Sample Preparation before Chromatography

Chromatography: General Considerations

FPLC System - FPLC Software

PURIFICATION STRATEGY

- Sequence of Events: Cell harvesting, Cell Disruption, Extraction and Clarification, Chromatography
- Sample Preparation before Chromatography: Cell Debris Removal, Clarification and Concentration: dialysis, filtration, ultrafiltration , others
- FPPLC, columns, resolution, selectivity, efficiency and capacity
- Three Phase Strategy: Linking Chromatography Techniques
- FPLC System - FPLC Software

Cell Disruption considerations

- Stability of the released protein
- Location of target protein within the cell (membrane, nucleus, mitochondria, etc.)
- Yield and kinetics of the process. Extent of disruption: possible use of marker substances, measure protein concentration. Balance: volume & lysis efficiency.
- Suggested lysis volume for bacterial cells: 10-20% of original cell culture
- Scale-up
- Option: perform protein purification without cell debris clarification step, directly from the cell lysate (bed absorption chromatography)

Methods to monitor lysis

- Reduction of whole cells: decrease of Abs660nm before and after treatment.
- Weight of cell pellet before & after lysis
- Monitor nucleic acid release : Increase in the Abs260nm during lysis

  (This method could be difficult because of the "haze" generated, which can alter absorbance readings.)
- Microscopically : Compare cells before and after treatment

CELL DESINTIGRATION AND EXTRACTION: METHODS THAT DO NOT NEED SPECIAL EQUIPMENT

- **Freezing and thawing**: Repeated cycles (can denature protein). For cells without a cell wall (animal cells). Not suitable for large scale. Not reliable method

- **Osmotic shock:** Transfering cells from a high to a low osmotic pressure. **Useful to release periplasmic proteins** from Gram negative bacteria. Not reliable method

- **Chaotropic agents (urea, GuHCl):** Extremely denaturative. Not suitable for large scale. Use for extremely insoluble proteins or inclusion bodies

- **Detergents (Brij, NP40, DDM, etc.):** Anionic and non-ionic detergents permeabilize Gram negative cells. Can interfere in downstream process. Dissolve membrane-bound proteins. Use in combination with mechanical methods. Problematic!!! *Bacterial Expression Screen - DDM (Dodecyl Maltoside) lysis - Small Affinity binding* *http://wolfson.huji.ac.il/purification/TagProteinPurif/DDM_Bacterial_Expr_screen.html*

- **Organic solvents**: Toluene, ether, chloroform, isoamyl alcohol, etc at different concentration can release different materials from the cell. Extremely denaturative. Use only for solvent resistent proteins. Not reliable method

- **Enzymatic lysis:** Lysozyme hydrolyze linkages in the peptido-glycan of bacterial cell walls. Used for pretreatment of cells in combination with mechanical methods.
Yeast cell walls can be hydrolyzed with snail gut enzymes and glucanases

CELL DESINTIGRATION AND EXTRACTION: METHODS THAT NEED SPECIAL EQUIPMENT

- **Combine with chemical treatment: lysozyme, detergents, Dnase, etc.**
- **Mixers and blenders:** Useful for animal and plant tissues (Warring-blender)
- **Coarse grinding** Grinding with a pestle and mortar of frozen mycelium**. Fine grinding in a bed mill:** Useful for yeast, larger cells, algae and filamentous fungi. Use of different glass beads (Bead-beater)
- **Homogenization:** Animal cells. Piston/plunger device. Wheaton-Dounce homogenizer
- **Sonication:** Bacterial cells disrupted by high frequency sound and share forces. Low scale. Very vigorous process. Heat generation. Not reliable method
- **High pressure lysis:** Pumping cell suspension through a narrow orifice at high pressure. Mainly for bacterial cells. **Very reliable and efficient method.** French-press, Microfluidizer, Avestin, etc**:** medium scale (20-100ml). Microfluidizer, Maunton-Gaulin**:** For larger volumes

Cell Lysis

Equipment in LSI

HTP – Low scale:

Bacterial Expression Screen - DDM (Dodecyl Maltoside) lysis - Small Affinity binding

http://wolfson.huji.ac.il/purification/TagProteinPurif/DDM_Bacterial_Expr_screen.html

As French-press but for medium/larger volumes

For bacterial and yeast cells

High speed

Other applications.

Avestin Emulsiflex C3

Microfluidizer

Microfluidizer low volume benchtop machine

One Shot Model

Clarification

removing cell debris
unbroken cells
nucleic acids
“colloids”
and more

Centrifugation

Normal
Continuous flow

Filtration

Normal (Dead end)

Tangential Flow Filtration (Cross flow)

No clarification

Dialysis

Ultrafiltration

Concentration, dia-filtration

Buffer Exchange

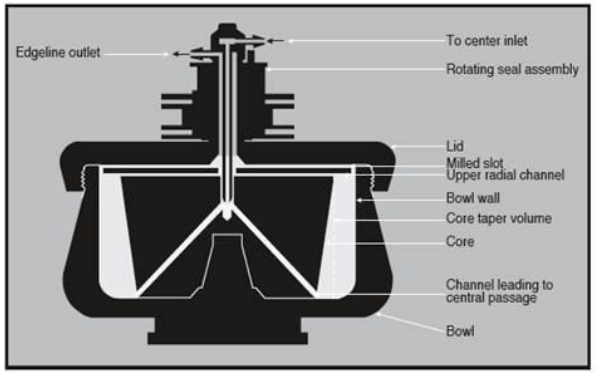
Fractional Precipitation

Ammonium SO_4
PEG
Polyethyleneimine
Streptomycin Sulphate

Sample Preparation before Chromatography: Cell Debris Removal, Protein Clarification and Concentration

- Centrifugation For small sample volumes 15min 10,000g .

  For very turbid cell homogenates: 30min 50,000g



- Continuous Centrifugation (Industrial)
- Filtration before loading in chromatographic column

Pore size filter: 1µm for particle size of chromatographic medium 90 µm and upward

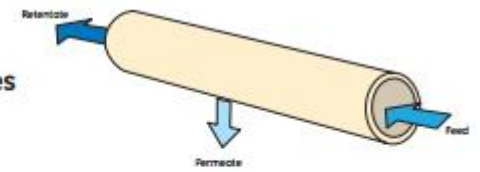
Pore size filter: 0.45µm for particle size of chromatographic medium 30 or 34 µm

Pore size filter: 0.22µm for particle size of chromatographic medium 3, 10, 15 µm

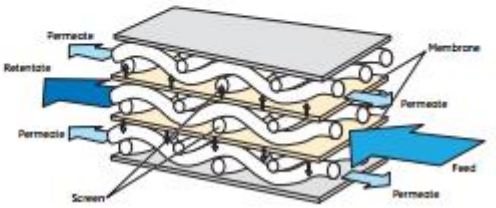
Pore size filter: 0.1µm for SEC-MALS & IEX-MALS

- Filtration large scale, Normal (Dead end): Filters. H
- TFF: Tangential Flow Filtration (Cross flow)
- Expanded Bed Adsorption: no clarification

Open Channel
Hollow Fiber Cartridges



Screen Channel
Flat Sheet Cassettes



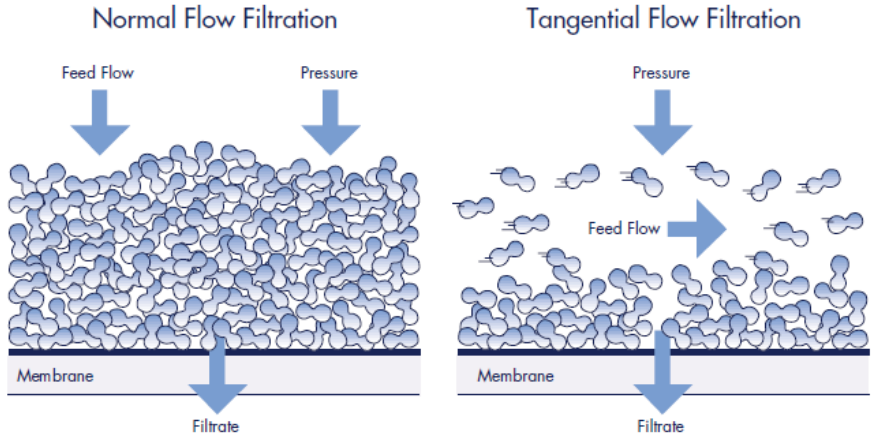
Membrane-Based Systems

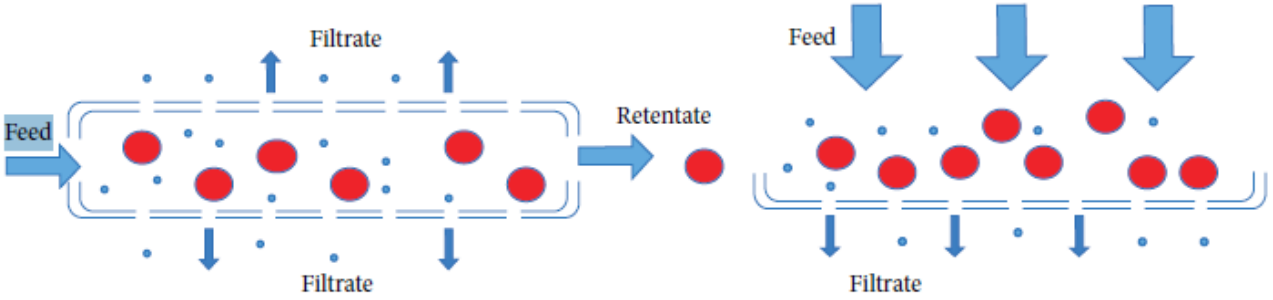
➢Pressure-driven processes, such as ultrafiltration (UF), microfiltration, virus filtration, and nanofiltration . Or electric field (electro-ultrafiltration, EUF)

➢They are mainly used for protein concentration and buffer exchange replacing SEC on an industrial scale.

➢There are charge membranes that can use as IEX, RPC, Affinity, HIC (Pall, Mustang, etc)

➢Another emerging technology in membrane separation processes is high-performance tangential flow filtration (HPTFF).





Tangential or cross flow and Normal or dead end filtration

Normal / Cross Flow Filtration / Ultrafiltration

GE Healthcare

Normal flow filtration products

Syringe filters

Bottle-Top Filters

Membrane filtration capsules

Filter capsules

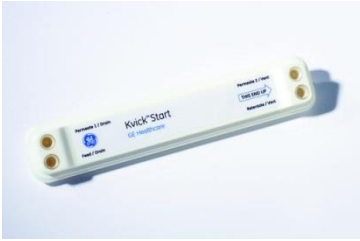
Cross flow filtration products

ÄKTAcrossflow™

- Fully automated filtration system for cross flow membrane screening, process development, and small scale processing.
- Enable automation at very small scale, with capacity ranging from liters down to 25 ml.

Hollow fiber ultrafiltration cartridges

Available with ten different molecular weight



Kvick Cassette family

- Membrane surface area from 50 cm^2 to 2.5 m^2
- MW cutoffs (5k, 10k select, 10k, 30k, 50k, and 100k)

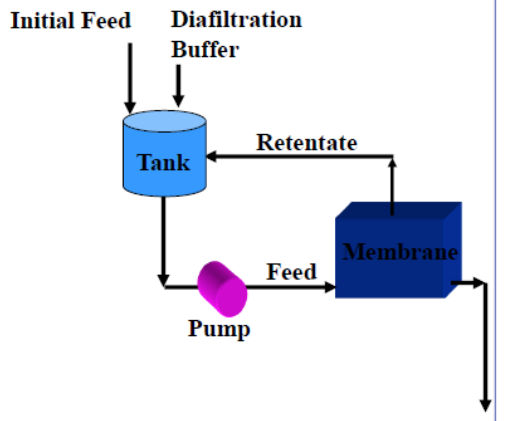
TFF: Tangential or cross flow filtration

Merck

Basic TFF Operation

Basic Components

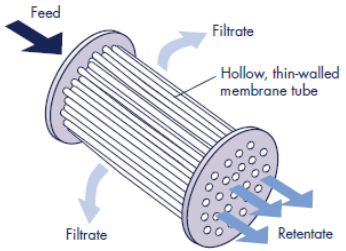
- Membranes
- Pump
- Tank
- Piping



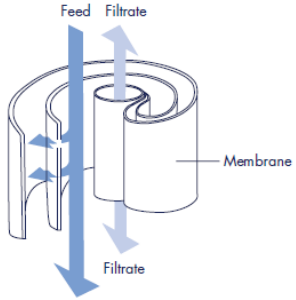
	Microfiltration	Virus Filtration	High-Performance Filtration	Ultrafiltration TFF	Nanofiltration/ Reverse Osmosis
Components retained by membrane	Intact cells Cell debris	Viruses	Proteins	Proteins	Antibiotics Sugars Salts
membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane					
Components passed through membrane	Colloidal material Viruses Proteins Salts	Proteins Salts	Proteins Salts	Small Peptides Salts	(Salts) Water
Approximate membrane cutoff range	0.05 µm – 1 µm	100 kD – 0.05 µm	10 kD – 300 kD	1 kD – 1000 kD	<1 kD

TFF: Tangential or cross flow filtration

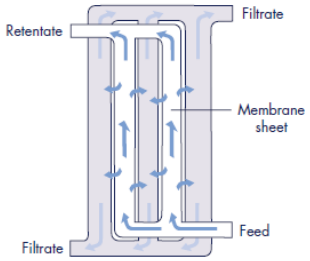
Merck



Hollow Fiber



Spiral-Wound



Flat Plate

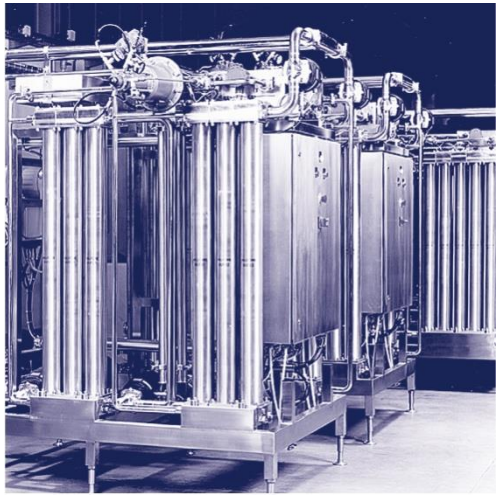
Prep/Scale filter modules

Pellicon cassettes

Labscale™ Benchtop TFF System with Pellicon XL module

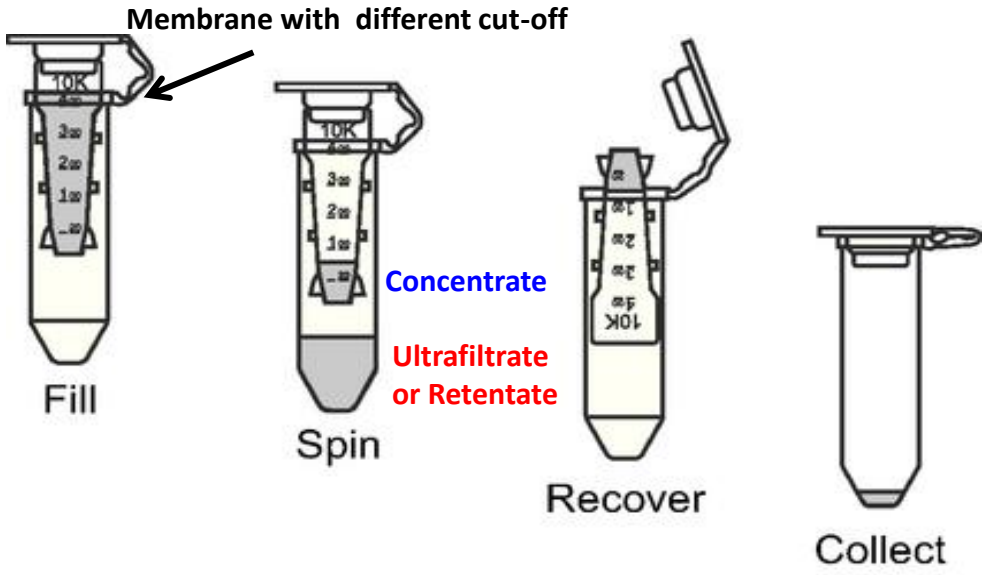
ProFlux® M12 Benchtop TFF system with spiral wound modules

Fully automated 80 m2 Pellicon system for concentration and diafiltration



Large-scale spiral wound UF/DF system

Ultrafiltration

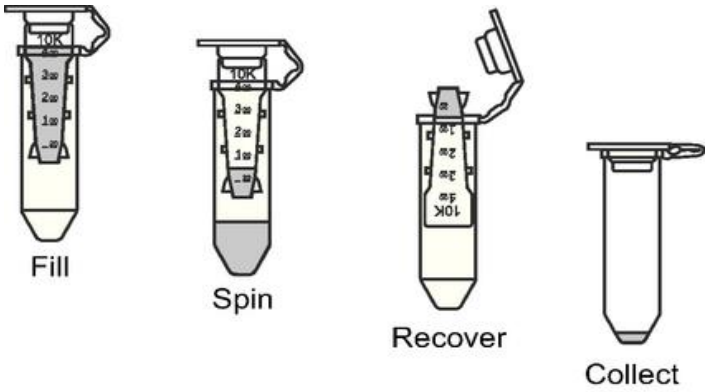


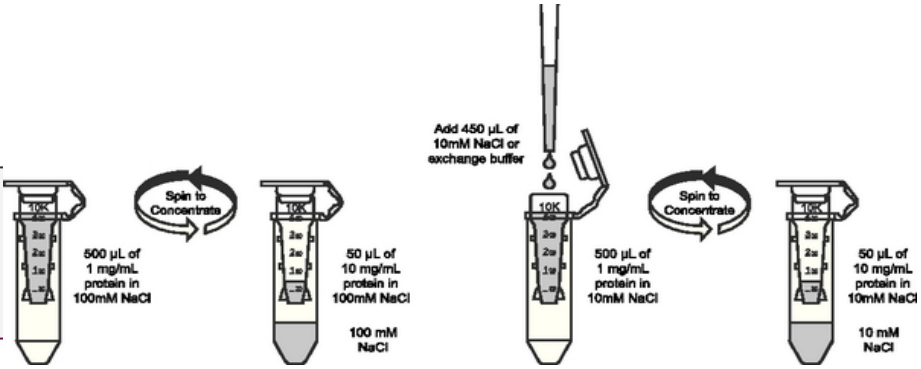
- ✓ A process that uses semi-permeable membranes to separate molecules on the basis of size.
- ✓ It is particularly appropriate for concentration, partial purification or for buffer exchange.
- ✓ Is a gentle and non denaturing method.
- ✓ The ultrafiltrate is cleared of macromolecules which are significantly larger than the cutoff of the filter
- ✓ The buffer concentration in the ultrafiltrate will be exactly the same as in the concentrate
- ✓ Do not replace GF, although the principles are the same: separation according to ratio of the molecule
- ✓ Proteins with MW lower than the cut-off, will be retained in the concentrate if they aggregate, or are part of a complex
- ✓ Cut-off at least two or three times of the protein size
- ✓ Some proteins can stick to the membranes

Ultra Centrifugal Devices

Amicon / Millipore - Merck

Product		Amicon® Ultra–0.5	Amicon® Ultra–2	Amicon® Ultra–4	Amicon® Ultra–15
Maximum initial sample volume (mL)		0.5	2	4	15
Final concentrate (retentate) volume (µL)		15–20	15–70	30–70	150–300
MWCO	**Qty/Pk**				
3,000 MWCO	8	UFC500308		UFC800308	UFC900308
	24	UFC500324	UFC200324	UFC800324	UFC900324
	96	UFC500396		UFC800396	UFC900396
	500	UFC5003BK			
10,000 MWCO	8	UFC501008		UFC801008	UFC901008
	24	UFC501024	UFC201024	UFC801024	UFC901024
	96	UFC501096		UFC801096	UFC901096
	500	UFC5010BK			
30,000 MWCO	8	UFC503008		UFC803008	UFC903008
	24	UFC503024	UFC203024	UFC803024	UFC903024
	96	UFC503096		UFC803096	UFC903096
	500	UFC5030BK			
50,000 MWCO	8	UFC505008		UFC805008	UFC905008
	24	UFC505024	UFC205024	UFC805024	UFC905024
	96	UFC505096		UFC805096	UFC905096
	500	UFC5050BK			
100,000 MWCO	8	UFC510008		UFC810008	UFC910008
	24	UFC510024	UFC210024	UFC810024	UFC910024
	96	UFC510096		UFC810096	UFC910096
	500	UFC5100BK			





