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

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).

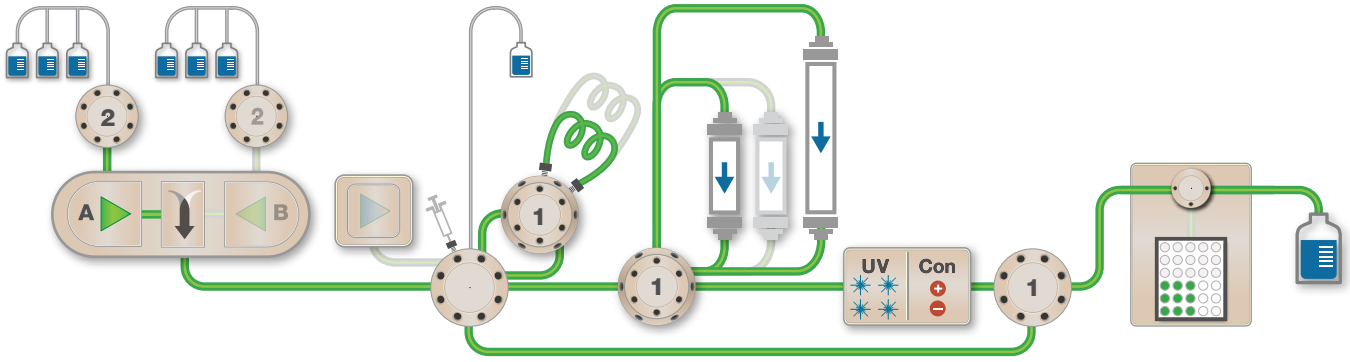


Fig. 1. NGC™ Quest Plus 10 Chromatography System used for this purification workflow.

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.

BIO-RAD

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.

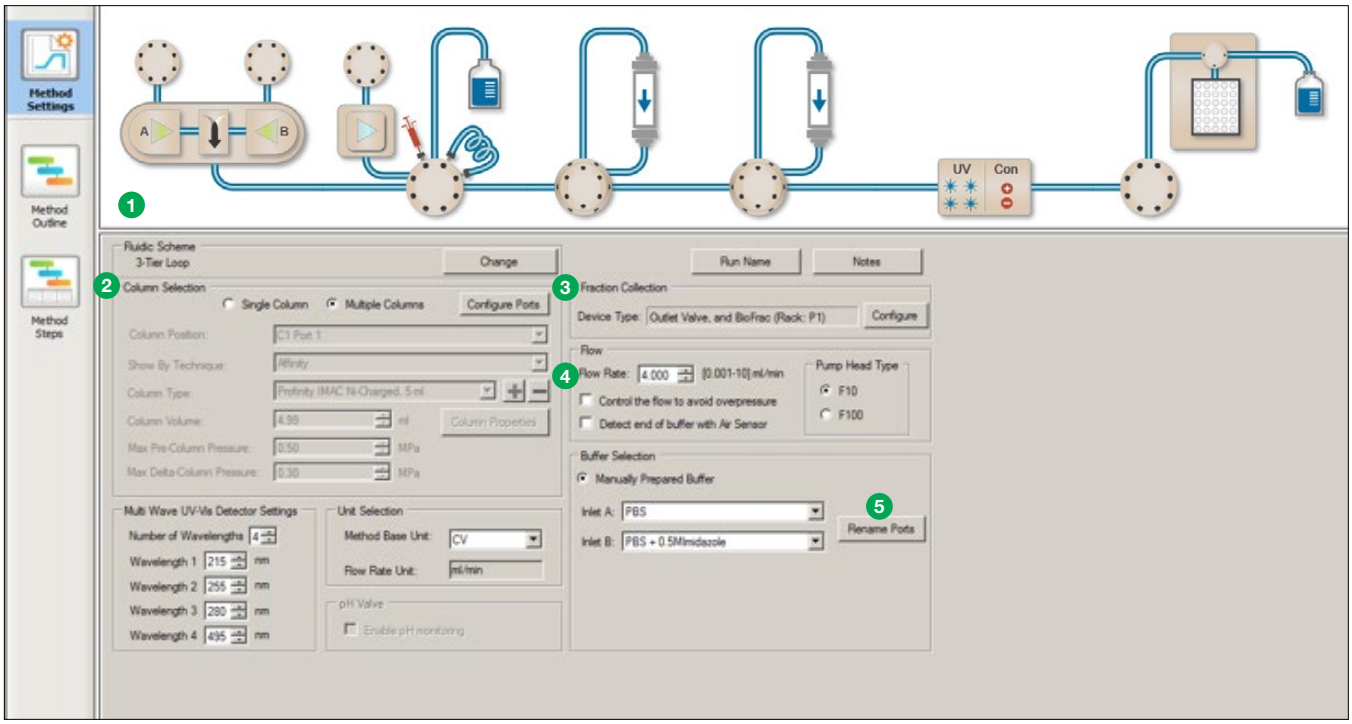


Fig. 2. Method settings for this purification workflow.

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).

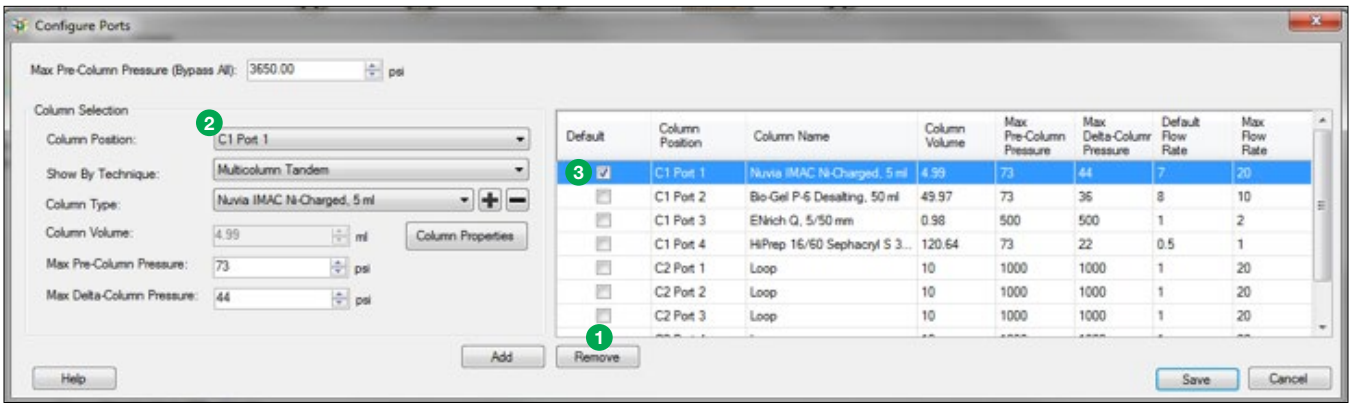


Fig. 3. Configuration of the columns used in this purification workflow.

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.

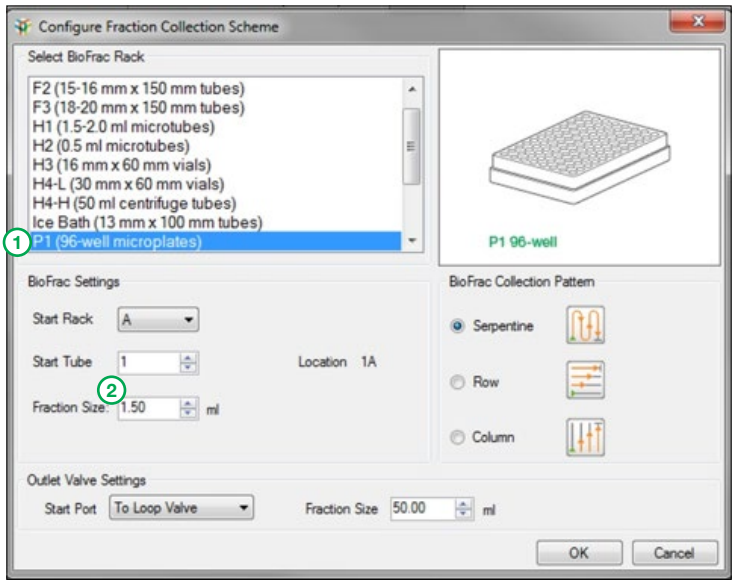


Fig. 4. Configuration of the fraction collection scheme.

