Transforming a ChromLab[™] Software 2-D Purification Template into an Automated Multidimensional (Multi-D) Purification Workflow — An Instructional Guide

Chromatography	Bulletin 6735

ChromLab Software has prewritten templates to simplify the process of automating routine purification processes. The templates allow for two dimensions of purification with an affinity step followed by a buffer exchange or size exclusion step. This step-by-step instructional guide describes how to expand upon these templates to create customized multidimensional (Multi-D) methods to include multiple steps as needed. Specifically, we use the **2-D Affinity (5 ml)** - **Desalting (50 ml)** template as a starting point to generate an automated four-step immobilized metal affinity chromatography purification workflow method. The results from this IMAC purification process are detailed in bulletin 6725.

System Considerations

The application is run on an NGC Quest[™] Plus 10 Chromatography System with the following additions: third tier, a sample pump, two buffer inlet valves, two column switching valves (CSVs), and an outlet valve (Figure 1). The recommended module placement and fluidics setup can be found on page 6 (3-Tier + 2 CSV + 1 Outlet) of the NGC Multidimensional (Multi-D) Chromatography Plumbing Guide (bulletin 6674).

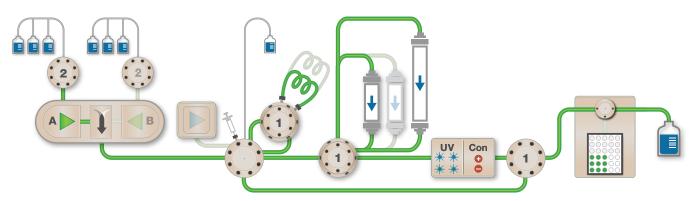


Fig. 1. NGC[™] Quest Plus 10 Chromatography System used for this purification workflow.

These additional modules play a vital role in making sure the Multi-D method is successful. The buffer inlet valves allow switching of the buffer systems between the different columns, one of the CSVs is utilized as a loop valve allowing for separate loops of differing volumes, and the outlet valve is needed to shuttle the eluted protein back to the inject valve for loop storage between columns.



Method Settings

The 2-D Affinity (5 ml) - Desalting (50 ml) template was used for this workflow. The Method Settings window in Figure 2 shows the parameters that were changed.

Hethod Settings Method Outrine		
	Rudic Scheme 3-Tier Loop Change	Run Name Notes
Method Steps	Column Selection Single Column Multiple Columns Configure Posts Column Posts Column Prostor: Column Prostor: Column Prostor: Column Prostor: Column Violume 439 det Column Pressure: 530 MPa Max Deta Column Pressure: 530 MPa	Device Type: Outlet Valve, and BioFrac (Rack: P1) Configure Row Row Pump Head Type Image: Image: Image: Image: Control the flow to avoid overpressure Image: Image: Device trand overpressure <td< td=""></td<>
	Number of Wavelength 4 Out Section Wavelength 1 215 mm Wavelength 2 255 mm Wavelength 3 200 mm Wavelength 4 435 mm	Rename Pota

Fig. 2. Method settings for this purification workflow.

- 1 Select the correct fluidics scheme to match the system.
- 2 Configure the columns for the purification workflow, assigning them to their correct positions on the CSV (Figure 3).
- 3 Select the correct fraction collection type (Figure 4).
- 4 Change the default flow rate to 4 ml/min to minimize the risk of overpressure.
- 5 Rename the buffer and outlet ports for clarity (Figure 5).

fax Pre-Column Pressure (Bypa	as All): 3650.00	÷ (osi								
Column Selection	2				Column		Column	Max	Мах	Default	Мак
Column Position:	umn Postion: C1 Pot 1		Default	Position	Column Name	Volume	Pre-Column Pressure	Delta-Colum Pressure	Flow Rate	Rate Rate	
Show By Technique:			•	3 🗹	C1 Port 1	Nuvia IMAC N-Charged, 5 ml	4.99	73	44	7	20
Column Type:	Nuvia IMAC N-Charger	5, 5 ml	-+-	13	C1 Port 2	Bio-Gel P-6 Desalting, 50 ml	49.97	73	36	8	10
Column Volume:	4.99	÷		- 23	C1 Port 3	ENrich Q, 5/50 mm	0.98	500	500	1	2
	4.39	mi	Column Properties	10	C1 Port 4	HiPrep 16/60 Sephacryl S 3	120.64	73	22	0.5	1
Max Pre-Column Pressure:	73	psi		13	C2 Port 1	Loop	10	1000	1000	1	20
Max Delta-Column Pressure:	44 10	es.		10	C2 Port 2	Loop	10	1000	1000	1	20
				10	C2 Port 3	Loop	10	1000	1000	1	20
Max Delta-Column Pressure:	44	psi								1	

Fig. 3. Configuration of the columns used in this purification workflow.

1 Remove the default columns from the template by selecting the checkboxes next to the column names and clicking Remove.

2 Add the four columns being used by selecting the Column Position from the dropdown menu and then selecting the correct column using both the Show By Technique and Column Type dropdown menus.

- C1 Port 1: Nuvia IMAC Ni-Charged
- C1 Port 2: Bio-Gel P-6 Desalting
- C1 Port 3: ENrich Q
- C1 Port 4: HiPrep 16/60 Sephacryl S 300 HR

3 Select the Nuvia IMAC Ni-Charged Column as the default column.

Configure Fraction Collection Scheme	
Select BioFrac Rack	
F2 (15-16 mm x 150 mm tubes) F3 (18-20 mm x 150 mm tubes) H1 (1.5-2.0 ml microtubes) H2 (0.5 ml microtubes) H3 (16 mm x 60 mm vials) H4-L (30 mm x 60 mm vials) H4-H (50 ml centrifuge tubes) Ice Bath (13 mm x 100 mm tubes) P1 (96-weil microplates)	 P1 96-well
BioFrac Settings	BioFrac Collection Pattern
Start Rack 🖌 👻	Serpentine
Start Tube 1 Location 1A	⊘ Row
Fraction Size: 1.50 🗼 ml	© Column
Outlet Valve Settings	
Start Port To Loop Valve Fraction Size 5	i0.00 🗢 ml
	OK Cancel

Fig. 4. Configuration of the fraction collection scheme.

- 1 Select the P1 (96-well microplates) rack.
- Increase the Fraction Size to 1.5 ml to collect all fractions from the size exclusion (SEC) column. To hold this fraction volume, a deep well 96-well plate was used.

