# Comprehensive characterization of an antibody with multiple technologies, CE, iCIEF-MS and novel alternative fragmentation

**Andy Mahan/ Kristen Nields**
Cell Engineering & Early Development– Janssen

T cell lymphocyte with receptors to kill cancer cell in cancer immunotherapy 3D render

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# About our group

- Cell Engineering and Early Development
- Part of the Biopharmaceutical Discovery, Product Development and Supply Organization (BTDS )
- Based in Spring House, PA USA
- 20 miles north of Philadelphia
- Johnson & Johnson, 130 year old company, > 130,000 employees.

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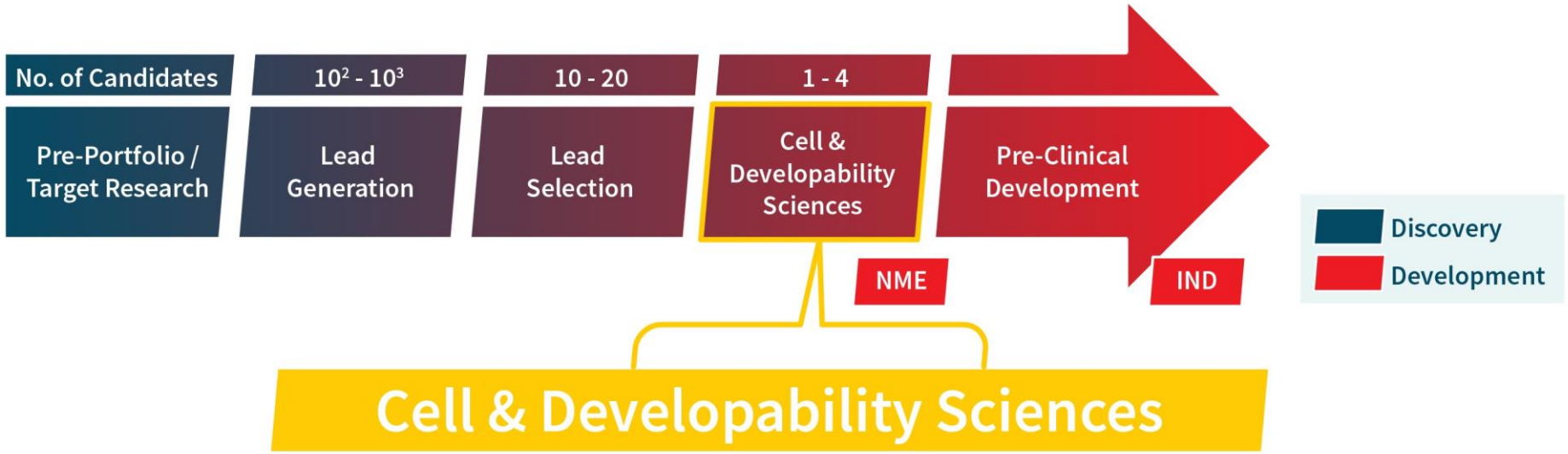
# Steps in large molecule early development




- Bridges discovery and preclinical development
- Delivers the manufacturing cell line for lead candidates

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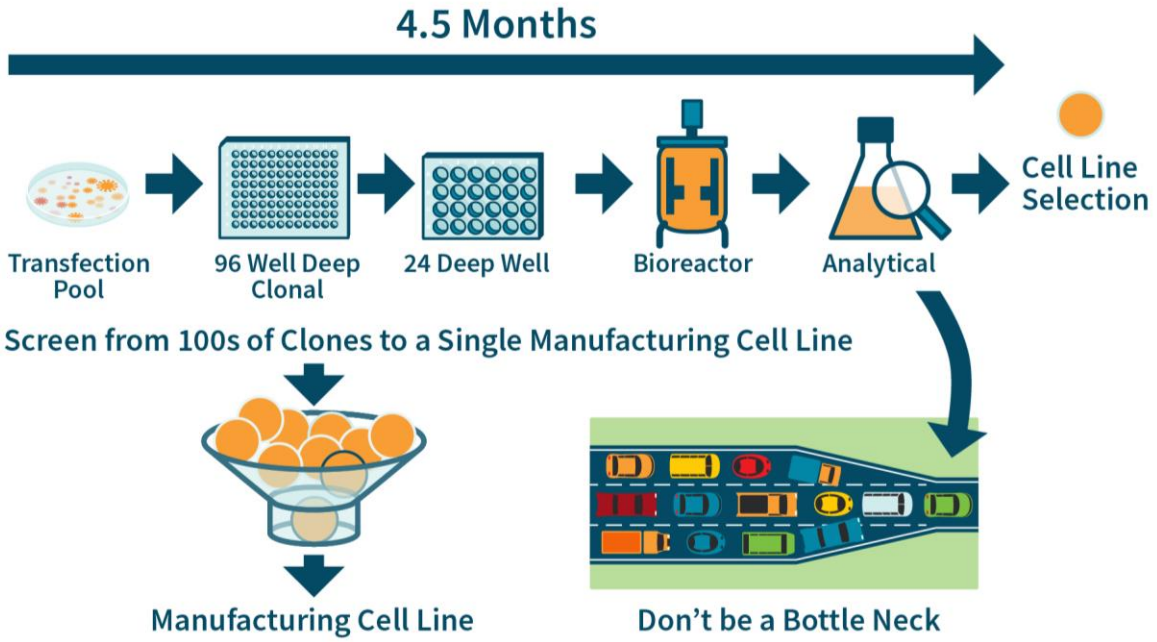
# Cell line development process




| Product quality assessment | |
|---|---|
| HMWS, fragments, %Heterodimer | SEC |
| Purity | GXII, cSDS |
| Charge heterogeneity | cIEF |
| Molecule ID | Intact mass |
| Glycosylation | Reduced intact mass |
| Glycation | Deglycosylated |
| Sequence variants, uSP, clipping, PTMs | Peptide mapping |

- Deliver comprehensive characterization of the lead cell lines
- Shorten timelines and increase bandwidth—*Even for new protein platforms*

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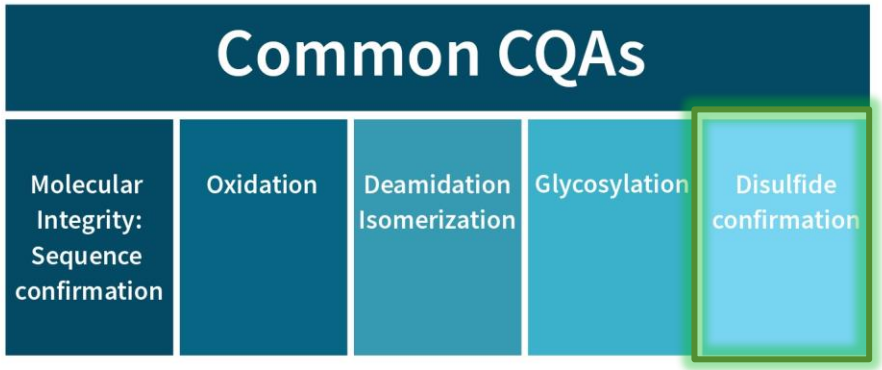
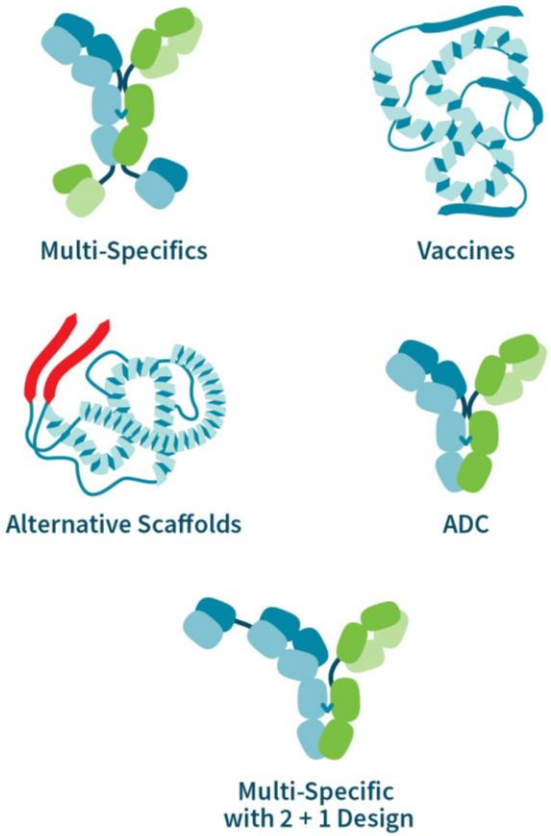
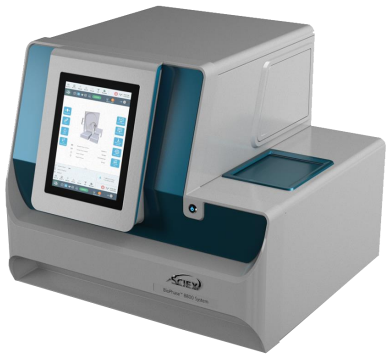
# Challenges of next generation biologics

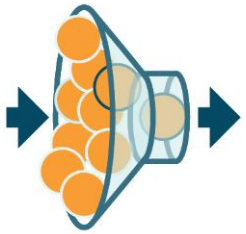
- Mixture of antibodies, multi-specific antibody, vaccines and fusion proteins.
- Lack of correlation between complex charge profiling in cIEF and intact characterization
- Complex 3D structure, and convoluted PTMs bring additional challenges to comprehensive characterization of NGBs.
- Complicated disulfide linkage to maintain 3D structure



# Unified protein therapeutics characterization workflow






**High throughput cIEF screening**

**Detailed intact level peak assignment**

**Thorough peptide map level confirmation of PQAs**

# Charge variant analysis of an antibody

Viral exacerbation at 40x magnification

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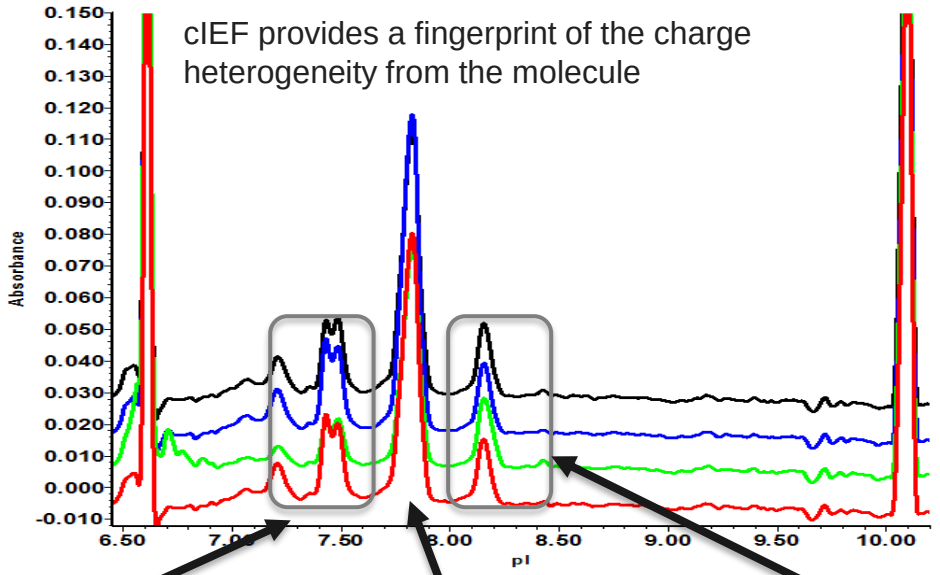
# Critical components in charge analysis




## Acidic variants

Sialic Acid, deamidation, non-classical disulfide linkage, trisulfide bonds, high mannose, glycation, modification by maleuric acid, cysteinylation, reduced disulifde bonds, non reduced species and fragments

## Main peak

Cyclization of the N-terminal glutamine to pyroGlu, removal of the heavy chain C-terminal Lys,

and glycosylation of the conserved asparagine residue in the CH2 domain with neutral oligosaccharides

## Basic variants

**C-terminal Lys**, N-terminal Glu, Isomerization of Asp, Succinimide, Met oxidation, Amidation, Incomplete disulfide bonds, incomplete removal of leader sequence, mutations from Ser to Arg, Aglycosylation, fragments and aggregates

Mabs 2012

Yi Du, Alison Walsh, Robin Ehrick, Wei Xu, Kimberly May, and Hongcheng Liu

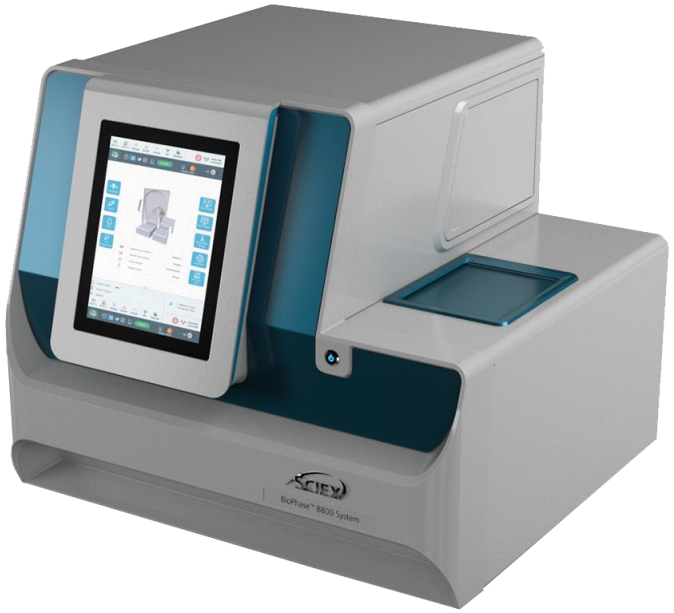
Merck Research Laboratories; Union, NJ USA

# BioPhase 8800 system

***The SCIEX BioPhase 8800 system gives you the agility to analyze up to 8 samples simultaneously, with automated capacity of up to 96 samples.***

## Robustness

Hardware and software designed for high repeatability and uptime.




