Strategies and Case Studies on the Bioanalysis of Protein Therapeutics and Biomarkers by LC-MS/MS

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BioAgilytix

AGENDA

- Workflows for Large Molecule LC/MS
- Strategies
- Case Studies
- Summary

Workflows for large molecule LC/MS

LC/MS Approaches for Proteins

DIRECT DIGESTION: SURROGATE PEPTIDE-BASED

HYBRID IA-LC/MS: SURROGATE PEPTIDE-BASED

HYBRID IA-LC/MS: INTACT PROTEIN-BASED

LC/MS Approaches for Proteins

Direct Digestion LC-MS	Surrogate Peptide IA-LC-MS	Intact IA-LC-HRMS
• Bottom-up approach (protease cleavage) • Recommended SPE clean-up • Surrogate-peptide based	• Immunocapture followed by Bottom-up approach (protease cleavage) • Generic – anti-human Fc, Protein A/G, anti-human kappa/lambda, etc • Targeted – anti-idiotypic antibody or target antigen-based enrichment	• Immunocapture followed by Top-down approach • Targeted – anti-idiotypic antibody or target antigen-based enrichment • High resolution mass spectrometry • Deconvolution or XIC summing
PROS: Generic, Less complex **CONS: Less selective; matrix effect**	**PROS: High selectivity and sensitivity** **CONS: Complex, Need reagents**	**PROS: Complete structural information, special applications** **CONS: Complex, Need reagents**
**Typical LLOQ ~0.2 µg/mL*	**Typical LLOQ ≤ 0.025 µg/mL*	**Typical LLOQ ~0.5-5.0 µg/mL*

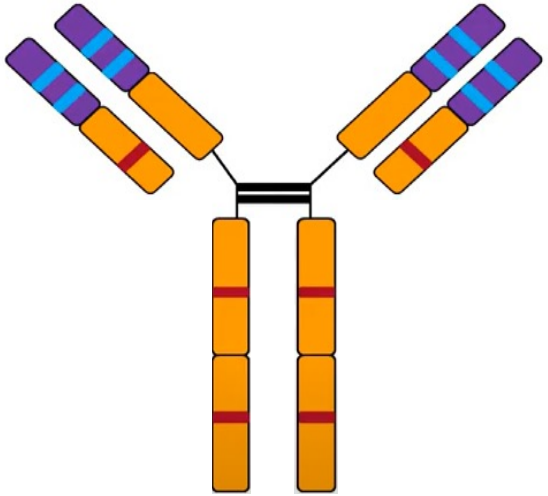
**LLOQ is dependent on analyte, matrix, and capture system.*

Strategies

Identification of Surrogate Peptide

- Combination of open source bioinformatic tools
- BLAST is performed based on the biological organism/species

Choice of Surrogate Peptide



Monoclonal Antibody (mAb)

UNIQUE PEPTIDES (Variable region)

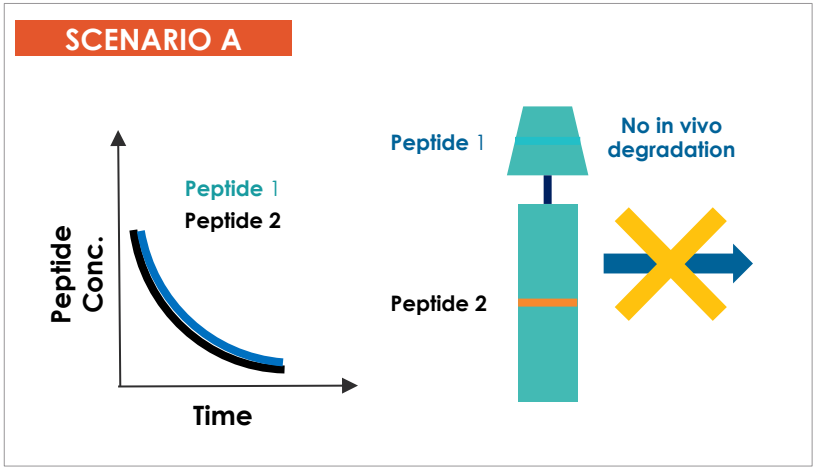
- Specific to analyte/drug only
- Preclinical/clinical

GENERIC PEPTIDES (Constant region)

- Common within class of mAbs
- Discovery/early development
- Suites preclinical with anti-human capture reagents
- Not for clinical unless anti-idiotypic capture reagents are used

Dual Peptide Approach

- Second peptide as diagnostic probe in discovery-grade and method development phases
- Limitations in regulated studies



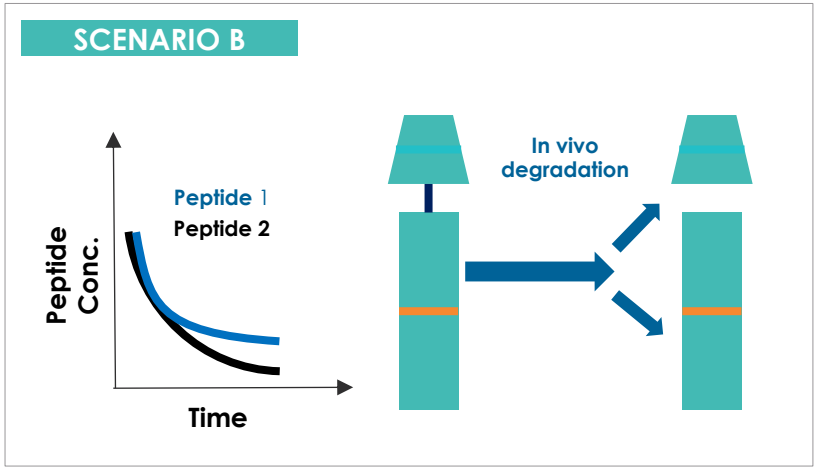
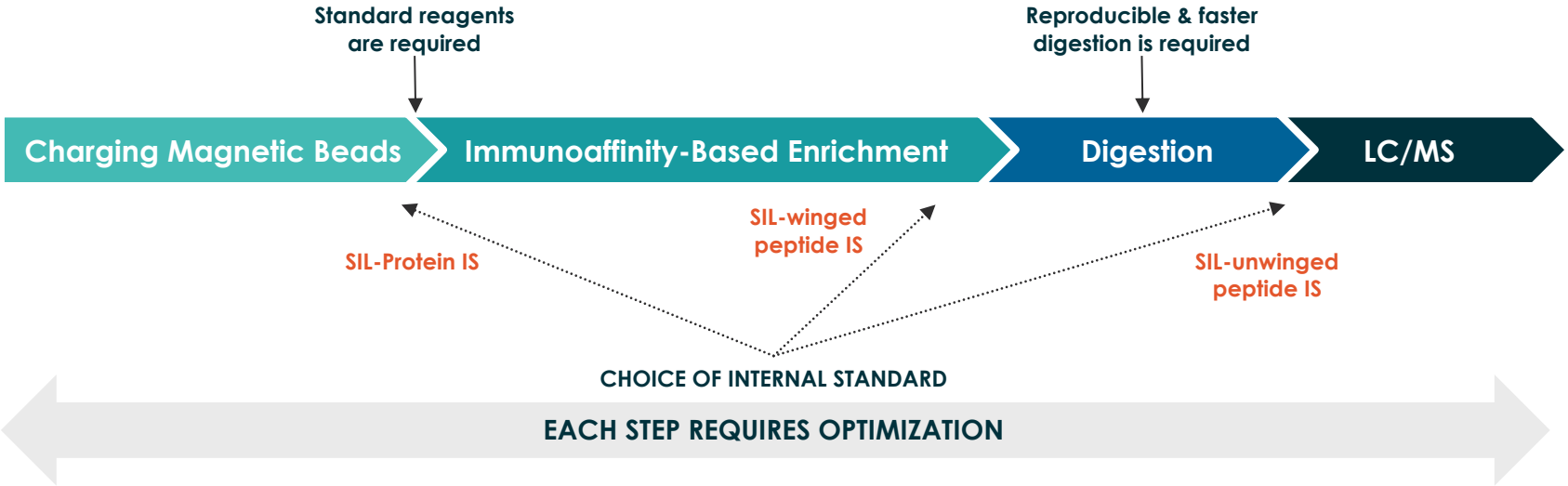
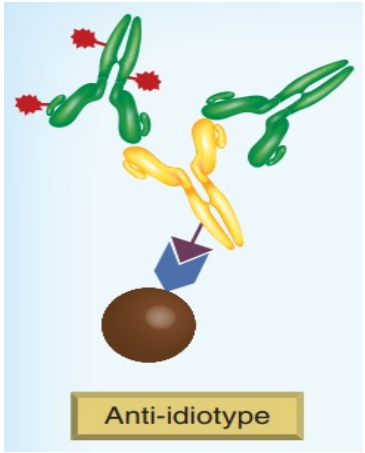


