Bioanalysis of Intact Biotherapeutics by Hybrid LBA/LCMS: Challenges & Solutions

Regulated Bioanalysis Interest Group (RBIG)
Tuesday June 5th 2018

Presiding:
Dian Su (Genentech), Jian Wang (Bristol-Myers Squibb) and Fabio Garofolo (Angelini Pharma)

Panelists:
Kevin Bateman (Merck), Matthew Szapacs (GlaxoSmithKline), John Mehl (Bristol-Myers Squibb) and Shawna Hengel (Seattle Genetics)

Summary of program and discussion

- **Estimate of attendance:** ~150
- Agenda: **presentation followed by panel discussion** (please see page 3)
- **Presentations** (Please see pages 4-36, about 5min/presentation)
- **Pre-workshop survey questions for discussion** (Please see pages 37-42)
- **Discussion was pretty live. Topics ranged from data process to sample preparation. Challenges were brought up in the aspects in sensitivity. Hot topics are summarized as below:**
  - **Subunits vs. Intact detection**: different observation by difference organization: increase vs. similar in MS signal at low level; increase in MS signal at higher level
  - **Benefits** of intact quantification compared to surrogate peptide quantification: capture important biotransformation such as oxidation (***Matt Szapacs from GSK***)
  - **General challenges** for intact quantification:
    1. Overall sensitivity is about 10x lower for intact LC-MS quantification vs ELISA assay, what can we do to improve the sensitivity: affinity capture step for enrichment;/subunits for quantification (***Kevin Bateman from Merck***) Some audience proposed to use HCD technique to measure product ion
    2. Not pretty chromatography of intact quantification relative to peptide quantification
  - **Data processing:** Deconvolution vs. charge states
    1. Deconvolution: deconvolution should be used with caution-compound dependent (***Matt Szapacs from GSK***); some deconvolution software result in ghost peaks.
    2. Charge states: Should just a couple most intense m/z ions or more should be used for quantification (***Matt Szapacs from GSK***)
    3. Deconvolution vs. charge states for intact quantification: some didn't observe significant difference between the two approaches however, there is a need for software improvements to enable more efficient and complete use of deconvolution for intact mass quantification. (***John T. Mehl from BMS***)
    4. Sample preparation is important to mitigating detection bias by intact quantification (***Shawna Hengel from Seattle Genetics***)

Agenda

05:45pm-05:55pm – Introduction (Discussion Topics & Panelists)

05:55pm-06:00pm - *Intact MS of Proteins for Quantitative Analysis at Merck*

- Kevin Bateman (Merck)

06:00pm-06:05pm – *Intact and Subunit-level Antibody Analysis from In-life Samples at GSK* -

Matthew Szapacs (GlaxoSmithKline)

06:05pm-06:10pm – *Intact mAb Quantification -Does Deconvolution Make a Difference?*

- John Mehl (Bristol-Myers Squibb)

06:10pm- 06:15pm – *Intact MS of Proteins for Quantitative Analysis at Seattle Genetics*

- Shawna Hengel (Seattle Genetics)

06:15pm-07:00pm – Panel Discussion

Introduction

- Pros/Cons of using HRMS (**QTOF & Orbitrap**) for bioanalysis of intact therapeutic proteins and/or subunits
- ***"Bottom-up"*** (signature peptide) and ***"Top-down"*** strategies in Bioanalysis
- Limitation of **signature peptide** approach to provide sufficient information on the biotherapeutics measured
- ***"Lost in digestion"*** - how to preserve the therapeutic protein for intact quantification

Introduction (Cont.)

- Identification and quantitation of **catabolites** for a better understanding of the various circulating biotherapeutic forms, biotransformation, glycoforms quantitation and post-translational modifications.
- How to overcome **sensitivity** issues in therapeutic intact protein quantification
- Advantages of **summing isotope signals** on charge state & isotope effects on S/N;
- Optimizing extraction window (**XIC**) for quantitation,
- Intact biotherapeutics bioanalysis by **Hybrid LBA/LCMS** in a fully **Regulated Environment**.

INTACT MS OF PROTEINS FOR QUANTITATIVE ANALYSIS AT MERCK

Lisa O'Callaghan, Daniel Spellman, Kevin Bateman
Pharmacokinetics, Pharmacodynamics and Drug Metabolism (PPDM)

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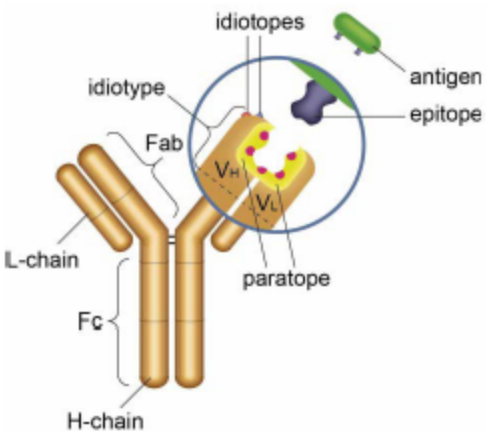
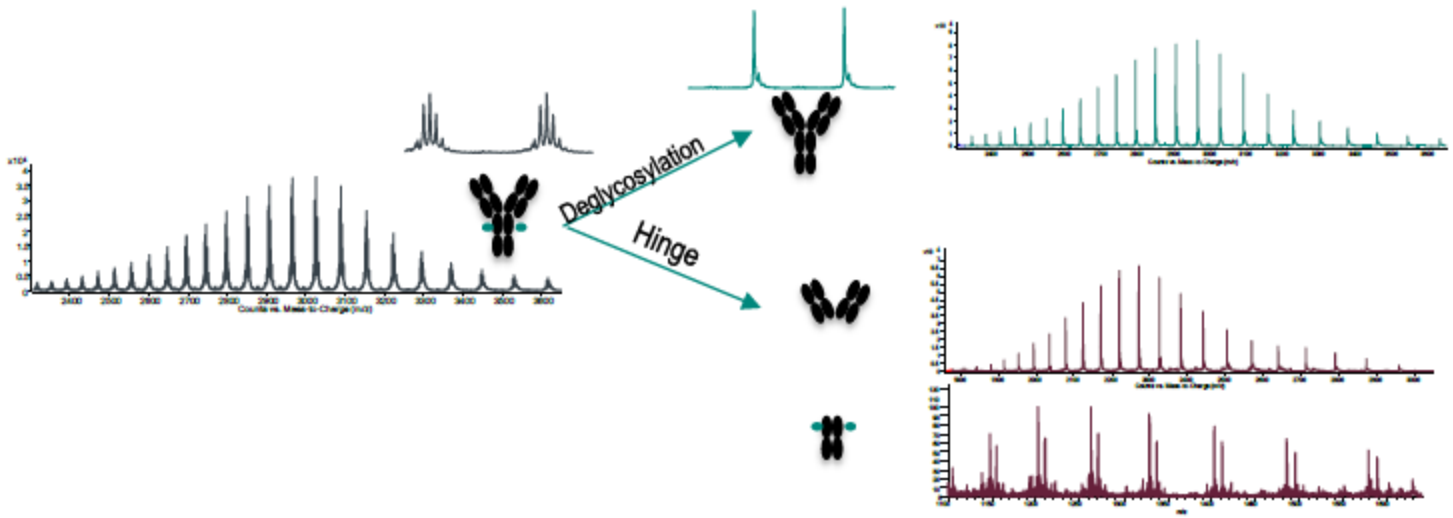
June 5, 2018
ASMS Conference on Mass Spectrometry and Allied Topics

How can we Increase Sensitivity?

Increase amount of analyte
- Process more sample
- More specific capture (antigen)

Make analyte more detectable
- Deglycosylation
- Hinge digestion

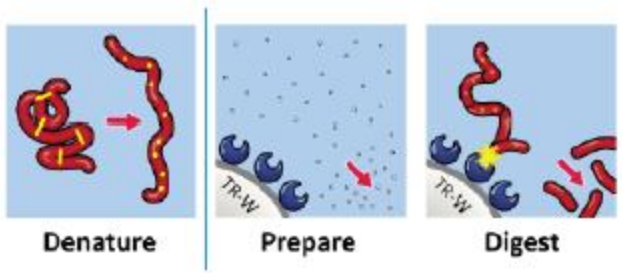


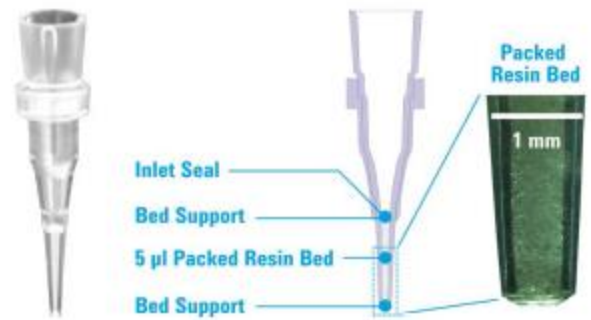


Sample Automation – Agilent AssayMap

Increase amount of analyte
- 20 → 30 µL sample volume
- 50 → 25 µL elution
- Target Capture

Make analyte more detectable
- On-cartridge deglycosylation (PNGase F)
- On-cartridge hinge digestion (IDES)





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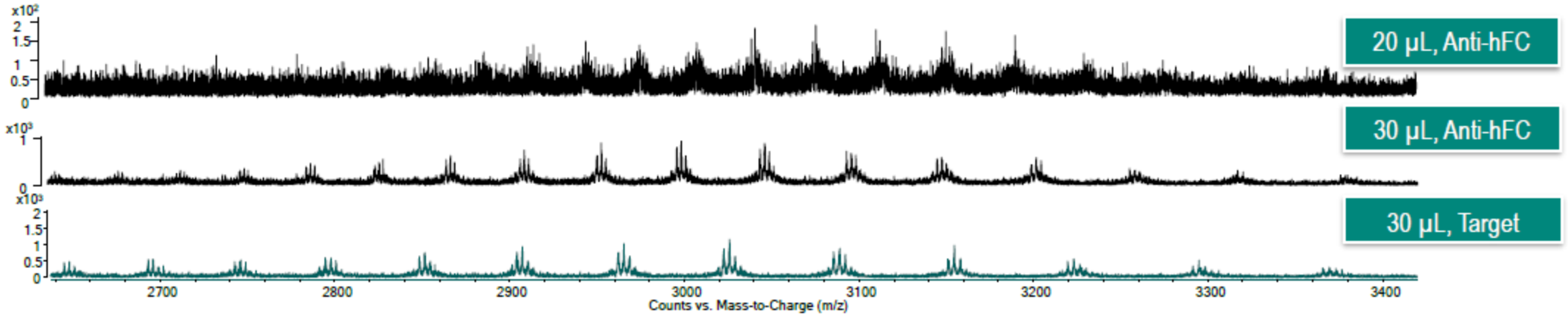
Increase in Sample Volume; Anti-human Fc vs Target Capture



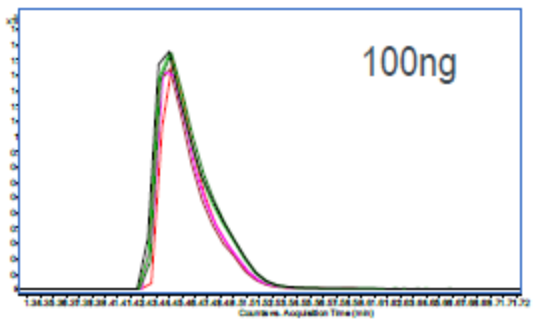
10x increase in LLOQ by sample volume increase and automated AM IA

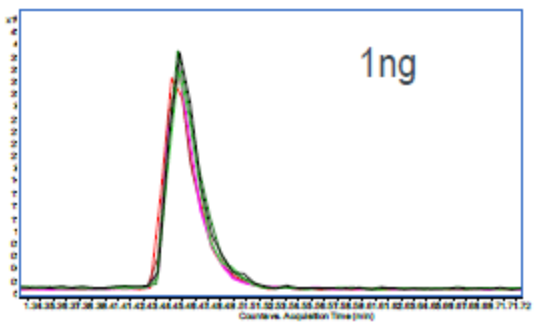
No improvement from anti-hFC to target capture

Detection Limit = 0.1 µg/mL

Reds = anti-FC

Greens = Target





Can we improve further with middle-down approach?