1. Select the P1 (96-well microplates) rack.
2. 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.

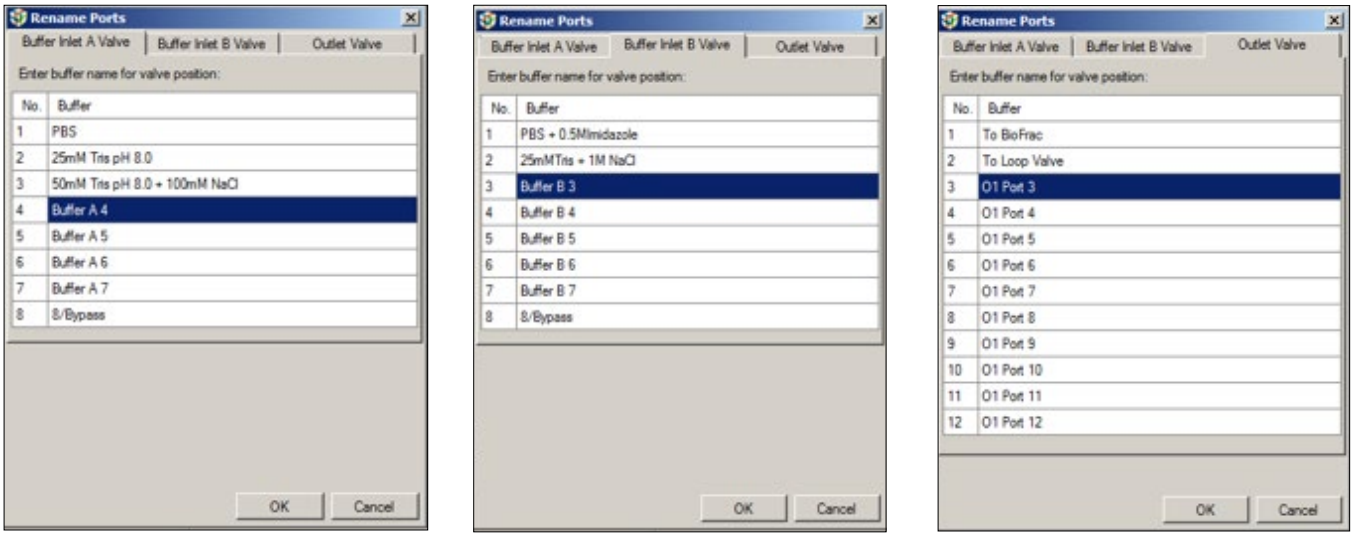


Fig. 5. Optional renaming of buffer and outlet ports for clarity.

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).

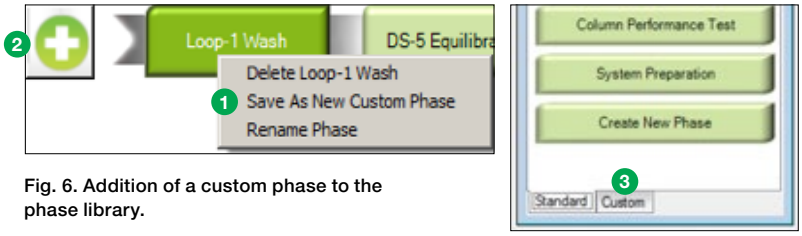


Fig. 6. Addition of a custom phase to the phase library.

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).

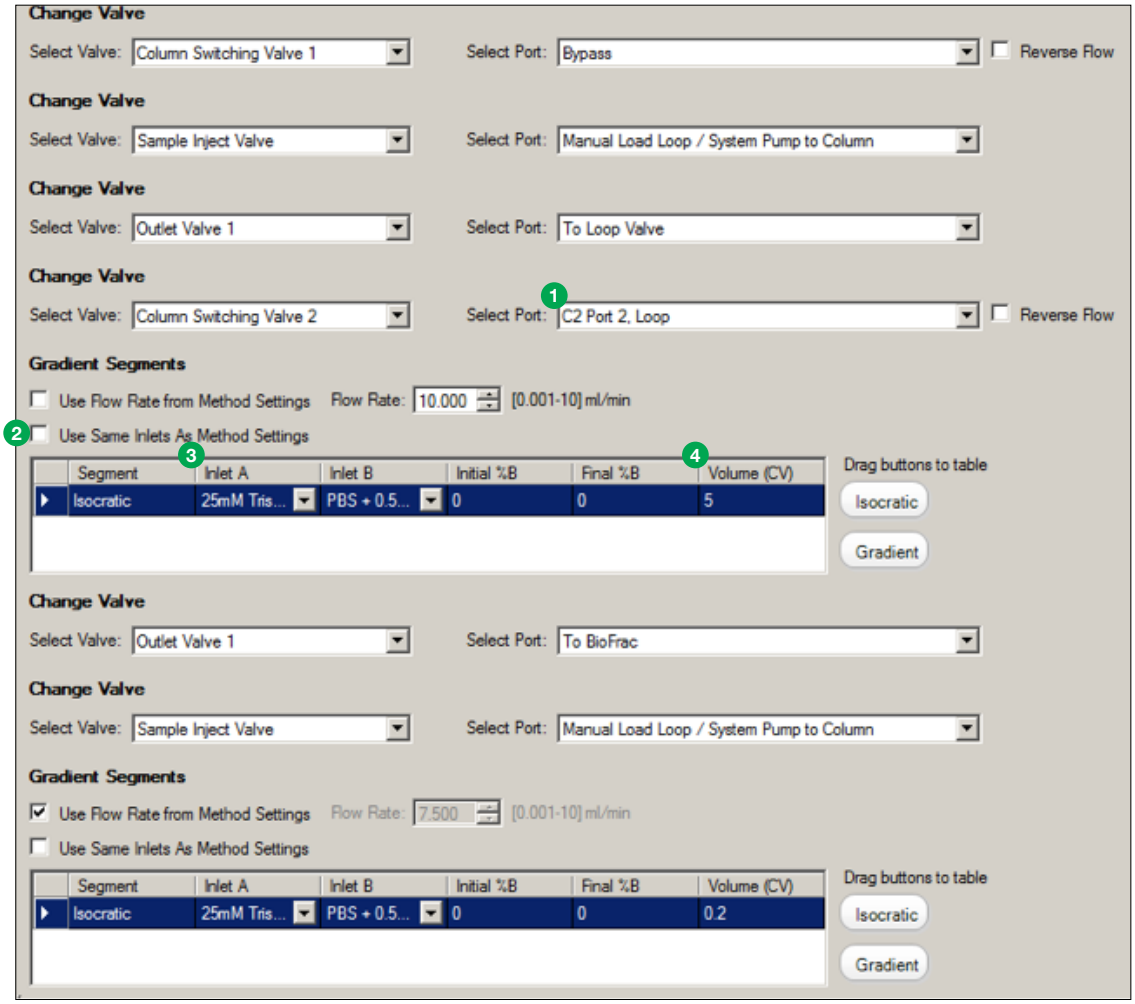


Fig. 7. Conversion of the Loop-1 Wash phase to a Loop-2 Wash phase.

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.

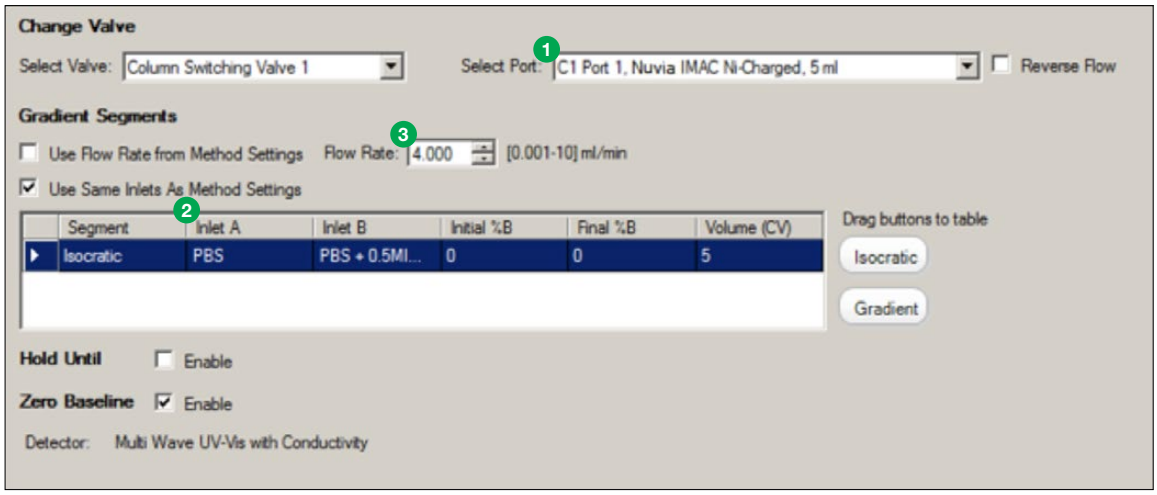
To wash Loop 3, insert another Loop-1 Wash custom phase as before. Change the Column Switching Valve 2 position to C2 Port 3, Loop, change the buffer system to the SEC buffer (50 mM Tris pH 8.0 + 100 mM NaCl), and reduce the isocratic volume to 2 CV to flush the system with buffer (5 ml) and wash the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

Equilibrating the Columns

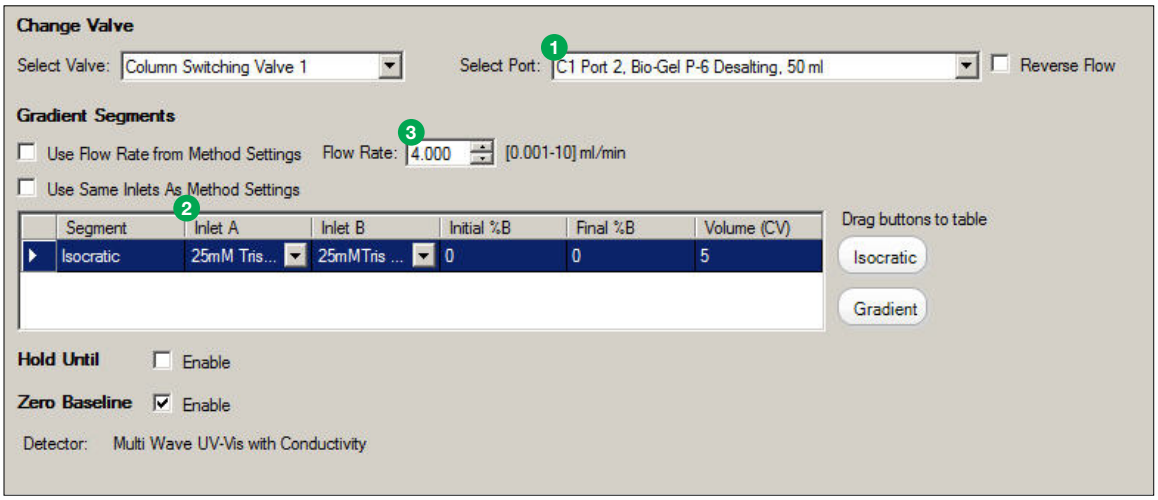
The **2-D Affinity (5 ml) - Desalting (50 ml)** template comes with two column equilibration phases, DS-5 and AC-1 equilibration, which differ by their Column Switching Valve 1 position and buffer system. When equilibrating the columns, equilibrate the low-pressure IMAC, desalting, and SEC columns before 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.

