



Multidimensional (Multi-D) Chromatography Success Guide



Table of Contents

Chapter 1	Introduction	1
	Workflow Setup	2
	System Requirements	3
Chapter 2	Experimental Considerations	5
	Buffer Composition Factors	6
	Column Compatibility	7
	Complementary Column Chemistries	8
Chapter 3	Multi-D Method Creation in ChromLab™ Software	9
	Method Creation Parameters	10
	Examples of Automated Multi-D Purification Workflows	11
	Three-column mAb purification	11
	Four-column IMAC purification	12
	Other Applications of Automated Purification Workflows	13
	Scale Up	13
	Tangential Flow Filtration (TFF)	14
	Resources	14

List of Abbreviations

AEX, anion exchange; **CSV**, column switching valve; **DMSO**, dimethylsulfoxide; **HIC**, hydrophobic interaction chromatography; **IEX**, ion exchange; **IMAC**, immobilized affinity chromatography; **mAB IgG**, monoclonal antibody immunoglobulin G; **MM**, mixed mode; **NDSB**, non-detergent sulfobetaine; **pI**, isoelectric point; **PIPES**, piperazine-N,N'-bis(2-ethanesulfonic acid); **SEC**, size exclusion chromatography

1 Introduction

This guide has been created to help set up and run two or more single-column chromatography methods as a multidimensional (Multi-D) purification workflow. Our goal is to cover as many of the upfront considerations and variables as possible in order to make creating and validating these workflows easier.

Multi-D purifications include tandem and automated multicolumn methods. We define a tandem method as having two columns in linear sequence with no detection between them so the eluate from column 1 flows directly onto column 2 (Figure 1A). An automated multicolumn method involves two or more columns where the eluate from one column flows through a detector, is shuttled into secondary storage (for example, a static loop), and then applied to the next column (Figure 1B). This process of elution, detection, shuttling, and application onto a new column can be repeated several times. The theory behind automated multicolumn chromatography is based on the concept of orthogonality in which complementary but different purification types are used to improve protein purification.

An automated workflow provides one-push functionality that enables the user to start a method and walk away. Thus, the user is able to focus on other work concurrently, maximizing productivity. The automated nature of the workflow also ensures that the purification methods are highly consistent and reproducible, providing confidence that the end product will meet purity and yield requirements run after run.

In this chapter, we focus on some important parameters to consider before setting up a Multi-D chromatography workflow and pinpoint the various system components or modules needed.

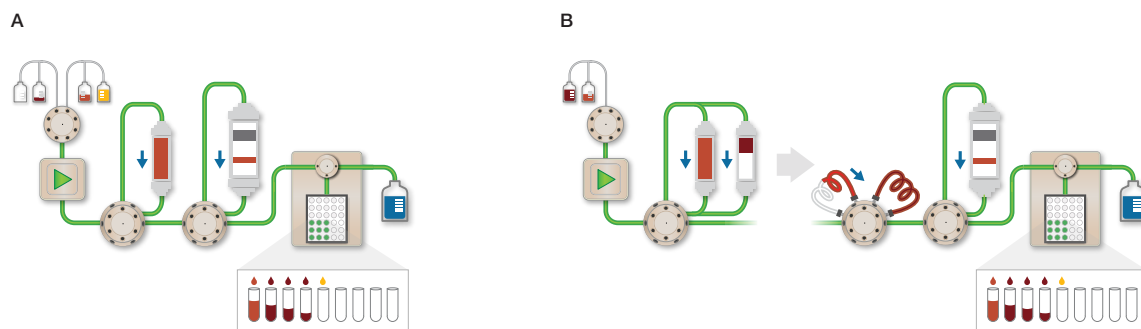


Fig. 1. Representation of tandem (A) and automated multicolumn (B) workflows.



Workflow Setup

At the heart of successful Multi-D workflows are well-developed single-column chromatography methods. In creating these purification methods, the following factors should be known and optimized:

- Column flow rates
- Elution windows
- Elution volumes
- pH
- %B elution (gradient or isocratic)

Single-variable analysis of these parameters is often straightforward and can be achieved through software automation such as with scouting features.

