ThermoFisher SCIENTIFIC

Rapid Assay Development and Refinement for Targeted Protein Quantitation Using an Intelligent SRM (iSRM) Workflow

September 2, 2010

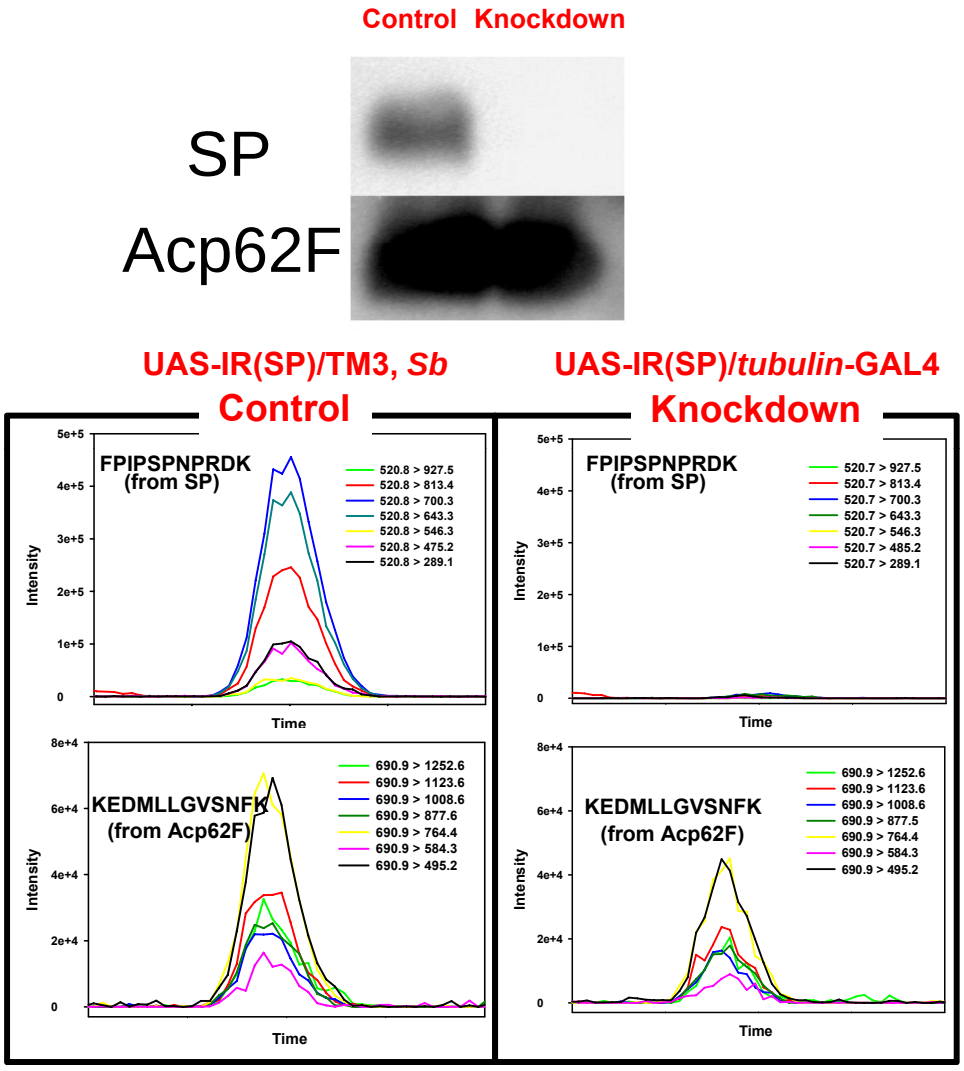
Reiko Kiyonami, Ph. D.
Senior Strategic Marketing Specialist – Proteomics
Thermo Fisher Scientific

What is Targeted Quantitative Proteomics?

Discovery
Fishing for differentially expressed proteins

Targeted
Measuring proteins in known pathways

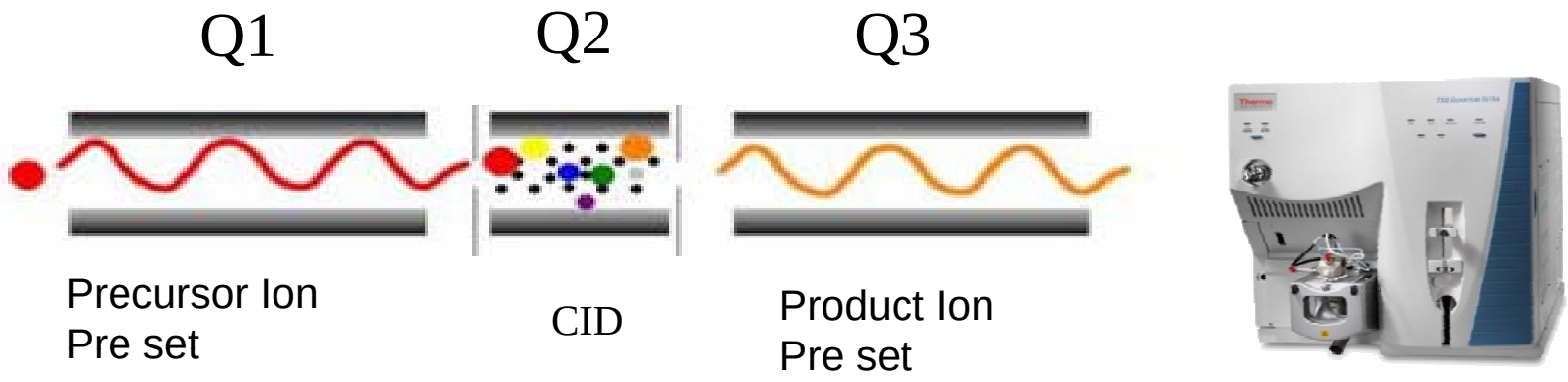
Why Targeted Quantitative Proteomics?



Data provided by M. J. MacCoss from Uni. of Washington

- Need to verify potential biomarkers which have been found in the discovery experiments to narrow down the long biomarker candidate list to a few of real promising ones for validation stage.
- Need to acquire consistent quantitative data for large protein sets to facilitate modeling in systems biology studies.
- The traditional immunoassay method is too expensive to implement on such a large scale.
- Alternatively, a multiple SRM assay on a triple quadrupole mass spectrometer can be used for targeting hundreds of biomarker candidates from hundreds of complex samples in a high throughput way.

SRM (Selected Reaction Monitoring) assay



1. A “proteotypic” peptide is selected as being quantitative surrogate of a targeted protein.
2. Q1 is set to transmit only the precursor ion of the selected peptide.
3. Q2 is used to induce fragmentation of the precursor ion.
4. Q3 is set to transmit a specific product ion,

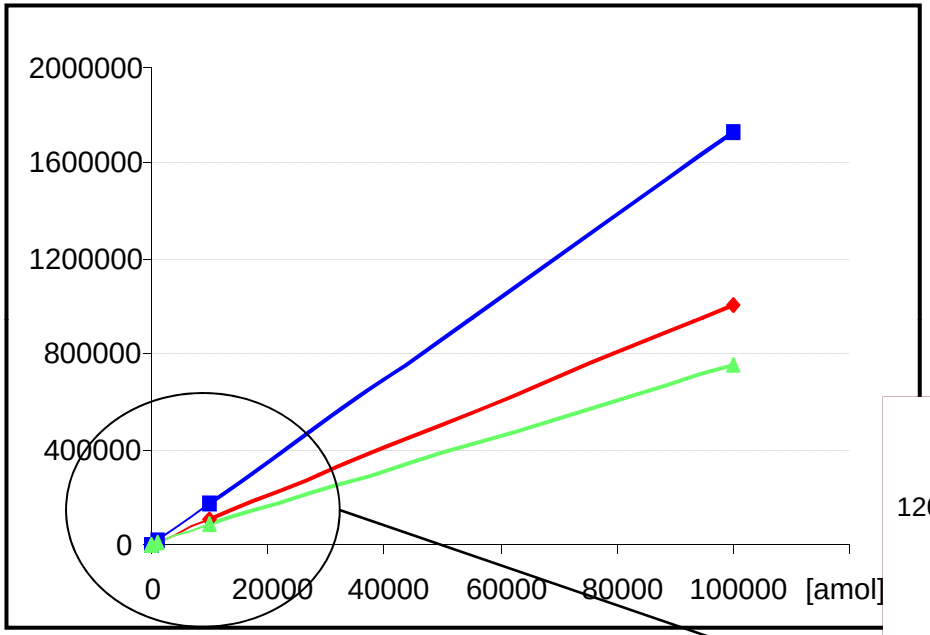
   Each precursor to product ion set is called an SRM transition.

Why SRM Analysis for Targeted Protein Quantitation?

- Highest specificity/sensitivity – necessary for low abundant peptides in complex matrices like plasma
- Largest linear dynamic range for quantitation
- Well-established as the quantitative method of choice for small molecule applications

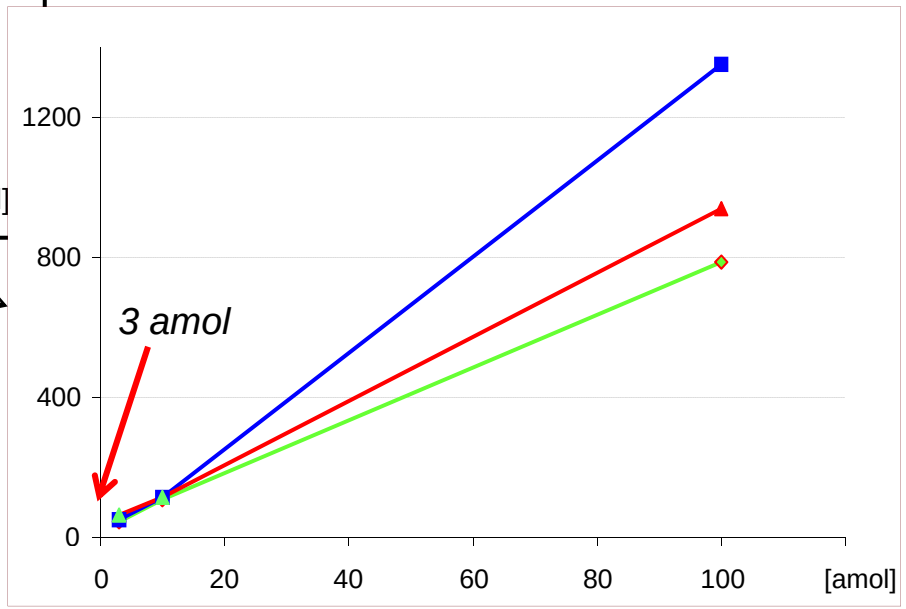
TSQ Vantage allows Low Level Quantitation in Background Matrix

Linear response over wide dynamic range



Stable isotope labeled peptides spiked into whole yeast lysate

LOD in low attomole range



GILFVGSGVSGGEEGAR	801.4	1072.5
LTILEELR	498.8	782.5
LVEDPQVIAPFLGK	767.4	569.4

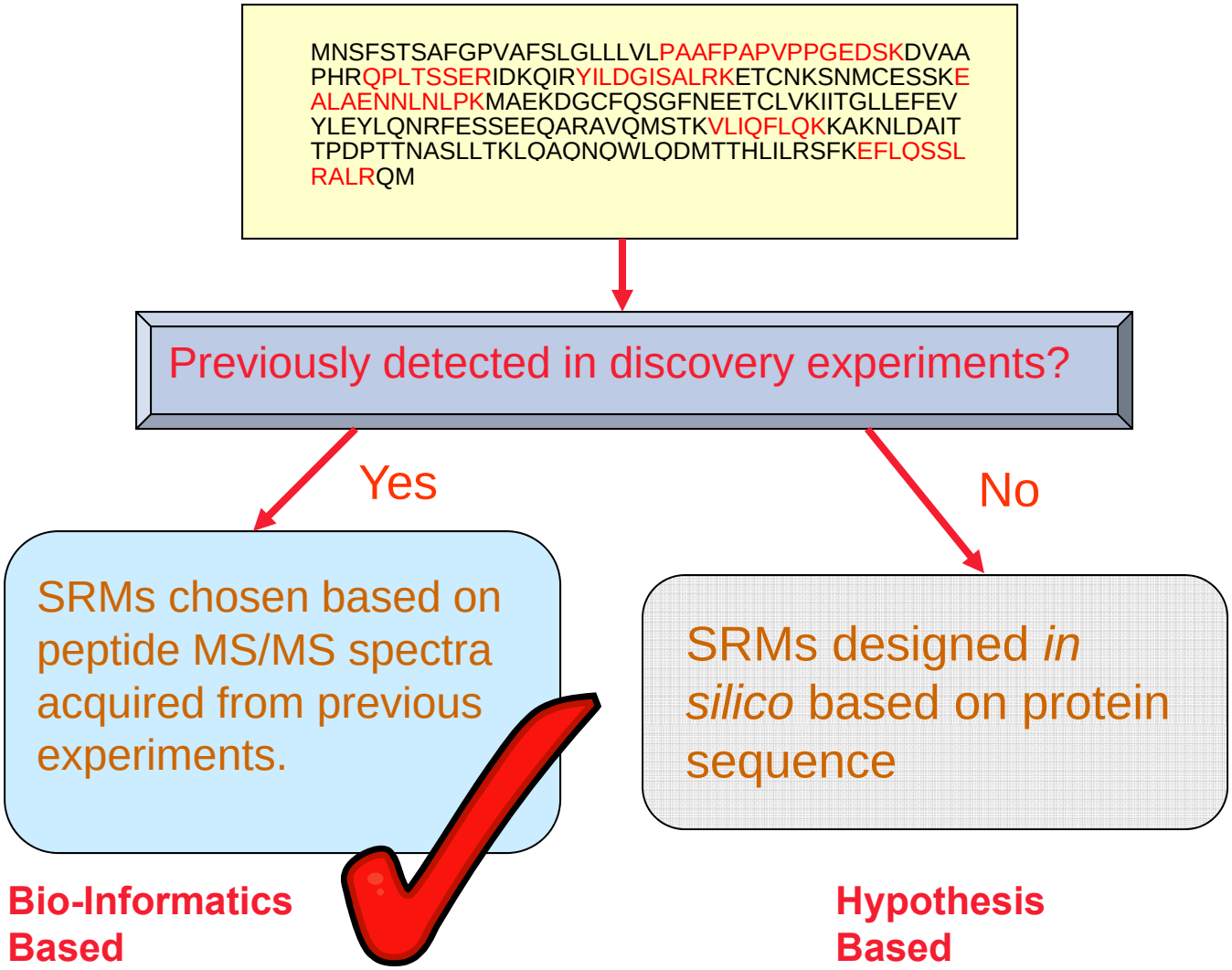
ThermoFisher SCIENTIFIC

Peptide Selection and SRM Assay Design

The success and confidence of an SRM experiment directly depends on the selected proteotypic peptides and the specific transitions.

