Demonstration of SRM Method Visualization and Automated On-the-fly Retention Time Updating for Targeted Peptide Quantitation

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ABSTRACT

It is a challenge to target hundreds of peptides in a single LC run by SRM-MS. Here we look at two software features that assist in better method definition and improved data acquisition:

- SRM visualization to observe concurrent transitions and approximate dwell times in a given method
- Real-Time RT updating during method acquisition to minimize chances of missing a schedule SRM transition due to chromatographic shifts

Results indicate successful "on-the-fly" updating of scheduled retention time windows as the LC gradient causes peptides to shift, allowing for improved data quality, easier method development, and fewer missed data.

INTRODUCTION

Peptide quantitation using LC-SRM-MS methodologies has progressed over the years to help answer increasingly difficult questions in biology. Targeting hundreds of peptides in a single LC run has resulted in technical challenges that require manual data evaluation to ensure all peptides eluted within their RT windows. Both sample composition and sample load can cause changes in peptide retention times from sample to sample, causing significant RT shifts. Increasing RT windows to ensure peptide detection comes at the cost of either lower dwell times or longer cycle times, either of which can have deleterious effects on LC-SRM-MS precision and sensitivity. These challenges have identified the need for more sophisticated acquisition software. Here we present the application of two software features that assist in visualizing the SRM method design as well as ensuring successful detection of peptide targets in a timed LC-SRM-MS method in the event of chromatographic shifts. The algorithms were evaluated on a set of PRTC peptides in HeLa lysate with several different reversed-phase gradients intended to cause chromatographic shifts. Use of the new features enabled better method design and resulted in a higher success rate of peptide detection.

MATERIALS AND METHODS

A sample set consisting of 15 13C/15-labeled peptides (PRTC peptides from Pierce) in HeLa lysate (Pierce) were evaluated on a Thermo Scientific™ TSQ Quantiva™ Triple Quadrupole MS. The LC-SRM-MS method targeted 467 transitions (60 precursors) using timed SRM with variable retention time windows. Out of 467 transitions, 15 were identified as "Retention Time Standards" with Reference Intensity Threshold set as 5.0e4 for 5 precursor *m/z* (493.768, 444.547, 695.832, 558.326, 567.973).

- A Thermo Scientific™ EASY-nLC™ 1000 system was used with a trapping column configuration for sample introduction and gradient delivery.
- Precolumn equilibration was set to 10 uL at 2.5 uL/min or a maximum pressure of 500 bar.
- Analytical column equilibration was set to 10 uL at 0.5 uL/min or a maximum pressure of 500 bar.
- Sample loading was performed at 2.5 uL/min for a total of 10 uL (from a 1 uL sample injection volume).
- Mobile phase A: 2% acetonitrile/0.1% formic acid (v/v); Mobile phase B: 90% acetonitrile/0.1% formic acid (v/v)
  .
- Columns
  - Trap column: ProntoSil C18AQ (Bischoff Chromatography), 3um beads, 150 um x 3 cm
  - Analytical column: Picochip ProntoSil C18AQ (Bischoff Chromatography), 3um beads, 75 um x 10 cm
- Gradients: Seven different gradients were used to artificially shift peptide RTs, and are shown in Figure 3. Flow rate during gradient delivery was 300 nL/min.

To evaluate performance of the instrument control software, LC-SRM-MS data were acquired using three different approaches with each of the 7 chromatographic gradients:

- Unscheduled (all 467 transitions acquired in a 1 second cycle time)
- Scheduled (3 minute RT windows for all transitions with a 1 sec cycle time)
- dRT (1.5 minute RT windows for all transitions except those identified as "RT Standards", which would have longer RT windows)

Data were analyzed in Thermo Scientific™ Xcalibur™ software and Skyline (University of Washington).

SRM Visualization

The use of timed SRM acquisition is often a requirement for the detection and quantitation of a large number of peptides in complex sample matrices to increase the dwell time of transitions and improve analyte detection. Determination of the retention time windows used for each analyte is a balancing game to ensure adequate dwell times per transition without inflating the cycle time, causing reduced sampling across the chromatographic peak. SRM visualization in the method editor allows the user to see the dwell time and retention time windows of all the precursor masses by plotting it in a graph.

