Science Together

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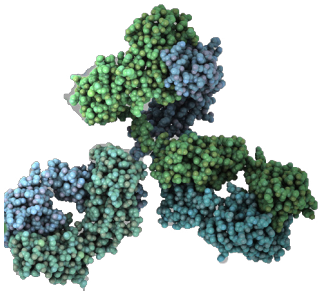
Two step purification with the sample pump set up



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WHAT IS TWO STEP PURIFICATION?

Two step purification is a special multicolumn chromatography solution. Two independent methods, each with their associated specific column, are used to realize the purification of the target molecule without manual interference. The principle here is that the protein sample is applied on the first column. During elution of the protein, the protein peak is detected triggering the collection of the eluted protein in a storage loop or storage vessel/container. The protein is then automatically applied on the second column to further enhance the quality and or purity of the purified protein. Several system set ups can be used to automate the purification. In this TechNote a two step purification with the sample pump set up is discussed.

SET UP WITH SAMPLE PUMP: WHAT DO YOU NEED?

In the sample pump set up a Lab standard KNAUER Multi Method FPLC system for all Bio-Chromatography methods is adapted. Hereby, a column selection valve, an outlet valve, as well as a sample pump must be added to the system. Alternatively, the AZURA Bio Lab System Advanced with an outlet valve can be used **(Fig. 1, Tab. 3)**.

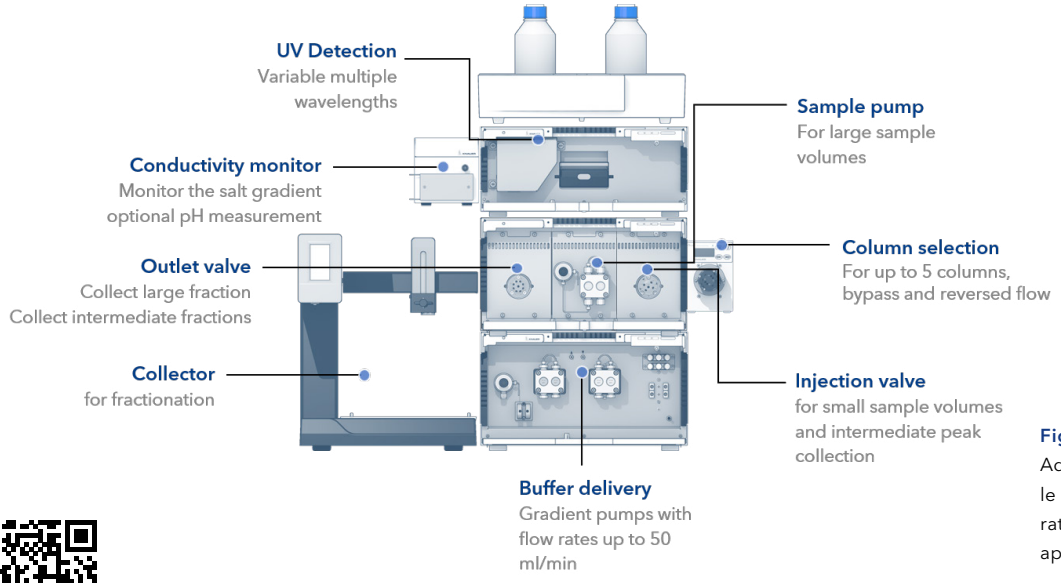

Fig. 1 AZURA Bio Lab System Advanced in set up with sample pump: exemplary configuration for automated two step applications.

Additional Information

Two step purification with the sample pump set up

The sample pump is used to apply the sample on the first column. The peak eluting from the first column is collected in the sample loop of the injection valve and redirected to the second column. This set ups allows the loading of large sample volumes and minimizes the risk of cross contamination during first peak collection because the sample loop is only used for the eluting peak. The injection of small sample volumes is not supported **(Fig. 2)**.

