A Method for Automated Multistep (Multidimensional) Purification Processes for Protein Recovery

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Chromatography is the most powerful separation tool available to protein scientists and plays a central role in proteomics and functional biology. Problem solving in both areas requires purified proteins, but the purification requirements are different. In both types of research the samples are often complex mixtures derived from tissue homogenates, body fluids, or cell cultures.

In proteomics, chromatography is primarily used to prepare samples for mass analysis and subsequent identification. A chromatographer in that environment focuses on high-thoughput analysis. The multidimensional aspect of chromatography in proteomics can involve ion exchange, affinity, and size exclusion as the first dimension and desalting by reversed-phase chromatography as the second dimension. Proteins are separated from complex mixtures and desalted before mass spectrometry, an area that has been recently reviewed by Wang and Hanash (1) and Shi and coworkers (2). The importance of high throughput to these strategies is reflected in the development of chip technology for such separations (3).

Product Focus: Proteins

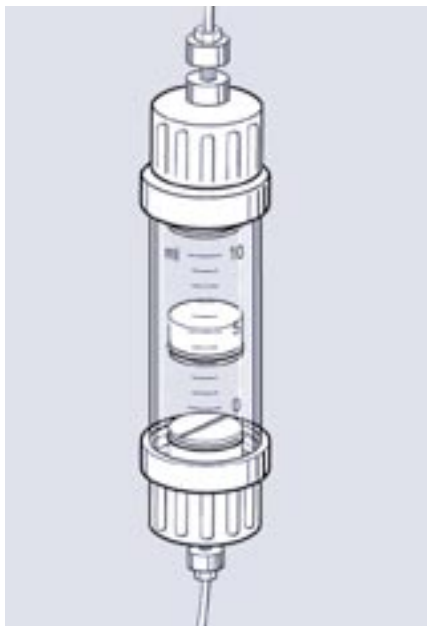
Process Focus: Downstream processing

Who Should Read: Process development

Keywords: Automation of multi-step protein purification processes

Level: Intermediate

ÄKTAexplorer GE Healthcare (www.amershambiosciences.com)



Superloop GE Healthcare (www.amershambiosciences.com)

In functional biology, a chromatographer has a different focus. Here it is more important to recover the purified protein than to identify it. Multidimensional chromatography in this area uses desalting as the first dimension and some adsorptive technique (ion exchange, hydrophobic interaction, or affinity) as the second dimension. Multidimensional chromatography is not a new strategy for protein recovery. In the early 1960s, Lawrence and Benjamin used it to study blood proteins (4), and it has since been a consistent strategy for purifying proteins in cases when protein recovery is important (5, 6).

Fully automating multidimensional chromatography for protein recovery requires a capital outlay for new instrumentation. However, in the absence of such capital, the prevalence of chromatographs with sophisticated chromatography control software packages makes it easier for many laboratories to automate multi-dimensional chromatography. We describe here a protocol to configure existing instruments for multidimensional chromatography. Because it does not require new instrumentation, it can be done with a minimal outlay of capital.

Materials and Methods

Reagents: PBS (phosphate buffered saline) was purchased as a 10X stock from

EMD (www.emdchemicals.com) and diluted as required. All reagents were purchased from Sigma-Aldrich (www.sigmaaldrich.com) except where noted.

Our test substance was bovine serum albumin (BSA) conjugated to cibacron blue (blue-BSA) and was made in house. Briefly, BSA (USB, www.usbweb.com) was dissolved in 100 mM $NaHCO_3$ pH 9.0 (J.T. Baker, www.jtbaker.com) at a concentration of 20 mg/mL. The monochlorotriazine dye cibacron blue 3G-A was dissolved at a concentration of 10 mg/mL in 100 mM $NaHCO_3$ pH 9.0. One hundred mL of the dye was added slowly to the BSA solution. After an overnight incubation at room temperature with constant stirring, the unreacted dye was removed with 50 g of activated charcoal. The solution was filtered on Whatman #1 filter paper (www.whatman.com) then through a 0.45 µm filter (Nalgene, www.nalgenelabware.com).

