Automated mAb Workflows: Combining Multidimensional (Multi-D) Purifications with Product Analysis

Tech Note

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Chromatography

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Abstract

The purification of biologics, specifically monoclonal antibodies (mAbs), frequently involves multiple chromatography columns and buffer systems. The prevalence of small-batch biologic production allows the use of smaller chromatographic columns, which can be amenable to completely automated purification. A single Multi-D method combining both purification and analysis on the NGC™ Chromatography System allows for hands-free, reproducible, and high-fidelity purifications with minimal user intervention.

Here, a typical mAb protein purification workflow was fully automated using the NGC System, and host cell protein (HCP) contamination was monitored by ELISA. This automated purification strategy utilizes a Protein A affinity capture and a buffer exchange column run in tandem followed by a pair of ion exchange (IEX) columns run in tandem before aggregation analysis using size exclusion chromatography (SEC). Final HCP-ELISA analysis demonstrated the ability to generate an automated single-purification method on the NGC System that is as effective as a more traditional sequential approach but with a much shortened time to completion.

Introduction

Protein biologics, including human mAbs, represent a growing percentage of new medicines used to treat diseases ranging from cancer to autoimmune disorders. Biologics are frequently produced by expressing the protein in tissue cultures of both human and nonhuman cell lines, such as HEK 293 and Chinese hamster ovary (CHO) cells, respectively. In the case of nonhuman cell lines, the subsequent purification is extremely important as even low levels of HCP contamination can cause an immune response in the patient after delivery of the drug. That an immune response is possible with HCP concentrations below the limit of detection on typical SDS-PAGE gel visualization can lead to a false sense of purity (Wang et al. 2015). These HCPs can copurify by “hitchhiking” or binding to the target mAb through weak, low-affinity interactions. Obtaining a clean biologic requires its separation from these low-affinity contaminants as well as from the other cellular material in the original cell culture.

Here, we demonstrate the development of a robust, high-quality, and automated Multi-D chromatography purification utilizing both tandem and multi-column configurations using ChromLab™ Software on an NGC Chromatography System. The initial mAb capture step was performed using a Protein A column. As these columns are eluted at low pH to minimize contact with the low pH buffer, the eluted protein was immediately neutralized using a simple desalting column run in tandem for buffer exchange. The neutralized protein sample was captured into a static loop prior to injection onto a pair of IEX columns again configured in tandem. An anion exchange (AEX) column was utilized in flow-through mode to clear co-eluting HCPs. It was paired with a cation exchange (CEX) column in bind and elute mode. The protein eluted from the CEX column was then captured in a second static loop where a small portion underwent analytical SEC aggregation analysis while the majority was sent to the fraction collector for subsequent ELISA analysis. Throughout the purification workflow, HCP-ELISA was used to verify product purity of the individual steps and at the end of the automated purification on the NGC System.

This lab-scale purification was designed to be representative of a typical mAb purification workflow but is by no means all encompassing. Larger diameter/volume columns with different resins can easily be used without affecting the functionality of the method. Ultimately, we are demonstrating how the NGC System and ChromLab Software are effective tools in the automation of small-batch mAb production.

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Materials and Methods

mAb Sample

To mimic an expressed unpurified protein, a purified human monoclonal antibody immunoglobulin G (mAb IgG) was mixed with CHO tissue culture fluid (TCF) to a final concentration of 1 mg/ml antibody in 1.1 mg/ml TCF.

NGC System Configuration

The NGC Discover™ 10 Pro Chromatography System (Bio-Rad Laboratories, catalog #7880011) with an extra column switching valve (CSV) (Bio-Rad Laboratories, catalog #7884012) was used for all purifications and the methods were generated in ChromLab Software. The system consists of three inlet valves, a buffer blending module, two F10 pumps, a mixer module, an injection valve, a sample pump, two CSVs, a multiwavelength detector, a pH module, an outlet valve, and the BioFrac™ Fraction Collector (Bio-Rad Laboratories, catalog #7410002).

