



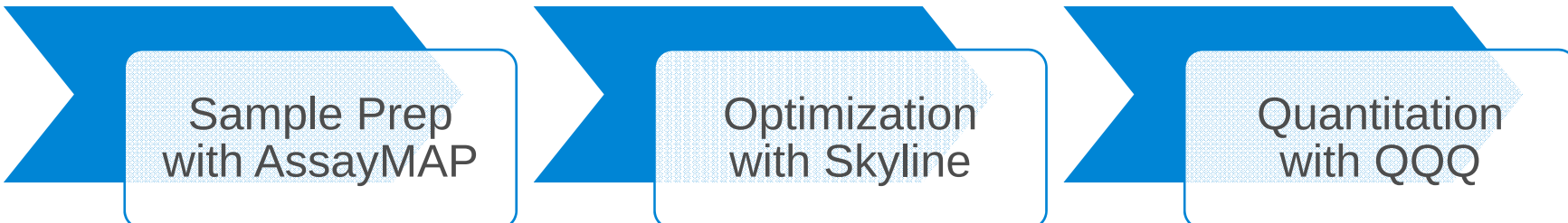
Bringing Precision to Peptide Quantitation Using Agilent Automation and LC/MS Solutions

Ning Tang, Ph.D.

Agilent Technologies

Santa Clara, CA

Outline



Sample Prep
with AssayMAP

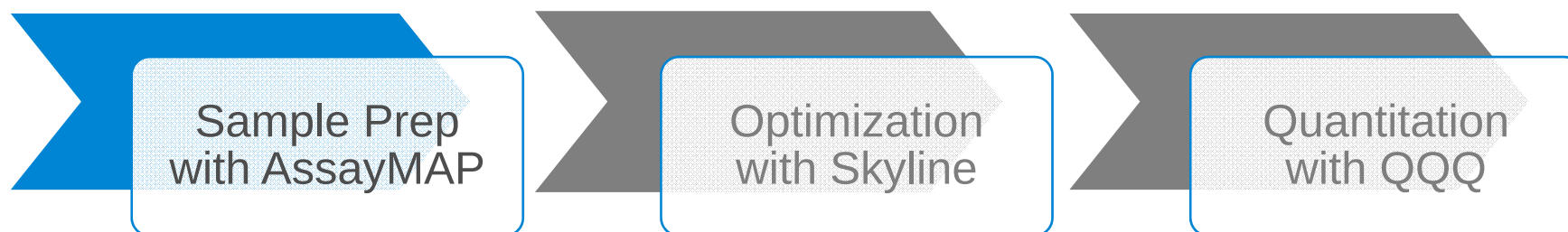
Optimization
with Skyline

Quantitation
with QQQ

- 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

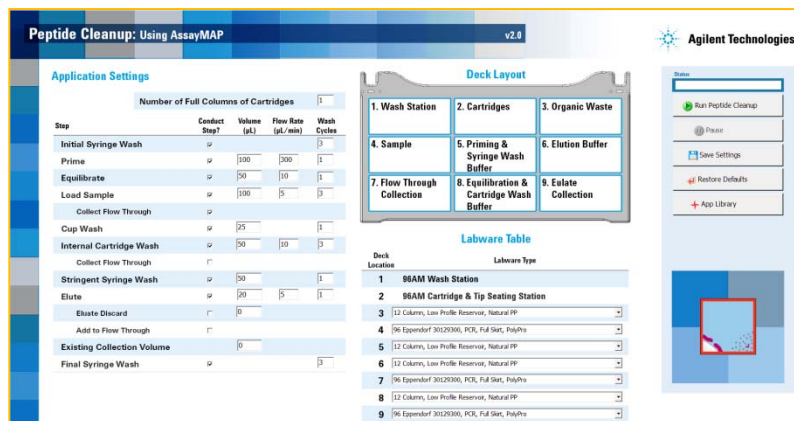


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AssayMAP Technology Components

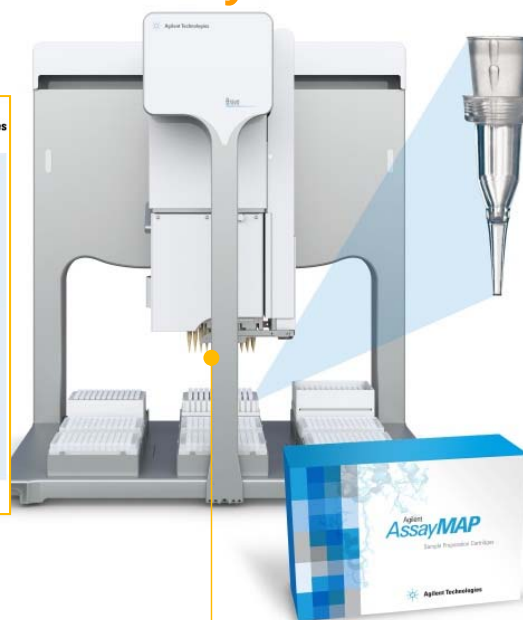
Automated workflows designed for analytical chemists



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



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Workflow Library

Protein Sample Prep Workbench: WORKFLOW LIBRARY

v1.0



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

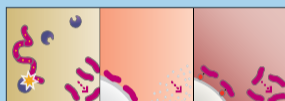
SISCAPA[®]
assay technologies

SISCAPA[®] Workflow

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

Open

Post-Translational Modification Workflows



Phosphopeptide Enrichment Workflow - TiO₂

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 glycans. 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 glycans. Using AssayMAP Bravo and ProZyme GlykoPrep-plus[®] APTS Chemistry Kit.

Open

Workflow Library

App Library

Utility Library

Literature Library



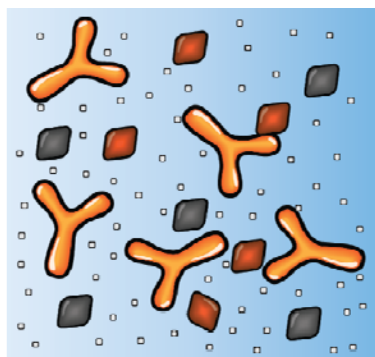
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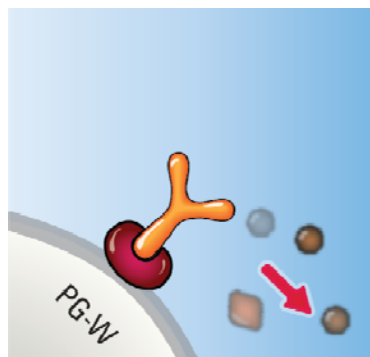
5

AssayMAP mAb Quantitation Workflow

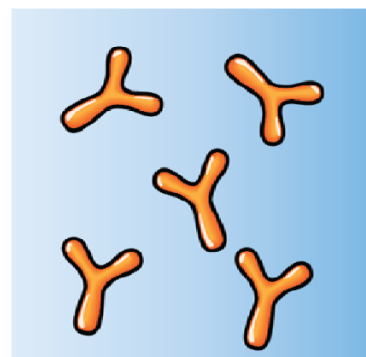
Each step in the workflow was performed using the AssayMAP Bravo