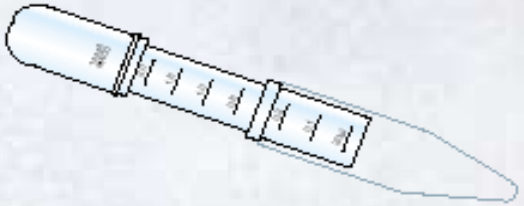
Ultrafiltration devices VIVASPIN

VIVASPIN 500

Process Volume: 500µl to 5µl
Operating Mode: Centrifuge
Pages: 6 - 9

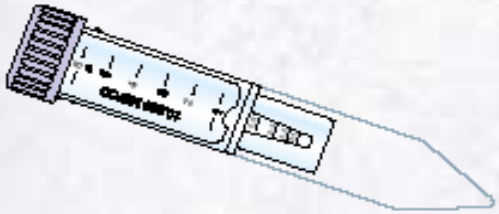
VIVASPIN 2

Process Volume: 2ml to 8µl
Operating Mode: Centrifuge
Pages: 6 - 9



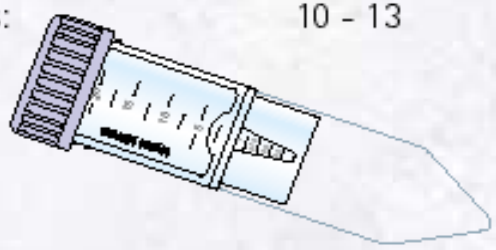
VIVASPIN 6

Process Volume: 6ml to 30µl
Operating Mode: Centrifuge
Pages: 10 - 13



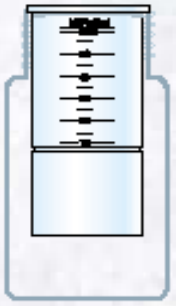
VIVASPIN 20

Process Volume: 20ml to 50µl
Operating Mode: Centrifuge
Gas Pressure
Pages: 10 - 13



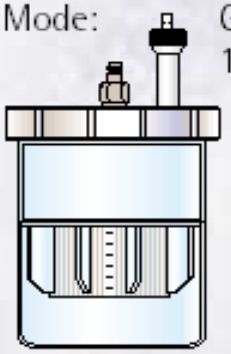
VIVACELL 70

Process Volume: 70ml to 150µl
Operating Mode: Centrifuge
Gas Pressure
Pages: 14 - 15



VIVACELL 250

Process Volume: 250ml to 600µl
Operating Mode: Gas Pressure
Pages: 16 - 17



Selecting Hollow Fiber Cartridges and Systems

According to GE Healthcare

Application	Ultrafiltration (NMWC)	Microfiltration (µm)
Bacterial/pyrogen removal	10,000	
Protein concentration	3,000, 5,000, 10,000, 30,000	
Enzyme concentration	10,000, 30,000, 50,000	
Virus concentration/purification/removal	100,000, 300,000, 500,000, 750,000	
Protein/antigen recovery from fermentation broth	500,000, 750,000	0.1, 0.2, 0.45, 0.65
Bacterial cell concentration	500,000	0.1, 0.2
Insect cell concentration		0.1, 0.2
Mammalian cell concentration		0.2, 0.45, 0.65
Yeast concentration		0.1, 0.2, 0.45
Continuous cell culture perfusion		0.1, 0.2, 0.45
Red blood cell washing		0.45, 0.65
Red blood cell stroma removal	500,000	0.1
Hemoglobin concentration	5,000, 10,000	
Peptide concentration	1,000, 3,000	

Table 2. Recommended pore sizes for select applications

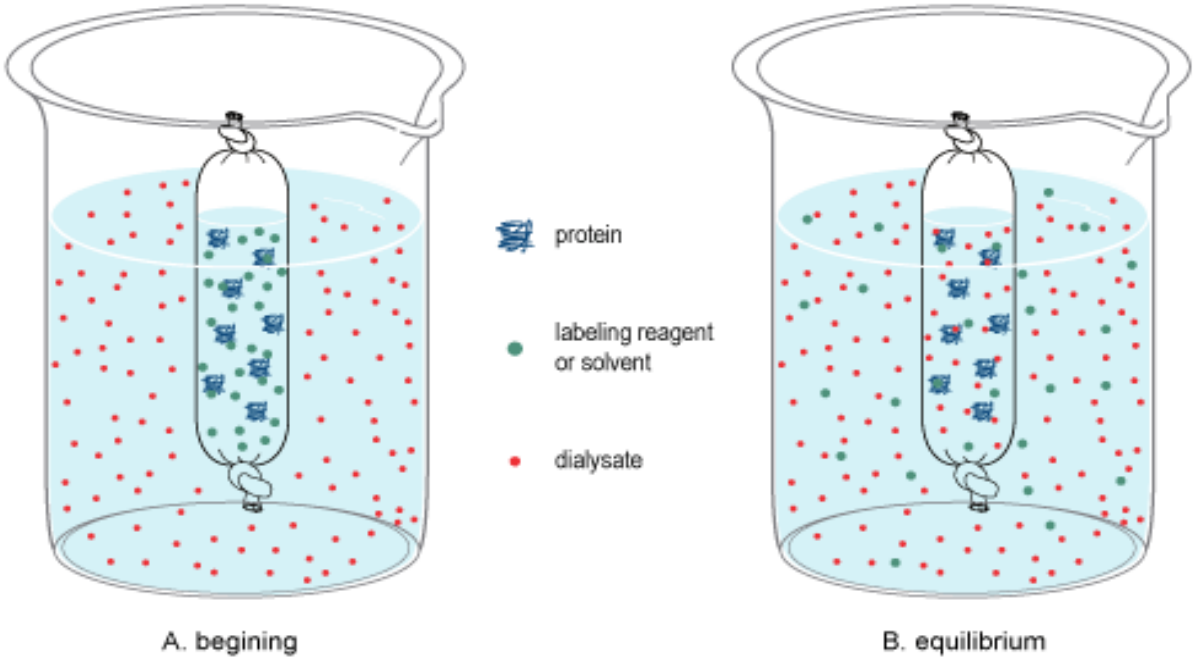
"How can I maximize recovery using Ultrafiltration?" Merck

- ✓ Pick an appropriate NMWL:

  Example: For a 60 kDa protein: two potential membrane choices are 10 kDa or 30 kDa NMWL

- ✓ Pick devices with low non-specific binding
- ✓ Check the chemical compatibility of your device
- ✓ Devices can be use many times (Check before- Don't spin to dryness)
- ✓ Use an invert spin for small volumes
- ✓ Use devices with vertical membrane panels
- ✓ Ensure the protein is soluble at the desired final concentration
- ✓ Allows simultaneous concentrating and desalting
- ✓ Requires much less buffer volumes than dialysis
- ✓ Allows multiple sample processing
- ✓ Easy to use and relatively fast (if buffer is not viscous)

Dialysis



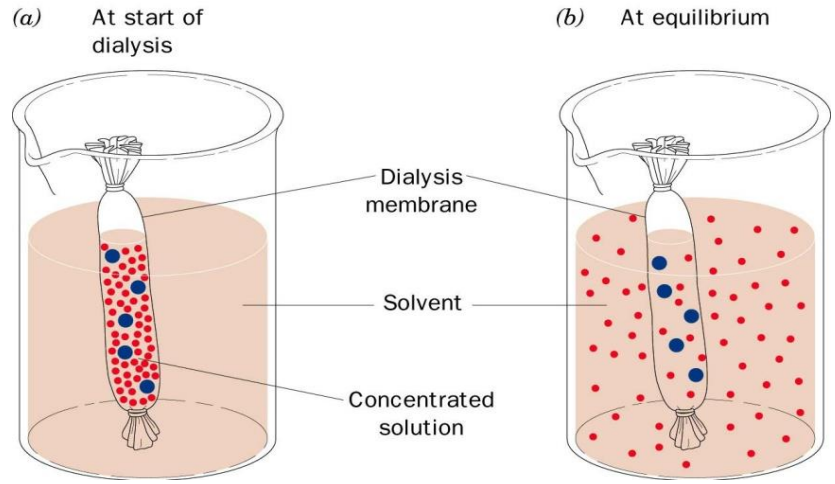
- ✓Time
- ✓Temperature
- ✓Solvent
- ✓Volume
- ✓Cut-off

A process of separating molecules in solution by the difference in their rate of diffusion

Uses of dialysis

- To remove unwanted small molecules from a protein solution

  - DNA
  - salts
  - high CMC detergents
  - small proteins



- For buffer exchange
- “Desalting”

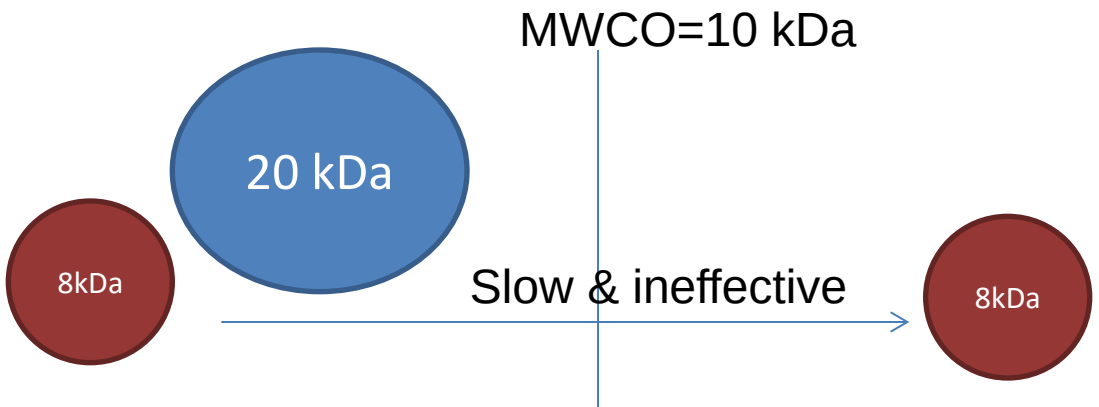
The dialysis membrane

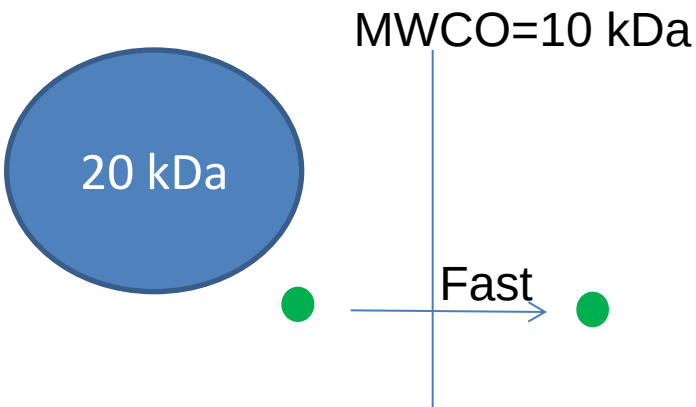
- Molecular weight cutoff (MWCO)- the average pores size

  MW>MWCO - molecule **will not** cross membrane

  MW<MWCO – molecule will cross membrane

- MW<<MWCO cross membrane faster than MW<MWCO





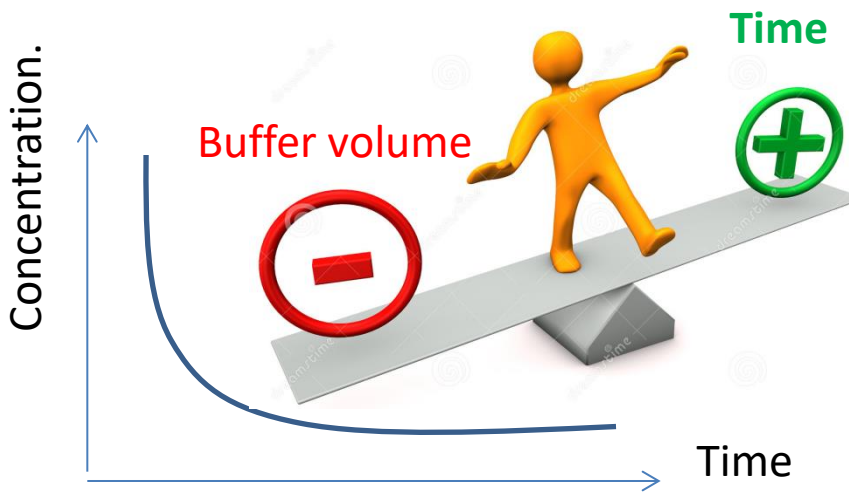
Dialysis General Considerations

- **Protocol:**
- Choose the membrane due to protein size.
- The “old” membranes are with cut-off of 13 kDa
- Load the sample into dialysis tubing (wash membrane and check for holes).
- Place sample into an external chamber of dialysis buffer (with gentle stirring of the buffer).
- Dialyze for 2-4 hours
- Change the dialysis buffer and dialyze for another 2-4 hours
- Change the dialysis buffer and dialyze for 2 hours - ON.

Example:

- For 10ml sample of 1M in 10L buffer – sample will reach to 1mM at equilibrium (~4h)
- Same sample in 1L – 10mM after 4h

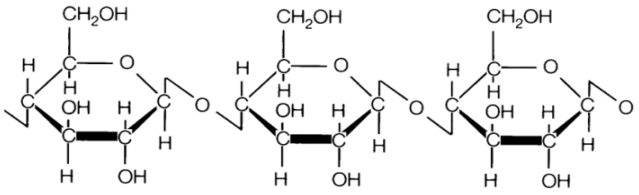
  Then replace buffer 1L – 0.1mM after 4h.



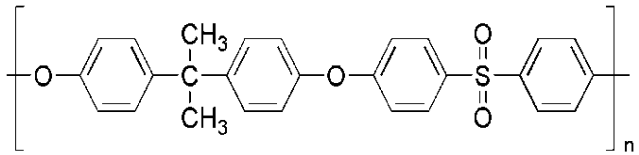
Types of membrane

- There are more then 30 types of synthetic and natural dialysis membranes

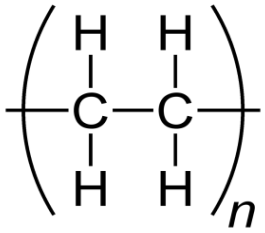
Cellulose



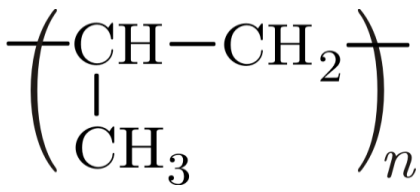
Polysulfone



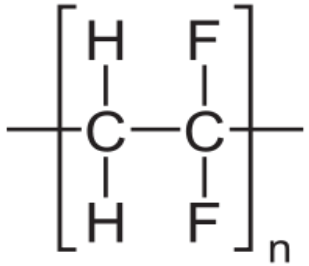
Polyethylene



Polypropylene



Polyvinylidene fluoride

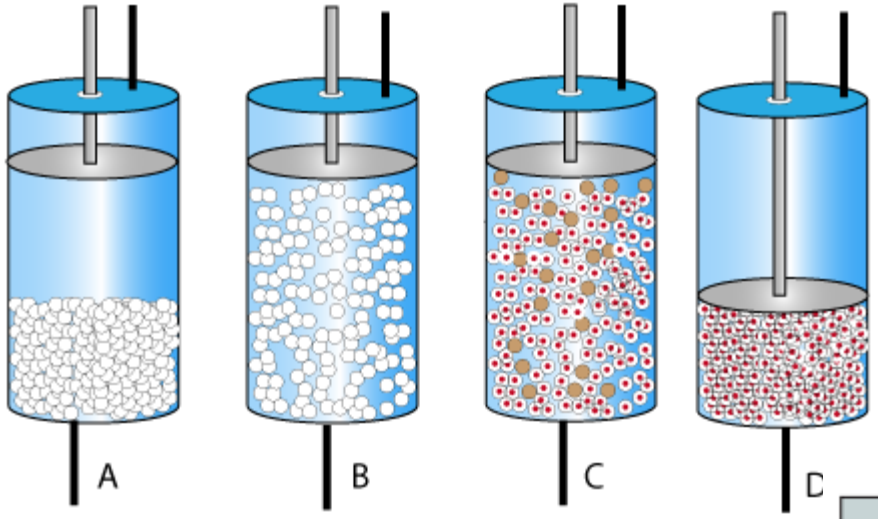


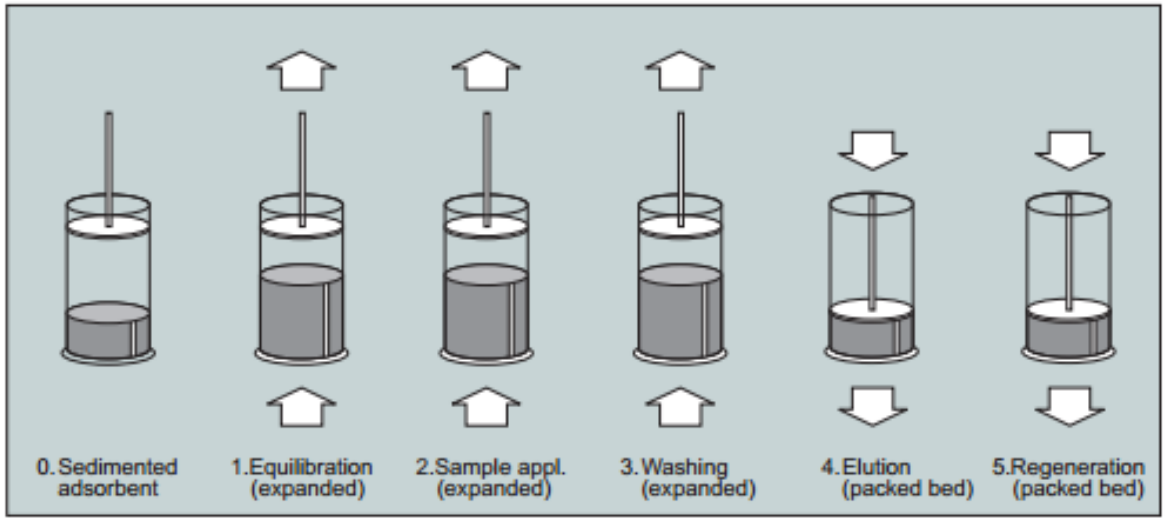
Ultrafiltration or dialysis

- Protein Desalting or Buffer Exchange
- The protein solution may be purified from low MW materials , like salts, low MW reagents, etc
- Multiple solvent exchanges, will progressively purify macromolecules from contaminating solutes.
- Ultrafiltration is faster than dialysis and requires less buffer
- Protein will be concentrated during ultrafiltration
- Diafiltration: Microsolutes are removed most efficiently by adding buffer to the solution being ultrafiltered at a rate equal to the speed of filtration.

Expanded Bed Adsorption Chromatography

Protein capture to resins without clarification (HIC, IEX and AC)





Sample Preparation: Fractional Precipitation

- **Ammonium Sulphate (salting-out):** Stabilizes proteins. Non denaturative. Useful before HIC or to concentrate proteins before GF
- **Dextran Sulphate or Polyvinylpyrrolidine:** Precipitates lipoproteins
- **Polyethylene glycol - PEG > 4000 up to 20%w/v:** Non-denaturative. Supernatan can be used directly to IEX or AC
- **Acetone/Ethanol:** Up to 80%. Useful for peptide or protein concentration. Highly denaturative.
- **Polyethyleneimine 0.1%, Protamine Sulphate or Streptomycin Sulph. 1% :** Removal of nucleic acids. Precipitation of nucleoproteins. Can precipitate negatively charge proteins

Ammonium Sulfate Precipitation

- Solubility of proteins varies according to the ionic strength of the solution
- "Salting in" at low ion concentrations (<0.5 M), the solubility of proteins increases with increasing [salt]
- Solubility of the protein begins to decrease at higher [salt].
- "Salting out": At a sufficiently high ionic strength, the protein will precipitate out of the solution

By adding salt, there is an increase in the surface tension of the water

increasing hydrophobic interactions between water and the protein of interest

The protein then reduces its surface area, which diminishes its contact with the solvent.

