Rapid Low-level Identification and Quantitation of Host Cell Proteins

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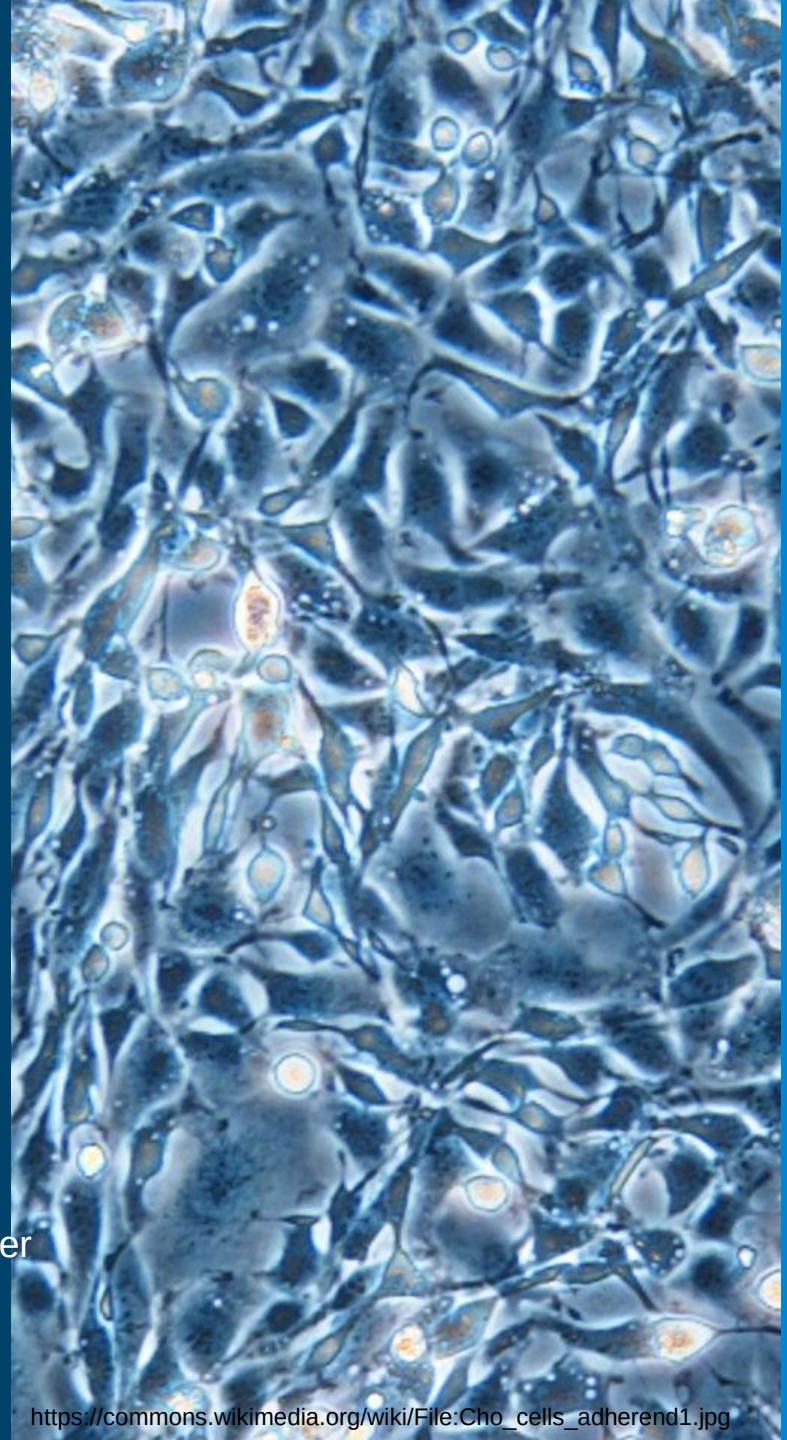


https://commons.wikimedia.org/wiki/File:Cho_cells_adherend1.jpg

Agilent
Trusted Answers

HCP Introduction

Residual Host Cell Proteins (HCP) are process impurities remained in a purified drug product

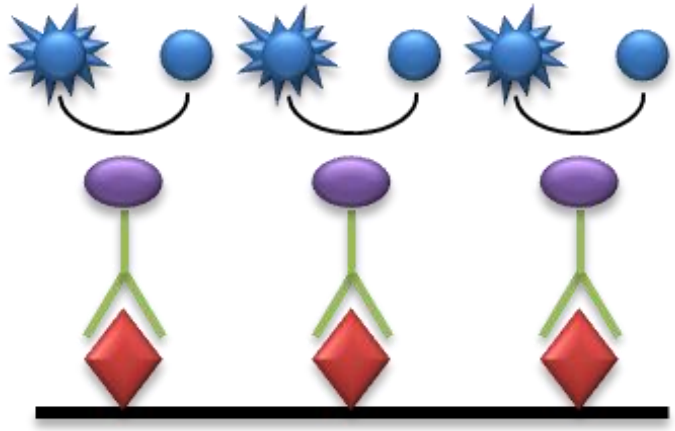
- HCPs can influence product stability as well as other unwanted effects in final product
- FDA requires that HCP contaminants in the final product are measured and reported
- ELISA is still a widely accepted method for HCP quantification

Strengths

- Very sensitive
- High level of reproducibility
- High-throughput (plate format, automation)

Challenges

- Lack of specificity, no identification of individual HCPs
- Quantitation is based on a cohort of HCPs
- Lack of coverage for non-immunoreactive HCPs



Modified with courtesy of Wikipedia
https://en.wikipedia.org/wiki/ELISA#/media/File:ELISA_diagram.png

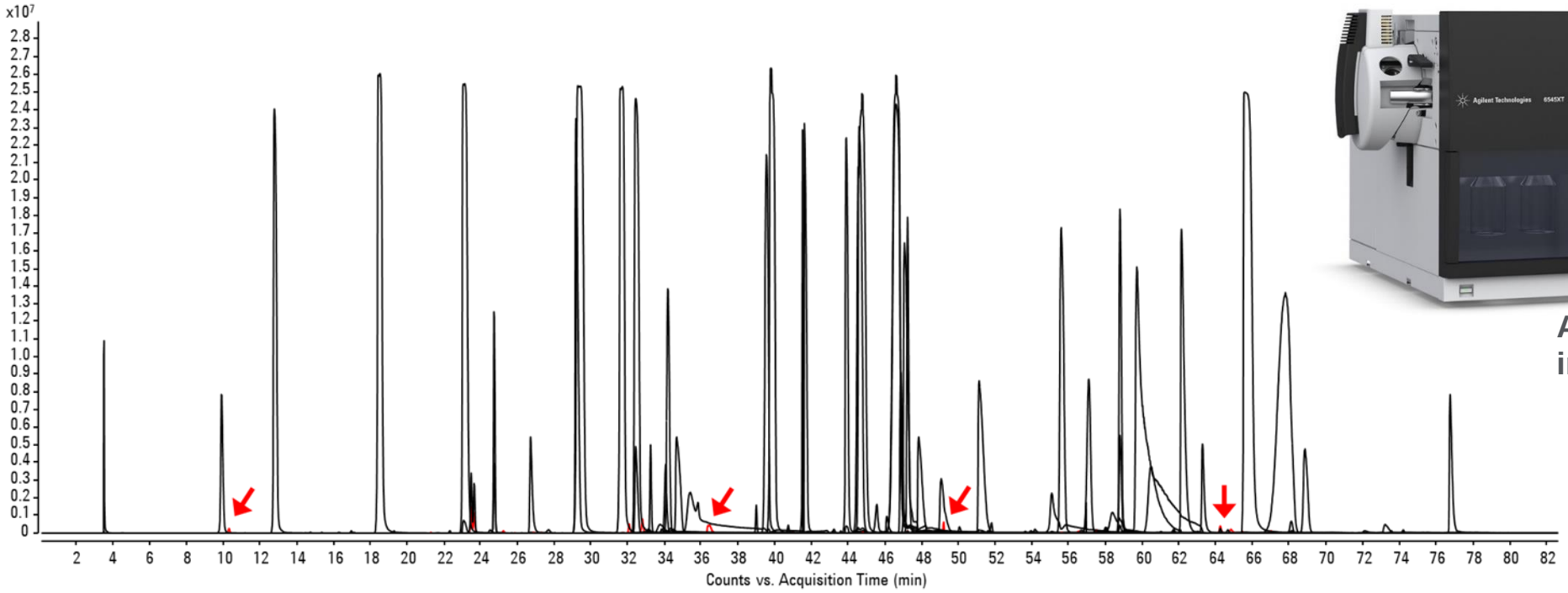
LC/MS as a Solution for Host Cell Protein Analysis

Advantages

- Doesn’t require protein specific antibodies
- Improve early purification process
- Identify individual protein including immunogenic HCPs
- High analytical sensitivity (low ppm)
- Provides both qualitative and quantitative information

Challenge for LC/MS Analysis of HCPs

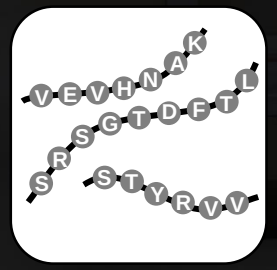
- Low abundant HCP peptides co-elute with very intense “product” mAb peptides
  - Need broad dynamic range and great separation
  - Reproducible chromatography needed for good quantitation



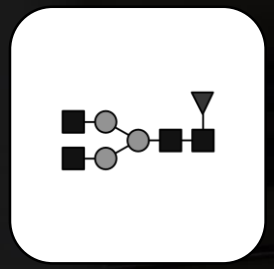
Able to measure 5 orders in-spectrum dynamic range

