Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

BioResolve SEC mAb Guard and Columns

CONTENTS

I. INTRODUCTION

II. SEC ANALYSIS OF MONOCLONAL IgG ANTIBODY AGGREGATES, MONOMERS, AND FRAGMENTS
- a. Considerations
- b. Determining Extra-Column LC System Dispersion
- c. Selecting the Appropriate BioResolve SEC mAb Column

III. COLUMN USE CONSIDERATIONS
- a. SEC Buffer Preparation
- b. Column Installation
- c. Column Equilibration
- d. Confirming Column Performance
- e. BioResolve SEC mAb Guard

IV. COLUMN SPECIFICATIONS

V. TROUBLESHOOTING

VI. COLUMN CLEANING, REGENERATION, AND STORAGE
- a. Cleaning and Regeneration
- b. Storage

VII. CAUTIONARY NOTE

I. INTRODUCTION

Waters™ BioResolve™ SEC mAb Columns and BioResolve SEC mAb Guards are specifically manufactured and quality control tested to deliver reproducible, accurate quantitation of monoclonal antibodies (mAbs), associated high molecular weight aggregates (≥300,000 Da) and lower molecular weight fragments (≤100,000 Da). A range of available column sizes provides flexibility for performance optimization on a variety of chromatographic platforms, ranging from higher dispersion HPLC to lower dispersion UPLC™ systems. A guard column is also available which can provide effective trapping of insoluble particulates and excipients sometimes present in samples and/or eluents, thereby extending analytical column lifetime.

BioResolve SEC mAb Columns and associated guards are manufactured in a cGMP, ISO 9001 certified plant using stringent manufacturing protocols and ultra-pure reagents. Each batch of stationary phase must pass a series of stringent QC tests. Individual packed columns are tested for efficiency and then for mAb performance using the Waters mAb Size Variant Standard to ensure column-to-column consistency for this challenging application. The results for the mAb Size Variant Standard separation and uracil efficiency for each individual column is included in the column box.

For important information on extending SEC column life, see Section III, part e.

II. SEC ANALYSIS OF MONOCLONAL IgG ANTIBODY AGGREGATES, MONOMERS, AND FRAGMENTS

a. Considerations

Historically, native size-exclusion chromatography (SEC) has been the most widely used methodology for the assessment of non-covalent protein aggregation (high molecular weight species [HMWS]) in recombinant protein based biotherapeutic products. However, in recent years due to the improved capabilities of SEC columns and LC systems, there has also been a greater interest in using SEC for the non-denatured analysis of protein fragments (i.e., Low Molecular Weight Species [LMWS1&2 (~100 kDa), LMWS3 (~50 kDa), and LMWS4 (~50 kDa)]) in these samples (Figure 1). Most notably, the analysis of the largest IgG mAb fragment (LMWS1), resulting from the hydrolytic degradation of the hinge region, has been targeted. In comparison to the more traditional separation of the dimer and higher molecular weight forms of HMWS (≥300 kDa) from the monomer (~150 kDa), the separation of the LMWS1 fragment, a predominant form of which for a mAb is two-thirds the molecular weight of the monomer (~100 kDa), can be more challenging. This is due to the LMWS1 and monomer being more similar in size (hydrodynamic radius) versus the size comparison between the monomer and HMWS protein forms. An additional difficulty is presented by the elution order of the various mAb sourced proteins where the low-abundance LMWS1 peak elutes as a trailing shoulder from the main mAb monomer peak (Figure 1).

The Waters mAb Size Variant Standard is supplied with a certificate of analysis for each prepared standard lot. It is comprised of the NISTmAb Reference Material (RM) 8671 (a humanized monoclonal antibody) and nonreduced IdeS (Fabricator®) digested NISTmAb fragments LMWS2 (~100,000 Da) and LMWS3 (~50,000 Da), two mAb fragments with similar molecular weights as the LMWS1 and LMWS4, respectively. All four of the LMWS are present in the Waters mAb Size Variant Standard. The NISTmAb RM contains only the hydrolytic degradation fragments (LMWS1 and LMWS4). More information on the Waters mAb Size Variant Standard can be found on waters.com, search p/n: 720006811EN.

Note: Figure 1 shows the differences between the intact monomer and its size variants for the NISTmAb sample and Waters mAb Size Variant Standard. In particular, the hydrolytic degradant fragment (Fab/c) for the NIST mAb sample like those for other mAbs appears slightly larger than the IdeS digestion fragment although they are of similar molecular weight. In contrast, the size of the Ides ~50 kDa fragment $(Fc/2)_2$ appears larger than the ~50 kDa hydrolytic fragments most likely due to a partial expansion around the glycan-containing region due to the absence of the hinge.

