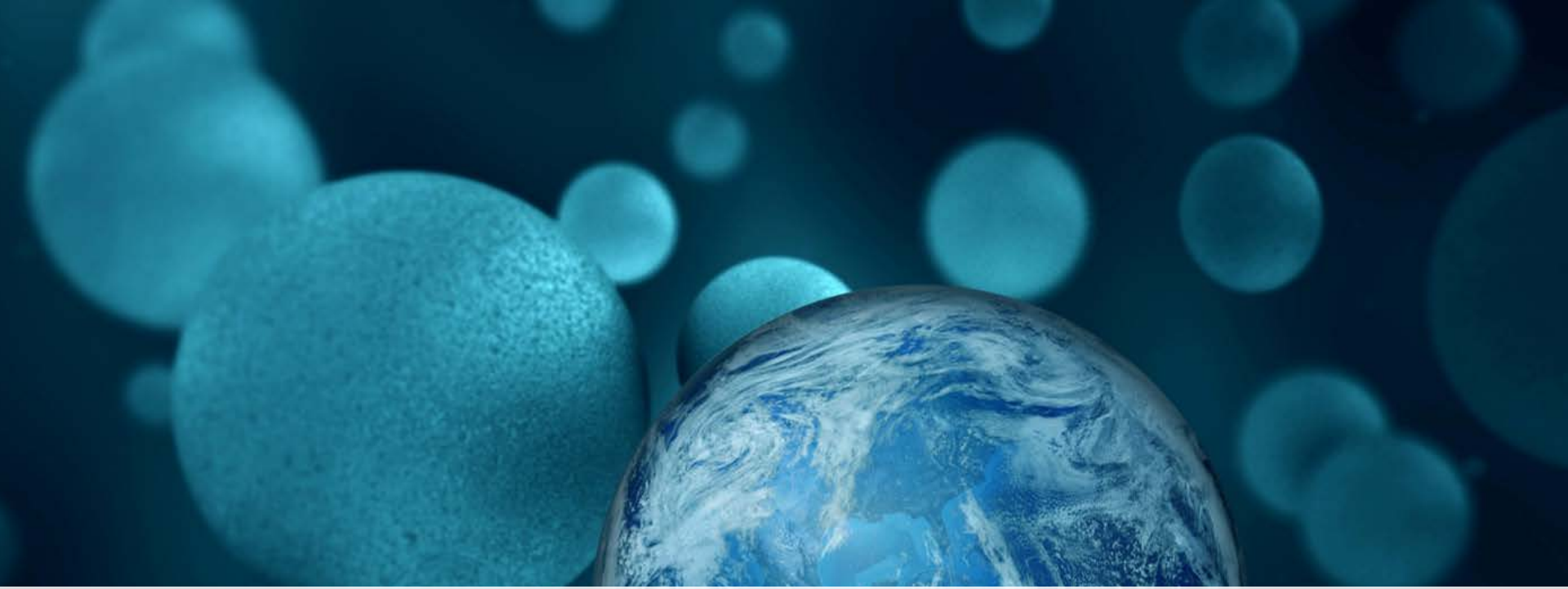

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iQuan: Best Practices For Peptide Quantitation On a Triple Quadrupole Mass Spectrometer

Application outline for peptide quantitation

- **(1) Background and Workflows**
- **(2) Peptide selection and standards**
- **(3) Collision energy (CE) optimization**
- **(4) Liquid chromatography (LC) gradient optimization**
- **(5) Thermo Scientific™ TSQ Quantiva™ method editor and parameter selection**
- **(6) Experimental set up for peptide quantitation – an example workflow**
- **(7) Results**
- **(8) Data processing with Thermo Scientific™ TraceFinder 4.1™**

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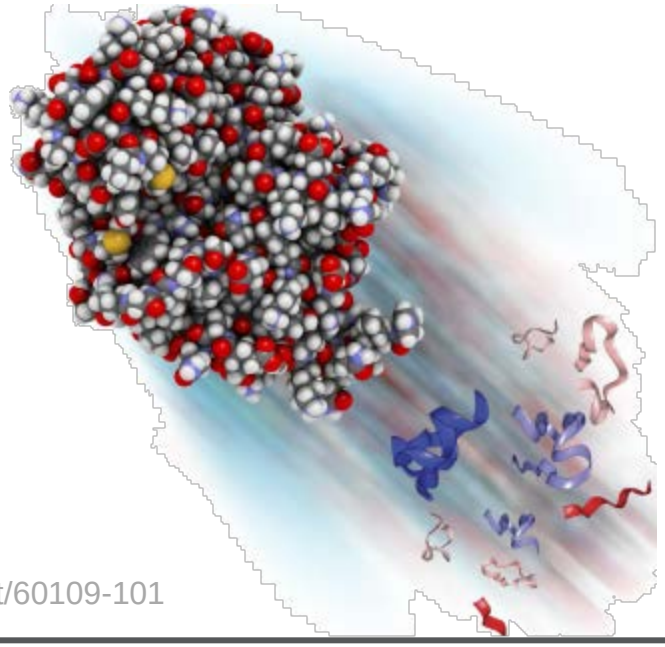
Protein Quantitation with Confidence

- **Goal:** Addressing protein and peptide quantitation with the same ease as that of traditional small molecule quantitation
- **Challenges:**
  - Transitioning from traditional workflows for small molecules
  - Developing robust, sensitive, reproducible methods regardless of expertise
  - Addressing sample preparation complexity
  - Reducing cost/sample
- **Confidence from Sample Preparation, LC-MS, to data analysis and reporting**

Sample Prep with SMART Digest Kit: Why?

- Ensuring reproducibility is a critical challenge
  - Existing in-solution trypsin digestion of proteins is
    - Time consuming
    - Requires multiple steps (protein assay, denaturation, reduction and alkylation)
    - Labor resulting in increased chances of errors, and lack of reproducibility

- SMART Digest Kit
  - Highly reproducible
  - Quick and easy to use
  - Detergent free
  - Less prone to chemically-induced PTMs
  - Autolysis-free
  - Amenable to automation

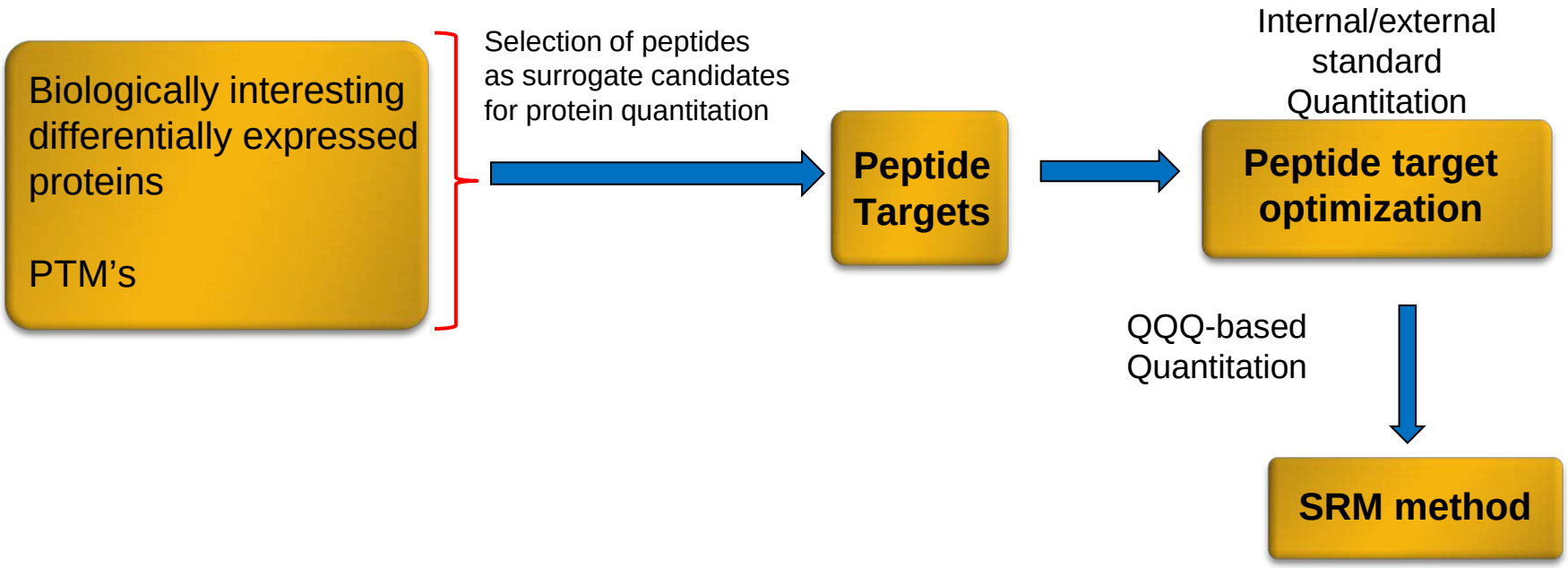


https://www.thermofisher.com/order/catalog/product/60109-101

Four basic components of Peptide Quantitation

Challenge:

- Quantifying with confidence multiple targets
- Complex matrices
- Limited sample volume



How Do We Get Started?

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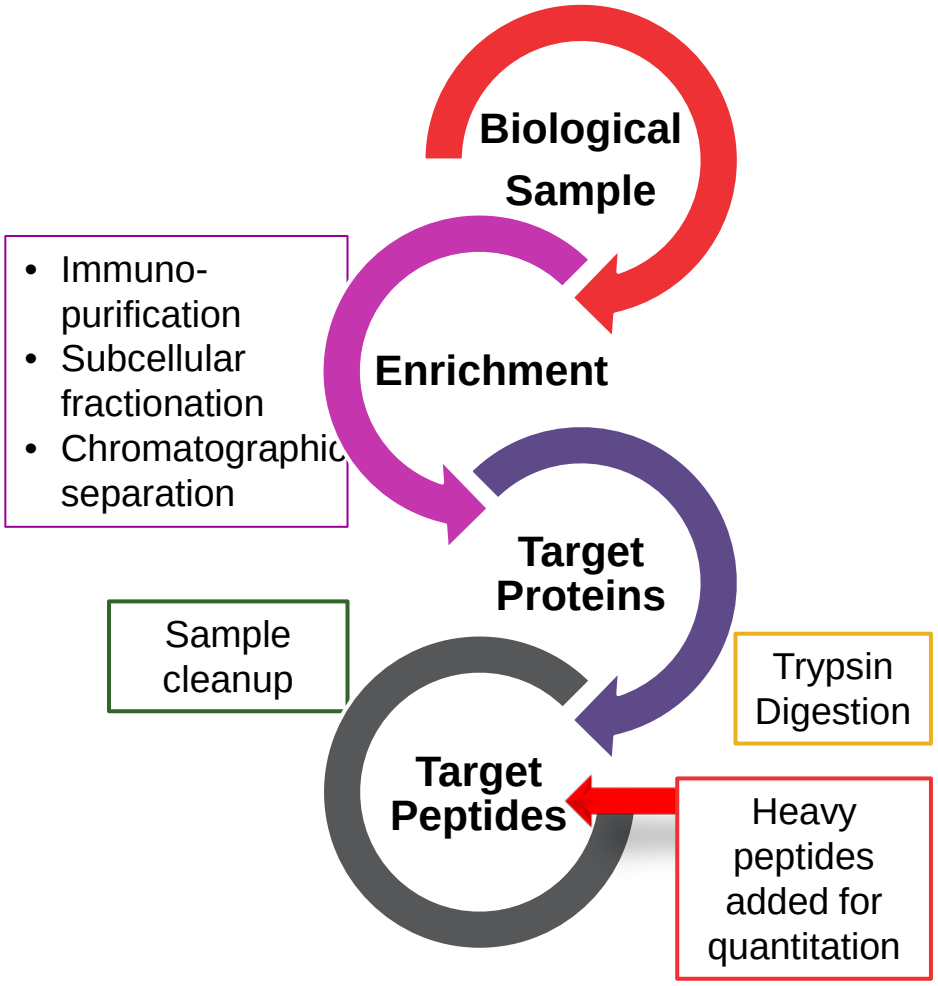
Optimization Process



SRM Method Optimization

- Gradient optimization
- RT Scheduling
- Transition refinement
- Internal Standard Spike Level
- Cycle Time
- CID Gas Pressure
- Collision Energy

Final SRM-MS Method

- Run Standards
- Quantify targets in samples
- Process data

For Targets Without Empirical MS (Discovery data)…

Do the math!

- For the Top-Class QqQ available today, 10's-100's amol can be detected

Questions

- How much total sample do you need to load to get this range (or higher) of your target peptides?
- What do you need to do to enrich your sample to get ~100 amol on column?

Sample Enrichment	Equivalent Sample Volume
1 ug neat plasma (no enrichment)	~ 15 nL
Plasma Depletion (protein level)	~220 nL per inj.
Orthogonal Chromatography (peptide level)	~500 - 1500 nL per inj.
Antibody Enrichment (peptide level)	~100-1000 µL per inj.

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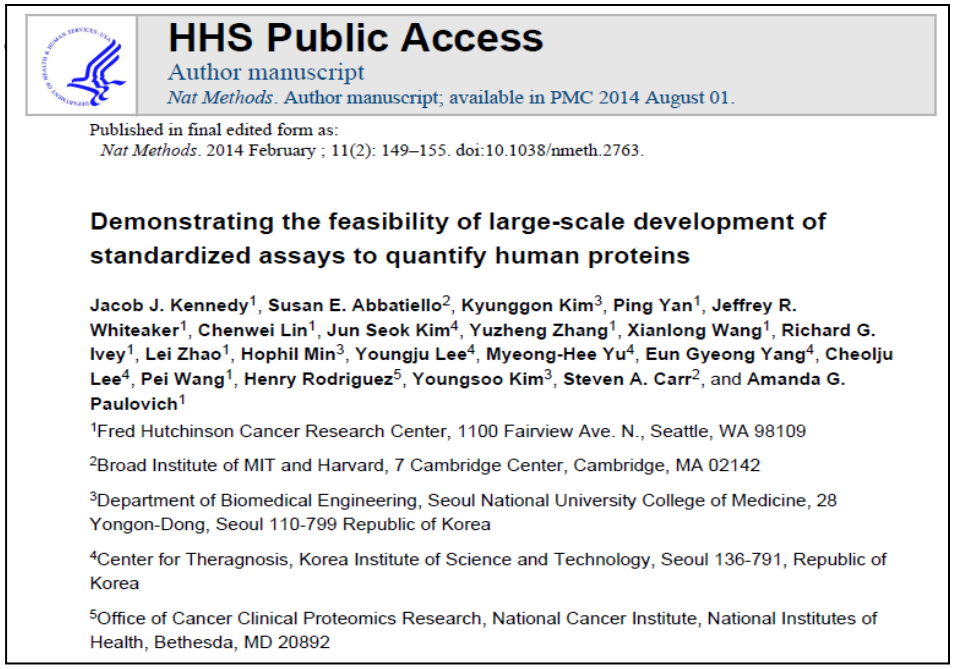
How Many Peptide Candidates per Protein is Ideal?

- Considerations:
  - Can you get multiple peptides from each protein?
  - Do you need to look at site-specific PTMs?
  - What do you do if you can only detect 1-2 peptides per protein?



HHS Public Access

Author manuscript

Nat Methods. Author manuscript; available in PMC 2014 August 01.

Published in final edited form as:

Nat Methods. 2014 February ; 11(2): 149–155. doi:10.1038/nmeth.2763.

Demonstrating the feasibility of large-scale development of standardized assays to quantify human proteins

Jacob J. Kennedy[1], Susan E. Abbatiello[2], Kyunggon Kim[3], Ping Yan[1], Jeffrey R. Whiteaker[1], Chenwei Lin[1], Jun Seok Kim[4], Yuzheng Zhang[1], Xianlong Wang[1], Richard G. Ivey[1], Lei Zhao[1], Hophil Min[3], Youngju Lee[4], Myeong-Hee Yu[4], Eun Gyeong Yang[4], Cheolju Lee[4], Pei Wang[1], Henry Rodriguez[5], Youngsoo Kim[3], Steven A. Carr[2], and Amanda G. Paulovich[1]

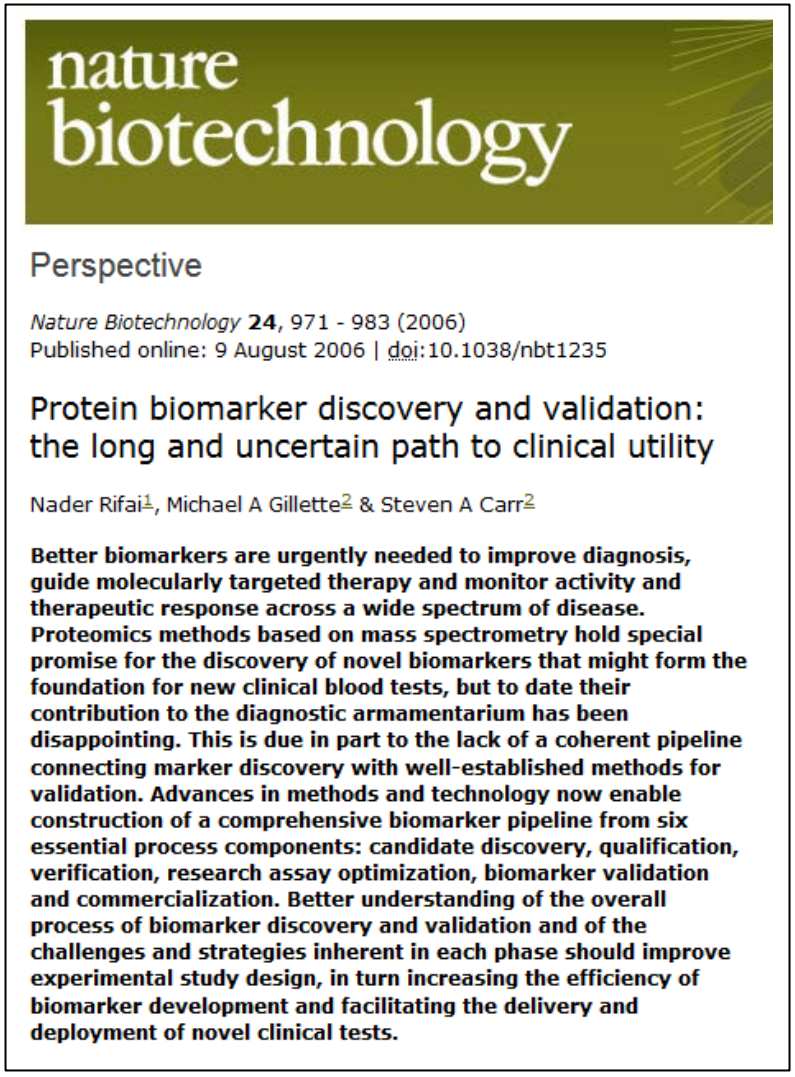
[1]Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., Seattle, WA 98109

[2]Broad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge, MA 02142

[3]Department of Biomedical Engineering, Seoul National University College of Medicine, 28 Yongon-Dong, Seoul 110-799 Republic of Korea

[4]Center for Theragnosis, Korea Institute of Science and Technology, Seoul 136-791, Republic of Korea

[5]Office of Cancer Clinical Proteomics Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892



nature biotechnology

Perspective

Nature Biotechnology **24**, 971 - 983 (2006)
Published online: 9 August 2006 | doi:10.1038/nbt1235

Protein biomarker discovery and validation: the long and uncertain path to clinical utility

Nader Rifai[1], Michael A Gillette[2] & Steven A Carr[2]

Better biomarkers are urgently needed to improve diagnosis, guide molecularly targeted therapy and monitor activity and therapeutic response across a wide spectrum of disease. Proteomics methods based on mass spectrometry hold special promise for the discovery of novel biomarkers that might form the foundation for new clinical blood tests, but to date their contribution to the diagnostic armamentarium has been disappointing. This is due in part to the lack of a coherent pipeline connecting marker discovery with well-established methods for validation. Advances in methods and technology now enable construction of a comprehensive biomarker pipeline from six essential process components: candidate discovery, qualification, verification, research assay optimization, biomarker validation and commercialization. Better understanding of the overall process of biomarker discovery and validation and of the challenges and strategies inherent in each phase should improve experimental study design, in turn increasing the efficiency of biomarker development and facilitating the delivery and deployment of novel clinical tests.