- Peptides have to be unique to the targeted protein
- Peptides should not contain Cys, Met or other commonly modified residues
- Peptides should yield excellent mass spectral signal
- Selected product ions used for SRM analysis should have higher mass over charge values for increased selectivity

From Protein…to SRM
– How can you select your peptides/transitions?



ThermoFisher SCIENTIFIC

Can we use CID MS/MS data for selecting SRM transitions?

research articles Journal of proteome research

Correlation between y-Type Ions Observed in Ion Trap and Triple Quadrupole Mass Spectrometers

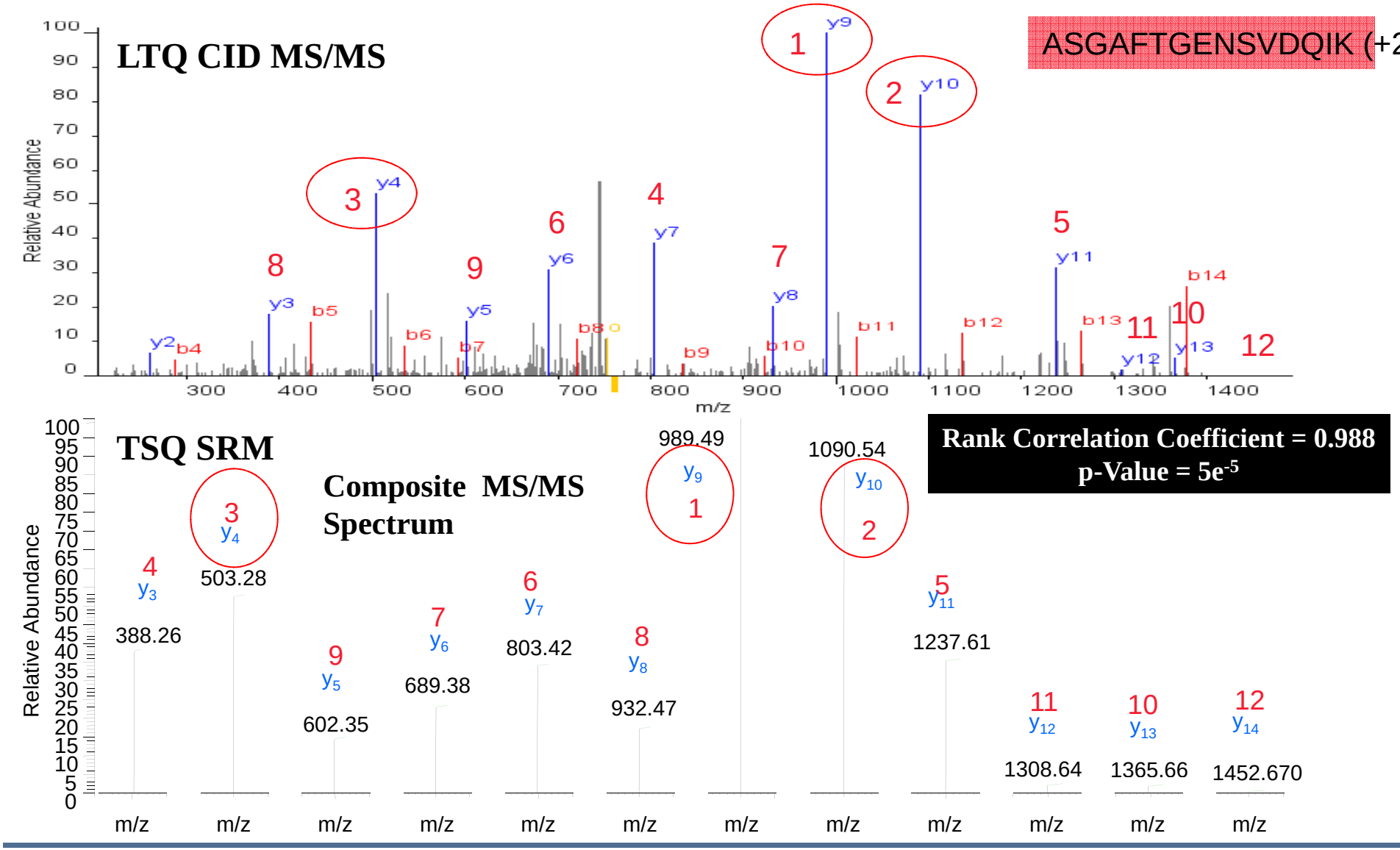
Carly A. Sherwood,[†] Ashley Eastham,[‡] Lik Wee Lee,[†] Jenni Risler,[§] Olga Vitek,[||] and Daniel B. Martin*,[†]

Institute for Systems Biology, 1441 North 34th Street, Seattle, Washington 98103, Amgen, 1201 Amgen Court West, Seattle, Washington 98119, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue, Seattle, Washington 98112, and Purdue University, 250 North University Street, West Lafayette, Indiana 47907

Received March 31, 2009

Multiple reaction monitoring mass spectrometry (MRM-MS) is a technique for high-sensitivity targeted analysis. In proteomics, MRM-MS can be used to monitor and quantify a peptide based on the production of expected fragment peaks from the selected peptide precursor ion. The choice of which fragment ions to monitor in order to achieve maximum sensitivity in MRM-MS can potentially be guided by existing MS/MS spectra. However, because the majority of discovery experiments are performed on ion trap platforms, there is concern in the field regarding the generalizability of these spectra to MRM-MS on a triple quadrupole instrument. In light of this concern, many operators perform an optimization step to determine the most intense fragments for a target peptide on a triple quadrupole mass spectrometer. We have addressed this issue by targeting, on a triple quadrupole, the top six y-ion peaks from ion trap-derived consensus library spectra for 258 doubly charged peptides from three different sample sets and quantifying the observed elution curves. This analysis revealed a strong correlation between the y-ion peak rank order and relative intensity across platforms. This suggests that y-type ions obtained from ion trap-based library spectra are well-suited for generating MRM-MS assays for triple quadrupoles and that optimization is not required for each target peptide.

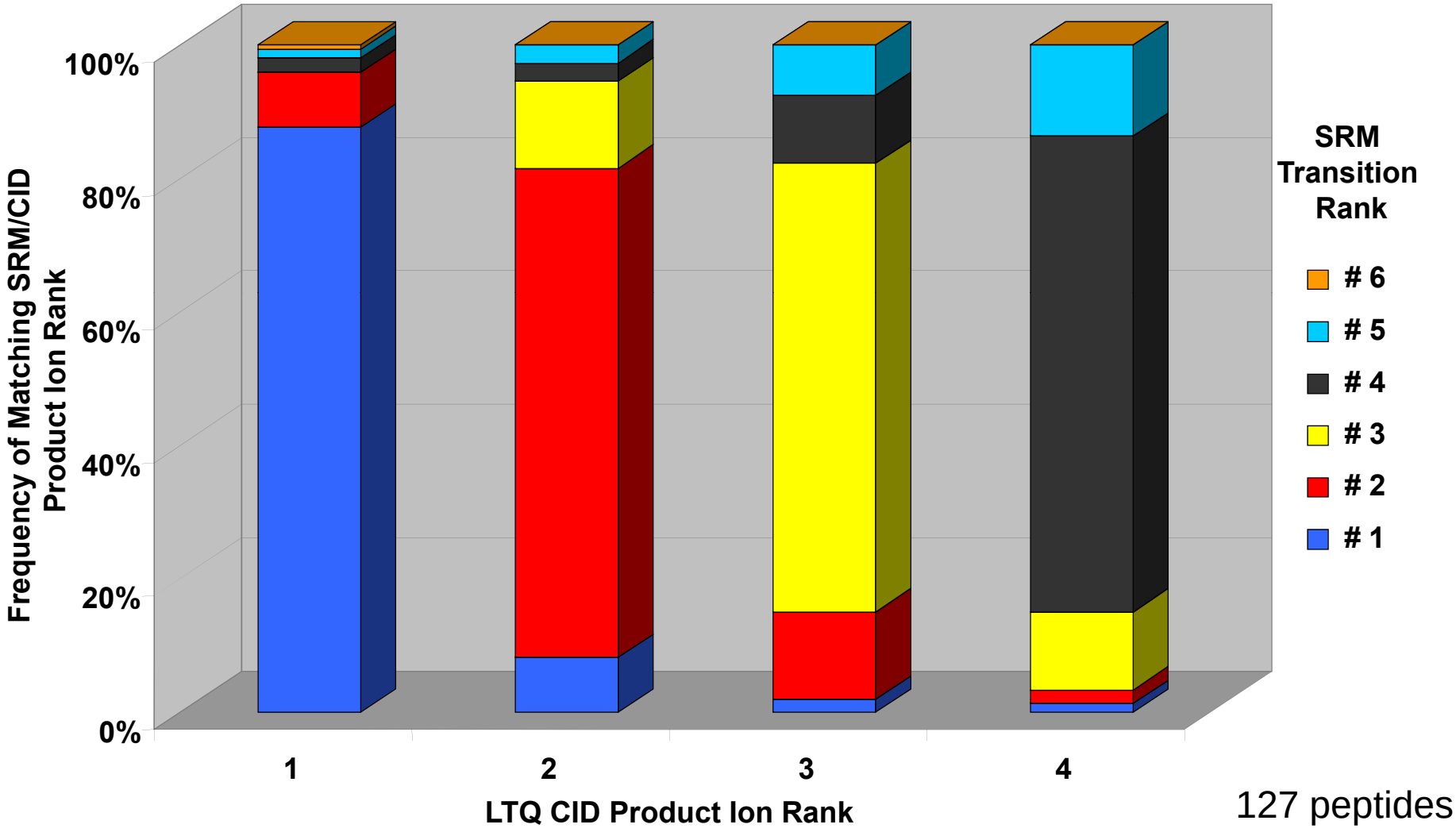
Comparing Product Ion Rankings – Linear Ion Trap CID vs. Triple Quadrupole SRM Transitions



