Coupling Mass Spectrometry (MS) with non-MS Assays for Automated Profiling of Antibody Impurities

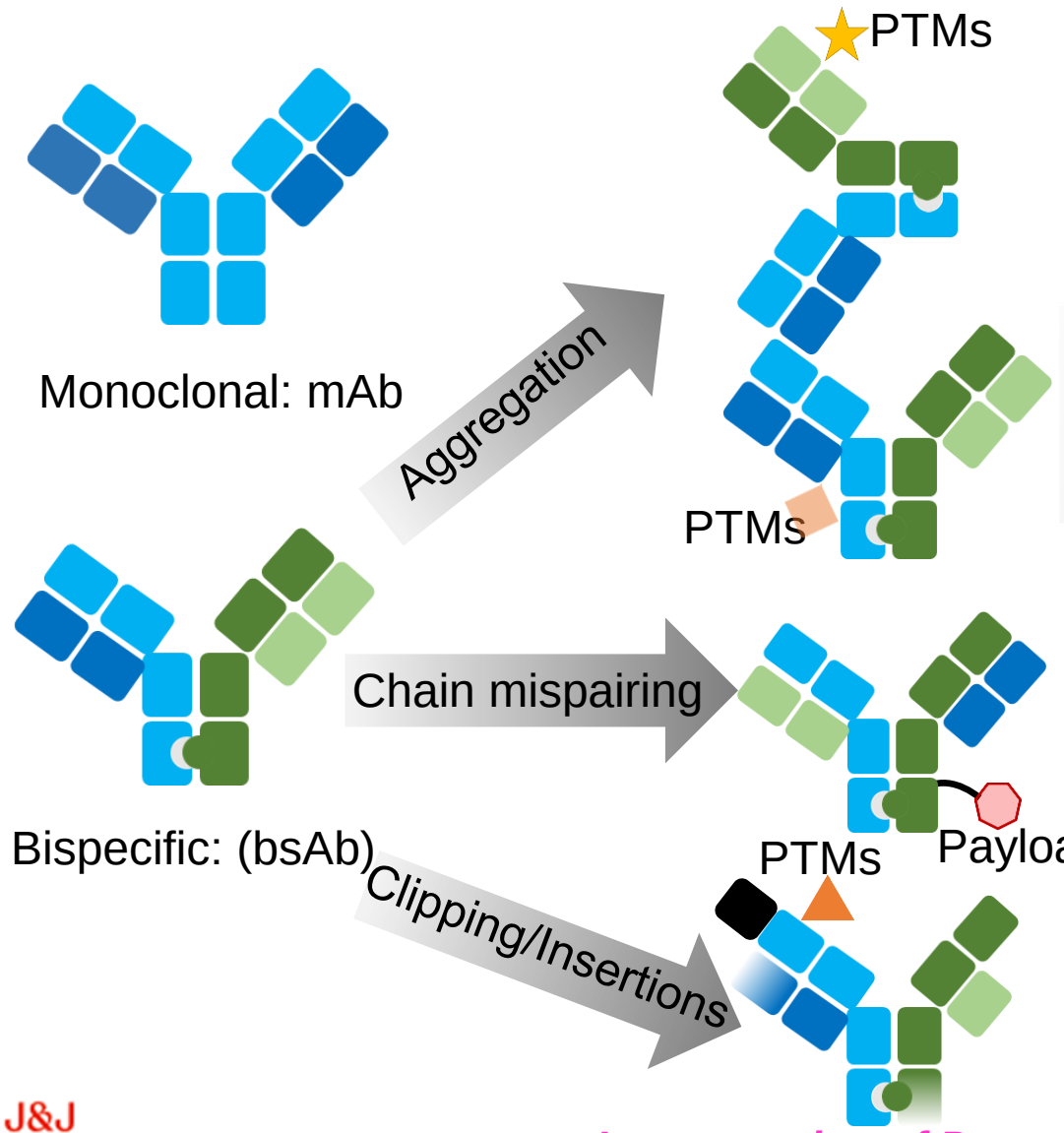
Harsha Gunawardena

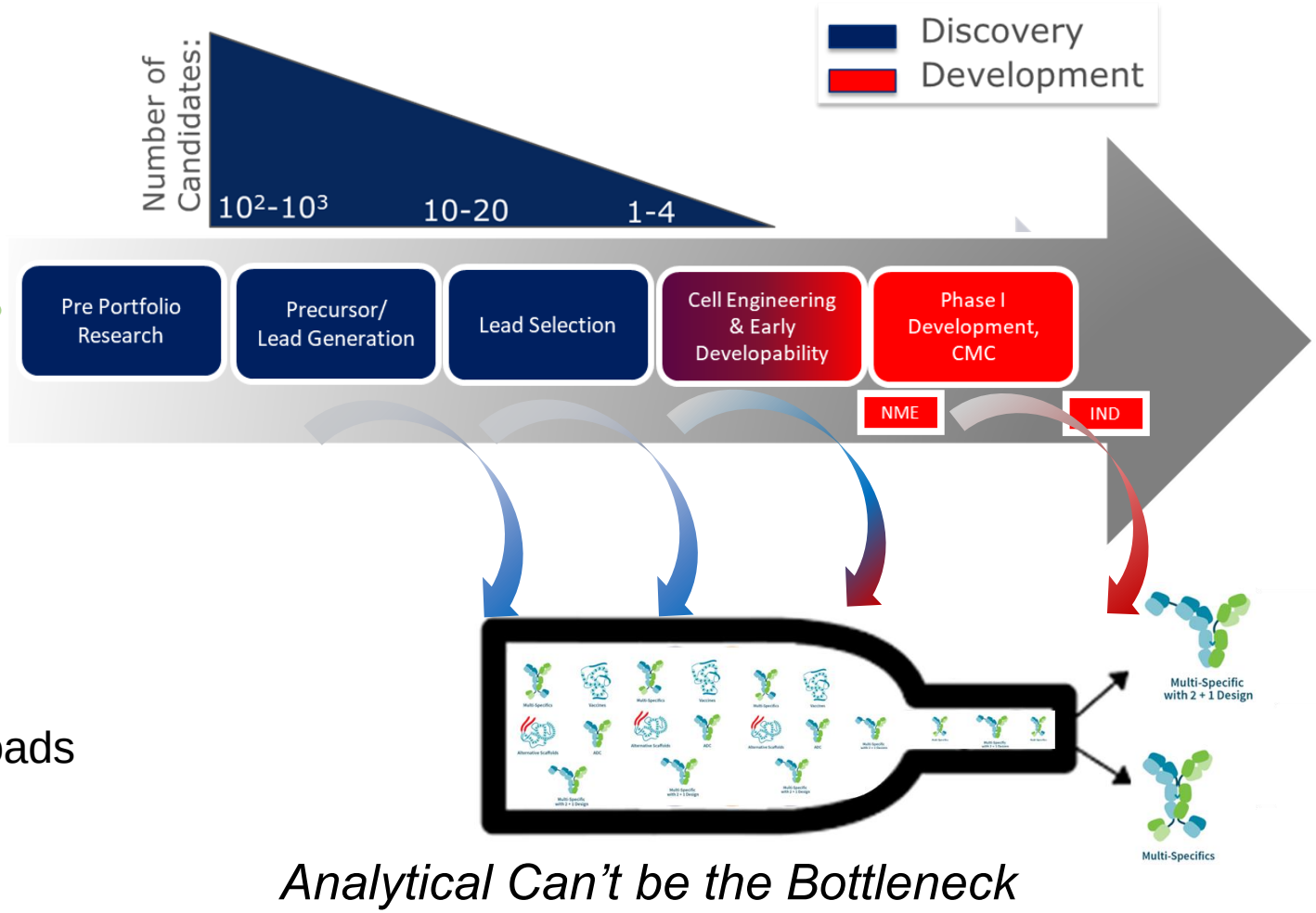
CASSS WCBP Conference 2025

Outline

- Need for speed and comprehensive characterization in pharmaceutical R&D
- Part 1: **Proposed peak identification strategy: concepts and experiments**
  - Non-MS-assays and peak detection a historical perspective
  - Developing mass/size calibration ruler: A case for forced trisulfide degradation products (that mimic bioreactor H_2S-induced mAb degradation)
  - Cathepsin D- and L-induced mAb clipping to mimic cell culture harvest conditions
  - High-pH Rituximab clipping to mimic structural instability under forced degradation
- Part 2: **MS and non-MS Data Automation to couple Byosphere**
  - Coupling RapidFire data with non-MS assays
  - Data analysis in the Byosphere pipeline
  - Automated analysis and reporting of Impurities
- Conclusions and Future directions

Multispecific Modalities Requires Faster and Comprehensive Characterization Workflows in Drug Discovery & Development





Analytical Can't be the Bottleneck

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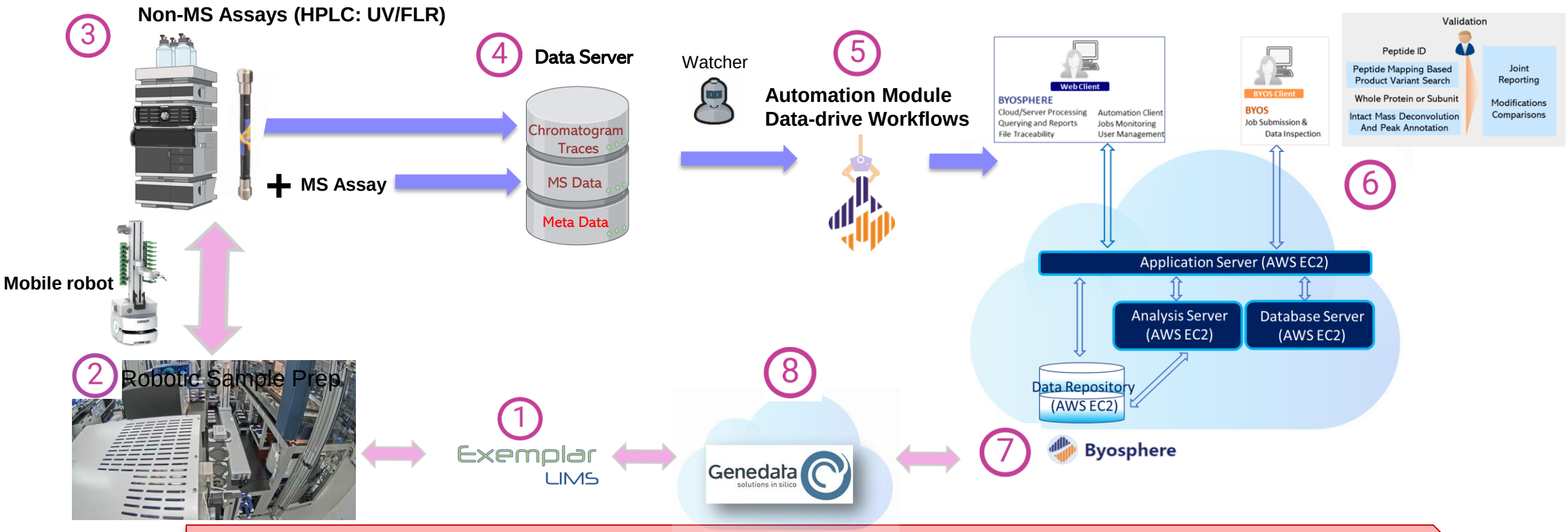
Analytical Assays Automation with Rapid Informatics Pipeline



E2E Automated Sample Preparation, Acquisition and Data Analysis

1. LIMS system supports the retrieval of samples
2. Automated protein A purification using robotics and robotic hand-off (Future state)
3. Non-MS Assays (i.e. Proteometer Trace Chromatograms)
4. Data severs storing data
5. Automated data sweep to *Enterprise Byos Software* with fully integrated intact MS Data-driven workflows
6. Automated export of results and joint reporting of modifications

7&8 Aggregate data with molecule information to build 'In-house' knowledge base (also auto QC)

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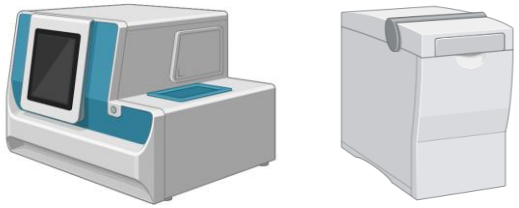
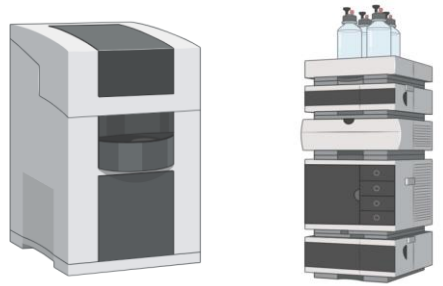
The problem: peak detection of non-MS Assays

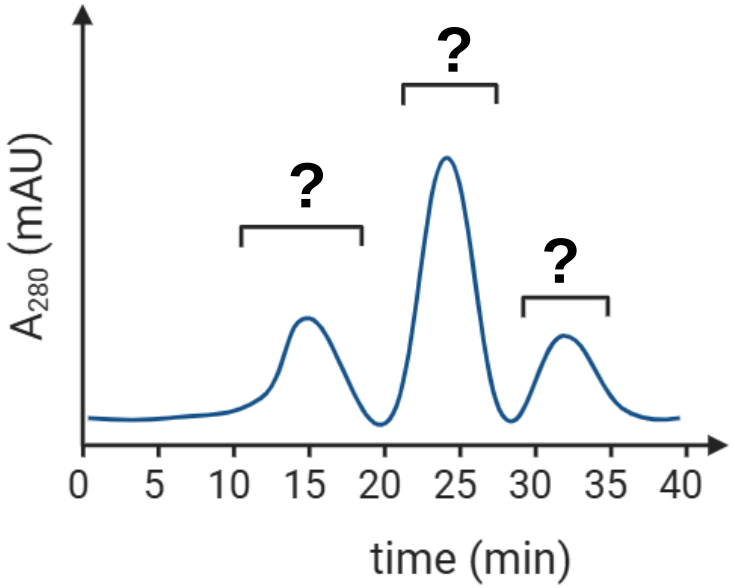
CE-SDS (UV 220 nm)



Chip-Electrophoresis

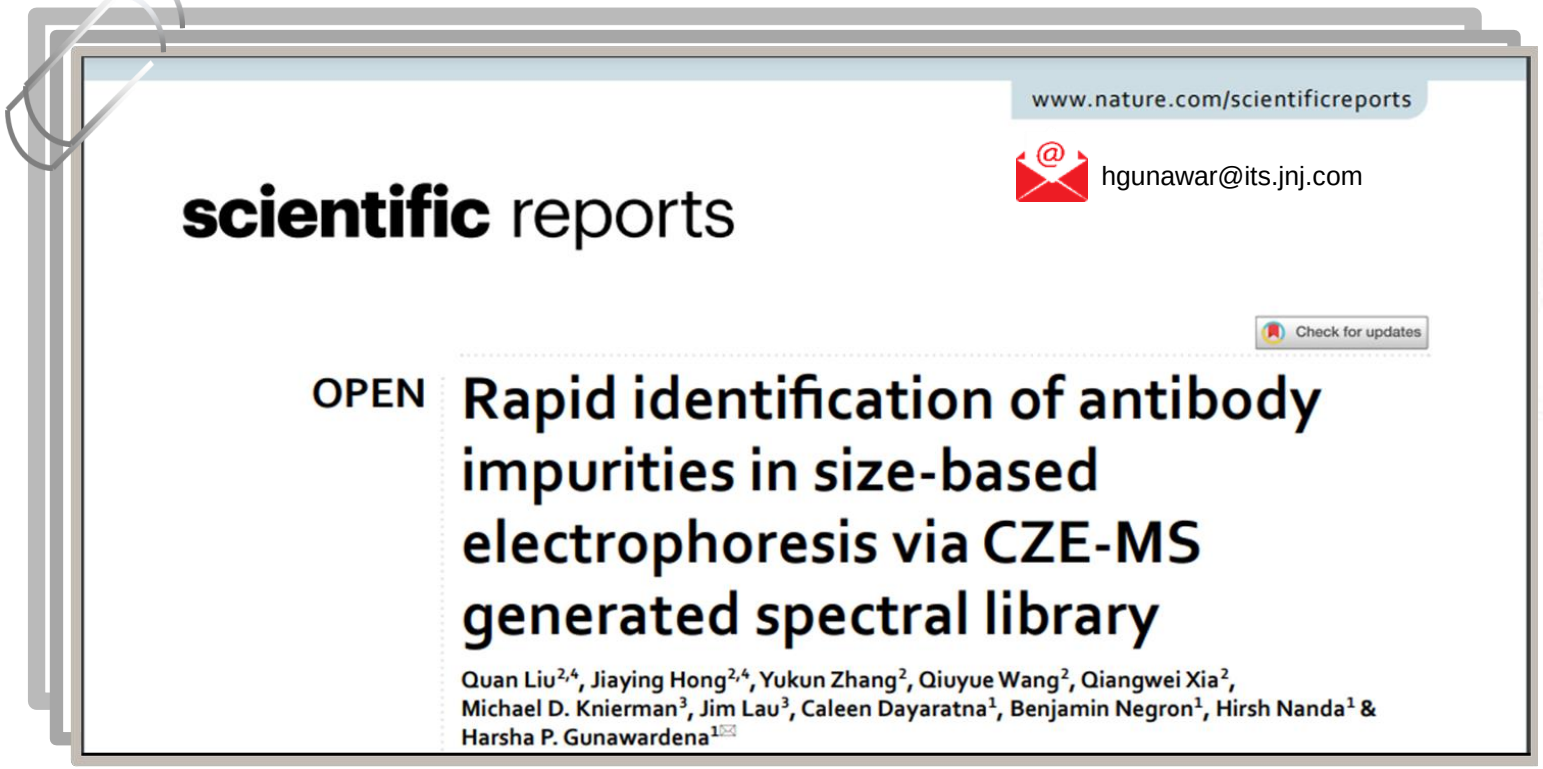
CZE, HPLC

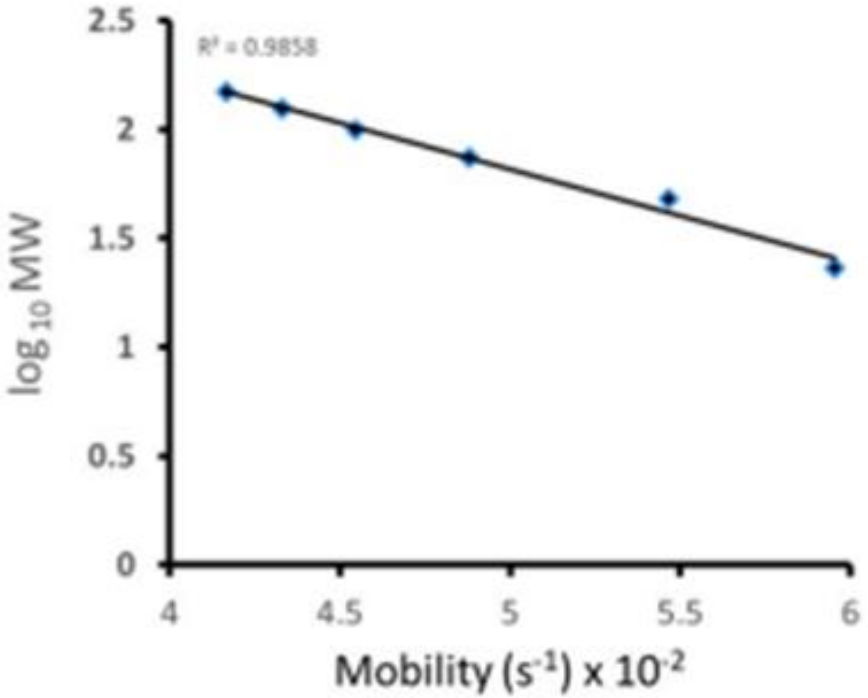




Peak ID is the missing puzzle !

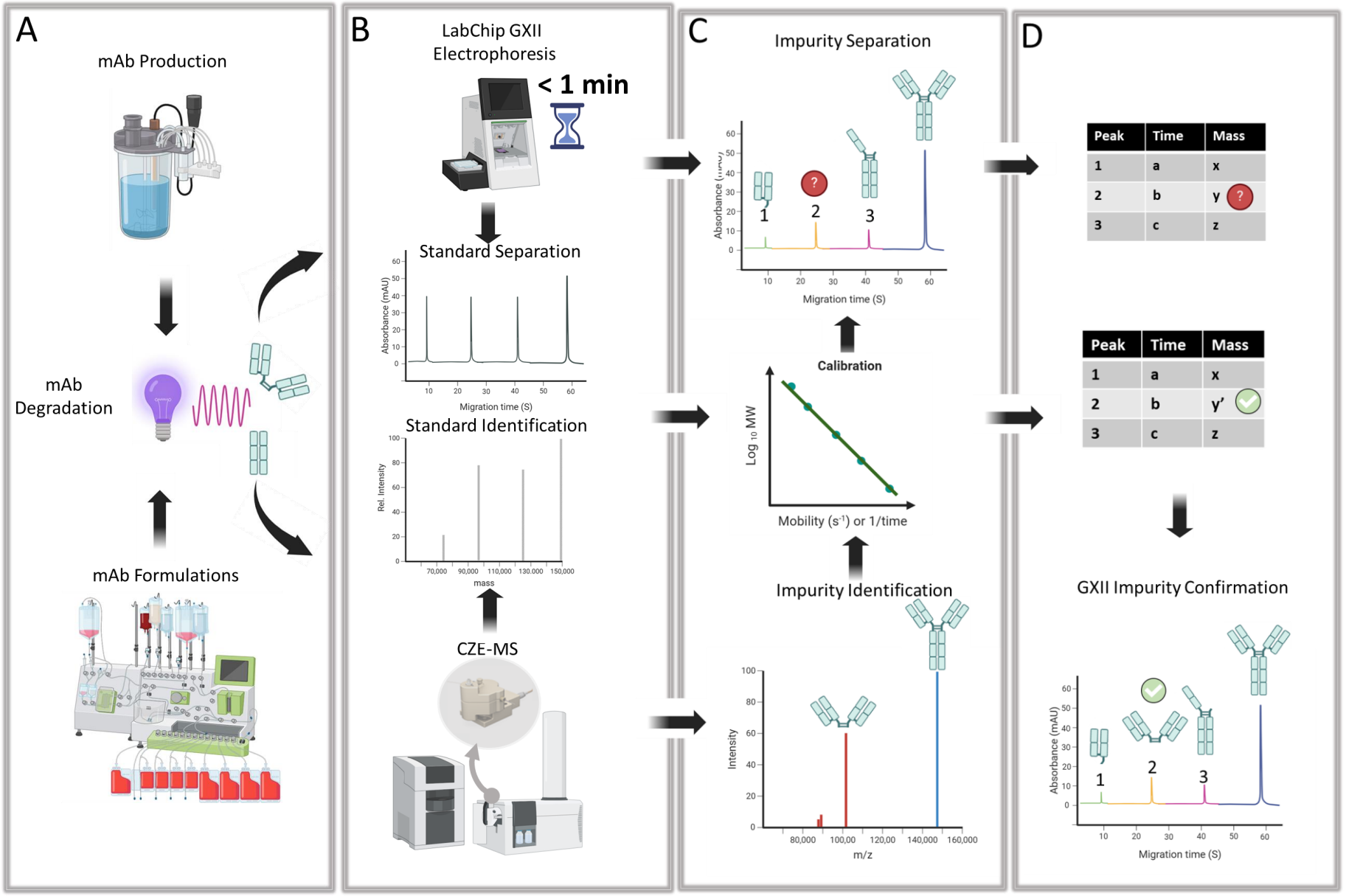
Estimating Molecular Weight by Mobility Calibration





- Molecular weight of proteins estimated by calibrating mobility with mass from SDS-PAGE (Zwan. 1966, ***Anal. Biochem.)***
- Molecular weight of IgG clipping estimated by calibrating mobility with mass from CE-MS (Li et al., 2022, ***J. Chromatography A.)***
- Molecular weight of unknown IgG impurities estimated by calibrating mobility using mass spectral library (Liu et al., 2023, ***Scientific Reports***)) **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License: https://creativecommons.org/licenses/by-nc-nd/4.0/deed.en.

