Direct Quantitation of Therapeutic Antibodies for Pharmacokinetic Studies using Immuno-Purification and Intact Mass Analysis

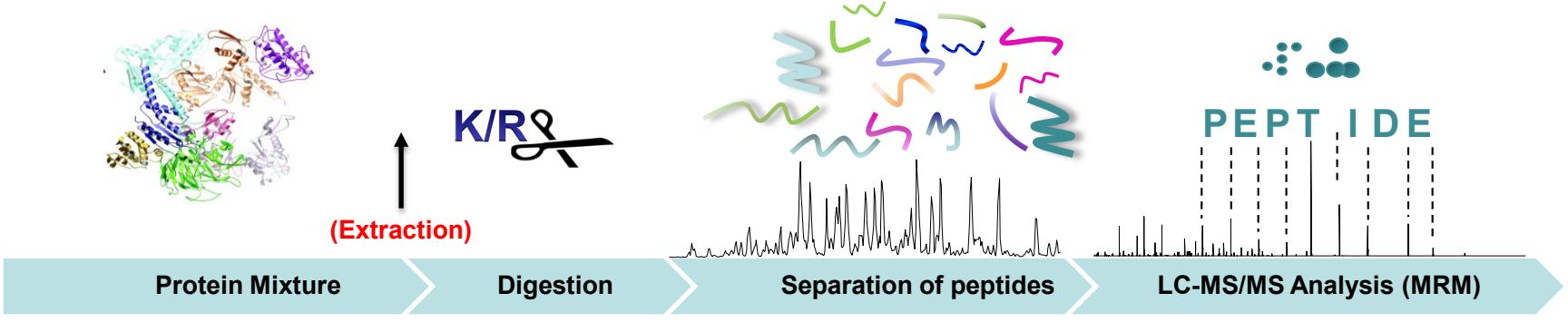
Kevin Bateman, Lisa O'Callaghan and Dan Spellman

Merck & Co., Inc.

CASSS MS 2020

MERCK
Be well

Bottom-up vs Top-Down Methods



MERCK
Be well

Why Surrogate Peptide Approach?

- Make the protein assay into a small molecule assay
- Enables the use of well-established small molecule tools:
  - Chromatography is robust and reproducible
  - Peptide fragmentation well understood and predictable
  - Sensitive and selective MRM based quantitation on validated QQQ
  - Data processing software is in place
  - Standard LIMS workflows is in place

Surrogate peptide-based approaches have proven robust and reliable

Challenges of Surrogate Peptide Approach

- Complex sample preparation that can lead to assay variability if not well controlled
  - Affinity based enrichment
  - Digestion to release peptides
- Complicated MS method development
  - Need to select appropriate peptides (unique/selective/sensitive)
  - Optimization of multiple peptides with multiple transitions per peptide
- Need appropriate internal standard
  - Labeled intact protein is better than labeled peptide IS
- Assumption that the peptide(s) represent the intact protein
  - Data processing challenges, what happens when peptides give different concentrations for the same protein?

FDA Presentation at 2017 AAPS Short Course

Questions for the audience

1. How do you demonstrate the 'uniqueness' of the signature peptide?
2. How do you demonstrate that an enzymatic digestion was complete after incubation?
3. How do you demonstrate that a signature peptide is exclusively from intact therapeutic protein?
4. How do you demonstrate that the catabolic or biotransformed therapeutic protein retains the same efficacy and safety as the unmodified product?
5. How are internal standards chosen?

Protein Bioanalysis by Mass Spectrometry: Regulatory Perspectives

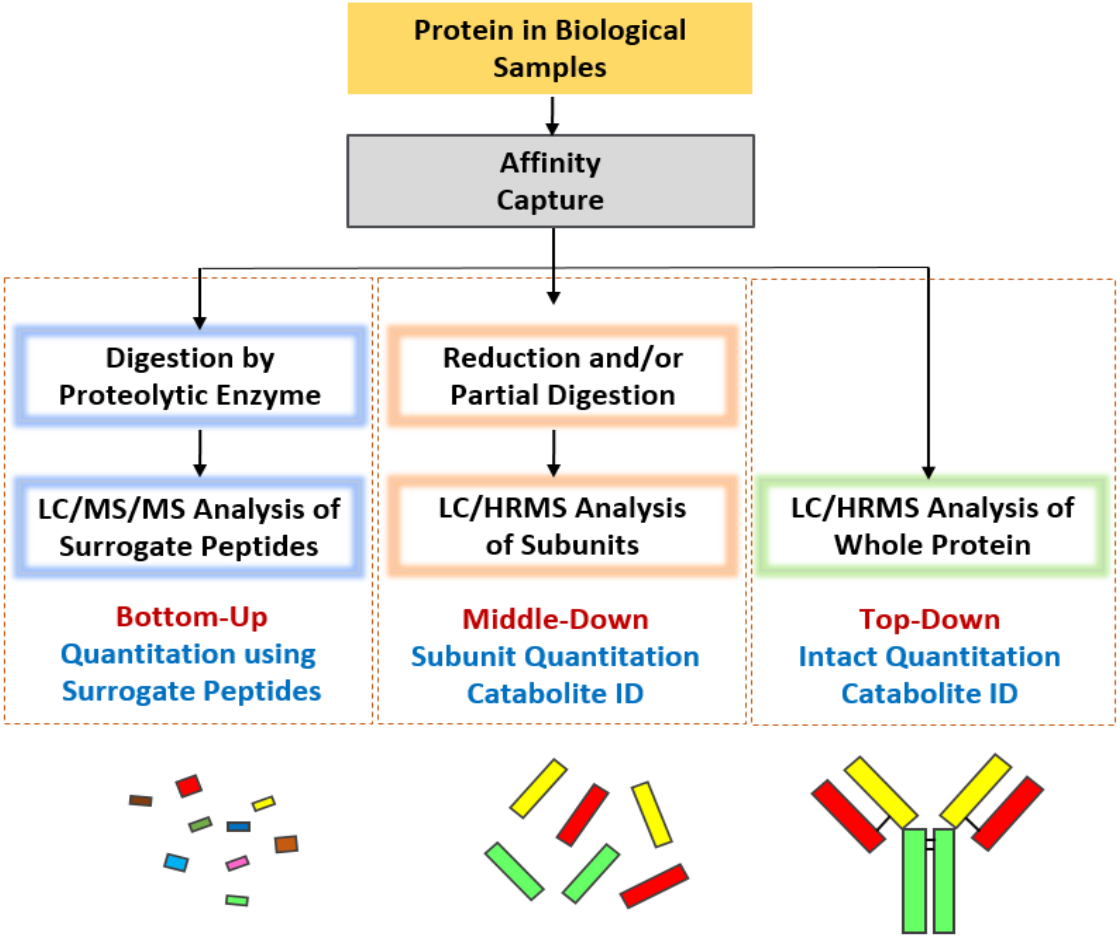
Brian Furmanski, Ph.D.
Division of Clinical Pharmacology V
FDA/CDER/OTS/OCP

Concerns with Surrogate Peptide Approach

- Single step of affinity capture (versus capture and detection for LBA)
  - What are we capturing? Or not capturing?
- Surrogate peptide, not the intact protein
  - What are we measuring? Or not measuring?
- Surrogate IS, not the labeled protein
  - Is extraction robust? Is digestion consistent?
- Does this approach truly represent the dosed molecule?
- What methods could be developed to address these concerns?

Surrogate peptide approach has potential limitations if the method is not well understood

Other Approaches for LC-MS Based Analysis

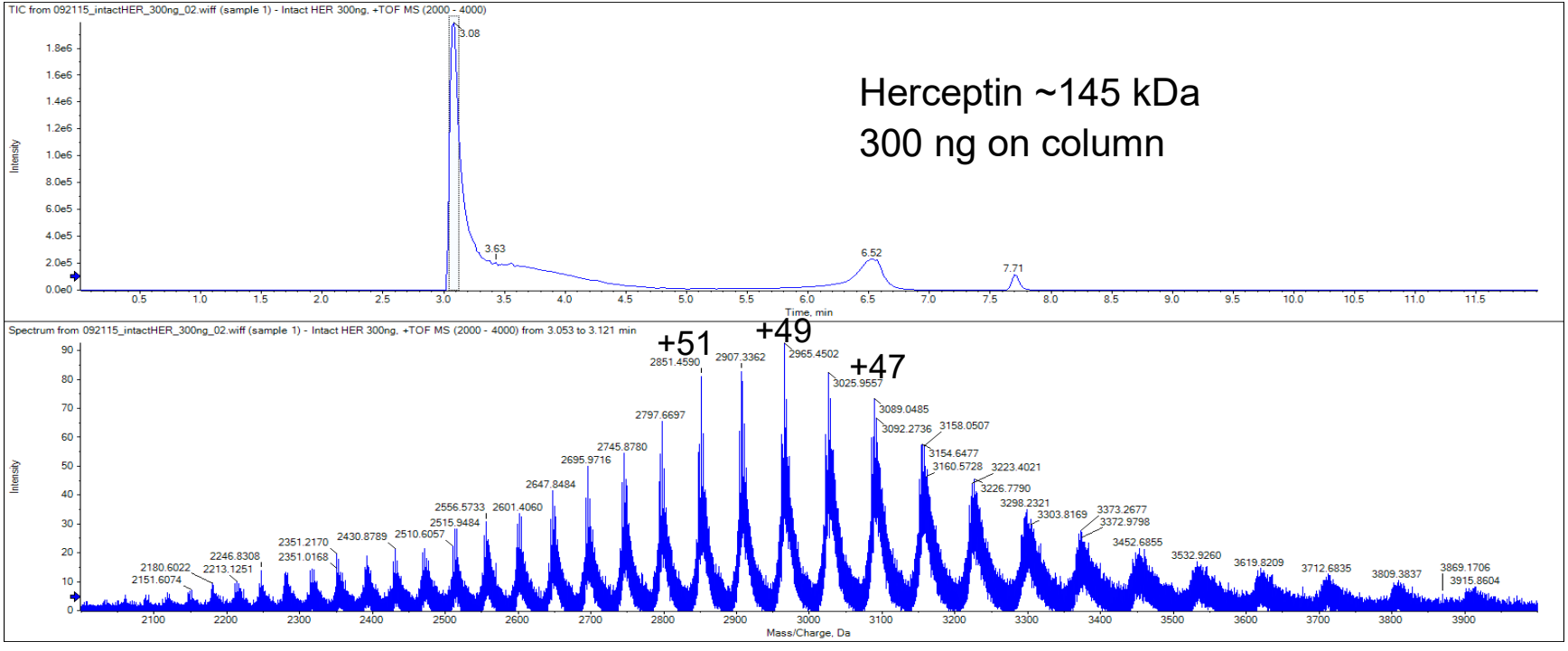


MERCK
Be well

Intact Protein Analysis

- Instead of digesting the protein into smaller peptides, the protein is analyzed with minimal pretreatment.
  1. No pretreatment
  2. De-glycosylation
  3. Reduction
  4. Limited proteolysis (IdeS)
  5. Combination of 2 and 3 or 4
- Potential Benefits
  - Truly represents the molecule you are dosing/quantitating
  - Less complex sample preparation
  - Ability to identify changes (catabolites) of your molecule

Intact Protein Mass Spectrometry



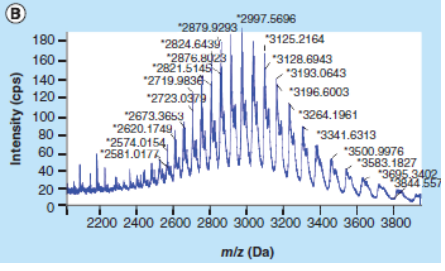
Intact mass based quantitative analysis for large proteins (i.e. mAbs) in bio-fluids (serum, plasma, etc.) has not been generally practical.

