Proteoform Specific Microheterogeneity Assessment of Biopharmaceuticals Using a Modified Orbitrap Tribrid mass spectrometer

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Conflict of Interest Statement:
C. Beaumal, S. Carillo, S. Millán-Martín and J. Bones are employees of NIBRT. K. Srzentić, A. Norris and R. Melani are employees of Thermo Fisher Scientific, the manufacturer of instrumentation, consumables and certain softwares used in the generation of data presented here. J. Bones received funding as part of a collaborative research agreement between NIBRT and Thermo Fisher Scientific. C. Beaumal, S. Carillo and S. Millán-Martín are funded through that collaborative research agreement. The authors know of no other information that may affect the impartiality or subjectivity of the scientific study.

Moving Beyond mAbs

- Manufacturing of monoclonal antibodies (mAbs) is becoming platform, certain analytical strategies for mAb characterization are becoming routine.
- Developments in analytical instrumentation, separation chemistries and software solutions have been the driving force, underpinning this move towards platform.
- However, nothing is ever that simple. Understanding charge heterogeneity is still complex for mAbs and characterisation of emerging formats, such as multi-specifics, conjugated forms and Fc fusion proteins is highly challenging.
- Can we simplify characterisation workflows through the implementation of high-resolution MS and MS/MS detection, especially on the intact level?

Native Intact MS analysis is powerful for complex formats

- Native intact mass analysis, especially when combined with upfront separation, is a powerful approach for characterisation of complex biopharmaceutical entities:
  - Multi-specifics – correct chain pairing
  - Conjugates – preventing loss of labile functionalities or domains
  - Proteoform specificity – how individual molecular entities are composed

- Challenges remain, especially for highly glycosylated heterogenous molecules, such as Fc fusion proteins, many of which contain multiple N- and O-glycosylation sites, making spectral deconvolution difficult if not impossible.

- Ideally, instrument configuration should enable efficient transmission of native protein ions, high m/z capability, efficient isolation of ions corresponding to proteoforms of interest, multiple ion activation for native top-down sequencing and gas phase fractionation for spectral simplification.

Orbitrap Ascend Biopharma Tribrid MS

Back Ion Routing Multipole
Enables parallel analysis; performs HCD at MS^{3+} stage

Front Ion Routing Multipole
Enable parallel analysis, performs HCD at MS^2 stage

Recommended: Native MS option*
Enables isolation in the quadrupole up to *m/z* 8,000

Advanced Active Ion Beam Guide
Prevents neutrals and high velocity clusters from entering mass resolving quadrupole

QR5 Segmented Quadrupole Mass Filter with Hyperbolic Surfaces
Improved sensitivity with 0.4 *m/z* precursor isolation widths

Recommended: EASY-IC/ETD and PTCR Ion Source options
Based on Townsend discharge, reliable and easy to use

Electrodynamic Ion Funnel
Efficient ion transfer
Broad tuning curves
Optimized for labile compounds

High-Capacity Ion Transfer Tube
Increased ion flux

Recommended UVPD
Unique fragmentation mode for analyte structure elucidation

Modified Dual-Pressure Linear Ion Trap Mass Analyzer
Up to 50 Hz MS^n and sensitive mass analysis
Six fragmentation types: CID, HCD, ETD/EThcD/ETciD, and UVPD

Recommended Native MS option*
Enables isolation up to *m/z* 8,000

Ultra-High-Field Orbitrap Mass Analyzer
Offers resolution > 480K FWHM and acquisition rates up to 45 Hz, TurboTMT

Recommended: Native MS option*
Detection in the Orbitrap analyzer to *m/z* 16,000

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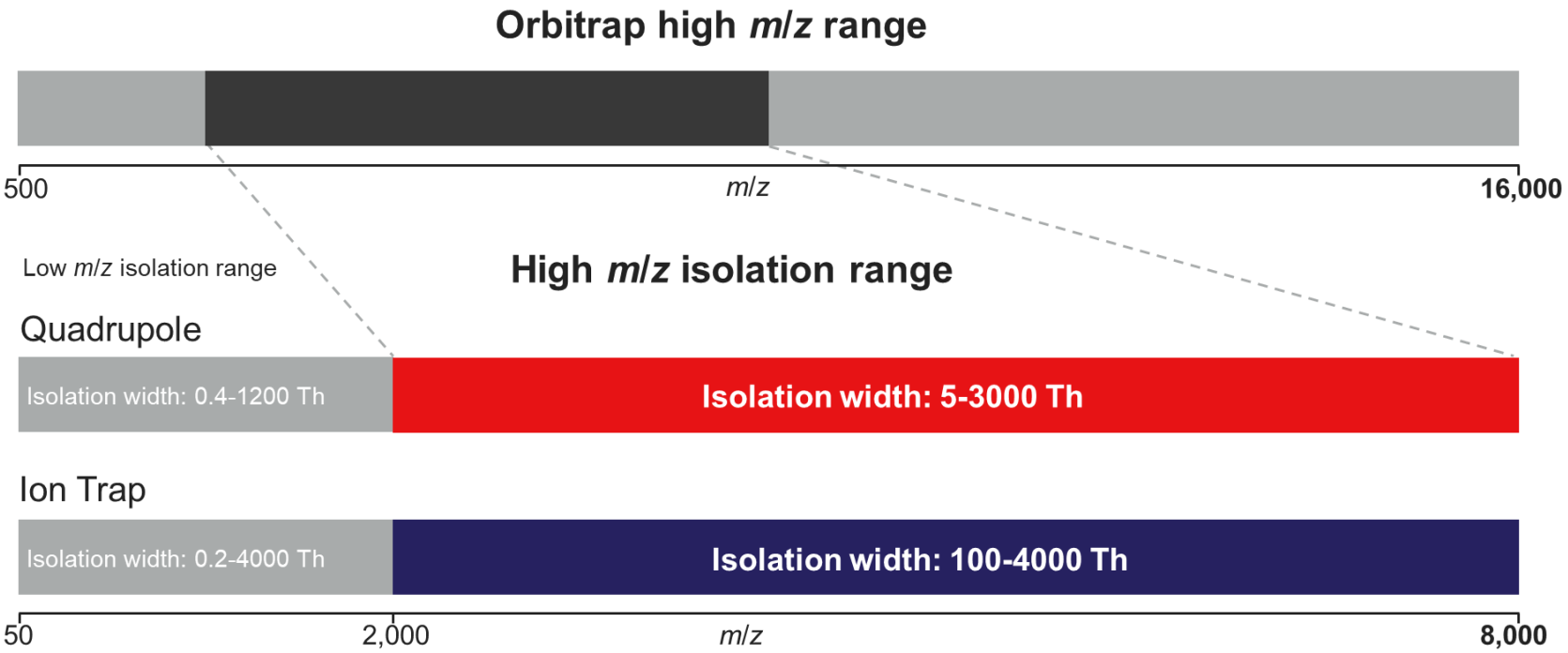
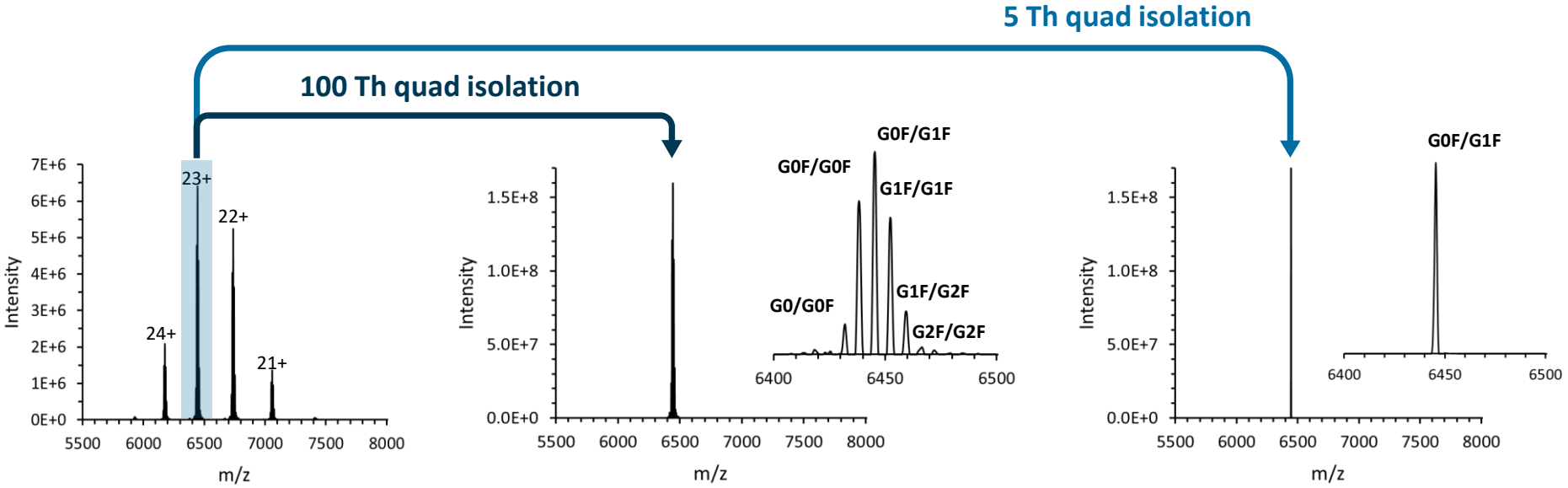
Orbitrap Ascend Biopharma with Native MS option