🇊 Re	ename Ports	1 R	ename Ports	🧐 R	ename Ports 2
	er Inlet A Valve Buffer Inlet B Valve Outlet Valve r buffer name for valve position:	1	er Inlet A Valve Buffer Inlet B Valve Outlet Valve I rbuffer name for valve position:		fer Inlet A Valve Buffer Inlet B Valve Outlet Valve er buffer name for valve position:
No.	Buffer	No.	Buffer	No	Buffer
1	PBS	1	PBS + 0.5Mimidazole	1	To BioFrac
2	25mM Tris pH 8.0	2	25mMTits + 1M NaCl	2	To Loop Valve
3	50mM Tris pH 8.0 + 100mM NaCl	3	Buffer B 3	3	O1 Part 3
4	Buffer A 4	4	Buffer B 4	4	01 Port 4
5	Buffer A 5	5	Buffer B 5	5	01 Port 5
6	Buffer A 6	6	Buffer B 6	6	O1 Port 6
7	Buffer A 7	7	Buffer B 7	7	01 Port 7
8	8/Bypass	8	8/Bypass	8	O1 Port 8
		-		9	O1 Port 9
				10	01 Port 10
				11	01 Port 11
		2		12	01 Port 12
	OK Cancel	3	OK Cancel		OK Cancel

Fig. 5. Optional renaming of buffer and outlet ports for clarity.

Editing the Method Outline

The quickest way to generate a custom method is by replicating phases. Common tasks performed more than once during a method (for example, equilibrating a column, washing a loop, flushing the system with buffer) represent opportunities with which to create a single functional phase that can be replicated.

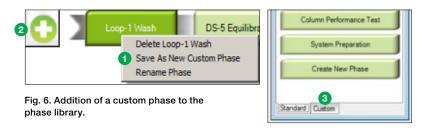
Washing the Loops



The Loop-1 Wash phase is necessary in the Multi-D method to ensure each eluate is fully loaded onto the next column. The IMAC purification workflow is a four-column purification so three different volume loops are needed. Using a different loop for each step is preferred to minimize contamination between columns. Typically, the loop is flushed with the buffer A of the column the sample will be loaded onto.

- Loop 1 (10 ml): used to load the IMAC column eluate onto the desalting column; use PBS buffer
- Loop 2 (20 ml): used to load the desalting column eluate onto the ion exchange (IEX) column; use Tris buffer
- Loop 3 (5 ml): used to load the IEX column eluate onto the SEC column; use PBS buffer

Replicate the Loop-1 Wash phase in the template to accommodate washing all the loops (Figure 6).



- 1 Right click the phase and select Save As New Custom Phase to save the phase in your custom phase library.
- 2 Click the large green plus sign to access the custom phase library.
- 3 Select the Custom tab in the phase library window.

Insert the Loop-1 Wash custom phase into the method after the original Loop-1 Wash phase. Convert this phase to a Loop-2 Wash (or any loop position) phase (Figure 7).

Change Valve	
Select Valve: Column Switching Valve 1	Select Port: Bypass
Change Valve	
Select Valve: Sample Inject Valve	Select Port: Manual Load Loop / System Pump to Column
Change Valve	
Select Valve: Outlet Valve 1	Select Port: To Loop Valve
Change Valve	•
Select Valve: Column Switching Valve 2	Select Port: C2 Port 2, Loop 🔽 🗖 Reverse Row
Gradient Segments Use Row Rate from Method Settings Row Rate:	0.000 🛫 [0.001-10] ml/min
2 Use Same Inlets As Method Settings	
Segment Inlet A Inlet B Isocratic 25mM Tris ▼ PBS + 0.5	Initial %B Final %B Volume (CV) Drag buttons to table ▼ 0 0 5 Isocratic
	Gradient
Change Valve	
Select Valve: Outlet Valve 1	Select Port: To BioFrac
Change Valve	
Select Valve: Sample Inject Valve	Select Port: Manual Load Loop / System Pump to Column
Gradient Segments	
Use Row Rate from Method Settings Row Rate: 7	500 === [0.001-10] ml/min
Use Same Inlets As Method Settings	
Segment Inlet A Inlet B	Initial %B Final %B Volume (CV) Drag buttons to table
▶ Isocratic 25mM Tris ▼ PBS + 0.5	• 0 0 0.2 Isocratic
	Gradient
,	

Fig. 7. Conversion of the Loop-1 Wash phase to a Loop-2 Wash phase.

- 1 Change the Column Switching Valve 2 position to C2 Port 2, Loop.
- 2 Uncheck the Use Same Inlets As Methods Settings checkbox to change the buffer system.
- 3 Change the buffer system from PBS to 25 mM Tris pH 8.0 using the dropdown menu for Inlet A.
- 4 Set the Volume to 5 CV (25 ml) to ensure the 20 ml loop is fully flushed.
- 5 Rename the phase to make it easier to track which phase performs which function. To do so, right click the phase, select Rename Phase, and enter Loop-2 Wash.

To wash Loop 3, insert another Loop-1 Wash custom phase as before. Change the Column Switching Valve 2 position to C2 Port 3, Loop, change the buffer system to the SEC buffer (50 mM Tris pH 8.0 + 100 mM NaCl), and reduce the isocratic volume to 2 CV to flush the system with buffer (5 ml) and wash the 5 ml loop. Rename the phase Loop-3 Wash.



The **2-D Affinity (5 ml) - Desalting (50 ml)** template comes with two column equilibration phases, DS-5 and AC-1 equilibration, which differ by their Column Switching Valve 1 position and buffer system. When equilibrating the columns, equilibrate the low-pressure IMAC, desalting, and SEC columns before the higher pressure IEX column to avoid possibly overpressuring and damaging a column. Furthermore, choose the correct column position, buffer system, and flow rate for each column.

Since the provided AC-1 Equilibration and DS-5 Equilibration phases can be used for equilibration of the IMAC and desalting columns, respectively, rename them as IMAC Equilibration and P6 Equilibration, respectively. Use the P6 Equilibration phase to create two new phases for equilibrating the IEX and SEC columns. For all four phases, change the column, buffer, and flow rate as shown in Figure 8A–D and rename accordingly.

A. IMAC column

Change Valve									
Select Valve: Column Switching Valve 1 Select Port: C1 Port 1, Nuvia IMAC Ni-Charged, 5 ml									
Gradient Segments									
Use Row Rate from	n Method Settings	Flow Rate: 4.0	00 - [0.001-1	10] ml/min					
Use Same Inlets A									
Segment	2 Inlet A	Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table			
▶ Isocratic	PBS	PBS + 0.5MI	0	0	5	Isocratic			
						Gradient			
Hold Until Examine									
Hold Until Enable									
Zero Baseline 🔽 Enable									
Detector: Multi War	ve UV-Vis with Cor	ductivity							

B. Desalting column

Change Valve				•		
Select Valve: Co	lumn Switching Valve	1 💌	Select Port	: C1 Port 2, Bio-G	iel P-6 Desalting, 50 n	nl 💽 🗖 Reverse Flow
- William Contraction	e from Method Setting ets As Method Setting		000 🛨 (0.00)1-10] ml/min		
Segment	2 ∣ Inlet A	Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table
► Isocratic	25mM Tris	25mMTris	0	0	5	Isocratic
						Gradient
Hold Until	Enable					
Zero Baseline	Finable					
Detector: Multi	i Wave UV-Vis with Co	onductivity				

