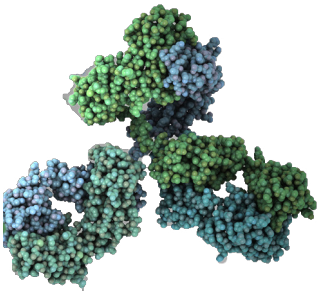
Science with Passion

Two step purification with a basic 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 a basic set up is discussed.

BASIC SYSTEM SET UP: WHAT DO I NEED?

In the basic set up a Lab standard KNAUER AZURA® Bio Lab system for all biochromatography methods is adapted. Just the column selection valve and the outlet valve are added to the system. **(Fig. 1)**.

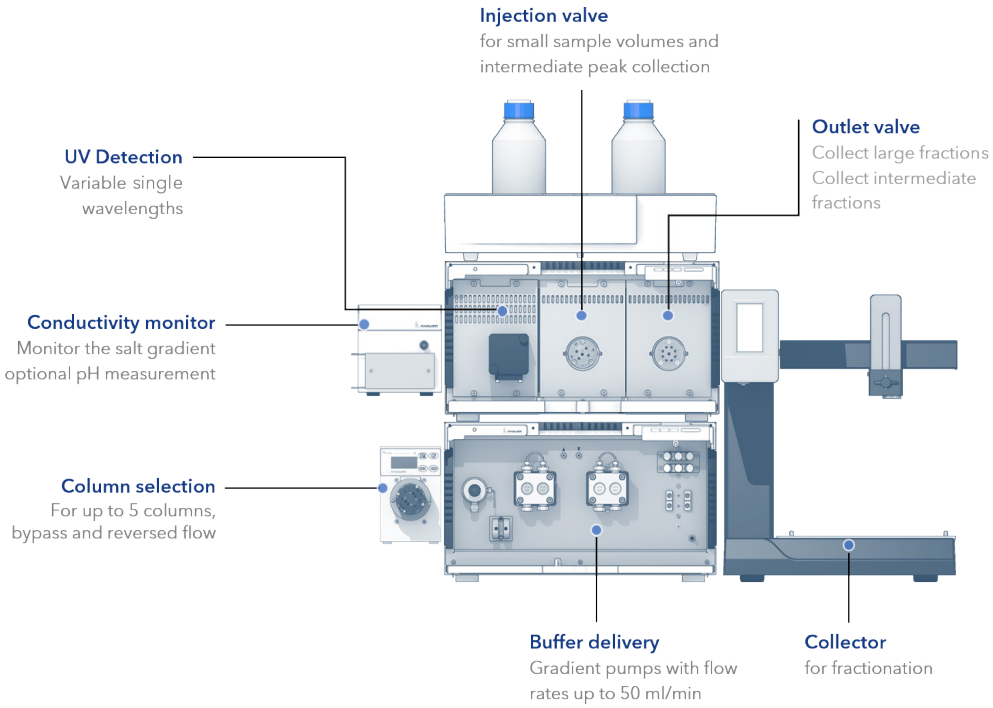

Fig. 1 AZURA Bio Lab system in basic set up: Exemplary configuration for automated two step applications.

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In the basic two step set up the sample is injected via the sample loop of the injection valve. The first peak is collected in the sample loop as well and redirected to the second column. This set up is limited to small sample volumes. The volume of the elution peak of the first column should be equal or even smaller than the injection volume due to diffusion effects that will result in sample loss **(Fig. 2)**.