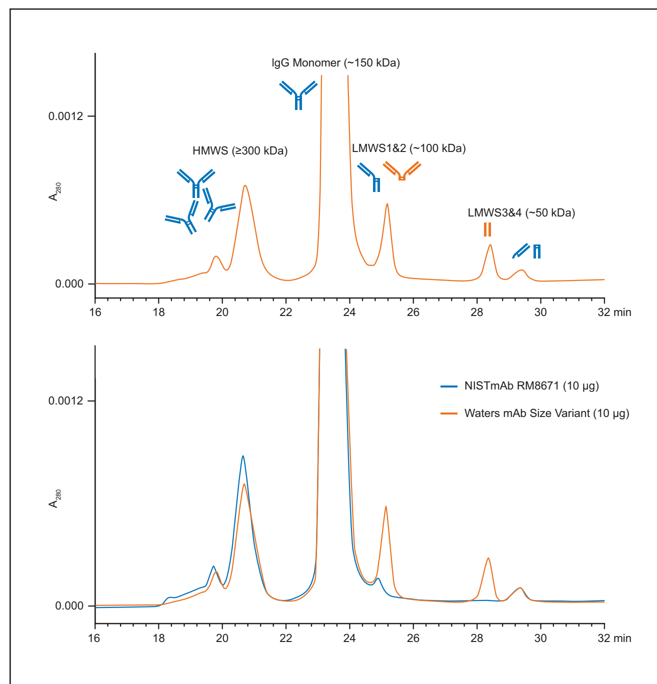

Figure 1. Separation of mAb aggregates, monomers, and fragments on NISTmAb RM 8671 and Waters mAb Size Variant Standard using a Waters BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 x 300 mm Column. LMWS: Ides fragments (orange); hydrolytic degradation fragments (blue). Conditions: Ambient temperature and 0.3 mL/min.

System dispersion is an important consideration when choosing the best BioResolve SEC mAb Column dimension for a particular mAb separation. Very low bandspread systems provide the best efficiency and clip resolution, especially if shorter columns are used. Smaller particle columns, such as sub-2-µm, will quickly lose resolution as system dispersion increases. When using higher bandspread HPLC and UHPLC systems, however, very good results can still be obtained by using longer and larger I.D. columns, such as the BioResolve SEC mAb, 2.5 µm 7.8- and 4.6-mm I.D. x 300 mm length columns.

Extra-column dispersion causes an increase in the sample volume relative to the amount of sample injected. This occurs as it travels through the flow path of an LC system without a column in place. Additional information may be found in Waters Application Note "Impact of LC System Dispersion on the Size-Exclusion Chromatography Analysis of Monoclonal IgG Antibody Aggregates and Fragments: Selecting the Optimal Column for Your Method Waters" (p/n: 720006336EN).

b. Determining Extra-Column LC System Dispersion

In SEC separations, the analytes elute within a single SEC column volume during the isocratic separation. It is critical to determine the LC system dispersion volume without the SEC column attached. This value includes the volume of the system injector, post injector tubing, and detector flow cell. The quality of the connections made between these components and proper setting of ferrules is critical to performance. This information will help ensure that the most appropriate BioResolve SEC mAb Column is used for the analysis.

The following procedure can be used to determine the LC system dispersion volume, often referred to as the 5-Sigma bandspread:

1. Replace the column with a Zero-Volume Union (p/n: 700002636). The LC tubing should be 0.0025" I.D. x 8.5" L (p/n: 700009971). Check that ferrules are set to the optimal depth to ensure that no additional dead volume has been introduced by poor connection.
2. Purge all LC solvent, wash, and purge lines with water, then 50/50 water/acetonitrile.
3. Set the detector to 273 nm and collect data at >40 points per second with a filter setting of none.
4. Flow rate: 0.5 mL/min and equilibrate the system for 10 min.
5. Run time: 1 min.
6. Sample: 0.16 mg/mL caffeine in 50/50 water/acetonitrile.
7. Injection volume: 0.5 µL.
8. Inject (three) mobile-phase blanks followed by (five) caffeine sample injections.
9. To calculate the LC system volume:
   a. Measure the caffeine peak width (in minutes) at 4.4% peak height (i.e., 5-Sigma).
   b. Multiply the peak width by the flow rate to determine the peak volume width in mL.
   c. Multiply the peak volume width in mL by 1000 to determine the peak volume width in µL.

Note: The average ACQUITY™ UPLC H-Class System dispersion volume, when measured using 5-Sigma Method, should be <12 µL when used with CH-A, <16 µL for CM-A and <22.0 µL for 30-CH-A column heater. If your value is greater, determine the source(s) of the deleterious extra peak dispersion volume and correct.

c. Selecting the Appropriate BioResolve SEC mAb Column

The following table provides suggested column sizes based on LC system dispersion volume and degree of mAb component separation difficulty. Additional information on developing a robust, mAb SEC separation may be found in the Waters Applications Note "Method Development for Size-Exclusion Chromatography of Monoclonal Antibodies and Higher Order Aggregates" (p/n: 720004076EN).

Table 1. BioResolve SEC mAb Column Recommendations Based on Application and LC System Dispersion

Separation Type	LC System Dispersion Volume	BioResolve SEC mAb Column Recommendation
mAb Monomer from Aggregates	≤20 µL	4.6 x 150 mm
	>20 µL	7.8 x 150 mm
mAb Monomer from Aggregates and Fragments	≤20 µL	4.6 x 300 mm
	>20 µL	7.8 x 300 mm

Note. The most robust separations are obtained on the 7.8 x 300 mm columns.

