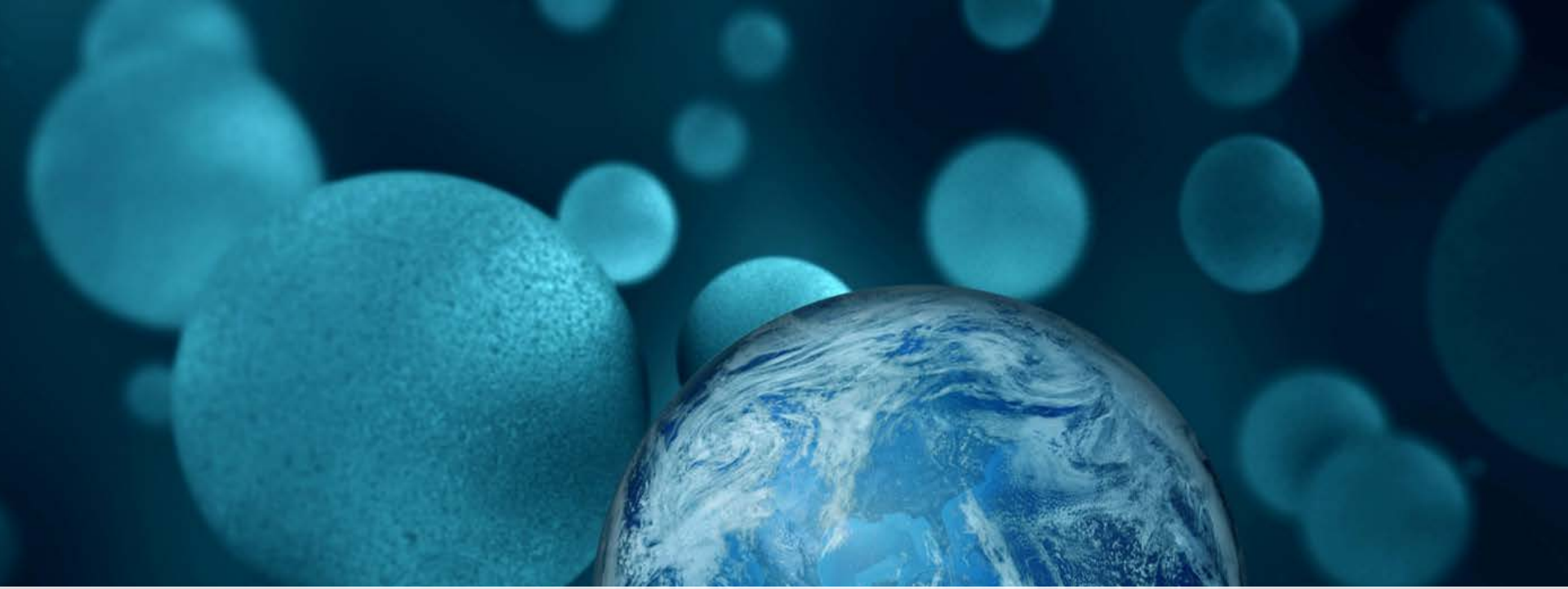

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High Resolution Accurate Mass Peptide Quantitation on Thermo Scientific™ Q Exactive™ Mass Spectrometers

Goals

- Explore the capabilities of **H**igh **R**esolution **A**ccurate **M**ass for Peptide Quantitation
  - Balancing the benefits of speed, selectivity, and number of analytes
- Go through an example of peptide quantitation workflow
  - Discovery-based, data dependent MS for target selection
  - Setting up the targeted SIM (single ion monitoring) or PRM (parallel reaction monitoring) method
  - Data evaluation
- Tips and tricks for optimal performance
  - Best practice techniques for acquiring reliable data
  - Reference guides for HRAM MS set-up

HRAM Peptide Quantitation on Q Exactive Series Mass Specs

- Q Exactive MS instruments have several key features:
  - High resolution precursor measurements
  - High resolution fragment measurements
  - Efficient precursor window isolation
  - Multiplexing capabilities
- Targeted quantitation experiment possibilities:
  - Full MS
  - t-SIM
  - PRM (with and without MS scan)
- Untargeted quantitation
  - DIA

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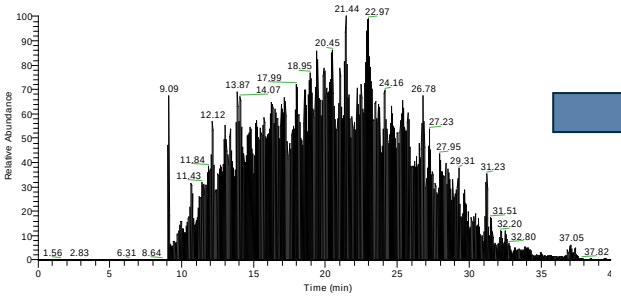
An Example HRAM Experiment: Discovery to Quan

Top 20 dd-MS2 Screen



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Peptide Selection

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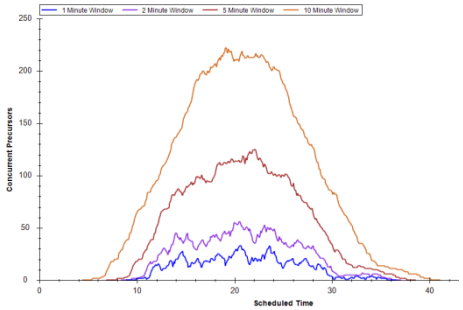
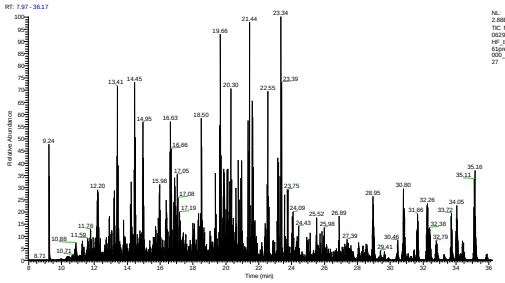


- Balance RT windows and number of targets
- Adjust MS parameters for best cycle time

Quan Method



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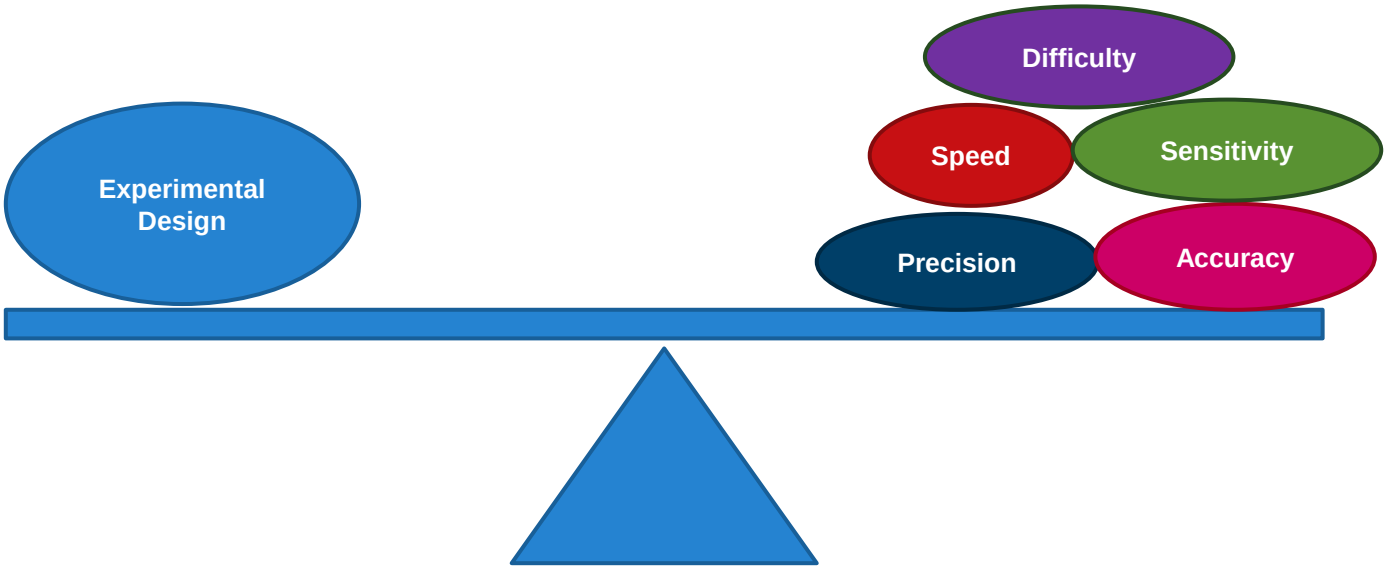
Define the Experiment

- What are your goals?
  - *Are you trying to quantitate 1000 peptides?*
  - *Is absolute quantitation of only 5 targets your priority?*
  - *Are you interested in only targeting, or do you also want to survey the sample?*

- There will always be a sacrifice within the targeted experiment
  - *Choose the right balance for your experiment*



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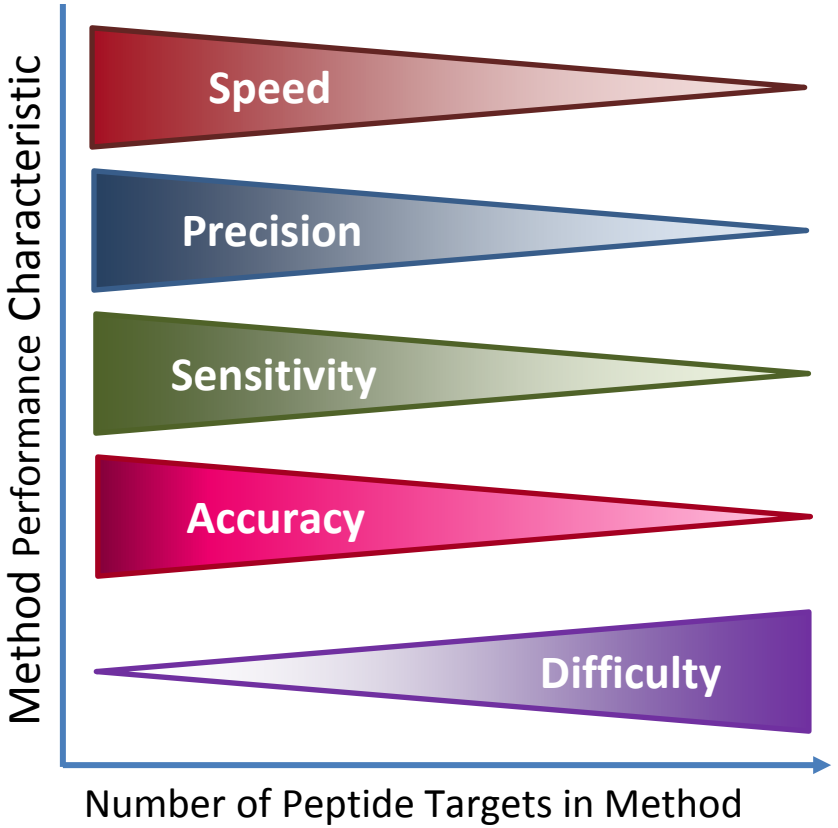
Considerations for Number of Targets

As numbers of peptide targets increase:

- LC method may need to be longer for best separation
  - Affects method throughput
- Fewer measurements can be made for each target
  - May affect reproducibility, sensitivity and accuracy
- More targets means RT scheduling
  - Choosing RT window widths and balancing concurrent precursors can be challenging



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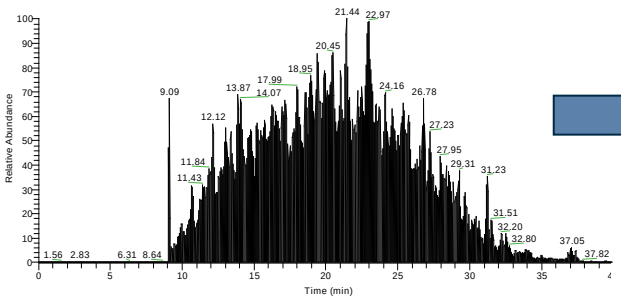
An Example HRAM Experiment: Discovery to PRM

Top 20 dd-MS2 Screen



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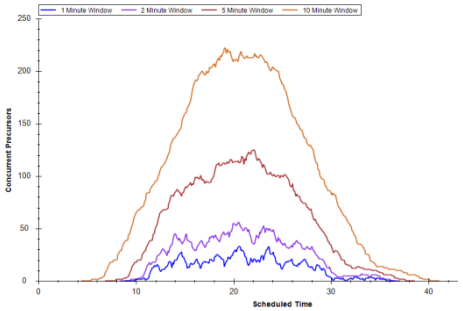

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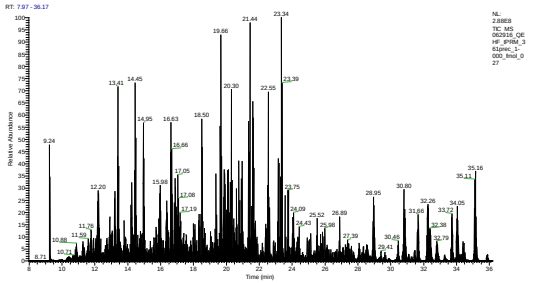
Quan Method



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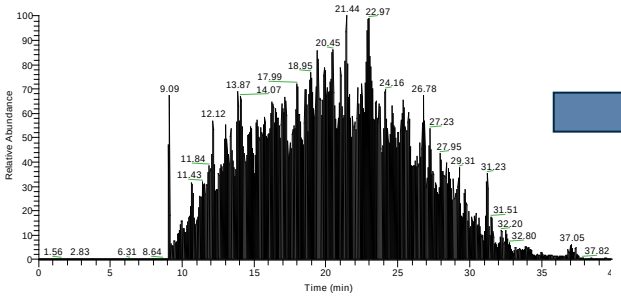

Database Search

Peptide Selection

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Example Experimental Design: Data Dependent Acquisition

Sample Details

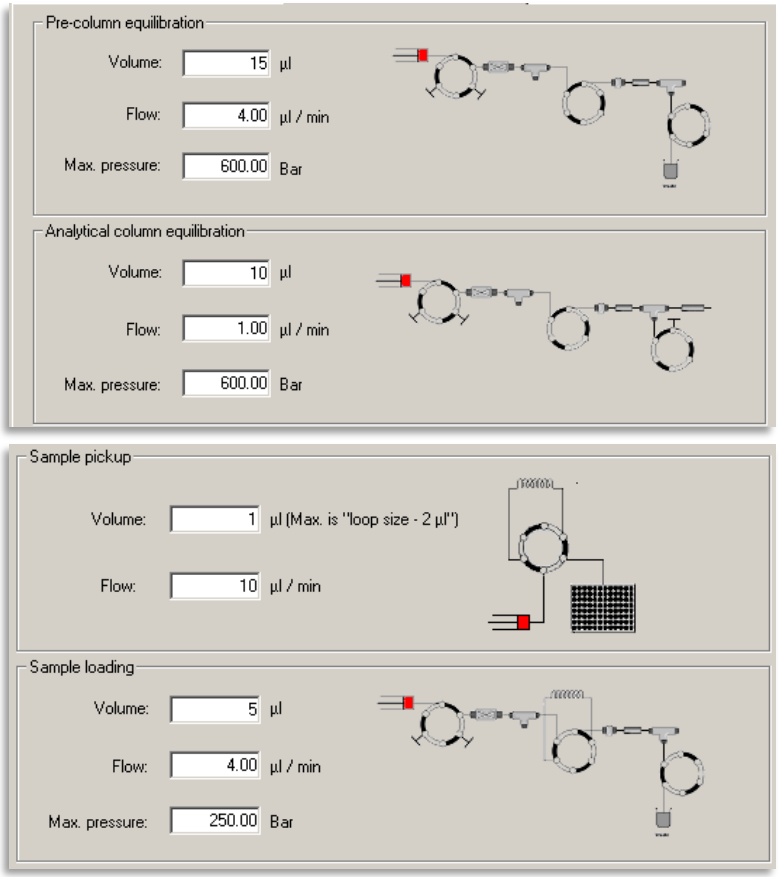
- Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (Part #88328)
  - 0.5 µg/µL
- Thermo Scientific™ Pierce™ Peptide Retention Time Calibration Mixture (Part #88320)
  - Response curve from 25 amol -100 fmol/µL
  - Light versions spiked at a fixed 10 fmol/µL
- 6×5 LC-MS/MS Peptide Reference Mix (Promega, Madison, WI, Part #V7495)
  - 20 amol – 200 fmol/uL
- MS Qual/Quant QC Mix (Sigma®, Saint Louis, MO, Part #MSQC1-1VL)
  - 14 light and heavy peptides at various L:H ratios

Thermo Scientific™ Q Exactive™ HF Mass Spectrometer Conditions

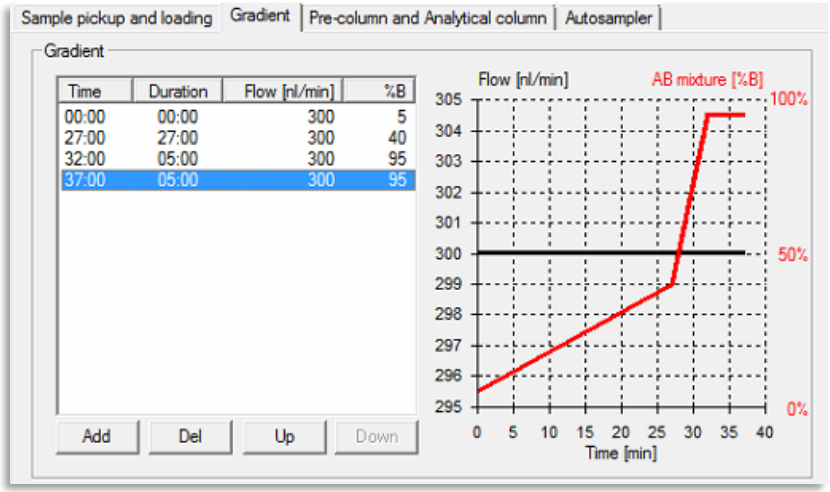
- Top 20 data dependent acquisition
- Full MS resolution: 120k
- Full MS AGC target: 3e6
- MS2 Resolution: 15k
- MS2 max injection time: 20 ms
- MS2 AGC target: 1e5
- NCE: 27

Example LC Conditions – EASY-nLC 1000

- Trap-Elute configuration
  - Trap column: Thermo Scientific™ Acclaim™ PepMap™ 100 C18 (100 µm ID x 2 cm L, 5 µm particle)
  - Analytical column: Thermo Scientific™ EASY-Spray™ C18 (75 µm ID x 25 cm L, 2 µm particle)



- Gradient
  - A = 2% ACN/0.1% formic;
  - B = 90% ACN/0.1% formic



- Time per injection: 60 minutes

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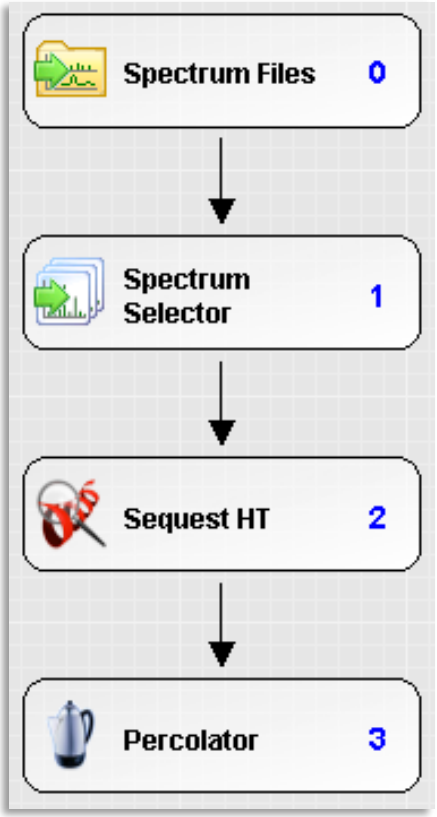
Proteome Discoverer – Identifying Peptide Candidates

- Search discovery data in Thermo Scientific™ Proteome Discoverer™ Software
  - Processing workflow



1. Input Data	
Protein Database	Homo sapiens (SwissProt TaxID=9606)
Enzyme Name	Trypsin (Full)
Max. Missed Cleavage Sites	2
Min. Peptide Length	6
Max. Peptide Length	144
2. Tolerances	
Precursor Mass Tolerance	10 ppm
Fragment Mass Tolerance	0.05 Da
Use Average Precursor Mass	False
Use Average Fragment Mass	False
3. Spectrum Matching	
Use Neutral Loss a Ions	True
Use Neutral Loss b Ions	True
Use Neutral Loss y Ions	True
Use Flanking Ions	True
Weight of a Ions	0
Weight of b Ions	1
Weight of c Ions	0
Weight of x Ions	0
Weight of y Ions	1
Weight of z Ions	0
4. Dynamic Modifications	
Max. Equal Modifications Per Peptide	3
1. Dynamic Modification	Oxidation / +15.995 Da (M)
2. Dynamic Modification	None
3. Dynamic Modification	None
4. Dynamic Modification	None
5. Dynamic Modification	None
6. Dynamic Modification	None
5. Dynamic Modifications (peptide terminus)	
6. Dynamic Modifications (protein terminus)	
7. Static Modifications	
Peptide N-Terminus	None
Peptide C-Terminus	None
1. Static Modification	Carbamidomethyl / +57.021 Da (C)
2. Static Modification	None
3. Static Modification	None
4. Static Modification	None
5. Static Modification	None
6. Static Modification	None

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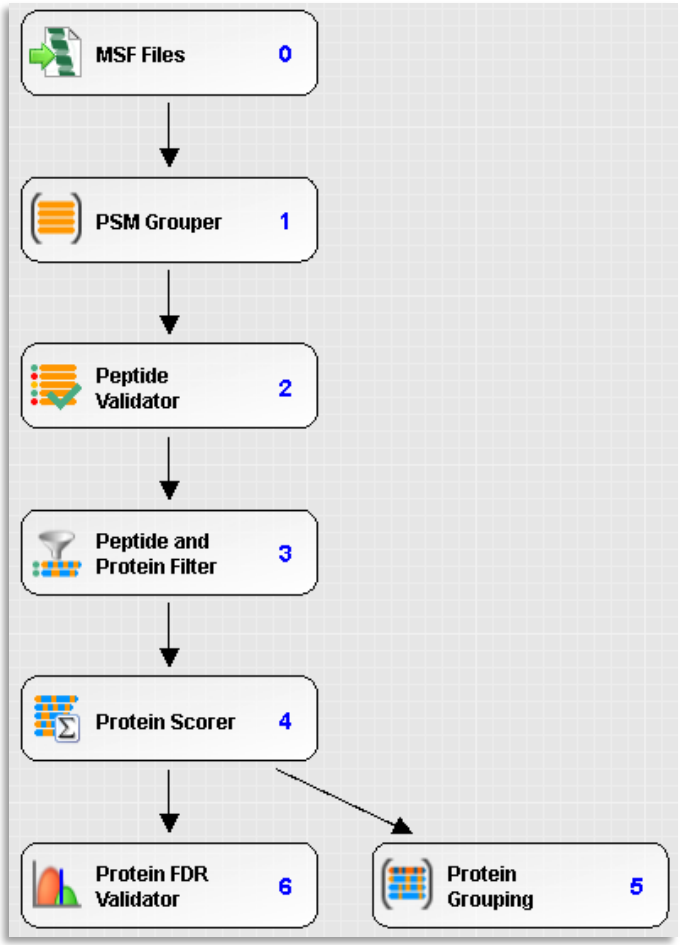
Proteome Discoverer – Identifying Peptide Candidates