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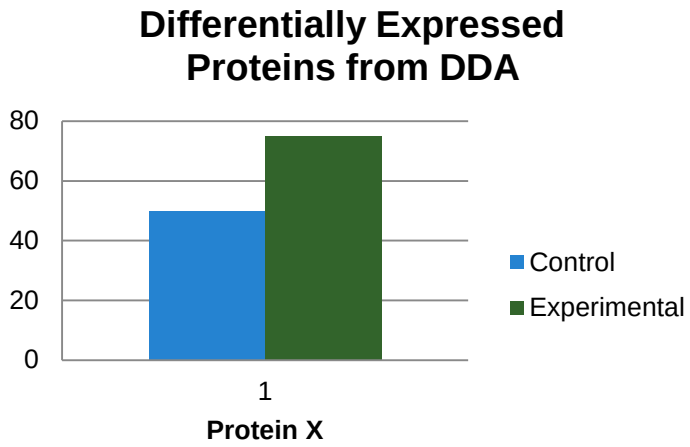
Peptide Selection Criteria



Identified Peptides

Unique only to Target Protein?

If yes

No peptides with Methionine*

Select higher abundance peptides

Good chromatographic peak shape?

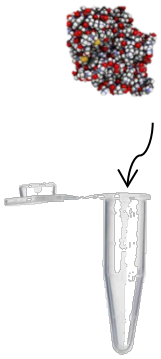
If yes

Surrogate peptide candidates for quantitation

Multiple peptide targets per protein is ideal

External Standards

Sample



SMART Digest Kit

LC/MS

Peak Integration

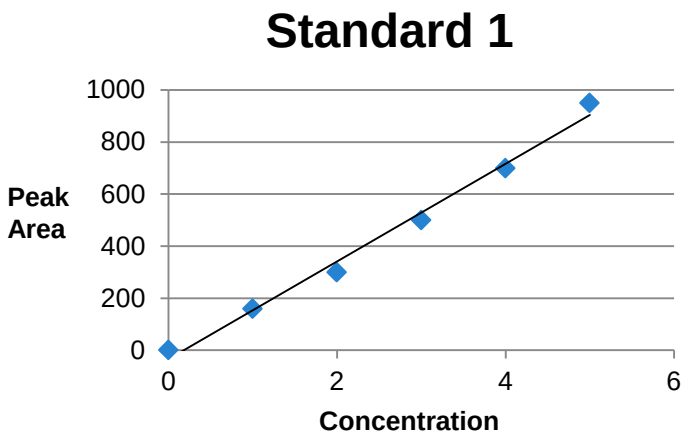
Target Protein Concentration

Standard

SMART Digest Kit

LC/MS

Peak Integration



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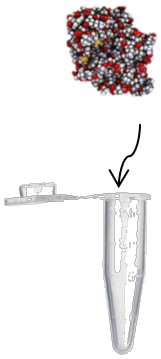
Stable Isotope Labeled (SIL) Internal Standards

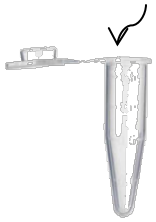
Sample



SMART Digest Kit

R (Δ10Da)
K (Δ8Da)

ABCDEF**X**



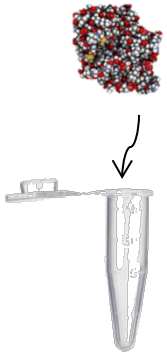
LC/MS

Peak Integration

Unlabeled / SIL

Target Protein Concentration

Standard



SMART Digest Kit

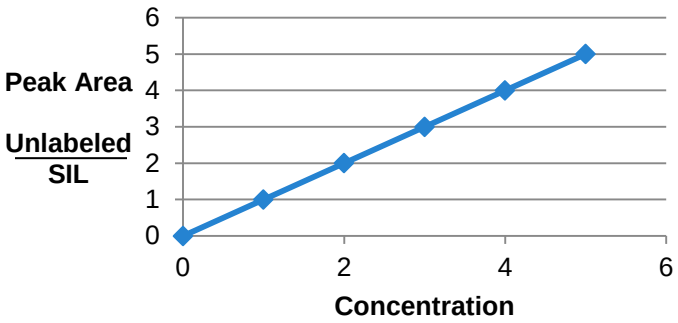
R (Δ10Da)
K (Δ8Da)

ABCDEF**X**

LC/MS

Peak Integration

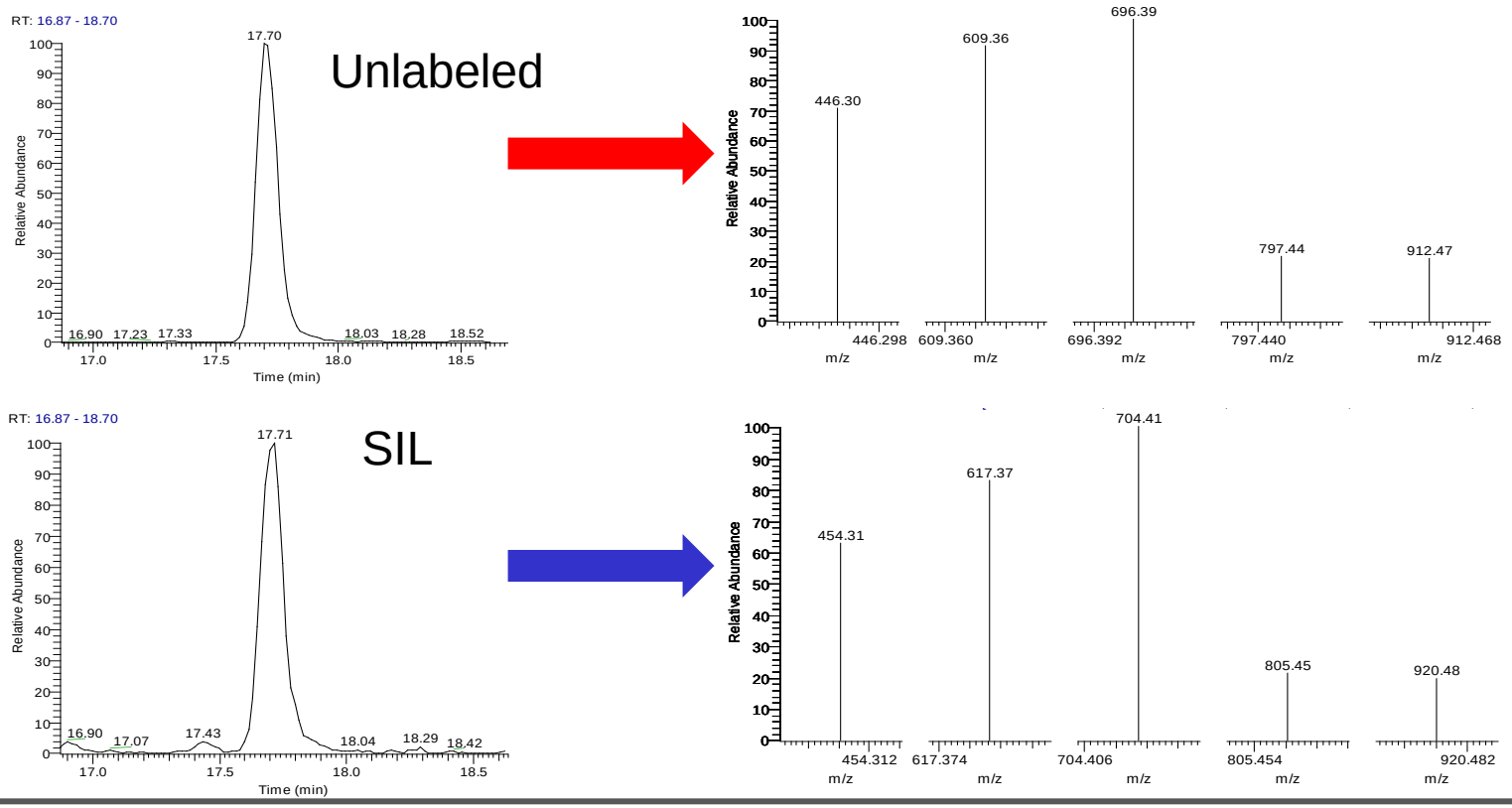
Standard 1



https://www.thermofisher.com/br/en/home/life-science/protein-biology/peptides-proteins/custom-peptide-synthesis-services/peptides-targeted-quantitation.html

Advantages of SIL Standards

- Helps with ID due to same RT and fragment ratio pattern
- Serves as Internal Standard for Sample Prep
  - Each SIL spiked in at same concentration, every sample should have similar SIL Peak Area
  - Using SIL's for each peptide can improve precision (reproducibility) of quantitation



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Questions

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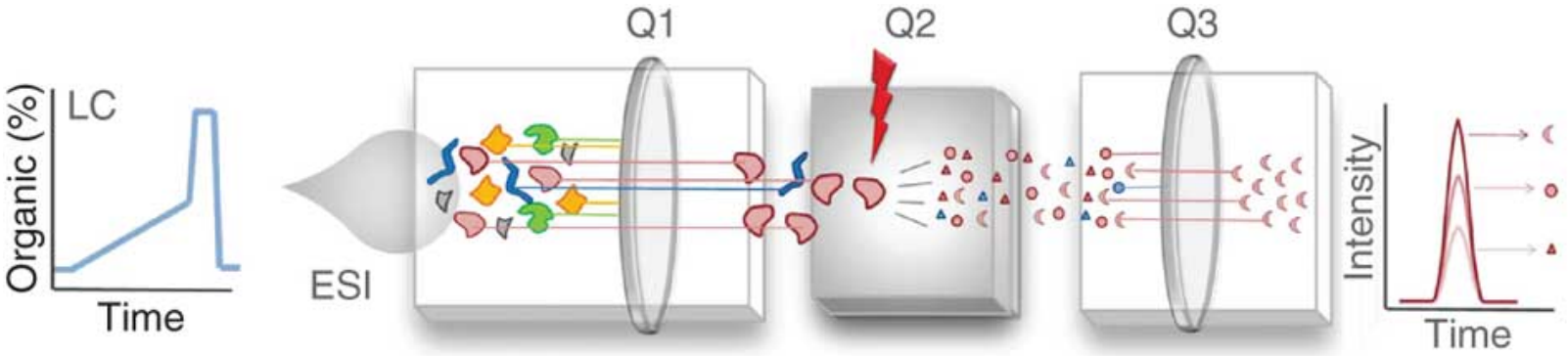
Two methods for CE optimization

- Large number of targets
  - “Automated”
  - Specific softwares (Skyline)
  - LC multiple targets/run
- Few targets
  - Manual Optimization
  - Direct infusion
  - One target at a time

Collision Energy (CE) Optimization

- On a triple quadrupole MS, each product ion is made separately by CID, and transferred to Q3 and the detector

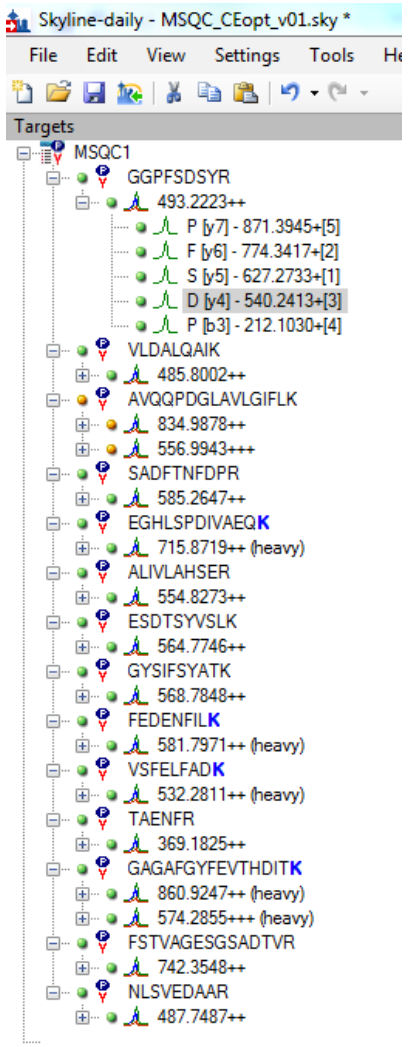


- CE can be optimized for each Q3 product ion individually
- Multiple product ions should be monitored in complex matrices
  - Confirms peptide ID and provides a means to determine if there are interferences

http://www.nature.com/nmeth/journal/v9/n6/full/nmeth.2015.html

CE optimization for large number of precursors

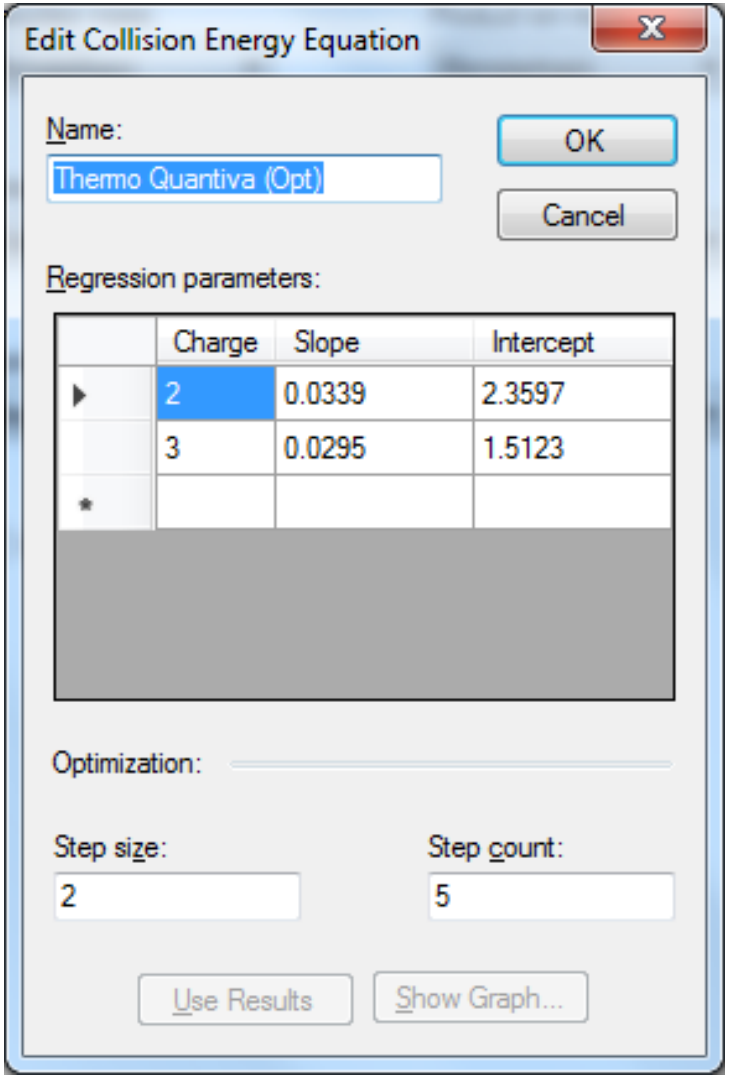
- Efficiently performed utilizing the LC system and software such as Skyline
- Start with >5 fmol on-column for nanoflow (above LOQ)
- Select fragment ions to target
- If >10 peptides require CE optimization, first find RTs for the LC method, then schedule CE optimization runs
- Most vendors provide a starting “CE regression equation” for their platform



https://skyline.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_optimize_ce

Start with Recommended CE Regression Equations

- TSQ Quantiva™ has the following recommended CE regression equations for peptides at CID gas pressure of 1.5 mTorr.
  - 2+ precursors: CE = m/z * 0.0339 + 2.3597
  - 3+ precursors: CE = m/z * 0.0295 + 1.5123
- CE regression equations can be adjusted based on your peptide set.



