TECHNICAL NOTE 73671

Maximize productivity in proteome profiling

Combining intelligent tandem nanoLC-MS methods and a novel double-barrel ESI source for 100% MS utilization

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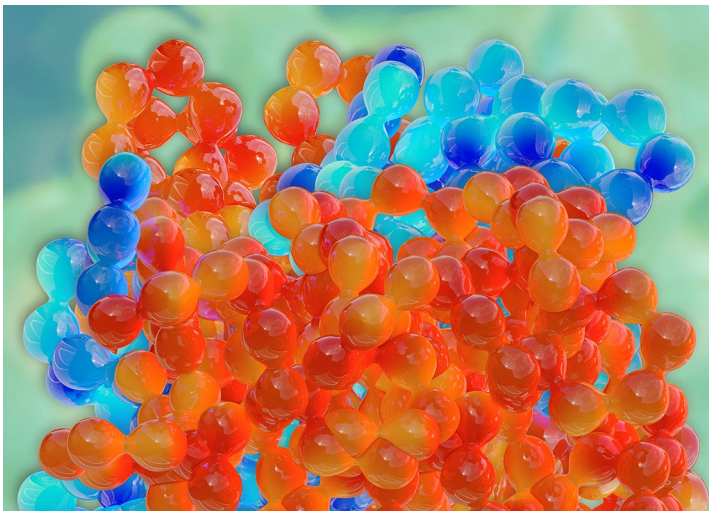
Keywords: Deep-dive proteomics, intelligent tandem nanoLC-MS, Sonation double-barrel column oven, 100% MS utilization, UltiMate 3000 RSLCnano



Goal

Develop a novel, easy-to-use, intelligent tandem nanoLC-MS setup capable of 100% MS utilization that is compatible with the new Sonation double-barrel nano ESI source

1. Introduction

The field of bottom-up proteomics is primarily aimed at identifying and quantifying complete cell or tissue proteomes or even those of entire organisms. NanoLC-MS, employing long columns and long shallow gradients, has long since been recognized as the gold standard for “deep-dive” proteomic workflows due to its sensitivity and separation capacity.

The advantages of resolution and sensitivity brought about by adopting long columns and low flowrates do however come at a price. Such columns have high volume to flow rate ratios, resulting in slow analyte migration times. This, coupled with long washing and equilibration steps, the speed of which is limited by the backpressure generated by the column, means that the productivity, i.e., the time spent collecting sample data, relative to the total analysis time, is relatively low.

One way to drastically increase productivity (otherwise known as MS utilization) is by adopting a tandem nanoLC system. Tandem nanoLC employs a second separation path comprising a pump and analytical column into the system. By offsetting the gradient runs on the two column sets, it is possible to generate an almost continuous feed of sample analytes into the mass spectrometer[1].

Despite its long-term availability, the uptake of tandem nanoLC-MS workflows has been minimal. Detractors from widespread adoption include: (i) complex fluidic and method setup, (ii) challenges in making dead volume free post-column connections, (iii) dispersion effects associated with post-column valves and fluidics, and (iv) variation in LC peak properties between columns, due to even minor variation in gradient delivery by separation pumps.

Here is presented a robust and simple-to-use tandem nanoLC-MS setup comprising a Thermo Scientific™ UltiMate™ 3000 RSLCnano system in tandem nanoLC configuration coupled to a new double-barrel nano ESI source, which is compatible with Thermo Scientific™ mass spectrometers (Figure 1).

This enhanced, easy-to-use setup has the following advantages:

- Absence of post-column fittings and connections eliminates post-column dispersion
- One dedicated pump for gradient delivery on both columns for higher inter-column retention time consistency
- Easy sequence setup resulting from intelligent LC method design, which automatically aligns columns, pumps, and switching valves
- Facile fluidic setup due to Thermo Scientific™ nanoViper™ fingertight fittings
- Full compatibility with direct injection as well as *trap-and-elute* (pre-concentration) types of nanoLC-MS setups

The proof-of-principle data for the new tandem nanoLC-MS setup were obtained by running sequential 90 min gradient separations of 200 ng HeLa protein digest in direct injection mode. The optimized method afforded >73,000 peptides and >6,100 protein group identifications consistently from both columns. Furthermore, MS utilization was increased from 51% to 98%, affording an increase in throughput from around 8 to 14 samples per day.

