Sample preparation and columns run-working practics

Fig 1. ÄKTA avant is a preparative chromatography system designed for process development, method optimization, and scale-up.

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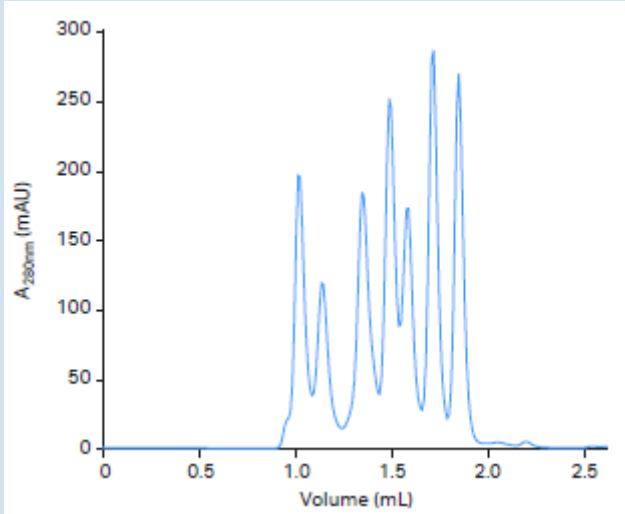
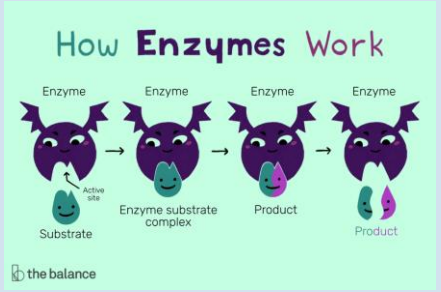
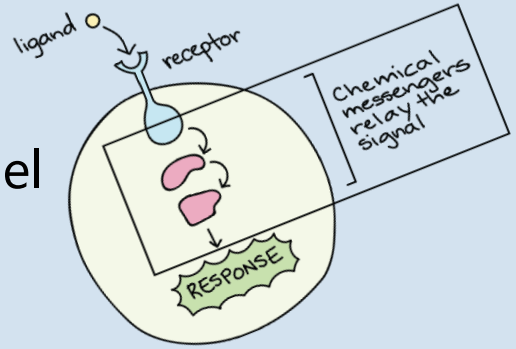


Classes of proteins to be purified by chromatography:

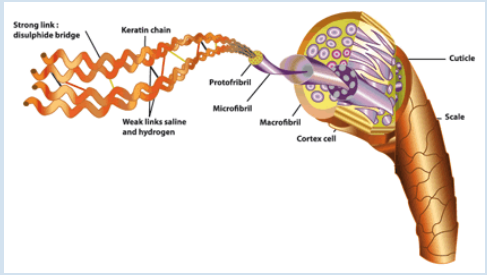
Antibodies. Modulate immune system



Enzymes. Lock-and-key mechanism with substrate



Receptors. Transfer a signal or membrane channel



Structure of cells and organisms. (kerain, fibrin, actin/myosin)

Hormones. Transfer remote signal (insulin, FSH, HGH)

***Other biomolecules can be separated as well: Viral particles, DNA, RNA, Plasmids, exosomes and more**

What are they purified for ?

- **Drugs & Vaccines**

  - 8 out of the 10 top-selling drugs globally are proteins, 7 are antibodies, used mainly as cancer immunotherapy but also for psoriasis and as a vaccines. Among the known ones – Avastin and Rituxan

- **Research in companies and academia**

  - Use proteins to understand their mechanism of action
  - Understand mechanisms of action of diseases

- **Diagnostic**

  - Hospitals use enzymes to detect metabolites (like cholesterol) to identify disorders and malignancies

Getting started
which column should I use for purifying my sample?

- Size exclusion SEC–when my material differs from most other sample materials in size
- Affinity-when my material has a unique characteristic over the utilized; e.g. tag, specific group
- Ion exchange- when the pI of my protein is known and differs from most other materials in the sample
- HIC – When non of the above works…
- Reverse Phase- when my material can withstand organic solvents (rare)

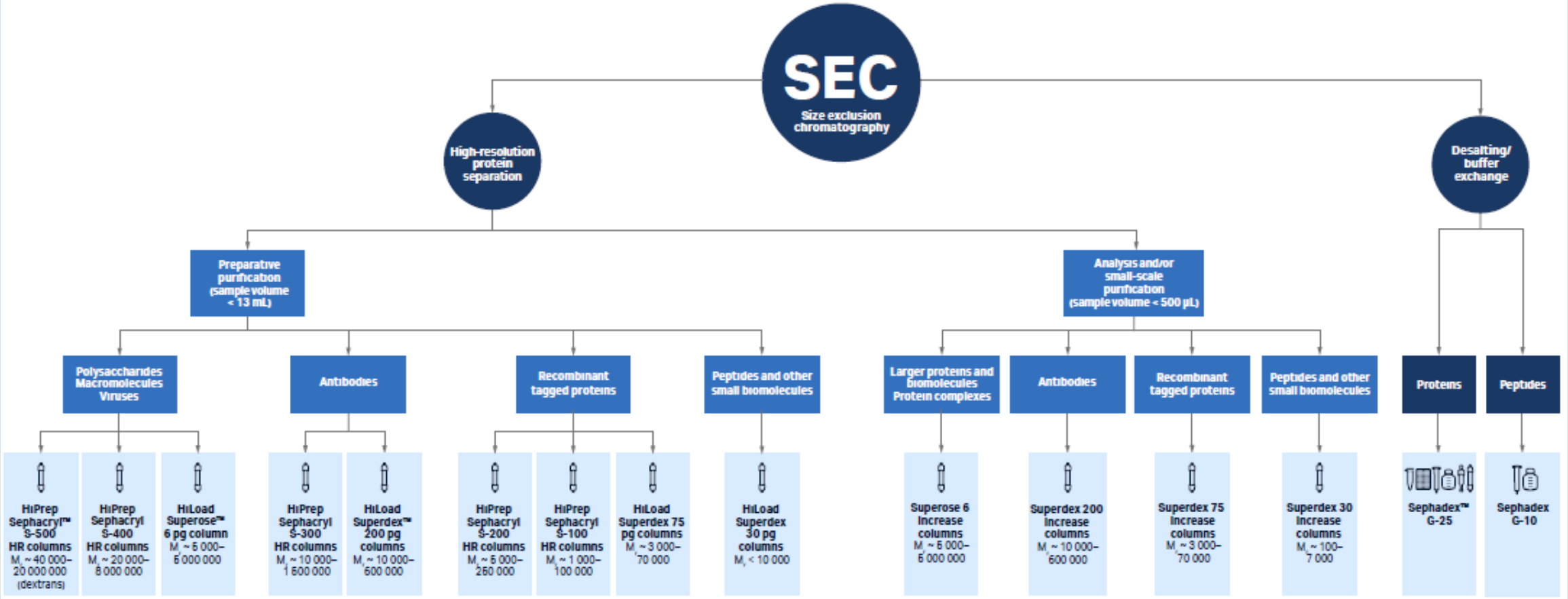
General guidance for all chromatography columns

