

An Integrated Workflow for Automated Calculation of Antibody-Drug Conjugate (ADC) Drug-to-Antibody Ratio (DAR)

Using Automated Sample Preparation,
Agilent MassHunter Walkup, and Agilent MassHunter
BioConfirm DAR Calculator Software

Application Note

Biotherapeutics and Biosimilars

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Abstract

An easy-to-use and fully integrated method for the determination of drug-to-antibody ratios (DARs) of antibody-drug conjugates (ADCs) has been developed using the Agilent AssayMAP Bravo, Agilent MassHunter Walkup software, and the Agilent BioConfirm DAR calculator.



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Introduction

Antibody-drug conjugates (ADCs) represent a new generation of targeted biotherapeutics that make up a rapidly growing segment of the drug discovery pipeline. They are created by attaching potent cytotoxic drugs through a linker to monoclonal antibodies (mAbs) that target specific cells. They deliver those drugs to the desired tissue while limiting toxicity to nontargeted tissues¹. Different types of conjugation chemistries are used to create ADCs, including cysteine-linked, lysine-linked, and site-specific conjugation. Depending on the type of conjugation chemistry, different methodologies are used for the characterization of ADCs^{2,3}.

The production and characterization of potent, highly selective, and low-toxicity ADCs are more complicated than the processes used for traditional mAbs. The number of drug molecules attached to each antibody can vary considerably depending on the conjugation chemistry and reaction conditions. Achieving the proper average drug-to-antibody ratio (DAR) is crucial to optimizing the efficacy, and minimizing the toxicity of the ADC. Rigorous quality control is required to ensure that ADCs have a consistent DAR, which is frequently determined using liquid chromatography coupled to mass spectrometry (LC/MS).

Reduction and deglycosylation are often done offline before analysis to reduce the complexity of the ADC LC/MS results used for the DAR calculation. Such sample preparation prior to LC/MS and subsequent calculation of the DAR values are often manually intensive, limiting throughput and introducing variability. In addition, the scientists developing and analyzing ADCs are often not experts in LC/MS analysis. This Application Note describes an integrated workflow for ADC DAR determination that automates sample preparation and DAR calculation, while enabling operators who are not experts in LC/MS to obtain high quality results.

The Agilent AssayMAP Bravo is an automated sample preparation platform whose simple user interface gives non-automation experts the ability to efficiently purify, reduce, and deglycosylate ADCs prior to LC/MS analysis. Automated sample preparation enables robust ADC DAR analysis by minimizing variability in sample preparation, which is a major source of data variability in the ADC DAR workflows. Agilent MassHunter Walkup Software allows users to walk up to the LC/MS instrument with their processed samples, input minimal sample information, choose the appropriate ADC analysis method, place the samples in position as directed by the system, and walk away. The users are not required to know how to set up or optimize the LC/MS system for their application. This is done by the system administrator, also using the MassHunter Walkup Software.

Following the LC/MS run, Agilent MassHunter BioConfirm DAR Calculator software provides an easy-to-use solution for the calculation of DAR, which is then sent to the analyst as part of an email report on the analysis results.

Experimental

Material

Rapid PNGase F was obtained from New England Biolabs. Eppendorf 96-well PCR plates were from Eppendorf, and Tris chloride and Tris(hydroxymethyl) aminomethane were obtained from EMD Millipore. All other chemicals were sourced from Sigma-Aldrich. The formulated ADC was from Genentech.

In-solution reduction and deglycosylation

Lyophilized ADC was reconstituted in deionized (DI) water to 5 mg/mL, aliquoted and stored at -80°C until used. Reduction and deglycosylation were performed manually using the Agilent AssayMAP Bravo Platform and Protein Sample Prep Workbench software. For the automated workflow, 5 μL of ADC were dispensed into each well of the first four columns of a 96-well Eppendorf PCR plate (position A1-D4). Subsequently, 10 μL of 10 mM Tris buffer (pH = 7.5) (position A1-D2), or 35 mM DTT in 10 mM Tris buffer (pH = 7.5) (position A3-D4) were added to the samples. Finally, 20 μL of 10 mM Tris buffer (pH = 7.5) (positions A1-D1 and A3-D3) or rapid PNGase F (diluted 1:40) in 10 mM Tris buffer (pH = 7.5) (A2-D2 and A4-D4) were added to the samples. The sample plate was then sealed using an Agilent PlateLoc Thermal Plate Sealer, and incubated at 50°C for 10 minutes.