## Flexibility

Flexible for your workflow requirement, switching between UV and LIF detection is simple and seamless. And Integrated detection modules.

## Software

New workflow-oriented software design for touch screen and direct control.

## Compatibility

96 well plates are designed to ANSI/SLAS standards and are conveniently compatible with commercial liquid handling systems.

## Pre-assembled reagent/consumables

Simplifies operation and minimizes user error

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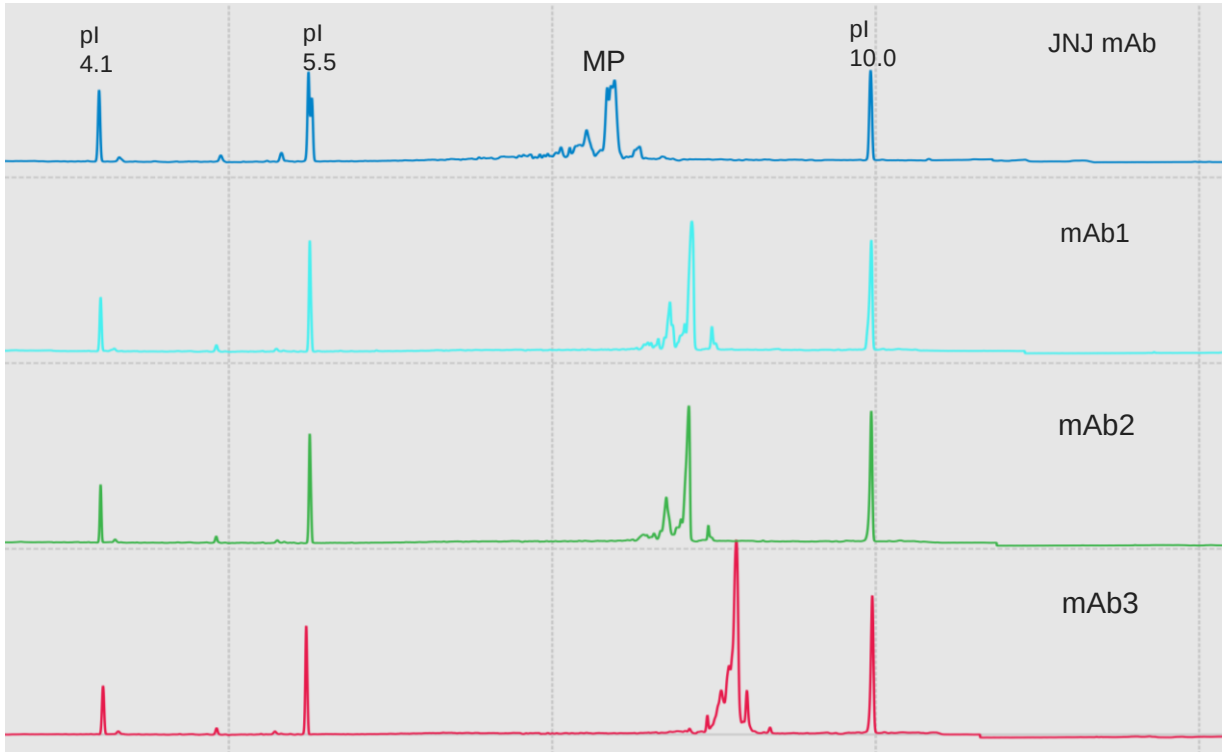
# Multiplexed cIEF analysis using BioPhase 8800 system




8 parallel capillaries allow for rapid screening of multiple protein therapeutics for charge heterogeneity

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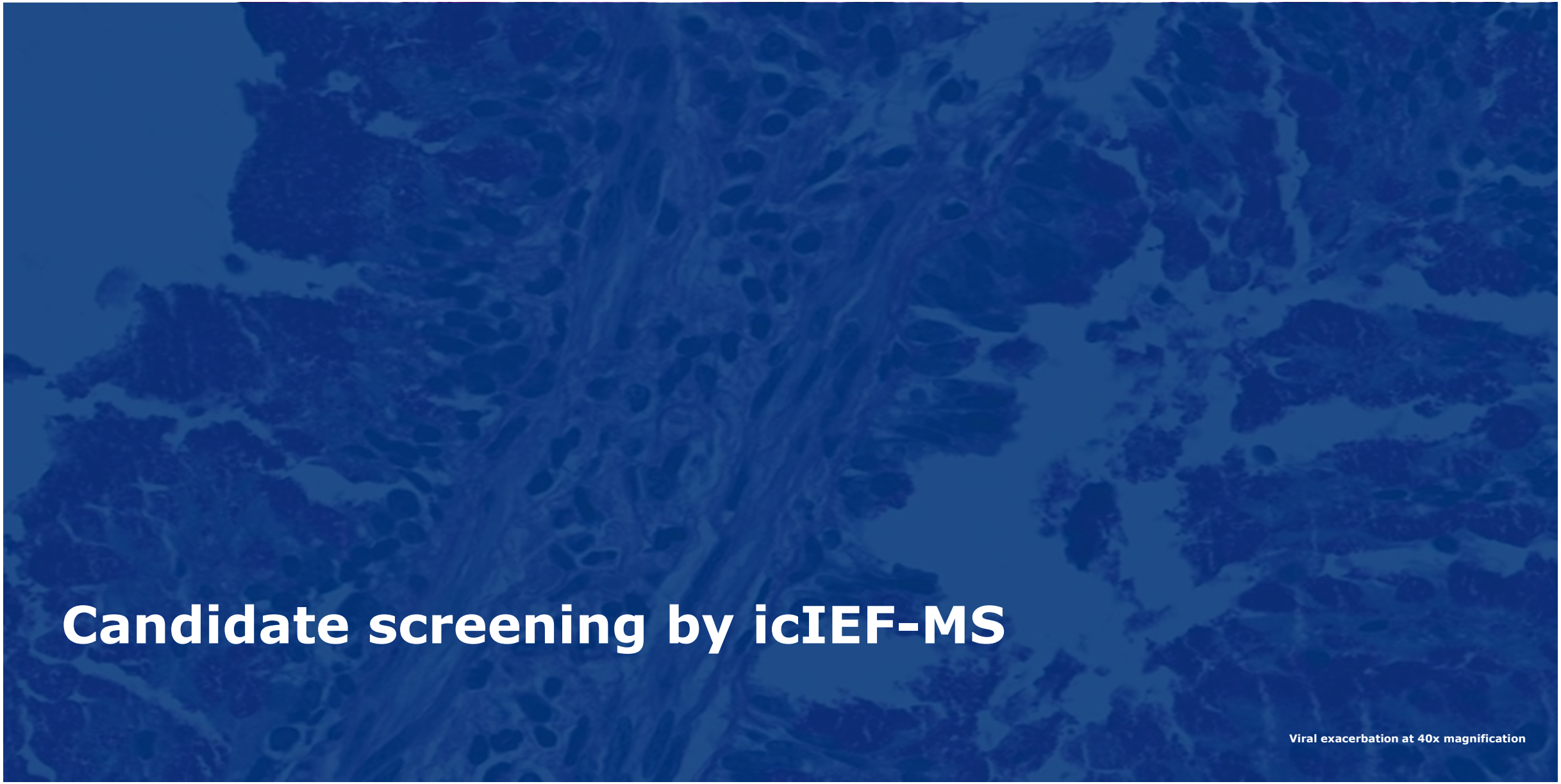
# Candidate screening by icIEF-MS



Viral exacerbation at 40x magnification

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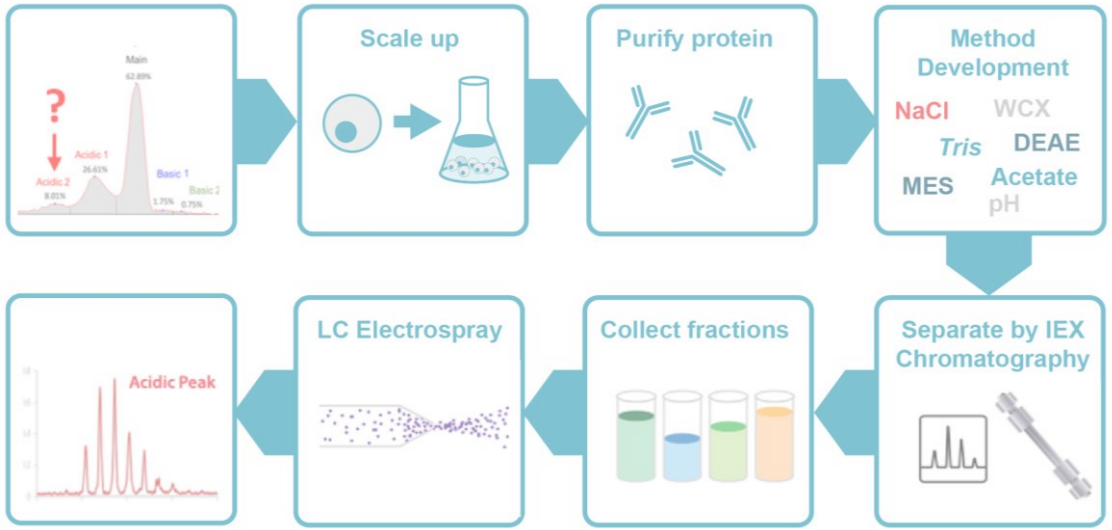
# IntaBio icIEF-MS System: Rapid, comprehensive icIEF peak identification by MS

***Once an interesting profile is identified through high throughput screening with the BioPhase 8800 system, the identification of the separated peaks is required.***