Typically, after single chromatography runs some form of analysis is performed to investigate the quality of the separation and/or structural analysis of the target protein. This presents an interesting challenge since the Multi-D workflows automate several single-column methods and potentially reduce the chances of testing intermediate protein products. Hence, it is important to incorporate standard analysis techniques — such as SDS-PAGE, host cell contamination evaluation, analytical SEC — into the Multi-D method validation process and compare the final results from the single and Multi-D runs to confirm comparable purification performance and final recovery. Due to the automated nature of the Multi-D purifications, these results should be consistently reproducible, as human errors and general inconsistencies between successive purifications will be minimized.

System Requirements

Setting up an effective purification method is an important step in the Multi-D workflow. To ensure purification success, both hardware components and software capabilities need to be carefully considered. Here, we address the benefits of each system component to the process of automating chromatography purification. Though not every module is necessary, each addresses a specific need, depending on the purification methodology.

Tandem:

- CSV
 - Plumbing the columns to two CSVs allows the eluate from the first column to be loaded directly onto the second column
 - Enables pre- and delta-column pressure monitoring of every column, forward- and reverse-flow elution, and column bypass for system priming or cleaning
- Sample Pump
 - Allows the application of large sample volumes without contaminating the system pumps
 - Can be used in conjunction with air sensors to maximize the volume of applied sample and with a sample inlet valve for multiple sample loadings
- Sample Inlet
 - For the sequential application of multiple samples and washing the sample pump with buffer
 - Can be used in conjunction with air sensors to maximize the volume of applied samples
- Buffer Inlet
 - Used to change the buffer composition to accommodate different buffer requirements
 - Makes it easier to clean and maintain the system

Automated Multicolumn:

- Outlet Valve
 - For shuttling the flowthrough or eluted protein from a column to a fraction collector (final fraction collection); secondary storage, such as static loop or large container (application onto another column); or a large open container (flowthrough or column wash collection)
- CSV
 - 2 CSVs can support up to 10 columns and 1 CSV can be used as a loop valve to hold up to 5 static loops, thus increasing the throughput capability for multiple samples and columns

- Sample Pump
 - Allows the application of large sample volumes without contaminating system pumps
 - Can be used in conjunction with air sensors to maximize the volume of applied sample
- Sample Inlet
 - For the application of multiple samples and washing the sample pump with buffer
 - Can be used in conjunction with the outlet valve for large-volume elutions or application of the sample from one column onto another
 - Can be used in conjunction with air sensors to maximize the volume of applied samples
- Buffer Inlet
 - Used to change the buffer composition to accommodate the different buffer requirements of each column in the purification workflow
 - Simplifies system cleaning and maintenance

2 **Experimental Considerations**

In sequential single-column chromatography purifications, the columns used for the various steps of the purification can, to a certain extent, be treated as separate entities. However, if the buffer used for one column is not compatible with the next column, then the fractions collected from the first column often have to be pooled and dialyzed before the user can proceed with the purification process. This can add variability and prolong the time to results.

Multi-D chromatography can make this process easier by eliminating the time-consuming steps between sequential single-column runs and can improve reproducibility of the data by minimizing the potential for human error at each step. However, due to the automated nature of the workflows and the fact that multiple column steps are linked together, managing the composition of the buffers required for each column and the properties of the actual columns themselves becomes very important. In this chapter, we highlight some common parameters to consider while setting up automated purification workflows. These tips could help make the difference between successful and failed runs.



Buffer Composition Factors

The traditional buffer composition considerations that apply to single-column chromatography are just as applicable when setting up Multi-D chromatography workflows. Specifically, be mindful of the following:

- Minimize the ionic strength of the sample before applying it to an IEX column to maximize binding
- Do not use Tris buffer with a Ni-IMAC column as the free electrons on the primary amine of Tris will outcompete the target protein for the nickel ions of the resin
- Make sure the pH of the binding buffer is appropriate for the target's pI and the type of column being used (above the pI for anion/Q columns and below the pI for cation/S columns)
- Be conscious of the following UV absorbers as they could mask the presence of small elution peaks or peak separation
 - imidazole (280 nm)
 - Tris buffer (214 nm)
 - acetone (214 nm)
 - Triton X-100 (280 nm)
 - NP-40 substitute (280 nm)
 - NDSB (280 nm)
 - DMSO (255 nm)
 - PIPES buffer (214 nm)

Additional considerations need to be addressed when dealing with tandem methods because the elution buffer of column 1 will be the sample application buffer for column 2. This is especially important when considering the pH of the buffers since changing the pH can alter the binding affinity of the proteins to the resin. This is why desalting columns are frequently used as the second column in tandem chromatography; no buffer considerations need be taken into account other than the sample volume for buffer exchange.

