Gluck Equine Research Center

College of Agriculture, Food and Environment

Efficient Generation of Highly Multiplexed Serum Biomarker Panels using Gas Phase Fractionation and DIA Libraries

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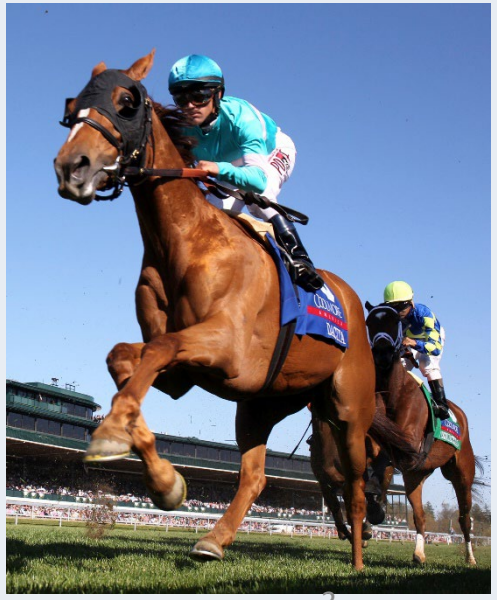
Overview

- Introduction
- Workflow development using Orbitrap Exploris
- Method and quality control evaluations
- Transferring methodology to Triple Quadrupole for high throughput analyses

Introduction – Equine Biological Passport

- Equine athletes are subject to drug abuse, however, there are strong mitigation efforts to deter doping practices

- Development of Equine Biological Passport (EBP)
  - In-direct means of monitoring drug abuse in equine athletes
  - Measure differential expression of proteins and peptides following drug administration
  - EBP is a valuable tool for drugs that are rapidly incorporated into tissues, designer drugs, and drugs which are poorly detected via traditional small molecule detection methods



Introduction – Benefits of DIA for Equine anti-doping

- DIA methods are very useful in evaluating thousands of peptides from complex proteomes, but typically require increased analytical time compared to targeted methods

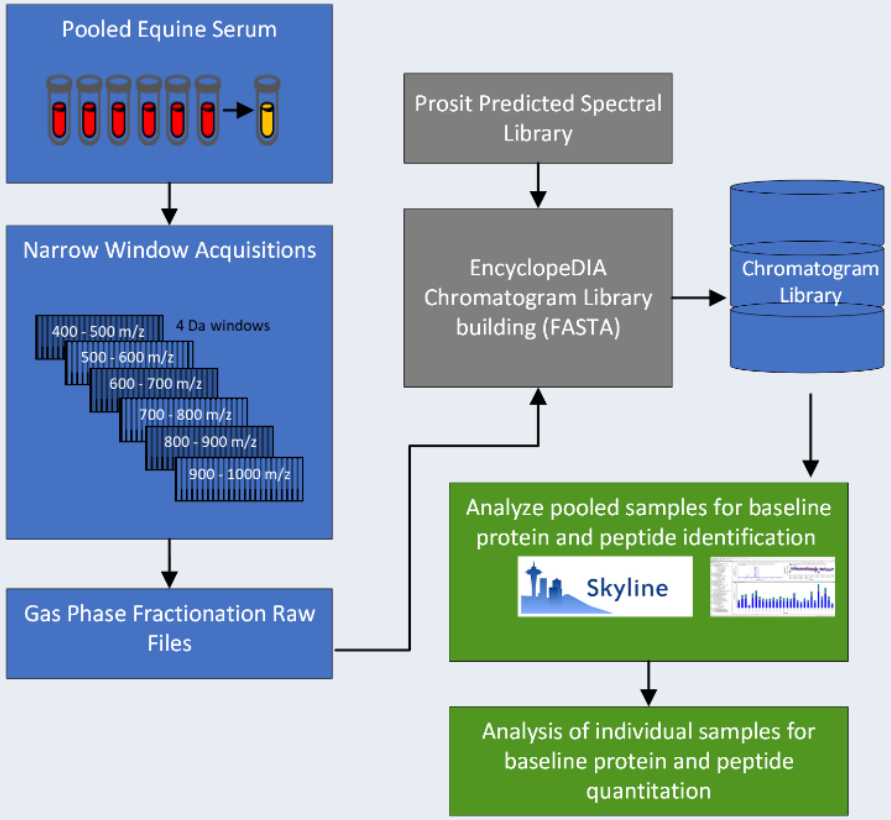
- The overall **aim** of this work was to develop a ***robust, reproducible peptide and protein monitoring method for routine anti-doping analyses***

  - Specific Aim 1: Build gas-phase fractionated library of equine peptides on the Orbitrap Exploris 480 coupled with nano-flow LC

  - Specific Aim 2: Transfer methodology to TSQ Altis triple quadrupole coupled with a Vanquish Horizon high-flow LC for routine screening

