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Ligand Binding Assay Critical Reagent Characterization via LC-MS

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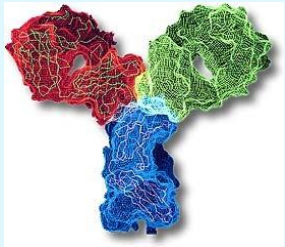
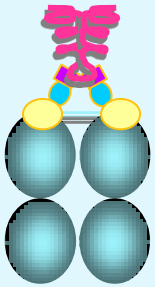
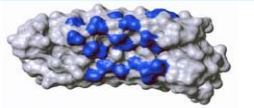
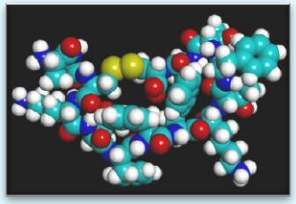
Advancement of Large Molecules Therapeutics

- Typical size in range of 3-200 kDa
- **Includes:** mAbs/derivatives, cytokines, peptides, fusion proteins, etc.
- Distinguished by manufacturing process: biological system vs. chemical synthesis
- Attributes:
  - Physical composition limits routes of administration
  - Half-life range from minutes (peptides) to weeks (mAbs)
  - Catabolism to endogenous amino acids limits toxicity

Antibodies	Antibody-like Platforms	Novel Protein Platforms	Therapeutic Peptides and Peptidomimetics
	Mimetibody™ 	Centyrins™  DARPins™ 	

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Bioanalytical Approaches: Large Molecule Therapeutics

- Bioanalytical gold standard - ligand binding assays (LBAs)
  - Pharmacokinetic assessment, immunogenicity testing, "free vs. total" measurement.
  - Multiple platforms from multiple vendors (MSD, EnVision, etc.)
  - Straightforward equipment training /use

*** Performance is dependant on quality of critical assay reagents for sensitivity, reproducibility, robustness, etc.**

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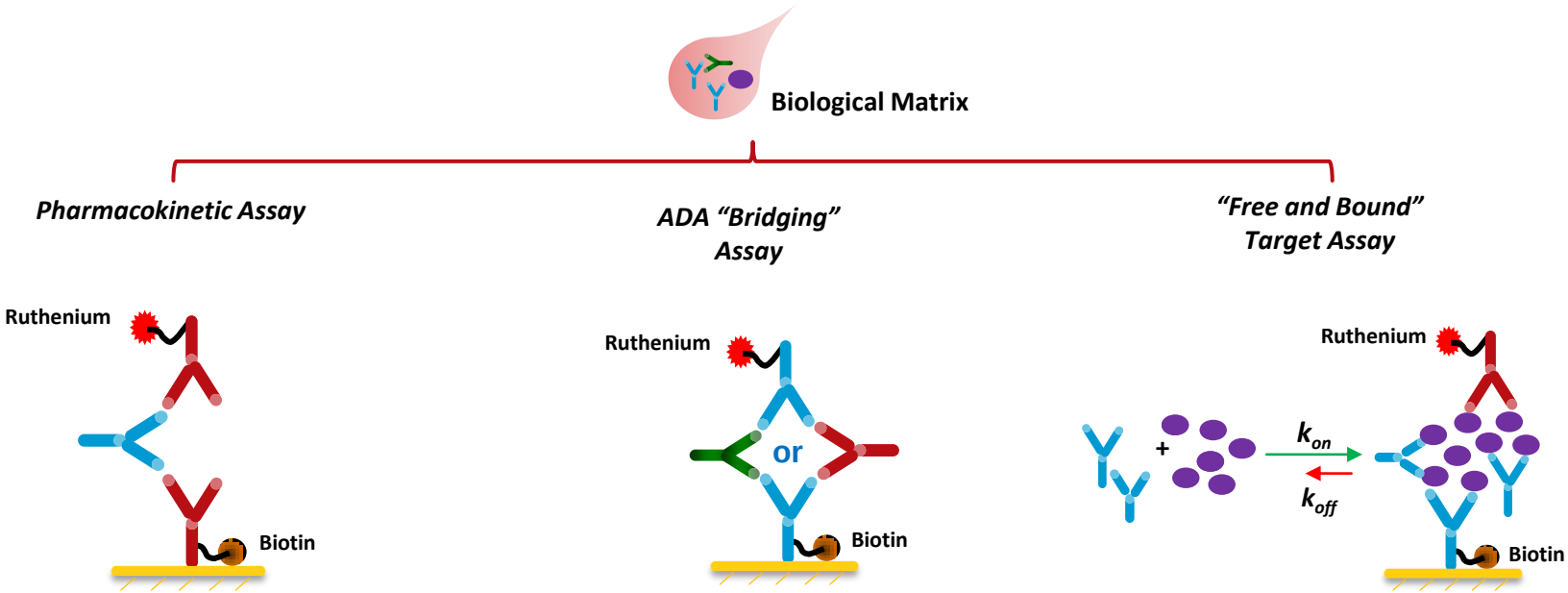
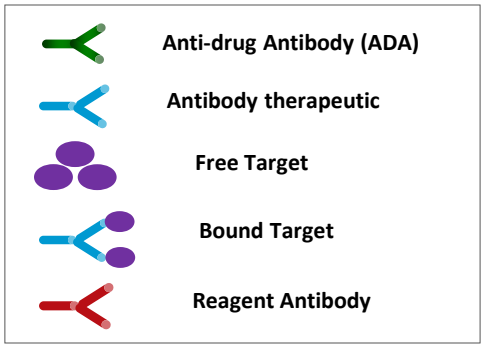
Ligand Binding Assay Methods - MSD





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LBA vs. LC-MS Large Molecule Summary

Parameter	LBA	LC-MS
Sensitivity	✓	
Selectivity/Specificity		✓
Dynamic Range		✓
Development Time		✓ ?
Sample Preparation	✓	
Throughput	✓	
Molecular Characterization		✓
Biological Activity	✓	
Regulatory Acceptance	✓	

Applications for LC-MS in Large Molecule Development

Quantitative Protein/Peptide Analysis:

- From complex matrices → support pharmacokinetic studies /ADME development
- Quantify "free" vs. "total" therapeutics → support of target engagement studies
- Quantify anti-therapeutic antibody response

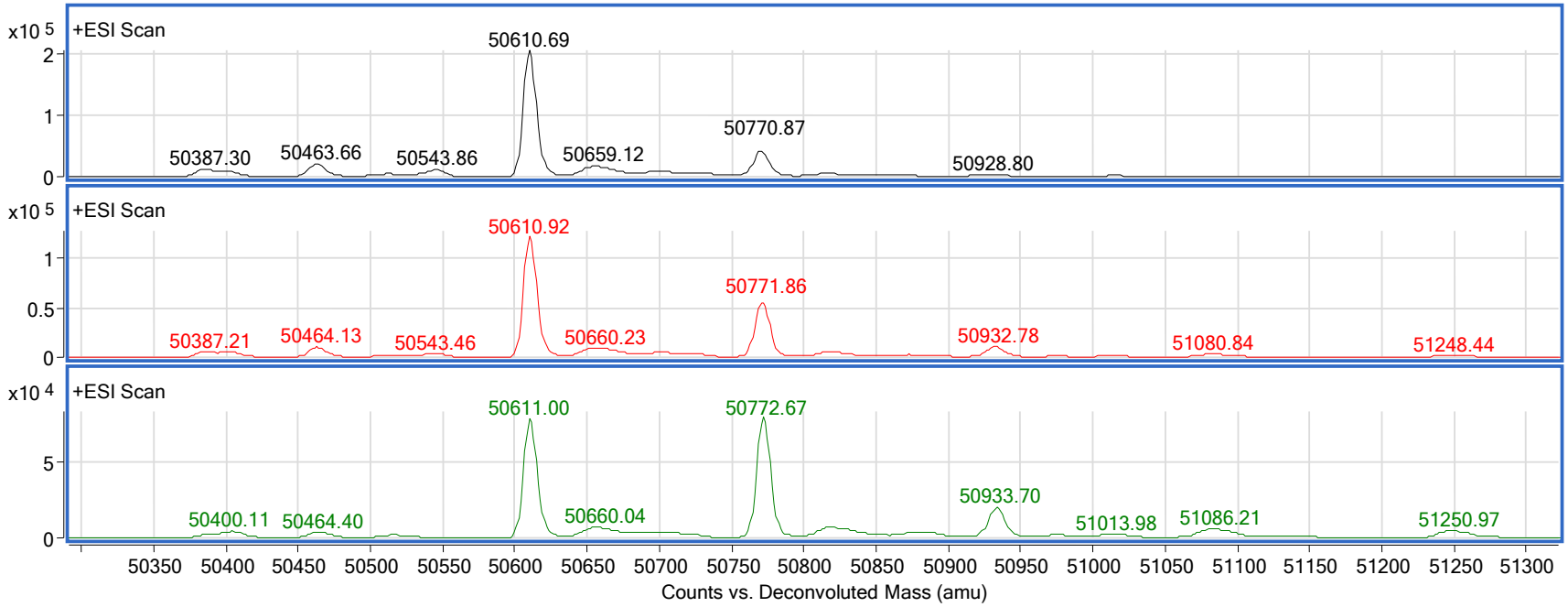
Physical Characterization:

