

Multi Dimensional HPLC Separation of Monoclonal Antibody Mixtures from Cell Culture Supernatants



Therapeutic monoclonal antibodies (mAbs) come with structural complexity highly demanding towards analytics. Combining multiple analytical techniques on a system enables simultaneous multi-attribute analysis (MAA) such as molecular weight (MW), amino acid sequence, N-glycosylation, N- and C-terminal processing, deamidation, oxidation, fragmentation, and aggregation.¹

An automated 2D-LC/MS multi-attribute analyzer incorporating Protein A affinity chromatography in the first dimension with a multimethod option (SEC, CEX, or HIC) in the second dimension, with desalting SEC-MS for glycan analysis.²⁻⁶ This setup enables purification of mAbs from cell culture supernatants and determination of mAb titer, size/charge/hydrophobic variants, molecular weight, amino acid sequence, and post-translational modifications. The setup can automatically switch between the different 2D methods without impacting resolution.

New to 2D-LC?	Agilent offers 2-Day in-person training on 2D-LC ProtA-SEC application for new users. Contact your local sales representative and ask about service package (p/n R3995C).
Want to try 2D-LC on your own?	Agilent offers the InfinityLab 2D-LC ProtA-SEC kit (p/n G4245A), which includes columns, standards and a media kit containing application notes, workflow guide, method file, and report template. Call your local sales representative for details.