Literature is growing in this area

A workflow for absolute quantitation of large therapeutic proteins in biological samples at intact level using LC-HRMS

Wenying Jian*,[1], Lijuan Kang[1], Lyle Burton[2] & Naidong Weng[1]

Bioanalysis (2016) 8(16), 1679–1691



15 ug/mL from
100 uL of plasma

Direct quantitation of therapeutic antibodies for pharmacokinetic studies using immuno-purification and intact mass analysis

Lisa A Vasicek[1], Xin Zhu[2], Daniel S Spellman[1] & Kevin P Bateman*,[1]
[1]Pharmacokinetics, Pharmacodynamics & Drug Metabolism, Merck & Co., Inc., 770 Sumneytown Pike, West Point, PA 19486, USA
[2]Agilent Technologies, 2850 Centerville Rd, Wilmington, DE 19808, USA
*Author for correspondence: kevin_bateman@merck.com

Bioanalysis (2019) 11(03), 203–213

0.1 ug/mL from
30 uL of plasma

A whole-molecule immunocapture LC–MS approach for the *in vivo* quantitation of biotherapeutics

John F Kellie*,[1], Jonathan R Kehler[1], Thomas J Mencken[1], Richard J Snell[2] & Charles S Hottenstein[1]

Bioanalysis (2016) 8(20), 2103–2114

IdeS digestion and reduction
0.1 – 0.25 ug/mL

Generic Hybrid Ligand Binding Assay Liquid Chromatography High-Resolution Mass Spectrometry-Based Workflow for Multiplexed Human Immunoglobulin G1 Quantification at the Intact Protein Level: Application to Preclinical Pharmacokinetic Studies

Christian Lanshoeft,[†,‡] Sarah Cianférani,[‡] and Olivier Heudi*,[†]
[†]Novartis Institutes for Biomedical Research, Drug Metabolism and Pharmacokinetics, Novartis Campus, 4056 Basel, Switzerland
[‡]Laboratoire de Spectrométrie de Masse BioOrganique, Université de Strasbourg, CNRS, IPHC UMR 7178, 67000 Strasbourg, France

DOI: 10.1021/acs.analchem.6b04997
Anal. Chem. 2017, 89, 2628–2635

Overnight deglycosylation
0.1 ug/mL from 50 uL serum

MERCK
Be well

Challenges of Intact MS Analysis

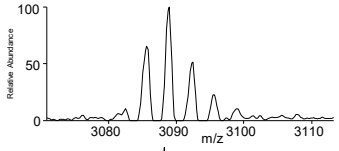
- Requires an affinity capture sample preparation step
- Requires a high-resolution mass spectrometer
  - Qtof or Orbitrap
- Chromatography of intact protein at low concentrations in a biomatrix
- Sensitivity at the intact level not as good as MRM based methods
- Labeled intact protein IS can be expensive and time consuming to procure
- Data processing approaches and tools for quantitative analysis at the intact level not well established

Sample Preparation Formats

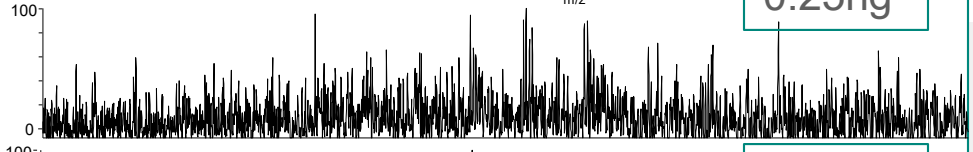
	Magnetic Beads	Tips	Immunoassay Plates	Membrane/resin Based Plates
Capture Reagent Capacity	Up to 100 µg, depending on bead volume	Up to 100 µg	1-2 µg	Up to 100 µg
Automation Strategy	Magnetic Beads Handler or Pipette tip-based Liquid Handler with plate-based magnet	Pipette tip-based Liquid Handler	Plate washer	Positive or negative vacuum pressure, centrifugation.
Typical Elution Volume	50-200 µL	10-100 µL	50-100 µL	50-100 µL
Immunocapture Reagents/Formats	Many different vendor choices of beads, beads-antigen binding chemistry, binding capacity, and bead volumes are possible	Different choices of resin, loading capacity, and resin volume available	Limited to streptavidin or amine-based coupling and low capacity/surface area of the plate	Limited to options provided specifically by commercial vendors
Cost	High – need beads, antibody, and liquid handler	High – need tips, antibody, and liquid handler	Low – uses little antibody and plate washer	Medium – requires commercial plate product
Example Commercial Products	Dynabeads ™ sold by Thermo Fisher	AssayMAP Cartridges sold by Agilent	Pierce™ Streptavidin Plates sold by Thermo Fisher	Capturem™ sold by Takara Bio