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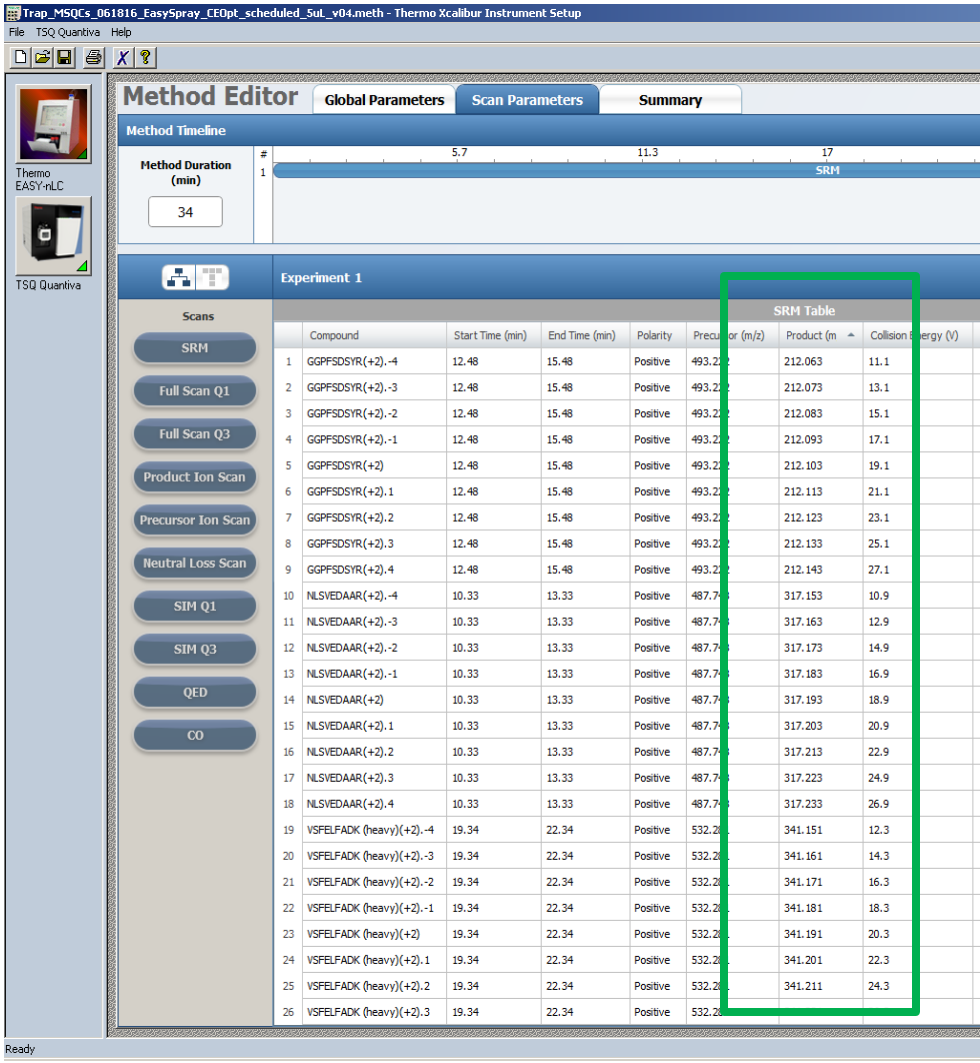
LC-SRM-MS Methods Can Be Generated and Imported

- Skyline generates the CE step gradient for each transition
  - Transition lists can be imported into the TSQ Method Editor
  - Automatic Method Exports from Skyline are available in TSQ v 2.1 software
- Product ion m/z is stepped in 0.01 m/z to associate steps in CE
- Large numbers of peptides (>10) may require scheduled methods for CE optimization



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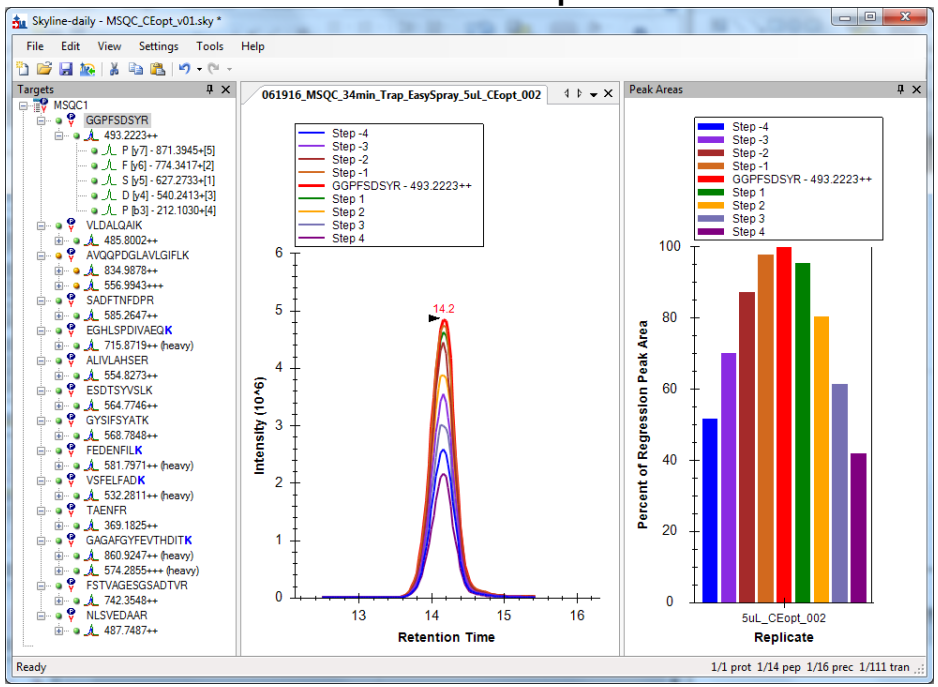
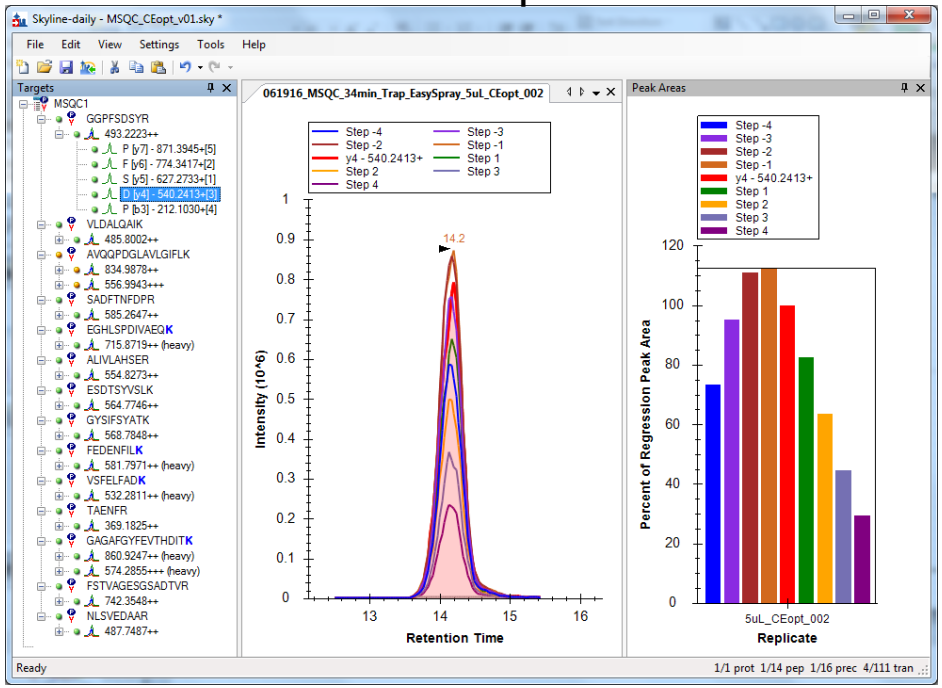
CE Step Gradient for On-Line Optimization

- Import data for acquired CE optimization runs back into Skyline
- Choose for “precursor” or “product” optimization (optimal CEs may be different)
- Save as a CE Optimization Library, and the settings will be exported for all methods with these optimized peptides.

Precursor Level Optimization



Product Level Optimization



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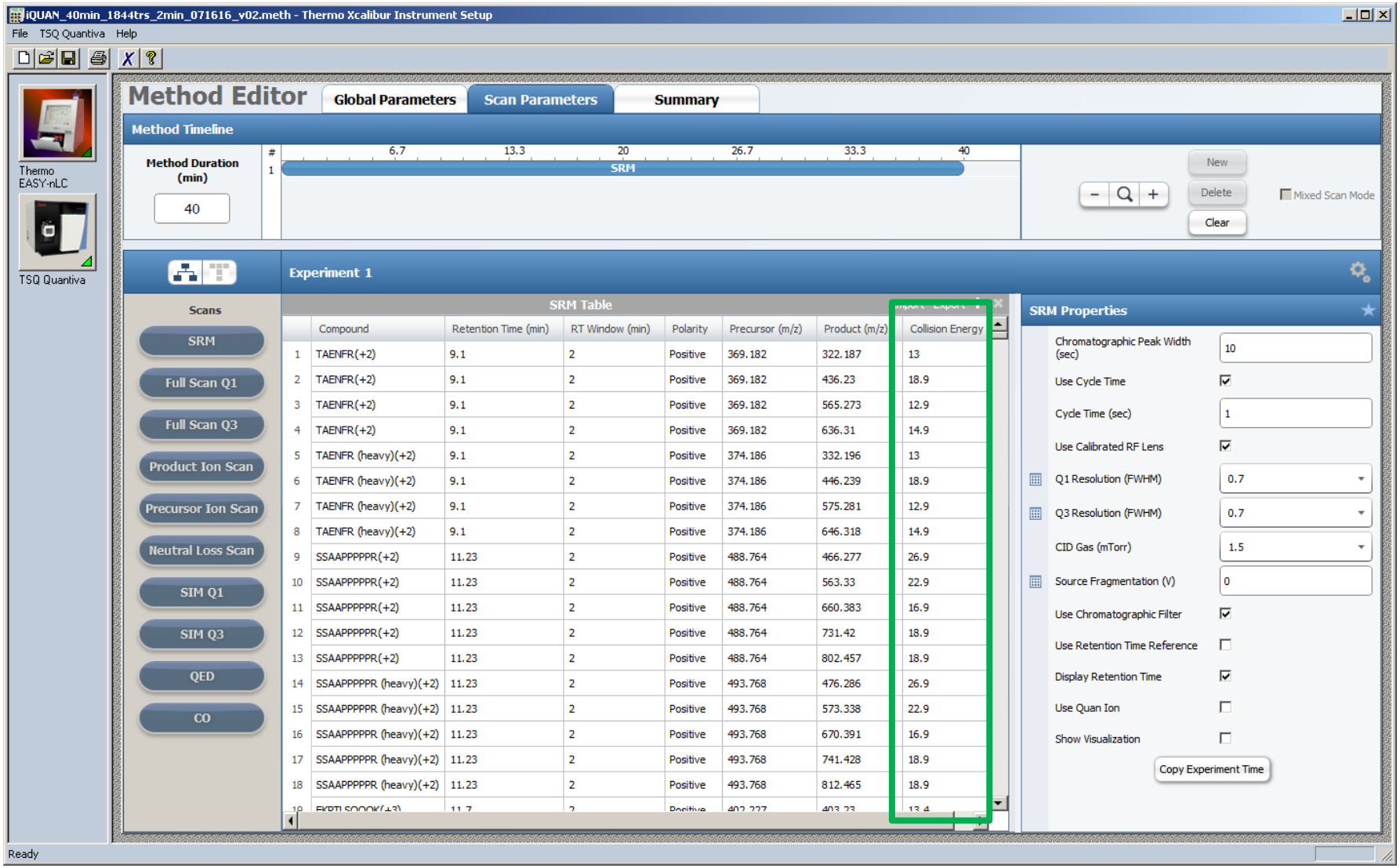
Optimal CE for Each Fragment

Automated CE optimization complete using LC



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Questions

Manual Tune Page Optimization

Select precursor m/z

Quad resolution

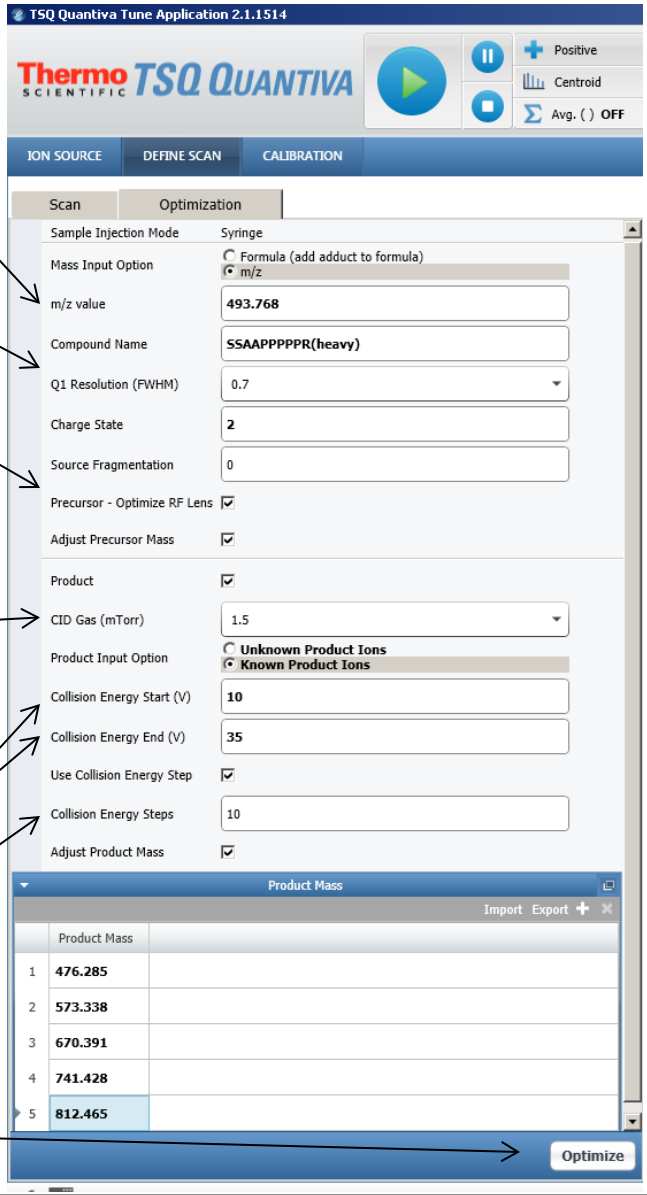
Optimize RF lens (best ion transmission for each precursor)

Choose CID gas Pressure

Collision energy range

CE step number

Click optimize to start



- Infusion-based approach
- Samples must be more concentrated and have some organic solvent for better ESI performance
- MS ramps voltages
- XICs are plotted to determine optimal settings
- PDF report is generated
- Optimized settings can be pasted into Method Editor

Optimization Without Known Fragment Ions

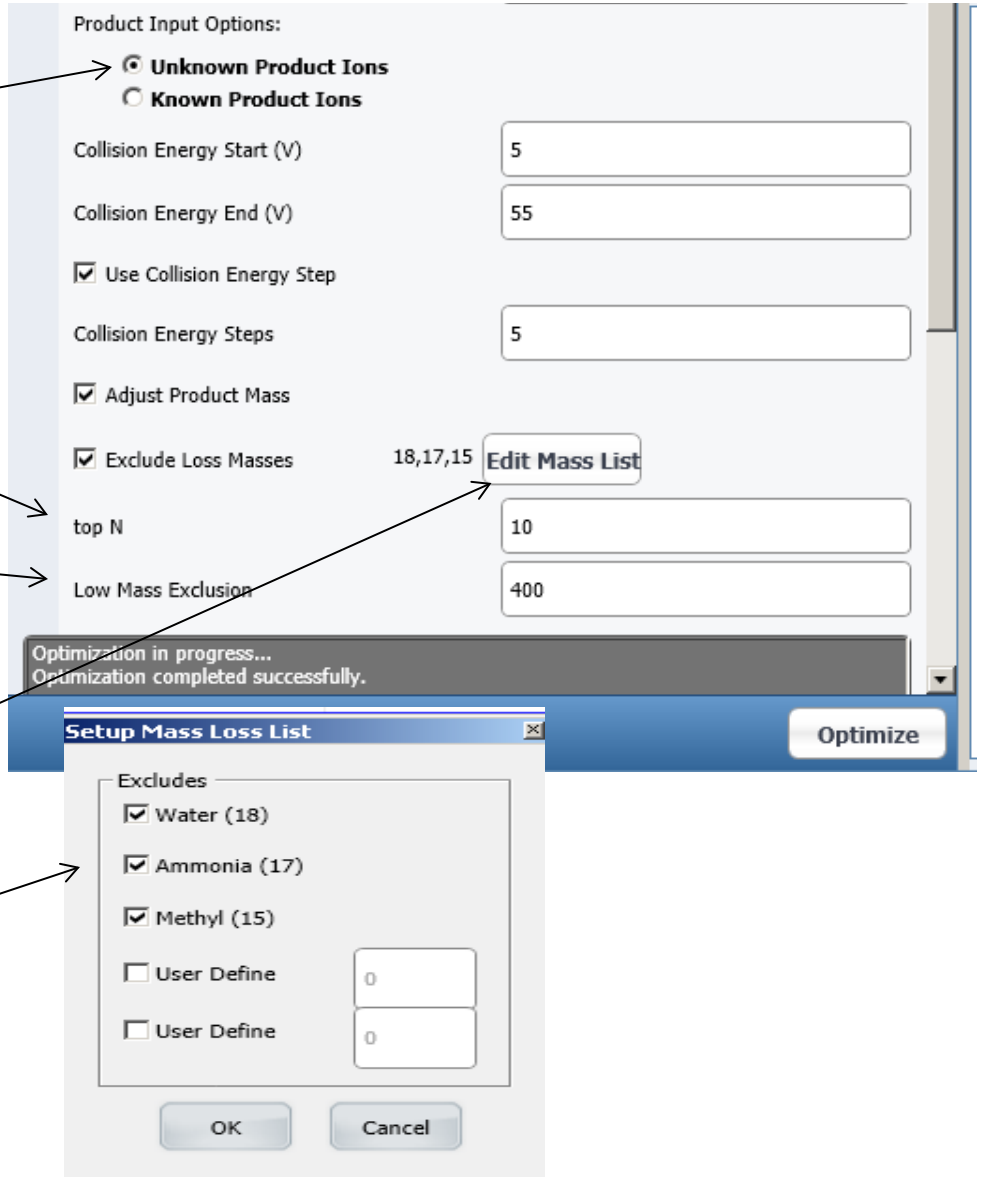
Select Unknown Product Ions

Top N
(how many fragment ions to optimize)

Low mass exclusion
(doesn't select ions below this m/z)

Minor mass losses can also be excluded.

Click on Edit Mass List, select masses to exclude.



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Example Optimization Report

Compound Optimization Report

Thermo SCIENTIFIC

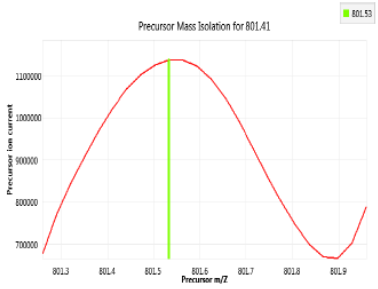
Date & Time	Monday, April 11, 2016 11:45 AM
Instrument Model	TSQ Quantiva
Instrument Serial	TQH-Q1-0188
Software Version	2.0.1292.15

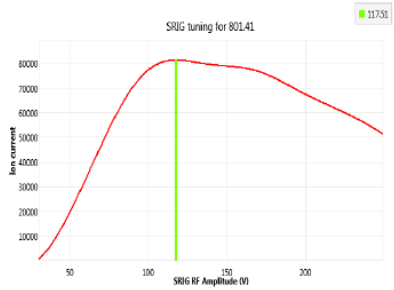
Source Parameters	
Source Type	H-ESI
Spray Voltage (V)	3500
Sheath Gas (Arb)	10
Aux Gas (Arb)	0
Sweep Gas (Arb)	0
Ion Transfer Tube Temp (°C)	350
Vaporizer Temp (°C)	25

Compound Optimization Input	
Source Fragmentation (V)	0
Step Collision Energy Value	5
Collision Energy Start (V)	5
Collision Energy End (V)	55
Charge State	2
Step Collision Energy	Yes
Unknown/Known Products	Known
Exclude Loss Masses	None
Compound Name	compound
Product	Yes
CID Gas (mTorr)	1.5
Adjust Precursor Mass	Yes
m/z Value	801.411
Q1 Resolution	0.7
Adjust Product Mass	Yes
Number of Products	5
Optimize RF Lens	Yes

Compound Optimization Results

Compound Name	Precursor m/z	Product m/z	Collision Energy (V)	RF Lens (V)	Intensity	Source Fragmentation (V)
compound	801.533	772.315	28.202	117.506	5182.305	0
compound	801.533	928.45	28.051	117.506	2476.773	0
compound	801.533	1015.491	29.466	117.506	1815.119	0
compound	801.533	1072.531	29.77	117.506	7327.675	0
compound	801.533	1171.53	27.747	117.506	2064.839	0





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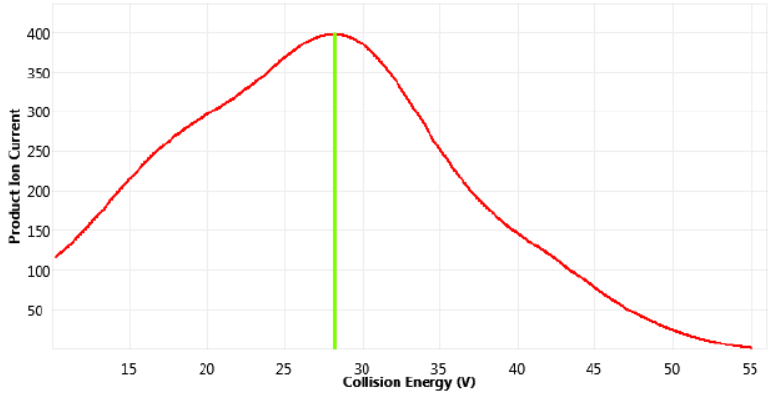