- Glycoform/PTM/disulfide characterization of therapeutics
- Characterization of critical reagents to support PK/IR assessment

LC-MS Characterization of Large Molecules - Glycosylation

Characterization of Glycoform Distribution:

- Analysis of therapeutic proteins from different culture conditions or cell lines
- Identification of shift in glycoform distribution
  - Impact on clearance, immunogenicity, or effector function



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LC-MS Characterization of LBA Critical Reagents

- Ligand binding assays primarily use anti-idiotypic mAbs generated from hybridoma cell lines as capture/detection reagents
- Two-tier approach to characterize and qualify new reagent lots prior to use in validated immunoassays:
  - Characterization with size-exclusion HPLC, SDS-PAGE
  - Qualification with QC analysis in validated immunoassay
- Determine incorporation efficiency of new lots of labeled LBA reagents

LBA Critical Reagents - Observed issues

1. No lot-to-lot difference observed by HPLC/SDS-PAGE, but reagent failed qualification in validated assay
2. Reagents pass qualification, but abnormal cross reactivity observed when tested in study subjects

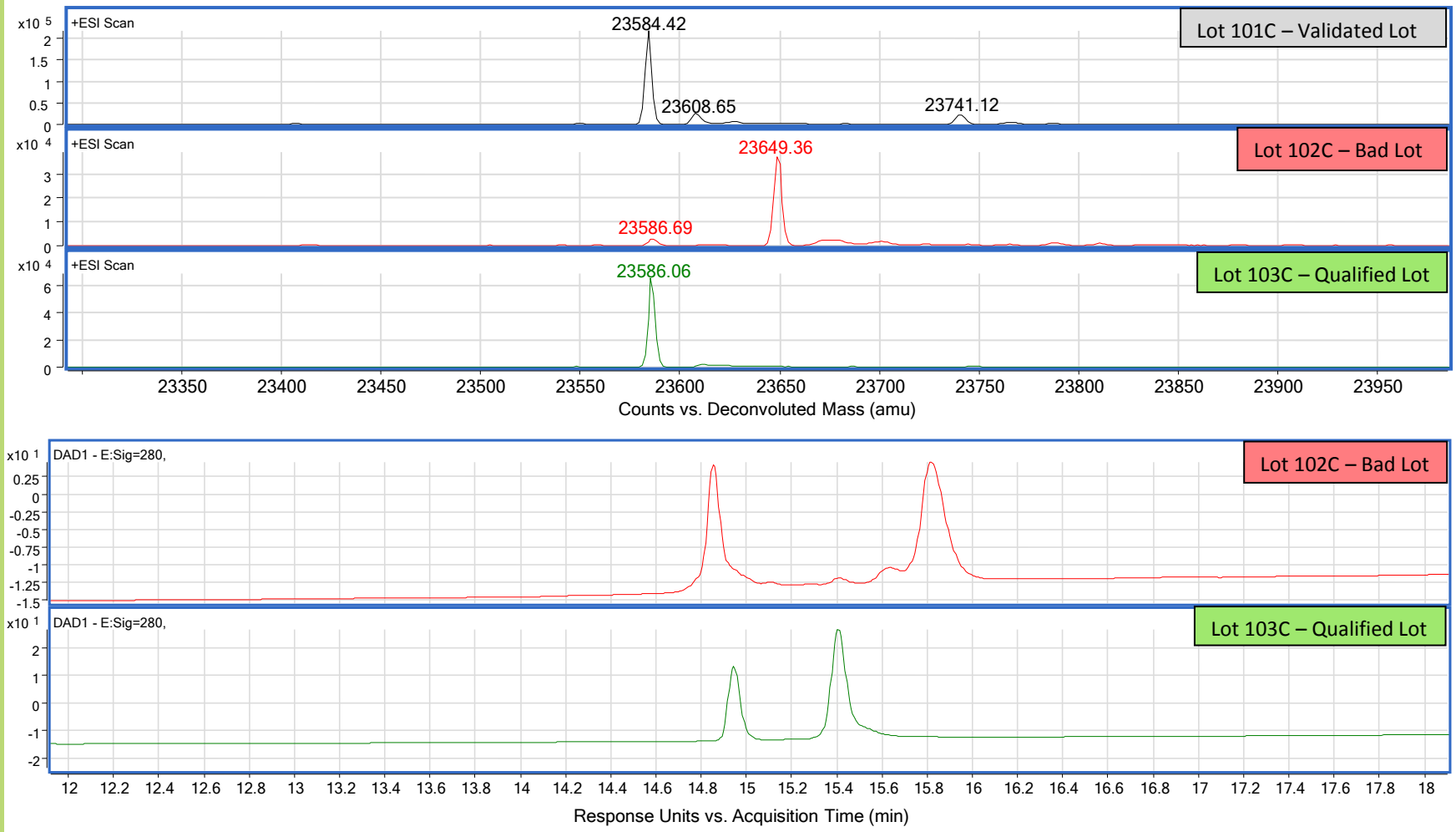
LC-MS Characterization of LBA Critical Reagents – Case #1

CNTO xx1: Capture and Detection Reagent Antibodies – Overview

- Anti-CNTO xx1 mAbs serve as the capture and detection reagents for PK assay following biotin and ruthenium labeling, respectively.
- Original lots depleted → new lot from same clones requested → qualification passed
- Assay generated abnormally high concentration results > LLOQ for placebo/pre-dose samples in two clinical studies (31% and 34%) → **False "positives"**
- Reagent characterization by LC-MS revealed a clear distinction of reduced molecular structures between reagent lots.
  - Light chain
  - Heavy chain
  - Glycosylation pattern

LC-MS Characterization of LBA Critical Reagents – Case #1

Anti-CNTOxx1 mAb Capture – Light Chain and DAD @ 280nm:



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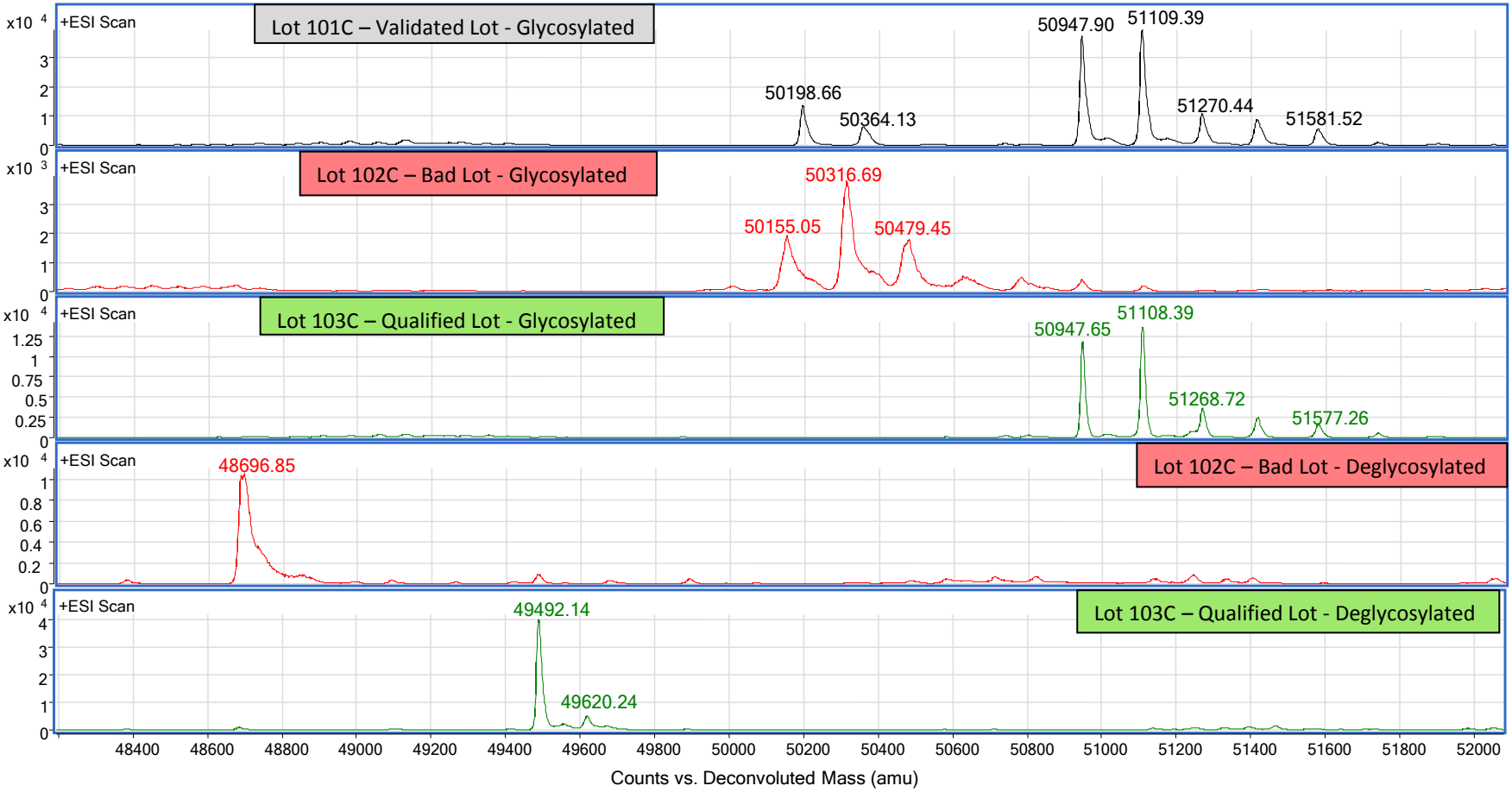
LC-MS Characterization of LBA Critical Reagents – Case #1

Anti-CNTOxx1 mAb Capture – Heavy Chain (Glycosylated and Deglycosylated):



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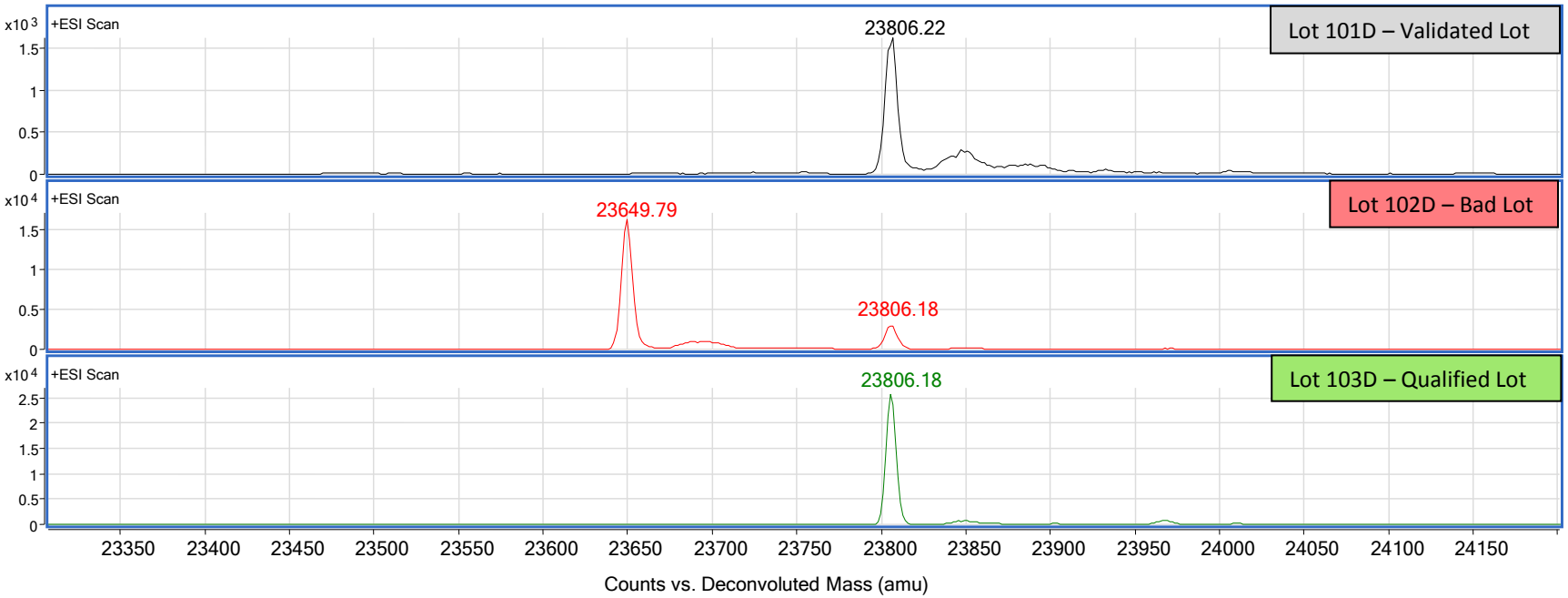
LC-MS Characterization of LBA Critical Reagents – Case #1

Anti-CNTOxx1 mAb Detection – Light Chain:



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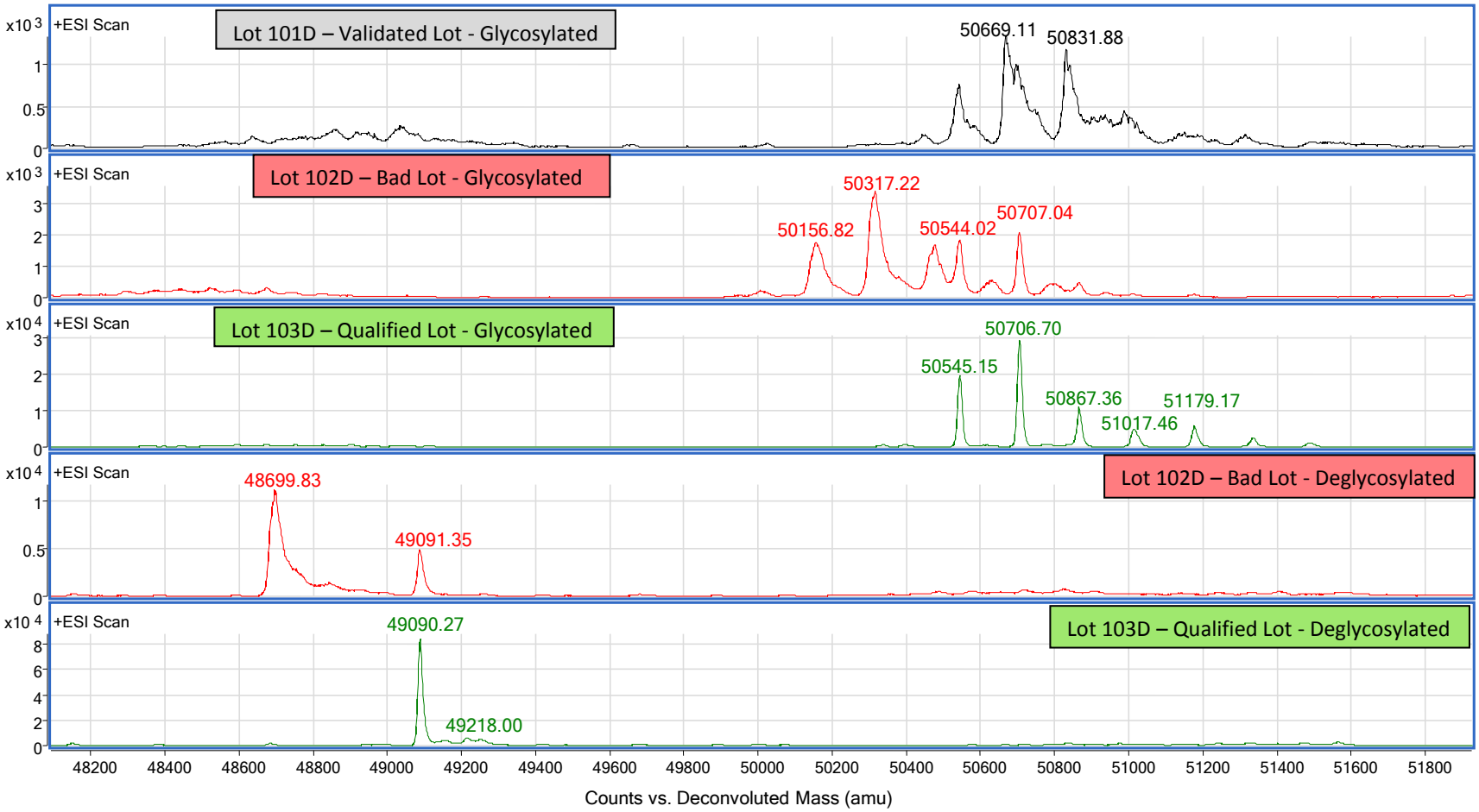
LC-MS Characterization of LBA Critical Reagents – Case #1

Anti-CNTOxx1 mAb Detection – Heavy Chain (Glycosylated and Deglycosylated):



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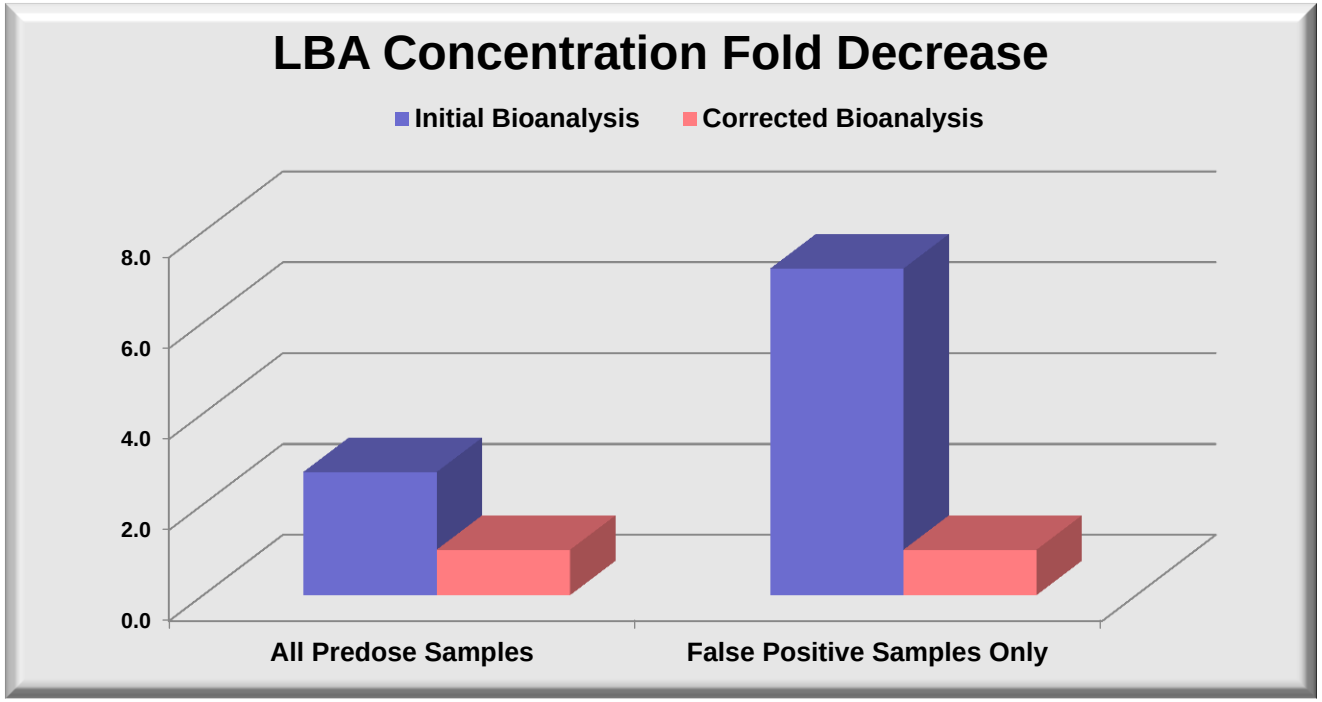
LC-MS Characterization of LBA Critical Reagents – Case #1

False "Positive" Results:

- Initial & corrected bioanalytical mean predose sample concentration (2-study average) :
  - All predose samples → 2.7-fold decrease
  - False positive predose samples → 7.2-fold decrease
- **Total predose false "positives"**: Initial bioanalysis = 153 → Corrected bioanalysis = 4



* Corrected bioanalysis concentrations normalized to 1.

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Case #1 Summary

CNTO xx1 Study Flow Summary

LC-MS analysis of anti-CNTO xx1 mAbs revealed mass alteration for light/heavy chain