Orbitrap mass range up to *m/z* 16,000 and quadrupole/ion trap isolation up to *m/z* 8,000



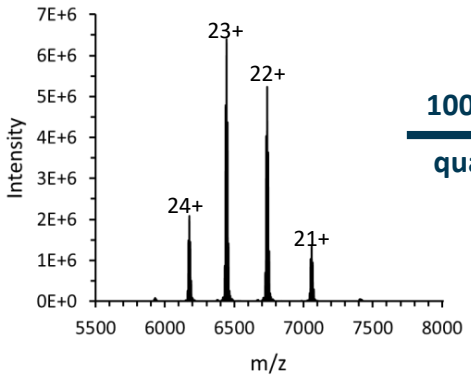
Static infusion of Trastuzumab – Quad isolation



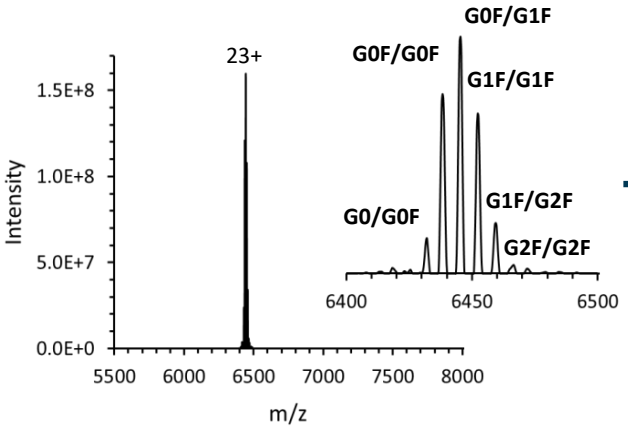
New quadrupole technology in Orbitrap Ascend Biopharma facilitates transmission and narrow isolation of ions at high *m/z*, ability to isolate ions corresponding to individual proteoforms

Native TD-MS using Quad isolation: 100 Th window

Trastuzumab MS1 spectrum



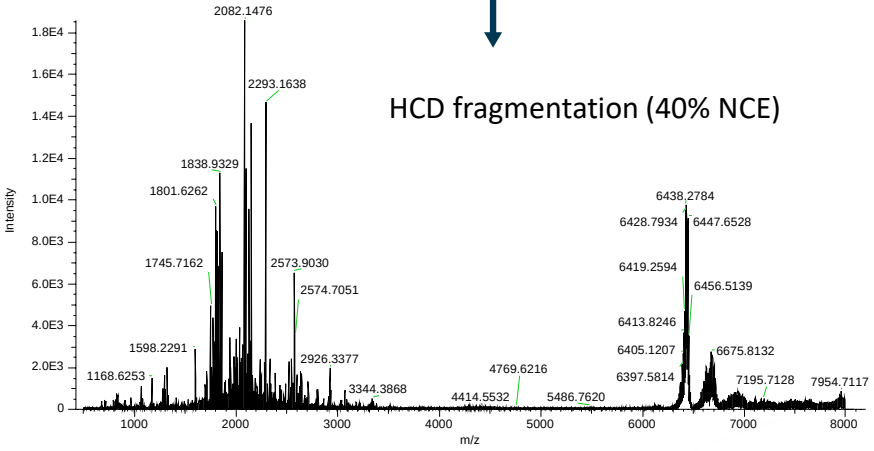
100 Th window quad isolation



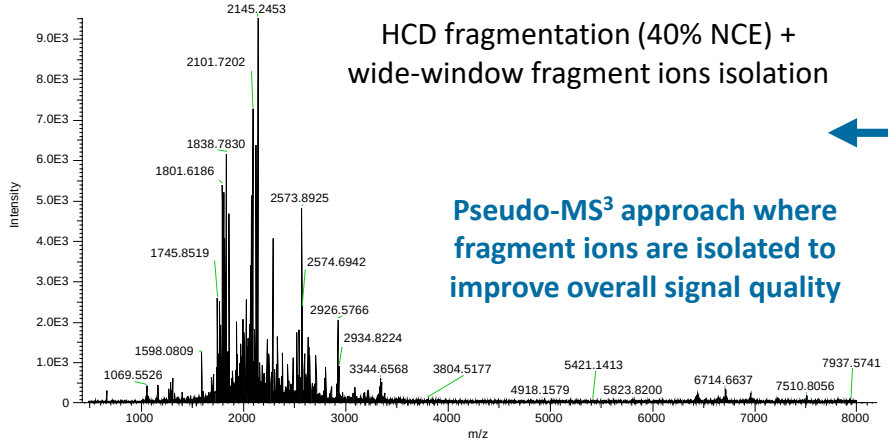
Native TD-MS using HCD of the isolated 23+ ion

High S/N, albeit with significant precursor signal remaining

HCD fragmentation (40% NCE)



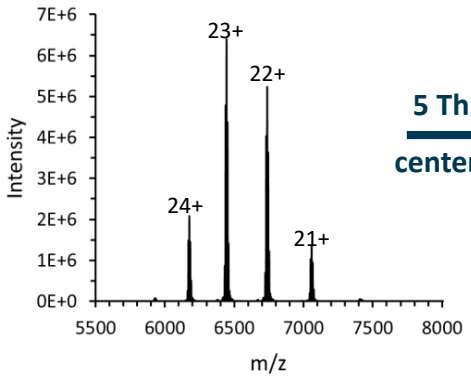
HCD fragmentation (40% NCE) + wide-window fragment ions isolation



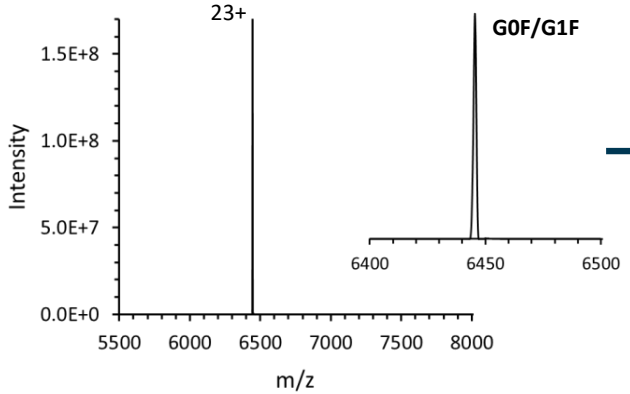
Pseudo-MS^3 approach where fragment ions are isolated to improve overall signal quality

Native TD-MS using Quad isolation: 5 Th window

Trastuzumab MS1 spectrum



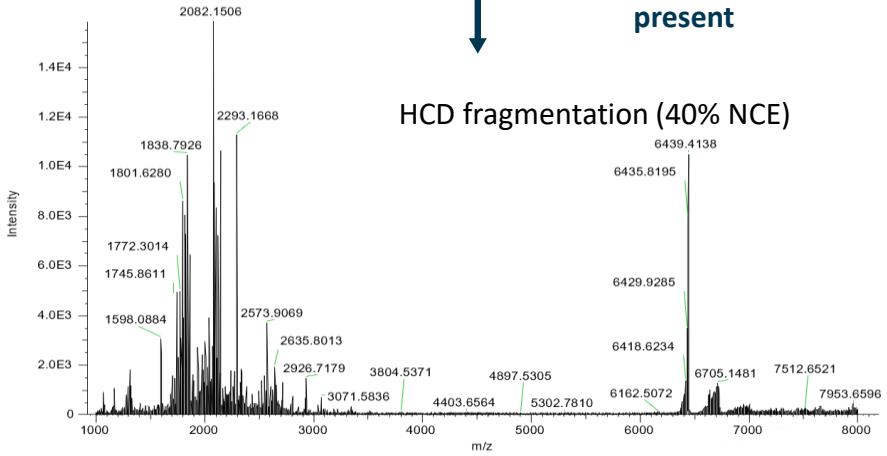
5 Th quad isolation centered on 6445 *m/z*



Similar observation as for the 100 Th isolation window.