Column Compatibility

As mentioned before, the chemical and physical characteristics of the various columns will affect Multi-D chromatography methods. Since multiple columns are linked together in these workflows, the differences in their properties are amplified and need to be managed. These columns (especially those in tandem) cannot be at odds with one another and must work together like gears in a machine to effect the desired outcome. Due to the different fluidic schemes involved in tandem and automated multicolumn purifications, the column considerations are also different.

Tandem:

Column factors are more critical in tandem methods since the eluate from one column flows directly onto the second column. Consider the following column factors when creating tandem methods.

- **Binding capacities** — since the two columns are in line, the second column must have sufficient binding capacity to hold all of the eluted protein from the first column. If it does not, the second column will become saturated and the extra target protein will not bind
- **Column pressures** — equilibrate lower pressure columns before higher pressure columns in order to avoid accidentally overpressuring the system or damaging a column
- **pH** — ensure the elution pH of column 1 is compatible with column 2 since it is not possible to change buffer systems between columns

Automated Multicolumn:

The inclusion of secondary sample storage is inherent in automated multicolumn chromatography, so each column can almost be viewed independently. However, since multiple columns are connected together in sequence, some factors still need to be taken into consideration.

- **Binding capacities** — lower capacity columns may require multiple injections from a secondary loop or container to ensure that all of the sample is processed
- **Column pressures** — as with tandem methods, equilibrate lower pressure columns before higher pressure columns in order to avoid accidentally overpressuring the system or damaging a column
- **pH** — if the buffer requirements of the columns are different, buffer inlet valves can be used to seamlessly switch between different buffers for each column
- **Column volumes/elution volumes** — one of the reasons to have well-defined single-column protocols is to fully characterize the elution profile (when) and elution volume (how much) of the target protein from each column

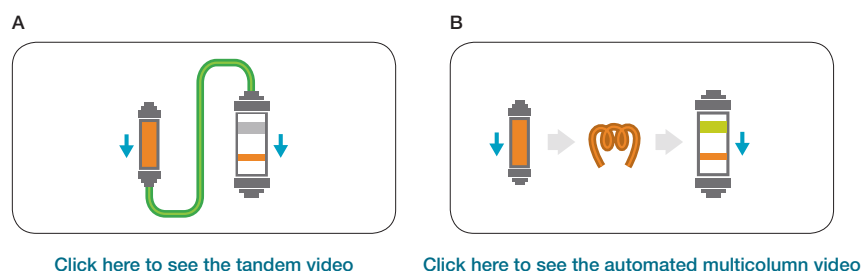


Fig. 2. Illustrations and videos depicting tandem (A) and automated multicolumn (B) workflows.

Complementary Column Chemistries

Listed below are column types that can be paired successfully for the specified applications and the benefits of such a column pairing.

Application	1st Column	2nd Column	Benefit
mAb purification	Protein A	Desalting / buffer exchange	Rapid neutralization of low pH elution buffer
mAb purification	Protein A	SEC	Rapid neutralization of low pH elution buffer and aggregation analysis
Polyhistidine-tagged protein purification	IMAC	Desalting / buffer exchange	Buffer exchange to remove imidazole and/or lower ionic strength buffers
Protein purification or abundant contaminant protein removal	IEX (flowthrough)	IEX (capture)	Rapid contaminant protein removal; the contaminants bind to the first column while the target protein comes off in the flowthrough
Protein purification or abundant contaminant protein removal	IEX (flowthrough)	MM	As above, with enhanced binding and elution selectivity of mixed-mode resin
Polishing steps or separation of active from inactive protein forms	IEX	HIC	Eluate from IEX column in high salt binds well to the HIC column
Concentration of low abundance target followed by size analysis	IEX	SEC	Peak separation and analysis

