Data Independent Acquisition (DIA)

Early Days in the Application of DIA

SWATH Presentation from 2014 – Early Days for DIA
Data Independent Acquisition

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MS/MS^{ALL} WORKFLOW WITH SWATH™ ACQUISITION

TARGETED PROTEIN QUANTITATION

Original Presentation - 2014

AB SCIEX TRIPLETOF® 5600$^+$ SYSTEM

AB SCIEX TripleTOF® 5600+ System is the fastest and most sensitive high-resolution mass spectrometer for high performance qualitative and quantitative analysis.

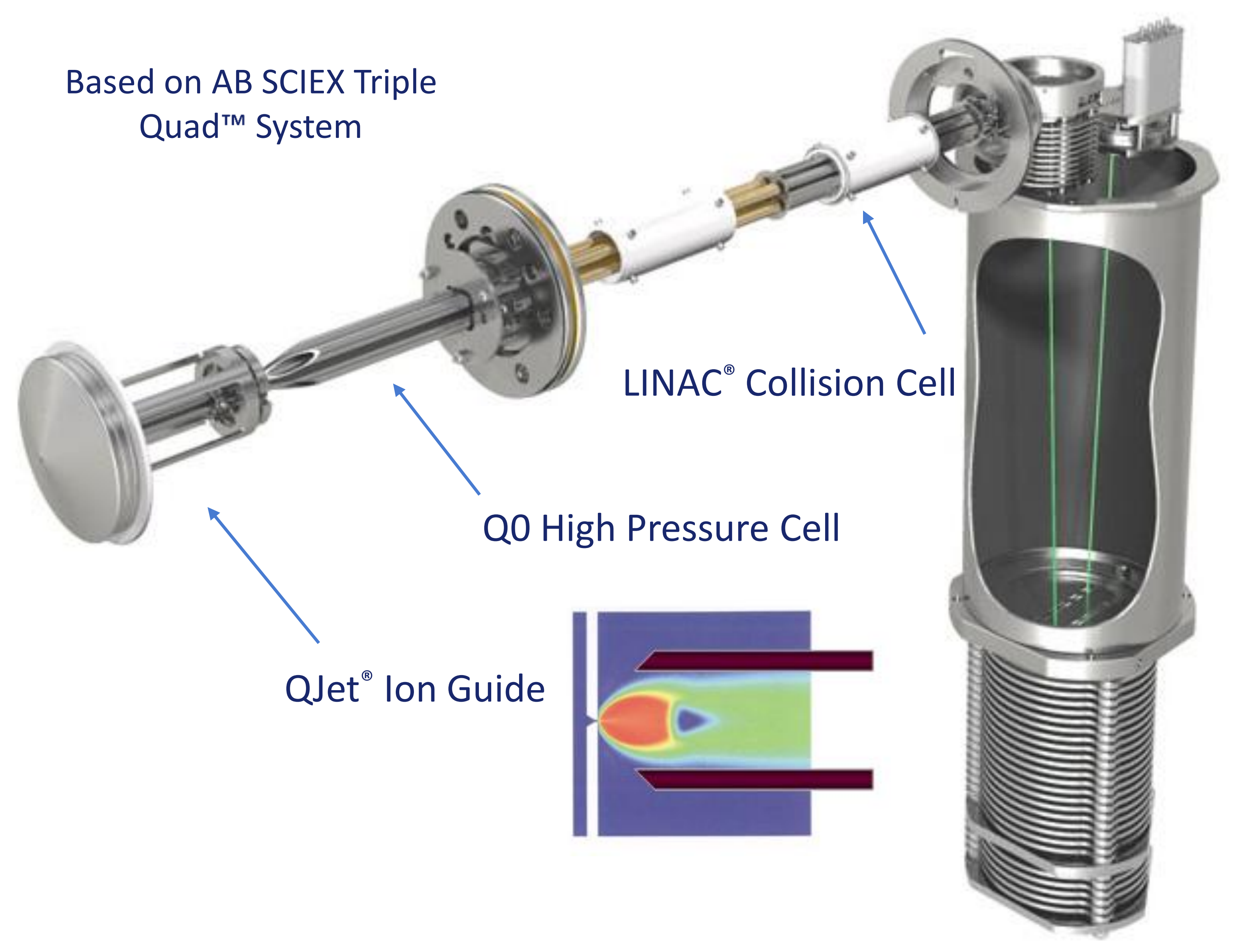
Key System Attributes:

- Sensitivity
- Speed
- Linear Dynamic Range
- Resolution
- Mass Accuracy
- Reliability

Original Presentation - 2014

AB SCIEX TRIPLETOF® 5600$^+$ SYSTEM

ION PATH INNOVATIONS – HIGH SENSITIVITY FRONT END



Based on AB SCIEX Triple Quad™ System

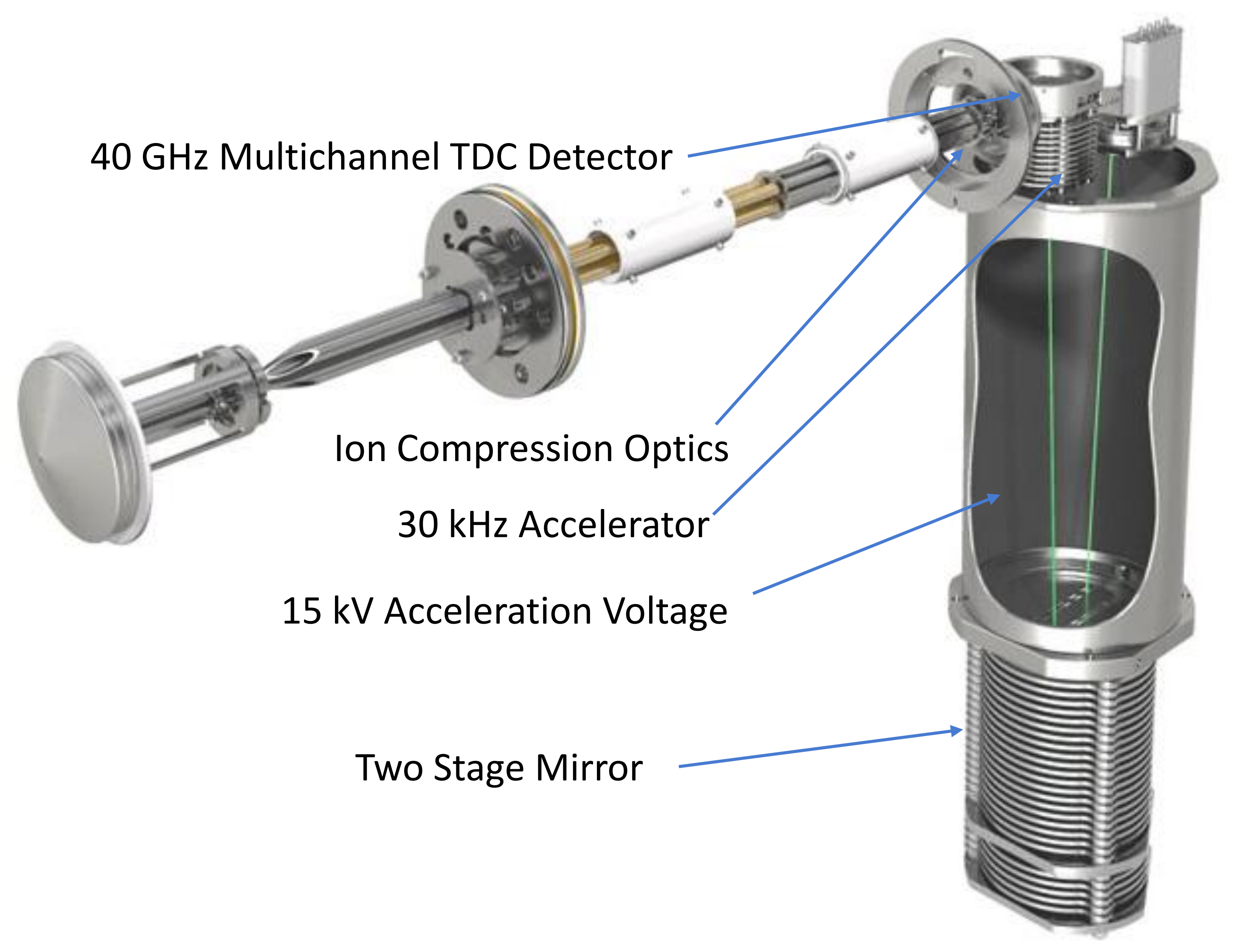
LINAC® Collision Cell

Q0 High Pressure Cell

QJet® Ion Guide

AB SCIEX TRIPLETOF® 5600+ SYSTEM

ION PATH INNOVATIONS – ACCELERATOR TOF™ ANALYZER



40 GHz Multichannel TDC Detector

Ion Compression Optics

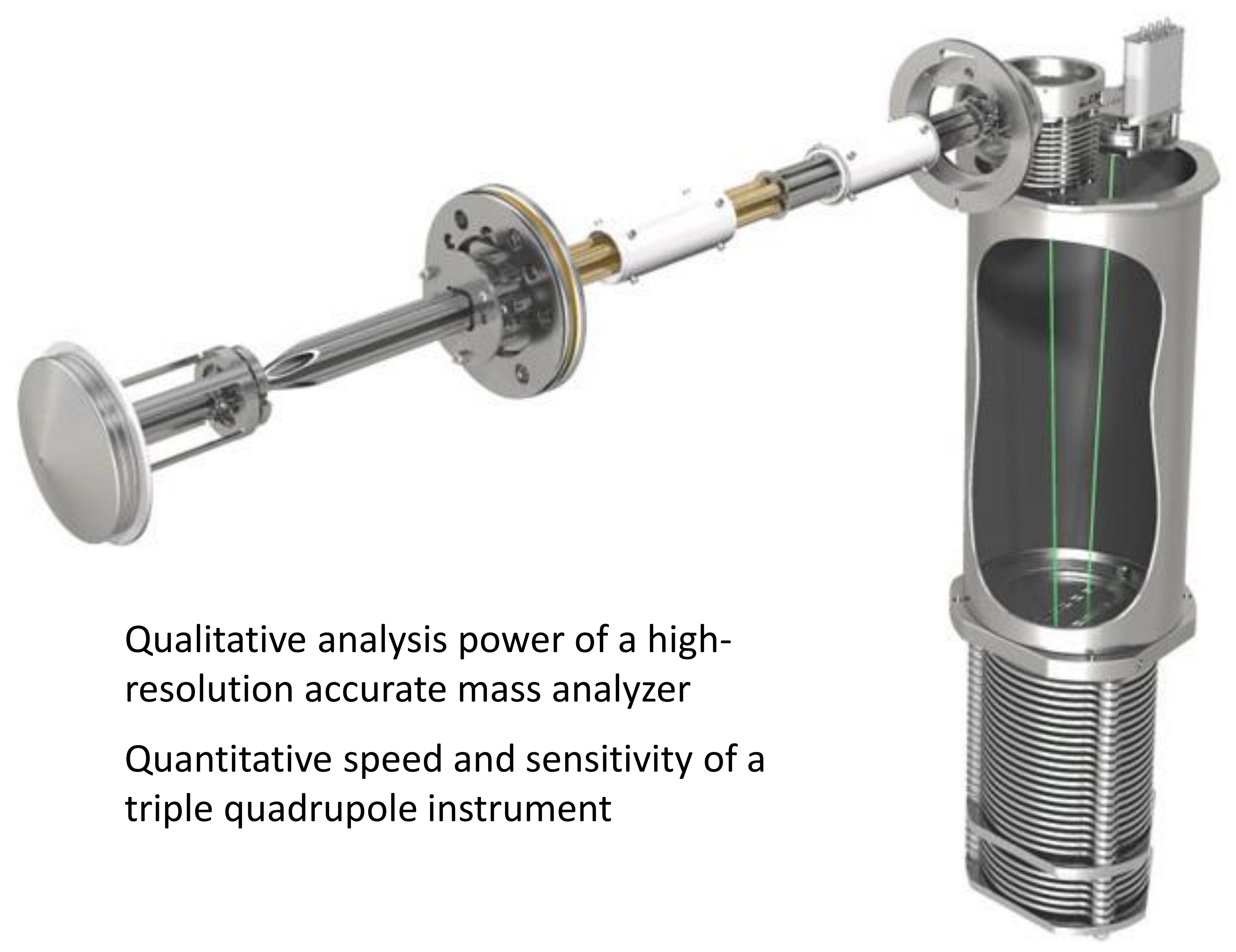
30 kHz Accelerator

15 kV Acceleration Voltage

Two Stage Mirror

AB SCIEX TRIPLETOF® 5600+ SYSTEM

ION PATH INNOVATIONS – THE BEST OF BOTH WORLDS



Qualitative analysis power of a high-resolution accurate mass analyzer

Quantitative speed and sensitivity of a triple quadrupole instrument

AB SCIEX TRIPLETOF® 5600+ SYSTEM

KEY FEATURES FOR TARGETED QUANTITATION

- MS/MS Sensitivity
  - Able to obtain high quality MS/MS on low level analytes
- MS/MS Speed
  - Up to 100 MS/MS in a second
- MS/MS Resolution:
  - High sensitivity mode ~15,000
  - High resolution mode ~30,000
  - Better mass accuracy and specificity
- Dynamic Range
  - ~3 - 4 orders

Original Presentation - 2014

MS/MS^{ALL}

Goal: Collect a MS and MS/MS spectrum at high resolution on every analyte in your sample

What does this enable?

Digital record of everything in your sample

Quantitation and confirmation of everything in the sample

Single method for acquiring all your data

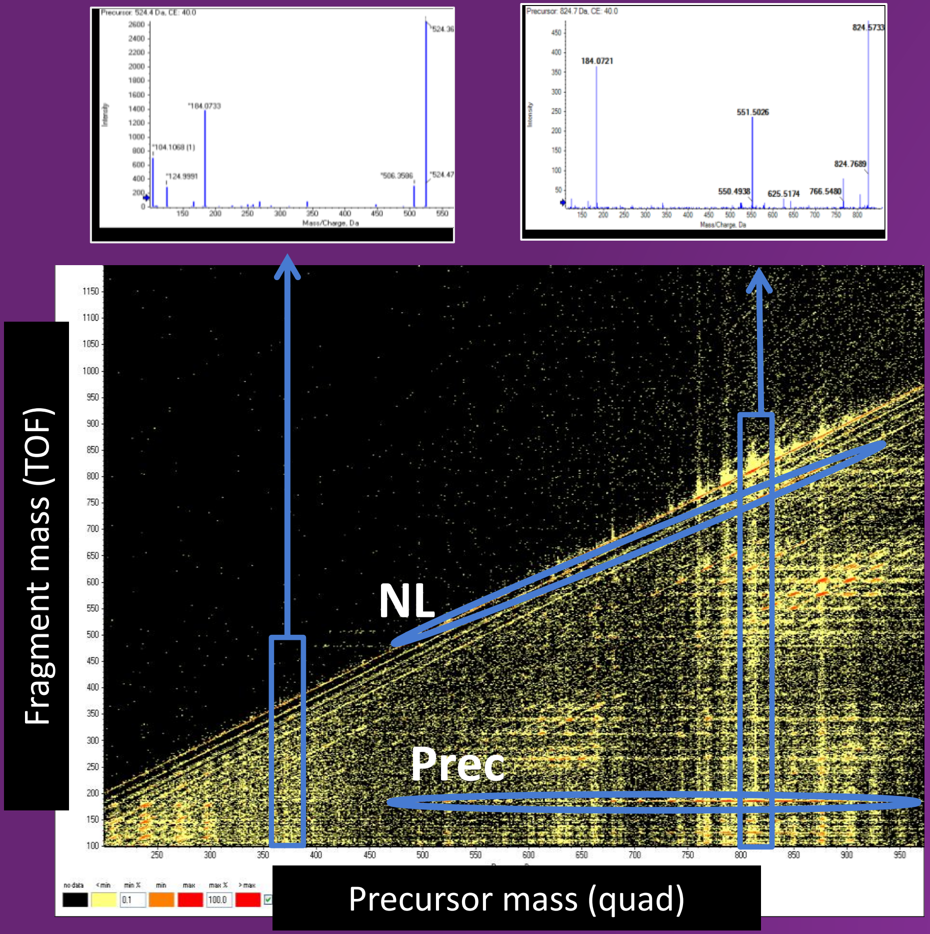
> “All truths are easy to understand once they are discovered; the point is to discover them” – Galileo Galilei