	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Starting percent saturation	Amount of ammonium sulfate to add (grams) per liter of solution at 20°C																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Leads to precipitation

PURIFICATION STRATEGY

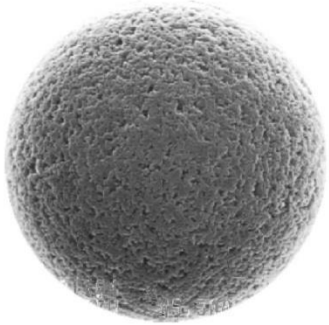
- Sequence of Events: Cell harvesting, Cell Disruption, Extraction and Clarification, Chromatography
- Sample Preparation before Chromatography: Cell Debris Removal, Clarification and Concentration: dialysis, filtration, ultrafiltration , others
- FPPLC, columns, resolution, selectivity, efficiency and capacity
- Three Phase Strategy: Linking Chromatography Techniques
- FPLC System - FPLC Software

Each chromatography media…

Is a unique combination between a certain Base Matrix and Ligand

Base matrices

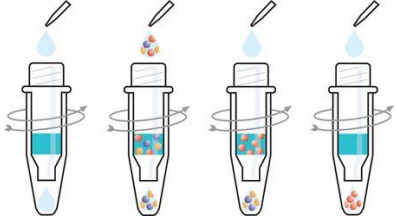
- Capto™
- Sepharose™
- Sephadex™
- Sephacryl™
- SOURCE™
- Etc



Ligands

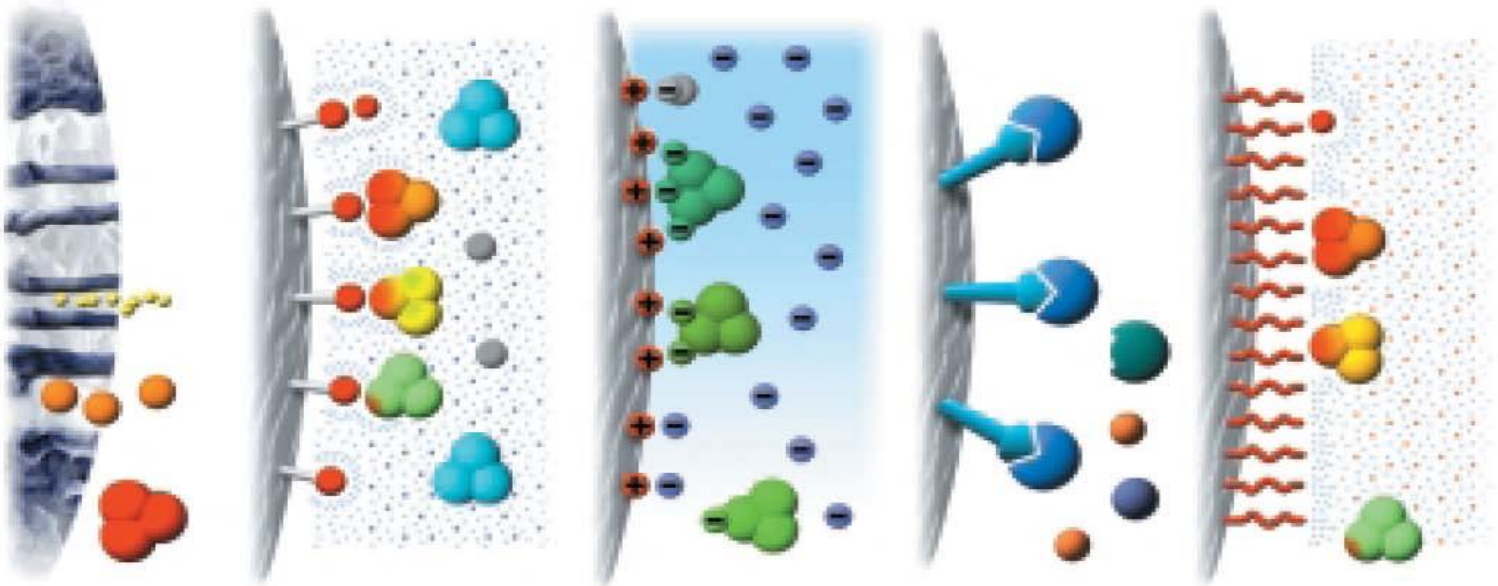
- IEX
- HIC
- SEC
- Affinity
- Multimodal
- Etc

Batch binding



Spin columns with filter

Gravity Desalting Columns

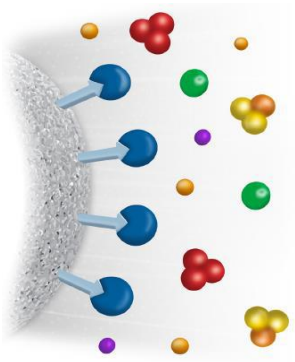


Gel filtration | Hydrophobic interaction | Ion exchange | Affinity | Reversed phase

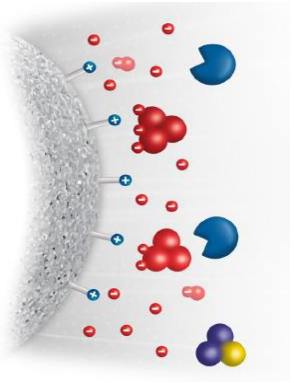
FPLC columns

The principles of chromatography techniques

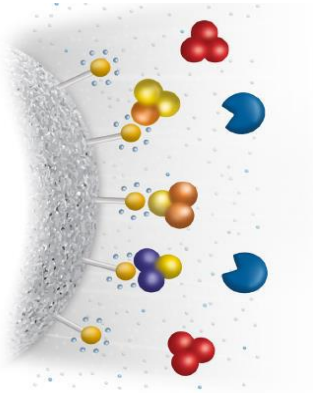
Affinity Chromatography (AC)



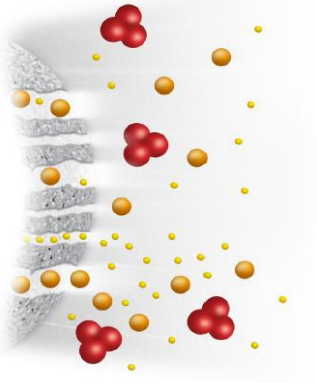
Ion exchange Chromatography (IEX)



Hydrophobic interaction Chromatography (HIC)



Size exclusion Chromatography (SEC)



- Bind – elute principle
- Requires specific elution conditions
- Concentrating effect

- Diffusion – no binding
- Any elution conditions
- Diluting effect

Overview: separation techniques

Technique	Parameter for separation	Based on
Gel filtration	Size/Shape	MW, shape, ratio and oligomeric state of the molecule
Ion exchange/ Hydroxyapatite/ Chromatofocusing	Charge interaction on surface	Asp, Glu, Lys, Arg, His
Hydrophobic/ Reversed phase	Hydrophobic sites interaction	Trp, Phe, Ile, Leu, Tyr, Pro Met, Val, Ala
Affinity	Biological function	Antibody – antigen. Protein A/G/L
IMAC (Immobilized Metal ion Affinity Chromatography)		poly His
Multimodal	Mixture: a) HIC + AIEX b) HIC + CIEX c) Size + HIC + AEIX d) Hydroxyapatite: Ca^{2+} and PO_4^- interactions	
Covalent	Covalent interaction	SH groups (Cys)

Linking Chromatography Techniques into a Purification Protocol - General Rules

- Combine techniques with complementary selectivity

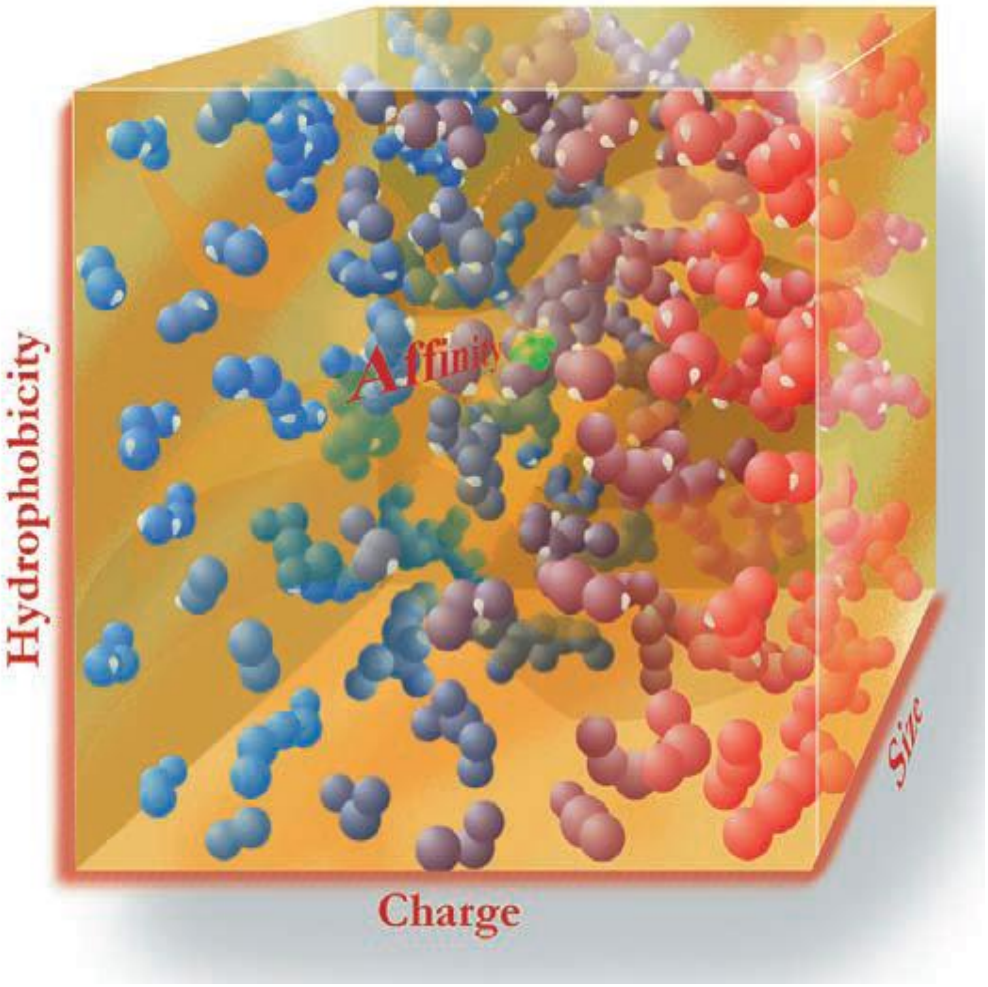
  IEX, HIC and GF

- or different selectivity

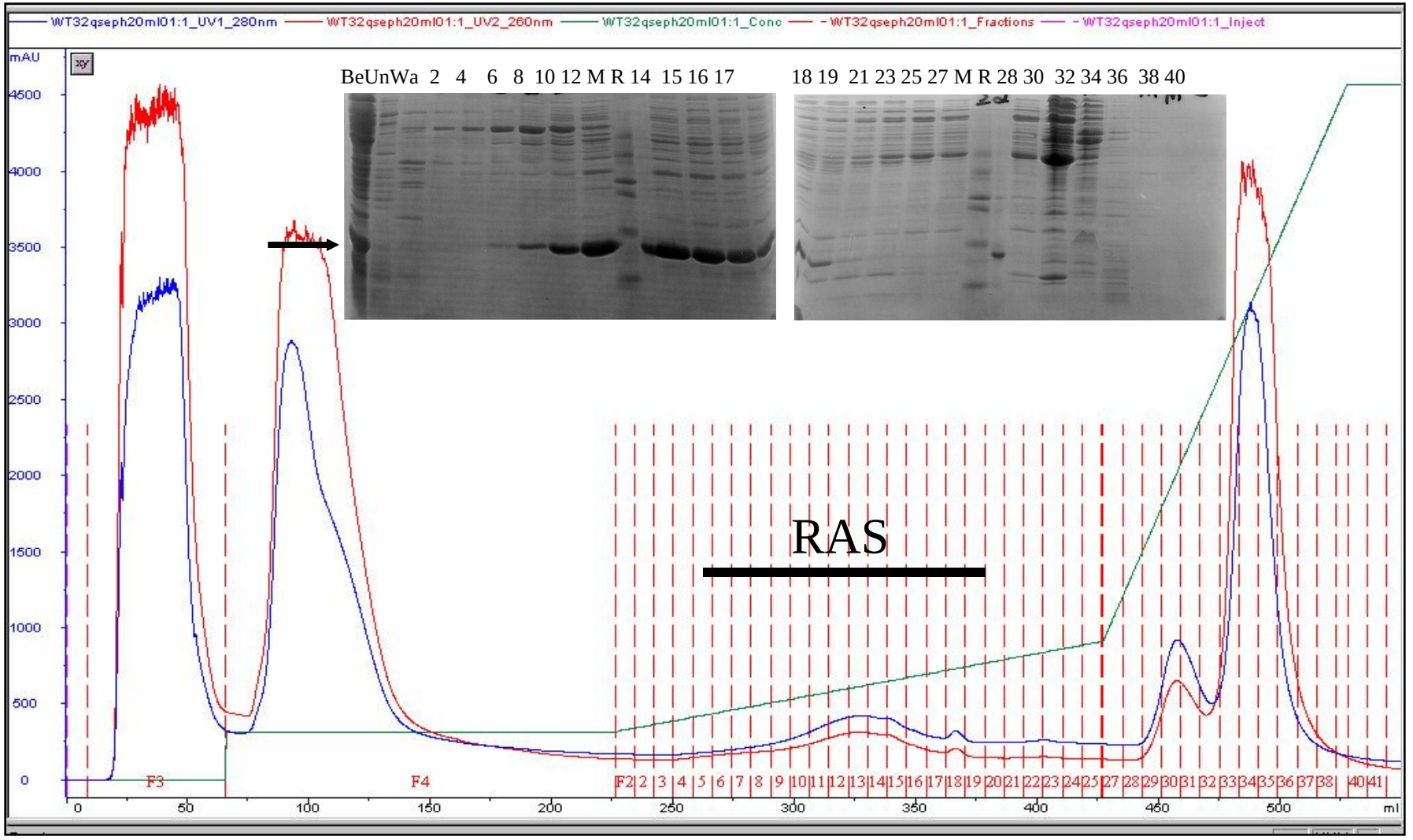
  different pH, buffers, salts, etc.

- Minimize sample handling between purification steps

  (like concentration, dialysis, long assays, non-working days, etc.)



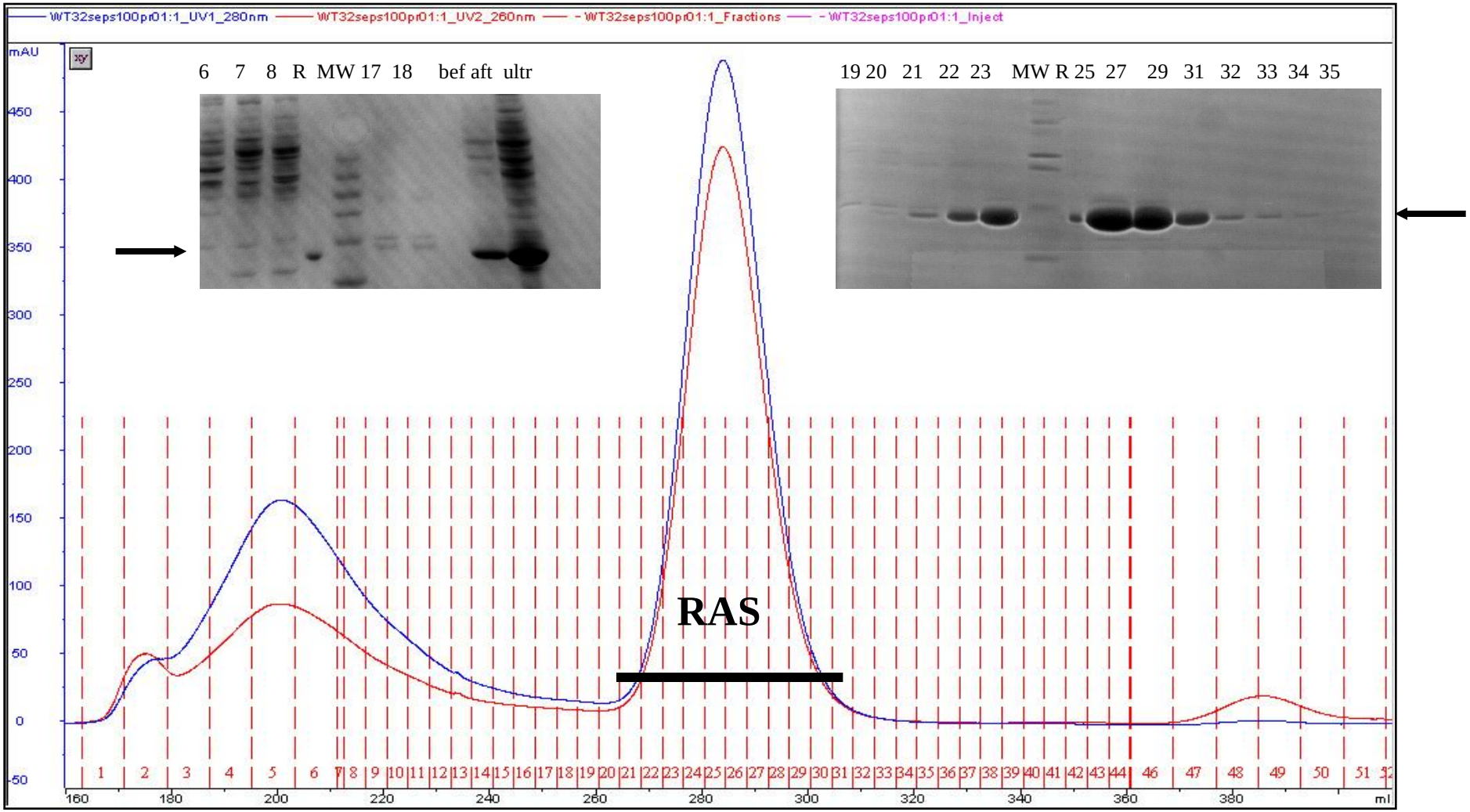
CAPTURE - Anion Exchange



500ml culture after lysis and sonication. Q-Sepharose FF 100x16mm (~20ml) in 25mM TrisHCl pH8.0 buffer + additives.

