

A Skyline Tool for Creating Robust Large Scale Targeted MS/MS Assays

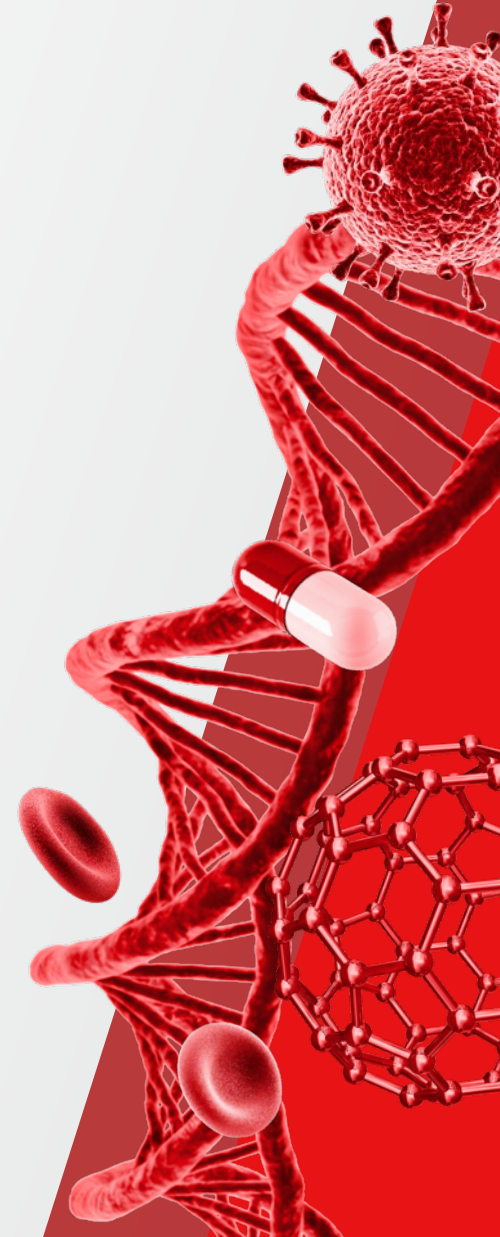
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June 4, 2023

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²University of Washington, Department of Genome Sciences, WA

 The world leader in serving science



Data Independent Analysis vs Targeted MS2



Data Independent Analysis (DIA)

Maximized Ease of Use, High Coverage

Compromised Sensitivity / Selectivity

Hardware and Processing Improvements

Targeted MS2

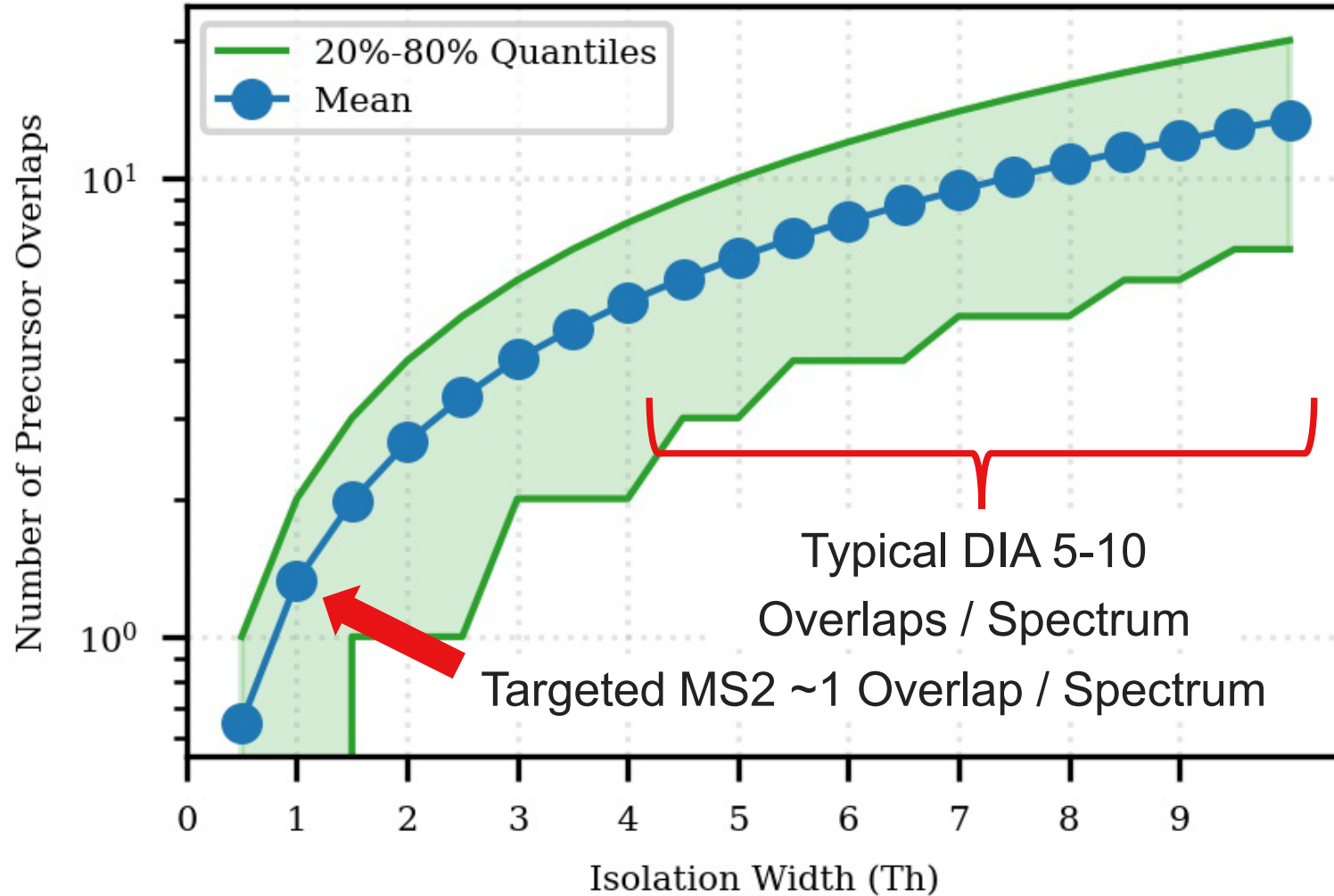
Maximized Sensitivity / Selectivity

Limited Number of Compounds / Hard to Setup & Maintain

Advancing at Slower Rate

Targeted MS2 Yields Highest Quality Spectra

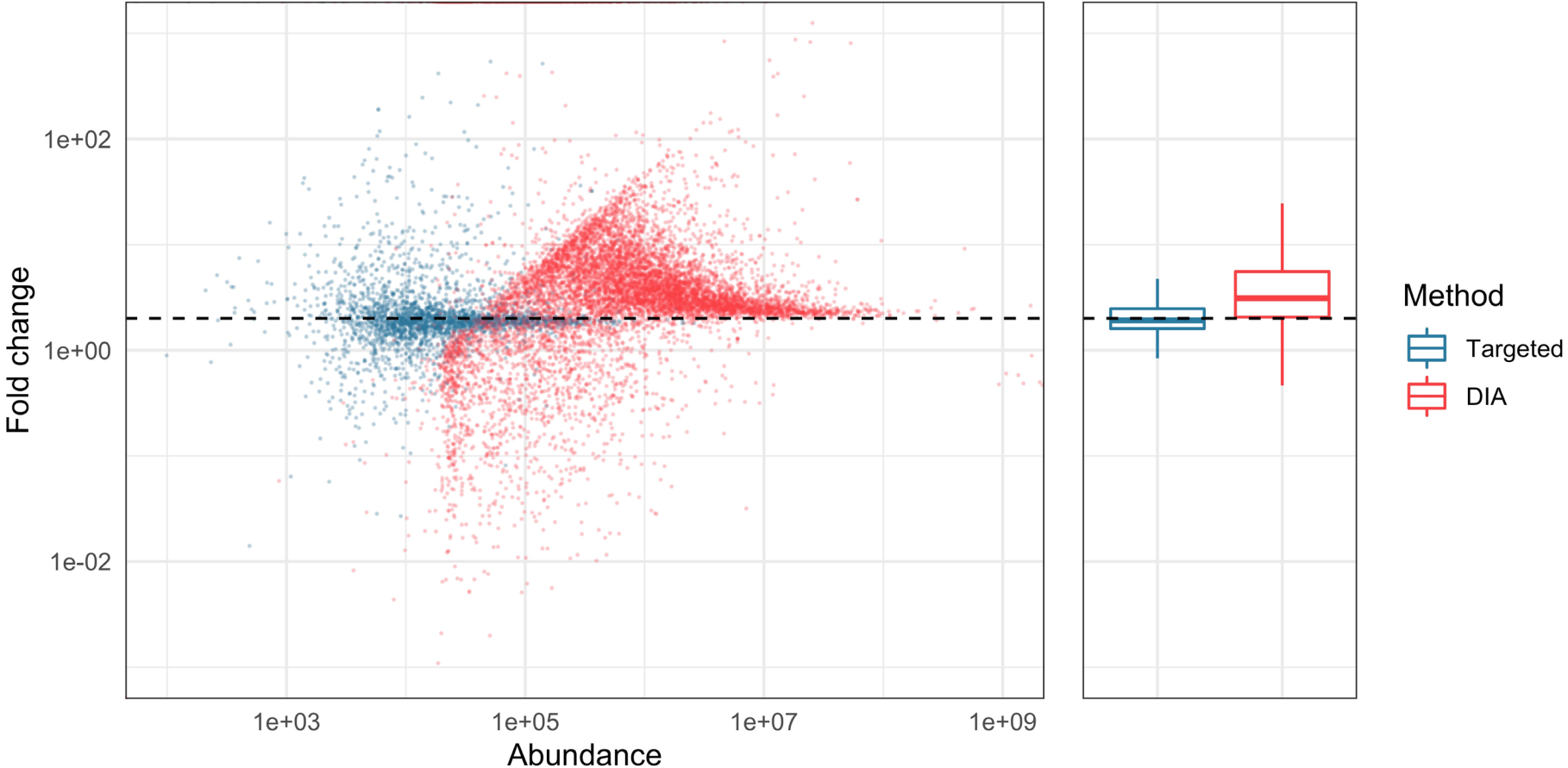
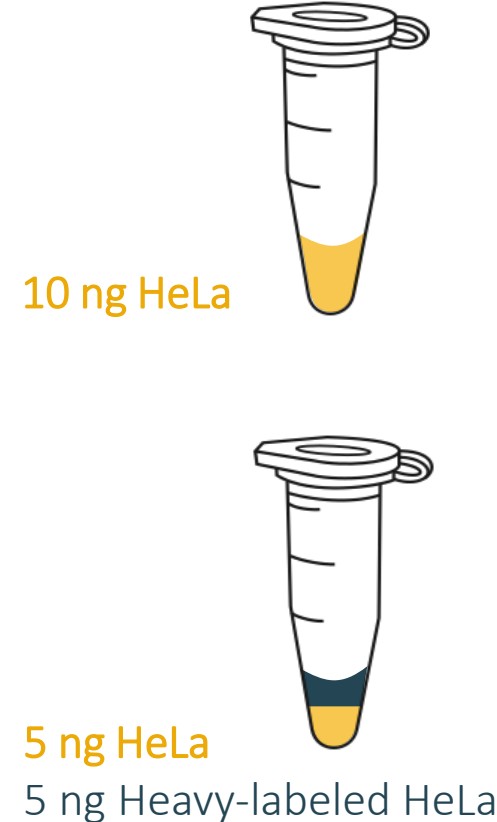
Overlapping Precursors Compete for Space in Multipoles, Can Also Cause Signal Processing Issues



9k Proteins, 83k Peptides in 30 min Gas-Phase Fraction Library

Targeted MS2 Sometimes Has Better Coverage than DIA

2x Dilution Example



DIA: 1,359 Peptides Correct Ratio +/- 25%

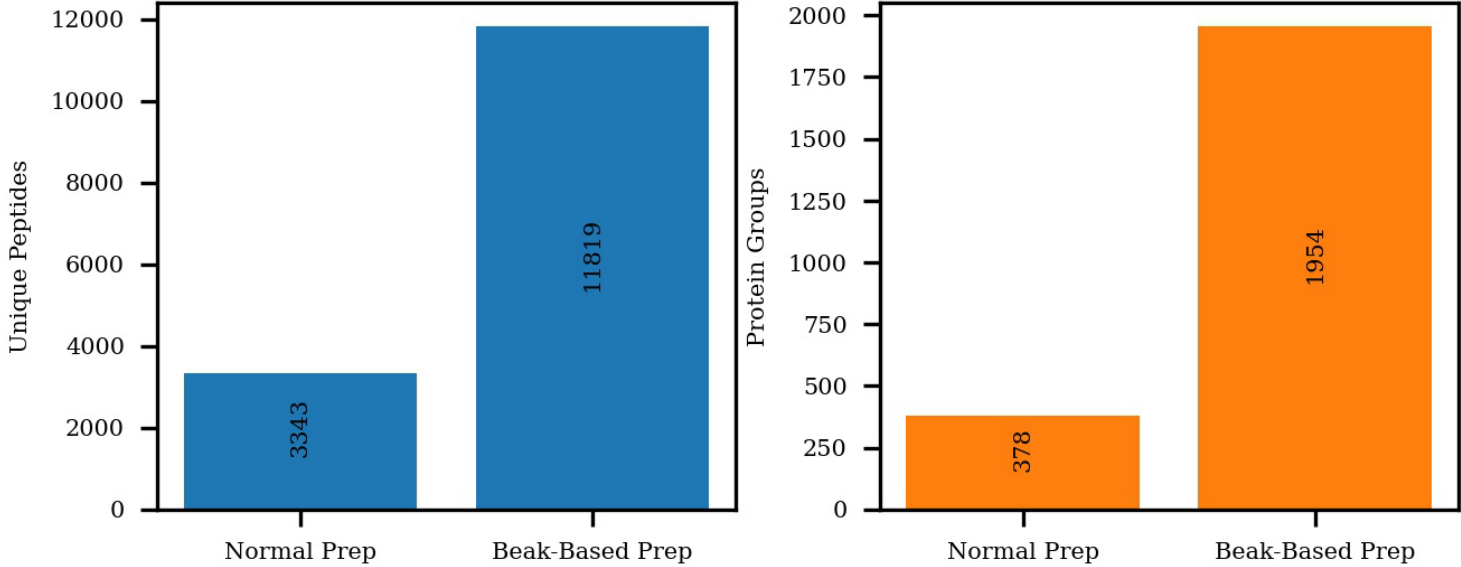
Targeted: 1,414 Peptides Correct Ratio +/- 25%

New, Bead-Based Protocol: Suitable for High-Throughput Target MS2 Assay?

Christine Wu Poster Tues 589

Mag-Net: Bead based capture of membrane particles from plasma enables liquid biopsy measurements for >4,500 proteins

Bead-Based Plasma Prep Gas-Phase Fractions, LIT Analyzer



DIA is the Current Technique of Choice for Such a Sample, But Let's Try Targeted MS2

Results are from 6x Gas-Phase Fractions, Using LIT with 1 Th Isolation Width, Peptide Searching with SEQUEST + Inferys

Why is Targeted MS2 Not Used at Large Scale?

Challenge

Fiddly and Manual Processes

Selecting High Quality Targets / Transitions

Limited Coverage

Peak Integration

Drifting Retention Times

Why is Targeted MS2 Not Used at Large Scale?

Challenge	Solutions
Fiddly and Manual Processes	Automation
Selecting High Quality Targets / Transitions	Chromatogram Library, Automated Filters, [Possible Dilutions]
Limited Coverage	RT Alignment, [Faster Instruments, Brighter Source]
Peak Integration	RT Alignment, Library Spectral Comparison, mProphet
Drifting Retention Times	RT Alignment

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Peak Integration	RT Alignment , Library Spectral Comparison, mProphet
Drifting Retention Times	RT Alignment

More About RT Alignment at the End!

We've Made a Skyline External Tool

To Enable Large-Scale Targeted Experiments

1. To Eliminate (or at Least Drastically Reduce)
 - Manual Editing of Spreadsheets
 - Manual Review of Selected LC Peaks
2. Implement New Features
 - Automated Transition Selection
 - Precursor Load Balancing
3. Share Our Advances While Maintaining Control of a Few Proprietary Algorithms

External Tools for Skyline

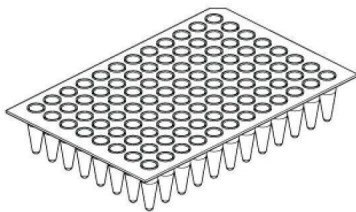
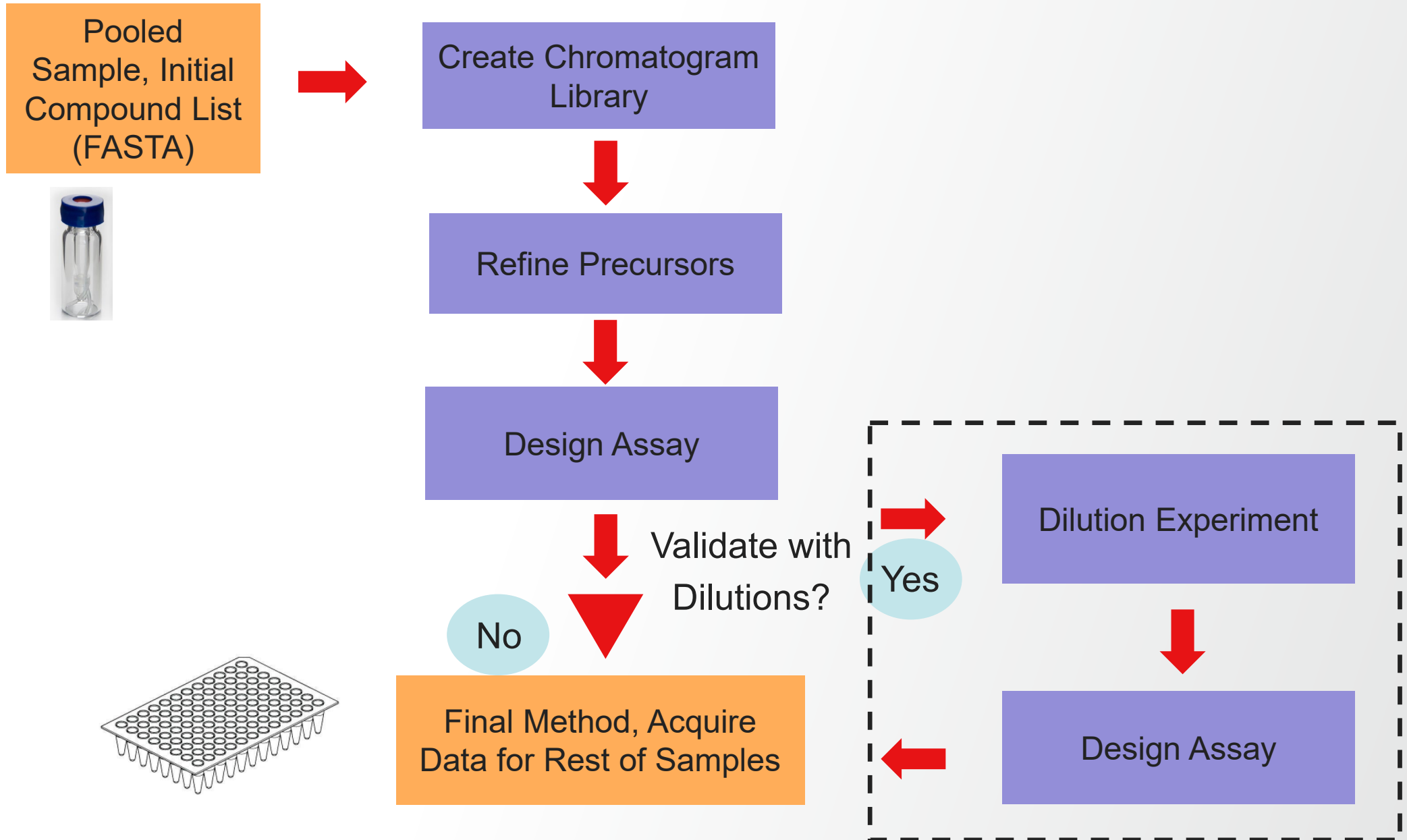
To learn more about creating External Tools of your own and making them easy to install and share with others, please consult the resources on the Skyline **Documentation** page.

To submit an External Tool to the Skyline Tool Store, [click here](#).

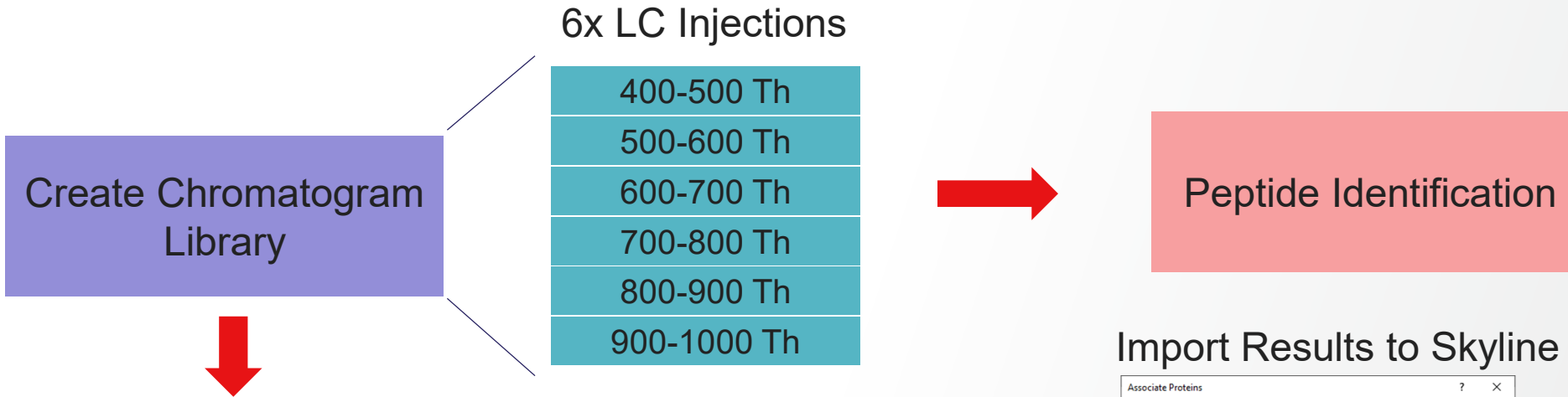


DALL-E A shiny, golden hammer with its head embedded in a rock, like Excalibur. It is illuminated by a brilliant sun.

