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Top-Down CDR Sequencing of Native Intact mAbs Through Deconvolution of HC+LC Mixture Spectra

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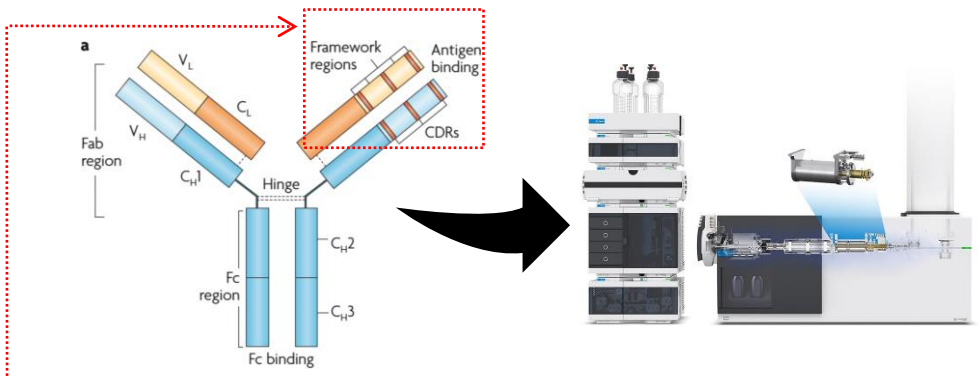
Introduction

Recent investigations into electron-based fragmentation (ExD) of native intact monoclonal antibodies (mAbs) have demonstrated cleavage of disulfide bonds connecting heavy chain (HC) and light chain (LC), exposing Complementarity-Determining Regions (CDR) and framework domains of both subunits to efficient top-down (TD) fragmentation [1]. This allows for sequence confirmation of the HC+LC CDRs within minutes, without introducing artifacts related to sample preparation. Here we introduce the ability to confirm multiple sequence hypotheses inside ExDViewer against a single TD mixture spectrum. We have also added automated methods that infer the correct ion assignment when multiple are plausible from HC+LC mixture spectra. HC and LC subunits are simultaneously interrogated against a single mixture spectrum in seconds and resulting fragmentation is visualized with customizable graphics.
ExDViewer available as Freeware [3] at *exdviewer.agilent.com*

Experimental

Sample Preparation

NIST mAb was purchased from NIST (Gaithersburg, MD). Intact mAbs were buffer exchanged into 100 mM ammonium acetate using Amicon Ultra 0.5 mL 10 kDa centrifugal molecular weight cutoff filters (Sigma, St. Louis, MO, USA). Working solutions of intact mAb were prepared at 1 mg/mL before introduction into the mass spectrometer [2].

