The goal of drug discovery is to find drugs that have a wide differential between the effective dose and the dose where serious side effects are observed. One common approach to finding safe and effective drugs is to make them as specific as possible. While there are many excellent small-molecule drugs, finding highly specific small-molecule drugs is difficult. This is due, in part, to the limited binding specificity that can be engineered into these molecules. Biologics, on the other hand, can be developed with exquisite selectivity.

An exciting new approach to generating effective biotherapeutics with minimal side effects is antibody-drug conjugates (ADCs) that combine the selectivity of biologics with the potency and wide target diversity of small-molecule drugs. The approach is to attach a small-molecule drug to an antibody that binds with high specificity to a population of target cells. This allows the ADC to create a high local concentration of drug at the target cells, with a low level of drug exposure at non-target cells. While the concept of an ADC is relatively straight forward, the analytical work required to fully characterize ADCs is quite complex.

To develop an ADC, antibodies with the proper affinity and specificity must be engineered, and an effective drug/cross-linker combination must be conjugated to the antibody. Additionally, an optimal number of drugs per antibody must be achieved to produce the desired potency, minimize off-target effects, and exhibit good pharmacokinetic properties. Unlike small molecules, ADCs are heterogeneous populations that vary by the number drugs attached to the antibody. The drug-to-antibody ratio (DAR) is a critical quality attribute of ADCs that must be carefully monitored during the development process. A low DAR value can result in poor efficacy whereas a high DAR value can result in undesired toxicity and poor pharmacokinetic properties.

This workflow guide focuses on the characterization of ADCs using multiple complementary approaches.

One of the most common and information rich ADC DAR characterization workflows is the analysis of intact ADCs by LC/MS. This approach reveals the distribution of antibodies with different numbers of drug conjugates within the ADC sample and the mass of various antibody glycoforms. The data provided by this workflow is powerful but the complex spectra generated can be challenging to interpret.
Other ADC DAR characterization workflows involve preparing a sample of the purified ADC before analysis. By adding a deglycosylation step, this workflow removes the glycans from the ADC, resulting in greatly simplified spectra and giving you greater confidence in picking peaks to determine the DAR value. It also enables you to determine antibody mass without the complexity of added glycan masses. In addition, deglycosylation gives you the opportunity to separately examine the glycan profile of the antibody.

Apart from deglycosylation, adding a reduction step prior to ADC analysis allows mass determination of the light and heavy chains and the relative distribution of drug conjugates on each chain. Combining antibody reduction with deglycosylation simplifies the interpretation of the spectra and allows mass determination of deglycosylated light and heavy chains.

To help you with data analysis and reporting—vital aspects of characterizing ADCs—we have integrated a DAR calculator into Agilent MassHunter software. This addition can rapidly calculate DAR values for both intact and reduced ADCs with minimal user input. The reports from this software summarize the theoretical mass, measured mass, drug/linker mass, and overall DAR value for the ADC. The software also tabulates all the DAR species that were detected with their respective theoretical masses, measured masses, and percent area or height, along with the spectra used for the calculation. The report provides an easy electronic record where you can review, archive, and share different analyses.
INTACT ADC DAR DETERMINATION

- Deconvolution
- DAR calculation
- Separation with UHPLC
- High resolution and accurate mass determination

DATA PROCESSING
- Deconvolution
- DAR calculation

REPORT GENERATION
Determining and monitoring ADC DAR values throughout the drug discovery process will guide your choice of which candidate ADCs to move forward with in the drug discovery process. We have developed an intact ADC DAR workflow in both sample-limited and nonsample-limited situations as there are minimal sample handling steps.

The first step in ADC analysis is separation by liquid chromatography. Due to the diverse chemical nature of ADCs, Agilent offers a variety of columns so you can select the one that provides the best performance for a specific ADC of interest. Choices include high-resolution columns such as the PLRP-S 1000Å column, Poroshell 300Å, 5µm columns, or the AdvanceBio RP-mAb (450Å, 3.5µm).

Agilent TOF and Q-TOF mass spectrometers are typically used to analyze intact ADCs. These instruments deliver the high resolution and accurate-mass measurements that you will need to calculate drug-to-antibody ratios downstream. Using Agilent MassHunter Bioconfirm software, you can easily deconvolute raw mass spectra to obtain accurate intact protein molecular weight that is reproducible over multiple runs. Finally, using the deconvoluted spectra and a small number of user inputs, the software’s DAR calculator automatically picks peaks representing ADCs with different drug loads. The calculator also integrates the area of those peaks or measures their height, determines the percentage of the total for each of these ADC peaks, and generates an average DAR value.

The parameters used can be saved in the DAR calculation as method file to enable high-throughput sample processing, and the results can be used to generate a DAR report containing the key information needed to make data-driven decisions.

