mAb Titer Analysis with the Agilent Bio-Monolith Protein A Column

Application Note

Biopharmaceuticals and Biosimilars

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Abstract

Monoclonal antibodies (mAbs) are becoming increasingly important in the treatment of various diseases. During development of recombinant mAbs, protein titer and yield from various cell culture supernatants must be monitored. This application note describes how the Agilent Bio-Monolith Protein A column was successfully applied in the determination of mAb concentrations.

Introduction

Protein A from *Staphylococcus aureus* has a very strong affinity for the Fc domain of immunoglobulins (IgG), allowing its capture from complex matrixes such as cell-culture supernatants. Affinity chromatography making use of Protein A is the gold standard in therapeutic monoclonal antibody (mAb) purification, and typically represents the first chromatographic step in downstream processing. Protein A chromatography finds applications beyond this large-scale purification. At the analytical scale, it is used early in the development of mAbs for the high-throughput determination of mAb titer and yield directly from cell culture supernatants, and to purify µg amounts of material for further measurements, for example, by mass spectrometry (MS), ion-exchange (IEX), size exclusion chromatography (SEC), or hydrophobic interaction chromatography (HIC).

Agilent Technologies

This application note describes the use of the Agilent Bio-Monolith Protein A column in mAb titer analysis. This HPLC column (Figure 1) has a 5.2-mm id, a column length of 4.95 mm, and is composed of a highly cross-linked poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolithic disk coated with native Protein A from *S. aureus*. Its monolithic nature, characterized by well-defined channels of 1,200 to 1,500 nm, and by the absence of pores and voids, delivers fast and efficient separations with negligible carryover and excellent robustness. These are features typically expected from a column for mAb titer analysis, to successfully guide clone selection and cell-culture optimization. We present the best practice for use of the column in the determination of absolute mAb concentrations in Chinese hamster ovary (CHO) cell-culture supernatants. Data from a trastuzumab biosimilar project are used for illustration purposes. Trastuzumab, marketed as Herceptin since 1998, is used in the treatment of HER2 positive breast cancer, and comes out of patent in 2014 in Europe, and 2018 in the United States.

Experimental

Materials

Water was obtained from Biosolve (Valkenswaard, The Netherlands). Citric acid, acetic acid, NaH_2PO_4, and Na_2HPO_4 were purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). Trastuzumab biosimilar CHO cell culture supernatants were obtained from a local biotechnology company.

Sample preparation

Herceptin stock solution present at 21 mg/mL was diluted in mobile phase A prior to injection. Cell supernatants were diluted 1:1 in 50 mM Na_2HPO_4. Supernatants were centrifuged at 5,000 g for 5 minutes prior to injection.

Instrumentation

Agilent Bio-Monolith Protein A measurements were performed on:

- Agilent 1100 Series Quaternary Pump (G1311A)
- Agilent 1100 Series Autosampler (G1313A)
- Agilent 1100 Series Diode Array Detector (G1315A)

Software

Agilent Technologies OpenLAB CDS ChemStation revision C01.05 (35)

Conditions

Column:	Agilent Bio-Monolith Protein A (p/n 5069-3639)	
Mobile phase:	A) 50 mM phosphate, pH 7.4; B) 100 mM citric acid, pH 2.8 mM acetic acid, pH 2.6	
Gradient:	Time (min)	%B
	0 to 0.5	0 (binding)
	0.6 to 1.7	100 (elution)
	1.8 to 3.5	0 (regeneration)
Flow rate:	1 mL/min	
Injection volume:	Variable (50 µL, optimized for CHO cell culture supernatants)	
Detection:	UV at 280 nm	

