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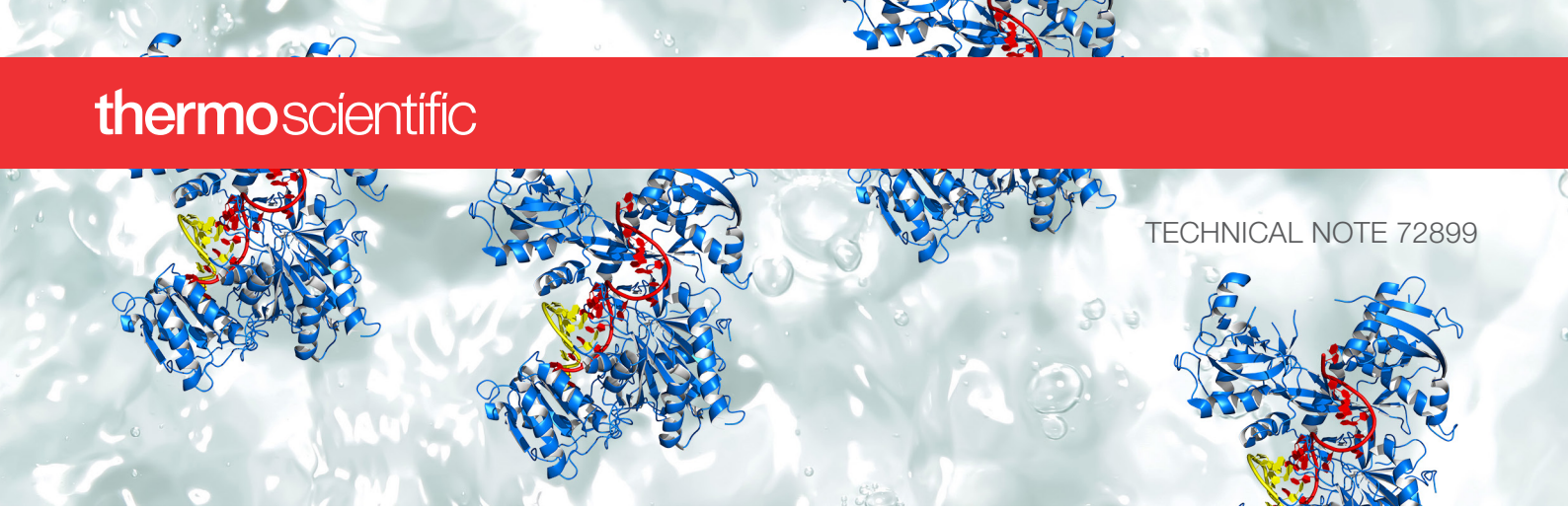

TECHNICAL NOTE 72899

Tandem nanoLC-MS: maximum MS utilization for deep-dive proteomic analysis

Authors

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Keywords

Tandem nano-flow LC, nanoLC-MS proteomics, continuous MS utilization

Goal

Create tandem nano-flow LC-MS based proteomics methods for deep-dive proteomics experiments using long columns and shallow gradients, which afford maximum MS utilization.

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1. Introduction

1.1. NanoLC-MS – deep into the proteome with time-intensive methods

Nano-flow LC-MS proteomics applications using long columns and shallow gradients coupled to high-resolution mass spectrometers are well established as the gold standard for deep-dive "discovery"-based proteomics workflows. While permitting unsurpassed levels of sensitivity, such methodology continues to suffer in terms of productivity due to the inherent MS idle time associated with each analytical run. In extreme cases, actual MS sample data acquisition can account for as little as half of the total analysis time. The remainder is taken up with column washing, equilibration, and sample loading as well as with void time (time taken for sample components that do not interact with the stationary phase to travel from the sample loop through the column and associated fluidics to the MS detector). While some of these delays can be reduced, for example, through the adoption of trap columns and high sample loading flow rates, the analysis time lost during which the sample migrates through the column plus the delays associated with column washing and equilibration remain.

1.2. Tandem nanoLC workflows for maximum productivity in deep-dive proteomics

One way to significantly reduce the latency in nanoLC is by incorporating a second separation path comprising an extra analytical separation pump and column (analytical and trap) into the setup, which is then operated in tandem. By temporally offsetting the gradient steps of the two columns (see Figure 1), it is possible to ensure a continuous feed of sample to the mass spectrometer. Furthermore, the introduction of a post-column divert valve ensures that only eluted sample components are directed to the mass spectrometer, while all contaminant components eluted during the wash phase are diverted to waste. Here we describe such a tandem nanoLC-MS method with pre-concentration onto a trap column using the Thermo Scientific™ UltiMate™ 3000 RSLCnano platform.[1]

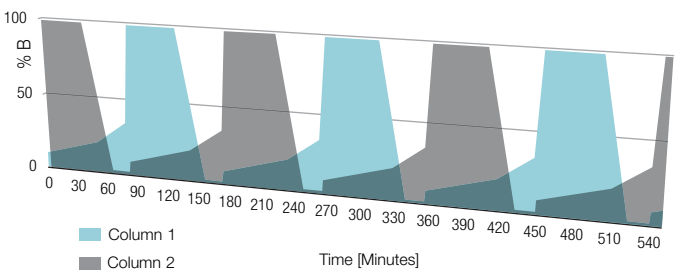


Figure 1. Flow-gradient schematic depicting the concept of tandem nanoLC operation. Note that the column wash phase, represented by an organic hold here for simplicity, is replaced by multiple "saw-tooth" wash cycles in the method described below.

