

## Automated High-Throughput Analytical-Scale Monoclonal Antibody Purification Using Production-Scale Protein A Affinity Chromatography Resin

---

Stephan M. Koza, Caitlin M. Hanna, Albert H. W. Jiang, Ying Qing Yu

Waters Corporation

---

### Abstract

An automated analytical-scale Protein A affinity based purification of monoclonal antibody (mAb) from Chinese hamster ovarian (CHO) cell conditioned media is described. This cost effective procedure was demonstrated to purify between 120 µg to 240 µg of mAb using the analyst's choice of process-scale Protein A resin, a 96-well 0.2 µm filtration plate, and an orbital plate shaker. While the multiple pipetting steps (12 steps/sample) of the purification method can be performed manually, the demands on the analyst's time, tediousness of the purification, and potential for errors, are greatly reduced when using the Andrew+ robotic platform, taking approximately 1 hr for 48 samples. In addition to deployment of a developed and vetted purification protocol, the intuitive OneLab visual programming interface for the Andrew+ robot can facilitate the optimization and evaluation of this and similar plate-based procedures.

The finalized procedure yields a purified and neutralized mAb sample with a concentration of 1.02 µg/µL or greater when loading 120 µg (≥85% recovery in 100 µL) and 2.27 µg/µL when loading 240 µg (≥94% recovery in 100 µL). An assessment of this procedure as a sample pretreatment for size variant analysis (size-exclusion

---

chromatography, SEC) and released N-glycan profiling using LC-MS is also presented.

## Benefits

- High throughput, automated analytical-scale (120 µg to 240 µg) Protein A affinity batch purification of mAb from cell culture with high sample recovery (≥85%)
- Use of the analyst's choice of process-scale Protein A or other affinity chromatography resin (Protein L or G)
- Preparation time of approximately 1 hr for 48 samples
- Effective removal of host cell proteins and other interferences for SEC and released N-Glycan analysis

---

## Introduction

The high-throughput analytical-scale purification of therapeutic recombinant monoclonal antibodies (mAb) from cell culture samples can be essential to support the development of their cell culture processes.<sup>1</sup> Reliable and reproducible purification can add to the success of analytical methods such as size-exclusion chromatography (SEC), released N-glycan analysis, peptide mapping, and other methods where the conditioned media components, host-cell proteins, and nucleic acids may interfere with sample preparation or the analysis.

Numerous approaches and formats for the analytical-scale purification of mAbs have been developed in the preceding decades using Protein A affinity capture. The primary goals of this Protein A purification study was to develop an automated method that minimizes mAb mass requirements and maximizes recovery along with final mAb concentration, all while using the analyst's choice of process-scale Protein A resin.

---

## Experimental

Preparations of mAb (trastuzumab) were obtained from various sources and diluted into phosphate buffered saline (PBS) or non-transfected conditioned (14-day) CHO cell media (NTM) to indicated concentrations. NTM was prepared with the assistance of Syd Labs, Inc. using non-transfected CHO-K1 cells in a spinner flask. Spent media was collected from the flask on days 2 through 15 (~90% average cell viability), pooled, and 0.2 µm filtered.

## Protein A

Robotic system:	Andrew+ Pipeting Robot with Extraction+ Module
Filter plate:	Pall™ AcroPrep™ Advance 96-well Filter Plates - 350 µL, 0.2 µm Supor™ membrane (Product ID: 8019)
Collection plate:	Waters QuanRecovery™ 700 µL 96-well plate (p/n: 186009184)
Protein A Resin:	Cytiva MabSelect™ (p/n: 17519901), slurries are ~25%  (1:1, PBS:50%). for 50% resin, centrifuge at 1000 g for 3 minutes and replace supernatant with volume of 400 mM NaCl, 20% ethanol equal to resin volume.
PBS:	phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 8 mM Na <sub>2</sub> HPO <sub>4</sub> , and 2 mM KH <sub>2</sub> PO <sub>4</sub> , pH 7.4
NB:	neutralization buffer: 1M Tris, pH 7.5
EB:	elution buffer: 100 mM glycine, pH 3.0
Orbital shaker:	Eppendorf ThermoMixer® C (8 °C)
Software:	OneLab (Andew Alliance/Waters)

