XBridge Premier Protein SEC 250 Å, 2.5 µm Columns

CONTENTS

I. INTRODUCTION

Thank you for choosing a Waters™ SEC Column. The XBridge™ Premier Protein SEC 250 Å, 2.5 µm Columns help scientists obtain reliable protein separations, from approximately 10,000 to 650,000 Daltons, made possible through the use of Waters MaxPeak™ High Performance Surfaces (HPS) and novel BEH-based SEC particle (i.e., BEH-polyethylene oxide) technologies (Figure 1). Advancements in SEC column hardware and particle technology work to minimize secondary ionic or hydrophobic interactions between proteins and the column to allow chromatographers to use a "generic" or "platform-type" method for many protein samples using a simple SEC eluent formulation (e.g., phosphate buffered saline).

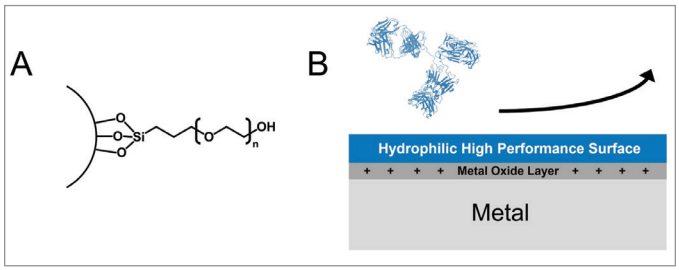

Figure 1. Use of ACQUITY™ and XBridge Premier Protein SEC 250 Å Column Technology to reduce undesired secondary interactions. (A) Hydroxy-terminated PEO bonded BEH particles with low ionic and low hydrophobic secondary interactions. (B) A MaxPeak High Performance Surface with hydrophilic properties to minimize secondary interactions between biomolecules and column hardware.

A Waters XBridge Premier Protein SEC 250 Å, 2.5 µm Guard Column is also available, which can provide effective trapping of insoluble particulates and excipients sometimes present in samples and eluents, thereby extending the analytical column's lifetime.

XBridge Premier Protein SEC 250 Å, 2.5 µm Columns and recommended XBridge Premier Protein SEC 250 Å, 2.5 µm Guards are manufactured in a cGMP, ISO 9001 certified plant using stringent manufacturing protocols and ultra-pure reagents. Each batch of SEC 250 Å stationary phase must pass a series of stringent QC tests that includes testing with Waters mAb Size Variant Standard (p/n: 186009429) to help ensure consistent performance for challenging size-based protein separations.

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

Special Reminder: Optimum separation performance is achieved using the 4.6 mm I.D. columns on low dispersion LC systems (see Section II. b). When using higher band spread HPLC and UHPLC systems, it should be noted that very good results can still be obtained XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 I.D. x 300 mm length columns. Additionally, the ACQUITY Premier System is uniquely suited to perform SEC separations with XBridge Premier Protein SEC Columns as it too is designed with MaxPeak High Performance Surfaces.

II. SEC ANALYSIS OF PROTEIN AGGREGATES, MONOMERS, AND FRAGMENTS

a. Considerations

Historically, native size-exclusion chromatography (SEC) has been widely used to assess non-covalent protein aggregation (high molecular weight species [HMWS]). In recent years ,it has also become of interest to use SEC for the non-denatured analysis of protein fragments (i.e., low molecular weight species [LMWS]). A monoclonal antibody sample can be taken as an example (Figure 2). There is often an IgG mAb fragment (LMWS1) present that is formed by the hydrolytic degradation of the hinge region. This fragment is two-thirds the molecular weight of the monomer. In comparison to a more traditional monomer/dimer separation, obtaining resolution of the LWMS1 species can be more challenging. This is due to the LMWS1 and monomer being more similar in size. monomer/dimer separation, obtaining resolution of the LWMS1 species can be more challenging. This is due to the LMWS1 and monomer being more similar in size.