Overview of High Throughput (>1k) Targeted Workflow



Step 1: Acquire Chromatogram Library



Refine

Design Assay

Import Peptide Search

Spectral Library

Build (selected) Use existing

Start from: Search results (build library directly)

File	Score Type	Score Threshold
220119_P2_1neo_30min_uPAC_DIA11	Percolator q...	0.01

IRT standard peptides: None

Workflow: DIA (selected)

Associate Proteins

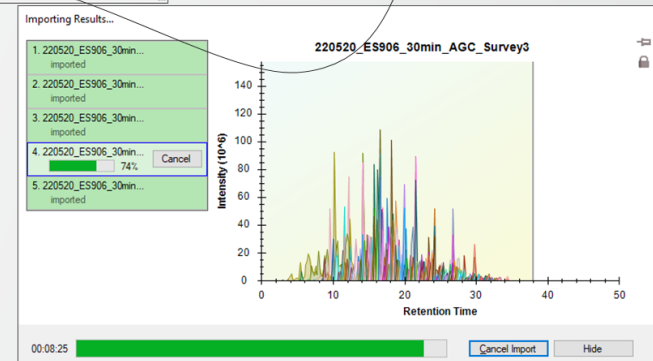
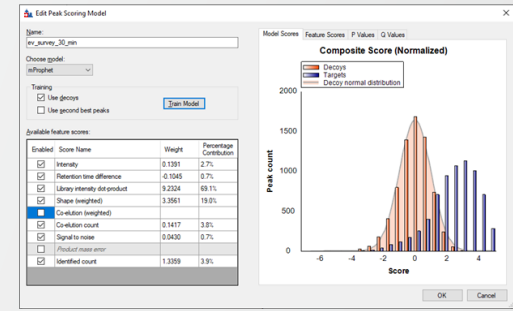
Organize all document peptides into associated proteins or protein groups.

Protein parsimony options

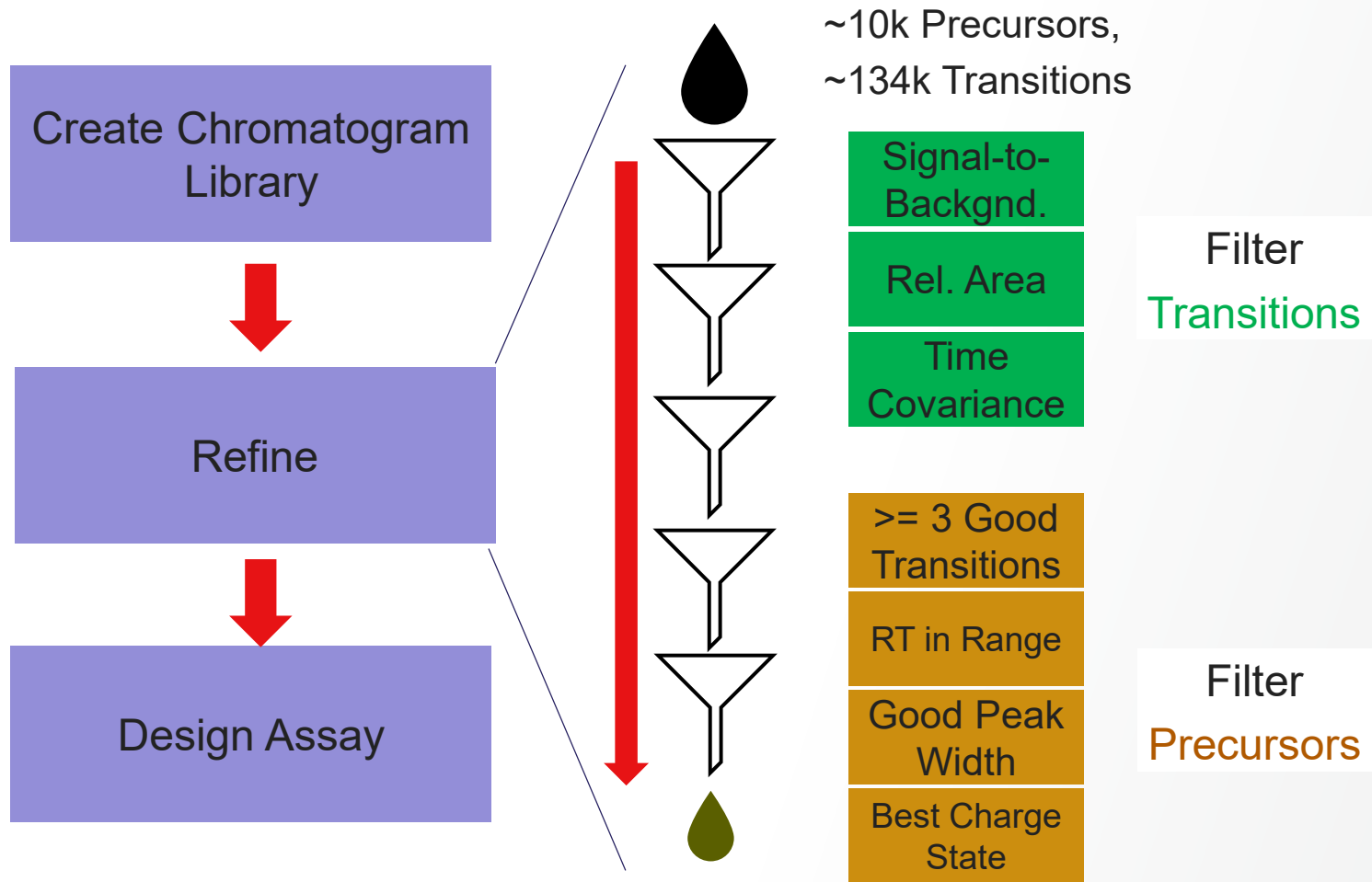
- Create protein groups
- Find minimal protein group list that explains all peptides
- Remove subset protein groups

Protein association results:	Mapped	Unmapped	Targets
Proteins	2336	18,081	1856
Peptides	8844	0	8137
Shared Peptides	1887		0

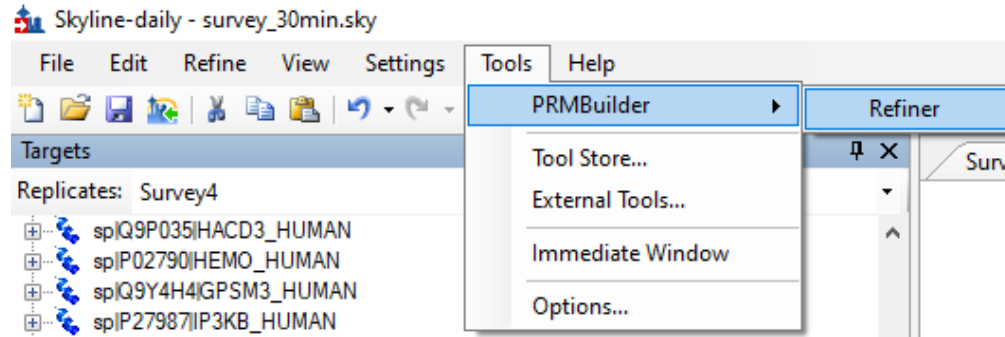
1856 proteins, 8137 peptides, 20,099 precursors, 267,913 transitions



Step 2: Refine Results with Additional Targeted Injections



Start the External Tool



Exports a Custom Report
to External Tool

Document Grid: PRM Builder: Precursor Refine

Reports ▾ | 1 of 198625 | Export... Actions ▾ | Find: A

	File Name	Peptide Modified Sequence	Precursor Mz	Precursor Charge	Protein	Product Mz	Fragment Ion	Product Charge	Retention Time	Area	Background	Raw Times	Raw Intensities	Fwhm	Library Ion Mobility Value	Protein Locator	Peptide Locator	Transition Locator
▶	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	1216.483367	y11	1	10.52	529	0	10.26,10.3,10.34...	48,76,358,0,0,99...	0.09	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	1115.435688	y10	1	10.52	1319	0	10.26,10.3,10.34...	66,0,0,0,0,0,287...	0.05	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	968.367274	y9	1	10.52	4367	3	10.26,10.3,10.34...	0,0,0,0,0,335,94...	0.06	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	881.335246	y8	1	10.52	1452	1	10.26,10.3,10.34...	51,0,156,0,0,112...	0.07	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	794.303218	y7	1	10.52	1741	0	10.26,10.3,10.34...	0,0,0,0,0,116,40...	0.06	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	680.26029	y6	1	10.65	1597	1825	10.26,10.3,10.34...	403,350,505,57...	0.23	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	520.229642	y5	1	10.52	1426	2	10.26,10.3,10.34...	98,138,0,181,53...	0.1	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	449.192528	y4	1	10.57	590	939	10.26,10.3,10.34...	82,212,0,0,94,0...	0.06	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	232.140415	y2	1	10.52	2019	0	10.26,10.3,10.34...	0,0,0,0,0,411,0...	0.04	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	608.745321	y11	2	10.48	1525	2004	10.26,10.3,10.34...	3039,1264,0,128...	0.08	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	397.655247	y7	2	10.48	1341	1	10.26,10.3,10.34...	28,0,0,0,0,134,1...	0.1	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	260.618459	y5	2	10.65	2349	1	10.26,10.3,10.34...	0,0,0,93,0,65,20...	0.18	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	332.145226	b3	1	10.65	1855	0	10.26,10.3,10.34...	0,124,0,64,0,0,1...	0.17	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	566.245669	b5	1	10.65	1684	0	10.26,10.3,10.34...	302,269,308,127...	0.18	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...
	220520_ES906_...	ETTFSSNC(+57)...	723.790457	2	sp O14639 ABL...	283.626472	b5	2	10.65	3980	735	10.26,10.3,10.34...	92,150,0,0,46,15...	0.07	#N/A	MoleculeGroup:/...	Molecule:/sp O1...	Transition:/sp O1...

Refine the Chromatogram Library

Filter
Transitions

Set Method
Parameters

Export
Method

Refine Targets

Skyline Report File

TMT Modifications Present? **No**

Min Signal/Backgd.

Min Rel. Area

Min Time Corr.

Min/Max Width (s) -

Min/Max RT (min) -

Min Good Trans. Keep All Precs.

Define Method

Analyzer

Scan Rate (kDa/s)

Min Dwell (msec)

MS3

Optimize Scan Range

LC Peak Width (s)

Min. Pts. per Peak

Cycle Time (s)

Acq. Window (min)

Max Peps. / Prot.

Protein Priority File

Create Method

Balance Load 1 Charge/Prec.

Base Name

Method

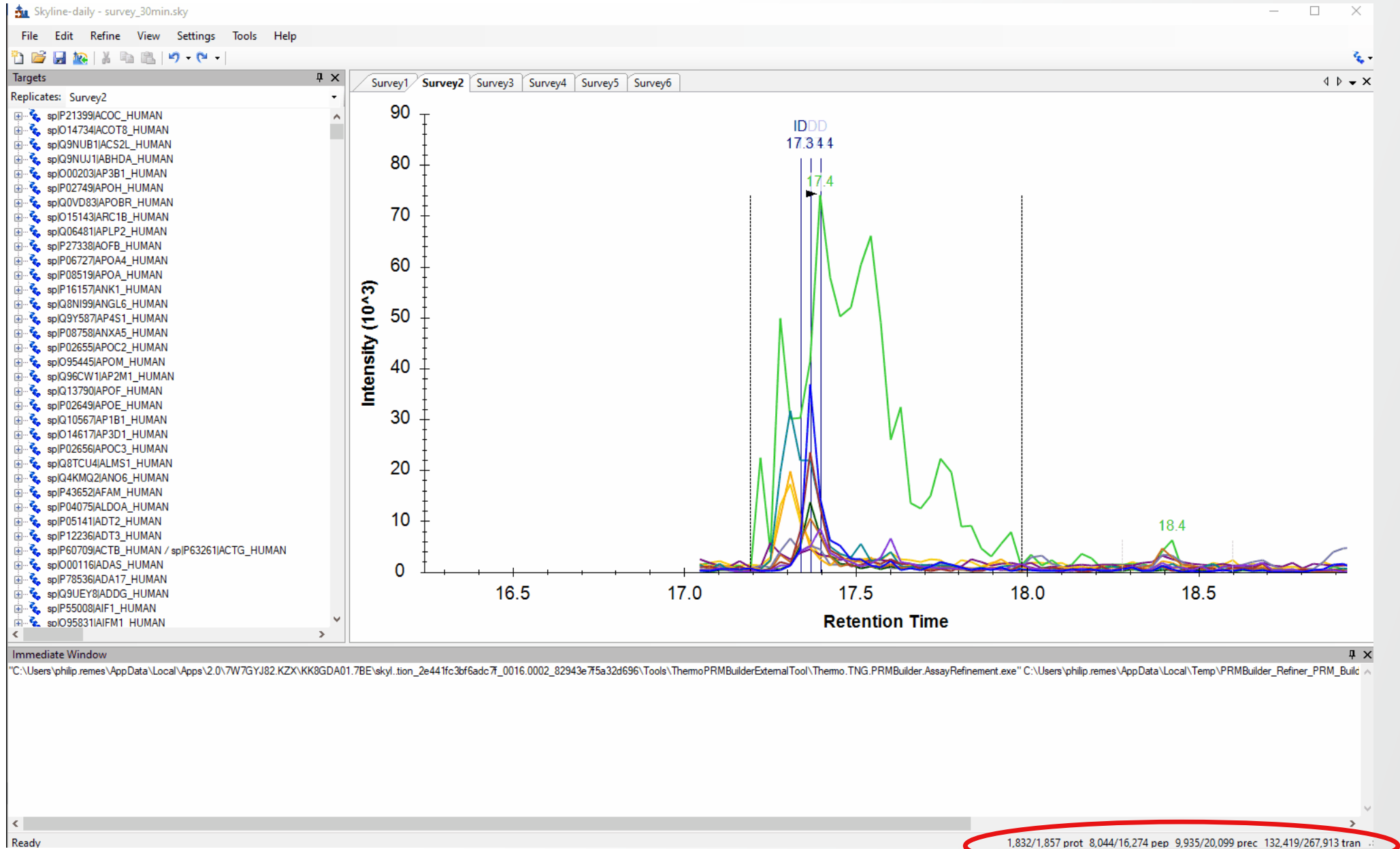
Template

Skyline Connection **Connected**

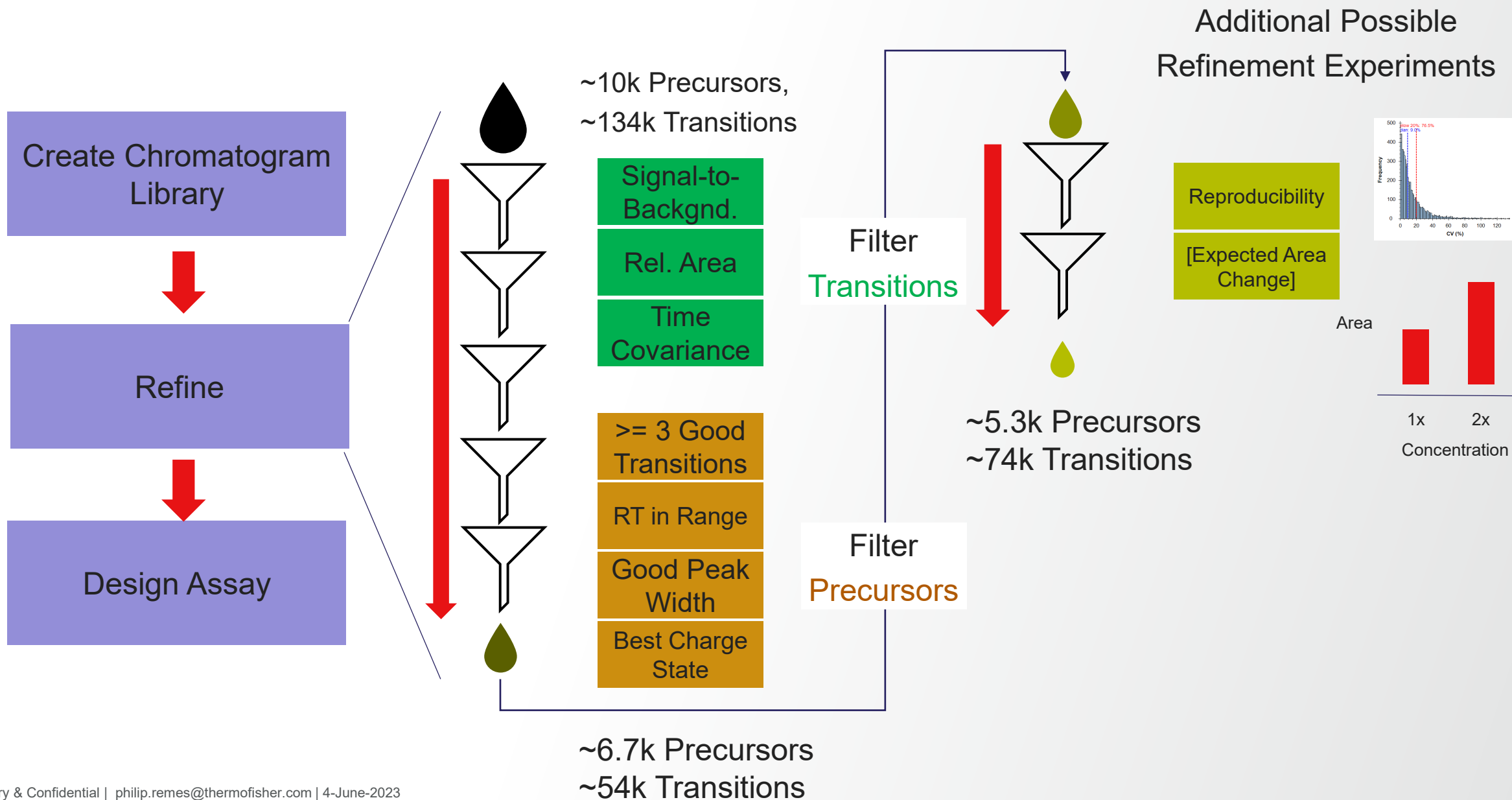
[Searle et. al. Chromatogram libraries improve peptide detection and quantification by data independent acquisition mass spectrometry | Nature Communications](#)



Using Tool to Update Skyline Document

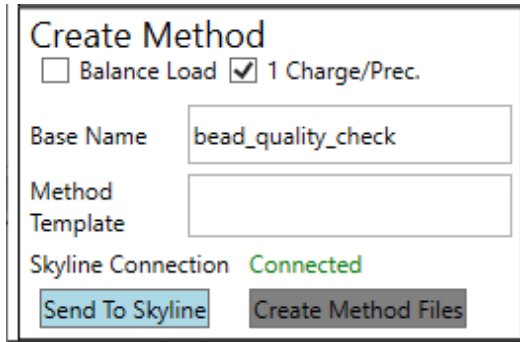


Step 2: Refine Results with Additional Targeted Injections

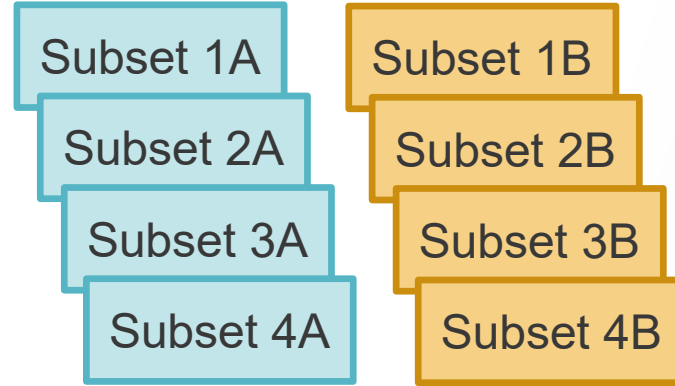


Checking the Precursor Quantitative Precision

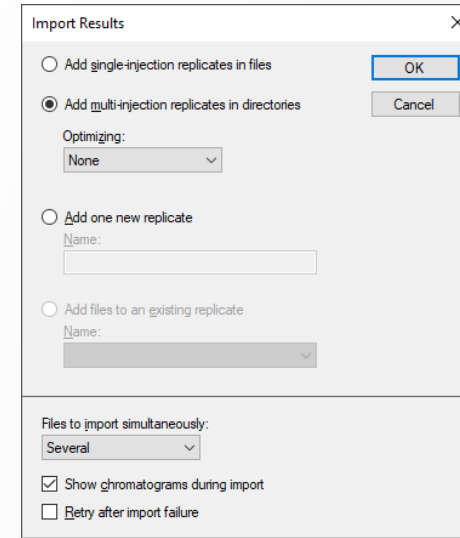
Particularly if there are Many Precursors, We Can Filter Out the Ones with Poor Precision



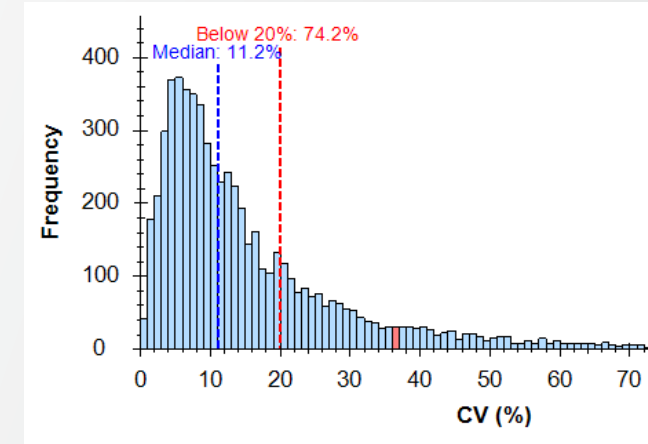
Press to Create Instrument Methods for N Assay Subsets



Acquire 2-5 Replicates



Load Multi-Injection Results



Filter ~25% of Targets by CV

Situation: Library Filtered, CV Filtered

Most Peptides Look Beautiful

A Minority Are Low Abundance
(psst...CV isn't a Perfect Metric)

