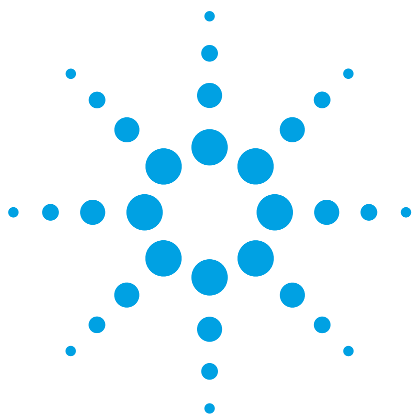

Agilent Bio-Monolith Protein A Monitors Monoclonal Antibody Titer from Cell Cultures

Application Note

Biotherapeutics & Biosimilars

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Introduction

In the biopharmaceutical industry, downstream processing for monoclonal antibody production typically includes three chromatographic steps; capture, intermediate purification, and polishing. Protein A is frequently used as a capturing step, which results in excellent throughput (that is, capacity and speed) while playing an important role in concentrating the target molecule, immunoglobulin. To monitor monoclonal antibody titer and yield from cell-culture supernatants before expensive preparative and large amounts of protein A are employed, a small (analytical) scale procedure is necessary to determine the titer of monoclonal antibody for the optimal time for harvest of the monoclonal antibody product. In this application note, prepacked Agilent Bio-Monolith Protein A columns were used to illustrate the quick capture of monoclonal antibody titer from cell supernatant.

Methods and Materials

Sodium phosphate (monobasic and dibasic), citric acid monohydrate (Sigma-Aldrich (p/n C1901), and an *Escherichia coli* cell-lysis kit were purchased from Sigma-Aldrich Corp. (p/n CB0500). Humanized CHO-cell derived monoclonal antibody (IgG1) was purchased from Bio-Creative Labs.

Eluent A is used for equilibration, binding, and re-equilibration. This buffer contained 20 mM sodium phosphate buffer, pH 7.4. To make 1 L of 20 mM sodium phosphate buffer, pH 7.4, briefly, dissolve 3.1 g of $NaH_2PO_4 \cdot H_2O$ and 10.9 g of Na_2HPO_4 (anhydrous) in distilled H_2O to make a volume of 1 L. This is 0.1 M (100 mM) sodium phosphate buffer with pH 7.4 at 25 °C. This buffer can be stored for up to 1 month at 4 °C. This stock solution is then diluted 1:5 with deionized water (take 200 mL of stock solution and add 800 mL deionized water) to obtain 20 mM sodium phosphate buffer, pH 7.4. Eluent B is used for protein elution and contains 0.1 M citric acid, pH 2.8. To make 1 L of citric acid buffer, weigh out 21 g of citric acid monohydrate, dissolve it in approximately 600 mL of water with gentle strirring, and adjust the pH to 2.8. Finally, dilute the solution with deionized water to the 1 L mark in a volumetric flask.

The manufacturer's recommended protocol was followed to obtain *E. coli* supernatant. Briefly, 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). Weigh out 1.0 g cell paste, mix with 10 mL Cellytic B reagent, 0.2 mL lysozyme, 0.1 mL protease inhibitors, and 500 units benzonase. The mixture is vortexed briefly and mixed for 10 minutes (by hand or on the shaker) to ensure extraction of the soluble proteins. The mixture is then centrifuged at 5,000 x g for 10 minutes to pellet any insoluble materials. Carefully remove the soluble protein fraction (supernatant) 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.

The estimated concentration of protein was 40 mg/mL. The supernatant was then spiked with IgG1 as described; 40 mg/mL *E. coli* supernatant spiked with 2.5 mg/mL purified humanized IgG1. After mixing, the mixture was diluted further with mobile phase A (20 mM sodium phosphate buffer, pH 7.4) with a 1:1 ratio to a final concentration of 1.25 mg/mL of IgG1 and 20 mg/mL *E. coli* supernatant.