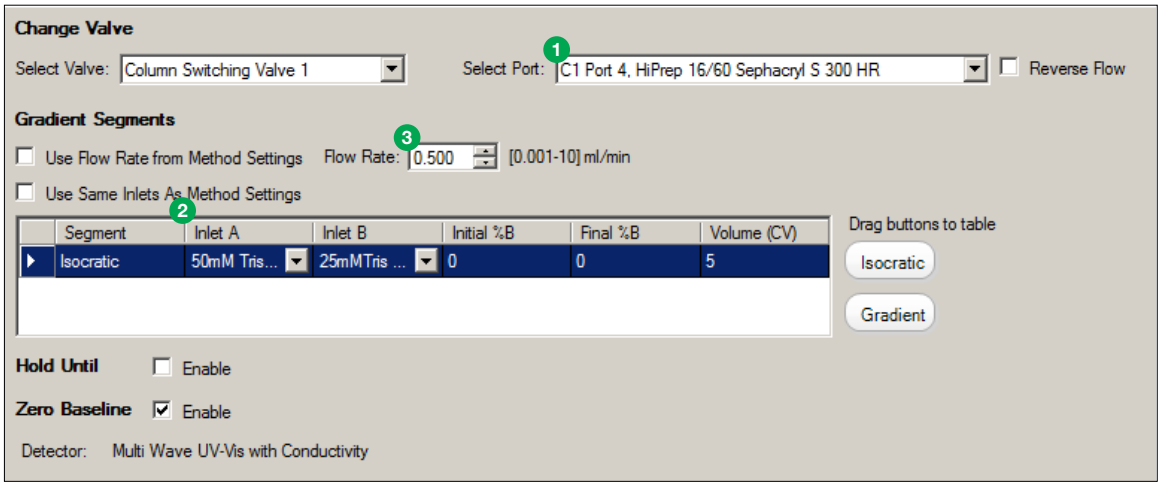
A. IMAC column



B. Desalting column



C. SEC column



D. IEX column

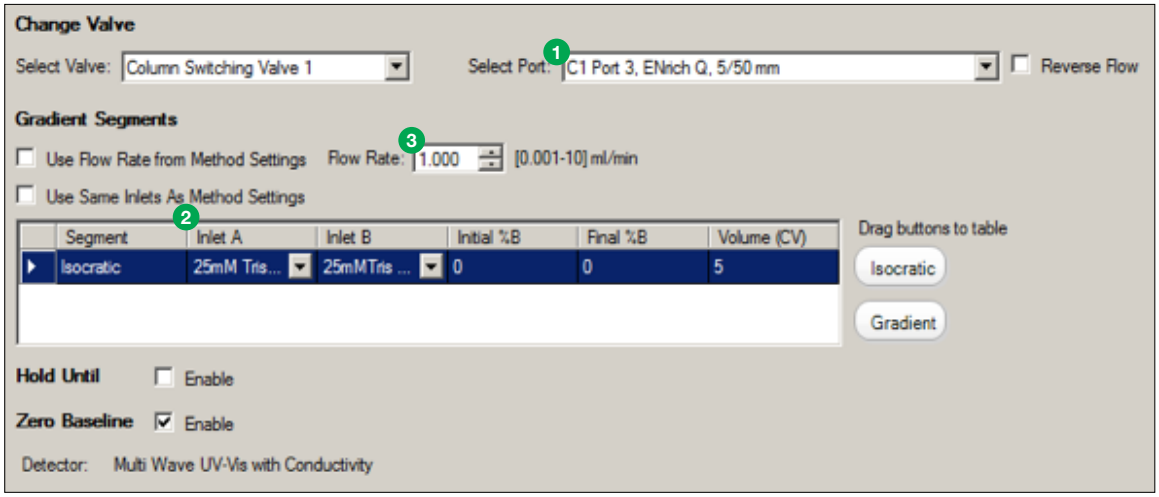


Fig. 8. Configuration of the four different equilibration phases.

1	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.

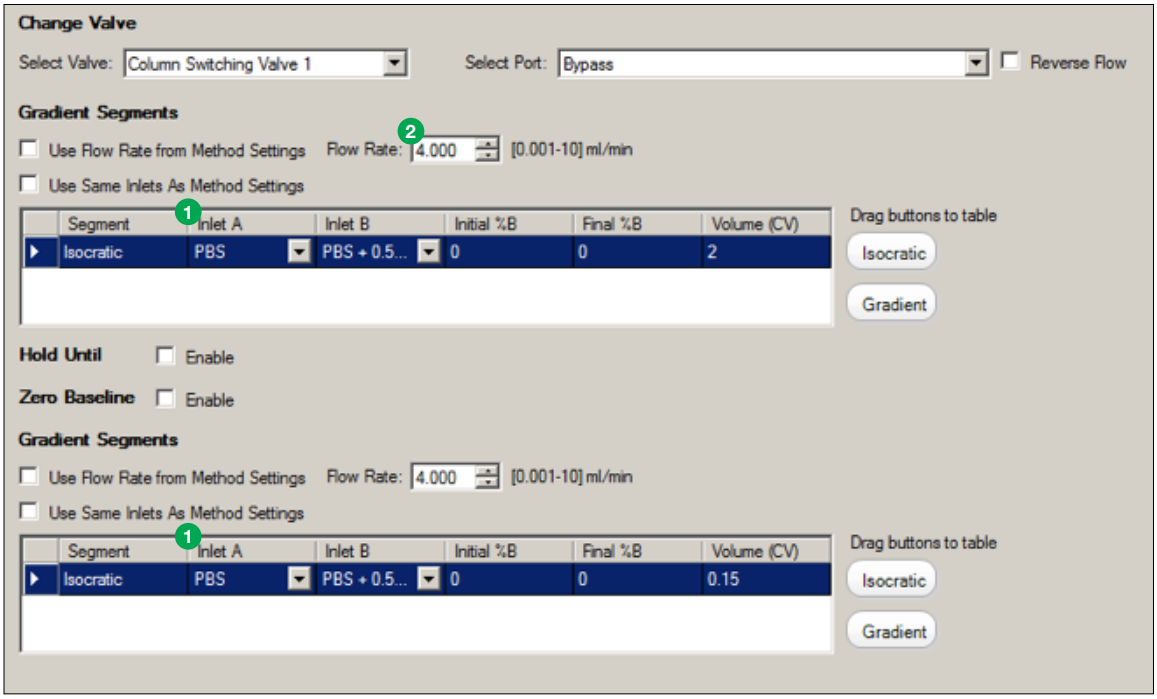


Fig. 9. Configuration of the IMAC Buffer Change phase.

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.

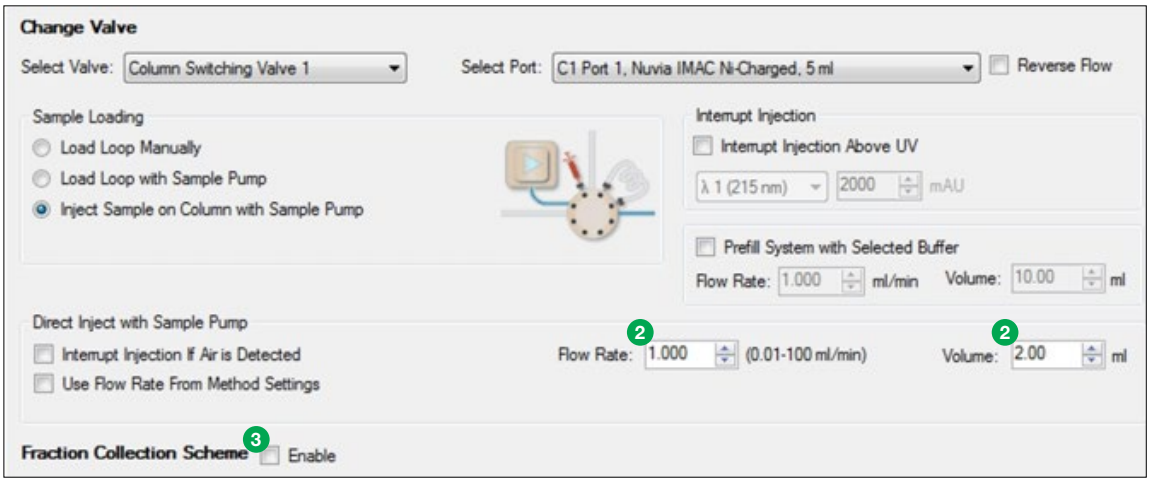


Fig. 10. Configuration of the IMAC Sample Application phase.

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.

