Application Note

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Closing the Loop: An Integrated Software Ecosystem for mAb and ADC Characterization from Data Acquisition Through Reporting

Authors

Ruoji Luo, Guannan Li, Lichen Xiu, Jordy Hsiao, Linfeng Wu, and David L. Wong

Agilent Technologies, Inc.

Abstract

Large and complex biomolecules, such as monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs), constitute a critical class of biopharmaceuticals with diverse therapeutic applications. Comprehensive analytical characterization of these molecules necessitates advanced instrumentation, including specialized hardware, consumables—particularly separation columns—and robust software solutions.

This application note focuses on developing an integrated software ecosystem that enables end-to-end analysis of mAbs and ADCs at multiple levels, including intact molecules and peptide fragments. The results demonstrate the software's ease of use and its capability to provide a complete solution for analyzing complex biopharmaceutical molecules and their critical quality attributes (CQAs).

Introduction

The analysis of complex monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), and their critical quality attributes (CQAs) is challenging.[1] It requires not only advanced analytical instrumentation and fit-for-purpose consumables, but also a well-integrated digital ecosystem that connects all components, including hardware, software, consumables, and personnel. Furthermore, such a digital ecosystem should be capable of supporting workstation-based solutions for individual laboratories or users while also being scalable to accommodate multiple laboratories and users. In addition, it should facilitate the secure sharing and updating of data, methods, results, and reports in a compliant manner.

Here, we demonstrate an integrated software platform (Agilent OpenLab CDS, with Agilent OpenLab ECM XT, Agilent MassHunter Qualitative Analysis, and Agilent MassHunter BioConfirm software), incorporating new hardware (Agilent 6230C LC/TOF), inert flow path (Agilent 1290 Infinity III Bio LC), and consumables (Agilent Altura Ultra Inert columns), to enable end-to-end data acquisition, analysis, and reporting of mAbs and ADCs at both the intact and peptide levels (Figure 1).

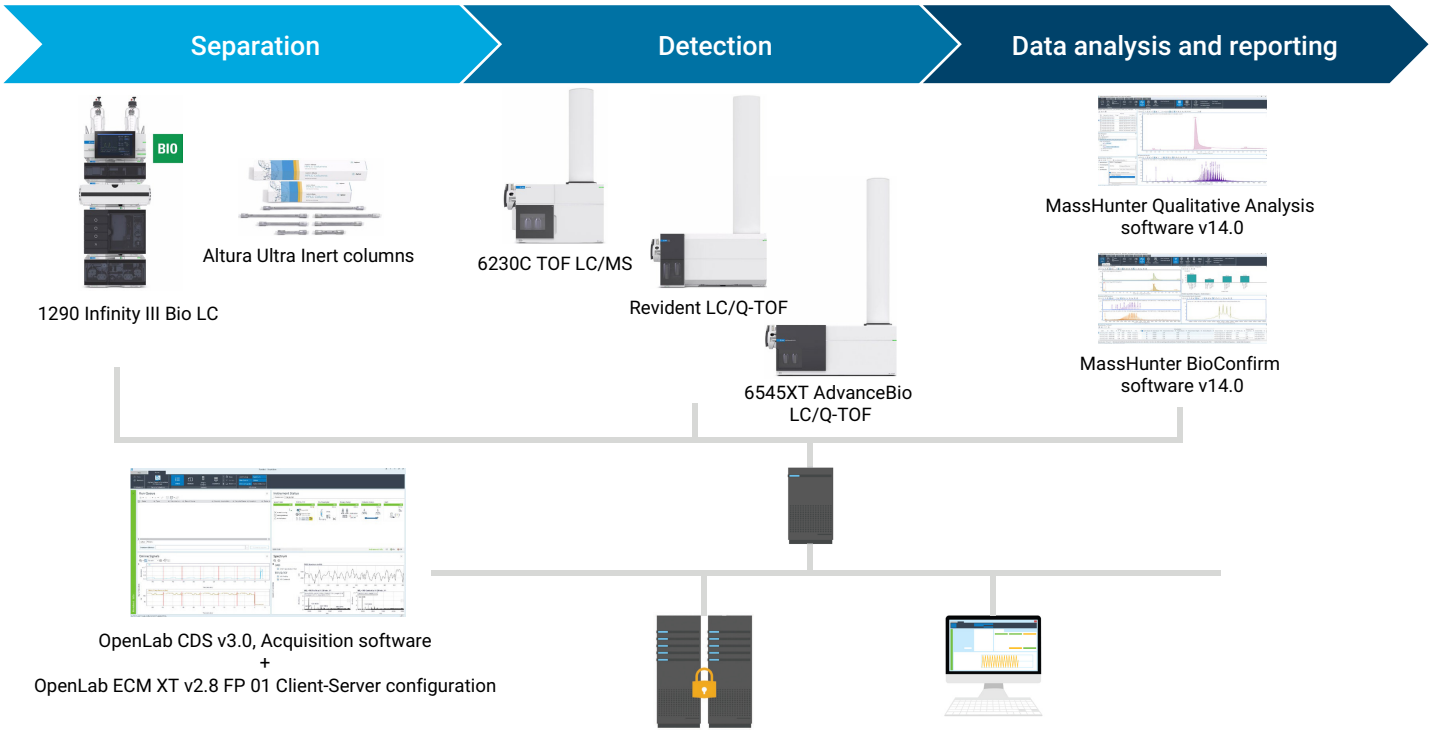


Figure 1. Solution overview.

