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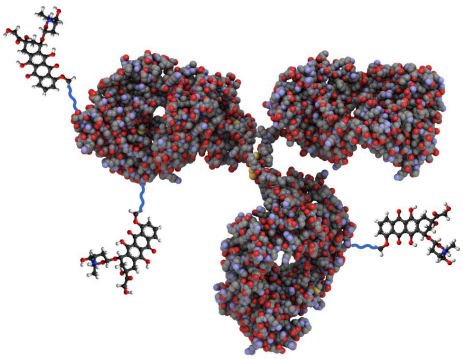
Consumables Workflow Ordering Guide

Improving the Quality of Antibody Drug Conjugates by Orthogonal Analytical Methods



Antibody Drug Conjugates

Antibody-drug conjugates (ADCs) represent a new generation of targeted biotherapeutics that make up a rapidly growing segment of the drug discovery pipeline. Created by attaching potent cytotoxic drugs through a linker to monoclonal antibodies (mAbs) that target specific cells. ADCs approved by the US FDA in 2019/2020 are based on conjugation at cysteine and lysine, with cysteine linker being the majority.

Table 1. Approved ADCs from 2019 to 2020.

Name	IgG isotype	Target	Linker site	Payload
Gemtuzumab ozogamicin	IgG4	CD33	Lysine	Calicheamicin
Brentuximab vedotin	IgG1	CD30	Cysteine	Auristatin (MMAE)
Trastuzumab emtansine	IgG1	HER2	Lysine	Maytansine (DM1)
Inotuzumab ozogamicin	IgG4	CD22	Lysine	Calicheamicin
Polatuzumab vedotin	IgG1	CD79b	Cysteine	Auristatin (MMAE)
Enfortumab vedotin	IgG1	Nectin 4	Cysteine	Auristatin (MMAE)
Trastuzumab deruxtecan	IgG1	HER2	Cysteine	Topoisomerase I inhibitor
Sacituzumab govitecan	IgG1	TROP-2	Cysteine	Active metabolite of irinotecan (SN-38)
Belantamab mafodotin	IgG1 afucosylated	BCMA	Cysteine	Auristatin (MMAF)

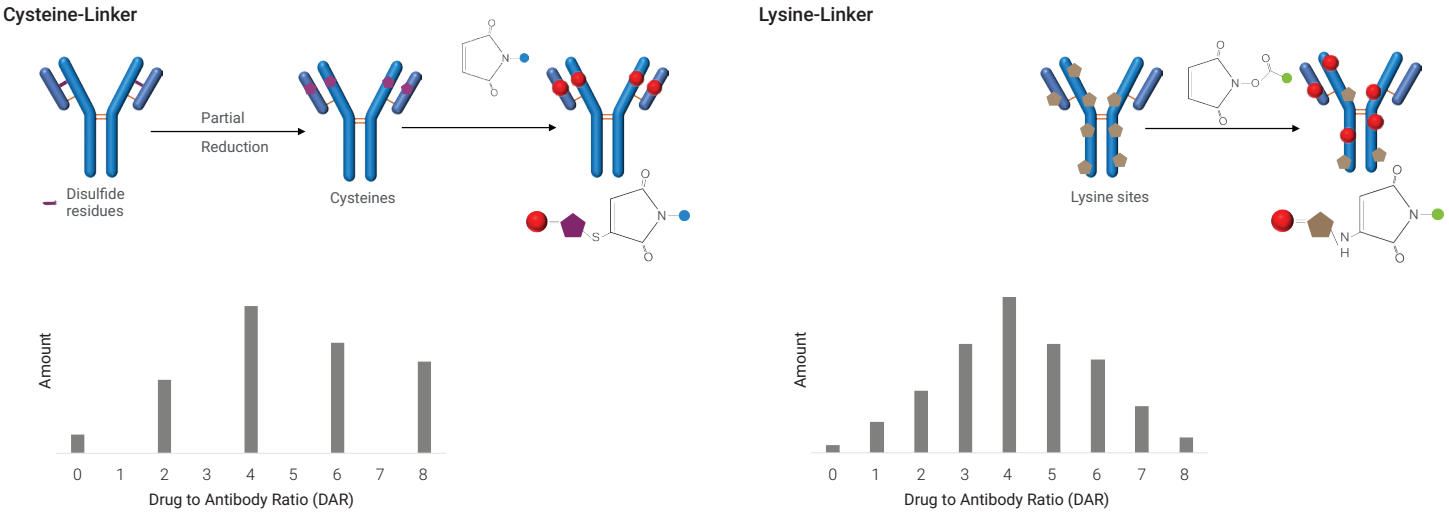


Figure 1. ADC Conjugation Types.