The blue-BSA was further purified by anion-exchange chromatography on Q-Sepharose Big-Beads packed in a XK 50/7.5 column (both from GE Healthcare, www.gehealthcare.com). The column was equilibrated in 10mM $NaHCO_3$ pH 9.0, and than the sample was applied. The unbound protein was washed out with five column volumes of the equilibration buffer. The blue-BSA was eluted with 500 mM NaCl in 50 mM Tris-HCl pH 8.2. The peak was collected and stored at 4 °C until used. In some experiments, blue BSA was mixed with lysozyme (0.05 mg/mL) and ribonuclease (0.15 mg/mL). When blue BSA was used alone as the sample, it was diluted to approximately 1–2 mg/mL.

Two columns were used in demonstrating the multidimensional chromatography functionality of the ÄKTAexplorer 100. The desalting column was HIPrep 26/10, and the adsorption column was either HiTrap Q Sepharose or HiTrap SP Sepharose, both from GE Healthcare.

Instrumentation: All hardware was from GE Healthcare. The ÄKTAexplorer 100 is a common instrument in protein science laboratories that focus on recovery of purified proteins. A Superloop is used, which can be configured for multidimensional chromatography as shown in Figure 1. Figure 1 shows the

Figure 1: All tubing connections for performing multidimensional chromatography on ÄKTAxplorer 100; a Superloop is used for sample injection and collection; the desalting column is shown as well as positions for several adsorption columns.

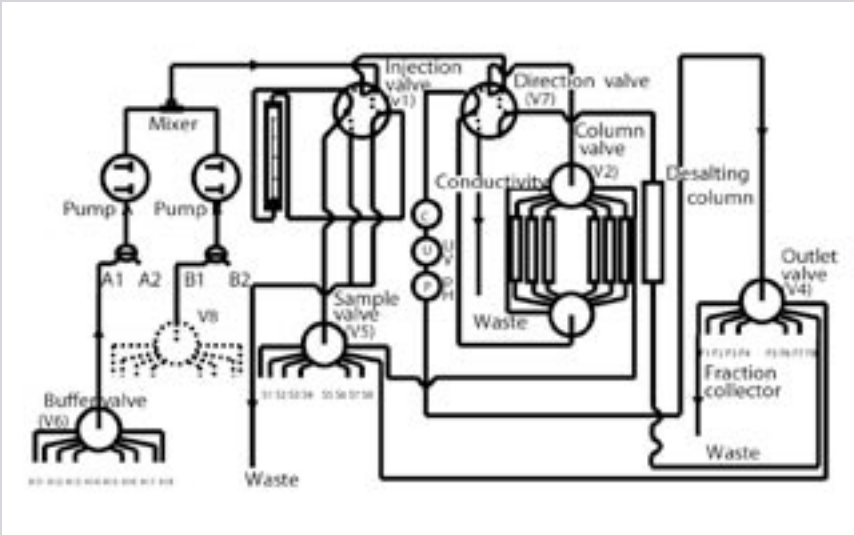


Figure 2: The default flow scheme for ÄKTAexplorer 100; the Superloop takes the place of the sample loop.

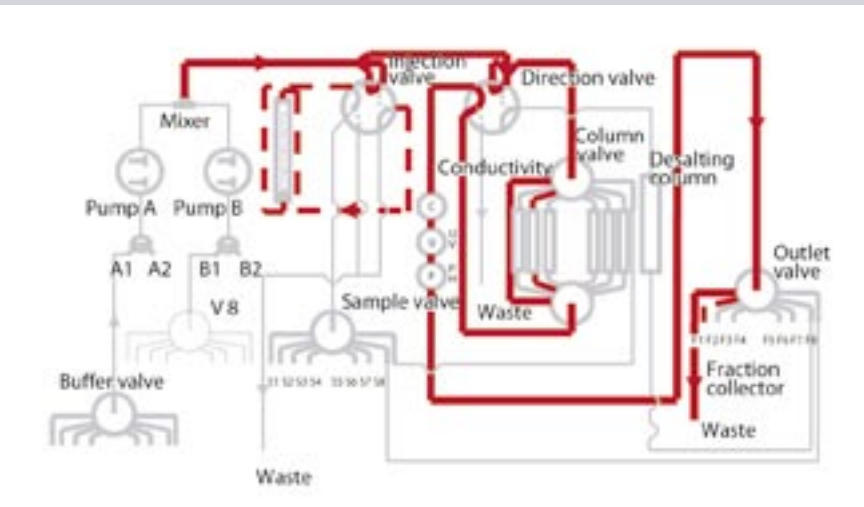


Figure 3: The flow scheme for the integrated desalting configuration; flow from the desalting column can be diverted to waste, by using the column bypass, or flow can be applied to the adsorption column.

