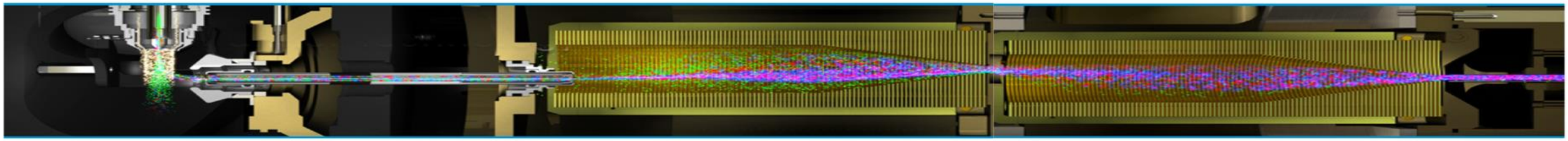


Targeted Protein and Peptide Quantitation Using Agilent LC/MS and Skyline Software

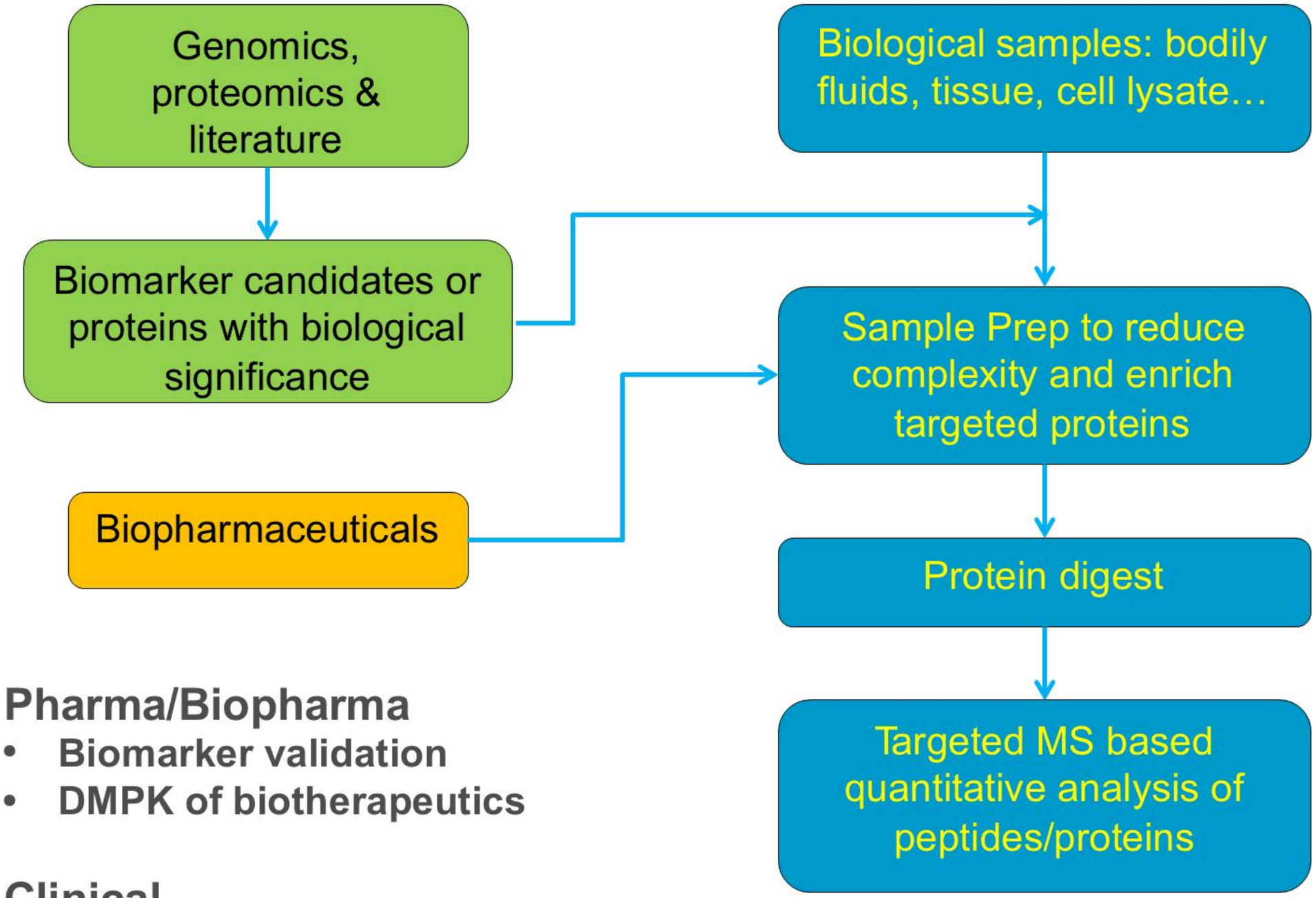
Alex Zhu, Ph.D.

LC/MS Applications Scientist

Agilent Technologies

Wilmington, DE

Targeted Protein Quantitation



Pharma/Biopharma

- **Biomarker validation**
- **DMPK of biotherapeutics**

Clinical

- **Translational research**

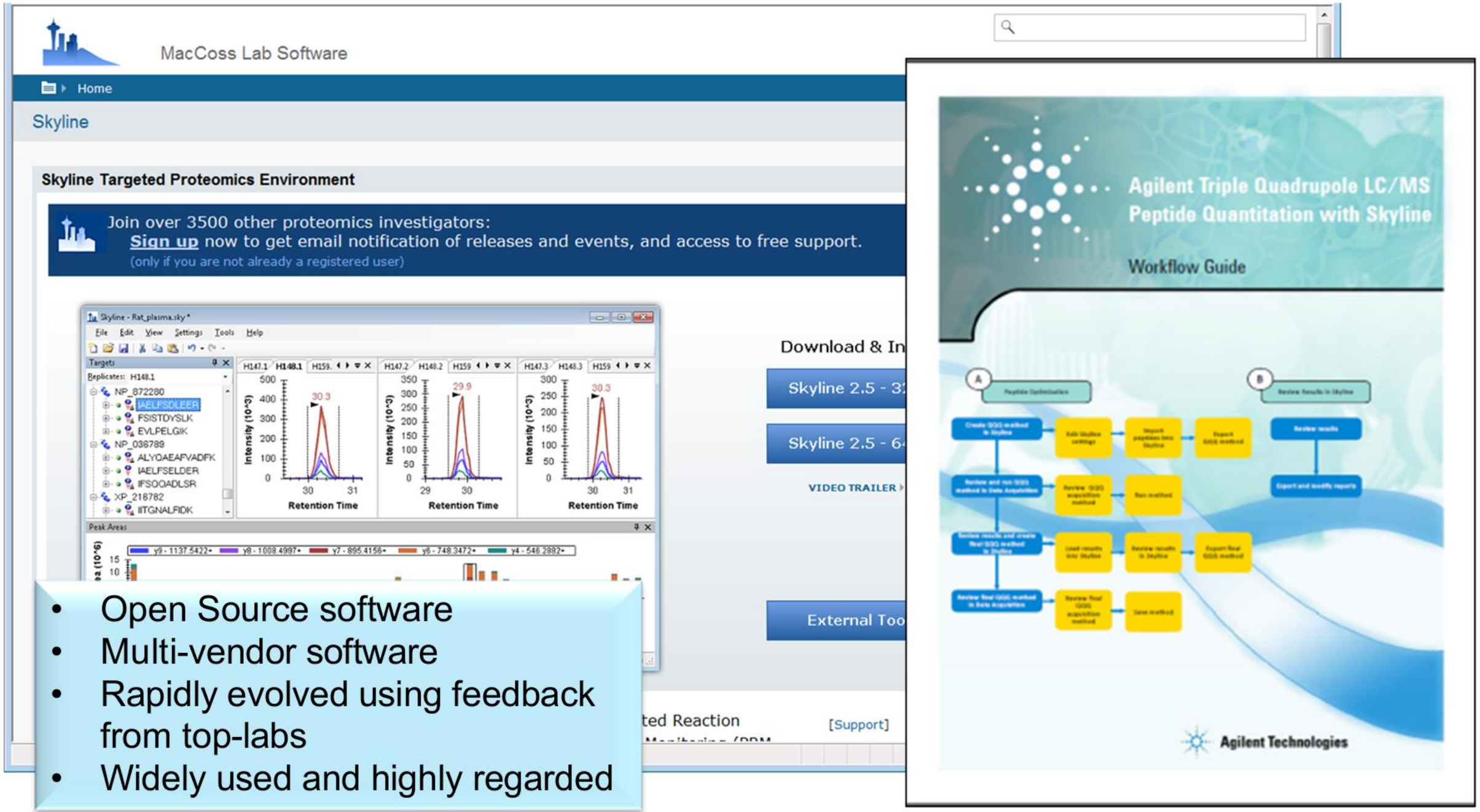
MRM Based Quantitation Workflow Overview

- Selection of target proteins
- Selection of target peptides that represent the protein list
- Selection of best charge states and MRM transitions for each targeted peptide
- Optimization of collision energy for each MRM transition
- Application of the assays to the detection and quantitation of the proteins

MRM Assay Development – Current Approaches

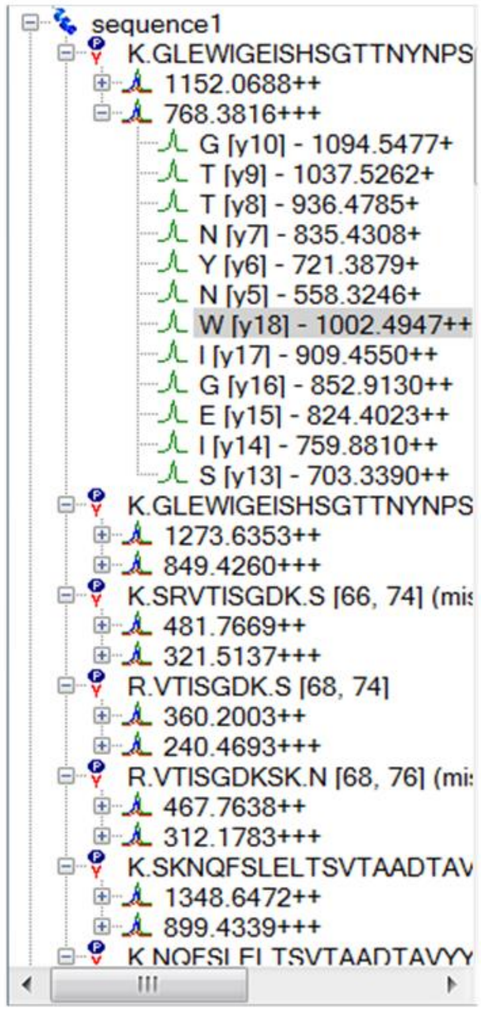
1. Data dependent acquisition (Auto MS/MS on a QTOF) → Database search → Create MRM list or spectral library to use in Skyline
2. MS2 Scan followed by Product ion scans on a QQQ
3. Publically available databases (PeptideAtlas, the Global Proteome machine Database, etc.).
4. Skyline MRM predictions.