III. COLUMN USE CONSIDERATIONS

a. SEC Buffer Preparation Guidance

Only use high quality, filtered water (i.e., Milli-Q Millipak 0.22 µm filtered water) when preparing the SEC mobile phase. It should be filtered with a <0.22 µm filter. Sterile units containing 0.2 µm nylon filters have been successfully used for this purpose (i.e., Fisher Scientific catalog no. 09-740-46). Solutions that are susceptible to microbial growth should be replaced at regular intervals to avoid column contamination. Do NOT refill partially full SEC eluent bottles with new eluent, as this can lead to the rapid propagation of microbial organisms in the fresh mobile phase. Rather, use a new bottle containing freshly prepared SEC eluent.

Solvent inlet filters (a.k.a., sinker filters), are often a source of bacterial contamination and it is highly recommended that they not be used. It is also recommended to avoid the use of silica-based sintered glass filter supports when filtering mobile phases of pH > 6.8 due to the potential of introducing soluble and/or insoluble silicates into the SEC eluent which could alter column performance.

b. Column Installation

1. Prior to placing the column on the system, purge the system of any organic or water-immiscible mobile phases. It is important to purge the entire fluidic path through the detector to waste prior to column installation to ensure that any dislodged debris is not deposited on the head of the column. When connecting the column inlet, orient it in the proper direction as noted by the arrow on the column inlet side and the column label. Check that ferrules are adjusted to the optimal depth to ensure that no additional dead volume has been introduced by poor connection.
2. BioResolve SEC mAb 200 Å Columns are shipped in a solution containing 10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM KCl.
3. It is important to ensure mobile-phase compatibility before changing to a different mobile-phase system. A 1:5 dilution of your buffer or water may be used to replace the storage solvent, if compatibility is a concern. Use a flow rate of 0.2 mL/min for 4.6 mm I.D. columns and 0.5 mL/min for 7.8 mm I.D. columns, increasing slowly in 0.1 mL/min increments, to purge the solvent from the column for three-column volumes (refer to Table 2 for column volumes and pressure limits). Use of the dilute buffer solution has provided faster column equilibration while better maintaining column conditioning.

   Note: Storing a column in 100% water or 100% buffer is NOT recommended since this may compromise column performance and allow microbial growth. It is also highly recommended to use 10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM KCl for long-term guard or column storage.

4. Ensure that the mobile phase flows freely from the column outlet. Stop flow and attach the column outlet to the detector using 0.0025" I.D. x 8.5" L PEEK tubing (p/n: 700009971), 0.004" I.D. x 8.5" L PEEK tubing (p/n: 700009972), or .005" I.D. x. 25" L MP35N tubing (p/n: 430002575). Slowly resume flow and monitor the system pressure to ensure the column is within its pressure limitations.
5. Continue to equilibrate the column at desired flow rate in your buffer, gradually increasing the flow in 0.1 mL/min increments, using a minimum of 10-column volumes prior to use, until a stable baseline and column pressure is reached. Monitor the system pressure to ensure the column is within its pressure limit.
6. Once the system pressure has stabilized, ensure that there are no leaks at either the column inlet or outlet.

c. Column Equilibration

BioResolve SEC mAb Columns and Guards are shipped in a solution containing 10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM KCl.

It is important to ensure mobile-phase compatibility before changing to a different mobile-phase system. A 1:5 dilution of your buffer or water can serve as a transition mobile phase, if compatibility concerns are questioned. Please refer to details in the Column Installation section. Equilibrate the column with a minimum of 10-column volumes of the buffer to be used.

Table 2. Empty Analytical Column Volumes in mL* and Column Pressure Limits

*(*multiply by 10 for equilibration solvent volume)*

Column Dimension	Approximate Volume	Pressure Limits
4.6 x 150 mm	2.5 mL	4500 psi (310 bar)
4.6 x 300 mm	5.0 mL	6500 psi (448 bar)
7.8 x 150 mm	7.0 mL	4500 psi (310 bar)
7.8 x 300 mm	14.0 mL	6500 psi (448 bar)

The maximum pressure limits listed above are exclusive of system contributions and refers only to pressure across the column. If operating at pressures above the recommendation, column life can be affected.

d. Confirming Column Performance

Upon receiving a new BioResolve SEC Column and throughout its lifetime usage, Waters recommends performing a benchmark test. By using Waters mAb Size Variant Standard (p/n: 186009284), you can:

- Verify the performance of the column upon receipt.
- Monitor the condition of the columns for extended use.
- Troubleshoot resolution or peak shape difficulties that may arise.

In lieu of the mAb Size Variant standard, a 0.1 mg/mL solution of uracil prepared in your buffer can be used to benchmark the columns' performance by performing an injection using the conditions given on the Column Test Report. The Column Test Report contains uracil retention time, 5-Sigma efficiency, USP tailing, and column pressure data as a convenient benchmarking reference. The results may vary based upon the system dispersion. For more information, see the following figures.

Note: The flow rate used for both the mAb and uracil in the Column Test Report has been optimized for the mAb separation. For this reason, the data for uracil is not comparable to those reported for XBridge™ Protein SEC BEH Columns.

The information below details how to successfully prepare and use the included Waters mAb Size Variant Standard for benchmarking or troubleshooting purposes. Note that uracil is not added to this standard. Section III (e) provides separation conditions, and Figures 3–7 provide the results you should expect to obtain with the Waters mAb Size Variant Standard on a variety of LC systems.

