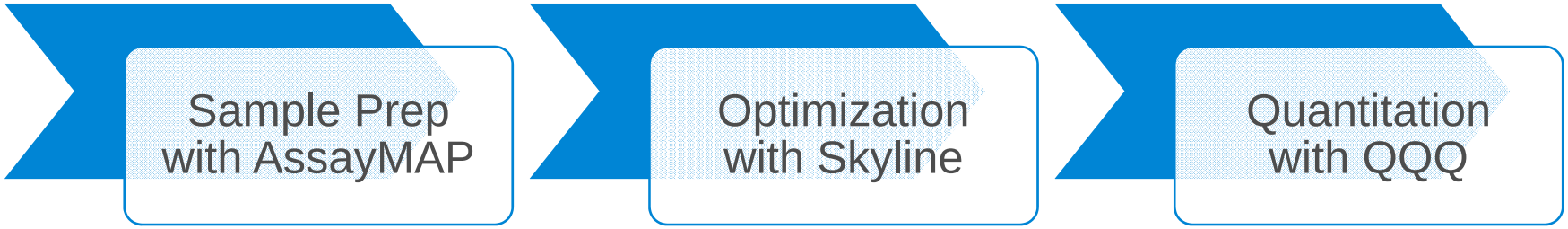
Bringing Precision to Peptide Quantitation Using Agilent Automation and LC/MS Solutions

Ning Tang, Ph.D.

Agilent Technologies

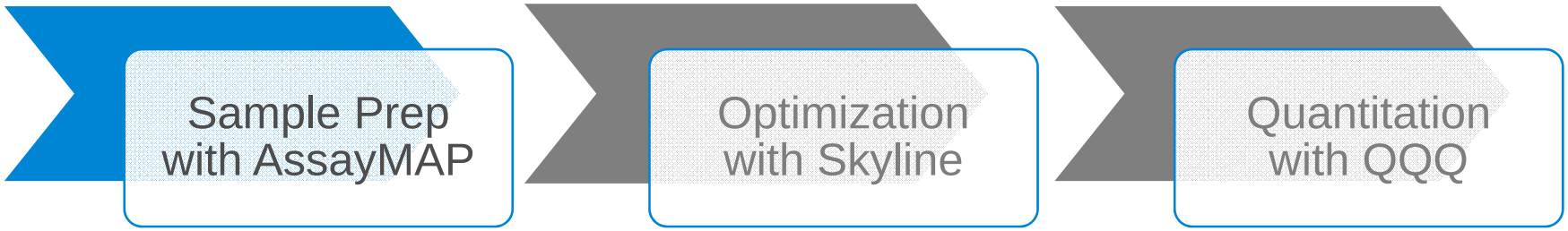
Santa Clara, CA

Outline



- Sample preparation using AssayMAP
  - Affinity purification
  - In solution digestion and peptide cleanup
- Skyline workflow for targeted protein quantitation
  - Skyline based MRM method development
  - MassHunter and Skyline automation workflow
  - All ions DDA and Skyline workflow
- Ultimate sensitivity and robustness with Agilent 6495 QQQ
  - Low attomole LOQ for peptides
  - Six orders of linearity
  - Great robustness
  - Excellent precision at the lowest levels
  - Extended mass range benefits glycopeptides

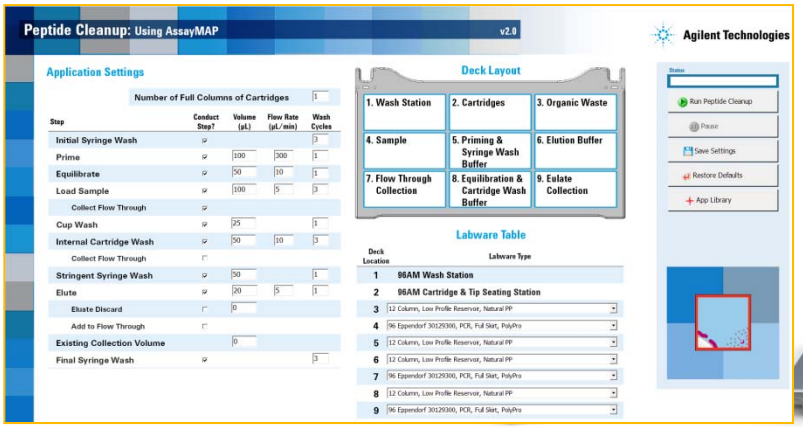
Outline



- Sample preparation using AssayMAP
  - Affinity purification
  - In solution digestion and peptide cleanup
- Skyline workflow for targeted protein quantitation
  - Skyline based MRM method development
  - MassHunter and Skyline automation workflow
  - All ions DDA and Skyline workflow
- Ultimate sensitivity and robustness with Agilent 6495 QQQ
  - Low attomole LOQ for peptides
  - Six orders of linearity
  - Great robustness
  - Excellent precision at the lowest levels
  - Extended mass range benefits glycopeptides

AssayMAP Technology Components

Automated workflows designed for analytical chemists



Simple User Interface

Uses customer language - not automation language

- Affinity (protein) Purification
- In-Solution Digestion
- Peptide Cleanup (desalting)
- Phosphopeptide Enrichment
- Fractionation
- Liquid handling utilities

Microchromatography Cartridges

quantitative binding & elution

Protein purification

- *PA-W (protein A)*
- *PG-W (protein G)*
- *SA-W (streptavidin)*

Reversed-phase cleanup:

- *C18 (silica)*
- *RP-S (polymeric)*

Fractionation:

- *SCX*
- *RP-S*
- *C18*

Phosphopeptide enrichment:

- *TiO_2*

Positive Displacement Pipetting

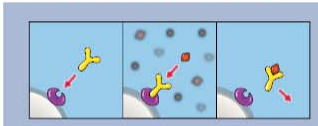
Syringes interface directly with cartridges and enable precise, controlled liquid flow through cartridges with no air bubbles to disrupt binding

Workflow Library

Protein Sample Prep Workbench: WORKFLOW LIBRARY v1.0

Agilent Technologies

General Workflows



Affinity Purification Workflow

Create custom affinity cartridges and use them to enrich for target molecules. Using AssayMAP Bravo and Cartridges.

Open

Peptide Sample Prep Workflow

Digest proteins, desalt, and optionally fractionate peptides. Using AssayMAP Bravo and Cartridges.

Open

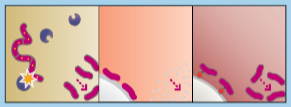
SISCAPA® assay technologies

SISCAPA® Workflow

Addition-only trypsin digest and immunocaputre of target peptides and internal standards. Using SISCAPA® antibodies and Bravo 96LT Head.

Open

Post-Translational Modification Workflows



Phosphopeptide Enrichment Workflow - TiO_2

Digest proteins, desalt and enrich for phosphopeptides. Using AssayMAP Bravo and Cartridges.

Open

PROzyme®

N-Glycan Sample Prep, RX Digestion & 2-AB Labeling v1.0

Denature glycoproteins, then release, label and cleanup gylcans. Using AssayMAP Bravo and ProZyme GlykoPrep-*plus*® 2AB Chemistry Kit.

Open

PROzyme®

N-Glycan Sample Prep, RX Digestion & APTS Labeling v1.0

Denature glycoproteins, then release, label and cleanup gylcans. Using AssayMAP Bravo and ProZyme GlykoPrep-*plus*® APTS Chemistry Kit.

Open

Workflow Library

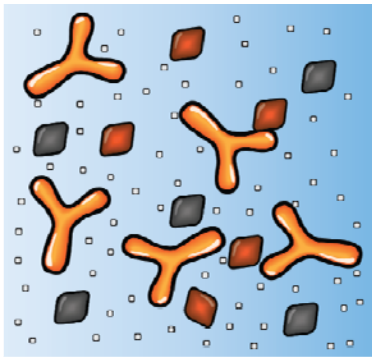
App Library

Utility Library

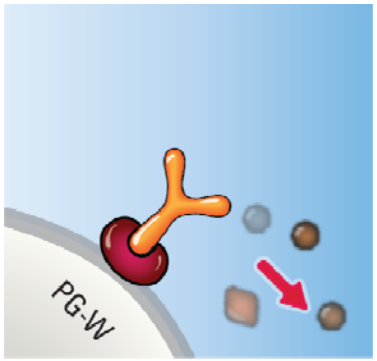
Literature Library

AssayMAP mAb Quantitation Workflow

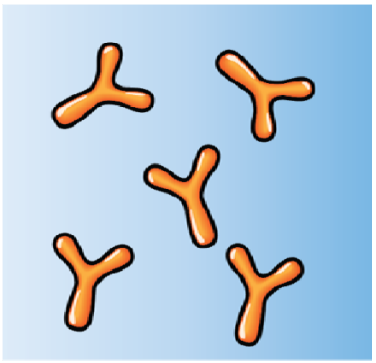
Each step in the workflow was performed using the AssayMAP Bravo