A workflow for mAb impurity analysis combining electrophoretic separation and accurate assignment of masses



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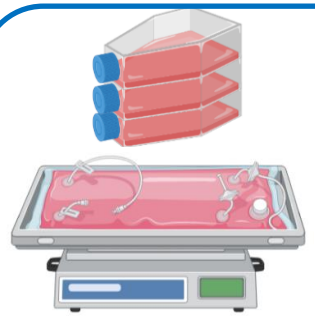
Simulating Bioprocessing Systems to Examine mAb Impurities

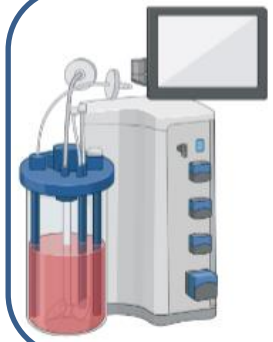
mAb Trisulfides and degradation in bioreactor settings

Monitoring NIST mAb H2S-Induced degradation products by GXII and CZE-MS



mAb Cathepsin degradation during cell culture

Monitoring NIST mAb Cathepsin degradation products by GXII and CZE-MS



mAb High-pH degradation during bioprocessing

Monitoring Rituximab high-pH degradation products by GXII and CZE-MS

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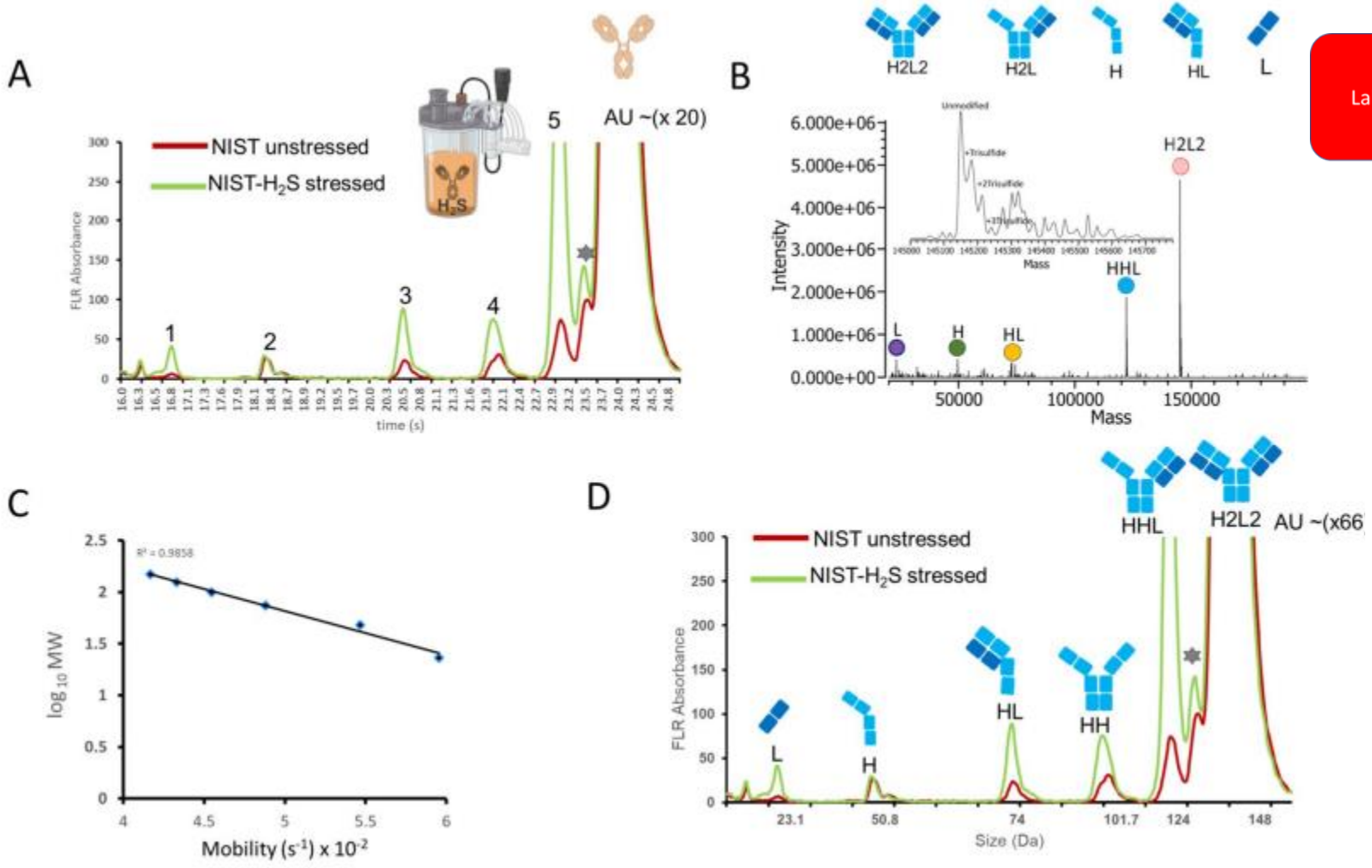
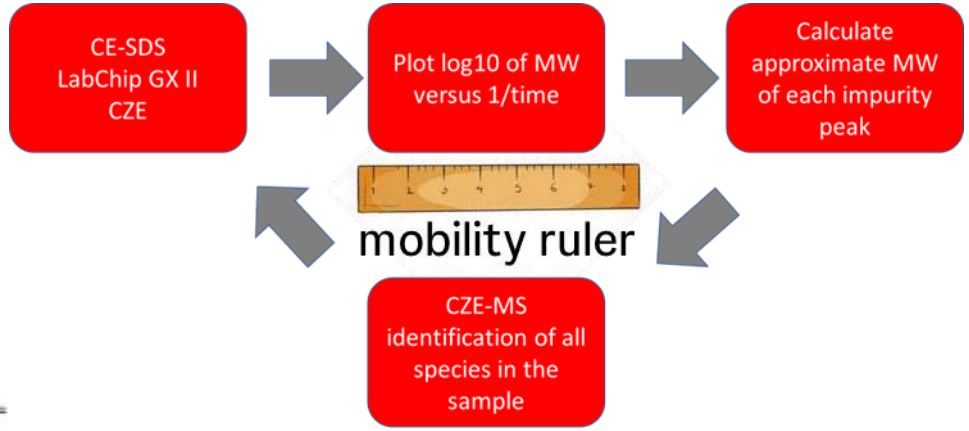
H_2S-induced degradation products of IgG as a GXII mobility ruler





Analysis of degradation products of NISTmAb
A) LabChip GXII electropherogram of unknown mAb fragments peak 1-5 under unstressed (native condition.
B) Deconvoluted mass spectrum resulting from H_2S-induced degradation products of NISTmAb.
C) Linear regression analysis via NISTmAb fragments.
D) Assignment of masses to GXII peaks based on calibration in C

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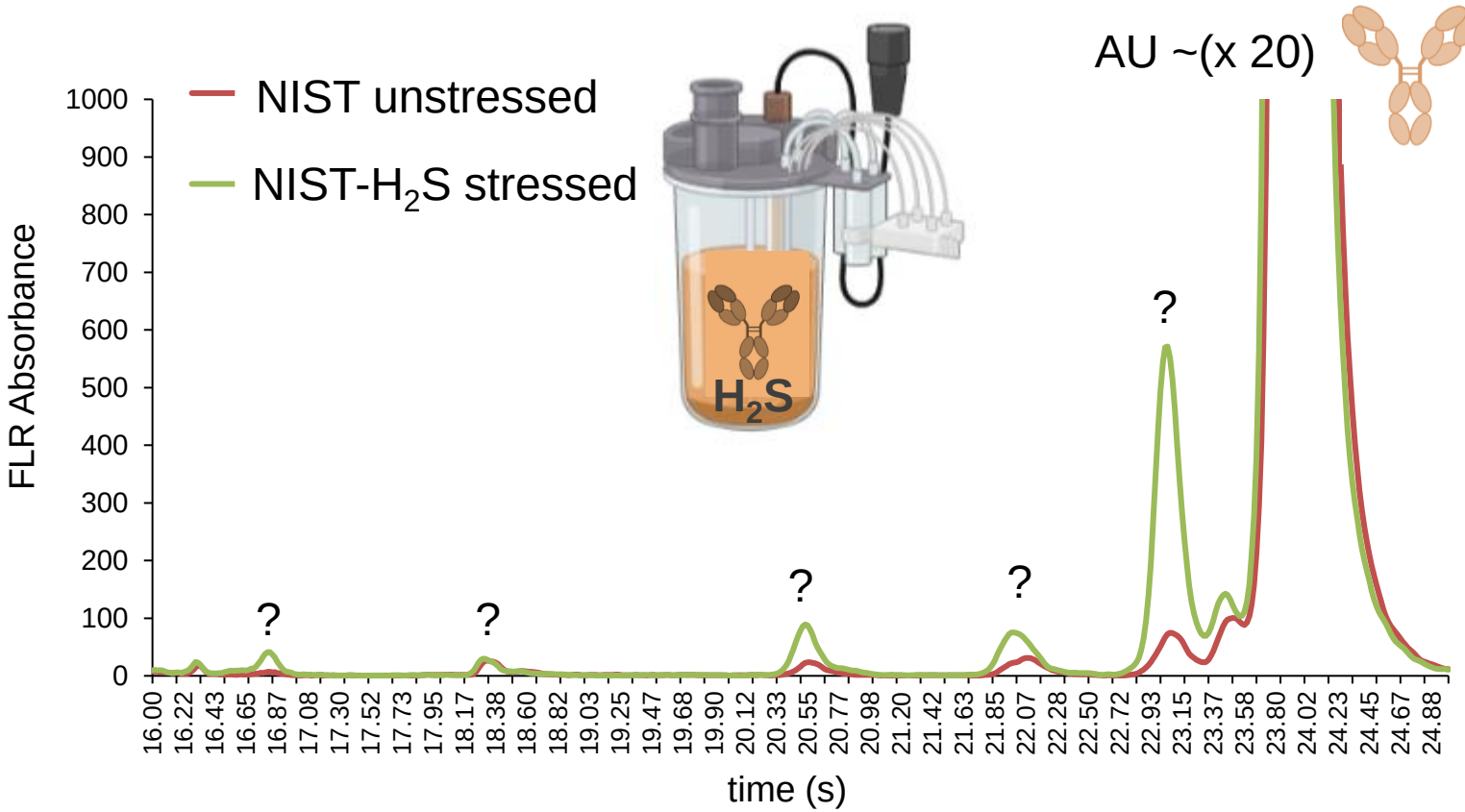
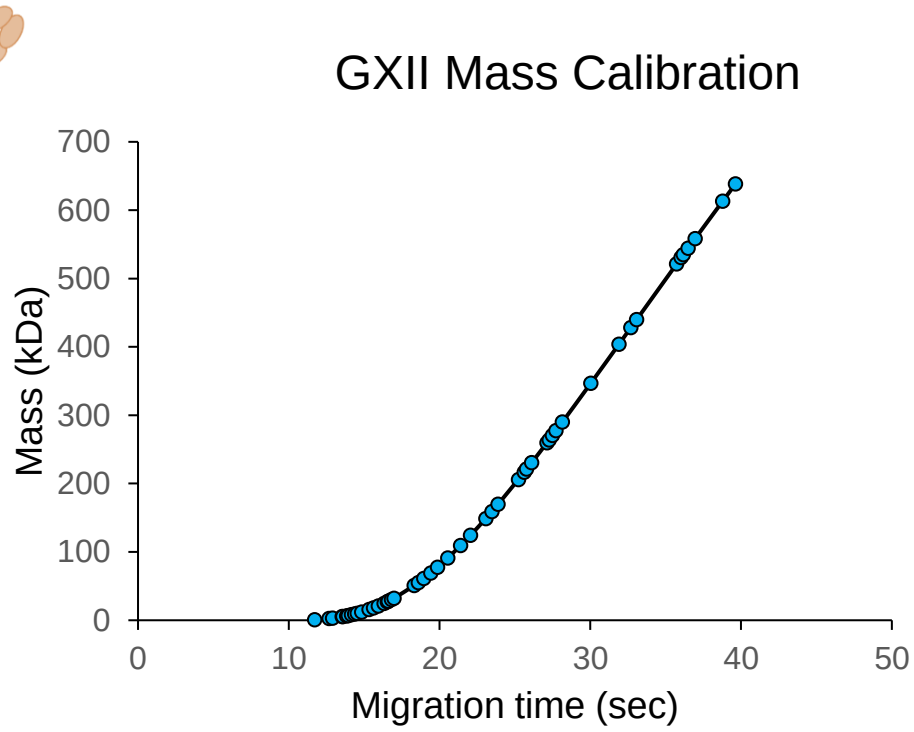
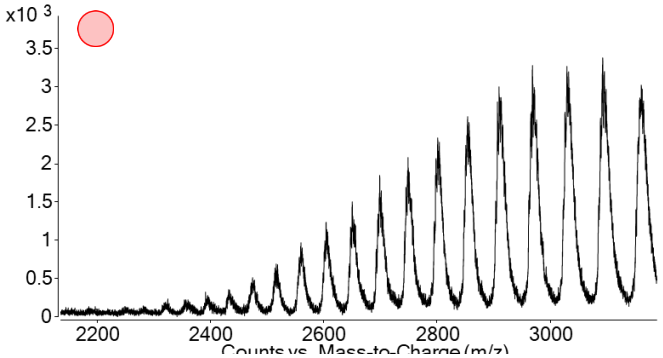
GXII Electropherogram Analysis of NIST IgG1 Fragments

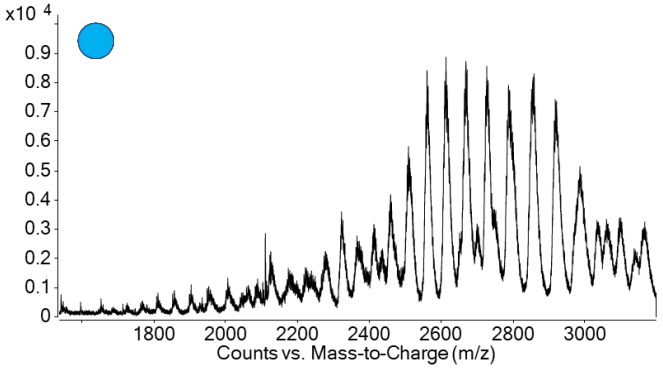


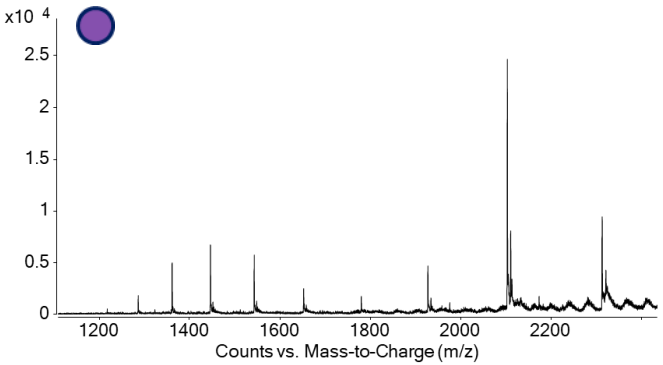


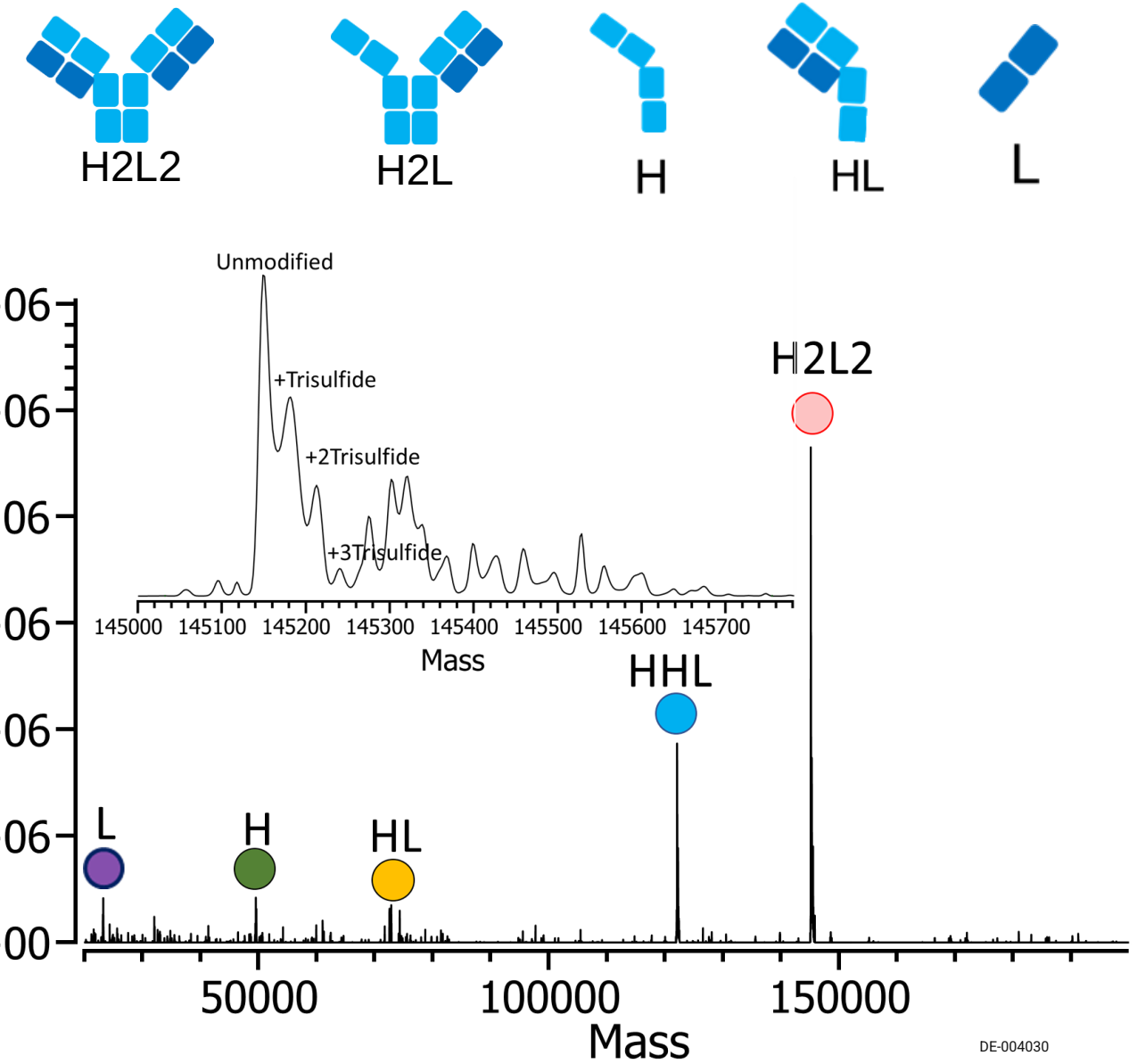
- x-axis time variance is important for the accuracy of size (kDa) estimation (Peak ID)
- Each second in GXII time is ~ 15 KDa in size
- GXII Calibration of higher masses is an extrapolation of low molecular weight markers

CZE-MS of NIST H_2S-Induced Degradation Products









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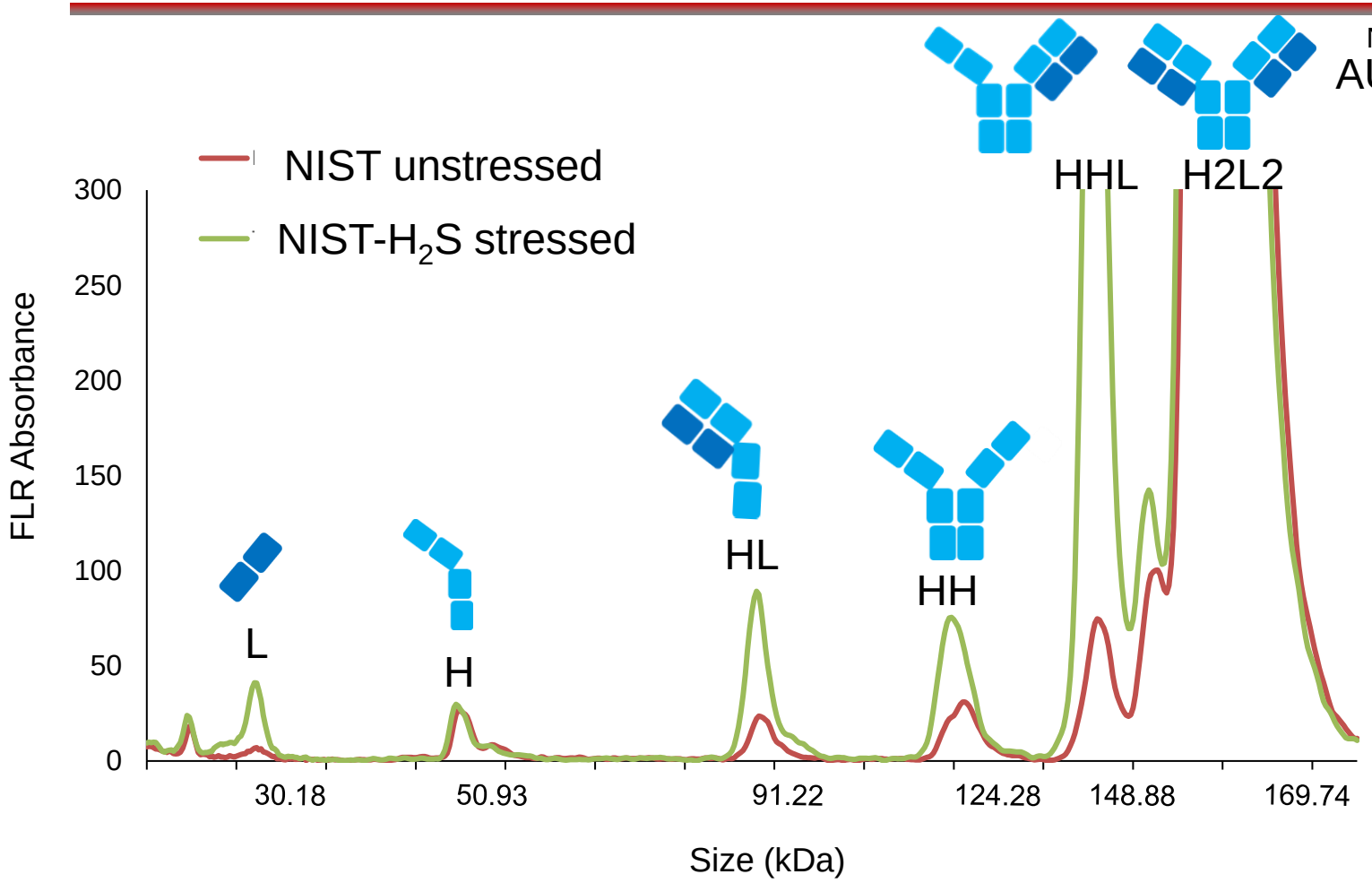
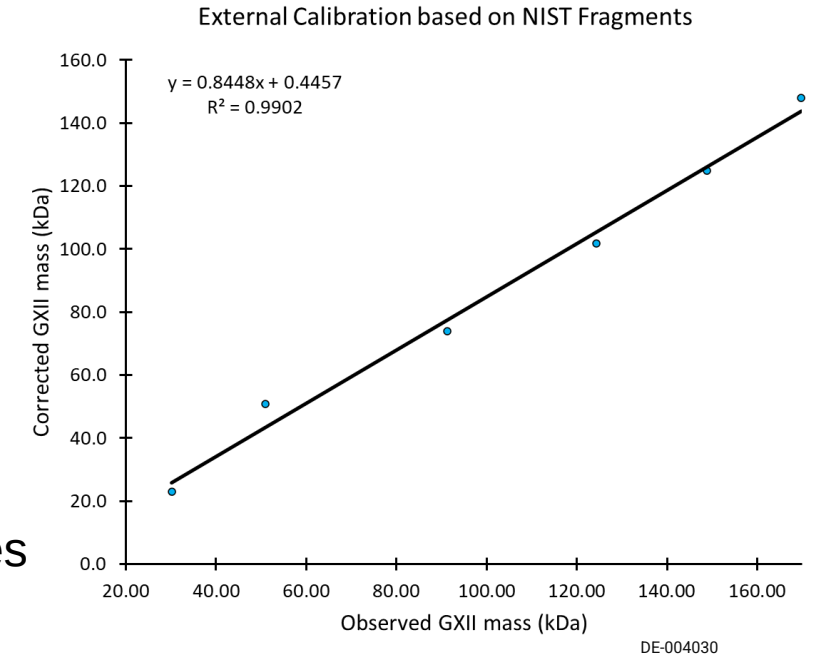
GXII Electropherogram Analysis of NIST IgG1 Fragments



Corrected Mass (Da)	Observed Mass (Da)	Species
23.1	30.18	L
50.8	50.93	H
74.0	91.22	HL
101.7	124.28	HH
124.8	148.88	HHL
148.0	169.74	H2L2