Conditions

Column:	Agilent Bio-Monolith Protein A (p/n 5069-3639)
Sample:	Humanized IgG1 and *E. coli* lysate
Eluent:	A) 20 mM sodium phosphate, pH 7.4 B) 0.1 M citric acid, pH 2.8
Injection:	see chromatograms
Flow rate:	1.0 mL/min, unless specified otherwise
Gradient:	Time (min) % B 0 0 0.5 0 0.6 100 1.7 100 1.8 0 3.5 0
Temperature:	25 °C
Detector:	UV, 280 nm
System:	Agilent 1260 Infinity Bio-inert Quaternary LC System

Results and Discussion

Quantitation for titer of monoclonal antibody

Accurate quantitation of the titer and yield of monoclonal antibody will help to determine the amount of monoclonal antibody in the cell-culture lysate (supernatant) and indicate the harvest time. This is a very important step in manufacturing antibody. To demonstrate the ability of the Bio-Monolith Protein A column in the quantitation of IgG1, different amounts (µg) of purified IgG1 were injected onto the column. Figure 1 shows the linearity areas (constructed by humanized IgG1 ranging from 0.625-5 µg) to the amounts of humanized IgG1 loaded on the column. This linear calibration curve is a standard curve. This correlation shows that the Bio-Monolith Protein A column can be used for quantitation of monoclonal antibody in harvest cell-culture media with different concentration ranges. The column was also injected with 0.312 µg humanized IgG1 (data point not shown). The signal-to-noise (S/N) ratio here was higher than 1:1 ratio for the amount of 0.312 µg. The maximum loading capacity is approximately 400-500 µg IgG.

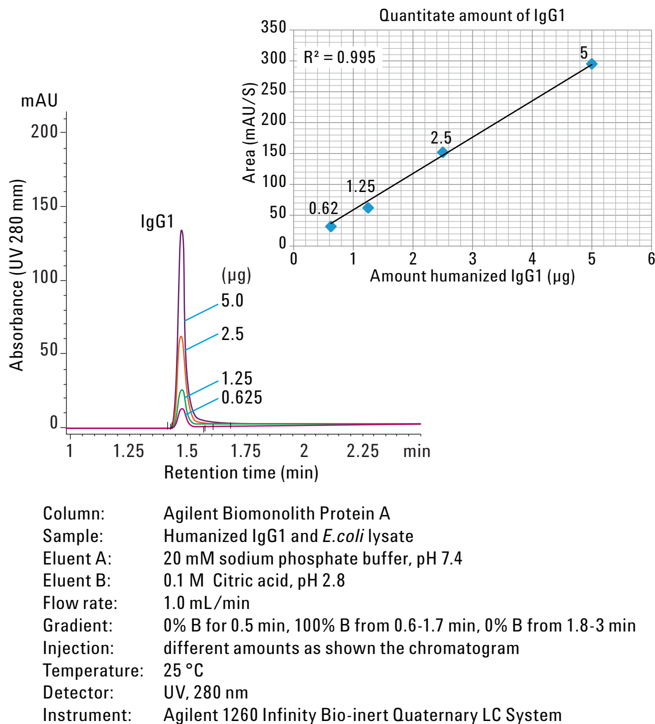


Figure 1. Agilent Bio-Monolith Protein A column quantitated monoclonal antibody.

In this example, the data from Figure 2 shows 5–0.625 µg humanized IgG1 ranges. The column was also injected with 0.312 µg humanized IgG1 (data point not shown). The S/N ratio was higher than a 1:1 ratio in the amount of 0.312 µg. The maximum loading capacity is approximately 400–500 µg IgG.

Determination of IgG1 titer from harvested cell culture supernatant

The first step of purification for monoclonal antibody is to determine its presence in the cell-culture lysate (supernatant). This requires columns that have a very specific affinity for monoclonal antibody and only retain and quantitate monoclonal antibody.

The specificity of the Bio-Monolith Protein A column is evident in Figure 2. When the supernatant (proteins from *E. coli* lysate) did not contain monoclonal antibody (IgG1), the chromatogram in Panel A shows no retention of any proteins. All proteins from the supernatant flowed through the column. The expansion of the baseline from Panel A provides further proof. Panel B shows the column captured and separated only monoclonal antibody (supernatant from *E. coli* was spiked with IgG1). The amount of IgG1 was pre-determined and re-confirmed using the above standard curve (Figure 1), it was about 1.25 µg/µL. The Bio-Monolith Protein A column captured only the monoclonal antibody and eluted at approximately 1.4 minutes.

