High Sensitivity Protein Quantitation Using a Triple Quadrupole with a Dual Ion Funnel

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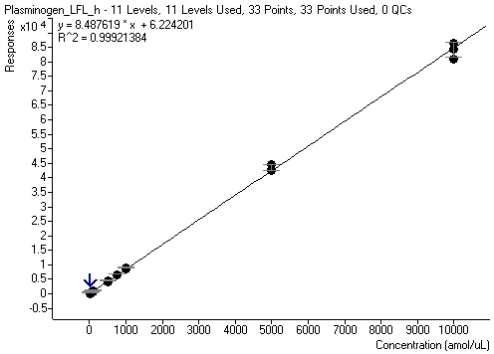
6490 Triple Quadrupole

Overview

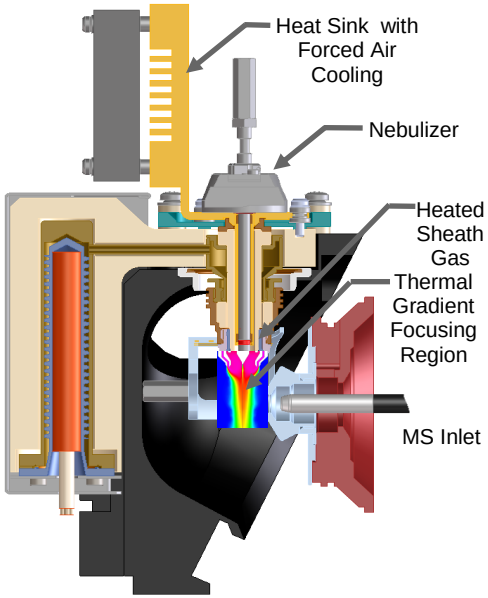
1. Improvements in LC/MS instrumentation
2. Impact of flow rate and column i.d. on sensitivity
3. Robustness of analysis for complex samples
4. Reduced sample complexity using SISCAPA
5. Automation of sample preparation using Bravo system



iFunnel Technology in the Agilent 6490 – 3 Key Elements

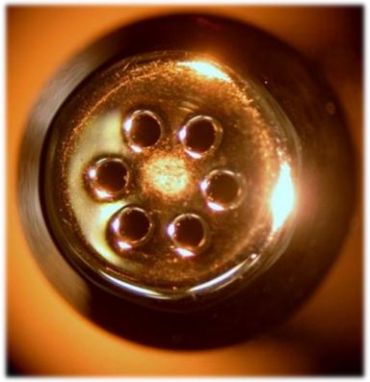
More efficient ionization

- Thermal confinement of ESI ion plume
- Efficient desolvation to create gas phase ions



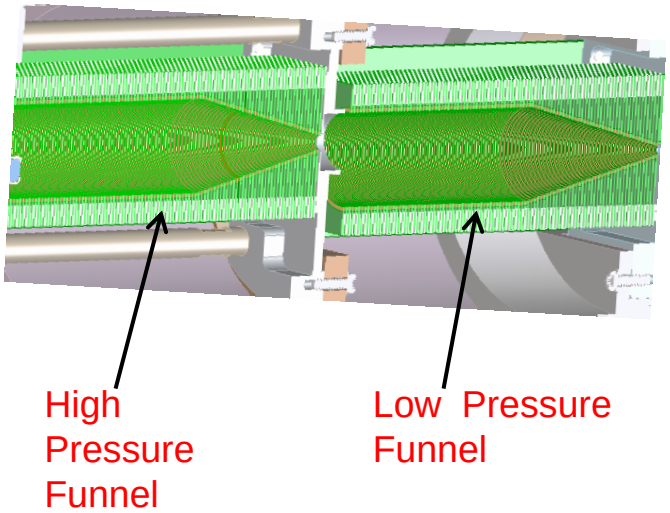
Increased ion sampling

- 6 capillary inlets
- Samples 12x more ion rich gas from the source

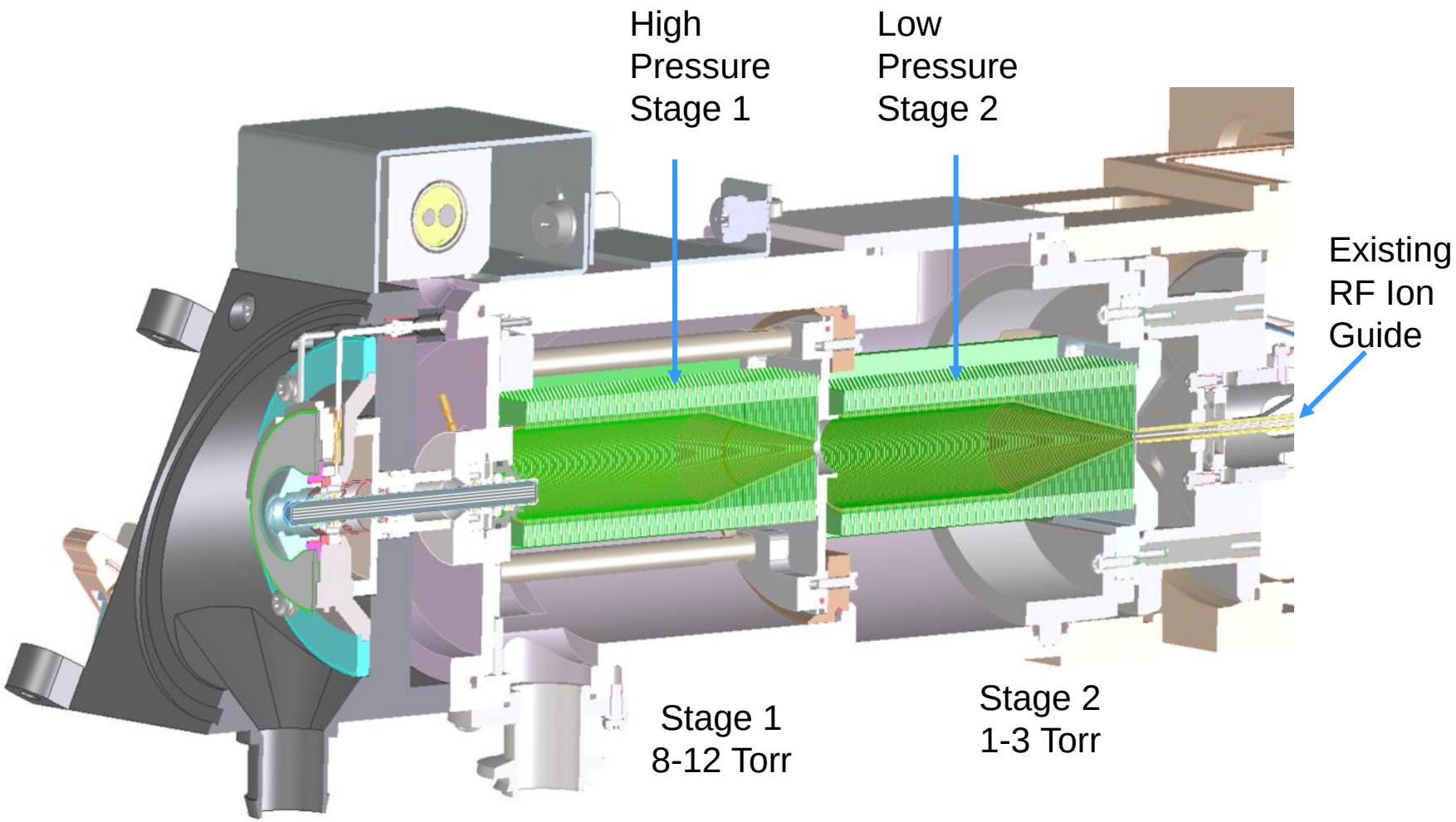


Greater ion transfer

- Removes the gas but captures the ions
- Helps to remove source generated noise



Two Stage Ion Funnel Manages the Gas Load



Stage 1 offset breaks up the high pressure gas exiting the Hexabore Capillary

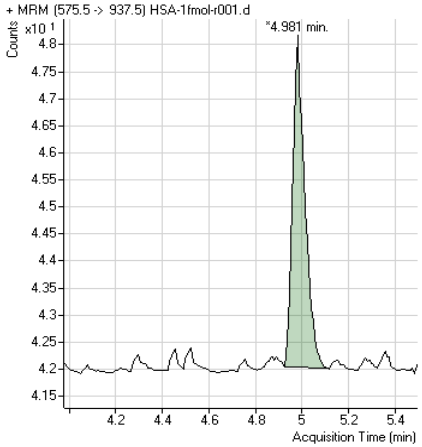
High and Low Pressure Funnels

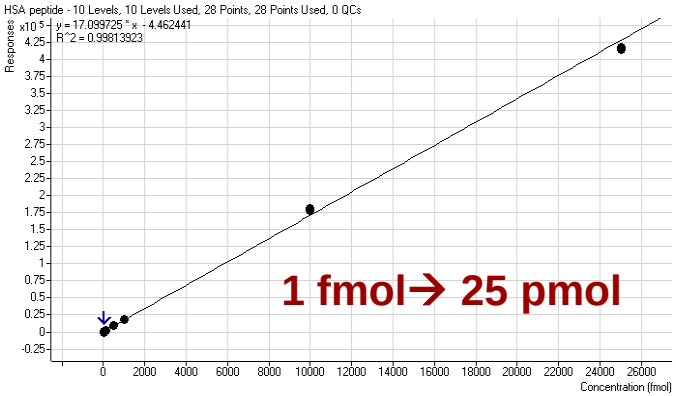
How much difference does the iFunnel make?

