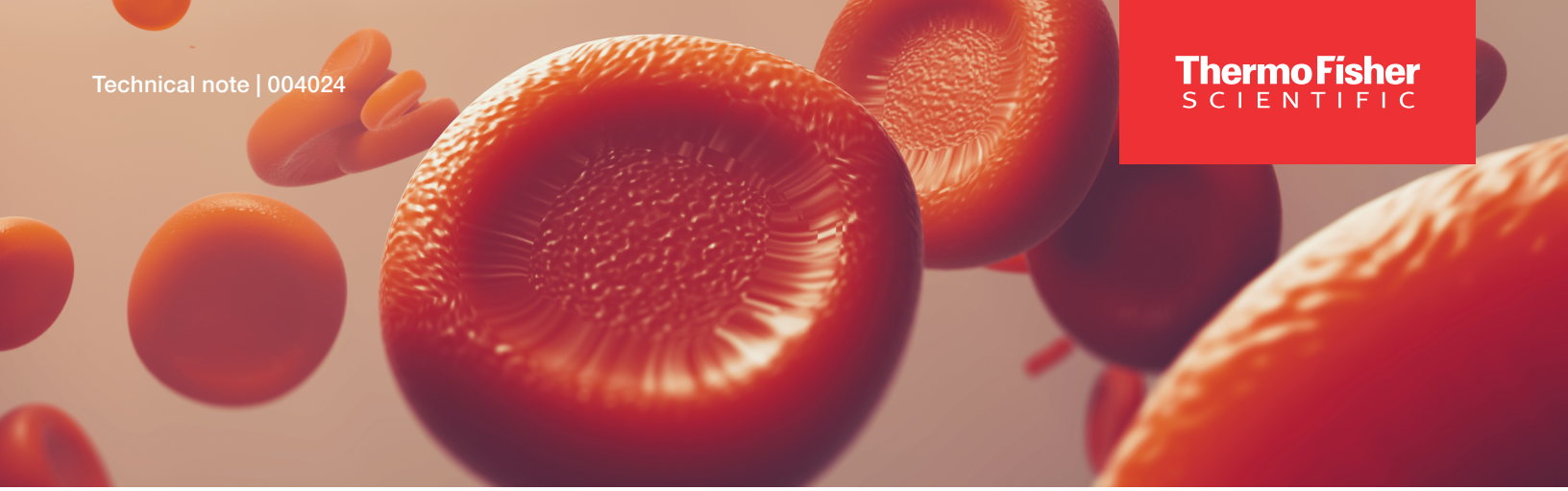

Technical note | 004024

Leveraging Adaptive RT on the Orbitrap Ascend MultiOmics Tribrid mass spectrometer for improved sensitivity and throughput in targeted workflows

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Keywords

Orbitrap Ascend, MultiOmics, Tribrid, targeted workflow, PRM, Adaptive RT, retention-time alignment

Goal

To utilize the Adaptive RT feature on the Thermo Scientific™ Orbitrap™ Tribrid™ platform for targeted assay developments. By adding an Adaptive RT data-independent acquisition (DIA) experiment to the traditional PRM method, a background retention time reference file is generated for downstream retention time alignment in targeted experiments. The result is a completed targeted workflow, from discovery to target, in one mass spectrometer with improved sensitivity, throughput, and high reproducibility.

Introduction

First available on Thermo Scientific™ Stellar™ mass spectrometers (MS) in 2024, Adaptive RT is an innovative algorithm designed to dynamically align retention time windows by utilizing reference retention time data stored in the instrument's method file. This allows for narrow retention time windows with an accompanying increase in the number of targets and/or an increase in the available injection time-per-target.[1] This method eliminates the need for using traditional spiked-in retention time calibration standards used for alignment and improves overall sensitivity and throughput.[2]

Here, we evaluate the Adaptive RT features on the Thermo Scientific™ Orbitrap™ Ascend MultiOmics Tribrid™ MS using Tune version 4.3 instrument control software, with the goal of improving sensitivity and throughput for targeted (PRM) workflows by using Ion Trap and/or Orbitrap analyzers available on the Tribrid platform.

Materials and methods

Consumables

- Thermo Scientific™ UHPLC-MS grade water (Cat. No. W81)
- Thermo Scientific™ UHPLC-MS grade 100% acetonitrile (Cat. No. A9561)
- Thermo Scientific™ Optima™ LC-MS Grade Formic Acid (Cat. No. A11710X1AMP)
- Thermo Scientific™ EASY-Spray™ HPLC Column, PepMap™ RSLC C18, 2 µm, 150 µm x 15 cm (Cat. No. ES906)
- Thermo Scientific™ PepMap™ Neo Trap Cartridge, 5 µm C18 300 µm x 5 mm (Cat. No. 174500)
- Thermo Scientific™ SureSTART™ 0.2 mL TPX Screw Top Microvial with Glass Insert (Cat. No. 60180-1655)
- Thermo Scientific™ SureSTART™ 9 mm Screw Caps, Level 3 High Performance Applications (Cat. No. 6PSC9STB1)
- Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard (Cat. No. A47996)
- Biognosys™ PQ500™ Reference Peptide Kit (Biognosys Cat. No. 1915523)
- Thermo Scientific™ Pierce™ neat digested plasma, 100 µg per vial

Instrumentation

- Thermo Scientific™ Vanquish™ Neo™ UHPLC system
- Thermo Scientific Orbitrap Ascend MultiOmics Tribrid MS
- Thermo Scientific™ EASY-Spray™ source

Data analysis

- Thermo Scientific™ Proteome Discoverer™ software, version 3.1 SP1
- Skyline software, University of Washington, MacCoss Lab, version 24.1.1.398

Sample preparation

The Pierce HeLa Digest/PRTC standard was resuspended with 0.1% formic acid in water at a concentration of 200 ng/µL for evaluation of targeted assay development with Ion Trap and Orbitrap using the Adaptive RT feature.

