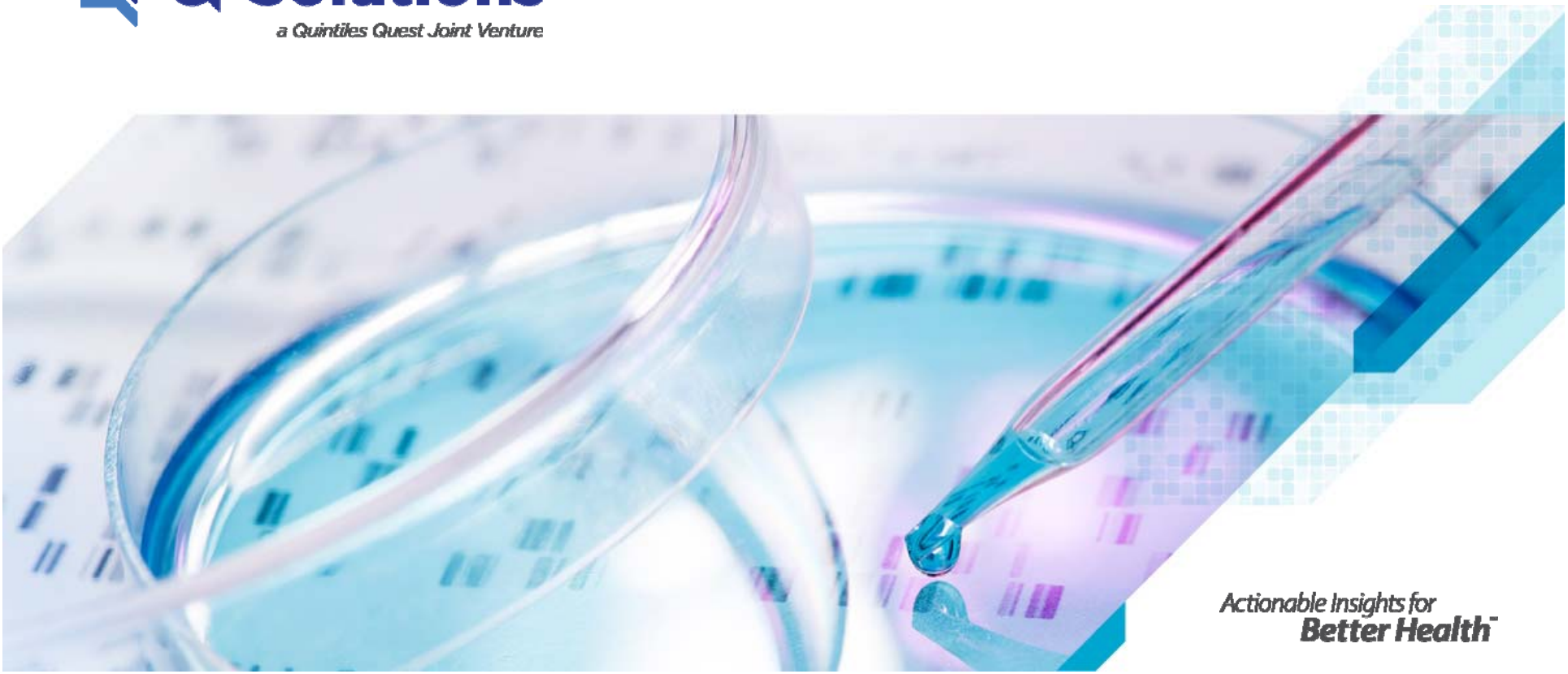
Q² Solutions
a Quintiles Quest Joint Venture



What can LC-MS offer beyond LBA approaches in the field of large molecules bioanalytics?

Global BioPharma Summit, ThermoFisher, Basel, September 12, 2017

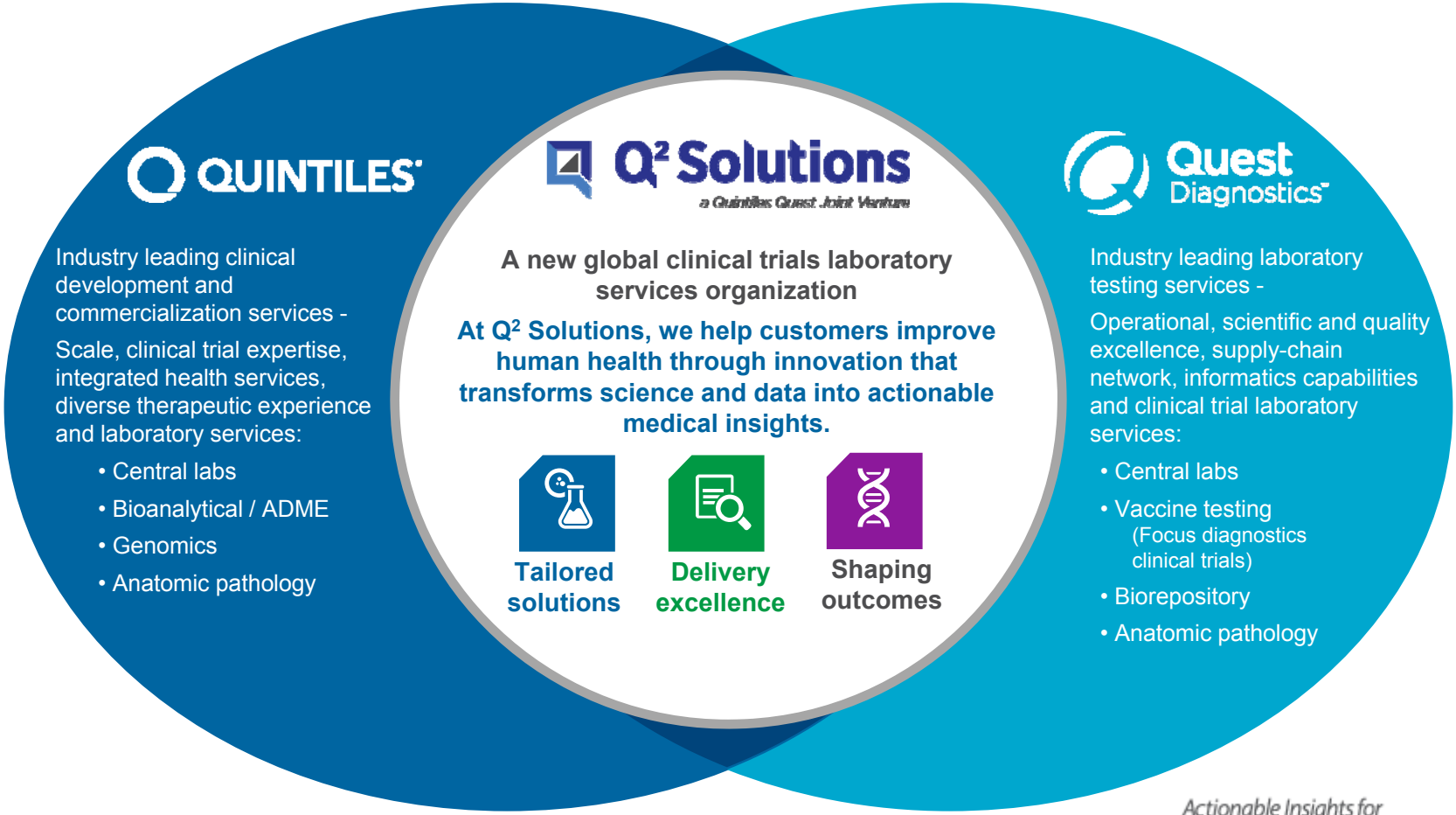
Benno Ingelse, Scientific Director / business development Europe
benno.Ingelse@q2labsolutions.com

Introducing

Q² Solutions - a new global clinical trials laboratory services joint venture formed to provide greater innovation, quality and value for our customers

Q² Solutions
a Quintiles Quest Joint Venture

This joint venture was borne out of a shared commitment to quality, customer service and – above all – helping bring new treatments to patients



Actionable Insights for Better Health™

Our Scientific Heritage and Progression

Advion QUINTILES Q² Solutions a Quintiles Quest Joint Venture

1985
Beginnings of LC/MS
- Ion-Spray LC/MS invented in Jack Henion's Cornell University lab

1993
LC/MS Lab, Ithaca, NY
- Jack Henion and Tom Kurz establish Advion bioanalytical lab in 1993; Founders remain engaged and passionate with the laboratory today

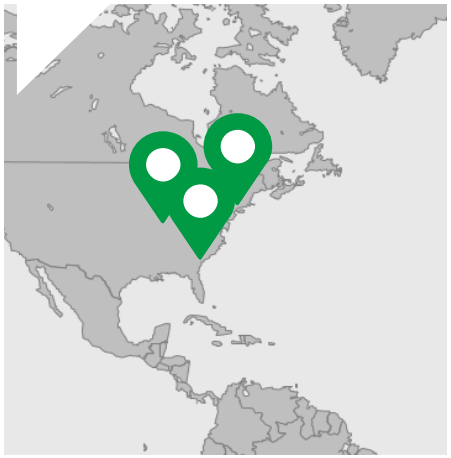
2007
Immunoassay Lab, Manassas, VA/Marietta, GA
- ELISA, MSD, Cell Culture Lab
- PK/TK, Immunogenicity, Biomarker, Vaccines