Original Presentation - 2014

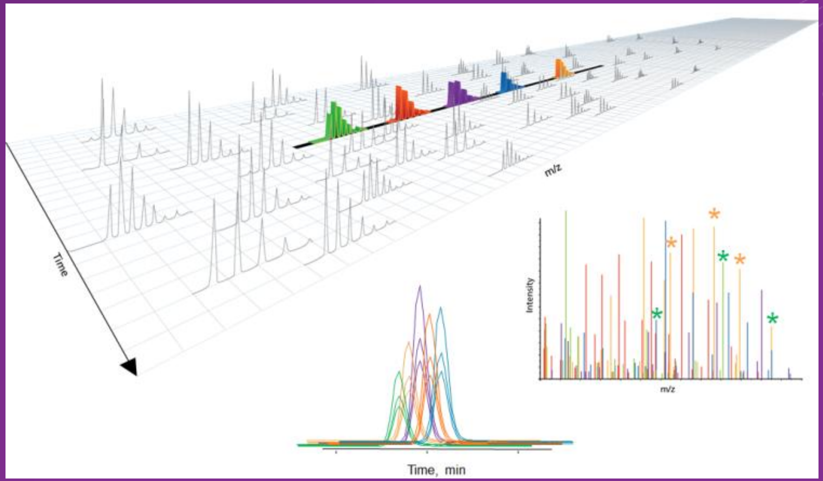
MS/MS^{ALL} WORKFLOWS

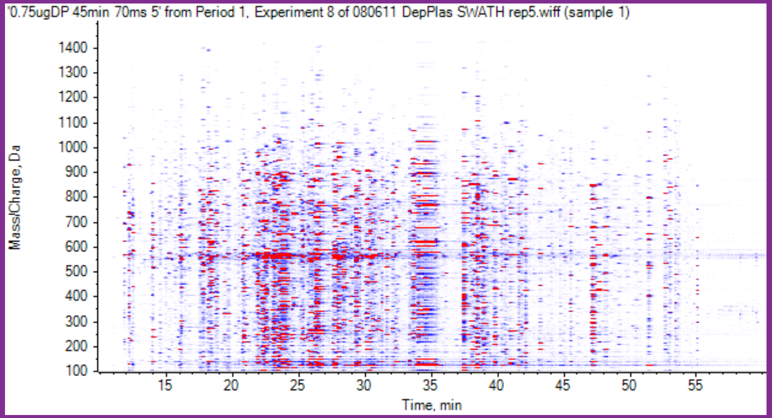
AB SCIEX DATA INDEPENDENT WORKFLOWS

Infusion MS/MS^{ALL} for Lipidomics



MS/MS^{ALL} with SWATH™ Acquisition for Proteomics





MS/MS^{ALL} WITH SWATH™ ACQUISITION

KEY WORKFLOW BENEFITS

1. Comprehensive quantitation
   - Comprehensive – acquires MS/MS of everything
   - Quantitation – ‘MRMs’ of everything

2. High quality quantitation – QQQ like
   - Excellent depth of coverage
   - High specificity

3. Easy quantitation
   - Single acquisition method – no method development
   - Re-analysis without re-acquisition

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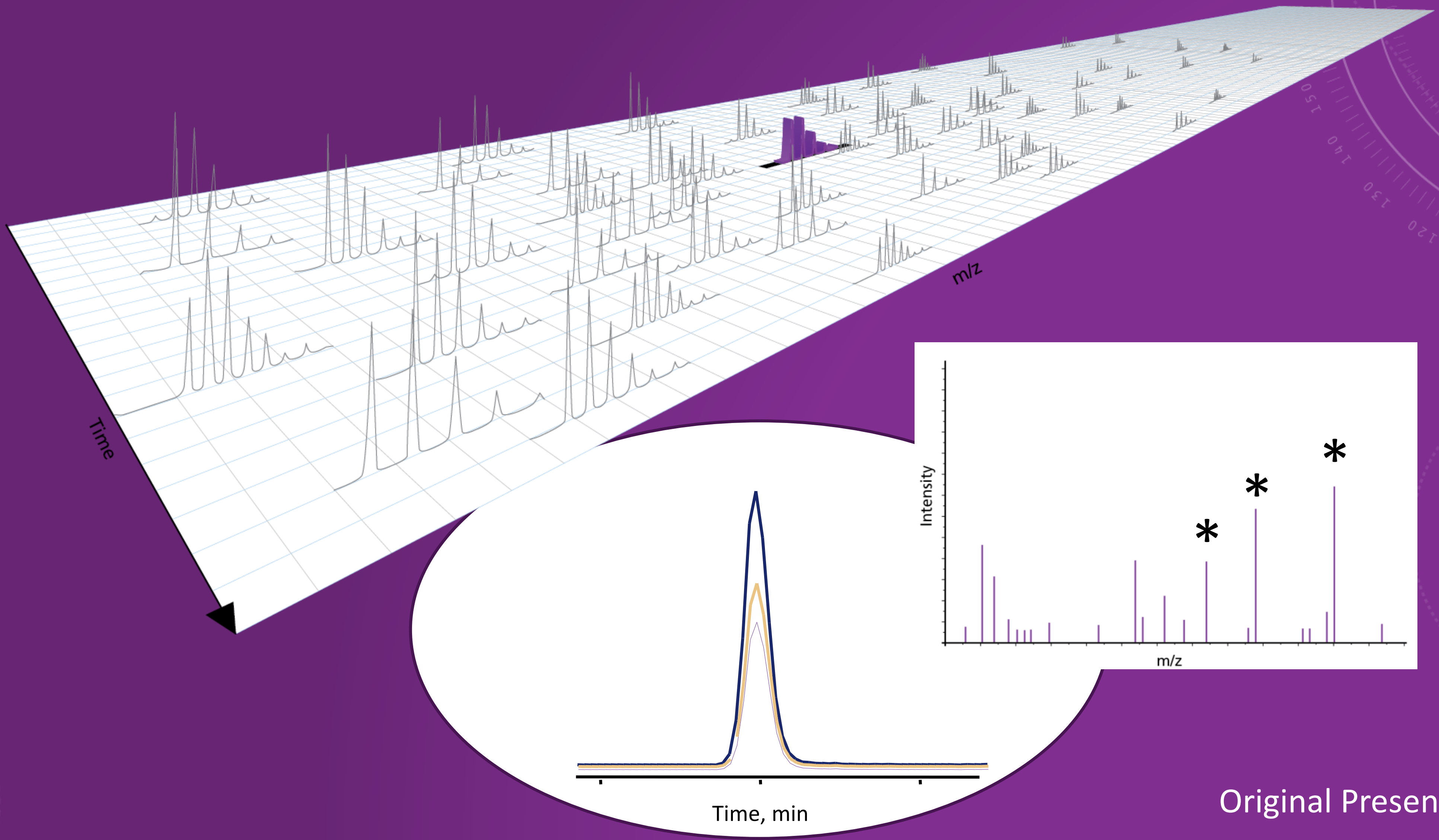
TRADITIONAL ACQUISITION STRATEGIES

IDA, MRM, MRM^{HR}



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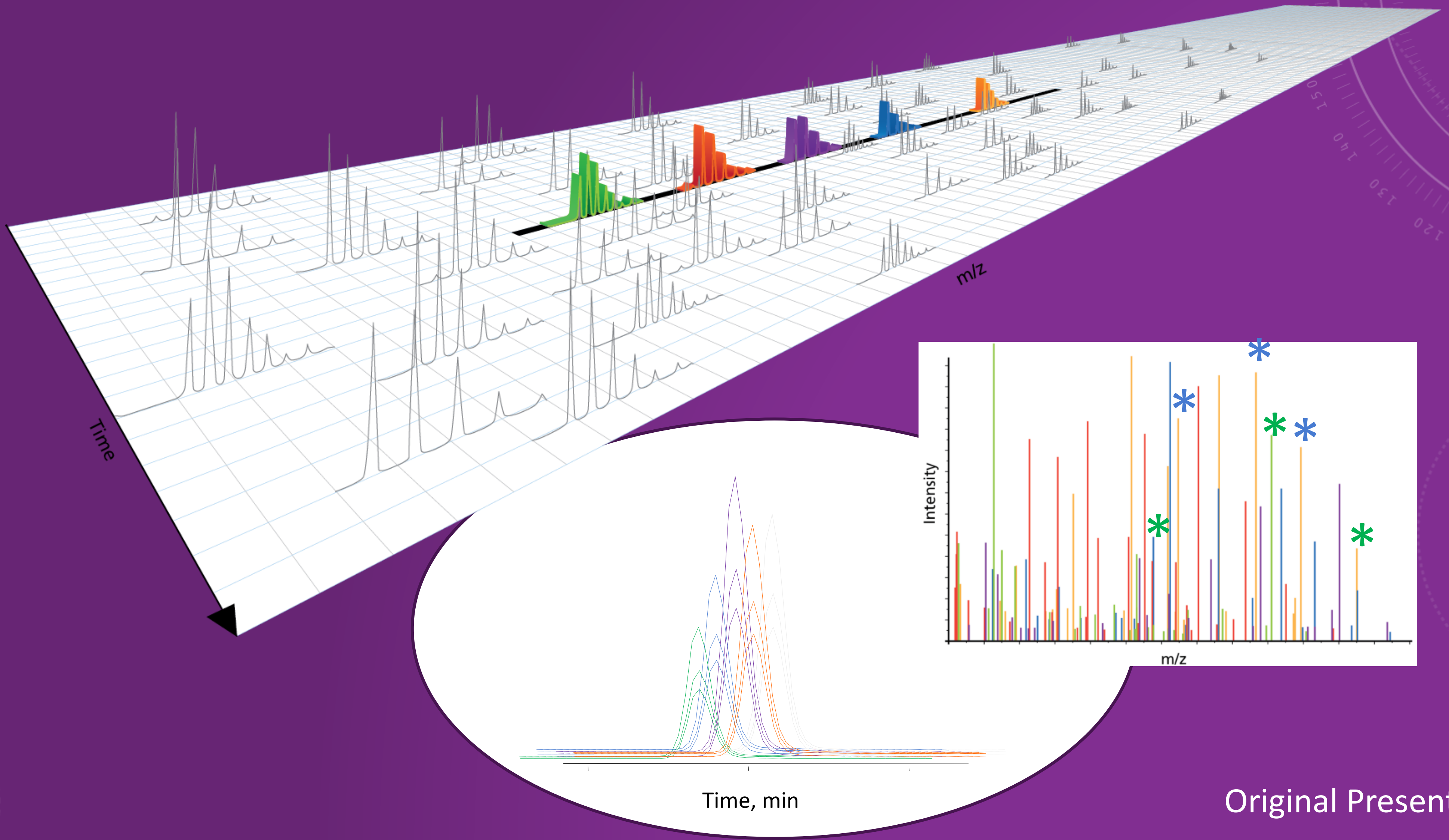
MS/MS^{ALL} WITH SWATH™ ACQUISITION



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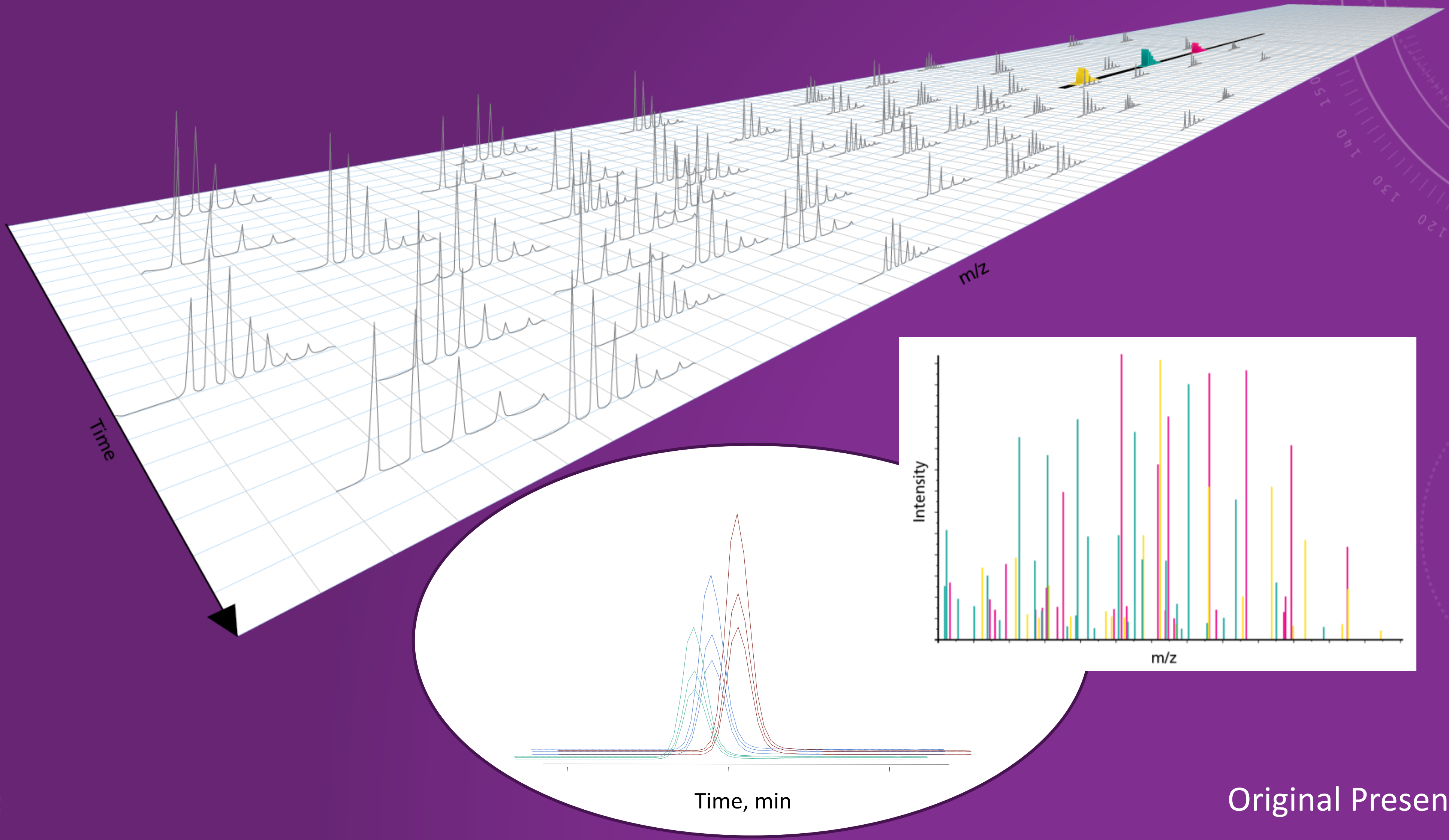
MS/MS^{ALL} WITH SWATH™ ACQUISITION



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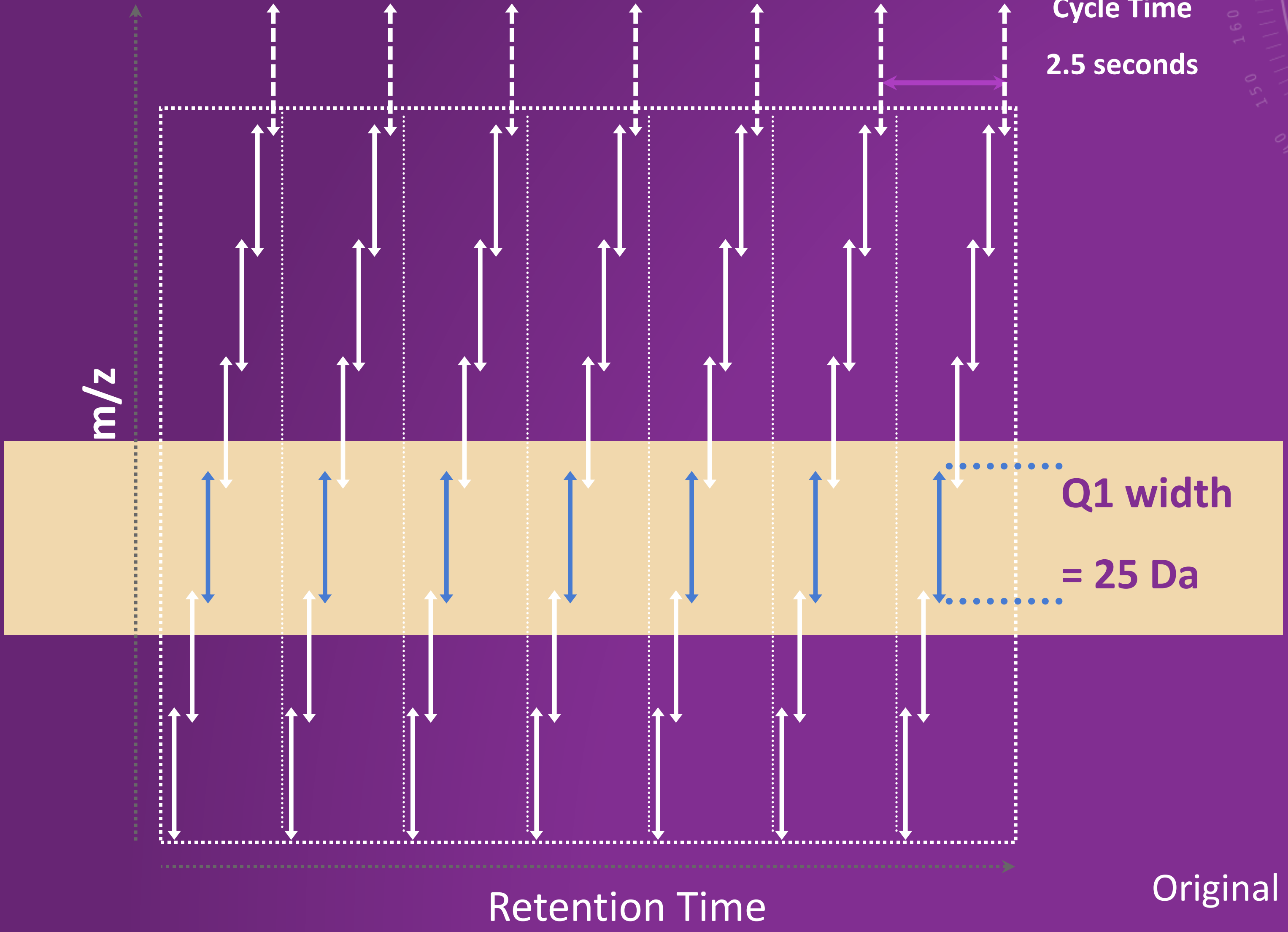
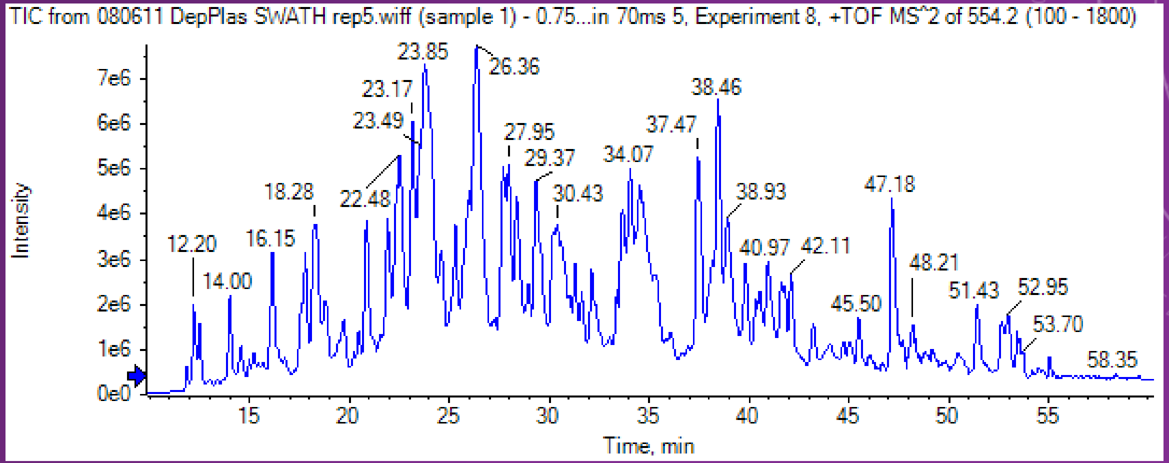
Q1 ISOLATION STRATEGY

