Poster Reprint

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# Combining Microdroplet Reactions with ExD Fragmentation for Fast Characterization of Proteins and Antibody Subunits

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## Introduction

Monoclonal antibodies (mAb) represent an increasingly large share of disease treatment strategies which hold promise for combating many existing ailments. In-depth characterization of mAbs with mass spectrometry is crucial for ensuring safety and efficacy after development. Current analysis methods involve extensive offline reduction and digestion reactions which are time-consuming and increase the risk of introducing artifacts. The unique design of the Agilent AJS source enables fast, mAb characterization with microdroplet reactions which has been demonstrated to accelerate chemical reduction and digestion reactions.[1] Here, we further enhance microdroplet characterization by utilizing electron-capture dissociation (ECD) for sequence and post translation modification analysis. Combining microdroplet accelerated reactions with ECD fragmentation capabilities, the characterization of mAbs can be improved while reducing time and cost.

## Experimental

NIST mAb was buffer exchanged into 5 mM ammonium bicarbonate using BioRad p-6 micro biospin columns and diluted to 1 mg/mL in 5 mM ammonium bicarbonate. 10 mg/mL dithiothreitol (DTT) dissolved in 5 mM ammonium bicarbonate and dispensed in a separate vial for loading onto the LC. The reduction reaction was conducted using an autosampler injection program which drew up 10 µL of DTT solution and then 5 µL of the NIST mAb sample. The volume is then mixed in the autosampler needle and injected into the flow path of the instrument directly to the mass spectrometer ion source. Upon arrival at the ion source the mixture is aspirated, initiating the microdroplet reaction where DTT reduces the disulfide bonds in microseconds. The resulting product ions are analyzed using the 6545XT AdvanceBio LC/Q-TOF (Figure 1). Reaction products were analyzed in MS1 mode to verify reaction efficiency then targeted MS2 was used to isolate the light chain (LC) subunits for ECD fragmentation analysis. ExD viewer software was then used to analyze ECD fragments for total fragmentation coverage. In some cases, additional collision induced dissociation (CID) was used to increase fragmentation coverage. Table 1 and Table 2 delineate the conditions used for the LC/MS system and the injector program used to automate mixing of the reagents prior to injection. Tuning of the ExD cell was conducted using the autotune feature with the steps outlined in Figure 2. Figure 3 shows the schematic of the lenses that were tuned.

## Experimental

Figure 1. Agilent 6545XT Advance Bio LC/Q-TOF paired with Agilent 1290 Infinity II Bio LC fitted with new generation of ExD cell.

| Agilent 1290 Infinity II Bio LC | |
|---|---|
| **Column** | Union |
| **Thermostat** | 4 °C |
| **Mobile Phase A** | 5mM Ammonium Bicarbonate |
| **Column Temp** | 25 °C |
| **Flow Rate** | 0.025 to 0.300 mL/min |
| **Injection Volume** | 5 µL |
| **Mass on Column** | 5 µg |

| Injector Program | | |
|---|---|---|
| **Function** | **Parameter** | |
| Draw | 10 µL of 10 mg/mL DTT | |
| Draw | 5 µL of 1 mg/mL NIST mAb | |
| Mix | Mix from air 4 times | |
| Remote | Set remote line "start" for 125 ms | |
| Wait | Wait 0.1 min | |
| Inject | Inject | |
| **Time (min)** | **%A** | **Flow Rate (mL/min)** |
| 0.0 | 100% | 0.300 |
| 0.1 | 100% | 0.300 |
| 0.2 | 100% | 0.025 |
| 1.9 | 100% | 0.025 |
| 2.0 | 100% | 0.300 |
| 3.5 | 100% | 0.300 |

Table 1. LC conditions and injector program used for microdroplet reactions.

