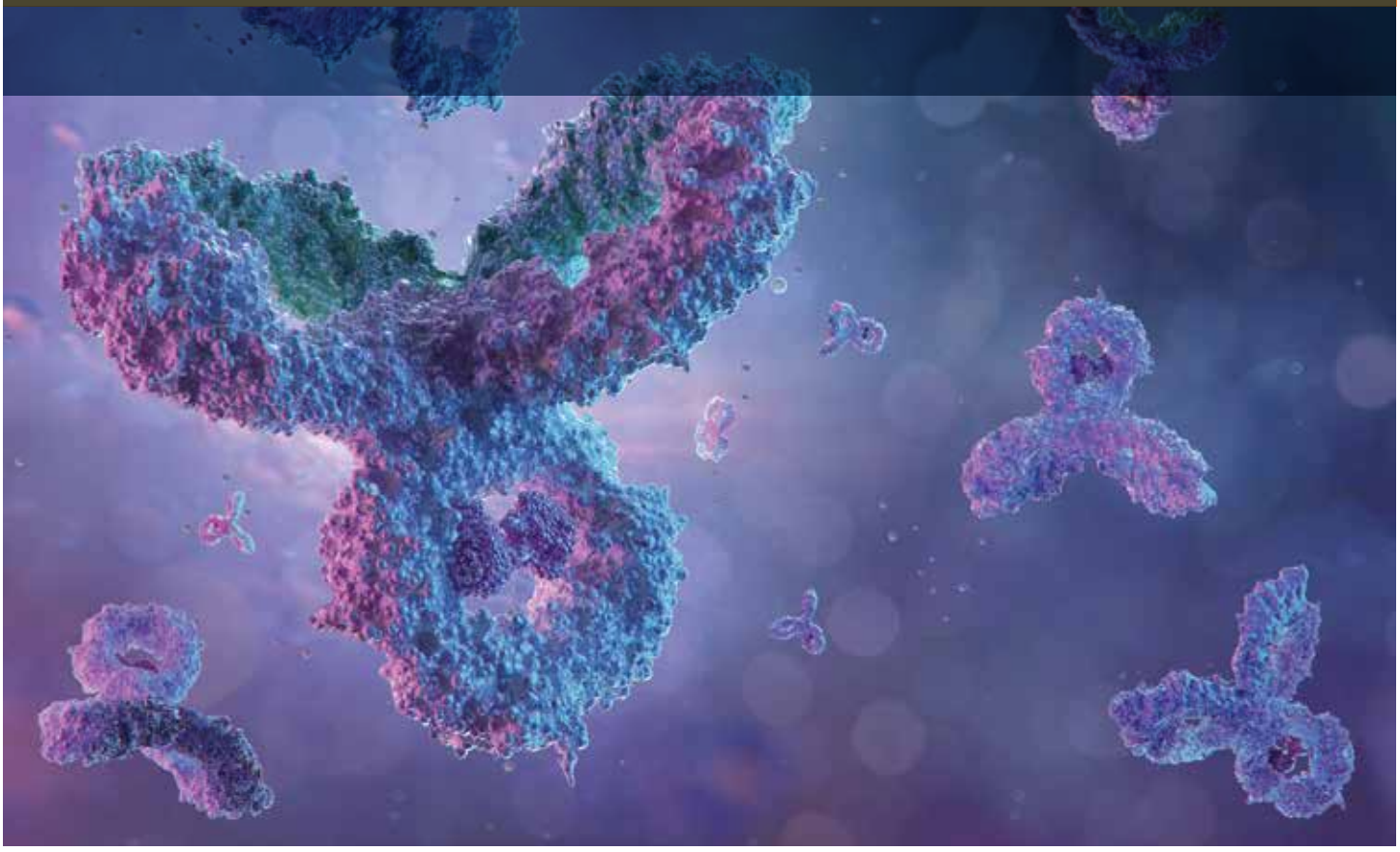
Agilent

Trusted Answers

Agilent Biocolumns

Titer Determination

Application Compendium



Contents

Titer Determination

Background

In biotherapeutic manufacture, titer determination is the measurement of the concentration of the target protein in the fermentation broth. There are two notable occasions when accurate titer determination is required. The first is during the clone selection process, selecting only those transfected clones that provide sufficient amounts of the target protein, since not all clones will be equally effective. The second is during scale up of the fermentation process to monitor the concentration of the target protein. Optimization of the cell culture conditions and determining the best harvest time relies on accurate titer determination.

For monoclonal antibodies, one of the most effective ways of titer determination is to use affinity chromatography. By absorbing the IgG molecule onto a Protein A or Protein G affinity chromatography column, the remaining impurities and byproducts from the fermentation broth can be removed. Elution of the purified monoclonal antibody and quantification by comparing the peak area to a calibration curve allows rapid measurement of the protein concentration.

Employing a monolithic column helps to eliminate the risk of clogging from cell culture debris and provides rapid (sub 1 minute) results.

These columns may also be used for purifying sufficient material for subsequent CQA analysis by another complementary technique, such as Aggregate Analysis or Charge Variant Analysis, and can easily be combined into a 2D workflow.

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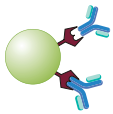

Titer Determination

Affinity chromatography

Ideal for mAb titer determination during process development

BioMonolith Protein A and BioMonolith Protein G

Native Protein A or Protein G

Attribute	Advantage
Fast separation	Shorter method development times
High binding capacity	Greater application flexibility
Minimal clogging	Less system down time

Getting Started

In selecting an affinity column for titer determination, the first point to consider is the target protein to be purified or analyzed. Different immunoglobulins (IgG 1, 2, etc.) from different sources (human, mouse, etc.) have different affinities for Protein A versus Protein G. For example, Human IgG3 binds tightly to Protein G, but not at all to Protein A. Guidelines on selecting a Protein A or a Protein G column, as well as suggested mobile phases and a sample method, can be found in the "How- to-Guide" that follows. Mobile phase B, the eluting buffer in an affinity experiment, is one method parameter that can be optimized.

Affinity Chromatography for Titer Determination: A "How-To" Guide

Introduction

Affinity chromatography is a powerful technique, which takes advantage of highly specific molecular interactions, frequently between specific proteins (e.g. antigen/antibody). Agilent offers several specialty affinity products, including monolithic Protein A and Protein G columns for the isolation and quantitation of monoclonal antibodies (mAbs).

In recent years, 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 make up for better targeting of disease agents. During the development of these antibodies, Protein A and G analytical affinity columns are used to determine antibody titer or concentration from various cell culture supernatants, to select the high-yield clone.

Column Selection

Protein A and G columns have high affinity for antibodies, and so they bind only to antibodies in cell-culture supernatants. However, they have different selectivity. For example, Agilent Bio-Monolith protein A columns have high affinity for human subclasses IgG1, IgG2, and IgG3 and no affinity for IgG3, whereas Agilent Bio-Monolith Protein G columns have high affinity for human subclasses IgG1, IgG2, IgG3, and IgG4. IgG 1 is the most commonly used as biotherapeutic for its stability (long half life), abundance in serum, and less aggregation formation. IgG 3 is often not used because it's prone to aggregate and less stable. Conversely, the Protein G column has no affinity for human subclass monoclonal antibodies such as IgA and IgD, but the Protein A column binds to both these antibodies (Table 1). Together, these columns complement each other, so Protein G has affinity for mAbs that do not bind to Protein A and vice versa (Figure 1). They therefore and enable titer determination of the various mAb subclasses and fragments currently in development as biotherapeutics.

Table 1. Binding affinity of Protein A and G to different human and mouse IgG subclasses [(1), (2)].

Antibody	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	Protein A	Protein G
Human Fab	+	+
Human F(ab')2	+	+
Human scFv	+	-
Human Fc	++	++
Human K	-	-
Human λ	-	-

Key code for relative affinity of Protein A & G for respective antibodies:
++++ = Strong affinity
+++ = Moderate affinity
++ = Weak affinity
+ = Slight affinity
- = No affinity

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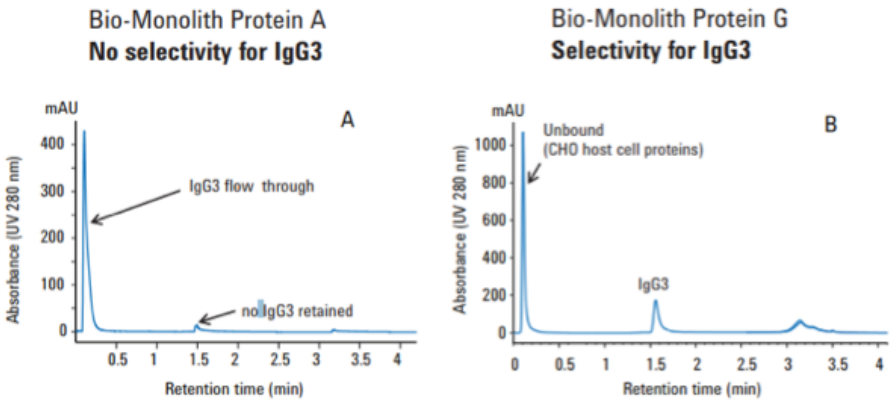


The Bio-Monolith Protein A has no affinity for IgG3 but the Bio-Monolith Protein G does.

Figure 1. From 5991-6087EN or application note 5991-6094EN.

HPLC system considerations - Protect your proteins during analysis

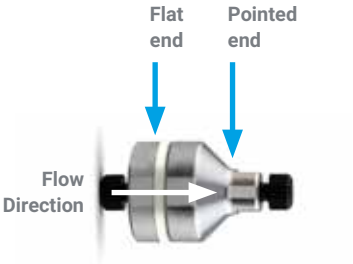
These monolithic columns are compatible with HPLC and UHPLC systems, however an ideal choice for this type of analysis is the Agilent 1260 Infinity II bio-inert LC. It handles challenging solvent conditions with ease, such as extreme pH values of pH 1 to pH 13, and buffers with high salt concentrations. Corrosion resistant titanium in the solvent delivery system and metal-free materials in the sample flowpath create an extremely robust instrument.

Sample Preparation

Sample preparation for affinity chromatography is similar to that for any protein analysis for HPLC. With some minor sample preparation being required before injection to optimize column performance and extend column lifetimes.

- Centrifuge or filter samples to remove host cell debris and particulates from the supernatant or lysate, to prevent blockage of the columns.
- For serum/plasma samples, it is also best to remove lipids from sample. Lipids will strongly bind to the columns and can cause fouling of columns and instruments.

The Bio-Monolith protein A column has a white band and Bio-Monolith Protein G has a yellow band around the column.



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Conditions

Sample injection

For samples containing 1 to 5 mg/mL of mAb injection volumes of 1 to 5 µL are recommended. Samples can be dissolved in H_2O or mobile phase A. Up to 50 µL or up to 400 to 500 mg mAb/injection can be injected on the columns.

Flow rate

Columns can be run at 1.0 to 3.0 mL/min for high speed.

Temperature

For successful separations, 25 °C is a typical temperature. Columns can be operated from 4 to 40 °C.

Detection

Detection by UV at 280 nm being is recommended, at this wavelength absorbance is due to amino acids with aromatic or more conjugated side chains.

Mobile phase

Mobile phase A Binding and washing buffer

Mobile phase A is the binding buffer: 50 mM sodium phosphate buffer, pH 7.4.

Binding/washing buffers should be freshly made. In addition, filtration of buffers through a 0.22 or 0.45 µm membrane is recommended to reduce buffer impurities that build up on the frits inside the column. This filtration will help to prevent column blockage.