Experimental

Materials and methods

Trastuzumab (Herceptin), trastuzumab emtansine (T-DM1) and monoclonal antibody standard RM 8671 (NISTmAb, p/n 5191-5745) were obtained from Genentech (South San Francisco, California, USA) and Agilent Technologies, respectively. Both trastuzumab and T-DM1 were digested with trypsin for the peptide mapping analysis. Other consumables including LC columns used are summarized in Table 1.

Table 1. Consumables for the solution.

Description	Part Number
Agilent Altura SEC 200 Å, 4.6 × 30 mm, 1.9 µm	PL1580-1201A
Agilent Altura PLRP-S 1000 Å, 2.1 × 50 mm, 5 µm	PL1912-1502A
Agilent Altura ZORBAX Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm	204215-308
Agilent InfinityLab bio solvent bottle, PP, 1 L, with cap	9301-6028
Agilent InfinityLab bio solvent inlet filter, 10 µm, PEEK	5320-0070
Agilent InfinityLab Quick Connect LC fitting	5067-5965
Agilent InfinityLab Acetonitrile for LC/MS	5191-5101
Agilent InfinityLab Water for LC/MS	5191-5121
Agilent LC/MS Grade Formic Acid	5191-4549
Agilent Polypropylene Vials	5188-2788
Agilent Snap Top Caps	5182-3458

A sample sequence was set up to run without human intervention for several days (Table 2). This was achieved through a combination of task actions, wait actions, and custom sample parameters. Unattended data analysis allowed samples to be processed automatically immediately after data acquisition was complete, using predefined data analysis and reporting methods.

Instrumentation

- Agilent 1290 Infinity III LC system including:
  - Agilent 1290 Infinity III Bio High-Speed Pump (G7132A)
  - Agilent 1290 Infinity III Bio Multisampler (G7137A)
  - Agilent 1290 Infinity III Multicolumn Thermostat (G7116B)
- Agilent 6230C LC/TOF
- Agilent Revident LC/Q-TOF
- Agilent 6545XT AdvanceBio LC/Q-TOF

Software

- Agilent OpenLab CDS version 3.0, Acquisition software
- Agilent OpenLab ECM XT, version 2.8 feature pack 01
- Agilent MassHunter BioConfirm software, version 14.0
- Agilent MassHunter Qualitative Analysis software, version 14.0

Table 2. A sequence for automated data acquisition, processing, reporting, and instrument control over several days.

						Sample Custom Parameters			
	Action	Sample Name	Inj/Vial	Acquisition Method	Processing + Reporting Method	BioConfirm Sequence	BioConfirm DAR 0 Mass	BioConfirm Drug/Linker Mass	BioConfirm Modification Profiles, and more...
Day 1	Inject	T-DM1	25	IntactProtein.amx	IntactProtein-DAR.bcpmx		148,059	957.5	
	Inject	Water	3	IntactProtein.amx					
	Inject	Herceptin	25	IntactProtein.amx	IntactProtein.bcpmx	Herceptin sequence			
	Inject	Water	3	IntactProtein.amx					
	Inject	NISTmAb	25	IntactProtein.amx	IntactProtein.bcpmx	NISTmAb sequence			
	Inject	Water	3	IntactProtein.amx					
	Task	Turn off the LC modules, switch MS to Eco Standby							
	Wait	Wait until next morning, e.g. 8:00							
	Task	Turn on all instruments, run mass calibration for MS							
Day 2	…								
Day 3	Inject	T-DM1	25	IntactProtein.amx	IntactProtein-DAR.bcpmx		148,059	957.5	
	Inject	Water	3	IntactProtein.amx					
	Inject	Herceptin	25	IntactProtein.amx	IntactProtein.bcpmx	Herceptin sequence			
	Inject	Water	3	IntactProtein.amx					
	Inject	NISTmAb	25	IntactProtein.amx	IntactProtein.bcpmx	NISTmAb sequence			
	Inject	Water	3	IntactProtein.amx					

LC/MS analysis

For intact-level analysis, trastuzumab, T-DM1 and NISTmAb were diluted to 1 µg/µL in deionized water prior to injection. Samples were separated using Altura SEC column (4.6 × 30 mm, 200 Å, 1.9 µm) and Altura PLRP-S column (2.1 × 50 mm, 1000 Å, 5 µm), for the native and denatured analysis, respectively. LC/MS analyses were conducted on a 1290 Infinity III LC coupled with a 6230C LC/TOF system equipped with a Jet Stream source. For peptide-level analysis, both trastuzumab and T-DM1 were digested with trypsin. The resulting peptides were separated using Altura ZORBAX Eclipse Plus C18 column (2.1 × 150 mm, 1.8 µm) and analyzed by 1290 Infinity III LC coupled with a 6545XT AdvanceBio LC/Q-TOF or Revident LC/Q-TOF system. Table 3 lists the LC/MS parameters used.

Table 3. LC and TOF/Q-TOF conditions.