Development of DIA-only chromatogram library

- Performing non-targeted LFQ DIA acquisition
  - Narrow-window gas phase fractionation to build DIA-only chromatogram library
  - See Thursday Poster: **Efficient Generation of Highly Multiplexed Serum Biomarker Panels using Gas Phase Fractionation and DIA Libraries**
- To boost data confidence using LFQ DIA acquisition, need to implement a rigorous **system suitability and quality control protocol**
- In addition to peptide targets, all SRM information is empirically determined from the LFQ DIA experiment



Methodology adapted from Pino et al., 2020

Samples Prepared for DIA Workflow on Orbitrap Exploris 480 Mass Spectrometer

Matrix Matched (quality control) - pooled horse sera mixed 1:1 with chicken sera

Reference - pooled serum from healthy, un-treated, ‘control’ performance horses

Pre-administered samples – serum collected prior to drug administration to establish expected protein profile(s)

Post-administered samples – limited number of horses with sera collected at defined time points post administration

System Suitability Test (SST)

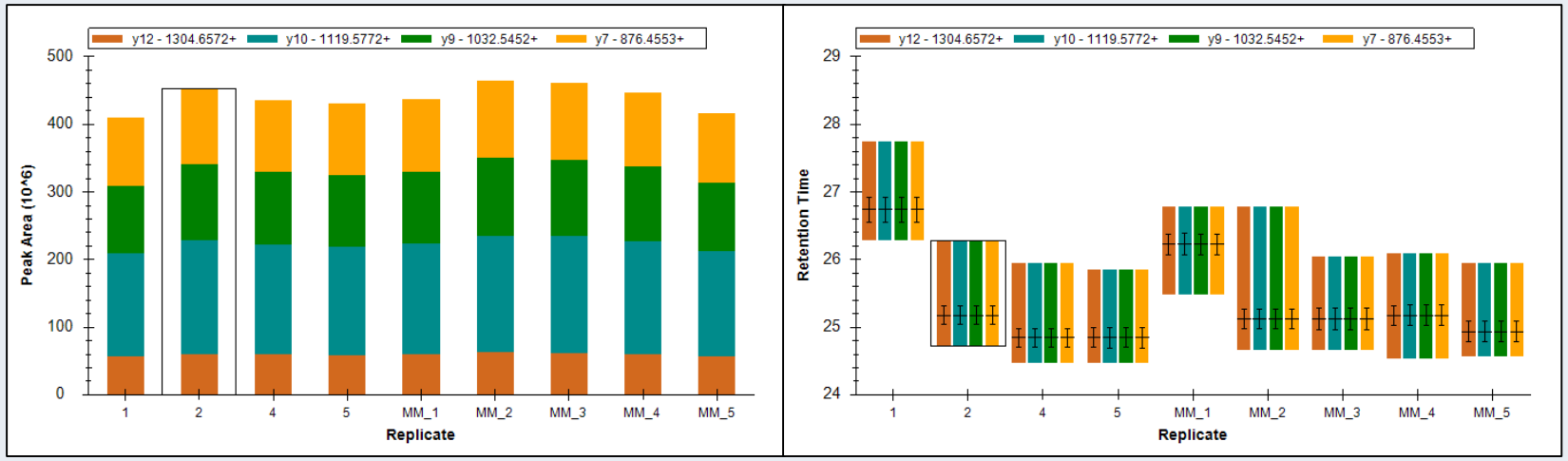
Digestion Performance – Intact protein standard (soy lectin, yeast enolase)