- Always filter your material before loading to column- 0.2 micron, or centrifuge (take only supernatant)
- Keep air bubbles away from column- make sure you know how to connect the column to the chromatography system (AKTA)
- Keep your column clean- perform CIP (cleaning in place after each column use)
- Apply appropriate flow rate to each column- usually given in the column instructions
- Choose the most efficient column for your needs- column type, column size

Size exclusion chromatography

Guide to chromatography resins

Click on the icons to be directed to the corresponding web page.



SEC

- Sample preparation- sample should be 0.2 micron filtered or centrifugated (take supernatant) before loaded onto column
- Sample volume – max. 5% of column volume
- Flow rate- if resolution is poor, lower flow rate. If resolution is good, flow rate can be increased as long the max. pressure is not exceeded
- To verify molecule size, a size calibration curve can be created- use standard size markers which are in the resolution range for your specific column
- Make sure pressure is normal. High pressure in SEC column usually indicates clogged filters- replace filters every ~30 column uses.
- Track column performance to know when it has ended its life…(columns are not immortal)

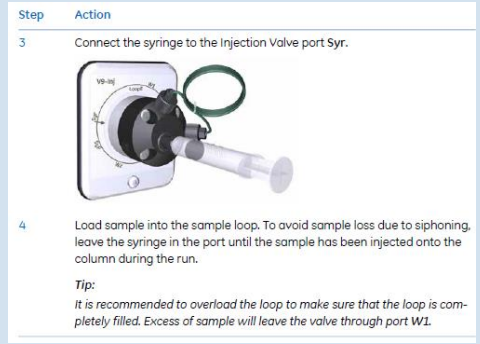
Typical SEC column run parameters :

- **Column- Superdex™ 200 Increase 10/300 GL** 
- **Column volume 24 ml, sample volume range 25 µl – 500 µl; lower sample volume=higher resolution**
- **Flow rate- 0.3-0.7 ml/min. depending on :**
  1. **Temperature- low temp. (minimum 4^0 C) results in higher pressure, hence lower flow rate is required**
  2. **Running solution viscosity, e.g. 20% EtOH has higher viscosity than water hence requires lower flow rate**
  3. **Required resolution- normally, lower flow rates result in slightly better resolution**
- **Material concentration – between *0.1mg/ml and 50mg/ml; lower concentration= higher resolution. *below this concentration, material may not be detected by UV**
- **Normal running pressure – given in the column instructions; between 15 bar and 30 bar**

Always read column instructions before starting work !

Typical SEC column run protocol :

- **Wash column with 1 CV (column volume) of DDW**
- **Column Equilibrate with 1 CV of running buffer, or until the following readings are stable in the graph:**
  1. **Stable conductivity reading**
  2. **Stable pH reading**
  3. **Stable UV reading**
  4. **Stable pressure reading**
- **Connect a loop to the AKTA- The loop volume should be a bit smaller than your sample volume so that you fill the loop with excess volume**
- **First wash the loop with sample buffer to make sure it's clean**
- **Load your sample into the injection loop, don't inject to the column yet !**
- **Once all run parameters are stable inject the sample, empty loop with at least 1.5 loop volumes**
- **To collect the eluted materials automatically, you should have a running method, or collect manually by instructing the AKTA when to collect each peak**



Step	Action
3	Connect the syringe to the Injection Valve port **Syr**.
4	Load sample into the sample loop. To avoid sample loss due to siphoning, leave the syringe in the port until the sample has been injected onto the column during the run. *Tip:* *It is recommended to overload the loop to make sure that the loop is completely filled. Excess of sample will leave the valve through port **W1**.*

Typical SEC column chromatogram:

Column Superdex 200 Increase 10/300 GL

Check the performance of the column using the following procedure:

Sample:	100 µL 2% acetone (20 mg/mL) in buffer or water
Eluent:	Buffer or water
Flow rate:	1.0 mL/min, room temperature
Detection:	280 nm

Function test

As an alternative to the above efficiency test, check the column performance by running a function test.

Column Superdex 200 Increase 5/150 GL

Sample:
1. Thyroglobulin (M_r 669 000) 3 mg/mL
2. Aldolase (M_r 158 000) 3 mg/mL
3. Conalbumin (M_r 75 000) 3 mg/mL
4. Carbonic anhydrase (M_r 29 000) 3 mg/mL
5. Ribonuclease A (M_r 13 700) 3 mg/mL

Sample volume:	10 µL
Eluent:	0.01 M phosphate buffer, 0.14 M NaCl, pH 7.4
Flow rate:	0.45 mL/min, room temperature
Detection:	280 nm

Result is shown in Fig. 3.