2008
Macromolecule/Biomarkers LC/MS, Ithaca, NY
- Proteins, peptides, antibodies, lipids, small molecule biomarkers
- Immunoaffinity chromatography, nano LC, accurate mass bioanalytical

2011
ADME Lab, Indianapolis, IN
- Center of excellence for in vitro ADME and metabolite ID
- Former Eli Lilly ADME group

2015
Quintiles and Quest form Joint Venture
- Q² Solutions brings together bioanalytical, ADME, central, and genomic labs services

Q^2 Solutions Bioanalytical and ADME Locations



Indianapolis, IN, USA

- Home to our in vitro ADME, discovery bioanalytical, and metabolite identification capabilities
- Scientists and equipment acquired from Eli Lilly
- In vitro ADME screening, discovery, and definitive ADME assays

Ithaca, NY, USA

- GLP compliant bioanalytical liquid chromatography mass spectrometry (LCMS) laboratory
- LCMS biomarker assays in support of PKPD studies
- TK and PK studies
- Dried Blood Spot R&D and testing
- Bioanalytical method development, validation and sample analysis

Ithaca Lab Facility

65
LC/MS systems

Marietta, GA, USA

- Home to our Immunoassay Bioanalytical (PK and immunogenicity) capabilities
- GLP-compliant & discovery bioanalytical/ligand binding assays – biologics and biosimilars
- Neutralizing Immunogenicity cell-based assays

Why do LC-MS for proteins

Specialized Tools

Case studies

Conclusions

Why do LC-MS at all for Protein Bioanalysis?

- Higher accuracy & Precision (ISTD)
- Multiplexing
- No immunological reagents required
- Signature peptide vs. binding epitope

- Higher operational costs
- Lower sample throughput
- Lower sensitivity
- Loss of 3D structure (bottom-up)

Why do LC-MS at all for Protein Bioanalysis?

- Mass spectrometry offers a structural handle on the analyte at the elemental level (allows tracking of specific modifications)
- Presence of drug binding endogenous components in incurred samples can complicate analysis
- (Nanoflow) LC offers an additional means to obtain selectivity
- LBA is very much the dominant technique for biologics bioanalysis … and still for good reason

Why Measure Proteins and Peptides by LC/MS?

- Difficulty with *selectivity* of LBA method
  - Example, DX-2930 - developed hybrid IA-LC/MS method for mAb in parallel with struggling LBA MD
- Difficulty with *sensitivity* of LBA method
  - Example - developed hybrid IA-LC/MS method using nanoelectrospray to improve sensitivity ~60X over LBA method for mAb therapeutic
- Better understand biotransformation and how that impacts bioanalysis (LBA or LC/MS)
  - Example - PEGylated peptide, with pharmacologically important C- and N-termini
    - Use bottom-up approach and measure both terminal peptides in preclinical studies
- Multiplexing advantages
  - Several proprietary examples of multiple analyte biomarker assays or co-IP workflows

What FDA Says Regarding Bioanalysis of Biologics

FDA BMV Draft Guidance (2013)

"When possible, the LBA should be compared with a validated reference method (such as LC-MS) using incurred samples and predetermined criteria to assess the accuracy of the LBA method."

Very similar to FDA 2001 BMV Guidance:

"When possible, the LBA should be compared with a validated reference method (such as LC-MS) using incurred samples and predetermined criteria to assess the accuracy of the LBA method."

Interestingly, no other global regulatory authority has taken this position

Why do LC-MS for proteins

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Hybrid IA-LCMS

Sensitivity need drives chromatographic flow rate drives sample preparation

Immunopurification techniques coupled with Mass Spectrometry

- Offline bead-based immunoprecipitation (All Processing Using Hamilton Microlab Star)
  - Magnetic Streptavidin Beads
  - Biotinylated antibody
  - Incubate and elute with low pH
  - Digest or assay intact
- Online immunoaffinity
  - Antibody immobilized to protein G slurry
  - Load with neutral aqueous, elute with low aqueous pH
  - Target signature peptide in bottom-up workflow – “Sequential IP”
    - Orthogonal selectivity to extraction and C18 separation
  - Target intact analyte (no digestion)

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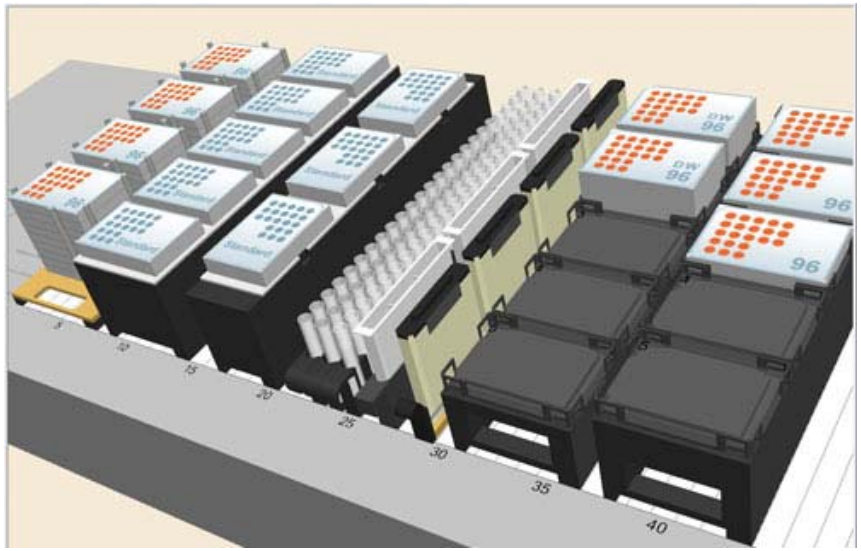
Jones BR and Schultz GA (2016), Adaptation of Hybrid Immunoaffinity LC-MS Methods for Protein Bioanalysis in a Contract Research Organization. *Bioanalysis*. 8(15): 1545–1549.

Hamilton Microlab STAR

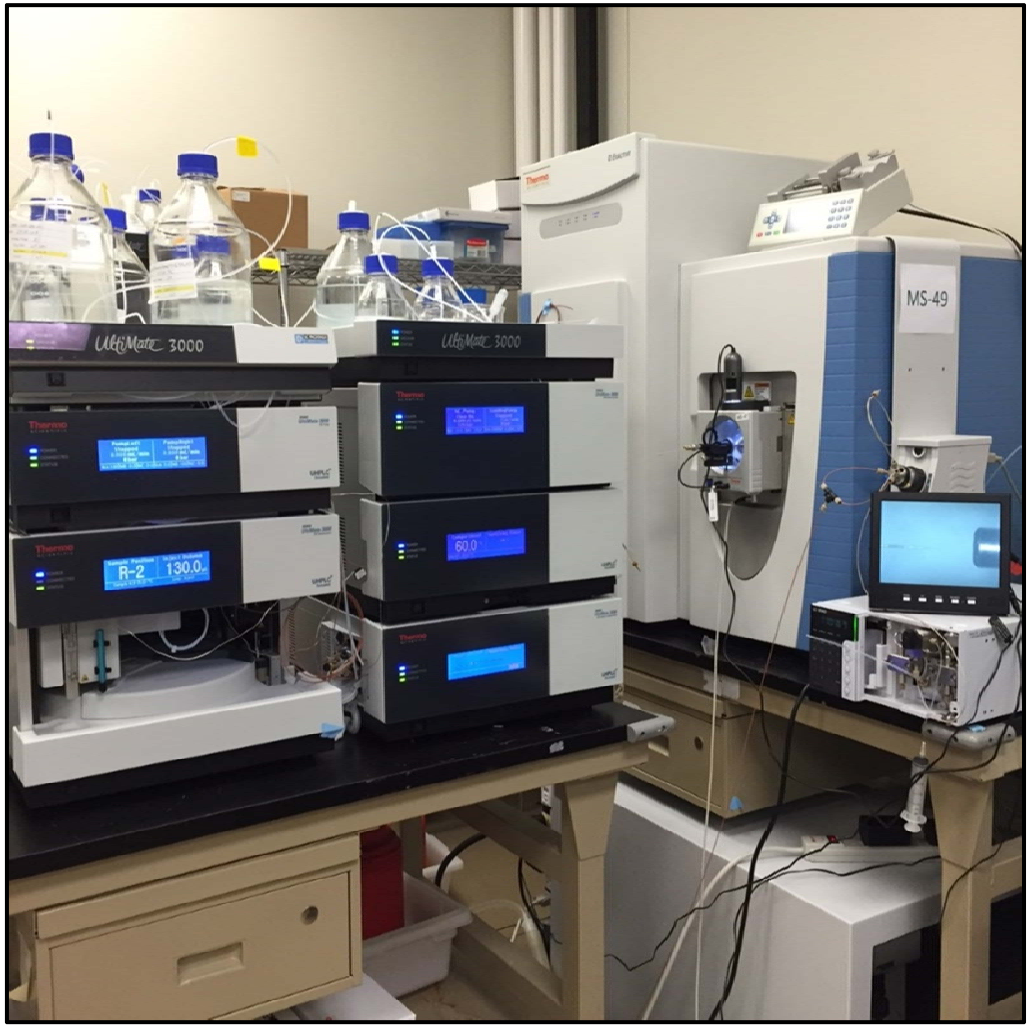
S.T.A.R. – Sequential Transfer Automation Robot

