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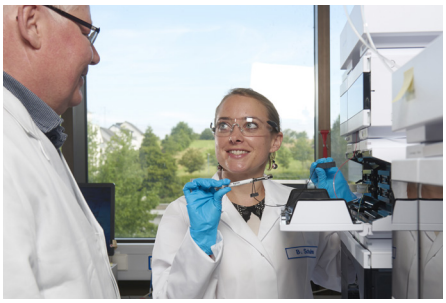
Consumables Workflow Ordering Guide

# mAb Charge Variant Analysis by Weak Cation Exchange (WCX) Chromatography

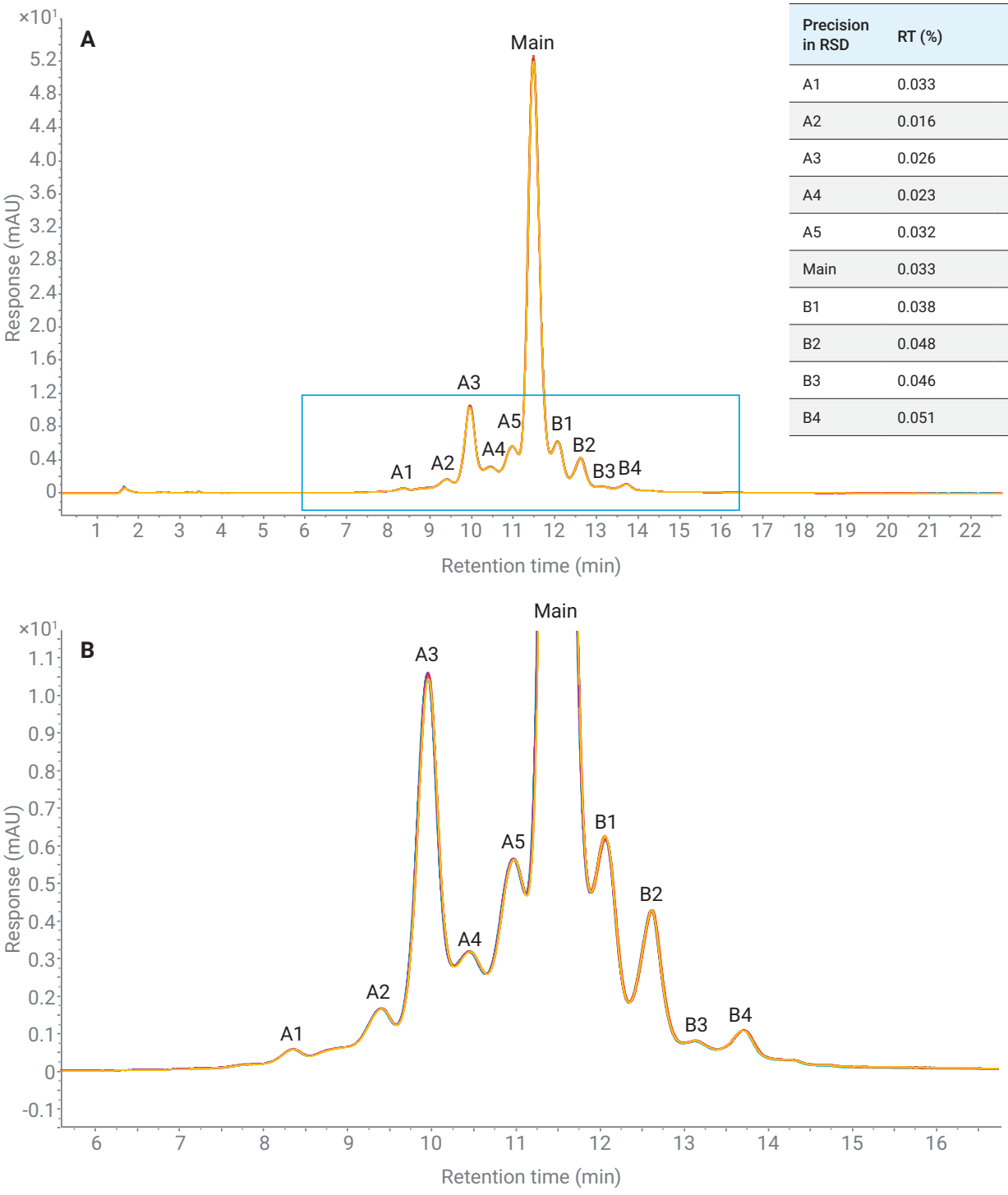


Monoclonal antibodies (mAbs) are a prominent biotherapeutic that are produced by most major pharmaceutical companies. mAbs are about 150,000 Daltons and have several modifications that add to the complexity of this class of macromolecule. Modifications can occur as the protein is produced or as they are manufactured and stored. Modifications such as C-terminal truncation, deamidation, changes in glycosylation, and amino acid deletion or substitution affect the overall charge of a mAb and are considered charge variants. Charge variants are typically monitored via ion exchange chromatography.[1,2] Monitoring critical quality attributes (CQAs) such as charge variants is essential to ensure efficacy, safety, and immunogenicity of the final product is not compromised. Government agencies such as the U.S. Food and Drug Administration (FDA) require charge variant data when submitting a Biologics License Application.

Although charge variant analysis is an established technique, challenges remain for this assay. The buffers that are frequently used have a high salt concentration and can be corrosive to traditional stainless-steel hardware in liquid chromatography systems and columns. Using a bio-compatible or bio-inert system like the Agilent 1290 Infinity II Bio LC or the Agilent 1260 Infinity II Bio-Inert system mitigates this issue. The flow path is completely iron and