LC/MS analysis

LC/MS analyses were conducted on an Agilent 1290 Infinity II UHPLC system coupled with an Agilent 6230 Accurate-Mass TOF LC/MS system equipped with an Agilent Dual Jet Stream ESI source. Tables 1 and 2 list the LC/MS parameters used. Approximately 1.4 µg of sample was injected for each run.

Data Analysis

Data analysis was performed automatically by MassHunter BioConfirm B.08.00 and DAR Calculator B.01.01 software following sample submission and data acquisition through the MassHunter Walkup. BioConfirm automation was enabled in the acquisition method by linking a BioConfirm method containing DAR Calculator parameters.

Raw data obtained from LC/MS were processed automatically by the Intact Protein Deconvolution with the DAR Calculation custom feature in BioConfirm. This process deconvoluted the data using the Maximum Entropy deconvolution algorithm (Table 3), exported the deconvoluted spectra to a csv file, and calculated the average DAR for the ADC sample (Table 4).

The drug-to-antibody ratio was calculated using the following formula:

$$DAR = \sum_{i=0}^n i \times \left(\frac{A_i}{A_{total}} \right)$$

Equation 1.

Where i denotes the number of drugs attached to the antibody (DAR species), and A_i denotes the area under each DAR species peak cluster, which includes all glycan peaks.

If necessary, peak limits can be manually adjusted in the DAR Calculator software after reviewing the automated data analysis results.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System				
Column	Agilent PLRP-S, 300 Å, 2.1 × 50 mm, 5 µm (p/n PL 1912-1501)			
Thermostat	5 °C			
Solvent A	0.1 % Formic acid in water			
Solvent B	0.1 % Formic acid in acetonitrile			
Gradient	Intact ADC		Reduced ADC	
	Time (min)	%B	Time (min)	%B
	0–2	3–10	0–5	3–20
	2–10	10–45	5–6	20–95
	10–13	45–70	6–10	95
	13–15	70		
Column temperature	80 °C			
Flow rate	0.5 mL/min			
Injection volume	2 µL			

Table 2. Mass spectrometer parameters.

Agilent 6230 Accurate-Mass TOF LC/MS system		
	Intact ADC	Reduced ADC
Source	Dual Agilent Jet Stream	Dual Agilent Jet Stream
Gas temperature	325 °C	290 °C
Gas flow	13 L/min	13 L/min
Nebulizer	60 psig	35 psig
Sheath gas temperature	350 °C	350 °C
Sheath gas flow	12 L/min	12 L/min
VCap	4,000 V	4,000 V
Nozzle voltage	2,000V	2,000 V
Fragmentor	400 V	175 V
Skimmer	65 V	45 V
Mass range	800–8,000 m/z	300–3,200 m/z
Scan rate	1.0 spectra/sec	1.0 spectra/sec
Acquisition mode	Positive, High (10,000 m/z) mass range	Positive, Standard (3200 m/z) mass range

Table 3. BioConfirm deconvolution parameters.

	Intact ADC	Reduced ADC
Deconvolution tab		
Deconvolution algorithm	Maximum entropy	Maximum entropy
Mass range (Da)	140,000–160,000	20,000–60,000
Mass step	1	1
Use limited m/z range	1,800–5,000	1,000–3,200
Subtract baseline	7	7
Adduct	Proton	Proton
Isotope width	Automatic/20 Da	Automatic/20 Da
Maximum entropy tab		
Peak signal-to-noise (S/N)	≥30	≥30
Maximum number of peaks	100	100
Calculate average mass using top % of peak height	25 %	25 %

Results and Discussion

Integrated workflow using AssayMAP Bravo, Walkup Software, and the DAR Calculator

The AssayMAP Bravo Platform enables the automated reduction and deglycosylation of antibodies and ADCs, thus reducing human error, assuring reproducibility, and allowing the analyst to walk away and perform other tasks (Figure 1). This open access automation solution is specifically designed for biomolecule sample preparation using miniaturized, packed-bed chromatography cartridges, simple and reliable automated processes, and an application-based user interface.

