

Intact Protein LC-MS for Pharmacokinetic & In-Life Study Support

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Topics & questions to answer

- Background
- “Quantitative” case study
 - Example Data
 - Mock Bioanalytical Method Validation
 - Sample analysis – different assays
 - Glycoform monitoring
- Summary and conclusions

Key Questions:

- Can the Intact LC-MS assay be held to traditional PK assay performance standards from a GLP validation perspective?
- How do sample results from the Intact LC-MS assay compare to established formats (LBA, surrogate peptide)?
- Can advantages of Intact Protein Mass Spectrometry (e.g. mass variant monitoring) be retained?

MS of Intact Proteins in a Bioanalysis Setting

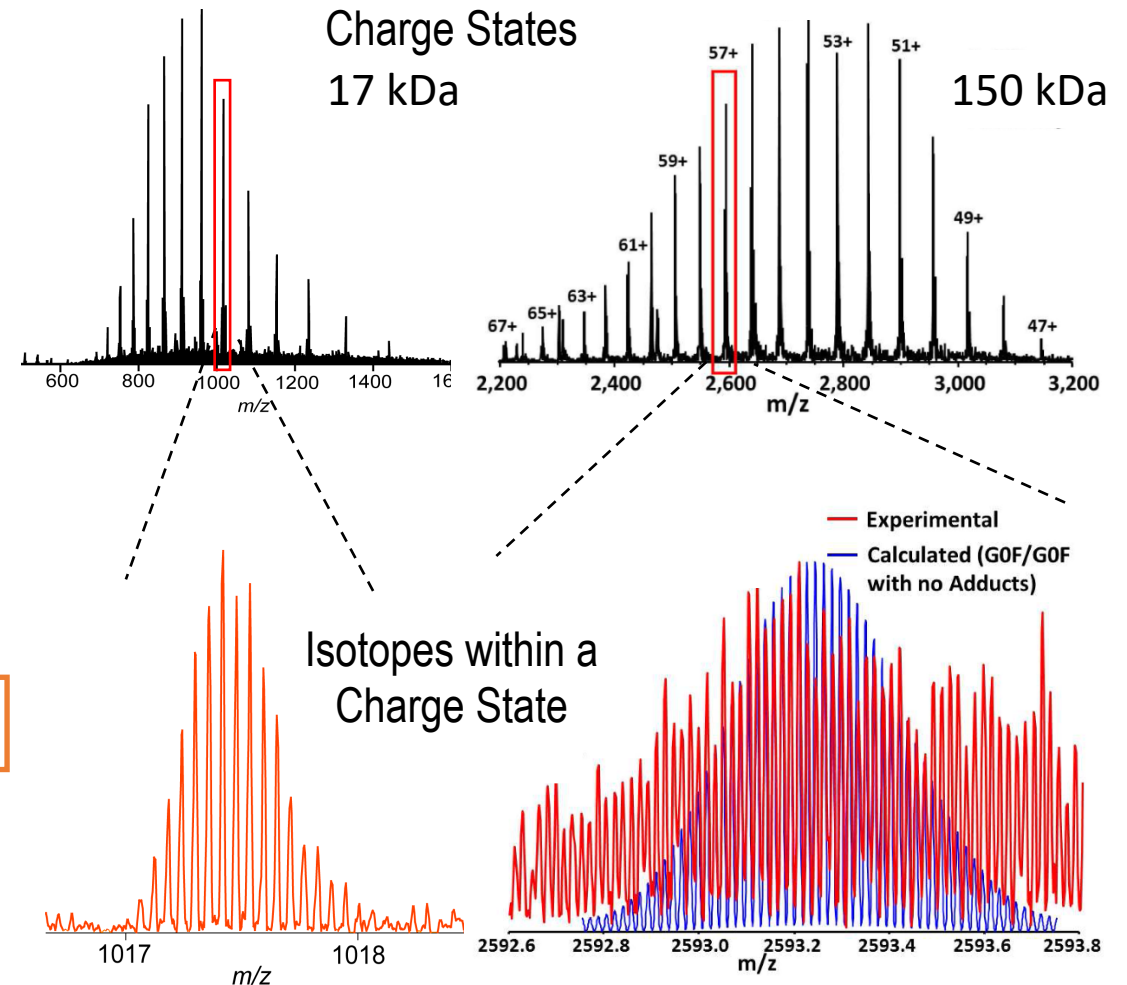
- Small molecules (typically) have few charge states, with few naturally occurring isotopes
- Protein digestion “levels the playing field” – while the mixture is more complex, molecules are smaller & as a result...
 - They can be better purified based on size or other properties
 - It's easier to perform LC separation (and MS analysis)
 - For MS: Few charge states and few isotopes
- Intact proteins have many charge states, with many isotopes under each charge state

Best Practise for Mass spectrometry:

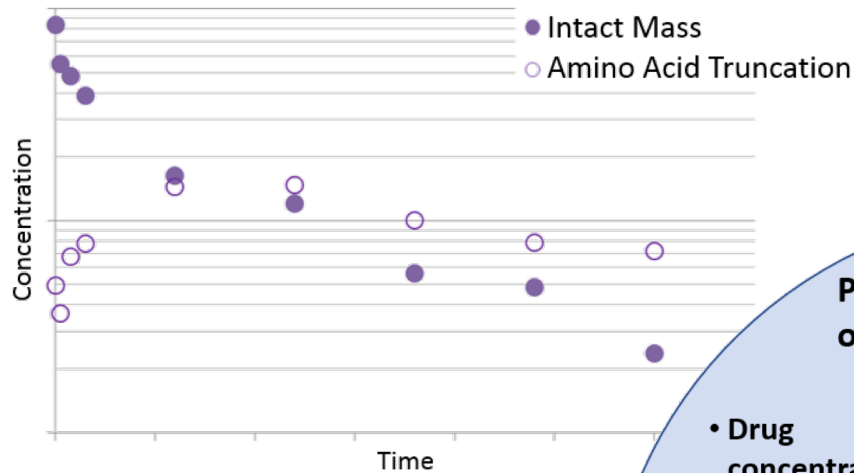
Small Molecules - quantify the whole molecule & metabolites

Large molecules - quantify a small, surrogate peptide to infer whole molecule concentration

Information about the whole molecule is lost during digestion

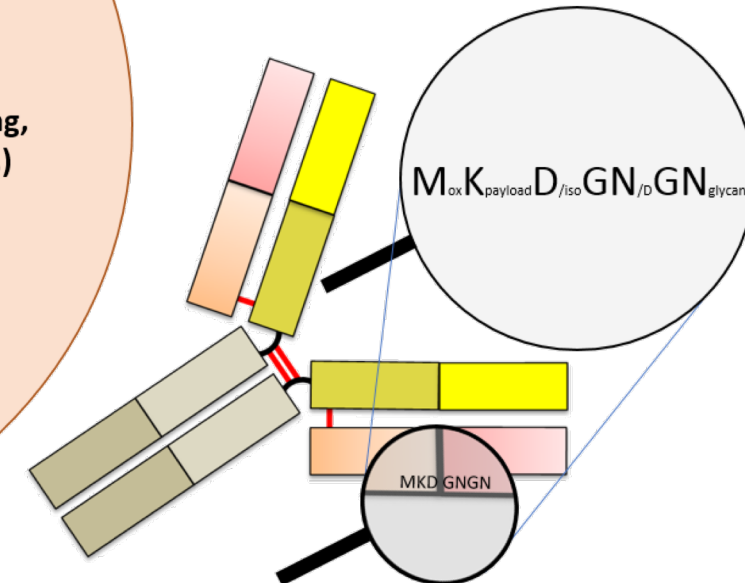
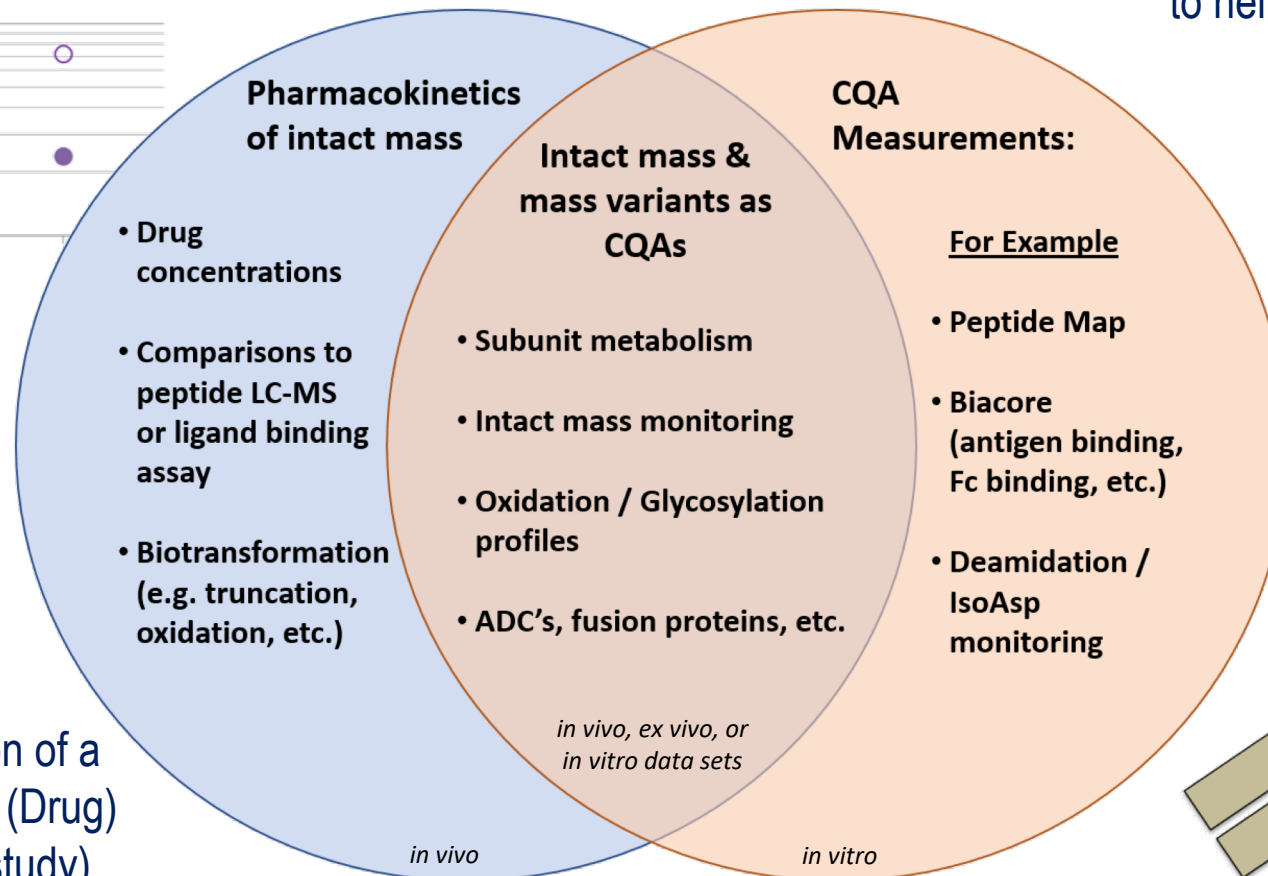