- **Advanced Liquid Handling:**
  - > Monitored Air Displacement
  - > Liquid Level Detection (cLLD / pLLD)
- **Integrated Third-Party Devices:**
  - > Tele-shakers
  - > Inheco heater/cooler modules
  - > Magnetic bead plates
- **“Walk Away” Methods:**
  - > Error Handling
  - > Network Capability

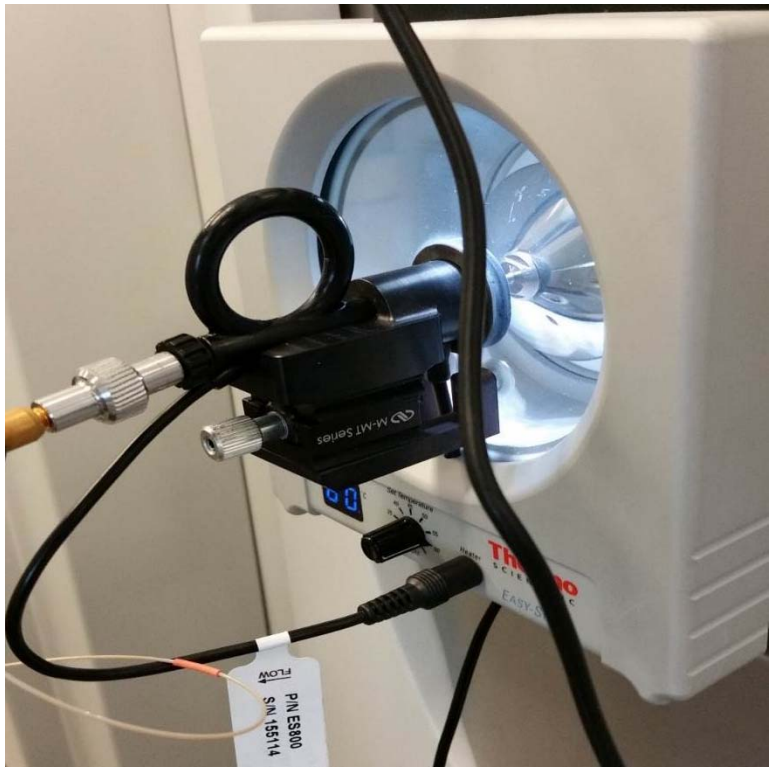


Dionex RSLC Ultimate 3000 Nano LC Systems

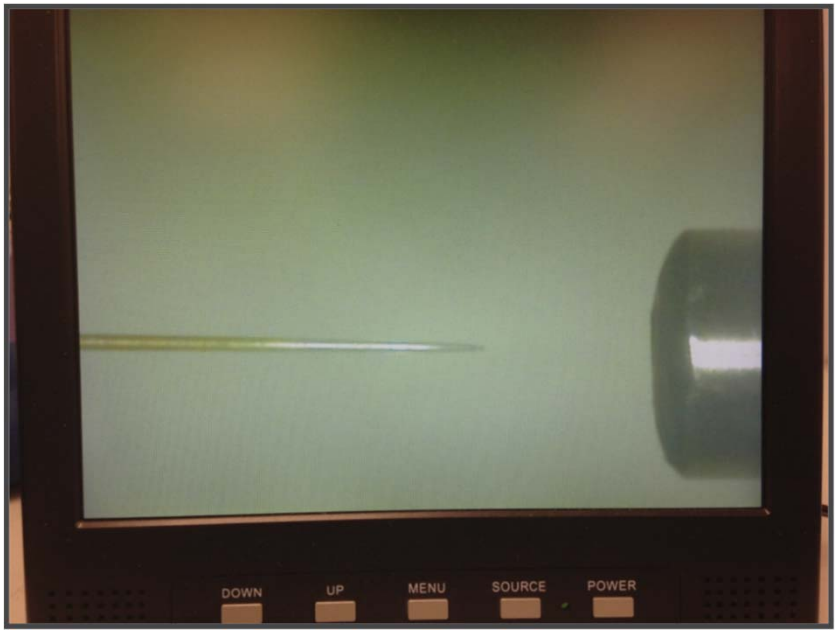
- Allows for multi-dimensional liquid chromatography
- Low flow rates
- Low system volumes
- Large injection volume possible, use multi-dimensionality to step-down flow rates and column sizes



Thermo EASY-Spray Source

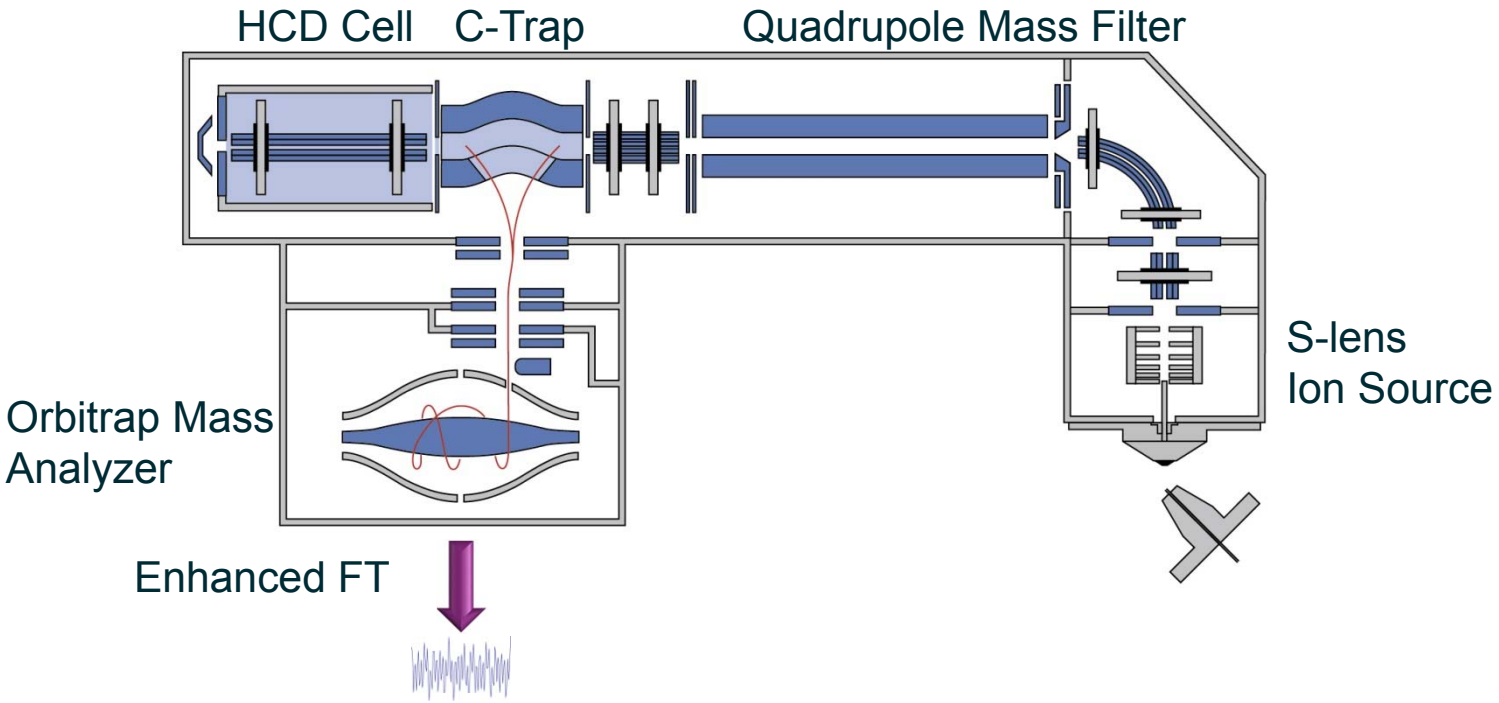


- Integrated column/Sprayer
- No additional software
- Closed source
- Robust
- Easy to use



High Resolution Accurate Mass Spectrometry

Thermo QExactive+



Sturm RM, Jones BR, Mulvana DE, Lowes S (2016), "HRMS Using a Q-Exactive Series Mass Spectrometer for Regulated Quantitative Bioanalysis: How, When, & Why to Implement." *Bioanalysis*. 8(16): 2343-2356.

