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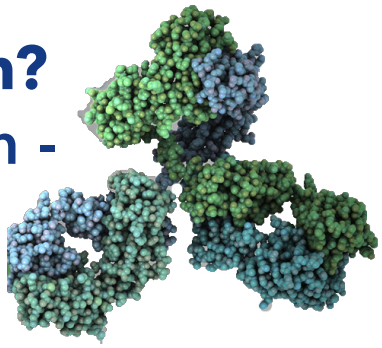
How to optimize your purification? Your guide for two step purification - principles and system set up



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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 **(Fig. 1)**.

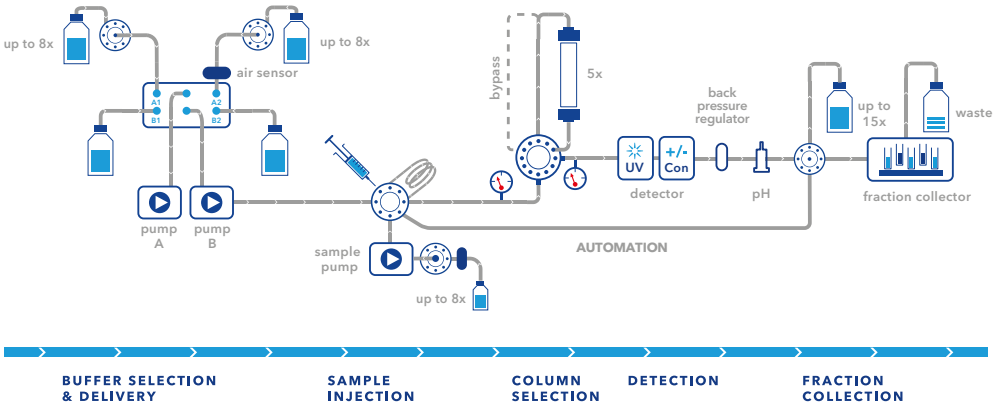

Fig. 1 Example of a typical two step purification system.

WHY SHOULD I USE TWO STEP PURIFICATION?

For many protein purifications a sequence of different methods is used to achieve pure protein. The protein purification strategy describes the combination of several method steps and divides them into capture, intermediate and polishing step **(Fig. 2)**. The "capture" step purifies the protein from the crude extract. The "intermediate" step removes further contamination, while the aim of the final "polishing" step is to get rid of all remaining impurities to gain a highly purified product. Depending on the yield and purity of the protein which is needed for further analysis and /or use of the protein, several steps are combined. Nevertheless, purification strategies with just one step or more than three steps are possible as well.

Fig. 2 Purification strategy.

Additional Information

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WHICH METHODS CAN I COMBINE?

A two step purification system can easily be used to combine two methods of your purification strategy. A classical approach is the combination of a capture with a polishing step. Some examples for the first and the second step are listed in **Tab. 1**. In principle any method can be combined. Please consider while designing your two step strategy that the elution conditions of the first step should match the starting conditions of the second step. A typical example for two step purification is the purification of His tagged proteins. The capture step for these proteins is done by immobilized metal ion affinity chromatography. The elution is achieved by imidazole. The next logical step which can be easily integrated in a two step set up is a desalting column to exchange the buffer. Hereby the imidazole is removed, and the ionic strength can be reduced.

Tab. 1 Overview of methods used for the first and second step of two step purification.

Step	Mode	Method
1. Step	Capture	Tag-affinity (e.g. GST, Strep-Tag) Antibody affinity (e.g. Protein A, Protein G) Immobilized metal ion affinity (IMAC) (e.g. Ni-NTA, Talon) Ion exchange chromatography
2. Step	Intermediate/ Polishing	Size exclusion chromatography Desalting/buffer exchange Ion exchange chromatography Hydrophobic interaction chromatography

WHAT ARE THE BENEFITS?

The benefits for this set up are manifold. The transition from one to another step generally involves manual interaction and thus is time consuming. Automation by combining these steps increases the efficiency and optimizes the workflow. The quick and automated linkage of multiple chromatographic purification steps into one method eliminates manual sample handling and minimizes time spent between steps and simple handling errors. This automation strategy can be easily adapted to several purification task.

WHAT ARE THE SYSTEM REQUIREMENTS AND WHICH SYSTEM SET UPS ARE AVAILABLE?

Several system set ups can be used to automate the purification. Some of the most common set ups are discussed. We will explain in detail the benefits and limitations of each set up. **(Tab. 2)**. We assume that the biocompatible multi-injection valve (**AVN94CE**) is included within the FPLC system. For all set ups a column selection valve (**AVZ52CE**) to select the different columns and an outlet valve (**AVS34CE**) to redirect the flow and collect the first peak must be installed in the system.