Affinity Chromatography with Protein A

A 1 ml Bio-Scale™ Mini UNOsphere SUPrA™ Affinity Cartridge (Bio-Rad Laboratories, catalog #7324200) was used for the Protein A affinity purification step. The wash and equilibration buffer consisted of 10 mM sodium phosphate, pH 7.8, 150 mM sodium chloride, and 10 mM EDTA while the elution buffer was 100 mM sodium citrate, pH 3.0. For the single-column method, 1 ml fractions were collected during the sample application and elution steps. For the Multi-D method, the protein was eluted off the affinity column directly onto the P-6 Desalting Cartridge for pH neutralization and buffer exchange.

Buffer Exchange Chromatography

A 10 ml Bio-Scale™ Mini Bio-Gel® P-6 Desalting Cartridge (Bio-Rad Laboratories, catalog #7325304) was used for buffer exchange during the single-column method development chromatography while a 50 ml cartridge (Bio-Rad Laboratories, catalog #7325312) was used for the Multi-D method. The desalting buffer consisted of 20 mM Bis-Tris/Tris, pH 6.5. This buffer was generated using the buffer blending valve during single-column desalting and via the inlet valve during the Multi-D method. Fractions (1 ml) were collected during the sample application and elution steps of the single-column runs while the outlet valve directed the eluate back to the static loop on the loop valve during the Multi-D method.

Anion Exchange Chromatography

An ENrich™ Q 5 x 50 Column (Bio-Rad Laboratories, catalog #7800001) in flow-through mode was used for AEX chromatography. A flow rate of 0.9 ml/min was used during the Multi-D method runs because the method calls for two medium pressure columns in tandem. IEX buffer A consisted of 20 mM Bis-Tris/Tris while IEX buffer B was composed of buffer A + 1 M NaCl. During the single-column runs and pH scouting a buffer blending valve was used to generate the buffers at the appropriate pH (6.5–8.5). For the Multi-D method, pH 6.5 buffers were introduced using the two buffer inlet valves. For the single-column method, 1 ml fractions were collected during the sample application, elution, and final wash steps. For the Multi-D method, the protein in the flowthrough from the AEX column was loaded directly onto the CEX column.

Cation Exchange Chromatography

An ENrich S 5 x 50 Column (Bio-Rad Laboratories, catalog #7800021) in bind and elute mode was used for CEX chromatography. Using the same buffers as for the ENrich Q Column, the final %B concentration of the linear gradient step was scouted during single-column development, creating a series of runs with end points ranging from 30 to 50%B. Fractions (1 ml) were collected during the sample application, elution, and final wash steps of the single-column purification development runs. For Multi-D chromatography, the flow rate was set to 0.9 ml/min as for the AEX column. During the Multi-D method, the eluted protein from the CEX column was captured in a static sample loop for storage prior to injection onto the SEC column.

Size Exclusion Chromatography

An ENrich SEC 650 10 x 300 Column (Bio-Rad Laboratories, catalog #7801650) was used for analytical purposes to assess the aggregation state of the purified product. The SEC buffer consisted of 20 mM Bis-Tris/Tris buffer, pH 6.5, with 150 mM NaCl. A portion of the sample eluted from the CEX column (0.7 ml) was loaded onto the SEC column from the static loop and eluted with an isocratic wash of buffer A at 1 ml/min for 1.3 CV; 1 ml fractions were collected. During the Multi-D method runs, after SEC analysis was complete, the remaining protein in the static loop on the loop valve was sent directly to the fraction collector.