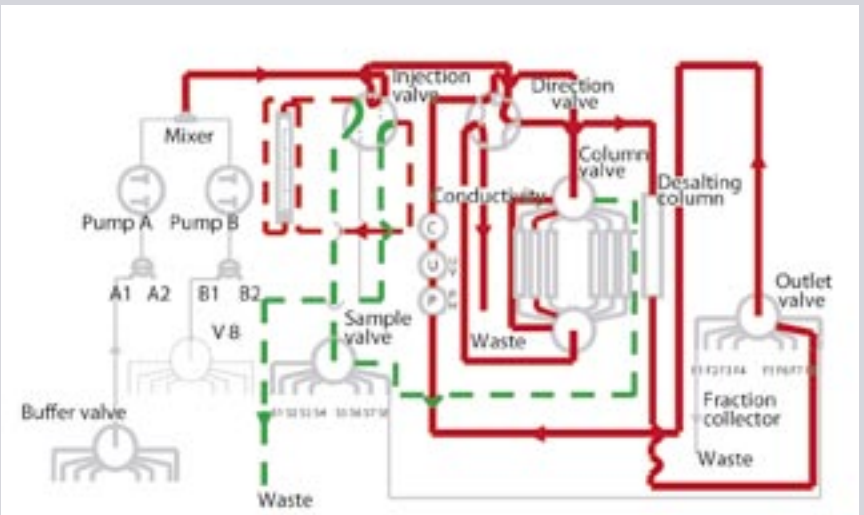


Figure 4: The flow scheme for reloading a peak from the adsorption column into the SuperLoop

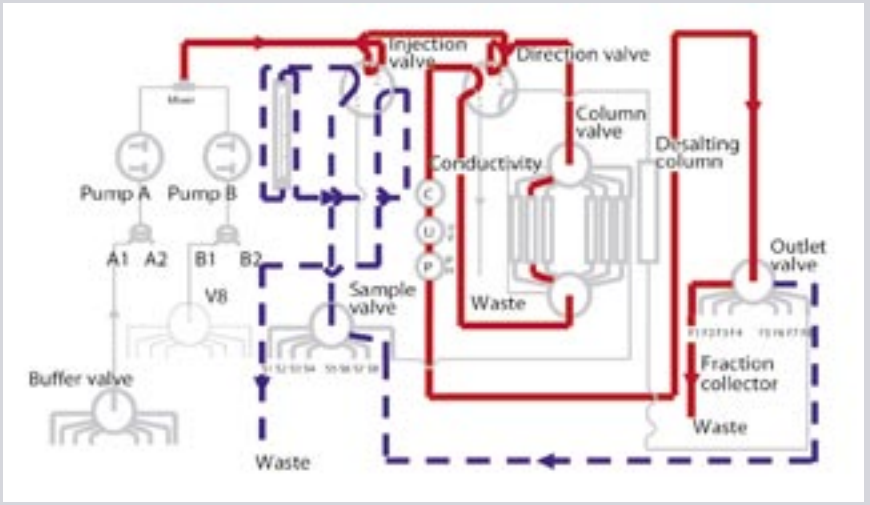


Figure 5: Direct loading using integrated desalting; sample was blue-BSA; adsorption column was Q Sepharose; buffer A was PBS, pH 7.4; buffer B was 1 M NaCl in buffer A.