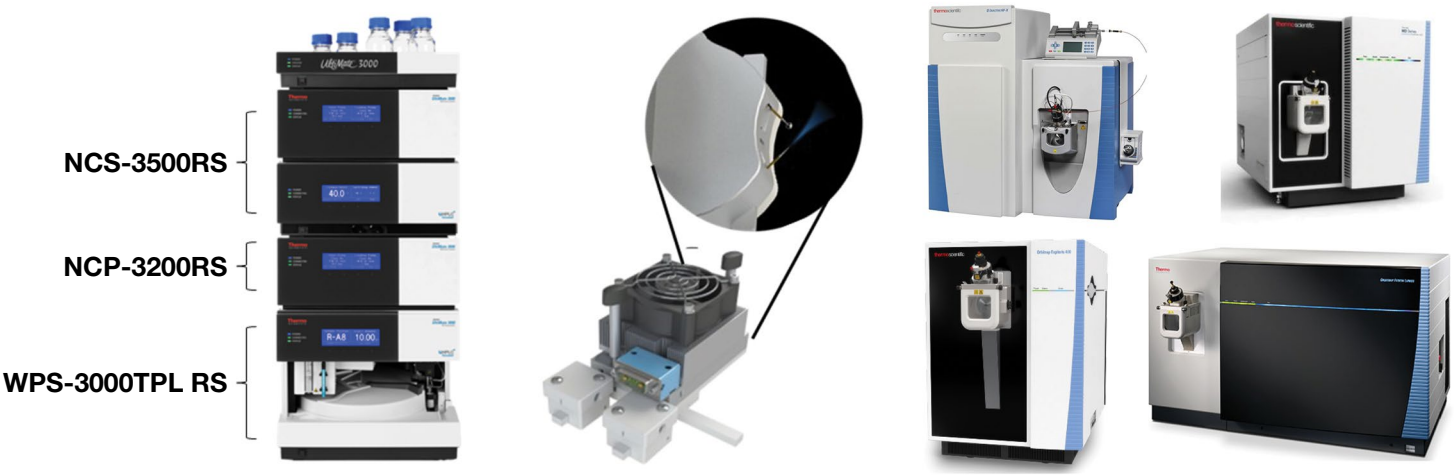


Figure 1. The principal hardware components of the novel tandem nanoLC-MS setup comprise a) UltiMate 3000 RSLCnano in tandem configuration, b) Sonation GmbH Double-Barrel Oven for the Thermo Scientific™ NanoSpray Flex™ ion source, c) Thermo Scientific mass spectrometers

2. Experimental materials and methods

2.1 Sample preparation

Thermo Scientific™ Pierce™ HeLa protein digest (P/N 88328, 20 µg/vial) was reconstituted to a final concentration of 200 ng/µL by dissolving the pellet with 100 µL 0.1% formic acid (FA) in water.

2.2 Consumables

- Fisher Scientific™ LC-MS grade water with 0.1% formic acid (P/N 85171)
- Fisher Scientific™ LC-MS grade 80% acetonitrile with 0.1% formic acid (P/N 15431423)
- Fisher Scientific™ LC-MS grade formic acid (FA) (P/N 10596814)
- Fisher Scientific™ LC-MS grade isopropanol (P/N 10684355)
- Thermo Scientific™ Pierce™ trifluoroacetic acid (TFA), LC-MS grade, (P/N 85183)
- Fluidics and columns used to setup the tandem nanoLC system are given in Table 1 and Figure 2 for direct injection mode and Table 2 and Figure 3 for trap-and-elute mode.

2.3 Tandem nanoLC hardware and fluidic configurations

The UltiMate 3000 RSLCnano system for tandem nanoLC-MS comprises the NCS-3500RS Binary Rapid Separation Nano/Capillary Pumps, NCP-3200RS Binary Rapid Separation Nano/Capillary ProFlow Pumps, and WPS-3000TPL RS Rapid Separation Pulled-Loop Nano/Capillary Thermostatted Well Plate Autosampler modules (Figure 1a). Either one or two, 10-port 2-position switching valves installed in the column compartment are required, depending on whether the system is being operated in direct injection (Table 1 and Figure 2) or trap-and-elute mode (Table 2 and Figure 3).

Table 1. Fluidics and accessories for tandem nanoLC-MS operation in direct injection mode

	Part number	Description	#
A	6041.5240	Thermo Scientific™ nanoViper™ capillary, 20 µm × 350 mm	1
B	6041.5121	Thermo Scientific™ nanoViper™ capillary, 20 µm × 150 mm	2
C	6041.5260	Thermo Scientific™ nanoViper™ capillary, 20 µm × 550 mm	1
D	6041.5275	Thermo Scientific™ nanoViper™ capillary, 20 µm × 650 mm	1
E*	N/A	75 µm × 40 cm C18, 1.9 µm self-packed column with pulled emitter, Evotec GmbH	2
F	6826.2410	Thermo Scientific™ nanoViper™ sample loop 10 µL	1
G	6040.2303	Thermo Scientific™ Viper™ plug	2
H**	6041.5293	Thermo Scientific™ nanoViper™ column to MS tubing i.d. × o.d. × l, 20 µm × 360 µm × 1 m	2
Other components	6041.0001A	10-port 2 position switching valve	1
	6820.0027	Polypropylene vials 250 µL with glass insert	1
	6820.0028	Polypropylene caps for WPS vials	1
	6820.0023	10 mL vials (headspace) with crimp caps and septum (for transport liquid)	1

The letter designations refer to Figure 2.
*Consumables are all from Thermo Fisher Scientific except for item "E". These self-packed columns with pulled emitter were packed by Evotec GmbH, München.
**This one-sided nanoViper capillary was cut to 55 cm in length.

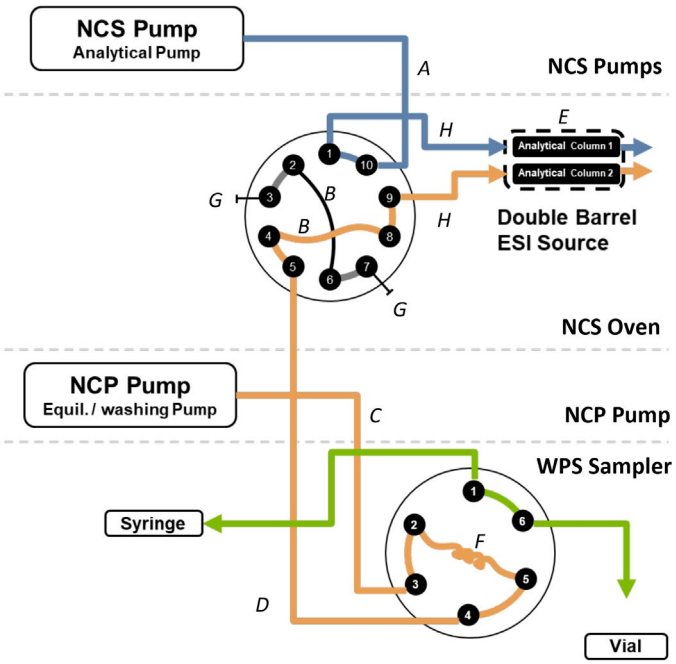


Figure 2. Fluidic configuration scheme for the tandem nanoLC in direct injection mode (letter description are given in Table 1).