2. Experimental

2.1. Consumables

- Fisher Scientific™ LC-MS grade Water (P/N W6-212)
- Fisher Scientific™ LC-MS grade Acetonitrile (P/N 10616653)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), LC-MS grade, (P/N 85183)
- Thermo Scientific™ Pierce™ Formic acid (FA), LC-MS grade, (P/N 28905)

Fluidics and columns used to setup the application are listed in Table 1.

2.2 Sample

Thermo Scientific™ Pierce™ HeLa protein digest (P/N 88328, 20 µg/vial) reconstituted to a final concentration of 200 ng/µL in loading buffer (see Table 2 for details).

2.3 NanoLC-MS configuration and separation conditions

Measurements were carried out using an UltiMate 3000 RSLCnano system in tandem pre-concentration setup, comprising a Thermo Scientific™ UltiMate™ NCS-3500RS, Thermo Scientific™ UltiMate™ NCP-3200RS, and Thermo Scientific™ UltiMate™ WPS-3000TFC autosampler in a single stack (see Figure 2A). When installing the solvent feed lines for the NCP-3200RS pump, it is important that they are plumbed around the outside of the instrument and not fed through the column oven as warming of the solvent can lead to outgassing. Both NCS-3500RS and NCP-3200RS pumps were configured with a ProFlow™ flow meter. Two 2-position 10-port low dispersion valves (6041.0001A) were installed in the column compartment. The WPS-3000TFC autosampler was fitted with a post-column 1/32" nano valve (P/N 6820.6232) in the lower position and a 900 bar compatible 2pos-6port injection valve (P/N 6826.0011A) in the upper position and configured for low-flow applications using the upgrade nano/cap kit for WPS-3000TFC (P/N 6824.0030). Both the 1/32" nano valve and upgrade kit are included in the UltiMate 3000 RSLCnano tandem nanoLC kit (see Table 1 and the UltiMate 3000 RSLCnano Standard Application Manual[2] for details).

Table 1. Contents of the Thermo Scientific™ UltiMate™ 3000 RSLCnano Tandem nanoLC kit. The letter and number assignments are given in Figure 2B.

#	Item	P/N
a	Thermo Scientific™ Acclaim™ PepMap™ RSLC C18 column, 2 µm, 100 Å, 75 µm × 15 cm*	164534
b	300 µm I.D. × 5 mm packed with Acclaim PepMap 100 C18, 5 µm, (set of 5 cartridges)	160454
	µ-Precolumn holder, 5 mm, with 30 µm I.D. connecting tubing, Thermo Scientific™ nanoViper™ fingertight fittings	164649
1	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" I.D. × L 20 µm × 350 mm	6041.5240
2,3	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" I.D. × L 75 µm × 650 mm	6041.5775
4	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" I.D. × L 75 µm × 250 mm	6041.5730
	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" I.D. × L 20 µm × 750 mm	6041.5280
	Thermo Scientific™ nanoViper™ sample loop 20 µL, FS/PEEK sheathed	6826.2420
5	PTFE tubing, 500 µm I.D., 100 cm, used as waste tubing	6720.0077
	1/16” Universal Fingertight Fitting, one-piece design, long thread	6720.0015
6	Fused silica tubing I.D. 20 µm O.D. 280 µm, 5 m for nanoLC connections	160475*
	Cutter for fused silica tubing (cleavage stone)	6720.0016
	Upgrade kit nano/cap WPS-3000TFC	6824.0030
	1/32” 2 pos 6 port nano switching valve (C2N series)	6820.6232
	Fittings for nano valve	6720.0080
	1/32” PEEK sleeve, 3 cm, 300 µm I.D. (6 pieces)	6820.1320
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pcs.	6820.0027
	Polypropylene caps for WPS vials, 25 pcs.	6820.0028
	Cytochrome C digest, 1.6 nmol, Lyophilized	161089

* This capillary must be cut to the appropriate length using the cleavage stone supplied in the kit.
Note: Consumables are from Thermo Fisher Scientific unless stated otherwise.