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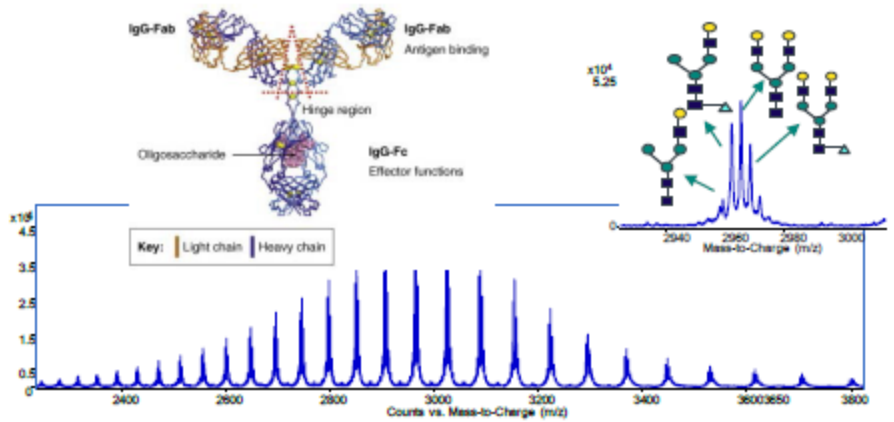
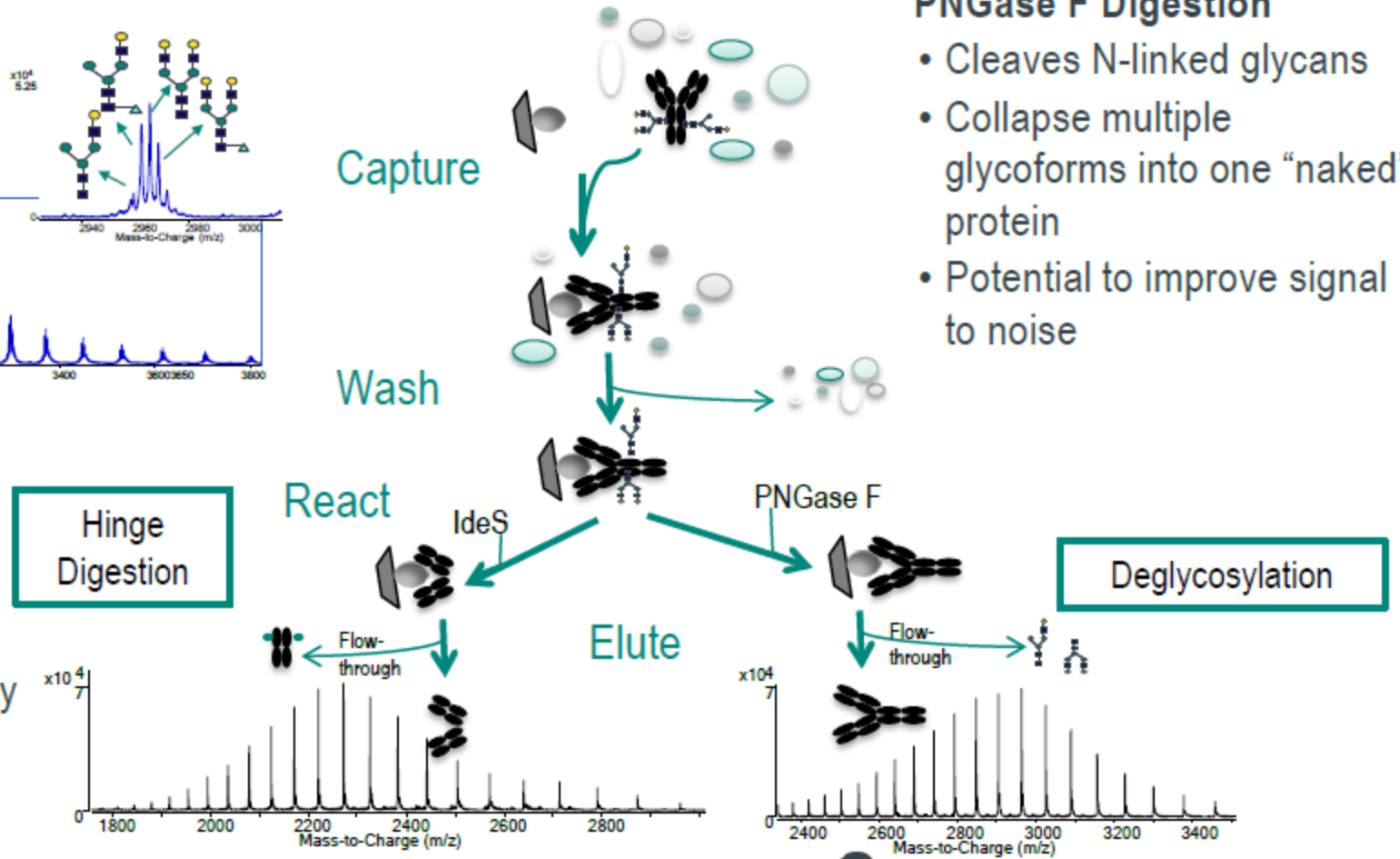
AssayMap On-Cartridge Reactions



IdeS Digestion

- Cleaves IgG at hinge region
- Smaller fragment has the potential to improve sensitivity



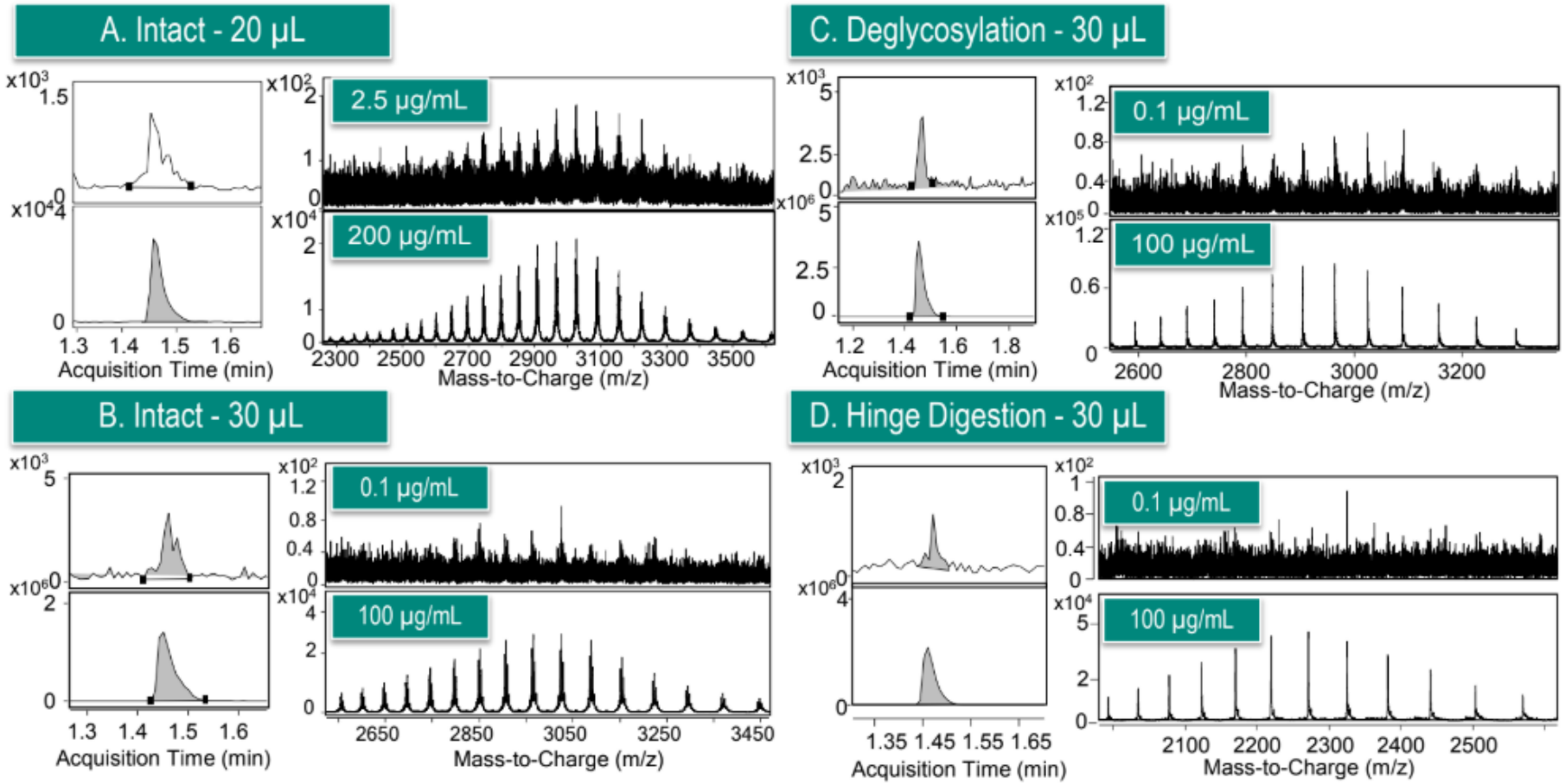
PNGase F Digestion

- Cleaves N-linked glycans
- Collapse multiple glycoforms into one "naked" protein
- Potential to improve signal to noise

Middle-Down Compared to Fully Intact

Highly concentrated samples showed strong signal increase for middle down approaches

Low concentration samples showed no improvement



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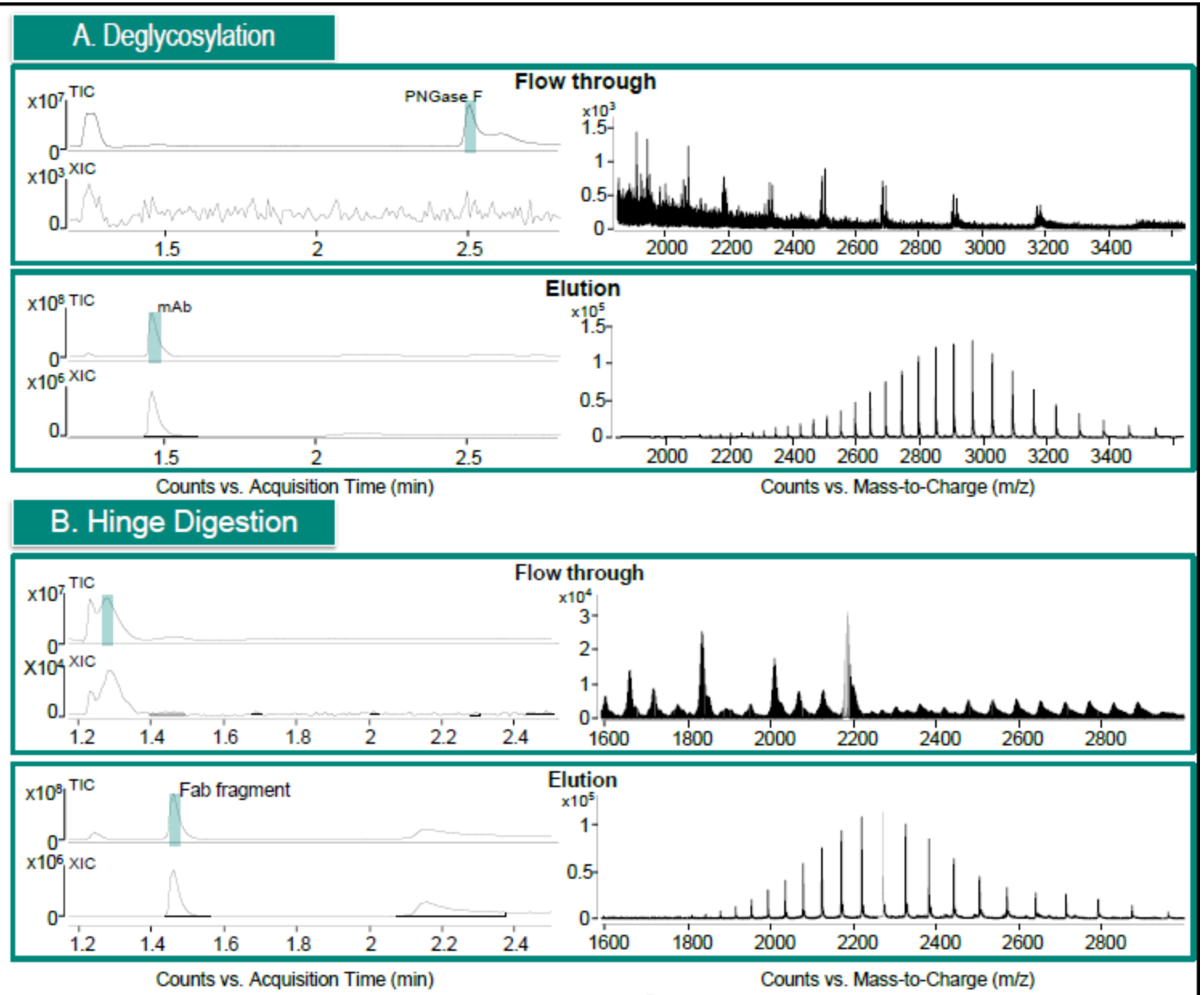
Flow Through Evaluation

Previous hypothesis: No increase in sensitivity due to losses from on-cartridge reactions

Flow through captured and assessed

No intact or digested protein captured in flow through from either on-cartridge reaction



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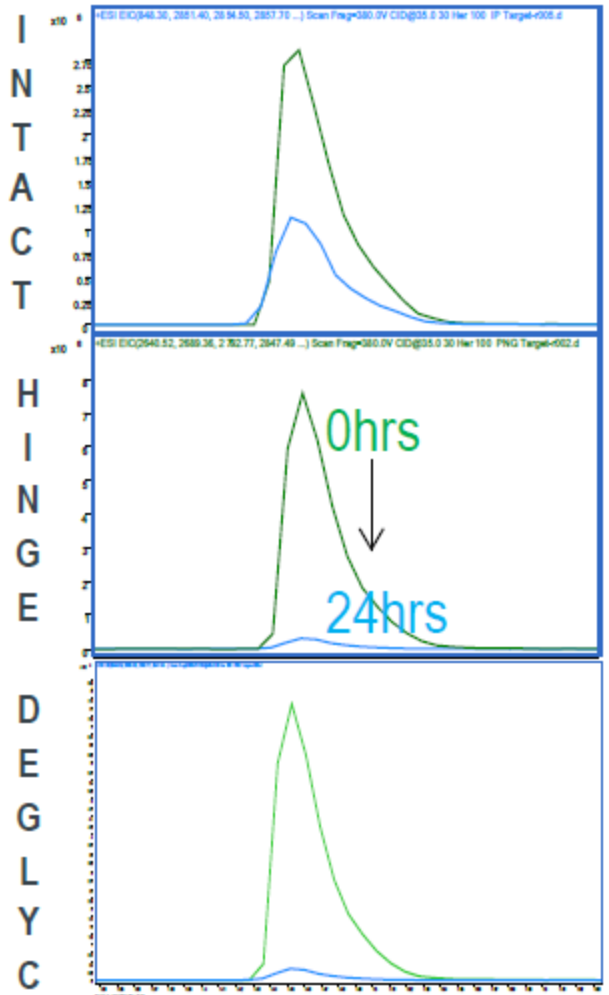
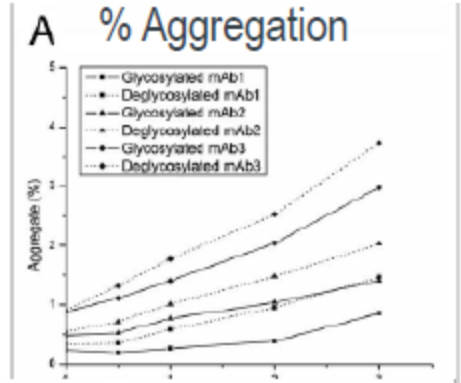
Middle-down Stability