- Mass differences unrelated to N-linked heavy chain glycosylation

Hybridoma system generated abnormal mAbs → aberrant LBA results

New lot production of reagents → LBA qualified with similar mass profiles

Bioanalysis timelines upheld

➢ Critical reagent antibodies produced in stably expressed cell lines

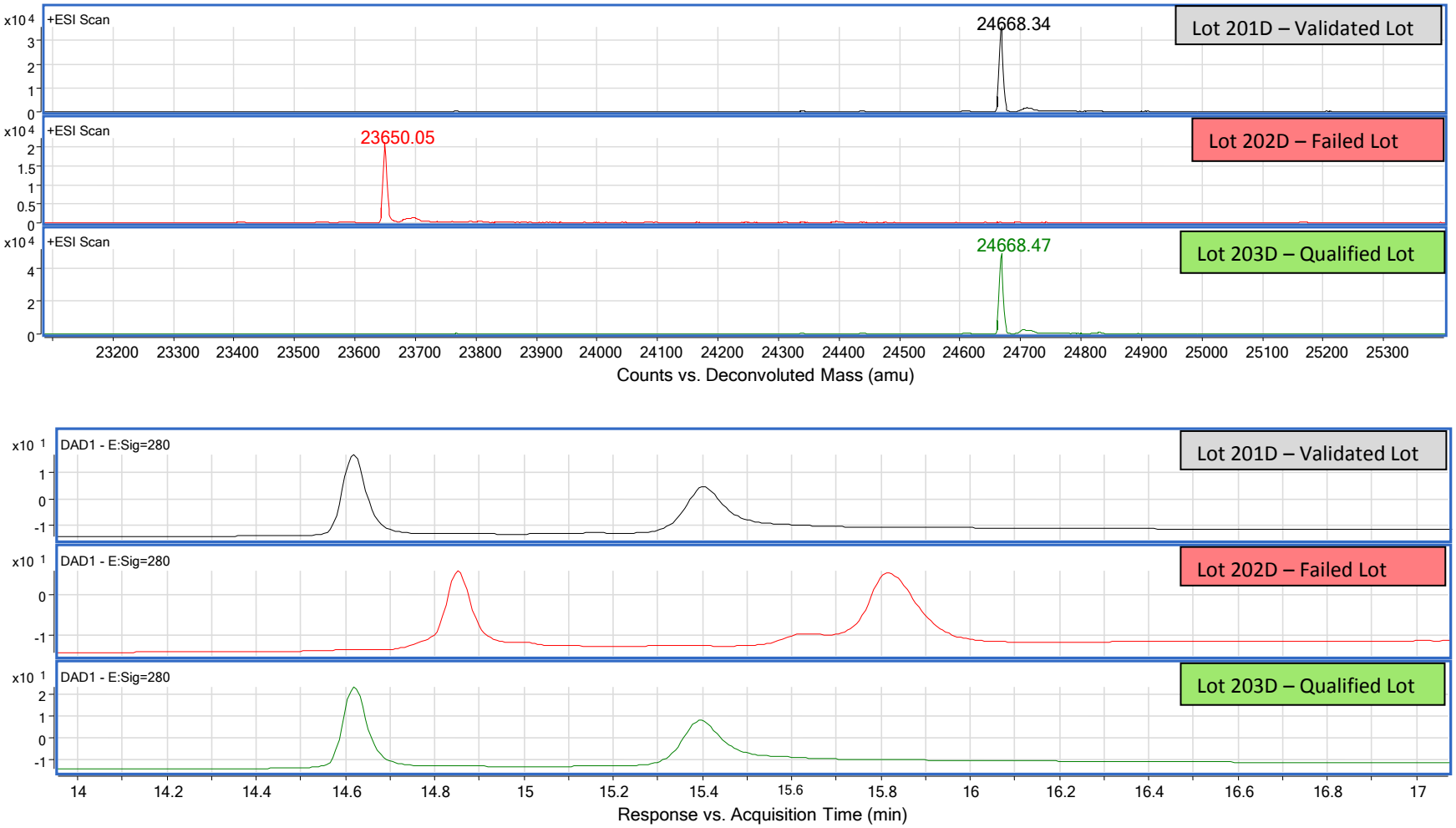
LC-MS Characterization of LBA Critical Reagents – Case #2

CNTO xx2: Detection Reagent Antibody – Overview

- Anti-CNTO xx2 mAb serves as the detection reagent for PK assay ruthenium labeling
- Original lot depleted → new lot from same clone requested → qualification failed
- Reagent characterization by LC-MS revealed a clear distinction of reduced molecular structures between reagent lots
  - Light chain
  - Heavy chain

LC-MS Characterization of LBA Critical Reagents – Case #2

Anti-CNTOxx2 mAb Detection – Light Chain and DAD @ 280nm:



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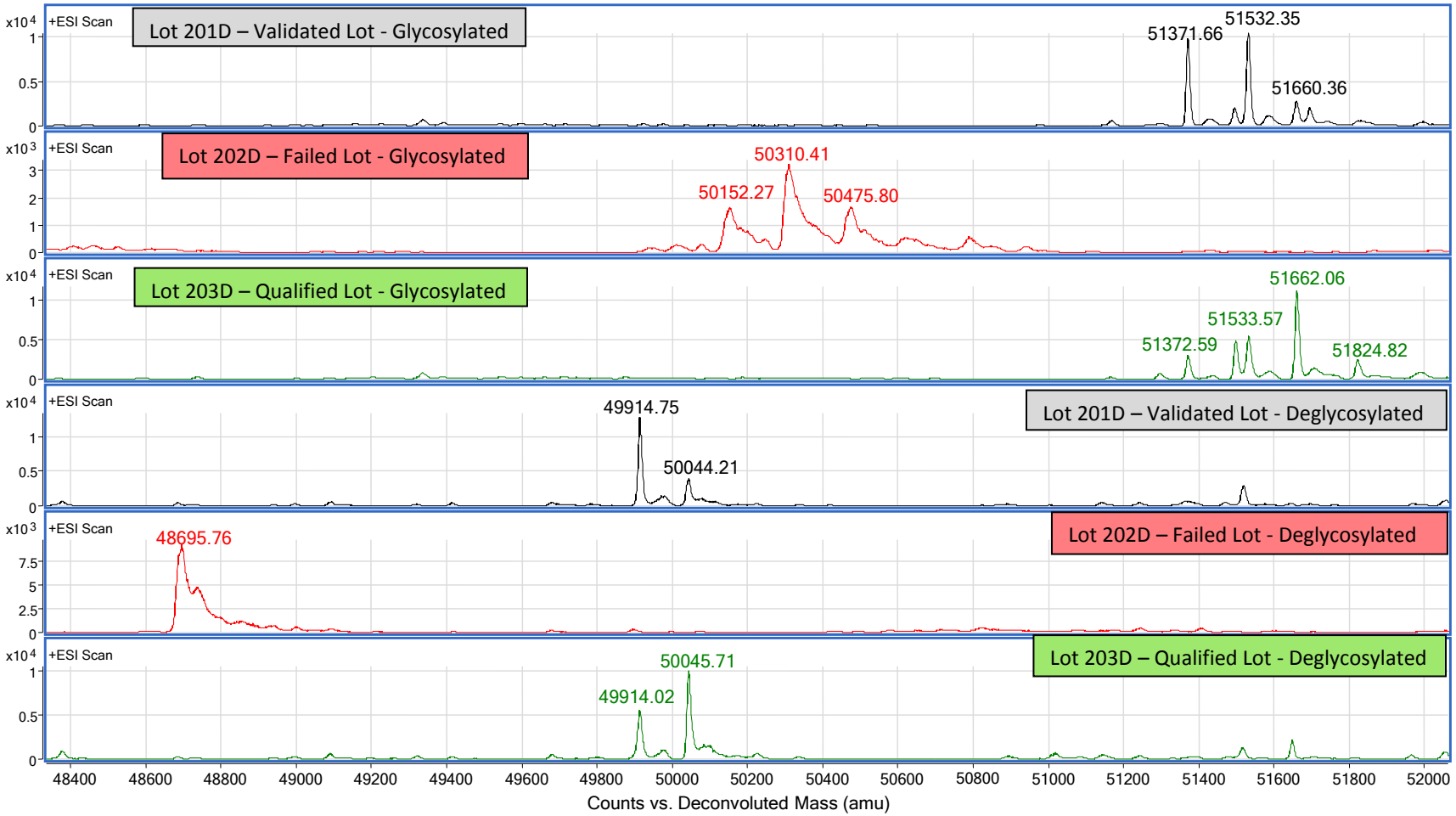
LC-MS Characterization of LBA Critical Reagents – Case #2

Anti-CNTOxx2 mAb Detection – Heavy Chain (Glycosylated and Deglycosylated):