6545XT AdvanceBio LC/Q-TOF

Intact Protein
Industry leading data quality and sensitivity at the intact level



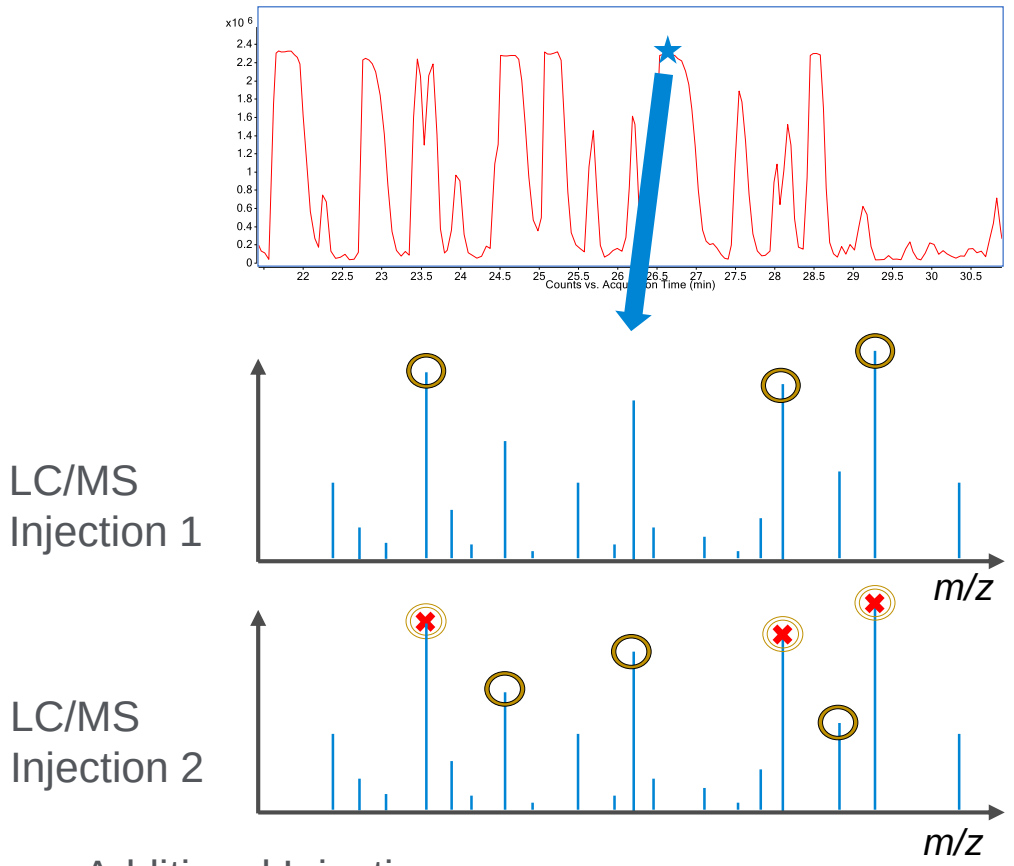
Peptide Mapping and PTMs
Ready for routine confirmation or digging deeper with Iterative MS/MS



Glycan Profiling
Full released glycan workflow with BioConfirm B.09

Iterative MS/MS Acquisition

An Easy way to Dig Deeper



Automatically look for different peptides with each injection

Host Cell Protein Analysis

Dr. Linfeng Wu Dr. Shuai Wu

https://en.wikipedia.org/wiki/File:BTEC_Bioreactors.jpg

Experiment to Evaluate HCP Analysis

- Spike-in UPS2 standards in purified CHO-cultured mAb before digestion
  - UPS2 mix of 48 proteins spanning 6 orders of magnitude
  - standard protein levels from 0.0004 to 313 ppm
  - mAb without UPS2 was used as a negative control
- Automated sample preparation using AssayMAP Bravo
  - Denaturation, reduction, alkylation, digestion, desalting, fractionation
- 60min LC method on an AdvanceBio Peptide Plus column (2.1x150 mm)
- Data-dependent acquisition by 6545XT AdvanceBio LC/Q-TOF
  - Iterative MS/MS vs. Auto MS/MS

AssayMap Bravo

6545XT AdvanceBio LC/Q-TOF

Iterative MS/MS Decision Engine Improves Protein Identification

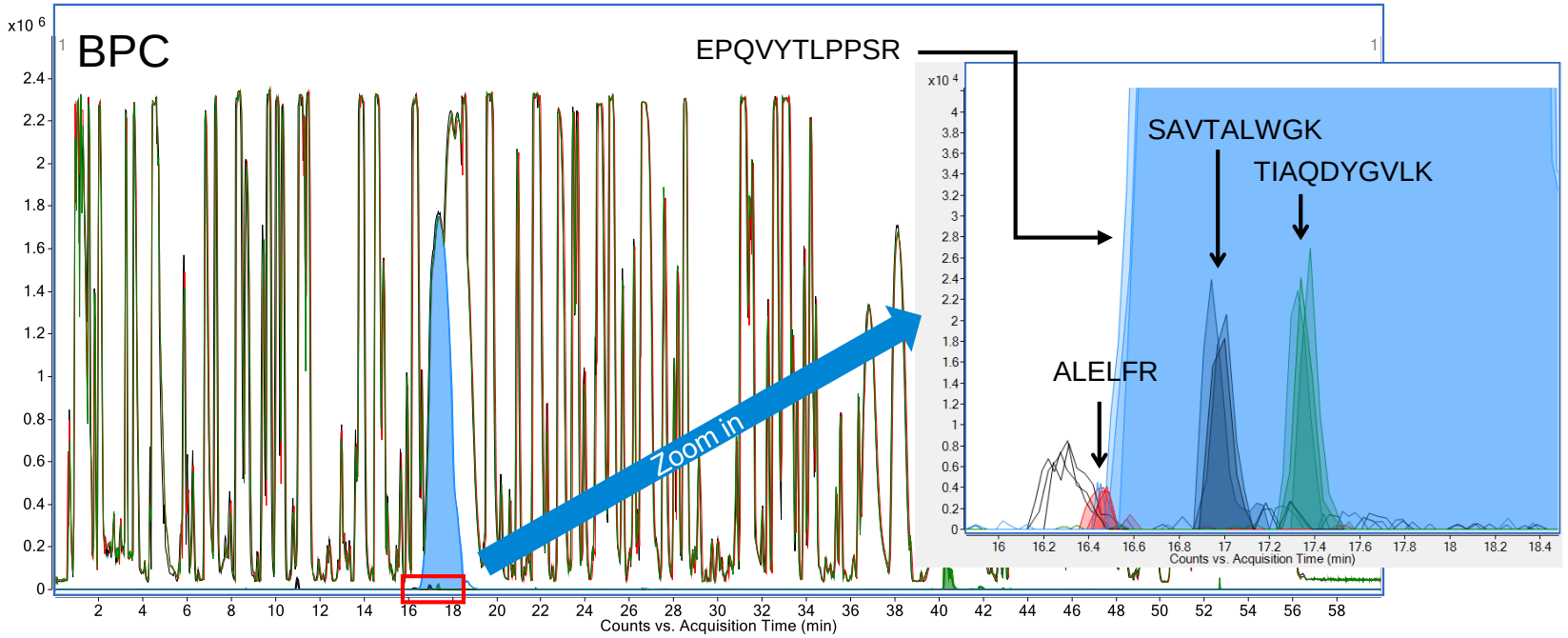
Comparison of Unique Peptide Sequences between Iterative MS/MS and Auto MS/MS

Protein Accession	Protein Spiking Level (ppm)	3 Injections per Method	
		Iterative MS/MS	Auto MS/MS
mAb_HC	NA	419	382
mAb_LC	NA	201	186
ALBU_HUMAN_spike	313.0	46	43
CAH2_HUMAN_spike	137.3	19	15
CAH1_HUMAN_spike	135.6	9	9
LEP__HUMAN_spike	76.2	4	1
HBB_HUMAN_spike	74.8	12	10
HBA_HUMAN_spike	71.3	7	6
UBIQ_HUMAN_spike	50.0	6	6
CO5_HUMAN_spike	40.3	4	4
CATA_HUMAN_spike	28.1	2	2
SUMO1_HUMAN_spike	18.3	3	1
NQO1_HUMAN_spike	14.5	2	0
PRDX1_HUMAN_spike	10.4	3	0
PPIA_HUMAN_spike	9.5	4	4
MYG_HUMAN_spike	8.0	2	1

- Iterative MS/MS acquisition method identified more unique peptide sequences

Excellent Chromatography Reproducibility and Dynamic Range

Overlaid chromatograms of three LC-MS/MS runs



In-spectrum Dynamic Range

~ 4.3 orders of magnitude

Peptide	Mass Error (ppm)	Intensity	Intensity %RSD	Protein spiking Level (ppm)	Protein Name
ALELFR	-1.1	6.76E+03	10.3%	8	Myoglobin
TIAQDYGVLK	-1.8	1.51E+05	6.2%	10.4	Peroxiredoxin 1
SAVTALWGK	4.8	1.36E+05	6.0%	74.8	Hemoglobin subunit beta
EPQVYTLPPSR	1.0	1.38E+08	1.2%	NA	mAb