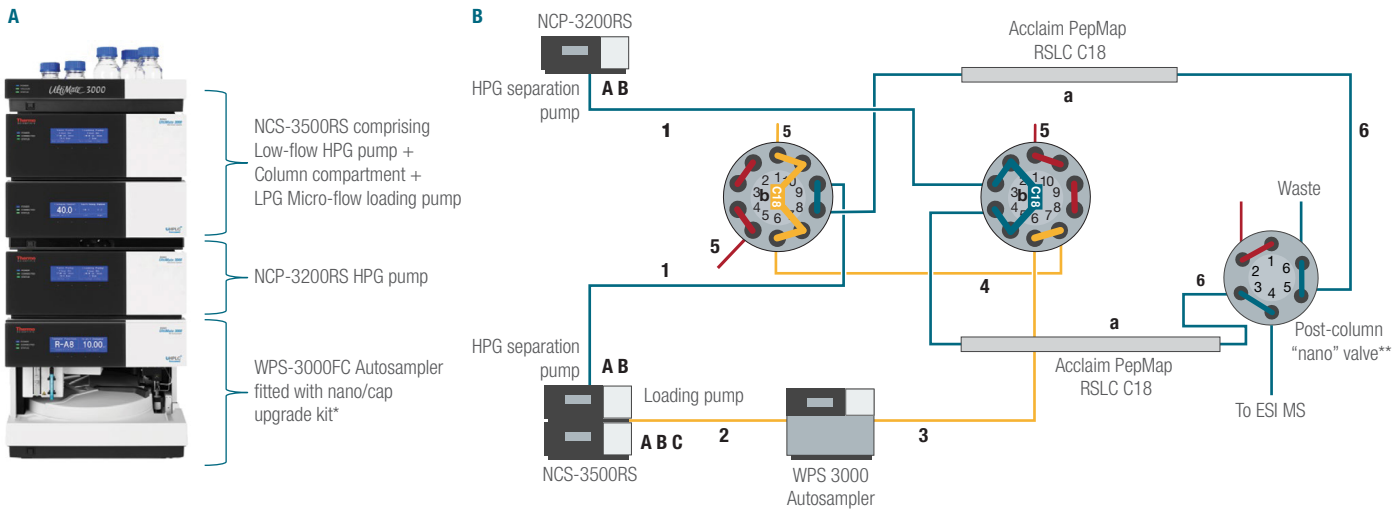
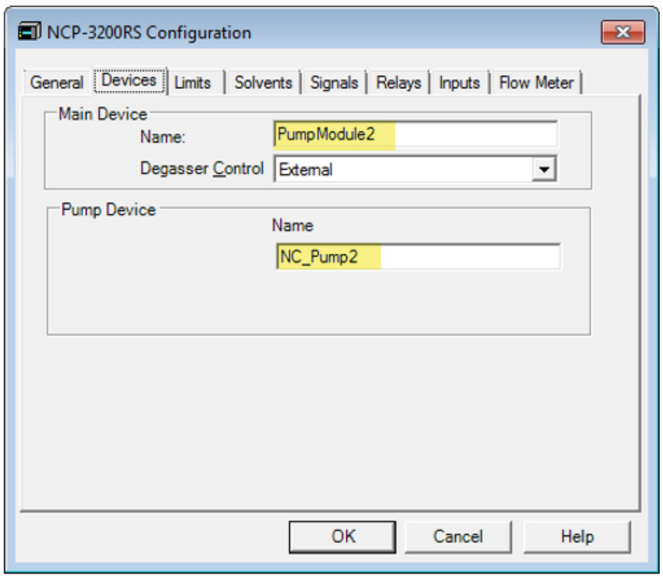


Figure 2. UltiMate 3000 RSLCnano system (A) and fluidic (B) configurations for the tandem nano pre-concentration of sample onto a trap column LC setup. Note: The number and letter descriptions for each of the fluidic components are given in Table 1. *All components are included in the application kit except the high pressure injection valve (P/N 6826.0011A), which must be ordered separately and installed in the upper position in the FC autosampler. **Alternatively, the post-column 1/32” nano valve can be switched using an external VICI® USB controller. This enables existing installations to be upgraded from a standard to a tandem configuration (see section 2.4 for details).

The device names and signals of the NCP-3200RS pump were modified through the addition of the number “2” after the standard abbreviations in the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) instrument configuration manager as shown in Figures 3A and 3B, respectively.

A



B

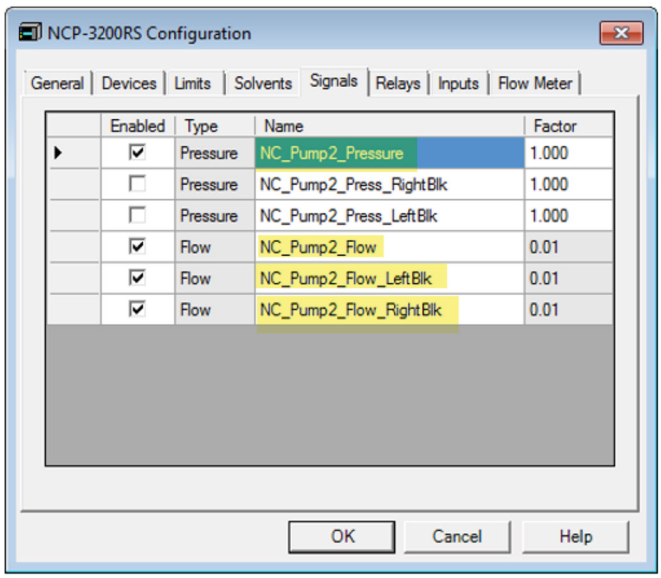


Figure 3. NCP-3200RS (A) device configuration settings and (B) signal configuration settings

When configuring the WPS-3000TFC autosampler, the number of detection channels must be set to “0” under the Fraction Collection tab and the “bridge volume” should also be set to “0” under the Options tab.

The system fluidics listed in Table 1 were configured as shown in Figure 2B. For the purposes of the experiments described here, the 15 cm long Acclaim PepMap columns provided in the kit (P/N 164534) were replaced with two 50 cm long Acclaim PepMap columns (75 µm x 50 cm, 2 µm, P/N 164942), which must be ordered separately. In order to interface the respective linear Acclaim PepMap column outlet with the post-column nano valve in the autosampler, the fused silica outlet supplied with the columns must be removed and replaced with a piece of extended fused silica supplied in the kit (see item 6 in Table 1 and Figure 2B). The column outlet is connected to the nano fused silica tubing using a nano connector. Before the capillary is installed it must first be **cut to a length of 60 cm** using the cleavage stone. The capillary is connected to the nano valve using the PEEK sleeves (P/N 6820.1320) and fittings (P/N 6720.0080) provided in the Tandem nanoLC kit (Table 1).

