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# Characterization of Antibody-Drug Conjugate Critical Quality Attributes Using the Agilent Cary 3500 UV-Vis Multizone Temperature Capability

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## Abstract

Antibody-drug conjugates (ADCs) are a growing class of biotherapeutics. During manufacturing, it is important to monitor critical quality attributes (CQAs) of the ADCs such as drug-to-antibody ratio (DAR) and aggregation. In this application note, we demonstrate the functional benefits of the Agilent Cary 3500 UV-Vis Multicell spectrophotometer for analysis of DAR and aggregation index of ADCs with its unique capability of analyzing samples at multiple temperatures simultaneously. With the custom equation function in the software, simple methodology, and accurate temperature control, the Cary 3500 UV-Vis allows for the determination of DAR and aggregation index with confidence and ease.

## Introduction

ADCs represent a rapidly growing portion of the drug discovery pipeline in pharmaceutical companies due to their specific and targeted mode of action.[1] The measurement of DAR provides the average number of cytotoxic small molecules conjugated to each antibody and is generally recognized as a CQA as it greatly impacts the safety and efficacy of the product. The DAR is also known to strongly influence the tendency of the product to form aggregates[2], which may elicit immune responses. Hence, analyzing both DAR and aggregation of ADCs during development is essential.

As a common workhorse technique in the biopharmaceutical laboratory, UV-Vis spectroscopy is often used as a robust and convenient technique to determine protein concentration. In this application note, we show that UV-Vis spectroscopy can also be used to determine the DAR of an ADC so long as the cytotoxic small molecule contains a UV-visible chromophore with an absorption maximum distinct from that of the antibody scaffold.[1]

The Cary 3500 UV-Vis is simple and quick to use, with high throughput and accurate temperature control that is possible across four pairs of cuvettes at four different temperatures simultaneously. Simultaneous measurement of UV absorbance for multiple samples removes unwanted variables and increases confidence in the results that are generated. The easy method setup and the custom calculation features make it user friendly. The Cary 3500 UV-Vis spectrophotometer is compatible with the Agilent OpenLab software suite. When configured with OpenLab software, it supports laboratories to follow the compliance guideline of FDA 21 CFR Part 11. In this study we demonstrate the benefits of Cary 3500 UV-Vis to determine DAR and aggregation index.

## Experimental

### Instrumentation
The Agilent Cary 3500 UV-Vis Multicell spectrophotometer was fitted with eight positions. Data acquisition was carried out using Cary UV Workstation software (version 1.0.1284) with the Cary 3500 Multizone software add-on, using the parameters shown in Table 1.

**Table 1.** Experimental parameters for the Agilent Cary 3500 UV-Vis spectrophotometer.

| Parameters | Value |
|---|---|
| X Mode | nm |
| Y Mode | Absorbance |
| Collect Mode | Scan |
| Scan Range Start | 400 nm |
| Scan Range Stop | 200 nm |
| Averaging Time | 0.020 s |
| Data Interval | 1.0 nm |
| Scan Rate | 3,000 nm/min |
| Spectral Bandwidth | 1.0 nm |
| Detector Module | Multicell Peltier UV-Vis |

### Materials
Herceptin and its ADC analog were bought from a local distributor in Singapore. Both monoclonal antibody (mAb) samples were concentrated using Vivaspin 500 centrifugal concentrator spin columns (10 kDa MWCO; Sartorius).

An Agilent rectangular cell, UV 10 mm, 700 µL open cuvette (part number 5061-3391) was used with the Cary 3500 UV-Vis.

Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Merck Millipore).

