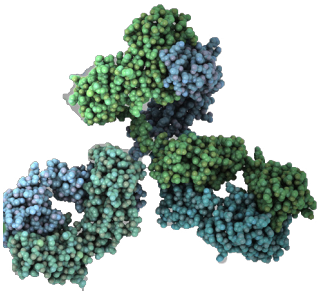
Science with Passion

Two step purification with the sample pump set up and PurityChrom 6



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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)**.

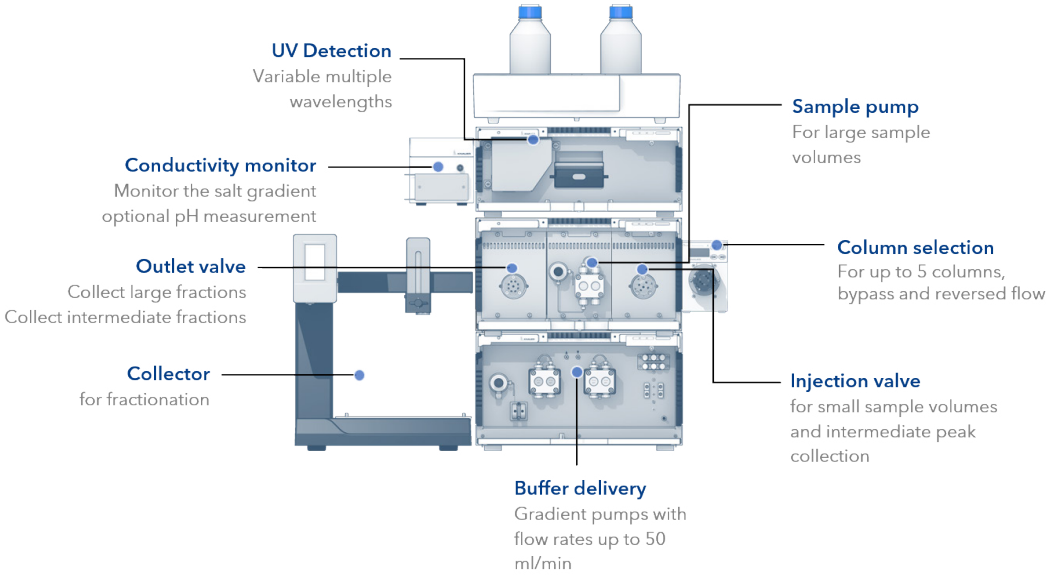


Fig. 1 AZURA Bio Lab System Advanced in set up with sample pump: exemplary configuration for automated two step applications.

Two step purification with the sample pump set up and PurityChrom 6

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)**.

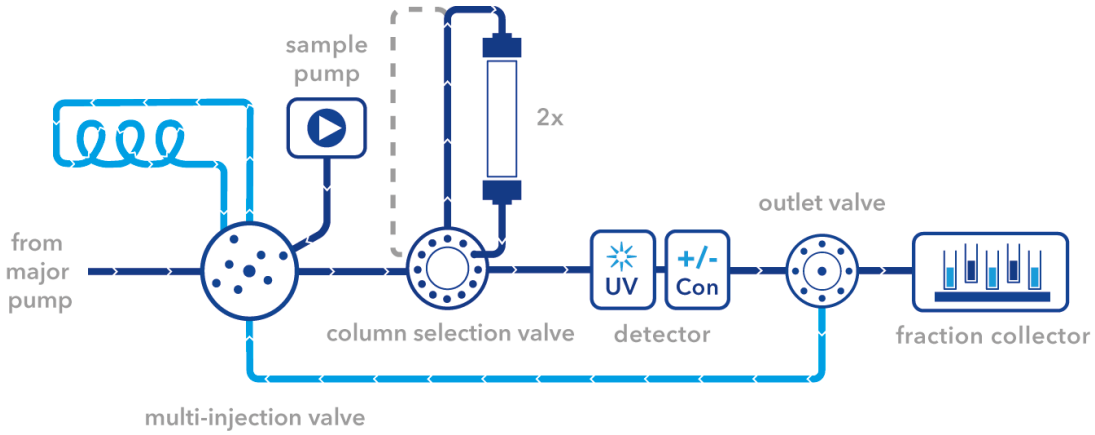


Fig. 2 Flow scheme for the sample pump set up for two step purification.