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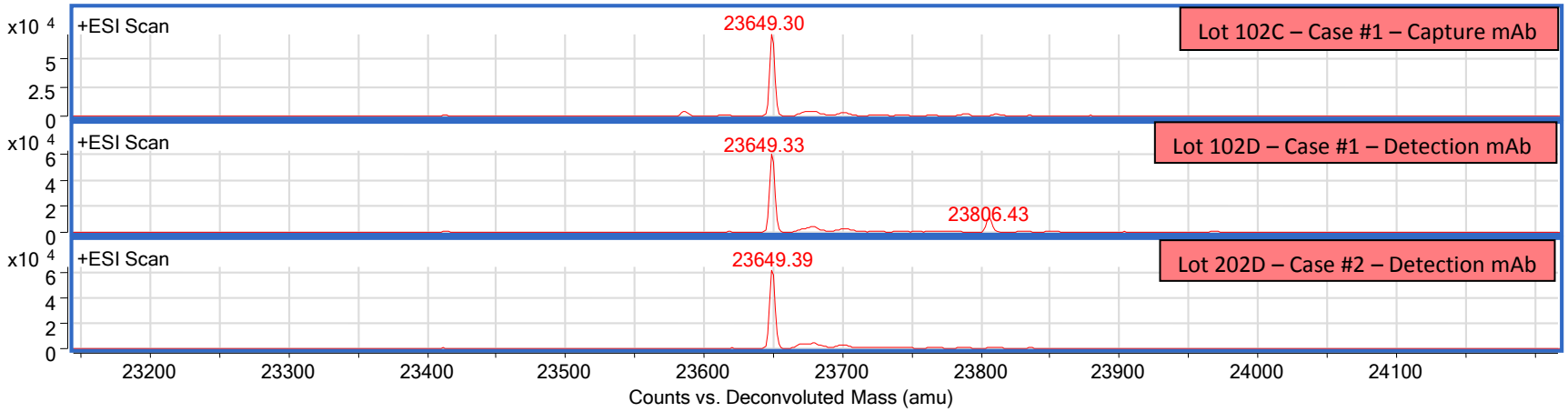
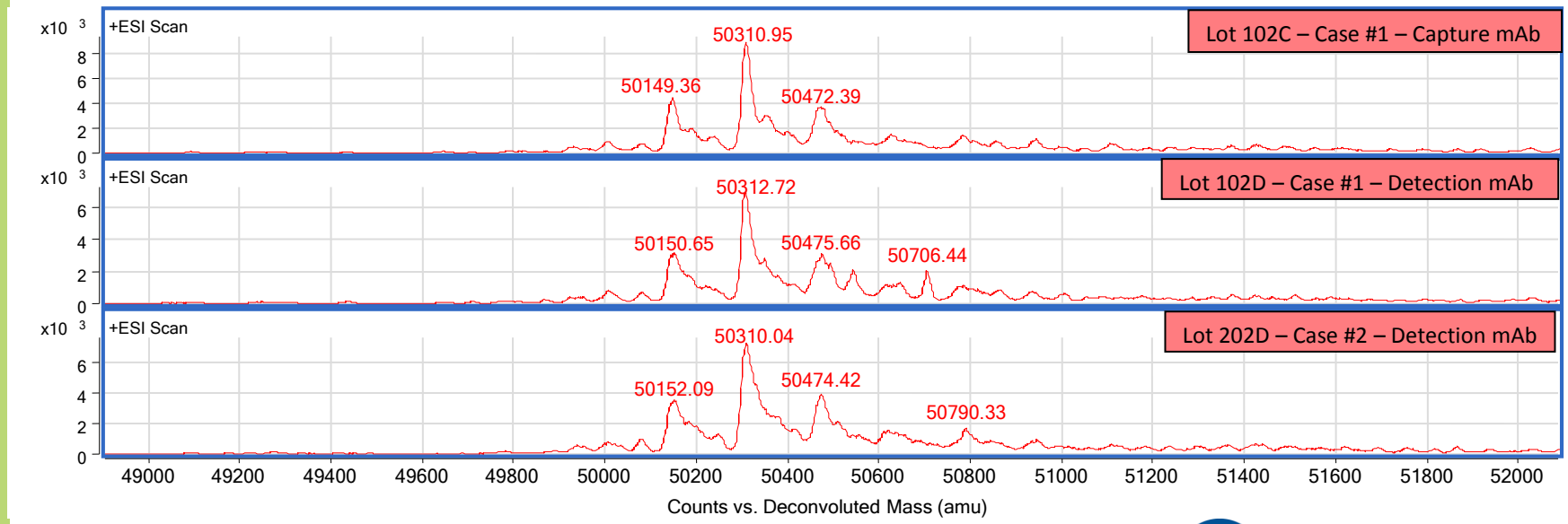
Case #1 vs. #2

Poor Performing Lots – Light and Heavy Chain:





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Case #1 vs. #2 Summary – Hybridoma Clone Integrity

Anti-CNTOxx1 Capture
Anti-CNTOxx1 Detection
Anti-CNTOxx2 Detection

All three lots produced within 6 months from 3 independent hybridoma clones

** **Result:** Determined that reagent generation corresponded with change in media components → previous media solution returned to use.

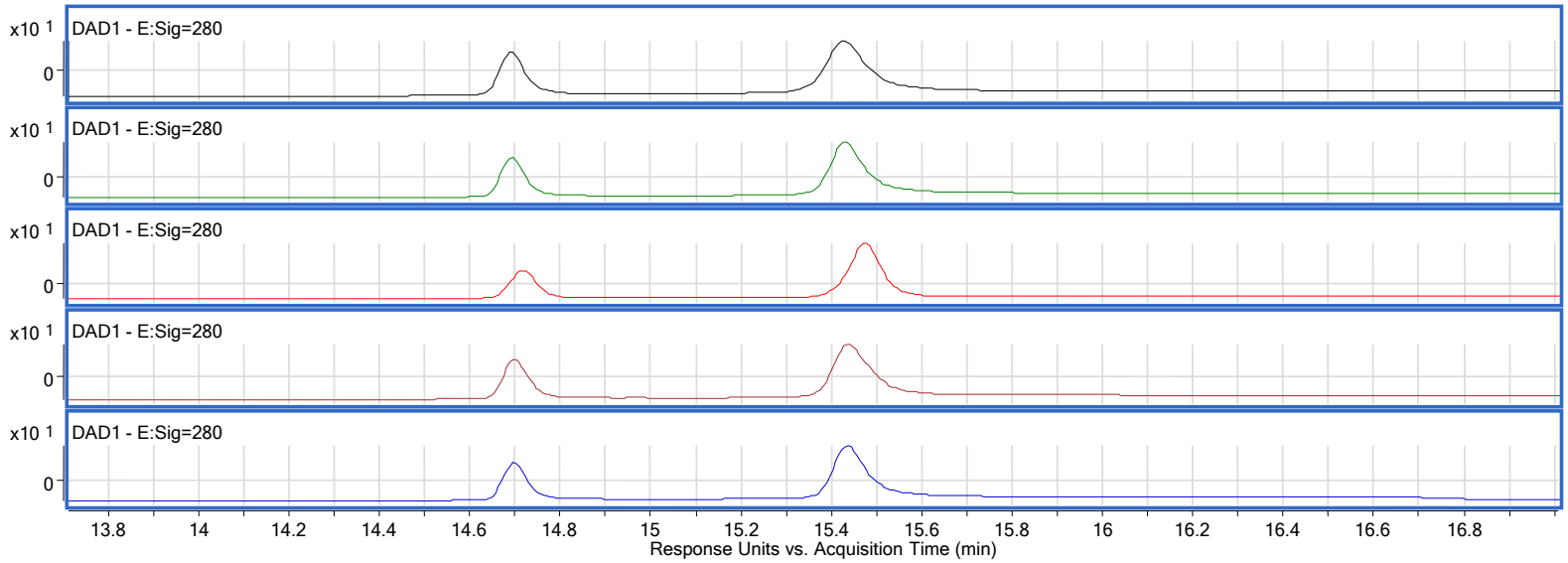
LC-MS Characterization of LBA Critical Reagents – Case #3