## Current workflow – time consuming




## 30 minutes per sample

**IntaBio icIEF-MS system streamlines and accelerates product quality analysis**

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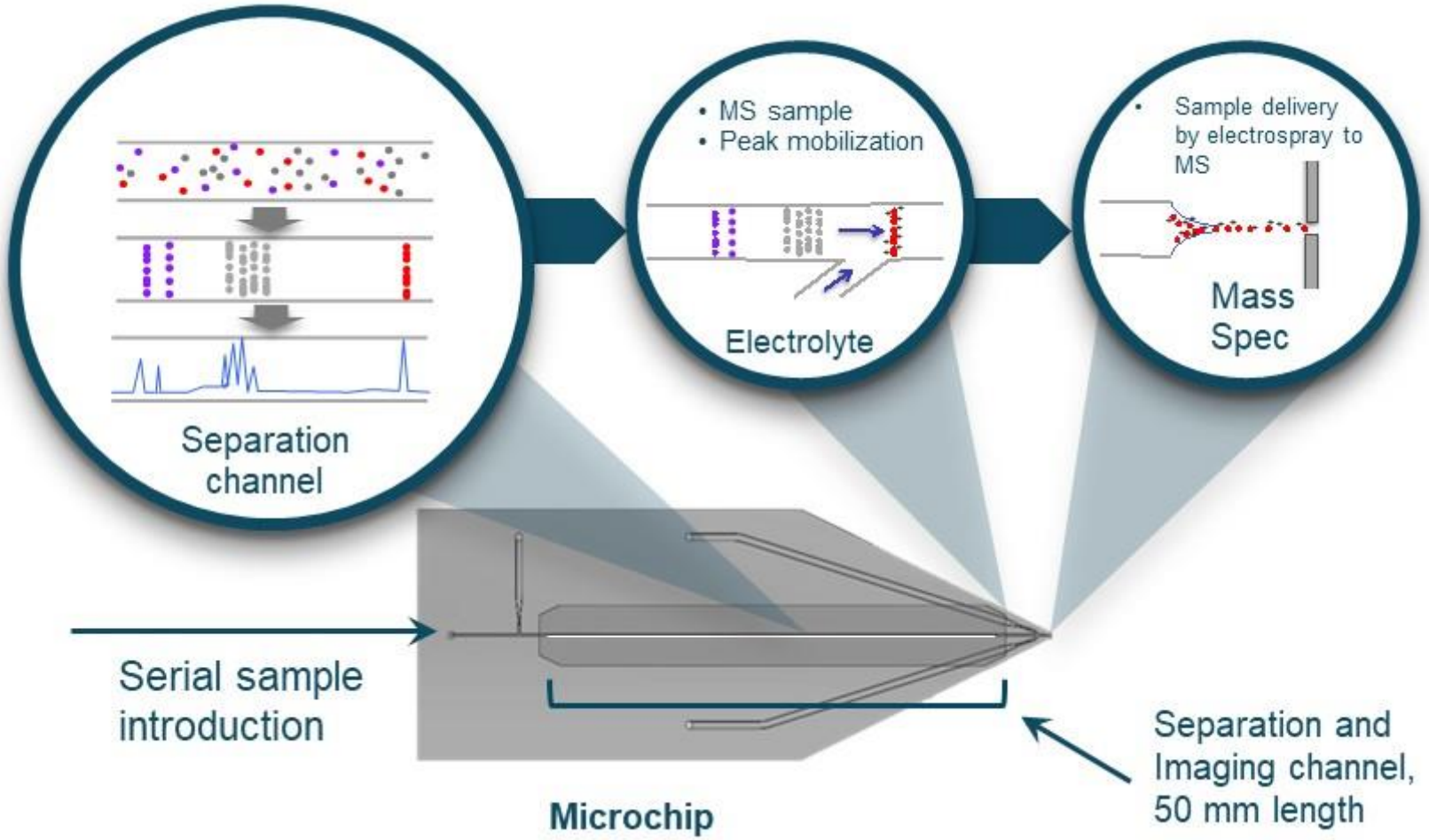
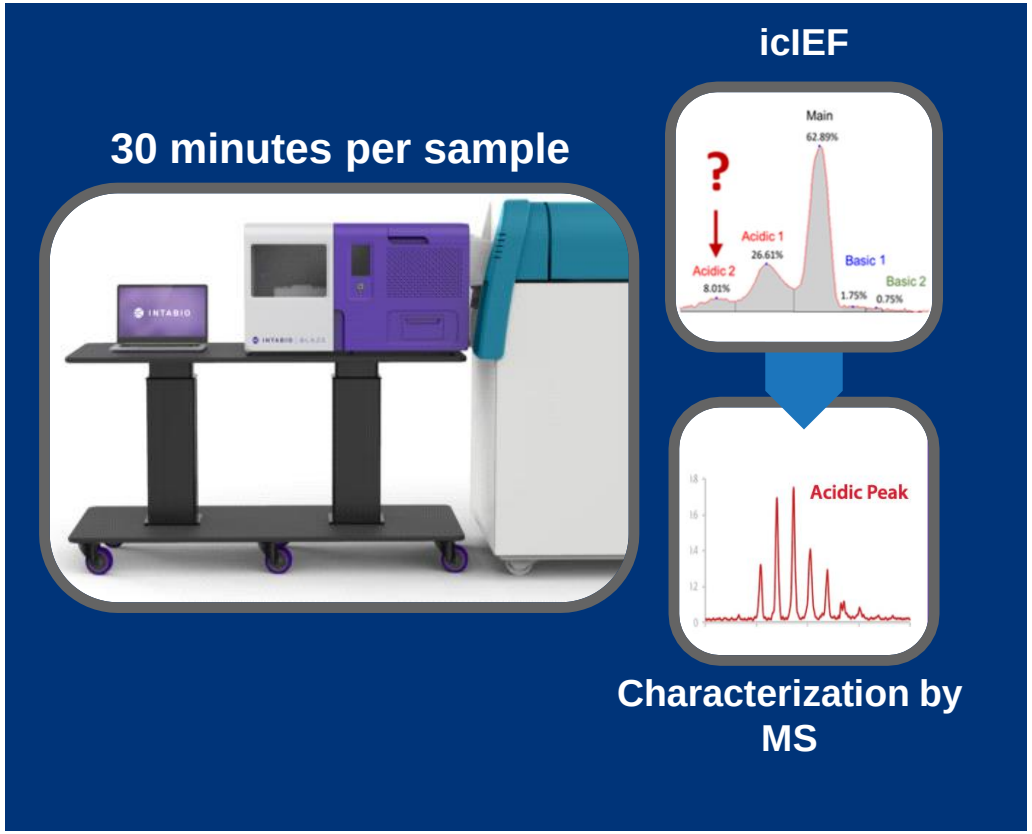
# Integrated cartridge for high-resolution icIEF and MS



# IntaBio icIEF-MS system technology




Detailed characterization of charge variants, including:

- pI
- UV quantitation
- Mass and PTMs by MS
- N-linked glycan pairs

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# Lead candidate selection in early biotherapeutic development

## Analytical challenge:

- Established icIEF UV method showed unusual “split-peak” profile
- Identification of split peaks unknown

## Goals of icIEF-MS analysis

- Reproduce previous iCE 3 results
- Mass analysis and charge variant identification
- Determine source of peak splitting
- Evaluate the suitability of clone candidates for production

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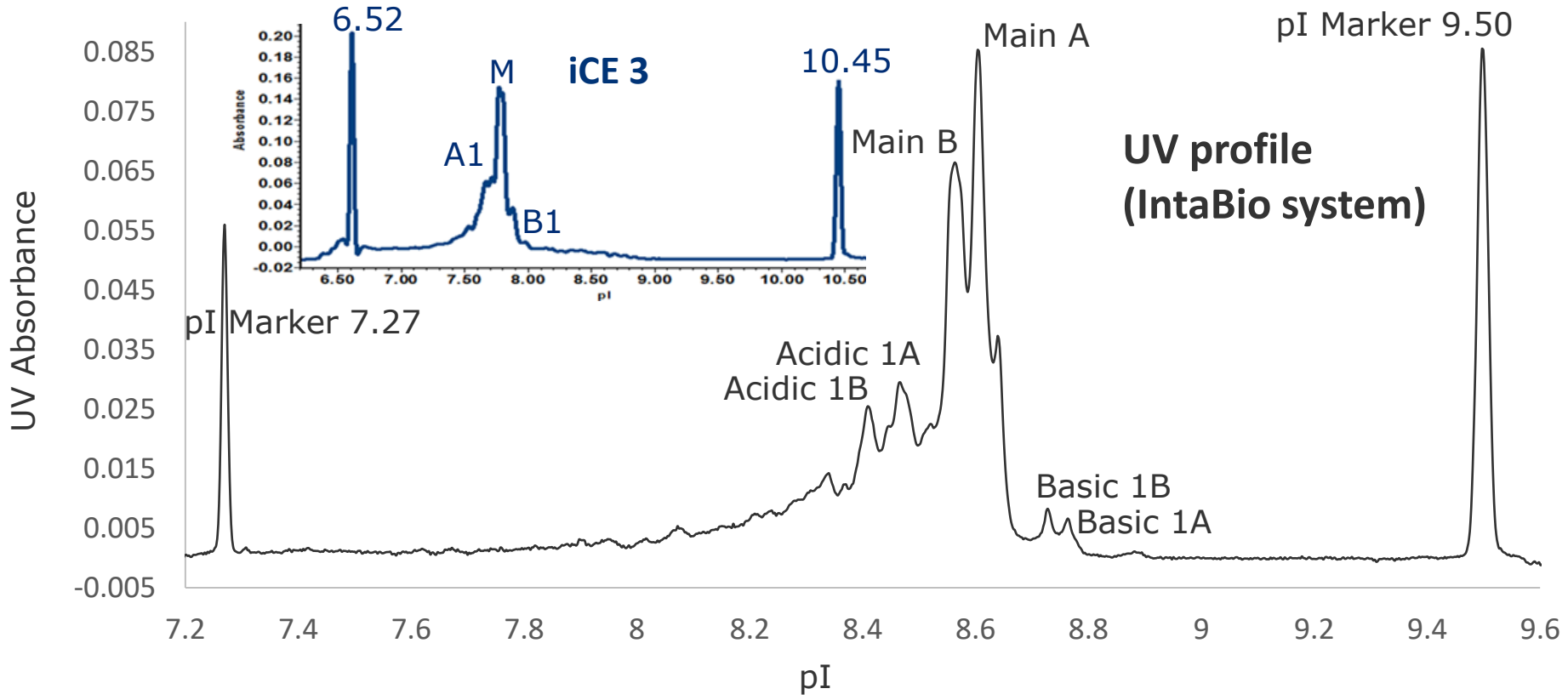
# Charge profile – IntaBio system better resolves split peaks by icIEF-UV

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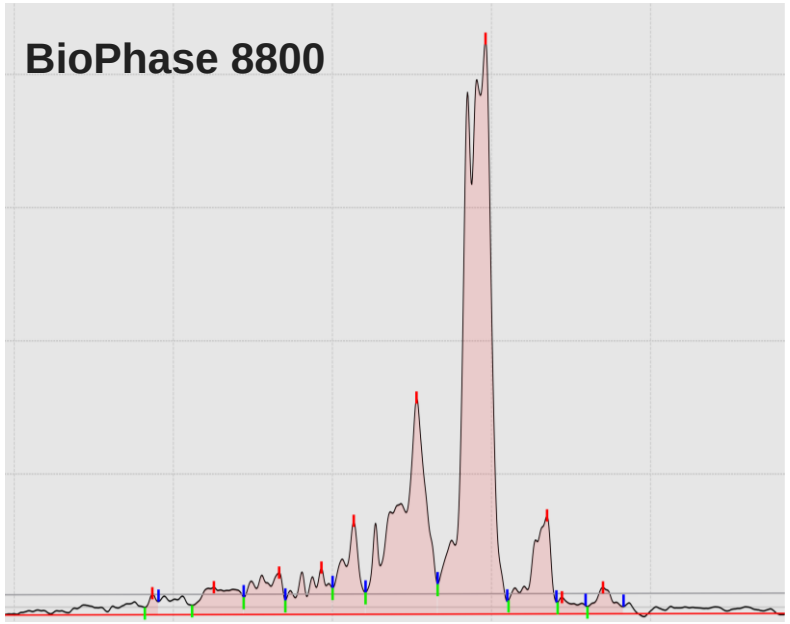
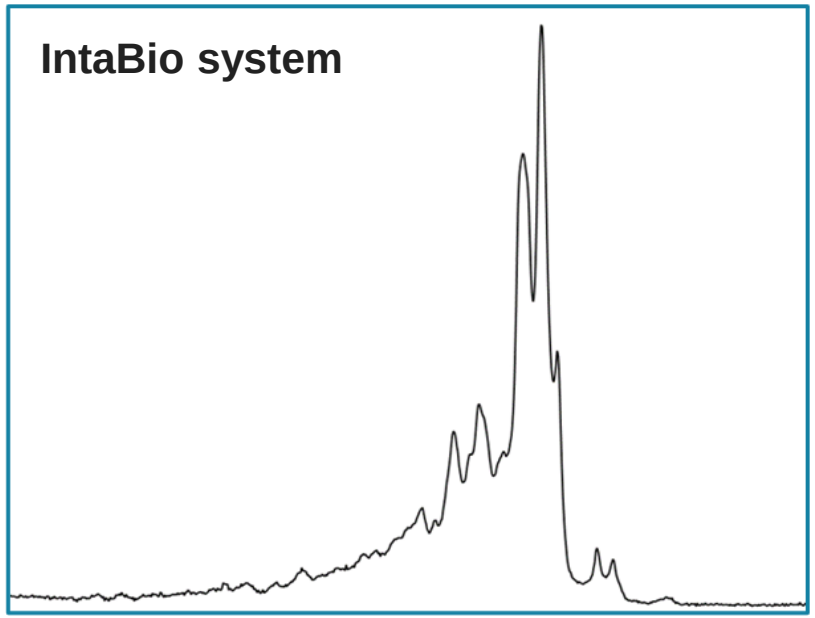
# Comparison of BioPhase 8800 system and IntaBio system