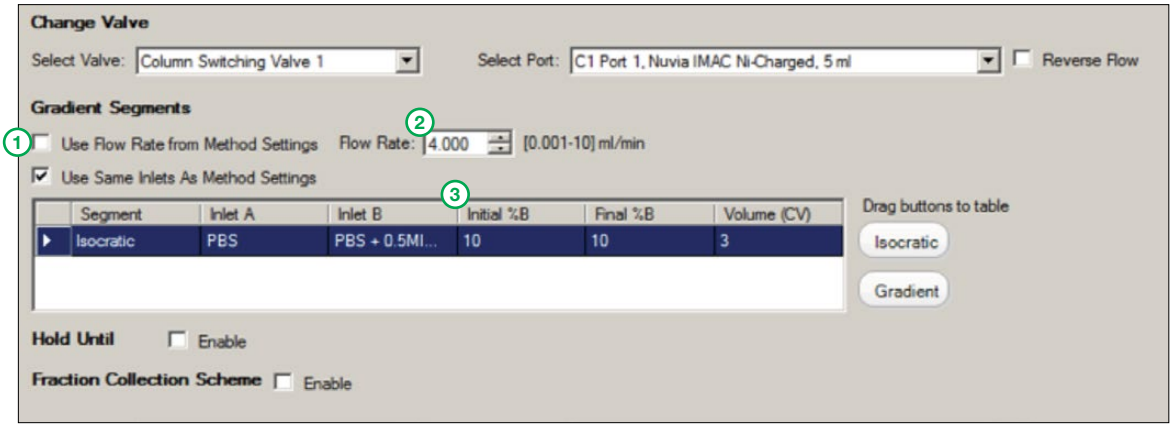


Fig. 11. Configuration of the IMAC Column Wash phase.

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).

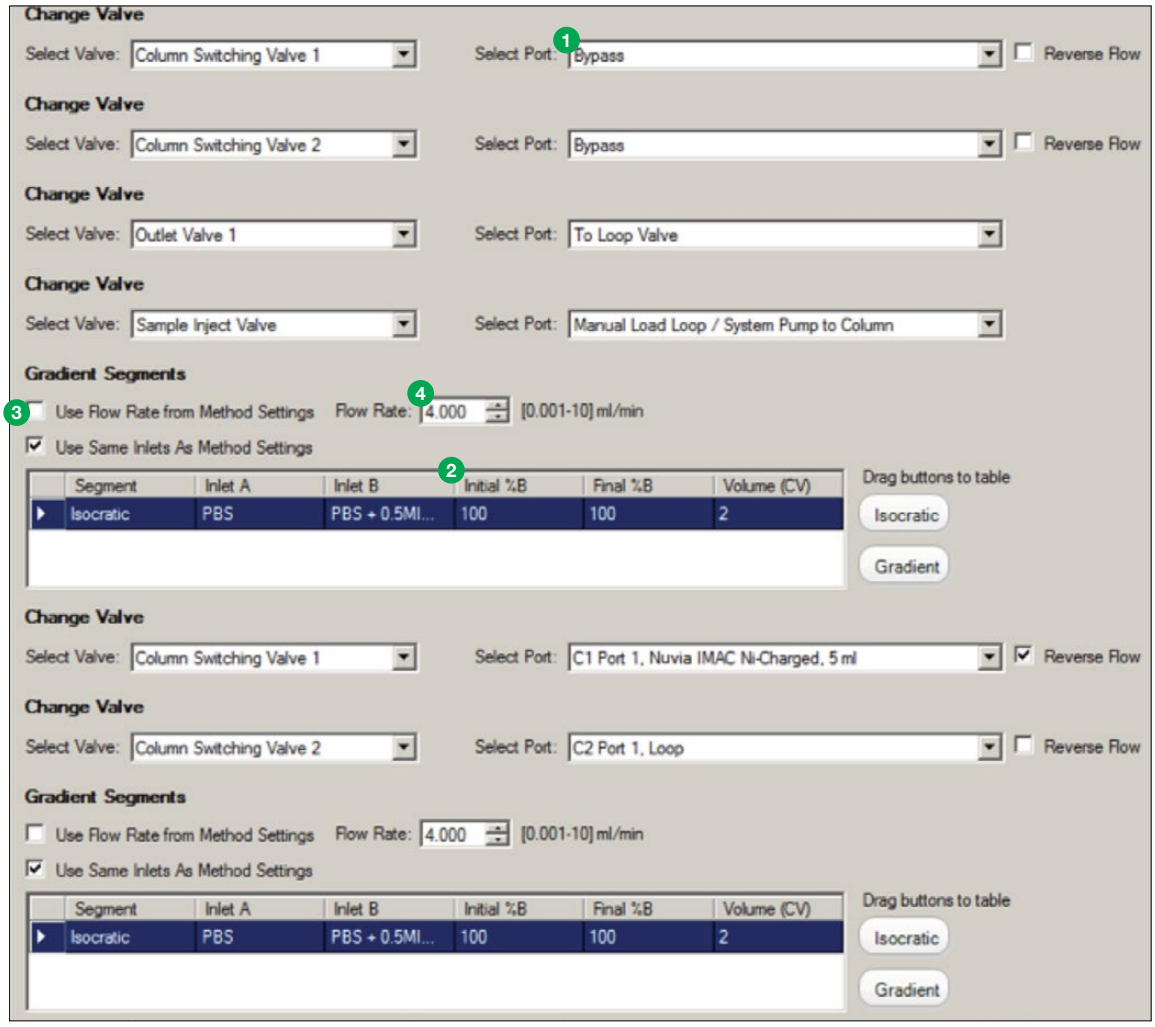


Fig. 12. Configuration of the IMAC Elution phase.

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.

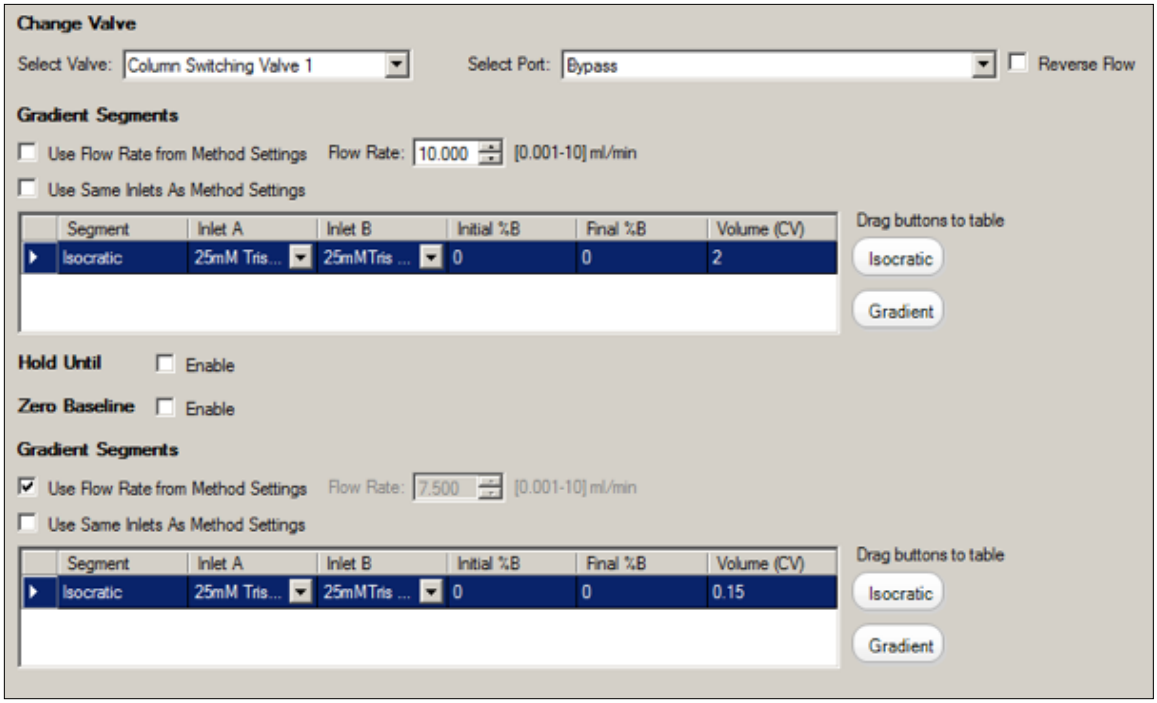


Fig. 13. P6 Buffer Change phase.

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).

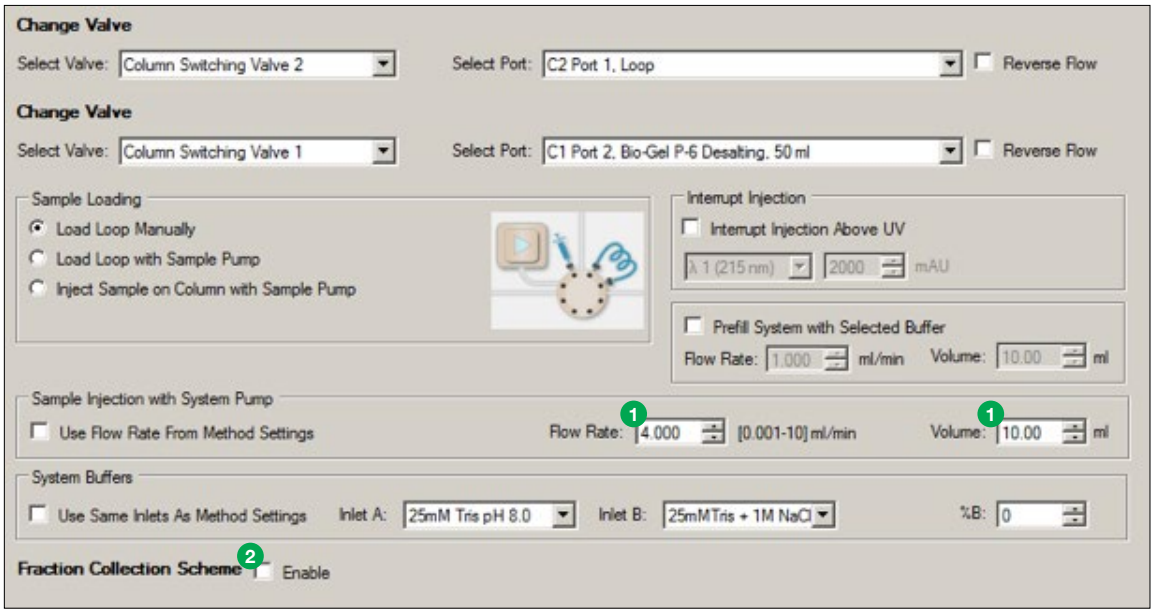


Fig. 14. Configuration of the P6 Sample Application phase.