Impact of iFunnel on Sensitivity: Standard Flow LC/MS with JetStream Source

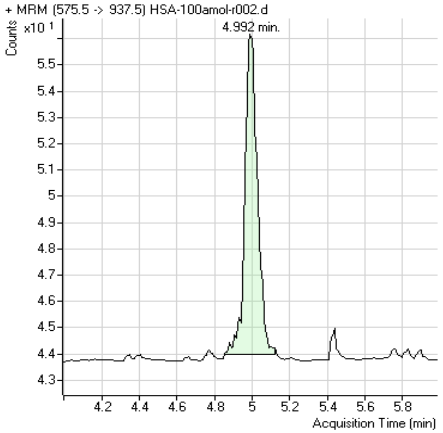
Calibration Curves

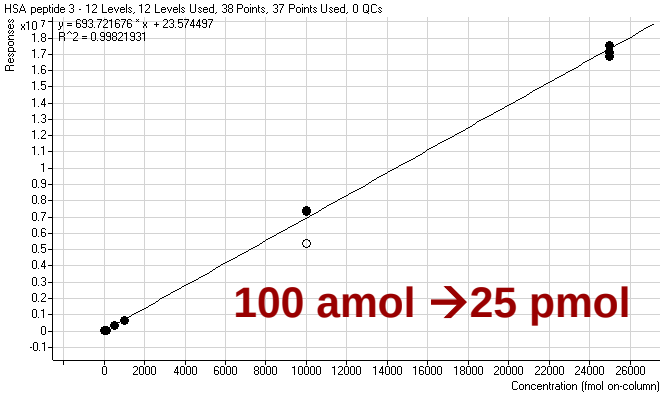
QQQ (6460)
1 fmol on-column





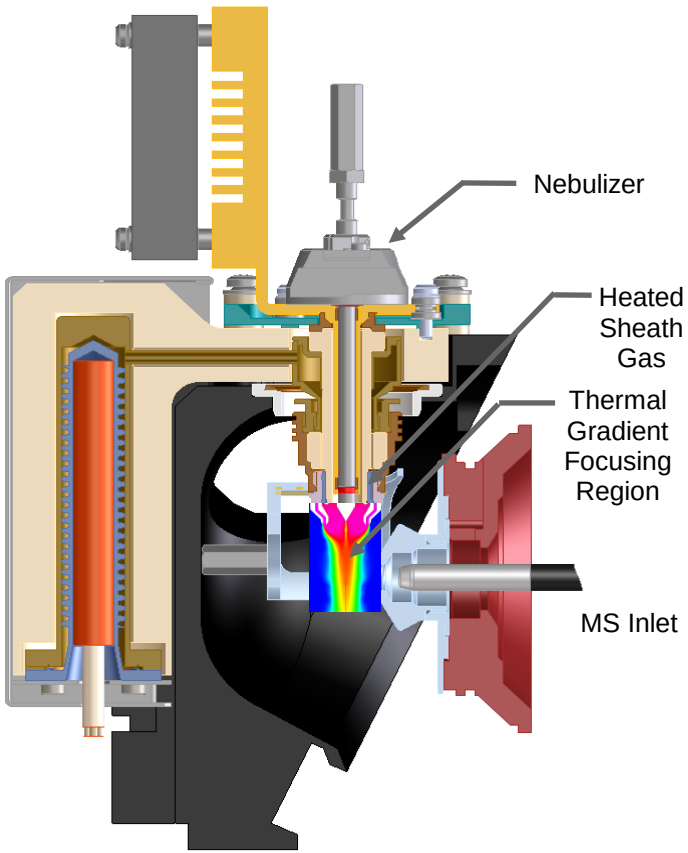
QQQ with iFunnel
100 amol on-column



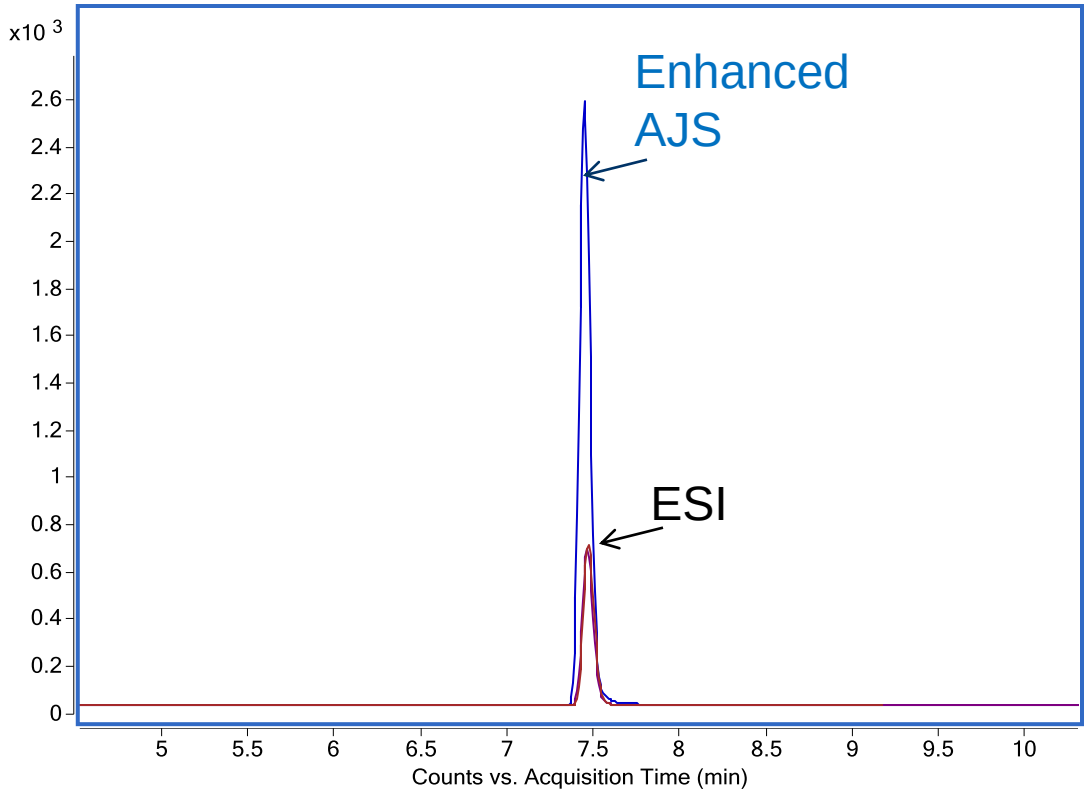


HSA Standard Peptide (LVNEVTEFAK, 575.5 → 937.5), Poroshell 120 2.1 x 150 mm column at 0.5 mL/min

JetStream Source vs. ESI at Capillary Flow Rates (17 µL/min)