Spectral Quality for Pure Protein

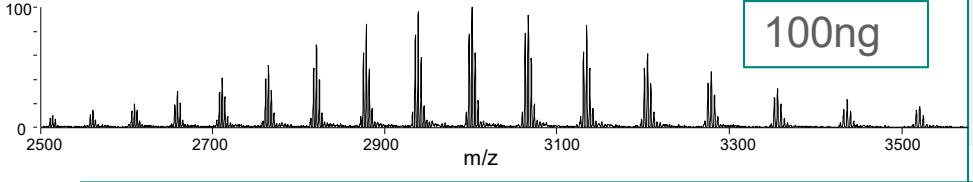
Thermo QEHF Biopharma



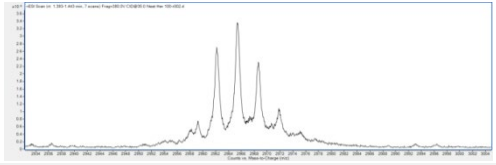
0.25ng



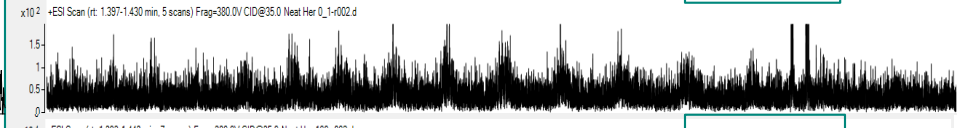
100ng



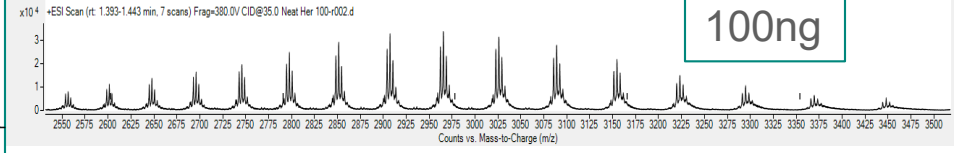
Agilent 6545XT



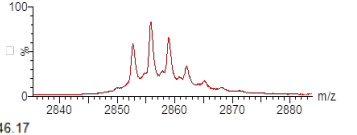
0.1ng

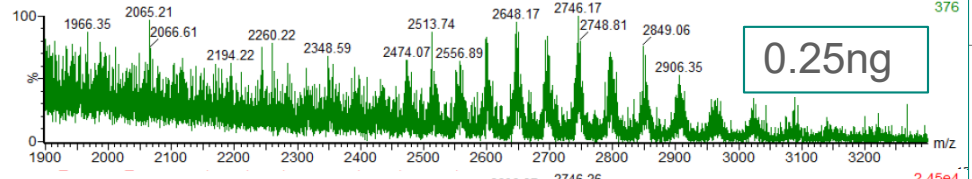


100ng

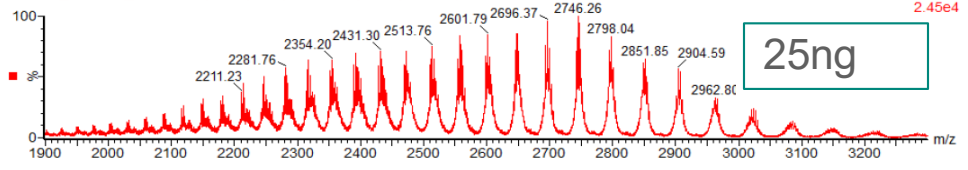


Waters I-Class/Xevo G2-XS



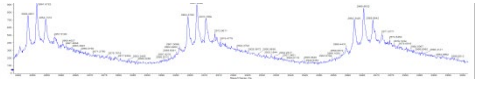


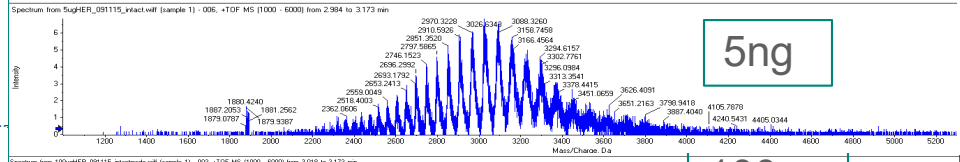
0.25ng



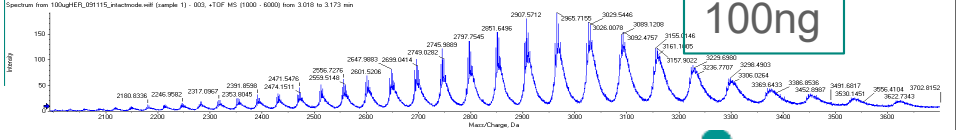
25ng

Sciex 6600





5ng

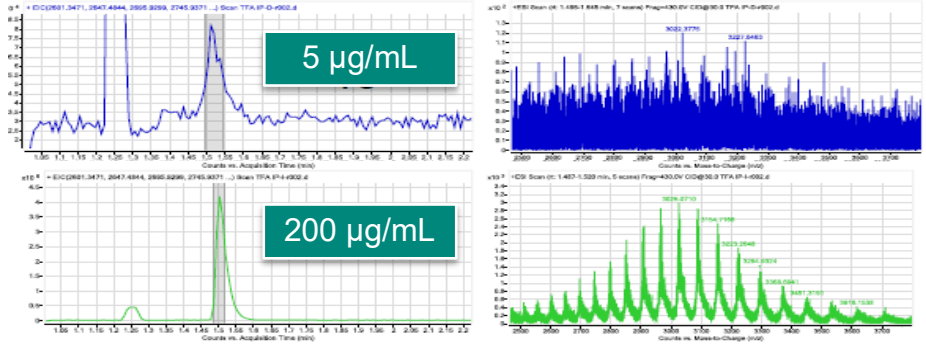


100ng

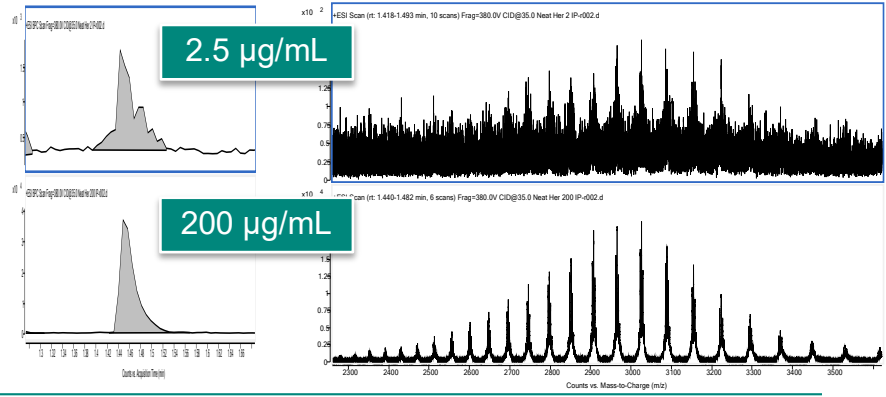
MERCK

Matrix Curves

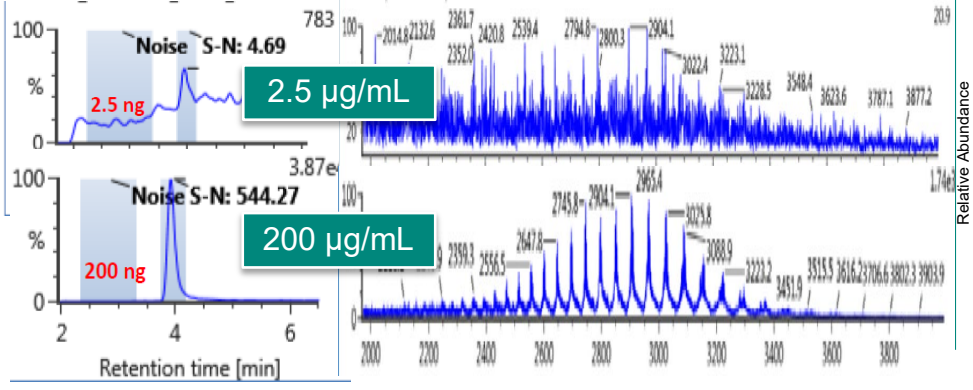
Agilent 6550



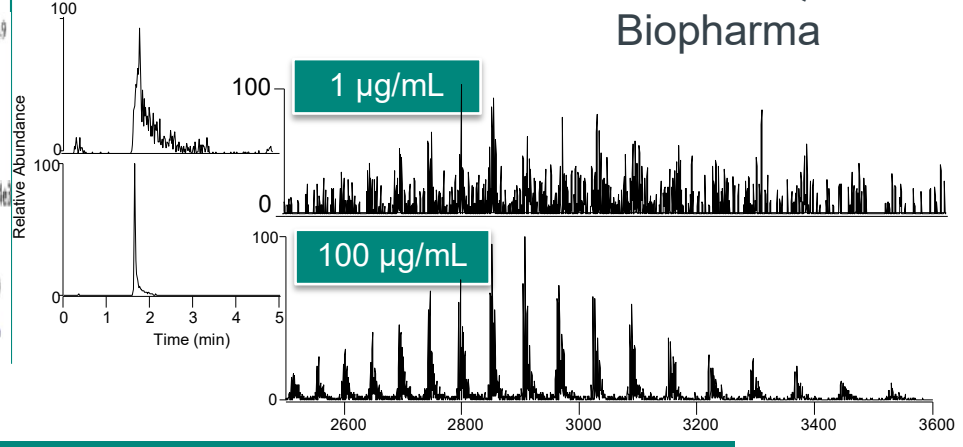
Agilent 6545XT



Waters Vion



Thermo QEHF Biopharma

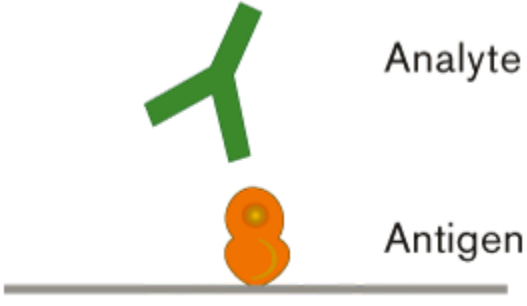


Modern HRMS systems can provide data of sufficient quality

MERCK

Anti-Human Fc Capture versus Target Capture

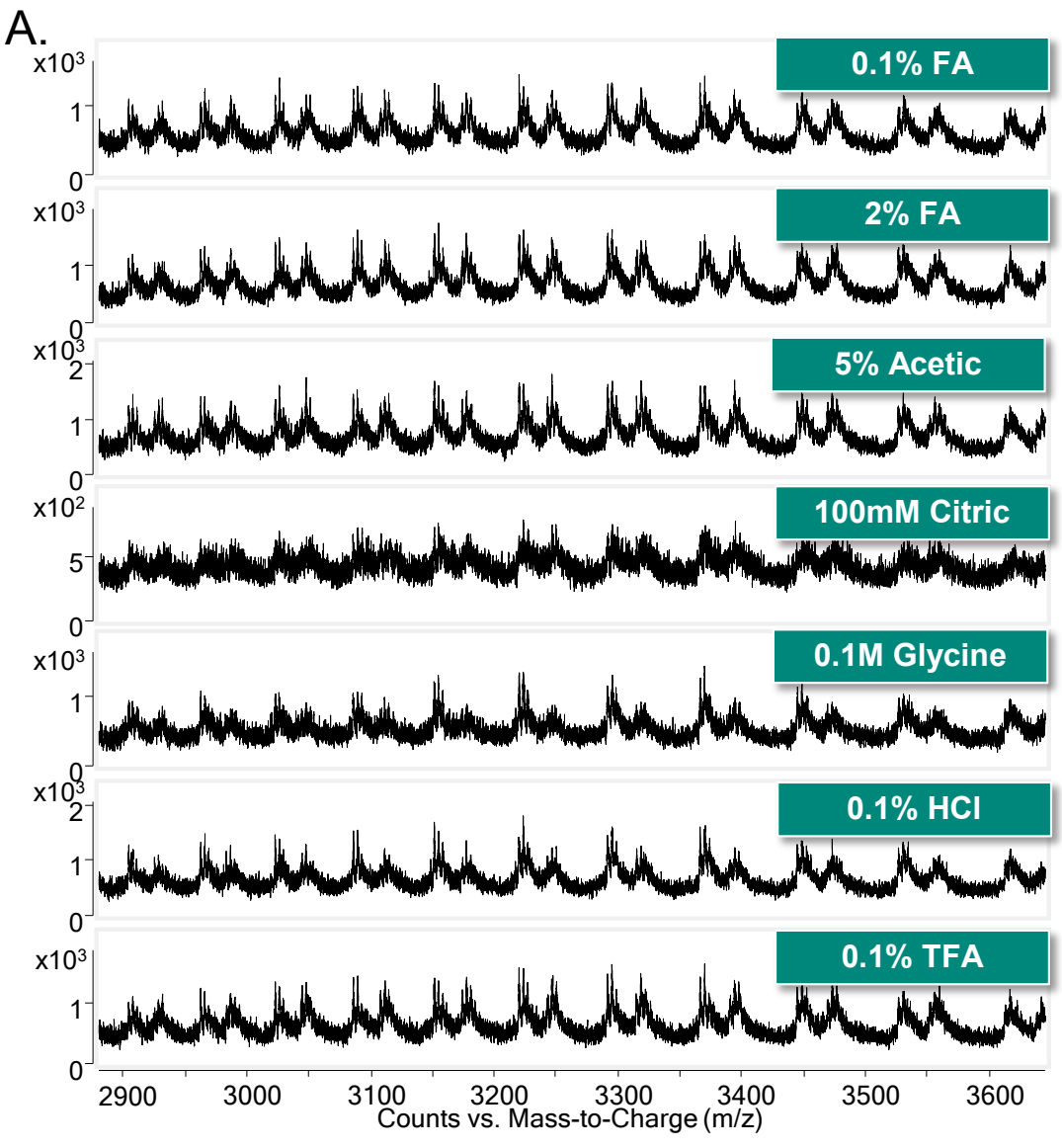
Less Specific

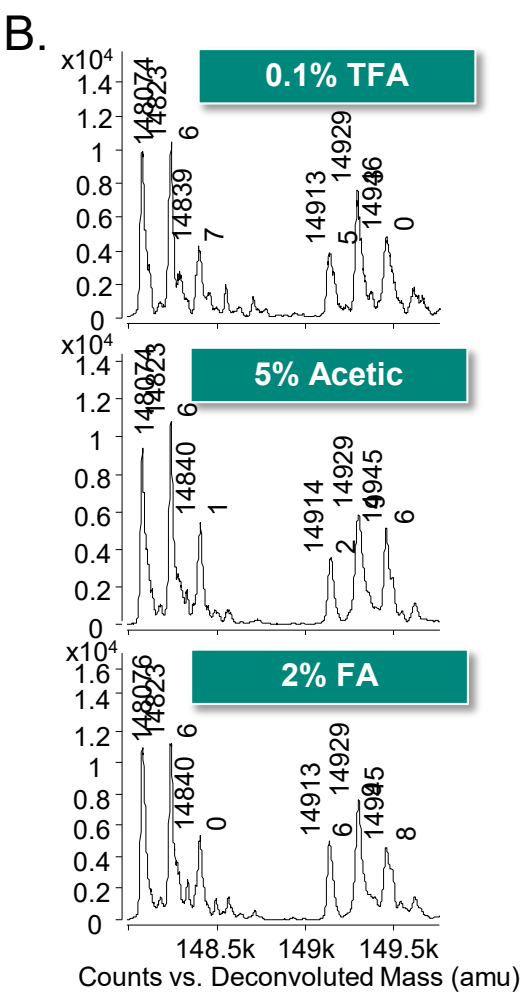


More Specific

- Human specific “generic” capture is suitable for preclinical studies
- Analyte specific capture is required for human studies
- Elution buffer from capture molecule can impact intact MS analysis