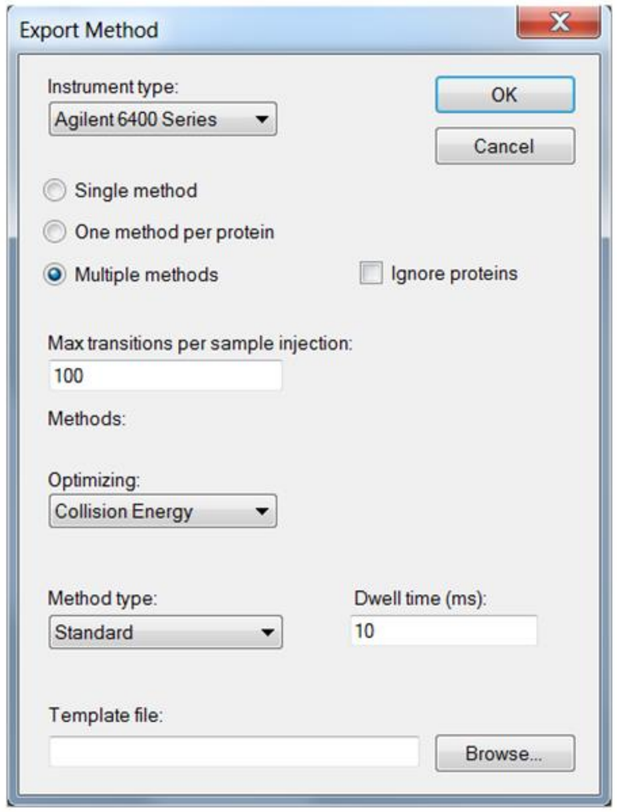
MRM Assay Development – Current Approaches From Skyline Prediction

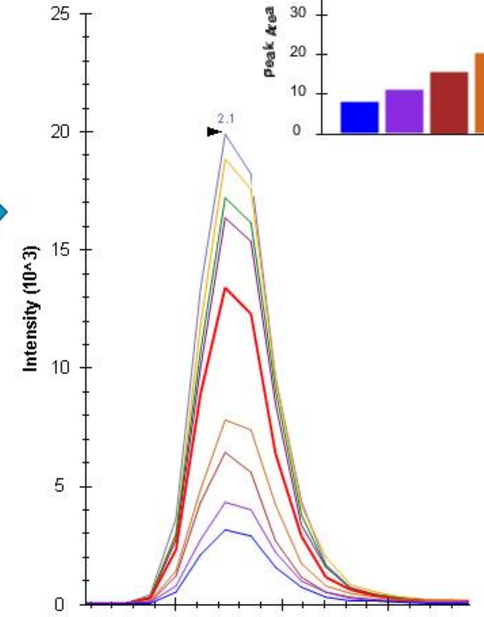


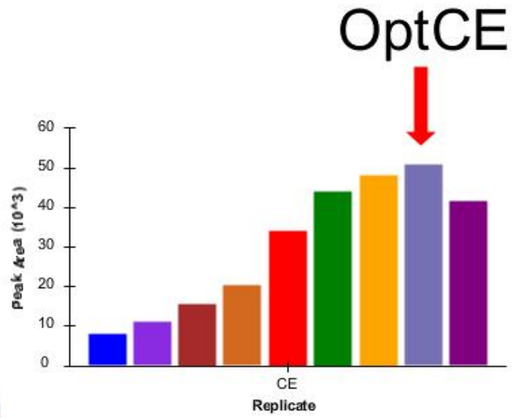
- Open Source software
- Multi-vendor software
- Rapidly evolved using feedback from top-labs
- Widely used and highly regarded

MRM Assay Development – Current Approaches From Skyline Prediction





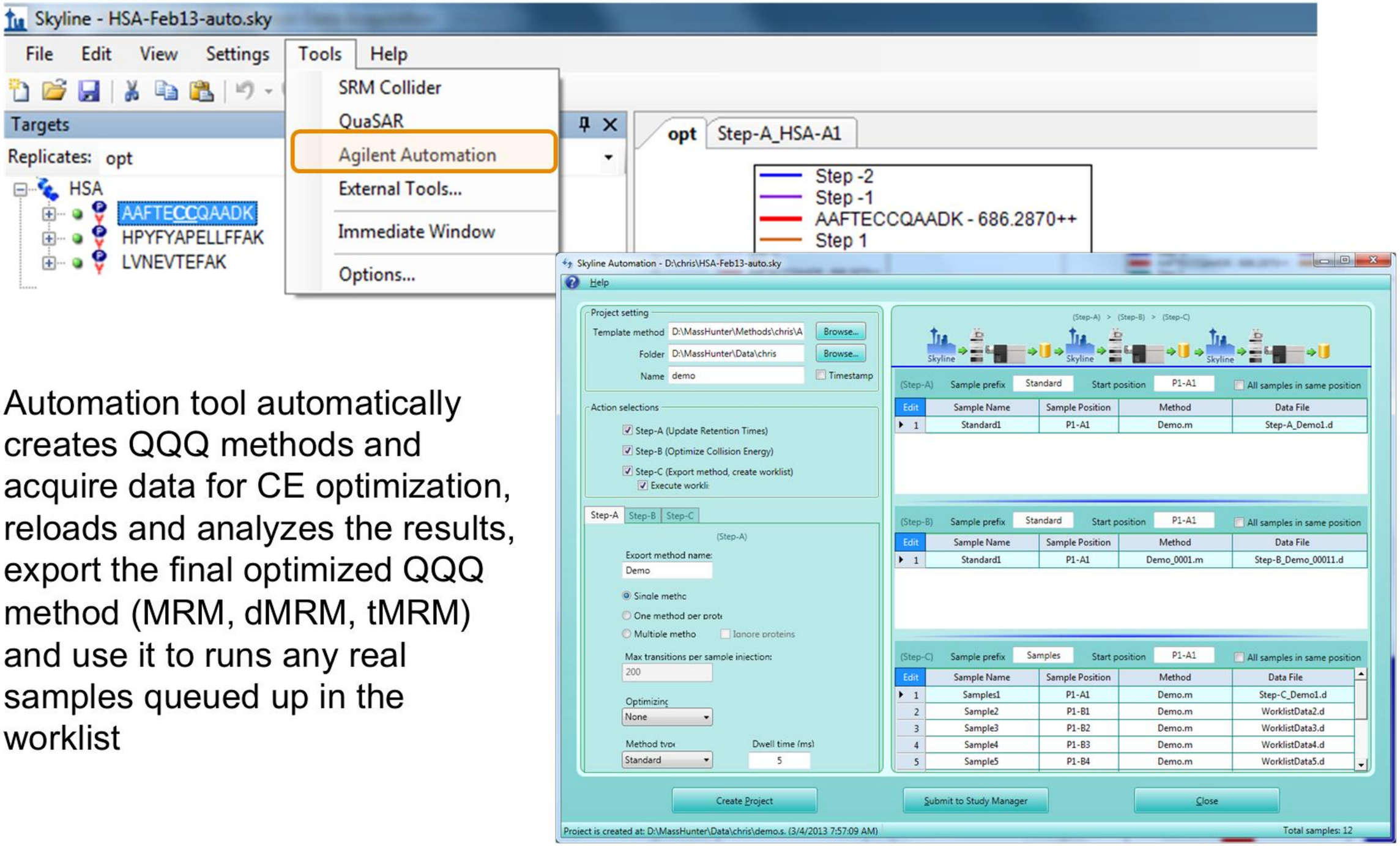




For this mAb, the entire list of > 40 peptides need to be evaluated. The number can be even bigger if including peptides with miscleavages.

CE Optimization Using Agilent Automation Tool

Automation tool automatically creates QQQ methods and acquire data for CE optimization, reloads and analyzes the results, export the final optimized QQQ method (MRM, dMRM, tMRM) and use it to runs any real samples queued up in the worklist



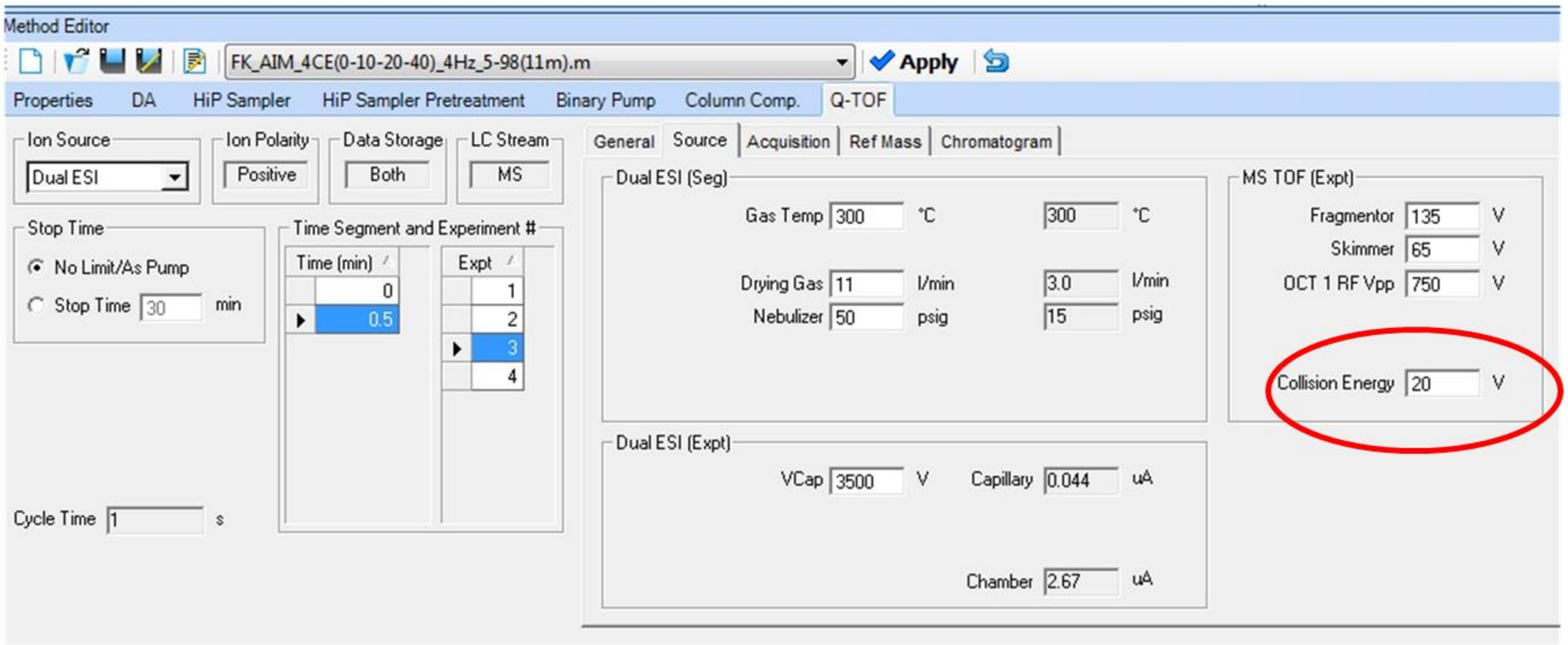
Challenges

- No prediction for representative peptides/preferred charge state.
- Questionable prediction for modified peptides.
- Numerous runs for collision energy optimization based on the number of proteins and peptides.

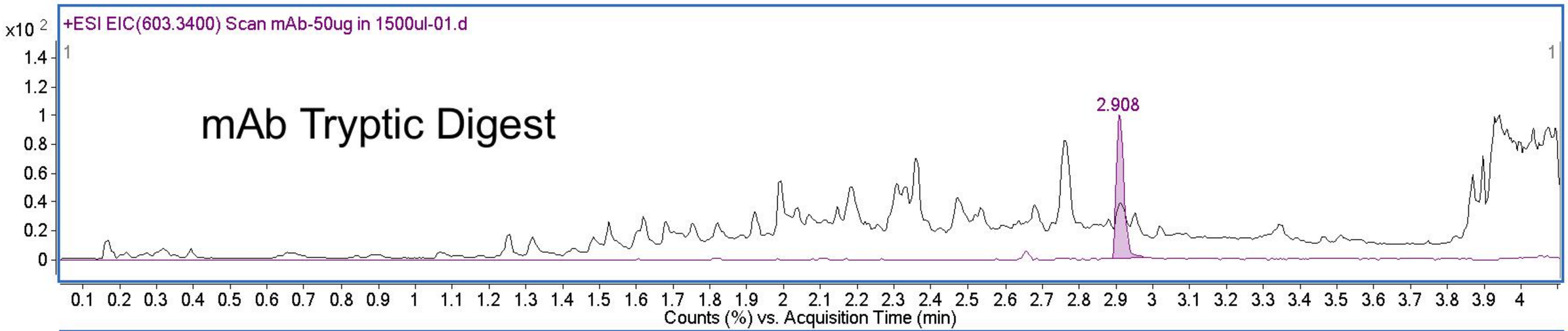
Simplified MRM Method Development:
Agilent All Ions MS/MS on a QTOF + Skyline SW