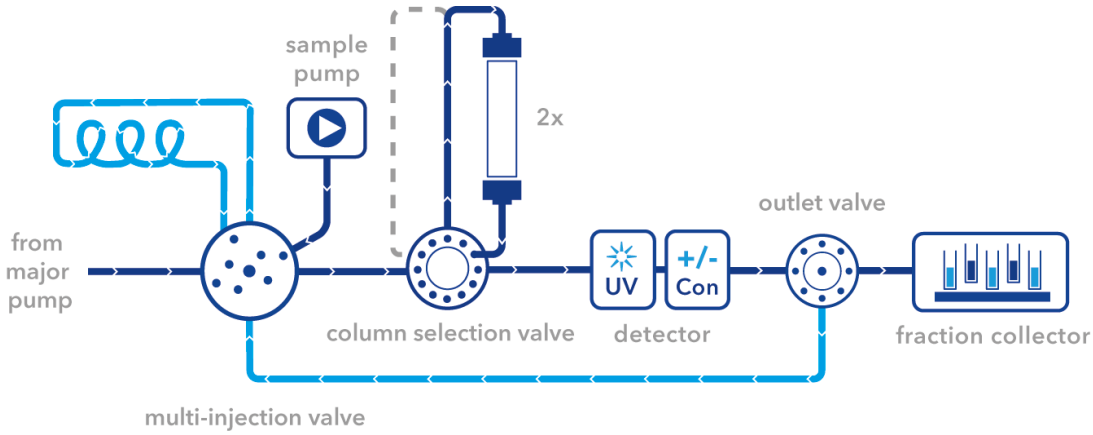

Fig. 2 Flow scheme for the sample pump set up for two step purification.

The column selection valve is placed between the multi-injection valve and the UV detector. The column outlet port (Col) of the multi-injection valve is connected via the PEEK capillary with the inlet port (IN) of the column selection valve. The outlet port (OUT) is connected to the UV detector flow cell, which in turn is connected to the conductivity monitor. The columns are installed according to **Fig. 3**.

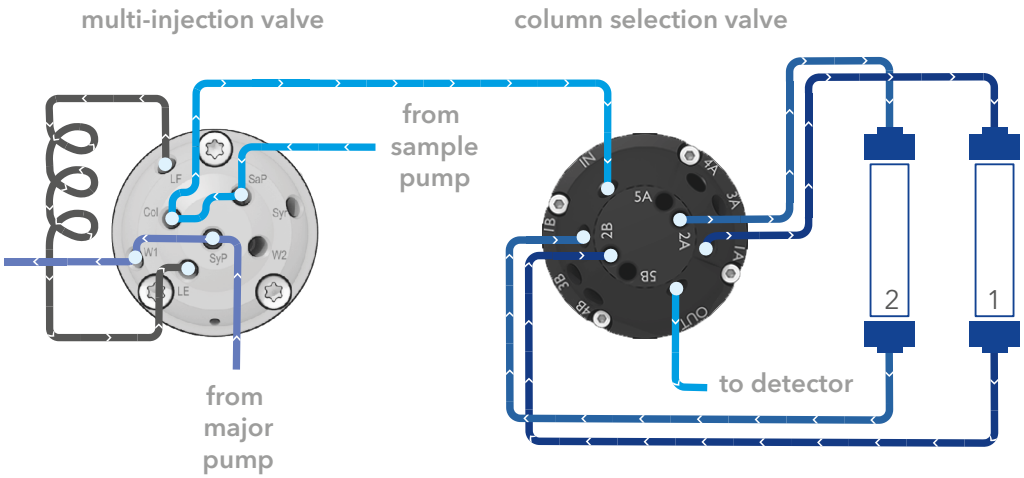

Fig. 3 Connection of the column selection valve.