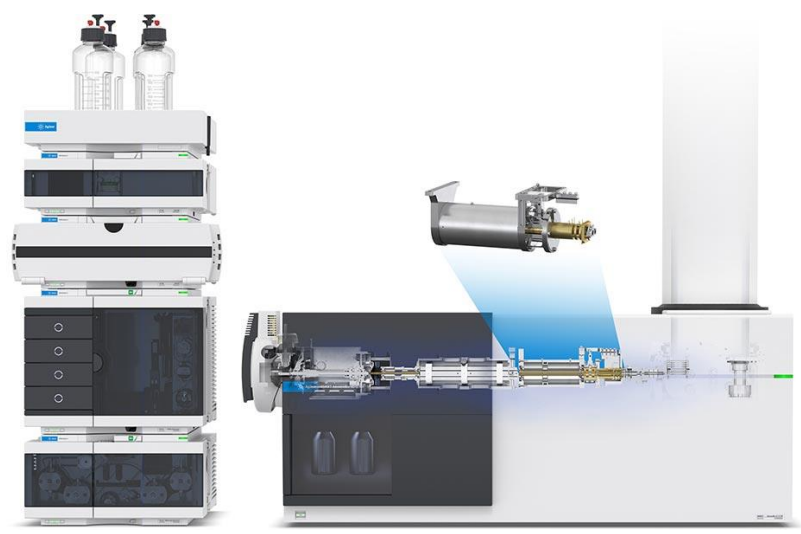


- Direct infusion of intact mAb in minutes
- Compatible with Capillary Electrophoresis (CE) and nano-Flow infusion techniques
- *No introduction of sample-handling artifacts*, like Post-Translational Modifications (PTMs), related to enzyme digestion and reduction/alkylation
- Top-down ECD fragmentation is most efficient at fragmenting the exposed CDR3 variable regions, as intact disulfide bonds hinder fragmentation of the constant region that would otherwise compete for ion signal.

Experimental

Instrument Analysis

Electron Capture Dissociation (ECD) experiments were performed using the 6545XT AdvanceBio LC/Q-TOF (Agilent, USA) mass spectrometer modified to enable ECD by installation of a second generation ExD cell (Agilent, USA). Static nanospray was used to introduce the mAb (1 mg/ml 100 mM ammonium acetate). Native Carbonic Anhydrase was used for tuning the ExD cell. ECD spectra of antibodies were recorded in MS1 mode using a low mass cutoff to eliminate ions below approximately *m/z* 4,000. Supplemental collision energy of 100V was added to aid fragmentation [2].



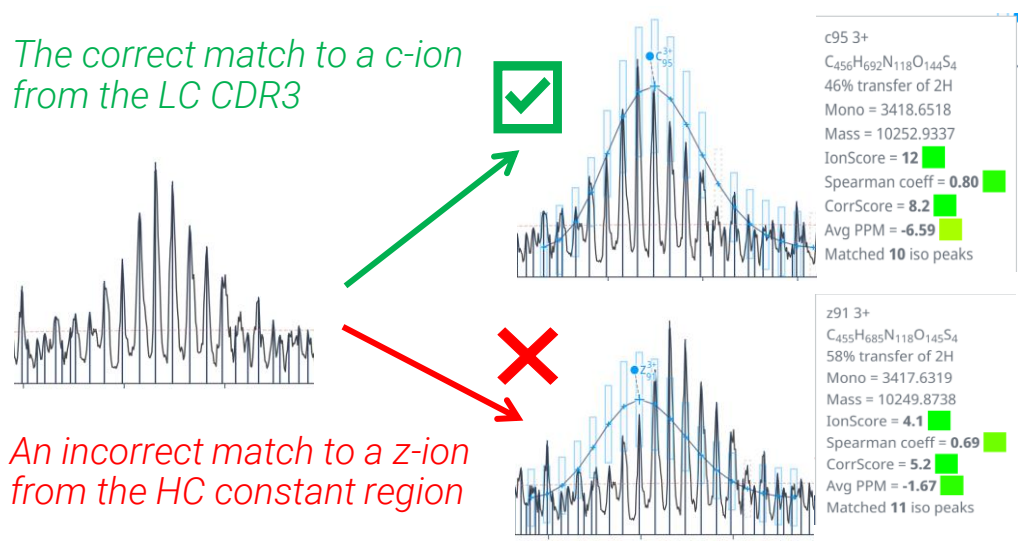
Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer with ECD cell shown in insert.