Anti-CHO ELISA

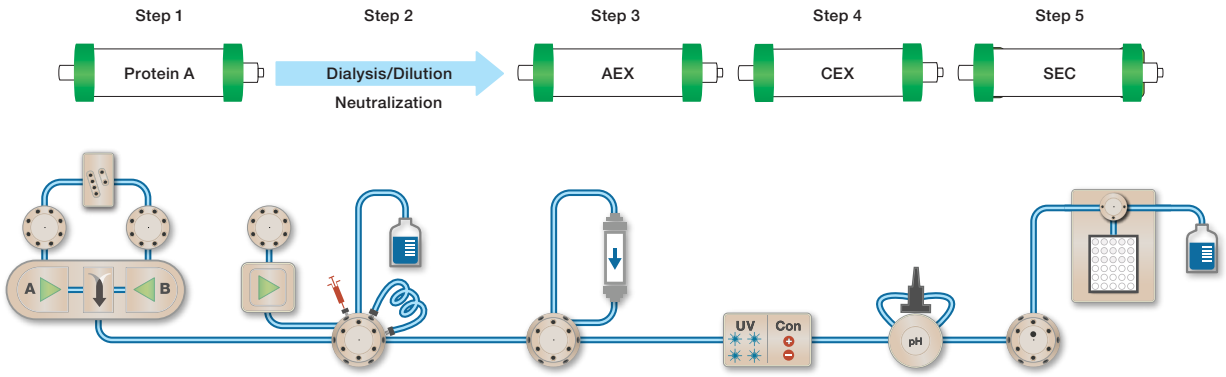
The goat anti-CHO HCP ELISA kit (Cygnus Technologies, catalog #F550) included anti-CHO–coated microstrips, the anti-CHO:HRP conjugate, CHO HCP standards (0, 1, 3, 12, 40, and 100 ng/ml), tetramethylbenzidine (TMB) substrate, wash solution (20x Tris buffered saline), and the stop solution (0.5 M sulfuric acid). The assay was carried out following the manufacturer's protocol. The plates were read using dual-wavelength analysis at 450 nm (test wavelength) and 650 nm (reference wavelength). The standard curve was fit with a second order polynomial and the corresponding sample values were interpolated to determine the total immunoreactive HCP equivalents in ng/ml. The HCP equivalent concentration was normalized to the mAb concentration and reported in parts per million (ppm).

Results

Purification Overview and Optimization Criteria

Figure 1 provides a schematic representation of both traditional sequential and automated Multi-D purification workflows. The purification scheme for both workflows includes four differentiated steps. An affinity purification column (Protein A) captures the antibody at high pH. The antibody is then eluted at low pH. Typically, in sequential purification, the antibody fractions are then neutralized by either overnight dialysis or simple dilution into IEX buffer A before column-loading. The Protein A–purified sample is then loaded onto an AEX column and the flowthrough is collected, as mAb G does not bind to the column. Finally, the sample is bound to a CEX column and eluted with a salt gradient. During the automated purification, the Protein A column is followed in tandem by a desalting column to immediately neutralize the sample and reduce the ionic strength of the buffer in preparation for the IEX columns. In addition, the two IEX steps are accomplished as a second tandem purification step. As the mAb does not bind to the AEX column under these buffer conditions, it is found in the flow-through volume, which is loaded directly onto the CEX column. To minimize contamination from any AEX-bound proteins or nucleic acids, the AEX column is taken out of line prior to elution of the mAb from the CEX column.