From Surrogate Peptide LC-MS to the Intersection of Pharmacokinetic and Attribute Monitoring



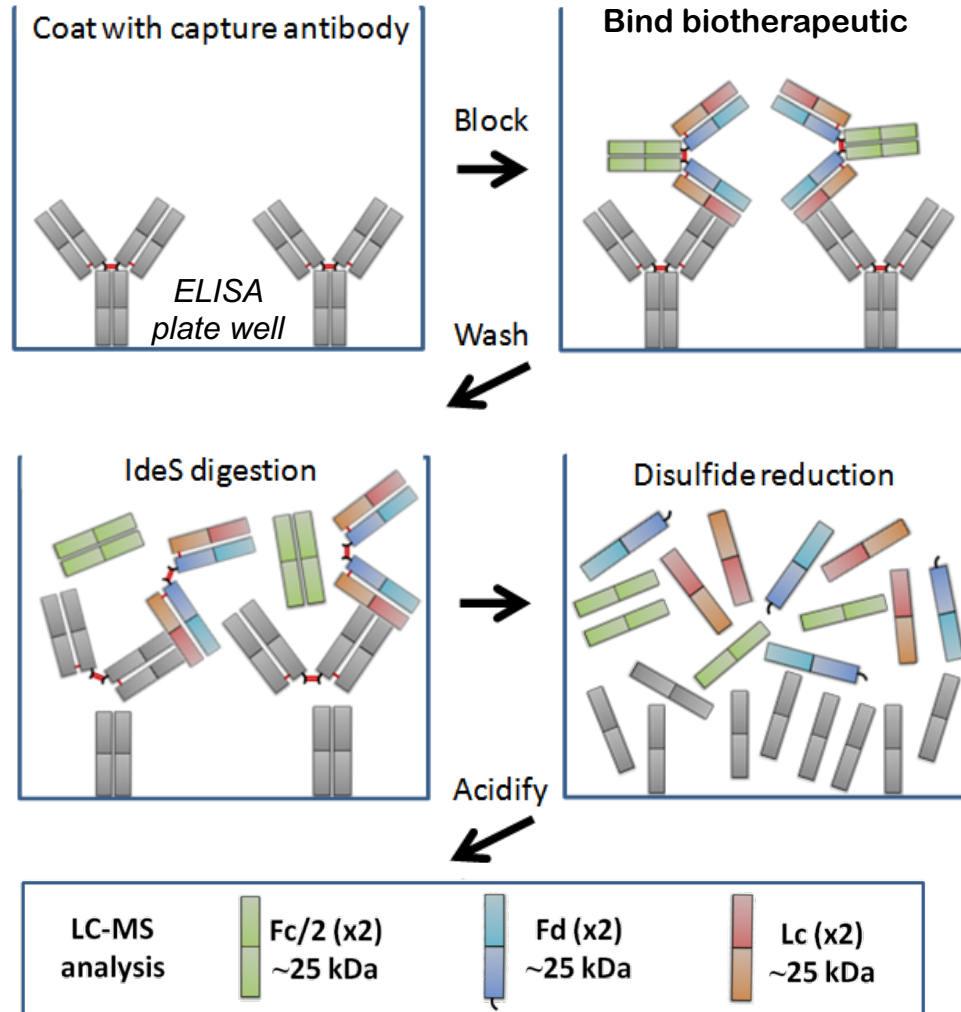
CQA - Chemical or other attribute that can be clearly defined and monitored to help ensure product quality

Biotransformation –Modification of a chemical or biological compound (Drug) occurring *in vivo* (during in-life study)



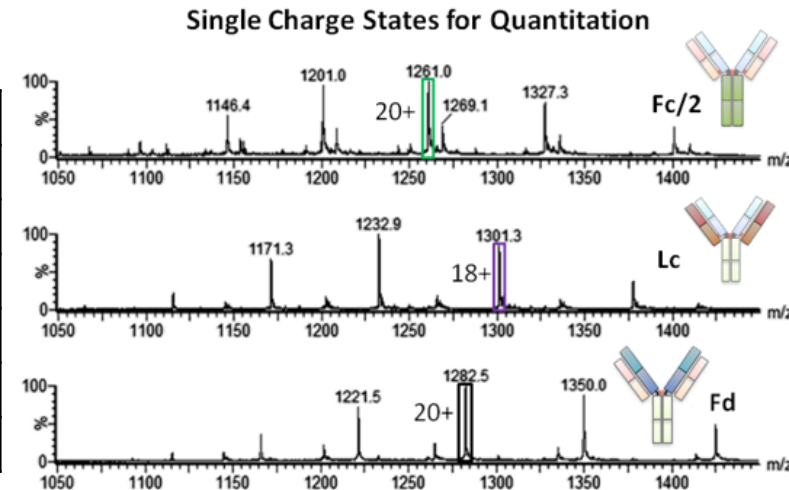
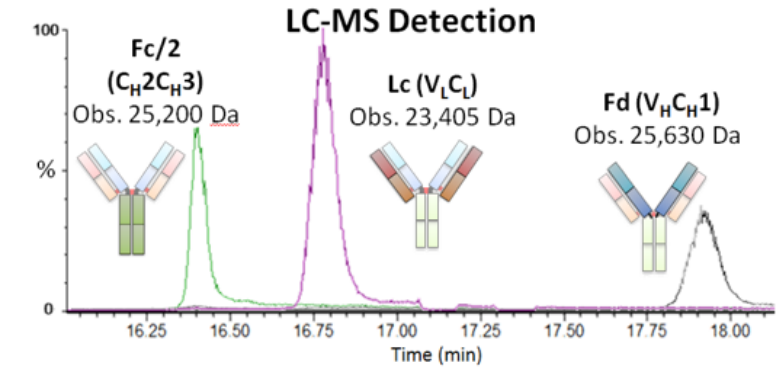
Whole Molecule (mAb Subunit) analysis platform

Immunocapture method from plasma for ex-vivo, pre-clinical, clinical samples



Synapt G2-Si

ATTRIBUTE	Intact	Subunit	Peptide
Intact Mass	YES	NO	NO
Clipping	Maybe	YES	Maybe
Oxidation	NO	Maybe	YES
IsoAsp/DeAm	NO	NO	YES
Glyco profile	YES	YES	NO
DAR	Maybe	YES	Maybe

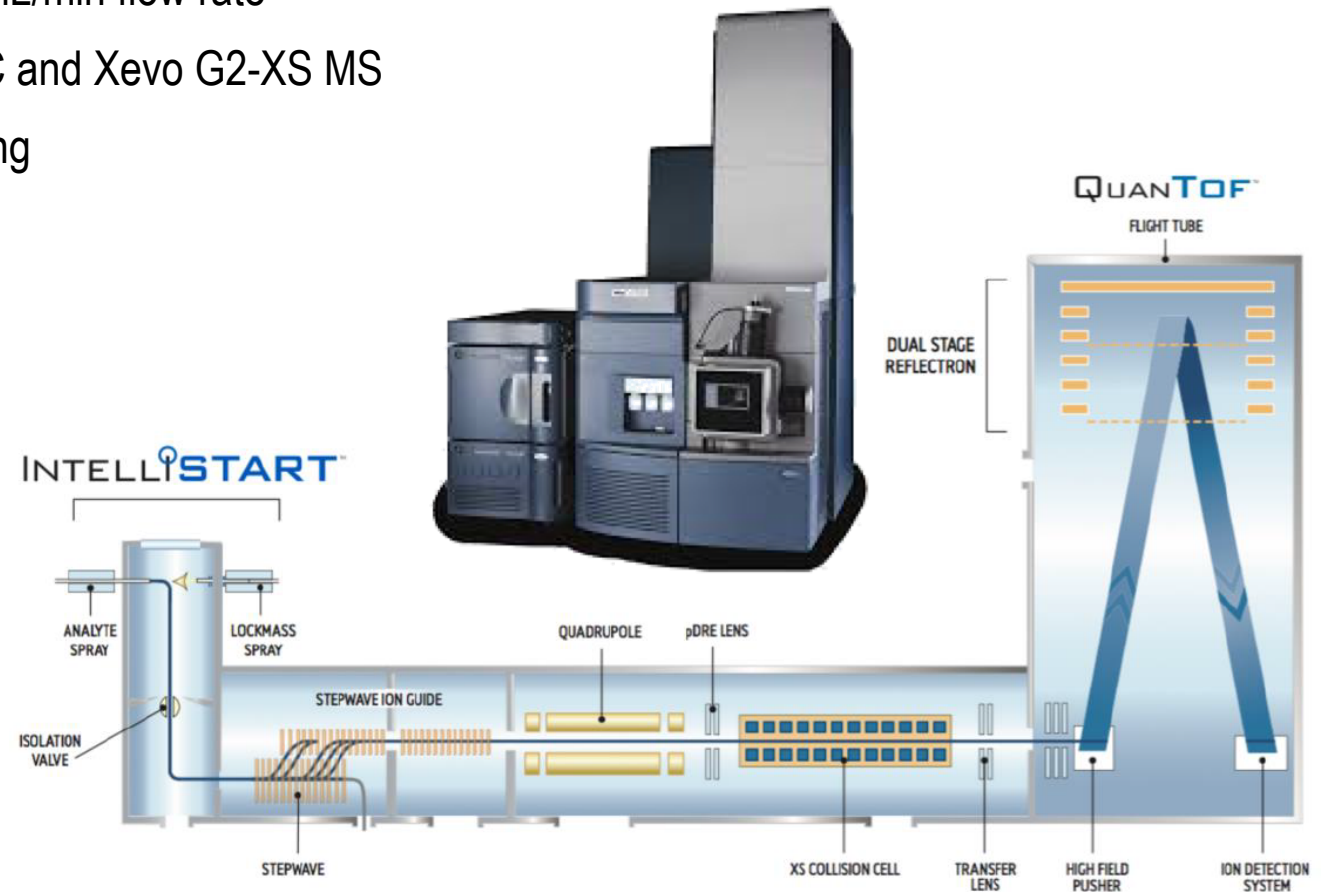
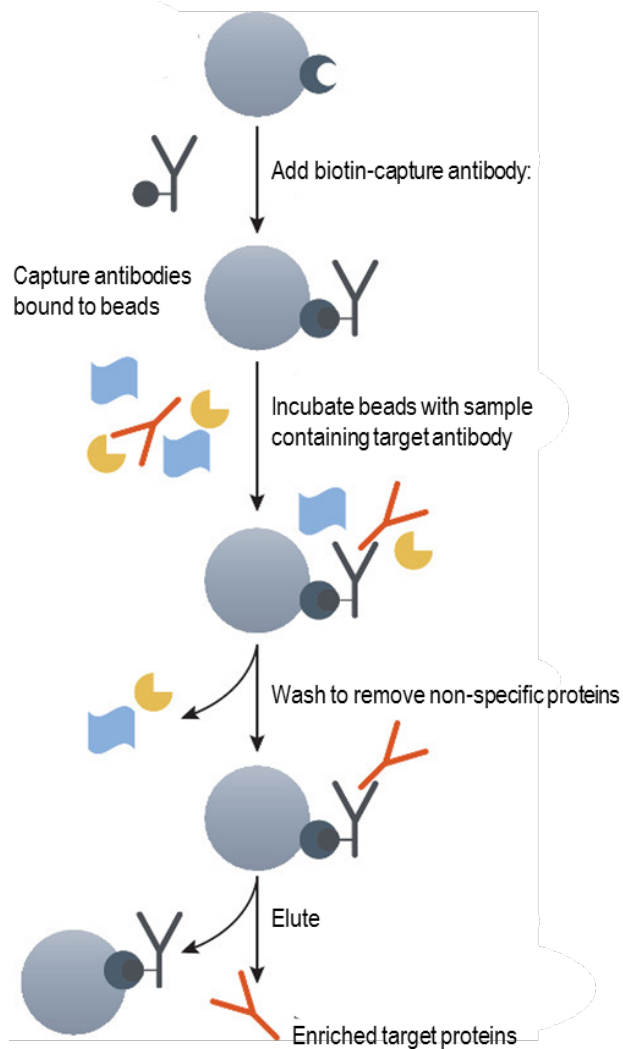