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).

A

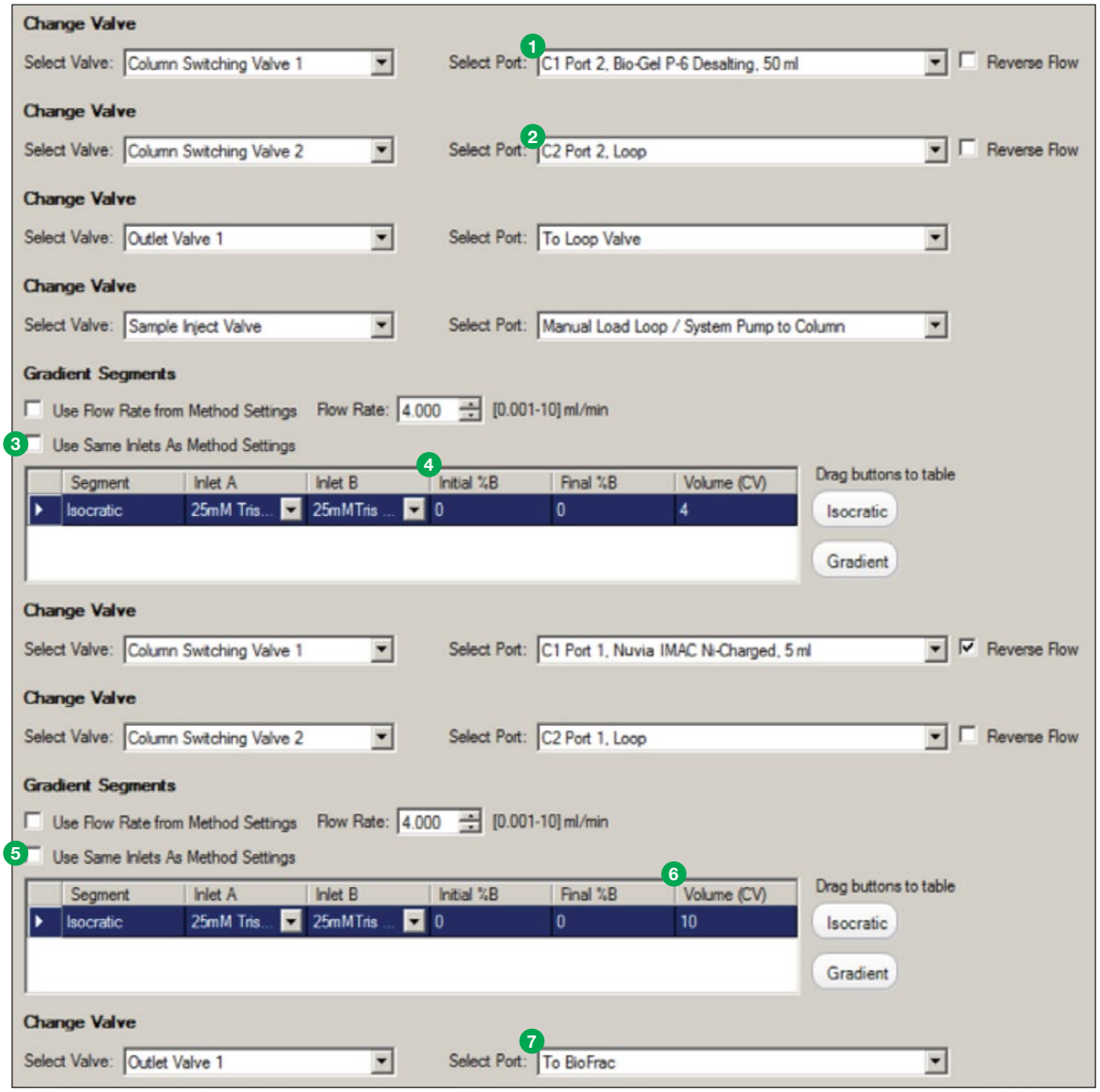
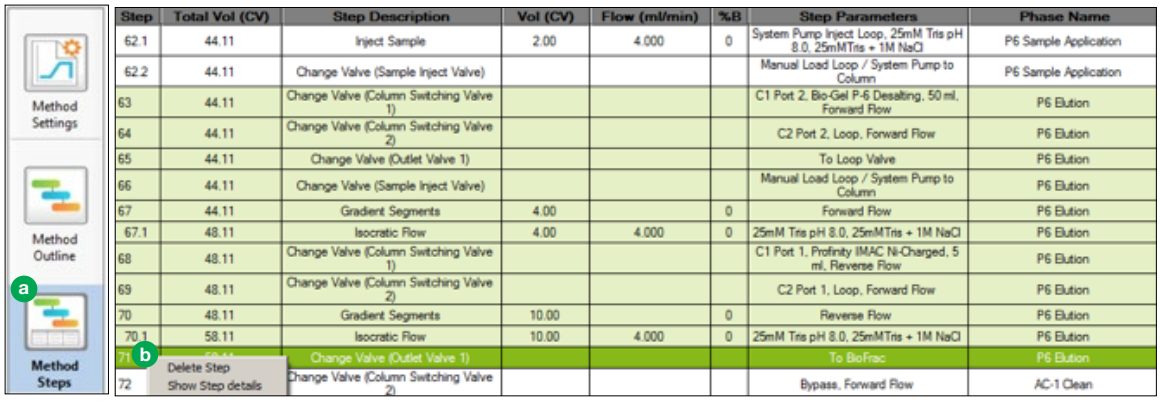


Fig. 15A. Configuration of the P6 Elution phase.

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.
3. 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.
5. 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.
7. 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.

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

B



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
63	44.11	Change Valve (Column Switching Valve 1)				C1 Port 2, Bio-Gel P-6 Desalting, 50 ml, Forward Flow	P6 Elution
64	44.11	Change Valve (Column Switching Valve 2)				C2 Port 2, Loop, Forward Flow	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
67.1	48.11	Isocratic Flow	4.00	4.000	0	25mM Tris pH 8.0, 25mMTris + 1M NaCl	P6 Elution
68	48.11	Change Valve (Column Switching Valve 1)				C1 Port 1, Profinity IMAC Ni-Charged, 5 ml, Reverse Flow	P6 Elution
69	48.11	Change Valve (Column Switching Valve 2)				C2 Port 1, Loop, Forward Flow	P6 Elution
70	48.11	Gradient Segments	10.00		0	Reverse Flow	P6 Elution
70.1	58.11	Isocratic Flow	10.00	4.000	0	25mM Tris pH 8.0, 25mMTris + 1M NaCl	P6 Elution
71		Change Valve (Outlet Valve 1)				To BioFrac	P6 Elution
72		Change Valve (Column Switching Valve 2)				Bypass, Forward Flow	AC-1 Clean

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

a Click Method Steps on the left side of the screen and make sure the P6 Elution phase is highlighted.

b Right 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).

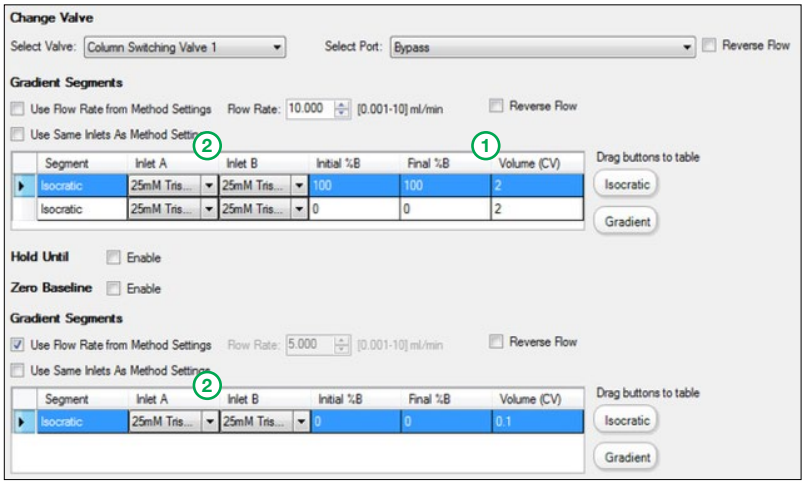


Fig. 16. Configuration of the IEX Buffer Change phase.

(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.