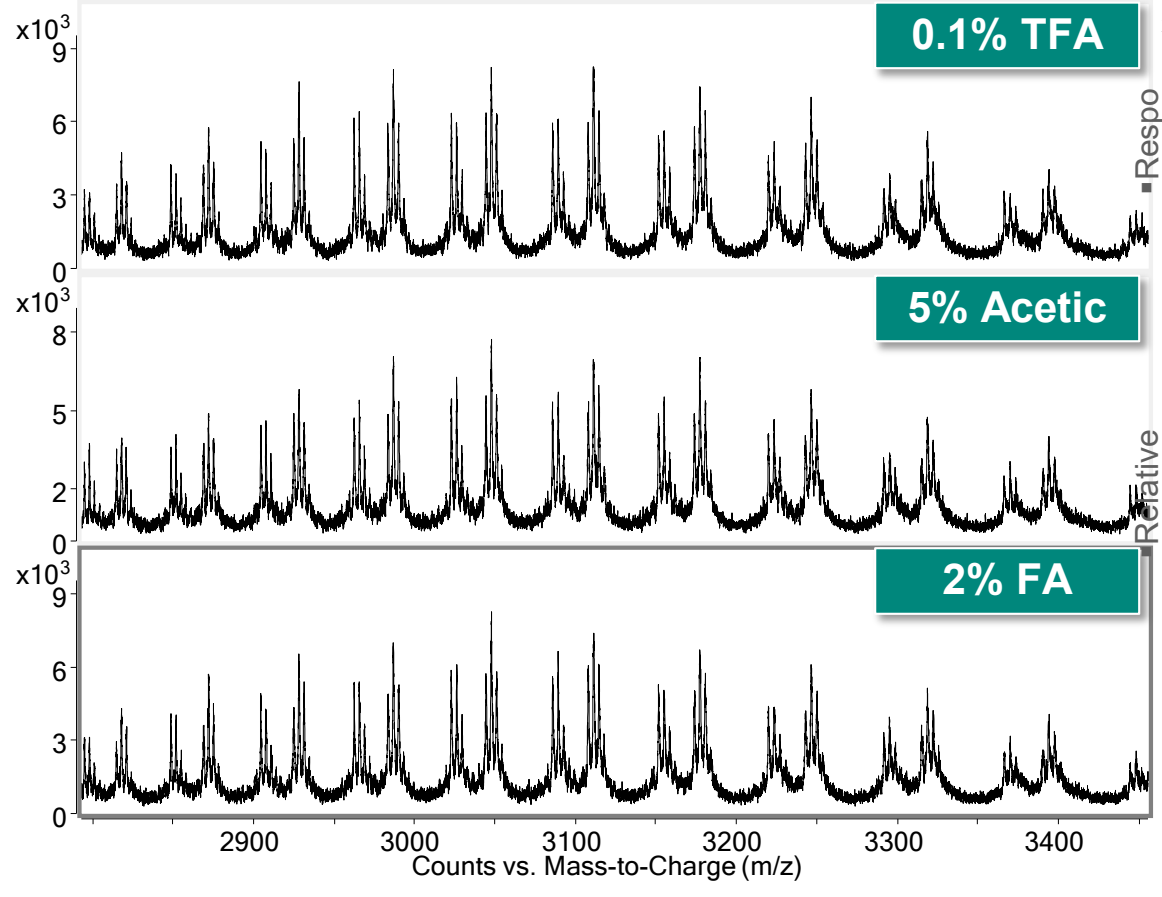
MERCK
Be well

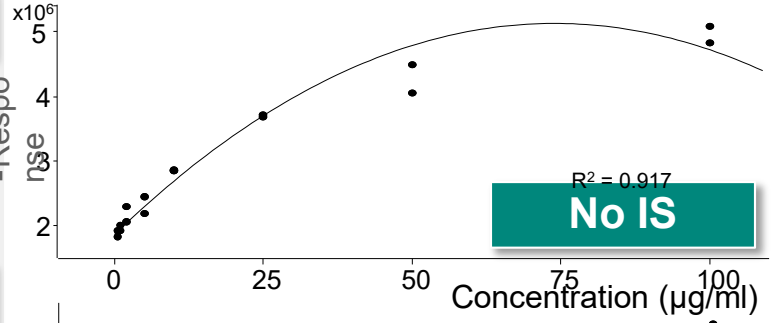


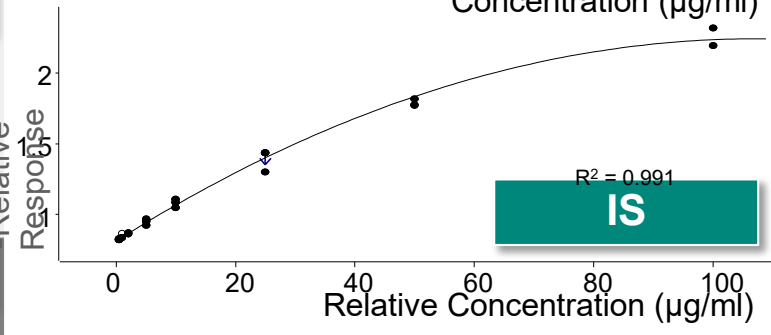


Full MS for seven elution buffers using 10µg/mL MK-8226 and SIL-MK-8226

Impact of Internal Standard

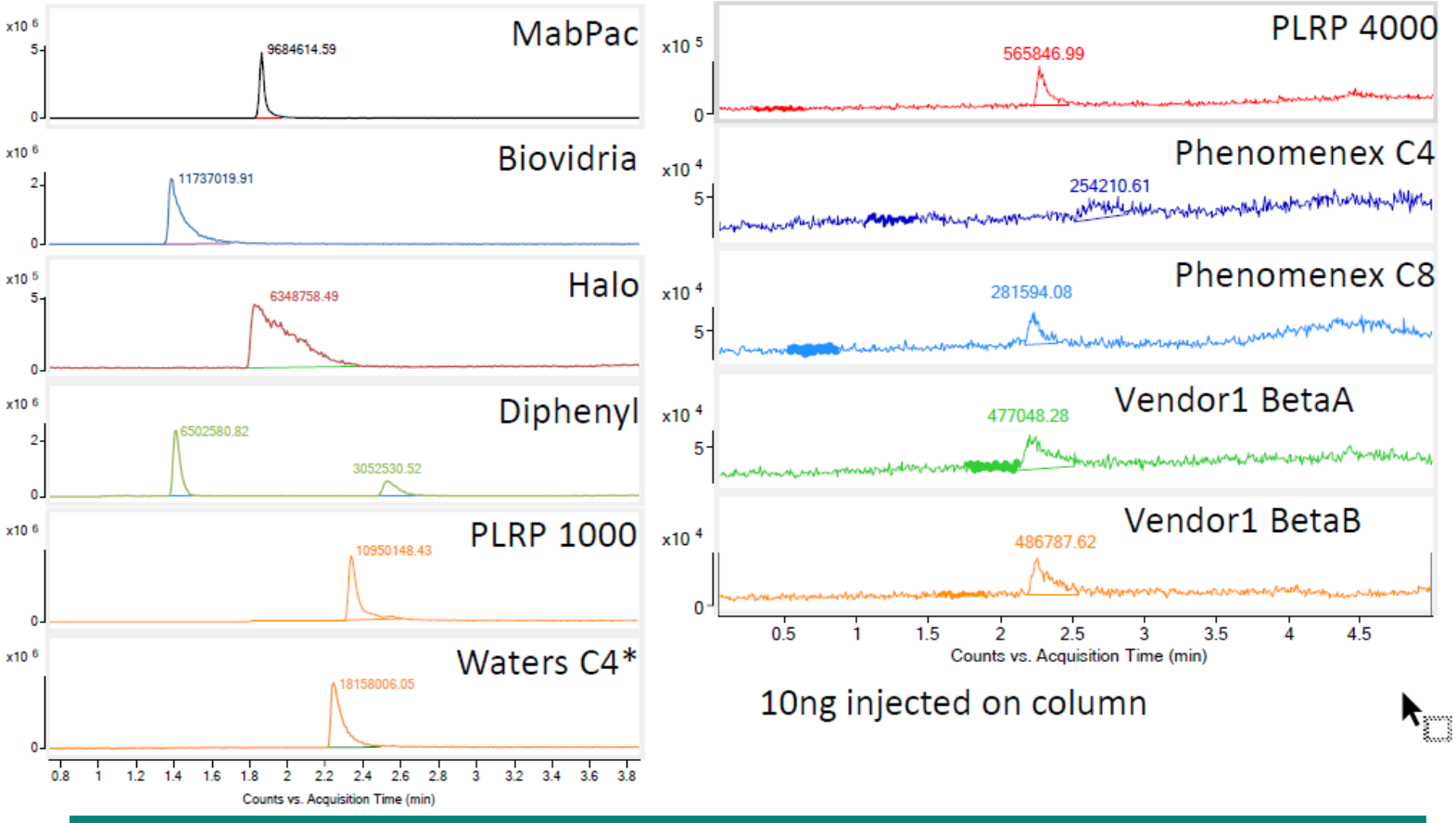






MERCK
Be well

Chromatography Comparison



Extensive column conditioning (repeat injections of high concentration mAB) is required to achieve acceptable performance in many cases

MERCK Be well

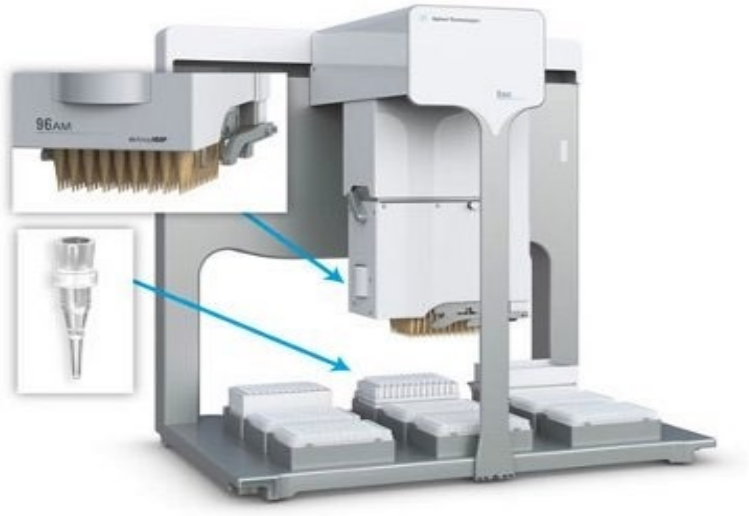
How Can We Increase Sensitivity?