Table 2. Fluidics and accessories for tandem nanoLC-MS operation in trap-and-elute mode

	Part number	Description	#
A	6041.5240	Thermo Scientific™ nanoViper™ capillary, 20 µm × 350 mm	4
B	6041.5121	Thermo Scientific™ nanoViper™ capillary, 20 µm × 150 mm	1
C	6041.5780	Thermo Scientific™ nanoViper™ capillary, 75 µm × 750 mm	1
D	6041.5775	Thermo Scientific™ nanoViper™ capillary, 75 µm × 650 mm	1
E*	N/A	75 µm x 40 cm C18, 1.9 µm self packed emitter column, Evotec GmbH	2
F	160454	300 µm i.d. x 5 mm cartridge packed with Acclaim PepMap 100 C18, 5 µm (set of 5)	2
	164649	µ-Precolumn holder, 5 mm with 30 µm i.d. connecting tubing, Thermo Scientific™ nanoViper™ fingertight fittings	2
G	6826.2420	Thermo Scientific™ nanoViper™ sample loop 20 µL	1
H**	6720.0077	PTFE tubing, 500 µm i.d., 100 cm, used as waste tubing	1
	6720.0015	1/16" Universal Fingertight Fitting, one-piece design, extra-long thread (4 pieces)	1
I**	6041.5293	Thermo Scientific™ nanoViper™ column to MS tubing i.d. x o.d. x l, 20 µm x 360 µm x 1 m	2
Other Components	6041.0001A	10-port 2 position switching valve	2
	6820.0027	Polypropylene vials 250 µL with glass insert	2
	6820.0028	Polypropylene caps for WPS vials	2
	6820.0023	10 mL vials (headspace) with crimp caps and septum (for transport liquid)	1

The letter designations refer to Figure 3.
*Consumables are all from Thermo Fisher Scientific except for item "E". These self-packed columns with pulled emitter were packed by Evotec GmbH, München.
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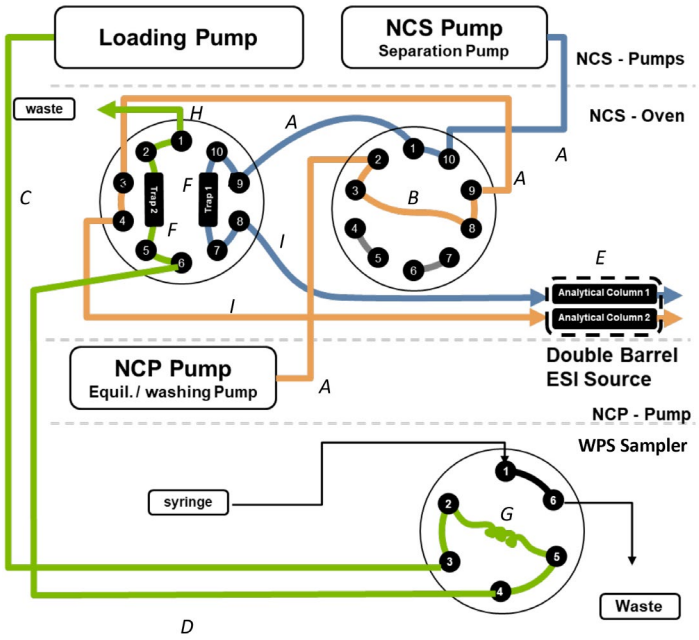

Figure 3. Fluidic configuration scheme for the tandem nanoLC in trap-and-elute mode (letter descriptions are given in Table 2).

2.4 Installing and connecting the Sonation double-barrel ESI source

The Sonation double-barrel column oven (P/N DBO-TF-FIS) was fitted onto the Nanospray Flex ion source (P/N ES071) using the corresponding source conversion kit (P/N PRSO-V2-KES71) according to the manufacturers instructions[2]. The double-barrel column oven is also fully compatible with Nanospray Flex NG ion sources (P/N ES072). In this case, the conversion kit for the Nanospray Flex NG ion source is required (P/N PRSO-V2-KES72) along with the column oven (P/N DBO-TF-FIS). Two 75 µm i.d. x 40 cm self-packed columns with pulled emitter were installed in the source according to the manufacture instructions. The interface between the LC and the columns was made using 2 x UHPLC liquid junction kits (P/N ES269). The source was then connected to a Thermo Scientific™ Q Exactive™ HF-X mass spectrometer as shown in Figure 4.

Sonation column oven control software (COControl 3.4) was installed onto the PC according to the manufacturer instructions[2]. The high voltage switch was configured and connected as described in the user manual. A contact closure cable P/N 6000.1004 was connected between Port 3 of the WPS-3000TPL RS autosampler and the HV switch of the source. Intelligent commands included in the script ensure that the valve positions and the high voltage are synchronized such that the packed emitter column running the gradient is always that to which the high voltage is applied (Figure 5).

