

## Application Note

# Lowering Quantitation Limits for mAb Titer Measurements Using Small Volume 3.5 $\mu$ m Particle-Size Protein-A Affinity Columns

---

Stephan M. Koza, Steve Shiner, Matthew A. Lauber

Waters Corporation, United States

*Published on May 27, 2025*

Contact Sales

---

## Abstract

Protein A (ProA) affinity chromatography (PAAC) is often used for the measurement of monoclonal antibody (mAb) titer in cell culture conditioned media (CM) samples. Here we introduce high-pressure capable and low volume (70  $\mu$ L) ProA columns with efficient 3.5  $\mu$ m diameter non-porous particles packed into hydrophilic organosilica modified metallic hardware in either 2.1 x 20 mm or 3.9 x 5 mm configurations. These columns were compared to a commercially available ProA column comprised of 20  $\mu$ m diameter fully porous particles packed into 2.1 x 30 mm PEEK™ hardware. Linear ranges and lower limits of quantitation (LLQ) were evaluated for pure mAb standard loads (0.02 to 10  $\mu$ g and 0.01 to 0.2  $\mu$ g) and mock Chinese hamster ovary (CHO) CM samples representing a range of mAb titers (0.037 to 1.033 mg/mL). Total analysis cycle time per sample is approximately

2.3 minutes at a flow rate of 1.0 mL/min and 1.3 minutes at a flow rate of 2.0 mL/min.

## Benefits

- Up to a 5-fold to 7-fold reduction in lower limits of quantitation when low mAb titers and limited sample volumes restrict mAb loads
- Improved elution peak profile simplifies integration
- 5-fold to 9-fold higher signal-to-noise for lower limits of detection

---

## Introduction

Analytical ProA affinity columns are used to provide critical cell culture titer information during the manufacturing of mAb and other recombinant proteins with ProA affinity binding sites (*e.g.* fusion proteins). Additionally, higher throughput analytical PAAC is often deployed during mAb manufacturing process development, particularly when higher precision titer measurements are required.

Here, we present two recently developed high efficiency, low volume (70  $\mu$ L) ProA columns that are packed with 3.5  $\mu$ m non-porous particles in either a 2.1 x 20 mm or a 3.9 x 5 mm column configuration. The metal alloy hardware used to construct these columns is modified with hydrophilic MaxPeak™ High Performance Surfaces and is capable of operating at significantly higher column pressures (6,000 PSI, 414 bar) than PEEK hardware.<sup>1</sup> The 3.5  $\mu$ m non-porous polymer particle surfaces are also modified with a hydrophilic layer prior to attaching ProA ligands. While these surface modifications are critical to inhibit undesired interactions with the protein of interest, there are also a myriad of components in the CM sample that must also be thwarted from binding to the column including host-cell-proteins, DNA, RNA, and surfactants among others.

The two ProA column configurations were evaluated for linear range and LLQ alongside a commercially available ProA column comprised of 20  $\mu$ m diameter fully porous particles packed into 2.1 x 30 mm PEEK hardware (Column P). An extended NISTmAb calibration curve from 10  $\mu$ g to 0.02  $\mu$ g and a lower load calibration from 0.2  $\mu$ g to 0.01  $\mu$ g were assessed along with mock low-viability CHO CM samples representing titers from 1.033 mg/mL to 0.037 mg/mL NISTmAb.

---

## Experimental

### Sample Description

NISTmAb reference material, RM 8671 (10 mg/mL), and CHO conditioned media (clarified, 0.2 µm filtered, ~90% viability, with ~0.04 mg/mL trastuzumab).

Extended range calibration curves were generated for NISTmAb loads of 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05, and 0.02 µg, which were analyzed in duplicate. For this, NISTmAb was diluted to 1.0 and 0.1 mg/mL in PBS and intermediate points were generated by adjusting the injection volumes. Low range calibration curves were generated for NISTmAb loads of 0.2, 0.1, 0.05, 0.02, and 0.01 µg by adjusting the injection volumes of the 0.10 mg/mL standard, these were analyzed in triplicate.