A. Traditional Sequential Workflow



B. Automated Multi-D Workflow

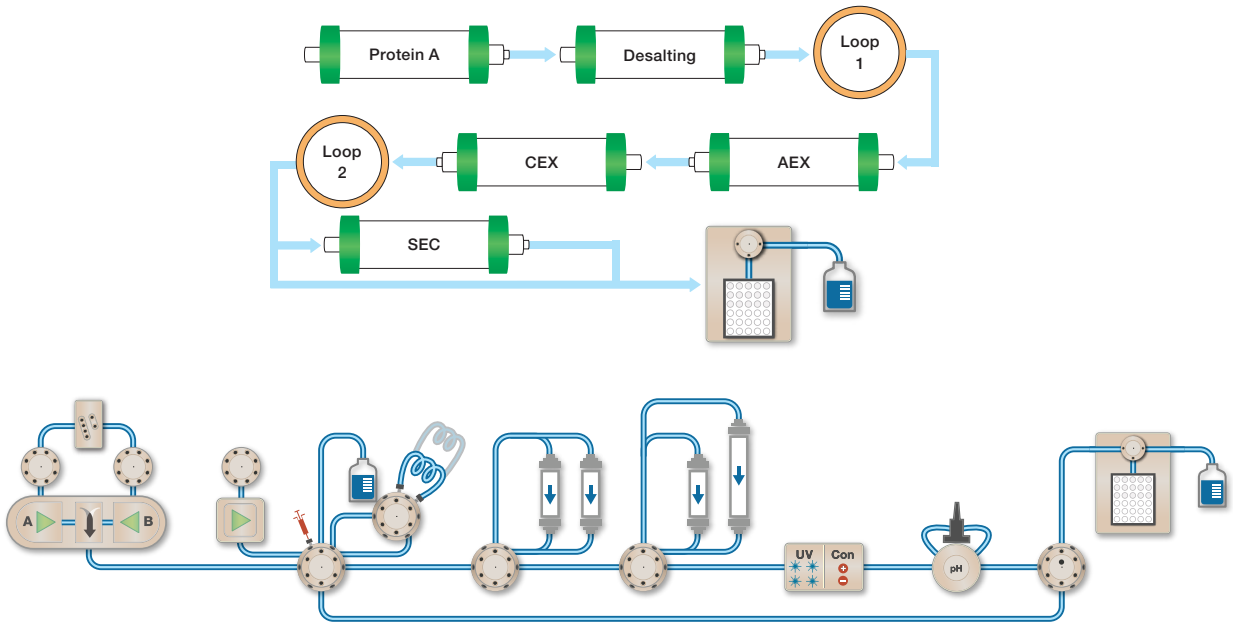


Fig. 1. Purification workflow and NGC System configuration. A, schematic representation of the traditional sequential purification. Five individual steps are required to purify the protein. In addition, protein and HCP analyses are required at each step in the process to monitor protein purity and assess HCP content. **B**, schematic representation of the automated Multi-D purification. The protein is purified in just one step. Protein and HCP analyses are carried out once, after final elution.

During the sequential single-column purification development, the efficacy of HCP removal, monitored by anti-CHO ELISA, was used primarily to determine the optimal purification conditions. To assess HCP removal, polyclonal antibodies raised to the host cell were used to quantitate the amount of HCP in the sample after each individual column run (Table 1). For the automated Multi-D workflow, ELISA evaluation was carried out once, after the final CEX column run. In addition, percent protein recovery was assessed by peak integration of the elution peaks at 280 nm. The assumption made is that the peak area from the Protein A column represents 100% protein recovered and each subsequent column elution is optimized to maximize protein recovery.

Table 1. HCP analysis of the optimized method.

	Concentration, ppm	Error (±)
Traditional Sequential		
TCF	1,100,000	—
Protein A	32.86	10.51
Desalting	11.90	3.81
AEX	2.38	0.76
CEX	0.71	0.23
Automated Multi-D		
CEX	1.09	0.38

Single-Column Method Development

During method development, 1 mg of mAb IgG in TCF was loaded onto the columns. The Protein A affinity column binding and elution conditions were previously optimized (Elms 2015) and the protein was eluted from the column, with pH 3.0 buffer, in a compact and concentrated peak (Figure 2A). Some mAbs are sensitive to pH, which can induce aggregation (acid hydrolysis). However, for the mAb IgG used in this experiment, no significant aggregation was induced at pH 3.0 (Elms 2015). To prevent the risk of aggregation, the sample was neutralized with 1.5 M Tris, pH 8.0, in the fraction tubes.

As most mAbs have an acidic isoelectric point (pI), the expectation with near neutral or lower pH buffer systems is that the mAbs would have an overall positive charge. Hence, the mAb would not bind to an AEX column, making the primary purpose of the AEX column in this purification scheme to bind negatively charged contaminant HCPs and host cell DNA. The unbound mAb IgG remains in the flowthrough for subsequent application to a CEX column. In order to minimize the need for buffer exchange between the two IEX chromatography columns, optimal pH conditions needed to be determined that would allow the mAb to flow though the AEX column but bind to the CEX column. pH scouting was carried out with the Protein A–neutralized sample on the AEX column using a pH range of 6.5–8.5. Figure 2B shows the variation in contaminant retention on the column as a function of pH. Using the measurements from the NGC System and integration of the peak volumes by ChromLab Software, the percent recoveries were calculated and ranged from 98% at pH 6.5 to 68% at pH 8.5, as shown in Table 2. The anti-CHO ELISA showed that, under all pH conditions, the HCPs in the CEX eluate were approximately an order of magnitude less than those in the eluate from the affinity column (data not shown). While the pH 6.5 flowthrough had more contaminant HCPs, the reduction from what was present in the starting material was considered acceptable while optimizing for mAb recovery.