MacCoss et al. *J. Proteome Research* **2008,** *8(6)*, 2733-2739

ThermoFisher SCIENTIFIC

Evaluation of Rank Overlap: LTQ CID to SRM

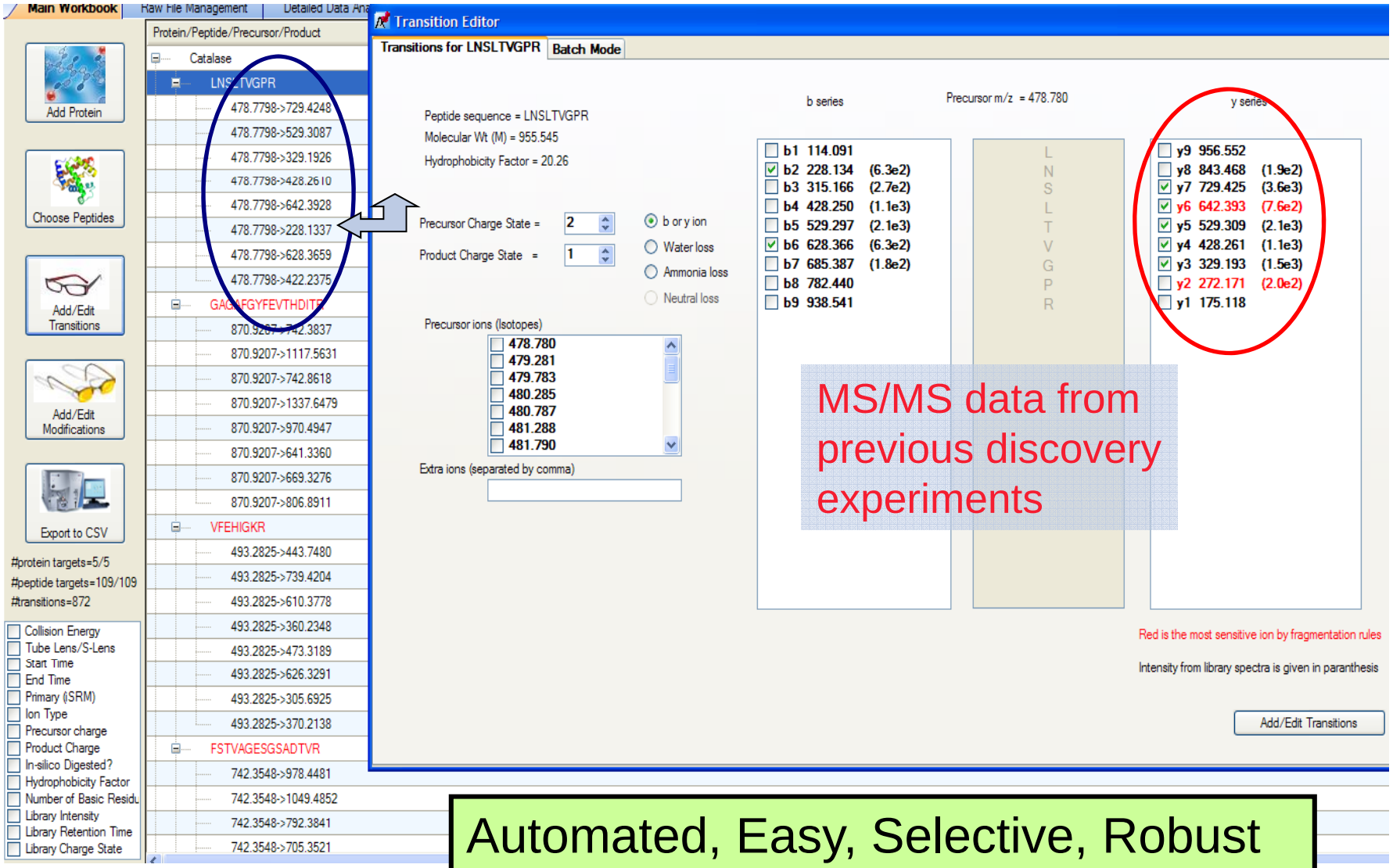


127 peptides

MacCoss et al. *J. Proteome Research* **2008,** *8(6)*, 2733-2739

ThermoFisher SCIENTIFIC

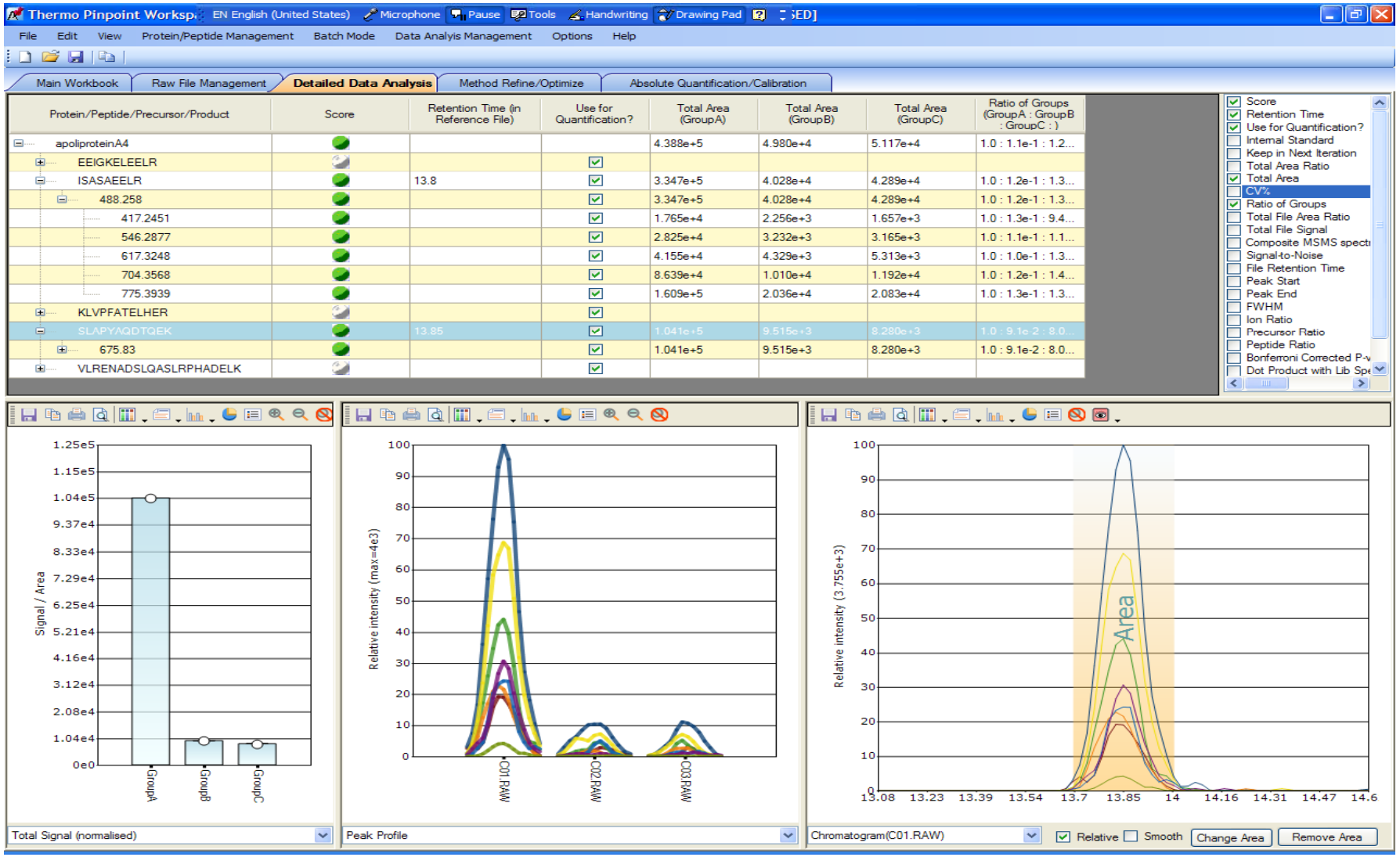
Automatic Peptide and Transition Selection based on MS/MS spectral library by using Pinpoint



Automated, Easy, Selective, Robust

ThermoFisher SCIENTIFIC

Automated Data Processing and Peptide Verification using Pinpoint



ThermoFisher SCIENTIFIC

Biomarker Workflow

List of ***candidate*** markers generated

Need to ***verify and validate*** in expanded population

LTQ Velos

LTQ Orbitrap Velos

SIEVE

Proteome Discoverer 1.0

Ion Trap-based mass specs

Unbiased

TSQ Vantage

Pinpoint 1.0

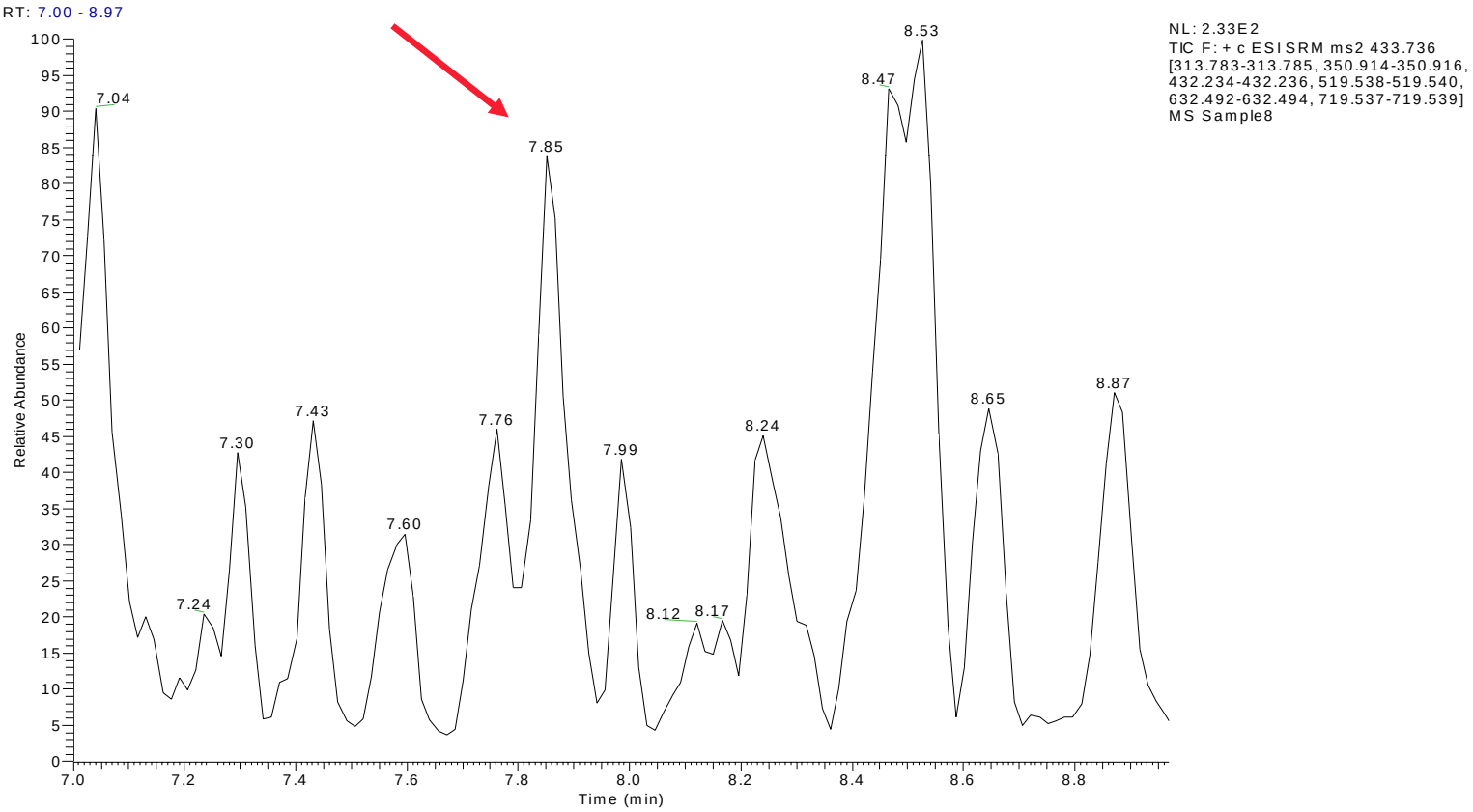
Targeted Analysis: SRM Assays

ThermoFisher SCIENTIFIC

Challenge for Peptide Quantitation using SRM
How to verify if the detected peak is the right targeted peptide

Which peak is my peptide?

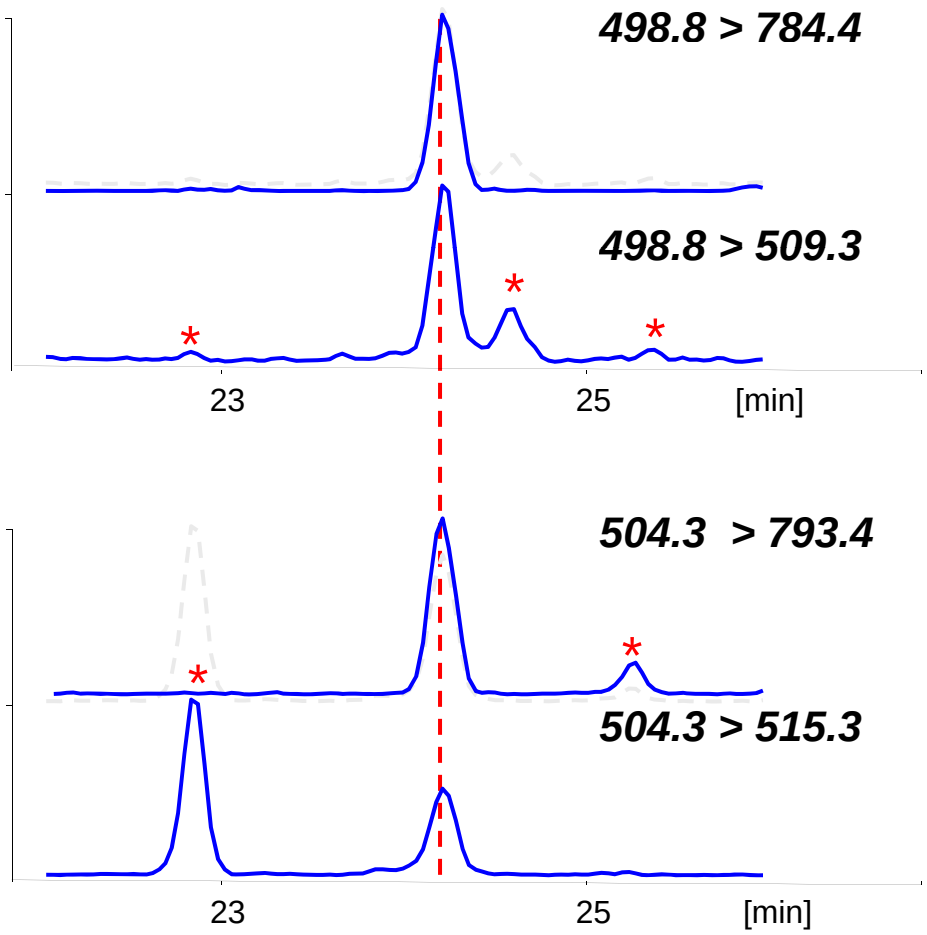
Samples are very complex and background interferences require verification of the targeted peptide peak.



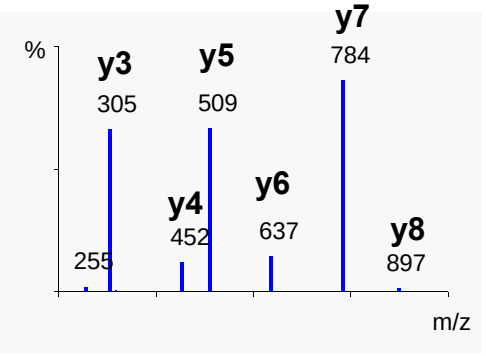
ThermoFisher SCIENTIFIC

Peptide verification using isotopically labeled peptides as internal standards

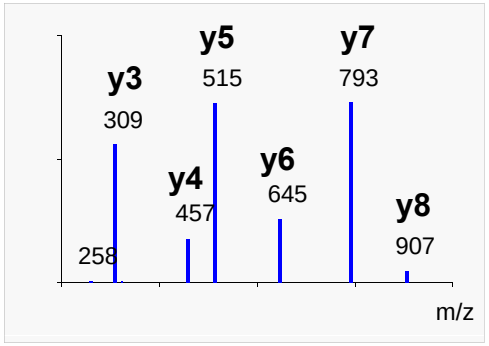
- Not practical for large scale quantitative proteomics considering the cost and time to make the internal standards



