

A Trap & Elute Style 2D Protein A–SEC Heart-Cut Method for the Analysis of Monoclonal Antibody Titer and Size Variants in Cell Culture

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Abstract

In this application note, simplified trap-and-elute style single heart-cut Protein A affinity and size-exclusion chromatography (ProA-SEC) 2D methods for the analysis of monoclonal antibody (mAb) titer in CHO-cell conditioned media (CM) are presented. In addition to titer, a high-throughput (HT) version of the ProA-SEC method is used to monitor high molecular weight size variants (HMWS) while a high-resolution (HR) version is used to monitor both HMWS and low molecular weight size variants (LMWS) possessing ProA affinity. These methods employ high-pressure-capable (414 bar or 6000 PSI) and efficient small volume (~70 µL) ProA columns (nonporous 3.5 µm, 2.1 X 20 mm, and 3.9 X 5 mm) that enable the direct loading of eluted mAb onto high performance 1.7 µm and 2.5 µm particle-size SEC columns. Direct loading allows for increased recovery and more timely pH neutralization. In addition, these small volume ProA columns can deliver a highly concentrated mAb peak to a subsequent SEC column, which increases the resolution of the SEC size variant separation. Method set-up and analytical performance metrics are highlighted, including linearity, recovery, and the veracity of the

size variant determinations.

Benefits

- Simplified 2D ProA-SEC method of mAb titer and size variants using a BioResolve™ Protein A Affinity Column, a single 6-port valve, two UPLC™/UHPLC pumps, and no holding loops
- Rapid 5-minute analysis of mAb titer and HMWS of CM samples
- Analysis of mAb titer, HMWS, and LMWS (100 KDa fragment) in under 20 minutes

Introduction

Analytical ProA affinity columns have been routinely used to monitor the titer of mAb and other Fc containing constructs such as fusion proteins and bi-specific antibodies in CM. In addition, ProA columns have also been deployed as an online 2D purification for SEC to monitor both mAb titer and mAb size variant levels in cell culture by either connecting the two columns in series or via switching valves.¹⁻³

Here, presented, are two 2D methods that make use of two recently developed high efficiency, low volume ProA columns. These ProA columns are packed with 3.5 µm non-porous particles in 2.1 X 20 mm and 3.9 X 5 mm column configurations and are capable at operating at column pressures of 6000 PSI (414 bar). In addition, the metal surfaces of the column hardware and frits, and the packed particles are modified to minimize undesired protein-surface interactions under a broad range of conditions.⁴⁻⁵ For a high-resolution 2D ProA-SEC (HR ProA-SEC) separation, the 2.1 X 20 mm ProA column was paired with a 1.7 µm, 4.6 X 300 mm SEC column. For a high-throughput 2D ProA-SEC (HT ProA-SEC) separation, the 3.9 X 5 mm ProA column was paired with a 2.5 µm, 7.8 X 150 mm SEC column. The LC system and valve configuration for these methods are shown in Figure 1. This configuration allows for the direct loading of the mAb peak eluted from the ProA column onto the SEC column, which is made possible by the high-pressure capabilities of the ProA columns.

The 2D HR ProA-SEC and HT ProA-SEC methods were assessed for titer determination and HMWS abundance over a range of NISTmAb concentrations spiked into clarified CM. Additionally, the HR ProA-SEC configuration was evaluated for the analysis of LMWS.