C. SEC column								
Change Valve Select Valve: Column Switching Valve 1	Select Port: C1 Port 4, HiPrep 16	6/60 Sephacryl S 300 HR	Reverse Row					
Gradient Segments 3 □ Use Row Rate from Method Settings □ Use Same Inlets As Method Settings								
	Initial %B Final %B 0 0	Volume (CV) 5 Isocratic Gradient	: to table					
Hold Until Enable Zero Baseline Enable Detector: Multi Wave UV-Vis with Conductivity								

D. IEX column

Change Valve									
Select Valve: Column Switching Valve 1	Select Port	1 Port 3, ENrich Q), 5/50 mm	Reverse Row					
Gradient Segments Use Row Rate from Method Settings Row Rate: 1.000 [0.001-10] ml/min Use Same Inlets As Method Settings									
Segment Inlet A Inlet I	B Initial %B	Final %B	Volume (CV)	Drag buttons to table					
🕨 Isocratic 25mM Tris 💌 25mM	ITris 💌 0	0	5	Isocratic					
				Gradient					
Hold Until Enable									
Zero Baseline 🔽 Enable									
Detector: Multi Wave UV-Vis with Conductivit	у								

Fig. 8. Configuration of the four different equilibration phases.

0	Select the appropriate column from the Select Port dropdown menu	C1 Port 1, IMAC	C1 Port 2, Desalting	C1 Port 4, HiPrep	C1 Port 3, ENrich Q
2	Select the appropriate buffer from the Inlet A dropdown menu	PBS	25 mM Tris, pH 8.0	50 mM Tris, pH 8.0 + 100 mM NaCl	25 mM Tris, pH 8.0
3	Set the Flow Rate	4 ml/min	4 ml/min	0.5 ml/min	1 ml/min

This results in four phases to equilibrate the four different columns.

IMAC Purification



After equilibration, each column during a Multi-D method follows the same basic tenets of sample load, column wash, and elution. However, frequently before the sample can be loaded, the system needs to be primed with the correct buffer. These buffer change phases are unique to the Multi-D method but can be replicated from the templates as well.

At this point, the last phase the method completed was equilibration of the IEX column, so the system contains IEX buffer A. The system needs to be flushed with IMAC buffer A before the sample can be loaded using the sample pump. The **2-D Affinity** (5 ml) - Desalting (50 ml) template contains a phase called DS Buffer Change that places Column Switching Valve 1 in Bypass and then flushes the system with buffer at 10 ml/min before reducing the flow rate (and lowering the system pressure) before sample application.

In order to replicate the buffer change functionality, save the DS Buffer Change phase as a custom phase as previously described. Insert the phase into the method between the IEX Equilibration and Wait for Sample Load phases and edit as in Figure 9.

Change Valve									
Select Valve: Column Switching Valve	1 💌	Select Port:	Bypass		Reverse Flow				
Gradient Segments	Gradient Segments								
Use Row Rate from Method Settings	Row Rate: 4.0	00 🛨 [0.001-1	0] ml/min						
Use Same Inlets As Method Settings									
Segment Inlet A	Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table				
🕨 Isocratic 🛛 PBS 🖉	PBS + 0.5 💌	0	0	2	Isocratic				
					Gradient				
Hold Until Enable									
Zero Baseline 🔲 Enable									
Gradient Segments									
Use Row Rate from Method Settings	How Rate: 4.0	00 🕂 [0.001-1	0] ml/min						
Use Same Inlets As Method Settings									
Segment Inlet A	Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table				
Isocratic PBS	PBS + 0.5 💌	0	0	0.15	Isocratic				
					Gradient				
1									

Fig. 9. Configuration of the IMAC Buffer Change phase.

- 1 Select the correct buffer for the IMAC column from the Inlet A dropdown menu in both Gradient Segments sections.
- 2 Set the Flow Rate to 4 ml/min.
- 3 Rename and save the phase as IMAC Buffer Change.

The Wait for Sample Load is an optional phase. The original intent of the phase was for users to be able to start the method and get the system ready while they prepared the sample. If this is not required, remove the phase by right clicking it and selecting Delete Wait for Sample Load.

Once the system has been primed with IMAC buffer, it is ready for sample loading onto the column. To do this, modify the AC-1 Sample Application phase as shown in Figure 10. After loading the sample onto the column, the unbound material is subsequently washed away prior to the elution step.

Change Valve		
Select Valve: Column Switching Valve 1	Select Port: C1 Port 1, N	Nuvia IMAC Ni-Charged, 5 ml 👻 🔲 Reverse Flow
Sample Loading		Interrupt Injection
Coad Loop Manually		Interrupt Injection Above UV
Load Loop with Sample Pump		λ 1 (215 nm) - 2000 🖨 mAU
Inject Sample on Column with Sample Pump	Pump	
		Prefill System with Selected Buffer
		Flow Rate: 1.000 📩 ml/min Volume: 10.00 📩 m
Direct Inject with Sample Pump		2 2
Interrupt Injection If Air is Detected	Row Rate:	
Use Row Rate From Method Setting	8	

Fig. 10. Configuration of the IMAC Sample Application phase.

- 1 Uncheck the Pre-Injection Sample Pump Wash box since it is not required for this application (after unchecking, the window will look as in Figure 10).
- 2 Set the Flow Rate to 1 ml/min and Volume to 2 ml.
- 3 If the flowthrough needs to be collected, enable the Fraction Collection Scheme and utilize the BioFrac[™] Fraction Collector.
- 4 Rename and save the phase as IMAC Sample Application.

To set up the wash step, edit the AC-1 Column Wash phase as in Figure 11.

Ch	ange Valve						
Sel	ect Valve: Column	n Switching Val	ve 1 💌	Select Port:	C1 Port 1, Nuvia	a IMAC Ni-Charged, 5	ml 💌 🗖 Reverse Row
Gr 1 □	adient Segments Use Row Rate fro Use Same Inlets A	m Method Settir	ngs		10] ml/min		
Г	Segment	Inlet A	Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table
Þ	Isocratic	PBS	PBS + 0.5MI	10	10	3	Isocratic
							Gradient
Ho	ld Until 🗖	Enable					
Fra	action Collection	Scheme 🖵	Enable				

Fig. 11. Configuration of the IMAC Column Wash phase.

(1) Uncheck the Use Flow Rate from Method Settings box.

- 2 Set the Flow Rate to 4 ml/min.
- 3 Change the Initial and Final %B to 10% as per the previously optimized IMAC wash conditions (see bulletin 6725 for the scouting development).
- 4 Rename and save the phase as IMAC Column Wash.

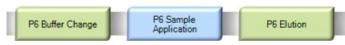
The AC-1 Elution phase in the template performs the exact function needed for the Multi-D method. Protein is eluted off of the IMAC column and shuttled back to a loop on the loop valve. Since the functions are the same, edit the phase for the Multi-D method (Figure 12).

