High throughput targeted proteomic workflows with Adaptive Retention Time on the Orbitrap Astral Zoom MS

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Abstract

Purpose: Live retention time (RT) correction using adaptive retention time alignment (aRT) on the Thermo Scientific™ Orbitrap™ Astral™ Zoom mass spectrometer (MS) to allow reproducible and robust targeted mass spectrometry at high throughput and large scale.

Results: aRT alignment allows for reproducible tracking of large numbers of targets, with high throughput gradients, narrow scheduling windows, and can be combined with DIA for a hybrid approach to targeting and discovery.

Introduction

Advances in MS instrumentation have accelerated the identification of biomarkers, but validating and translating these discoveries into assays for clinical use remains challenging and typically requires robust and accurate targeted approaches. While these often rely on predetermined reference target retention time (RT) windows, analytical column degradation or replacement, aging solvents, sample matrix effects, etc. can cause peptide RT shifts between injections and across longer-term analyses. To accommodate this, targeted MS experiments generally schedule wide target RT windows (>1 min) or rely on heavy labeled peptides to trigger acquisition of endogenous peptides. Both greatly limit the number of quantifiable targets per assay. To overcome this, we utilize adaptive RT (aRT) alignment for live correction of RTs together with the speed and sensitivity of the Orbitrap Astral Zoom MS. This allows reproducible tracking of >2000 targets with narrow RT windows, high throughputs, such as 300 samples per day (SPD), and hybrid workflows combining targeting, of up to 1000 targets, and discovery.

Materials and methods

LC-MS Experiments

Experiments were performed using Thermo Scientific™ Pierce™ HeLa protein digest resuspended in 0.1% TFA, or individual or pooled human plasma samples. Peptides were separated using a 15 cm Thermo Scientific™ PepMap™ analytical columns (150 µm inner diameter) and the Thermo Scientific™ Vanquish™ Neo UHPLC system at throughputs of 60 or 300 SPD. The liquid chromatography (LC) system was interfaced with the Orbitrap Astral Zoom MS. Reference runs using MS1 and/or data-independent-acquisition (DIA) scans were acquired to obtain reference aRT spectra, and to determine target RTs. For aRT alignment, methods consisting of aRT MS1 and/or DIA scans, as well as targeted MS2 scans with RT correction were optimized. For hybrid DIA workflows, we used aRT MS1 scans for reference acquisition and alignment, combined with narrow window DIA and a targeted MS2 experiment. Observed RT shifts were caused by natural column degradation, sample matrix effects or when indicated induced by variation in gradient flow rate and buffer composition.

Data analysis

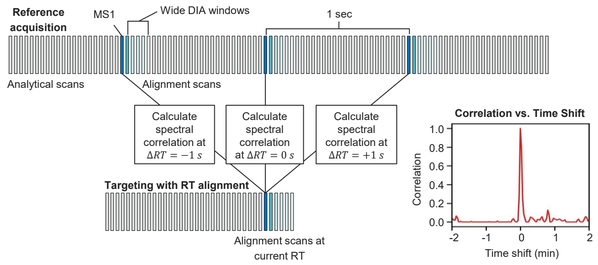
Reference DIA data was analyzed using Spectronaut™. Targets lists were then generated from the search output by selecting varying number of targets evenly distributed across the RT dimension. Target RTs for smaller scale assays were manually validated and exported from Skyline, while target RTs for large scale assays were exported from Spectronaut. For alignment runs, DIA and PRM data were analyzed using Spectronaut and Skyline respectively.

Results

Adaptive Retention Time Correction on the Orbitrap Astral Zoom MS

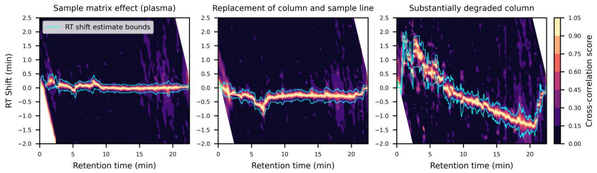
Adaptive RT alignment, as previously implemented on the Thermo Scientific™ Stellar™ and Thermo Scientific™ Orbitrap™ Excedion™ Pro MS, provides a computational approach to address this problem [1], allowing for increased target sampling reliability and narrower target windows. First a reference acquisition using the appropriate sample matrix, is recorded using adaptive RT MS1 and/or wide window DIA scans (**Figure 1**). Then in the actual targeted acquisition, spectra from these periodic "alignment" aRT scans (MS1 or wide window DIA) are cross-correlated in real time with spectra of the same scan type from the reference acquisition. This is used to determine the similarity of the spectra at the current time point to spectra at different time points in the reference data and estimate live shift boundaries, which will used to update target acquisition windows to correct for RT shifts and robustly track target analytes.

Figure 1. Adaptive RT alignment scan cross-correlations to estimate RT shifts.