WASH: 7cv 70mM NaCl ELUTION: gradient 10cv 70-200mM NaCl + 5cv 0.2-1M NaCl

POLISH - Size Exclusion



60 OD280nm (8ml) RAS after Q-Seph. - Load Sephacryl S100 920x26cm -

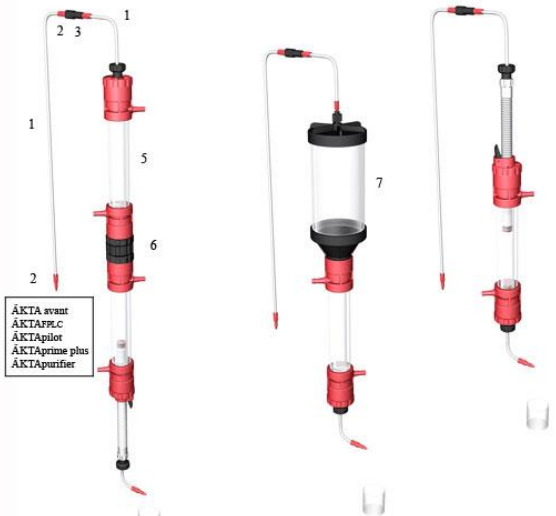
Flow 2.5ml/min - Pool RAS after GF: 36.8 OD280nm

GE Healthcare Chromatography Columns

Gravitation or centrifugation
Disposable plastic columns
Thermo, BioRad, etc

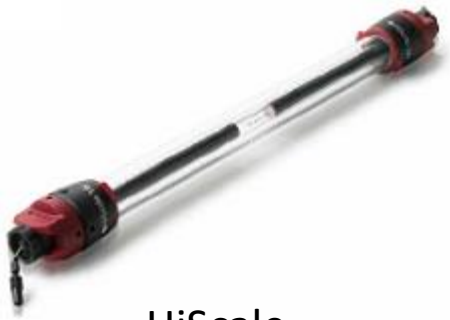
HiTrap columns 1 & 5ml

HiScreen columns



XK columns 1.6 & 2.6 cm

Prepacked Tricorn™ high-performance columns



HiScale

AxiChrom column

ReadyToProcess columns prepacked

Magnetic separation

Traditional purification

Centrifuge to pellet sample

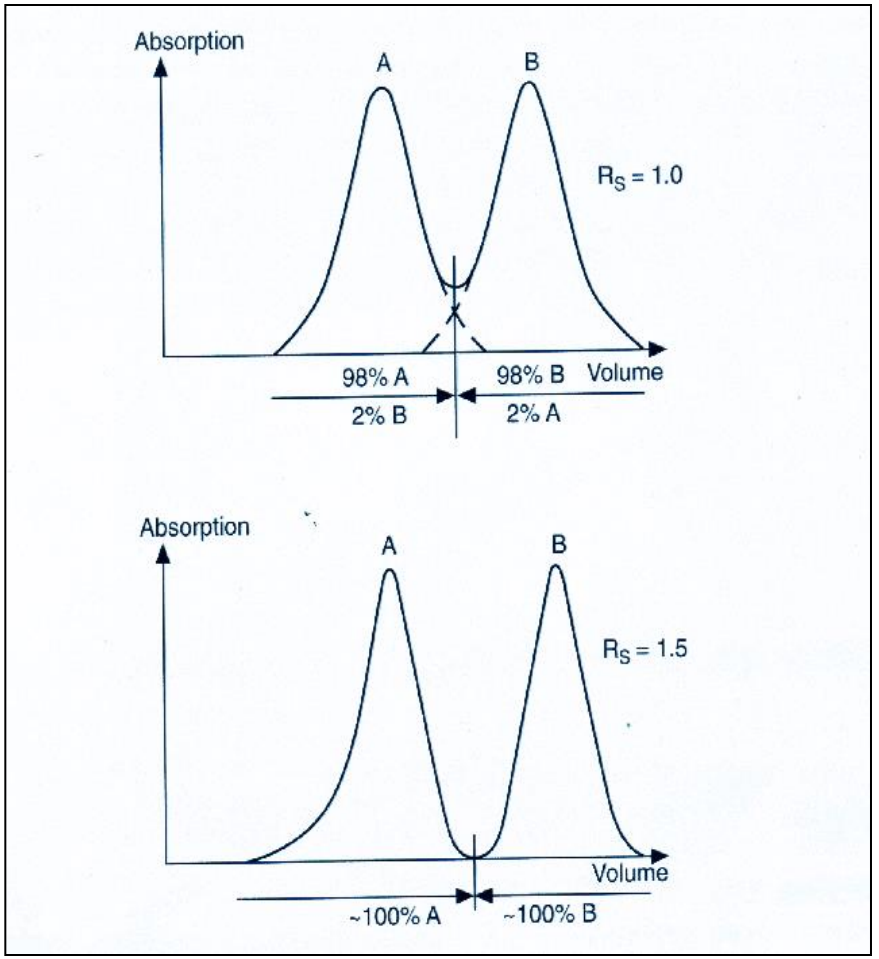
Careful removal of supernatant required to avoid sample loss

Magnetic bead purification

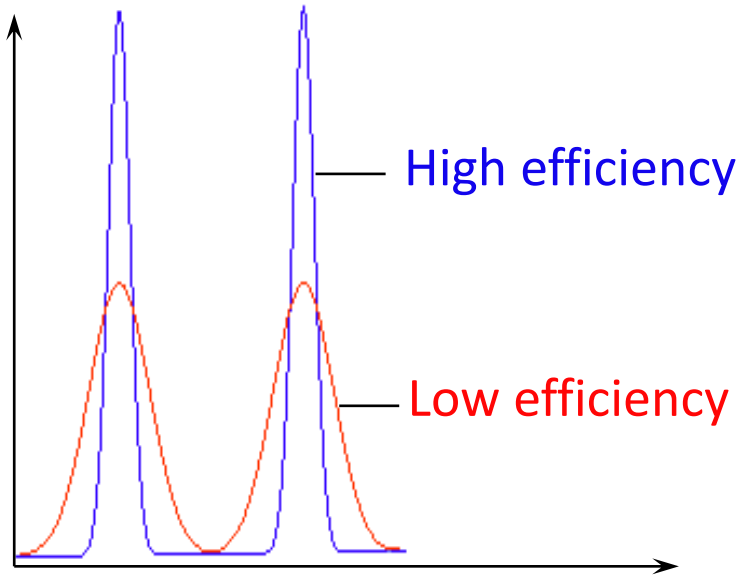
Supernatant can be easily removed with no sample loss

Resolution

- Is a measure of the relative separation between two peaks
- It shows if further optimization is necessary
- A complete resolve peak is not equivalent to a pure substance
- Resolution is proportional to:
  selectivity
  efficiency
  capacity



Resolution depends on efficiency and selectivity

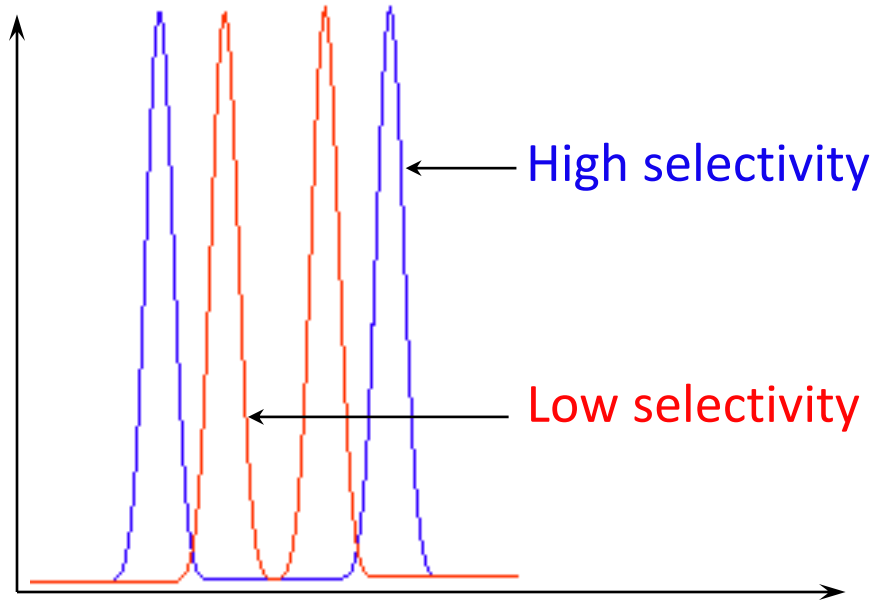


- Efficiency is a measure of peak width (ability to elute narrow, symmetrical peaks)
- Related to the zone broadening on the column (longitudinal diffusion of the molecules)
- Expressed as the number of theoretical plates for the column under specified experimental conditions.

Highest efficiency is achieved by:

- Using small uniform bead sizes with uniform size distribution (reduce diffusion)
- Good experimental technique (uniform packing, air bubbles, etc)

Resolution depends on efficiency and selectivity



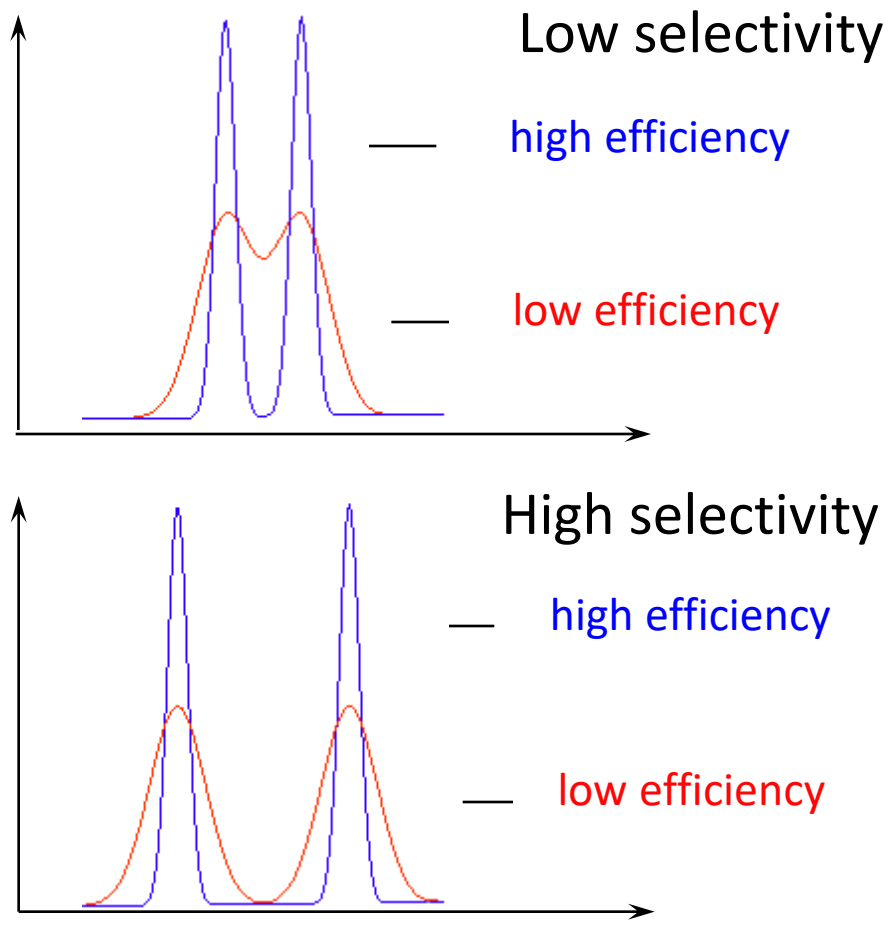
➢Selectivity is the ability of the system to separate peaks (distance between two peaks)

➢Selectivity depends:

1) IEX & HIC: nature and number of ligands and **experimental conditions** like pH, ionic strength, etc

2) GF: fractionation range

Good selectivity is more important than high efficiency for a good resolution



High efficiency can compensate for low selectivity…

But:

High cost

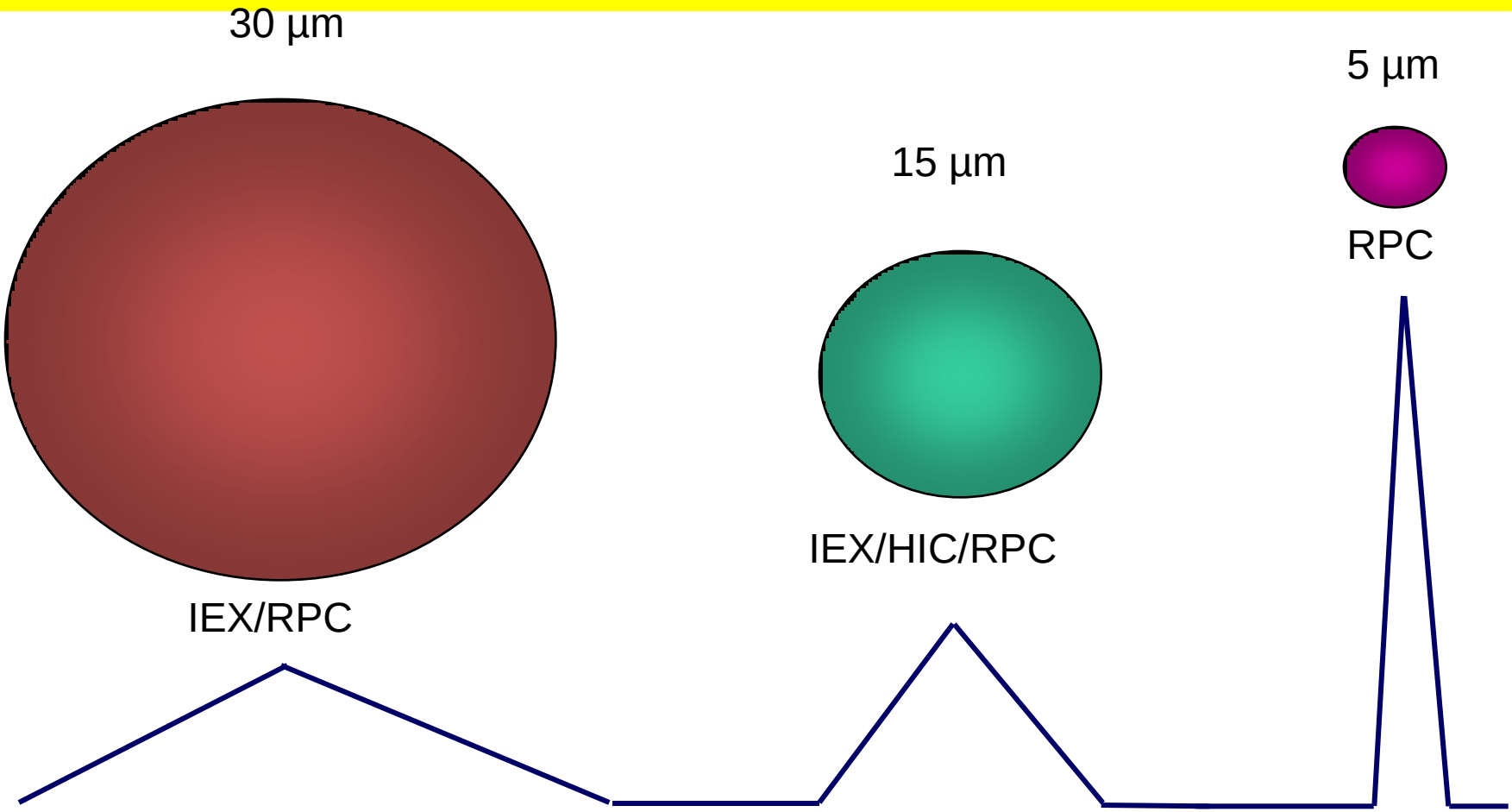
High Back Pressure

Low flow-rate

If selectivity is high, low efficiency can be tolerated

(if large peak volume is acceptable). **Lower cost**

SOURCE™



Types of capacity

- Total ionic capacity (e.g. 3.5 mM/ml)

- Available capacity (e.g. 25 mg HSA/ml)
  Varies with running conditions: pH, sample, ionic strength, etc

- Dynamic capacity (e.g 25 mg HSA/ml, 300 cm/h)
  flow rate dependent
  also varies with pH, sample, ionic strength

Capacity

- *Available capacity* is the amount of protein that can be bound under defined experimental conditions
- *Dynamic capacity* is available capacity at a defined flow rate.
- Both capacities depend upon:

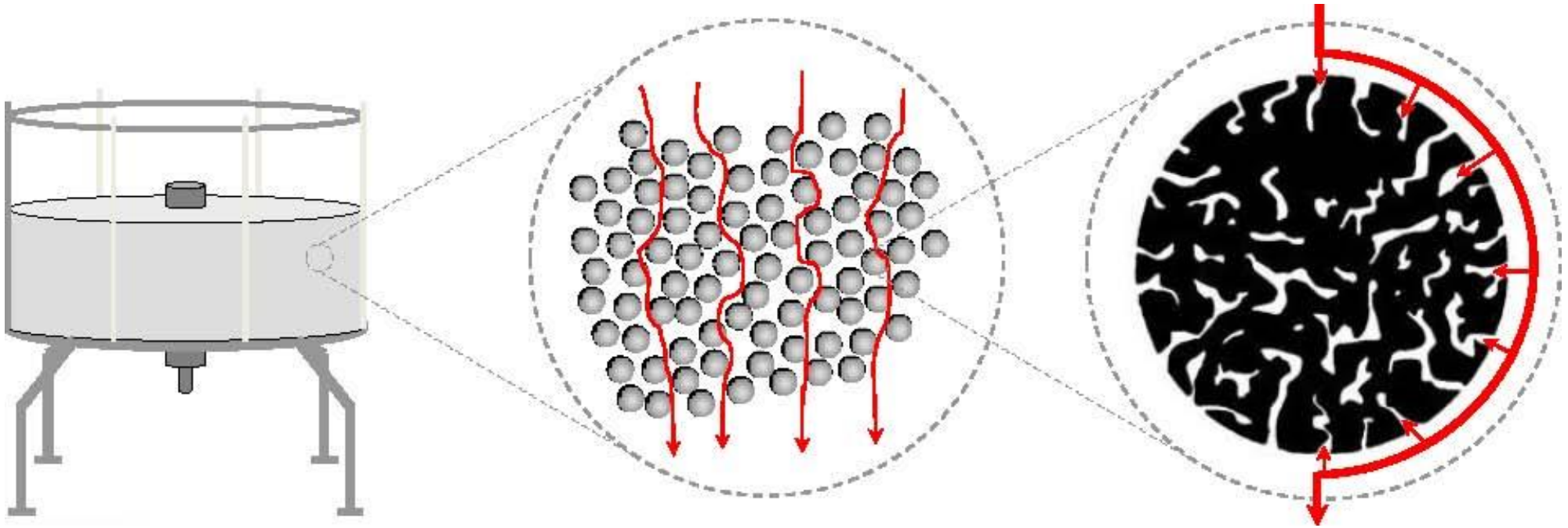
  The chosen experimental conditions: pH, ionic strength of the buffer, the nature of the counter-ion, the flow rate and the temperature.

  The properties of the protein (molecular size, charge/pH relationship).

  Presence of contaminants

  The properties of the resin (small molecules that enter the porus matrix will have an higher capacity).
- Macroporus and highly substituted with many pores to increase surface area
- Non-porus matrices have considerable lower capacity, but higher efficiency due to shorter diffusion distances.

Packed bed of porous particles
Two types of void volume exist!