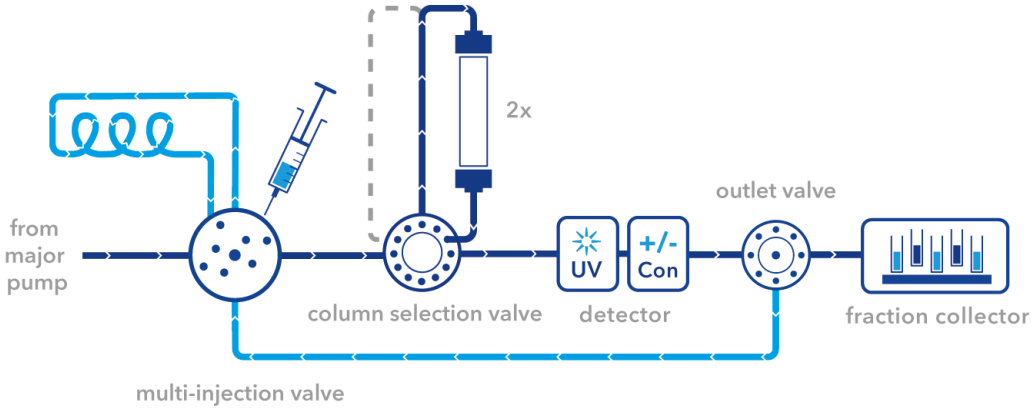


Fig. 2 Flow scheme for the basic set up for two step purification.

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 the connected to the UV detector flow cell which in turn is connected to the conductivity monitor. The columns are installed according to **Fig. 3**.

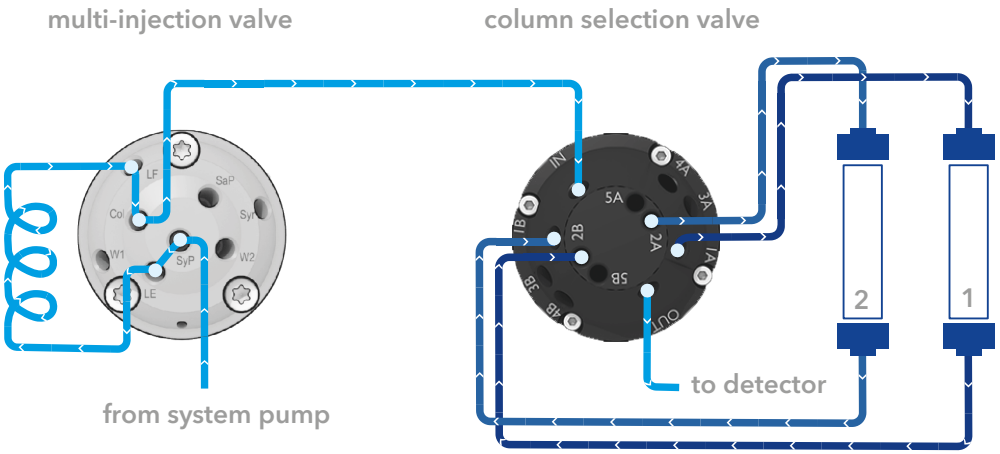


Fig. 3 Connection of the column selection valve.

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 sample pump port (SaP) of the multi-injection valve **(Fig. 4)** The syringe port is still accessible for sample injection.

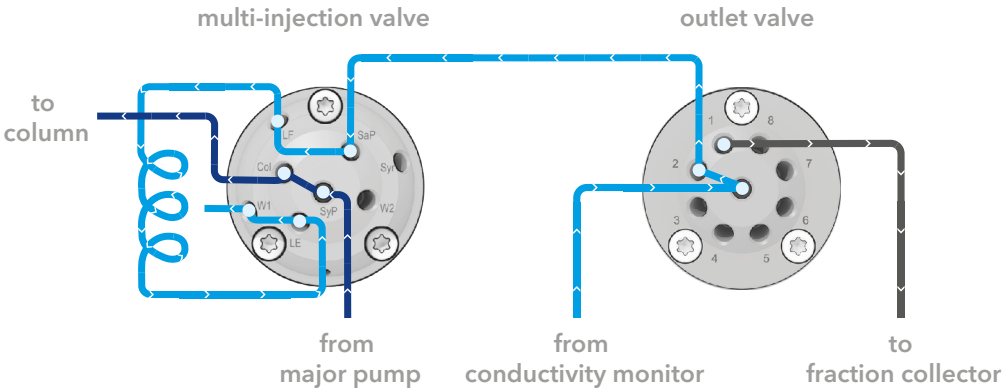


Fig. 4 Connection of the outlet valve.