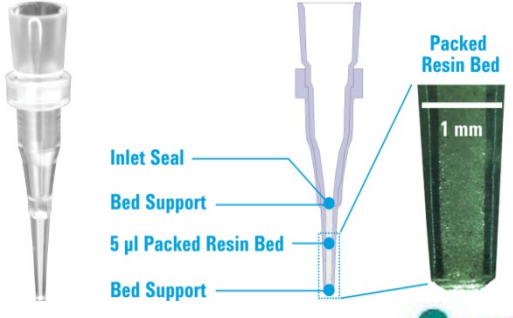
- Increase amount of analyte
  - Process more sample

- Make analyte more detectable through sample preparation
  - More specific capture (antigen)
  - De-glycosylation
  - Hinge digestion

Sample Automation – Agilent AssayMap

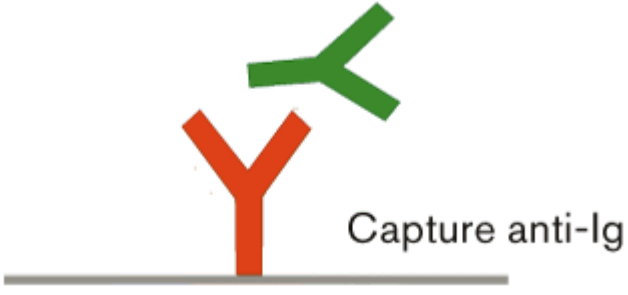
- Cartridge based immunoaffinity purification
  - 96 50 µL samples in 1 hr
  - 150 µg capacity tips
  - High precision and accuracy
    - Intra-assay variability <2%
    - Sample and Elution volumes <10 µL
- Sample preparation
  - Immunocapture
    - 30 µL sample volume
    - 50 µL elution
    - Target Antigen Capture
  - Partial Digestion
    - On-tip de-glycosylation (PNGase F)
    - On-tip hinge digestion (IdeS)





MERCK
Be well

Anti-Human Fc Capture versus Target Capture



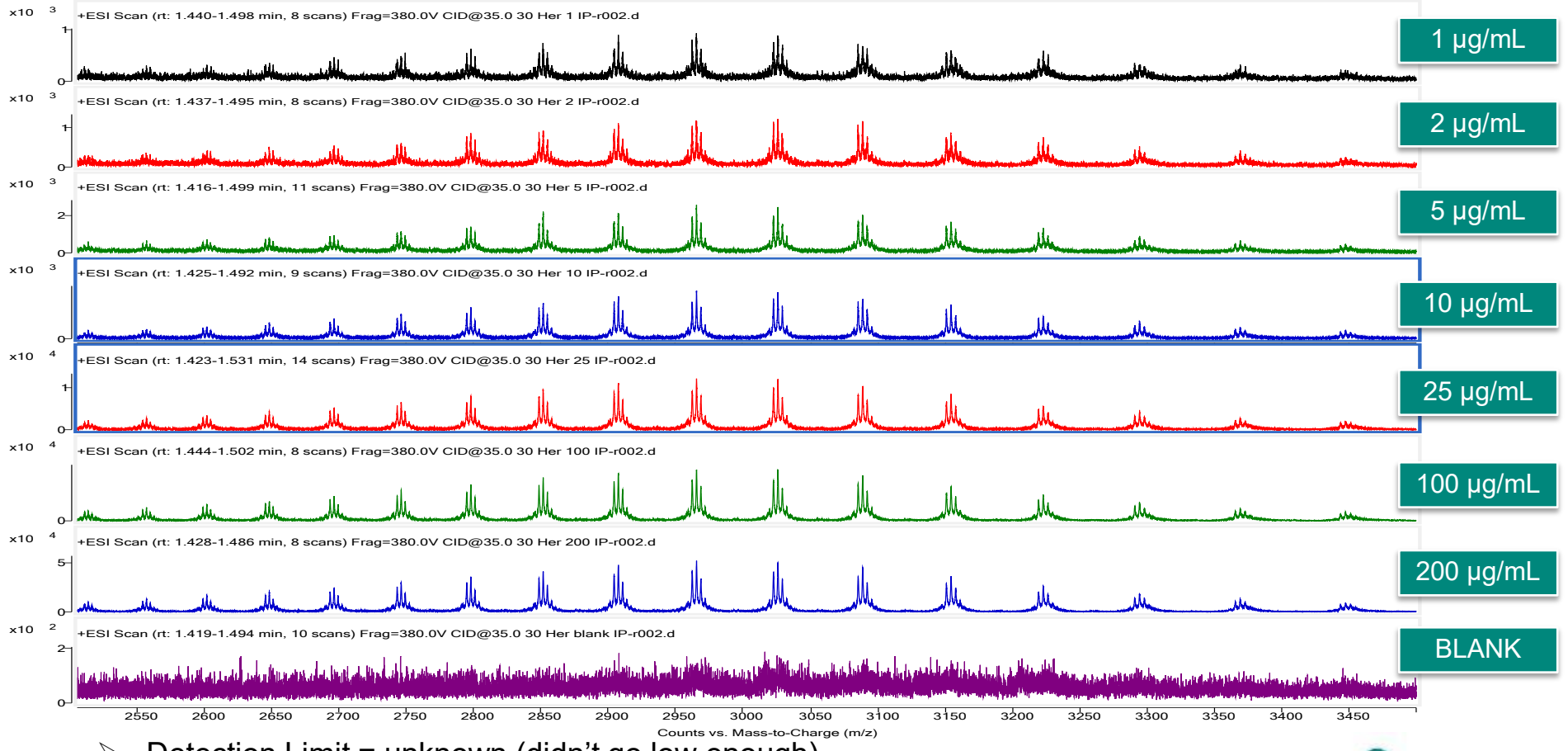
Less Specific

More Specific

Target (antigen) based capture has the potential to be more specific, enabling more sample to be processed