ExDViewer Data Analysis

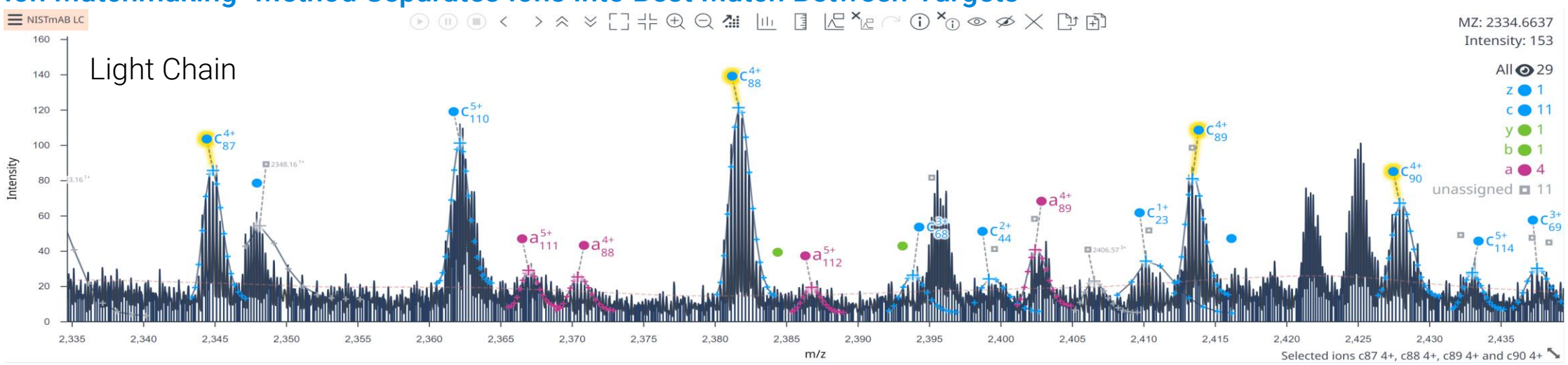
Download and Install Freeware [3] at *exdviewer.agilent.com*

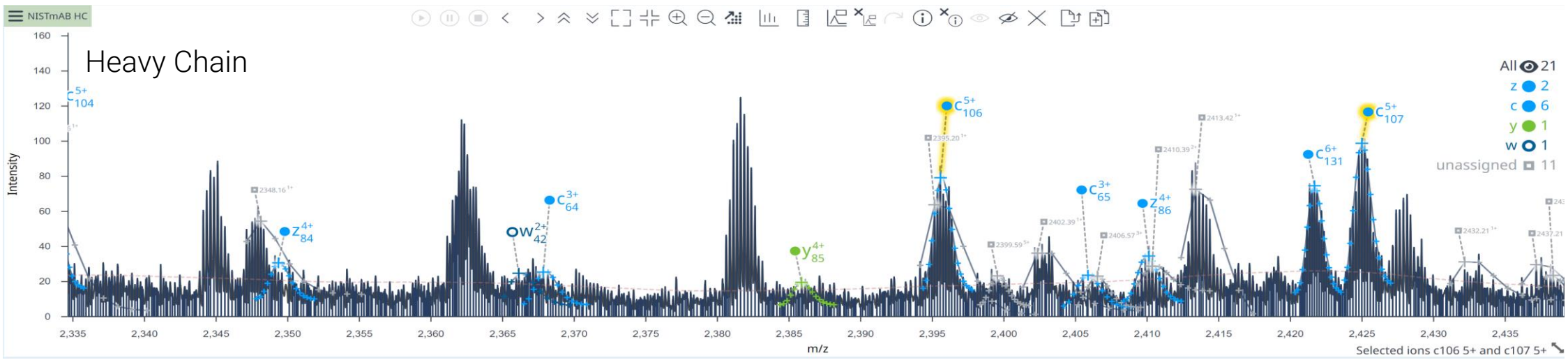
Input → Scan Selection → Peak Picking → Deconvolution → Matching

- Specify input spectra, including .d format
- Assign to co-eluting HC and LC target sequences
- Enable automatic averaging of profile data to improve signal-to-noise ratio (SNR)
- Default peak picking and deconvolution parameters
- Optionally enable ***Ion Matchmaking***