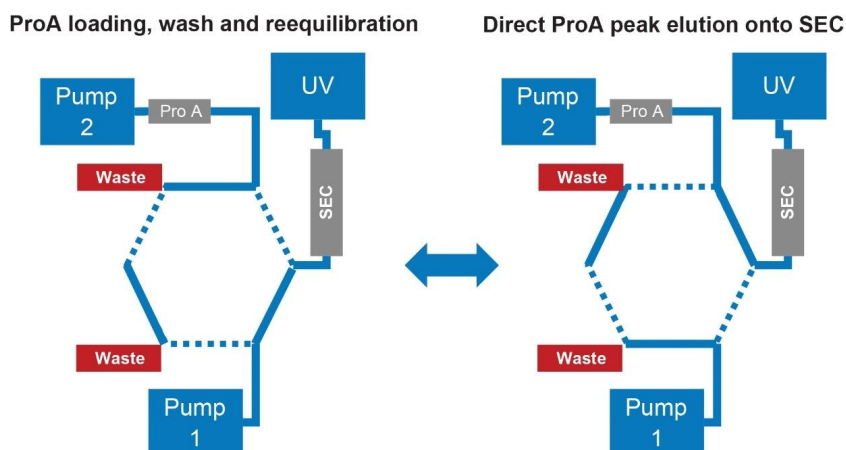


Figure 1. The trap-and-elute single 6-port valve 2D configuration with direct loading of a heart-cut of the ProA elution peak onto the SEC column is diagrammed.

Experimental

Sample Description

NISTmAb reference material, RM 8671 (10 mg/mL) and CHO cell CM, clarified, 0.2 μ m filtered, ~90% viability, with ~0.035 mg/mL trastuzumab).

Method Conditions

LC Conditions

LC system:

ACQUITY™ UPLC H-Class System with ACQUITY Column Manager (w/ 6-port valve) and additional ACQUITY UPLC I-Class Binary Solvent Manager (pump), ACQUITY Tunable UV (TUV) Detectors (5

	mm flow cell)
Columns:	<p>BioResolve Protein A Affinity Column, MaxPeak™ Premier, 3.5 µm, 2.1 x 20 mm (p/n: 186011369)</p> <p>BioResolve Protein A Affinity Column, MaxPeak Premier, 3.5 µm, 3.9 x 5 mm (p/n: 186011379)</p> <p>ACQUITY Premier Protein SEC Column, 250 Å, 1.7 µm, 4.6 x 300 mm (p/n: 186009964)</p> <p>XBridge™ Premier Protein SEC Column, 250 Å, 2.5 µm, 7.8 x 150 mm (p/n: 186009961)</p> <p>“Column P”: 20 µm porous, 2.1 X 30 mm PEEK™ Protein A column</p>
Column temperature:	ambient
Sample temperature:	6 °C
Injection volume:	10 µL or as indicated
ProA loading mobile phase:	1.0 mM KH ₂ PO ₄ , 3.0 mM Na ₂ HPO ₄ , and 155 mM NaCl, pH 7.4
ProA elution mobile phase:	100 mM KH ₂ PO ₄ titrated to pH 3.0 (HCl)
SEC mobile phase:	35 mM K ₂ HPO ₄ , 65 mM KH ₂ PO ₄ , 100 mM KCl, pH 7.0
Detector:	TUV, 280 nm or 214 nm as noted
Data management:	Empower™ Chromatography Data System

3.9 X 5 mm ProA HT Gradient Table

Time (min)	Flow (mL/min)	%A	%B
Initial	1.0	100	0
0.65	1.0	100	0
0.66	1.0	0	100
1.16	1.0	0	100
1.17	1.0	100	0
1.5	1.0	100	0

2.1 X 20 mm ProA HR Gradient Table

Time (min)	Flow (mL/min)	%A	%B
Initial	0.2	100	0
1.75	0.2	100	0
1.76	0.2	0	100
3.25	0.2	0	100
3.26	0.2	100	0
5.00	0.2	100	0

Results and Discussion

As a first step in developing a 2D ProA-SEC method, the chromatographic profiles produced by step elution were evaluated from the 2.1 X 20 mm and 3.9 X 5 mm ProA column configurations (Figure 2). These profiles were also compared to a commercially available ProA column comprised of 20 μ m porous particles packed into 2.1 X 30 mm PEEK hardware (Column P). The mobile phases used (see Experimental) were adapted from published results for the ProA-SEC analysis of NISTmAb.³ Here, observations determined that the 2.1 X 20 mm ProA column generated peak heights approximately 3-fold higher than Column P and 2-fold higher than the 3.9 X 5

mm column, along with concomitantly smaller peak volumes.