| Agilent 6545XT Q-TOF w/ExD Cell | |
|---|---|
| **Source** | Dual Agilent Jet Stream |
| **Gas Temp** | 360 °C |
| **Gas Flow** | 12 L/min |
| **Nebulizer** | 60 psi |
| **Sheath Gas Temp** | 400 °C |
| **Sheath Gas Flow** | 12 L/min |
| **Capillary Voltage** | 5000 V |
| **Nozzle Voltage** | 2000 V |
| **Fragmentor** | 380 V |
| **Skimmer** | 45 V |
| **Octupole RF Voltage** | 750 $V_{p-p}$ |
| **Mass Range** | 200-3200 *m/z* |
| **Scan Rate** | 2 scans/s |
| **Slicer Position** | High Resolution |
| **Acquisition Mode** | Positive, Standard (3200 *m/z*) Mass Range |
| **Detector Mode** | ADC: 2 GHz |

Table 2. MS conditions used for microdroplet reactions with ExD cell.

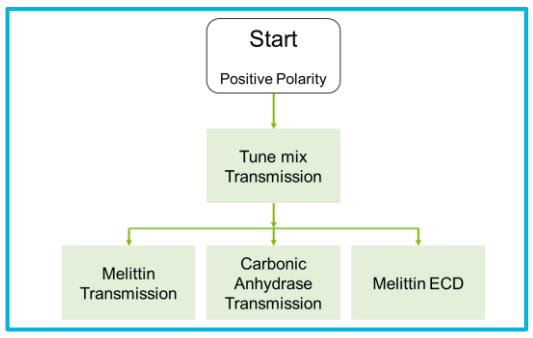


Figure 2. Tuning steps for optimizing the ExD cell.

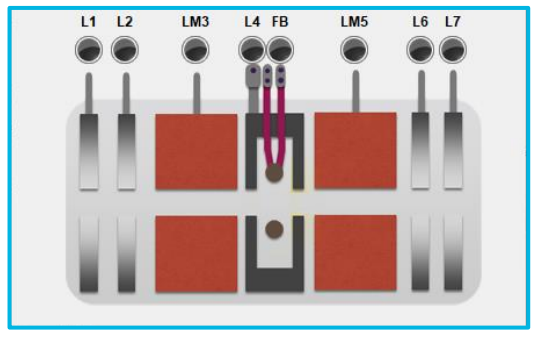


Figure 3. Schematic of ExD cell lenses and filament

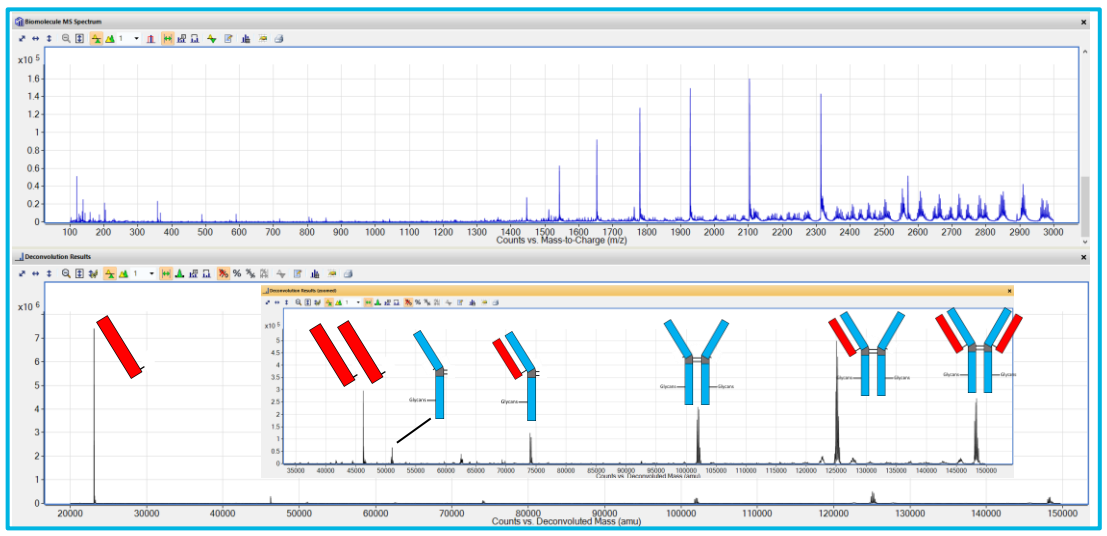


Figure 4. (*top*) Raw mass spectrum of products of NIST mAb microdroplet reduction reaction. (*bottom*) Deconvoluted mass spectrum; the most abundant product is the LC.

