

Drug-to-Antibody Ratio (DAR) Calculation of Antibody-Drug Conjugates (ADCs)

Using Automated Sample Preparation and Novel DAR
Calculator Software

Application Note

Authors

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Introduction

Antibody-drug conjugates (ADCs) are a new class of biotherapeutics that represent a rapidly growing portion of the drug discovery pipeline in pharmaceutical companies. ADCs are monoclonal antibodies chemically linked to biologically active small molecule drugs. By combining potent cytotoxic drugs with target-specific antibodies, ADCs deliver cytotoxic drugs to the diseased tissue while limiting the toxicity in the nontargeted tissues.

Drug-to-antibody ratio (DAR) is the average number of drugs conjugated to the antibodies, which is an important attribute of ADCs. The DAR value affects the efficacy of the drug, as low drug loading reduces the potency, while high drug loading can negatively affect pharmacokinetics (PK)¹ and toxicity. With the current conjugation chemistry, that is lysine side-chain amidation or cysteine interchain disulfide bond reduction, a drug load of 0 ~8 drugs (D0 ~ D8) per antibody is commonly observed.

LC/MS is a popular analytical method for measuring the DAR and the drug load distribution of ADCs. It is an essential method used in the identification of various drug-loaded ADCs species. In most cases, the intact ADC can be analyzed directly using LC/MS to determine the DAR value. The ADCs may be reduced before LC/MS analysis when specific DAR information on the light and heavy chains is needed. Additionally, the ADCs may be deglycosylated prior to LC/MS analysis to further reduce spectrum complexity.

ADC sample preparation prior to LC/MS analysis is typically done manually which can introduce variability and limit throughput. The Agilent AssayMAP Bravo is an easy-to-use automated sample preparation system that gives great reproducibility, labor savings in the form of reduced hands-on time, scalability (8–96 samples can be run simultaneously), simple person-to-person and site-to-site method transfer, and it minimizes the possibility of human error. AssayMAP Bravo is an excellent platform for providing automated sample preparation of the aforementioned reactions.



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The AssayMAP Bravo automated sample preparation platform together with the Agilent LC/MS and MassHunter/BioConfirm/DAR Calculator software provides a reproducible and easy solution for the DAR calculation of ADCs. In this Application Note, intact and reduced ADCs, with or without deglycosylation, were processed in parallel using the Agilent AssayMAP Bravo platform, and analyzed using the Agilent LC/MS. The DAR was then determined with the Agilent DAR calculator.

Experimental

Material

Rapid PNGase F was purchased from New England Biolabs (Ipswich, MA). Eppendorf 96-well PCR plates were from Eppendorf (Hauppauge, NY); Tris chloride and Tris(hydroxymethyl)aminomethane were purchased from EMD Millipore (Billerica, MA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

In-solution reduction and deglycosylation using Agilent AssayMAP Bravo

Lyophilized ADCs were reconstituted in deionized (DI) water to 5 mg/mL, aliquoted and stored at -80°C until used. All reagent transfers were performed using the Agilent AssayMAP Bravo platform (Santa Clara, CA) through Protein Sample Prep Workbench software. Five microliters of ADCs were dispensed into each well of the first four columns of a 96-well Eppendorf PCR plate (position A1-H4). Subsequently, 10 μL of 10 mM Tris buffer (pH = 7.5) (position A1-H2), or 35 mM DTT in 10 mM Tris buffer (pH = 7.5) (position A3-H4) were added to the samples. Finally, 20 μL of 10 mM Tris buffer (pH = 7.5) (positions A1-H1 and A3-H3) or rapid PNGase F (diluted 1:40) in 10 mM Tris buffer (pH = 7.5) (A2-H2 and A4-H4) were added to the samples. The sample plate was sealed using an Agilent PlateLoc Thermal Plate Sealer (Santa Clara, CA) and incubated at 50°C for 10 minutes.

LC/MS analysis

LC/MS analyses were conducted on an Agilent 6550 iFunnel Q-TOF (Santa Clara, CA) equipped with a Agilent Dual Jet Stream ESI source coupled with the Agilent 1290 Infinity UHPLC system (Waldbronn, Germany). Table 1 and Table 2 list the LC/MS parameters used. One microgram of sample was injected for each run.