pI




pI

**Separation profile conserved between both techniques**

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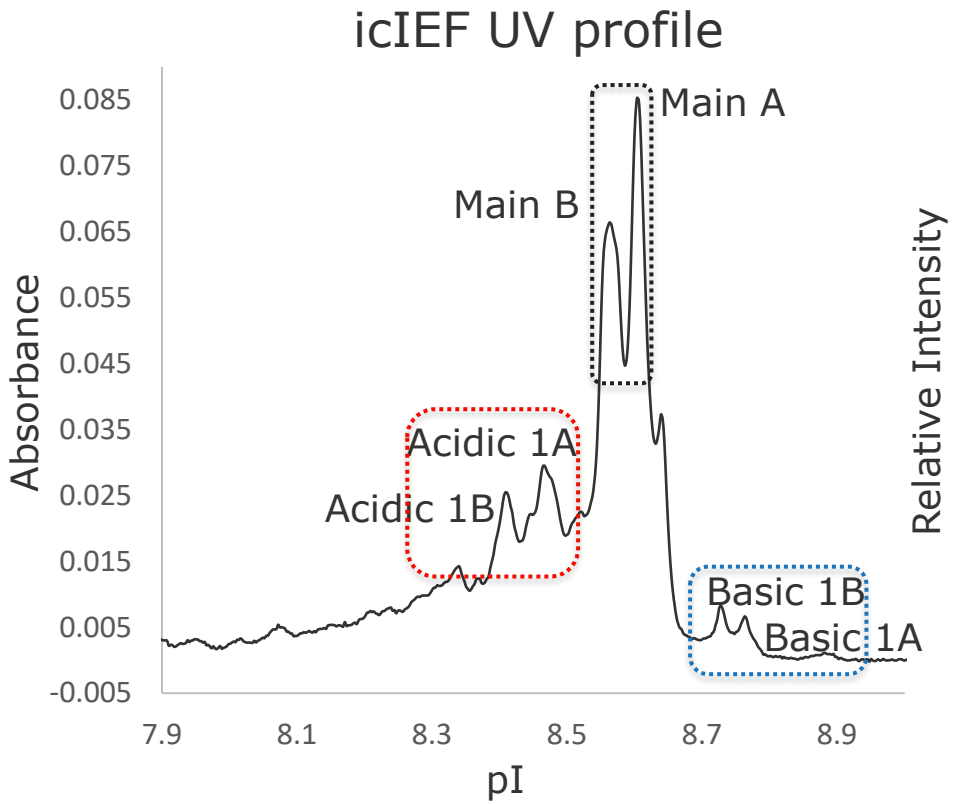
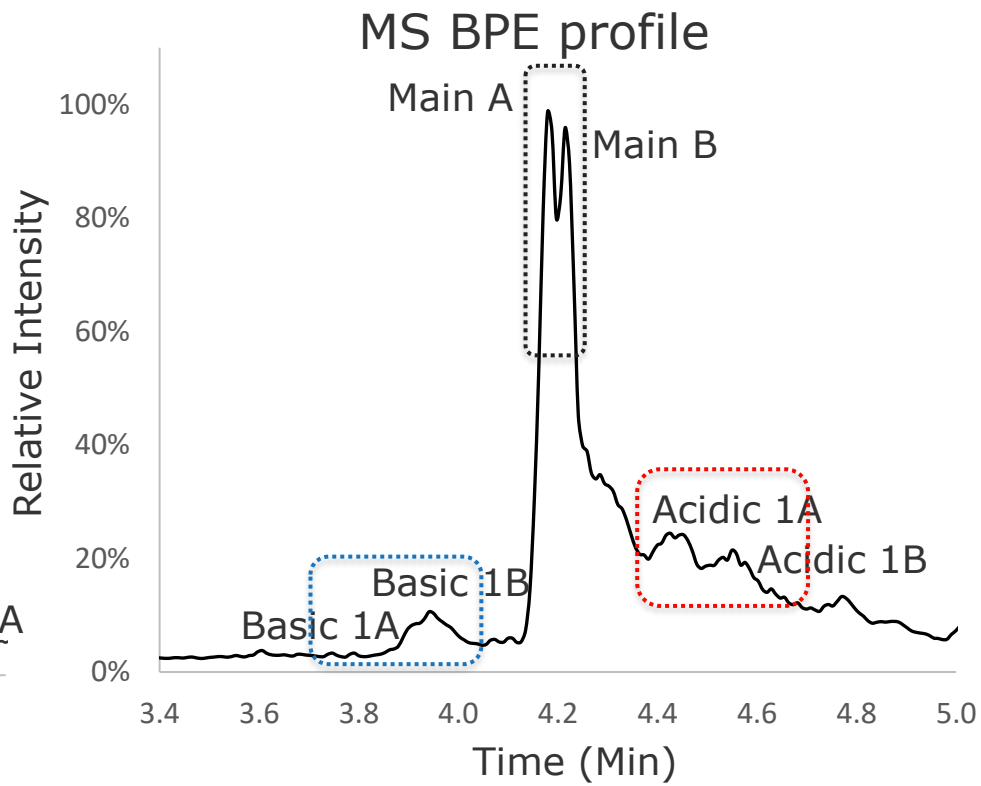
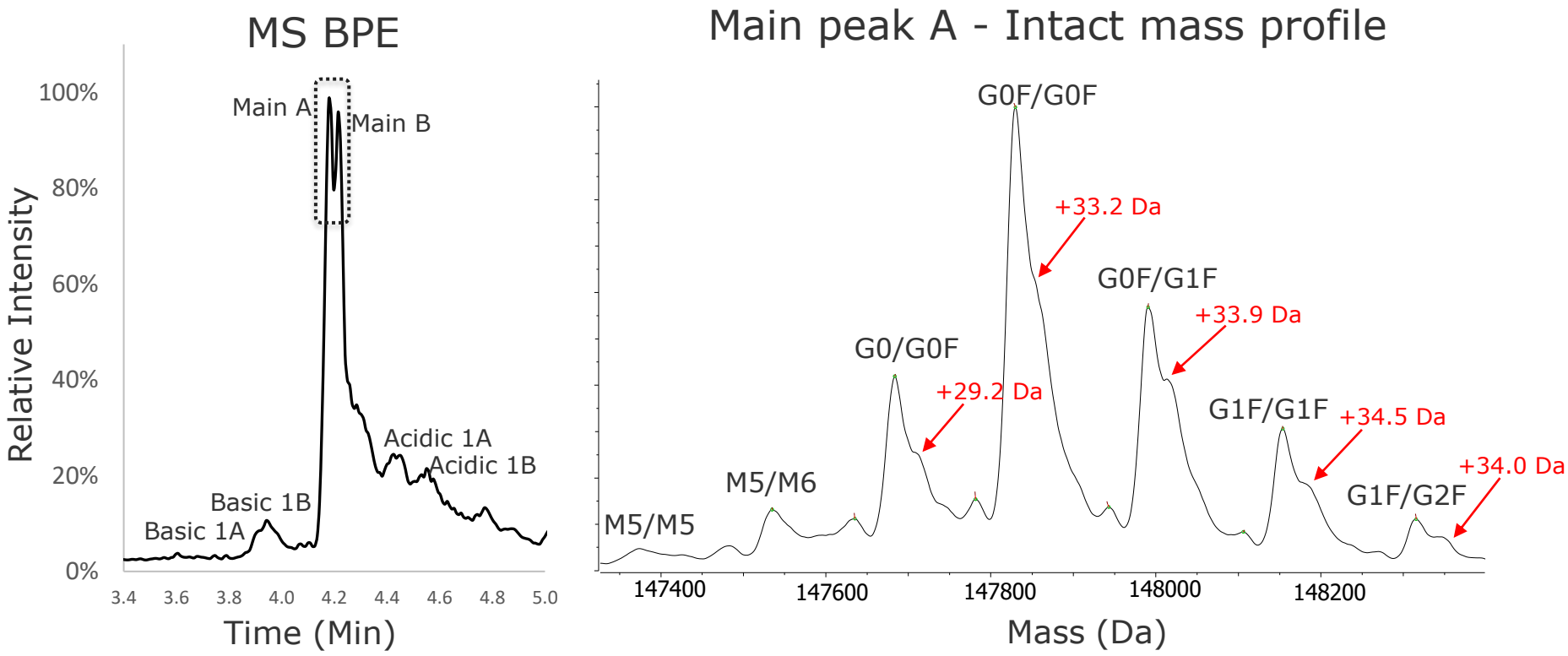
# Split peaks resolved in both UV and MS

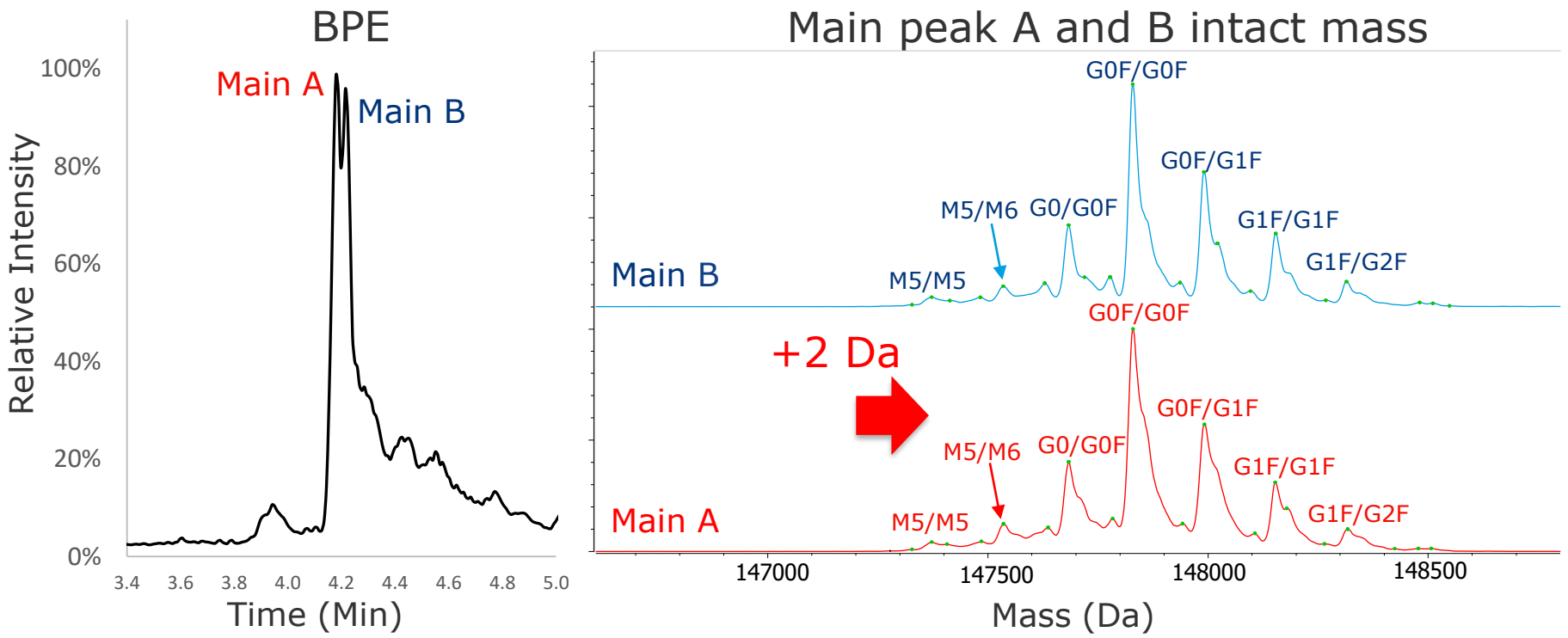


# Potential trisulfide bonds



# Potential free thiol groups



# EAD sheds new light on disulfides and trisulfides in an antibody

Viral exacerbation at 40x magnification

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# SCIEX ZenoTOF 7600 system

**Overcome duty cycle deficiencies**

>90% ions injected into the TOF

**Sensitivity gains of up to 5-20X**

Clearer unambiguous MS/MS spectral data

**Tuneable fragmentation of all molecule types**

Utilize controlled electron activated dissociation (EAD)

**MS/MS scan rates of up to 133Hz**

Improved DDA and High-resolution MRM ($MRM^{HR}$)

**Wide intra- and inter-scan dynamic range (LDR)**


ZenoTOF 7600 system

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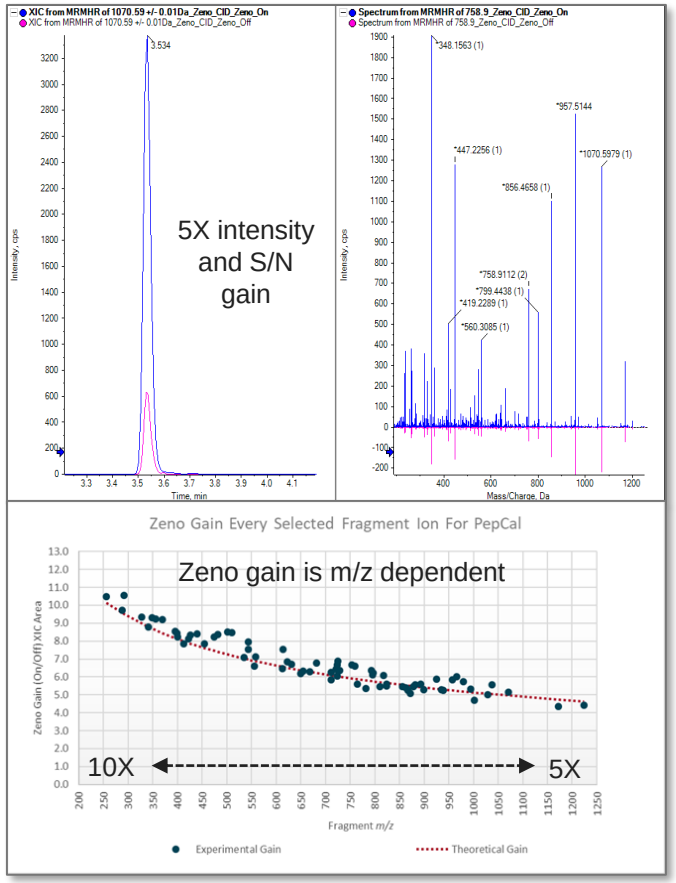
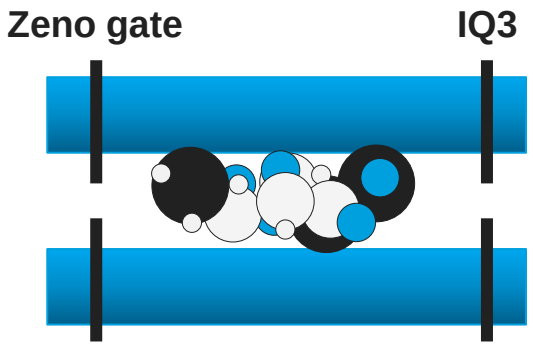
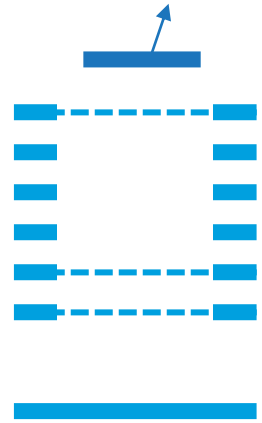
# SCIEX ZenoTOF 7600 system with Zeno trap