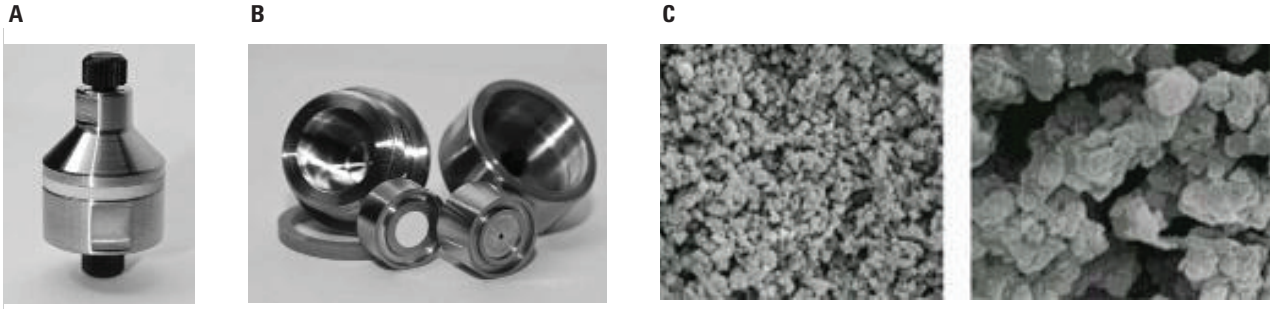

Figure 1. Agilent Bio-Monolith Protein A column (A), column housing and monolithic disk (B) and microscopic view on the monolith packing (C).

Results and Discussion

Buffer selection

Figure 2 shows a typical chromatogram from the Protein A column. The example chromatogram is one injection of the supernatant of a specific trastuzumab-producing CHO clone. The unbound material eluted in the flow-through while the mAb was retained at neutral pH (binding) and was only released (elution) after lowering the pH upon applying a step gradient. In this case, 50 mM Na-phosphate at pH 7.4 was used for binding/loading, and 100 mM citric acid at pH 2.8 for elution. This represents a good starting condition for any application.

When developing a new method for a Protein A column, both binding and elution buffers should be optimized. For binding buffers, 50 mM Na phosphate, pH 7.4, is a good starting point, and can be optimized between pH 7 and 8. For elution buffers, the 100 mM citric acid used here is a good starting point. Other possible elution buffers are 500 mM acetic acid, pH 2.6, 100 mM glycine HCl, pH 2.8, and 12 mM HCl, pH 1.9.

Figure 3 compares the elution of a Herceptin originator with acetic acid and citric acid. Very similar peak shape and area were observed, although peaks were slightly sharper using citric acid. In the case of this Herceptin originator, no material was seen in the flow-through, which was not surprising since this represented the marketed product and was devoid of host-cell proteins. In the chromatograms shown, the flow rate was set at 1 mL/min. The monolithic nature of the support, characterized by convective instead of diffusive mass transfer, allowed for near flow-rate independence and, hence, high-throughput separations. This is highly desirable in mAb titer determination, which typically requires the processing of a wide range of samples. The maximum flow rate that can be applied on the column is 2 mL/min, which allows fast, sub-2-minute separations.

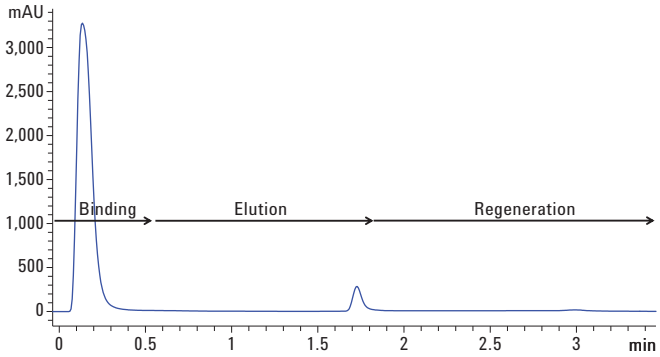

Figure 2. UV 280 nm Protein A chromatogram showing the supernatant of a trastuzumab-producing CHO clone. Injection volume was 50 µL. Peak width at half height was 0.10 minutes for the unbound material and 0.06 minutes for the retained mAb.

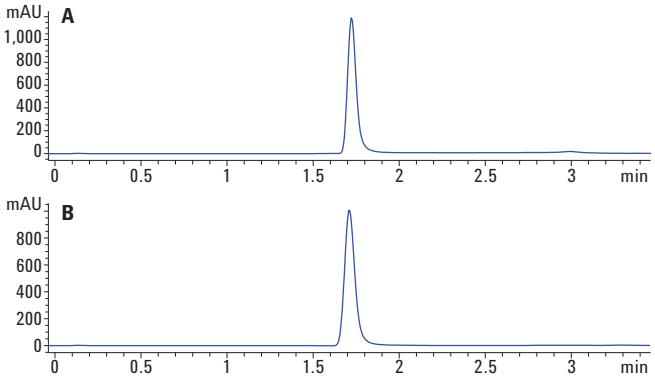

Figure 3. UV 280 nm Protein A chromatogram of Herceptin originator diluted in 50 mM Na-phosphate, pH 7.4, to 0.5 mg/mL (50-µL injection, 25-µg column load). Elution was achieved using citric acid (A) and acetic acid (B). Peak width at half height is 0.057 and 0.067 minutes for citric acid and acetic acid, respectively.