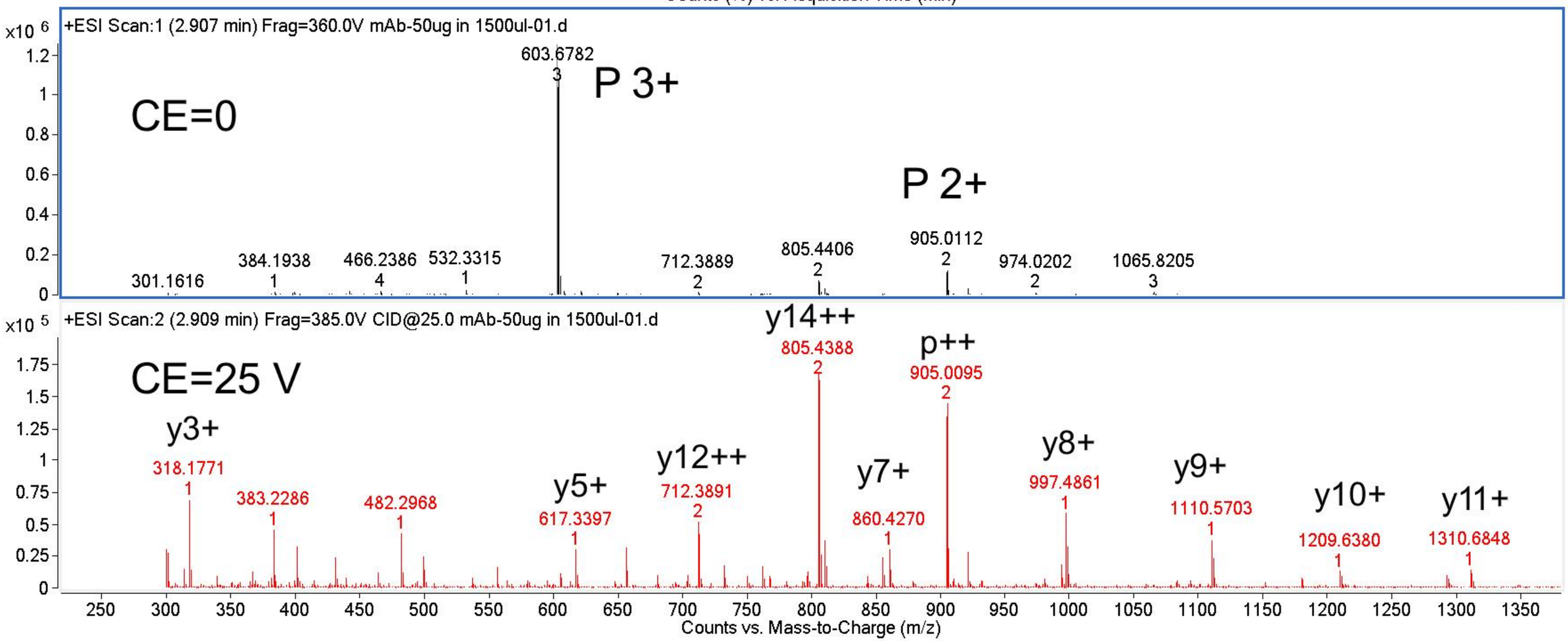
Agilent All Ions MS/MS :

- Data independent acquisition, no precursor selection, short cycle time
- Easy acquisition set up
- Stores MS and MS/MS spectra for all detectable peptides



All Ions MS/MS on Agilent QTOF

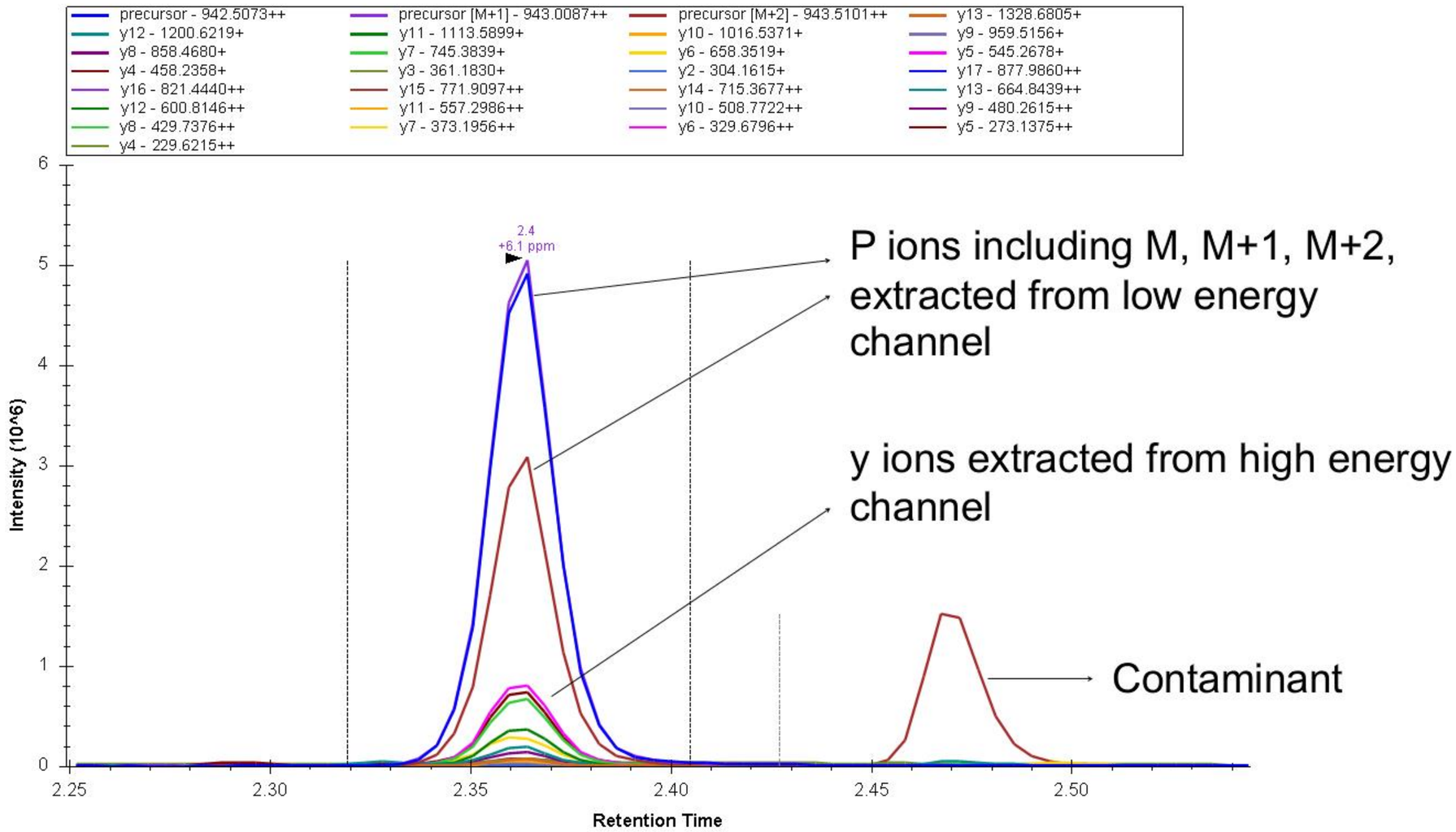




All Ions → Skyline Workflow

Step 1: Show p & y Ions for Peptide Confirmation

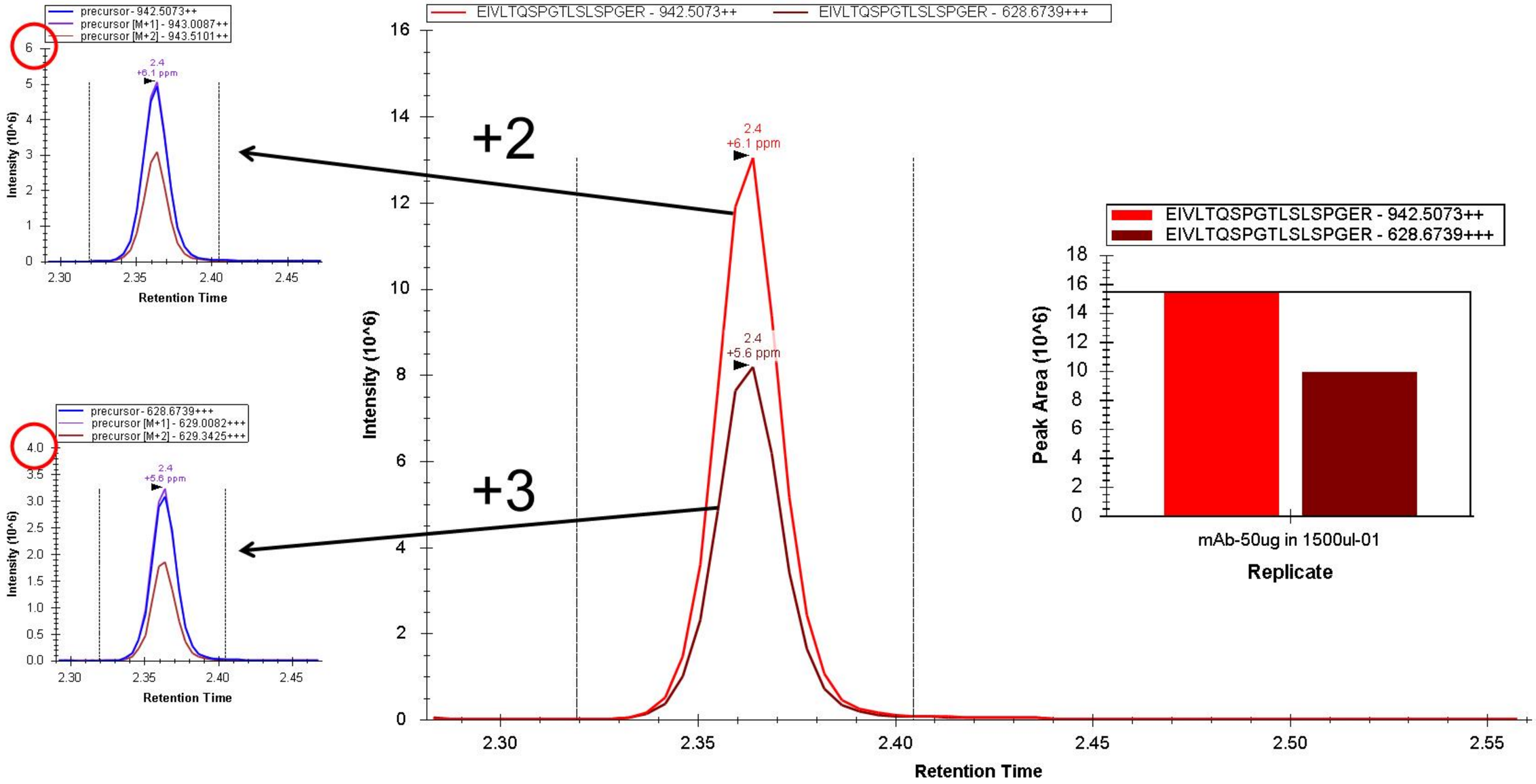
- Precursors (p ions) from low energy channel and products (y ions) from high energy channel are automatically extracted and plotted together to easily confirm the identity of the peptides.