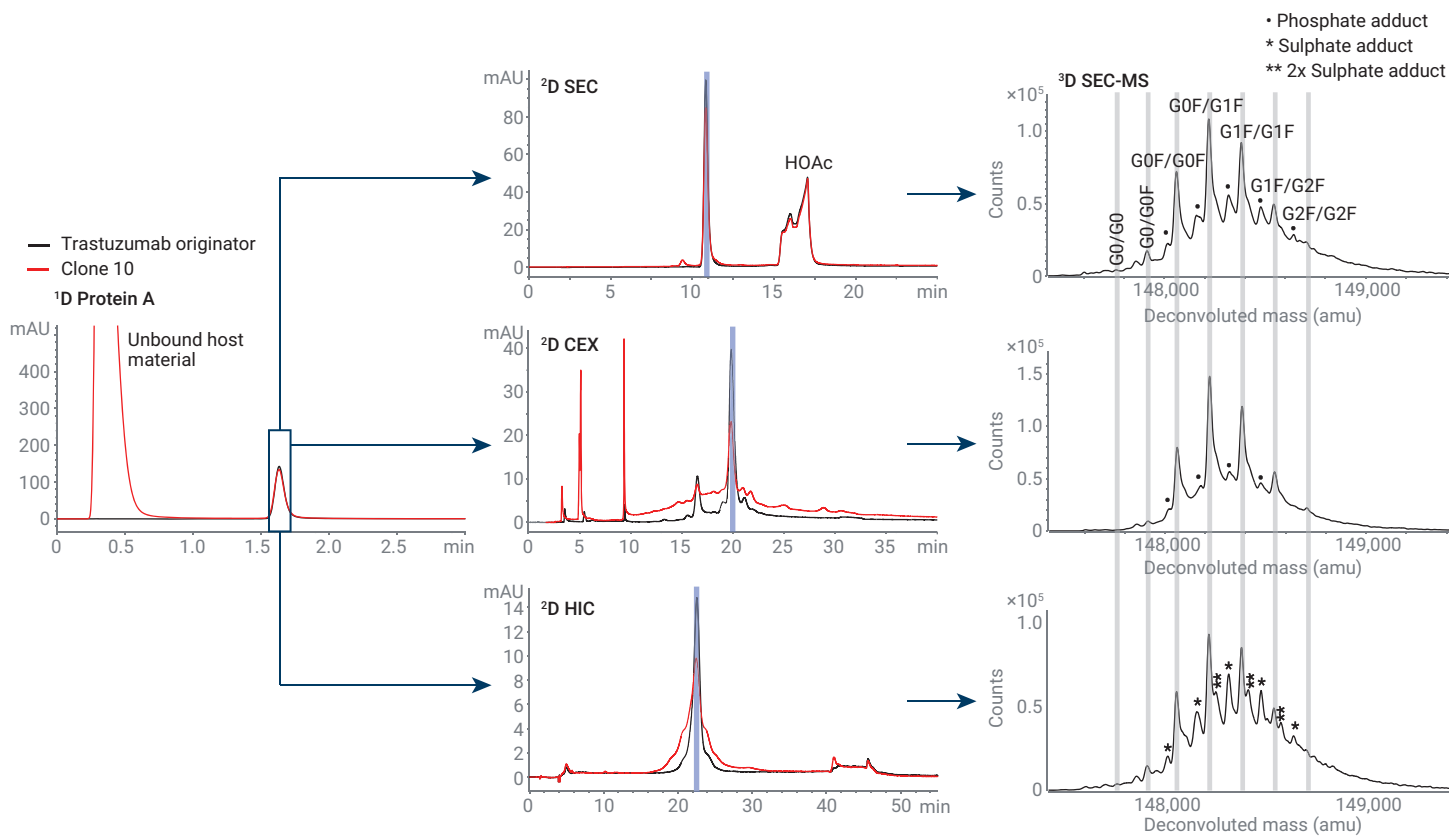


Figure 1. (A) 1D Protein A chromatogram of the trastuzumab originator and CHO clone 10 supernatants. (B) 2D SEC, CEX, and HIC chromatograms of the Protein A peak at 1.6 minutes. (C) Deconvoluted 3D SEC-MS spectra of the trastuzumab originator main 2D SEC, CEX, and HIC peak. 2D heart cuts taken are indicated in blue. (For details see application note 5994-3521EN)

Tips for optimizing your 2D and 3D separation

- Use Agilent polypropylene (PP) vial (p/n 5191-8150) with 250 mL insert (p/n 5182-0549) or deactivated glass vial (p/n 5182-0714) with 400 mL insert (p/n 5183-2086) for monoclonal antibody sample. We recommend using deactivated glass vials for biological or pharmaceutical compounds and using PP vials where glass vial is not an appropriate option.
- Always use high-quality chemicals i.e., HPLC grade, ACS grade, or reagent grade.
- Always filter the buffer mobile phase through 0.22 or 0.45 μm membrane filter. The 0.22 μm membranes are primarily used for solution sterilization while 0.45 μm membranes are typically used for particle removal or general filtration. Nylon membranes are hydrophilic and are resistant to a range of organic solvents and suitable for use with high pH samples. It's not suitable to acidic solutions. Regenerated cellulose membrane is resistant to a wide variety of solvents suitable for aqueous and organic samples.
- Check and calibrate pH meter to ensure the pH accuracy before measurement of buffers. Recalibrate if necessary.

Sample preparation

- Sample must be soluble in the eluent and should ideally be dissolved in the mobile phase itself.
- We recommend filtering samples using Captiva premium PES syringe filters to protect the column from possible damage.

Column

Storage of Protein A column⁷⁻⁸:

- Short term (overnight or less than two days):
 - Flush the column with binding buffer (mobile phase A).
 - Disconnect the column from the instrument.
 - Cap the column before storage at 4 to 8 °C. The column should be equilibrated before the first injection after short-term storage.
- Long term (over two days):
 - Flush the column with at least 1 mL (10 column volume (CV)) DI water.
 - Afterwards flush the column with at least 2 mL (20 CV) of 20 % Ethanol in 20 mM Tris buffer pH 7.4 at flow rate of 0.2 – 0.5 mL/min.
 - Cap the column before storage at 4 to 8 °C.

Storage of SEC column:

- Short-term storage (less than one week):
 - Store the column in the mobile phase with both ends capped.
- Long-term storage (more than one week):
 - Store the column in filtered 100 mM sodium phosphate, pH ≤ 7, with or without 0.02% NaN₃, or 20% methanol in water.
 - Flush the column with a minimum of 10 column volumes.
 - To switch to or from 20% methanol, column flushing must be done at low flow rates to avoid overpressuring the column due to high viscosity.
 - Starting at a lower flow rate, flush at no more than 0.1 mL/min for 4.6 mm columns, and no more than 0.2 mL/min for 7.8 mm columns, while also ensuring the pressure remains below 200 bar.

Storage of Bio MAb column:

- Short-term storage (less than one week): 20 mM phosphate buffer, pH 6.0.
- Long-term storage: Flush using 20 mM phosphate buffer (the shipping solution), with 0.1% NaN₃ (sodium azide) at pH 6.0. Flush for at least 15 times the column volume.

Storage of AdvanceBio HIC column:

- Short-term storage (overnight): Flush column with low salt-containing buffer.
- Long-term storage: Flush the salt-containing mobile phase from the column and transfer the column into 100% acetonitrile.

Easy selection and ordering information

This guide lists all the columns and supplies you will need for 2D-LC analysis using an appropriately setup system.¹

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.