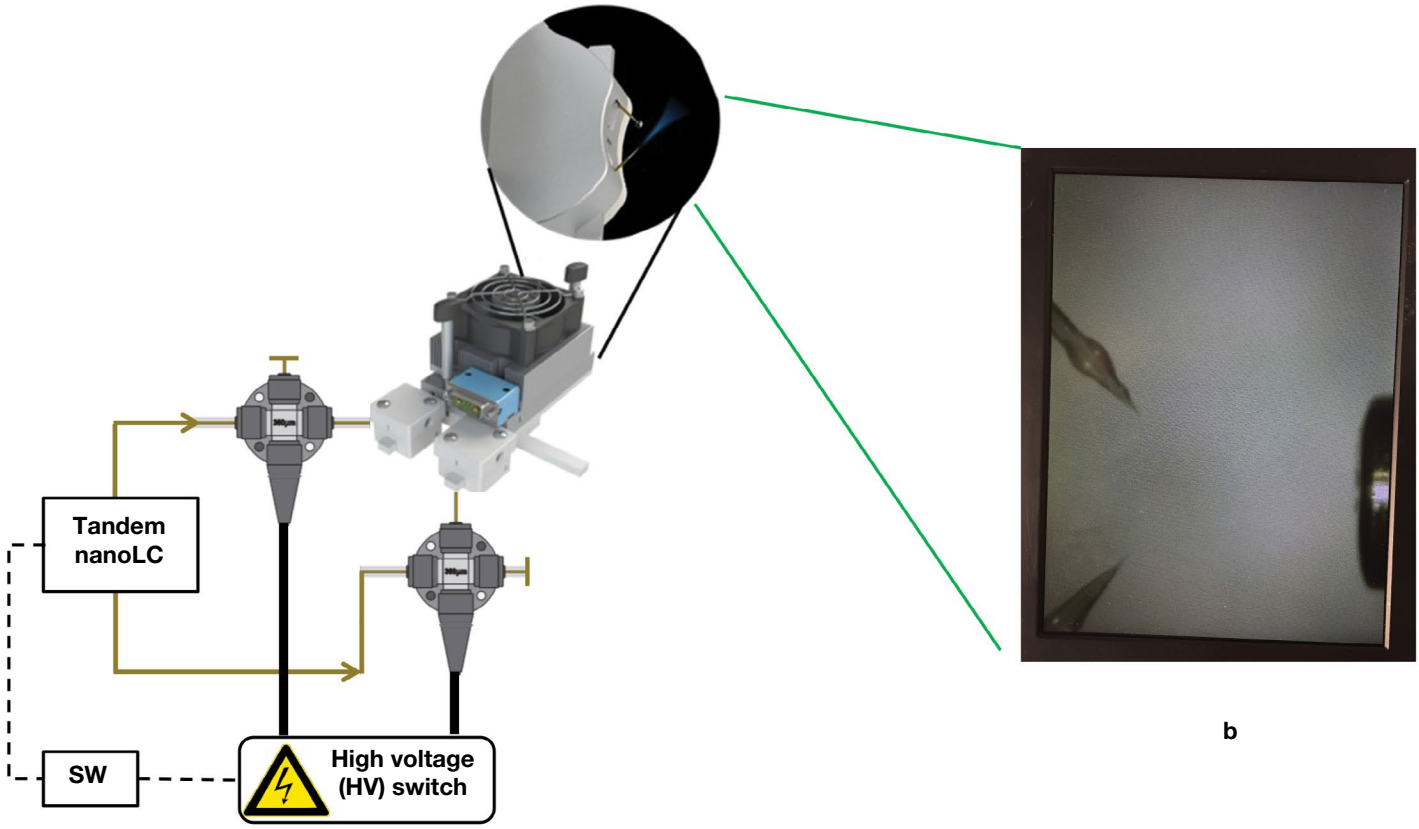

Figure 4. The Sonation double-barrel ion source mounted onto the tray of the Nanospray Flex ion source is connected to the LC system via two UHPLC liquid junction kits which are also connected to a high voltage switch (a). The voltage is switched between the two liquid junction interfaces (b) using contact closure cable controlled by LC.

	Time	Command	Value	Comment
50	0.000			
51	If		ColumnOven.ValveRight=10_1	
52		Sampler.Relay_3.State	On	
53	Else			
54		Sampler.Relay_3.State	Off	
55	End If			

Figure 5. Intelligent commands inserted into the LC script ensure that voltage is applied to the column that is running the gradient. The use of the "If-Else" clause links the column oven valve, which diverts the gradient between columns, to the relay state, which controls the source HV switch.

2.5 Tandem nanoLC solvent conditions

The solvents used to run this application are given in Table 3.

2.6 LC module configuration

The tandem nanoLC modules were configured using the "Chromeleon Instrument Configuration Manager" panel of Standard Instrument Integration (SII) for Thermo Scientifc™ Xcalibur™ software. Standard settings were used for configuring the NCS-3500RS and WPS-3000TPL RS modules. The NCP-3200RS pump is configured by inserting the digit "2" both after the module name and the word "pump" after each of the activated channels. It is important to configure NCP-3200RS pump according to these instructions to match the configuration designated in the optimized method templates available for download from AppsLab.

2.7 MS acquisition parameters

MS data were recorded with Q Exactive HF-X mass spectrometer. The MS tune and data acquisition parameters are shown in Tables 4 and Tables 5 and are available for download in AppsLab.

