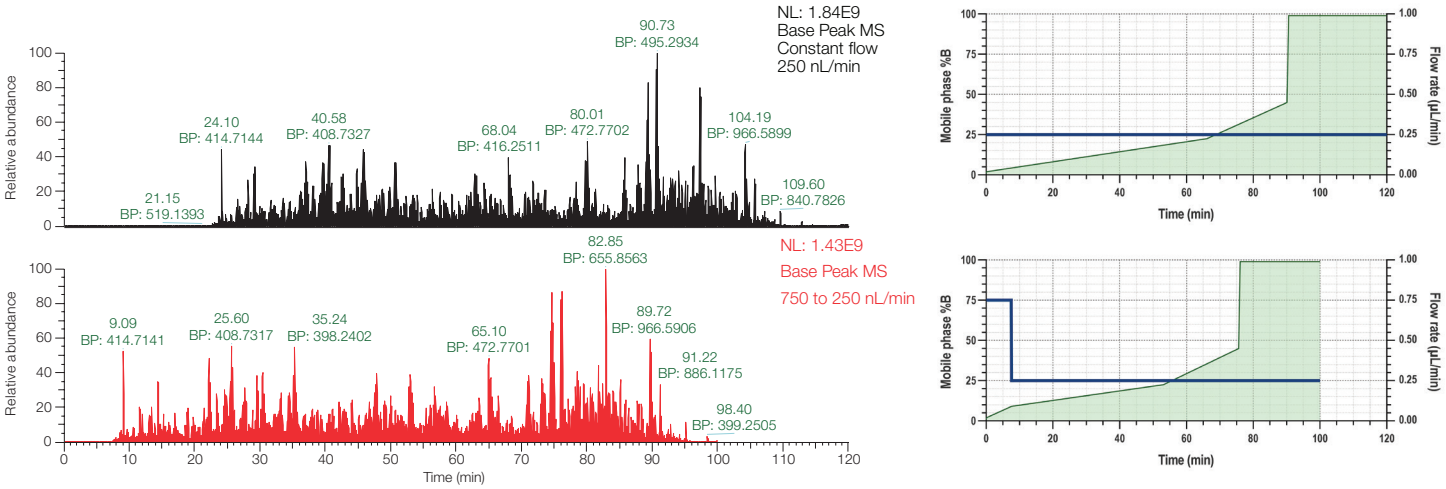


Figure 25. Base peak chromatograms obtained from separating 500 ng HeLa tryptic digest (2.5 µL injected). Upper panel: constant flow rate of 250 nL/min. Lower panel: optimized method with variable flow rates. After 7.5 min., the flow was reduced from 750 nL/min to 250 nL/min. Elution window = 67 min. Right: Solvent gradient and flow rate profiles. PepMap trapping column and 110 cm µPAC Neo column.

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