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Application Note

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MAb Quantitation: Protein A HPLC vs. Protein A Bio-Layer Interferometry

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Abstract

Rapid, accurate and cost-effective quantitation of monoclonal antibodies (MAbs) is essential for bioprocessing. High Pressure Liquid Chromatography (HPLC) and the Octet® are some of the commonly used techniques for MAbs titer determination. To ensure MAbs purification column efficiency, the dynamic binding capacity (DBC) of Protein A for Mab can be determined by loading feedstock onto the column until binding sites for the MAb become saturated and MAb begins to break through. An assessment of the relative merits of Protein A (HPLC) and the Sartorius Octet® R8 System to determine MAb concentration in a complex feedstock was performed using MAbs breakthrough as the analyte while monitoring method accuracy, precision, dynamic range, LoQ and cost per sample. The evaluation found that the Sartorius Octet® R8 system can be used to accurately quantitate 5%, 10% and 20% MAb breakthrough values in the presence of contaminant host cell proteins. Compared to HPLC analysis, the Octet® system had a slightly larger dynamic range and performed best at low concentrations with an almost 30-fold lower LoQ . The Octet® was further shown to provide a fast, accurate and economical means of quantifying MAbs, and decreases process development.

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Contents

Summary 3
Introduction 3
Materials and Methods 4
  Materials 4
  Methods 4
Results 6
  Comparison of Assay LoQ, LoD, Accuracy, Precision and Dynamic Range 6
  Comparison of Assay Costs 7
  Determination of 5%, 10% and 20% Breakthroughs 9
Conclusions 11

Summary

Rapid, accurate and cost-effective quantitation of monoclonal antibodies (MAbs) is essential for bioprocessing. Here we assess the relative merits of Protein A High Pressure Liquid Chromatography (HPLC) and Protein A biolayer interferometry using the Sartorius Octet® R8 system to determine MAb concentration in a complex feedstock. We perform this assessment within the context of determining a MAb breakthrough curve from a Protein A column loaded with a CHO culture feedstock.

The HPLC method has a limit of detection (LOD) 10-fold higher than that of the Sartorius Octet® R8 system (17 µg/mL vs. 0.1 µg/mL respectively), and a limit of quantitation (LOQ) more than 20-fold higher than the Octet® system (27 µg/mL vs. 1.3 µg/mL respectively). The HPLC and Octet® methods have a comparable dynamic range (spanning 1.9 versus 2.2 orders of magnitude, respectively). The Octet® method has two major benefits over the HPLC method: assay cost is reduced by >20%, and the total time of analysis is reduced by >6-fold. Assay cost was reduced from $1.21 per sample with the HPLC method to $0.93 per sample with the Octet® method. The greatest benefit in using the Octet® system was reduced process time — switching the assay format from HPLC to the Octet® platform reduced the total time for analysis from >24 hours with HPLC to <4 hours with the Octet® assay.

In summary, we find that HPLC and the Sartorius Octet® system can be used to generate comparable data, with the Octet® system providing significant improvements in assay cost, throughput and sample preparation time.

Introduction

There are currently 30 monoclonal antibodies approved by the FDA as biotherapeutic agents, representing the most rapidly growing class of new drugs. Despite advances in downstream processing technology, affinity purification of monoclonal antibodies using Protein A chromatography is still the industry standard. In order to use Protein A resin as productively as possible it is important to load the resin at close to its dynamic binding capacity (DBC). DBC is normally determined with small sorbent volumes before scaling up to the process level. Here we look at the effects of residence time and MAb concentration on Protein A dynamic binding capacity. The knowledge of DBC under these conditions allows us to scale our process robustly, while maintaining high resin productivity.

To measure the DBC of Protein A for MAb, feedstock is loaded onto the column until binding sites for the MAb become saturated and MAb begins to break through. As contaminant proteins in the CHO feedstock have a large absorbance, it is impossible to accurately determine the breakthrough of MAb using UV A280. To follow the MAb breakthrough, we collected fractions of the column flow-through and analyzed the samples by Protein A HPLC and Protein A Bio-Layer Interferometry assay, the latter using the Sartorius Octet® system. We compare these analytical methods using several metrics including process time, preparation time, cost per sample, dynamic range, precision, accuracy, limit of detection and limit of quantitation. We show here that the Octet® system provides a fast, accurate and economical means of quantifying MAb, and decreases process development.