Solvents and analysis conditions were used as described in Table 2. Two independent LC methods are required to set up a sequence with tandem nanoLC-MS operation.

Table 2 (Part 1). LC solvents and conditions for a 90-minute tandem nanoLC gradient on a 50 cm long column*

Property	Setting
NC pump mobile phase A	100% Water + 0.1% FA
NC pump mobile phase B	20%/80% Water/ACN + 0.1% FA
Loading solvent Loading pump A channel Loading pump B and C channels	 100% Water + 0.05% TFA 20%/80% Water/ACN + 0.1% FA
Sample	HeLa digest (200 ng/µL)
Autosampler wash solvent	100% ACN + 0.1% FA
Injection volume	5 µL
Loading time	0.5 min
Gradient flow rate	300 nL/min

NC pump settings

The left column shows the gradient for the column that is in line with the mass spectrometer. The right column shows the gradient for the column that is diverted to waste.

Column in line with MS

Time, min	%B	Comment
0	9.8	
51	20	Gradient
81	35	
86	99	Column wash cycle 1
92	99	

Column diverted to waste

Time, min	%B	Comment
0	99	
10	99	Column wash cycle 1**
20	1	
25	1	
35	99	Column wash cycle 2**
40	99	
50	1	
51	1	
55	99	Column wash cycle 3**
60	99	
64	1	Equilibration
82	1	Start of gradient
83	8	Gradient
92	9.8	

Oven temperature	60 °C
Sample temperature	5 °C

Loading pump settings

Time, min	Flow, µL/min	%B	Comment
0	120	99	Sample loop wash
1.0	120	99	
2.0	120	0	Sample loop equilibration
4.0	120	0	
5.0	5	0	Idle flow
69	5	0	
70	40	0	Set flow rate for sample loading
87	40	0	Start sample loop wash cycle
89	120	99	Sample loop wash
92	120	99	

*Examples of LC-MS method scripts are available for download at AppsLab (https://appslab.thermofisher.com/App/4213/tandemnanolcms)

**To reduce carryover, the columns are washed using "saw-tooth" gradients, i.e. rapid oscillations between aqueous and organic phases. These wash steps are denoted by the comment "wash cycle".

Table 2 (Part 2). LC solvents and conditions for a 90-minute tandem nanoLC gradient on a 50 cm long column

Property	Setting
Valve switching when Trap 1, Column 1 is in line with the mass spectrometer (Method 1)	
Valve switching when Trap 2, Column 2 is in line with the MS (Method 2)	
Commands manually inserted into the method Script Editor for Method 1 and Method 2	

Time, min	ValveLeft	ValveRight	Comment
0	1_2	10_1	Loading pump flow to waste
70	1_2	1_2	Loading pump flow to trap column 2
82	1_2	10-1	Trap column 2 in line with analytical column 2

Time, min	ValveLeft	ValveRight	Comment
0	1_2	10_1	Loading pump flow to waste
70	10_1	10_1	Loading pump flow to trap column 1
82	1_2	10_1	Trap column 1 in line with analytical column 1

Time, min	Command	Comment
0	Sampler.Drain (method 1)	Trap 1 column 1 in line with MS
0	Sampler.Collect (method 2)	Trap 2 column 2 in line with MS
69	Sampler.InjectValveToLoad	Loop switch in line with injection needle
69.5	Sampler.Wash	Loop and injection needle washed with high organic
72	Sampler.InjectValveToInject	Loop switched in line with trap column 20 µL organic solvent plug pushed through trap
79	Sampler.PrepareNextInjection	Sample loaded into sample loop
81.5	Sampler.InjectValveToInject	Sample pushed to the trap column
91.75	Sampler.Collect (method 1),	Trap 2 column 2 in line with mass spectrometer
91.75	Sampler.Drain (method 2),	Trap 1 column 1 in line with mass spectrometer

2.4 Upgrading the UltiMate 3000 RSLCnano system to a tandem setup

For users upgrading from a single to a tandem setup who wish to retain their WPS-3000TPL autosampler, post-column valve switching can be performed using a USB controlled Universal Electric Actuator (P/N EUHB) and corresponding mounting hardware (P/N CMH12H) from VICI Valco Instruments. For successful control of the external VICI valve using SII or Chromeleon CDS, the COM port being used by the controller must first be determined. This information is given in the Device Manager of the Microsoft® Windows® operating system (Figure 4A). The valve controller can then be configured in the Chromeleon/SII instrument controller. The VICI driver for the actuator can be found under VICI instrument → Module UEA in the Chromeleon/SII instrument configuration manager. The correct COM port is selected under the Communication tab and the valve configuration retrieved using the Retrieve Configuration button (Figure 4B). Under the Options tab, select Valve Mode: Two positions with stops and save the configuration. (Figure 4C).

Example commands for switching the actuator, which should be inserted into the method editor, are given in Table 3. The user will need to determine which valve position (A or B) corresponds to which column to MS connection when setting up the device.