For cysteine conjugation, reduction of the interchain disulfide bonds at the hinge region enables the attachment of up to eight drugs in multiples of two. Lysine linker often results in high a degree of heterogeneity. For example, trastuzumab emtansine has 90 Lys residues throughout the trastuzumab molecule, and each molecule may contain up to eight DM1 conjugates.

As industry adopts the "fail fast, fail cheap" strategy to increase the likelihood of final product approval, it is essential to gain a deep understanding of the structure-function relationship of ADCs early and quickly. This can only be achieved by employing a range of orthogonal analytical techniques to characterize each aspect of the structure and function of the molecule.

The small molecules that are conjugated to antibodies to produce ADCs are typically hydrophobic. For cysteine linked ADCs, the overall hydrophobicity increases as its DAR value becomes larger, making hydrophobic interaction chromatography (HIC) the perfect tool for DAR monitoring. Conversely, lysine linked ADCs have many Lys residues and consist of a mixture of positional isomers. HIC is not the suggested method to resolve lysine linked ADCs[7,8]. Reversed phase chromatography (RP) with mass spectrometry detection (MS) (RP-MS) is the method of choice. RP offers the selectivity for both intact mAb and fragment while MS provides the sensitivity and mass information both of which are critical for peak identification. This is essential for studying lysine linked ADCs because the fragments contain unconjugated and variably conjugated light and heavy chains as well as those with the linker alone[7].

The attachment of the hydrophobic payload to form the ADC also enhances hydrophobicity-driven aggregation[9]. Although aggregates and degradants are present in low concentrations, they have a big impact on the quality of biologics, leading to activity loss, decreased solubility, and increased immunogenicity. Size exclusion chromatography (SEC) is the standard method used to characterize protein aggregation.

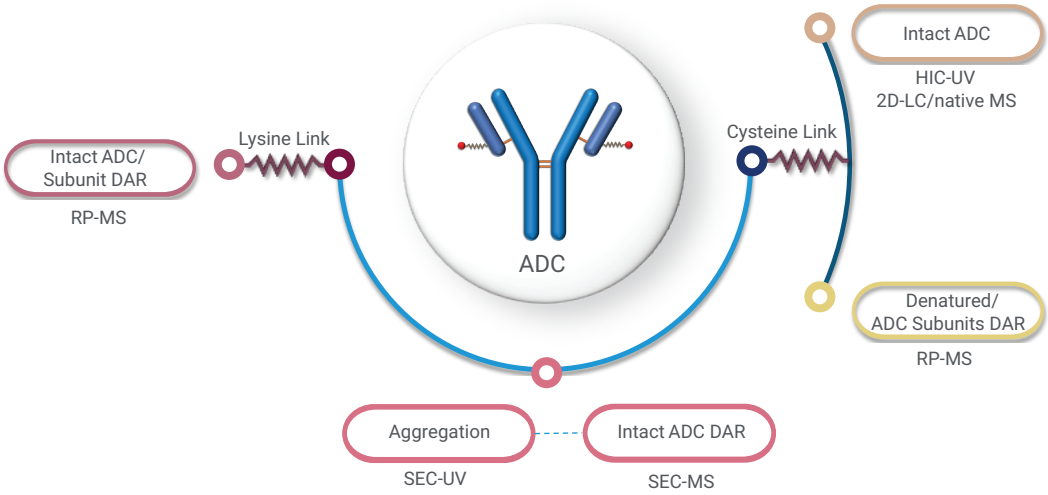


Figure 2. Orthogonal methods for characterizing ADCs.

Tips for optimizing your separation

Sample preparation

- ADC samples tend to be hydrophobic so it's critical to ensure solubility in the eluent. Samples should ideally be dissolved in the initial mobile phase.
- To protect the column from possible damage caused by aggregates and impurities, we recommend that samples are filtered using Captiva premium PES syringe filters (See Easy Selection and Ordering Information section) prior to HPLC analysis.
- When working with complex or "dirty" samples, use guard columns (See Easy Selection and Ordering Information section) to extend column lifetime.