The set up is, due to the use of the same sample loop for injection and reinjection, limited to small sample volumes. To overcome the problem of the small sample volume a variation of this set up is to inject the sample via the major pump. For this set up some additional changes must be implemented. The filter cartridge of the pressure sensor must be replaced by a dummy.

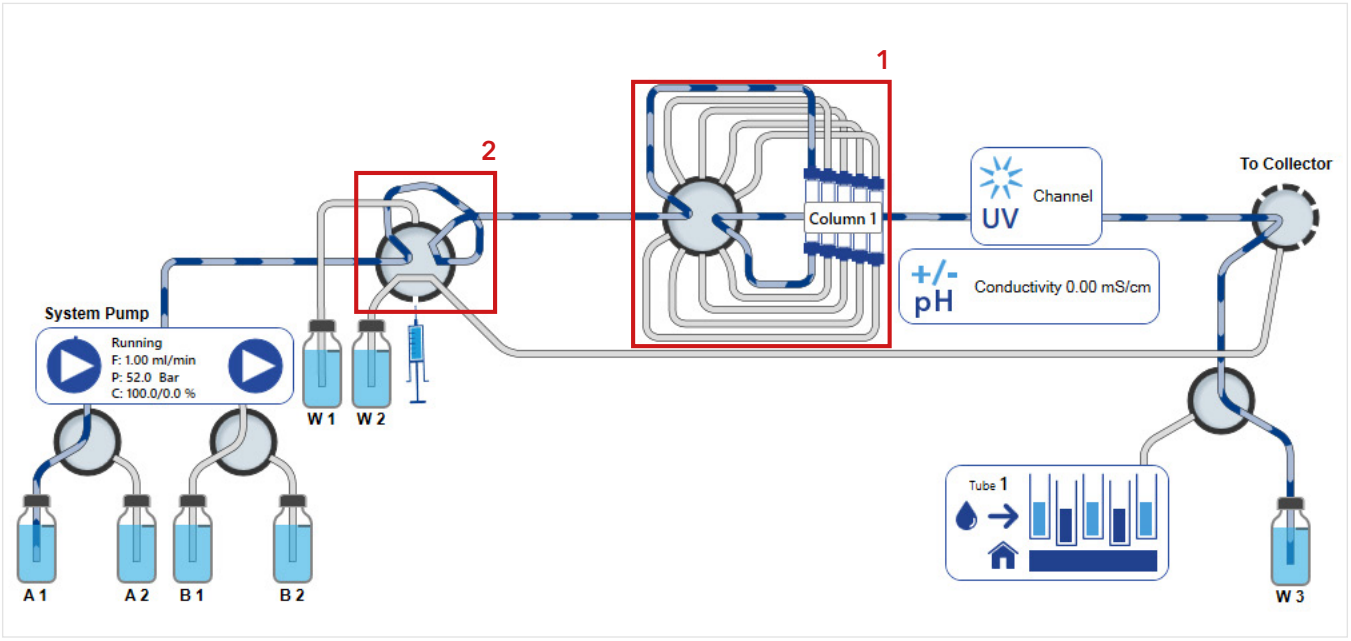
Please follow the manual for exchanging the pressure filter. When exchanging the pressure filter, we strongly advise to install an inline filter in front of the column selection valve **(Fig. 3)**. The filters frits of the inline filter should be cleaned or replaced from time to time. Additionally, the sample should be filtered via a 0,45 µm filter before injection.

HOW TO WRITE A METHOD

In the following section we will describe how to write an exemplary method for two step purification with PurityChrom6. 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. A 2 ml sample loop was used for sample injection and intermediate peak parking. Two separate methods were written for the two columns.