All Ions → Skyline workflow

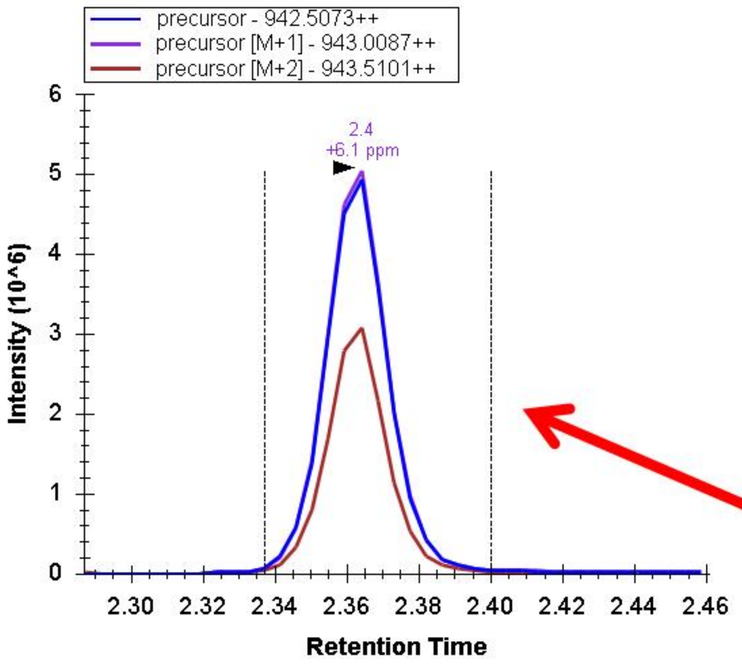
Step 2: Show only p Ions to Select Best Charge State

Best charge state for each peptide is easily determined by comparing p ions only

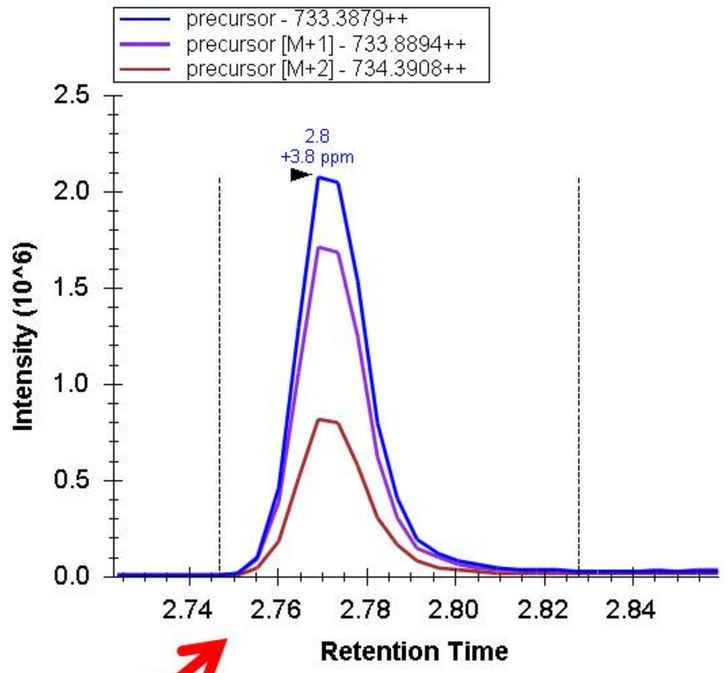


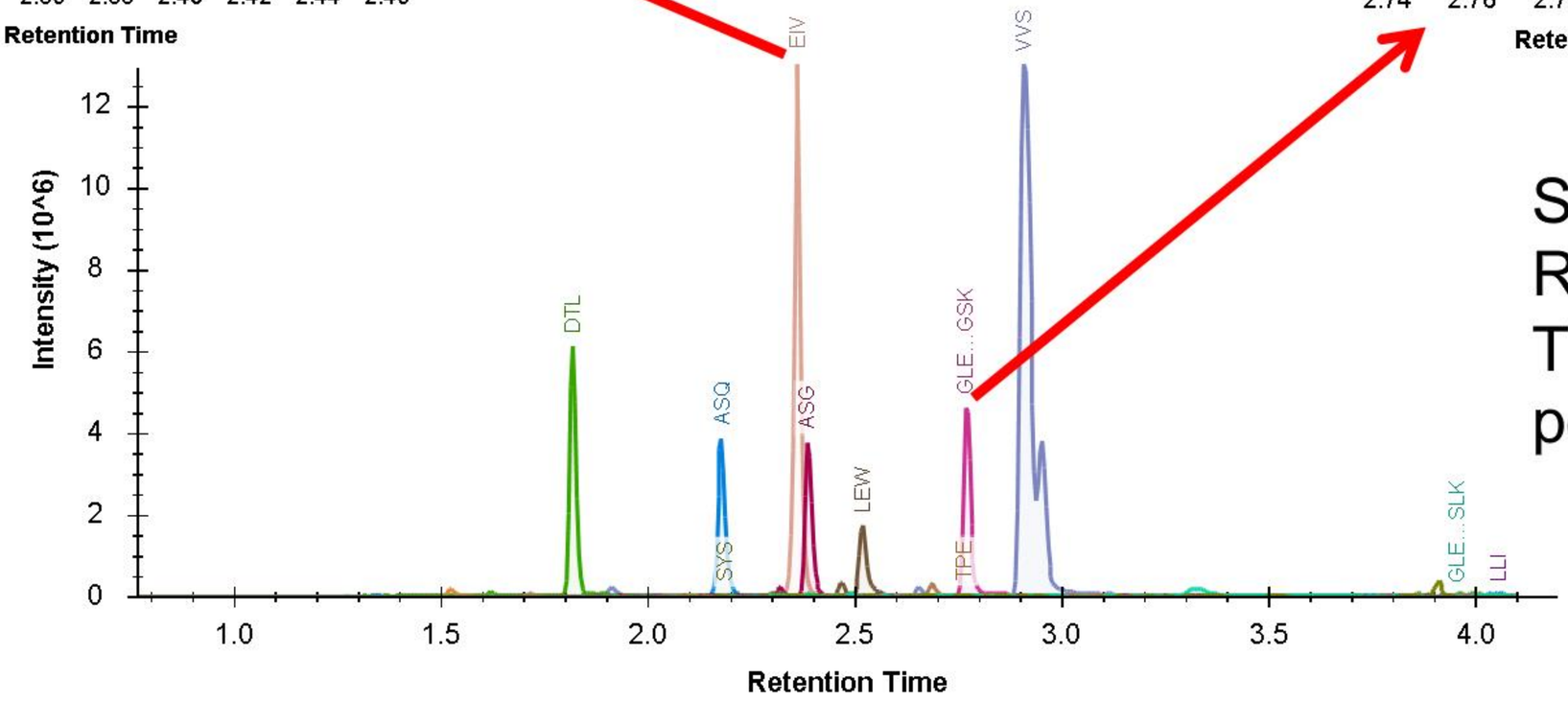
All Ions → Skyline workflow

Step 3: Show only p ions to Compare Peptide Abundance



Peptides with the highest signal are easily determined by comparing p ions from the best charge state of all peptides.

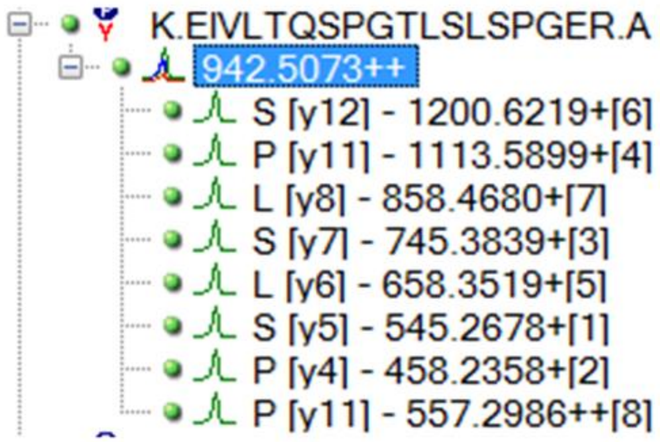




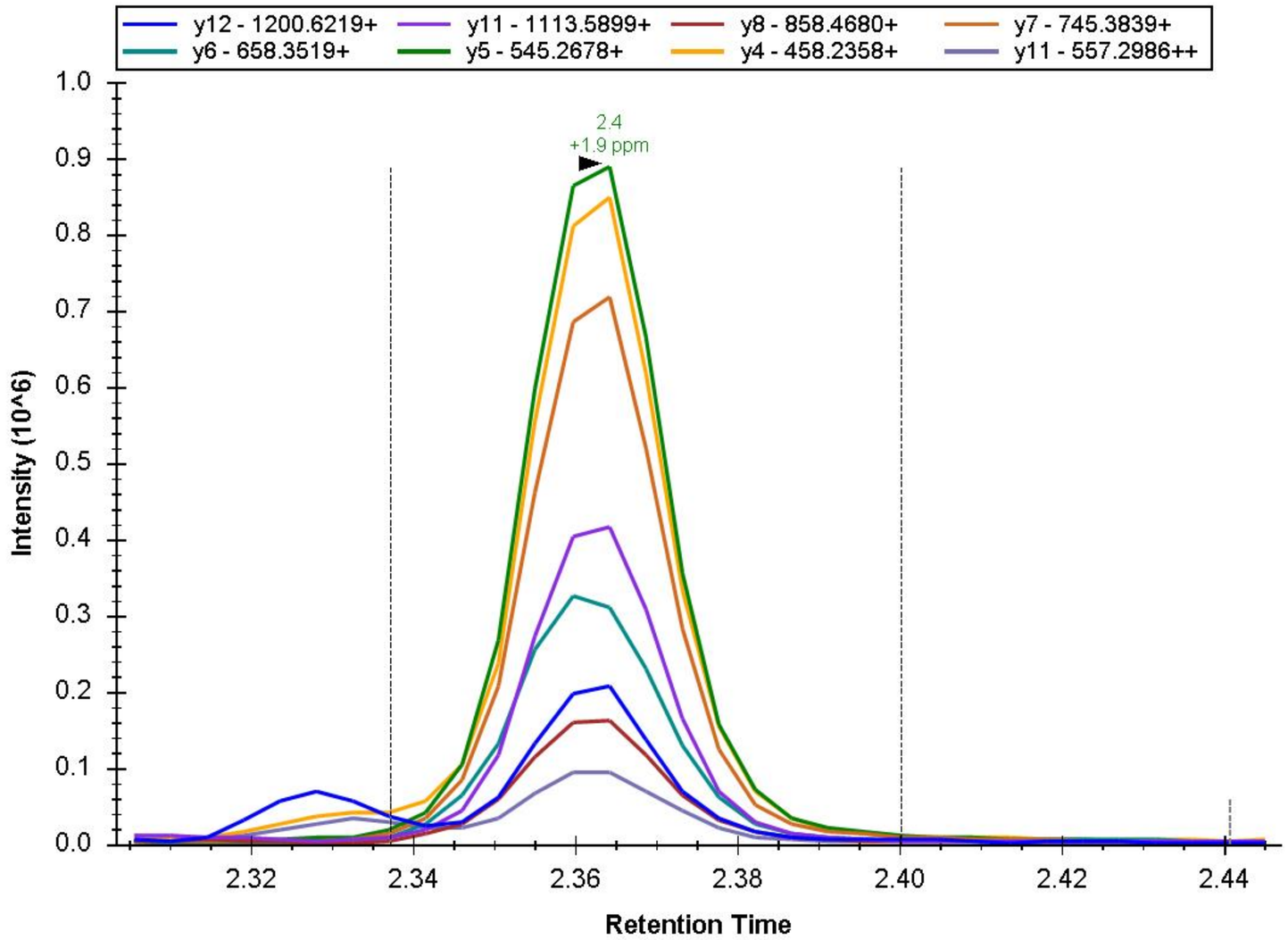
Skyline peptide Rank Refine Tool picks top n peptides