Agilent AdvanceBio HIC Columns:
DAR is monitored in the native form of ADCs

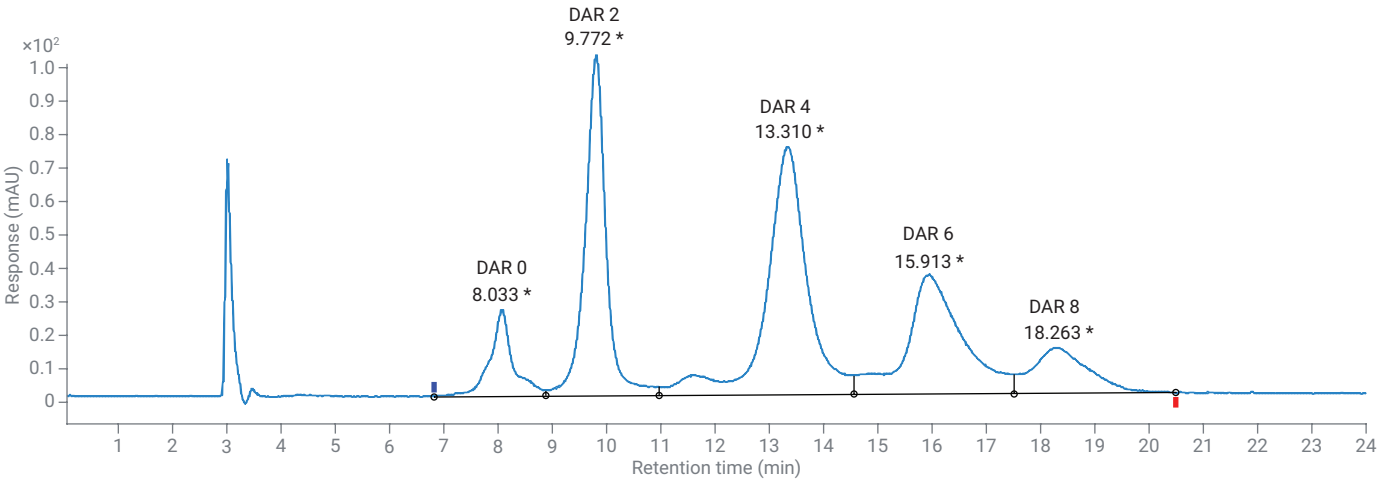


Figure 3. Separation of brentuximab vedotin using Agilent AdvanceBio HIC column. (5994-0149EN)

Hydrophobic Interaction Chromatography (HIC)

HIC utilizes high salts containing mobile phases that reduce biomolecule solubility. This encourages absorption onto the HIC stationary phase. Elution by salt gradient allows the molecules to elute in order of increasing hydrophobicity. Due to the high concentrations of salt used in HIC, a bio-inert LC is recommended. It is still important to avoid leaving either the LC system or the column in concentrated salt solution for any length of time. For that reason, using a quaternary LC system enables other channels to be used for organic modifiers and water or other flush solvents. Propan-2- ol is necessary to ensure accurate determination of higher order of DARs and extend column lifetime[1].

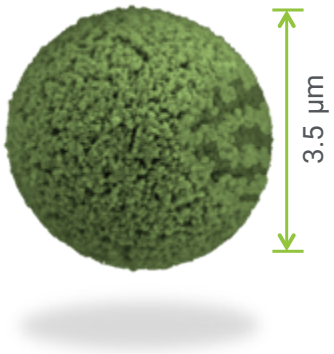


Figure 4. AdvanceBio HIC (Pore Size 450Å).