The outlet valve is placed between the conductivity monitor and the fraction collector. The conductivity monitor is connected via the PEEK capillary with the middle port of the outlet valve. Port 1 of the outlet valve goes by default to the fraction collector or waste container. Port 3 to 8 can be used for the collection of large fractions. Port 2 (reinjection) of the outlet valve is connected to the syringe port (Syr) of the multi-injection valve **(Fig. 4)** The syringe port is no longer accessible for sample injection.

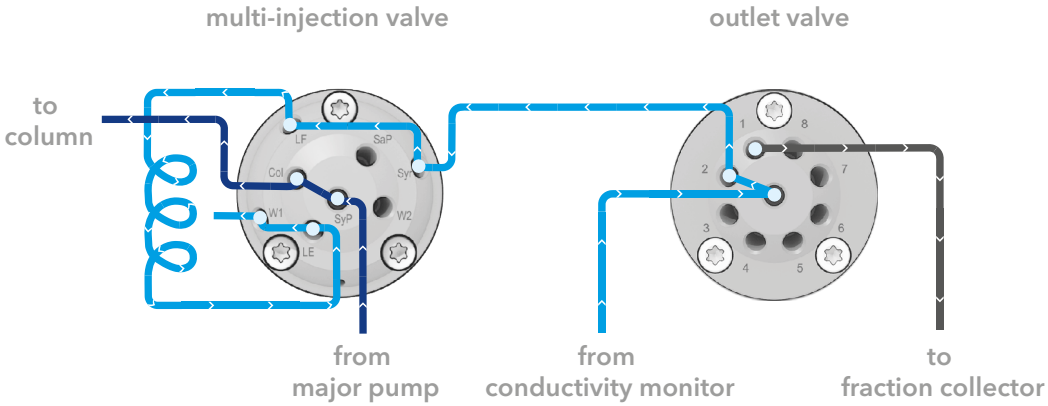

Fig. 4 Connection of the outlet valve.

HOW TO WRITE A METHOD

In the following section we will describe how to write an exemplary method for two step purification with PurityChrom. See also our checklist: „Creating methods in PurityChrom®" in the **PurityChrom Installation Information (V2655A)**. In our example, in the first step a 1 ml ion exchange column and in the second step a 5 ml desalting column was used. The sample pump with an airsensor is used for automatic sample application. A 2 ml sample loop was used for intermediate peak parking. Two separate methods were written for the two columns. First, important aspects of the ion exchange method with automatic sample application are highlighted for the time control file **(Fig. 5A - Fig. 7A)** and the changes in the flow are depicted in the flow scheme/visualization **(Fig. 5B - Fig. 7B)**. Make sure to choose the correct column in the beginning of the method **(Fig. 5**, 2) In the first step, the multi-injection valve is still in manual load position to equilibrate the ion exchange column with the major pump **(Fig. 5**, 1).

Two step purification with the sample pump set up

A

Volume [ml]	Function	Parameter	
0.00	Valve Position	A = A1	
0.00	Valve Position	B = B1	
0.00	Valve Position	Injection Valve = Manual Load/Rei	1
0.00	Valve Position	Outlet Valve = to Fraction Collector	
0.00	Valve Position	Column Selection Valve = Column 1	2
0.00	Valve Position	Fractionation = Waste	
0.00	Composition Major Pump	100.0 , 0.0	
0.00	Flowrate Major Pump	1.00 ml/min Constant Flow	
0.00	Composition Minor Pump	100.0	
0.00	Flowrate Minor Pump	0.00 ml/min Constant Flow	
0.02	UV Autozero		
0.02	Start Chromatogram	Channel 1,2,3,4,5,P,P (500 ms)	
5.00	Valve Position	Injection Valve = Direct Inject	
5.00	Flowrate Major Pump	0.00 ml/min Constant Flow	
5.00	Flowrate Minor Pump	1.00 ml/min Constant Flow	
5.00	Threshold	9.96 ml / Gameport Input 1	
10.00 + S	Flowrate Minor Pump	0.00 ml/min Constant Flow	
10.00 + S	Flowrate Major Pump	1.00 ml/min Constant Flow	
10.00 + S	Valve Position	Injection Valve = Manual Load/Rei	
15.00 + S	Composition Major Pump	100.0 , 0.0	
15.00 + S	Threshold	10.00 ml + S / 60.00 mAU	
25.00 + S	Composition Major Pump	60.0 , 40.0	
25.02 + S	Stop all		