Use of SRM visualization allowed for improved detection in the low abundance peptides by pinpointing congested regions of the chromatogram and indicating the average dwell time per transition in those regions. Retention time windows in these regions were easily reduced in duration so that the average dwell time increased.

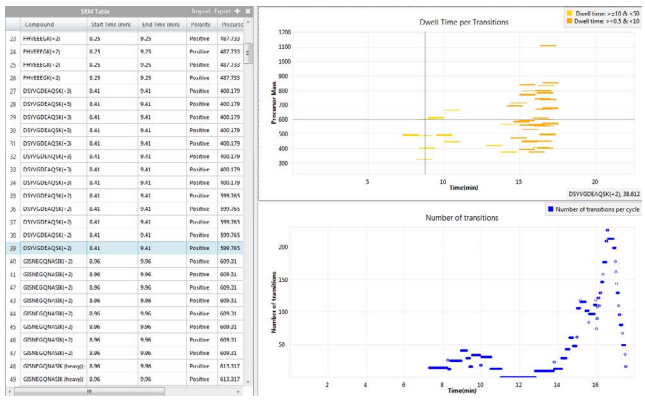

Figure 1. SRM Visualization Plots Visible in the Method Editor. Two new plots are displayed when "SRM Visualization" is enabled: "Dwell Time Per Transition" (upper right). And "Number of Transitions" (lower right). These plots help illustrate the approximate dwell times per precursor *m/z* and show where in the chromatographic gradient the most concurrent transitions elute, based on the scheduled RT method.

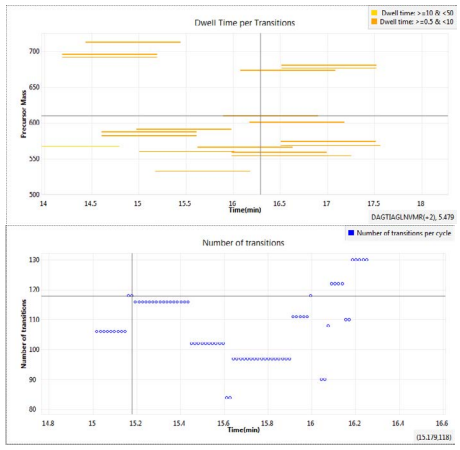

Figure 2. SRM Visualization – Zoomed in graphical display with tooltip

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Graphical Representation

The dwell time graph is plotted for each scan event (precursor/product ion) showing the start and end time. The dwell time for each scan event at a given time and precursor is shown in the tooltip as the mouse hovers over each plot (tooltip shown in the lower right corner of each plot). The x axis shows the time scaled at 0.001 minutes and sorted based on the start time, while the y axis is sorted by the precursor mass.

The transitions graph plots the number of transitions at any given time between the experiment start and end time. The x axis shows the time scaled at the cycle time and y axis is the number of transitions.

RESULTS

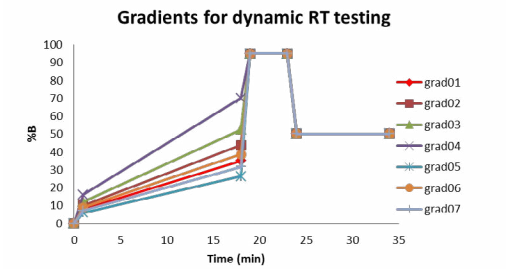

Figure 3. Seven reversed-phase gradients were used to test the ability of the dRT software feature to update scheduled RT windows "on-the-fly" during acquisition.

Samples were acquired on each of the 7 gradients in unscheduled methods to empirically determine the retention time of each of the 60 precursors (data not shown), then each gradient was re-run with a scheduled method. The chromatographic shifts from the different gradients in scheduled SRM mode are shown in Figure 4.