MS could not detect HH species !! But GXII size-recalibration does

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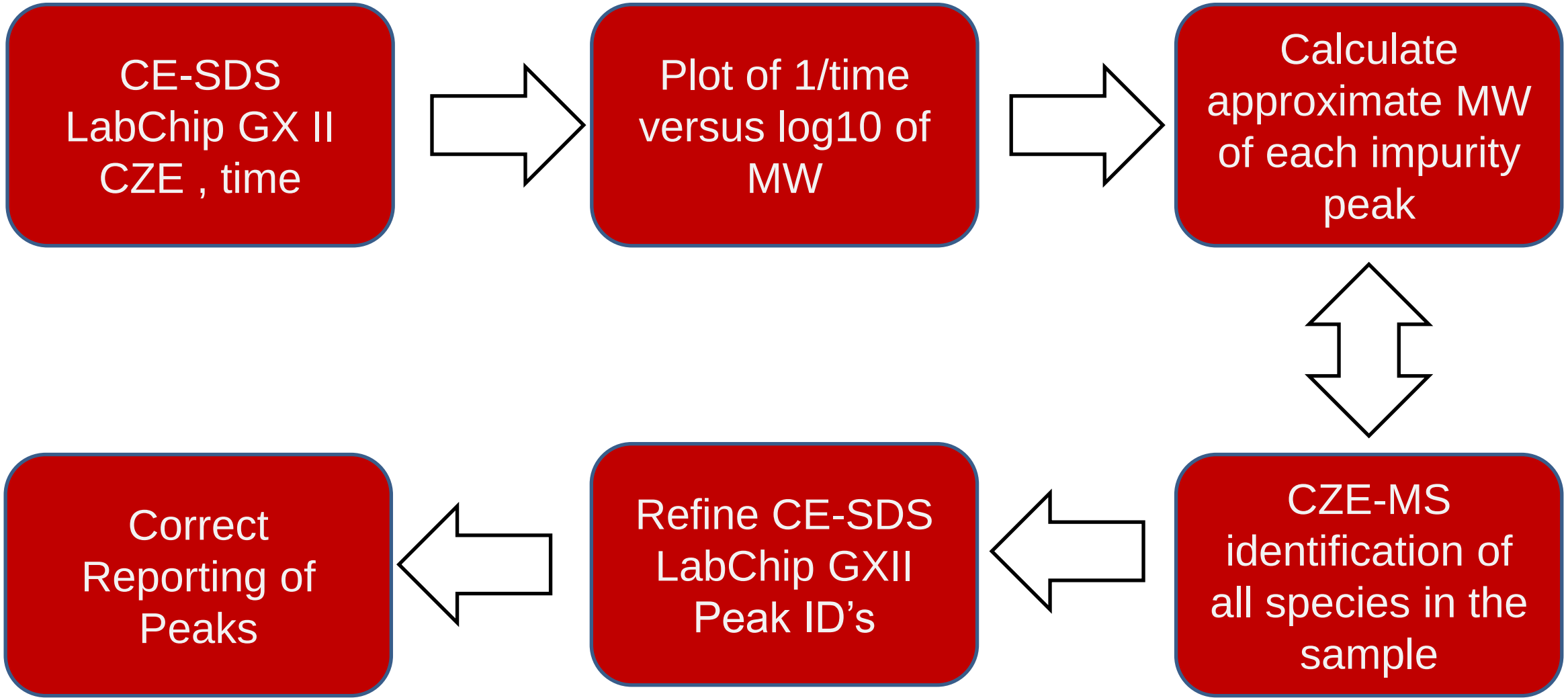
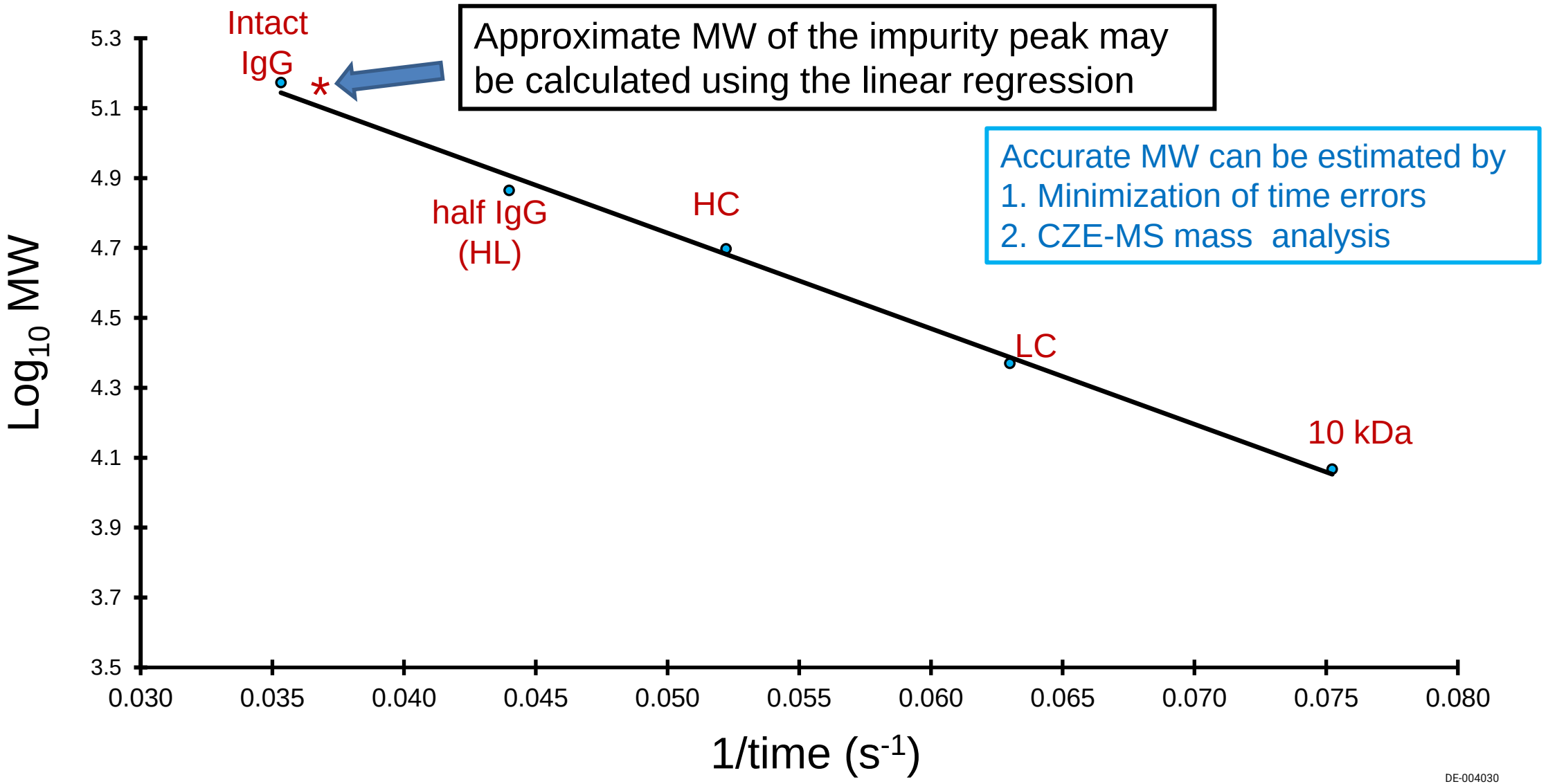
Peak Identification Strategy



Plot Mobility versus log10 of Molecular Weight (MW)



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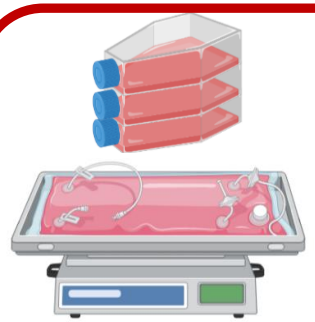
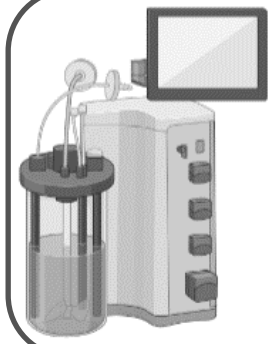
mAb Trisulfides and degradation in bioreactor settings

Monitoring NIST mAb H2S-Induced degradation products by GXII and CZE-MS



mAb Cathepsin degradation during cell culture

Monitoring NIST mAb Cathepsin degradation products by GXII and CZE-MS



mAb High-pH degradation during bioprocessing

Monitoring Rituximab high-pH degradation products by GXII and CZE-MS

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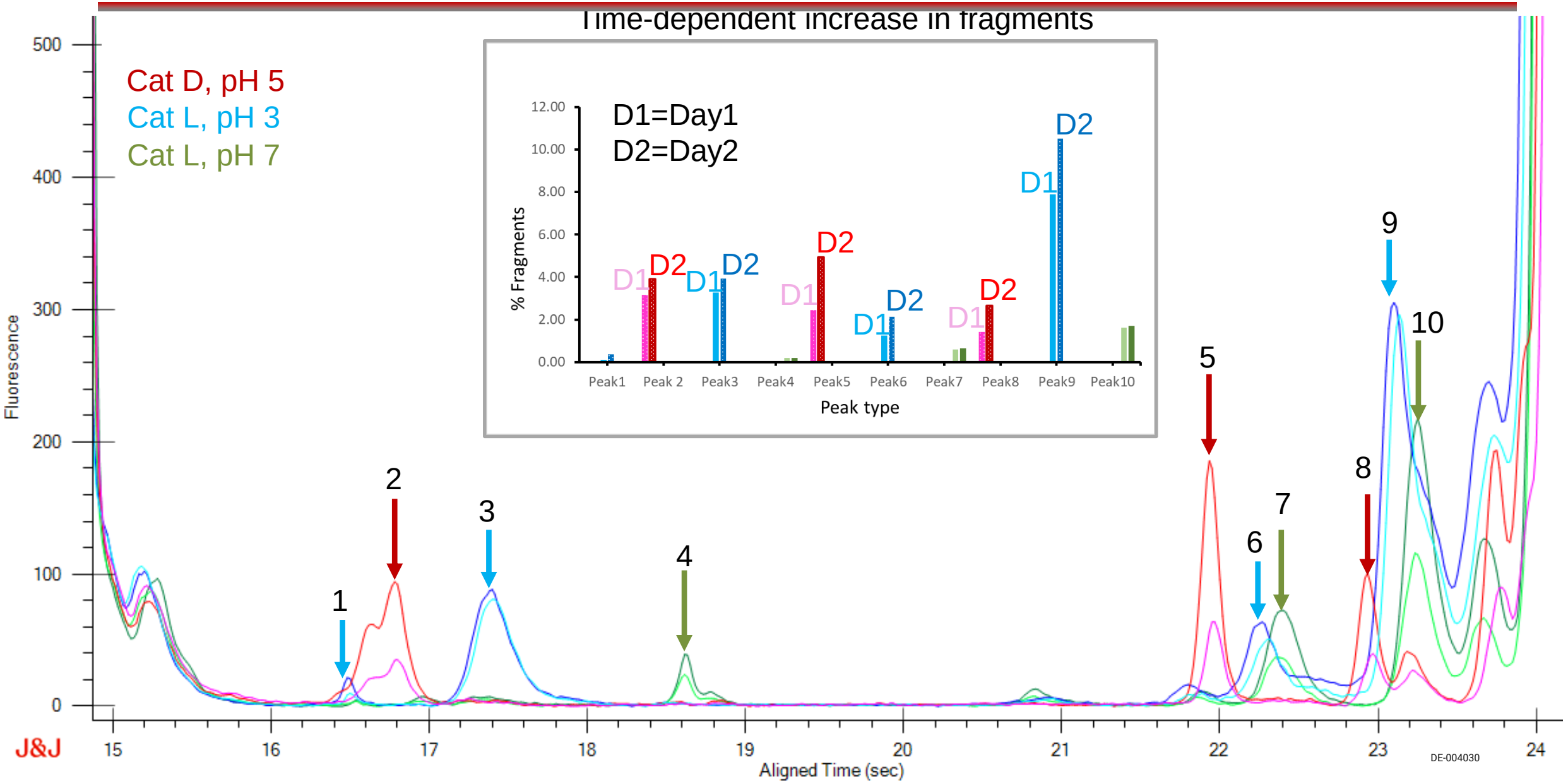
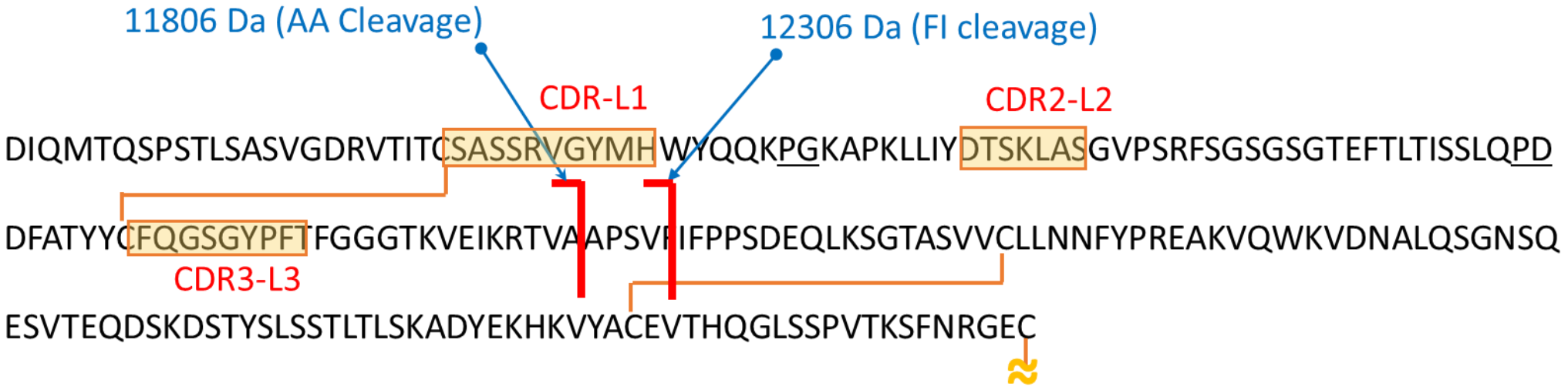
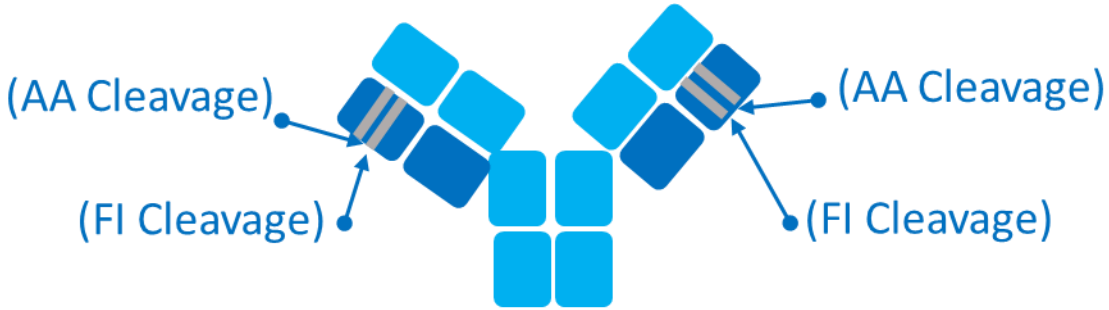
Overlayed GXII-FLR Traces of Cathepsin D and L at Day 1 and Day2



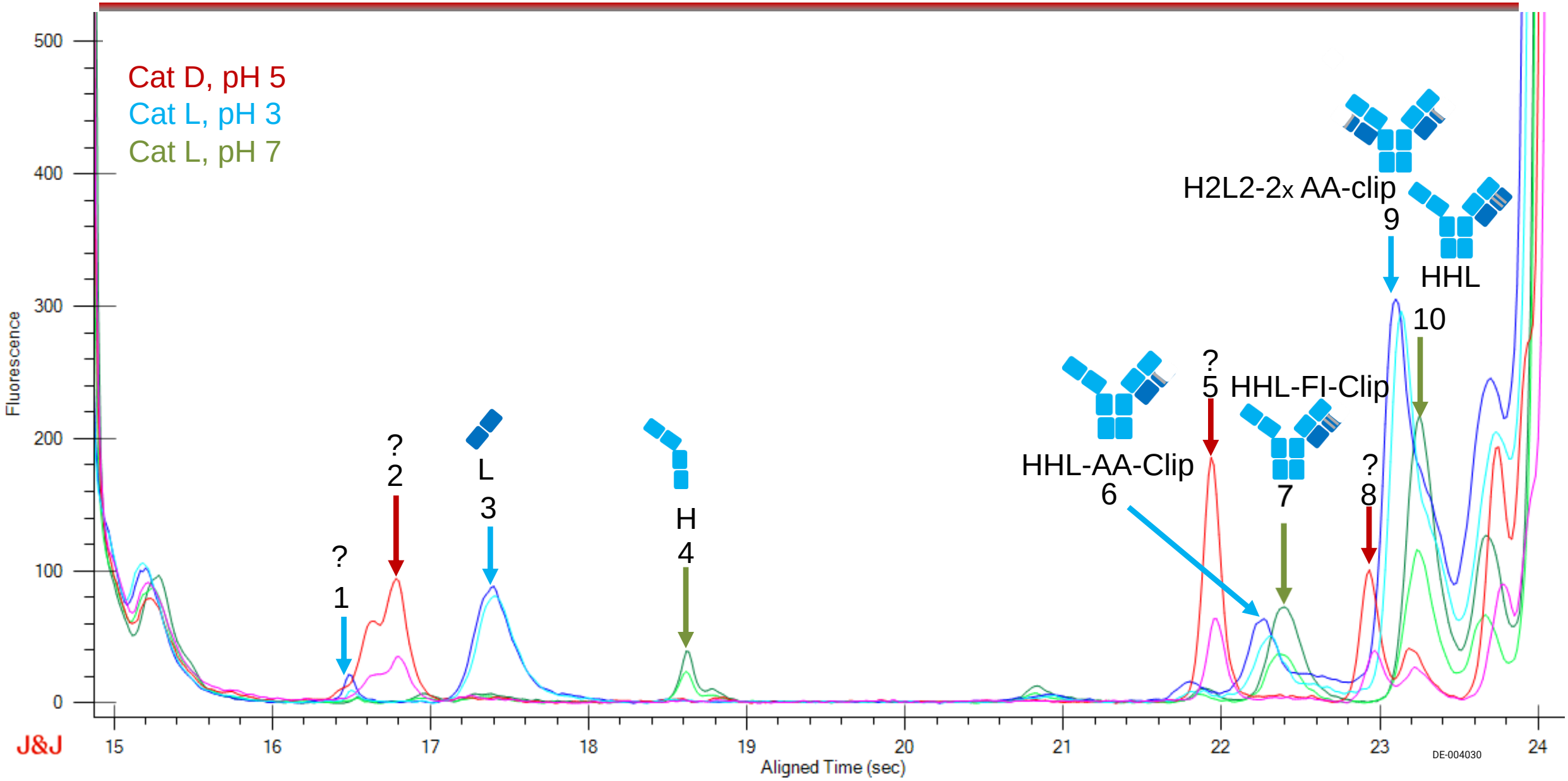
Cathepsin-D and -L Cleavages of Light Chain



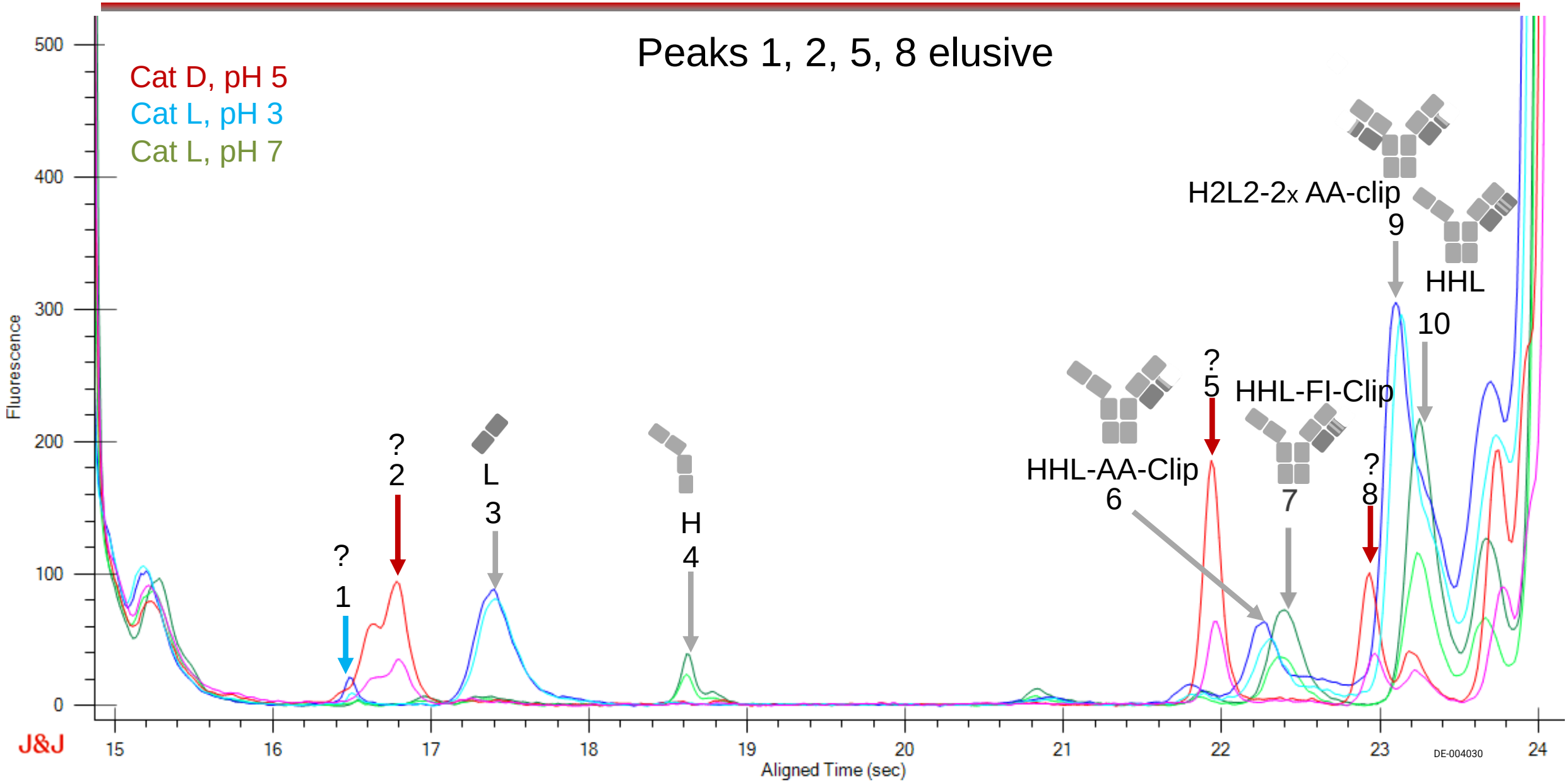


NIST IgG1

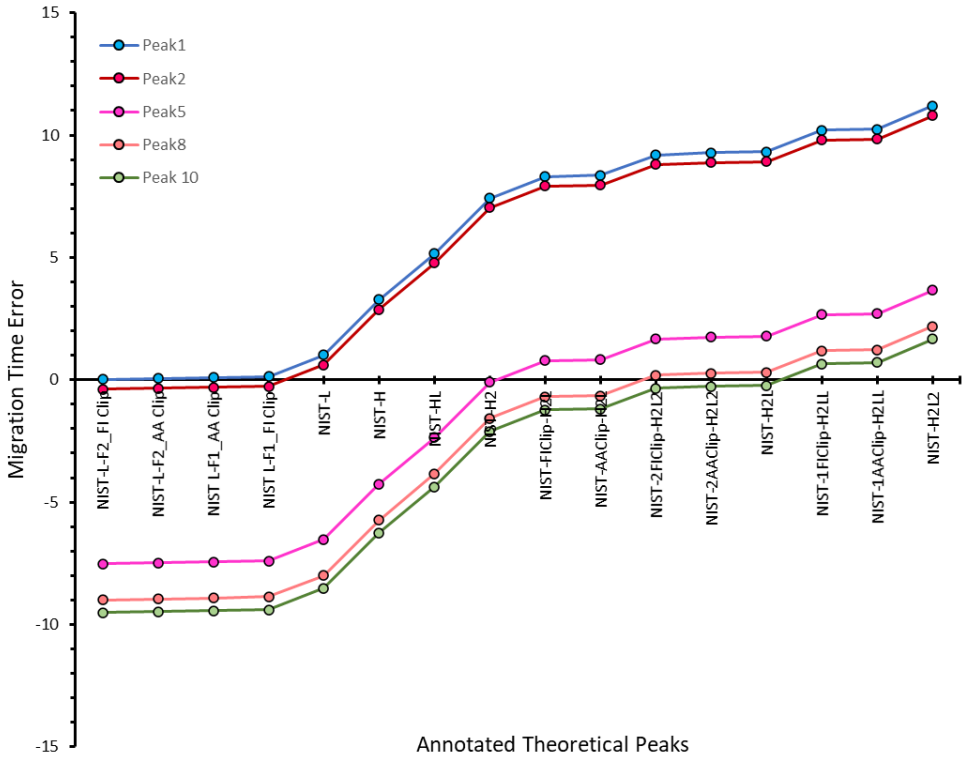
Peak Identification of Cathepsin-D, and -L Fragments