Interparticle void volume
(preferential flow path)

Intraparticle void volume
(contains majority of
binding sites: > 90 %)

For very big molecules there is a low binding capacity if porous are not big enough,

(behave like nonporous particles) giving wider peak & lower resolution

Membrane Chromatography

Single-use membrane adsorbers

Ready-to-use

Reduce hands-on time

Eliminate packing failures

No need for cold room storage

sartorius

Membranes with open pore structure

Allows high flow rates

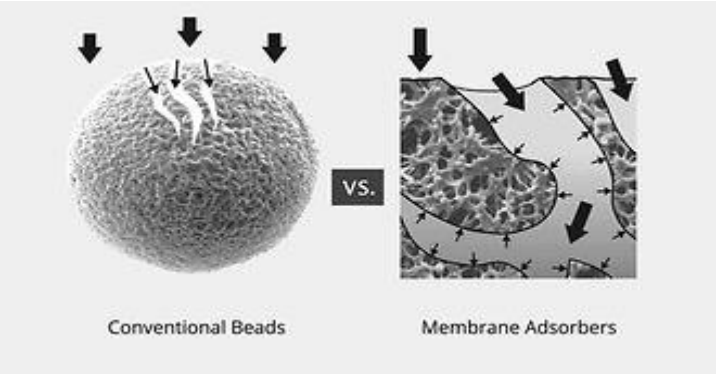
Very high throughput while keeping the bed volume small

Higher flow-rate than columns

Disposable (single-use)

Reduces storage space

Reduce validation costs

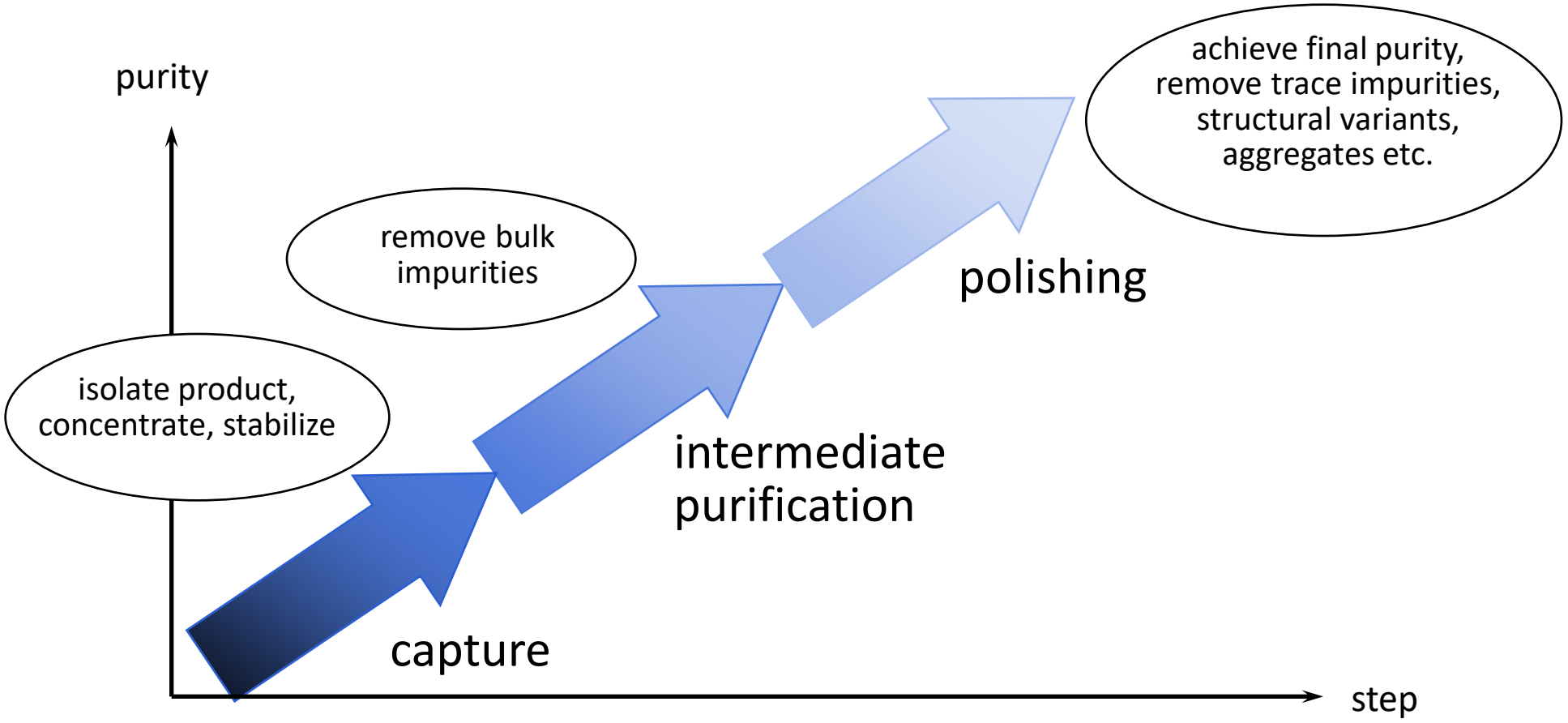


Conventional chromatography beads have a pore size < 100 nm. Sartobind® membranes > 3 µm

This allows large proteins, bioparticles, and viruses or virus-like particles to enter the macro-porous membrane structure

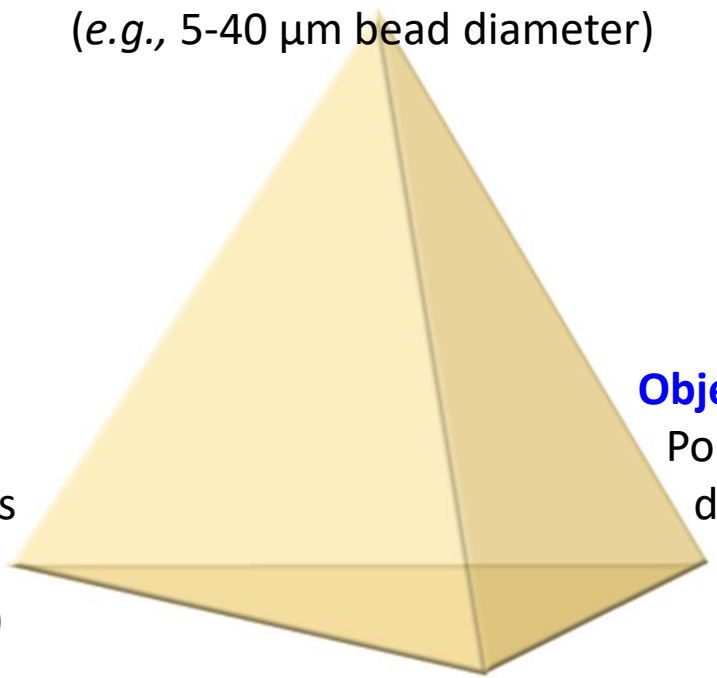
Makes chromatography as easy as filtration

Three Phase Strategy



Which type of chromatography resin provides the desired performance? How can I get the best?

Objective: High resolution
Small, uniformly sized beads
(*e.g.,* 5-40 µm bead diameter)



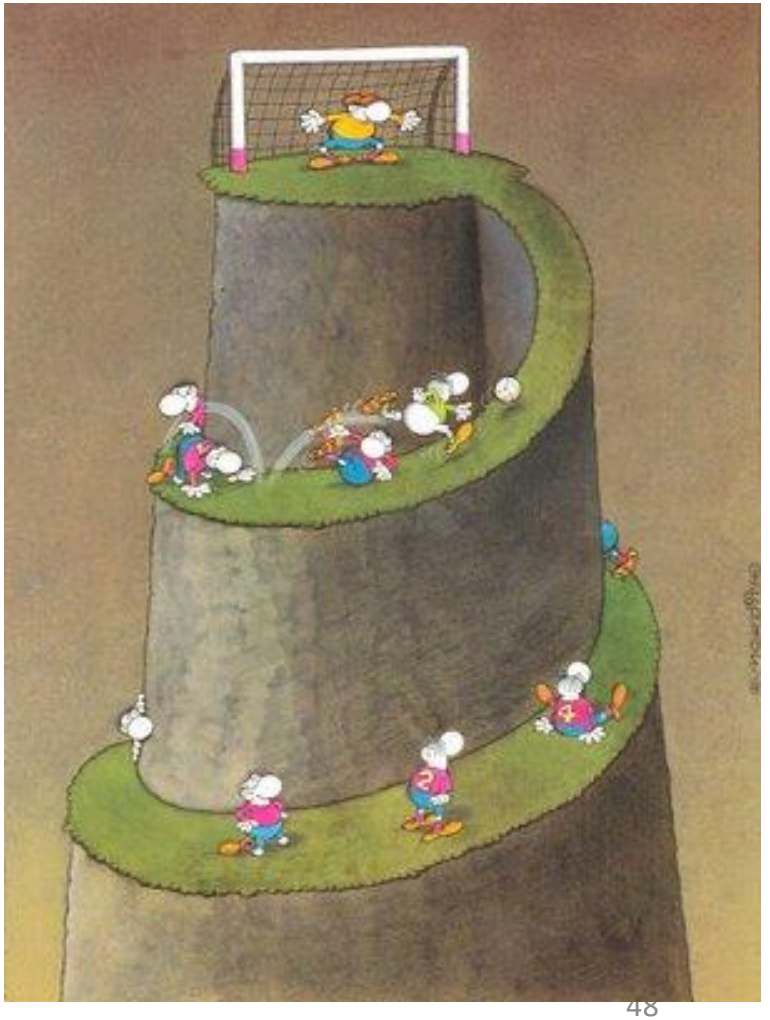
Objective: Speed
Large, rigid and uniformly sized beads provide the highest speed (*e.g.,* 50-100 µm, highly cross-linked agarose)

Objective: High binding capacity
Porous beads with high ligand density and directed ligand coupling

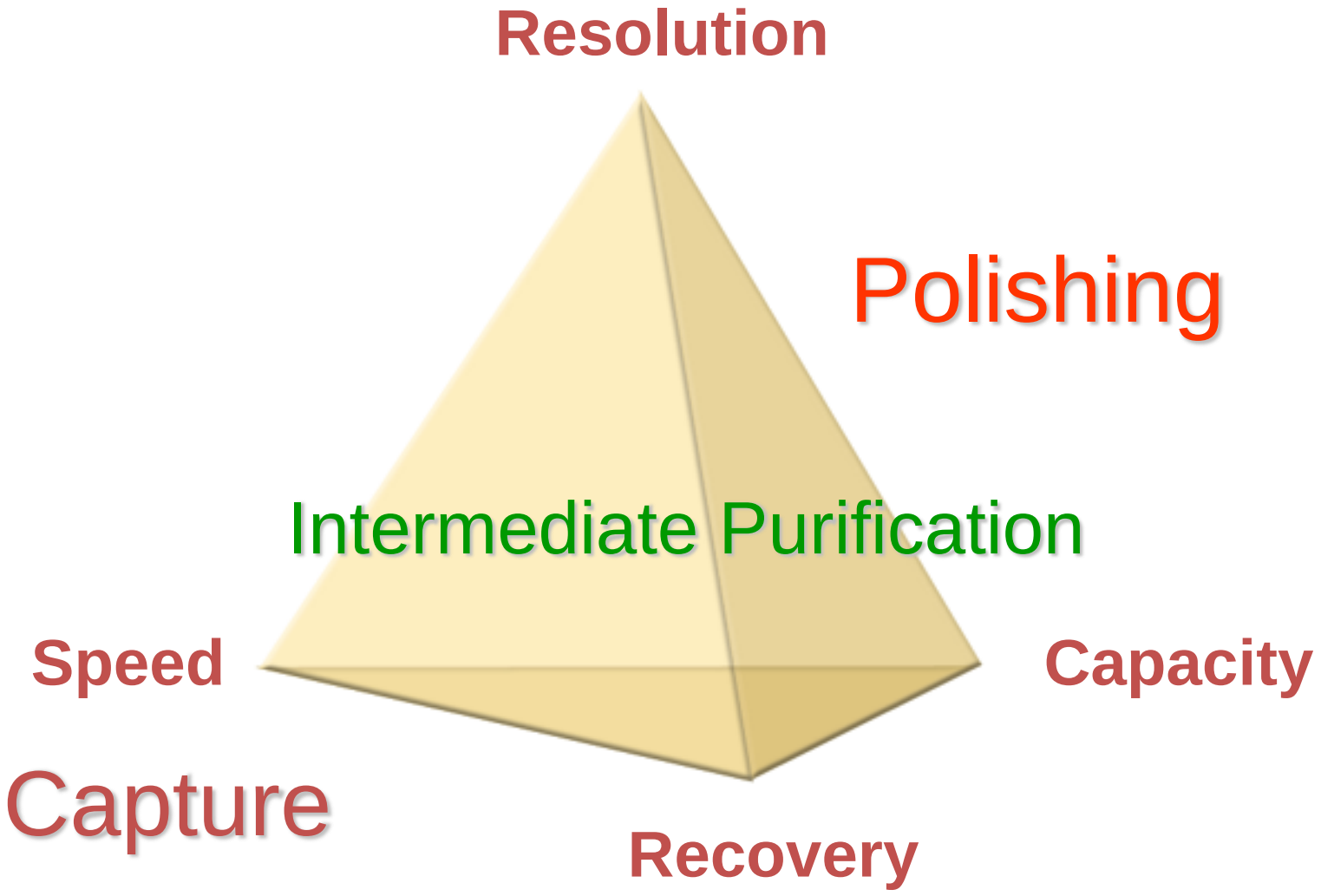
Objective: High recovery
Recovery is mostly dependent on buffer conditions and on how peaks are cut

Selection and combination of purification techniques

- Every technique offers a balance between resolution, capacity, speed and recovery
- So, resins should be selected to meet the objectives of the purification step
- GOAL: Fastest route to get a product of required purity

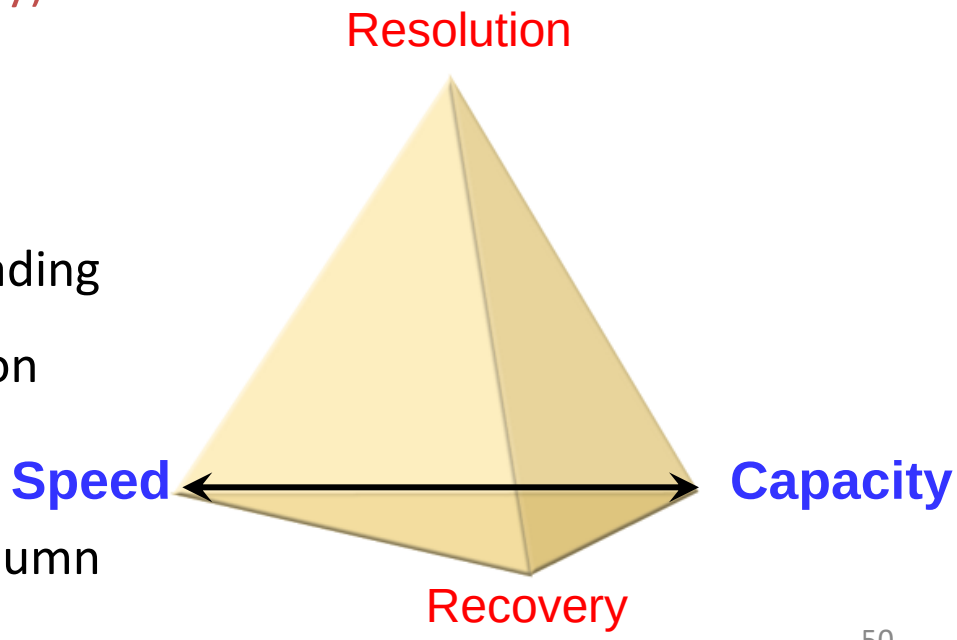


For Efficient Purification Strategies



Capture

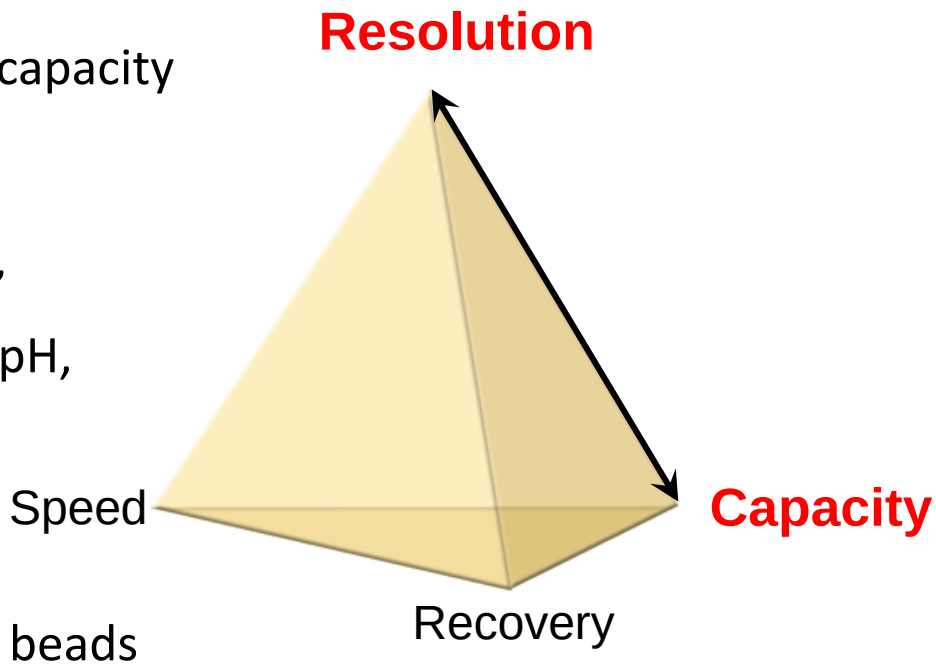
- GOAL:Initial purification of the target molecule from clarified source material.
- Rapid isolation, and concentration (volume reduction) of the target protein
- BONUS: Concentration (smaller and faster columns). Stabilization (removal of proteases)
- OPTIMIZATION: Speed and Capacity: Use Macroporus and Highly Substituted matrix
- Most suitable techniques: IEX / HIC / (Industry)
  or Affinity /IMAC/ IEX / HIC (Academics)
- Maximize binding of the target proteins and
  minimize binding of contaminants during loading
- Maximize protein purity during wash & elution
- Higher speed that do not affect
  considerably the dynamic capacity of the column



Intermediate Purification

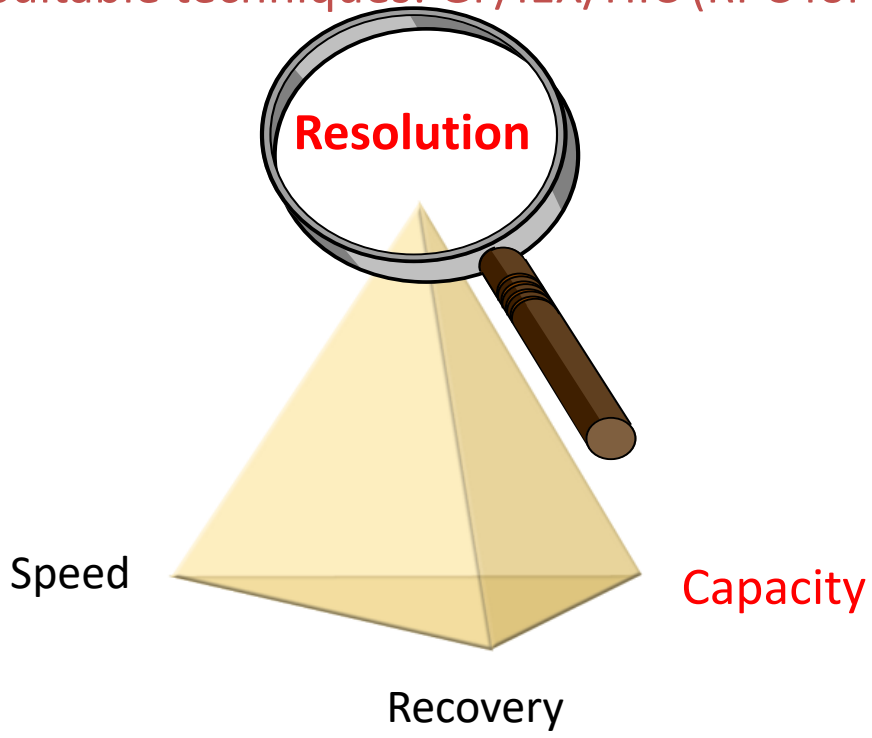
- **Goal:** Removal of major impurities
- Focus mainly on resolution
- Continuous gradient or multi-step elution
- Most suitable techniques: IEX / HIC or expensive affinity
- For good resolution use around 20% of column capacity with HIC or IEX
- Use a different technique (EIX, HIC, GF, Affinity),
- Or change the selectivity (same IEX at different pH, different ligands or salts concentr for HIC, etc.):

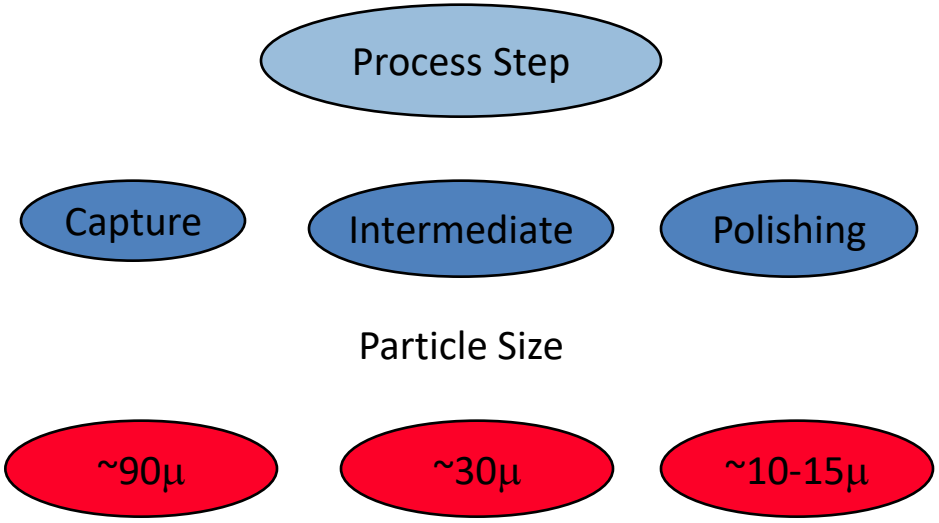
  Selectivity optimization
- Increase efficiency by using non-porous smaller beads