Two step purification with a basic set up and PurityChrom 6

A



B

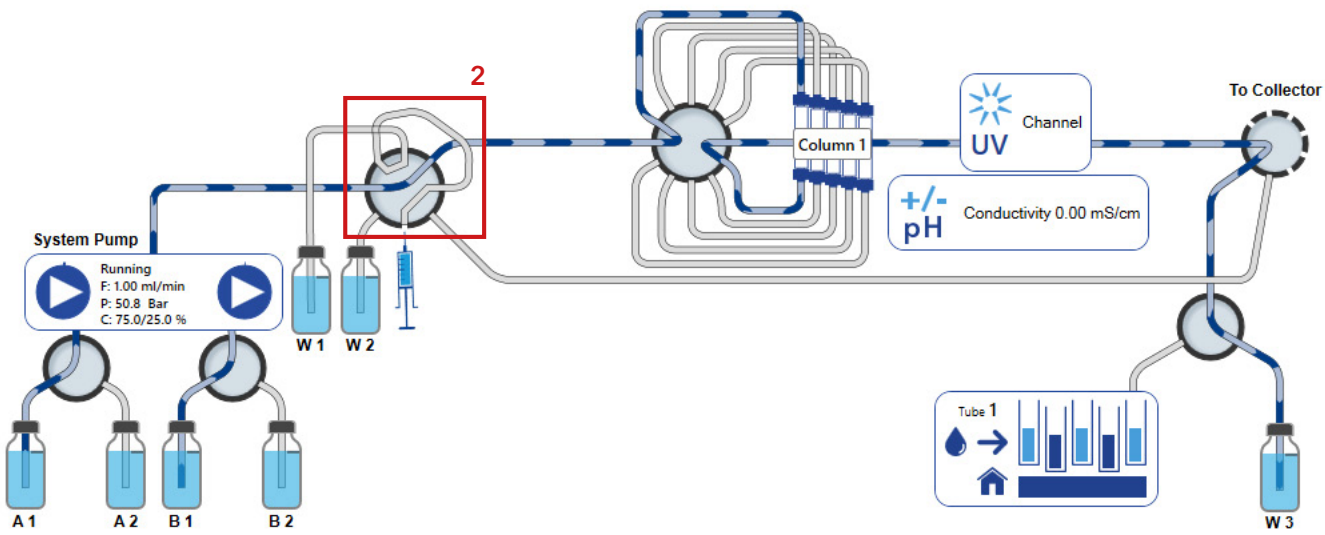
Separation

	Time [ml]	Function	Parameter
	0.00	Multi-Injection Valve**Valve Switch**	Position: **Manual Load**
1	0.00	Column Selection Valve**Valve Switch**	Position: **Column 1**
	0.00	Chromatogram**Start**	☒ System Pressure ☒ System Flow ☒ UV/Vis 1 ☒ Conductivity
	0.00	UV/Vis**Autozero**	
	0.00	System Pump**Flowrate**	Value: **1.00 ml/min**
	0.00	System Pump**Engine**	On
	0.00	System Pump**Solvents**	A: **100.0 %** B: **0.0 %**
	0.02	Multi-Injection Valve**Valve Switch**	Position: **Injection**
2	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* Multi-Injection Valve**Valve Switch** Position: **Pump Load/Reinject** 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** Multi-Injection Valve**Valve Switch** Position: **Manual Load** Process**Annotation** Text: **Peak Storage Stop**
	5.00	Multi-Injection Valve**Valve Switch**	Position: **Manual Load**
	15.00	System Pump**Solvents**	A: **60.0 %** B: **40.0 %**
	18.00	Process**Stop All Devices**	

Fig. 5 Ion exchange method during injection of sample (A) Visualization of AZURA Bio Lab System in basic set up (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 are highlighted **(Fig. 5-7 B)**. Make sure to choose the correct column in the beginning of the method **(Fig. 5 A+B.1)**. The sample is inserted in the manual load configuration of the multi-injection valve. During injection, the multi-injection valve switches to the inject position **(Fig. 5 A+B.2)**.