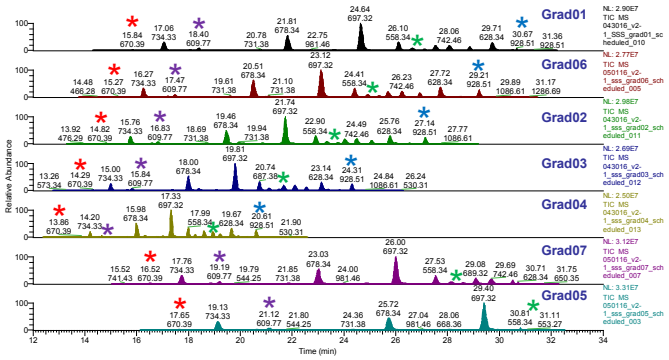

Figure 4. Scheduled SRM-MS chromatograms for each of the 7 gradients used in the study. Peaks are labeled with RT and *m/z* of the base peak. RT shifts from the "default" gradient (Grad01) were as much as 8 minutes. Four of the 5 peptides used as RT Standards are indicated with colored asterisks (*).

To test the ability of the "Dynamic Retention Time" software feature to adjust RT windows on-the-fly during a scheduled SRM method, the scheduled transition list used for Gradient 1 (Grad01 in Figures 3 and 4) was used for the other six LC gradients. RT windows were reduced to 1 minute except for transitions used as RT Standards, which were set to 5 minutes (see Figure 5).

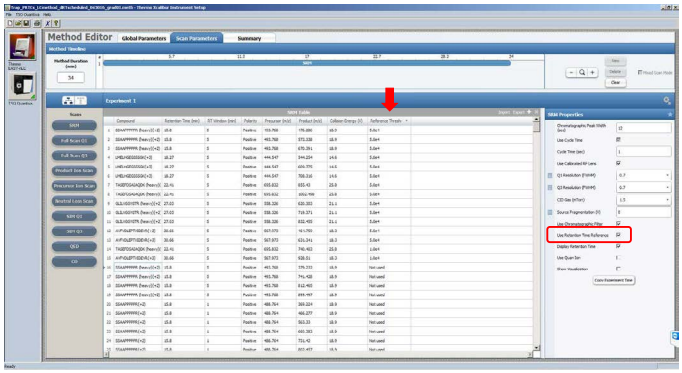

Figure 5. View of Method Editor including the option for using Retention Time References. Colored asterisks indicate peptide sequence and transitions noted in Figure 4.

Dynamic Retention Time (RT) Adjustment

The software is designed to automatically update the scheduled SRM retention time window during the acquisition, based on the specified reference threshold. The primary goal of this feature is to accommodate the shifts in the retention time due to chromatographic changes that would result in missing the analyte of interest. The reference peak detection thresholds are specified in the acquisition methods (Figure 5). The identification of these reference peaks and corresponding adjustment of retention time windows happens automatically during acquisition. If the reference peaks are not detected, no adjustments are made.

When the RT windows in certain regions are reduced in duration to increase the average dwell time, there is a drawback of ending up with a narrow RT windows, which could result in missing the analyte if the RT shifts outside of the specified window. The use of dynamic RT adjustment allows for increased success of peptide detection, even when small (≤1 min) RT windows are employed.