Mock CM samples were prepared by spiking a CM sample purified NISTmAb (10 mg/mL) to 1.00 mg/mL and then 1:1 serially diluting with CM to attain NISTmAb spike levels of 1.0, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/mL. The clarified CHO cell CM sample had ~90% cell viability and a concentration of approximately 0.04 mg/mL trastuzumab. In this study, we treated this endogenous trastuzumab as NISTmAb for demonstration purposes and assigned the concentration as 0.037 mg/mL based on a NISTmAb calibration curve and 50 µL injection volumes of CM. Accounting for the dilution of the endogenous mAb, the predicted spiked mock CM sample concentrations were 1.033, 0.535, 0.286, 0.161, 0.099, and 0.068 mg/mL.

## Method Conditions

### LC Conditions

LC system:

ACQUITY™ Premier QSM UPLC™ with TUV System  
with 100 µL mixer, 15 µL + 50 µL sample loop,  
0.004 PEEK TUV inlet

For Low Range LLQ: 15 µL sample loop and  
0.0025 PEEK TUV inlet

Columns and connector:

BioResolve™ Protein A Affinity Column, MaxPeak  
Premier, 3.5 µm, 2.1 x 20 mm (p/n: 186011369)

	BioResolve Protein A Affinity Column, MaxPeak Premier, 3.5 $\mu\text{m}$ , 3.9 x 5 mm (p/n: 186011379) "Column P": 20 $\mu\text{m}$ porous, 2.1 x 30 mm PEEK™ Protein A column
Column temperature:	ambient
Sample temperature:	6 °C
Injection volume:	10 $\mu\text{L}$ or as indicated
ProA loading mobile phase:	Dulbecco's Phosphate Buffered Saline (DPBS) at 2x concentration, pH 7.4 (0.1 $\mu\text{m}$ sterile-filtered)
ProA elution mobile phase:	100 mM $\text{KH}_2\text{PO}_4$ titrated to pH 3.0 (HCl)
Flow rate:	1.0 or 2.0 mL/min
UV absorbance:	280 nm, 80 points/sec
Data management:	Empower™ Chromatography Data System (CDS)

### 1.0 mL/min ProA Analytical Gradient Table

Time (min)	Flow (mL/min)	%A	%B
Initial	1.0	100	0
1.00	1.0	100	0
1.10	1.0	0	100
1.6	1.0	0	100
1.7	1.0	100	0
2.0	1.0	100	0

### 2.0 mL/min ProA Analytical Gradient Table

Time (min)	Flow (mL/min)	%A	%B
Initial	2.0	100	0
0.50	2.0	100	0
0.55	2.0	0	100
0.80	2.0	0	100
0.9	2.0	100	0
1.0	2.0	100	0

---

## Results and Discussion

Calibration curves were generated using the 1.0 mL/min gradient for both Waters ProA column configurations (2.1 x 20 mm and the 3.9 x 5 mm) and for Column P (20  $\mu$ m, 2.1 x 30 mm). For the 2.0 mL/min gradient, the 2.1 x 20 mm Waters ProA column was not evaluated due to excessive back pressure. Representative chromatograms

of the three highest loads (10, 5, and 2  $\mu\text{g}$ ) and three lowest loads (0.1, 0.05, and 0.02  $\mu\text{g}$ ) of the extended range NISTmAb calibration are shown for 1.0 mL/min (Figure 1) and for 2.0 mL/min (Figure 2). The peak heights observed are 5-fold to 9-fold greater for the Waters ProA columns in comparison to Column P for the low load samples with the 2.1 x 20 mm Waters ProA at 1 mL/min having the highest elution peaks. While the improved signal-to-noise ratio (s/n) delivered by the Waters ProA columns can proportionally decrease the limits of detection (e.g.  $\text{LOD} = 3 \times \text{s/n}$ ), where peaks start and end, are also made more clearly discernible for these columns. Depending on chromatographic conditions, this capability may improve peak integration reliability.