Waters mAb Size Variant Standard and Preparation for Use

Each vial of Waters mAb Size Variant Standard (p/n: 186009284) contains 160 µg of stabilized and lyophilized NISTmAb RM 8671 which has been supplemented with 2 µg of purified nonreduced IdeS digested NISTmAb fragments, respectively. NISTmAb was chosen as it is a well characterized benchmark and is used for evaluating the performance of methods for physicochemical and biophysical attributes. FabRICATOR® IdeS Protease is a unique enzyme that digests IgG at a specific site below the hinge region, generating $F(ab')_2$ and $(Fc/2)_2$ fragments under non-denaturing native SEC conditions.

Upon arrival and prior to reconstitution, please store the standard in its original packaging at -20 °C until preparation or its marked expiration date. After reconstitution, it is recommended to use the standard within 24 hours, as lengthier storage times can lead to changes in mAb size variant species levels noting that this standard is NOT intended to be used for mAb component quantitation.

If desired, the reconstituted standard can be frozen at -80 °C and thawed for later use noting that the relative amounts of mAb aggregate and fragment might change from when the standard was freshly prepared and used. For more information on Waters mAb Size Variant Standard, reference its Care and Use Manual (p/n: 720006811EN).

It is recommended to solubilize the standard to a concentration of 1–2 mg/mL with the addition of 18.2 MΩ water followed by using a standard benchtop vortex unit to fully mix the solution. To ensure complete solubilization, it is recommended to vortex for 5 seconds each in the upright, inverted position, then finally return to the upright position. For standard analysis with the mAb Size Variant Standard (1–2 µg/µL), it is suggested to use the following injection volumes:

Diameter (mm)	Length (mm)	Injection Volume (µL)
4.6	150	1.8–5
	300	3.5–5
7.8	150	5
	300	10

Larger injection volumes can be used but may result in slightly lower resolution between the monomer and the clip. If both monomer and clip relative quantification is desired, it is recommended that a 7.8 x 300 mm column be used. Injections can be made directly from the supplied vial containing the reconstituted sample.

Note: Due to low sample volume in the vial, it is recommended to set needle depth to 1 mm from the bottom of the vial. If injections lead to blank chromatograms, check the needle depth.

The difference between the intact monomer and its size variants are shown below for the NISTmAb sample and Waters mAb Size Variant Standard. The hydrolytic degradant fragment (Fab/c) for the NISTmAb sample, like those for other mAbs, appears larger than the IdeS digestion fragment although they are of similar molecular weight.

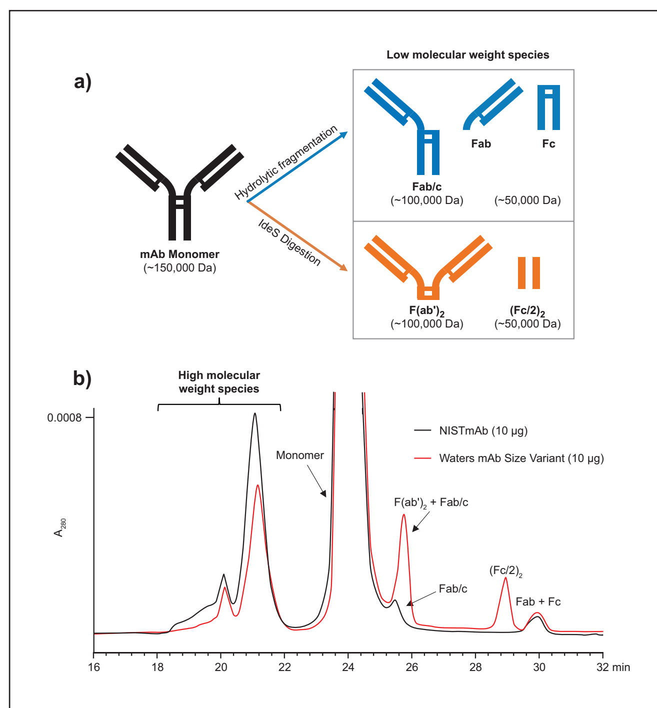

Figure 2. a) mAb graphic illustrating the difference between the intact monomer and fragments found in NISTmAb RM 8671 and Waters mAb Size Variant Standard. b) A representative A_{280} SEC chromatogram of NISTmAb (black trace) and modified Waters mAb Size Variant Standard (red trace) cropped to show both high and low molecular weight species, in addition to the monomer. Due to similarity in hydrodynamic radii, $F(ab')_2$ and Fab/c are not resolved. Data were collected with a BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 x 300 mm Column with absorbance measured at 280 nm.

e. Effect of System Dispersion

Selecting the best column configuration for your method depends on your LC system dispersion. An easy-to-follow and execute procedure is provided in Section II b. To illustrate the effects of medium and low system dispersion on SEC separations of the Waters mAb Size Variant Standard, four different BioResolve SEC mAb Columns of various configurations were run on an ACQUITY UPLC Arc™ Bio System and an ACQUITY UPLC H-Class Bio System.

The same sample and mobile phases were used on both systems. The conditions are shown below, and the SEC chromatograms are shown for the 4.6 x 150 mm column in Figure 3, the 4.6 x 300 mm column in Figure 4, the 7.8 x 150 mm column in Figure 5, and the 7.8 x 300 mm in Figure 6. Based on these data, Figure 7 clearly indicates the preferred use of a 7.8 x 300 mm column on a "high dispersion (Alliance™) LC system" for those applications where resolution and accurate quantitation of the mAb monomer (~150 kDa) from its closest lower molecular weight fragment (~100 kDa) is desired.