Prior to optimizing the CEX column conditions, the purification was repeated using pH 6.5 conditions for the buffer exchange column (Figure 2C) and the subsequent AEX column (data not shown) to simulate the final conditions that would be used in the Multi-D method. The ELISA assay was repeated to confirm that similar levels of HCPs remained. The resulting sample was then used to optimize the CEX column conditions. For this column, the steepness of the elution gradient was varied to an end point of 30%, 40%, or 50% of buffer B (Figure 2D) over a fixed elution volume. The anti-CHO ELISA showed that for all the gradients, the remaining HCPs were undetectable in the eluates (data not shown). The 50% gradient elution showed the most compact and concentrated mAb peak and was evaluated with a 20 mg/ml sample (data not shown); no increase in HCPs was seen in the more concentrated sample (Table 1).

A previous study demonstrated the ability of an ENrich SEC Column to separate mAb monomers from aggregates (Elms 2015), so a small amount of the CEX pooled fractions was loaded onto an SEC column for analysis. Figure 2E shows a single mAb elution peak from the SEC column, demonstrating the lack of higher multimeric states. Since the eluate from the CEX column had previously shown undetectable levels of HCPs, no HCP ELISA was performed on the fractions. After the single-column chromatography scheme was fully optimized, the purification was repeated with 20 mg of mAb IgG in TCF with anti-CHO HCP ELISA evaluation after each column (Table 1).

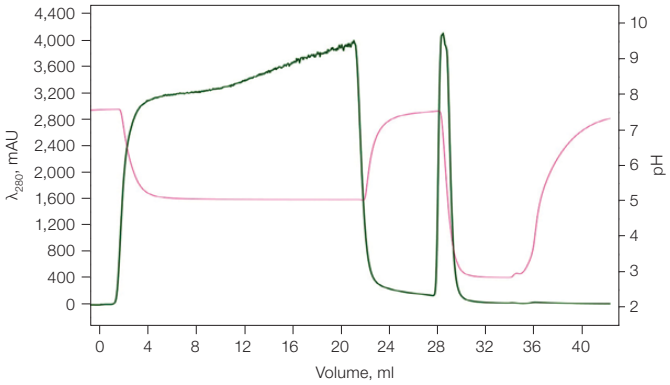


Fig. 2A. Sequential purification: Protein A elution chromatogram. Typical Protein A affinity purification in which the TCF-spiked sample is bound with a high pH buffer and eluted at low pH. IgG (20 mg) was added to 1.1 mg/ml CHO tissue culture fluid. OD 280 (—); pH (—).

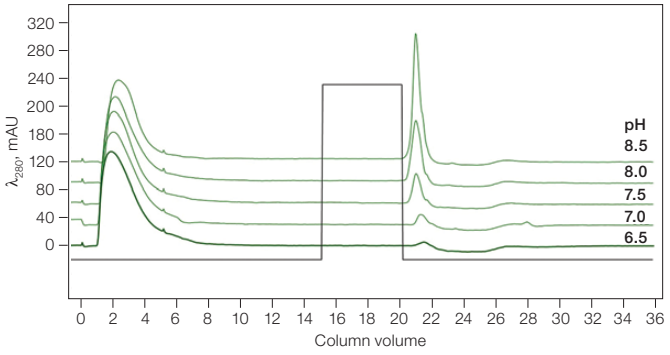


Fig. 2B. AEX pH scouting chromatogram. The AEX column was used in flow-through mode under increasing buffer pH conditions to optimize for protein recovery. As the pH increases, more mAb and HCP bind to the resin. To maximize protein recovery, buffer at pH 6.5 was selected for the optimized sequential and Multi-D methods. OD 280 (—); %B (—).

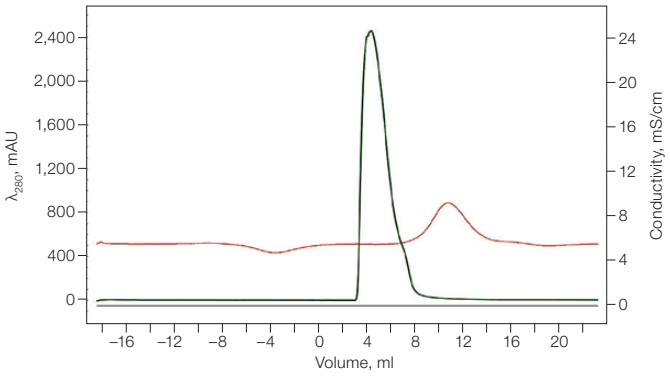


Fig. 2C. Sequential purification: desalting/buffer exchange elution chromatogram. Typical P-6 desalting elution showing the separation of the protein sample from the higher ionic strength affinity buffer. OD 280 (—); conductivity (—).