MERCK
Be well

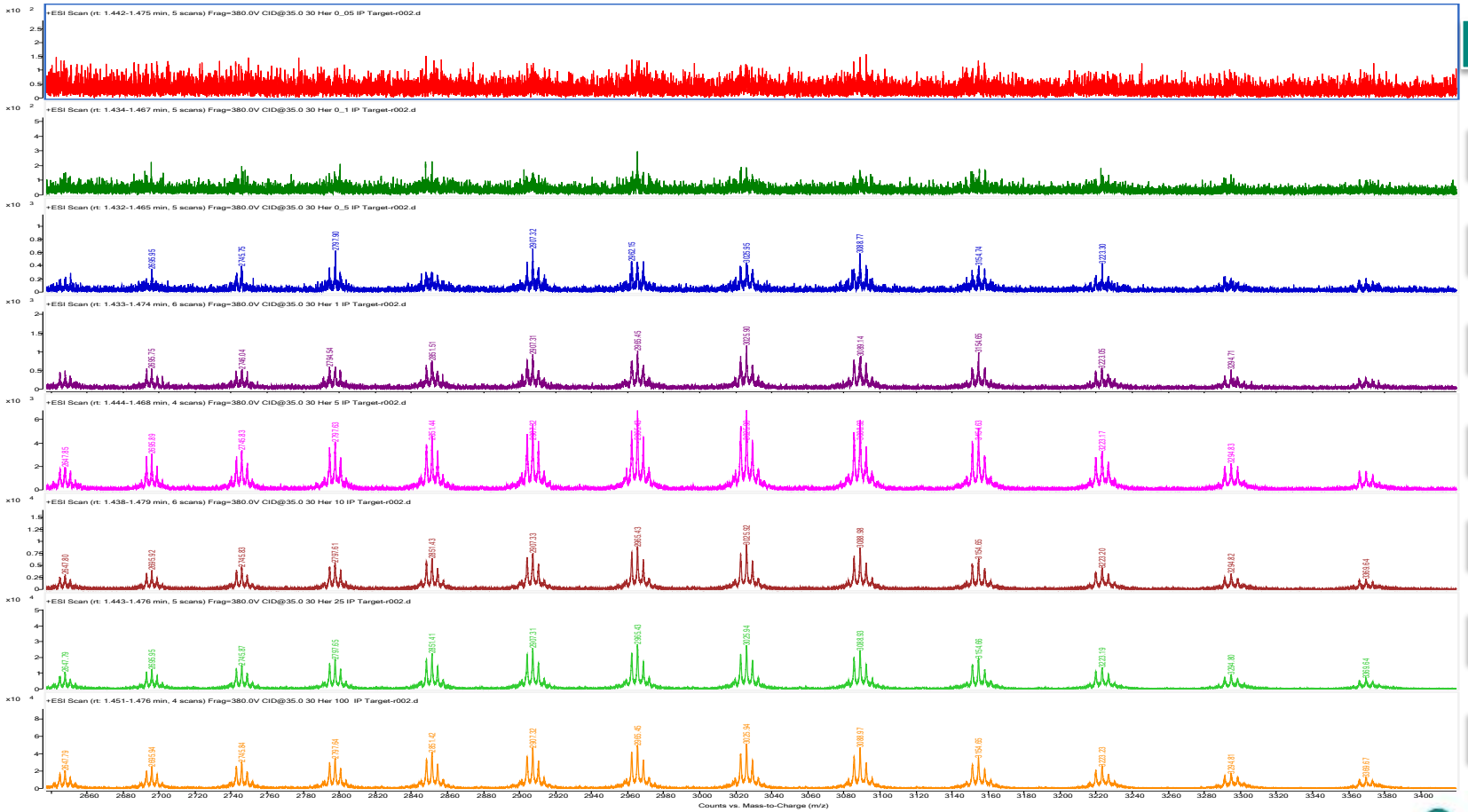
AssayMap 30uL, Anti-human Fc Capture



- Detection Limit = unknown (didn't go low enough)
- LDR = 1-100 µg/mL

MERCK
Be well

AssayMap 30µL, Target (antigen) Capture

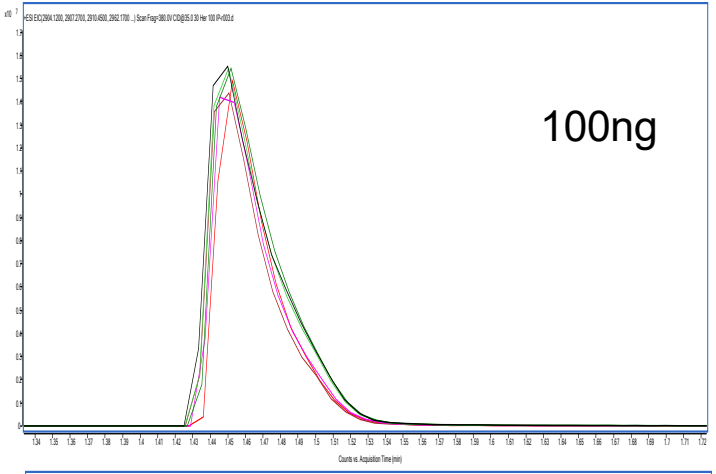


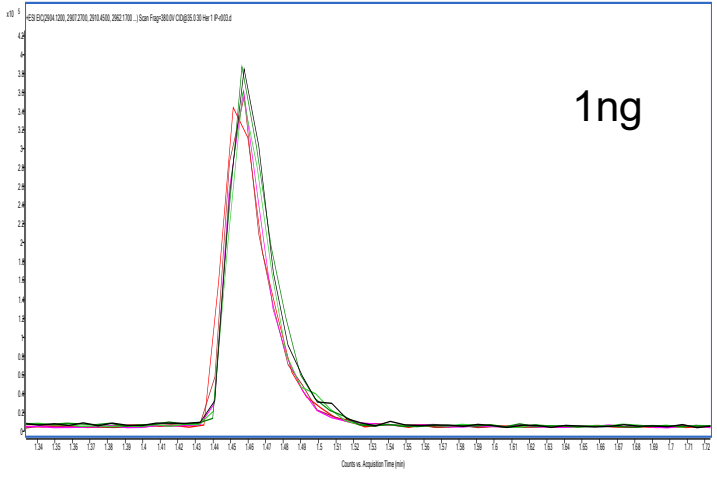
- Detection Limit = 0.1 µg/mL
- LDR = 0.1-100 µg/mL

MERCK
Be well

Anti-Human Fc Capture versus Target Capture

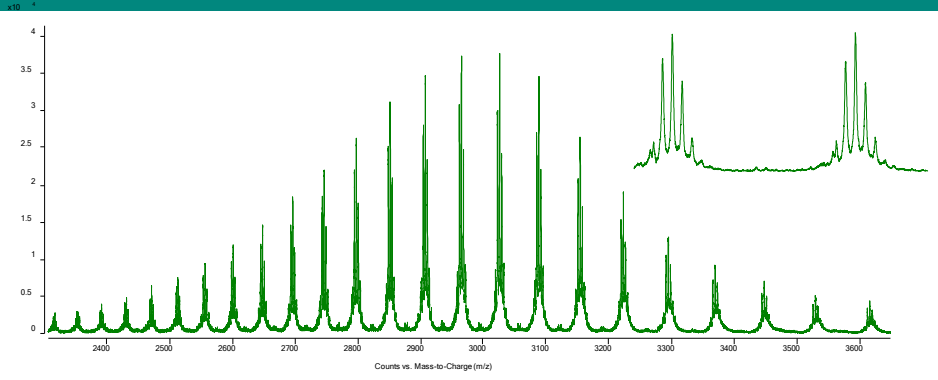
- Extracted ion chromatograms for both approaches overlaid
- Similar capture efficiency
  - Reds = anti-Fc
  - Greens = Target
- No improvement in LODs using target capture
- Improved LOD by processing more sample and reducing elution volume

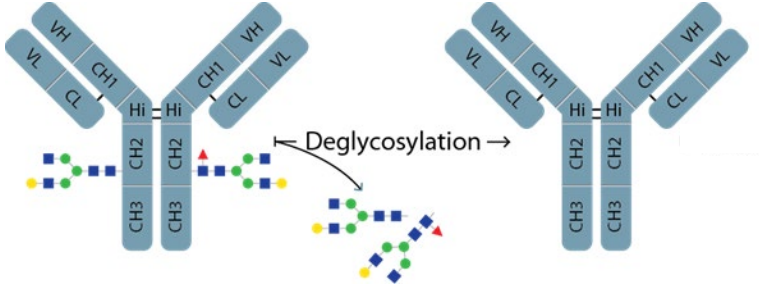


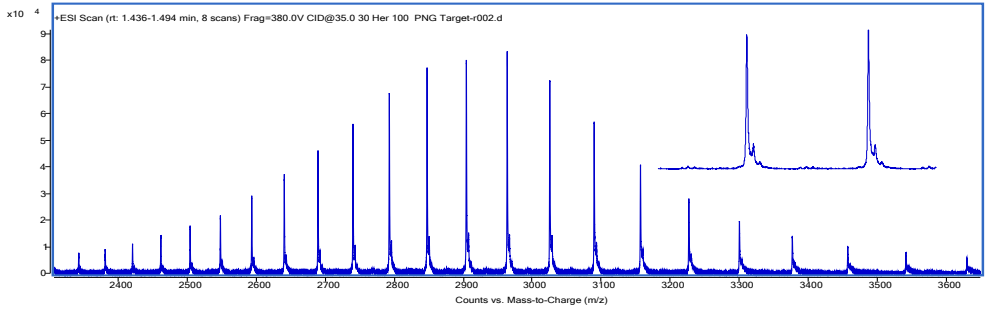


MERCK
Be well

PNGase F Digestion



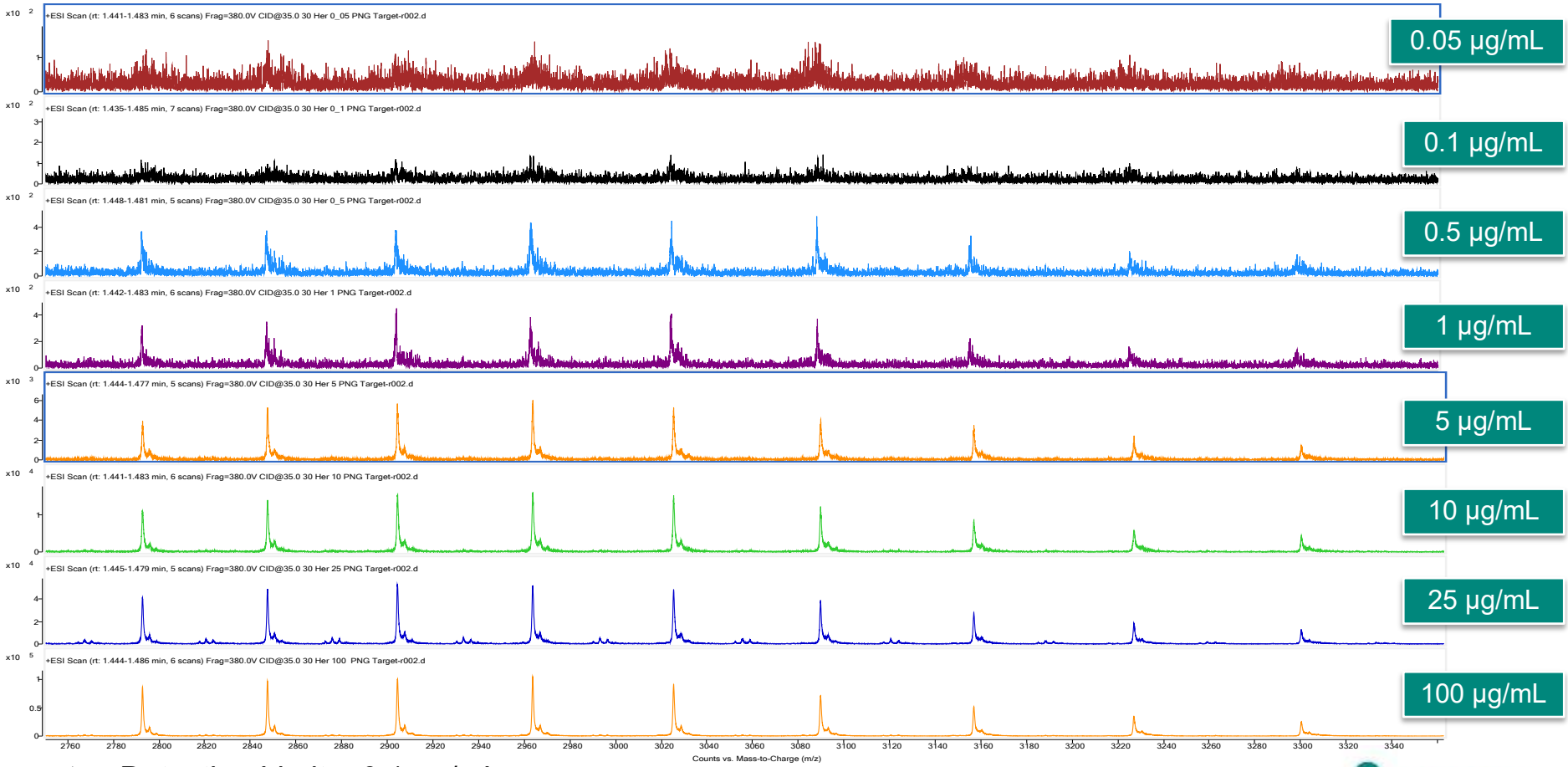




- Cleaves N-linked glycans
- Collapse multiple glycoforms into one “naked” protein
- Potential to improve signal to noise
- Benefit depends on complexity and extent of glycosylation pattern

MERCK
Be well

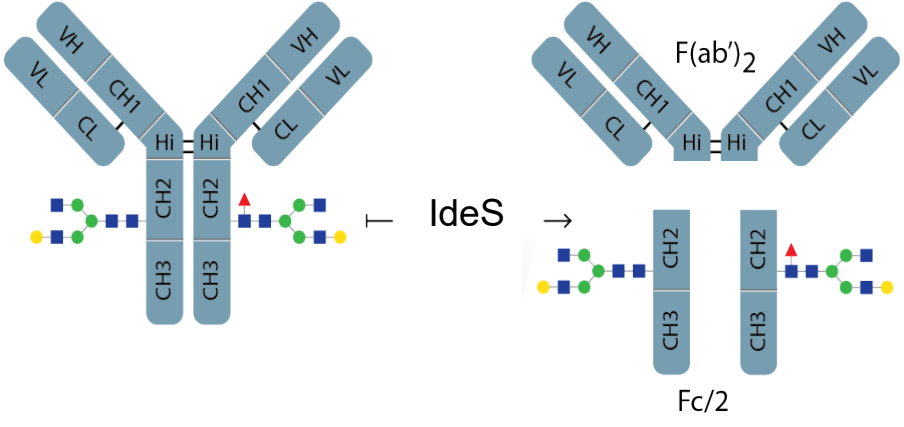
On-tip Deglycosylation, 30µL Sample, Target Capture