## SEC

LC system:	ACQUITY Premier UPLC with Binary Management
------------	---

(BSM or QSM) with CH-A column heater or  
a BioAccord™ LC-MS (ESI-ToF) system

Detection: ACQUITY UPLC TUV Detector with 5 mm titanium  
flow cell, wavelength: 280 nm

Vials: Polypropylene 12 x 32 mm Screw Neck Vial, with  
Cap and Pre-slit PTFE/Silicone Septum, 300 µL  
Volume, 100/pkg (p/n: 186002639)

Column(s): ACQUITY Premier Protein SEC 250 Å, 2.5 µm, 4.6 x  
150 mm, Column plus mAb Size Variant Standard  
(p/n: 176004783)

Column temp.: 25 °C

Sample temp.: 6 °C

Injection volume: 5 µL

Flow rate: 0.5 mL/min

Mobile phase: ammonium acetate, LC-MS grade (Supelco  
LiChropur™, eluent additive for LC-MS, 73594), 0.1  
µm sterile filtered, 200 mM or as indicated

Chromatography software: Empower™ 3 (FR 4)

## LC Conditions

LC system: ACQUITY UPLC I-Class PLUS

Sample collection: Waters QuanRecovery™ 700 µL 96-well plate p/n: 186009184

Column: ACQUITY UPLC Glycan BEH™ Amide Column p/n: 186004742 (1.7 µm, 2.1 mm x 150 mm, 130 Å)

Column temp.: 60 °C

Sample temp.: 6 °C

Injection volume: 15 µL

Mobile phase A: 50 mM Ammonium Formate, pH 4.4 (LC-MS grade, p/n: 186007081)

Mobile phase B: Acetonitrile

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	1.0	25	75	Initial
3.50	1.0	42	58	6
3.55	1.0	60	40	6
3.75	1.0	60	40	6
3.80	1.0	25	75	6
5.00	1.0	25	75	6

## ACQUITY RDa Detector Settings

Mass range: 400–7000 *m/z*

Mode:	ESI+
Sample rate:	10 Hz
Cone voltage:	45
Desolvation temperature:	300
Capillary voltage:	1.50 kV
Informatics:	Accurate Mass Screening using a glycan database

## Data Management

Chromatography software:	waters_connect
--------------------------	----------------

---

## Results and Discussion

### Method Development

An automated mAb purification method was successfully adapted from previously described filter plate based approaches for Protein A affinity and other modes of chromatography.<sup>1-3</sup> An outline of the basic protocol used is shown in Figure 1. This relatively simple procedure requires 12 pipetting steps and four incubation steps per sample. In brief, the optimization of the presented automated mAb Protein A purification included evaluations of binding and elution mechanics, volumes, and times, in addition to elution buffer pH.