Mobile phase B eluting buffer

Bio-Monolith Protein A and G columns are compatible with many low pH buffers that are used for mAb elution, see Table 2 for details. Citric acid, glycine, HCl, and acetate acid, are commonly used. If a low concentration sample is used and baseline noise and artifact peaks are of concern, HCl can be used as an eluent due to its low refractive index.

Note: Commonly, elution buffers for affinity columns have a refractive index (RI) that is very different from binding/washing buffers; therefore, baseline noise and an artifact peak could appear when the eluents start flowing. This peak could interfere with the quantitation of low concentration samples. To minimize this effect, high-quality chemicals are recommended to be used and blank runs should be included to establish the artifact peak. Blank runs can be used for baseline subtraction if desired.

Table 2. Compatible eluting buffers.

Column	Eluting Buffer	Concentration	pH
Bio-Monolith Protein A and rProtein A	Citric acid	0.1 M	2.5 to 3.0
	Glycine	0.1 M	2.5 to 3.0
	Acetic acid	5–20 %	
Bio-Monolith Protein G	Citric acid	0.1 M	2.5 to 3.0
	Glycine	0.1 M	2.5 to 3.0
	Acetic acid	5–20 %	

Fast separation protocols

The Agilent Bio-Monolith rProtein A (recombinant protein A) analytical column is the latest addition to the Bio-Monolith and affinity chromatography family. The column enables high-speed analysis of monoclonal antibody (mAb) titer and small-scale purification, and can easily be integrated into other analytical workflows, such as 2D-LC. This study tests the recombinant protein A column at the maximum flow rate and performs the bridging study against the native protein A column. A chromatography bind/elute method for mAb titering is demonstrated, which delivers an ultrafast run time (1 minute) suitable for high-throughput applications such as clonal selection, process development, and optimization.

High-throughput mAb titer analysis

Experimental

Chemicals and reagents

All chemicals and reagents were HPLC grade or higher and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Water was purified using a Milli-Q A10 (Millipore).

Sample

The sample was a crude Chinese Hamster Ovary (CHO) cell culture supernatant collected from a bioreactor that contained 1 mg/mL of recombinant IgG monoclonal antibody.

Instrumentation

Agilent 1260 Infinity II bio-inert LC comprising:

- 1260 Infinity II bio-inert pump (G5654A)
- 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option 100)
- 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option 019)
- 1260 Infinity II variable wavelength detector (G7114A)

HPLC Method Conditions

Parameter	Value	
Column:	Agilent Bio-Monolith rProtein A, 4.95 × 5.2 mm (p/n 5190-6903)	
Binding Buffer (Eluent A):	50 mM sodium phosphate, pH 7.4	
Binding Buffer (Eluent B):	100 mM citric acid, pH 2.6	
Gradient Profile:	Time (min) 0.0 to 0.2 0.3 to 0.65 0.66 to 0.90 (0.1 min postrun)	% B 0 (binding) 100 (elution) 0 (reconditioning)
Flow rate:	3 mL/min	
Column Temperature:	25 °C	
Detection:	UV, 280 nm	
Injection volume:	4 µL (10 µg loading)	

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Results and Discussion

High-throughput mAb titer analysis

With the high-throughput method, high-speed mAb titering with a 1-minute chromatography run time was demonstrated (Figure 1). The retention time of the purified (bound/eluted) mAb was approximately 0.61 minutes, well separated from the impurities peak at ~0.05 minutes containing host cell proteins from the CHO cell culture supernatant. In Figure 1, repeated injection of crude supernatant spiked with mAb showed consistent and robust performance of 60 samples/hour throughput with backpressure leveling at 125 bar.

Throughout the study, there was no noticeable change in peak shape, retention time, and backpressure. Figure 2 showed the chromatograms of different sample loading amounts. A calibration curve was then generated by plotting peak area versus injection quantity (Figure 3). Results indicated excellent linearity response (R2 = 0.9993), as shown in the calibration curve, and accurate measurement of mAb quantity from two separate sets of samples.

These data demonstrated the feasibility of accurate mAb titer measurement using this fast analysis method.

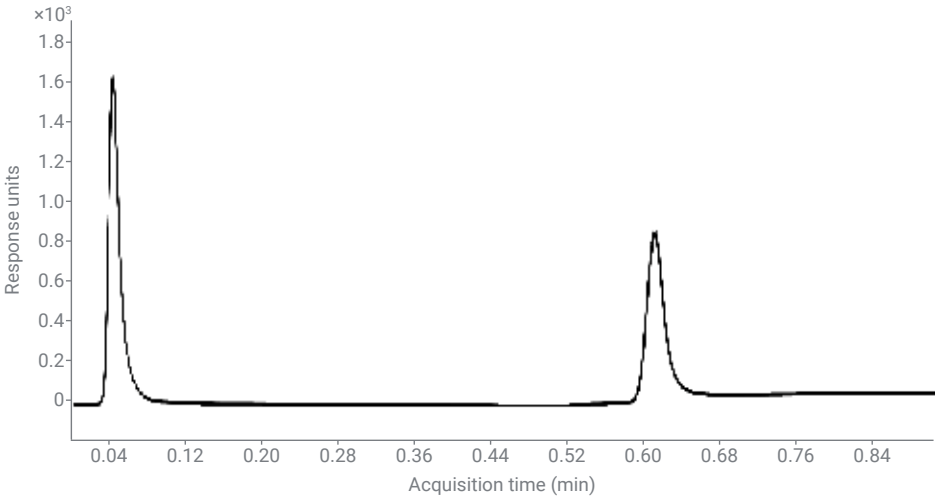

Figure 1. Agilent Bio-Monolith rProtein A column: Overlaid chromatograms of 60 consecutive injections. First peak indicates host cell protein impurities in culture supernatant; second peak is purified mAb.

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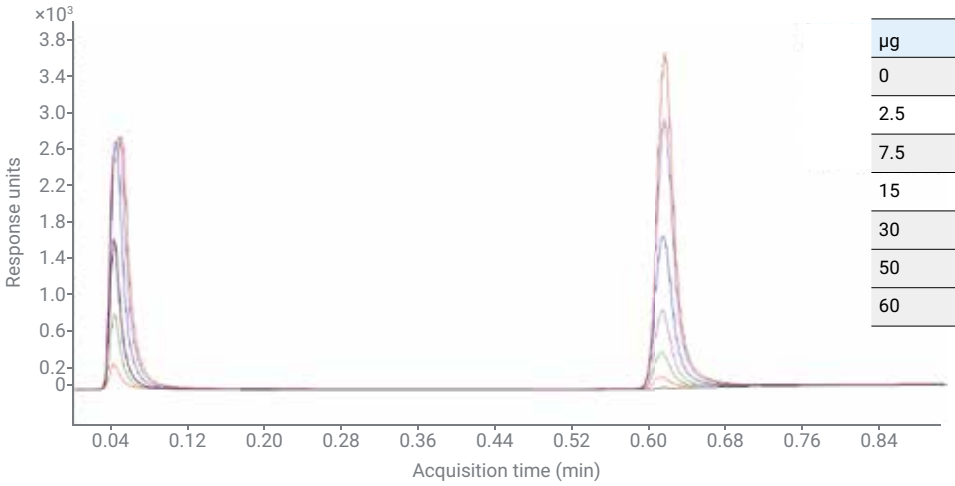
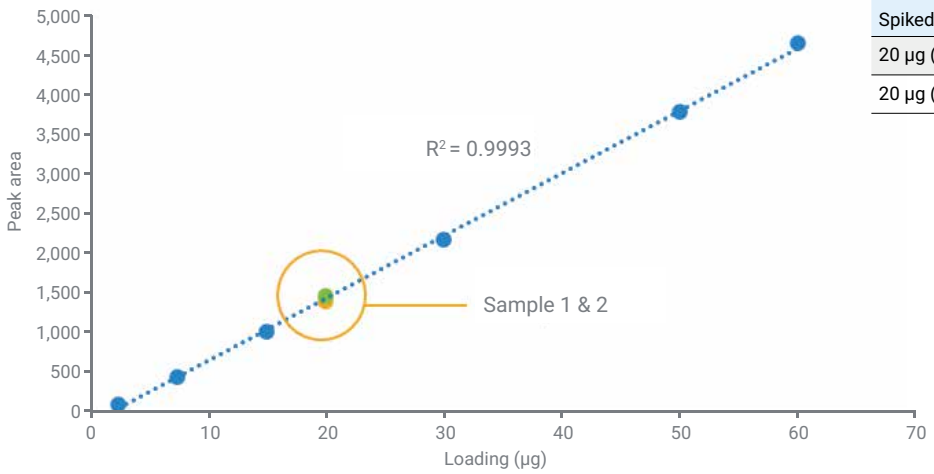


µg	Area	Height
0	0	0
2.5	105.8	97.3
7.5	453.3	365.0
15	1019.1	807.5
30	2186.5	1627.8
50	3811.8	2885.6
60	4696.1	3604.6

Figure 2. Agilent Bio-Monolith rProtein A column: Calibration curve. Overlay chromatograms of increasing sample loading amount for calibration curve generation.



Spiked Sample	Measured	% Deviation
20 µg (sample #1)	19.53 µg	-2.35%
20 µg (sample #2)	20.51 µg	2.53%

Figure 3. Agilent Bio-Monolith rProtein A column: Standard curve linearity response and % deviation.

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Bridging Study

In the bridging study, performance characteristics such as retention time, linearity and deviation of the standard curve, sample carryover, and recovery showed no detectable difference between the native and the recombinant columns. This work serves to give confidence to those who are transitioning from the native protein A column to the rProtein A column.

Experimental

Chemicals and reagents

All chemicals and reagents were HPLC grade or higher and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Water was purified using a Milli-Q A10 (Millipore).

Sample

The samples were crude Chinese Hamster Ovary (CHO) cell culture supernatant collected from a bioreactor that contained 1.5 mg/mL of recombinant IgG monoclonal antibody and purified recombinant IgG monoclonal antibody at the same concentration.