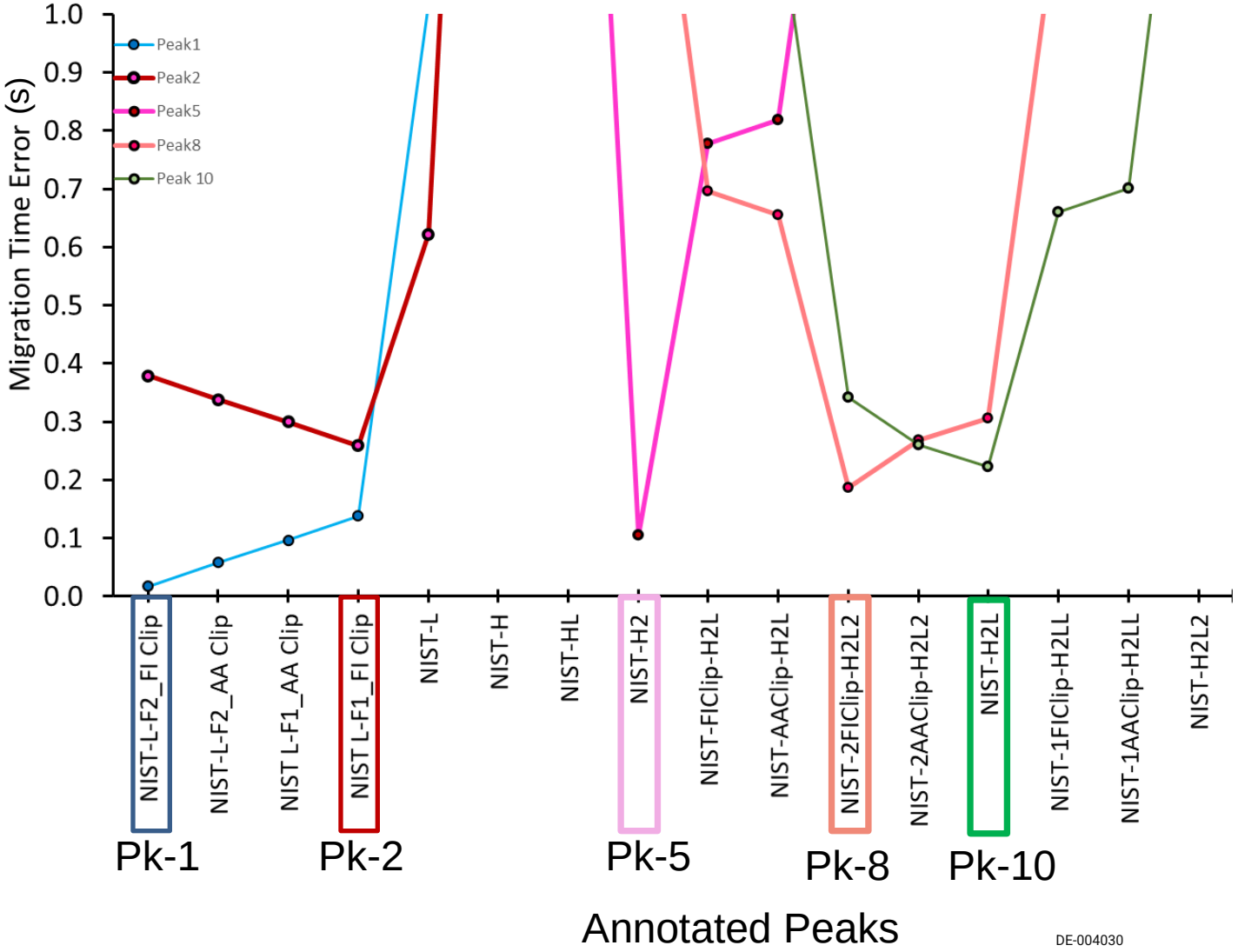


Unassigned Peaks in Cathepsin-D and -L Fragments



Minimization of Time Errors: An approach to increase Peak ID's





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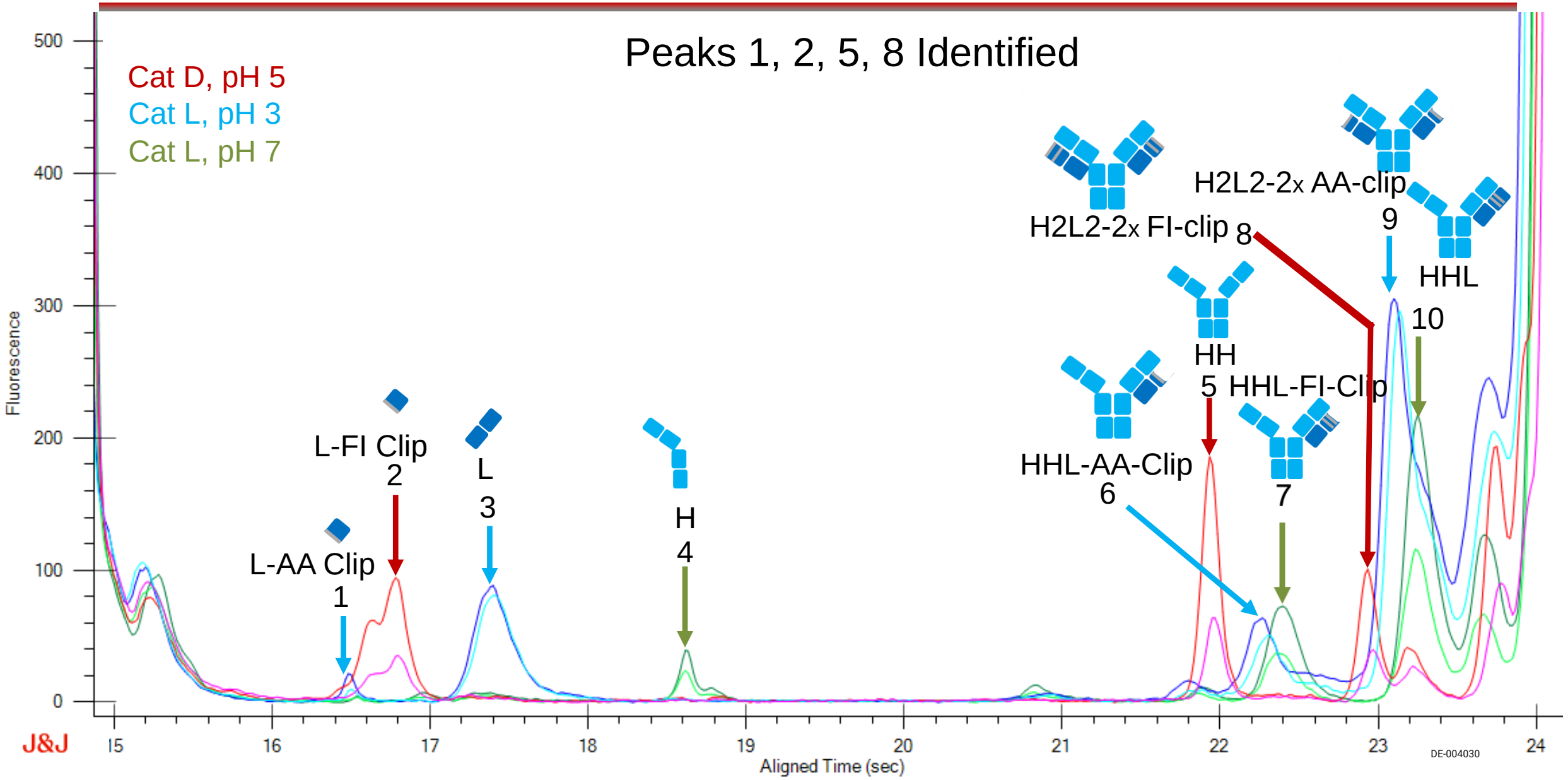
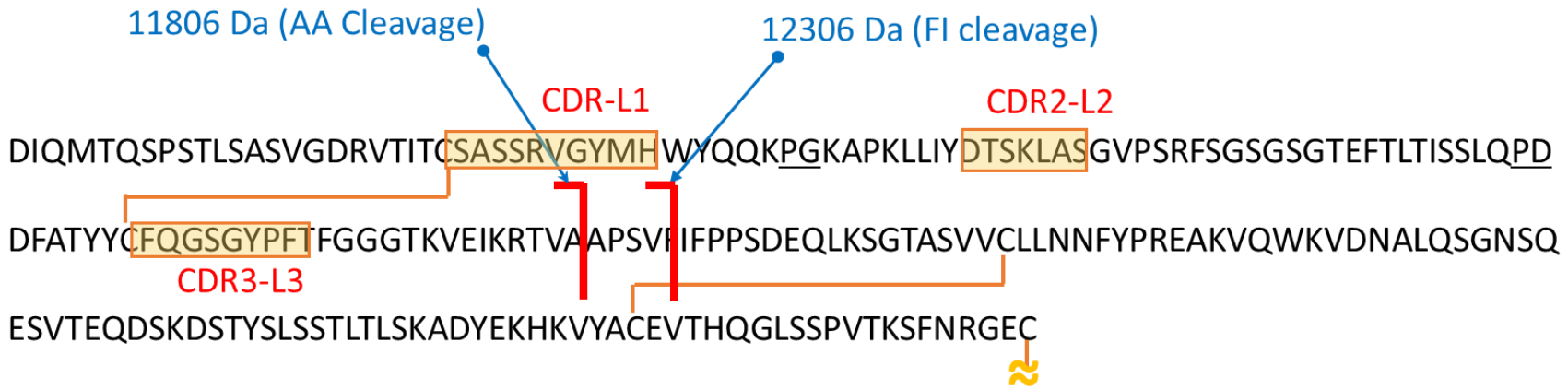
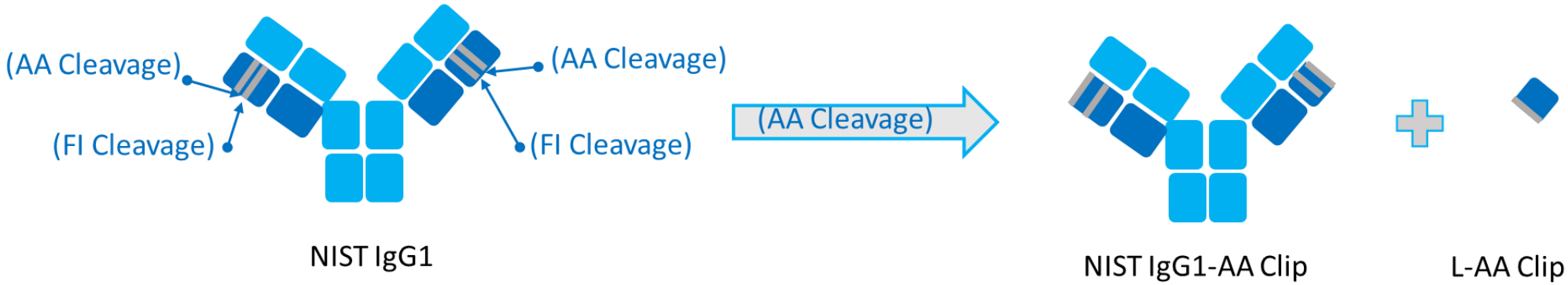
Assigning Unassigned Peaks in Cathepsin-D and -L Fragments



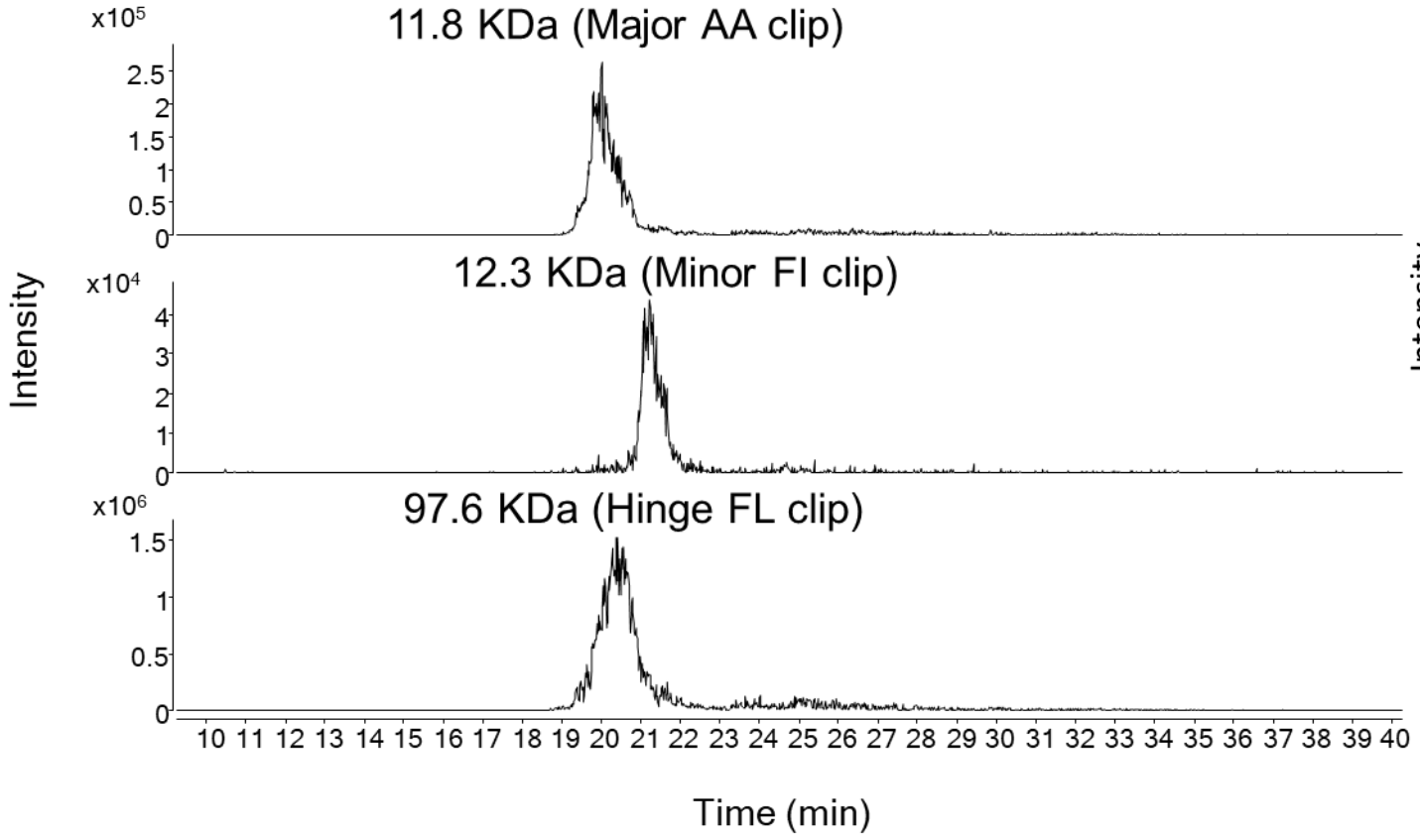
pH3 Cathepsin L-Induced NIST IgG1 Light Chain AA and FI Clips

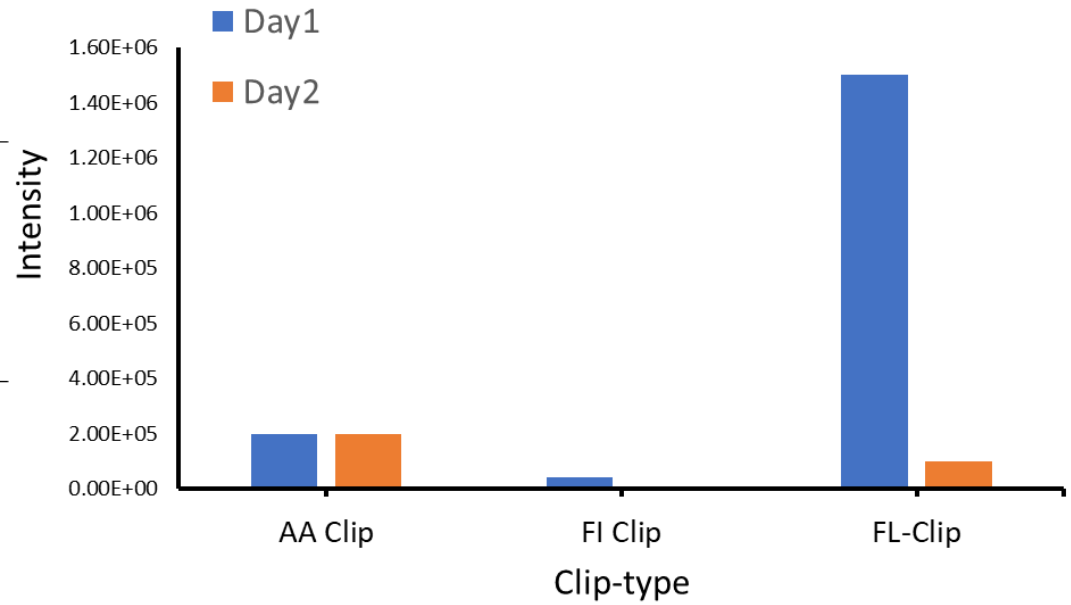


Cathepsin L Cleavage Sites
CDR-L1, CDR-L2, CDR-L3 of LC

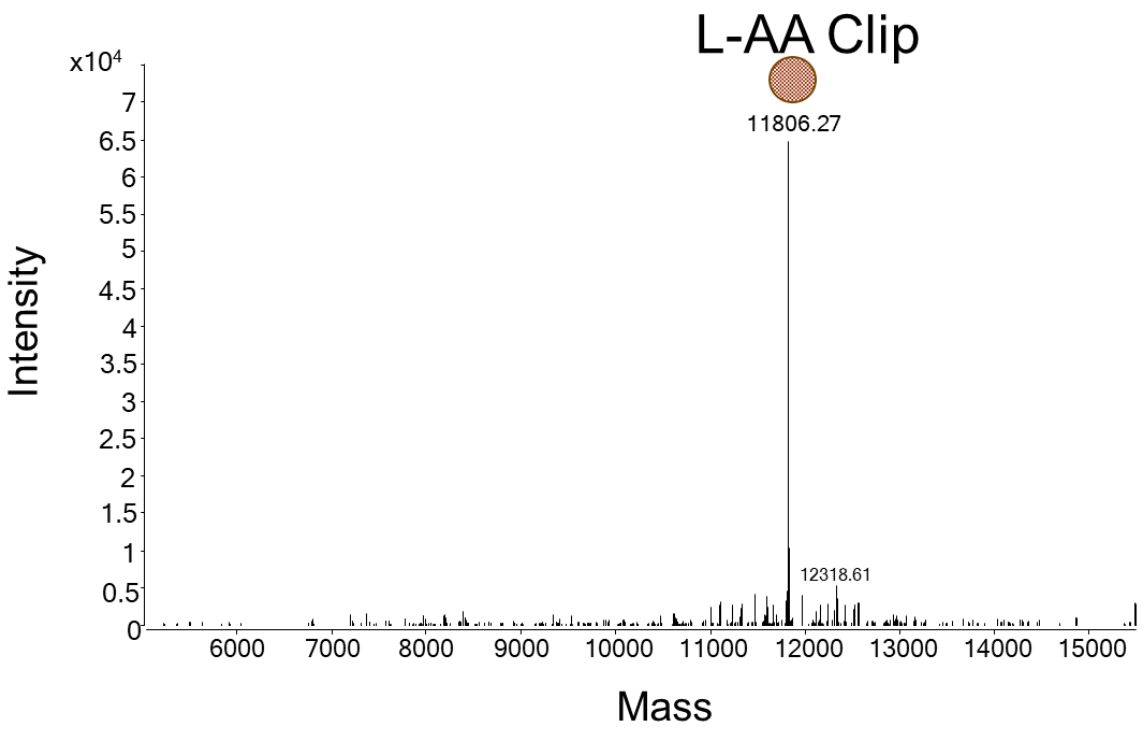


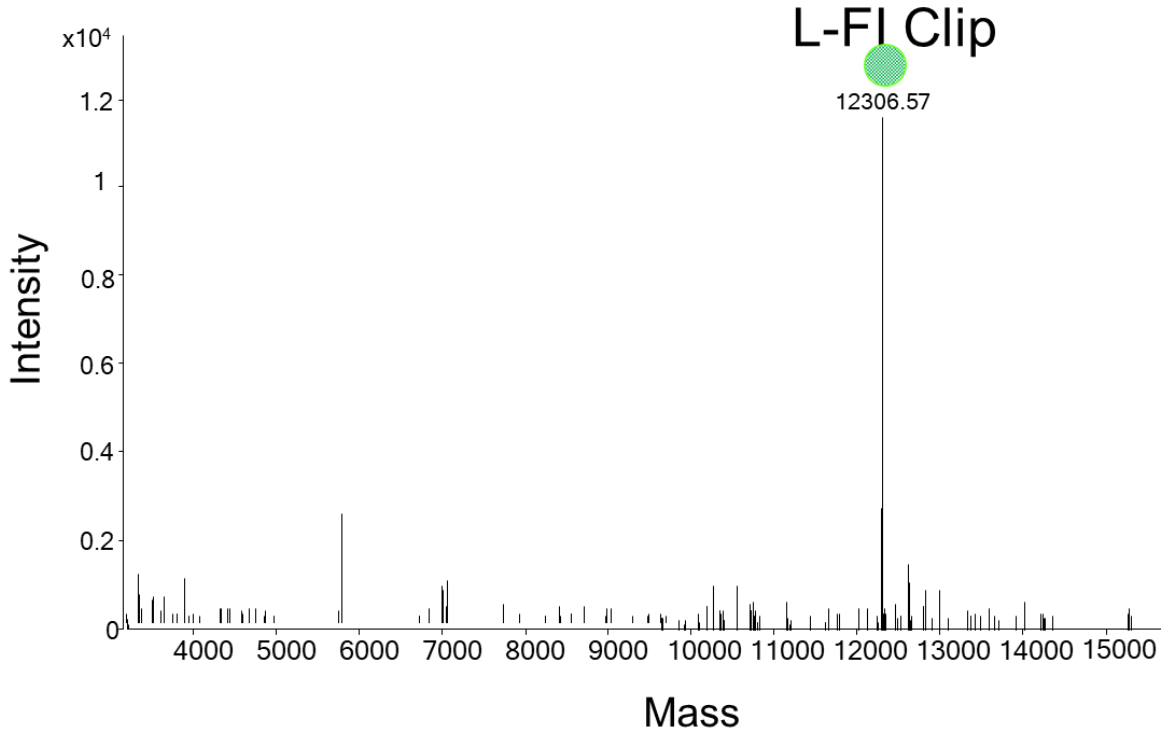
pH3 Cathepsin L CZE-MS Identification of Clips





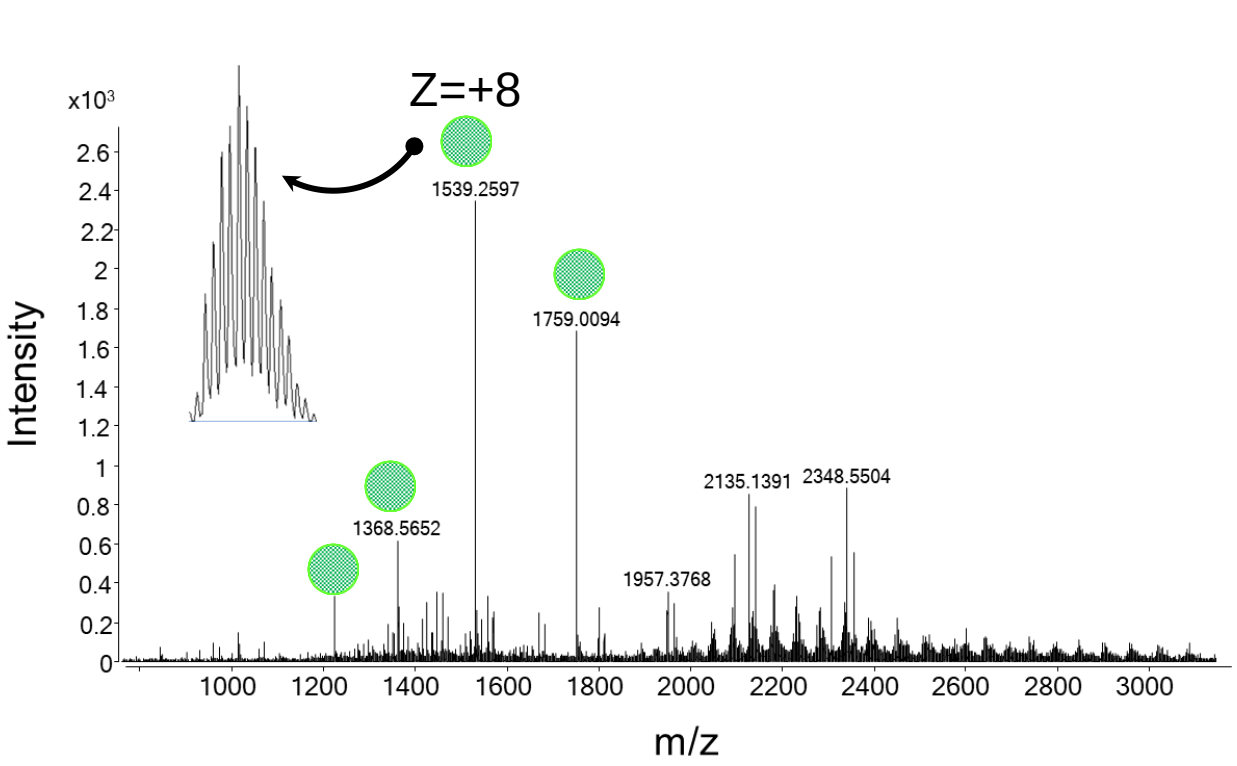
pH3 Cathepsin L CZE-MS Deconvolution Mass of AA and FI Clips

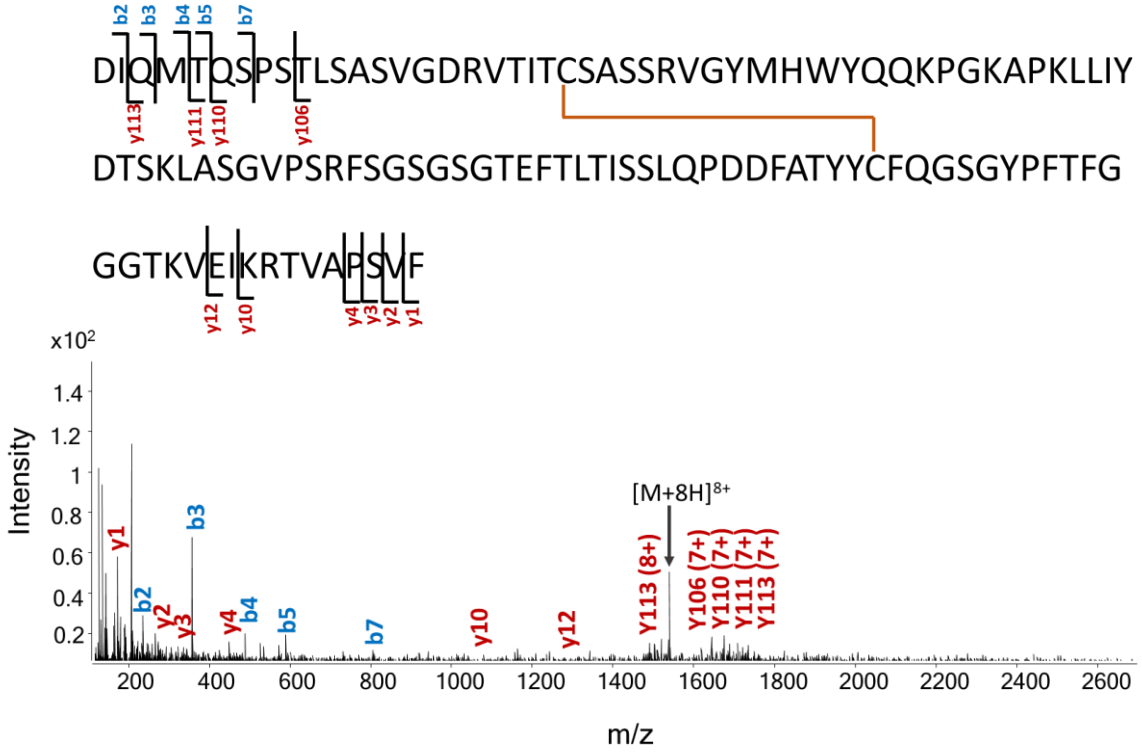




- MS1 Peak IDs of AA and FI Clip

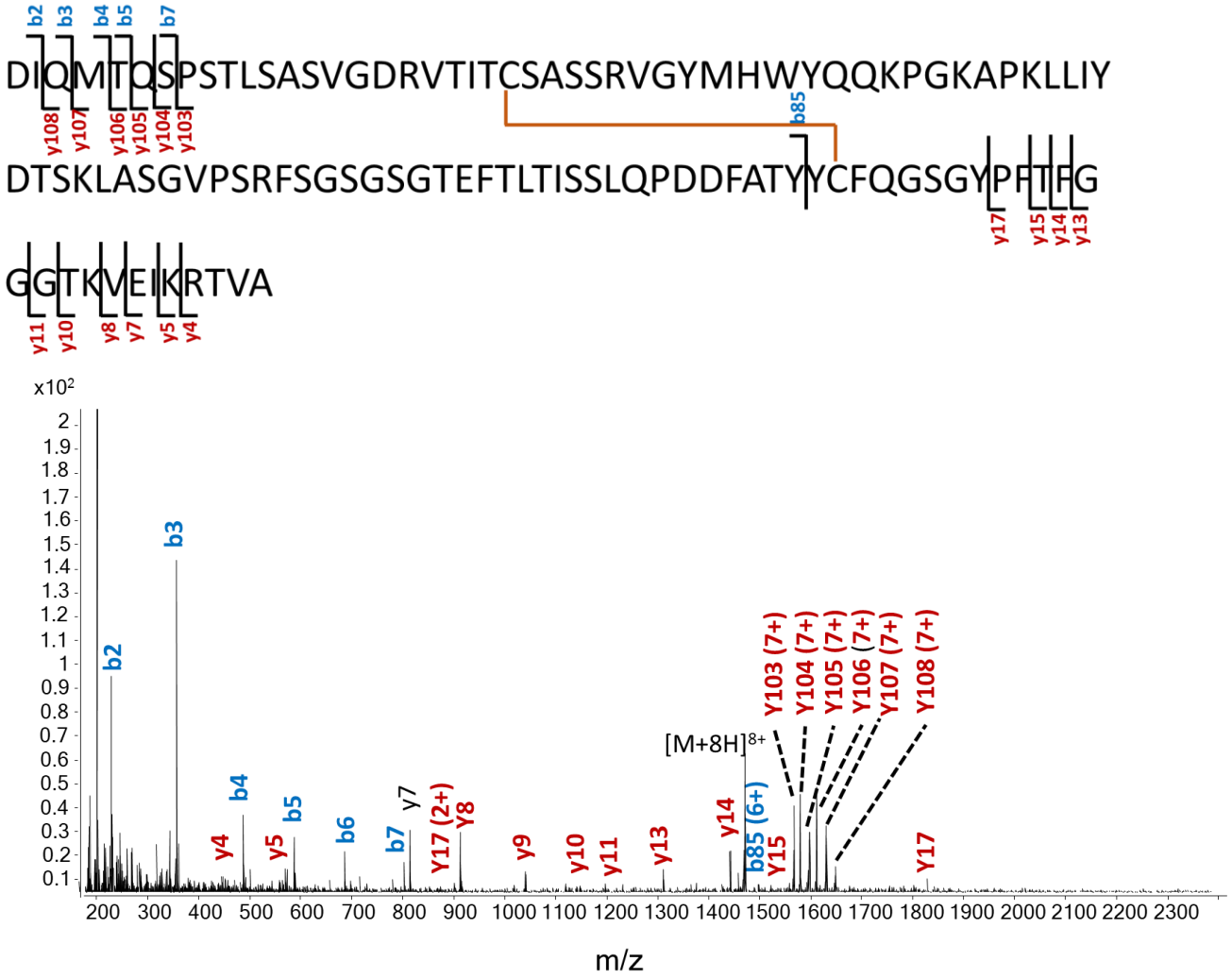
pH7 Cathepsin L CZE-MS Exclusively Shows Light Chain FI Clip