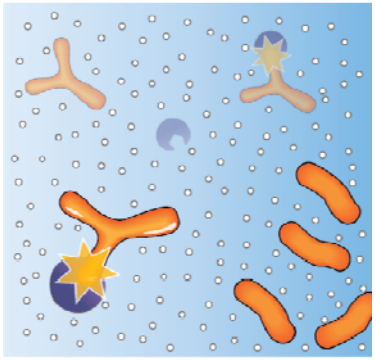
Input



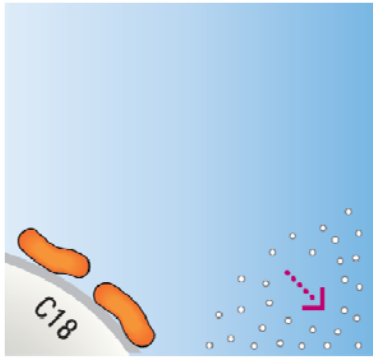
Purify



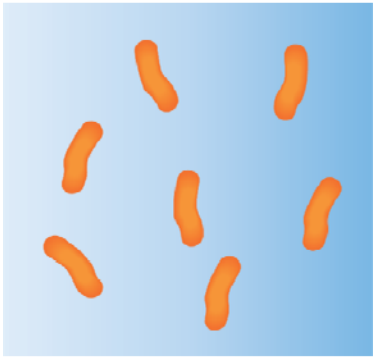
Output



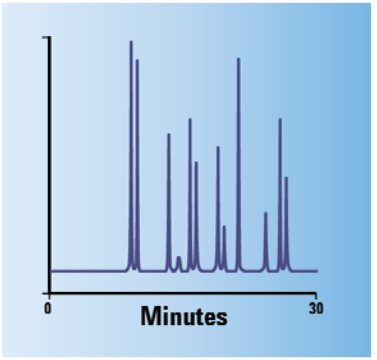
Digest



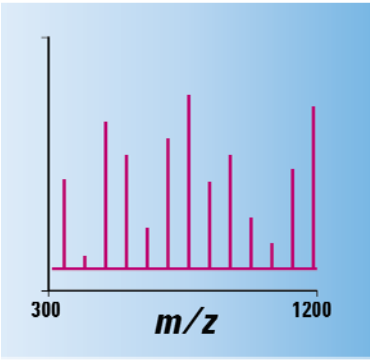
Cleanup



Output

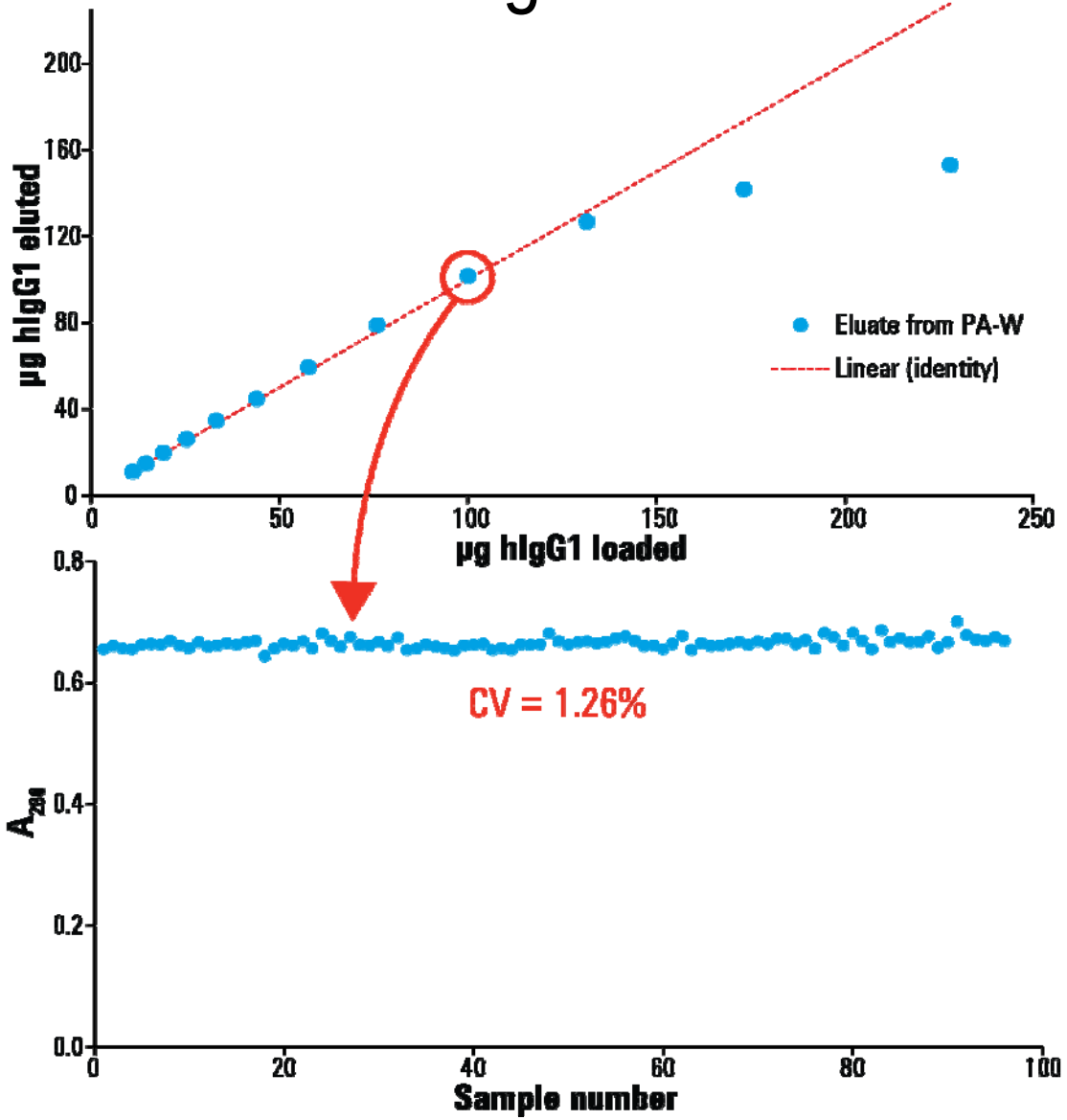


Separate



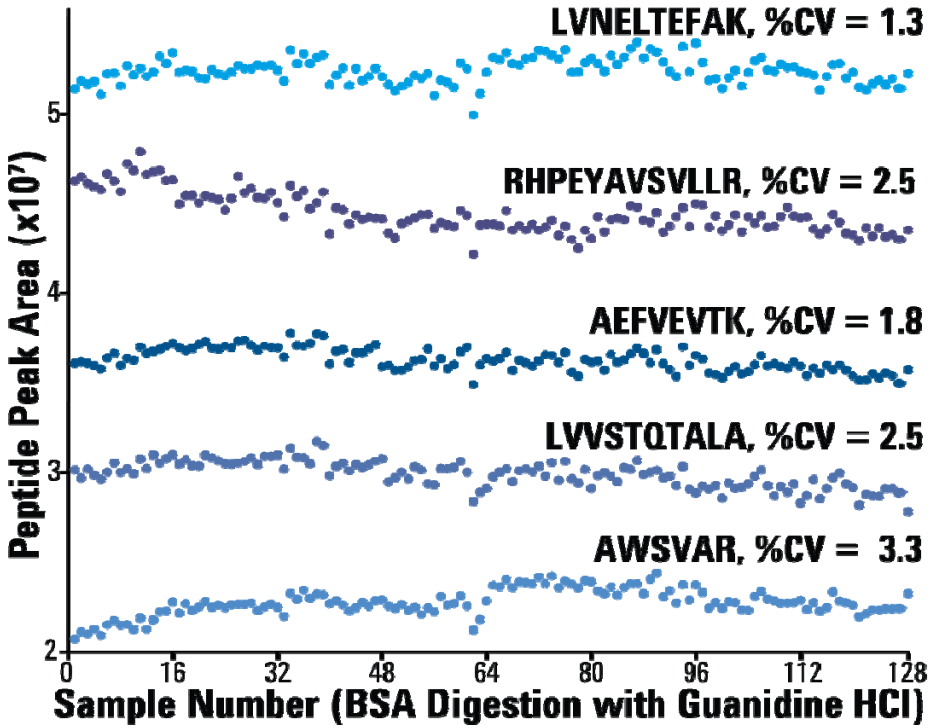
Analyze

Antibody Purification Using Protein A Cartridge



AssayMAP Digestion and Cleanup

Day 1 cleanup = C18
Day 2 cleanup = RP-S

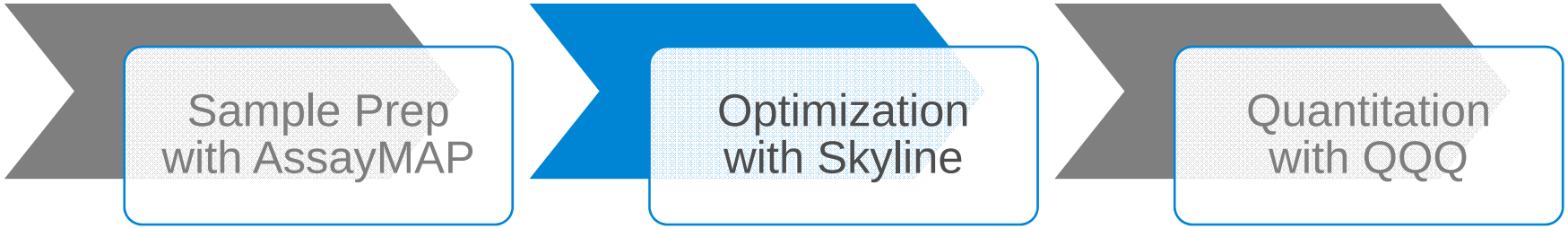


Urea	Day 1	Day 2
# of samples	64	62
# of peptides monitored	25	25
Avg. peak area %CV	3.3	3.7
# Peptides %CV <5	23	21
# Peptides 5>%CV<10	2	3
# Peptides %CV >10	0	1

Guanidine HCl	Day 1	Day 2
# of samples	64	64
# of peptides monitored	25	25
Avg. peak area %CV	2.3	2.6
# Peptides %CV <5	25	23
# Peptides 5>%CV<10	0	1
# Peptides %CV >10	0	1

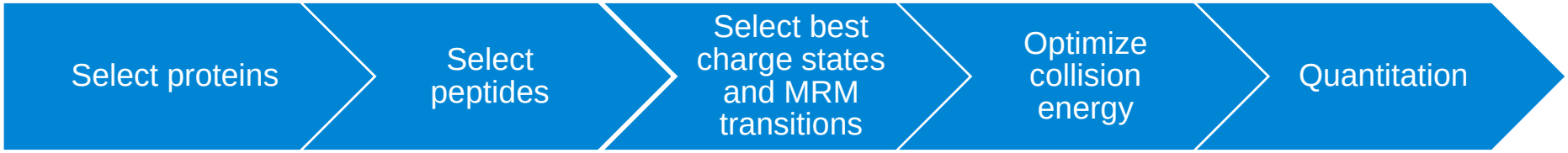
Low %CVs for both intra- and interday digestion and cleanup for samples prepared using urea or guanidine-based denaturation

Outline



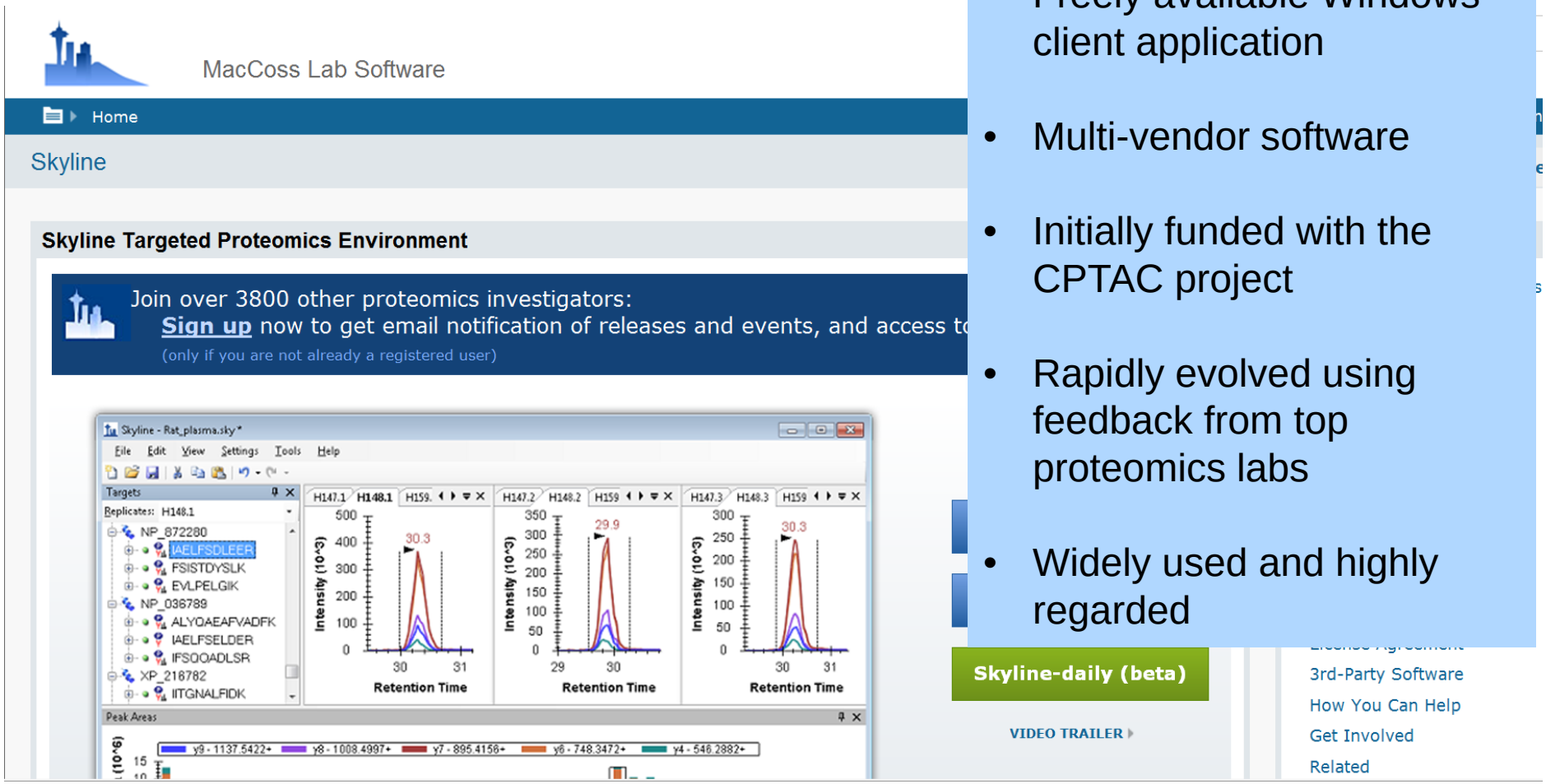
- Sample preparation using AssayMAP
  - Affinity purification
  - In solution digestion and peptide cleanup
- Skyline workflow for targeted protein quantitation
  - Skyline based MRM method development
  - MassHunter and Skyline automation workflow
  - All ions DDA and Skyline workflow
- Ultimate sensitivity and robustness with Agilent 6495 QQQ
  - Low attomole LOQ for peptides
  - Six orders of linearity
  - Great robustness
  - Excellent precision at the lowest levels
  - Extended mass range benefits glycopeptides