	Native MS	Intact Protein	Peptide Mapping
Column	Agilent Altura SEC 200 Å, 4.6 × 30 mm, 1.9 µm	Agilent Altura PLRP-S 1000 Å, 2.1 × 50 mm, 5 µm	Agilent Altura ZORBAX Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm
Column Temperature	25 °C	80 °C	50 °C
Injection Volume	5/10 µL	0.5/1 µL	5 µL
Multisampler Temperature	8 °C	8 °C	8 °C
Flow Rate	0.2 mL/min	0.4 mL/min	0.4 mL/min
Mobile Phase	A) Water + 100 mM ammonium acetate pH 7 B) –	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Gradient	100% A, no gradient	Time (min) %B 0 5 0.1 20 4 50 5 70	Time (min) %B 0 2 5 2 38 38 47 95 50 95 51 2 60 2
Stop Time	3 min	5 min	60 min
Post Time	1 min	1.5 min	-
Drying Gas Temperature	300 °C	350 °C	350 °C
Drying Gas Flow	12 L/min	13 L/min	13 L/min
Nebulizer	60 psi	60 psi	35 psi
Sheath Gas Temperature	300 °C	400 °C	375 °C
Sheath Gas Flow	12 L/min	12 L/min	12 L/min
Capillary Voltage	5,500 V	5,500 V	3,750 V
Nozzle Voltage	2,000 V	2,000 V	0 V
Fragmentor	380 V	380 V	45 V
Skimmer	220 V	140 V	35 V
Instrument Mode	High mass stable	High mass stable	System tune
Acquisition Mode	MS	MS	Auto MS/MS
Mass Range	*m/z* 1,000–10,000	*m/z* 800–6,000	*m/z* 100–3,000
Acquisition Rate	0.5 spectra/s	1 spectra/s	3 spectra/s
Reference Mass	–	–	*m/z* 322.048, 922.0097

Results and discussion

Native MS versus denatured intact protein and DAR analysis

Figure 2 shows the native raw and deconvoluted mass spectra of Herceptin and T-DM1. Here, a well-defined charge envelope spanning *m/z* 5,200 to 10,000, corresponding to charge states from 15+ to 28+, was observed in the raw mass spectrum of Herceptin, with the 23+ charge state being the most abundant. A similar charge-state distribution was observed for T-DM1. After deconvolution, all major glycoforms of Herceptin were identified. For T-DM1, DAR 0 through DAR 7 were detected, with an average DAR value of 3.4.

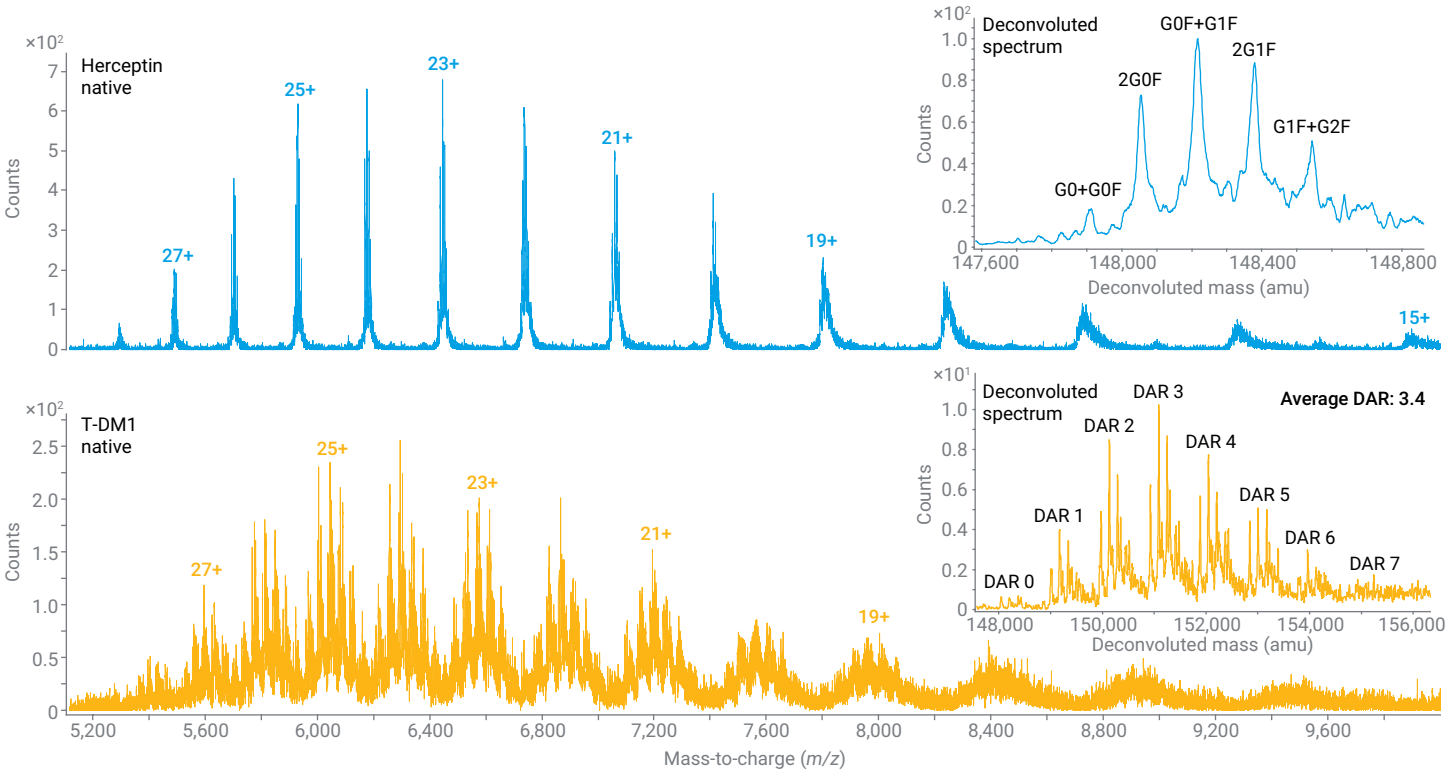


Figure 2. LC/MS analysis of Herceptin and T-DM1 under native MS conditions, measured by an Agilent 6230C TOF LC/MS with a sample load of 5 and 10 µg, respectively.