LC systems:	ACQUITY UPLC H-Class Bio, 5-Sigma system dispersion = 10 µL (Figures 3–6); ACQUITY Arc Bio, 5-Sigma system dispersion = 30 µL (Fig 3–6); Alliance HPLC System, 5-Sigma system dispersion = 49 µL (Fig 7)
Columns:	BioResolve SEC mAb Column, 200 Å, 2.5 µm, 4.6 x 150 mm (p/n: 176004592*) 4.6 x 300 mm (p/n: 176004593*) 7.8 x 150 mm (p/n: 176004594*) 7.8 x 300 mm (p/n: 176004595*) ** Includes column and one vial of mAb Size Variant Standard*
Column temp.:	35 °C active preheater CH-A (H-Class), CH-30A (H-Class), and 30-cm CH/C (Arc)
Mobile phase:	50 mM sodium phosphate pH 7.0, 200 mM KCl
Flow rate:	0.200 mL/min (for 4.6 mm I.D.)/ 0.575 mL/min (for 7.8 mm I.D.)
Sample manager temp.:	8 °C
Detector:	Tunable Ultraviolet (TUV) with a 5 mm Ti Flow Cell for ACQUITY UPLC H-Class Bio, 2489 UV/Vis with 10 mm Bio Inert Flow Cell for the ACQUITY Arc Bio and Alliance
UV detection:	280 nm, 10 Hz, fast filter
Seal wash:	10% HPLC grade methanol/ 90% 18.2 MΩ water v/v
Sample manager washes:	18.2 MΩ water
Reconstitution:	70 µL of 18.2 MΩ water
Sample:	2.28 mg/mL Waters mAb Size Variant Standard
Injection volume:	Varies: 1.8, 3.5, 5, or 10 µL depending on column configuration

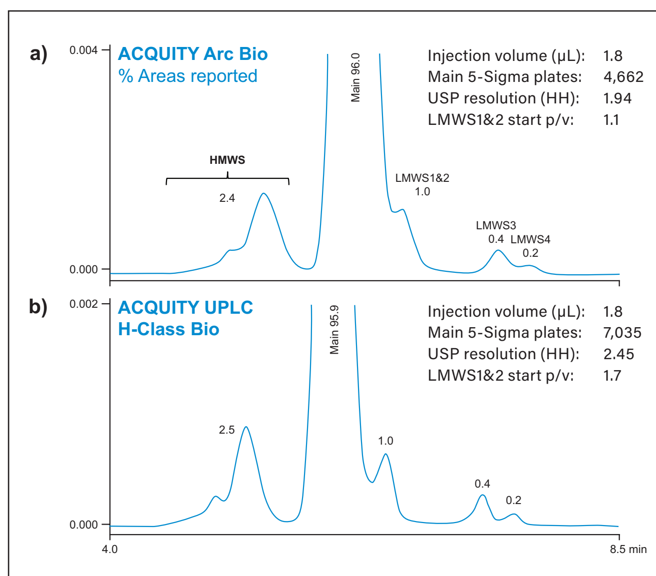

Figure 3. Separation of Waters mAb Size Variant Standard on a BioResolve SEC mAb, 4.6 x 150 mm Column on LC systems with 30 µL (ACQUITY Arc) and 10 µL (ACQUITY UPLC H-Class) system dispersions.

It is clear from the chromatograms shown in Figure 3 that the 100 kDa "clip" shows only a shoulder on the higher dispersion system due to the broadening of the main monomer peak. Quantification of the HMWS is adequate but not ideal, as the USP resolution at half height (R_s [USP, HH]) between the dimer and monomer is <2. The same 4.6 x 150 mm column provides very good R_s (USP, HH) between the dimer and monomer, but still is not ideal for robustly quantifying the clip. A start p/v value of >2 is desirable for the most robust and accurate quantification.

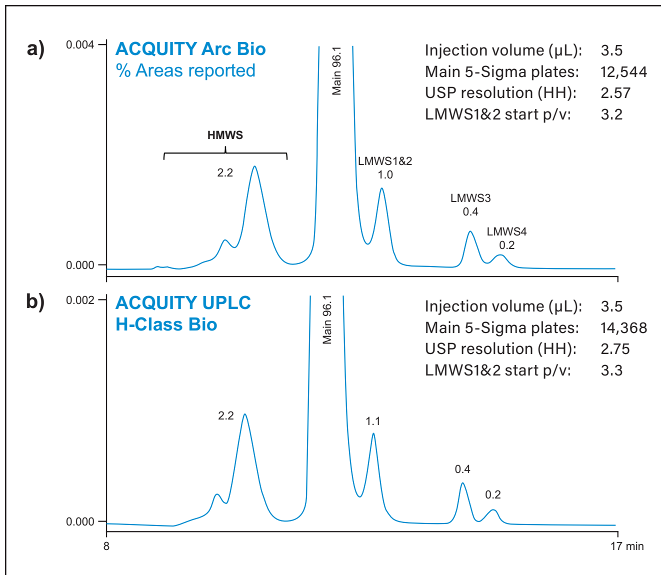

Figure 4. Separation of Waters mAb Size Variant Standard on a BioResolve SEC mAb, 4.6 x 300 mm Column on LC systems with 30 µL (ACQUITY Arc) and 10 µL (ACQUITY UPLC H-Class) system dispersions.