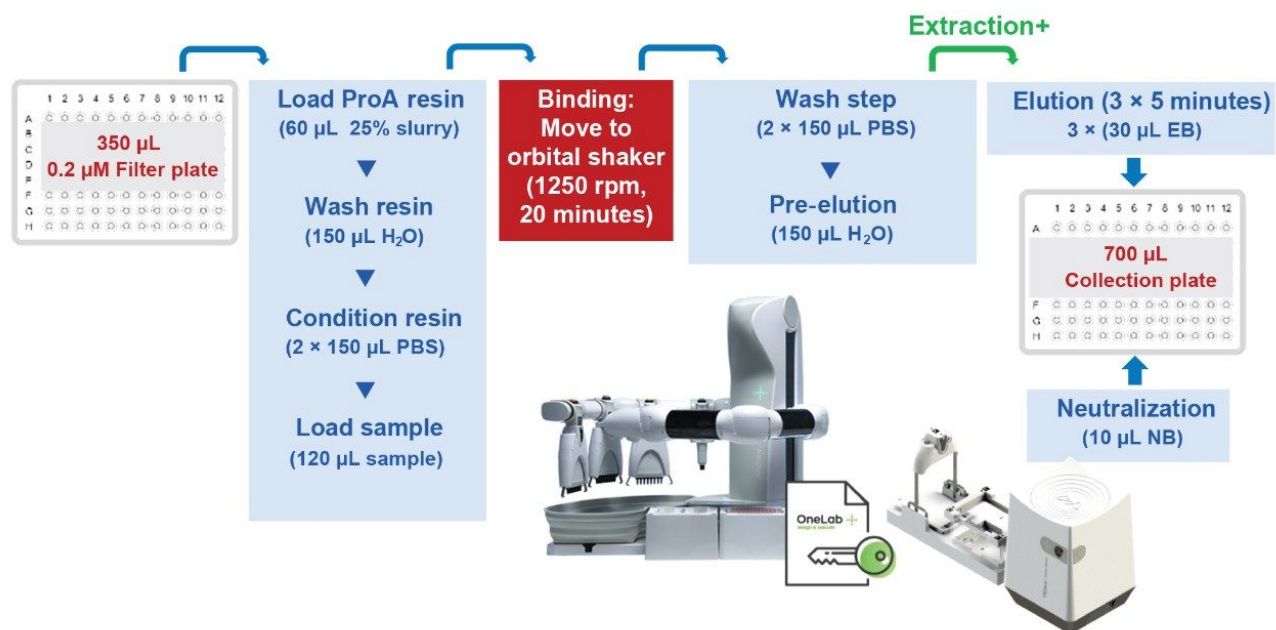


Figure 1. Shown is a diagram of the automated analytical-scale Protein A affinity-based purification of monoclonal antibody from CHO cell conditioned media using the Andrew+ robotic platform with the Extraction+ device and the OneLab visual programming interface. Elution buffer (EB) and neutralization buffer (NB) are defined in the experimental section.

It is critical that the Protein A resin is suspended during the loading step for effective binding. To accomplish this, the use of an orbital plate mixer (1250 RPM for 20 minutes, at 8 °C) produced greater mAb recoveries than repetitive pipet aspiration and dispensing (up to 2 minutes per loading step). When using the current Andrew+ robotic platform, transfer of the filter plate to and from an orbital shaker for the 20-minute binding step is the only action that the analyst needs to perform.

Elution buffer (glycine) pH is also a key factor in mAb recovery from Protein A. Here, we observed that while at pH values below 3.0 an increase in mAb recovery was observed, there was also a concomitant increase in artifactual multimeric aggregation (high molecular weight species, HMWS). As a result, in the final method 100 mM glycine, pH 3.0 was used for elution which could be effectively neutralized with 1.0 M TRIS, pH 7.5 added at a 1:9 ratio to the eluted sample. The volumes of buffer delivered for the equilibration, wash, and elution steps were also optimized. This process was greatly facilitated by the ease with which multiple volumes for a pipetting step can be programmed within the graphical OneLab interface.

## Method Evaluation

At a targeted sample titer of 1.0 µg/µL and load volume of 120 µL, the goal for this purification procedure of producing 100 µL of purified mAb sample at a concentration of 1.0 µg/µL or greater was achieved in day-to-day replicate experiments (Experiments B & C, Figure 2). The recoveries for these samples were 87%. At a lower titer of 0.5 µg/µL, and with a 120 µL load, we observed only 70% recovery, however when two separate 120 µL loadings (20 minutes each) of the 0.5 µg/µL sample were performed the recovery increased to 85% and the final concentration of the purified mAb sample was 1.02 µg/µL (Experiments A and D, Figure 2). The greater percentage of mAb lost for the lower loads indicates that unspecific losses due to the filter plate or Protein A resin are likely occurring. Phase ratios ( $\text{Volume}_{\text{sample}}/\text{Volume}_{\text{resin}}$ ) of 8 or 4 were used for these experiments as indicated and a previously described high-throughput SEC method was used to monitor the results of the Protein A purifications.<sup>4</sup>

The proposed method was also shown to be capable of purifying 240 µg of mAb on 15 µL of Protein A resin (Experiment E, Figure 2). Two experiments were conducted. One in which two 120 µL load steps at 1.0 µg/µL (20 minutes each, phase ratio=8) were performed, and a second in which the amount of Protein A resin and the concentration of the mAb was doubled (Experiments E and F, Figure 2). For the latter experiment the phase ratio is reduced two-fold to 4. Experiments doubling the volume of the sample load could not be accommodated by the 350 µL filter plate being used when placed on the orbital shaker. Both experiments resulted in recoveries of 95% or more, however, the purified sample is approximately two-fold higher in concentration when loading 240 µg of mAb on 15 µL of Protein A resin. This further demonstrates the utility of multiple binding steps in the event that the mAb titer of the cell culture is significantly lower than the desired concentration of the purified sample. Although not executed in this study, these data suggest that up to 480 µg mAb could be purified when using a higher sample concentration, or more binding steps, and 30 µL of resin per well.