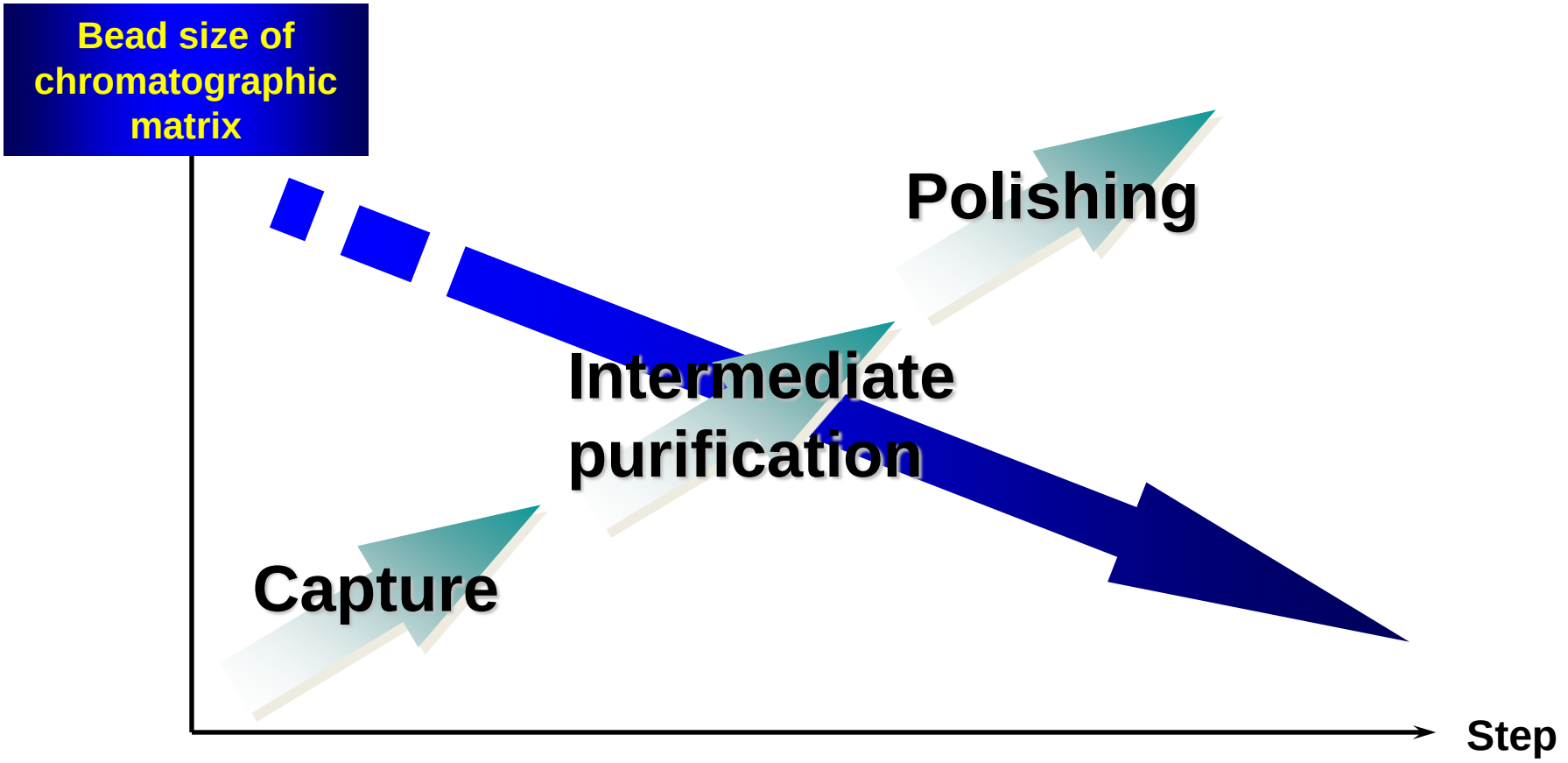
Polishing

- Final removal of trace contaminants, or separation of closely related substances, like structural variants of the target protein and aggregates.
- End product of required high level purity and homogenicity (oligomeric conformation, post-translational modificatons, phosphorilation, etc)
- Suitable techniques: GF/IEX/HIC (RPC for suitable proteins)

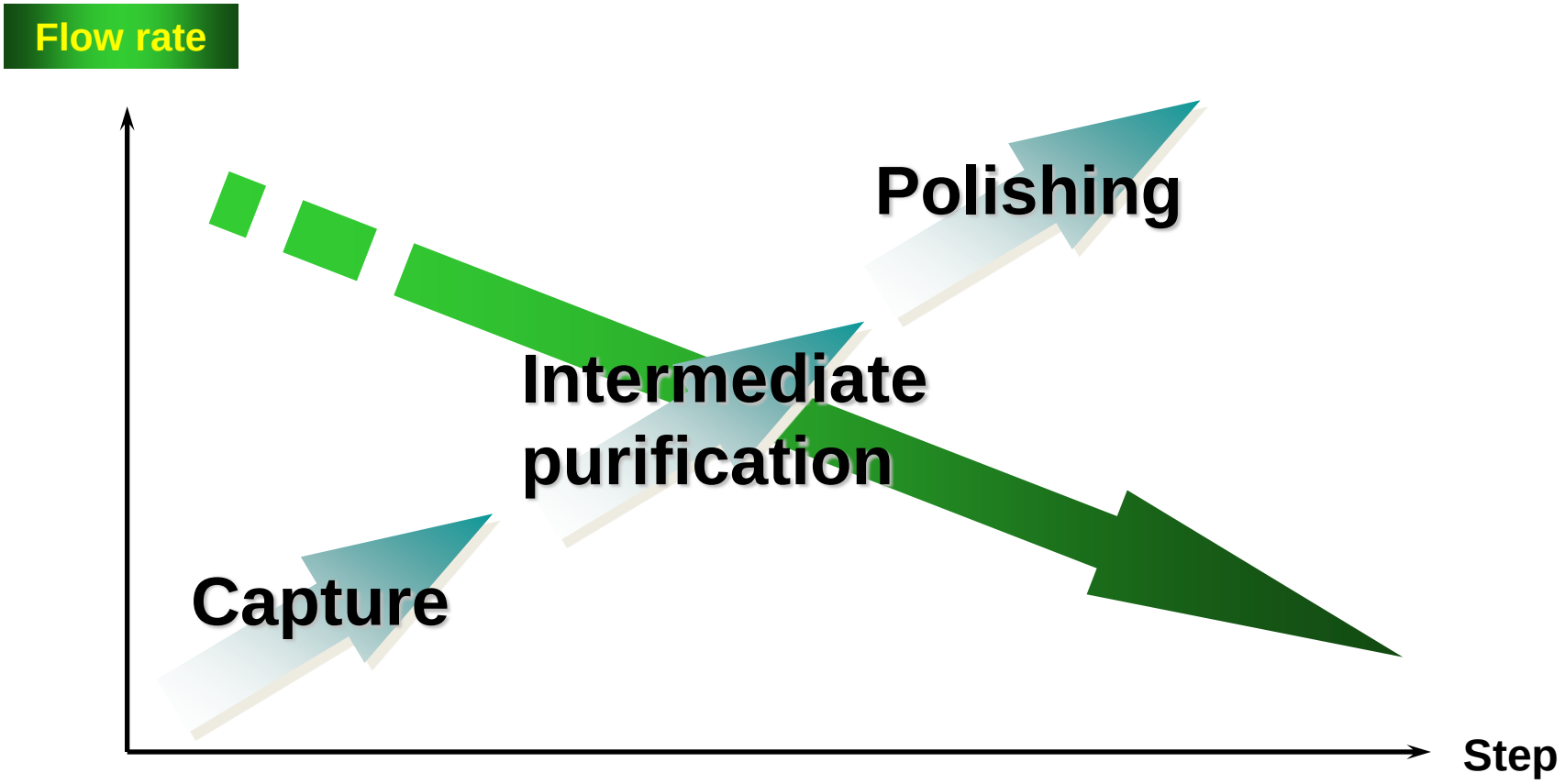




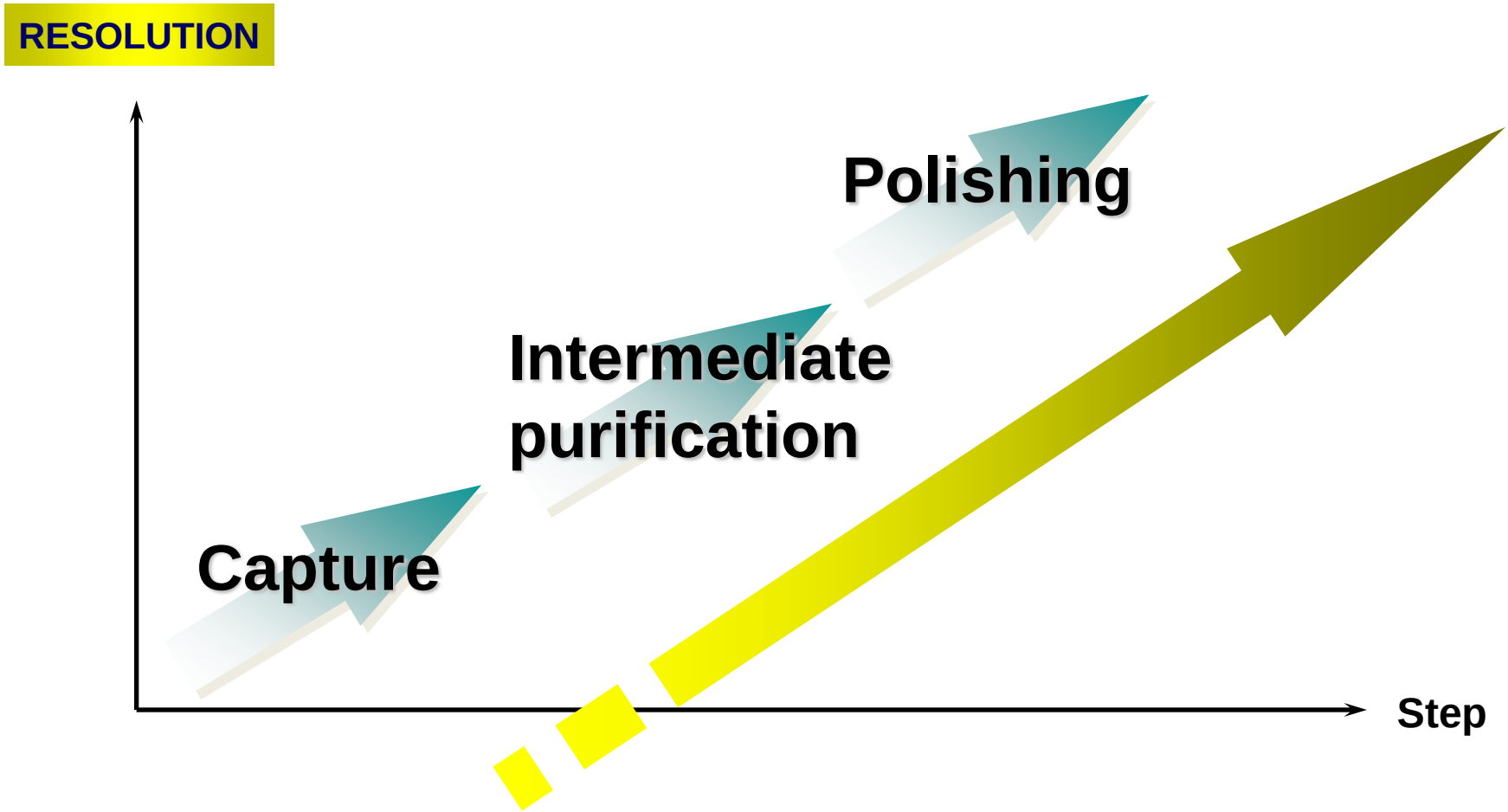
Three Phase Strategy



Three Phase Strategy



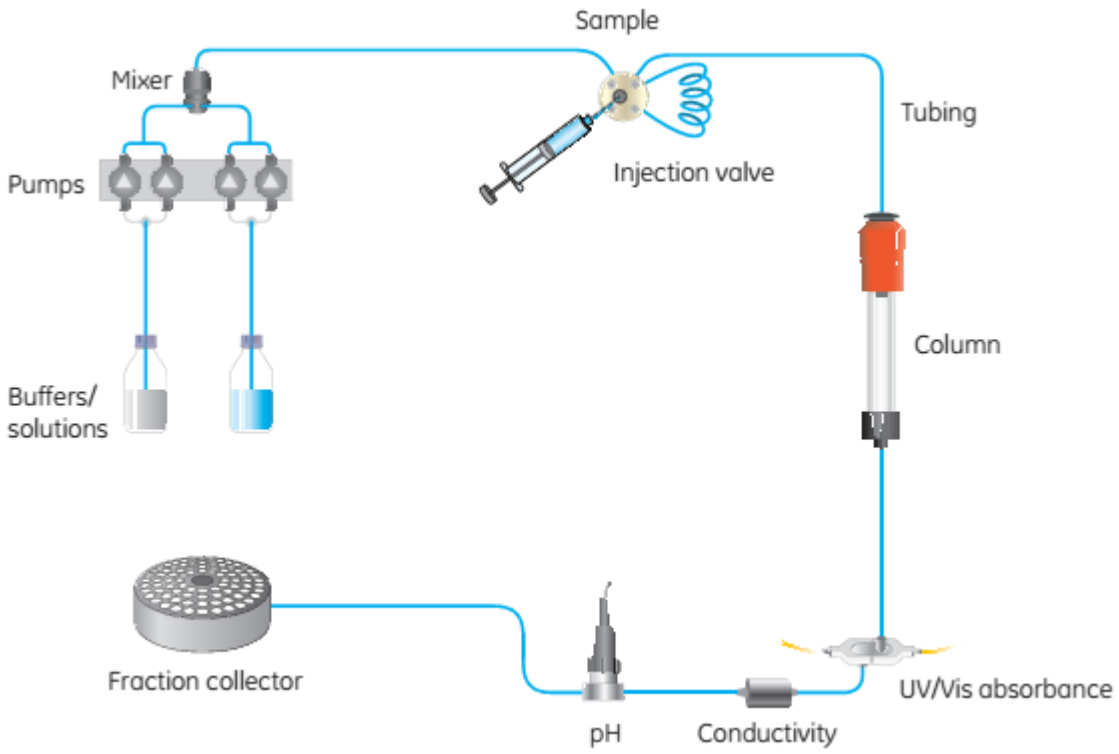
Three Phase Strategy



FPLC System

FPLC Software

Typical flow path for a chromatography system



ÄKTA explorer

ÄKTA Avant 25

ÄKTAxpress

ÄKTAxpress

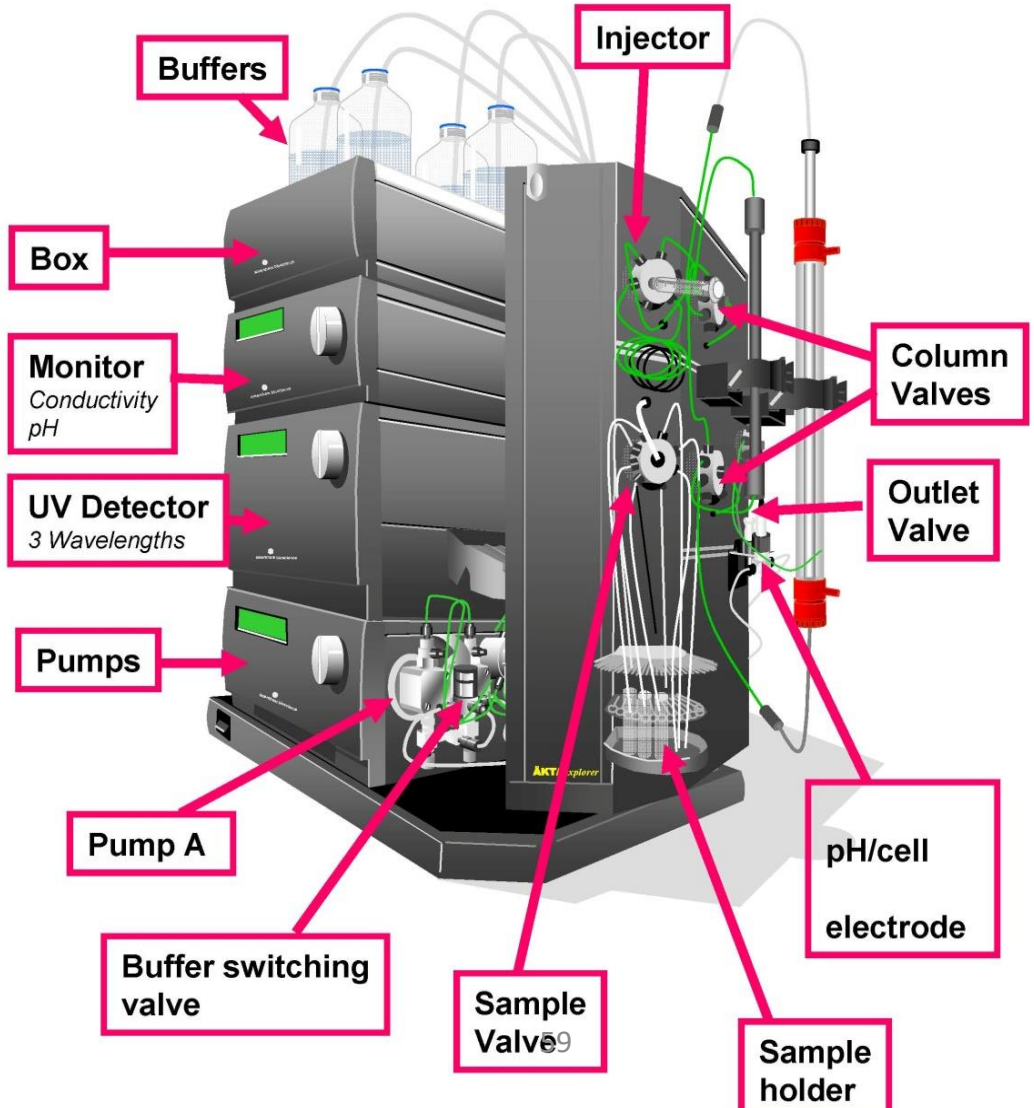
Chromatography system designed for automated, multistep protein purification of both single and multiple samples.