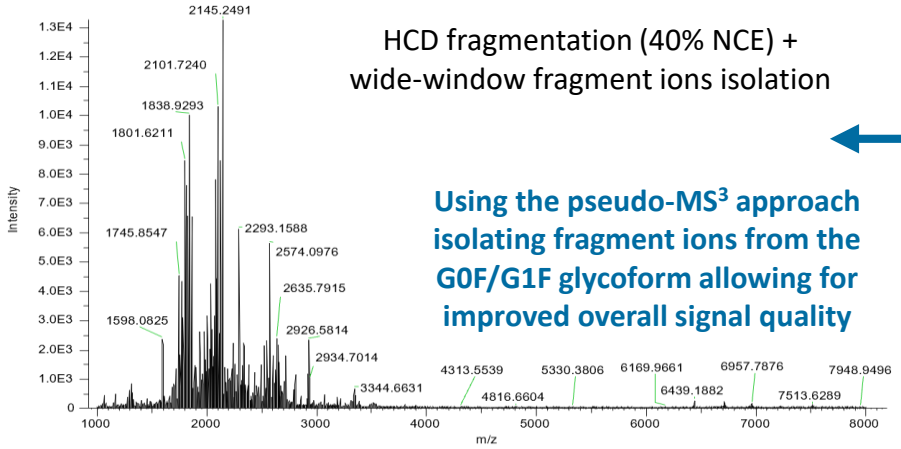
G0F/G1F glycoform fragmentation observed but with precursor signals still present

HCD fragmentation (40% NCE)



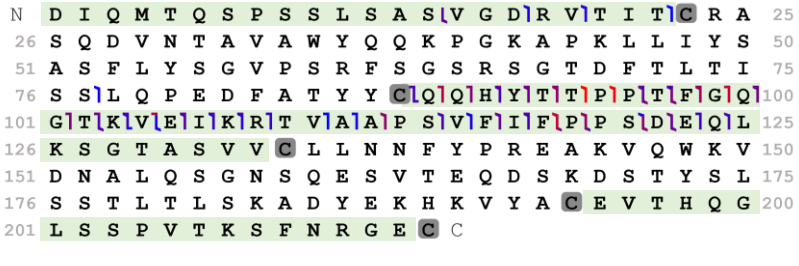
Using the pseudo-MS^3 approach isolating fragment ions from the G0F/G1F glycoform allowing for improved overall signal quality

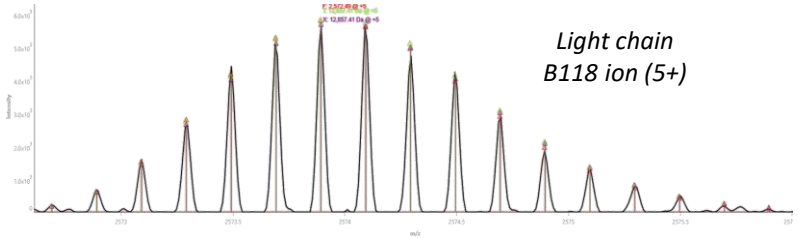
HCD fragmentation (40% NCE) + wide-window fragment ions isolation



Sequence coverage for nTD-MS of an isolated glycoform

Light chain – 17.8% sequence coverage



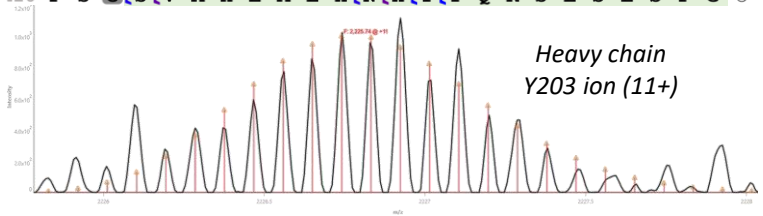


Light chain
B118 ion (5+)

Experimental conditions:

- 100 spectra averaging (5 microscans)
- Combination of ETD 25 ms, ETD 45 ms, UVPD 30 ms and HCD 40% acquisitions.
- 5 Th isolation windows centred on the G0F/G1F glycoform
- Data processing using ProSight native, 10 S/N threshold and score > 0.75
- Green part highlights areas where fragmentation is observed.

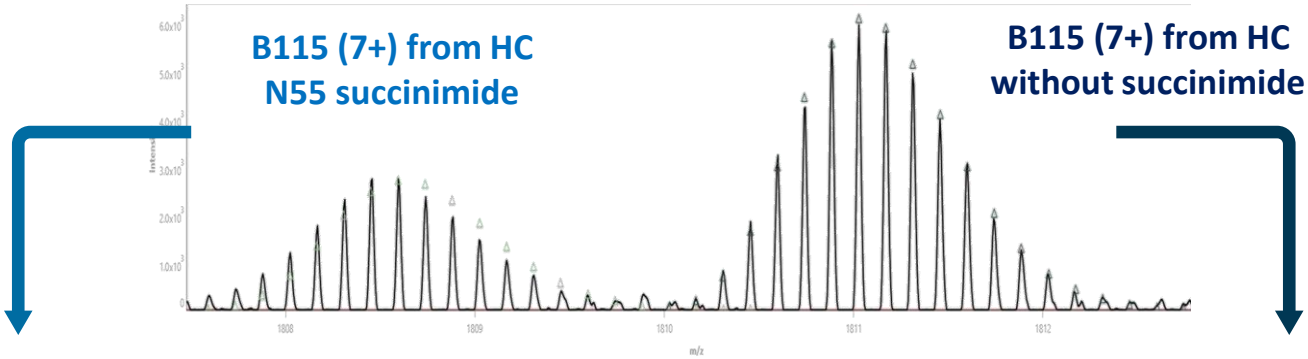
Heavy chain G0F – 14.5% sequence coverage



Heavy chain
Y203 ion (11+)

Use of multiple ion activation events on Orbitrap Ascend Biopharma for native top-down sequencing. Ability to confirm the glycan identity.

Identification of PTMs on individual glycoforms



Trastuzumab HC (1-140) – N55 succinimide

N	E	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	20
21	S	C	A	A	S	G	F	N	I	K	D	T	Y	I	H	W	V	R	Q	A	40
41	P	G	K	G	L	E	W	V	A	R	I	Y	P	T	N	G	Y	T	R	Y	60
61	A	D	S	V	K	G	R	F	T	I	S	A	D	T	S	K	N	T	A	Y	80
81	L	Q	M	N	S	L	R	A	E	D	T	A	V	Y	Y	C	S	R	W	G	100
101	G	D	G	F	Y	A	M	D	Y	W	G	Q	G	T	L	V	T	V	S	S	120
121	A	S	T	K	G	P	S	V	F	P	L	A	P	S	S	K	S	T	S	G	140

Trastuzumab HC (1-140)

N	E	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	20
21	S	C	A	A	S	G	F	N	I	K	D	T	Y	I	H	W	V	R	Q	A	40
41	P	G	K	G	L	E	W	V	A	R	I	Y	P	T	N	G	Y	T	R	Y	60
61	A	D	S	V	K	G	R	F	T	I	S	A	D	T	S	K	N	T	A	Y	80
81	L	Q	M	N	S	L	R	A	E	D	T	A	V	Y	Y	C	S	R	W	G	100
101	G	D	G	F	Y	A	M	D	Y	W	G	Q	G	T	L	V	T	V	S	S	120
121	A	S	T	K	G	P	S	V	F	P	L	A	P	S	S	K	S	T	S	G	140

Peptide mapping data identified succinimide formation at N55 on HC

Using 5 Th isolation, possible to identify and annotate succinimide presence using nTD-MS on an individual glycoform

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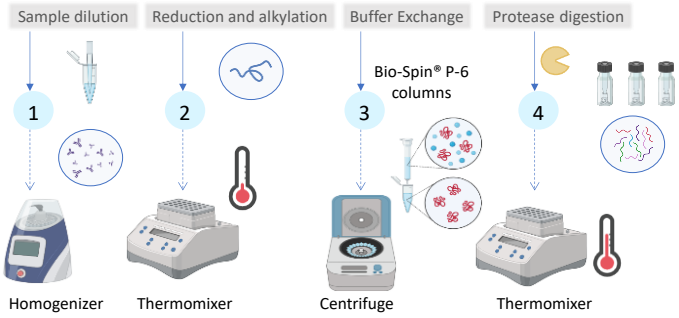
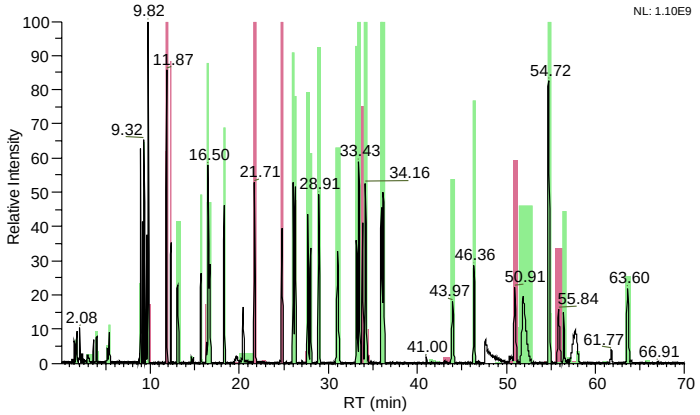
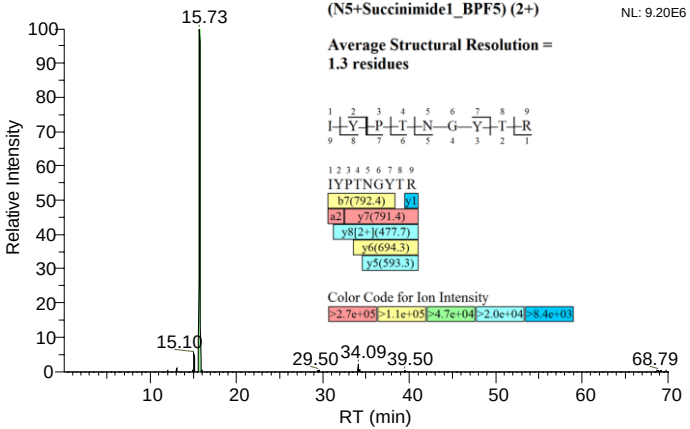
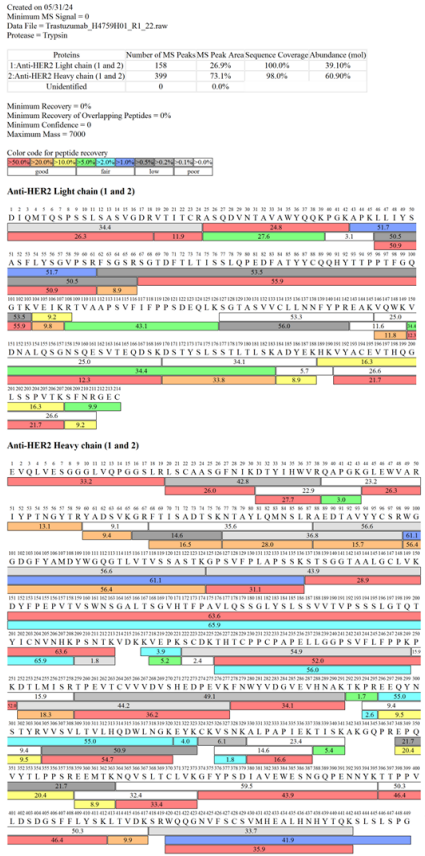
Confirmation of PTM annotation using peptide mapping