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**.

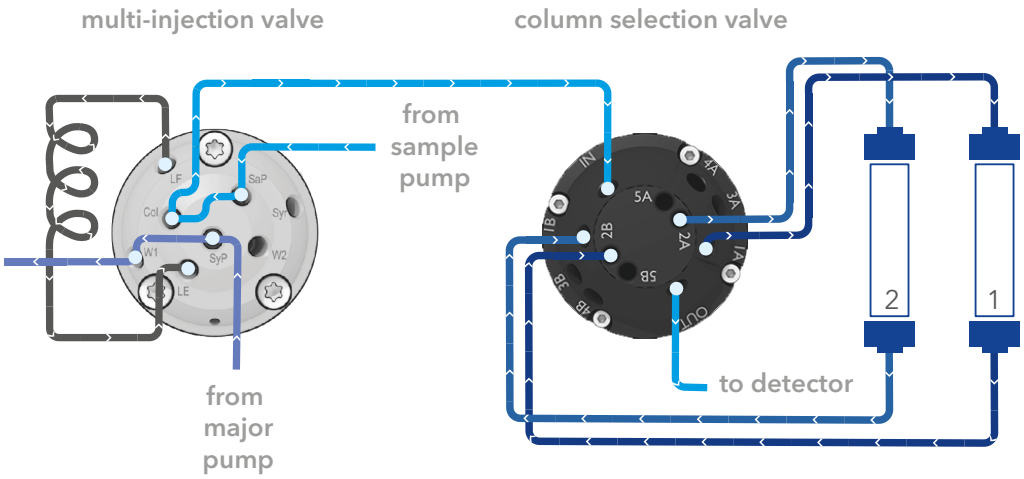


Fig. 3 Connection of the column selection valve.

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.