stainless-steel free. All fittings and capillaries are made of an alloy, MP35N, so corrosion is reduced. In addition, this prevents reactions from corrosion such as oxidation from occurring. The 1290 Infinity II Bio LC is available as a binary pump which is ideal for producing accurate and precise gradients, especially for shallow gradients which are typical of charge variant analyses. The quaternary pump option is also useful for charge variant analysis for testing different buffer conditions which is made easier with Agilent Buffer Advisor software.

The Agilent Bio MAb column is a weak cation exchange column specifically designed to characterize the charge heterogeneity of monoclonal antibodies.[3] It is available in PEEK hardware, which maintains an iron-free flow path throughout the 1290 Infinity II Bio LC system, essential for highly reproducible results (Figure1).[4]

Furthermore, the column itself was specifically designed for mAb separations and has an extra dense and highly uniform weak cation exchange layer that is bonded to the hydrophilic polymeric coating, which is designed to eliminate non-specific interactions, making it ideal for biosimilar comparability studies (Figure 2).[5]




| Precision in RSD | RT (%) | Area (%) |
|---|---|---|
| A1 | 0.033 | 1.793 |
| A2 | 0.016 | 0.701 |
| A3 | 0.026 | 0.403 |
| A4 | 0.023 | 0.813 |
| A5 | 0.032 | 0.327 |
| Main | 0.033 | 0.313 |
| B1 | 0.038 | 0.329 |
| B2 | 0.048 | 0.254 |
| B3 | 0.046 | 3.549 |
| B4 | 0.051 | 0.812 |

**Figure 1.** Acidic and basic variants of trastuzumab. Seven subsequent runs yields highly reproducible results in retention time and peak area.

