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

Application Note

Biopharmaceuticals and Biosimilars

Authors

Emmie Dumont, Isabel Vandenheede, Pat Sandra, Koen Sandra
Research Institute for Chromatography (RIC)
President Kennedypark 26
B-8500 Kortrijk
Belgium

James Martosella, Phu Duong, Maureen Joseph
Agilent Technologies, Inc

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.

Agilent Technologies

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 top-selling 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

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	
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:	50 µL	
Detection:	UV at 280 nm	
Fraction collection:	Time-based	

Conditions, LC/MS

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)	% B
	0	5
	0.5	5
	2	80.0
	3	80.0
	3.10	5
	5	5
Q-TOF source:	Agilent Jet Stream, positive ionization mode	
Drying gas temperature:	300 °C	
Drying gas flow rate:	8 L/min	
Nebulizer pressure:	35 psig	
Sheath gas temperature:	350 °C	
Sheath gas flow rate:	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:	1 spectrum per s	
Profile acquisition		
Diverter valve:	Time (min)	Flow to
	0	waste
	1	MS
	3.5	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.

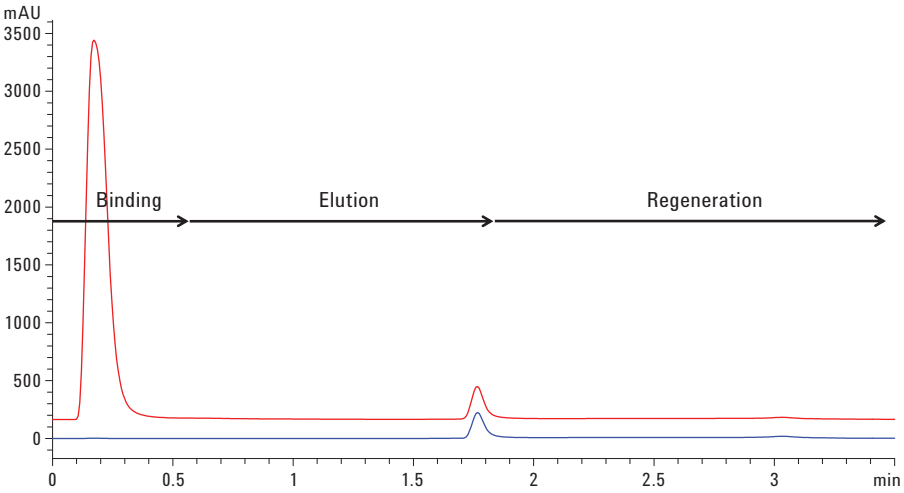

Figure 1. UV 280 nm Agilent Bio-Monolith Protein A chromatogram of a trastuzumab-producing CHO clone, clone 9 (red), and of a Herceptin originator diluted in 50 mM Na-phosphate pH 7.4 to 0.2 mg/mL (blue). Note that the supernatant was diluted 1:1 in phosphate buffer.

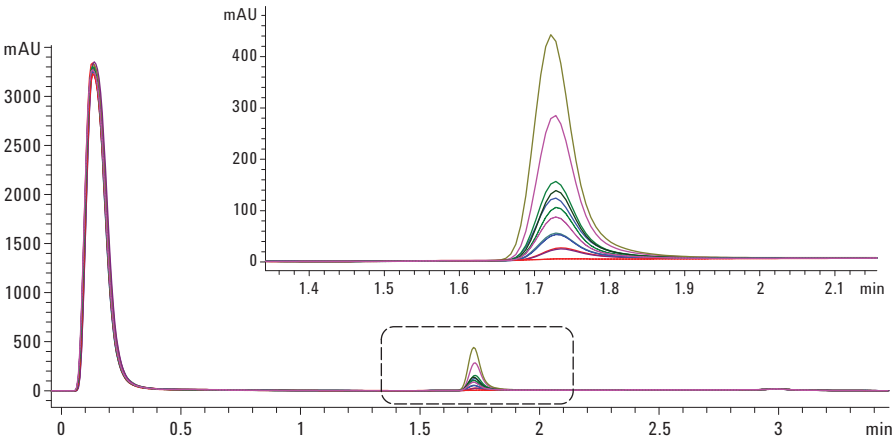

Figure 2. Overlaid UV 280 nm Agilent Bio-Monolith Protein A chromatograms of 12 trastuzumab-producing CHO clones.

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.