## The Zeno trap provides 5 to 10X sensitivity gains for MS/MS and MRM$^{HR}$ acquisition scan modes



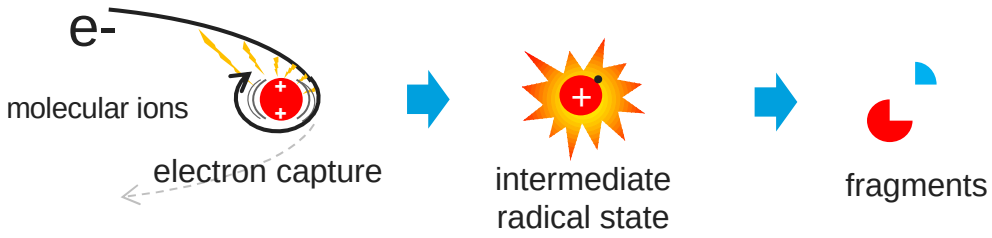




**Quadrupole region**
Continuous ion beam



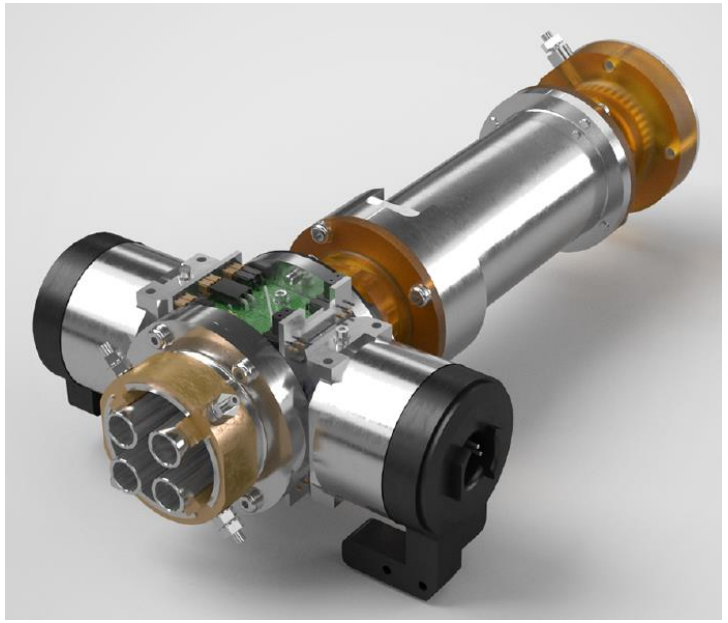
**TOF accelerator**
Ion pulsing

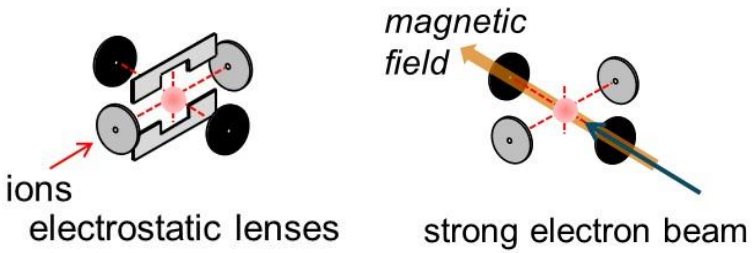
# Next generation alternative fragmentation




## Generic peptide map EAD IDA method

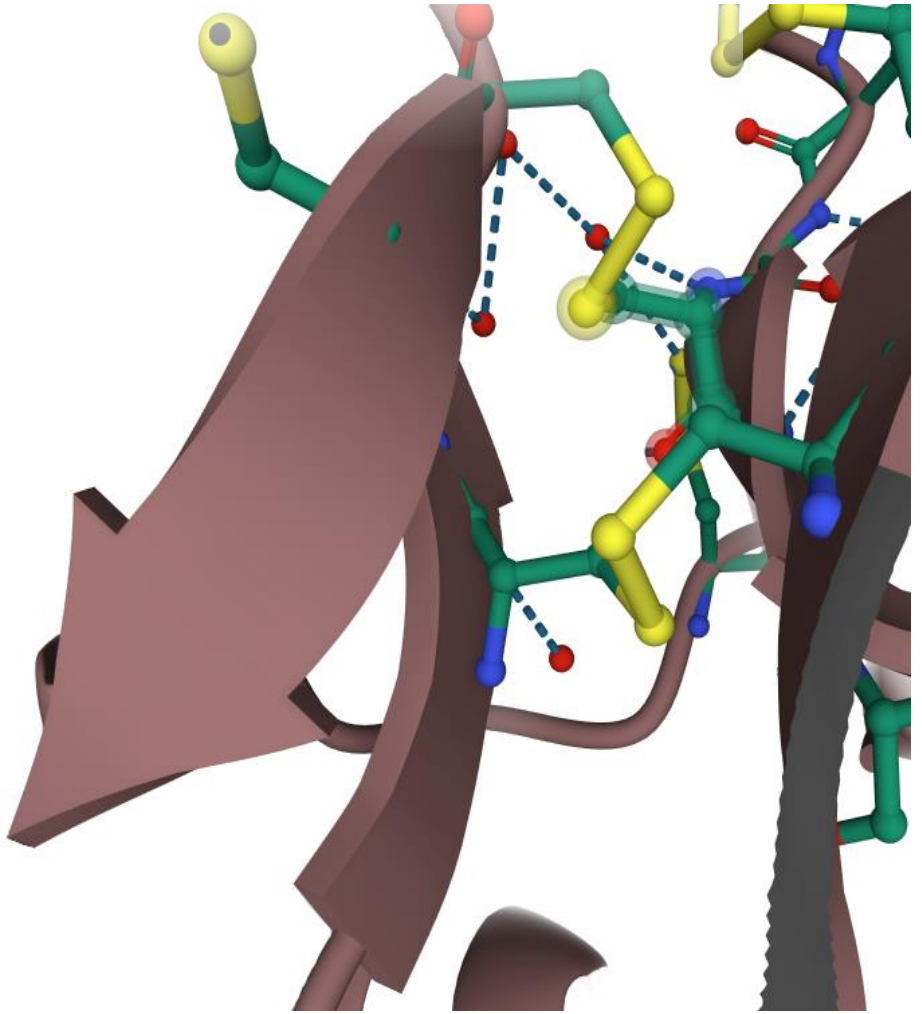
- Generic IDA method premade to cover peptide map analysis over wide range of PTMs and peptide size
- Optimized for peptide backbone fragmentation preserving side chain information (b, c, y, z + w ions)
- Confirms AA isomers
- Excellent MS/MS for all charge states in one experiment
- Quantitative performance like CID
- Zeno trap sensitivity gain for CID and EAD MS/MS
- Up to 20 Hz IDA EAD on LC time scale





# Challenges of disulfide analysis

- Disulfide linkages do not fragment routinely by CID
- This prevents complete sequencing – especially for amino acids close to the linkage
- Therapeutics modalities are becoming more complex in regard to PTMs, AA isomers and Disulfide bonds
- Disulfide peptides can be complex and ambiguines to assign with traditional ExD & CID



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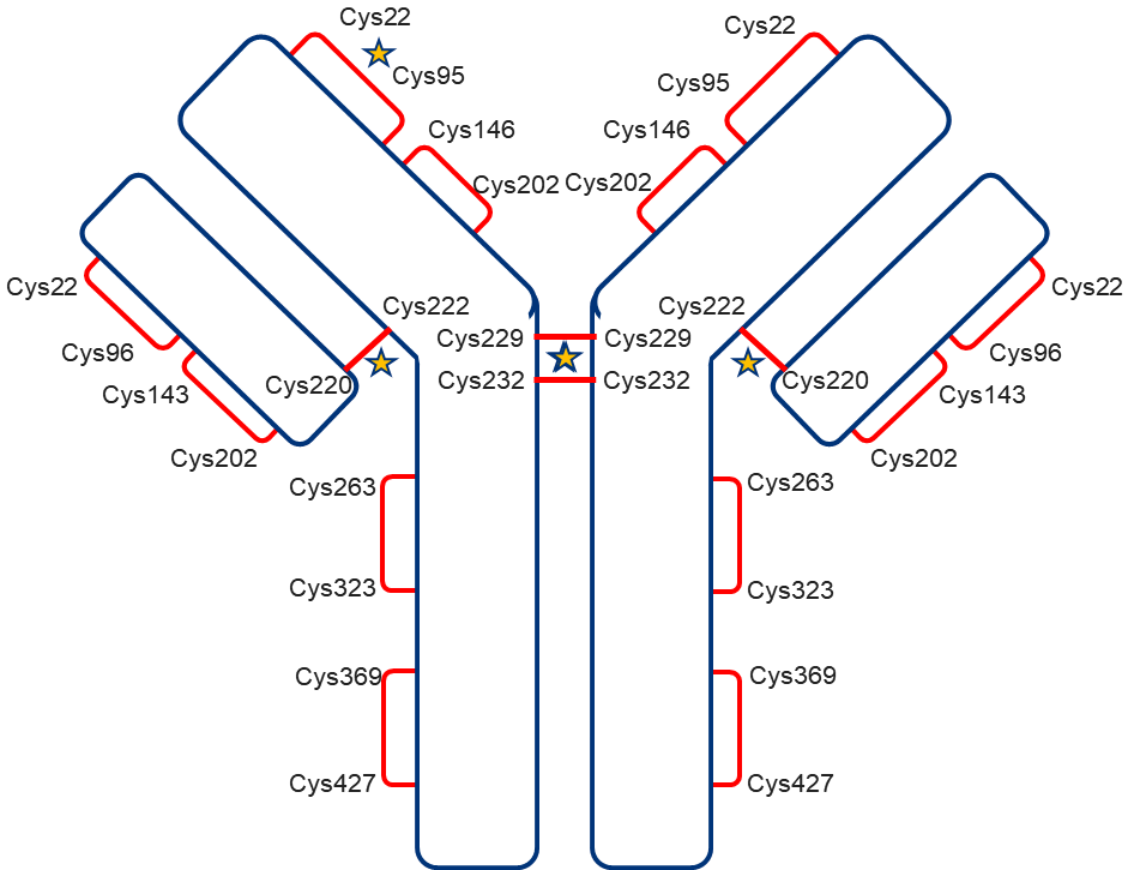
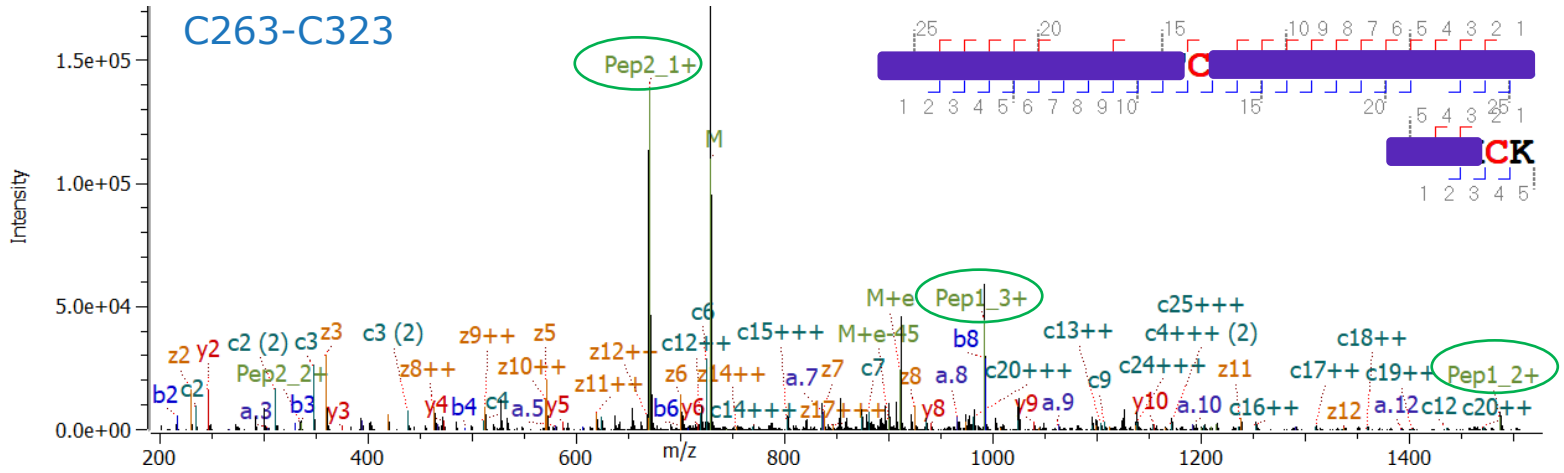
# Expected thiol isoforms of an antibody T-cell redirector