MassHunter Walkup software allows scientists not familiar with LC/MS to access instruments with ease. Even novice users can take full advantage of powerful LC/MS capabilities, without assistance from expert staff, by simply providing some basic information, choosing a method, and placing samples as directed. The results arrive in the user's email inbox automatically (Figure 1). Administrators of the LC/MS systems can take advantage of easy method setup and management, as well as management of the user queue.

The BioConfirm B.08.00 ADC DAR Calculator requires minimal input from the user, primarily the molecular weights of the unconjugated antibody and the drug/linker mass. It instantaneously calculates the average DAR from the deconvoluted ADC peaks produced by BioConfirm software, using auto peak integration. Walkup software can then send DAR calculator results to the user in an email report (Figure 1).

Table 4. DAR Calculator parameters.

	Intact ADC	Reduced ADC
Parameters tab		
Processing method	Intact	Reduced
Linker type	Lysine-linked	N/A
Intact DAR 0 mass (Da)	145170 (Degly)/148059 (Native)	N/A
Heavy chain DAR 0 mass (Da)	N/A	49,149 (Degly)/50,756 (Native)
Light chain DAR 0 mass (Da)	N/A	23,439
Drug/linker mass (Da)	957.5	957.5
Selected peaks	DAR 0–7	DAR 0–4
Properties tab		
Peak area	Use total area	Use total area
Peak baseline	Use y = 0	Use y = 0
Maximum DAR peak number	8	4
Report tab		
Report type	PDF	PDF

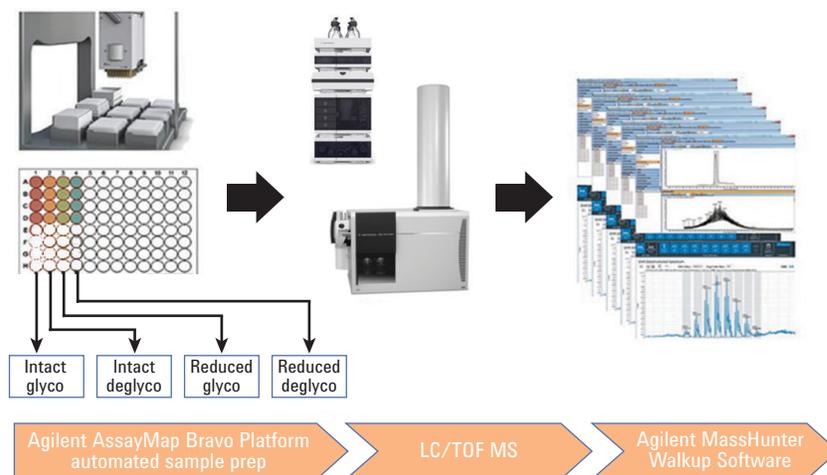


Figure 1. Integrated Agilent MassHunter Walkup ADC calculation workflow, from automated sample preparation on the Agilent AssayMAP Bravo Platform to LC/TOF MS analysis, automated DAR calculation, and delivery of email report.

Drug-to-antibody ratio determinations

The performance of the integrated workflow with automated sample preparation was compared to a similar workflow using manual sample preparation (reduction and deglycosylation), with a lysine-conjugated ADC sample. Four different sample preparation conditions were used for each workflow type. In the automated integrated workflow, four technical replicates were prepared on the AssayMAP Bravo for each of the four sample conditions (Figure 1). Each sample was then run three times on the LC/MS. In the manual integrated workflow, a single technical replicate was prepared manually for each of the four sample conditions, and each sample was run six times on the LC/MS. The four sample conditions for each workflow were as follows:

- Intact, glycosylated ADC
- Intact, deglycosylated ADC
- Reduced, glycosylated ADC
- Reduced, deglycosylated ADC

Automated integrated workflow results

Figure 2 shows the total ion chromatographs (TICs) and deconvoluted spectra of intact glycosylated (Figures 2A and 2B), and intact deglycosylated (Figures 2C and 2D) ADCs. For intact glycosylated ADCs, nine peak groups were observed with masses matching D0–D8. The four major peaks observed in each peak group correspond with

glycoforms G0F/G0F, G0F/G1F, G1F/G1F or G0F/G2F, and G1F/G2F. For intact deglycosylated ADC, nine peak groups were also observed with mass matching D0–D8 conjugation states. All glycoforms observed in glycosylated ADC samples were eliminated, and the peak mass was reduced correspondingly. The peak intensities were increased several fold compared to the intact ADCs.