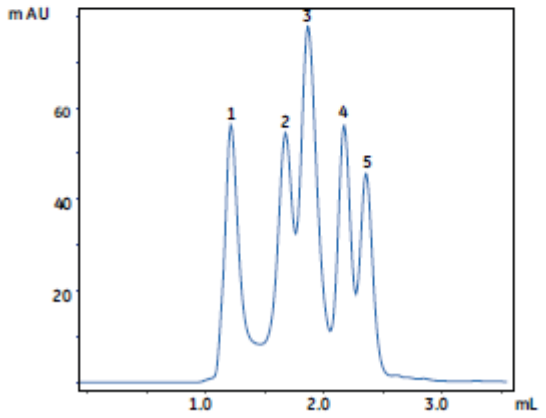
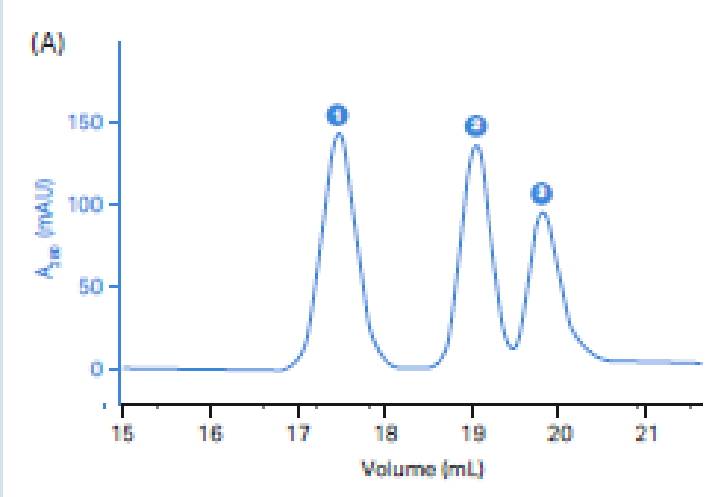
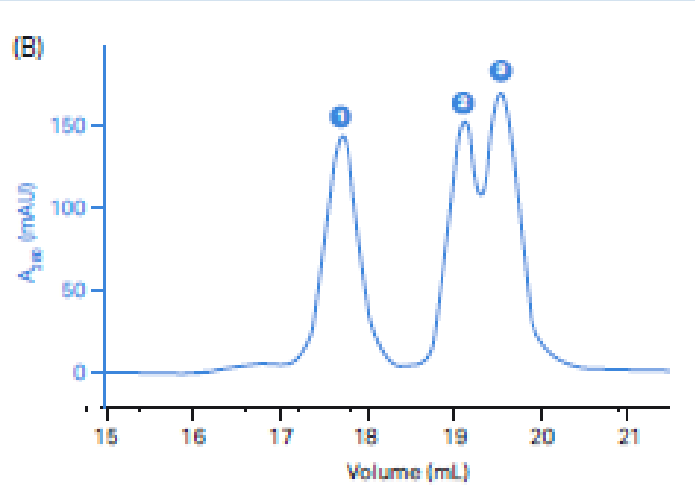


Fig 3. Typical chromatogram from a function test of Superdex 200 Increase 5/150 GL using ÄKTAmicro.



Good resolution



Poor resolution

Note that interactions in some cases may be used to improve resolution.

Some proteins can precipitate in low ionic strength solutions.

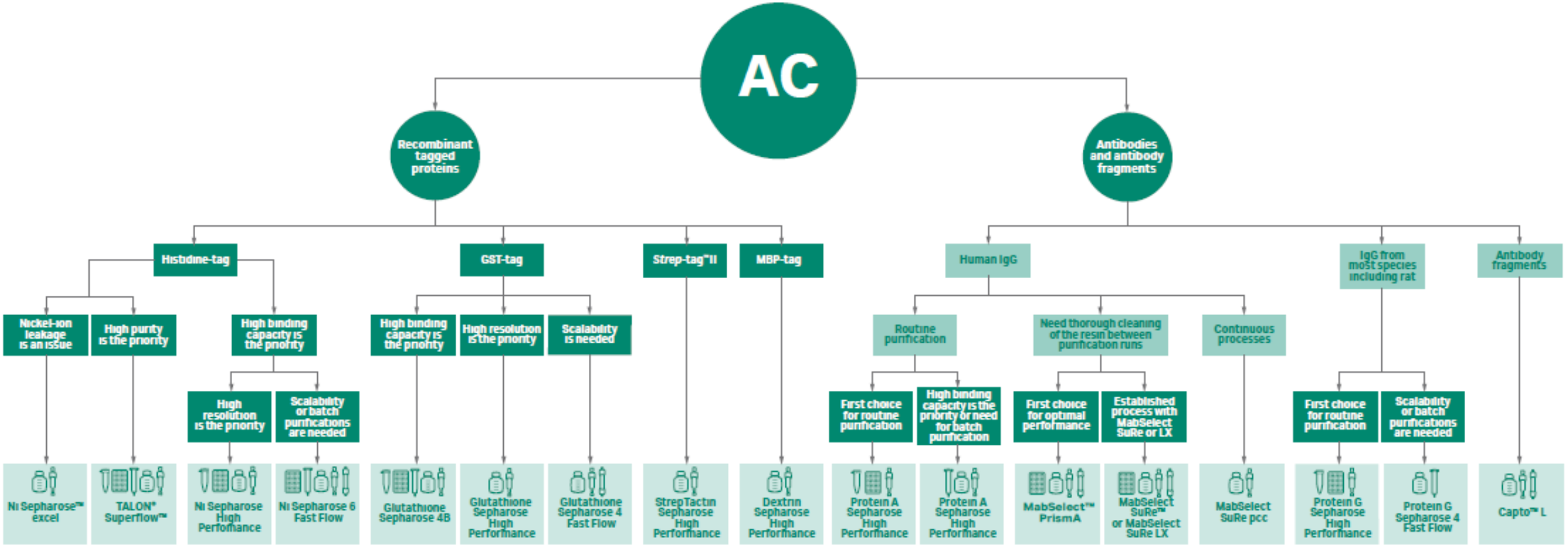
Avoid using unnecessarily high salt concentrations as this might increase hydrophobic interaction.

Addition of organic solvent can be beneficial for hydrophobic substances (Fig 2.11).

Affinity chromatography

Guide to chromatography resins

Click on the icons to be directed to the corresponding web page.