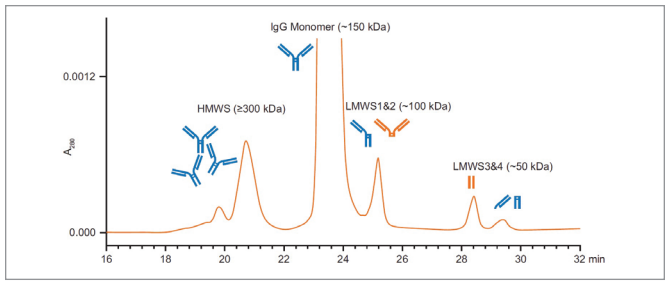

Figure 2. Differences between the intact monomer and its size variants for the Waters mAb Size Variant Standard. In particular, the hydrolytic degradant fragment (Fab/c) for the NISTmAb sample like those for other mAbs appears slightly larger than the IdeS digestion fragment although they are of similar molecular weight. More information about the Waters Size Variant Standard can be found in Section III. h.

System dispersion is an important consideration when using the XBridge Premier Protein SEC 250 Å, 2.5 µm Column. Very low band spread systems provide the best efficiency and clip resolution, especially if shorter columns are used. The apparent performance of smaller particle columns, such as sub-2-µm, will be significantly hindered if ever used with a higher dispersion system. When using higher band spread HPLC and UHPLC systems, it should be noted that very good results can still be obtained by using longer and larger I.D. columns, such as the XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 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, analytes elute within a single SEC column volume. 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 ACQUITY or XBridge Premier Protein SEC 250 Å 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 band spread:

1. Replace the column with a Zero-Volume Union (p/n: 700002636). The LC tubing to connect to the UV detector 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 connections.
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 and ACQUITY Premier FTN LC Systems have dispersion volumes, when measured using 5-Sigma Method, that 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.

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 SEC mobile phases. 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 (e.g., SEC eluents that do not contain an antimicrobial such as sodium azide, methanol, isopropanol, or acetonitrile) 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 (sinker filters) are often a source of bacterial contamination and it is highly recommended that they are not 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. Buffer Selection

XBridge Premier Protein SEC 250 Å, 2.5 µm Columns are designed to provide industry-leading method flexibility and are robust against a wide range of buffers. In many cases, a previously developed method may be used to achieve exceptional performance without further optimization. However, the inertness of these columns and their unique bondings afford the opportunity to explore reduced buffer and salt concentrations and other fine tuning for specific applications. Guidelines for enhancing performance of XBridge Premier Protein SEC 250 Å, 2.5 µm Columns are provided below.

1. Phosphate buffered saline (PBS), a commonly used biological buffer may be adequate in many general-purpose applications, providing simplicity and ease of mobile phase preparation at point of use. Columns will perform similarly across a pH range of 6.5 to 7.5, meaning that PBS packets and tablets from different vendors can be used. Regardless of preparation, it is critical to filter PBS buffers using a 0.2 µm (preferably sterile) filter prior to use to reduce the risk of particulate and/or microbial fouling of the column.