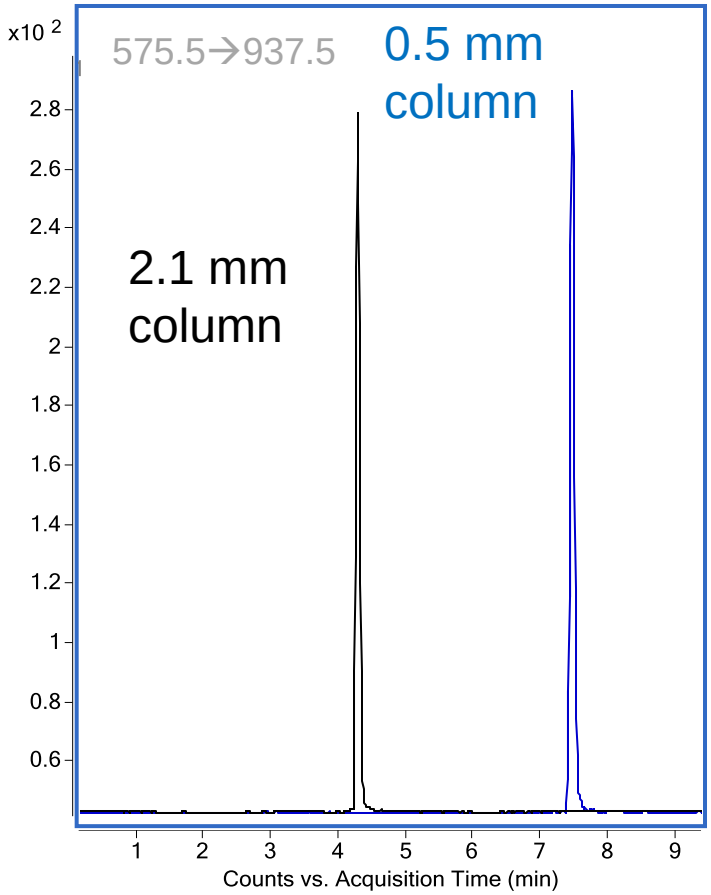


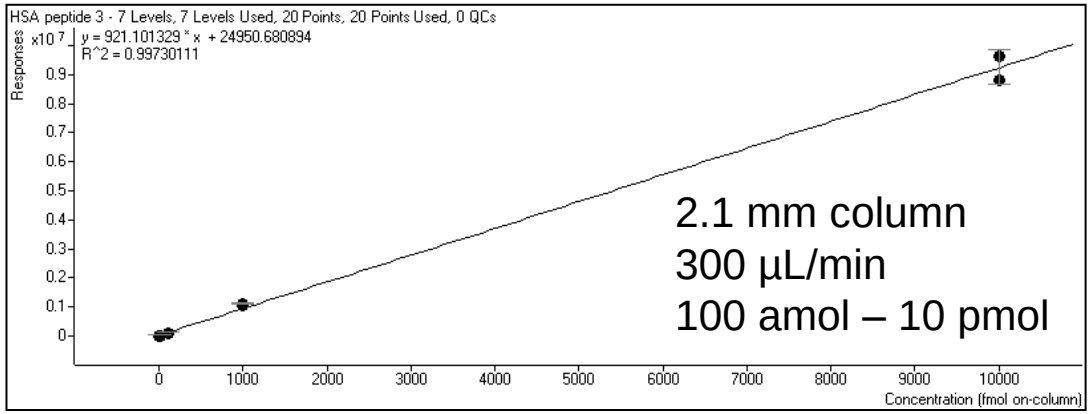
10 fmol on-column peptide LVNEVTEFAK

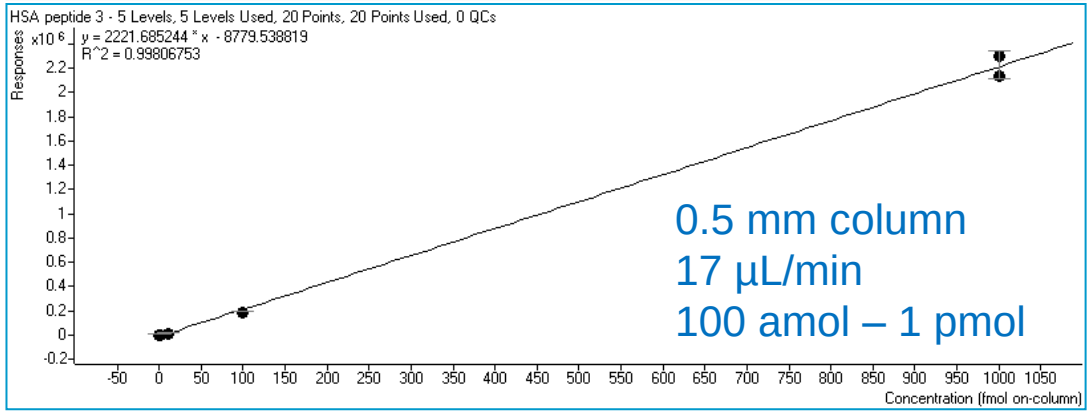


2.1 vs. 0.5 mm ID Columns: JetStream Source Shows Mass Dependent Behavior

1 fmol on-column

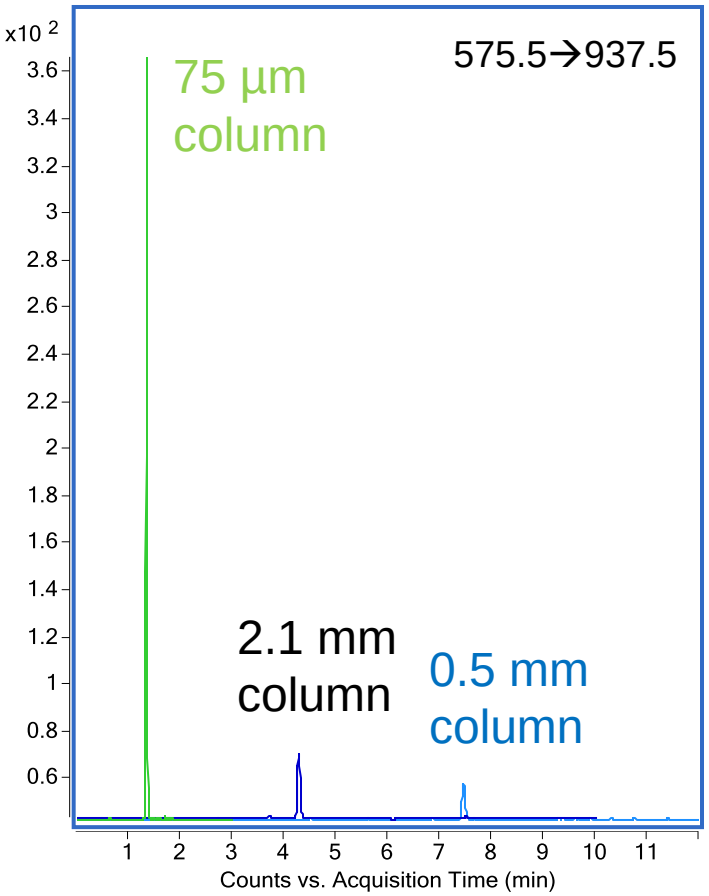




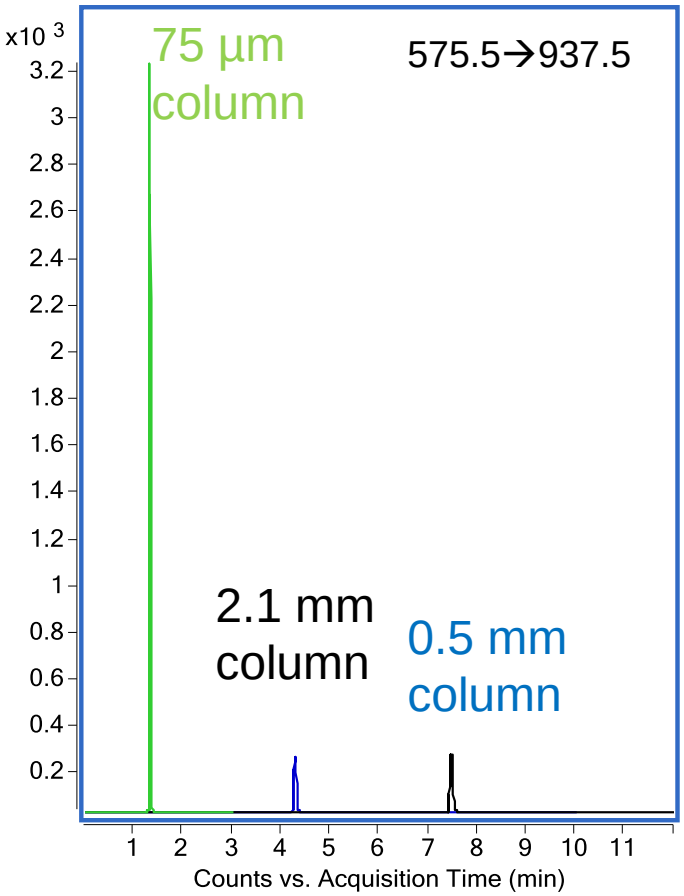


Comparison of Standard (0.3 mL/min), Capillary (17 µL/min) and Nanospray (600 nL/min)

100 amol on-column



1 fmol on-column

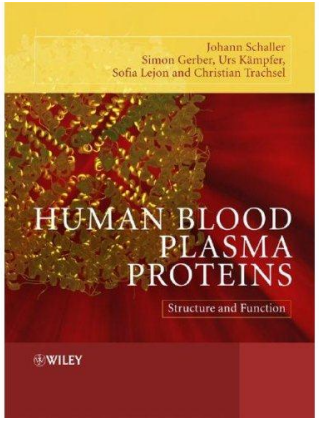


Robustness of LC/MS System

Goal: Demonstrate the performance of the instrumentation for the analysis of peptides in a complex matrix

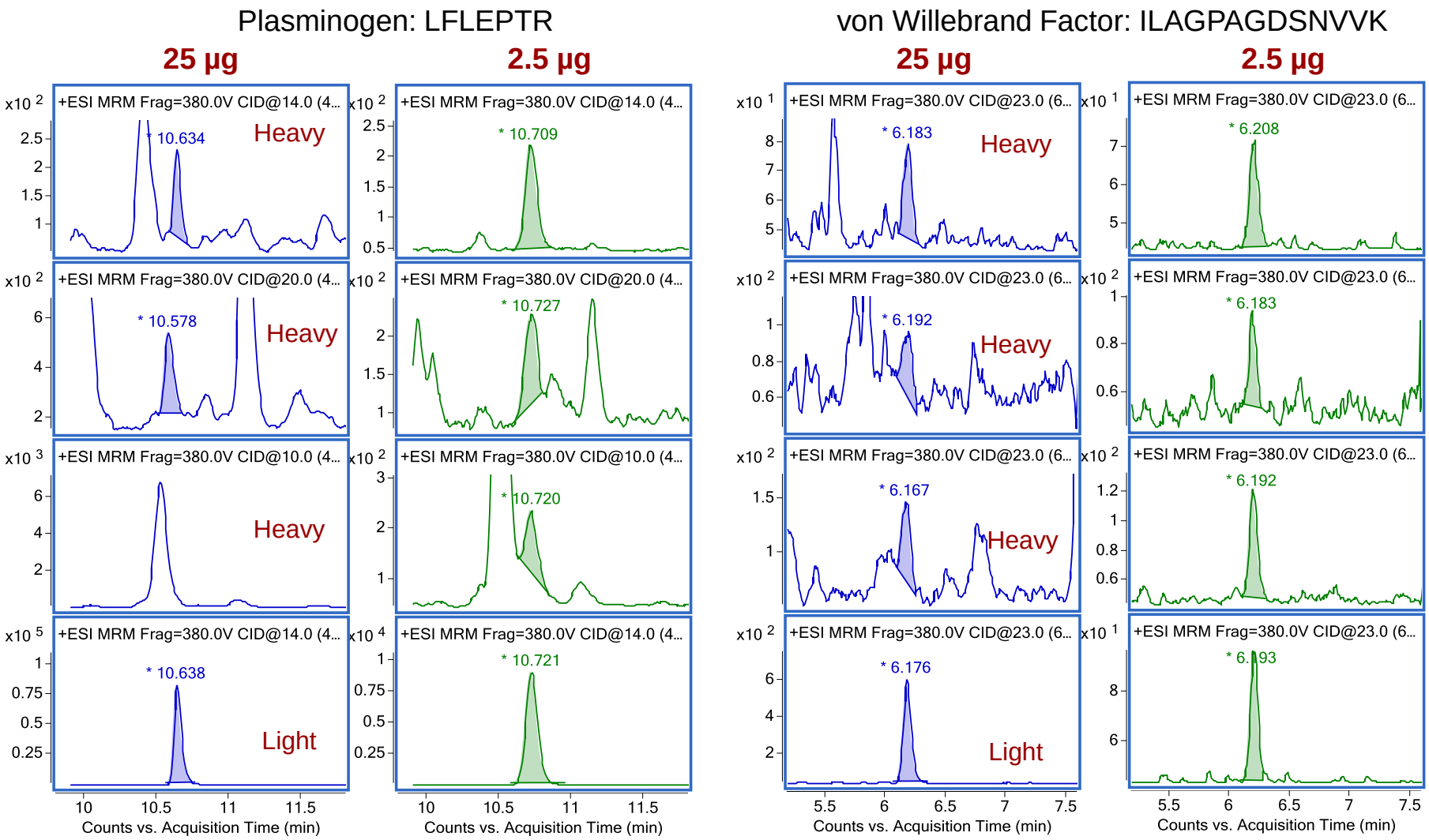
Instrumentation: 1290 Infinity LC system with 6490 QQQ

Samples: Stable isotope-labeled standard (SIS) peptides (12 total) in non-depleted plasma digest



These samples were kindly provided by Derek Smith and Christoph H. Borchers from the UVic-Genome BC Proteomics Centre

Injecting Less Sample Reduces Matrix Effects



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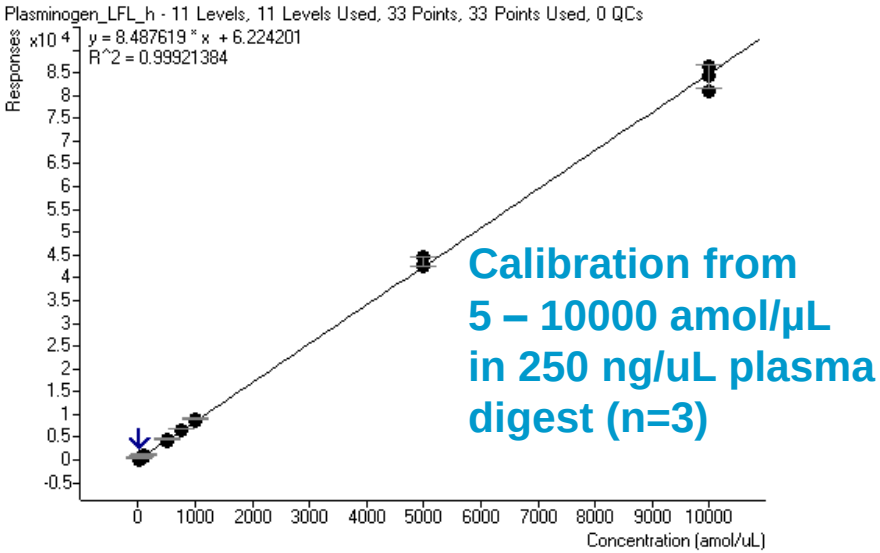
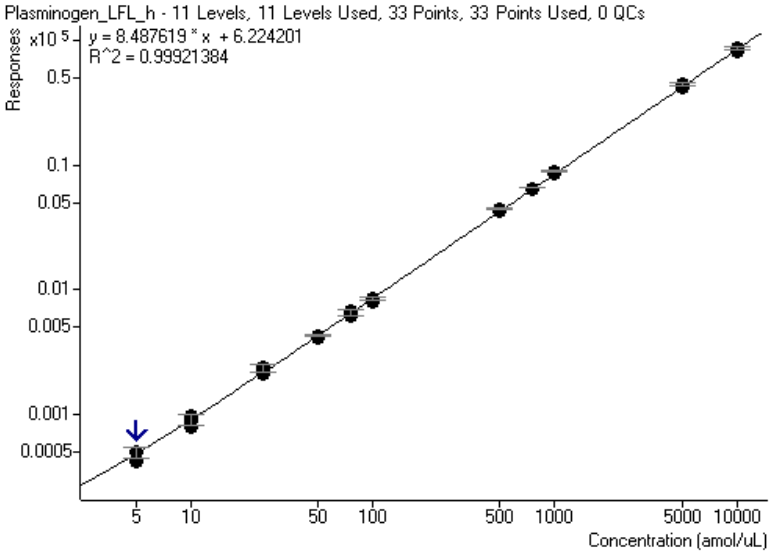
Standard Flow LC/MS: Quantitation of the Plasminogen Peptide in Plasma