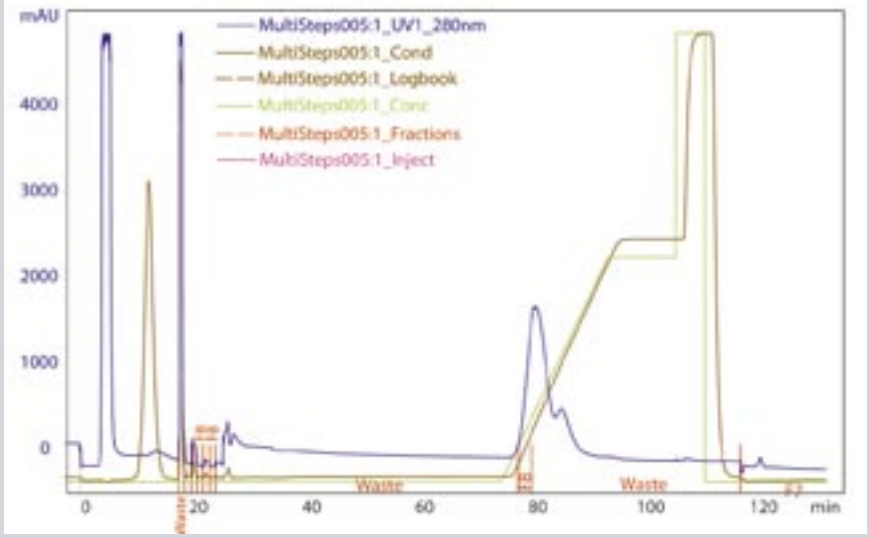
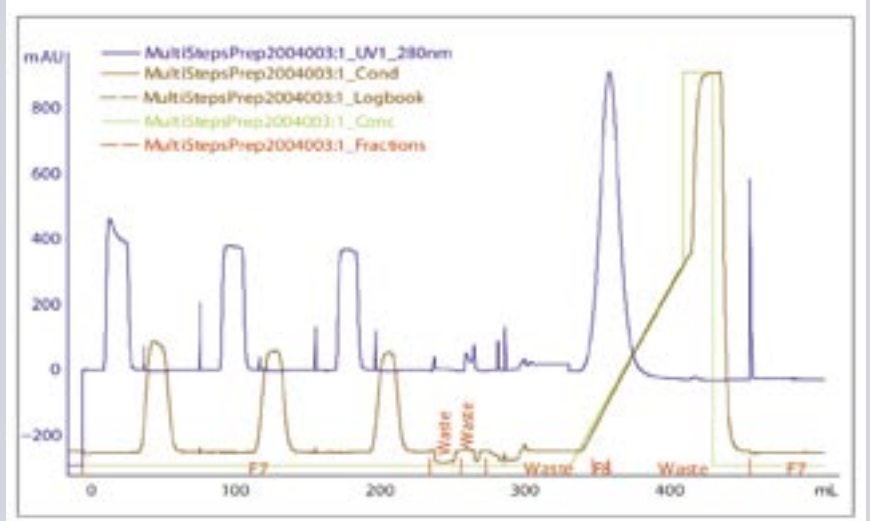


Figure 6: Using three desalting runs for loading; sample was three injections of blue-BSA; adsorption column was Q Sepharose. Buffer A was PBS, pH 7.4; buffer B was 1 M NaCl in Buffer A.



Superloop and the desalting column in place. Reconfiguring can be done without adding new components or changing control strategy. The flow direction valve and the sample selection valve were converted to function in the desalting step and buffer B selection valve (to provide the capability for eight pair of buffers), respectively.

When all valves are in their default positions, the system performs exactly the same as a regular ÄKTAexplorer. Figure 2 shows the default flow path. The injection valve, in addition to allowing injection from the Superloop, can be reloaded with elute from a chromatography step when the sample selection valve is switched to position F8. The desalting column is placed between position 6 of the direction valve and position 7 of the outlet valve. When the direction valve is switched to upflow and the outlet valve is switched to position 7, the flow coming from the pumps first goes through the desalting column and then the detectors. The flow can then either go through the bypass or go through a column, which allows direct loading of material from the desalting column onto the adsorption column based on its conductivity or ultraviolet (UV) reading.

Figure 3 shows the integrated desalting flow path. This desalting/loading process can be run repeatedly for the same adsorption column. The loading can be switched off based on either conductivity or UV reading. After loading, the flow direction valve is switched to downflow and the outlet valve is back to position F1 (default). A regular gradient/step elution can now be performed for the loaded column. Eluent can be reloaded onto the Superloop for the next chromatography step or collected by the fraction collector (if this is the final step). Such a configured system can automatically handle large amounts of untreated high salt dilute sample and perform multistep chromatography processes without human intervention in material transfer.

