Implementation of Real-Time Update for Time Scheduled Targeted Peptide Quantification (PRM) on a new Quadrupole Orbitrap Benchtop Mass Spectrometer

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ABSTRACT

Purpose: On-the-fly correction of scheduled retention time windows in order to allow more reliable profiling of more peptide targets

Methods: Dynamic retention time window correction for scheduled PRM was applied fully unattended and automated on a Thermo Scientific™ Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer.

Results: The dynamic correction of scheduled retention time windows in nano-LC setups allows for decreasing retention time windows to 60 seconds with increased reliability compared to static retention time window scheduling. This reduces the degree of overlapping of retention time windows, which in turn increases the amount of peptide targets that can be scheduled in an experiment.

INTRODUCTION

In targeted peptide quantification, it is necessary to cover hundreds of targets with high absolute sensitivity at the early stages of the biomarker development pipeline. This is commonly achieved by combining nano-LC with time-scheduled retention time (RT) lists. To maximize the number of scheduled targets and enable their detection with high sensitivity, the number of overlapping RT windows has to be minimized by keeping the windows as narrow as possible. However, this is rendered difficult by the relatively high retention time variations of nano-LC systems.

In parallel reaction monitoring experiments (PRM), the feature "*Dynamic retention time*" (dRT) allows for dynamically adjusting the time windows of the *m/z* targets based on reference precursors, which are present in the sample. Upon detecting the product ions of a reference precursor during the experiment, a deviation of the actually observed RT from the expected RT (as defined previously by the user) of the reference precursor is calculated as a time offset, which is then used to adjust the RT windows of the *m/z* targets.

Retention time variations can be the result of (1) selectivity changes within the system (e.g. due to temperature changes), or (2) shifts and drifts caused by different sample composition, minor variations in solvent composition, or column aging. The first type of variations cannot be compensated by this method. Therefore it is recommended to run PRM studies under strict temperature control of the column compartment.

The second type of variations can be compensated successfully by applying the dRT feature. The following work demonstrates that the dRT feature dynamically corrects the scheduled time windows of 198 schedules in a way that 1-min time windows become applicable routinely.

Methods

LC Method	
LC system	Thermo Scientific™ EASY-nLC™ 1200 system
Column temperature	45° C
Mobile phase A	2% acetonitrile/0.1% formic acid (v/v)
Mobile phase B	80% acetonitrile/0.1% formic acid (v/v)
Column (1) ,(2) Column (3)	Thermo Scientific™ EASY-Spray™ Column C18, 3 um, 75 um x 25 cm Thermo Scientific™ EASY-Spray™ Column C18, 3 um, 75 um x 15 cm
Gradient(s)	See table 1
Inject volume Inject volume	3 uL (1) – for RT determination 1 uL (1), (2) and (3)- replicates

MS Method	
MS system	Q Exactive HF-X Hybrid quadrupole-Orbitrap MS
Mode	PRM
AGC Target	1e5
Isolation width	2 Th
Resolution	15,000
RT window width	Target compounds: 1 min PRTC landmark peptides: 6 min
Max. IT	40 ms

MATERIALS AND METHODS

Sample Preparation

For all measurements, a digested HeLa sample (500 ng/uL) was spiked with 50 fmol/uL PRTC-Kit peptides and injected directly on a nano-chromatography system. 198 peptides representing 150 proteins of different intensity were picked from a HeLa TopN discovery experiment and put on the PRM inclusion list. Additionally, 10 PRTC peptides were scheduled and used as landmarks for the dRT feature.

Methods

3 different experimental setups were chosen to mimic retention time variations of typical nano-LC experiments. For two of them, artificial interventions were applied in order to induce RT variations.

(1) Batch injection of 25 replicates with reference gradient A) upon RT determination with gradient F).

(2) Mimicking variations in solvent compositions by running gradient variations (table 2) randomly. RT determination was performed from the reference gradient A).

(3) Study of column 'Aging' by 70 consecutive injections (110 h batch) with gradient A) (table 2). The RT's were determined before the batch started from separate refinement runs.