Data analysis

Raw data obtained from LC/MS were deconvoluted with Agilent MassHunter Qualitative Analysis Software (Version B.07.00, Build 7.0.7024.0), with BioConfirm using the Maximum Entropy deconvolution algorithm. The deconvolution parameters were set as follows:

- For intact ADCs, mass range was set to 140,000–160,000 Da and mass step at 1 Da
- For reduced ADCs, mass range was set to 20,000–60,000 Da, mass step to 0.1 Da
- For both intact and reduced ADCs, average mass was calculated using top peak height at 25 %

The rest of the parameters were set to default. The deconvoluted spectra were then exported as a .csv file and imported into an Agilent DAR calculator. After inputting/selecting D0 mass and drug/linker mass, the DAR calculator automatically selects, annotates and integrates mass peak groups of ADCs with various drug loadings, and calculates the average DAR and generates a peak list table.

Results and Discussion

To determine the DAR of an example lysine conjugated ADC, ADCs were prepared in four different conditions (n = 8 for each condition); intact glycosylated, intact deglycosylated, reduced glycosylated, and reduced deglycosylated using the AssayMAP Bravo platform. They were analyzed with an Agilent 1290 Infinity UHPLC system equipped with a 2.1 mm ID 1000Å PLRP-S LC column which was coupled to the 6550 Q-TOF, followed by data analysis using Agilent MassHunter/BioConfirm/DAR Calculator software (Figure 1).

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity LC System		
Parameter	Intact	Reduced
Column	Agilent PLRP-S, 1000 Å 2.1 × 150 mm, 8 μm	Agilent PLRP-S, 1000 Å 2.1 × 150 mm, 8 μm
Sample thermostat	5 °C	5 °C
Mobile phase A	0.1 % Formic acid in water	0.1 % Formic acid in water
Mobile phase B	0.1 % Formic acid in acetonitrile	0.1 % Formic acid in acetonitrile
Gradient	0–5 minutes, 20 %B 5–10 minutes, 20–90 %B	0–5 minutes, 20 %B 5–6 minutes, 20–75 %B 6–10 minutes, 75–90 %B
Post time	None	None
Column temperature	80 °C	80 °C
Flow rate	0.4 mL/min	0.4 mL/min

Table 2. Mass spectrometer parameters.

Agilent 6550 Q-TOF LC/MS System		
Ion mode	Positive ion mode	Positive ion mode
Source	Agilent Dual Jet Stream	Agilent Dual Jet Stream
Drying gas temperature	290 °C	290 °C
Drying gas flow	14 L/min	14 L/min
Sheath gas temperature	400 °C	400 °C
Sheath gas flow	12 L/min	12 L/min
Nebulizer	40 psi	40 psi
Capillary voltage	4,500 V	4,500 V
Nozzle	1,500 V	1,500 V
Fragmentor voltage	250 V	250 V
Oct RF Vpp	750 V	750 V
Acquisition parameters MS mode	High (10,000 <i>m/z</i>) mass range, Extended mass range (2 GHz), MS only mode, Mass Range 1,800–6,000 <i>m/z</i>	High (10,000 <i>m/z</i>) mass range, Extended mass range (2 GHz), MS only mode, Mass Range 800–4,000 <i>m/z</i>