Based on these peak volume results, the 2.1 X 20 mm ProA column was paired with a 1.7 μm , 4.6 X 300 mm SEC column to create a 0.2 mL/min flow rate HR ProA-SEC method. In an alternative setup, a 3.9 X 5 mm ProA column was paired with the 2.5 μm , 7.8 X 150 mm SEC column to establish a 1.0 mL/min flow rate HT ProA-SEC method. Waters ACQUITY and XBridge Premier 250 Å SEC Columns were selected for this work due to their effective performance for mAb separations under a broad range of conditions.⁵ Representative chromatograms are shown in Figure 3 for the SEC and the 2D ProA-SEC separations. Comparable chromatographic profiles were observed for the 2D ProA-SEC separations versus SEC alone, and greater than 97% of the monomer was recovered. Low molecular weight species (LMWS2) were not observed in the ProA-SEC profiles as it is predominantly Fab, which in NISTmAb does not have affinity toward Protein A.

It has been previously reported that HMWS recovery and method-induced HMWS can be significant challenges for samples purified by ProA.³ The propensity of these 2D ProA-SEC methods to induce HMWS formation was assessed by capturing SEC-purified NISTmAb monomer directly onto ProA using a typical valve-based 2D configuration, and then eluting the trapped monomer onto the SEC column using the ProA-SEC method. The results of this study are shown in Figure 4 and Table 1. Less than 0.2% of method induced HMWS was observed, which is estimated to be near the limit of quantification (LOQ) of this method. Based on the monomer recoveries, method-induced HMWS levels, and the HMWS levels observed for SEC alone, the NISTmAb HMWS recoveries were estimated to be 85% for the proposed ProA-SEC methods. It was also noted that minor changes in the abundance of HMWS were observed for NISTmAb upon long-term storage in the autosampler. As a result, the relative recoveries of HMWS were based on same-day analyses.