VIFQGFTGK-^{14}N



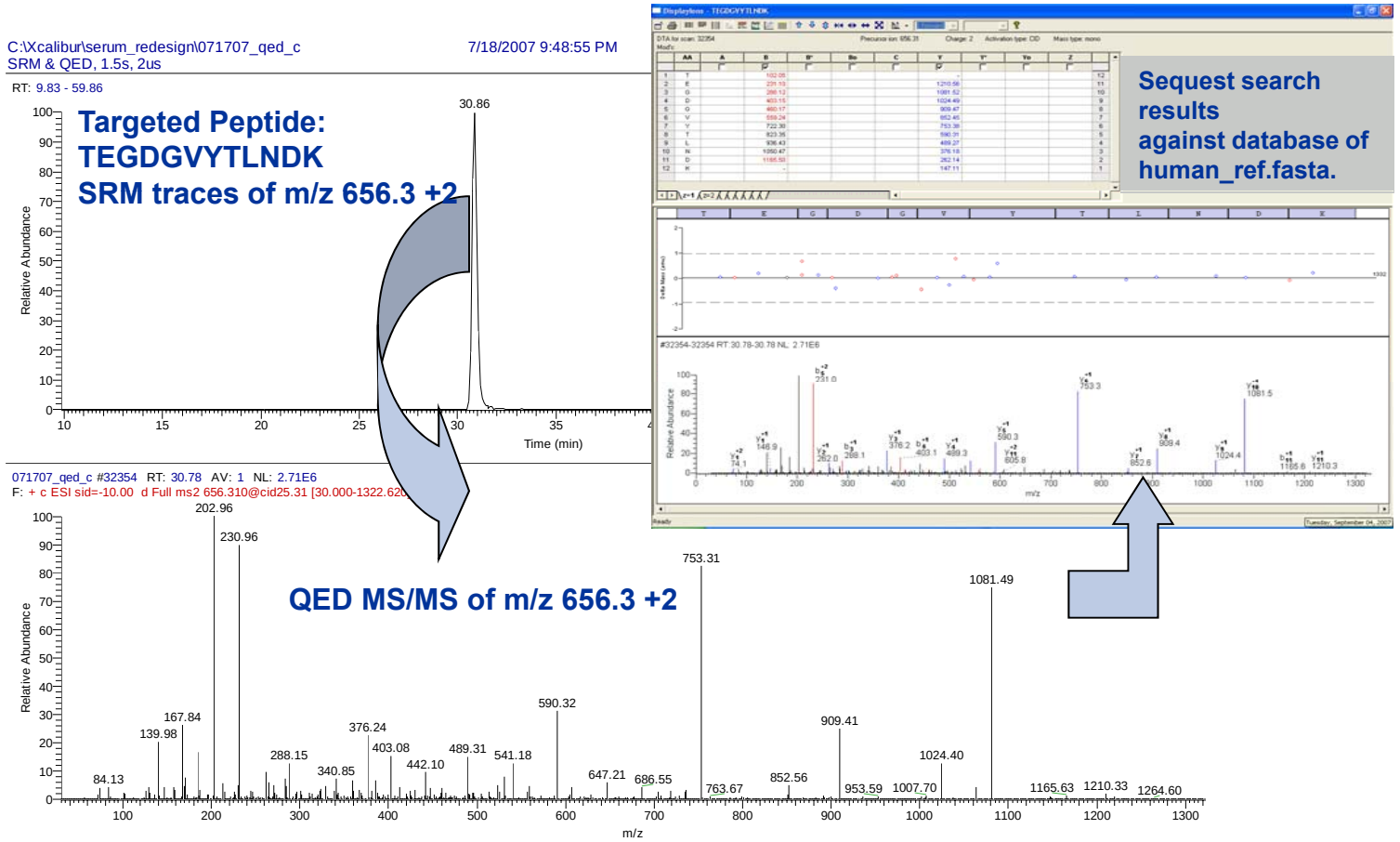
VIFQGFTGK-^{15}N



ThermoFisher SCIENTIFIC

Peptide verification using SRM triggered MS/MS spectrum

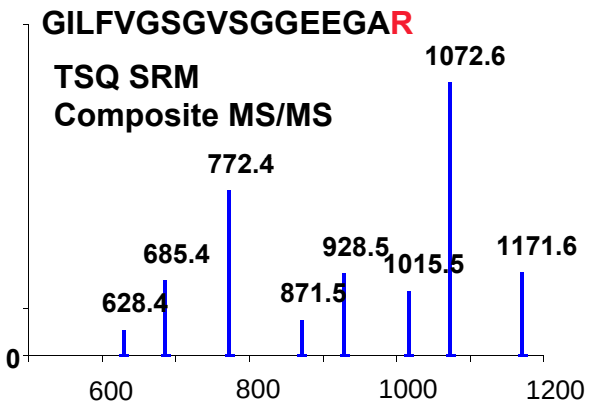
- Requires a broad mass selection window
- MS/MS spectra often contain multiple components for complex biological samples
- Need longer cycle time and less sensitive



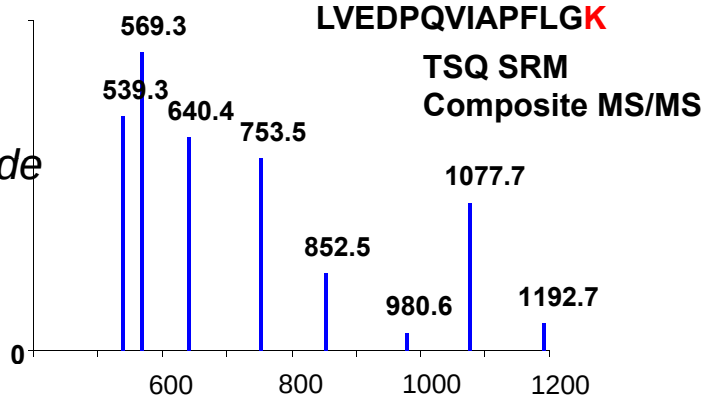
ThermoFisher SCIENTIFIC

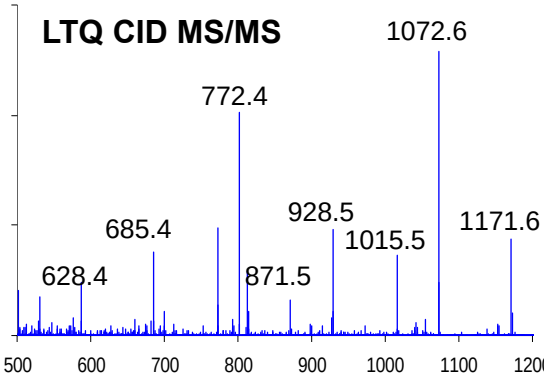
Peptide verification using composite MS/MS spectra (constituted with a large number of transitions per peptide)

- Measurements of multiple transitions per peptide - a composite tandem mass spectrum
- Narrow mass selection window (higher selectivity; Higher sensitivity
- Co-elution of all ions allows to eliminate false positive calls
- Limited throughput if constantly monitoring large number of transitions per peptide

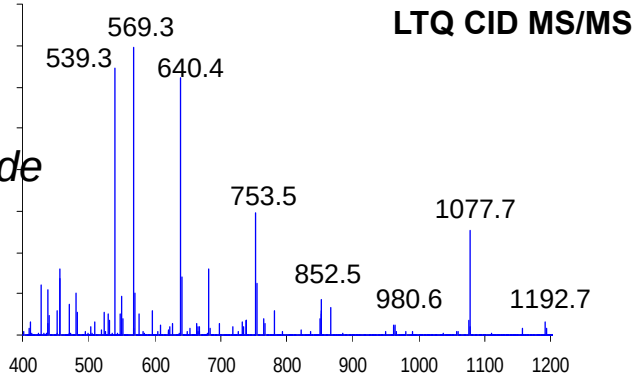


Heavy peptide





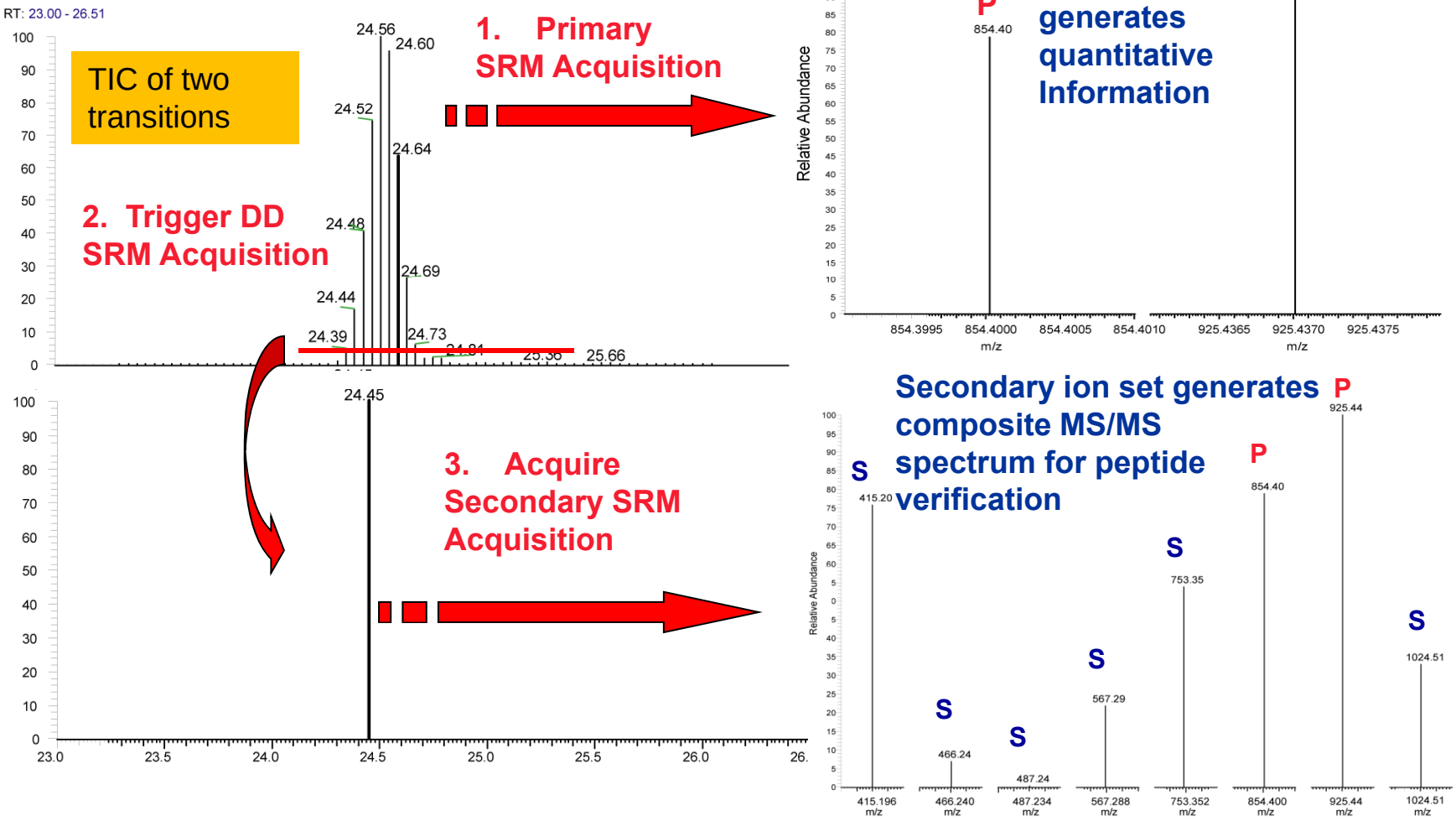
Heavy peptide



ThermoFisher SCIENTIFIC

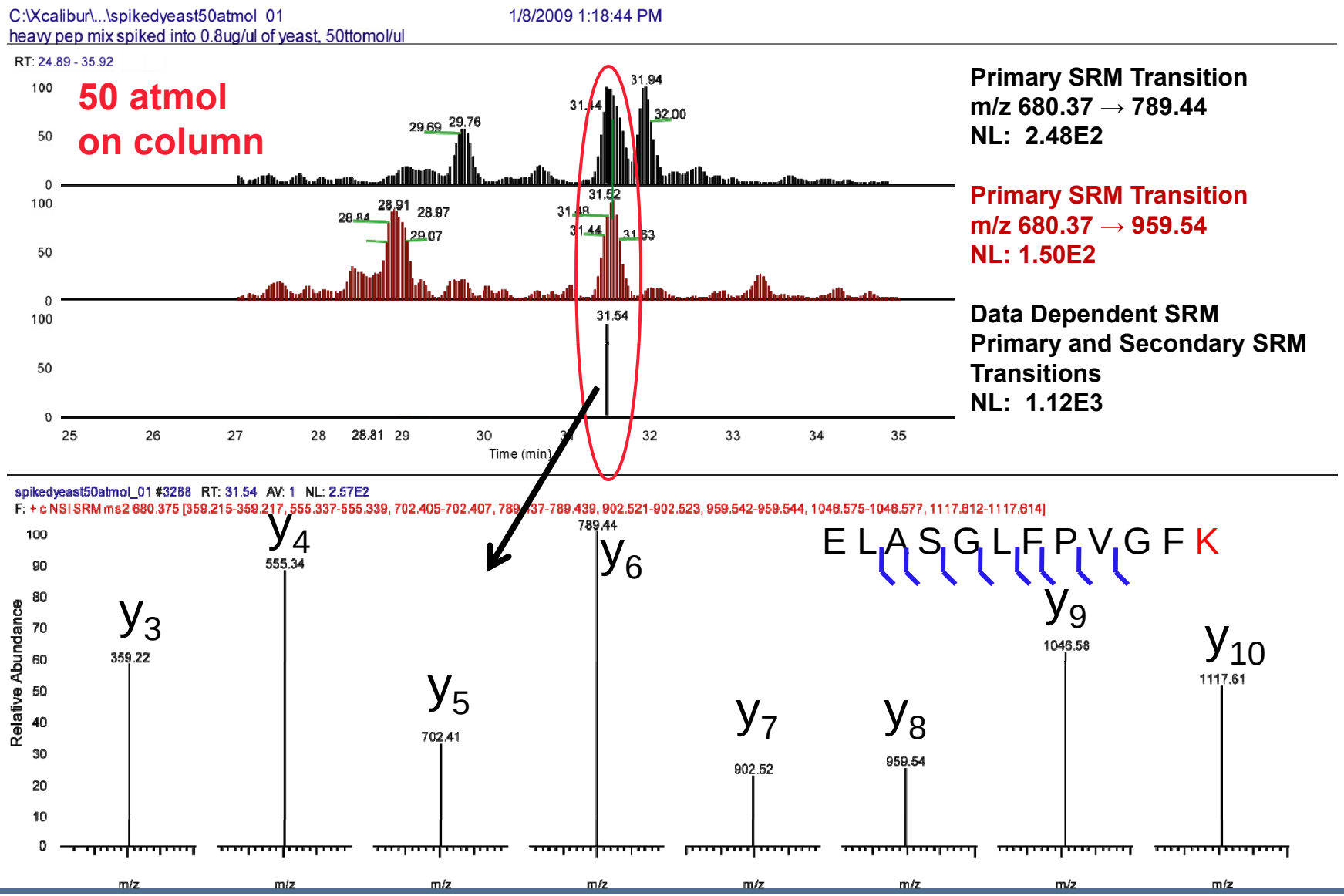
Introducing intelligent SRM (iSRM)