Why do LC-MS for proteins

Specialized Tools

Case studies

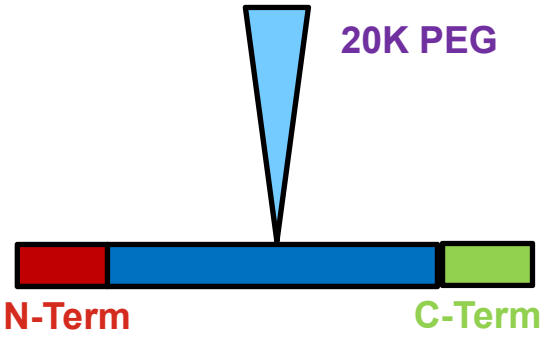
Conclusions

Peptide Bioanalysis

Bottom-up vs Intact
HRMS vs QqQ
Protein IP vs Conventional Extraction
Conventional Chromatography vs Nano-ESI

Triple Quadrupole for Peptide Therapeutic – Bottom-Up

Validated Assays to Support Preclinical Tox Studies



- ~6000 Da PEGylated peptide therapeutic
- Mid-peptide 20Kda PEG
  - PEG heterogeneity
- Amino- and carboxy-terminal domains both important for pharmacology
- In-vitro metabolism observed at both the N and C-terminal domains

<u>Two methods: C-term and N-term</u>

- Protein precipitation, trypsin digestion
- Conventional chromatography, HESI ionization, SRM Quant
- Validated to full BMV guidance

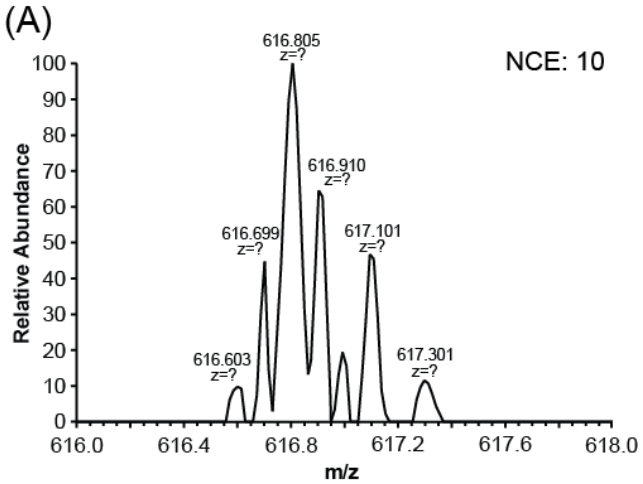
Results

- N-terminal AUC was **~2x lower** than the C-terminal, confirming greater metabolism at the N-terminus.

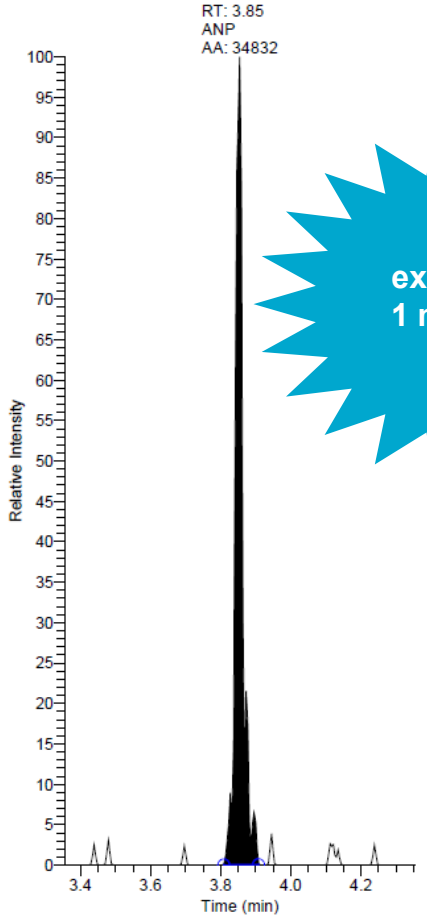
Cyclic Peptide Biomarker by HRMS - Intact

HRMS/MS scan mode, quantify on molecular ion

Atrial Natriuretic Peptide



immunoprecipitation and nanoelectrospray



100 fg/mL extracted from 1 mL synthetic matrix

*Ciccimaro E, Ranasinghe A, D'Arienzo C, Xu C, Onorato J, Drexler DM, Josephs JL, Poss M and Olah T (2014) Strategy to improve the quantitative LC-MS analysis of molecular ions resistant to gas-phase collision induced dissociation: application to disulfide-rich cyclic peptides. *Analytical chemistry* **86**:11523-11527.

Sequential Protein and Peptide Immunoaffinity Capture for Mass Spectrometry-Based Quantification of Total Human β-Nerve Growth Factor

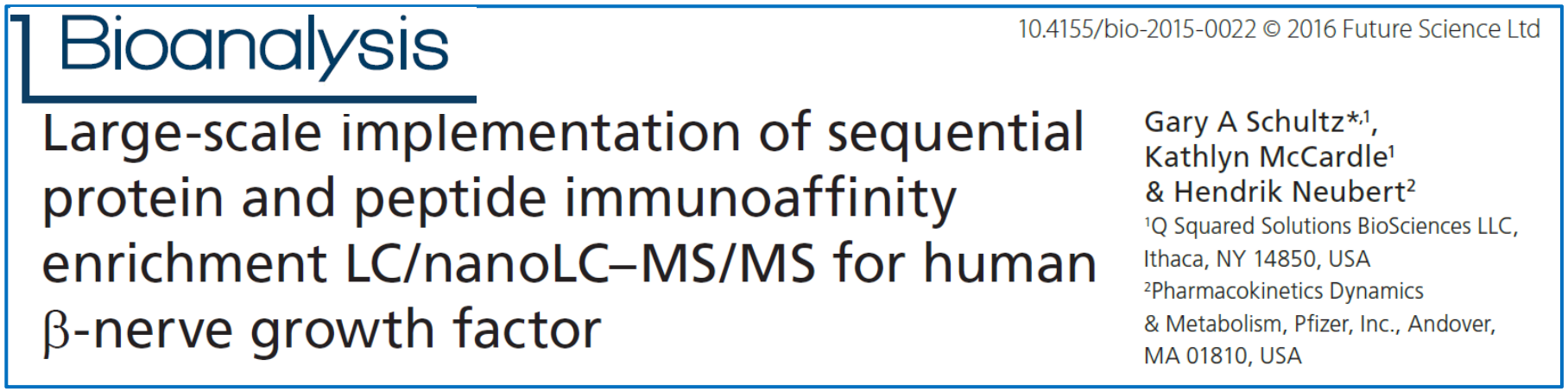
Bottom-up
Sequential IP (protein & peptide)
HRMS
Nano-ESI

Low-Abundance Protein Biomarker by IA-LCMS

Validated Assay to Support Clinical Studies

β-Nerve Growth Factor

- Important role in the modulation of pain through its interaction with p75 and TrkA receptors on neurons.
- β-NGF is a 13.5 kDa protein (dimer) that is endogenous in human serum.
- Therapeutic target engagement information needed



Bioanalysis

10.4155/bio-2015-0022 © 2016 Future Science Ltd

Large-scale implementation of sequential protein and peptide immunoaffinity enrichment LC/nanoLC–MS/MS for human β-nerve growth factor

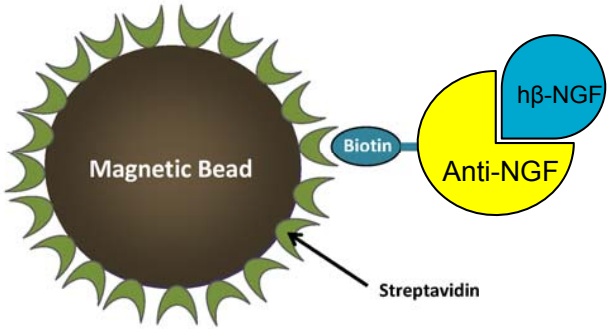
Gary A Schultz*,[1], Kathlyn McCardle[1] & Hendrik Neubert[2]