- Search discovery data in Proteome Discoverer
  - Consensus workflow



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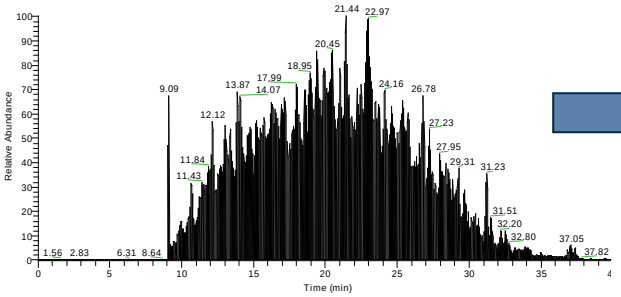
An Example HRAM Experiment: Discovery to PRM

Top 20 dd-MS2 Screen



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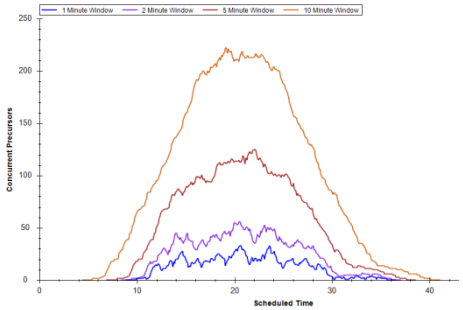

- Balance RT windows and number of targets
- Adjust MS parameters for best cycle time

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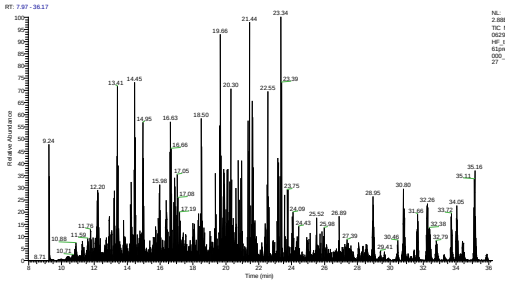

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An Example HRAM Experiment: Discovery to PRM

- Import .msf file into Skyline software
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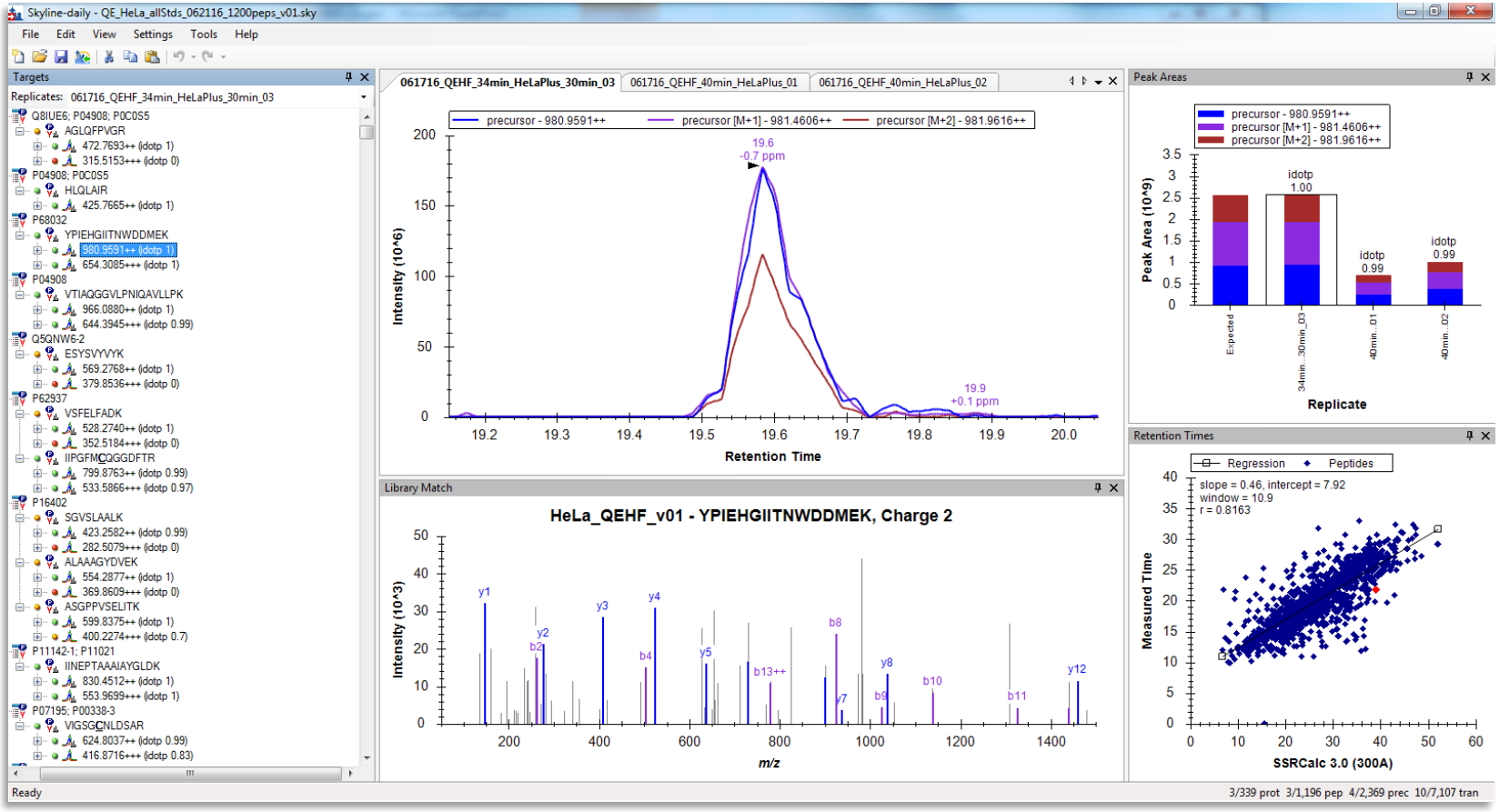
Evaluate Targets in Discovery Data with Skyline

- Raw files from discovery experiments can be re-analyzed to observe selected peptides from MS1 data in Skyline
- Once peptide targets are selected, import discovery data to ensure peptide is detected and to verify RT and isotopic correlation



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Proteotypic Peptide Selection – Refining the List

- "Proteotypic peptides are defined as the peptides that uniquely identify each protein and are consistently observed when a sample mixture is interrogated by a (tandem) mass spectrometer."[1]

- Key Criteria for peptide selection:

Unique sequence for target protein	Length of 6-25 amino acids	Not too hydrophilic or hydrophobic
No PTMs	No Missed cleavages	Ionizes well
Fragments generated are a variety of ions (i.e. not just 3-4 ions)	Not too close to N or C terminus (exoproteases)	

1. Mallick, P. et al. Nat. Biotechnol. 25, 125–131 (2007).

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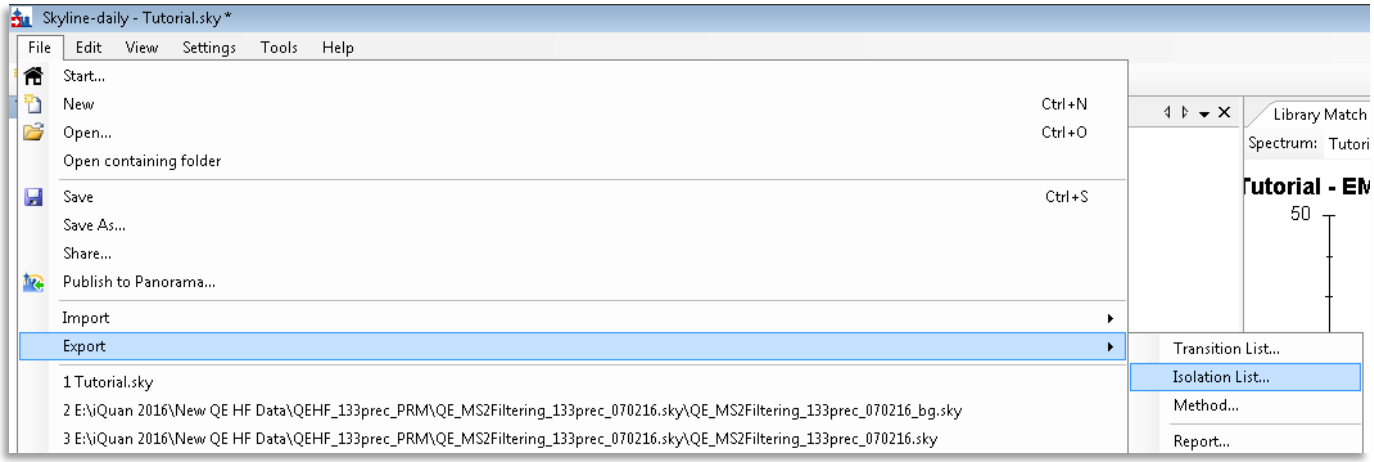
Generating a Q Exactive MS Inclusion List from Skyline

- Once you have refined your list, you can export the isolation list to set up your PRM instrument method.



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Generating a Q Exactive MS Inclusion List from Skyline

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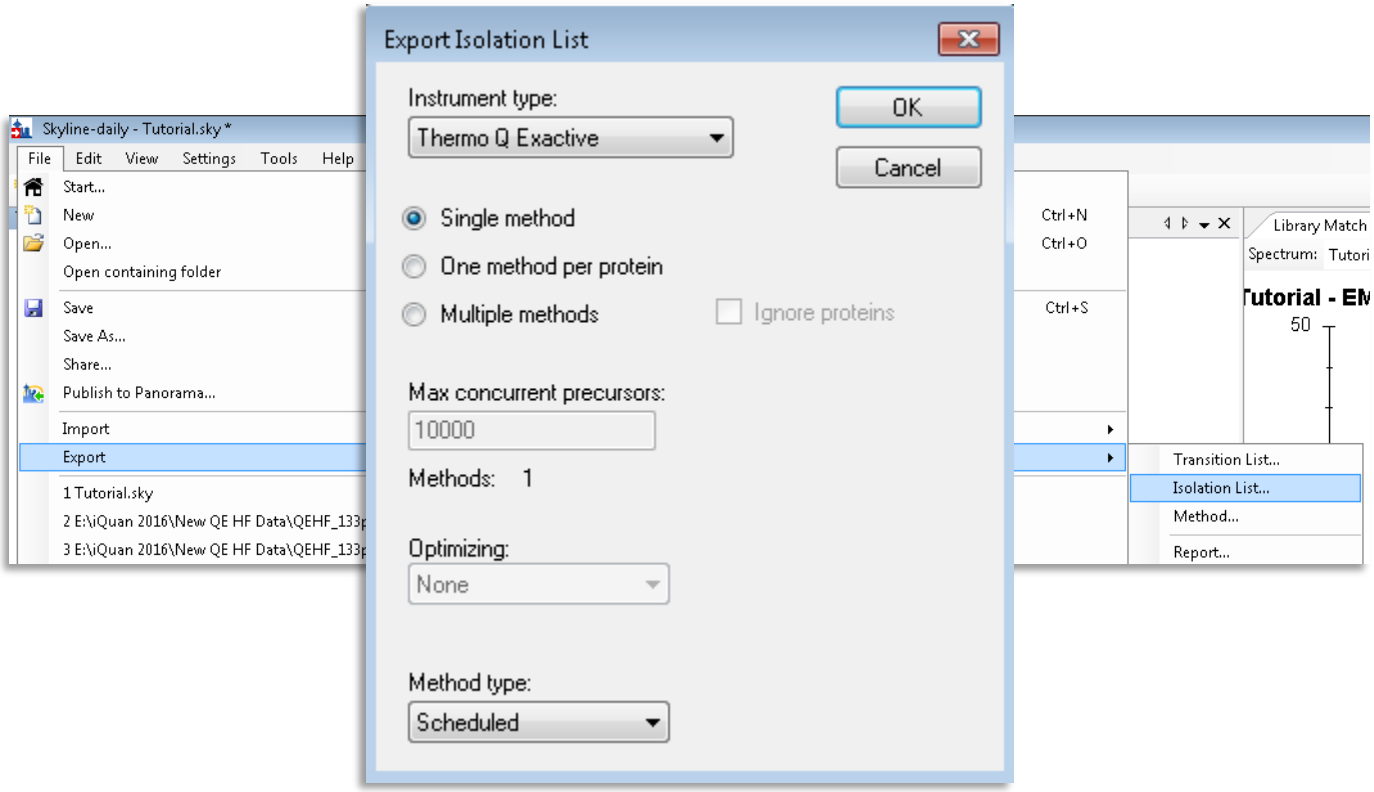
- Choose your instrument type and make sure to set method type to *Scheduled* if you have a high number of targets.



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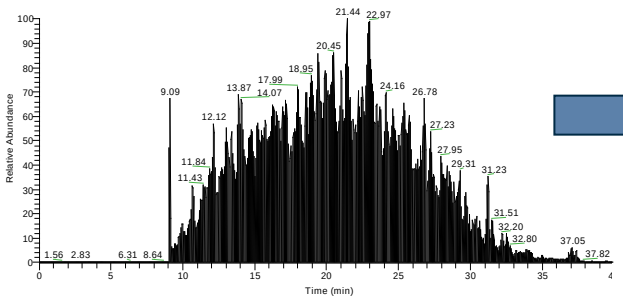

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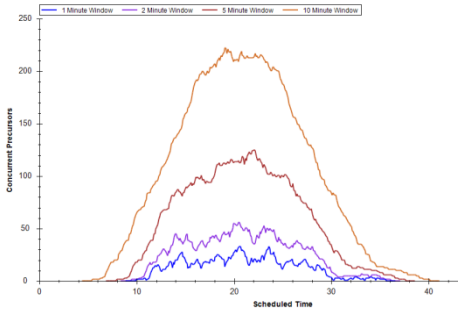

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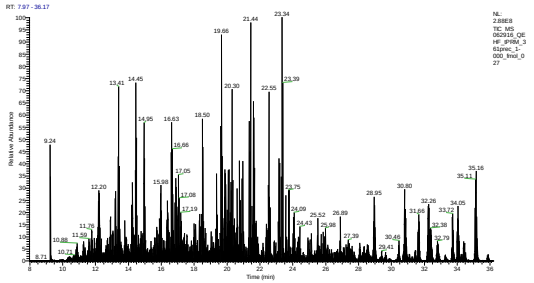
Quan Method



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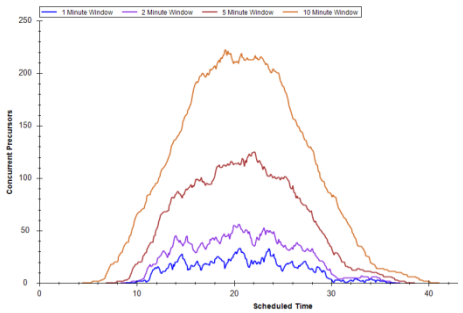

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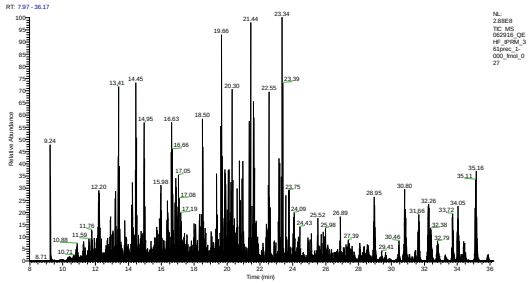

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Keys to a Successful Quantitation Experiment

- **Reproducibility!!**
  - Reproducibility in retention time
  - Reproducibility in overall signal within replicates
- Obtaining adequate scans across the peak
  - This is critical for accurate quantitation
  - *Tip: 10 scans across the peak for a middle level target will result in enough scans across the peak at your limit of detection*
- Sample complexity
  - Interferences will dictate your limit of detection

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Keys to a Successful Quantitation Experiment

- Reproducibility in nanoLC Applications
- Whichever method you choose, optimization of nanoLC can improve RT consistency and throughput
- Short gradients
  - May be optimal for low-complexity samples
    - Throughput can be significantly increased
    - S/N can benefit from sharper, taller chromatographic peaks
    - Ensure you can achieve sufficient separations
    - Ensure you can acquire enough points across the shorter peaks

Thermo Scientific™ UltiMate™ 3000 RSLCnano System

Thermo Scientific™ EASY-nLC™ 1000

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Setting up a Quan Method

Questions Before Starting:

- How wide are my chromatographic peaks?
- How many targets do I have?
- What is the complexity of my sample?

The answers to these simple questions will greatly impact the design of your targeted instrument method

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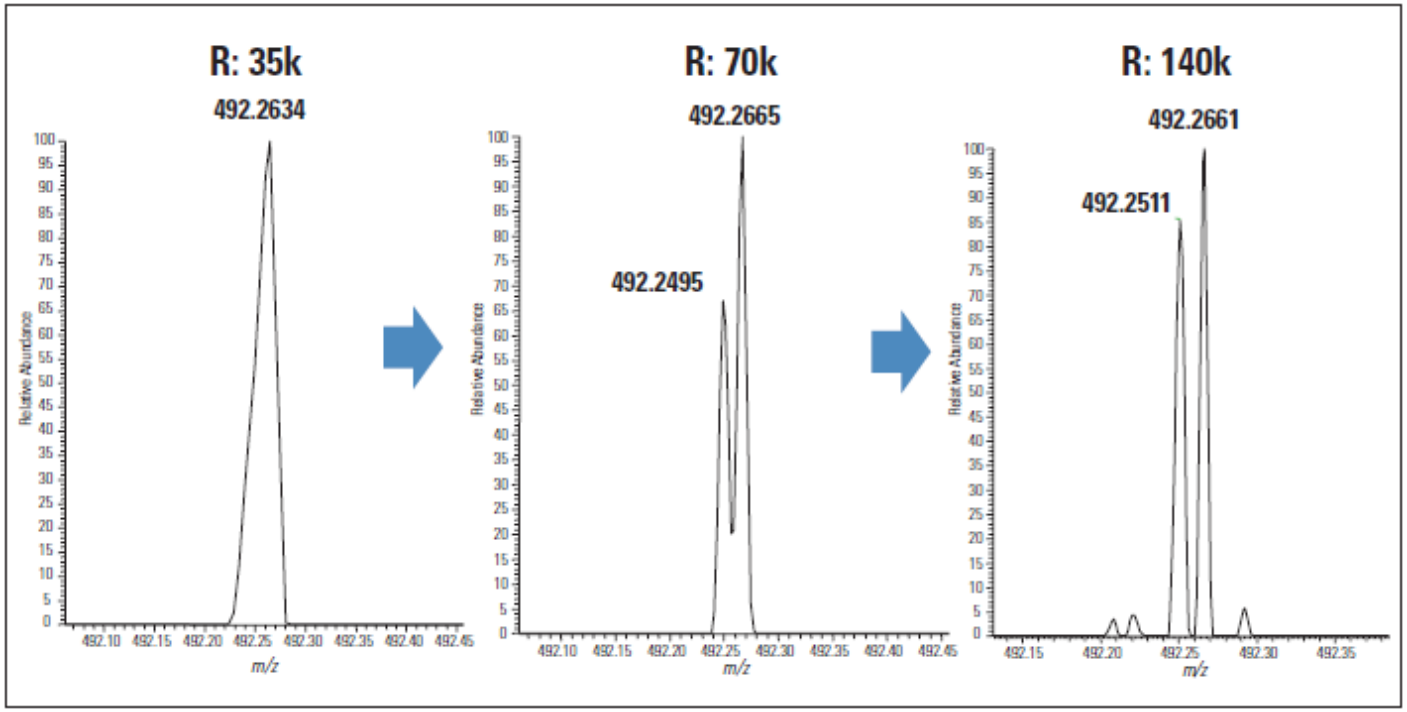
Benefits of HRAM



Resolution is the key to selectivity

HR/AM targeted peptide quantitation on a Q Exactive
MS. Zhang et al., *Thermo Scientific Application Note* 554

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Always Think About Total Cycle Time!

Thermo Scientific™ Q Exactive™ and Q Exactive Plus Mass Spectrometers

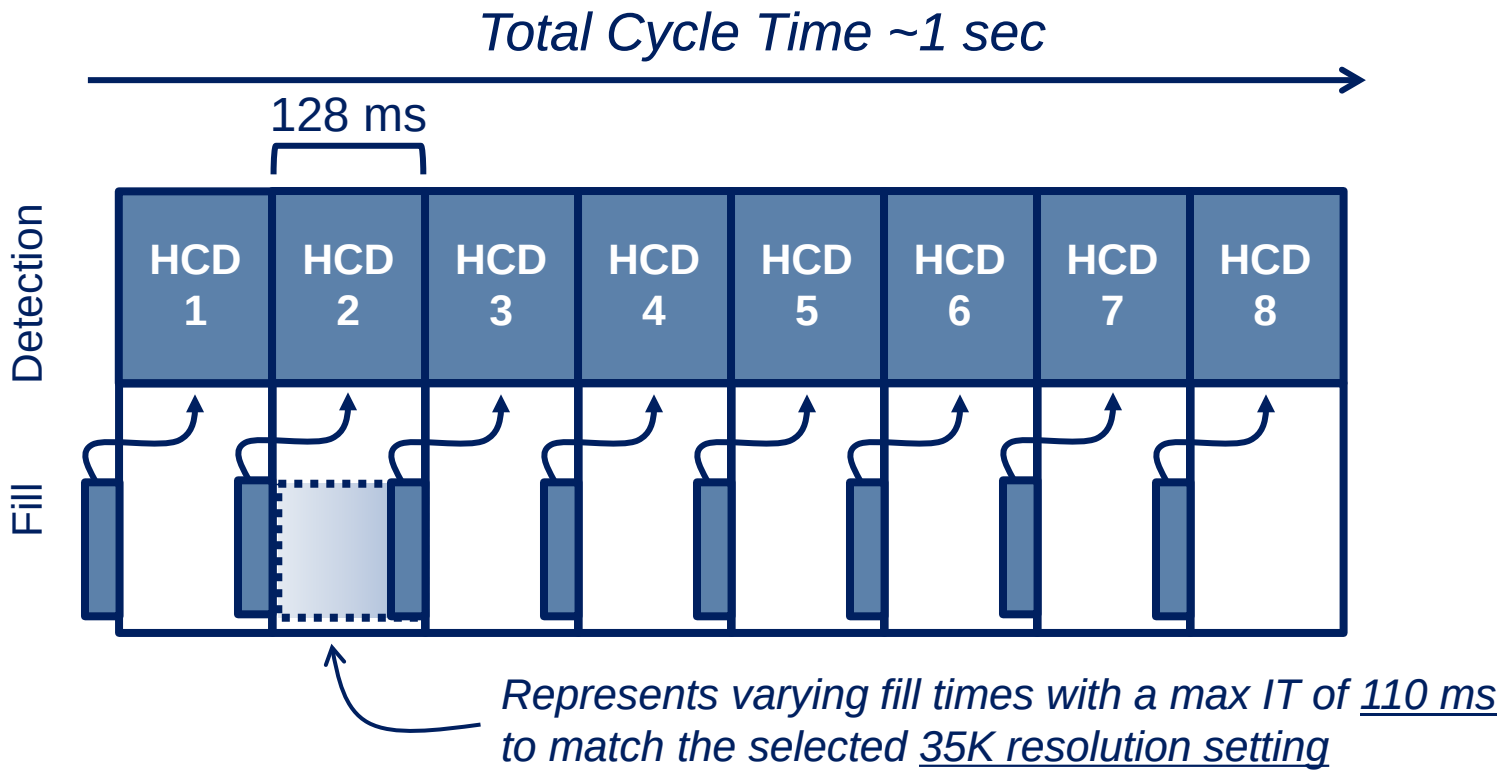
Resolving Power at m/z 200	Approximate scan speed (Hz)	Transient length (ms)	*Suggested Max Fill Time (ms)*
17,500	13	64	*50*
35,000	7	128	*110*
70,000	3	256	*240*
140,000	1.5	512	*500*

Q Exactive HF MS

Resolving Power at m/z 200	Approximate scan speed (Hz)	Transient length (ms)	*Suggested Max Fill Time (ms)*
15,000	18	32	*20*
30,000	12	64	*50*
60,000	7	128	*110*
120,000	3	256	*240*
240,000	1.5	512	*500*

ThermoFisher SCIENTIFIC

Always Think About Total Cycle Time!