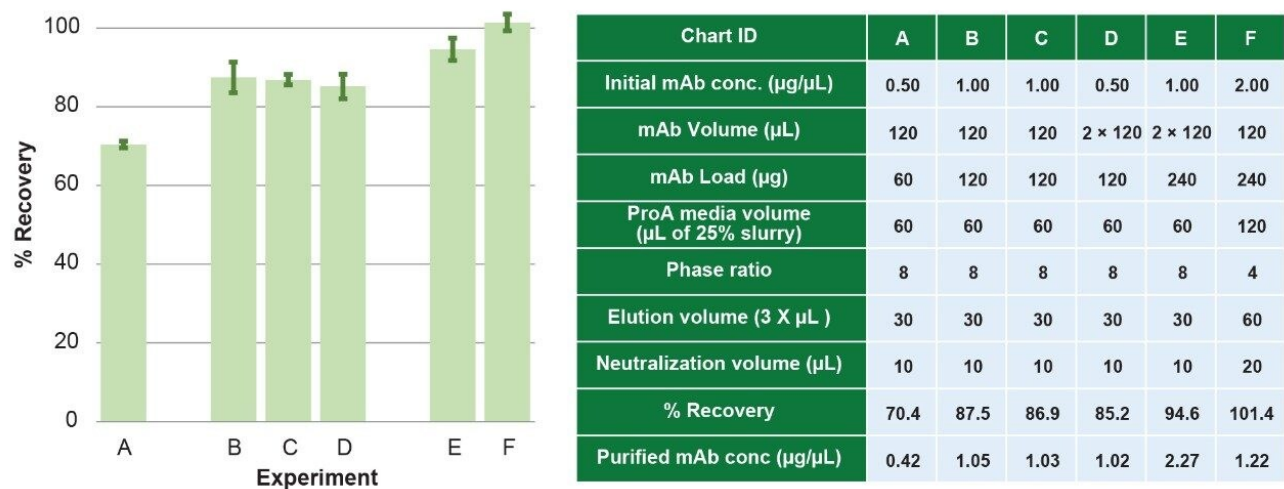


Figure 2. Loading study experiments and recoveries are presented. For sample loads of 120 µg or more 85% recovery or higher was observed. Details are provided in text. Error bars represent the range of values obtained (n=2). These data were collected on an ACQUITY Premier BSM UPLC.

In an expanded reproducibility study, the targeted mAb purification (120 µL at 1.0 µg/µL) achieved recoveries of 90% or greater. For this evaluation, the mAb sample was diluted with PBS to 1.0 µg/µL and a second sample was prepared by spiking mAb into NTM to the same concentration. Eight replicates of both were assessed based on total SEC peak areas (280 nm detection). Both samples resulted in comparable high sample recoveries with the PBS samples having a recovery of 94.4 ± 5.8% and the NTM samples having a recovery of 90.8 ± 5.4% (95% CI) with both resulting in a purified mAb concentration of greater than 1.09 µg/µL.

In addition to reliable recovery, the Protein A method also provided a relatively effective purification for mAb samples to be further analyzed for native size variants and released N-glycans. Due to a low average cell viability (90%) the NTM exhibited significant levels of components (host cell protein, DNA, etc.) that could interfere with both of these analyses as observed in the SEC chromatograms presented in Figure 3.