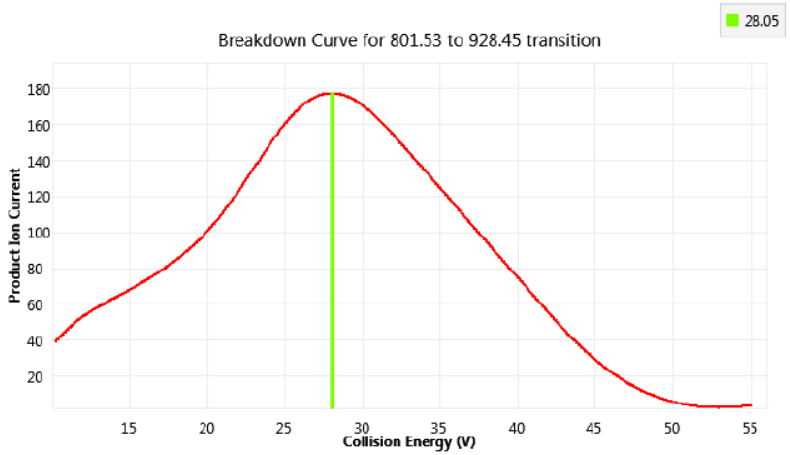

Serial Number: TQH-Q1-0188

Signature:
Monday, April 11, 2016 11:45 AM

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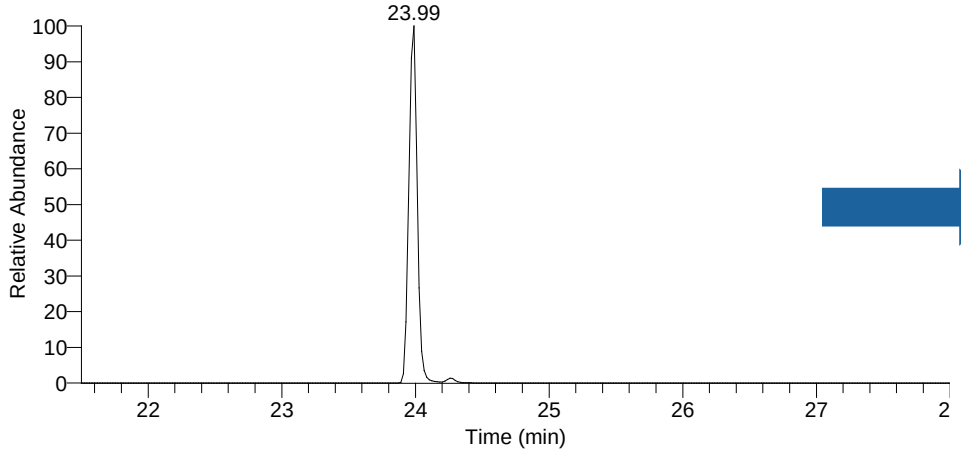
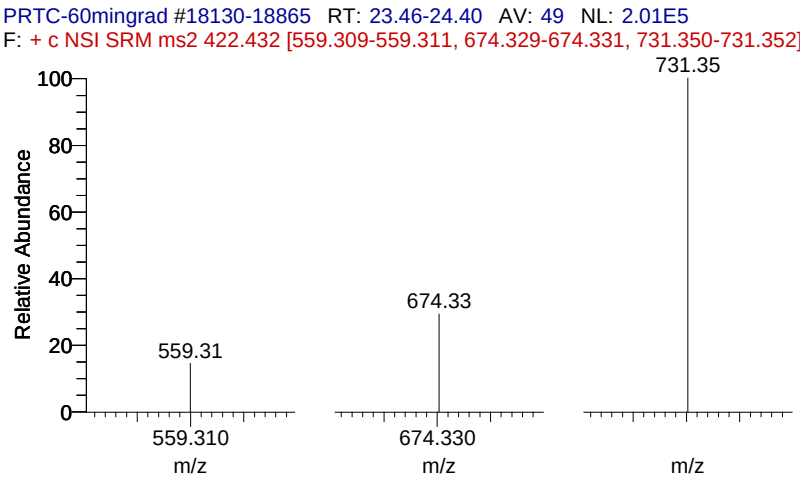
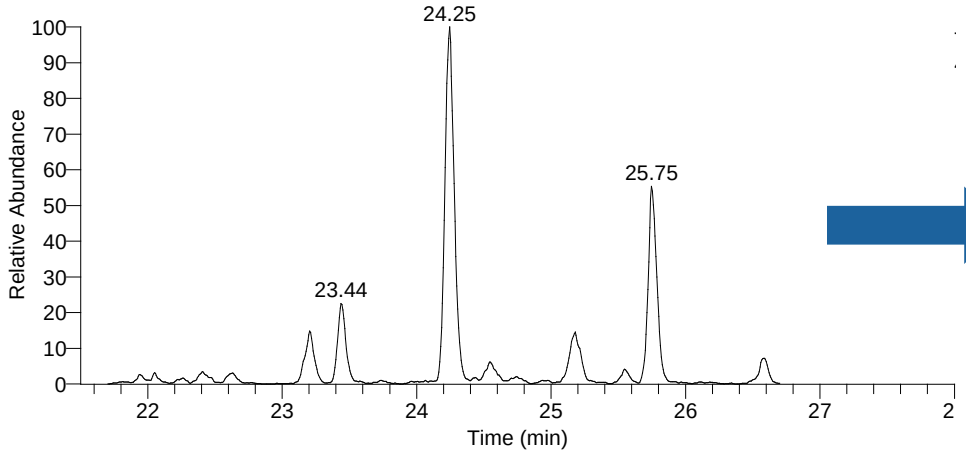
Ion ratio confirmation in matrix background

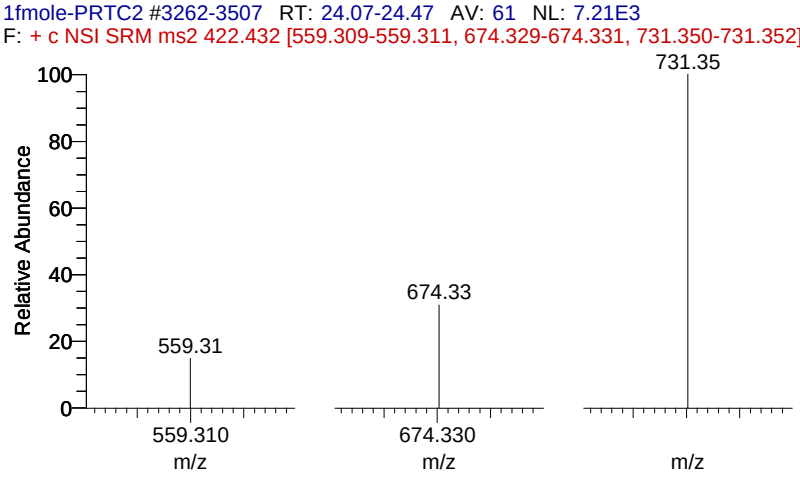
Neat Standard





Standard in Matrix





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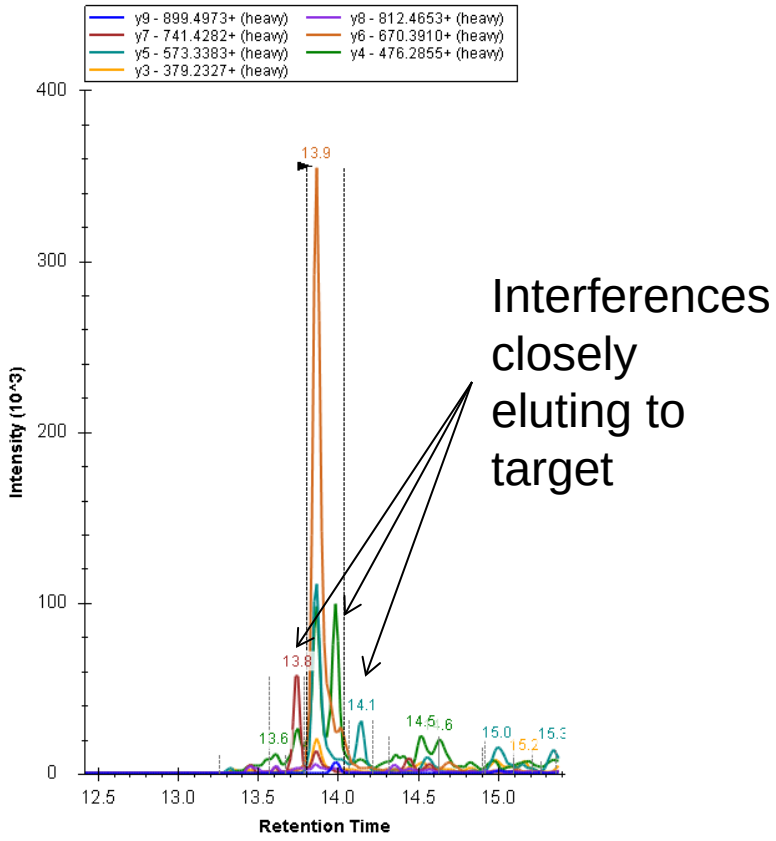
If CE optimization done neat, check signal in the matrix

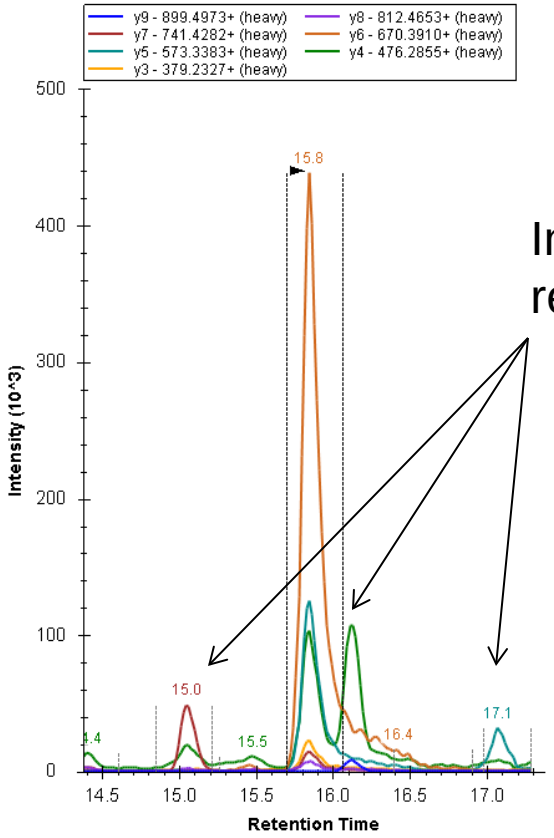
- Ensure none of the target peaks have interference from the matrix background (peak splitting, shoulders).
- Modify the gradient to help shift interferences

Short Gradient



Interferences closely eluting to target

Long Gradient



Interferences resolving

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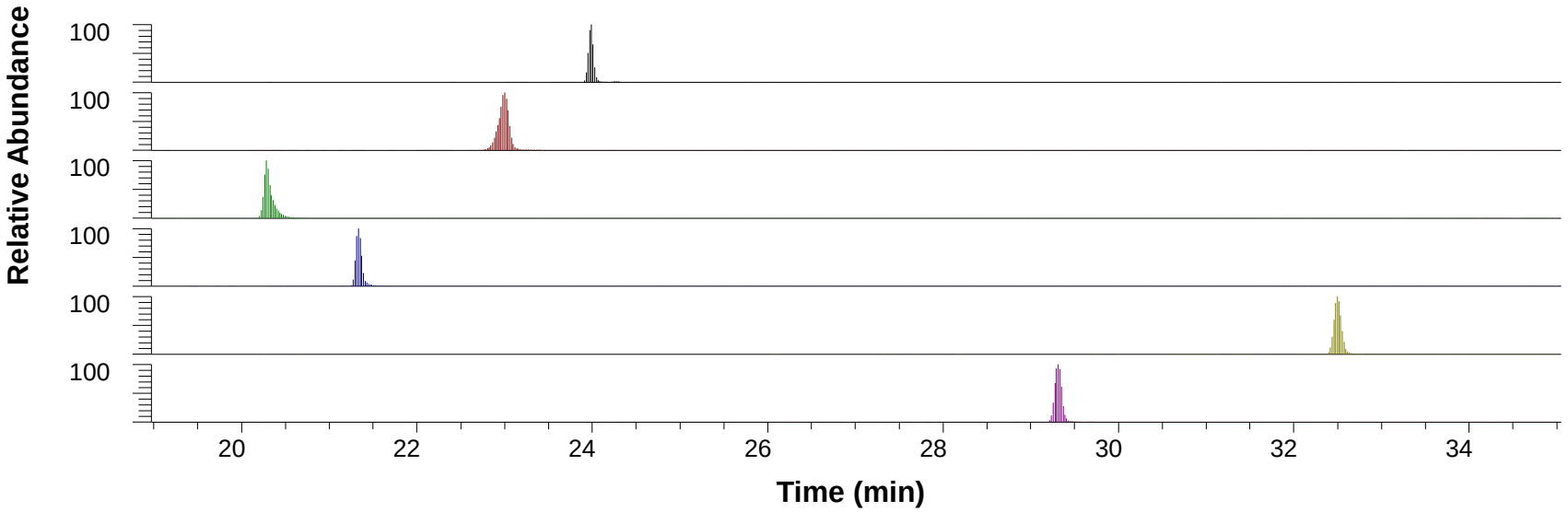
Optimization of LC gradient

Goal: Maximize the time between targets and minimize the effect of the background matrix, while eluting in the shortest time possible.

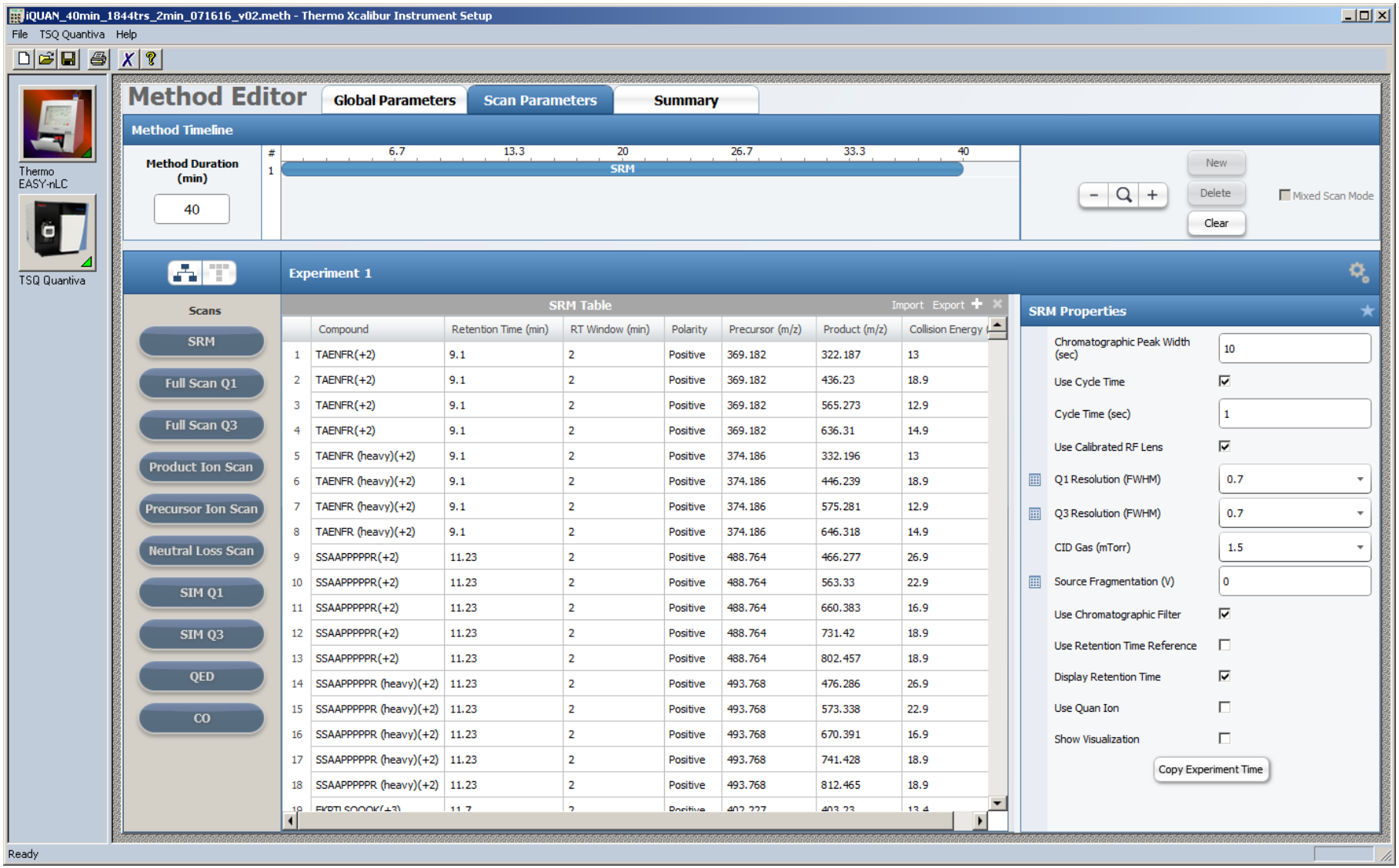


Gradient Length	Chromatographic Resolution	Sensitivity	Reproducibility	Sample Throughput
Short	↓	↓	↓	↑
Long	↑	↑	↑	↓

Application outline for peptide quantitation

- (1) Background and Workflows
- (2) Peptide selection and standards
- (3) Collision energy (CE) optimization
- (4) Liquid chromatography (LC) gradient optimization
- **(5) Thermo Scientific™ TSQ Quantiva™ method editor and parameter selection**
- (6) Experimental set up for peptide quantitation – an example workflow
- (7) Results
- (8) Data processing with Thermo Scientific™ TraceFinder 4.1™