Linear plot



Calibration from 5 – 10000 amol/µL in 250 ng/uL plasma digest (n=3)

Log-Log plot

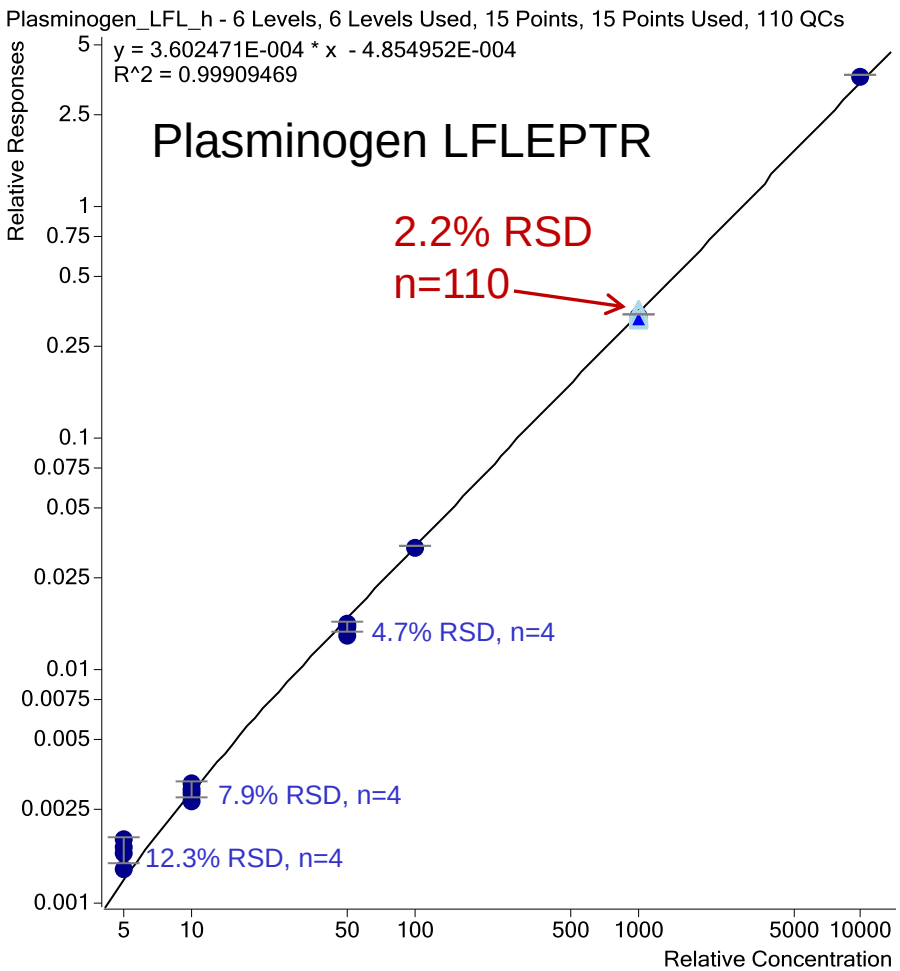


441.3>621.4	ESTD Calibration standards (amol/uL)										
	5	10	25	50	75	100	500	750	1000	5000	10000
%Accuracy (average, n =3)	98.2	97.5	104.6	98.1	99.2	97.4	101.3	101.8	101.3	101.8	98.8
Reproducibility (%RSD, n = 3)	11.85	10.78	7.21	1.17	6.22	2.74	1.41	0.48	1.52	2.58	3.24
Response factor	8.33	8.28	8.87	8.32	8.42	8.27	8.60	8.64	8.60	8.64	8.39
Precision (%RSD, n = 11)	2.30										

The samples were provided by Derek Smith and Christoph H. Borchers from the UVic-Genome BC Proteomics Centre

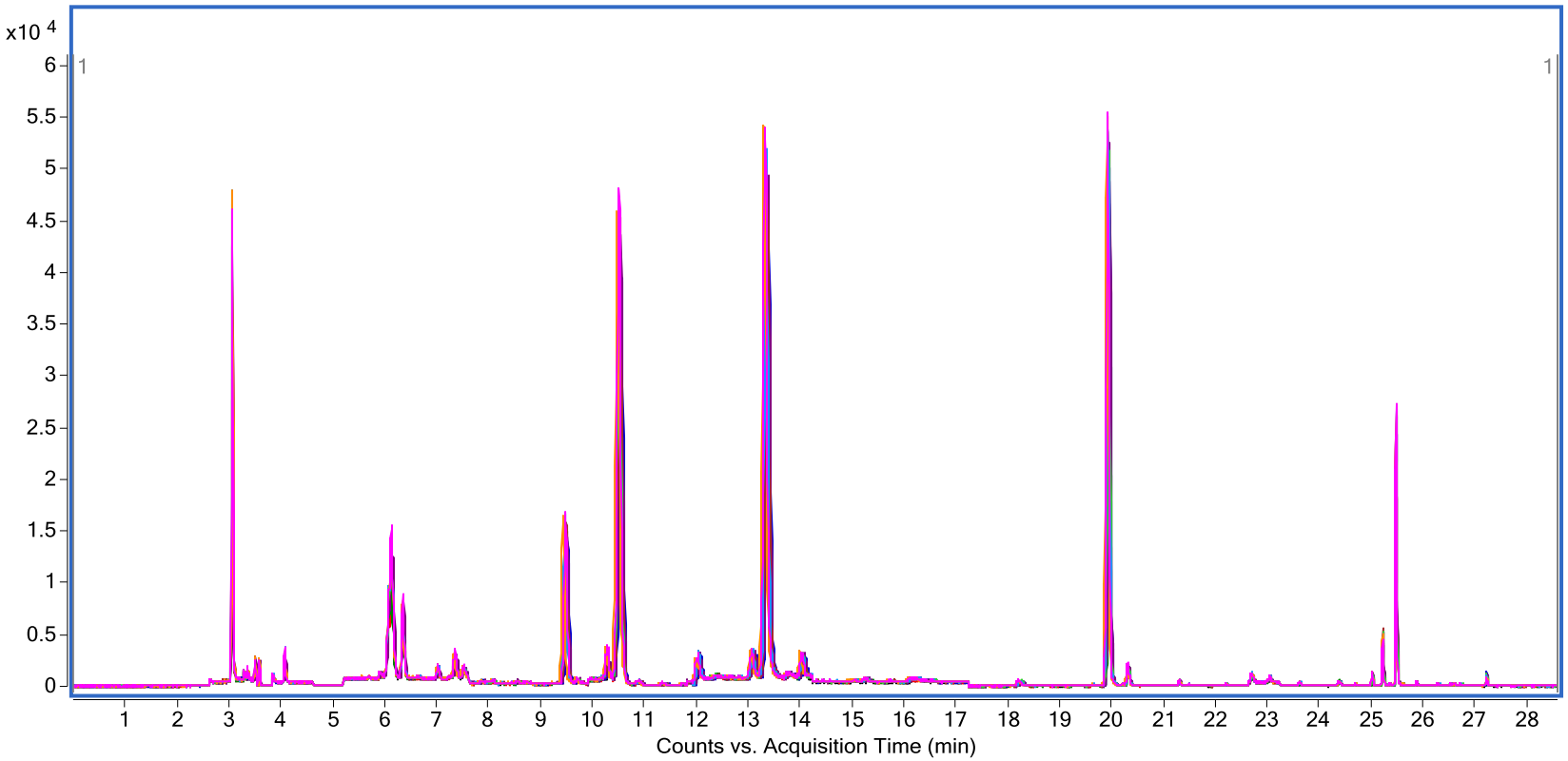
Reproducibility for 110 Injections (10 fmol SIS Peptides and 2.5 µg Plasma Digest On-column)

Protein	Response %RSD	Ret. Time %RSD
Adiponectin: IFYNQQNHYDGSTGK	9.8	0.13
Antithrombin-III : DDLYVSDAFHK	4.7	0.16
Apolipoprotein A-II precursor: SPELQAEAK	6.7	0.12
Apolipoprotein C-III: GWVTDGFSSLK	2.3	0.08
Ceruloplasmin : EYTDASFTNR	9.6	0.14
Heparin cofactor II: TLEAQLTPR	6.1	0.15
Histidine-rich glycoprotein: DGYLFQLLR	3.4	0.02
Kininogen-1: TVGSDTFYSFK	3.3	0.13
L-selectin: AEIEYLEK	9.5	0.15
Plasminogen: LFLEPTR	2.2	0.13
Vitamin D-binding protein: THLPEVFLSK	3.0	0.12
von Willebrand Factor: ILAGPAGDSNVVK	9.5	0.15