The PQ500 sample was resuspended with 120 µL diluent from the kit using manufacturer's protocol, then further diluted four times with background plasma to a concentration of 300 ng/µL for targeted method development. The above PQ500 sample was then serially diluted threefold with 300 ng/µL background plasma in 11 steps.

LC-MS analysis

The Vanquish Neo UHPLC system was used with a trap-and-elute configuration, utilizing the EASY-Spray PepMap HPLC column, in a 60 samples-per-day (SPD) method. HPLC gradients and parameters are shown in Table 1.

Table 1. LC method for 60 SPD method (24 min method duration).

	Time (min)	Flow (µL/min)	%B
Gradient	0.0	Run	
	0.0	3.0	4.0
	0.5	1.3	4.0
	0.6	0.8	8.0
	0.9	0.8	
	13.9	0.8	22.5
	20.8	0.8	35.0
	21.2	2.0	55.0
	21.2	Column wash	
	21.7	3.0	99
	24.0	3.0	99
	24.0	Stop run	
	24.0	Column equilibration	
LC parameters	Column temperature		45°C
	Mobile phase A		0.1% formic acid in water
	Mobile phase B		0.1% formic acid in 80% acetonitrile
	Sampler temperature		7°C

Mass spectrometry

The mass spectrometer used for all of the experiments was an Orbitrap Ascend Tribrid MS with MultiOmics options. It was controlled by Orbitrap Tribrid series Tune version 4.3 software. The MS parameter settings are summarized in Tables 2A through 2E.

Table 2A. MS method global parameters.

Application mode	Peptide
Expected LC peak width (s)*	12
Advanced peak determination	True
Default charge state	2
Ion source type	NSI
Positive spray voltage (V)*	2,200
Ion transfer tube temperature (°C) *	275

*Parameters are subjected to optimization

Table 2B. Adaptive RT DIA parameters.

Precursor mass range (*m/z*)**	400–800
Isolation window (*m/z*)**	50
Window overlap (*m/z*)	0
Window placement optimization	Off
Ion Trap scan rate	Turbo
Scan range (*m/z*)**	350–2,000
RF lens (%)	30
Activation type	CID
Collision energy (%)	30
AGC target	Standard
Maximum injection time mode	Auto
Acquire reference**	Checked for scheduled PRM method to generate reference file

**Parameters that can be changed or optimized

Table 2C. MS experiment parameters.

Detector type†	Ion Trap/ Orbitrap
Ion Trap scan rate/Orbitrap resolution†	Turbo/60K
Scan Range (*m/z*)	350–2,000
RF lens (%)	60
AGC target	Standard
Maximum injection time mode	Auto

†Using Ion Trap MS1 in conjunction with Ion Trap PRM experiments and Orbitrap MS1 with Orbitrap PRM experiments

Table 2D. GPF-DIA experiment parameters.

Detector type	Ion Trap/Orbitrap
Ion Trap scan rate/Orbitrap resolution	Turbo/45,000
Precursor mass range (*m/z*)‡	400–500, 500–600, 600–700, 700–800, 800–900, 900–1,000
Isolation window (*m/z*)	1 (Ion Trap) /4 (Orbitrap)
Windows overlap (*m/z*)	0
Window placement optimization	On
Activation type	HCD
Normalized collision energy (%)	30
Scan range (*m/z*)	145–1,450
RF lens (%)	60
AGC target	Standard
Maximum injection time (ms)	35 (Ion Trap)/91 (Orbitrap)

‡Each GPF-DIA experiment covered 100 *m/z* precursor mass range

Table 2E. Targeted MS^n experiment parameters.

MS^n level	2
Detector type	Ion Trap/Orbitrap
Ion Trap scan rate/Orbitrap resolution	Turbo/15,000/7,500
Isolation mode	Quadrupole
Isolation window (*m/z*)	1 (Ion Trap)/ 0.7 (Orbitrap)
Activation type	HCD
Normalized HCD Collision energy (%)	30
Scan range§	Defined in table
RF lens (%)	60
Normalized AGC target (%)	300
Maximum injection time mode	Dynamic
Desired minimum points across the peak	7
Loop control	All
Dynamic time scheduling	Off (during target refinement); Adaptive RT (when final reference file is ready)
Time mode††	Unscheduled or start/end time
Reference file	.rtbin file generated from Adaptive RT DIA experiment that has Acquire Reference checkbox checked for data acquisition
Targeted mass list	Exported from PRM Conductor with user-defined precursor filtering and method settings; or imported from known targeted mass list, e.g., PQ500 transition list

§Scan range defined in table with optimized scan range for Ion Trap experiments and fixed range 200–1,500 *m/z* for Orbitrap experiments

††Unscheduled time mode was only applied for the initial PQ500 PRM experiment to decide the precursor targets' elution profile; start/end times were defined in the mass list table based on the experiment plan

All experimental data were acquired with a 60 SPD LC gradient using the Vanquish Neo UHPLC coupled with an EASY-Spray HPLC column (Cat. No. ES906, 2 µm, 150 µm x 150 mm) and the PepMap Neo Trap cartridge (Cat. No. 174500, 5 µm, 300 µm x 5 mm).

The Pierce HeLa digested protein with PRTC standard was used for developing label-free targeted assays from discovery results. A set of gas-phase fractionation data-independent acquisition (GPF-DIA) data were collected with a narrow isolation window (1 Th for Ion Trap DIA and 4 Th for Orbitrap DIA) to generate a chromatogram library. GPF-DIA data were searched by Proteome Discoverer software. The search result file was imported into Skyline-daily software to generate a spectra library along with 14 PRTC peptides. Untargeted discovery data were sequentially refined for targeted PRM candidates utilizing Skyline software and the PRM Conductor tool.