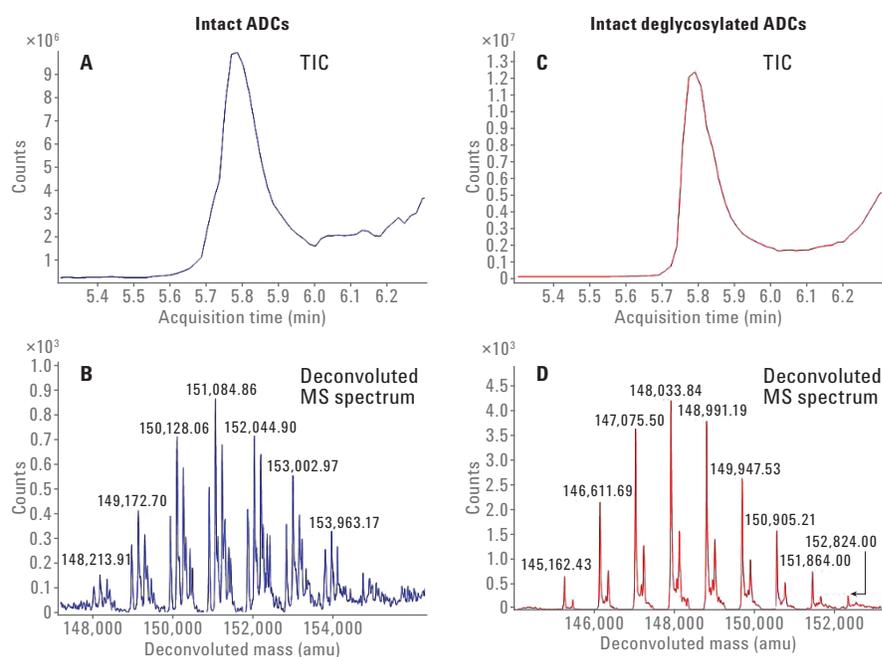


Figure 2. Total ion chromatographs (TICs) and deconvoluted spectra of intact glycosylated (A,B) and intact deglycosylated (C,D) ADC prepared using the Agilent Bravo AssayMAP Platform.

The deconvoluted spectra were then analyzed in the BioConfirm DAR calculator. Figure 3 shows representative graphs of the DAR calculations of intact glycosylated (left) and intact deglycosylated ADC (right). The average DAR value calculated from four technical replicates was 3.5 for the intact glycosylated ADC. The DAR value calculated for the intact deglycosylated ADC was also 3.5.

Figure 4 shows the TICs and deconvoluted spectra of reduced glycosylated (Figure 4A–4C), and reduced deglycosylated ADC (Figure 2D–2F). For light chains, four peak groups were observed (D0–D3) for both glycosylated and deglycosylated reduced ADC.

For heavy chains, four peak groups were observed for glycosylated and reduced ADC, while five peaks were observed for deglycosylated and reduced ADCs (D0–D4). For each peak group, the four major peaks observed in the glycosylated and reduced ADCs represent G0, G0F, G1F, and G2F glycoforms. After deglycosylation, only one major peak was observed in each peak group representing fully deglycosylated ADCs. The minor peak observed for deglycosylated heavy chains is due to ADC with an extra linker. The peak intensities for the reduced and deglycosylated chains were increased approximately 2 to 4-fold compared to the reduced and glycosylated ADC chains.