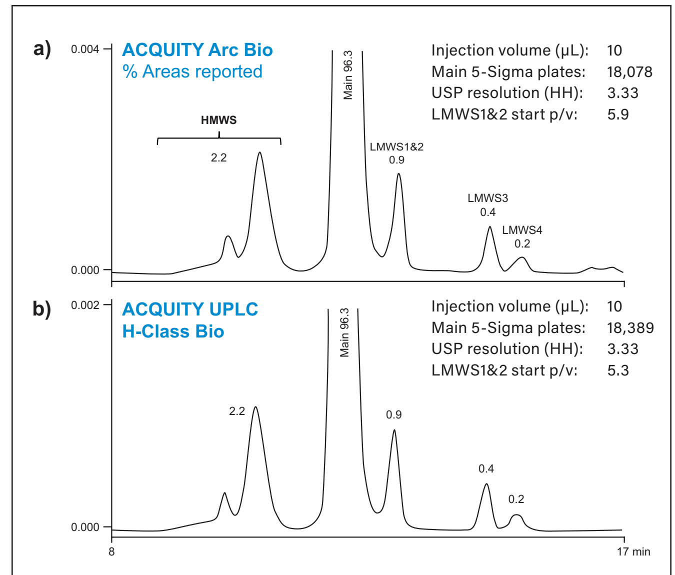

Figure 6. Separation of Waters mAb Size Variant Standard on a BioResolve SEC mAb, 7.8 x 300 mm column on LC systems with 30 µL (ACQUITY Arc) and 10 µL (ACQUITY UPLC H-Class) system dispersions.

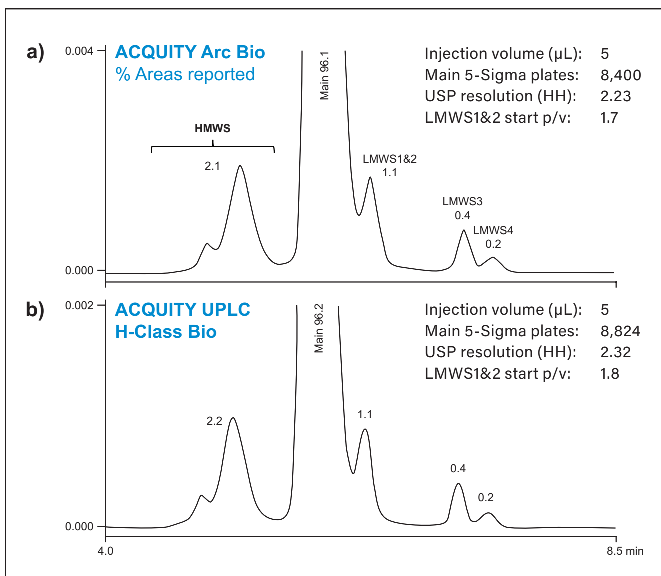

Figure 5. Separation of Waters mAb Size Variant Standard on a BioResolve SEC mAb, 7.8 x 150 mm Column on LC systems with 30 µL (ACQUITY Arc) and 10 µL (ACQUITY UPLC H-Class) system dispersions.

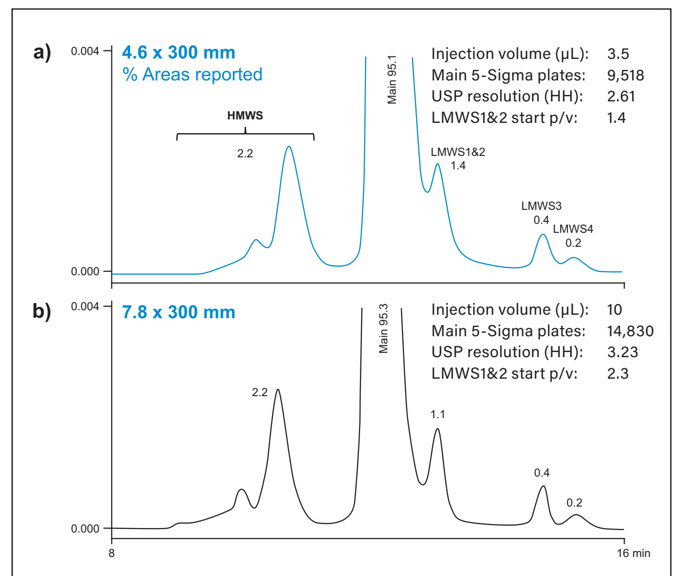

Figure 7. Separation of Waters mAb Size Variant Standard on a BioResolve SEC mAb, 4.6 x 300 mm and 7.8 x 300 mm Columns on an LC system with a 49 µL (Alliance) system dispersion.

e. BioResolve SEC mAb Guard

Use of the specially developed BioResolve SEC mAb Guard can effectively prolong analytical column lifetime. The useful lifetime of the guard column can be influenced by many factors including:

- Mobile phase cleanliness/microbial contamination
- Sample precipitates/aggregation
- Excipients in sample formulations
- Working at extremes of pressure, pH, and/or temperature

Guard columns may need replacement if the following are observed:

- Significant increase in column pressure
- Wide tailing or split peaks

Injection of particulates as well as excipients contained in the mAb sample matrix onto any SEC column can shorten its useful life as detailed in Waters "Size-Exclusion Chromatography (SEC) Optimization Guide" (p/n: 720006067EN).