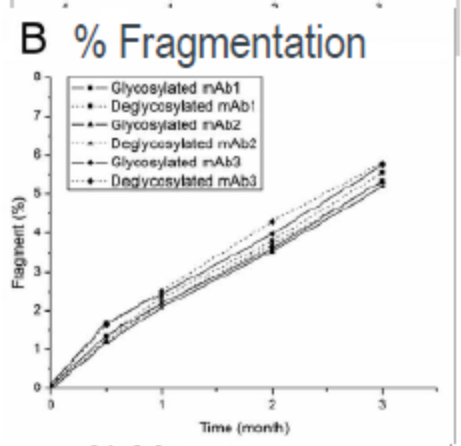


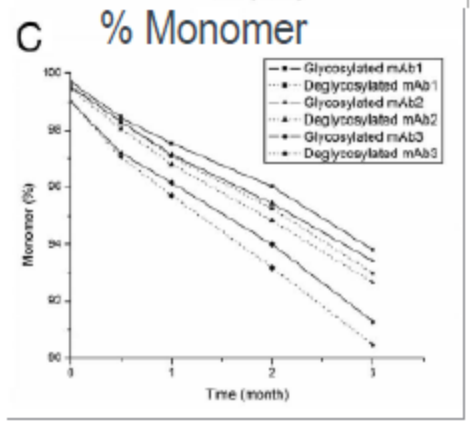
Samples reassayed 24 hrs later and large signal decrease observed

- Deglycosylated species less stable than intact?
- Deglycosylation may result in increased aggregation and decreased stability

	% loss
Intact	67%
Hinge	88%
Deglyco-sylation	96%







Bayer et al., mAbs 2011, 3:6, 568-576.

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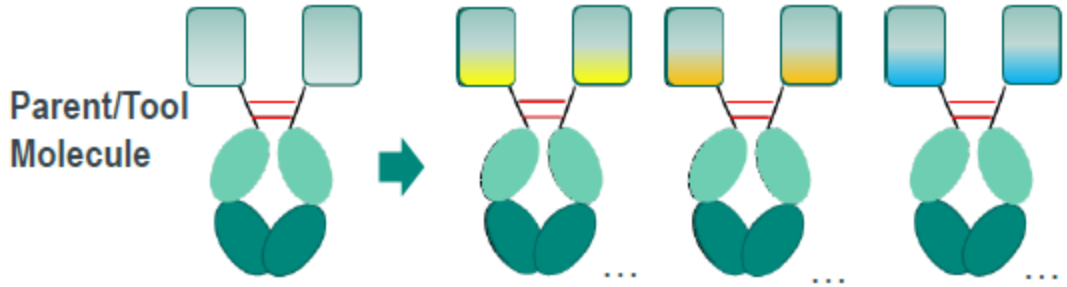
Fusion Protein Optimization



Surrogate peptide approach requires changes in detection for each analyte

Intact detection does not change

	Intact HRMS	Surrogate Peptide
Sample Volume	30 µL	5 µL
Sample Prep Time	2.5 hrs	8 hrs
LLOQ	0.5 µg/mL	0.5 µg/mL
ULOQ	60 µg/mL	100 µg/mL

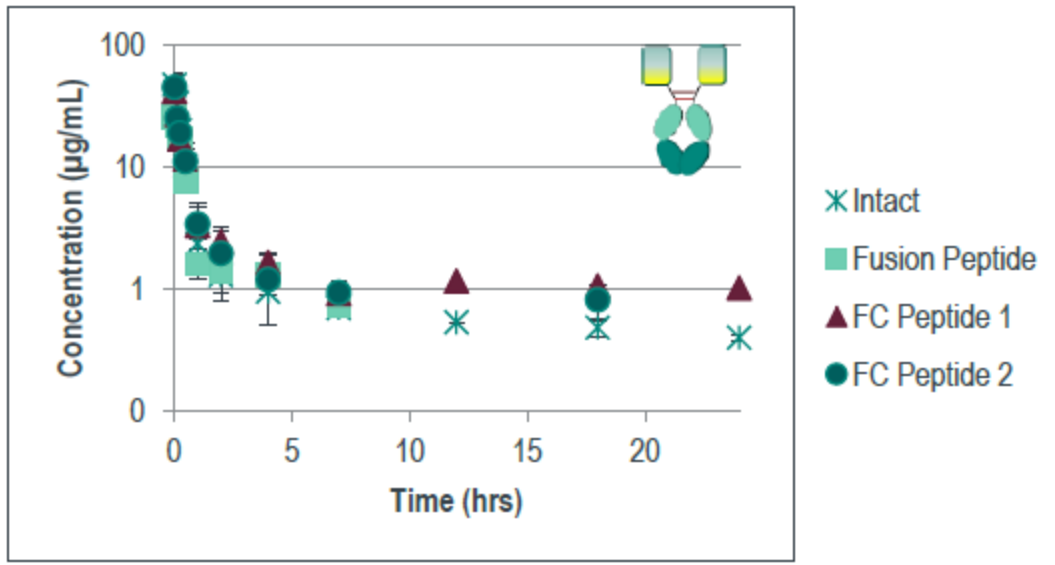
	Intact HRMS		Surrogate Peptide	
STD	Avg. Calc. Conc. (µg/mL)	Accuracy	Avg. Calc. Conc. (µg/mL)	Accuracy
LLOQ	0.5	108%	0.5	98%
1	1.0	97%	1.1	107%
2	1.9	95%	1.9	98%
3	4.8	97%	5.5	110%
4	10.4	104%	10.5	105%
5	24.8	99%	18.0	90%
6	50.7	101%	53.9	108%
ULOQ	58.9	98%	84.2	84%
LQC	1.1	107%	1.2	116%
MQC	5.3	106%	5.1	102%
HQC	24.9	100%	25.1	101%

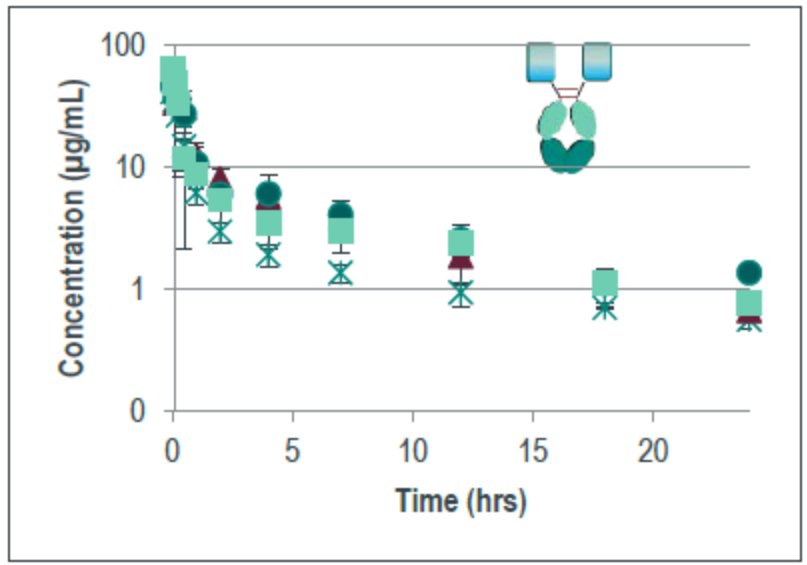
Fusion Protein PK

Intact PK achieved for several protein variants without any method changes

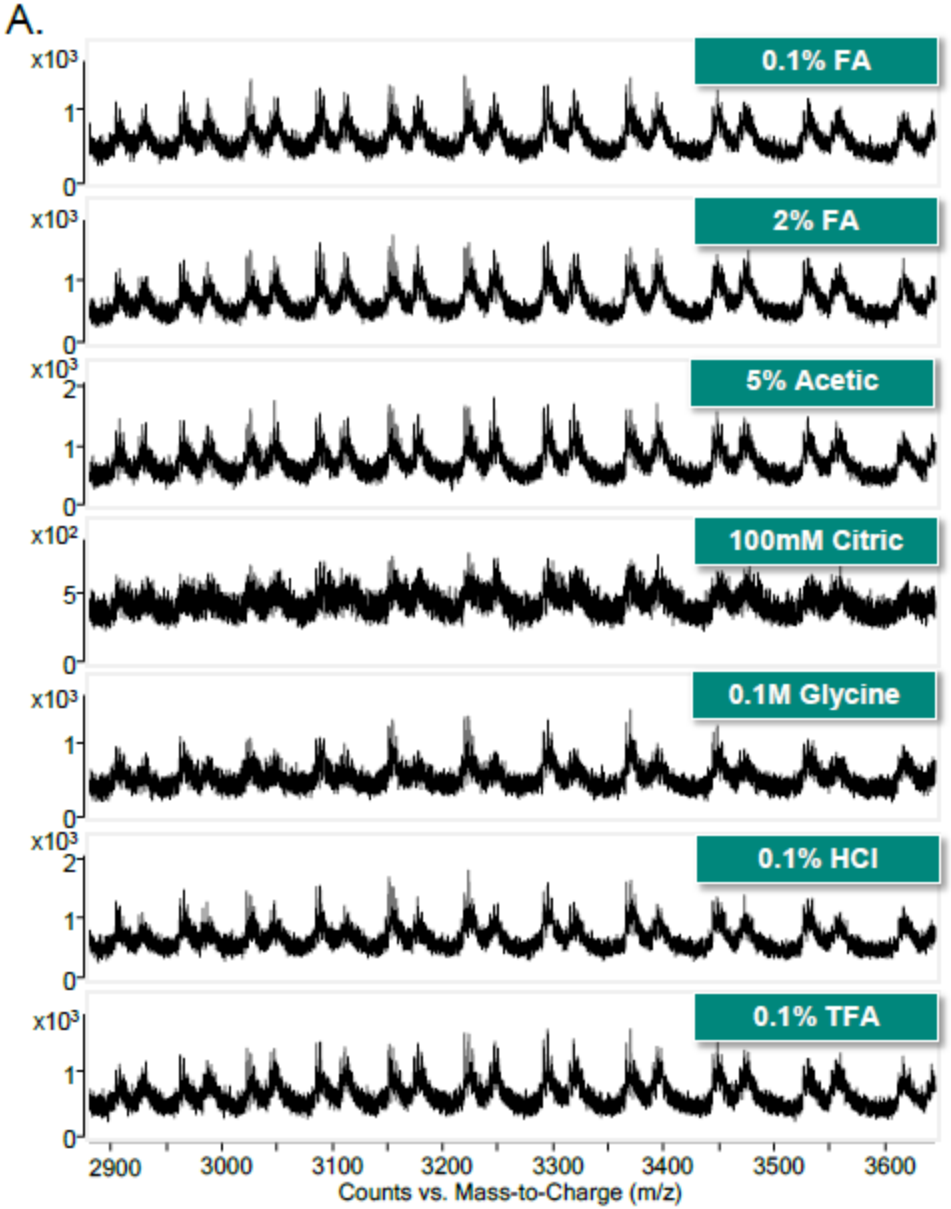
Intact shows slightly different PK at later time points