Intact protein LC-MS: the rationale

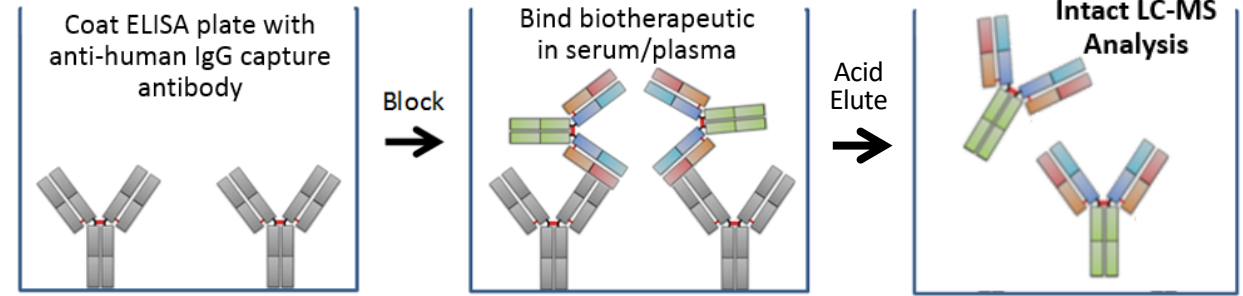
- Used streptavidin immunoassay plate
 - Lower capacity than magnetic beads; requires less serum from study
 - Clean background for LC-MS
 - Preferred assay format in-house
- No internal standard
 - Favorable assay performance without I.S.
- High flow rate, minimal separation in gradient
 - Sample highly purified from immunocapture
 - Need sharp elution peak for best detection
 - Method was robust (e.g. low carry over, no LC column pressure issues)
- Assay range
 - 5 to 50 $\mu\text{g/mL}$ – match sample ranges (& dilution schemes) of previous assays; match expected in-life sample concentration amounts
- Quant scheme
 - Used all charge states in range (observed higher variance by using fewer charge states)

Intact protein LC-MS: the basics

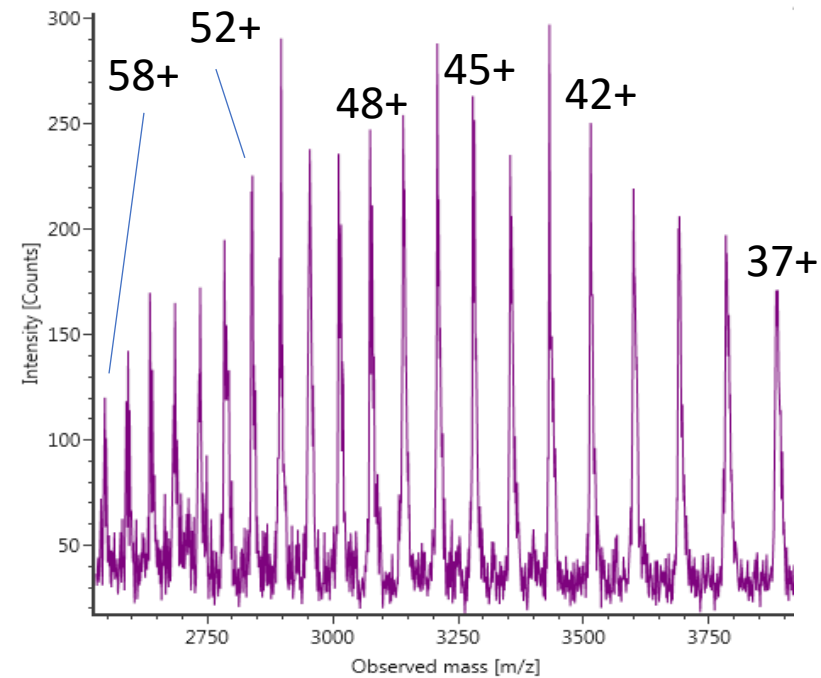
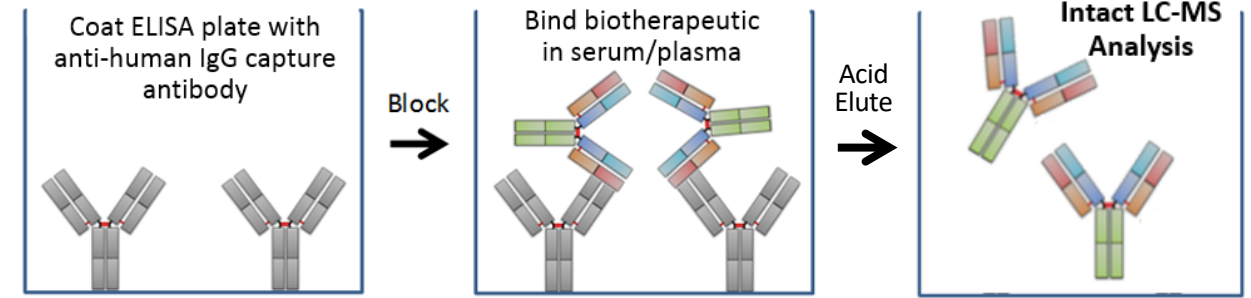
- 2.5 hour for sample preparation
- LC-MS: 12 samples per hour (5 min/sample)
- LC conditions: 0.25 mL/min flow rate
- Waters Acquity UPLC and Xevo G2-XS MS
- UNIFI Data Processing



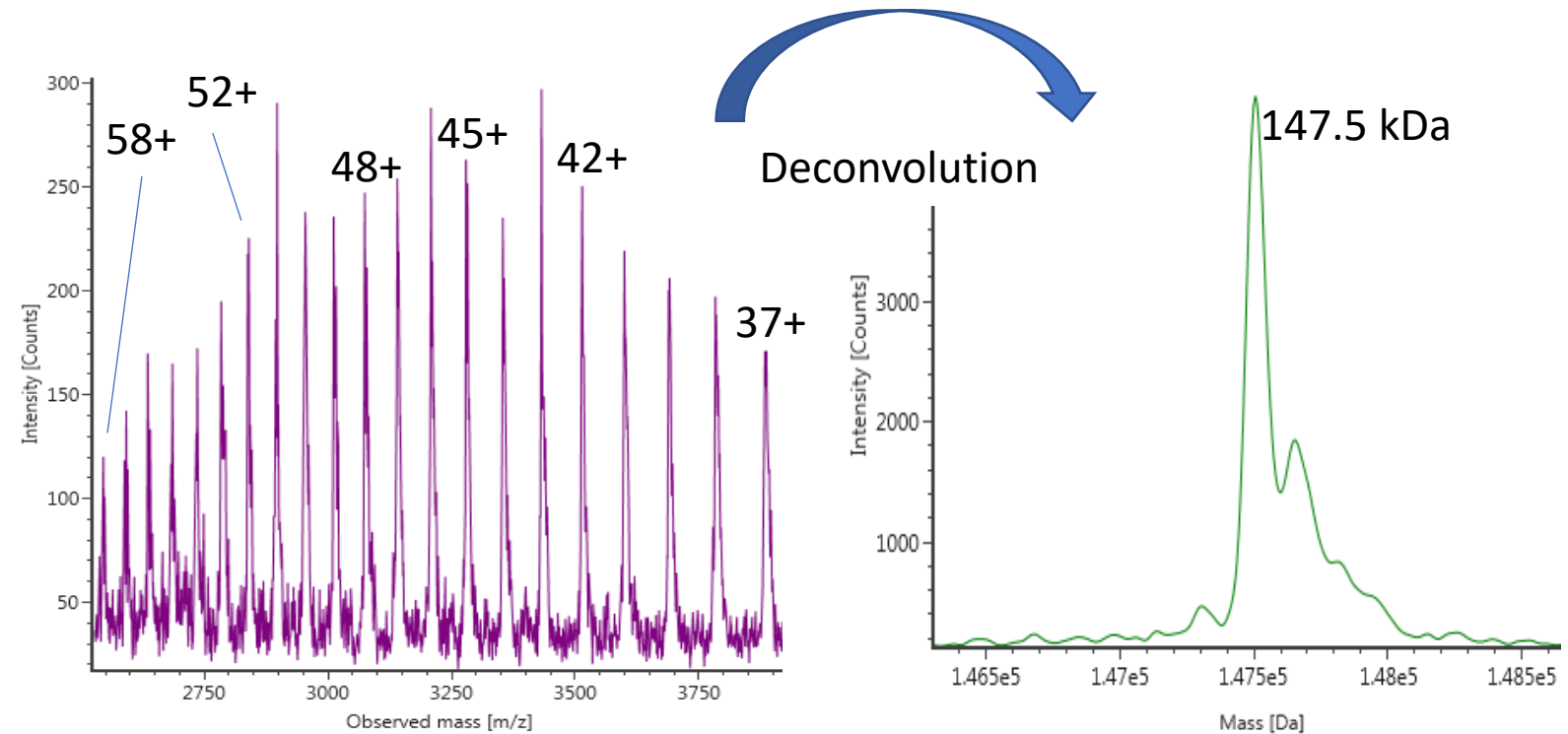
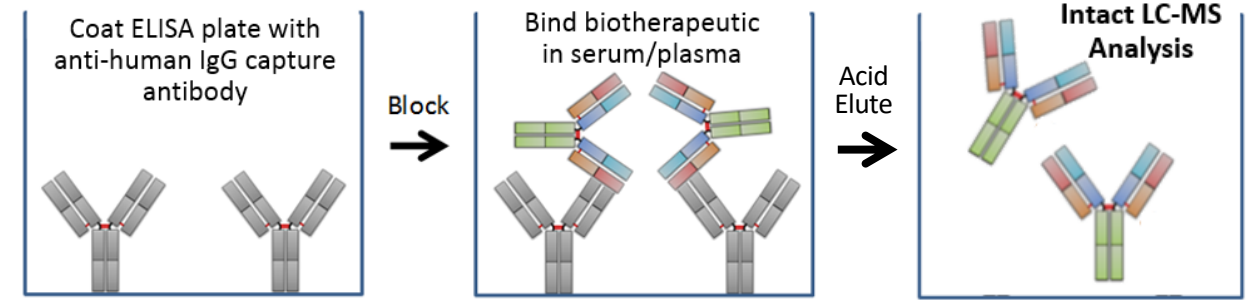
Intact protein LC-MS: Workflow and Data