Method Editor for Thermo Scientific™ TSQ Quantiva™



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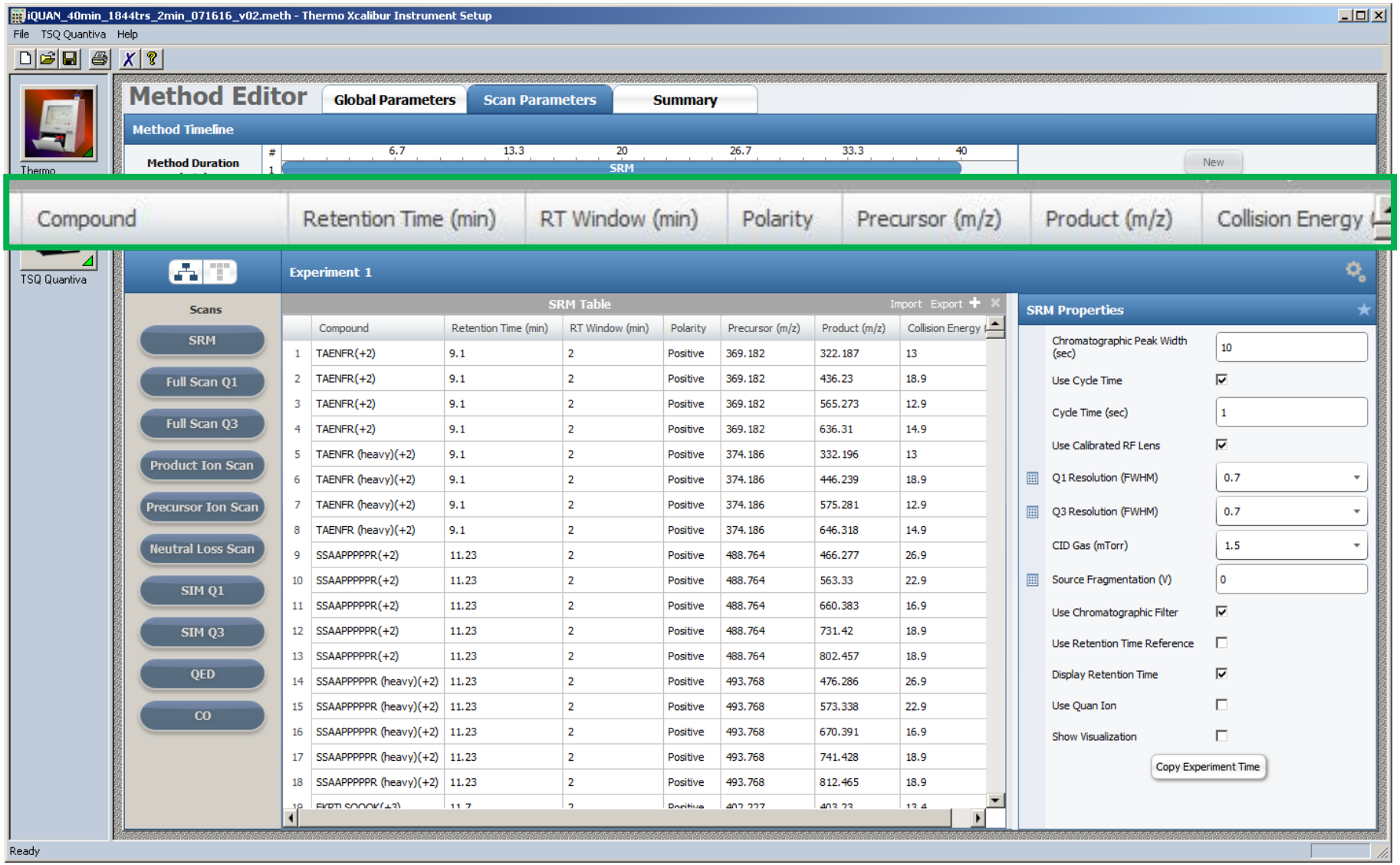
Method Editor for Thermo Scientific™ TSQ Quantiva™



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Parameter Selection for Targeted Peptide Quantitation

Set chromatographic peak width

Use cycle time for best sampling frequency

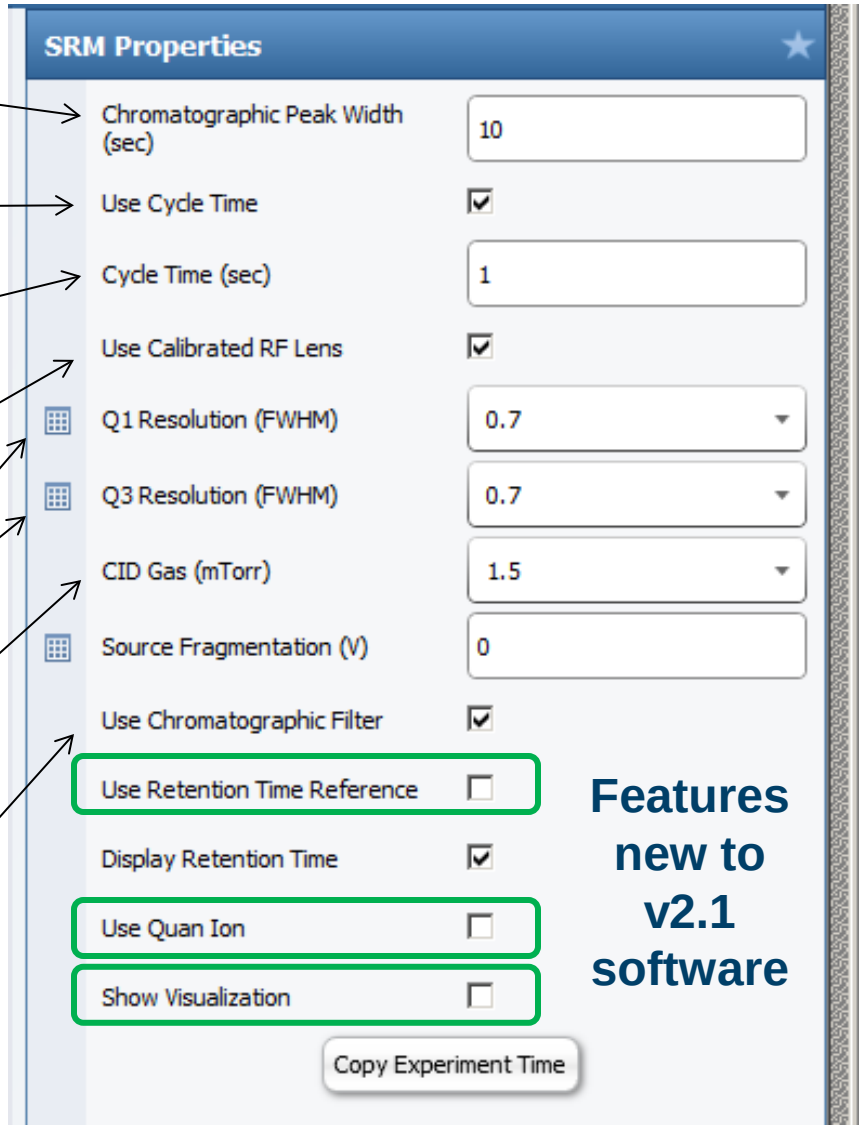
Set cycle time to get 10-15 points across the peak

If RF Lens was not optimized for each target, use calibrated value

Q1 and Q3 Resolution can be individually set for targets in the SRM table, or globally here

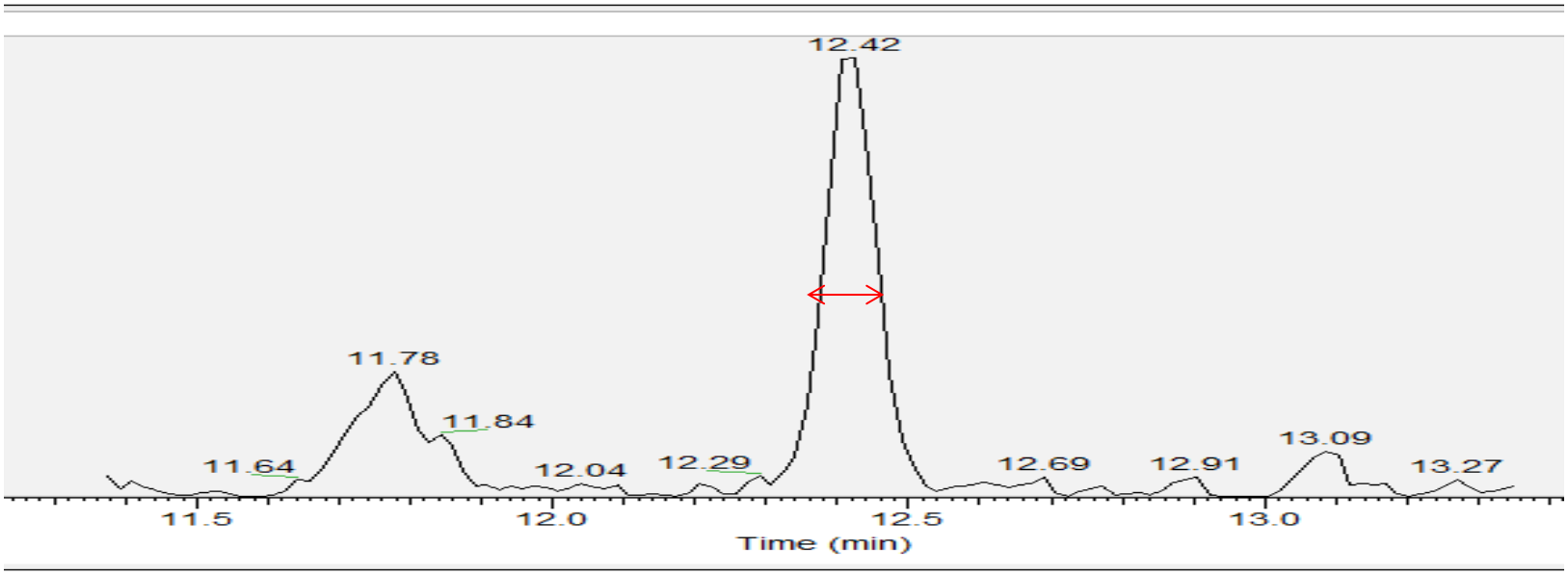
Choose the CID gas pressure you optimized with

Chromatographic Filter provides “on the fly” signal averaging



Cycle time determination

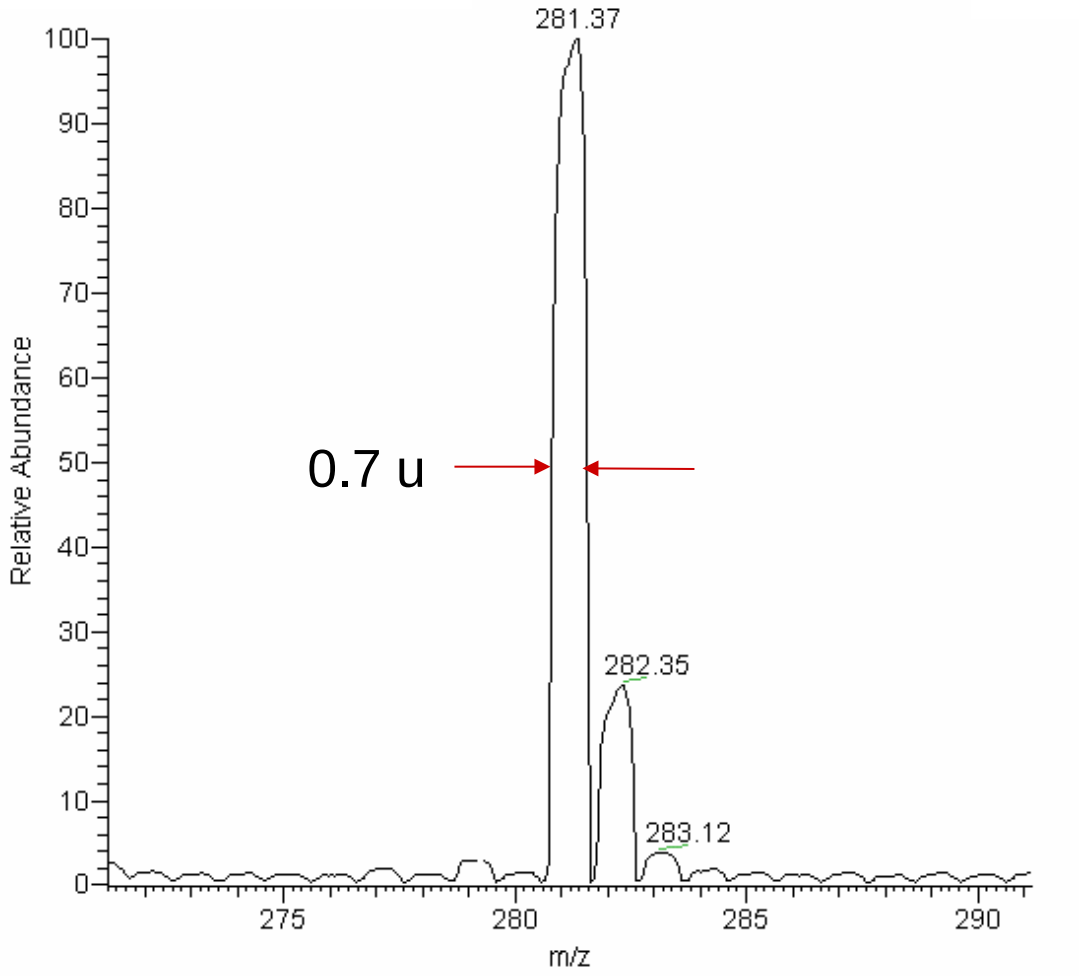
Use the peak widths from narrowest target peaks (at lower concentrations).



$$\frac{\text{peak width}}{\text{cycle time}} = \text{Scans across the peak}$$

9.6 seconds/0.64 seconds = 15 scans

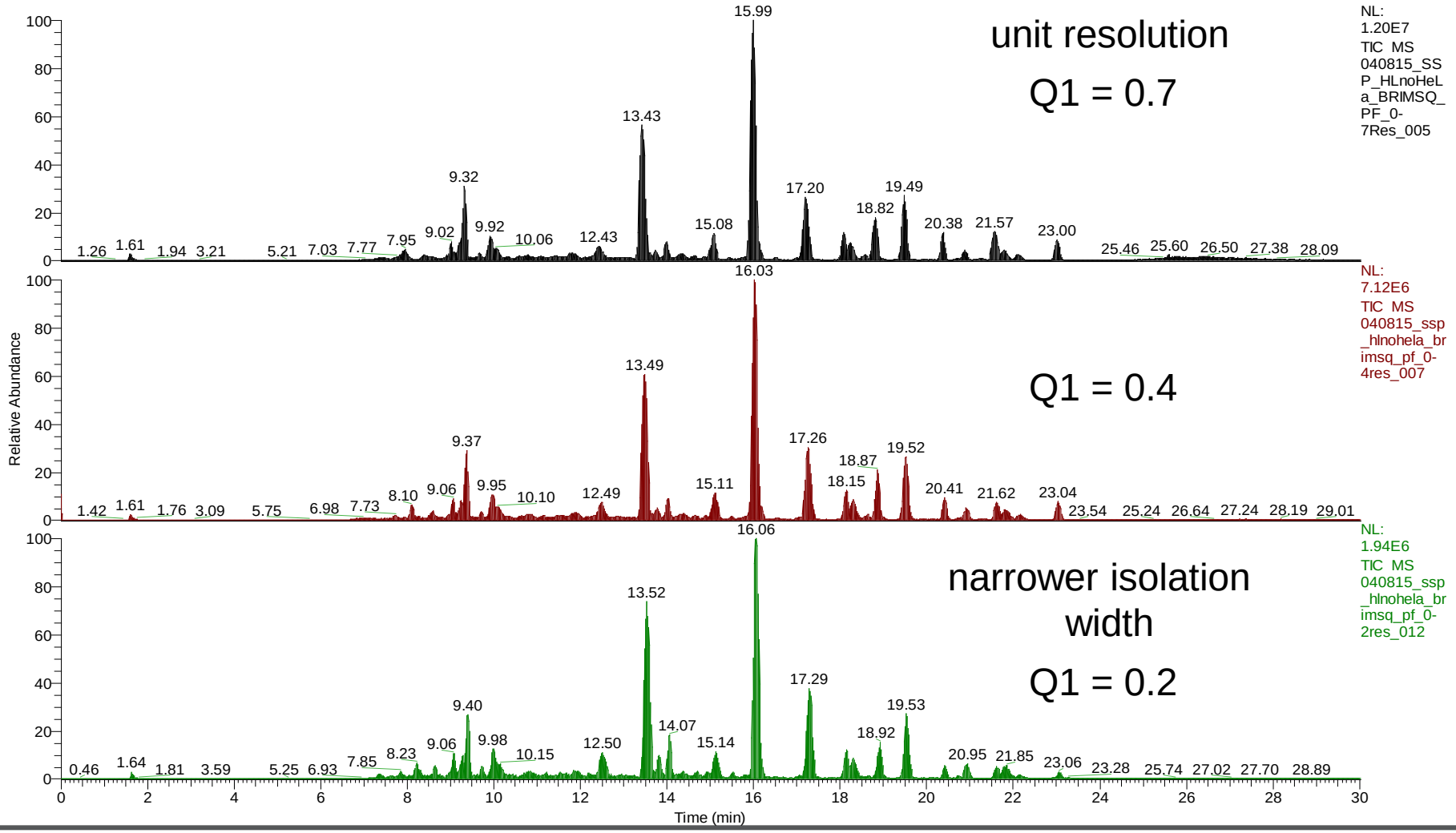
Quadrupole Unit Resolution (0.7 FWHM)



Note: 0.7 FWHM is equivalent to 1.0 mass width at base of peak (*m/z* scale)

The Effect of Q1 Resolution on Intensity

- PRTC peptide mix spiked into HeLa and analyzed with the same gradient and method, only Q1 resolution was changed



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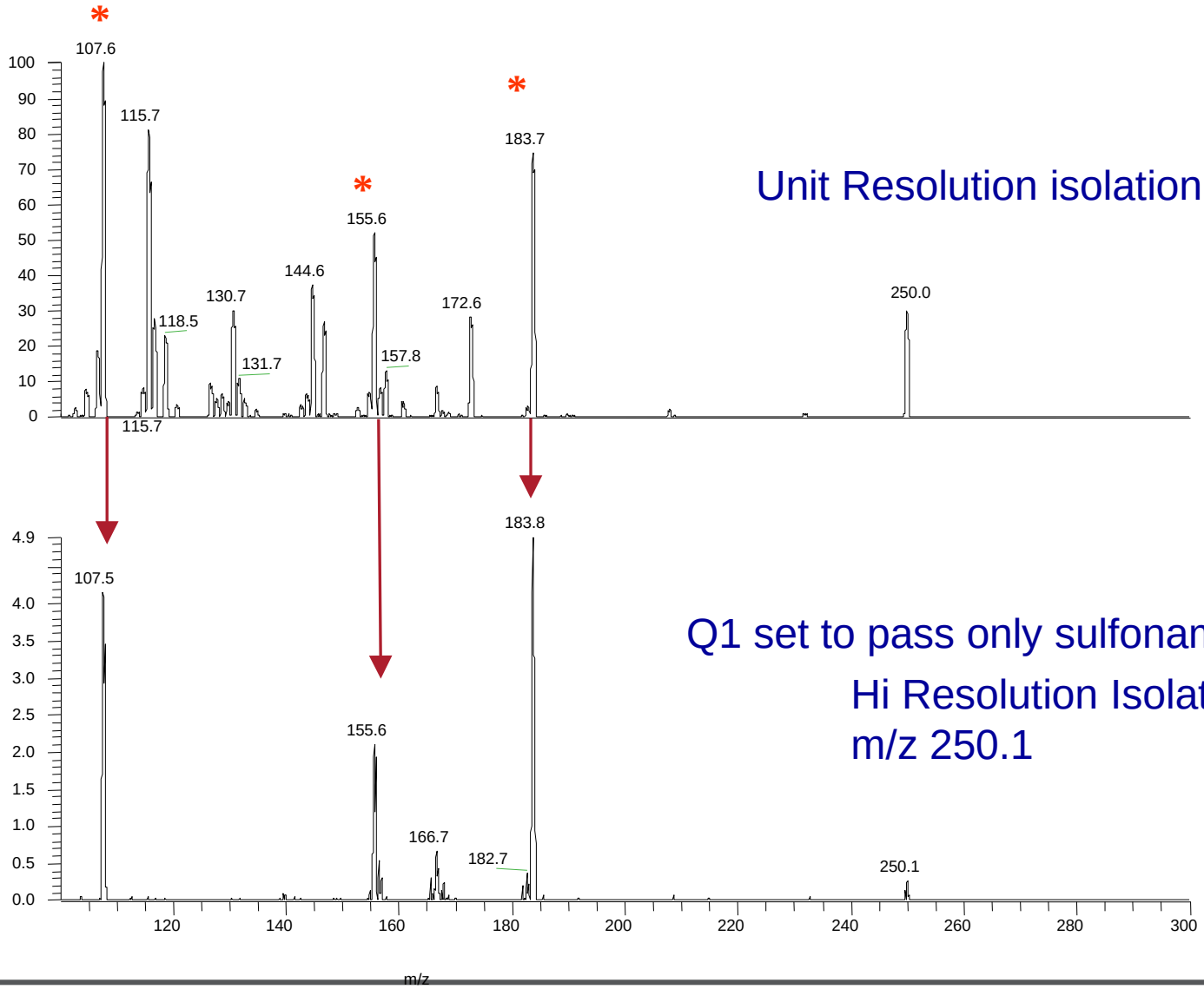
Isobaric Discrimination – Effect of Increasing Resolution