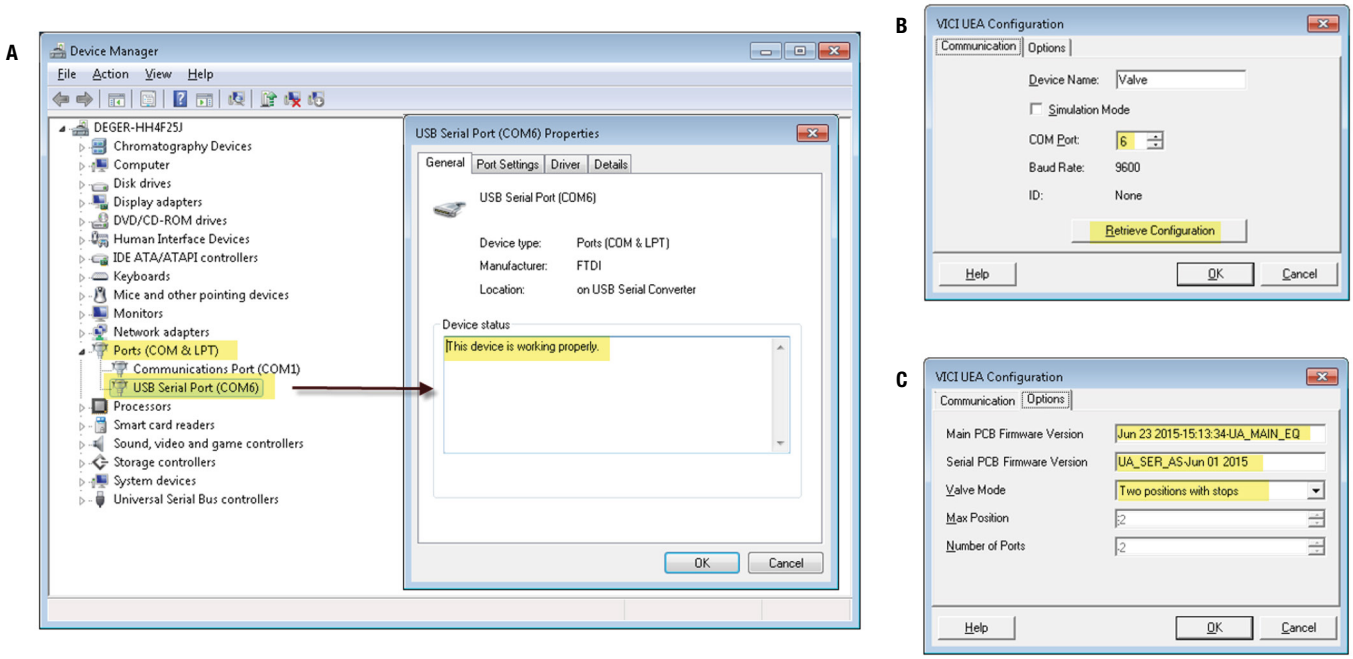


Figure 4. Configuring the external VICI USB controller. (A)The COM Port is displayed under the Device Manager of the Windows operating system. (B) The assigned COM port is selected in the Communication tab in the instrument configuration. (C) After retrieving the configuration, the Valve Mode is set to "Two positions with stops" in the Options tab.

Table 3. Commands inserted into the Method Editor when using the Universal Electric Actuator from VICI Valco Instruments to control the 1/32" nano valve.

Time, min	Command
0	Valve.positionA
91.75	Valve.positionB

Autosampler method settings are given in Table 4.

Table 4. Autosampler settings for fast autosampler routines

Property	Setting
Draw speed	1 µL/s
Draw delay	2 s
Dispense speed	8 µL/s
Dispense delay	2 s
Dispense to waste speed	8 µL/s
Sample height	2 mm
Puncture depth	8 mm
Wash volume	25 µL
Wash speed	8 µL/s

2.5 MS conditions

Tables 5 and 6 show MS tune settings and parameters of the MS method for data-dependent acquisition (DDA) experiments.

Table 5. MS tune settings

Parameters / Components	Settings / Details
Source settings	
ESI source	Thermo Scientific™ Nanospray Flex™ Ion Source
Polarity	Positive
Ion transfer tube temperature	275 °C
Spray voltage positive ion	1.9 kV
Ion funnel RF level	40

Table 6. MS settings for DDA experiments

Parameters / Components	Setting
MS 1 resolution	60,000
AGC target	3e6
Maximum IT	50 ms
Scan range	375–1500 *m/z*
DDA	
MS 2 resolution	15,000
AGC target	2e5
Maximum IT	25 ms
TopN	20
Isolation window	1.4 *m/z*
Fixed first mass	100 *m/z*
NCE	30
Minimum AGC target	8e3
Charge exclusion	Unassigned, 1, 7, 8, >8
Peptide match	Preferred
Dynamic exclusion	15 s

2.6 Data acquisition and processing

Data were acquired using Thermo Scientific™ Xcalibur™ 4.1 software. The UltiMate 3000 RSLCnano system was controlled using Standard Instrument Integration (SII) 1.4 software. Chromatographic peak characteristics of extracted ion chromatograms (EICs) of peptides from HeLa cell protein digest were evaluated using Thermo Scientific™ Chromeleon™ Chromatography Data System 7.2.9. DDA data for HeLa cell protein digest were processed with Thermo Scientific™ Proteome Discoverer™ 2.2 software using the Sequest™ HT search algorithm. The false discovery rate (FDR) was below 1% at the peptide and protein level.