- Total Cycle time is the length of time it takes to cycle through your entire target list
- This determines how many scans across the peak are obtained
- Sufficient amount of data points defines the peak to increase quantitative reproducibility

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Setting up a Quantitative Method

Questions Before Starting:

- How wide are my chromatographic peaks?
- How many targets do I have?
- What is the complexity of my sample?

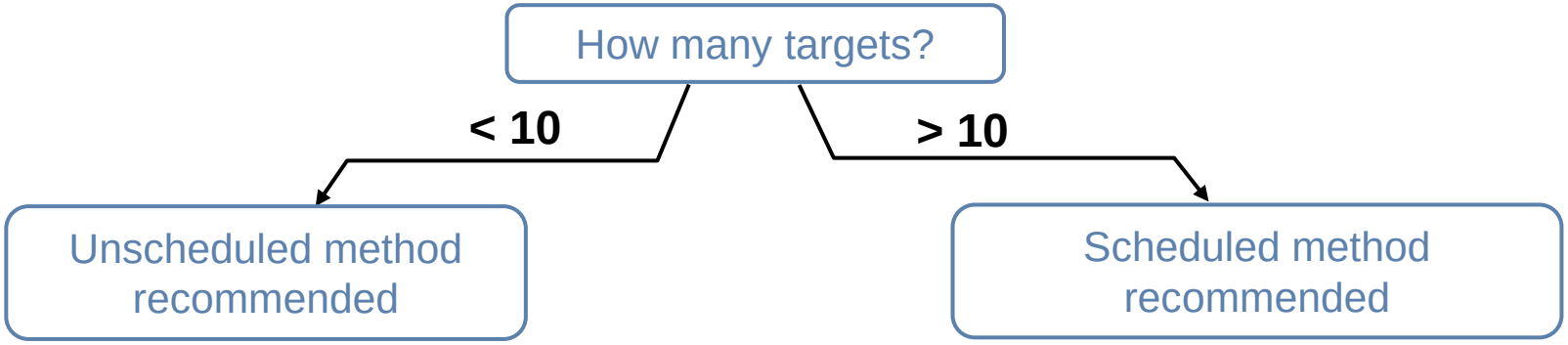
ThermoFisher SCIENTIFIC

Scheduled or Unscheduled? Q Exactive HF MS Example

How many targets?

ThermoFisher SCIENTIFIC

Scheduled or Unscheduled? Q Exactive HF MS Example



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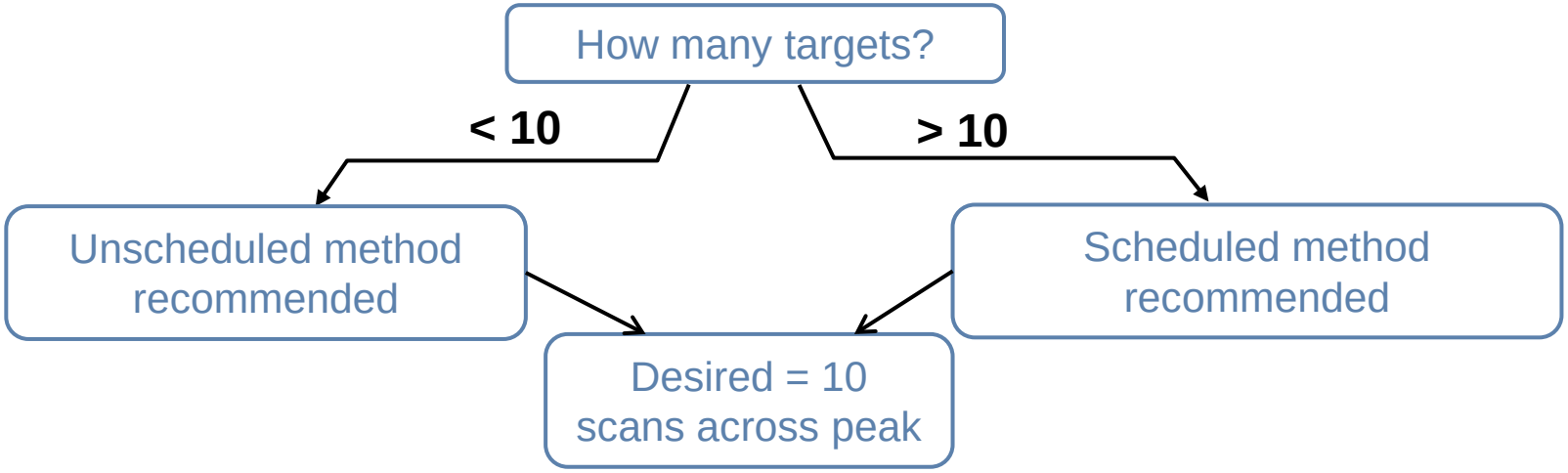
Scheduled or Unscheduled? Q Exactive HF MS Example



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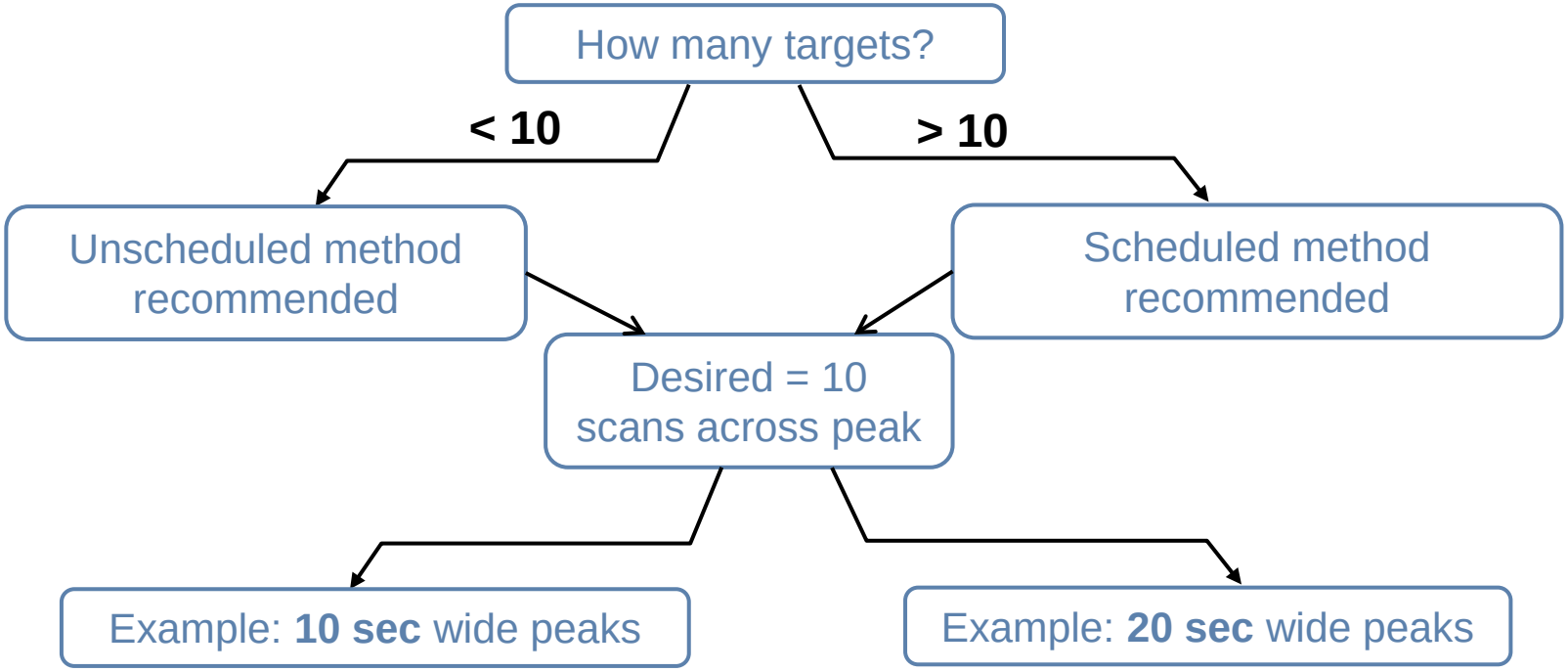

ThermoFisher SCIENTIFIC

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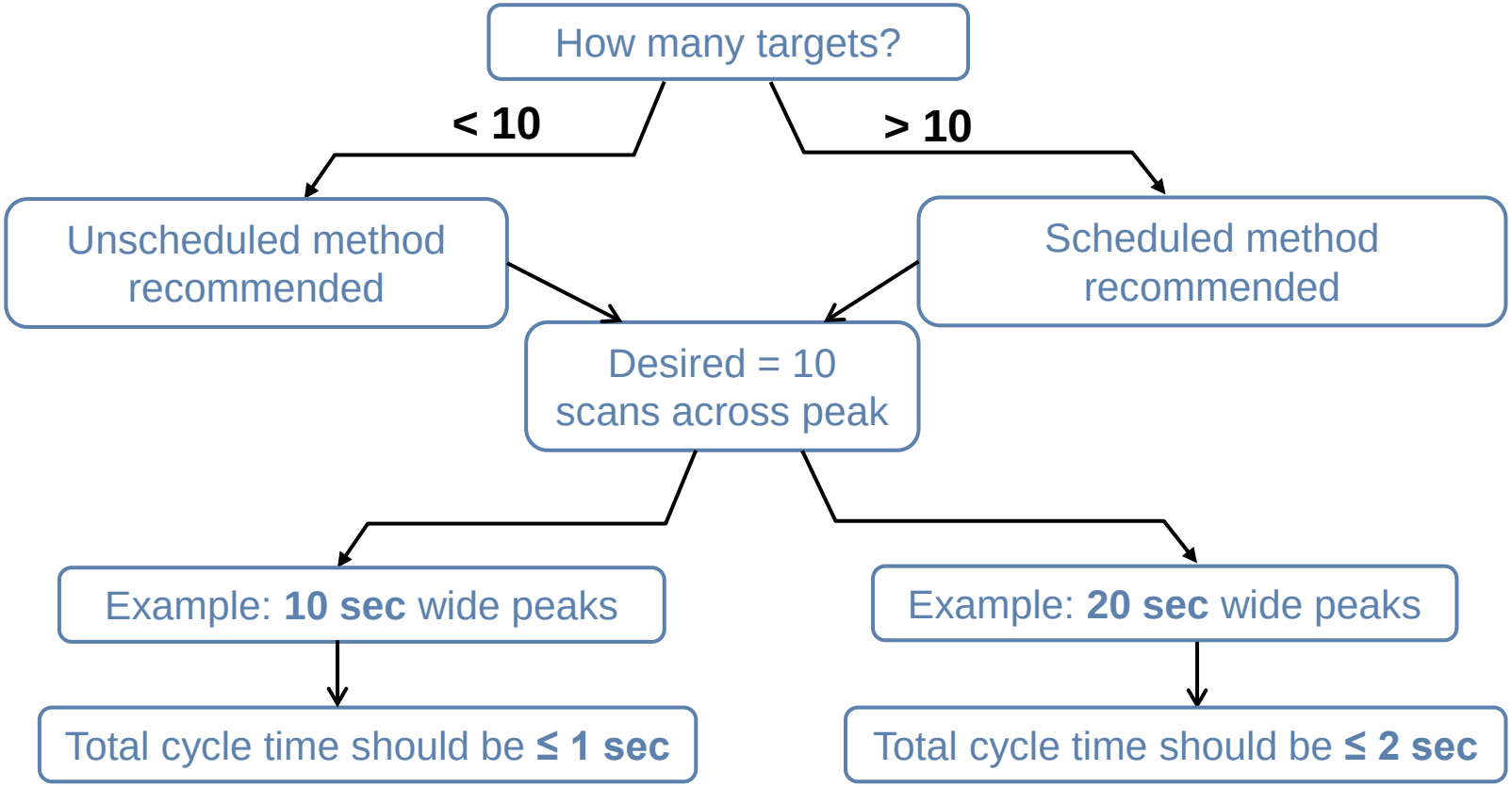

ThermoFisher SCIENTIFIC

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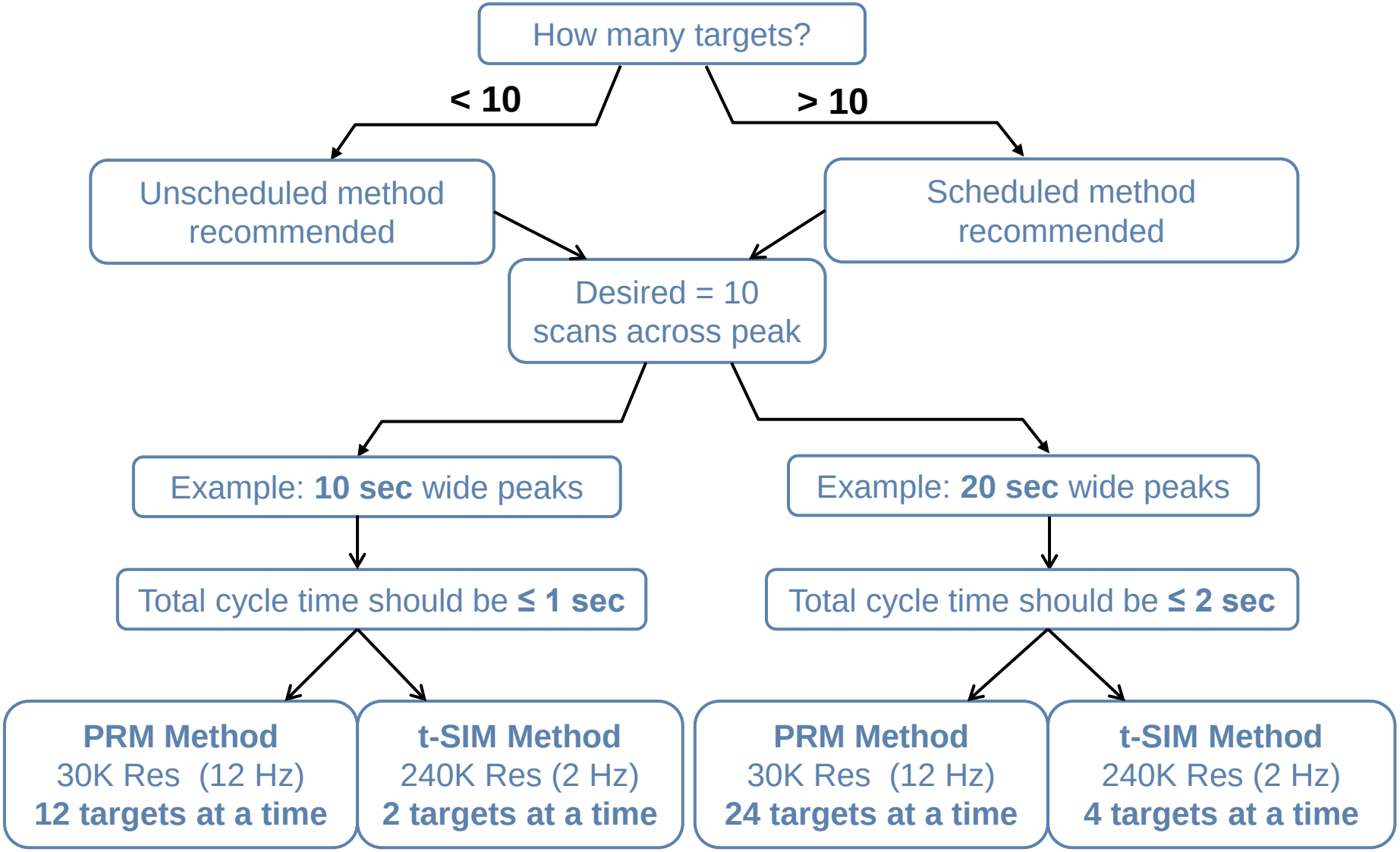
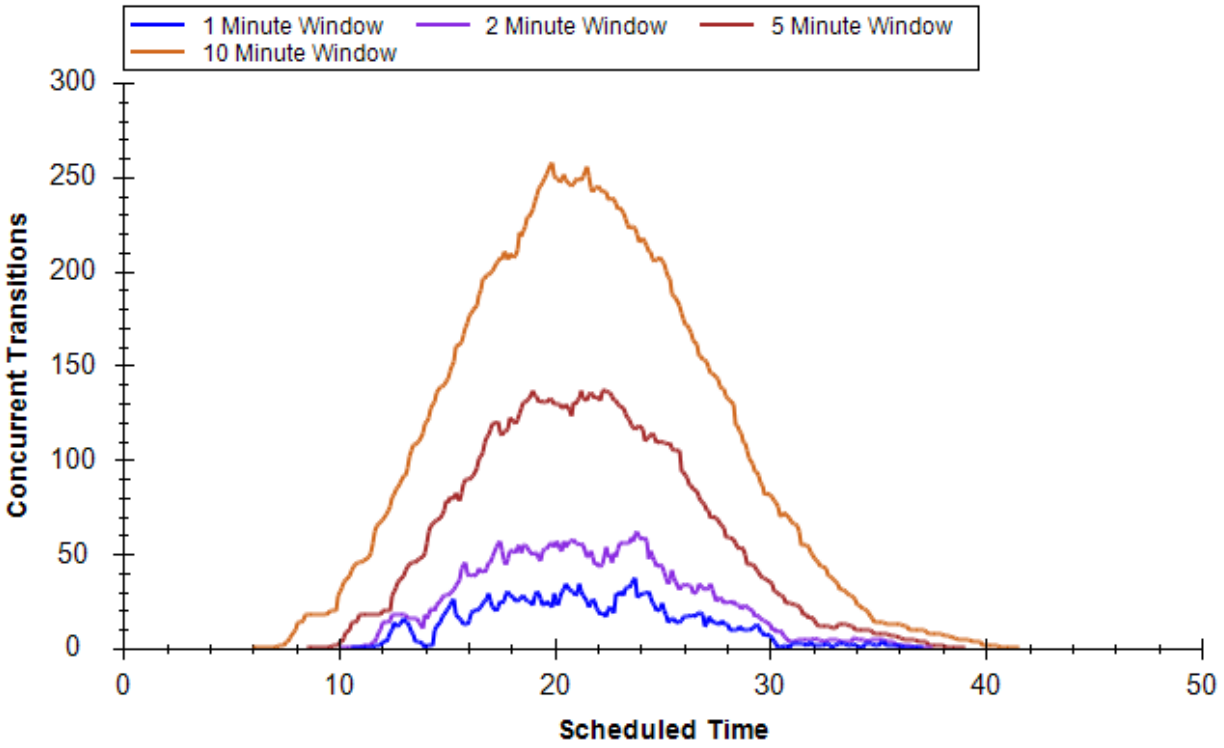


ThermoFisher SCIENTIFIC

Scheduled or Unscheduled?