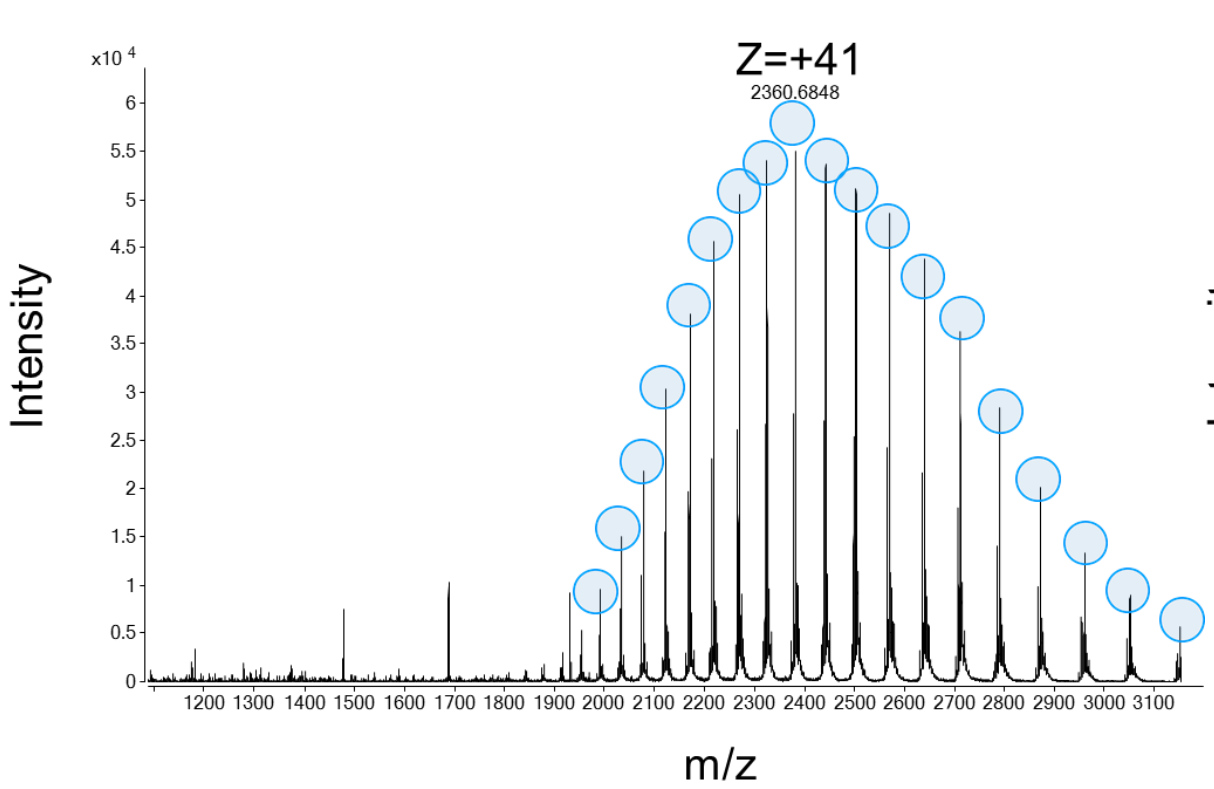
- Unambiguous MS2 Sequence Confirmation of FI Clip by CE-MS/MS

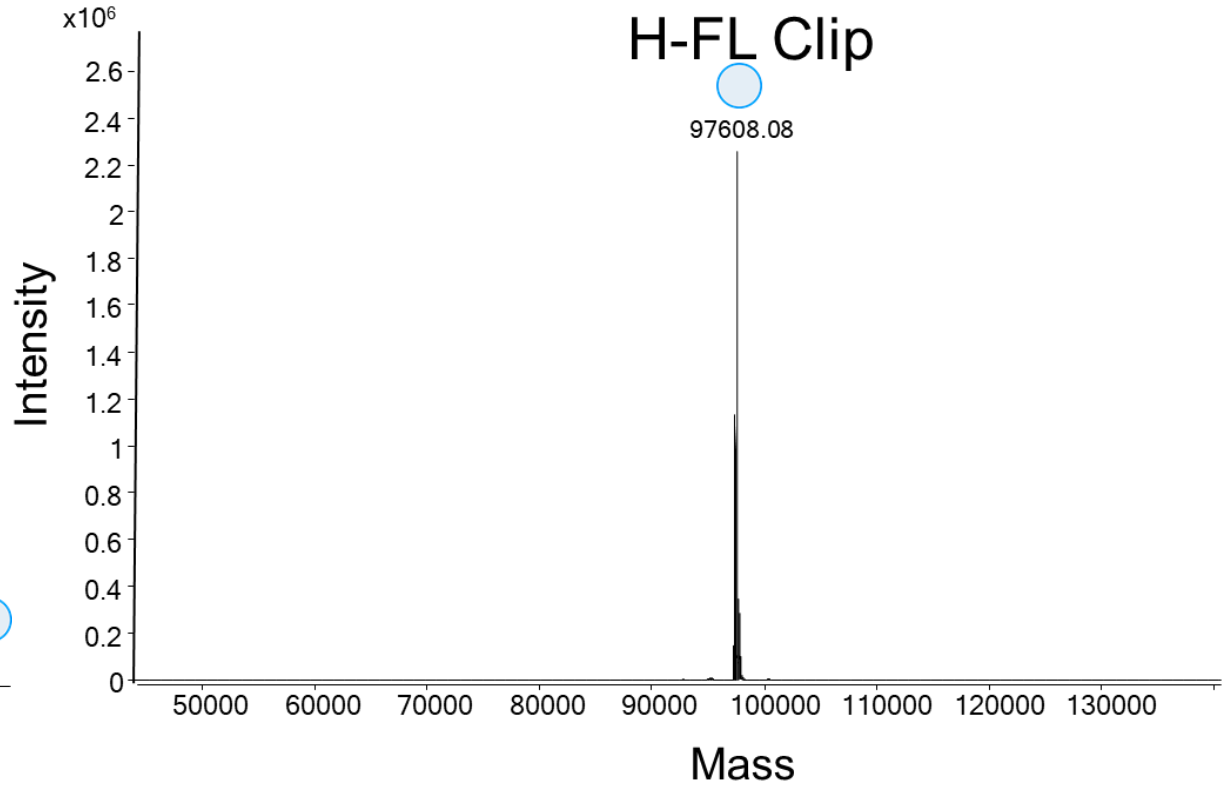
pH3 Cathepsin L CZE-MS/MS of Light Chain AA Clip



- Unambiguous MS2 Sequence Confirmation of AA Clip by CE-MS/MS

pH3 Cathepsin L CZE-MS Identification FL Clip





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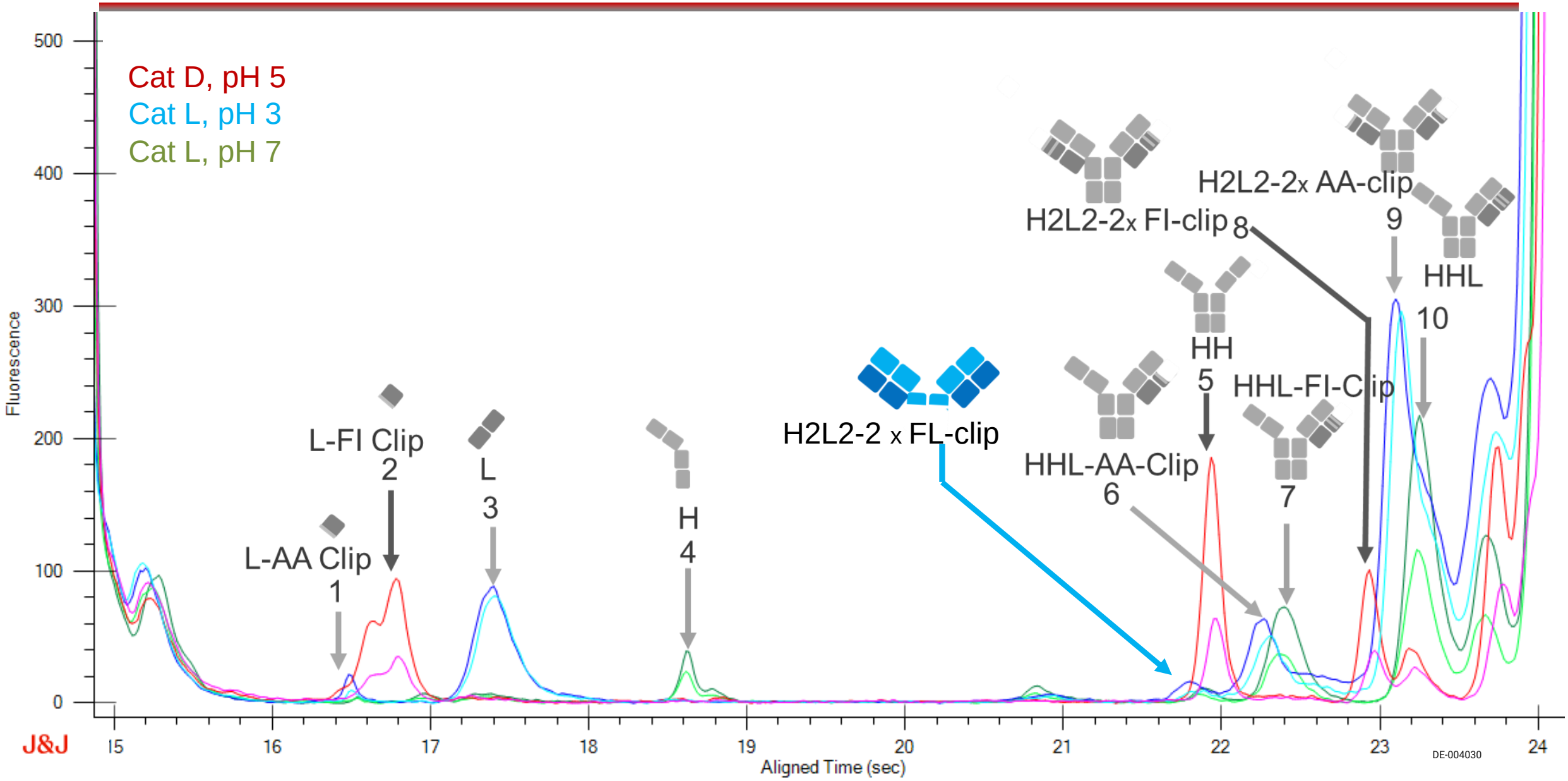
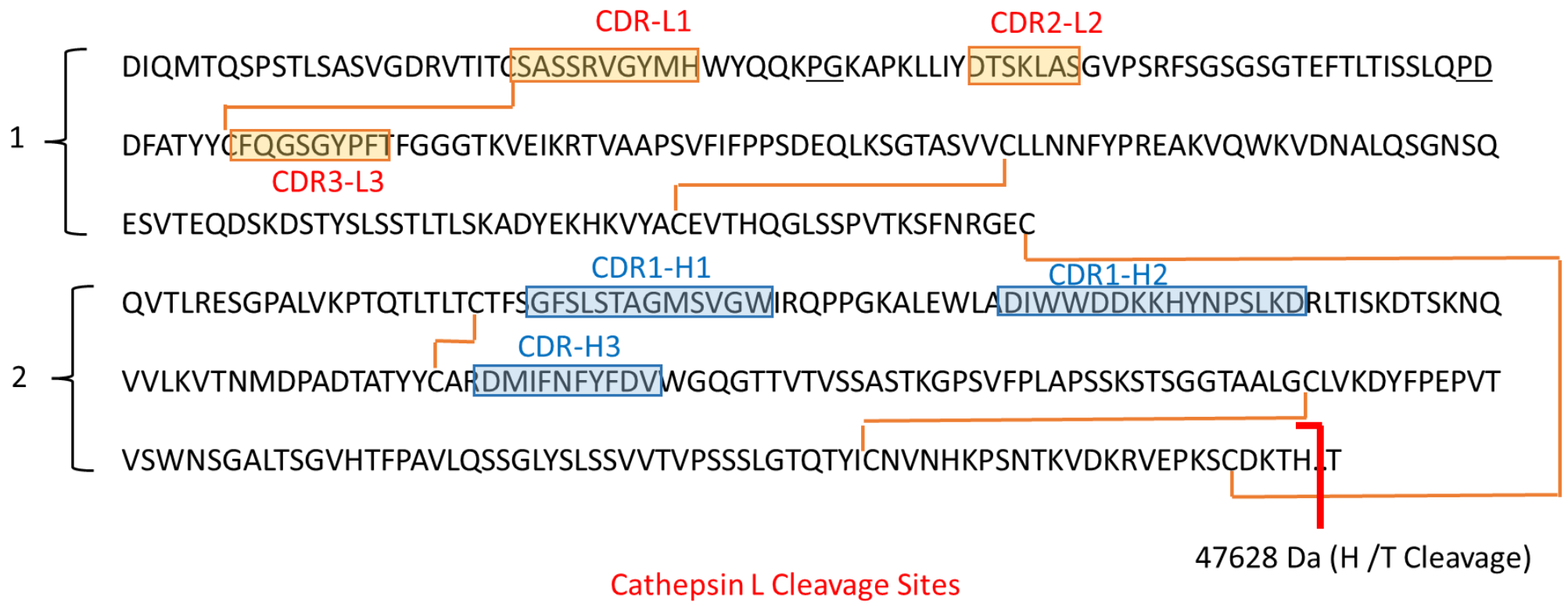
Peak Assignment of pH3 Cathepsin L CZE-MS Identification FL Clip



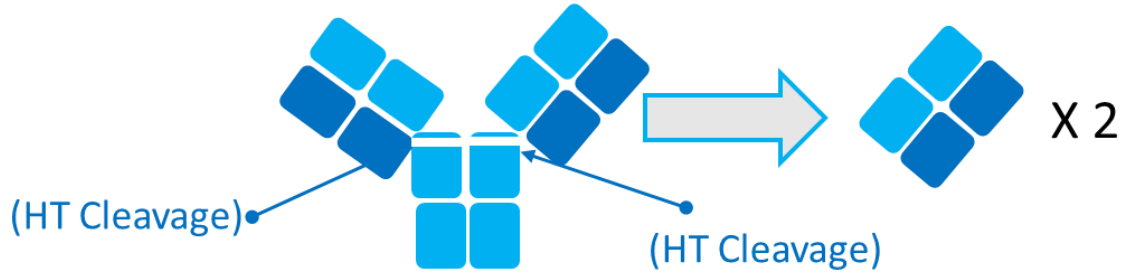
pH3 Cathepsin L-Induced 47.6 Da, HT Clips



1 = LC and 2 = Fd'

CDR-L1, CDR-L2, CDR-L3 of LC

CDR-H1, CDR-H2, CDR-H3 of HC



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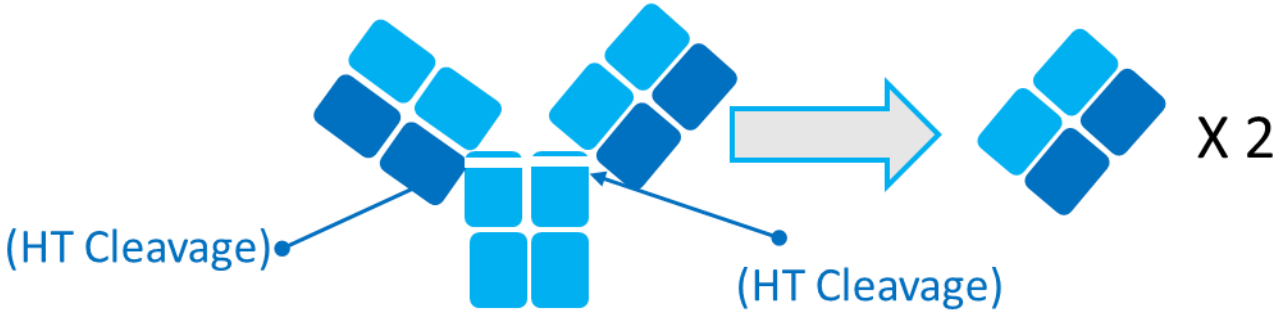
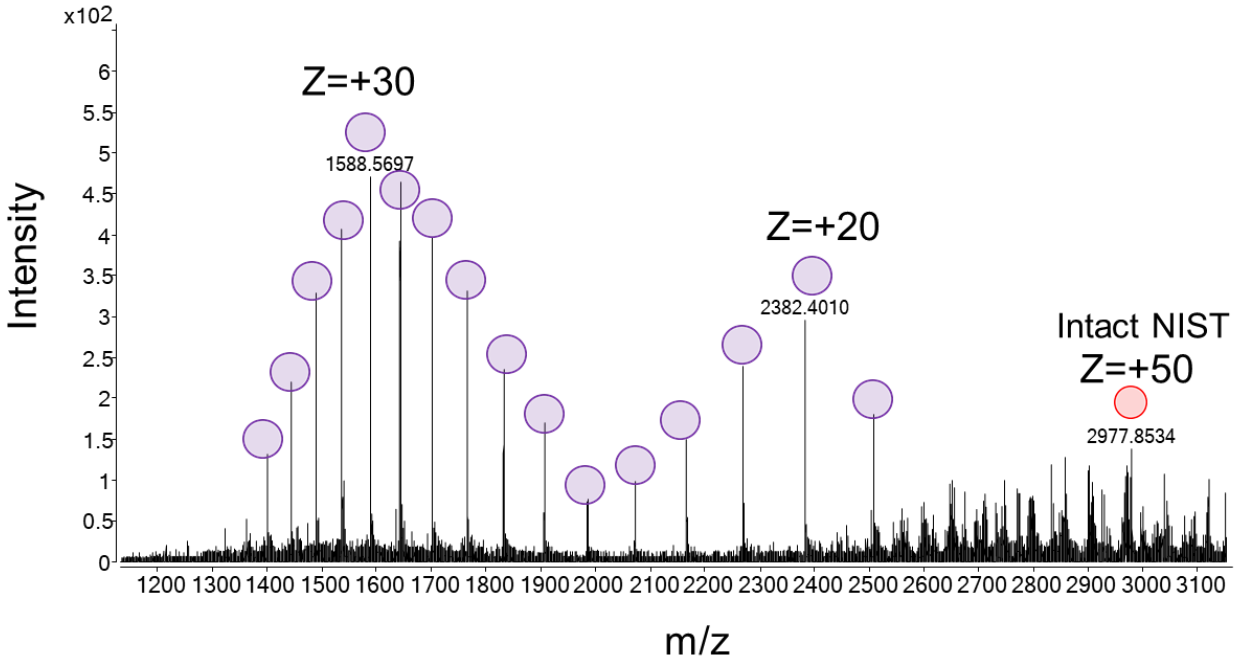
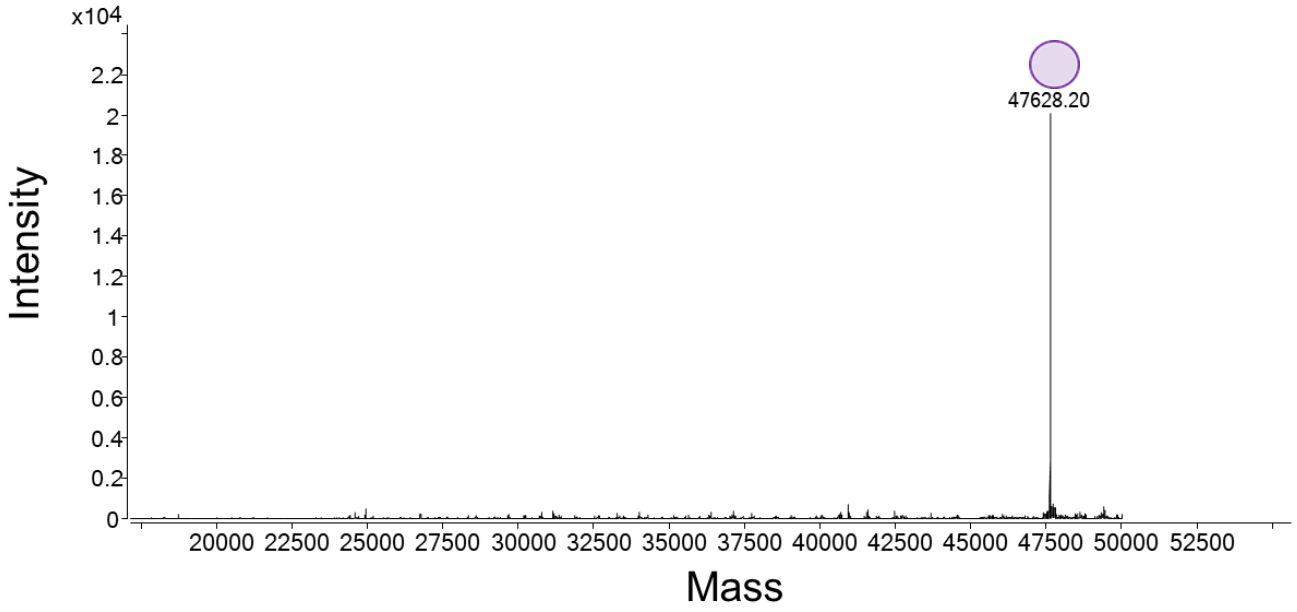
pH3 Cathepsin L CZE-MS of 47.6 kDa, Fragment due to HT Clip