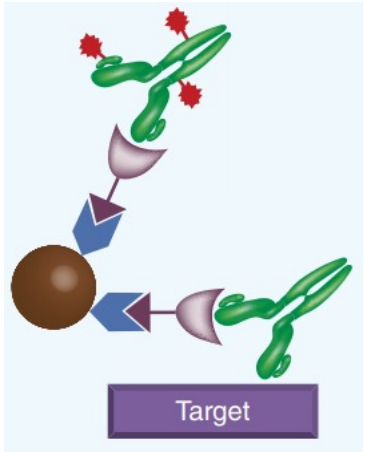
Image: Biomed. Chromatogr. 2012; 26: 1024–1032

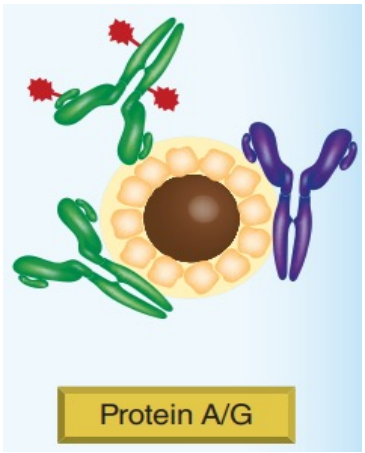
Choice of Internal Standard in Hybrid Assays



Choice of Capture System in mAb Therapeutics



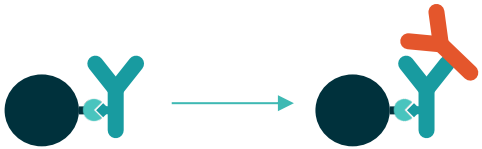




1. **Anti-ID Ab**: Paratope and idiotope overlap → drug antigen and capture system compete = [unbound drug]
2. **Anti-ID Ab**: Paratope and idiotope *do not* overlap → drug antigen and capture system *donot* compete = [unbound drug] + [bound drug] = [total drug]
3. **Anti-ID Ab**: complex specific: [bound drug]
4. **Target**: [unbound drug]
5. **Protein A/G/Fc-based**: [total drug]

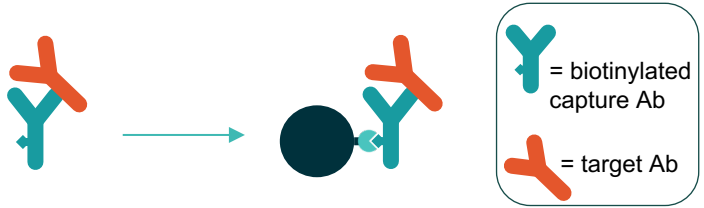
Image: Bioanalysis 2016; VOL. 8, NO.13

Immunocapture Approaches



Direct capture

- ✓ Streptavidin coated magnetic beads are charged with biotinylated capture antibody before sample incubation
- ✓ Operationally friendly
- ✓ Charged beads can be prepared in bulk
- ✓ Charged beads are typically stable in refrigerated conditions



Indirect capture

- ✓ Biotinylated capture antibody is incubated with sample before charging the streptavidin coated magnetic beads
- ✓ Elongated sample preparation time compared to direct capture
- ✓ Works well for low concentration analytes (eg: biomarkers)

Case Studies

CASE STUDY #1

Platform Assay: Bispecific Therapeutic Program

The molecule was designed to form a complex to assist in drug delivery.

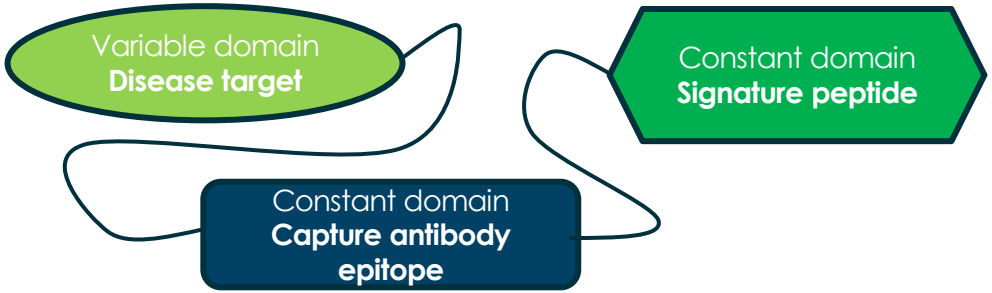
A PK method to measure the drug monomer was needed.

Multiple drug candidates in the program. Candidates share common domains.

Need an LC/MS platform assay for multiple drug candidates

CASE STUDY #1

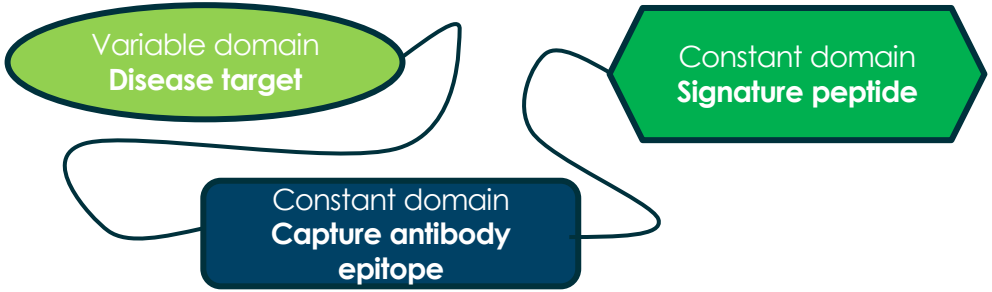
Signature Peptide Selection



- Peptide candidates from **variable region**
  ASQ----------
  LLI------
- Peptide candidates from **constant region**
  GLI---NN-------- (best in sensitivity)
  FSG------
  ELN------

CASE STUDY #1

Signature Peptide Selection

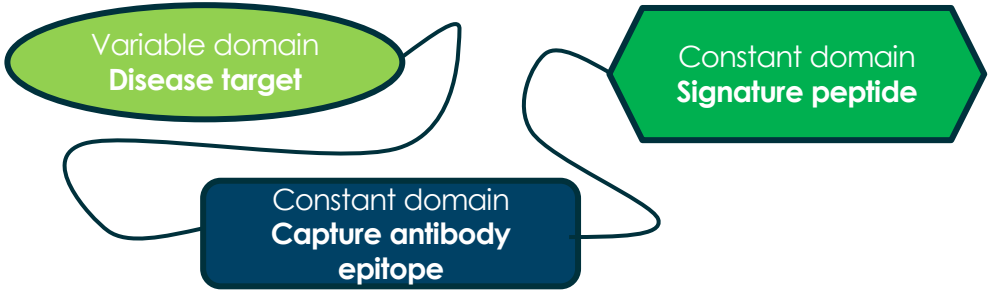


- Peptide candidates from variable region
  ASQ----------
  LLI------
- Peptide candidates from **constant region**
  GLI---NN-------- (best in sensitivity)
  FSG------
  ELN------
- GLI-peptide contains PTM on asparagine according to CMC data
- ELN-peptide was selected

- PTM proportion difference batch to batch.
- PTM *in vivo* after drug is dosed?