Instrumentation

The Agilent 1290 Infinity II Bio LC system consists of the following components:

- 1290 Infinity II bio high-speed pump (G7132A)
- 1290 Infinity II bio multisampler (G7137A)
- 1290 Infinity II multicolumn thermostat with bio heat exchanger (G7116B)
- 1290 Infinity II diode array detector (G7117B) and variable wavelength detector with respective bio flow cell

HPLC Method Conditions

Parameter	Value	
Column:	Agilent Bio-Monolith rProtein A, 4.95 × 5.2 mm (p/n 5190-6903) Agilent Bio-Monolith Protein A, 4.95 × 5.2 mm (p/n 5069-3639)	
Binding Buffer (Eluent A):	50 mM sodium phosphate, pH 7.4	
Binding Buffer (Eluent B):	100 mM citric acid, pH 2.6	
Gradient Profile:	Time (min) 0.0 to 0.5 0.6 to 2.6 2.7 to 4.0	% B 0 (binding) 100 (elution) 0 (reconditioning)
Flow rate:	1.5 mL/min	
Column Temperature:	25 °C	
Detection:	UV, 280 nm	
Injection volume:	5 to 50 µL (25 µg loading)	

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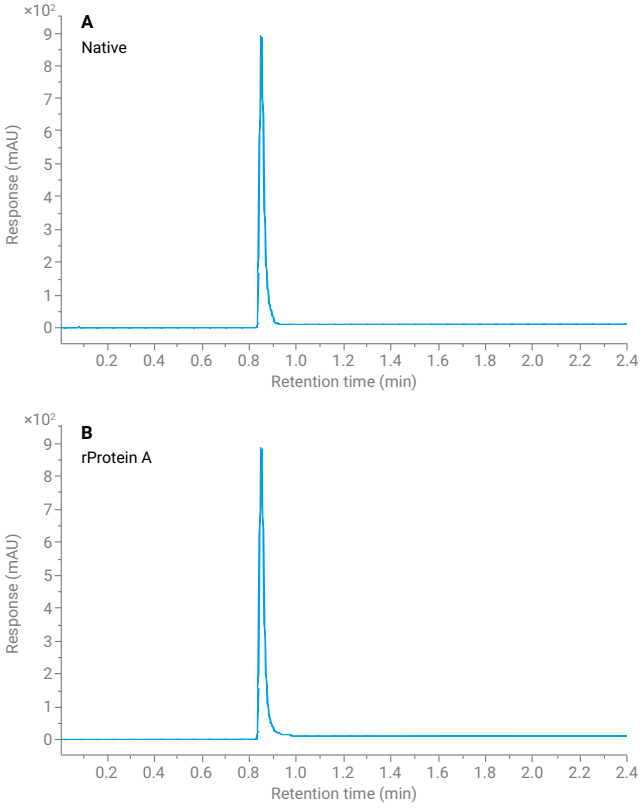
Results and Discussion

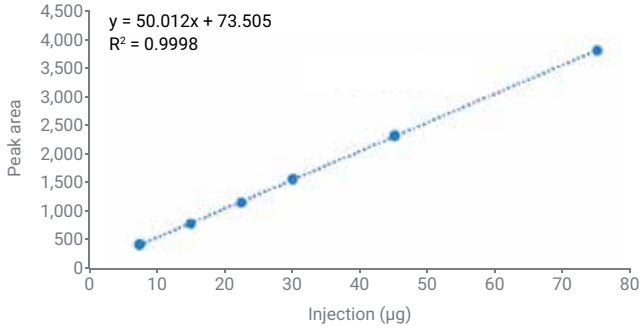
Bridging study

Performance of the two Bio-Monolith protein A columns were tested under the same conditions. All characteristics showed little or no difference between the native and the rProtein A columns, including retention time and peak shape of the purified mAb (Figure 4), linearity response of the standard curve and spiked sample recovery (Figures 5 and 6), and sample carryover (Figure 7).



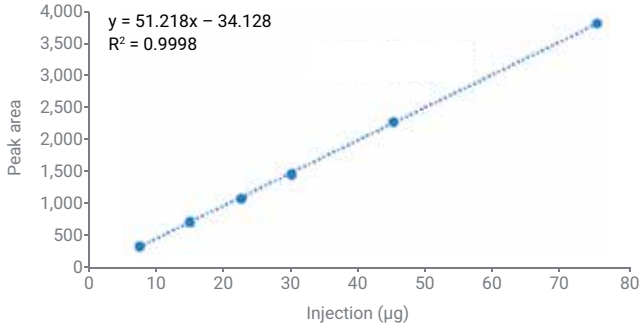
	Native	rProtein A
Retention Time (Min)	0.850 ± 0.001	0.850 ± 0.001
Peak Height	880.9 ± 8.1	881.8 ± 5.3

Figure 4. Chromatogram and mAb peak result comparison between Native and rProtein A columns.



Spiked Sample	Measured	% Deviation
25 µg (pure)	25.25 µg	0.99%
25 µg (in sup.)	26.09 µg	4.35%

Figure 5. rProtein A column: Linearity response



Spiked Sample	Measured	% Deviation
25 µg (pure)	24.94 µg	-0.23%
25 µg (in sup.)	25.78 µg	3.12%

Figure 6. Native Protein A column: Linearity response

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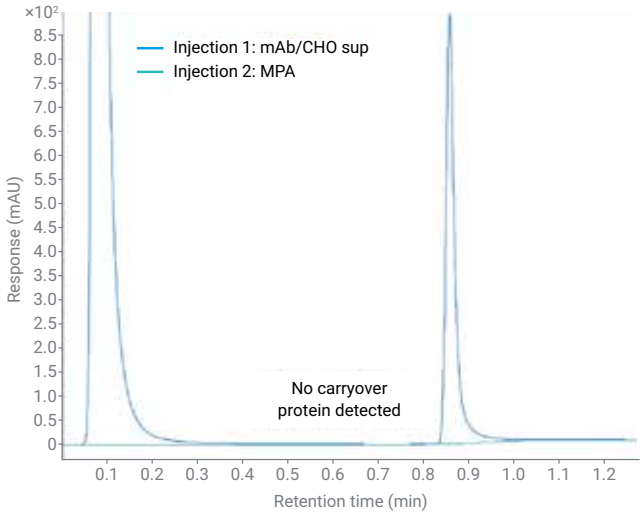

Figure 7. rProtein A column: Carryover Analysis. Subsequent injection of binding buffer (MPA) showed no detectable protein carryover.

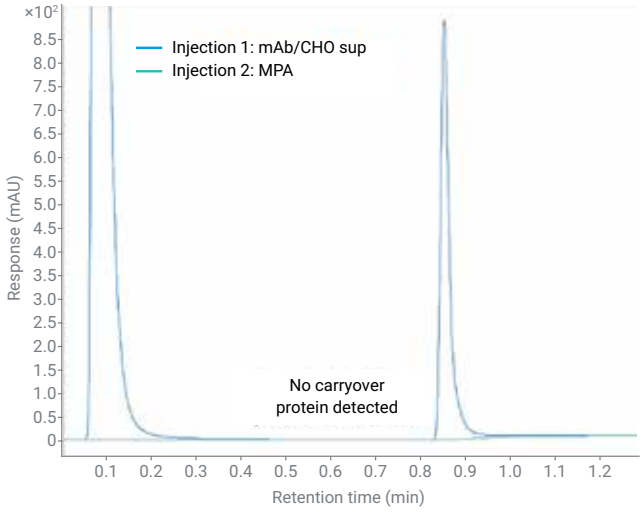

Figure 8. Native Protein A column: Carryover analysis. Subsequent injection of binding buffer (MPA) showed no detectable protein carryover.

Recovery analysis

In addition to comparing the recovery between native and the rProtein A columns, two non-Agilent rProtein A columns were included in this study. Flow rate was adjusted to 2 mL/min to accommodate a non-Aglient column's operating flow rate. Additional mAb samples were included:

- Agilent-NISTmAb (part number 5191-5744)
- Sigma SiLu mAb from Sigma-Aldrich (SiLu Lite, part number MSQC4)

Baseline area under the curve (AUC) of the mAb peak was obtained by injecting purified mAb sample, which was diluted with mobile phase B, without a column (with a union). The column was applied and AUC of eluted mAb was obtained. The same amount of mAb sample as baseline AUC was used.

Recovery % = (AUC of eluted mAb/Baseline AUC) × 100

HPLC Method Conditions

Parameter	Value	
Column:	Agilent Bio-Monolith rProtein A, 4.95 × 5.2 mm (p/n 5190-6903) Agilent Bio-Monolith Protein A, 4.95 × 5.2 mm (p/n 5069-3639)	
Binding Buffer (Eluent A):	50 mM sodium phosphate, pH 7.4	
Binding Buffer (Eluent B):	100 mM citric acid, pH 2.6	
Gradient Profile:	Time (min) 0.0 to 0.4 0.5 to 1.3 1.31 to 4.0	% B 0 (binding) 100 (elution) 0 (reconditioning)
Flow rate:	2 mL/min	
Column Temperature:	25 °C	
Detection:	UV, 280 nm	
Injection volume:	4 µL (10 µg loading)	

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Result analysis

The average recovery of the rProtein A column was 1% lower than the native protein A column but still showed better recovery than the two non-Agilent columns. While the nProtein A column took the slight lead in recovery, it was the rProtein A column that demonstrated the most robust recovery across the three mAb samples.

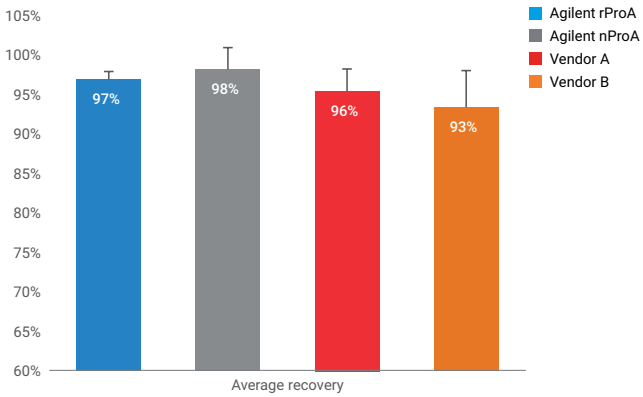

Figure 9. mAb recovery results comparison.

Conclusion

The bridging study between native column and the rProtein A column has demonstrated that rProtein A delivers a similar or equivalent performance to the native protein A column.

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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 30–50 samples.

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

Column regeneration

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

Clean-in-place

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

If the impurities are highly hydrophobic or lipidic, and are not easily removed from the column, 2-propanol (up to 30 %), or guanidine hydrochloride (up to 3 M) can be used to remove these impurities. After using these alternative cleaning solutions, follow steps 1 through 4.

WARNING: When you wash the column with these cleaning solutions, always decrease the flow rate on the column to avoid generation of high pressures that might exceed the maximum allowed pressure over the column.

Short-term storage

For storage, overnight or for a few days, the columns can be flushed with binding buffer, disconnected from the instrument, capped, and stored at 4 to 8 °C. Columns should be equilibrated before the first injection after short-term storage.

Long-term storage

If the column will not be in use for more than two days, it should be washed with at least 1 mL (10 CV) of DI water and afterwards flushed with at least 2 mL (20 CV) of 20 % ethanol with 20 mM Tris buffer, pH 7.4 at a flow rate of 0.2 to 0.5 mL/min. It should then be sealed with column end stops and stored at 4 to 8 °C (39 to 46 °F).

References

1. Richman, D. D., Cleveland, P. H., Oxman, M. N., and Johnson, K. M. 1982. *"The binding of 1. Staphylococci protein A by the sera of different animal species."* J. Immunol. 128: 2300–2305.
2. Frank, M. B. 1997. *"Antibody Binding to Protein A and Protein G beads".* 5. In: Frank, M. B., ed. Molecular Biology Protocols. Oklahoma City.

Ordering information

Part Number	Description
5190-6903	Bio-Monolith Recombinant Protein A, 4.95 x 5.2 mm
5069–3639	Bio-Monolith Protein A, 4.95 x 5.2 mm
5190–6900	Bio-Monolith Protein G, 4.95 x 5.2 mm

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Robust, Reliable, Recombinant Protein A Monolith Column for Antibody Titer Determination

Authors

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Andrew Coffey

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Abstract

Rapid screening of crude cell culture supernatant allows decisions to be made on the optimum time for harvest during the manufacture of biotherapeutic antibodies. There are several advantages to using a Bio-Monolith column with recombinant Protein A affinity ligand. First, the Bio-Monolith structure has wide through-pores that minimize the risk of clogging. Second, the use of recombinant Protein A provides the selectivity towards IgG that is associated with native Protein A, but with a higher ligand purity and a more robust structure. Finally, the column can also be used for small-scale purification so that other analytical techniques can be applied, particularly in the determination of critical quality attributes (CQAs).