A



B

Separation

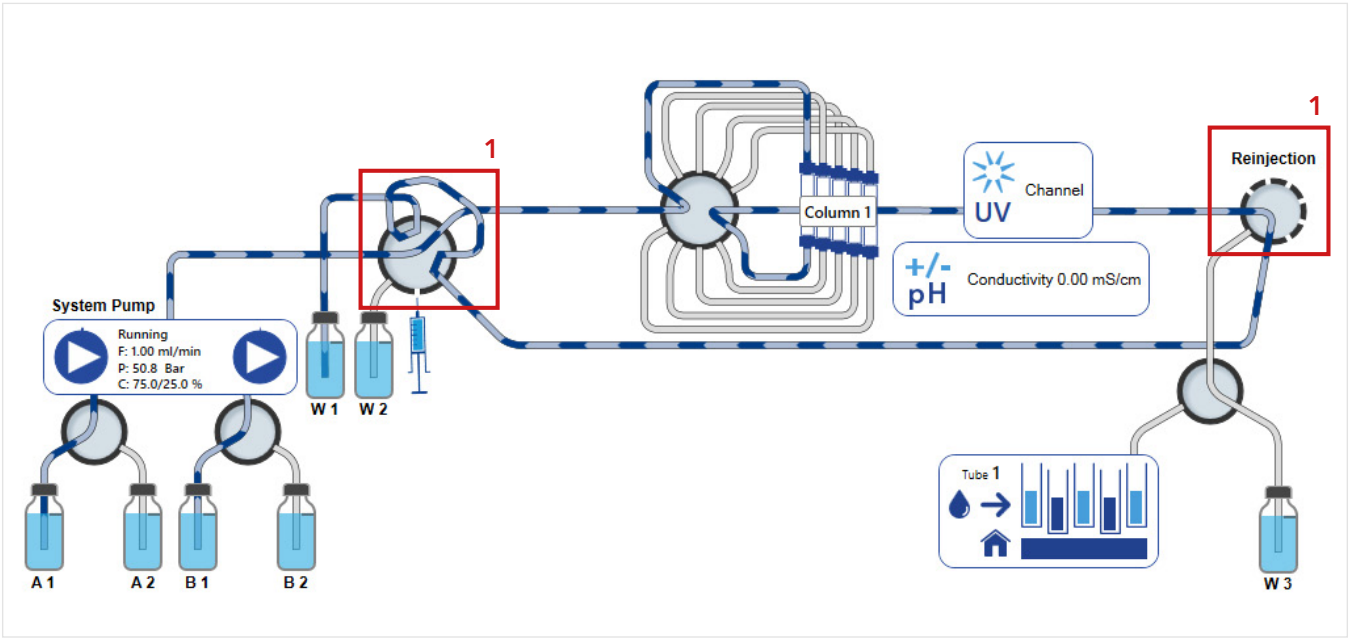
Time [ml]	Function	Parameter	
0.00	Multi-Injection Valve**Valve Switch**	Position: **Manual Load**	
0.00	Column Selection Valve**Valve Switch**	Position: **Column 1**	
0.00	Chromatogram**Start**	☒ System Pressure ☒ System Flow ☒ UV/Vis 1 ☒ Conductivity	
0.00	UV/Vis**Autozero**		
0.00	System Pump**Flowrate**	Value: **1.00 ml/min**	
0.00	System Pump**Engine**	On	
0.00	System Pump**Solvents**	A: **100.0 %** B: **0.0 %**	
0.02	Multi-Injection Valve**Valve Switch**	Position: **Injection**	
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 Multi-Injection Valve**Valve Switch** Position: **Pump Load/Reinject** 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** Multi-Injection Valve**Valve Switch** Position: **Manual Load** Process**Annotation** Text: **Peak Storage Stop**	
5.00	Multi-Injection Valve**Valve Switch**	Position: **Manual Load**	2
15.00	System Pump**Solvents**	A: **60.0 %** B: **40.0 %**	1
18.00	Process**Stop All Devices**		

After the sample injection and an isocratic washing step, the elution starts after 5 ml with beginning of the gradient **(Fig. 6 B.1)**. At this time point the multi-injection valve should be switched back to the manual load position **(Fig. 6 A+B.2)**.