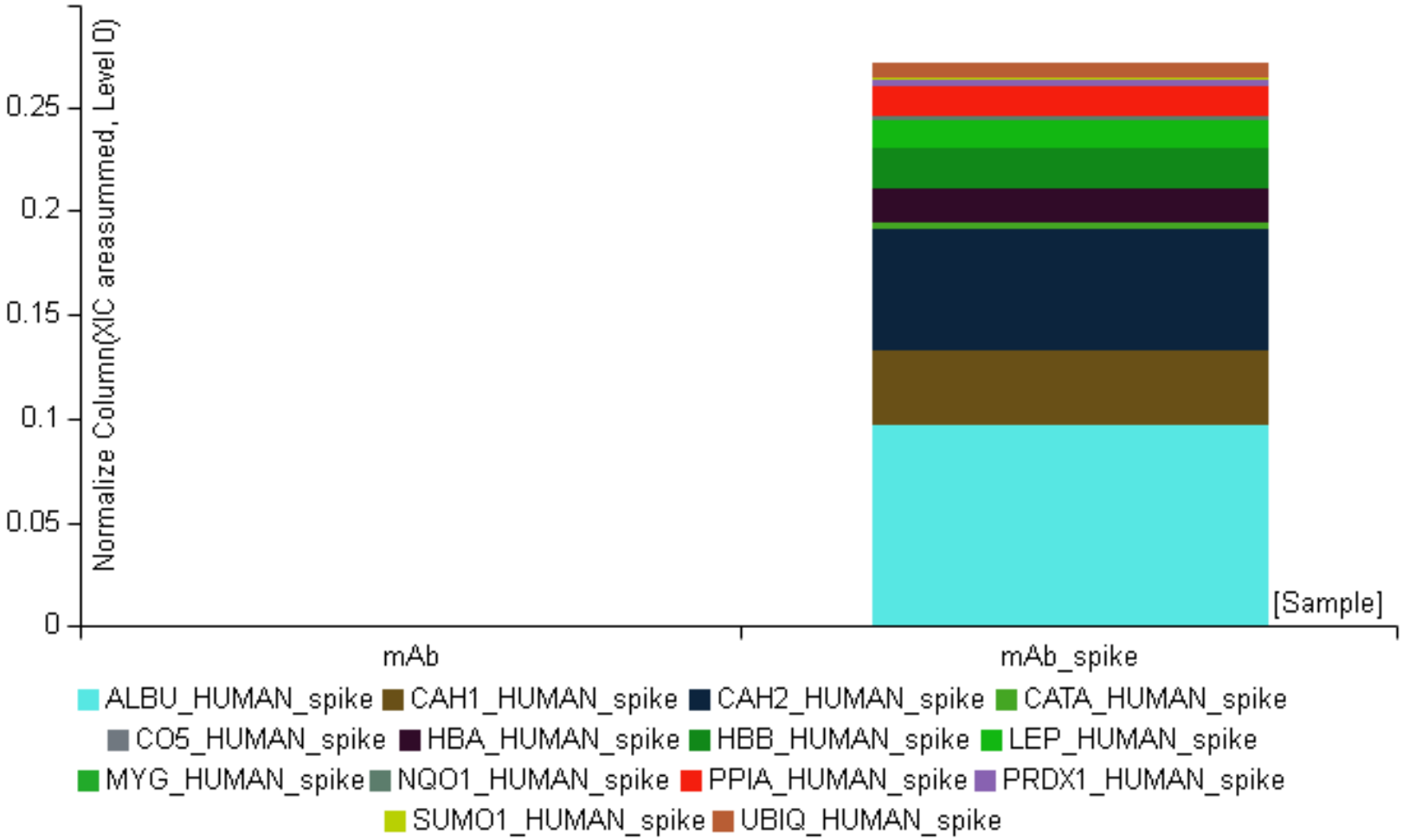
Report Directly from Protein Metrics Software -- Byologic

Protein alias name	[Sample] ←	mAb	mAb_spike ↓
mAb_HC		100	100
mAb_LC		41.2	31.6
ALBU_HUMAN_spike			0.0973
CAH2_HUMAN_spike			0.0586
CAH1_HUMAN_spike			0.0354
HBB_HUMAN_spike			0.0199
HBA_HUMAN_spike			0.0157
PPIA_HUMAN_spike			0.0146
LEP_HUMAN_spike			0.0133
UBIQ_HUMAN_spike			0.0073
PRDX1_HUMAN_spike			0.00319
CATA_HUMAN_spike			0.00266
NQO1_HUMAN_spike			0.00117
SUMO1_HUMAN_spike			0.000967
CO5_HUMAN_spike			0.000752
MYG_HUMAN_spike			0.000467

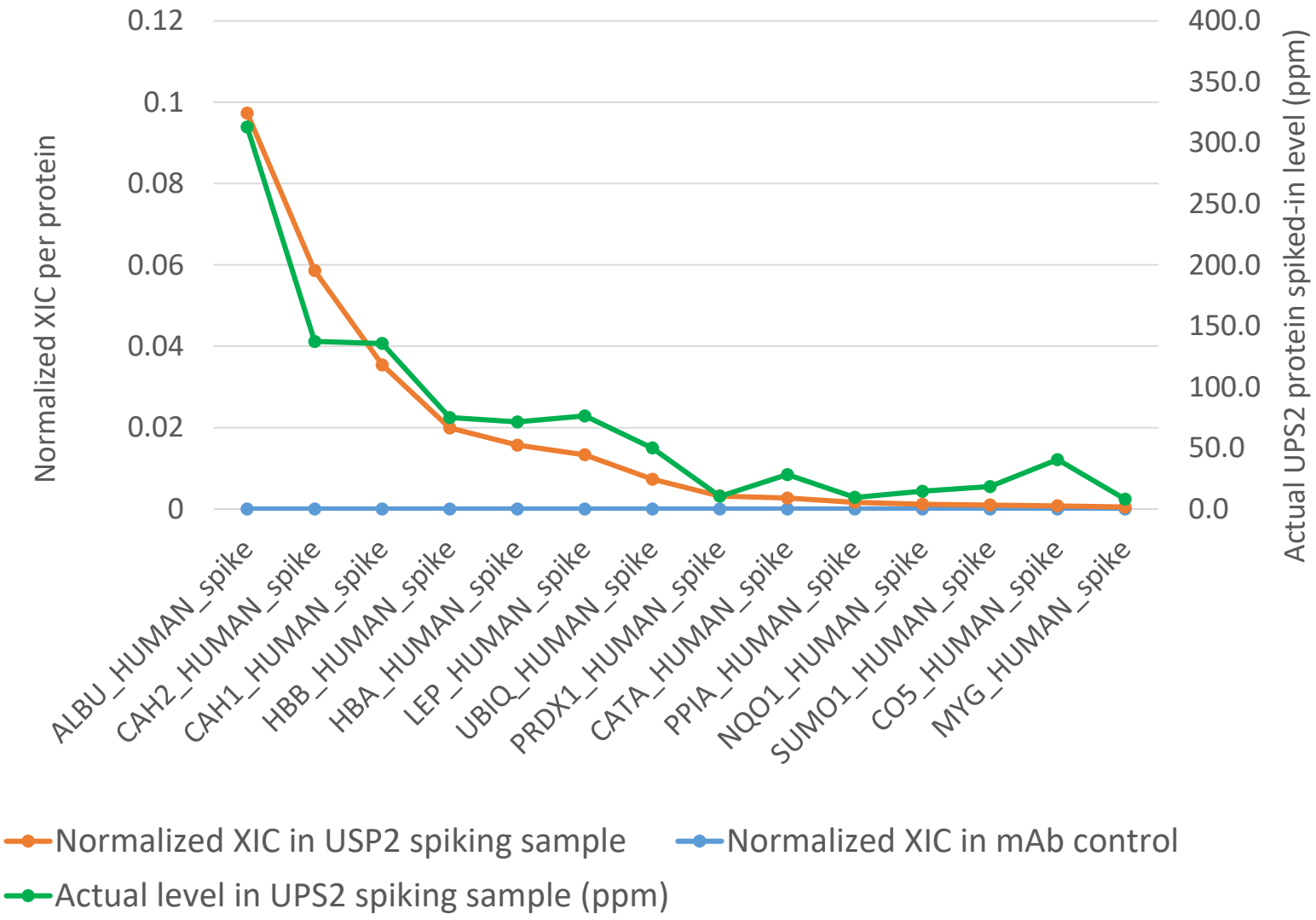
← **8 ppm spiking level**

Identification of all the spiking proteins above 8 ppm coupling with Iterative MS/MS

Stacked Barchart of Normalized Protein per Sample



Label-free Quantification



Improve Identification by Fractionation using AssayMAP Bravo

Fractionation: Using AssayMAP v1.0

A. Run Plate Stacking Utility

Number of Fractions: 6

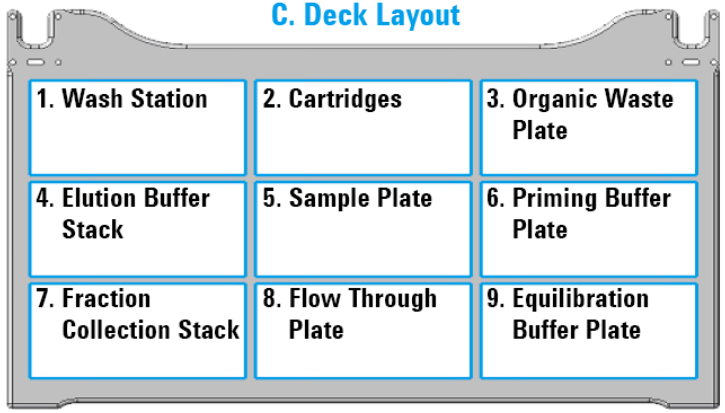
B. Application Settings

Number of Full Columns of Cartridges: 1

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☑			3
Prime Cartridges	☑	100	300	1
Equilibrate Cartridges	☑	50	10	1
Load Sample	☑	100	5	3
Collect Flow Through	☑			
Cartridge Cup Wash	☑	50		1
Internal Cartridge Wash	☑	25	5	3
Add Wash to Flow Through	☑			
Predispense Elution Buffer	☑	25		
Elute Fraction 1		25	5	1
Elute Fraction 2		25	5	1
Elute Fraction 3		25	5	1
Elute Fraction 4		25	5	1
Elute Fraction 5		25	5	1
Elute Fraction 6		25	5	1
Final Syringe Wash	☑			3