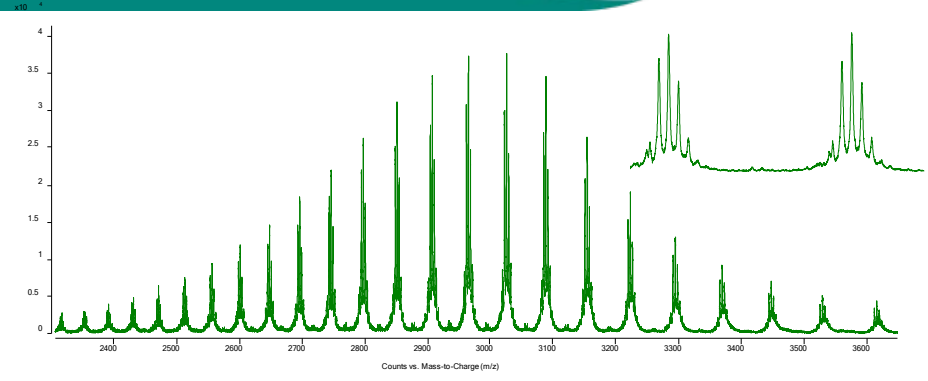


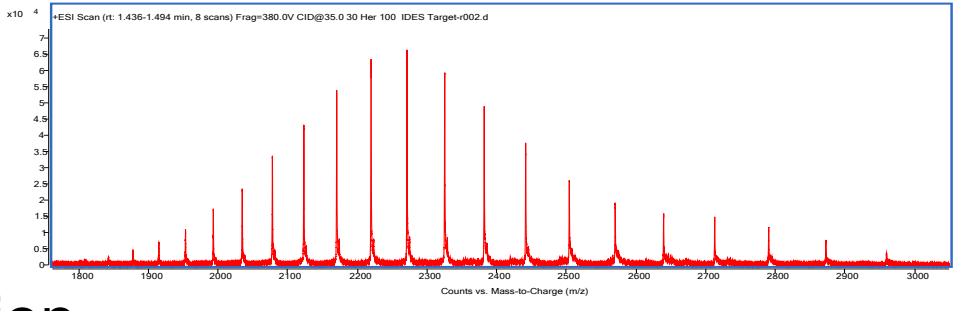
- Detection Limit = 0.1 µg/mL
- LDR = 0.1-25 µg/mL

MERCK
Be well

IdeS Digestion



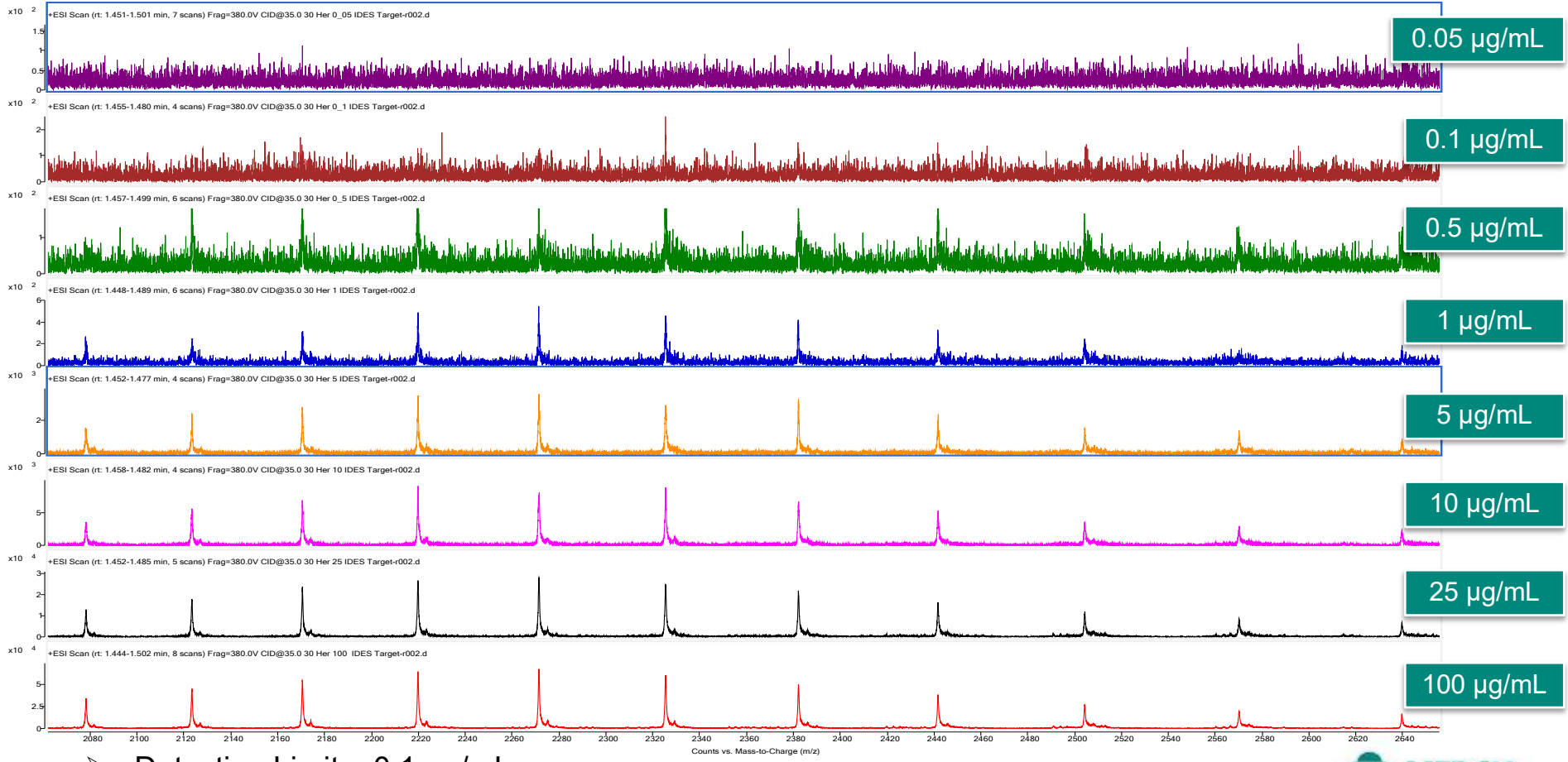




- Cleaves IgG at hinge region
- Smaller fragment has the potential to improve sensitivity. How small is small enough?

MERCK
Be well

Target Capture of 30µL Sample, On-tip IdeS Digestion

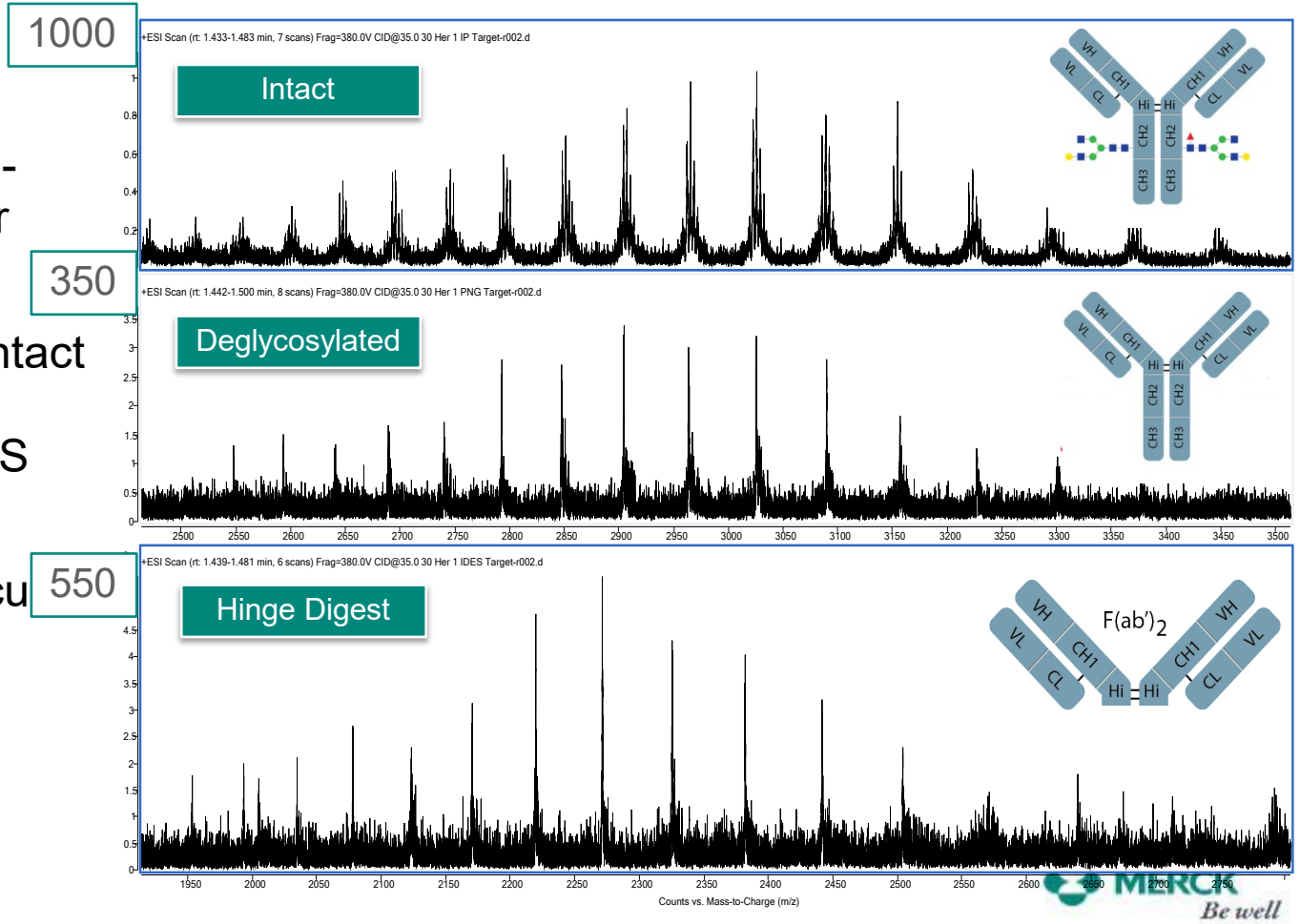


- Detection Limit = 0.1 µg/mL
- LDR = 0.1-25 µg/mL

MERCK
Be well

Improved Sensitivity?