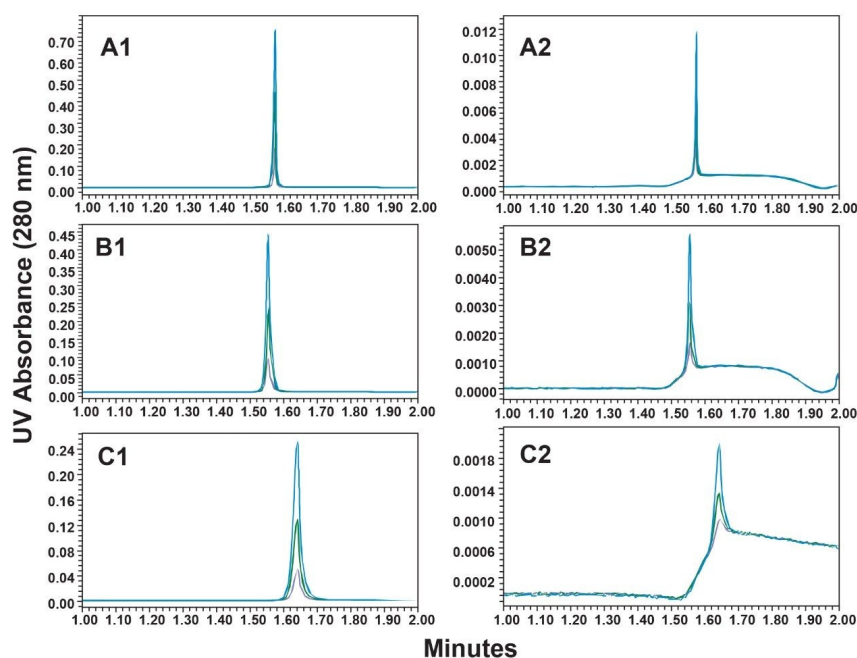


Figure 1. Overlays of high (10, 5, and 2  $\mu\text{g}$ ) and low load (0.10, 0.05, and 0.02  $\mu\text{g}$ ) ProA chromatograms from the extended calibration curve of NISTmAb are shown. The columns used are: Waters 2.1 x 20 mm column (A1 and A2), Waters 3.9 x 5 mm column (B1 and B2), and Column P (20  $\mu\text{m}$ , 2.1 x 30 mm PEEK column, C1 and C2) at a flow rate of 1.0 mL/min. Separation conditions are provided in text.

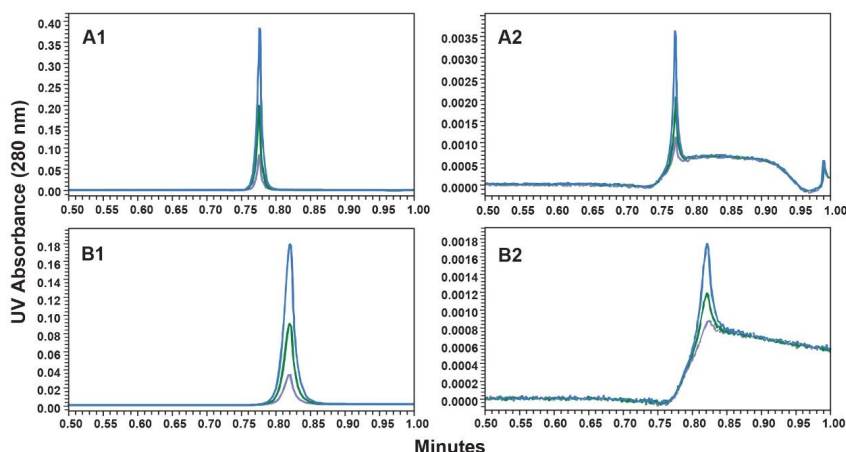


Figure 2. ProA chromatograms with a 1-minute analysis time at a flow rate of 2.0 mL/min are depicted for higher (10, 5, and 2 µg) and lower loads (0.10, 0.05, and 0.02 µg) from the extended calibration curve of NISTmAb. The columns used are: Waters 3.9 x 5 mm column (A1 and A2), and Column P (20 µm, 2.1 x 30 mm PEEK column, B1 and B2). Additional separation conditions are provided in text.