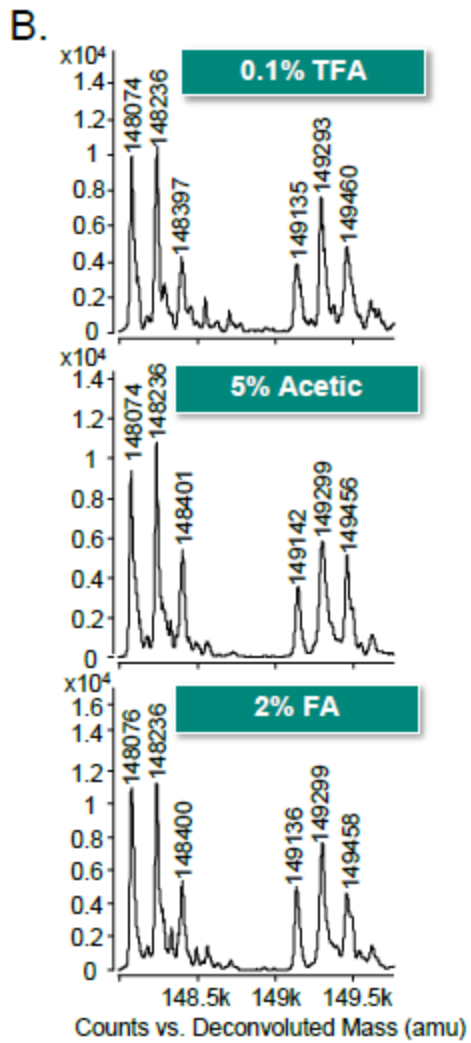
- Possible catabolism





Backups





Optimization of Elution Conditions

Seven common IA elution buffers evaluated

Max Signal: 0.1% TFA, 5% Acetic and 2% FA

Least Interference:5% Acetic and 2% FA

Most Stable: 0.1% TFA and 2% FA

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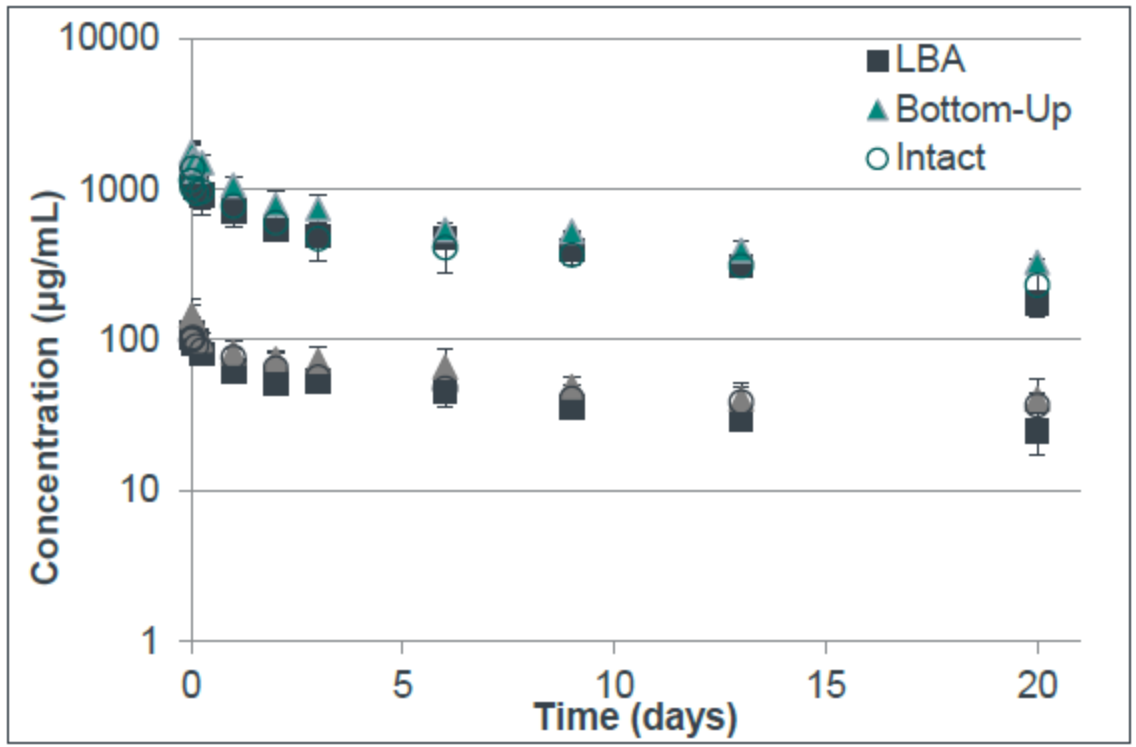
PK Study mAb IgG1: LBA vs Surrogate vs Intact

Similar PK demonstrated by all analysis techniques

Less than 30% variability across all measurements



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

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Current Sensitivity for Intact mAb PK Quantitation

Sample	Capture	Volume (µL)	LLOQ (µg/mL)	Dynamic Range (µg/mL)
Bottom-Up	Anti-Fc	5	0.05	0.05 - 200
Top-Down	Anti-Fc	20	2.5	2.5 - 200
		30	0.1	0.1 – 50
	Target	30	0.1	0.1 – 100
Deglycosylation	Target	30	0.1	0.1 – 50
Hinge Digestion	Target	30	0.1	0.1 - 100

No improvement with hinge digestion or deglycosylation

Increased sensitivity with larger sample volume and on-cartridge enrichment

- 2.5 → 0.1 µg/mL

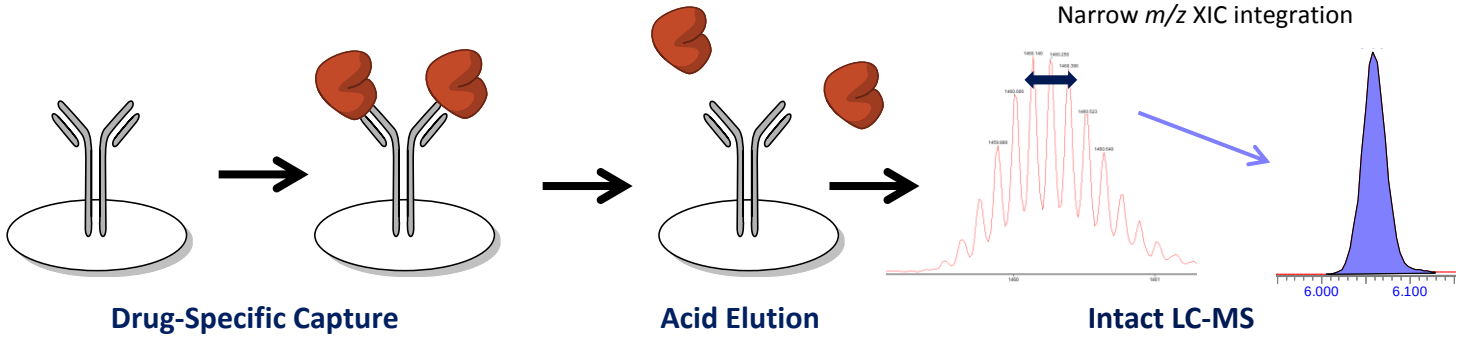
Intact and Subunit-level Antibody Analysis from In-life Samples at GSK

Matt Szapacs

Outline

- Intact domain antibody example – method validation
- mAb subunit LC-MS review
- Measuring intact and subunit concentration by MS: 2 assays, 3 analytes
- Large & intact mass quantitation strategies

Intact Mass Quantitation of Domain Antibody (~15 kDa)



Overall Statistics

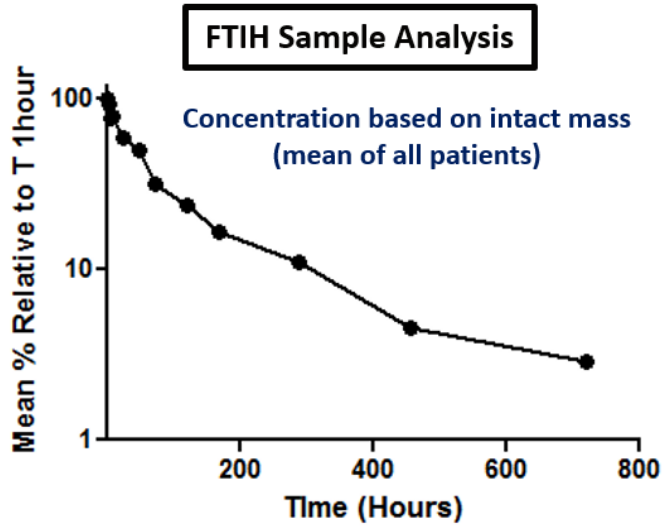
	QC 10 10 ng/mL	QC 30 30 ng/mL	QC 150 150 ng/mL	QC 1600 1600 ng/mL	QC 2000 2000 ng/mL
Mean	10.0	29.8	154.8	1441.5	1705.3
Std Dev	0.9	2.4	8.1	106.5	114.0
Precision (%CV)	8.7	8.2	5.3	7.4	6.7
Bias (%)	-0.5	-0.6	3.2	-9.9	-14.7
n	12.0	12.0	12.0	12.0	12.0
Average Within-run Precision (%)	9.1	7.9	5.7	7.8	7.4
Between-run Precision (%)	Negligible	2.5	Negligible	Negligible	Negligible

3 P&A runs: **Passed acceptance criteria**
(5 QC levels: LLQ/low/mid/high/HLQ), n=4
+/- 20% bias & Precision at each STD and QC level
+/- 25% bias & Precision at LLQ STD and QC level

Selectivity: **Passed acceptance criteria**
- Total blanks n=1 for 6 lots (no greater than 20% of the analyte response at the LLQ)
- Spike recovery at 3x LLQ in selectivity lots; n=3 per lot (+/- 20% bias and precision)

Stability: **Passed acceptance criteria** (+/- 20% bias and precision)
- Long term plasma stability @ -80°C: established 220 days
- 3 Freeze/Thaw cycles @ -80°C
- RT plasma stability for 24 hours
- Whole blood stability : Passed RT and wet ice.

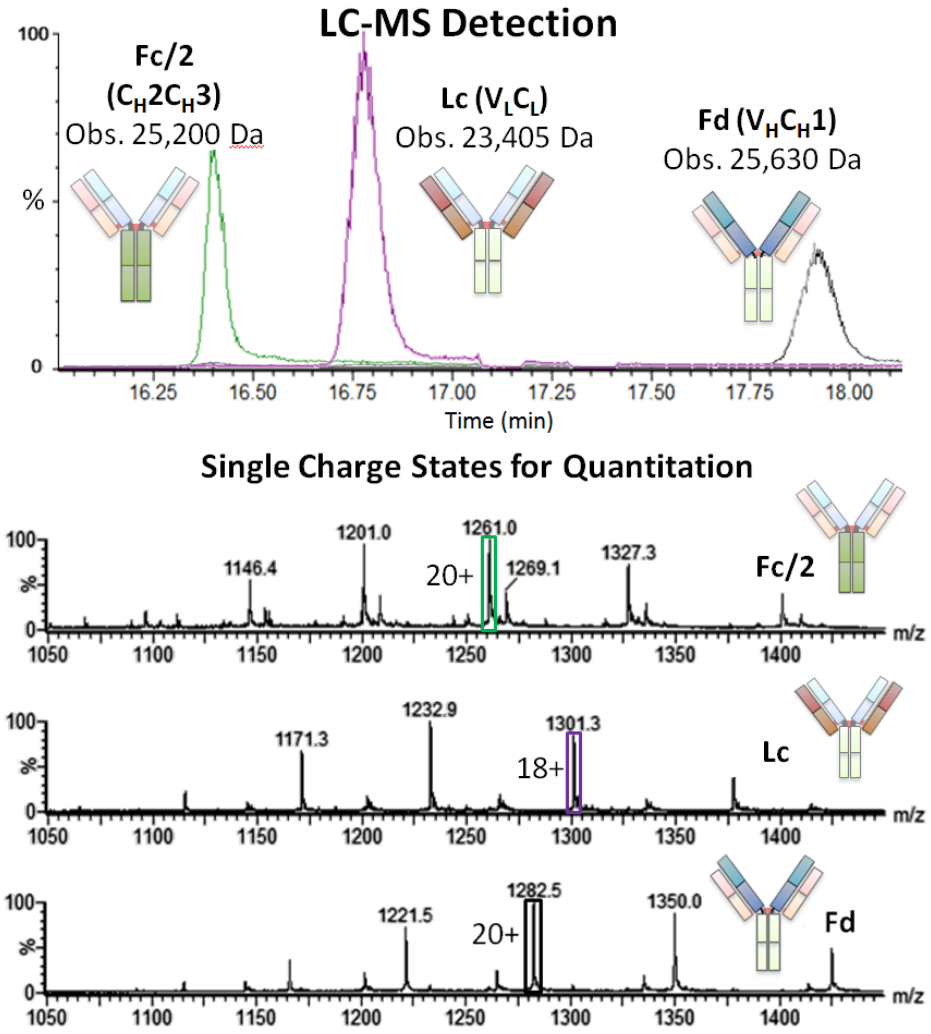
- Evidence for robust assay performance at intact mass level
- Promising for application at larger masses
- Long-term stability an important consideration

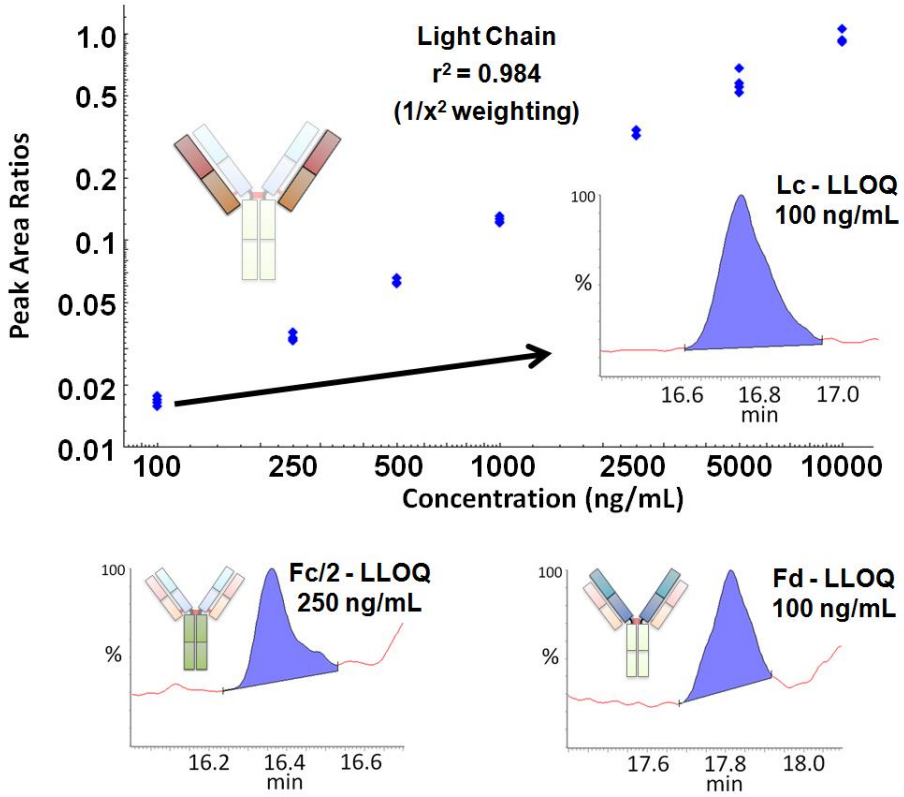


Kehler *et al.* Manuscript in Preparation

LC-MS Quantitation of the Whole Protein

Demonstrate Selectivity and Linearity in Quantitation Down to 100 ng/mL



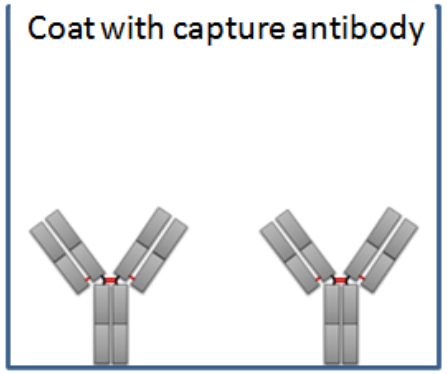


Advantages of mAb Subunit MS

- Easily monitor mass variants as low as +16 Da (oxidation)
- Localize mass variants to a particular subunit
- Subunits should agree in concentration measurement – if not then indication of unknown biotransformation on subunit(s)