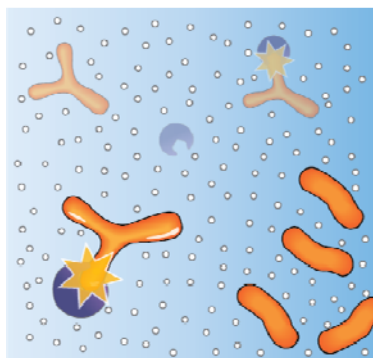
Input



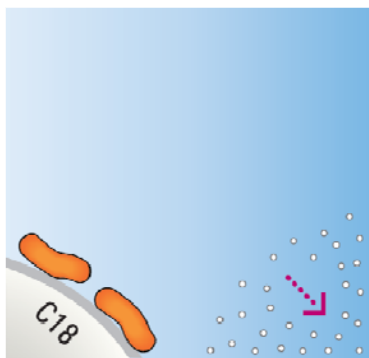
Purify



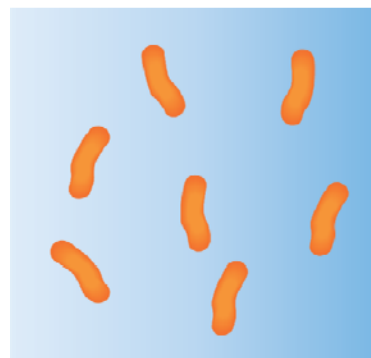
Output



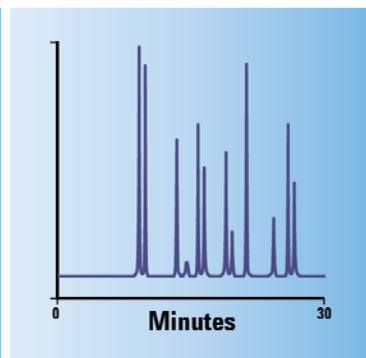
Digest



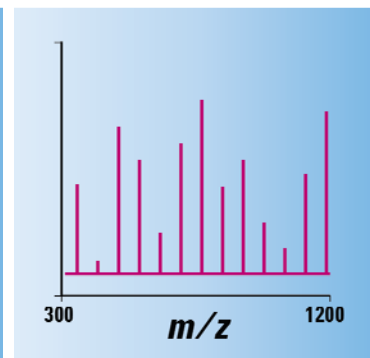
Cleanup



Output



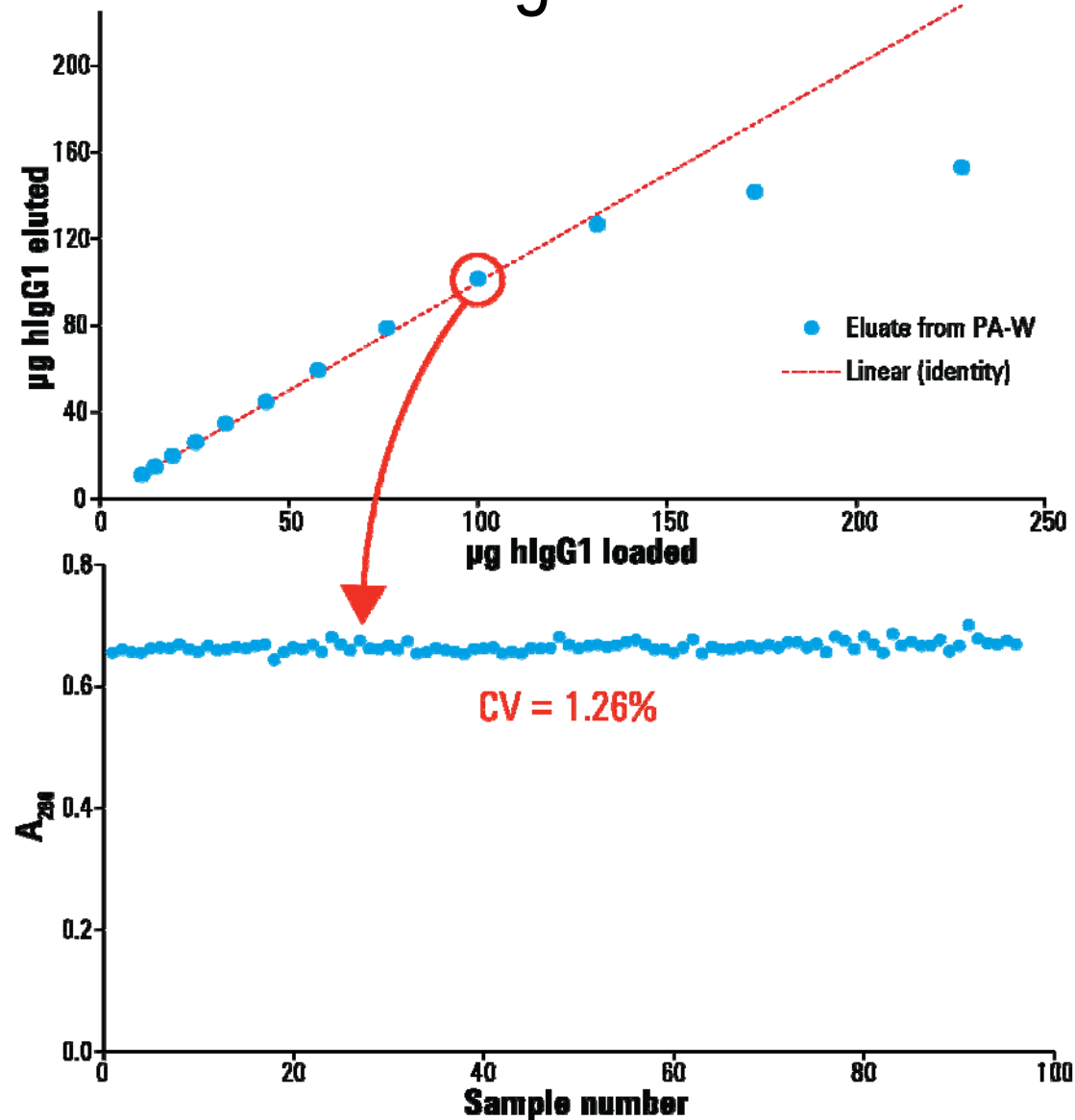
Separate



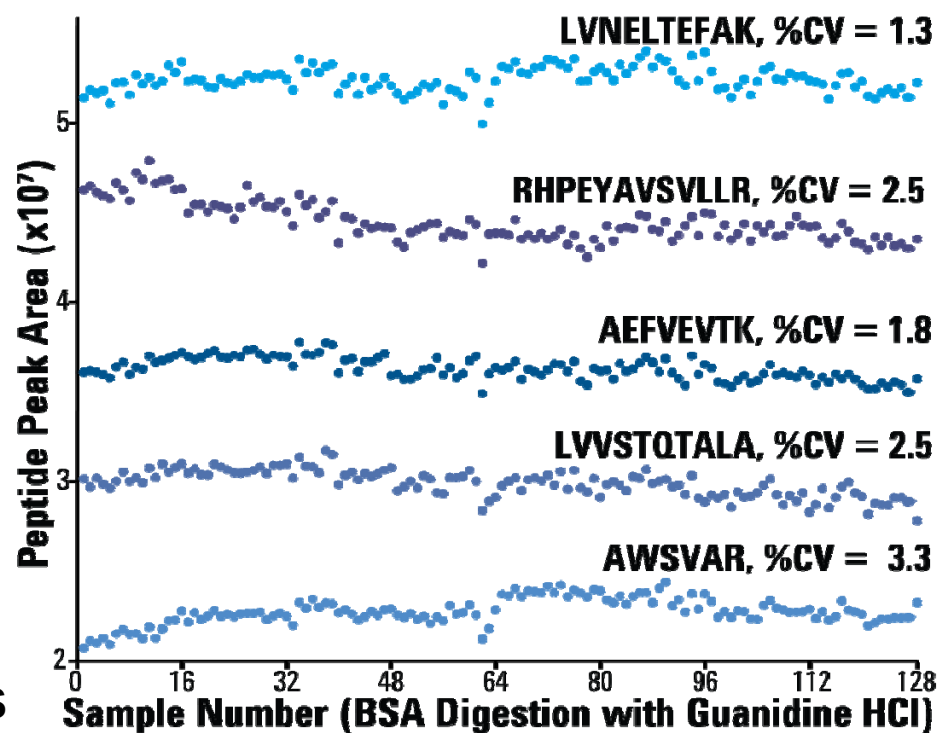
Analyze



Antibody Purification Using Protein A Cartridge



AssayMAP Digestion and Cleanup

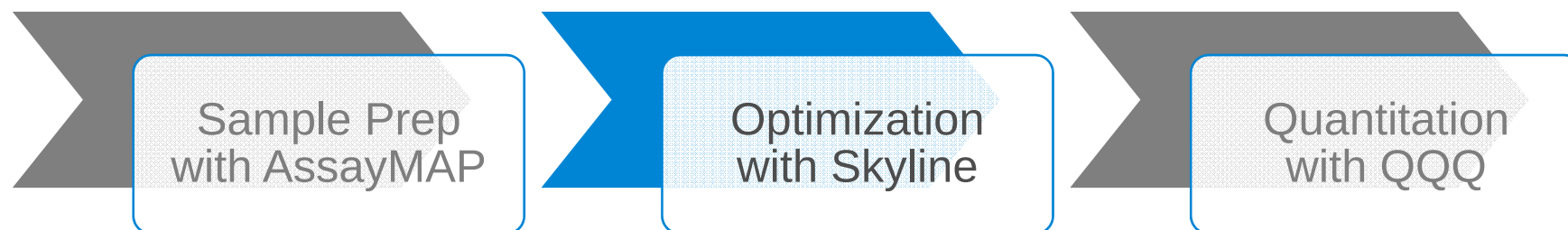


Day 1 cleanup = C18
Day 2 cleanup = RP-S

Urea	Day 1	Day 2	Guanidine HCl	Day 1	Day 2
# of samples	64	62	# of samples	64	64
# of peptides monitored	25	25	# of peptides monitored	25	25
Avg. peak area %CV	3.3	3.7	Avg. peak area %CV	2.3	2.6
# Peptides %CV <5	23	21	# Peptides %CV <5	25	23
# Peptides 5>%CV<10	2	3	# Peptides 5>%CV<10	0	1
# Peptides %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

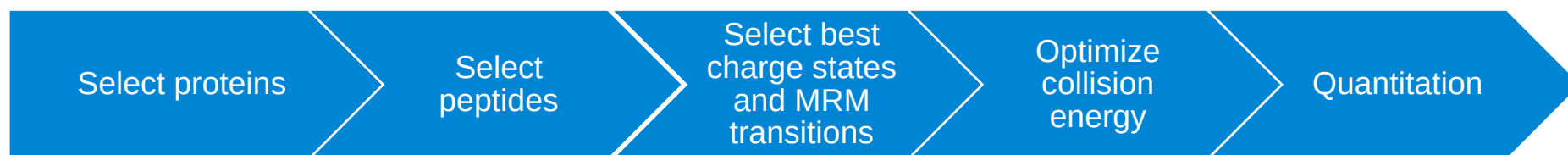
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MRM Based LC/MS Protein Quantitation



- 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

The screenshot shows the Skyline Targeted Proteomics Environment software interface. At the top, there is a logo for MacCoss Lab Software and a navigation bar with 'Home'. Below this, the title 'Skyline Targeted Proteomics Environment' is displayed. A banner encourages users to join over 3800 other proteomics investigators and sign up for email notifications. The main window displays a list of targets on the left, including 'NP_872280' and 'NP_036789'. The right side shows three chromatograms for different targets: H147.1, H148.1, and H159. Each chromatogram plots Intensity (10⁻³) against Retention Time. The peaks are labeled with their retention times: 30.3, 29.9, and 30.3. A legend at the bottom indicates the peak areas for each target.

- 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

Skyline-daily (beta)

[VIDEO TRAILER](#)

[License Agreement](#)
[3rd-Party Software](#)
[How You Can Help](#)
[Get Involved](#)
[Related](#)