Variation in analyte elution times presents a major challenge for targeted MS using retention time scheduling. Here we show that aRT can effectively track and compensate for mild RT shifts caused by plasma matrix effects (**Figure 2, left**), i.e. using a plasma pool for RT correction of individual plasma samples, routine replacement of a blocked analytical column and sample line (**Figure 2, middle**), as well as and more extreme shifts caused by transferring a targeted assay to a different LC-MS setup with a severely degraded column (**Figure 2, right**).

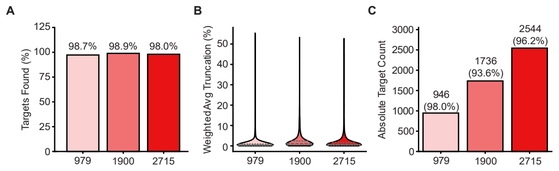
Figure 2. aRT can track shifts related to plasma matrix effects, column/ sample line replacements, and a severely degraded column.



Target recovery for large scale assays

After confirming robustness against different RT shift sources, we investigated target recovery for larger scale assays up to 2715 targets. In all tested assays, we were able to find ≥ 98% of targets, with the remaining 2% potentially being artifacts from the initial RT determination using Spectronaut (**Figure 3A**). Evaluating potential target peak truncation, we saw consistent low truncation, with median truncation values of 0.4% (979 targets), 1.1% (1900 targets), and 0.7% (2715 targets), respectively (**Figure 3B**). Considering only targets captured with less than 10% peak truncation, we were still able to recover 98%, 93.3% and 96.2% of targets for the 979, 1900 and 2715 targets assays, respectively (**Figure 3C**).

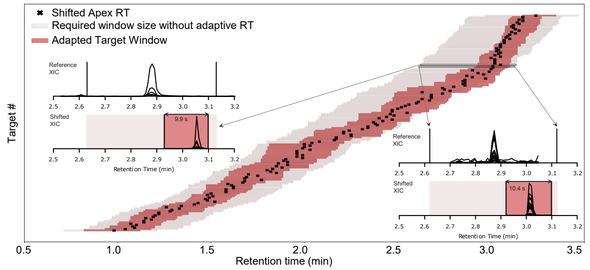
Figure 3. Target recovery for large scale assays using aRT. Overall target recovery (A), target peak truncation levels (B), and number of targets captures with <10% peak truncation (C).



Adaptive RT allows for robust targeting with high throughput gradients

With an increasing focus on large scale cohort studies, comes an increased need for throughput. However, the decreasing peak width and increasing peptide density seen with fast higher throughput gradients can be challenging for targeting; compounding the need for RT shift correction and narrow target scheduling windows. Here, we evaluated the performance of targeted proteomics using aRT at a throughput of 300 SPD. We induced an RT shift by varying flow rates across the gradient in order to delay elution times relative to the reference acquisition. We found that shorter gradients reduce the standard deviation in RT shifts and allow for very narrow target acquisition windows. Using aRT for RT correction, we were able to capture targets with target acquisition windows of less than 15 s, which greatly increases the target capacity in comparison to the 30 s static windows that would be required to capture all targets without adaptive RT (**Figure 4**).

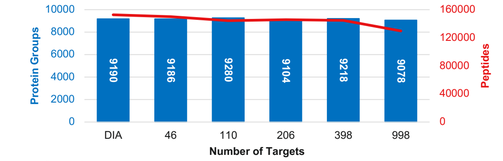
Figure 4. Targeting at 300 SPD with aRT. Adaptive target acquisition windows (red) in comparison to static RT windows (grey) with example XICs.



Adaptive RT-based Hybrid DIA to combine the strengths of targeting and discovery

The high MS2 scan speed and analyzer parallelization of the Astral Zoom MS in combination with aRT correction additionally opens the possibility to combine targeted and discovery proteomics with little cost to discovery performance. Comparing the performance of standard narrow window DIA (nDIA, 4Th) with Hybrid DIA combining nDIA with targeting of increasing target numbers, we found that even when including ~1000 targets (5 ms max. inject time) and near complete target recovery, we maintained high DIA performance, with less than a 2% decrease in protein group IDs and ~13% decrease in peptide IDs (**Figure 5**). While we focused on higher abundant targets for the initial test, this shows the potential to combine discovery with targeted acquisition of peptides of interest, low abundant species with poor DIA coverage or potentially even PTM analysis in un-enriched samples.

Figure 5. Identification rate from 200 ng HeLa digest comparison for DIA and Hybrid DIA.



Conclusions

- Adaptive RT alignment on Orbitrap Astral Zoom MS shows robustness against various sources of RT shifts and has high target recovery even for thousands of targets
- aRT enables the use of target acquisition windows <15 s for robust targeting at high throughput
- aRT Hybrid DIA allows targeting of up to 1000 targets with minimal cost to DIA depth

References

1. Remes et al., Highly Multiplex Targeted Proteomics Enabled by Real-Time Chromatographic Alignment, Analytical Chemistry, 2020.

Conflict of interest

All authors, apart from M.M. and M.B.L, are employees of Thermo Fisher Scientific, the manufacturer of instrumentation used in this study.

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