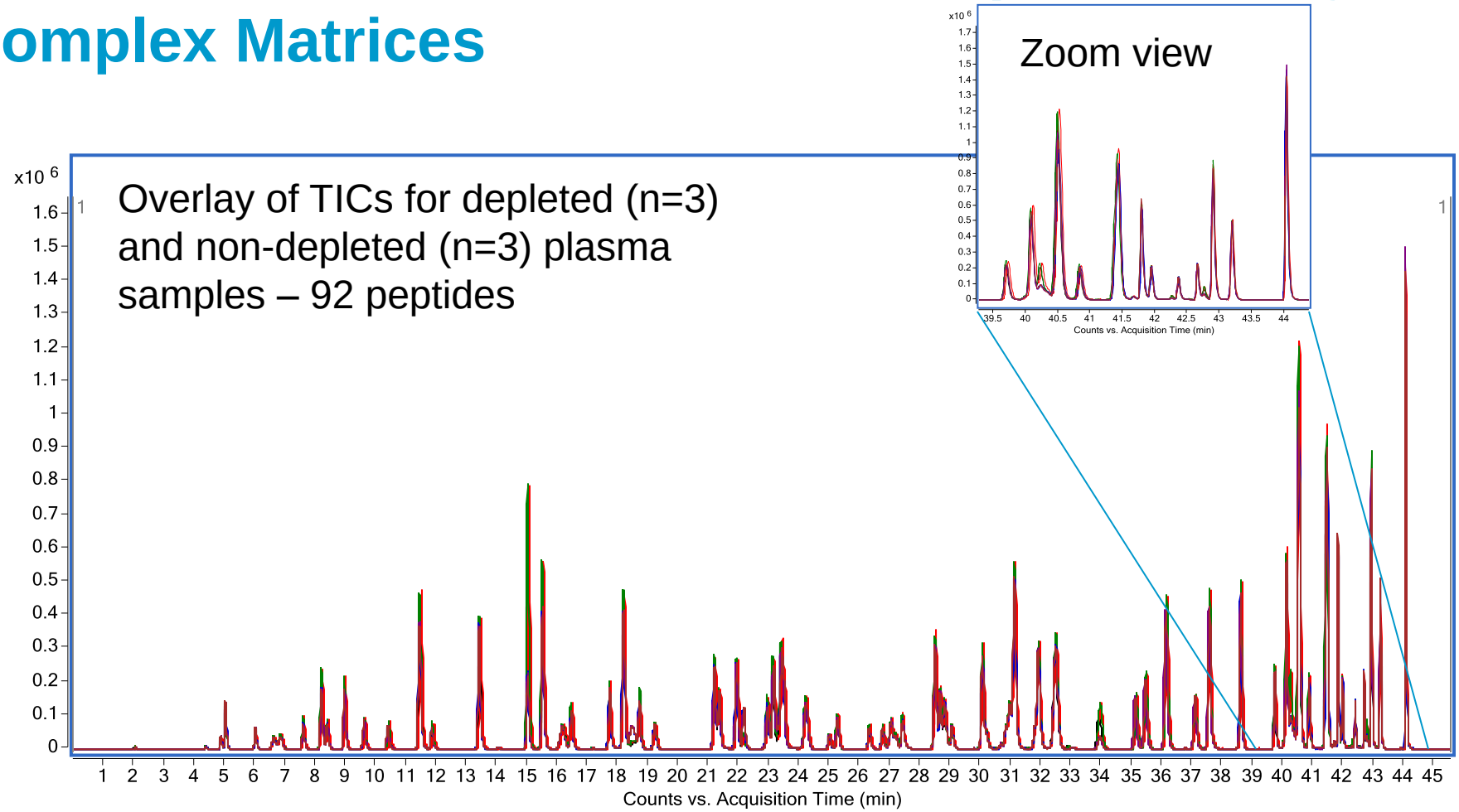


The samples were provided by Derek Smith and Christoph H. Borchers from the UVic-Genome BC Proteomics Centre

Reproducibility for 110 Injections: Overlay of TIC for Runs 1, 10, 20, 30, 40, 50, 60, 70, 80 and 90

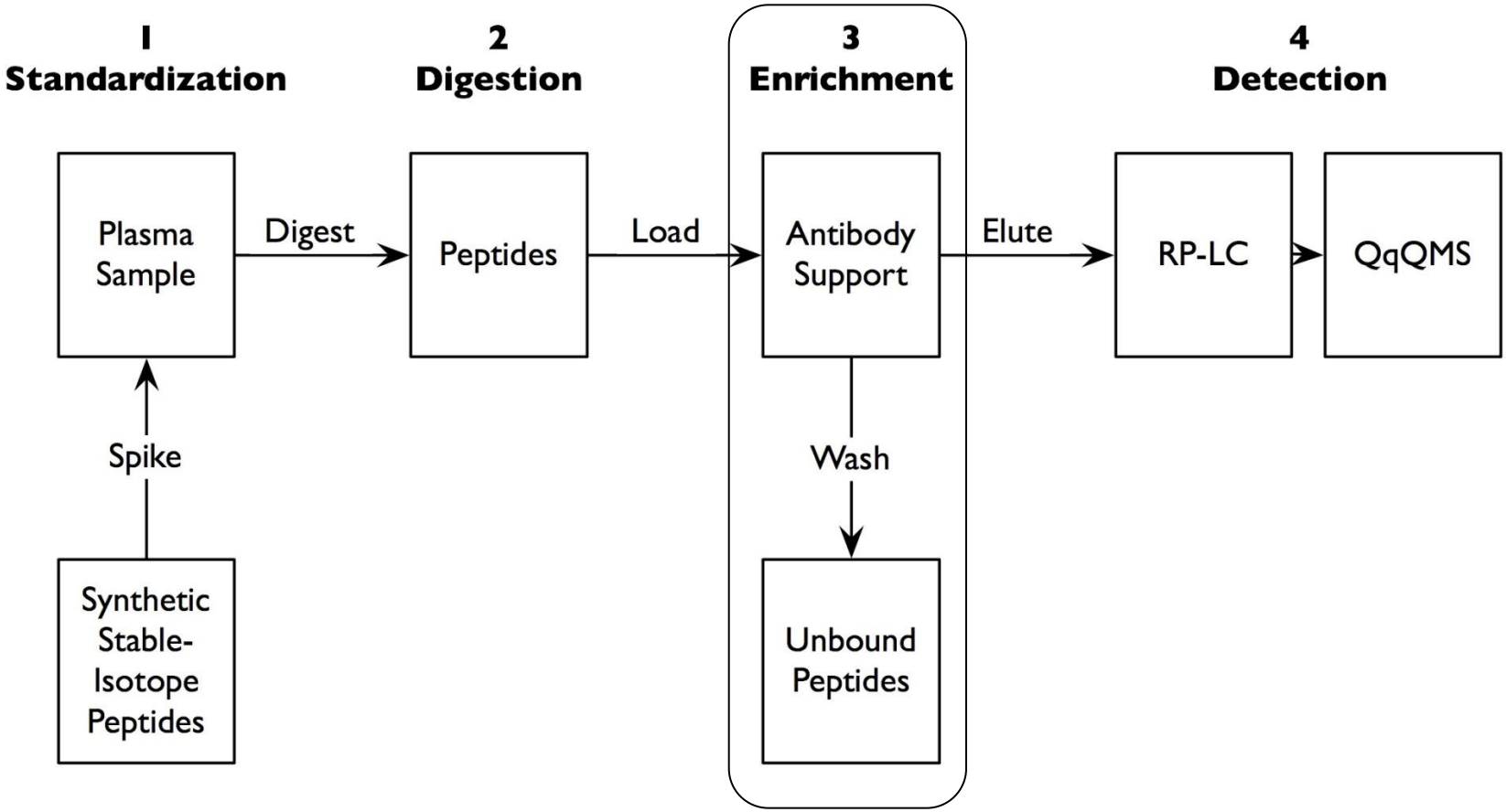


Standard Flow LC: Excellent RT Reproducibility in Complex Matrices



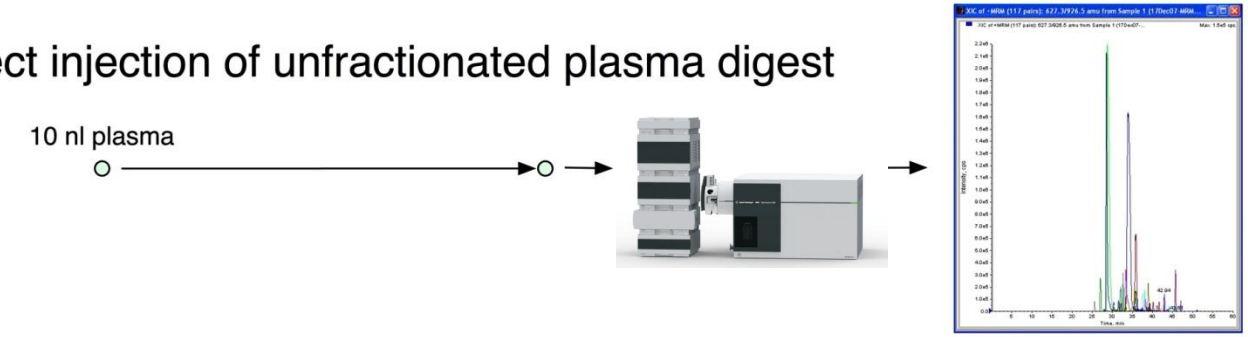
SISCAPA™ Process Schematic Diagram:

Stable Isotope-labeled Standards with Capture on Anti-Peptide Antibodies

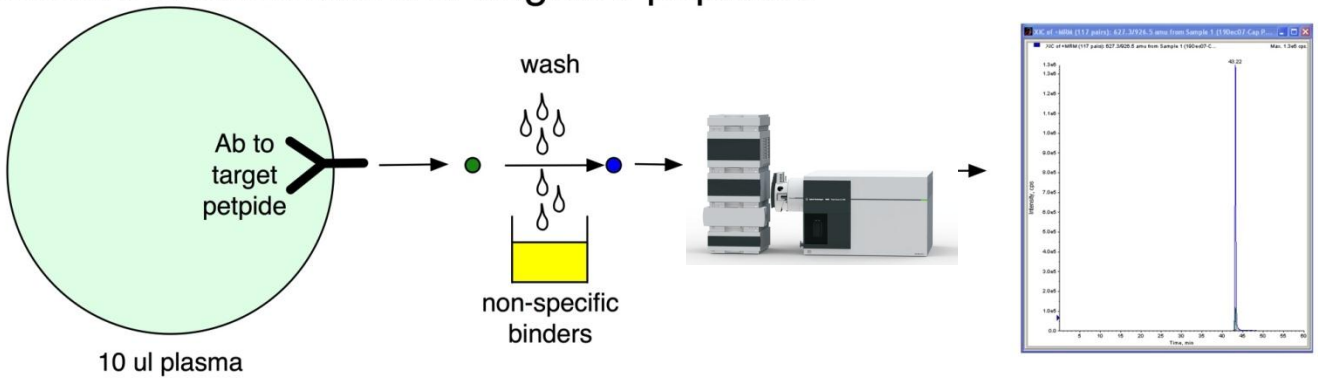


SISCAPA: Enrich Target Peptides and Decrease Sample Complexity

Direct injection of unfractionated plasma digest



SISCAPA enrichment of targeted peptides



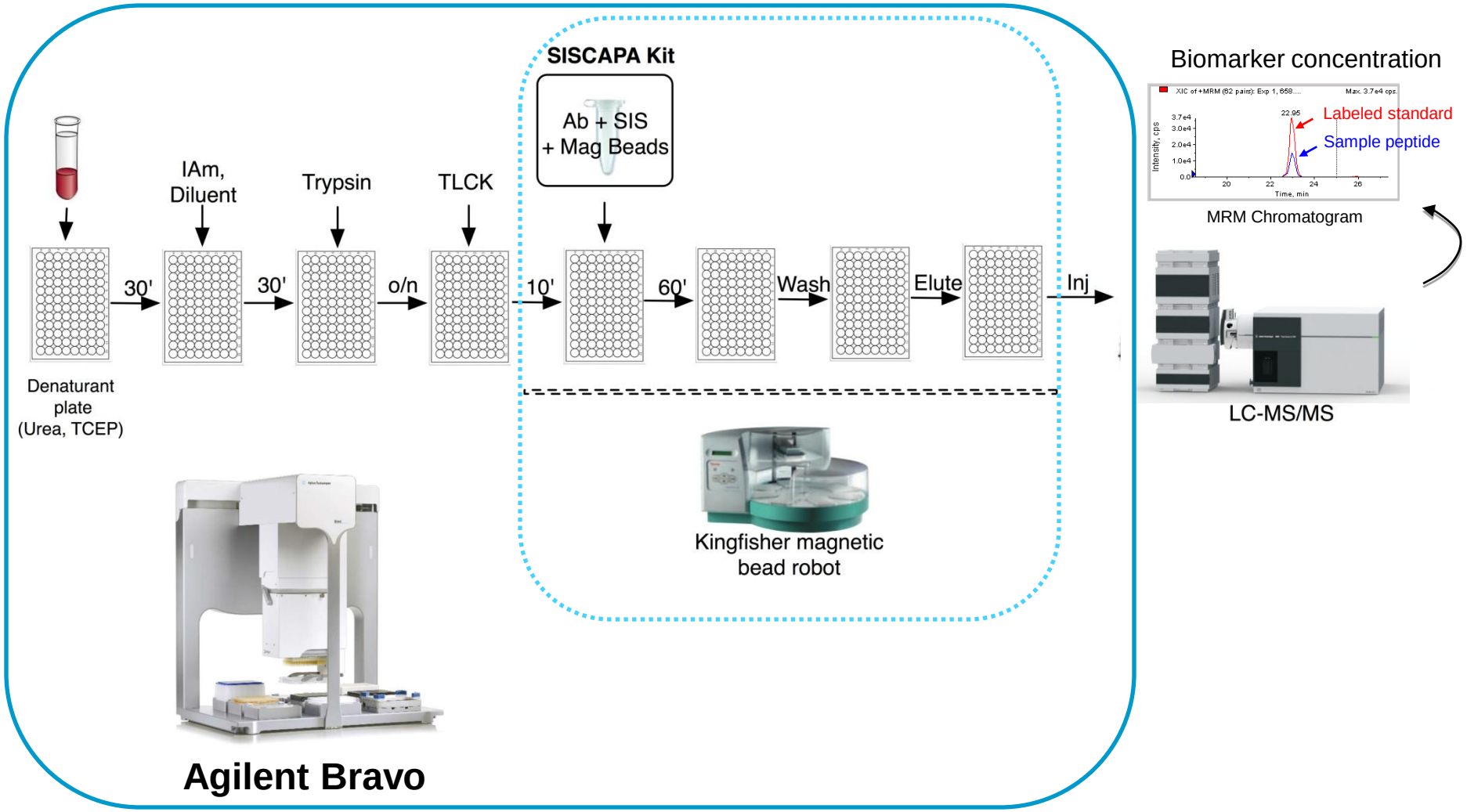
1000x larger digest input volume	+	Reduced ion suppression	+	Same LC-MS/MS	=	>1,000-fold increased MRM assay sensitivity

Two Key Components of SISCAPA Automation

1. Automation of sample processing steps to
   - Increase throughput to hundreds of samples per day
   - Improve assay precision (replacing hand pipetting etc)

2. Robust, high-throughput MS platform
   - Overcome robustness limitations of nanoflow chromatography
   - Reduce LC/MS cycle times from 30-45 min to 5 min/sample

Magnetic Bead Implementation of SISCAPA Assay Technology



Automated Sample Handling for Protein Biomarker Studies

Automation of a SISCAPA Magnetic Bead Workflow for Protein Biomarker Quantitation by Mass Spectrometry Using the Agilent Bravo Automated Liquid Handling Platform

Application Note

Authors

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Christine Miller
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Abstract

An automated protocol has been developed implementing SISCAPA immunoaffinity enrichment of biomarker peptides prior to quantitation by mass spectrometry. In this protocol, magnetic beads coated with anti-peptide antibodies bind specific target peptides from tryptic digests of samples such as plasma and serum, after which the beads are washed to remove unbound peptides, and the bound, purified peptides are eluted in small volumes for injection into an LC/MS/MS system. Internal standards (stable isotope-labeled versions of the same peptides) allow accurate quantitation. The Agilent Bravo implementation allows SISCAPA processing of 96 samples in less than 30 minutes.

Introduction

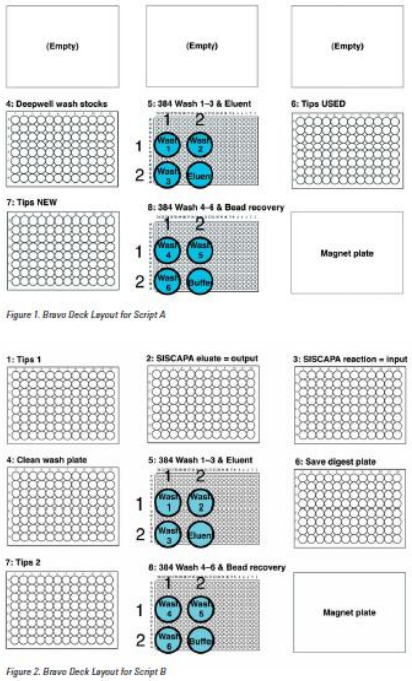
Mass spectrometry (MS) has become the benchmark technology for analysis of peptides based on three important advantages: 1) precise quantitation (particularly when used with internal standards); 2) near-absolute structural specificity (based on unique molecular fragmentation patterns); and 3) facile multiplexing of hundreds of measurements in a single analytical run. These advantages can be exploited in the analysis of "proteotypic" peptides, which occur uniquely in a single protein sequence, to reveal the concentrations of the parent proteins in proteolytic digests of complex samples such as patient plasma or serum. The principal limitation of the technology in research on protein biomarkers has been sensitivity for low-abundance proteins, which may be present at levels 10 orders of magnitude below the major plasma proteins such as albumin. This problem has been solved through specific capture of target peptides by purpose-designed anti-peptide antibodies (the SISCAPA technology), yielding >100,000-fold enrichment of target vs albumin peptides, and thus extending the sensitivity of quantitative mass spectrometry to match that of immunoassays. When implemented using established affinity carriers such as magnetic beads, large numbers of samples can be processed with efficiency and precision. As a result, the inherent quality advantages of MS measurement can be obtained over the full range of existing and candidate proteins biomarkers.

Agilent Technologies

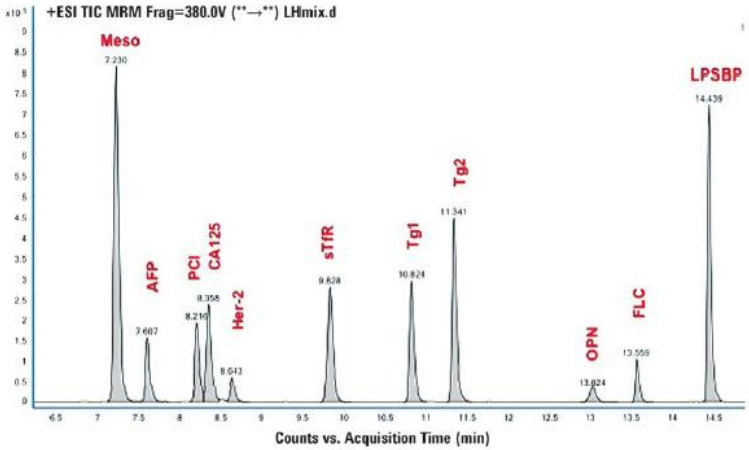
SISCAPA Protocol

The Bravo can be set up to perform

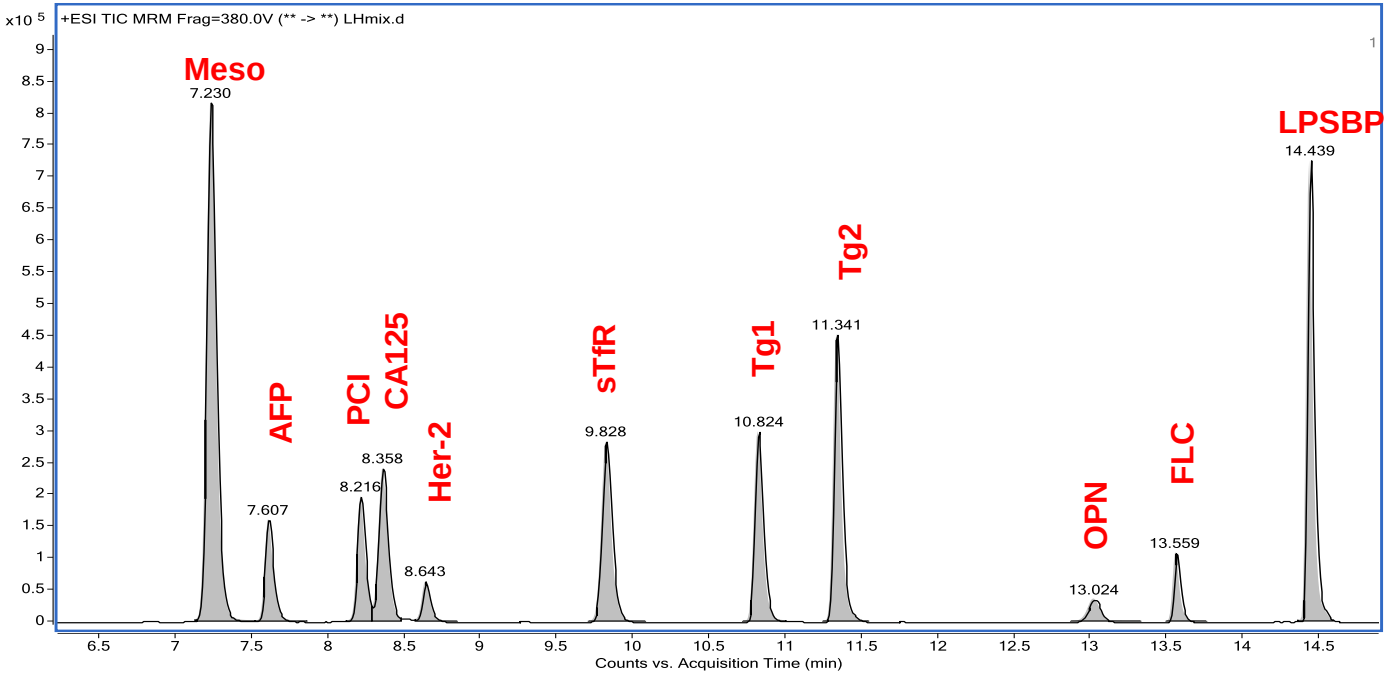
1. Enzymatic digestion, reduction and alkylation steps
2. The SISCAPA process in 2 scripts to fully utilize the magnetic bead-based antibody capture and to elute off the same beads