Though the applications listed in the table above relate to simpler workflows, multiple columns can be linked to create automated purification strategies to achieve optimal purifications. The tables below list the specifics of two such Multi-D applications. For a more in-depth look at these two workflows, refer to the examples in the following chapter.

mAb Screening

Column	Function	Benefit
Protein A	Affinity capture	Rapid automated screening of mAbs, in line buffer exchange to neutralize Protein A eluate, and analysis of mAb peak to determine monomer from aggregate and heavy light chain fragmentation
Desalting	Buffer neutralization/exchange	
SEC	Antibody integrity analysis and resolution from aggregates	

IMAC Purification

Column	Function	Benefit
IMAC	Affinity capture of histidine-tagged recombinant proteins	Automated IMAC purification from crude sample to purified target protein. Multi-D approach minimizes hands-on time and maximizes reproducibility
Desalting	Removal of imidazole and reduction of buffer ionic strength	
AEX	Polishing step for further resolution from co-eluting proteins	
SEC	Separation of intact protein from fragments based on size	

3 Multi-D Method Creation in ChromLab™ Software

Developing a Multi-D method is not as straightforward as developing a single-column method. The NGC™ Chromatography System has been designed to ease the adoption of more automated chromatography and is scalable to fit the user's throughput requirements. The key to the NGC System's convenience is its integrated software package, ChromLab Software. The software provides a simplified approach to programming tandem and automated multicolumn methods with minimal expertise. Prewritten templates included in the software provide model methods that can be used directly or adapted, depending on the user's requirements. A streamlined, successful, automated workflow will take into account the considerations emphasized in this document. Furthermore, following these guidelines will provide confidence that the data generated are both reproducible and accurate.

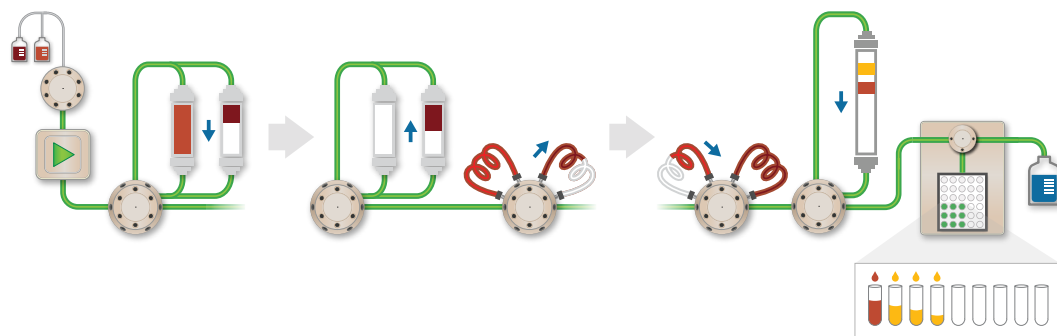


Fig. 3. Schematic of an automated multicolumn workflow in ChromLab Software.

Method Creation Parameters

Since different multi-column chromatography methods have their own unique requirements, it would be difficult to try and account for all of the different variables a user might face while creating a new Multi-D method or when using one of the ChromLab Software templates (Figure 4). The following suggestions highlight many of the common factors encountered during automated purification method creation.

- If the buffer requirements of the columns are different, buffer inlet valves will be needed
- Equilibrate the columns in order of increasing pressure to avoid accidentally overpressuring the system or damaging a column
- With automated multicolumn methods, the positions of the valves (CSV1, CSV2, Outlet, and Inject) will need to be considered at various phases during the run
- Place the CSV in bypass to prime the system with elution buffer if an isocratic elution gradient is needed
 - Along with reverse-flow elution, this will help minimize elution volume and increase run-to-run reproducibility
- Place the CSVs in bypass when changing buffer systems
 - This allows an increase in flow rate, reduces method time, and eliminates overpressure risks
- Confirm optimized elution volumes for each step to ensure complete elution of the target protein into the static loop
 - Factor in additional tubing volumes to ensure the protein is fully eluted through the detector outlet valve and inject valve into the loop

All new methods will require validation testing and optimization to ensure that the entire sample is captured and shuttled through the workflow efficiently.