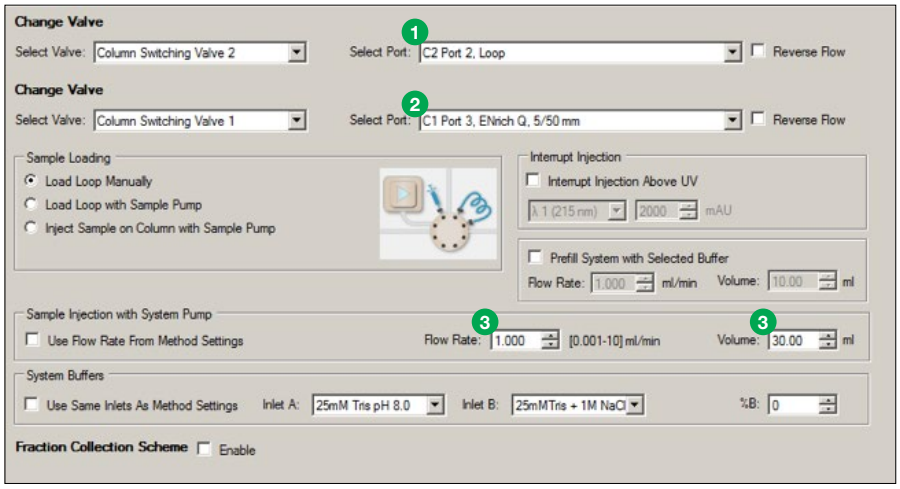


Fig. 17. Configuration of the IEX Sample Application phase.

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.

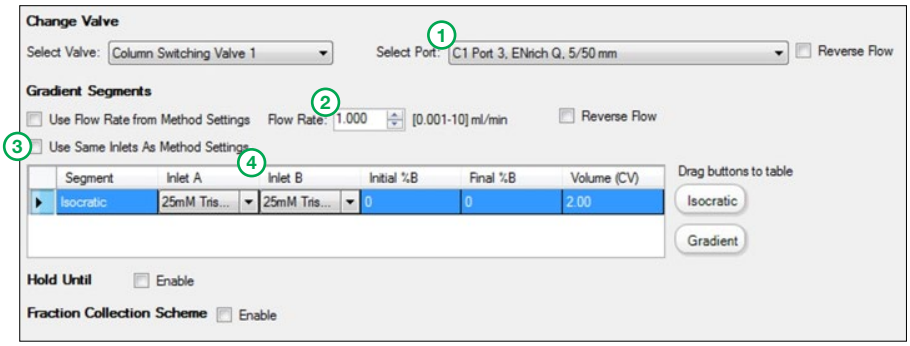


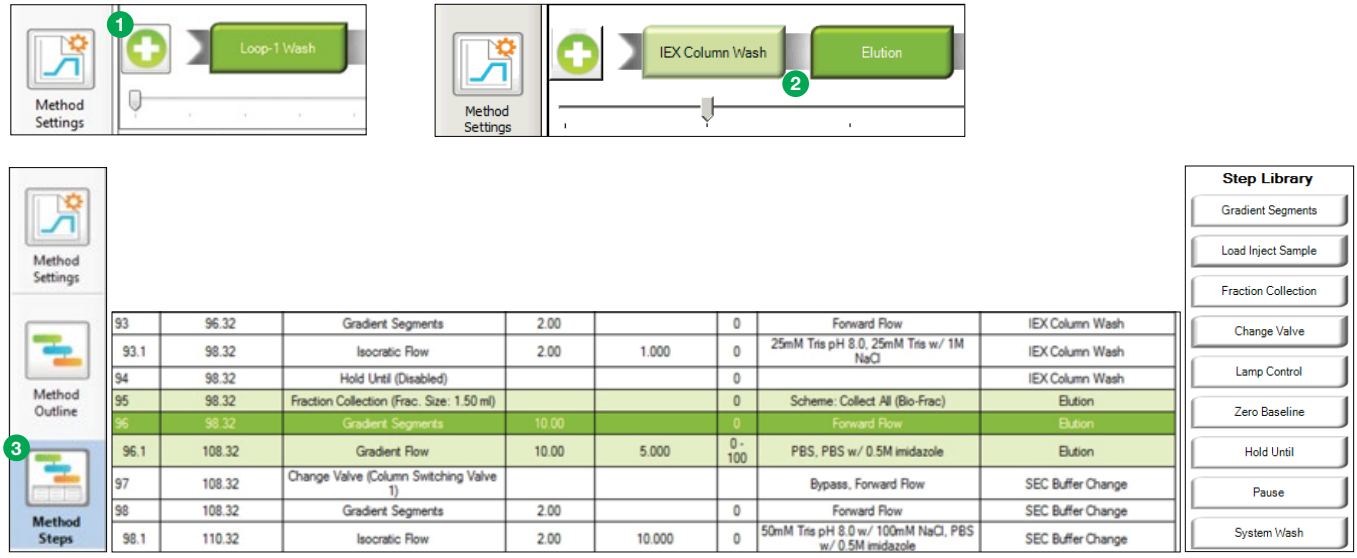
Fig. 18. Configuration of the IEX Column Wash phase.

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.
4. 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)

A



93	96.32	Gradient Segments	2.00		0	Forward Flow	IEX Column Wash
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
96	98.32	Gradient Segments	10.00		0	Forward Flow	Elution
96.1	108.32	Gradient Flow	10.00	5.000	0 - 100	PBS, PBS w/ 0.5M imidazole	Elution
97	108.32	Change Valve (Column Switching Valve 1)				Bypass, Forward Flow	SEC Buffer Change
98	108.32	Gradient Segments	2.00		0	Forward Flow	SEC Buffer Change
98.1	110.32	Isocratic Flow	2.00	10.000	0	50mM Tris pH 8.0 w/ 100mM NaCl, PBS w/ 0.5M imidazole	SEC Buffer Change

1. 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.

B

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
4 96	98.32	Change Valve (Inlet Valve-Buffer Inlet A)				PBS	Elution- 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.

4. Drag 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).

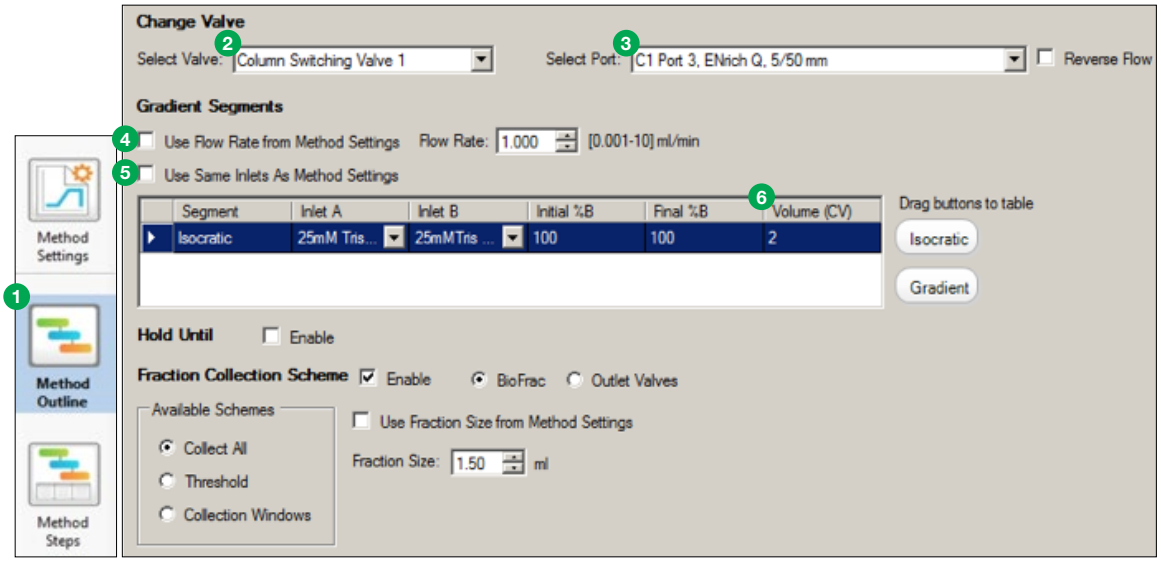


Fig. 20. Modification of the Change Valve section in the IEX Elution phase.

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.
5. 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).

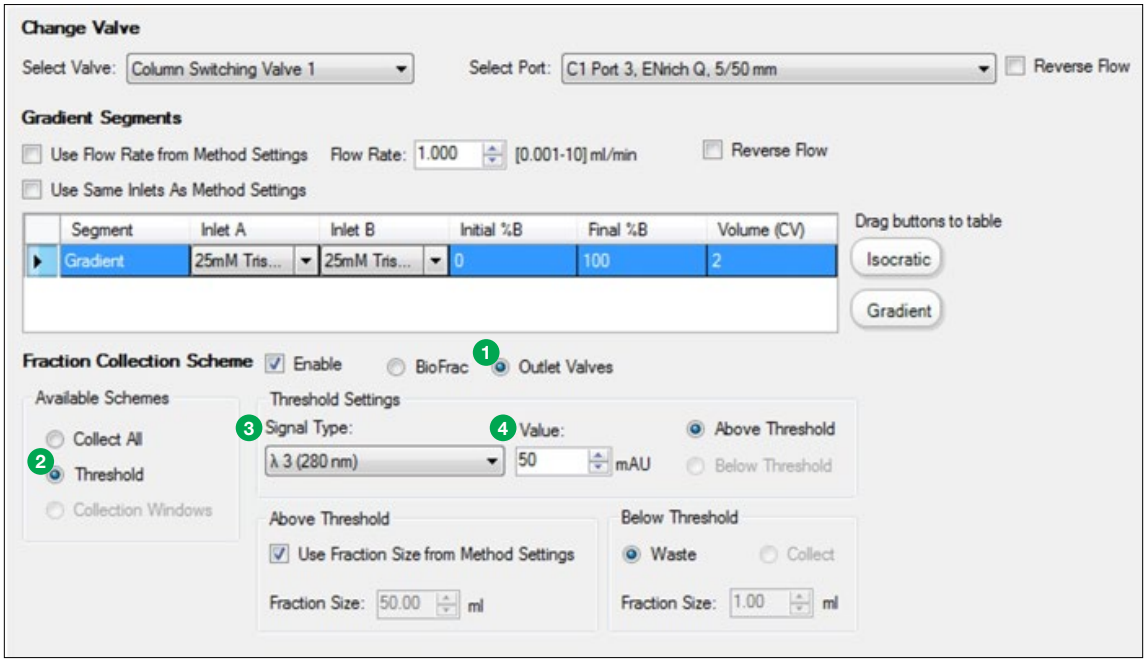


Fig. 21. Modification of the Fraction Collection Scheme in the IEX Elution phase.