CASE STUDY #1

Signature Peptide Selection



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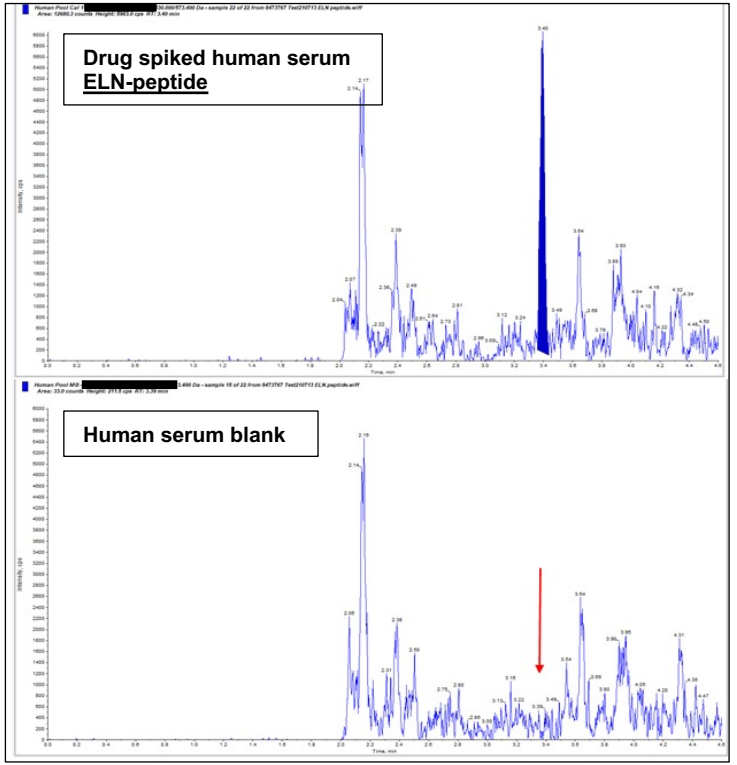
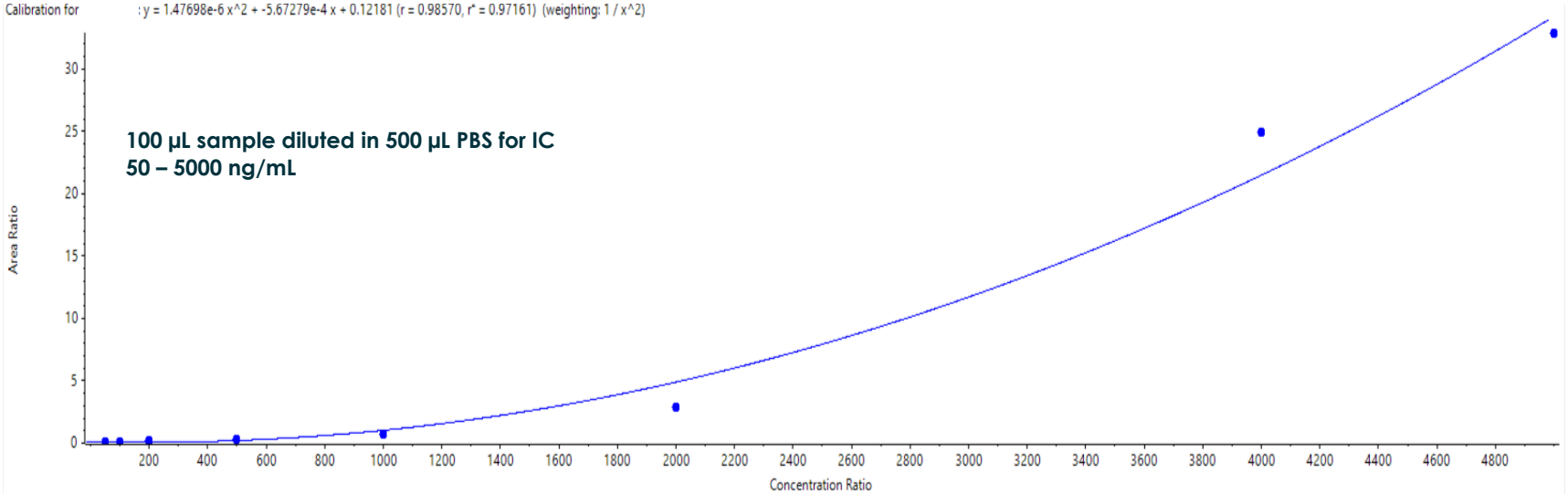


CASE STUDY #1

Immunocapture Condition Optimization

Parabola quadratic response

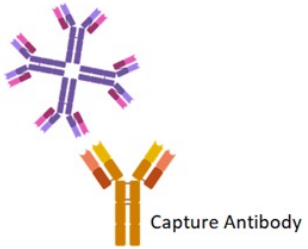


CASE STUDY #1

Immunocapture Condition Optimization

Reasons behind the abnormal curve response

HIGH CONCENTRATION

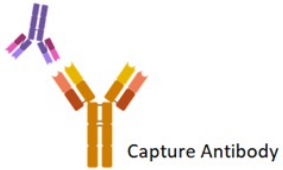


Antibody Tetramer

Formation of protein complex was **pH-** and **concentration**-dependent

Antibody captured more proteins in high concentration when protein-complex formed

LOW CONCENTRATION



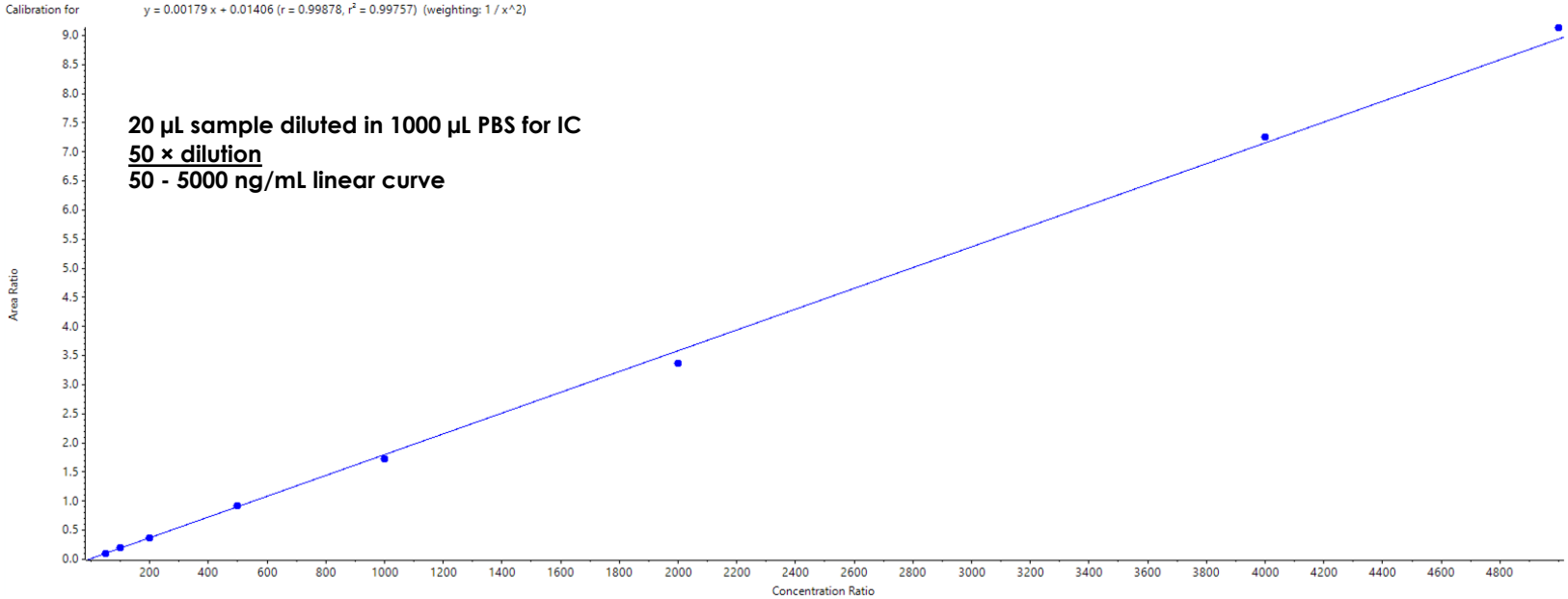
Antibody Monomer

Solution: excessive dilution for IC

CASE STUDY #1

Immunocapture Condition Optimization

Linear response



CASE STUDY #1

Assay Performance

Inter-day A&P comparison of two drug candidates using the same signature peptide from the constant region

DRUG CANDIDATE #1

Analytical Run	Replicate	Reported Concentration (ng/mL)			
		50	150	1500	3750
AR01	1	56.2	194 [a]	1820 [a]	4,340
	2	61.5	165	1,680	3,880
	3	56.3	184 [a]	1,500	4,230
	4	58.4	162	1,670	3,980
	5	62.1	167	1,580	4,500
	6	55.0	139	1,670	4,290
AR02	1	49.8	140	1,510	3,630
	2	45.1	149	1,450	3,610
	3	47.0	160	1,470	3,710
	4	48.6	147	1,420	3,570
	5	44.4	143	1,350	3,490
	6	45.8	152	1,310	3,340
AR03	1	44.7	133	1,380	3,580
	2	47.7	141	1,390	3,630
	3	45.5	129	1,400	3,610
	4	39.8	140	1,460	3,550
	5	44.4	143	1,440	3,800
	6	42.7	143	1,380	3,750
	Mean	49.7	152	1,490	3,810
	%CV	13.6	11.4	9.23	8.69
	%DEV	-0.600	1.33	-0.667	1.60

DRUG CANDIDATE #2

Analytical Run	Replicate	Reported Concentration (ng/mL)			
		50	150	1500	3750
AR01	1	58.5	174	1,550	4,130
	2	56.3	166	1,610	4,170
	3	56.0	162	1,710	3,830
	4	58.1	164	1,470	4,370
	5	55.5	167	1,650	4,220
	6	62.5	162	1,500	3,990
AR02	1	47.5	140	1,610	3,750
	2	49.8	149	1,640	3,830
	3	55.8	138	1,590	3,720
	4	49.0	144	1,440	3,680
	5	50.0	143	1,410	3,640
	6	50.6	151	1,540	3,670
AR03	1	56.1	173	1,640	4,280
	2	57.5	170	1,640	4,140
	3	57.6	166	1,510	3,950
	4	51.9	160	1,530	3,970
	5	57.8	162	1,530	4,100
	6	52.1	163	1,470	3,950
	Mean	54.6	159	1,560	3,970
	%CV	7.47	7.20	5.33	5.68
	%DEV	9.20	6.00	4.00	5.87