Figure 3 shows the deconvoluted mass spectra of Herceptin and T-DM1 under denaturing conditions. A well-defined charge envelope ranging from *m/z* 2,000 to 6,000, with charge states from 25+ to over 70+, was observed for Herceptin under denaturing conditions. After deconvolution, the same glycoforms identified under native conditions were also observed for Herceptin under denaturing conditions. For T-DM1, DAR 0 through DAR 8 were detected, with an average DAR value of 3.7.

Excellent intact protein analysis of Herceptin and accurate DAR analysis of T-DM1 were achieved under both native and denaturing conditions using newly developed Altura Ultra Inert columns coupled to a new 6230C TOF LC/MS system and the OpenLab CDS and MassHunter software suites.

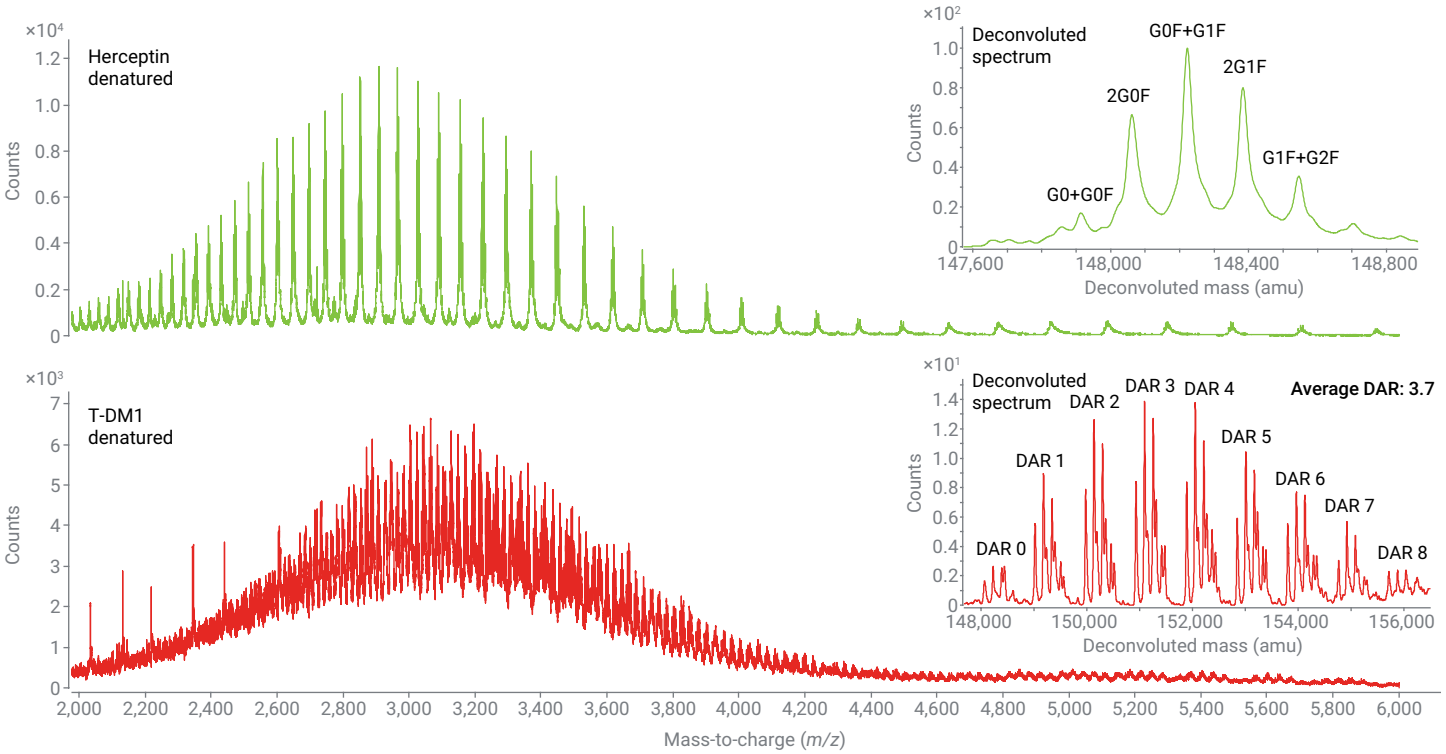


Figure 3. LC/MS analysis of Herceptin and T-DM1 under denaturing MS conditions, measured by an Agilent 6230C TOF LC/MS with a sample load of 1 µg.

Robustness of the solution

To demonstrate the robustness of the overall solution, a sequence of 75 replicate injections of T-DM1, Herceptin and NISTmAb was performed, as illustrated in Table 2. Figure 4 demonstrates minimal retention time shifts. Over the course of the 75 injections conducted across a three-day period, the solution achieved relative standard deviations of 0.25% and 0.26% for Herceptin and NISTmAb, respectively.