[1]Q Squared Solutions BioSciences LLC, Ithaca, NY 14850, USA

[2]Pharmacokinetics Dynamics & Metabolism, Pfizer, Inc., Andover, MA 01810, USA

NGF Extraction Procedure Methods

Incubate with biotinylated Ab, capture with streptavidin coated magnetic beads



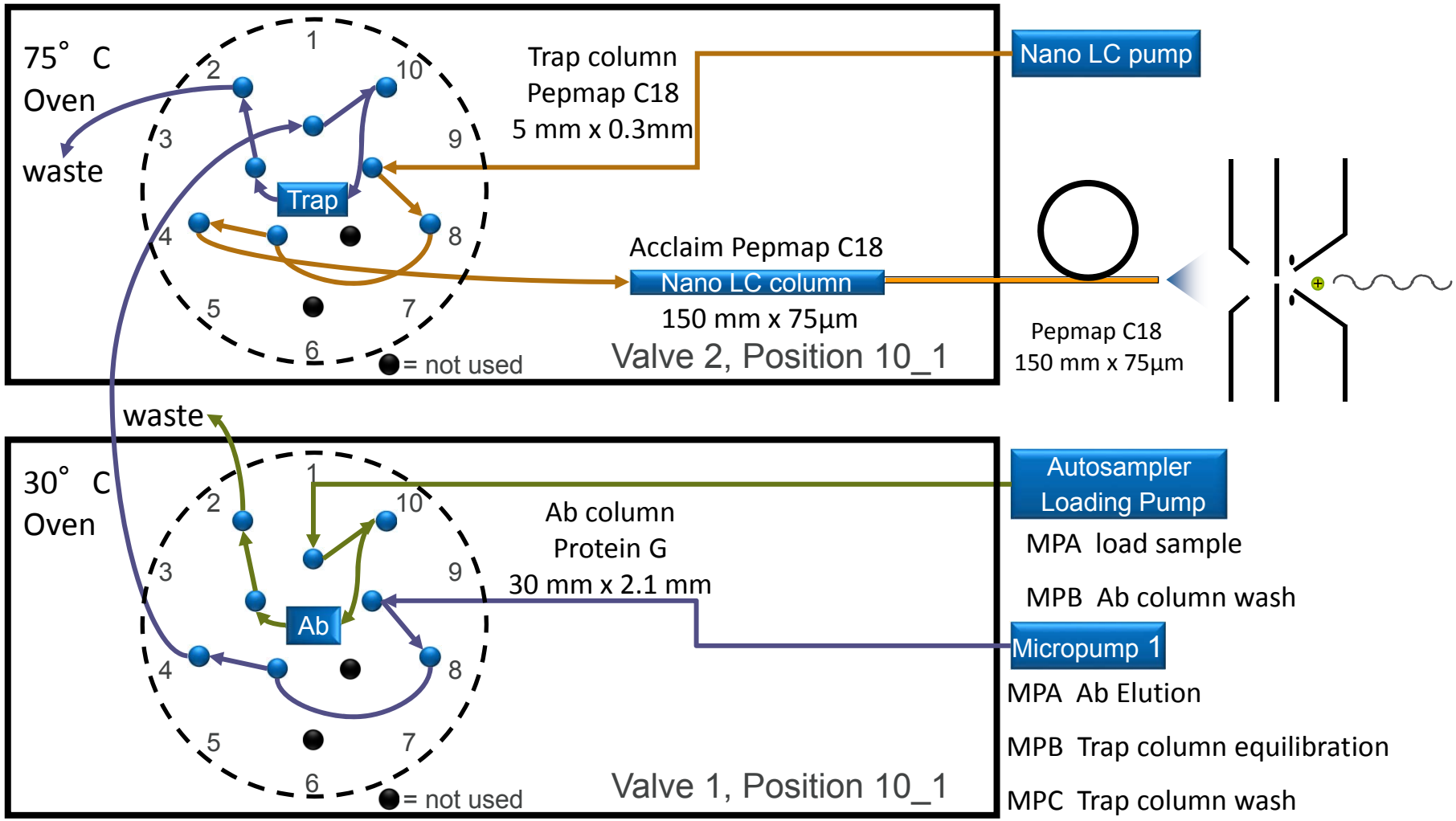
Elute with acid

Reduction, alkylation, and enzymatic digestion to the signature peptide and SIL peptide

SSSHPIFHR GEFSVCDSVS VWVGDKTTAT
DIKGKEVMVL GEVNINNSVF KQYFFETKCR
DPNPVDSGCR GIDSKHWNSY CTTTHTFVKA
LTMDGKQAAW RFIRIDTACV CVLSRKAVRR A

Extended Sequence SIL-Internal Standard:
AWRFIR-IDTACVC[$V^{15}N^{13}C_5$][$L^{15}N^{13}C_6$]SR-KAVRRA

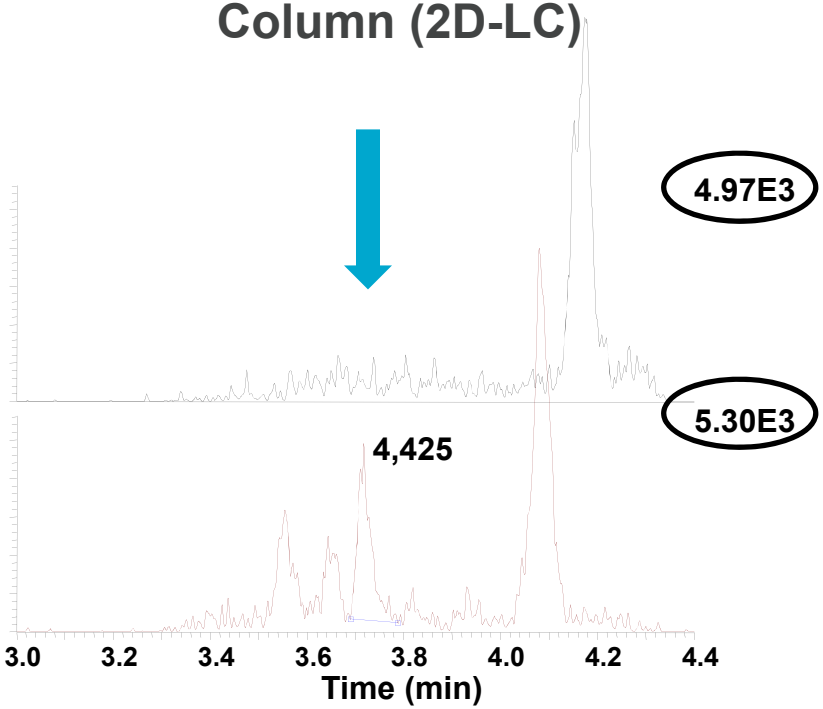
Immunoaffinity-LC/nanoLC-MS/MS



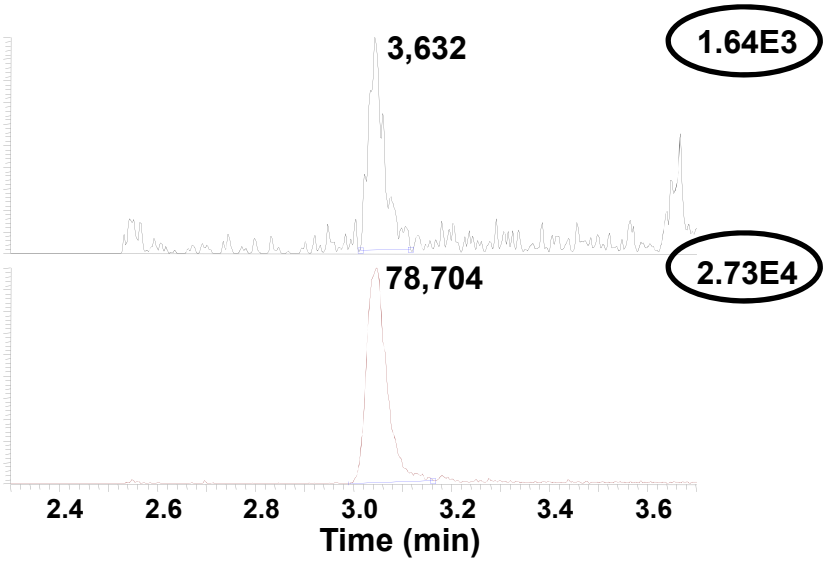
Value of Sequential IP

7 pg/mL LLOQ NGF Standard

Without Anti-peptide Antibody Column (2D-LC)



With Anti-peptide Antibody Column (3D-LC)