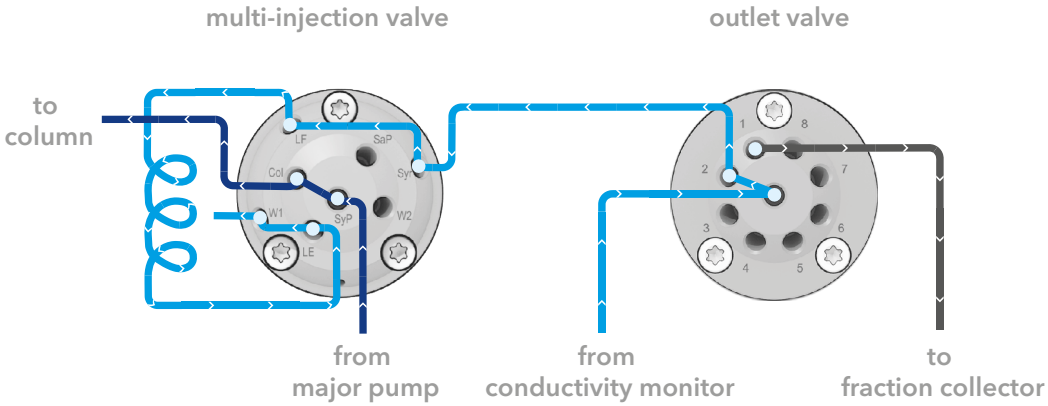


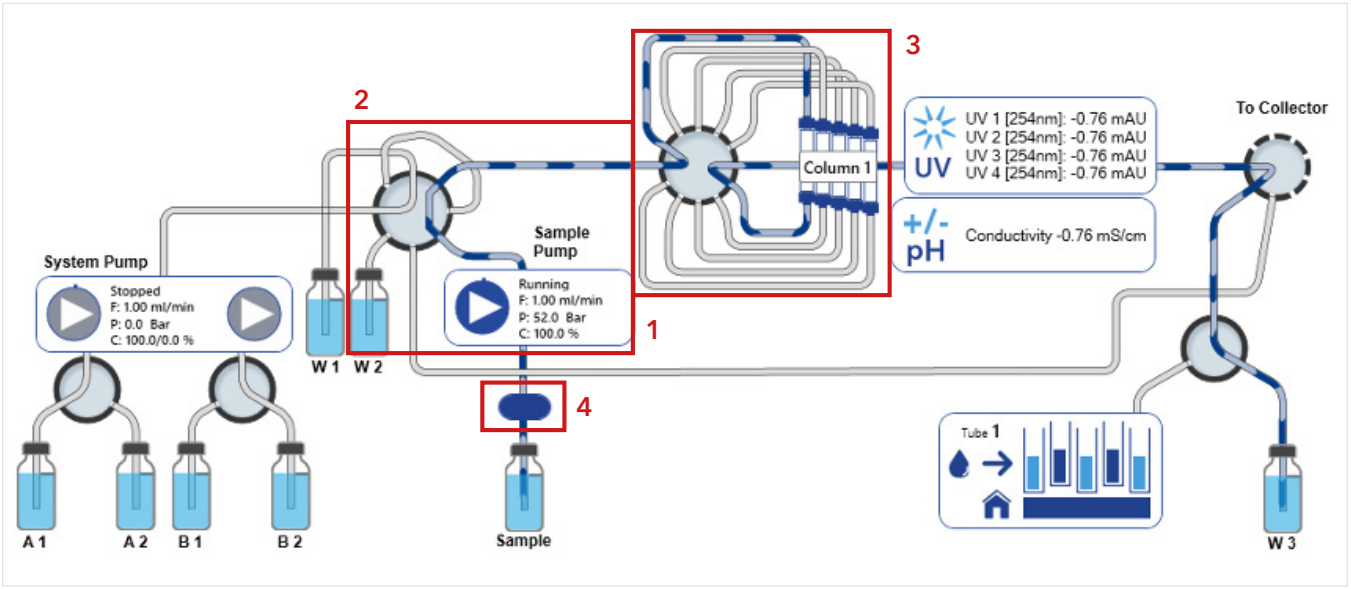
Fig. 4 Connection of the outlet valve.

HOW TO WRITE A METHOD

In the following section we will describe how to write an exemplary method for two step purification with PurityChrom 6. 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.