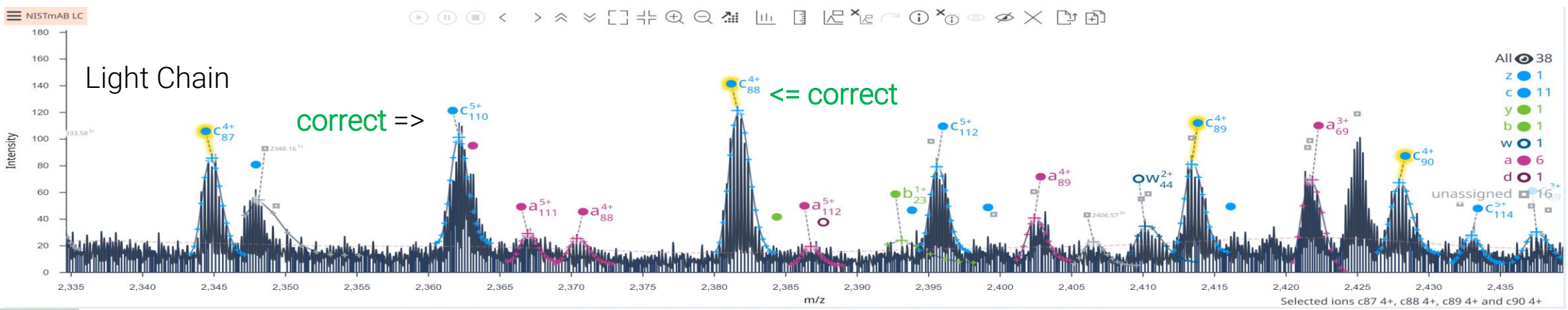
"Ion Matchmaking" Method Separates Ions Into Best Match Between Targets

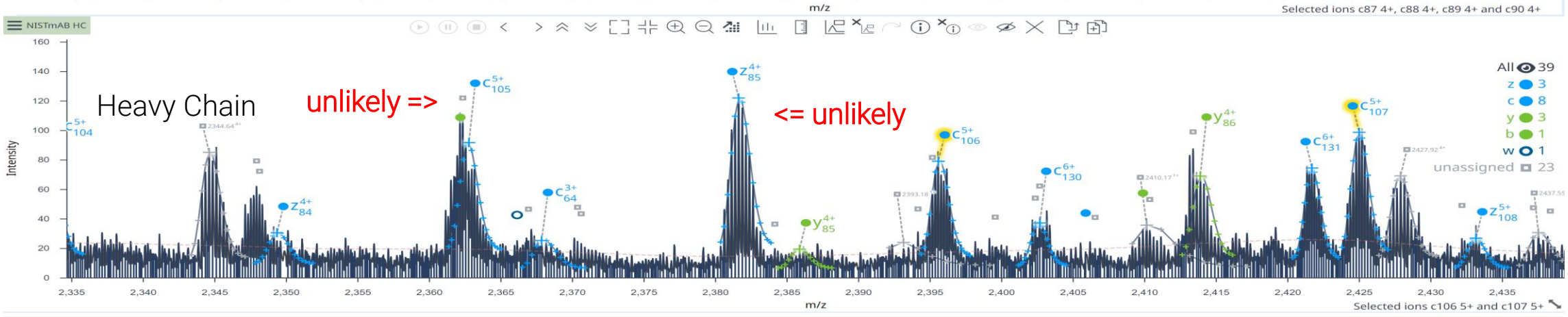




c-ions matching CDR3 domains are highlighted. "Ion Matchmaking" method enables confidence in automatic identification of ambiguous ion assignments matching both HC and LC. CDR3 binding domain has low ion overlap in LC and HC due to disulfide bonds in the effector region blocking C-terminal fragments.