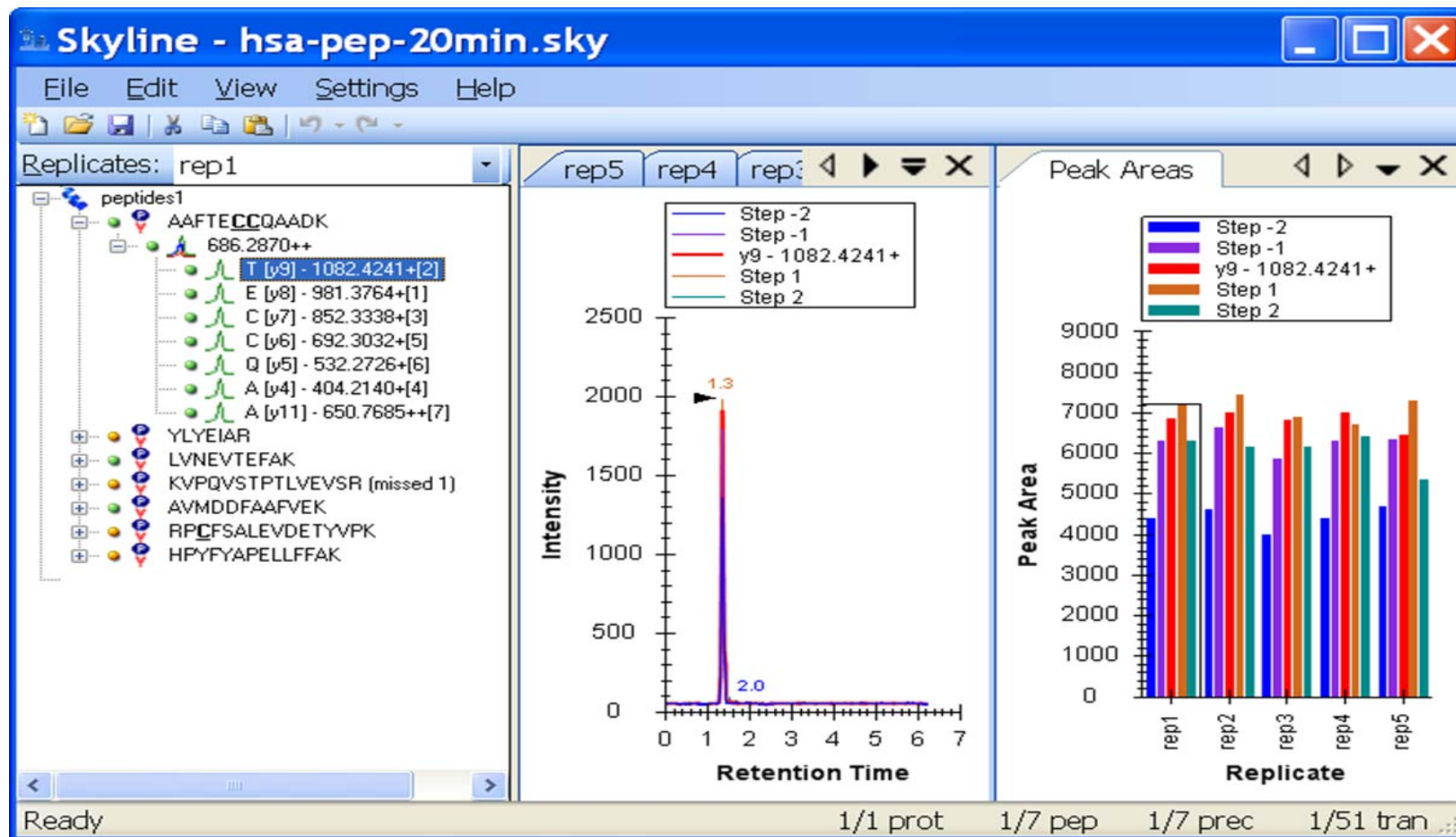


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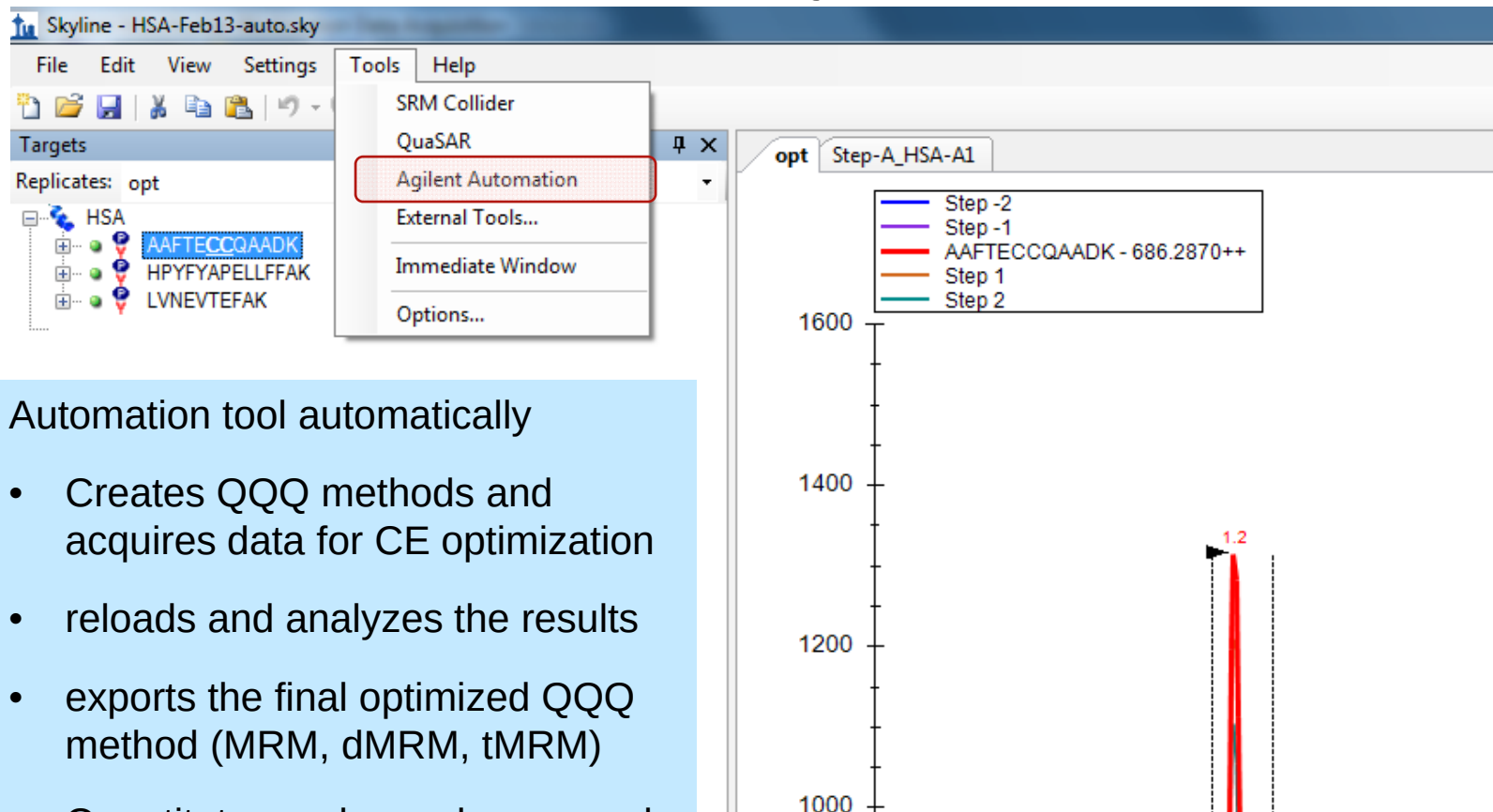
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11

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

Skyline Automation - D:\chris\HSA-Feb13-auto.sky

Help

Project setting

Template method: D:\MassHunter\Methods\chris\A

Folder: D:\MassHunter\Data\chris

Name: demo ☐ Timestamp

Action selections

☒ Step-A (Update Retention Times)

☒ Step-B (Optimize Collision Energy)

☒ Step-C (Export method, create worklist)

☒ Execute workli:

Step-A Step-B Step-C

(Step-A)

Export method name: Demo

☒ Single methc

☐ One method per prote

☐ Multiple metho ☐ Ignore proteins

Max transitions per sample injection: 200

Optimizing: None

Method type: Standard Dwell time (ms): 5

Project is created at: D:\MassHunter\Data\chris\demo.s. (3/4/2013 7:57:09 AM) Total samples: 12

MRM→RT dMRM CE opt dMRM/tMRM→Quant

(Step-A) Sample prefix: Standard Start position: P1-A1 ☐ All samples in same position

Edit	Sample Name	Sample Position	Method	Data File
▶ 1	Standard1	P1-A1	Demo.m	Step-A_Demo1.d

(Step-B) Sample prefix: Standard Start position: P1-A1 ☐ All samples in same position

Edit	Sample Name	Sample Position	Method	Data File
▶ 1	Standard1	P1-A1	Demo_0001.m	Step-B_Demo_00011.d

(Step-C) Sample prefix: Samples Start position: P1-A1 ☐ All samples in same position

Edit	Sample Name	Sample Position	Method	Data File
▶ 1	Samples1	P1-A1	Demo.m	Step-C_Demo1.d
2	Sample2	P1-B1	Demo.m	WorklistData2.d
3	Sample3	P1-B2	Demo.m	WorklistData3.d
4	Sample4	P1-B3	Demo.m	WorklistData4.d
5	Sample5	P1-B4	Demo.m	WorklistData5.d



