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Liquid chromatography

ZebraWash: An innovative approach in the Vanquish Neo UHPLC system to reduce trap column carryover

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Keywords

ZebraWash, bottom-up proteomics, trap-and-elute, carryover, HeLa protein digest, Orbitrap mass spectrometry

Goal

Demonstrate the superior performance of the ZebraWash procedure in Thermo Scientific™ Vanquish™ Neo UHPLC systems for rapid and effective reduction of the trap column carryover in the trap-and-elute workflow for low-flow LC-MS applications

Introduction

Two main workflows are used in low-flow LC-MS proteomics experiments: (i) direct injection of the sample onto the separation column and (ii) trap-and-elute injection when the sample is first injected onto the shorter trap column and then the separation gradient is delivered through the trap and separation column. The limitation of the direct injection workflow in low-flow LC analysis is the long loading times that are a result of the requirement to load relatively large sample volumes (several microliters) onto the very narrow (75 and 150 µm internal diameter) and often long (15–75 cm) columns. These nano columns generate high backpressure even at low nano-flow rates (typically below 1,000 nL/min). The overhead time increases significantly if the liquid chromatography (LC) system cannot accelerate sample loading with elevated pressure capabilities due to LC hardware limitations. The trap-and-elute workflow allows substantial acceleration of the sample loading. In this case, the sample is loaded onto the short trap column (5–20 mm) with the same or slightly larger internal diameter than the separation column in higher flow rates. As a result, the sample loading onto the trap column takes seconds rather than minutes as required for the direct injection setup. An additional advantage of loading onto the trap column is sample desalting, which reduces the contamination of the electrospray ionization interface (ESI).

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The trap and elute workflows can be used for any flow range from nano- to capillary- and micro-flow (1 nL/min–100 µL/min), but the most significant improvements are observed for nanoLC separations. The trap column set-up can be operated in forward or backward flush elution mode. In forward flush mode, the analytes are eluted in the same direction as loaded onto the trap column. Forward flush is recommended when samples contain insoluble particulates, e.g., from sample preparation routines. The trap column protects the separation column in this mode, behaving similarly to a guard column by accumulating insoluble particles and hydrophobic contaminants. In backward flush mode, after analytes are loaded onto the trap column, they are eluted by reversing the flow direction. This usually results in sharper chromatographic peaks in comparison with forward flush elution. The switch between these two setups usually requires replumbing of fluidics. However, in the Vanquish Neo UHPLC system, the desired mode is selected during the method set up by simply checking the box in the method editor.

While adding the trap column to the flow path has many advantages, it also has some drawbacks that should be considered. Sample loading onto the short trap column might result in losses of very hydrophilic peptides if the loading volume or loading buffer are not optimized. Adding fluidic lines and a column with different inner dimensions and lengths might lead to increased carryover if washing is not done properly. Carryover—resulting from sample overloading, insufficient washing, nonspecific sample binding, or column chemistry—can prevent having consistent results for each injection. In the past, to reduce the carryover on the trap column and injection fluidics, users ran matrix blanks between sample runs or manually created special washing procedures using an external pump. This action reduced the sample throughput and increased the method's complexity.

Thus, a fully automated, optimized, and easy-to-use solution to remove carryover caused by the trap column while maintaining sample throughput and confidence in results is required. Here, we developed and introduced an innovative ZebraWash procedure for rapid and effective reduction of the trap column carryover in trap-and-elute workflows.

Experimental

Sample preparation

Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard (P/N A47996, 10 µg/vial) was reconstituted by adding 50 µL of 0.1% formic acid (FA) in water. Subsequently, the vial was sonicated for 2 min, followed by multiple cycles of sample aspiration and release with a pipette to dissolve it completely. The final sample concentration was 200 ng/µL HeLa digest with 100 fmol/µL PRTC.

Consumables

- Fisher Scientific™ LC-MS grade water with 0.1% formic acid (P/N LS118-500)
- Fisher Scientific™ Optima™ LC-MS grade 80% acetonitrile with 0.1% formic acid (P/N LS122-500)
- Fisher Scientific™ Optima™ LC-MS grade 100% acetonitrile with 0.1% formic acid (P/N LS120-212)
- Fisher Scientific™ Optima™ LC-MS grade formic acid (P/N 10596814)
- Fisher Scientific™ Optima™ LC-MS grade isopropanol (P/N 10684355)

Fluidics and consumables used to set up the Vanquish Neo UHPLC system for trap-and-elute injections are given in Table 1.