Change Valve								
Select Valve: Colu	mn Switching Valve 1	•	Select Port:	Bypass			•	Reverse Rov
Change Valve								
Select Valve: Colu	mn Switching Valve 2	•	Select Port:	Bypass			•	Reverse Rov
Change Valve								
Select Valve: Outle	et Valve 1	•	Select Port:	To Loop Valve			•	
Change Valve								
Select Valve: Sam	ple Inject Valve	•	Select Port:	Manual Load Lo	oop / System Pump	to Column	•	
Gradient Segmer	its							
Use Row Rate f	from Method Settings	Row Rate: 14	000 - [0.001-	10] ml/min				
	As Method Settings							
Segment	Inlet A	Inlet B	2 Initial %B	Final %B	Volume (CV)	Drag buttons to	table	
Isocratic	PBS	PBS + 0.5MI	100	100	2	Isocratic		
I						Gradient		
Change Valve								
Select Valve: Colu	mn Switching Valve 1	•	Select Port:	C1 Port 1, Nuvi	a IMAC Ni-Charged	. 5 ml	• •	Reverse Row
Change Valve								
	mn Switching Valve 2		Salart Part	C2 Port 1, Loop			Tel c	Reverse Row
Select valve. [Colu	mn Switching Valve 2	-	Select Foit.	C2 Port 1, Loop	1		<u> </u>	rveverse now
Gradient Segmen	its							
Use Row Rate f	from Method Settings	Row Rate: 4.	000 🛨 [0.001-	10] ml/min				
Use Same Inlets	As Method Settings							
Segment	Inlet A	Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to	table	
▶ Isocratic	PBS	PBS + 0.5MI	100	100	2	Isocratic		
1						Gradient		

Fig. 12. Configuration of the IMAC Elution phase.

- 1 Place Column Switching Valve 1 in Bypass.
- 2 Select 100%B buffer for an isocratic elution.
- 3 Uncheck the Use Flow Rate from Method Settings box.
- 4 Set the Flow Rate to 4 ml/min.
- 5 Rename and save the phase as IMAC Elution.

P-6 Desalting



At this point in the method, the protein has been eluted from the IMAC column using PBS + 0.5 M imidazole. Before loading the sample onto the anion exchange (AEX) column, the amount of imidazole and ionic strength of the eluate must be lowered to allow binding to the AEX column. The system still contains IMAC buffer B, so the first thing to do is flush out the system with desalting buffer. Earlier, we saved the DS Buffer Change as a custom phase. Here, since the original phase can be used for its intended purpose, it can be left unchanged (Figure 13). Rename and save the phase as P6 Buffer Change.

Change Valve				
Select Valve: Column Switching Valve 1	Select Port:	Bypass		▼ □ Reverse Row
Gradient Segments				
Use Row Rate from Method Settings Row Rate:	10.000 - 10.001	101 ml /min		
	10.000 - 10.001	-roj monari		
Use Same Inlets As Method Settings		10.100		Drag buttons to table
Segment Inlet A Inlet B	Initial %B	Final %B	Volume (CV)	
Isocratic 25mM Tris 25mMTris	. 💌 0	0	2	Isocratic
				Gradient
Hold Until Enable				
Zero Baseline 🗌 Enable				
Gradient Segments				
Use Flow Rate from Method Settings Flow Rate:	7.500 [0.001-	10] ml/min		
Use Same Inlets As Method Settings				
Segment Inlet A Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table
▶ Isocratic 25mM Tris 💌 25mMTris	. 💌 0	0	0.15	Isocratic
				Gradient
·				

Fig. 13. P6 Buffer Change phase.

In the existing template, the desalting sample application and elution steps are combined in a phase called DS-5-1 Sample Application/Elution since eluting from the desalting column is just an isocratic flow of buffer A. All you need to do in this sample application phase is to increase the sample volume loaded to equal the volume of your loop plus the volume of the fluidics from the loop to the column (Figure 14).

Select Port: C2 Port 1, Loop	Reverse Row
Select Port: C1 Port 2, Bio-Gel P-6 Desating, 50 ml	Reverse Row
Interrupt Injection	
Interrupt Injection	Above UV
1 (215 nm) -	2000 🚔 mAU
Prefill System with	Selected Buffer
Row Rate: 1.000	🗄 mil/min Volume: 10.00 📑 m
Flow Rate: 4.000 🛨 [0.001-10] ml/	min Volume: 10.00 🛨 m
25m M TrispH 8.0 ▼ Inlet B: 25m M Tris + 1 M NaCl ▼	%В: 0 🛨
	Select Port: C1 Port 2, Bio-Gel P-6 Desating, 50 ml

Fig. 14. Configuration of the P6 Sample Application phase.

- 1 Set the Flow Rate to 4 ml/min and the Volume to 10 ml.
- 2 Uncheck the Enable box for the Fraction Collection Scheme.
- 3 Rename and save the phase as P6 Sample Application.

The sample volume injected onto the column is a critical variable to consider. The void volume of the 50 ml P-6 Desalting Column is 15 ml. Normally, we inject 2x the loop volume to make sure the loop is flushed sufficiently. However, this means that the protein will elute off the column before loading is complete. For ease of use and Multi-D method development, it is best to make the elution step its own phase. Hence, when we add an elution phase we need to remember that 10 ml of the 15 ml void volume has already been used. If we do not account for this, we run the risk of not capturing the protein into a second loop as it is being eluted.

At this point, we start deviating from the template. Since we need to elute the protein from the P-6 Desalting Column and have it shuttle back to the loop valve, we can replicate the IMAC Elution phase, which is set up to do just that. As previously described, right click the IMAC Elution phase, save it as a custom phase, access the custom phase library, and insert the IMAC Elution custom phase into the method after the P6 Sample Application phase.

There are several different ways that the eluted protein can be captured back into a loop. The method described here is a simple "window" method based on the knowledge accrued during the optimization steps. The volume window that the protein will be eluted in can be calculated and used to generate the method. Other threshold collection methods use hold steps to monitor the 280 nm UV trace. The latter is preferred when different volumes of material might be eluted. However, in this purification, the elution volume is fixed.

Several steps are needed to convert the IMAC Elution phase to the P6 Elution phase (Figure 15). We can use the first Gradient Segments step to elute the protein from the P-6 Column (remembering to account for 10 ml of the 15 ml void volume during the P6 Sample Application phase), shuttle it into the correct loop, and then switch the outlet valve back to the default position, continuing to flush the column (Figure 15A).