All Ions → Skyline workflow

Step 4: Show Only y Ions to Select Top MRMs

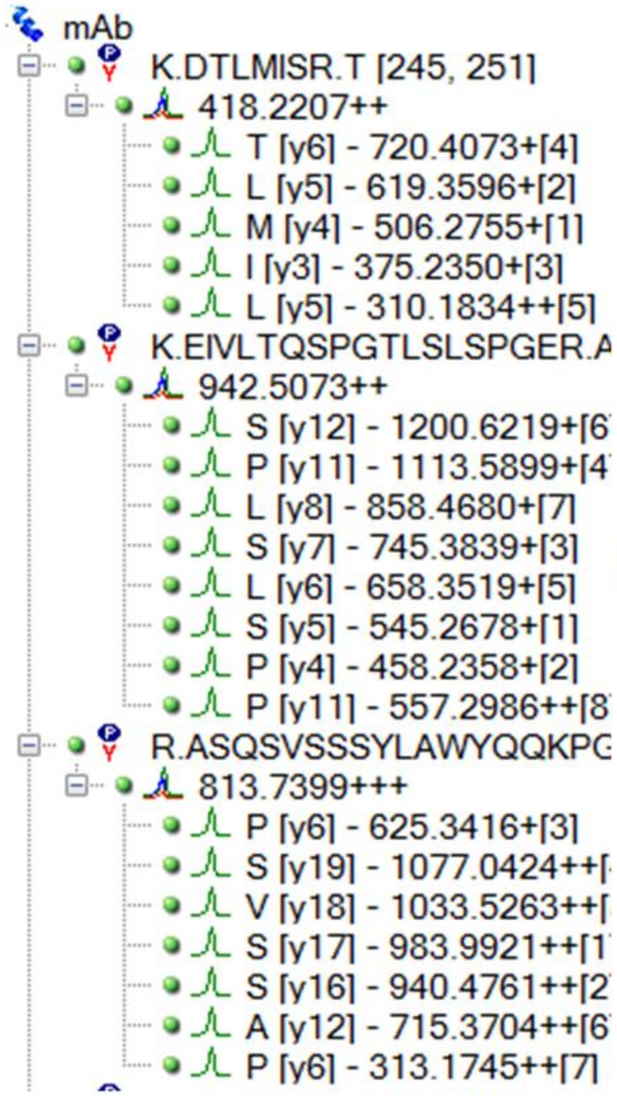


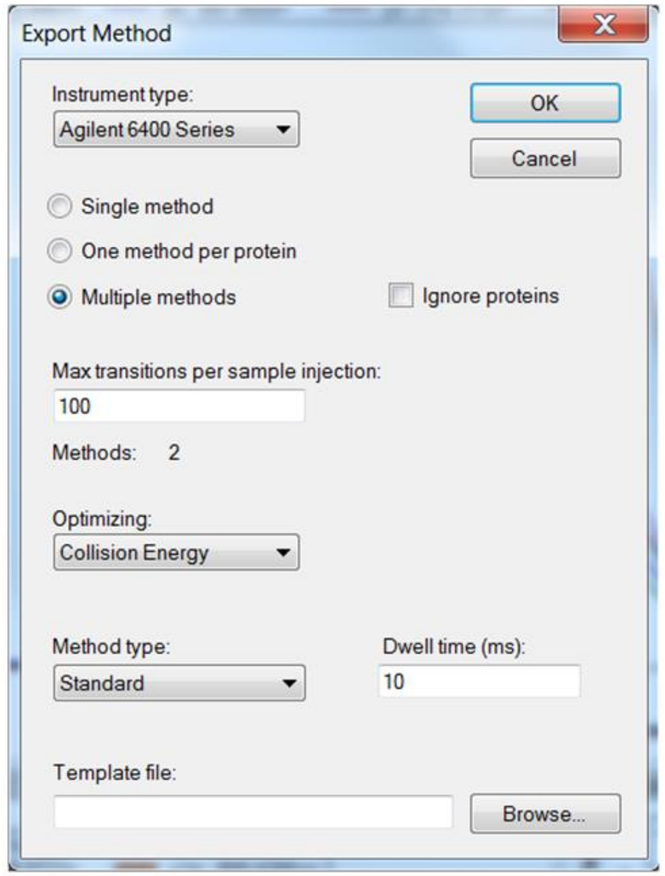
Skyline Transition rank refine tool picks top n MRMs

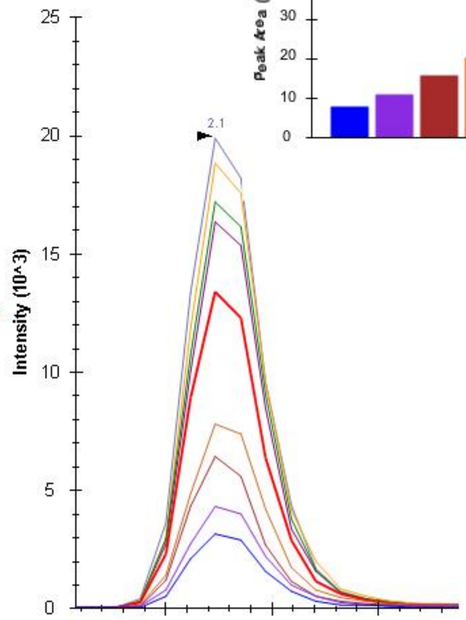


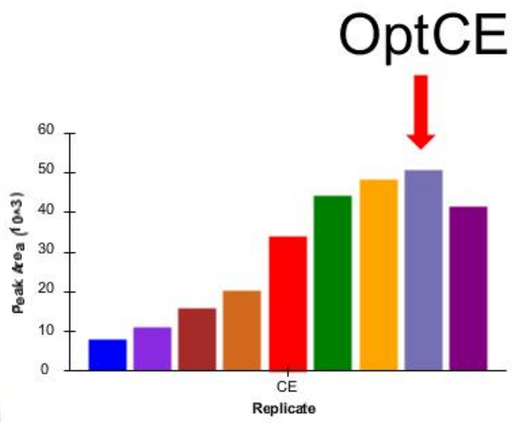
All Ions → Skyline workflow

Step 5: CE Optimization









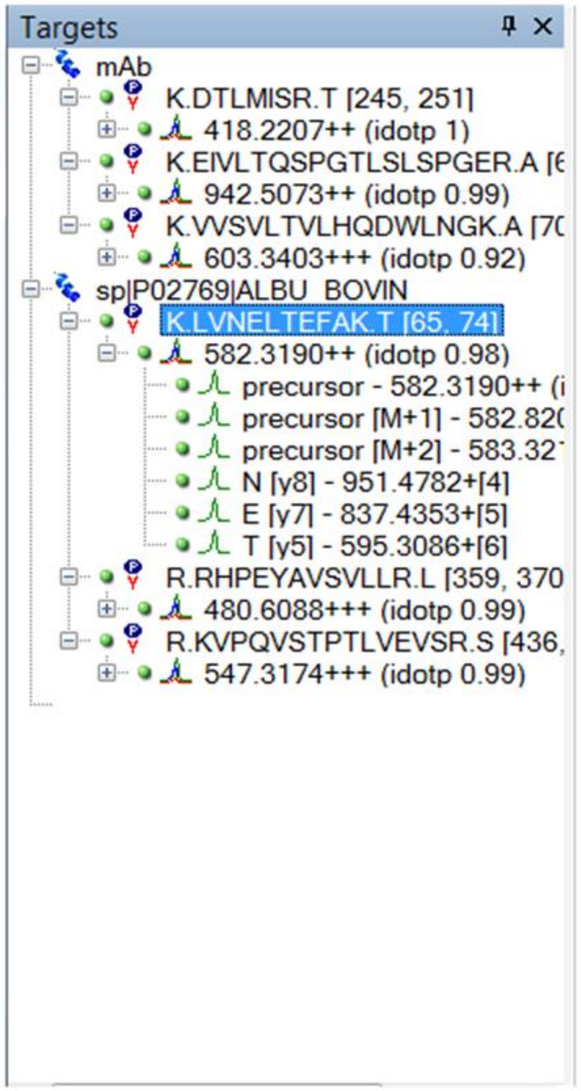
CE optimization process can be automated using new Agilent Automation Tool integrated into Skyline SW

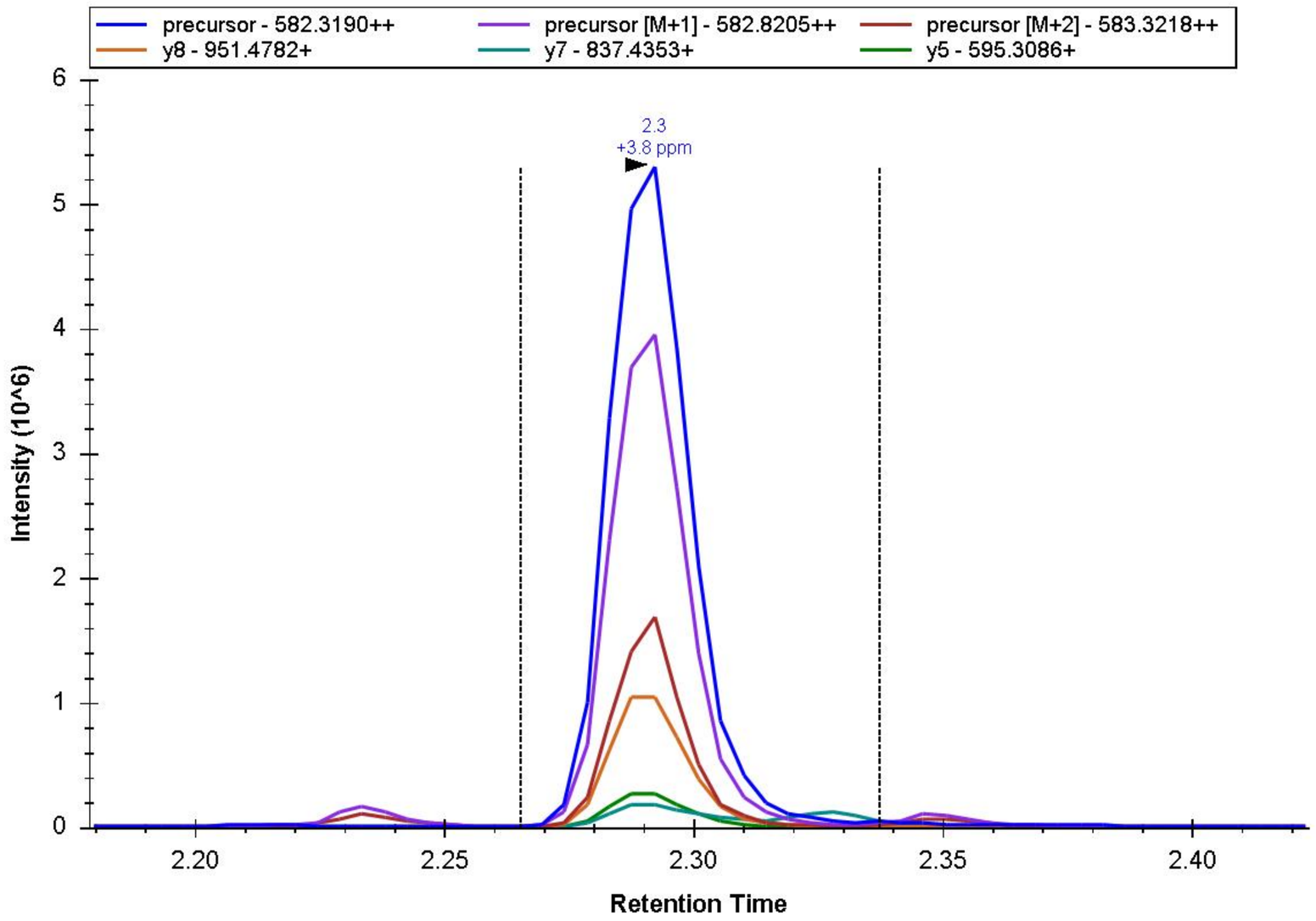
Skyline Prediction Workflow vs. All Ions-Skyline Workflow

mAb Tryptic Digest	Skyline Prediction Workflow	All Ions – Skyline Workflow
Number of peptides for CE optimization	40	3 (Selected by All Ions run)
Injections required for CE optimization	40 (One inj. for one peptide)	3
Total time required for MRM method development	40×10 = **400** min	(3+1)×10=**40** min (1 is for All Ions run)

Save up to 90% of time and 90% of samples needed for CE optimization

Method Development for Multiple Proteins

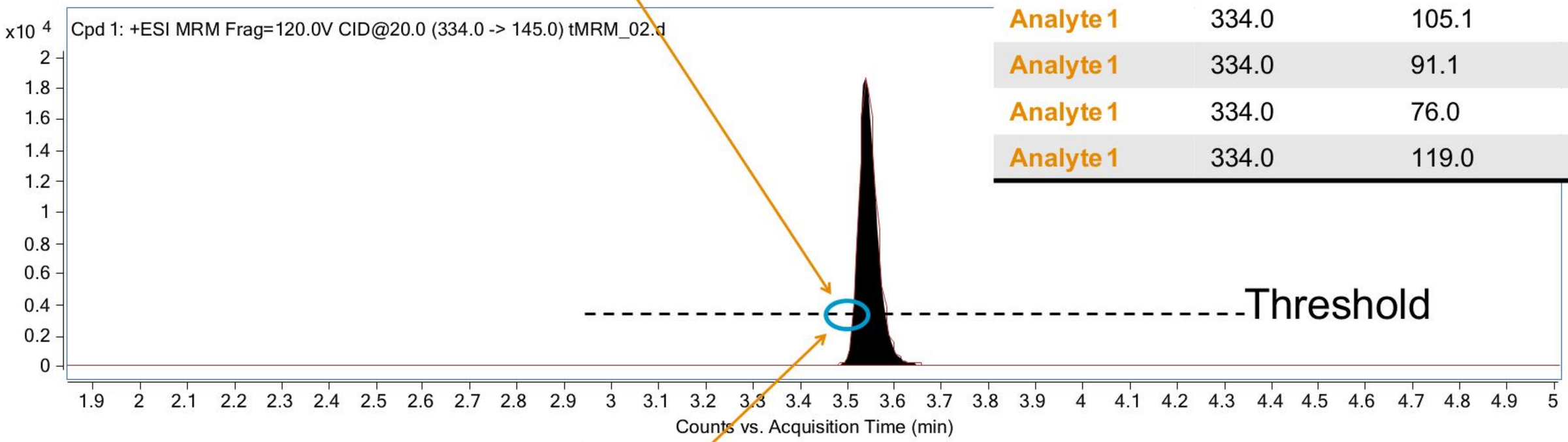




- Method development for multiple proteins can be done from the same All Ions result, simultaneously or at a later time.
- Import a different protein sequence and load the same All Ions data. Same procedure followed.