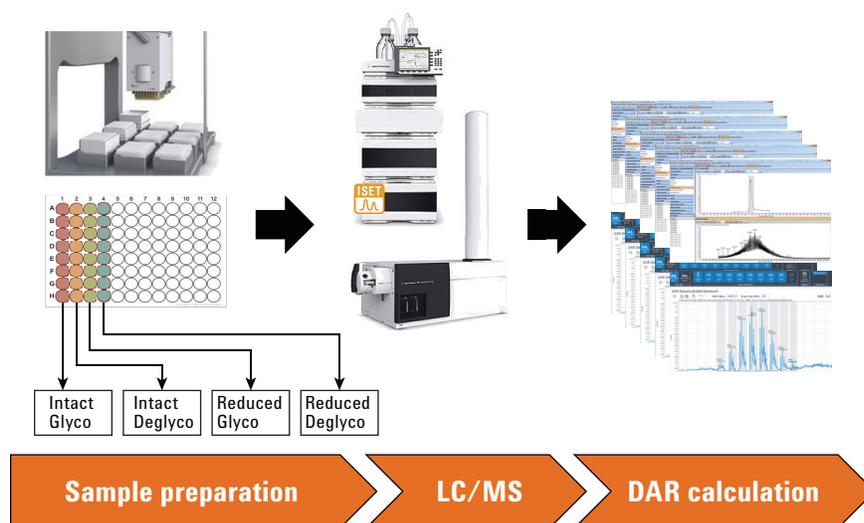


Figure 1. Workflow of the DAR calculation of ADCs.

Figure 2 shows the overlaid total ion chromatograph (TIC), extracted spectra, and the deconvoluted spectra of intact glycosylated (Figure 2 panels A-C), and intact deglycosylated (Figure 2 panels D-F) ADCs of eight replicates. Both intact glycosylated and intact deglycosylated ADCs eluted in less

than 1 minute with a mass envelope centered around 3,000 Da with charge states between +35 and +66. For intact glycosylated ADCs, nine peak groups were observed with masses matching D0–D8. The four major peaks observed in each peak group corresponds with glycoforms G0F/G0F, G0F/G1F, G1F/G1F

or G0F/G2F, and G1F/G2F. For intact deglycosylated ADC, 10 peak groups were observed with mass matching D0–D9. All glycoforms observed in glycosylated ADC samples were eliminated, and the peak mass was reduced correspondingly. The peak intensities were increased about 2-fold compared to the intact ADCs.