MRM Based LC/MS Protein Quantitation



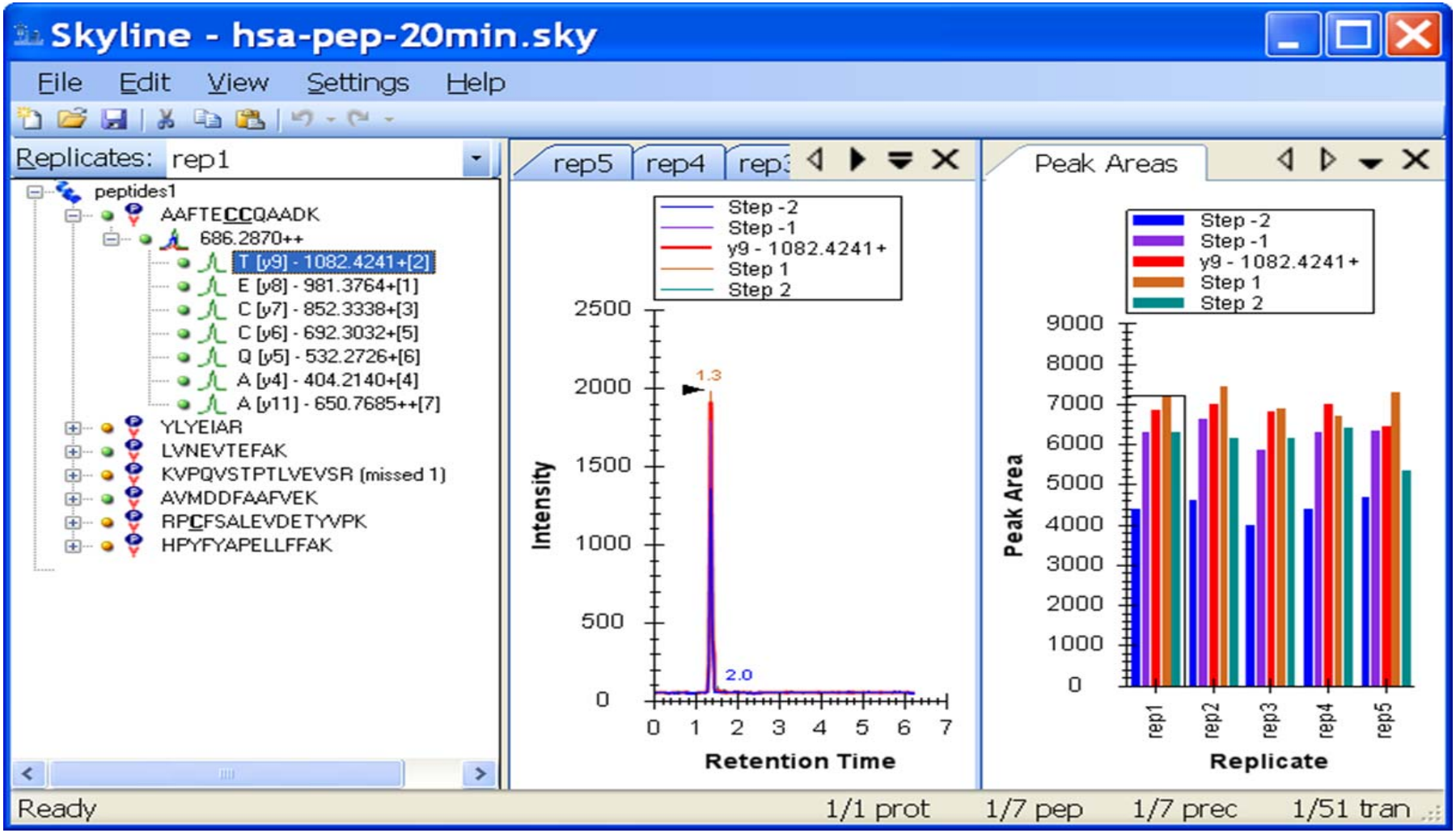
- Selection of target proteins
- Selection of target peptides that represent the protein list
- Selection of best charge states and MRM transitions for each targeted peptide
- Optimization of collision energy for each MRM transition
- Application of the assays to the detection and quantitation of the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

Skyline Targeted Proteomics Environment

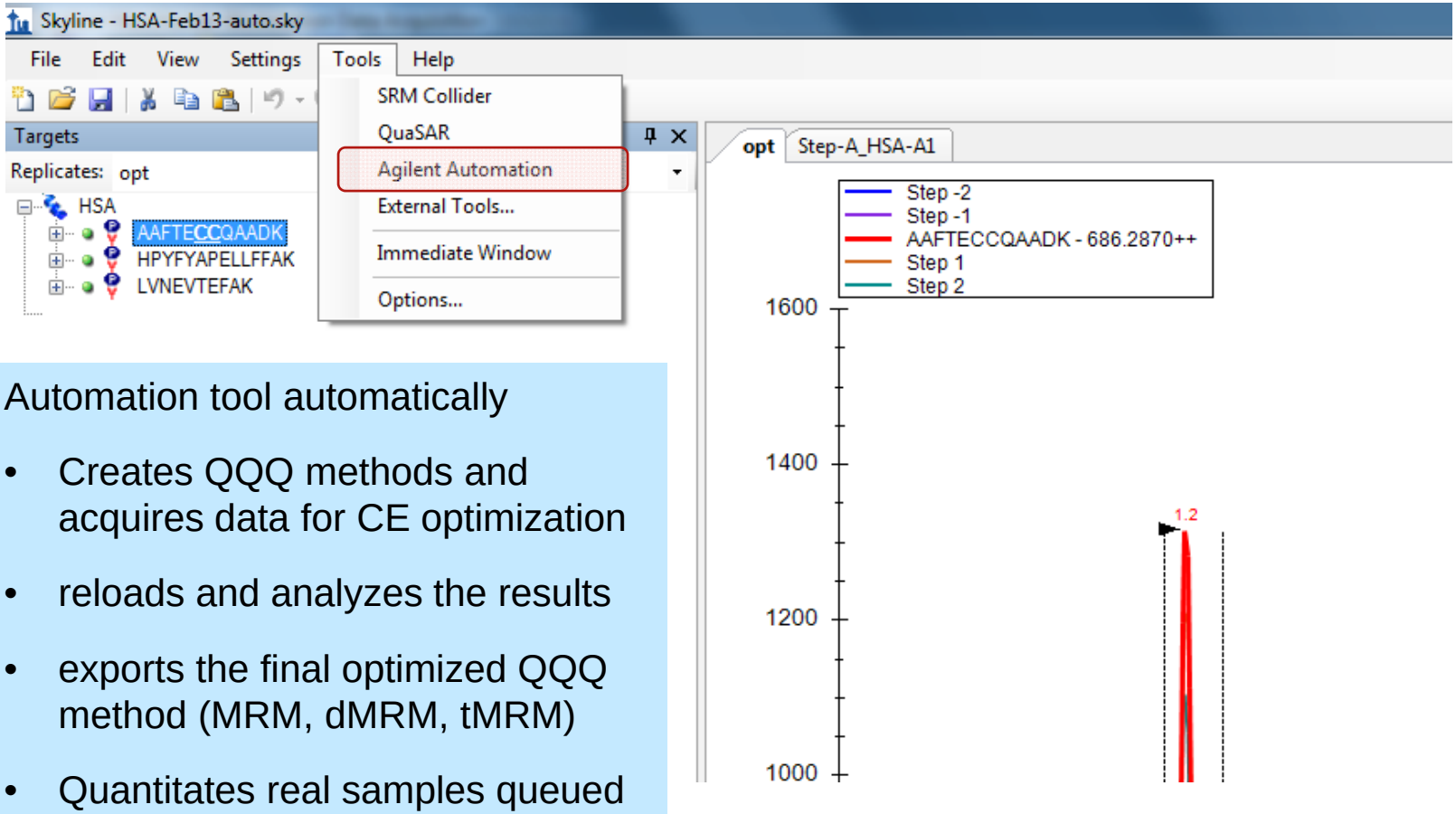


- Freely available Windows client application
- Multi-vendor software
- Initially funded with the CPTAC project
- Rapidly evolved using feedback from top proteomics labs
- Widely used and highly regarded