- Peptide mapping performed according to Millán-Martín et al. on Orbitrap Ascend Biopharma with data processing in BioPharma Finder, simple column and mobile phase change
- PTMs identified by nTDMS were confirmed on the peptide level, example shown is the same N55 succinimide as noted on previous slide

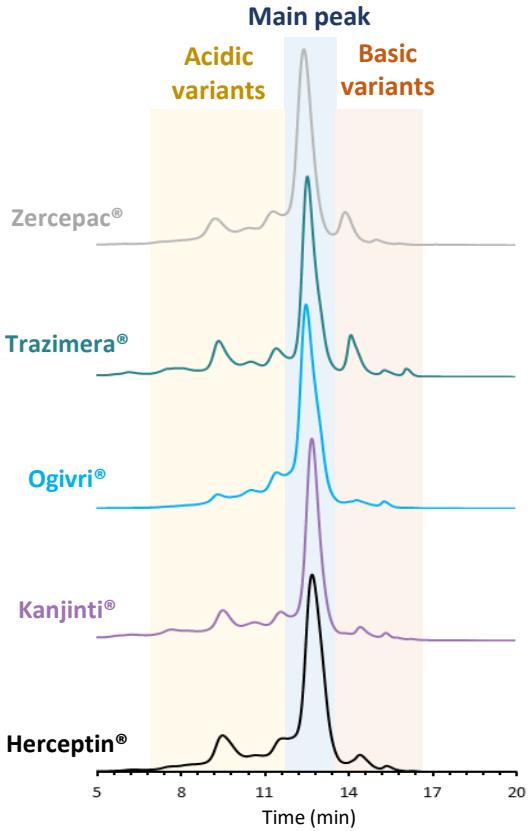


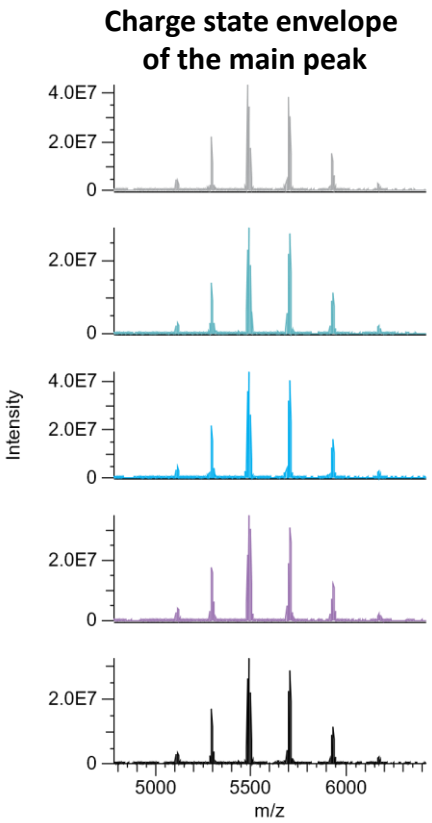


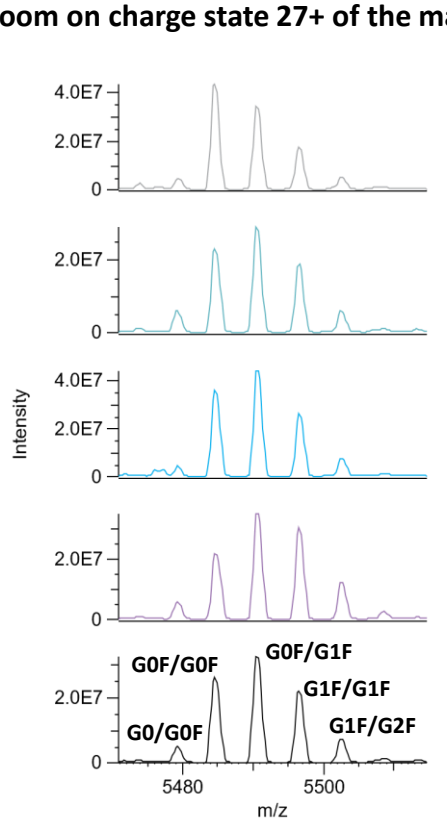


Millán-Martín *et al. Nature Protocols*. 2023, 18 (4):1056-1089.

Hyphenation with pH gradient ion exchange chromatography







Identification of different glycosylation profiles depending on the biosimilars and different levels of acidic and basic species in the chromatogram

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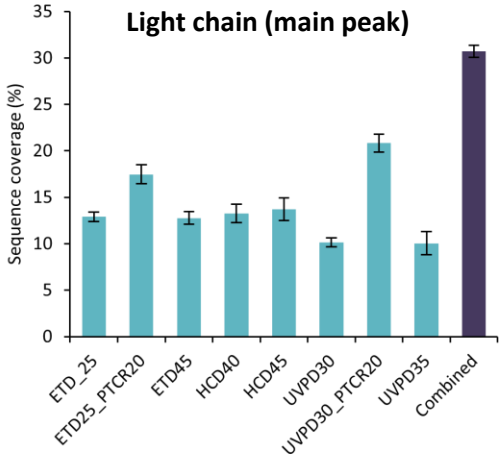
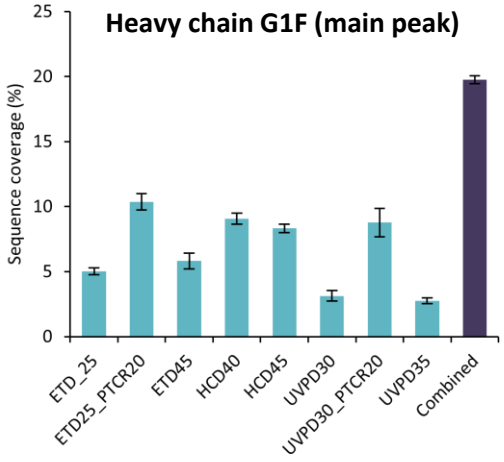
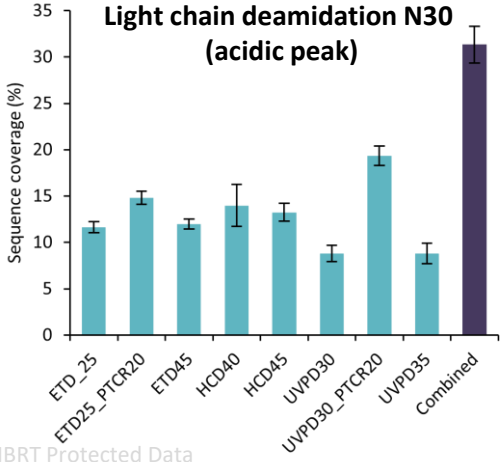
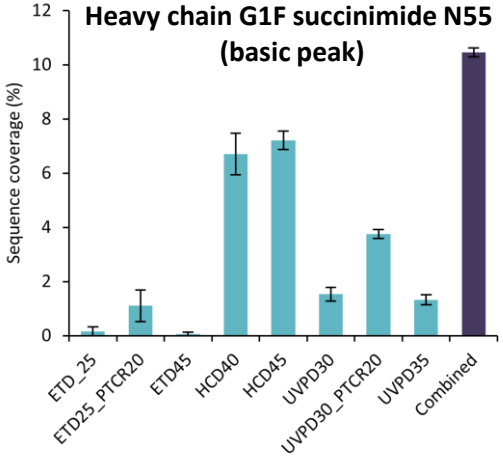
nTD-MS on chromatography timescales