Table 3. Solvents and instrument conditions for tandem nanoLCMS application in direct injection or trap-and-elute mode

Module	Property	Setting
NCS-3500RS pump	Mobile phase A	H_2O with 0.1% FA (pH ~3.0)
	Mobile phase B	80/20 (v/v) ACN / H_2O with 0.1% FA (pH ~3.0)
Loading pump (trap-and-elute-mode only)	Loading buffer channel A	H_2O with 0.05% TFA (pH ~1.0)
	Loading buffer channel B	ACN with 0.05% TFA (pH ~1.0)
NCP-3200RS pump	Mobile phase A	H_2O with 0.1% FA (pH ~3.0)
	Mobile phase B	80/20 (v/v) ACN / H_2O with 0.1% FA (pH ~3.0)
NCS-3500RS; NCP-3200RS; Loading pump	Rear seal washing buffer	90/10 (v/v) H_2O / isopropanol with 0.1% FA
WPS-3000RS	Sampler washing buffer	100% ACN with 0.1% FA
WPS-3000RS	Temperature control	5 °C
Column oven		60 °C
Dual source		60 °C

FA= Formic acid, TFA = Trifluoroacetic acid, ACN = Acetonitrile

Table 4. MS tune settings

Parameters / Components	Settings / Details
Source settings	
Polarity	Positive
Ion transfer tube temperature	275 °C
Spray voltage positive ion	1.9 kV
Ion funnel RF level	40

Table 5. MS data acquisition parameters

Parameters / Components	Settings / Details
MS1 Resolution	60,000
AGC target	3e6
Maximum IT	50 ms
Scan range	375–1500 *m/z*
DDA	
MS2 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

2.8 Data acquisition and processing

Data were acquired using the Xcalibur software package. The Tandem UltiMate 3000 RSLCnano system was controlled using SII for Xcalibur software. Acquired in data-dependent acquisition (DDA) mode data for HeLa protein digest were processed with Thermo Scientific™ Proteome Discoverer™ 2.4 software using the spectral library search and the Thermo Scientific™ Sequest™ HT search algorithm. The false discovery rate (FDR) was set to 1% at the peptide and the protein level. Protein abundances in multiple injections were normalized to "Total Peptide Amount" and then processed with R script[3].

3. Results and discussion

3.1 Tandem nanoLC-MS methods explained

Fully optimized tandem nanoLC method templates have been created for direct injection and trap-and-elute modes for different gradient lengths, each affording ~100% MS sample data acquisition. In addition to the benefits associated with conventional tandem nanoLC setups as described in reference 1, these novel methods confer the following extra attributes:

- Comprise a single intelligent LC method that automatically aligns the active column to the ESI voltage supply
- Easy sample sequence setup due to automated alignment of sample and active column
- Assignment of one nanoLC analytical pump for sample separation on both columns, thereby reducing inter-column retention time variability

A comparison of a conventional nanoLC method and a tandem nanoLC method is shown in Figure 6. A typical method can be considered to comprise three main stages: (i) injection and sample transfer, (ii) sample separation, and (iii) washing and equilibration.

In conventional nanoLC-MS setups, these three stages have to be executed sequentially (Figure 6a). The introduction of a second pump permits these operations to be synchronized between columns and executed in parallel (Figure 6b). As soon as the last peptide has been eluted from column 1, it is switched offline, while column 2, which by this time has been loaded with new sample, is switched online. After this, column 1 is washed, equilibrated, and loaded with new sample, while the sample on column 2 is being eluted and detected with MS. The cycle then repeats, alternating between sample elution on one column, and washing, equilibration and sample loading on the second column.

This allows all redundant MS time to be virtually eliminated from the LC-MS run (compare Figure 7a and Figure 7b), corresponding to an increase in productivity of more than 80%. This equates to an increase from approximately eight samples per day (Figure 7a) to around 14 samples per day (Figure 7b) for the example analysis employing 90 min gradients in Direct Injection mode.

3.2 Superior run-to-run reproducibility afforded by single gradient pump

Comparison of triplicate sample analysis on alternate columns revealed very reproducible peptide profiles and signal intensities between columns and also low retention time variation, as little as 0.2% for the example peptide shown in Figure 8.

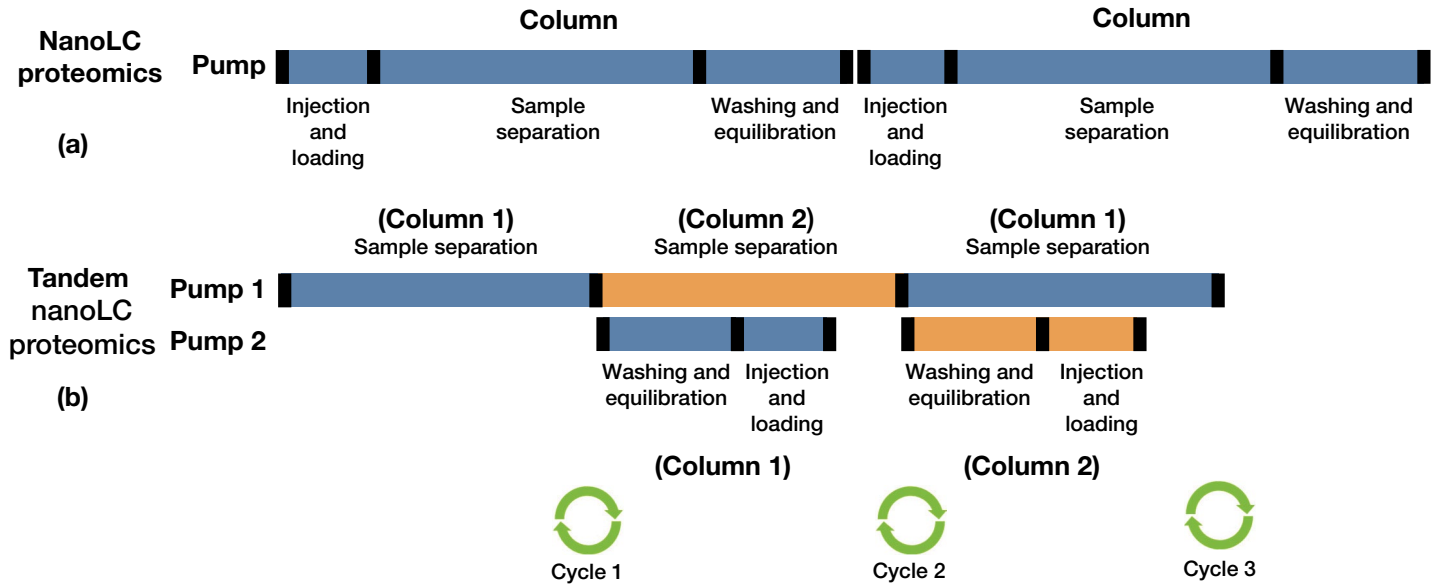

Figure 6. Schematic depicting a conventional nanoLC method (a) with a tandem nanoLC method (b)