BASIC SET UP

In the basic set up a Lab standard KNAUER Multi Method FPLC system for all Bio-Chromatography methods is adapted. Next to the column selection valve and the outlet valve no other components must be added to the system **(Fig. 3)**.

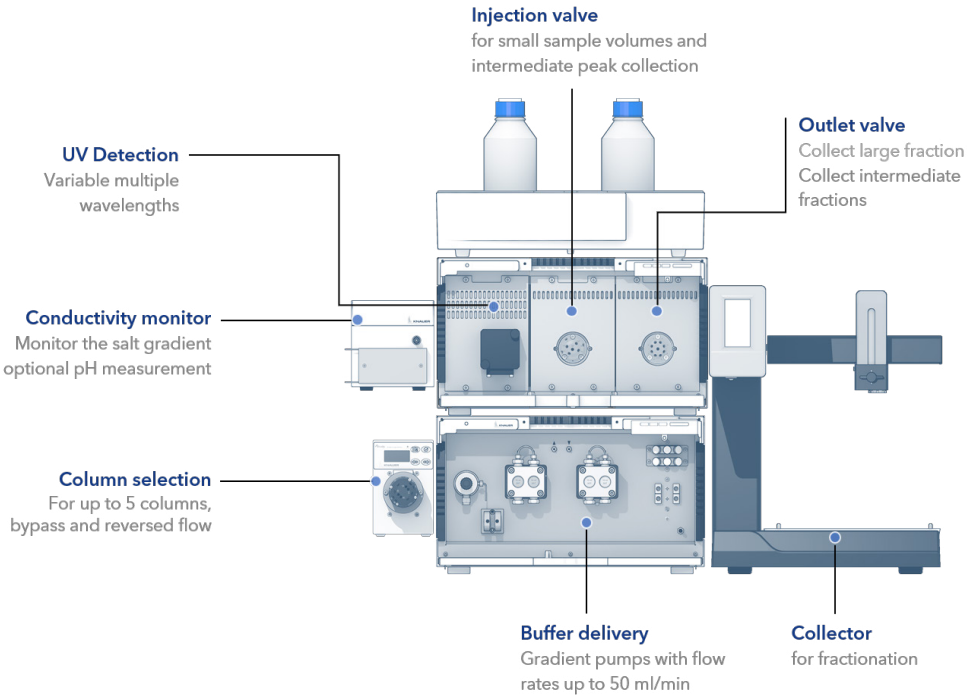

Fig. 3 AZURA Bio Lab system in basic set up: exemplary configuration for automated two step applications.

The reinjection position of the outlet valve is connected to the sample pump inlet of the injection valve so that the syringe port is still accessible for sample injection. The sample is injected via the sample loop of the injection valve. The first peak is collected in the sample loop of the injection valve as well and reinjected onto the second column **(Fig. 4)**.

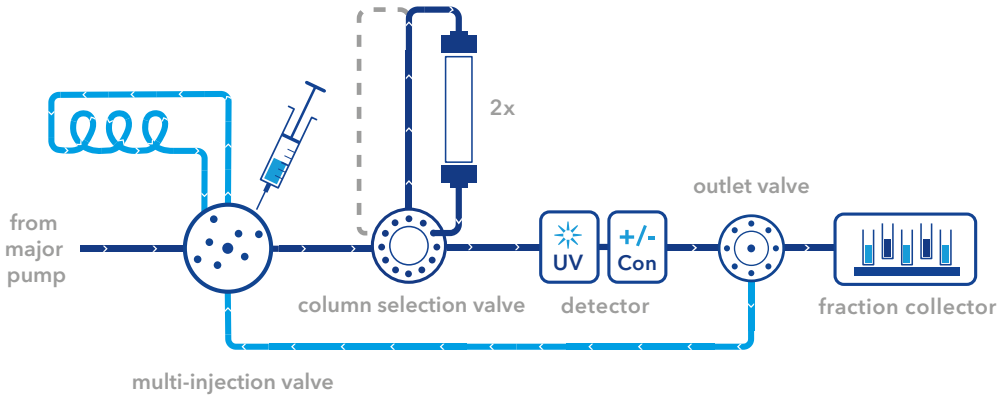

Fig. 4 Flow scheme for the basic set up for two step purification.

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Consideration: 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. 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 and we strongly advise to install an inline filter in front of the column selection valve. You can find more details about this set up including the connection of the capillaries and information about PurityChrom plus method writing in section **Two step purification with the basic set up (VNT0014)**.