Table 2. Gradients for experiment (2)

Time(min)	A)	B)	C)	D)	E)	F)	G)	%B
	0	0	0	0	0	0	0	3
	5	4	6	6	4	5.5	4.5	3
	40	39	41	40	40	40.5	39.5	40
	45	44	46	45	45	45.5	44.5	90
	50	49	50	50	50	50.5	49.5	90
	51	50	51	51	51	51.5	50.5	3
	59	58	59	59	59	59.5	58.5	3

Data Analysis

All data processing was performed using Skyline software (University of Washington, Seattle). 3 transitions of each peptide were monitored for confirmation purposes. Quantifiable peptides were determined by manual inspection. If a peptide was only partially in the measured RT window or completely absent it was considered "not quantifiable". Values for static mode were calculated from the actual retention time in dRT mode, assuming that no correction had been applied.

RESULTS

(1) Offset generation

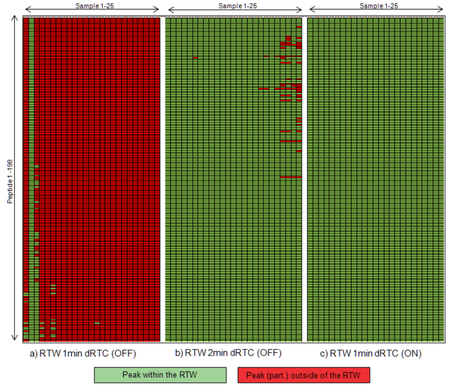
A measurement with an "offset" gradient (table 2, gradient F)) was used to determine the RT's of targets and PRTC landmarks. Afterwards, 25 replicates were measured in one batch with gradient A). Fig. 1 shows results from different RT window widths with the dRT feature switched ON and OFF. When applying 1 min windows in static mode (dRT=OFF) nearly all signals were missed except in run 2 (2a). In retrospective analysis an offset of 30-40 seconds compared to the reference was determined for nearly all scheduled peptides causing this behaviour.

The offset can be compensated by increasing the RT window width to 2 min (Fig. 1b). But this comes at the expense of data points being spread across the chromatographic peak due to more overlapping RT windows. In addition we observed an increasing number of non-quantifiable of peptides in the later runs.

In contrast, the right pane (Fig. 1c) shows the same experiment with 1-minute windows, but with dRT activated. Here all 198 peptides could be monitored throughout all 25 replicate runs.

RESULTS cont'd

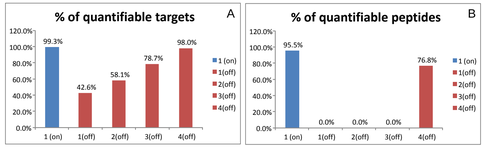
Figure 1. 198 peptides monitored under three different conditions with 25 concurrent injections each. Left: RT window (RTW) 1 min, dRT=OFF. Middle: RT window 2 min, dRT=OFF. Right: RT window 1 min, dRT=ON. Red color: Peptide is (at least partially) outside of the specified window and not quantifiable. Green color: peptide is inside the specified window and quantifiable.



(2) Gradient variation

The reference gradient A) was used to determine the RT's of targets and PRTC landmarks. Afterwards, 30 measurements were performed with a retention time window of 1 minute, dRT switched ON, and applying gradients B) to G) in random order (5 times each gradient). Here, 99.3% of all targets and 95.5% of all peptides were covered throughout all 25 replicates. For comparison, the success rates of the static mode (dRT=OFF) with broader windows were measured (Fig. 2).

Figure 2. Percentages of quantifiable targets (A) and peptides (B) throughout all 25 injections for experiment (2). Red color: Static mode (dRT=OFF) with different RT window widths. Blue color: dRT switched ON with a fixed RT window width of 1 min.



(3) Long-term performance (70 injections)

After the retention times were determined and refined from three replicates, 70 injections were performed in one batch without any refinement or adjustment. Two outliers resulting from an injection failure (run 59 and 63) were removed. Fig. 3 shows the retention time and determined peak width of the peptide DGNVLLHEMQIQHPTASLIAK, which elutes between 29.7 and 28.5 min and has an expected retention time of 29.45 min (black line). With dRT=ON the peptide was quantifiable throughout all 70 injections, although the peptide showed an RT variation of 48 seconds. As shown in Fig. 4 for six different runs (in Fig. 3 marked with an asterisk), the apex is always located in the middle of the RT window, independent of the actual retention time.

In contrast, as demonstrated by the yellow (static 1-minute window) and green & yellow (static 2-minute window) areas in Fig. 3, a static 1-minute window is not sufficient and has to be expanded to 2 minutes in order to capture this peptide in 69 of the 70 runs.