Affinity

- **Most popular affinity columns:**

HisTrap™-for Histidibe tagged proteins. Contains Nickel ions
For other tagged proteins- GST, Streptavidin, MBP use the appropriate column
MabSelect™ - for purifying monoclonal antibodies
Other affinity resins

- **Make sure you choose the right column in terms of column volume; affinity column has a specific capacity (and limited) with respect to the type of molecules it can bind, e.g. 40mg of His tagged protein per 1 ml of HisTrap™ column**

- **Two types of chromatography columns are usually available :**
  1. **FF- Fast Flow, normal size chromatography beads, ~90 micron, for regular resolution**
  2. **HP- High Performance, 40 micron beads for higher resolution. Works at lower flow rates**

- **Flow rate- too high flow rate in binding stage may cause part of the sample will not bind to the column. Binding time is minimum 2 min.**

- **Make sure you sanitize (clean) the column after the use**

Affinity columns (partial list)

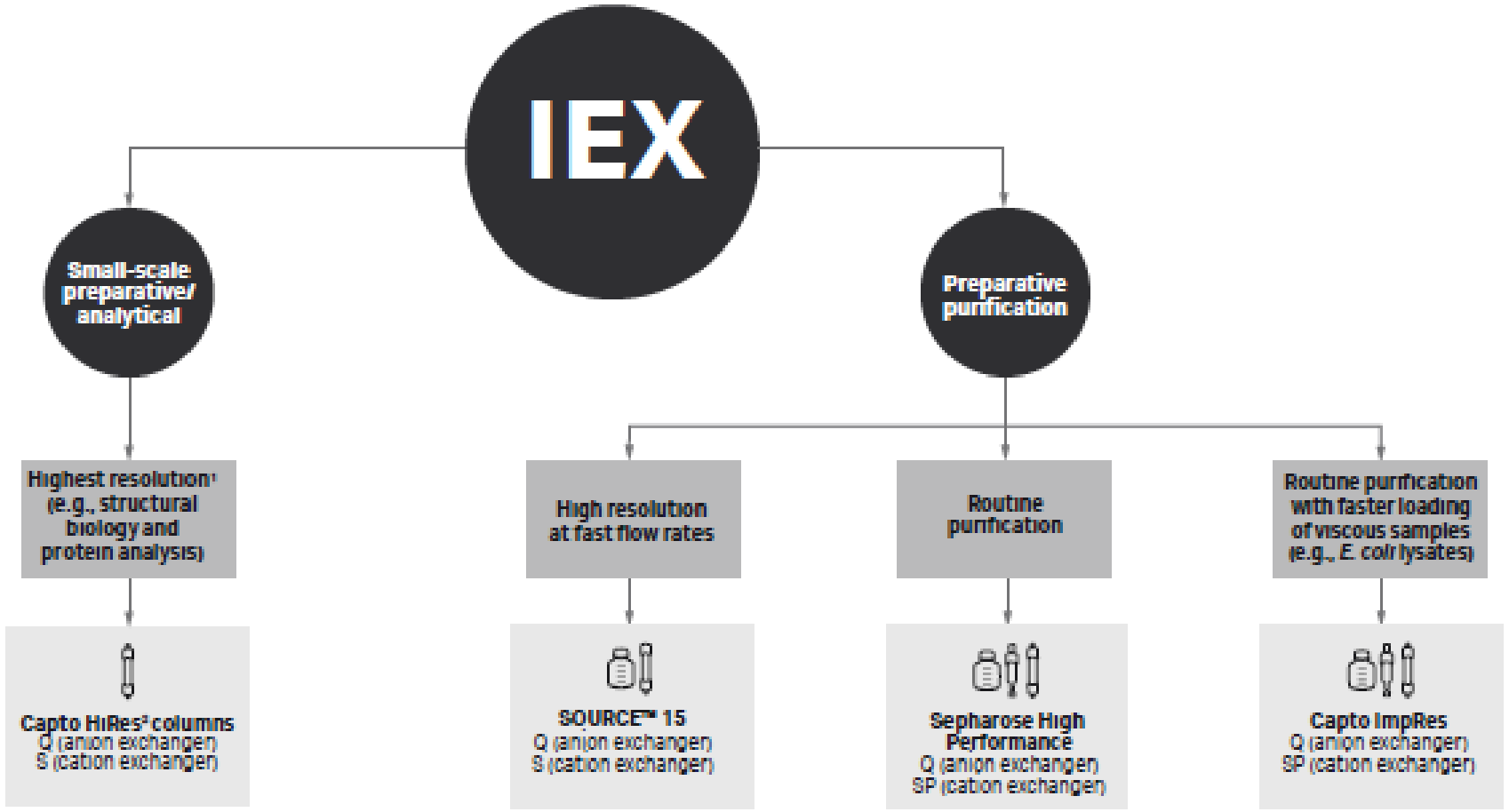
Prepacked columns and media for isolation and purification of immunoglobulins