Triggered MRM (tMRM) Analysis

Secondary MRM
Transitions are “Triggered”



Triggered cycle (above threshold)

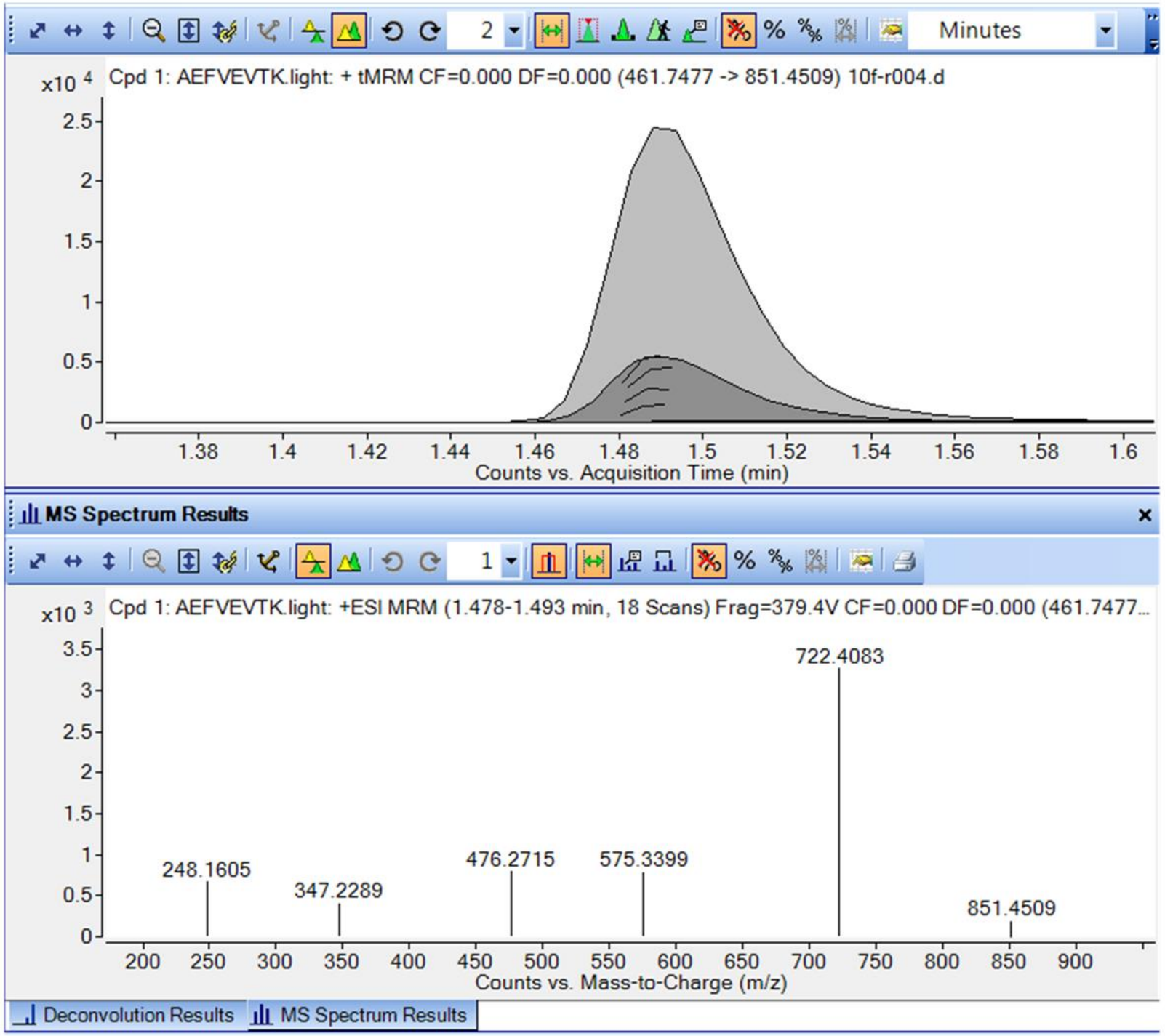
Analyte 1	334.0	145.0
Analyte 1	334.0	117.0
Analyte 1	334.0	132.1
Analyte 1	334.0	105.1
Analyte 1	334.0	91.1
Analyte 1	334.0	76.0
Analyte 1	334.0	119.0

Primary cycle (below threshold)

Analyte 1	334.0	145.0
Analyte 1	334.0	117.0

Less cycle time comparing to product scan
Secondary transitions only collected for compounds found to be present

tMRM Example

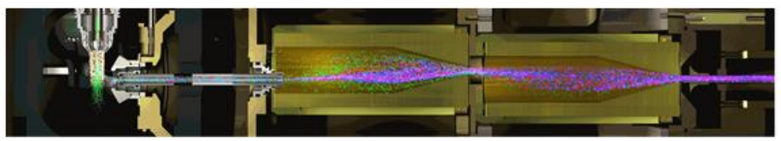


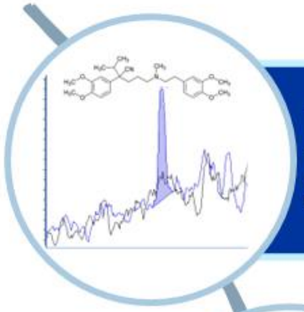
All Ions-Skyline Workflow Summary

With new All Ions – Skyline method development workflow:

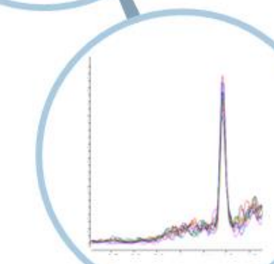
- MRM method development is much faster and simpler
- Agilent automation tool in Skyline SW automatically optimizes CE, creates and exports final QQQ method and runs the worklist
- Database search or spectral library is not needed
- Can be used for single protein or multiple proteins method development, as well as revisit for different proteins without more sample runs

6495 QQQ LC/MS - Premium Performance

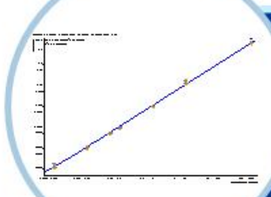




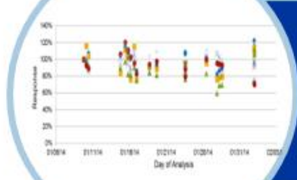
Improved sensitivity (IDL / MDL) – Average 3x in S/N specifications and applications



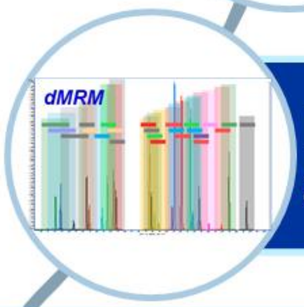
Improved precision and excellent accuracy at the lowest levels – 4 to 5x in IDL spec



Proven 6 orders of linear dynamic range

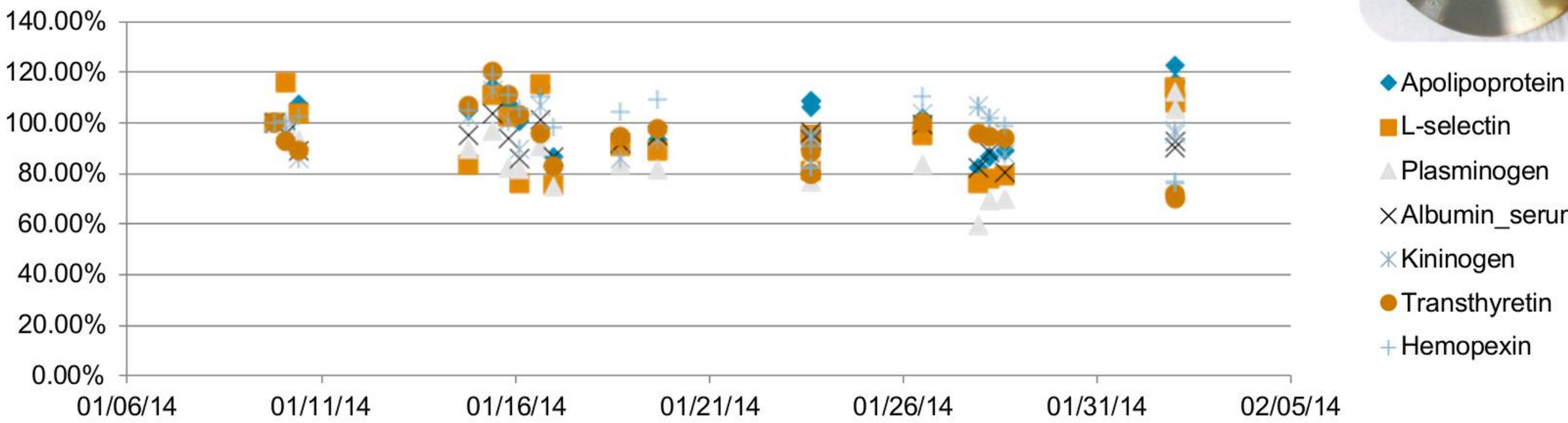


Proven robustness in complex matrix – Food matrix and biological matrix (plasma)



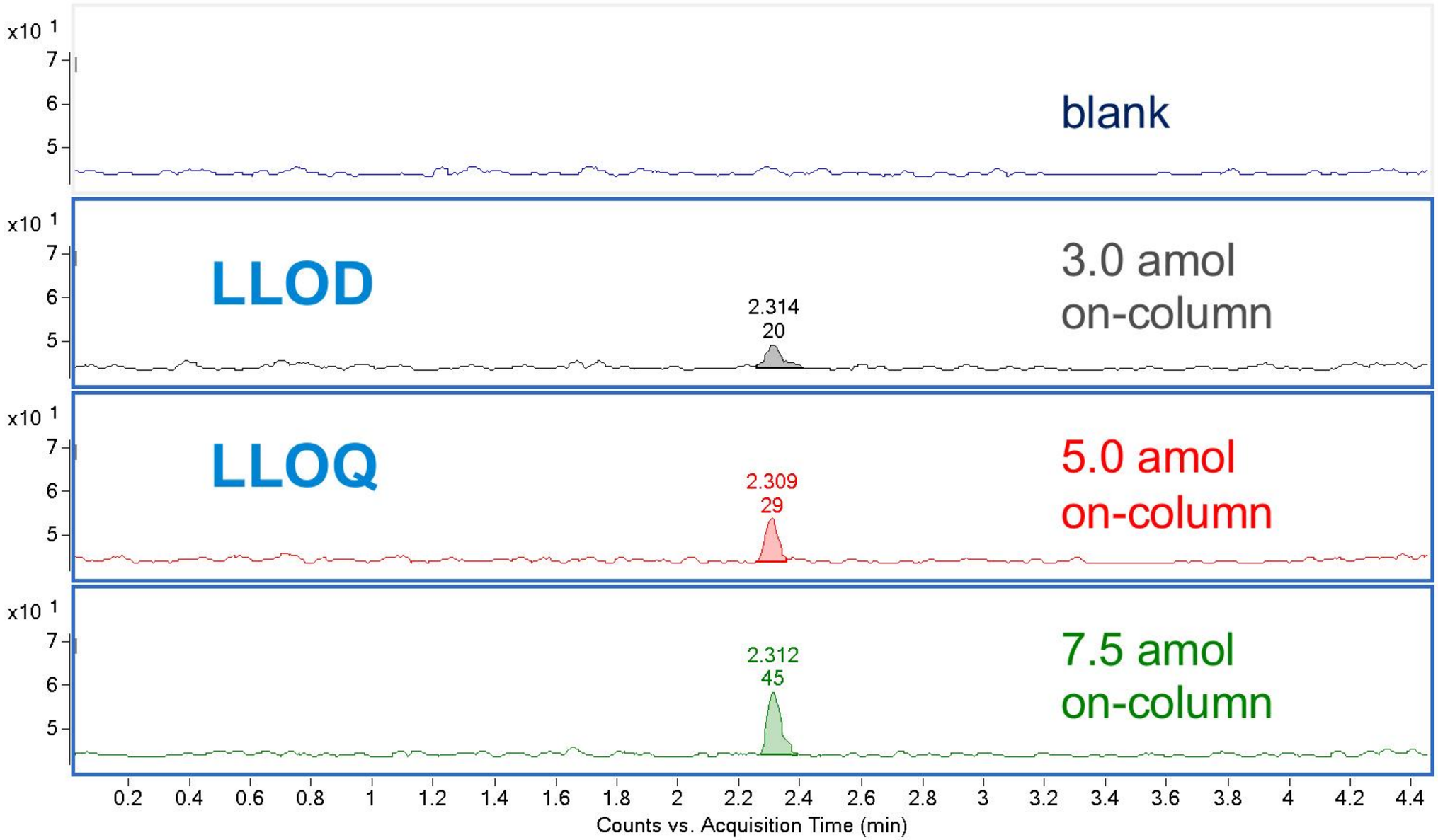
Improved mass range, fast scan speed and MRM acquisition rate