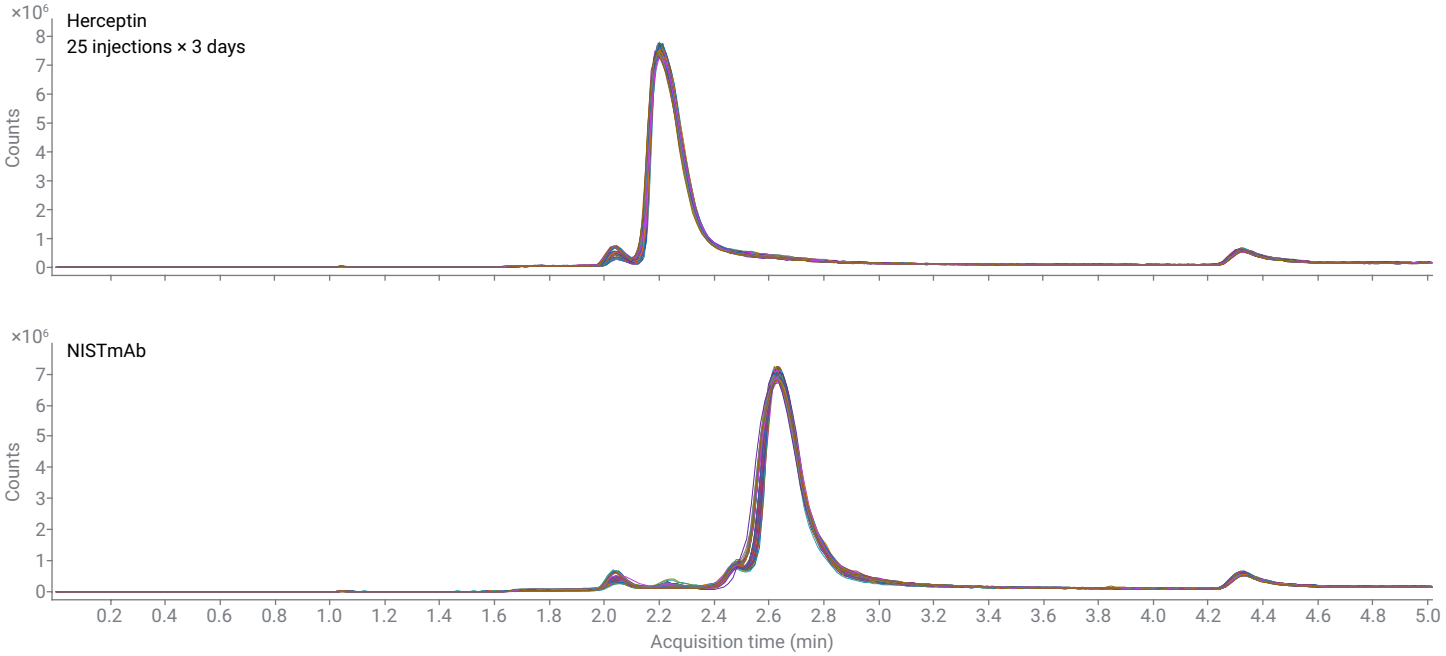


Figure 4. Overlay of 75 total ion chromatograms acquired over 3 days for reversed-phase separation of Herceptin and NISTmAb, measured by an Agilent 6230C TOF LC/MS with a sample load of 0.5 µg.

Peptide mapping

The total ion chromatograms (TICs) of trypsin-digested Herceptin, acquired using the Revident LC/Q-TOF and the 6545XT AdvanceBio LC/Q-TOF systems, are shown in Figure 5. The corresponding sequence coverage results are presented in Figure 6. Both instruments delivered similar TICs. A sequence coverage of 91.0% was obtained with the 6545XT AdvanceBio LC/Q-TOF, which is slightly higher than the 88.9% achieved with the Revident LC/Q-TOF. The sequence coverage patterns largely overlap between the two instruments.