Kellie *et al.* Bioanalysis, 2016, 8 (20), 2103-2114

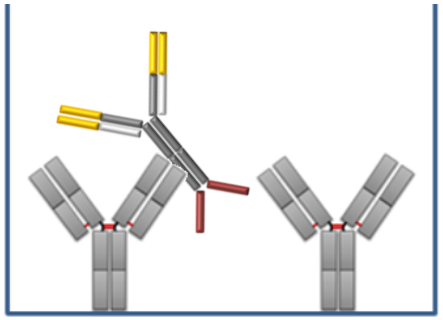
2 Assays, 3 Analytes: Intact and Reduced LC-MS Assays



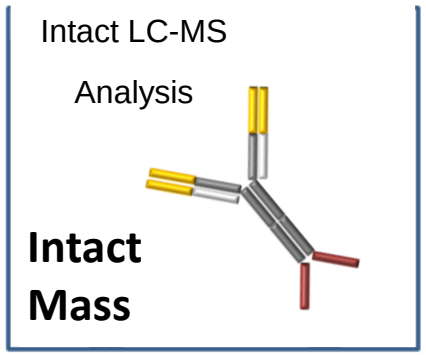
Block

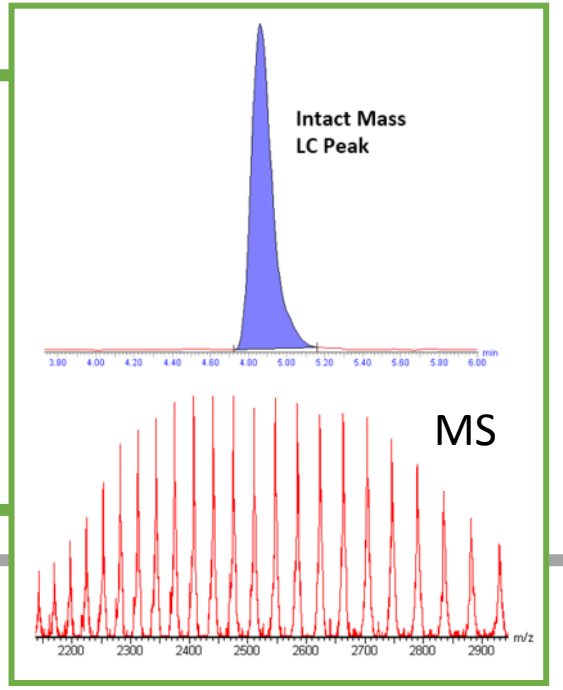
Wash

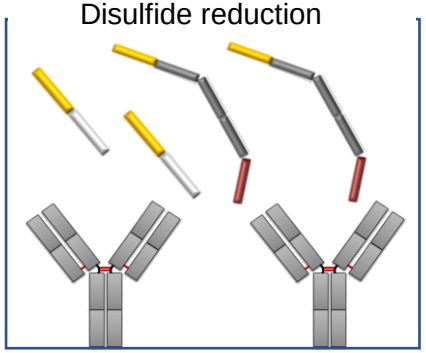
Bind Drug (1000 – 10,000 ng/mL in Rhesus Serum)



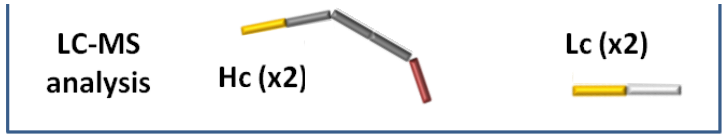
Acid Elute





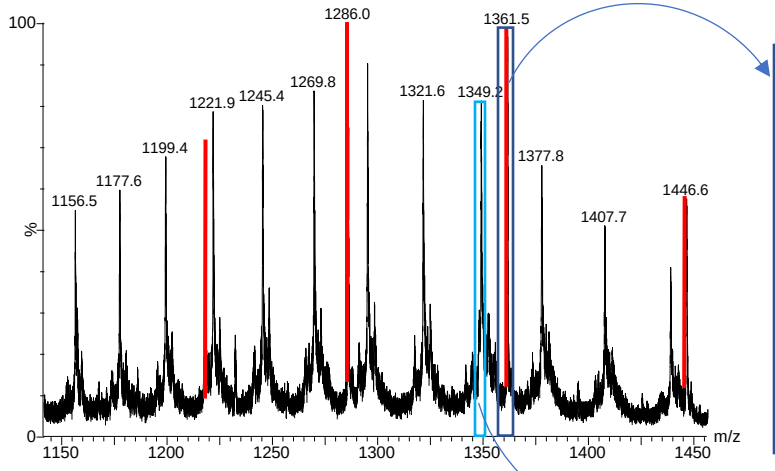


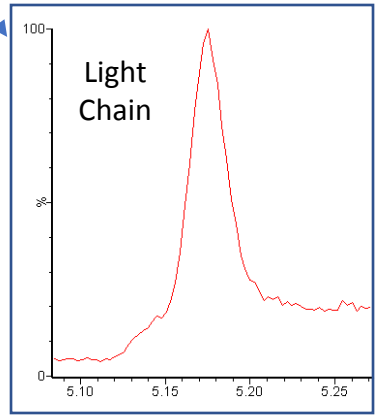
Acidify



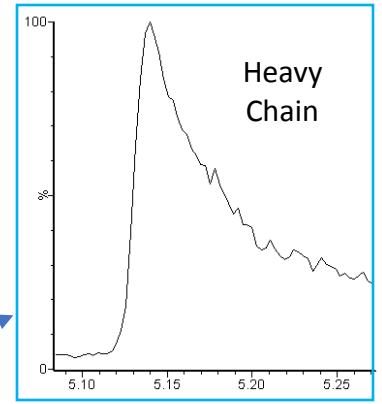
Reduced Subunits

Heavy (black) & Light Chain (red) MS



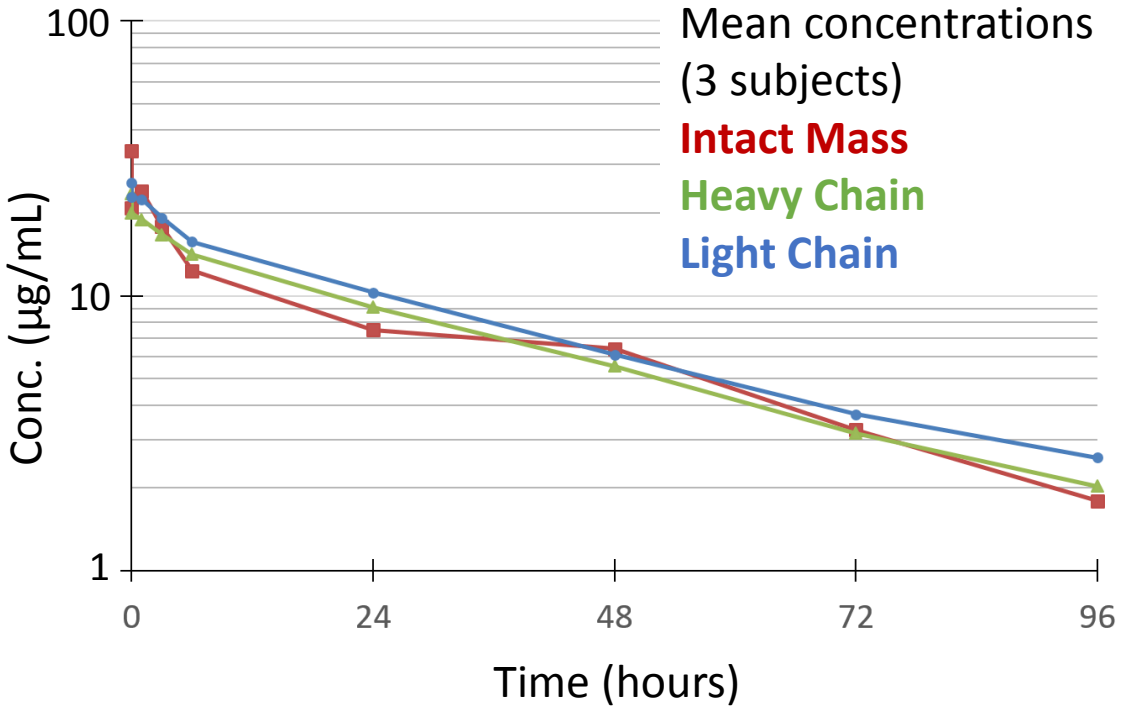


LC
Chromatograms



Pannullo, Wednesday Poster 060

Intact and Reduced Assay: Sample Results



- Reasonable agreement within assay tolerance
- Heavy Chain slightly lower than Light Chain
- Ongoing research to better characterize & understand these relationships

Individual Subject Concentration Differences (%)

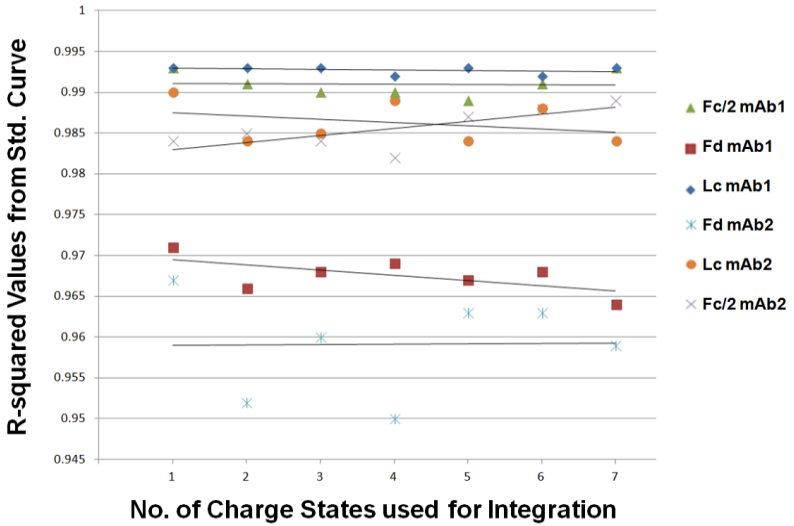
Time (hr)	HC (vs. LC) % Difference	Intact (vs. HC/LC mean) % Difference
0.083	-9.53	2.79
0.083	-13.89	64.07
0.083	-4.10	23.59
0.5	-12.52	-8.05
0.5	-13.02	17.14
0.5	-15.68	-19.29
1	-15.84	1.99
1	-18.30	23.55
1	-16.87	20.34
3	-21.28	-24.03
3	-9.86	14.85
3	-9.48	7.23
6	-13.80	-10.39
6	-5.66	-16.65
6	-10.96	-33.41
24	-7.56	-36.99
24	-19.67	-16.52
24	-9.57	-22.22
48	-2.85	8.89
48	-9.86	7.29
48	-16.73	13.39
72	-12.71	-9.59
72	-17.76	4.67
72	-15.97	-19.67
96	-28.22	-40.49
96	-19.23	-6.47
96	-25.74	-41.09

Pannullo, Wednesday Poster 060

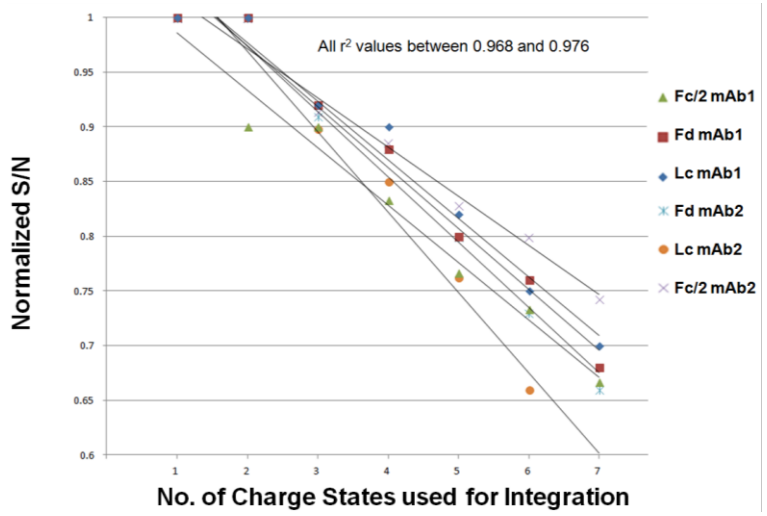
LC-MS Data: Quantitation Strategies

- Try to use “simpler is better” approach - Use the fewest peaks / charge states possible for quantitation
- Similar assay performance for using few vs. many charge states
- Use of many peaks places greater burden on data review (e.g. is that peak real?)
- Deconvolution use is appropriate sometimes, but may be problematic in a regulated setting

Assay performance is similar regardless of number of charge states used…

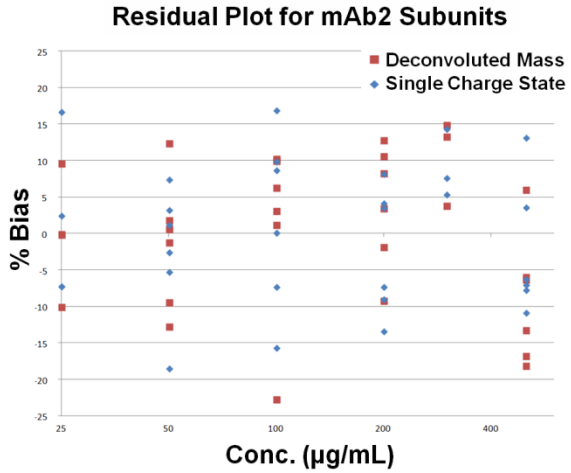


…But LLOQ S/N decreases with increasing charge states used

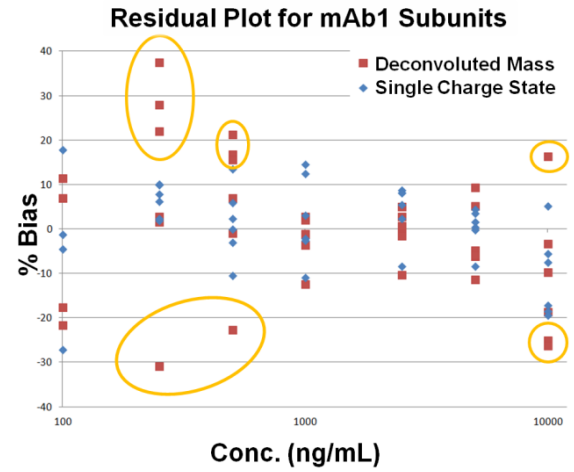


Kellie, *et al.* Bioanalysis, 2017, 9(23), 1883-1893.