Materials and Methods

Materials

Biological Sample
MAb used in this study was purified from CHO feedstock (Clone 38, Pall Life Sciences) using two chromatographic steps: Protein A (MAbSelect SuRe, GE Healthcare), followed by cation exchange chromatography (S HyperCel, Pall Life Sciences).

Experimental Equipment
- Shimadzu HPLC system
- POROS® A 20 µm column (2.1 x 30 mm, 0.1 mL) (Invitrogen, part no. 2-1001-00)
- Sodium phosphate dibasic HPLC grade (Sigma Aldrich, part no. 10028-24-7)
- Sodium phosphate monobasic HPLC grade (Sigma Aldrich, part no. 7558-80-7)
- Glycine HPLC grade (Sigma Aldrich, part no. 56-40-6)
- Sartorius Octet® R8 system
- Octet® Protein A Biosensors (Sartorius, part no. 18-5010)
- Octet® Sample Diluent (Sartorius, part no. 18-5028)
- AKTA Explorer chromatography system with Unicorn software
- GE Healthcare HiTrap MabSelect SuRe Protein A sorbent, 1 mL column

Methods

Octet® System Analytical Method
Samples for Octet® analysis were diluted 1:10 in diluent buffer (0.1% / wt. BSA, 0.02% / vol. Tween 20 in PBS) to bring the sample concentrations below 300 µg/mL. Analytic runs were set up on the Octet® system using Octet® Analysis Studio Software. The analytical procedure used here is adapted from Technical Note found on the Sartorius website High Sensitivity Detection of Human IgG Using Protein A Biosensors. The critical parameters can be found in Table 1.

Protein A HPLC Analytical Method
Samples for HPLC analysis were 0.2 µm filtered by centrifugation (3,000g, 10 minutes) using Pall 0.2 µm AcroPrep™ 96-well filter plates (Pall Life Sciences, part no. 8019). Protein A HPLC analysis was performed using a 100 µL Poros A 20 µm chromatography column (Invitrogen) and a HPLC chromatography system (Shimadzu). Important parameters are summarized in Table 2.

Table 1: Assay parameters for Sartorius Octet® system.

Category	Parameter	Value
Quantitation	Quantitation time	60 sec
	Shake speed	1,000 rpm
Regeneration	pH	1.1
	Buffer concentration	10 mM Glycine
	Tween concentration	0.02% / vol.
	Regeneration cycles	3 cycles
	Regeneration time	5 sec/cycle
	Shake speed	1,000 rpm
Neutralization	Neutralization buffer	Sartorius Sample Diluent
	Neutralization cycles	3 cycles
	Neutralization time	5 sec/cycle
	Shake speed	1,000 rpm

Table 2: Assay parameters for HPLC.

Category	Parameter	Value
Buffer	Load buffer	25 mM phosphate + 300 mM sodium chloride, pH 7.2
	Elution buffer	10 mM glycine, pH 3.0
	Strip buffer	10 mM glycine, pH 2.5
Time program	Inject / wWash	30 column volumes
	Elute	20 column volumes
	Strip	20 column volumes
	Re-equilibrate	30 column volumes
System	Flow rate	2 mL/min
	Injection volume	50 µL

Method for Determination of Assay Accuracy, Precision, and Theoretical LoQ and LoD
For each assay, 6 replicates of 8 point standard curves were generated. For the HPLC assay, standards were prepared in loading buffer. For the Octet® assay, standards were prepared in a 90% Sartorius Sample Diluent, 10% mock CHO feedstock solution. These standards were measured using different biosensors and at different stages in regeneration to simulate how unknown samples are measured on a 96-well plate.