Heavy Chain (H) Assignment is truly a Fab' generated via two HT Clips

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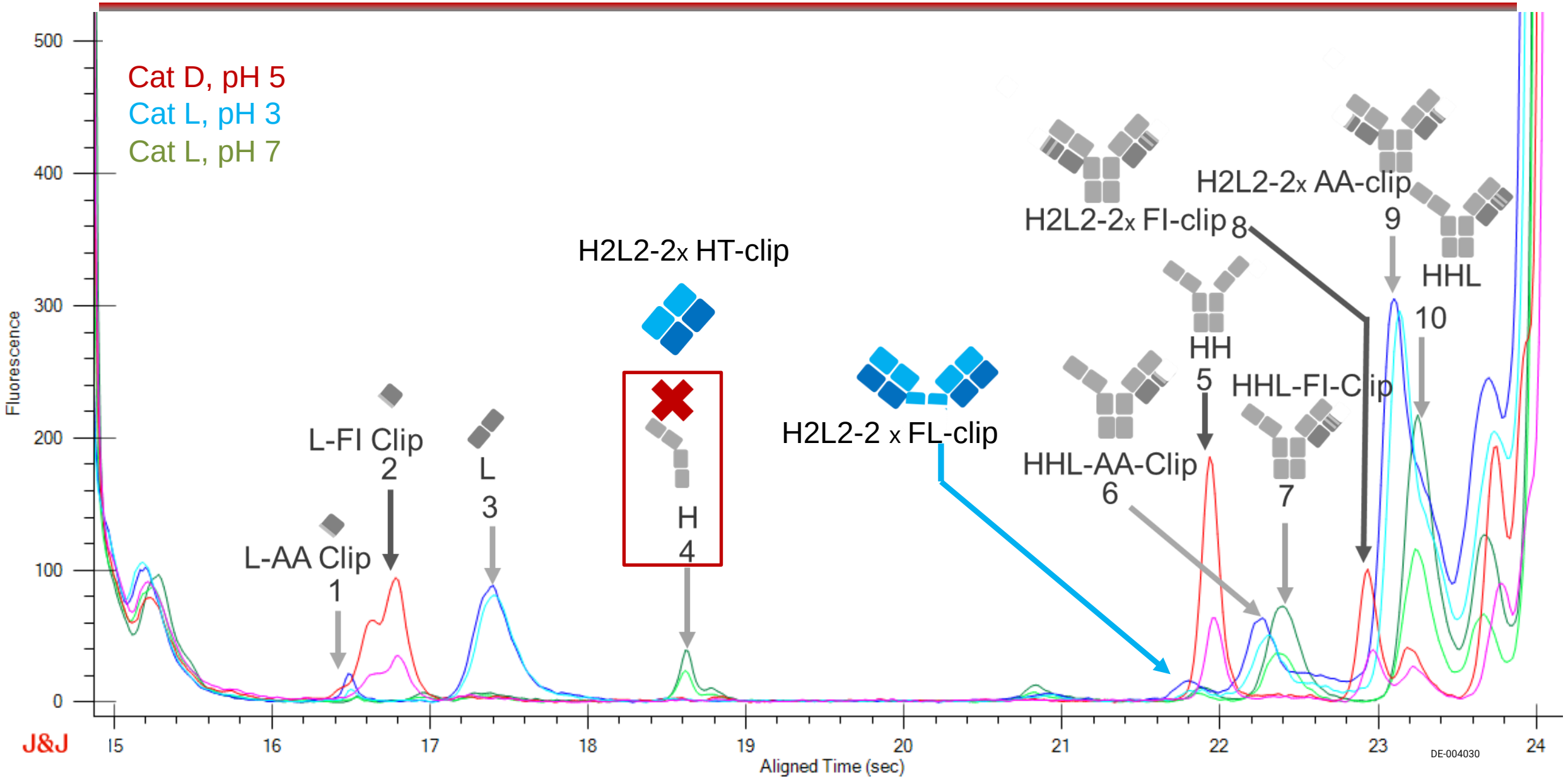
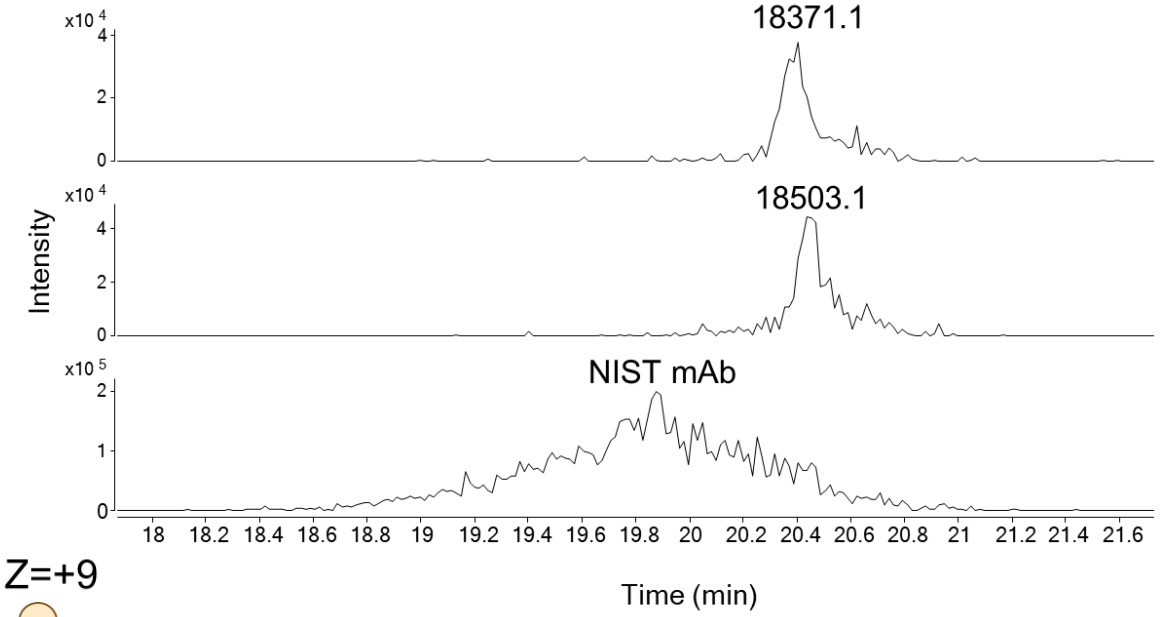
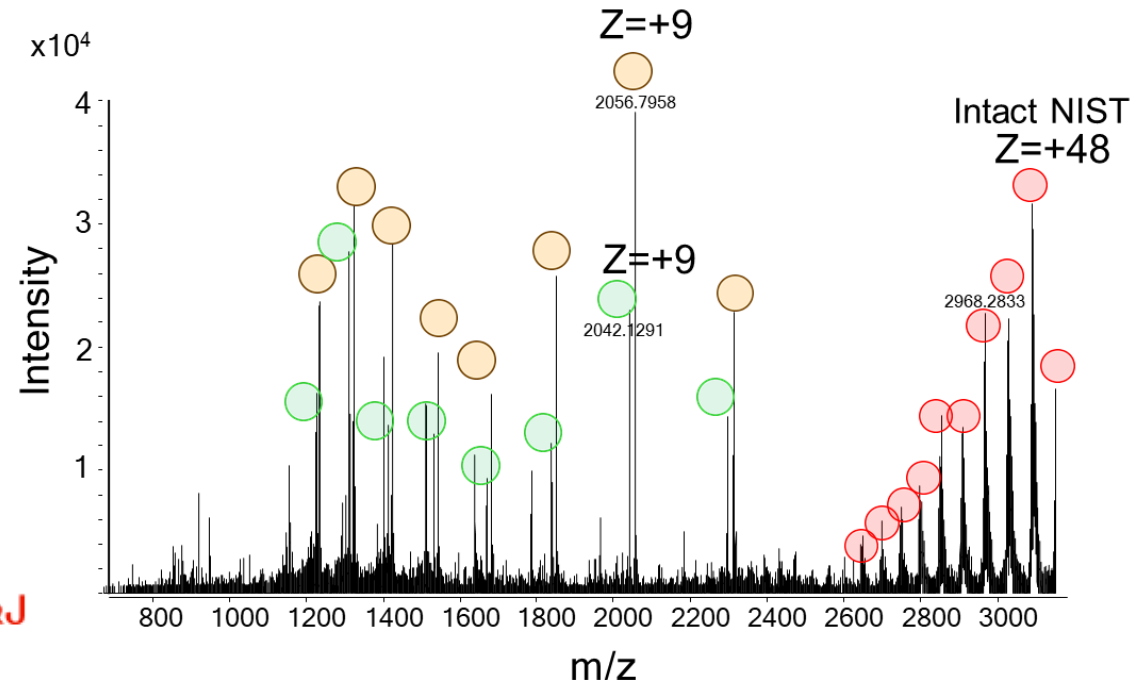
DE-004030

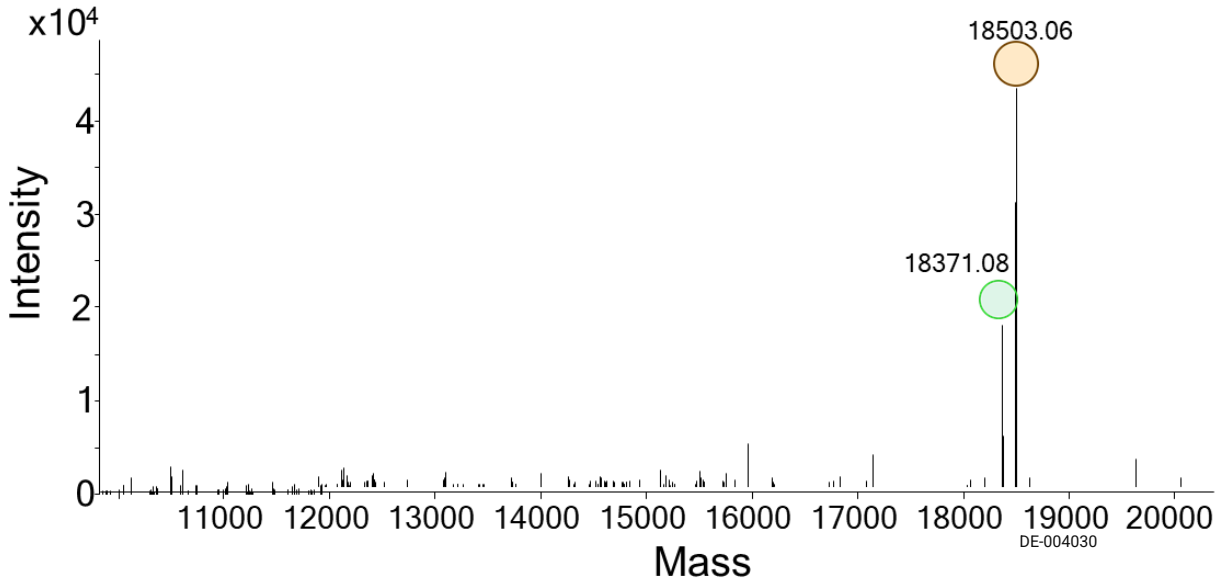
Reassignment of Misidentified peaks 47.6 kDa, Fragment HT Clip



pH 5 Cathepsin D, CZE-MS Unidentified Peaks







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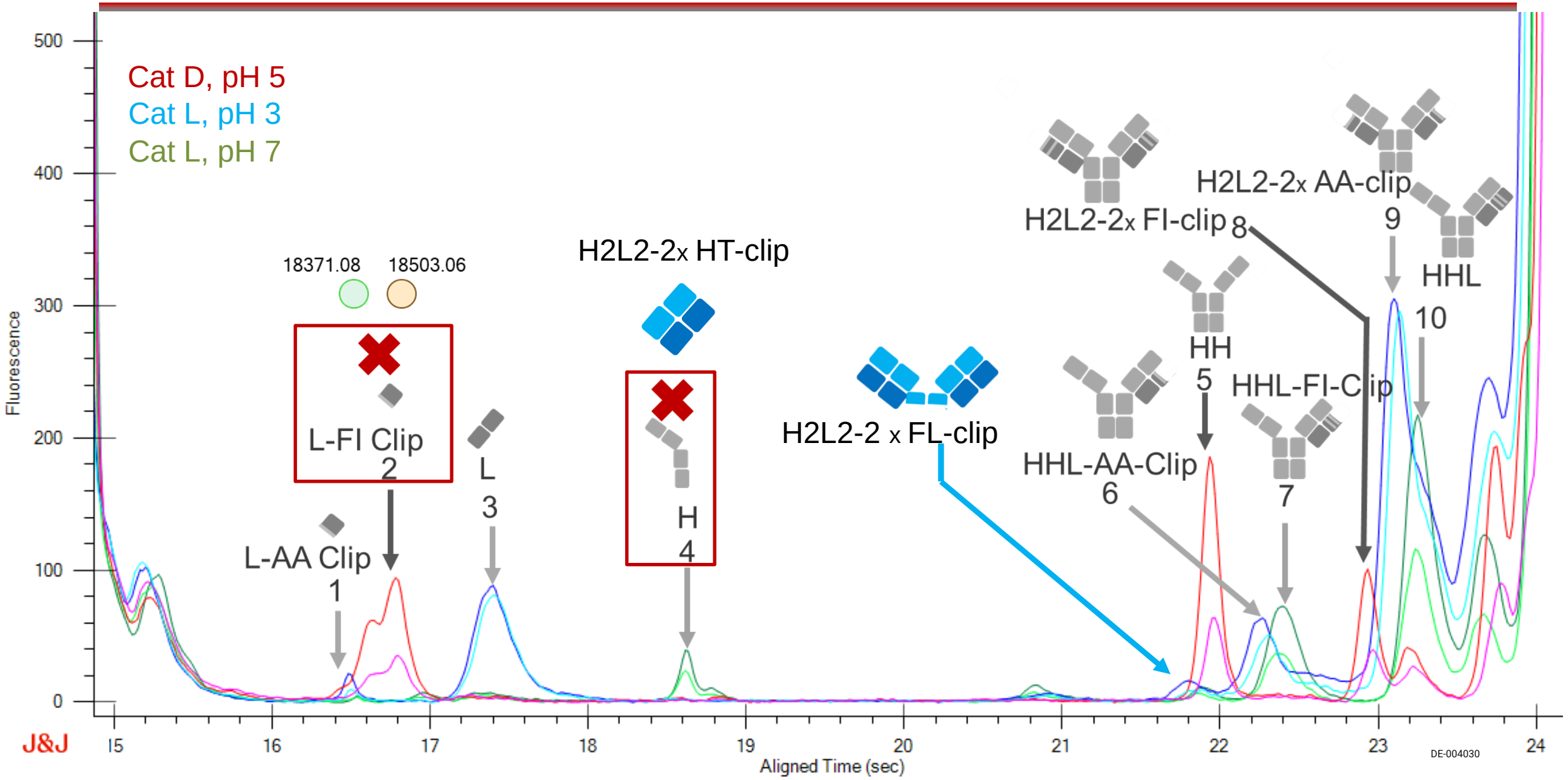
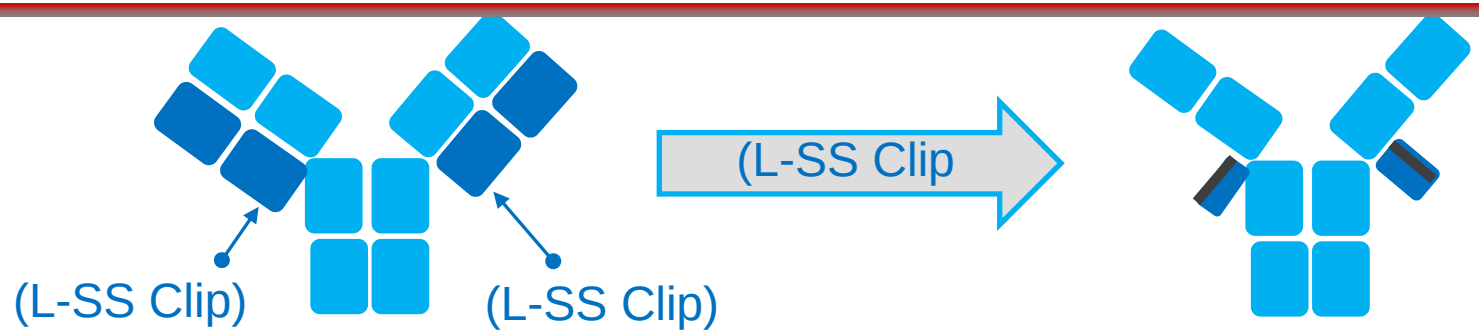
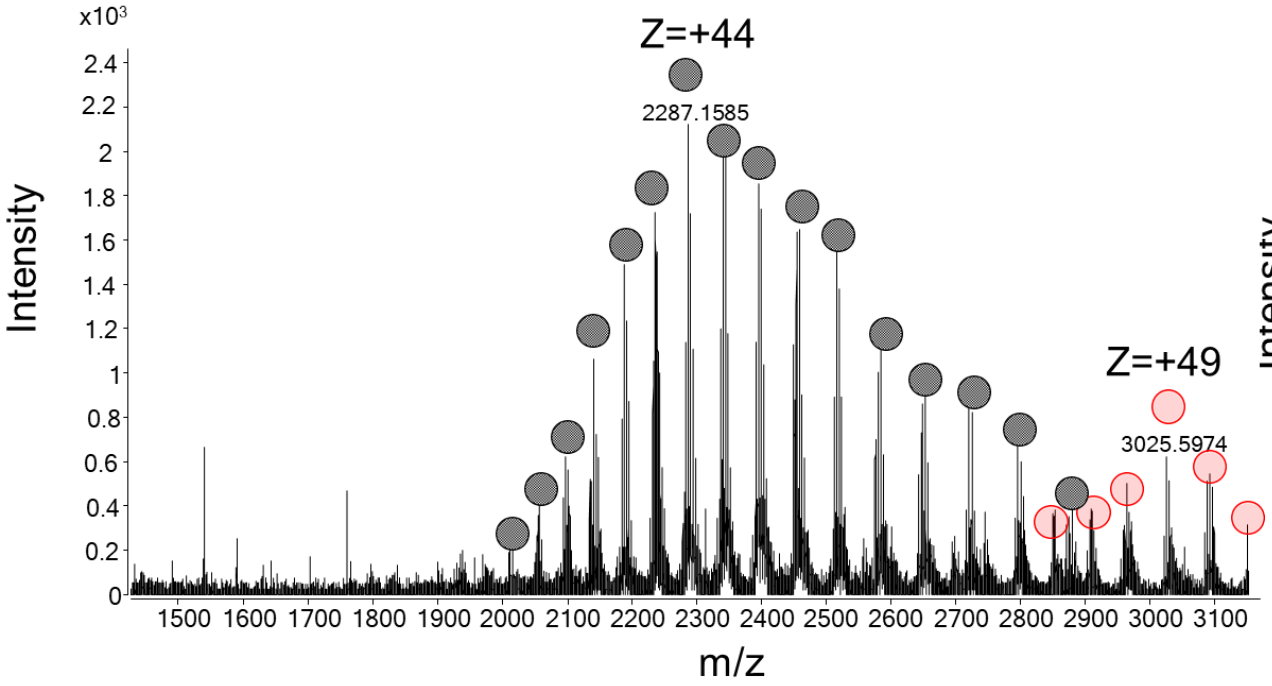
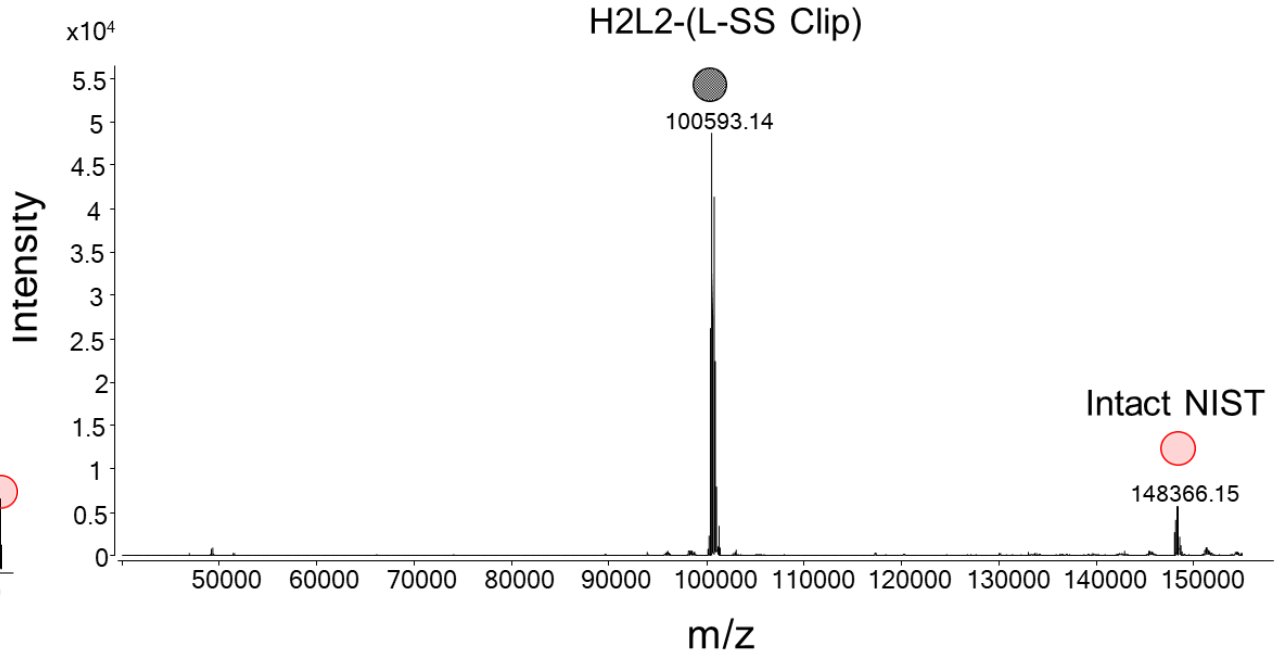
Reassignment of Misidentified peaks 18 kDa, Fragment Pair



pH7 Cathepsin L CZE-MS of 105.9 kDa Fragment







(HH) Assignment is a mAb with truncated light chains via two SS Clips

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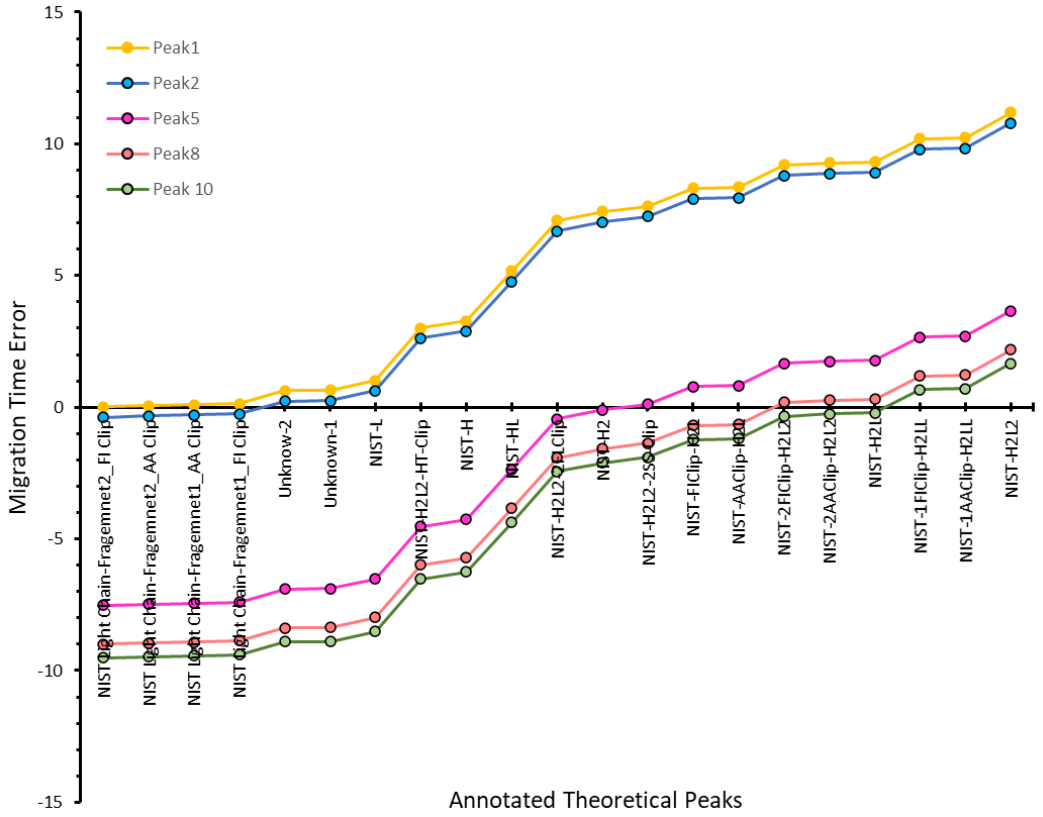
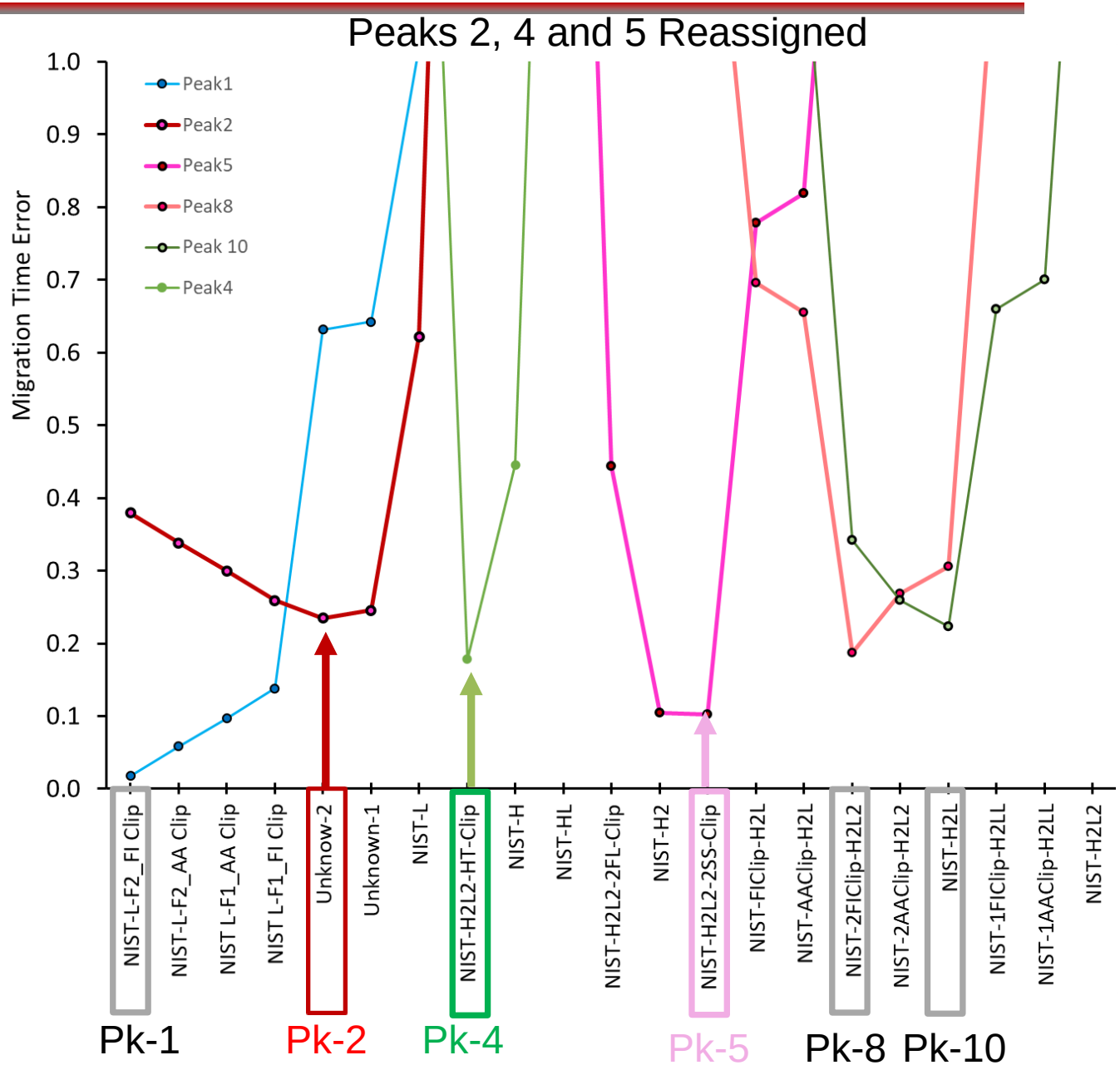
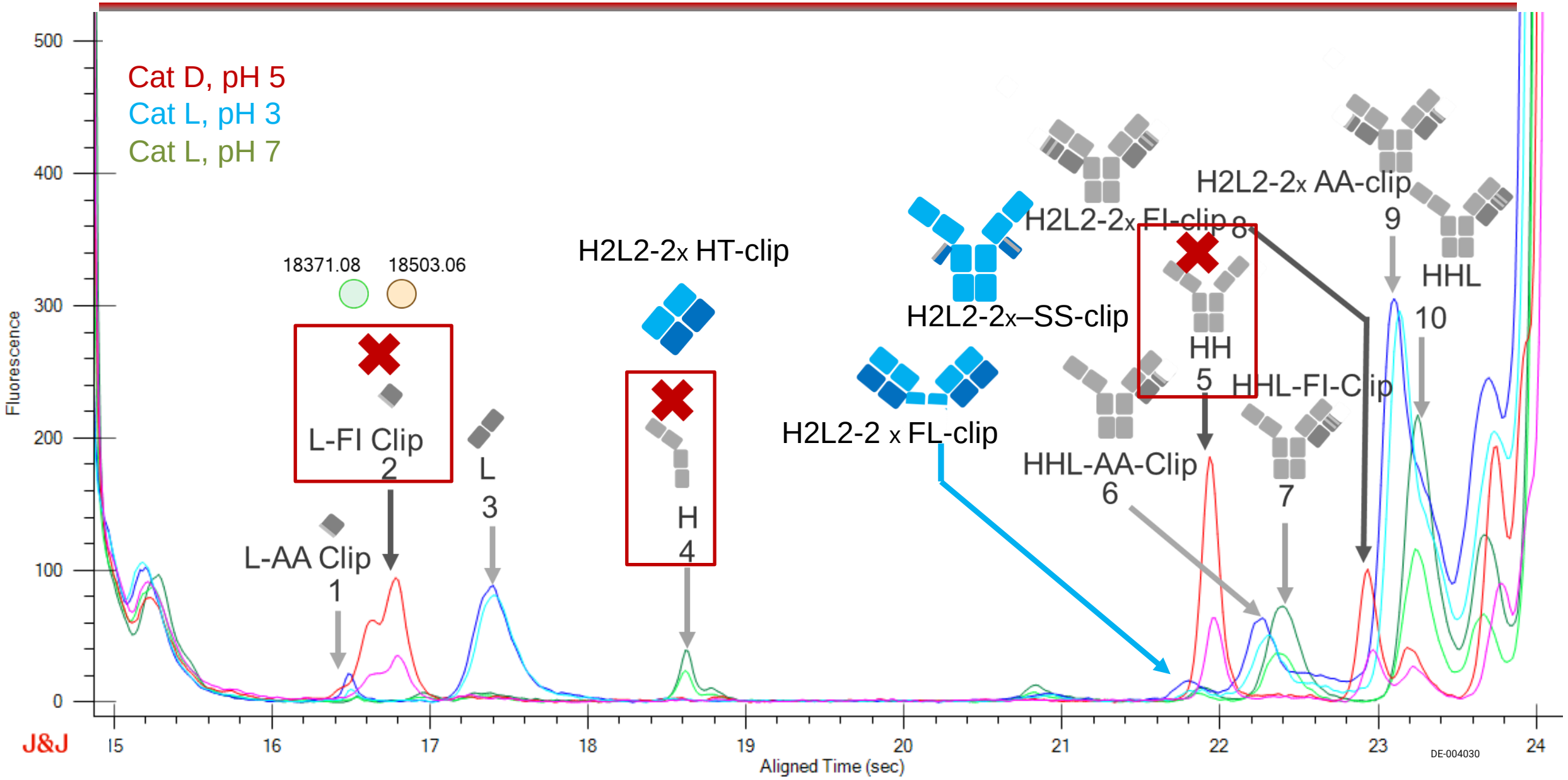
Reassignment of Peak ID's via Minimization of Time Errors