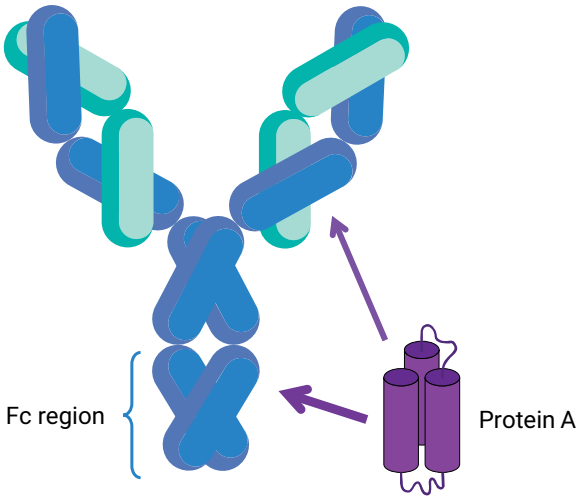

Figure 1. Protein A interaction with immunoglobulin G (IgG).

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Introduction

Native Protein A is a surface protein isolated from Staphylococcus aureus, which has a high binding affinity for the Fc region of many different types of immunoglobulin from different species. Native Protein A affinity chromatography has become the method of choice for the purification of monoclonal antibodies from crude cell culture supernatant. Recombinant Protein A can provide some extra benefits since it can be produced in a purer form and can be engineered to ensure that its immobilization onto a stationary phase creates the ideal orientation for optimum binding.

It is also helpful for improving column lifetime, which can otherwise be compromised due to the crude nature of cell culture supernatant. This is because it can withstand the harsh conditions required for column cleanup better than native Protein A columns.

This application note tests the lifetime of a new Agilent recombinant Protein A Bio-Monolith column.

Experimental

Reagents and chemicals

All chemicals and reagents were HPLC grade or higher and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Water was purified using a Milli-Q A10 (Millipore).

Sample

The sample was crude Chinese Hamster Ovary (CHO) cell culture supernatant collected from a bioreactor that contained 1 mg/mL of recombinant IgG monoclonal antibody.

Instrumentation

Agilent 1260 Infinity II bio-inert LC comprising:

- Agilent 1260 Infinity II bio-inert pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II variable wavelength detector (G7114A)

Conditions, HPLC

Parameter	Value	
Column:	Agilent Bio-Monolith rProtein A, 4.95 × 5.2 mm (p/n 5190-6903)	
Binding Buffer	Eluent A) 50 mM phosphate, pH 7.4	
Eluting Buffer	Eluent B) 100 mM citric acid, pH 2.6	
Cleanup Buffer	1) 1 M NaCl in 100 mM sodium phosphate, pH 7.4 2) 20% isopropanol in 50 mM sodium phosphate, pH 7.4	
Gradient:	Time (min) 0 to 0.5 0.6 to 1.8 1.9 to 4.0	% B 0 (binding) 100 (elution) 0 (reconditioning)
Flow rate:	1 mL/min	
Injection volume:	As required (1 to 20 µL)	
Column temp:	24 °C	
Detection:	UV at 280 nm	

Results and discussion

A crude cell culture supernatant solution containing much higher levels of host cell proteins than in the previous work[1] was chosen to investigate the robustness of Bio Monolith rProtein A columns.

A repetitive sequence involving step gradients for binding, elution, and column reconditioning was used and the results from every 250th injection are shown in Figures 2, 3, and 4. After 1,500 injections, a column regeneration step (see Method conditions) was introduced using a cleanup buffer, which was performed every 500 injections thereafter. As expected from such a challenging crude sample matrix, a gradual build-up of pressure was observed (Figure 5).

However, with regular cleanup, the column continued to provide consistent, reliable peak area and titer analysis during the 3,000 injections, as shown in Figures 6 and 7.

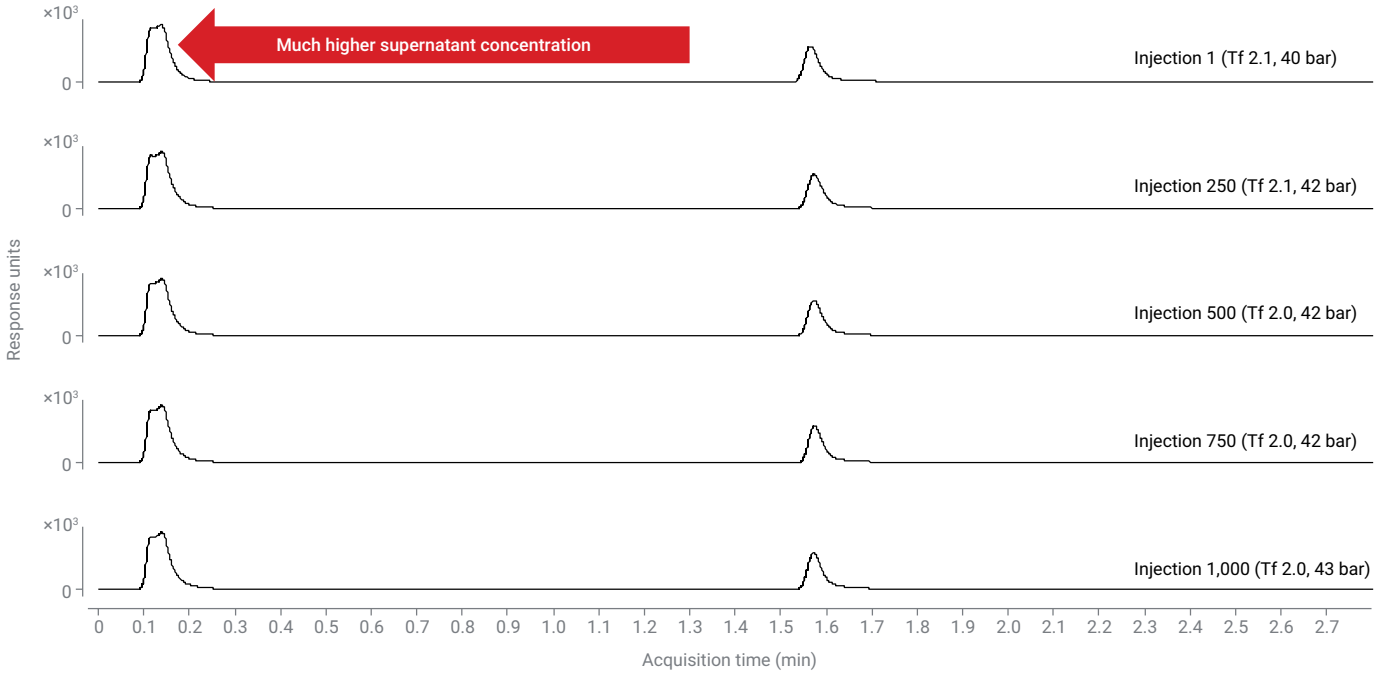

Figure 2. Agilent Bio-Monolith rProtein A column lifetime: Injections 1 to 1,000..

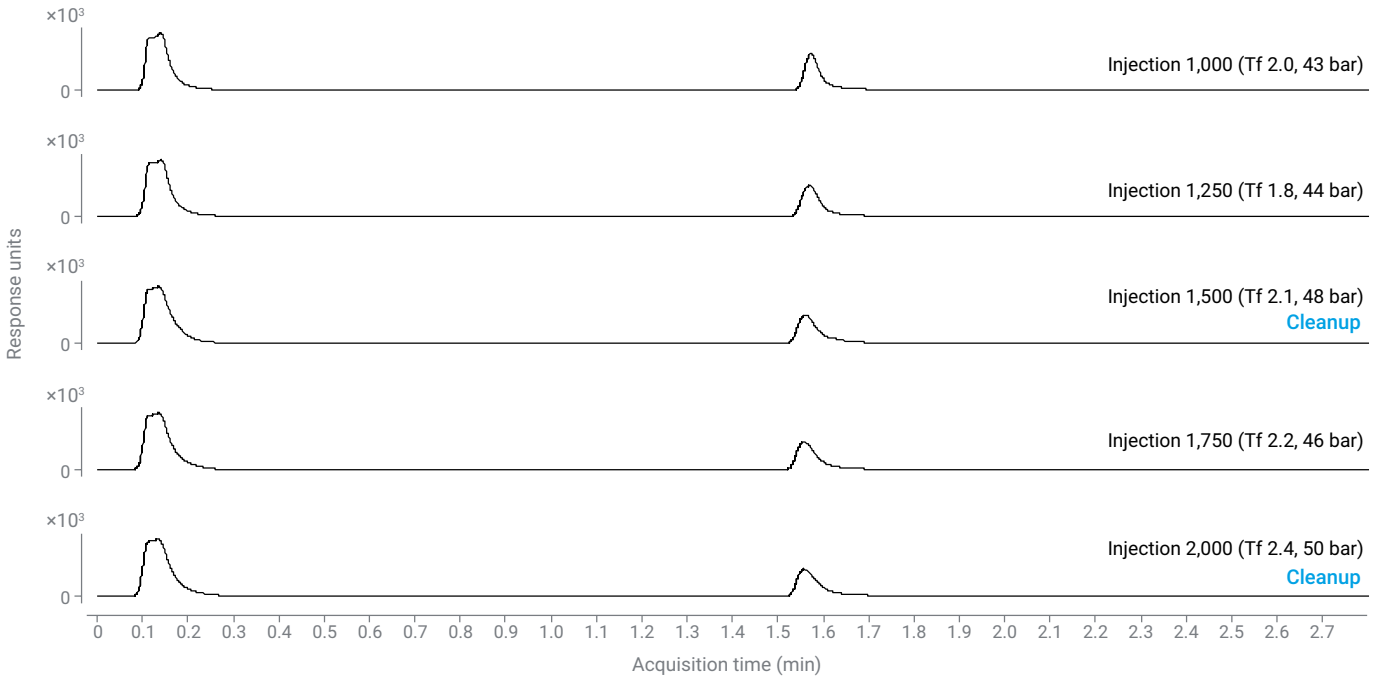

Figure 3. Agilent Bio-Monolith rProtein A column lifetime: Injections 1,000 to 2,000.