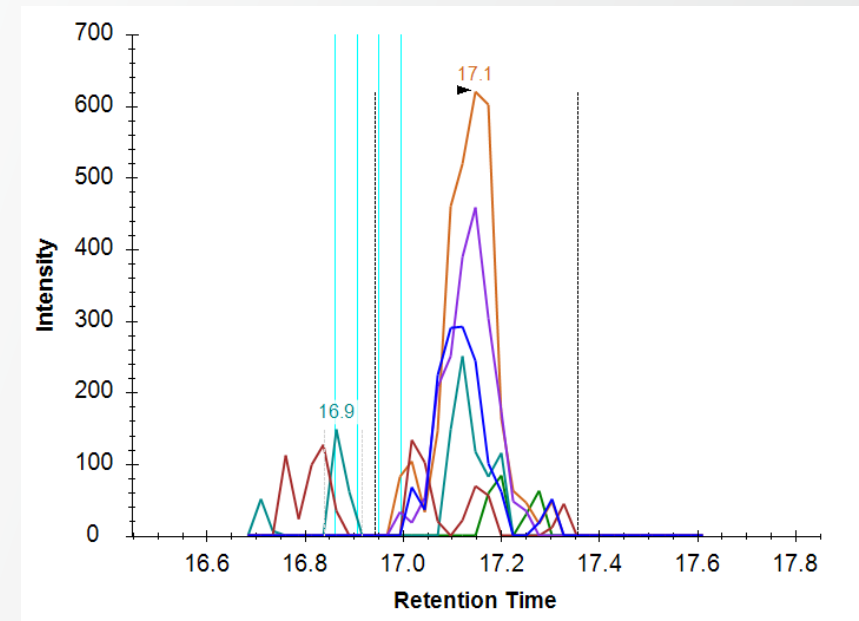
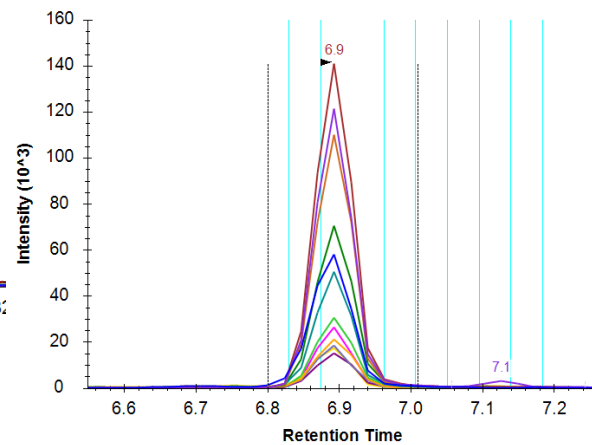
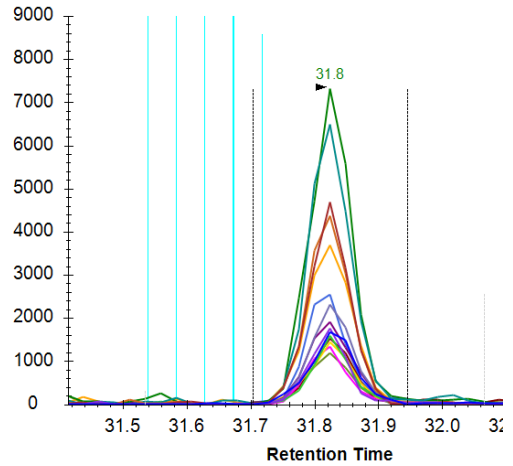
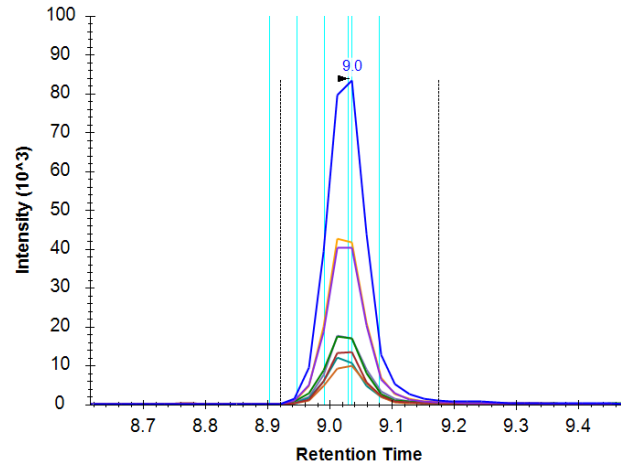
Skyline-daily - quality_check_cv_filtered.sky

File Edit Refine View Settings Tools Help

Targets

Replicates: Replicate1

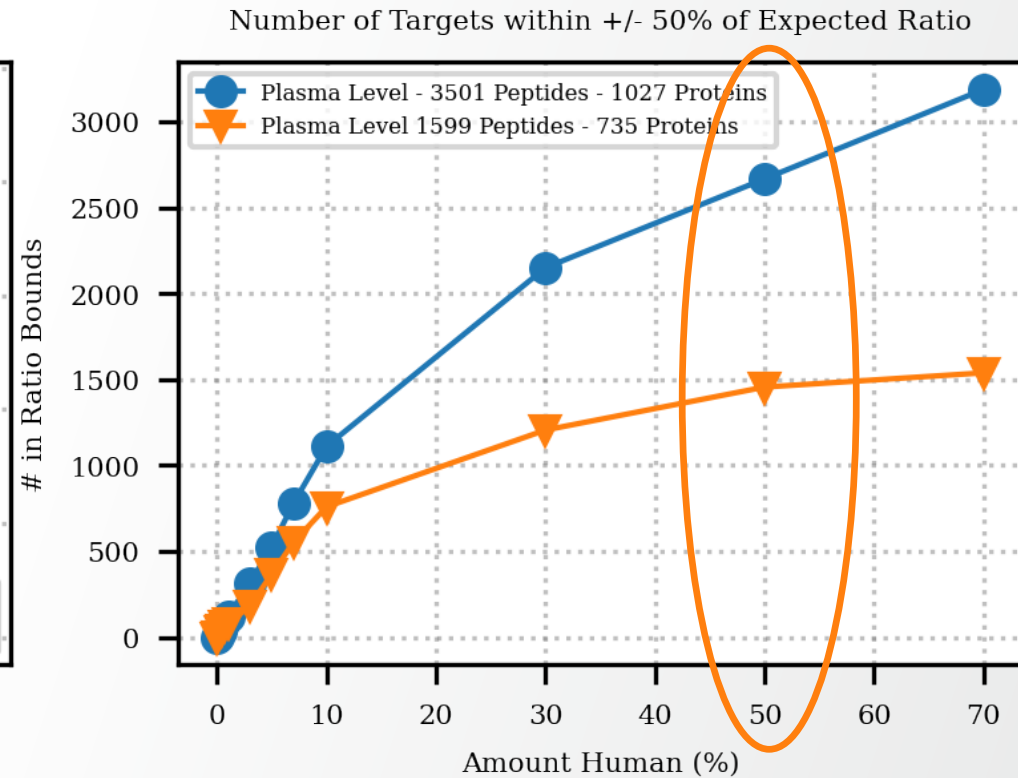
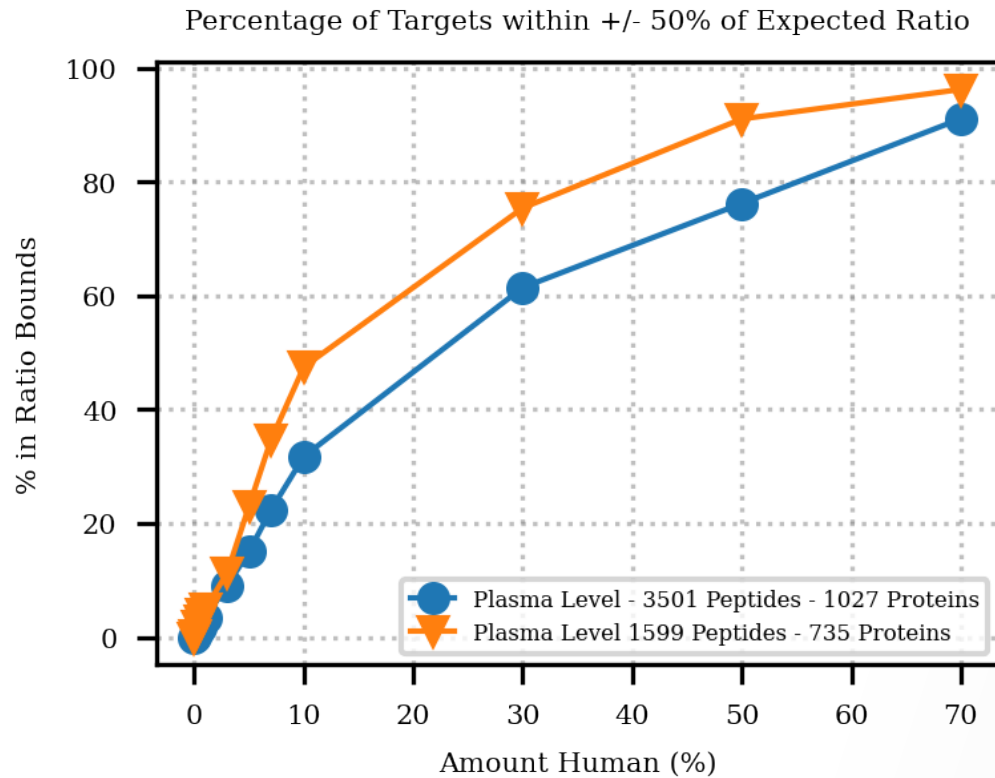
- R.QATLEGLQEVVGR.L [529, 541]
- R.LQQEATEHATESEER.F [692, 706]
- K.LVGGQAGLGR.R [779, 788]
- R.VLLNDGGYDPETGVFTAPLAGR.Y [892, 914]
- spIP0DP2IGAL3A_HUMAN / spIA0A0B4J2D5IGAL3B_HUMAN
- K.ITDLANLSAANHDAIFPGGFGAAK.N [117, 141]
- K.NLSTFAVDGK.D [142, 151]
- K.EVVEAHVDQK.N [224, 233]
- spIP50395GDIB_HUMAN
- K.VPSTEAEALASSMLGFEK.R [119, 137]
- R.TDDYLDGPCYETINR.I [194, 208]
- R.LSAIYGGTYMLNKPRIEIVNGK.V [241, 264]
- K.FVSIQDLVVK.D [380, 390]
- K.DLGTESQIFISR.T [391, 402]
- spIP05155IC1_HUMAN
- K.LYHAFSANK.K [153, 161]**
- K.DFTCVHQALK.G [202, 211]
- K.GVTSVSIQIFHSPDLAIR.D [217, 233]
- R.LLDSLPSDTR.L [277, 286]
- K.YPVAHFIDQTLK.A [330, 341]
- R.LEDMEQALSPSVFK.A [367, 380]
- K.FQPTLLTLPRI.I [391, 400]
- K.VTTSQDMLSIK.L [403, 415]
- K.FPVMGR.V [488, 494]
- spIP48735IDHP_HUMAN
- K.VAKPVVMDGDMTR.I [46, 60]
- R.IIWQFIK.E [61, 67]
- K.LILPHVDIQLK.Y [70, 80]
- K.CATITPDEAR.V [113, 122]
- R.LVPGWTKPITIGR.H [160, 172]
- K.TIEEAAHGTVTR.H [341, 353]
- K.GRPTSTNPIASIFAWTR.G [361, 377]
- K.VQVETVESGAMTK.D [401, 413]
- K.DLAGCIHGLSNVK.L [414, 426]
- K.LNEHFLNTDFLDITK.S [427, 442]
- spIP34932HSP74_HUMAN
- K.VLATAFDTLLGGR.K [222, 234]
- K.STNEAMEWNNK.L [737, 748]
- spIP52566GDIR2_HUMAN
- K.TLLGDGPVVTDPK.A [51, 63]
- K.APNVVVTR.L [64, 71]
- K.ATFMVGSYGRPEEYFLTPVEEAPK.G [139, 164]
- spIP06396GELS_HUMAN
- R.GASQAGAPQGR.V [33, 43]
- R.EVQGFESATFLGYFK.S [148, 162]
- K.HVVPNEVVQR.L [178, 188]
- K.VNSGAGTMSVSLVADENPFQAGALK.S [303, 327]
- K.TASDFITK.M [361, 368]
- K.QTQVSVLPEGGETPLFK.Q [374, 390]
- R.DPDQTDGLSLYSSHIANVER.V [398, 419]
- R.VPDAATLHTSTAMAAQHGMDDDGTGQK.Q [420, 447]
- K.AGALNSNDAFVLT.T [585, 597]
- K.TPSAAYLVVGTGASEAK.T [598, 615]
- R.AQPQVAEGSEPDGFWEALGGK.A [627, 648]
- R.YIETPANR.D [730, 738]



Advanced Filtering: Using Dilution Curve

For Example, Dilute Human in Chicken Plasma

One Could Accept Peptides with Correct Ratio at 50% Dilution



One Could Additionally Optimize Transitions Using Dilution Curve LOQ Results

Nick Shulman Poster Tues 552

Optimizing lower limits of quantification and detection by choosing transitions in Skyline

Step 3: Design Assay

Account for Finite Instrument Speed

Create Chromatogram Library

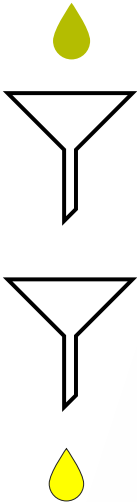


Refine



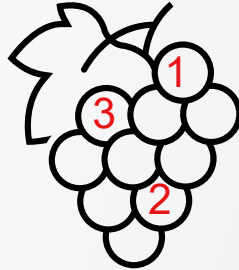
Design Assay

~5.3k Precursors
~74k Transitions



Choose Best
N Peptides
per Protein

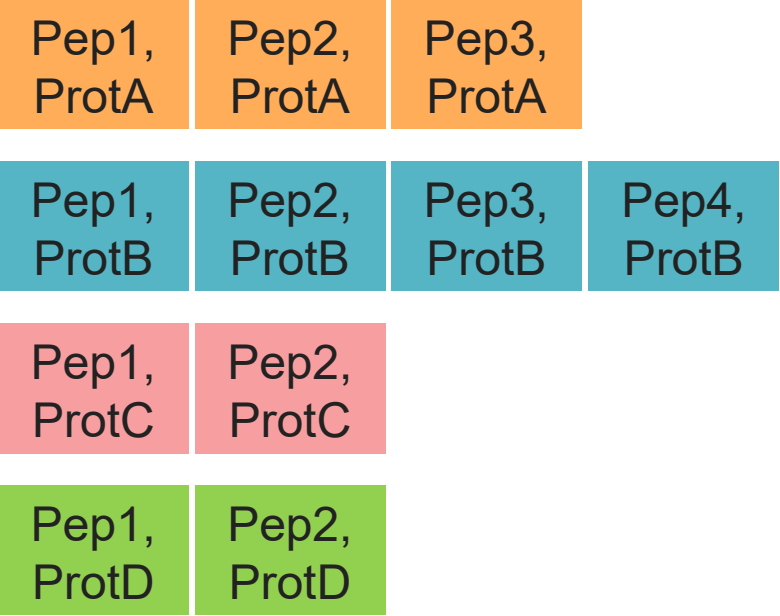
Load Balance



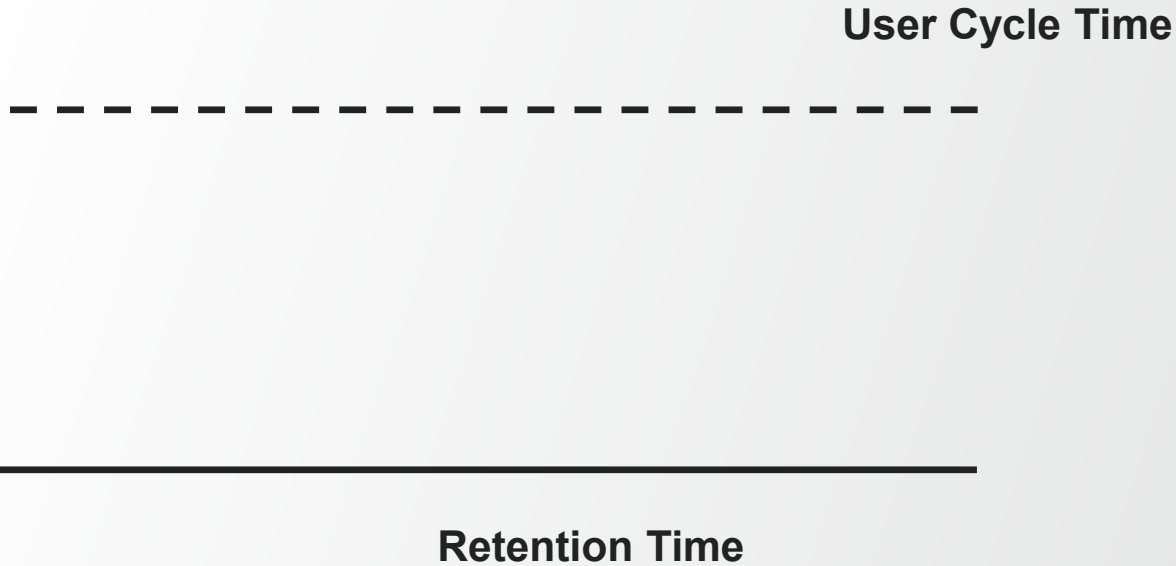
3.5k Precursors / 1k Proteins
or
1.6k Precursors / 0.7k Proteins