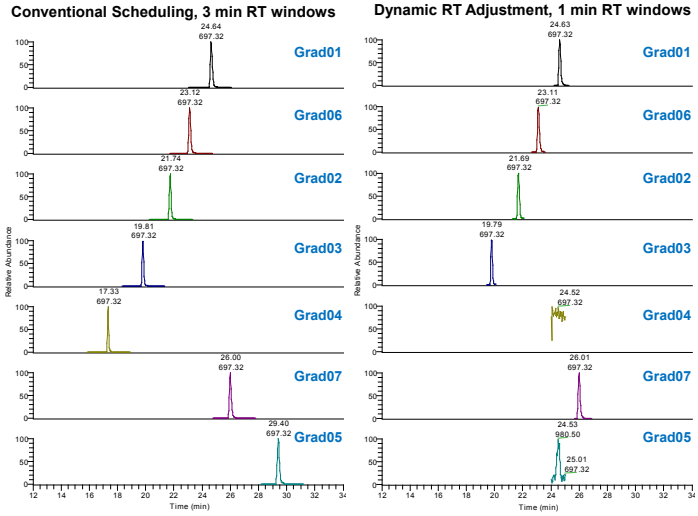

Figure 6. Extracted Ion Chromatogram for peptide ISGLIYEETR (+2) to demonstrate the ability of Dynamic RT Adjustment to move the 1 min wide RT windows due to chromatographic shift. Conventional RT scheduling is shown on the left, with 3 min RT windows and manually updated retention time data to allow for successful detection of the peptide over different gradients. On the right, Dynamic Retention Time Adjustment was used so that the RT windows could be shortened to 1 min for most transitions, resulting in higher dwell times overall (See Figure 7). Gradients 04 and 05 had RT shifts too large for the RT Standard peptides to be detected with 5 min RT windows, so detection defaulted to the RT defined from Gradient 01.

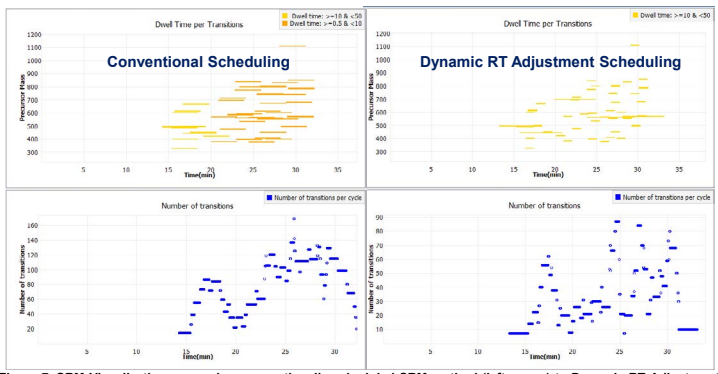

Figure 7. SRM Visualization comparing conventionally scheduled SRM method (left panes) to Dynamic RT Adjustment scheduling (right panes). Not only does the average dwell time increase when using Dynamic RT Adjustment, but the number of concurrent transitions decreases, due to being able to use smaller RT windows for scheduling.

Considerations for Dynamic Retention Time (RT) Adjustment

These experiments were conducted as a proof-of-concept to evaluate the Dynamic RT Adjustment feature. The RT windows for the RT Standards were set to 5 minutes, therefore any chromatographic changes causing a RT shift of greater than +/- 2.5 minutes for the RT Standards would result in no peak detected.

Additional options for improving flexibility of the method would be to increase the RT windows of the RT Standard peptides, even monitoring for these peptides during the entire chromatographic run.

In these experiments, RTs were empirically determined by running an unscheduled method to observe where each peptide eluted. Not only is this time consuming, but lower abundant peptides may not be reliably detected at dwell times near 1 msec. If the same RT Standard peptides are used in all samples, real-time RT updating of scheduled SRMs will ensure a higher success rate of analyte detection with smaller RT windows. The benefit of this approach is increased dwell times (see Figure 7).

CONCLUSIONS

Here we introduced two software features that provide clarity and flexibility to targeted peptide quantitation method design and experiments.

SRM Visualization

- Plots dwell time per transition and concurrent transitions as a function of retention time
- Provides interactivity, providing links between the SRM table and visualization plots
- Improves method design to minimize chances of too many overlapping transitions and/or dwell times that are too low

Dynamic Retention Time Adjustment

- Updates scheduled RT windows during a method if significant chromatographic shifts are observed
- Allows for much narrower RT scheduling windows to be used, which increases dwell times for most transitions
- Minimizes the need for manual data evaluation and re-scheduling of SRM methods
- Minimizes the incidence of “missing data” due to peaks shifting outside of their RT windows

REFERENCES

1. Escher, C., Reiter, L., MacLean., Ossola, R., Herzog, F., Chilton, J., MacCoss, M.J., Rinner, O. “Using iRT, a normalized retention time for more targeted measurement of peptides”. *Proteomics* 2012, (12(8):1111-1121. doi: 10.1002/pmic.201100463.