B

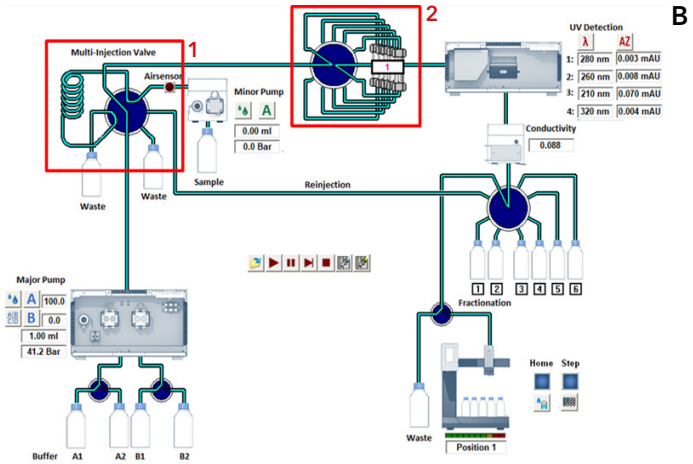

Fig. 5 Ion exchange method during column equilibration. Time control file of ion exchange method (A). Visualization of AZURA Bio Lab system in set up with sample pump (B).

After equilibrating the column the sample is automatically applied via the sample (minor) pump. Therefore, the multi-injection valve switches to direct inject **(Fig. 6**, 1) and the flow of the sample pump is set to 1 ml/min **(Fig. 6**, 2).

A

Volume [ml]	Function	Parameter	
0.00	Valve Position	A = A1	
0.00	Valve Position	B = B1	
0.00	Valve Position	Injection Valve = Manual Load/Rei	
0.00	Valve Position	Outlet Valve = to Fraction Collector	
0.00	Valve Position	Column Selection Valve = Column 1	
0.00	Valve Position	Fractionation = Waste	
0.00	Composition Major Pump	100.0 , 0.0	
0.00	Flowrate Major Pump	1.00 ml/min Constant Flow	
0.00	Composition Minor Pump	100.0	
0.00	Flowrate Minor Pump	0.00 ml/min Constant Flow	
0.02	UV Autozero		
0.02	Start Chromatogram	Channel 1,2,3,4,5,P,P (500 ms)	
5.00	Valve Position	Injection Valve = Direct Inject	1
5.00	Flowrate Major Pump	0.00 ml/min Constant Flow	
5.00	Flowrate Minor Pump	1.00 ml/min Constant Flow	2
5.00	Threshold	9.96 ml / Gameport Input 1	
10.00 + S	Flowrate Minor Pump	0.00 ml/min Constant Flow	
10.00 + S	Flowrate Major Pump	1.00 ml/min Constant Flow	
10.00 + S	Valve Position	Injection Valve = Manual Load/Rei	
15.00 + S	Composition Major Pump	100.0 , 0.0	
15.00 + S	Threshold	10.00 ml + S / 60.00 mAU	
25.00 + S	Composition Major Pump	60.0 , 40.0	
25.02 + S	Stop all		