The PQ500 in plasma sample was used to build the PRM method on the Orbitrap Ascend MultiOmics MS. The PQ500 peptide transition listed (from Biognosys) was imported into Skyline-daily software to generate a spectra library for 804 PQ500 peptides, along with 14 spiked in PRTC peptides, which were used as reference peptides by enabling the Pierce PRTC as the Indexed Retention Time (iRT) calculator. Multiple unscheduled isolation lists of the PQ500 sample were exported from Skyline-daily software to create a set of 10 unscheduled PRM methods to gauge the elution profile of each peptide on the mass list. The combined unscheduled PRM results were further refined in Skyline-daily software utilizing PRM Conductor to create downstream wide/narrow targeted window PRM methods. Once the final PQ500 PRM method was ready, serial-diluted PQ500 in plasma samples were used to access the figures of merit of the targeted PQ500 assay.

Data analysis and post-processing

Data were processed by Proteome Discoverer software (version 3.1 SP1) with the CHIMERYS™ search algorithm for GPF library generation and Skyline-daily software (version 24.1.1.398), utilizing the PRM Conductor tool for target refinement and targeted list generation.

Results and discussion

Adaptive RT is a two-step process

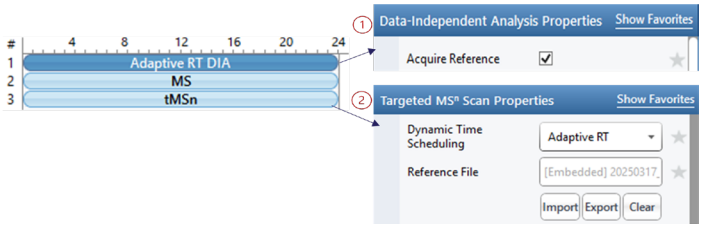


Figure 2. How Adaptive RT works.

Acquire reference—the spectra generated from the background Adaptive RT DIA experiment are compressed into a .rtbin file that can be used in Align-to-Reference experiments. The retention times of the targets should be determined as they usually are and entered into the targeted table.

Dynamic time scheduling with Adaptive RT—the MS instrument can adjust retention times automatically according to the retention time drifts detected in background analytes eluting into the system in real time, compared to a reference experiment.

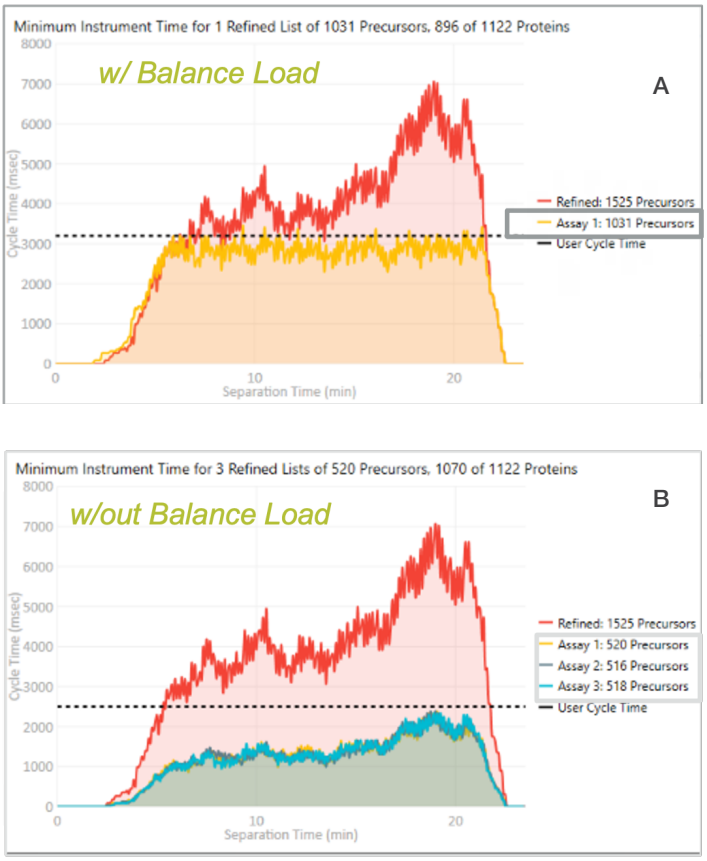


Figure 3. "Balance Load" strategy in PRM Conductor target assay refinement.

(A) Using "balance load", only a single assay will be created using only the N peptides per protein that best utilize the instrument acquisition resources.

(B) Not using "balance load", exporting as many assays as are required to acquire data for all the precursors that passed the user filters.