Fig. 6 Ion exchange method during gradient elution (A) Visualization of AZURA Bio Lab System in basic set up (B) Ion exchange method

Two step purification with a basic set up and PurityChrom 6

A



B

Separation

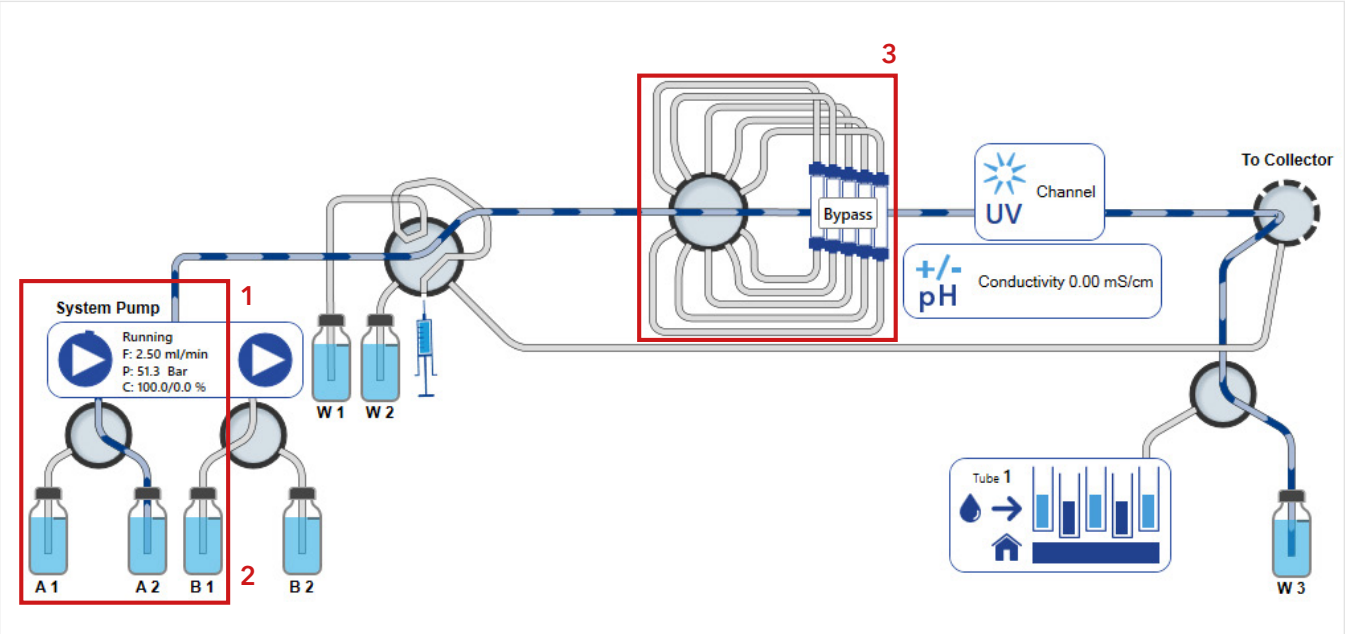
	Time [ml]	Function	Parameter
	0.00	Multi-Injection Valve**Valve Switch**	Position: **Manual Load**
	0.00	Column Selection Valve**Valve Switch**	Position: **Column 1**
	0.00	Chromatogram**Start**	☒ System Pressure ☒ System Flow ☒ UV/Vis 1 ☒ Conductivity
	0.00	UV/Vis**Autozero**	
	0.00	System Pump**Flowrate**	Value: **1.00 ml/min**
	0.00	System Pump**Engine**	On
	0.00	System Pump**Solvents**	A: **100.0 %** B: **0.0 %**
	0.02	Multi-Injection Valve**Valve Switch**	Position: **Injection**
	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**
			Function / Parameter
			Overstepping Actions
			Multi-Injection Valve**Valve Switch** Position: **Pump Load/Reinject**
			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**
			Function / Parameter
			Understepping Actions
			Outlet Valve**Valve Switch** Position: **To Collector**
			Multi-Injection Valve**Valve Switch** Position: **Manual Load**
			Process**Annotation** Text: **Peak Storage Stop**
	5.00	Multi-Injection Valve**Valve Switch**	Position: **Manual Load**
	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 basic set up (B) Ion exchange method

To recognize the eluting peak threshold functions are used. The threshold is active during the gradient elution **(Fig. 7 B.1)**. Once a peak above 100 mAU is detected (Peak storage start), this peak is rerouted to the sample loop. For this the outlet valve switches to the reinjection position and the multi-injection valve switches to pump load/ reinject **(Fig. 7 A+B.1)**. If the peak is below 100 mAU (Peak storage stop) the outlet valve switches back to waste and the multi-injection valve back to manual load **(Fig. 7 B.2)**.