**Featured Application Note**

- Analysis of Antibody-Drug Conjugate Through the Characterization of Drug Antibody Ratios Using an Agilent 1290 Infinity Binary LC System Coupled to an Agilent 6550 iFunnel Q-TOF (5991-6242EN)
PROCESSED ADC DAR CHARACTERIZATION

ADC

SAMPLE PREPARATION
- Deglycosylation
- Reduction
- Reduction & deglycosylation

ANALYSIS
- Separation with UHPLC
- High resolution and accurate mass determination

DATA PROCESSING
- Deconvolution
- DAR calculation

REPORT GENERATION
As a complement to the intact ADC DAR characterization workflow, you can obtain additional information by chemically processing the ADC before performing your DAR analysis. Common ADC processing steps include deglycosylation, reduction of ADC to light and heavy chains, and reduction plus deglycosylation. You can perform all these manipulations manually, or you can increase throughput using the Agilent AssayMAP Bravo automated sample-preparation platform.

The AssayMAP Bravo platform delivers unparalleled reproducibility, labor savings, scalability (you can run 8 to 96 samples simultaneously), and simple person-to-person and site-to-site method transfers—all while minimizing human error during sample preparation. What’s more, the AssayMAP Bravo platform comes with Protein Sample Prep Workbench software. The software provides a simple user interface that facilitates rapid implementation so that you can take advantage of the power of automation in days rather than months. You can also use the AssayMAP Bravo platform to purify ADCs from complex matrices such as serum.

The first variation of this workflow is ADC deglycosylation. This analytical approach provides the intact mass of the ADC with its various drug loads, without the complexity added by glycans.

The second variation is reduction of ADC disulfide bonds, which enables you to separate the light and heavy chains of the antibody prior to MS analysis. This provides another level of detail for characterizing ADCs. The DAR calculator in our MassHunter software is designed to analyze either intact or reduced ADCs with simple commands. The calculator determines the DAR values for the light and heavy chains individually as well as providing the total DAR value for the reduced ADC sample.

The third variation involves sequential reduction and deglycosylation of the ADC to yield glycan-free light and heavy chains. This variation simplifies the deconvoluted spectra for the heavy chain, potentially revealing details obscured when glycans are present.

For comprehensive ADC characterization, one or more of these sample preparation workflows may be required. The relative success of each may differ depending on the chemical characteristics of the ADC.

**Featured Application Note**
- Drug-to-Antibody Ratio (DAR) Calculation of Antibody-Drug Conjugates (ADCs) Using Automated Sample Preparation and Novel DAR Calculator Software (5991-6263EN)
- Determination of Drug-to-Antibody Ratio for Antibody-Drug Conjugates Purified from Serum Using Automated Affinity Purification, LC/MS Analysis, and Novel DAR Calculation Software (5991-6621EN)

**Recommended system configuration**

**HARDWARE – LC/MS SYSTEM**
- Agilent 1290 Infinity LC System including:
  - Agilent 1290 Infinity Binary Pump (p/n G4220A)
  - Agilent 1290 Infinity TCC (p/n G1316C)
  - Agilent 1290 Infinity Sampler (p/n G4226A)
  - Agilent 1290 Infinity FC/ALSTherm (p/n G1330B)
- TOF/Q-TOF Options:
  - Agilent 6230 TOF LC/MS (p/n G6230BA)
  - Agilent 6530 Q-TOF LC/MS (p/n G6530BA)
  - Agilent 6550 iFunnel Q-TOF LC/MS (p/n G6550AA)

**SOFTWARE**
- Agilent MassHunter Qualitative Analysis Software (p/n G3337AA)
- Agilent MassHunter BioConfirm Software (p/n G6829A)
- Agilent MassHunter DAR Calculator (p/n G6829A-64001)

**ASSAYMAP BRAVO & CONSUMABLES**
- AssayMAP Bravo (p/n G5542BA)
- Consumables for antibody purification
  - PA-W Cartridges (p/n G5496-60000)
  - SA-W Cartridges (p/n G5496-60010)

**ANALYTICAL COLUMNS**
- Agilent PLRP-S 1000Å
  - 2.1 x 150 mm, 8 µm (p/n PL1912-3802)
- Agilent Poroshell 300SB-C8
  - 2.1 x 75 mm, 5 µm (p/n 660750-906)
- Agilent Poroshell 300SB-C3
  - 2.1 x 75 mm, 5 µm (p/n 660750-909)
- Agilent Advance RP-mAb
  - 2.1 x 100 mm, 3.5 µm (p/n 785775-906)
You can also use Agilent MassHunter Walkup software to achieve ADC DAR characterization. Agilent Walkup software allows you to access LC/MS instruments with ease. So now even novice users can take full advantage of the powerful analytical capabilities of LC/MS to obtain DAR values, without assistance from expert staff. All they have to do is input some basic information (such as D0 value and a drug/linker mass), choose a method, and insert samples as directed. Results show up in the submitter’s in-box automatically.
MassHunter Walkup is designed to provide powerful yet straightforward system administration to meet the needs of even the most diverse laboratory team.