Change Valve	0	
Select Valve: Column Switching Valve 1	Select Port: C1 Port 2, Bio-Gel P-6 Desalting, 50 ml	Reverse Flov
Change Valve		
Select Valve: Column Switching Valve 2	Select Port. C2 Port 2, Loop	Reverse Rov
Change Valve		_
	Select Port: To Loop Valve	
Select Valve: Outlet Valve 1	J Select Port. To Loop Varve	<u>·</u>
Change Valve		
Select Valve: Sample Inject Valve	Select Port: Manual Load Loop / System Pump to Col	lumn 💌
Gradient Segments		
Use Row Rate from Method Settings Row Rate:	4.000 * [0.001-10] ml/min	
Use Same Inlets As Method Settings	0	
Segment Inlet A Inlet B	Titidi 40 Titidi 40 Voldine (CV)	Drag buttons to table
▶ Isocratic 25mM Tris 💌 25mM Tris	🔽 0 0 4	Isocratic
		Gradient
Change Valve		
Select Valve: Column Switching Valve 1	Select Port: C1 Port 1, Nuvia IMAC Ni-Charged, 5 ml	▼ I▼ Reverse Flow
Change Valve		
Select Valve: Column Switching Valve 2	Select Port: C2 Port 1, Loop	Reverse Rov
Gradient Segments		
Use Row Rate from Method Settings Row Rate: Use Same Inlets As Method Settings	4.000 🛨 [0.001-10] mi/min	
Segment Inlet A Inlet B	Initial %B Final %B Volume (CV)	Drag buttons to table
► Isocratic 25mM Tris ► 25mMTris .		Isocratic
		Confinal
		Gradient
Change Valve	0	
Select Valve: Outlet Valve 1	Select Port: To BioFrac	•

Fig. 15A. Configuration of the P6 Elution phase.

- 1 Change the Column Switching Valve 1 position from Bypass to C1 Port 2 to place the P-6 Desalting Column in line.
- 2 Change the Column Switching Valve 2 position from Bypass to C2 Port 2, Loop since there is a large volume difference in the loops.
- In the upper Gradient Segments section, uncheck the Use Same Inlets As Method Settings box and select 25 mM Tris buffers from the Inlet A and B dropdown menus.
- 4 Set the volume in the first Gradient Segments section to 4 CV (20 ml) and the Initial and Final %B to zero.
- In the lower Gradient Segments section, uncheck the Use Same Inlets As Method Settings box and select the 25 mM Tris buffers from the Inlet A and B dropdown menus.
- 6 Set the volume in the second Gradient Segments section to 10 CV (50 ml) to buffer exchange the system and tubing.
- Switch the outlet valve back to default position 1 (To BioFrac) to avoid pushing the protein sample out of the loop and into the waste.

	Step	Total Vol (CV)	Step Description	Vol (CV)	Flow (ml/min)	% B	Step Parameters	Phase Name
	62.1	44.11	Inject Sample	2.00	4.000	0	System Pump Inject Loop, 25mM Tris pH 8.0, 25mMTris + 1M NaCl	P6 Sample Application
~	62.2	44.11	Change Valve (Sample Inject Valve)				Manual Load Loop / System Pump to Column	P6 Sample Application
Method	63	44.11	Change Valve (Column Switching Valve 1)				C1 Port 2, Bio-Gel P-6 Desalting, 50 ml, Forward Flow	P6 Elution
Settings	64	44.11	Change Valve (Column Switching Valve 2)				C2 Port 2, Loop, Forward Row	P6 Elution
	65	44.11	Change Valve (Outlet Valve 1)				To Loop Valve	P6 Elution
-	66	44.11	Change Valve (Sample Inject Valve)				Manual Load Loop / System Pump to Column	P6 Elution
	67	44.11	Gradient Segments	4.00		0	Forward Flow	P6 Elution
Method	67.1	48.11	Isocratic Row	4.00	4.000	0	25mM Tris pH 8.0, 25mMTris + 1M NaCl	P6 Elution
Outline	68	48.11	Change Valve (Column Switching Valve 1)				C1 Port 1, Profinity IMAC Ni-Charged, 5 ml, Reverse Row	P6 Elution
a	69	48.11	Change Valve (Column Switching Valve 2)				C2 Port 1, Loop, Forward Row	P6 Elution
	70	48.11	Gradient Segments	10.00		0	Reverse Flow	P6 Elution
	70.1	58.11	Isocratic Row	10.00	4.000	0	25mM Tris pH 8.0, 25mMTris + 1M NaCl	P6 Elution
Method	71 b	Delete Step	Change Valve (Outlet Valve 1)				To BoFrac	P6 Elution
Steps	72	Show Step details	Change Valve (Column Switching Valve 2)				Bypass, Forward Row	AC-1 Clean

8 Delete the last Change Valve step as it is not needed (Figure 15B).

Fig. 15B. Deletion of the Change Valve step.

в

- a Click Method Steps on the left side of the screen and make sure the P6 Elution phase is highlighted.
- Bight click the Change Valve (Outlet Valve 1) step and select Delete Step from the menu. Confirm the action to remove the step from the phase.
- 9 Rename and save the phase as P6 Elution.

Ion Exchange Purification



At this stage, the protein is in the second loop, ready to be loaded onto the IEX column, and the system contains desalting buffer, which is the same as IEX buffer A. However, system pump B is filled with IMAC buffer B, which we need to replace with IEX buffer B. We can replicate the DS Buffer Change custom phase to accomplish this (Figure 16).

elect Valve: Colu	mn Switching Val	ve 1	•	Select Port:	Bypass		▼ Reve
radient Segmer	ts						
	rom Method Setti	ngs Ro	w Rate: 10	.000 🔶 [0.001	-10] ml/min	Reverse Row	
Use Same Inlets	As Method Settin	6				3	
Segment	Inlet A		et B	Initial %B	Final %B	Volume (CV)	Drag buttons to table
Isocratic	25mM Tris	▼ 25n	M Tris	100	100	2	Isocratic
Isocratic	25mM Tris	▼ 25n	nM Tris 🔻	0	0	2	Gradient
ero Baseline	Enable						
iradient Segmer		100	w Rate: 5.0	000 👘 (0.001	-10] ml/min	🕅 Reverse Row	
iradient Segmer	rom Method Settin As Method Settin Inlet A	2	et B	000 🗼 (0.001	-10] ml/min Final %8	Volume (CV)	Drag buttons to table
iradient Segmer 7 Use Row Rate f 9 Use Same Inlets	rom Method Setti As Method Settin	2	et B	trand *			Drag buttons to table Isocratic

Fig. 16. Configuration of the IEX Buffer Change phase.

- (1) Set the upper isocratic segment for 100%B to 2 CV (10 ml).
- (2) Select the correct buffers from the Inlet A and Inlet B dropdown menus for all of the Gradient Segments.
- 3 Rename and save the phase as IEX Buffer Change.

For sample application onto the IEX column, we can replicate the P6 Desalting Sample Application phase from earlier in the method. Right click the P6 Sample Application phase and save it as a custom phase. Insert this custom phase after the IEX Buffer Change phase and edit as in Figure 17.