Desalting can be an integrated part of any chromatography run on ÄKTAexplorer 100. Elute (peak) can be stored in Superloop and loaded onto the next chromatography step. The flow path for automatically loading a peak form the adsorption column into the Superloop is shown in Figure 4. Seven adsorption steps

(not including their buffer change steps) can be run in a single Unicorn method on such a configured ÄKTAexplorer 100. No extra component or strategy change is needed for the reconfiguration. The configured ÄKTAexplorer 100 works as a regular ÄKTAexplorer 100 at its default position, which maintains all the original functions of ÄKTAexplorer for process development.

Key Control Strategies: The following technical details are given because they are important in making the reconfigured ÄKTAexplorer 100 a robust system. Two parameters are critical: A control strategy for defining a peak must be identified so that peak can be either loaded into the Superloop or passed to the second column; and a control strategy must be defined for preventing contamination in the Superloop. The latter is critical to prevent crossover of samples in the Superloop.

A control strategy for peak-selective reloading of elutes by Superloop should allow selective reload of the target peak back to the Superloop from the chromatography elution. There are four descriptors of a targeted peak: a minimum retention volume before starting a watch command for collection, a threshold UV value for turning on collection, a UV value describing the height of the vicinity of the valley between the two peaks (assuming the peaks are so close), and a maximum retention volume before turning off the collection.

The control strategy contains the following six commands: A watch command for the threshold UV value to turn on the collection is initiated at the minimum retention volume; a watch command for negative UV Slopes will be initiated as soon as the collection is activated; a watch command for the vicinity of the valley will be initiated as soon as the first negative slope is being detected; a watch command for a positive UV Slope will be initiated as soon as the vicinity of the valley is reached; the collection will be turned off either by a positive UV slope or at the maximum retention volume; and if the peaks are separated enough apart, a watch command for UV less than a threshold can be used to turn off the collection.

A control strategy for Superloop cleaning between chromatography steps is also critical because the eluates downstream should not be contaminated by the residual from the upstream material. There are five descriptors for the cleaning: leftover volume in the Superloop, buffer reloading volume, buffer push-out volume, number of load/push-out loop, and final push-out volume.

The suggested control strategy contains the following five commands: Push out (injection position) leftover feed with pressure watch command (stop at the end); Load the Superloop from position 8 of outlet valve with buffer A; Push out (injection position) buffer A with pressure watch command (stop at the end);Loop multiple times from b to c; and push out (injection position) left-over with pressure watch command (stop at the end).

The Unicorn method was programmed modularly. Details are available elsewhere (7).