Agilent's QQQ + Skyline: Collision Energy Optimization



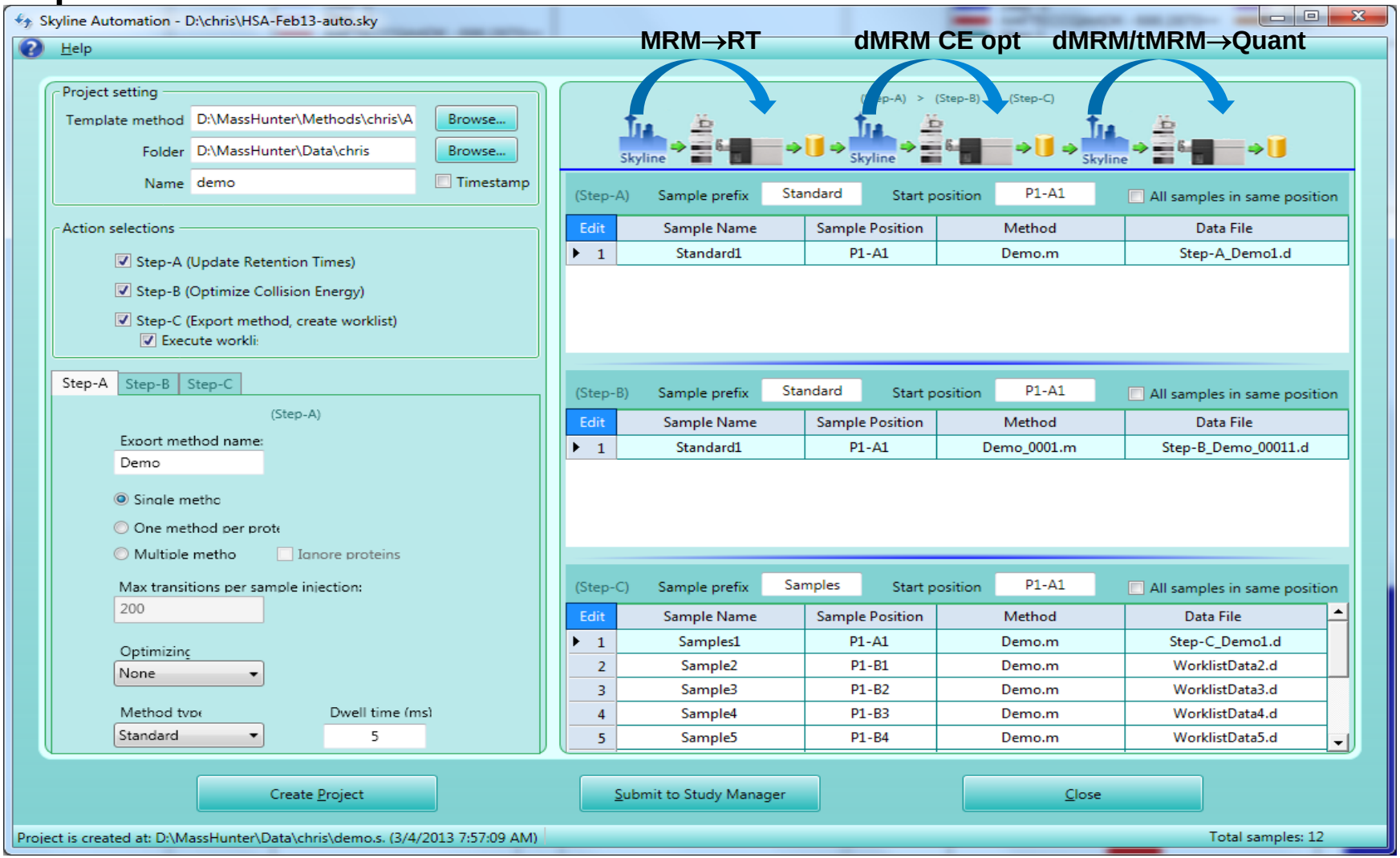
New Automation Link in Skyline



Automation tool automatically

- Creates QQQ methods and acquires data for CE optimization
- reloads and analyzes the results
- exports the final optimized QQQ method (MRM, dMRM, tMRM)
- Quantitates real samples queued up in the worklist

New Agilent Automation Tool For Skyline and the QQQ Acquisition Software



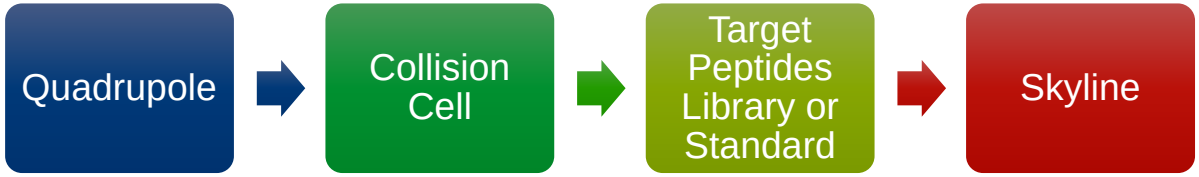
Agilent's QTOF and Skyline

- Data Dependent Acquisition (DDA): MS1 Filtering
- Data Independent Acquisition (DIA)

All ions MS/MS

- ✓ No isolation in quadrupole
- ✓ 2 or more collision energies applied
- ✓ Simplify the MRM method development

Wide band isolation

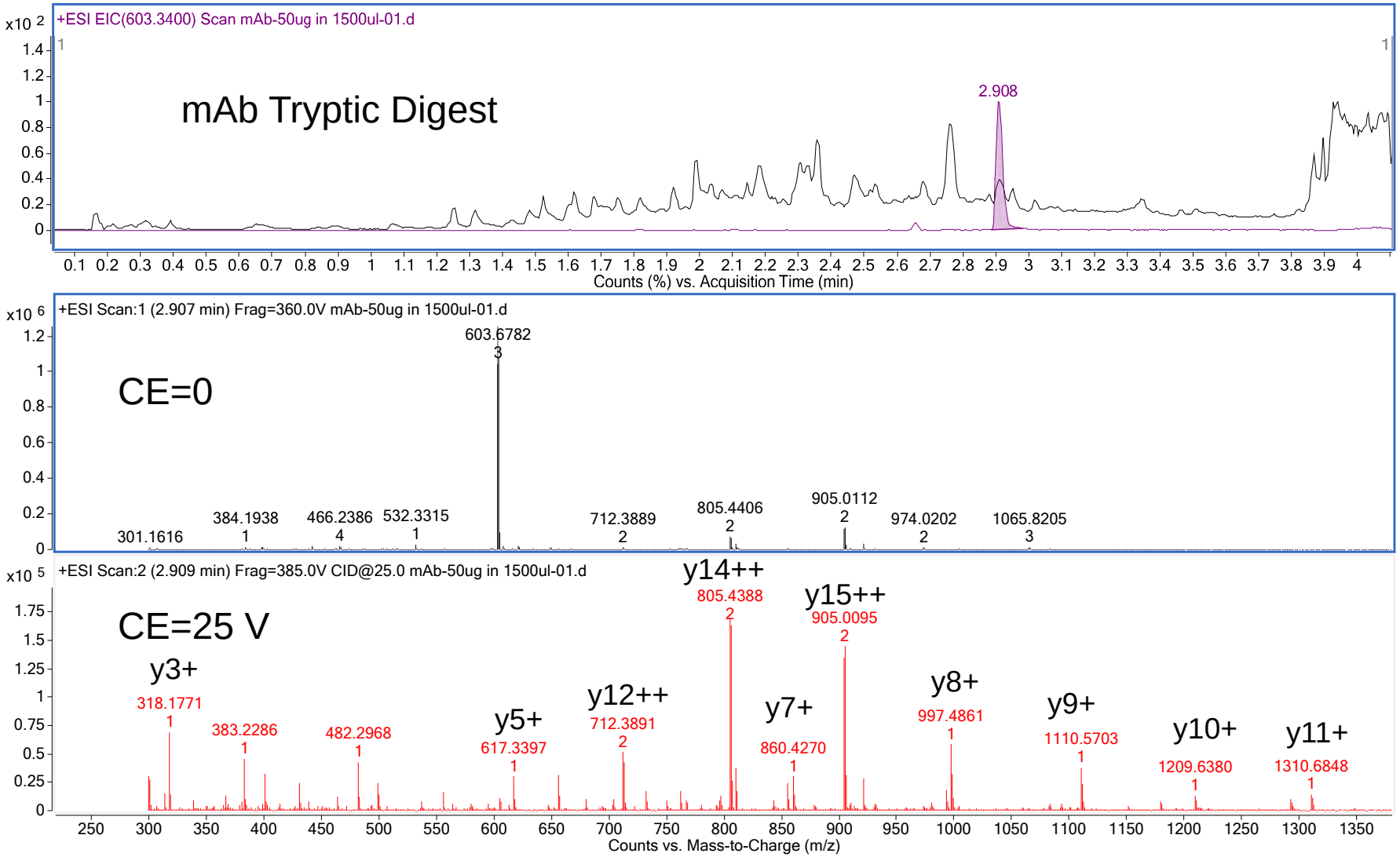


- ✓ Isolate 10 Da
- ✓ Typically use same formula as DDA

All Ions MS/MS → Skyline → QQQ MRM Workflow

- All Ions acquisition on a Q-TOF
  - Two channels: 0 V and 25 V
- Load All Ions data into Skyline
  - Create new Skyline project and import FASTA
  - Select target peptides for each protein of interest
  - Select charge state for each peptide precursor
  - Select multiple transitions for each peptide (>6)
- Export MRM list or method with CE opt to run on a QQQ
- Quantitation using the optimized method on a QQQ

