Bio-Monolith Protein G Column - More Options for mAb Titer Determination

Application Note

Biologics and Biosimilars

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Introduction

In recent years, monoclonal antibodies (mAbs) have become one of the major biopharma products in response to the need to treat various diseases. These antibodies have been engineered with a specific genetic makeup for better targeting of disease agents. During the development of these antibodies, Protein A and G analytical affinity columns are used for determining their titer or concentration from various cell culture supernatants, to select the high-yield clone. An inert polymeric monolith is used as the support for both the Protein A and Protein G columns. Both columns have high affinity for antibodies, and so, they bind only to antibodies in cell-culture supernatants. However, they have different selectivity, as indicated in Table 1.

This application note introduces the Agilent Bio-Monolith Protein G column. The column is designed for high speed and high loading capacity. Data are presented to show the linearity with high specificity. Linearity analysis reveals the column's capability for accurate quantitation analysis of mAbs in cell-culture supernatant. In addition, lifetime analysis data indicate that the column can be highly reproducible, and has a long lifetime with stable and low backpressure. The Bio-Monolith Protein G column is complementary to the Bio-Monolith Protein A column, to provide more options for titer determination of monoclonal antibodies.

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Table 1. Binding affinity of Agilent Bio-Monolith Protein A and G for different IgG subclasses [1,2].

Antibody	Protein A	Protein G
Human		
Human IgG1	++++	++++
Human IgG2	++++	++++
Human IgG3	–	++++
Human IgG4	++++	++++
Human IgA	++	–
Human IgD	++	–
Human IgE	++	–
Human IgM	++	–
Mouse		
Mouse IgG1	+	++
Mouse IgG2a	++++	++++
Mouse IgG2b	++++	+++
Mouse IgG3	+	+++
Mouse IgM	+/–	–
Antibody fragments		
Human Fab	+	+
Human F(ab′)2	+	+
Human scFv	+	+
Human Fc	+	+
Human κ	+	+
Human λ	+	+

++++ = Strong affinity
+++ = Moderate affinity
++ = Weak affinity
+ = Slight affinity
– = No affinity

Materials and Methods

- Sodium phosphate monobasic monohydrate (Sigma-Aldrich, Corp., p/n S3522) (MW 137.99)
- Sodium phosphate dibasic dihydrate (Sigma, p/n 71643) (MW 177.99)
- Citric acid monohydrate (Sigma, p/n C7129) (MW 210.14)
- Sodium chloride (Sigma, p/n S5886) (MW 58.44)
- Phosphoric acid, 85 wt % in H_2O, 99.99% trace metal basis (Aldrich, p/n 345245)
- Sodium hydroxide solution, 50 to 52% in water, eluent for IC (FLUKA 72064) (MW 40)
- Glycine, for electrophoresis, ≥99% (Sigma, p/n G8898) (MW 75.07)
- Glacial acetic acid (Sigma, p/n A9967) (MW 60.05)
- Hydrochloric acid, 36.5 to 38.0%, BioReagent, for molecular biology (Sigma, p/n H1758)
- *Escherichia coli* cell-lysis kit (Sigma p/n CB0500).
- Chinese hamster ovary (CHO)-cell supernatant and lysate, insect-cell supernatant, humanized CHO-cell derived monoclonal antibody (IgG2 and IgG3) from CreativeBio Labs.

Method

Mobile phase A is the binding and washing buffer, containing 50 mM sodium phosphate buffer, pH 7.4. Two stock solutions (0.2 M) are made from 27.6 g sodium phosphate monobasic monohydrate in 1 L deionized water, and 35.6 g sodium phosphate dibasic dihydrate, also in 1 L of deionized water.

To make 2 L sodium phosphate containing 50 mM sodium phosphate buffer and 50 mM sodium chloride, pH 7.0:

1. Mix 195 mL of sodium phosphate monobasic monohydrate stock solution and 305 mL sodium phosphate dibasic dihydrate stock solution. Agitate using a magnetic stirrer.
2. Add 5.8 g sodium chloride, and continue stirring.
3. Add 1 L deionized water.
4. Measure the pH of the solution, and adjust to pH 7.0 with NaOH or 3 M phosphoric acid.
5. Pour the solution into a 2 L volumetric flask, and add water to the mark.

Mobile phase B is an eluting buffer containing 0.1 M citric acid, pH 2.0, made by dissolving 21 g citric acid monohydrate in about 600 mL of water with gentle stirring. Adjust to pH 2.0 with 1 M HCl, and then dilute solution with water to 1 L in a volumetric flask.