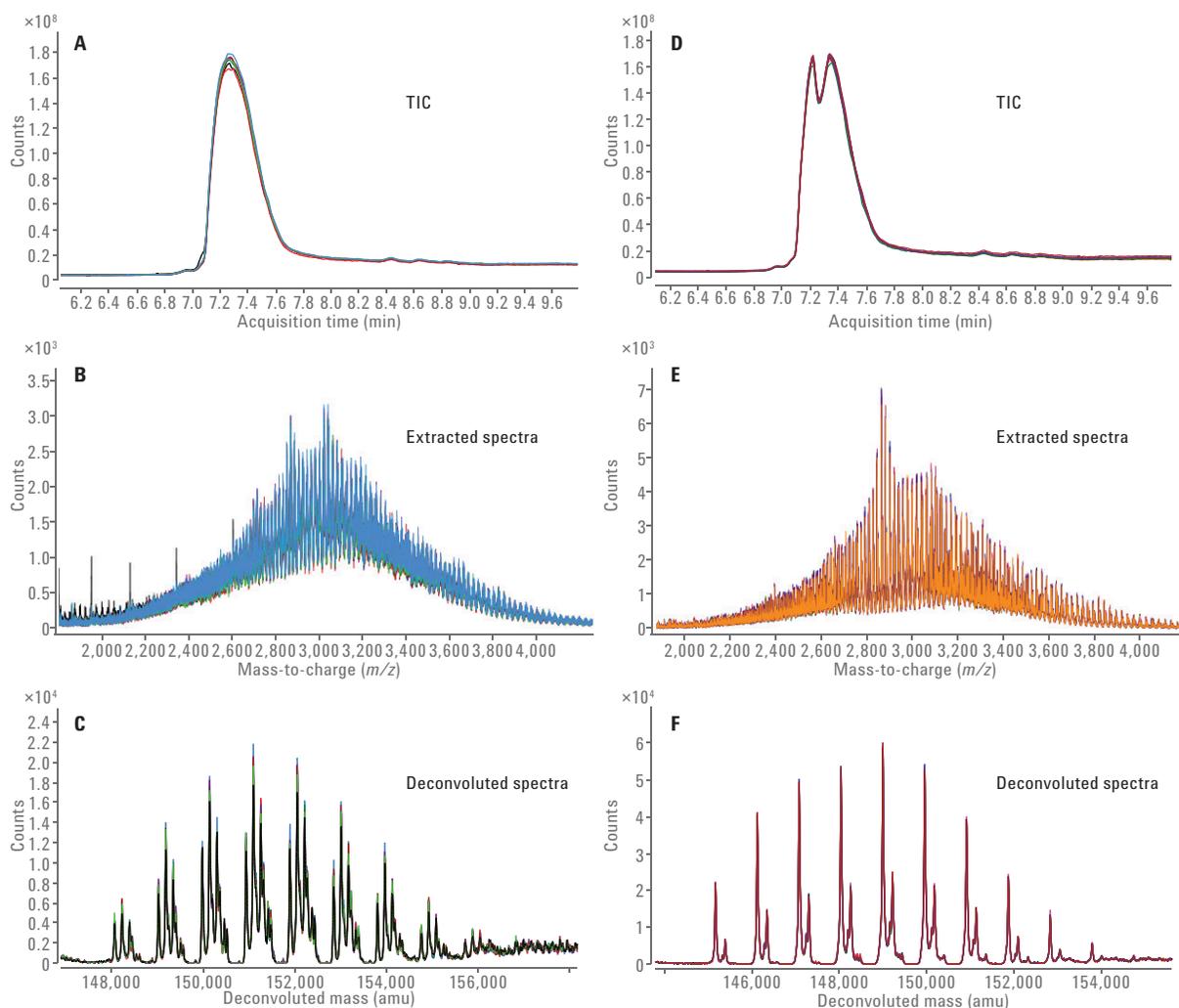


Figure 2. Total ion chromatograph, extracted spectra, and deconvoluted spectra of intact glycosylated (panels A-C) and intact deglycosylated (panels D-F) ADCs.

The deconvoluted spectra were then analyzed in an Agilent MassHunter DAR calculator. Figure 3 shows a representative graph of the DAR calculation of intact glycosylated ADC. The DAR values calculated from eight replicates were 3.6 (%CV = 0) for the intact glycosylated ADCs and 3.88 (%CV = 1.1) for the intact deglycosylated ADCs.