Table 1 provides the coefficients of determination ( $R^2$ ) and LLQ values of the extended and low range NISTmAb calibration curves generated for this study (see *Experimental* section). LLQ was calculated from the standard error of regression ( $s_{y/x}$ ) and the slope ( $m$ ) of the linear regression ( $LLQ = 10(s_{y/x}/m)$ ). The lowest loads were removed for the Column P LLQ and  $R^2$  determinations due to their low s/n resulting in excessive linear fit residuals. These results indicate that the Waters ProA columns deliver a 2-fold to 3-fold lower linear range for calibrations extending downward from a 10 µg load (Extended Range) and a remarkable 5-fold to 7-fold decrease in LLQ for 0.2 µg loads and lower (Low Range) in comparison to Column P. It should be noted that to obtain the 1 mL/min low range LLQ results the mixing and dispersion volumes of the UPLC were minimized (see *Experimental* section). Although not evaluated, Column P would be predicted to provide a higher upper linear loading range before exceeding the UV absorbance limit of the detector due to its reduced peak heights.

Table 1.	Calibration Curve					Mock CHO conditioned media		
Column	Flow rate (mL/min)	R <sup>2</sup> Extended range	R <sup>2</sup> Low range	LLQ Extended range (µg)	LLQ Low range (µg)	Flow rate (mL/min)	R <sup>2</sup> Extended range	LLQ Extended range (µg)
Waters 2.1 × 20 mm	1	0.99997	0.9998	0.16	0.05	1	0.99997	0.19
Waters 3.9 × 5 mm	1	0.99998	0.9999	0.13	0.06	1	0.99998	0.17
Column P, 2.1 × 30 mm	1	0.9997	0.9986	0.33	0.12	1	0.9997	0.41
Waters 3.9 × 5 mm	2	0.99998	0.9994	0.14	0.10	2	0.99999	0.20
Column P, 2.1 × 30 mm	2	0.9998	0.9991	0.44	0.18	2	0.9997	0.37

1. Extended range ≤10 µg NISTmAb and Low range ≤1 µg NISTmAb
2. Concentration levels (duplicate analyses): Waters Extended (9), Column P Extended (7), Waters low (6), Column P low (5), All mock (7)

The LLQ values of the two Waters ProA columns were comparable despite the s/n being 2-fold higher for the 2.1 × 20 mm configuration. This may be due in part to the narrower peak widths ( $W_{0.5} \approx 0.25$  sec) produced by the 2.1 × 20 mm column allowing for the collection of only 20 points across the top half of the peak when using a data collection rate of 80/sec. The Waters ProA columns also deliver greater peak area reproducibility at the lower load range as indicated by the significantly lower LLQ and higher R<sup>2</sup> values.

Analyses of a series of 7 mock CM samples (see *Experimental* section) ranging in predicted NISTmAb concentrations from 0.037 mg/mL to 1.033 mg/mL demonstrated comparable trends in R<sup>2</sup> and LLQ values as those observed between the columns for the extended calibration curves (Table 1). Representative chromatograms for the lowest concentration CM with 0.037 mg/mL of mAb (Figure 3) also show comparable mAb peak profile differences to those of the calibration standards. At this low load, a low-level pre-peak is observed for all three columns consistent with trace level retention of non-mAb CM components.

---

*Figure 3. ProA chromatograms of a CHO conditioned media sample containing 0.037 mg/mL mAb are compared. 1.0 mL/min (left frame) and 2.0 mL/min (right frame) separations are depicted. The Waters 2.1 × 20 mm, Waters 3.9 × 5 mm, and Column P (20 µm, 2.1 × 30 mm PEEK) columns were evaluated. Additional separation conditions are provided in text.*

---

Correlation plots shown in Figure 4 comparing the predicted concentration for the CM samples to the values

---



determined using the corresponding calibration curve demonstrate high correlation ( $R^2$ ) and minimal bias with slopes near 1.00 for linear fits forced through the origin. Deviations from the ideal slope value of 1.00 can be due to the stability of the mAb in CM as well as interactions of the mAb with components of the CM such as surfactants, DNA and RNA, and host-cell proteins, as well as variations in dilutions of the samples.