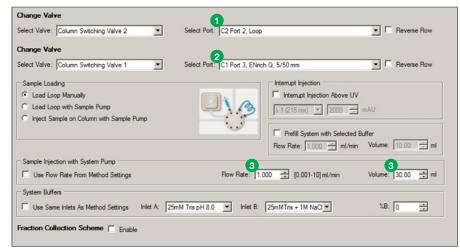


Fig. 17. Configuration of the IEX Sample Application phase.

- **1** Set Column Switching Valve 2 to C2 Port 2, Loop.
- 2 Set Column Switching Valve 1 to position 3 (C1 Port 3).
- 3 Set the Flow Rate to 1 ml/min and the Volume to 30 ml.
- 4 Rename and save the phase as IEX Sample Application.

To ensure complete sample loading and removal of unbound proteins, we can replicate the IMAC Column Wash phase. Right click the IMAC Column Wash phase and save it as a custom phase. Insert this custom phase after the IEX Sample Application phase and edit as in Figure 18.

100000	nge Valve ct Valve: Colur	nn Switching Valve	1 🔹	Select Port	C1 Port 3, ENric	ch Q, 5/50 mm	▼ Reverse Row
		om Method Settings As Method Settings	_	000 🚖 (0.00)1-10] ml/min	Reverse Row	
	Segment	Inlet A	4) Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table
Þ	Isocratic	25mM Tris	25mM Tris	• 0	0	2.00	Isocratic
	d Until	Enable					Gradient

Fig. 18. Configuration of the IEX Column Wash phase.

- (1) Set Column Switching Valve 1 to position 3 (C1 Port 3).
- 2 Set the Flow Rate to 1 ml/min.
- ③ Uncheck the Use Same Inlets As Method Settings box.
- Select the IEX buffers from the Inlet A and Inlet B dropdown menus.
- 5 Rename and save the phase as IEX Column Wash.

Unlike the preceding columns, the eluate from the IEX column will be eluted back into a loop using a linear gradient and threshold collection scheme. A Change Valve step needs to be added to ensure the CSV is in the correct position to allow this.

Adding Steps into the Elution Phase (Figure 19)



Click the big green plus sign to open the phase library.

2 Drag an Elution phase to follow the IEX Column Wash phase.

3 Click Method Steps on the left side of the screen to bring up the steps view. The individual steps that can be inserted into the phase are listed on the right side of the screen in the Step Library.

в							
93.1	98.32	Isocratic Flow	2.00	1.000	0	25mM Tris pH 8.0, 25mM Tris w/ 1M NaCl	IEX Column Wash
94	98.32	Hold Until (Disabled)			0		IEX Column Wash
95	98.32	Fraction Collection (Frac. Size: 1.50 ml)			0	Scheme: Collect All (Bio-Frac)	Elution- Modified
96	98.32	Change Valve (Inlet Valve-Buffer Inlet A)				PBS	Bution- Modified
97	98.32	Gradient Segments	10.00		0	Forward Flow	Elution- Modified
97.1	108.32	Gradient Flow	10.00	5.000	0 - 100	PBS, PBS w/ 0.5M imidazole	Elution- Modified
98	108.32	Change Valve (Column Switching Valve 1)				Bypass, Forward Flow	SEC Buffer Change
99	108.32	Gradient Segments	2.00		0	Forward Flow	SEC Buffer Change

Fig. 19. Addition of a Change Valve step into the IEX Elution phase.

Orag a Change Valve step into the phase and place it between Fraction Collection (step 95) and Gradient Segments (step 96). Since Fraction Collection spans the entire phase, it is always the first step of a phase. Adding a step changes the name of the phase to Elution-Modified. Inserting the step defaults to a Buffer Inlet A change, which needs to be edited to Column Switching Valve 1 (Figure 20).

	Change Valve
	Select Valve: Column Switching Valve 1 Select Port: C1 Port 3, ENrich Q, 5/50 mm Reverse Row Gradient Segments Use Row Rate from Method Settings Row Rate: 1.000 10.001-10] ml/min 5 Use Same Inlets As Method Settings Use Same Inlets As Method Settings Use Same Inlets As Method Settings
Method Settings	Segment Inlet A Inlet B Initial %B Final %B Orag buttons to table Isocratic 25mM Tris 25mM Tris 100 100 2
	Hold Until Enable
Method Outline	Fraction Collection Scheme Image: Enable Image: BioFrace O Outlet Valves Available Schemes Image: Use Fraction Size from Method Settings
	Collect All Fraction Size: 1.50 in ml
Method Steps	C Collection Windows

Fig. 20. Modification of the Change Valve section in the IEX Elution phase.

- 1 Click Method Outline on the left side of the screen to return to the phase overview.
- 2 Select Column Switching Valve 1 from the Select Valve dropdown menu under Change Valve.
- 3 Select C1 Port 3 from the Select Port dropdown menu.
- 4 Uncheck the Use Flow Rate from Method Settings box and set the Flow Rate to 1 ml/min.
- Uncheck the Use Same Inlets As Method Settings box and use the dropdown menus under Inlet A and Inlet B to select IEX buffers A and B, respectively.
- 6 Set the volume of the gradient based on your optimization process (see bulletin 6725 for more information).

To elute the protein from the IEX column into the sample loop, modify the Fraction Collection Scheme (Figure 21).

Change Val	ve					
Select Valve:	Column Switching	Valve 1 👻	Select Port: C	1 Port 3, ENrich (Q, 5/50 mm	 Reverse Flow
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	Settings Flow Rate:	1.000 🚖 [0.001-10	0] ml/min	Reverse Flow	
Segme	nt Inlet A	Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table
Gradien	25mM Ti	is 🔻 25mM Tris	- 0	100	2	Isocratic
			•			Gradient
Available Sc		Threshold Settings	BioFrac 🚺 Outlet V	alves		
Collect	t All	Signal Type:	4 Value:		Above Threshold	
2 Thresh	blo	λ 3 (280 nm)	▼ 50	mAU () Below Threshold	
Collect	tion Windows	Above Threshold	from Method Settings	Below Three		
		V Use Hacuon Size	nom metrioù Settings	· Waste	O collect	

Fig. 21. Modification of the Fraction Collection Scheme in the IEX Elution phase.

- Select Outlet Valves.
- 2 Under Available Schemes, select Threshold.
- 3 Under Threshold Settings, set the Signal Type to 280 nm using the dropdown menu.
- 4 Set the Value to 50 mAU Above Threshold.

To ensure that the loop valve is in the correct position to collect the protein as it elutes from the IEX column, insert and edit another Change Valve step after the Column Switching Valve 1 change valve step (Figure 22).