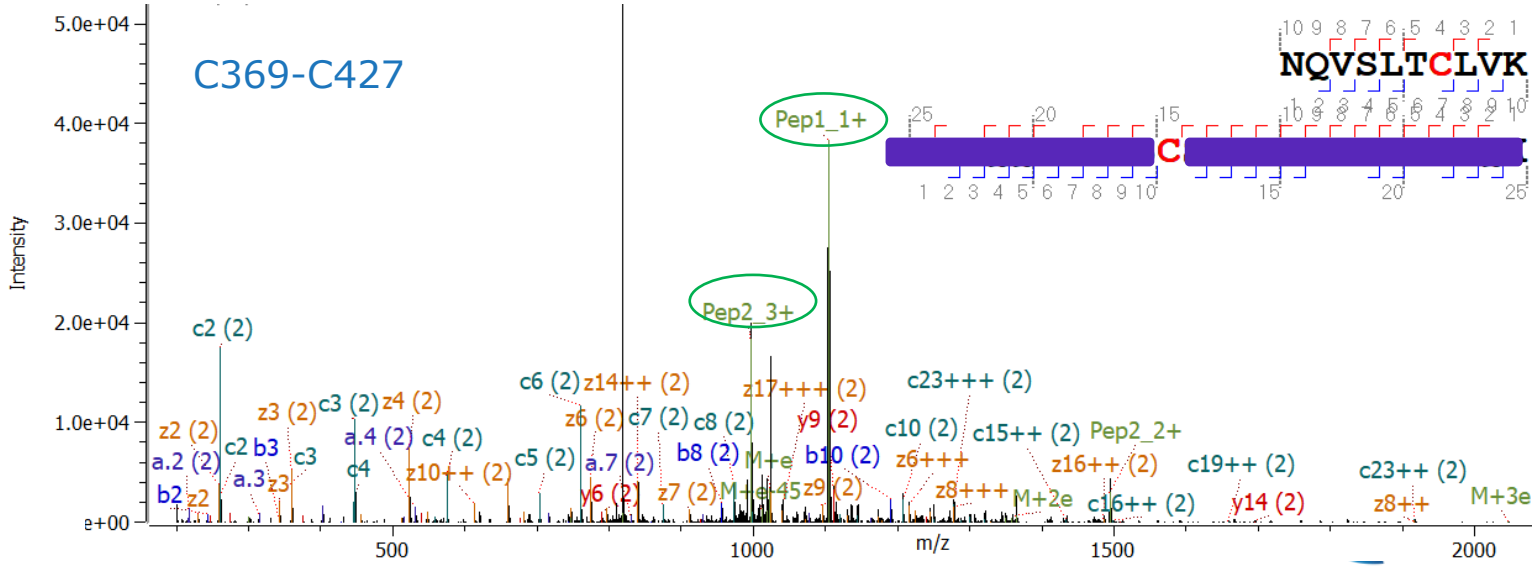


# Disulfide analysis- expected C263-C323

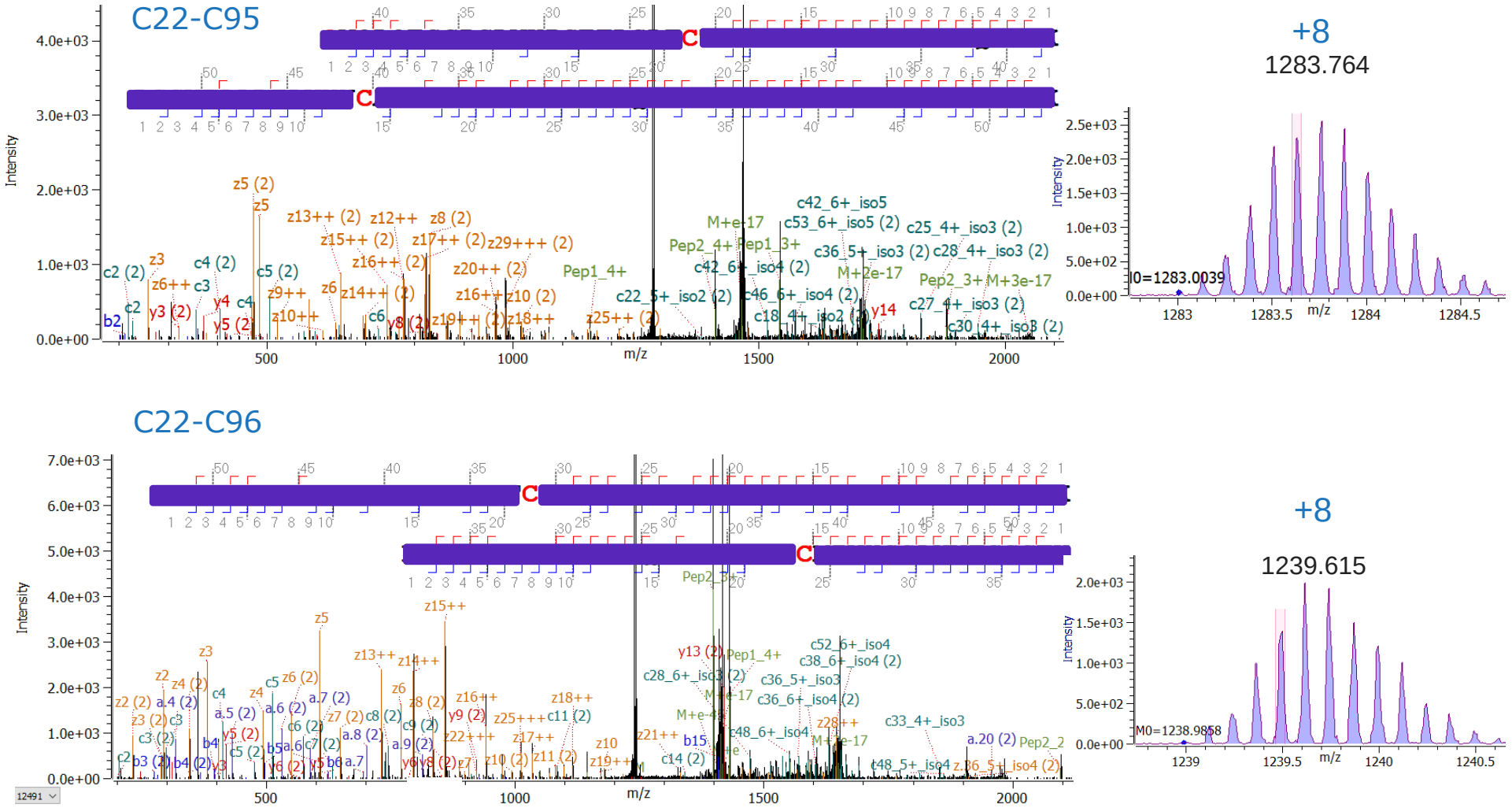
EAD provides a complete sequence coverage for regular size disulfide peptide




Dissociated peptide 1 and peptide 2 are dominant fragments in EAD disulfide spectra