Proven System Robustness in Complex Matrix: Protein Quantitation in Plasma



- Selected peptides from 42 peptides in the QC sample – normalized to Day 1 response
- **Peptide QC samples** analyzed daily after every ~25 plasma digest injections
- No significant signal degradation observed after **853 injections** of **40 µg plasma digest per injection** and **3.5 weeks of continuous operation**
- Response %RSD: 6 - 15

Peptide Quantitation: Outstanding Sensitivity with Standard Flow Chromatography

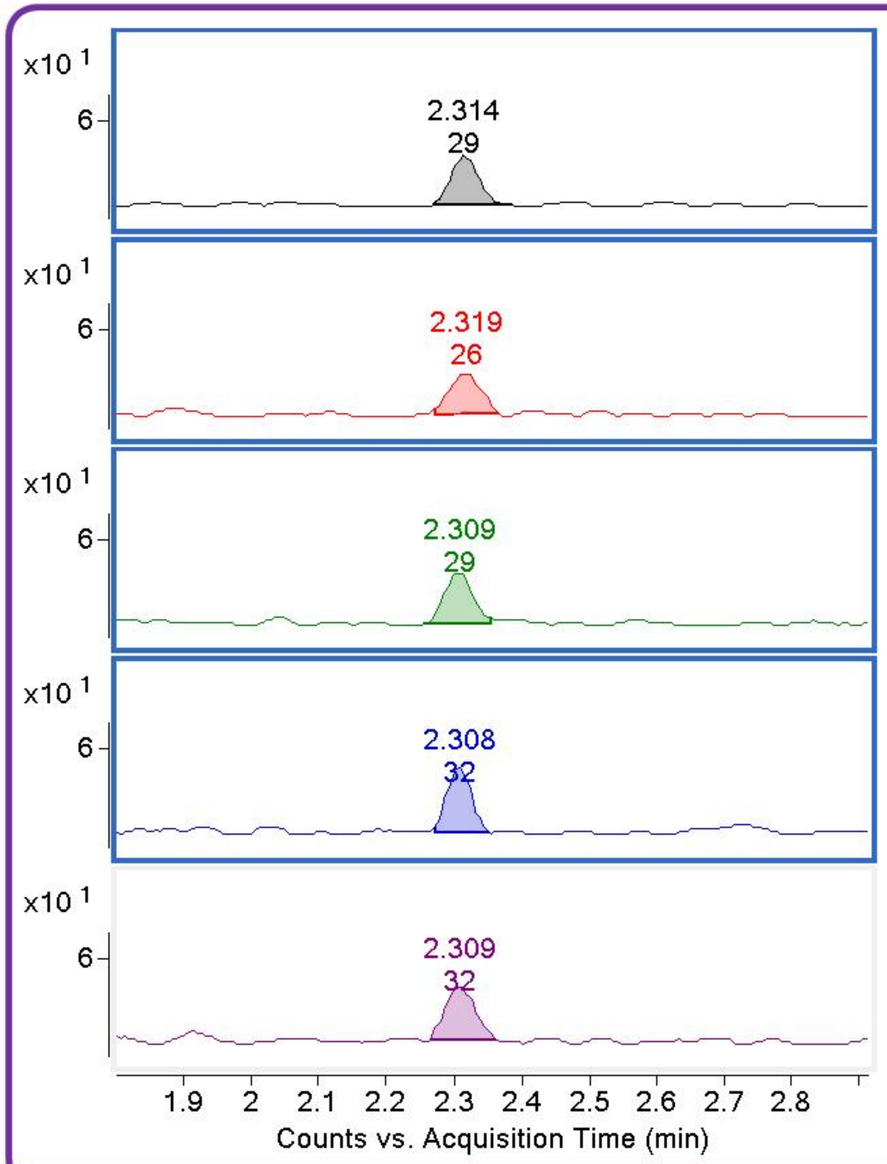


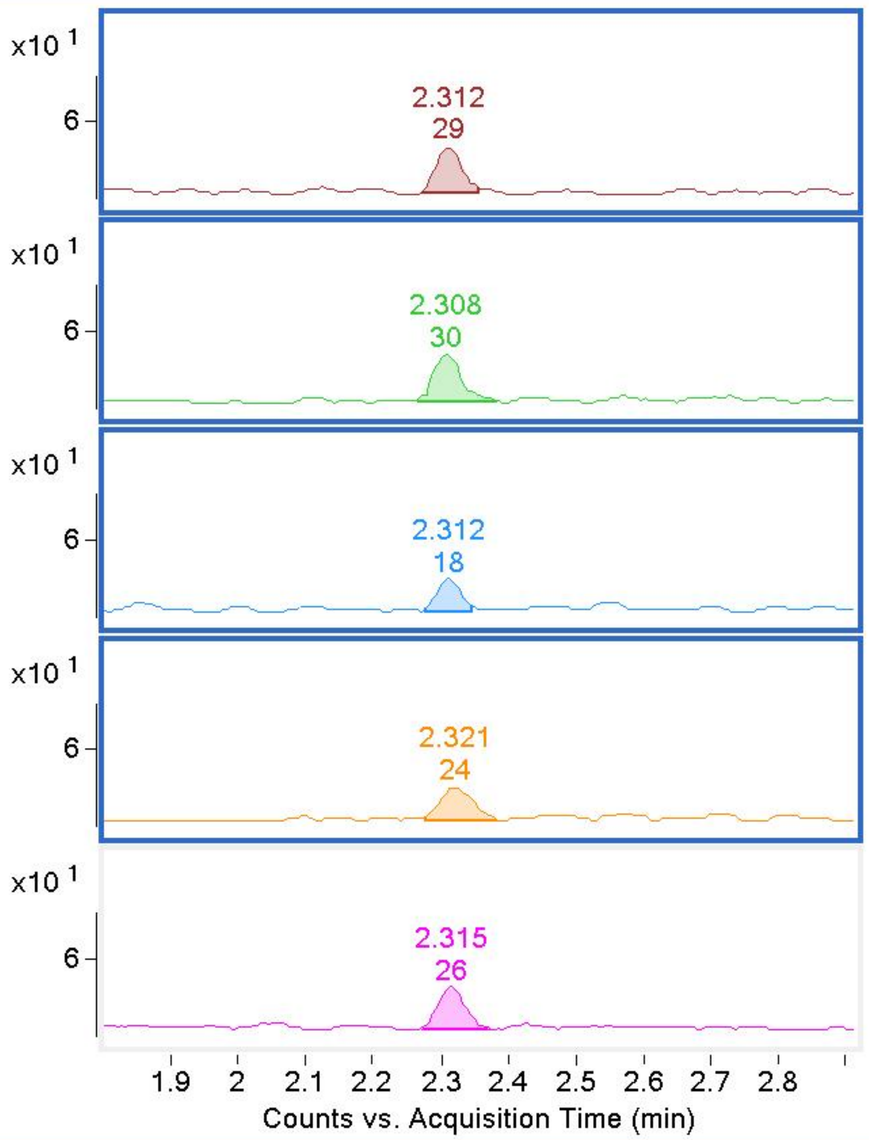
Instrumentation: Agilent JetStream + 6495 QQQ + 1290 UHPLC (2.1mm column, 0.6ml/min)
Sample: synthetic peptide standard (LVNEVTEFAK) spiked into enolase tryptic digest
Injection volume: 1 µL

LVNEVTEFAK: Excellent Precision and Low Amol IDL

Amount measured	Replicates	%RSD	t (99%)	IDL
5.0 amol (LLOQ)	n = 10 injections	14.0	**2.821**	**2.0 amol**

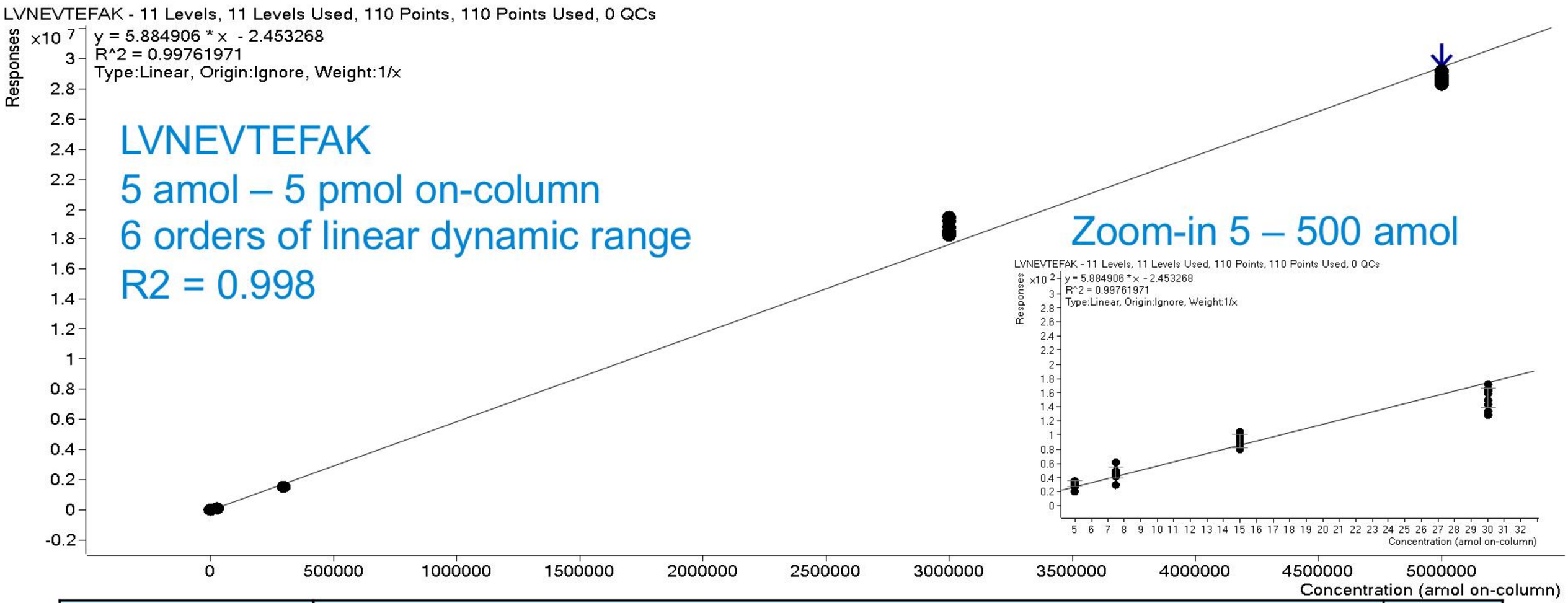
IDL = t x (%RSD/100) x Amount = 2.821 x (14.0/100) x 5.0 amol = 2.0 amol