Optimization 1: N Peptides per Protein

Ranked Best to Worst Score

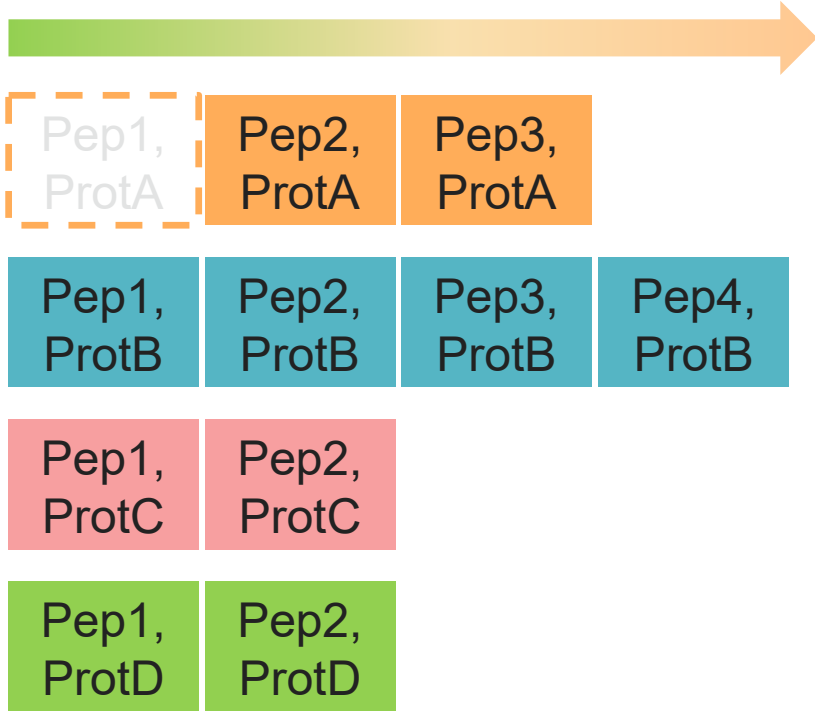


Instrument Time

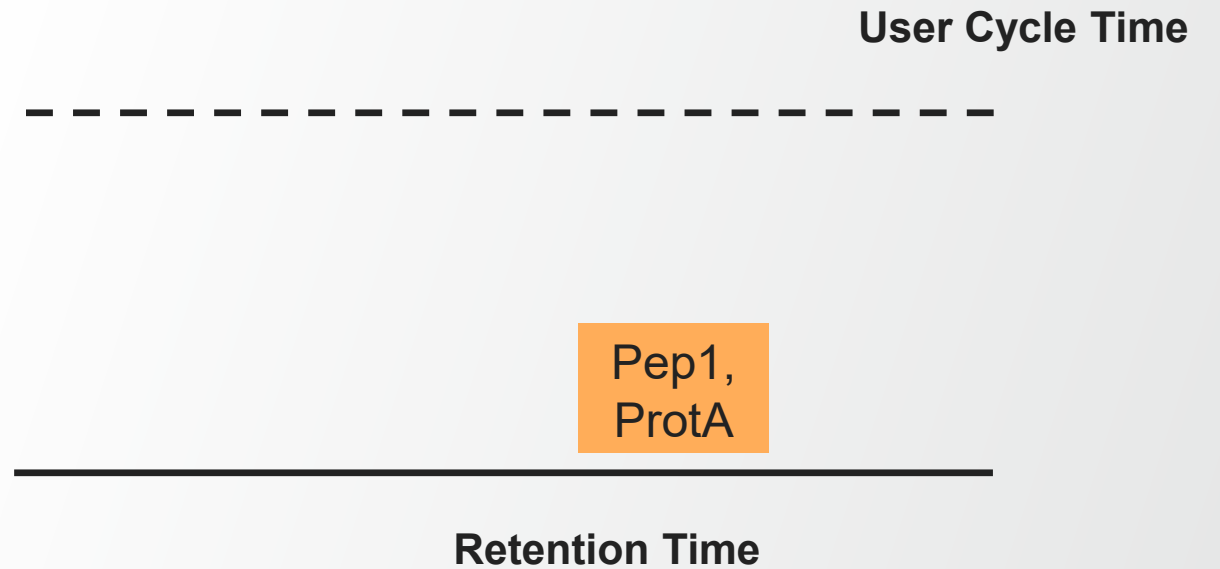


Optimization 1: N Peptides per Protein

Ranked Best to Worst Score

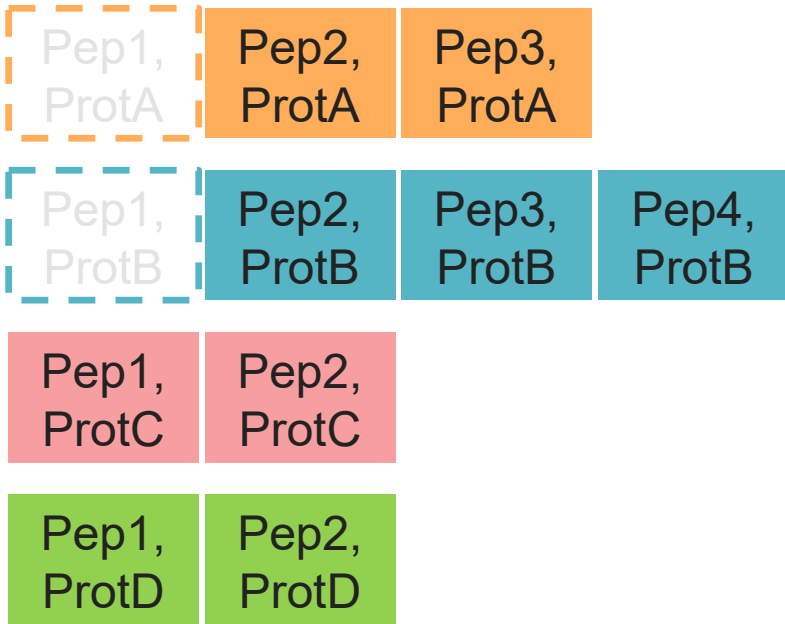


Instrument Time

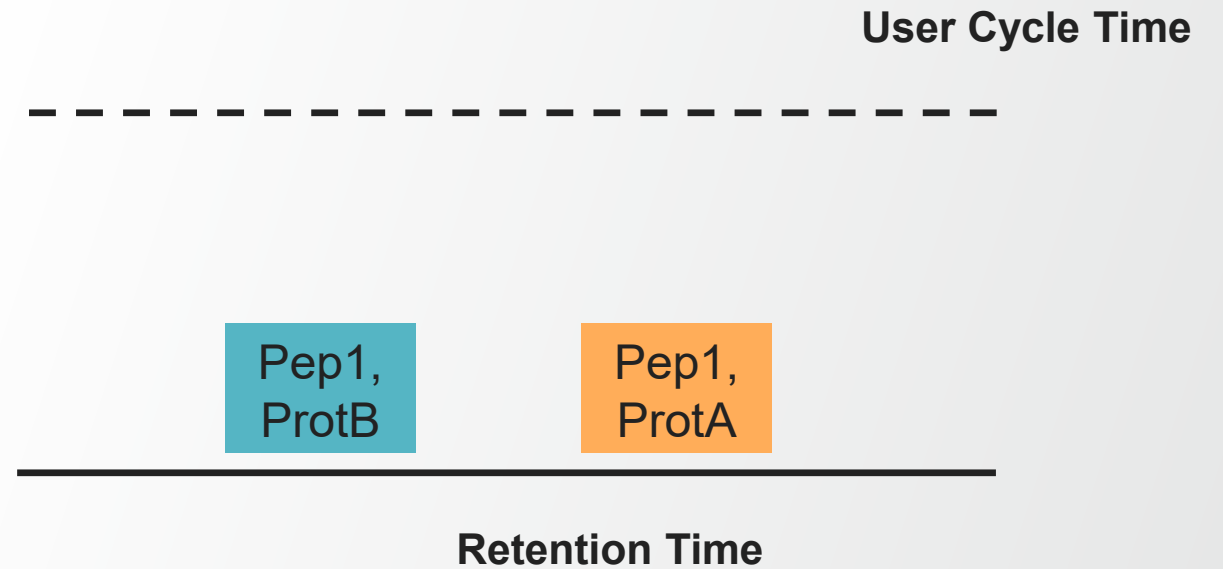


Optimization 1: N Peptides per Protein

Ranked Best to Worst Score

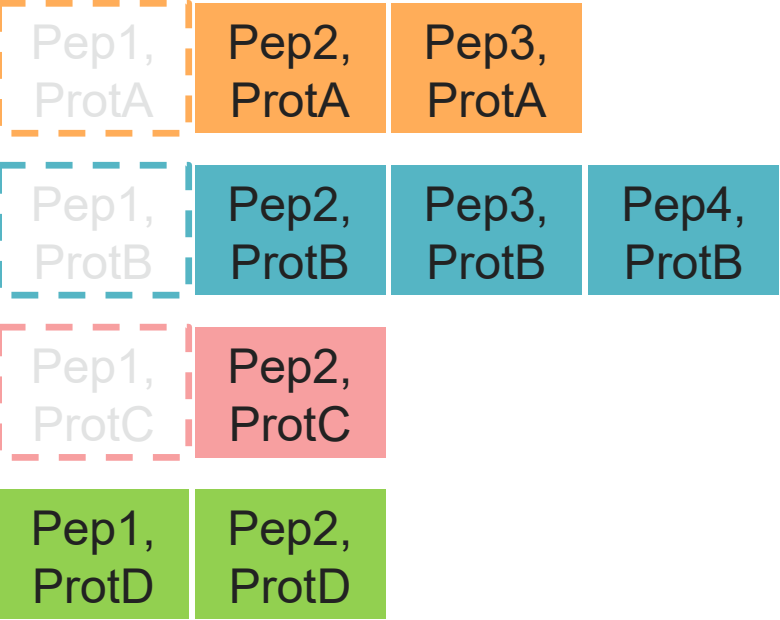


Instrument Time

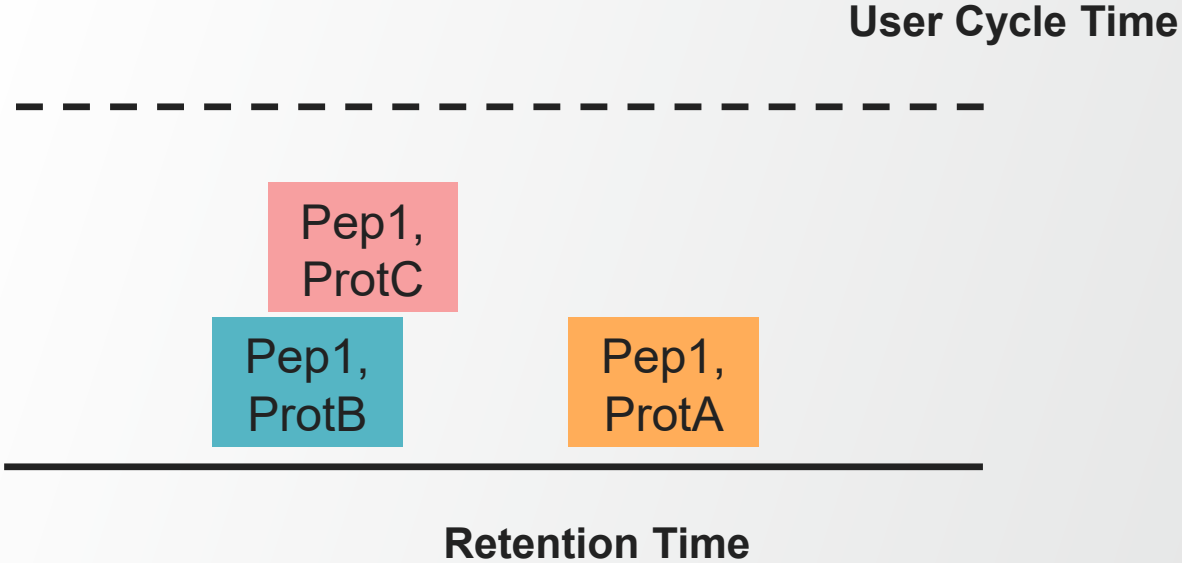


Optimization 1: N Peptides per Protein

Ranked Best to Worst Score

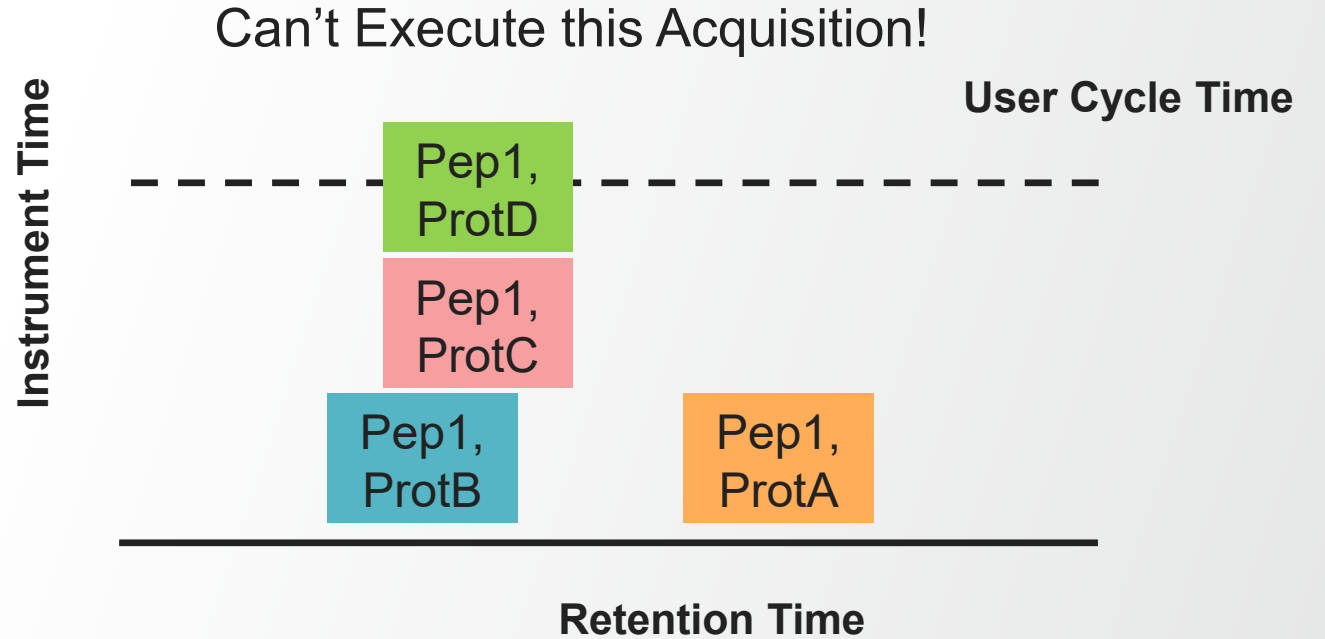
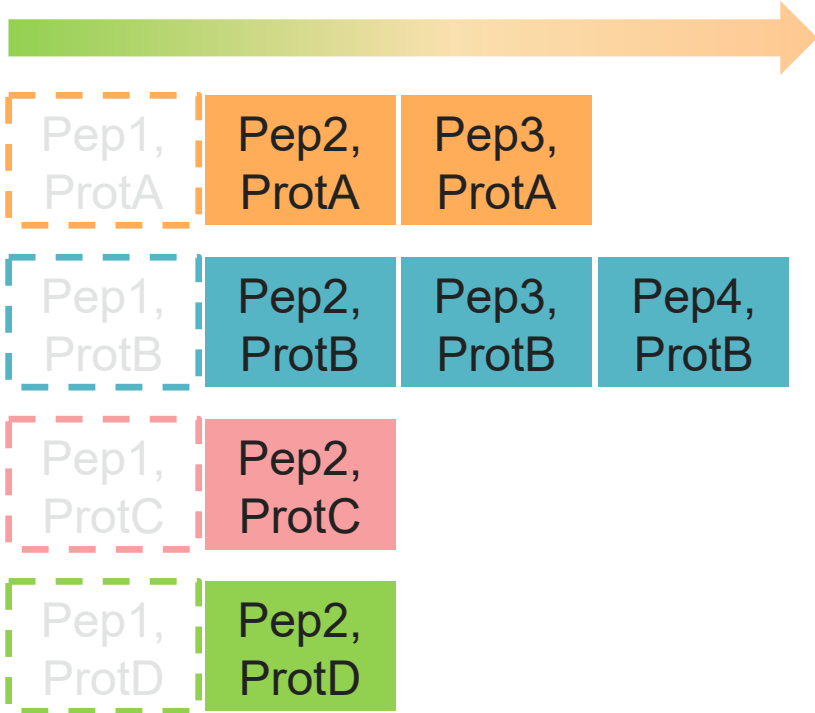


Instrument Time



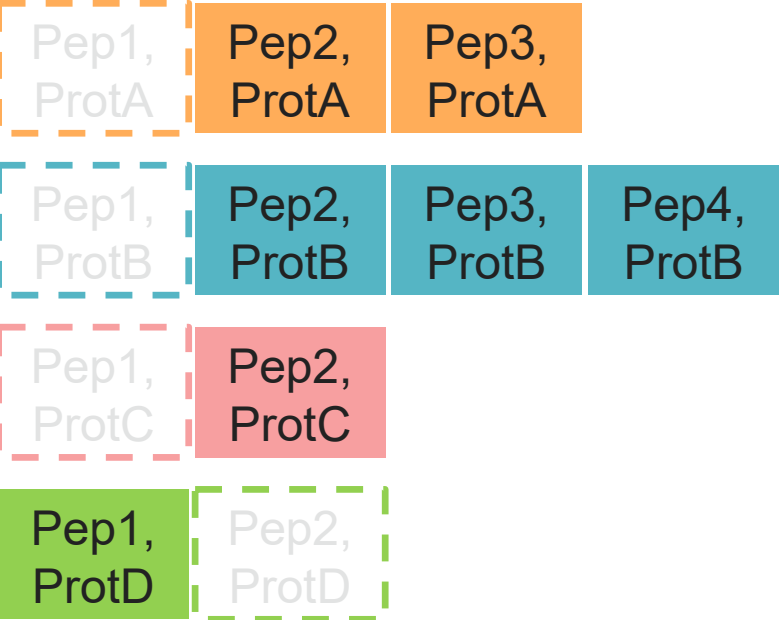
Optimization 1: N Peptides per Protein

Ranked Best to Worst Score



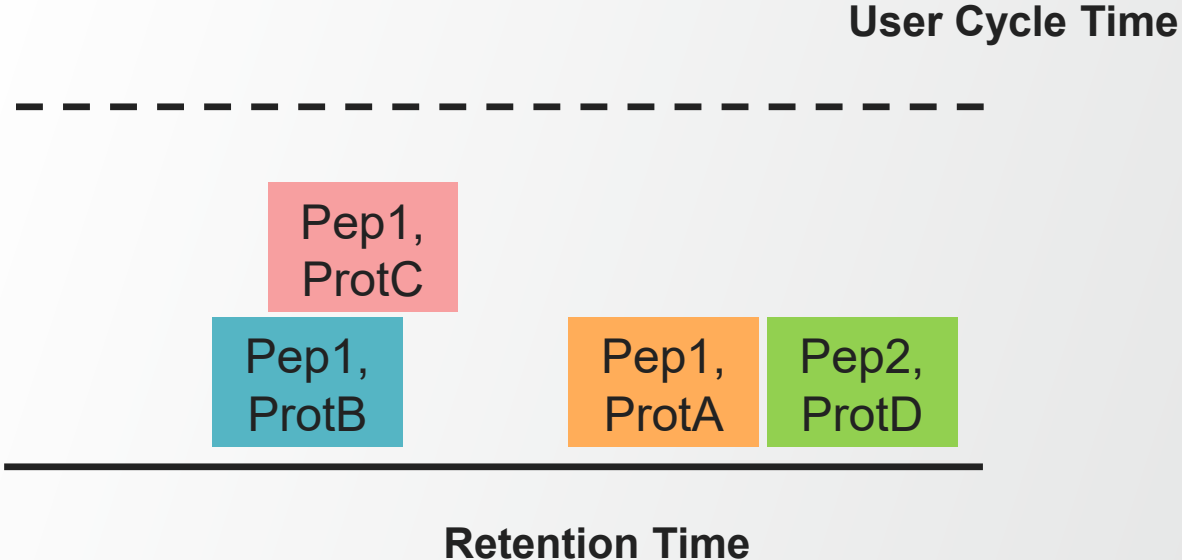
Optimization 1: N Peptides per Protein

Ranked Best to Worst Score



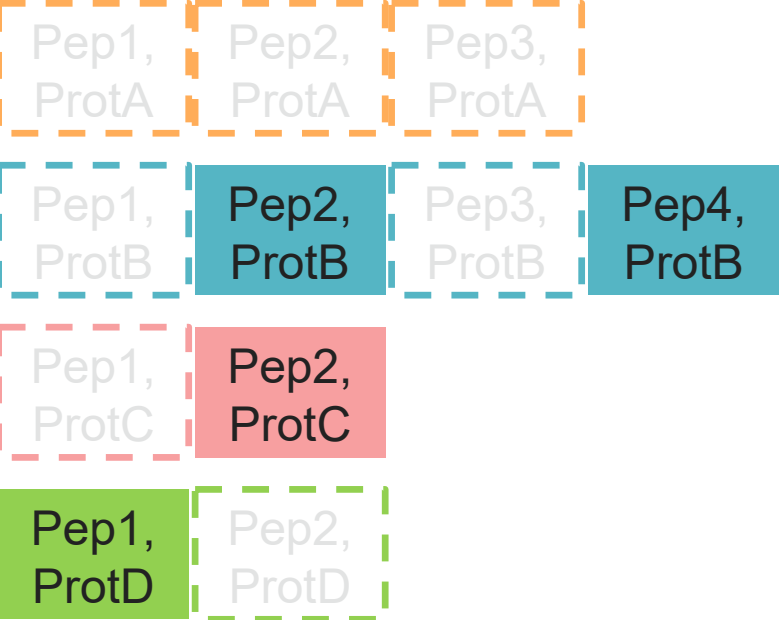
We Take the Peptide2 Instead

Instrument Time



Optimization 1: N Peptides per Protein

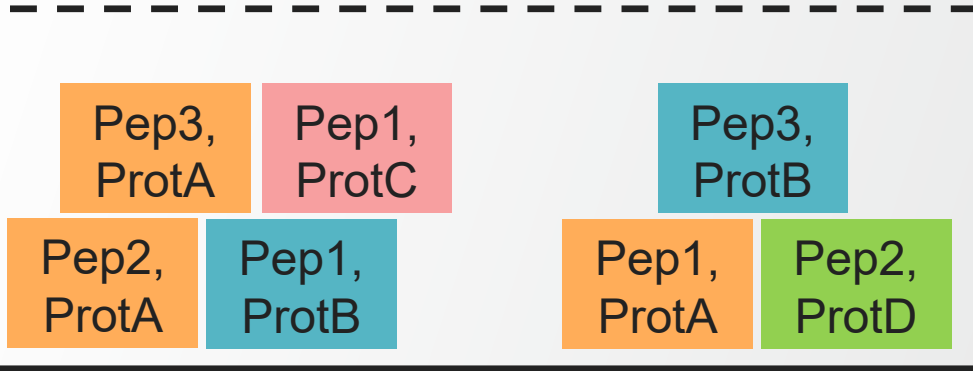
Ranked Best to Worst Score



Add Peptides Until No More Fit

Instrument Time

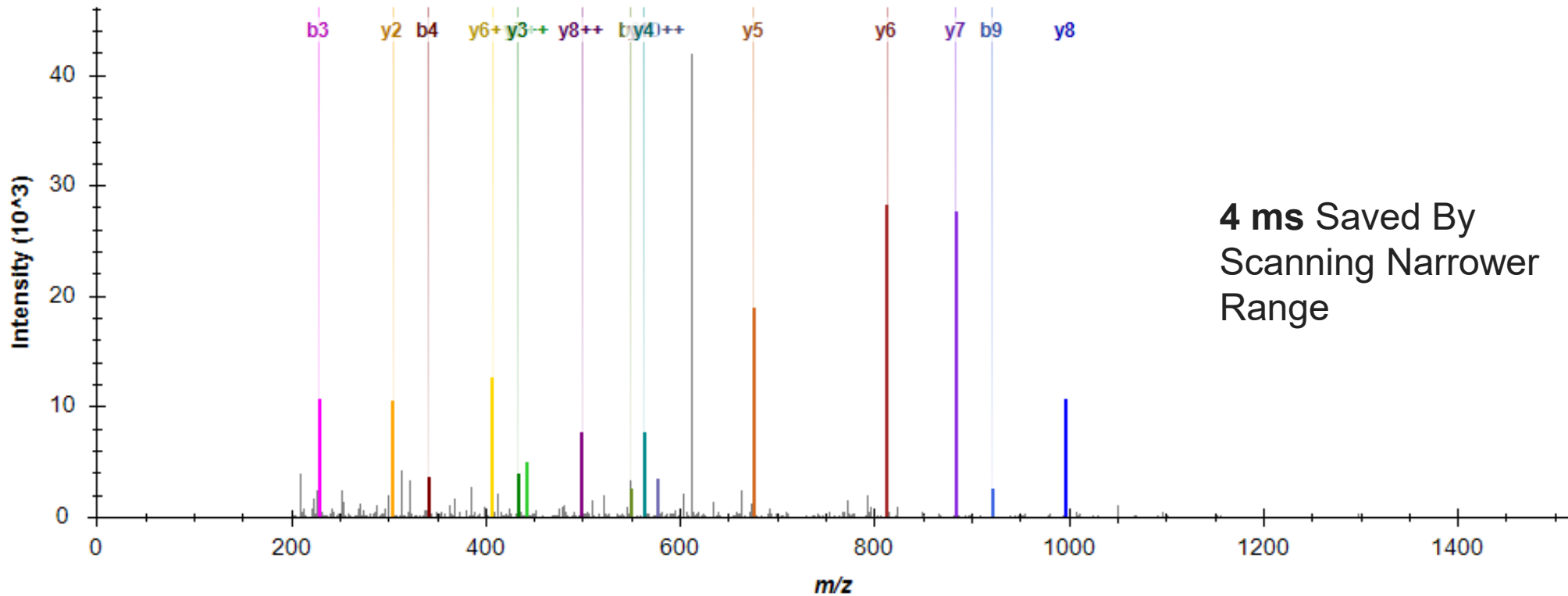
User Cycle Time



Retention Time

Optimization 2: Scan Range Minimization

Example: LIT Scan Rate 0.008 ms/Th



Survey Range m/z 200-1500

Optimized Range m/z 218-1006

$$\text{MinAcqTime} = C + 0.008 * 1300 = C + \mathbf{10.4\ ms}$$

$$\text{MinAcqTime} = C + 0.008 * 788 = C + \mathbf{6.3\ ms}$$

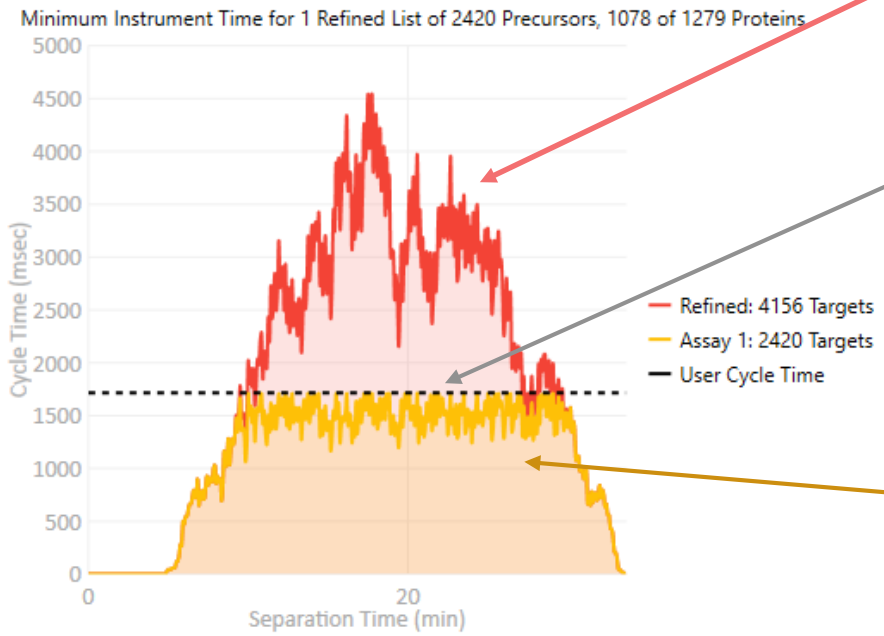
Optimization 2: Scan Range Minimization

Define Method	
Analyzer	Iontrap
Scan Rate (kDa/s)	125
Min Dwell (msec)	5.00
<input type="checkbox"/> MS3	
<input type="checkbox"/> Optimize Scan Range	
Scan Range	200.0 - 1500.0
LC Peak Width (s)	12.00
Min. Pts. per Peak	7
Cycle Time (s)	1.71
Acq. Window (min)	0.75
Max Peps. / Prot.	1000
Protein Priority File	