Standard Ion Matching for Each Target Results in incorrect assignment of Ions Overlapping with the CDR3 domain

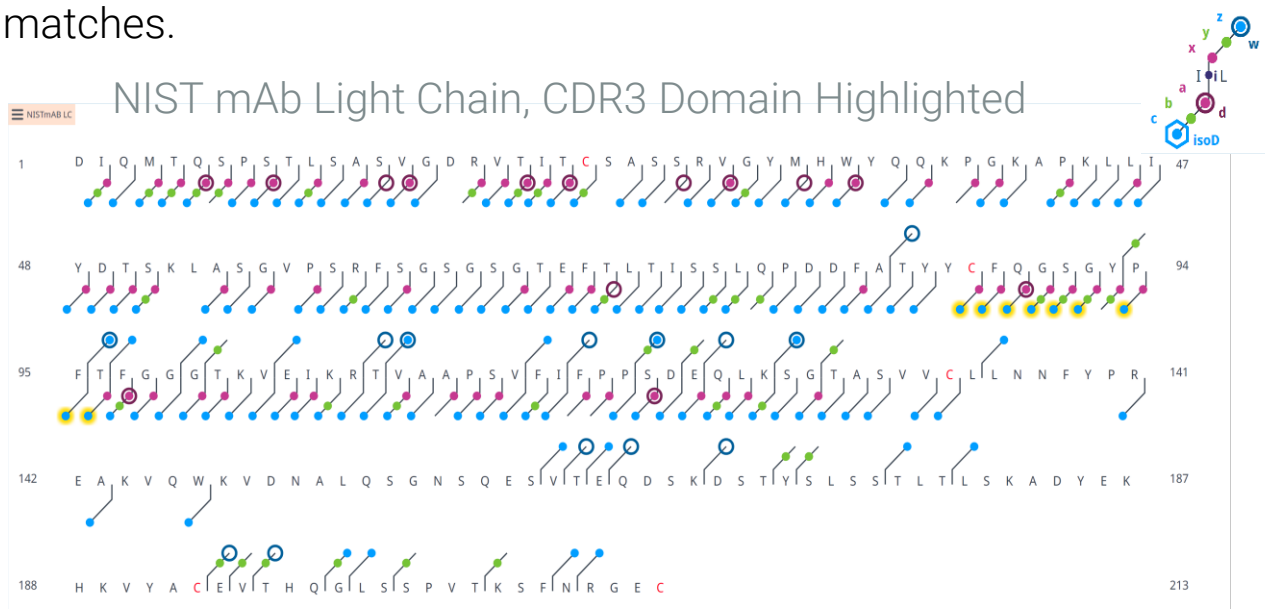


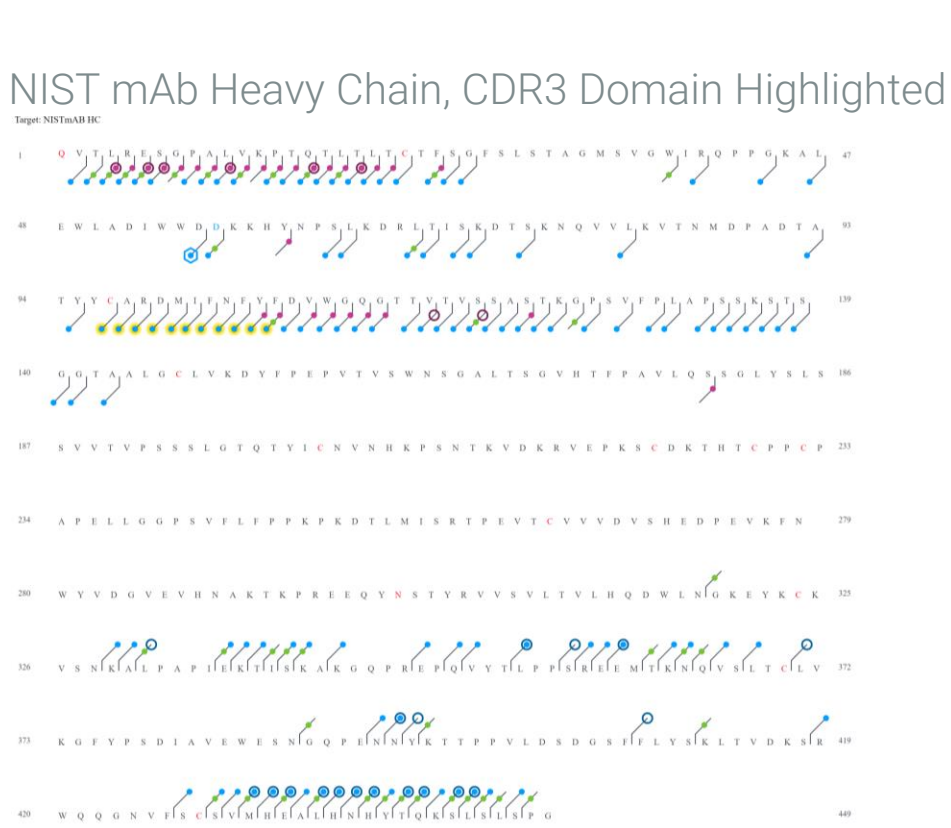


Highlighted ions showcase *c*-ion overlap between heavy and light chain. As compared to "Ion Matchmaking" method, overlap can be seen not only between *c/z*-ions but also assignment of other ions in place of *c*-ions. Ion coverage of the CDR3 binding domain results in some ions being assigned incorrectly between LC and HC.

Results and Discussion

ExDViewer produced de-charged and de-isotoped masses that formed a nearly-complete series of fragments corresponding to those expected for CDR sequences. Due to the complex nature of HC+LC mixture spectra, “ambiguous” overlapping isotopic clusters can be frequent between both chains. Here we can easily verify that 3-7+ charge states were commonly detected for each *c*-ion and *b*-ion series over CDRs and were not compromised by ambiguous ion matches.





Chain	Ion Matchmaking	Overlapping CDR3 Pos	Charge	#True	#False
HC	Enabled	97-106	3 – 6+	92	5
LC	Enabled	87-95	3 – 6+	171	11
HC	Disabled	97-106	3 – 6+	99	16
LC	Disabled	87-95	3 – 6+	182	29

All ions in the *m/z* range defined by c-ions 97-60 (HC) and 87-95 (LC) (3-6+) were manually inspected for accurate assignment. Assignments can vary between individuals.

Conclusions

- ECD fragmentation yielded 45-50% and 75-85% coverage of the HCs and LCs, respectively, in the variable region.
- Ion Matchmaking significantly reduced the proportion of false ion matches overlapping in the region of the spectrum containing expected CDR3 *c*-ions, enabling faster verification by manual inspection.