b

C. Deck Layout

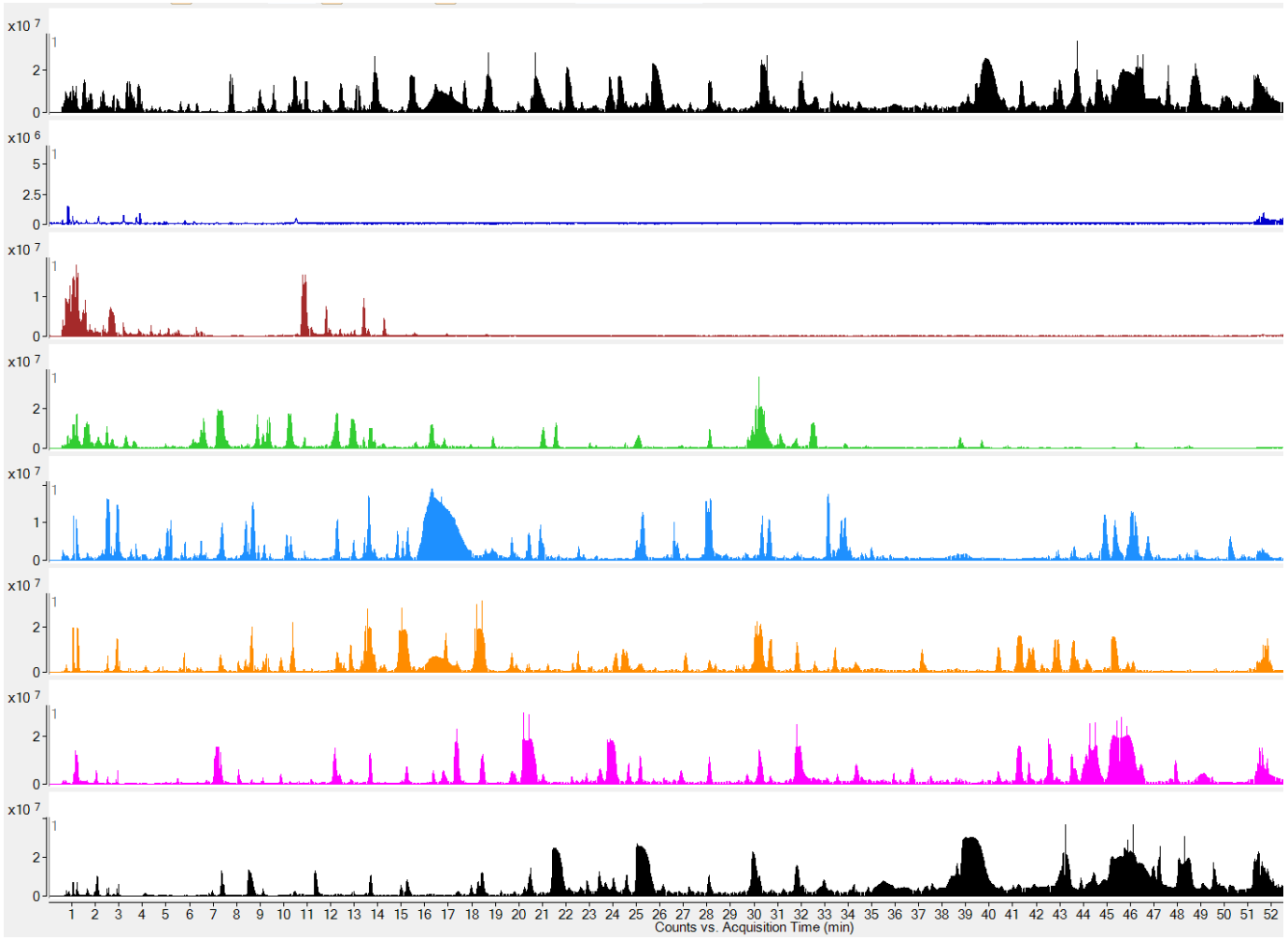


D. Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station
3	96 AbGene 1127, 1 mL Deep Well, Square Well, Round Bottom
4	Stack of n*: 96 Greiner 650201, U-Bottom Standard PolyPro
5	96 Greiner 650201, U-Bottom Standard PolyPro
6	96 Greiner 650201, U-Bottom Standard PolyPro
7	Stack of n*: 96 Greiner 650201, U-Bottom Standard PolyPro
8	96 Greiner 650201, U-Bottom Standard PolyPro
9	96 Greiner 650201, U-Bottom Standard PolyPro

* The number of plates in a stack equals the Number of Fractions (0 to 6).

Improve Identification by Fractionation using AssayMAP Bravo



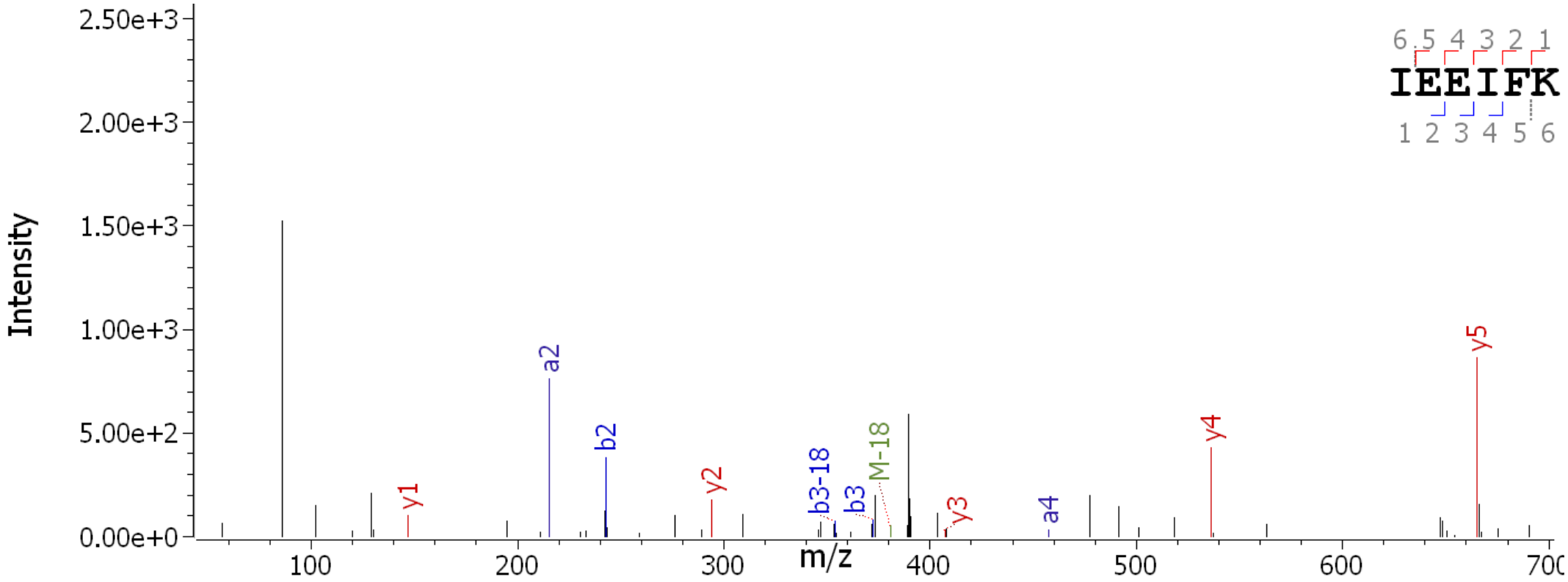
Improve Identification by Fractionation using AssayMAP Bravo

Protein Accession	Protein Spiking Level (ppm)	# Unique Peptide Sequences	
		1D LC-MS/MS	HPRP + LC-MS/MS
ALBU_HUMAN_spike	313.0	46	**79**
CAH2_HUMAN_spike	137.3	19	**32**
CAH1_HUMAN_spike	135.6	9	**15**
LEP_HUMAN_spike	76.2	4	**6**
HBB_HUMAN_spike	74.8	12	**22**
HBA_HUMAN_spike	71.3	7	**14**
UBIQ_HUMAN_spike	50.0	6	**9**
CO5_HUMAN_spike	40.3	4	**6**
CATA_HUMAN_spike	28.1	2	**14**
SUMO1_HUMAN_spike	18.3	3	**11**
NQO1_HUMAN_spike	14.5	2	**8**
PRDX1_HUMAN_spike	10.4	3	**9**
PPIA_HUMAN_spike	9.5	4	**11**
MYG_HUMAN_spike	8.0	2	**2**
CYB5_HUMAN_spike	7.6	0	**2**
EGR_HUMAN_spike	3.0	0	**1**
SYHC_HUMAN_spike	2.7	0	**5**
KCRM_HUMAN_spike	2.0	0	**3**