1. 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).

A

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: 50.00 ml)			0	Signal: λ 3 (280 nm), collect if threshold is > 50.00 mAU otherwise waste (Outlet Valve)	Elution- Modified
96	98.32	Change Valve (Column Switching Valve 1)				C1 Port 3, ENrich Q, 5/50 mm, Forward Flow	Elution- Modified
97	98.32	Change Valve (Inlet Valve-Buffer Inlet A)				PBS	Elution- Modified
98	98.32	Gradient Segments	2.00		0	Forward Flow	Elution- Modified
98.1	100.32	Gradient Flow	2.00	1.000	0 - 100	25mM Tris pH 8.0, 25mM Tris w/ 1M NaCl	Elution- Modified
99	100.32	Change Valve (Column Switching Valve 1)				Bypass, Forward Flow	SEC Buffer Change
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).

B

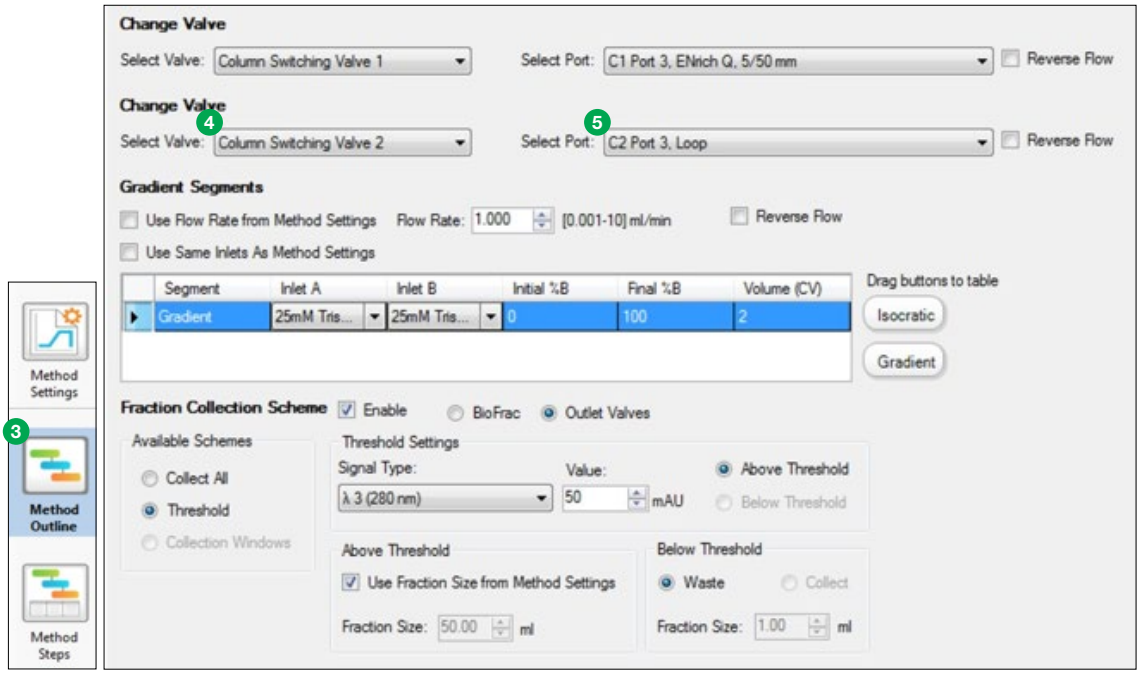
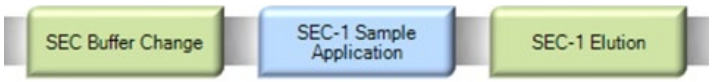


Fig. 22. Configuration of the IEX Elution phase.

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).

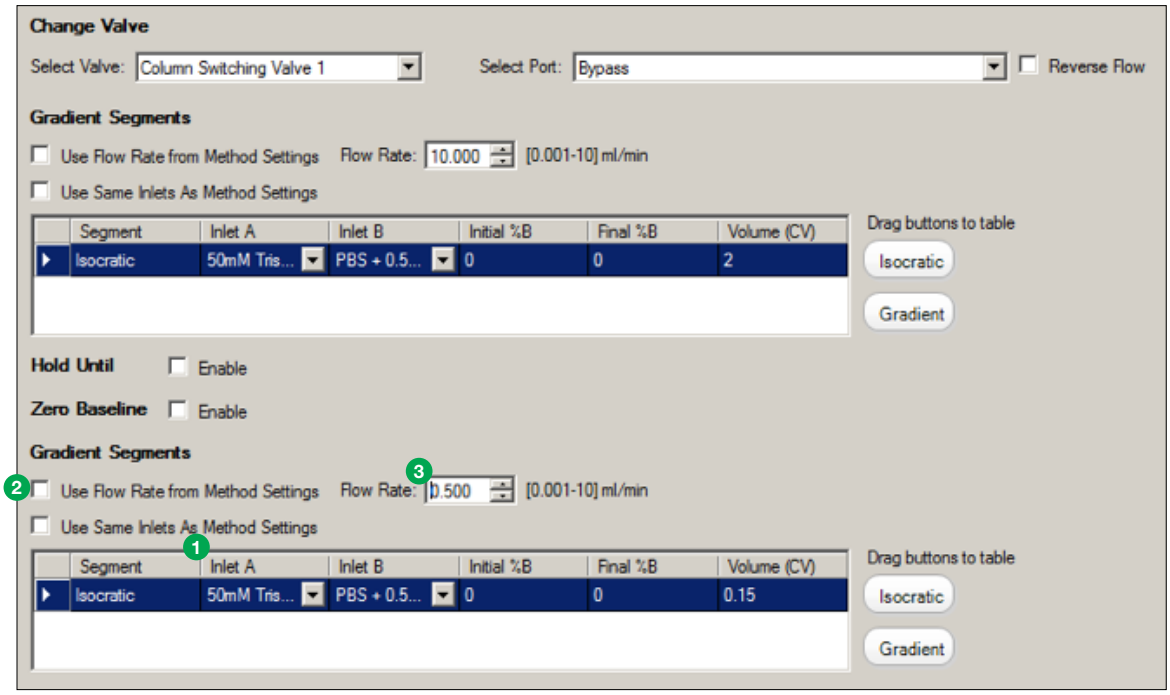


Fig. 23. Configuration of the SEC Buffer Change phase.

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.

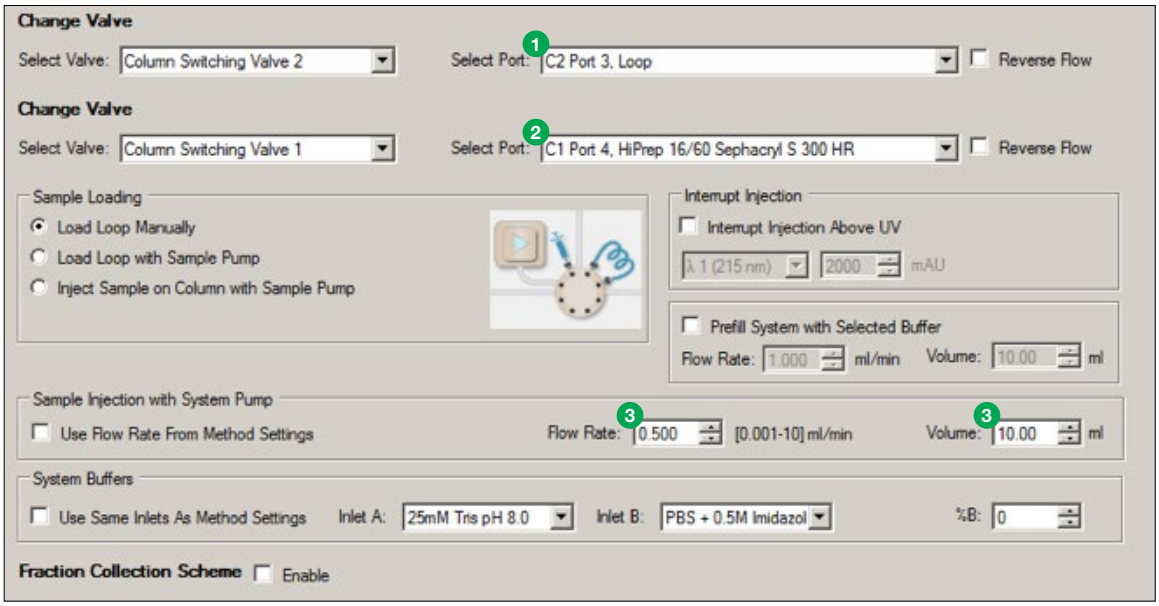


Fig. 24. Configuration of the SEC Sample Application phase.

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.