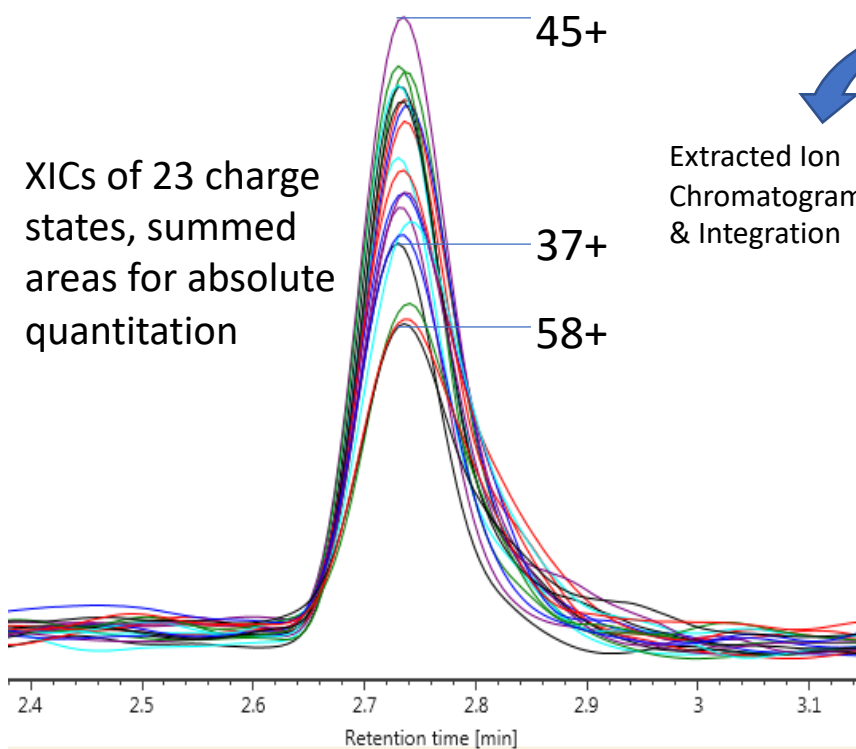
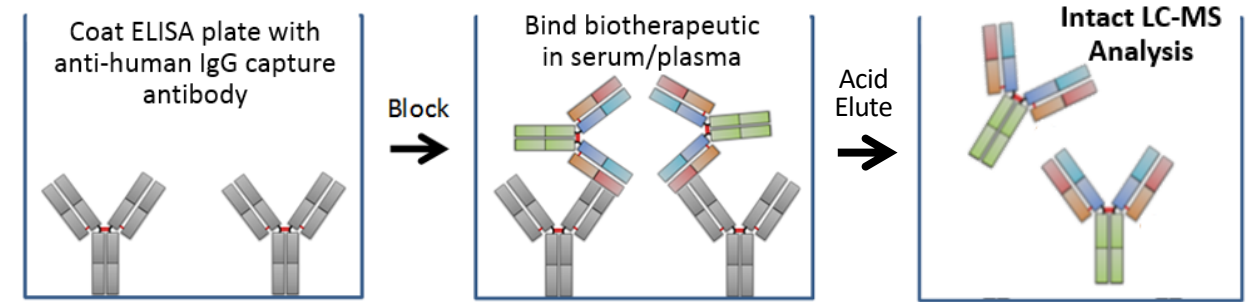
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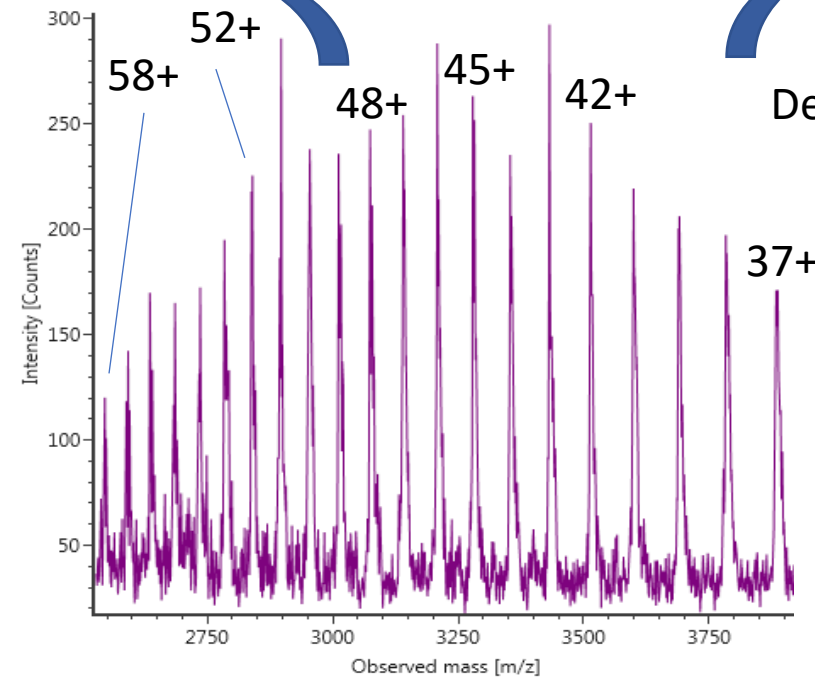
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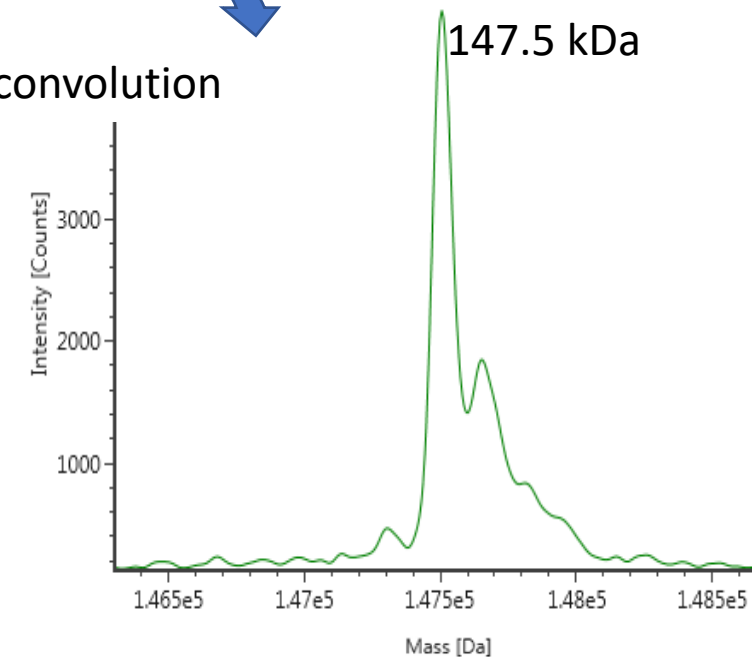
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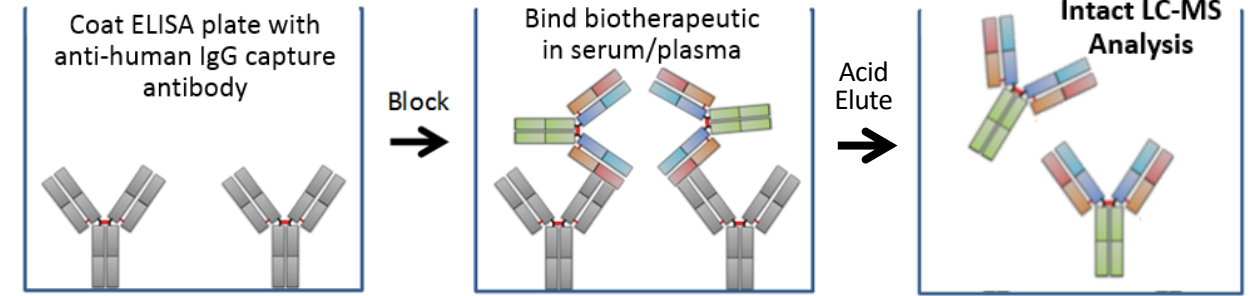
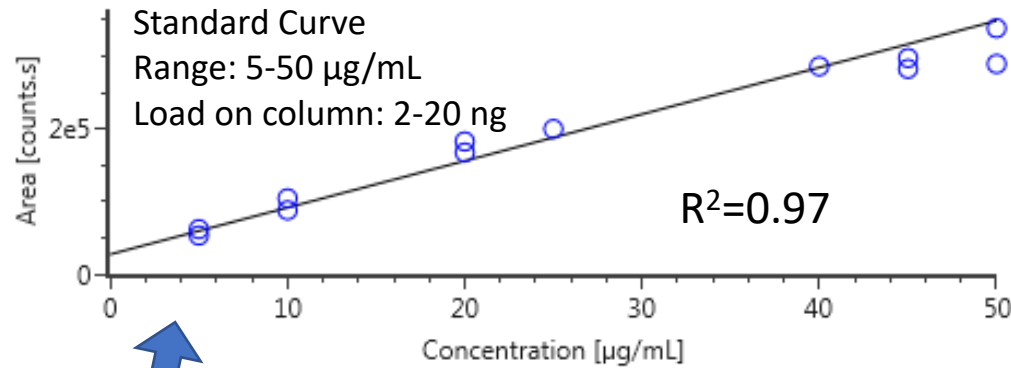
Extracted Ion Chromatograms & Integration