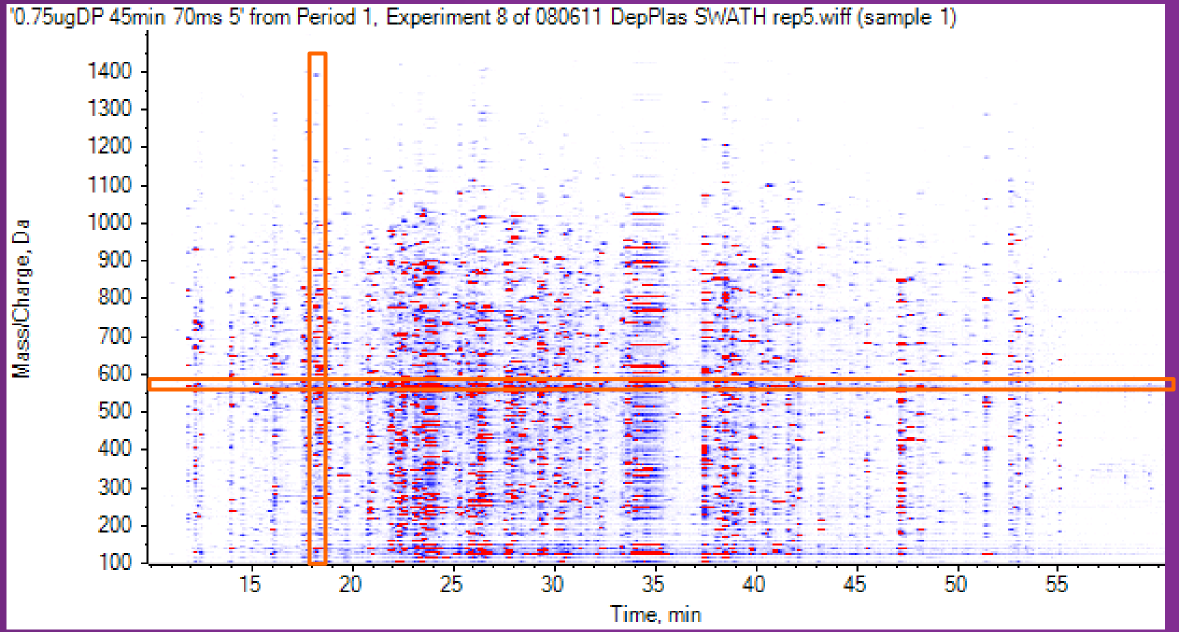


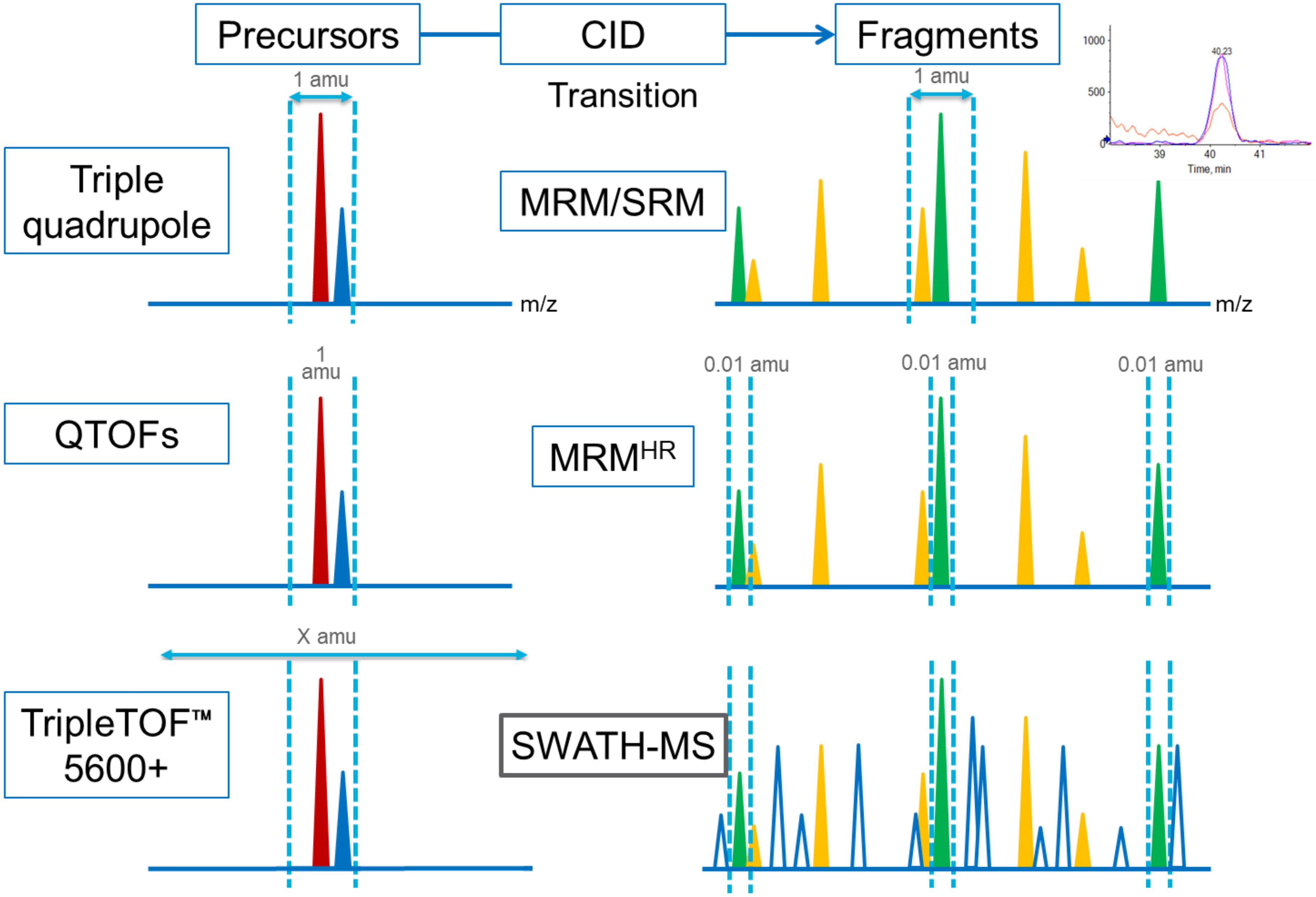
Original Presentation - 2014

SINGLE SWATH™ ACQUISITION WINDOW

- Current strategy uses 25 Da window to cover the peptide mass range in a LC time frame
- 3 D data
- MS/MS on all precursors between 550 – 575 m/z


TIC from 080611 DepPlas SWATH rep5.wiff (sample 1) - 0.75...in 70ms 5, Experiment 8, +TOF MS^2 of 554.2 (100 - 1800)


'0.75ugDP 45min 70ms 5' from Period 1, Experiment 8 of 080611 DepPlas SWATH rep5.wiff (sample 1)



Precursors

CID

Fragments

Transition

Triple quadrupole

MRM/SRM

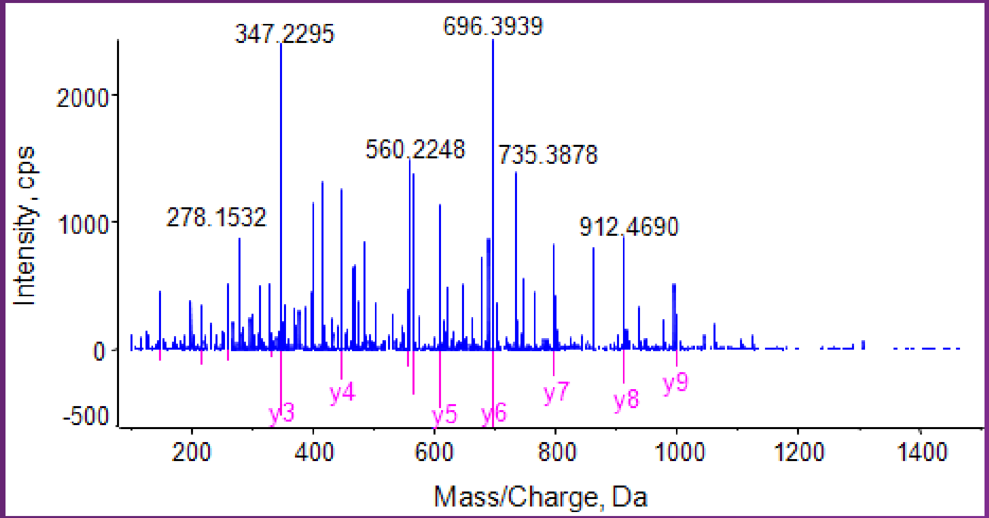
QTOFs

MRM^{HR}

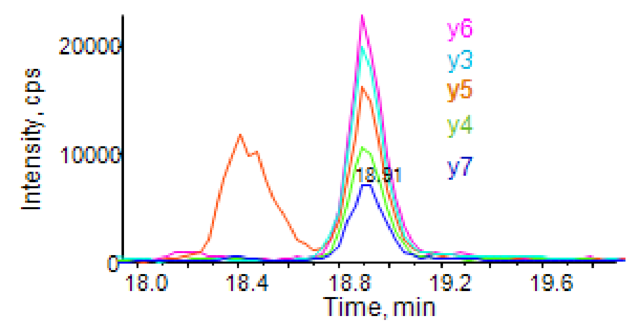
TripleTOF™ 5600+

SWATH-MS

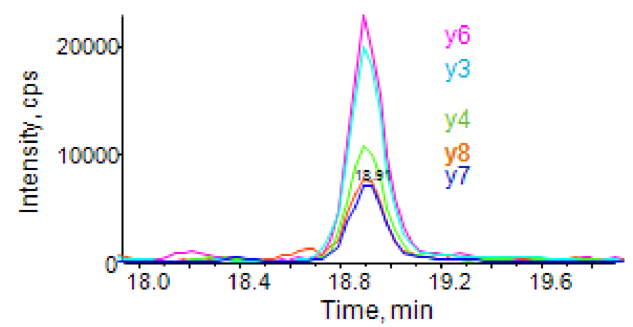
POST-ACQUISITION XICS FROM MS/MS



- MS/MS from Ion Library CRP ESDTSYVSLK $^{2+}$
- Full Scan MS/MS at 18.9 mins
- Choose best fragment ion set for quantification – post-acquisition extraction
- Multiple measurements per analyte



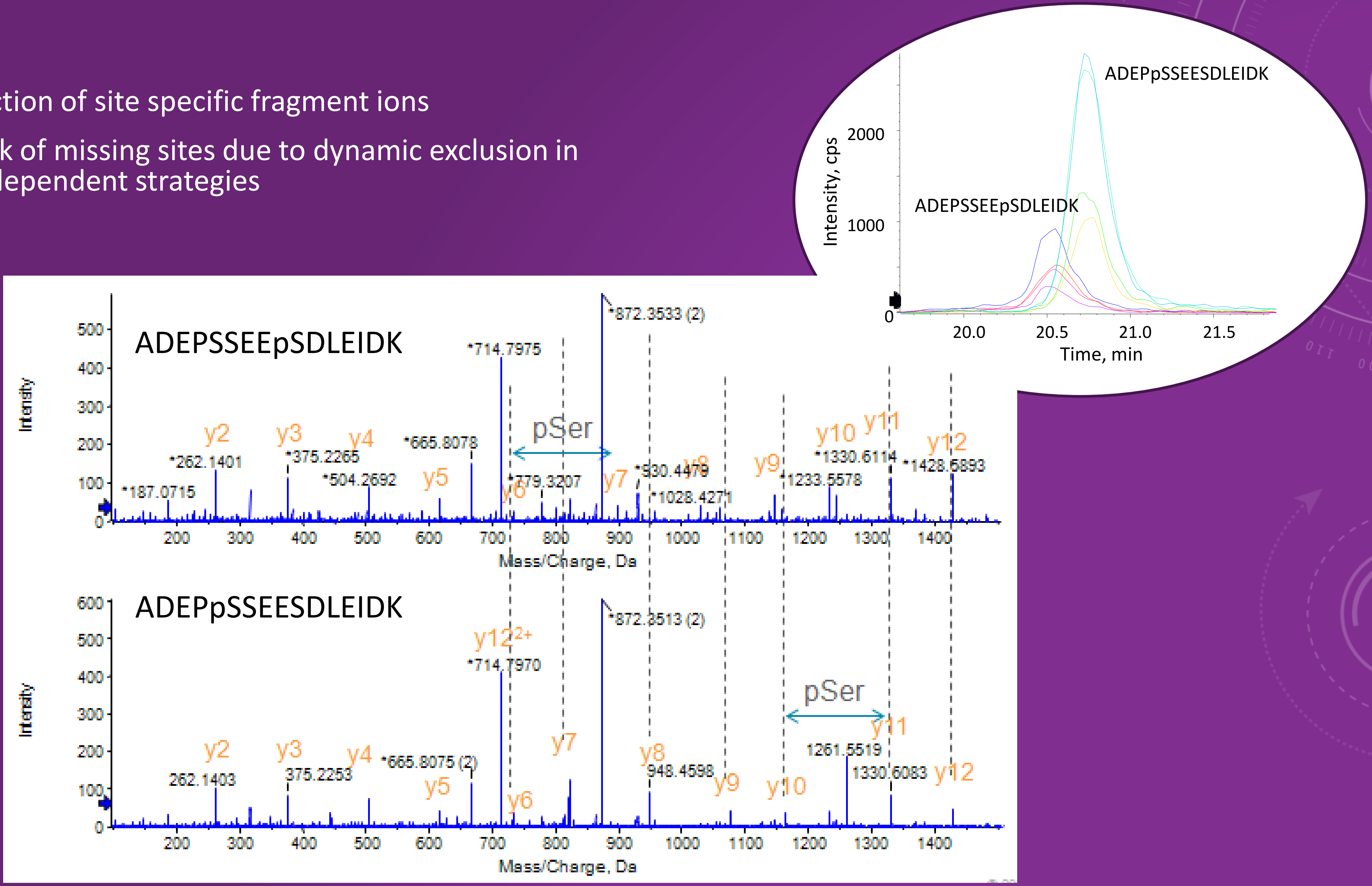
XICs from Full Scan MS/MS at 18.9 mins



Refined to reduce interferences
y5 replaced with y8

TARGETED EXTRACTION OF FRAGMENT IONS

- Extraction of site specific fragment ions
- No risk of missing sites due to dynamic exclusion in data dependent strategies



CHANGING THE WAY WE DO OMICS RESEARCH?