- Ammonium sulfate is the commonly used salt for HIC due to its ability to induce hydrophobic protein interaction onto the column but it also increases the likelihood of precipitation. The best way to avoid precipitation is to dilute sample with concentrated ammonium sulfate bringing the sample matrix as close as possible to the initial mobile phase[2]. Here are the advantages:
  - Best peak shapes and sensitivity
  - Determine in advance whether the sample precipitates before injection and avoid sample precipitation onto the head of the column
- At the end of the gradient, use a relatively slow reverse gradient over several minutes . Re-equilibrate with 2- 3 column volumes. (User guide)
  - Drastic change of viscosity due to change in salt concentration requires a gradual return to initial mobile phase to prevent column damage
- Elevated temperature is a common approach to running high-viscosity mobile phases, however it is not recommended for HIC due to degradation of protein peak shape
- 2 M ammonium sulfate is a considerable quantity. If a less pure salt is used, baseline of the chromatogram can drift.
  - OpenLab CDS Blank Subtraction can be applied to filter out the baseline drift[3]

Agilent PLRP-S Columns:
Monitor DAR of intact ADCs and subunits

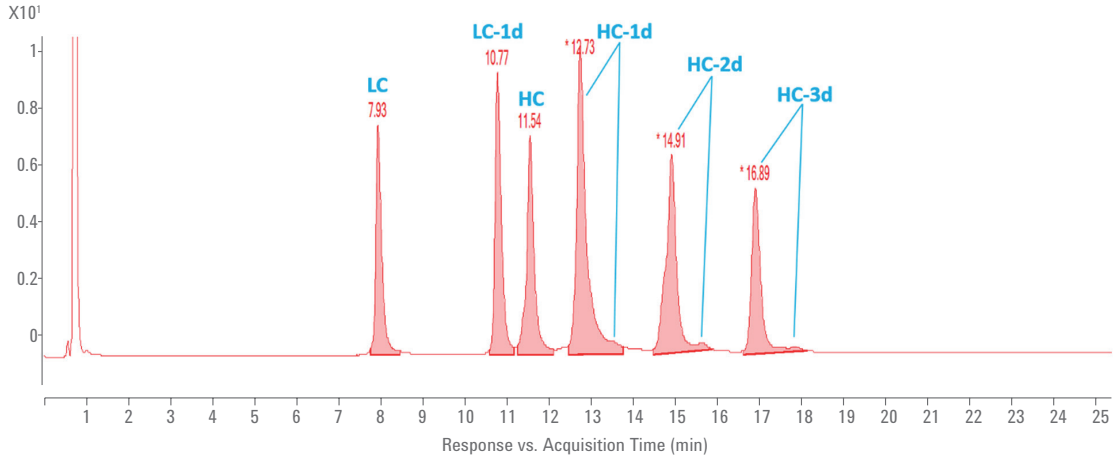


Figure 5. UV absorption spectrum at 280 nm wavelength for reduced brentuximab vedotin separated by reversed phased chromatography and peak identities was determined through mass spectrometry. (5991-6559EN)

PLRP-S

- Reverse flow will not usually harm the column but should be avoided except when trying to clear a clogged frit (see "column care").
- Start the flow rate at a reduced rate and gently increase it to the desired operating flow rate to prevent overpressure.
- Always use high purity reagents and chromatography grade solvents to prepare your mobile phase. Degas and filter all mobile phase before use.
- Use an inline filter to protect your column and increase its lifetime.
- Avoid using 100% aqueous eluents with PLRP-S columns as they will significantly reduce the column lifetime and may result in a rapid deterioration in peak width and symmetry.

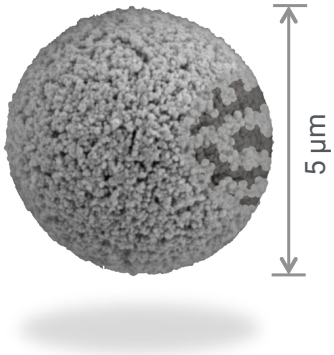


Figure 6. PLRP-S (Pore Size 1000Å).

Agilent AdvanceBio SEC Columns:

Monitors monomers, dimers, aggregates, and degradants

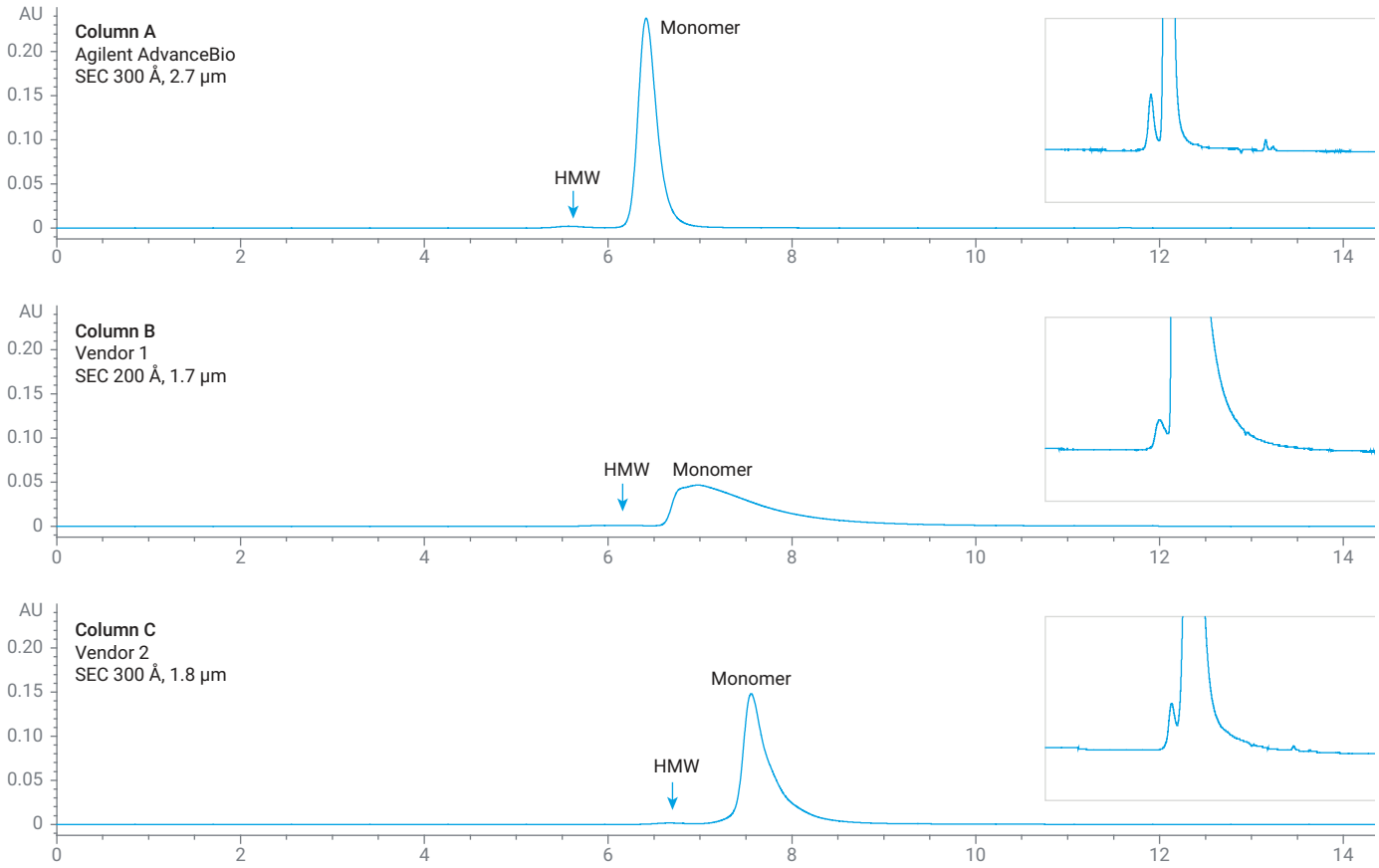


Figure 7. AdvanceBio SEC 300 Å, 2.7 µm for analyzing the Lys-linked trastuzumab emtansine. Column B exhibits increased secondary interactions, as shown by a loss of peak resolution. Column C gives a slightly more narrow peak shape, but the resolution is also inferior to the AdvanceBio column. (5994-3276EN)

Size Exclusion Chromatography (SEC)

Aggregate analysis is another critical quality attribute of ADC characterization. This analysis is complex due to the presence of the cytotoxic drugs attached to the antibody that can induce aggregation and create more complex impurity profiles. SEC is effective, but still challenging, for the quantification of aggregates and fragments. ADCs are frequently more hydrophobic than mAbs alone and are therefore more susceptible to nonspecific interactions. It is important to select a stationary phase that offers an inert hydrophilic bonding surface chemistry to minimize secondary interactions without the need for organic modifier that could influence aggregation state.