## Verifying Microdroplet Reaction Efficiency

To verify the effectiveness of the microdroplet reaction tests were conducted in various buffer conditions, concentrations, and injection volumes to achieve optimal reduction efficiency and detection of reaction products. Under the conditions outlined in Tables 1 and 2 the NIST mAb samples were successfully reduced via microdroplet reactions prior to being detected in the mass spectrometer. The most abundant product detected was the reduced light chain; other products were also detected such as the intact NIST mAb, heavy chain, and other partially reduced species. The deconvoluted spectrum of the microdroplet reduction reaction and the raw mass spectral data are displayed in Figure 4.

## ECD Fragmentation of NIST mAb LC with ExD Cell

To show the effectiveness of the ExD cell in characterizing middle down products of microdroplet ractions a charge state of the NIST mAb LC (1779 *m/z*; $13^{+}$) was set to be isolated by the quadrupole for ECD fragmentation. The resulting fragmentation pattern was analyzed using the ExD Viewer software, which is displayed in Figure 5. Many of the observed fragments are reported as c/z ions, typically associated with ECD fragmentation, with some ions being reported as b/y ions

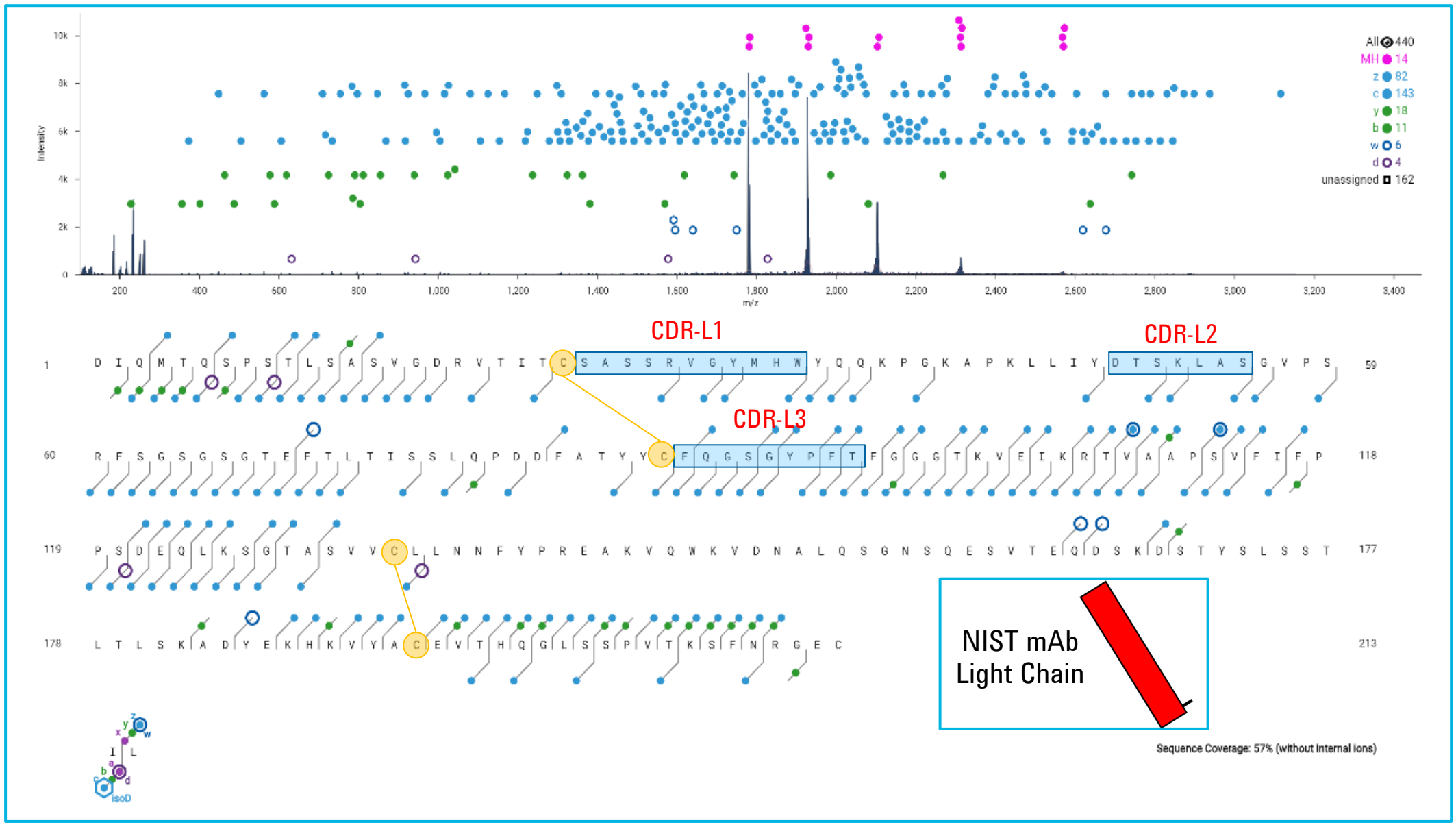