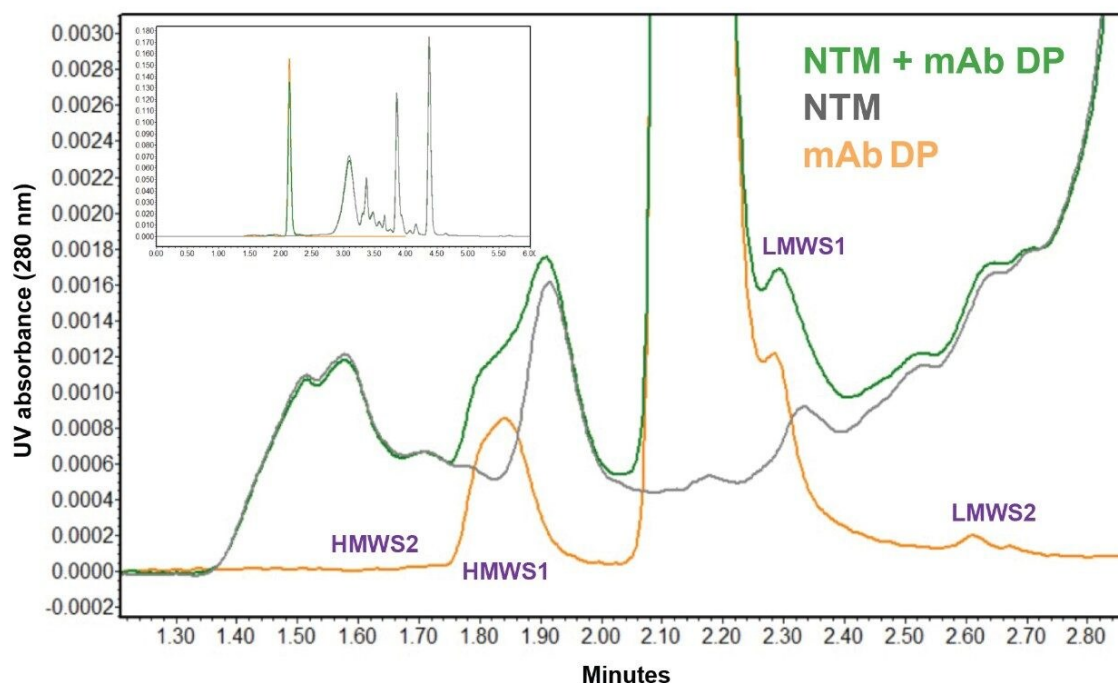


Figure 3. Shown are full-scale and zoomed views of SEC chromatograms of non-transfected CHO cell media both neat (NTM, gray) and spiked with mAb drug product (NTM+DP, green). A  $1 \mu\text{g}/\mu\text{L}$  dilution of drug product (DP, orange) diluted in PBS is also shown. Additional details provided in the text. These data were collected on an ACQUITY Premier BSM UPLC.

SEC was used to evaluate the relative abundances of HMWS and low molecular weight size variants (LMWS) for the Protein A purified samples as compared to the original sample as a control (Figure 4). When comparing to the chromatograms presented in Figure 3, a significant removal of interfering components is achieved. However, it is noted that the Protein A purification procedure alters the mAb HMWS levels. Trace level amounts ( $<0.05\%$ ) of multimeric HMWS2 are artifactually generated while dimeric HMWS1 is partially recovered. Absolute HMWS1 size variant recoveries were estimated as 68% for the spiked NTM sample and 59% for the spiked PBS sample, assuming that additional HMWS1 forms were not also generated by the protein A purification process. Challenges with the quantitative recovery of HMWS mAb variants when using Protein A affinity chromatography purification, even when deploying a more precise LC-based methodology, have been previously reported.<sup>6</sup> Despite this bias, the Protein A method presented may still be able to provide valuable information with respect to the level of HMWS in conditioned media samples. Although out of the scope of this study, further optimization

of the purification method may also increase the accuracy of the size variant assessment.

