Protein Purification with the Äkta Avant

This guide describes how to use the Äkta Avant 25, which is available for protein purification and located in the Protein Production and Purification Core Facilty (http://research.med.helsinki.fi/corefacilities/akta/index.html). This manual is a work in progress and if you find anything missing, please let us know. This manual does not go into theory at all, but only describes the steps needed to perform a two step purification. In this guide, three often used examples of affinity chromatography methods are described: Ni^{2+}NTA affinity chromatography, Protein A affinity chromatography and gel filtration (also called size exclusion chromatography; desalting = gel filtration with low resolution that only separates low molecular weight constituents like salts and amino acids from proteins, but doesn't separate proteins by their size). In our examples, we purify secreted proteins from conditioned cell culture supernatant. Because we have a rather large sample volume (400 ml), the sample is loaded directly to the column with the sample pump in the first (affinity chromatography) step. The second step is the size exclusion chromatography/gel filtration. In this step, the sample (the peak of choice from the elution of the first chromatography step) is loaded with a syringe into the capillary loop, from which it is injected into the gel filtration column.

1. Buffers needed

For Ni2+NTA affinity chromatography

· Ni2+NTA binding buffer: 30 mM phosphate buffer pH 8.0, 400 mM NaCl. How much? ≥250 ml for a 1-ml-column, ≥500 ml for a 5-ml-column

· Ni^{2+}NTA elution buffer: 30 mM phosphate buffer pH 8.0, 400 mM NaCl, 250 mM imidazole. How much? ≥100 ml for a 1-ml-column, ≥250 ml for a 5-ml-column

· column storage buffer: 20% ethanol

For Protein A affinity chromatography

· PBS-T (0.1% Tween-20). How much? ≥250 ml for a 1-ml-column, ≥500 ml for a 5-ml-column

· PAS elution buffer: 0.1 M citrate pH 3.0. How much? ≥100 ml for a 1-ml-column, ≥250 ml for a 5-ml-column

· column storage buffer: 20% ethanol

· neutralization buffer: 1M Tris/HCl, pH 8.5 How much? 5-10 ml.

For Gel Filtration chromatography

· Any buffer that is compatible with the protein you want to purify. PBS, TBS or HBS are commonly used buffers. How much? Depending on the column volume: For 10/100 columns (10/100 = 10 mm inner diameter, 100 mm column height): ≥100ml, for 10/300 columns: ≥250 ml, for 16/600 columns: ≥1 liter, for 26/600 columns: ≥2 liter

· column storage buffer: 20% ethanol

2. Columns needed

For Ni2+NTA affinity chromatography

Depending on the amount of protein that you want to purify:

HisTrap FF 1 ml (GE Healthcare, 17-5319-01) for up to 40 mg of protein; HisTrap FF 5 ml (GE Healthcare, 17-5255-01) for more than 40 mg. Alternative columns for special cases are: HisTrap FF Crude (for purification from cell lysate), HisTrap HP (higher binding capacity at same column dimensions), HisTrap Excel (chemically more stable and higher affinity for his-tagged proteins, but only about 25% binding capacity)

For Protein A affinity chromatography

Depending on the amount of protein that you want to purify:

HiTrap rProtein A FF 1 ml (GE Healthcare, 17-5079-01) for up to 40 mg of human IgG_1; HiTrap rProtein A FF 5 ml(GE Healthcare, 17-5080-01) for more than 40 mg of human IgG_1

For Gel filtration/Desalting

Depending on the volume of protein that you want to purify/desalt, you need differently sized columns:

Column	***max. sample volume***
10/300 Superdex 200 (Increase)	1 ml
HighLoad 16/600* or 16/60*	2 ml
HighLoad 26/600* or 26/60*	5 ml
FastDesalting HR 10/10*	1 ml

3. Reservation, logging in and starting the program

3.1 Do not forget to reserve the machine at http://jeltsch.akta.org/ → 'Online reservation system'. You need to log in with your University login/password in order to make a reservation. Please leave your phone number in case we need to contact you.

3.2 Switch on the Äkta Avant and the computer if they are not running already. It does not matter in which order you switch them on. The Äkta ON/OFF switch is on the left side of the machine, near the bottom edge:

3.3 You need to login twice: First into the computer and then into the UNICORN program. To login into the computer, you may use either your own university account or if more than one person need to control the FPLC device, you can login in using a local account (user: Unicorn, pw: see the note attached to the computer). Then start the UNICORN program via the desktop icon. To login to the program, you need to use your own university account. In the login window, make sure that the Adminstrator box is not checked:

3.4 The machine should now already connect to the computer. If this doesn't happen automatically (usually the first time you log in) you can connect manually by clicking 'system' → 'Connect to systems' from the Unicorn program.