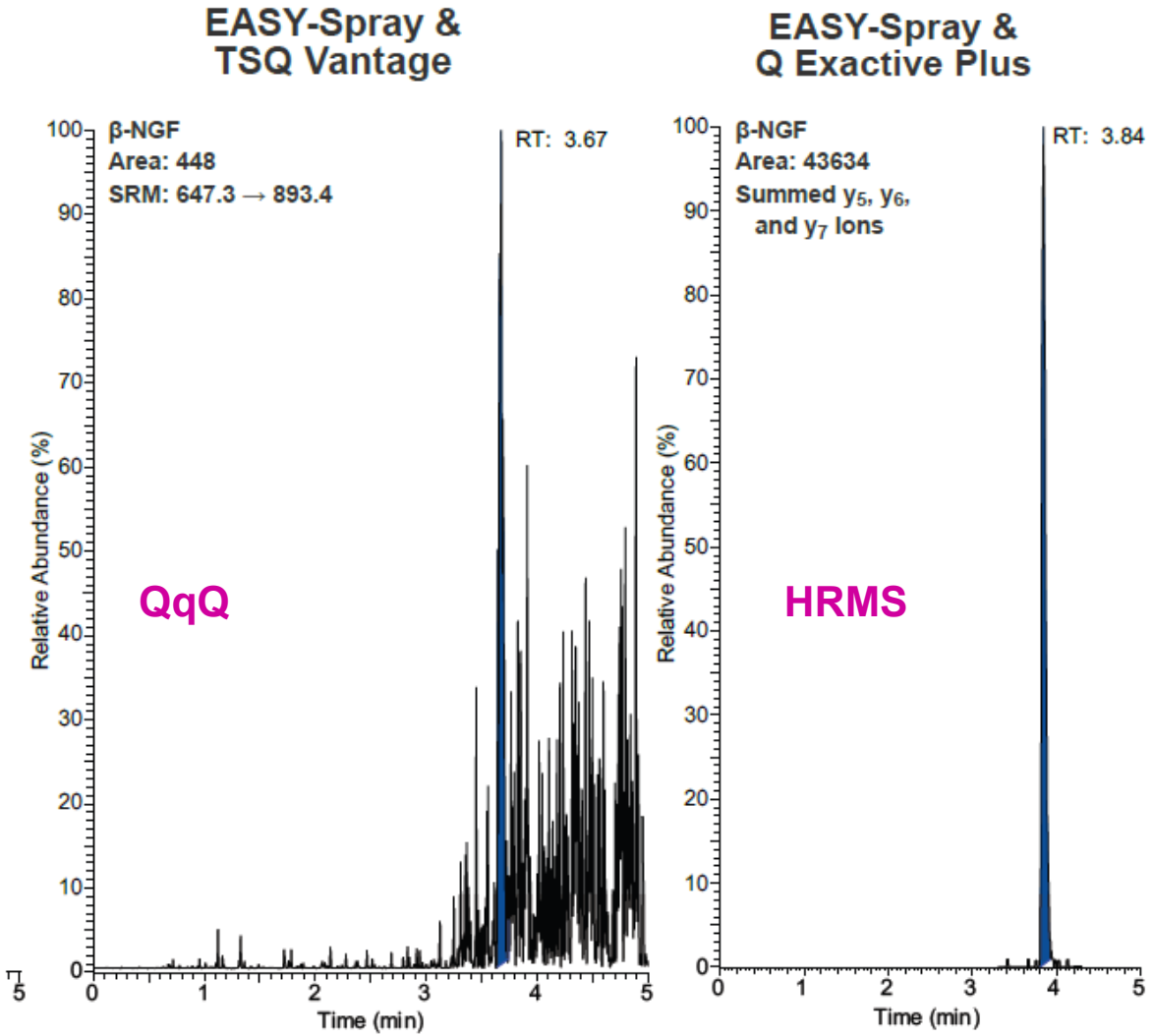


18-fold improvement

High Resolution Accurate Mass Spectrometry

Selectivity Advantage

- The orthogonal selectivity offered by accurate mass measurements using a Q Exactive Plus MS enabled the EASY-Spray ion source to accurately quantify β-NGF at a 7 pg/mL level



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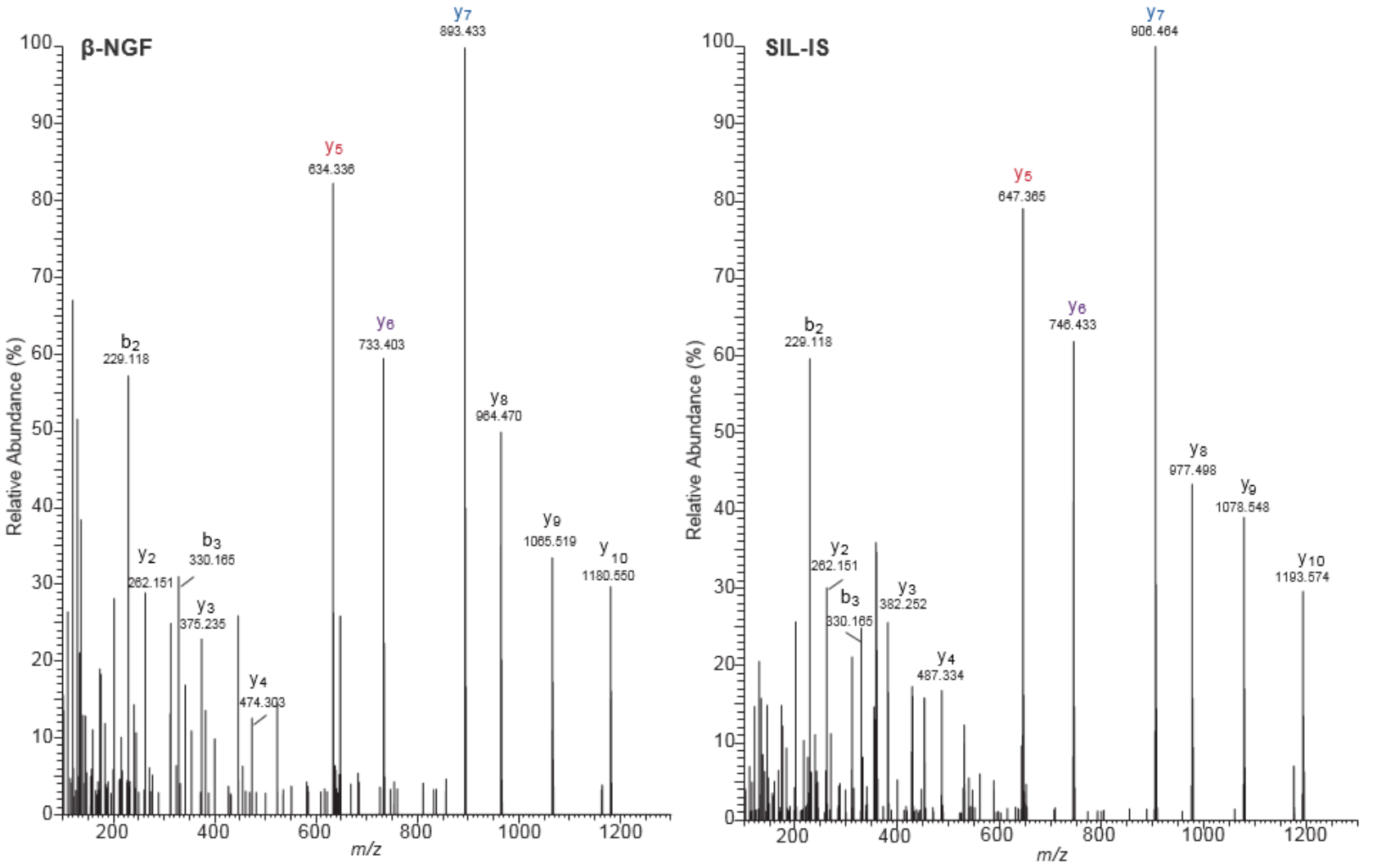
Representative HCD (MS/MS) Spectra Q Exactive Plus MS



Three product ions (y_5, y_6, and y_7) were selected to generate an XIC for β-NGF and two product ions (y_5 and y_7) to generate an XIC for SIL-IS using EASY-Spray ion source and Q Exactive Plus MS.

Q² Solutions
a Customer Center Joint Venture

Representative accuracy and precision data for Q-Exactive Plus (EASY-Spray source) total β-NGF assay

QC (pg/mL)	LLOQ (7.000)	Low (21.00)	END (41.55)	Low (241.6)	High (1042)	ULOQ (1280)
	Surrogate			Serum		Surrogate
Replicate 1	7.193	25.69	37.68	257.0	1048	947.9
Replicate 2	6.320	18.98	43.01	250.3	905.4	1163
Replicate 3	5.857	23.73	38.52	240.3	1063	1189
Replicate 4	7.770	21.86	40.27	243.1	1071	1038
Replicate 5	5.042	20.70	46.24	255.5	1089	1273
Replicate 6	5.534	16.34	43.58	242.9	1134	1280
Mean	6.286	21.22	41.55	248.2	1052	1148
CV (%)	16.4	15.7	7.9	2.9	7.4	11.5
RE (%)	10.2	-1.0	NA	-2.7	-1.0	10.3
n	6	6	6	6	6	6

CV (%) = (SD/Mean) × 100
RE (%) = [(Mean - Nominal)/Nominal] × 100
END: Endogenous
NA: Not applicable
ULOQ: Upper limit of quantitation

Formic Acid Digestion of a Antibody Biotherapeutic. Can this be Quantitative?