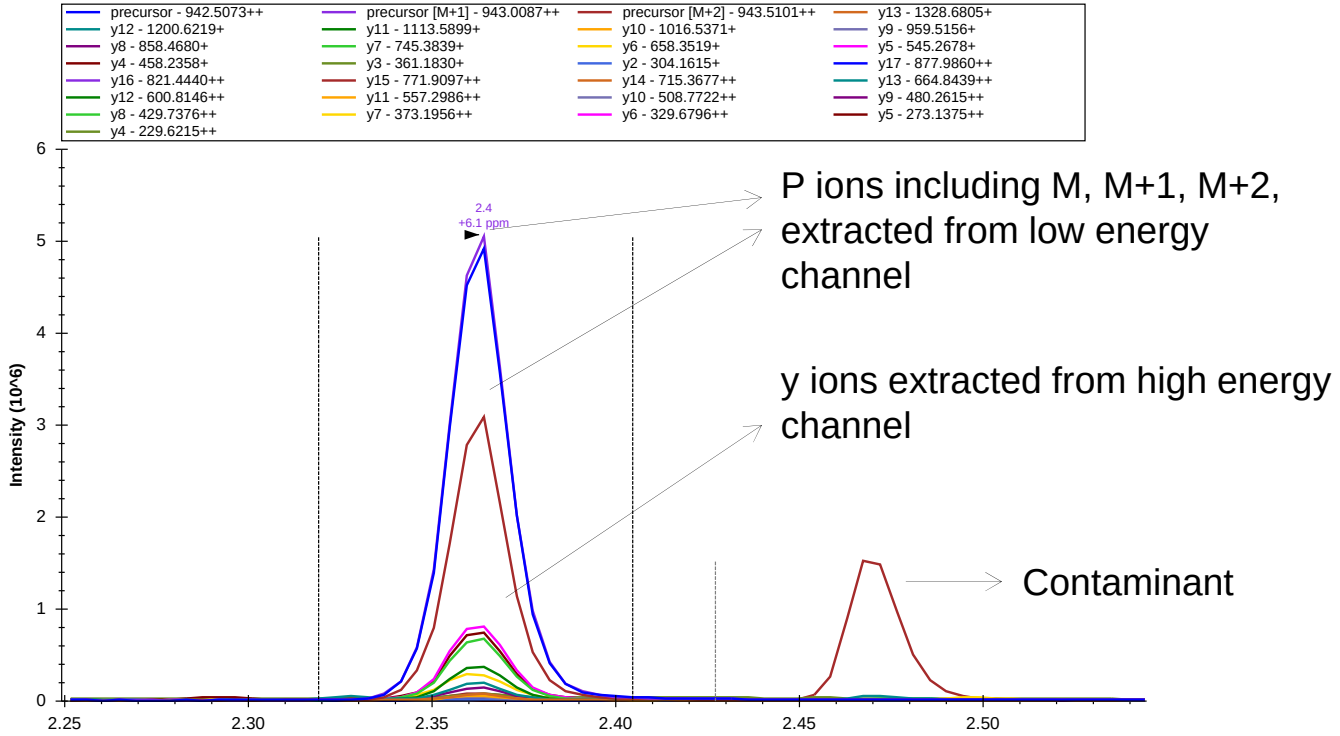
All Ions MS/MS on Agilent QTOF



All Ions → Skyline Workflow

Step 1: Show p & y Ions for Peptide Confirmation

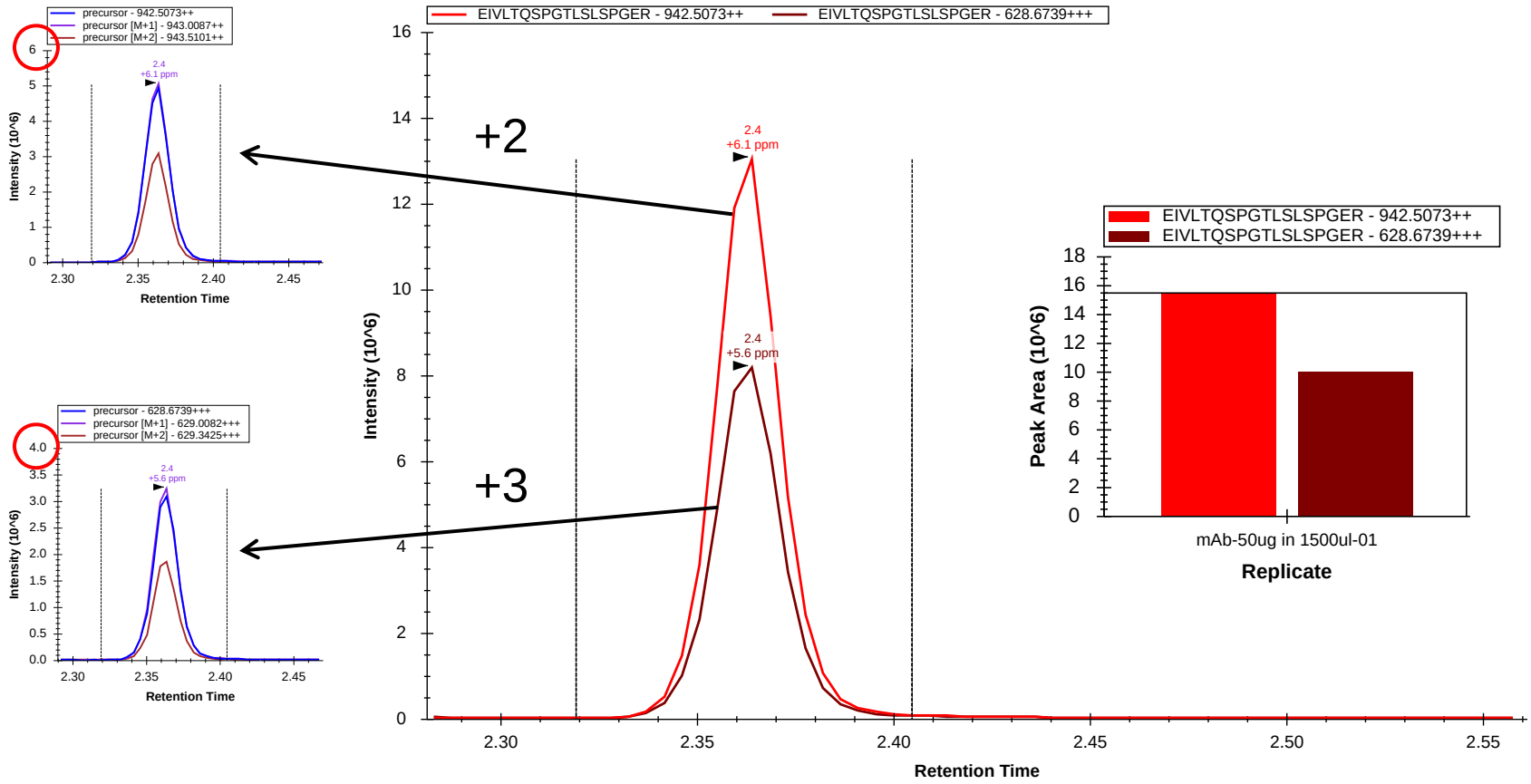
Precursors (p ions) from low energy channel and products (y ions) from high energy channel are automatically extracted and plotted together to easily confirm the identity of the peptides.



Retention time and peptide sequence confirmation

All Ions → Skyline workflow

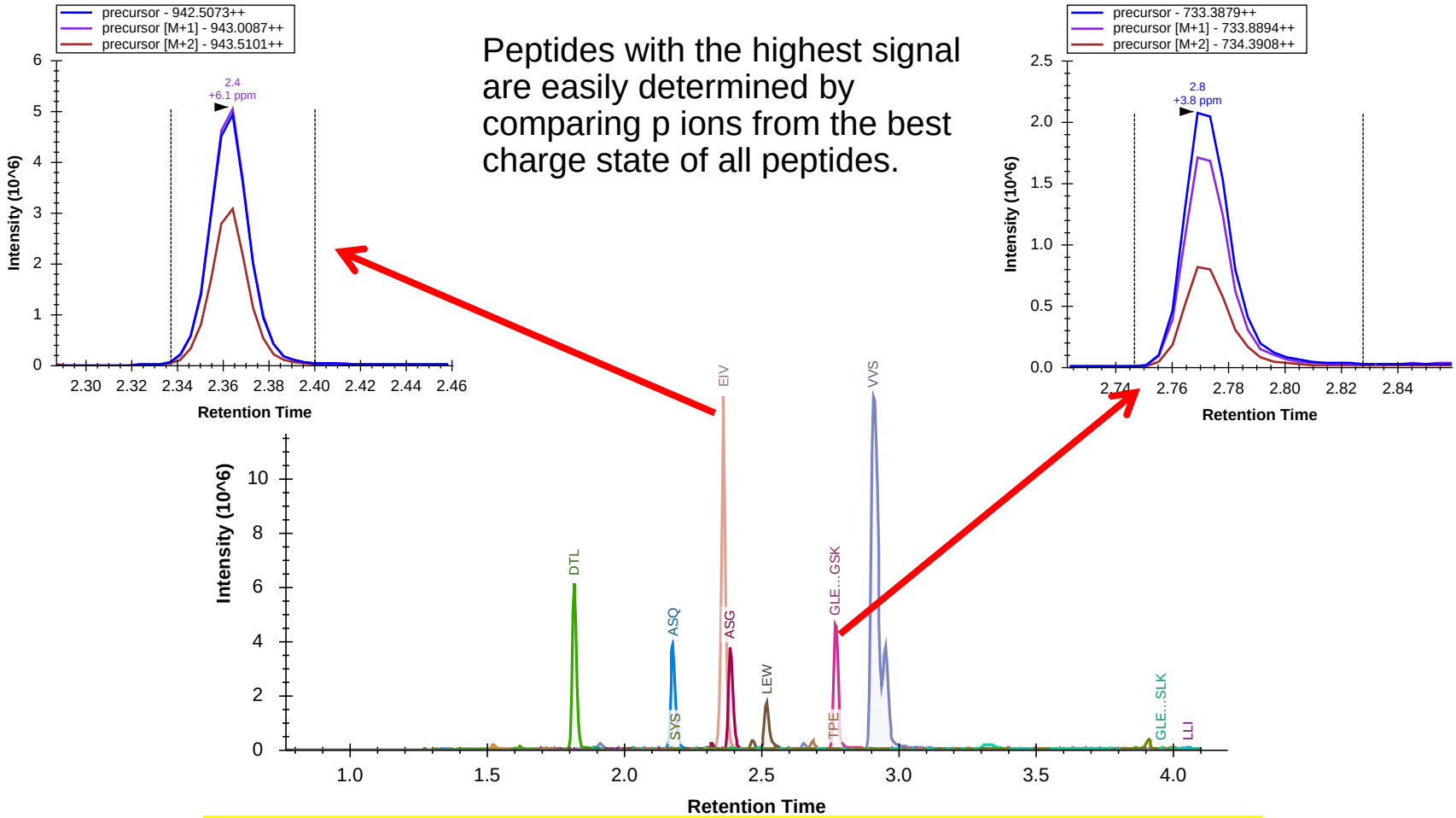
Step 2: Show only p Ions to Select Best Charge State



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

All Ions → Skyline workflow

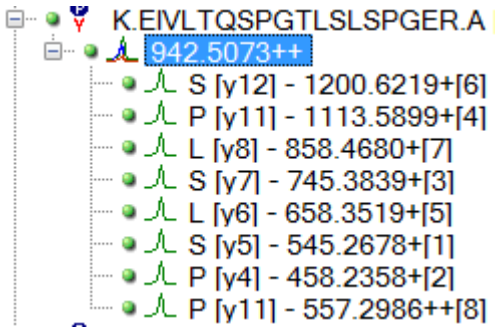
Step 3: Show only p ions to Compare Peptide Abundance