Ordering information	Product		Binding capacity per ml chromatography medium (approx.)	Average particle diameter µm	Maximum operating flow rate[1]	Maximum operating pressure	pH stability[2]		Application areas
Code No.	Prepacked columns	Column size					Long term	Short term	
29-0485-76	HiTrap Protein A HP	1 × 1 ml	20 mg human IgG	34	4 ml/min	0.5 MPa, 5 bar	3–9	2*–10	Isolation and purification of classes, subclasses and fragments of IgG from many different species.
17-0402-01		5 × 1 ml			4 ml/min				
17-0402-03		2 × 1 ml			4 ml/min				
17-0403-01		1 × 5 ml			20 ml/min				
17-0403-03		5 × 5 ml			20 ml/min				
29-0485-81	HiTrap Protein G HP	1 × 1 ml	25 mg human IgG	34	4 ml/min	0.5 MPa, 5 bar	3–9	2*–10	Protein G and protein A have different IgG binding specificities, dependent on the origin of the IgG. Binds to all IgG subclasses from human, mouse, and rat; binds total IgG from guinea pig, goat, cow, sheep, and horse. Unlike protein A, protein G binds human IgG3. Applications of protein G include practically all applications of protein A.
17-0404-01		5 × 1 ml			4 ml/min				
17-0404-03		2 × 1 ml			4 ml/min				
17-0405-01		1 × 5 ml			20 ml/min				
17-0405-03		5 × 5 ml			20 ml/min				
29-0486-65	HiTrap Protein L	1 × 1 ml	Approx. 25 mg human Fab	85	4 ml/min	0.5 MPa, 5 bar	2–10	15 mM NaOH	Purification of antibodies and antibody fragments such as Fab fragments, scFv, and Dabs containing kappa light chains.
17-5478-51		5 × 1 ml			4 ml/min				
17-5478-15		1 × 5 ml			20 ml/min				
17-5478-55		5 × 5 ml			20 ml/min				
17-5478-14	HiScreen Capto L	1 × 4.7 ml	Approx. 25 mg human Fab	85	3.9 ml/min	0.3 MPa, 3 bar	2–10	15 mM NaOH	Optimization of chromatography conditions in process development
17-5079-01	HiTrap rProtein A FF	5 × 1 ml	50 mg human IgG	90	4 ml/min	0.5 MPa, 5 bar	3–10	2*–11	Recombinant protein A exhibits similar Fc region specificity to that of native protein A but shows enhanced binding capacity.
17-5079-02		2 × 1 ml			4 ml/min				
28-9464-89		100 × 1 ml*			4 ml/min				
17-5080-01		1 × 5 ml			20 ml/min				
17-5080-02		5 × 5 ml			20 ml/min				
28-4082-53	HiTrap MabSelect™	5 × 1 ml	min 30 mg human IgG	85	4 ml/min	0.5 MPa, 5 bar	3–10	2*–12	For high-throughput capture of monoclonal antibodies.
28-4082-55		1 × 5 ml			20 ml/min				
28-4082-56		5 × 5 ml			20 ml/min				
29-0491-04	HiTrap MabSelect SuRe™	1 × 1 ml	min 30 mg human IgG	85	4 ml/min	0.5 MPa, 5 bar	3–12	2*–14	Designed to tolerate harsh cleaning-in-place protocols.
11-0034-93		1 × 5 ml			4 ml/min				
11-0034-94		5 × 1 ml			20 ml/min				
11-0034-95		5 × 5 ml			20 ml/min				
28-4082-58	HiTrap MabSelect Xtra™	1 × 5 ml	Approx. 40 mg human IgG	75	4 ml/min	0.5 MPa, 5 bar	3–10	2–12	For capture of high-titer monoclonal antibody feedstreams.
28-4082-60		5 × 1 ml			20 ml/min				
28-4082-61		5 × 5 ml			20 ml/min				
17-5110-01	HiTrap IgM Purification HP	5 × 1 ml	5 mg human IgM	34	4 ml/min	0.5 MPa, 5 bar	3–11	2*–13	Purification of monoclonal IgM from hybridoma cell culture and human IgM.
17-5115-01	HiTrap IgY Purification HP	1 × 5 ml	20 mg pure IgY/ml medium or 1/4 egg yolk/5 ml medium	34	20 ml/min	0.5 MPa, 5 bar	3–11	2*–13	Purification of IgY from egg yolk.
28-9269-73	HiScreen MabSelect	1 × 4.7 ml	min 30 mg human IgG	85	3.9 ml/min	0.3 MPa, 3 bar	3–10	2*–12	Optimization of chromatography conditions in process development
28-9269-76	HiScreen MabSelect Xtra	1 × 4.7 ml	Approx. 40 mg human IgG	75	2.3 ml/min	0.3 MPa, 3 bar	3–10	2*–12	Optimization of chromatography conditions in process development
28-9269-77	HiScreen MabSelect SuRe	1 × 4.7 ml	min 30 mg human IgG	85	3.9 ml/min	0.3 MPa, 3 bar	3–12	2*–14	Optimization of chromatography conditions in process development
17-5474-15	HiScreen MabSelect SuRe LX	1 × 4.7 ml	Approx. 60 mg human IgG	85	3.9 ml/min	0.3 MPa, 3 bar	3–12	2*–14	Optimized for high binding capacity at long residence time.

Ion exchange chromatography

Guide to chromatography resins

Click on the icons to be directed to the corresponding web page.



Ion exchange – Cation or Anion

- **Most popular ion exchange columns:**

Q- Quaternary amine – strong anion exchanger
DEAE- Diethyl amino Ethyl- weak anion exchnager
SP –Sulfopropyl –strong cation exchanger
S-Sulfonyl – strong cation exchanager
CM- Carboxy methyl – weak cation exchanger

- **Make sure you choose the right column in terms of column volume; ion exchange column has a specific capacity (and limited) with respect to the type of molecules it can bind, e.g. 70mg of anionic protein per 1 ml of Q Sepharose™ column**

- **Two types of chromatography columns are usually available :**
  1. **FF- Fast Flow, normal size chromatography beads, ~90 micron, for regular resolution**
  2. **HP- High Performance or ImpRes (improved resolution), 40 micron beads for higher resolution. Works at lower flow rates**

- **Flow rate- too high flow rate in binding stage may cause part of the sample will not bind to the column. Binding time is minimum 2 min.**

- **Make sure your protein is in the right buffer when loaded onto the column; pH below the pI for cation exchanger, above the pI for anion exchanger**

Ion exchange – Cation or Anion (continued)

- **Typical run protocol for an anion exchanger:**

  1. **Wash out the 20% ethanol from the column with 2CV DDW**
  2. **Equilibrate the column until a stable conductivity, pH, and UV reading are obtained, about 2 to 5 CV's, depending on the equilibration buffer salt concentration**
  3. **Load your sample at a maximum flow rate which is 2 min. residence time on column**
  4. **Wash with 2-5 CV of binding buffer. This is to wash out proteins which bind non-specific**
  5. **Elute your protein: either by Increasing salt concentration gradually, e.g. from 20mM NaCl to 500 mM NaCl throughout 20 CV's, or by one step of high NaCl concentration- this will result in less resolution compared with gradient elution**