B

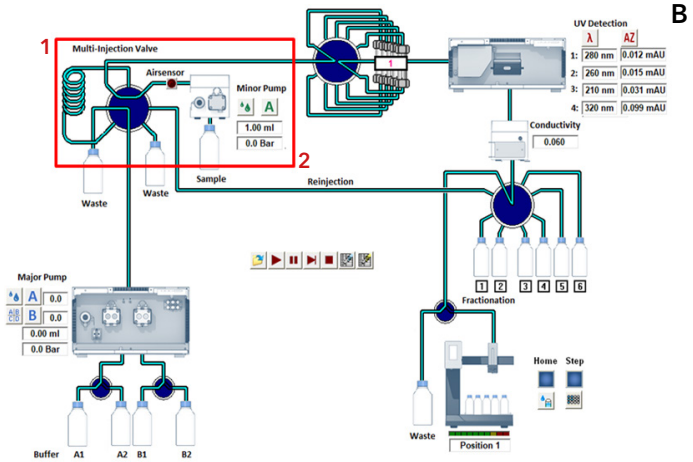

Fig. 6 Ion exchange method during automatic sample injection. Time control file of ion exchange method (A). Visualization of AZURA Bio Lab system in set up with sample pump (B).

During automatic sample injection the threshold function is used to detect air via the air sensor and then automatically moves to the next step in the method **(Fig. 7)**. The variable time shift was used to design the method more flexible and to adjust the sample amount accordingly. For more information please check the chapter "Air sensor usage for sample application with the sample pump (with chromatogram)" in the **AZURA Air Sensor Supplement (V6879)**.

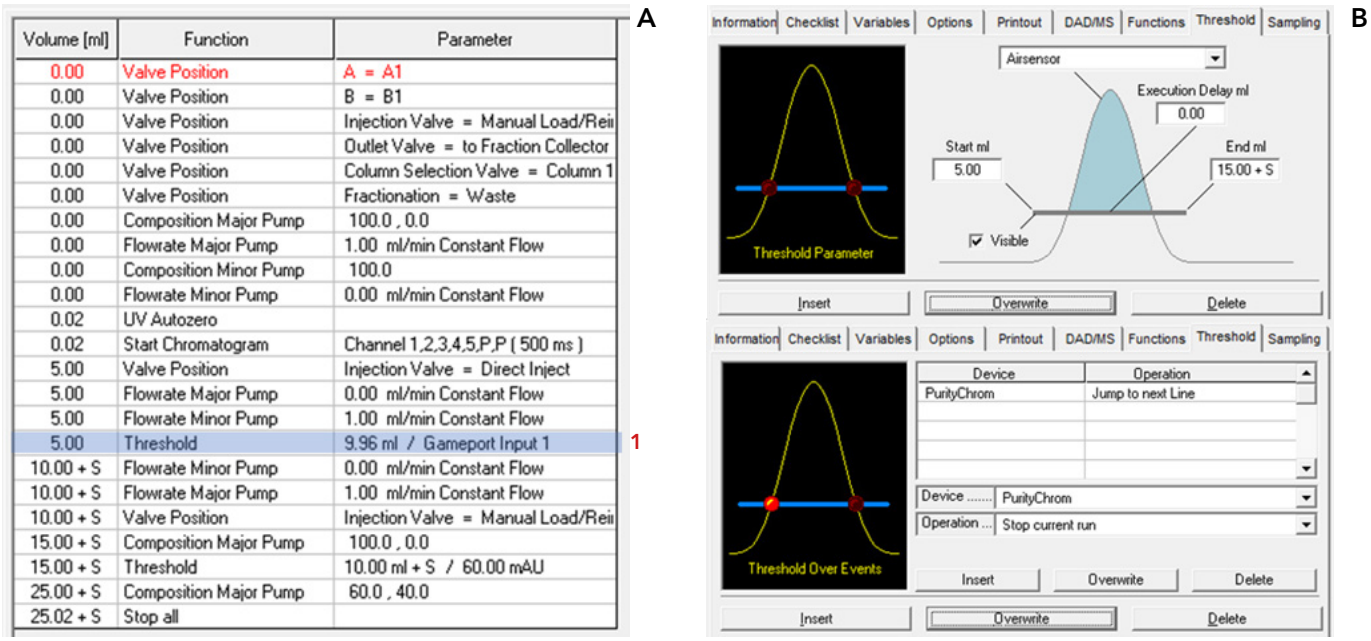

Fig. 7 Ion exchange method airsensor detection during automatic sample injection with Time control file of ion exchange method (A) and threshold function (B).