From discovery to target in one MS

Using Ion Trap for PRM assay development

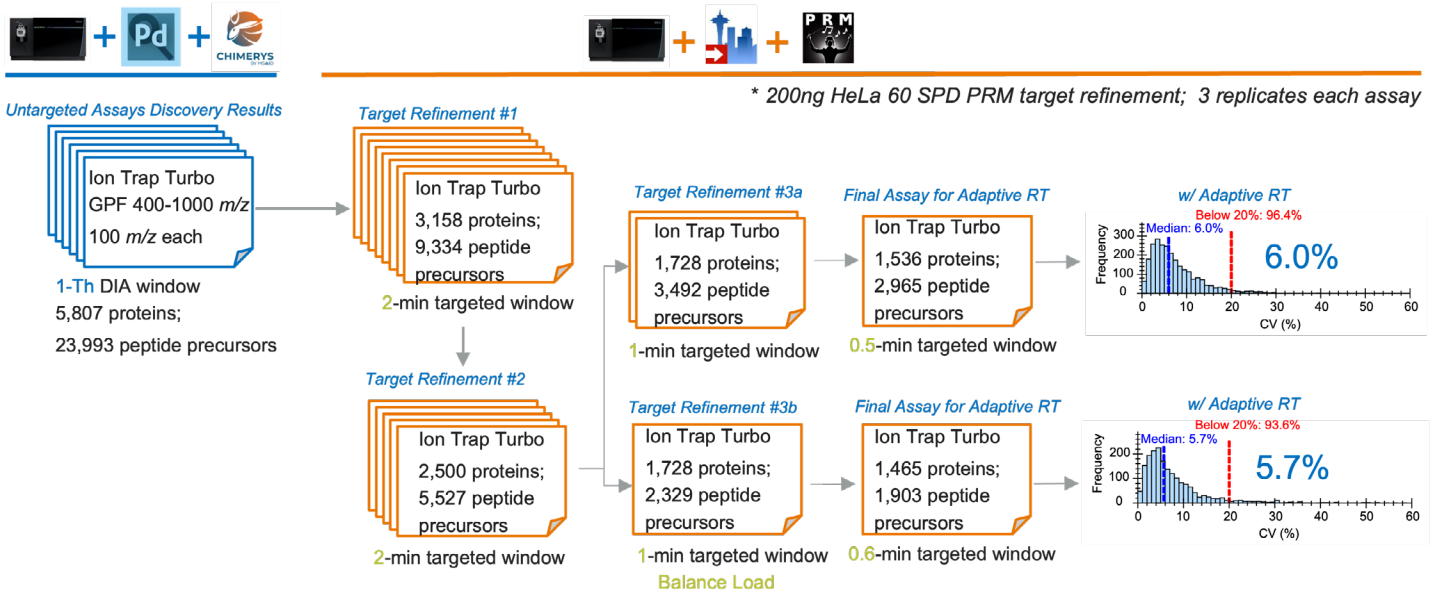


Figure 4. Workflow for assay development using Ion Trap in Orbitrap Ascend MultiOmics Tribrid MS. 200 ng HeLa digest with 100 fmol PRTC was loaded onto column for every injection during assay development.

Untargeted assay discovery data collection was performed with a set of GPF-DIA experiments using 1 Th DIA window and Ion Trap turbo scan rate. Six fractions were collected to cover the overall precursor range 400–1,000 *m/z*, with each fraction covering a 100 *m/z* precursor range consecutively. GPF-DIA data were searched by Proteome Discoverer software and the CHIMERYS search engine, with 0.5 Da fragment mass tolerance for Ion Trap detection and 0.01 target FDR. Untargeted discovery data were imported into Skyline-daily software for spectra library building and generation of draft assays using PRM Conductor for initial potential target candidates. Each draft assay was acquired in triplicate for downstream target refinements, with qualified targets that passed user-defined filters to build final assays with Adaptive RT. The final assays with Adaptive RT have a CV% around 6%, indicating extremely high reproducibility for these assays.

Using Orbitrap and Ion Trap for PRM assay development

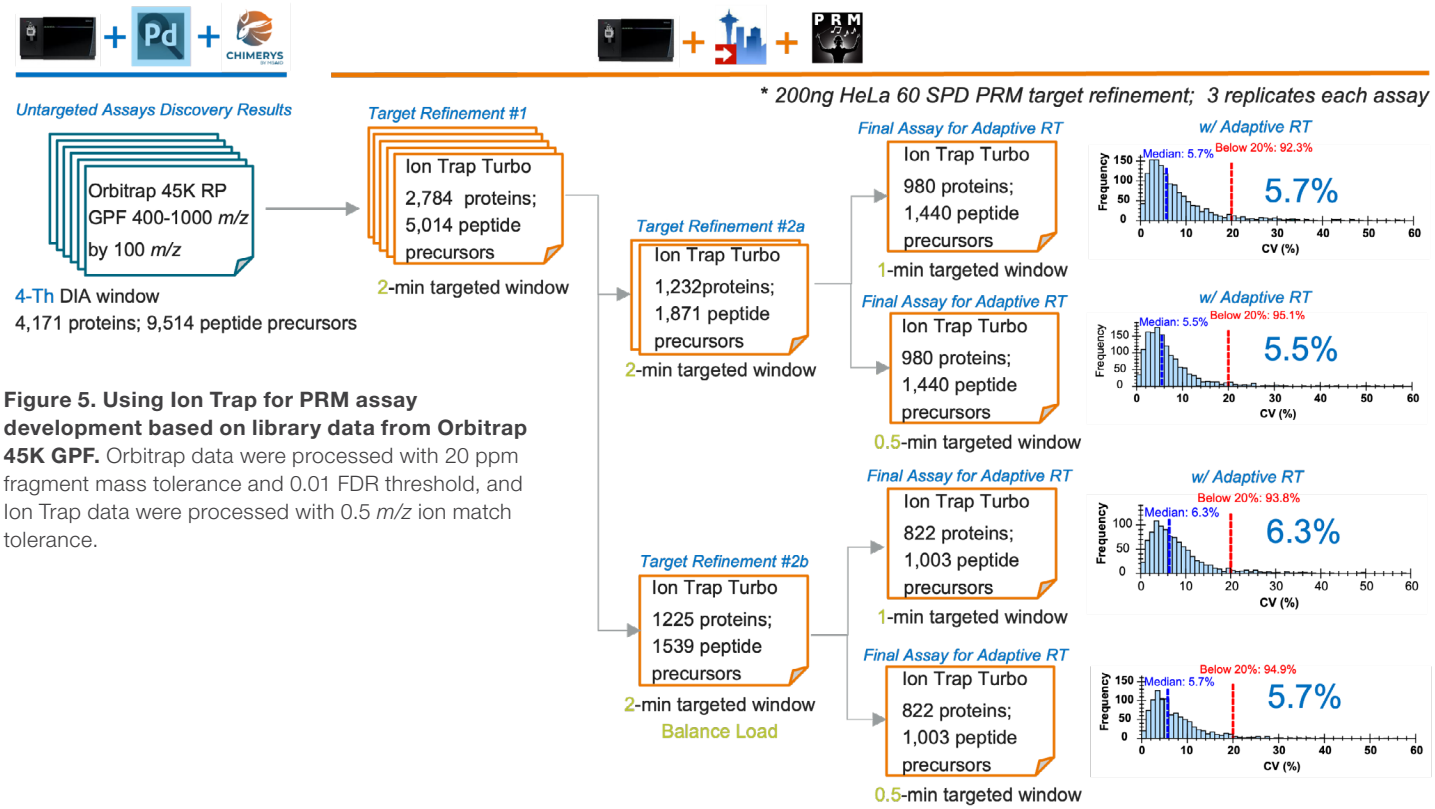


Figure 5. Using Ion Trap for PRM assay development based on library data from Orbitrap 45K GPF. Orbitrap data were processed with 20 ppm fragment mass tolerance and 0.01 FDR threshold, and Ion Trap data were processed with 0.5 *m/z* ion match tolerance.