3. Results and discussion

3.1 Tandem nano-flow LC method explained

An optimized LC method is used for tandem LC operation that enables ~100% MS sample data acquisition (Figure 5). This method contains the following attributes:

- Simultaneous washing of separation column 2 and trap column 2 parallel to gradient elution on separation column 1
- Parallel washing of injection fluidics and sample loop during sample elution to eliminate carryover
- Pre-loading of sample 2 onto the trap column 2, using the “prepare.nextinjection” command
- Transfer of the sample to separation column 2 while sample 1 is undergoing gradient elution in parallel
- The delay in sample peptide elution is removed using post-column flow diversion
- Minimal post column peak dispersion achieved by implementing a 1/32” nano valve with low port to port volume

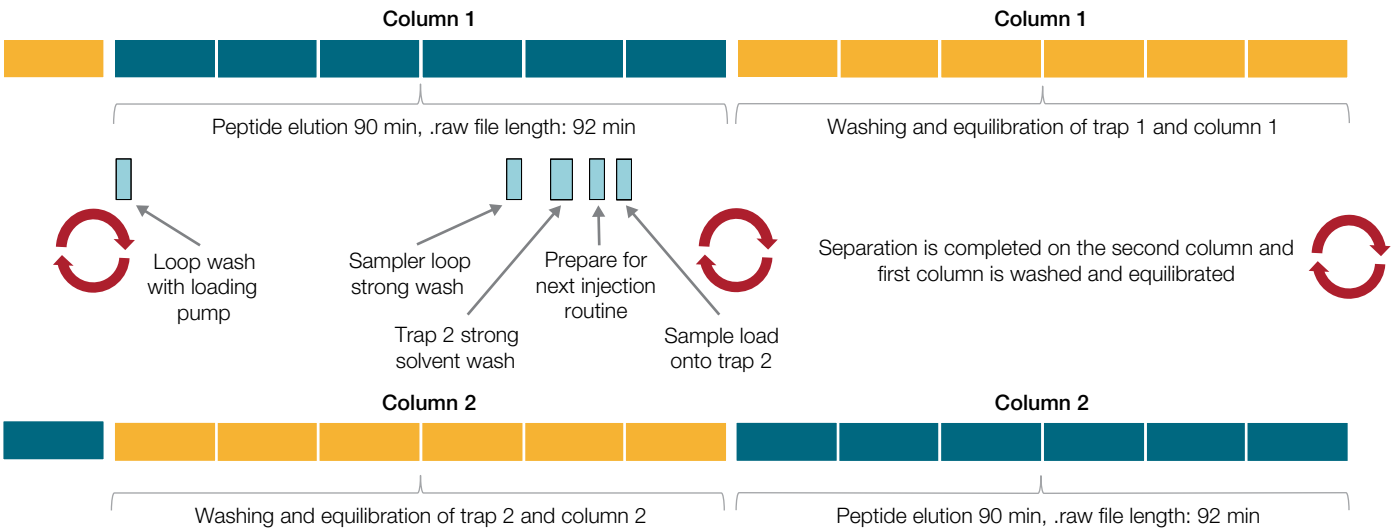


Figure 5. Schematic of the pre-concentration tandem high-throughput low-flow LC method

Example method scripts containing complete LC-MS parameters are available for download from the Thermo Scientific AppsLab Library.[3]

A “start” method is required to enter into the tandem LC cycle. This is used to:

- Equilibrate trap column 1 and analytical column 1
- Inject the first sample and load onto trap column 1
- Switch to analytical column 1
- Start the gradient

For a 50 cm analytical column using a 300 nL/min flow rate a 15-minute method is sufficient to fulfill all of these criteria. An example sequence table showing the initial injection and subsequent tandem cycle is show in Figure 6.

Note: For tandem nanoLC applications run using the WPS-3000FC sampler: a ready check error will occur when attempting to start the sequence if vial positions are not given for the fraction collection “split point” and either the initial tube “tube position” or “tube number” in the e-panel before the sequence is started. Although these parameters are not relevant for tandem nanoLC operation, they are required by the WPS-3000FC. Any vial position can be selected, for example, “RA1” may be inserted as the tube position and “1” as the tube number.

3.2 Continuous MS utilization with tandem LC-MS

Optimized tandem nanoLC-MS methodology using “Prepare.nextInjection” commands enabled reproducible chromatography both intra-column (Figure 7A) and inter-column (Figure 7B) with peptide data elution covering 90 of the 92 minutes of each sample run, equivalent to over 97% MS utility.