This set of 6 standard curves was used to provide an estimate of assay accuracy and precision. To determine assay accuracy, an average standard curve was determined using the first 3 replicates of the 6 standard curves measured. The next 3 replicate curves were then treated as experimental points, and tested for their fit to the average standard curve based on their expected value. Percentage Bias (%Bias) could then be calculated over the range of the assay using the following simple formula:

$$\%Bias = \frac{|Experimental - Expected| * 100}{Expected}$$

To determine assay precision, all 6 curve replicates were used to produce an average standard curve. Each of the 48 points used to generate this curve could then be assigned a concentration. The six measurements at each of the 8 known concentrations could then be used to calculate the coefficient of variance (CV), a common measure of precision, over the range of the assay. The formula for CV is as follows:

$$\%CV = \frac{Standard\ Deviation * 100}{Average\ Value}$$

From these estimates of accuracy and precision, an estimate of total error (TE) was obtained using the following commonly used formula:

$$\%TE = \%Bias + 1.96 * \%CV$$

The set of 6 standard curves described in this section were then used to obtain theoretical values for the assay Limit of Detection (LoD) and Limit of Quantitation (LoQ). LoD is defined as the minimum concentration at which an analyte's presence can be detected by a given assay, whereas LoQ is defined as the minimum concentration at which an analyte can be reliably quantified. A method from ICH 1996 "Validation of Analytical Procedures: Methodology" was used. In this document, the following equations are used to calculate LoD and LoQ.

$$LoD = \frac{3.3\sigma}{M} \qquad LoQ = \frac{10\sigma}{M}$$

Where:
σ = the standard deviation of the response
M = the slope of the calibration curve

Here the standard deviation of the response is the standard deviation of the y intercepts of the 6 standard curves generated above. The calculations of LoD and LoQ give values in terms of machine parameters, which must be converted back to concentrations using the calibration curve of the assay in question.

Application DoE and Procedure for Generating Samples

A two-factor, two-level factorial design was used to test for the effect of residence time and MAb titer on sorbent DBC. The experimental space is described in Table 3.

Table 3: Factors and levels used to define experimental space.

Factor	Low level	High level	Units
Feedstock titer	1	2	mg/mL
Residence time	3	5	min

Purified MAb was spiked into depleted CHO feedstock at 1 and 2 mg/mL and adjusted to pH 7.2 before loading. The spiked CHO feedstock was applied to a 1 mL Protein A column (MAb Select SuRe, GE Healthcare) with 3- and 5-minute residence times. Flow-through from the column was collected in 1 mL fractions. Buffer compositions and experimental steps are listed in Table 4.

Table 4: Experimental conditions for generation of breakthrough curves.

Category	Parameter	Value
Buffer	Loading	20 mM sodium phosphate, 150 mM NaCl, pH 7.2
	Equilibrate	10 column volumes of loading buffer, 1 minute residence time
Process	Load	80 column volumes of sample at desired concentration and residence time, pH 7.2
	Wash	5 column volumes of loading buffer, 1 minute residence time

Results

Comparison of Assay LoQ, LoD, Accuracy, Precision and Dynamic Range

Theoretical LoQ and LoD values for each assay were calculated as described in the Methods section. Results are summarized in Table 5.

As can be seen from a comparison of Tables 5 and 6, the theoretical calculations are confirmed. For both assays, total error increases far outside the acceptable range at the LoD, but is comfortably within the range at the LoQ.

Table 5: Theoretical assay LoD and LoQ.

HPLC		Octet® assay	
LoD (µg/mL)	**LoQ (µg/mL)**	**LoD (µg/mL)**	**LoQ (µg/mL)**
20.69	29.58	0.11	1.32

Table 6: Sample measurements at LoD and LoQ.

HPLC					
	Loaded conc. (µg/mL)	**Average value (µg/mL)**	**Bias (%)**	**CV (%)**	**Total error (%)**
LoD	17	21.53	26.67	2.33	31.32
LoQ	27	28.19	4.40	0.64	5.69
Octet® assay					
	Loaded conc. (µg/mL)	**Average value (µg/mL)**	**Bias (%)**	**CV (%)**	**Total error (%)**
LoD	0.1	0.18	82.37	39.63	161.63
LoQ	1.3	1.29	0.96	0.79	2.55

Accuracy and precision were then estimated over the expected dynamic range of the assay. %Bias, %CV and %error were calculated as described in the Methods section. Table 7 summarizes the findings over the dynamic range tested and includes the measurements made at the theoretical LoQ.

From these data, we can make an assessment of assay dynamic range. The arbitrary value of 10% total error was chosen as a cut-off point for either the upper or lower range of the assay. If no measurements in the lower range were above 10% total error, the LoQ was chosen as the assay's lower limit.