### Calculations
The ADC analog of Herceptin is covalently conjugated to the antitubulin cytotoxic agent, DM1, using 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (MCC) as a linker.[3] Using the extinction coefficients of each component at these wavelengths ($\varepsilon_{280\ DM1}$ = 5,700 $M^{-1}\ cm^{-1}$, $\varepsilon_{252\ DM1}$ = 26,790 $M^{-1}\ cm^{-1}$ and $\varepsilon_{280\ mAb}$ = 218134 $M^{-1}\ cm^{-1}$, $\varepsilon_{252\ mAb}$ = 76,565 $M^{-1}\ cm^{-1}$), the average DAR can be derived using Equation 1, where R is the ratio of absorbance at 252 to 280 nm of the ADC.[4]

**Equation 1.**

$$\text{Average DAR} = \frac{(\varepsilon_{252\ mAb} - R \times \varepsilon_{280\ mAb})}{(R \times \varepsilon_{280\ DM1} - \varepsilon_{252\ DM1})}$$

To calculate the aggregation index, use Equation 2, where OD350 represents the absorbance at 350 nm and OD280 − OD350 is the difference in the absorbance at 280 and 350 nm respectively.[5]

**Equation 2.**

$$\text{Aggregation index} = \left(\frac{OD350}{OD280 - OD350}\right) \times 100$$

## Methods

The experiments were performed with Herceptin and its ADC analog at 0.5 mg/mL. To demonstrate changes in DAR value, Herceptin and its ADC analog were mixed at different percentages (Table in Figure 2) and were measured using the Cary 3500 UV-Vis. The DAR was calculated using Equation 1 in the Cary UV Workstation software.

To study effects of temperature on DAR, four experiments were carried out in parallel with the Multizone software add-on (Figure 1). The concentration of "spin-column concentrated" ADC used in this experiment was estimated to be 25 mg/mL using UV absorbance at 280 nm. Of the 25 mg/mL sample, 10 µL was 40x diluted with 390 µL of water. Diluted ADC, 400 µL, was transferred to four cuvettes and incubated at temperatures of 60, 70, 80, and 90 °C for 90 minutes in the Cary 3500 UV-Vis simultaneously. The absorbances of the samples were measured after doing a baseline correction and DAR values were calculated using Equation 1.
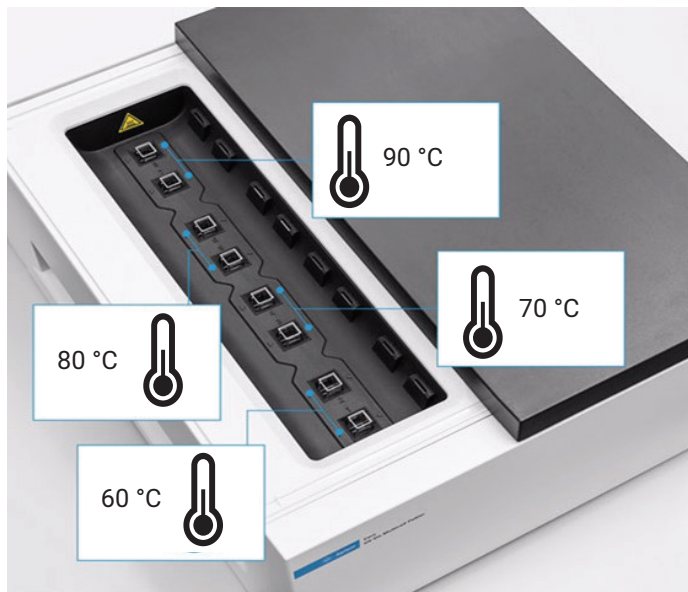


**Figure 1.** Monitoring DAR using the Cary 3500 Multizone temperature control functionality in a single experiment.

To study the effects of aggregation over time, 10 µL of the 25 mg/mL sample was 40x diluted with 390 µL of water. Diluted ADC, 400 µL, was transferred to a cuvette and incubated at 90 °C in the Cary 3500 UV-Vis and the absorbance of the sample was measured at different time intervals. Aggregation index was calculated using Equation 2.