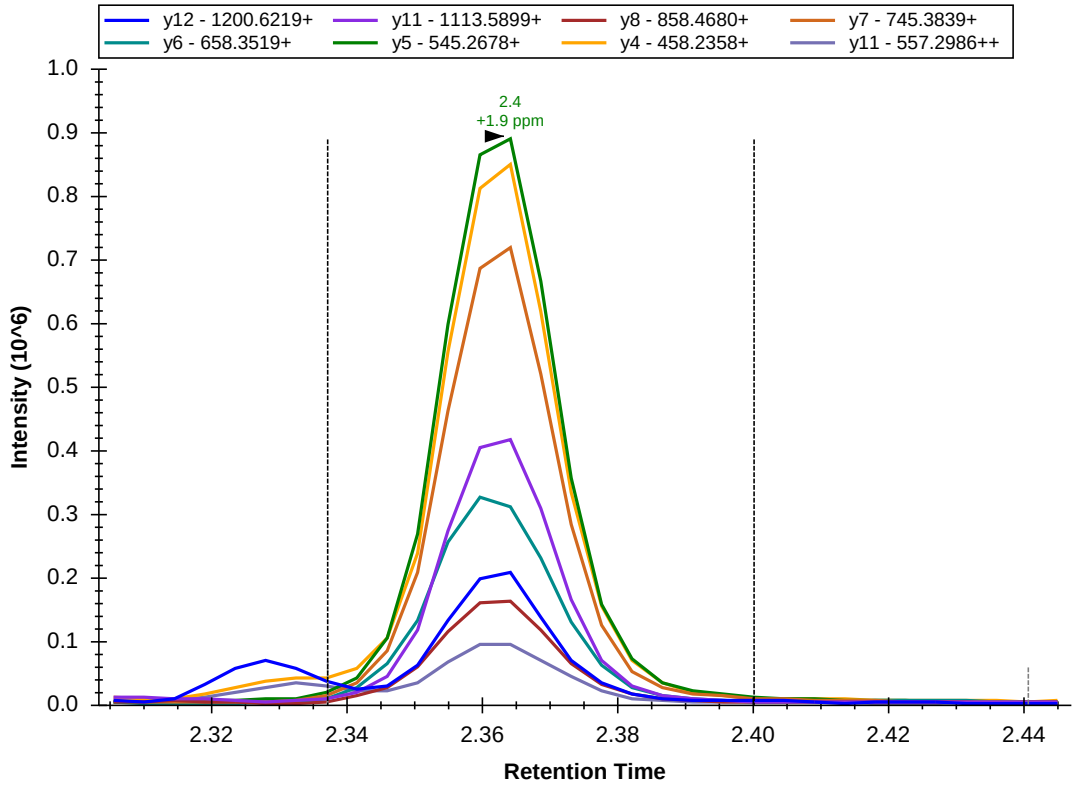


Skyline peptide rank refine tool picks top n peptides

All Ions → Skyline workflow

Step 4: Show Only y Ions to Select Top MRMs

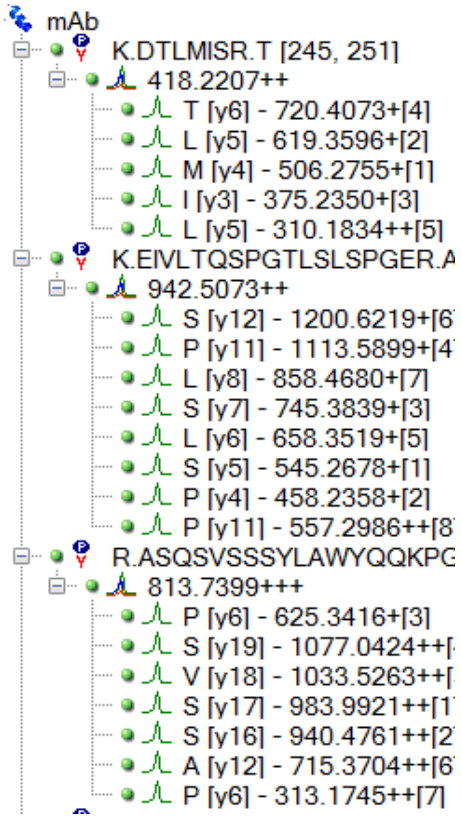


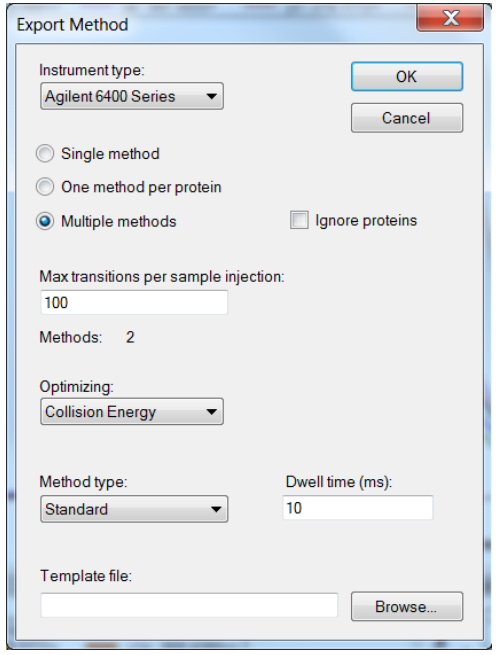


Skyline transition rank refine tool picks top n MRMs

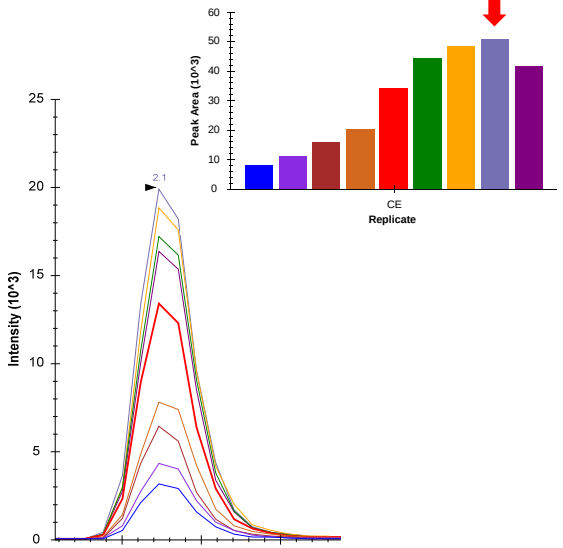
All Ions → Skyline workflow

Step 5: CE Optimization



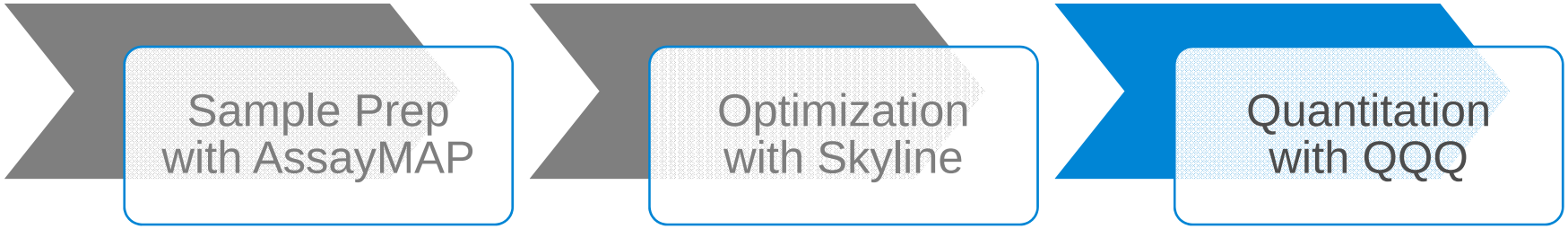


Opt CE



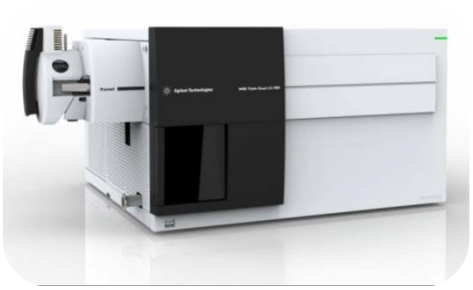
CE optimization process can be automated using new Skyline Automation Tool

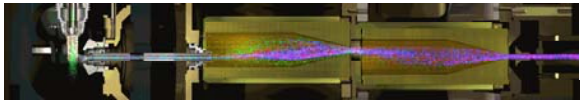
Outline

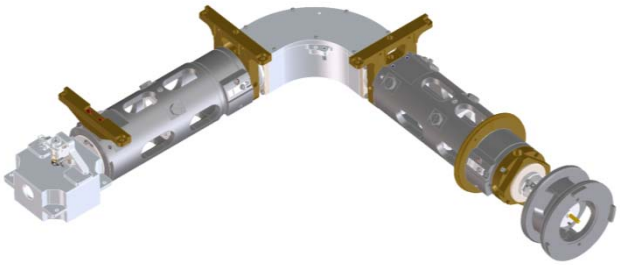


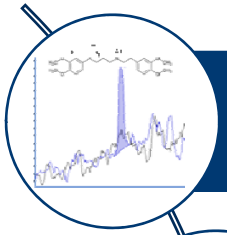
- Sample preparation using AssayMAP
  - Affinity purification
  - In solution digestion and peptide cleanup
- Skyline workflow for targeted protein quantitation
  - Skyline based MRM method development
  - MassHunter and Skyline automation workflow
  - All ions DDA and Skyline workflow
- Ultimate sensitivity and robustness with Agilent 6495 QQQ
  - Low attomole LOQ for peptides
  - Six orders of linearity
  - Great robustness
  - Excellent precision at the lowest levels
  - Extended mass range benefits glycopeptides

6495 QQQ LC/MS - Premium Performance

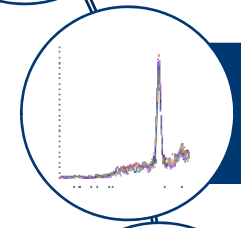




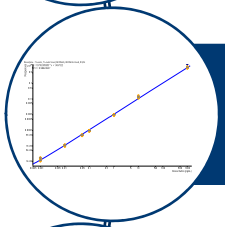




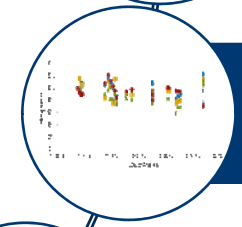
Improved sensitivity (IDL / MDL) – Average 3x in S/N specifications and applications



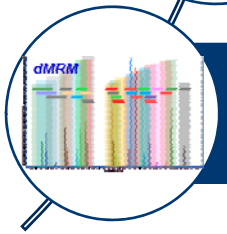
Improved precision and excellent accuracy at the lowest levels – 4 to 5x in IDL spec



Proven 6 orders of linear dynamic range



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

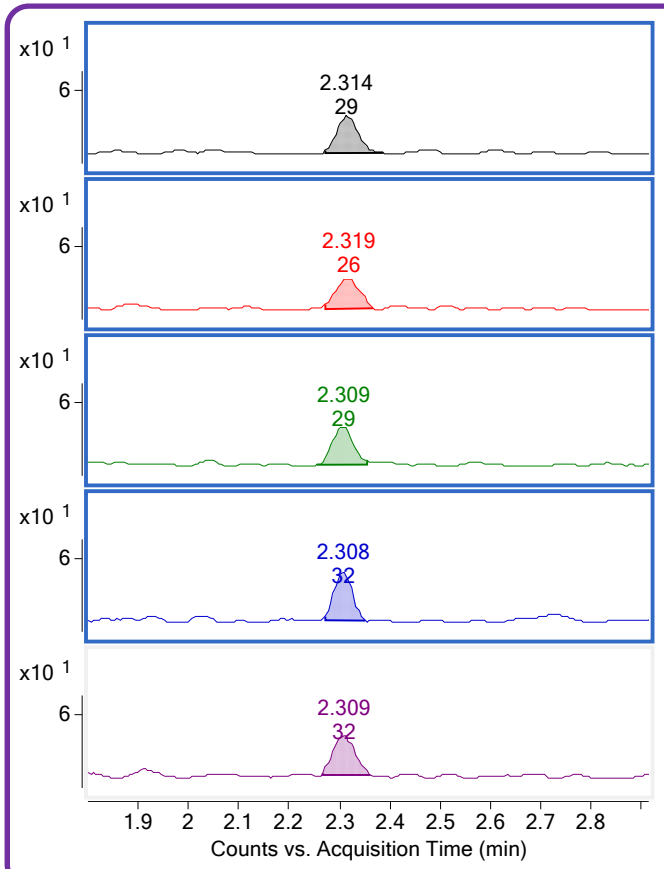


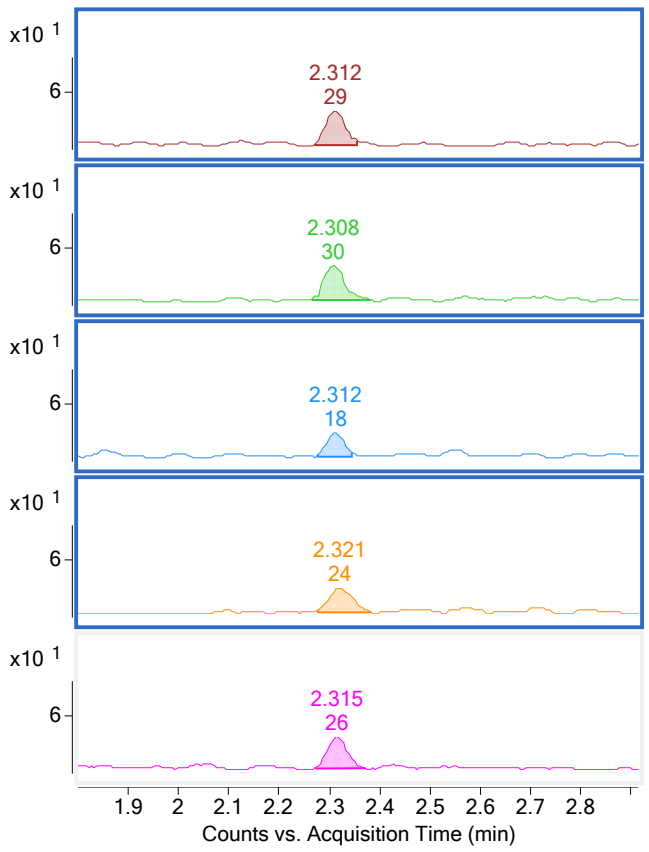
Improved mass range, fast scan speed and MRM acquisition rate