CHO-cell supernatant, lysate, insect-cell lysate (all samples were spun), and humanized monoclonal IgG1, IgG2, and IgG3 were purchased from Creative BioLabs, New York. *Escherichia coli* lysate was prepared according to the recommended protocol of Sigma-Aldrich, Corp [3]. The kit contains CelLytic B, a bacterial lysis reagent (500 mL), lysozyme solution (10 × 1 mL), benzonase (25,000 units), and protease inhibitor cocktail (5 mL). The lysate is prepared by adding 1.0 g cell paste to 10 mL CelLytic B reagent, 0.2 mL lysozyme, 0.1 mL protease inhibitors, and 500 units of benzonase. The mixture is vortexed briefly, and mixed for 10 minutes (by hand or on the shaker) to ensure further extraction of the soluble proteins. The mixture is then centrifuged at 5,000 g for 10 minutes to pellet any insoluble materials. The soluble protein fraction (supernatant) is carefully removed from the cell debris (pellet at the bottom of the tube). The protein concentration in the supernatant can be estimated using the Bradford protein assay, in this case 40 mg/mL.

Some supernatant was then spiked with IgG1, IgG2, or IgG3 as described. Supernatants were diluted with binding buffer (or buffer A) to a final concentration of 10 mg/mL, and spiked with 2 mg/mL purified humanized single monoclonal antibody such as IgG1, IgG2, or IgG3.

Conditions

Column:	Agilent Bio-Monolith Protein G, 5.2 mm diameter, 4.95 mm long (p/n 5069-3639)
Binding buffer:	A, 50 mM sodium phosphate, pH 7.4
Eluting buffer:	B, 0.1 M citric acid, pH 2.0
Sample:	see chromatograms
Inj vol:	see chromatograms
Flow rate:	1.0 mL/min (or see chromatograms)
Gradient:	Time (min) % A 0 100 0.4 100 0.5 0 2.0 0 2.1 100 4.2 100
Temp:	25 °C
Detector:	UV, 280 nm
System:	Agilent 1260 Infinity Bio-inert Quaternary LC

Results and Discussion

Specificity and selectivity

As indicated in Table 1, the Protein G column has higher binding affinity for different humanized monoclonal antibody subclasses than the Protein A column, and only Protein G has affinity for the IgG3 subclass. Data in Figure 1A demonstrate the specificity of the Bio-Monolith Protein G column and its ability to monitor titer, that is, the presence and concentration of antibodies in the supernatant. The column was injected with the sample containing purified recombinant humanized mAb, IgG3, which was spiked in the supernatant of CHO cells. This mAb was expressed in the CHO cell line. The data show that IgG3 was the only protein captured and eluted from the column, at about 1.6 minutes at 1.0 mL/min, whereas all host-cell proteins were not captured by the column, and eluted at the flow-through peak.

To show the different selectivity between Protein A and Protein G columns, both were separately injected with monoclonal antibodies IgG1, IgG2, and IgG3. Both IgG1 and IgG2 were captured by the two columns (data not shown) but IgG3 was not captured by Bio-Monolith Protein A. IgG3 was eluted as a flow-through peak, and IgG3 was only captured and eluted by the Bio-Monolith Protein G column (compare Figures 1A and 1B).