Using Orbitrap 7.5K RP for PRM assay development

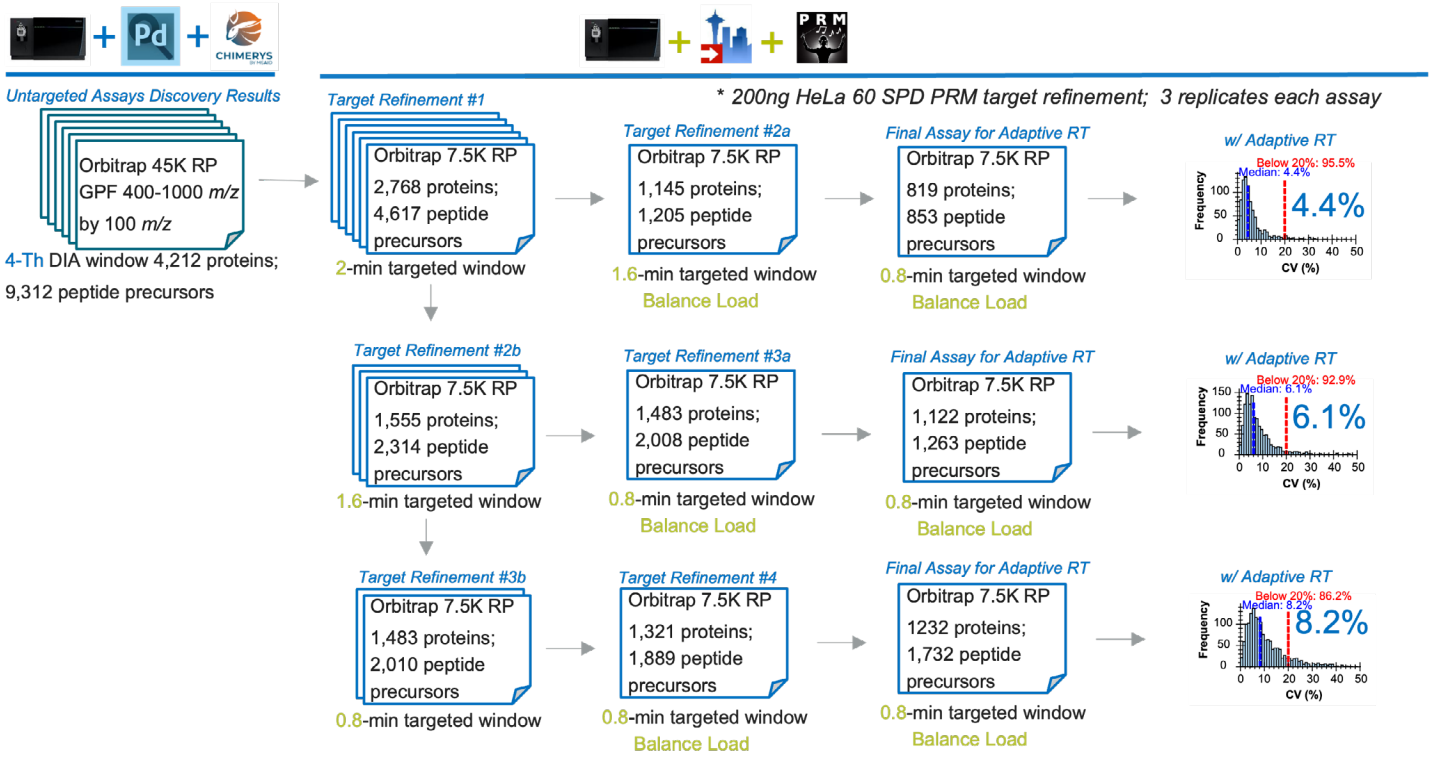


Figure 6. Using Orbitrap 7.5K RP for PRM assay development based on library data from Orbitrap 45K GPF. Orbitrap data were processed with 20 ppm fragment mass tolerance and 0.01 FDR threshold.