- Scheduled acquisition increases the number of possible targets
- Narrow RT windows further increase the number of possible targets
  - Insufficient LC reproducibility can result in lost data if RT windows are too narrow
  - Balance RT window size to maximize number of targets without exceeding desired cycle time



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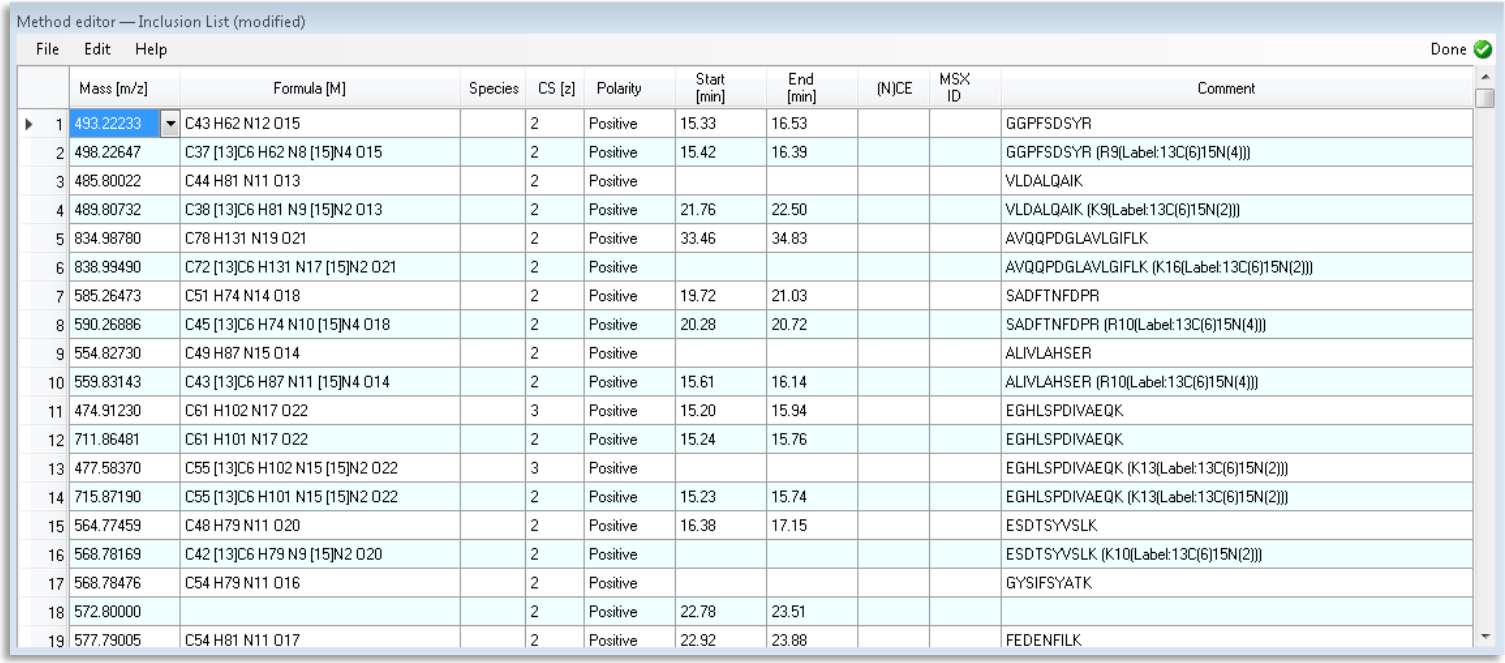
Incomplete Scheduling

- Often, the data-dependent acquisitions can provide accurate retention times
  - Not all targets may be acquired, though, due to interferences
- Constructing an incomplete scheduled list can help acquire the RTs of those not detected in ddMS2 runs
  - The RTs included on the list will mitigate lost cycle time that would result from a completely unscheduled target list
  - Sensitivity for the undetected targets is increased as they are scanned for during each cycle
  - RTs for the previously unscheduled targets can then be added to the targeted list – optimizing cycle time to get sufficient sampling across the peak


Method editor — Inclusion List (modified)

File Edit Help Done

	Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	Start [min]	End [min]	(N)CE	MSX ID	Comment
1	493.22233	C43 H62 N12 O15		2	Positive	15.33	16.53			GGPFSDSYR
2	498.22647	C37 [13]C6 H62 N8 [15]N4 O15		2	Positive	15.42	16.39			GGPFSDSYR (R9(Label:13C(6)15N(4)))
3	485.80022	C44 H81 N11 O13		2	Positive					VLDALQAIK
4	489.80732	C38 [13]C6 H81 N9 [15]N2 O13		2	Positive	21.76	22.50			VLDALQAIK (K9(Label:13C(6)15N(2)))
5	834.98780	C78 H131 N19 O21		2	Positive	33.46	34.83			AVQQPDGLAVLGIFLK
6	838.99490	C72 [13]C6 H131 N17 [15]N2 O21		2	Positive					AVQQPDGLAVLGIFLK (K16(Label:13C(6)15N(2)))
7	585.26473	C51 H74 N14 O18		2	Positive	19.72	21.03			SADFTNFDPR
8	590.26886	C45 [13]C6 H74 N10 [15]N4 O18		2	Positive	20.28	20.72			SADFTNFDPR (R10(Label:13C(6)15N(4)))
9	554.82730	C49 H87 N15 O14		2	Positive					ALIVLAHSER
10	559.83143	C43 [13]C6 H87 N11 [15]N4 O14		2	Positive	15.61	16.14			ALIVLAHSER (R10(Label:13C(6)15N(4)))
11	474.91230	C61 H102 N17 O22		3	Positive	15.20	15.94			EGHLSPDIVAEQK
12	711.86481	C61 H101 N17 O22		2	Positive	15.24	15.76			EGHLSPDIVAEQK
13	477.58370	C55 [13]C6 H102 N15 [15]N2 O22		3	Positive					EGHLSPDIVAEQK (K13(Label:13C(6)15N(2)))
14	715.87190	C55 [13]C6 H101 N15 [15]N2 O22		2	Positive	15.23	15.74			EGHLSPDIVAEQK (K13(Label:13C(6)15N(2)))
15	564.77459	C48 H79 N11 O20		2	Positive	16.38	17.15			ESDTSYVSLK
16	568.78169	C42 [13]C6 H79 N9 [15]N2 O20		2	Positive					ESDTSYVSLK (K10(Label:13C(6)15N(2)))
17	568.78476	C54 H79 N11 O16		2	Positive					GYSIFSYATK
18	572.80000			2	Positive	22.78	23.51			
19	577.79005	C54 H81 N11 O17		2	Positive	22.92	23.88			FEDENFILK

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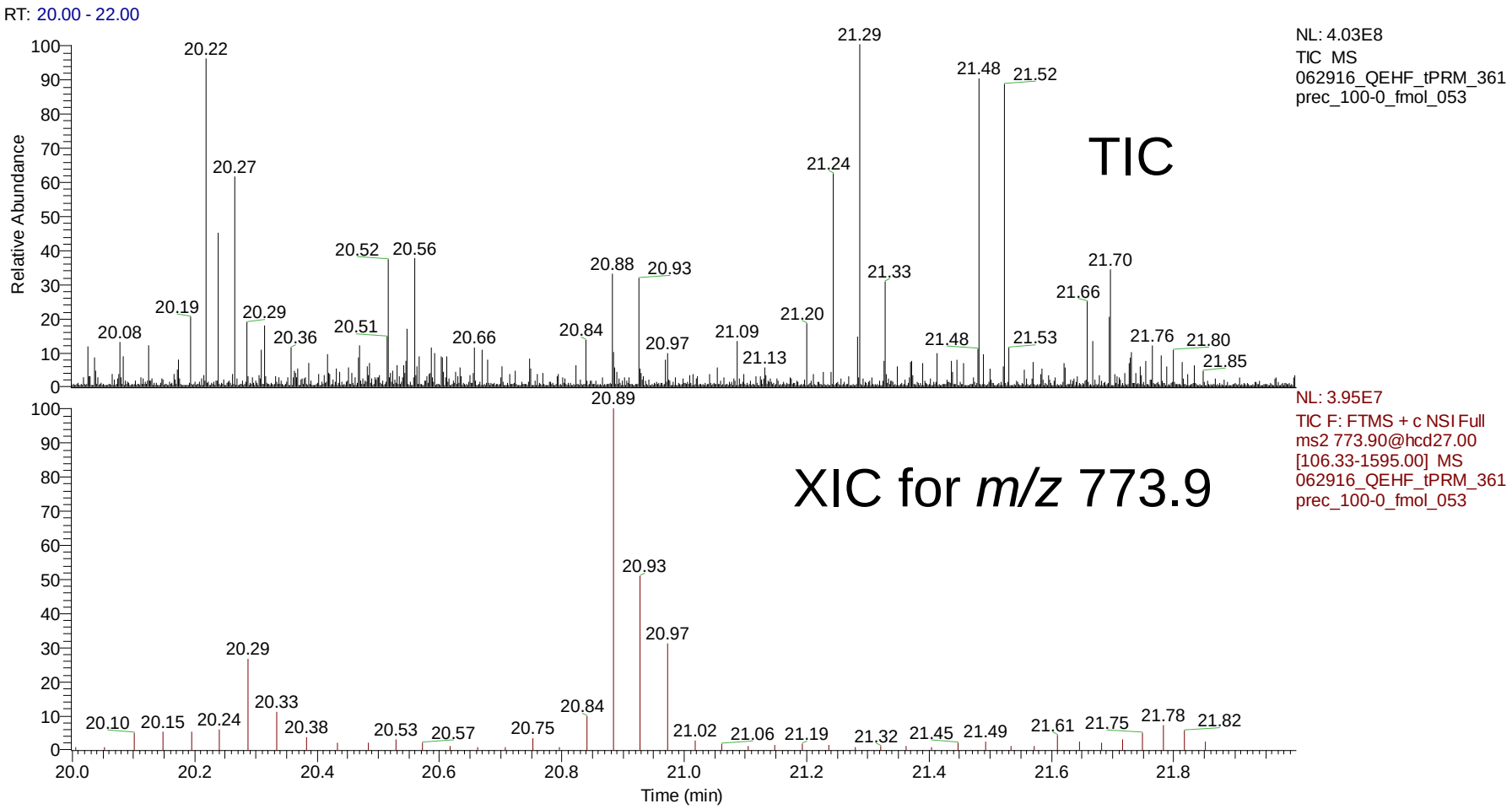
Worst case scenario: too many concurrent precursors



For a 361 precursor method, the 20 minute mark had too many concurrent precursors scheduled, and could only generate ~6 points across the chromatographic peak with a 2 minute RT window.

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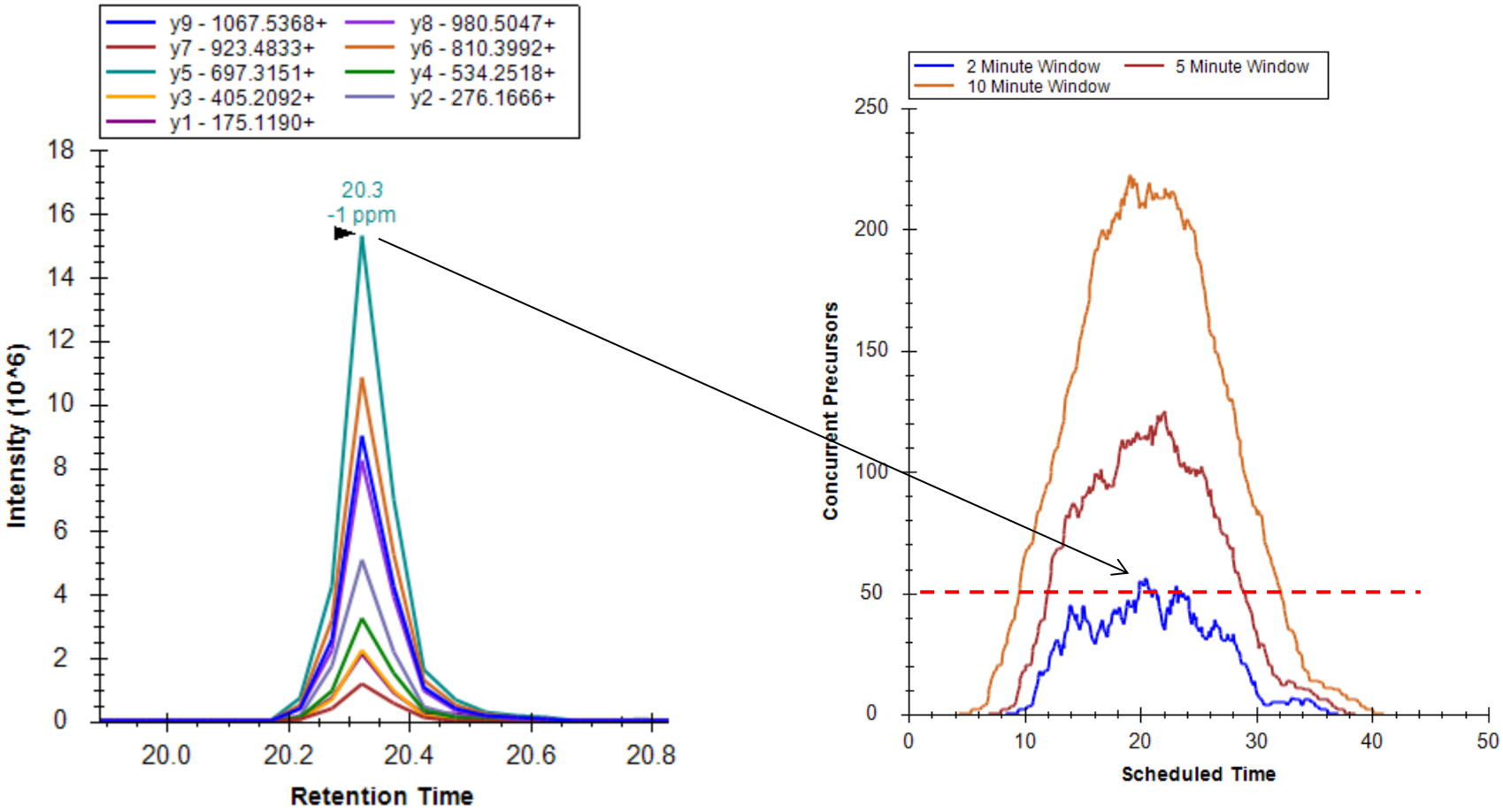
Same data, in Skyline



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Setting up a Quantitative Method

Questions Before Starting:

- How wide are my chromatographic peaks?
- How many targets do I have?
- What is the complexity of my sample?

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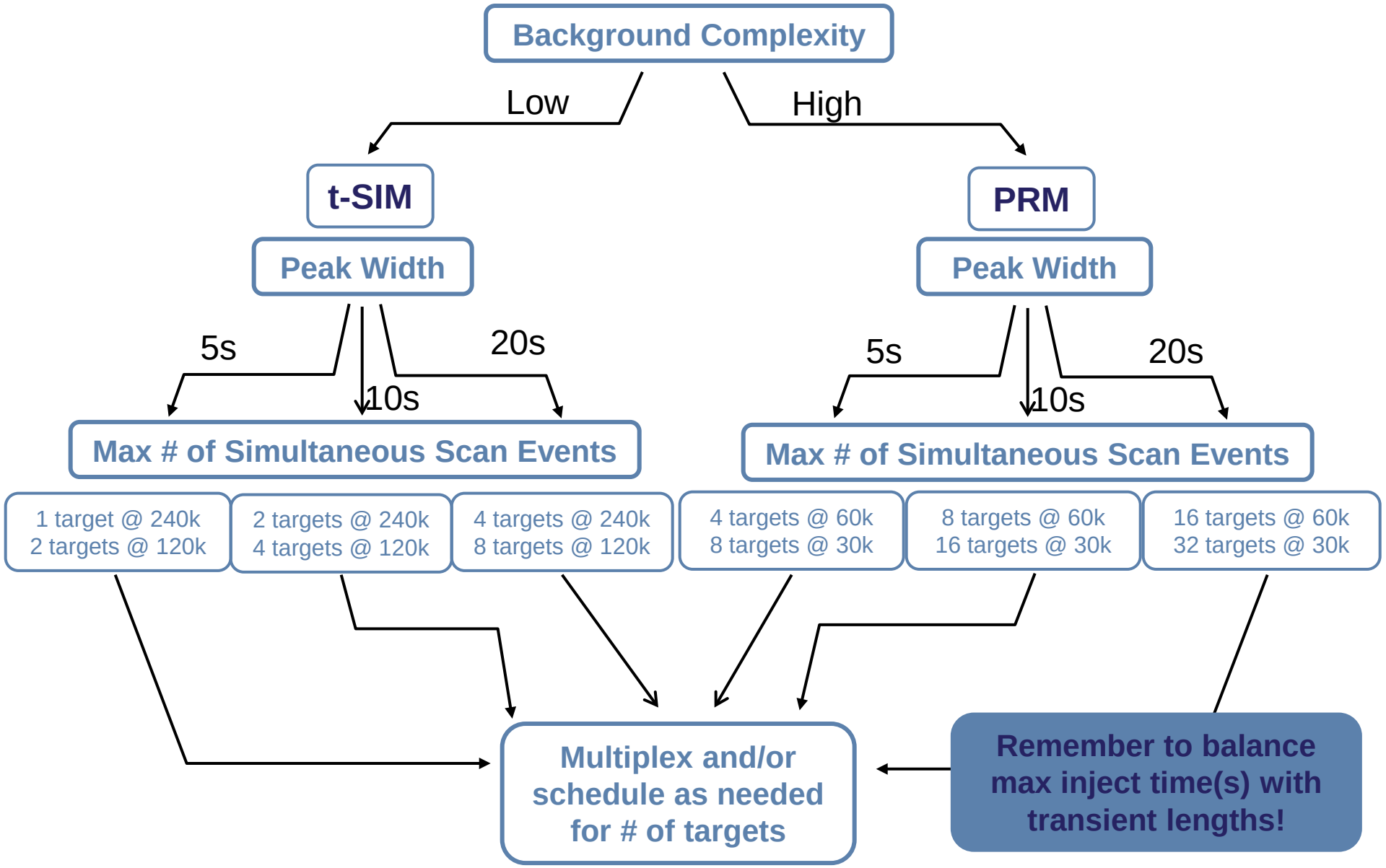
Method Selection Flowchart



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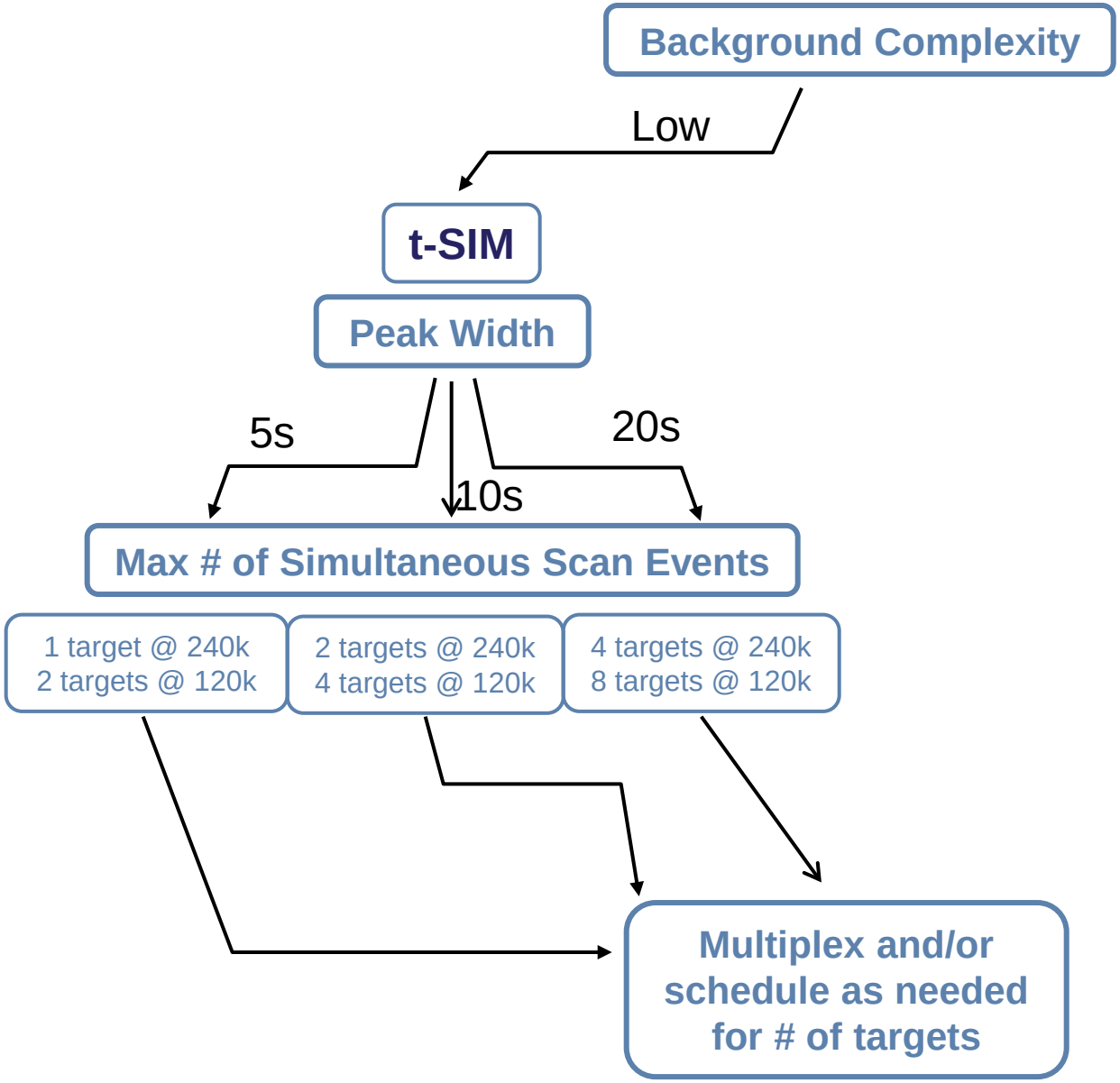
Method Selection Flowchart



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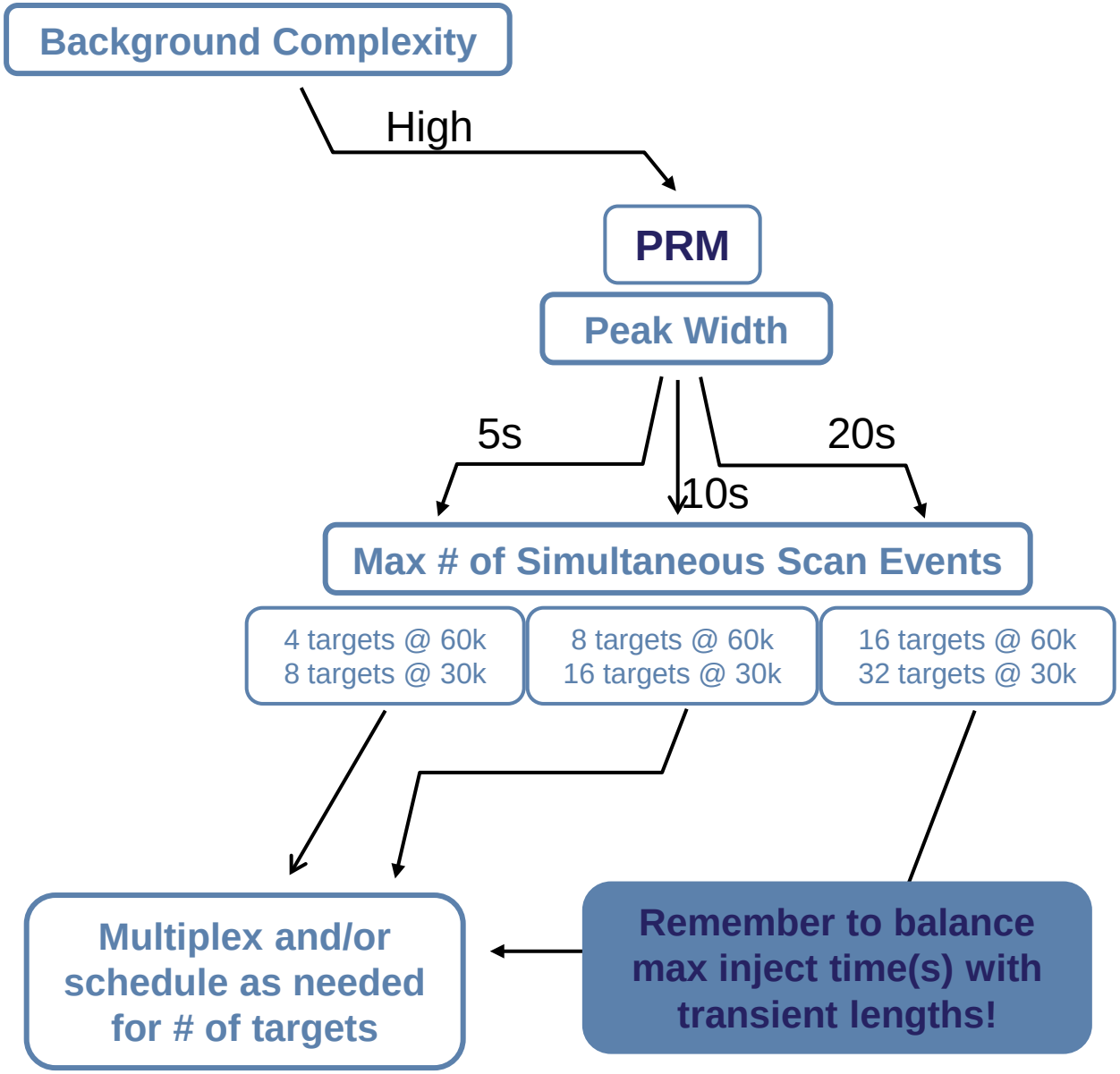
Method Selection Flowchart