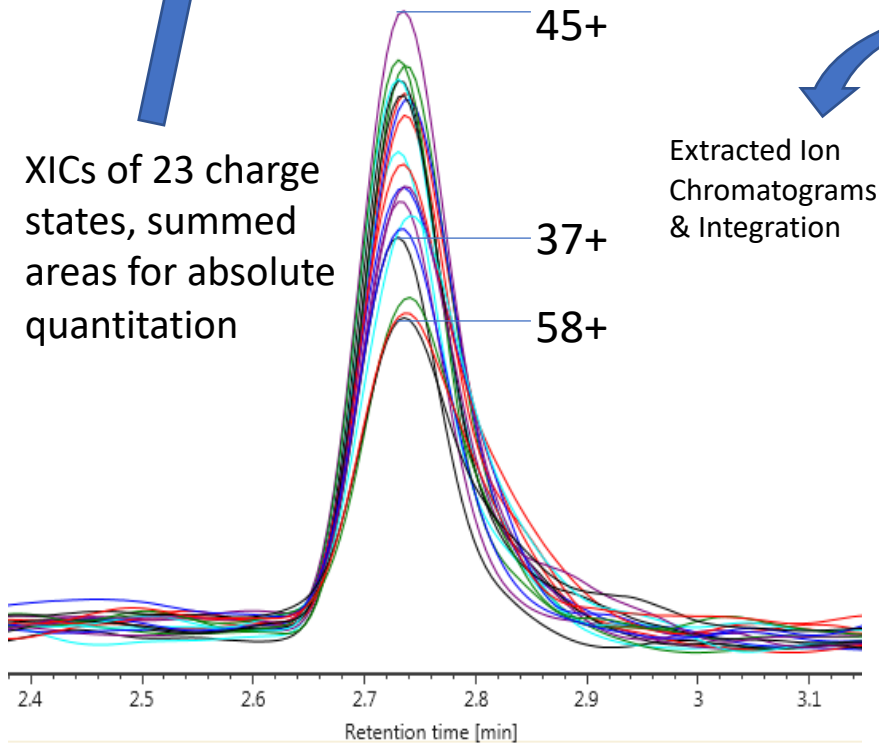
Deconvolution



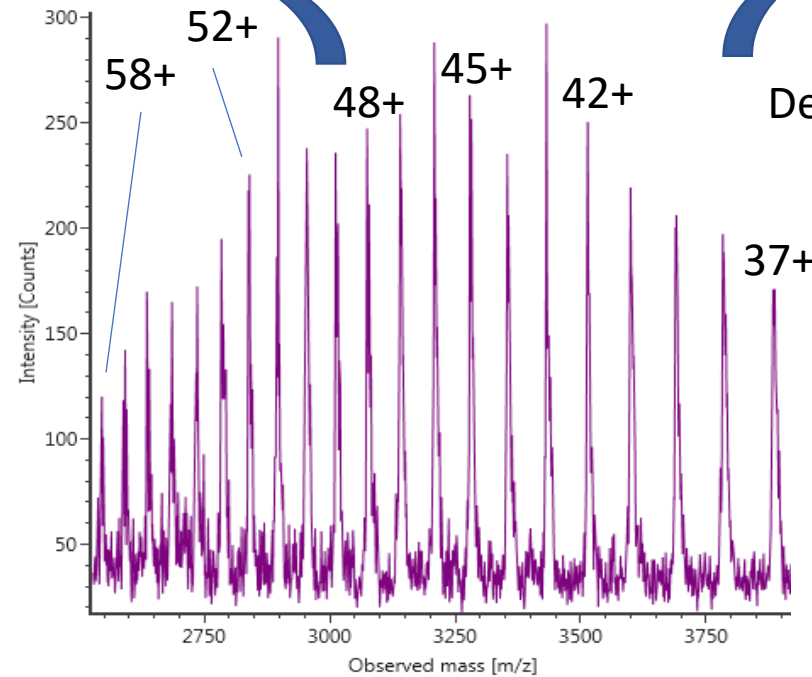
Intact protein LC-MS: Workflow and Data



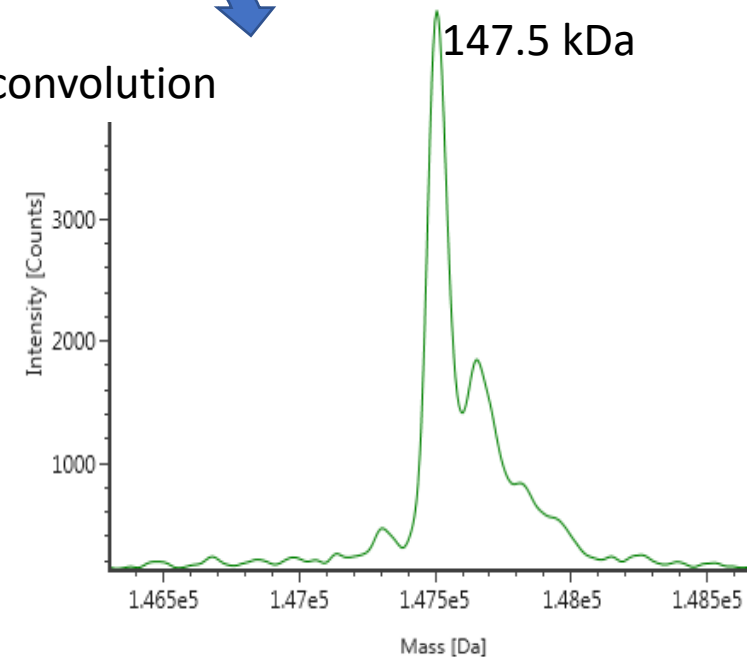
XICs of 23 charge states, summed areas for absolute quantitation



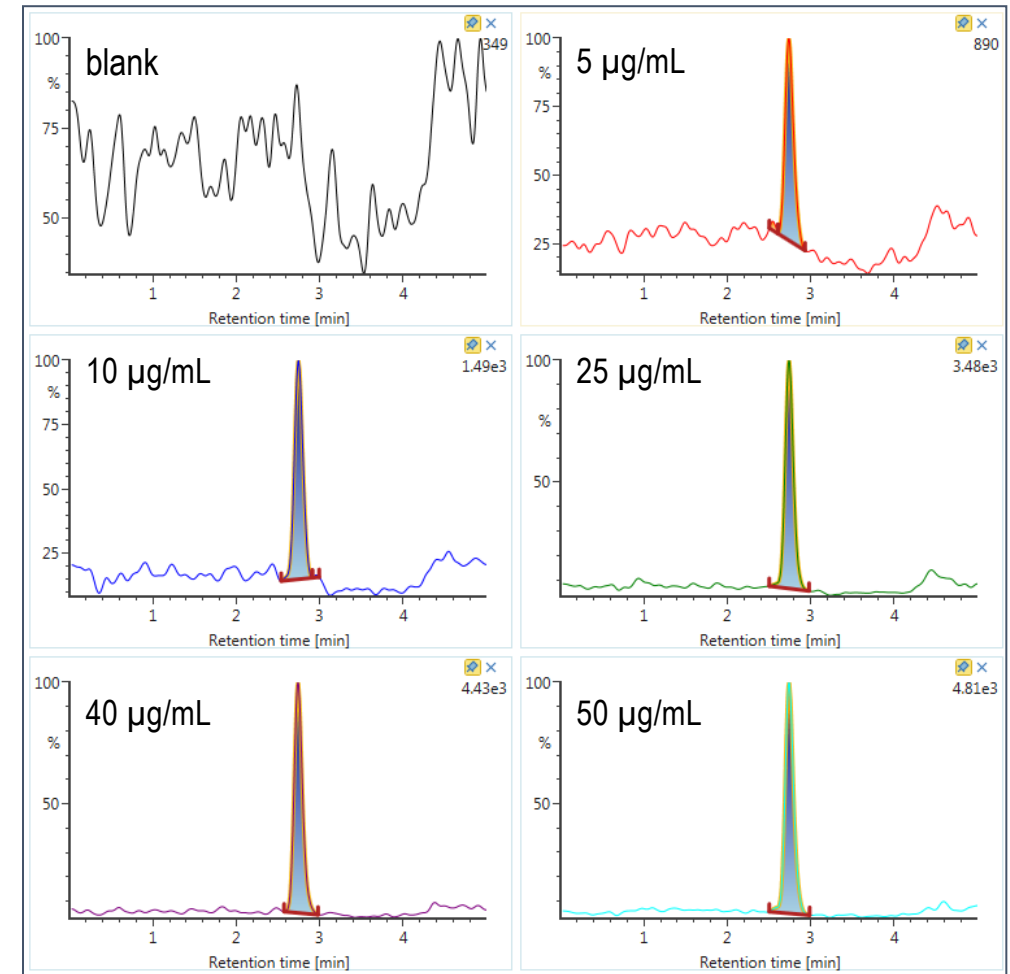
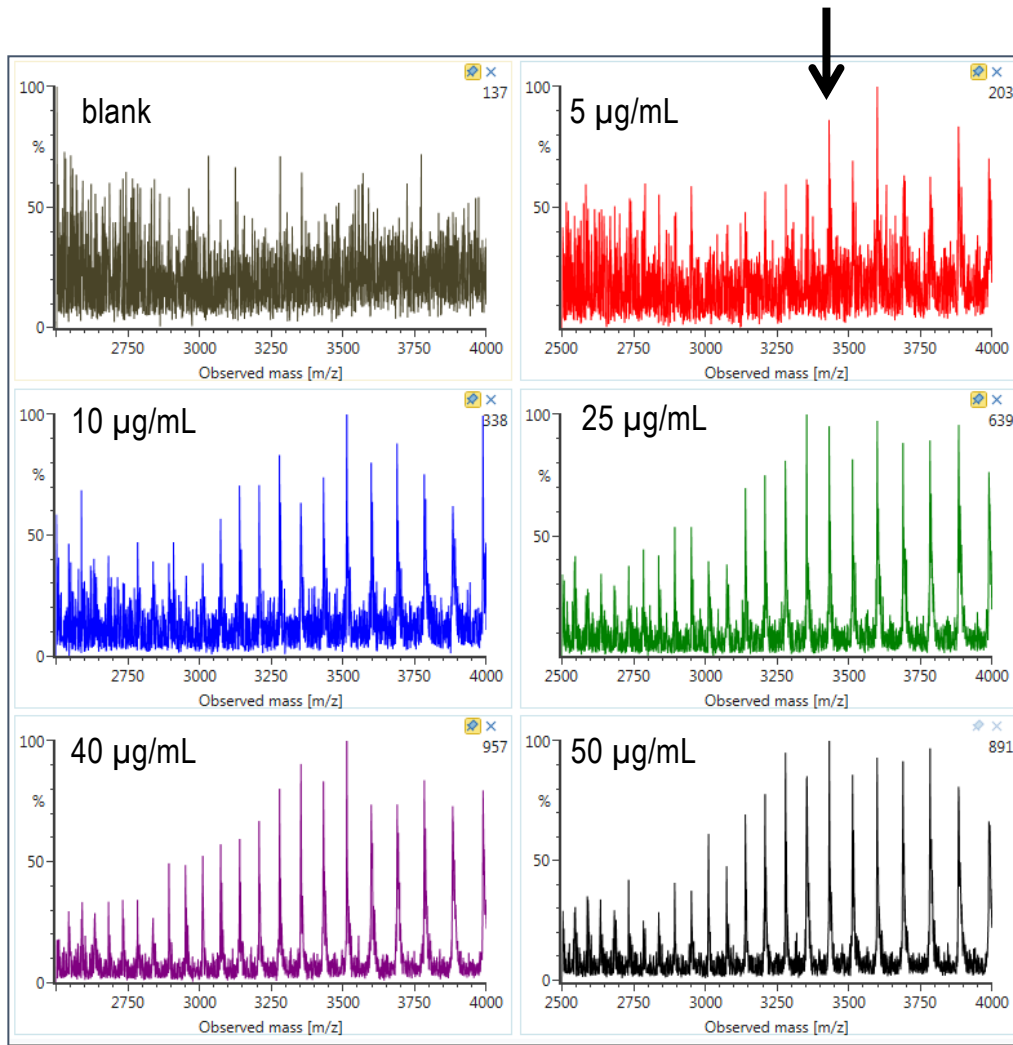
Extracted Ion Chromatograms & Integration