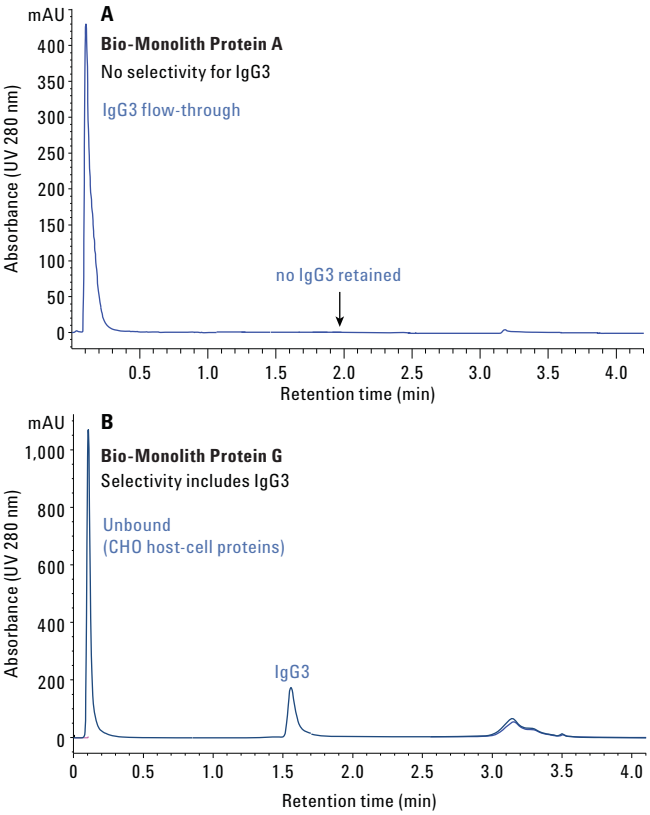

Figure 1. A) No binding of IgG3 on Bio-Monolith Protein A column. IgG3 was eluted as the flow-through peak. 2 mg/mL humanized IgG3 mixed with binding buffer (3 µL was injected onto the column). B) The Agilent Bio-Monolith Protein G column quickly captured only IgG3 from a harvested cell culture spiked with IgG3 (5 µL of 2.0 mg/mL IgG3 mixed with 10 mg/mL CHO-cell supernatant was injected onto the column).

A more rugged test was performed to confirm that the specificity of the Bio-Monolith Protein G column was such that it had no binding affinity for host-cell proteins. Host-cell protein samples from *E. coli* cell lysate, CHO-cell lysate, supernatant, and insect-cell lysate were used. These lysates contained host-cell proteins that were extracted by lysing buffers that contained sodium dodecyl sulfate, a chemical that greatly influences nonspecific binding to the column. These samples did not contain antibody, only host-cell proteins. The test was run because rugged samples give nonspecific results if the column is not well designed. Figure 2 shows no evidence of any protein from any host-cell supernatant being absorbed by the column. All host-cell proteins were eluted as flow-through peaks. The data suggest that the Bio-Monolith Protein G column did not have any affinity for host-cell proteins.

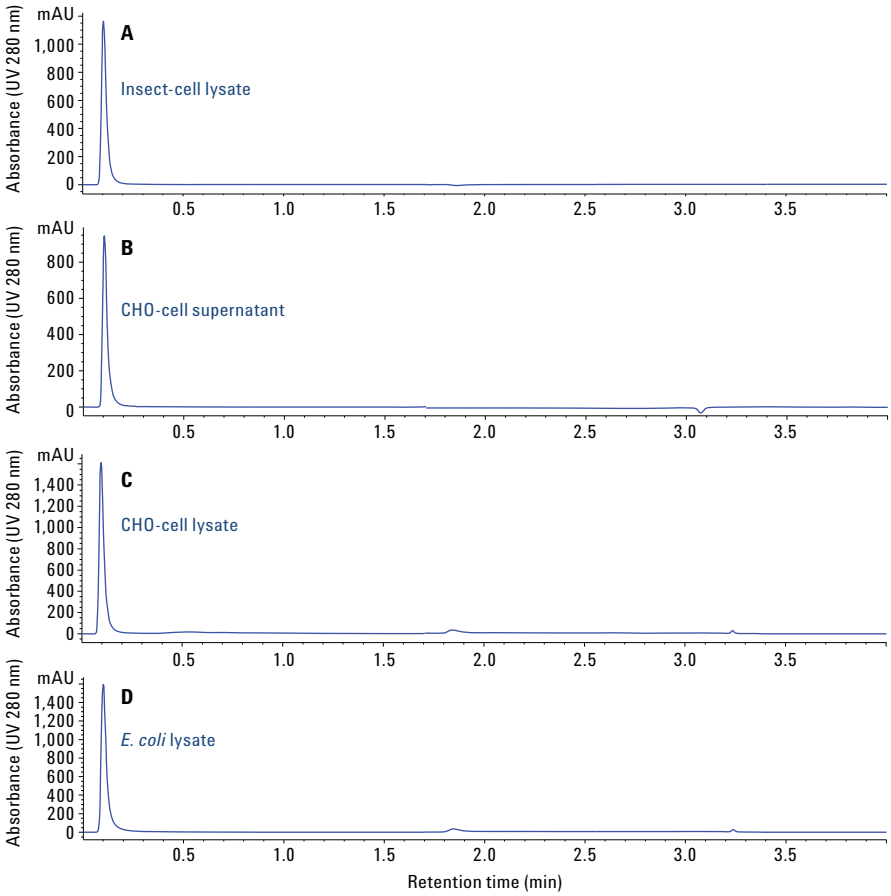

Figure 2. Specificity analysis using the Agilent Bio-Monolith Protein G column with 10 mg/mL host-cell proteins diluted with binding buffer. The injection volume was 5 µL for all samples. A) insect cell lysate, B) CHO-cell supernatant, C) CHO-cell lysate, D) *E. coli* lysate.