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**

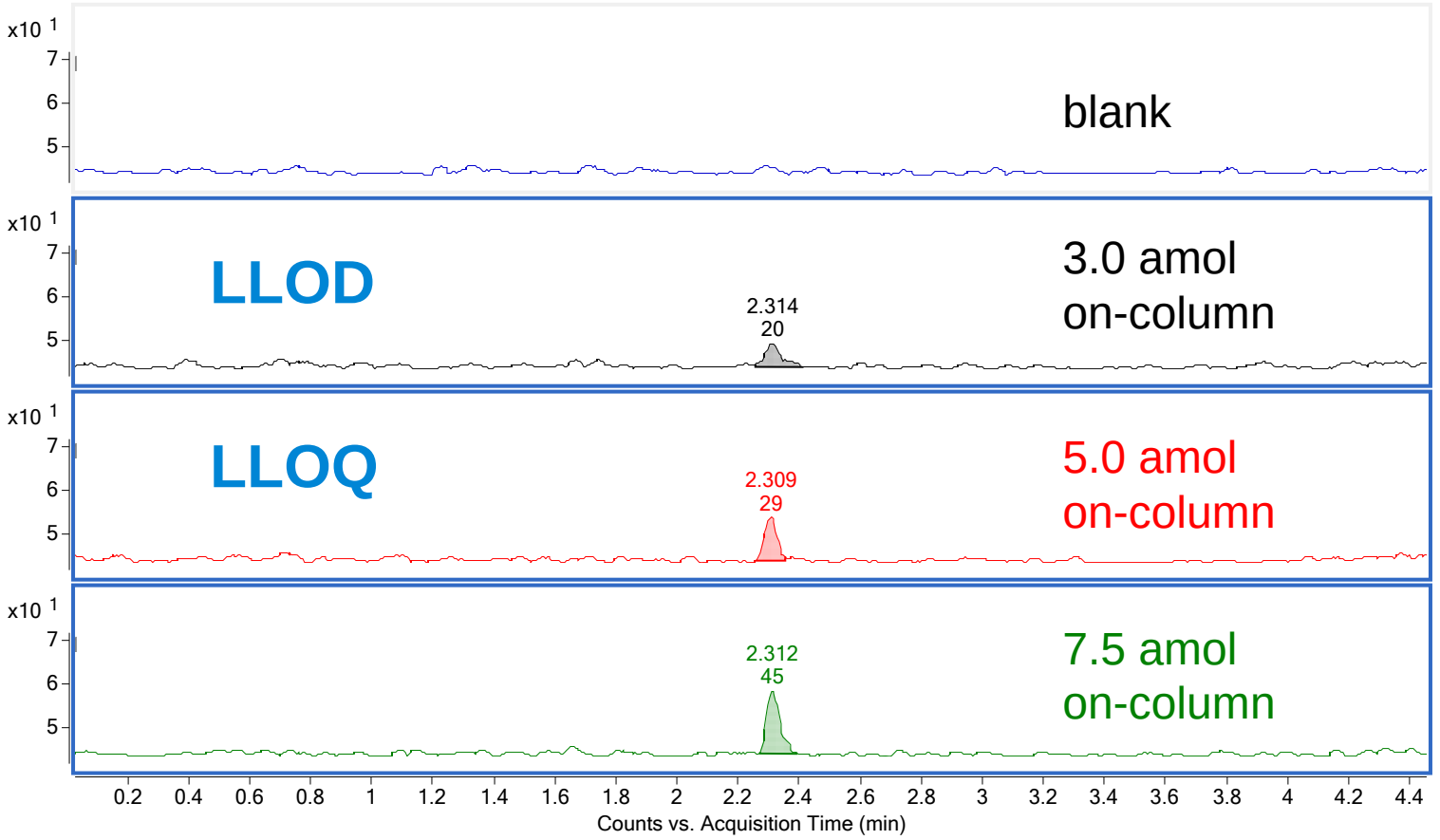
MDL = t x (%RSD/100) x Amount = 2.821 x (14.0/100) x 5.0 amol = 2.0 amol





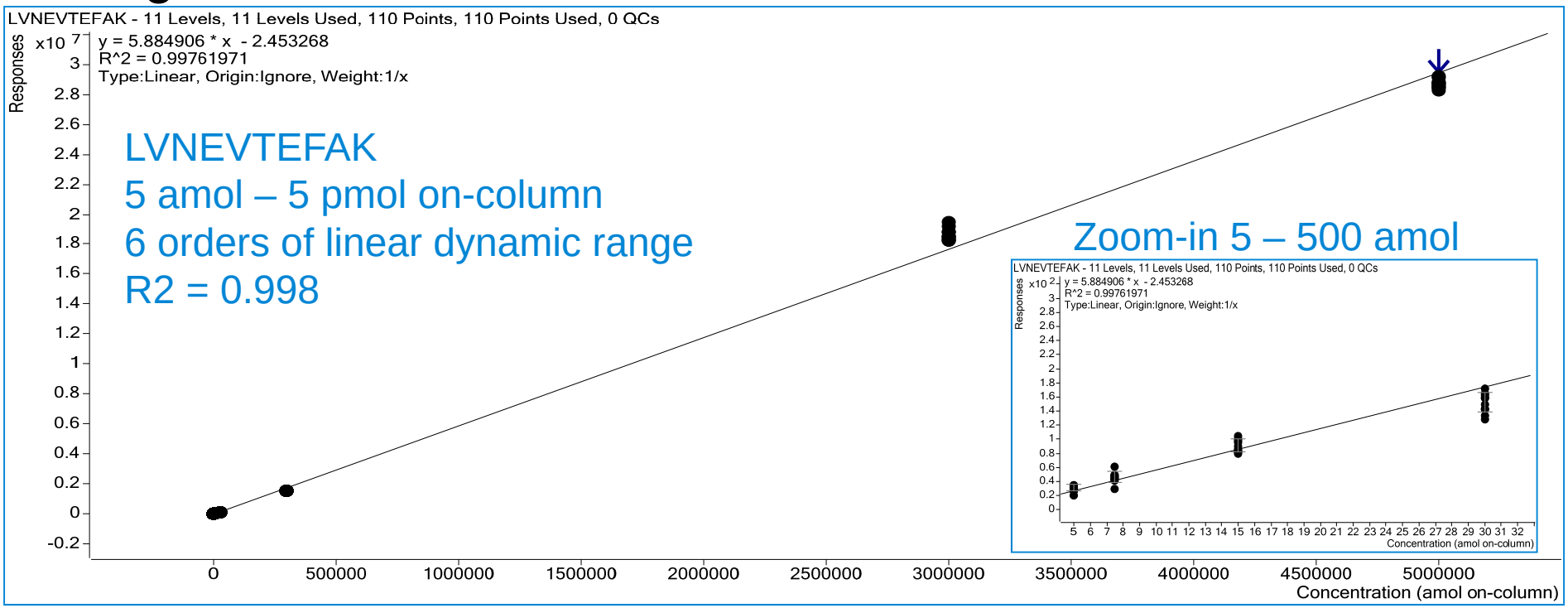
Inj #	Peak Area
1	30.5
2	28.7
3	30.4
4	33.91
5	35.1
6	31.5
7	34.5
8	20.7
9	26.7
10	26.6
%RSD	14.0

Peptide Quantitation: Outstanding Sensitivity with Standard Flow Chromatography



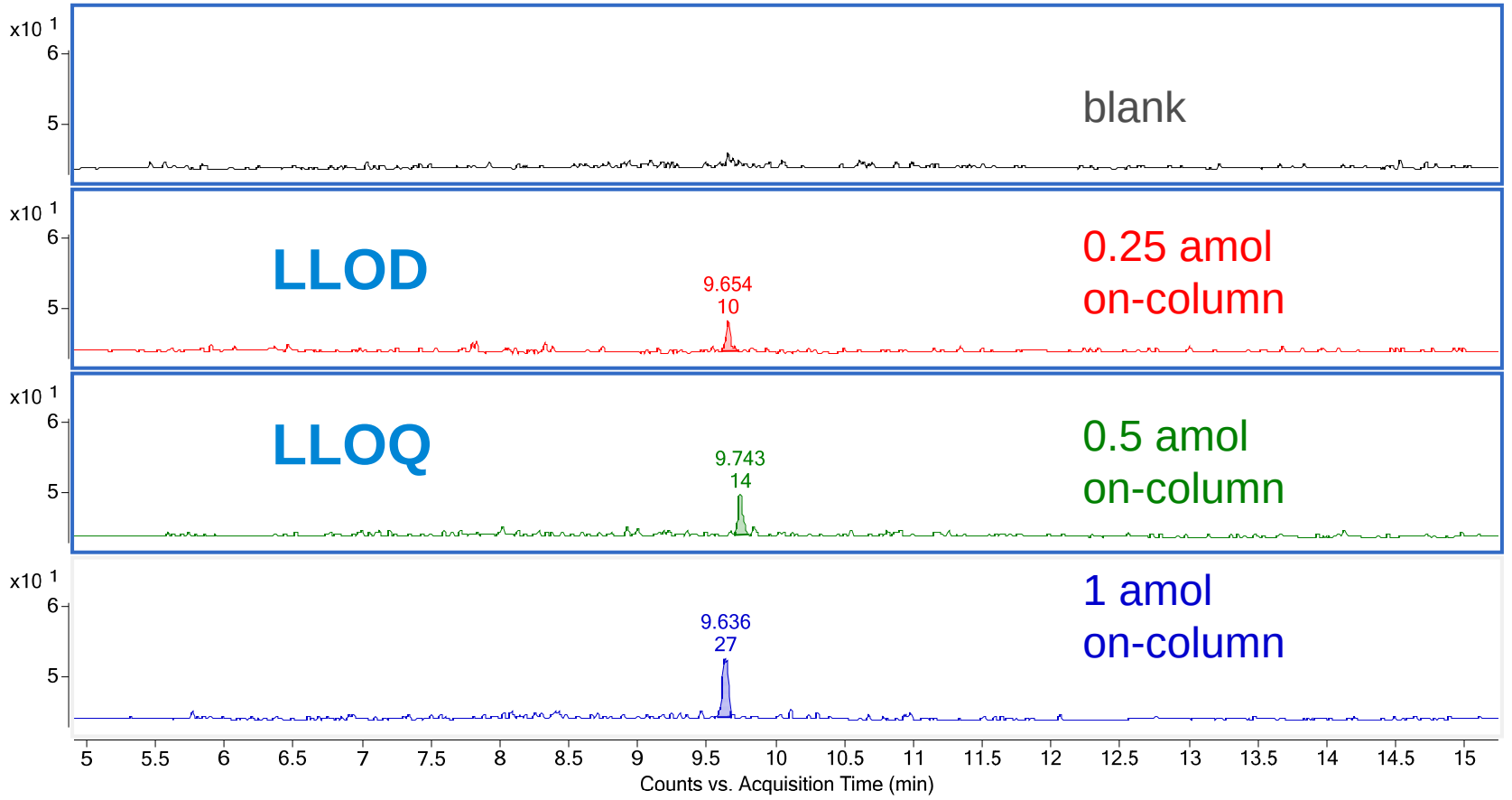
Instrumentation: 1290 UHPLC + Agilent JetStream + 6495 QQQ
Sample: synthetic peptide standard (LVNEVTEFAK) spiked into enolase tryptic digest
Injection volume: 1 µL