- **Make sure you sanitize (clean) the column after the use**

Your chromatography system

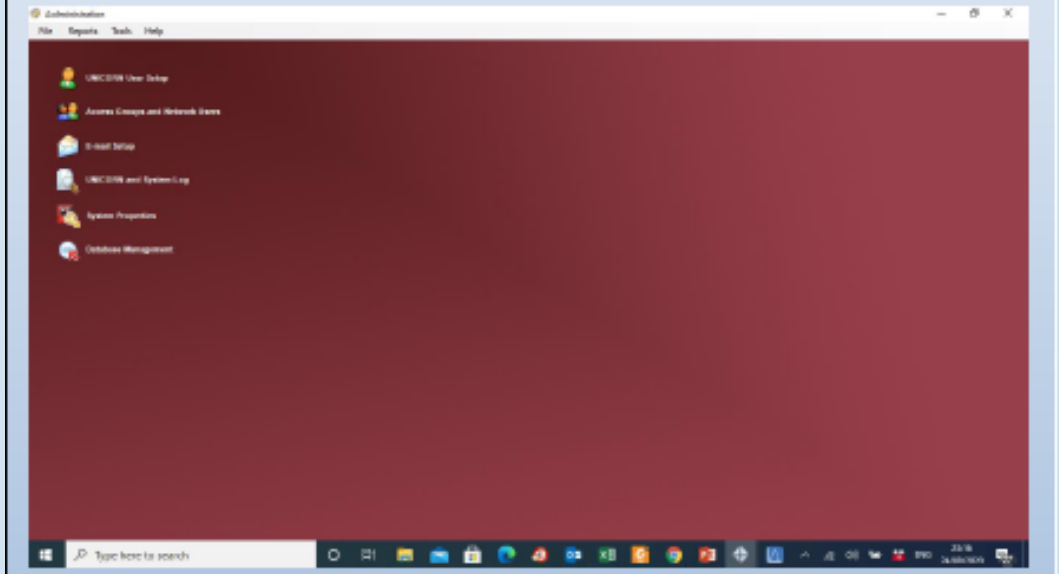
ÄKTA™ avant

Unicorn™ control software

Unicorn™ has 4 screens:

1. Administration
2. Method editor
3. Evaluation
4. System control

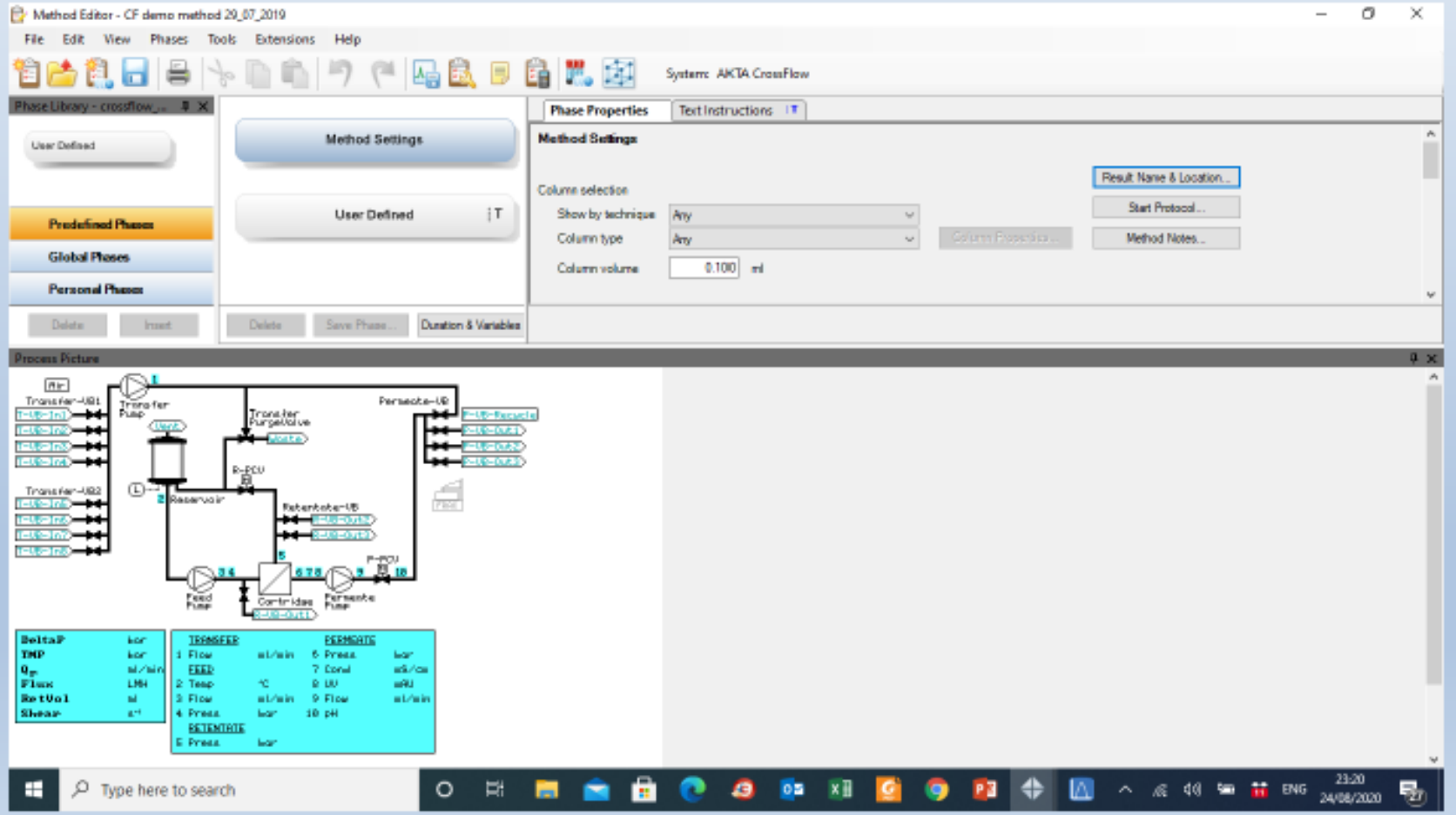
1.Unicorn™ Administration screen



Area	Concerns
User administration	User properties and authorization of access to the system, see *Section 3.2 UNICORN User setup, on page 127* and *Section 3.3 Access groups and network users, on page 142*. **Note:** *It is recommeded to have one responsible person, or a small group, at least in larger installations.*
System administration	• Maintenance of software aspects of UNICORN, including - definition of connected systems, see *Section 3.1.1 System properties, on page 102*. - monitoring of system usage (logs), see *Section 3.1.4 UNICORN and System logs, on page 121*.
Database administration	Set up and maintenance of one or many instances of the UNICORN database, see *Chapter 4 Database management, on page 163*.
Network administration	Setup of the network functions relevant to UNICORN, see *Section 2.4 Network installation and configuration, on page 53*. **Note:** *In a network installation, this is normally carried out by the IT staff responsible for the company's network.*
E-mail Setup	Setup of administrator e-mail accounts for sending and recieving messages. See *Section 3.5 E-mail Setup, on page 160*.