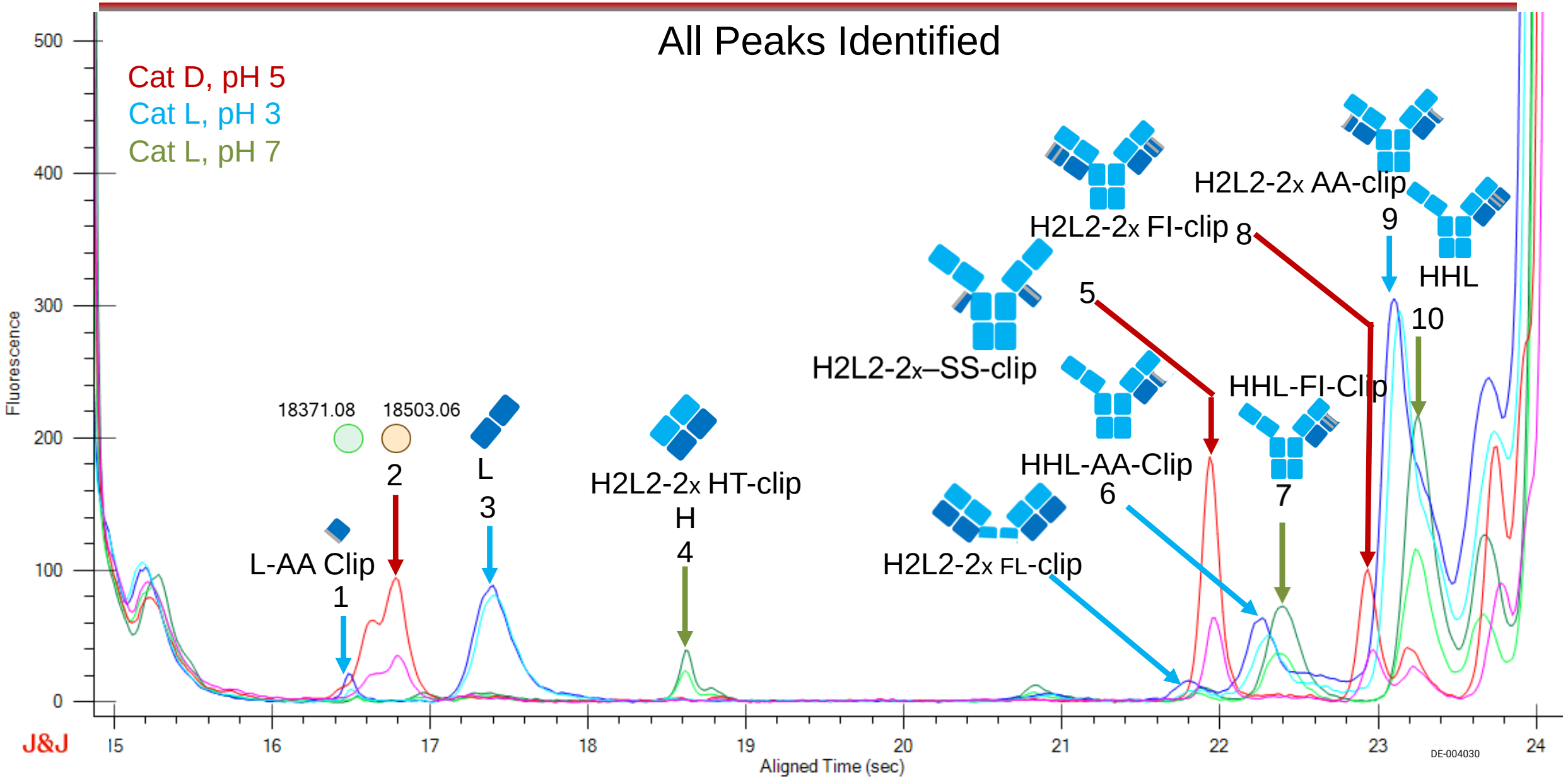




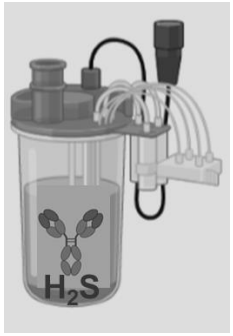
Reassignment of Misidentified peaks Light Chain SS Clips



Overlayed GXII-FLR Traces of Cathepsin D and L Impurities

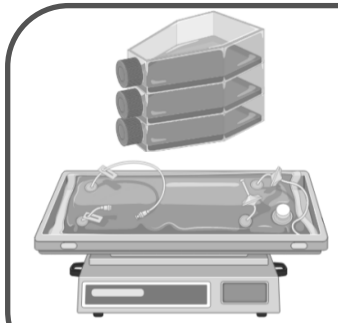


Simulating Bioprocessing Systems to Examine mAb Impurities



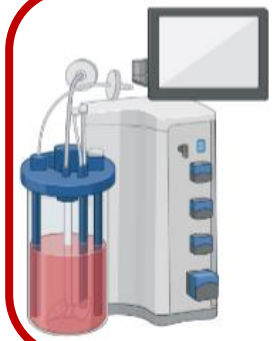
mAb Trisulfides and degradation in bioreactor settings

Monitoring NIST mAb H2S-Induced degradation products by GXII and CZE-MS



mAb Cathepsin degradation during cell culture

Monitoring NIST mAb Cathepsin degradation products by GXII and CZE-MS



mAb High-pH degradation during bioprocessing

Monitoring Rituximab high-pH degradation products by GXII and CZE-MS

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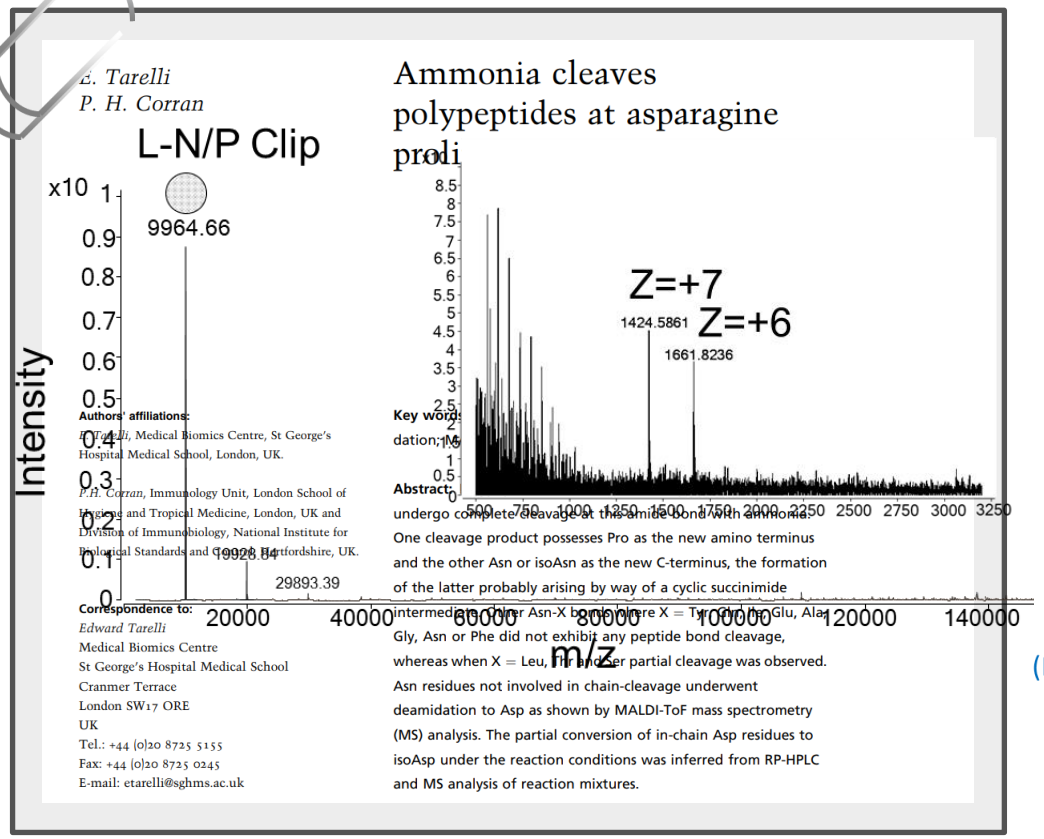
pH 10 Ammonia-Induced Rituximab Light Chain Clip



E. Tarelli
P. H. Corran

Ammonia cleaves polypeptides at asparagine prodi

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Key words: dation; MS

Abstract:
undergo complete cleavage at this amide bond with ammonia. One cleavage product possesses Pro as the new amino terminus and the other Asn or isoAsn as the new C-terminus, the formation of the latter probably arising by way of a cyclic succinimide intermediate. Other Asn-X bonds where X = Tyr, Gly, Thr, Glu, Ala, Gly, Asn or Phe did not exhibit any peptide bond cleavage, whereas when X = Leu, Ile, Met or Ser partial cleavage was observed. Asn residues not involved in chain-cleavage underwent deamidation to Asp as shown by MALDI-ToF mass spectrometry (MS) analysis. The partial conversion of in-chain Asp residues to isoAsp under the reaction conditions was inferred from RP-HPLC and MS analysis of reaction mixtures.

Rituximab Light Chain:

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYA

TSNLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFG

GGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQW

KVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT

HQGLSSPVTKSFNRGEC

(NP Cleavage) (MP Cleavage) (NP Cleavage)

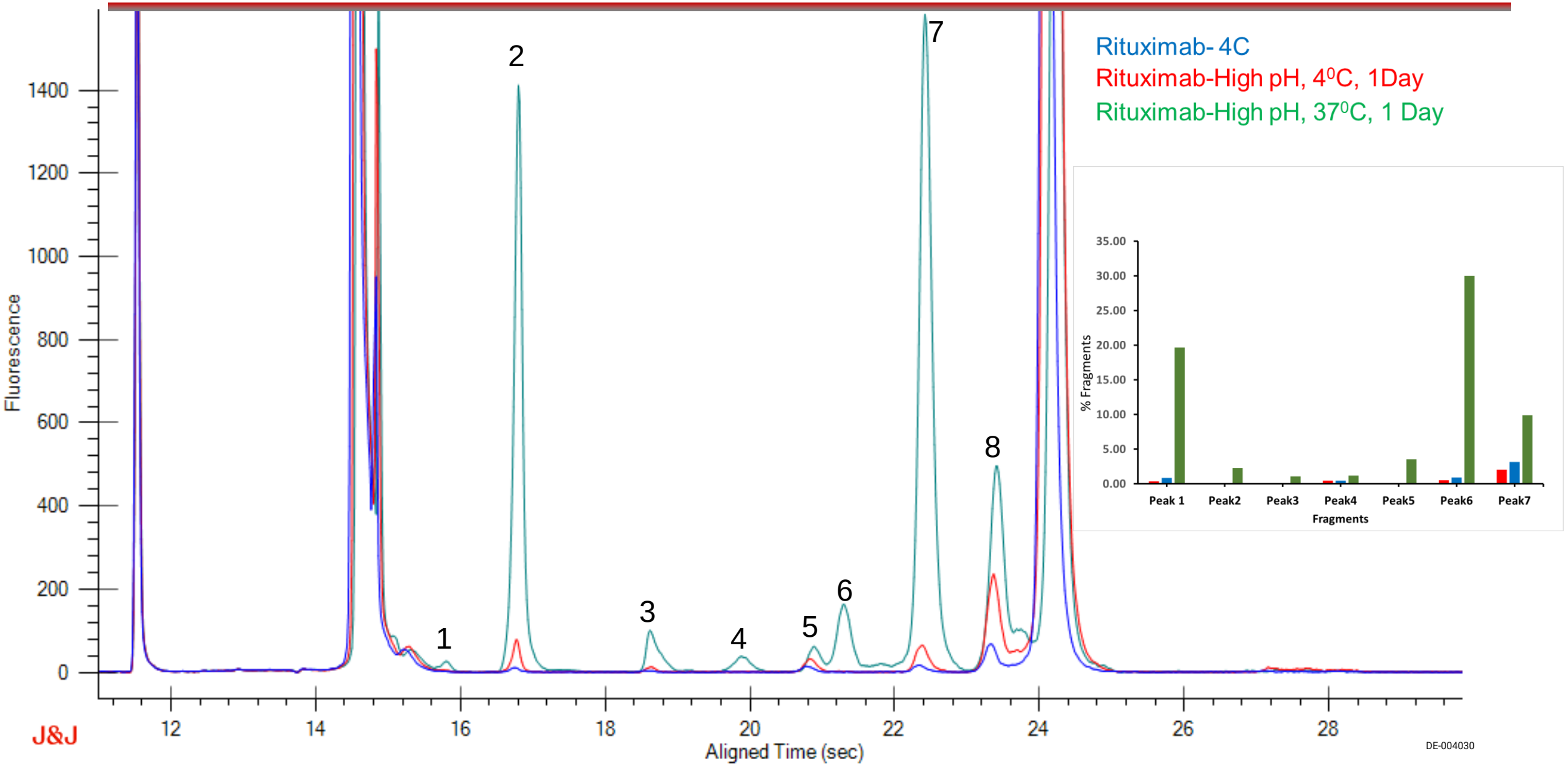
Rituximab IgG1

Rituximab IgG1-NP Clip

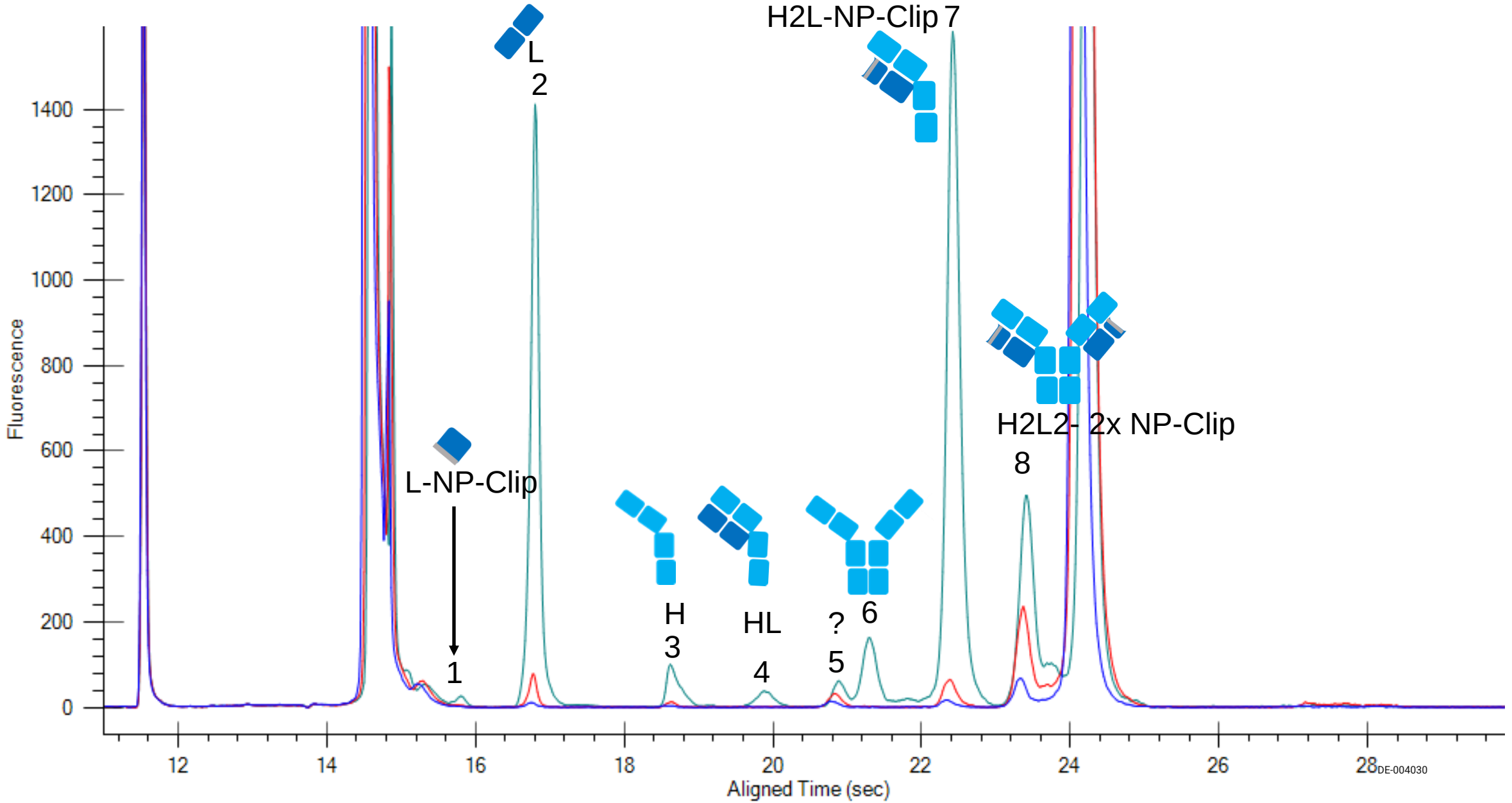
L-NP Clip

Rituximab Light Chain has a NPP motif that is susceptible to NP-cleavage at high pH

Overlayed GXII-FLR Traces of Rituximab pH-Induced Forced Degradation



Identification of Rituximab pH-Induced Degradation Products



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Agilent ExD cell for 6545XT AdvanceBio LC/Q-TOF

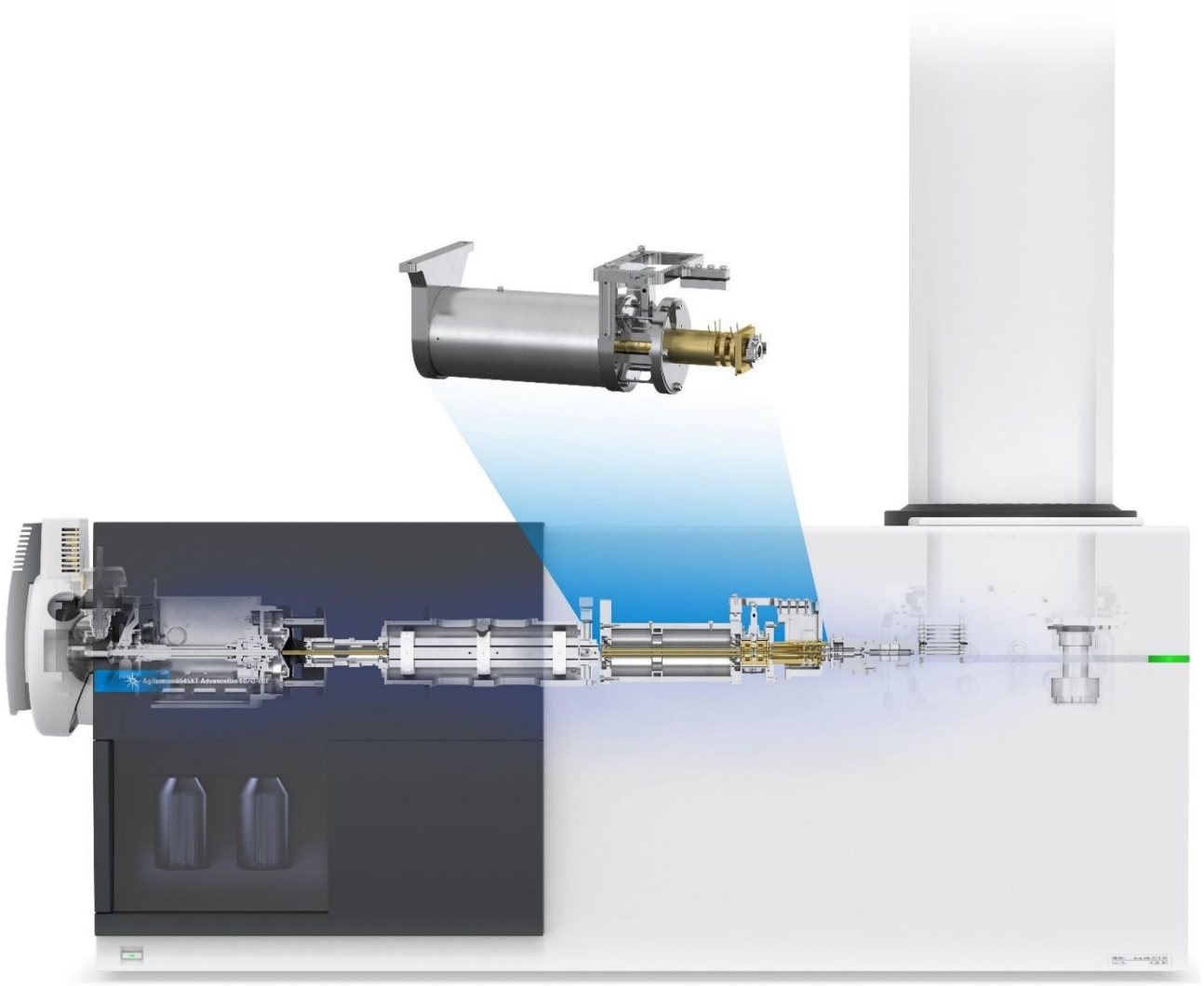
- Key Features

Simplified Operation

Enhanced ECD Efficiency

Synergy with 6545XT

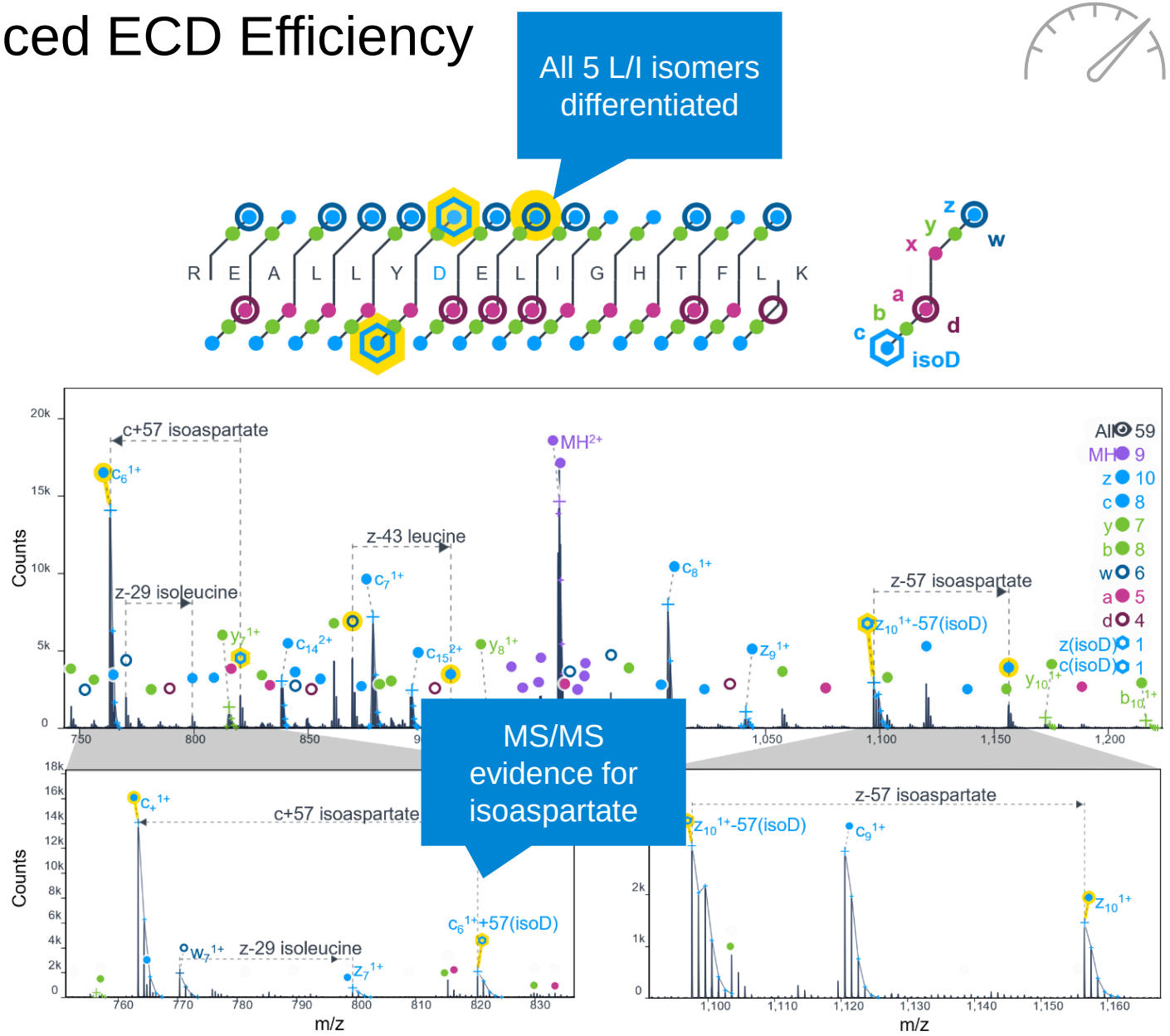
ExDViewer Software



DE-004030

Enhanced ECD Efficiency

- ✓ Greater confidence in results
- ✓ Less time spent fine-tuning
- ✓ Greater applicability for:
  - Lower-abundance/impurities
  - Lower-charge cations
  - Difficult analytes