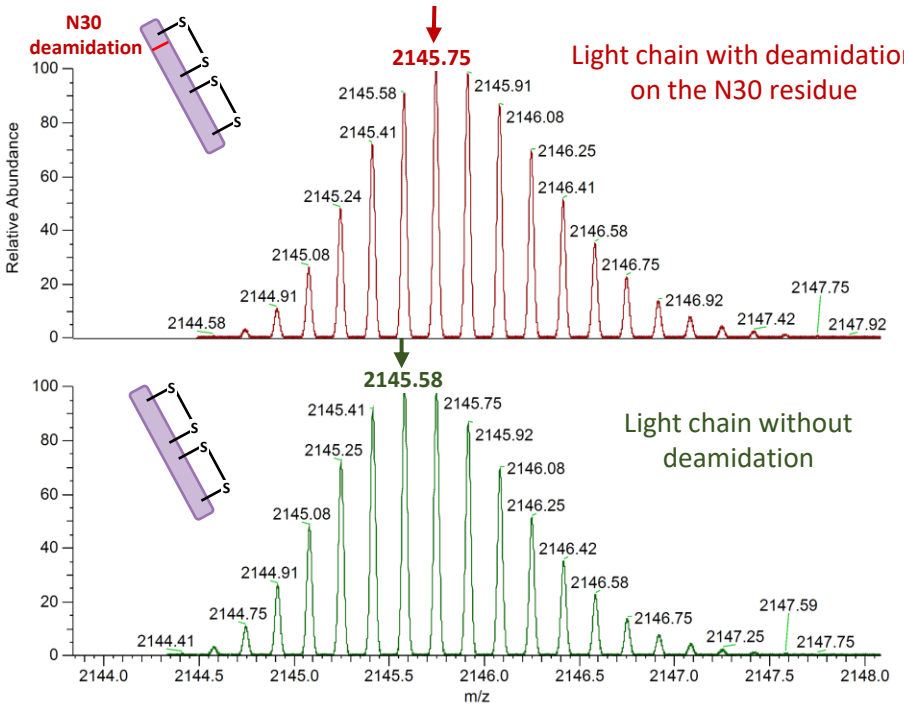




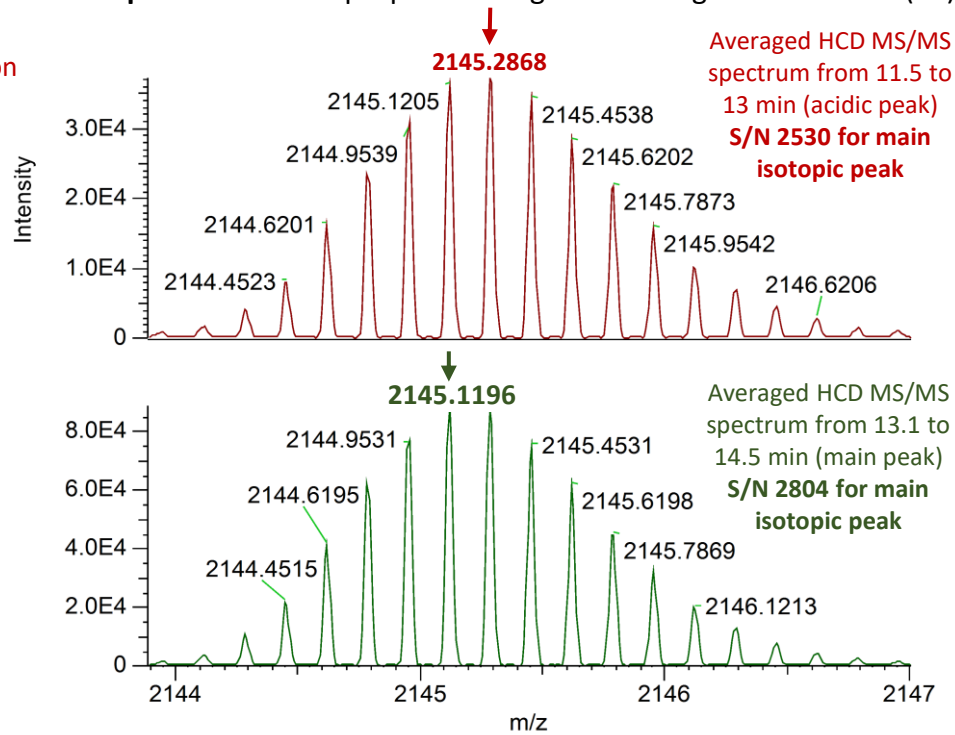
- Sequence coverage assessed using various ion activation strategies (ETD, HCD and UVPD)
- Numbers in labels refer to activation times in milliseconds for ETD and UVPD
- Data shown is the average for the various biosimilar molecules studied and for different species present in the CEX chromatogram
- PTM distributions examined, excellent degree of comparability across analysed biosimilars and innovator molecules

Identification of Deamidation on Trastuzumab innovator

Theoretical isotopic profile of light chain fragment ion B118 (6+)



Experimental isotopic profile of light chain fragment ion B118 (6+)



Combination of CEX separation with narrow window isolation and subsequent nTD-MS across the chromatographic peak enables confident identification of modifications such as deamidation, difference in Tr, *m/z* and isotopic distribution of precursor and product ions

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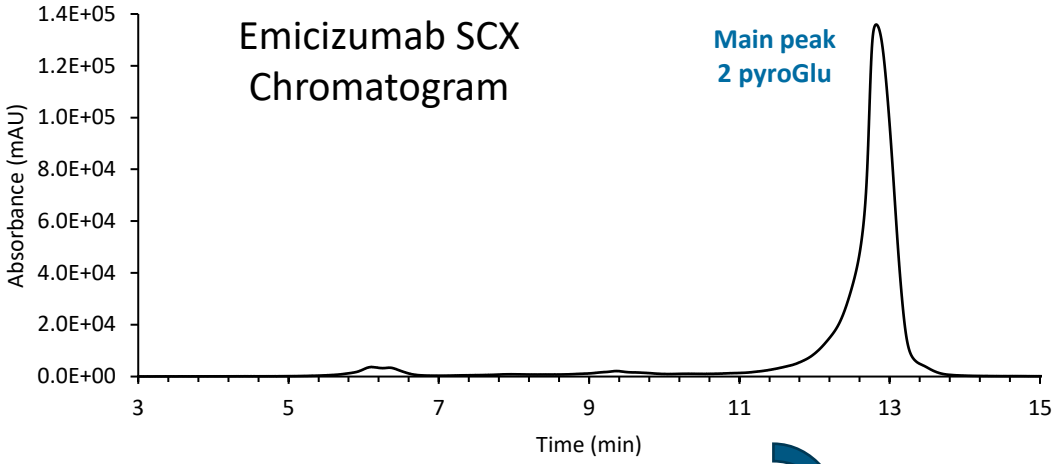
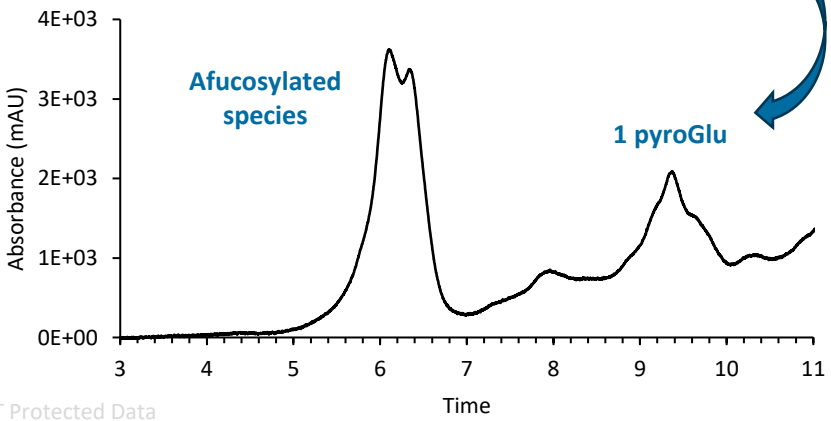
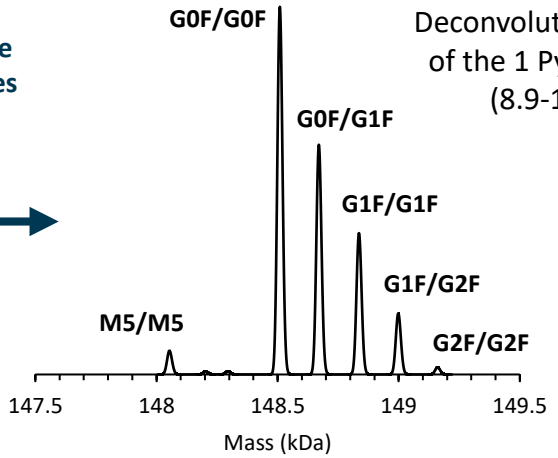
Annotation of PyroGlu on Emicizumab heavy chains





Zoom on the acidic species

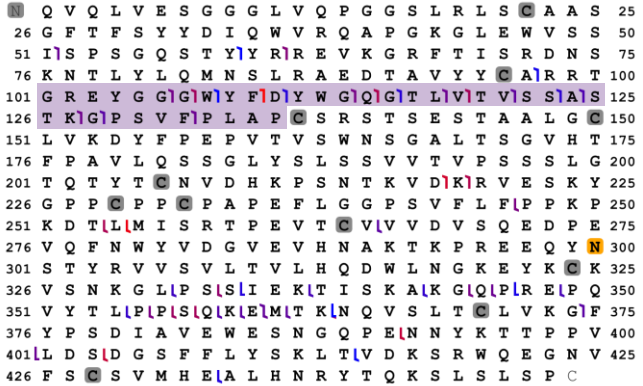


Deconvoluted spectrum of the 1 PyroGlu peak (8.9-10 min)