Instrument Performance – Peptide Retention Time Calibration (PRTC) mix

Evaluating Experimental Methodology

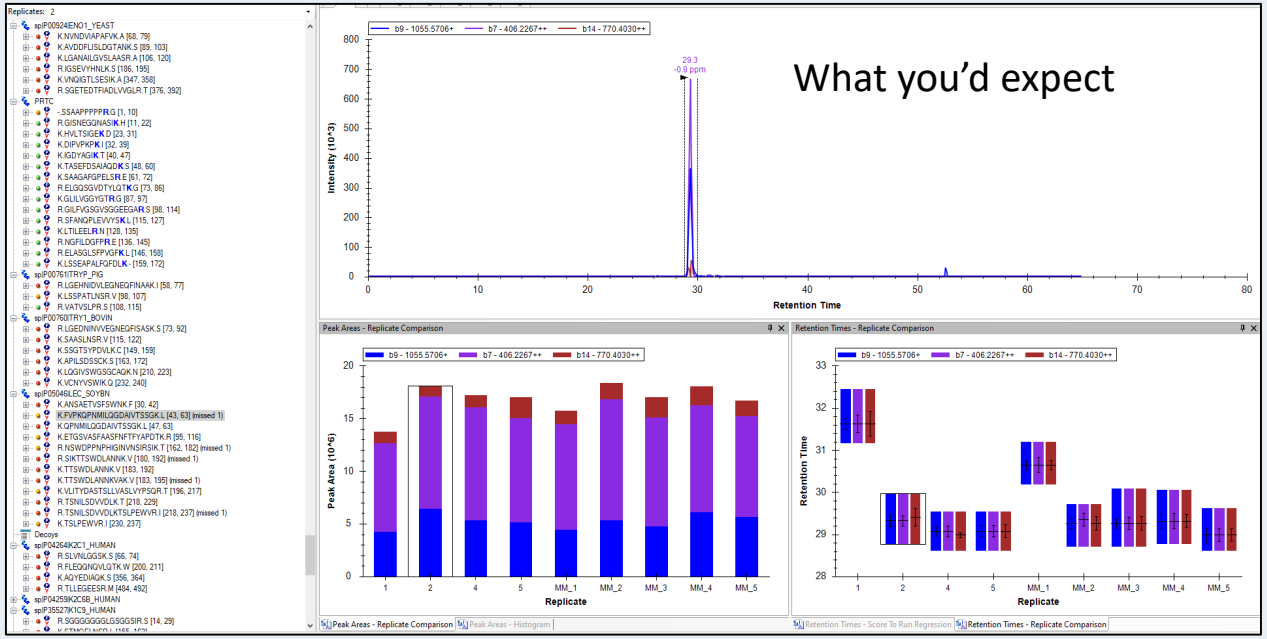
Orbitrap Exploris 480 System Suitability – PRTC

ELGQSGVDTYLQT**K**



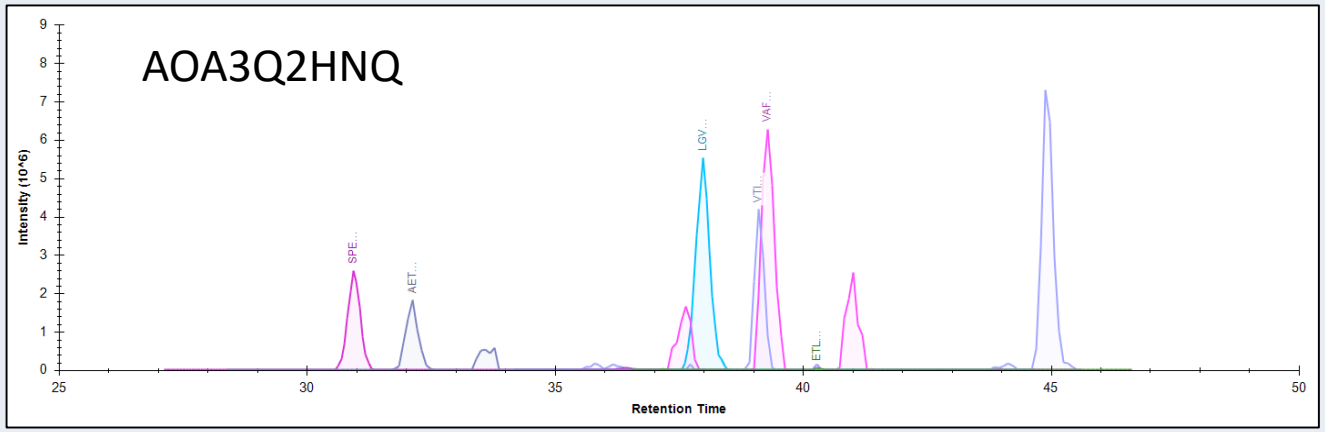
Evaluating Experimental Methodology

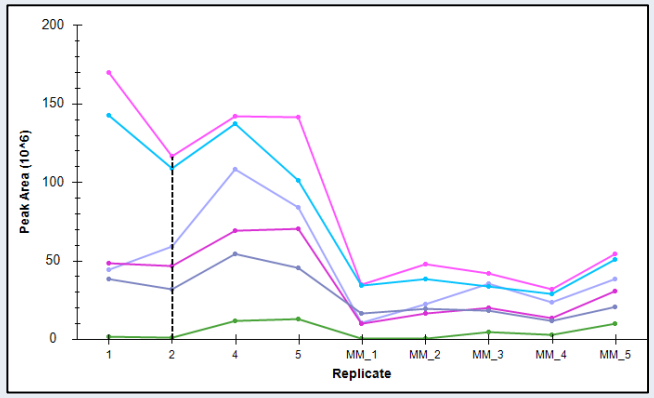
Digestion Performance – Easy Pep Maxi Kits
Evaluation of soy lectin or yeast enolase