Deconvolution



Example of quantitative data for Intact LC-MS Assay



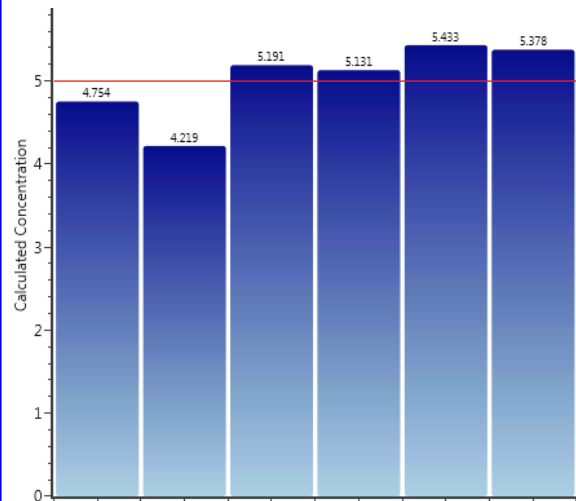
Mass Spectra (multi charge state pattern)

Extracted ion chromatograms,
single charge state (m/z 3401)

Mock validation: Qc results from 3 Precision and accuracy runs

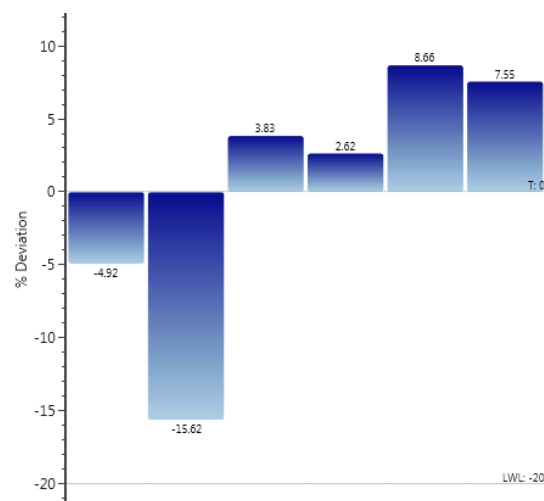
Conc.	Day 1			Day 2			Day 3			Total		
µg/mL	n	Accuracy (% Bias)	Precision (% CV)	n	Accuracy (% Bias)	Precision (% CV)	n	Accuracy (% Bias)	Precision (% CV)	n	Accuracy (% Bias)	Precision (% CV)
5	6	1.7	7.8	6	0.4	9.1	6	2.1	13.2	18	1.4	10.0
15	6	7.4	3.7	6	18.8	2.2	6	8.3	6.9	18	11.5	4.3
25	6	4.2	4.5	6	18.5	6.2	6	6.3	2.3	18	9.7	4.3
40	6	-2.7	4.3	6	0.8	3.8	6	-5.1	4.6	18	-2.3	4.2
50	6	-11.9	6.8	6	-12.3	4.7	6	-17.0	3.7	18	-13.7	5.1

Calculated Concentration



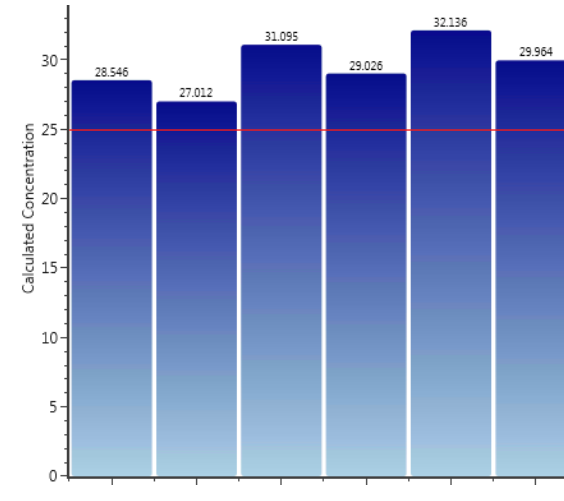
QC Replicates (n=6)

% Bias



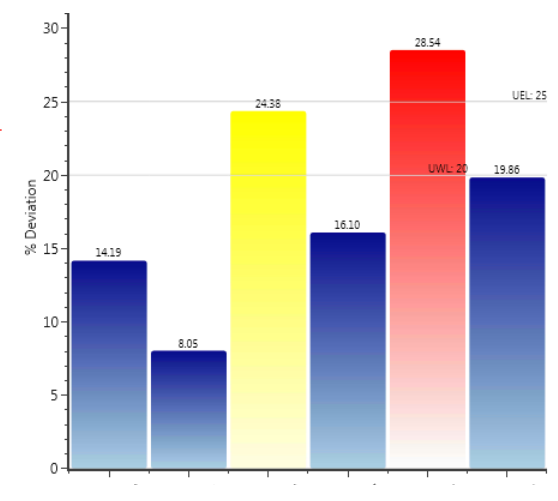
QC Replicates (n=6)

Calculated Concentration



QC Replicates (n=6)

% Bias



QC Replicates (n=6)

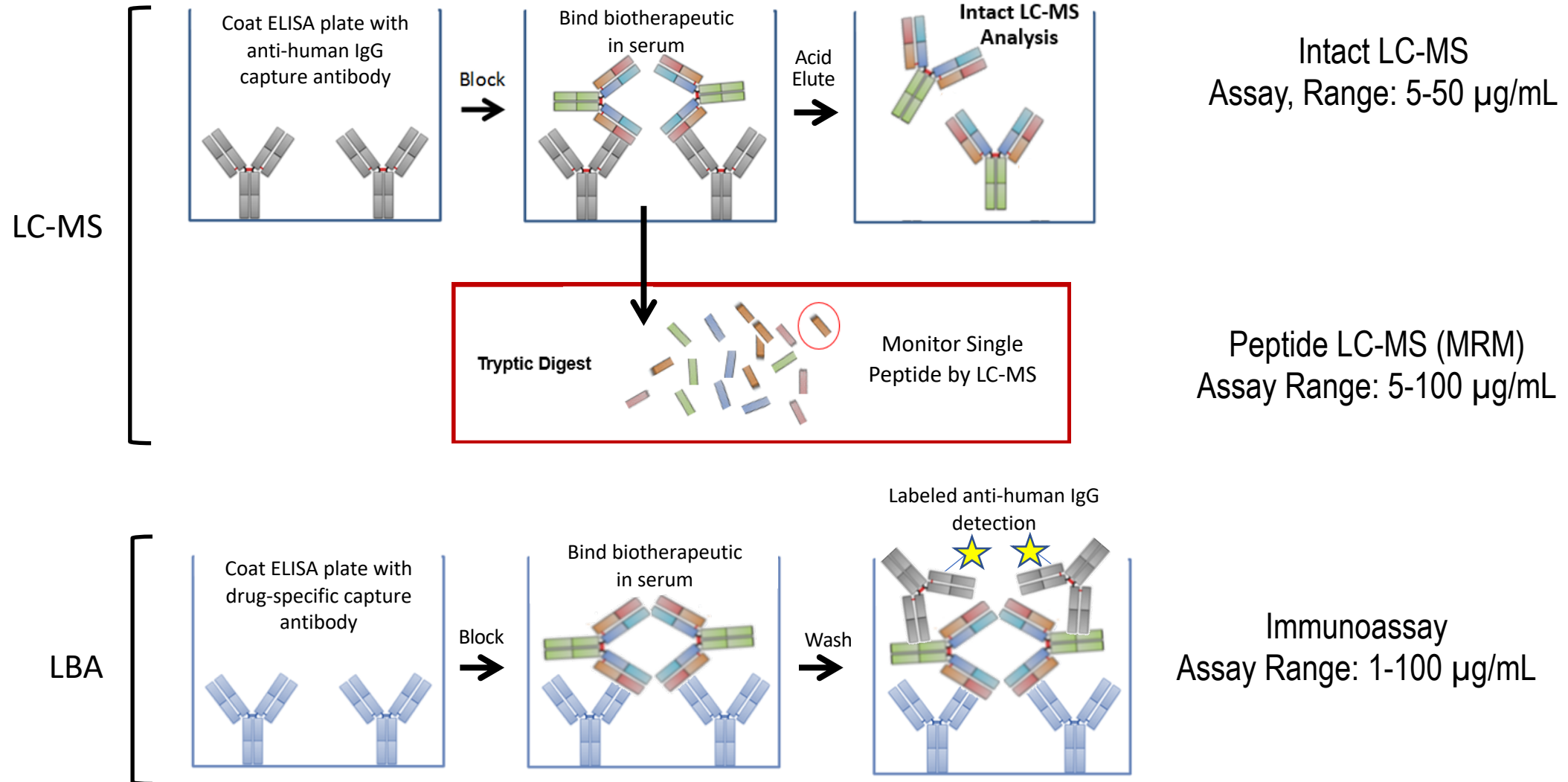
Mock validation: Stability results

Stability Condition	Storage Temperature	Duration	n	QC 15 µg/mL		QC 25 µg/mL		QC 40 µg/mL	
				Accuracy (% Bias)	Precision (% CV)	Accuracy (% Bias)	Precision (% CV)	Accuracy (% Bias)	Precision (% CV)
Post-Process Reinjection	4 °C	36 h	6	12.1	7.2	13.0	4.4	6.7	2.5
Room Temp.	~25 °C	24 h	6	10.5	3.4	9.0	3.9	-12.8	2.7
Freeze-Thaw	-80 °C	5 cycles	6	11.0	2.7	5.3	4.3	-6.8	5.3
Long-Term	-80 °C	408 days	6	10.3	6.6	1.8	3.9	-10.0	1.8