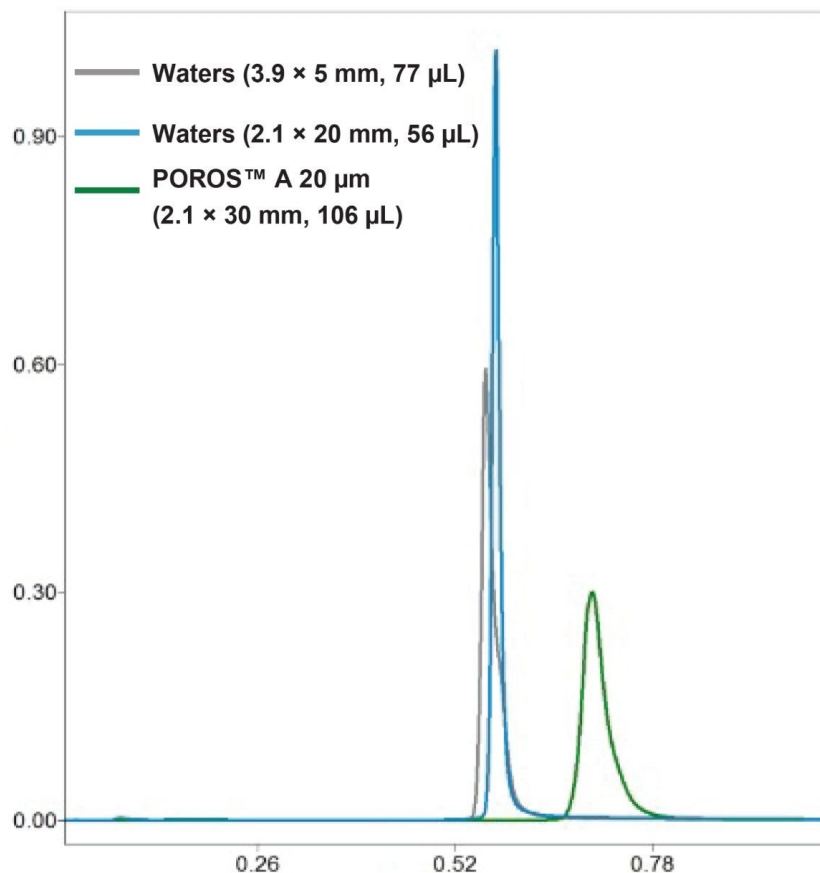


Figure 2. Separations for the ProA columns evaluated are shown for NISTmAb (10 µg). Flow rates were 0.5 mL/min and the proA columns were eluted with a 0.01 min gradient. ProA mobile phases are provided in the text. Estimated peak volumes (µL) are provided in the legend.

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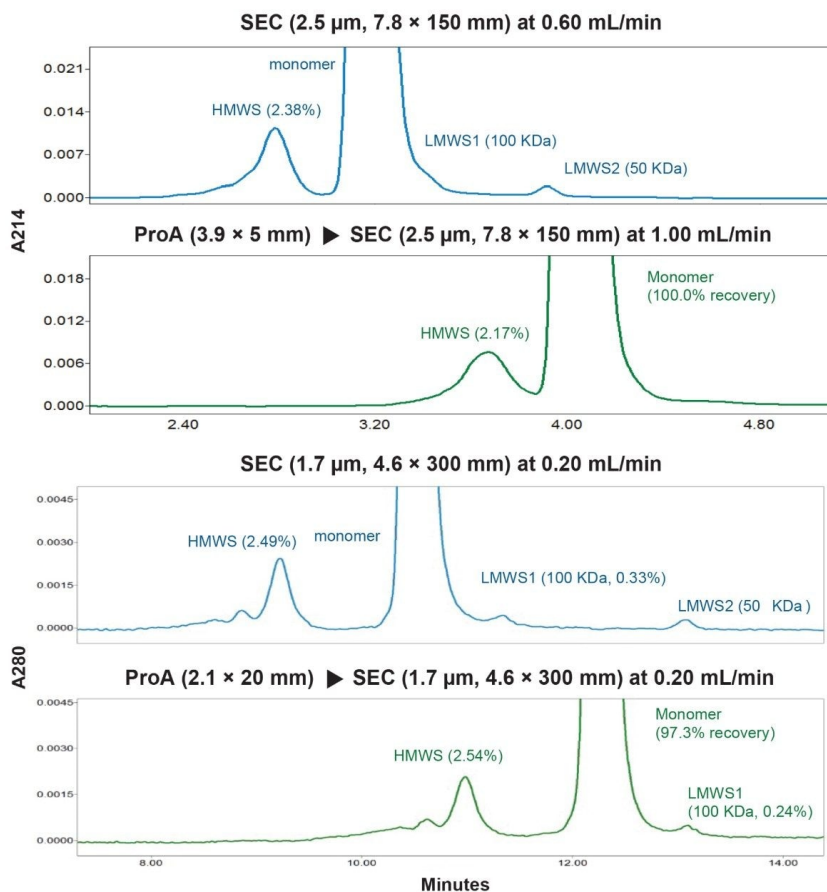


Figure 3. SEC and 2D ProA-SEC comparisons for the analysis of HMWS are shown for NISTmAb (10 µg). Columns and flow rates are shown in the figure. The ProA columns were eluted with a 0.01 min gradient. ProA mobile phases are provided in the text.