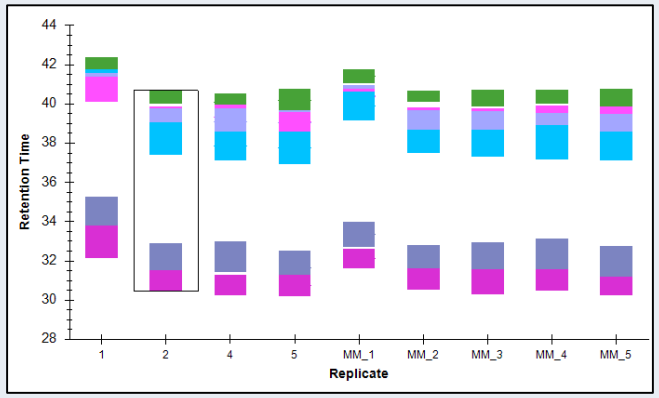


Evaluating Experimental Methodology

Reference vs. QC at Protein Level





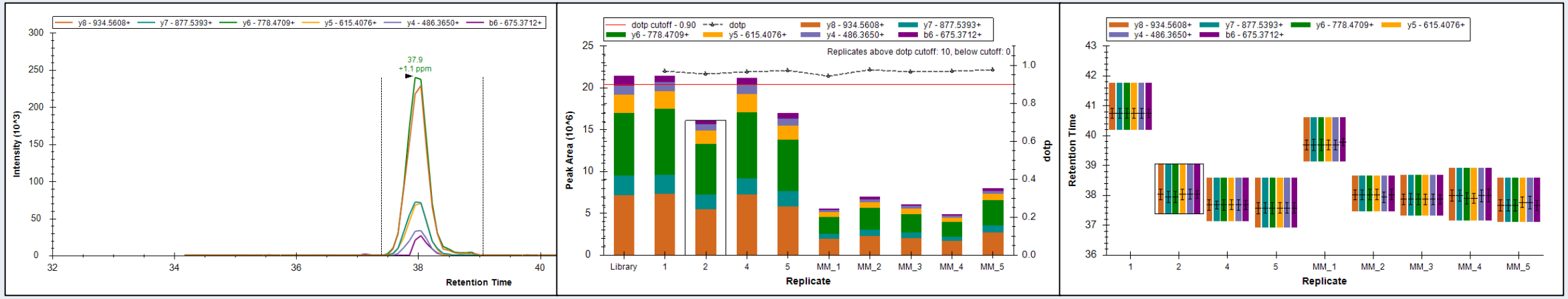


- 6 good peptides identified for the protein
- Relative AUC values look consistent across the replicates and samples
- Average AUC values from Reference samples are 2-3x more abundant than average AUC values from QC sample
- RT values look very consistent

Evaluating Experimental Methodology

Reference vs. QC at Peptide Level

AOA3Q2HNQ - LWVYELLLK



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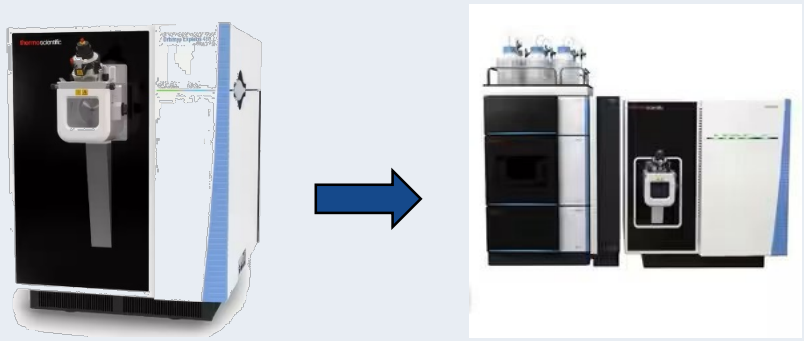
Transitioning to High Throughput Methodology using the Altis Triple Quadrupole

- ✓ Instrument functioning properly
- ✓ Digestion adequate
- ✓ Data analyses – proteins and peptides are detected in ratios expected
- ✓ Generated chromatogram library, selected high quality peptide targets

Utilize triple quadrupole for high throughput screening methods of relevant peptides and proteins

Key Considerations when Transitioning to Triple Quadrupole

- Experimental scale
  - Multiplexed peptide detection
  - Large number of samples
  - Cost per sample
  - Sample throughput critical
- Maintaining high data confidence
- Method changes to sample prep and data processing
- Determining peptide retention times



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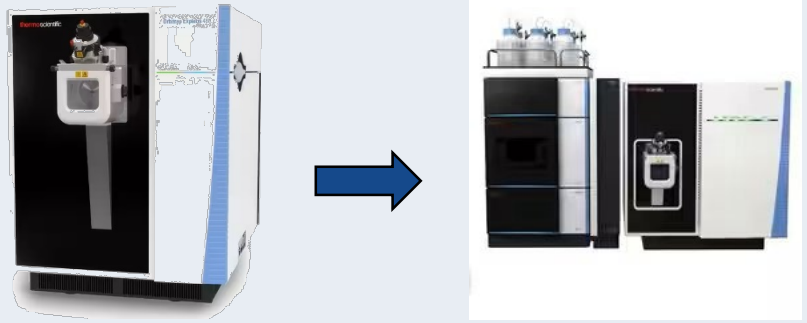