Deconvolution can yield similar results as single charge states…



…But not for all compounds/assays



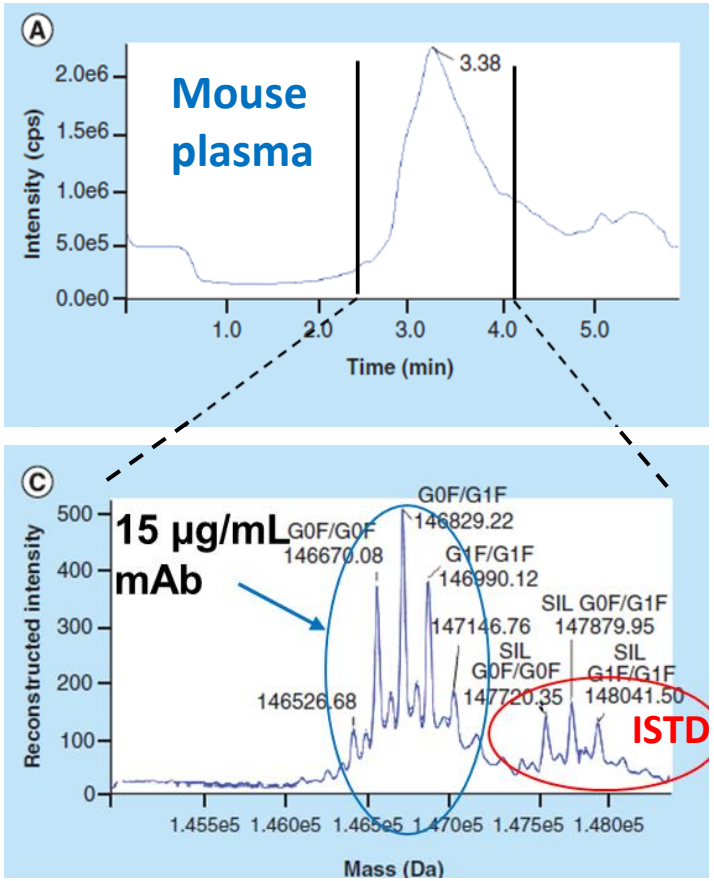
Intact mAb Quantification
Does Deconvolution Make a Difference?

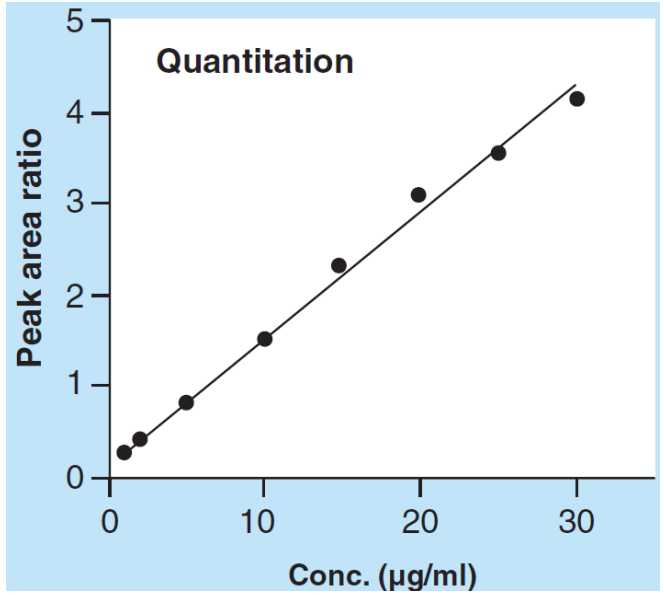
John T. Mehl, Ph.D.
Bioanalytical Research
Bristol-Myers Squibb
Princeton, NJ

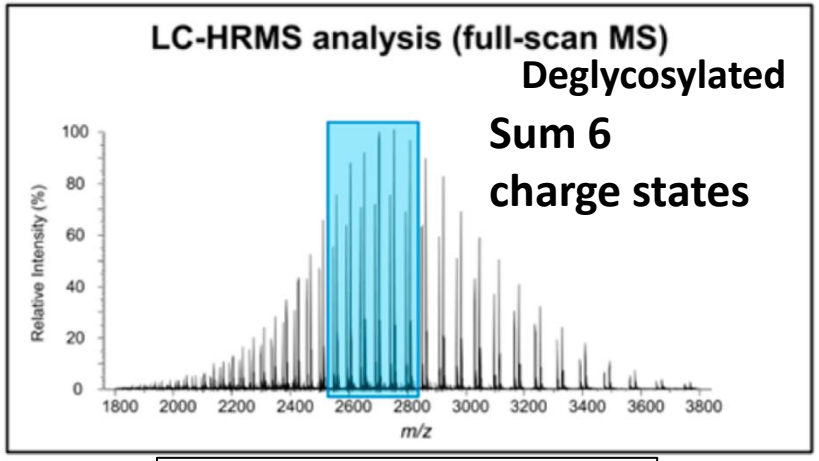
ASMS RBIG Workshop, San Diego, CA, June-5 2018

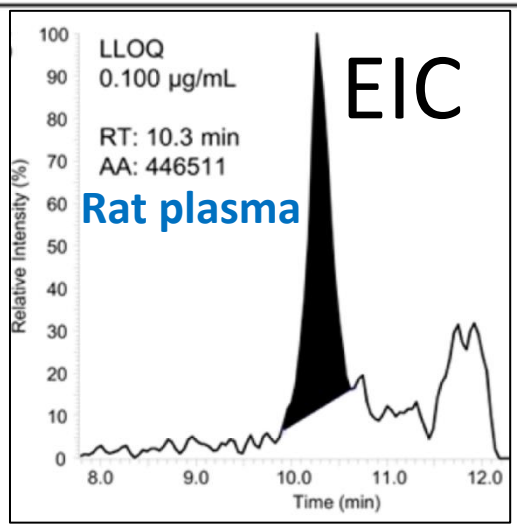
Quantitative analysis of mAbs using Intact Mass

Spectrum Deconvolution or Extracted-ion chromatogram







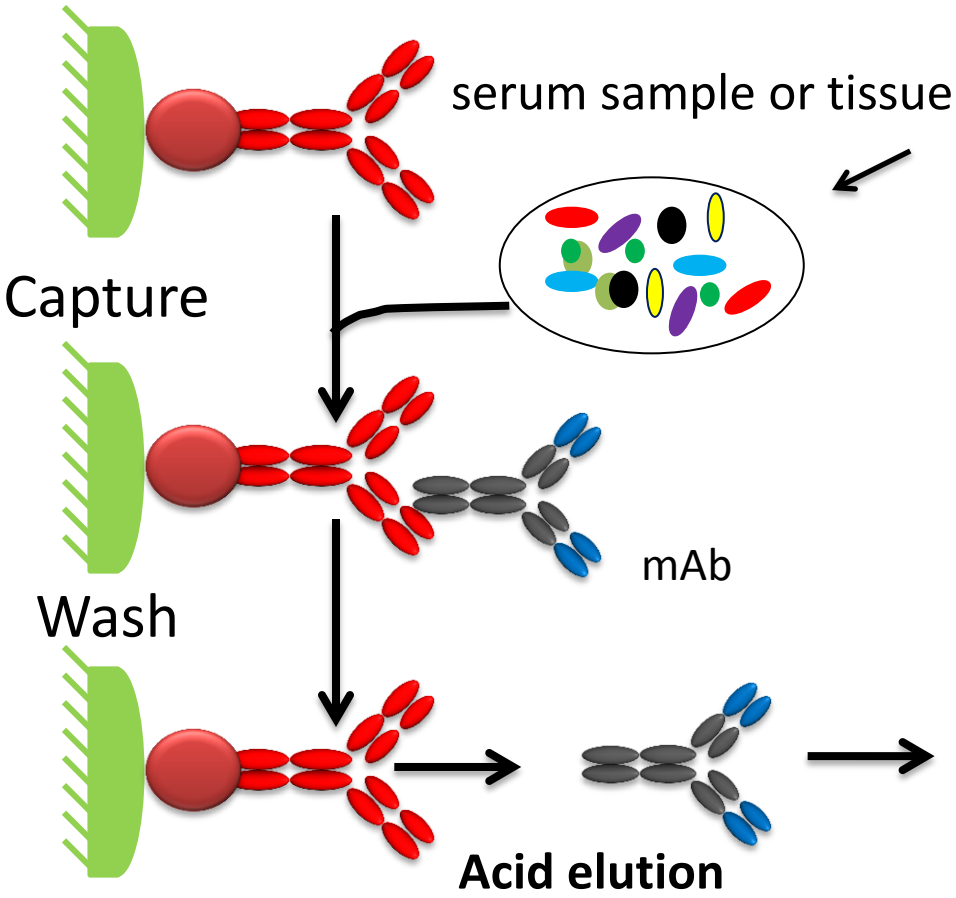


Jian, W., *et al., Bioanalysis* (**2016**) 8(16), 1679-1691

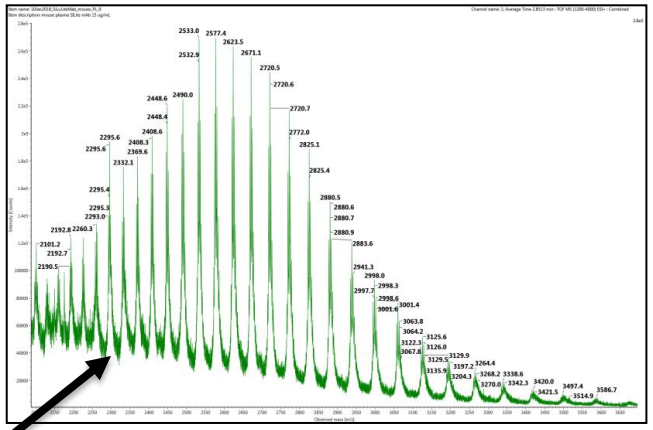
Lanshoeft C., *et al., Anal Chem* (**2017**) 89, 2628-2635

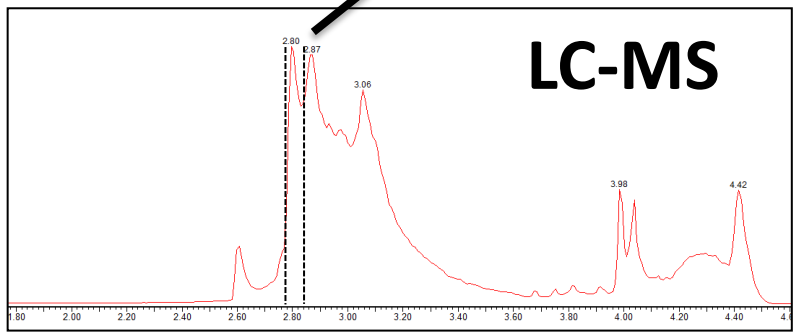
Immunocapture sample preparation; Hybrid LC-MS

Immobilized anti-human-Fc capture

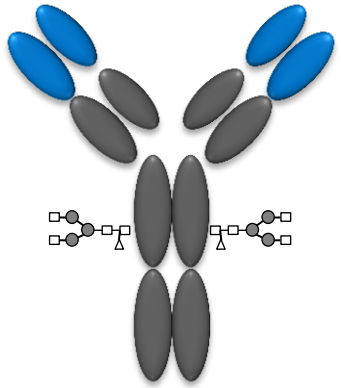


HRMS Full-scan





Intact mAb Quantification



SiLu™MAb
Human mAb
From Sigma-Aldrich

What level of sensitivity can be achieved ?

How to process data; EIC or Deconvolution ?