Table 1. Vanquish Neo UHPLC system, fluidics, and accessories for trap-and-elute workflow

Part number	Description	#
VN-S10-A-01	Vanquish Neo UHPLC System • Binary Pump N, Split Sampler NT, Solvent Rack, Vanquish System Controller, and System Base with drawer	1
6036.1180	Vanquish Display	1
6PK1655	Vial and septa kit, 100/pack of: • Vial 0.2 mL amber TPX screw 9 mm short thread conical glass insert • Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	1
ES75500PN	Thermo Scientific™ EASY-Spray™ PepMap™ Neo UHPLC column, 75 µm × 500 mm, 2 µm, 1,500 bar	1
164535	Thermo Scientific™ Acclaim™ PepMap™ C18 column, 3 µm, 75 µm × 150 mm, 20 mm packing bed	1
6252.1950	Vanquish Split Sampler Sample Loop, 100 µL	1

LC solvents and system temperature settings

The recommended solvents in the trap-and-elute workflow are described in Table 2.

Table 2. Solvents for instrument operation

	Solvent	Composition
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
Split Sampler NT 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
Split Sampler NT Wash Port	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	100% ACN with 0.1% FA
Binary Pump N and Split Sampler NT	Rear seal wash buffer	25/75 (v/v) H_2O / IPA* with 0.1% FA

*FA = formic acid, ACN = acetonitrile, IPA = isopropanol

Vanquish Neo UHPLC method parameters

The generic parameters for sample aspiration, loading, and column equilibration are shown in Table 3. The typical nanoLC-MS method with a 60 min gradient is described in Table 4.

Table 3. Generic LC parameters for the nanoLC-MS method

	Parameter	Value
Sample loading	Mode	PressureControl
	Pressure	800 bar
	Loading volume*	Automatic (5 µL)
Sample pick–up*	Outer needle wash mode	After draw
	Outer needle wash time (Strong)	3.0 s
	Outer needle wash speed (Strong)	80.0 µL/s
	Outer needle wash time (Weak)	5.0 s
	Outer needle wash speed (Weak)	80.0 µL/s
	Draw speed	0.2 µL/s
	Draw delay	2.0 s
	Dispense speed	5.0 µL/s
	Vial bottom detection	Enabled
Column equilibration	Fast equilibration	Enabled
	Mode	PressureControl
	Pressure	1,500 bar
	Equilibration factor	3
Trap column	Fast wash and equilibration	Enabled
	ZebraWash**	Disable / Enabled
	Fast Wash factor / ZebraWash cycles**	Factor 27 / 2, 4, 8, 16 cycles
	Equilibration factor*	Automatic (2)
	Mode	PressureControl
	Pressure	800 bar
	Trap flush direction	Forward flush
Temperature	EASY-Spray column temperature	50 °C
	Autosampler temperature*	7 °C
	Trap column	Room temperature (~23 °C)

*System default value

** Fast Wash factor is optional only when ZebraWash is disabled and vice versa.

Table 4. LC gradients for 75 µm × 500 mm column in trap-and-elute workflow

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0	0	0.3	1
0.1	0.1	0.3	6
40.1	40	0.3	20
60.1	20	0.3	35
61.1	1	0.3	50
Column wash phase			
62.1	1	0.3	99
70	7.9	0.3	99

MS acquisition parameters

MS data were acquired with a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer in data-dependent acquisition (DDA) mode with MS parameters described in Table 5.

Data processing and analysis

Acquired .raw files of HeLa sample were processed with Thermo Scientific™ Proteome Discoverer™ 2.5 software using a 2-step SEQUEST™ HT search algorithm and INFERYS rescoring node. The false discovery rate (FDR) was set below 1% at both the peptide and the protein level. Further data analysis and plotting were performed with R script.

Table 5. MS acquisition parameters for DDA methods

Category	Property	Setting
Method settings	Application mode	Peptide
	Method duration (min)	70
Ion source	Positive ion (V)	1,900
	Ion transfer tube temp. (°C)	275
MS global settings	Infusion mode	Liquid chromatography
	Expected LC peak width (s)	15
	Advanced peak determination	TRUE
	Default charge state	2
	Internal mass calibration	Off
Full scan	Scan range (*m/z*)	375–1,200
	AGC target	300
	RF lens (%)	45
	Data type	Profile
	Polarity	Positive
	Source fragmentation	Disabled
	Orbitrap resolution	60,000
	Maximum injection time (ms)	Auto
MIPS	Monoisotopic peak determination	Peptide
	Relax restrictions when too few precursors are found	TRUE
Intensity	Intensity threshold	1E4
Charge state	Include charge state(s)	2–5
	Include undetermined charge states	FALSE
Dynamic exclusion	Perform dependent scan on single charge state per precursor only	TRUE
	Exclusion duration (s)	30
ddMS2	Multiplex ions	FALSE
	Isolation window (*m/z*)	2
	Collision energy type	Normalized
	HCD collision energy (%)	26
	Scan range mode	Define first mass
	First mass (*m/z*)	120
	Normalized AGC target (%)	50
	Data type	Centroid
	Number of dependent scans	25
	Orbitrap resolution	15,000
	Maximum injection time (ms)	Auto