	Orbitrap	Altis (QQQ)
Stock serum analyzed	0.00625 ul serum	0.375 ul serum
Protein amount analyzed	0.5 ug serum protein	30 ug protein
Method Run Time	65 minutes	20 minutes
LC flow rates	300 nl/min (0.3 ul/min)	150 ul/min
Analytical column	EASY-Spray™ C18 Reversed Phase HPLC Column, 3um, 75um x 150 mm	Two columns connected Acclaim™ PepMap™ C18 Reversed Phase HPLC Column, 3 um, 1 mm x 150 mm Total column length: 300 mm
Data software	Skyline, EncyclopeDIA	Skyline, TraceFinder

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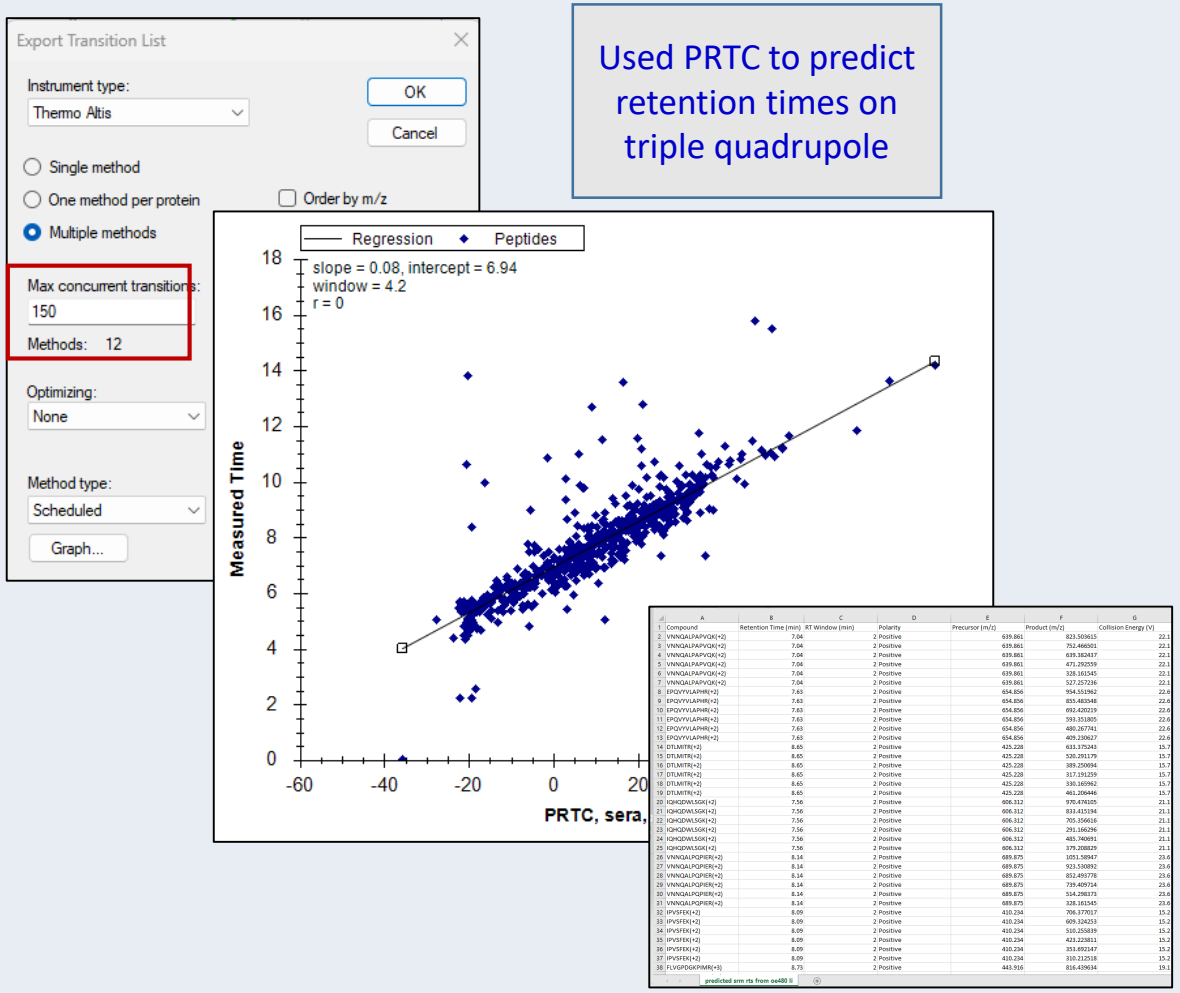
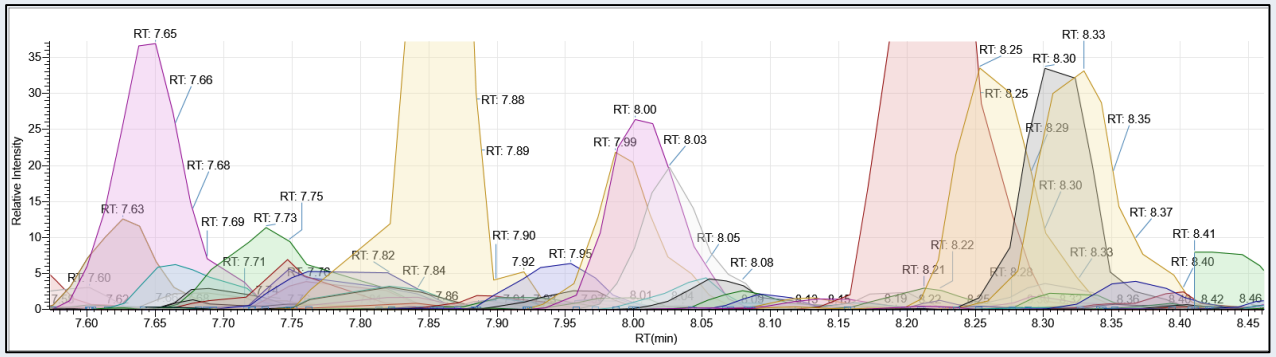
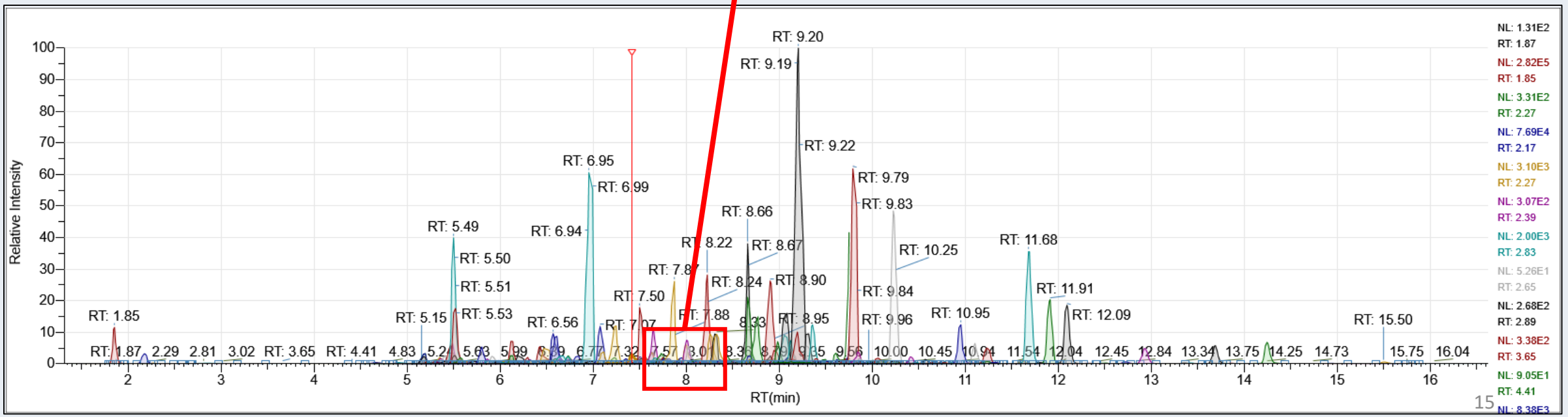