SET UP WITH SAMPLE PUMP

A more advanced set up is the set up with sample pump **(Fig. 5)**. Hereby, the already mentioned column selection and outlet valve, as well as a sample pump must be added to the Advanced Bio Purification system.

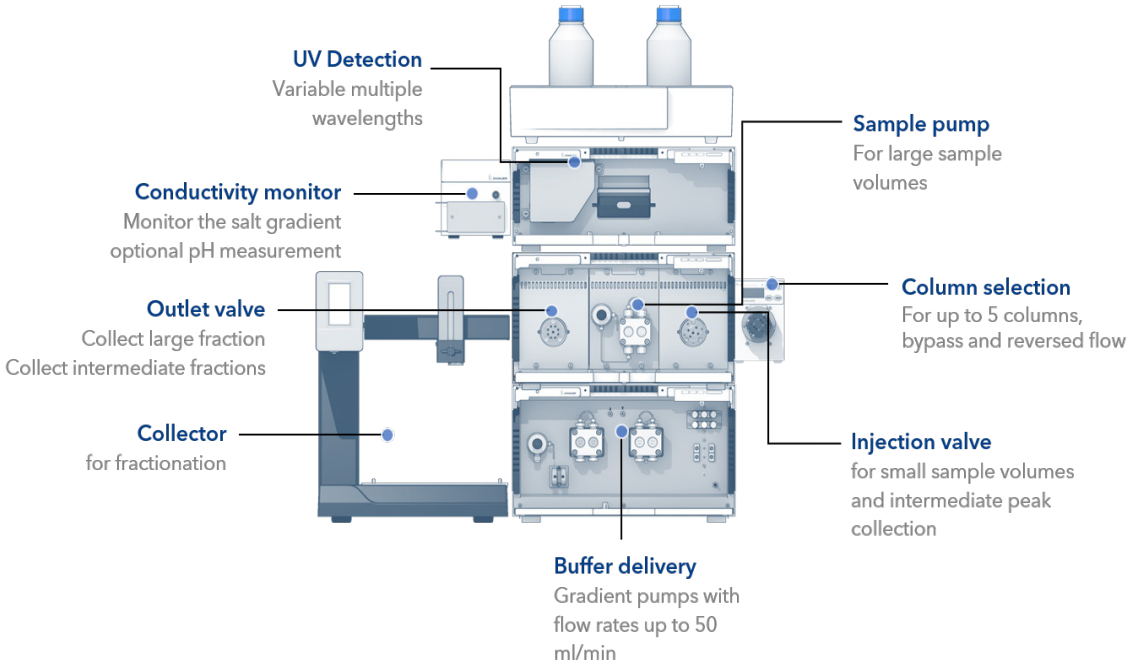

Fig. 5 AZURA Bio Lab system in set up with sample pump: exemplary configuration for automated two step applications.

The sample pump is used to apply the sample onto the column. The peak eluting from the first column is collected in the clean sample loop of the injection valve minimizing the risk of cross contamination during first peak collection. Therefore, the reinjection position of the outlet valve is connected to the syringe port of the injection valve **(Fig. 6)**. This set up allows the loading of large sample volumes. The injection of small sample volumes is not supported. You can find more details about this set up including the connection of the capillaries and information about PurityChrom plus method writing in section **Two step purification with the sample pump set up (VTN0015)**.

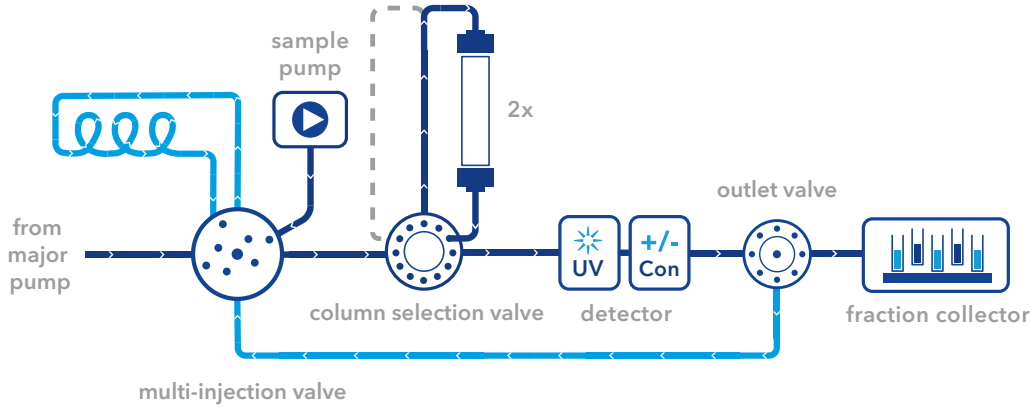

Fig. 6 Flow scheme for the sample pump set up for two step purification.