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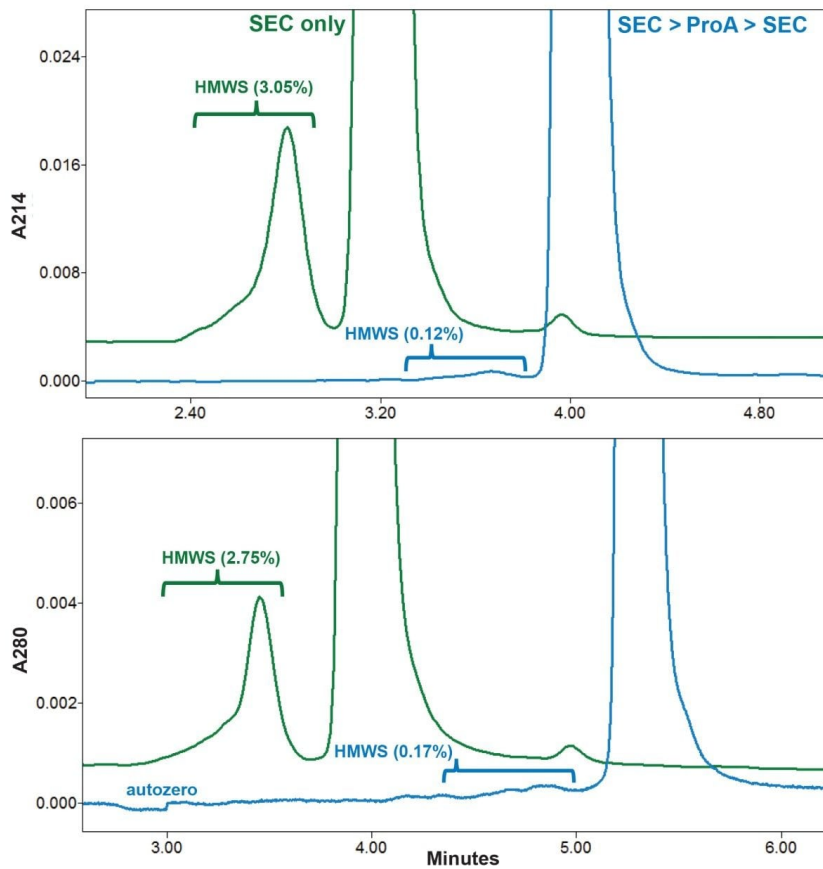


Figure 4. Evaluations of method-induced HMWS for 2D ProA-SEC are shown for NISTmAb (10 μ g). A heart-cut of the mAb monomer from the SEC separation (green) is first captured on ProA, column positions are swapped and then mAb is eluted from ProA onto SEC (blue). Top frame: 3.9 X 5.0mm ProA and 2.5 μ m, 7.8 X 150 mm SEC at 1.0 mL/min. Bottom frame: 2.1 X 20 mm ProA and 1.7 μ m, 4.6 X 150 mm SEC at 0.3 mL/min. ProA columns eluted with a 0.1 min gradient. SEC and ProA mobile phases are provided in the text.

Table 1

Method	ProA	SEC	%HMWS (method induced)	%HMW	HMWS % recovery	Monomer % recovery
UP-SEC	none	4.6 × 150		2.75		
2D UPLC	2.1 × 20	4.6 × 150	0.16	2.75	84	97
HP-SEC	none	7.8 × 150		3.05		
2D HPLC	3.9 × 5	7.8 × 150	0.12	2.73	86	100

This was a marked improvement in comparison to the previously published holding-loop based 2D ProA-SEC results for NISTmAb, demonstrating recoveries of 94% for monomer and 32% for HMWS, while method-induced HMWS was not reported for NISTmAb.³ This improvement is likely due to the greater precision with which the ProA elution peak is delivered to the SEC column, thereby reducing the overall volume of low pH buffer being introduced to the SEC column. It is also predicted that the more immediate dilution and neutralization afforded by directly loading the ProA peak onto the SEC column would help minimize method-induced HMWS formation. Regardless of these improvements, these results highlight that compromises between HMWS recovery and the generation HMWS artifacts may be difficult to circumvent when developing a 2D ProA-SEC method and underscores the importance of understanding the recoveries and method-induced artifacts when developing such a method. Accordingly, the HMWS levels determined by ProA-SEC methods may often be more appropriate for comparing relative differences in HMWS between samples.