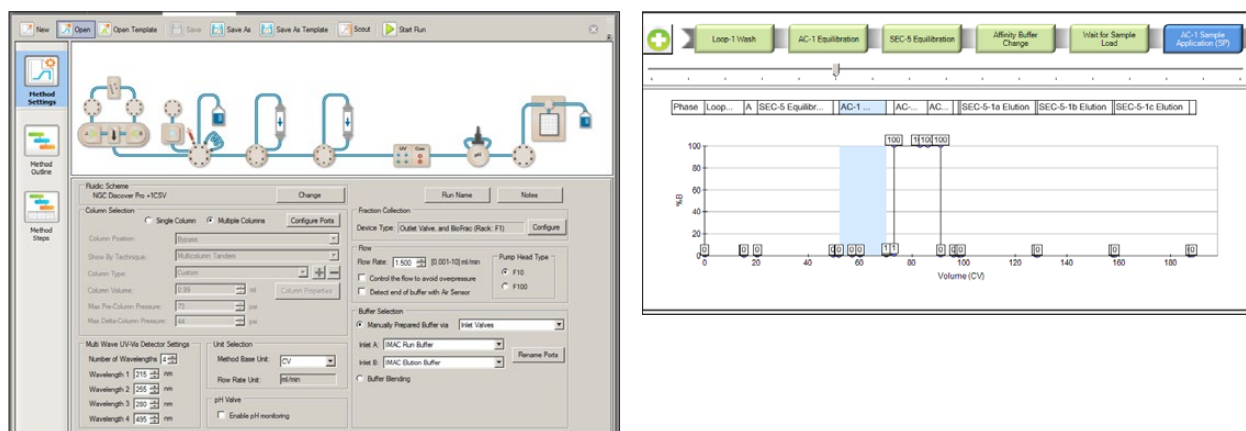


Fig. 4. Example of a 2-D template in ChromLab Software.

Examples of Automated Multicolumn Purification Workflows

The following examples illustrate the data generated from ChromLab Software during automated multicolumn purifications. Furthermore, they emphasize that the automated nature of the workflow provides one-push functionality to protein purification, which enables the user to start a method and walk away to focus on other work.

Three-column mAb purification

In this example, we focus on a common application in the production of biologics for pharmaceutical applications: screening aggregation levels of a monoclonal antibody in tissue culture fluid. During the production of biologics, it is not uncommon to observe aggregation of the target protein during expression. Aggregation often can be prevented, or at least reduced, by optimizing the media and/or growth conditions and by optimal clone selection. To optimize the production method to reduce aggregation, multiple experimental conditions must be analyzed. Typically, the monoclonal antibody is first separated from the tissue culture matrix by affinity purification on a Protein A column. The sample is then analyzed by SEC to resolve and quantitate the ratio of monomer to aggregate.

Using an automated multicolumn workflow on the NGC System (Figure 5), a mAb IgG expressed in Chinese hamster ovary (CHO) cells was automatically loaded onto affinity and SEC columns, purified, and analyzed for aggregates; fractions from the SEC column were then collected. Specifically, the sample was loaded onto the column by the sample pump, purified on a Protein A column, and then buffer exchanged on the desalting column. The sample was then circulated through the detector and returned through the outlet valve and sample injection module to the sample loop. Part of the sample was then injected onto an SEC column for aggregate analysis. Following this, the sample stored in the sample loop was sent to the fraction collector. The chromatogram in Figure 5A shows the various phases of the purification and depicts the overlay of three samples containing 0%, 10%, and 25% aggregation. The highlighted peaks (blue circle) represent the degree of aggregation in the different samples and the chromatogram in Figure 5B illustrates that the varying levels of aggregation can be clearly distinguished and quantified.

For more details about this particular purification, refer to bulletin 6694. A link to this bulletin can be found in the Resources section at the end of this chapter.