The annotation in the threshold function is used to mark the start and stop of the peak sampling 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

A



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

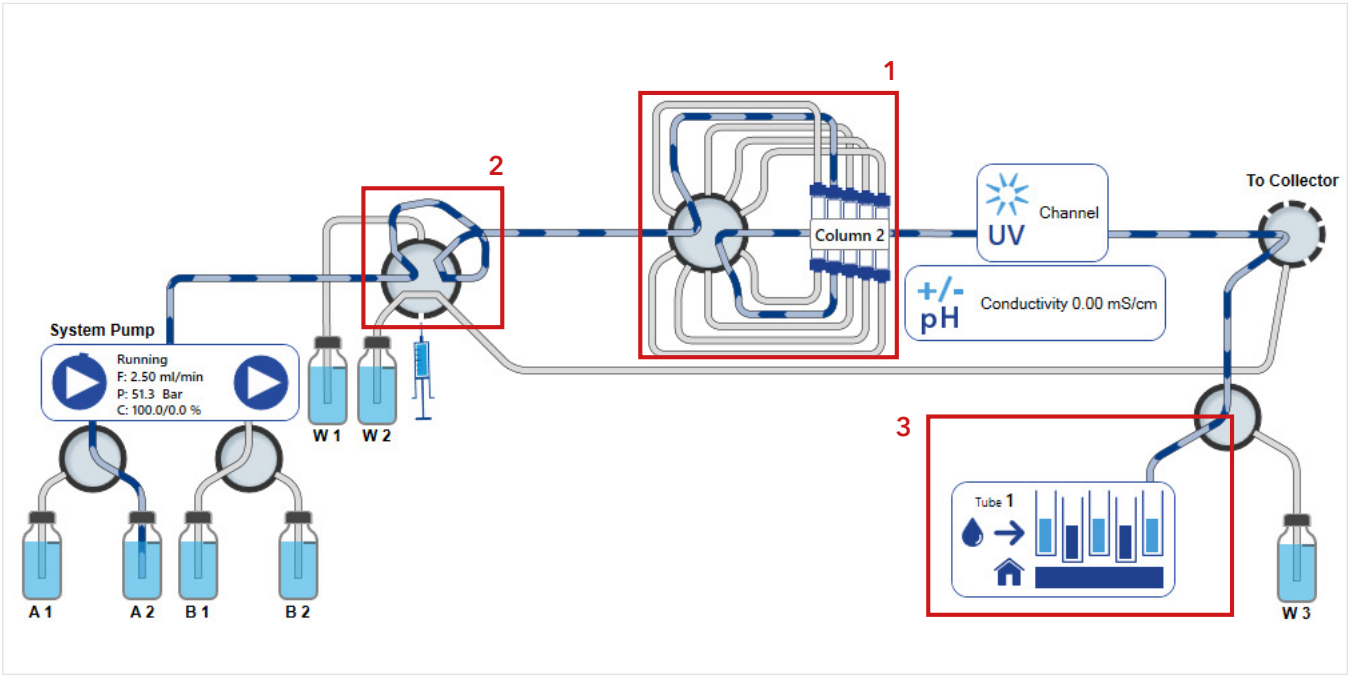
Fig. 8 Desalting method during system wash (A) Visualization of AZURA Bio Lab System in basic set up (B) Desalting method Conditioning

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.

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)**.

Two step purification with a basic 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 ☒ UV/Vis 1 ☒ Conductivity
	0.00	UV/Vis\Autozero	
2	0.02	Multi-Injection Valve\Valve Switch	Position: Injection
3	0.02	Table\Threshold	Name: Fractionation Duration: 10.00 ml Delay: 0.00 ml Channel: Threshold: 50
			Function / Parameter *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 basic set up (B) Desalting method

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.

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.

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

VTN0026 – Two step purification with the sample pump set up and PurityChrom6