Instrument Needs
~4500 msec to
Complete a Cycle
with Default Range
m/z 200-1500

Based on LC Peak
Width and Pts-per-
Peak, Cycle is 1710
msec

Assay has
2.4k of 4.1k Peptides,
1.0k of 1.2 Proteins



Optimization 2: Scan Range Minimization

Define Method

Analyzer: Iontrap

Scan Rate (kDa/s): 125

Min Dwell (msec): 5.00

MS3

Optimize Scan Range

Scan Range: 200.0 - 1500.0

LC Peak Width (s): 12.00

Min. Pts. per Peak: 7

Cycle Time (s): 1.71

Acq. Window (min): 0.75

Max Peps. / Prot.: 1000

Protein Priority File:

Pressed "Optimize"

Define Method

Analyzer: Iontrap

Scan Rate (kDa/s): 125

Min Dwell (msec): 5.00

MS3

Optimize Scan Range

LC Peak Width (s): 12.00

Min. Pts. per Peak: 7

Cycle Time (s): 1.71

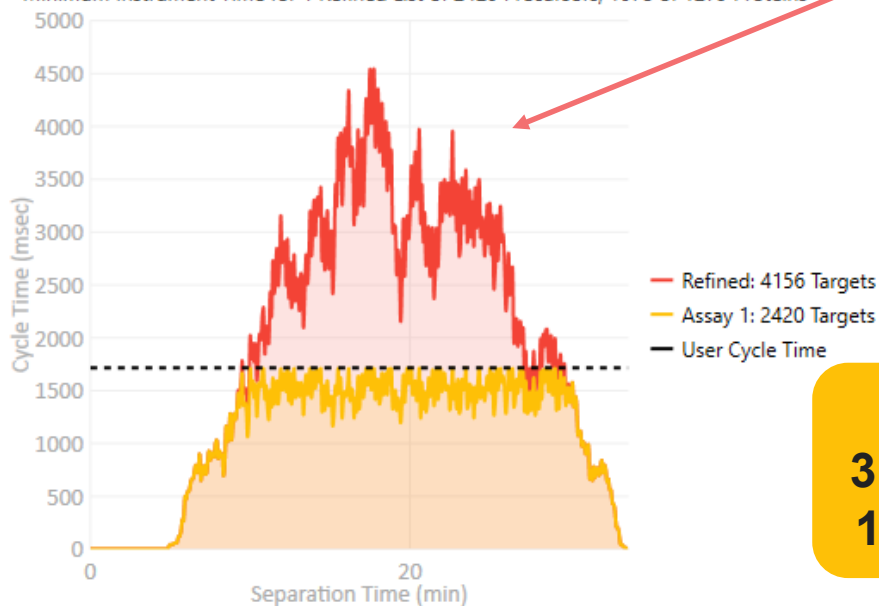
Acq. Window (min): 0.75

Max Peps. / Prot.: 1000

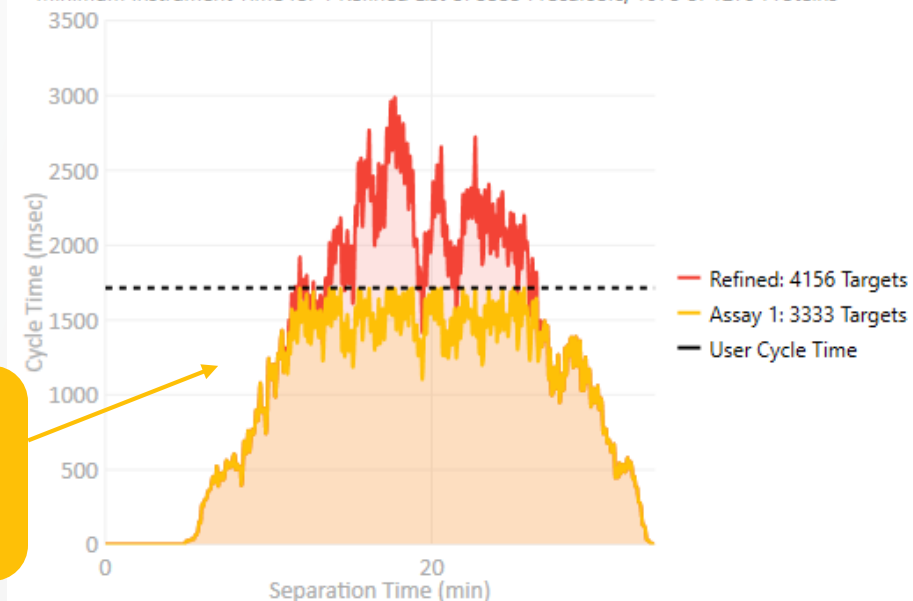
Protein Priority File:

~1500 msec Saved
by Optimizing Scan
Range for All
Precursors

Minimum Instrument Time for 1 Refined List of 2420 Precursors, 1078 of 1279 Proteins

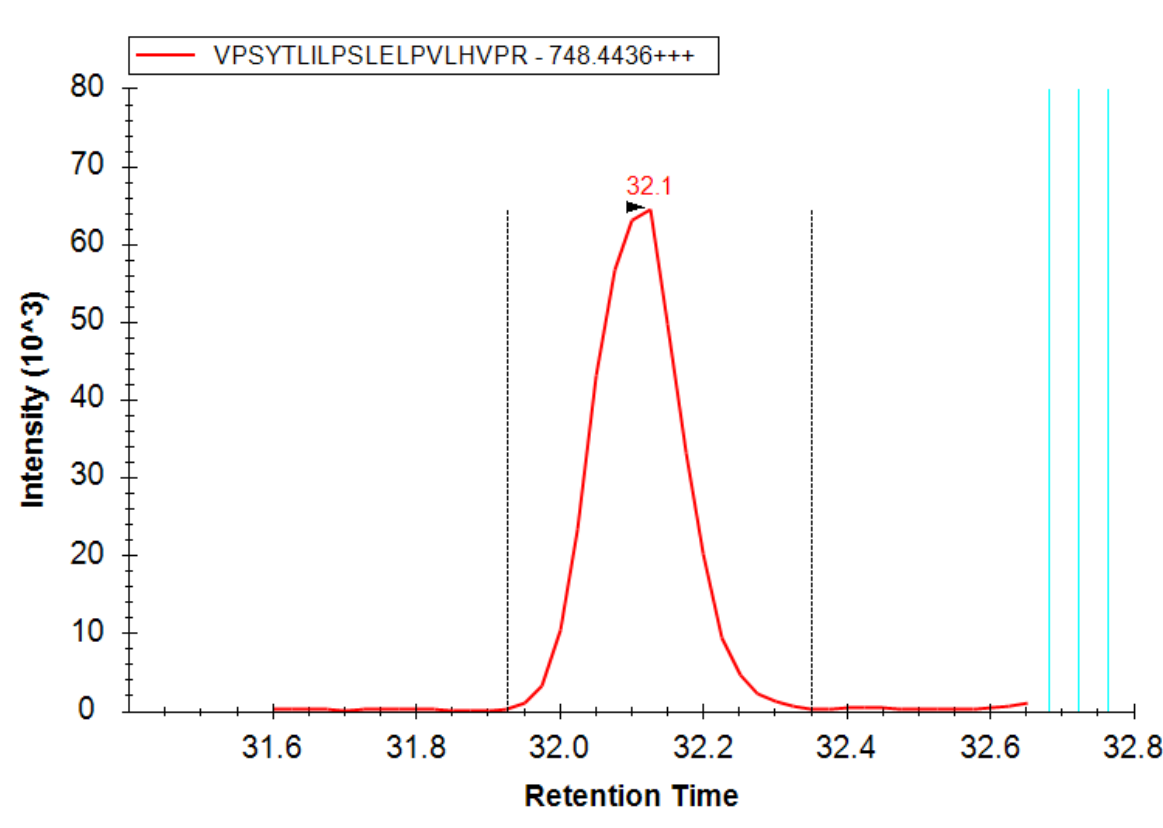


Minimum Instrument Time for 1 Refined List of 3333 Precursors, 1078 of 1279 Proteins



Assay is Now
3.3k of 4.1k Peptides
1.0k of 1.2k Proteins

Effect of Acquisition Segment Width



RT Alignment Enables Narrow Acquisition Segments (3-4 Peak Widths)

Acquisition Segment Time Width

Narrower Acquisition Segment Width Increases Throughput

Define Method

Analyzer: Iontrap

Scan Rate (kDa/s): 125

Min Dwell (msec): 5.00

MS3

Optimize Scan Range

LC Peak Width (s): 13.00

Min. Pts. per Peak: 7

Cycle Time (s): 1.86

Acq. Window (min): 3.00

Max Peps. / Prot.: 1000

Protein Priority File: []

Changed from 3.0 to 0.75 min

Define Method

Analyzer: Iontrap

Scan Rate (kDa/s): 125

Min Dwell (msec): 5.00

MS3

Optimize Scan Range

LC Peak Width (s): 13.00

Min. Pts. per Peak: 7

Cycle Time (s): 1.86

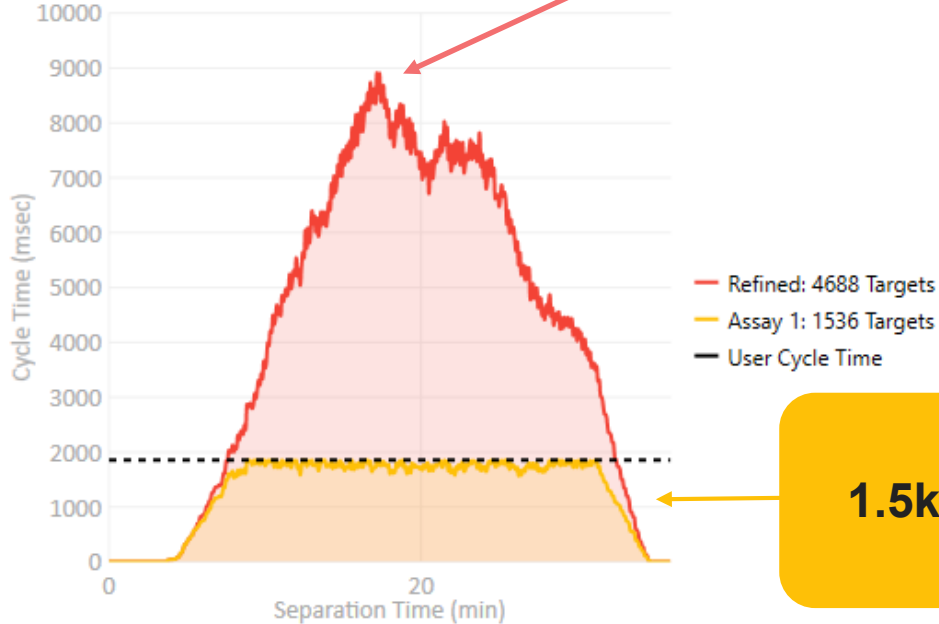
Acq. Window (min): 0.75

Max Peps. / Prot.: 1000

Protein Priority File: []

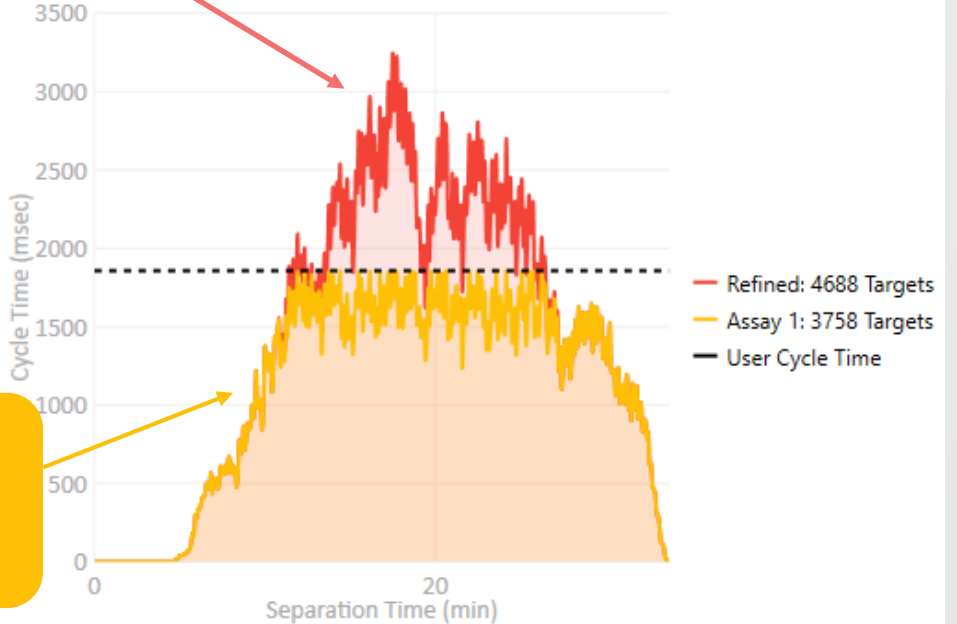
~6000 msec Saved

Minimum Instrument Time for 1 Refined List of 1536 Precursors, 1115 of 1412 Proteins



Instead of 1.5k Assay is 3.7k Peptides

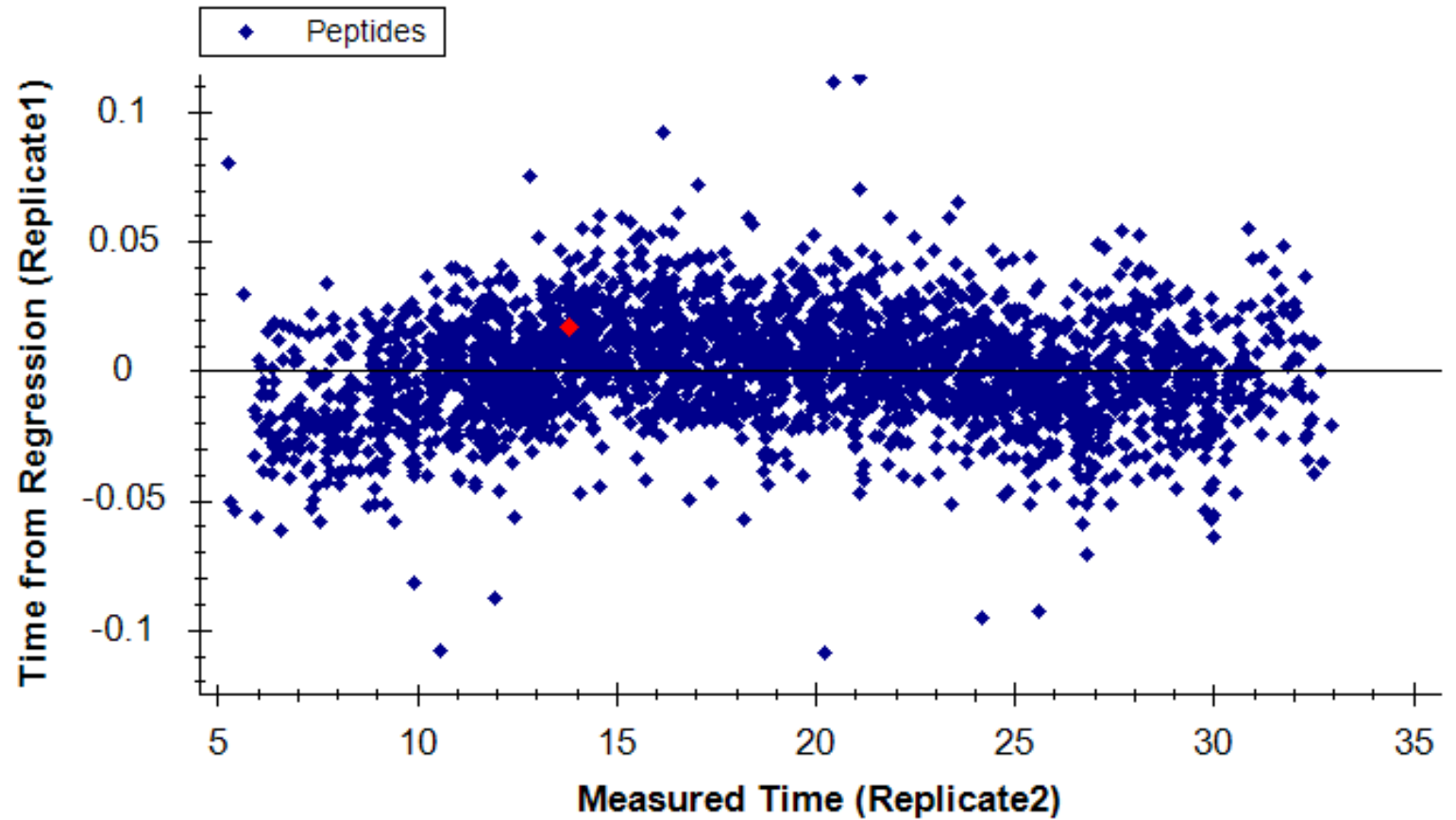
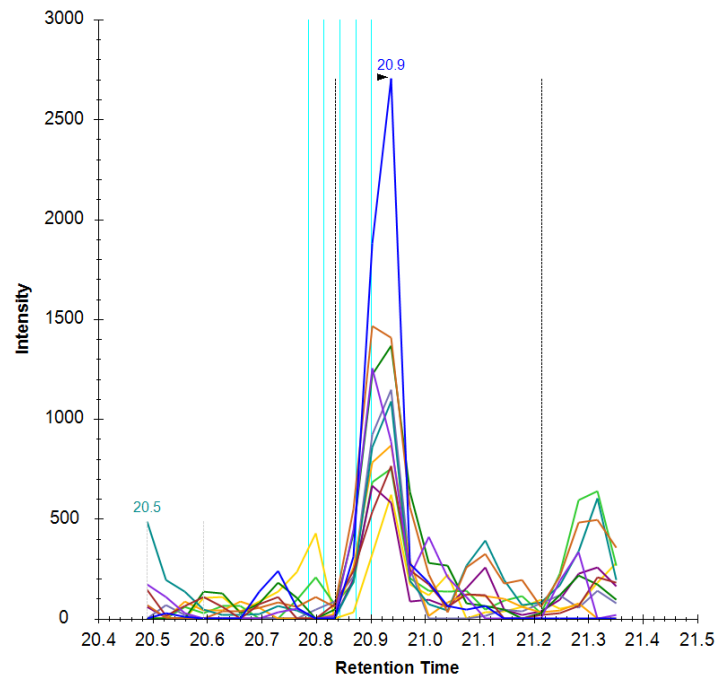
Minimum Instrument Time for 1 Refined List of 3758 Precursors, 1178 of 1412 Proteins



Narrower Acquisition Widths Improves Peak Picking

Generally, is Very Good, Due to Narrow Acquisition Windows

Consistent Peak Picking Between Replicates



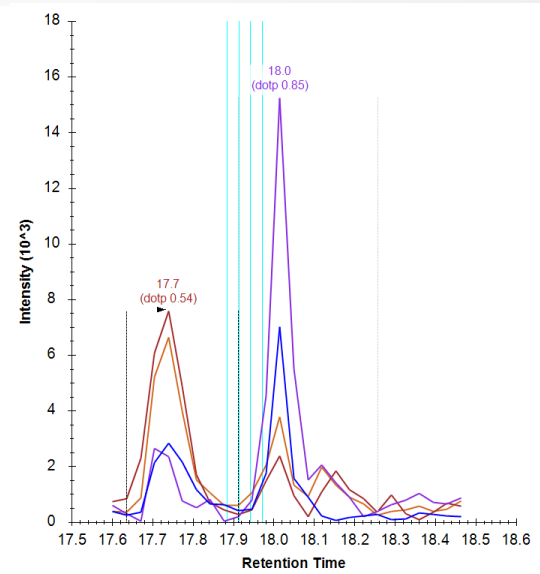
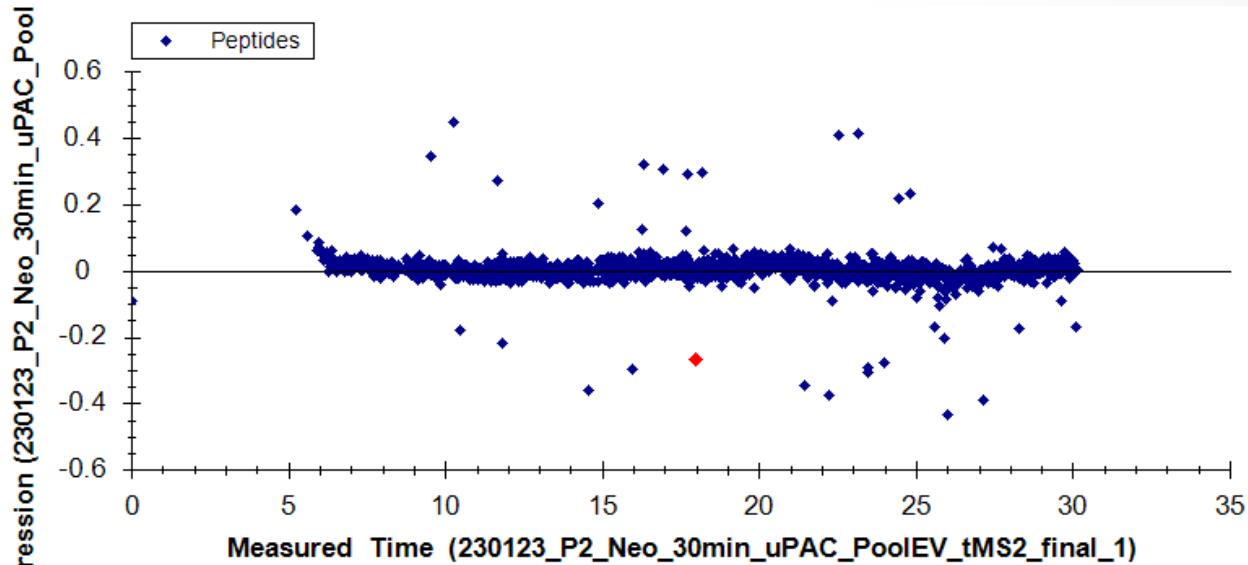
Fraction of Questionable Peaks is ~1% with Larger Data Sets

Room for Improvement Still!