An 11-plex SISCAPA assay run on the Bravo and 6490 QQQ combination



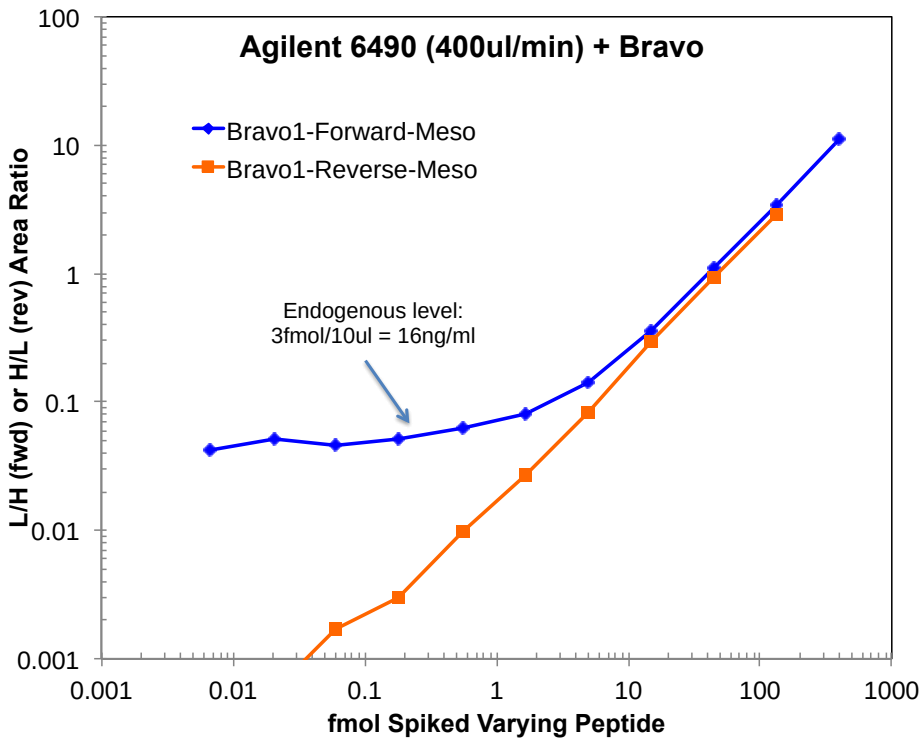
11-Plex SISCAPA Using iFunnel QQQ-MS at Standard Flow Rate



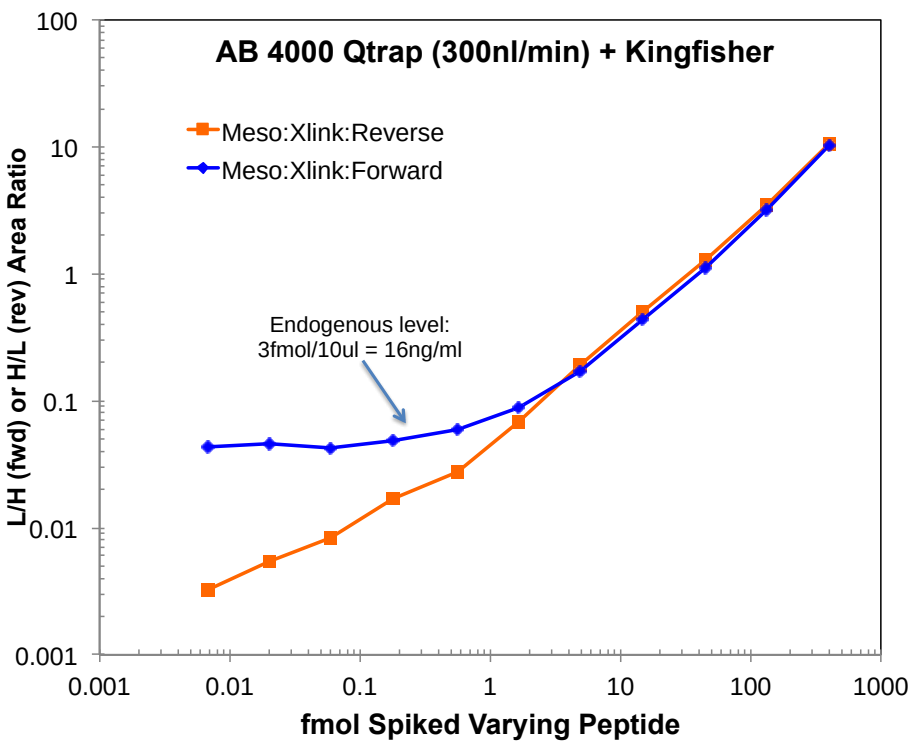
Column: ZORBAX Eclipse Plus RRHD C18, **2.1x150mm**, 1.8 um, 95Å
Injection volume: 10 µL containing 200 fmol each peptide
Flow rate: 0.4 mL/min
MS: 6490 QQQ with iFunnel technology
MS Mode: Dynamic MRM

Comparison of Standard Flow Ion Funnel QQQ-MS to Nanoflow QQQ-MS

Mesothelin forward and reverse curves (log/log)