CASE STUDY #2

Therapeutic Transgene Expression Product

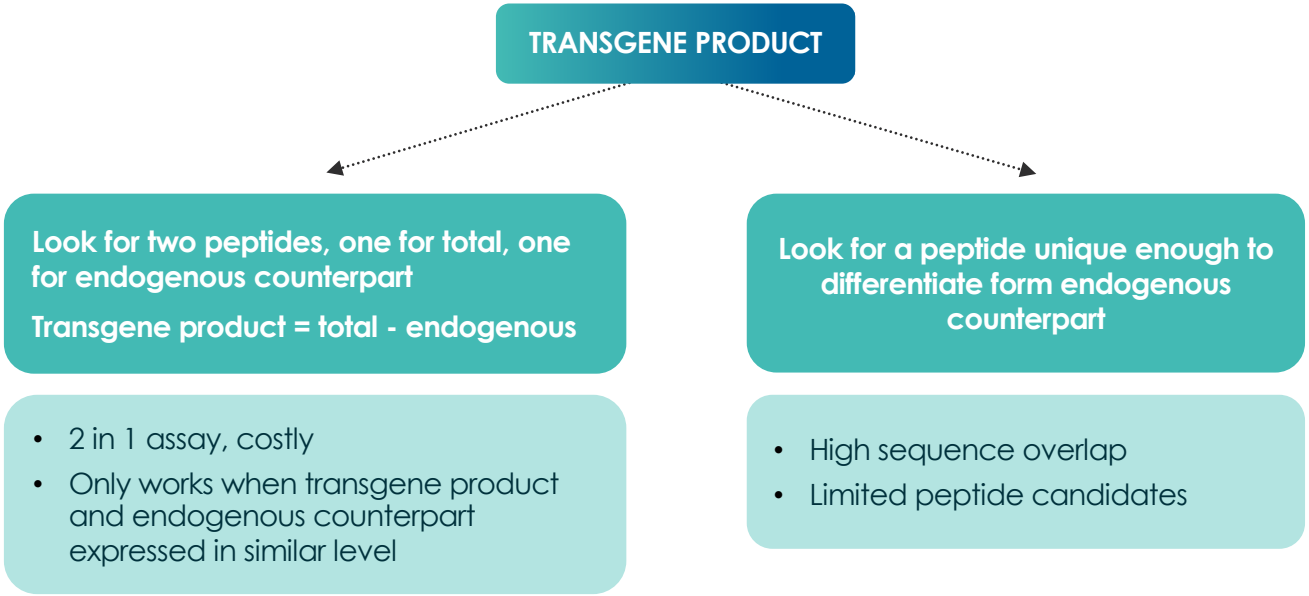
- Truncated version of a human soluble protein
- Method development in both NHP and human serum
- High sequence overlap compared to the endogenous counterpart, especially for human

CHALLENGE: Need signature peptides to differentiate from the endogenous counterpart

CASE STUDY #2

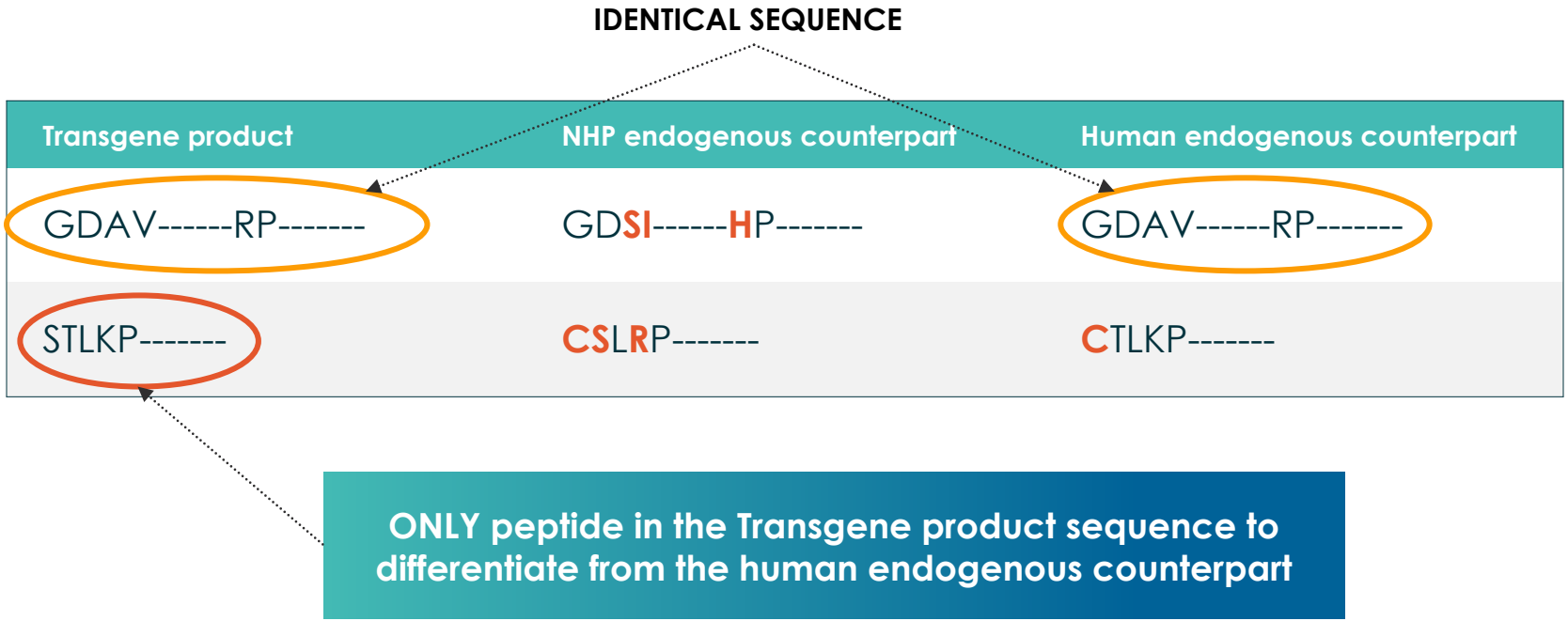
Signature Peptide Selection

Strategy options



CASE STUDY #2

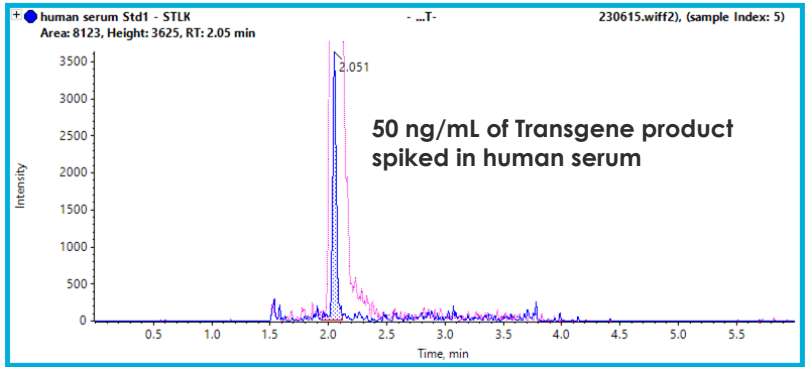
Signature Peptide Selection

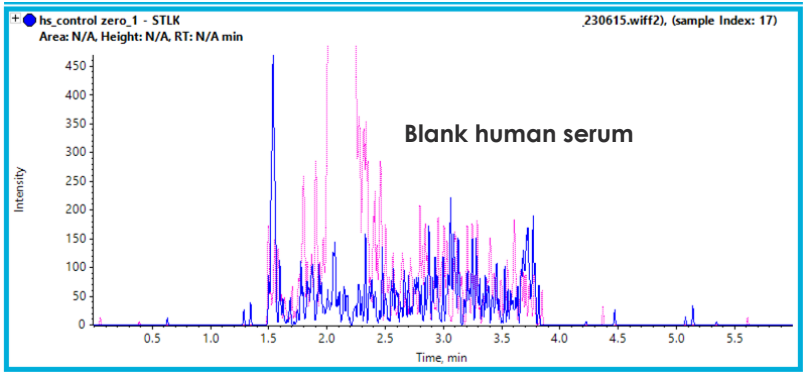


CASE STUDY #2

Signature Peptide Selection

STLK-peptide selectivity in human serum



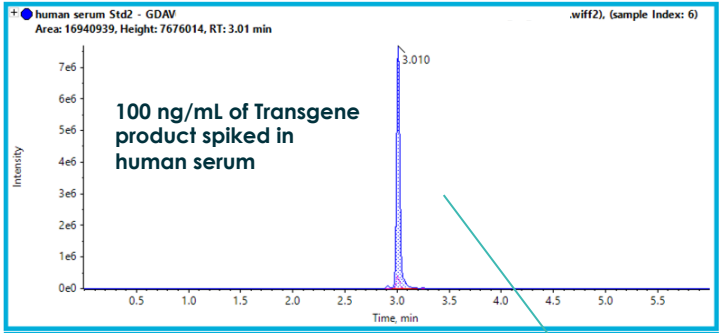


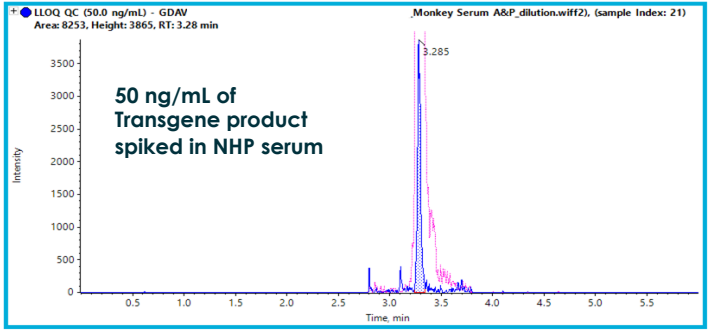
STLK-peptide is the **only** signature peptide can be used to distinguish the transgene expressed product from endogenous counterpart in human serum