Peptide Quantitation – 6 Orders of Linear Dynamic Range



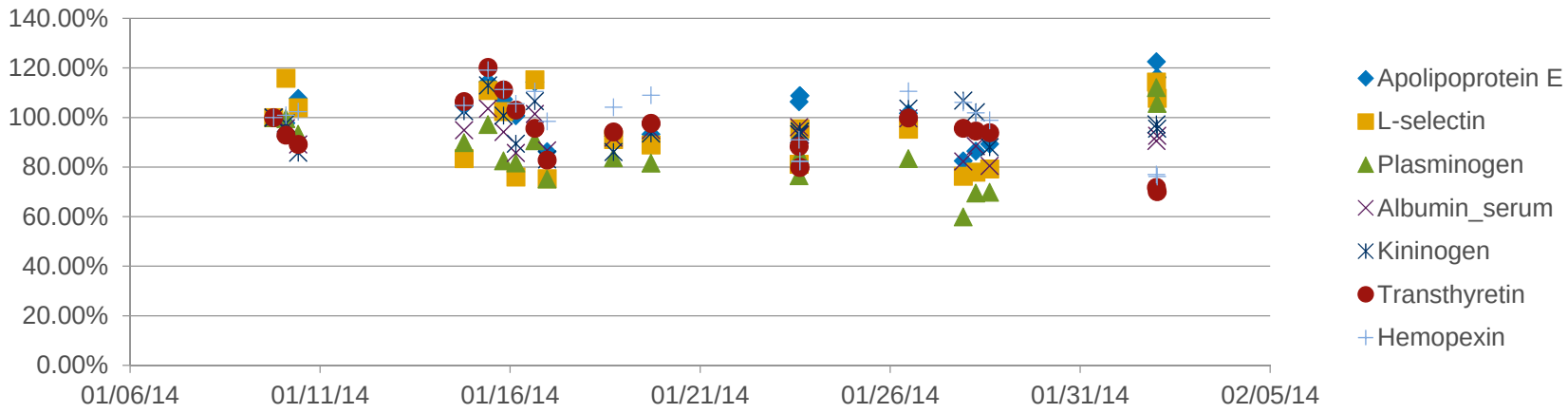
LVNEVTEFAK	Calibration Standards (amount on-column; 1 µL injected)									
	5.0 amol	7.5 amol	15 amol	30 amol	300 amol	3 fmol	30 fmol	300 fmol	3 pmol	5 pmol
%Accuracy	109.8	108.7	105.0	87.1	85.2	81.4	86.4	87.4	105.6	97.5
Reproducibility (%RSD, n=10)	14.0	16.0	9.4	9.0	1.6	1.2	0.6	0.7	2.1	1.0
RT (%RSD,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

Peptide Quantitation: Ultimate Sensitivity with Nanoflow Chromatography



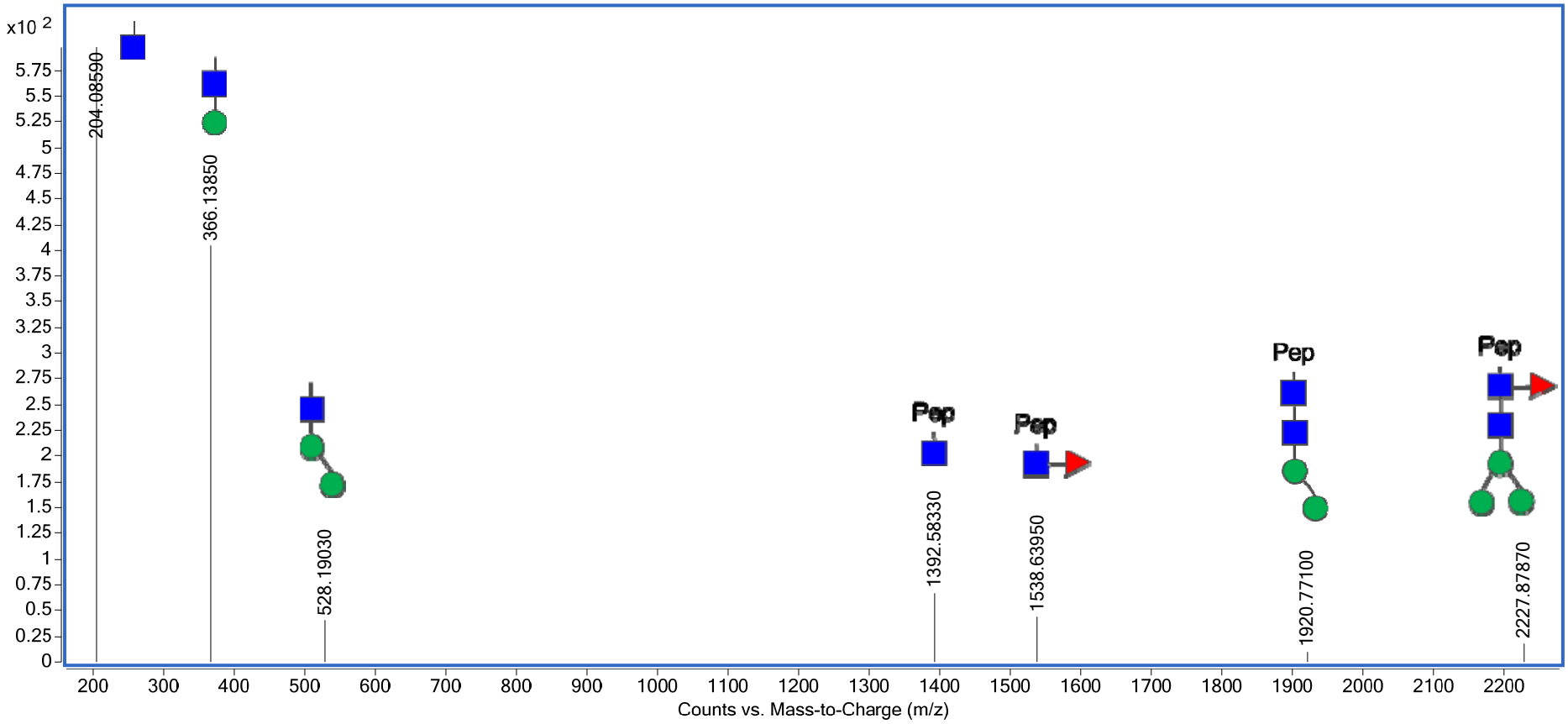
Instrumentation: 1260 HPLC-Chip/MS + 6495 QQQ
Sample: synthetic peptide standard (LVNEVTEFAK) spiked into enolase tryptic digest
Injection volume: 1 µL

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

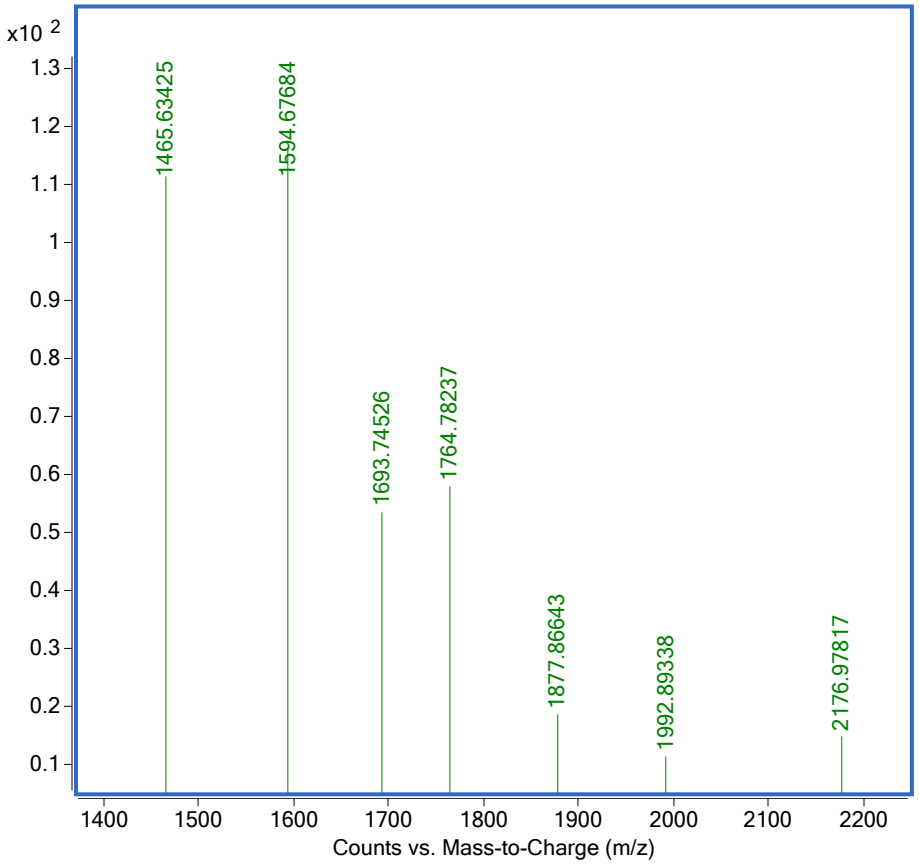
Increased Mass Range Useful for Peptides



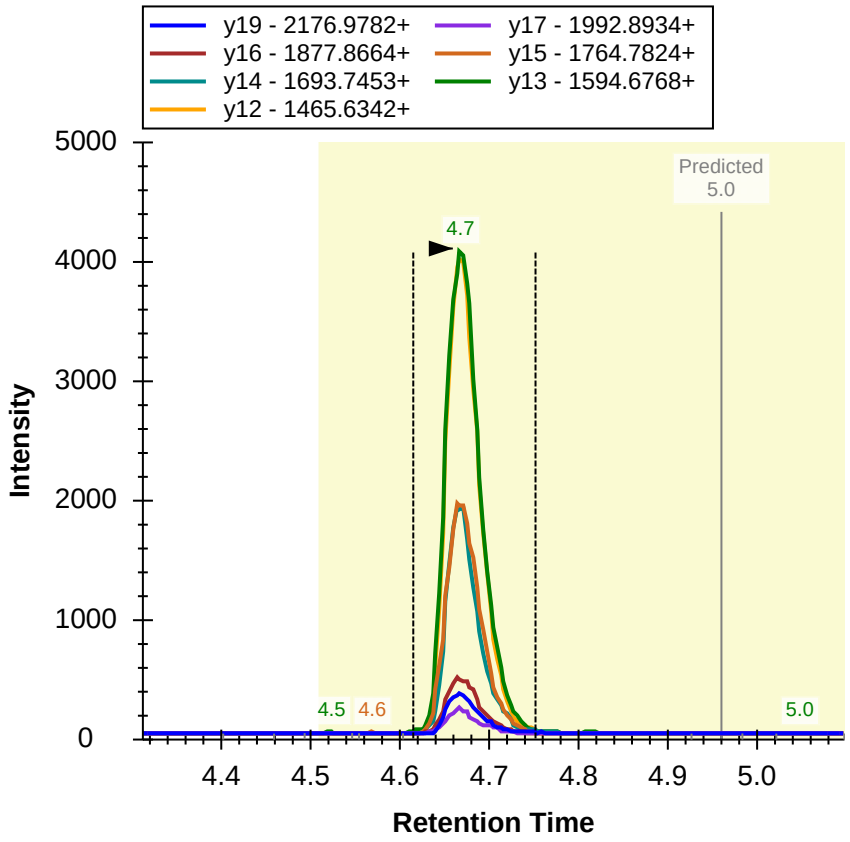
QQQ MRM spectrum for glycopeptide: EEQYN[+1606.6]STYR (G1F)

Increased Mass Range Useful for Peptides

MRM Spectrum



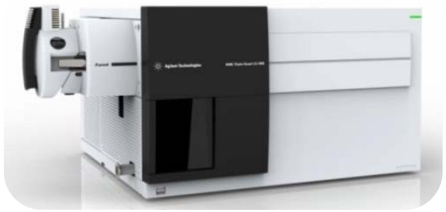
Skyline Overlay of MRM Chromatograms



MRM Spectrum for IgG Peptide: GFYPSDIAVEWESNGQPENNYK

Summary

- The ability to perform reproducible protein sample preparation on a versatile instrument platform which enables scaling of sample preparation makes robust, high-throughput, protein quantification attainable.
- Agilent LCMS coupled with Skyline software enables a simple automated method development workflow for targeted proteomics and Biopharma research.
- 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.



Acknowledgement

Alex Zhu

Steve Murphy

Zach Van Den Heuvel

Michael Bovee

Adam Krahenbuhl

Jason Russell

Christine Miller

Yanan Yang

THANK YOU !