Using Orbitrap 15K RP for PRM assay development

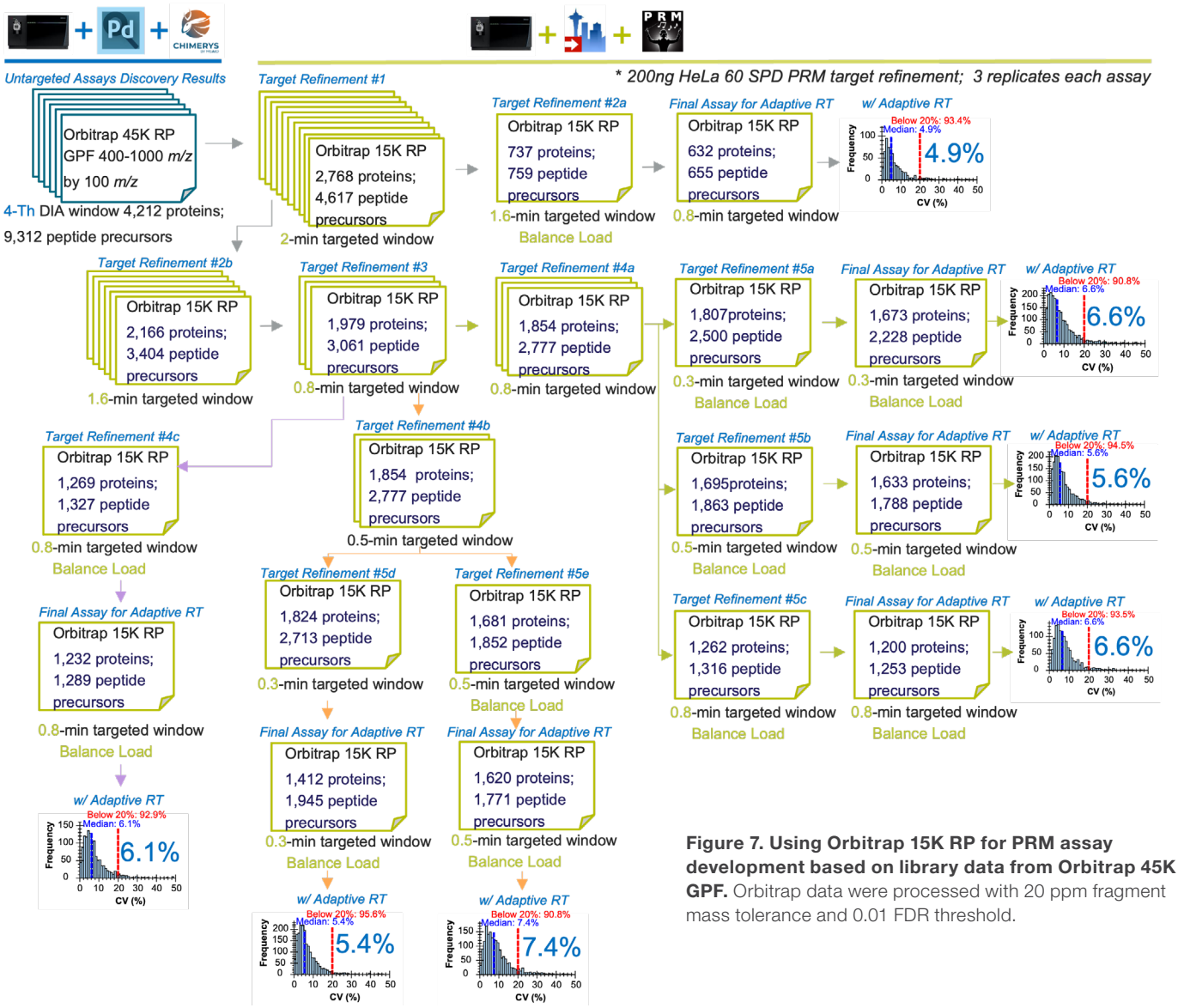


Figure 7. Using Orbitrap 15K RP for PRM assay development based on library data from Orbitrap 45K GPF. Orbitrap data were processed with 20 ppm fragment mass tolerance and 0.01 FDR threshold.

One cannot step in the same river twice

But Adaptive RT helps ensure you reel in your catch in the chromatographic river

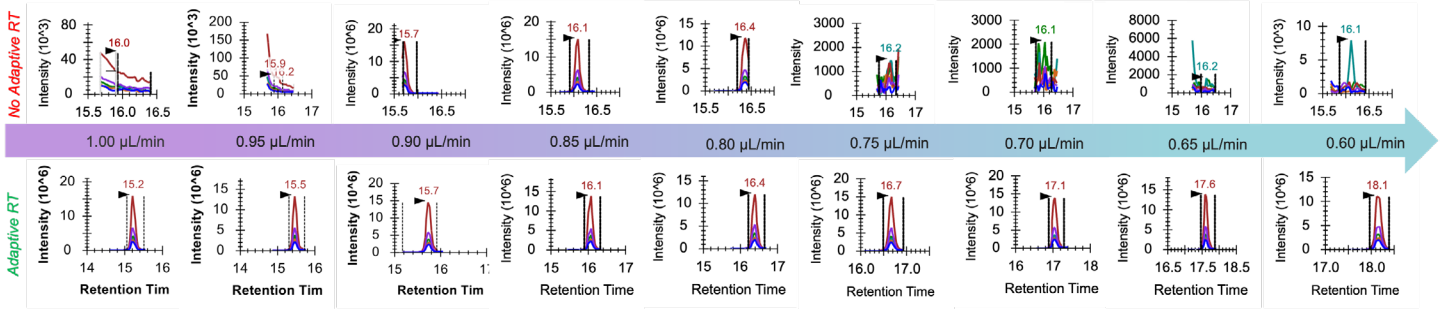


Figure 8. Evaluating Adaptive RT performance when LC retention time shifted based on a targeted assay with 0.8-min targeted acquisition window. Even minor changes in LC flow rate would result in shifts of target retention time. Without using Adaptive RT, the target would be missed whenever the target's retention time is outside the designated acquisition window. In comparison, using Adaptive RT under the same circumstance, the target would still be captured with consistent peak quality, as the system automatically adjusted the targeted acquisition window in real time.