#Peaks > +/- 0.2 min

Default Skyline

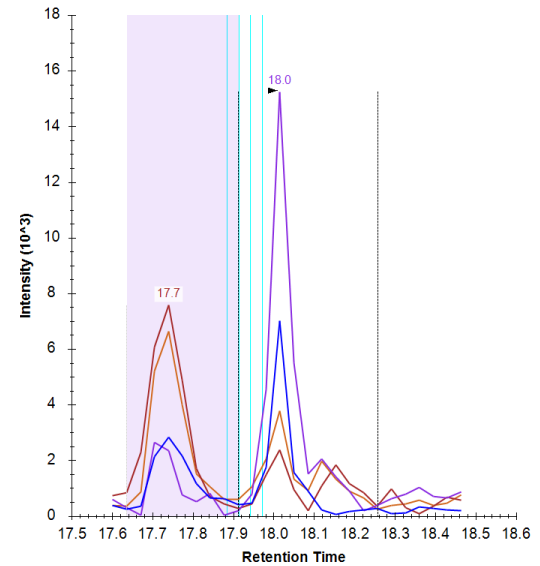
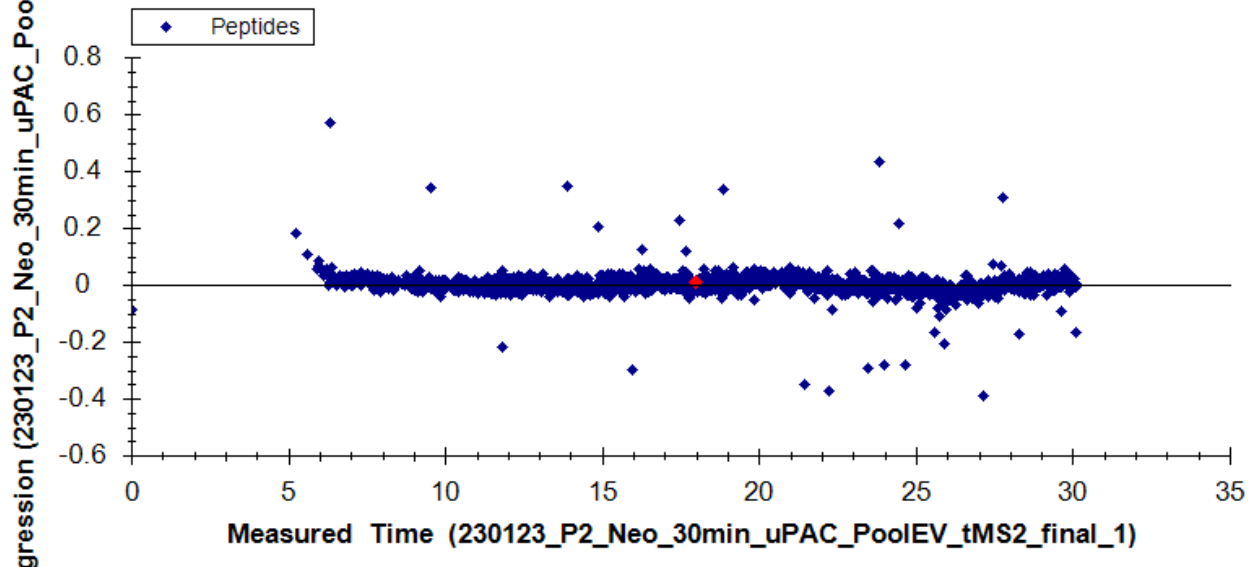
• ~27/2500



Default Skyline

mProphet

• ~17/2500

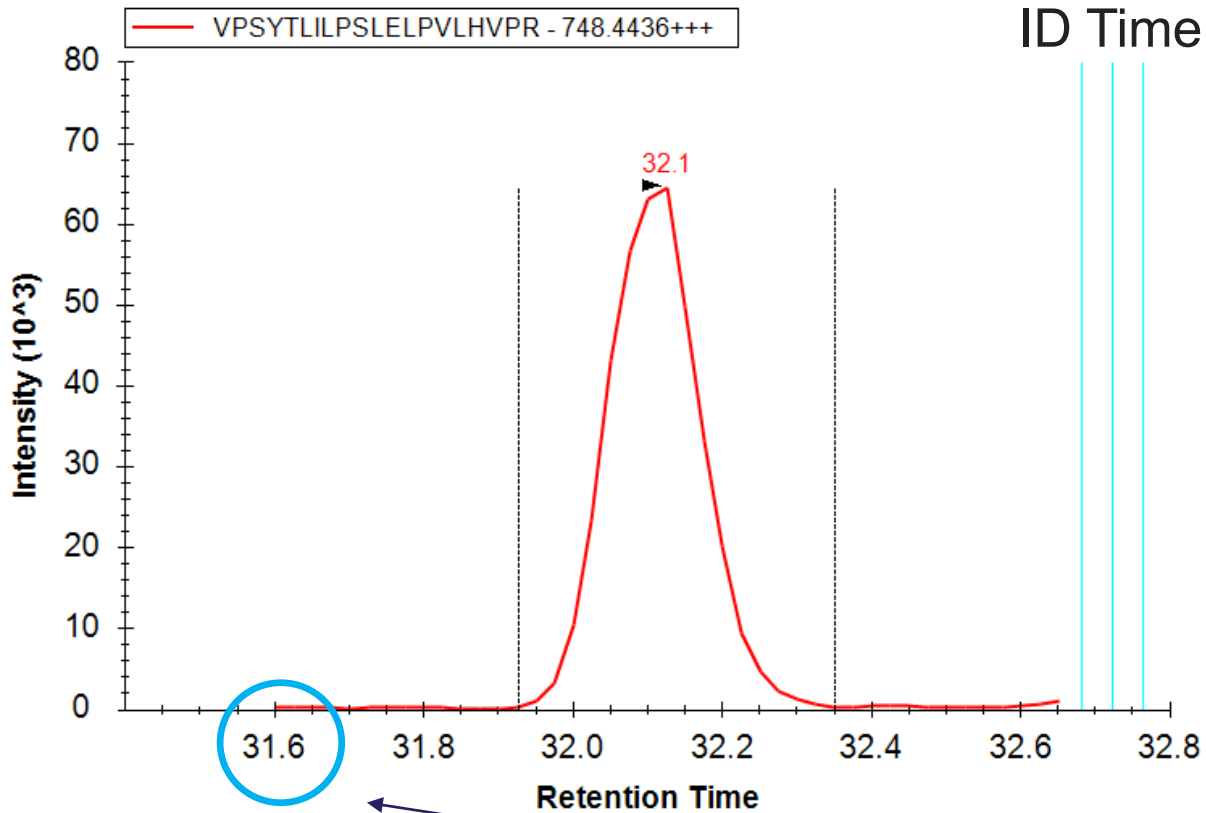


mProphet Reintegration

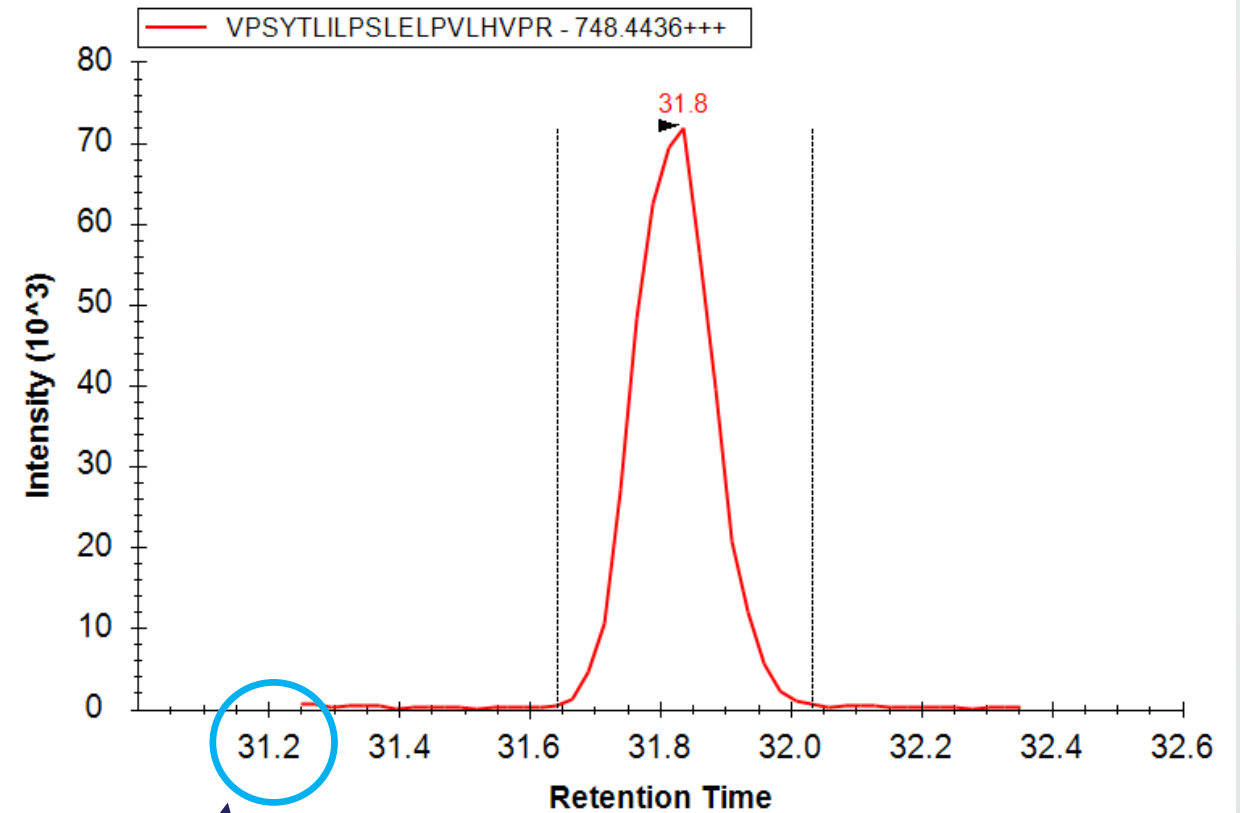
Real-Time Alignment: Overview

Acquisition Windows 2-3x Narrower Than Traditional Targeted MS2 → 2-3x More Targets

Replicate 1

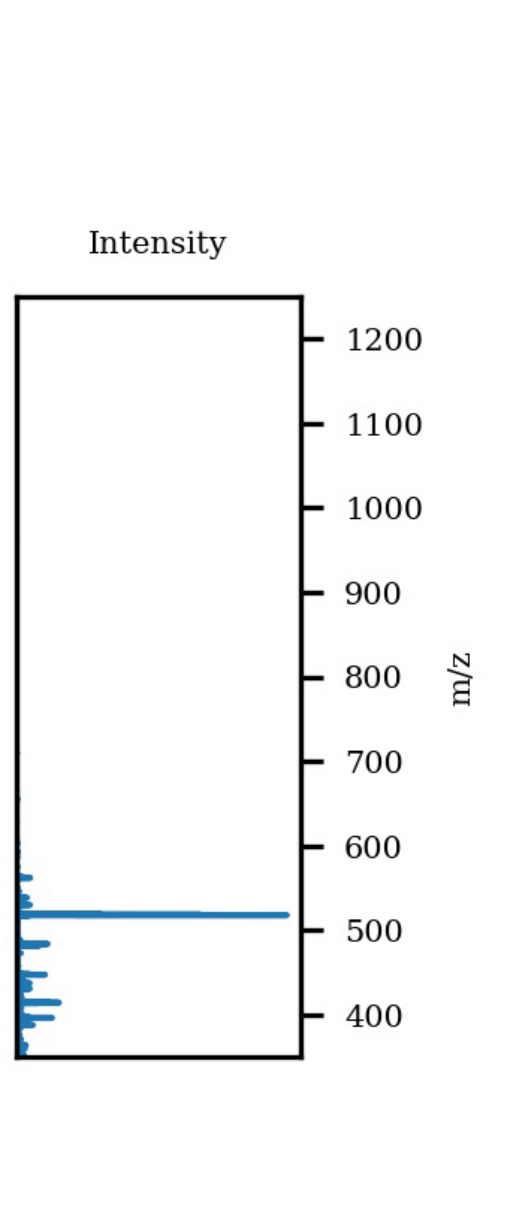
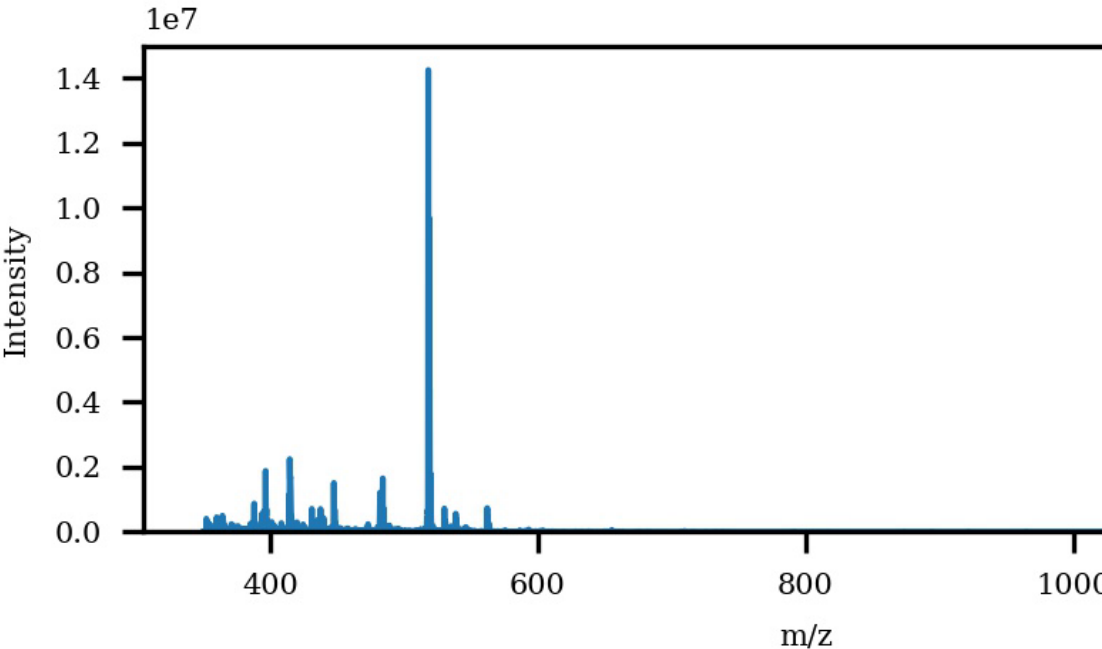