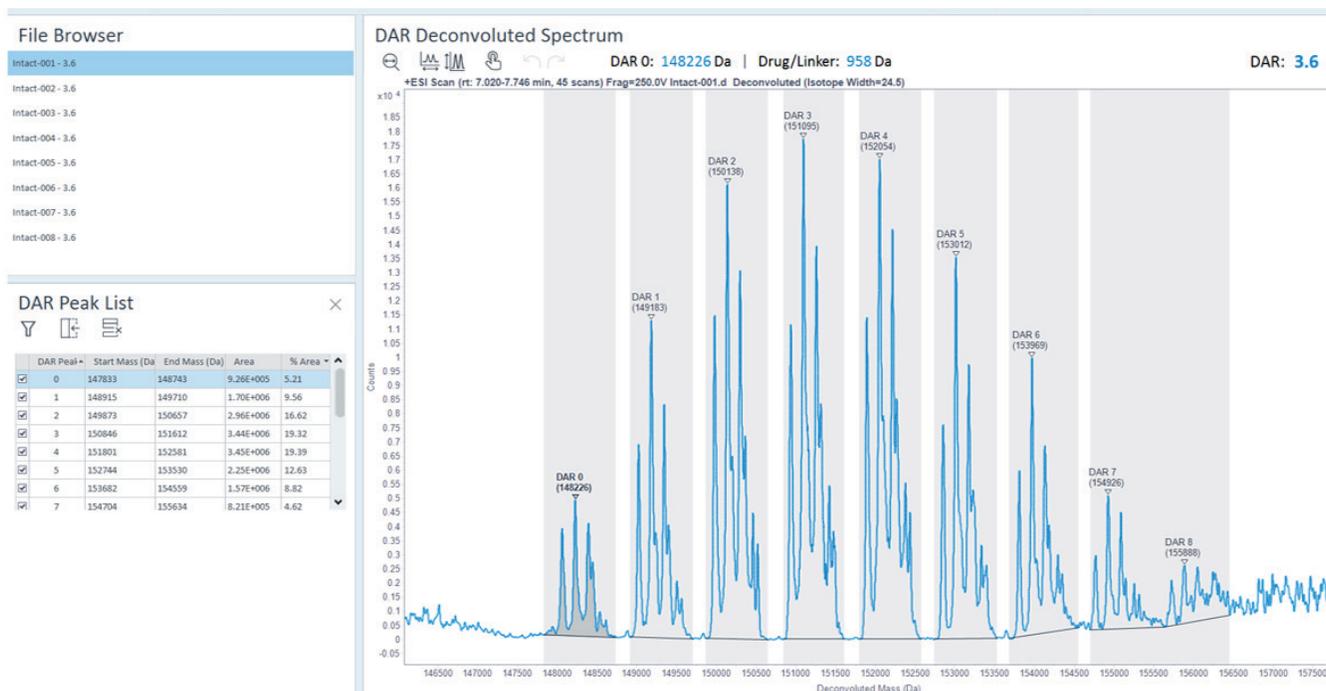


Figure 3. Representative DAR calculation of intact glycosylated ADCs.

Figure 4 shows the overlaid TIC, extracted spectra, and the deconvoluted spectra of reduced glycosylated (Figure 4 panels A-D), and reduced deglycosylated (Figure 4 panels E-H) ADCs of eight

replicates. Both reduced glycosylated and reduced deglycosylated ADCs eluted in less than 1 minute with a mass envelope centered around 1,400 Da, with light chain charge states between +6 and +27

and heavy chain charge states between +13 and +53. For light chain ADCs, four peak groups were observed (D0–D3) for both glycosylated and deglycosylated ADCs. The signal intensities are similar between the two conditions.