Figure 7:. Superloop flushing; sample washed out was blue-BSA

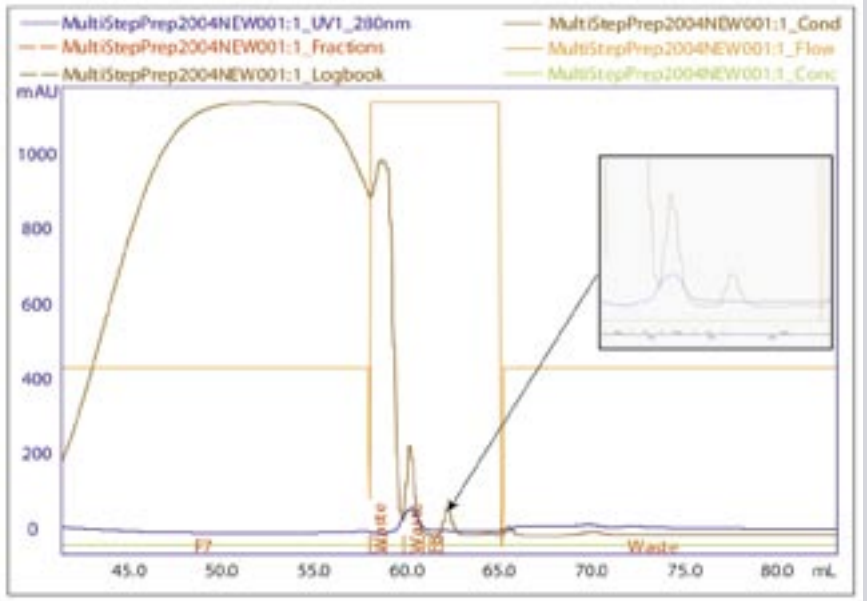


Figure 8: Peak separation, loading the first peak to the Superloop; this sample contains three proteins that separate into three peaks on SP Sepharose. Buffer A was 20 mM Na-phosphate, pH 6.8; Buffer B was 0.5 M NaCl in buffer A. The three proteins blue-BSA, lysozyme, and ribonuclease were mixed. Blue-BSA was used at 2 mg/mL, lysozyme at 0.05 mg/mL, and ribonuclease 0.15 at mg/mL.

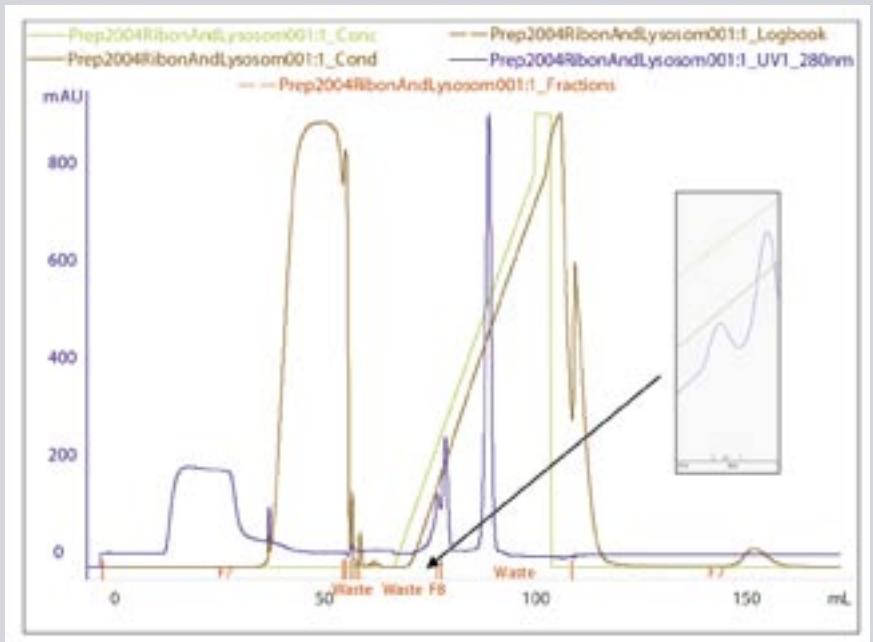


Figure 9: Peak separation, loading the second peak to the Superloop, with samples and conditions as described for Figure 8