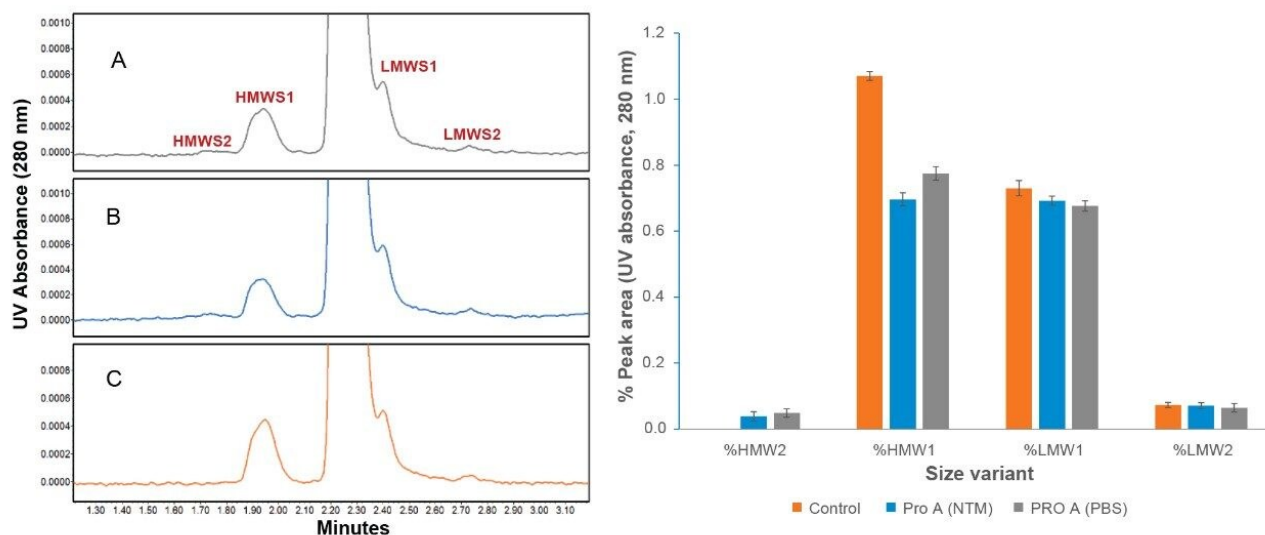


Figure 4. Shown is an evaluation the SEC-UV quantitative results for protein A purified mAb spiked into A; PBS ( $n=8$ , gray) and B; non-transfected CHO cell conditioned media (NTM,  $n=8$ , blue) are compared to C; a spike control ( $n=3$ , orange). Chromatographic conditions are provided in the text. Error bars represent the uncertainty (95% CI) of values obtained. These data were collected on an ACQUITY Premier BSM UPLC.

The effectiveness of the Protein A purification was also effective in conjunction with released N-glycan analysis. Over 6000 proteins and glycoproteins have been identified as part of the CHO cell proteome, many of which are not entirely removed even during the Protein A purification of mAb.<sup>7</sup> To address the utility of the proposed mAb purification for N-glycan analysis a comparison was made between mAb samples that were Protein A purified from NTM and PBS (Figure 5). These results were generated using a high-throughput LC-MS method with ESI-ToF detection as previously reported.<sup>7</sup> Consistent with the extent of overall purification observed by SEC, comparable results were observed for the major mAb glycoforms. Also noted were three trace level glycoforms, that may be of interest for mAb product development as they can impact product safety (FA2BG1) or efficacy (FA2G2S1 and M5). Of these, the only significant change observed was a measurable increase in the relative abundance of the high-mannose glycan (M5) from 0.38% to 0.58% for the mAb purified from NTM. This increase is likely due to low abundance co-purified HCP, and although not pursued in this work modifications to the volumes and solutions used in the Protein A washing step (Figure 1) may further improve HCP removal.

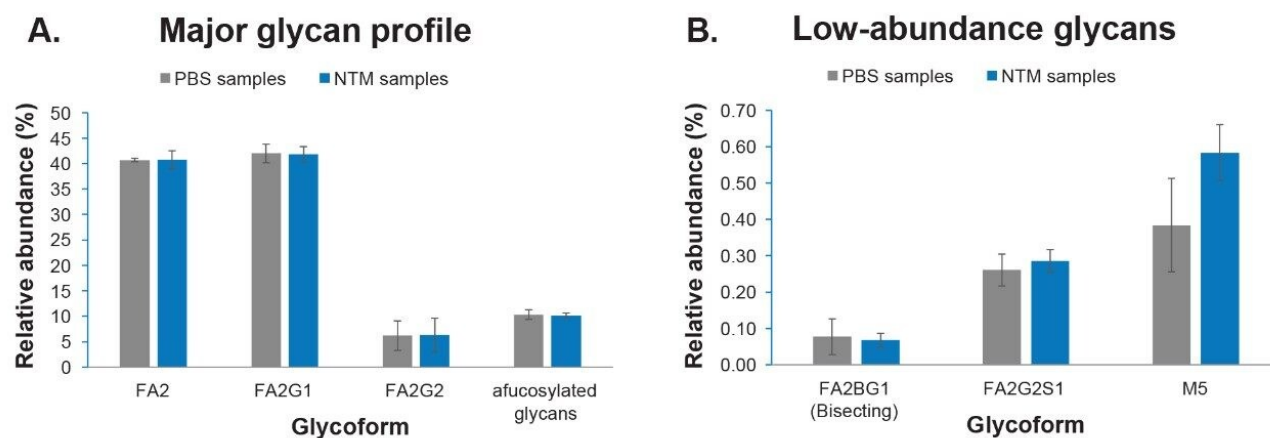


Figure 5. Comparison of UPLC-MS N-glycan profiles of mAb purified from PBS (gray) and NTM (blue). Error bars show the 95% confidence interval (n=4). The method enables comparison of N-glycan profiles between multiple sample types for high- and low-abundance glycans.