Consequently, it is important to help ensure samples are free of particulates before injecting onto the SEC column. If samples appear cloudy or turbid, they should not be injected, as this could lead to column pressure increases. Sample preparation such as filtration or centrifugation may be used, if appropriate.

To address the desire to extend the operating lifetime of the BioResolve SEC mAb Column, the BioResolve SEC mAb Guard was designed to prevent sample or eluent-based particulates, as well as some excipients contained in sample, from compromising the analytical column performance without compromising the desire to adequately resolve the 150 kDa mAb monomer from the 100 kDa fragment (Figure 8).

LC system:	ACQUITY UPLC H-Class Bio
Column:	BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 x 300 mm (p/n:186009439)
Column temp.:	Ambient
Mobile phase:	50 mM sodium phosphate pH 7.0, 200 mM KCl
Flow rate:	0.575 mL/min
Sample manager temp.:	8 °C
UV detection:	280 nm, 10 Hz, no filter
Seal wash:	10% HPLC-grade methanol/ 90% 18.2 MΩ water v/v
Sample manager washes:	18.2 MΩ water
Reconstitution:	70 µL of 18.2 MΩ water
Sample:	2.28 mg/mL Waters mAb Size Variant Standard
Injection volume:	10 µL of Waters mAb Size Variant Standard

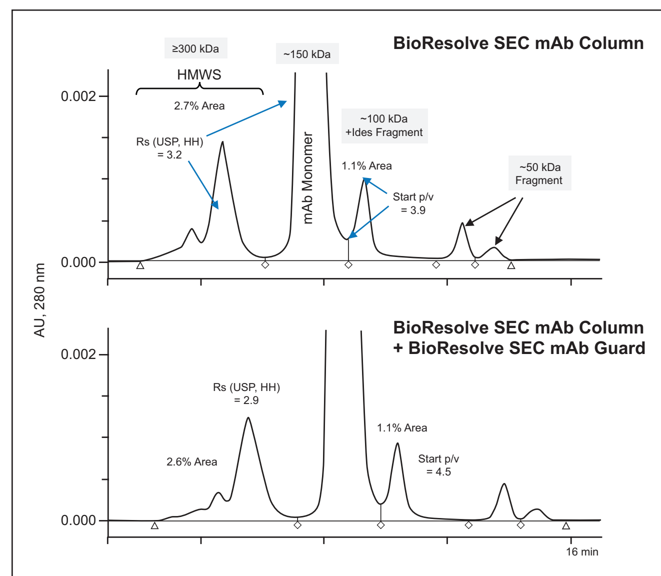

Figure 8. Separation of Waters mAb Size Variant Standard on a BioResolve SEC mAb, 7.8 x 300 mm Column with (bottom) and without (top) a BioResolve SEC mAb, 4.6 x 30 mm Guard.

IV. COLUMN SPECIFICATIONS

- Shipping solvent: BioResolve SEC mAb, 200 Å, 2.5 µm Columns are shipped in a solution containing 10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM KCl.
- pH range: 2.5–8.0.
- Temperature range: 4–60 °C.
- Reduce flow rate when operating at low temperatures (e.g., 10 °C) to avoid excessive column pressure.
- Recommended salt concentration: 100–500 mM KCl or NaCl.
- Recommended buffer concentration: 25–200 mM.
- Organic concentration: <10% acetonitrile, <20% methanol.

  Note: It is important to ensure mobile phase and sample compatibility when changing to a different mobile phase. Addition of organic solvents can adversely affect protein solubility.

- Recommended maximum injection volumes and mass loads:

Column Length	4.6 x 150 mm	4.6 x 300 mm	7.8 x 150 mm	7.8 x 300 mm
Volume Load	<10 µL	<20 µL	<30 µL	<60 µL
Mass Load	<100 µg	<200 µg	<300 µg	<600 µg

The injection volumes and mass loads listed above are reflective of legacy methods on columns and systems that suffered from substantial and prolonged protein losses. Today, much lower injection volumes and mass loads provide better resolution for harder to resolve peaks. This can be confirmed for well resolved peaks by making a series of increasing injection volumes and obtaining equivalent characterization data. The use of a 2489 UV/Vis Detector on an ACQUITY UPLC H-Class Bio System in conjunction with 7.8 mm I.D. columns provides better sensitivity and signal-to-noise ratios due to the 10 mm path length in its Bio-inert flow cell. However, this larger volume flow cell will compromise the resolution on 4.6 x 150 mm columns. Keep in mind that not all 10 mm flow cells are equivalent. Waters standard analytical flow cells contain Teflon wetted parts that can produce tailing peaks for proteins. When possible, always select Bio-inert or Bio-compatible parts for your systems.