Example 1

Intact mAb Sample Prep and LC-MS conditions

Mouse plasma (100µL)
anti-Human Fc magnetic beads

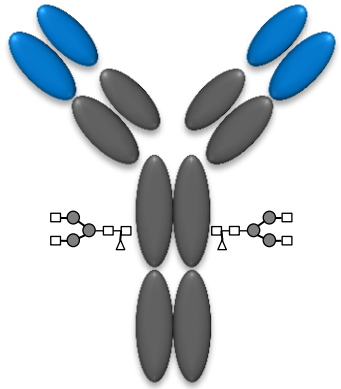
Immunocapture

↓

Elution

↓

LC-MS analysis (Xevo G2-XS Q-TOF),

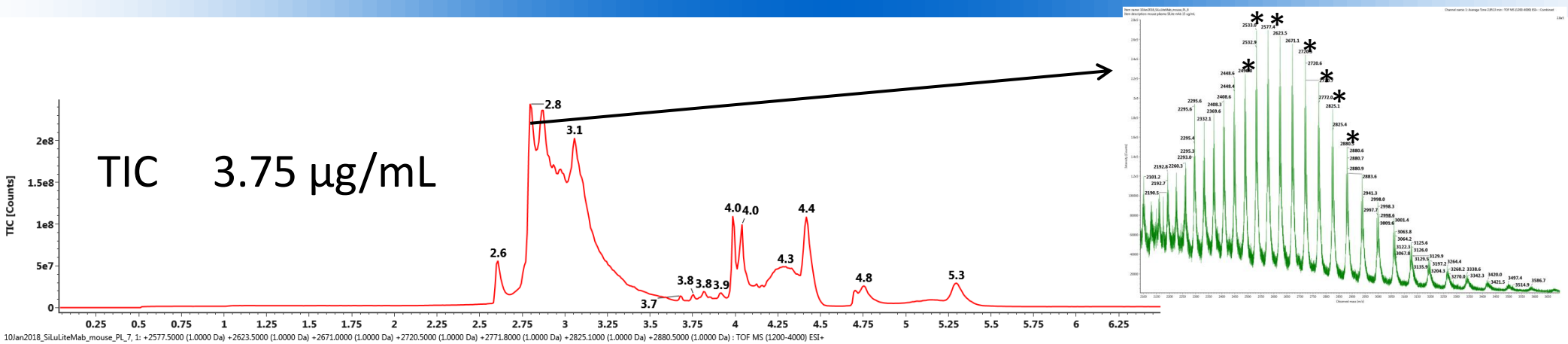


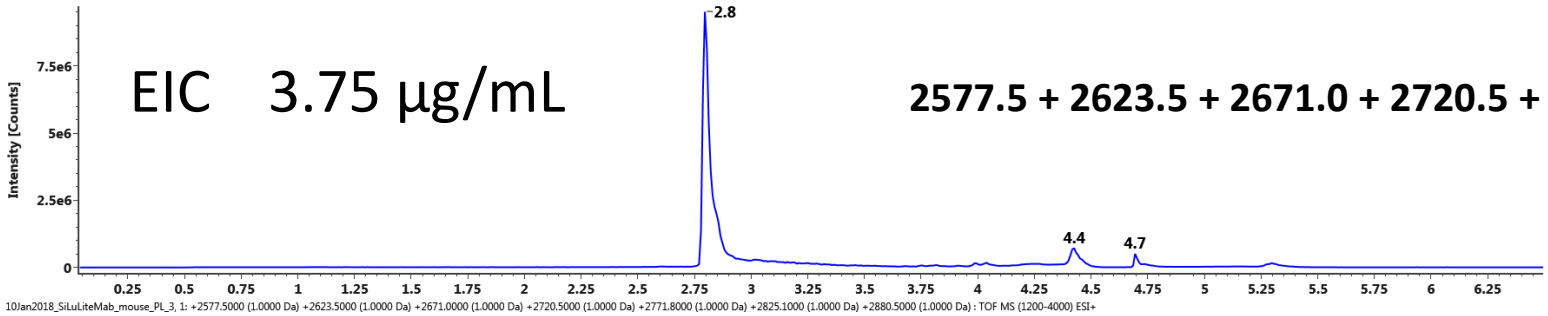
SiLu™MAb
Human mAb
From Sigma-Aldrich

6.5 min LC method

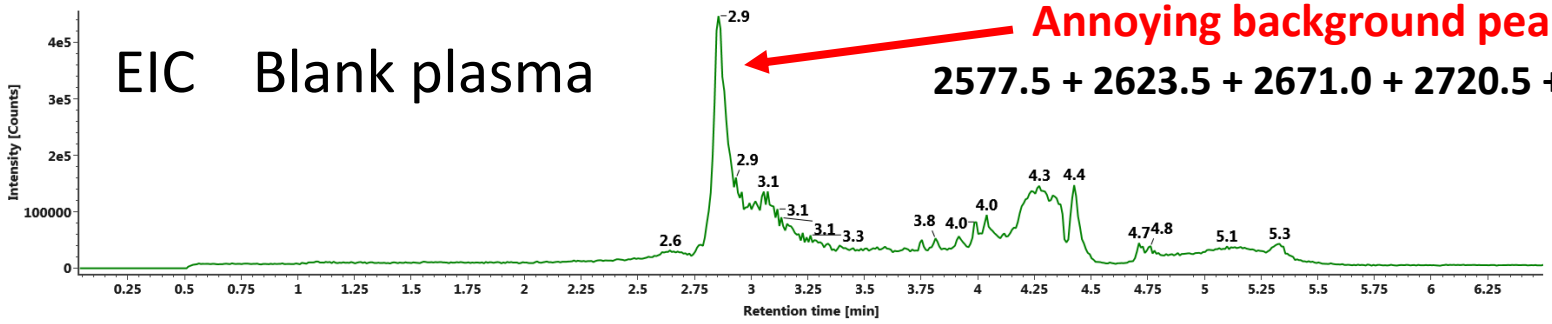
Acquity UPLC Protein BEH C4 Column 300A, @ 80°C

Intact Mass Quantification of SiLu™MAb immunocaptured from mouse plasma





2577.5 + 2623.5 + 2671.0 + 2720.5 + 2771.8 + 2825.1 + 2880.5 m/z



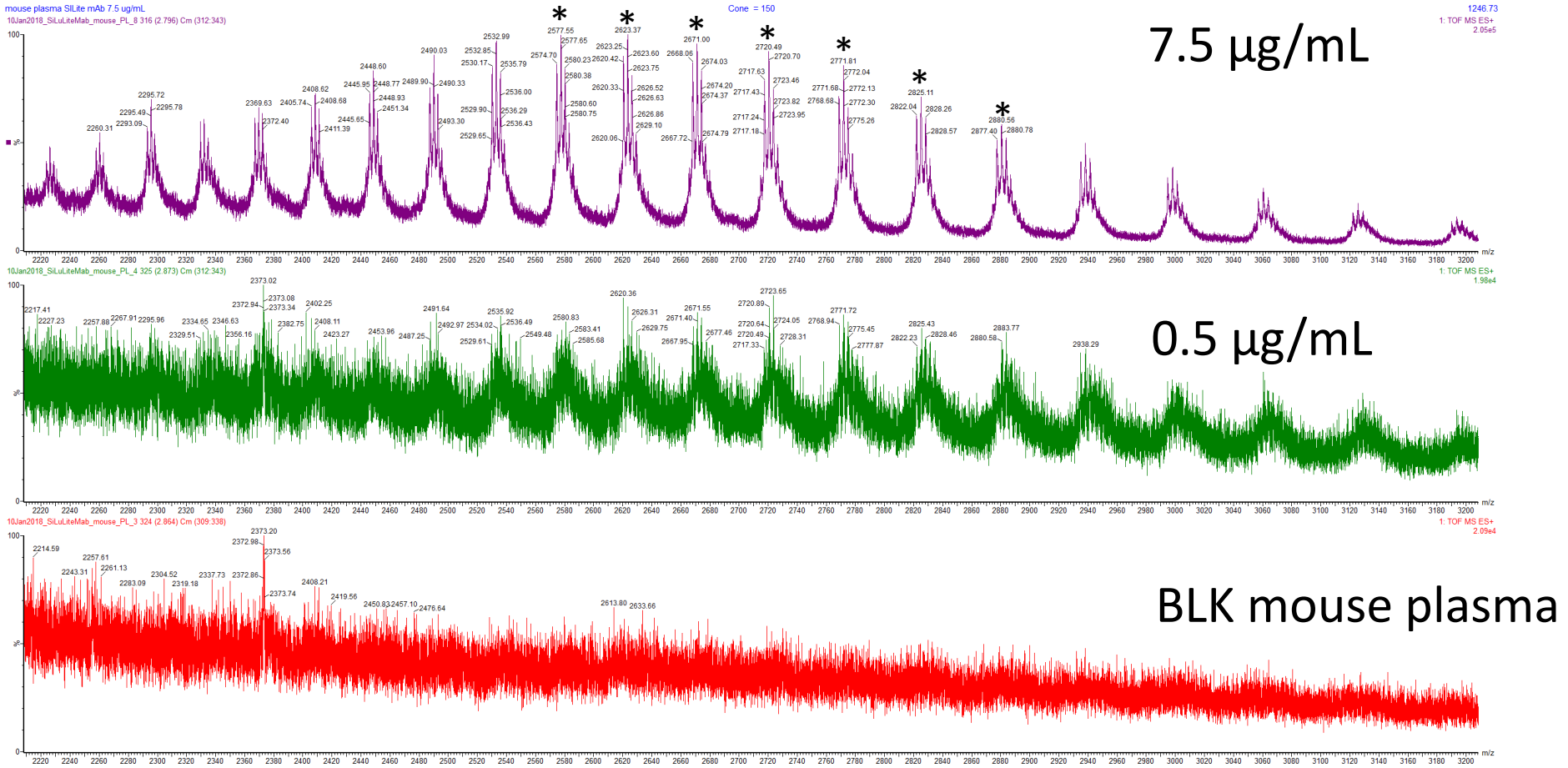
Annoying background peak in blank

2577.5 + 2623.5 + 2671.0 + 2720.5 + 2771.8 + 2825.1 + 2880.5 m/z

Bristol-Myers Squibb

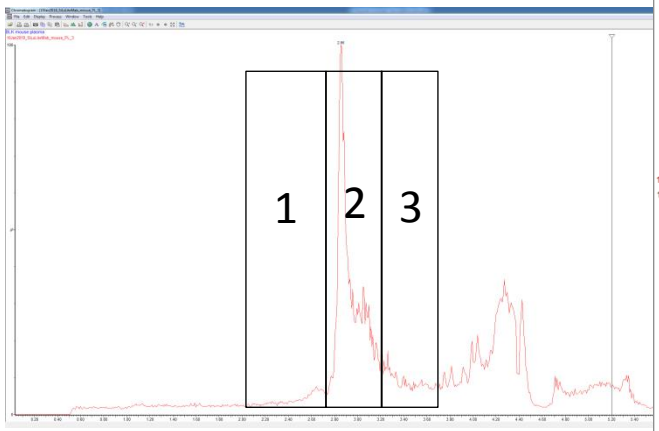
Intact Spectrum of SiLu™MAb
immunocaptured from mouse plasma