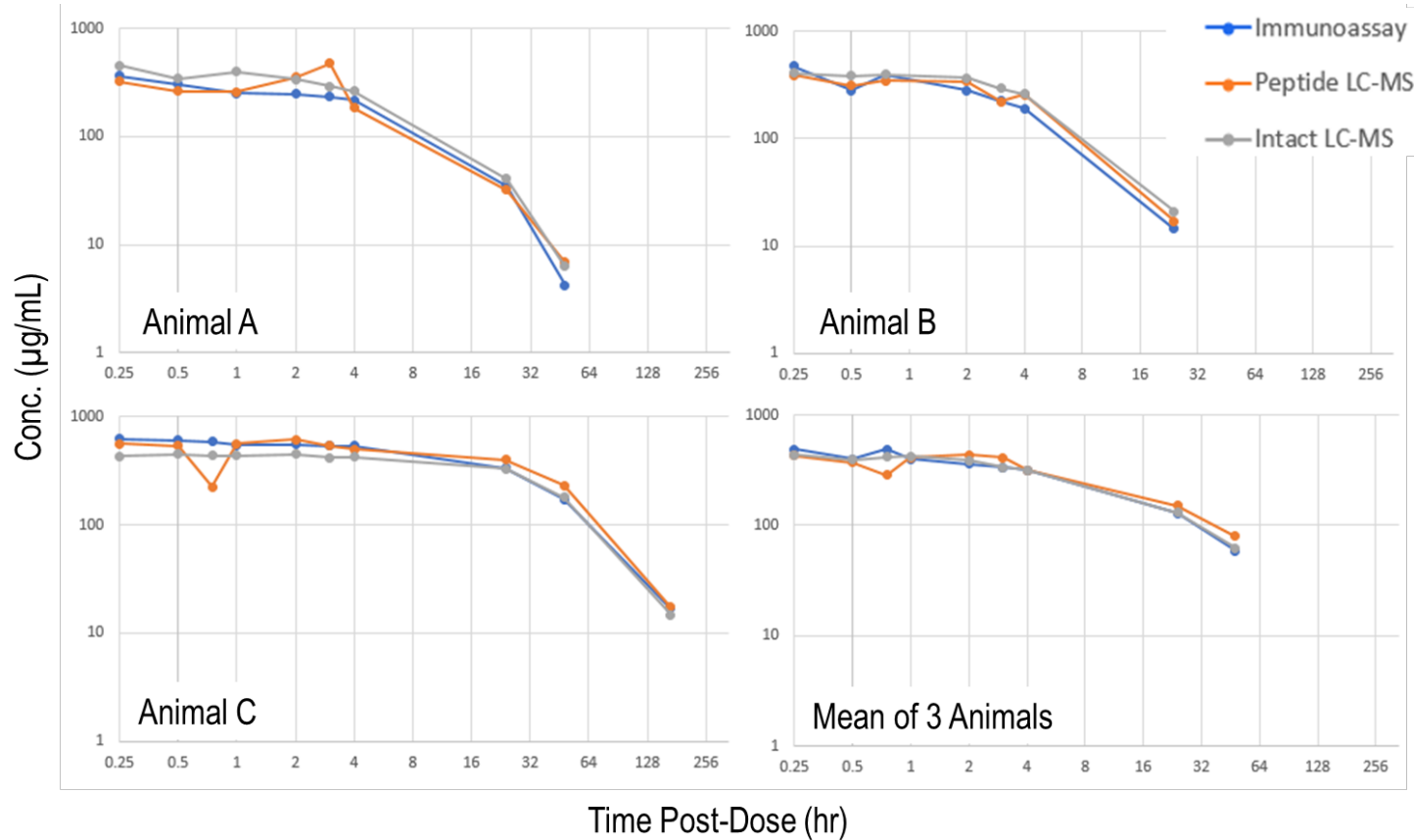
- Intact mass & detection is stable under standard stability test conditions
- Long-term stability especially important for future long-term study support

In-life Sample Analysis: Study details & assay formats utilized

- Cynomolgus monkeys (n=3) dosed with GSKmAb (10 mg/kg)
- Samples analyzed to 168 hours post dose



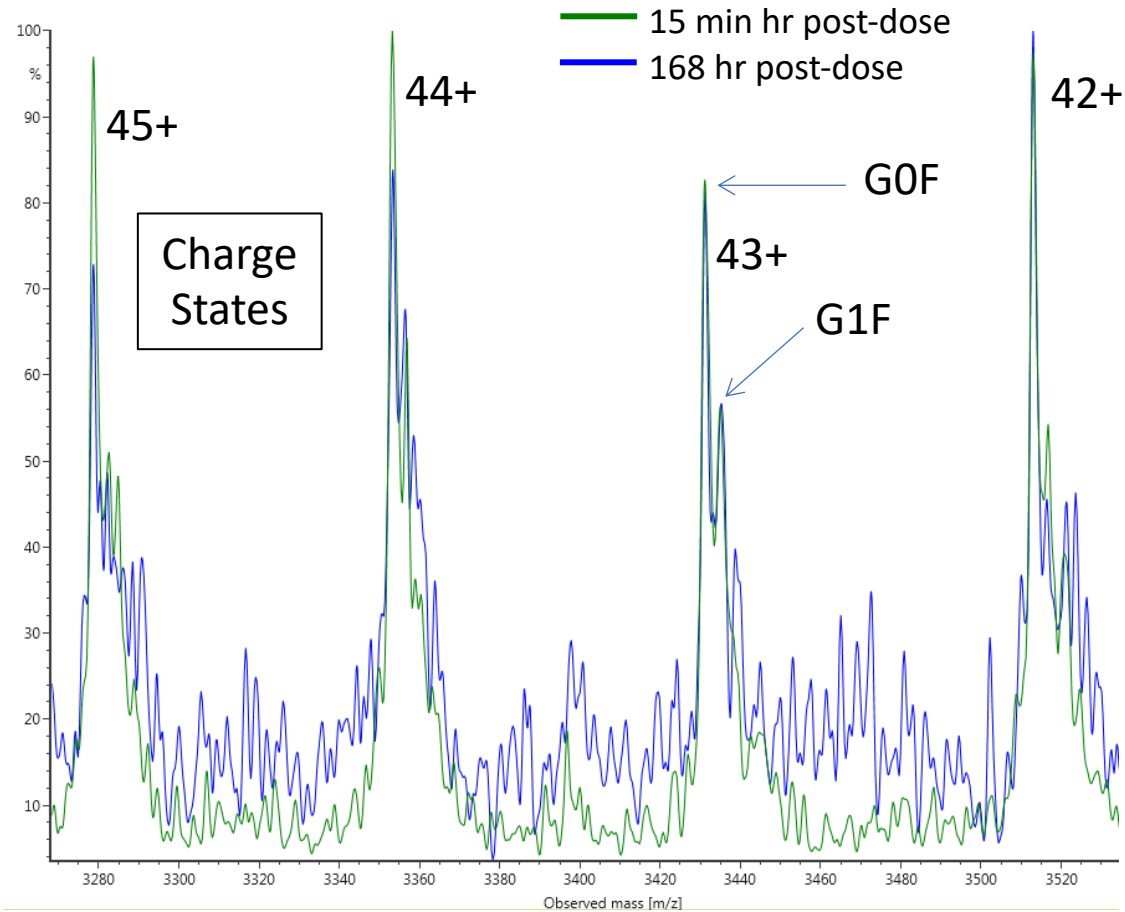
In-life Sample results: Plots for assay comparison



Difference (%) for Mean Sample Concentrations		
Time (hr)	Intact vs. LBA	Intact vs. Peptide
0.25	-12.0	1.5
0.5	-1.1	6.1
0.75	-15.6	37.8
1	4.6	1.8
2	6.7	-12.3
3	0.8	-20.6
4	0.2	0.9
24	2.8	-13.6
48	5.5	-24.2

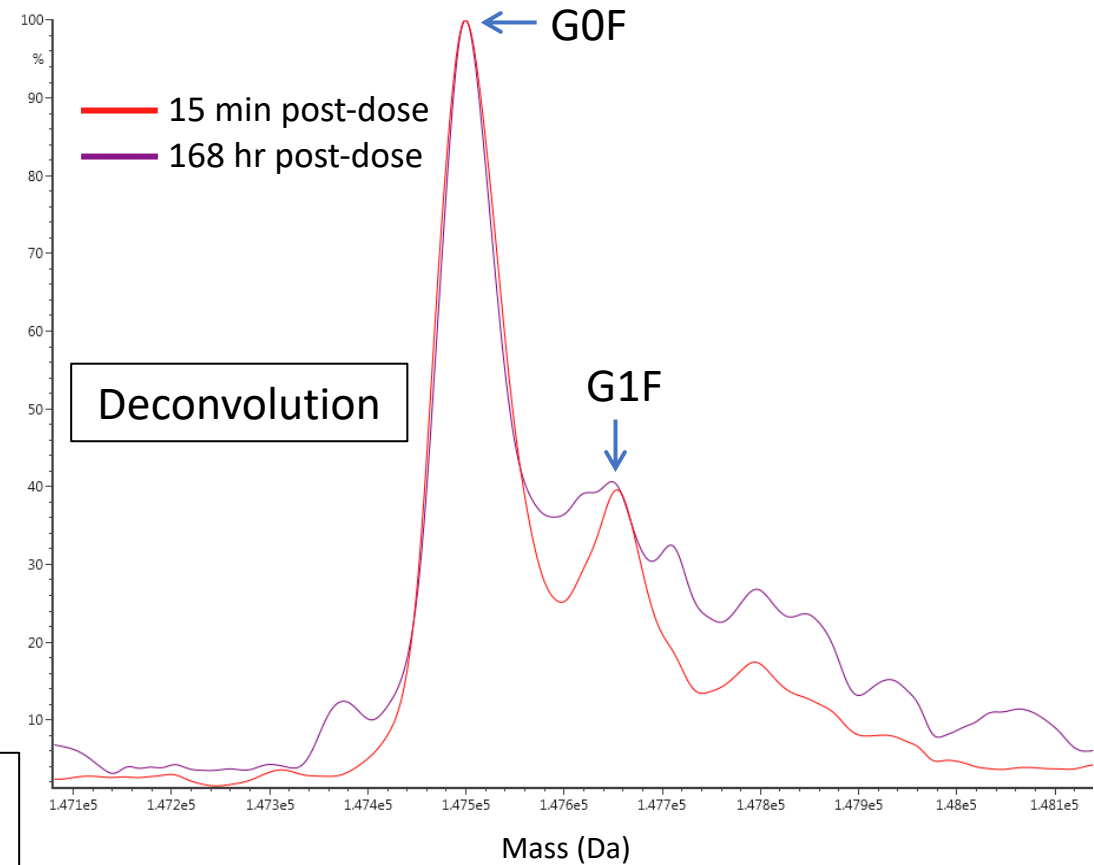
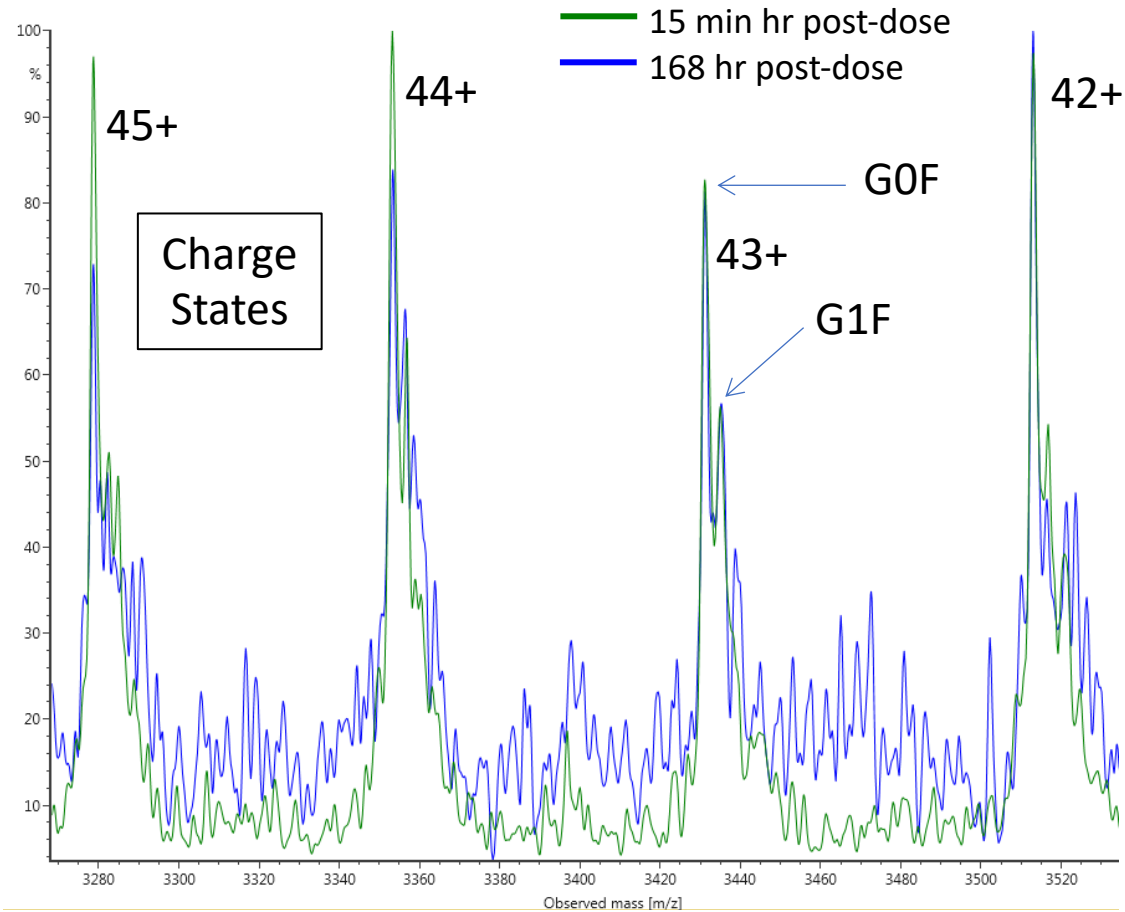
AUC _{last} (h*µg/mL)	Subject A	Subject B	Subject C	Mean (AUC _{48 hr})
Intact	4140	3240	23170	7899
Peptide	3411	2487	28475	8659
LBA	3356	2942	24634	7749