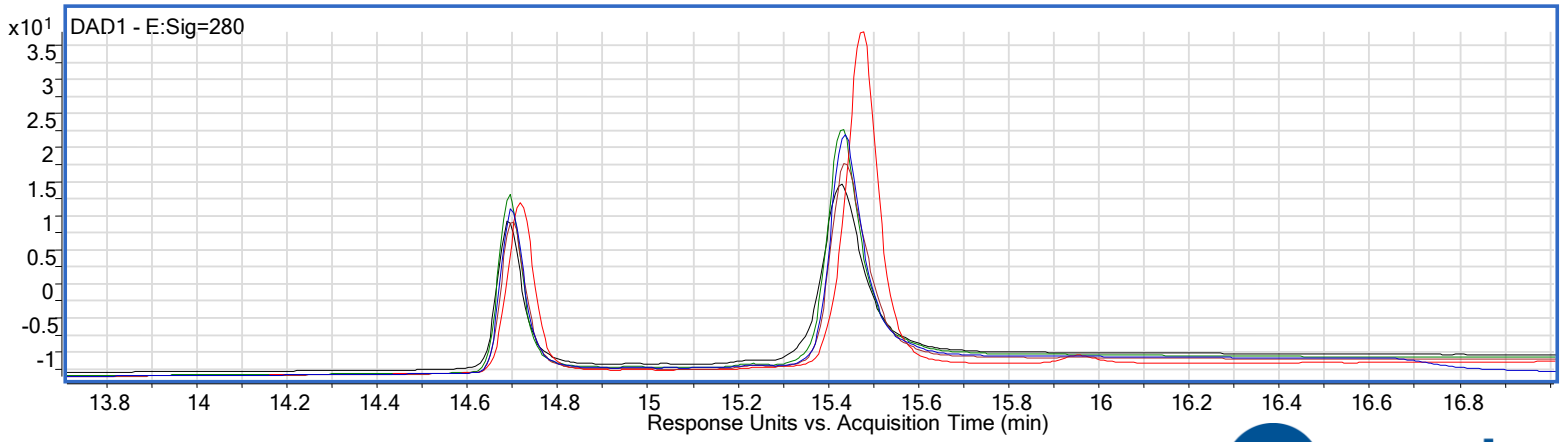
CNTO xx3: Detection Reagent Antibody – Overview

- Anti-CNTO xx3 mAb serves as the detection reagent for PK assay ruthenium labeling
- Original lot depleted → new lot from same clone requested → qualification failed
- Second new lot requested → qualification passed at 2x original LBA concentration
- Reagent characterization by LC-MS revealed a clear distinction of reduced heavy chain between reagent lots; no light chain alteration.

LC-MS Characterization of LBA Critical Reagents – Case #3

Anti-CNTOxx3 mAb Detection – DAD @ 280nm:





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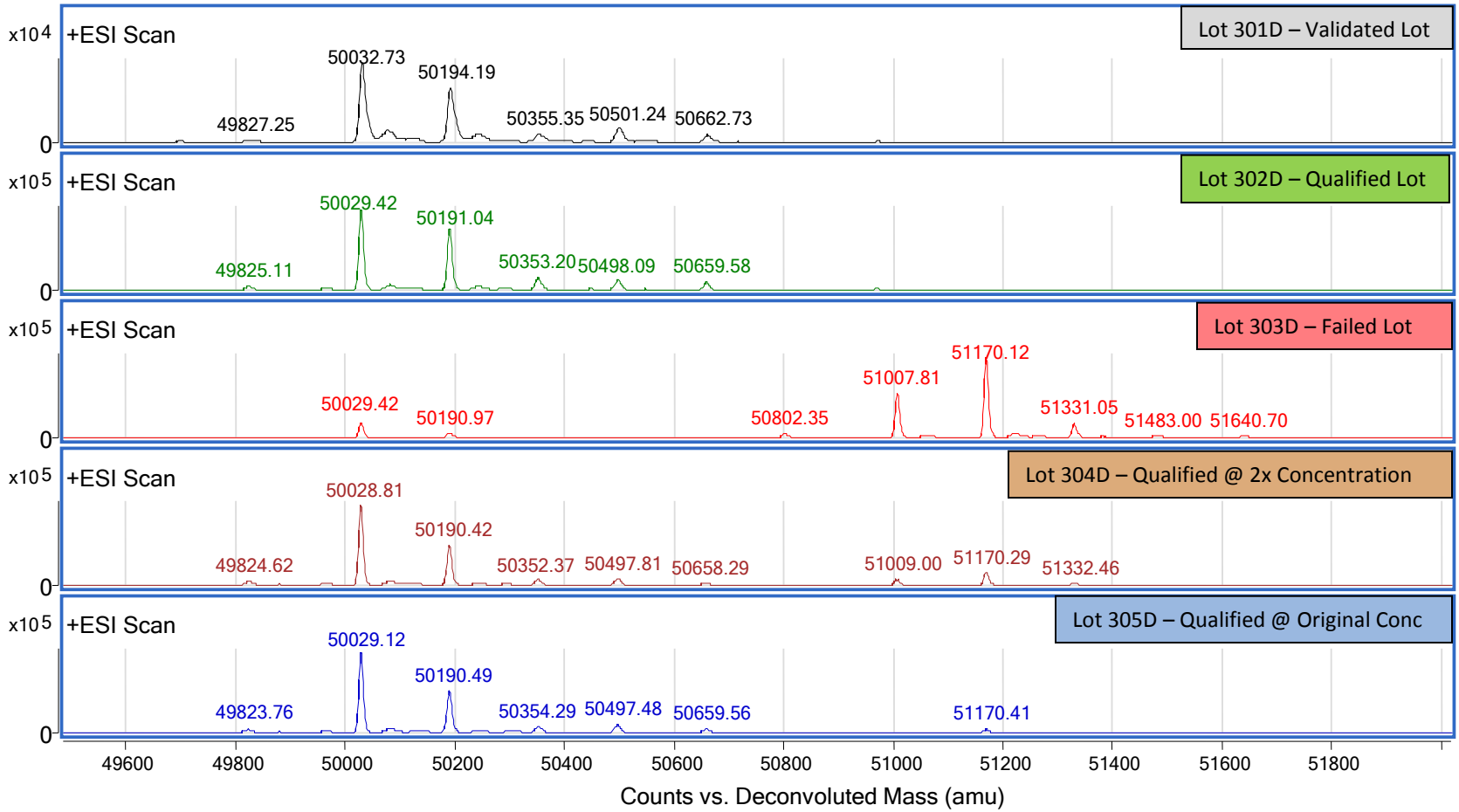
LC-MS Characterization of LBA Critical Reagents – Case #3

Anti-CNTOxx3 mAb Detection – Glycosylated Heavy Chain:



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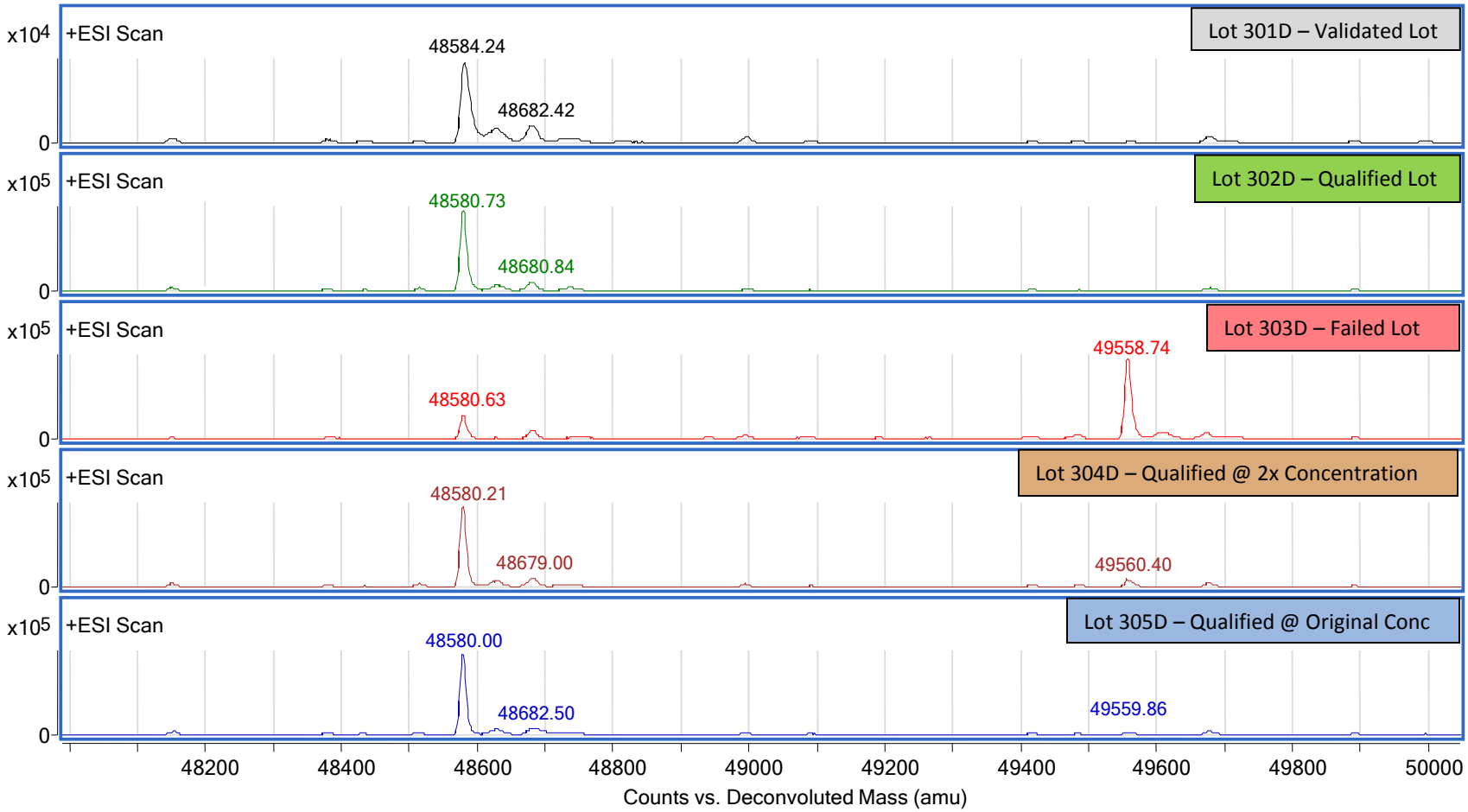
LC-MS Characterization of LBA Critical Reagents – Case #3

Anti-CNTOxx3 mAb Detection – Deglycosylated Heavy Chain:



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Case #3 Summary

CNTO xx3: Detection Reagent Antibody:

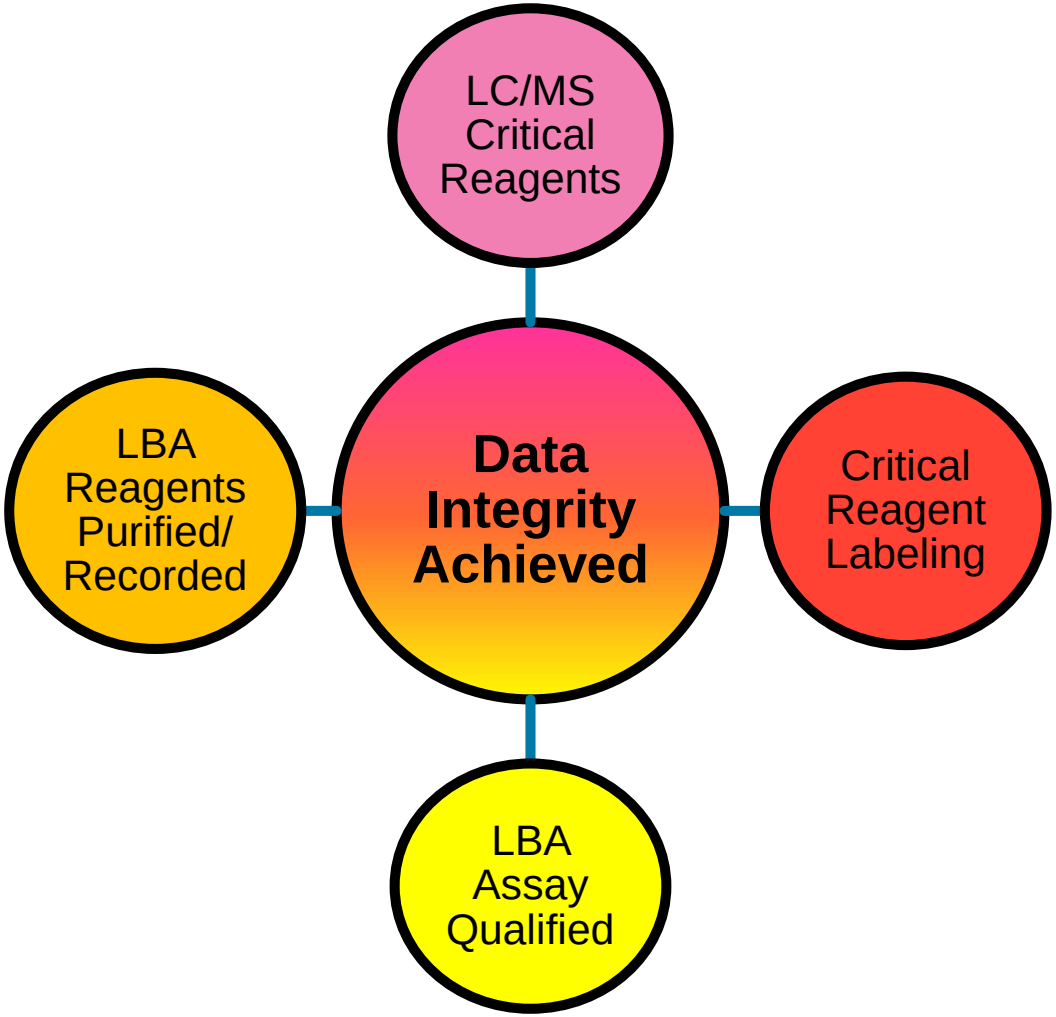
- LC-MS analysis of replacement lot mAb showed sub-species as primary population
- Primary sequence alteration responsible for mass difference → not glycosylation
- Second new lot → qualified at 2x concentration → contained minority amount of same sub-species
- Most recent lot contained minimal sub-species population → qualified at original concentration
- Direct correlation between purity of primary species and LBA assay performance

Conclusions:

Large Molecule Applications:

- LC-MS analysis can be used as quality control check for primary mass and post-translational modifications for:
  - large molecule therapeutics
  - LBA critical reagents
- LC-MS analysis of LBA critical reagents can expedite assay troubleshooting efforts
- MS characterization can help distinguish molecular differences when alternate methods cannot (HPLC, SDS-PAGE, etc.)
- "Jack of all trades" instrument?
- MS can be used as sensitive tool to "finger print" molecular profiles of large molecules

Existing Model:



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Thank You

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