Precision, linearity, carryover, and injection size

Precision is critically important in the determination of the mAb titer. Table 1 shows the peak area and retention time repeatability that can typically be expected upon injecting a Herceptin originator 10 times. Chromatograms are shown in Figure 4. More than acceptable relative standard deviation (RSD) values were obtained for both citric acid and acetic acid as elution buffers. Carryover was simultaneously assessed by injecting a buffer blank after the mAb injection sequence (Figure 5). At a 10-fold column load of 5 µg, carryover appeared to be nonexistent, which can again be attributed to the use of a monolithic support. Carryover at 1% levels became apparent upon a single load of 500 µg of mAb onto the column. This represents the maximum column load and is one typically not encountered in real-life experiments. It is worth noting that carryover was eliminated after the injection of a second buffer blank.

Table 1. Retention time and peak area RSD values obtained for the 10-fold analysis of a Herceptin originator at 0.5 mg/mL (5-µL injection volume).

	Acetic acid		Citric acid	
	Peak area	**RT (min)**	**Peak area**	**RT (min)**
1	361	1.669	383	1.666
2	362	1.668	372	1.666
3	373	1.668	365	1.665
4	365	1.669	389	1.667
5	370	1.669	383	1.666
6	373	1.669	378	1.666
7	367	1.671	379	1.668
8	365	1.668	377	1.666
9	366	1.670	376	1.667
10	360	1.670	377	1.667
Mean	366	1.669	378	1.667
S	4.64	0.001	6.52	0.001
% RSD	**1.27**	**0.06**	**1.73**	**0.06**

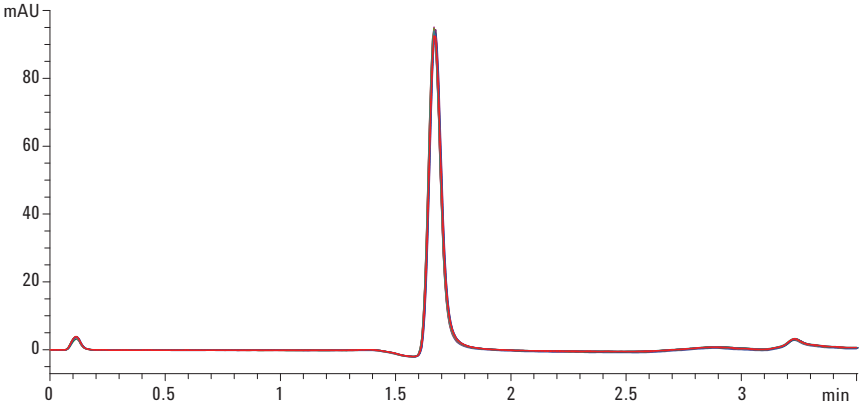

Figure 4. Replicate (n = 10) UV 280 nm Protein A chromatograms of a Herceptin originator diluted in 50 mM Na phosphate, pH 7.4, to 0.5 mg/mL (injection volume 5 µL). Elution was achieved using acetic acid.