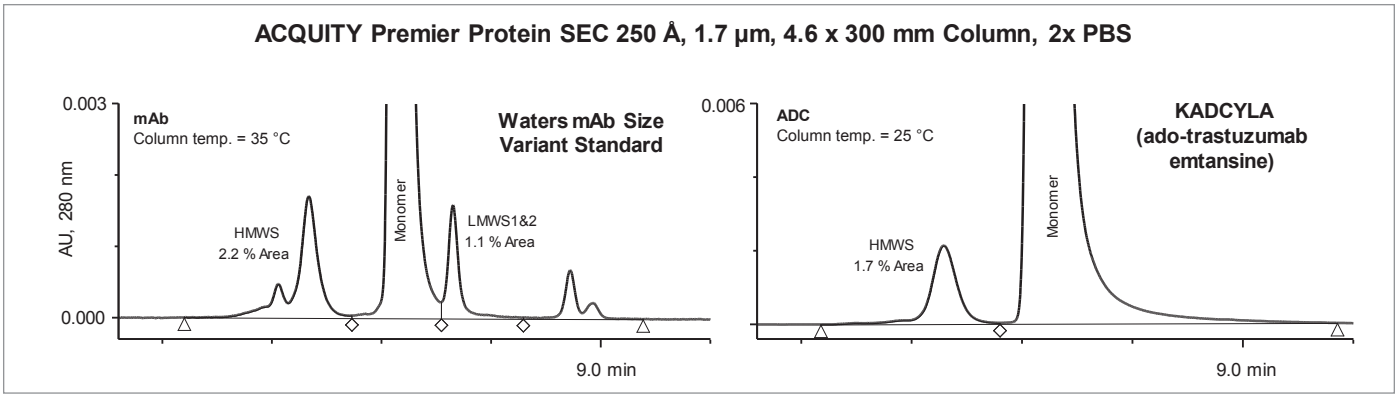

Figure 3. Exemplary separations of both the Waters mAb Size Variant Standard and the antibody drug conjugate (ADC) KADCYLA obtained on an ACQUITY UPLC H-Class Bio System using 2x PBS (20 mM phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4, 0.200 mL/min, TUV = 280 nm). Excellent resolution and quantitation were achieved for aggregates and fragments.

2. Organic co-solvents are commonly used in SEC mobile phases to mitigate hydrophobic secondary interactions. XBridge Premier Protein SEC 250 Å, 2.5 µm Columns exhibit very low levels of such interactions and in many cases will not need organic modifier to resolve or recover species that typically require it, like ADC's. If an organic modifier is employed, isopropanol (IPA) is the recommended solvent for these columns. Begin with a concentration no greater than 5% of the mobile phase composition and increase, only if necessary, up to a maximum of 15%. Most situations will not require more than 5% to realize benefit.

Note: The addition of organic co-solvent increases viscosity and thus column backpressure. Prolonged use at excessive pressure will reduce column lifetime. Refer to Section III. d. for maximum operating pressures.

3. The lower dependence on high concentrations of buffer or salt mean that XBridge Premier Protein SEC 250 Å, 2.5 µm Columns are well suited for LC-MS applications where low ionic strength volatile buffers are preferred. This aspect is discussed in detail in Section III. g.

c. Column Installation

1. Prior to placing the column on the system, purge the system of any organic or water-immiscible mobile phases. Purge the entire fluidic path through the detector to waste prior to column installation to fully clear away any dislodged debris. 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. XBridge Premier Protein SEC 250 Å, 2.5 µm 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 1 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. Furthermore, storage in 100% buffer under refrigeration can cause buffer precipitation, leading to shorter column lifetimes. It is highly recommended to use 10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM KCl for long-term guard or column storage. When using this recommended storage solution, it is best to store the column at room temperature.

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 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.

d. Column Equilibration

XBridge Premier Protein SEC 250 Å, 2.5 µm Columns and XBridge Premier Protein SEC 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 1. 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)

Table 1. 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.

e. Applying Optimal Column Temperatures

Column temperature can greatly influence separation performance when using XBridge Premier Protein SEC 250 Å, 2.5 µm Columns and is thus an important consideration in method optimization. While increases in resolution can often be achieved at elevated temperatures for monoclonal antibody separations, higher column temperatures can have a deleterious effect on separations where analytes are highly susceptible to hydrophobic secondary interactions, such as in the case of antibody drug conjugates. As shown in Figure 4, reasonably good performance can be achieved using 2x PBS for the Waters mAb Size Variant Standard at temperatures ranging from 25 °C to 45 °C. A modest increase in resolution may be realized at higher temperature. However, for the ADC Kadcyla, elevated column temperatures have a profound negative effect on efficiency and resolution. This is likely due to preservation of PEO segment hydration, and thus hydrophilicity, at lower temperatures. This is not to imply that all mAbs and ADCs will perform as shown in this example. As mAbs and ADCs can vary greatly, and behavior can be impacted by factors such as mobile phase composition and pH, it is important to screen column temperature to ensure the best possible performance in each application.