Figure 3. Retention time profile of the peptide DGNVLLHEMQIQHPTASLIAK measured with dRT=ON. Retention time is indicated by a horizontal black bar for each injection. Peak width is depicted by a red bar. The expected RT is 29.45 min. The yellow area shows a 1-minute window in static mode; the green areas together with the yellow area form a 2-minute window.

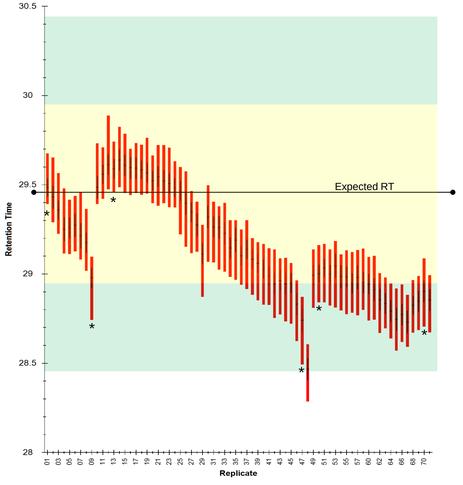


Figure 4. XIC's of the sum of three transitions of the peptide DGNVLLHEMQIQHPTASLIAK in six different injections (1, 9, 13, 47, 50, 69; marked by an asterisk in Fig. 3). Although the retention time varied between 28.7 and 29.6 min, dRT was able to successfully adjust the RT windows around the chromatographic peak.

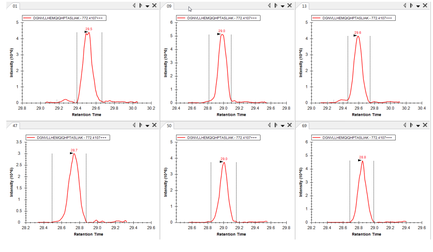
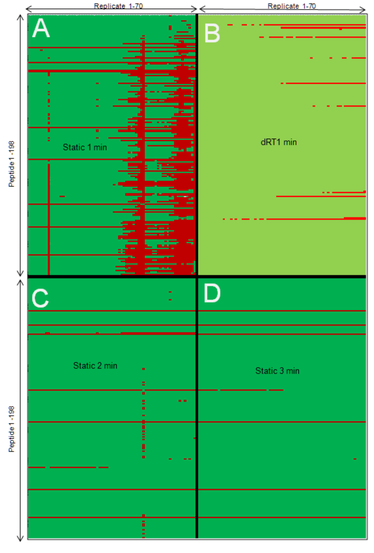


Fig. 5 shows the differences between static scheduling and dRT for 70 replicate injections. In dRT mode the ratio of detected targets reaches 94% (186/198) (Fig. 5B). In static scheduling mode, only 7 of the 198 peptides could be detected within the specified window of 1 min (Fig. 5A). Broadening windows to 2 min and 3 min increased success rates to 77% and 96% (C&D), respectively, but at the expense of a higher number of overlapping windows and thus a significantly increased cycle time (Fig. 5C,D).

Figure 5. Quantifiable (green) and not quantifiable (red) peptide targets over 70 consecutive PRM runs with dRT=ON (B) and dRT=OFF (A, C, D). Conditions: A, C, D: static scheduling with 1, 2 and 3 min windows. B: dRT scheduling with 1 min windows.



CONCLUSIONS

- The dynamic correction of scheduled retention time windows in nano-LC setups allows for decreasing windows to 60 seconds with increased reliability compared to static retention time window scheduling.
- dRT reduces the overlapping of windows and can therefore either increase the number of quantifiable targets per run or improve the sensitivity due to longer inject times compared to static scheduling.
- Extending the application to other peptide retention time standards would increase the flexibility.
- The implementation of dRT could be the foundation for more sophisticated triggering algorithms like isPRM[2].

REFERENCES (if necessary)

1. Gallien S, Duriez E, Crone C, Kellmann M, Moehring T, Domon B. *Targeted proteomic quantification on quadrupole-orbitrap mass spectrometer. Mol Cell Proteomics.* 2012 Dec;11(12) 1709-1723.
2. Sebastien Gallien, Bruno Domon, *Detection and quantification of proteins in clinical samples using high resolution mass spectrometry Methods, Volume 81, 15 June 2015, Pages 15-23*

TRADEMARKS/LICENSING