These comparisons highlight that the two Waters ProA columns can allow for reduced sample volumes while still providing comparable analytical performance at higher sample loads when compared to a leading ProA column constructed with 20  $\mu\text{m}$  porous particles packed into 2.1 x 30 mm PEEK hardware. The lower LLQ of the Waters ProA columns are primarily the result of the smaller volumes of the columns and of a more efficient packed bed without mass transfer due to the 3.5  $\mu\text{m}$  non-porous ProA media.

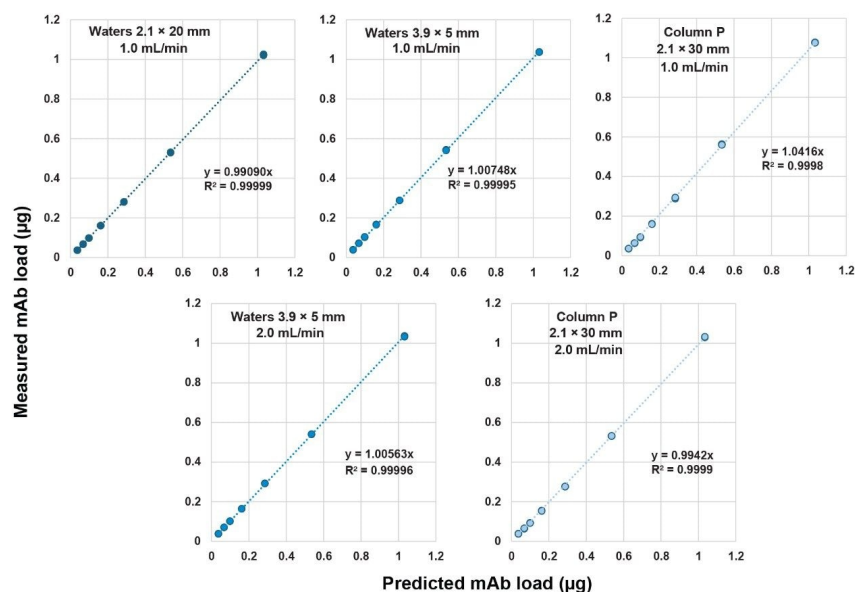


Figure 4. Correlation plots for the predicted NISTmab concentrations spiked into CHO cell culture sample versus the concentrations measured for the spiked samples using external NISTmAb ProA calibration curves. Column P is a commercially available 20  $\mu\text{m}$ , 2.1 x 30 mm PEEK ProA column. The characteristics of the external calibration curves are provided in Table 2. The columns and flow rates used are indicated. Linear fits for the correlation plots are forced through the origin.

---

## Conclusion

Waters BioResolve Protein A Affinity Columns can provide:

- Up to 5-fold to 7-fold lower LLQ values versus a ProA column constructed with 20 µm porous particles packed into 2.1 x 30 mm PEEK hardware while also delivering comparable performance at higher sample loads. A reduced LLQ can be advantageous when mAb concentrations are low and sample volumes are extremely limited, as when determining titer for milliliter scale micro-bioreactor samples
- Improved peak profiles that can increase peak integration reliability at lower sample loads
- 5-fold to 9-fold higher s/n for lower limits of detection

---

## References

1. Kizekai, L., Shiner, S.J. and Lauber, M.A. Waters ACQUITY and XBridge Premier Protein SEC 250 Å Columns: A New Benchmark in Inert SEC Column Design. Waters Application Note. [720007493](#). January 2022.

---

## Featured Products

Empower Chromatography Data System (CDS) <<https://www.waters.com/nextgen/global/products/informatics-and-software/chromatography-software/empower-software-solutions/empower-cds.html>>

ACQUITY Premier Tunable UV Detector <

<https://www.waters.com/nextgen/global/products/chromatography/chromatography-detectors/acquity-uplc-and-acquity-premier-tunable-uv-detectors.html>>

720008775, May 2025



© 2025 Waters Corporation. All Rights Reserved.

[Terms of Use](#) [Privacy Notice](#) [Trademarks](#) [Careers](#) [Legal and Privacy Notices](#) [Cookies](#)  
[Cookie Preferences](#)