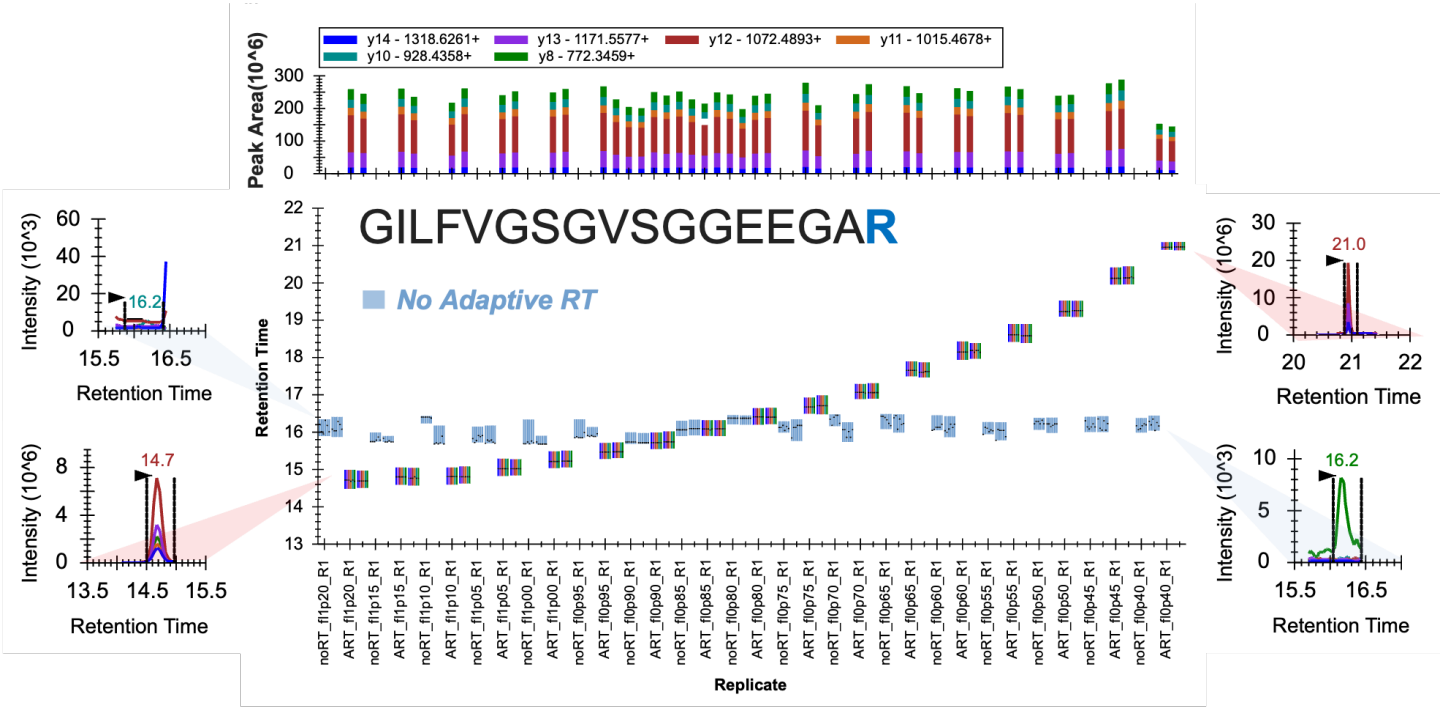


Figure 9. Evaluating Adaptive RT performance when LC retention time is shifted on a targeted assay with a 0.8-min targeted acquisition window. Two replicates were performed for each LC flow rate and MS setting. LC flow rate varied from 1.20 µL/min down to 0.40 µL/min with a 0.05 µL/min step size. MS methods were the same, except with and without using Adaptive RT for each LC flow rate setting. With Adaptive RT, the targeted retention time automatically shifted in real time to fit the changes and capture the quality targets, even when retention time shifted more than 5 min in this 24-min gradient.