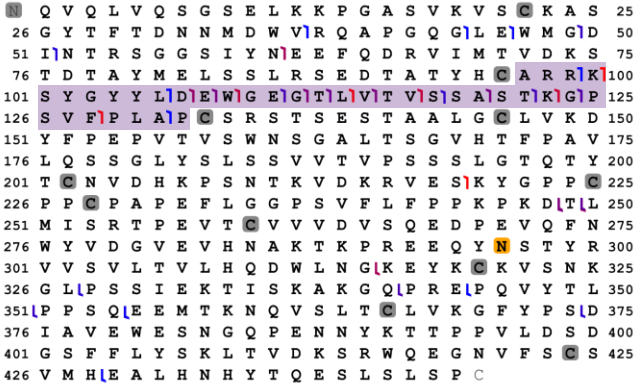
- The main peak is identified as Emicizumab with the two heavy chains bearing a PyroGlu
- Minor species in the acidic region are also observed, including afucosylated species and Emicizumab bearing only one PyroGlu

Annotation of PyroGlu on Emicizumab heavy chain

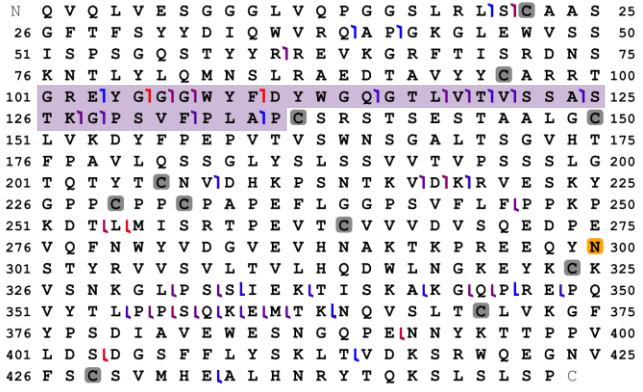
Emicizumab heavy chain A - **PyroGlu**



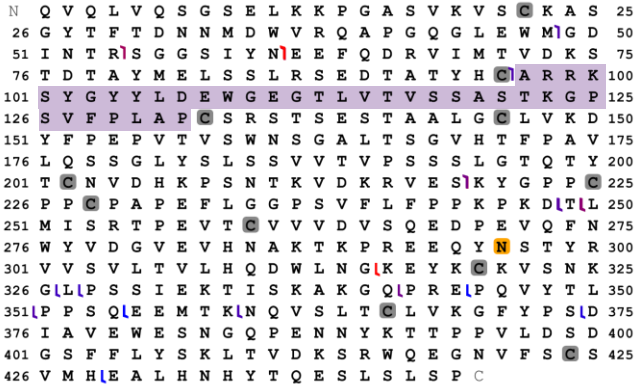
Emicizumab heavy chain B - **PyroGlu**



Emicizumab heavy chain A – **no PyroGlu**



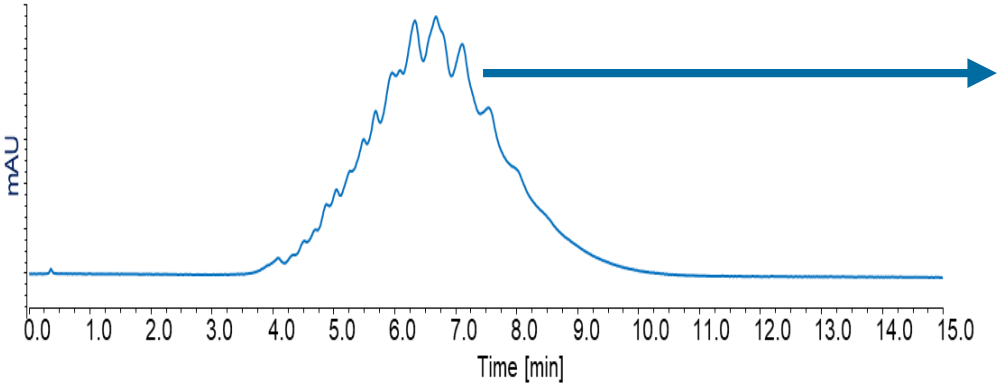
Emicizumab heavy chain B – **no PyroGlu**

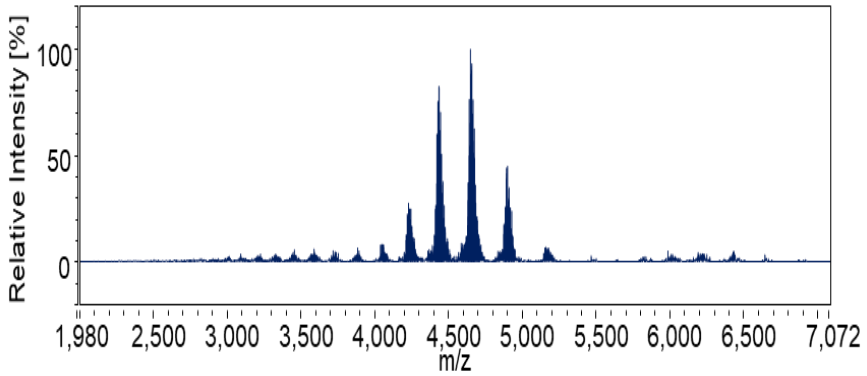


- Emicizumab is composed of 2 different heavy chains and 2 identical light chains
- Presence of more than 20 fragments confirming the presence of the pyroGlu on both heavy chain A and B
- More than 20 fragment ions confirming the absence of pyroGlu on heavy chain A, while almost no ions pointing in this direction for heavy chain B, leading to the conclusion that pyroGlu loss is more likely to happen on heavy chain A

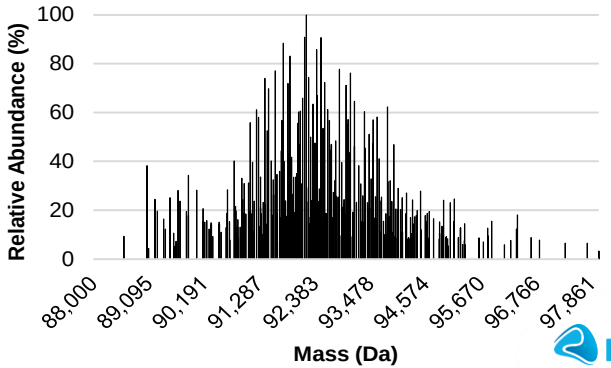
Characterisation of highly heterogeneous Fc fusion proteins

Fc fusion proteins (FcFP's) consist of desired linker protein joined to the hinge region and Fc domain of an immunoglobulin. Being unnatural proteins, they are often highly glycosylated to protect the molecule from proteolytic digestion



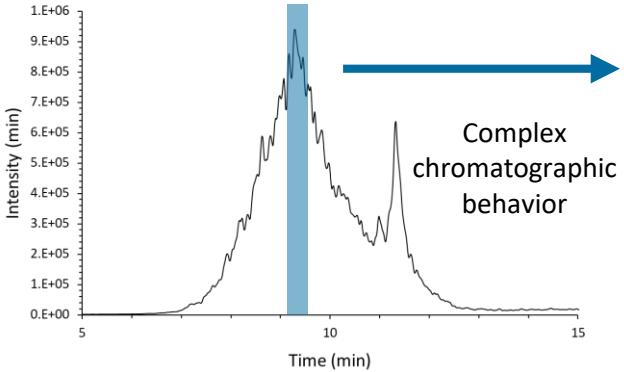


Resulting heterogeneity from *N*- and *O*-glycosylation, in addition to other modifications present in the primary sequence, makes chromatographic separation very difficult and results in highly complex spectra and deconvolution challenging

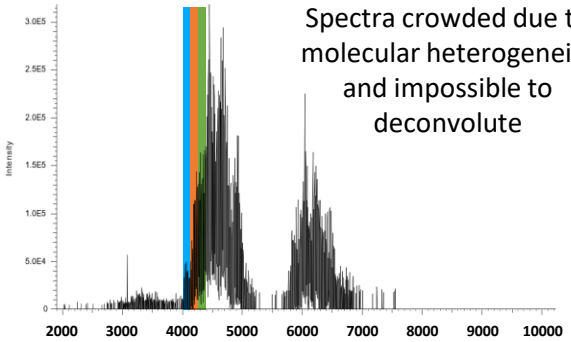


LC-DIA-PTCR for FcFP characterisation

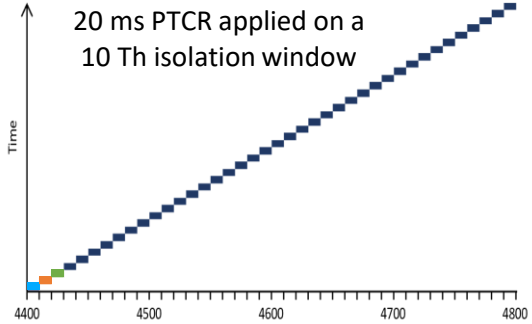
DIA-PTCR recently introduced by Schachner *et al*. Here, application of DIA-PTCR on the LC timescale for complex FcFP characterization was explored