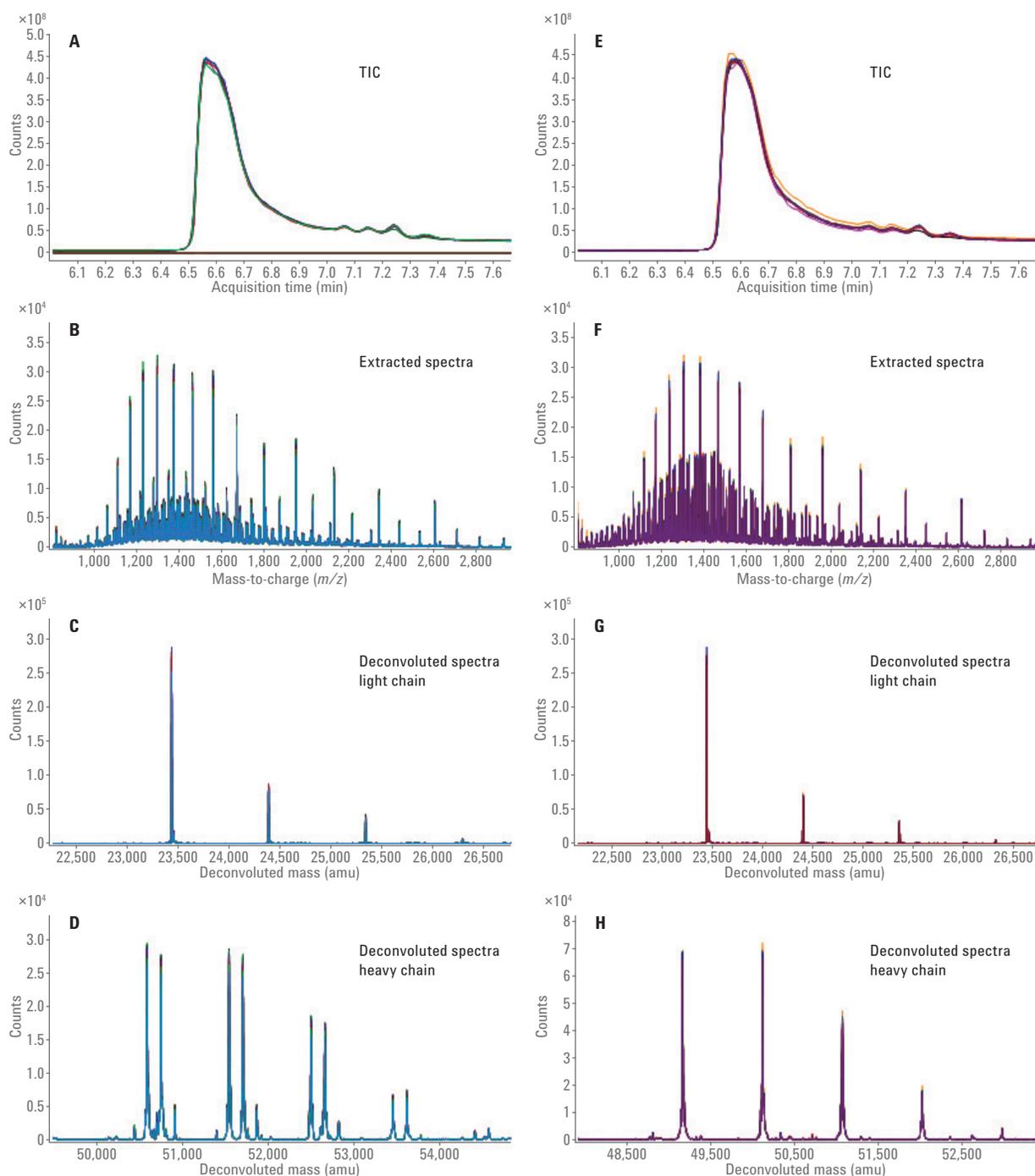


Figure 4. TIC, extracted spectra, and deconvoluted light and heavy chain spectra of reduced glycosylated (panels A-D) and reduced deglycosylated (panels E-H) ADCs.

For heavy chain ADCs, five peak groups were observed for both glycosylated and deglycosylated ADCs (D0–D4). For each peak group, the four major peaks observed in the glycosylated ADCs represent G0, G0F, G1F, and G2F glycoforms. After deglycosylation, only one major peak was observed in each peak group representing fully

deglycosylated ADCs; a minor peak was also observed in each peak group representing ADCs with an extra linker. The peak intensities were also increased about 2-fold compared to the reduced glycosylated ADCs. The exported .csv files were processed by the Agilent DAR Calculator. Figure 5 shows a representative report from the DAR

calculator of reduced glycosylated ADCs. The DAR calculated eight replicates of reduced glycosylated and deglycosylated ADCs were 3.23 (%CV = 2.2), and 3.21 (%CV = 2.6), respectively. The DAR value calculated from the reduced ADC was slightly different from that of the intact ADC as reported previously².