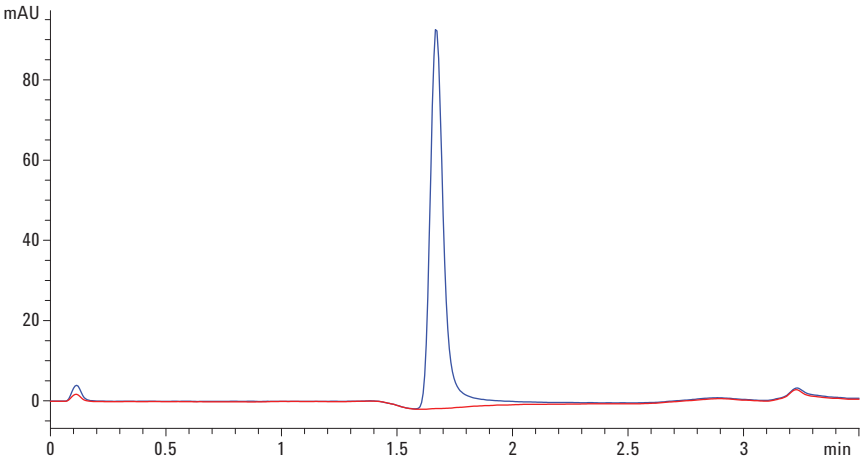

Figure 5. UV 280 nm Protein A chromatograms of a Herceptin originator diluted in 50 mM Na phosphate, pH 7.4, to 0.5 mg/mL, and a blank buffer analyzed after a sequence of 10 Herceptin injections. Elution was achieved using acetic acid, and injection volumes were 5 µL.

The limit of detection (LOD) was around column loads of 0.5 µg. This put some demands on injection volume. If samples have low mAb levels, high volume injections are required. Figure 6 shows the linearity obtained when increasing the injection volume from 5 to 50 µL for a 1 mg/mL Herceptin originator. With the knowledge that 50 µL injections are perfectly feasible and that the lowest detectable amount on-column is 0.5 µg, samples with mAb concentrations at 10 µg/mL are within reach.

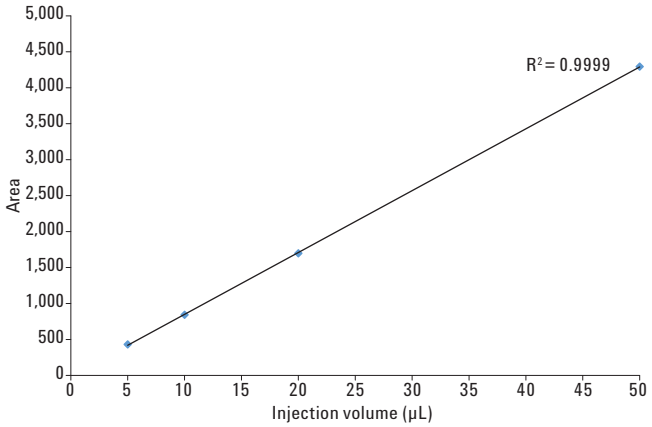

Figure 6. Linearity obtained by increasing the injection volume of a Herceptin originator (0.5 mg/mL) from 5 to 50 µL.

In mAb titer determination, it is important to be able to assess absolute mAb concentrations. These can be found by linking the peak areas measured in cell-culture supernatants to an external calibration curve constructed by diluting a mAb standard. For the Herceptin biosimilar project, this standard was found in the originator product, which was accurately formulated at 21 mg/mL. The calibration curves of a dilution series of Herceptin originators using citric acid and acetic acid as elution buffers are shown in Figure 7. The corresponding chromatograms are shown in Figure 8. In both cases, linearity was excellent, between 0.02 mg/mL and 2 mg/mL, which is the typical mAb titer range in CHO cells.

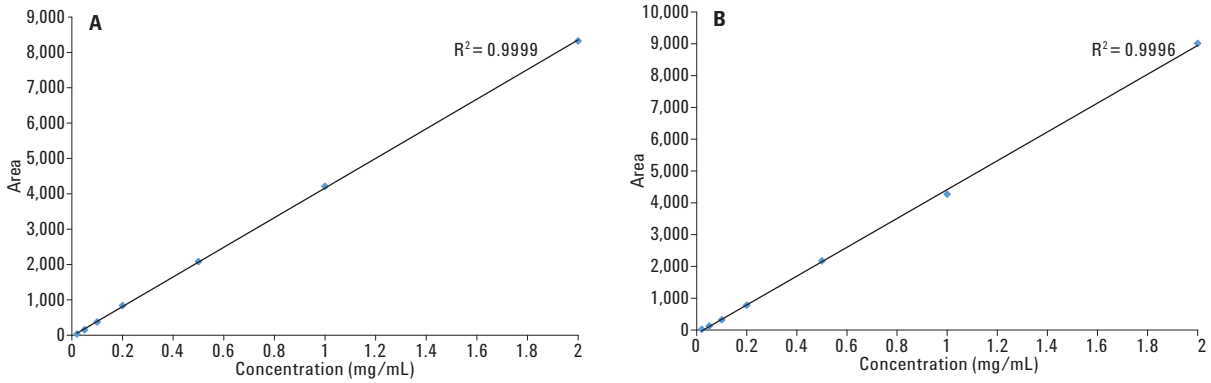

Figure 7. Herceptin Protein A calibration curve (0.02 to 2 mg/mL) using citric acid (A) and acetic acid (B) as elution buffer.