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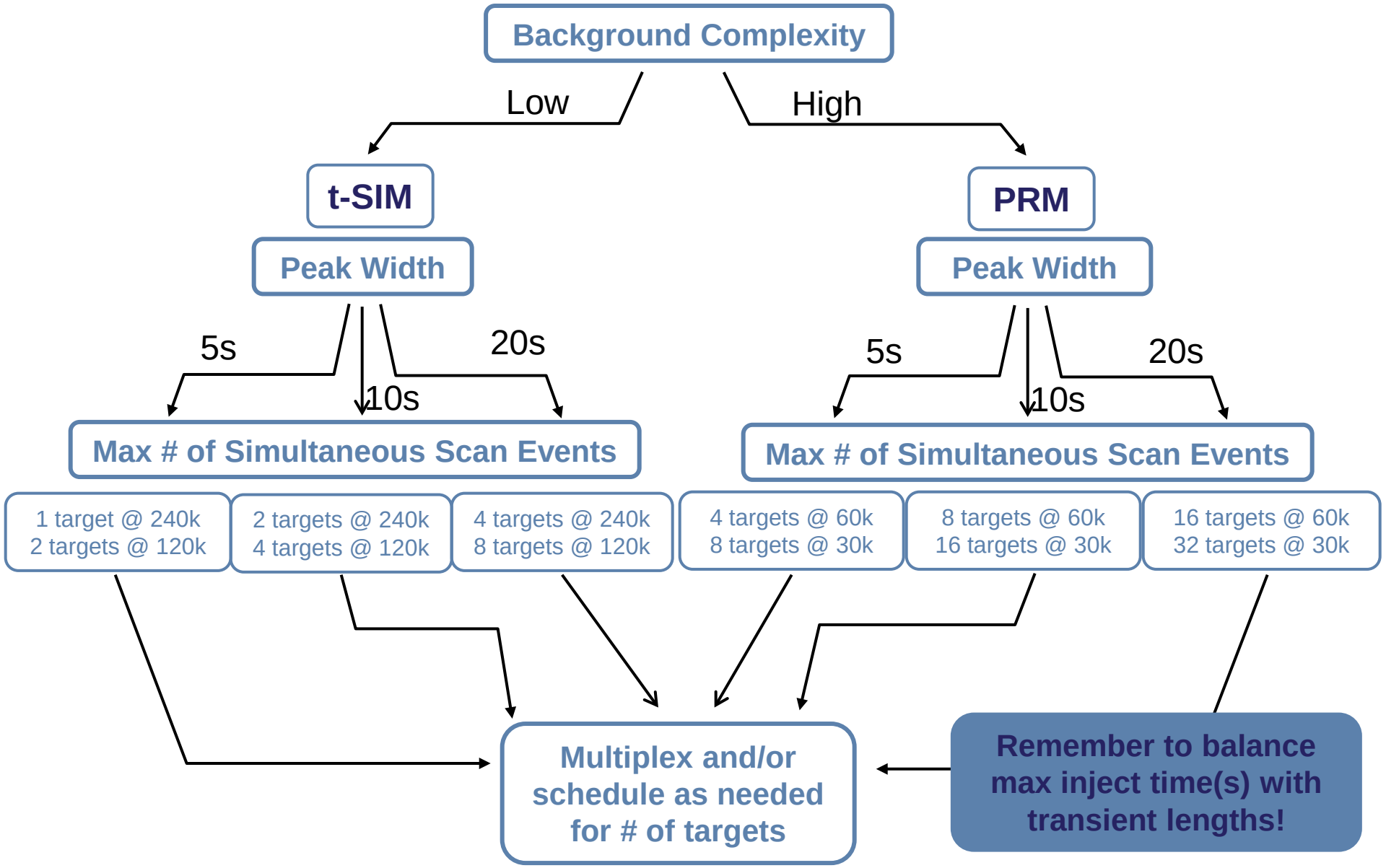
Method Selection Flowchart



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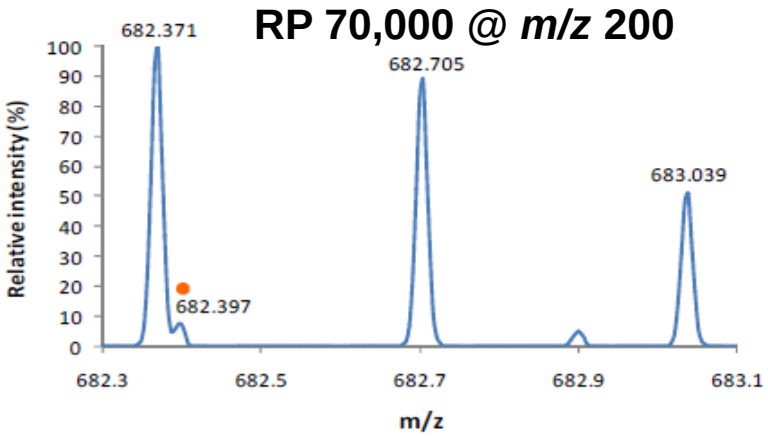
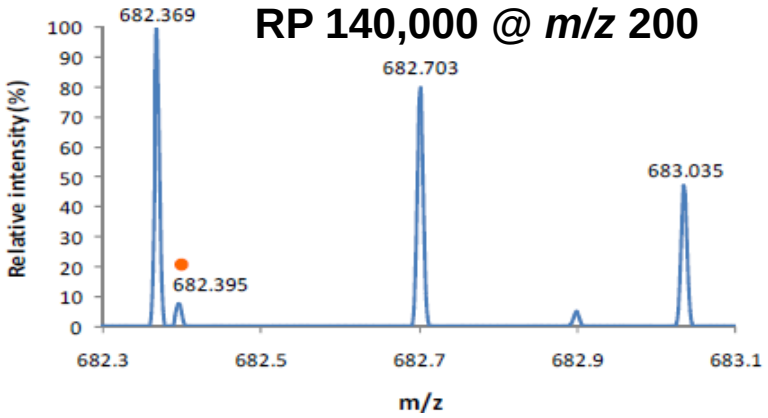
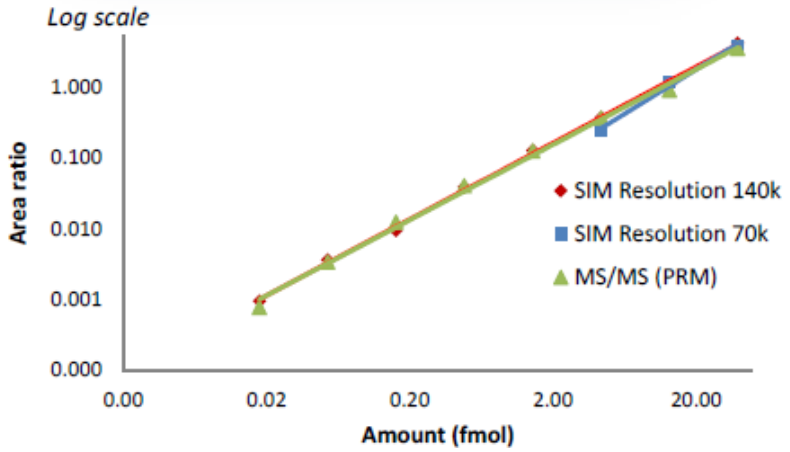
HRAM: Sensitivity Gain Through High Resolution and MS2

PRM (t-MS2) provides the same sensitivity as t-SIM analysis, but with more selectivity





- SDLAVPSELALLK
- *m/z* 682.40
- Urine digest matrix



Targeted Proteomic Quantification on Quadrupole-Orbitrap Mass Spectrometer
Gallien et al., MCP 2012, O112.019802

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Different Scan Mode Options

- Quantitation by…
  - *Full MS*
  - *t-SIM (targeted-Selected Ion Monitoring)*
  - *PRM (Parallel Reaction Monitoring or t-MS2)*
  - *Combination of MS1 with PRM (both MS1 and MS2 data can be evaluated)*

- All Scan modes utilize high resolution and accurate mass (HRAM)
  - Extract ions with narrow mass window (<5ppm)

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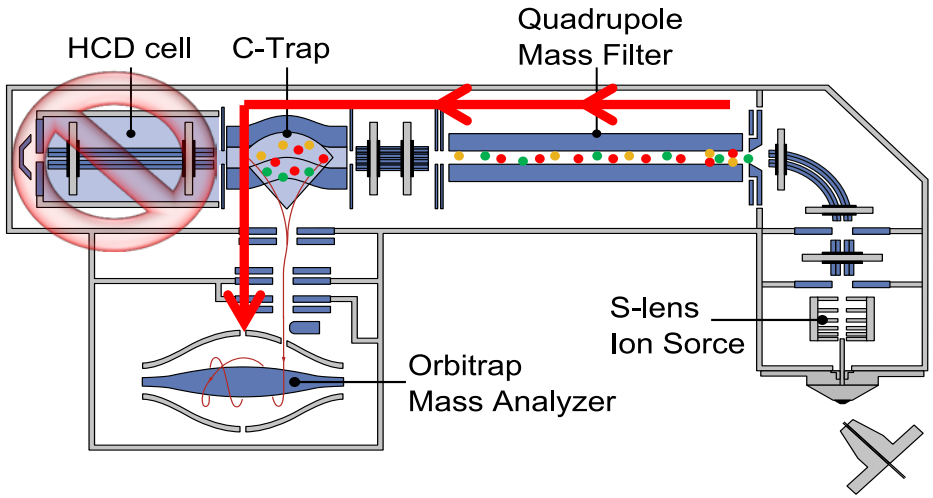
Global Quantitation: Full MS

- Complexity: High charge density (AGC 1e6 to 3e6)
- High resolution across dynamic range (up to 240K on Q Exactive HF MS)
- Option of acquiring confirmatory MS2

- Qualitative Attributes: RT, accurate mass, and isotopic distribution
- Quantitative Attribute: Peak areas of precursor isotopes



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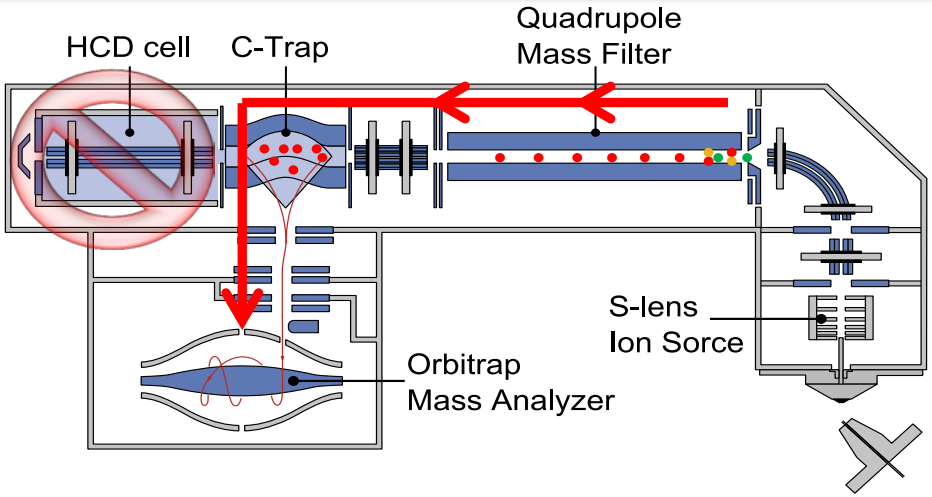
Scan Mode: t-SIM

- Use the highest resolution setting to resolve your target from co-eluting species in the isolation window
- Select number of isotopes for quantitation post acquisition
- Option of multiplexing (MSX) to maximize cycle time

- Qualitative Attributes: RT, accurate mass, and isotopic distribution
- Quantitative Attribute: Peak areas of precursor isotopes



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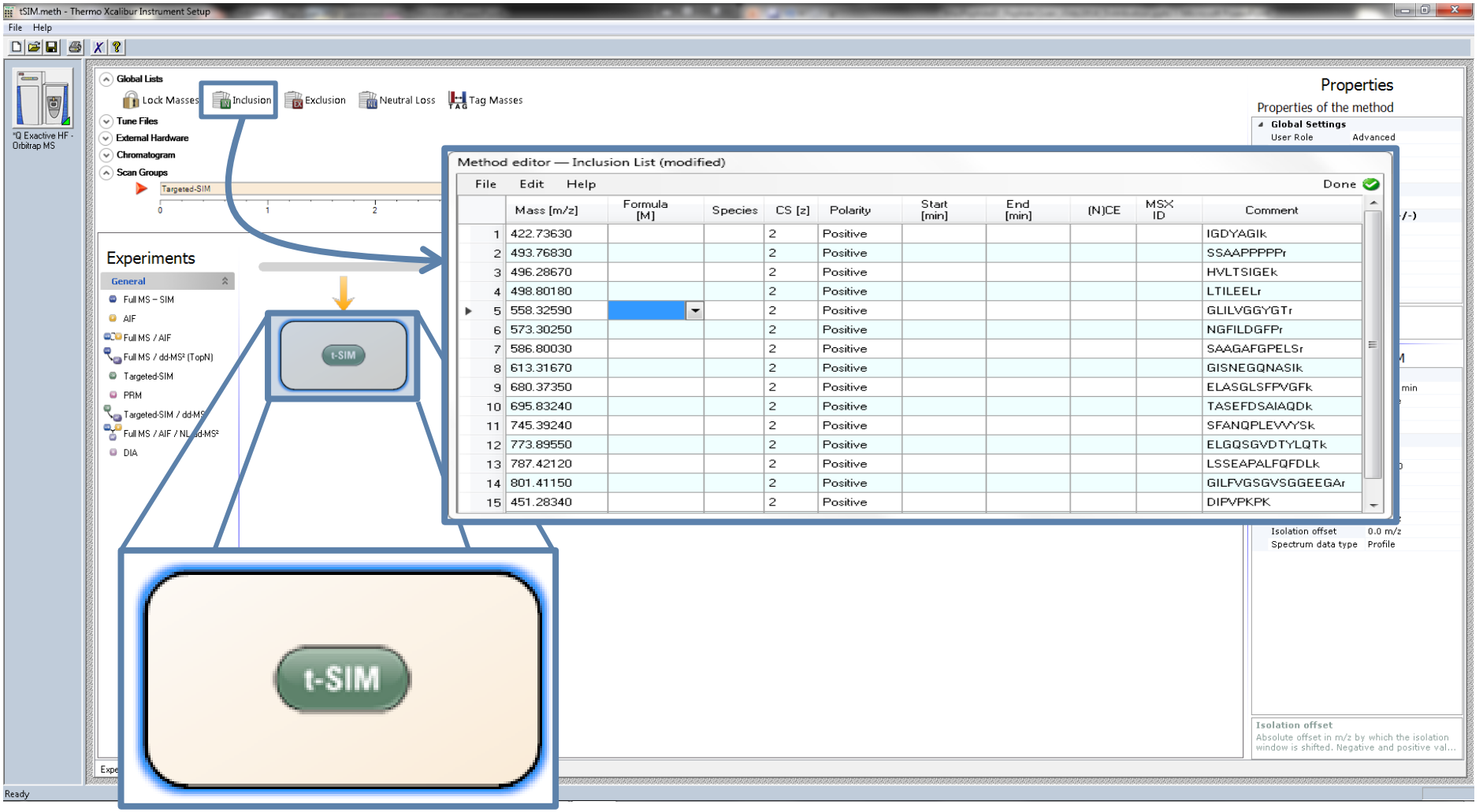
t-SIM Method – Q Exactive HF MS Example



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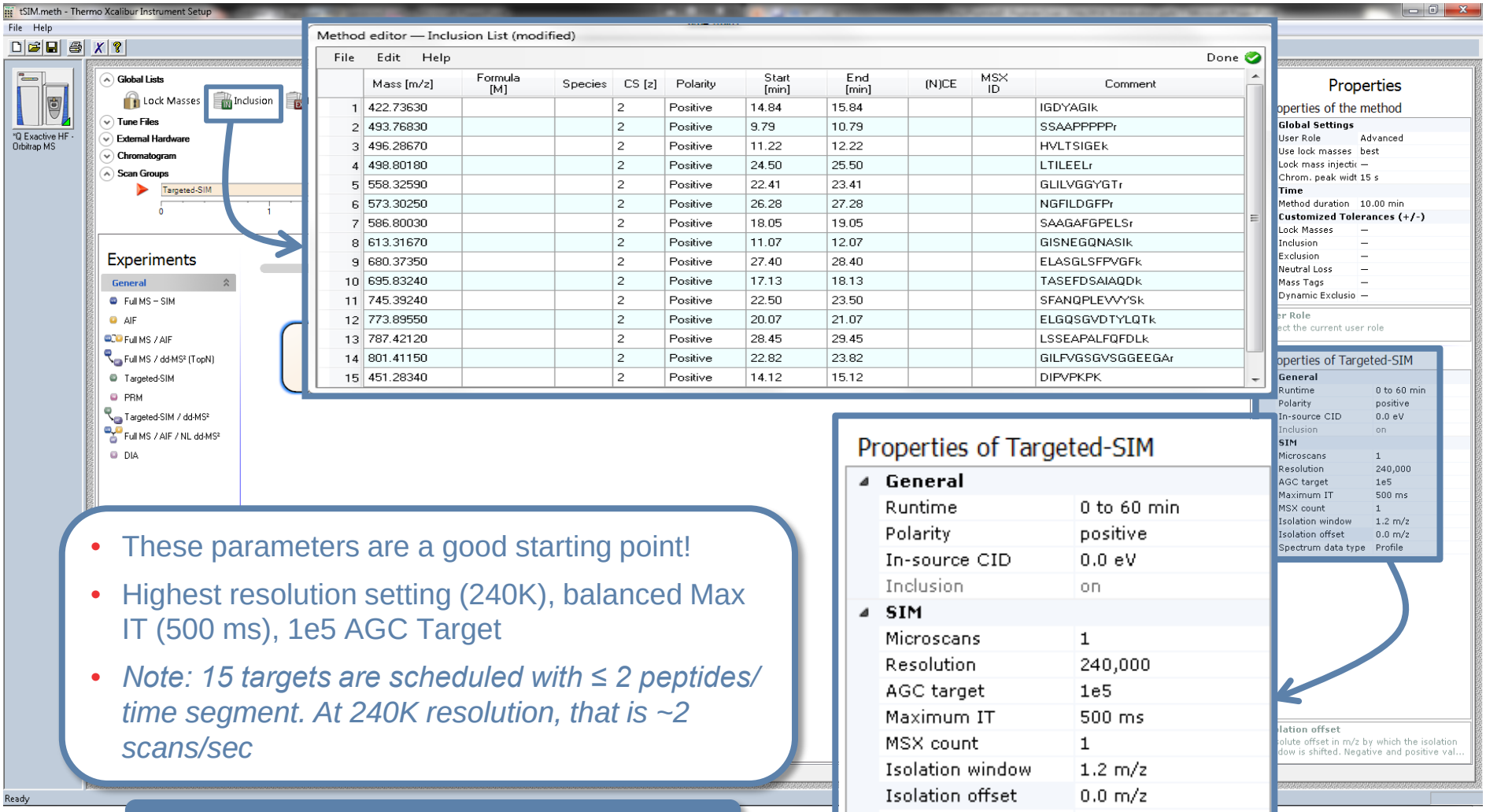
t-SIM Method – Q Exactive HF MS Example



Method editor — Inclusion List (modified)

File Edit Help Done

	Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	Start [min]	End [min]	(N)CE	MSX ID	Comment
1	422.73630			2	Positive	14.84	15.84			IGDYAGIk
2	493.76830			2	Positive	9.79	10.79			SSAAPPPPPr
3	496.28670			2	Positive	11.22	12.22			HVLTSIGEk
4	498.80180			2	Positive	24.50	25.50			LTILEELr
5	558.32590			2	Positive	22.41	23.41			GLILVGGYGTr
6	573.30250			2	Positive	26.28	27.28			NGFILDGFPr
7	586.80030			2	Positive	18.05	19.05			SAAGAFGPELSr
8	613.31670			2	Positive	11.07	12.07			GISNEGQNASIk
9	680.37350			2	Positive	27.40	28.40			ELASGLSFPVGFk
10	695.83240			2	Positive	17.13	18.13			TASEFDSAIAQDk
11	745.39240			2	Positive	22.50	23.50			SFANQPLEVVYSk
12	773.89550			2	Positive	20.07	21.07			ELGQSGVDTYLQTk
13	787.42120			2	Positive	28.45	29.45			LSSEAPALFQFDLk
14	801.41150			2	Positive	22.82	23.82			GILFVGSGVSGGEEGAr
15	451.28340			2	Positive	14.12	15.12			DIPVPKPK

Properties of Targeted-SIM

General	
Runtime	0 to 60 min
Polarity	positive
In-source CID	0.0 eV
Inclusion	on
SIM	
Microscans	1
Resolution	240,000
AGC target	1e5
Maximum IT	500 ms
MSX count	1
Isolation window	1.2 m/z
Isolation offset	0.0 m/z
Spectrum data type	Profile

- These parameters are a good starting point!
- Highest resolution setting (240K), balanced Max IT (500 ms), 1e5 AGC Target
- *Note: 15 targets are scheduled with ≤ 2 peptides/ time segment. At 240K resolution, that is ~2 scans/sec*

Tip: Reaching max IT is your goal!