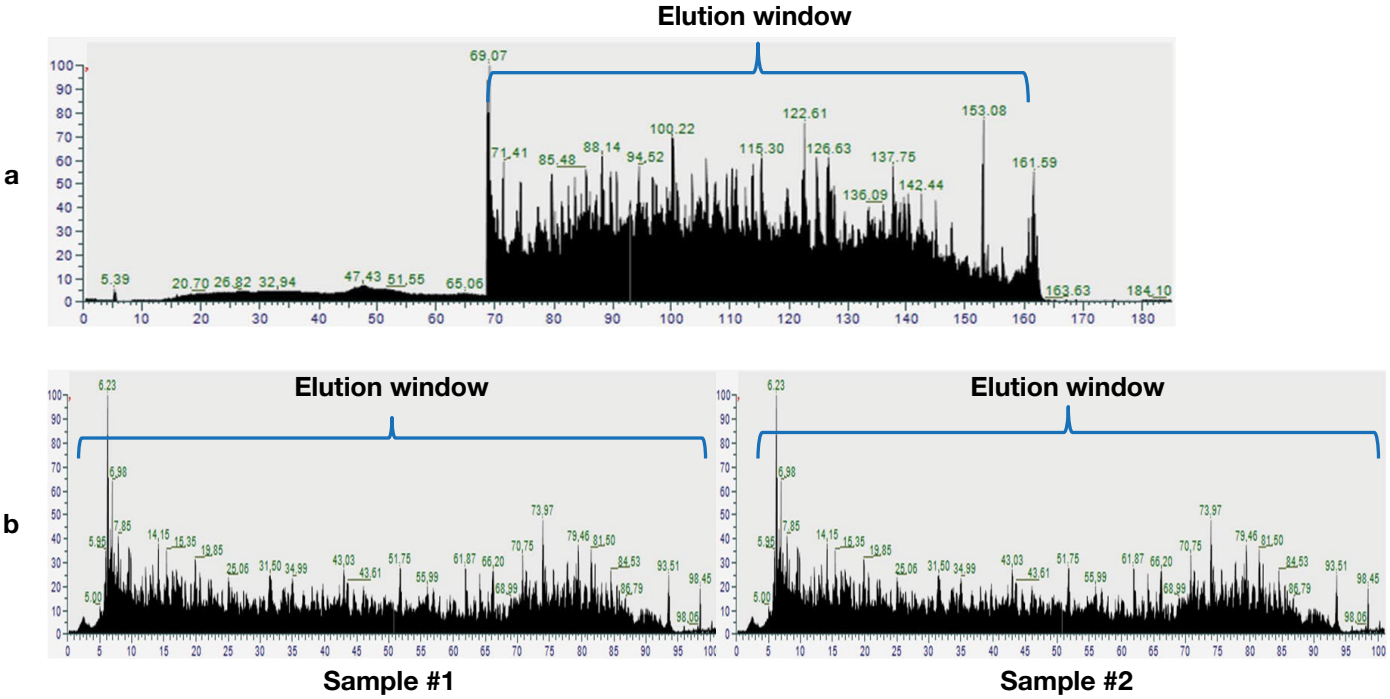

Figure 7. Typical nanoLC-MS analysis of 200 ng HeLa protein digest on a 75 µm × 40 cm column (a) compared to the results with tandem nanoLC-MS (b)