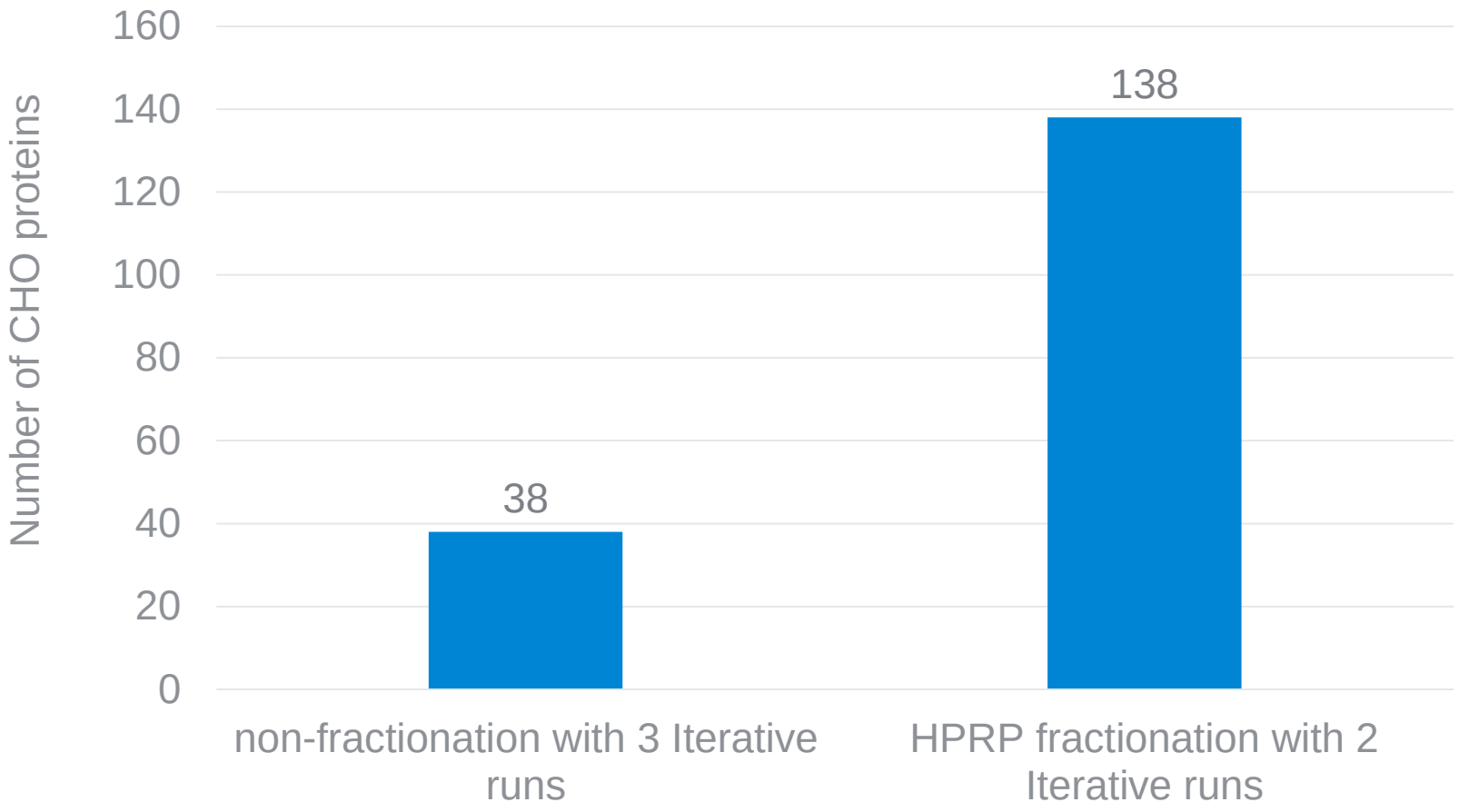
← **2 ppm**

Identification of all the spiking proteins above 2 ppm with Iterative MS/MS acquisition

MS/MS Spectrum of a Peptide from a Protein Spiked at 2 ppm



Improved Identification of Endogenous HCPs by AssayMAP Bravo Fractionation



HCP Discovery

LC/Q-TOF + Protein Metrics SW

- Ideal platform for HCP Discovery
- Excellent chromatographic reproducibility and dynamic range
- Easy processing of DDA data for simultaneous ID and semi-quant

Iterative MS/MS

- Automated approach to easily dig deeper
- Identified all spiked proteins down to **8ppm**

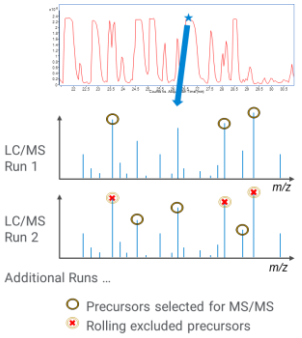
AssayMAP Bravo

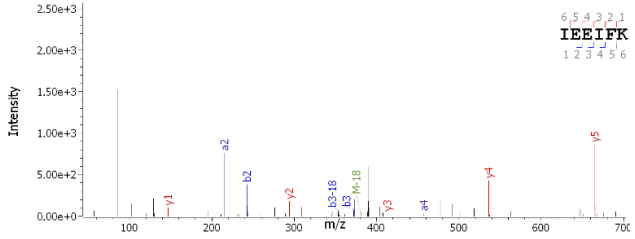
- Personal sample prep workbench
- Fractionation allowed detection down to **2ppm**