With MassHunter Walkup, a single administrator can easily manage many systems and applications. View instrument status, add user profiles, and modify methods remotely. Get system error notices on your smartphone via either text message or email. You can even take an instrument offline remotely – useful for accommodating scheduled preventative maintenance – with minimal negative impact to the sample submitters.

Featured Application Note

- An Integrated Workflow for Automated Calculation of Antibody-Drug Conjugate (ADC) Drug-to-Antibody Ratio (DAR) (5991-7366EN)

Additional software required

SOFTWARE

- Agilent MassHunter Walkup (p/n G2725CA, version C.02.02 or later)
ADC PAYLOAD LOCALIZATION

ADC SAMPLE PREPARATION
- Digestion
- Cleanup

ANALYSIS
- Separation with UHPLC
- High resolution and accurate mass determination

DATA PROCESSING
- Peptide mapping
Determining the drug-to-antibody ratio gives you valuable information about the average number of drugs conjugated to an ADC. However, this approach does not typically provide information about the location of the drug on the ADC. For that, you’ll need to digest the purified ADC, clean up the resulting peptide mixture, and analyze the peptides using LC/MS. This analysis can be either qualitative (to simply determine the sites of conjugation) or quantitative (to assess the percent occupancy of the conjugation sites and better understand the structure-activity relationship or pharmacokinetics of the ADC).

To access information about which amino acid residues have been conjugated by drug or linker, or both, drug-conjugated peptides can be identified from proteolyzed samples using MS/MS techniques.

Examining ADCs at the peptide level enables you to confidently assign site-specific conjugation and determine relative occupancy for residues modified at sub-stoichiometric levels. In this workflow, a purified ADC is denatured, reduced, alkylated, and digested. The resulting peptide mixture is then cleaned up. These steps can be performed manually or in an automated fashion using AssayMAP Bravo (see Bovee et al 2015, Russell et al 2014). Following cleanup, a reversed-phase LC separation is performed. We recommend the Agilent AdvanceBio C18 peptide mapping column for this step. It is specifically designed to deliver rapid, high-resolution separation of peptides, which can be easily coupled to mass spectrometry. The peptides can be analyzed using a high-resolution, accurate-mass Q-TOF system. Finally, MassHunter Bioconfirm software is used to determine which peptides are conjugated to the drug.

**Recommended system configuration**

**HARDWARE – LC/MS SYSTEM**

- Agilent 1290 Infinity LC System including:
  - Agilent 1290 Infinity Binary Pump (p/n G4220A)
  - Agilent 1290 Infinity TCC (p/n G1316C)
  - Agilent 1290 Infinity Sampler (p/n G4226A)
  - Agilent 1290 Infinity FC/ALSTherm (p/n G1330B)

- Q-TOF Options:
  - Agilent 6530 Q-TOF LC/MS (p/n G6530BA)
  - Agilent 6550 iFunnel Q-TOF LC/MS (p/n G6550AA)

**SOFTWARE**

- Agilent MassHunter Qualitative Analysis Software (p/n G3337AA)
- Agilent MassHunter BioConfirm Software (p/n G6829A)

**ASSAYMAP BRAVO & CONSUMABLES**

- AssayMAP Bravo (p/n G5542BA)
- PA-W Cartridges (p/n G5496-60000)
- SA-W Cartridges (p/n G5496-60010)
- C18 Cartridges (p/n 5190-6532)

**ANALYTICAL COLUMNS**

- AdvancedBio Peptide Map 2.1 x 150 mm, 2.7 µm (p/n 653750-902)
- RRHD Eclipse Plus C18, 2.1 x 100 mm, 1.8 µm (p/n 959758-902)
- RRHD Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm (p/n 959757-902)

**Featured Application Notes**

- Mapping the Drug Conjugation Sites of an Antibody-Drug Conjugate Using Automated Sample Preparation and LC/MS Analysis (5991-6389EN)
- Automation of Sample Preparation for Accurate and Scalable Quantification and Characterization of Biotherapeutic Proteins Using the Agilent AssayMAP Bravo Platform (5991-4872EN)
- Automation for LC/MS Sample Preparation: High Throughput In-Solution Digestion and Peptide Cleanup Enabled by the Agilent AssayMAP Bravo Platform (5991-2957EN)