Tab. 2 Detailed overview of system set up

System		Basic	Sample pump	With loop valve
Injection via	Sample loop of injection valve	Major pump	Sample pump	Loop valve via injection valve
Collection of first peak	Sample loop of injection valve via sample pump inlet.		Sample loop of injection valve via syringe port	Loop valve via sample pump inlet of injection valve
Benefits	Free syringe port Easiest set up	Free syringe port Large sample volumes No extra sample pump needed	Large sample volumes Loading of sample via sample pump (minimized cross contamination)	Multipurpose (injection) Minimized risk of cross contamination
Limitations	Sample loop is used for injection and collection Volume of the first elution peak should be equal or smaller than the injection volume	Major pump used for injection	Syringe port is used for first peak collection in sample loop Small sample volumes not supported	Large delay volume Not optimal for small scale purification
System changes	Column selection and outlet valve			
		Change filter of pressure sensor Inline filter	Sample pump	Sample loop valve

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WHICH SET UP SHOULD I USE?

All set ups have their benefits and limitations. The easiest and cost-attractive set up is the basic set up. Only a few changes need to be included in the system. The drawback is that this set up is restricted to small sample volume and that the sample volume and the first elution peak should be in the same volume range. The limitation with the small sample volume can be overcome by using the major pump for sample injection. This bears the risk of cross contamination as the sample passes the mixing chamber. From our experience for most of our customers this was not a problem and did not affect their purification. Nevertheless, if you are frequently applying large sample volumes, we recommend using the sample pump set up. For better clarity we put together pros and cons of the different set up in **Tab. 2**.

ARE THERE OTHER OPTIONS POSSIBLE? CAN I DO MULTI STEP PURIFICATION?

Of course, additional set ups are possible. One option for example is to include an additional loop valve used for sample application and the collection of the peaks. With this set up multiple two step purifications could be started in a row without manual interaction or even multi step purifications are possible. Please keep in mind that this set up will increase the delay volume of your system and complexity of the application. The methods should be well established to ensure excellent purification results. If you have specific ideas, please contact our sales team to discuss your purification challenge.

TIPPS AND TRICKS FOR TWO STEP PURIFICATION

Know your method! For good results and a successful two step purification, the methods should be well established to ensure excellent purification results. You should know the elution volume/peak of the first purification to guarantee that the first peak is stored in an appropriate vessel. A vessel that is too small will result in sample loss as some parts of the sample is transported to the waste. A vessel too big will result in sample dilution, which can affect the second purification step. An option for medium up to high sample volumes is the use of variable sample loop. These variable sample loops can be emptied completely or partially as well as filled completely or partially. This allows you to work very flexible and easily switch between different sample sizes.

Use automatic sample loading! For sample loading by a pump, an air sensor can be used to detect the end of your sample. This protects the column from damage by running dry and supports more importantly automatic sample loading. Upon air detection, various functions can be programmed. In the case of automatic sample loading, once air is detected, the software will continue with column washing and elution.

Measure the pressure! Use pressure control that allows you to determine the pressure difference over your column bed. The first pressure sensor measures the pressure before the column, while the second sensor measures the pressure after the column. The software PurityChrom® automatically calculates the pressure difference over the media bed. If the pressure differential value (DP) exceeds the preset limit during the run, either the run pauses or another action is applied. Thus, you can protect your valuable columns and media from overpressure.

ADDITIONAL DEVICES

Tab. 3 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/Titanium, biocompatible, 10 µm	A3379-1
Sample pump in an ASM2.2L or stand alone	AZURA P4.1S Compact pump with 50 bar pressure sensor, 50 ml/min ceramic pump head for ASM 2.2L	DPG12FB
	Compact pump with 50 bar pressure sensor, 10 ml/min ceramic pump head for ASM 2.2L	DPG12EB
	Compact pump with pressure sensor and 50 ml/min ceramic pump head, stand alone	APG20FB
	Compact pump with pressure sensor and 10 ml/min ceramic pump head, stand alone	APG20EB
Column selection valve/ Loop 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
Air sensor	Air sensor for 1/8" tubing	A70093
Pressure control	Pressure control set for pressure difference determination	AZG10

RELATED KNAUER APPLICATIONS

VTN0014 - Two step purification with a basic set up

VTN0015 - Two step purification with the sample pump set up