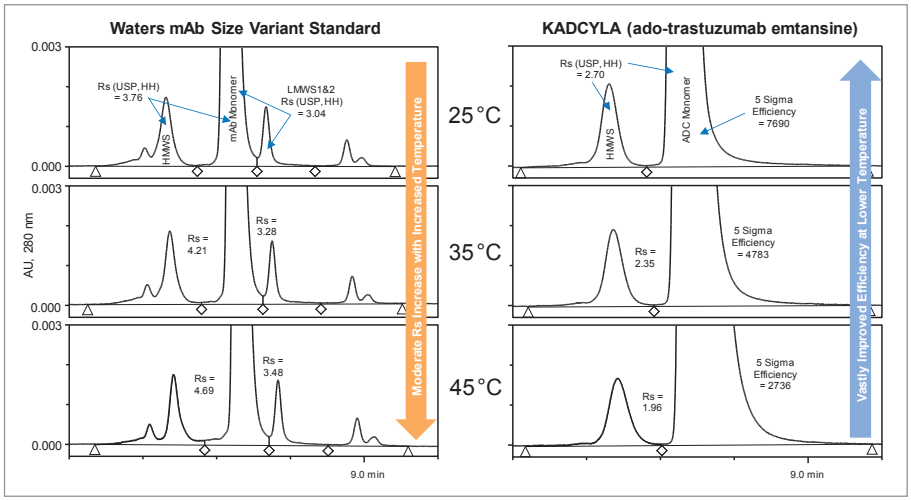

Figure 4. Column temperature effects on separation performance using an ACQUITY Premier Protein SEC 250 Å, 1.7 µm, 4.6 x 300 mm Column for both mAb (Waters mAb Size Variant Standard) and ADC (KADCYLA) samples. Performed on an ACQUITY UPLC H-Class Bio System, 2x PBS (20 mM phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4, 0.300 mL/min, TUV = 280 nm). While a modest resolution increase was observed for the mAb at elevated temperature, the ADC monomer peak shape and 5-Sigma efficiency was significantly better at 25 °C compared to 45 °C. An ACQUITY Column is used in this example, though behavior will be similar for the XBridge Premier Protein SEC 250 Å, 2.5 µm Column, as it shares the same PEO-based particle surface chemistry.

f. Confirming Column Performance

Upon receiving a new XBridge Premier Protein SEC 250 Å, 2.5 µm Column and throughout its lifetime usage, Waters recommends performing a benchmark test. By using Waters mAb Size Variant Standard (p/n: 186009429), 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 Waters mAb Size Variant Standard, a 0.1 mg/mL solution of uracil prepared in your buffer can be used to benchmark the column's 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.

Note: Section III. h. details how to successfully prepare and use the included Waters mAb Size Variant Standard for benchmarking or troubleshooting purposes.

g. MS Compatibility

Native SEC separations can now be readily coupled to MS to facilitate deep characterization of protein therapeutics and the study of non-covalent protein complexes. The inertness of the XBridge Premier Protein SEC 250 Å, 2.5 µm Columns improves upon the chromatography that to-date has been obtainable with ammonium acetate mobile phases. This can be seen in the form of improved sample recoveries, the preservation of non-covalent protein complexes, and lower limits of detection. Figure 5 provides a comparison study wherein it was found that an XBridge Premier Protein SEC 250 Å, 2.5 µm Column was able to yield protein recoveries in ammonium acetate mobile phase at a level significantly lower than that of an alternative state-of-the-art column technology.

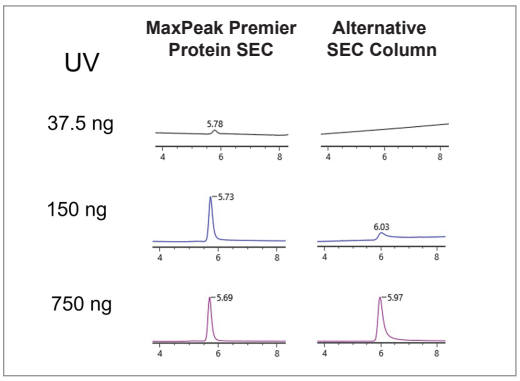

Figure 5. UV chromatograms of NISTmAb as obtained with pH 6.8 ammonium acetate mobile phase (40:60 mixture of 1x IonHance™ CX-MS A/B Buffers), 0.2 mL/min mobile phase, and an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column. Results from an alternative commercially available SEC column are shown for comparison.

To perform a high sensitivity MS experiment, it is important to start with high quality mobile phases. Waters IonHance Concentrates are manufactured and QC tested for this exact purpose. Ammonium acetate mobile phases can be prepared from IonHance Concentrates, such as those listed below. Ammonium acetate concentrations as low as 50 mM have been found to be reliable for SEC-MS with ACQUITY or XBridge Premier Protein SEC 250 Å Columns.