# Results and discussion

### Use of Cary 3500 UV-Vis for drug-to-antibody ratio determination

To mimic the manufacturing of ADC conjugation, different percentages of ADC and free antibody were mixed, and DAR was determined using the Cary 3500 UV-Vis. Figure 2 shows the UV-Vis spectra of the ADC and unconjugated antibody mixtures. Using Equation 2, the DAR was calculated. The drug molecule DM1 of ADC showed a strong absorbance at 252 nm, which was different from the absorbance of the major chromophores of Herceptin at 280 nm[4], as shown in Figure 2. As expected, DAR values changed upon different percentages of mAb and ADC in the solution.
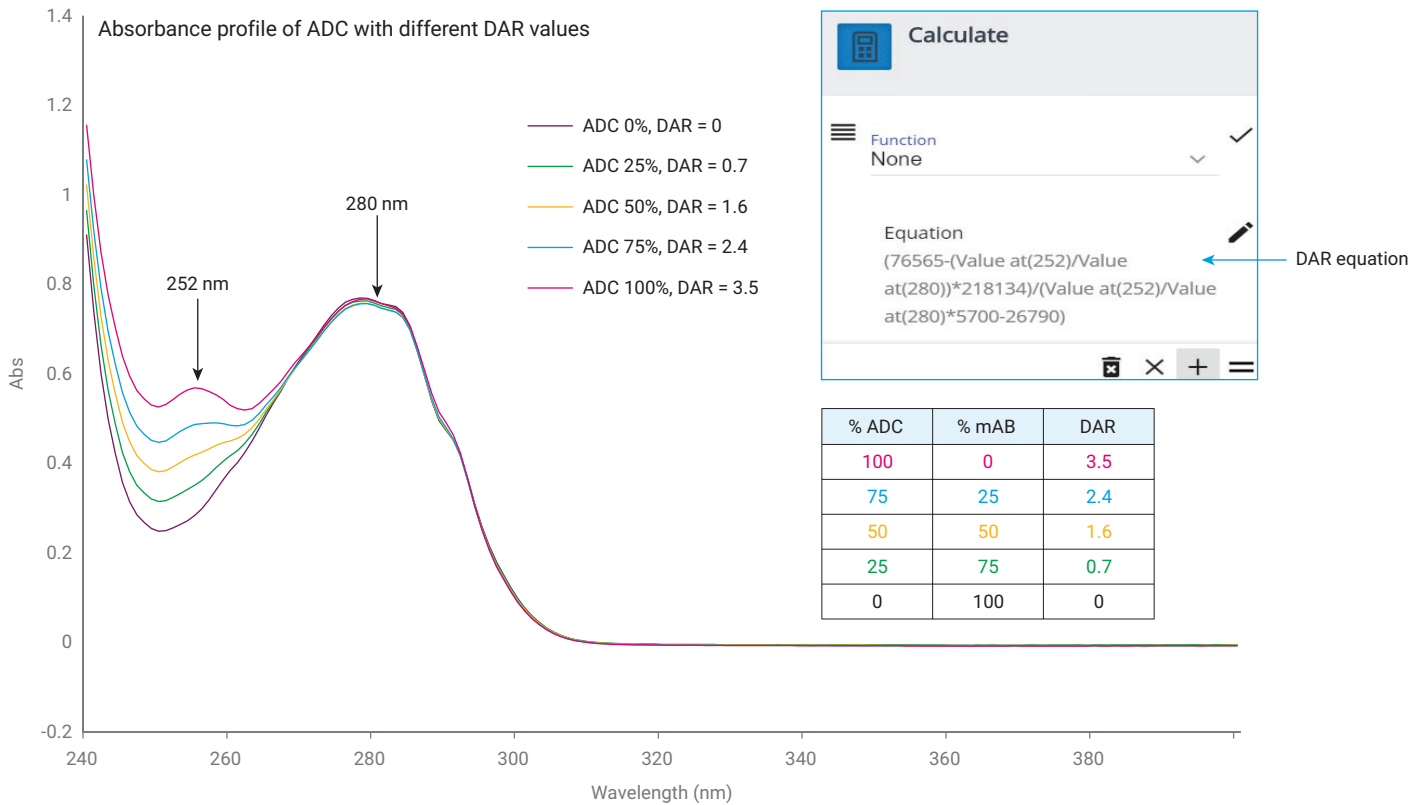
**Figure 2.** UV-Vis spectra of the ADC and unconjugated antibody mixtures with different average DAR values.