Accurate quantitation

Accurate quantitation of mAb titer is essential during the early stages of development when the cell line is selected, and also during manufacture when the amount of mAb in the cell-culture supernatant determines the optimum harvest time. To demonstrate the ability of the Bio-Monolith Protein G column in the accurate quantitation of mAbs, different amounts (µg) of purified IgGs were injected onto the column. Data of peak areas versus amounts of IgGs generated were used to construct the linearity lines, to determine the accuracy of the analysis. Figure 3 shows the linearity of peak areas from the Protein G column, showing that the column can be used for quantitation of mAb in harvest cell-culture media with different concentration ranges. The column was injected as low as 2 µg IgG. The signal-to-noise ratio was no higher than 1:1 for 2 µg (data not shown). The maximum loading capacity for this column is approximately 400 to 500 µg IgG (data not shown), which covers the range of concentrations achieved during cell-line selection and production.

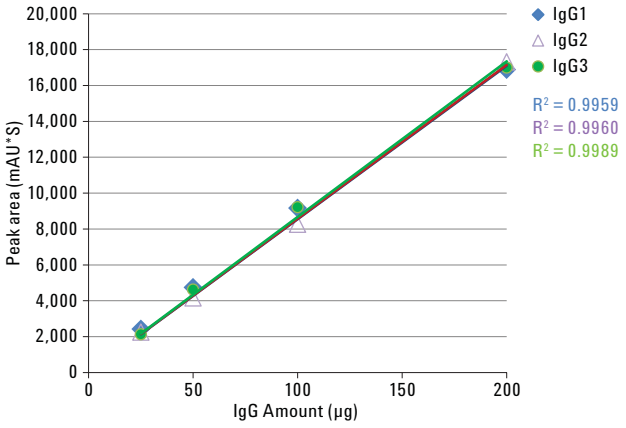

Figure 3. Agilent Bio-Monolith Protein G column quantitates monoclonal antibodies. The linearity lines include peak area data from 25 to 200 µg IgG.

Wider loading range than another vendor's protein G column

Figure 4A shows the linearity comparison for a wide loading range of IgG3 between the Bio-Monolith Protein G column and another vendor's 2.1× 30 mm, 4,000Å, protein G column. The Agilent column generated a linear loading range from 25 to 200 µg IgG3, whereas the other column could only generate linear data between 25 and 100 µg IgG3, as recommended by the other vendor's literature. The reason for not having a linear line at a higher loading range was because the column was not able to retain all material at the higher loading range. In fact, at a 200 µg loading range, the other vendor's protein G column had a break-through peak for the mAb (some IgG3 was not retained on the column and eluted as a flow-through peak), as shown in Figure 4B.