2								
Method Settings	93.1	98.32	Isocratic Row	2.00	1.000	0	25mM Tris pH 8.0, 25mM Tris w/ 1M NaCl	IEX Column Wash
	94	98.32	Hold Until (Disabled)			0		IEX Column Wash
-	95	98.32	Fraction Collection (Frac. Size: 50.00 ml)			0	Signal: \lambda 3 (280 nm), collect if threshold is > 50.00 mAU otherwise waste (Outlet Valve)	Bution- Modified
Method	96	98.32	Change Valve (Column Switching Valve 1)				C1 Port 3, ENrich Q, 5/50 mm, Forward Flow	Bution- Modified
Dutline	2 97	98.32	Change Valve (Inlet Valve-Buffer Inlet A)			12 2	PBS	Eution-Modified
	98	98.32	Gradient Segments	2.00		0	Forward Row	Eution-Modified
-	98.1	100.32	Gradient Row	2.00	1.000	0.100	25mM Tris pH 8.0, 25mM Tris w/ 1M NaCl	Bution- Modified
Method	99	100.32	Change Valve (Column Switching Valve 1)				Bypass, Forward Row	SEC Buffer Change
Steps	100	100.32	Gradient Segments	2.00		0	Forward Flow	SEC Buffer Change

1 With the modified Elution phase selected, click Method Steps.

2 Drag in a Change Valve step between Change Valve (Column Switching Valve 1) (step 96) and Gradient Segments (step 97).

А

Select Valve: Column Switch	ing Valve 1 🔹	Select Port: C1	Port 3, ENrich Q, 5/50 mm	✓ Reverse F
Change Valve		6		
Select Valve: Column Switch	ing Valve 2 🔻	Select Port: C2	Port 3, Loop	💌 🖂 Reverse F
Gradient Segments				
	d Settings Flow Rate: 1.000	[0.001-10]	mi/min Reverse Flor	w
		[0.001-10]		
Use Same Inlets As Metho	-			Drag buttons to table
Segment Inlet			Final %B Volume (CV)	
Gradient 25mM	Tris 🔻 25mM Tris 💌 0	,	100 2	Isocratic
Gradient 25mM	Ins • 25mM Ins •		100 2	
Gradient 25mM	ins • 25mM ins • U		2	Gradient
Fraction Collection Schem		c @ Outlet Val		
Fraction Collection Schem	ne 👽 Enable 💿 BioFra			Gradient
Fraction Collection Schen Available Schemes © Collect All	ne 📝 Enable 💿 BioFra Threshold Settings	c Outlet Value:	lves	Gradient
Fraction Collection Schen Available Schemes Collect All Threshold	ne V Enable BioFra Threshold Settings Signal Type:	c Outlet Value:	Above Thresho	Gradient
Fraction Collection Schen Available Schemes © Collect All	ne V Enable BioFra Threshold Settings Signal Type:	c Outlet Value:	Above Thresho	Gradient
Fraction Collection Schen Available Schemes Collect All Threshold	ne ♥ Enable ◎ BioFra Threshold Settings Signal Type: (λ 3 (280 nm)	c Outlet Value: 50	e Above Thresho nAU	Gradient
Fraction Collection Schen Available Schemes Collect All Threshold	ne ✓ Enable ⊙ BioFra Threshold Settings Signal Type: λ 3 (280 nm) Above Threshold	c Outlet Value:	e Above Thresho nAU Below Thresho Below Threshold	old Id

Fig. 22. Configuration of the IEX Elution phase.

- 3 Click Method Outline.
- 4 Under Change Valve, select Column Switching Valve 2 from the Select Valve dropdown menu.
- 5 From the Select Port dropdown menu, select C2 Port 3, Loop.
- 6 Rename and save the phase as IEX Elution.

Now the correct column and loop will be in position at the beginning of the phase, which is set up to elute with a 10 ml 0–100%B linear gradient of IEX buffers and monitor the 280 nm wavelength, diverting the protein to the loop when the UV absorbance exceeds the threshold. This will successfully elute the protein from the IEX column and store it in the loop until sample application onto the final SEC column.

Size Exclusion Purification



At this point, the protein is in Loop 3 and the system contains IEX buffer. Before loading the sample onto the SEC column, the system needs to be flushed with SEC buffer. This can be accomplished by replicating the DS Buffer Change phase again (Figure 23).

Change Valve										
Select Valve: Column	Switching Valve 1	•	Select Port:	ypass		▼ □ Reverse Flow				
Gradient Segments	Gradient Segments									
Use Flow Rate from	□ Use Row Rate from Method Settings Row Rate: 10.000 🚔 [0.001-10] ml/min									
Use Same Inlets A	s Method Settings									
Segment	Inlet A	Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table				
▶ Isocratic	50mM Tris 💌	PBS + 0.5 💌	0	0	2	Isocratic				
						Gradient				
Hold Until	Enable									
Zero Baseline	Enable									
Gradient Segments		3								
2 Use Flow Rate from	n Method Settings	Row Rate: 0.50	00 🗧 [0.001-1	0] ml/min						
Use Same Inlets A	s Method Settings									
Segment	Inlet A	Inlet B	Initial %B	Final %B	Volume (CV)	Drag buttons to table				
► Isocratic	50mM Tris 💌	PBS + 0.5 💌	0	0	0.15	Isocratic				
						Gradient				
1										

Fig. 23. Configuration of the SEC Buffer Change phase.

- 1 Select the SEC buffer from the Inlet A dropdown menus in the upper and lower Gradient Segments sections.
- 2 Uncheck the Use Flow Rate from Method Settings box.
- 3 Set the Flow Rate to 0.5 ml/min (the manufacturer's recommended flow rate for the SEC column).
- 4 Rename and save the phase as SEC Buffer Change.

To load the protein onto the SEC column, access the custom phase library and drag in a P6 Desalting Sample Application phase after the SEC Buffer Change phase. Edit the phase as shown in Figure 24.

Change Valve		
Select Valve: Column Switching Valve 2	Select Port C2 Port 3, Loop	Reverse Row
Change Valve		
ielect Valve: Column Switching Valve 1	Select Port C1 Port 4, HiPrep 16/60 Sephacryl S 300 HR	Reverse Row
Sample Loading	Interrupt Injection	
Load Loop Manually	Interrupt Injection Abov	ve UV
C Load Loop with Sample Pump	λ 1 (215 nm) Ξ 2000	mAU
C Inject Sample on Column with Sample Pump		
	Prefill System with Sele	ected Buffer
	Flow Rate: 1.000 n	nl/min Volume: 10.00 📻 ml
Sample Injection with System Pump		•
Use Row Rate From Method Settings	Flow Rate: 0.500 🚖 [0.001-10] ml/min	Volume: 10.00 🕂 ml
System Buffers		
Use Same Inlets As Method Settings Inlet A:	25mM Tris pH 8.0 V Inlet B: PBS + 0.5M Imidazol V	%B: 0 🕂

Fig. 24. Configuration of the SEC Sample Application phase.