From Discovery to Targeted Protein Quantification

Protein Discovery

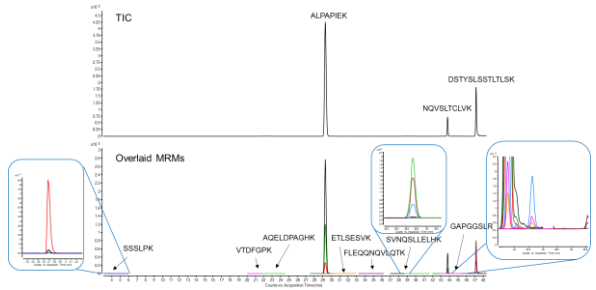
6545XT
AdvanceBio
LC/Q-TOF



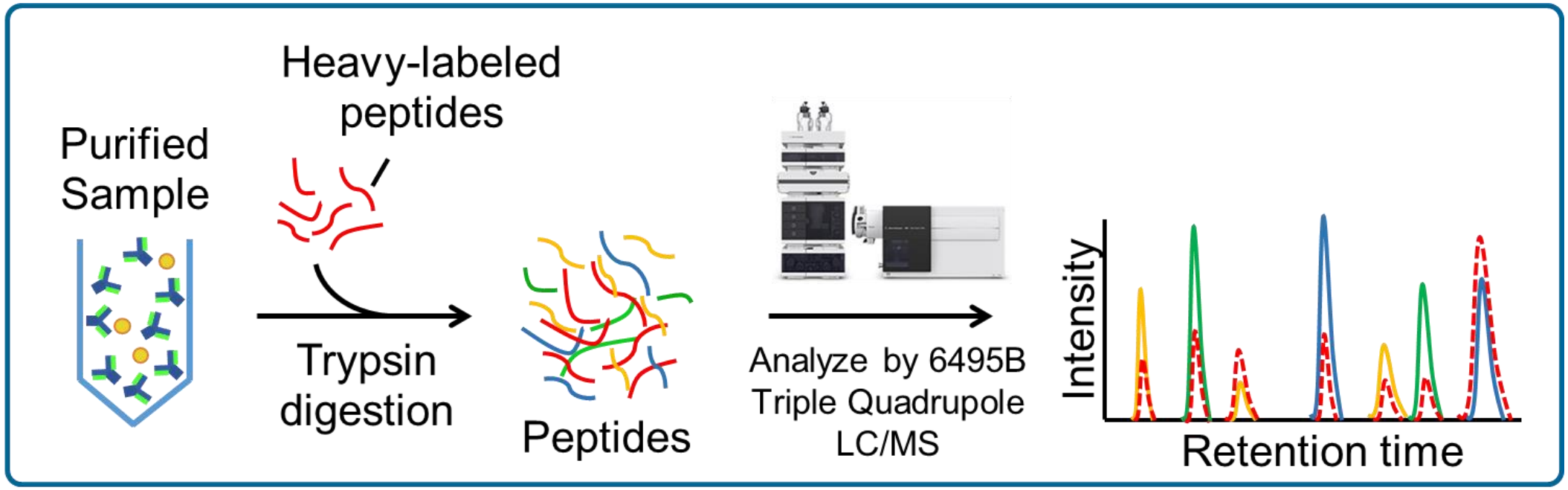


Targeted Quantification by MRM

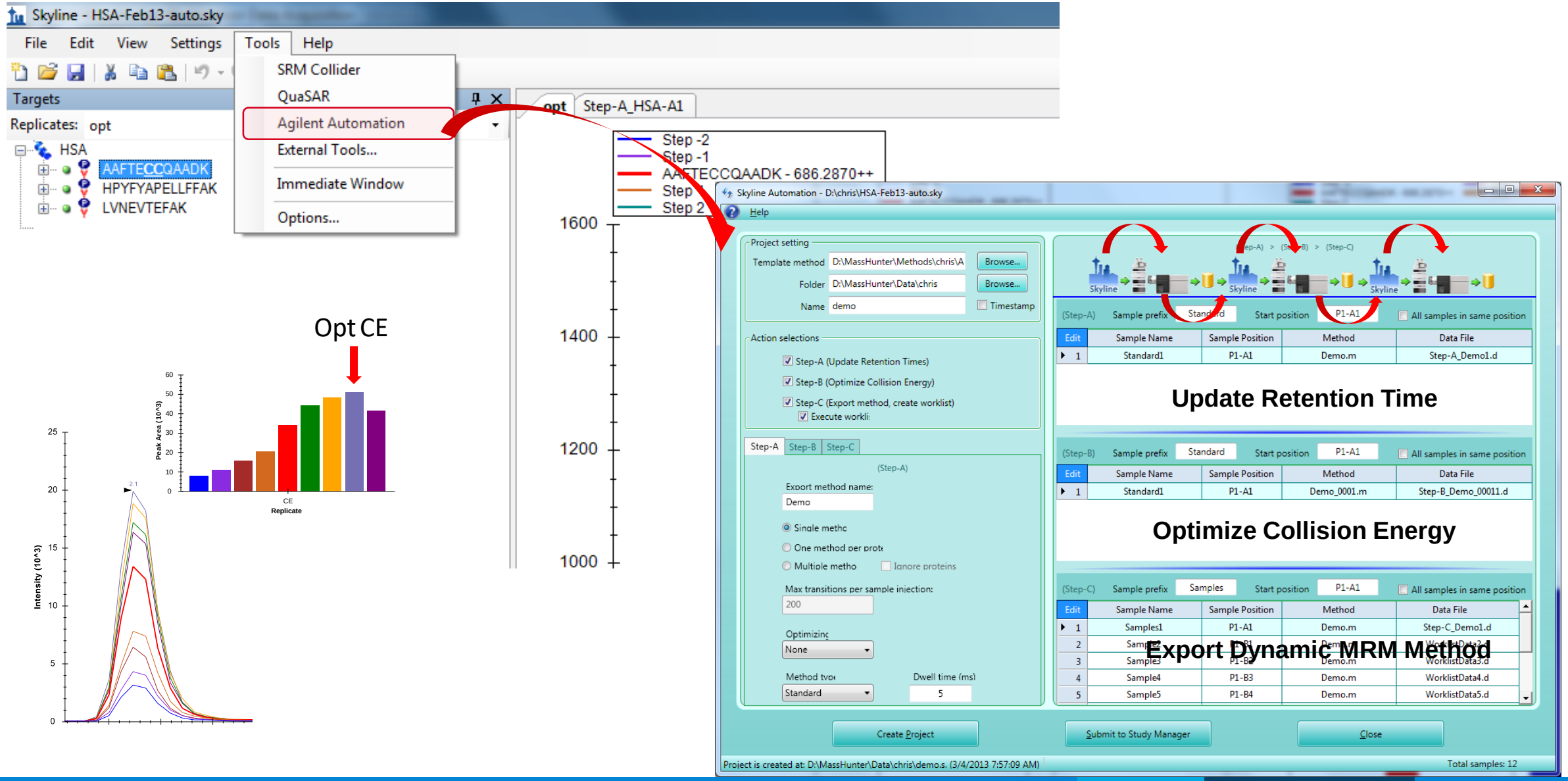
6495B
Triple Quadrupole
LC/MS



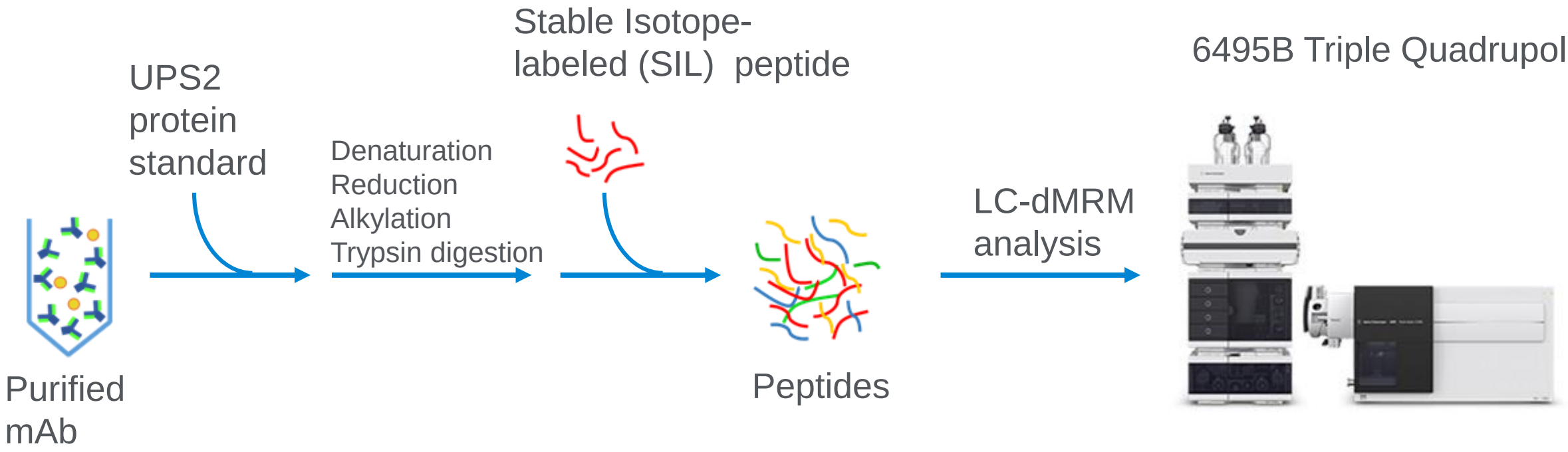
Targeted Protein Quantification by Isotope Dilution Strategy and MRM



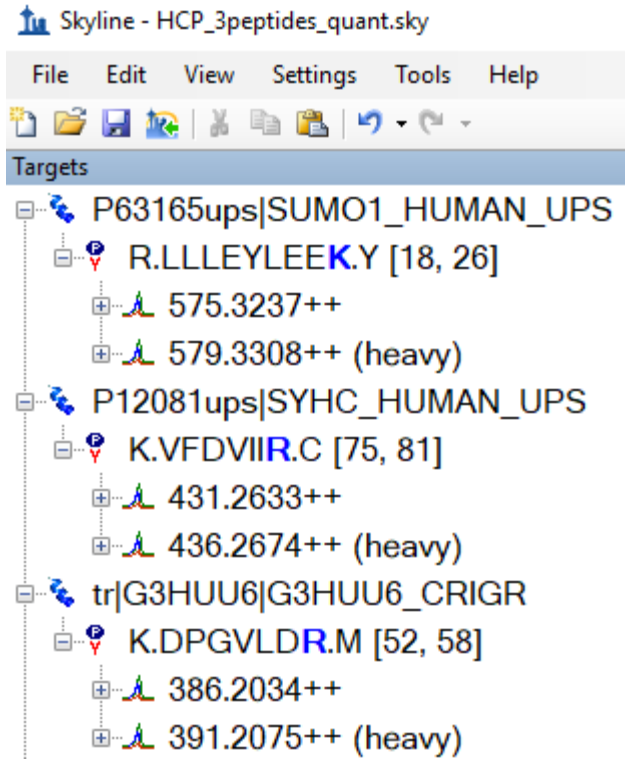
Skyline Automation

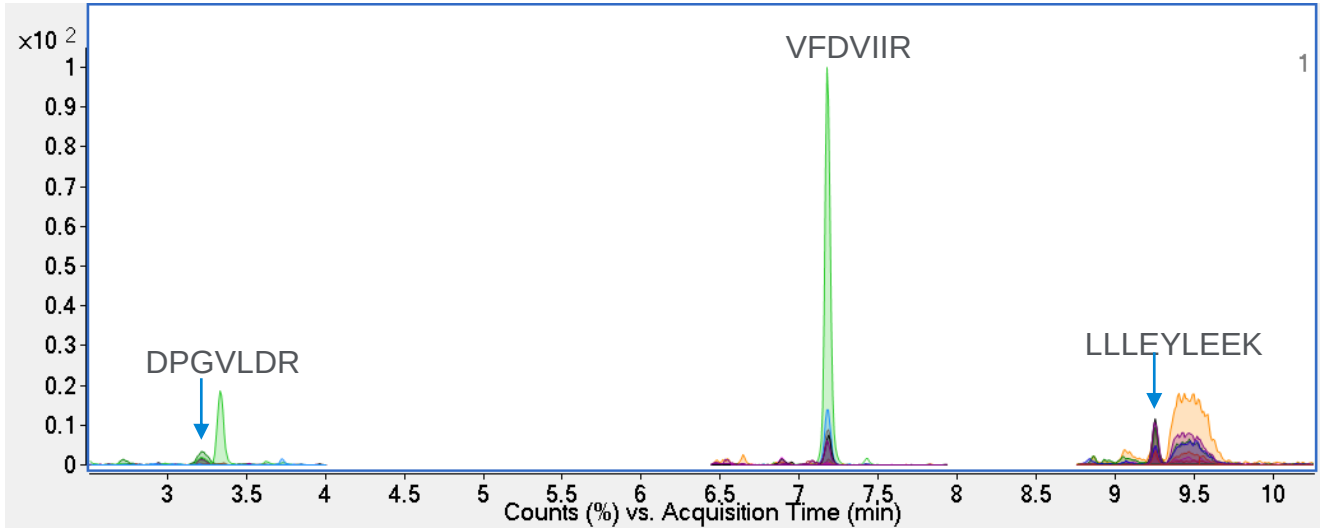


MRM Assay to Evaluate Targeted HCP Quantification



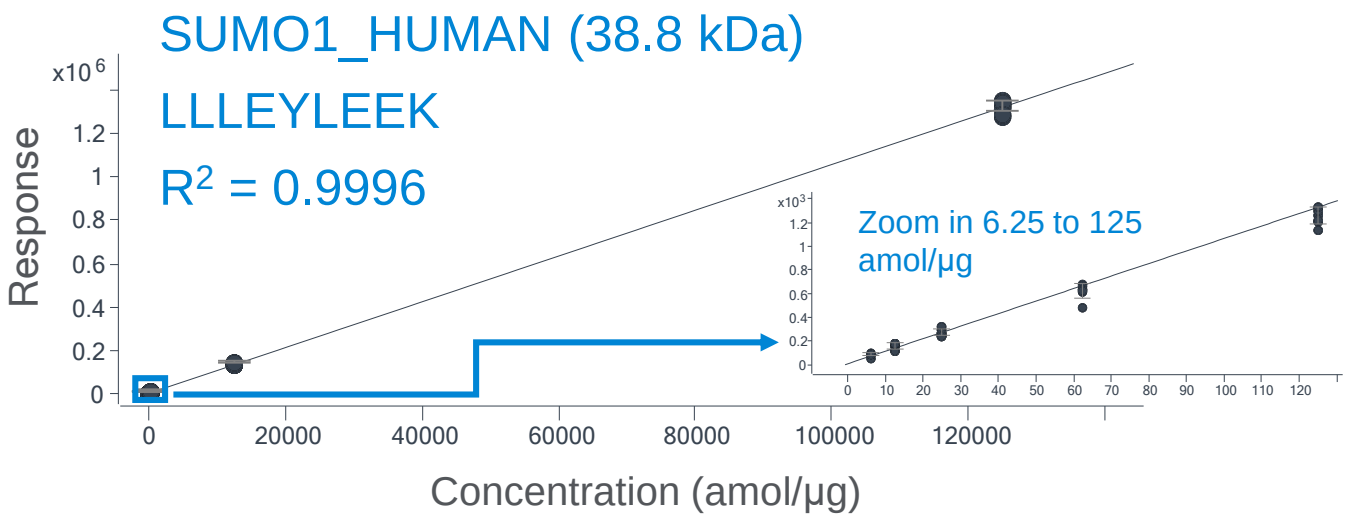
Three Peptides Selected to Evaluate Targeted HCP Quantitation