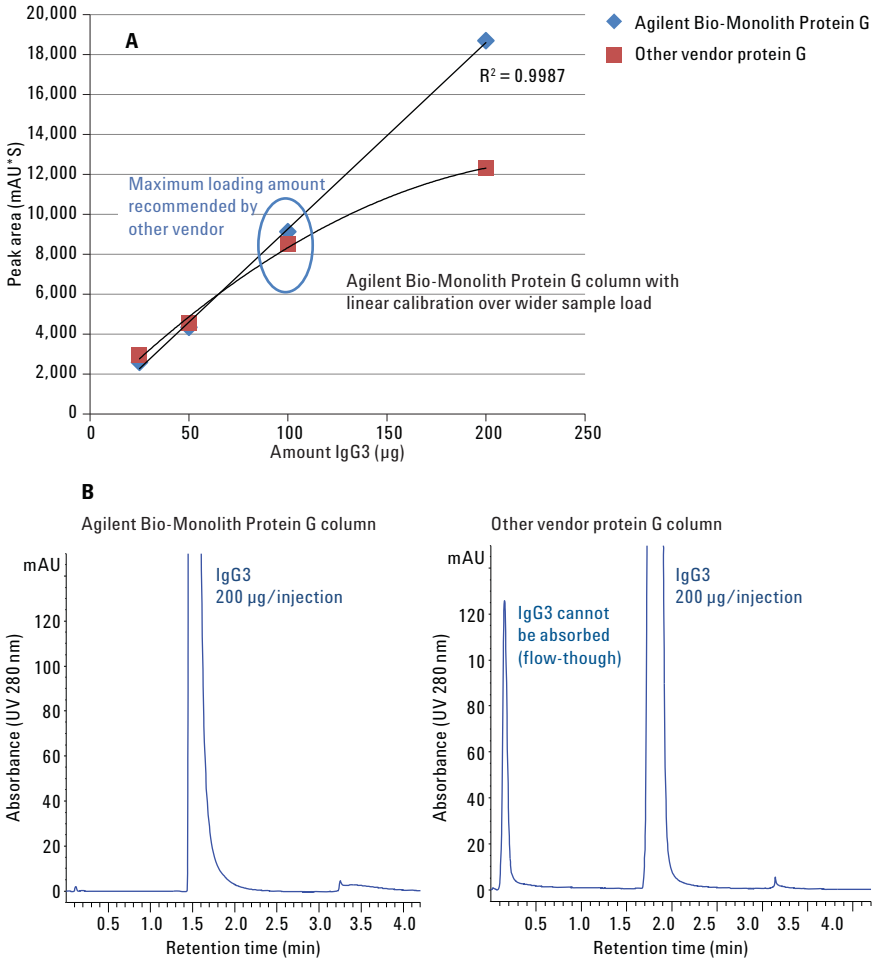

Figure 4. A) Linear loading range comparison between Agilent Bio-Monolith Protein G column and another vendor's protein G column. B) Under a 200 µL load, the other vendor's column shows flow-through that is not observed with the Bio-Monolith Protein G. Thus, the Bio-Monolith Protein G has a higher loading capacity.

Rapid separations

The Bio-Monolith Protein G is designed for rapid separations. Its capability for fast mAb capture and elution at various flow rates is demonstrated with IgG3 in Figure 5, with 1.0, 1.5, 2.0, and 2.5 mL/min flow rates (The column can be operated up to 3.0 mL/min, data not shown.). Table 2 shows the flow rates and operating gradients. The retention of the IgG3 peak was shortened when the flow rate increased. However, the relative peak areas between all flow rates were similar. This indicates that the column generated similar recovery at all flow rates (Table 3).

Table 2. Flow rates and operating gradients used in an assessment of retention capability of the Agilent Bio-Monolith Protein G column.

Time (min)	%A	%B
1.0 mL/min		
0	100	0
0.4	100	0
0.5	0	100
1.7	0	100
1.8	100	0
4.2	100	0
1.5 mL/min		
0	100	0
0.3	100	0
0.4	0	100
1.3	0	100
1.4	100	0
3.0	100	0
2.0 mL/min		
0	100	0
0.2	100	0
0.3	0	110
0.9	0	100
1.0	100	0
2.1	100	0
2.5 mL/min		
0	100	0
0.1	100	0
0.3	0	100
0.7	0	100
0.8	100	0
1.7	100	0

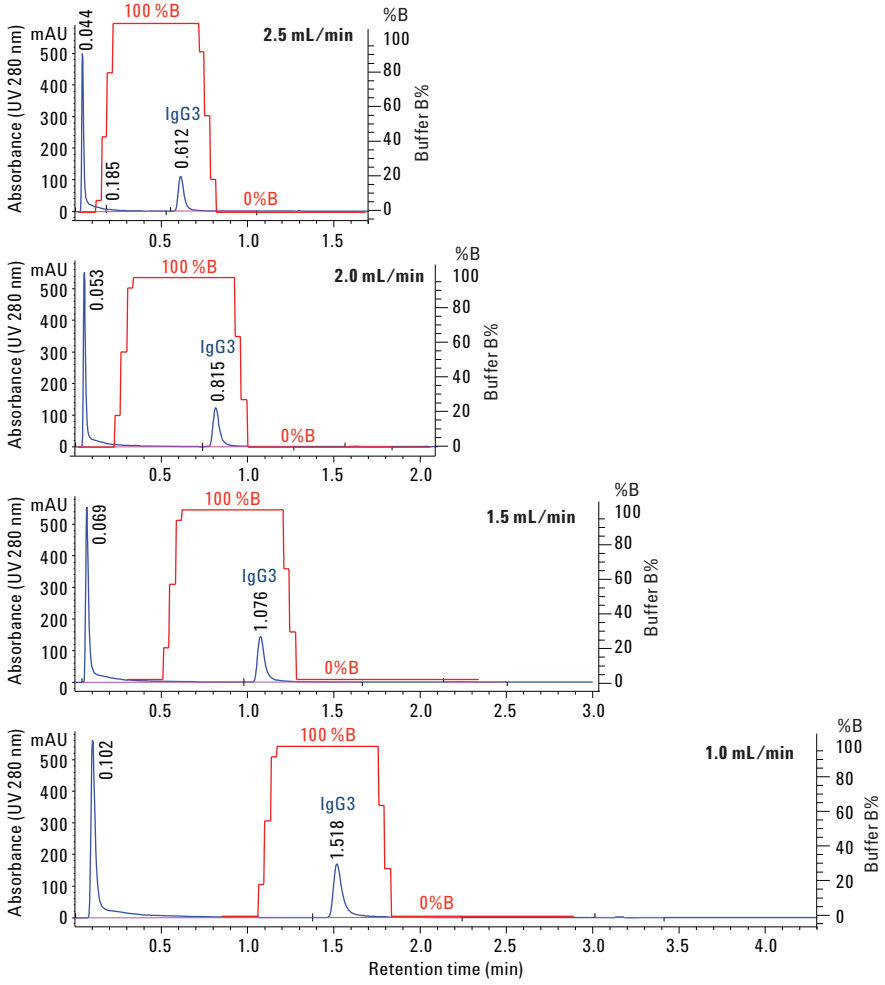

Figure 5. Binding of IgG3 on the Agilent Bio-Monolith Protein G column evaluated at several flow rates. Injection 5 µL (2.0 mg/mL IgG3 mixed with 5 mg/mL CHO-cells host proteins).