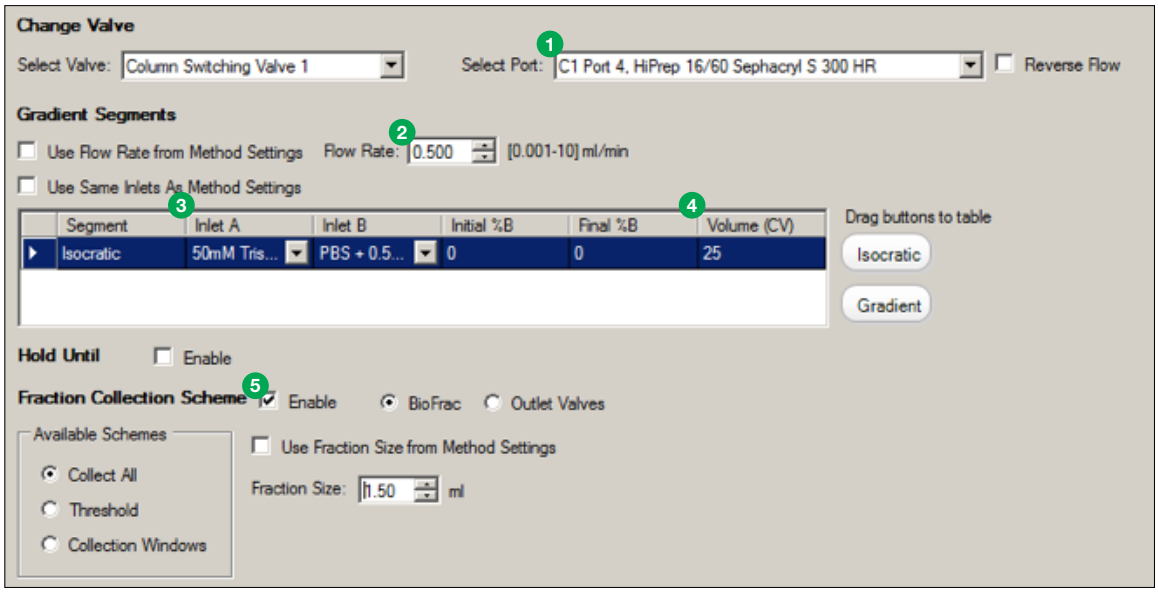


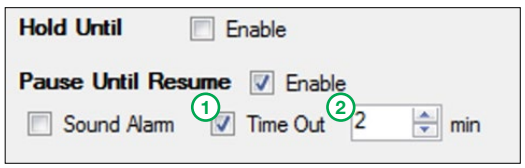
Fig. 25. Configuration of the SEC Elution phase.

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.



1. Check the Time Out box.
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.

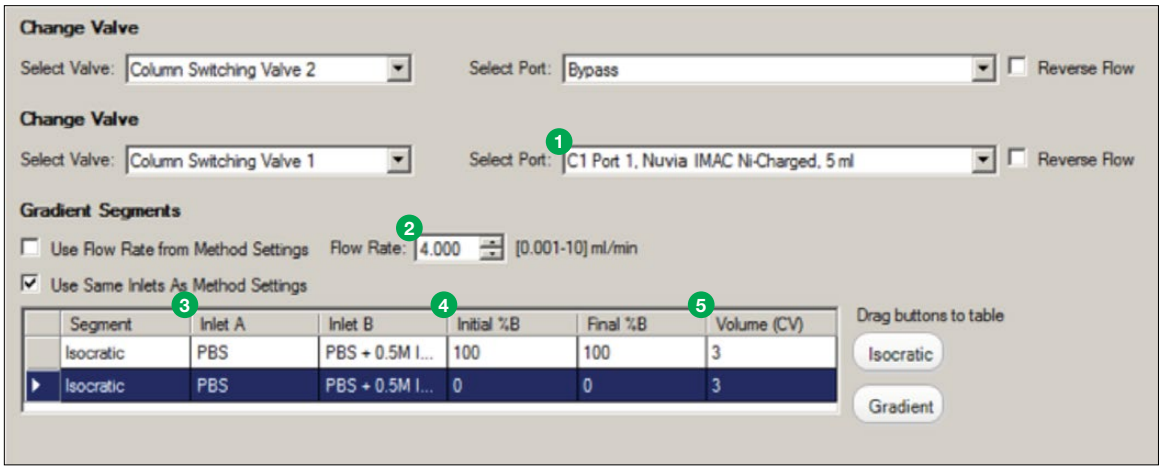


Fig. 27. Conversion of the AC-1 Clean phase to a cleaning/storage phase for each column.

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).

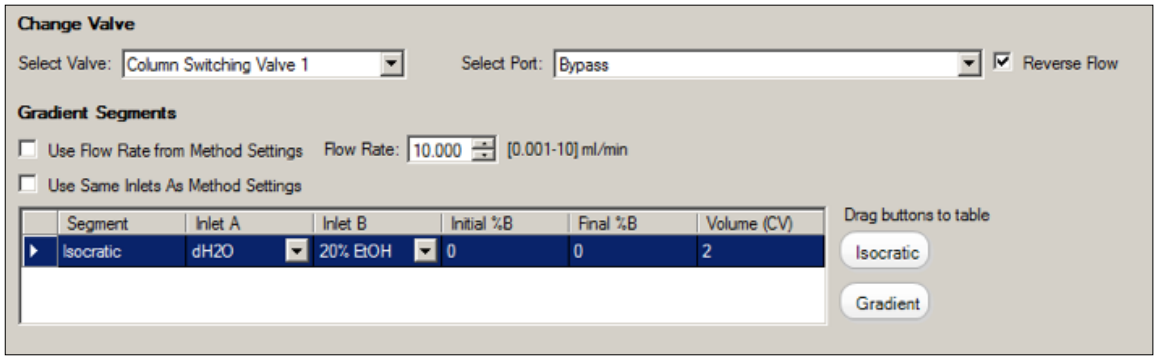


Fig. 28. End Run phase.

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.

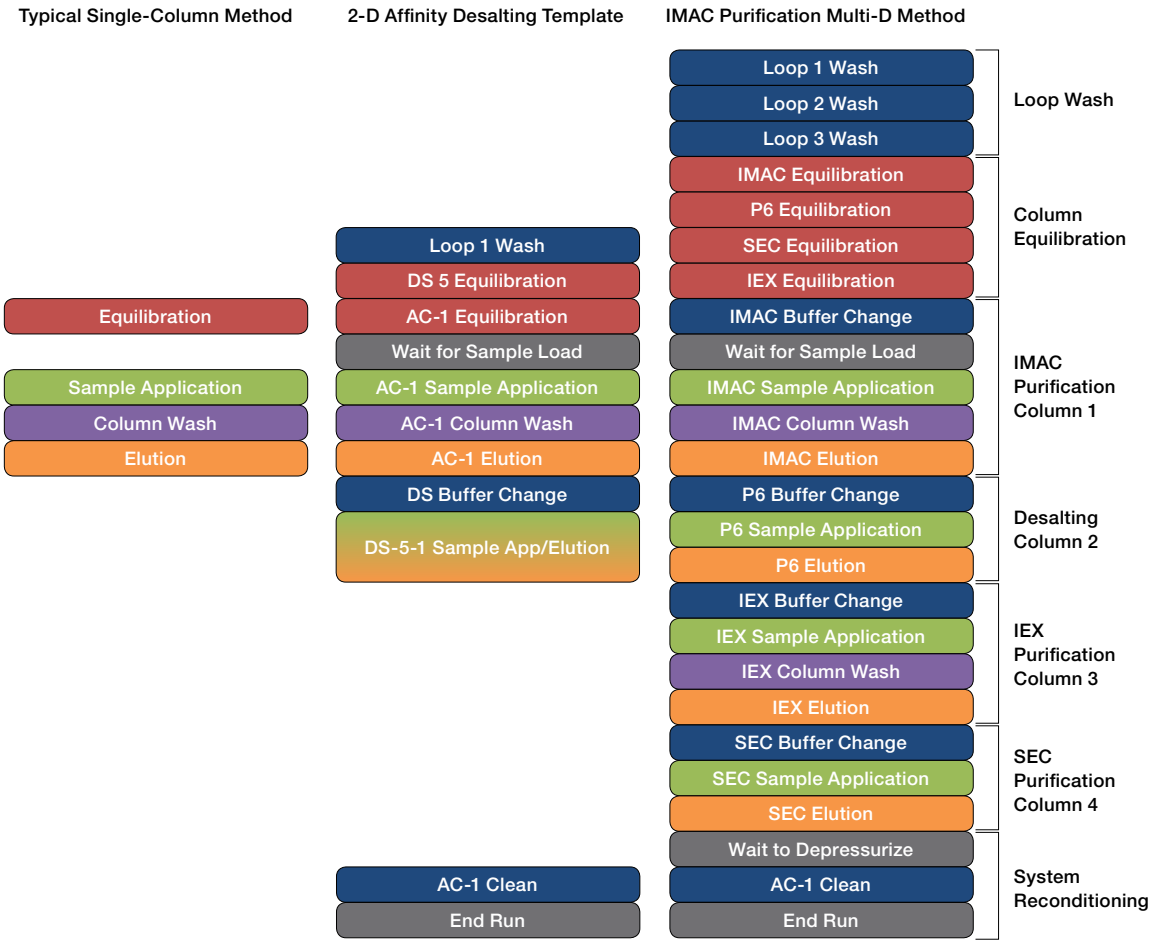


Fig. 29. Evolution of the Multi-D IMAC purification method.

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