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t-SIM Method: Multiplexing

- Multiplexing (MSX) of t-SIM scans can optimize cycle times for quantitation of ***co-eluting*** peptides
  - Q Exactive MSX function allows *up to* 10 precursors to be analyzed in the Orbitrap simultaneously
    - Only one transient required
  - However, *each* precursor has a separate *injection time*
  - Thus, we recommend MSX of four or fewer precursors to ensure cycle times remain optimal

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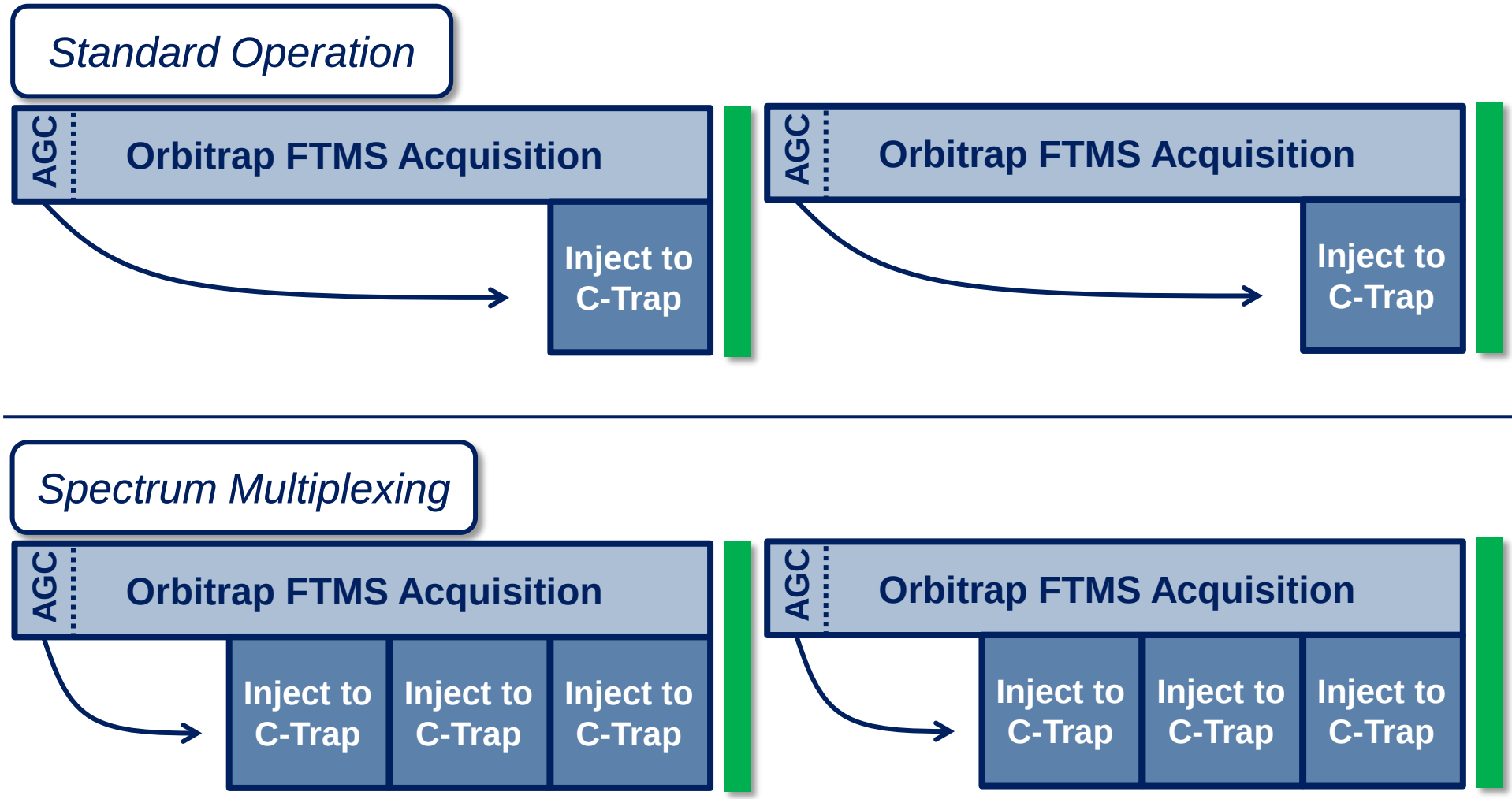
MSX Functionality and Parallel C-Trap Filling



Multiplexing Maximizes the Cycle Time of the Q Exactive

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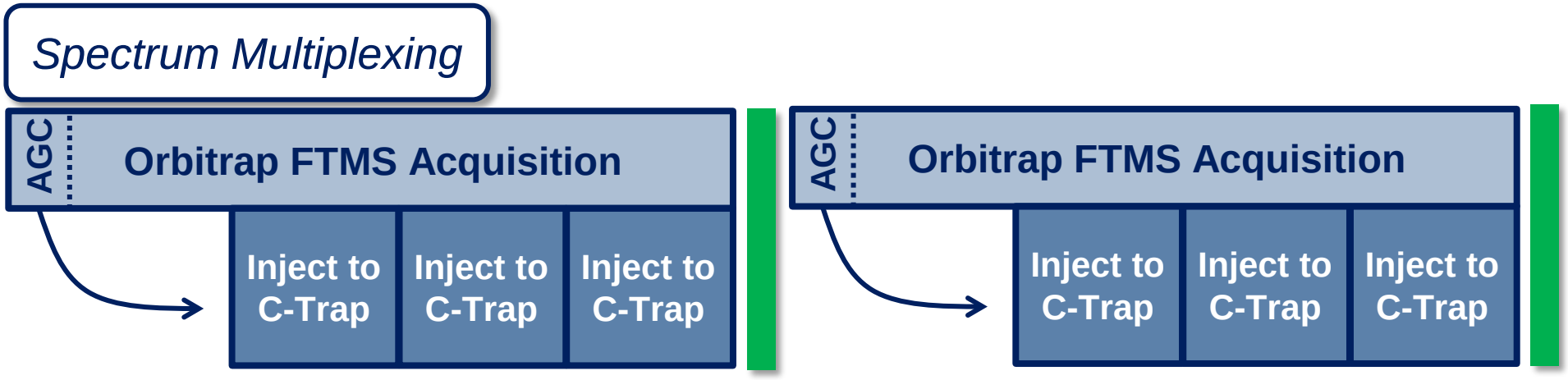
MSX Functionality and Parallel C-Trap Filling

Things to consider…

- When multiplexing, the AGC setting and max IT is for each fill event.
- Higher order multiplexing require lower max IT
  - (max IT)/(multiplex count) = per target max IT
- Target Value Recommendations for t-SIM (240K Resolution)…
  - Targeting without multiplexing (Target Value: 1e5-2e5)
  - Multiplexing 2-5 Targets (Target Value: 1e5)
  - Multiplexing 5-10 Targets: (Not recommended due to fill time/scan time relationship)



Multiplexing Maximizes the Cycle Time of the Q Exactive MS

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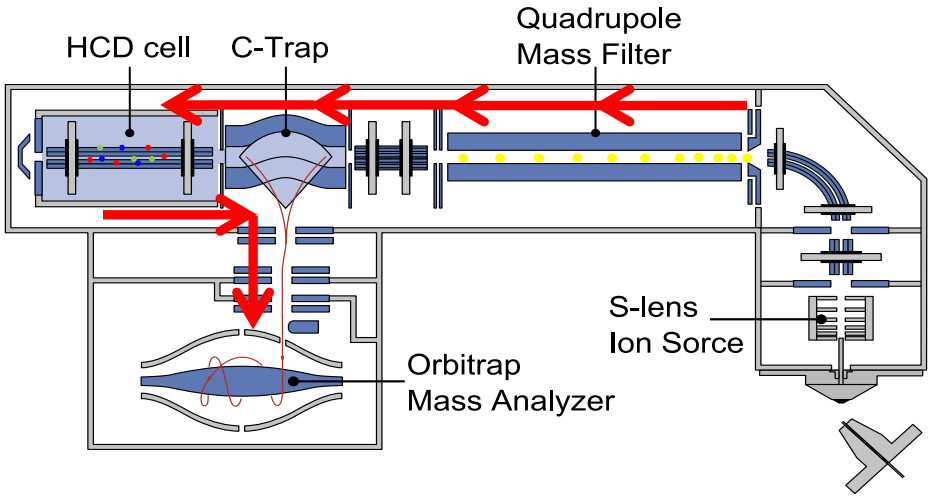
PRM (t-MS2)

- Generates high resolution full mass range MS2 spectra
- Flexibility to choose the specific fragment ions for quantitation post-acquisition
- Since it is a high resolution scan, you can extract your ion with a narrow ppm mass window achieving high selectivity
- The Orbitrap analyzer permits parallel detection of all target product ions in one, concerted high resolution mass analysis

- Qualitative Attribute: RT, fragment accurate mass, and fragment ion ratios
- Quantitative Attribute: Peak areas of selected fragment ions



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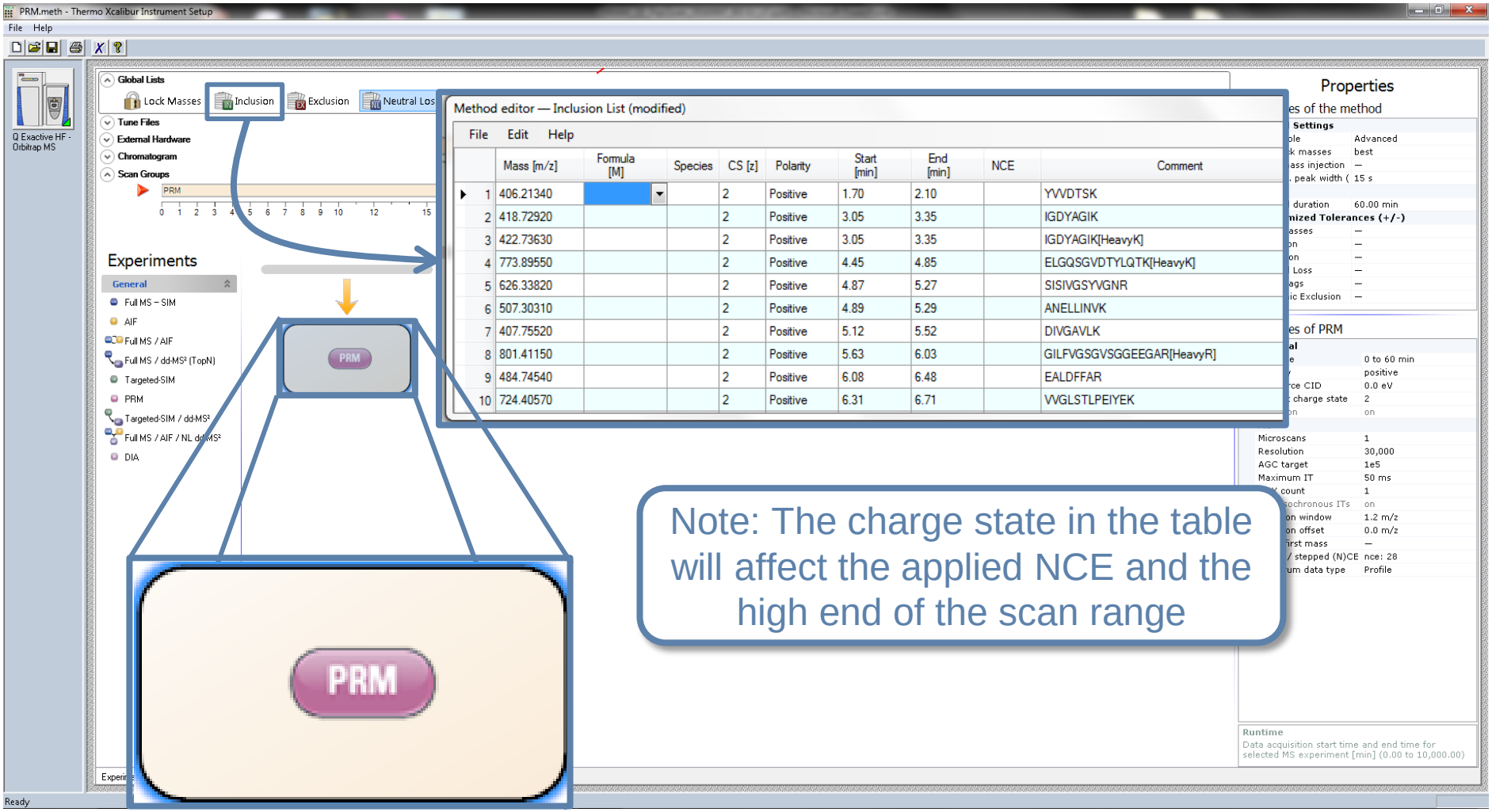
PRM Method – Q Exactive HF MS Example



Note: The charge state in the table will affect the applied NCE and the high end of the scan range

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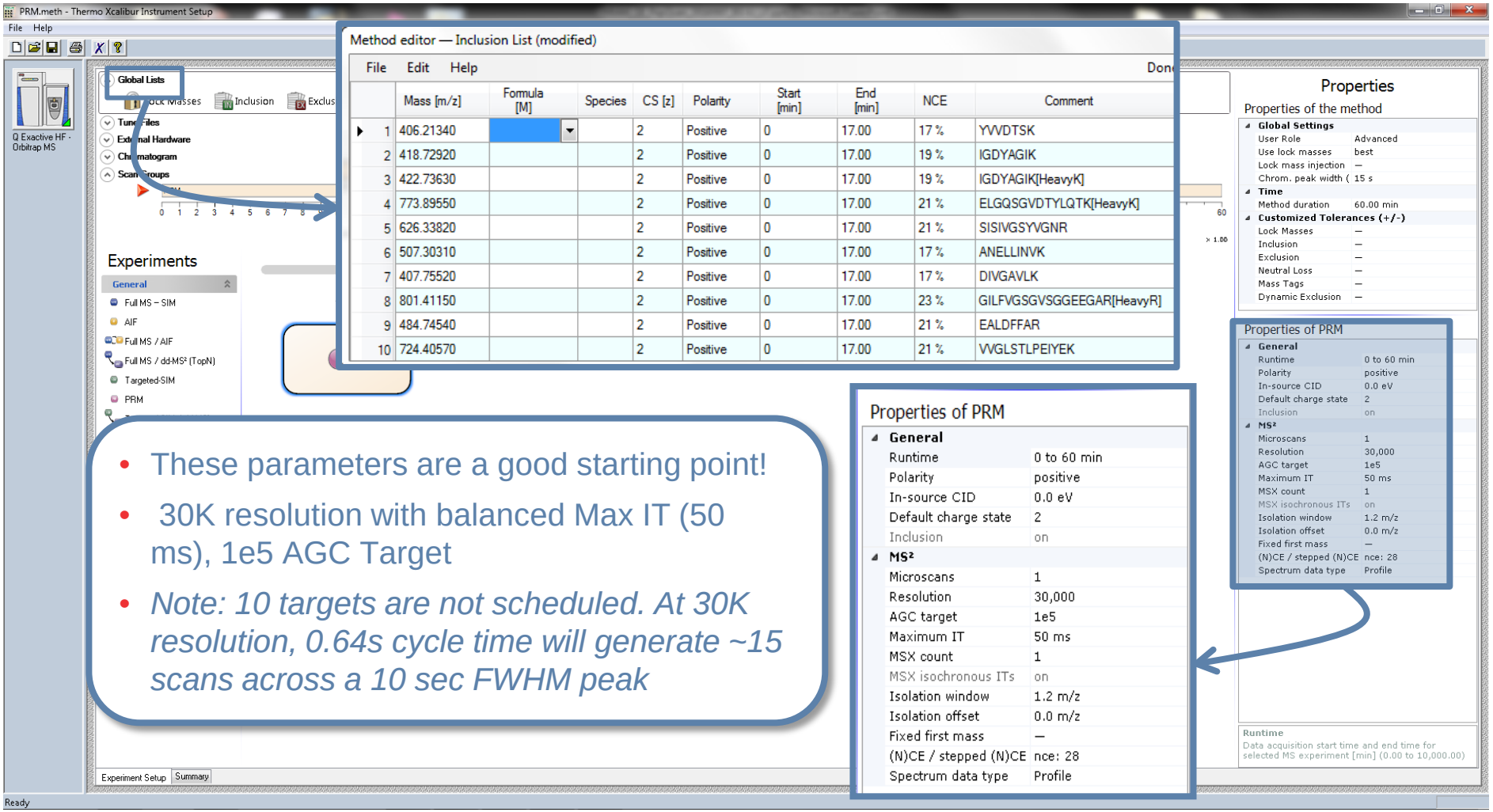
PRM Method – Q Exactive HF MS Example



- These parameters are a good starting point!
- 30K resolution with balanced Max IT (50 ms), 1e5 AGC Target
- *Note: 10 targets are not scheduled. At 30K resolution, 0.64s cycle time will generate ~15 scans across a 10 sec FWHM peak*

Properties of PRM

General	
Runtime	0 to 60 min
Polarity	positive
In-source CID	0.0 eV
Default charge state	2
Inclusion	on
MS²	
Microscans	1
Resolution	30,000
AGC target	1e5
Maximum IT	50 ms
MSX count	1
MSX isochronous ITs	on
Isolation window	1.2 m/z
Isolation offset	0.0 m/z
Fixed first mass	–
(N)CE / stepped (N)CE	nce: 28
Spectrum data type	Profile

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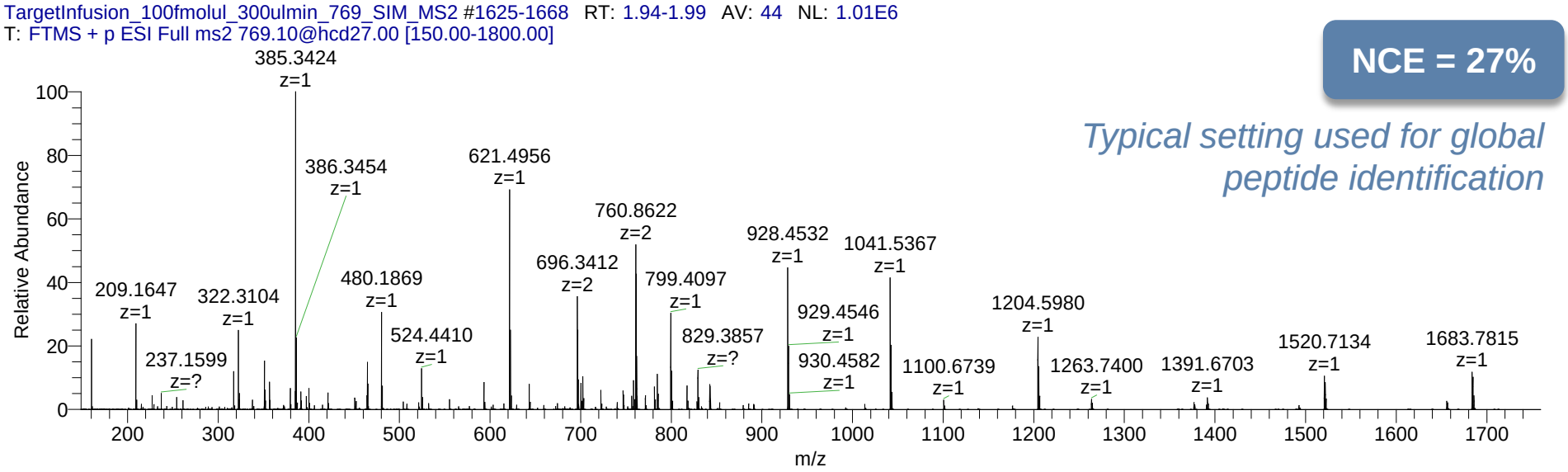
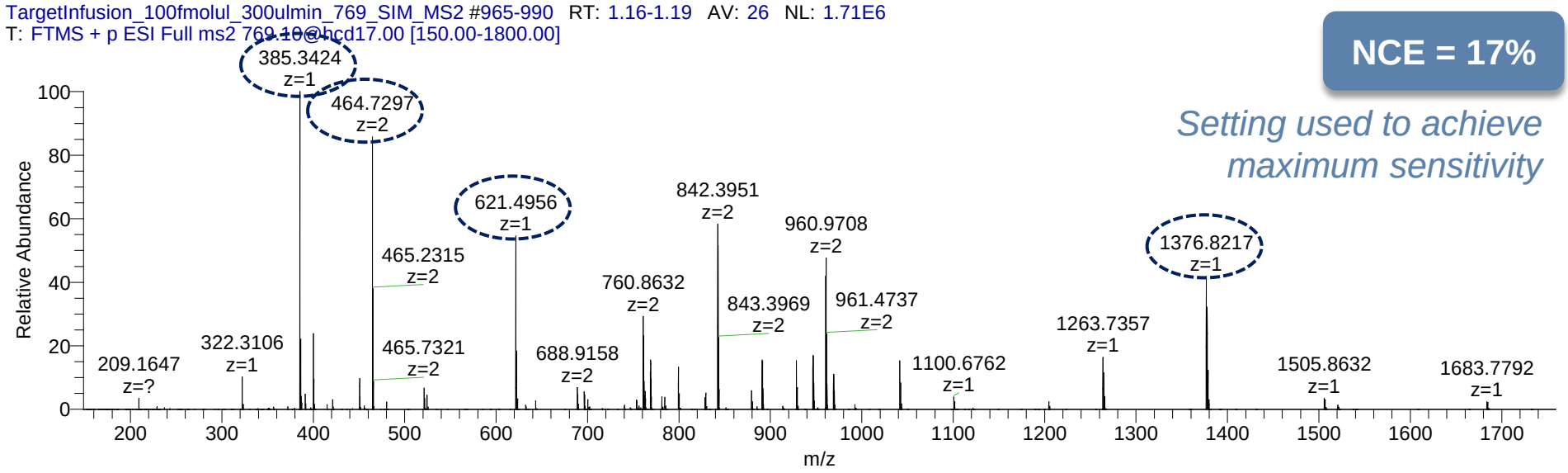
NCE Optimization