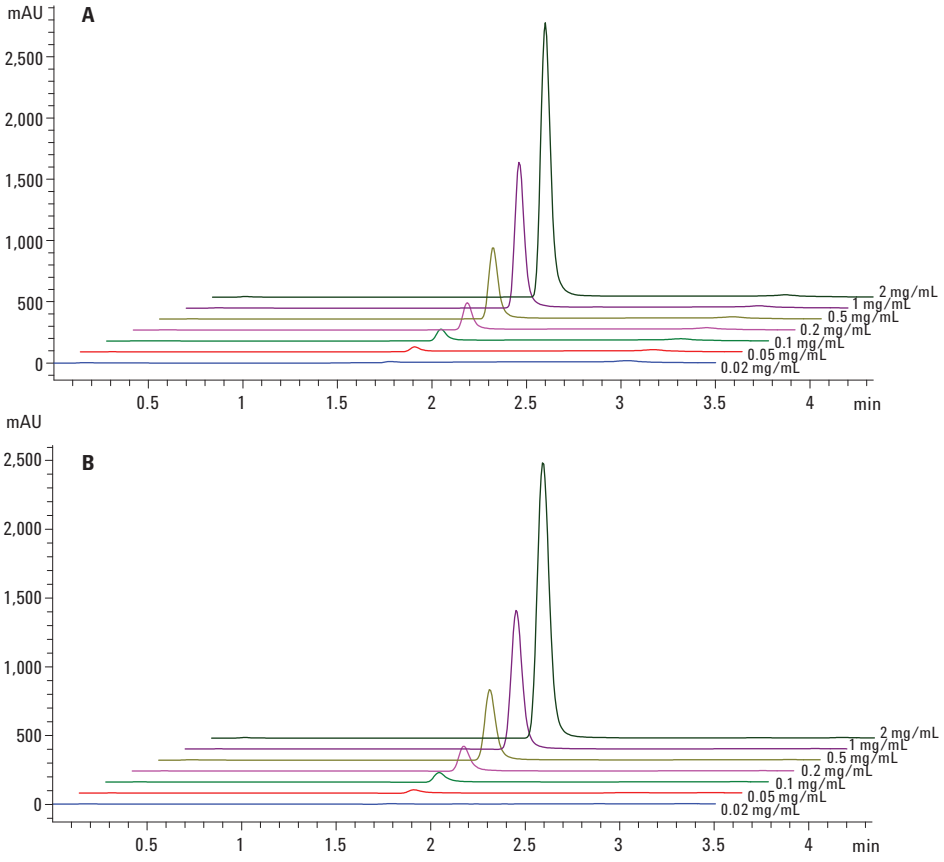

Figure 8. Overlaid UV 280 nm Protein A chromatograms of Herceptin calibration points using citric acid (A) and acetic acid (B) as elution buffer.

Application in mAb titer determination

The method possesses all the characteristics for the determination of mAb titer in cell-culture supernatants. It is fast, precise, and linear in the expected mAb concentration range and does not suffer from carryover. To illustrate this, nine trastuzumab-producing clones, generated in the framework of a Herceptin biosimilar development program, were analyzed using the Bio-Monolith Protein A column to determine absolute mAb concentrations. Chromatograms are displayed in Figure 9, and Table 2 reports the obtained mAb titers using both citric acid and acetic acid as elution buffer. Very consistent data were generated using both elution buffers. These results allow clear decisions to be made early in the development of mAbs. High-producing clones can be readily selected and subjected to further development.

Table 2. Comparison between the absolute mAb concentrations in the different trastuzumab CHO clones obtained using either citric acid or acetic acid as elution buffer.