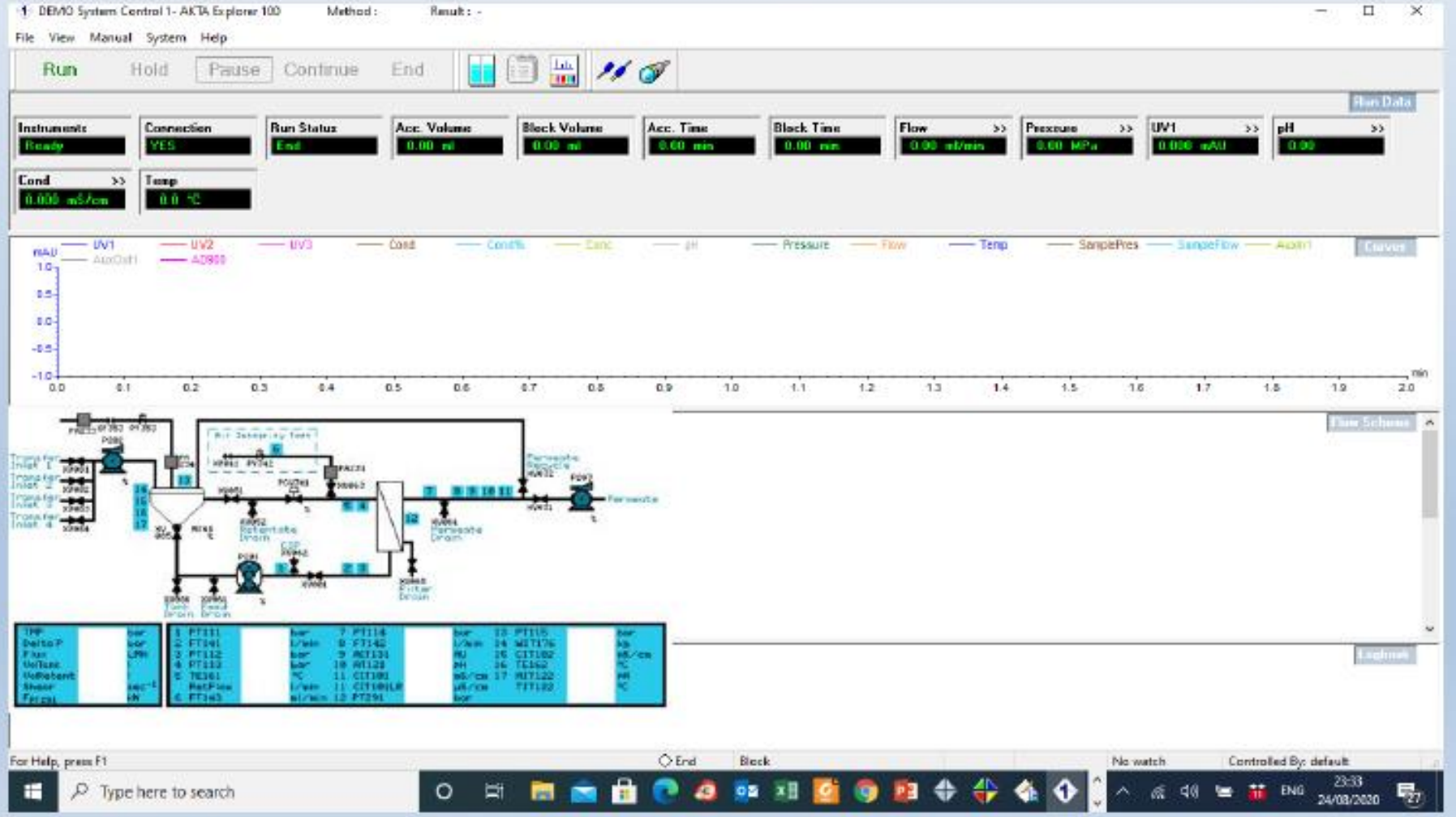
2.Unicorn™ Method Editor

Write and edit methods in this screen



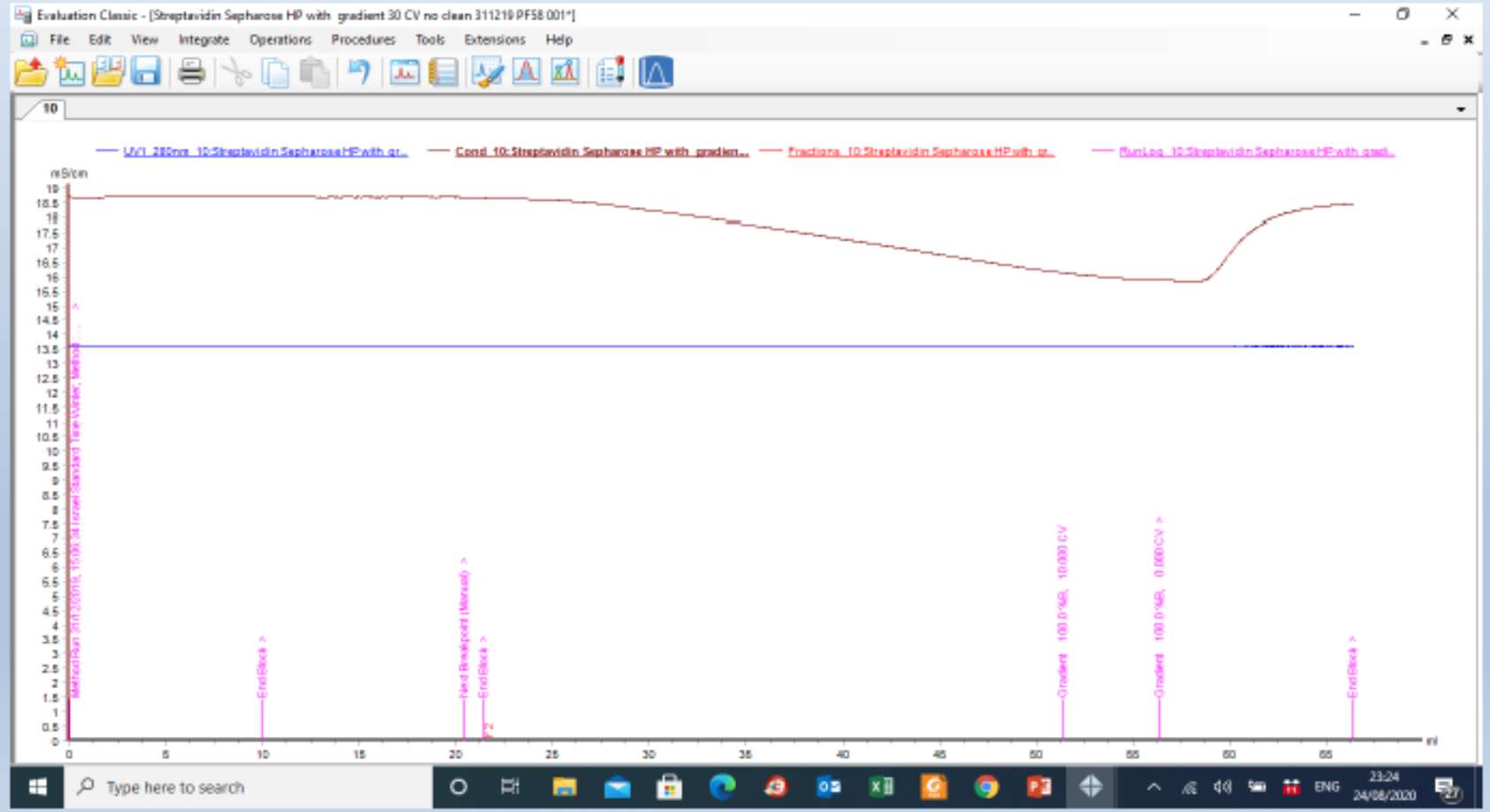
4. Unicorn™ system control screen

Operate the system manually or with a method by this screen. View online run parameters



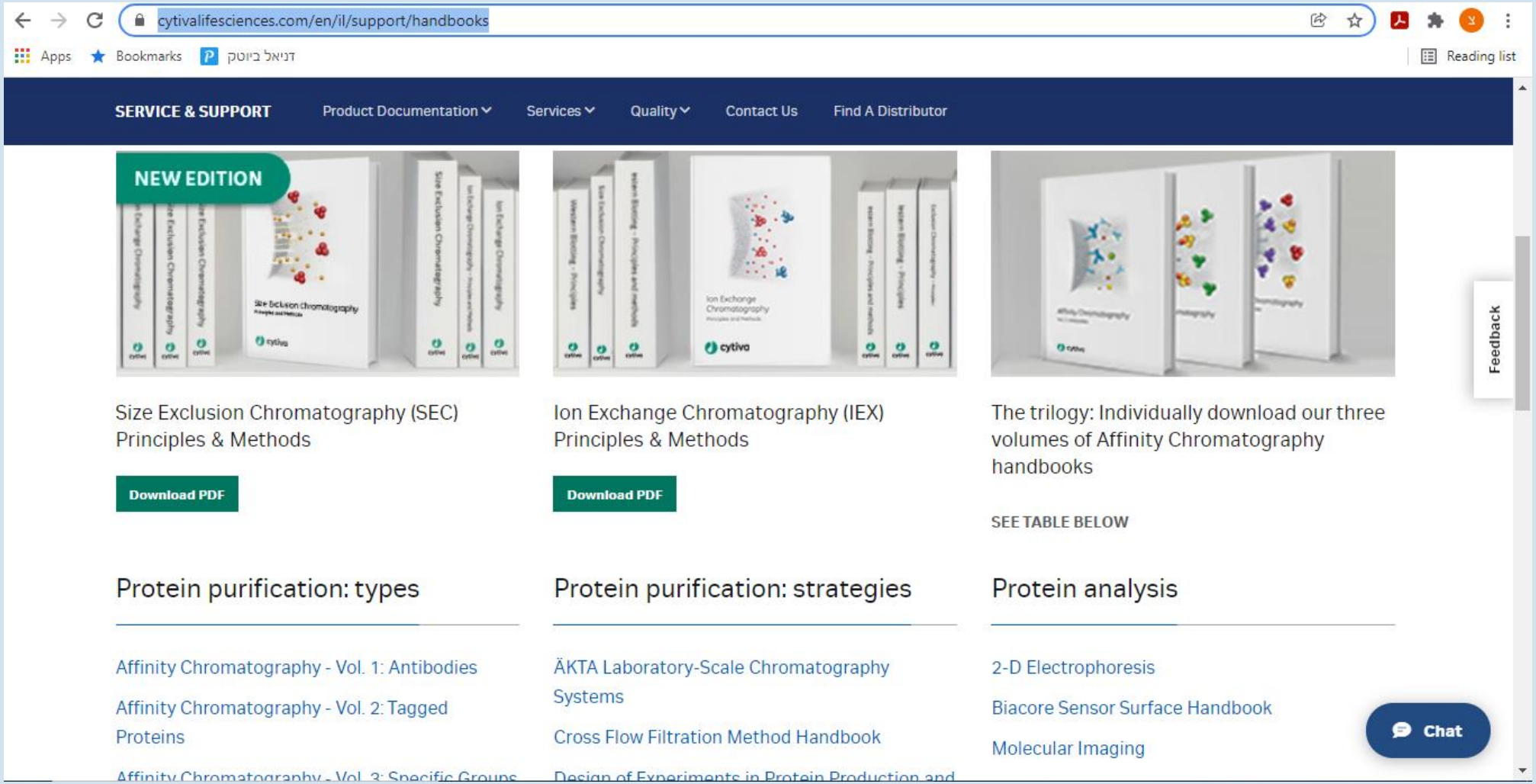
3.Unicorn™ Evaluation screen

For viewing post-run results graphically, comparing runs, results analysis etc.



Knowledge resources

https://www.cytivalifesciences.com/en/il/support/handbooks



Thanks for your attention !

DanyelBIOTECH

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LIFE SCIENCES & BIOMANUFACTURING