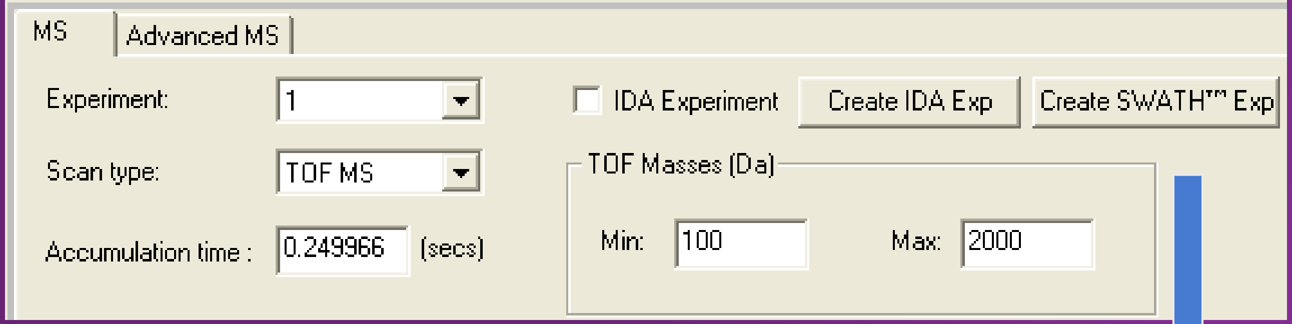
MS/MS^{ALL} USING SWATH™ ACQUISITION

	Discovery (IDA)	Targeted Quantitation (MRM)	MS/MS^{all} with SWATH™ Acquisition
Identification	✓✓✓		✓✓*
Reproducibility	✓✓	✓✓✓	✓✓✓
Sensitivity	✓	✓✓✓	✓✓
Analyte Coverage	✓✓	✓	✓✓✓
Quantitation	✓	✓✓✓	✓✓
Retrospective Interrogation	✓✓	✓	✓✓✓

* Future

MS/MS^{ALL} WITH SWATH™ ACQUISITION

ACQUISITION METHOD BUILDER



Experiment: 1

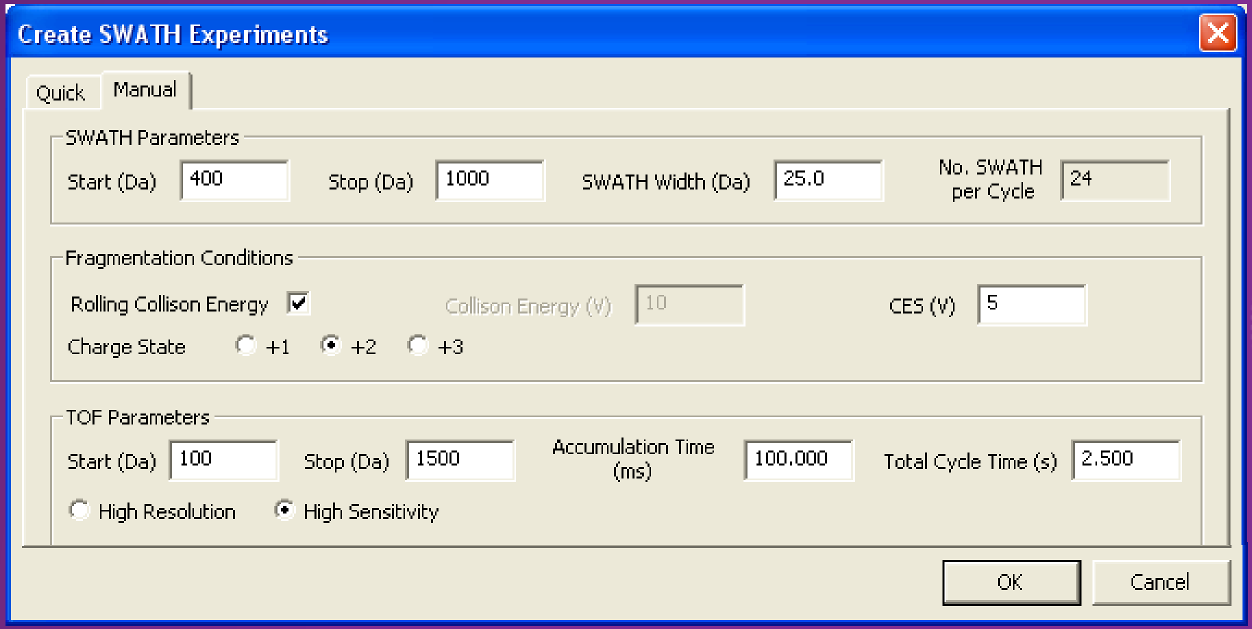
Scan type: TOF MS

Accumulation time : 0.249966 (secs)

☐ IDA Experiment

TOF Masses (Da)

Min: 100 Max: 2000



SWATH Parameters

Start (Da) 400 Stop (Da) 1000 SWATH Width (Da) 25.0 No. SWATH per Cycle 24

Fragmentation Conditions

Rolling Collison Energy ☑ Collison Energy (V) 10 CES (V) 5

Charge State ○ +1 ● +2 ○ +3

TOF Parameters

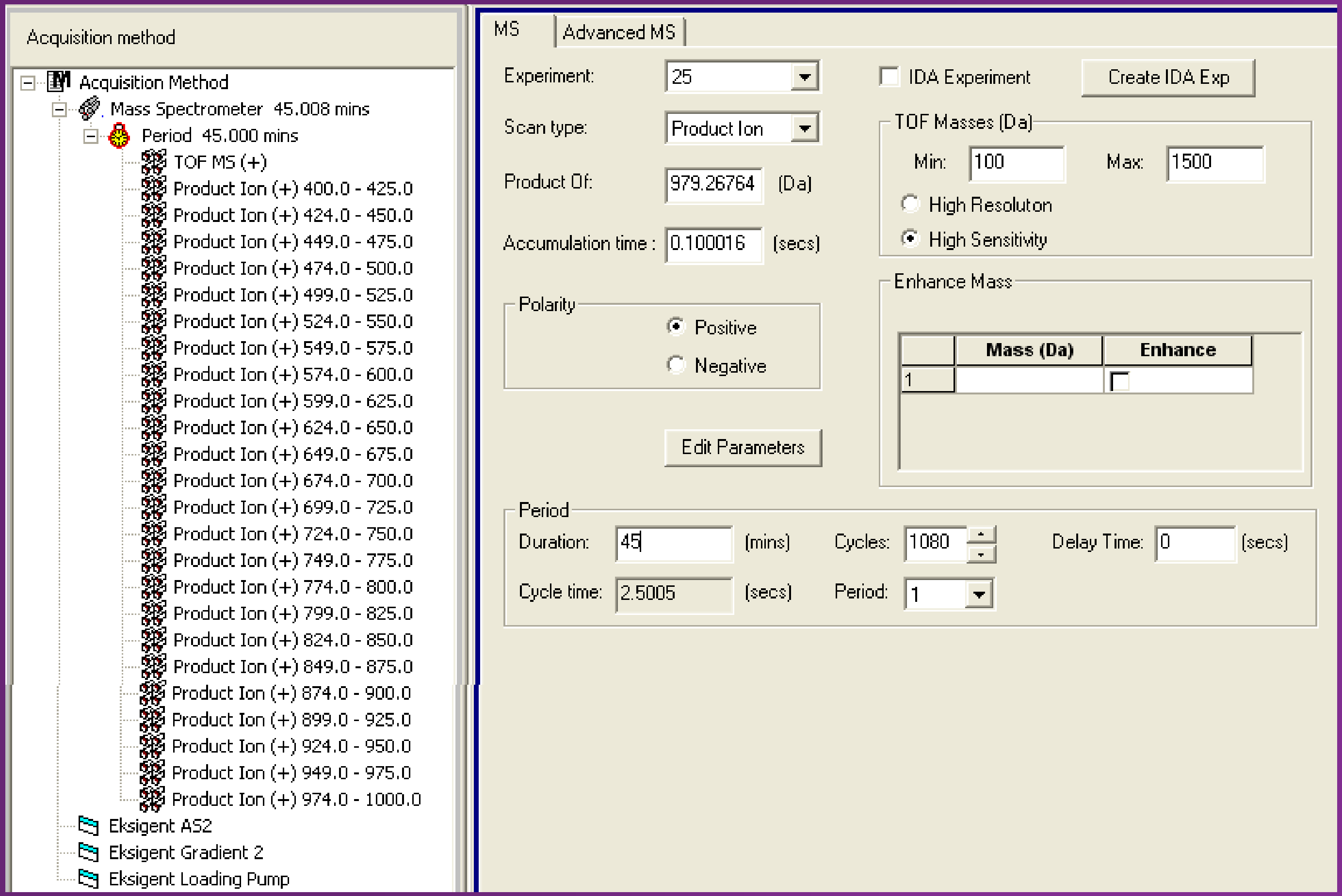
Start (Da) 100 Stop (Da) 1500 Accumulation Time (ms) 100.000 Total Cycle Time (s) 2.500

○ High Resolution ● High Sensitivity

- Set mass range and window width for Q1 mass selection
- Set CE and MS/MS parameters
- Easy method creation

MS/MS^{ALL} WITH SWATH™ ACQUISITION

ACQUISITION METHOD BUILDER



Acquisition method

- Acquisition Method
  - Mass Spectrometer 45.008 mins
    - Period 45.000 mins
      - TOF MS (+)
      - Product Ion (+) 400.0 - 425.0
      - Product Ion (+) 424.0 - 450.0
      - Product Ion (+) 449.0 - 475.0
      - Product Ion (+) 474.0 - 500.0
      - Product Ion (+) 499.0 - 525.0
      - Product Ion (+) 524.0 - 550.0
      - Product Ion (+) 549.0 - 575.0
      - Product Ion (+) 574.0 - 600.0
      - Product Ion (+) 599.0 - 625.0
      - Product Ion (+) 624.0 - 650.0
      - Product Ion (+) 649.0 - 675.0
      - Product Ion (+) 674.0 - 700.0
      - Product Ion (+) 699.0 - 725.0
      - Product Ion (+) 724.0 - 750.0
      - Product Ion (+) 749.0 - 775.0
      - Product Ion (+) 774.0 - 800.0
      - Product Ion (+) 799.0 - 825.0
      - Product Ion (+) 824.0 - 850.0
      - Product Ion (+) 849.0 - 875.0
      - Product Ion (+) 874.0 - 900.0
      - Product Ion (+) 899.0 - 925.0
      - Product Ion (+) 924.0 - 950.0
      - Product Ion (+) 949.0 - 975.0
      - Product Ion (+) 974.0 - 1000.0
  - Eksigent AS2
  - Eksigent Gradient 2
  - Eksigent Loading Pump

MS | Advanced MS

Experiment: 25

☐ IDA Experiment

Create IDA Exp

Scan type: Product Ion

TOF Masses (Da)

Min: 100

Max: 1500

○ High Resoluton

● High Sensitivity

Product Of: 979.26764 (Da)

Accumulation time : 0.100016 (secs)

Polarity

● Positive

○ Negative

Enhance Mass

	Mass (Da)	Enhance
1		☐

Edit Parameters

Period

Duration: 45 (mins)

Cycles: 1080

Delay Time: 0 (secs)

Cycle time: 2.5005 (secs)

Period: 1

- TOF MS with 24 looped product ion scans
- 25 Da window
- 1 Da overlap between windows for complete coverage

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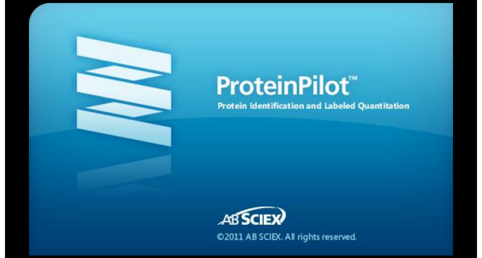
MS/MS$^{\text{ALL}}$ WITH SWATH™ ACQUISITION WORKFLOW

DIFFERENTIAL PROTEIN EXPRESSION ACROSS SAMPLES

1. Data Acquisition

IDA and SWATH™ Acquisition Data Collection

2. Protein Identification



ProteinPilot™

Protein Identification and Labeled Quantitation

AB SCIEX

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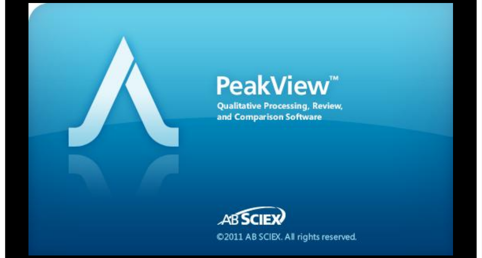
Database Search of IDA data

↓

ProteinPilot® Software Results (*.group file) + SWATH™ Acquisition data file

3. MS Peak Extraction



PeakView™

Qualitative Processing, Review, and Comparison Software

AB SCIEX

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Protein / Peptide Selection with MS/MS$^{\text{ALL}}$ with SWATH™ Acquisition MicroApp