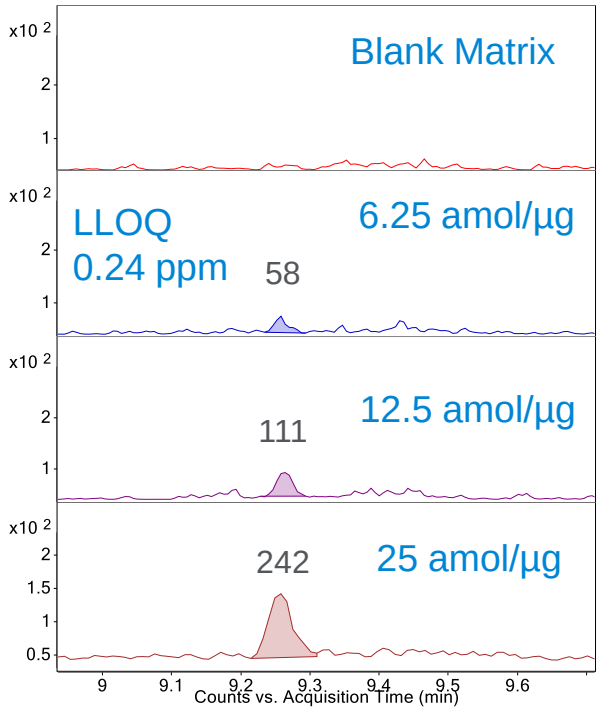




Standard Curve by Heavy SIL Peptide Standard

Peptide sequence: LLLEYLEEK



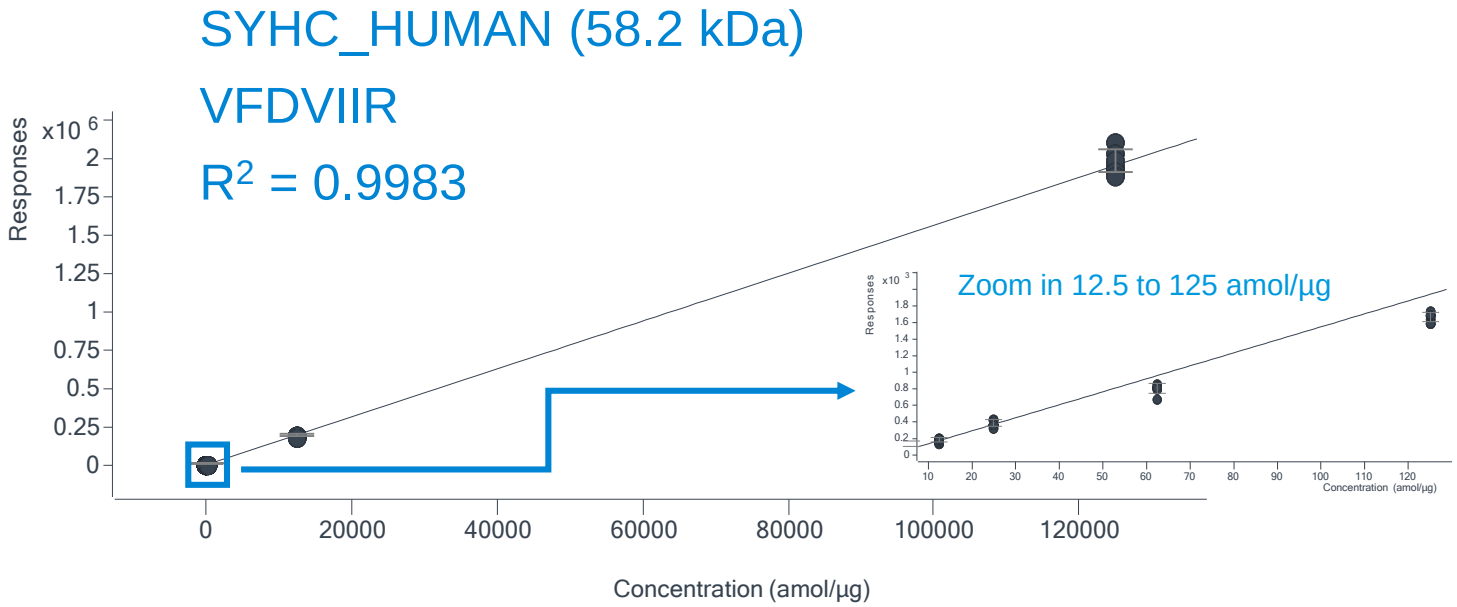


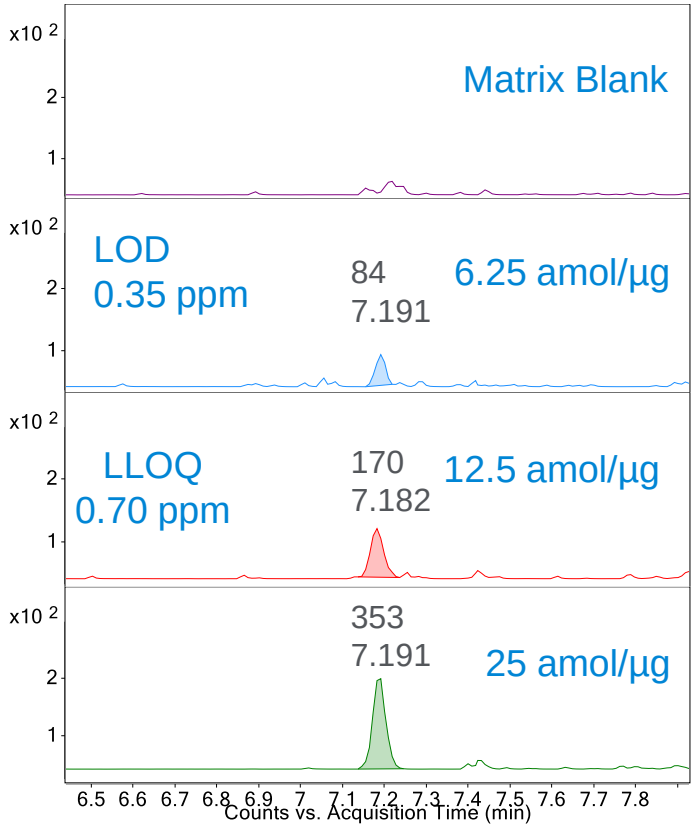
13 min LC-MS analysis that includes sample on-line desalting

Standard curve ranges from 6.25 amol/µg to 125 fmol/µg (0.24 ppm ~ 4765 ppm)