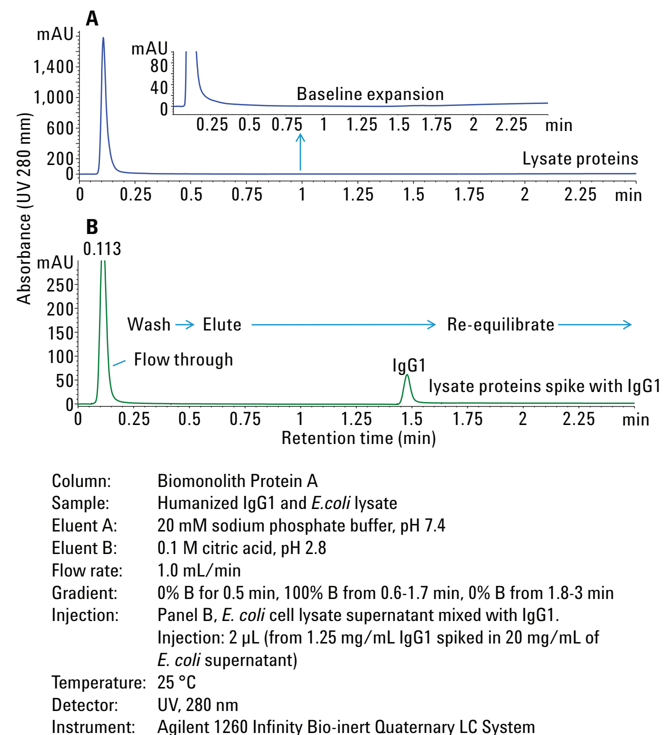


Figure 2. The Agilent Bio-Monolith Protein A column quickly captures only IgG1 from a harvested cell culture spiked with IgG1. Panel A, *E. coli* cell lysate supernatant; Panel B, *E. coli* cell lysate supernatant mixed with IgG1. Injection: 2 µL (from 1.25 mg/mL IgG1 mixed with 20 mg/mL of *E. coli* supernatant).

Effect of flow rate on IgG1 binding, peak area, and column back pressure

Three different flow rates, 1.0, 1.5 and 2.0 mL/min, were used to understand the effect of flow rate on IgG1 binding and column performance (Figure 3). Two times the concentration of IgG1 and *E. coli* was loaded for this study in order to observe changes easily. There was very little effect of IgG1 binding on the column when the flow rate was increased (Table 1). The percentages of unbound proteins (*E. coli* proteins), relative area, and bound IgG1 relative area remained unchanged at different flow rates (Table 1).

Data from Table 1 also shows that increasing flow rate linearly increases the column backpressure (Figure 4). Typically, the Bio-Monolith Protein A column is recommended for operation at 1.0 mL/min. However, the column is rated to a maximum backpressure of 72 bar. Based on this particular instrument, at 1.0 mL/min, the column backpressure was 32 bar. When the flow rate was increased to 2.0 mL/min, the column back pressure increased to 68 bar. There was minimal effect on IgG1 binding onto the column at maximum backpressure, as indicated earlier. Therefore, it is possible to operate at a higher flow rate for faster analysis.