Table 3. Agilent Bio-Monolith Protein G column delivers similar relative percentages of flow-through and IgG3 peaks for all flow rates.

Flow rate (mL/min)	Total area (mAuU*S)	Flow-through peak area (mAU*S)	Flow-through peak relative area (%)	IgG3 peak area (mAU*S)	IgG3 peak relative area (%)
2.5	798	521	65.3	277	34.7
2.0	1,056	709	67.1	347	32.9
1.5	1,390	932	67.1	458	32.9
1.0	2,069	1392	67.3	677	32.7

Figure 6 shows the linearity line of the column's backpressure. When the column was tested at 0.5 mL/min intervals, increasing from 1 to 2.5 mL/min, the backpressure increased linearly. The column has a maximum backpressure of 150 bar. A typical separation flow rate of the Bio-Monolith Protein G column is 1.0 mL/min. With the instrument flow rate set at 1.0 mL/min, the column backpressure was ~24 bar. When the flow rate was increased to 2.5 mL/min, the column backpressure increased to ~60 bar. There was minimal effect on IgG binding on the column at maximum backpressure, as indicated earlier.

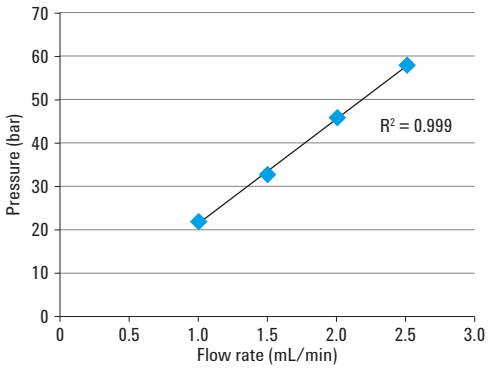

Figure 6. Flow rate versus back pressure. The backpressure of the Agilent Bio-Monolith Protein G column increased linearly when the flow rate also increased linearly.

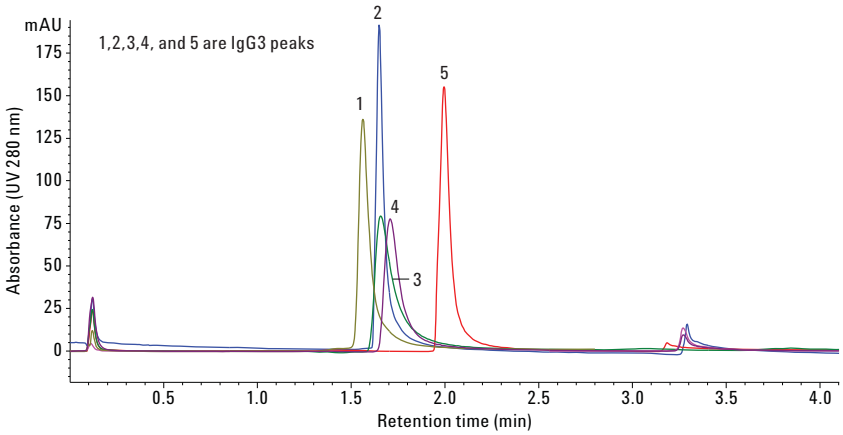

Figure 7. Humanized IgG3 peak eluted by different acidic eluents from the Agilent Bio-Monolith Protein G column.

The compatibility of different elution buffers

Figure 7 shows compatibility with different eluting buffers for the Bio-Monolith Protein G column. The IgG3 peak can be eluted by many different acidic eluents. Table 4 shows the strengths and pH of the acidic eluents. These eluents were capable of eluting IgG3 from the column with similar retention times (similar data were also observed for other IgGs), except with 12 mM HCl. The retention of the IgG3 peak was longer, compared to the retention time of the IgG3 peak eluted by other eluting buffers. This eluting buffer, when its concentration was increased to 0.1 M, gained enough strength to elute IgG3 with retention similar to other eluting buffers.