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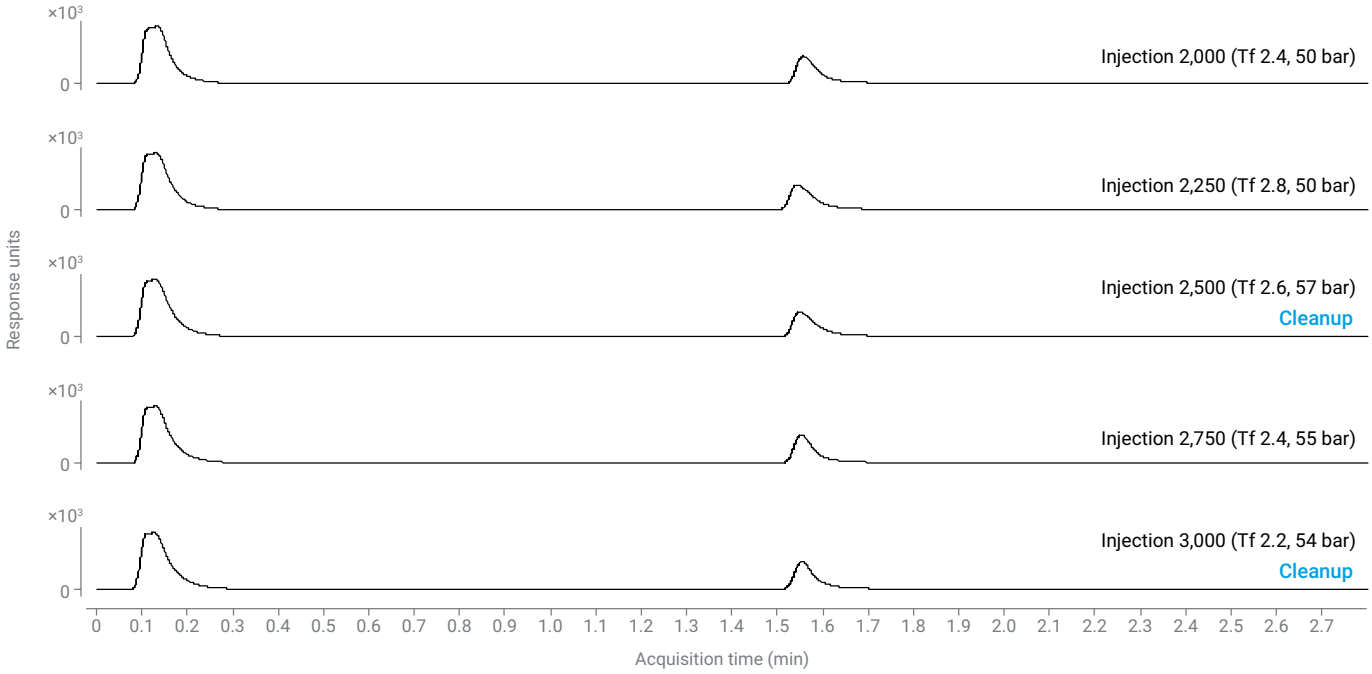

Figure 4. Agilent Bio-Monolith rProtein A column lifetime: Injections 2,000 to 3,000.

Table 1. Column pressure versus injection number during lifetime.

N-glycans	Innovator	Biosimilar
1	38.0	40.0
250	41.0	41.5
500	43.0	42.0
750	48.0	42.0
1,000	-	43.0
1,250	-	44.0
1,500	-	48.0

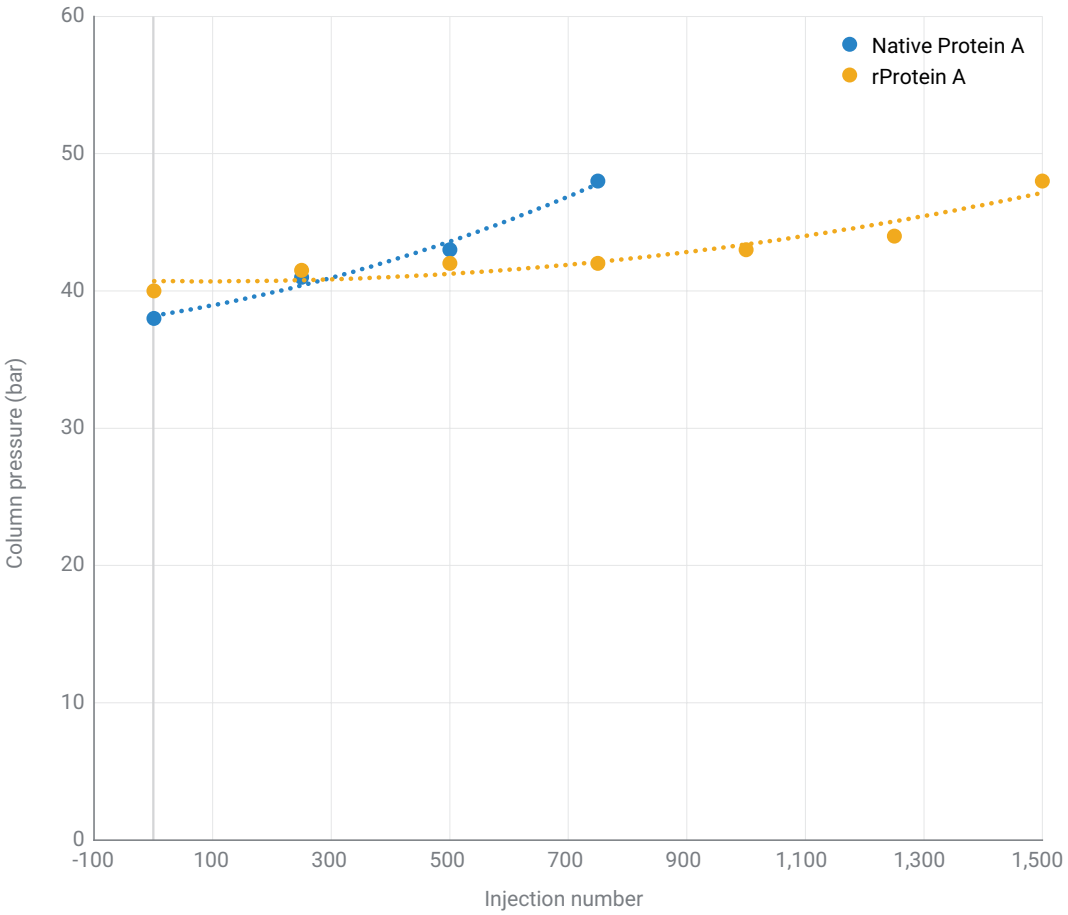

Figure 5. Column pressure versus injection number during lifetime.

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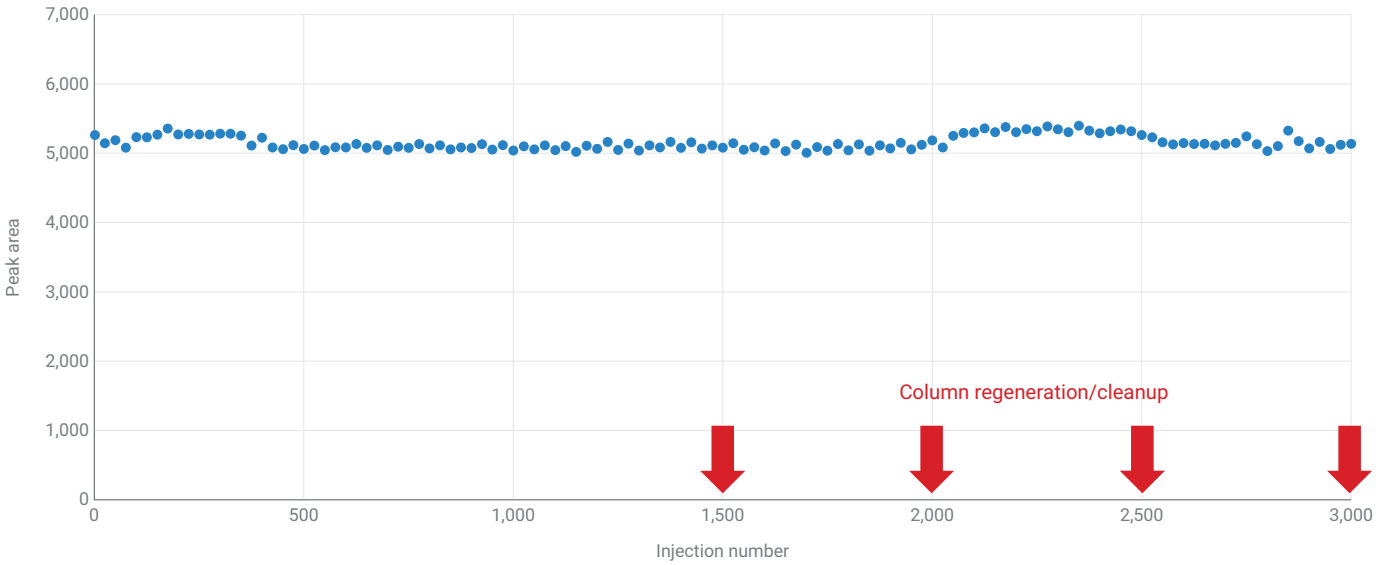

Figure 6. Peak area versus injection number during column lifetime.

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Table 2. Peak area versus injection quantity (µg) during column lifetime.

Quantity (µg)	Initial	After 2,000 Injections	After 3,000 Injections
1	654	633	688
2	1,363	1,323	1,308
5	2,766	2,984	2,979
10	5,526	5,699	5,666
15	7,706	7,653	7,699
20	10,541	10,268	10,347

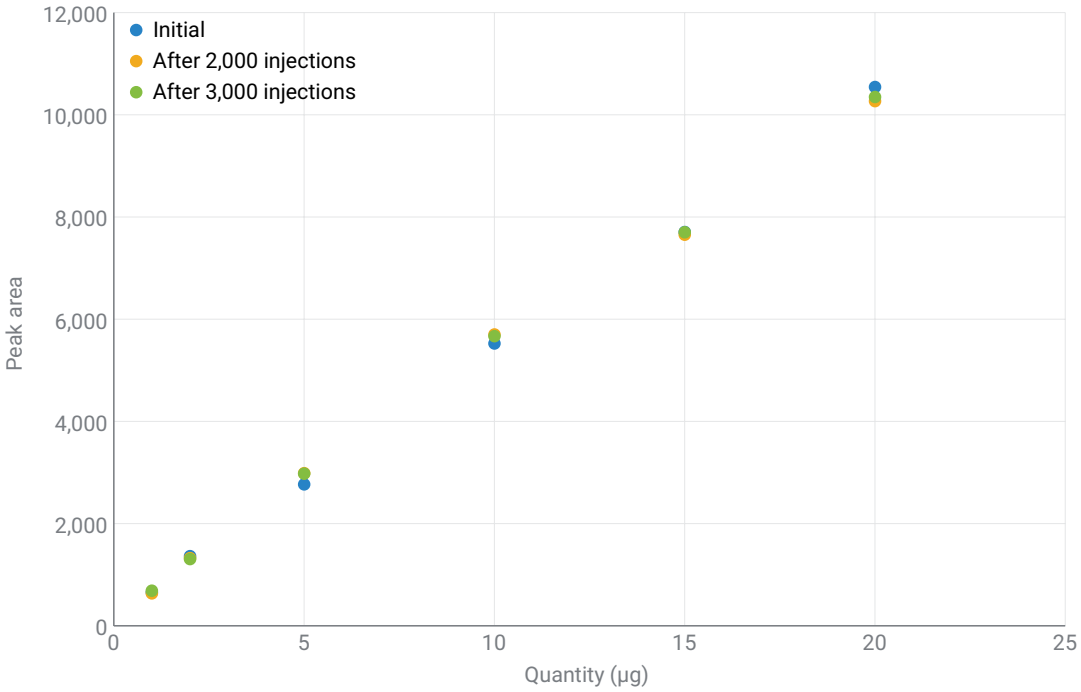

Figure 7. Peak area versus injection number during column lifetime.

Conclusions

This application note has shown that the Bio-Monolith rProtein A column is capable of consistent and reliable performance in titer analysis beyond what we have observed with equivalent native Protein A columns.

References

1. Coffey, A.; Kondaveeti. Improved Lifetime of Bio-Monolith Protein A Columns for Titer Determination. *Agilent Technologies Application Note*, publication number 5994-2168EN, **2020**.

Application Note

Titer Determination

Agilent

Trusted Answers

Cell Clone Selection Using the Agilent Bio-Monolith Protein A Column and LC/MS.

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Abstract

This application note describes how the Agilent Bio-Monolith Protein A column was applied to determine recombinant monoclonal antibody titer in Chinese hamster ovary cell-culture supernatants, and how the column was used to enrich µg amounts of antibody for further structural characterization by mass spectrometry. The workflow provides guidance for the clone selection process in biopharmaceutical and biosimilar development.