Two step purification with the sample pump set up and PurityChrom 6

A



B

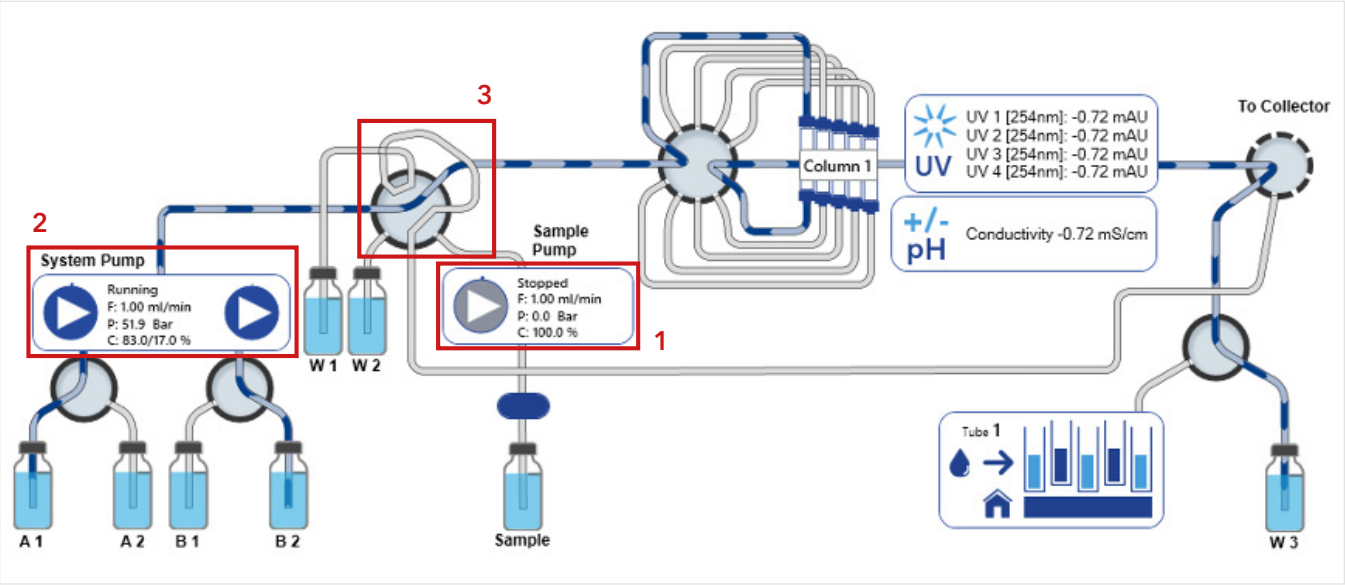
	Time [ml]	Function	Parameter
1	0.00	Sample Pump**Engine**	On
	0.00	Sample Pump**Flowrate**	Value: **1.00 ml/min**
	0.00	System Pump**Engine**	Off
	0.00	System Pump**Flowrate**	Value: **1.00 ml/min**
	0.00	System Pump**Solvents**	A: **100.0 %** B: **0.0 %**
	0.00	UV/Vis**Autozero**	
2	0.00	Multi-Injection Valve**Valve Switch**	Position: **Direct Injection**
3	0.00	Column Selection Valve**Valve Switch**	Position: **Column 1**
4	0.00	Process**Wait**	Airsensor**Input 1** = On Mode: **Hold**
	0.00	Chromatogram**Start**	☒ System Pressure ☒ System Flow ☒ Sample Pressure ☒ Sample Flow ☒ UV/Vis 1 ☒ UV/Vis 2 ☒ UV/Vis 3 ☒ UV/Vis 4 ☒ Conductivity
	0.02	Sample Pump**Engine**	Off
	0.02	System Pump**Engine**	On
	0.02	Multi-Injection Valve**Valve Switch**	Position: **Manual Load/Reinject**
	5.00	System Pump**Solvents**	A: **100.0 %** B: **0.0 %**
	5.00	Table**Threshold**	Name: **Peak Storage Start** Duration: **10.00 ml** Delay: **0.47 ml** Channel: **UV/Vis 1** Threshold: **100 mAU** *Overstepping Actions* Outlet Valve**Valve Switch** Position: **Reinjection** Process**Annotation** Text: **Peak Storage Start**
	5.00	Table**Threshold**	Name: **Peak Storage Stop** Duration: **10.00 ml** Delay: **0.55 ml** Channel: **UV/Vis 1** Threshold: **100 mAU** *Understepping Actions* Outlet Valve**Valve Switch** Position: **To Collector** Process**Annotation** Text: **Peak Storage Stop**
	15.00	System Pump**Solvents**	A: **60.0 %** B: **40.0 %**
	18.00	Process**Stop All Devices**	

Fig. 5 Ion exchange method during automatic sample injection [A] Visualization of AZURA Bio Lab System in set up with sample pump [B] Ion exchange method

First, the changes in the flowpath are depicted in the flow scheme/ visualization **(Fig. 5-7 A)** and important aspects of the ion exchange method with automatic sample application are highlighted **(Fig. 5-7 B)**.

The sample is automatically applied via the sample pump. Therefore, the flow of the sample pump is set to 1 ml/min **(Fig. 5 A+B.1)** and the multi-injection valve switches to direct injection **(Fig. 5 A+B.2)**. Make sure to choose the correct column in the beginning of the method **(Fig. 5 A+B.3)** and that the column is equilibrated with buffer A. During automatic sample injection the wait function is used to detect the end of the sample application via the air sensor **(Fig. 5 A+B.4)**.

A



When the sample application is finished, the sample pump is turned off **(Fig. 6 A+B.1)** and the system pump starts running **(Fig. 6 A+B.2)**. The multi-injection valve is switching to the Manual Load position **(Fig. 6 A+B.3)**. After an isocratic washing step, the elution starts after 5 ml with the beginning of the gradient **(Fig. 6. B.4)**.