Evaluation of the quantitative performance of the ProA-SEC methods focused on size-variant and titer measurements of NISTmAb spiked into CM. Mock CM samples were generated by spiking purified NISTmAb (10.0 mg/mL) at concentrations of 1.0, 0.50, 0.25, and 0.125 mg/mL into a CM sample containing 0.035 mg/mL trastuzumab and with ~90% cell viability. However, for demonstration purposes, this endogenous mAb is treated as an unknown.

Representative chromatograms of the mock CM samples are presented in Figure 5. UV absorbance for the HT configuration was set to 214 nm (A214), while for the HR configuration, 280 nm (A280) was used to monitor the monomer and A214 was used to monitor the size variants for improved detection limits. The peak areas were then normalized using an A214/A280 ratio of 14.10 determined by SEC. As shown in Figure 6, comparable HMWS abundances were observed at varying mAb concentration levels for both methods. While LMWS1 appears as a shoulder for the HT method, it could only be reliably integrated with the HR method.

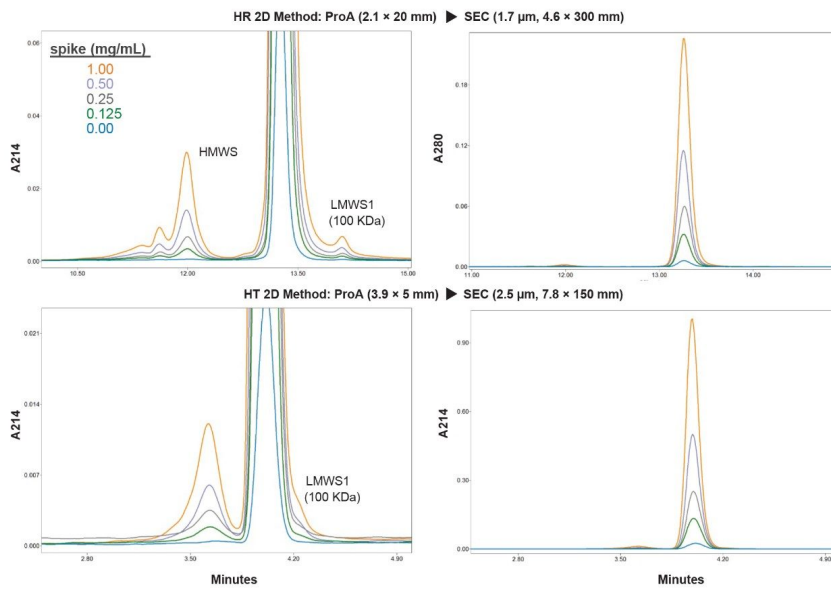


Figure 5. Zoomed and full scale views of 2D ProA-SEC analyses of titer and HMWS are presented for NISTmAb spiked into CM at levels of 0.125, 0.25, 0.50, and 1.00 mg/mL. The concentration of mAb in the unspiked CM sample was 0.035 mg/mL. LMWS1 was also assessed with the HR 2D method. The flow rates were 0.20 mL/min for the HR 2D method and 1.00 mL/min for the HT 2D method, and injection volumes were 10 μ L. Quantitative results are shown in Figures 5 and 6.