Relative detection of glycoforms



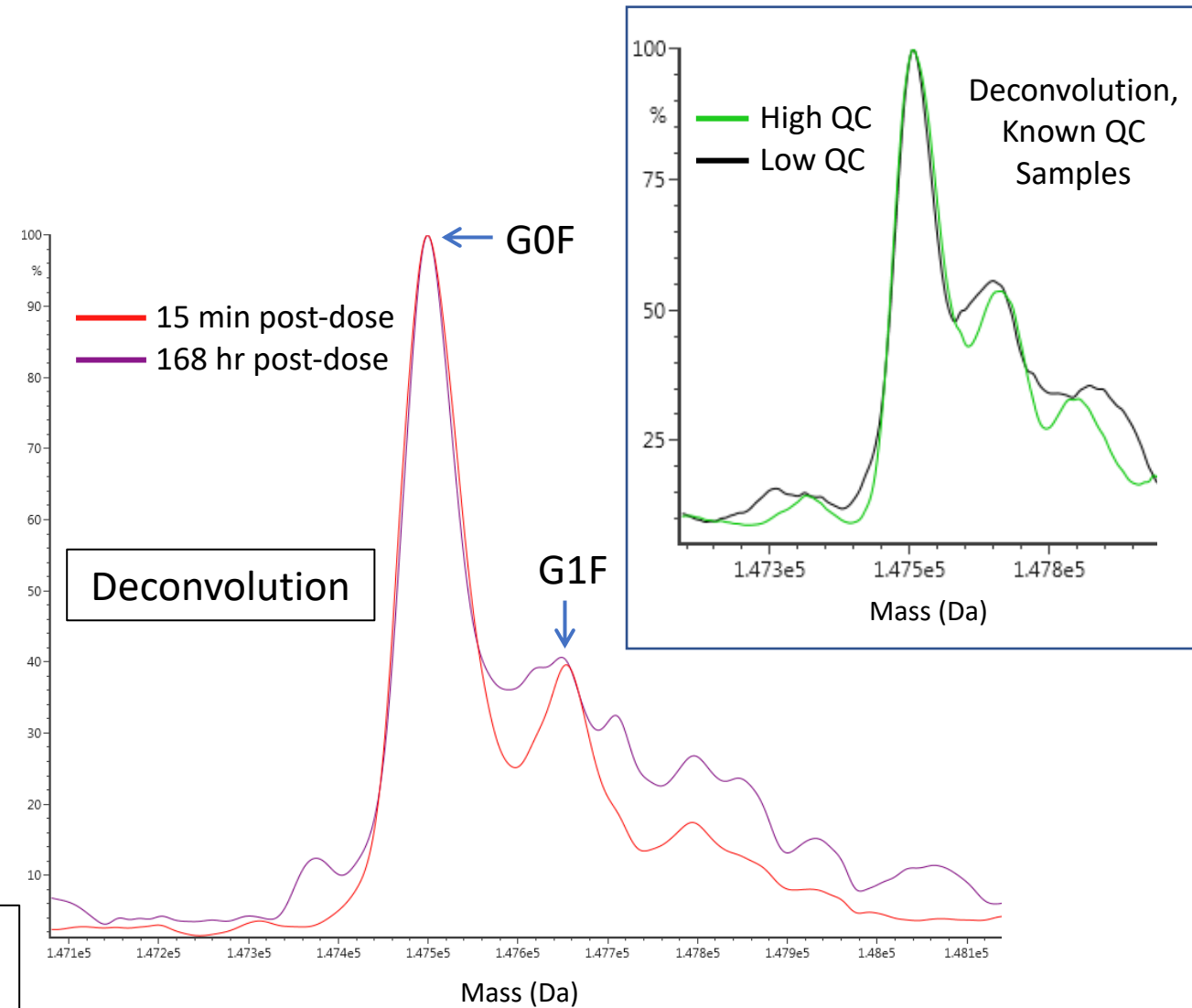
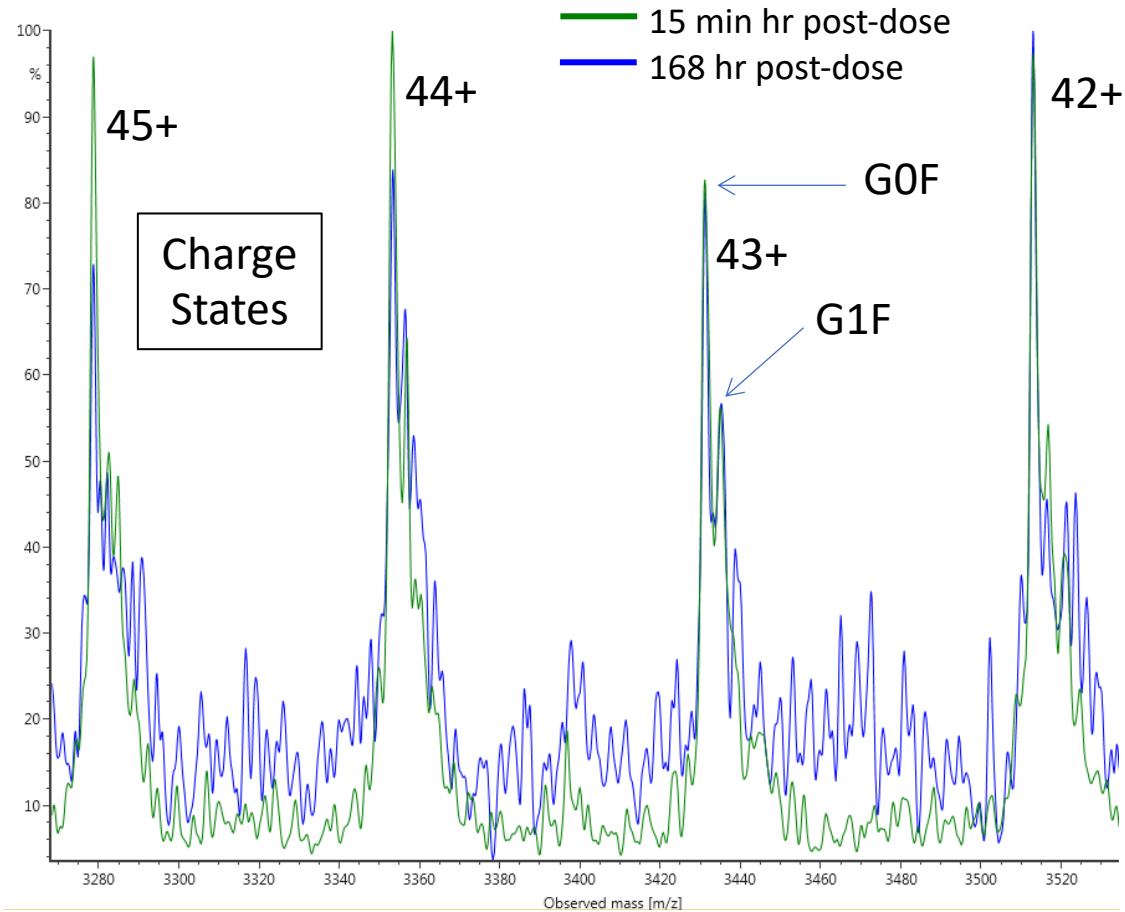
G0F/G1F ratios do not drastically change over time, but data quality (e.g. S/N) for reliable relative quantitation is worse at lower concentrations

Relative detection of glycoforms from in-life samples



G0F/G1F ratios do not drastically change over time, but data quality (e.g. S/N) for reliable relative quantitation is worse at lower concentrations

Relative detection of glycoforms



G0F/G1F ratios do not drastically change over time, but data quality (e.g. S/N) for reliable relative quantitation is worse at lower concentrations

Different quantification modes: charge states and deconvolution

Varying number of Charge States in QC Data: % CV

QC Conc. (µg/mL)	n	1 Charge State	3 Charge States	6 Charge States	23 Charge States	Deconvolution
5	6	20.55	8.22	10.66	10.66	25.92
15	6	12.64	6.95	8.12	6.53	9.78
25	6	5.33	5.50	3.82	2.27	6.33
40	6	10.58	4.03	3.89	4.03	7.66
50	6	12.03	3.90	4.32	3.70	7.24
Calibration R ²		0.978	0.973	0.986	0.970	0.985
Calibration exclusion	Data points	2	0	1	0	0

All settings may be viable, but multiple charge state strategies minimize % CV, particularly at the LLOQ

Summary & Conclusions

- Intact protein LC-MS for PK and other in-life study support is a viable alternative to LBA and peptide LC-MS assays (but might not be as sensitive)
- Intact protein LC-MS assays can generate comparable data packages for bioanalytical method validation and study support
- Additional data & for mass variants possible
- Data processing is not standardized across industry (work in progress)

Acknowledgements & Statements

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Waters

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- Henry Shion
- Ian Edwards
- Mark Wrona

- All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed.
- All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.