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Introduction

Monoclonal antibodies (mAbs) are currently in widespread use for the treatment of life-threatening diseases, including cancer and autoimmune diseases. Over 30 monoclonal antibodies are marketed, nine displayed blockbuster status in 2010, and five of the 10 top-selling biopharmaceuticals in 2009 were mAbs [1]. mAbs are currently considered the fastest growing class of therapeutics. The knowledge that the topselling mAbs are, or will become, open to the market in the coming years has resulted in an explosion of biosimilar activity. The first two monoclonal antibody biosimilars were approved in 2013, and both contain the same active substance, infliximab [2].

Whether developing innovator or biosimilar mAbs, well thought out clone selection is critical early on in the development process. This application note describes how the Agilent Bio-Monolith Protein A column can guide this process. This HPLC column is composed of a poly(glycidyl methacrylate-co-ethylene dimethacrylate) highly porous monolithic support coated with Protein A from Staphylococcus aureus. It combines the advantages of monoliths, that is, fast and efficient separations with limited carry-over, with the selectivity of the Protein A receptor for the Fc region of immunoglobulin G (IgG). As such, it represents an ideal tool for the high-throughput determination of mAb titer and yield directly from cell-culture supernatants, and for purifying mAbs at analytical scale for further measurements, for example by mass spectrometry (MS), ion exchange (IEX), size-exclusion chromatography (SEC), or hydrophobic interaction chromatography (HIC).

We have illustrated the selection of trastuzumab- biosimilar-producing Chinese hamster ovary (CHO) clones, based on titer and structural characteristics, using the Bio-Monolith Protein A column. Trastuzumab has been marketed as Herceptin since 1998, and is still in widespread use in the treatment of HER2 positive breast cancer [3]. This major biotherapeutic becomes open to the market in 2014 in Europe and 2018 in the US. To select clones based on biosimilar mAb titer, absolute concentrations were determined making use of a calibration curve generated with the Herceptin originator. To assess the structural characteristics and to compare with the originator molecule, the Protein A column was used to enrich analytical-scale quantities of the mAbs prior to mass spectrometric analysis.

Experimental

Materials

Acetonitrile, water, and isopropanol were obtained from Biosolve (Valkenswaard, The Netherlands). Citric acid, formic acid, NaH_2PO_4. Na_2HPO_4, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) 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 for construction of the calibration curves. 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. Collected fractions were reduced at room temperature for 1 hour by adding 10 mM TCEP.

Instrumentation

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)
- Agilent 1200 Infinity Series Analytical-scale Fraction Collector (G1364C)

LC/MS measurements were performed on:

Agilent 1290 Infinity Binary LC equipped with:

- Agilent 1290 Infinity Binary Pump (G4220B)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF with Agilent Jet Stream LC/MS (G6540A)

Software

- Agilent Technologies OpenLAB CDS ChemStation revision C01.05 (35)
- Agilent Technologies MassHunter for instrument control (B05.01)
- Agilent Technologies MassHunter for data analysis (B06.00)
- Agilent Technologies BioConfirm software for MassHunter (B06.00)

Conditions, Bio-Monolith column

Parameter	Value	
Column:	Agilent Bio-Monolith Protein A (p/n5069-3639)	
Mobile phase:	A) 50 mM phosphate, pH 7.4 B) 100 mM citric acid, pH 2.8	
Gradient:	Time (min) 0 to 0.5 0.6 to 1.7 1.8 to 3.5	% B 0 (binding) 100 (elution) 0 (regeneration)
Flow rate:	1 mL/min	
Injection volume:	50 µL	
Detection:	UV at 280 nm	
Fraction collection:	Time-based	

Conditions, LC/MS

Parameter	Value	
Cartridge:	Online desalting cartridge, 2.1 × 10 mm	
Mobile phase:	A) 0.1% formic acid in water (v:v) B) 0.1% formic acid in acetonitrile (v:v)	
Flow rate:	400 µL/min	
Injection volume:	Variable (corresponding to a protein amount of 1 µg)	
Needle wash solvent:	60 % acetonitrile, 35 % water, 5 % isopropanol	
Autosampler temperature:	7 °C	
Gradient:	Time (min) 0 0.5 2 3 3.10 5	% B 5 5 80.0 80.0 5 5
Q-TOF source:	Agilent Jet Stream, positive ionization mode	
Drying gas temperature:	300 °C	
Drying gas flow rate:	8 L/min	
Drying gas flow rate:	35 psig	
Nebulizer pressure:	350 °C	
Sheath gas temperature:	11 L/min	
Nozzle voltage:	1,000 V	
Capillary voltage:	3,500 V	
Fragmentor voltage:	200 V	
Q-TOF detection:	Mass range 3,200 amu	
Data acquisition range:	500 to 3,200 m/z	
High-resolution mode	(4 GHz)	
Data acquisition rate: mode	1 spectrum per s	
Profile acquisition		
Diverter valve:	Time (min) 0 1 3.5	Flow to waste MS waste

Results and Discussion

Clone selection through determination of trastuzumab titer

Figure 1 shows an overlay of the Protein A chromatograms of the supernatant of a specific trastuzumab-producing clone and a Herceptin originator. The unbound material eluted in the flow-through while the mAb was only released after lowering the pH. In the case of the originator, no material was observed in the flow-through, which is not surprising since this represents the marketed product. In the case of the supernatant, a substantial signal resulting from the unbound material was seen.

Figure 2 shows an overlay of the Protein A chromatograms of 12 trastuzumab-producing clones, generated in the framework of a biosimilar development program. From these chromatograms, a distinction can already be made between low and high producing clones. Absolute mAb concentrations can be determined by linking the peak areas to an external calibration curve constructed by diluting Herceptin originators.

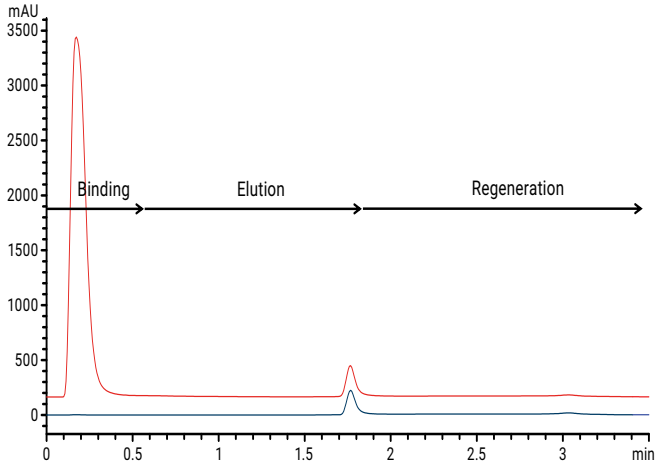

Figure 1. UV 280 nm Agilent Bio-Monolith Protein A chromatogram of a trastuzumab-producing CHO clone, clone 9 (red), and of a Herceptin originator diluted in 50 mM Na-phosphate pH 7.4 to 0.2 mg/mL (blue). Note that the supernatant was diluted 1:1 in phosphate buffer.

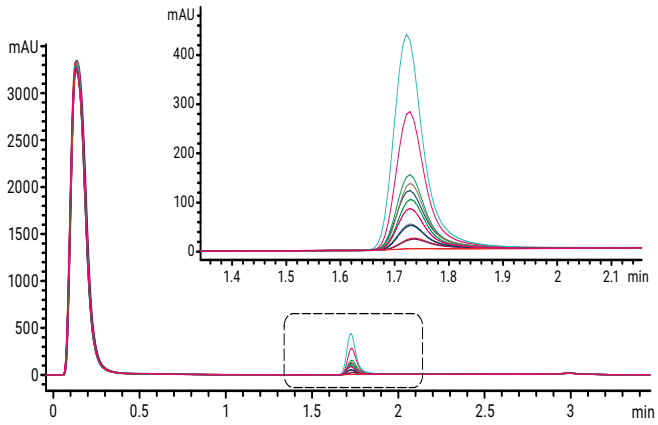

Figure 2. Overlaid UV 280 nm Agilent Bio-Monolith Protein A chromatograms of 12 trastuzumab-producing CHO clones.

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The calibration curve and corresponding chromatograms of the Herceptin calibration points are shown in Figures 3 and 4. Good linearity was obtained between 0.02 and 2 mg/mL, which is the typical mAb titer range in CHO cells. Obtained mAb titers are reported in Table 1 and are pictured graphically in Figure 5. From these findings, clear decisions could be made for further biosimilar development, that is, high-producing clones 9 and 10 could readily be selected and sub cloned. Table 1 also shows the titers obtained when growing the CHO clones in two different cell-culture media, and clearly shows the benefit of one over the other, linking the peak areas to an external calibration curve constructed by diluting Herceptin originators.

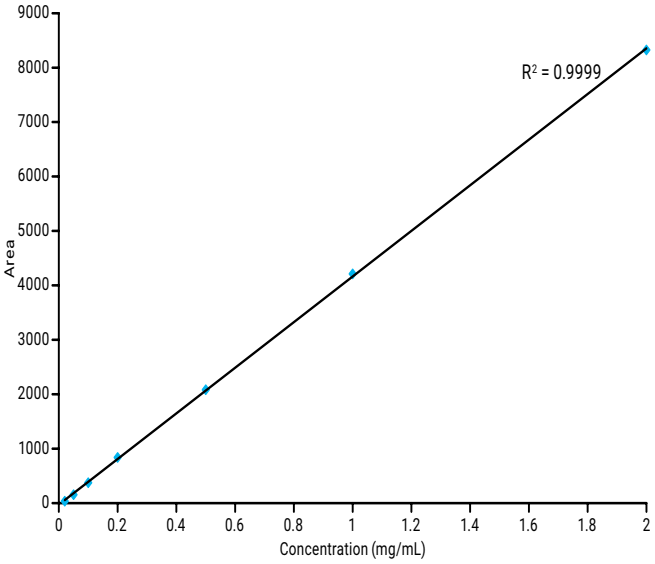

Figure 3. Herceptin Agilent Bio-Monolith Protein A calibration curve, 0.02 to 2 mg/mL.

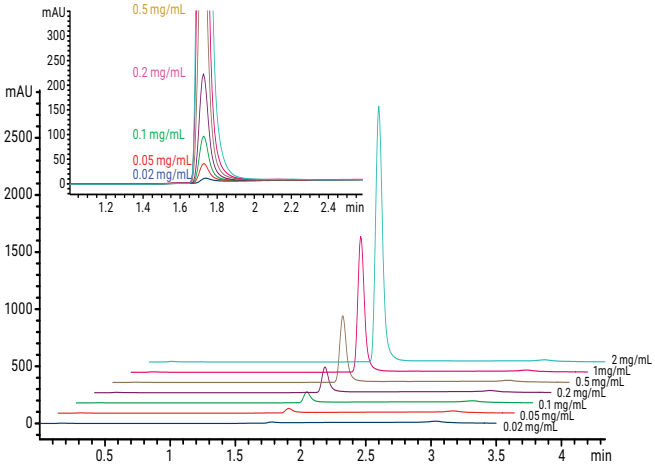

Figure 4. Overlaid UV 280 nm Agilent Bio-Monolith Protein A chromatograms of Herceptin calibration points.