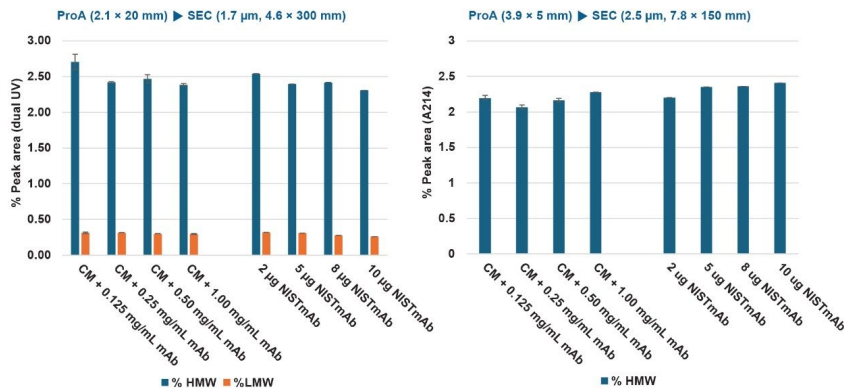


Figure 6. Shown are 2D ProA/SEC %HMWS and %LMWS1 determinations for the HR 2D (left) and HT 2D (right) experiments presented in Figure 5. LMWS1 could only be determined reliably for the HR 2D method for sample loads $\geq 2 \mu\text{g}$. The values determined for the HR 2D method used normalized A280 monomer peak area and A214 HMWS and LMWS1 peak areas to determine the reported relative abundances. Error bars represent the range of the result ($n=2$).

The capability to determine mAb titer was also assessed using the mock CM samples. The spiked CM standard-additions response curves for the HT and HR ProA-SEC methods are shown in Figure 7. Here, high coefficients of correlation (R^2) for a linear fit are seen. The X-intercepts, calculated from the linear equation, resulted in CM titer assignments of 0.030 mg/mL of mAb for the HT method and 0.036 mg/mL for the HR method. Based on these results, the predicted titers for the spiked mock CM samples were calculated. The titers of the mock CM samples were then measured using external calibration curves generated with unadulterated NISTmAb for both ProA-SEC methods. The slopes of correlation plots comparing the predicted mAb concentrations in the mock CM samples and the measured concentrations were 0.94 for the HT method and 0.97 for the HR method, indicating minimal bias in the analyses (Figure 7). Deviations from the ideal slope value of 1.00 can be attributed 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 that may result in loss of protein sample prior to analysis or attenuated protein A binding.

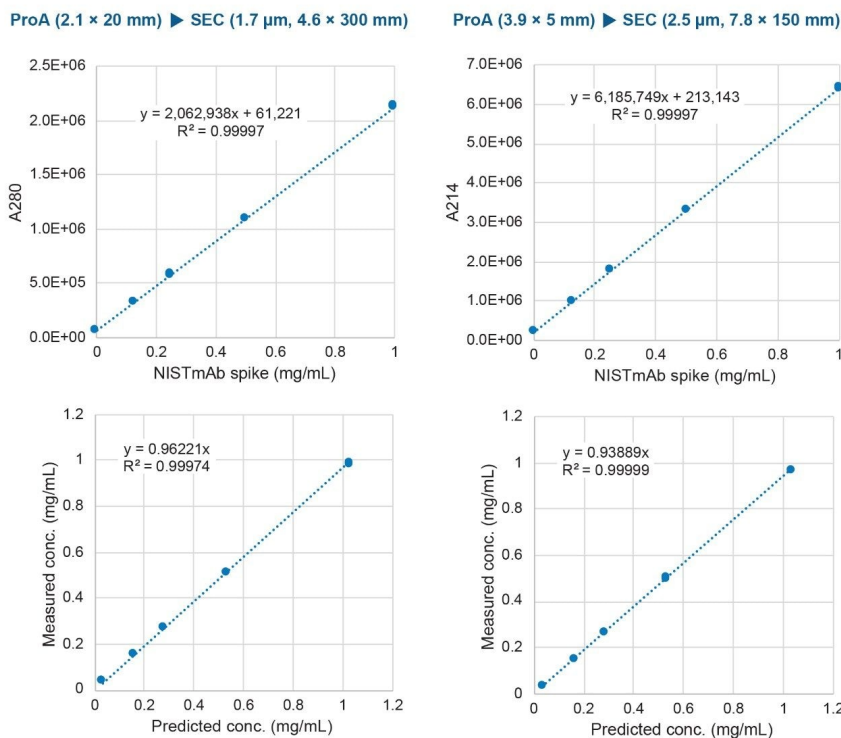


Figure 7. Shown are 2D ProA-SEC titer determinations for the HR and HT 2D experiments presented in Figure 5 ($n=2$). The NISTmAb spiked CM sample linearity (standard additions) curves are presented in the top graphs. The bottom graphs show the correlation between the titer determined for the spiked samples using an external calibration curve of NISTmAb compared to the predicted values. Injection volumes were 10 μL .