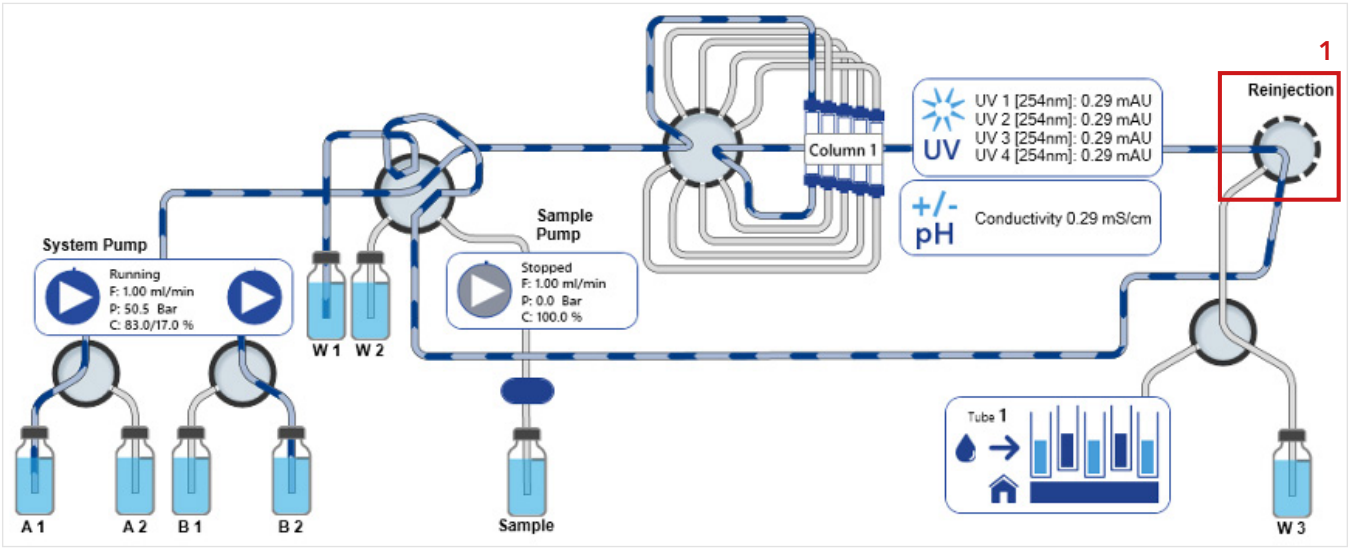
B

Time [ml]	Function	Parameter	
0.00	Sample Pump**Engine**	On	
0.00	Sample Pump**Flowrate**	Value: **1.00 ml/min**	
0.00	System Pump**Engine**	Off	
0.00	System Pump**Flowrate**	Value: **1.00 ml/min**	
0.00	System Pump**Solvents**	A: **100.0 %** B: **0.0 %**	
0.00	UV/Vis**Autozero**		
0.00	Multi-Injection Valve**Valve Switch**	Position: **Direct Injection**	
0.00	Column Selection Valve**Valve Switch**	Position: **Column 1**	
0.00	Process**Wait**	Airsensor**Input 1** = On Mode: **Hold**	
0.00	Chromatogram**Start**	☒ System Pressure ☒ System Flow ☒ Sample Pressure ☒ Sample Flow ☒ UV/Vis 1 ☒ UV/Vis 2 ☒ UV/Vis 3 ☒ UV/Vis 4 ☒ Conductivity	
0.02	Sample Pump**Engine**	Off	1
0.02	System Pump**Engine**	On	2
0.02	Multi-Injection Valve**Valve Switch**	Position: **Manual Load/Reinject**	3
5.00	System Pump**Solvents**	A: **100.0 %** B: **0.0 %**	4
5.00	Table**Threshold**	Name: **Peak Storage Start** Duration: **10.00 ml** Delay: **0.47 ml** Channel: **UV/Vis 1** Threshold: **100 mAU** *Overstepping Actions*: Outlet Valve**Valve Switch** Position: **Reinjection**; Process**Annotation** Text: **Peak Storage Start**	
5.00	Table**Threshold**	Name: **Peak Storage Stop** Duration: **10.00 ml** Delay: **0.55 ml** Channel: **UV/Vis 1** Threshold: **100 mAU** *Understepping Actions*: Outlet Valve**Valve Switch** Position: **To Collector**; Process**Annotation** Text: **Peak Storage Stop**	
15.00	System Pump**Solvents**	A: **60.0 %** B: **40.0 %**	4
18.00	Process**Stop All Devices**		