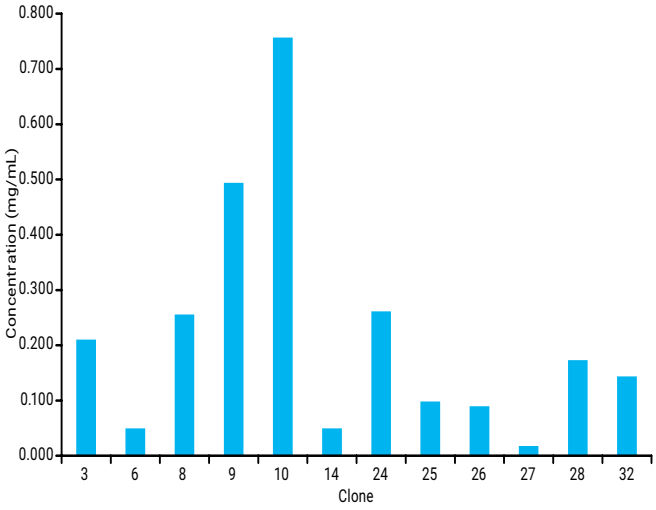

Figure 5. Graphical representation of the biosimilar mAb titer, expressed in mg/mL, in the different trastuzumab CHO clones.

Table 1. Absolute trastuzumab biosimilar concentrations determined in the different CHO clones grown on two different media.

CHO Clone Medium A	Concentration (mg/mL)	CHO Clone Medium B	Concentration (mg/mL)
3	0.156	3	0.210
6	0.048	6	0.050
8	0.155	8	0.256
9	0.215	9	0.494
10	0.311	10	0.757
14	0.038	14	0.050
24	0.082	24	0.262
25	0.049	25	0.098
26	0.037	26	0.090
27	-	27	0.018
28	0.117	28	0.173
32	0.156	32	0.144

Clone selection by assessing structural characteristics

Next to the mAb titer, the second important criterion in clone selection is based on the structural aspects. In the case of biosimilar development, the structure should be highly similar to the originator product, within the originator batch-to-batch variations. Therefore, Protein A fractions were collected and measured on high-resolution mass spectrometry following disulfide-bond reduction giving rise to the light and heavy chain. This strategy allowed verification of the amino acid sequence and revealed the glycosylation pattern.

To be able to reduce the mAb directly in the collection vial containing acidic buffer, TCEP was chosen instead of the more common reductant dithiothreitol (DTT). The former allows reduction over a broad pH range including low pH values, while the latter's reducing capacities are limited to pH values above seven. Reduced fractions were delivered to the MS system following online desalting. Figures 6 and 7 show the deconvoluted light and heavy chain spectra of one Herceptin originator and two high yield trastuzumab biosimilar-producing clones.

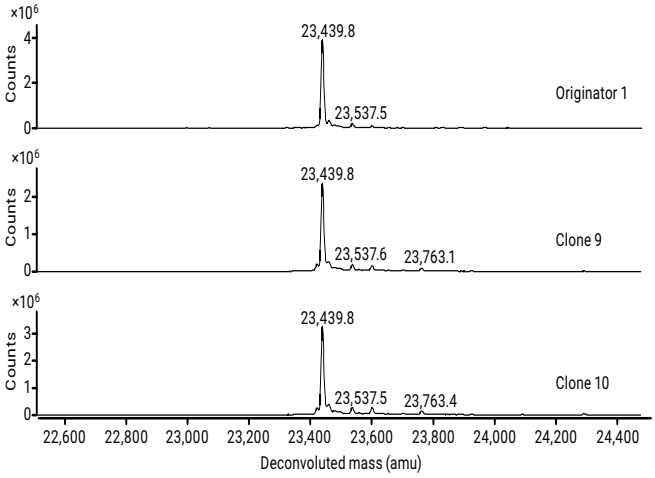

Figure 6. Deconvoluted light chain spectra of a Herceptin originator and two trastuzumab-producing clones.

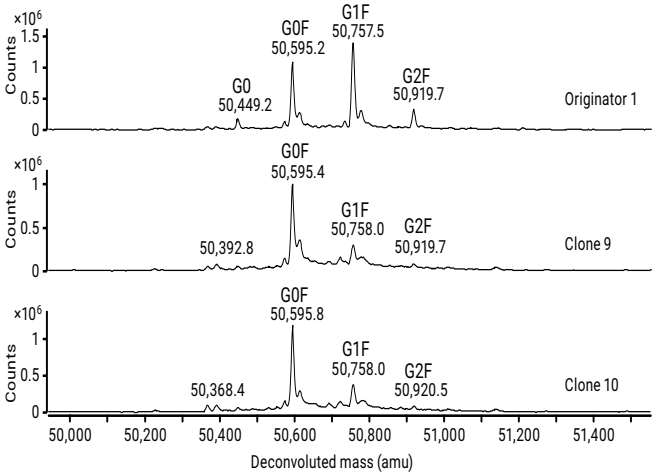

Figure 7. Deconvoluted heavy chain spectra of a Herceptin originator and two trastuzumab producing clones. The abbreviations G0, G0F, G1, and G2F refer to the N-glycans attached to the mAb backbone..

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Tables 2 and 3 display the measured MW values and relative intensity of the main glycoforms in four originator production batches and 12 trastuzumab clones. From this, it can be concluded that the Herceptin originators and clone derived trastuzumab displayed the same light and heavy chain molecular weight values.

In addition, the same N-glycans, which are of the complex type, were observed on the heavy chain of the originators and clone derived mAbs.

These are considered the most important attributes of biosimilarity according to US and European regulatory authorities (the primary sequence should be identical and glycosylation should be preserved). While glycosylation is similar from a qualitative perspective, quantitative differences were seen. A separate application note describes how the Protein A Bio-Monolith was used in the tuning of the growth medium to fit the glycosylation to the originator specifications [4].

Table 2. Measured light and heavy chain MW values in the originators and trastuzumab clones.

MW (Da)	Originator 1	Originator 2	Originator 3	Originator 4	Clone 3	Clone 6
Light chain	23,439.8	23,439.8	23,439.8	23,439.8	23,439.8	23,440.2
Heavy chain *	49,149.9	49,150.2	49,150.1	49,150.1	49,150.5	49,151.0

MW (Da)	Originator 8	Originator 9	Originator 10	Originator 14	Clone 24	Clone 25
Light chain	23,439.8	23,439.8	23,439.8	23,439.9	23,439.8	23,439.9
Heavy chain *	49,150.6	49,150.1	49,150.5	49,150.2	49,150.6	49,151.1

MW (Da)	Originator 26	Originator 27	Originator 28	Originator 32
Light chain	23,440.0	23,441.4	23,439.8	23,439.9
Heavy chain *	49,150.9	49,151.9	49,150.7	49,150.9

*Theoretical deglycosylated MW values.

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Table 3. Relative intensity of the main glycoforms in four originator production batches and trastuzumab clones.

Glycoform	Originator 1	Originator 2	Originator 3	Originator 4	Clone 3	Clone 6
% Man 5	1.6	1.6	1.3	1.1	2.7	1.6
% G0F-GlcNAc	1.5	2.7	3.3	2.4	3.2	3.2
% G0	5.7	5.9	5.0	4.9	2.8	3.3
% G0F	35.2	44.8	50.5	48.2	66.1	56.2
% G1F	45.2	38.4	34.0	36.8	20.6	27.7
% G2F	10.7	6.6	5.9	6.7	4.7	8.1

Glycoform	Clone 8	Clone 9	Clone 10	Clone 14	Clone 24	Clone 25
% Man 5	2.6	3.3	5.0	1.2	1.9	5.1
% G0F-GlcNAc	3.8	4.8	4.6	2.1	3.6	4.2
% G0	1.7	2.9	2.9	3.9	2.2	2.3
% G0F	69.9	66.1	64.1	64.6	68.6	60.7
% G1F	18.4	18.5	19.5	22.9	19.4	20.9
% G2F	3.6	4.3	3.8	5.3	4.3	6.7

Glycoform	Clone 26	Clone 27	Clone 28	Clone 32
% Man 5	5.4	0.0	1.5	3.1
% G0F-GlcNAc	5.8	0.0	2.9	4.3
% G0	1.8	0.0	1.2	2.7
% G0F	61.6	67.2	61.6	64.3
% G1F	19.5	32.8	26.3	20.3
% G2F	5.8	0.0	6.5	5.3

Conclusions

The Agilent Bio-Monolith Protein A column was successfully applied in the selection of trastuzumab-biosimilar-producing clones based on both titer and structural similarity to the originator. This clone selection process is of utmost importance early in the development of innovator and biosimilar mAbs.

References

1. 1. K. Sandra, I. Vandenheede, P. Sandra. *J. Chromatogr. A.,* **1335**, 81 (2014).
2. www.ema.europa.eu
3. www.gene.com
4. 4. E. Dumont, *et al.,* Cell Culture Optimization Using an Agilent Bio-Monolith Protein A Column and LC/MS, *Agilent Technologies Application Note,* publication number 5991-5124EN (**2014**).

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Application Note

Titer Determination

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Trusted Answers

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

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Abstract

Monoclonal antibodies (mAbs) are becoming increasingly important in thetreatment of various diseases. During development of recombinant mAbs, proteintiter and yield from various cell culture supernatants must be monitored. Thisapplication 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).

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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, Bio-Monolith column

Parameter	Value	
Column:	Agilent Bio-Monolith Protein A (p/n5069-3639)	
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) 0 to 0.5 0.6 to 1.7 1.8 to 3.5	% B 0 (binding) 100 (elution) 0 (regeneration)
Flow rate:	1 mL/min	
Injection volume:	Variable (50 µL, optimized for CHO cell culture supernatants)	
Detection:	UV at 280 nm	

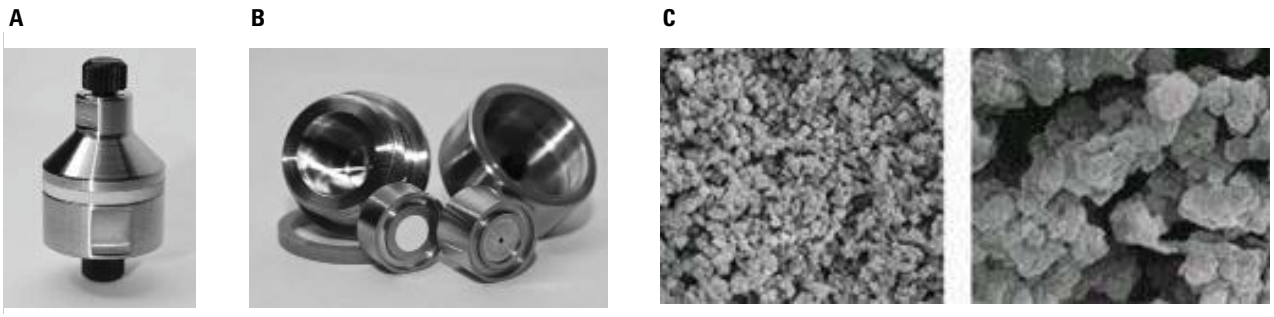

Figure 1. UV 280 nm Agilent Bio-Monolith Protein A chromatogram of a trastuzumab-producing CHO clone, clone 9 (red), and of a Herceptin originator diluted in 50 mM Na-phosphate pH 7.4 to 0.2 mg/mL (blue). Note that the supernatant was diluted 1:1 in phosphate buffer.

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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.