It is worth noting the difference in total error between the measured values on the HPLC assay at 31.25 µg/mL and 27 µg/mL (Table 7). The 6 readings used to validate the HPLC assay's theoretical LoQ were all taken in succession and thus have a very low CV. The six readings taken at 31.25 µg/mL were spread out over the 48 samples used to generate the six standard curves, and are therefore more indicative of how an actual assay is performed. For this reason the value of 62.5 µg/mL was used as the lower range of the HPLC assay.

For the Octet® assay, both the standard curve measurements and the measurements for the validation of the assay's theoretical LoQ were taken using different biosensors and at different stages of biosensor regeneration. As a result, the LoQ measurements and the standard curve measurements show better agreement. For this reason, the LoQ was chosen as the assay's lower limit.

Table 7: Error estimates for each analytical method over its dynamic range.

HPLC				**Octet® assay**			
Expected value (µg/mL)	**Bias (%)**	**CV (%)**	**Total error (%)**	**Expected value (µg/mL)**	**Bias (%)**	**CV (%)**	**Total error (%)**
2000	0.67	1.56	3.74	300	3.83	6.52	16.62
1000	2.51	1.66	5.76	150	0.87	3.19	7.12
500	0.25	1.05	2.30	75	0.27	1.57	3.34
250	1.45	1.68	4.74	37.5	0.80	1.70	4.13
125	2.79	0.99	4.74	18.8	0.35	2.42	5.11
62.50	4.38	1.45	7.23	9.38	0.25	0.70	1.62
31.25	6.08	5.43	16.71	4.69	1.42	0.64	2.68
27	4.40	0.64	5.69	1.3	0.96	0.79	2.55

Although no data were collected above 2000 µg/mL for the HPLC assay, the method has been validated up to 5000 µg/mL, so this was used as the top of the assay's analytical range. On the Octet® assay, 150 µg/mL was the highest value not to exceed 10% total error, so this measurement was used as the top of the assay's analytical range.

Considering this information, the HPLC assay dynamic range has minimum of 62.5 µg/mL and a maximum of 5000 µg/mL. This gives an 80-fold difference, which spans 1.9 orders of magnitude. The Octet® R8 system dynamic range has a minimum of 1.3 µg/mL and a maximum of 150 µg/mL. This gives a slightly larger 115-fold difference, which spans 2.1 orders of magnitude.

Comparison of Assay Costs

In generating the data for this application, 220 samples and 48 standards were analyzed, along with 2 blanks for each Octet® sample plate, and 4 blanks total for the HPLC Protein A run. Each assay also required its own preparatory work. For this comparison, it is assumed that the methods have been pre-written and saved, so programming time is not considered. Table 8 summarizes total time required to perform both assays.

Using the slowest process as a reference, we can see that the Octet® assay overall is almost 9 times faster than the HPLC assay. The Octet® assay offers savings not only in process time (11 times faster), but in prep time as well (3 times faster).

Using this approach we can also determine cost per sample. Buffer costs were determined from prices listed on the Sigma Aldrich catalog. Price per sample was calculated assuming 20% more buffer was made than was absolutely required to run the samples. Consumable costs include the Octet® Biosensors, HPLC Protein A column averaged over its usable life (3000 runs), and any filters and microtiter plates used in sample preparation. Operator costs were determined based on time required performing preparatory work, billed at a rate of $100 per person, per hour. Results are given in Table 9 in terms of cost per sample.

The data show that the Octet® cost per assay is significantly less than that of HPLC. The cheapest HPLC assay, using an auto-sampler that accepts 96-well plates, costs $1.21 per sample. The cheapest Octet® assay, employing user-prepared sample diluent, costs $0.93 per sample. This represents a cost reduction of >20%. This does not take into account column cleaning, regeneration or theoretical plate testing which add to the overall cost per sample when using HPLC.