CHO clone	Concentration (mg/mL) acetic acid	Concentration (mg/mL) citric acid
3	0.251	0.210
8	0.296	0.256
9	**0.492**	**0.494**
10	**0.728**	**0.757**
24	0.256	0.262
25	0.133	0.098
26	0.126	0.090
28	0.204	0.173
32	0.178	0.144

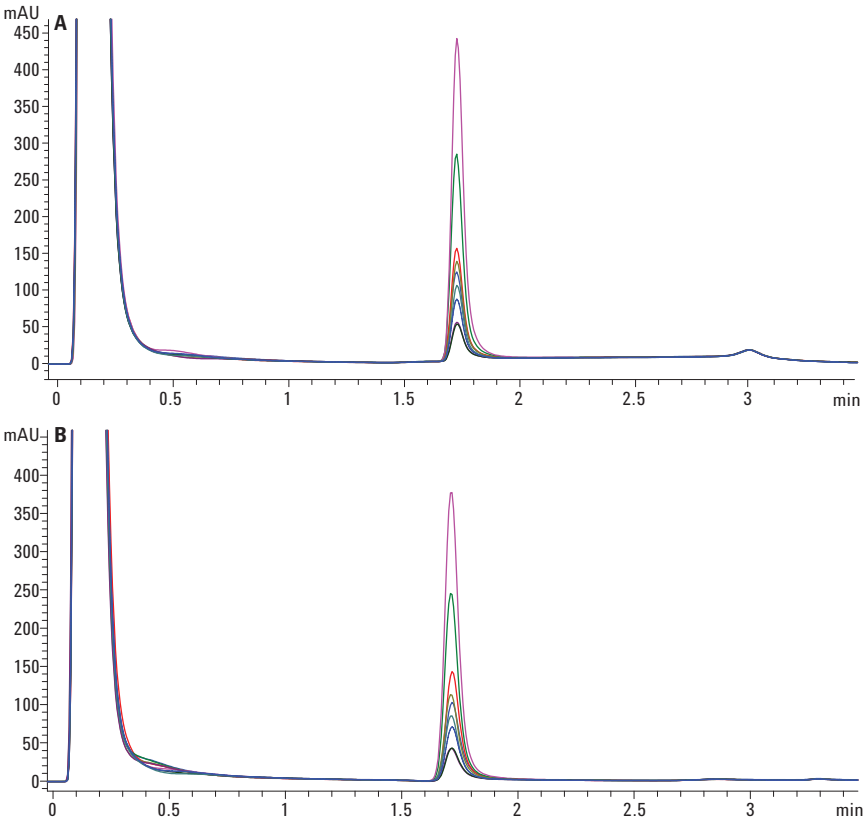

Figure 9. Overlaid UV 280 nm Protein A chromatograms of nine trastuzumab-producing CHO clones using citric acid (A) and acetic acid (B) as elution buffer.

Maximizing column lifetime

Column regeneration

A major benefit of using a monolithic disk is that the presence of channels instead of pores decreases the likelihood of column clogging when injecting cell-culture samples. This increases robustness and reduces cleaning efforts. Column contamination can be reduced by running a blank gradient injection after every 30 to 50 samples.

If column deterioration is observed (tailing or broad peaks), the following cleaning procedure is recommended. Column regeneration is the first step. If performance is still suboptimal, the clean-in-place procedure can be used, which will reduce the amount of Protein A available.

Column regeneration

1. Wash with 2 mL (20 column volumes (CV)) of 100 mM phosphate buffer + 1 M NaCl, pH 7 to 8, at 0.5 to 1.0 mL/min.
2. Wash with 2 mL (20 CV) of low-pH solution (such as elution buffer).
3. Re-equilibrate with binding buffer.

Clean-in-place

1. Wash with 1 to 2 mL (10 to 20 CV) of 0.1 M NaOH (reverse flow direction) at 0.2 to 0.5 mL/min.
2. Wash with 1 to 2 mL (10 to 20 CV) of DI water at 0.5 to 1.0 mL/min.
3. Wash with 1 to 2 mL (10 to 20 CV) of concentrated buffer (0.1 to 0.5 M) to restore normal pH (7.0 to 7.4).
4. Re-equilibrate with 5 mL (50 CV) of binding buffer.

Conclusions

The Agilent Bio-Monolith Protein A column is an ideal tool for the determination of mAb titer in cell-culture supernatants. Several method parameters can be optimized for the best method on the Protein A column, including binding and elution buffers and injection size (volume and concentration). As demonstrated here, the column delivers high-throughput, precise, accurate, linear, and robust separations in the concentration range encountered in real-life situations, and allows clear decisions to be made towards clone selection and cell-culture optimization.

For More Information

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