Product	Part Number	10x Diluted Composition
IonHance Ammonium Acetate pH 6.8 Concentrate	186009705	100 mM ammonium acetate, 2% ACN, pH 6.8
IonHance CX-MS pH Concentrate A, pH 5	186009280	50 mM ammonium acetate, 2% ACN, pH 5
IonHance CX-MS pH Concentrate B, pH 8.5	186009281	160 mM ammonium acetate, 2% ACN, pH 8.5

All LC columns exhibit some level of background ions when coupled to mass spectrometry. Optimizing experimental parameters can help any analysis yield better results. ACQUITY and XBridge Premier Protein SEC 250 Å Columns have very high coverage bondings. This affords very high recoveries and compatibility with lower concentrations of ammonium acetate in the mobile phase. Users should expect to see some level of background ions between 600 and 900 *m/z*. These background ions have been carefully studied, and it is confirmed that they will not prematurely foul a mass spectrometer nor interfere with native protein signal that is inherently observed at a significantly higher mass range (>1000 *m/z*). Where possible, it is advised to perform native SEC-MS with an acquisition window set to 1000 *m/z* or higher. When low mass ion signal is acquired, as with the BioAccord™ System, an analyst may find it beneficial to assess experimental runs using extracted ion chromatograms. Background subtraction can also be employed on summed mass spectra. Example analyses with each one of these approaches are shown on the next page.

Experimental settings for SEC-MS with a Waters BioAccord System

Range:	High mass range 400–7000 *m/z*
Cone voltage:	150 V
Capillary voltage:	1.5 kV
Mobile phase:	40:60 mixture of 10x diluted IonHance CX-MS A/B Concentrates
Flow rate:	0.2 mL/min
Column dimension:	4.6 x 150 mm
Column temp.:	30 °C
TUV:	280 nm

Experimental settings for SEC-MS with a Waters Xevo™ G2-XS QTof System

Range:	1000–7000 *m/z* (7000 *m/z* or higher is recommended to comprehensively collect native charge state envelopes)
Cone voltage:	60 V
Offset voltage:	100 V
Capillary voltage:	2.5 kV
Source temp.:	100 °C
Desolvation temp.:	4500 °C
Desolvation gas flow:	800 L/hr
Cone gas flow:	50 L/hr
Collision energy:	6 eV
Mobile phase:	10x diluted IonHance Ammonium Acetate pH 6.8 Concentrate
Flow rate:	0.2 mL/min
Column dimension:	4.6 x 150 mm
Column temp.:	25 °C
TUV:	280 nm