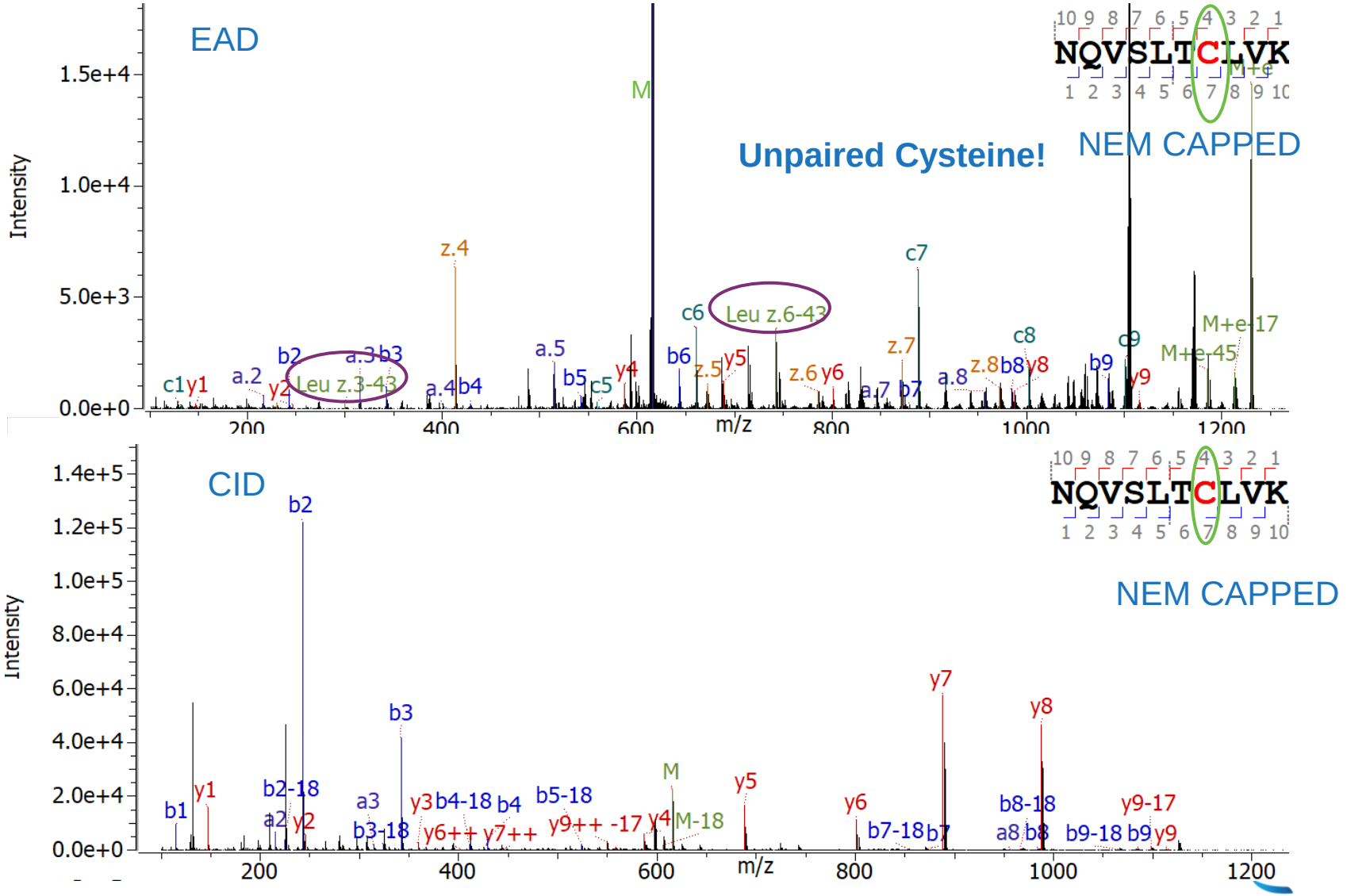


# Disulfide analysis - large peptides



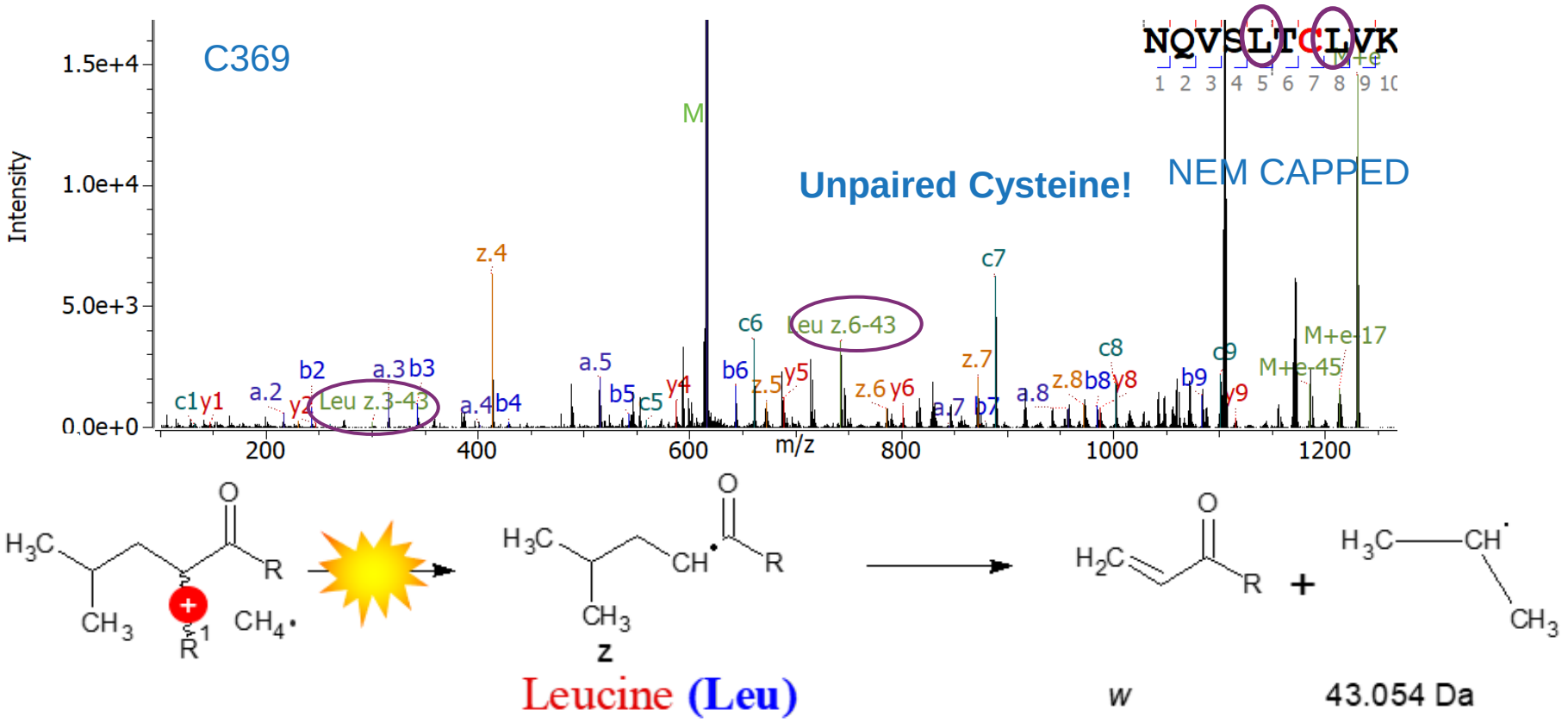

EAD provides complete sequence coverage for bigger peptide

# Disulfide analysis - free thiol



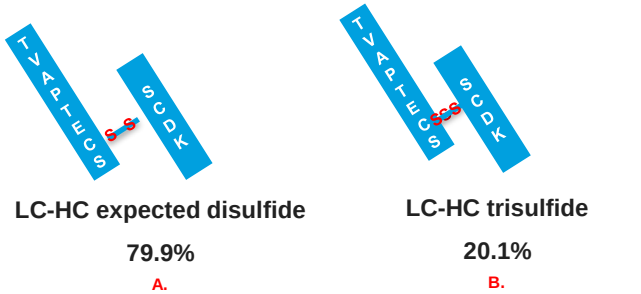

Both EAD and CID can detect trace level of free thiol

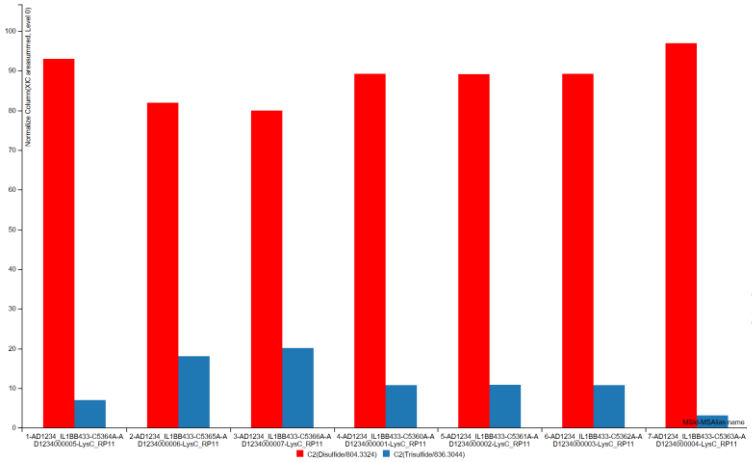
# Disulfide analysis - free thiol

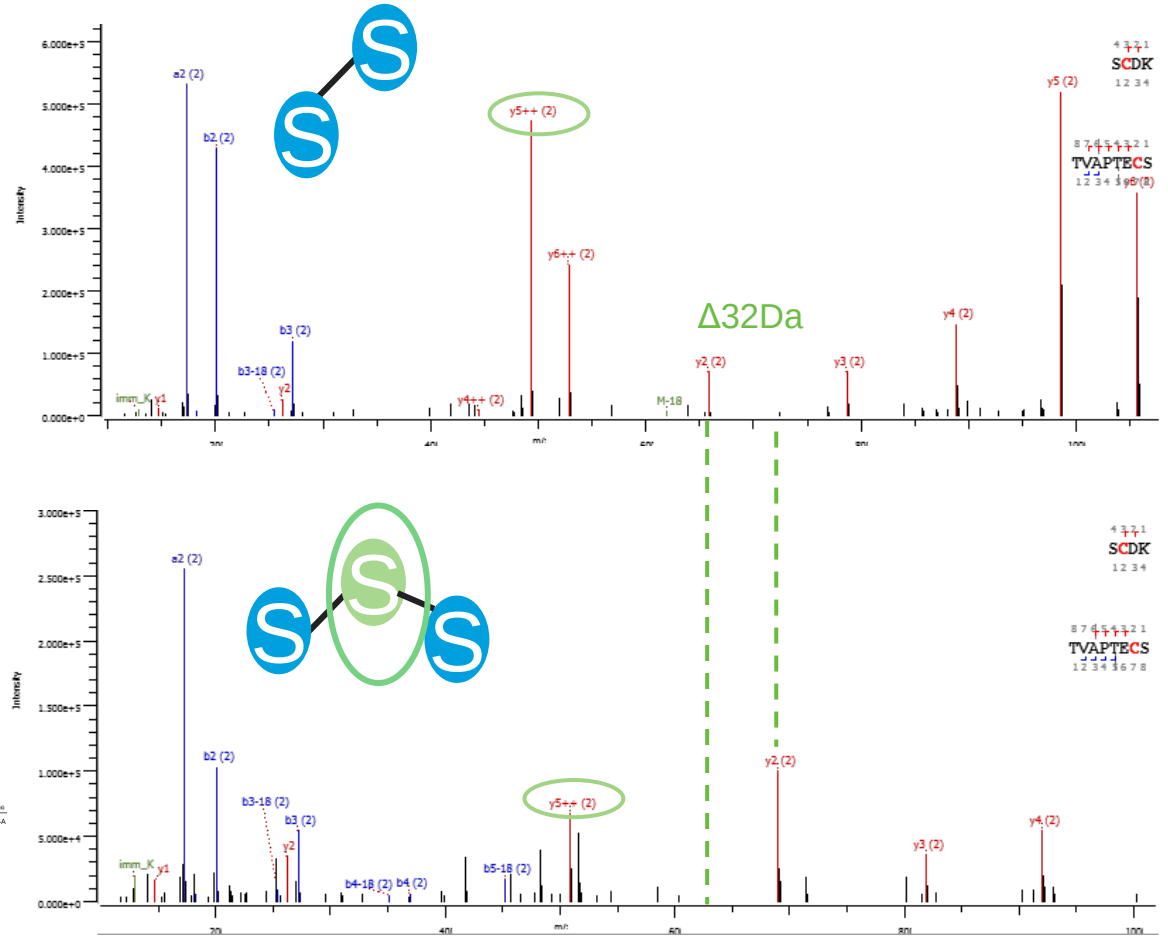



Both EAD and CID can detect trace level of free thiol

# Disulfide analysis - trisulfide analysis



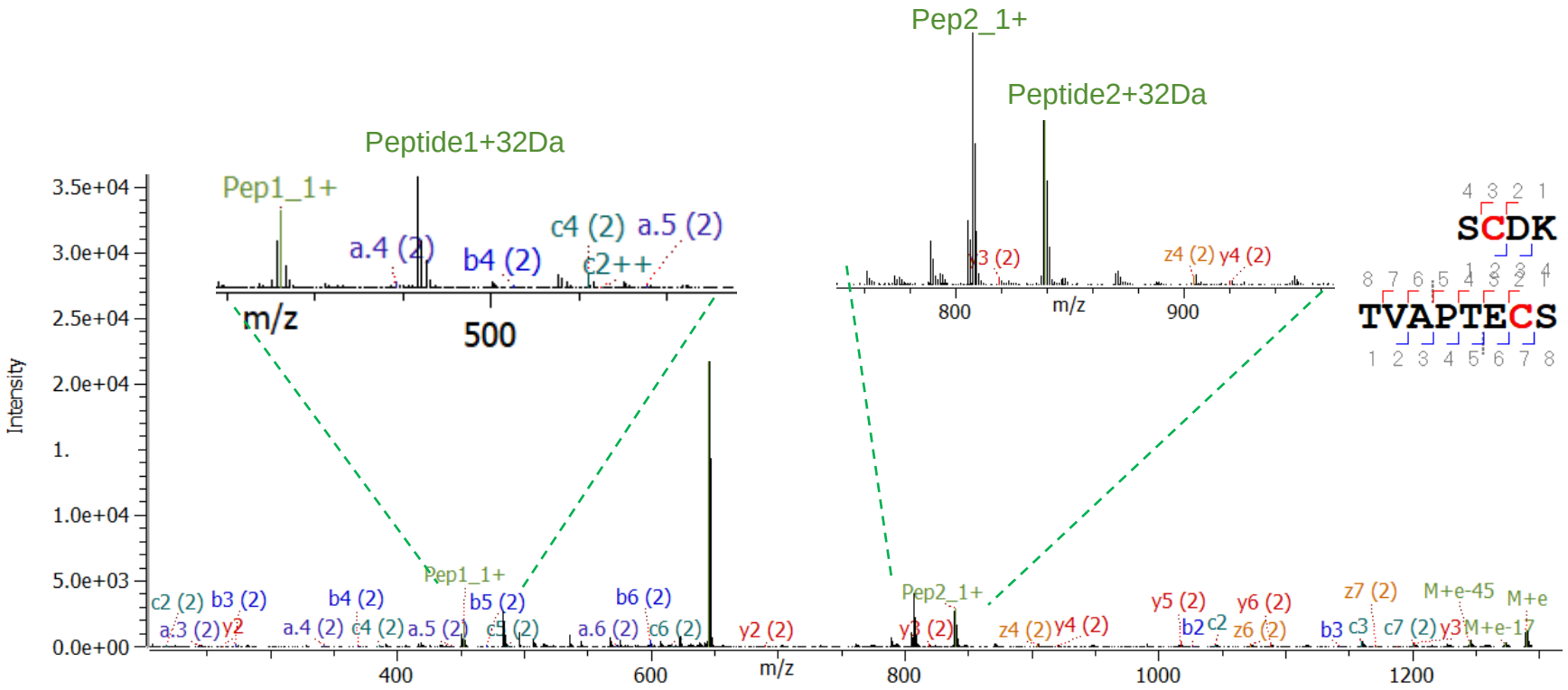





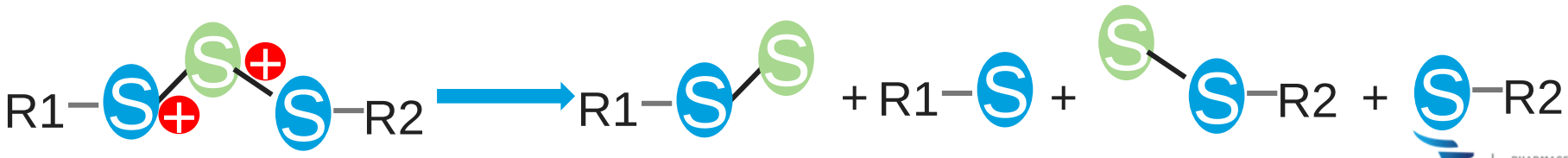

Comparison of disulfide and trisulfide peptide in CID

# Disulfide analysis - trisulfide analysis

## Characterization of trisulfide peptide in EAD

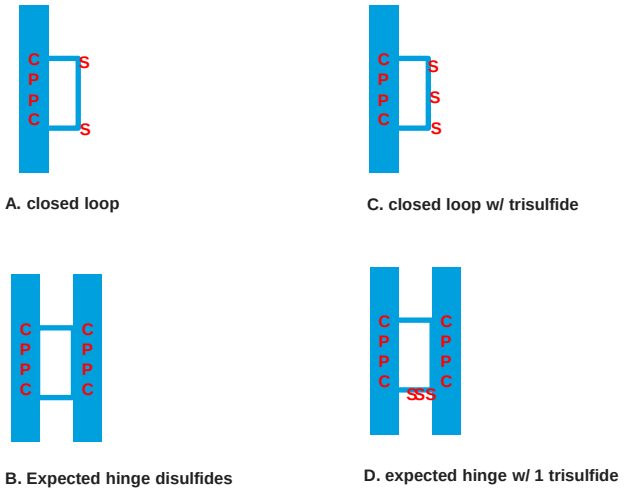


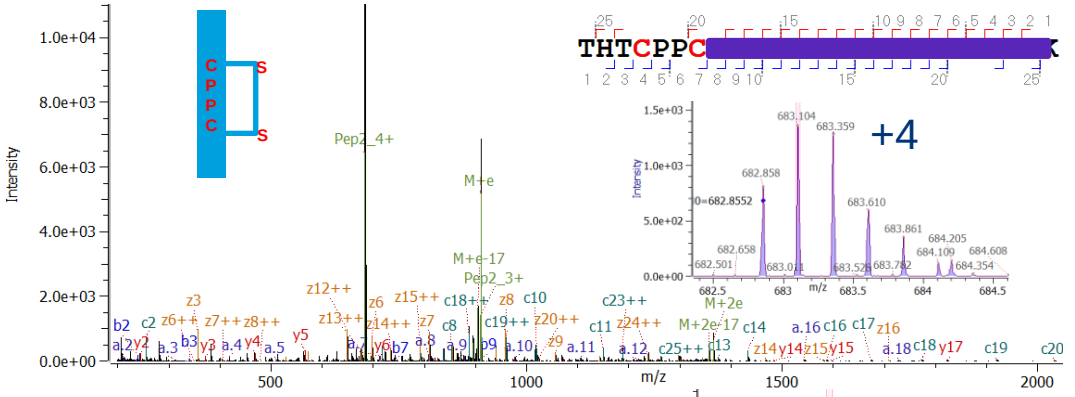

EAD results in two pairs of dominant fragments (P and P+32da) for confident trisulfide peptide confirmation