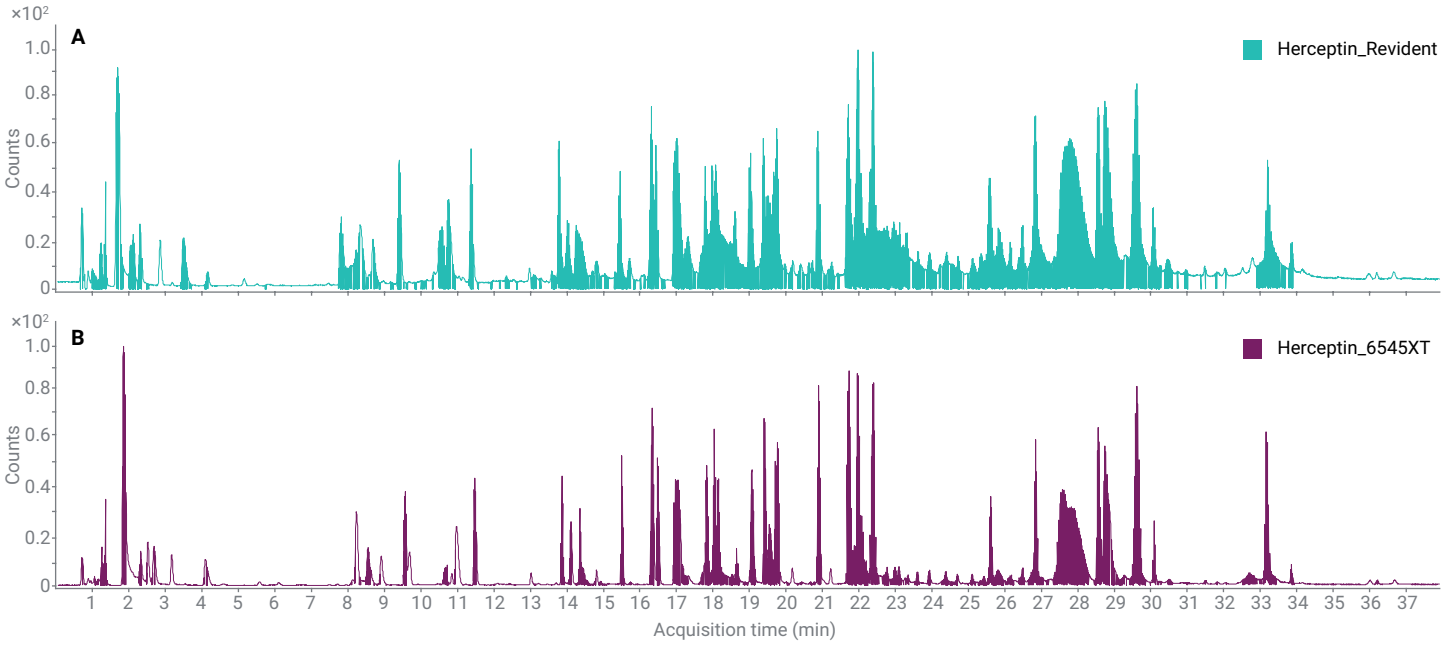


Figure 5. Total ion chromatograms for trypsin-digested Herceptin, measured by an Agilent Revident LC/Q-TOF (A) and Agilent 6545XT AdvanceBio LC/Q-TOF (B).

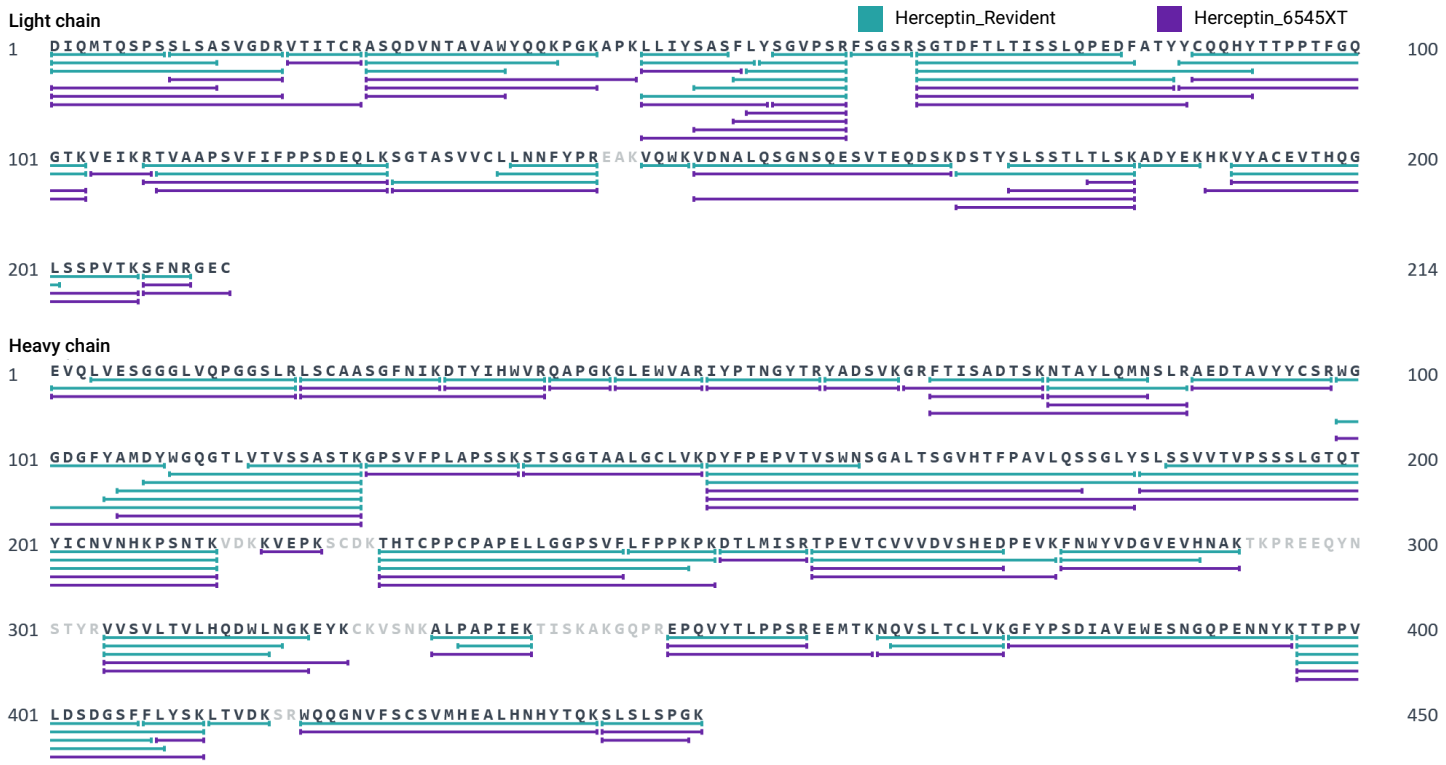


Figure 6. Sequence coverage map for Herceptin, measured by an Agilent Revident LC/Q-TOF (88.9%) and Agilent 6545XT AdvanceBio LC/Q-TOF (91.0%).

Figure 7 presents the collision induced dissociation (CID) MS/MS spectra for the peptide VVSVLTVLHQDWLNGK in its unmodified form and in a deamidated form, along with the corresponding fragment coverage maps. Both the native and deamidated forms achieved great MS/MS fragment ion coverage. Comparable results were obtained using 6545XT AdvanceBio LC/Q-TOF.