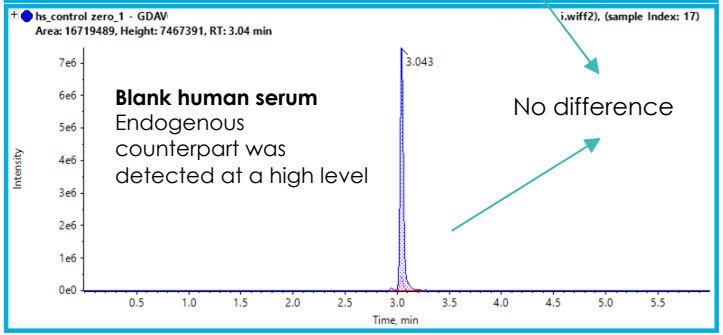
CASE STUDY #2

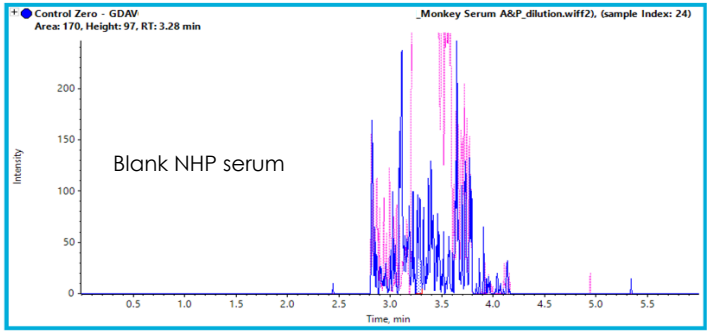
Signature Peptide Selection

GDAV-peptide selectivity in human and NHP serum





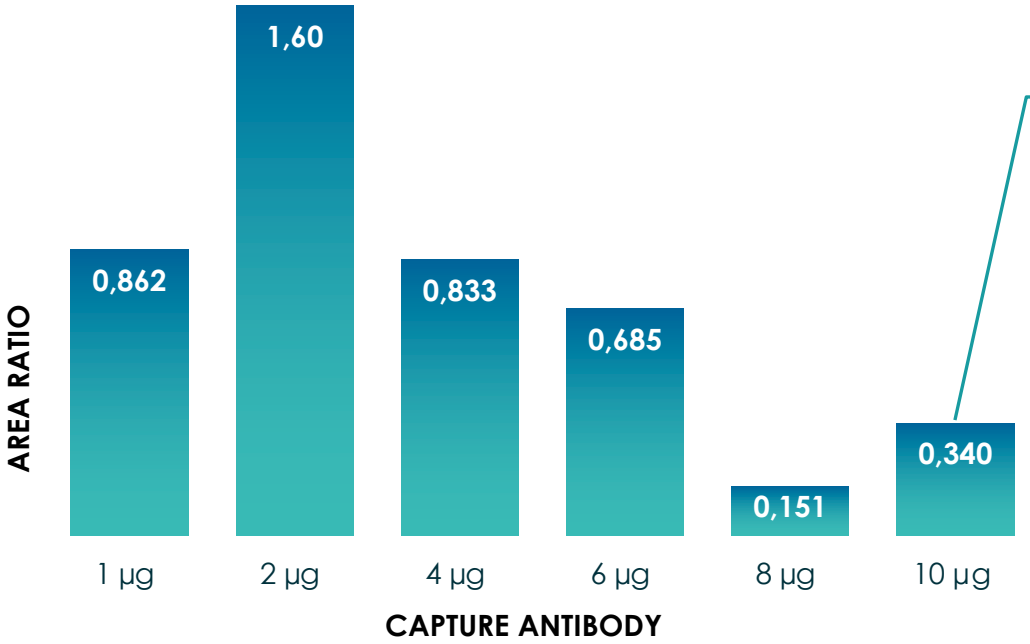




GDAV-peptide is the **most sensitive** signature peptide among all peptides tested. It was selected for the NHP study.

CASE STUDY #2

Capture Antibody Hook Effect

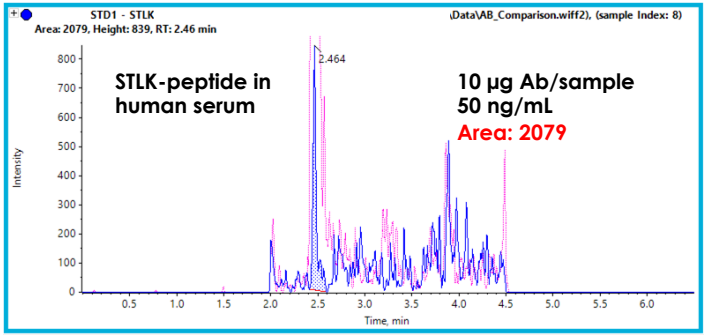


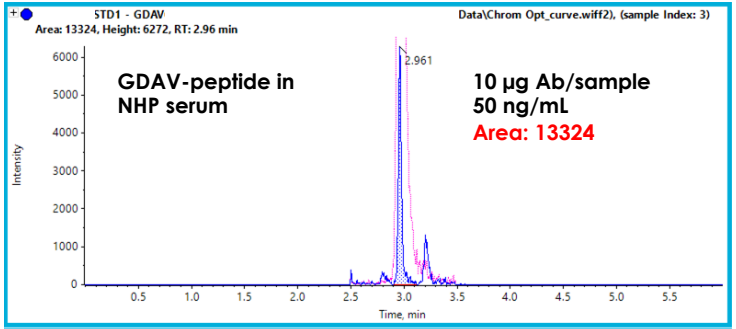
- Capture antibody is commercially available anti-human endogenous counterpart antibody
- Considering the high endogenous level, 10 µg antibody/sample was used as initial capture condition
- 3 antibodies with different epitope tested. All showed **low recovery** at 10 µg antibody/sample, therefore, **compromised the sensitivity** of the assay