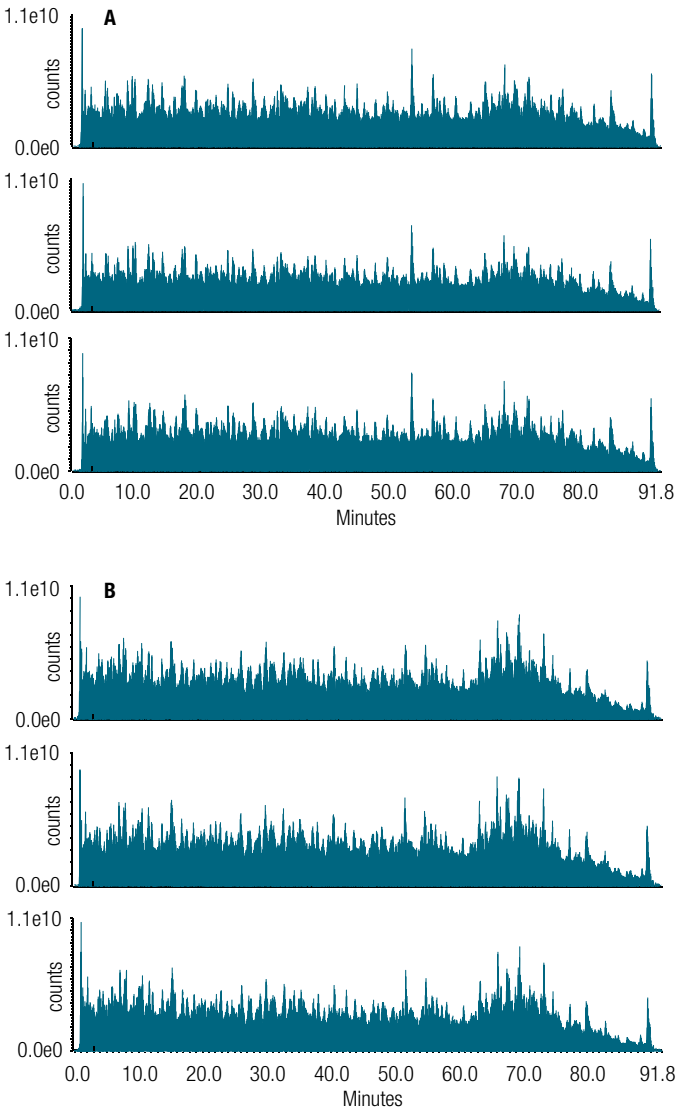


Figure 7. Typical TIC profile of HeLa protein digests for repeat 1 µg injections on (A) trap 1 column 1 and (B) trap column 2, analytical column 2

Retention time variation for individual components between columns is to be expected due to tolerances in fluidic capillary and column I.D.s. Inter-column RT differences ranged from 1.8% to 7.1% for the selected individual components accordingly.

	Sample Type	File Name	Sample ID	Path	Inst Meth	Proc Meth	Position
▶ 1	Unkno...	20181024_Seq_START	1	D:\P	C:\Xcalibur\methods\Tandem_nano_1st_inj_col1_50cm_v7		RA2
2	Unkno...	20181024_HeLa_Col_A_1ug_01	1	D:\P	C:\Xcalibur\methods\Tandem_nano_inj_col1_50cm_v11		RA4
3	Unkno...	20181024_HeLa_Col_B_1ug_01	1	D:\P	C:\Xcalibur\methods\Tandem_nano_inj_col2_50cm_v11		RA4
4	Unkno...	20181024_HeLa_Col_A_1ug_02	1	D:\P	C:\Xcalibur\methods\Tandem_nano_inj_col1_50cm_v11		RA4
5	Unkno...	20181024_HeLa_Col_B_1ug_02	1	D:\P	C:\Xcalibur\methods\Tandem_nano_inj_col2_50cm_v11		RA4
6	Unkno...	20181024_HeLa_Col_A_1ug_03	1	D:\P	C:\Xcalibur\methods\Tandem_nano_inj_col1_50cm_v11		RA4
7	Unkno...	20181024_HeLa_Col_B_1ug_03	1	D:\P	C:\Xcalibur\methods\Tandem_nano_inj_col2_50cm_v11		RA4

Figure 6. Example Sequence table for nano tandem LC

An increase in peak width is also to be expected when comparing tandem to single nanoLC-MS due to the contribution of the 1/32” nano valve to post-column dispersion.

The method is fully adaptable to longer or shorter gradients as required. It should also be noted that there is no restriction on the size of the peptide retention window with respect to the overall analysis time. In the example presented here, a one-minute elution delay was intentionally inserted before sample elution commenced and an extra minute added at the end of the peptide elution window to show the reader that the entire peptide elution envelope was captured during the run (Figure 7).

The amount of carryover was assessed by comparing the peak areas for select HeLa peptides from a 1 µg HeLa injection (Figure 8A) with a blank run (Figure 8B). Carryover was less than 0.2% for all selected peptides.

The consistency in chromatography data (Figure 7) is accompanied by highly consistent MS DDA results reflected in the number of PSMs and peptide and protein groups from run to run (Figure 9) with over 4200 protein groups detected during each run independent of the column.