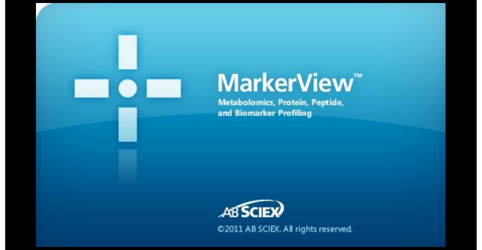
↓

XIC Peak Area Extraction

↓

Export MarkerView™ Software Feature Tables

4. Statistical Analysis



MarkerView™

Metabolomics, Protein, Peptide, and Biomarker Profiling

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Normalize MS Data

↓

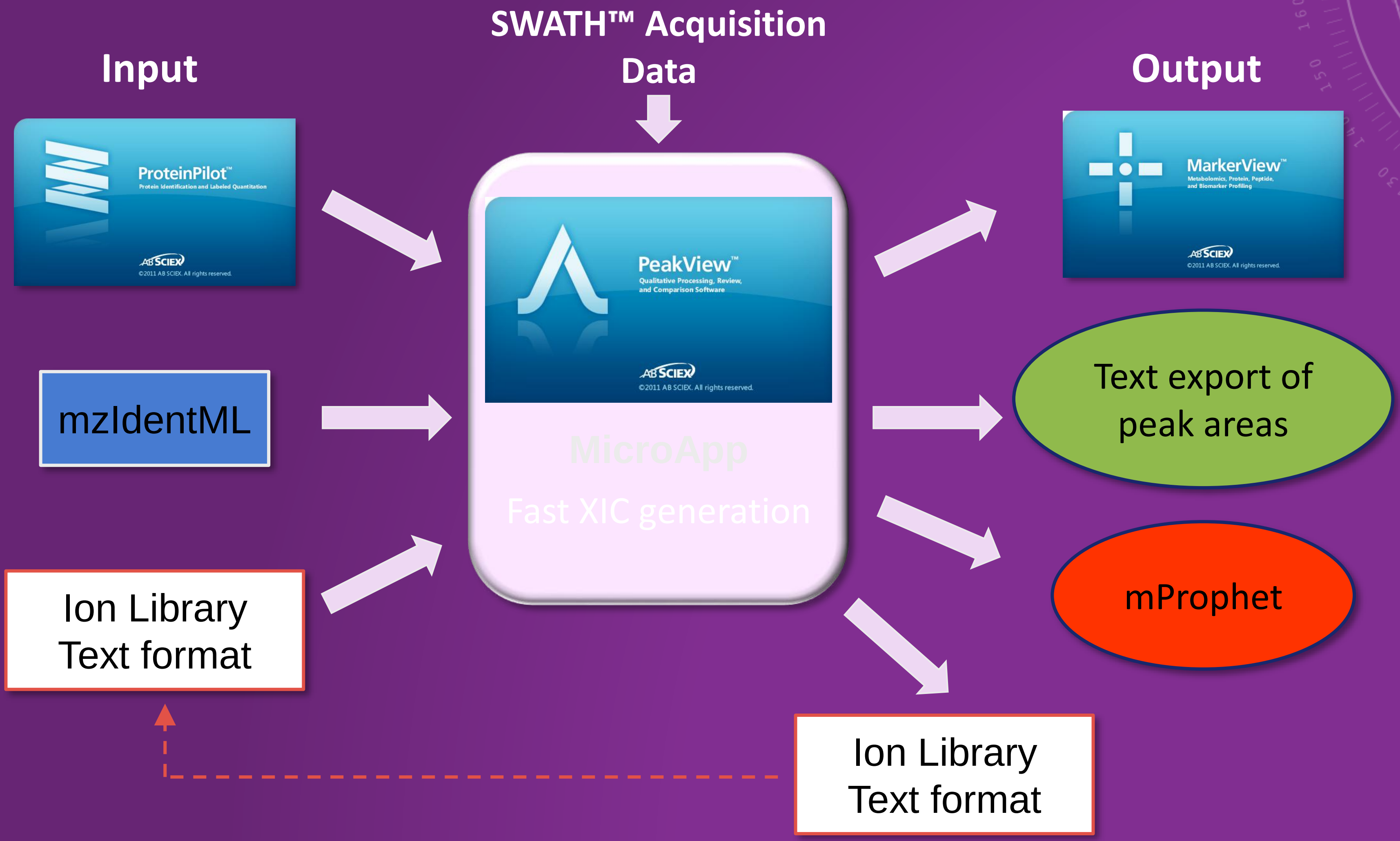
Perform PCA / PCVG Analysis

↓

Results Interpretation

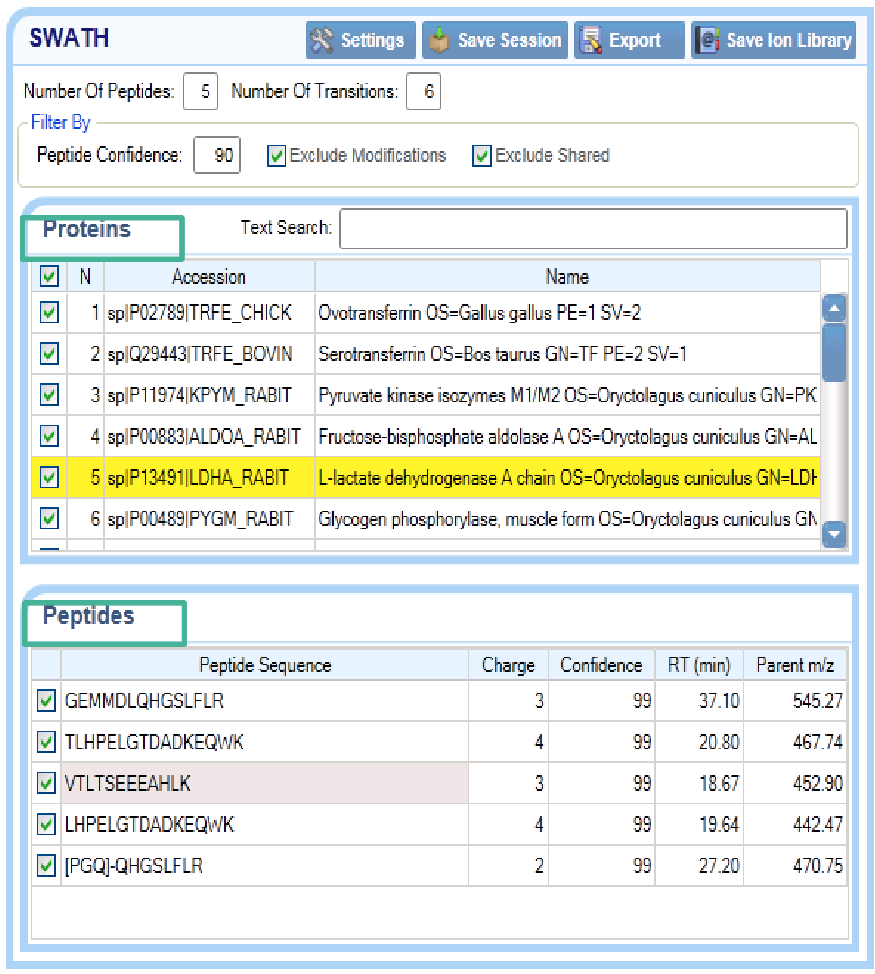
MS/MS^{ALL} FOR SWATH™ ACQUISITION

PEAK EXTRACTION ENGINE



DATA PROCESSING

LOAD PROTEINS/PEPTIDES AND WIFF FILES



Number Of Peptides: 5 Number Of Transitions: 6

Filter By

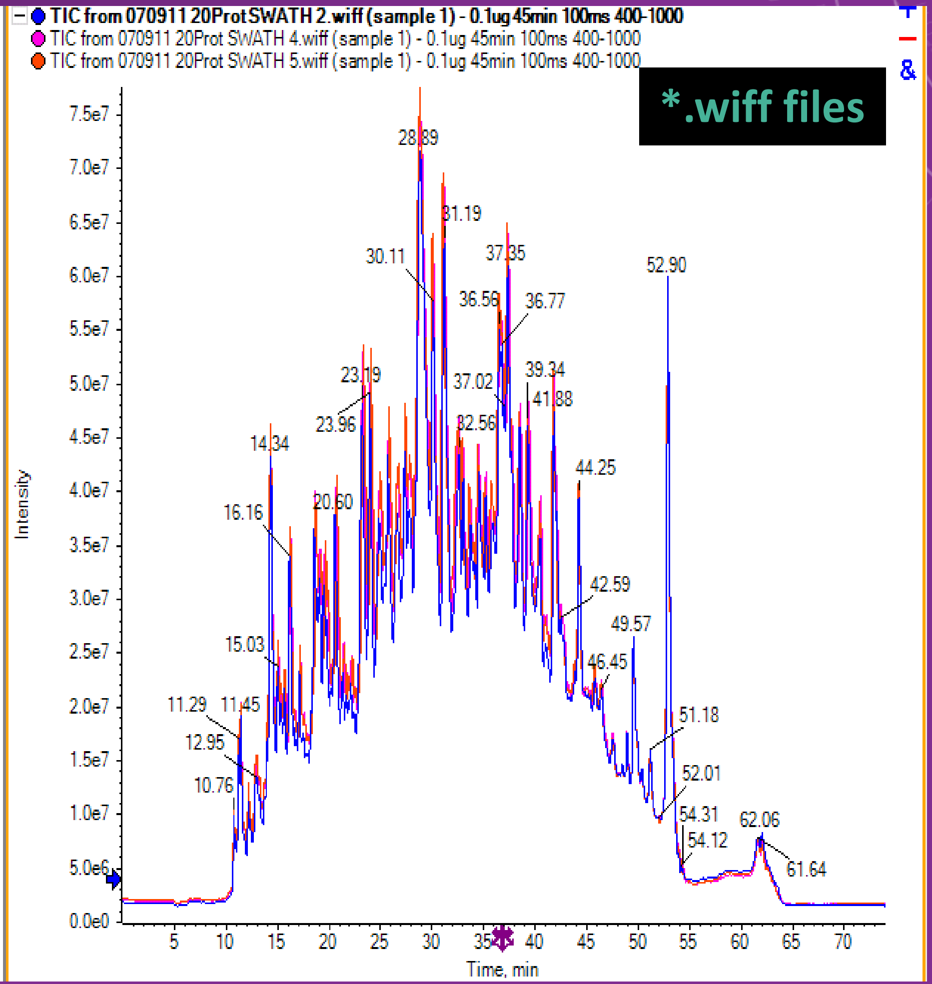
Peptide Confidence: 90 ☑ Exclude Modifications ☑ Exclude Shared

Proteins Text Search:

☑	N	Accession	Name
☑	1	sp|P02789|TRFE_CHICK	Ovotransferrin OS=Gallus gallus PE=1 SV=2
☑	2	sp|Q29443|TRFE_BOVIN	Serotransferrin OS=Bos taurus GN=TF PE=2 SV=1
☑	3	sp|P11974|KPYM_RABIT	Pyruvate kinase isozymes M1/M2 OS=Oryctolagus cuniculus GN=PK
☑	4	sp|P00883|ALDOA_RABIT	Fructose-bisphosphate aldolase A OS=Oryctolagus cuniculus GN=AL
☑	5	sp|P13491|LDHA_RABIT	L-lactate dehydrogenase A chain OS=Oryctolagus cuniculus GN=LDH
☑	6	sp|P00489|PYGM_RABIT	Glycogen phosphorylase, muscle form OS=Oryctolagus cuniculus GN

Peptides

	Peptide Sequence	Charge	Confidence	RT (min)	Parent m/z
☑	GEMMDLQHGSLFLR	3	99	37.10	545.27
☑	TLHPELGTDADKEQWK	4	99	20.80	467.74
☑	VTLTSEEEAHLK	3	99	18.67	452.90
☑	LHPELGTDADKEQWK	4	99	19.64	442.47
☑	[PGQ]-QHGSLFLR	2	99	27.20	470.75

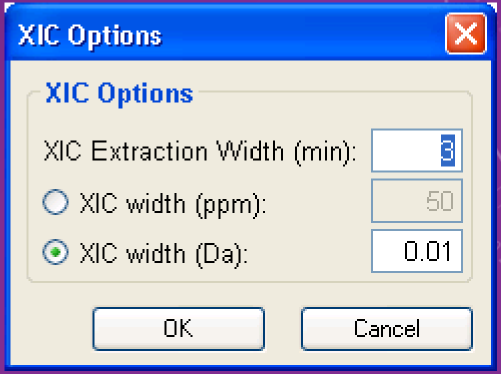


*.wiff files

DATA PROCESSING

FRAGMENT ION EXTRACTION

- For each selected peptides, fragment ion XICs are generated
    - Algorithm finds RT where fragment ions overlap and peak area generated for each
- Ion library spectrum (pink) and SWATH™ Acquisition MS/MS from peak apex (blue) aligned for comparison

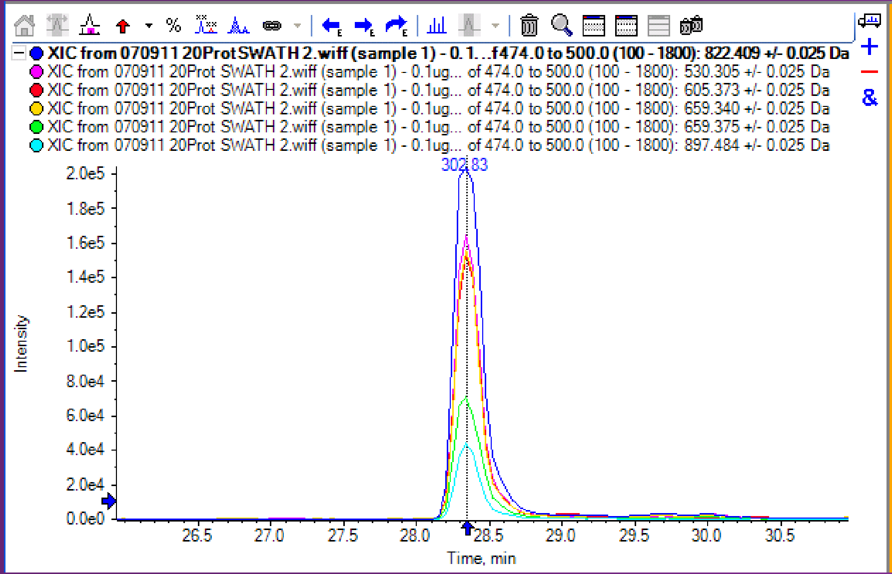

XIC Options

XIC Options

XIC Extraction Width (min): 3

○ XIC width (ppm): 50

● XIC width (Da): 0.01


● XIC from 070911 20Prot SWATH 2.wiff (sample 1) - 0.1…f474.0 to 500.0 (100 - 1800): 822.409 +/- 0.025 Da

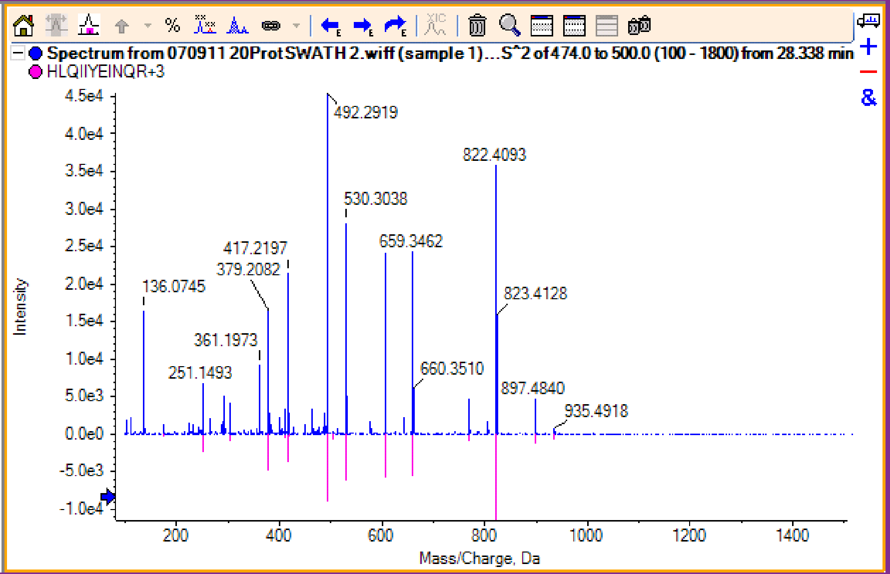
● XIC from 070911 20Prot SWATH 2.wiff (sample 1) - 0.1ug… of 474.0 to 500.0 (100 - 1800): 530.305 +/- 0.025 Da

● XIC from 070911 20Prot SWATH 2.wiff (sample 1) - 0.1ug… of 474.0 to 500.0 (100 - 1800): 605.373 +/- 0.025 Da

● XIC from 070911 20Prot SWATH 2.wiff (sample 1) - 0.1ug… of 474.0 to 500.0 (100 - 1800): 659.340 +/- 0.025 Da

● XIC from 070911 20Prot SWATH 2.wiff (sample 1) - 0.1ug… of 474.0 to 500.0 (100 - 1800): 659.375 +/- 0.025 Da

● XIC from 070911 20Prot SWATH 2.wiff (sample 1) - 0.1ug… of 474.0 to 500.0 (100 - 1800): 897.484 +/- 0.025 Da

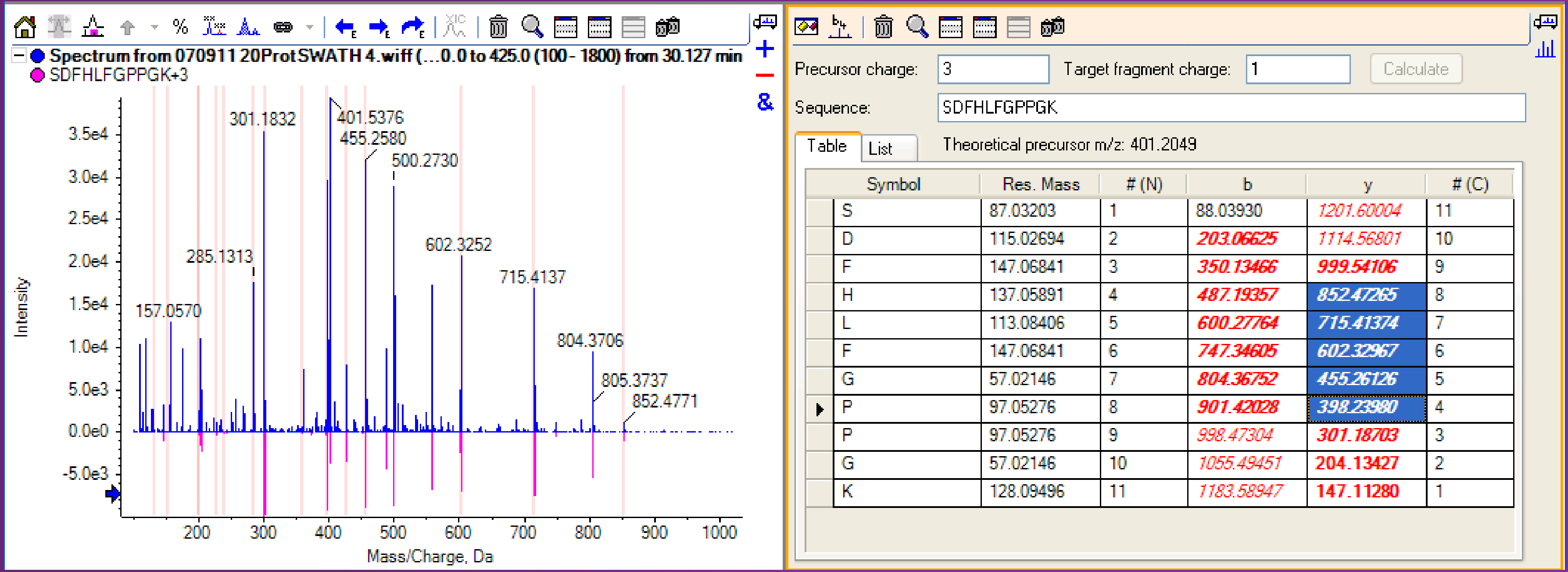

● Spectrum from 070911 20Prot SWATH 2.wiff (sample 1)…S^2 of 474.0 to 500.0 (100 - 1800) from 28.338 min

● HLQIIYEINQR+3

DATA PROCESSING

FRAGMENT ION CONFIRMATION

- Peptide MS/MS can be matched to the theoretical sequence using the Peptide Fragment pane



● Spectrum from 070911 20ProtSWATH 4.wiff (…0.0 to 425.0 (100 - 1800) from 30.127 min

● SDFHLFGPPGK+3

Precursor charge: 3

Target fragment charge: 1

Sequence: SDFHLFGPPGK

Table | List

Theoretical precursor m/z: 401.2049

Symbol	Res. Mass	# (N)	b	y	# (C)
S	87.03203	1	88.03930	1201.60004	11
D	115.02694	2	203.06625	1114.56801	10
F	147.06841	3	350.13466	999.54106	9
H	137.05891	4	487.19357	852.47265	8
L	113.08406	5	600.27764	715.41374	7
F	147.06841	6	747.34605	602.32967	6
G	57.02146	7	804.36752	455.26126	5
P	97.05276	8	901.42028	398.23980	4
P	97.05276	9	998.47304	301.18703	3
G	57.02146	10	1055.49451	204.13427	2
K	128.09496	11	1183.58947	147.11280	1

MS/MS^{ALL} WITH SWATH™ ACQUISITION

CHANGING THE PARADIGM IN PROTEOMICS

1. Comprehensive quantitation
    - Comprehensive – acquires MS/MS of everything
    - Quantitation – ‘MRMs’ of everything
2. High quality quantitation – QQQ like
    - Excellent depth of coverage
    - High specificity
3. Easy quantitation
    - Single acquisition method – no method development
    - Re-analysis without re-acquisition