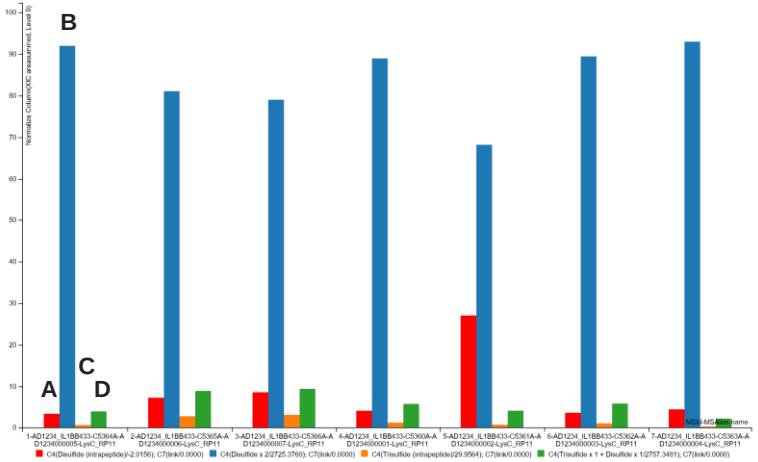


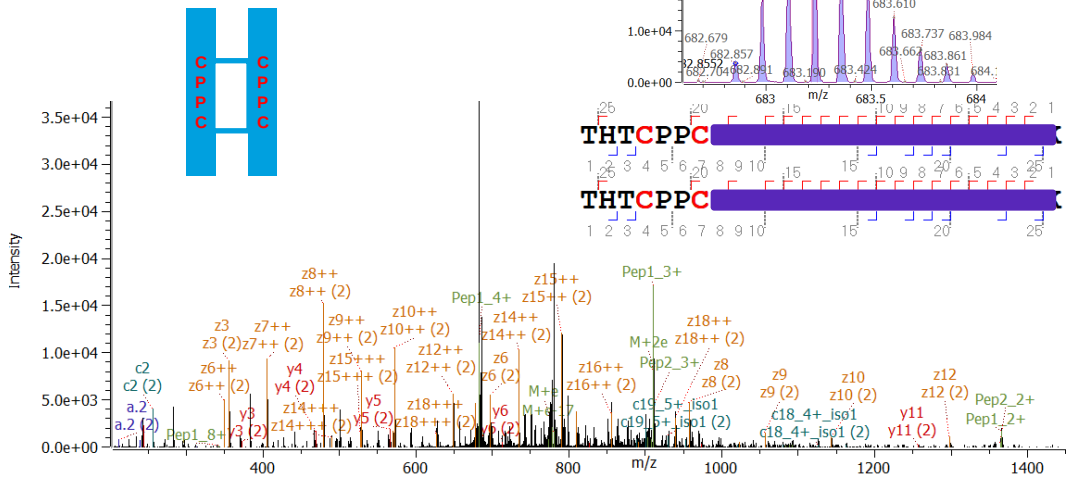
# Disulfide analysis - trisulfide analysis

## Systematically analysis of complex disulfide formation



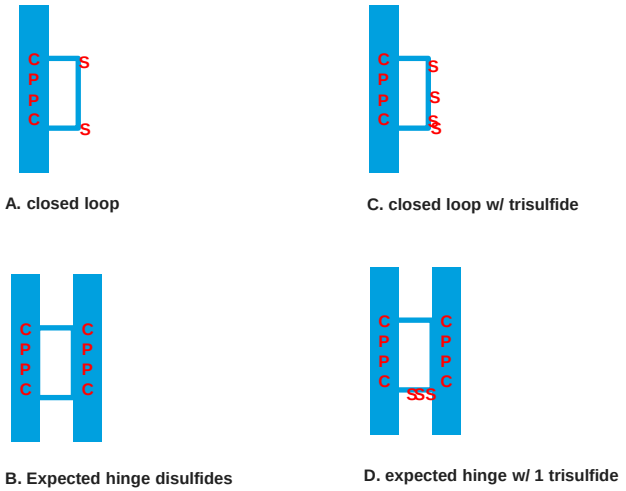



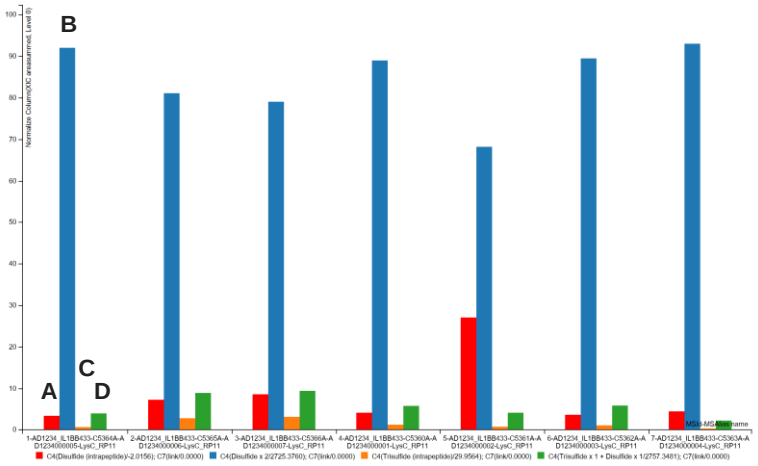



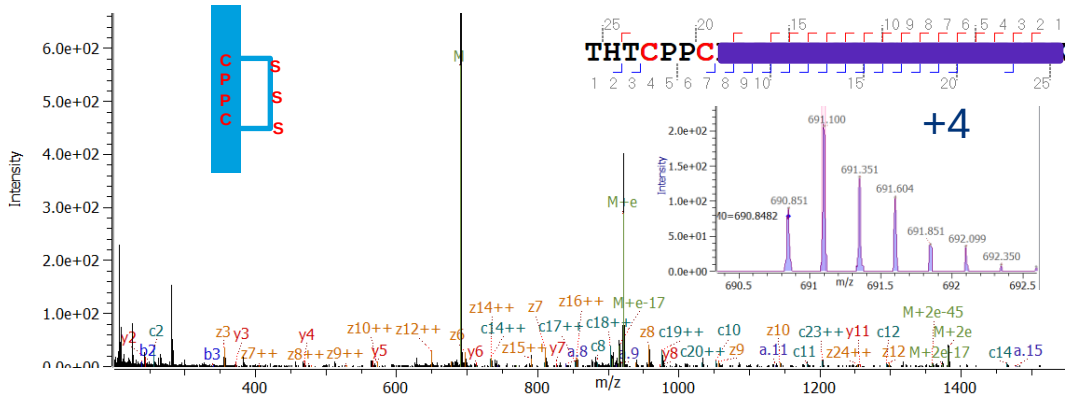



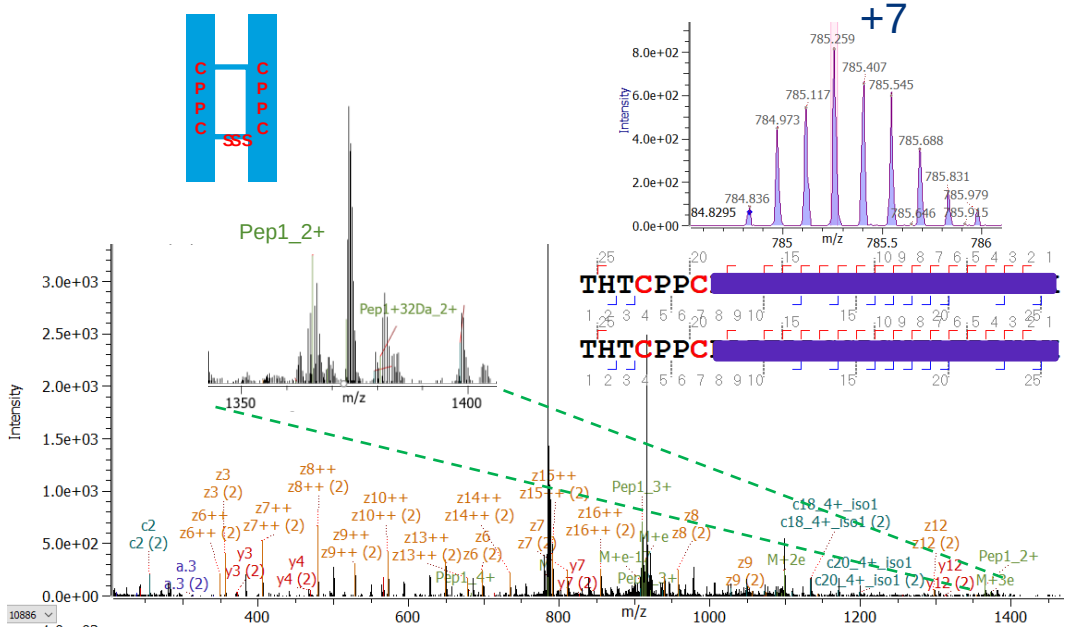
# Disulfide analysis - trisulfide analysis

## Systematically analysis of complex disulfide formation

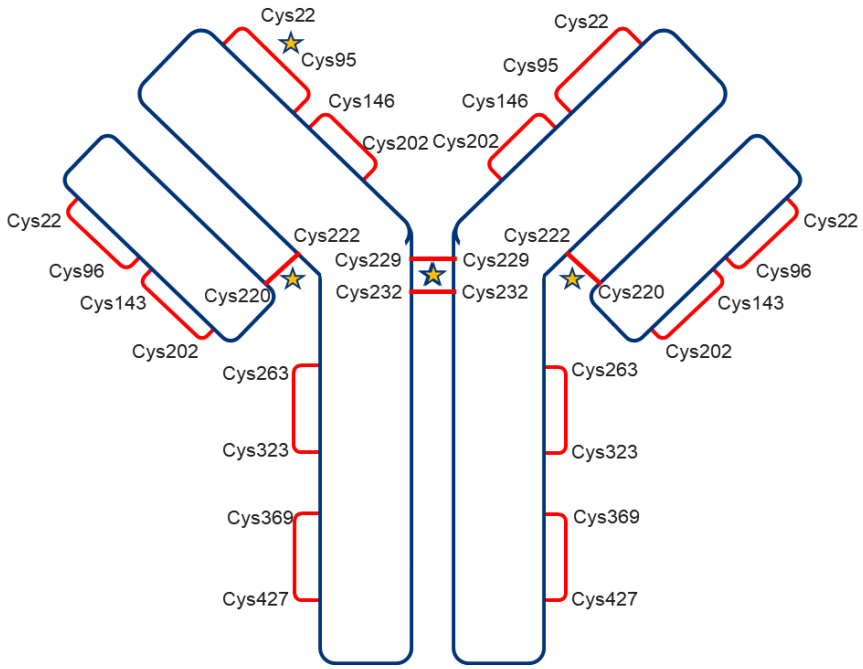


# Thiol isoforms confirmed by non-reduced peptide mapping




| Disulfide # | Cys position-1 | Cys position-2 | Observation |
|---|---|---|---|
| 1 | HC - Cys22 | HC - Cys95 | 25.0% Expected disulfide<br>75.0% Trisulfide |
| 2 | HC - Cys146 | HC - Cys202 | 98.4 % Expected disulfide<br>1.6% Free thiol |
| 3 | HC - Cys263 | HC - Cys323 | 98.9 % Expected disulfide<br>1.1% Free thiol |
| 4 | HC - Cys369 | HC - Cys427 | 97.1 % Expected disulfide<br>2.9 % Free thiol |
| 5 | LC - Cys22 | LC - Cys96 | 97.9 % Expected disulfide<br>2.1 % Free thiol |
| 6 | LC - Cys143 | LC - Cys202 | 99.7 % Expected disulfide<br>0.3% Free thiol |
| 7 | HC – Cys 228<br>HC – Cys 231 | HC – Cys 228<br>HC – Cys 231 | 68.0% Expected disulfides<br>26.9% Intra-peptide Disulfide<br>1.0% Intra-peptide Trisulfide<br>4.1% Disulfide,Trisulfide |
| 8 | LC – Cys 222 | HC – Cys 220 | 87.8% Expected disulfide<br>0.6% Free thiol – cysteinyl<br>0.3% Free thiol – glutathionyl<br>10.4% Trisulfide<br>0.9% LC-LC (scrambled) |

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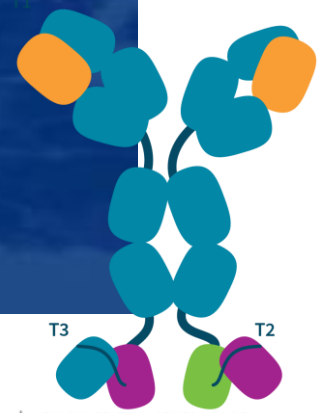
# Summary

- The antibody T-cell redirector candidate shows a complicated linkage, which is different from original design.
- High abundance of trisulfide linkage and free thiol was detected and confirmed with multiple technologies, BioPhase, Intabio® system and ZenoTOF 7600 system.
  - BioPhase 8800 system provides high throughput ciEF screening.
  - The Intabio system offers automatic on-line cIEF MS peak confirmation
  - ZenoTOF 7600 system enhances detailed information of PTMs, including disulfide linkage, isomer identification etc.
- EAD cleaves disulfide bonds resulting in intact peptide peaks, providing additional information on the linkage
- High fragment ion coverage for large peptides or highly charged peptides
- EAD provides confident confirmation on the existence of trisulfide in the MS/MS spectra

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# Thank you!

## Collaboration to validate performance of the ZenoTOF 7600 system for biotherapeutics

SCIEX
- Scott Mack
- Zoe Zhang
- Zhichang Yang
- Mariam ElNaggar
- Maggie Ostrowski
- Elliott Jones
- Sahana Mollah

Protein Metrics
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- St. John Skilton
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- Tom Kelly
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