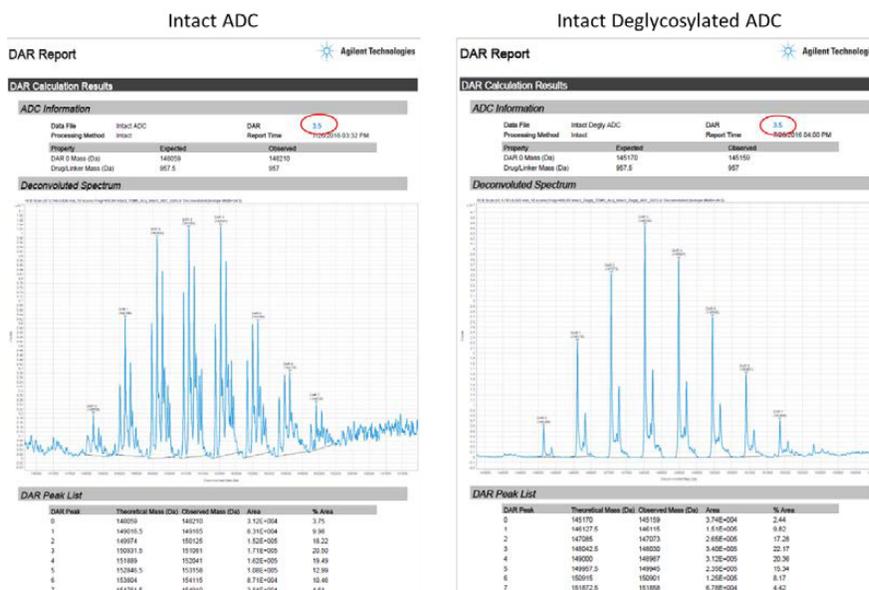


Figure 3. Representative DAR calculation of intact glycosylated and deglycosylated ADC prepared using the Agilent Bravo AssayMAP Platform.

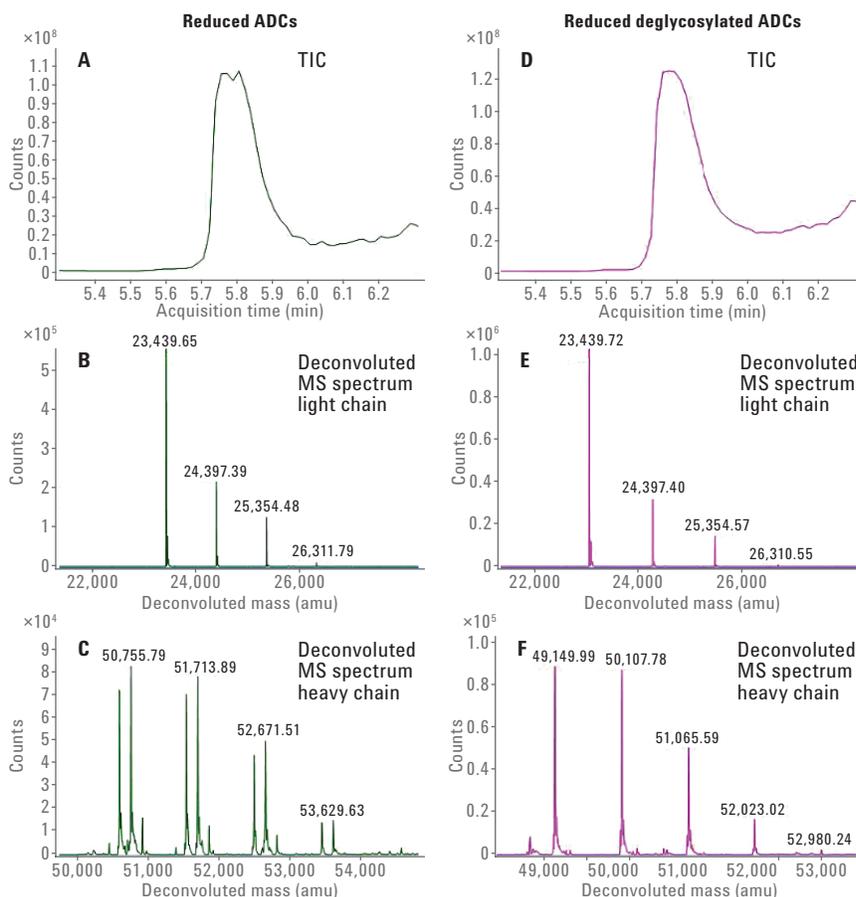


Figure 4. TIC and deconvoluted light and heavy chain spectra of reduced glycosylated (panels A–C) and reduced deglycosylated (panels D–F) ADC prepared using the Agilent Bravo AssayMAP Platform.