Combined spectra from 2.75 – 2.95 min

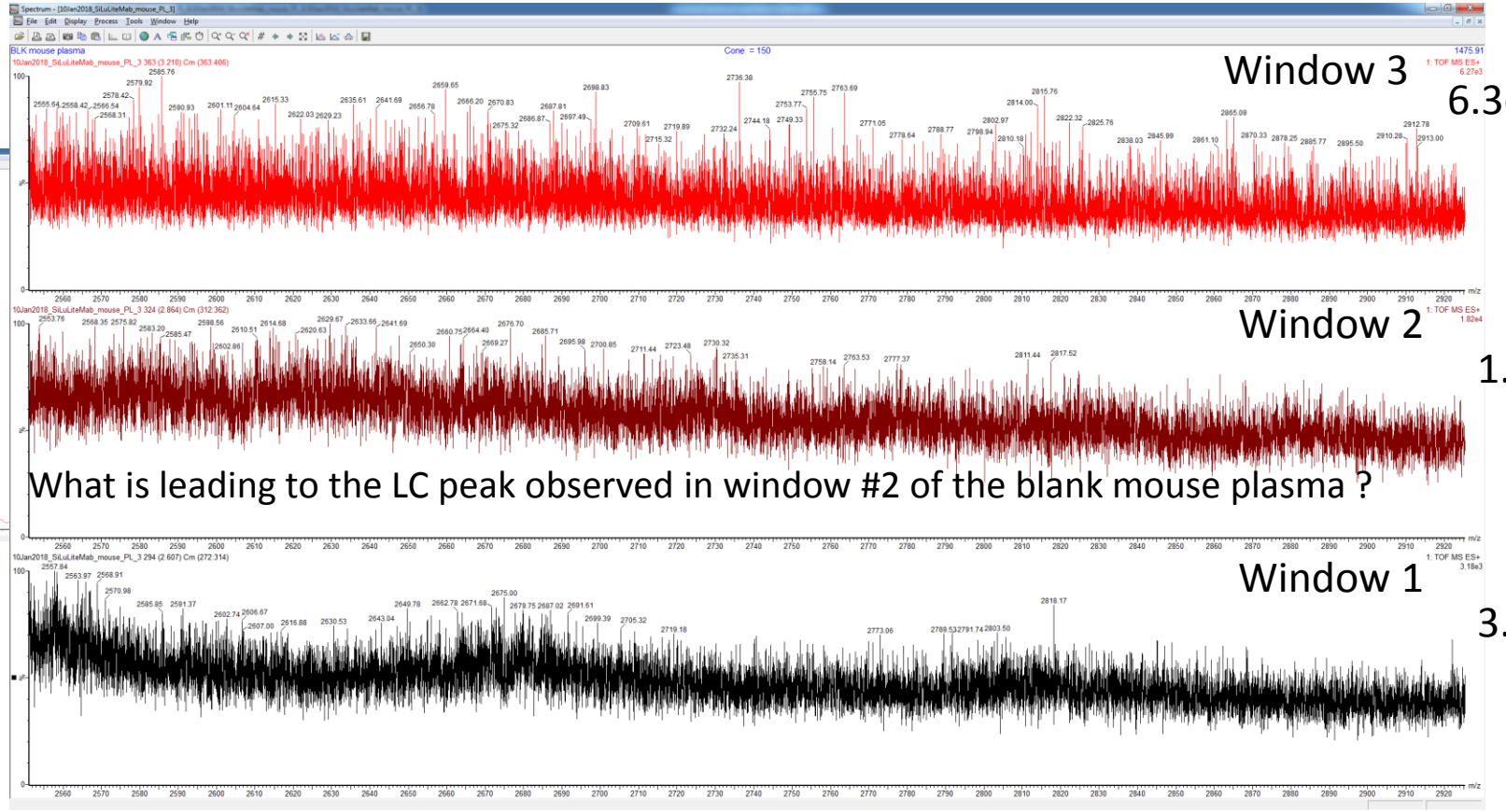


Peak in EIC is from Increased Baseline Signal

EIC, Blank Plasma

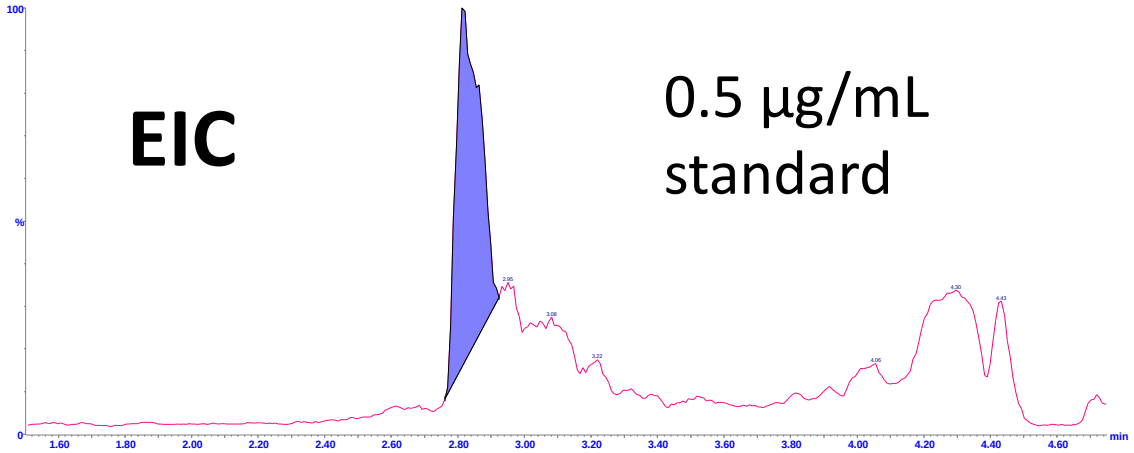


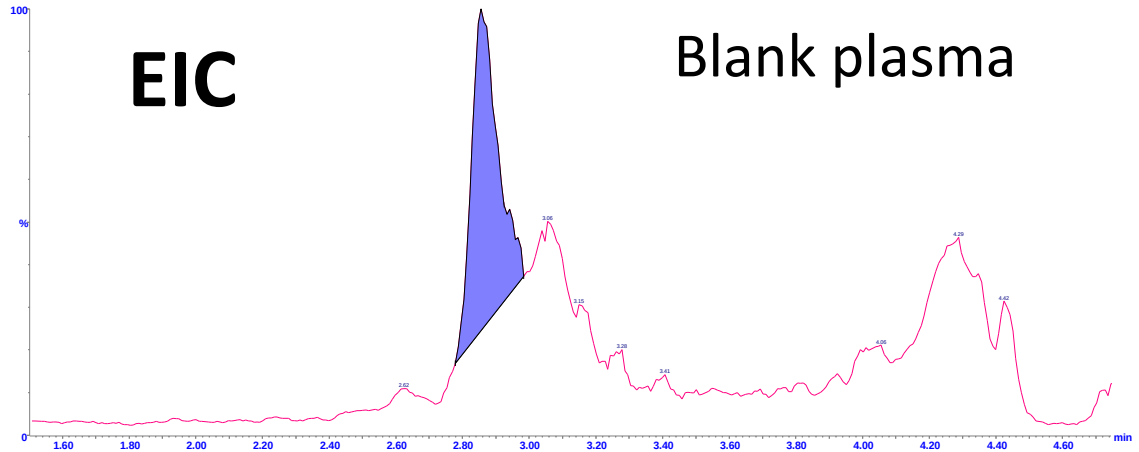
Summed Spectrum



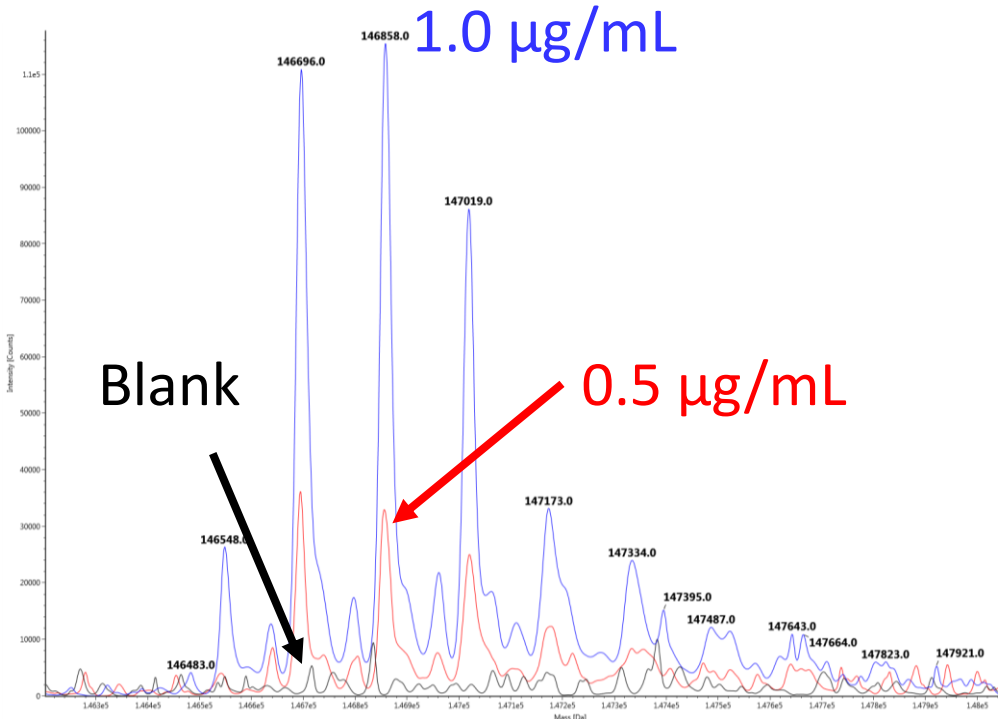
What is leading to the LC peak observed in window #2 of the blank mouse plasma ?

Intact Mass mAb Quantification
EIC or Deconvolution ?





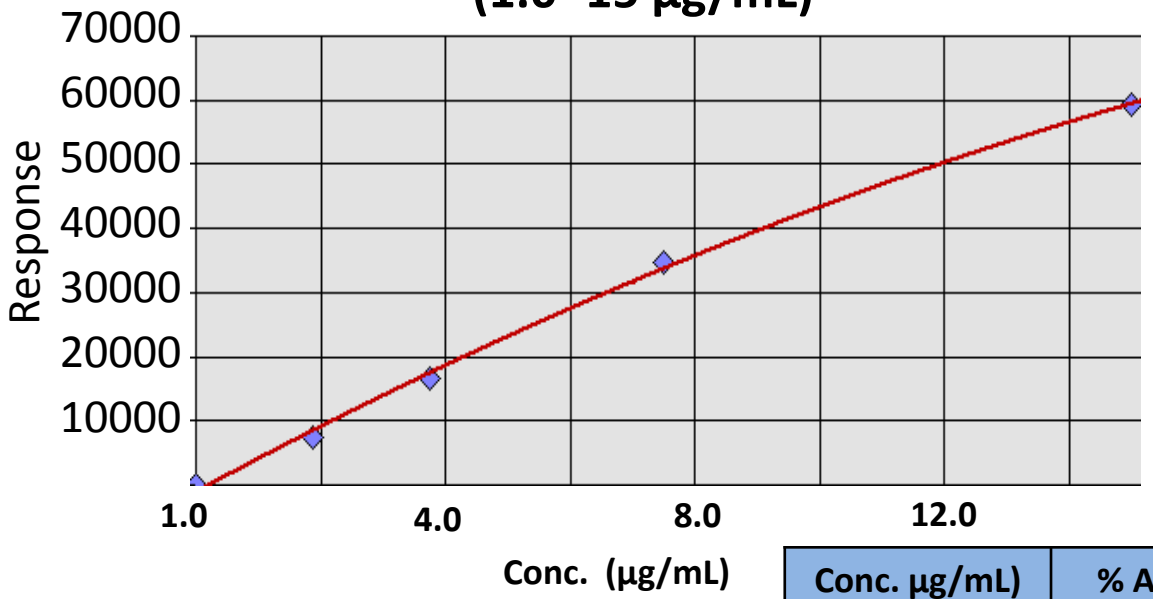
Deconvolution



Bristol-Myers Squibb

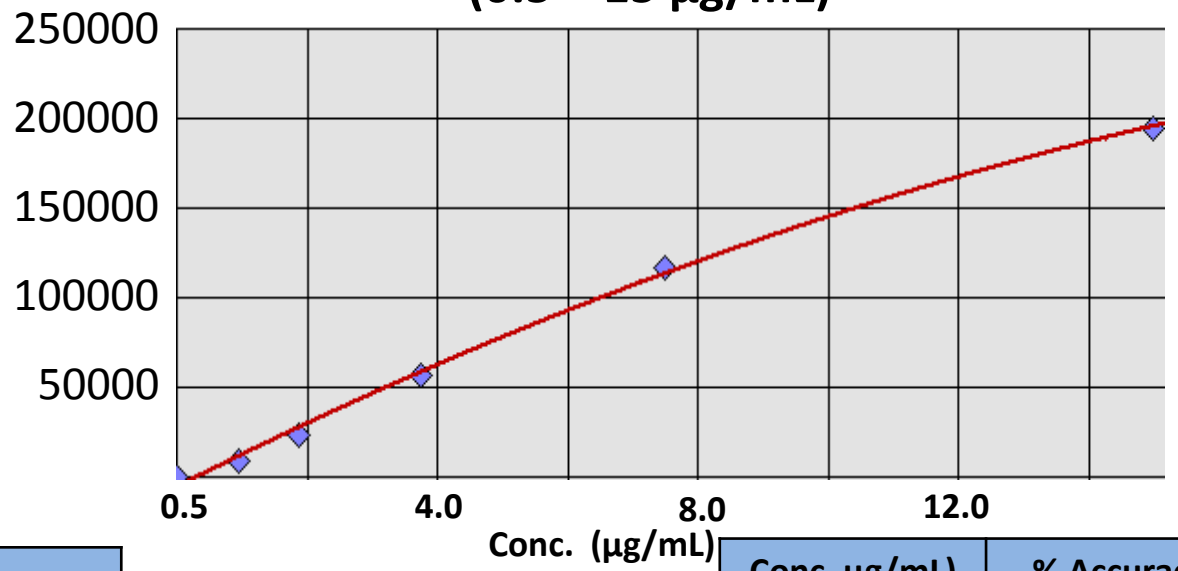
Standard Curve of Intact SiLu™MAb in mouse plasma
EIC and Deconvoluted Standard Curves

EIC Standard Curve
(1.0 -15 µg/mL)



Conc. µg/mL)	% Accuracy
0.5	N/A
1.0	102.0
1.9	98.1
3.8	98.5
7.5	101
15	99.2

Deconvolution Standard Curve
(0.5 – 15 µg/mL)



Conc. µg/mL)	% Accuracy
0.5	108.2
1.0	96.2
1.9	98.3
3.8	99.2
7.5	102.1
15	98.7

RBIG Panel Discussion

1A. What are the **current industry strategies** for overcoming **sensitivity issues** in intact protein quantification?

1B. Which are the ones really working and which are not?

- Maximizing enrichment by IA by using best capturing antibody for improving mass spectrometry S/N?
- Deglycosylation?
- Subunits quantification?
- Summing isotope signals
- Charge state coalescence with DMSO
- Optimizing extraction window (XIC) for quantitation?
- Declustering potential & accumulation time?
- Optimization of chromatographic condition for intact proteins
- Others

RBIG Panel Discussion

2. **Why** intact protein quantification should be performed?

3. **How** intact protein quantification should be performed?

4. What are the **Pros/Cons** of using **HRMS** for quantitation of intact proteins and/or subunits?

RBIG Panel Discussion

5. "**Lost in digestion**" fact or myth?

6. Is the use of the "**Bottom-up**" (**signature peptide**) approach impacting the bioanalysis of biotherapeutics?

7. Are the risks of **not providing sufficient information** on the biotherapeutics bioanalysis by Hybrid LBA/LCMS **signature peptide** approach confirmed by actual data?

RBIG Panel Discussion

8. What are the **most common applications** for intact proteins quantification?

- Identification of intact biotherapeutics and their catabolites?
- Quantitation of intact biotherapeutics and their catabolites?
- Studies for better understanding the various circulating biotherapeutic forms?
- Biotransformation studies?
- Glycoforms quantitation?
- Post-translational modifications evaluations?
- Others….

RBIG Panel Discussion

9. What are the **best stationary phases** for intact proteins quantification?

10. Is the use of **stable isotope-labeled internal standard** version of the protein crucial?

11. Is there any concern raised by **Regulatory Agencies** when the intact quantification is used instead of the traditional bottom-up approach?

RBIG Panel Discussion

12. What are the current **industry standards** in “Top-down” protein Bioanalysis?