ÄKTAprocess

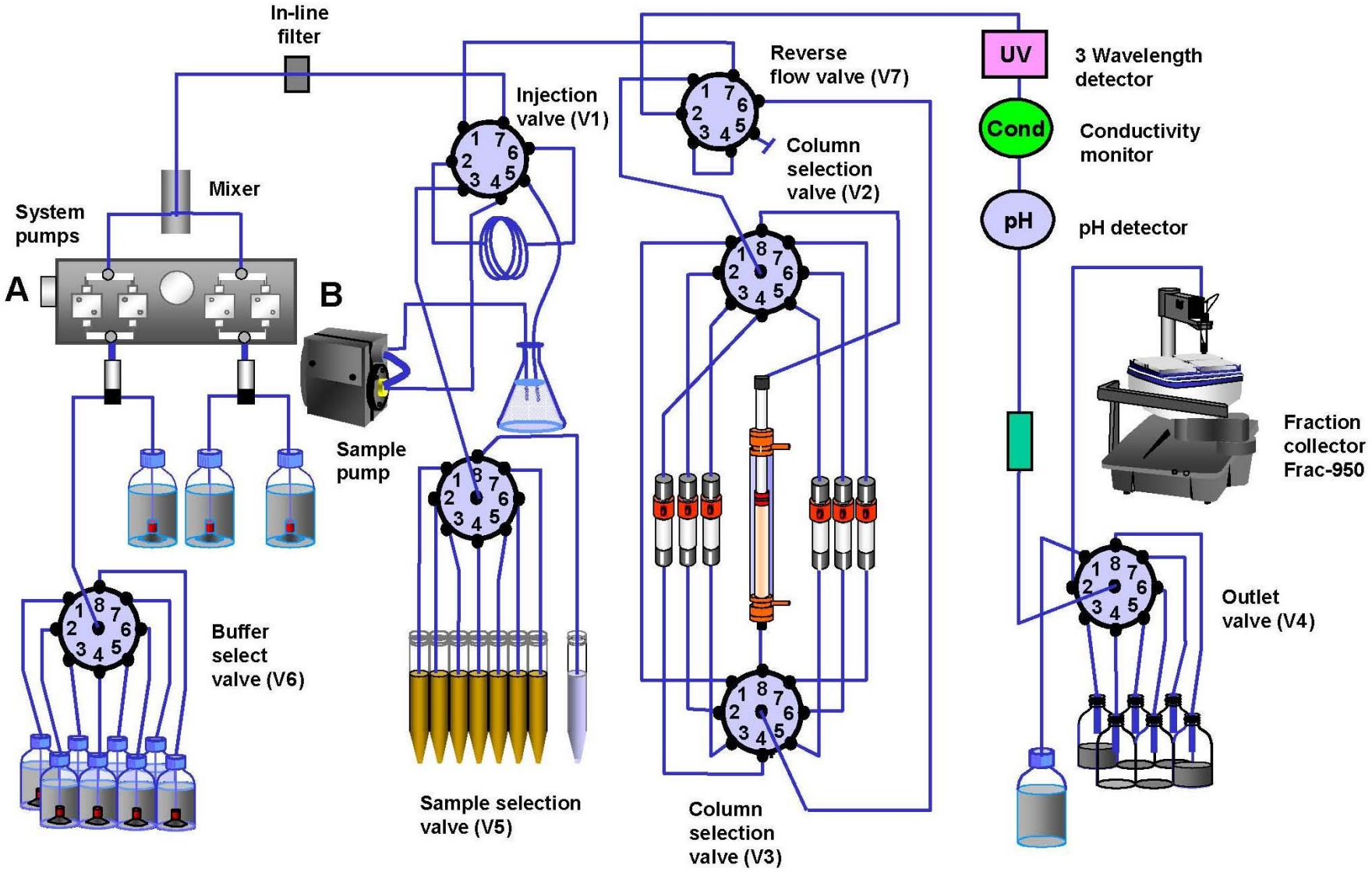
System for Process Scale-up, and Large-scale manufacturing

Flow rates up to 2000 l/h for large-volume manufacturing

AKTA Explorer



AKTA Explorer



System Orientation

Interactive instrument display delivers realtime information.

Buffer tray for convenient, large capacity storage.

One wet side with clear view of flow path for convenience and usability. Module-

Cooled, built-in fraction collector prevents local heating of samples.

Snap-on column connection * provides additional convenience

Modular design for easy addition / exchange of valves, pumps, and other components

Swivel foot for easy access to all parts of the instrument

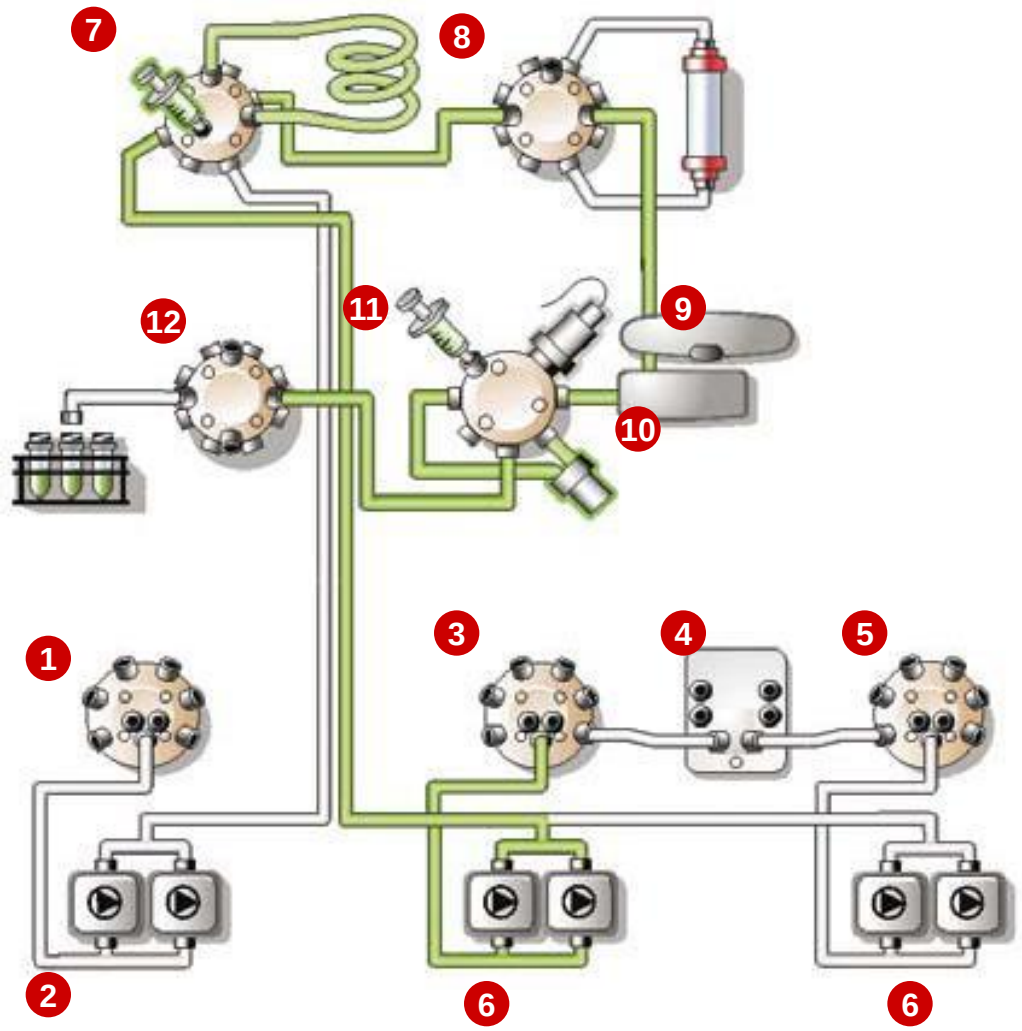
Automatic sample application eliminates manual re-plumbing.

High precision system pumps with integrated air sensors.

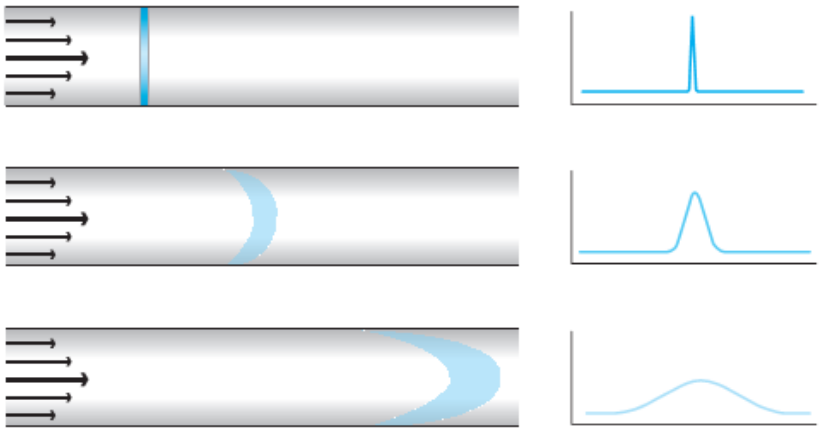
* To be released at a later date

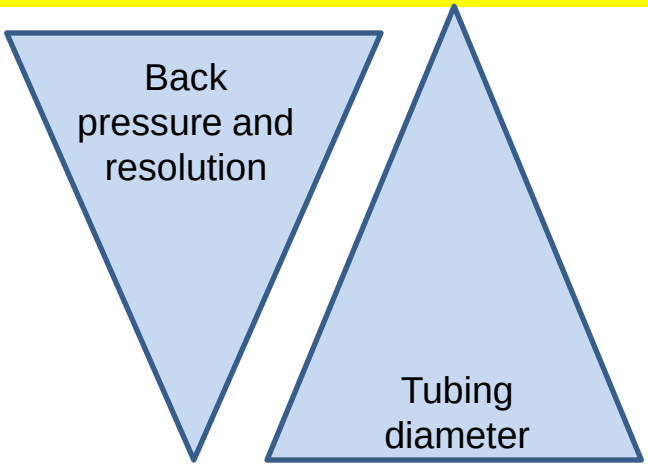
System flow path for optimized performance

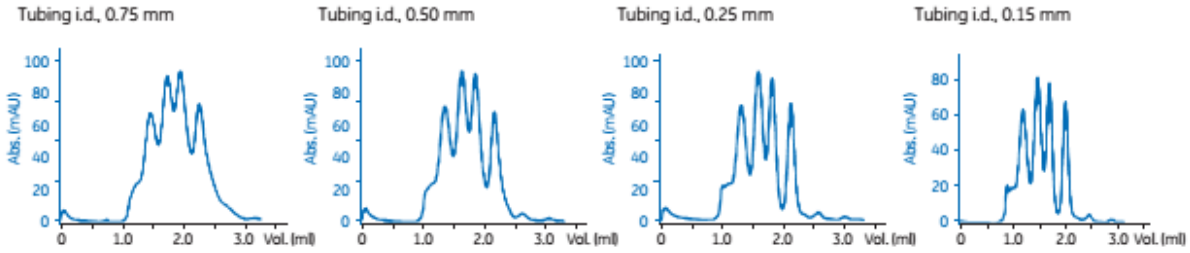
1. Sample Inlet valve
2. Sample pump
   - Flow rate: 0.001 to 25 ml/min
   - Pressure range: 0 to 10 MPa
3. Inlet valve A
4. Quaternary valve
5. Inlet valve B
6. System pump
   - Flow rate: 0.001 to 25 ml/min
   - Pressure range: 0 to 20 MPa
7. Injection valve
8. Column valve
9. UV monitor
   - Wavelength range: 190 to 700 nm
   - in steps of 1 nm, up to 3 wavelengths.
   - Flow cells: 2 or 10 mm optical path
10. Conductivity monitor
    - Conductivity reading range:
    - 0.01 mS/cm to 999.99 mS/cm
11. pH valve
12. Outlet valve



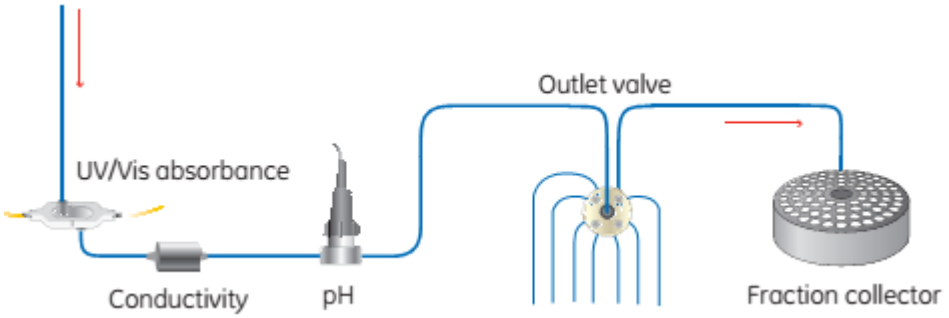
Tubing dimensions affect peak broadening and resolution







Peak broadening in tubing
Liquid flows faster in the middle of a tube



Peak broadening after the UV/Vis detector

Injection Valve

- Sample pump with many sample inlets for different samples
- System pump and Sample pump can be run simultaneously

Loop

Manual injection valve

pH and conductivity Valve

- Bypass of pH flow cell
- Positions for pH calibration
- Flow restrictor

Auto-sampler

Superloop

Column Valves

- Up to five column positions
- Bypass position
- Flow direction change (reversed flow)
- Pressure sensors before and after column

Inlet Valves

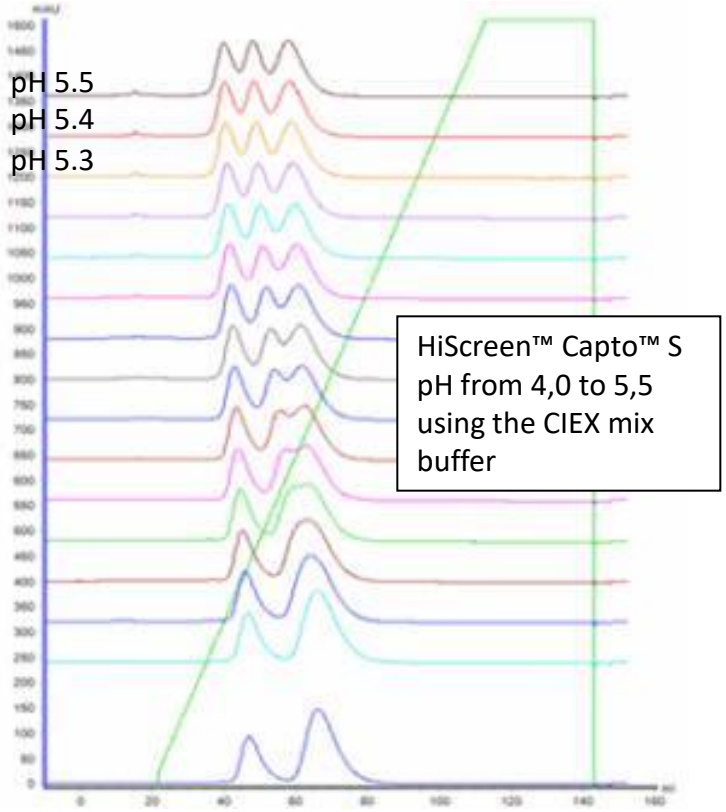
- Air sensor useable with all inlets

Outlet Valve

- Waste
- Fraction collector
- Up to 10 outlet positions

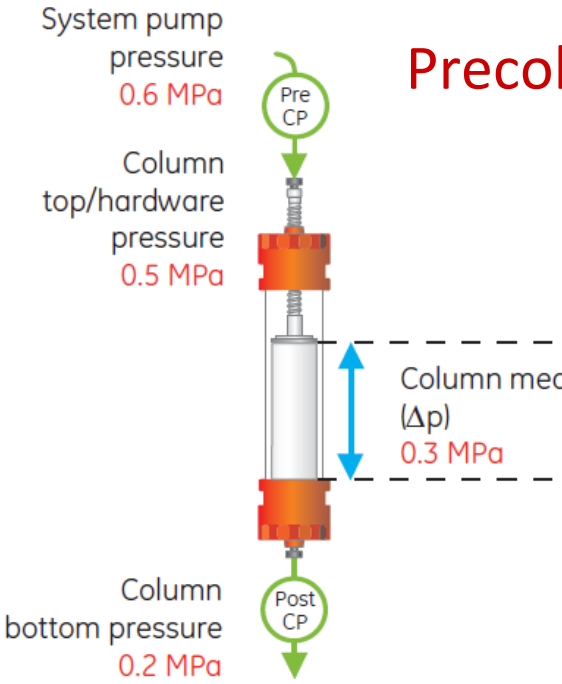
BufferPro – Automatic buffer preparation

- Completely automated, advanced on-line buffer preparation
- Increases throughput and accuracy
- Buffer mixing with corresponding acid and base
- Easy and quick pH-scouting
- 26 buffer systems available



16 pH steps 0.1 units apart,
with no manual buffer titration at all!

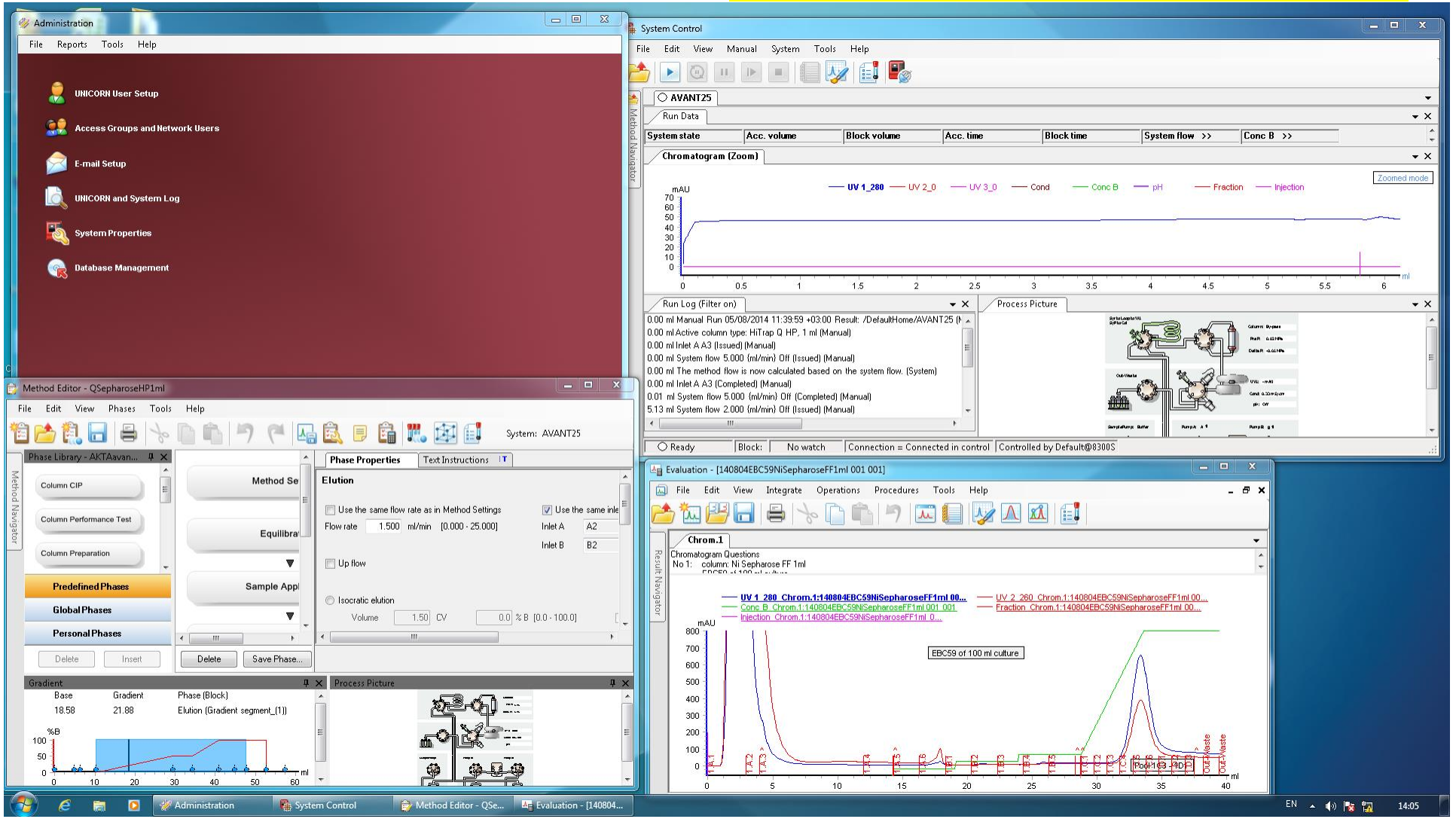
Precolumn pressure and delta pressure (ΔP)



- Pressure sensors integrated into the column valve
  - Before the column to protect column hardware
  - After the column, the pressure difference over the packed media bed (ΔP) is calculated
- Flow regulated mode automatically decreases flow rate when pressure exceeds the preset limit
- Pressure sensors are also connected to the system and sample pumps