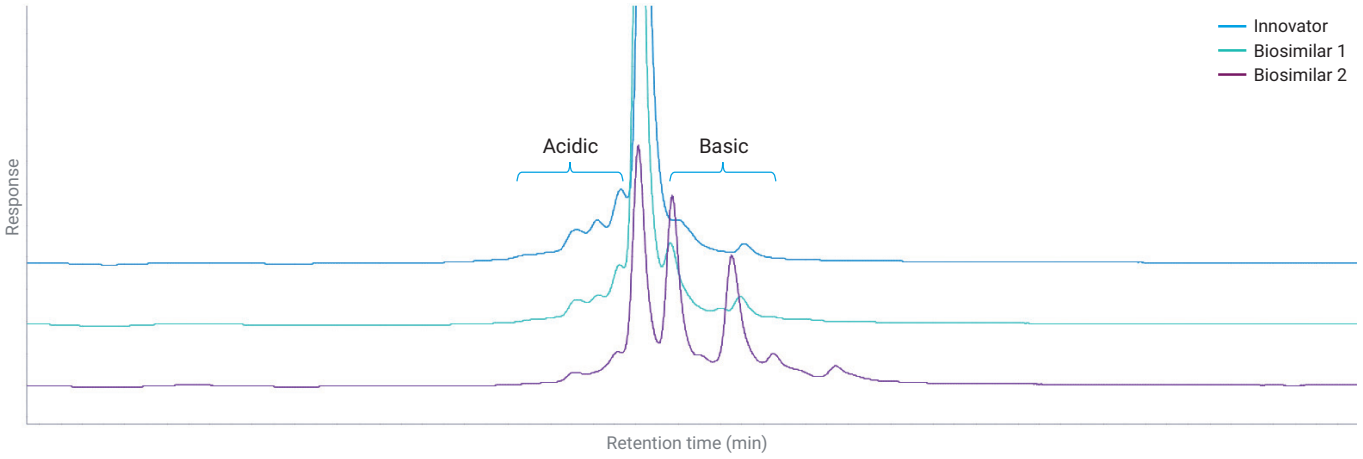



**Figure 2.** Comparison of charge variants between rituximab and two biosimilars.

## Best Practices for Optimizing Chromatographic Conditions

Two parameters are essential to achieve the desired resolution and enable optimal separation of charge variants: determining the optimal pH of the mobile phase as well as the optimal gradient slope. Both factors can have a major impact on the separation. Table 1 suggests parameters from where to start optimization.

| | Salt Gradient | pH Gradient |
|---|---|---|
| **Parameter** | **Value** | **Value** |
| Column | Bio MAb, NP5, 2.1 x 250 mm, PEEK<br>(part number 5190-2411) | Bio MAb, NP5, 2.1 x 250 mm, PEEK<br>(part number 5190-2411) |
| Suggested LC System | Agilent 1290 Infinity II Bio LC System with<br>High Speed Pump | Agilent 1290 Infinity II Bio LC System with<br>Flex (Quaternary) Pump |
| Mobile Phase | A: 30 mM phosphate buffer, pH 6.8<br>B: 30 mM phosphate buffer, pH 6.8, 500 mM NaCl | A: Water<br>B: 1.6 M NaCl<br>C: 100 mM $NaH_2PO4$<br>D: 100 mM $Na_2HPO4$ |
| Gradient | 0-100 mM NaCl from 0-30 minutes;<br>100-500 mM from 30-31 min:<br>Isocratic 500 mM from 31-35 min.<br>Post-time 15 minutes | pH 6.0 to 8.0, 0 to 20 minutes<br>0 to 800 mM NaCl, 20 to 25 minutes<br>800 mM NaCl, 25 to 30 minutes |
| Flow Rate | 0.25 mL/min | 0.25 mL/min |
| Injection Volume | 1-5 µL | 1-5 µL |
| Temperature | Ambient | Ambient |
| Detection | 280 nm | 280 nm |

**Table 1.** Suggested starting conditions

### Mobile Phase Considerations

- The mobile phase should contain buffer to maintain the desired operating pH as well as consistent charge, the concentration is typically 20-30 mM.
- Phosphate buffers are very common in the range of pH 6 to 7. Other compatible buffers include acetate, Tris, and MES-containing buffers as well as acetonitrile and methanol. mAb samples must be soluble in the mobile phase.
- A competing ion must be introduced to elute the mAb from the column which is typically accomplished with 100 mM to 500 mM sodium chloride.
- Addition of sodium chloride will alter the pH of the mobile phase, so the pH will need to be readjusted.
- For optimal results, buffers should be made fresh and stored in the refrigerator unless being actively used as bacterial growth is common in dilute buffer. In addition, it is best practice to filter buffers to prevent column clogging.

### pH Gradient Buffer Considerations

- Salt gradients are more common in ion-exchange chromatography; however, pH gradients are an alternate method[6,7] that may result in higher resolution. The Bio MAb column is stable from pH 2-12.
- mAb pH gradients typically start at pH 6 and run up to pH 7-8 and have a sodium chloride salt column cleanup/ equilibration step.
- Monosodium phosphate and disodium phosphate are typical elution buffers.