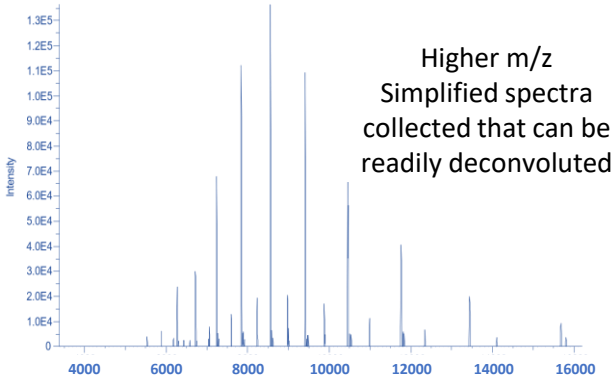
Complex chromatographic behavior



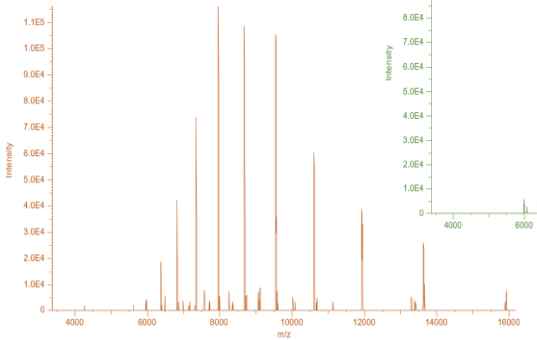
Spectra crowded due to molecular heterogeneity and impossible to deconvolute

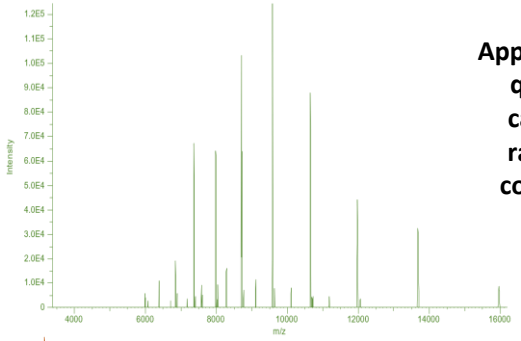


20 ms PTCR applied on a 10 Th isolation window



Higher m/z Simplified spectra collected that can be readily deconvoluted

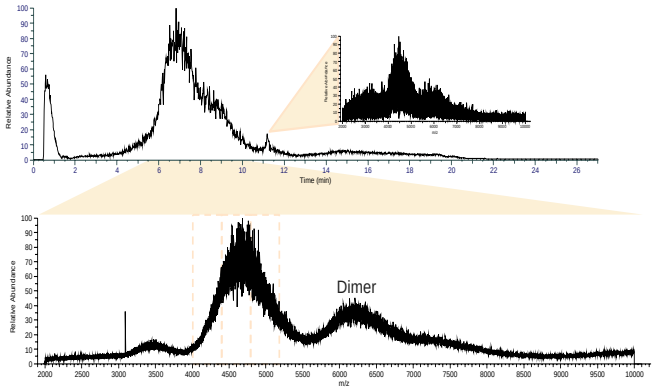




Application exploits the new quad technology, PTCR capability and high m/z range to simplify highly complex biotherapeutics characterization

Schachner *et al. Nature Communications.* 2024, 15(1): 3259.

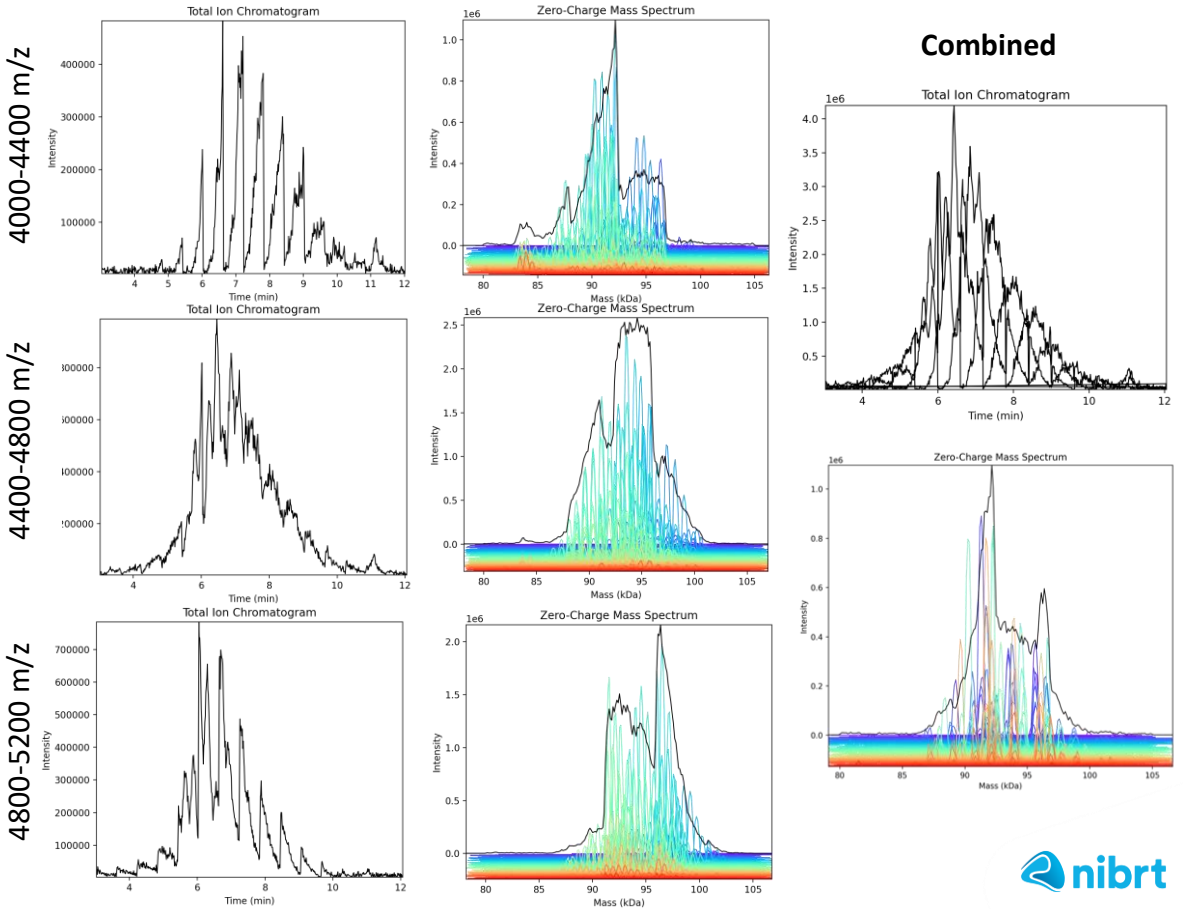
Application to Luspatercept (pI 5.4)



- FcFP combining a modified extracellular domain of activin receptor with IgG1 Fc domain

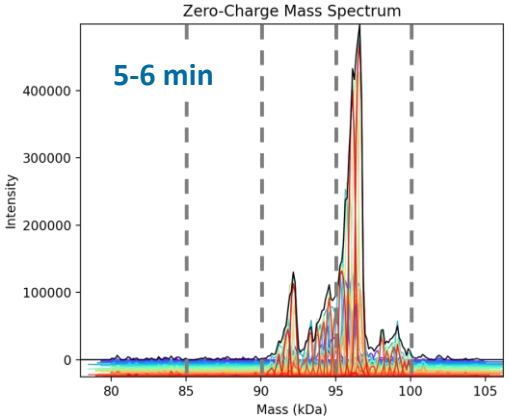
- LC-DIA-PTCR data processed using UniDec UniChrom:
  - 4000-5200 composite parameters
  - Sliding window (min): 0.0000001 offset 2
  - Time start 7 to 12 min
  - Charge range 5-20
  - Mass range 85000-105000 Da
  - Sample every 120 Da
  - Peak FWHM (Th) 50

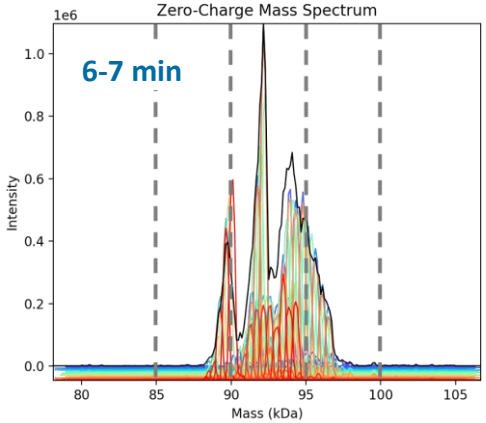
- Composite zero charge spectrum generated illustrating resolution of proteoforms present