Bottom-up
QqQ
Protein IP
Nano-ESI

CPSA 2015

Bottom-Up Quantitative Measurement of an Antibody Therapeutic in Human Plasma by LC/MS/MS using Formic Acid Digestion

Lian Shan[1], Robert M. Sturm[1], Raymond F. Biondolillo[1], John E. Buckholz[1], Barry R. Jones[1], Ryan Faucette[2], Daniel J. Sexton[2]
[1]Q[2] Solutions, Ithaca, NY; [2]Dyax Corp., Burlington, MA

DX2930 Background

- Large Molecule mAb Biotherapeutic, DX2930
  - Difficulty with human plasma LB Assay
- Hybrid IA-LC/MS method feasibility initiated.
  - Bottom-up digestion approach.
- No suitably selective tryptic signature peptides.
  - Required alternative digestion.
- *In silico* sequence homology searches utilizing alternative enzymatic digestion reagents did not provide unique peptides
- Putative unique peptides identified from *in-silico* formic acid chemical digestion (cleavage at aspartic acid residues)

Assay Details

Extraction *(Hamilton Microlab STAR)*

- Bead-based immunocapture of 100 µL plasma with Ab
- Elute at low pH, neutralize
- Add denaturant (Guanidine-HCl) and internal standard (winged SIL)
- Reduce (TCEP)
- Add formic acid (~2%)
- Incubate overnight at 95ºC

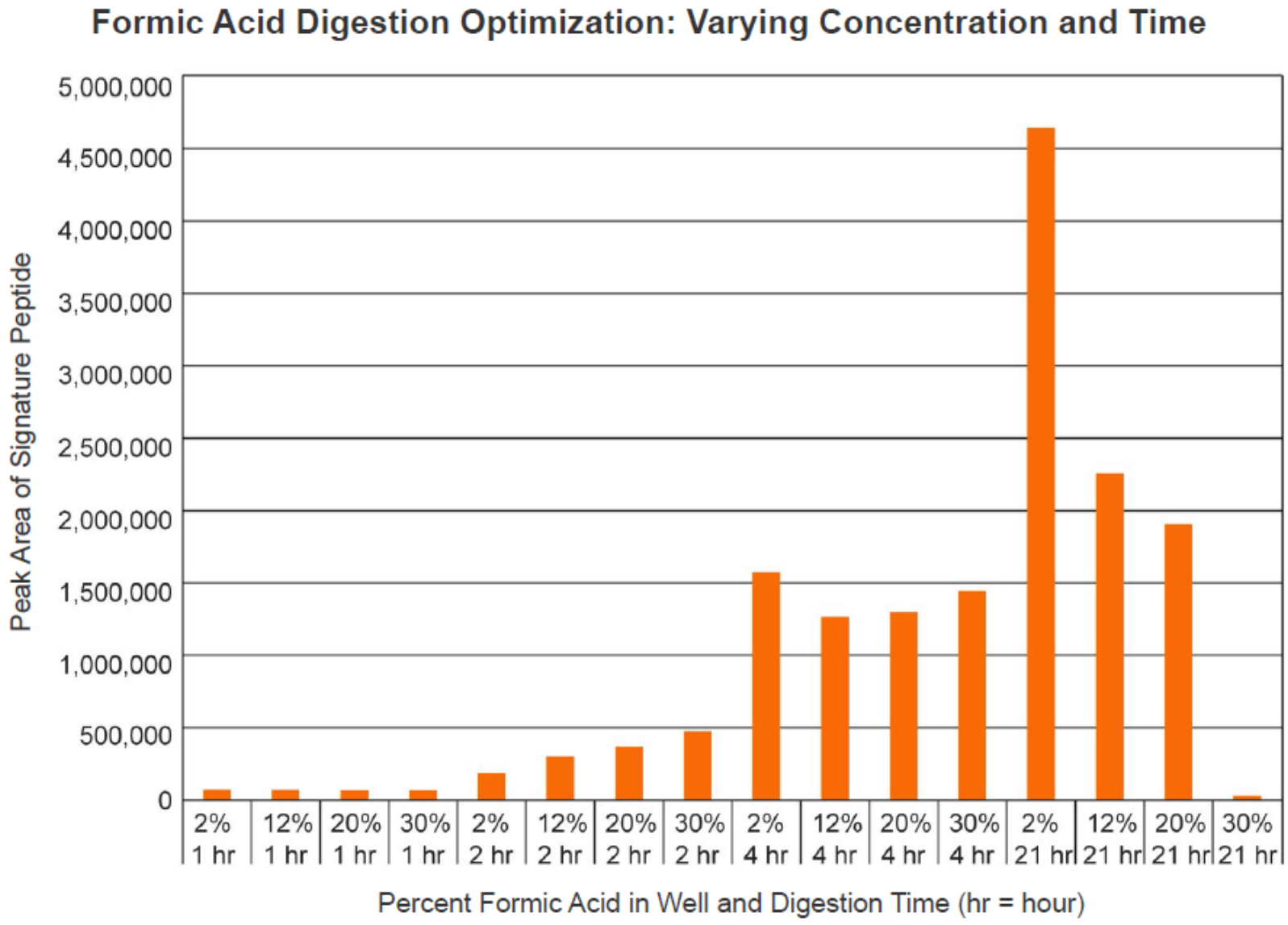
LC *(Dionex Ultimate 3000 multidimensional nano-LC system)*

- 40 µL injection to C18 trap µ-Precolumn™, 0.3 mm x 5 mm at 300 µL/min
- Elute to the nano-LC column (PepMap C18, 75 µm x 15 cm) at 600 nL/min

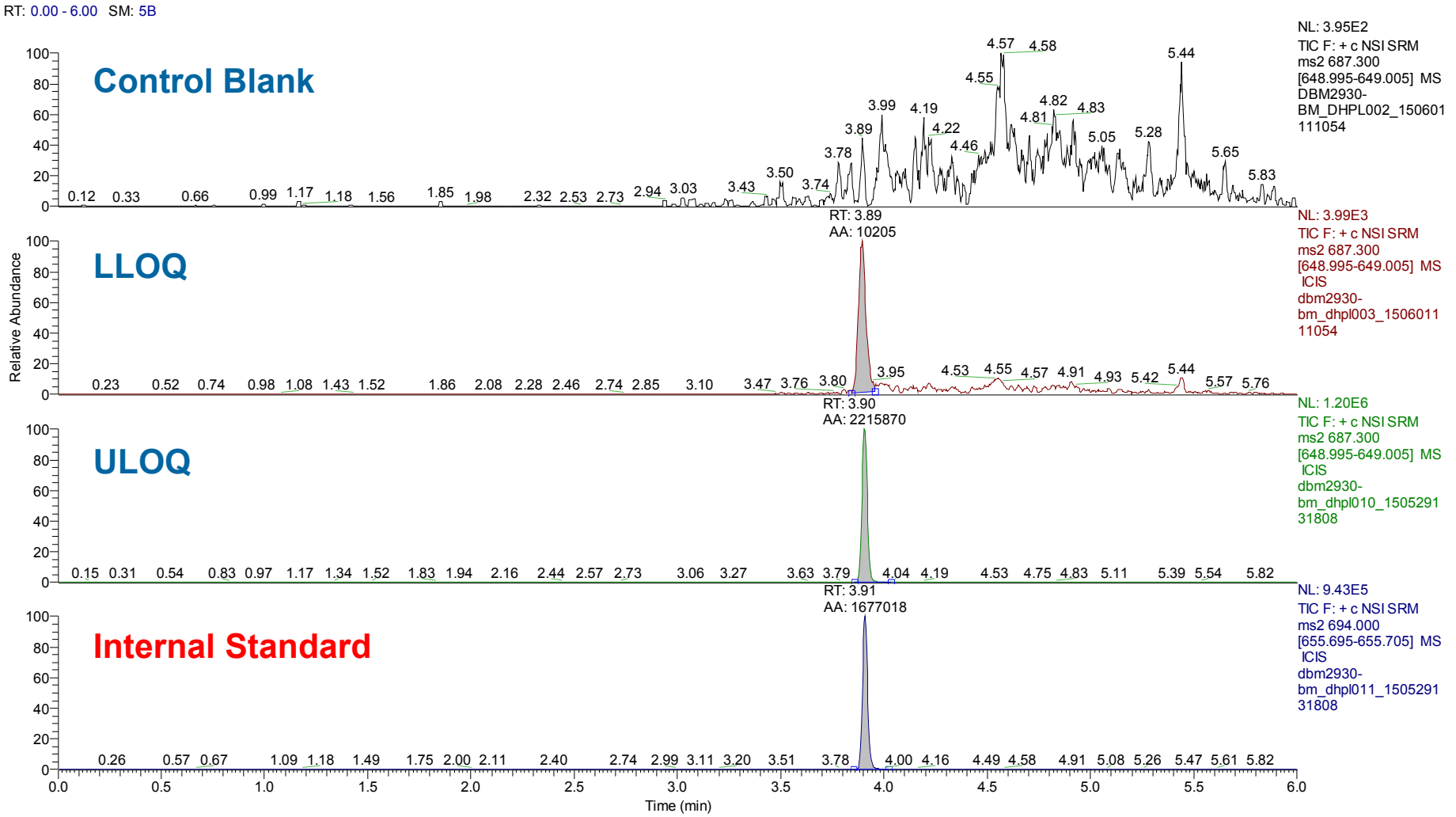
MS *(Thermo TSQ Vantage QQQ MS)*

- Thermo EASY-Spray source

Formic Acid Digestion Conditions



Representative XIC for Formic Acid Digestion Assay



Inter-Run Statistics

Two A&P test batches

		LLOQ	QC1	QC2	QC3	ULOQQC	100X DilQC
Theoretical Conc. (ng/mL)		**100**	**300**	**900**	**1200**	**1500**	**100000**
Run	3	111	302	1030	1170	1390	NA
		124	309	1030	1160	1450	NA
		117	310	1020	1150	1350	NA
		110	305	1030	1550	1560	NA
		120	305	1020	1160	1510	NA
		120	321	1090	1220	1440	NA
Run	4	108	332	951	1240	NA	93000
		107	357	971	1200	NA	94700
		111	337	944	1220	NA	92700
		111	346	1260	1240	NA	96000
		107	342	949	1240	NA	97600
		114	360	917	1230	NA	93100
Mean		**113.5**	**329.5**	**1016.5**	**1237**	**1462**	**94517**
S.D.		**5.66**	**21.20**	**91.12**	**105.99**	**76.68**	**1967**
%CV		**5.0**	**6.4**	**9.0**	**8.6**	**5.2**	**2.1**
%Theoretical		**113.5**	**109.8**	**112.9**	**103.1**	**97.5**	**94.5**
n		**12**	**12**	**11**	**11**	**6**	**6**