Original Presentation - 2014

QUANTITATION STRATEGIES BY MS

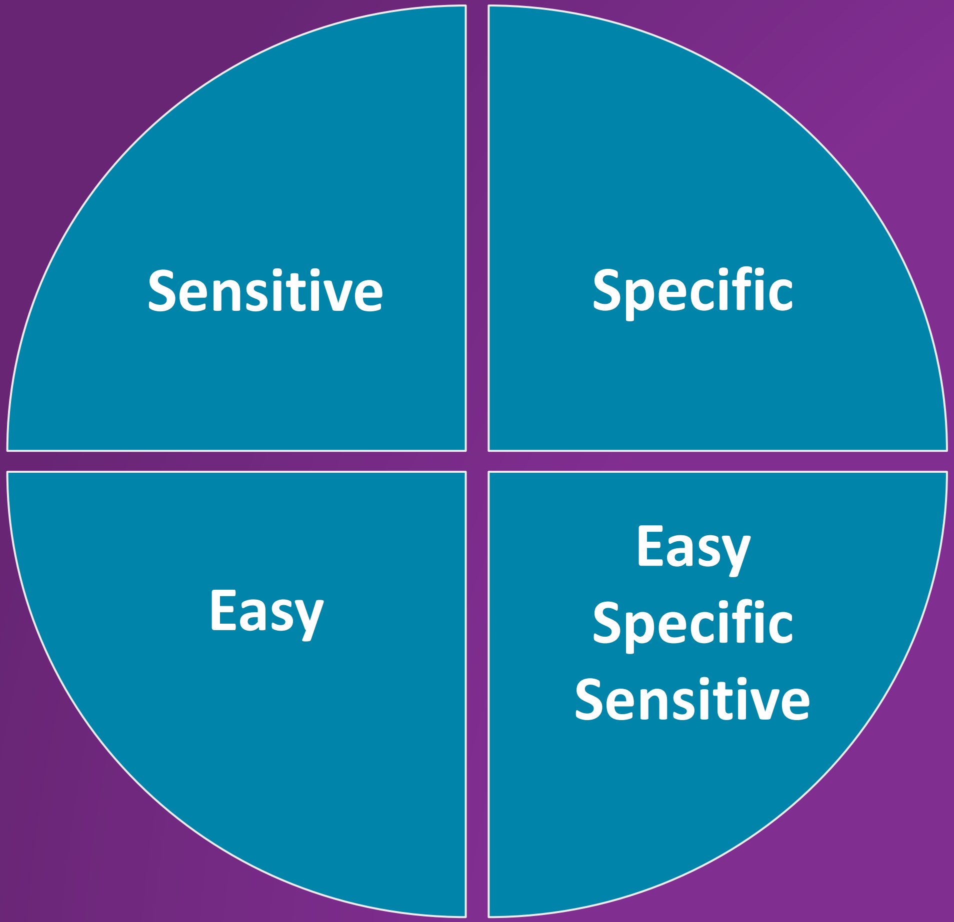
***Scheduled* MRM™**
Algorithm

Upfront assay development
Low multiplexing
High sensitivity

Targeted Acquisition

***Scheduled* MRM^{HR}**
Workflow

Upfront assay development
Low multiplexing
Highest specificity



Lowest specificity
High multiplexing
No assay development

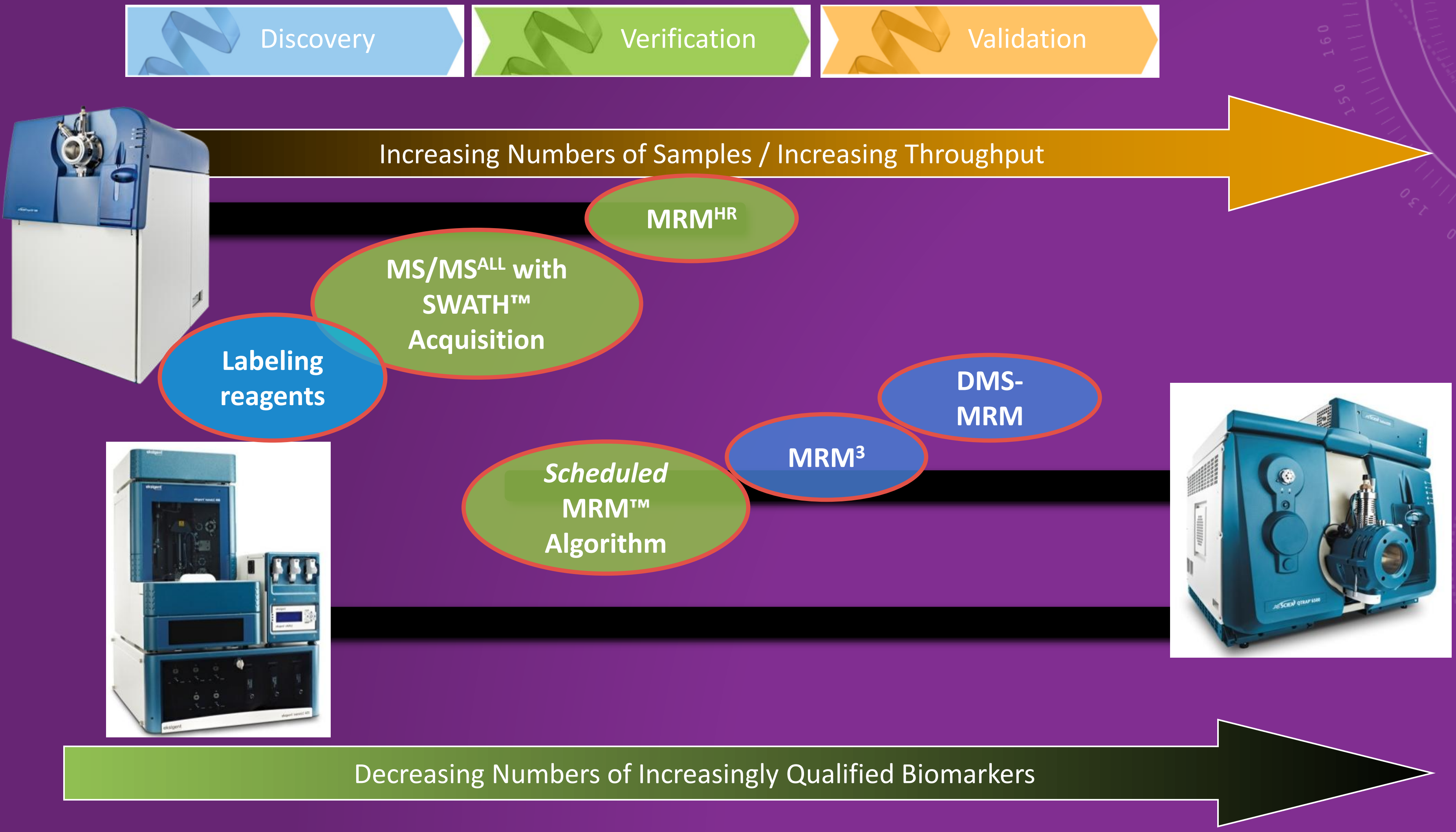
MS (MS1)

Untargeted Acquisition

Good specificity
High multiplexing
No assay development
Permanent record

MS/MS^{ALL} with SWATH™
Acquisition

PROTEIN BIOMARKER RESEARCH PIPELINE



Discovery

Verification

Validation

Increasing Numbers of Samples / Increasing Throughput

MRM^{HR}

MS/MS^{ALL} with SWATH™ Acquisition

Labeling reagents

DMS-MRM

MRM^{3}

Scheduled MRM™ Algorithm

Decreasing Numbers of Increasingly Qualified Biomarkers

DATA EXAMPLES

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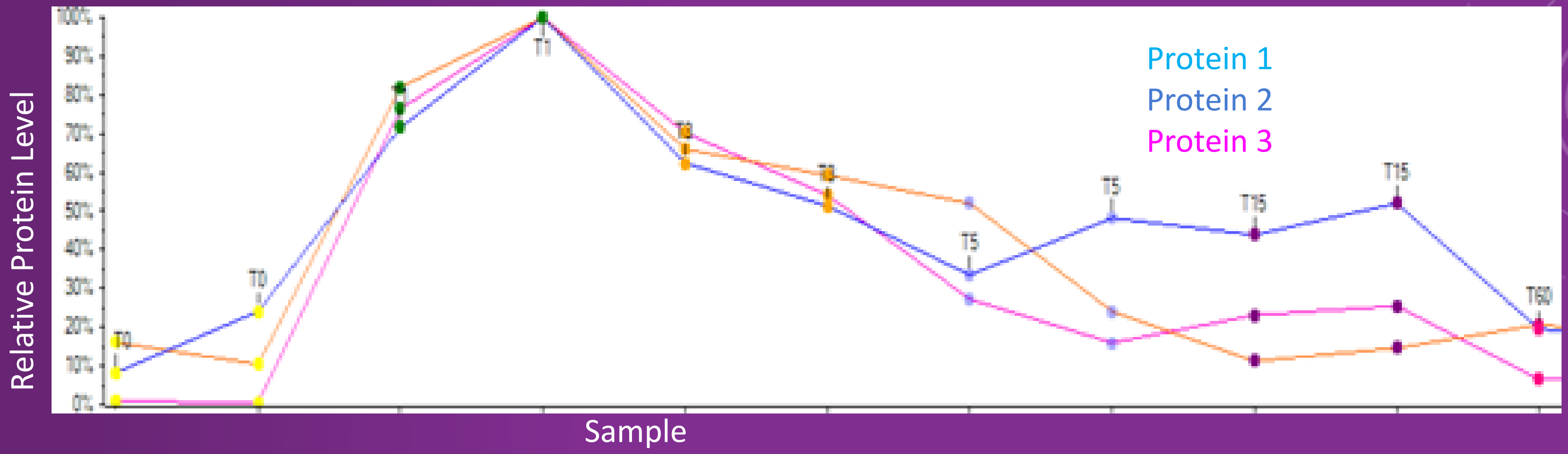
DIFFERENTIAL PROTEIN EXPRESSION ANALYSIS

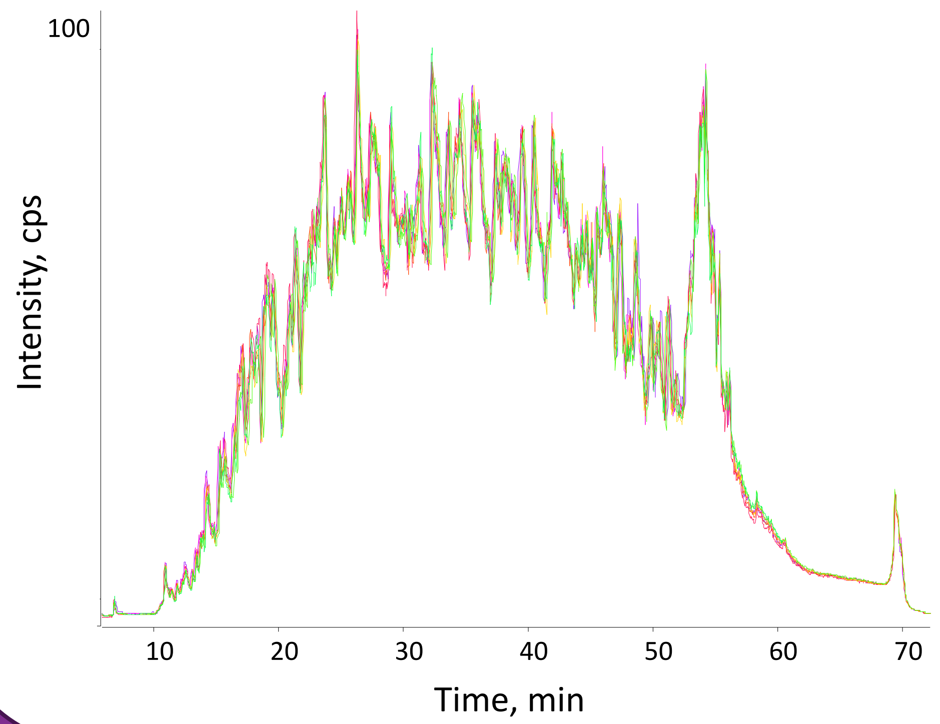
- The goal of quantitative proteomics is to both quantify and confirm a broad range of proteins and peptides across complex biological samples
- Data independent acquisition in combination with the targeted data extraction provides a powerful workflow for protein / peptide quantitation
- Exploring quantitative reproducibility at high multiplexing
  - Replicate analysis on depleted plasma and other complex proteomes
  - Dynamic range comparisons



REPLICATE INJECTIONS OF DEPLETED PLASMA

HIGH NANOLC REPRODUCIBILITY

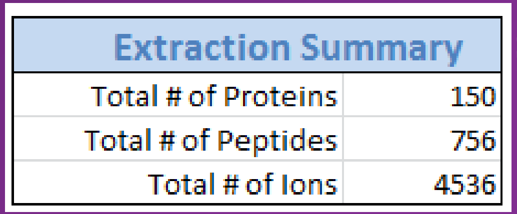
- High reproducibility is a key component of targeted quantitation experiments
- Eksigent nanoLC-Ultra® with the cHiPLC®-nanoflex provides long term retention time stability for analyzing many samples
- Reproducibility of cHiPLC columns ensures every new column maintains the same retention times of the previous studies



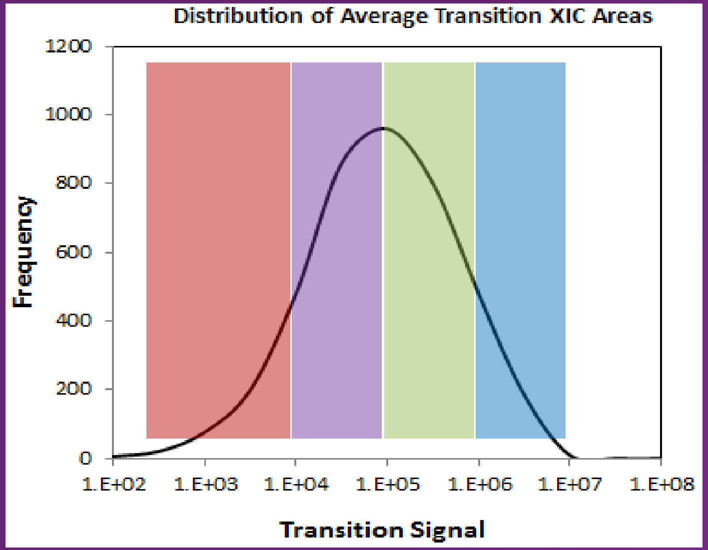
REPRODUCIBILITY OF XIC DATA

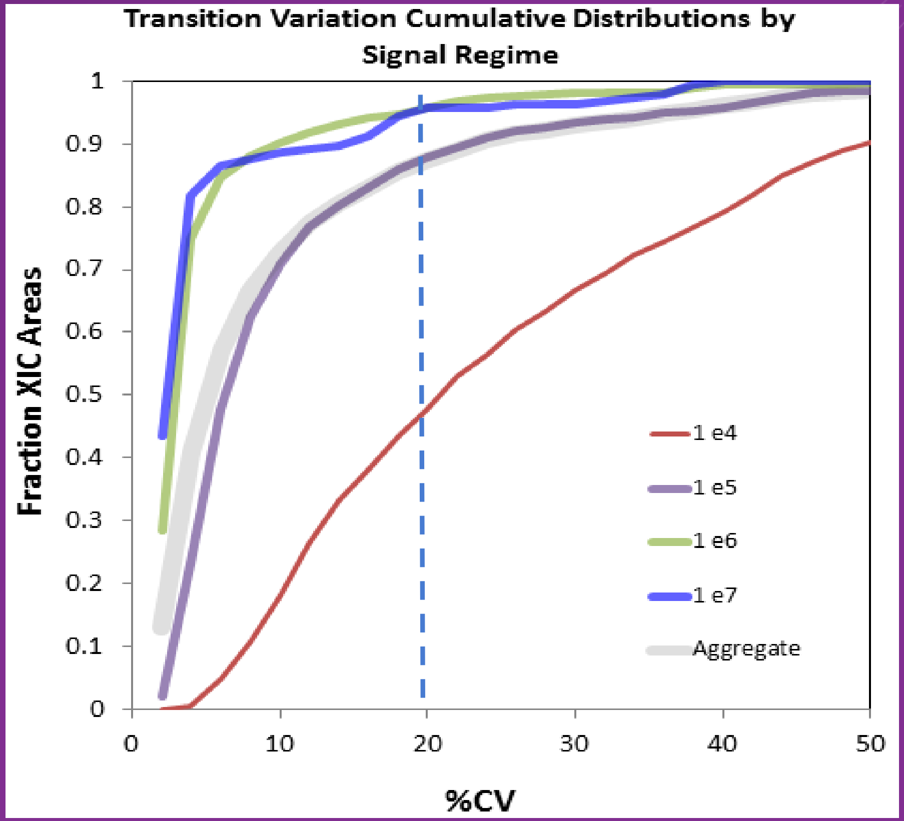
DEPLETED PLASMA

- Cumulative frequency plots showing reproducibility at the different XIC peak areas
- Distribution of XICs in the different intensity bins (bottom)

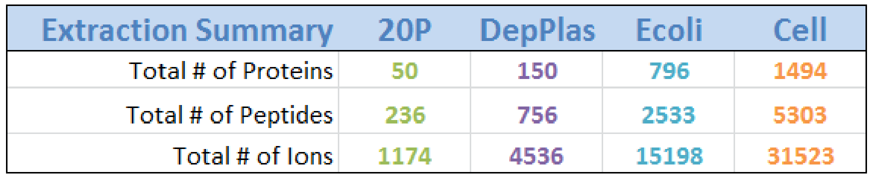


Extraction Summary	
Total # of Proteins	150
Total # of Peptides	756
Total # of Ions	4536