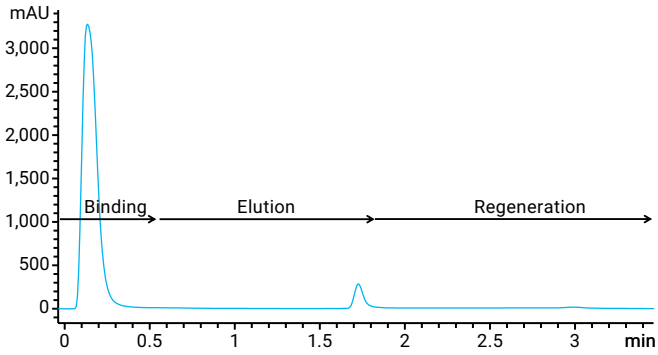

Figure 2. UV 280 nm Protein A chromatogram showing the supernatant of a trastuzumab-producing CHO clone. Injection volume was 50 µL. Peak width at half height was 0.10 minutes for the unbound material and 0.06 minutes for the retained mAb.

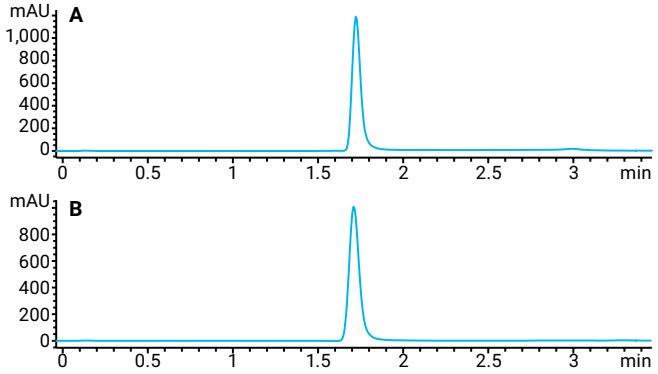

Figure 3. UV 280 nm Protein A chromatogram of Herceptin originator diluted in 50 mM Na-phosphate, pH 7.4, to 0.5 mg/mL (50-µL injection, 25-µg column load). Elution was achieved using citric acid (A) and acetic acid (B). Peak width at half height is 0.057 and 0.067 minutes for citric acid and acetic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

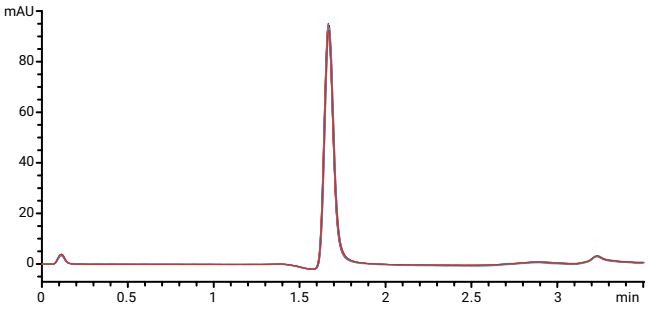

Figure 4. Replicate (n = 10) UV 280 nm Protein A chromatograms of a Herceptin originator diluted in 50 mM Na phosphate, pH 7.4, to 0.5 mg/mL (injection volume 5 µL). Elution was achieved using acetic acid.

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	1.678
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

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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.

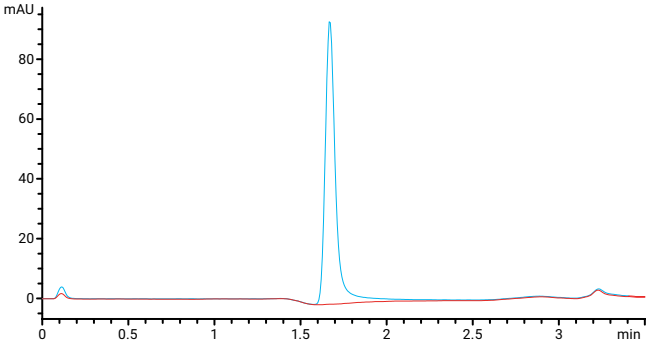

Figure 5. UV 280 nm Protein A chromatograms of a Herceptin originator diluted in 50 mM Na phosphate, pH 7.4, to 0.5 mg/mL, and a blank buffer analyzed after a sequence of 10 Herceptin injections. Elution was achieved using acetic acid, and injection volumes were 5 µL.

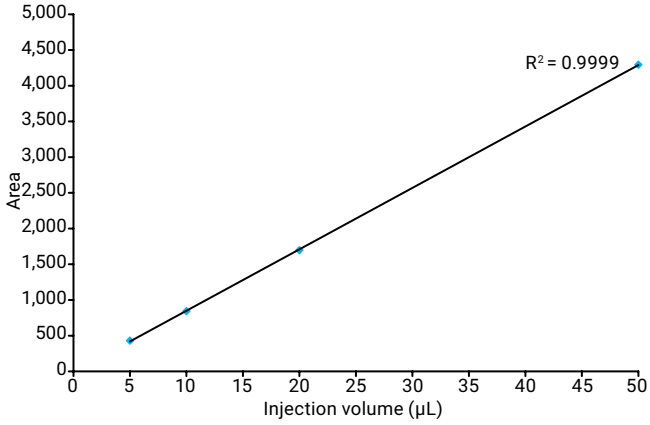

Figure 6. Linearity obtained by increasing the injection volume of a Herceptin originator (0.5 mg/mL) from 5 to 50 µL.

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.

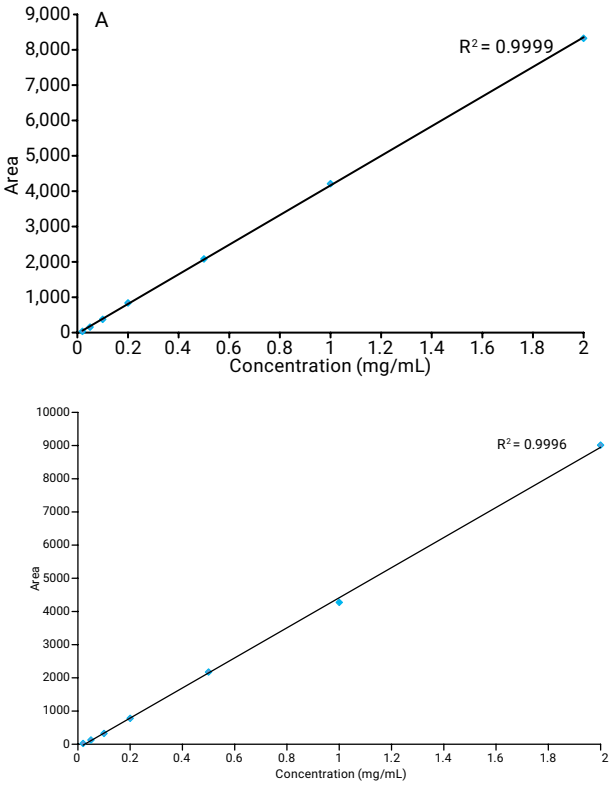

Figure 7. Herceptin Protein A calibration curve (0.02 to 2 mg/mL) using citric acid (A) and acetic acid (B) as elution buffer.

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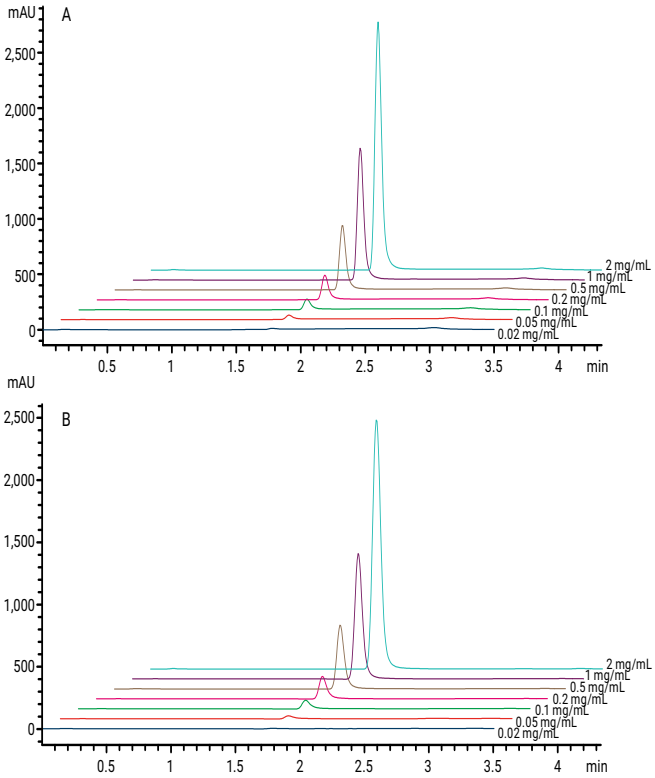

Figure 8. Overlaid UV 280 nm Protein A chromatograms of Herceptin calibration points using citric acid (A) and acetic acid (B) as elution buffer.

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.

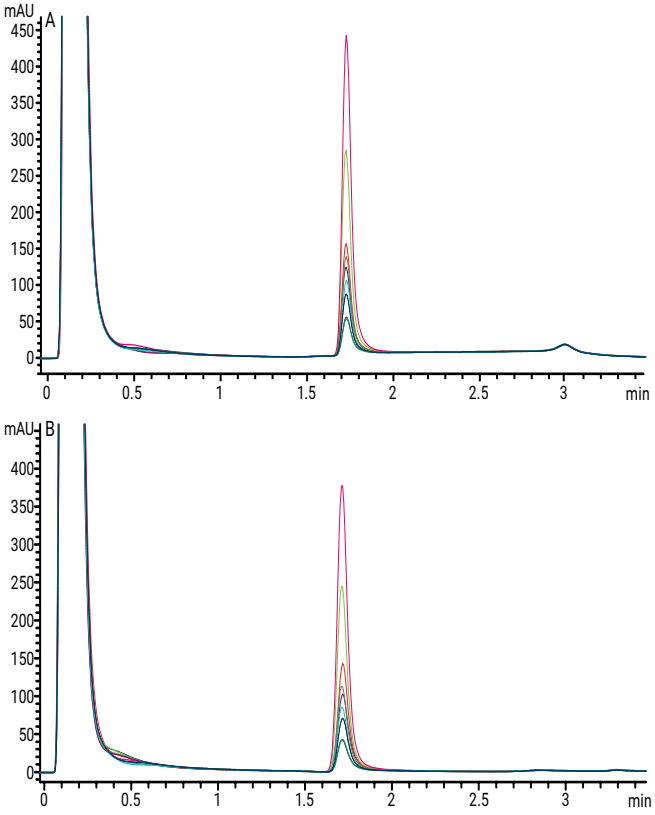

Figure 9. Overlaid UV 280 nm Protein A chromatograms of nine trastuzumab-producing CHO clones using citric acid (A) and acetic acid (B) as elution buffer.

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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. 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 was successfully applied in the selection of trastuzumab-biosimilar-producing clones based on both titer and structural similarity to the originator. This clone selection process is of utmost importance early in the development of innovator and biosimilar mAbs

For More Information

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Additional Application Notes

Publication Number	Title
5991-2990EN	Agilent Bio-Monolith Protein A Monitors Monoclonal Antibody Titer from Cell Cultures
5991-4723EN	Reducing Cycle Time for Quantification of Human IgG Using the Agilent Bio-Monolith Protein A HPLC Column
5991-5125EN	Cell Culture Optimization Using an Agilent Bio-Monolith Protein A Column and LC/MS
5991-6094EN	Bio-Monolith Protein G Column - More Options for mAb Titer Determination

Additional Information

Agilent Bio-Monolith columns are also available in ion exchange formats.

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