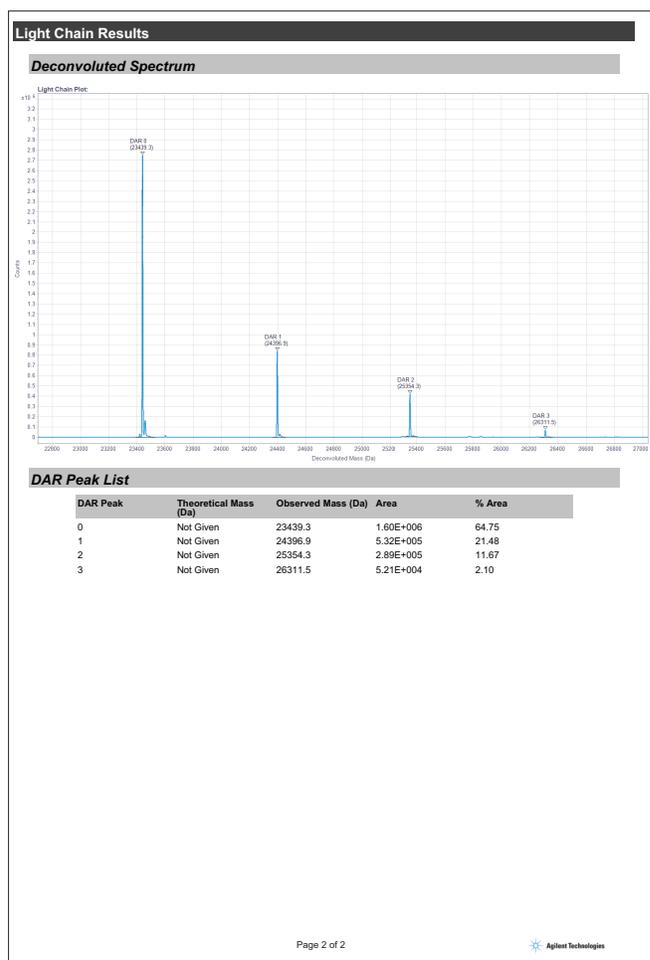
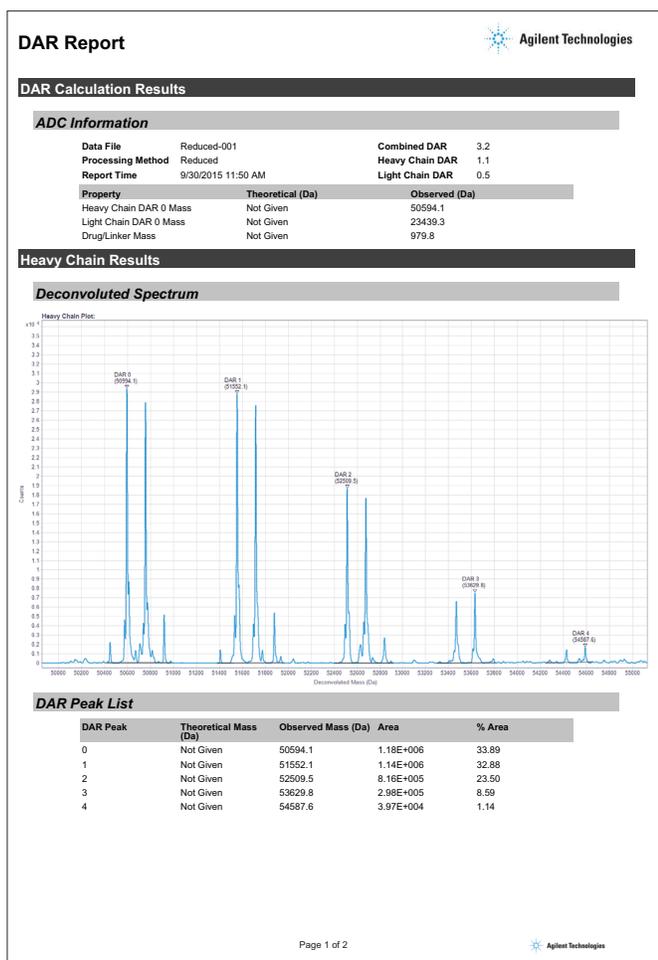


Figure 5. Representative report from the Agilent DAR calculator of reduced ADCs.

Conclusion

Together with the use of rapid PNGase F, automated sample preparation with the AssayMAP Bravo allows for rapid deglycosylation in a high-throughput manner (less than one hour). The user-friendly control software Protein Sample Prep Workbench and the precision of the AssayMAP Bravo platform enable easy and reproducible reduction and deglycosylation of ADC samples for DAR calculation. The Agilent 1290 Infinity UHPLC/6550 Q-TOF mass spectrometer generates high resolution accurate mass spectra for accurate and straightforward DAR calculation with MassHunter/BioConfirm/DAR Calculator software. The Agilent PLRP-S column delivers excellent peak shape in the formic acid mobile phase, enhancing sensitivity. Agilent's automated robotics, LC/MS, software package, and consumables provide a complete solution for rapid, easy, accurate and reproducible DAR calculation of ADCs.

References

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2. Basa, L. Chapter: Drug-to-Antibody Ratio (DAR) and Drug Load Distribution by LC-ESI-MS. Antibody Drug Conjugates. *Methods in Molecular Biology*; L. Dury (Ed.); Humana Press: New York, 2013; 1045, pp 285-293.

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