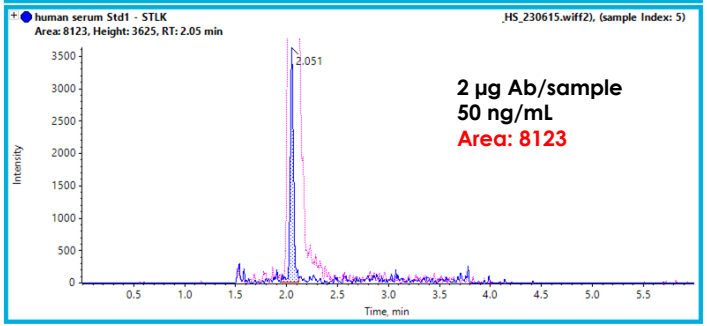
CASE STUDY #2

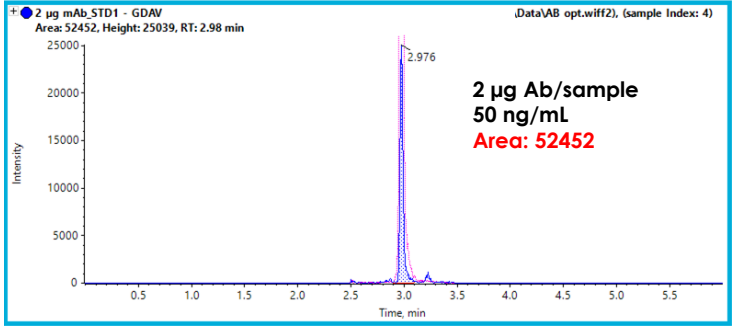
Capture Antibody Hook Effect

More sensitive with only 2 µg antibody/sample







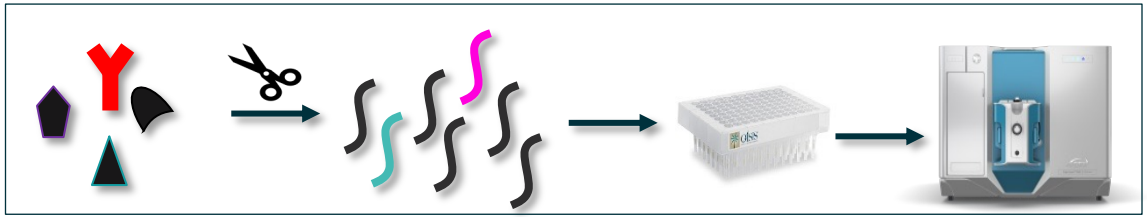


CASE STUDY #3

IgG1 Total PK Assay in Clinical Study

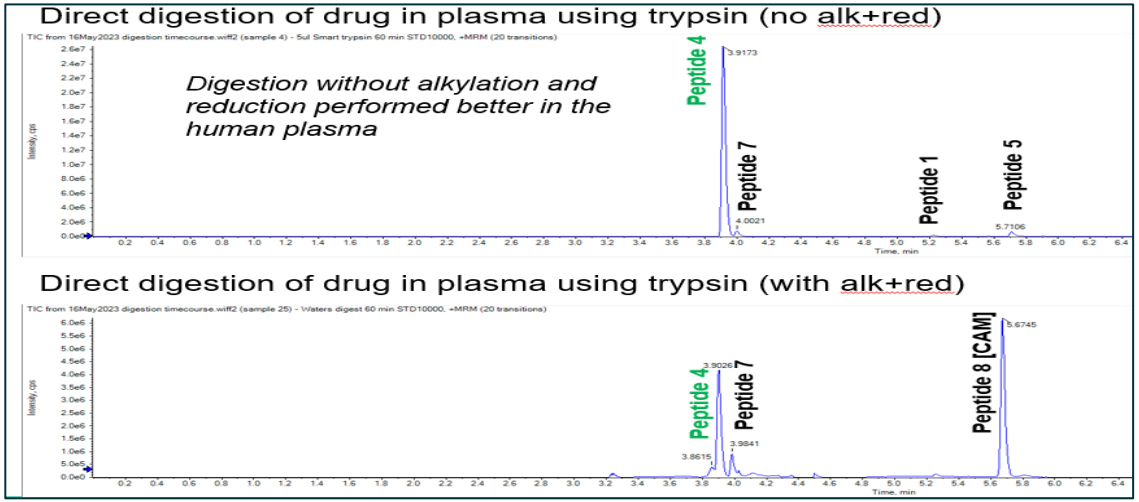
Workflow

Signature peptides from CDR region of IgG1 mAb therapeutic



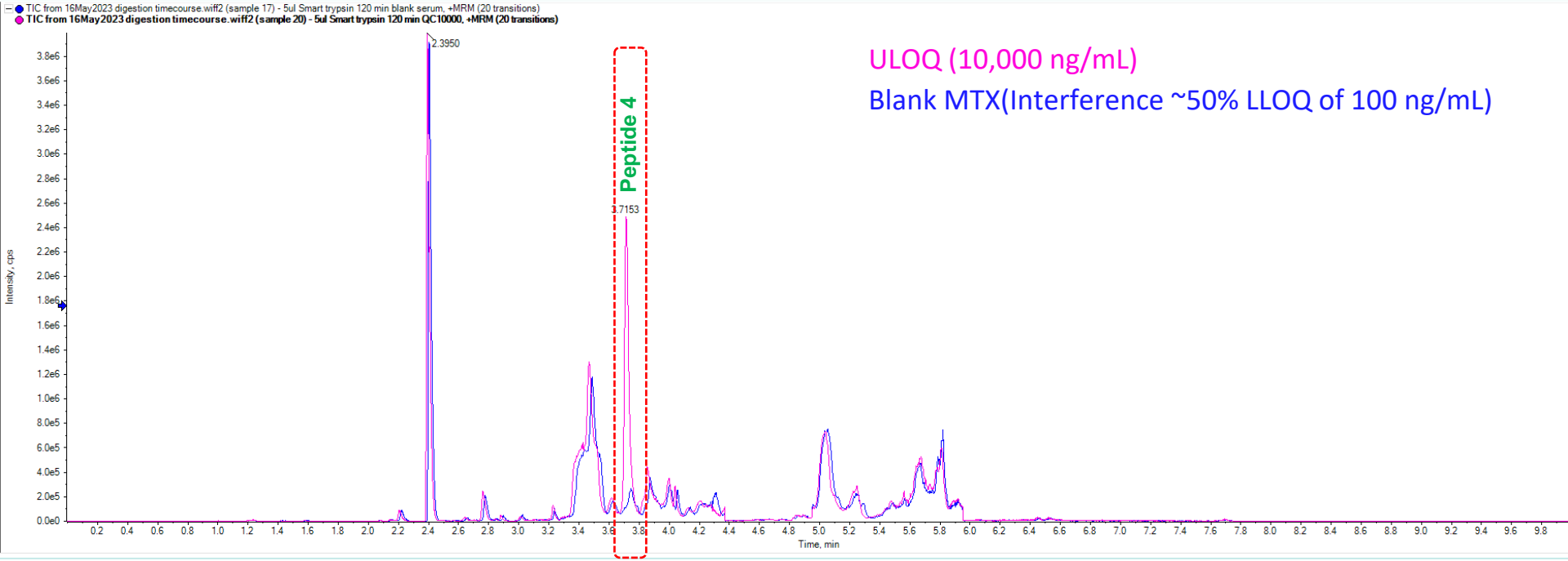
Signature peptide candidates

Tryptic peptide	Chain	#AA	BLASTp Human % identity
Peptide 1	LC	26	81%
Peptide 2	LC	26	88%
Peptide 3	HC	19	89%
Peptide 4	**LC**	**9**	**89%**
Peptide 5	HC	15	90%
Peptide 6	HC	26	92%
Peptide 7	HC	21	93%
Peptide 8	LC	24	96%



CASE STUDY #3

Interference in Blank Matrix



ULOQ (10,000 ng/mL)

Blank MTX(Interference ~50% LLOQ of 100 ng/mL)

CASE STUDY #3

Troubleshoot Interference

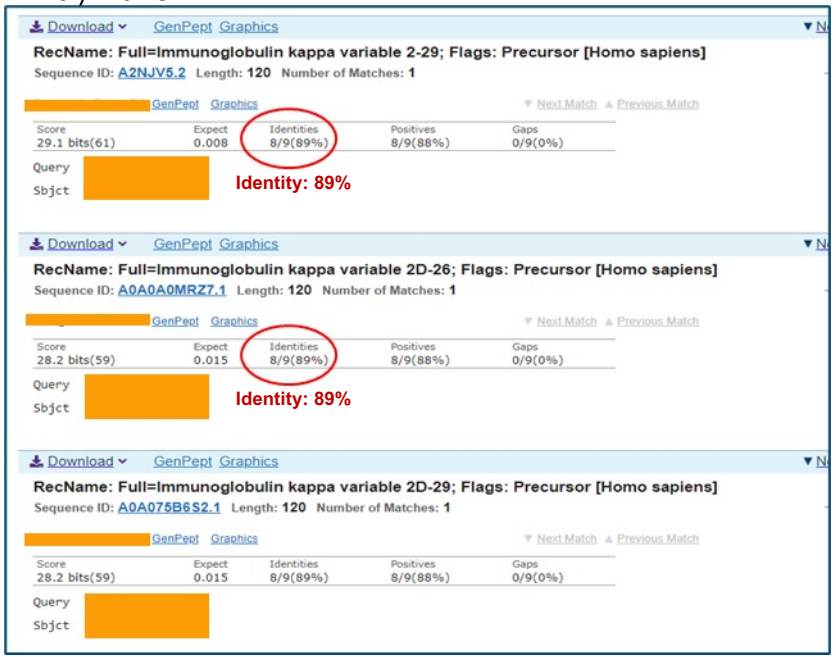
Several troubleshooting events were attempted

1. SPE elution optimization
2. LC gradient optimization
3. Alternate MRM transitions
4. High resolution modes on Q1 and Q3
5. TCA precipitation
6. LC-HRMS
7. Revisit the database search

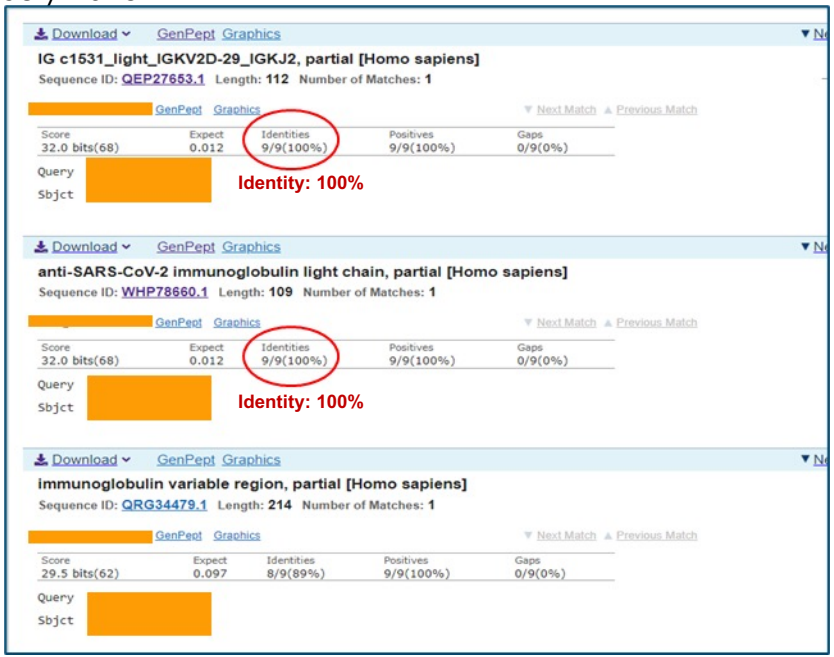
CASE STUDY #3

Revisit BLAST

May 2023



July 2023



- Hypothesis on interference

Omicron boosting induces de novo B cell response in humans. Nature. 2023 May;617(7961):592-598. doi: 10.1038/s41586-023-06025-4. Epub 2023 Apr 3. PMID: 37011668.