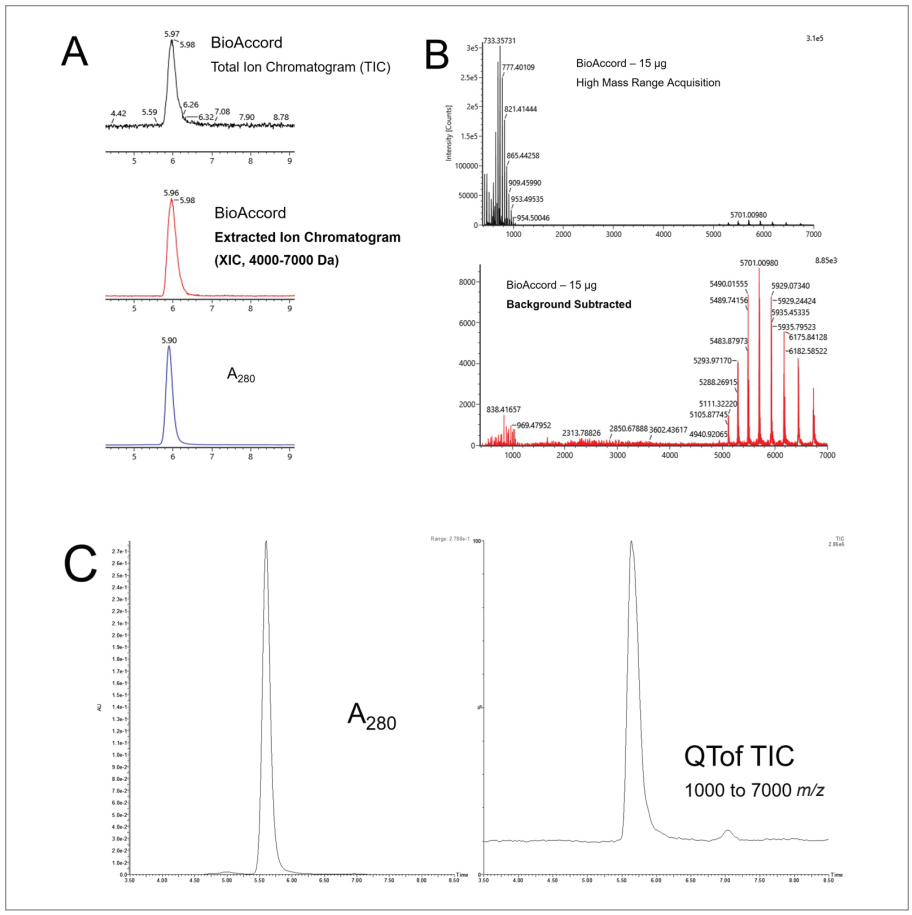

Figure 6. Native SEC-MS on 15 µg of NISTmAB as performed with an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column and either a BioAccord or Xevo G2-XS QTof using several different data acquisition and data analysis approaches. (A) BioAccord high mass range acquisition (400 to 7000 m/z) to produce a total ion chromatogram and an extracted ion (1000 to 7000 m/z) chromatogram. (B) BioAccord summed mass spectrum without and with background ion subtraction. (C) Xevo G2-XS QTof total ion chromatogram as obtained with a mass acquisition from 1000 to 7000 m/z.

h. Waters mAb Size Variant Standard and Preparation for Use

The Waters mAb Size Variant Standard (p/n: 186009284) 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 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).

Each vial of Waters mAb Size Variant Standard 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. 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, 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.

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 vortexing until the solution is mixed. 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 Waters mAb Size Variant Standard (1–2 µg/µL), it is suggested to use the following injection volumes:

Table 2. Injection Volume Recommendations

Diameter (mm)	Length (mm)	Injection Volume (µL)
4.6	150	1.8–5
	300	3.5–5
7.8	150	10–20
	300	15–25

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 300 mm length 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.

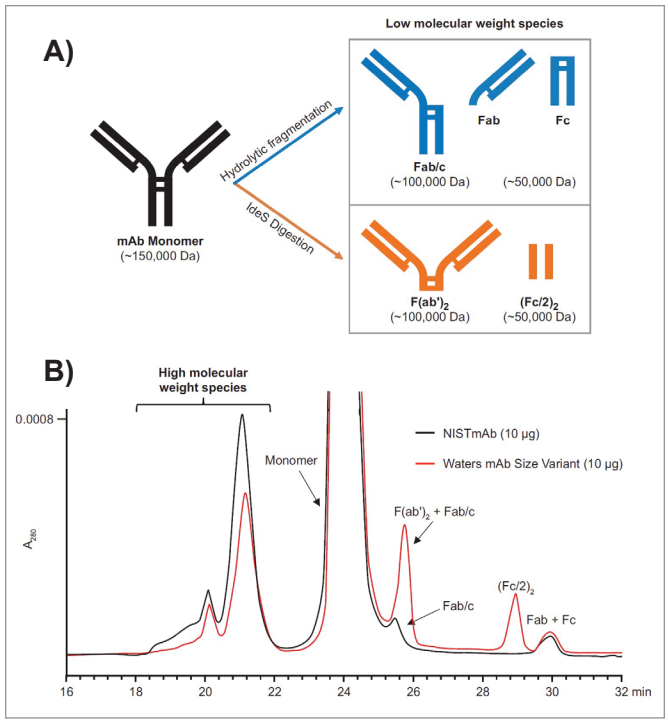

Figure 7. Example SEC separations of the Waters mAb Size Variant Standard a) mAb graphic illustrating the difference between the intact monomer and fragments found in NISTmAb RM 8671 and Waters mAb Size Variant Standard. b) A representative A280 SEC chromatogram of NISTmAb (black trace) and modified 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.

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.

More information on the Waters mAb Size Variant Standard can be found on waters.com, search p/n: 720006811EN.