Replicate 2

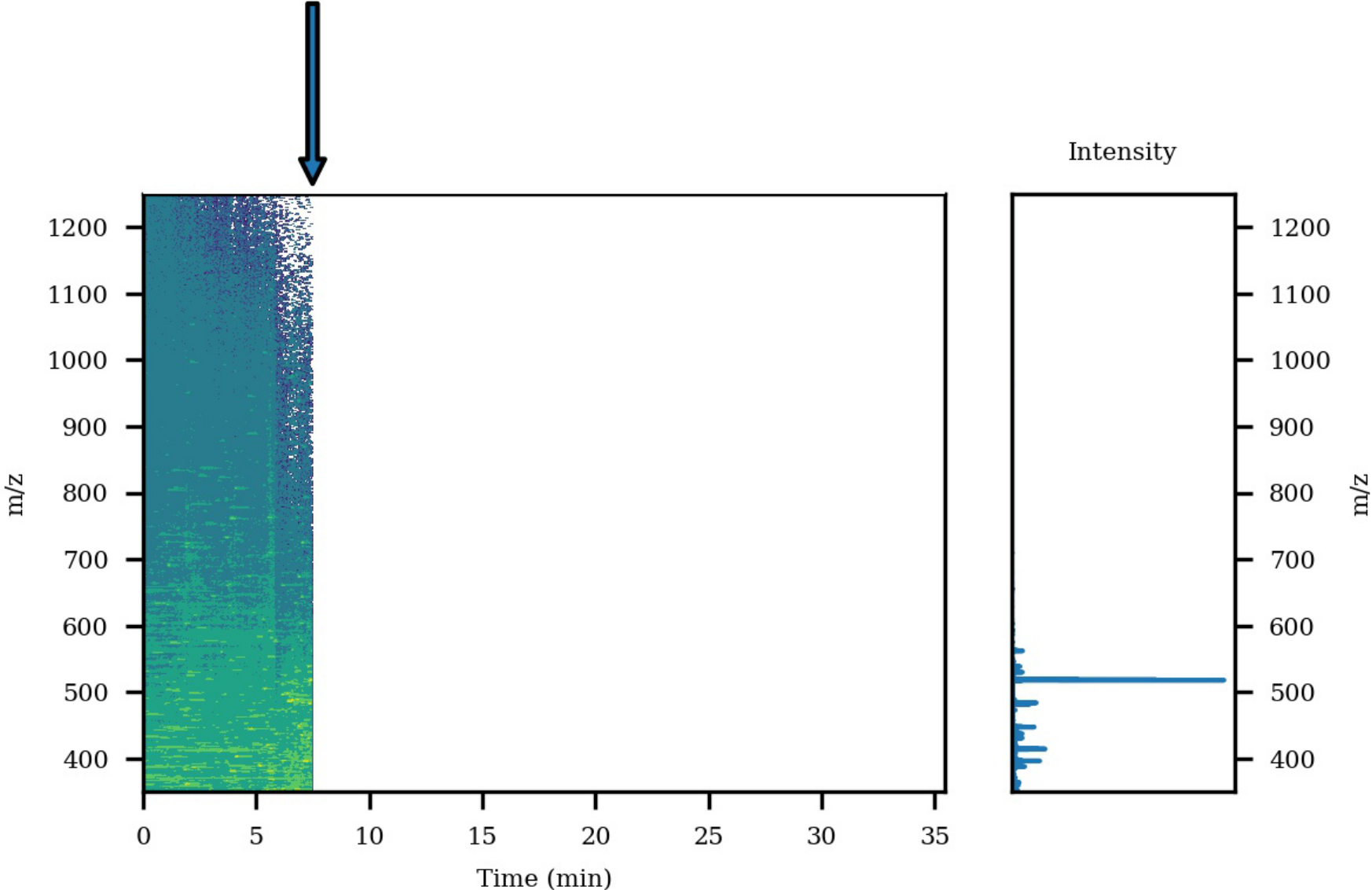


Acquisition Time Window Has Shifted

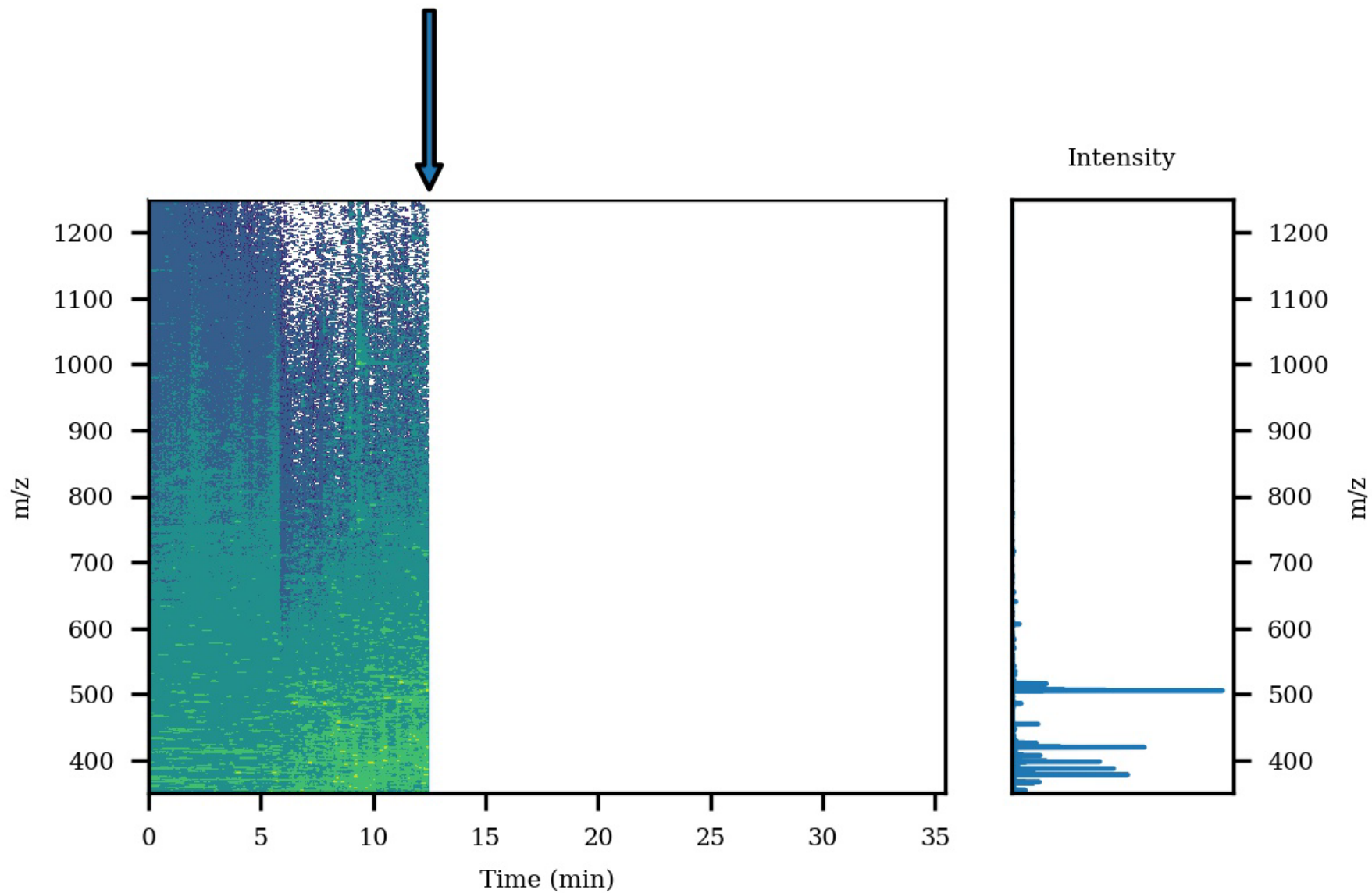
Real-Time Alignment: Reference Spectra



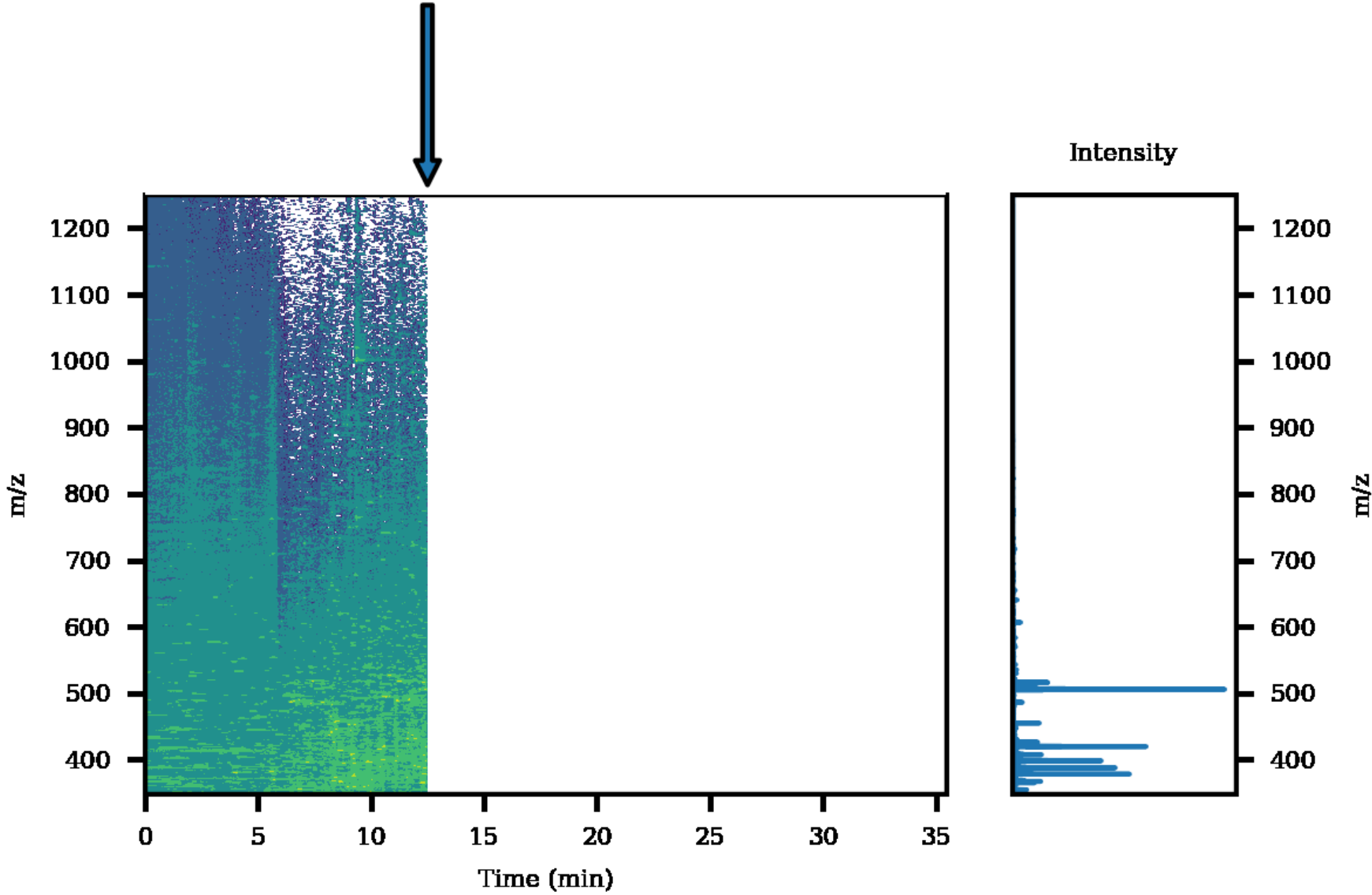
Real-Time Alignment: Reference Spectra



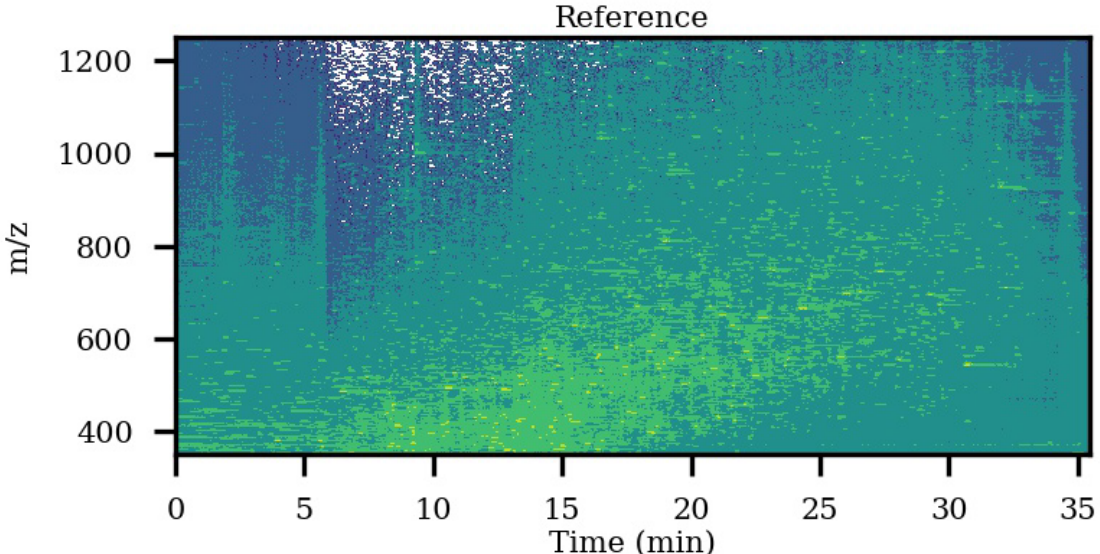
Real-Time Alignment: Reference Spectra



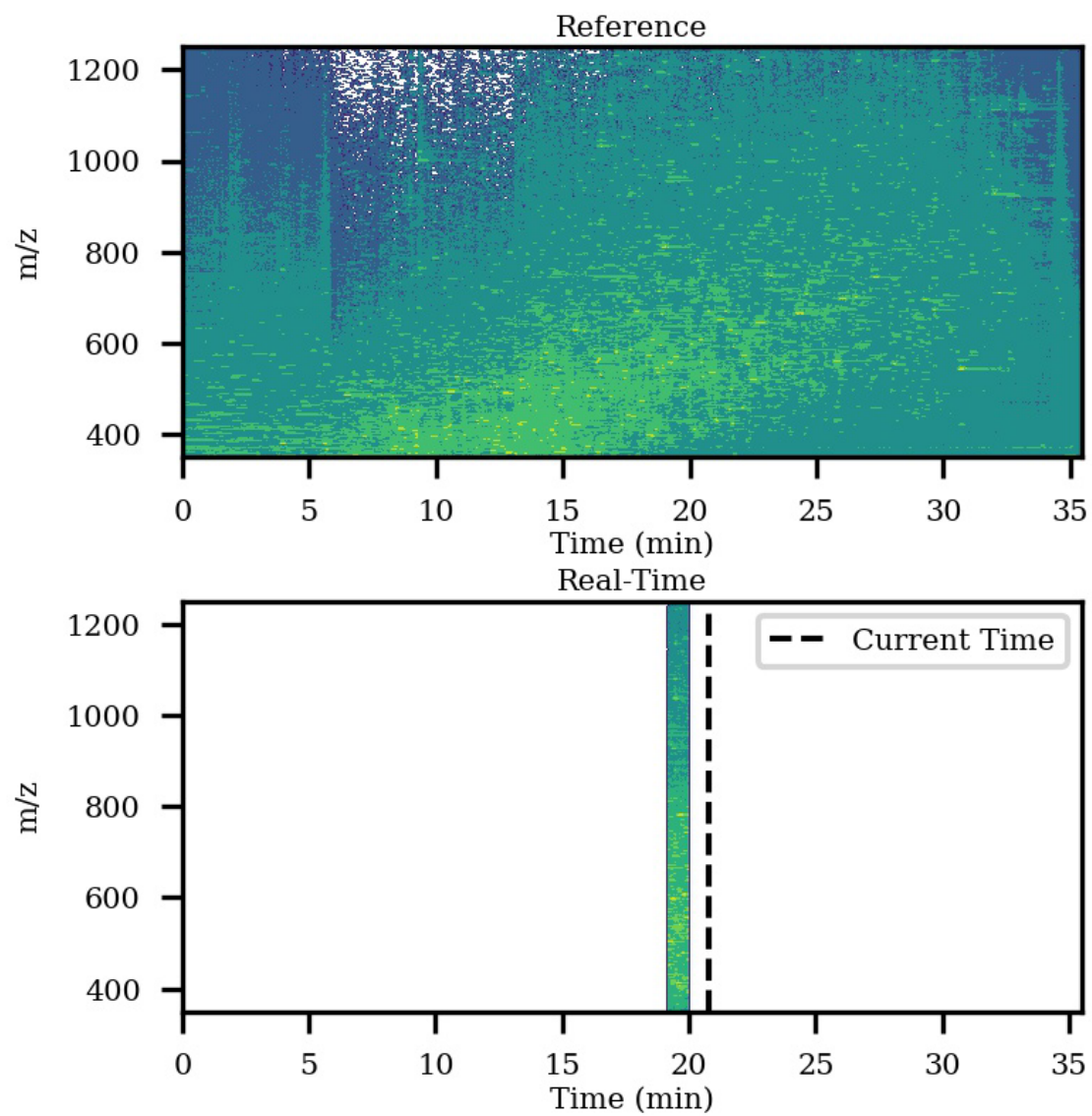
Real-Time Alignment: Reference Spectra



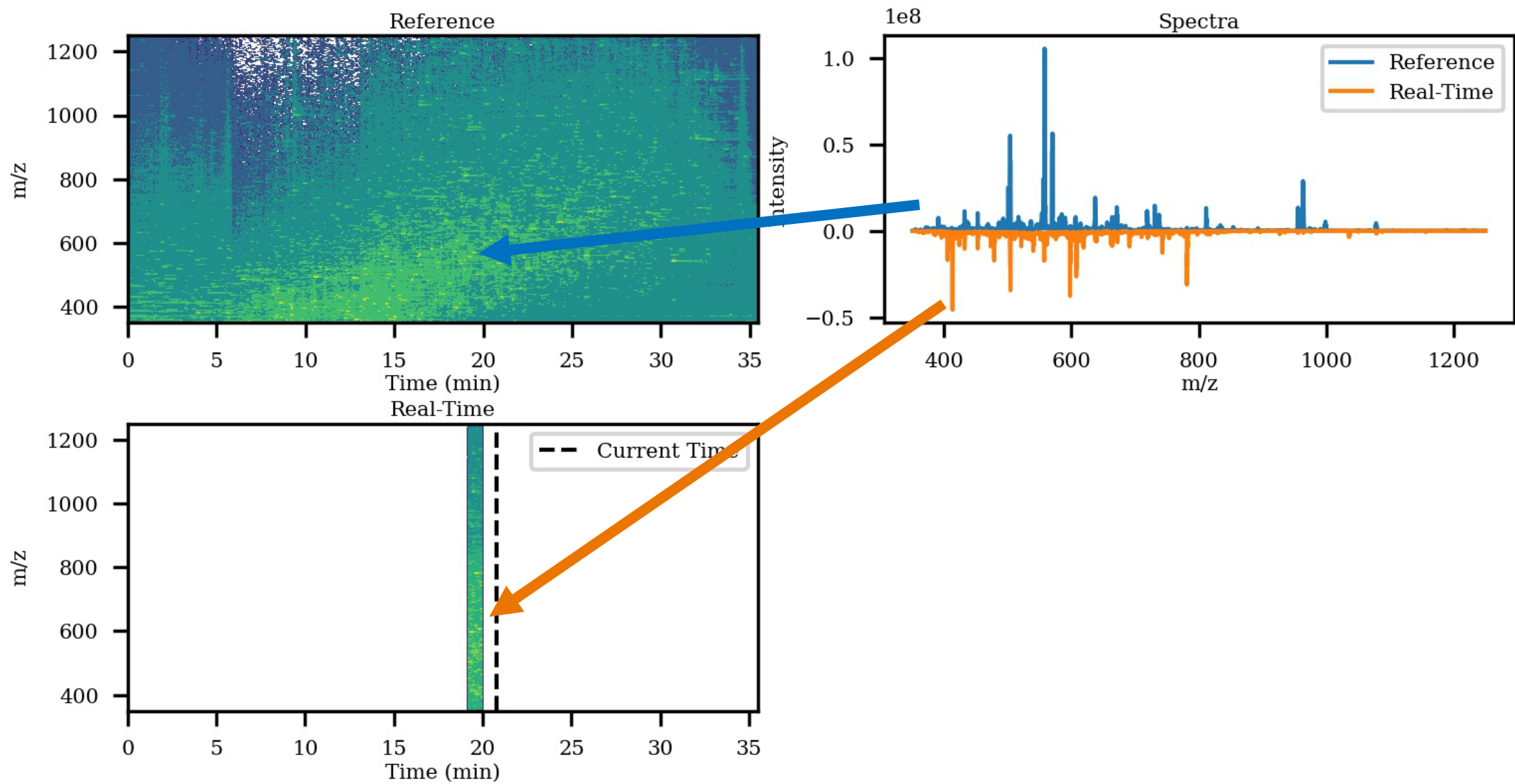
Real-Time Alignment: Cross Correlation



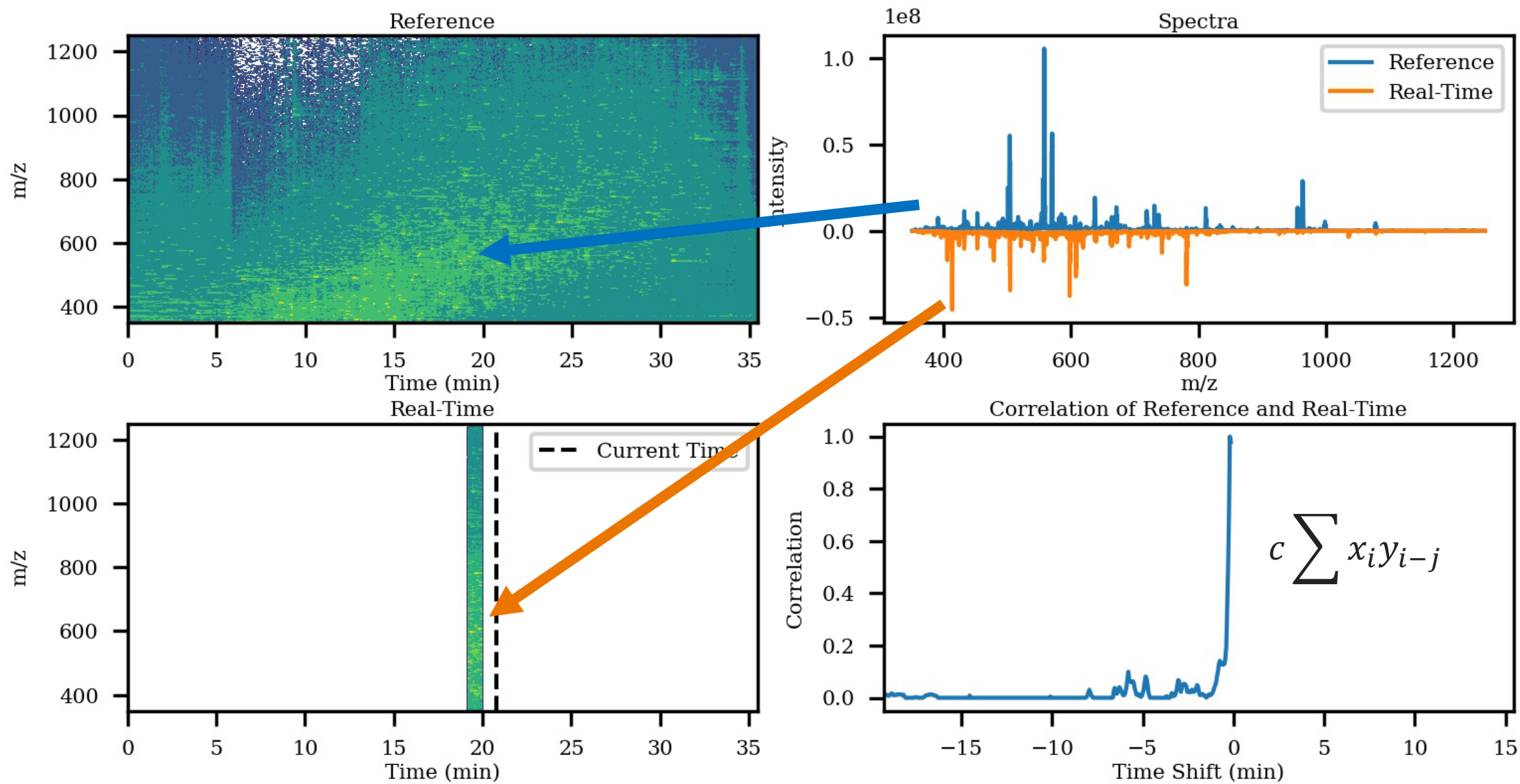
Real-Time Alignment: Cross Correlation



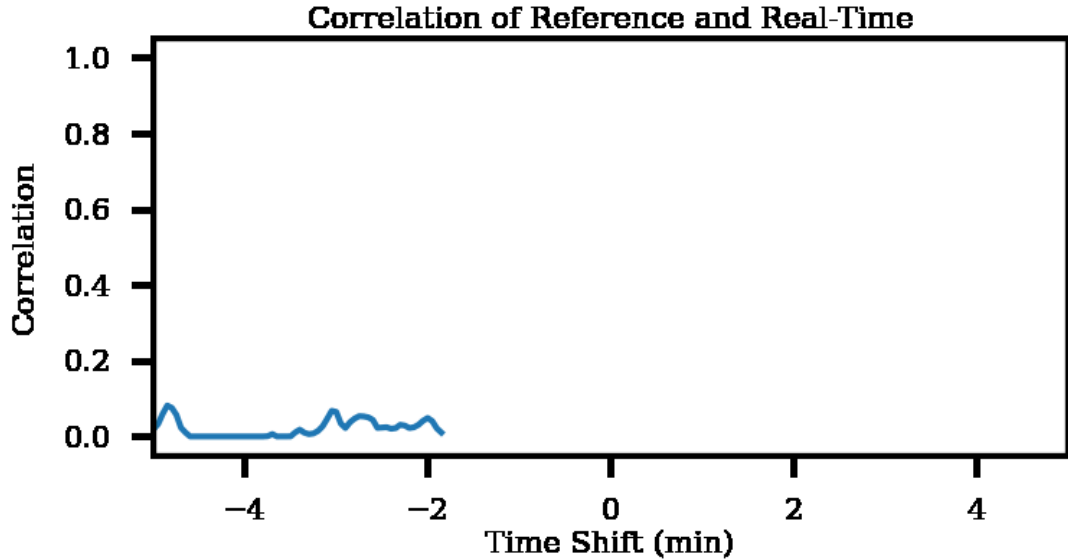
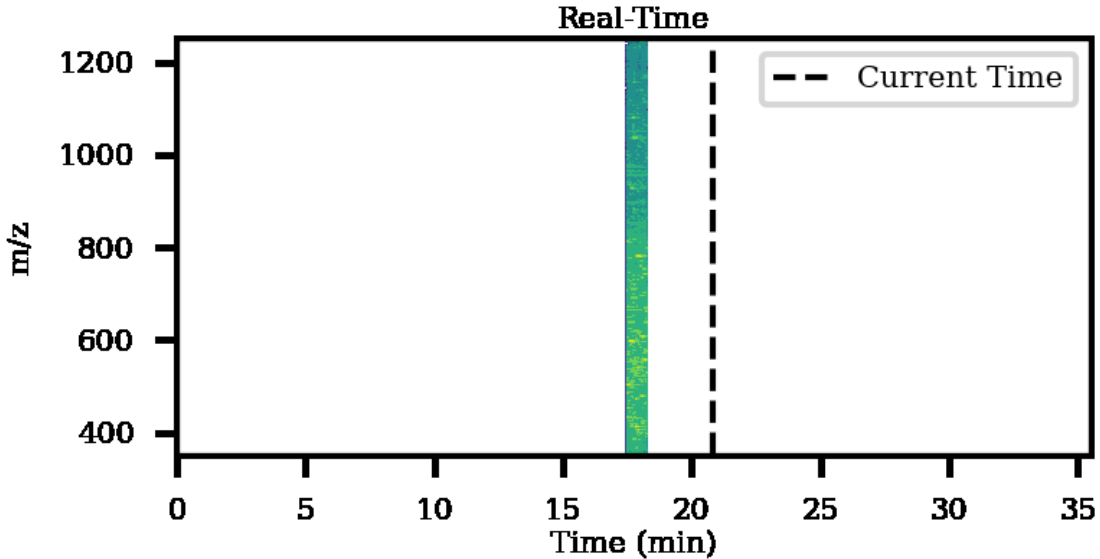
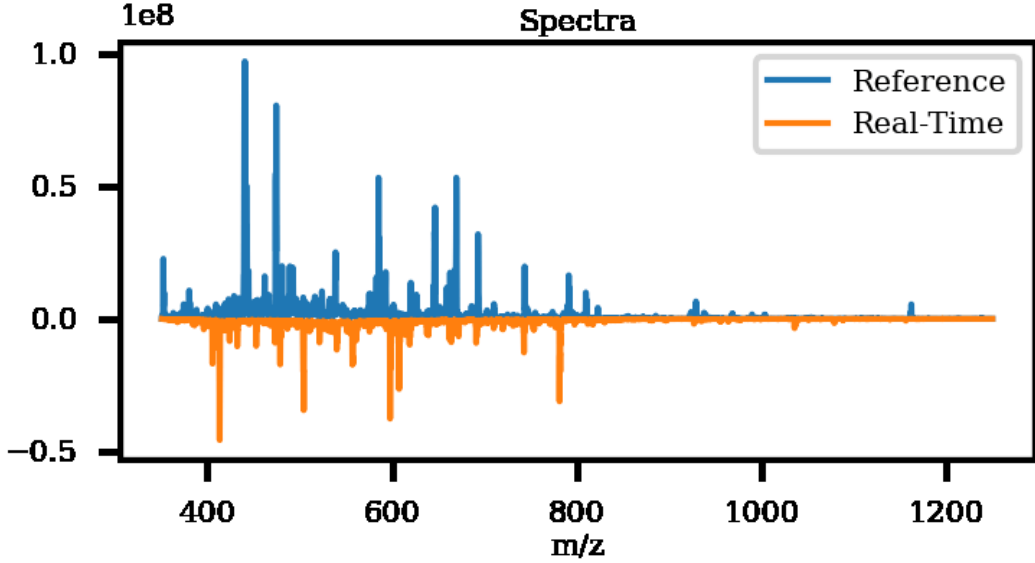
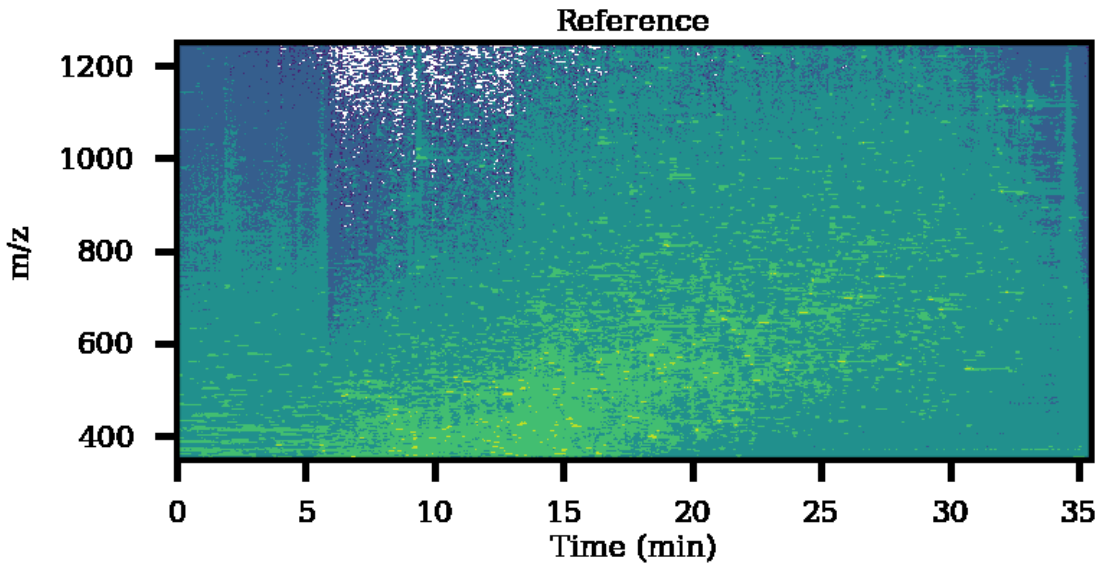
Real-Time Alignment: Cross Correlation