Results and discussion

Working principle of ZebraWash

The automated ZebraWash wash procedure was developed to reduce the carryover with samples containing strongly adsorbing analytes or contaminants. Alternating strong and weak wash liquids are drawn into the needle and sample loop from the wash port during the washing process. After that, this solvent plug is pushed over the trap column at a pre-set pressure limit. The alternation between the strong and weak wash solutions facilitates the removal of even strongly bound contaminants rom the trap column and reduces carryover on the fluidics and trap column.

Each switch between the strong and weak solvent is defined as one ZebraWash cycle. It consists of 3 µL strong wash liquid and 3 µL of weak wash liquid. The number of cycles can be adjusted in the instrument method editor. The integrated system intelligence automatically controls the entire washing procedure. No further user interaction is needed. A maximum number of wash cycles of 2, 4, and 16 are allowed for 10, 25, and 100 µL sample loops, respectively. When all washing cycles are completed, the trap column and fluidics are equilibrated with weak wash liquid delivered from the metering device.

We used a 100 µL sample loop to compare ZebraWash with the standard Fast Wash procedure and to assess how the increased number of ZebraWash cycles affects carryover levels. It should be noted that the sample loop volume does not contribute to either gradient delays or overhead time in the trap-and-elute workflow. Additionally, the composition of the wash liquids can be adjusted to achieve more comprehensive washing if applications require it. It gives the flexibility to customize the washing strength and remove contaminants from the trap column by using different proportions of acetonitrile, methanol, isopropanol, water, etc.

Decoupling the LC fluidics, trap column, and separation column carryover

The overall carryover in the LC-MS analysis combines contributions from the fluidics, trap column, and separation column. In the standard trap-and-elute workflow in the Vanquish Neo UHPLC system, the washing of the trap column is completed independently from the washing of the separation column. The system automatically switches the trap column offline to wash and re-equilibrate it using the metering device at the end of the gradient separation while the separation column is washed with the separation pump. As ZebraWash minimizes the carryover on the trap column, we developed an experimental procedure to decouple the carryover of the separation and trap columns.

The standard nanoLC-MS run was followed by two consecutive gradients through the separation column, while the trap column was washed with the ZebraWash procedure. As the fluidics were not switched and the trap column stayed offline, the blank injection afterward was used to measure the carryover that originated from the trap column and injection fluidics.

The ZebraWash procedure was tested with 16 cycles (maximum supported number of cycles with a 100 µL loop) and achieved an ultra-low level of carryover (< 0.004%) on the trap column independent of the injection amount (Figure 1). In total, ZebraWash pushed 48 µL of strong wash liquid and 48 µL of weak wash liquid through the trap column during the washing procedure.

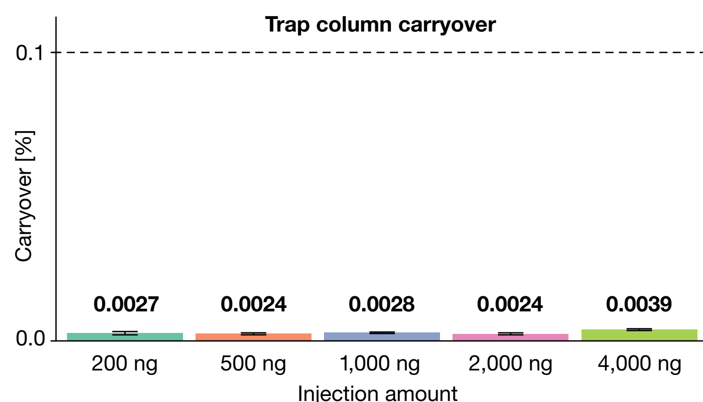

Figure 1. Trap column carryover after injection of HeLa protein digest with amounts from 200 to 4,000 ng, 3 replicates for each injection amount. The carryover was estimated based on the sum intensity of all quantified HeLa peptides.