Unit Resolution isolation

Q1 set to pass only sulfonamide

Hi Resolution Isolation
m/z 250.1

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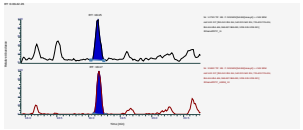
SRM vs HSRM

- 200 amol on column GISNEGQNASIK[HeavyK]
- While the signal drops with Q1 setting of 0.2, the background and chemical noise are drastically reduced



SRM

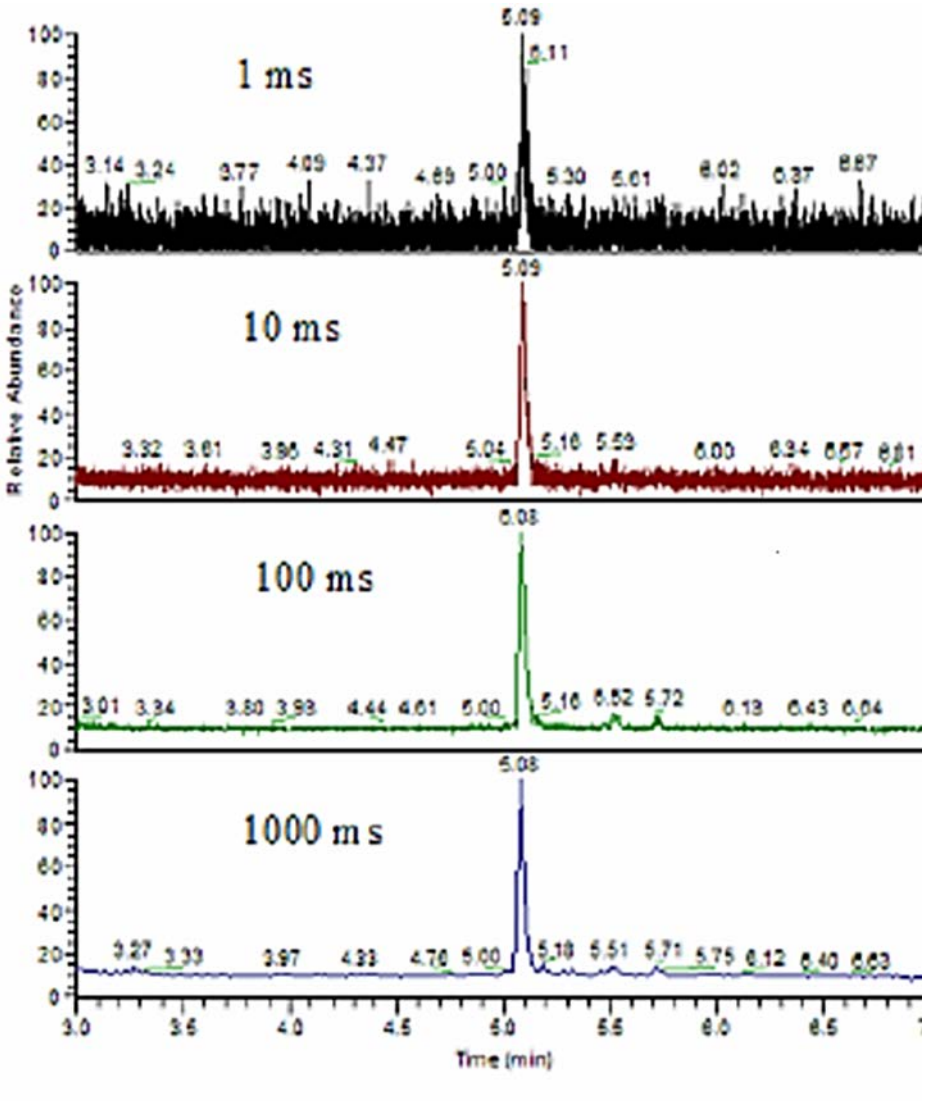
Q1 = 0.7

H-SRM

Q1 = 0.2

Parameters to consider- Dwell Time

- Increasing Dwell Time improves S/N by increasing ion statistics
- Reproducibility of signal is also improved
- Dwell Times of 10 msec or greater provide high quality data for low abundant analytes
- TSQ Quantiva™ can detect signal with dwell times as low as 1 msec
- RT scheduling will increase dwell times vs unscheduled SRM methods



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Dwell time can be variable using cycle time

$$\frac{\text{cycle time}}{\text{\# product ions}} = \text{dwell time per fragment}$$

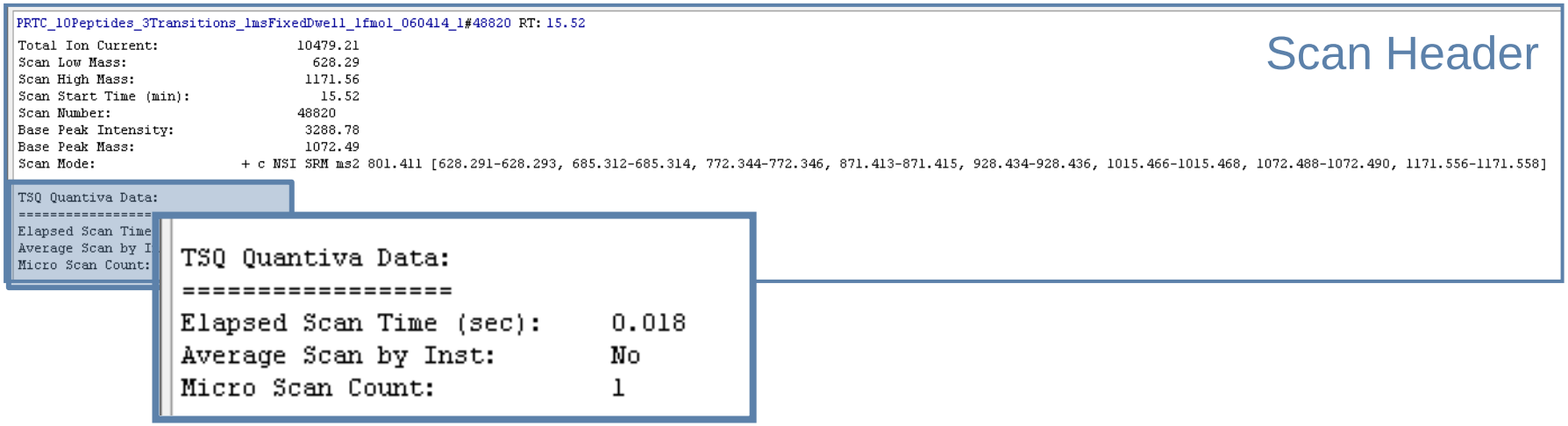
0.5/10 = 0.05 seconds

0.5/50 = 0.01 seconds

- Minimum dwell time should be 10 milliseconds for low abundance targets.
- Shorter dwell times have poor ion statistics and can make quantitation more difficult.
- We want to have optimal chromatographic resolution of targets and minimal RT overlap.

Determining Cycle Time when “setting” Fixed Dwell

Method: 1 ms Fixed Dwell
Transition List: 10 Peptide, 8 transitions each (80 Transitions total)



- Elapsed scan time of 18 ms for 8 transitions
- 18 ms / 8 transitions = 2.25 msec dwell time per transition
- With a setting of 1 ms dwell, an interscan delay of ~1.25 ms is calculated
- Elapsed scan time of 18 ms x 10 peptides = ~180 ms Cycle Time

- *Note: The interscan delay will be dynamic and specific to the mass jump*

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Experimental Design Example for Peptide Quantitation

Goal: To quantify >360 peptides in a single QqQ method

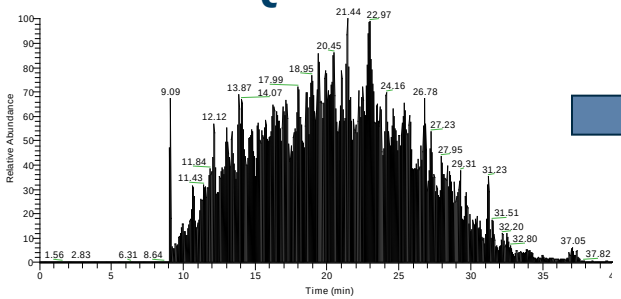
- **Sample Details**
  - HeLa Lysate Digest (Pierce, 88328)
    - 0.5 ug/uL
  - Retention Time Calibration Peptides (Pierce, 88320)
    - Response curve from 25 amol-100 fmol/uL)
    - Light versions spiked at a fixed 10 fmol/uL)
  - 6 x 5 LC-MS/MS Peptide Reference Mix (Promega, V7495)
    - Isoforms for each peptide ranged: 20 amol – 200 fmol/uL
  - MS Qual/Quant QC Mix (Sigma, MSQC1-1VL)
    - 14 light and heavy peptides at various L:H ratios
    - Peptide concentrations ranged from 160 amol/uL – 300 fmol/uL

Experimental Design Example for Peptide Quantitation

- **Discovery Experiment to Select Target Peptides**
  - Top 20 data dependent acquisition on a QE HF
    - 30K Resolution
    - Max fill time: 20 msec
    - AGC target: $1e^6$

Peptide Selection and Spectral Library Generation

Top 20 dd-MS2 Screen QE HF



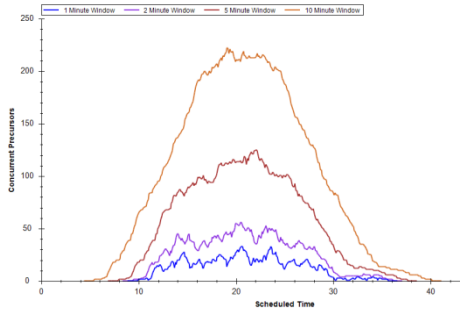
Database Search

Peptide Selection

- Select peptides from search engine results
- Import list into Skyline Software

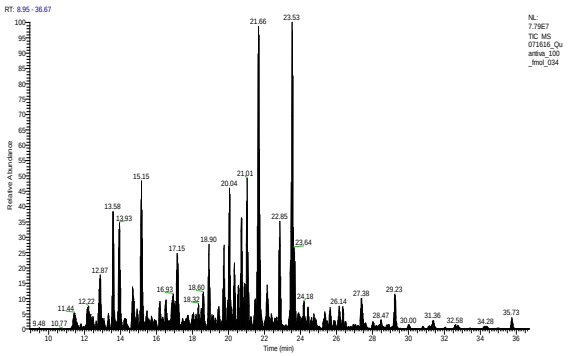
Skyline

- Generate Spectral Library
- Select and refine peptide list
- Export targeted list of transitions



- Balance RT windows and number of transitions
- Adjust MS parameters for best cycle time
- Optimize CE (optional)

tSRM Method TSQ Quantiva



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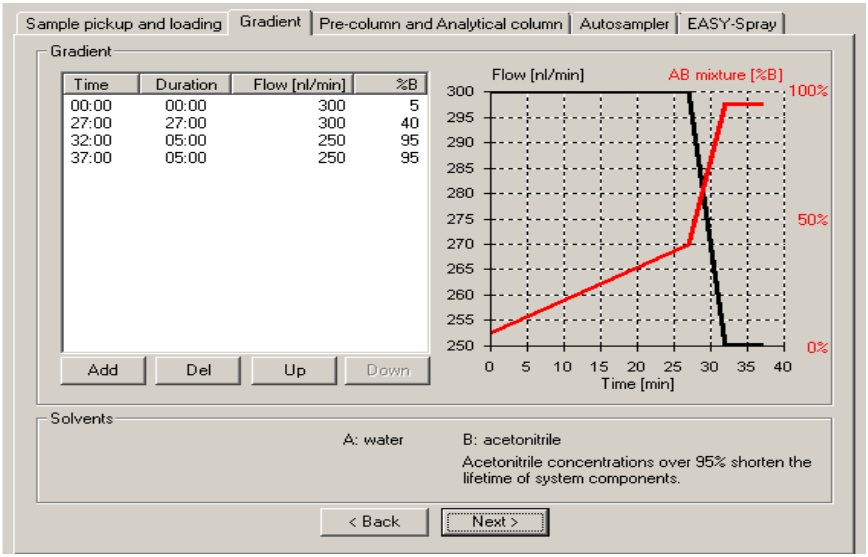
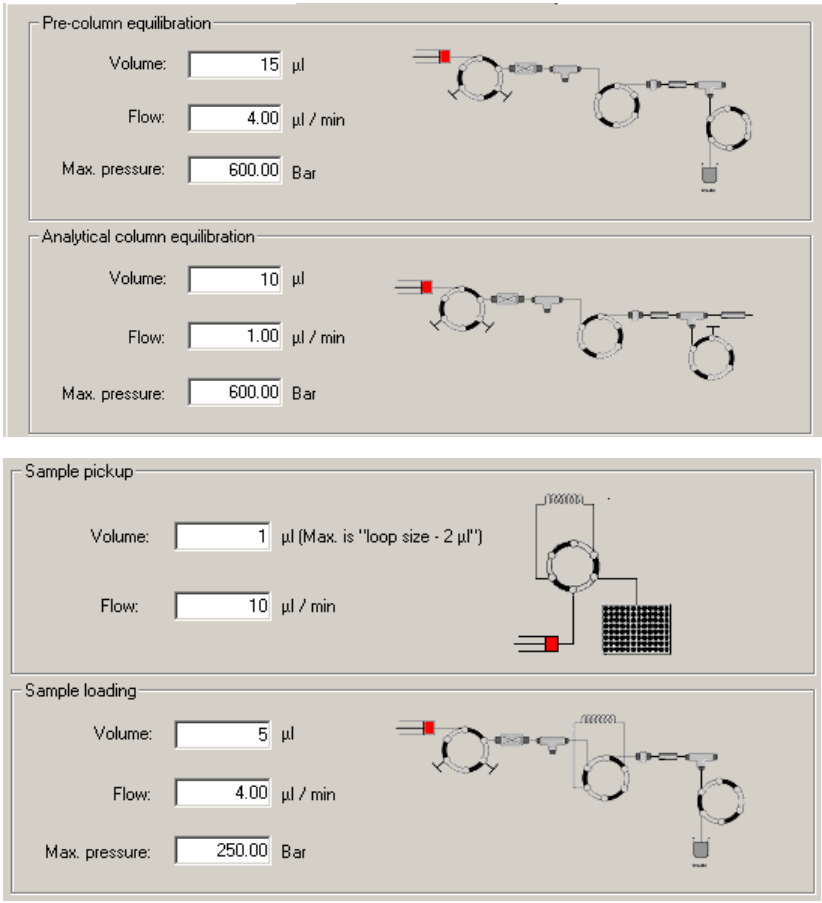
LC Conditions

- Trap-Elute configuration (Easy nanoLC 1000)
  - Trap column: Acclaim PepMap 100, 100 um x 2cm, 5 um beads
  - Analytical column: EasySpray PepMap, 75 um x 25 cm, 2um beads, 50˚C.
- Gradient
  - A = 2% ACN/0.1% formic;
  - B = 90% ACN/0.1% formic



- Total run time = 60 minutes



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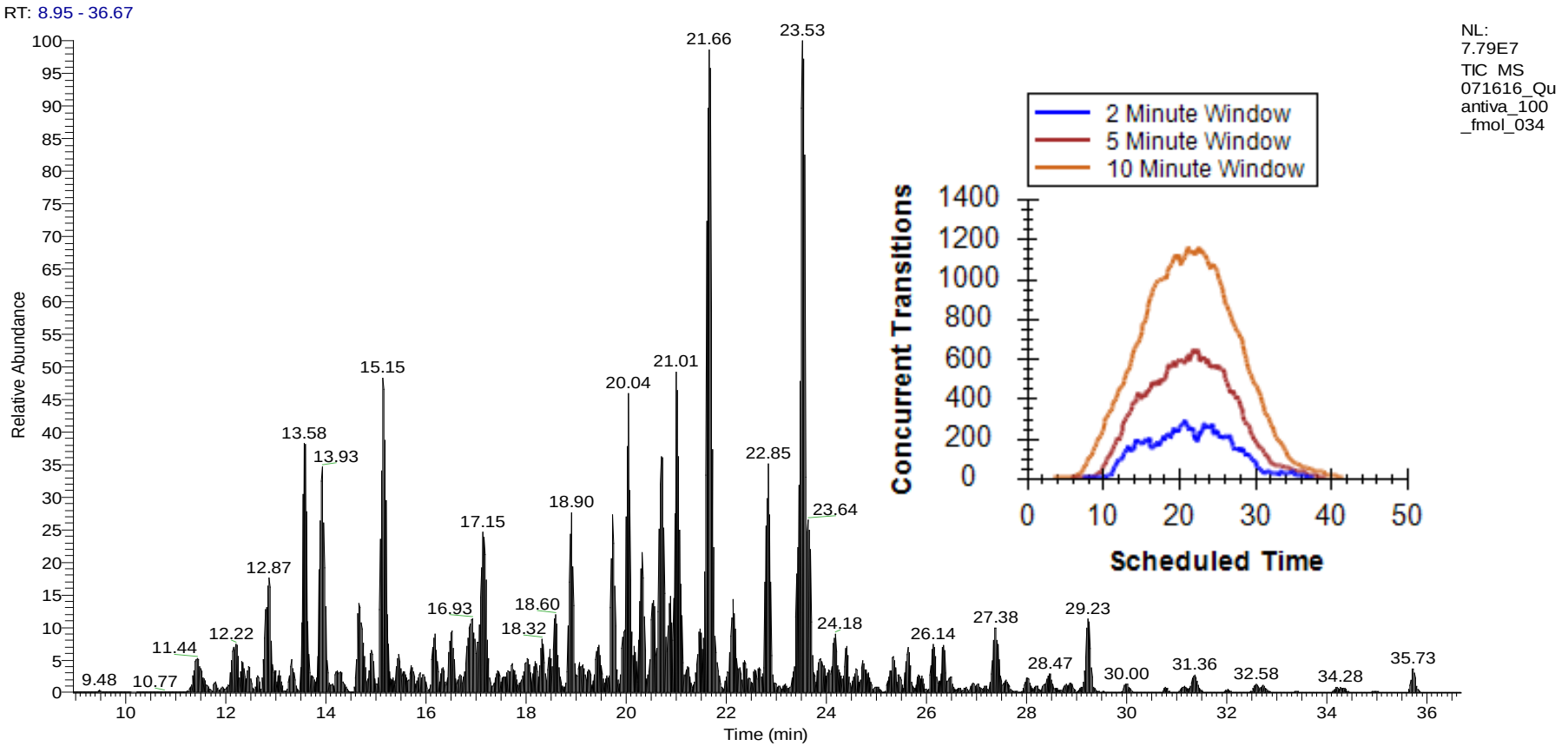
Targeted SRM for 361 peptides (1842 transitions)