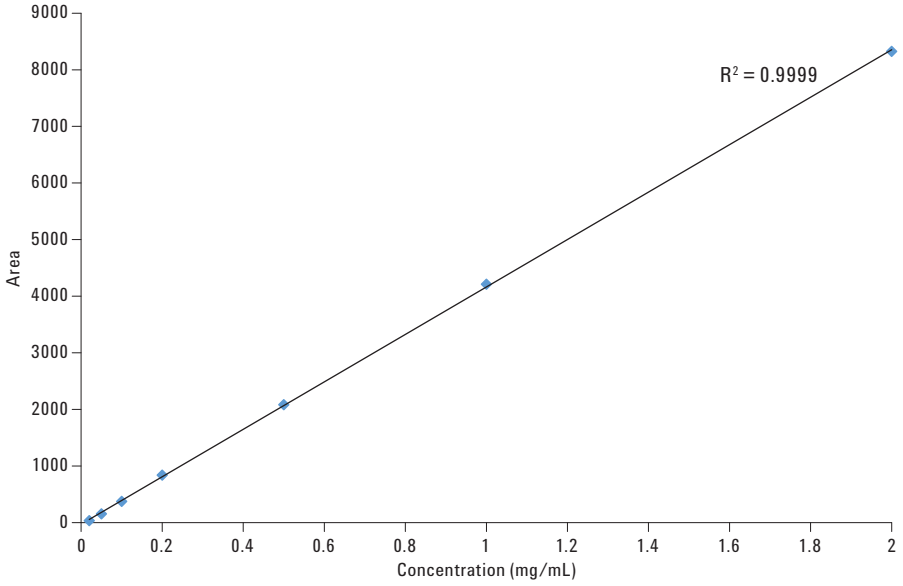

Figure 3. Herceptin Agilent Bio-Monolith Protein A calibration curve, 0.02 to 2 mg/mL.

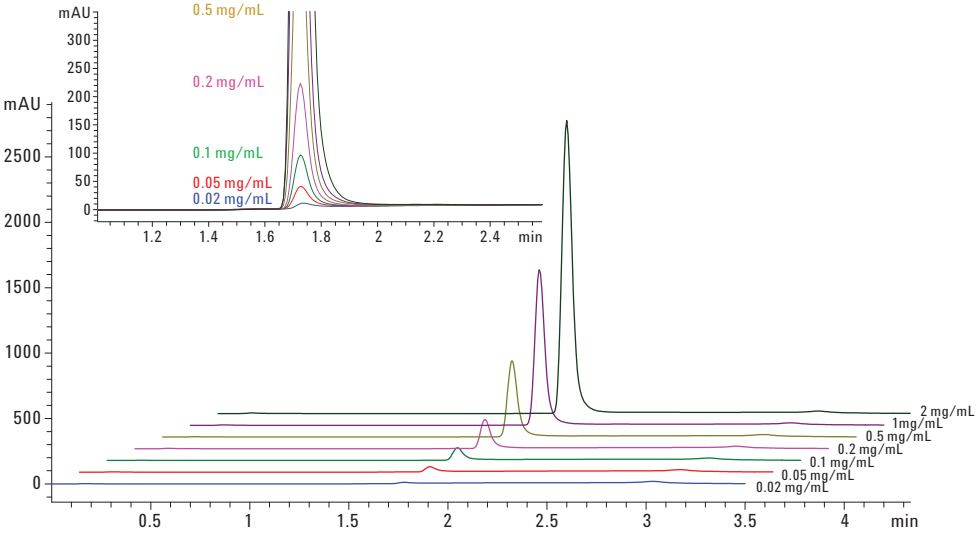

Figure 4. Overlaid UV 280 nm Agilent Bio-Monolith Protein A chromatograms of Herceptin calibration points.