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

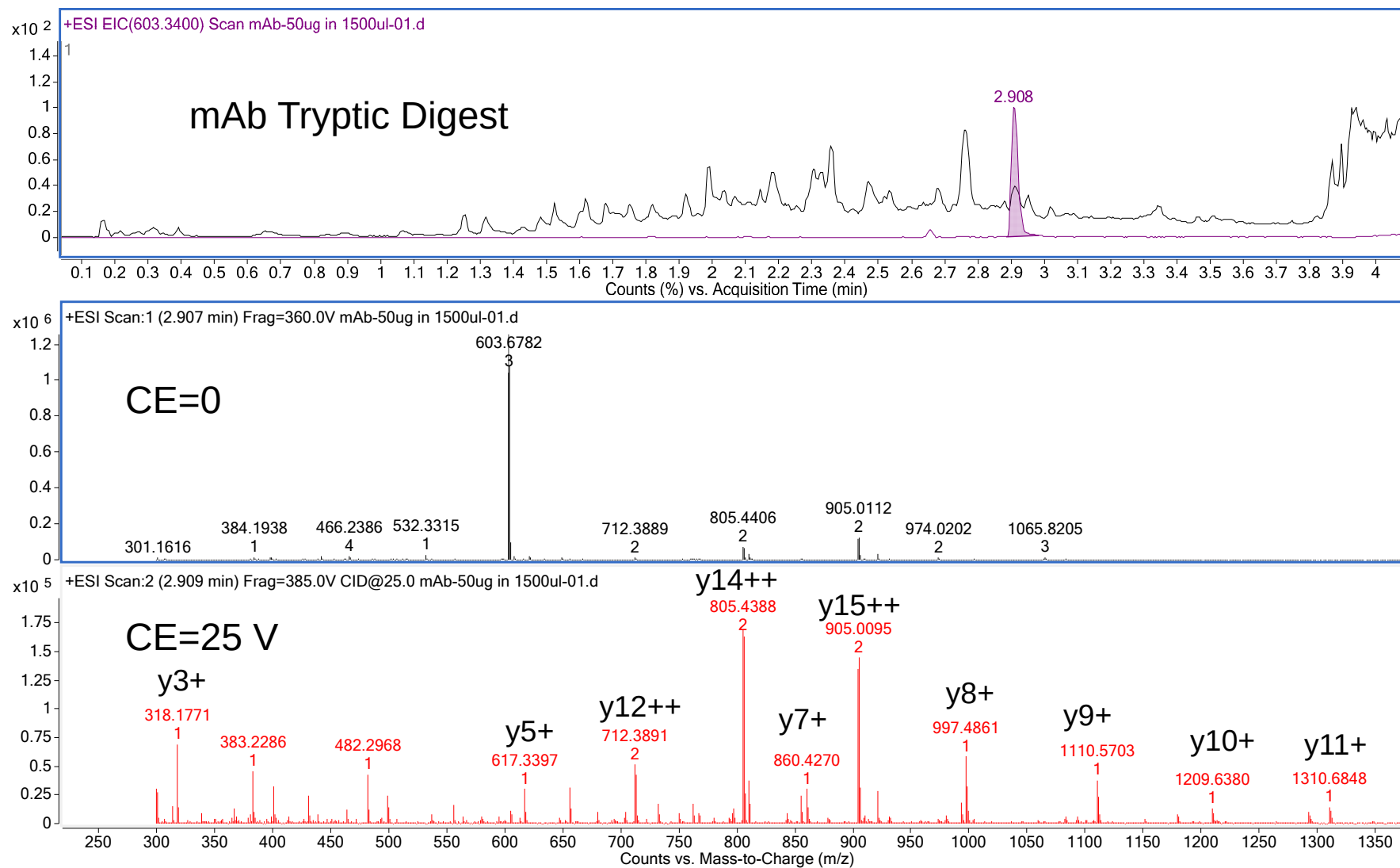


All Ions MS/MS → Skyline → QQQ MRM Workflow



- 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

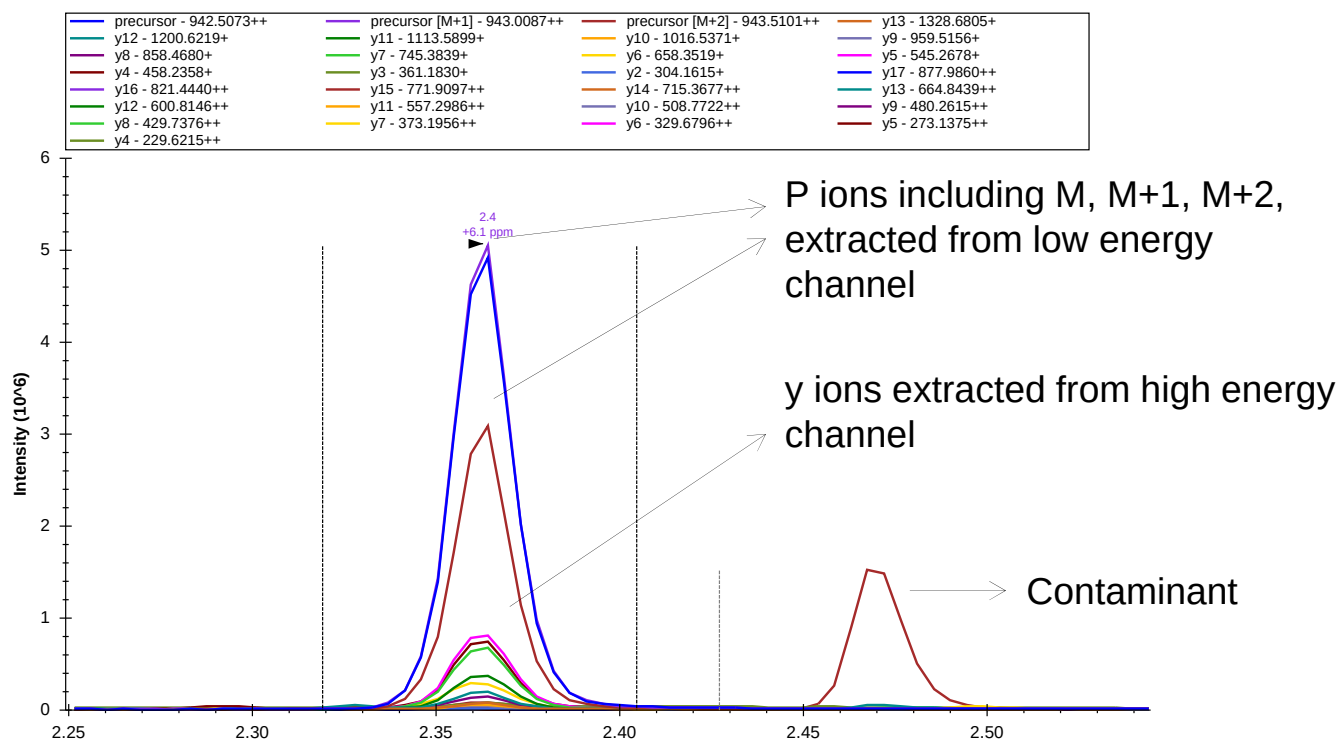
All Ions MS/MS on Agilent QTOF



All Ions → Skyline Workflow

Step 1: Show p & y Ions for Peptide Confirmation

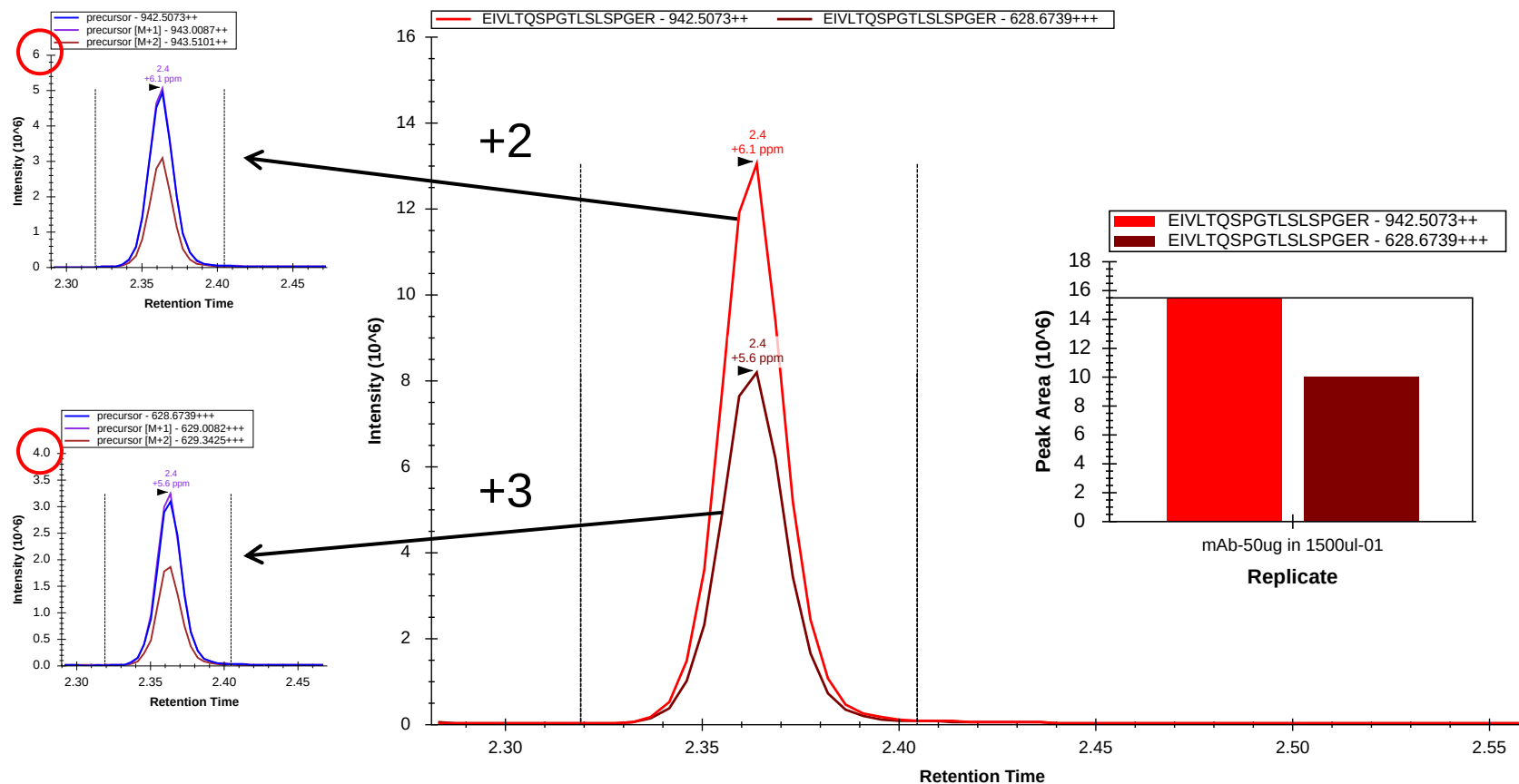
Precursors (**p ions**) from low energy channel and products (**y ions**) from high energy channel are automatically extracted and plotted together to easily confirm the identity of the peptides.