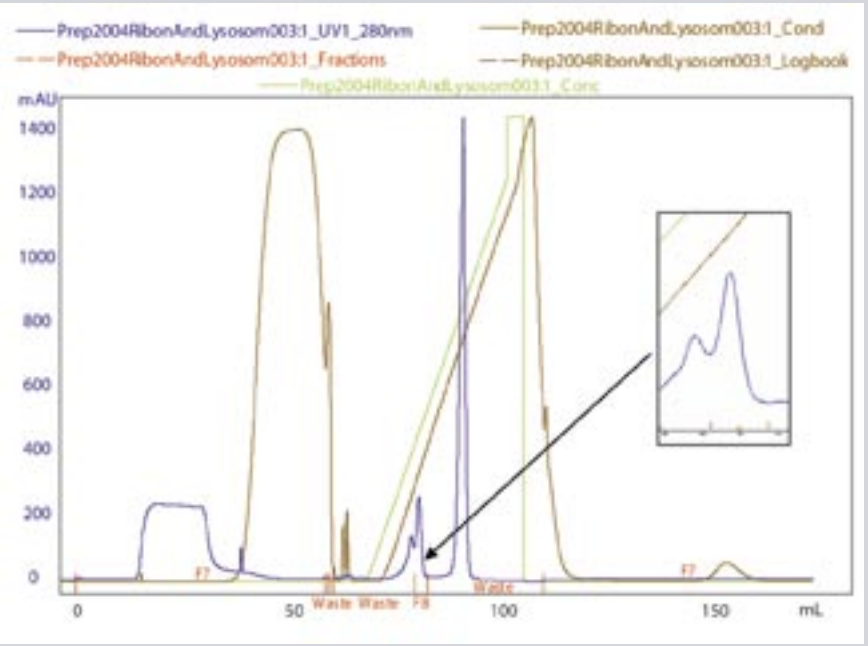
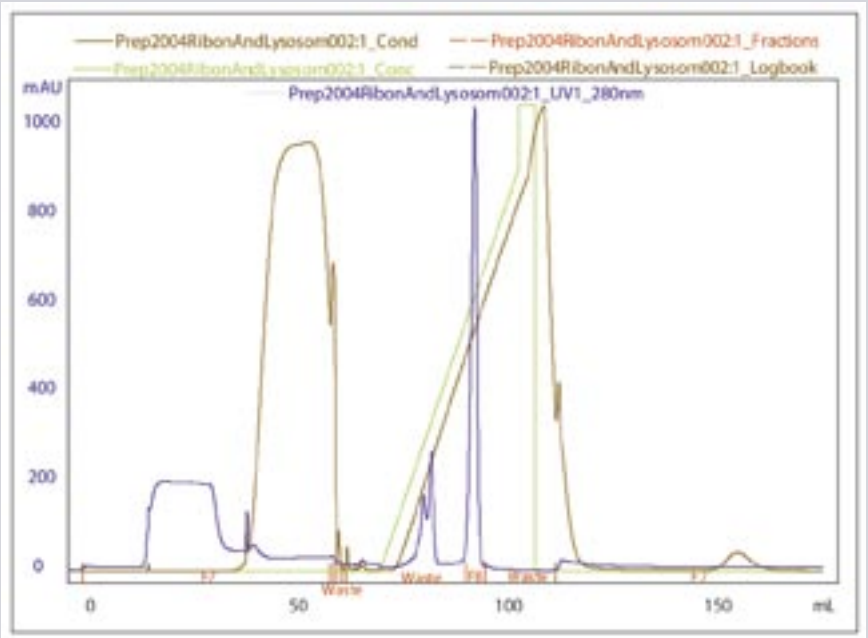


Figure 10: Peak separation, loading the third peak to the Superloop, with samples and conditions as described for Figure 8



Results

Integrated Desalting Function: Blue BSA was used as the sample for the demonstration. Buffer A, for column equilibration, loading, and washing, was PBS, pH 7.4. Buffer B, for elution of the adsorption column, was buffer A with 1 M NaCl.

The desalting column is HiPrep 26/10, and the adsorption column is HiTrap Q-Sepharose Fast Flow (5 mL). The flow rate for was 4 mL/min.

Figure 5 shows the chromatogram. The first UV peak was from the desalting column, and a conductivity peak comes out a little later. The adsorption column position switched off to bypass when conductivity went up to the threshold level. The second sharp UV peak represents washing of the Superloop. The Superloop seemed to be clean based on UV (280 and 215) after three repeated washing circles. A regular chromatography wash and a gradient elution for the adsorption column were then performed. The Superloop collection was turned on correctly when UV reading reached the threshold but was soon turned off because of the oversensitivity of the UV slope (the valley vicinity watch was not programmed in this run). The collection function was demonstrated in a separate run. Detailed information regarding the operation variables can be found in the Unicorn method (7).

Repeated Desalting Runs: If the feed is a very large amount of dilute material, the desalting column needs to run multiple times to process the entire amount of feed material. Such a situation was demonstrated for three injections from the Superloop onto the desalting column with the same blue BSA material. This is shown in Figure 6, showing the three peaks eluting form the desalting column. The peak eluting from the adsorption column is correspondingly higher than the one seen in Figure 5. Notice that the scale on Figure 6 is different from the scale on Figure 5, to compensate for the higher protein mass in the sample. Buffer A for column equilibration, loading and washing was PBS, pH 7.4. Buffer B, for elution of the adsorption column, was buffer A with 1 M NaCl. The desalting column is HiPrep 26/10 and the adsorption column is HiTrap Q-Sepharose Fast Flow (5 mL).