LC system:	ACQUITY UPLC H-Class Bio
Column:	XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 300 mm; MaxPeak Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 30 mm Guard; and Waters mAb Size Variant Standard (p/n: 176004795)
Column temp.:	Ambient
Mobile phase:	50 mM sodium phosphate pH 7.0, 200 mM KCl
Flow rate:	0.200 mL/min
Sample manager temp.:	10 °C
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:	3.5 µL of Waters mAb Size Variant Standard

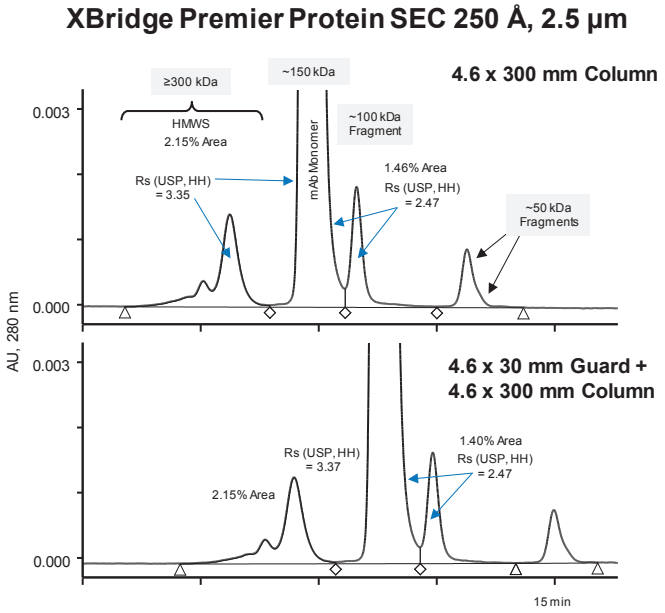

Figure 8. Separation of Waters mAb Size Variant Standard on an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 300 mm Column with (bottom) and without (top) a MaxPeak Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 30 mm Guard. The % area and resolution values are virtually unchanged when running with the guard.

i. MaxPeak Premier Protein SEC 250 Å, 2.5 µm Guard

Use of the specially developed MaxPeak Premier Protein SEC 250 Å, 2.5 µm 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 ACQUITY Premier Protein SEC 250 Å, 1.7 µm, the MaxPeak Premier Protein SEC 250 Å, 2.5 µm Guard was designed to prevent sample or eluent-based particulates, as well as some excipients contained in sample, from fouling the analytical column performance without compromising the ability to adequately resolve the 150 kDa mAb monomer from the 100 kDa fragment (Figure 8).

IV. COLUMN SPECIFICATIONS

- Shipping solvent: XBridge Premier Protein SEC 250 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 (Although the XBridge Premier SEC 250 Å, 2.5 µm particles are chemically stable up to 60 °C, their hydration layer can be affected by elevated temperatures. Best native state chromatography has been obtained with separations controlled at ± 5 °C from room temperature.)
- 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. (See Section III.b. for considerations on selecting mobile phase composition.)
- Recommended buffer concentration: 25–200 mM.
- Organic concentration: <15% isoproanol, <15% acetonitrile. (See Section III. b. for more considerations on the use of organic co-solvents.)

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
Volume Load	≤0.5 mL/min	≤0.5 mL/min	≤1.5 mL/min	≤1.5 mL/min
Mass Load	≤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, and 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) on waters.com.

VI. COLUMN CLEANING AND STORAGE

a. Column Cleaning

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.
   - When storing the column in the shipping solvent listed above, it is preferable to keep the column at ambient (room) temperature.
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.
   - Refrigeration is not recommended as buffer salts can precipitate and lead to shorter column lifetimes.

VII. COLUMN QR CODE

The quick reference (QR) code that is located on the column label provides column-specific information (i.e., the part and serial numbers that are unique identifiers for the column), an its encoding follows a widely adopted industry-standard.

1. Scan QR code using any device that is capable of scanning QR codes (i.e., for smart phones and tablets, use the built-in camera app).
2. Be directed to the column's information hub on waters.com.
3. Access technical and scientific information for the column (i.e., certificate of analysis, application notes).

IX. 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 waters.com/sds.

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