## Conclusion

Taken together, these data demonstrate that the Andrew+ robotic platform can be effectively adapted to perform a filter-plate based Protein A affinity purification of 120  $\mu$ g to 240  $\mu$ g quantities of mAb from clarified cell culture samples with high recovery (>90%). Amounts of mAb as low as 60  $\mu$ g can also be purified with lower recovery (70%). The method has a predicted upper purification limit of at least 480  $\mu$ g when using MabSelect resin, however, this value may vary depending on the binding capacity of the manufacturing scale Protein A resin selected. The method produces a 0.2  $\mu$ m filtered sample that can be concentrated up to 2.2  $\mu$ g/mL depending on sample load.

The automated procedure performs 12 separate pipetting steps per sample and the only user action required is to move the filter plate to and from an orbital shaker for the 20-minute binding step. The method has a preparation time of approximately 1 hr for 48 samples, with 35 minutes attributable to the 20-minute binding step and three hold times of 5 minutes each during mAb elution. And finally, the effective removal of host cell proteins and other SEC and released N-Glycan analysis interferences along with minimal generation of artifactual aggregation was demonstrated.

---

## References

1. Hopp J, Pritchett R, Darlucio M, Ma J, Chou JH., "Development of a High Throughput Protein a Well-Plate Purification Method for Monoclonal Antibodies". *Biotechnol Prog.* 2009 Sep-Oct; 25(5):1427–32.
2. Coffman, Jonathan L., Jack F. Kramarczyk, and Brian D. Kelley., "High-Throughput Screening of Chromatographic Separations: I. Method Development and Column Modeling." *Biotechnology and bioengineering* 100.4 (2008): 605–618.
3. Bergander, T., Nilsson-Välimaa, K., Öberg, K. and Lacki, K.M., "High-Throughput Process Development: Determination of Dynamic Binding Capacity Using Microtiter Filter Plates Filled With Chromatography Resin". *Biotechnology progress*, 24(3), pp.632–639. 2008.
4. Stephan M. Koza, Albert H. W. Jiang, and Ying Qing Yu, "Rapid SEC-UV Analysis of Monoclonal Antibodies Using Ammonium Acetate Mobile Phases" *Waters Application Note 720007852*, February 2022.
5. Dunn ZD, Desai J, Leme GM, Stoll DR, Richardson DD. "Rapid two-dimensional Protein-A Size Exclusion Chromatography of Monoclonal Antibodies for Titer and Aggregation Measurements from Harvested Cell Culture Fluid Samples. *MAbs.* 2020;12(1):1702263.
6. Jones, M., Palackal, N., Wang, F., Gaza-Bulseco, G., Hurkmans, K., Zhao, Y., Chitikila, C., Clavier, S., Liu, S., Menesale, E. and Schonenbach, N.S., 2021. ""High-Risk" Host Cell Proteins (HCPS): A Multi-Company Collaborative View". *Biotechnology and Bioengineering*, 118(8), pp.2870–2885.
7. Caitlin M. Hanna, Stephan M. Koza, and Ying Qing Yu, "Automated High-Throughput N-Glycan Labelling and LC-MS Analysis for Protein A Purified Monoclonal Antibodies". *Waters Application Note 720007854*, February 2023.

---

## Featured Products

[ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>](https://www.waters.com/134613317)

[ACQUITY UPLC Tunable UV Detector <https://www.waters.com/514228>](https://www.waters.com/514228)

[Biopharmaceutical Platform Solution with UNIFI <https://www.waters.com/waters/10195515>](https://www.waters.com/waters/10195515)

---

[waters\\_connect <https://www.waters.com/waters/nav.htm?cid=135040165>](https://www.waters.com/waters/nav.htm?cid=135040165)

720007861, February 2023



© 2023 Waters Corporation. All Rights Reserved.

[Termos de Uso](#) [Privacidade](#) [Marcas comerciais](#) [Carreiras](#) [Cookies](#) [Preferências de cookies](#)