Fig. 6 Ion exchange method during gradient elution [A] Visualization of AZURA Bio Lab System in set up with sample pump [B] Ion exchange method

Two step purification with the sample pump set up and PurityChrom 6

A



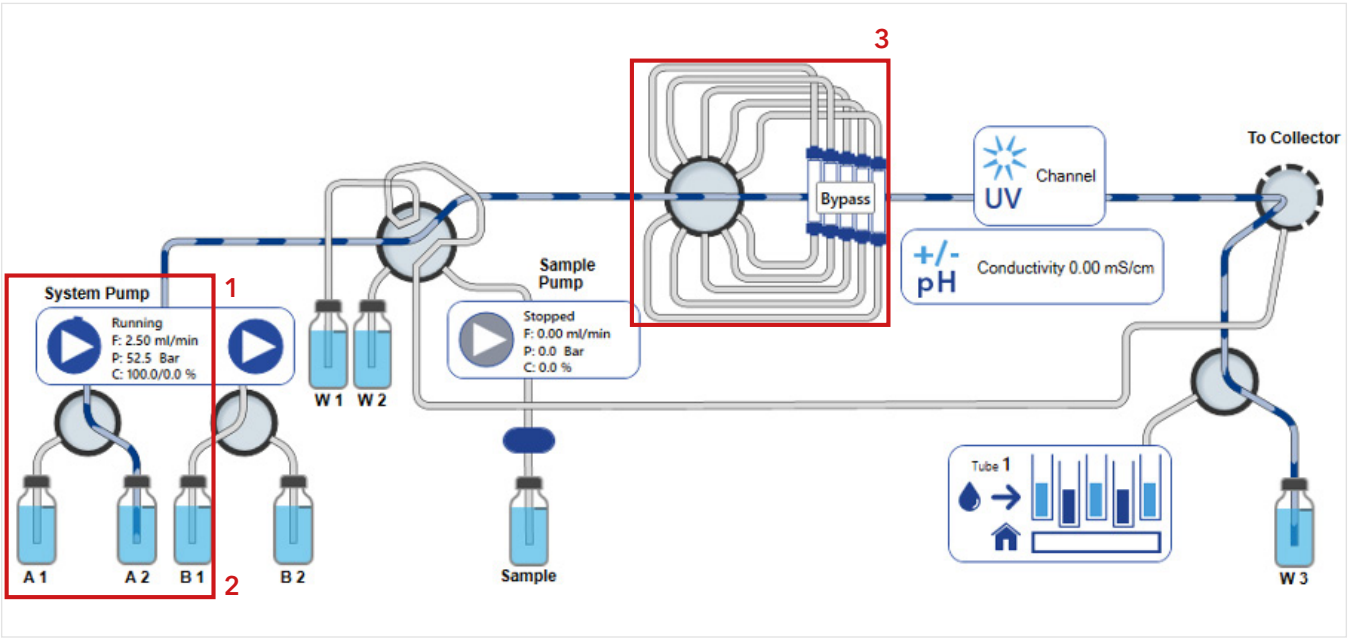
B

	Time [ml]	Function	Parameter
	0.00	Sample Pump**Engine**	On
	0.00	Sample Pump**Flowrate**	Value: **1.00 ml/min**
	0.00	System Pump**Engine**	Off
	0.00	System Pump**Flowrate**	Value: **1.00 ml/min**
	0.00	System Pump**Solvents**	A: **100.0 %** B: **0.0 %**
	0.00	UV/Vis**Autozero**	
	0.00	Multi-Injection Valve**Valve Switch**	Position: **Direct Injection**
	0.00	Column Selection Valve**Valve Switch**	Position: **Column 1**
	0.00	Process**Wait**	Airsensor**Input 1** = On Mode: **Hold**
	0.00	Chromatogram**Start**	☒ System Pressure ☒ System Flow ☒ Sample Pressure ☒ Sample Flow ☒ UV/Vis 1 ☒ UV/Vis 2 ☒ UV/Vis 3 ☒ UV/Vis 4 ☒ Conductivity
	0.02	Sample Pump**Engine**	Off
	0.02	System Pump**Engine**	On
	0.02	Multi-Injection Valve**Valve Switch**	Position: **Manual Load/Reinject**
	5.00	System Pump**Solvents**	A: **100.0 %** B: **0.0 %**
1	5.00	Table**Threshold**	Name: **Peak Storage Start** Duration: **10.00 ml** Delay: **0.47 ml** Channel: **UV/Vis 1** Threshold: **100 mAU** *Overstepping Actions* Outlet Valve**Valve Switch** Position: **Reinjection** Process**Annotation** Text: **Peak Storage Start**
2	5.00	Table**Threshold**	Name: **Peak Storage Stop** Duration: **10.00 ml** Delay: **0.55 ml** Channel: **UV/Vis 1** Threshold: **100 mAU** *Understepping Actions* Outlet Valve**Valve Switch** Position: **To Collector** Process**Annotation** Text: **Peak Storage Stop**
	15.00	System Pump**Solvents**	A: **60.0 %** B: **40.0 %**
	18.00	Process**Stop All Devices**	