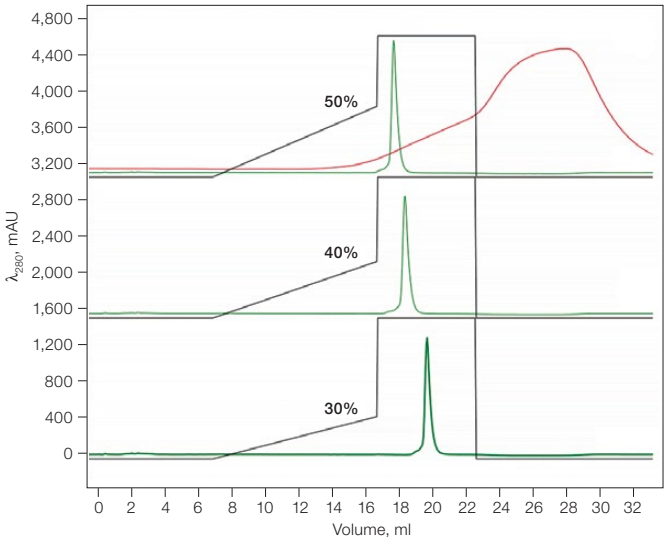


Fig. 2D. CEX %B scouting chromatograms. %B scouting was carried out under increasing %B conditions. All three separations showed undetectable levels of HCPs. The 50%B elution profile yielded the most compact peak and was used for the optimized sequential and Multi-D methods. OD 280 (—); conductivity (—); %B (—).

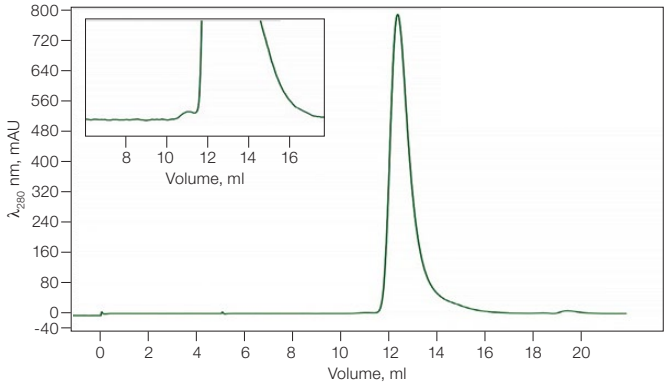


Fig. 2E. Sequential purification: analytical SEC elution chromatogram. A small portion of the pooled CEX fractions was injected into the ENrich 650 SEC Column for aggregation analysis. The inset shows a magnified portion of the absorbance trace, highlighting the separation of the aggregates (leading dimer peak) from the monomer.

Table 2. Percent recovery under AEX pH scouting conditions.

pH	% Recovery
6.5	98
7.0	96
7.5	91
8.0	81
8.5	68

Multi-D Method Development

Following optimization of the individual column purification steps, the NGC System was plumbed into a Multi-D configuration (Figure 1B), and the optimized buffer and elution conditions from each individual run were combined into a single method within ChromLab Software. After initial validation, 20 mg of mAb IgG in TCF was purified using this automated Multi-D method (Figure 3); purification and SEC analysis were completed in just over 5 hours. As was the case with the single-column purification, the final eluate from the CEX column was evaluated using the anti-CHO HCP ELISA (Table 1). The remaining HCP contamination for the single column and Multi-D chromatography schemes was 0.7 ± 0.2 and 1.2 ± 0.4 ppm of HCP equivalent (errors reported as 95% confidence), respectively. As the results from the two schemes are within the margins of error of each other, the final purity of the samples is indistinguishable, demonstrating that the automated Multi-D chromatography scheme can produce similar high-quality results in a much shorter length of time.