NCE = 27%

Typical setting used for global peptide identification



NCE = 17%

Setting used to achieve maximum sensitivity

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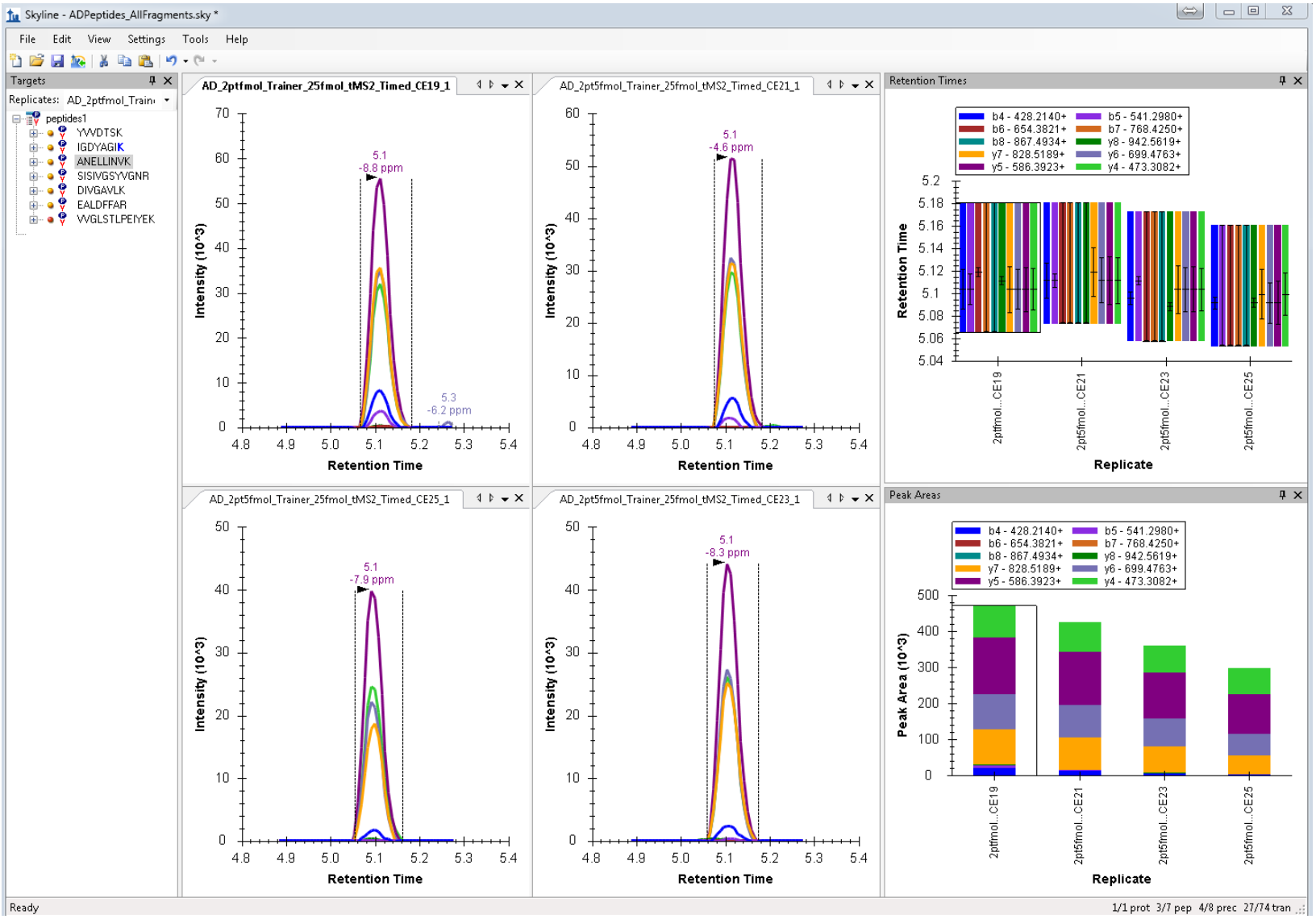
NCE Optimization



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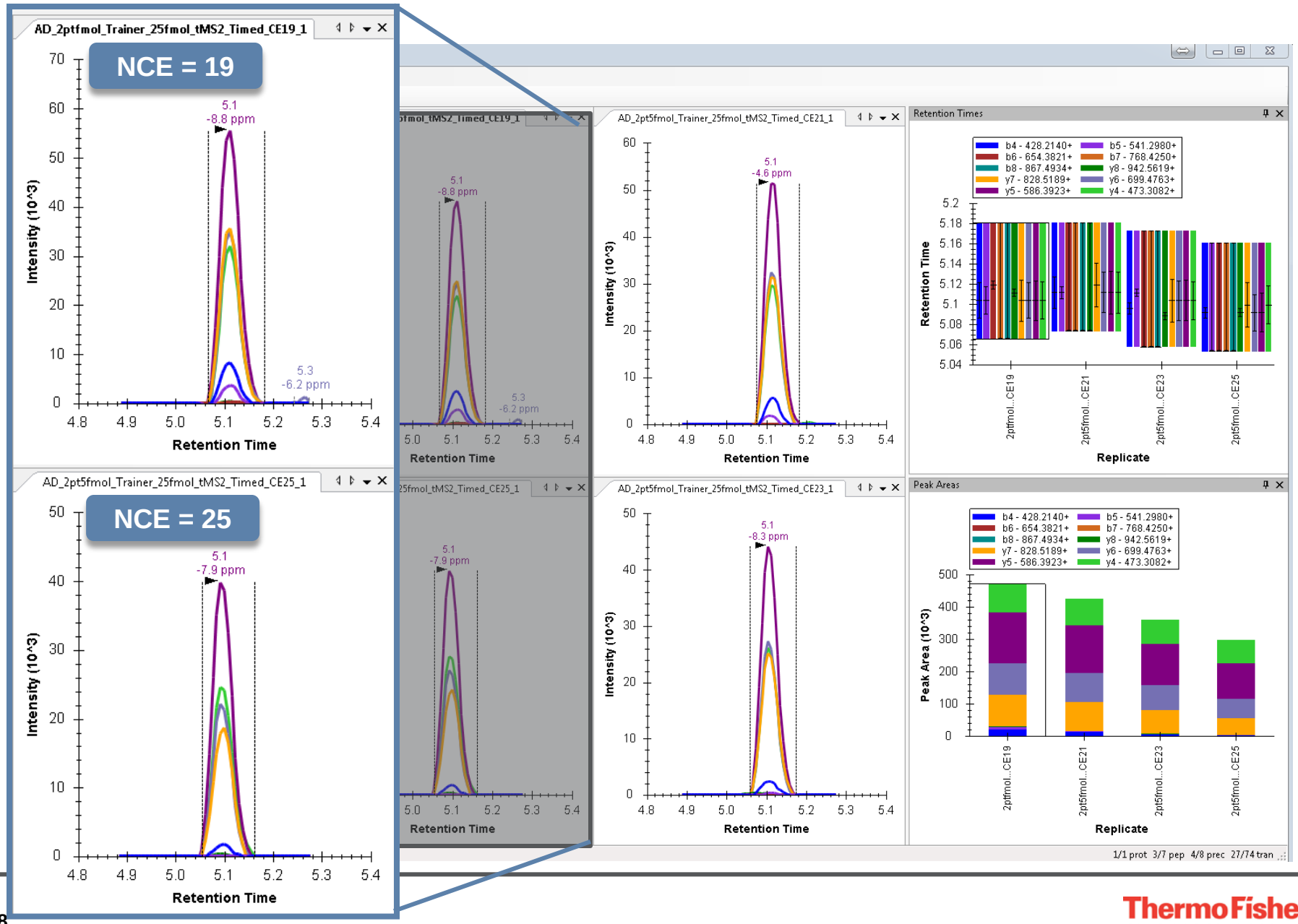
NCE Optimization



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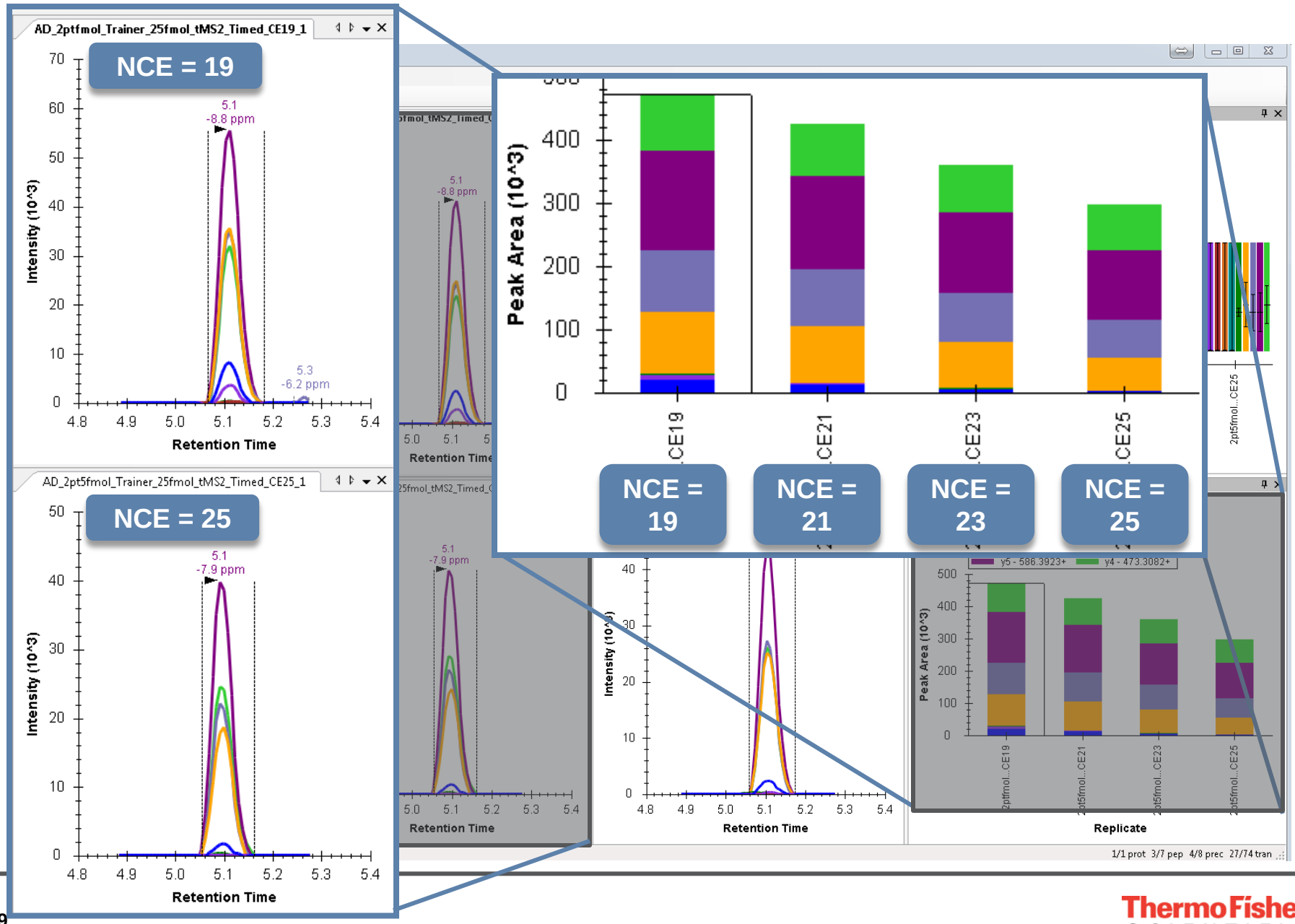
NCE Optimization



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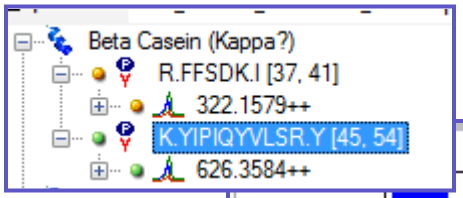
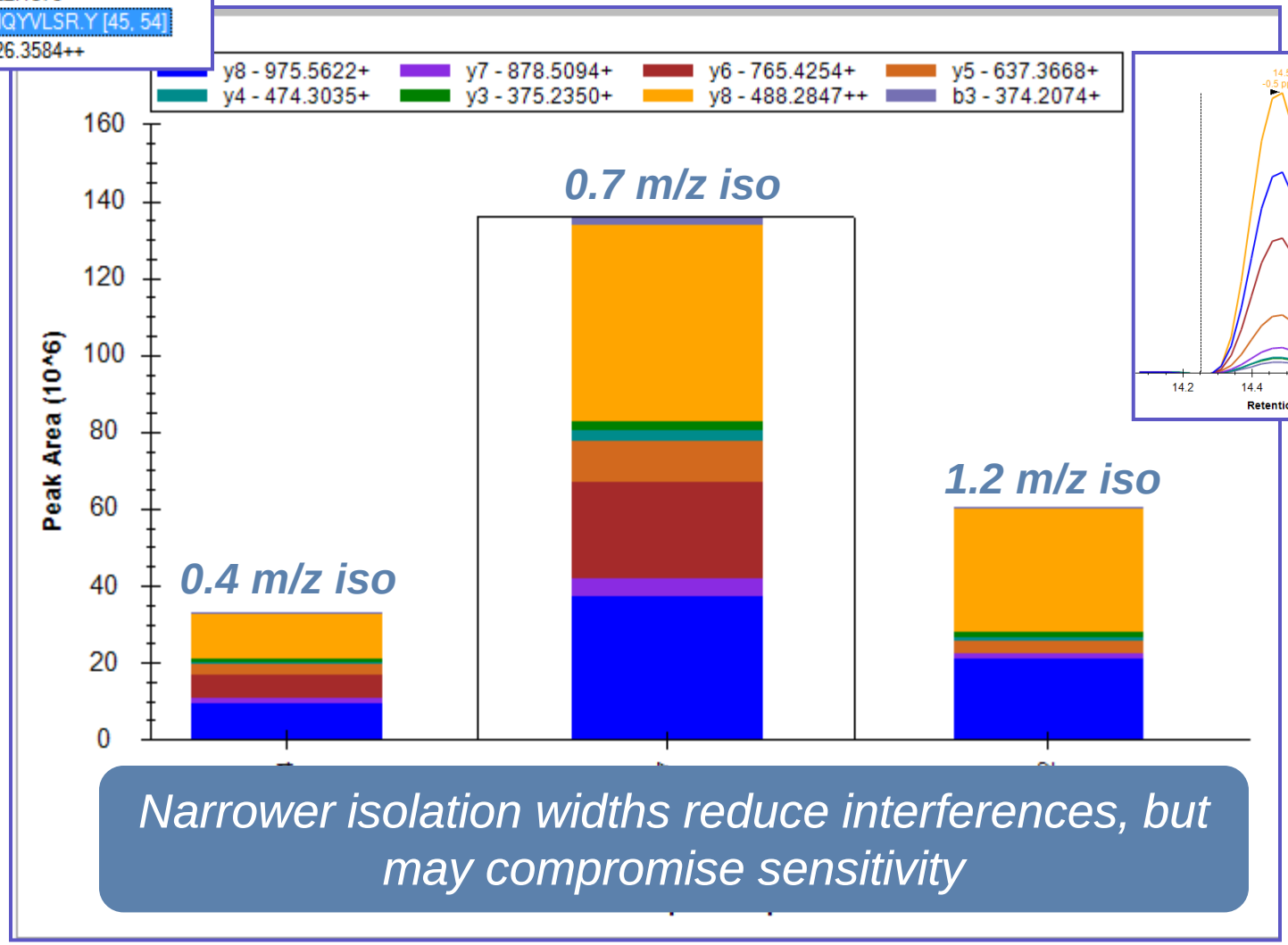
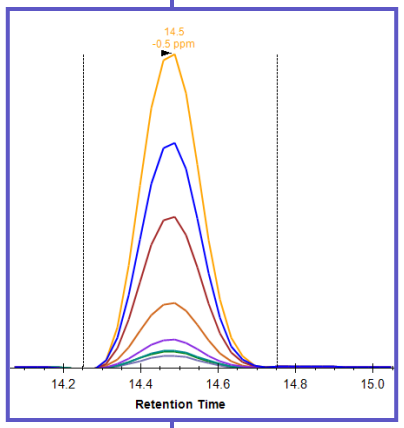
Isolation Window Optimization



Triplicate injections at each isolation width





Narrower isolation widths reduce interferences, but may compromise sensitivity

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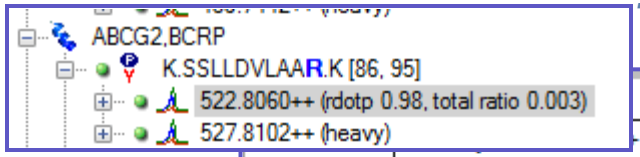
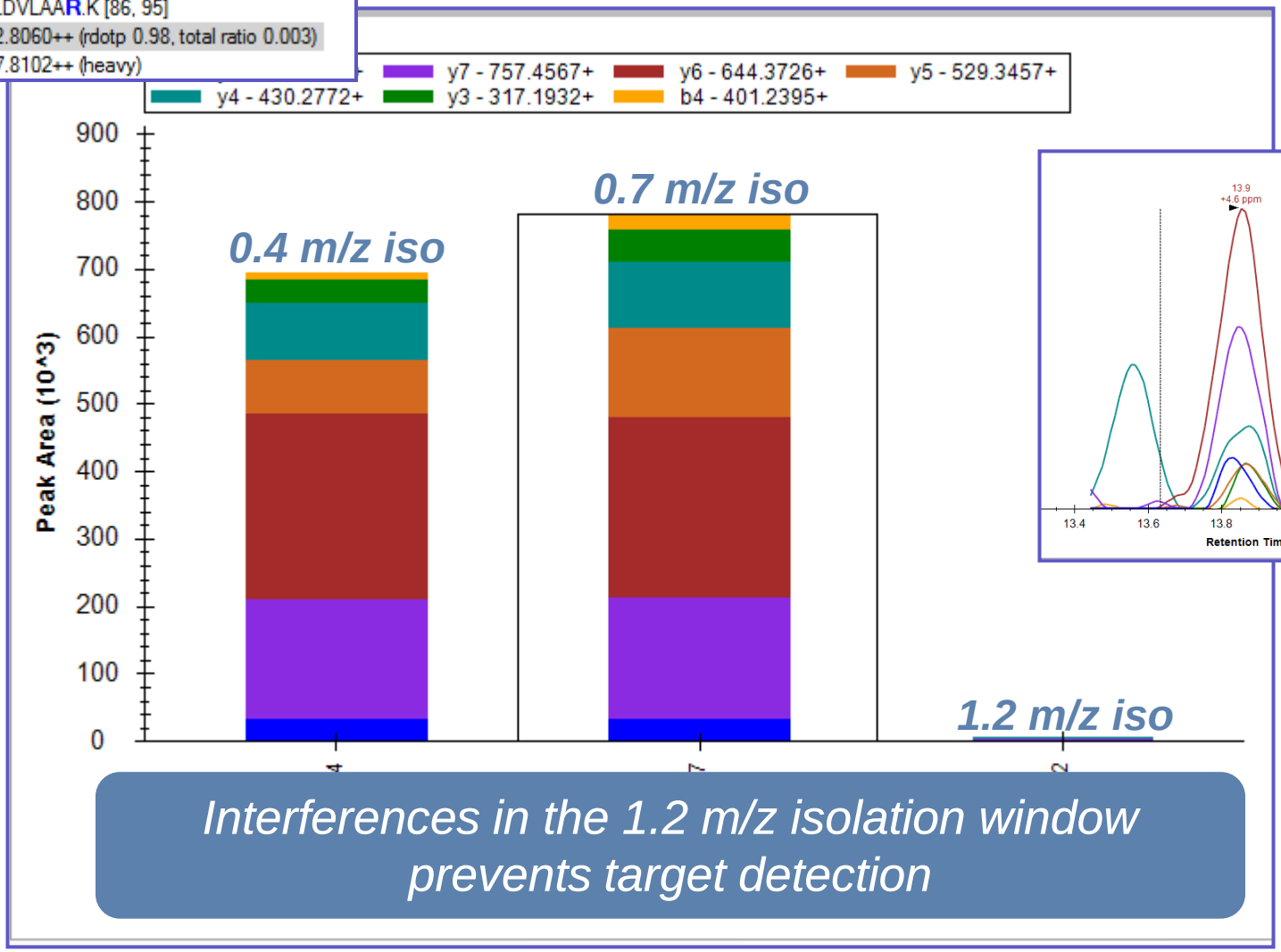
Isolation Window Optimization



Triplicate injections at each isolation width



Interferences in the 1.2 m/z isolation window prevents target detection

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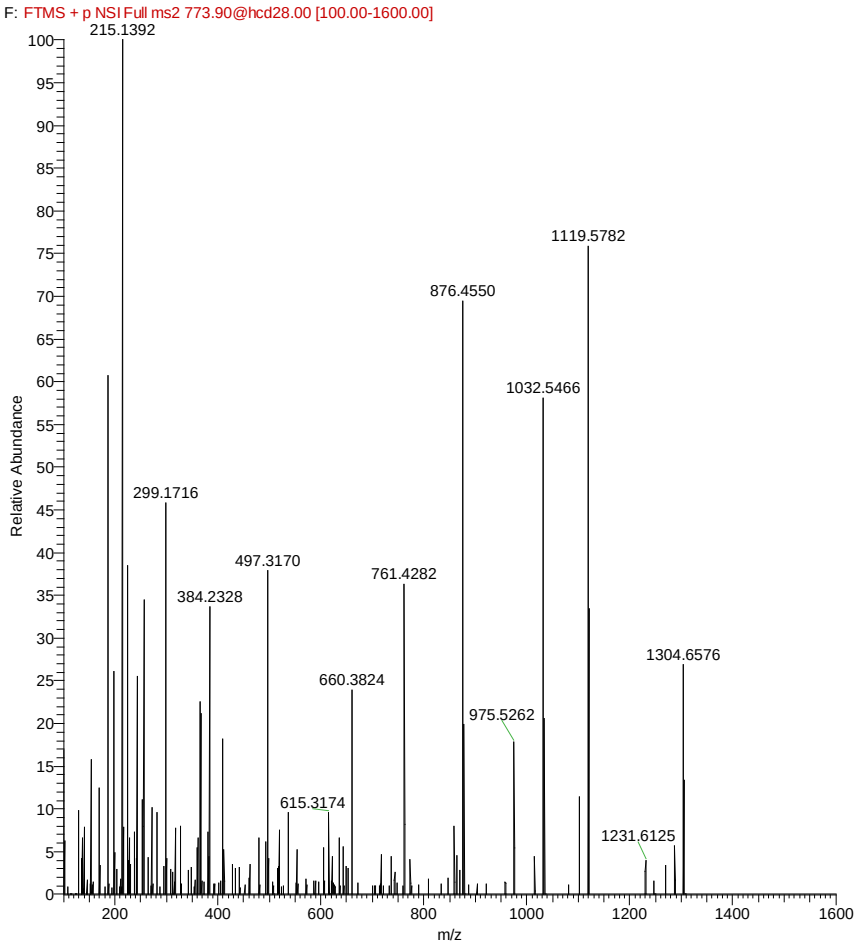
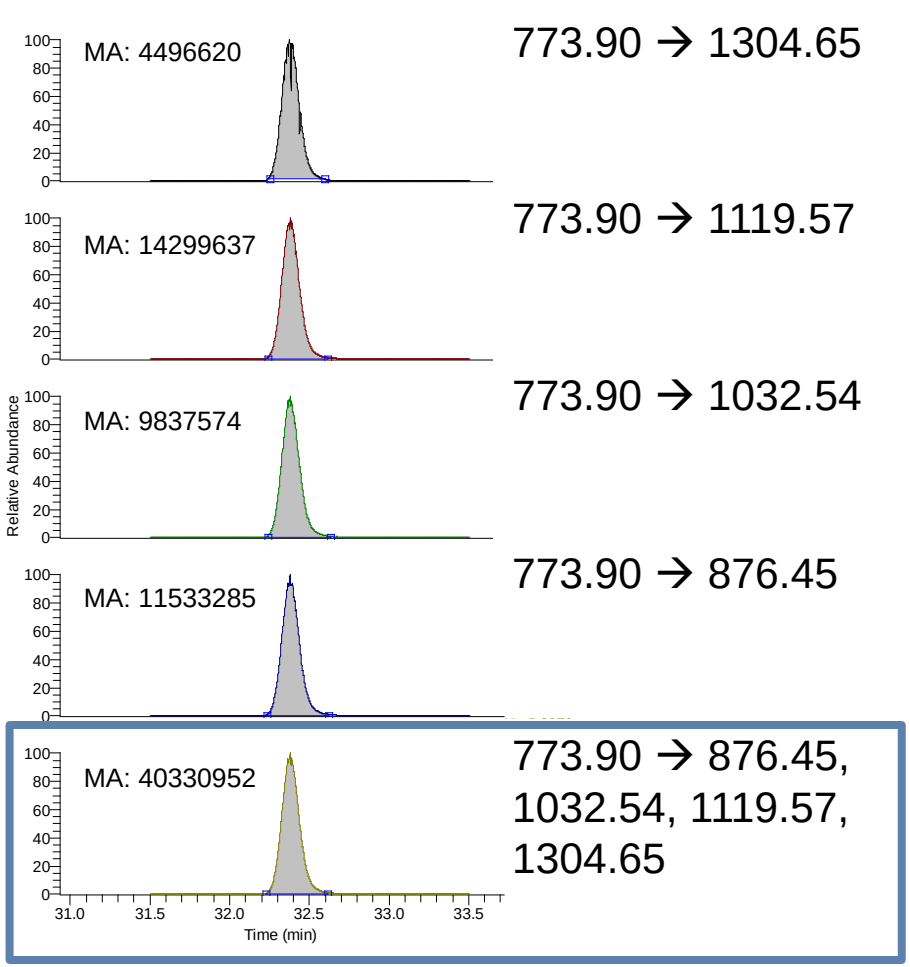
Example: Quantitation using PRM