Large number of transitions are split into two sets



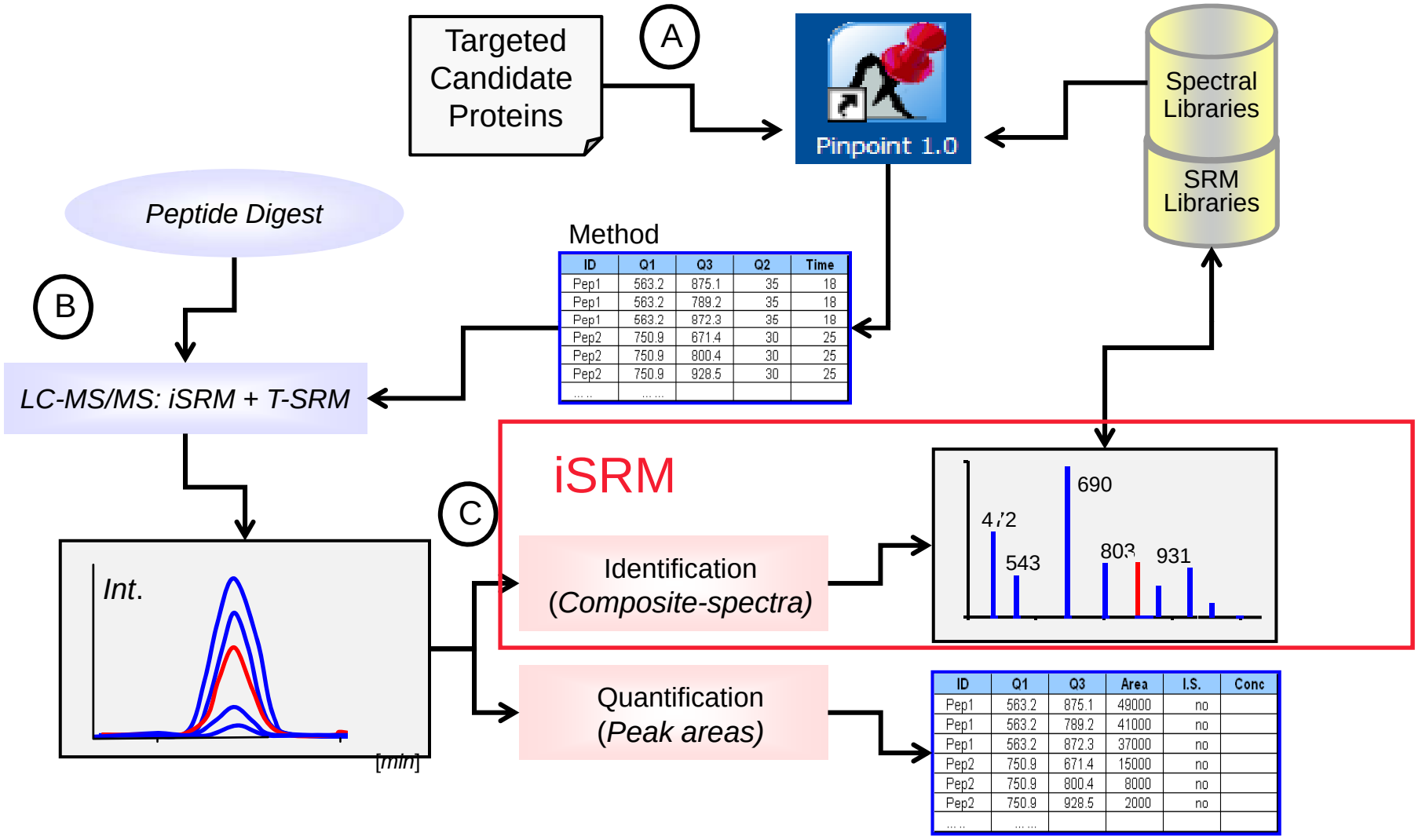
ThermoFisher SCIENTIFIC

iSRM – An advantage for verifying low level targets in biological matrices



ThermoFisher SCIENTIFIC

Pinpoint software is integrated with iSRM workflow for automate assay design and data processing



ID	Q1	Q3	Q2	Time
Pep1	563.2	875.1	35	18
Pep1	563.2	789.2	35	18
Pep1	563.2	872.3	35	18
Pep2	750.9	671.4	30	25
Pep2	750.9	800.4	30	25
Pep2	750.9	928.5	30	25
...	...			

ID	Q1	Q3	Area	I.S.	Conc
Pep1	563.2	875.1	49000	no	
Pep1	563.2	789.2	41000	no	
Pep1	563.2	872.3	37000	no	
Pep2	750.9	671.4	15000	no	
Pep2	750.9	800.4	8000	no	
Pep2	750.9	928.5	2000	no	
...	...				

What can iSRM and Pinpoint software provide you?

The combination of Pinpoint and iSRM provides the capability to simultaneously verify and quantitate up to 1000 targeted peptides in a single HPLC/MS/MS experiment.

- Rapid SRM assay design and refinement for the targeted protein quantification based on the discovery data.

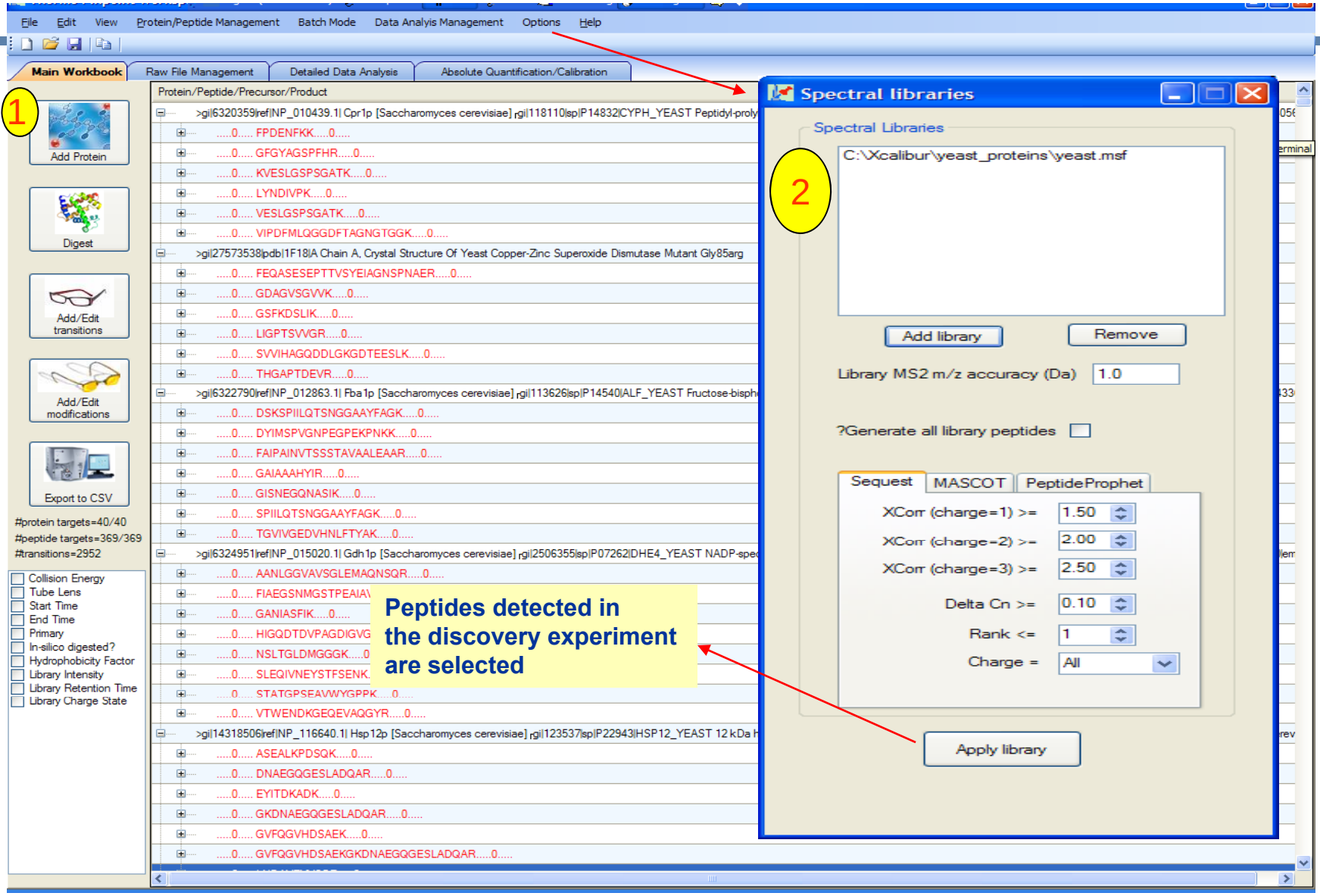
- Provide high throughput screening methods to narrow down a large number of candidate biomarkers coming out of discovery to the most promising few.

- Provide high throughput screening methods for signaling pathway studies.

ThermoFisher SCIENTIFIC

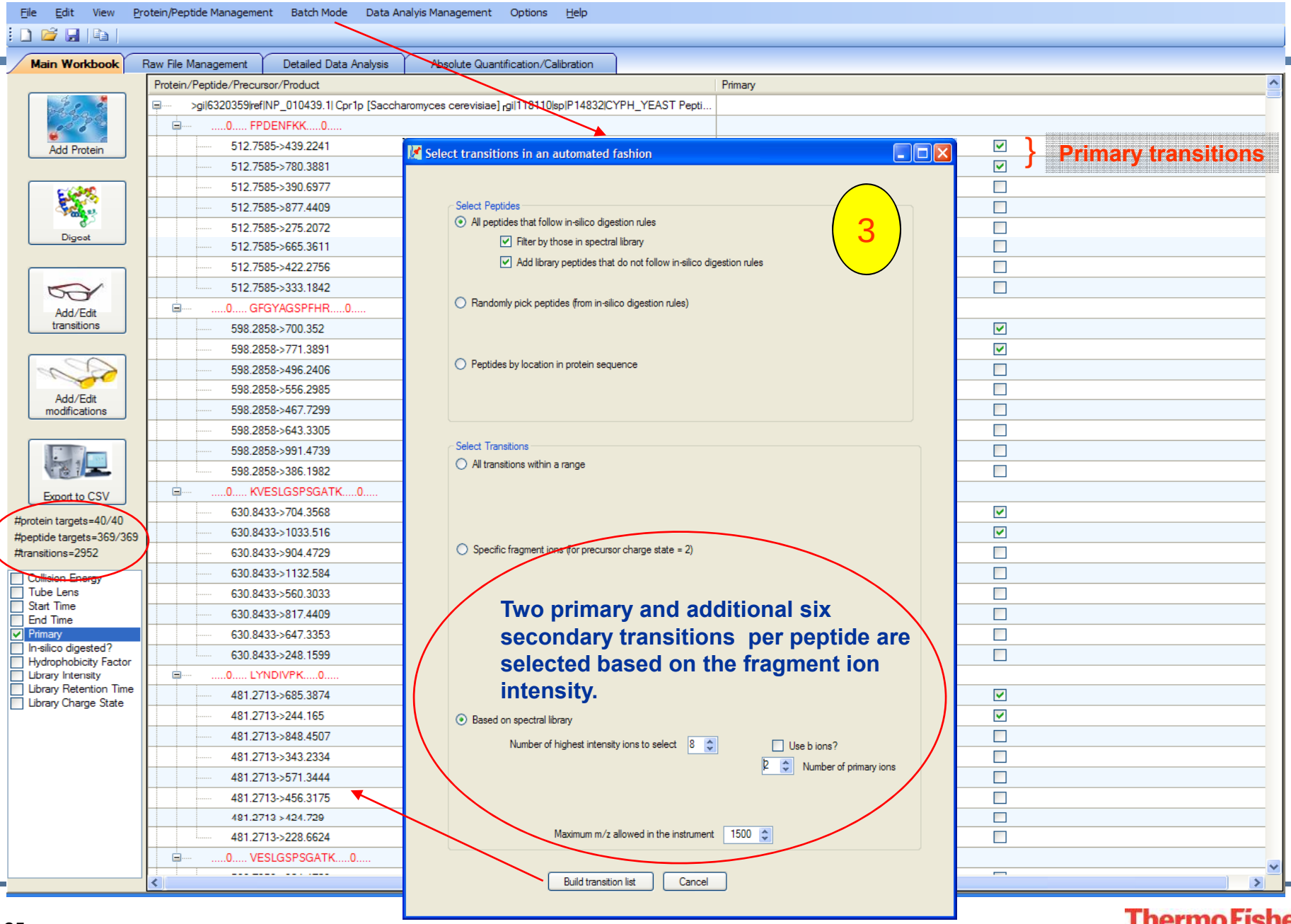
- **Rapid SRM assay development and refinement by using iSRM**
  - Targeting 40 known yeast proteins