- ECD fully fragmented all three CDRs for LCs quite consistently, whereas only the CDR3 region was determined with a high confidence for the HCs.
- *Manual verification of some low intensity ions can vary between individuals; thus we recommend drawing sequencing verification from the presence of multiple high scoring c-ions of consecutive charge state (3-6+) and default Ion Score threshold (> 1.5). This was observed for the complete CDR3 of each HC and LC.*

References

[1] Shaw JB, Liu W, Vasil Ev YV, Bracken CC, Malhan N, Guthals A, Beckman JS, Voinov VG. Direct Determination of Antibody Chain Pairing by Top-down and Middle-down Mass Spectrometry Using Electron Capture Dissociation and Ultraviolet Photodissociation. Anal Chem. 2020 Jan 7;92(1):766-773. doi: 10.1021/acs.analchem.9b03129. Epub 2019 Dec 12. PMID: 31769659; PMCID: PMC7819135.

[2] Vasil Ev YV, Franklin R, Harea MC, Guthals A, Beckman JS. Top-down characterization of native monoclonal antibodies obtained with electron capture dissociation on Q-TOF instruments. ASMS 2023.

[3] ExDViewer is Agilent proprietary software that is distributed at no monetary cost to users under specific terms of use defined in the End User License Agreement. ExDViewer is not open-source software. Download at exdviewer.agilent.com.

Conflict of Interest Disclosure

Authors Derrill Sturgeon, Stelios Gkekas, Panos Iatrou, and Alex Gavrilenko are employees of Devicepros, who work under contract of Agilent Technologies, which sells the instrumentation used in this analysis.

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