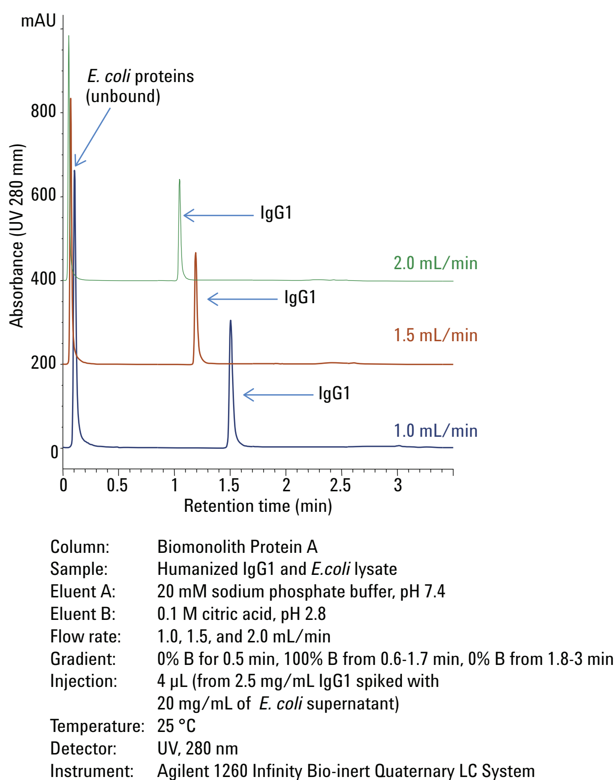


Figure 3. Binding of IgG1 with the Agilent Bio-Monolith Protein A column evaluated at several of flow rates. More sample was loaded for this study to easily observe changes in chromatogram and signal integration.

Table 1. Flow rate versus peak relative area on unbound proteins and IgG1.

Flow rate (mL/min)	Unbound area (mAu/S)	IgG1 area (mAu/S)	Unbound relative area (%)	IgG1 relative area (%)	Pressure (bar)
1.0	1230	738	63	37	32
1.5	840	492	63	37	47
2.0	636	363	64	36	68

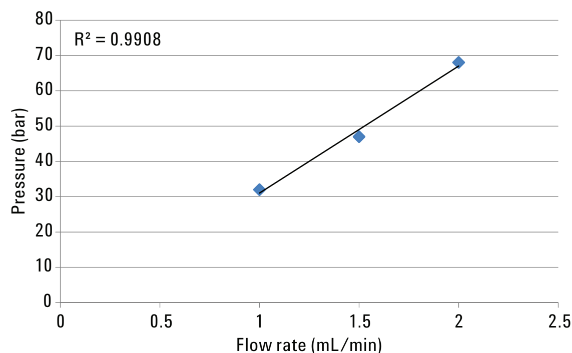
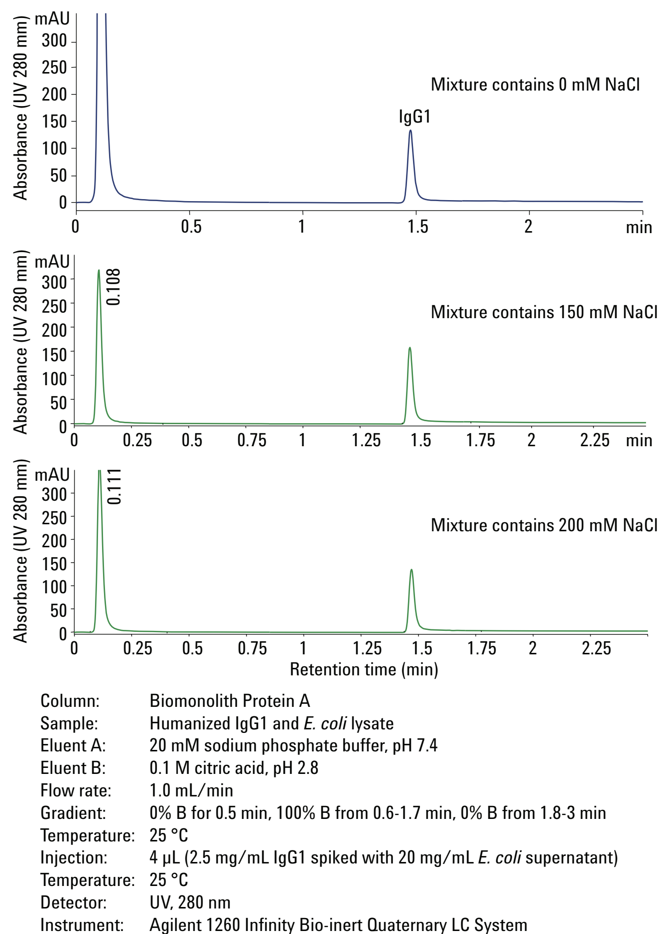


Figure 4. Flow rate versus back pressure. Column backpressure increased linearly when column flow rate increased linearly (1.0, 1.5, and 2.0 mL/min).