Initial peptide selection based on previous proteomics discovery data



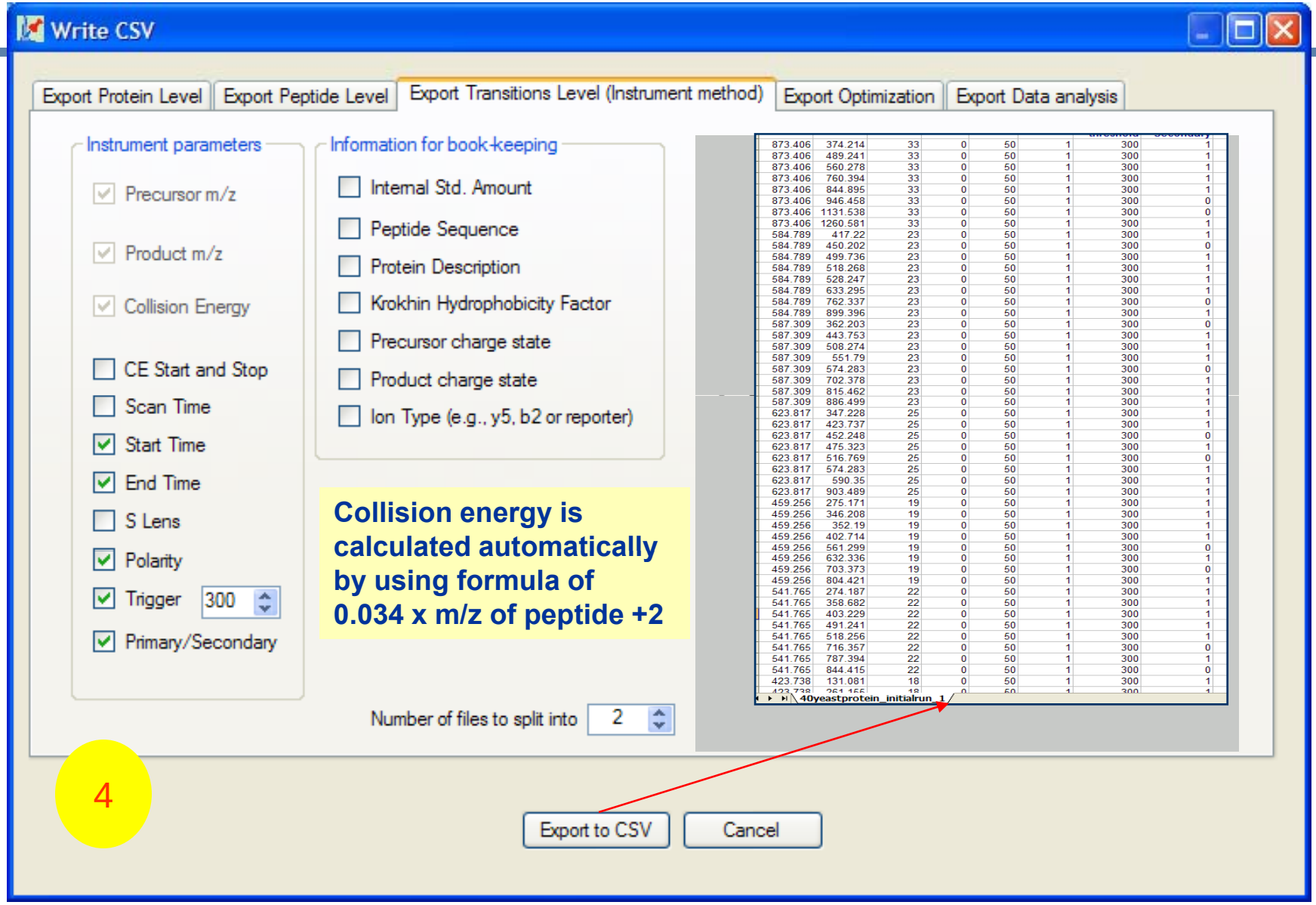
ThermoFisher SCIENTIFIC

Automatic transition selection based on the MS/MS spectral library



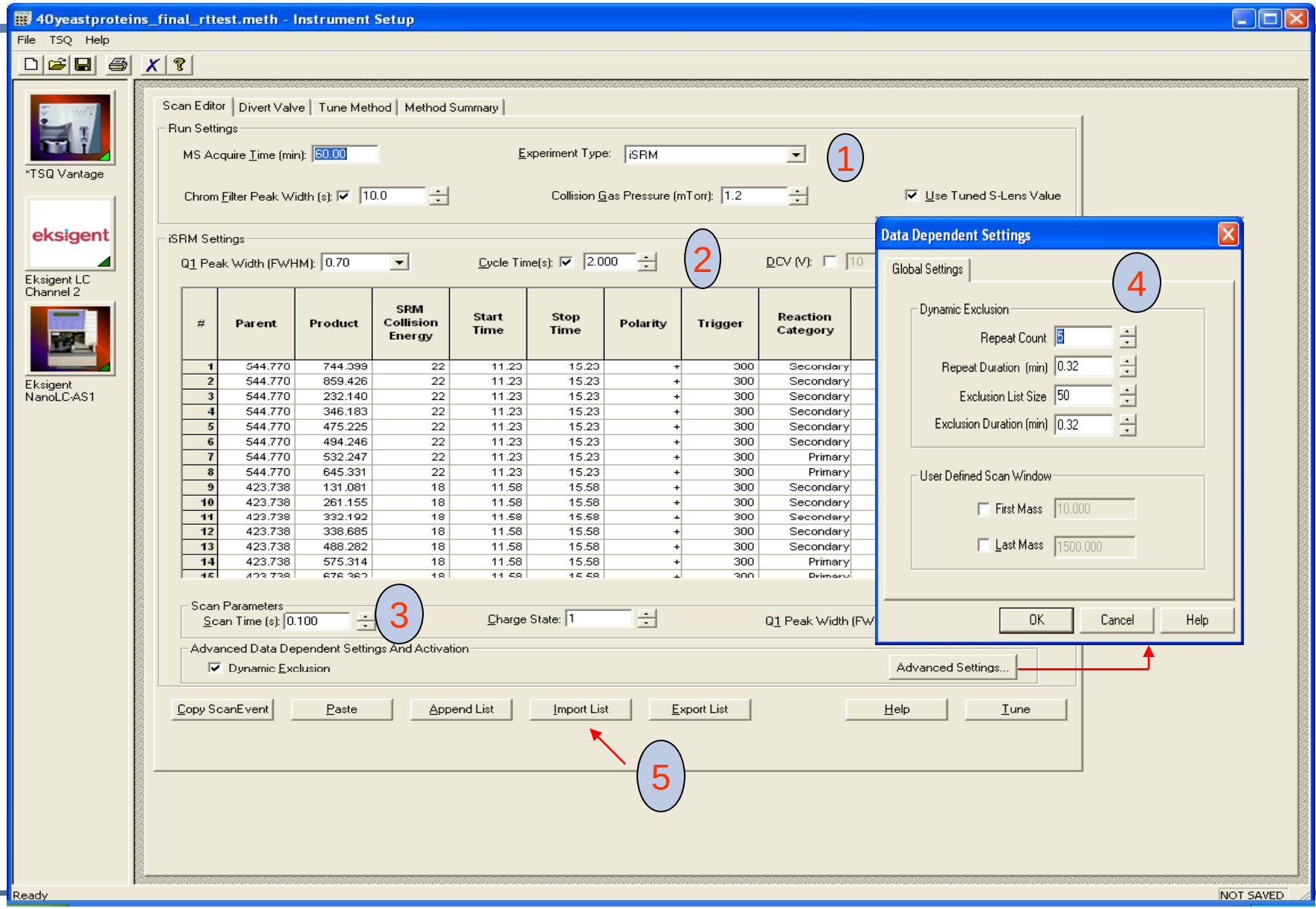
ThermoFisher SCIENTIFIC

Export initial transitions to csv file for generating the initial iSRM method



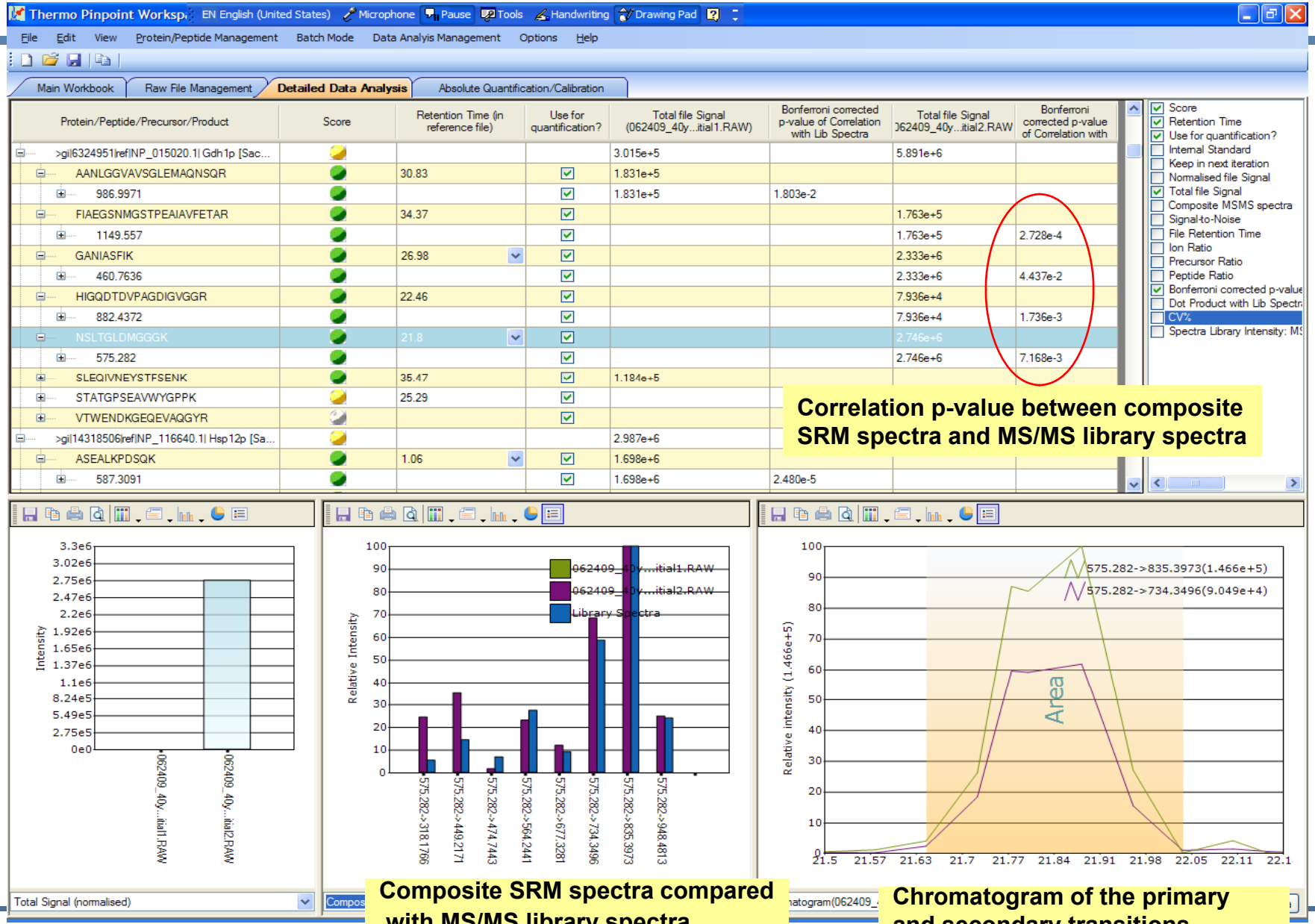
ThermoFisher SCIENTIFIC

iSRM instrument method set up

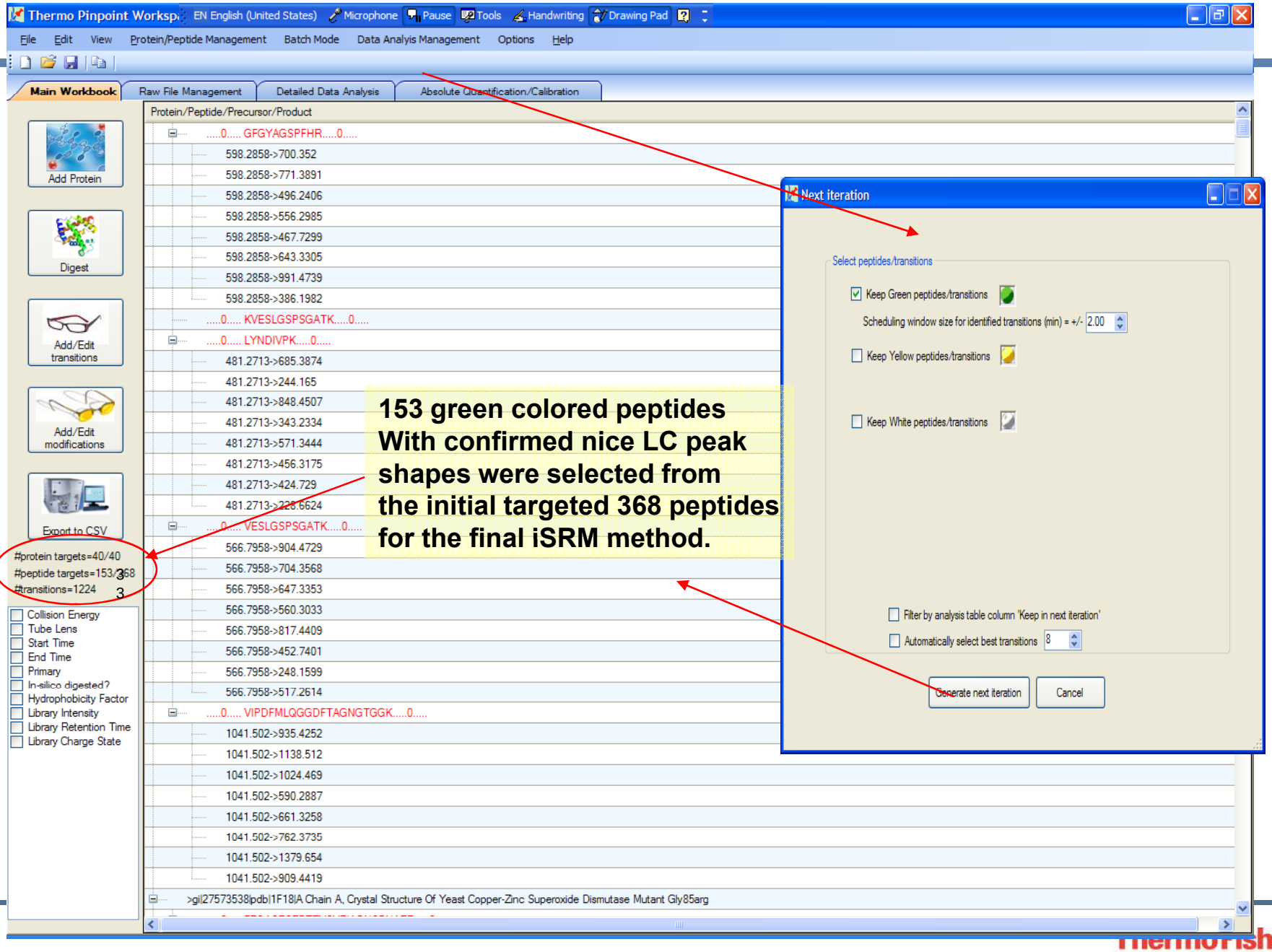


ThermoFisher SCIENTIFIC

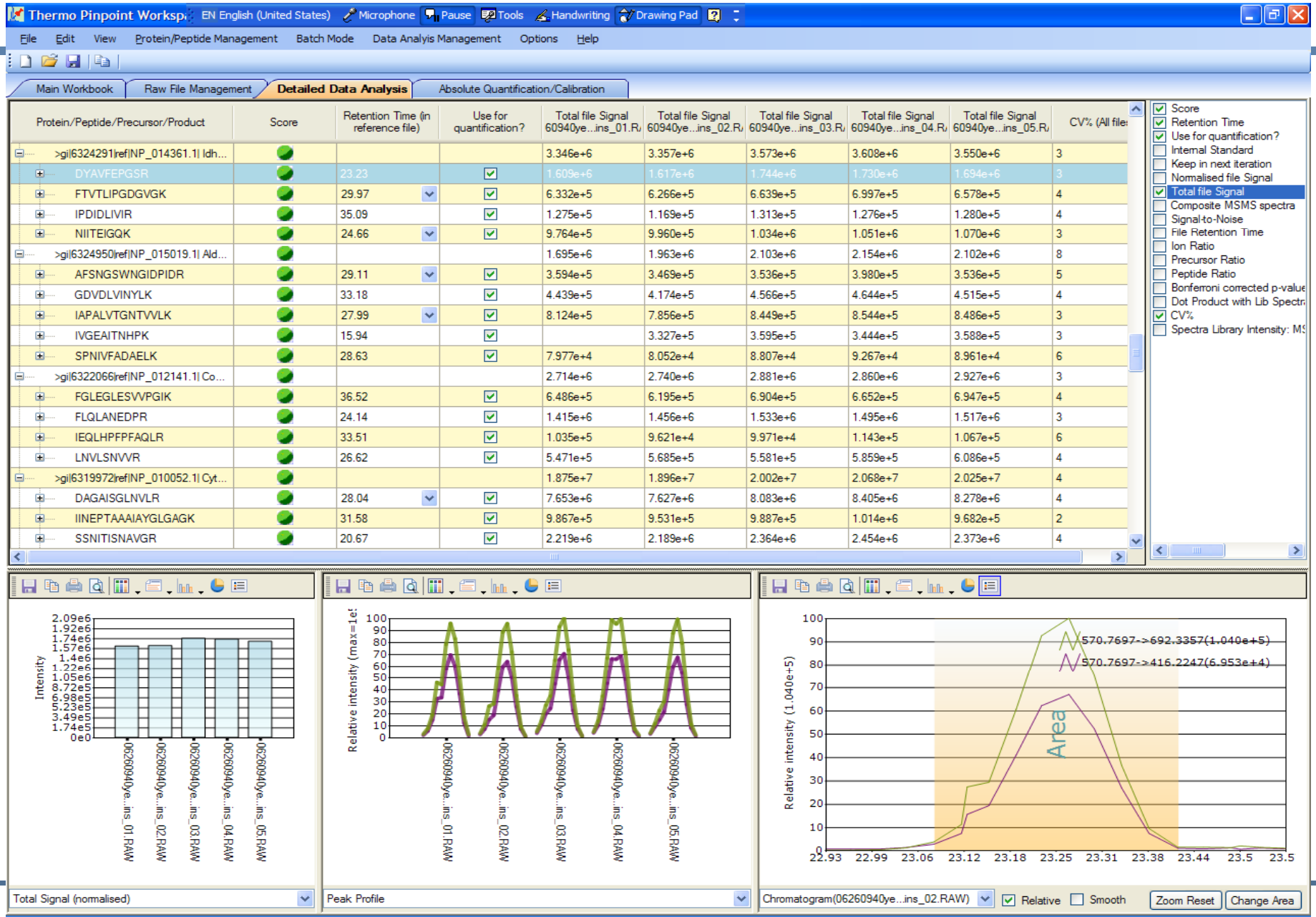
Peptide sequence verification and quantitation from the initial SRM assay



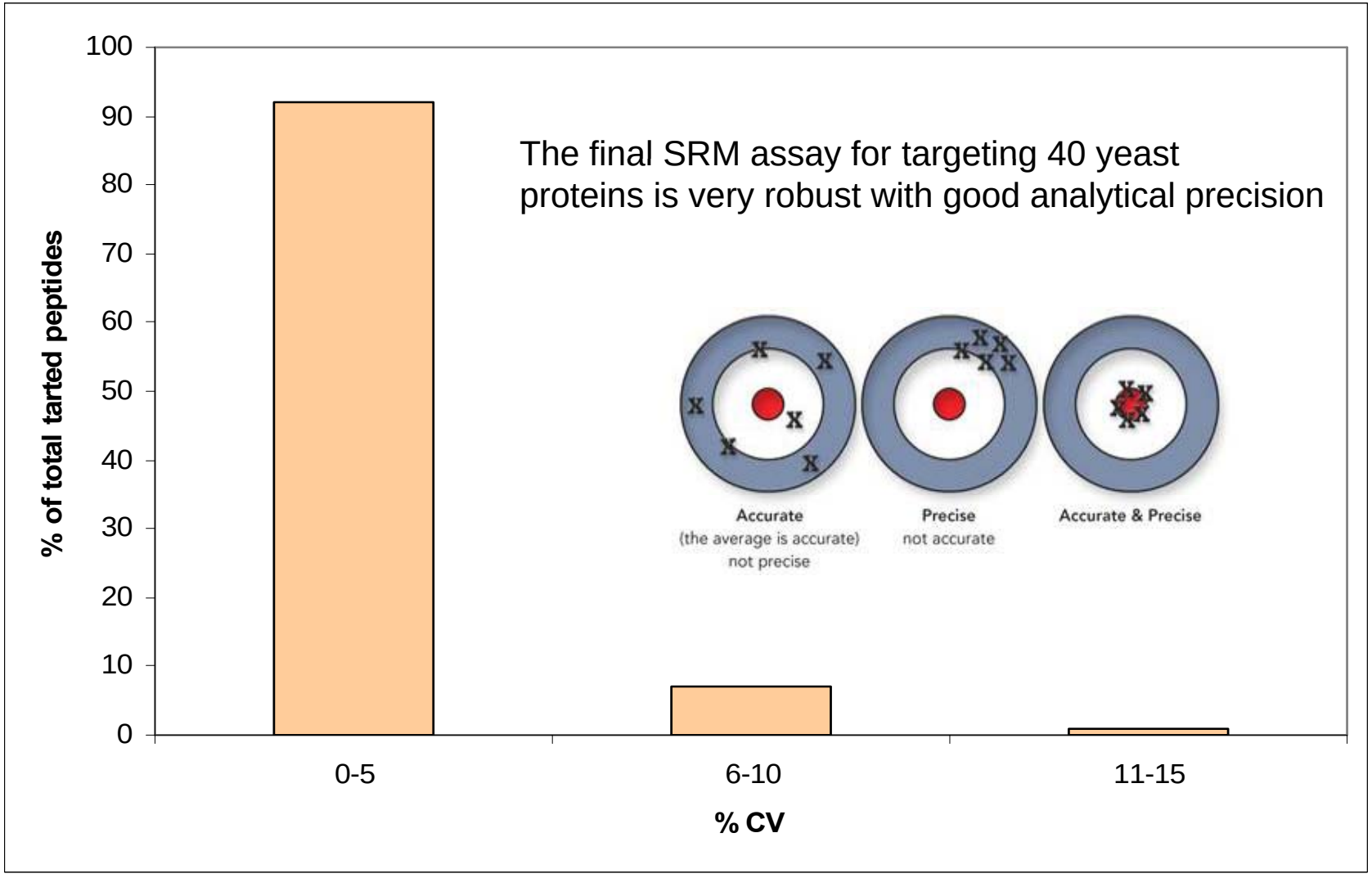
SRM method refinement based on the initial SRM assay data



Peptide sequence verification and quantitative precision from the refined iSRM assay

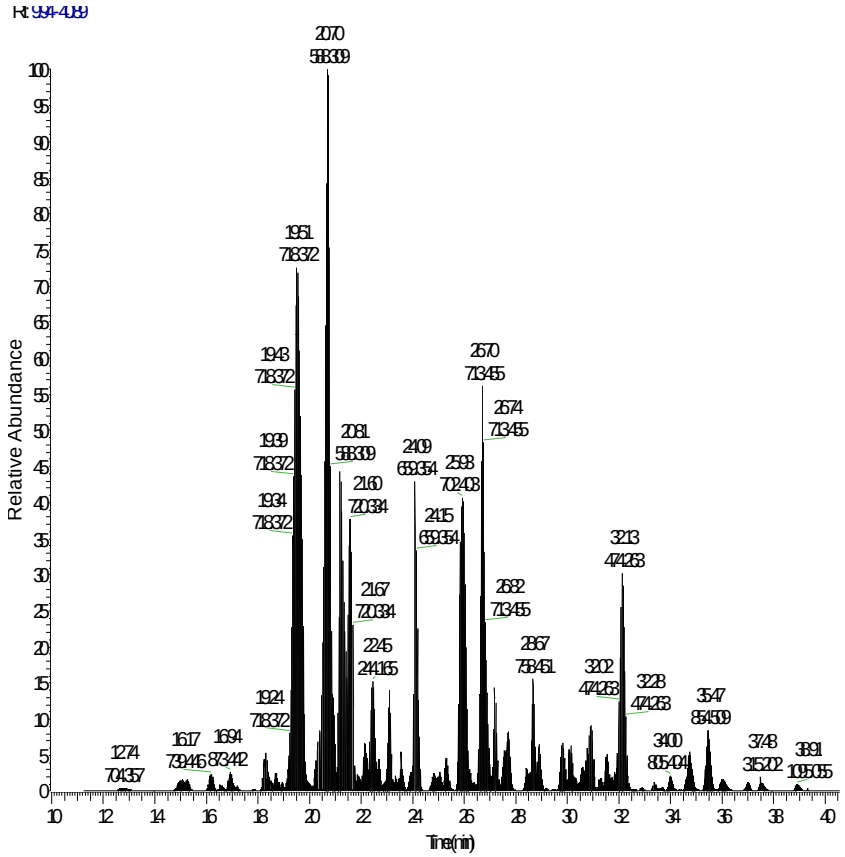


CV percentage for targeting 153 verified peptides using iSRM