Used PRTC to predict retention times on triple quadrupole

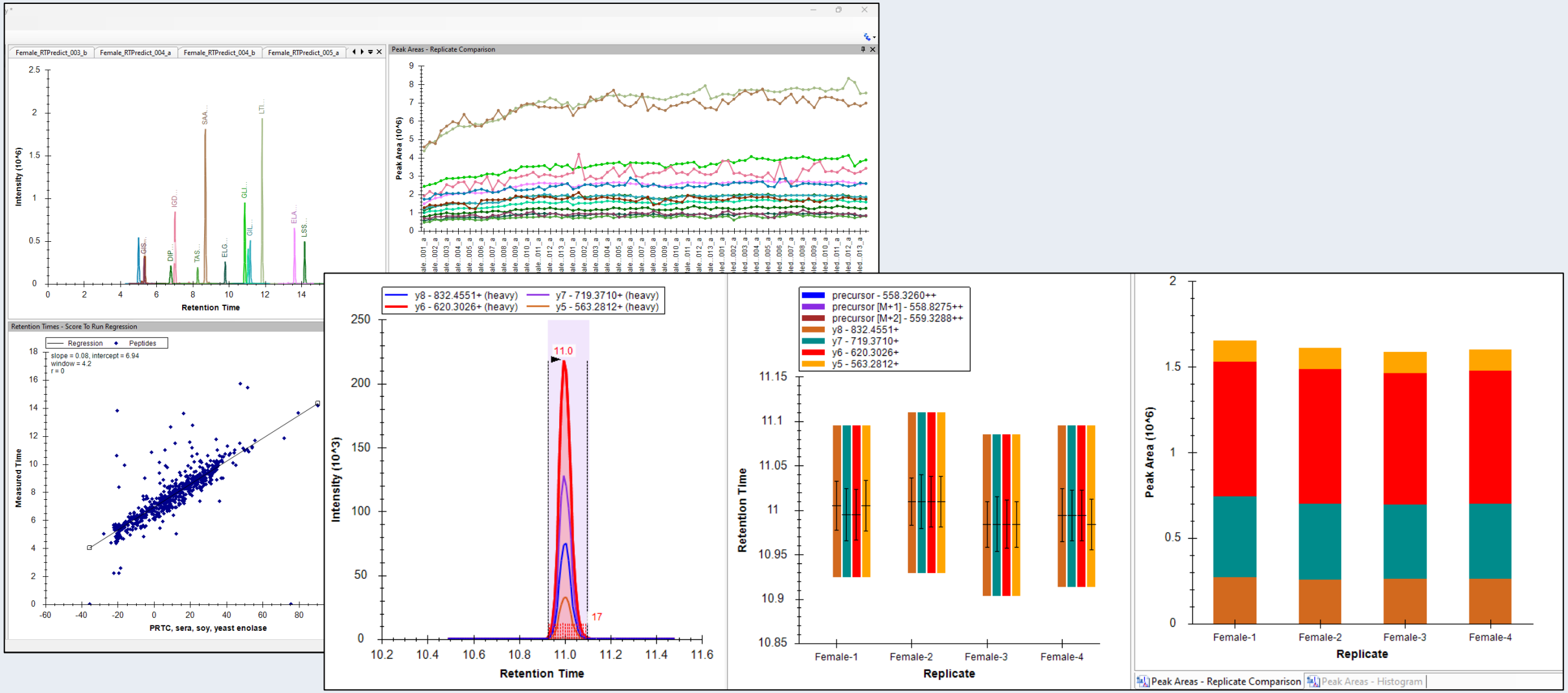
1400 SRM transitions from >400 peptides exported from the LFQ DIA experiments on the OE 480

- LFQ DIA data used to generate spectral libraries for all subsequent proteins, peptides, and LC-MS/MS data
- Relative RT values determined based on PRTC elution profile on discovery and targeted quantitation
- Each peptide has 3 SRM transitions based on rank order from LFQ DIA results
- More importantly, all CE values were derived directly from established equation based on precursor m/z and charge state in Skyline