Native LC/MS methods also enable determination of cysteine linked and lysine linked ADC DAR. Agilent has developed a 2D-LC/MS method[10] for the characterization of intact cysteine linked DARs under native LC/MS conditions. The workflow uses the Agilent AdvanceBio HIC column, the Agilent AdvanceBio SEC column, and highly sensitive MS method to accurately determine intact mass for all ADCs with various DARS. Similarly, Agilent developed a Native LC/MS method[11] using an Agilent AdvanceBio SEC 200 Å, 1.9 µm column and a 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent Jet Stream source. This method minimizes the interferences from organic solvent and acid in the mobile phase, it is ideal for lysine linked ADCs.

- Longer columns result in higher resolution – ideal for separating higher order of aggregates from monomers
- AdvanceBio SEC 300 Å, 2.7 µm columns are available in a variety of column lengths and diameters to provide fast and accurate quantitation of ADCs aggregates and monomers (User guide).
- Aqueous mobile phase PBS at pH 7.4 delivers the best resolution for both cysteine linked, and lysine linked ADCs[6]
- Higher salt concentration does not improve peak resolution of ADCs[6]

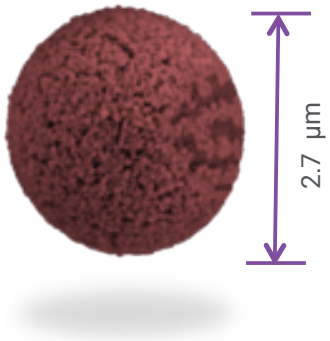


Figure 8. AdvanceBio SEC (Pore Size 300Å).

Easy Selection and Ordering Information

To order items listed in the tables below from the Agilent online store, add items to your Favorite Products list by clicking on the MyList # header links. You can then enter the quantities for the products you need, add the products to your Cart and proceed to checkout. Your list will remain under Favorite Products for your use with future orders.

If this is your first time using Favorite Products, you will be asked to enter your email address for account verification. If you have an existing Agilent account, you will be able to log in. However, if you don't have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are e-commerce enabled. All items can also be ordered through your regular sales and distributor channels.

Description	Part Number
MyList of Sample Preparation Supplies	
Captiva disposable syringe, 5 mL, 100/pk	9301-6476
Captiva Premium Syringe Filter, PES, 15 mm, 0.2 µm, 100/pk	5190-5096
MyList of Standards	
Agilent-NISTmAb, 25 µl	5191-5744
Agilent NISTmAb, 4 x 25 µL	5191-5745
300 Å AdvanceBio SEC calibration standard	5190-9417
MyList of AdvanceBio HIC Columns	
AdvanceBio HIC, 4.6 x 100 mm, 3.5 µm	685975-908
AdvanceBio HIC, 4.6 x 30 mm, 3.5 µm	681975-908
MyList of AdvanceBio PLRP-S Columns	
PLRP-S 1000Å, 1.0 x 50 mm, 5 µm	PL1312-1502
PLRP-S 1000Å, 2.1 x 50 mm, 5 µm	PL1912-1502
PLRP-S 1000Å, 4.6 x 50 mm, 5 µm	PL1512-1502
PLRP-S 1000A 5um, 2.1x50mm PEEK lined	PL1912-1502PK
PLRP-S 1000A, 5um. 2.1x100mm. PEEK lined	PL1912-2502PK

Description	Part Number
MyList of AdvanceBio SEC Columns	
AdvanceBio SEC 300Å, 4.6 x 150 mm, 2.7 µm, LC column	PL1580-3301
AdvanceBio SEC 300Å, 4.6 x 300 mm, 2.7 µm, LC column	PL1580-5301
AdvanceBio SEC 300Å, 7.8 x 150 mm, 2.7 µm, LC column	PL1180-3301
AdvanceBio SEC 300Å, 7.8 x 300 mm, 2.7 µm, LC column	PL1180-5301
AdvanceBio SEC 300Å, 4.6 x 50 mm, 2.7 µm, LC guard column	PL1580-1301
AdvanceBio SEC 300Å, 7.8 x 50 mm, 2.7 µm, LC guard column	PL1180-1301
AdvanceBio SEC 200 Å, 4.6 x 150 mm, 1.9 µm, LC column	PL1580-3201
AdvanceBio SEC 200 Å, 4.6 x 30 mm, 1.9 µm, LC guard column	PL1580-1201
AdvanceBio SEC 200 Å, 4.6 x 300 mm, 1.9 µm, LC column	PL1580-5201
AdvanceBio SEC 200A 1.9um 2.1x150mm PEEK lined	PL1980-3201PK
AdvanceBio SEC 200A 1.9um 2.1x50mm PEEK lined	PL1980-1201PK
MyList of HPLC Supplies	
Ultra low dispersion kit, bio, for use with 1290 Infinity II Bio System	5004-0007
Ultra-low dispersion kit for Agilent 1290 Infinity LC Series	5067-5189
MyList of Solvents & Reagents	
InfinityLab Ultrapure LC/MS acetonitrile, 1 L	5191-4496
InfinityLab Ultrapure LC/MS standard, water, 1L	5191-4498
Formic Acid - 99.5% purity	G2453-85060