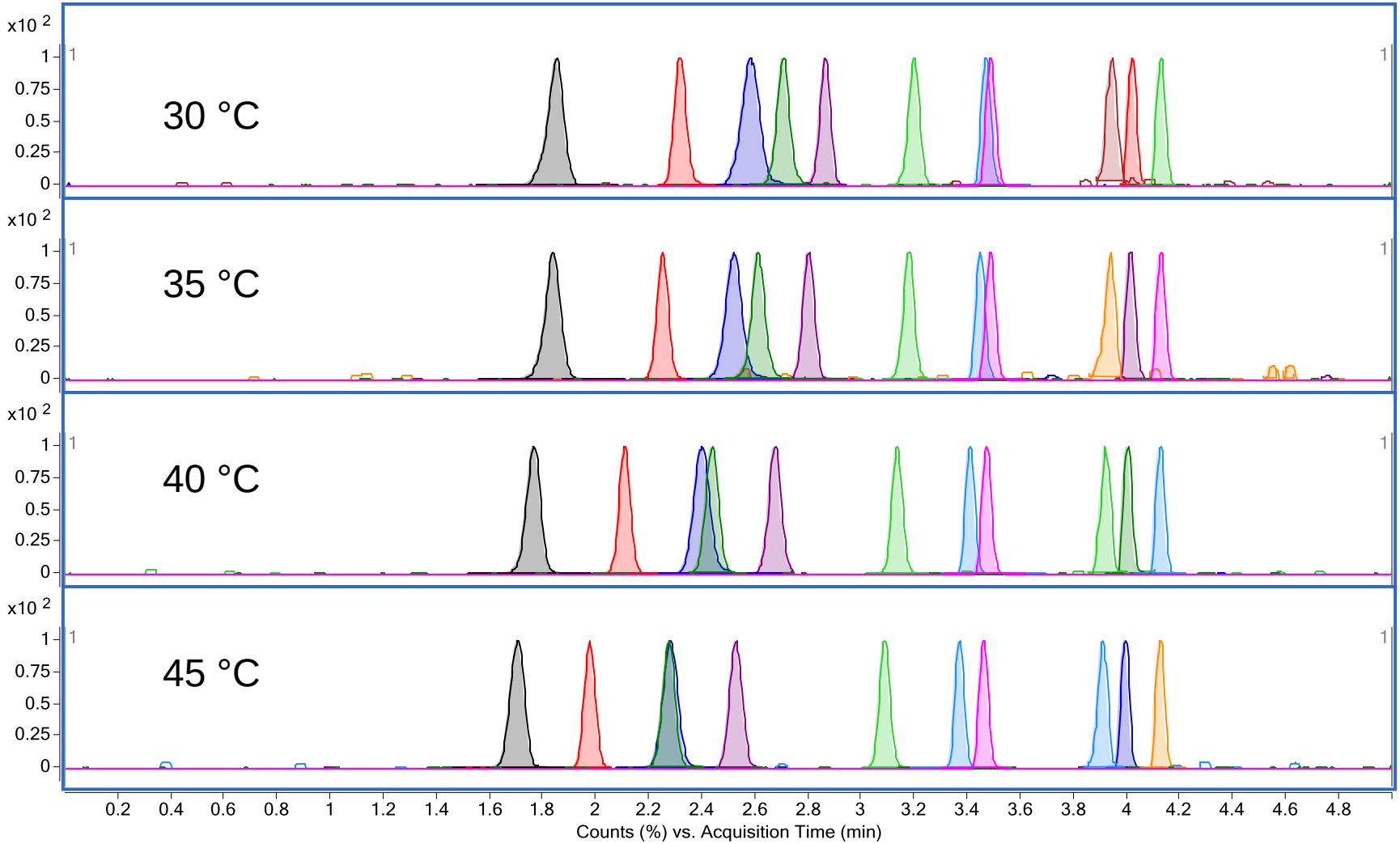
Standard flow ion funnel QQQ-MS



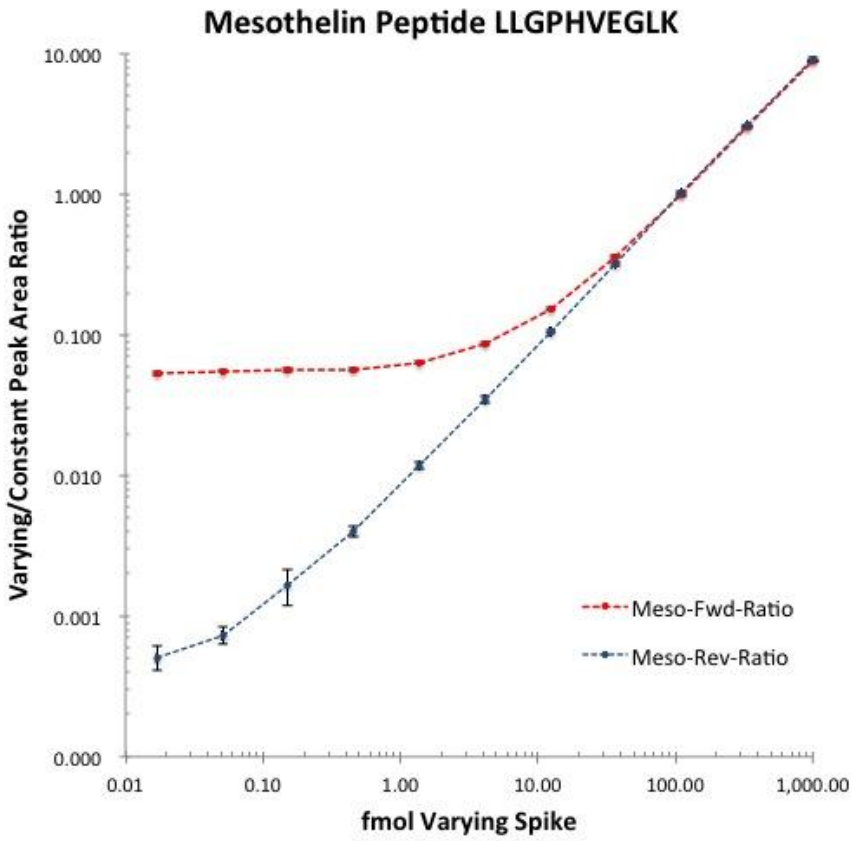
Nanoflow QQQ-MS

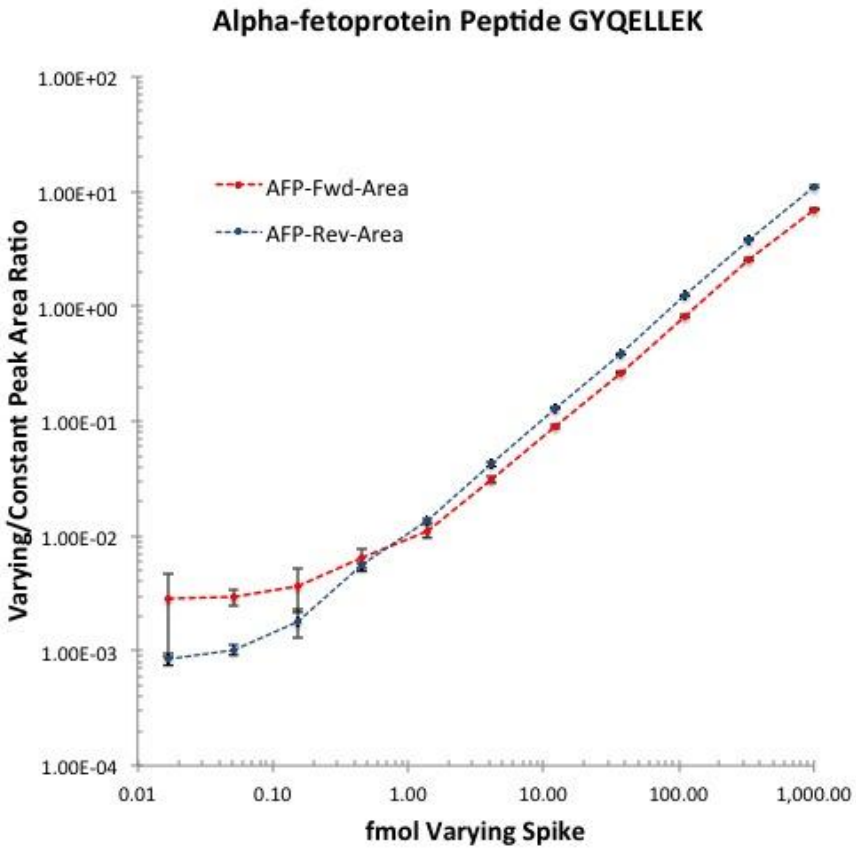
Forward curve: IS=heavy, standard addition above endogenous level with light
Reverse curve: IS=light, heavy peptide spiked in at known levels (no endogenous of heavy)

Effect of Column Temperature on Separation: Eclipse Plus EC-C18, 1.8 µm, 2.1 x 50 mm Column



Standard Flow Results: Rapid LC/MS Method Using Bravo-prepared SISCAPA Samples





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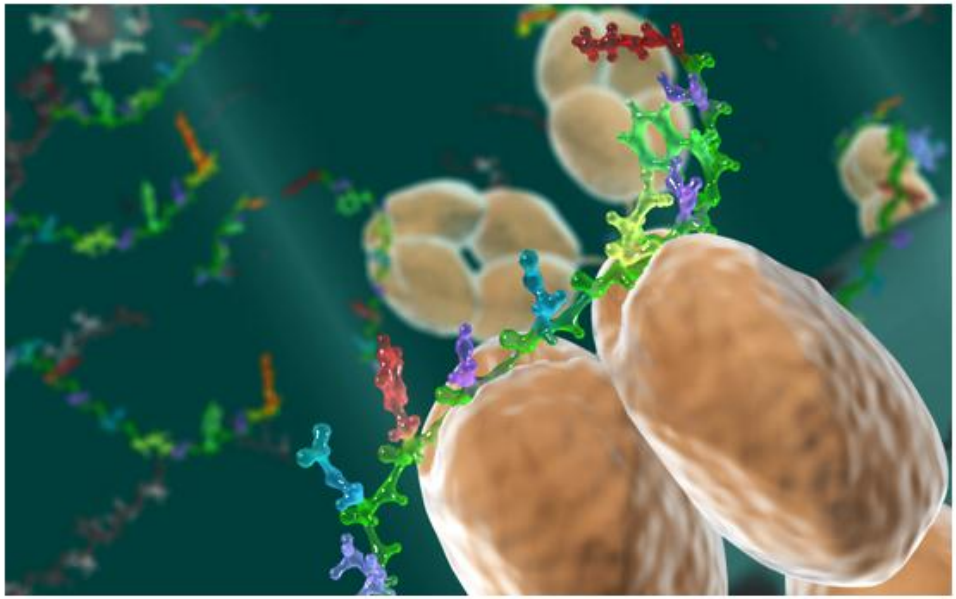
SISCAPA® technology – the smart shortcut to sensitive quantitation of protein biomarkers and targets.

SISCAPA assays combine the precision of MRM mass spectrometry with the power of affinity enrichment to deliver a superior alternative to conventional immunoassays for protein quantitation.

The **SISCAPA** workflow can be automated is several formats, and exploits familiar LC-MS/MS platforms widely used for drug and metabolite quantitation.



In comparison to conventional sandwich immunoassays, **SISCAPA** provides a range of practical advantages:

Done | Internet | 100%

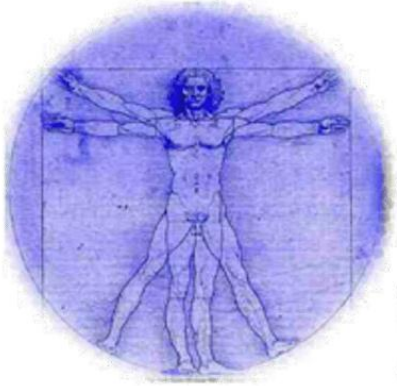
What's Coming? SRM Atlas

Agilent's collaboration with the Institute for Systems Biology will lead to the incorporation of the SRM Atlas into a product.

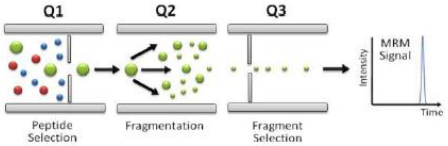
The Atlas is a look up facility for MRM transitions to form QQQ acquisition methods

The Atlas includes complete QTOF MS/MS spectra of chosen peptides for each protein

Human SRMAtlas



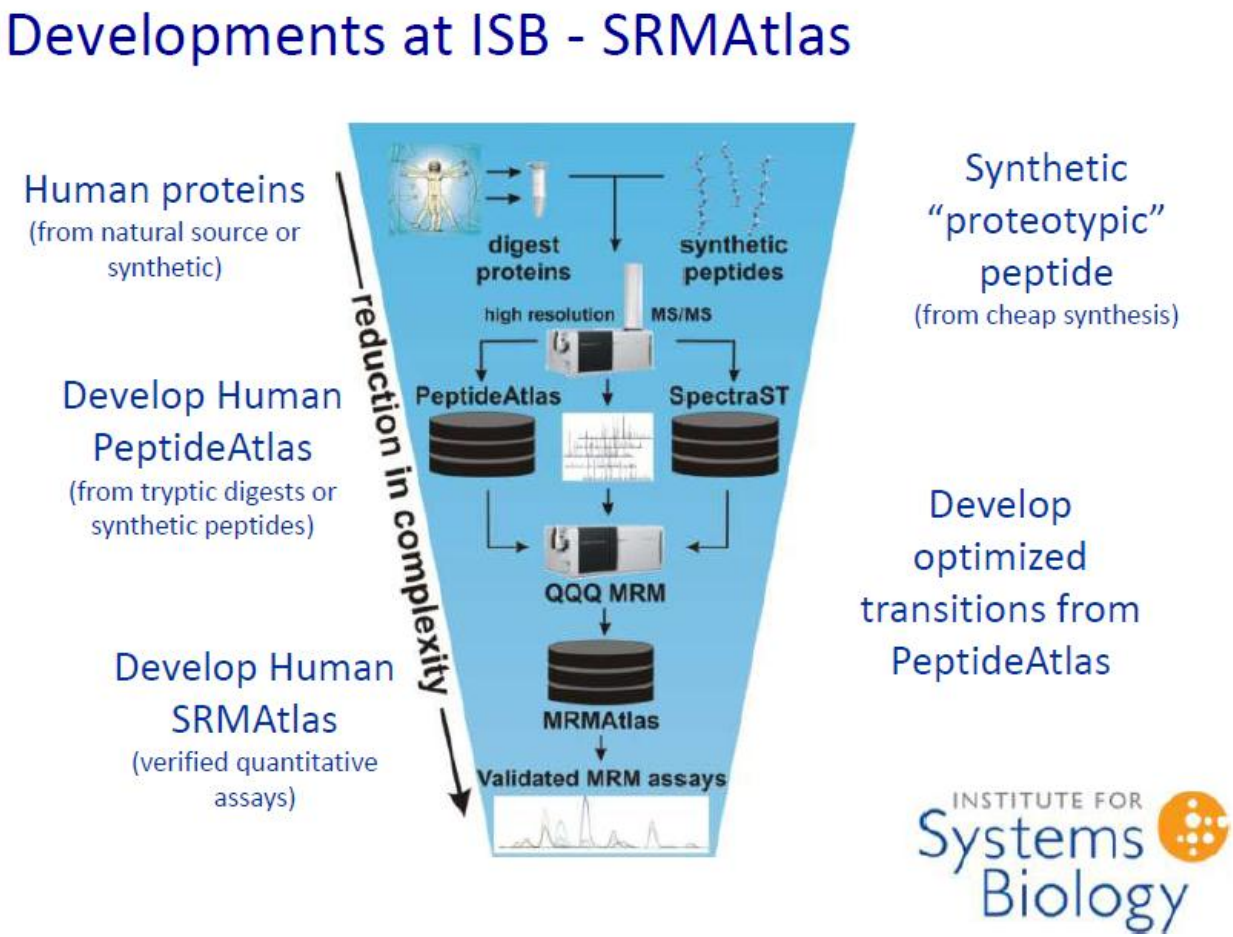
20,333 proteins (20,277 2010 version)
32,562 proteins incl. isoforms
658,684 tryptic peptides (any length)
480,284 distinct peptides (7-30aa)



INSTITUTE FOR Systems Biology

SRM Atlas - Background

Agilent is pleased to be working with ISB to be making a commercially available database by the end of 2011.



Acknowledgements

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Joe Slagel

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Swiss Federal Institute of Technology Zurich

Ruedi Aebersold
Christine Carapito
Paola Picotti

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Leroy Hood

Duchy of Luxembourg

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Conclusions

•The iFunnel system shows at least a 5x improvement in sensitivity compared to a standard QQQ

•The QQQ + iFunnel system demonstrated robust performance for peptide quantitation in plasma digests using standard flow LC/MS

•SISCAPA enrichment enhances sensitivity and reduces the complexity of the samples

•An integrated, automated, high-throughput SISCAPA assay is now feasible using Agilent components

Acknowledgements

Plasma Proteome Inst.

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Christoph H. Borchers

Agilent Technologies

Christine Miller

Yanan Yang

Moritz Wagner