Interlot Selectivity and Accuracy and Precision at LLOQ

6 individual lots

Inter-lot Accuracy and Precision at the LLOQ

	DX-2930 Concentration (ng/mL)					
	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
Replicate 1	108	117	116	114	113	115
Replicate 2	98.4	117	121	107	105	106
Replicate 3	109	117	113	111	93	104
Mean	105	117	117	111	104	108
%CV	5.6	0.0	3.5	3.2	9.7	5.4
%Theoretical	105.1	117.0	116.7	110.7	103.7	108.3

Inter-lot Selectivity

	DX-2930			
	Control Blank Peak Area	% of mean LLOQ area	Zero Sample Peak Area Ratio	% of mean LLOQ response
Lot 1	631	1.0%	0.001426	1.9%
Lot 2	166	0.3%	0.000401	0.5%
Lot 3	1235	2.0%	0.001335	1.8%
Lot 4	229	0.4%	0.000744	1.0%
Lot 5	405	0.7%	0.000593	0.8%
Lot 6	159	0.3%	0.000168	0.2%

Inter-lot selectivity was evaluated by analyzing six individual lots of sodium citrate-treated plasma.

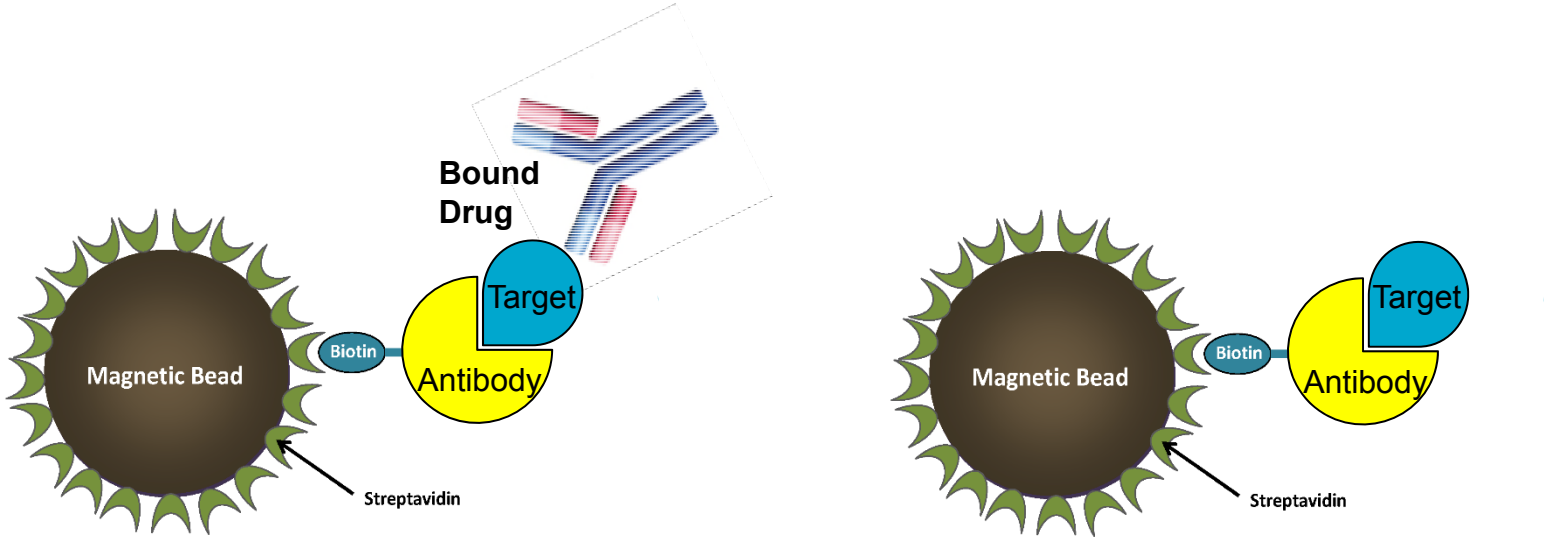
Carryover

Relative to mean LLOQ (100 ng/mL) response

STD 1 rep 1	0.111389
STD 1 rep 2	0.120343
Mean LLQ response (ratio)	**0.1159**
STD 1 rep 1	61364.37
STD 1 rep 2	40520.47
Mean LLQ response (area)	**50942**
First primary carryover zero	0
Second primary carryover zero	0.004932
Mean response	0.002466
% carryover response	**2.1**
First post carryover zero	0
Second post carryover zero	0.012715
Mean response	0.006358
%post carryover response	**5.5**

Where else can these technologies take us?

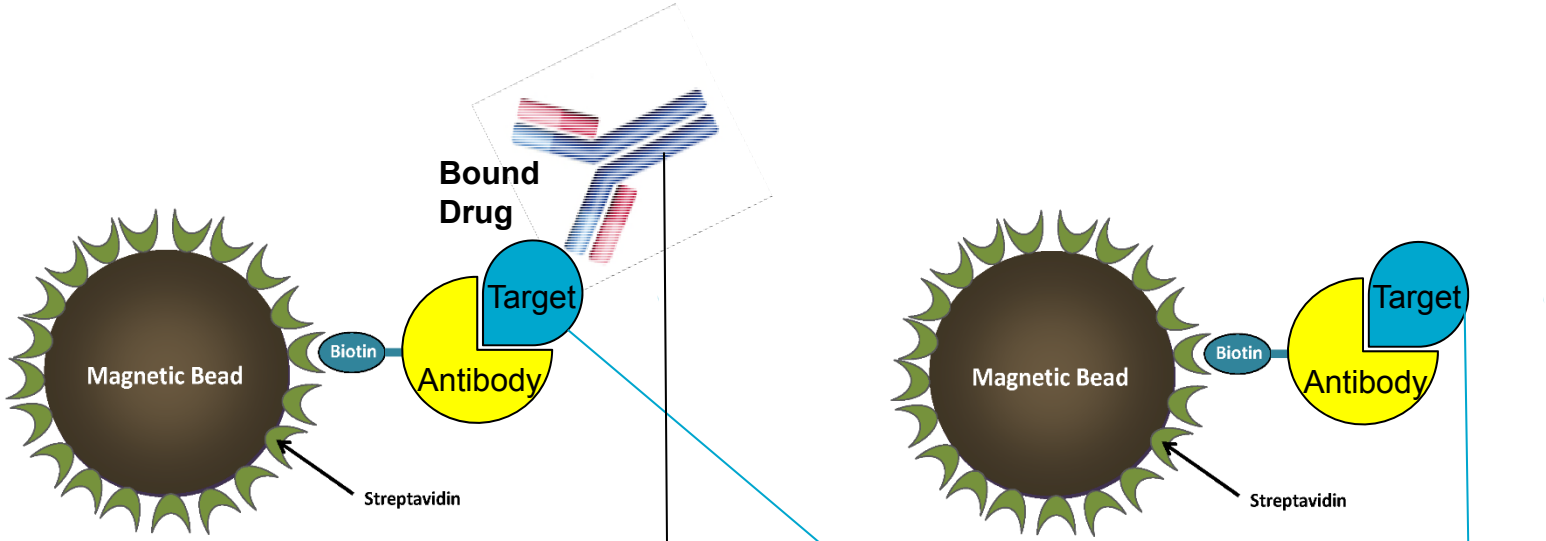
IA-LC/MS, HRMS, "bottom-up" quantification…



Can we measure total and bound target in same assay?

Where else can these technologies take us?

Can we measure total and bound target in same assay?



Possibility: PK and PD in same assay?

Signature peptide from **drug** – measure bound target concentration

Signature peptide from **target** – measure total target concentration

Why do LC-MS for proteins
Specialized Tools
Case studies
Conclusions

Conclusions

- LC-MS remains a complimentary approach to LBA and can demonstrate added advantages
- Wide set of tools / scan modes available to tune selectivity (IP, scan modes, nano-LC, HRMS)
- LC-MS can meet (and exceed) sensitivity obtained with LBA (but you need all available tools to get there)
- LC-MS for proteins meets all of the regulatory fundamentals in the practical environment of a modern bioanalytical laboratory

It's a little more capital intensive than plate-based LBA

But the results can be worth it …

Acknowledgement: Barry Jones, Steve Lowes