### More General Protein Mobile Phase Considerations

- The pH of the starting buffer should be 0.5 to 1 pH unit below the proteins' PI for cation-exchange chromatography.
- pH 6 is a good starting place if the pI of the protein is not known.

### Column Dimensions

- The inner diameter (id) of the column should be selected based on the amount of mAb being analyzed. A general guideline is that the volume injected should not exceed more than 1-2% of the entire column volume. For example, a 2.1 x 50 mm column should ideally have 1.7 µL or less injected while a 4.6 x 50 mm could handle an 8.3 µL injection. If a 2.1 mm id column is selected, the LC should be plumbed to minimize dispersion.
- A longer column such as 250 mm will yield higher resolution, while a shorter column like 50 mm can help reduce run time.

### Particle Size

- Generally, smaller particle sizes provide more efficient separation, but have higher operating pressures.
- Larger biomolecules like mAbs have a slower rate of diffusion, so particle sizes do not have as strong of an effect on resolution.
- Eluents with aqueous buffers are relatively viscous and can yield higher back pressures.
- Bio MAb particle sizes and max back pressures are as follows:
  - 1.7 µm: 689 bar
  - 3 µm: 551 bar
  - 5 µm: 413 bar
  - 10 µm: 275 bar
- Another consideration is that PEEK hardware has a pressure limit of 400 bar and is only available in the 5 and 10 µm particle sizes. Salt gradients can be corrosive to stainless steel, so using PEEK columns in addition to a bio-inert or bio-compatible LC system will maintain robustness of the system.

### Column Lifetime and Reproducibility

- Flow rates range from 0.1- 1.0 mL/min. 2.1 mm id columns typically run at 0.2-0.4 mL/min while 4.6 mm id columns typically run from 0.5-1.0 mL/min. Backpressure should be monitored and initially flow rates should be ramped slowly to ensure the column does not exceed the operating pressure limit.
- Bio MAb columns can withstand up to 80°C. However, to extend column lifetime, they should be regularly operated between 10-50°C.
- For maximum reproducibility, the equilibration/cleanup step in the gradient should be 5-10 column volumes.
- Consider using a guard column to extend the life of the analytical column.
- For extended storage, the column should be flushed for at least 15 column volumes and stored in 20 mM phosphate buffer with 0.1% sodium azide at pH 6.

## 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. Then, enter the quantities for the products you need, Add to 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 No. |
|---|---|
| MyList 1: Bio MAb columns for charge variant analysis | |
| Agilent Bio MAb, NP5, 2.1 x 50 mm, PEEK | 5190-2412 |
| Agilent Bio MAb, NP5, 2.1 x 250 mm, PEEK | 5190-2411 |
| Agilent Bio MAb, NP5, 4.6 x 50 mm, PEEK | 5190-2408 |
| Agilent Bio MAb, NP5, 4.6 x 250 mm, PEEK | 5190-2407 |
| Agilent Bio MAb, NP10, 2.1 x 50 mm, PEEK | 5190-2420 |
| Agilent Bio MAb, NP10, 2.1 x 250 mm, PEEK | 5190-2419 |
| Agilent Bio MAb, NP10, 4.6 x 50 mm, PEEK | 5190-2416 |
| Agilent Bio MAb, NP10, 4.6 x 250 mm, PEEK | 5190-2415 |
| Agilent Bio MAb, NP1.7, 4 x 10 mm, guard | 5190-2402 |
| Agilent Bio MAb, NP3, 4 x 10 mm, guard | 5190-2404 |
| Agilent Bio MAb, NP5, 4 x 10 mm, guard | 5190-2406 |
| Agilent Bio MAb, NP10, 4 x 10 mm, guard | 5190-2414 |
| Agilent Bio MAb, NP1.7, 4.6 x 50 mm | 5190-2401 |
| Agilent Bio MAb, NP3, 4.6 x 50 mm | 5190-2403 |
| Agilent Bio MAb, NP5, 4.6 x 250 mm | 5190-2405 |
| Agilent Bio MAb, NP10, 4.6 x 250 mm | 5190-2413 |
| Agilent Bio MAb, NP5, 10 x 250 mm | 5190-6884 |
| Agilent Bio MAb, NP5, 21.2 x 250 mm | 5190-6885 |
| MyList 2: Standards | |
| Agilent NIST mAb, 25 µL | 5191-5744 |
| Agilent NIST mAb, 4 x 25 µL | 5191-5745 |
| MyList 3: Supplies & Solvents | |
| Connections & Tubing | |
| InfinityLab Quick Connect LC fitting | 5067-5965 |
| Agilent InfinityLab Quick Turn Fitting (for connection on column outlet) | 5067-5966 |
| Quick Turn Capillary MP35N 0.12 x 200 mm | 5500-1595 |
| Fitting Union Bio compatible, MP35N 10-32 coned. zero dead volume | 5023-2625 |
| Quick Turn Capillary MP35N 0.12 x 280 mm | 5500-1596 |
| Mounting tool for quick turn fittings | 5043-0915 |
| Inline pressure relief valve kit (For use when another detector is used in series after the fluorescence flow cell) | G4212-68001 |
| 6-Column Selector Valve, analytical, Bio-compatible. 1290 Infinity II Bio LC System | 5320-0025 |
| Quick Change 2-Position/10-Port Bio Valve Includes 1300 bar Quick Change Bio valve head. | 5067-6682 |
| Ultralow dispersion tubing kit for Agilent 1290 Infinity II Bio | 5004-0007 |
| Capillary kit for 2 position/10 port switching valve bio | 5013-0002 |
| Capillary Kit for 6-Column Selector Valve. Bio-compatible, 0.12 mm id 1290 Infinity II Bio LC System | 5005-0070 |