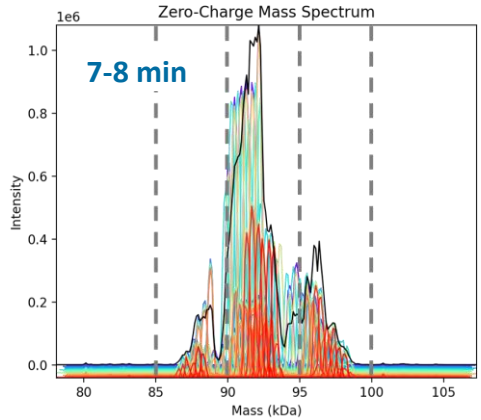


Assigning 1 min segments across the chromatogram

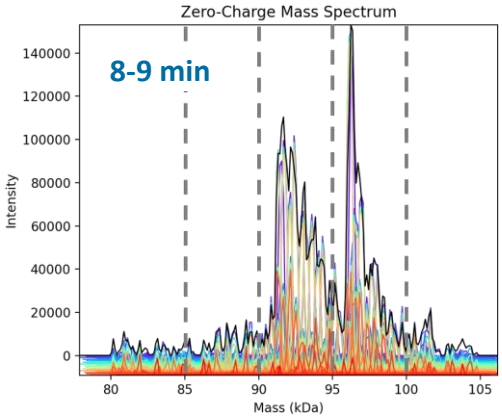
Deconvolution of 1 min chromatogram slices (4400-4800 m/z run) Same parameters outlined before were used for data processing

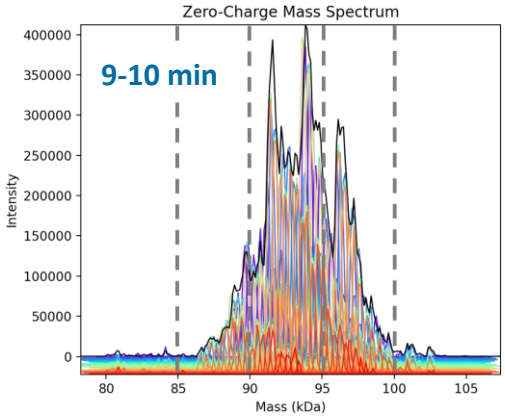






Identification of different major species depending on the retention time window selected, showing the difference in the glycosylation pattern.





Summary

- New instrumentation provides exciting approach for the development of complex characterisation solutions:
  - New high-performance quad technology for narrow window isolation
  - Different ion activation strategies available
  - PTCR combined with extended range to *m/z* 16,000

- Application for native TD-MS on the chromatographic time scale enabled assessment of various charge variant species present and provided insight into their identity with verification using peptide mapping.

- Ability to explore proteoform specific glycosylation, important for understanding complex formats such as bispecific antibodies.

- DIA-PTCR on the LC timescale very exciting for characterization of highly heterogenous molecules such as FcFP's, annotation of proteoforms identified ongoing.

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CASSS-MS Scientific Organizing Committee

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Proteoform Specific Microheterogeneity Assessment of Biopharmaceuticals Using a Modified Orbitrap Tribrid Mass Spectrometer

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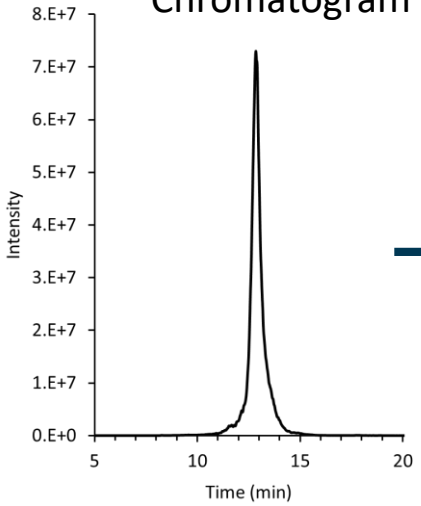
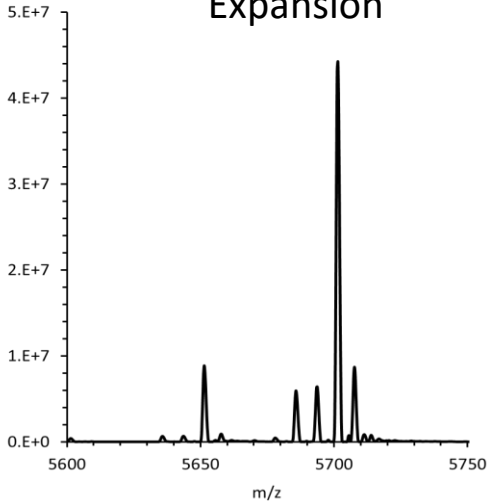
[2]Thermo Fisher Scientific, Reinach, Switzerland.
[3]Thermo Fisher Scientific, San Jose, California, United States.
[4]School of Chemical and Bioprocess Engineering, University College Dublin, Dublin, Ireland.

Evaluating chain pairing in bispecific antibodies

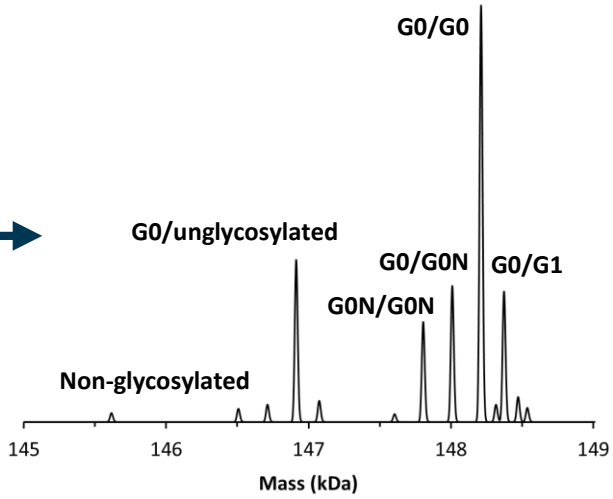
Amivantamab SCX Chromatogram



26+ Charge State Expansion



Deconvoluted masses

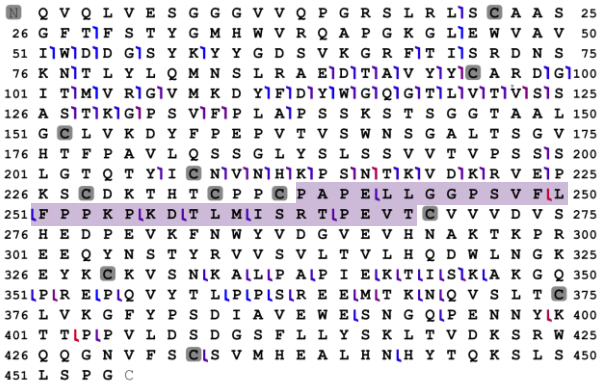


A key product quality attribute for bispecific antibodies is determination of miss paired chains. Here, amivantamab was analysed by pH gradient CEX-MS/MS using Orbitrap Ascend Biopharma. Major species identified post deconvolution are show, the question was then which chain of the molecule carried which identified modification?

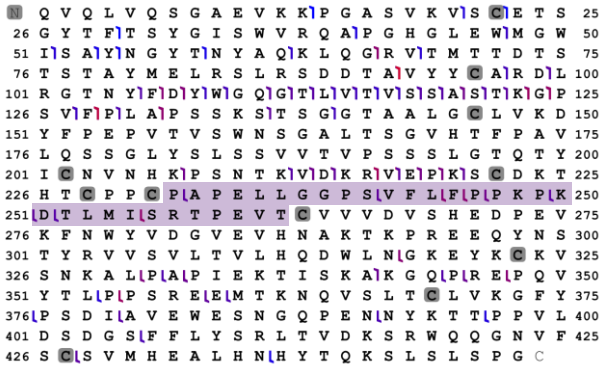
Annotation of modifications on Amivantamab bispecific

- Glycans present on amivantamab were all afucosylated
- Isolation of a specific glycoform using narrow quad isolation and fragmentation using various techniques cited before
- Fragmentation maps show that either chain of the bispecific could contain the glycan or be un-glycosylated, as demonstrated by the presence of fragment ions for each configuration, even in this poorly accessible region of the sequence

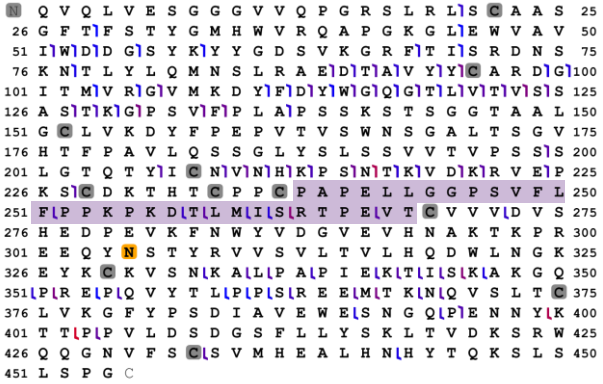
Amivantamab heavy chain A unglycosylated



Amivantamab heavy chain B unglycosylated



Amivantamab heavy chain A G0 glycoform



Amivantamab heavy chain B G0 glycoform