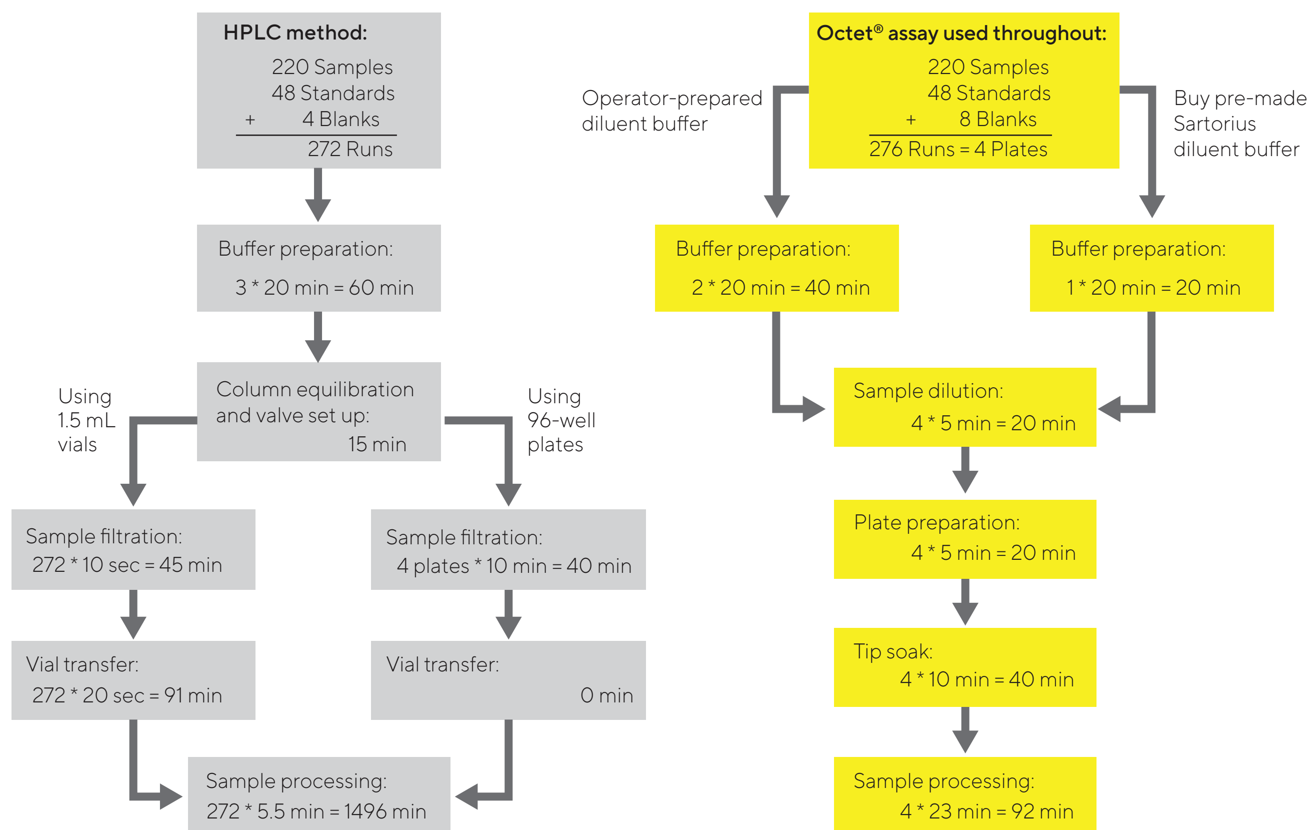

Table 8: Flow chart of process steps and step time summary.

	HPLC w/ vials	HPLC w/ plates	Octet® assay (prepare diluent)	Octet® assay (buy diluent)
Preparation time (min)	211	115	80	60
Process time (min)	1496	1496	132	132
Total time (min)	1707	1611	212	192

Table 9: Assay process cost comparison, given in terms of cost (in dollars) per sample.