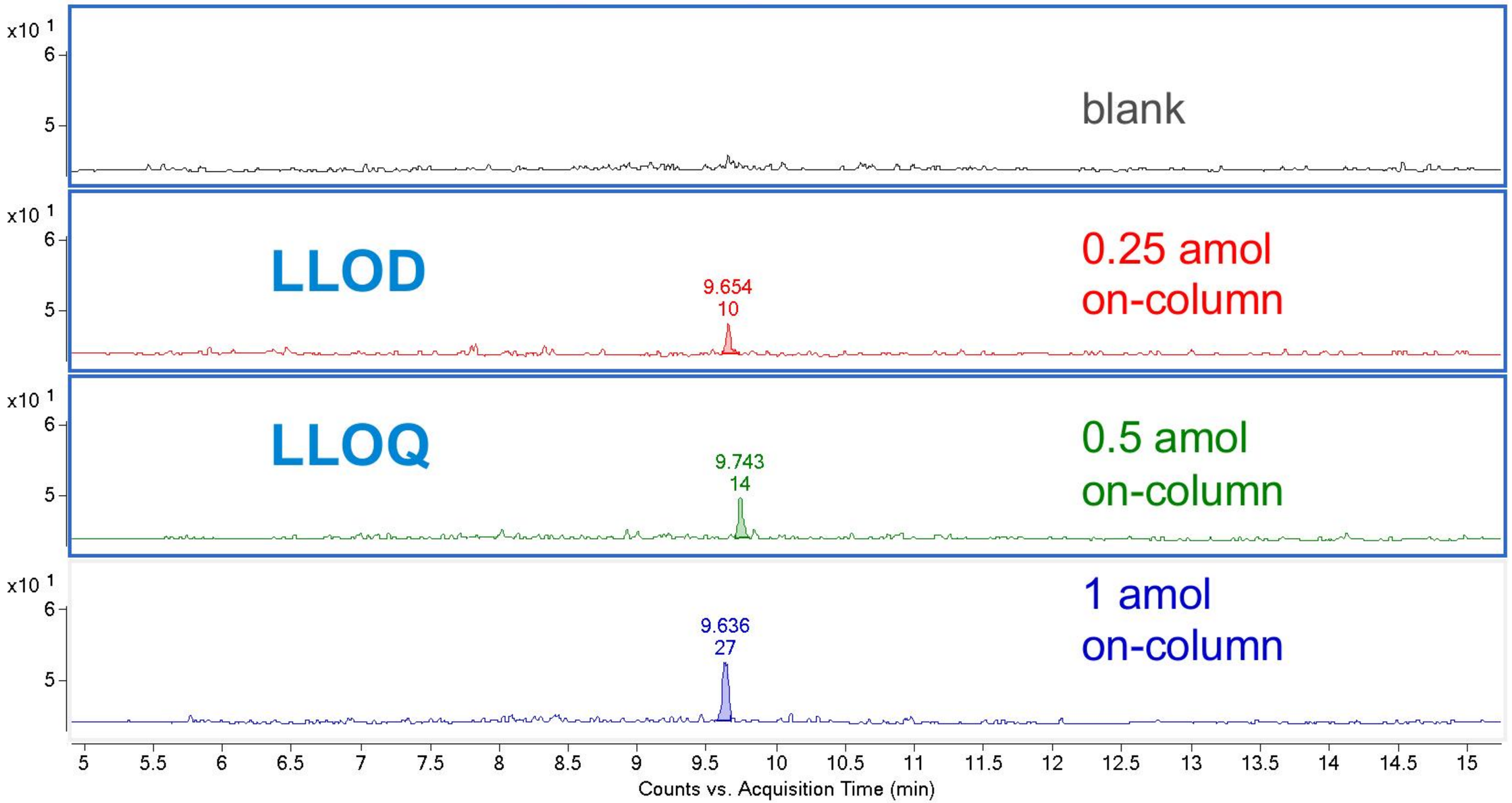
Inj #	Peak Area
1	30.5
2	28.7
3	30.4
4	33.91
5	35.1
6	31.5
7	34.5
8	20.7
9	26.7
10	26.6
%RSD	14.0

Peptide Quantitation – 6 Orders of Linear Dynamic Range



LVNEVTEFAK	Calibration Standards (amount on-column; 1 µL injected)									
	5.0 amol	7.5 amol	15 amol	30 amol	300 amol	3 fmol	30 fmol	300 fmol	3 pmol	5 pmol
%Accuracy	109.8	108.7	105.0	87.1	85.2	81.4	86.4	87.4	105.6	97.5
Reproducibility (%RSD, n=10)	14.0	16.0	9.4	9.0	1.6	1.2	0.6	0.7	2.1	1.0
RT (%RSD, n=100)	0.12									

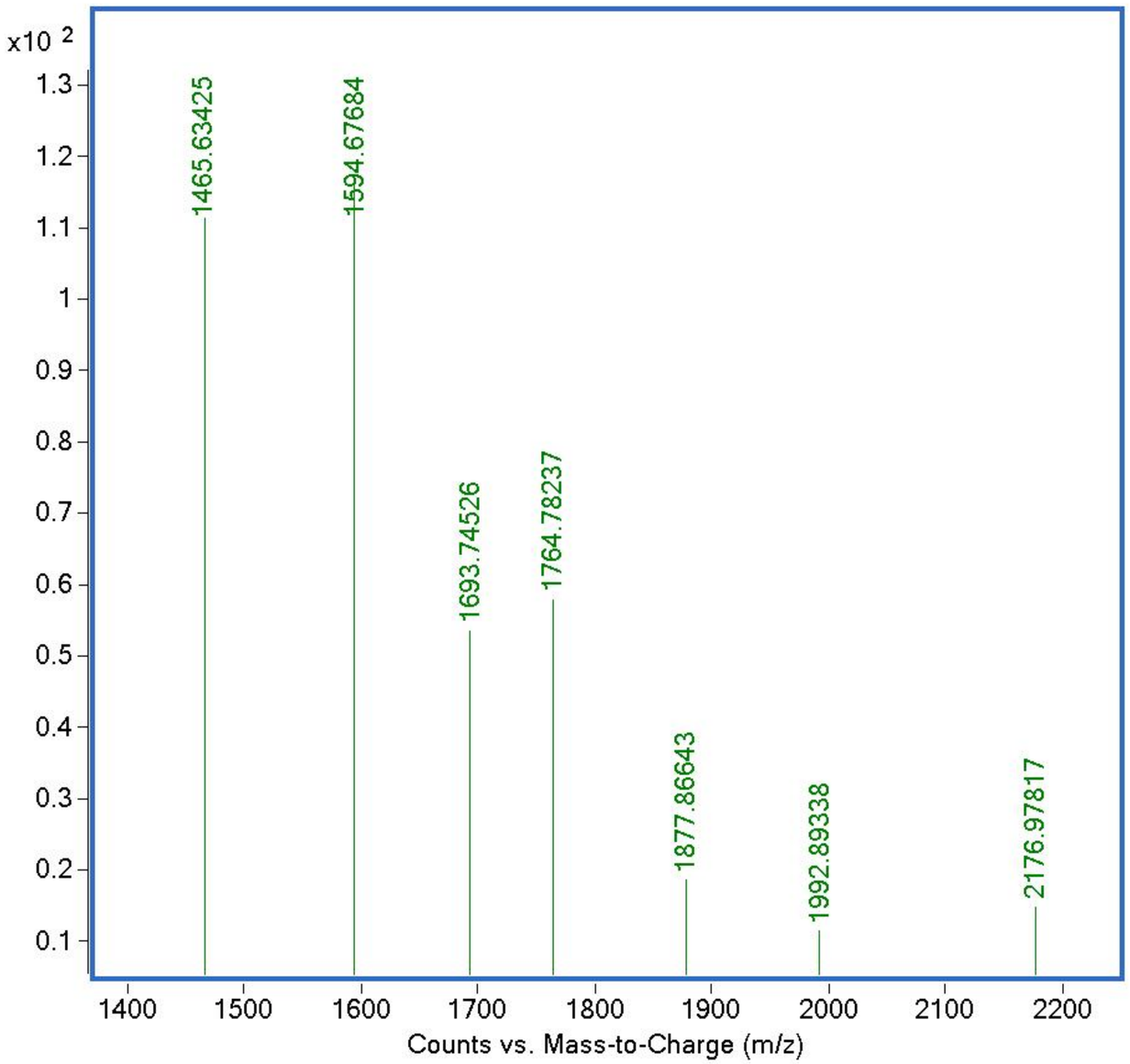
Peptide Quantitation: Ultimate Sensitivity with Nanoflow Chromatography



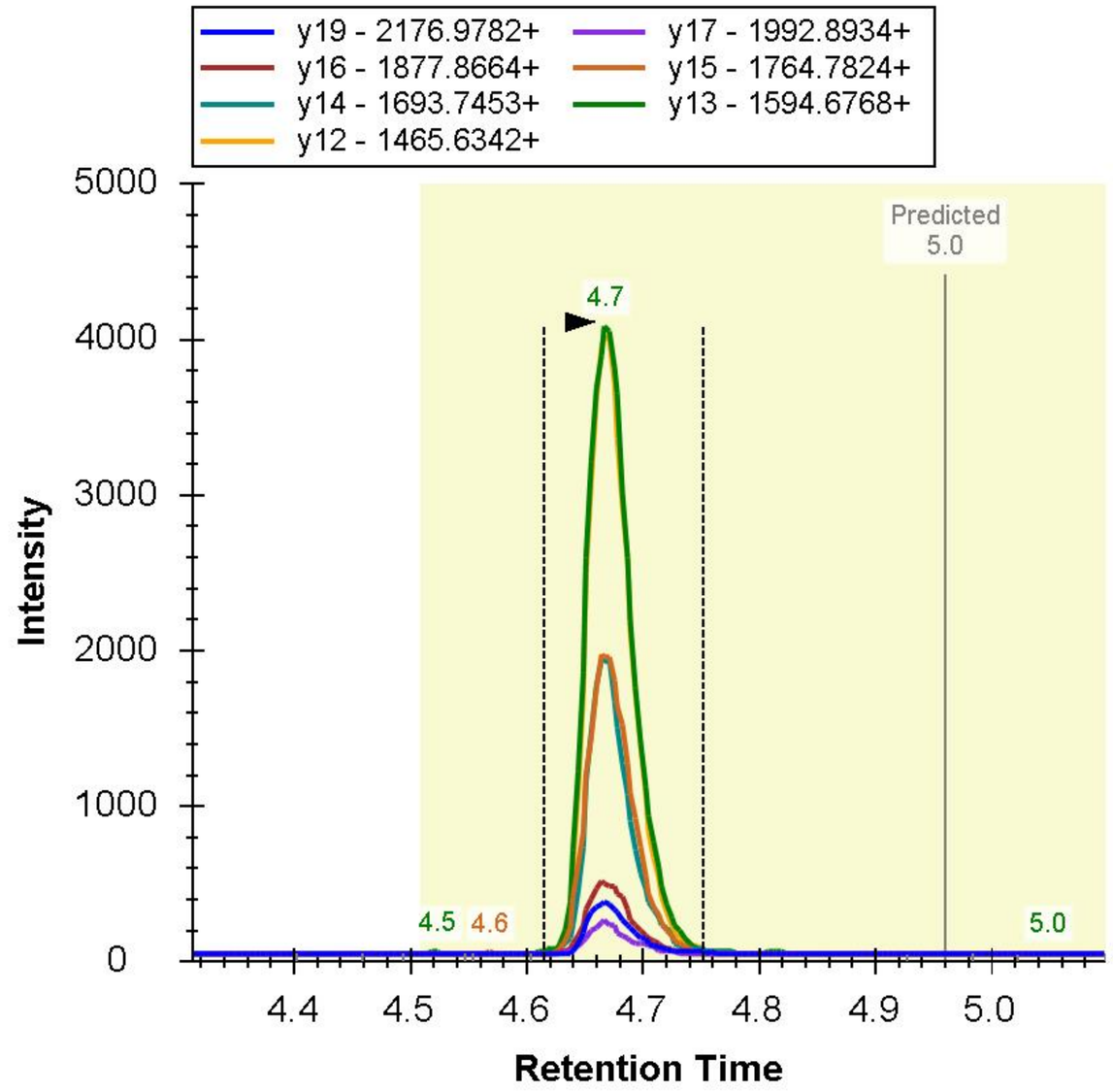
Instrumentation: 1260 HPLC-Chip/MS + 6495 QQQ
Sample: synthetic peptide standard (LVNEVTEFAK) spiked into enolase tryptic digest
Injection volume: 1 µL

Increased Mass Range Useful for Peptides

MRM Spectrum



Skyline Overlay of MRM Chromatograms



MRM Spectrum for IgG Peptide: GFYPSDIAVEWESNGQPENNYK

Summary

- The Agilent 6495 Triple Quadrupole LC/MS achieves low attomole sensitivity for peptides.
- The calibration curves show excellent linearity with six orders of dynamic range with great accuracy and reproducibility.
- With a complex plasma digest matrix, the 6495 QQQ showed excellent response and retention time reproducibility over an extended period (Total of 34mg of plasma digest injected over 3.5 weeks!)
- Improved performance in peptide quantitation can be achieved by the increased precursor ion transmission, enhanced detection efficiency (HED voltage up to -20kV) and extended m/z range of the 6495 QQQ

THANK YOU !