Reporting

Ready-to-use report templates are available for the analysis of intact protein, DAR, protein digest, released glycans, with built-in report builder supporting customized designs (Figures 8 through 10). This enables reports to be generated automatically and efficiently.

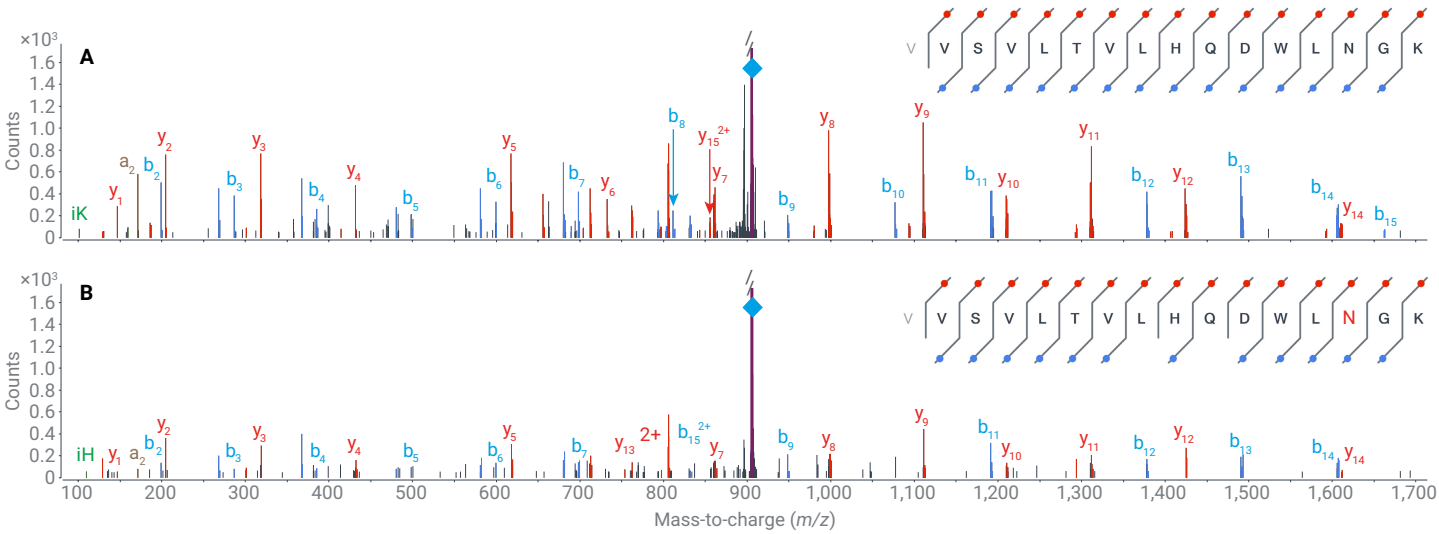


Figure 7. MS/MS spectra and fragment coverage maps for peptide VVSVLTVLHQDWLNGK in its unmodified form (A) and its N-14-deamidated form (B), measured by an Agilent Revident LC/Q-TOF.

BioConfirm Intact Protein Report

Agilent

Sample Information

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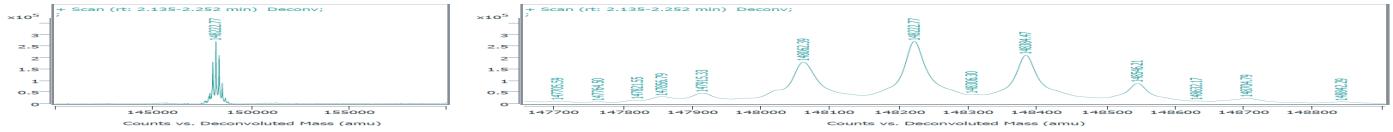
Confirmation Status	Confirmed	**Modifications**	||| mAb
		IRM Status	Success

Matched Sequences

Confirmation Status	Sequence Name	RT	Height	Mass	Tgt Seq Mass	Diff (Da)	Var Mods	Mods
Confirmed	HerceptinDigestAllLinksDefined	2.202	270213	148223	148221	2	1*G1F(1607.5013) + 1*G0F (NGA2F)(1445.3580) + 2*Lys-loss(-128.1750)	

...

Deconvoluted Spectra



...

Biomolecule Summary

Biomol	Sequence Name	Mass	Tgt Seq Mass	Diff (Da)	Area (MS)	Height (MS)	%Quant (Area)	%Quant (Height)	Mods	Var Mods
5	HerceptinDigestAllLinks Defined	147915	147913	3	2343895	46603	5.25	5.67		1*G0F (NGA2F)(1445.3580) + 1*G0 (NGA2)(1299.2142) + 2*Lys-loss(-128.1750)

...