CASE STUDY #3

Selectivity Assessment

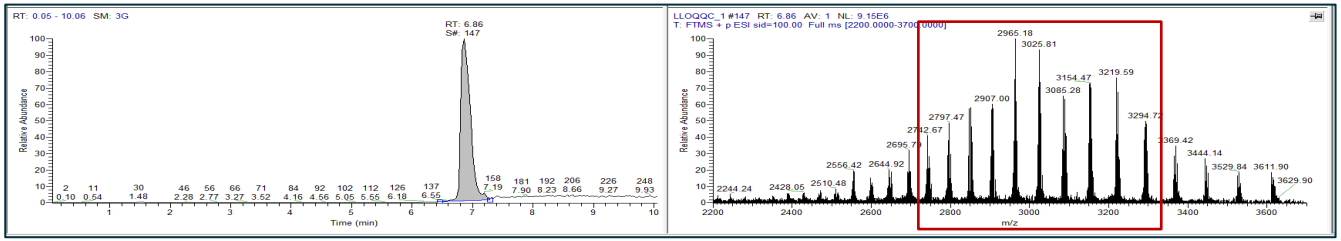
Matrix Lots	Interference based LLOQ of 250 ng/mL
Pooled BLK MTX	16%
BLK MTX Lot1	44%
BLK MTX Lot2	96%
BLK MTX Lot3	138%
BLK MTX Lot4	270%
BLK MTX Lot5	13%
BLK MTX Lot6	58%

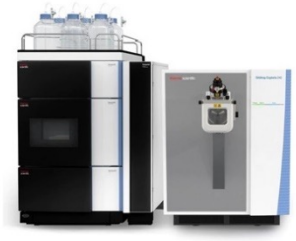
- Selectivity failed with varying levels of interference
- This data strengthened the hypothesis.
- Next steps: Hybrid IA approach using Anti-ID where paratope and idiotope *do not* overlap → drug antigen and capture system *donot* compete = [unbound drug] + [bound drug] = [total drug]

CASE STUDY #4

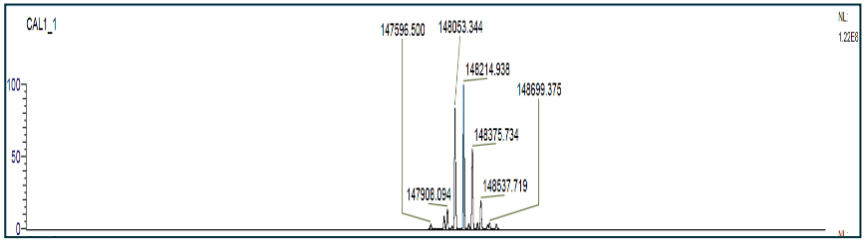
IgG Therapeutic Intact Analysis by LC/HRMS

XIC Approach





Deconvolution Approach



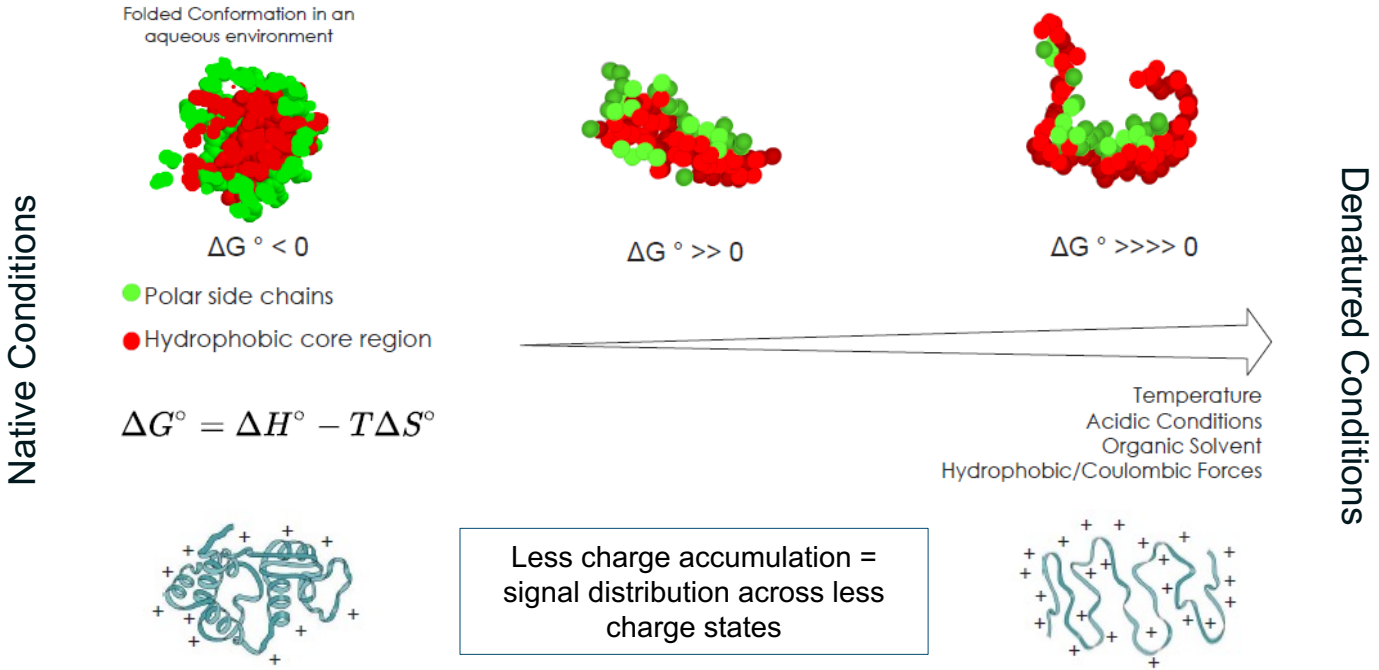
Quantitation by Intact LC-HRMS Analysis	
XIC approach	*Deconvolution*
Sum multiple charge states (typically 8-10)	Sum top intensity glycoforms (typically 2-3)
No proprietary algorithms applied post data acquisition	Deconvolution needs algorithms and differences exist in algorithms

- Application limited by sensitivity
- Dependent on ionization and charge state distribution (charge envelope)

CASE STUDY #4

How To Boost Intact Sensitivity:

Native vs Denatured Conditions



CASE STUDY #4

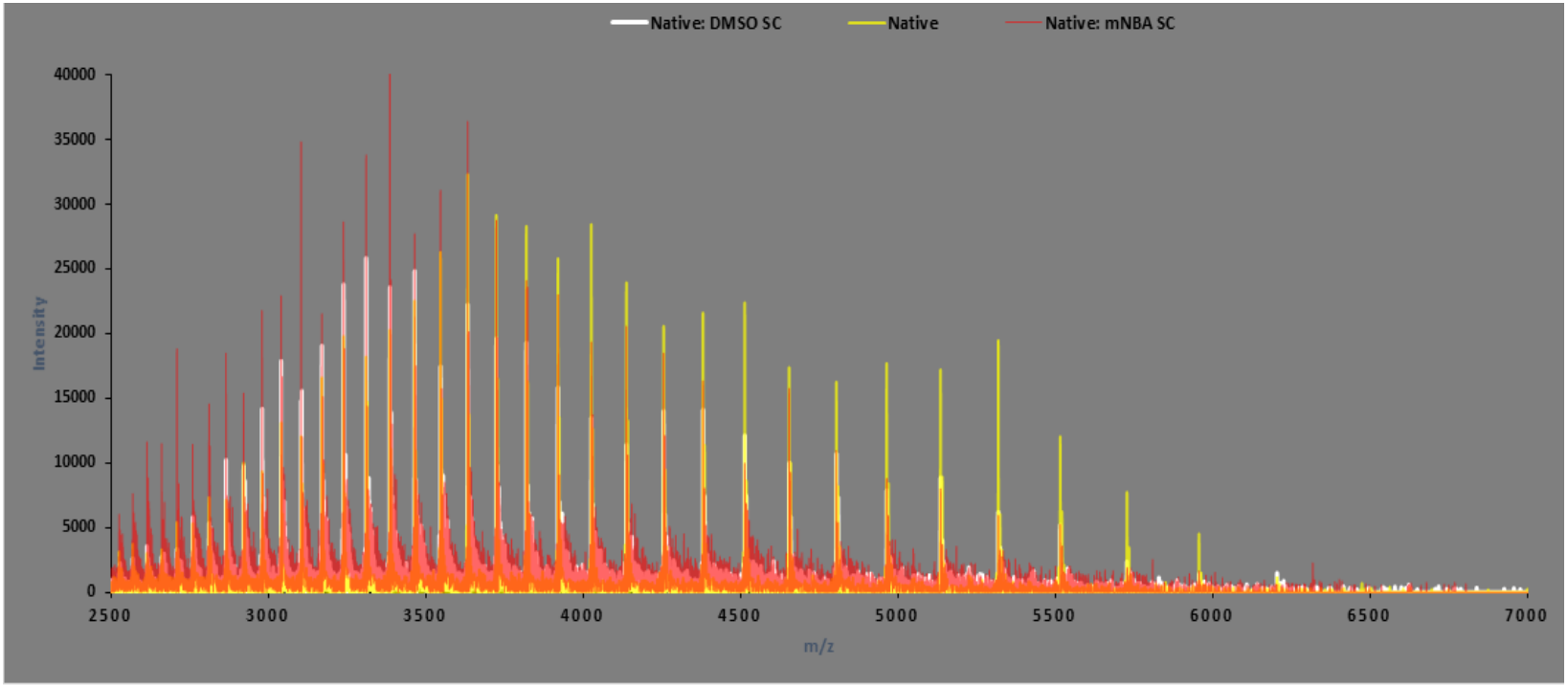
How To Boost Intact Sensitivity:

Can you supercharge your intact analysis?