- Cycle time = 1 sec
- CID gas pressure = 1.5 mTorr
- Q1/Q3 Resolution: 0.7/0.7
- RT windows: 2 minutes
- Chrom Filter = 8
- Collision Energy = optimized by transition
- Dwell time range = 3.5 – 125 msec
- Total method time = 1 hour



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Questions

Application outline for peptide quantitation

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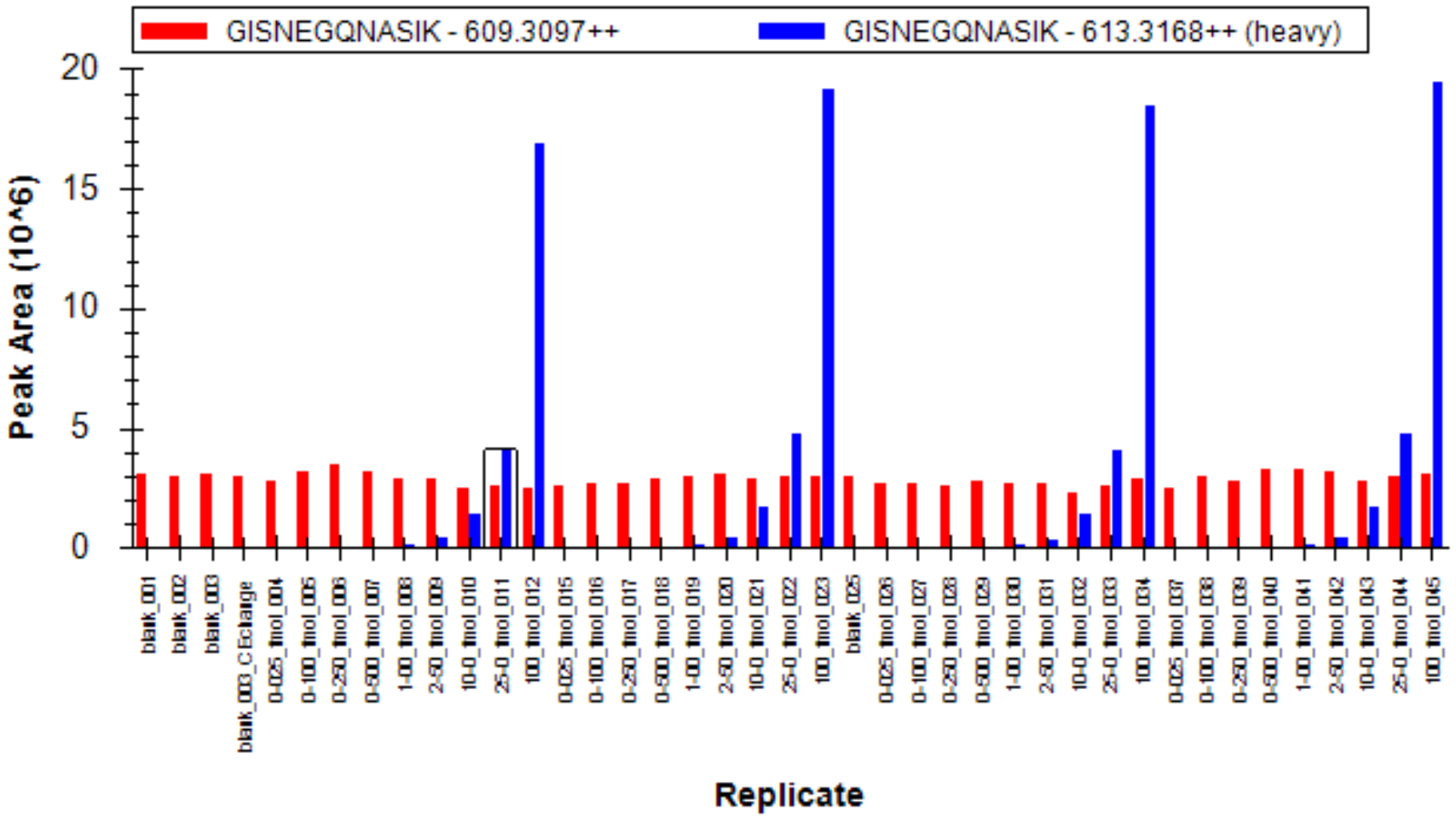
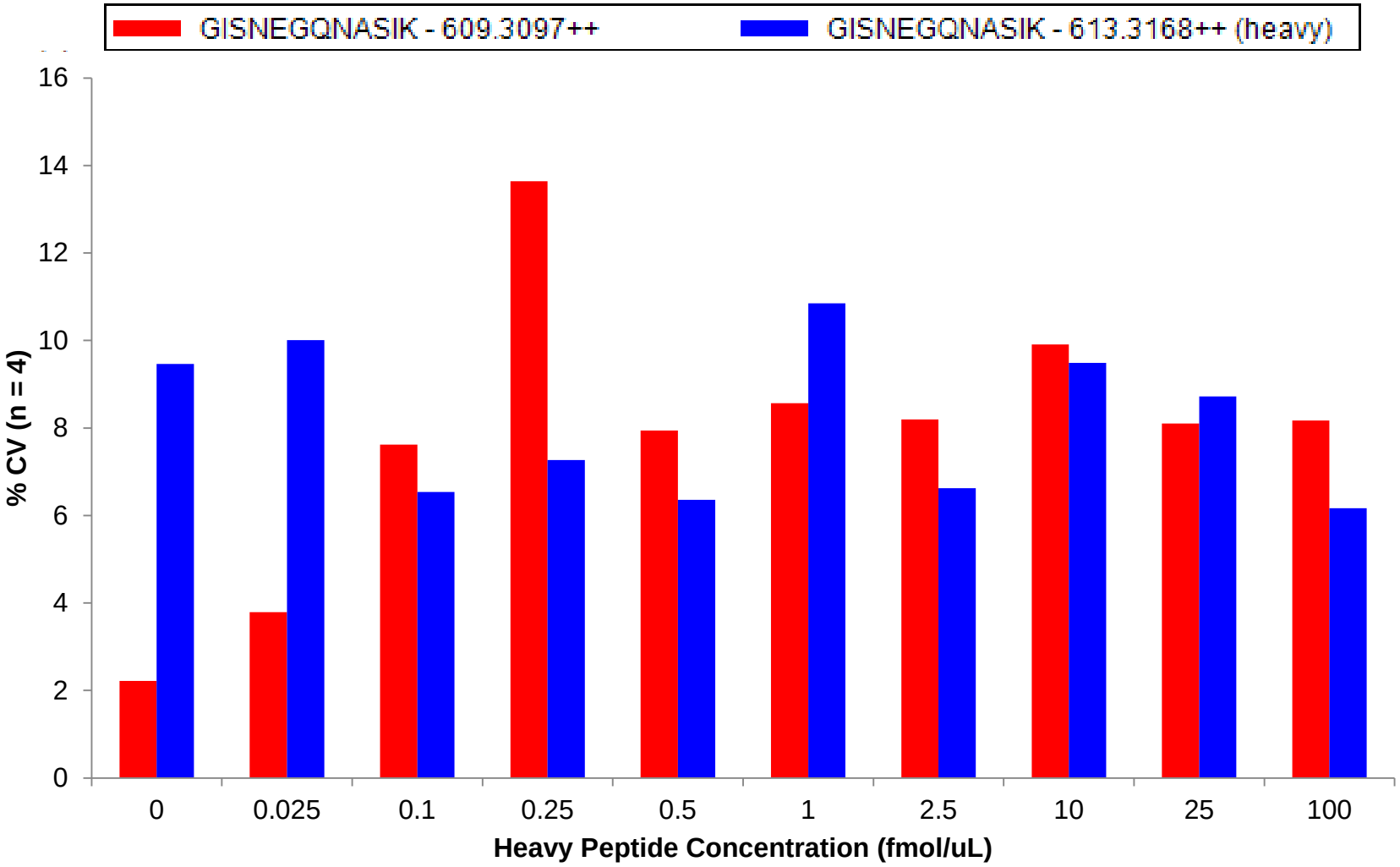
Reproducibility – Peak Areas



Reproducibility – Peak Areas



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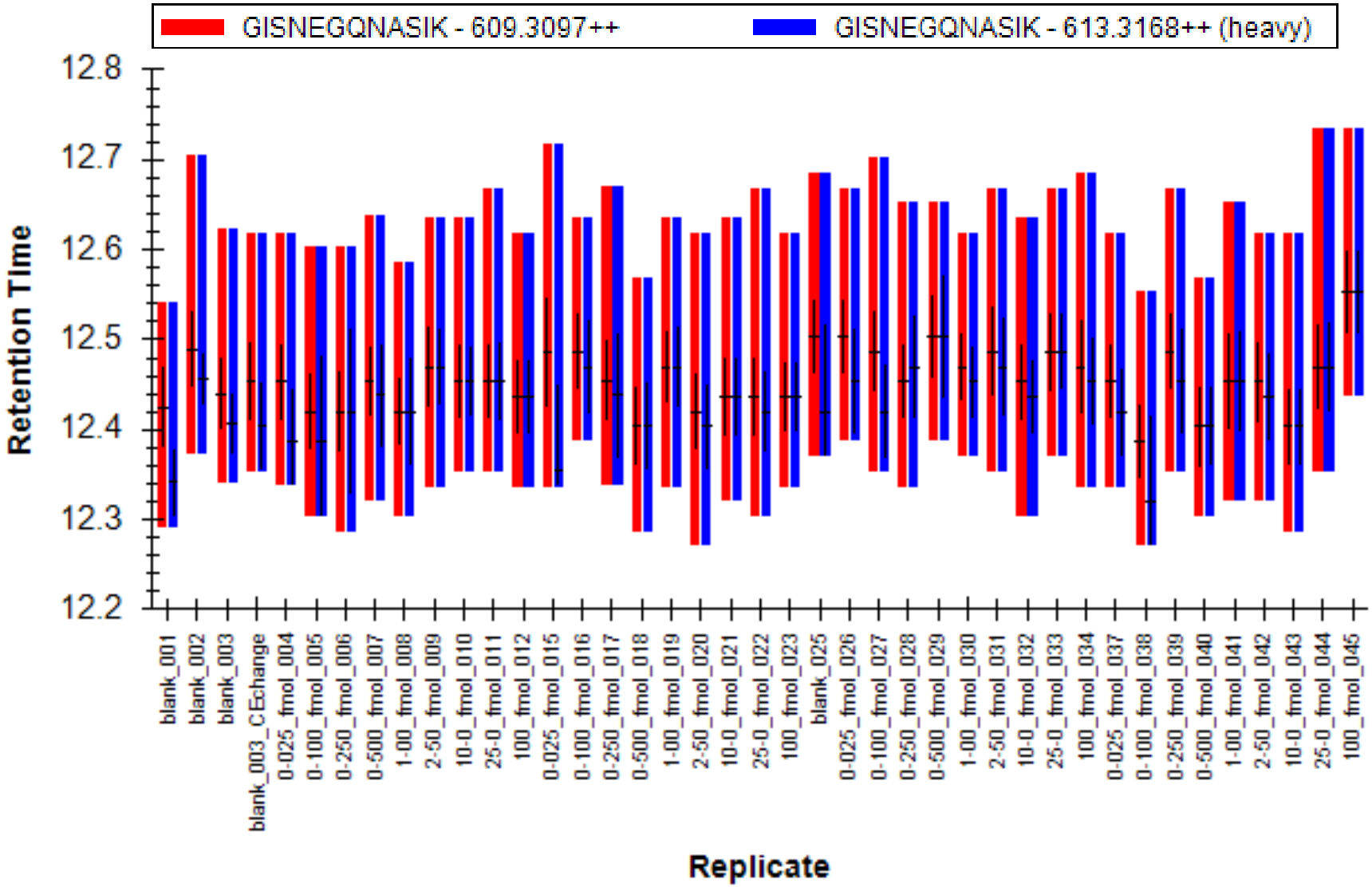
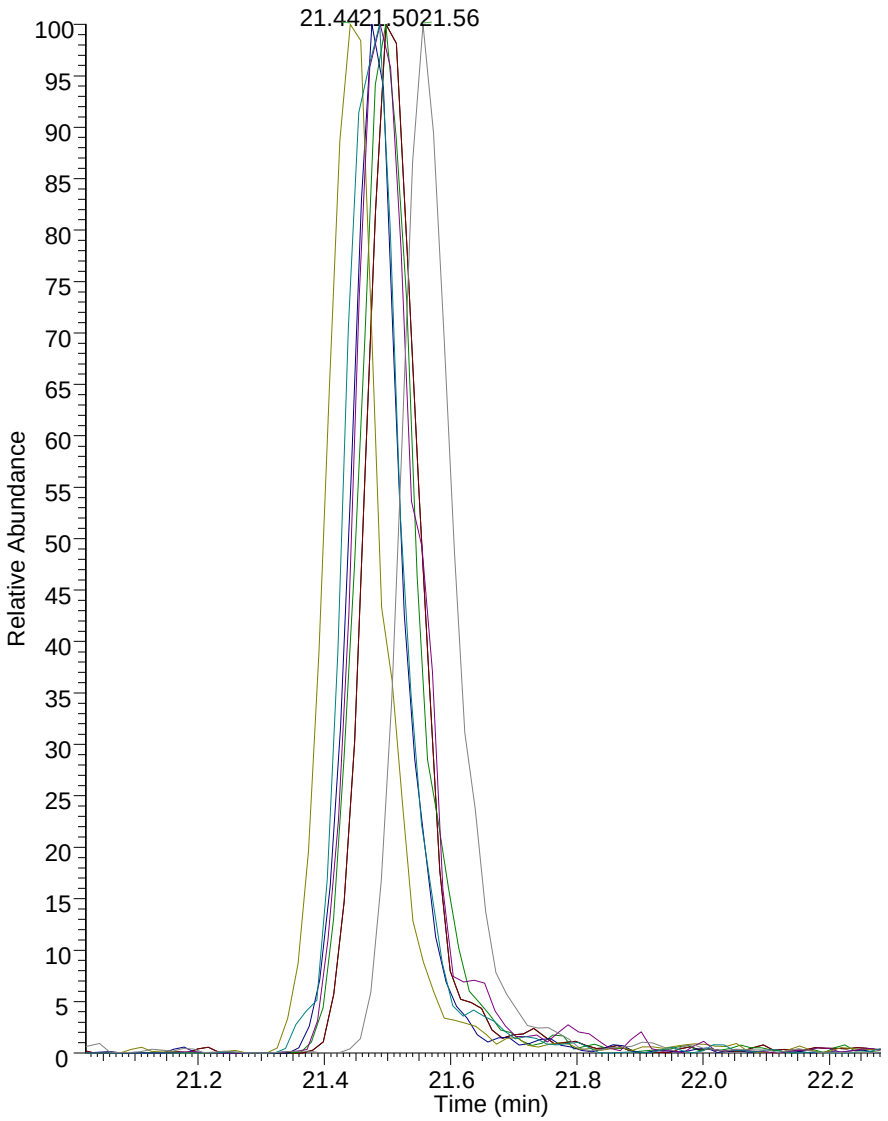
Reproducibility – Retention Times



Reproducibility – Retention Times

- Eight XICs overlaid, show very good RT reproducibility
- SIL peptide is plotted
- Time range for shown data is 48 hours
- RT windows of 2 minutes were used, but could reduce to 1.5 or 1 min.



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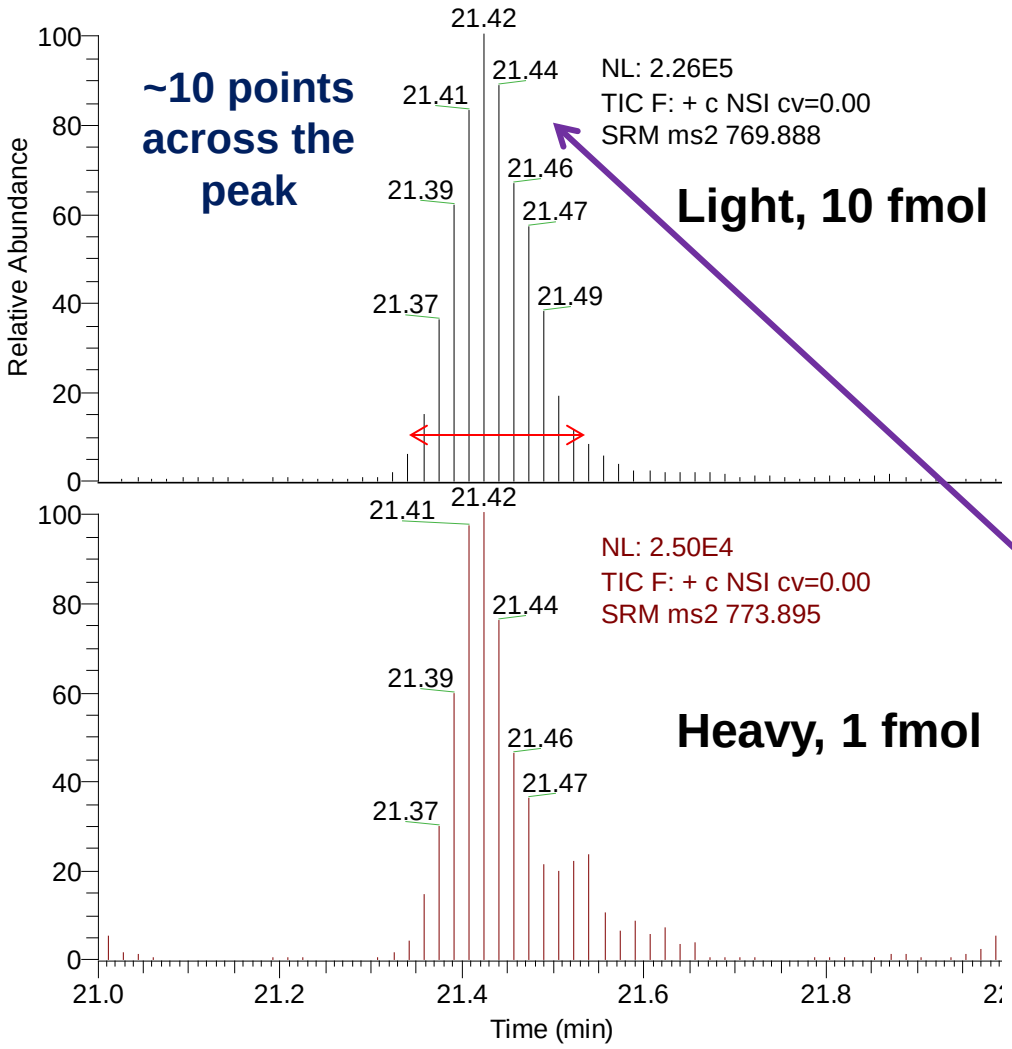
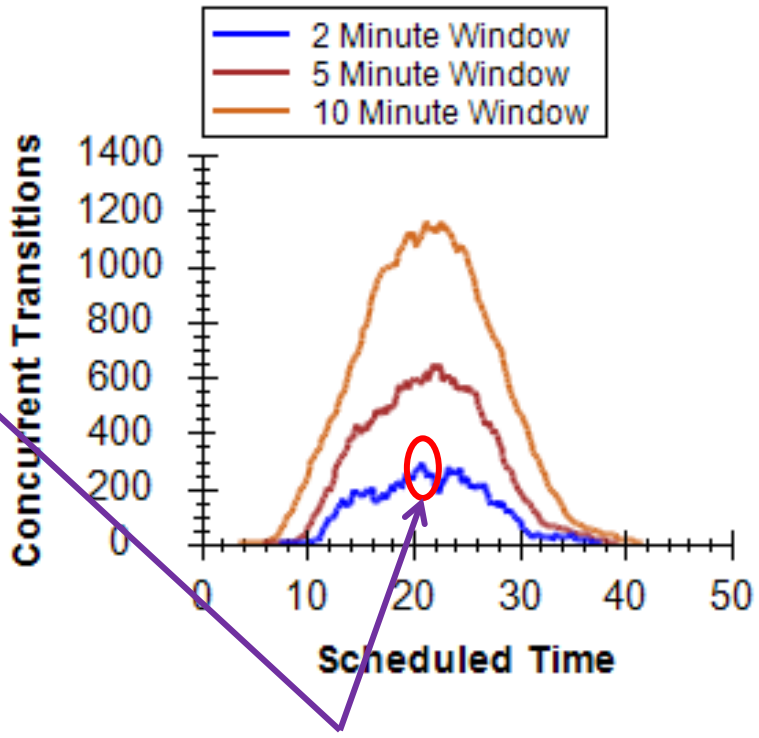
TSQ Quantiva™ : Consistent Sampling Frequency