Retention time and peptide sequence confirmation

All Ions → Skyline workflow

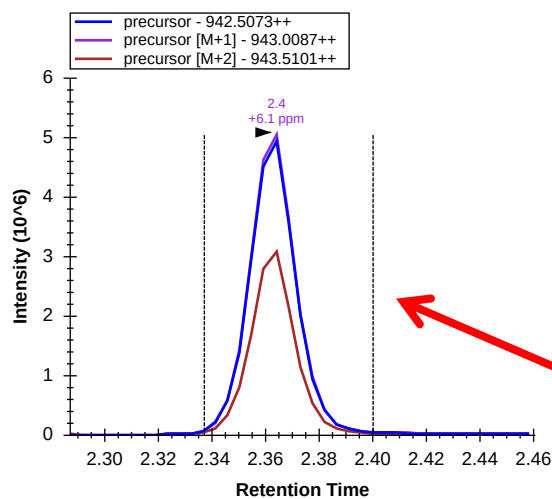
Step 2: Show only p Ions to Select Best Charge State



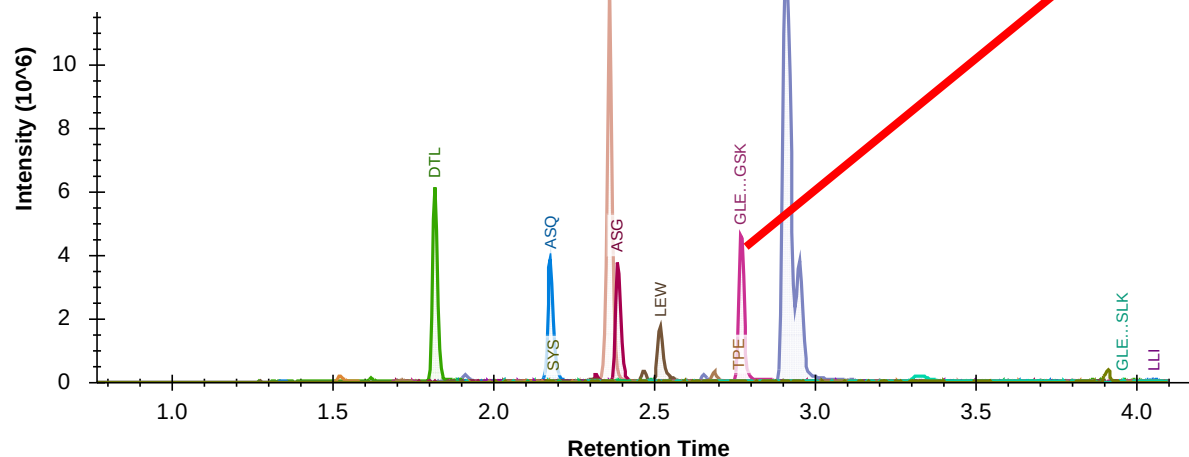
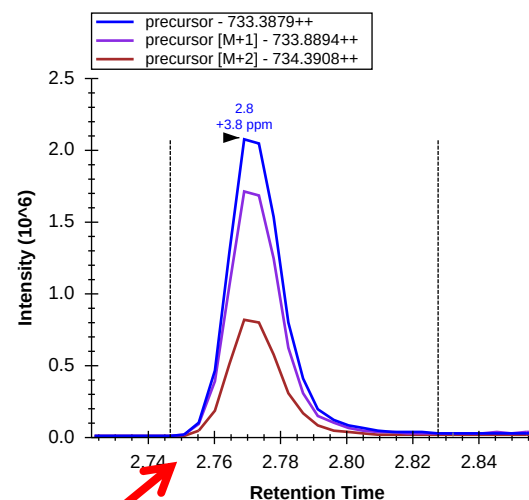
Best charge state for each peptide is easily determined by comparing p ions only

All Ions → Skyline workflow

Step 3: Show only p ions to Compare Peptide Abundance



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



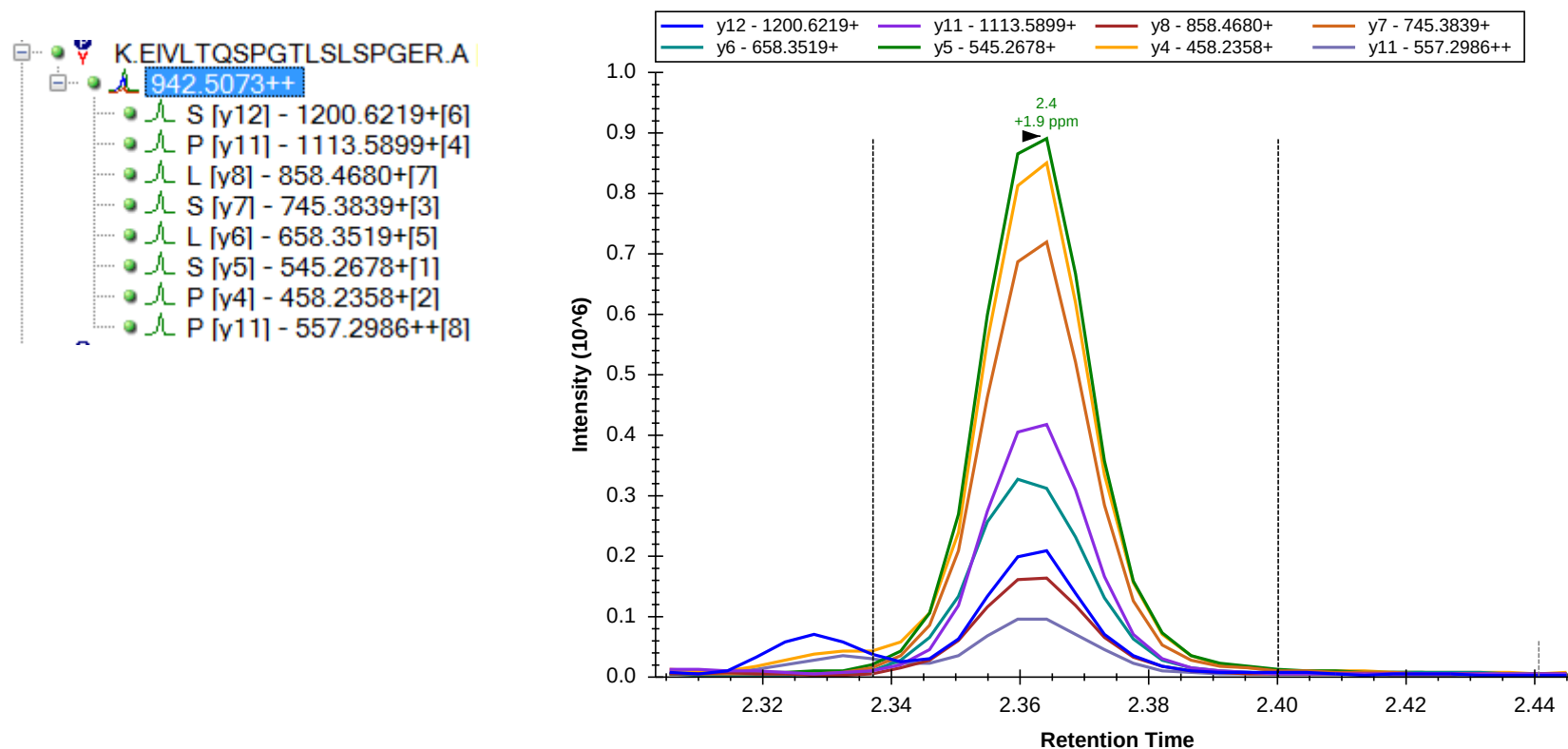
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20

All Ions → Skyline workflow

Step 4: Show Only y Ions to Select Top MRMs



Skyline transition rank refine tool picks top n MRMs



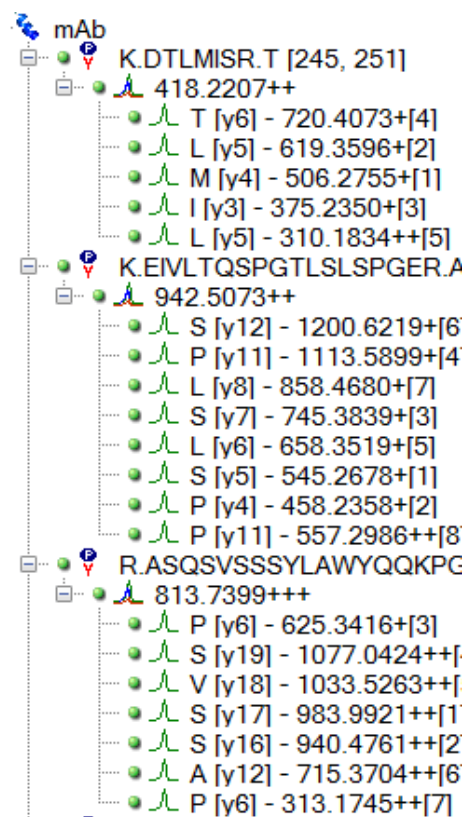
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21

All Ions → Skyline workflow

Step 5: CE Optimization



Export Method

Instrument type:
Agilent 6400 Series

OK
Cancel

☐ Single method
☐ One method per protein
☒ Multiple methods ☐ Ignore proteins

Max transitions per sample injection:
100

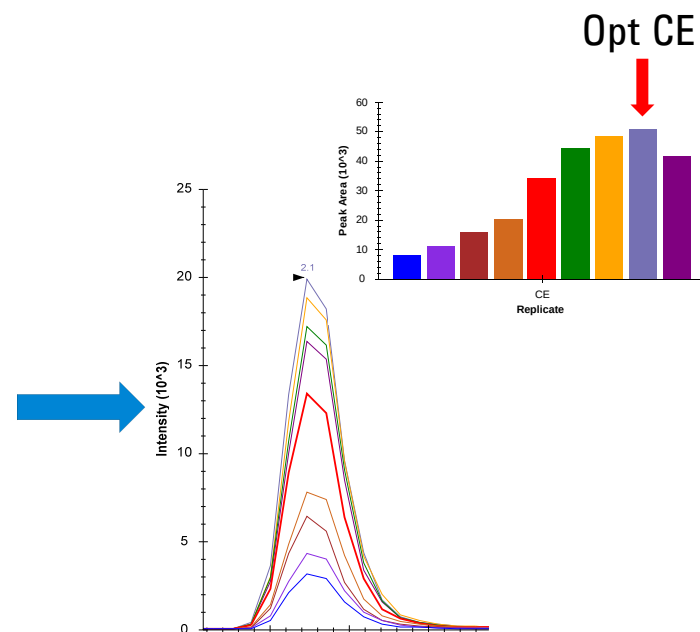
Methods: 2

Optimizing:
Collision Energy

Method type:
Standard

Dwell time (ms):
10

Template file:
Browse...