Figure 5. Coverage map of NIST mAb LC after microdroplet reduction. Results show 57% coverage using "restrictive scoring" parameter in ExD Viewer software. CDR-L3 domain has nearly complete ECD coverage. Native disulfide linkages are displayed in yellow.

## Results and Discussion

associated with CID. The overall coverage was reported at 57% using a restrictive scoring parameter outlined in the software. 20 V of CID energy was found to help improve ECD coverage. High levels of coverage were reported at the N- and C-termini and in between the intra-disulfide cysteines. The CDR-L3 domain also exhibited nearly complete coverage, which shows the advantage of ECD as the CDR-L3 domain is typically difficult to sequence via CID fragmentation. The MS analysis showed that some population of the NIST LC had internal disulfides reduced. This is confirmed by the fragmentation pattern observed in the coverage map.

### ECD Fragmentation of NIST mAb ½$F_c$ with ExD Cell

NIST mAb was digested offline with Fabricator obtained from Genovis. The ½$F_c$ fragments were the most abundant and the glycoform G0F (1403 *m/z*; $18^+$) was isolated in the quadrupole for ECD fragmentation. C1-C2 and C3-C4 disulfides remained intact for the ½$F_c$ . Fragmentation was limited in these disulfide-protected regions Figure 6. Similar to the intact NIST mAb microdroplet reduction experiments, internal disulfide bonds remain intact even if subjected to DTT reduction in the ion source.