*A few years back, GE Healthcare changed the way how to designate column sizes. The old style uses a mm/cm designation (e.g. a 10/30 column has an inner diameter of 10 mm and a length of 30 cm). The new style uses mm/mm (e.g. the old 10/30 column would be nowadays a 10/300 column). Hence the (old) HighLoad 16/60 Superdex 200 pg and (new) HighLoad 16/600 Superdex 200 pg have identical dimensions, as well as the (old) HighLoad 26/60 Superdex 200 pg and the (new) HighLoad 26/600 Superdex 200 pg.

4A. Preparation of the system for a run (Affinity chromatography)

4A.1 Rinse the inlet tubes by dipping them in clean PBS/H_2O before placing them in your buffer bottles. Put your buffers in their appropriate places and inlet tubes in the right buffer bottles.

	Ni2+NTA	***Protein A***
A1:	Ni2+NTA binding buffer	PBS-T
B1:	Ni2+NTA elution buffer	0.1 M citrate pH 3.0
B3:	column storage buffer	column storage buffer
Buff:	Ni2+NTA binding buffer	PBS-T
S4:	sample	sample

4A.2 Place the sample bottle into an ice-filled styrofoam box on top of the Äkta. You can additionally add 0.1% NaN_3 to the sample in order to prevent microbial growth.

4A.3 Place a bottle into the fridge to collect your flowthrough, connect tubing Out5 to the bottle.

4A.4 Empty the 2-liter waste bottle if needed.

4B. Preparation of the system for a run (Gel filtration)

4B.1 Rinse the inlet tubes (e.g. by dipping them in clean H_2O) before placing them in your buffer bottles. Put your buffers in their appropriate places and inlet tubes in the right buffer bottles.

A1:	Running buffer (PBS, TBS or HBS)
B3:	20% ethanol

4A.2 Empty the 2-liter waste bottle if needed.

5. Priming/purging the pumps

5.1 If the inlet tubings A1, B1, B3 and Buff (for affinity chromatography) and A1 and B3 (for gel filtration) are NOT filled with buffer, you MUST fille them! Otherwise the pump will run dry and break! To do this you attach a syringe to all 6 pump purge valves and draw about 20 ml buffer.

In order to fill inlet tubing B3 and S4, you need to switch from B1 to B3 and from Buf to S3, respectively, using the UNICORN software. For B3: Manual instructions → Flow path → Pump B inlet: choose inlet B3. For S4: Manual instructions → Flow path → Sample inlet: choose inlet S4.

5.2 Inlet tubings A1, B1 and Buff are the default positions of the valves and can be filled without changing valve positions with the UNICORN software. The sample you draw into your syringe while filling tube S4 can be returned into the sample bottle.

6. Attaching the column

6.1 Start a flow (1 to 2 ml/min) with the same buffer that has been used for column storage (mostly 20% EtOH):

Pumps and pressure -> System Flow; select for "Pressure control" D column pressure; Execute. You will be prompted to select the correct column from a list. Once you have selected the correct column, the instructions are in the execution window and you still

have to click "Execute"! Let the run proceed until all curves are running (nearly) horizontal.

Then switch the flow path to the column position that you want to use (see the table below for the column default position for the method you are going to use): Flow Path -> Column position -> Position 1 (or whatever default position your mehthod is using, in the following, we'll use position 1 as an example). Buffer will start to flow out from posiiton 1A of valve V9-C. Let the outflow continue for a few ml in order to clean the valve. Then connect the top of the column to position 1A. Before attaching the new column, fill the air-filled space at the top of the column by dripping buffer on it to remove the air that might be trapped inside. Once you have attached the column, the system will now go into overpressure and pause if you have have not opened the bottom of the column. That is totally fine, nothing to worry about. Just open the bottom of the column and connect it to position 1B of the same valve (V9-C).

7. Calibrate the pH monitor (optional)

Calibrating the pH monitor is not necessary if you just want to monitor the pH, but don't need absolute pH values. We do it about once a month, but this means that it may be off by 0.1 to 0.4 pH units if you leave it uncalibrated.