CE optimization process can be automated using new Skyline Automation Tool

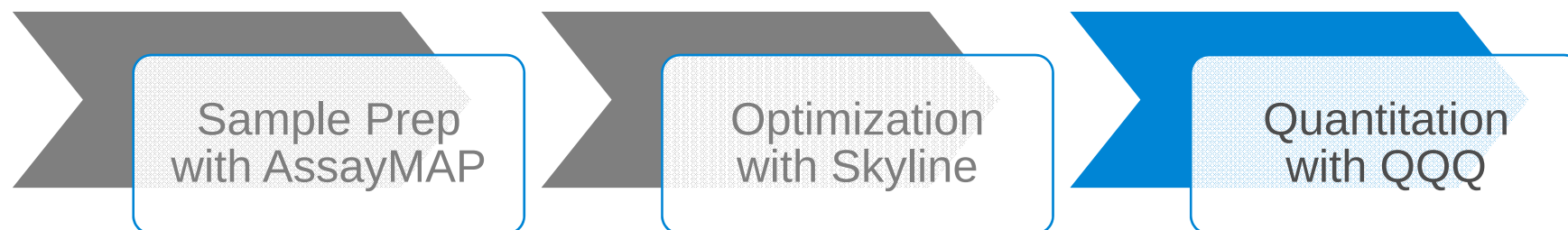


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22

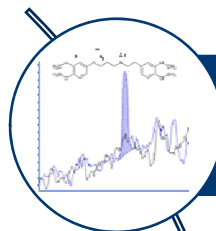
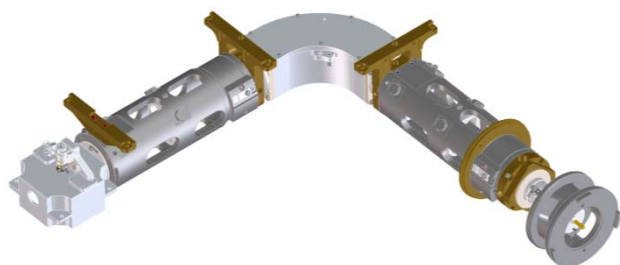
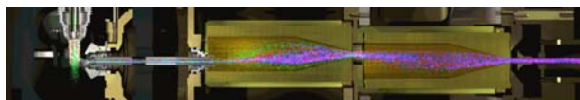
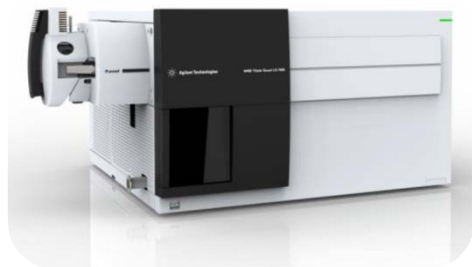
Outline



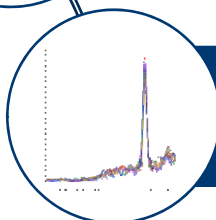
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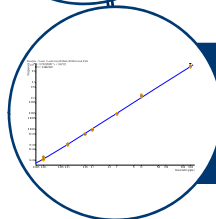
6495 QQQ LC/MS - Premium Performance



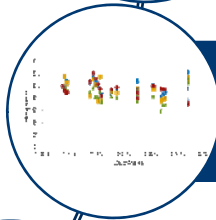
Improved sensitivity (IDL / MDL) – Average 3x in S/N specifications and applications



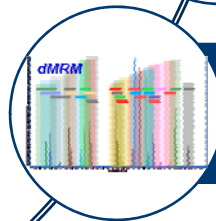
Improved **precision** and excellent **accuracy** at the lowest levels – 4 to 5x in IDL spec



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



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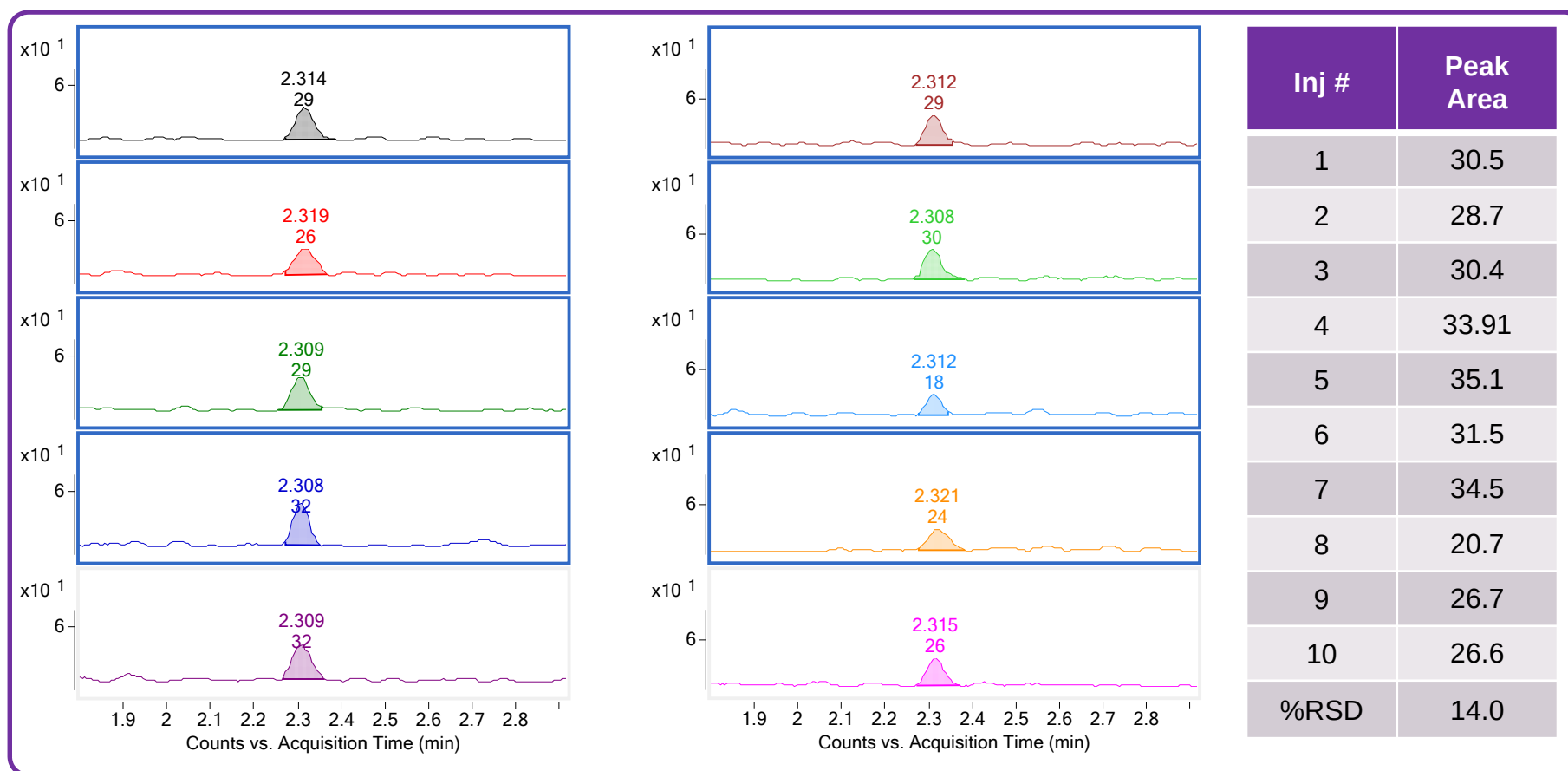
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24

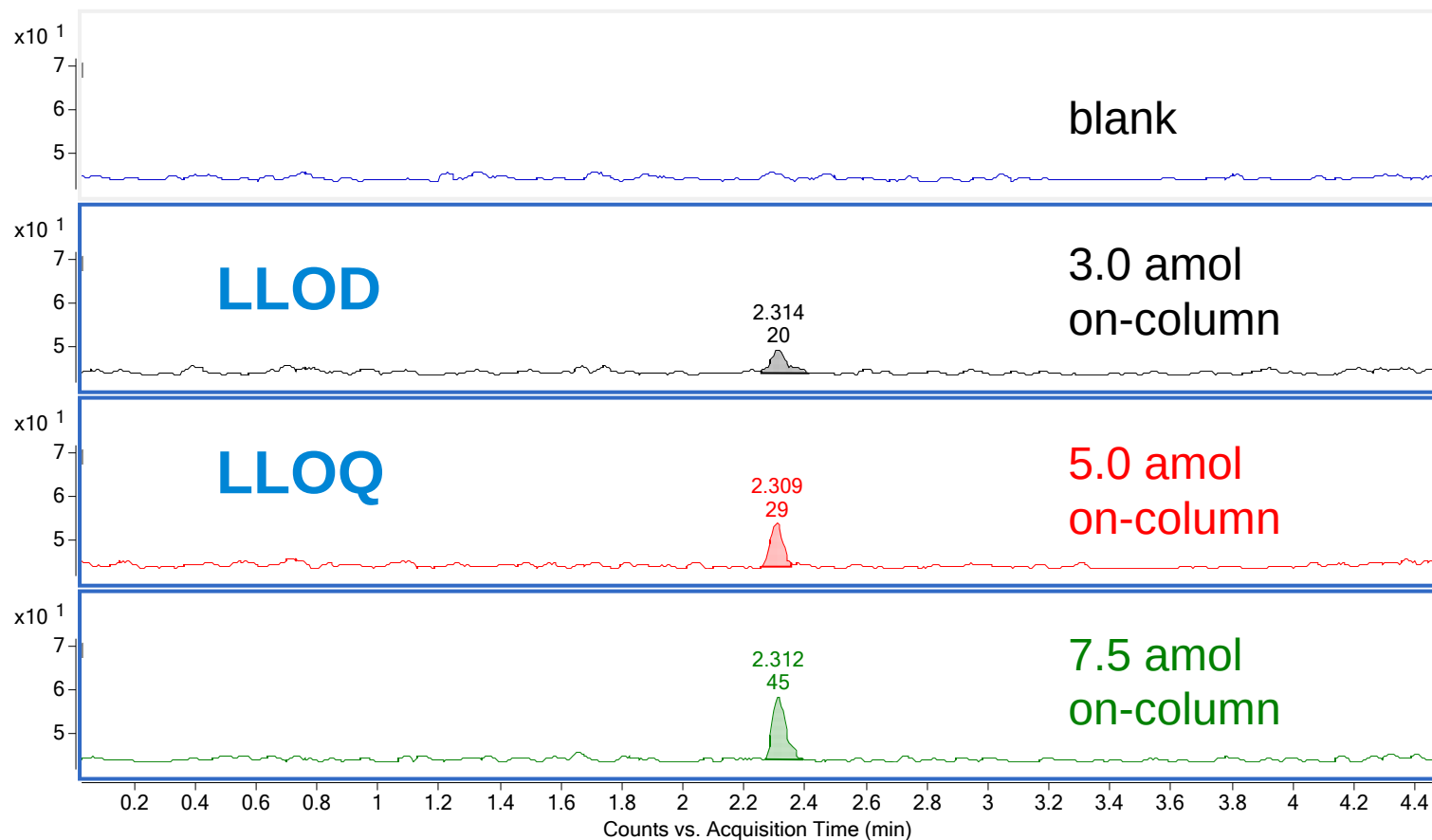
LVNEVTEFAK: Excellent Precision and Low Amol IDL