PRTC peptide ELGQSGVDTYLQTK





Most congested part of chromatogram

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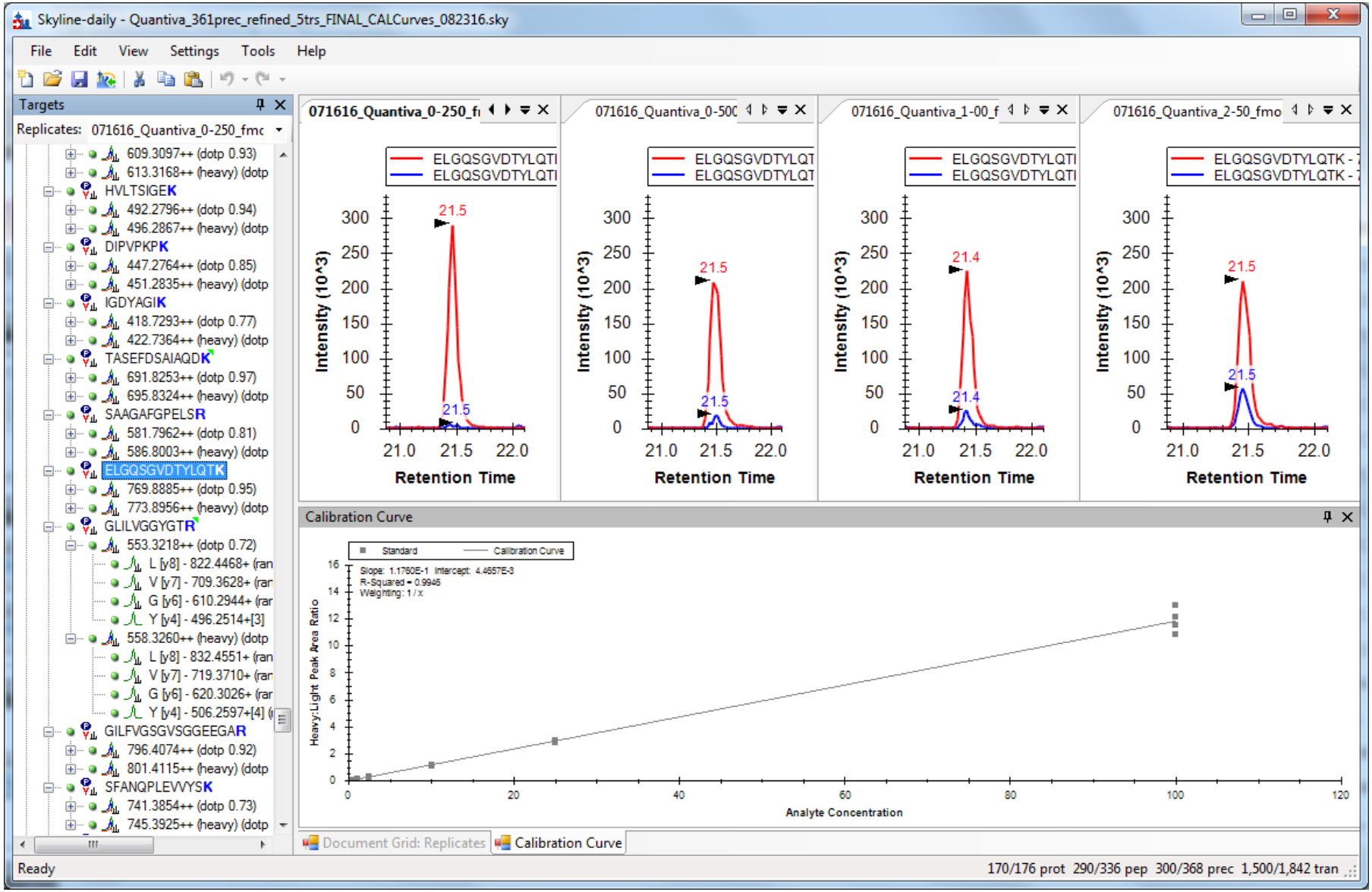
Linear Response While Monitoring 1842 Transitions



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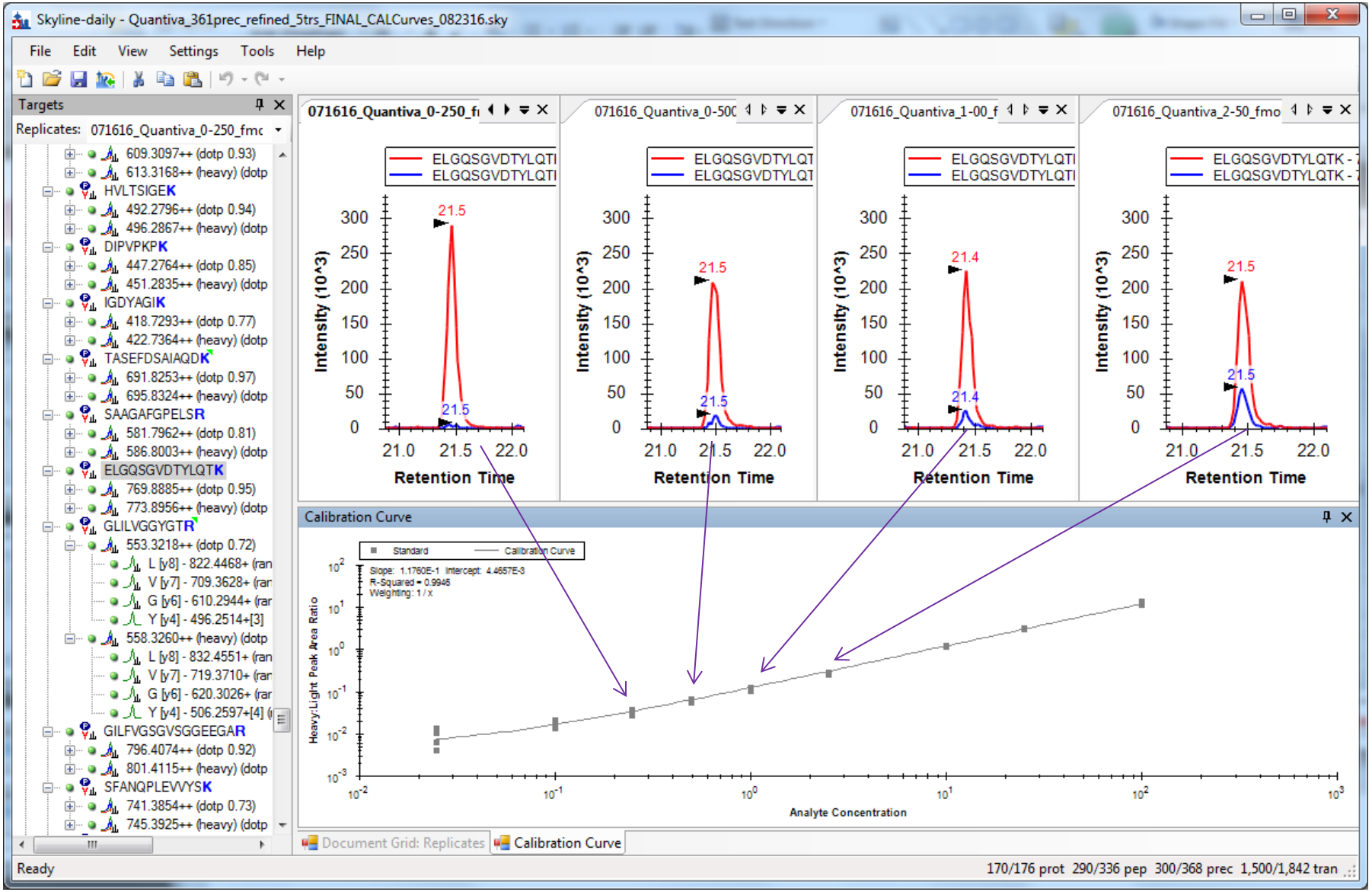

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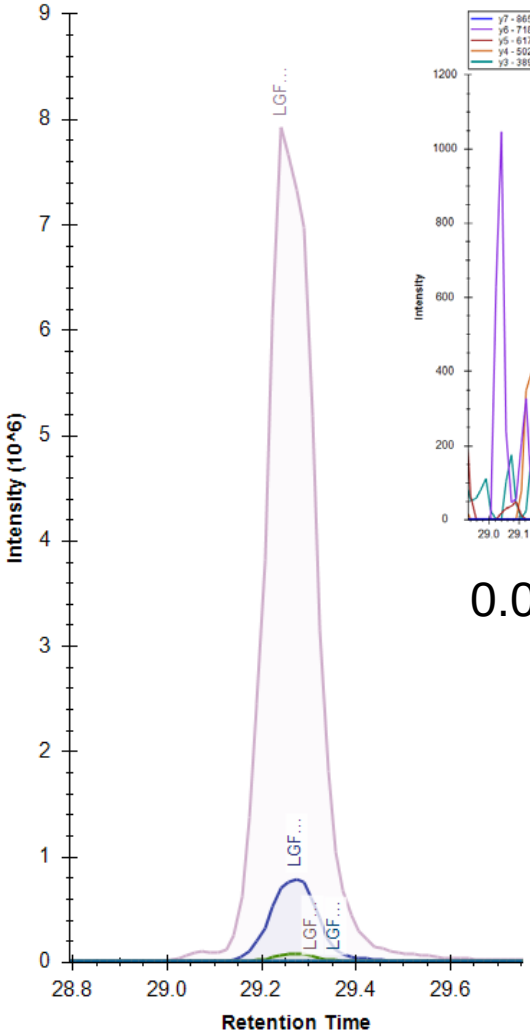
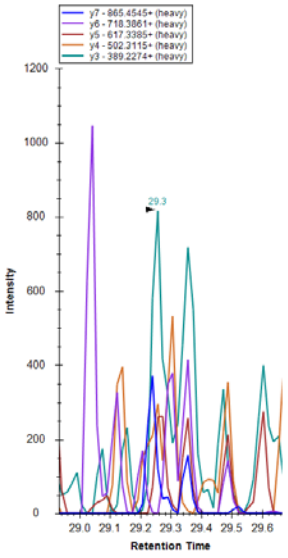
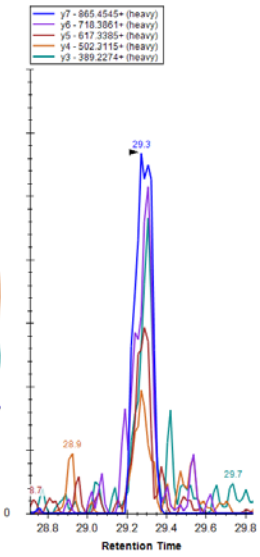
Promega 6 x 5 Peptide Standard in 500 ng HeLa

Peptide LGFTDLFSK

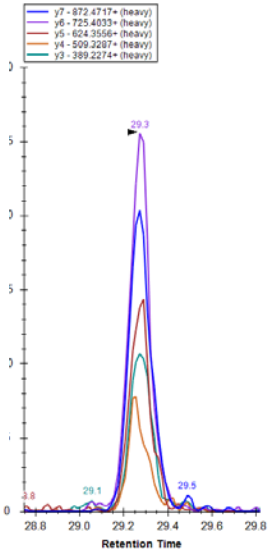




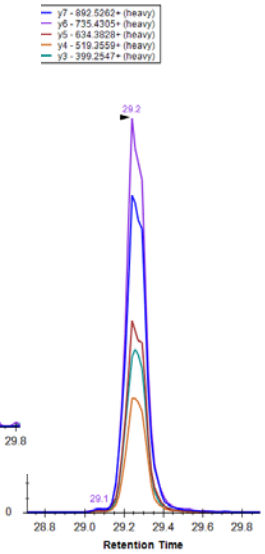
0.02 fmol



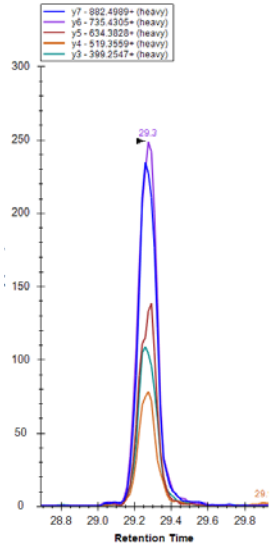
0.2 fmol



2 fmol



20 fmol



200 fmol

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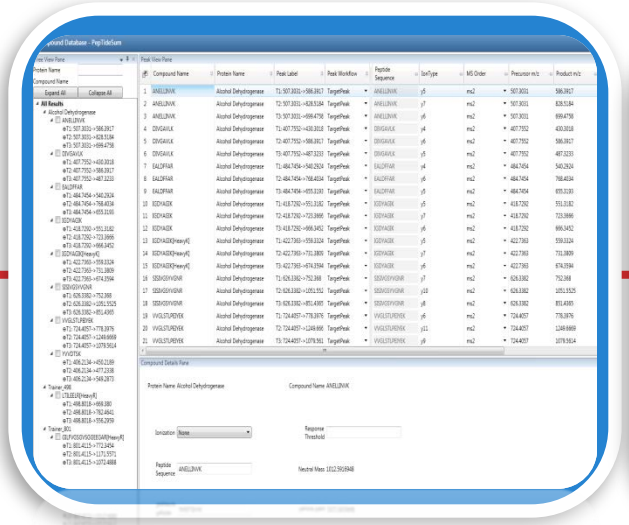
Processing peptide SRM data with TraceFinder 4.1™

Peptide Support



- Peptide predictor tool
- Amino acid formula support
- Import peptide and mass lists from Skyline, Pinpoint

Simplify Method Development

- Redesign/Update compound database, master method
- Autoscan filter selection
- Ratio confirmations for all peaks

Increase usability of Data Review

- Redesign/Update to include all peak information to review faster
- New advanced plots for peak review including group and batch plots

Enhancing Quan Workflows for Routine Bio/Pharma applications and Simplifying Method Development for all markets

Summary

- Remember to follow key points when establishing your assay.
  - Careful selection of peptide targets, give yourself multiple options.
  - Select m/z fragments above precursor and optimize CE's for each.
  - Optimize chromatography, ensure no interference with fragment ions.
  - Utilize SIL internal standards if possible.
  - Establish standards for endogenous protein concentration.
  - Run "Scheduled" with RT windows when possible to maximize dwell times.
  - Ensure you have enough scans across the peak.
  - Utilize ion ratio confirmation in your processing method.
  - Quantitate utilizing fragment with best signal/noise.

Acknowledgements

Susan Abbatiello

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Brad Groppe

Nick Molinaro

Kent Seeley

Tara Schroeder

Alan Atkins

Katie Southwick

Detlef Schumann

Parts – Reversed Phase Columns

Part Number	Description	Column Dimensions (ID x L)	Packing Phase	Bead Diameter (μm)
ES800	EASY-Spray	75 μm x 15 cm	PepMap C18	3
ES801	EASY-Spray	50 μm x 15 cm	PepMap C18	2
ES802	EASY-Spray	75 μm x 25 cm	PepMap C18	2
ES803	EASY-Spray	75 μm x 50 cm	PepMap C18	2

- http://www.proxeon.com/productrange/easy_spray/intro/index.html

Parts – Reversed Phase Columns

Part Number	Description	Analytical Column Dimensions (ID x L)	Packing Phase	Bead Diameter (μm); Pore Size (Å)
164738	Acclaim	75 μm x 15 cm	Acclaim PepMap 100 C18	3; 100
164739	Acclaim	75 μm x 50 cm	Acclaim PepMap 100 C18	3; 100
164940	Acclaim	75 μm x 15 cm	Acclaim PepMap 100 C18	2; 100
164941	Acclaim	75 μm x 25 cm	Acclaim PepMap 100 C18	2; 100
164942	Acclaim	75 μm x 50 cm	Acclaim PepMap 100 C18	2; 100
164568	Acclaim	75 μm x 15 cm	Acclaim PepMap 100 C18	3; 100
164569	Acclaim	75 μm x 25 cm	Acclaim PepMap 100 C18	3; 100

- https://www.thermofisher.com/order/catalog/product/160321

Parts – Reversed Phase Trap Columns

Part Number	Description	Trap Column Dimensions (ID x L)	Packing Phase	Bead Diameter (μm)
164535	Acclaim	75 μm x 2 cm	PepMap 100 C18	3
164564-CMD also known as 164564	Acclaim	100 μm x 2 cm	PepMap 100 C18	5

- https://www.thermofisher.com/order/catalog/product/160321

Parts – Metal Needle Kits and Ion Source Accessories

Part Number	Description
00950-00954	32-Gauge metal needle kit for high flow LC flow rates between 5µL/min to 400µL/min
97144-20040	34-Gauge metal needle kit for low flow LC flow rates between 500nL/min to 10µL/min
00106-10511	Tubing, 0.075mm ID x 0.193mm OD fused-silica capillary for low flow applications instead of metal needle
ES232	EASY-Spray Emitter Positioning Tool
ES235	EASY-Spray Emitter Wash Cap
106868-0064	Cleaving Stone, 1" x 1" x 1/32”