- Recommended flow rates and back pressures:

Column Length	4.6 x 150 mm	4.6 x 300 mm	7.8 x 150 mm	7.8 x 300 mm
Flow Rate Range	≤0.5 mL/min	≤0.5 mL/min	≤1.0 mL/min	≤1.0 mL/min
Max Column Back Pressure*	≤4500 psi	≤6500 psi	≤4500 psi	≤6500 psi

**The maximum recommended pressure drop across the column refers only to the column itself and NOT from the added pressure contribution from the LC System. To determine the pressure drop across the column: install a zero dead volume union in place of the column and determine the system pressure at operating conditions. Subtract that value from the system back pressure observed with the column installed.*

Column Back Pressure will be influenced by the temperature and buffer/salt concentrations used. For best column lifetime, flow rates should be chosen so as not to exceed the Max Column Back Pressure.

The maximum recommended pressure drop across the column is provided as a guideline to ensure longest possible column lifetimes. This guidance should not be interpreted as an absolute upper pressure limit. For example, some methods may exceed the suggested maximum pressure, yet deliver desired SEC separation performance, although shorter column lifetimes may be expected.

Working at extremes of pressure, pH, and/or temperature may result in shorter column lifetimes.

V. TROUBLESHOOTING

The first step in systematic troubleshooting is comparing the column performance in its current state to the performance when it was functioning properly. The functional tests with the protein mixture may reveal subtle changes in surface chemistry that affect the application.

There are several common symptoms of change in the column.

1. **An increase in pressure is often associated with decreased performance in the application.** The first step in diagnosis is to ensure that the elevated pressure resides in the column rather than somewhere else in the system. This is determined by monitoring system pressure as each connection is broken from the outlet end to the inlet. If the system is occluded, the blockage should be identified and removed. If the pressure increase resides in the column, it is helpful to know whether the problem was associated with a single injection or if it occurred over a series of injections. If the pressure gradually built up, it is likely that the column can be cleaned as described in Section VI. If a single sample caused the pressure increase, it likely reflects particulates or insoluble components, such as lipids or higher order insoluble aggregates. Cleaning is still an option but using the more aggressive options. If samples appear cloudy or turbid, they should not be injected, as this will lead to pressure increases. Sample preparation such as filtration or centrifugation may be used, but one should first check whether this impacts the results.
2. **Loss of resolution and increased peak tailing can be caused by microbial contamination.** It is important to follow good standard laboratory practices to prevent microbial contamination. This includes changing buffer bottles frequently, using high purity water, using a sterile filtration apparatus, and storing system and column under recommended conditions. If microbial contamination has occurred, cleaning the column will have no effect on performance. When changing the flow rate, ramp it at a rate of 0.1 mL/min and avoid immediate flow rate increases greater than 0.1 mL/min.
3. **Increased peak tailing can be caused by failure of a tubing connector or a buildup of material on the column inlet frit.** Before proceeding with diagnostic or corrective measures, check all connections, that the mobile phases have been correctly prepared, and the correct method has been selected. Then repeat the protein standard test. If the proteins show increased peak tailing, it is likely that there is significant buildup of material on the column inlet and the column will require replacement.
4. **Carryover is defined as the appearance of the constituents of one sample in the next analysis. In size-exclusion chromatography, carryover is typically due to system components or improper wash solvents.** Run a blank injection. If the protein peaks only appear when an injection is made, they likely originated from system component or inadequate wash solvents. Adsorption on system components most likely occurs in the loop or needle. In these instances, the component may need to be changed.

Note: Useful, general information on column troubleshooting problems may be found in "HPLC Columns Theory, Technology and Practice", U.D. Neue (Wiley-VCH, 1997), the Waters "HPLC Troubleshooting Guide" (p/n: wa20769), and on www.waters.com.

VI. COLUMN CLEANING AND STORAGE

a. Column Cleaning

1. Flush column at one half the normal flow rate. One or more of the following may be used:
   - 25–200 mM buffer solution, pH 3.0 + 0.5 M NaCl or KCl
   - 10% acetonitrile or 20% methanol combined with 25–100 mM buffer + 100 mM NaCl or KCl
   - Do not exceed 20% organic
   - Do not use ionic detergents and other surfactants

Reversal or back flushing can be tried, but may further damage the column, or only provide short lived improvement in column performance

b. Column Storage

Note: Storage of column in 100% water or 100% buffer is not recommended since this may compromise column performance and allow microbial growth.

1. Recommended storage solution is to purge the column with the shipping solvent.
   - 10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM KCl
   - A minimum of five-column volumes should be used
2. Alternative buffer combinations may also be used.
   - Buffers should contain 50–100 mM salt (KCl or NaCl) and a maximum phosphate concentration of 50 mM
   - Addition of 10% acetonitrile, 20% methanol, or 0.05% sodium azide should be added to eliminate microbial growth
   - Do NOT store in 100% water

Storage at 4 °C may also help minimize bacterial growth during short-term storage

VII. CAUTIONARY NOTE

Depending on user's application, these products may be classified as hazardous following their use, and as such are intended to be used by professional laboratory personnel trained in the competent handling of such materials. Responsibility for the safe use and disposal of products rests entirely with the purchaser and user. The Safety Data Sheet (SDS) for this product is available at www.waters.com/sds.

Research only. Not for IVD use.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

Waters, The Science of What's Possible, BioResolve, UPLC, ACQUITY, Alliance, XBridge, and Arc are trademarks of Waters Corporation. All other trademarks are the property of their respective owners

©2020 Waters Corporation. Produced in the U.S.A. July 2020 720006830EN IH-PDF

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com