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

$$\text{MDL} = t \times (\% \text{RSD} / 100) \times \text{Amount} = 2.821 \times (14.0 / 100) \times 5.0 \text{ amol} = 2.0 \text{ amol}$$



Peptide Quantitation: Outstanding Sensitivity with Standard Flow Chromatography

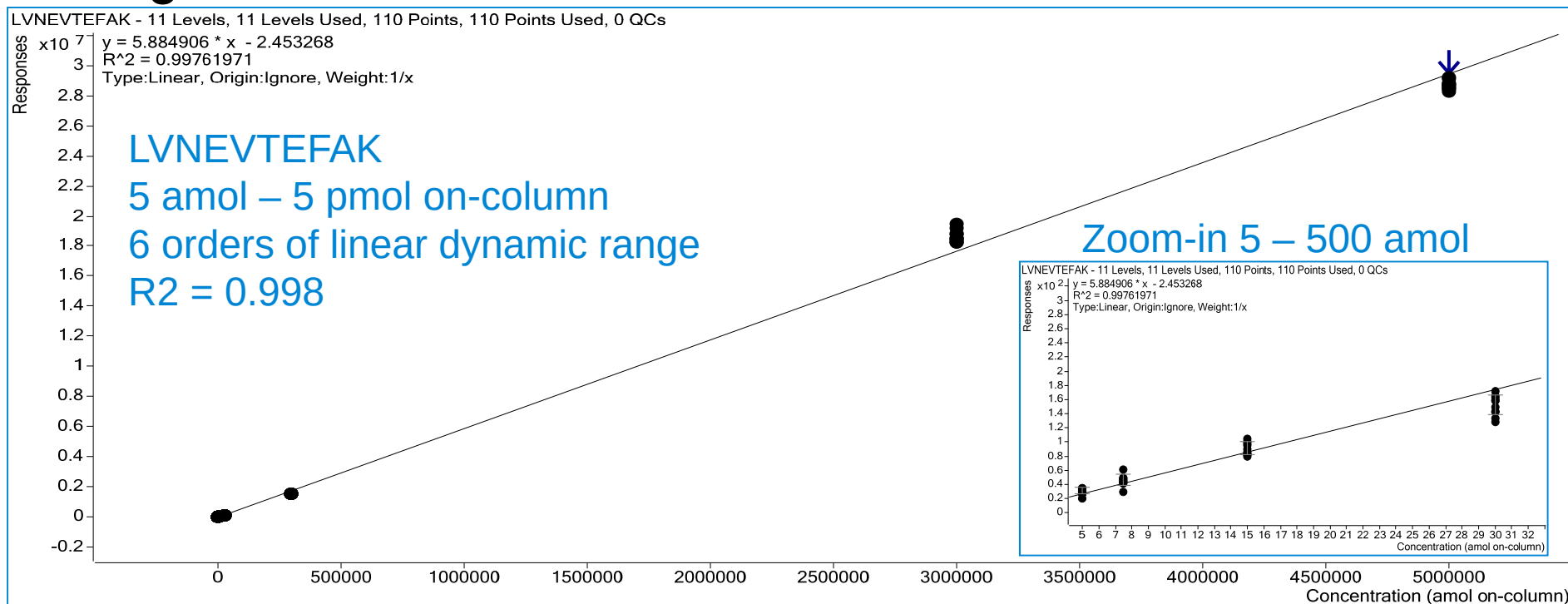


Instrumentation: 1290 UHPLC + Agilent JetStream + 6495 QQQ

Sample: synthetic peptide standard (LVNEVTEFAK) spiked into enolase tryptic digest

Injection volume: 1 μ L

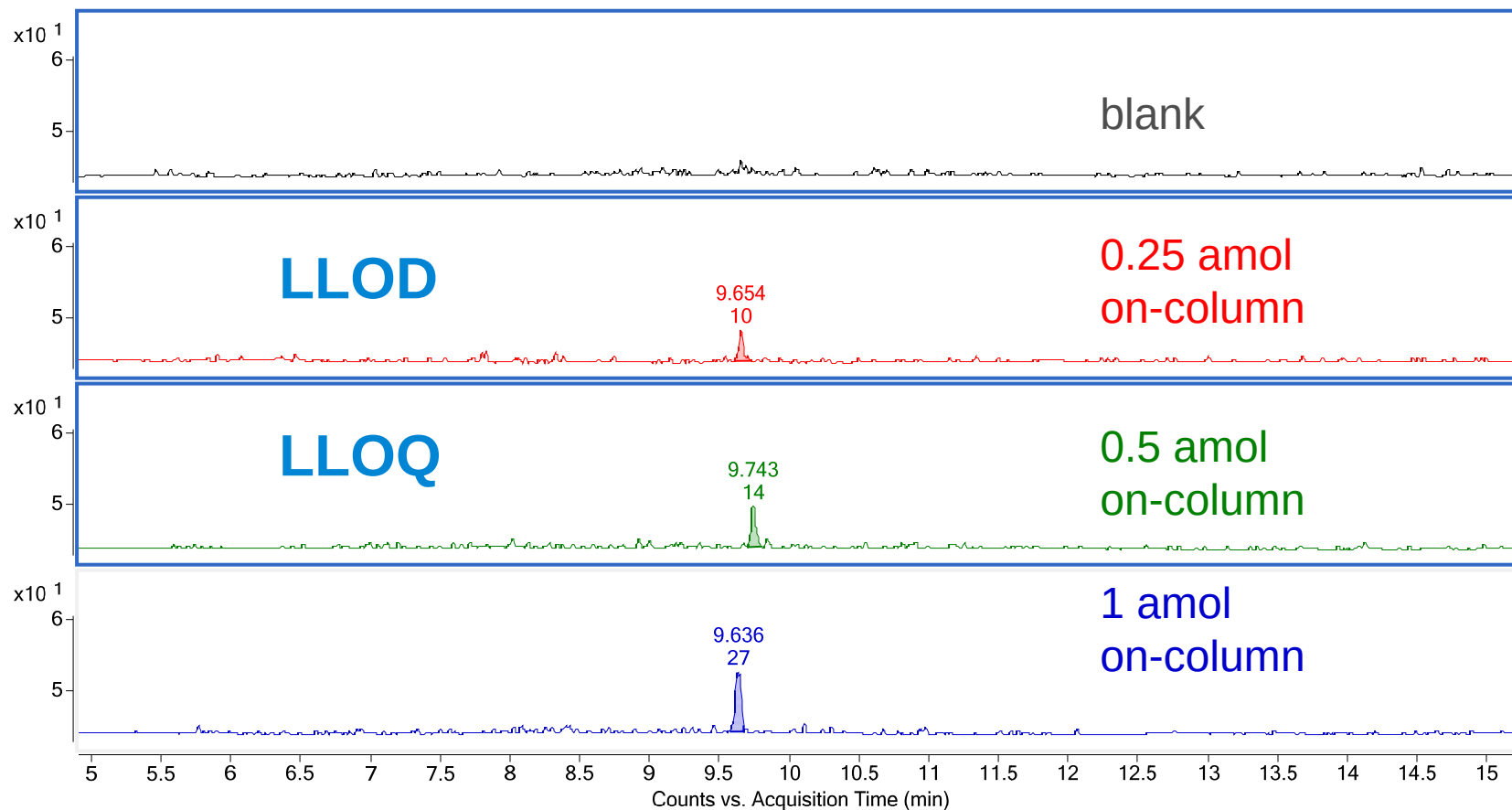
Peptide Quantitation – 6 Orders of Linear Dynamic Range



LVNEVTEFAK	Calibration Standards (amount on-column; 1 μ L 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

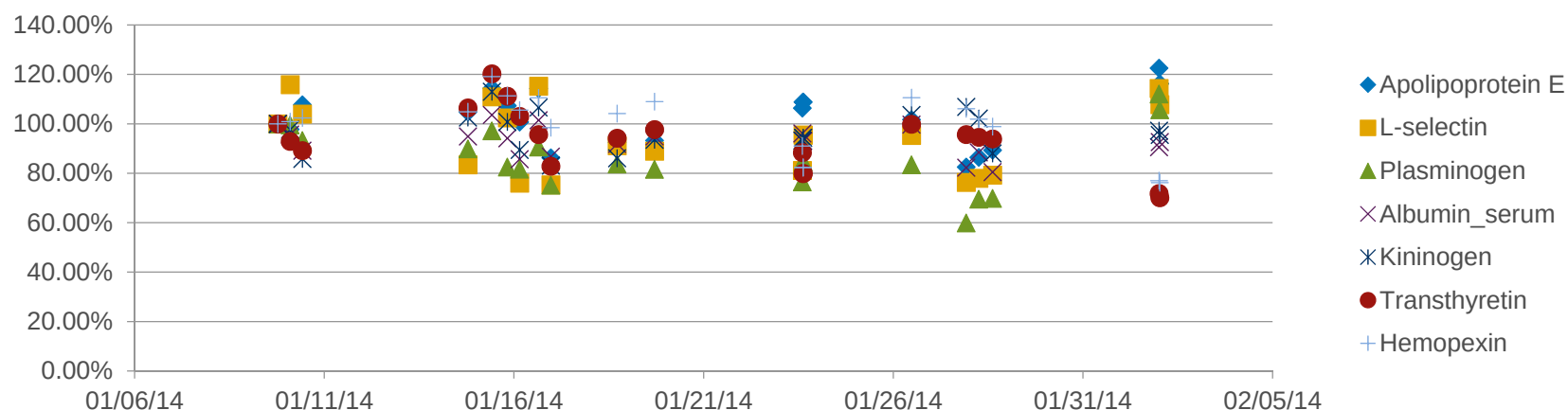
Sample: synthetic peptide standard (LVNEVTEFAK) spiked into enolase tryptic digest