Two step purification with the sample pump set up

After the sample injection and an isocratic washing step **(Fig. 8A**, 1**)**., the elution starts after 5ml with the beginning of the gradient **(Fig. 8A**, 2**)**. At this time point the multi-injection valve should be switched back to the manual load position **(Fig. 8**, 3**)**.

A

Volume [ml]	Function	Parameter	
0.00	Valve Position	A = A1	
0.00	Valve Position	B = B1	
0.00	Valve Position	Injection Valve = Manual Load/Rei	
0.00	Valve Position	Outlet Valve = to Fraction Collector	
0.00	Valve Position	Column Selection Valve = Column 1	
0.00	Valve Position	Fractionation = Waste	
0.00	Composition Major Pump	100.0 , 0.0	
0.00	Flowrate Major Pump	1.00 ml/min Constant Flow	
0.00	Composition Minor Pump	100.0	
0.00	Flowrate Minor Pump	0.00 ml/min Constant Flow	
0.02	UV Autozero		
0.02	Start Chromatogram	Channel 1,2,3,4,5,P,P (500 ms)	
5.00	Valve Position	Injection Valve = Direct Inject	
5.00	Flowrate Major Pump	0.00 ml/min Constant Flow	
5.00	Flowrate Minor Pump	1.00 ml/min Constant Flow	
5.00	Threshold	9.96 ml / Gameport Input 1	
10.00 + S	Flowrate Minor Pump	0.00 ml/min Constant Flow	
10.00 + S	Flowrate Major Pump	1.00 ml/min Constant Flow	1
10.00 + S	Valve Position	Injection Valve = Manual Load/Rei	3
15.00 + S	Composition Major Pump	100.0 , 0.0	1
15.00 + S	Threshold	10.00 ml + S / 60.00 mAU	
25.00 + S	Composition Major Pump	60.0 , 40.0	2
25.02 + S	Stop all		

B

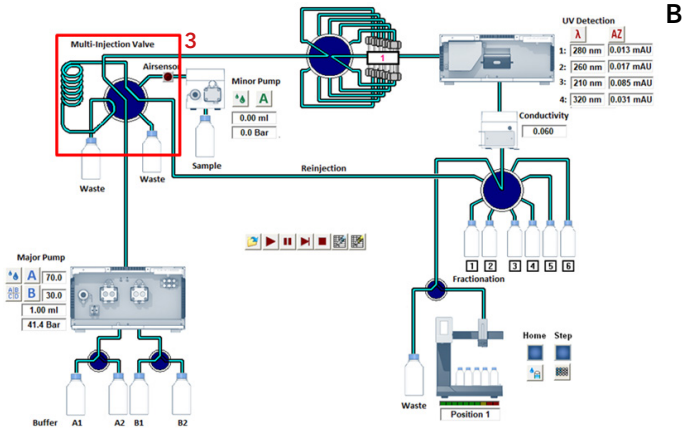

Fig. 8 Ion exchange method during gradient elution. Time control file of ion exchange method (A). Visualization of AZURA Bio Lab system in set up with sample pump (B).

To recognize the eluting peak a threshold function is used. The threshold is active during the gradient elution **(Fig. 9A)**. Once a peak above 60 mAU is detected (threshold over event), this peak is rerouted to the sample loop. For this the outlet valve switches to the reinjection position **(Fig. 9**, 1**)**. The multi-injection valve was already in the manual load/reinjection position. If the peak is below 60 mAU (threshold under event) the outlet valve switches back to waste. Please keep in mind to program an execution delay in the PurityChrom set up for the delay volume between the UV detector and the outlet valve.