It is also noteworthy that each acidic eluent generated an IgG3 peak with slightly different peak width and tailing factor. It is suggested that, depending on the IgG, acidic eluents and their strengths will have different influences on retention time, as well as peak width and tailing factor. Therefore, depending on the IgG and expectations of the data, acidic eluents and their concentrations must be determined experimentally.

Table 4. The compatibility and effect of different acidic eluents on IgG.

Peak no.	Acid	PW	5% TF	Pressure (bar) at 1 mL/min
1	0.1 M citric acid pH 2.0	0.058	1.68	24
2	0.1 M HCl	0.053	1.58	24
3	5% acetic acid	0.071	1.85	24
4	0.1 M glycine	0.075	1.82	24
5	12 mM HCl	0.068	1.69	24

Recovery of column performance after clean-in-place

Figure 8 shows the Bio-Monolith Protein G column can recover its full performance after clean-in-place (CIP). The column was injected with IgG3 after it was injected for more than 1,000 injections with IgG3 spiked in CHO-cell supernatant and lysate. Data show that when the column was dirty, peak width of IgG3 was broaden and peak height was reduced (compare Figure 8A and 8B, before and after clean-in-place). The data also show that some IgG3 was not captured by the column and was eluted as a flow-through peak (Figure 8, Panel A). After the column was cleaned, it regained its full performance and IgG3 was fully captured (see Figure 8B). Figure 9 shows the linearity comparison data of a wide loading dynamic range before and after the column was properly cleaned. IgG3 peak areas of before and after CIP were very comparable and highly linear. The data indicated that the column was effectively cleaned and compatible with the cleaning protocol described in Table 5.

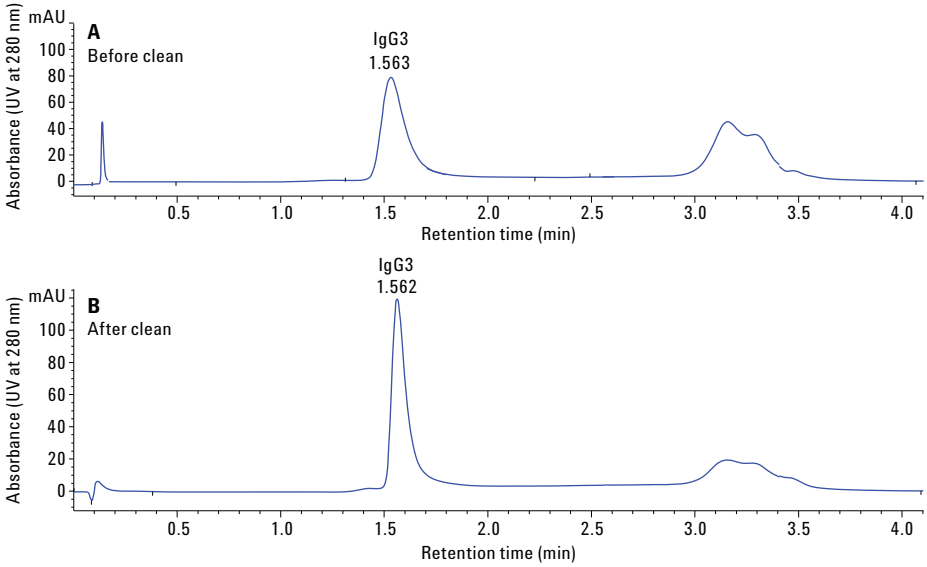

Figure 8. A) Agilent Bio-Monolith Protein G column injected with IgG3 after more than 1000 injections. Column was dirty. B) Column was cleaned and regained its full performance.

Table 5. Protocol for clean-in-place for the Agilent Bio-Monolith Protein G column. To prevent contaminants from entering the rest of the column, it should be in a reversed-flow direction for the first step of the protocol at 0.2 to 0.5 mL/min.

Step	Solution	Washing column volume (CV)
1	0.1 M NaOH	10 to 20
2	Deionized water	10 to 20
3	0.5 M Sodium phosphate buffer, pH 7.4	10 to 20
4	Re-equilibrate column with binding buffer	50

In some cases, simple regeneration of the monolithic column is not sufficient. Sample molecules might not fully elute from the column or might precipitate within it. This build-up of contaminants in the column can cause loss of resolution and binding capacity, increased backpressure, or complete blockage. A specific CIP protocol should be designed according to the type of contaminants that are present in the sample. More regeneration suggestions are in the user guide.