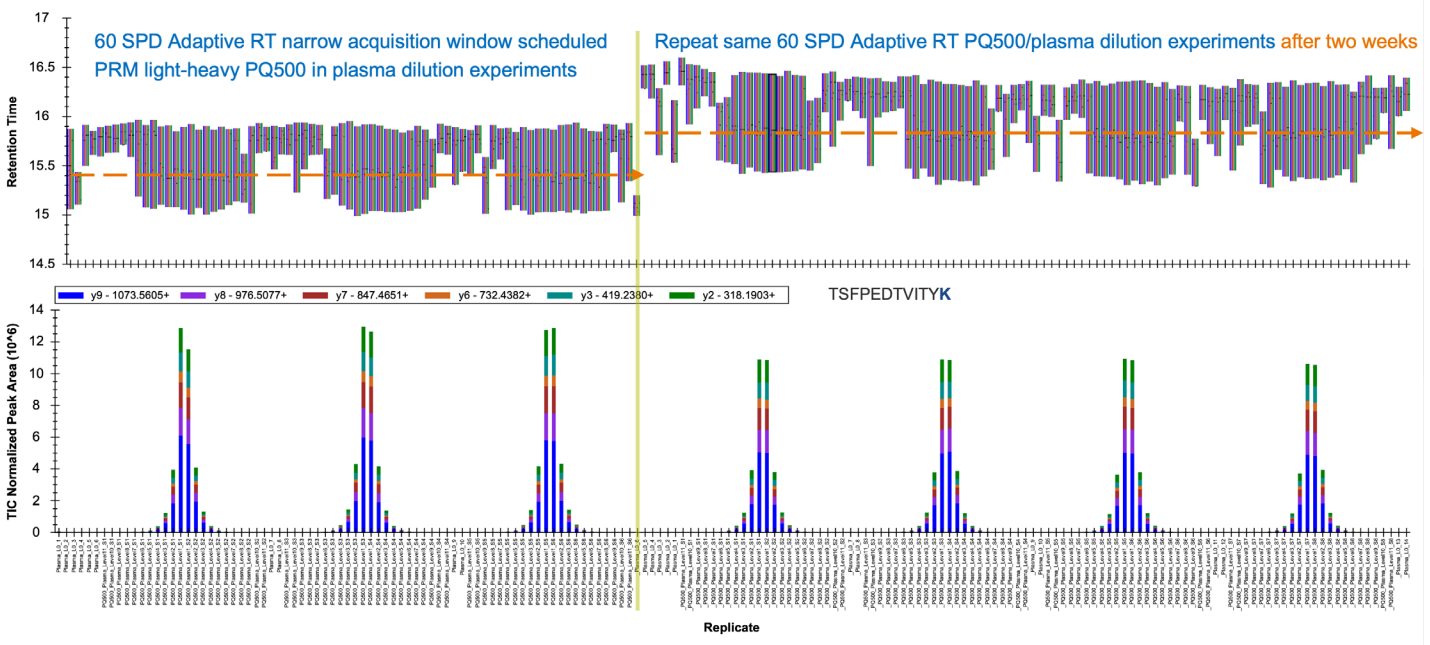
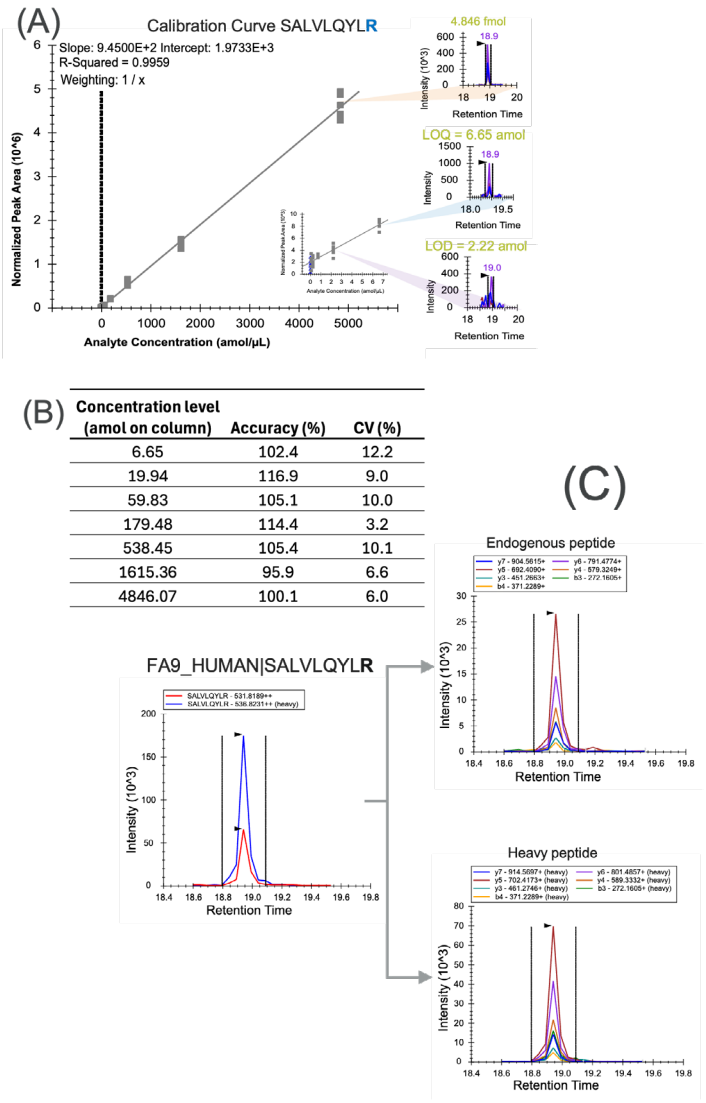


Figure 10. Evaluating Adaptive RT performance over time using a targeted PQ500/plasma assay with a narrow targeted acquisition window. The PQ500 peptide transition list was imported into Skyline software for assay development with PRM Conductor. PQ500 peptides were spiked into 300 ng of digested plasma matrix for analysis. A final assay containing 1,622 precursors (both light and heavy peptides for 804 PQ500 precursors, as well as 14 PRTC peptides as reference), utilizing a 0.8-min targeted window in a 24-min gradient, was used for dilution curve generation to characterize the assay's sensitivity for peptide quantification. Six technical replicates were performed at each concentration level. The serial-dilution experiment was repeated after 2 weeks on the same column. The Adaptive RT feature in the method helped ensure that the targeted RT windows were properly adjusted, even when there were slight changes in chromatographic conditions, such as column degradation.

Assessing method sensitivity for the PQ500 assay



(B)

Concentration level (amol on column)	Accuracy (%)	CV (%)
6.65	102.4	12.2
19.94	116.9	9.0
59.83	105.1	10.0
179.48	114.4	3.2
538.45	105.4	10.1
1615.36	95.9	6.6
4846.07	100.1	6.0

Figure 11. Evaluating the sensitivity of the PQ500/plasma assay with a 0.8-min targeted acquisition window using the Adaptive RT feature. (A) Example calibration curve of the SALVLQYLR peptide, showing estimated limit of quantitation (LOQ) at 6.6 attomoles and limit of detection (LOD) at 2.2 attomoles on the column. (B) Accuracy% and CV% of the SALVLQYLR peptide at different concentrations. (C) Identified endogenous proteins such as coagulation factor IX (FA9)—a vitamin K-dependent plasma protein that's involved in Hemophilia B disease with this workflow.

Conclusions

1. The Adaptive RT feature eliminates the cost and time of optimizing RT kits.
2. The method leverages a novel real-time mapping routine to dynamically adjust timed/scheduled data acquisition windows at narrower time intervals to eliminate missing values that compromise data quality and maximize data completeness.
3. With a narrower RT window, consistent median CV% values (~6%) were obtained, demonstrating extremely high reproducibility of these assays with the Adaptive RT real-time alignment feature.
4. The Orbitrap Tribrid platform provided flexibility for assay development; for 60 SPD (24-min gradient), about 3,000 qualified precursors can fit into one assay using Ion Trap or 2,200 precursors into one assay using Orbitrap.
5. Adaptive RT was proven to confidently align the retention time in real time, even when the retention time shifted up to 5 min.
6. The assay developed using this workflow can achieve great sensitivity and reproducibility, with an estimated LOD at 2.2 attomole and LOQ at 6.6 attomole with great accuracy.

References

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