8. Preparing the fraction collector

Fractions can be collected into many different tube types. The fraction collector holds six cassettes and we have casette types available for the following tubes/plates:

- 4 cassettes for 96-deep-well plates
- 2 cassettes for 5-ml-tubes (40 tubes/casette)
- 2 cassettes for 15-ml-tubes (15 tubes/casette)
- 2 cassettes for 50-ml-tubes (6 tubes/cassette)

For the Ni2+NTA affinity chromatography, we are using 15 ml Falcon tubes and for the Protein A affinity chromatography, we use Nunc 5 ml 75x12 Minisorp tubes. For the gel filtration, we use 96-deep-well plates with 1-ml-wells or 2-ml-wells. For a typical affinity purification you should place at least 10 tubes into the cassettes starting from position A1. For the Protein A affinity chromatography, you need to pipette 200 µl 1M Tris pH 8.5 into each fraction tube, because you need to neutralize the low pH of the elution buffer as soon as possible after the elution in order to prevent damaging your protein. For the gel filtration, place one or two (depending on your fraction size) 96-deep-well blocks into the 96-well-plate cassette. Always change the casettes only when the system is not running. (When the system is not running, it does a "full scan" and recognizes all cassettes. You can open the fraction collector also during a run, but you cannot swap different types of cassettes as the system will not recgnize the new order of the cassettes, this is called a "quick scan".)

9. Methods

A description of method creation is coming in a later version of this manual. For routine purifications (including Ni^{2+}NTA, Protein A affinity and gel filtration chromatography), there are ready methods available in the Unicorn software, which can be used:

Default methods:

method name	*chromatography*	*column*	*column default*
Ni2+NTA_HisTrap_1ml_default	Ni2+NTA affinity	1-ml-HisTrap or similar	position 5

Ni2+NTA_HisTrap_5ml_default	Ni2+NTA affinity	5-ml-HisTrap or similar	position 5
HiTrapPAS_1ml_default	Protein A affinity	1-ml-PAS or similar	position 5
HiTrapPAS_5ml_default	Protein A affinity	1-ml-PAS or similar	position 5
SEC200Increase_default	Gel filtration	10/300 Superdex 200 Increase with 0.5ml capillary loop	position 1
SEC200_16/60_default	Gel filtration	16/60 Superdex 200 with 2 or 2.5ml capillary loop	position 2
SEC200_26/60_default	Gel filtration	26/60 Superdex 200 with 2.5 or 5ml capillary loop	position 4
FastDesalting_default	Desalting/ buffer exchange	HR10/10 Sephadex 25 with 0.5ml capillary loop	position 1

10. Sample loading into the capillary loop (only for step 2: gel filtration)

For the gel filtration and desalting runs, you need to manually load the capillary loop with your sample before you start the run. Depending on your sample volume, you need to choose a volume-matched capillary loop. If you want to maximize your recovery, always use a capillary loop that is at least 30% larger than the volume of your sample. However, if peak separation is your goal, use a very small sample volume, a very small capillary loop and overfill it (e.g. fill a 0.5-ml-loop with 0.8 ml sample and resolve it on the HiLoad 16/600 Superdex pg).

10.1 Attach the correct capillary loop to positions LoopF and LoopE. Wash the loop by connecting a syringe to position Syr and pushing about 10 loop volumes running buffer through the loop.

10.2 Load your sample into a syringe (e.g. a 1-ml-syringe) avoiding/removing any air bubbles and insert the syringe to position Syr

10.3 Push the syringe content VERY SLOWLY into the sample loop. Excess sample exits at position W1 and you may recover it.

11. Starting the method run

11.1 Click the “folder” in the top left corner of the program window and choose a ready method.

11.2 When the program asks for the column information use the bar code scanner to scan the bar code on the column or find it on the list.

11.3 Double check that all tubes are in correct buffers.

11.4 Start the run by clicking the 'play' button.

11.5 After the run has finnished, you can remove your column. The default methods equilibrate the column in the end to 20% ethanol for storage. Even though it is sold as a single-use device, you can reuse it a couple of times. However, if you use it for a different protein, you need to do rigorous cleaning in between. Deattach the column from its tubings and firmly screw stop plugs to both ends to prevent the column from drying out. Store the column at +4°C.

12. Collecting, pooling and concentrating fractions from the affinity chromatography (first step)

After the run has finnished, you can look up from the evaluation module, in which fraction(s)