- No gain in signal intensity from middle-down approaches for this molecule
- Overall signal from intact higher than de-glycosylated and IdeS treatments
- More complex molecu may benefit from deglycosylation



Be well

Improvements in Sensitivity

Mass Spectrometer	LC	Sample Volume (µL)		IdeS (30 µL) (µg/mL)	Deglycosylation (30 µL) (µg/mL)
		20 (µg/mL)	30 (µg/mL)		
Agilent 6545XT	Infiniti 1290	2.5 - 100	0.1 - 100	0.1 - 100	0.1 - 100

- No improvement with hinge digestion or de-glycosylation, this will depend on complexity of molecule and size
- Increased sensitivity with larger sample volume and on-tip enrichment
  - 2.5 → 0.1 µg/mL

Data Processing Papers

Quantitation of intact monoclonal antibody in biological samples: comparison of different data processing strategies

Bioanalysis (2018) 10(13), 1055–1067

Xi Qiu[1], Lijuan Kang[1], Martin Case[2], Naidong Weng[1] & Wenying Jian*,[1]

[1]Pharmacokinetics, Dynamics & Metabolism (PDM), Janssen Research & Development, Pharmaceutical Companies of Johnson & Johnson, 1400 Mckean Road, Spring House, PA 19477

[2]Janssen Biotherapeutics, Janssen Research & Development, Pharmaceutical Companies of Johnson & Johnson, 3210 Merryfield Row, San Diego, CA 92121

Toward best practices in data processing and analysis for intact biotherapeutics by MS in quantitative bioanalysis

Bioanalysis (2017) 9(23), 1883–1893

John F Kellie*,[1], Jonathan R Kehler[1], Molly Z Karlinsey[1] & Scott G Summerfield[12]

[1]Bioanalysis, Immunogenicity & Biomarkers, In vitro/In vivo Translation Platform, R&D Platform Technology & Science, GSK, 709 Swedeland Rd. King of Prussia, PA, 19460, USA

[2]Bioanalysis, Immunogenicity & Biomarkers, In vitro/In vivo Translation Platform, R&D Platform Technology & Science, GSK, David Jack Centre for R&D, Park Road, Ware, Hertfordshire SG12 0DP, UK

Generic Hybrid Ligand Binding Assay Liquid Chromatography High-Resolution Mass Spectrometry-Based Workflow for Multiplexed Human Immunoglobulin G1 Quantification at the Intact Protein Level: Application to Preclinical Pharmacokinetic Studies

DOI: 10.1021/acs.analchem.6b04997
Anal. Chem. 2017, 89, 2628–2635

Christian Lanshoeft,[†,‡] Sarah Cianférani,[‡] and Olivier Heudi*,[†]

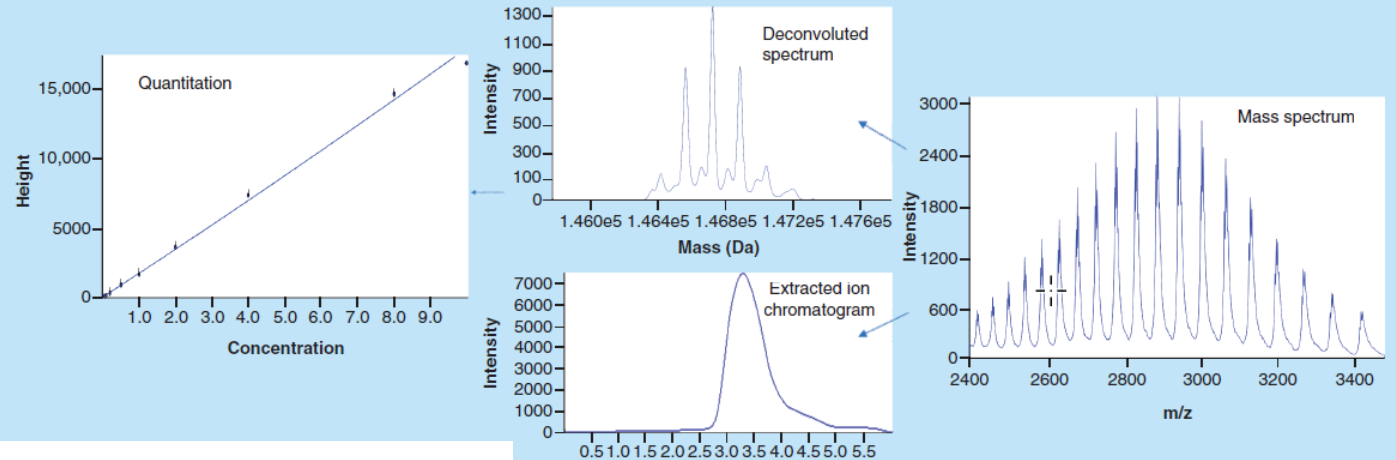
[†]Novartis Institutes for Biomedical Research, Drug Metabolism and Pharmacokinetics, Novartis Campus, 4056 Basel, Switzerland

[‡]Laboratoire de Spectrométrie de Masse BioOrganique, Université de Strasbourg, CNRS, IPHC UMR 7178, 67000 Strasbourg, France

MERCK
Be well

Data Processing Approaches

- Deconvolution of mass spectra from multiple charged ions and use the deconvoluted peak for quantitation
- Extracted ion chromatograms (XIC) of one or more charged ions using a defined mass extraction window (MXW)



Bioanalysis (2018) 10(13), 1055–1067

MERCK Be well

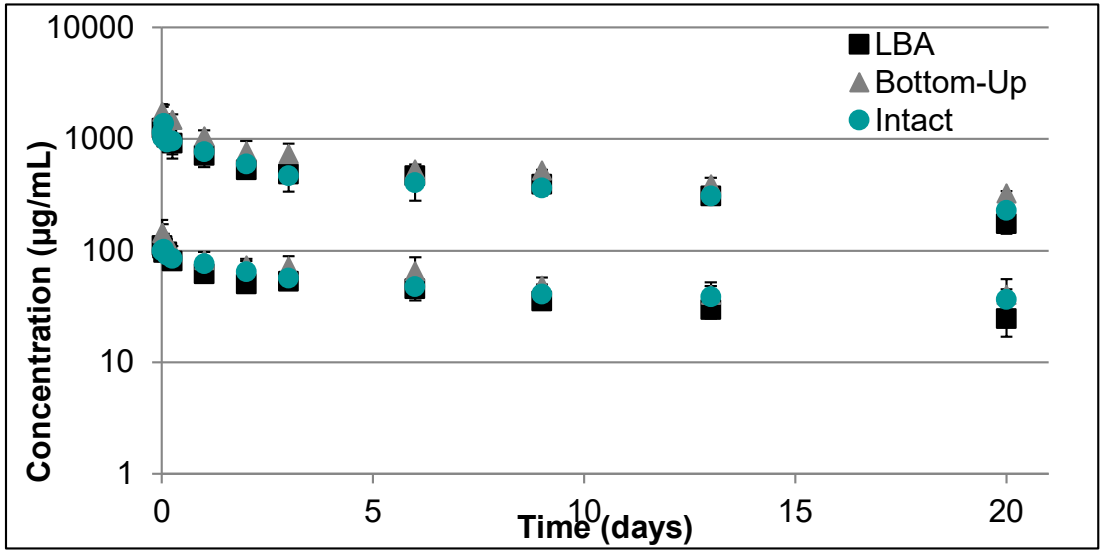
Challenges of Data Processing

- Deconvolution Approach:
  - Software is a “blackbox” and output is dependent on selection of starting parameters and varies by vendor
  - Settings used for contrived samples may not translate to incurred samples
  - Fully automated approaches are lacking, manual intervention and “tweaking” of settings will limit adoption in a regulated environment

Challenges of Data Processing

- Summed XIC Approach:
  - Currently requires manual selection of both extraction window and number of charge states to sum
  - No automated way to assess quality of data while varying extraction window and number of charge states
  - Most straightforward approach, but does not capture catabolite information that the deconvolution approach does

Linearity, Precision and Accuracy for Intact HRMS				
R^2 = 0.991	% Bias		%CV	
Conc. (µg/mL)	Interday	Intraday	Interday	Intraday
0.5	-15.6	-12.3	23.1	18.2
1	3.8	5.9	6.7	11.1
2	6.4	0.0	2.0	16.1
5	4.1	6.2	16.6	12.9
10	6.7	6.0	11.6	8.2
25	-1.8	-5.0	15.5	12.6
50	-13.3	-6.4	16.1	12.8
100	-4.4	-12.8	11.6	13.3
LQC	3.1	3.0	11.9	16.7
MQC	4.2	0.0	16.6	13.5
HQC	-7.7	-8.6	17.0	15.4



LBA data courtesy of SuChun Hsieh

Limitations of Intact Quantitative Analysis of Proteins

- Need improved mass spectrometry performance
  - Response for intact molecules not as good as for peptides as signal is spread over many charge states, limited dynamic range
- Chromatography of intact proteins is challenging
  - Sharper peaks would enhance MS detection
  - Proteins are sticky
- Interferences from bio-matrices
  - How do we look at human mAb in a human serum matrix?
- Routine data processing tools don't yet exist
  - Extract specific m/z values?
  - Deconvolute and use area?

Intact mass analysis for protein quantitation requires more research.

Conclusions

- Triple quadrupole approaches will continue to dominate mass spectrometry-based approaches for protein quantitation
- Current HRMS instrumentation has resolution and sensitivity for intact quantitation of biotherapeutics, limited dynamic range
- Middle-down approaches may provide some improvements, but on a case-by-case basis
- Areas for improvement
  - Chromatographic peak shape and robustness
  - Software for data processing

MERCK
Be well

Questions?

- **Intact Protein Mass Spectrometry for Therapeutic Protein Quantitation, Pharmacokinetics and Biotransformation in Pre-Clinical and Clinical Studies: An Industry Perspective**

  - John F. Kellie[a], John C. Tran[b], Wenying Jian[c], Barry Jones[d], John T. Mehl[e], Ying Ge[f], Jack Henion[g], and Kevin P. Bateman[h]
    - a. Bioanalysis, Immunogenicity & Biomarkers, GSK, Collegeville, PA
    - b. Biochemical & Cellular Pharmacology, Genentech Inc., South San Francisco, CA
    - c. DMPK, Janssen Research & Development, Johnson & Johnson, Spring House, PA
    - d. Q Squared Solutions, 19 Brown Road, Ithaca, NY 14850, USA
    - e. Bioanalytical Research, Bristol-Myers Squibb, Princeton, NJ
    - f. Department of Cell and Regenerative Biology, Department of Chemistry, Human Proteomics Program, University of Wisconsin-Madison, Madison, WI, 53705
    - g. Advion, Inc., 61 Brown Rd., Ithaca, NY 14850
    - h. PPDM, Merck & Co., Inc., West Point, PA, USA

MERCK
Be well