- 1 Select C2 Port 3, Loop from the Select Port dropdown menu on Column Switching Valve 2 as it contains the protein sample.
- 2 Select the HiPrep 16/60 Sephacryl S 300 HR Column on port 4 from the dropdown menu of Column Switching Valve 1.
- 3 Set the Flow Rate to the recommended 0.5 ml/min and the Volume to 10 ml since Loop 3 has a volume of 5 ml.
- 4 Rename and save the phase as SEC Sample Application phase.

The SEC column can be eluted with a simple isocratic flow across the column. To do this, replicate the AC-1 Column Wash custom phase and add fraction collection to it. Open the custom phase library and drag an AC-1 Column Wash phase into the method after the SEC Sample Application phase. Edit the phase as shown in Figure 25.

Change Valve								
Select Valve: Column Switching Valve 1 Select Port: C1 Port 4, HiPrep 16/60 Sephacryl S 300 HR 🔽 Reverse Flow								
Gradient Segments Use Row Rate from Method Settings Row Rate: 0.500 = [0.001-10] ml/min								
Use Same Inlets As Method Settings Segment Inlet A Inlet B Initial %B Final %B Volume (CV) Isocratic Isocratic 50mM Tris V PBS + 0.5 V 0 0 25 Isocratic Gradient								
Hold Until Enable Fraction Collection Scheme Enable Enable BioFrac Outlet Valves Available Schemes Collect All Collect All Collection Windows Collection Windows								

Fig. 25. Configuration of the SEC Elution phase.

- 1 Change the Column Switching Valve 1 position to C1 Port 4.
- 2 Set the Flow Rate to 0.5 ml/min (recommended flow rate for the SEC column).
- 3 Select the correct buffer for the isocratic elution from the dropdown menu under Inlet A.
- 4 Set an isocratic step of 25 CV to ensure all the sample buffer will be washed out of the column.
- 5 Since this is the last column in the purification, enable the Fraction Collection Scheme to collect fractions during elution.
- 6 Rename and save the phase as SEC Elution.

Following the steps outlined in this method will ensure that your protein is purified successfully using an automated Multi-D workflow utilizing IMAC, desalting, IEX, and SEC columns.

System Reset and Column Cleaning (optional)



After completing the Multi-D purification, it is possible to clean all the columns and flush them and the system with water and 20% ethanol. To avoid overpressuring any of the columns, a depressurization step can be added (Figure 26). Right click the Wait For Sample Load phase and save it as a custom phase. Open the custom phase library and drag the Wait For Sample Load phase into the method after the SEC Elution phase.

Hold Until Enable	(1) Check the Time Out box.
Pause Until Resume V Enable Sound Alarm V Time Out	② Set the Time Out duration to 2 min.

Fig. 26. Configuration of the depressurization step.

Use the AC-1 Clean phase to clean the IMAC column or load in storage buffer. The default phase washes a column with 100%B and re-equilibrates it with 100% buffer A. Edit the phase as shown in Figure 27 to clean/load storage buffer into the other columns.

ele	nge Valve ct Valve: Colu	mn Switching Val	ve 2 💌	Select Port:	Bypass		Reverse Flor
	nge Valve				0		
alei	ct valve: [Colu	mn Switching Val	ve 1 💌	Select Port:	C1 Port 1, NUVI	a IMAC Ni-Charged.	5 ml Reverse Ro
rad	dient Segmen	its	2				
			0.0.1.	10 00	4 4 65 1 4 4		
1	Use Flow Rate f	from Method Settin	ngs Row Rate: 4.0	00 = [0.00	11-10j ml/min		
		As Method Settin	-		/1-10j mL/min	6	_
			-	Initial %B	Final %B	5 Volume (CV)	Drag buttons to table
	Use Same Inlets	As Method Settin	ngs	4			Drag buttons to table Isocratic
	Use Same Inlets Segment	As Method Settin	Inlet B	Initial %B	Final %B	Volume (CV)	

Fig. 27. Conversion of the AC-1 Clean phase to a cleaning/storage phase for each column.

- 1 Change the Column Switching Valve 1 position to make sure the correct column is in line.
- 2 Set the Flow Rate to the recommended flow rate for the column.
- 3 Select the correct buffer system/cleaning solutions for the correct gradient segments.
- 4 Change the %B compositions of the gradient segments.
- **5** Change the volume of the gradient segments, ensuring the correct buffer is through the system and selected column.
- 6 Rename and save the phase as a custom phase and then insert a new phase for each column to be cleaned/stored.

The final End Run phase places Column Switching Valve 1 in the bypass position and flushes the system with the buffer of choice (buffer A1 by default). The Gradient Segments steps of the phase could be edited as needed to flush the system with water and load the system with a storage buffer such as 20% ethanol (Figure 28).

Change Valve										
Select Valve: Column Switching Valve 1 💌 Select Port: Bypass 💌 🔽 Reverse Row										
Gradient Segments □ Use Row Rate from Method Settings Row Rate: 10.000										
Segment Inlet A	Segment Inlet A Inlet B Initial %B Final %B Volume (CV) Drag buttons to table									
▶ Isocratic dH20 💌	▶ Isocratic dH2O ▼ 20% EIOH ▼ 0 0 2 Isocratic									
Gradient										

Fig. 28. End Run phase.

The generation of a Multi-D method involves the addition of many phases. However, the tandem and Multi-D templates in ChromLab Software offer a good starting point to build off of in terms of replicating method phases into the custom phase library. Regardless of the number of columns in the workflow, the individual columns follow a standard phase progression of system preparation with buffer, sample application, column wash, and column elution. In our current example, a majority of the IMAC purification workflow Multi-D phases were replicated from the template. Only the IEX Elution phase had to be generated by adding steps into a standard phase. The only other edits to the method were centered on selecting the correct valve positions, buffer systems, and flow rates.

Figure 29 depicts the evolution of the Multi-D IMAC purification method from a standard ChromLab Software method template.

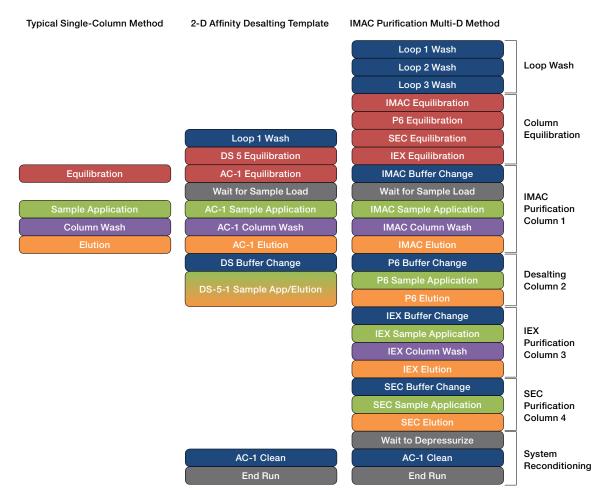


Fig. 29. Evolution of the Multi-D IMAC purification method.

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