Fig. 7 Ion exchange method during collection of the intermediate peak [A] Visualization of AZURA Bio Lab System in set up with sample pump [B] Ion exchange method

To recognize the eluting peak two threshold functions are used. The thresholds are active during the gradient elution. Once a peak above 100 mAU is detected (threshold "Peak Storage Start"), the peak is rerouted to the sample loop. For this the outlet valve switches to the reinjection position **(Fig. 7 A+B.1)**. The multi-injection valve was already in the manual load/reinjection position. If the peak is below 100 mAU (threshold "Peak Storage Stop") the outlet valve switches back to collector **(Fig. 7 B.2)**. The annotation in the threshold function is used to mark the start and stop of the peak storage in the chromatogram. Please keep in mind to program an execution delay for the delay volume between the UV detector and the outlet valve for "Peak Storage Start" and an execution delay for the delay volume between the UV detector and the multi-injection valve for "Peak Storage Stop". 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.

A



After the ion exchange method, the desalting method starts. First, the system and the tubing are primed with buffer used for the desalting step. The conditioning is used to wash the system at a flowrate of 2.5 ml/min with buffer A2 **(Fig. 8 A+B 1,2)** for 1 minute **(Fig. 8 B 4)**. The column selection valve is in the bypass position **(Fig. 8 A+B 3)**.

B

Conditioning | Separation

Actions

Function	Parameter	
System Pump**Engine**	On	
System Pump**Flowrate**	Value: **2.50 ml/min**	1
Sample Pump**Engine**	Off	
Sample Pump**Flowrate**	Value: **0.00 ml/min**	
Column Selection Valve**Valve Switch**	Position: **Bypass**	3
System Pump**SSV A**	Position: **A 2**	2
System Pump**Solvents**	A: **100.0 %** B: **0.0 %**	