- Supercharging agents increases positive/negative ion charging
- Supercharging agents are less volatile than the mobile phases
- Traditionally, supercharging agents are known to rescue the ionization suppression observed with TFA. Their role in intact biotherapeutics quantitation is not widely reported
- Few examples: m-nitrobenzyl alcohol (m-NBA), DMSO, sulfolane, formamide, etc.

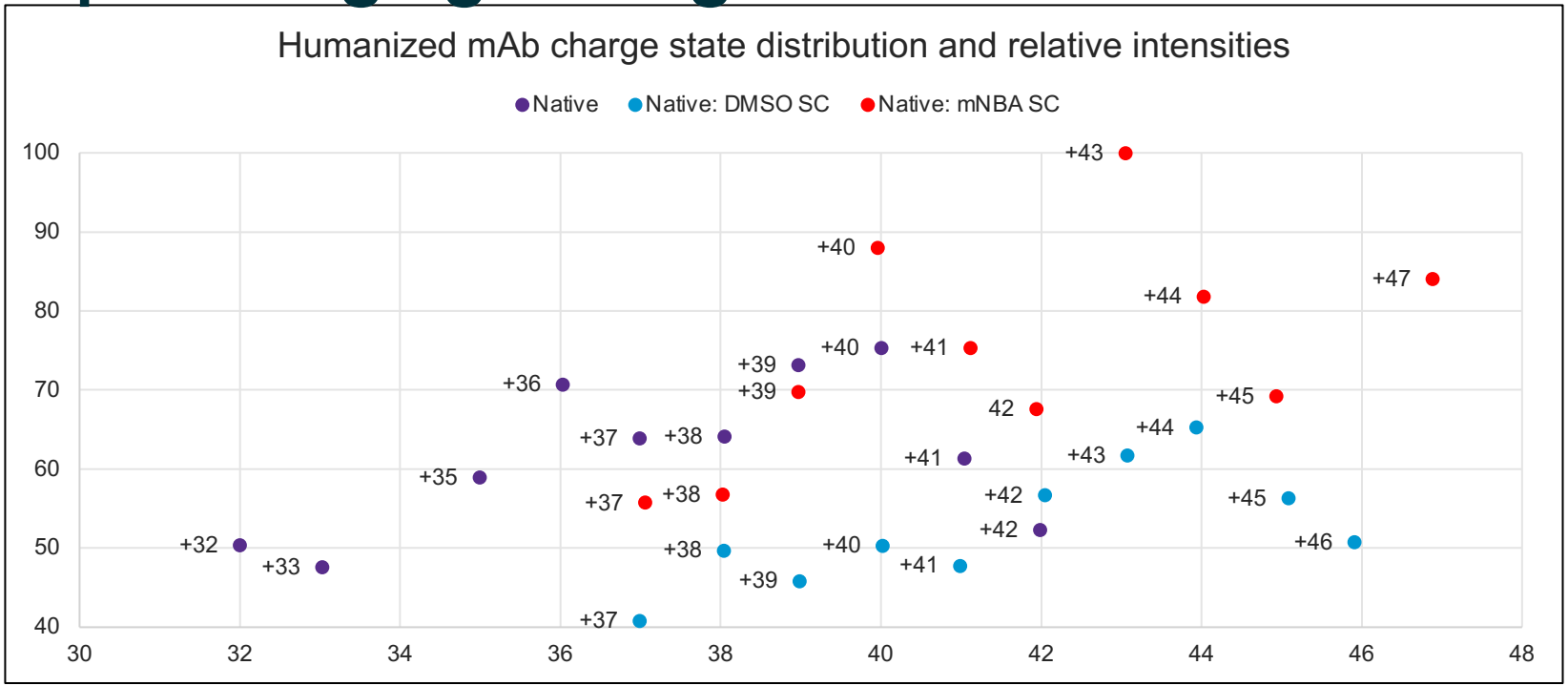
CASE STUDY #4

Native Chromatography and Supercharging



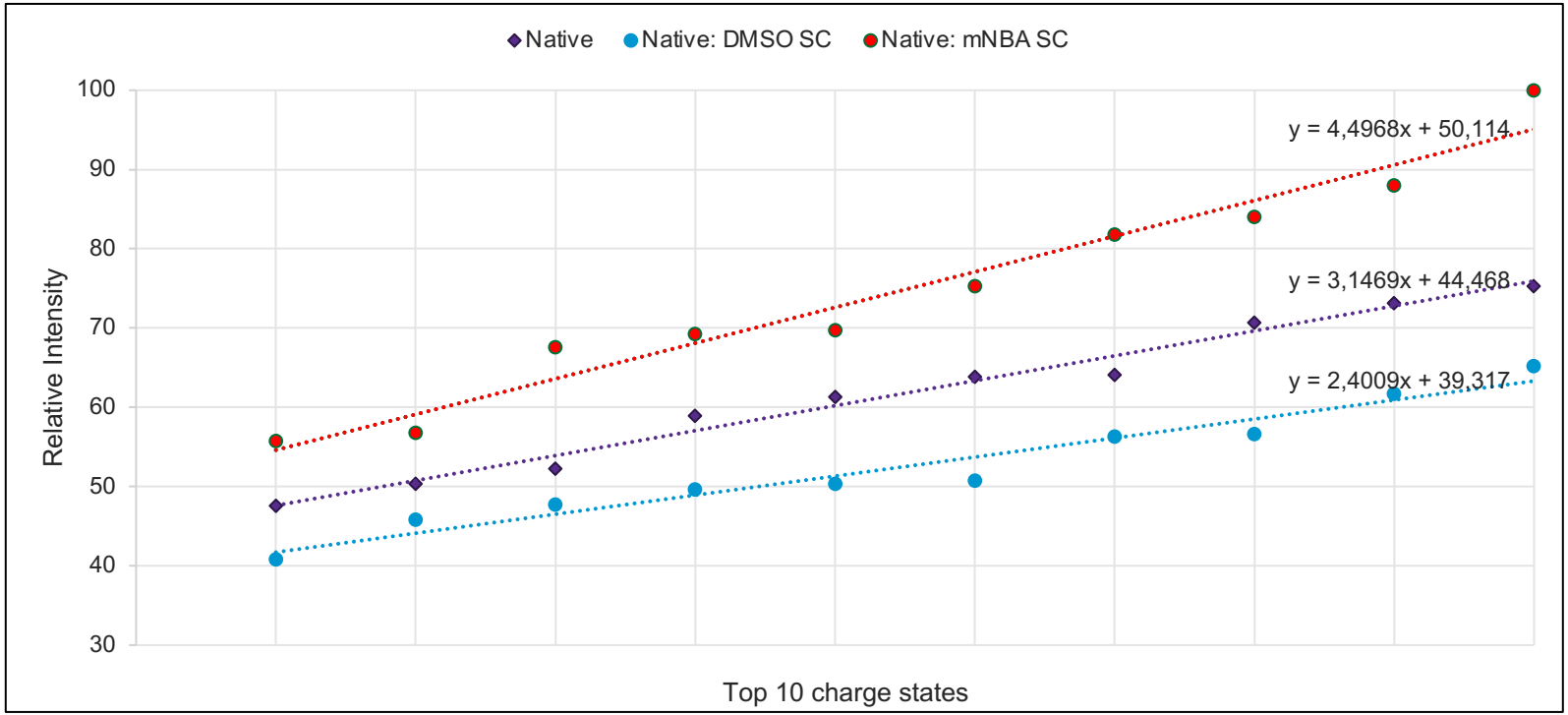
CASE STUDY #4

Supercharging: Charge State and Relative Intensity



CASE STUDY #4

Supercharging: Increased Signal Intensity



Summary

- Bottom-up approach is commonly used strategy for the quantification of proteins. Hybrid approaches provide additional selectivity and sensitivity.
- Selecting the appropriate internal standard is crucial and is dependent on assay needs, availability and performance.
- It is critical to understand the context of use and what do you intend to measure, when designing your hybrid assay to apply appropriate capture system
- Sensitivity in intact mass analysis is dependent on charge state envelope, that can be modulated by use of supercharging agents. Need more data to assess its impact and application.

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Thank you

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