| Description | Part No. |
|---|---|
| MyList 3: Supplies & Solvents | |
| Sample Containment | |
| High recovery vial, screw top, with fixed insert, clear, 300 µL insert volume, 100/pk. Vial size: 12 x 32 mm (12 mm cap) | 5188-6591 |
| Cap, screw, blue, PTFE/red silicone septa, 100/pk. Cap size: 12 mm | 5182-0717 |
| Vial, crimp/snap top, polypropylene, 250 µL, 1,000/pk. Vial size: 12 x 32 mm (11 mm cap)* | 5190-3155 |
| Cap, snap, clear, PTFE/silicone/PTFE septa, 100/pk. Cap size: 11 mm (for 5190-3155) | 5182-0566 |
| InfinityLab 96-well plate, 0.5 mL, 30/pk | 5043-9310 |
| InfinityLab 96-well plate closing mat, 50/pk | 5042-1389 |
| Solvents & Additives | |
| InfinityLab Ultrapure LC/MS Water, 1 L | 5191-4498 |
| Solvent Filtration | |
| 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 |
| Solvent Handling | |
| 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 waste can, GL45, 6 L with Stay Safe cap | 5043-1221 |
| InfinityLab charcoal filter with time strip, 58 g | 5043-1193 |
| Stay Safe starter kit and purging bottle, includes InfinityLab Stay Safe purging bottle (PN 5043-1339) and Stay Safe caps starter kit (PN 5043-1222) | 5043-1340 |
| InfinityLab Stay Safe cap starter kit | 5043-1222 |
| InfinityLab Stay Safe Purging Bottle | 5043-1339 |

## References:


1. Characterize mAb Charge Variants by Cation-Exchange Chromatography
   5991-5273EN
2. Charge Variant Analysis, Application Compendium
   5994-2074
3. Analysis of Intact and C-terminal Digested IgG1 on an Agilent Bio MAb 5 µm Column
   5991-0895EN
4. How Shallow Can You Go? Refining charge variant analysis of mAbs with the Agilent 1290 Infinity II Bio LC System
   5994-2692EN
5. Charge Variant and Aggregation Analysis of Innovator and Biosimilars of Rituximab
   5994-1496EN
6. pH Gradient Elution for Improved Separation of Monoclonal Antibody Charge Variants
   5990-9629EN
7. High-resolution Analysis of Charge Heterogeneity in Monoclonal Antibodies Using pH-gradient Cation Exchange Chromatography
   5991-1407EN

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