Superloop Cleaning Between Runs: The Superloop cleaning procedure was demonstrated in several of the runs (data not shown). Based on UV 215, the 3× loop usually cleans the loop completely. The wash buffer was buffer A, PBS, pH 7.4. The chromatogram in Figure 7 shows the small protein peak, absorbance at 280 nm, which is directed to waste in a Superloop flushing run. The desalting column is HiPrep 26/10, and the adsorption column is HiTrap Q-Sepharose Fast Flow (5 mL).

Selective Peak Collection: In this experiment, the blue BSA sample contains three proteins: blue-BSA, lysozyme, and ribonuclease. In this case, buffer A for column equilibration, loading, and washing, was Na-phosphate, pH 6.8. Buffer B for elution of the adsorption column was buffer A with 0.5 M NaCl. Lysozyme concentration was 0.05 mg/mL in buffer A, and ribonuclease concentration was 0.15 mg/mL in buffer A. The desalting column is HiPrep 26/10,

and the adsorption column in this case is HiTrap SP-Sepharose Fast Flow (5 mL).

Three peaks elute from a SP Sepharose FF column; two are very close to each other. It is possible to collect each of these three peaks separately. Figure 8 shows the collection of the first peak. The demonstration shows that the collection of Fraction 8 was cut off right when the second peak started to come up. Figure 9 demonstrates the collection of peak 2, and Figure 10 demonstrates the collection of peak 3.

Discussion

ÄKTAexplorer 100 can be reconfigured to provide many integrated functions without adding additional parts or changing the current strategies. An up-to-seven-step purification process can be automatically run on a standard ÄKTAexplorer 100 by a single Unicorn method (or a Queue method). The reconfigured ÄKTA 100 has an integrated desalting function that allows it to process an almost arbitrary amount of untreated dilute feed material because desalting can be run repeatedly while the feed is being absorbed on the adsorption column. The eluent from the adsorption chromatographic step can be reloaded to Superloop in this new configuration and be fed to a following column. The Superloop can be automatically cleaned between runs. These improvements make it practical to carry out a multistep purification process automatically on an ÄKTAexplorer 100. The reconfigured system works like a regular one at its default flow scheme and maintains all the function of an original explorer 100 for process development.

The above described reconfiguration of ÄKTAexplorer may have potential application in automated high throughput multistep purification processes for drug screening. A two-step process using two Q columns was carried out to show the functionality of handling multistep purification processes. This will be the subject of another article.

Unicorn programming includes a component called method Q to handle operation of multicolumns/multisystem processes. However, Q method has never before been practical because of the difficulty in physically transferring material from one step to another and because of the need for sample preparation (desalting) before each column loading. The traditional Q method depended on pumps for material transfer, which was unsuitable for relatively small amounts of material handling. This reconfigured system addressed the sample preparation issue by using desalting, which is a type of gel filtration, thus taking advantage of gel filtration's time differentiation of different molecular weights for loading of the Superloop. Superloop is better suited for handling small amounts of material than pump transferring as long as the cleaning issue can be addressed.

With the reconfiguration and the key control strategies, the corresponding Unicorn programming can be modularized. The new configuration preserves all the original features of ÄKTA design platform for process development and automates the step of sample preparation for chromatography. The system can control up to a seven-step purification processes with an enclosed material handling fashion. A multistep purification process performed on such a system will take less time than would running it in separate steps. Desalting costs no extra time because it is being done during loading of another column. Repeated runs of desalting allows a user to process arbitrary amounts of untreated dilute material limited only by the binding capacity of the adsorption column, which performs equivalently to the unit operation of concentrating/dialyzing using membranes that are often used in current practices. Changing the sample valve for pump B buffer selection allows the use of eight pairs of buffers for a purification process The original system can have only two buffer Bs.

In the future we will work to add a parallel processing capacity to vastly increase the possibility for using the system for high-throughput screening processes. A simple modification (adding two INV-908 valves) would give such a capacity to the system. Two additional valves will allow seven extra Sampleloops or Superloops to be used for the operations. This in theory could allow automatic processing of seven samples sequentially by an up-to-seven-step purification process. The Sampleloop could be made disposable to further simplify the screening task.

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7 Methods are available on CD suitable for installation by contacting the authors, ZJ at zuwei.jin@ge.com or RMK at bobkennedy@ge.com.

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