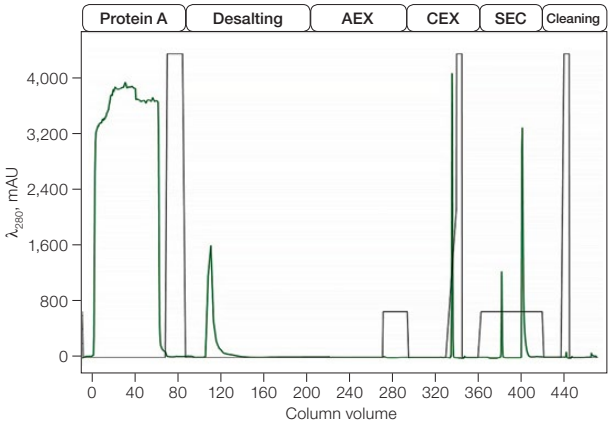


Fig. 3. Multi-D chromatograms showing 280 nm absorbance (—) and %B (—) traces. The single method runs two sets of tandem chromatography purifications resulting in undetectable HCP contamination. In addition to the purification, aggregation analysis using analytical SEC was also carried out (the first SEC peak) before sending the rest of the purified sample straight to the fraction collector (the second SEC peak).

Discussion

With the increase of small-batch process production, greater emphasis is being placed on improving the efficiency of purification. This Multi-D workflow automation represents one way to help accelerate the time to results of a common and time-consuming workflow. Developing a Multi-D single push-button solution to antibody purification involves two parts: (1) single column optimization, which is required for any method development, and (2) combining those individual steps into a single Multi-D method. As illustrated here, the scouting features within ChromLab Software combined with the NGC Chromatography System help facilitate the optimization process. With contaminant levels lower than the limit of detection using SDS-PAGE visualization, the HCP ELISA readout aids in determining the conditions with the lowest remaining HCPs to drive the decision-making process (best pH, elution profile, and so forth).

The advantages of automation include not only time savings but improved robustness and reproducibility of the workflow. Again, ChromLab Software helps facilitate the transition to an automated Multi-D process by providing numerous prewritten multi-column templates that can be used as the building blocks for larger, more complicated methods. The idea of a single method accomplishing multiple column separations in one workflow may initially seem daunting. However, just as with single columns, the Multi-D method has five general sequential phases: column equilibration, sample application, column wash, protein elution, and column cleaning. Once validated, the Multi-D method can be used with confidence and the knowledge that the final product will provide batch-to-batch consistency. The hands-off nature of the automated purification helps reduce the variability that is typically introduced by multiple users and allows users the time to focus on other work while the purification is taking place. Using a sample inlet valve on the NGC System, up to 14 different samples of varying volumes could be purified sequentially in an automated manner, even when using different column elution conditions.

This example demonstrates the incorporation of automated sample analysis into a purification workflow. While the mAb was analyzed for aggregates only at the end of the purification, it would be just as easy to monitor small portions at each column step if necessary. This could be accomplished by in-line analysis such as analytical SEC, as demonstrated here, with other external detectors (for example, dynamic light scattering), or even by incorporation onto a fluidic platform that could sample from the open fraction collector racks.

The NGC System and ChromLab Software were developed to help accelerate the time to results, to improve reproducibility of routine purifications through automation, and to simplify the conversion of these workflows into automated Multi-D procedures. This study gives an overview of just such a process, from optimization to automation.

References

Elms P (2015). Advantages of Multidimensional (Multi-D) Chromatography Using the NGC Chromatography System over Traditional Sequential Chromatography Bio-Rad Bulletin 6694.

Wang F et al. (2015). Host-cell protein measurement and control. BioPharm International 28, 32–38.

Additional Resources

These resources can be accessed by clicking the links or by visiting **bio-rad.com** and searching for the bulletin numbers.

NGC Chromatography Systems: Multidimensional (Multi-D) Chromatography Plumbing Guide (bulletin 6674) — helps configure and plumb an NGC System for Multi-D applications.

Transforming a ChromLab Software 2-D Purification Template into an Automated Multidimensional (Multi-D) Purification Workflow (bulletin 6735) — helps adapt the preprogrammed method templates in ChromLab Software to customized Multi-D methods.

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