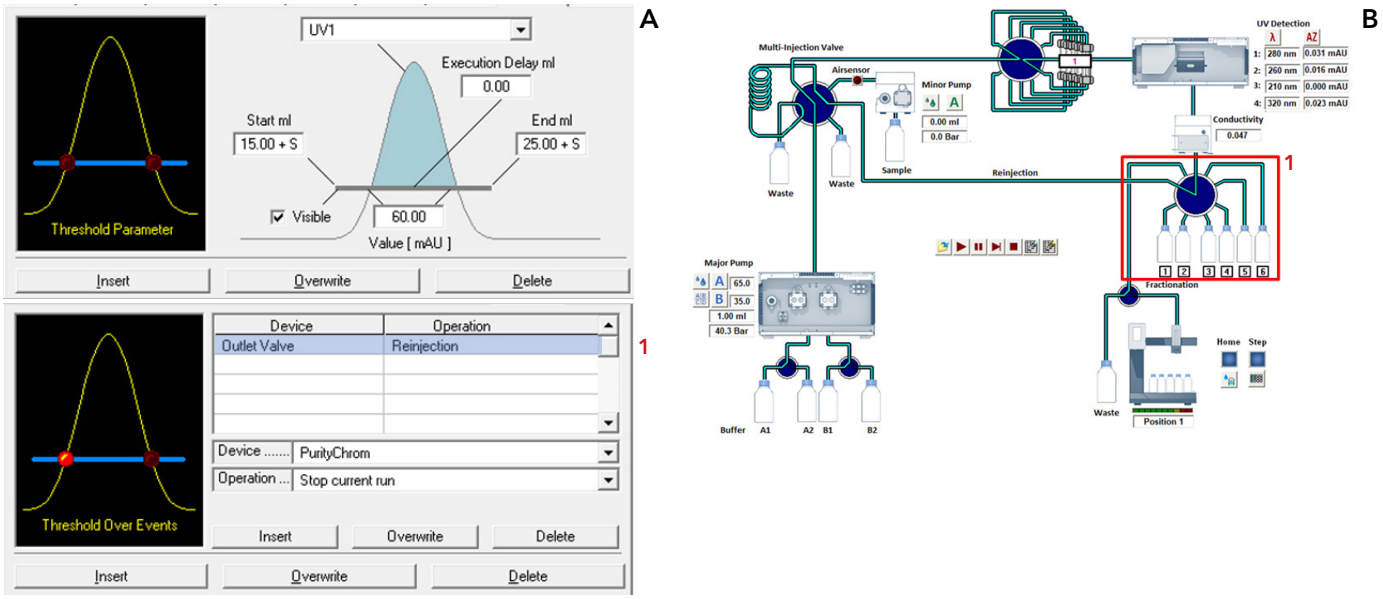

Fig. 9 Ion exchange method during collection of the intermediate peak. Time control file of ion exchange method definition of threshold function (A). Visualization of AZURA Bio Lab system in set up with sample pump (B).

At the end of the run purified protein is stored in the injection loop and can be further purified via the second column in the next step without manual interference. The time control file for the second method (Desalting) is shown in **Fig. 10A** and the flow scheme/ visualization is shown in **Fig. 10B**. Here, the buffer **(Fig. 10**, 1**)** and the column changes **(Fig. 10**, 2**)**.

To inject the intermediate peak onto the desalting column the multi-injection valve is set to the inject position **(Fig. 10**, 3**)**. The eluting peak is precisely fractionated with the help of the threshold function **(Fig. 10**, 4**)** using the fraction collector. At the end of the two step purification run the protein is purified and collected in small fractions.