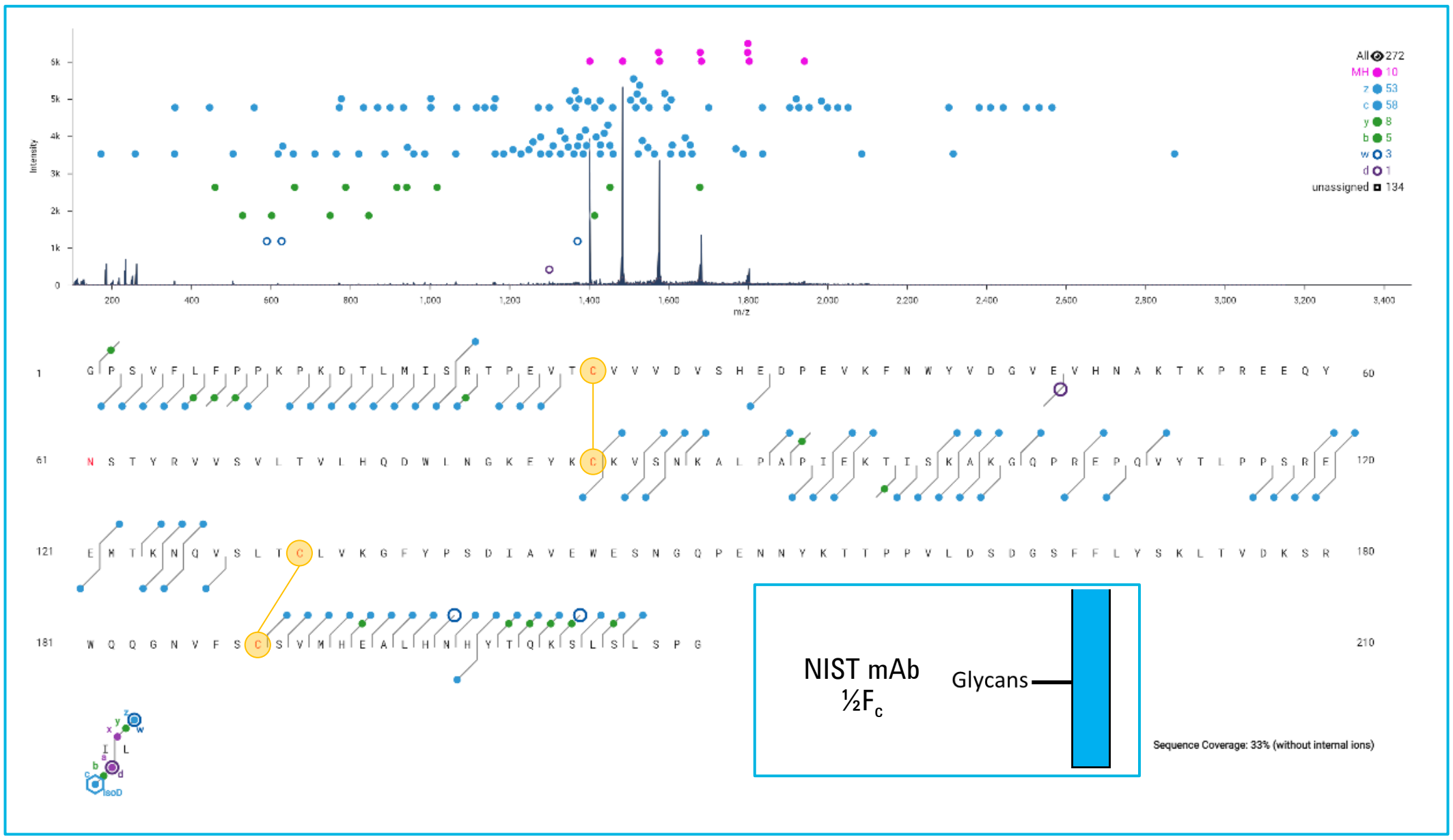


Figure 6. Coverage map of NIST mAb ½$F_c$ with offline digestion. Results show 33% coverage using “restrictive scoring” parameter in ExD Viewer software. Disulfide linkages are displayed in yellow.

## Conclusions

These experiments demonstrate the capabilities of the Agilent 6545XT AdvanceBio LC/Q-TOF with the ExD cell to do “middle-down” characterization of antibodies. The microdroplet reaction capabilities of the Agilent Jet Stream Source make possible the reduction of intact antibodies prior to ECD fragmentation. The results are encouraging and provide the impetus for continued experiments to expand the microdroplet reaction to enzymatic digestions to allow deeper characterization with ECD fragmentation in a time efficient and automated workflow.

## References

[1]Agilent App note: 5994-6752EN

https://www.agilent.com/en/promotions/asms



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