MyList 1 Chromatography columns used in application note ([5994-3521EN](#))

Description	Part No.
Bio-Monolith Protein A, 4.95 x 5.2 mm	5069-3639
Bio-Monolith Recombinant Protein A, 4.95 x 5.2 mm	5190-6903*
Agilent AdvanceBio SEC 300 Å, 7.8 x 300 mm, 2.7 µm	PL1180-5301
Agilent Bio MAb NP5 PK, 2.1 x 250 mm, 5 µm	5190-2411
Agilent AdvanceBio HIC, 4.6 x 100 mm, 3.5 µm	685975-908

* Agilent recently released the column, so it was not included in the study. It offers the same performance as the native Protein A column (5069-3639). See application note ["mAb Titer Determination in 60 Seconds Using the Agilent Bio-Monolith Protein A Column"](#) for more information.

MyList 2 Standards and controls

Description	Part No.
130 Å AdvanceBio SEC calibration standard	5190-9416
300 Å AdvanceBio SEC calibration standard	5190-9417
Agilent-NISTmAb, 4 x 25 µL	5191-5745

MyList 3 Sample preparation and Laboratory supplies, reagents and solvents

Description	Part No.
Sample filtration	
Captiva Disposable Syringe, 5 mL, 100/pk	9301-6476
Captiva Syringe Filter, polyethersulfone (PES) membrane, 4 mm, 0.45 µm, LC/MS certified, 100/pk	5190-5095
Captiva Syringe Filter, polyethersulfone (PES) membrane, 4 mm, 0.2 µm, LC/MS certified, 100/pk	5190-5094
Solvents & Reagents	
InfinityLab Ultrapure LC/MS acetonitrile, 1 L	5191-4496
InfinityLab Ultrapure LC/MS standard, water, 1L	5191-4498
MS solution, formic acid, 10 mL	US-700002341
Solvent Filtration Supplies*	
InfinityLab Solvent filtration assembly	5191-6776
InfinityLab solvent filtration flask, glass, 2 L	5191-6781
Filter membrane, Nylon 47 mm, 0.45 µm, 100/pk	5191-4338
Filter membrane, Nylon 47 mm, 0.2 µm, 100/pk	5191-4341
Filter membrane, regenerated cellulose 47 mm, 0.2 µm, 100/pk	5191-4340
Solvent bottle glass filter, solvent inlet, 20 µm	5041-2168

Description	Part No.
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	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap**	5043-1221
InfinityLab charcoal filter with time strip, 58 g**	5043-1193
Vials & Caps†	
Vial, screw, 2 mL, polypropylene, certified, 100 pk	5191-8150
9 mm screw style clear polypropylene cap, 100 pk	5191-8151
250 µL insert, polypropylene, 100 pk	5182-0549
Deactivated glass vial insert, 400 µL, deactivated glass, flat bottom, 500/pk	5183-2086
Cap, screw, blue, PTFE/red silicone septa, 100/pk	5182-0717
Deactivated glass vial, screw top, clear, certified, 2 mL, 100/pk	5182-0714

* Use the InfinityLab Solvent Filtration assembly prior to analysis if using solvents other than those listed in this table.

** Charcoal filter not included with waste can, order 5043-1221 and 5043-1193 together.

† Agilent recommends using a 250 µL vial insert (p/n 5182-0549) with the 2 mL polypropylene vial (p/n 5191-8150) to minimize dead volume.

Additional Resources

- Two-Dimensional Liquid Chromatography Principles, Practical Implementation and Applications authored by Peter W. Carr and Dwight R. Stoll ([5991-2359EN](#))
- Determination of Multiple Attributes of Monoclonal Antibodies ([5994-3521EN](#))
- Automated Monoclonal Antibody Subunit Analysis by Online Reduction Using 2D-LC/MS ([5994-4309EN](#))
- Multiple Heart-Cutting 2D-LC Analysis of Innovator versus Biosimilar Monoclonal Antibodies ([5991-6673EN](#))
- Agilent 1290 Infinity 2D-LC Solution user guide ([G2198-90001](#))
- [Agilent Heart-cutting 2D-LC Software Tutorial](#)
- mAb Titer Determination in 60 Seconds Using the Agilent Bio-Monolith rProtein A Column ([5994-3969EN](#))
- Agilent Bio-Monolith Protein A and G Columns Data Sheet ([5991-6040EN](#))

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This information is subject to change without notice.

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Printed in the USA, January 21, 2022
5994-4351EN