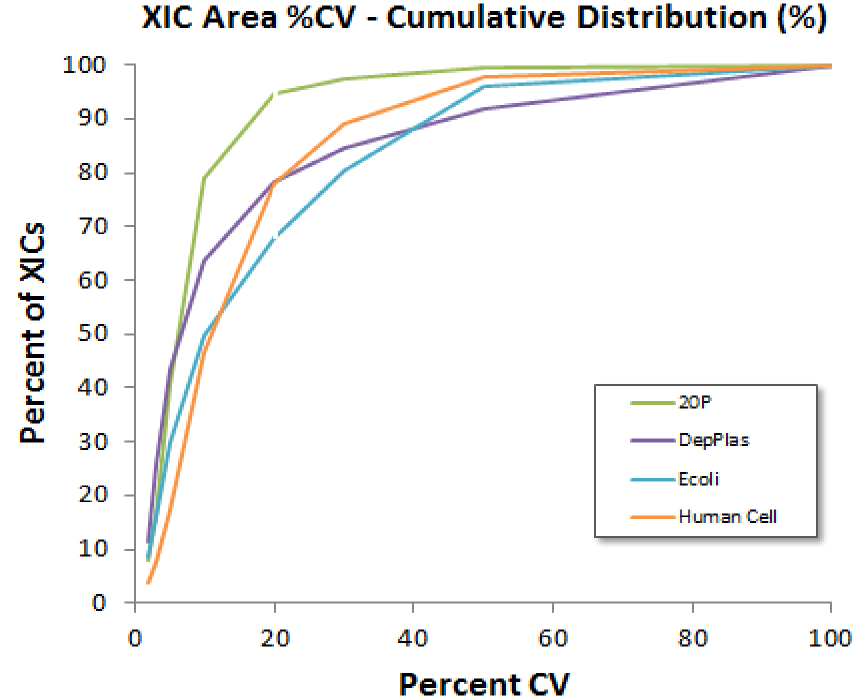


REPRODUCIBILITY ACROSS INCREASINGLY COMPLEX PROTEOMES



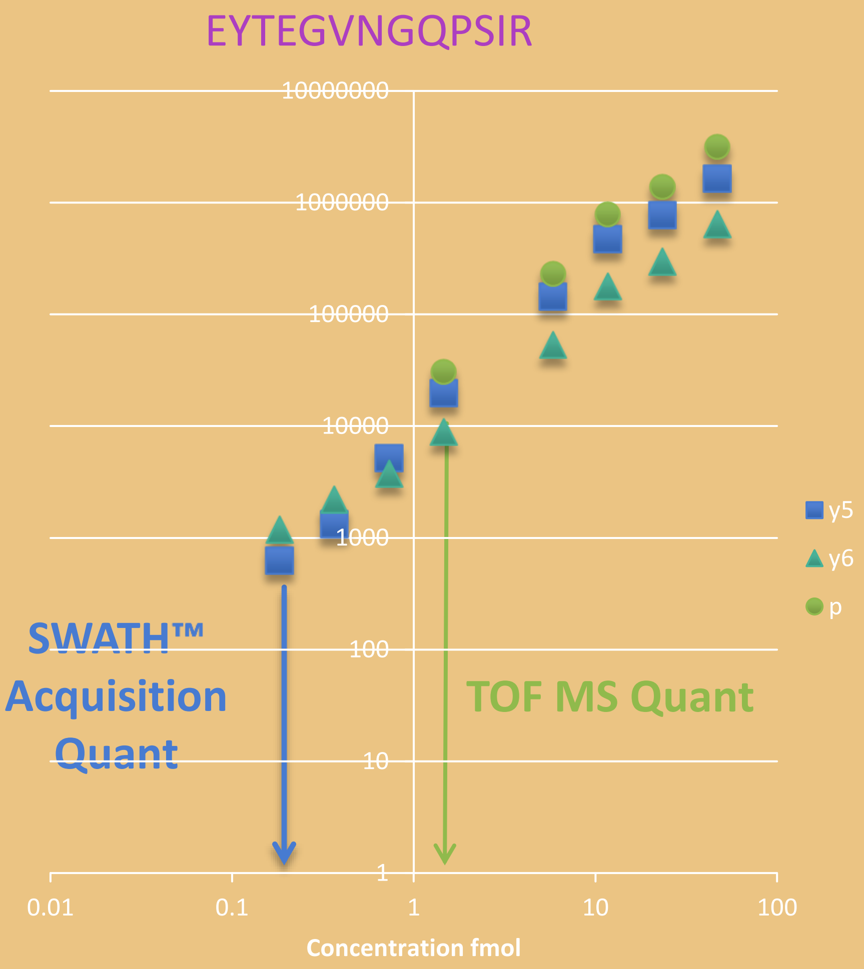
Extraction Summary	20P	DepPlas	Ecoli	Cell
Total # of Proteins	50	150	796	1494
Total # of Peptides	236	756	2533	5303
Total # of Ions	1174	4536	15198	31523

- Three LCMS replicate analyses using MS/MS^{ALL} with SWATH™ Acquisition were performed on more complex proteomes and the fragment ion XICs at two different XIC widths were assessed
- Large numbers of XICs can be generated with good reproducibility



HIGHER SELECTIVITY WITH SWATH™ ACQUISITION

PROVIDES BETTER QUANTITATION ON LOWER ABUNDANT PROTEINS



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MS vs SWATH™ Acquisition for quantitation:

- MS/MS^{ALL} with SWATH™ Acquisition quantitation provides a more sensitive detection of this peptide over TOF MS quantitation
- Improved dynamic range due to the selectivity provided by the Q1 isolation during SWATH acquisition

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DATA SUMMARY

- The data independent acquisition strategy, MS/MS^{ALL} with SWATH™ Acquisition, provides a comprehensive analysis of complex proteomes with good quantitative robustness
  - High reproducibility was demonstrated across replicate injections even in the most complex proteomes
- High resolution MS/MS provides higher resolution XICs with reduced chance of interferences
  - MS/MS^{ALL} with SWATH™ Acquisition provides better quantitation deeper into the proteome than MS1 quantitation strategies

MS/MS^{ALL} WITH SWATH™ ACQUISITION

CHANGING THE PARADIGM IN PROTEOMICS

- Comprehensive quantitative analysis with qualitative confirmation
  - Comprehensive ID and quant of all components within the dynamic range interrogated
  - Ask broader multidimensional questions (study more disease hypothesis, systems biology,etc)
- Quantitation with single acquisition method for all species in a single analysis
  - Transition from discovery to early verification sooner using SWATH Acquisition
- High resolution MS/MS quantification reduces potential for interferences
  - Higher quality quantitation than MS1 strategies - better quant, better dynamic range
- Quantitative performance comparable to leading triple quadrupole instruments
  - QQQ quant quality with no method development required
  - Highest multiplexing with high reproducibility
- Archive of all analytes enables retrospective *in silico* interrogation
  - Data re-interrogation of permanent record
  - When new hypothesis or new algorithms/data processing tools arise, go back and re-interrogate previous studies and find new answers from same acquisition

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THANK YOU

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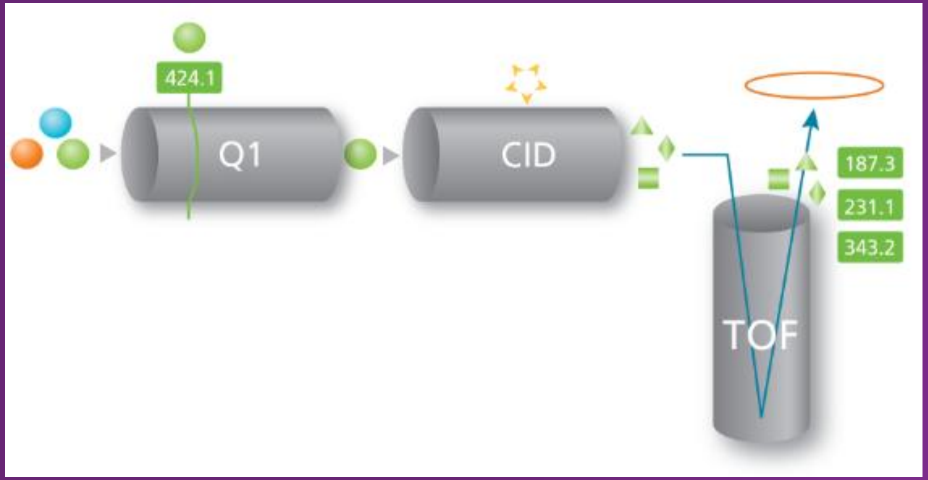
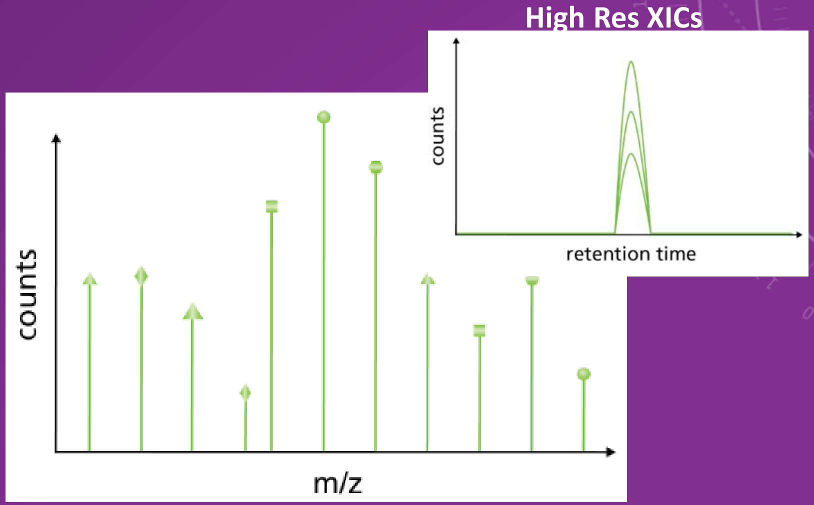
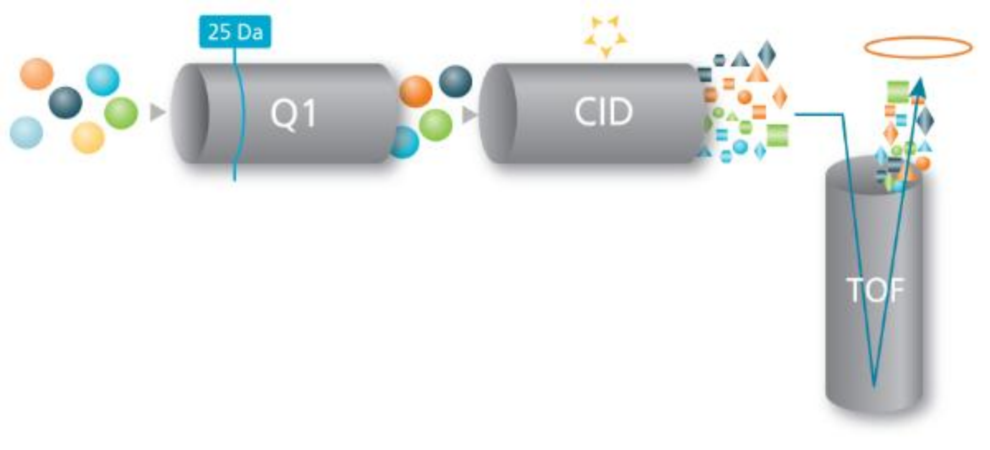
TARGETED HIGH RESOLUTION WORKFLOWS

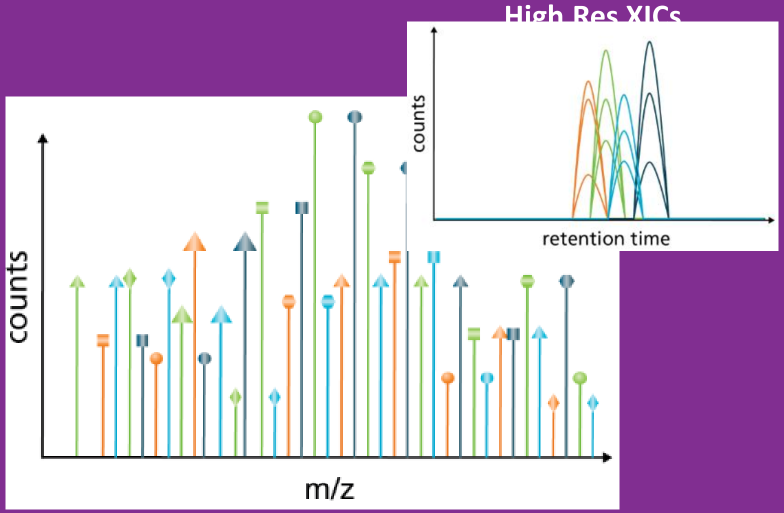
MRM^{HR} Workflow




High Res XICs

SWATH™ Acquisition

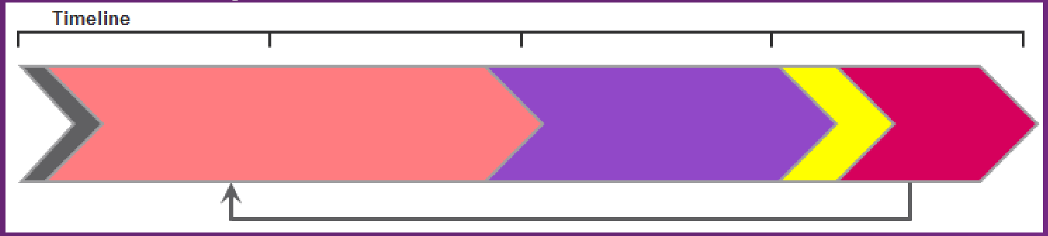



High Res XICs

STREAMLINE THE RESEARCH PIPELINE

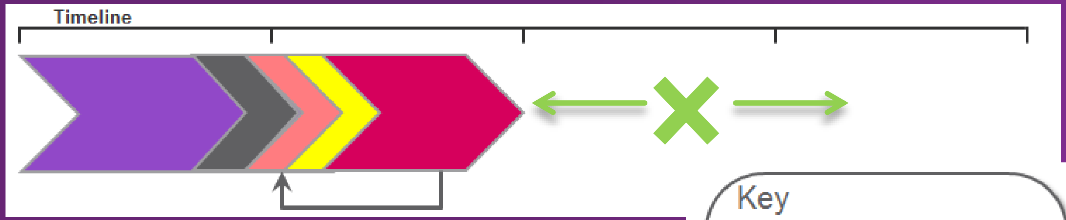
ACCELERATE VERIFICATION WITH SWATH™ ACQUISITION

Old Way with MRM

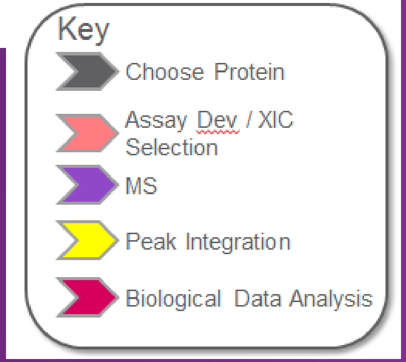


SWATH Acquisition provides a new strategy to significantly reduce the time to do verification work!

New Way with SWATH Acquisition



Verification in almost half the time! Verification feeds directly into Validation…!



- Minimize assay development
    - SWATH Acquisition data is collected, then best peptides and transitions are extracted - *in silico* assay optimization
- Easily achieve higher multiplexing
    - Any number of proteins and peptides can be extracted post-acquisition
- Analyze larger sample sets
    - Obtain the throughput needed for big biological questions

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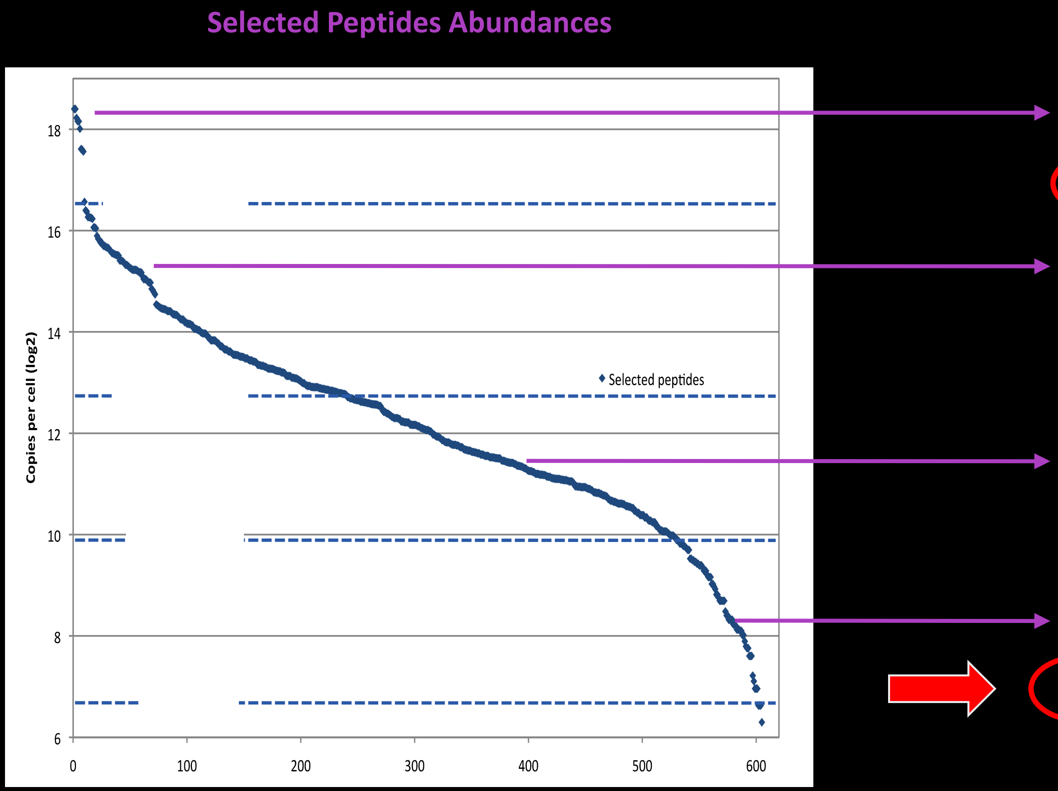
YEAST PROTEOLYTIC PEPTIDES DETECTED

SPANNING FOUR LOGS OF PROTEIN ABUNDANCE



SSAAGNTVIIGGGDTATVAK
YCR012W
3-phosphoglycerate kinase
314,024 copies per cell (log2=18.2)

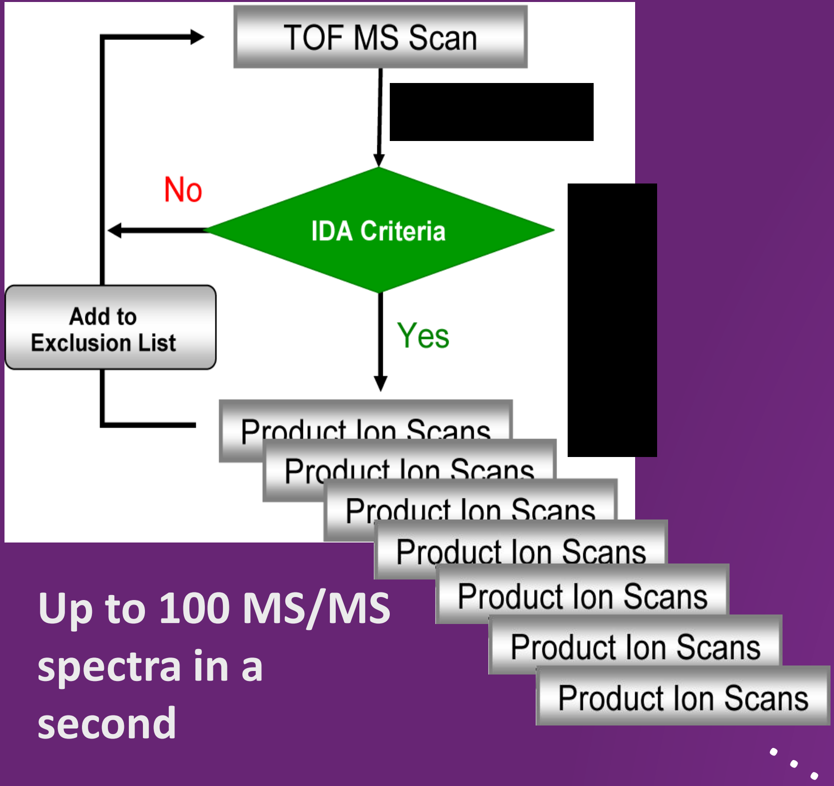
HLNDQPNADIVTIGDK
YBR039W
Gamma subunit of the F1 sector
of mitochondrial F1F0 ATP synthase
28,103 copies (log2=14.8)