Figures 5 and 6 show representative reports from the automated DAR calculator in BioConfirm for both reduced glycosylated and reduced deglycosylated ADC. The DARs calculated for the four technical replicates of reduced glycosylated and deglycosylated ADC were 3.2, and 3.0, respectively. The DAR value calculated from the reduced ADC was slightly different from that of the intact ADC reported previously¹.

Comparison to manual sample preparation

The same set of experiments was performed with the same ADC sample, using manual sample preparation instead of the AssayMAP Bravo Platform. As expected, the DAR values obtained using manual sample preparation and automated sample preparation were nearly identical (Table 5). Automation would not be expected to give a different DAR value, but automation does offer several advantages, including reduced labor and eliminating human error. The preconfigured software protocols allow users to run sample preparation with the click of a few buttons. With the 96-well format, the same amount of time is required to set up automated sample preparation for 1 or 96 samples.

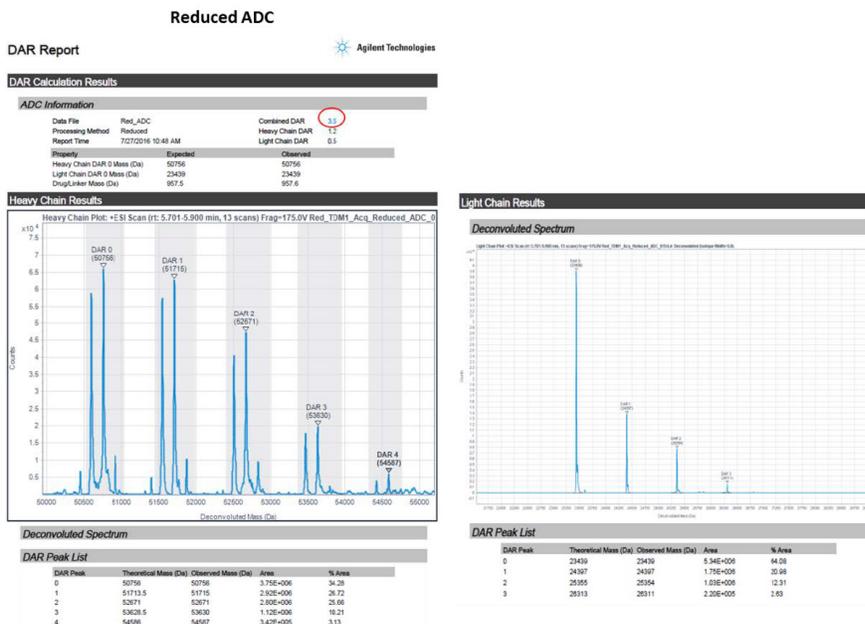


Figure 5. Representative report from the Agilent DAR calculator for reduced glycosylated ADC.



Figure 6. Representative report from the Agilent DAR calculator for reduced deglycosylated ADC.

Table 5. Comparison of results, using either automated (Bravo AssayMAP) or manual sample preparation.

	Intact ADC	Intact deglycosylated ADC	Reduced ADC	Reduced deglycosylated ADC
Automated sample prep (Total number of injections)	12	12	12	12
Average DAR	3.5	3.5	3.2	3.0
Manual sample prep (Total number of injections)	6	6	6	6
Average DAR	3.6	3.5	3.4	3.0

Conclusions

Scientists developing antibody-conjugated biotherapeutics need to obtain accurate and reliable DAR information without having to become experts in sample preparation, operating mass spectrometers, interpreting the data, or manually calculating the DAR.

Agilent provides an integrated workflow that delivers reliable DAR values right to the user's email, while requiring very little hands-on input. The Agilent AssayMAP Bravo Platform automates sample preparation, and Agilent MassHunter Walkup software ensures optimal MS data with little or no knowledge of operation of the instrument. Finally, Agilent BioConfirm software provides automatic calculation of the average DAR for all forms of the ADC, including intact and deglycosylated configurations, as well as reduced heavy and light chains. For reliable DAR results obtained with a minimum of effort or expertise, this integrated workflow using the AssayMAP Bravo Platform, MS instruments with Walkup software, and the Agilent MassHunter BioConfirm DAR calculator is ideal.

References

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