Salt tolerance - mixture of cell-culture supernatant and IgG1 containing different salt concentrations

Cell-culture supernatant often contains neutral salts such as sodium chloride (NaCl) and potassium chloride (KCl) for protein stabilization. Salt concentrations ranging from 100 to 200 mM are used, typically. However, salt is a strong eluting solvent for many purification processes, including affinity and ion-exchange methods. For some methods, as little as 50 mM NaCl can elute bound proteins from the column and prevent the column from retaining proteins for separation. Therefore, it is important to demonstrate that the affinity Bio-monolith Protein A column can tolerate a certain amount of salt. In this experiment, supernatant mixed with IgG1 was dissolved with 0 to 200 mM NaCl to show the salt tolerance of the column. Figure 5 shows data from a sample mixed with 0, 150, and 200 mM NaCl concentrations. Results indicate that the Bio-Monolith Protein A column can tolerate samples with high salt concentration without deterioration of peak shapes. Furthermore, calculation of peak areas from all three salt concentrations show that any change was negligible (Table 2).



Column:	Biomonolith Protein A
Sample:	Humanized IgG1 and *E. coli* lysate
Eluent A:	20 mM sodium phosphate buffer, pH 7.4
Eluent B:	0.1 M citric acid, pH 2.8
Flow rate:	1.0 mL/min
Gradient:	0% B for 0.5 min, 100% B from 0.6-1.7 min, 0% B from 1.8-3 min
Temperature:	25 °C
Injection:	4 µL (2.5 mg/mL IgG1 spiked with 20 mg/mL *E. coli* supernatant)
Temperature:	25 °C
Detector:	UV, 280 nm
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System

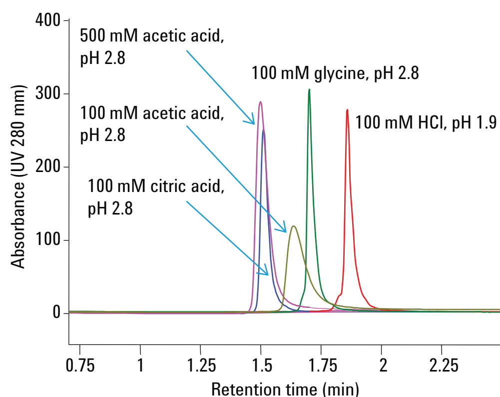
Figure 5. IgG1 and *E. coli* supernatant sample mixed with different salt concentrations and injected onto the Agilent Bio-Monolith Protein A column to evaluate its ability to work with samples containing salt. Injection: 2 µL (1.25 mg/mL IgG1 mixed with 20 mg/mL *E. coli* proteins).

Table 2. Minimal effect on column performances when salt is present in injected-sample.

NaCl (mM)	Area of IgG1 (mAu/S)
0	293
150	312
200	296

The influence of different elution buffers

Data from Figure 6 show the influence of the elution buffers on the Bio-Monolith Protein A column. The data clearly indicate that IgG1 peak can be eluted using many different acidic buffers. Peak heights and retention times of IgG1 peak were observed. HCl, glycine, citric acid, and acetic acid with pH 2.8 and 100 mM were used. These elution buffers provide very comparable peak height for IgG1, with variable retention times. Peak shapes and peak width are very similar in shape and width, except with 100 mM acetic acid. This elution buffer, when increased to 500 mM, gained enough strength to bring peak height and shape in line with other elution buffers. Therefore, depending on the acidic buffer, the concentration of the buffer needs to be determined.