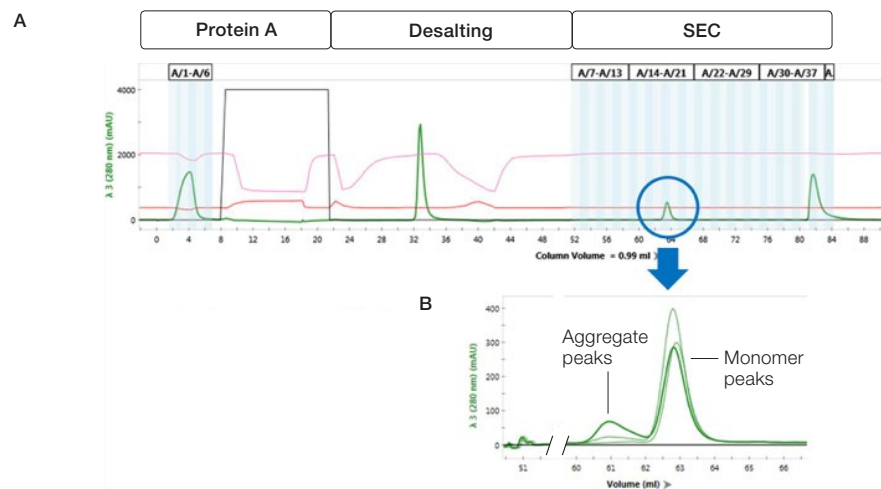


Fig. 5. Three-column Multi-D chromatography workflow (A) and visualization of aggregation levels (B).

Four-column IMAC purification

Polyhistidine tags (6x) are one of the most frequently used molecularly engineered protein tags used by research scientists. Depending on their application, a multi-step purification may be needed to achieve the desired purity level. Automating these workflows results in increased time savings and reproducibility between purifications. A simple three-column workflow for a 6x histidine-tagged protein (Figure 6A) would include IMAC, IEX, and SEC columns, representing the capture, intermediate, and polishing steps of the purification, respectively. It would be normal practice for researchers to dialyze or dilute their protein samples to remove/minimize the imidazole and salt content (ionic strength) before loading the samples onto the IEX column. In addition, to confirm where the target protein was eluted off the columns, SDS-PAGE analysis would be carried out after elution from the IMAC and AEX columns and before loading the samples onto the next columns in the purification process. These additional steps are easily accounted for in an automated multicolumn chromatography method (Figure 6B) since:

- a desalting column can be used to minimize the ionic strength of the buffer, and
- the entire sample is shuttled through the continuous workflow of the NGC System, eliminating the need for target protein confirmation

Hence, the automated multicolumn method can reduce the time for this routine purification technique from 2–3 days to approximately 5 hours by eliminating the need for time-consuming electrophoresis and dialysis procedures.

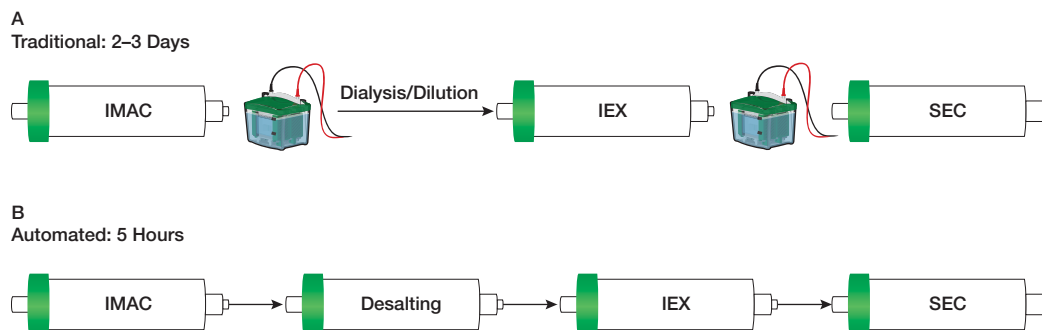


Fig. 6. Traditional (A) vs. automated (B) four-column purification workflow.

In this example, we see the results of a four-column automated Multi-D purification of SUMO-GFPsf-6xHis *E. coli* lysate (Figure 7). The target protein was labeled with both histidine and fluorescent tags, which facilitates its specific purification since absorbance of the target protein can be monitored simultaneously at 280 nm (absorbance of protein) and 495 nm (absorbance of fluorescent tag). The lysate (1 ml) was injected onto a 5 ml IMAC column and eluted in the reverse direction to yield a more concentrated eluate, and the target protein was captured into a static loop. The protein was then injected onto a 50 ml desalting column to reduce salt and imidazole levels prior to injection onto an anion exchange column. Following further polishing on this column, the sample was captured into a static loop and then sequentially injected (four smaller volume injections) onto an SEC column for final purification. The table shows the calculated percent recovery of the target protein from each column based on the peak area at 495 nm. This wavelength was used to specifically exclude the absorbance signal from imidazole, which is used to elute proteins from the IMAC column, and other non-target proteins at 280 nm.