NQQLEEDLEESDTK
YNL079C
Major isoform of tropomyosin
3,000 copies per cell (log2=11.6)

LNTGHEILQIQPR
YER024W
Carnitine acetyltransferase
319 copies per cell (log2= 8.3)

Sensitivity and Dynamic Range Equivalent to SRM Assays

DATA DEPENDENT ACQUISITION



TOF MS Scan

[redacted]

No

IDA Criteria

[redacted]

Yes

Add to Exclusion List

Product Ion Scans

Product Ion Scans

Product Ion Scans

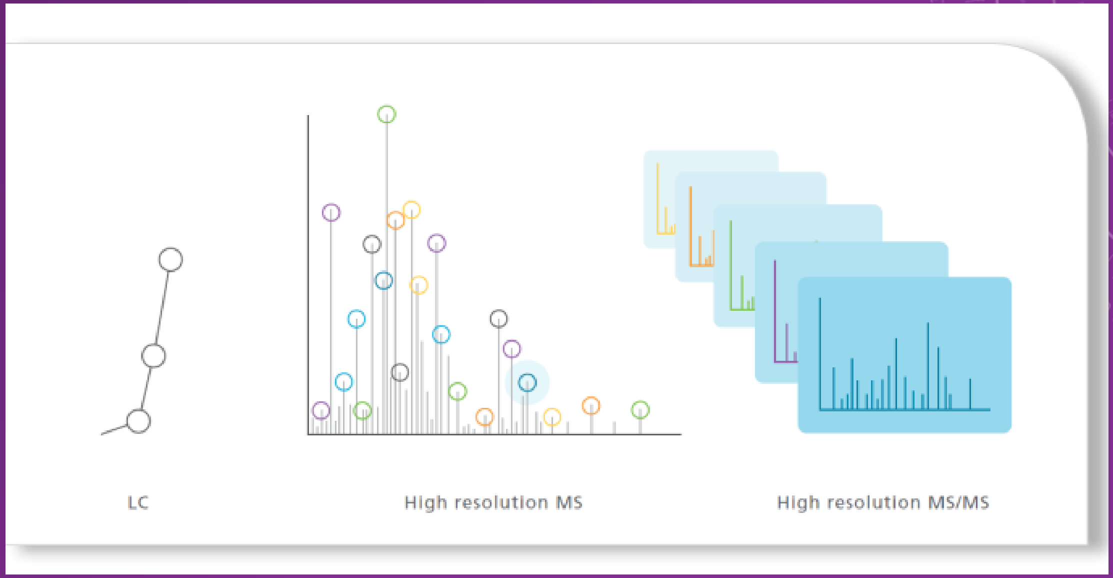
Product Ion Scans

Product Ion Scans

Product Ion Scans

Product Ion Scans

Up to 100 MS/MS spectra in a second



- Precursors are selected one by one based on MS intensity and sent for MS/MS using narrow mass isolation
- MS/MS is then used for peptide/protein identification

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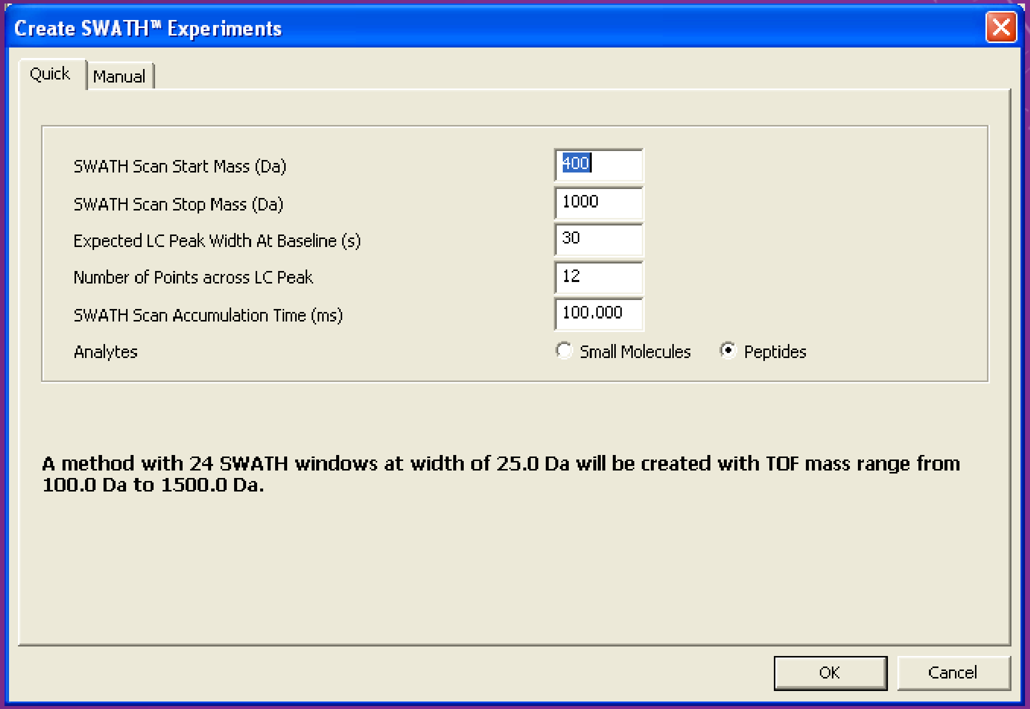
MS/MS^{ALL} WITH SWATH™ ACQUISITION

ACQUISITION METHOD BUILDER

- Quick tab

- Enter LC peak width for automatic computation of # points across the peak

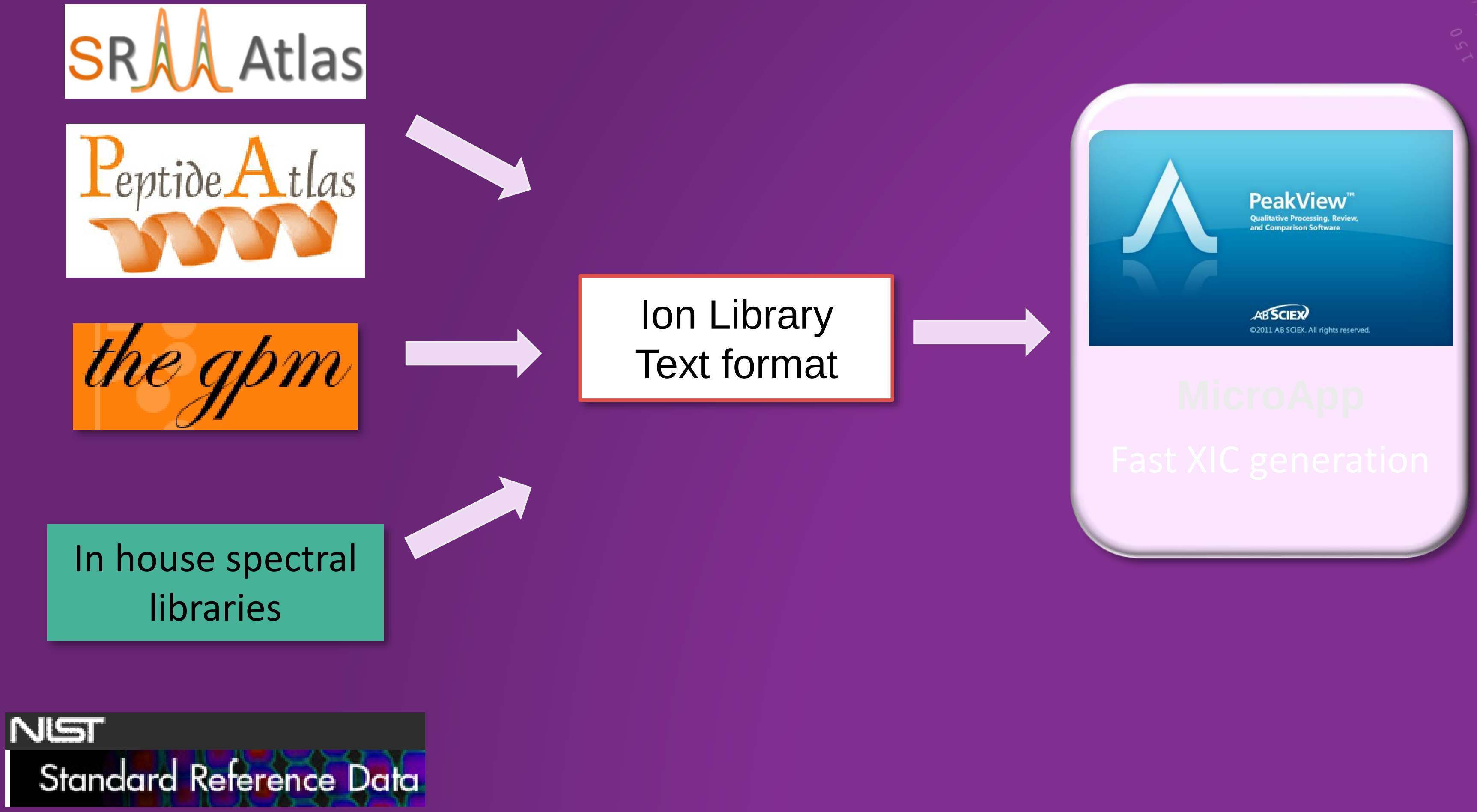


SWATH Scan Start Mass (Da) 400
SWATH Scan Stop Mass (Da) 1000
Expected LC Peak Width At Baseline (s) 30
Number of Points across LC Peak 12
SWATH Scan Accumulation Time (ms) 100.000
Analytes ○ Small Molecules ● Peptides

A method with 24 SWATH windows at width of 25.0 Da will be created with TOF mass range from 100.0 Da to 1500.0 Da.

MULTIPLE DATA SOURCES

INPUTS THROUGH THE ION LIBRARY TEXT INPUT



In house spectral libraries

Ion Library Text format

MicroApp

Fast XIC generation

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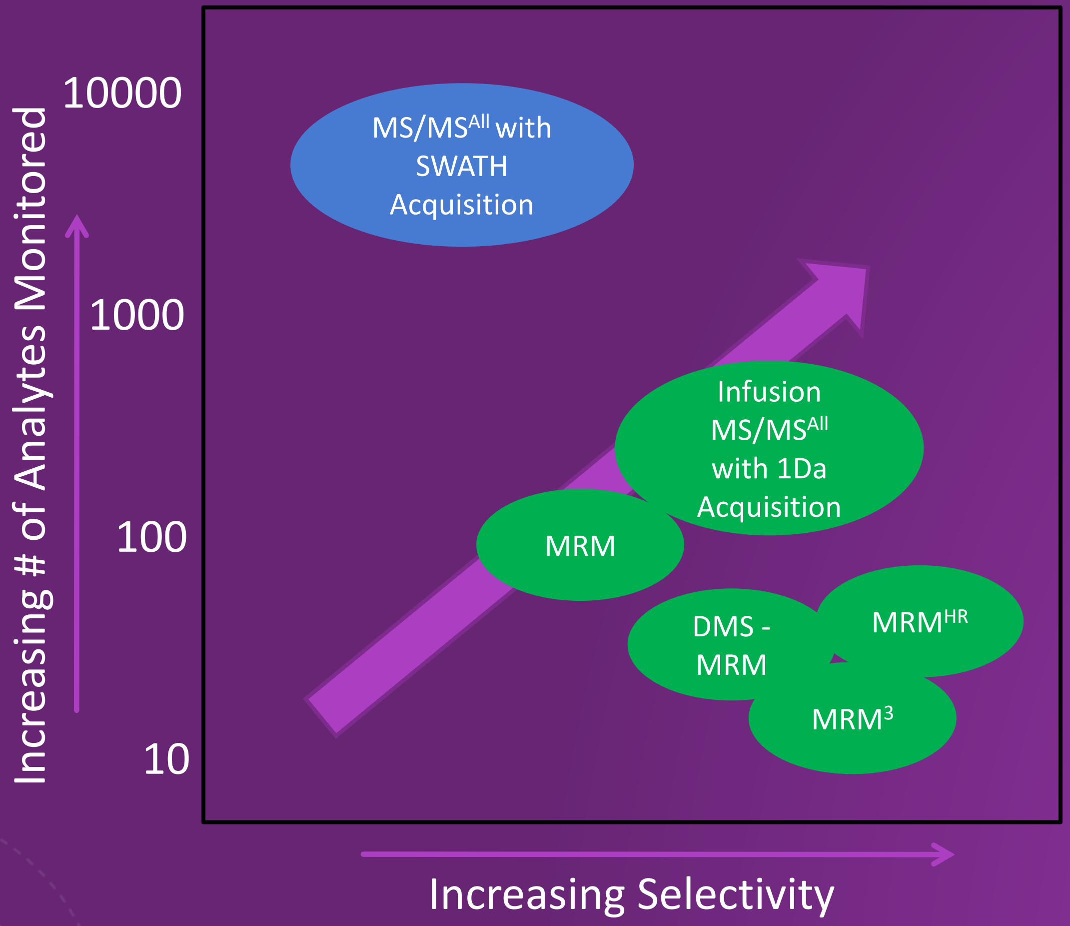
TARGETED WORKFLOWS



- The ideal experiment - full analyte coverage with highest specificity for quantitation
- Range of targeted quantitative workflows
- Infusion MS/MS^{All} workflow for lipid analysis
- Sequential Windowed Acquisition (SWATH)
- Coupled with high resolution, sensitivity and speed in MS/MS to provide quantification of all compounds

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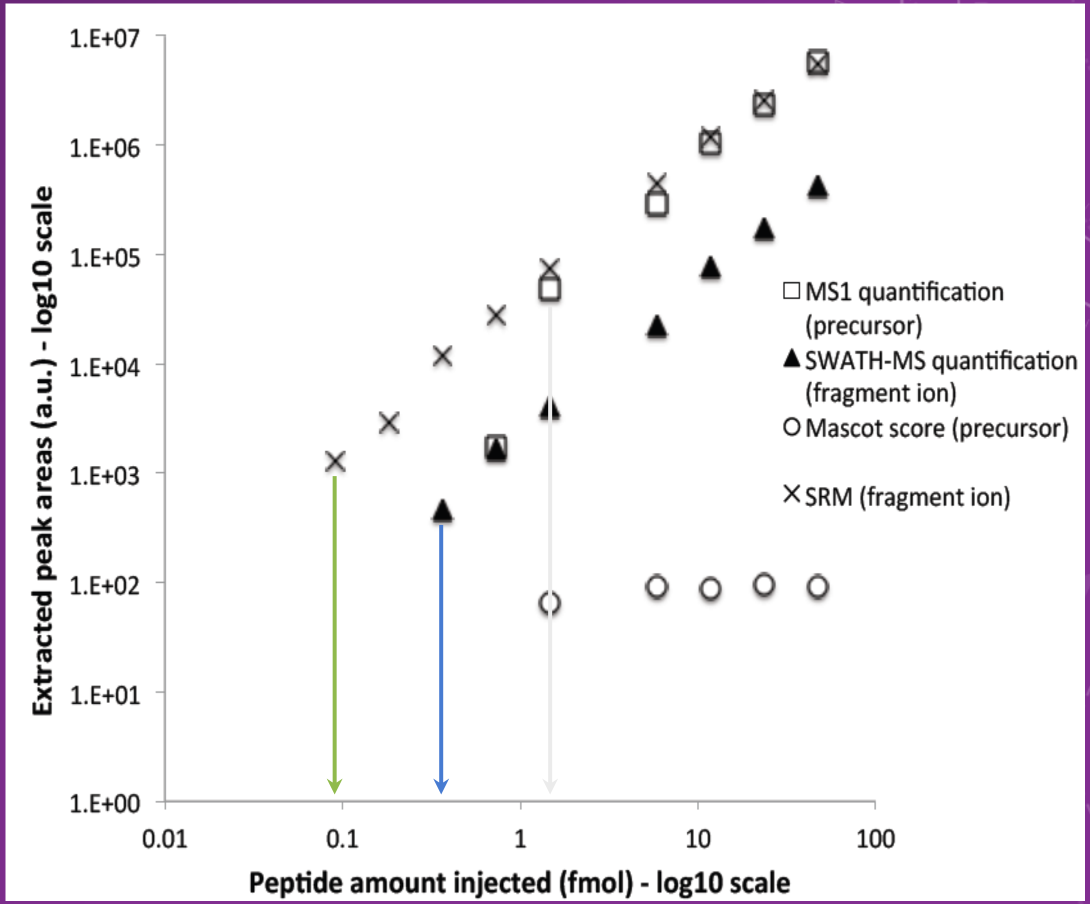
SENSITIVITY ASSESSMENT

- Comparison of the LLOQ of three quantitation techniques
- ELGQSGVDTYLQTK diluted into a yeast background
- MS1 – open squares is limited by background
- SWATH™ Acquisition provides better specificity by using fragment ions for quant
- MRM provides best LLOQ



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