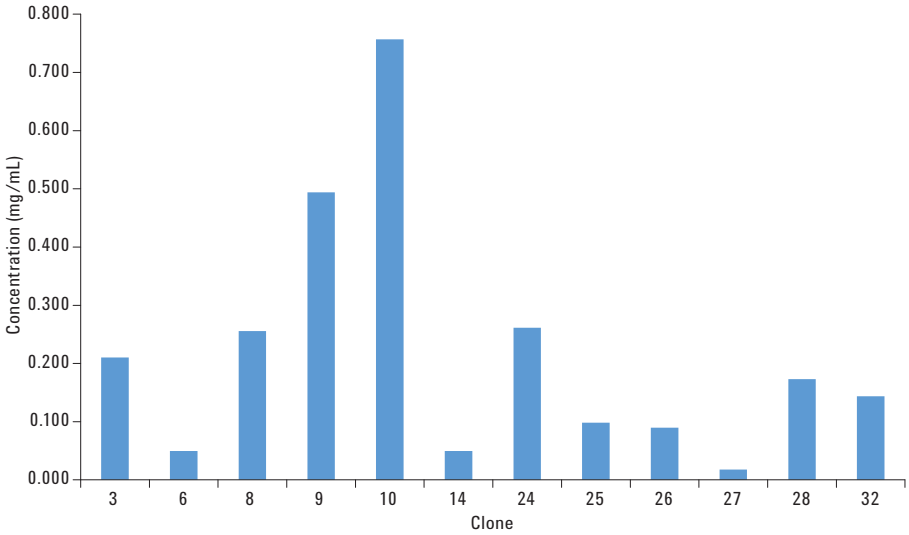

Figure 5. Graphical representation of the biosimilar mAb titer, expressed in mg/mL, in the different trastuzumab CHO clones.

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.

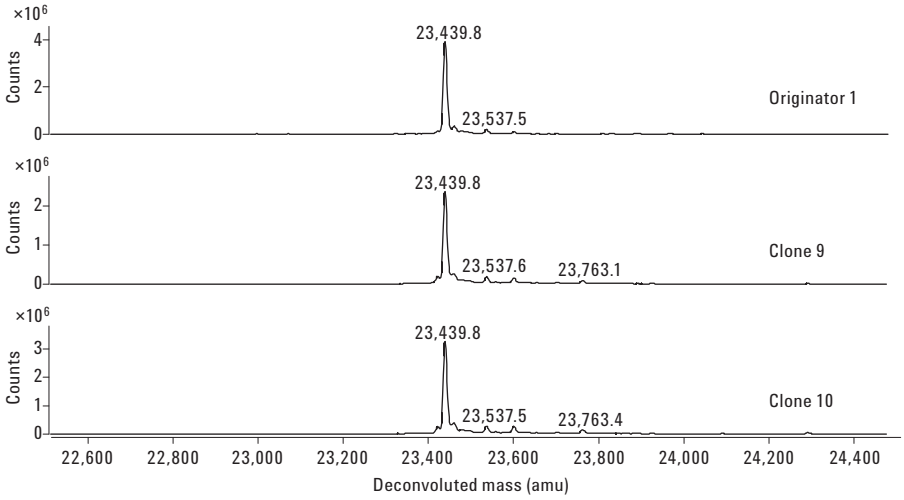

Figure 6. Deconvoluted light chain spectra of a Herceptin originator and two trastuzumab-producing clones.

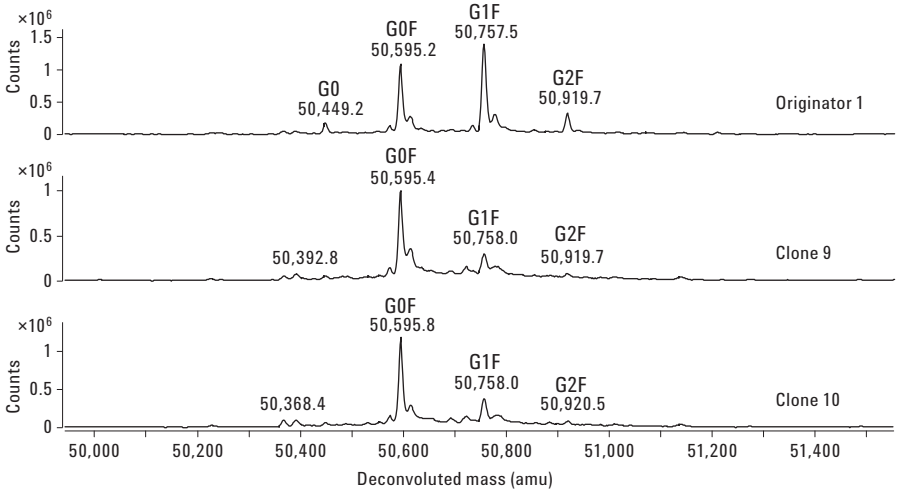

Figure 7. Deconvoluted heavy chain spectra of a Herceptin originator and two trastuzumab producing clones. The abbreviations G0, G0F, G1, and G2F refer to the N-glycans attached to the mAb backbone.

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)	Clone 8	Clone 9	Clone 10	Clone 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)	Clone 26	Clone 27	Clone 28	Clone 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.

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. K. Sandra, I. Vandenheede, P. Sandra. *J. Chromatogr. A.*, **1335**, 81 (2014).
2. www.ema.europa.eu
3. www.gene.com
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**).

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

Printed in the USA
August 21, 2014
5991-5124EN

Agilent Technologies