	HPLC w/ vials	HPLC w/ plates	Octet® assay (prepare diluent)	Octet® assay (buy diluent)
Buffer cost	$ 0.0028	$0.0028	$ 0.0042	$0.3478
Consumable cost	$1.58	$0.50	$0.44	$0.44
Operator cost	$ 1.29	$0.70	$0.48	$0.36
Total	$2.87	$1.21	$0.93	$1.15

Determination of 5%, 10% and 20% Breakthroughs

The two assays were used to analyze the fractions collected from the breakthrough curve experiment. These curves are plotted in Figure 1 (A–H). The breakthrough curves were used to determine the DBC at 5, 10 and 20% breakthrough. As can be seen in Figure 1, the breakthrough curves span a MAb concentration from 0 mg/mL to 2 mg/mL. All samples were undiluted when processed on the HPLC, and diluted 10-fold when processed on the Octet® system. This protocol puts all the samples within the dynamic range of the HPLC assay, but puts some samples outside the range of the Octet® assay (>150 µg/mL). As seen in Figure 1F and Figure 1H, assay error visibly increases when breakthrough MAb concentration becomes greater than 1.5 mg/mL. When a 1 mg/mL feedstock is loaded, breakthrough curves show less variability, as can be seen in Figure 1B. Figure 1D shows more variability at higher concentrations, but values are still within the range of 7% total error predicted at the upper end of the Octet® assay.

At lower concentrations the two assays show better agreement in curve shape. It follows that the 5%, 10% and 20% breakthrough values calculated using the Octet® system very closely match the values measured using HPLC. As can be seen in Table 10, DBCs never differ by more than 2 mg MAb/mL sorbent. To accurately quantify samples that contain higher concentrations of MAb it is necessary to perform dilutions to bring the MAb concentration <150 µg/mL. However, the Octet® assay is most accurate at concentrations from 1.3–75 µg/mL and samples that require the highest accuracy should be diluted to within this range.

Although no dilution experiments were performed, there is no reason to suspect that there is a constraint on the maximum dilution factor that can be used with either assay. However, as the magnitude of dilution and number of dilution steps increase so does the error. In our experience we find two consecutive 10-fold dilutions (to give a 100-fold dilution) acceptable. Employing this as the maximum dilution limits the Octet® assay to MAb concentrations <15 mg/mL. For higher MAb concentrations, other Octet® assay parameters could be further optimized, most notably sample plate shake speed. As shake speed is lowered, binding rate decreases, rendering high concentration measurements more accurate. Modifying this parameter, however, is likely to change the dynamic range and the total error of the analysis.

In this study, we focused on the initial breakthrough, so a minimal dilution (10-fold) was performed to give us the most accurate measurements at the beginning of the breakthrough curve. We see no reason that an additional 10-fold dilution could not be performed to more accurately determine higher percent breakthroughs that might be required for continuous chromatography.

The data show us that, as observed previously, sorbent capacity increases with both an increase in feedstock titer and an increase in retention time. It is also apparent that within the range tested these variables are independent of each other. The highest capacity we measured was ~55 mg product per mL sorbent. This was achieved at high residence time (5 min) and high feed titer (2 mg/mL).

Table 10: Sorbent DBC (mg protein / mL sorbent) as calculated using either HPLC or the Octet® system for each of the four separate breakthrough curves.

HPLC				
	3 min RT (1 mg/mL)	5 min RT (1 mg/mL)	3 min RT (2 mg/mL)	5 min RT (2 mg/mL)
5% DBC (mg/mL)	30.3	38.3	42.6	46.6
10% DBC (mg/mL)	39.3	42.3	46.6	50.6
20% DBC (mg/mL)	42.3	46.3	50.6	54.6
Octet®				
	3 min RT (1 mg/mL)	5 min RT (1 mg/mL)	3 min RT (2 mg/mL)	5 min RT (2 mg/mL)
5% DBC (mg/mL)	32.3	38.3	42.6	46.6
10% DBC (mg/mL)	38.3	42.3	46.6	50.6
20% DBC (mg/mL)	42.3	45.3	48.6	54.6