ThermoFisher SCIENTIFIC

A robust SRM assay for quantitation of 40 targeted yeast proteins was developed in a few short hours



Step 1- Select Target Peptides:

369 peptides/2952 transitions (738 primary, 2214 secondary) were selected by Pinpoint based on the discovery data (20 min).

Step 2- Acquire data:

Data acquisition using above initial SRM assay (60 min).

Step 3 – Verify data & optimize conditions:

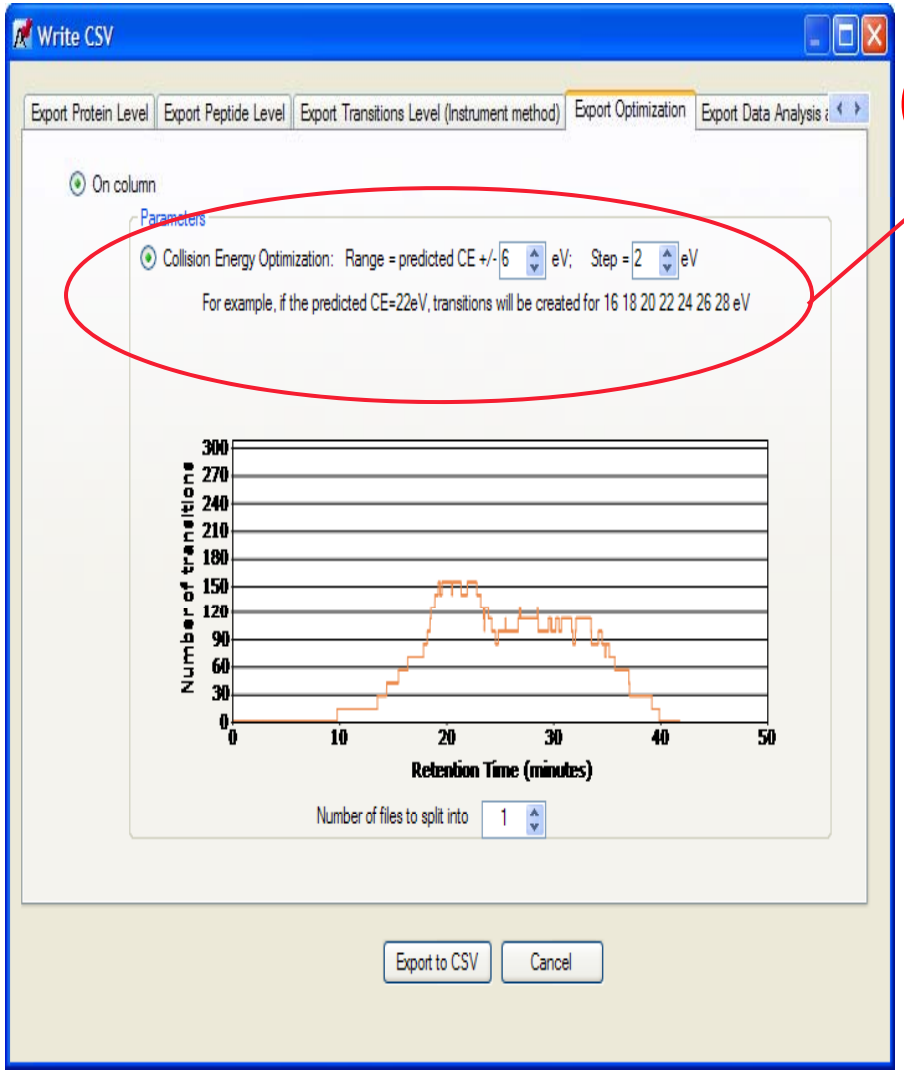
Data verification and generate optimized final SRM assay which kept only peptides that were confidently identified by composite MS/MS data and gave strong signal intensity. 153 peptides/1224 transitions (306 primary and 918 secondary) were retained in the final SRM assay (40 min) .

Step 4 – Validate final assay:

Validate the robustness and precision of final SRM assay by triplicate acquisition (60 min each, total 180 min).