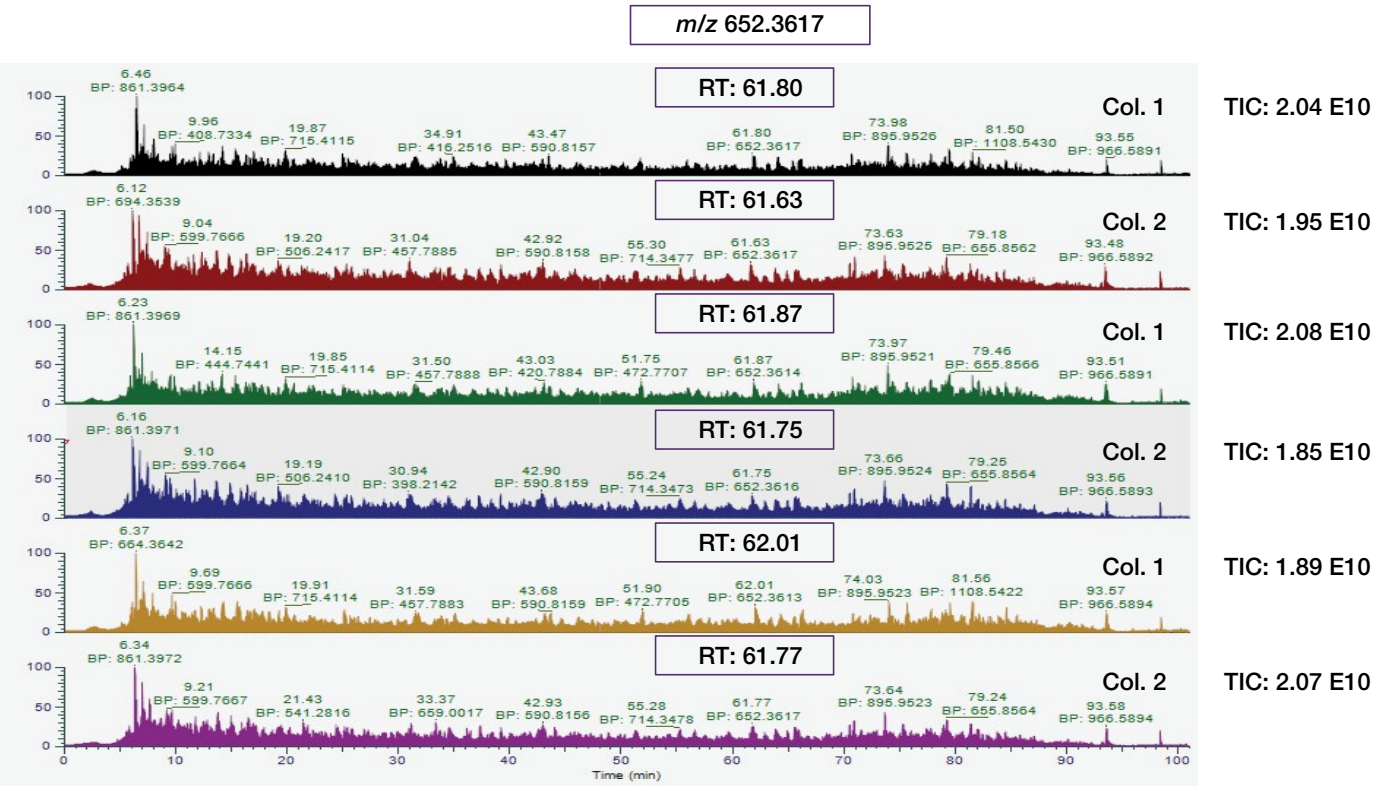

Figure 8. TIC from three consecutive runs on column 1 and column 2

3.3 Consistent data quality for the same sample measured on alternate columns

The analysis of 200 ng HeLa protein digest samples using a 90 min gradient resulted in over 73K peptide identifications. Approximately 90% of them are common across all runs (Figure 9a), 99% of which had less than 1 min retention times difference (Figure 9b).

3.4 Reproducible protein identification and quantification values

Reproducible inter-column chromatography corresponds to reproducible protein identification and quantification values. This is turn resulted in over 6,100 identified proteins with an approximate 98% overlap, irrespective of the column (Figure 10a). 93% of the 5,700 quantified proteins showed protein abundance variation of less than 25% (Figure 10b).

3.5 Template methods for direct injection and trap-and-elute applications

Eight standardized template methods applicable to column lengths ranging from 15 to 50 cm and yielding elution windows from 24 to 120 minutes were created. Each template method has a Quick Start method to initiate the tandem cycles. These templates are available for download from AppsLab.

3.6 Customization options for template methods

The method templates allow key stages of LC run to be tailored according to individual needs, e.g., adjusting gradient slope, column washing, and equilibration duration and solvent compositions.

Examples of method variants include:

- Saw-tooth column washing regimes to reduce carryover (Figure 11a)
- Adjustment of the column equilibration time (Figure 11b and c)
- Gradient optimization according to the sample type (Figure 11 d, e, and f)

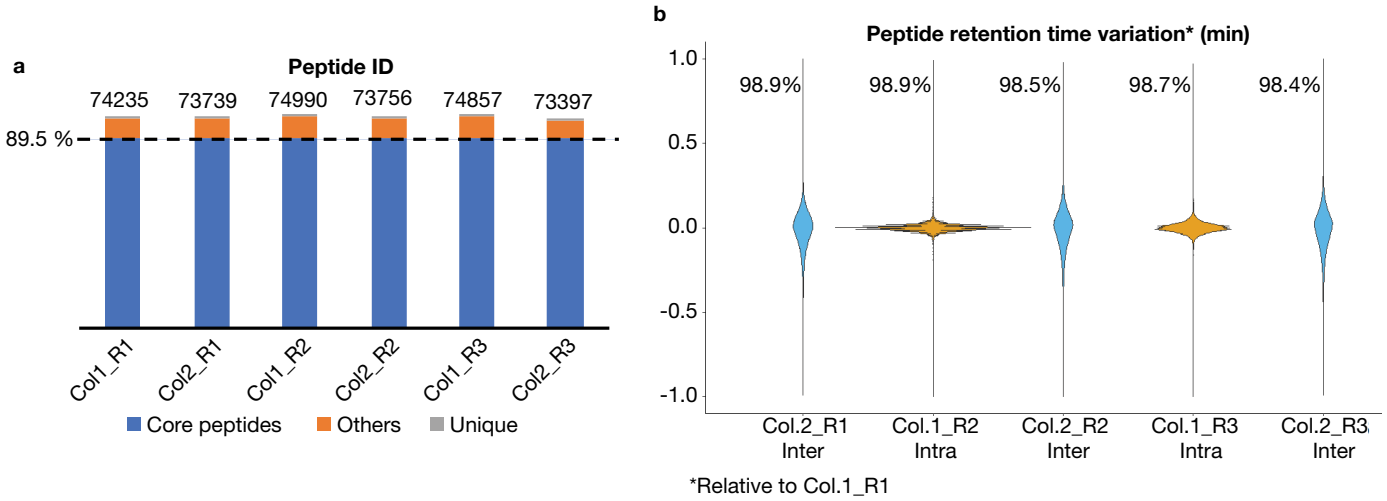

Figure 9. Consistent peptide identifications (a) and peptide elution (b) achieved both inter- and intra-columns