Description	Part Number
MyList of Column Fittings and Connectors	
Agilent InfinityLab Quick Connect Fitting (for connection on column inlet)"	5067-5965
Agilent InfinityLab Quick Connect Capillary MP35N 0.12 x 105 mm (for Quick Connect fitting)	5500-1578
Agilent InfinityLab Quick Turn Fitting (for connection on column outlet)	5067-5966
Quick Turn Capillary MP35N 0.12 x 280 mm (for Quick Turn fitting)	5500-1596
Mounting tool for quick turn fittings	5043-0915
Capillary MP35N 0.17 x 100 mm SL/SL ps/ps (for connecting SEC guard and column)	5500-1278
Capillary MP35N 0.12 x 90 mm SL/SL ns/ns (for connecting PLRP-S guard and column)	5004-0018
MyList of Solvent Handling Supplies	
InfinityLab Stay Safe cap starter kit	5043-1222
InfinityLab solvent bottle, clear, 1 L	9301-6524
InfinityLab solvent bottle, amber, 1 L	9301-6526
Solvent bottle, clear, 2 L	9301-6342
Solvent bottle, amber, 2 L	9301-6341
InfinityLab Stay Safe Purging Bottle, 1L	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap (Charcoal filter 5043-1193 not included)	5043-1221
InfinityLab charcoal filter with time strip, 58 g (use with 5043-1221)	5043-1193
MyList of Solvent Filtration Supplies	
InfinityLab Solvent filtration assembly	5191-6776
InfinityLab solvent filtration flask, glass, 2 L	5191-6781
Filter membrane, Nylon 47 mm, pore size 0.2 µm, 100/pk	5191-4341
Filter membrane, Regenerated Cellulose 47 mm, pore size 0.2 µm, 100/pk	5191-4340
Solvent bottle glass filter, solvent inlet, 20 µm	5041-2168
MyList Sample Containment	
A-Line screw top vial, 2 mL, amber, write-on spot, 100/pk	5190-9590
Screw cap, bonded blue, PTFE/silicone septa, 100/pk	5190-7021
Vial, screw top, clear, high recovery, 5 mL, for LC, 30/pk	5188-5369
Septa, preslit PTFE/silicone, 16 mm, 100/pk	5188-2758
Cap, screw, for 6 mL vials, 100/pk	9301-1379
InfinityLab 96-well plate, 2.0 mL, round wells, U shape, polypropylene, 45 mm, 30/pk	5043-9302
InfinityLab 96-well plate, 2.2 mL, square wells, U shape, polypropylene, 41 mm, 30/pk	5043-9300

Note: ADC DAR Calculator upgrade (part number G4994AA) is available for MassHunter DAR Calculator software designed to investigate DAR ratios of deconvoluted LC/MS sample data acquired from ADCs. Please contact your local Agilent Representative for ordering information.

References

1. An AdvanceBio HIC Column for Drug-to-Antibody Ratio (DAR) Analysis of Antibody Drug Conjugates (ADCs) 5994-0149EN
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9. Jakob W. Buecheler, Matthias Winzer, Jason Tonillo, Christian Weber, and Henning Gieseler Molecular Pharmaceutics 2018 15 (7), 2656-2664 DOI: 10.1021/acs.molpharmaceut.8b00177
10. Characterization of Antibody-Drug Conjugates Using 2D-LC and Native MS 5994-4328EN
11. Sensitive Native Mass Spectrometry of Macromolecules Using Standard Flow LC/MS 5994-1739EN

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DE73791623

Printed in the USA, November 17, 2022
5994-5089EN

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