ThermoFisher SCIENTIFIC

Additional sensitivity can be obtained by optimizing collision energy in a single injection



568.7904	341.116	16	9.8	29.8
568.7904	341.118	18	9.8	29.8
568.7904	341.12	20	9.8	29.8
568.7904	341.122	22	9.8	29.8
568.7904	341.124	24	9.8	29.8
568.7904	341.126	26	9.8	29.8
568.7904	341.128	28	9.8	29.8
568.7904	428.216	16	9.8	29.8
568.7904	428.218	18	9.8	29.8
568.7904	428.22	20	9.8	29.8
568.7904	428.222	22	9.8	29.8
568.7904	428.224	24	9.8	29.8
568.7904	428.226	26	9.8	29.8
568.7904	428.228	28	9.8	29.8
568.7904	434.216	16	9.8	29.8
568.7904	434.218	18	9.8	29.8
568.7904	434.22	20	9.8	29.8
568.7904	434.222	22	9.8	29.8
568.7904	434.224	24	9.8	29.8
568.7904	434.226	26	9.8	29.8
568.7904	434.228	28	9.8	29.8
568.7904	490.716	16	9.8	29.8
568.7904	490.718	18	9.8	29.8
568.7904	490.72	20	9.8	29.8
568.7904	490.722	22	9.8	29.8
568.7904	490.724	24	9.8	29.8
568.7904	490.726	26	9.8	29.8
568.7904	490.728	28	9.8	29.8
568.7904	557.216	16	9.8	29.8
568.7904	557.218	18	9.8	29.8
568.7904	557.22	20	9.8	29.8
568.7904	557.222	22	9.8	29.8
568.7904	557.224	24	9.8	29.8
568.7904	557.226	26	9.8	29.8
568.7904	557.228	28	9.8	29.8
568.7904	720.316	16	9.8	29.8
568.7904	720.318	18	9.8	29.8
568.7904	720.32	20	9.8	29.8
568.7904	720.322	22	9.8	29.8
568.7904	720.324	24	9.8	29.8
568.7904	720.326	26	9.8	29.8
568.7904	720.328	28	9.8	29.8
568.7904	867.316	16	9.8	29.8
568.7904	867.318	18	9.8	29.8
568.7904	867.32	20	9.8	29.8
568.7904	867.322	22	9.8	29.8
568.7904	867.324	24	9.8	29.8
568.7904	867.326	26	9.8	29.8
568.7904	867.328	28	9.8	29.8

ThermoFisher SCIENTIFIC

Retention time shift correction on-the-fly provides robust quantitative results across different columns and instruments

C:\Xcalibur\...\heavypep_25fmolmatrix05 9/1/2009 2:46:42 PM
heavy peptides,25fmol in matrix, 400nl

heavypep_25fmolmatrix05
TSQ Vantage

Experiment Type: iSRM
Chrom Filter Peak Width (s): 10.0
Collision Gas Pressure (mTorr): 1.2
Use Tuned S-Lens Value: Yes
Q1 Peak Width (FWHM): 0.70
Display Time Range for SRM table: Yes
Cycle Time (s): 2.000
DCV (V): Not used

SRM Table:

Parent	Product	CE	Start	Stop	Pol	Trigger	React Cat	Name
609.310	347.228	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
609.310	524.257	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
609.310	532.308	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
609.310	660.367	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
609.310	717.388	24	13.65	21.65	+	1.000e+03	Primary	(empty)
609.310	846.431	24	13.65	21.65	+	1.000e+03	Primary	(empty)
609.310	960.474	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
609.310	1047.506	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
613.317	355.243	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
613.317	528.264	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
613.317	540.323	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
613.317	668.381	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
613.317	725.403	24	13.65	21.65	+	1.000e+03	Reference	(empty)
613.317	854.445	24	13.65	21.65	+	1.000e+03	Reference	(empty)
613.317	968.488	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
613.317	1055.520	24	13.65	21.65	+	1.000e+03	Secondary	(empty)
521.793	451.266	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
521.793	588.325	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
521.793	659.362	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
521.793	730.399	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
521.793	801.436	22	20.33	24.33	+	1.000e+03	Primary	(empty)
521.793	914.520	22	20.33	24.33	+	1.000e+03	Primary	(empty)
521.793	985.557	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
526.797	461.274	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
526.797	598.333	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
526.797	669.370	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
526.797	740.407	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
526.797	811.444	22	20.33	24.33	+	1.000e+03	Reference	(empty)
526.797	924.528	22	20.33	24.33	+	1.000e+03	Reference	(empty)
526.797	995.565	22	20.33	24.33	+	1.000e+03	Secondary	(empty)
769.888	376.219	30	24.75	28.75	+	1.000e+03	Secondary	(empty)

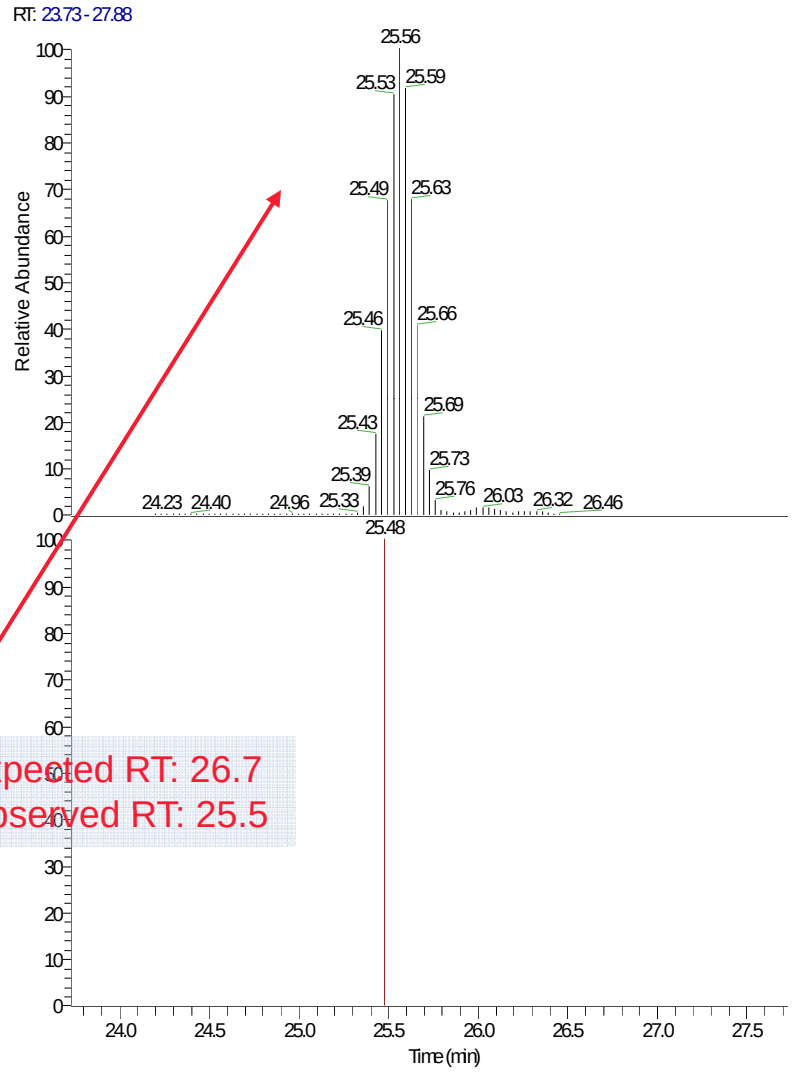
The TSQ series of triple quandrupole instruments allows user to assign single or multiple peaks as RT reference compounds. With the changes of chromatographic conditions, the retention time shifts from these reference compounds are captured and a linear regression curve will be generated on-the-fly that provides an offset and slope corredction to the tSRM windows of the compounds eluted afterwards. This approach enabels the use of narrow timed-SRM window without missing peaks due to retention time shifts, caused by column/mobile phase replacement, switching instrument, column clog or changing gradient conditions.

Targeted peak was detected by correcting the RT shift on the fly
The time shift was created by increasing the flow rate from 300 nl/min to 400 nl/min

heavypep_25fmolmatrix_2minwindow 9/1/2009 4:14:00 PM
heavy peptides,25fmol in matrix, 400nl

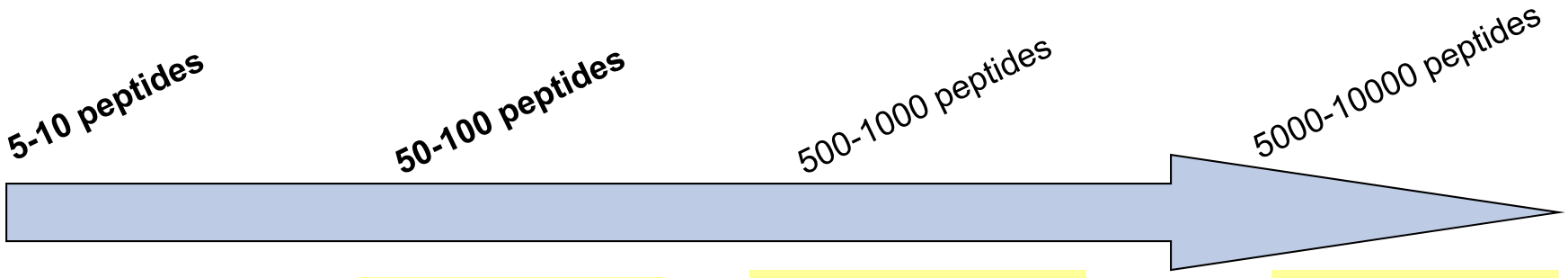
heavypep_25fmolmatrix_2minwindow
TSQ Vantage

613.317	854.445	24	11.65	23.65	+ 1.000e+03	Reference
613.317	968.488	24	11.65	23.65	+ 1.000e+03	Secondary
613.317	1055.520	24	11.65	23.65	+ 1.000e+03	Secondary
521.793	451.266	22	21.33	23.33	+ 1.000e+03	Secondary
521.793	588.325	22	21.33	23.33	+ 1.000e+03	Secondary
521.793	659.362	22	21.33	23.33	+ 1.000e+03	Secondary
521.793	730.399	22	21.33	23.33	+ 1.000e+03	Secondary
521.793	801.436	22	21.33	23.33	+ 1.000e+03	Primary
521.793	914.520	22	21.33	23.33	+ 1.000e+03	Primary
521.793	985.557	22	21.33	23.33	+ 1.000e+03	Secondary
526.797	461.274	22	21.33	23.33	+ 1.000e+03	Secondary
526.797	598.333	22	21.33	23.33	+ 1.000e+03	Secondary
526.797	669.370	22	21.33	23.33	+ 1.000e+03	Secondary
526.797	740.407	22	21.33	23.33	+ 1.000e+03	Secondary
526.797	811.444	22	21.33	23.33	+ 1.000e+03	Reference
526.797	924.528	22	21.33	23.33	+ 1.000e+03	Reference
526.797	995.565	22	21.33	23.33	+ 1.000e+03	Secondary
769.888	376.219	30	25.75	27.75	+ 1.000e+03	Secondary
769.888	489.303	30	25.75	27.75	+ 1.000e+03	Secondary
769.888	652.366	30	25.75	27.75	+ 1.000e+03	Secondary
769.888	753.414	30	25.75	27.75	+ 1.000e+03	Secondary
769.888	868.441	30	25.75	27.75	+ 1.000e+03	Primary
769.888	967.509	30	25.75	27.75	+ 1.000e+03	Secondary
769.888	1024.530	30	25.75	27.75	+ 1.000e+03	Primary
769.888	1111.562	30	25.75	27.75	+ 1.000e+03	Secondary
769.888	1296.642	30	25.75	27.75	+ 1.000e+03	Secondary
773.896	384.233	30	25.75	27.75	+ 1.000e+03	Secondary
773.896	497.317	30	25.75	27.75	+ 1.000e+03	Secondary
773.896	660.380	30	25.75	27.75	+ 1.000e+03	Secondary
773.896	761.428	30	25.75	27.75	+ 1.000e+03	Secondary
773.896	876.455	30	25.75	27.75	+ 1.000e+03	Primary
773.896	975.523	30	25.75	27.75	+ 1.000e+03	Secondary
773.896	1032.545	30	25.75	27.75	+ 1.000e+03	Primary
773.896	1119.577	30	25.75	27.75	+ 1.000e+03	Secondary
773.896	1304.657	30	25.75	27.75	+ 1.000e+03	Secondary
796.407	675.385	30	29.46	31.46	+ 1.000e+03	Secondary
796.407	762.337	30	29.46	31.46	+ 1.000e+03	Secondary
796.407	861.406	30	29.46	31.46	+ 1.000e+03	Secondary
796.407	918.427	30	29.46	31.46	+ 1.000e+03	Primary
796.407	1005.459	30	29.46	31.46	+ 1.000e+03	Secondary
796.407	1062.480	30	29.46	31.46	+ 1.000e+03	Primary
796.407	1161.549	30	29.46	31.46	+ 1.000e+03	Secondary



ThermoFisher SCIENTIFIC

Scaling Options using iSRM and Pinpoint



Regular multiple SRM	Scheduled SRM (tSRM) up to 3000 transitions	tSRM + iSRM (up to 16000 transitions	tSRM + iSRM + Split-n-stitch

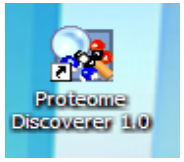
Automated scoring schemes to help prioritize large analysis into high, medium, low quality bins

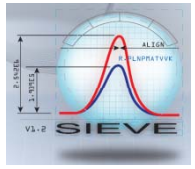
And more…

- **Single software to help iterative method building to go from protein list to absolute abundance**
- **Multi-threaded**
- **Extremely easy data and results sharing**
- **Customers can give video feedback**
- **Video help tutorials to get you started**

Optimizing assays for specificity & sensitivity

Bioworks
Proteome Discoverer
Mascot
SIEVE
BiblioSpec
PeptideAtlas

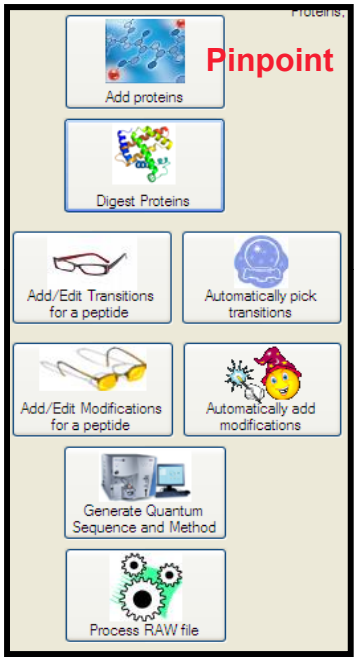




Build the SRM Assay

Acquire the Raw Files

SRM
H-SRM
T-SRM
iSRM



Process the Raw Files

Verify Targeted Peptide Information

Ion Ratio Calc.
Costas-Soras
P-Value
Heavy Labeled Peptides

Generate Reports

Protein Level
Peptide Level

Refine the Instrument Method

T-SRM, CE optimization
Heavy peptides
Protein Panels

ThermoFisher SCIENTIFIC

Summary

- Targeted protein quantitaiton using triple quadrupole mass spectrometers is complimentary to traditional western blots
- Thermo Fisher Scientific's workflow incorporates iSRM to facilitate rapid and robust method development for multiplexed assays.
- Pinpoint software allows initial SRM/iSRM assay to be designed automatically by using discovery MS/MS data or theoretical prediction. It also offers unique peptide verification scheme for SRM assay refinement.
- Thermo Fisher Scientific's workflow provides high-throughput peptide screens by simultaneously verifying and quantitating large number of peptides in a single HPLC MS/MS run.