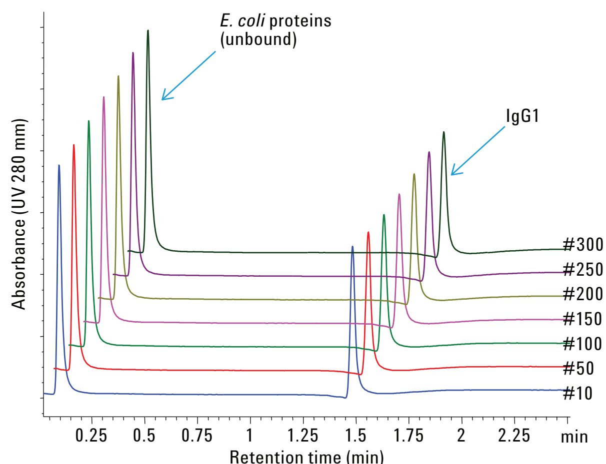


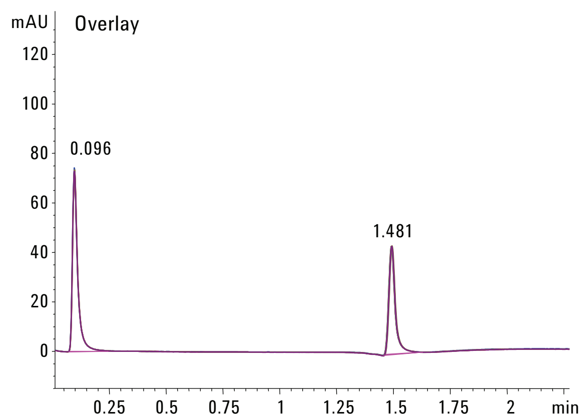
Column:	Biomonolith Protein A
Sample:	Humanized IgG1 and *E.coli* lysate
Eluent A:	20 mM sodium phosphate buffer, pH 7.4
Eluent B:	Various buffers with pH 2.8
Flow rate:	1.0 mL/min
Gradient:	0% B for 0.5 min, 100% B from 0.6-1.7 min, 0% B from 1.8-3 min
Injection:	4 µL (2.5 mg/mL IgG1)
Temperature:	25 °C
Detector:	UV, 280 nm
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System

Figure 6. Influence of different elution buffers on IgG1 peak height and shape. Injection: 4 µL (1.25 mg/mL IgG1 mixed with 20 mg/mL *E. coli* proteins).

Reproducibility – three hundred injections before clean-in-place

Data from Figure 7 and Table 3 show the results of 300 consecutive injections, to examine the column's reproducibility. The results indicate that the integrity of retention time, peak area, and peak width remained the same after 300 injections, without the column losing its binding and separating capacity for IgG1, and before clean-in-place.





Column:	Biomonolith Protein A
Sample:	Humanized IgG1 and *E.coli* lysate
Eluent A:	20 mM sodium phosphate buffer, pH 7.4
Eluent B:	0.1 M citric acid, pH 2.8
Flow rate:	1.0 mL/min
Gradient:	0% B for 0.5 min, 100% B from 0.6-1.7 min, 0% B from 1.8-3 min
Injection:	2 µL (1 mg/mL IgG1 spiked with 10 mg/mL of *E. coli* proteins)
Temperature:	25 °C
Detector:	UV, 280 nm
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System

Note: For every 20 injections, three cleaning cycles were used to make sure all residues eluted from column. Using above running condition without sample injection

Figure 7. Reproducibility of the Agilent Bio-Monolith Protein A column, 300 injections before clean-in-place.
Note: for every 20 injections, three injections without sample were used as a washing step to help elute as much residue from the column as possible (using the above running conditions without sample injection). The washing data (not shown) indicate that there was a negligible amount of residue left on the column from 20 injections.