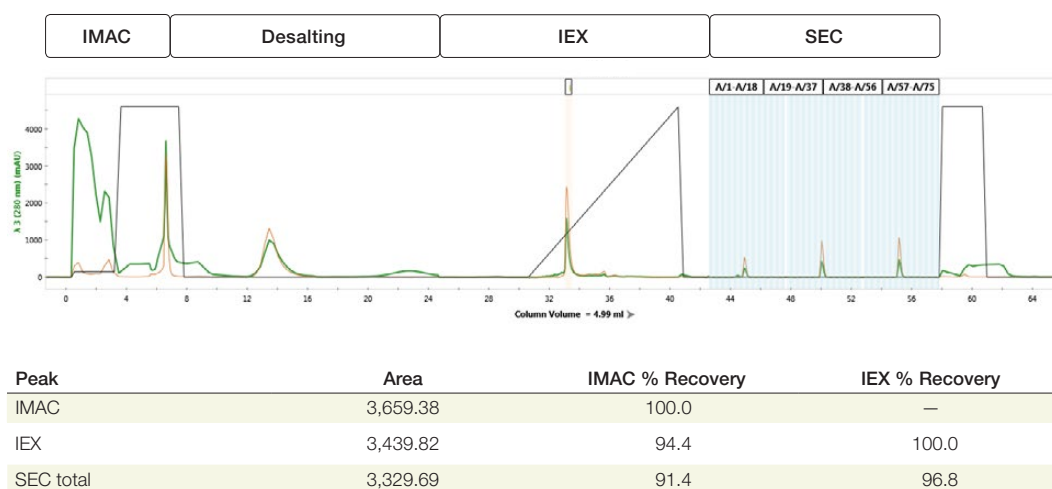


Fig. 7. Four-column Multi-D purification workflow and percent recovery of target protein.

Other Applications of Automated Purification Workflows

In addition to the given examples, there are a number of other ways in which the NGC System and ChromLab Software can be used for automated purification workflows. Though this is by no means a comprehensive list, it highlights the range of options available in automating protein purification.

Scale Up

Automated purification workflows are commonly seen as being useful for lab-scale purifications. However, as volumes of starting materials, columns, and eluates increase, so too can the scale of Multi-D protein purification processes. Following are some of the ways the NGC System can be scaled up to handle automated higher-throughput applications:

- Faster flow rates can be accomplished with 100 ml/min system pumps, which allow larger volume columns to be plumbed to the CSV

- Dynamic sample loops can be used for elution volumes up to 90 ml
- Larger elution volumes can be accommodated by shuttling the sample to an external container via one of two outlet valves and combining this with a sample inlet line to the sample pump

In addition, combining these features with wider-diameter tubing and a buffer blending valve can increase flow rates to 200 ml/min.

Tangential Flow Filtration (TFF)

TFF is used for the separation and purification of biomolecules and can be used to concentrate and desalt solutions ranging from ml to L volumes. Specifically, it can be used to fractionate large from small biomolecules and clarify cell lysates. Attaching a TFF cassette to a CSV can essentially make it function like a column, allowing the pressure to be monitored easily and the flow rate to be adjusted automatically. When using the TFF step as part of an automated purification workflow, the feedstock can be loaded onto the TFF “column”, the filtrate collected in an external container, and the retentate recirculated to the feed container. Utilizing an outlet valve and a sample inlet valve can create the closed loop necessary for continuous cycling of the feedstock.

Resources

Download the following resources by clicking on the links or visiting bio-rad.com and searching for the bulletin number.

- [Bulletin 6674—NGC Chromatography Systems: Multidimensional \(Multi-D\) Chromatography Plumbing Guide](#)

This guide highlights the purification templates and methods for which the various system configurations can be used and helps plumb your NGC System for Multi-D applications.

- [Bulletin 6694—Advantages of Multidimensional \(Multi-D\) Chromatography Using the NGC Chromatography System over Traditional Sequential Chromatography](#)

This application note compares the sequential and Multi-D workflows and illustrates that both yield proteins of equivalent purity.

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