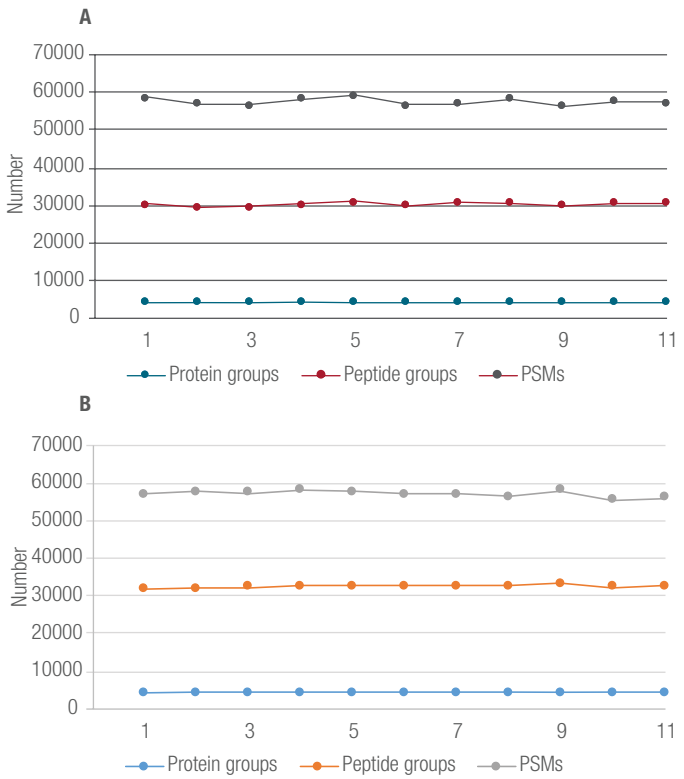


Figure 9. Number of protein groups, peptide groups, and PSMs from 22 × 1 µg HeLa cell digest injections using the 90-minute tandem nanoLC-MS method: (A) DDA results from column 1, (B) DDA results from column 2. Inter-column % RSDs were 2.0, 4.0, and 1.6 for the number of proteins, peptides, and PSMs, respectively.

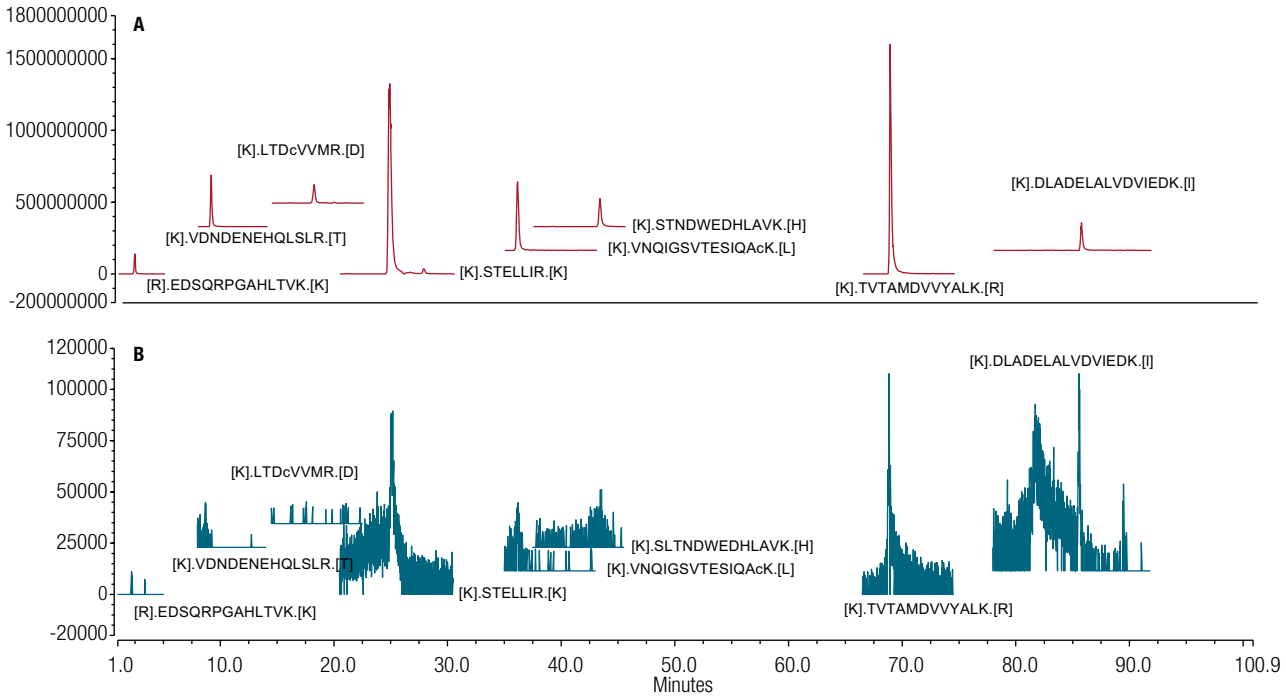


Figure 8. EICs for seven selected HeLa peptides from across the entire chromatographic range for both a 1 µg HeLa injection (A) followed by a blank injection (B)

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4. Conclusions

The versatility of the UltiMate 3000 RSLCnano system provides the flexibility necessary to meet the individual demands of modern proteomics research and beyond. Here we show that with a modest hardware extension to the UltiMate 3000 RSLCnano platform—the addition of a second nano-flow high-pressure gradient pump and 1/32" nano post-column switching valve plus the associated additional fluidics, columns, and column switching valve, all of which are commercially available—a tandem nanoLC stack can be created that enables up to twice the throughput capacity compared to a single nanoLC-MS system. Furthermore, due to post-column switching, contaminants can be diverted away from the MS while use of the "Prepare. nextinjection" command means that MS sample data acquisition time can be extended to 100% of the total analysis run time. The implementation of the tandem workflows also frees up time for extensive washing of the offline trap and analytical column, which helps reduce carryover to negligible values.

The implementation of the tandem nanoLC workflow combined with the proven robustness and class-leading retention time precision of the UltiMate 3000 RSLCnano system with ProFlow technology represents the optimum solution for true productivity in all nanoLC-MS proteomics workflows.

5. References

1. Thermo Scientific UltiMate 3000 RSLCnano System – Versatility and Performance –the Ultimate Solution for All Separation Workflows, BR-71898; 2016.
2. Thermo Scientific UltiMate 3000 RSLCnano Standard Applications Guide v3.0; 2018.
3. LC-MS methods available in AppsLab https://appslab.thermofisher.com/App/4213/tandemnanolcms

Find out more at **thermofisher.com/nanoLCMS**

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