Biomolecule Details

Biomolecule 1: A(1-214) + B(1-450) + C(1-214) + D(1-450) HerceptinDigestAllLinksDefined; 1*G1F(1607.5013) + 1*G0F (NGA2F)(1445.3580) + 2*Lys-loss(-128.1750)

Sequence Name	Mass	Tgt Seq Mass	Diff (Da)	Area (MS)	Height (MS)	%Quant (Area)	%Quant (Height)	Mods	Var Mods
HerceptinDigestAllLinksDefined	148223	148221	2	13832825	270213	30.99	32.88		1*G1F(1607.5013) + 1*G0F (NGA2F)(1445.3580) + 2*Lys-loss(-128.1750)

Biomolecule Spectra

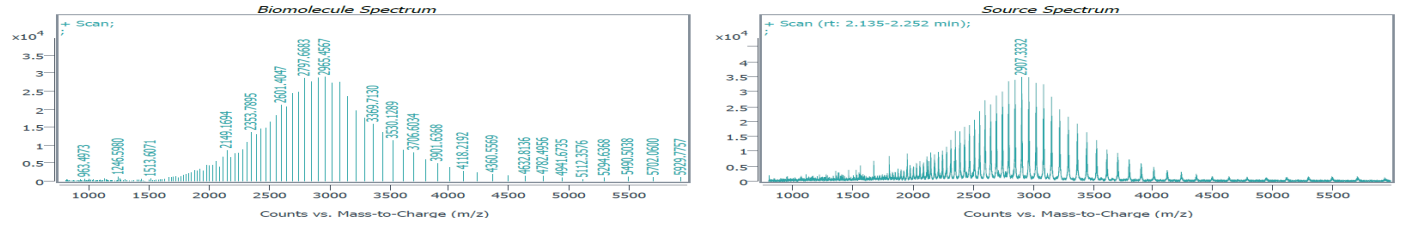


Figure 8. Report generated using the intact protein report template. A confirmation status of "Confirmed" indicates that the most abundant peak was matched to the intact protein.

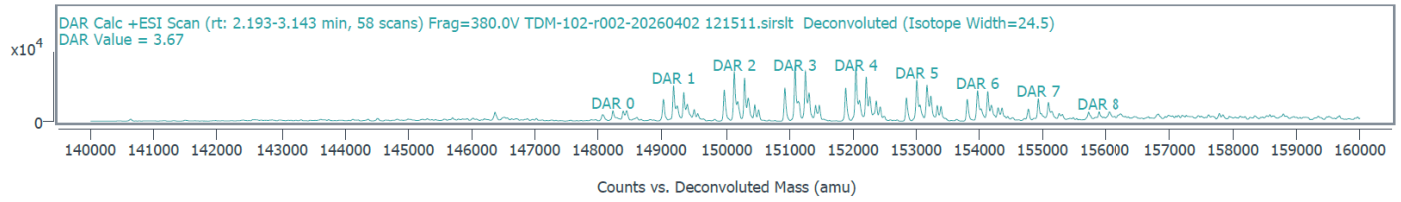
BioConfirm DAR Summary Report

Agilent

Sample Information

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DAR Summary



DAR Deconvoluted Peaks (max 10)

DAR Peak	Peak Mass (Da)	Area	% Area	Max Y
0	148226	330240	3.33	2076
1	149182	1174852	11.84	7010
2	150137	1541481	15.53	9852
3	151094	1728655	17.42	10825
4	152053	1753314	17.67	10770
5	153011	1461850	14.73	8164
6	153969	1136566	11.45	6029
7	154925	511893	5.16	4428
8	155884	284607	2.87	1857

Figure 9. Report generated using the DAR summary report template.

BioConfirm Protein Digest Report

Agilent

Sample Information

...

Sample Confirmation Status Confirmed **Enzyme** Trypsin

Sequence Summary

Confirmation Status	% Coverage	Sequence Name
Confirmed	90.96	HerceptinDigestAllLinksDefined

Sample Chromatogram List

...

Sequence Details

...

Coverage Map

...

Peptide Relative Quantitation Results

Sequence Name	Location	Variable Mods	File	Height	%Quant (Height)	Volume	%Quant (Volume)
HerceptinDigestAllLinksDefined	C214 [A\C]	Alkylation (iodoacetamide)	20251218_Herceptin_5uL_MAMHighFlow_CDS.sirslt	50569	100.00	509994	100.00

...

Biomolecule Summary

Biomol	Seq Loc	Mods	Var Mods	Score (Bio)	RT	Height	Mass	Tgt Seq Mass	Diff (Bio, ppm)
4	B(39-43)/D(39-43)			5.53	1.247	335268	499.2762	499.2754	1.58

...

Biomolecule Details

Biomolecule 4: B(39-43)/D(39-43) HerceptinDigestAllLinksDefined

Biomol	Seq Loc	Rule	Var Mods	RT	Height	Mass	Tgt Seq Mass	Diff (Bio, ppm)
4	B(39-43)/D(39-43)	Complete digest		1.247	335268	499.2762	499.2754	1.58

ECC (with sample chromatogram)

...

Mass Spectrum (with MFE spectrum, if available)

...

Fragment Spectra (if available)

...

Figure 10. Report generated using the protein digest report template.

Conclusion

An integrated software platform (OpenLab CDS version 3.0 Acquisition software, with OpenLab ECM XT, MassHunter Qualitative Analysis and MassHunter BioConfirm software), combined with new hardware (6230C LC/TOF) and consumables (Altura Ultra Inert columns), enables end-to-end workflows for the acquisition, analysis, and reporting of mAb and ADC data. The platform supports intelligent sequence management through task actions, wait actions, custom sample parameters, and other advanced features. Together, these capabilities deliver exceptional instrument robustness, reproducibility, and analytical accuracy.

Reference

1. Hao Liu; *et al.* Polarity- and Sequence-Dependent Ionization of Therapeutic Antibody–siRNA Conjugates: Enabling Intact Multi-Attribute Method for Comprehensive Characterization and Identity Release Assay. *Analytical Chemistry* **2026**, *98(10)*, 7422–7431.

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