Impact of sample amount and wash cycles on overall carryover

Minimal overall carryover levels from run to run prevent biased analysis. While ZebraWash reduces the carryover from the trap column, the contribution of the separation column becomes the major carryover source. Using the standard Fast Wash (FW) procedure or ZebraWash (ZW) resulted in overall carryover levels below 0.05% for injection amounts up to 4,000 ng (Figure 2). The increased number of ZebraWash cycles gradually reduced the contribution of trap column carryover, as seen from the intensity levels and the number of identified peptides in blank runs (Figures 2 and 3). The higher loading amounts lead to higher carryover levels, e.g., the carryover for the 4,000 ng sample was approximately 3–4 times higher for the 200 ng sample, which could be attributed to the overloading of the separation column. The washing of the trap column with standard Fast Wash is also an efficient method of carryover reduction. The volume of the strong wash liquid with the Fast Wash procedure (12 µL) is two

times larger than with 2 cycles of ZebraWash, which explains the slightly more efficient trap column washing (Figure 2). When consuming the same volume of strong wash liquid, the four cycles of ZebraWash consistently reduced more carryover than the Fast Wash procedure. It must be noted that the washing duration is dependent on the trap column dimension. The nano trap used here permits approximately 12 µL/min at 800 bar and room temperature (23 °C) during the washing procedure. As a result, it takes around 6 min to thoroughly wash and equilibrate the trap column with four cycles of ZebraWash and an equilibration factor of 2. Therefore, with a 25 µL sample loop in the standard configuration of the Vanquish Neo UHPLC system, it is recommended to have four cycles of ZebraWash for trap column washing. Users might use the other trap columns with larger i.d. for faster washing when considering more cycles for more comprehensive washing.

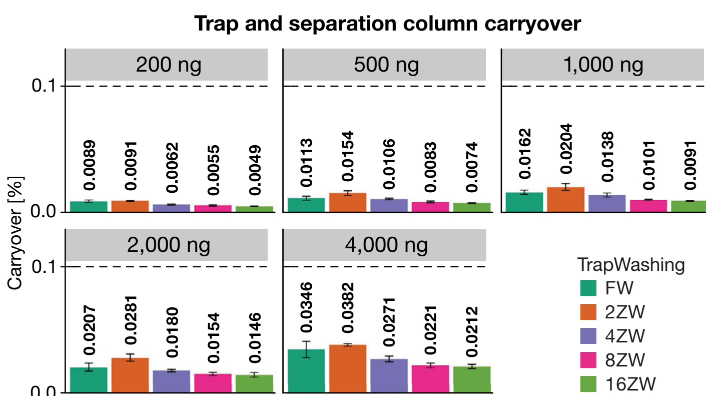

Figure 2. The level of overall carryover for different injection amounts and trap wash procedures, 3 replicates for each injection amount. The carryover was estimated based on the sum intensity of all quantified HeLa peptides.

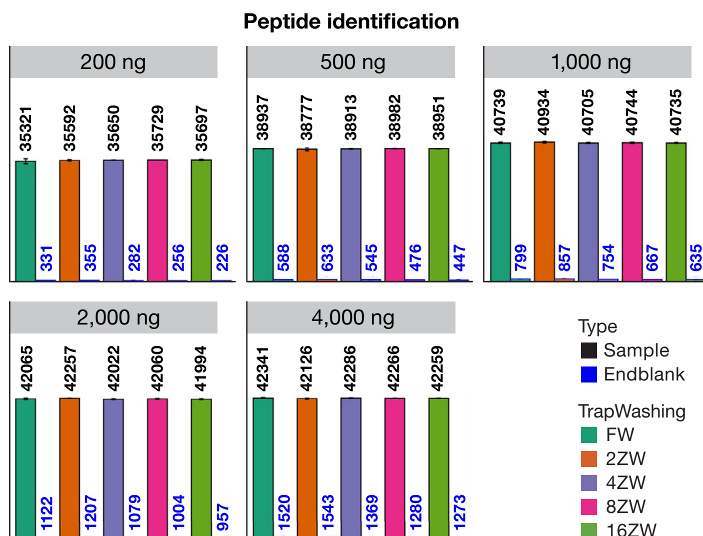

Figure 3. The number of peptides identified with the injection of 200 to 4,000 ng of HeLa protein digest and a blank injection afterward, 3 replicates. The trap column was washed with Fast Wash or ZebraWash (2, 4, 8, 16 cycles).

Conclusion

The ZebraWash procedure of the Vanquish Neo UHPLC system sets a new standard for efficient reducing of trap column carryover. This results in higher confidence using the trap-and-elute workflow. The independent and parallel washing of separation and trap columns delivers high-quality results without requiring intermediate wash runs, thus increasing sample throughput. The number of ZebraWash cycles and the composition of strong wash liquid can be optimized for different proteomics samples to ensure long-term robustness with minimal cross-contamination between samples.

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