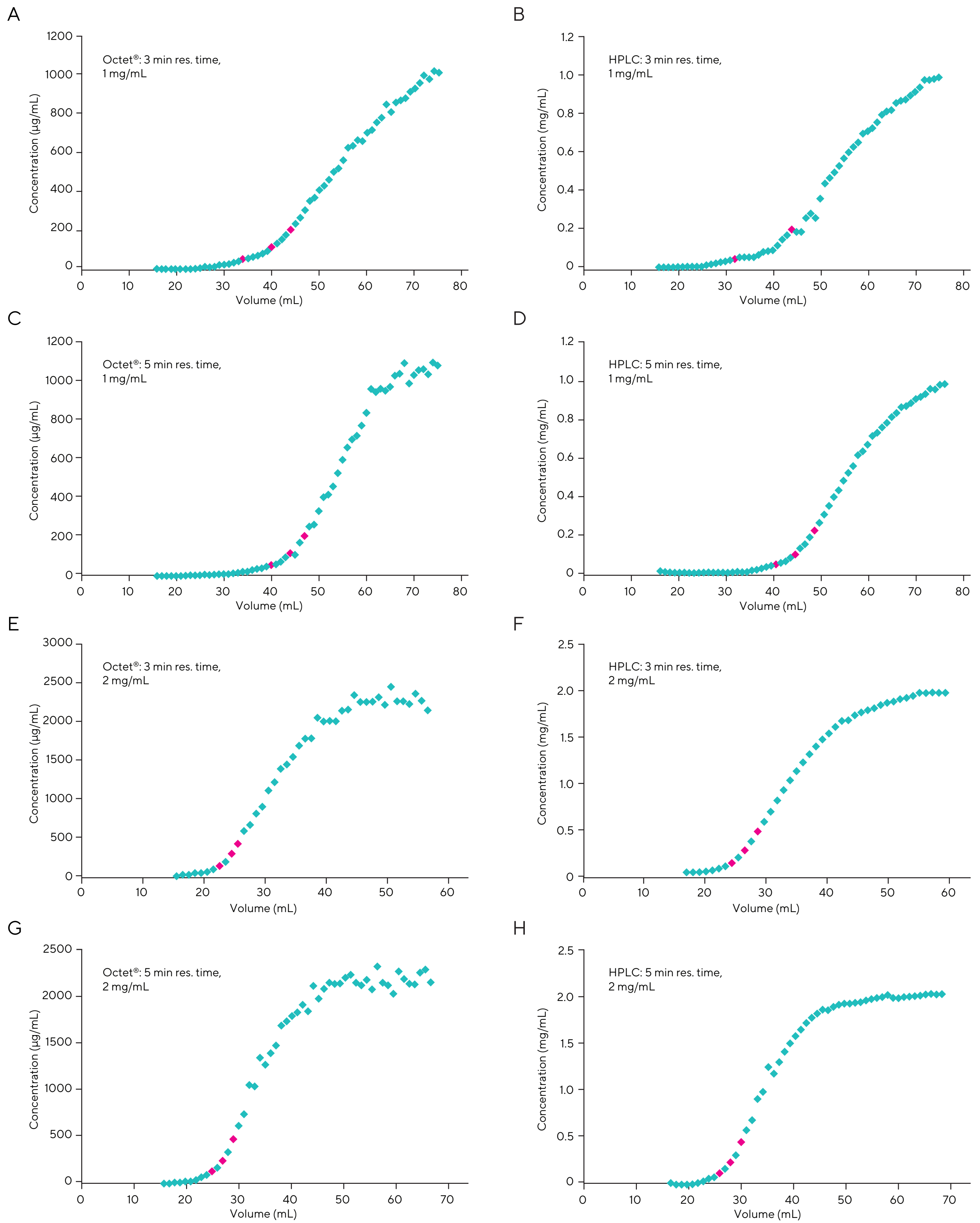

Figure 1: Breakthrough curves obtained using Octet® system or HPLC. Red points represent 5%, 10% and 20% breakthroughs as obtained from 1 mL fractions.

Conclusions

We have shown that the Sartorius Octet® R8 system can be used to accurately quantitate 5%, 10% and 20% MAb breakthrough values in the presence of contaminant host cell proteins. Compared to HPLC analysis, the Octet® system has a slightly larger dynamic range, and performs best at low concentrations with an almost 30-fold lower LoQ. This lower LoQ is an advantage for the Octet® platform when considering that sample dilution can be used to effectively expand the upper range of either assay. Another advantage to the Octet® system is the cost per assay, which is more than 20% lower in comparison to an equivalent HPLC assay. The greatest benefit in using the Octet® system is decreased process time. Switching from HPLC to Octet® R8 system reduced the total time for analysis from >24 hours to <4 hours.

The Octet® system is very well suited to the analysis of samples generated by process development. Here many conditions are screened to optimize the process. This generates a large number of samples which can be analyzed accurately, quickly and cheaply using the Octet® system and Protein A Biosensors.

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