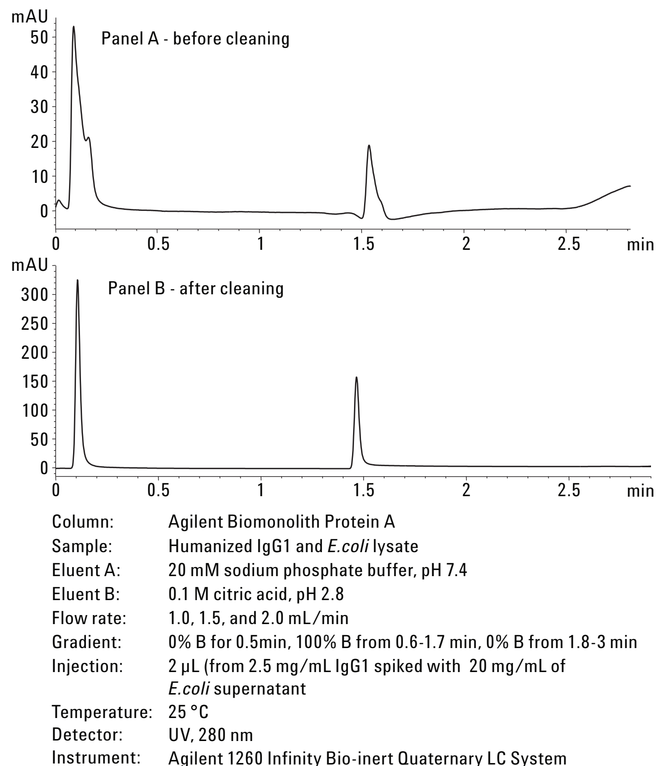
Table 3. Retention time, peak area, and peak width of IgG1 from 300 injections before clean-in-place (standard deviation of n = 300 was 0.75).

Injection number	IgG1 retention time (min)	IgG1 area (mAu/s)	IgG1 peak width (min)
10	1.48	89	0.02
50	1.48	88	0.02
100	1.48	88	0.02
150	1.48	87	0.02
200	1.48	88	0.02
300	1.48	87	0.02

Regeneration and clean-in-place

Panel A (Figure 8) shows deteriorated peaks of both unbound and IgG1. Retention times of peaks also shifted. This is a signal that the column needs to be regenerated. Panel B (Figure 8) shows column performance has been restored after cleaning with the suggested procedure. Briefly, the Bio Monolith Protein A column can be regenerated by washing with 20 column volumes of a buffer such as sodium phosphate buffer containing 1.0 M NaCl, pH 7.0 to 8.0, at 0.5 mL/min. The column should be washed with a low-pH solution (for example, 1.0 mM HCl or 1.0 M glycine-HCl, pH 2.5) at 0.5 mL/min. Then, wash with
10 column volumes of deionized water and re-equilibrate with buffer A (20 mM sodium phosphate buffer, pH 7.4) for 20 column volumes before the next injection.

In some cases, simple regeneration of the monolithic column is not sufficient. Sample molecules might not fully elute from the column or might even precipitate on the column. This buildup 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. Table 4 suggests an appropriate protocol.



Column:	Agilent Biomonolith Protein A
Sample:	Humanized IgG1 and *E.coli* lysate
Eluent A:	20 mM sodium phosphate buffer, pH 7.4
Eluent B:	0.1 M citric acid, pH 2.8
Flow rate:	1.0, 1.5, and 2.0 mL/min
Gradient:	0% B for 0.5min, 100% B from 0.6-1.7 min, 0% B from 1.8-3 min
Injection:	2 µL (from 2.5 mg/mL IgG1 spiked with 20 mg/mL of *E.coli* supernatant
Temperature:	25 °C
Detector:	UV, 280 nm
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System

Figure 8. Panel A shows Bio Monolith Protein column before cleaning; panel B shows column performance has been restored after cleaning.

Table 4. Suggested protocol for clean-in-place. Note: the column should be in a reversed-flow direction for the first step of the protocol at 0.2 to 0.5 mL/min to prevent contaminants from further entering the rest of the column.

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

Conclusions

The Agilent Bio-Monolith Protein A column has a very high affinity for monoclonal antibodies. It is evident that the column can capture and accurately quantitate monoclonal antibody exclusively from supernatant in less than 1.4 minutes to assess harvesting time. Columns can be used effectively to quantitate the amount of a monoclonal antibody with various flow rates without sacrificing data quality. The demonstrated flexibility of columns with different elution buffers as well as the tolerance for samples containing high salt concentrations allows one to easily design experimental procedures.

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