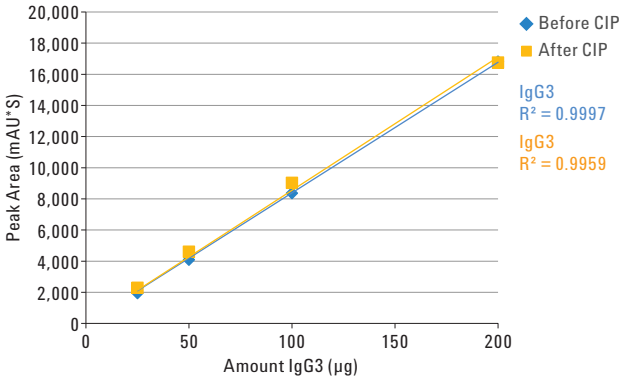

Figure 9. Comparison of linearity provided by the Agilent Bio-Monolith Protein G column across a wide dynamic loading range before and after clean-in-place.

Lifetime and reproducibility

Figures 10 and 11 show the results of 1,000 consecutive injections of IgG3 and CHO-cell supernatant plus lysate onto the Bio-Monolith Protein G column. The column received 40 injections with CHO-cell supernatant plus lysate, followed by 10 injections of IgG3. This sequence was set for 1,000 injections continuously without stopping for cleaning. The integrity of peak retention time and peak area (Figure 9), as well as peak tailing factor and peak width of IgG3 (Figure 10), remained nearly unchanged without compromising the column's performance in terms of binding, separation, and eluting capacity.

Figure 11 shows that the peak width and tailing factor had minimal impact during the course of the 1,000-injection study.

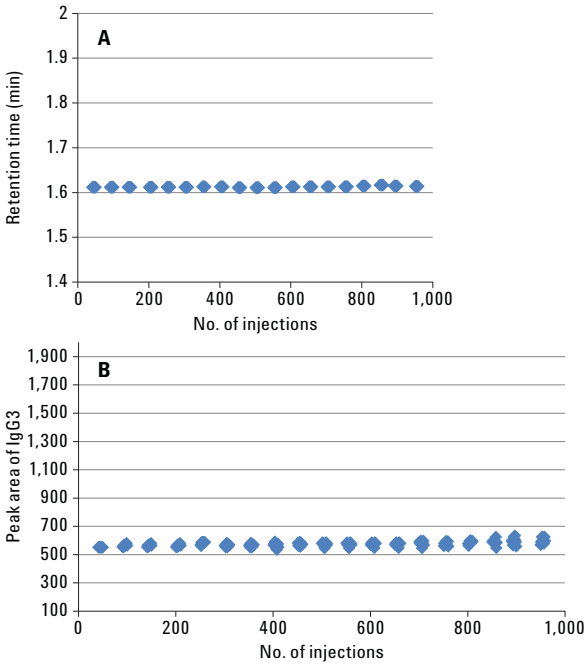

Figure 10. Reproducibility of the Agilent Bio-Monolith Protein G column over 1,000 injections without clean-in-place. A) Retention time. B) Peak area of IgG3. Ten injections were recorded after 40 injections, and over 1,000 injections were made. Retention time and peak width of IgG3 remained unchanged (standard deviation = 2.5, n = 100).

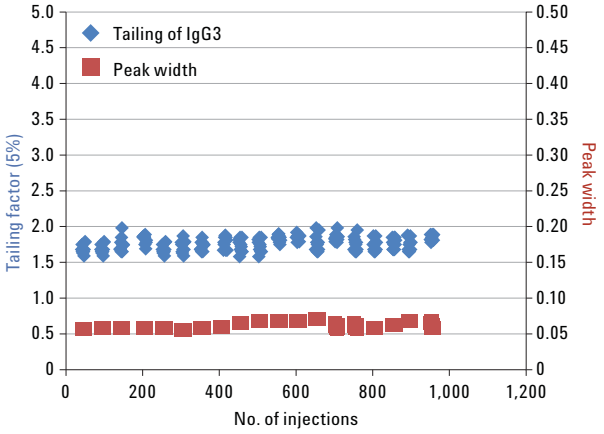

Figure 11. The consistency of peak width and tailing factor of the IgG3 peak over 1,000 injections on the Agilent Bio-Monolith Protein G column.

Conclusions

The Agilent Bio-Monolith Protein G column has a high affinity for monoclonal antibody subclasses. It is evident that the column can capture and accurately quantitate across a wide loading linearity range for mAb from supernatants. The columns can be used effectively to quantitate the amount of monoclonal antibody with various flow rates, without sacrificing data. The operating backpressure was significantly low, illustrating that the Bio-Monolith Protein G column can be operated with <600 bar HPLC instruments. The flexibility of the column with different acidic eluents enables easy and straightforward experimental design. Agilent Bio-Monolith Protein A and G columns are thus complementary, thus Protein G has affinity for mAbs that do not bind to Protein A, and *vice versa*. These columns provide more options for rapid titer determination for a wider range of mAb variants.

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