Standard Curve by Heavy SIL Peptide Standard

Peptide sequence: VFDVIIR



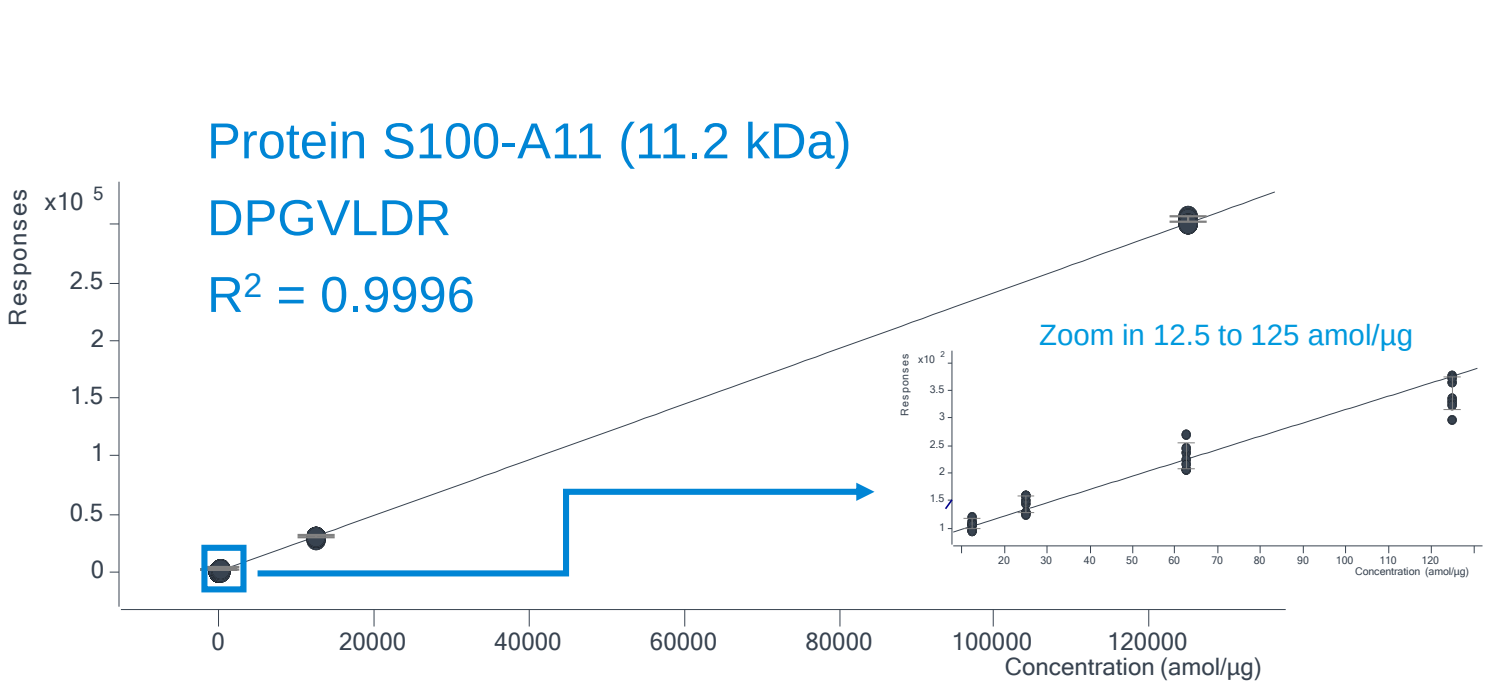


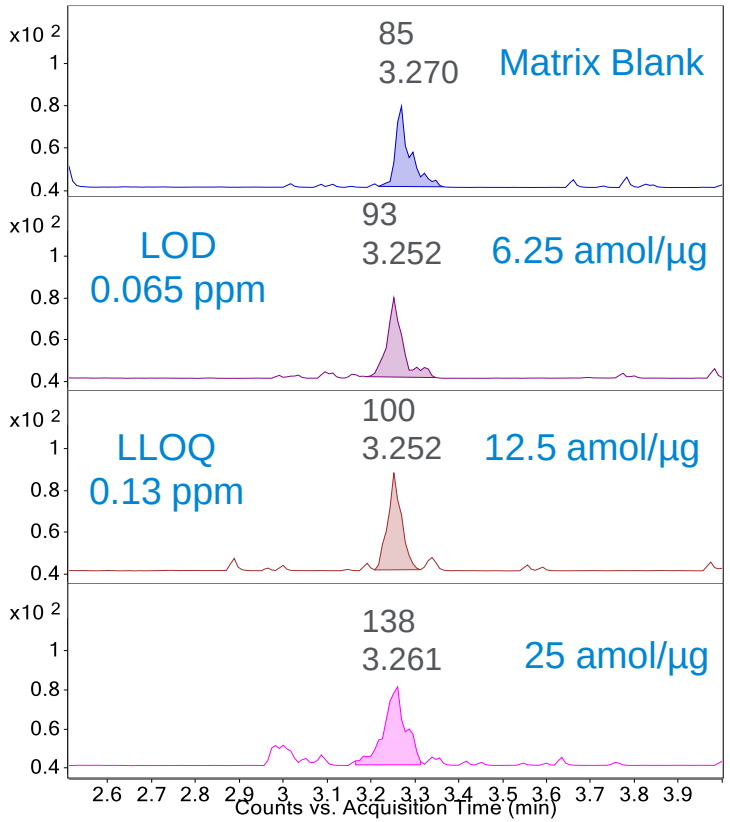
13 min LC-MS analysis that includes sample on-line desalting

Standard curve ranges from 6.25 amol/µg to 125 fmol/µg (0.35 ppm ~ 7002.5 ppm)

Standard Curve by Heavy SIL Peptide Standard

Peptide sequence: DPGVLDR

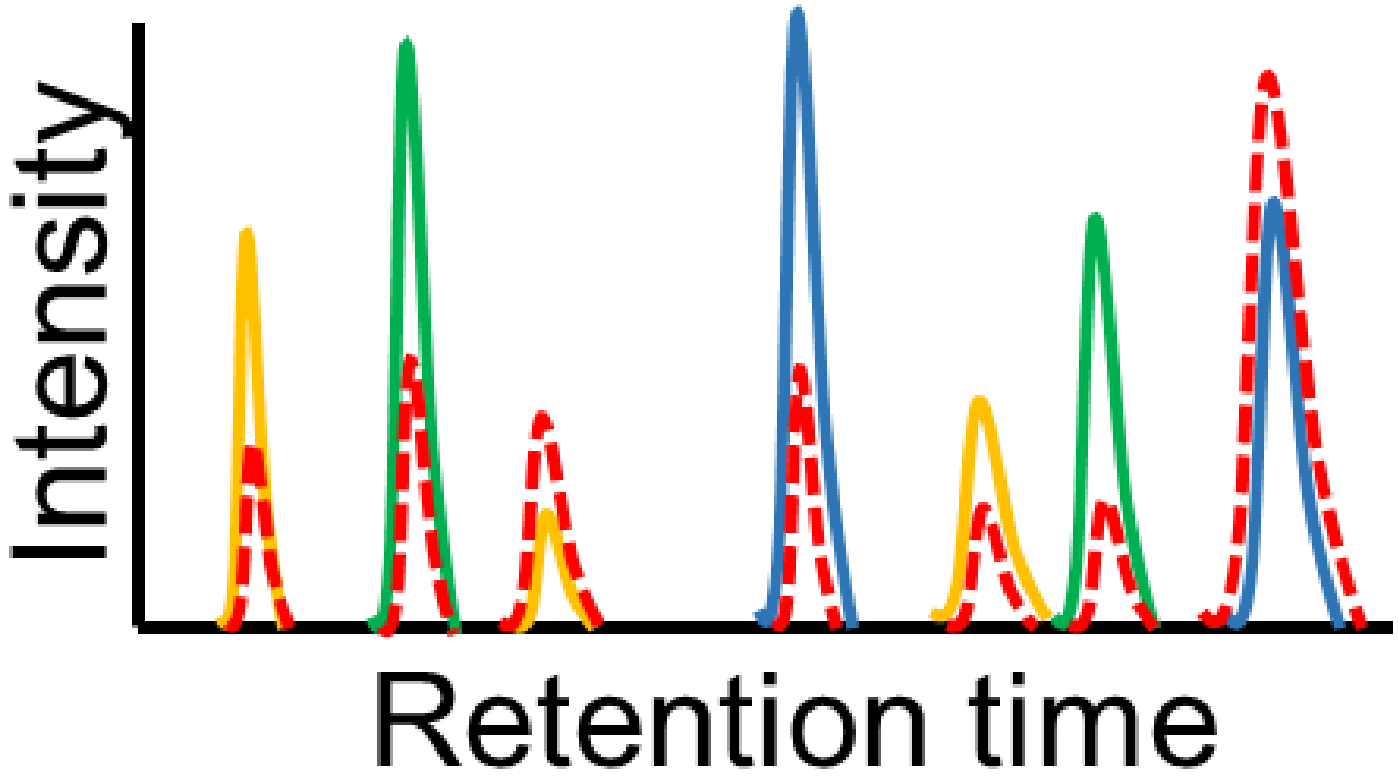




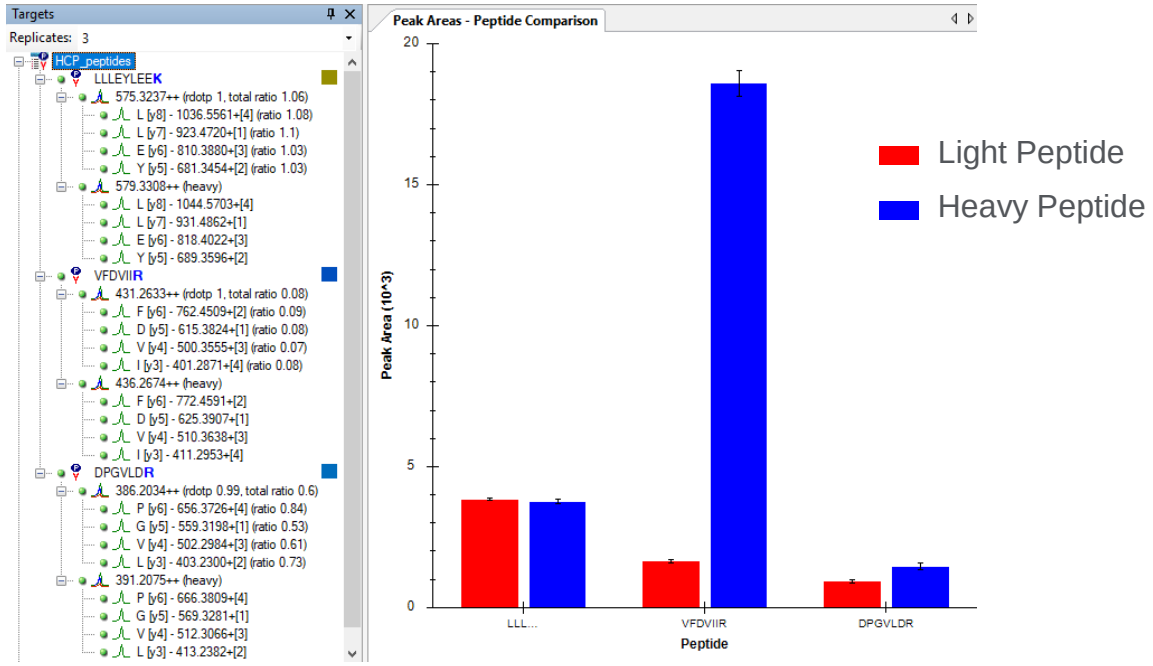
13 min LC-MS analysis that includes sample on-line desalting

Standard curve ranges from 12.5 amol/µg to 125 fmol/µg (0.13 ppm ~ 1336.3 ppm)

Absolute Quantification Using Heavy SIL Peptides as Internal Standards



Absolute Quantification of Targeted Proteins



Targeted Protein	SUMO1_HUMAN	SYHC_HUMAN	Protein S100-A11 (G3HUU6)
Protein MW	38,815 Da	58,233 Da	11,241Da
Peptide sequence	LLLEYLEEK	VFDVIIR	DPGVLDR
Spiked protein level	18.3 ppm	2.7 ppm	NA
Measured protein level	10.1 ppm	1.2 ppm	1.6 ppm

Summary of Workflows

HCP ID

Detection down to 2 ppm

Sample Prep
AssayMAP Bravo

Detection
6545XT AdvanceBio LC/Q-TOF

Processing
Protein Metrics

Iterative MS/MS

Targeted Quantitation

Sub-ppm monitoring

Sample Prep
AssayMAP Bravo

Detection
6495B Triple Quad

Processing
MassHunter Quant

Skyline integration for method development

Thank you!