A

Volume [ml]	Function	Parameter	
0.00	Valve Position	A = A2	1
0.00	Valve Position	B = B1	
0.00	Valve Position	Injection Valve = Manual Load/Rei	
0.00	Valve Position	Outlet Valve = to Fraction Collector	
0.00	Valve Position	Column Selection Valve = Bypass	
0.00	Valve Position	Fractionation = Waste	
0.00	Composition Major Pump	100.0 , 0.0	
0.00	Flowrate Major Pump	2.50 ml/min Constant Flow	
2.00	Start Chromatogram	Channel 1,2,3,4,5,P,P (500 ms)	
2.00	UV Autozero		
2.00	Valve Position	Injection Valve = Inject	3
2.00	Fraction Limiter	Limit = 2.00 ml	
2.00	Threshold	20.00 ml / 10.00 mAU	4
2.00	Valve Position	Column Selection Valve = Column 2	2
22.00	Stop all		

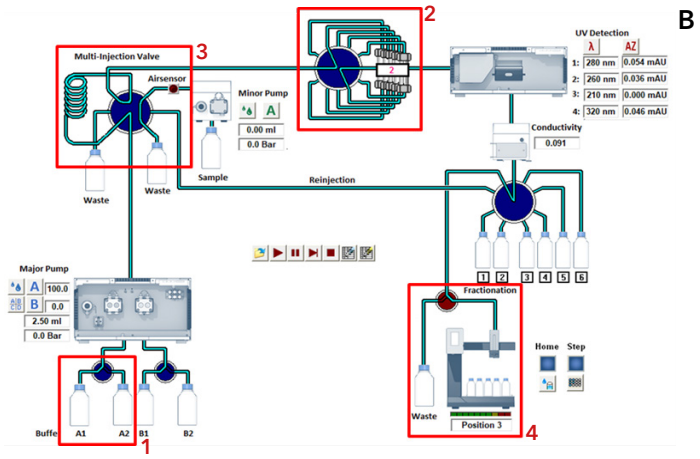

Fig. 10 Desalting method. Time control file of desalting method (A). Visualization of AZURA Bio Lab system in set up with sample pump (B).

To run both methods one after the other a sequence table should be used **(Fig. 11)**.

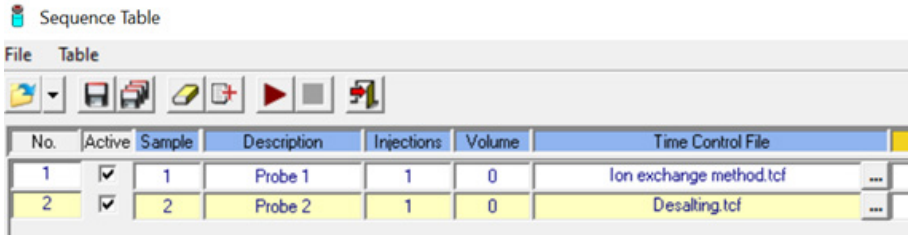

Fig. 11 Sequence table.

ADDITIONAL DEVICES

Tab. 1 List of additional devices

Device	Description	Article No.
Outlet valve in an ASM2.2L or stand alone	Smart valve drive with RFID-technology VU 4.1 valve drive for V 4.1 valves, stand alone	AWA01XA
	Universal valve drive for ASM 2.2L, assistant module VU 4.1 for valves V 4.1	EWA04
	Biocompatible multiposition valve with 8 Ports, 1/16"	AVS34CE
Filter cartridge	Filter cartridge for pump P 6.1L, high capacity, 2 µm titanium filter, 60 µl volume	A9661
Dummy filter cartridge	Empty cartridge, inline filter alternative	A9652
Inline filter	Inline Filter, PEEK/Titanium, 1/16", biocompatible, 10 µm	A3379
	Replacement Frits for inline filter, PEEK/itanium, biocompatible, 10 µm	A3379-1
Column selection valve	Smart valve drive with RFID technology VU 4.1 valve drive for V 4.1 valves, stand alone	AWA01XA
	Biocompatible column selection/sample loop selection valve, for 5 columns/sample loops and 1 bypass, reverse flow, 12 ports, 1/16", 50 bar	AVZ52CE

RELATED KNAUER APPLICATIONS

VTN0013 - How to optimize your purification? Your guide for two step purification - principles and system set up
VTN0014 - Two step purification with a basic set up