Based on regression analysis of the mock CM sample standard additions plots, the lower limits of quantitation (LLQ) for titer determinations using the HT and HR ProA-SEC methods were both approximately 0.2 μg mAb. LLQ was calculated from the standard error of regression ($s_{y/x}$) and the slope (m) of the linear regression at a 95% confidence level ($\text{LLQ}=10(s_{y/x}/m)$). It would be predicted that the monomer LLQ for the HR method would be lower if A214 monomer peak areas were used; however, that would reduce maximum sample loads, which would reduce the LLQ levels for the size variants, which are measured using A214 for the HR method.

Evaluation of relative size variants at a 90% confidence level will require estimated minimum sample loads of 0.06 μg HMWS for the HT method while the HR 2D method will require 0.04 μg HMWS and 0.004 μg LMWS. The

lower LLQ for LMWS is due to its decreased size heterogeneity versus the HMWS peaks observed. Higher sample loads resulting in peaks that remain within the linear range of the detector can deliver more precise results and should not exceed the more than 200 µg mAb dynamic binding capacity of these ProA columns.

Conclusion

An effective and easy-to-execute 2D ProA-SEC methodology using a standard UHPLC system outfitted with an auxiliary pump and a single switching valve is presented for the analysis of CM samples for both mAb titer and to comparatively assess size variant (HMWS and LMWS) abundances. While the initial results are promising, analysts should consider evaluating specific mAb and size-variant recoveries, in addition to method-induced HMWS, as a step toward establishing their own fit-for-purpose methods. The benefits of this approach are:

- Less complicated 2D ProA-SEC setup and more rapid method development versus 2D methods that rely on holding loops
 - Reduced peak dispersion prior to the SEC separation, more immediate ProA peak neutralization to minimize method-induced HMWS, and improved SEC performance due to lower acidic ProA peak volume transferred
 - A total high-throughput analysis time of approximately 5 minutes to determine mAb titer and HMWS levels in CHO cell conditioned media samples
 - High-resolution analysis of mAb titer, HMWS, and LMWS possessing Protein A affinity (*e.g.* 100 KDa fragment) in under 20 minutes
 - Applicability to other constructs with ProA affinity such as fusion proteins and multispecific mAb constructs
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References

1. Lemmerer, M., London, A.S., Panicucci, A., Gutierrez-Vargas, C., Lihon, M. and Dreier, P. 2013. Coupled affinity and sizing chromatography: a novel in-process analytical tool to measure titer and trend Fc-protein aggregation. *Journal of Immunological Methods*, 393(1–2), pp. 81–85.
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2. Gjoka, X., Schofield, M., Cvetkovic, A. and Gantier, R. 2014. Combined Protein A and size exclusion high performance liquid chromatography for the single-step measurement of mAb, aggregates and host cell proteins. *Journal of Chromatography B*, 972, pp.48–52.
3. Dunn, Z.D., Desai, J., Leme, G.M., Stoll, D.R. and Richardson, D.D. 2020, January. Rapid two-dimensional Protein-A size exclusion chromatography of monoclonal antibodies for titer and aggregation measurements from harvested cell culture fluid samples. *In MAb*s (Vol. 12, No. 1, p.1702263). Taylor & Francis.
4. Kizekai, L., Shiner, S.J. and Lauber, M.A. 2022. Waters ACQUITY and XBridge Premier Protein SEC 250 Å Columns: A New Benchmark in Inert SEC Column Design. Waters Application Note [720007493](#).
5. Koza, S. M., Yang, H. and Yu, Y.Q. 2022. Expanding Size-Exclusion Chromatography Platform Method Versatility for Monoclonal Antibody Analysis Using Waters XBridge Premier Protein SEC Columns. Waters Application Note [720007500](#).

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