The effect of temperature on DAR was also investigated using the Cary 3500 UV-Vis with the Multizone software add-on. Figure 3 shows the results of the effect of temperature on DAR. With the increase of temperature from 60 to 90 °C, there is a slight shift in DAR values. This decrease in DAR is probably due to aggregation of the ADC.[2]
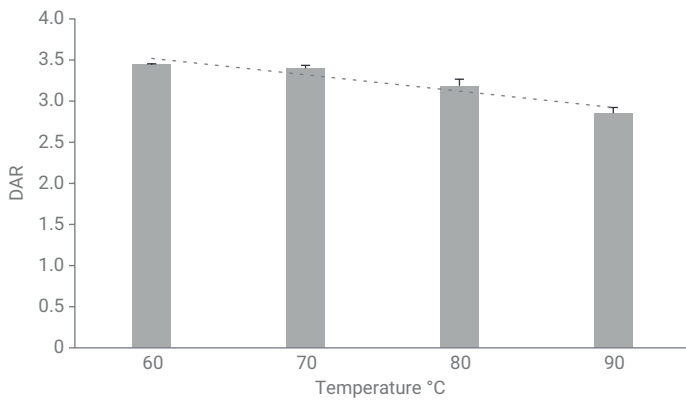
To further investigate the aggregation behavior, ADC aggregation index was determined at different time intervals at 90 °C. Using Equation 2 in Cary UV Workstation software, aggregation index was calculated. Figure 4 shows the aggregation index over different time points. As expected, aggregation increased with time at a higher temperature.
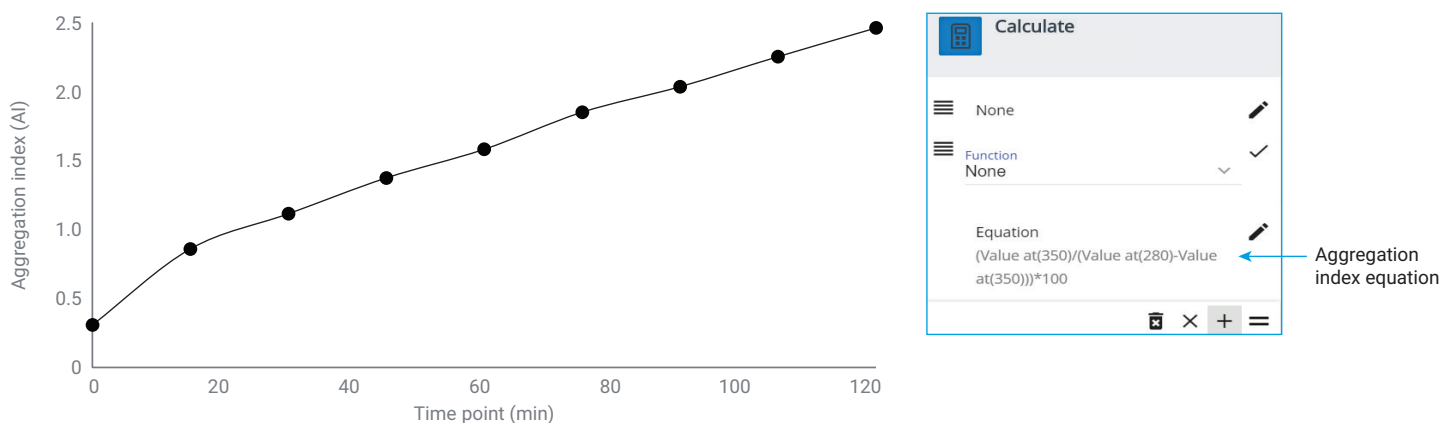


**Figure 3.** Effect of temperature on DAR (n = 3).

**Figure 4.** Aggregation index changes over time. Aggregation index equation was computed in the Cary UV Workstation software.

## Conclusion

This study demonstrates a simple yet powerful approach for ADC analysis using the Agilent Cary 3500 UV-Vis Multicell spectrophotometer. With simultaneous UV readouts and up to four independent temperature zones to be configured, the Cary 3500 UV-Vis with the Multizone software add-on is ideally suited to measuring DAR and aggregation index values. This application can be used in the biopharmaceutical industry for the analysis of these CQAs and with optional 21 CFR Part 11 software tools with the Agilent OpenLab software suite, can also be used in regulated environments.

## References

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