+ Add Function

Wait Condition

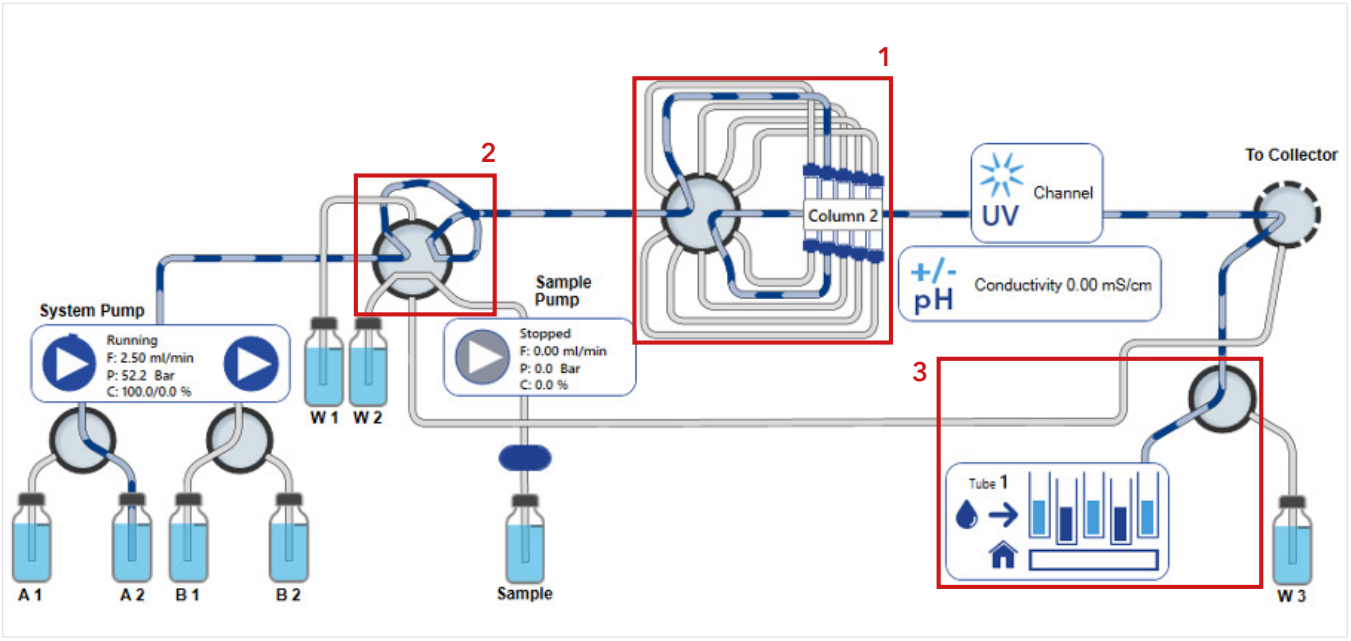
Type	Parameter	
Duration	Wait Time **1min**	4

+ Add Wait Condition

Fig. 8 Desalting method during system wash (A) Visualization of AZURA Bio Lab System in basic set up (B) Desalting method Conditioning

Two step purification with the sample pump set up and PurityChrom 6

A



B

Conditioning | Separation

	Time [ml]	Function	Parameter
1	0.00	Column Selection Valve**Valve Switch**	Position: **Column 2**
	0.00	Collection Unit**Set Volume Limit**	Volume limit **0.50 ml**
	0.00	Chromatogram**Start**	☒ System Pressure ☒ System Flow ☒ Sample Pressure ☒ Sample Flow ☒ UV/Vis 1 ☒ UV/Vis 2 ☒ UV/Vis 3 ☒ UV/Vis 4 ☒ Conductivity
2	0.00	UV/Vis**Autozero**	
3	0.02	Multi-Injection Valve**Valve Switch**	Position: **Injection**
	0.02	Table**Threshold**	Name: **Fractionation** Duration: **10.00 ml** Delay: **0.00 ml** Channel: **UV/Vis 1** Threshold: **50 mAU**
			Overstepping Actions: Collection Unit**Fractionation** Direction: **Fraction** *Understepping Actions*: Collection Unit**Fractionation** Direction: **Waste**
	20.00	Process**Stop All Devices**	

Fig. 9 Desalting method [A] Visualization of AZURA Bio Lab System in set up with sample pump [B] Desalting method

To run both methods one after the other a sequence table is used **(Fig. 10)**. Please remember to add a washing and reequilibration step for the ion exchange column.

Vial	SampleID	Injections	Volume	Active	Method	Browse
1		1	0.00 l	☑	\2Step_IEC	...
2		1	0.00 l	☑	\2Step_SEC	...
2		1	0.00 l	☑	\2Step_IEC_Wash+Equi	...
				☐		...

Fig. 10 Sequence table

After conditioning the system, the desalting step starts. Therefore, the column changes **(Fig. 9 A+B.1)**. To inject the intermediate peak onto the desalting column the multi-injection valve is set to the inject position **(Fig. 9 A+B.2)**. The eluting peak is precisely fractionated with the help of the threshold function **(Fig. 9 A+B.3)** using the fraction collector. At the end of the two-step purification run the protein is purified and collected in small fractions.

MATERIAL AND METHODS

System configuration

Instrument	Description	Article No.
Outlet valve	Smart valve drive with RFID-technology VU 4.1 valve drive for V 4.1 valves, stand alone	AWA01
	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/ Titanium, 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	AWA01
	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

VTN0015 – Two step purification with the sample pump set up

VTN0025 – Two step purification with the basic set up and PurityChrom6