Peak areas are calculated from the extracted ion chromatograms of parent → fragment ion





$[M+2H]^{2+}$ of ELGQSGVDTYLQTK[$^{13}C_6{}^{15}N_2$]

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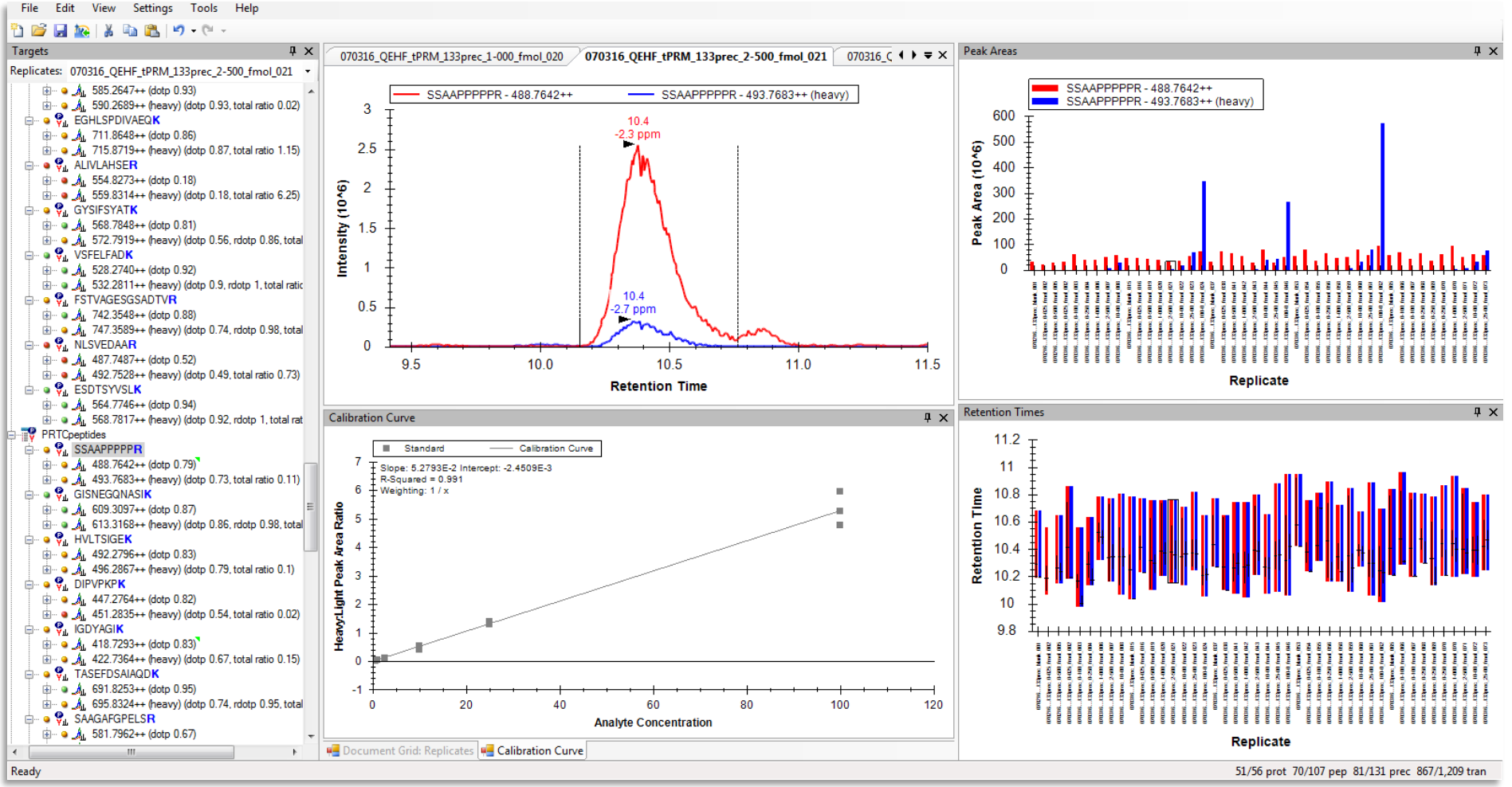
Example: Skyline – PRTC Peptide SSAAPPPPPR



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Summary

- You have options when deciding which instrument method to use. Pick which one best suits your needs.
  - Full MS
  - t-SIM → Low complexity
  - PRM (t-MS2) → High complexity
- Deciding on how many targets you want to go after will greatly affect your experiment set up
  - Number of targets will affect:
    - Scheduled vs unscheduled
    - Retention time windows, if scheduled
    - Scanning resolution
- The complexity of your sample will also affect your experiment set up:
    - Resolution
    - Maximum injection times

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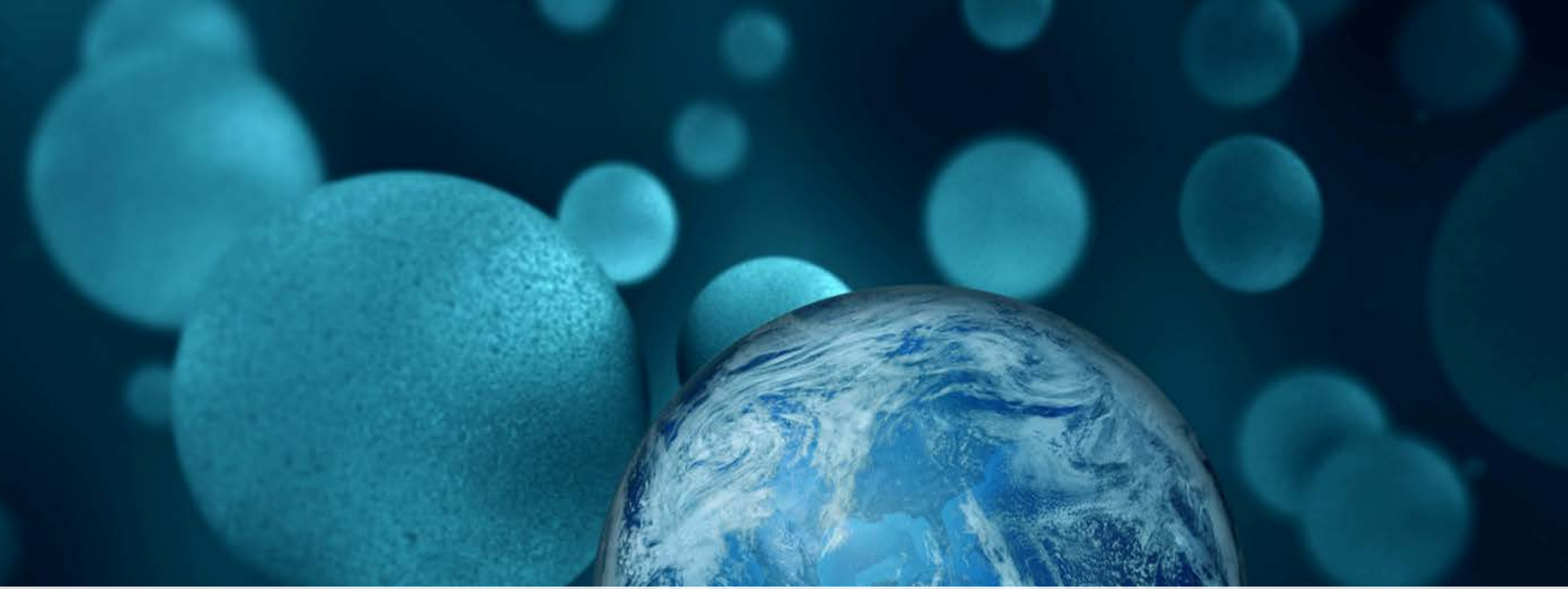

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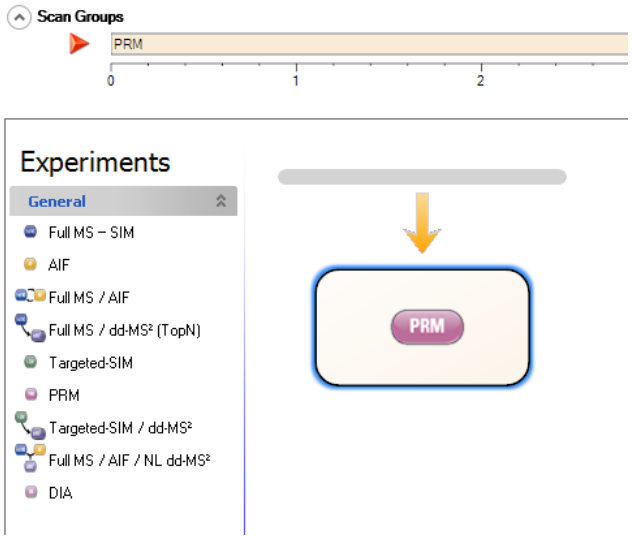
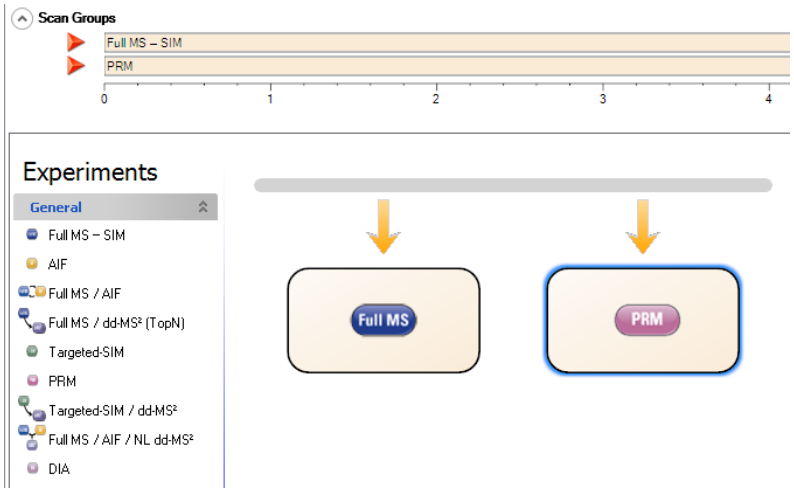
Tips, Tricks and Troubleshooting Help

Order of Operations (example of 5 targets)

- Type and number of experiment nodes will affect how the instrument scans



- Scan 1: MS2 of target 1
- Scan 2: MS2 of target 2
- Scan 3: MS2 of target 3
- Scan 4: MS2 of target 4
- Scan 5: MS2 of target 5
- Scan 6: MS2 of target 1



- Scan 1: Full MS
- Scan 2: MS2 of target 1
- Scan 3: Full MS
- Scan 4: MS2 of target 2
- Scan 5: Full MS
- Scan 6: MS2 of target 3
- Etc.

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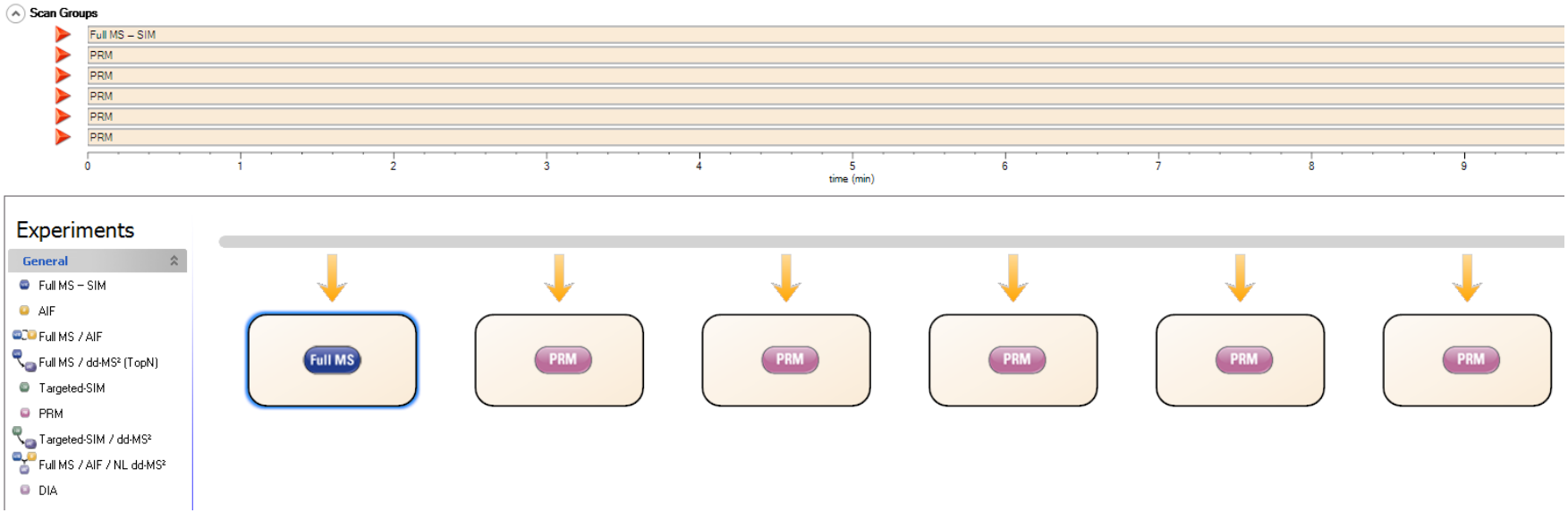
Order of Operations (example of 5 targets)

- Type and number of experiment nodes will affect how the instrument scans



- Scan 1: Full MS
- Scan 2: MS2 of target 1
- Scan 3: MS2 of target 2
- Scan 4: MS2 of target 3
- Scan 5: MS2 of target 4
- Scan 6: MS2 of target 5
- Scan 7: Full MS
- Scan 8: MS2 of target 1
- Etc.

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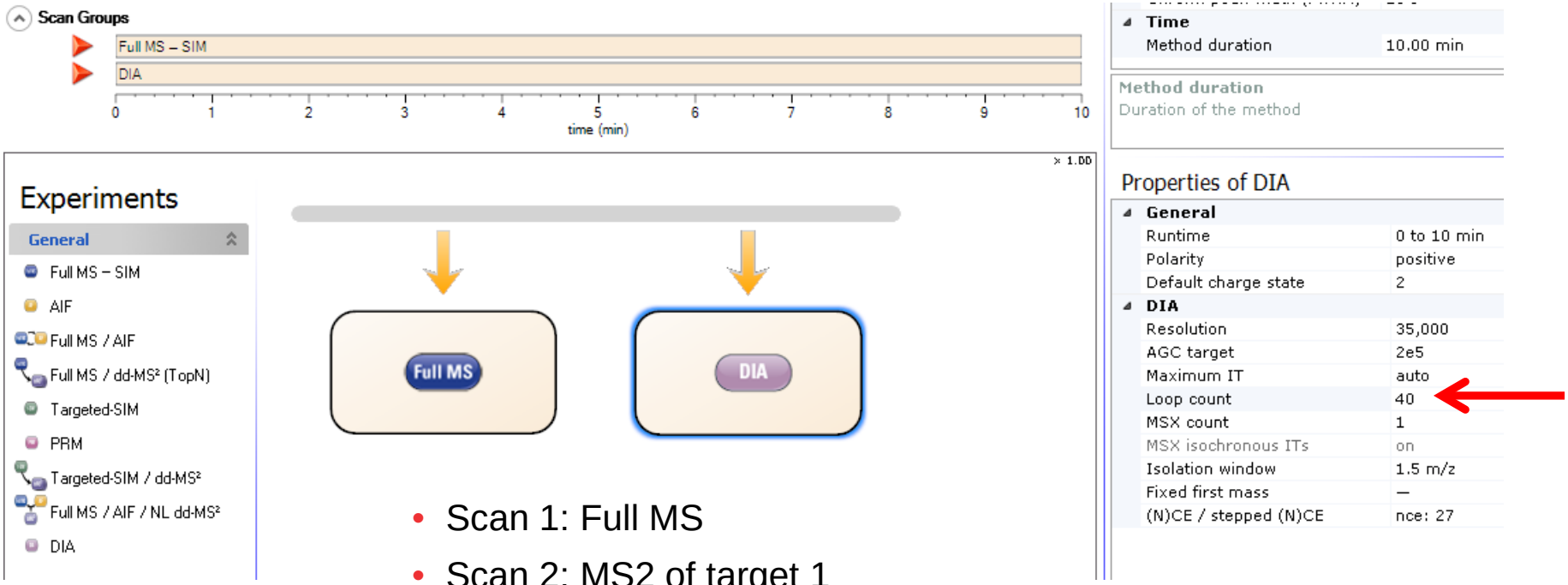
Order of Operations (example of 40 targets)

- Type and number of experiment nodes will affect how the instrument scans



- Scan 1: Full MS
- Scan 2: MS2 of target 1
- Scan 3: MS2 of target 2
- Scan 4: MS2 of target 3
- Scans 5 – 40: MS2s of targets 4 – 39
- Scan 41: MS2 of target 40
- Scan 42: Full MS

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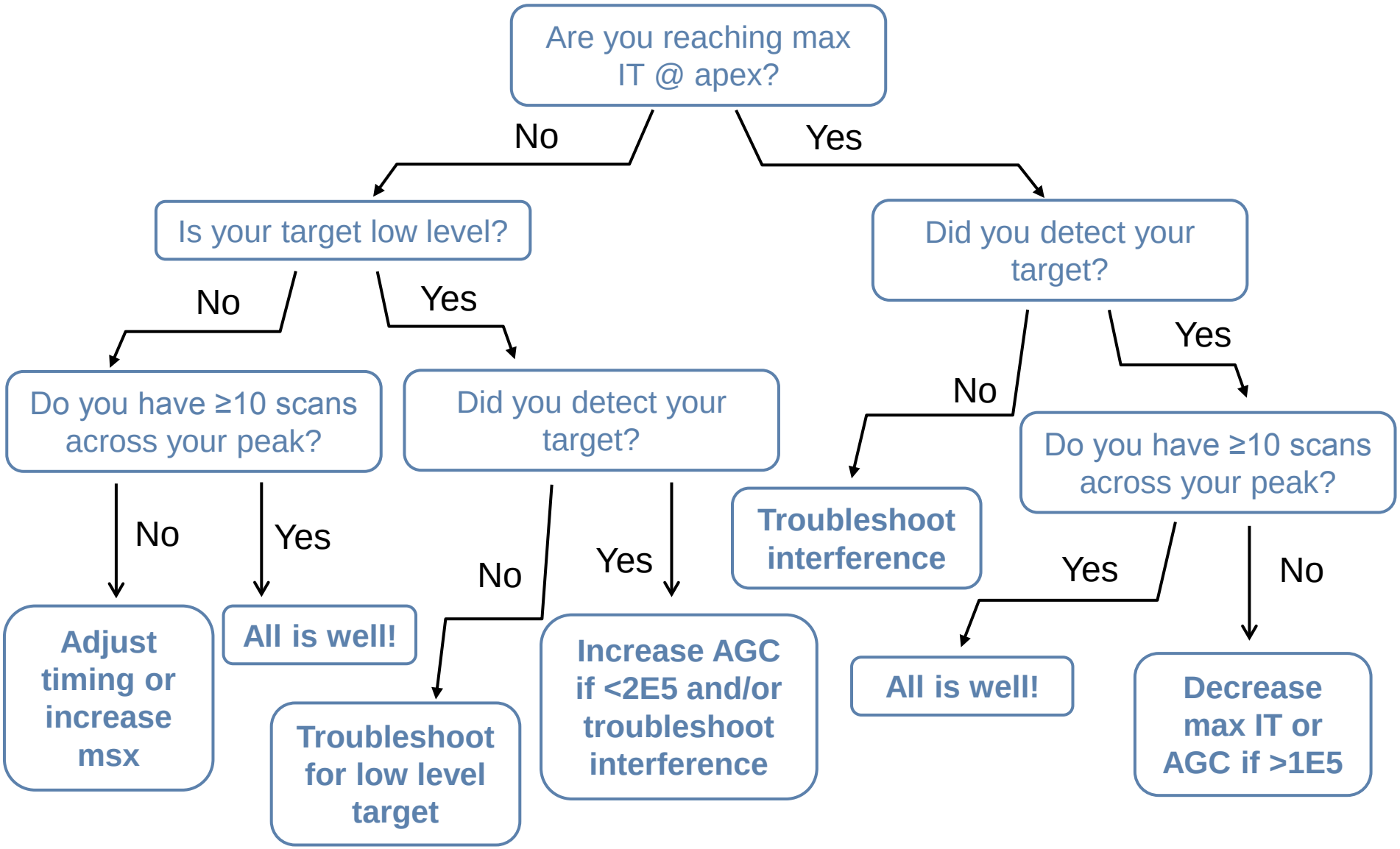
Method Optimization



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Method Troubleshooting

- Interference in your isolation window?
  - Decrease isolation width
  - Switch from t-SIM to t-MS2
  - Adjust gradient conditions
- Target is low level and close to limit of detection?
  - If you are reaching target value, increase target to 2e5
  - If you are not reaching target, then increase max IT as high as peak width allows
  - Play close attention to the timing of your targets. If you have very reproducible RT, then try to narrow your time segments which can allow you to increase your max IT.

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Where to get help!

- Technical Support-North America
  - Priority issues:
    - Call 800-532-4752,
    - Select option 2
  - Non-urgent issues:
    - Webform: **http://www.unitylabservices.com/contact.php**
    - Email: US.Techsupport.Analyze@ThermoFisher.com
      - Enter serial number in subject line
      - Provide brief Description of Issue
- Technical Support-Europe
  - Email:EU.techsupport.CMS@thermofisher.com