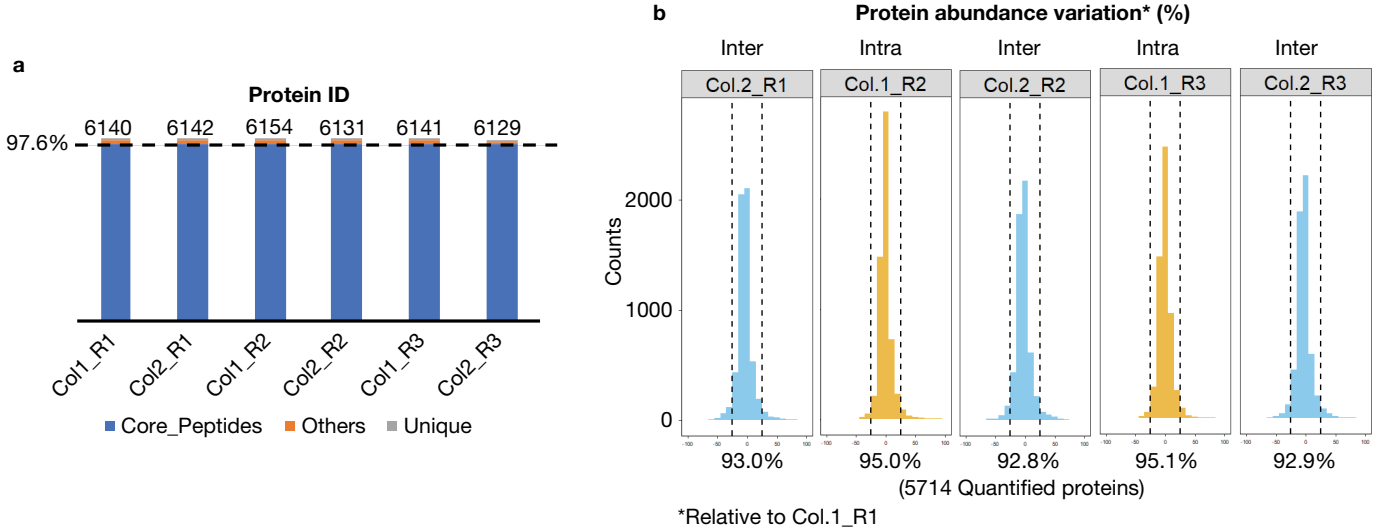

Figure 10. Reproducible protein identification (a) and quantification (b) inter- and intra- column

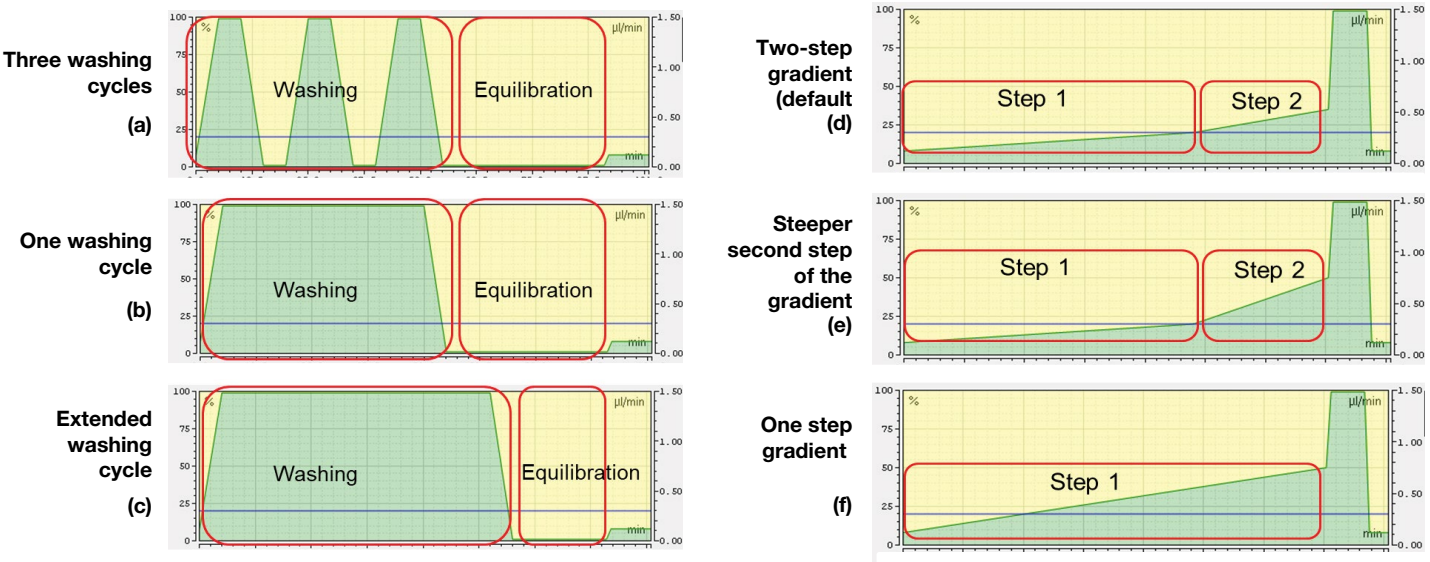

Figure 11. Possible template method customization options include type (a) and length (b,c) of column washing and equilibration steps and length and number of gradient steps (d,e,f)

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4. Conclusions

A novel tandem nanoLC-MS solution has been developed that is capable of achieving near 100% MS utilization for proteomic sample analysis for both direct injection and trap-and-elute (pre-concentration) modes.

The combination of new fluidics setups, intelligent method design, and a novel dual nano ESI source makes this robust workflow capable of producing high-quality data.

Several template methods, available for download, to support a wide range of tandem nanoLC-MS applications have been established.

References

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