Injection volume: 1 μ L



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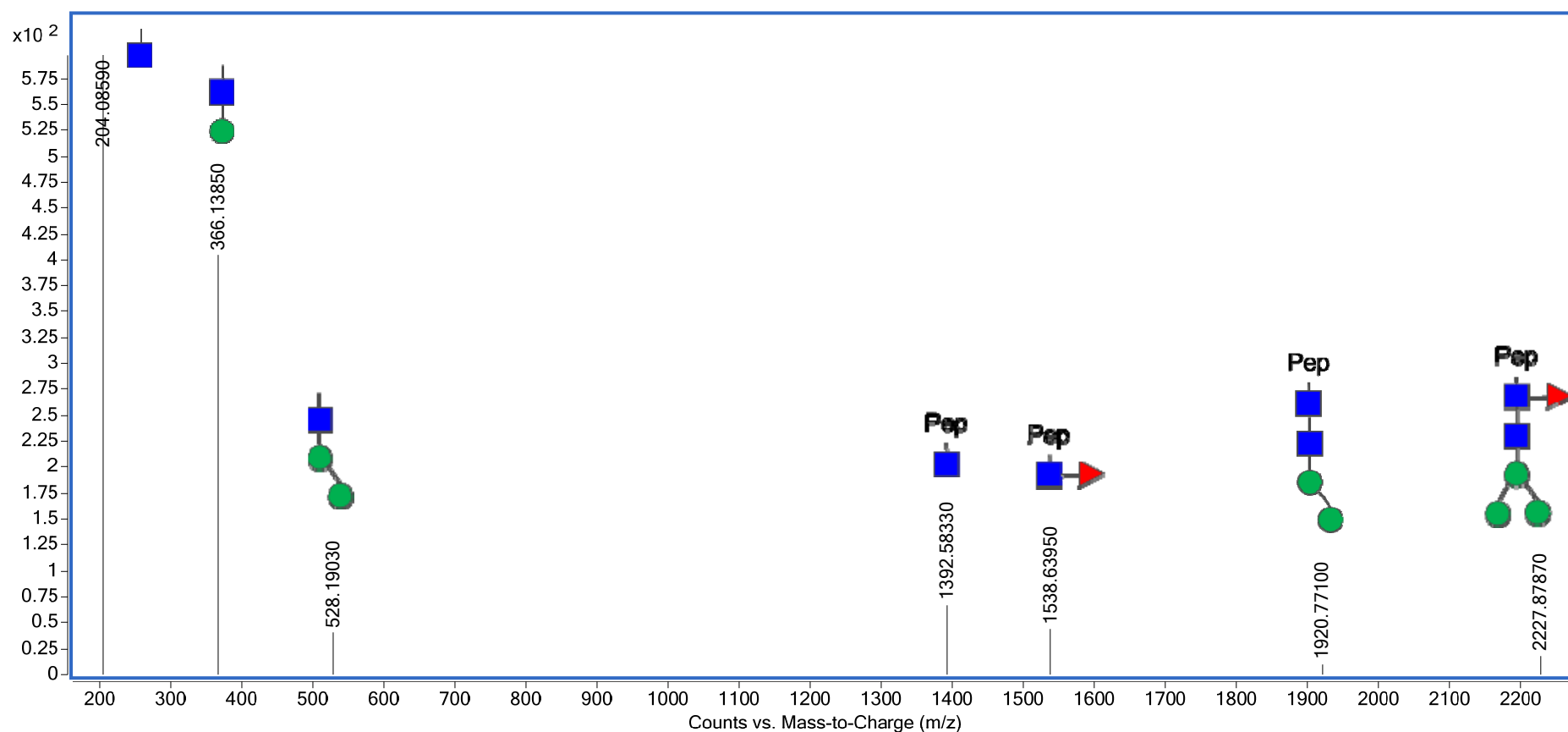
Proven System Robustness in Complex Matrix: Protein Quantitation in Plasma



- 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

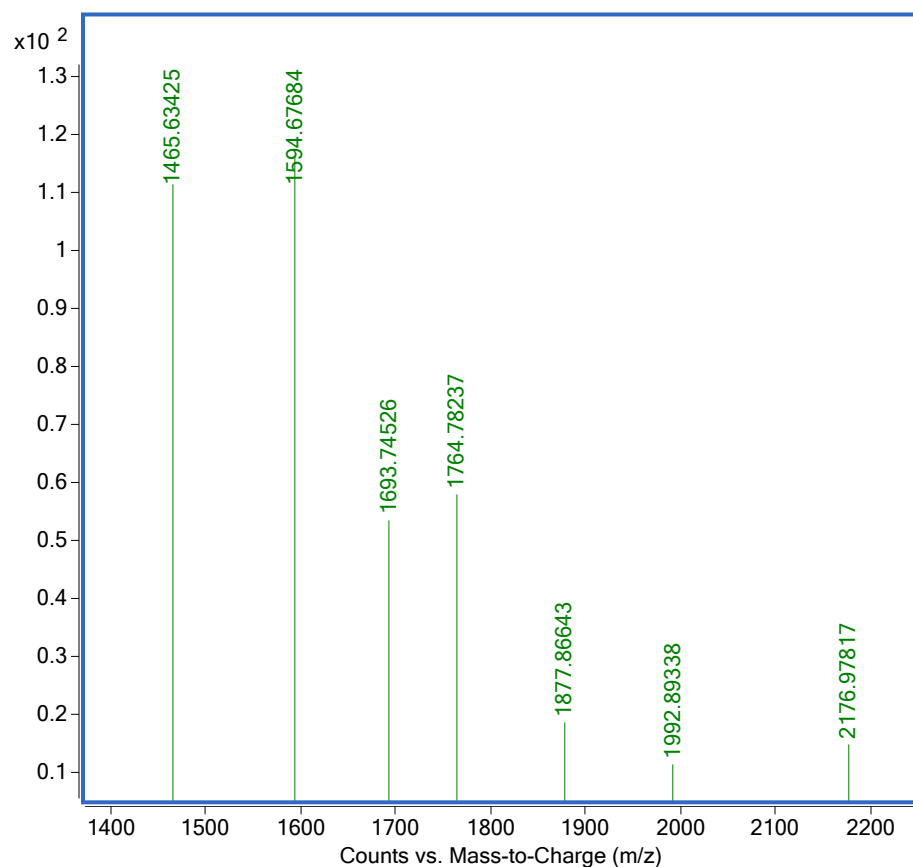


QQQ MRM spectrum for glycopeptide: EEQYN[+1606.6]STYR (G1F)

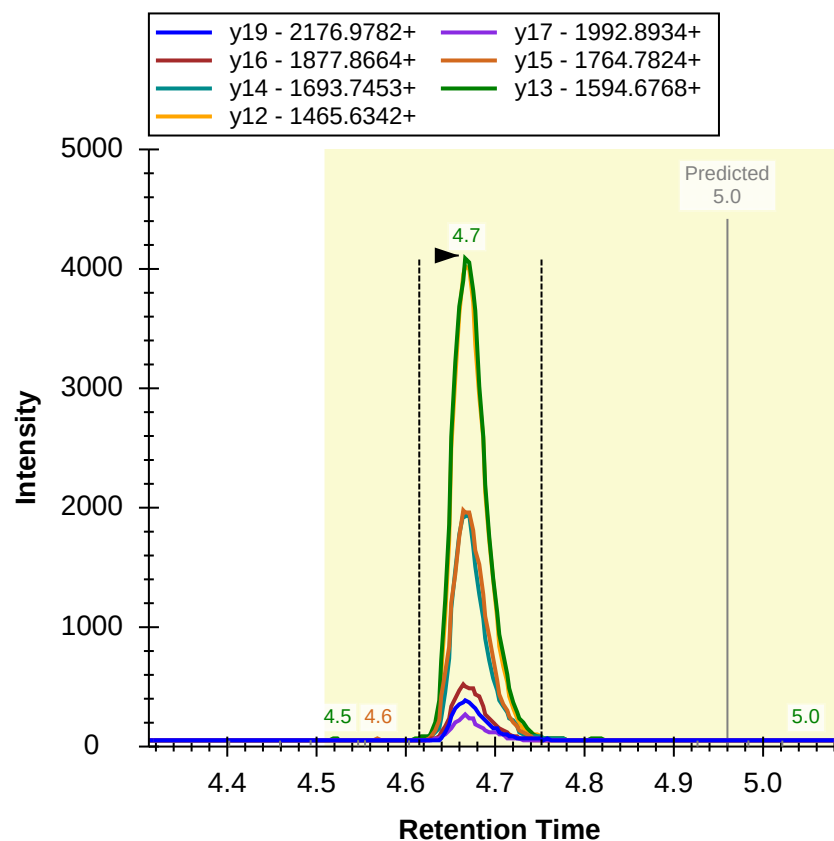


Increased Mass Range Useful for Peptides

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

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Jason Russell

Christine Miller

Yanan Yang

Speakers



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Associate Principal Scientist
AstraZeneca



Steve Murphy, Ph.D.
Director of AssayMAP Applications Development
Agilent Technologies

Moderator



Jeffrey Buguliskis, Ph.D.
Technical Editor
GEN

Wednesday
February 25, 2015
10:00 am ET

Automating MRM-Based Protein Quantification

GEN Webinars

Free Registration!

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A significant challenge to standardization in liquid chromatography-multiple reaction monitoring (LC-MRM) workflows is the variability caused by the multistep, highly manual nature of sample preparation. If MRM-based protein quantification is to enjoy widespread use for disease biomarker assessment studies—and ultimately secure acceptance for clinical analysts—the technique must be standardized to facilitate precise protein quantification. To address the issues of throughput and variability related to complex manual workflows, automation platforms are being developed to serve as the foundation for sample preparation in LC-MRM assays.

A typical sample preparation workflow for LC-MRM measurements of human plasma proteins involves several manual manipulations including protein solubilization/denaturation, reduction, alkylation, trypsinization, and subsequent solid-phase extraction (SPE). These manual tasks can be performed on the AssayMAP Bravo Platform, a state-of-the-art liquid handler equipped with an AssayMAP liquid-handling head. Tasso Miliotis, Ph.D., associate principal scientist at AstraZeneca, will discuss how his team has incorporated AssayMAP into their workflow and describe the results they've achieved.

Who Should Attend

- R & D scientists
- Proteomics scientists
- Bioanalytical scientists
- Researchers interested in protein quantification
- Biomarker assay developers

You Will Learn

- How LC-MRM is used for absolute quantification of proteins using stable isotope-labeled standards.
- Challenges in the workflow, and potential solutions.
- Important things to consider when automating the workflow.
- How the workflow can be improved using the AssayMAP Bravo Platform.

THANK YOU !



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