Real-Time Alignment: Cross Correlation

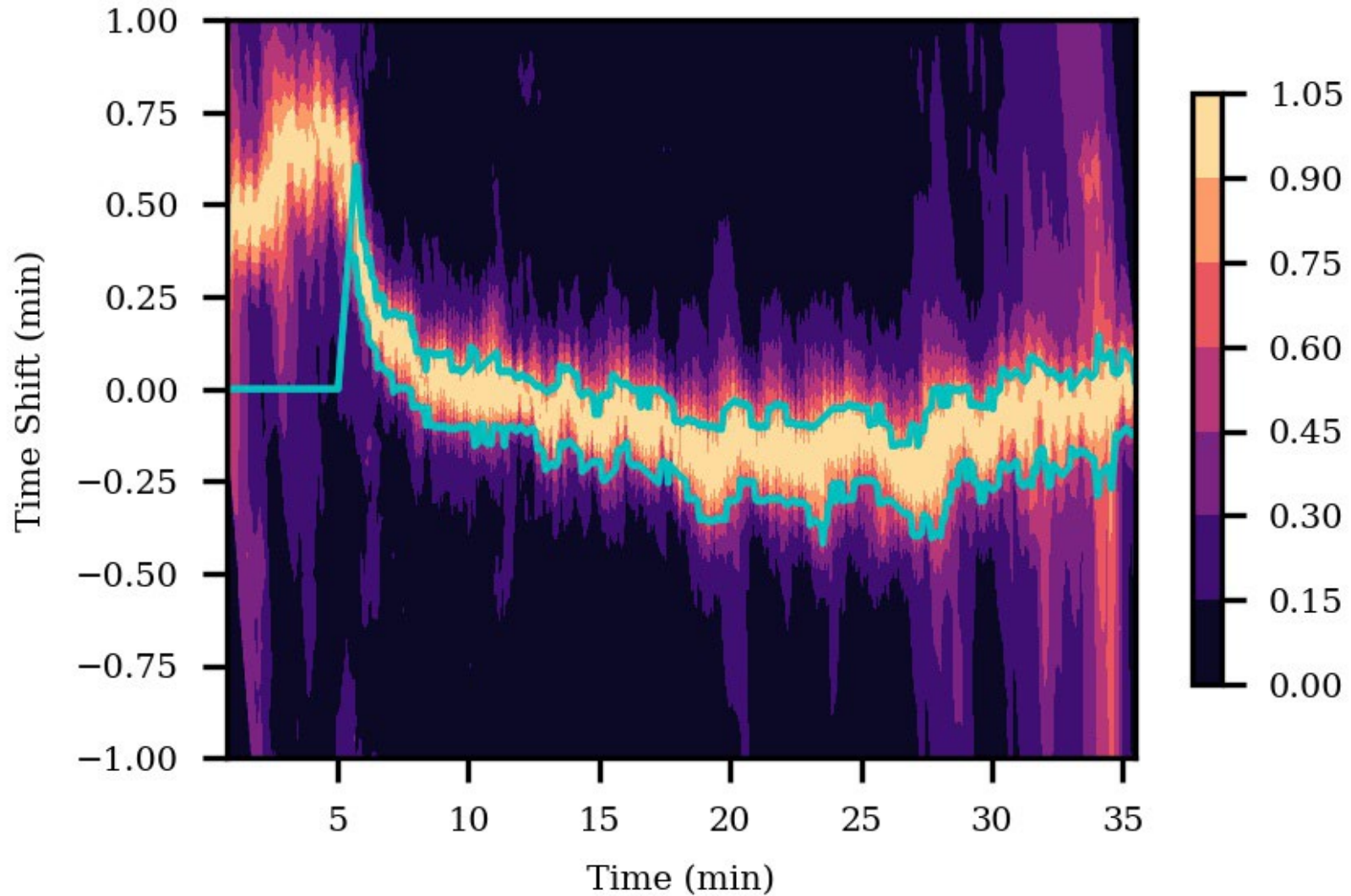


Real-Time Alignment: Cross Correlation



Real-Time Alignment with Uncertainty Bounds

Cyan Line Denotes Full Width Half Maximum of Cross Correlation

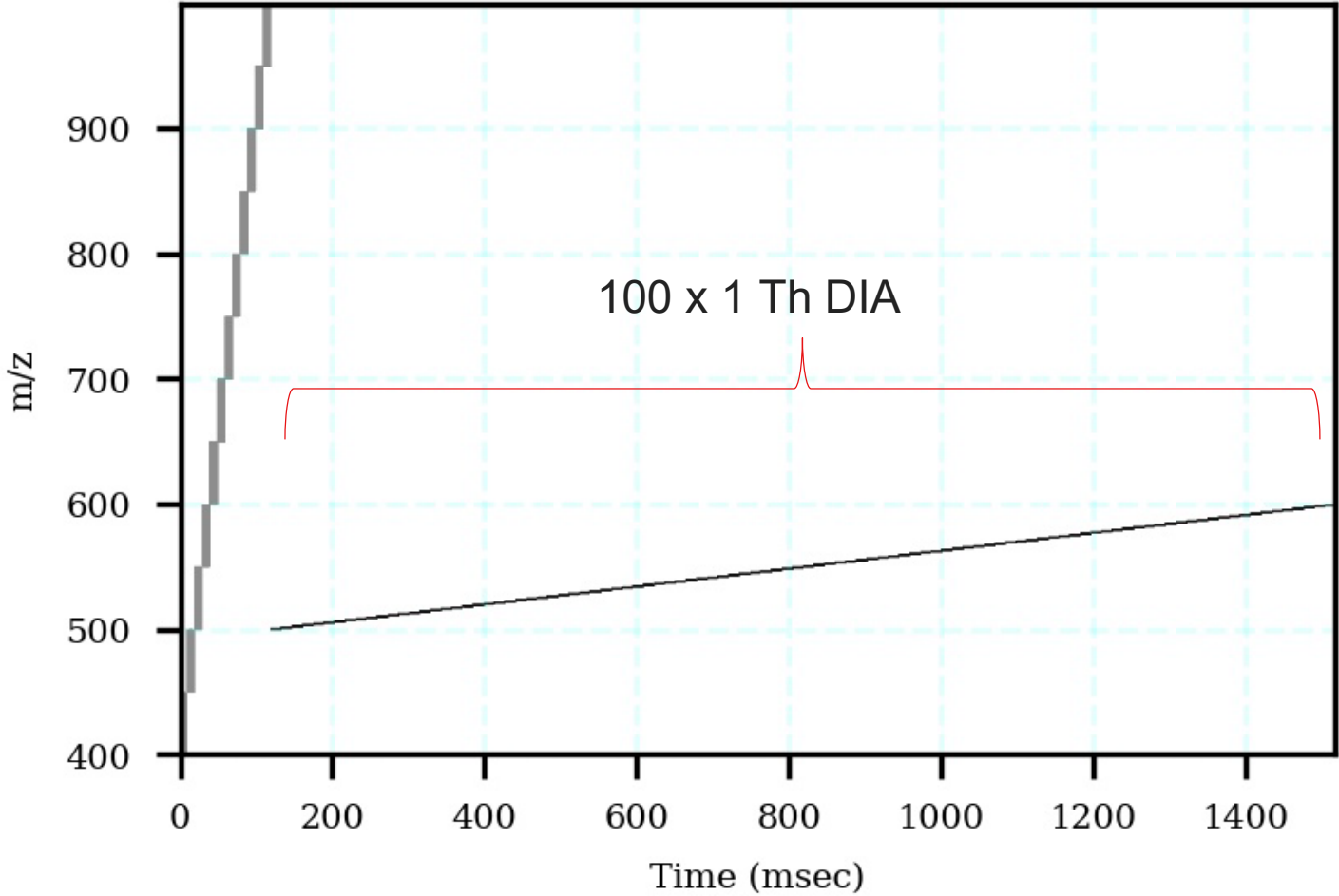


Alignment Acquisitions Are Inserted Into Methods

Gas-Phase Fractions

12 x 50 Th Alignment

One Cycle of Gas-Phase-Fraction DIA



LC Injections

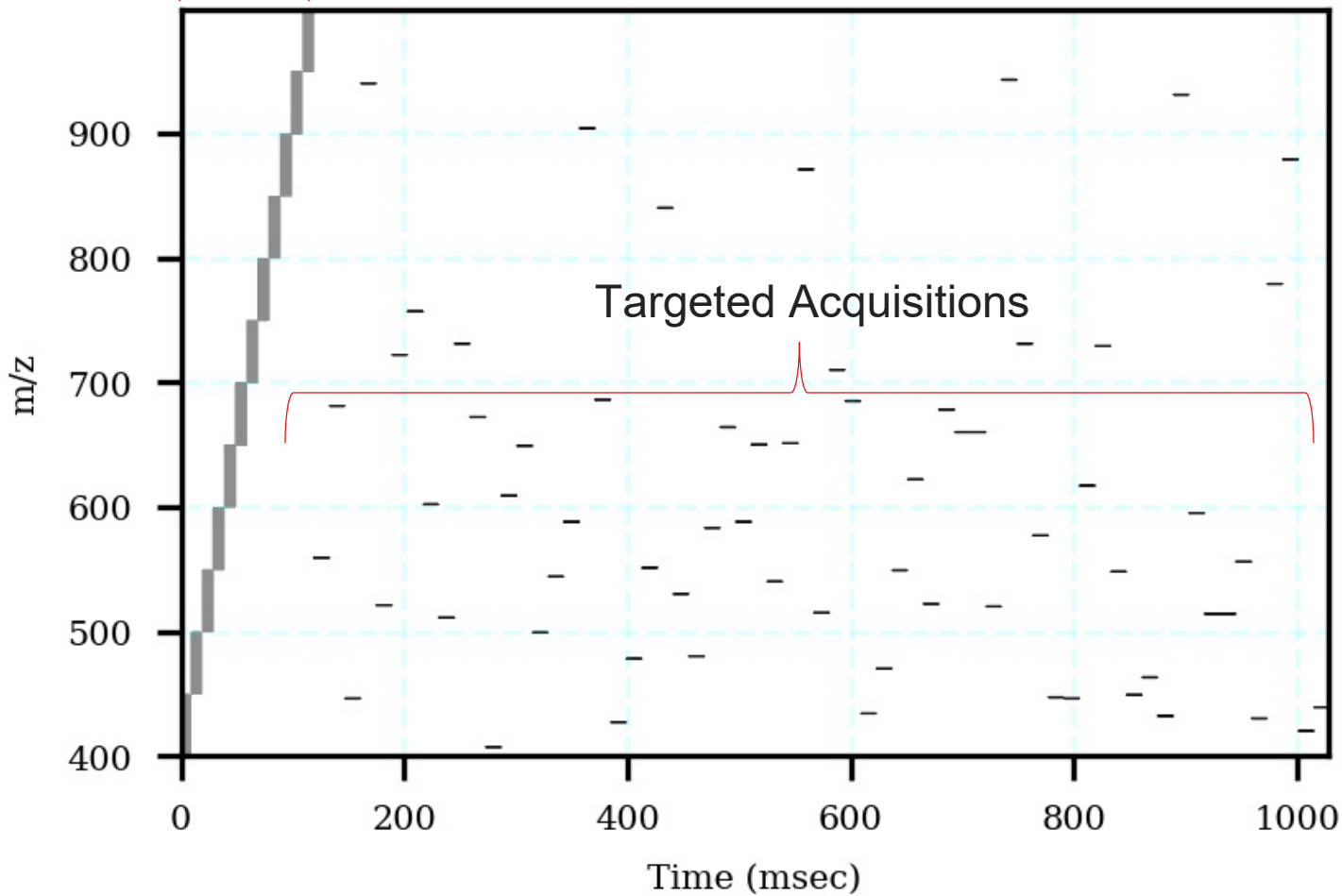
- m/z 900-1000
- m/z 800-900
- m/z 700-800
- m/z 600-700
- m/z 500-600
- m/z 400-500

Alignment Acquisitions Are Inserted Into Methods

Subsequent Targeted Methods

12 x 50 Th Alignment

One Cycle of Targeted MS2



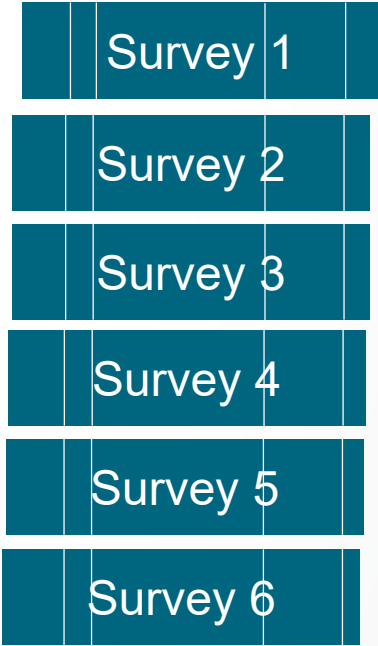
- Alignment Acquisitions Typically Performed at ~3 Points per LC Peak, i.e. Not Every Cycle

Automated Alignment Between Survey Runs and Creation of Reference File

Align Survey Runs to Last File

Create Method
 Balance Load 1 Charge/Prec.
Base Name: bead_quality_check
Method Template:
Skyline Connection: Connected
Send To Skyline Create Method Files

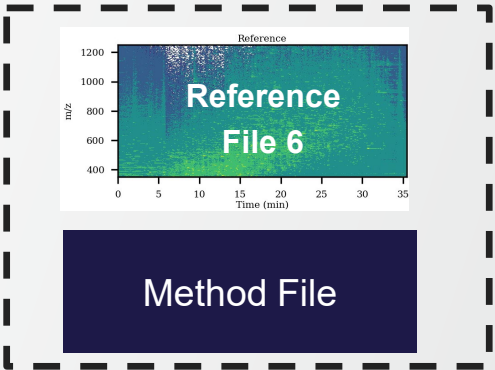
Press to Create Instrument Methods for N Assays Subsets



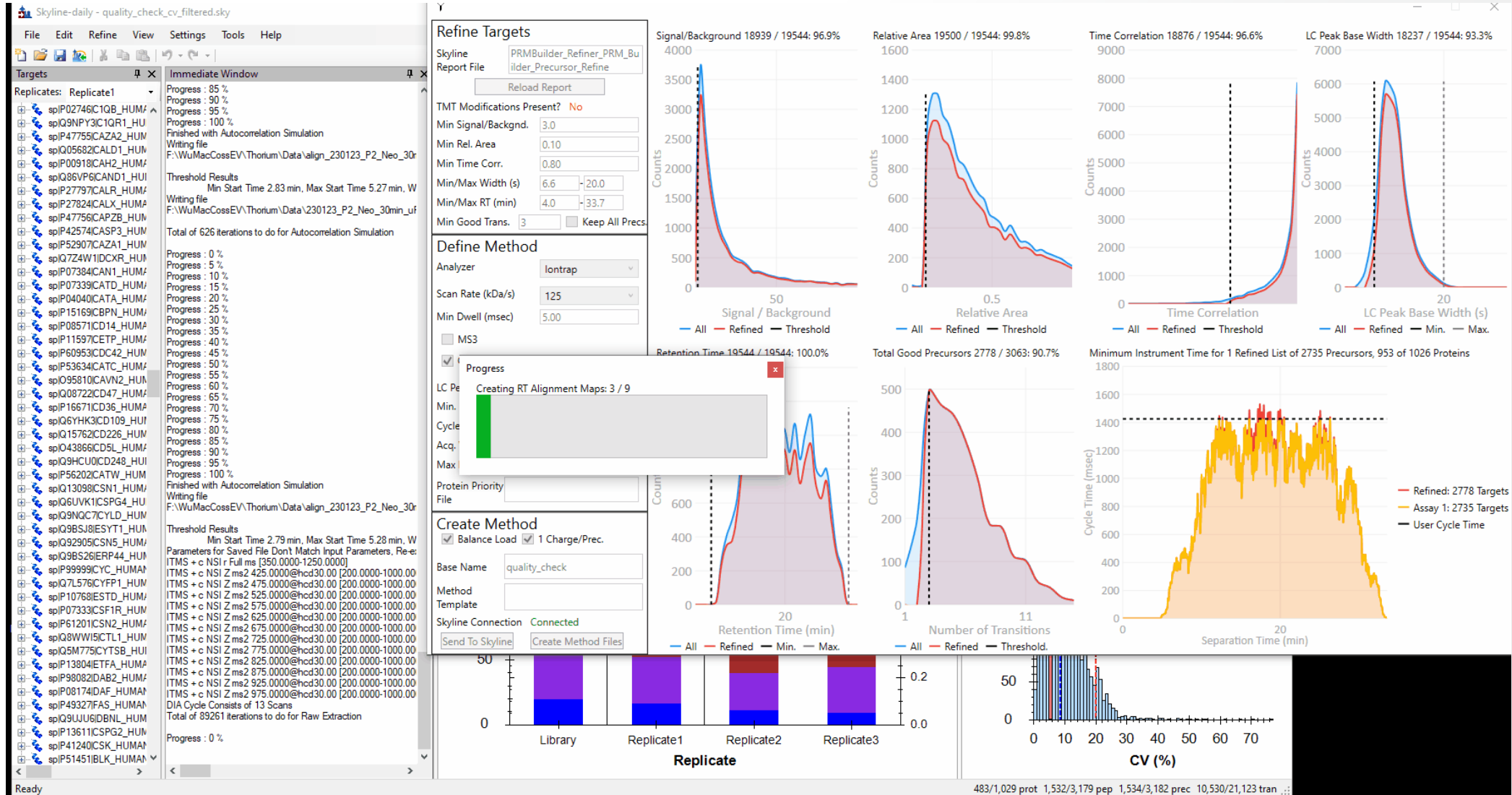
Create Method with Aligned RT's and Linked Reference File



Create Reference File



Automated Alignment Between Survey Runs and Creation of Reference File

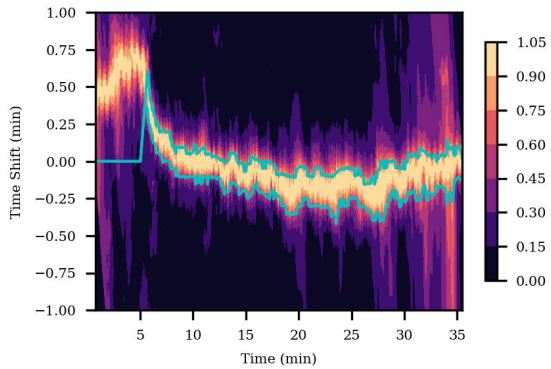


Large Scale Targeted Assays (5-7k Peptides/Hour) are Practical

Skyline Provides Excellent Platform and External Tools to Enable New Features

- m/z 900-1000
- m/z 800-900
- m/z 700-800
- m/z 600-700
- m/z 500-600
- m/z 400-500

Chromatogram Library

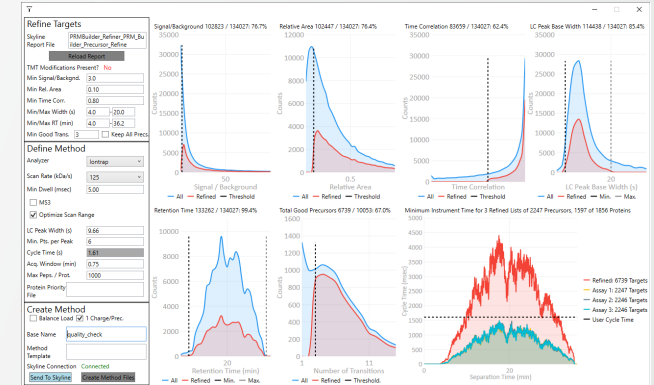


RT Alignment

Sufficient Data to Develop Assay



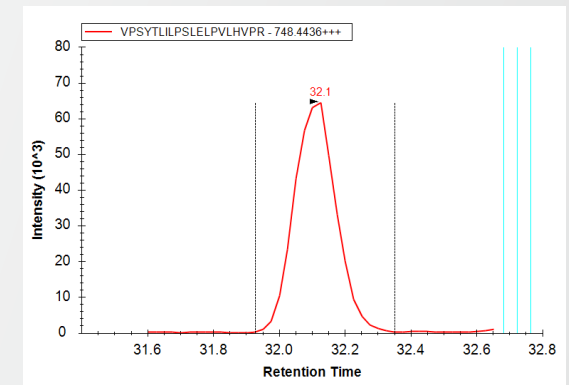
- Automated Transition Refinement
- Load Balancing
- Info About How Method Parameters Affect Assay



Narrow Acquisition Windows



- Increases Throughput 2-3x
- Improves Peak-Picking
- Eliminates Need to Update RT as LC Column Ages



Thank You!

Coauthors / Collaborators

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Mike MacCoss