Reproducibility of Methodology – PRTC SRM data

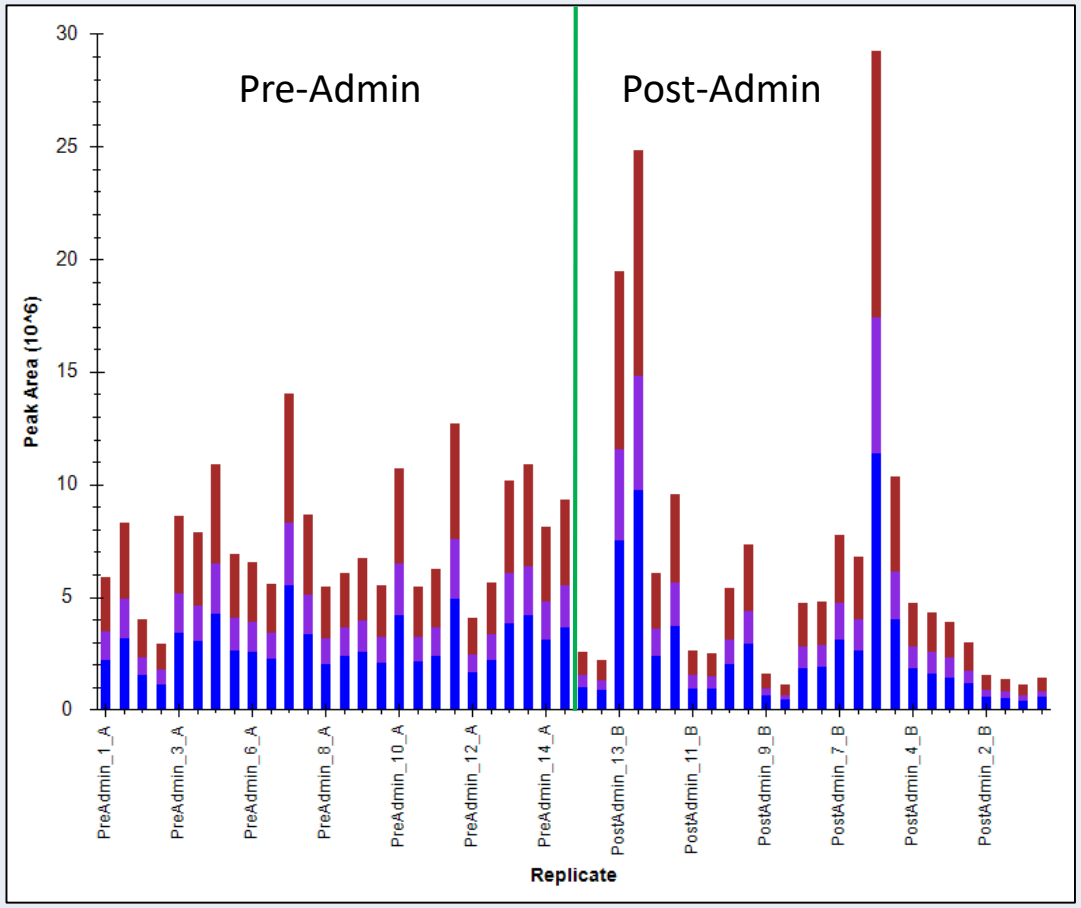


SRM Results Summary

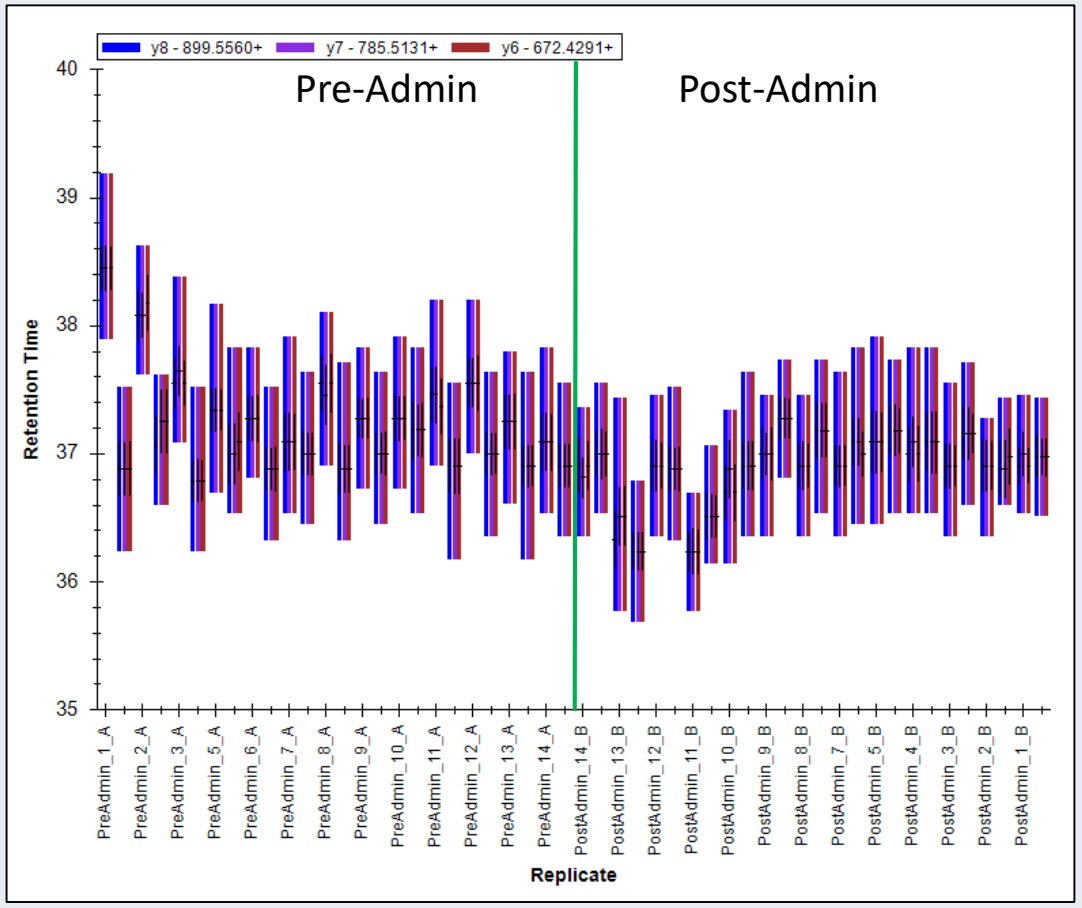
- Selected peptides for SRM had CV's < 30%, dotp value >0.5 were evaluated for use as biomarker
- Initially evaluated 175 proteins, 728 peptides, 6542 transitions
- Ultimately refined targets to 132 proteins, 472 peptides, 1402 transitions
  - Three transitions per peptide
  - Two peptides per protein

Detecting Bisphosphonate biomarkers of relevance - Fibronectin

Peptide Peak Area



Retention Time



Fibronectin, Q28377
R.GATYNIIVEALK.D

Conclusions

- The developed SRM method is able to **routinely detect thousands of peptides** in a **short analytical run time**, generate **reproducible and stable retention time**s
- We can **screen for thousands of peptides in a short analytical method**, enabling **higher throughput** of samples
- Routine detection of established peptide biomarkers allows us to **determine differential expression for thousands of peptides**

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