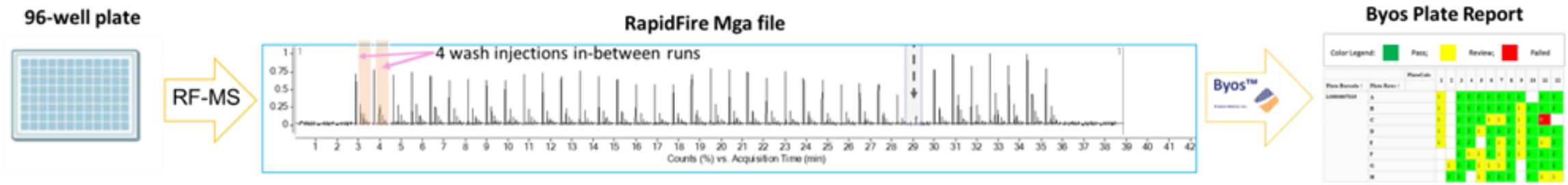
DE-004030

Outline

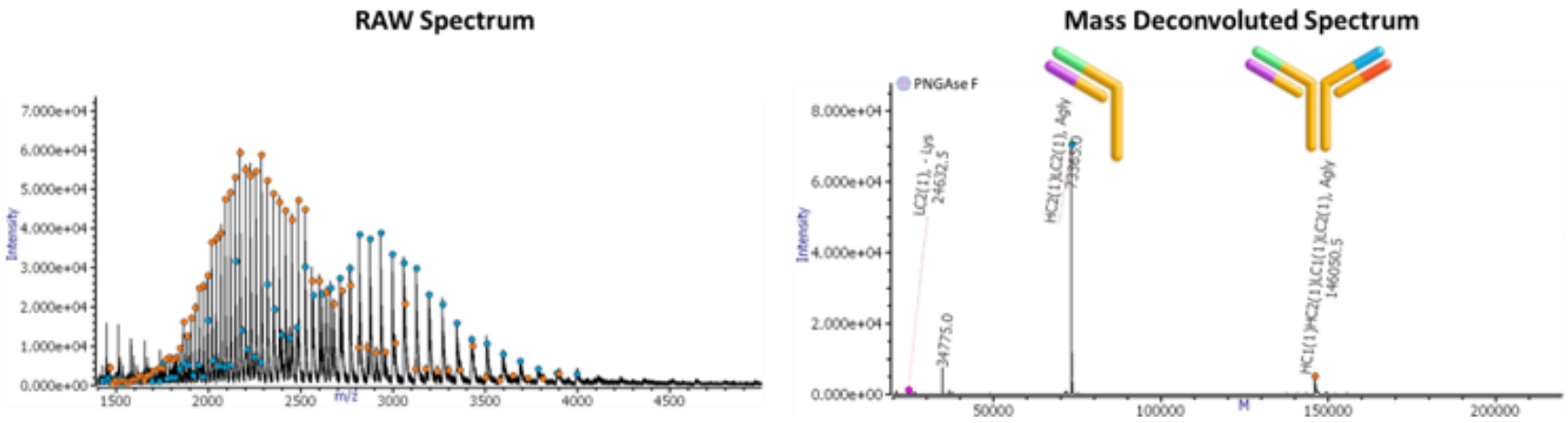
- Need for speed and comprehensive characterization in pharmaceutical R&D
- Part 1: **Proposed peak identification strategy: concepts and experiments**
  - Non-MS assays and peak detection a historical perspective
  - Developing mass/size calibration ruler: A case for forced trisulfide degradation products (that mimic bioreactor H_2S-induced mAb degradation)
  - Cathepsin D- and L-induced mAb clipping to mimic cell culture harvest conditions
  - High-pH Rituximab clipping to mimic structural instability under forced degradation
- Part 2: **MS and non-MS Data Automation to couple Byosphere**
  - Coupling RapidFire data with non-MS assays
  - Data analysis in the Byosphere pipeline
  - Automated analysis and reporting of Impurities
- Conclusions and Future directions

High-throughput MS data generation *via* RpidFire 360-6545XT Q/TOF

A



B



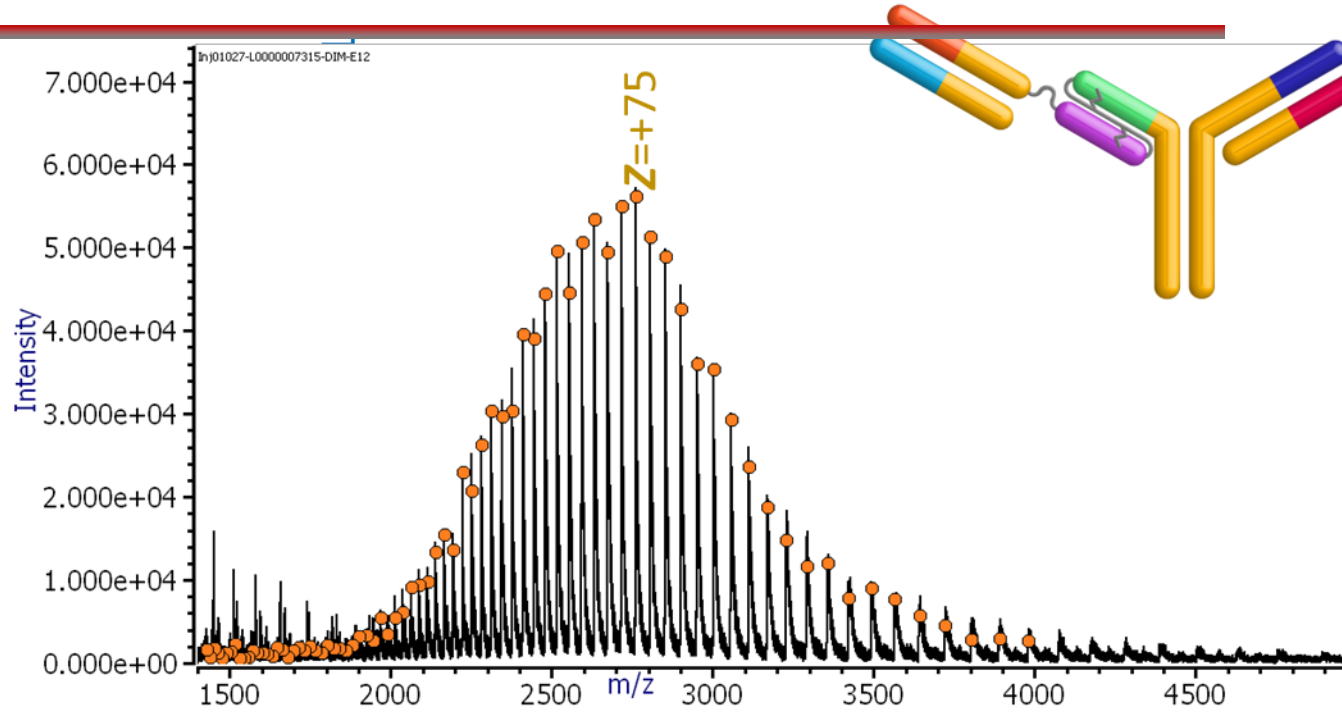
A) 96-well plate of bsAb subjected intact MS analysis via RapidFire-MS, spectral deconvolution and data visualization as Byos plate report

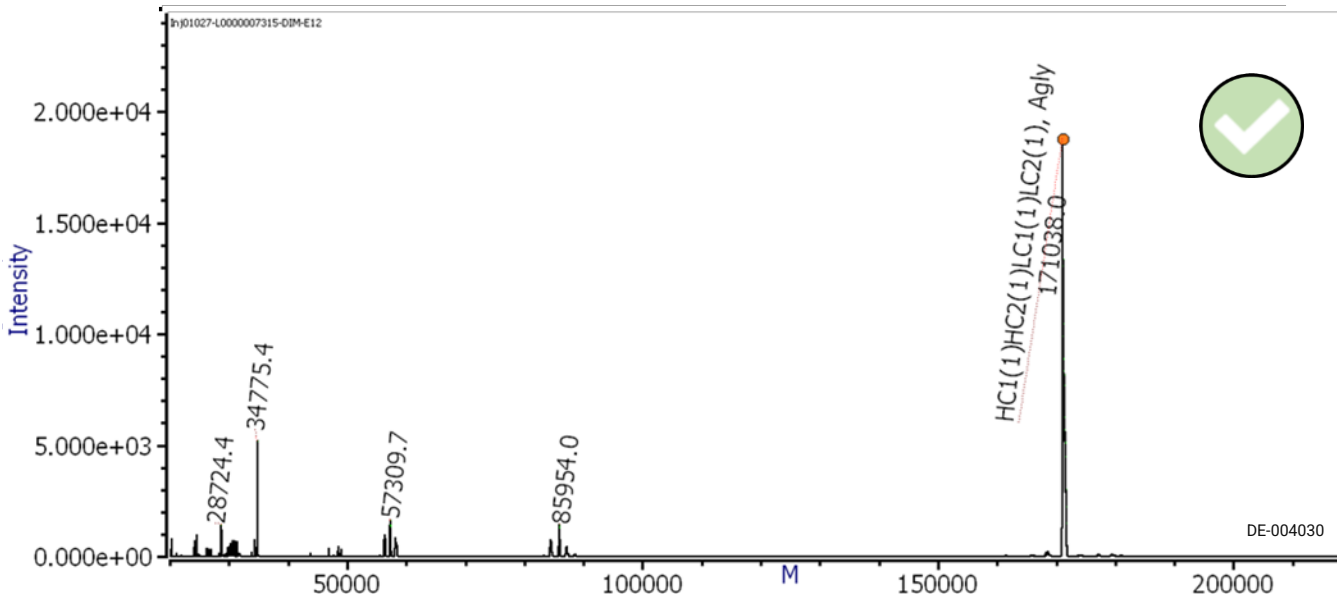
B) Representative RAW mass spectrum and corresponding deconvoluted mass spectrum

Byosphere Traffic Light Reports to Examine RapidFire Plates

Color Legend: Pass; Review; Failed

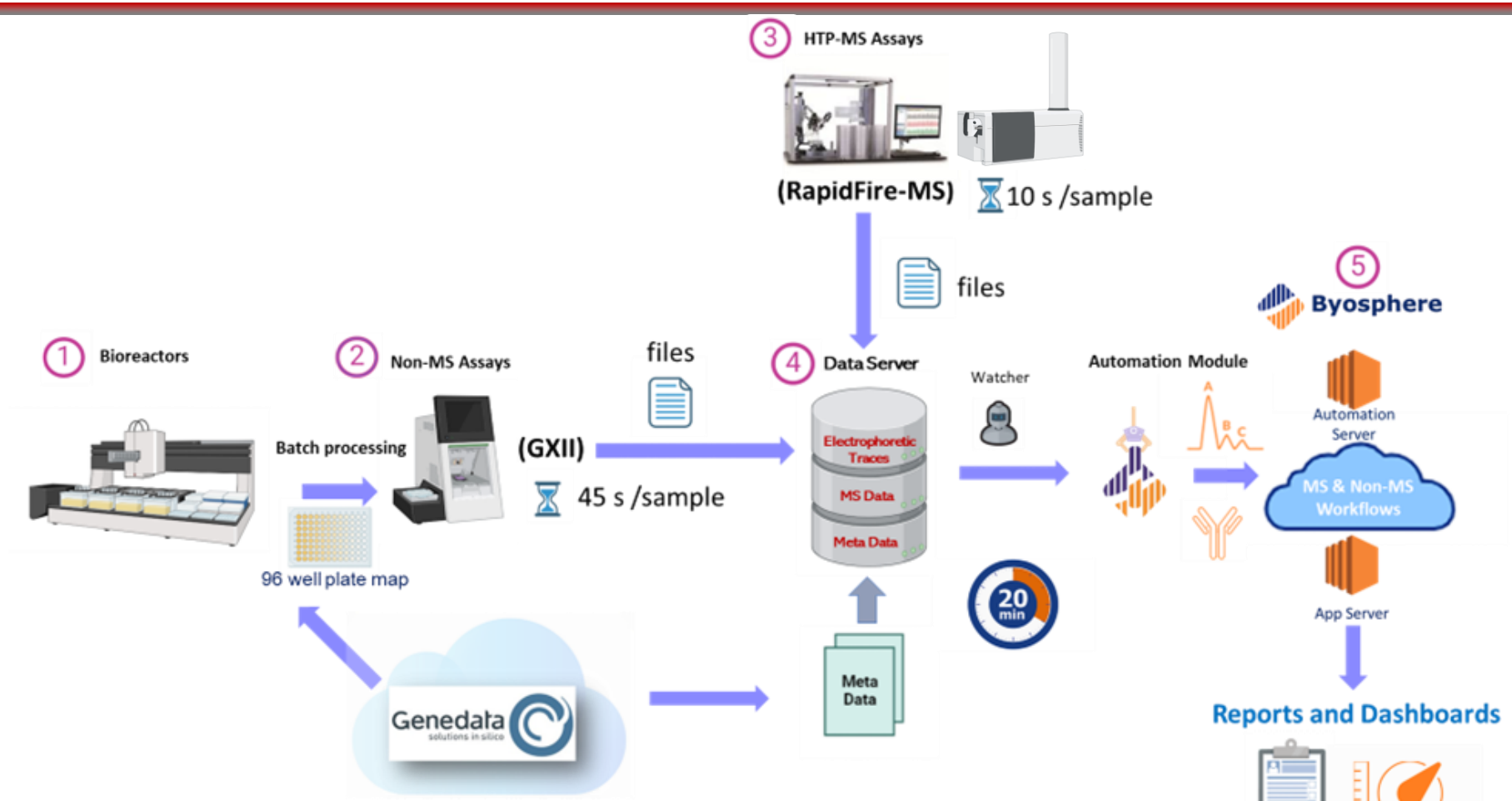
Plate Barcode ↑	Plate Rows ↑	PlateCols 1	2	3	4	5	6	7	8	9	10	11	12
L0000007315	A	1		2	2	2	2	2	2	2		2	2
	B	1		2	2	2	2	2	2	1	2	2	2
	C	1		2	2	2	1	1	2	1	2	0	
	D	1		2	2	1	2	2	2	1	2	2	2
	E	1		2	2		2	1	2	1	2	1	2
	F			2	1	1	2	1	2	1	2	2	2
	G		1	2	2	1	1	1	2		2	2	2
	H		2	2		1	2	2	2		2	1	1





- 96-well plate report for easy visualization of sample wells
- Reports annotated with plate barcodes
- Traffic lights to triage molecules based on identification

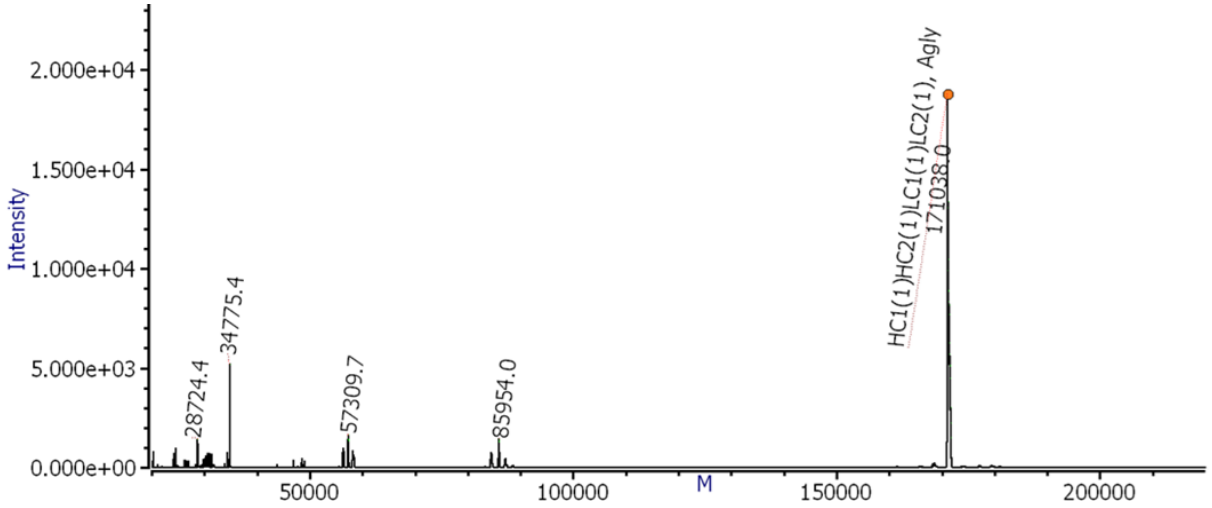
Byosphere Assay Automation of Non-MS with MS

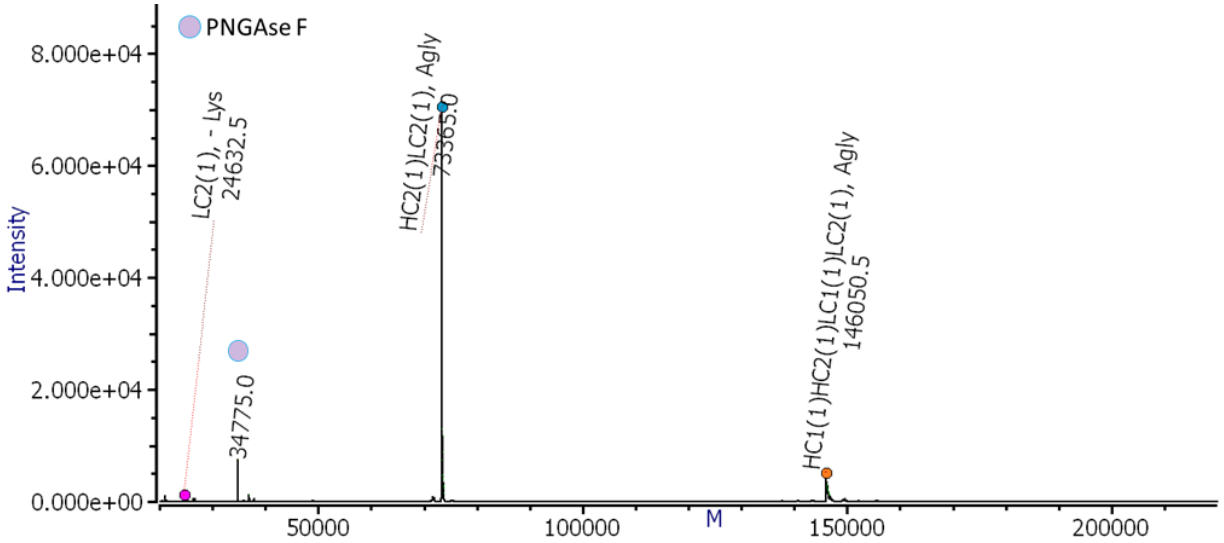


- Byosphere pipeline process both MS and non-MS data (i.e., GXII)
- Workflow processes MS and non-MS workflows and creates projects
- A hands-free automated report generation
- Deep querying capabilities with metadata fields
- Dashboards to monitor QC data

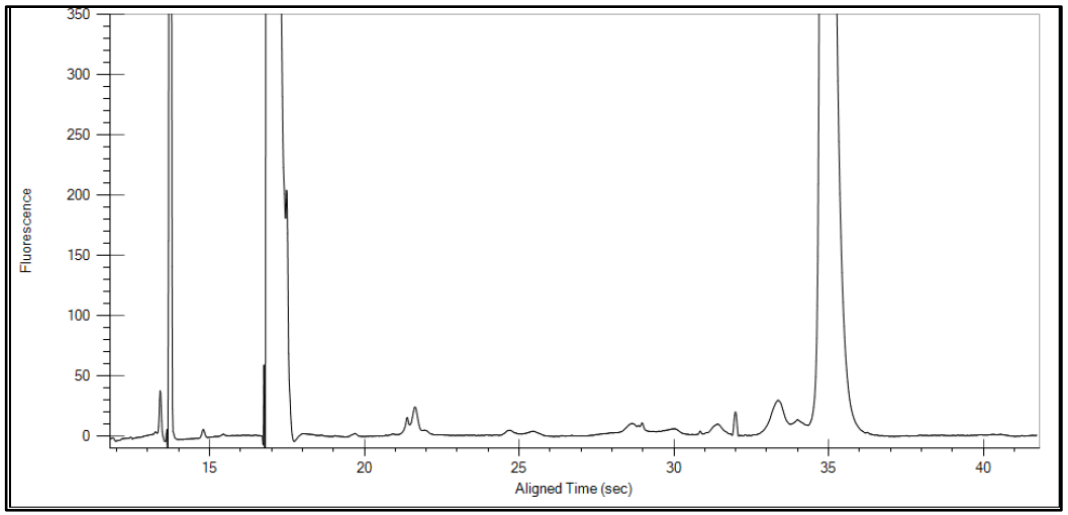
Future Directions: Software Automation for GXII Peak Annotation

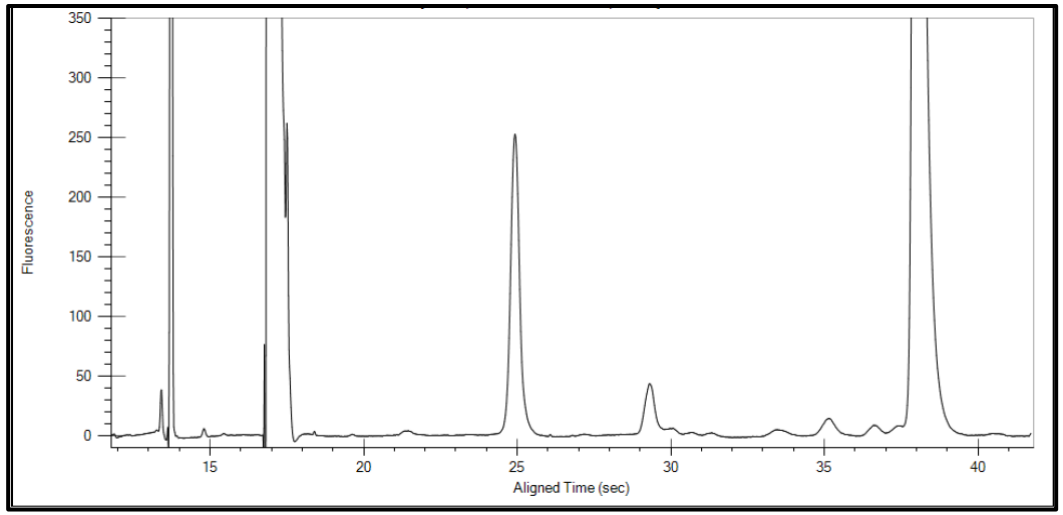
Fast MS (RapidFire–MS)





Fast Electropherogram (i.e., GXII)





DE-004030

Conclusions and Future Directions

- We successfully demonstrate a radical approach to obtain accurate peak identifications for size-based electrophoresis.
- We show the approach and its utility by applying to biopharmaceutical impurities.
- CZE-MS identifications greatly enhance GXII peaks assignment.
- We are currently exploring the integration RapidFire-MS for peak identification of non-MS assays we automated comprehensive impurity library generation.
- We envision that software automation will enable seamless peak identification of non-MS assays.
- Potential to add Alternative Fragmentation ECD - faster ID of unknowns.
- Consider a similar approach with downstream Development - enhance MW accuracy and ID of impurities in QC, updating calibration curves in QC.

J&J

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Biophysics Separations Team

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- Ilker Sen

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