Administration

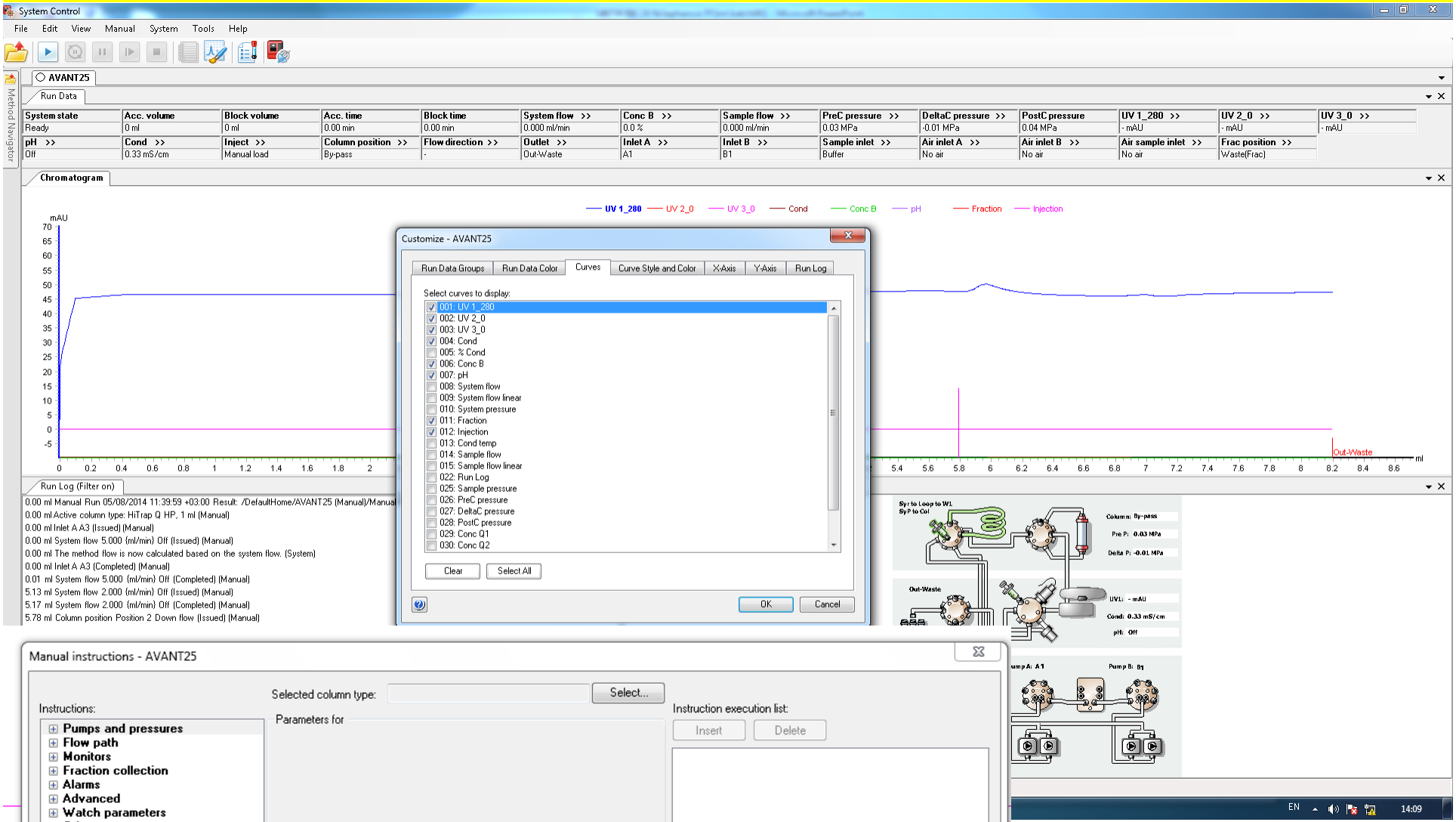
System Control

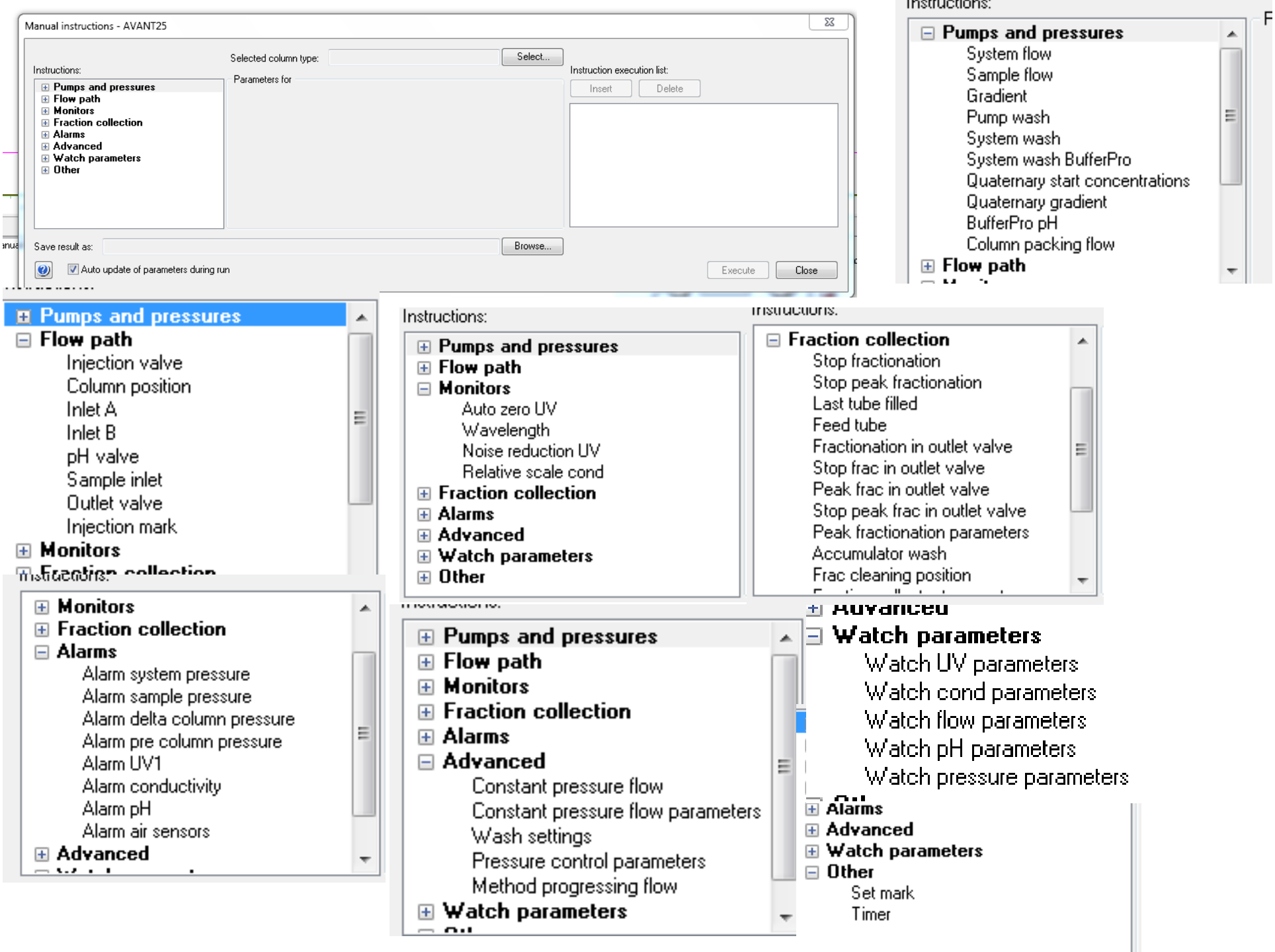


Method Editor

Evaluation

System Control

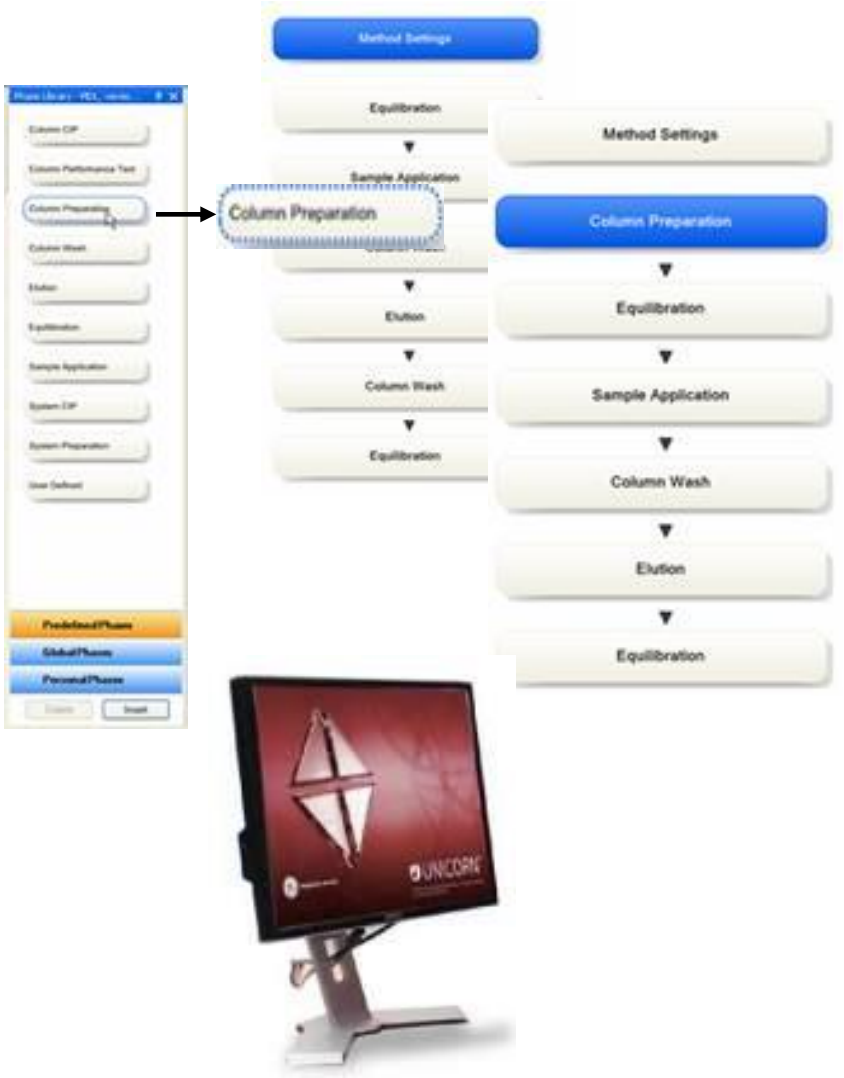



Manual instructions - AVANT25
Selected column type:
Select...
Instructions:
Parameters for
Instruction execution list:
Insert
Delete
Pumps and pressures
Flow path
Monitors
Fraction collection
Alarms
Advanced
Watch parameters
Other
Save result as:
Browse...
Auto update of parameters during run
Execute
Close
Instructions:
Pumps and pressures
System flow
Sample flow
Gradient
Pump wash
System wash
System wash BufferPro
Quaternary start concentrations
Quaternary gradient
BufferPro pH
Column packing flow
Flow path
Pumps and pressures
Flow path
Injection valve
Column position
Inlet A
Inlet B
pH valve
Sample inlet
Outlet valve
Injection mark
Monitors
Instructions:
Pumps and pressures
Flow path
Monitors
Auto zero UV
Wavelength
Noise reduction UV
Relative scale cond
Fraction collection
Alarms
Advanced
Watch parameters
Other
Instructions:
Fraction collection
Stop fractionation
Stop peak fractionation
Last tube filled
Feed tube
Fractionation in outlet valve
Stop frac in outlet valve
Peak frac in outlet valve
Stop peak frac in outlet valve
Peak fractionation parameters
Accumulator wash
Frac cleaning position
Monitors
Fraction collection
Alarms
Alarm system pressure
Alarm sample pressure
Alarm delta column pressure
Alarm pre column pressure
Alarm UV1
Alarm conductivity
Alarm pH
Alarm air sensors
Advanced
Pumps and pressures
Flow path
Monitors
Fraction collection
Alarms
Advanced
Constant pressure flow
Constant pressure flow parameters
Wash settings
Pressure control parameters
Method progressing flow
Watch parameters
Advanced
Watch parameters
Watch UV parameters
Watch cond parameters
Watch flow parameters
Watch pH parameters
Watch pressure parameters
Alarms
Advanced
Watch parameters
Other
Set mark
Timer

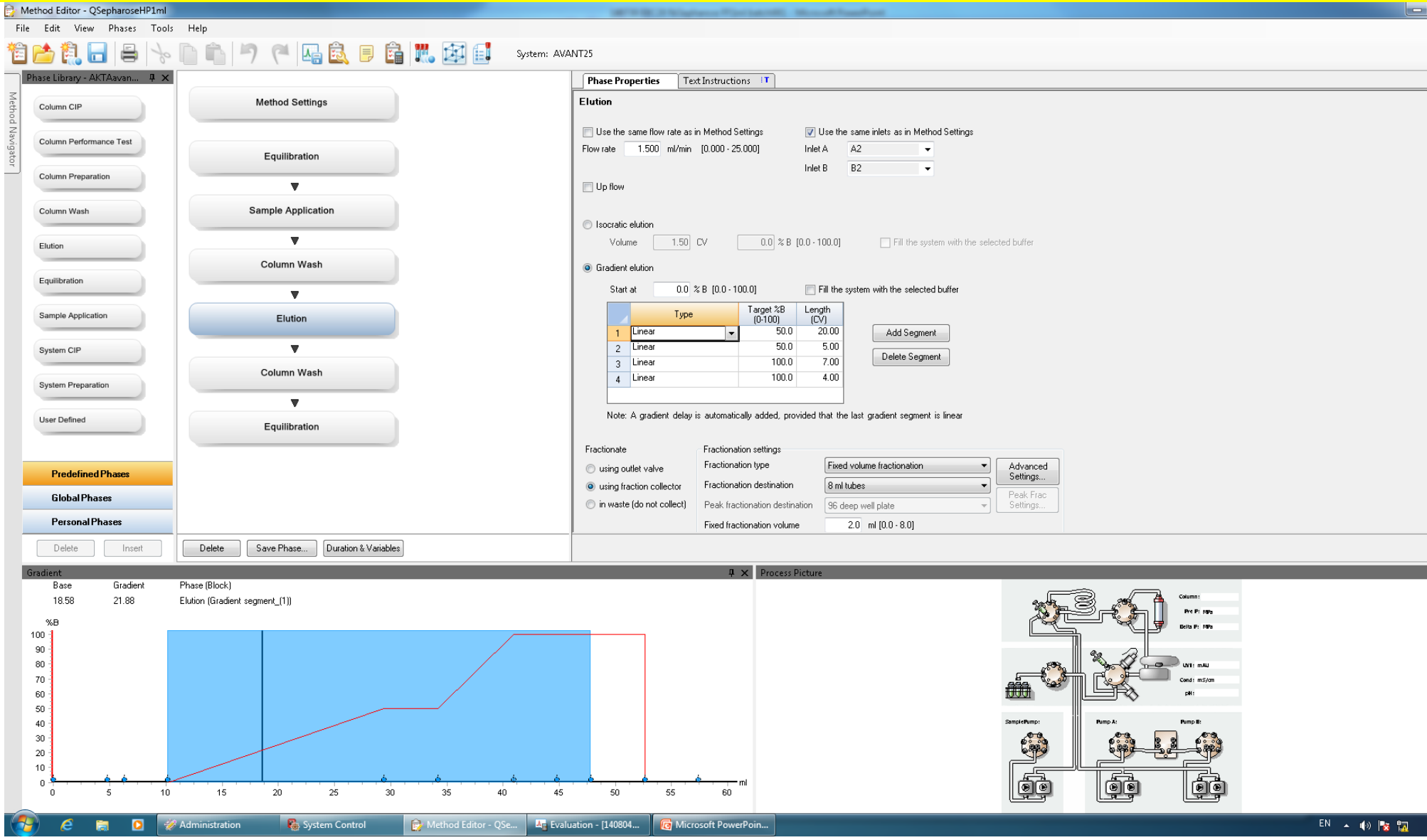
Method Editor

UNICORN™ 6

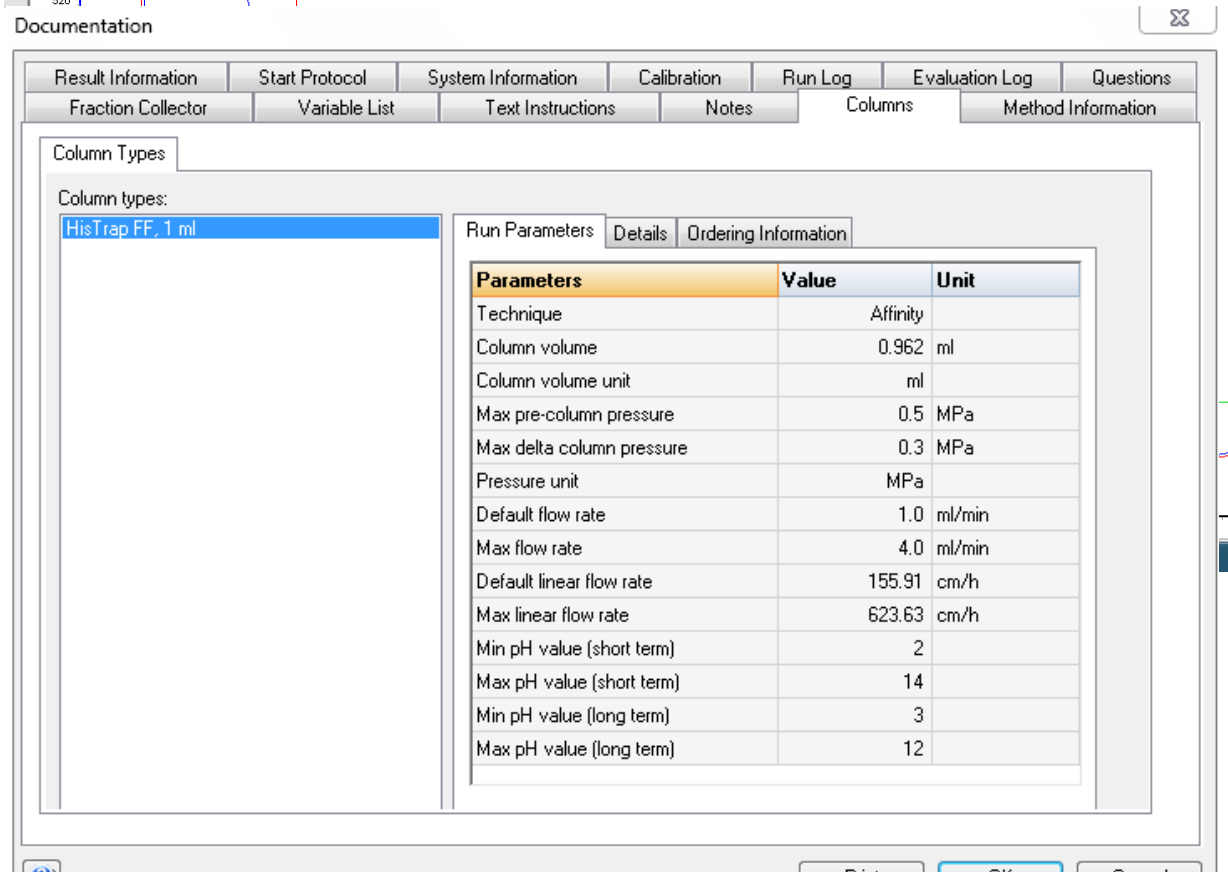
- Contains all instructions used for controlling the chromatographic run
- Easy and flexible method creation - Drag and drop from the phase library into the method outline
- Use pre-defined methods / phases or program your own
- Automatic calculation of volume, pressure and flow rate settings for columns
- Built-in application support



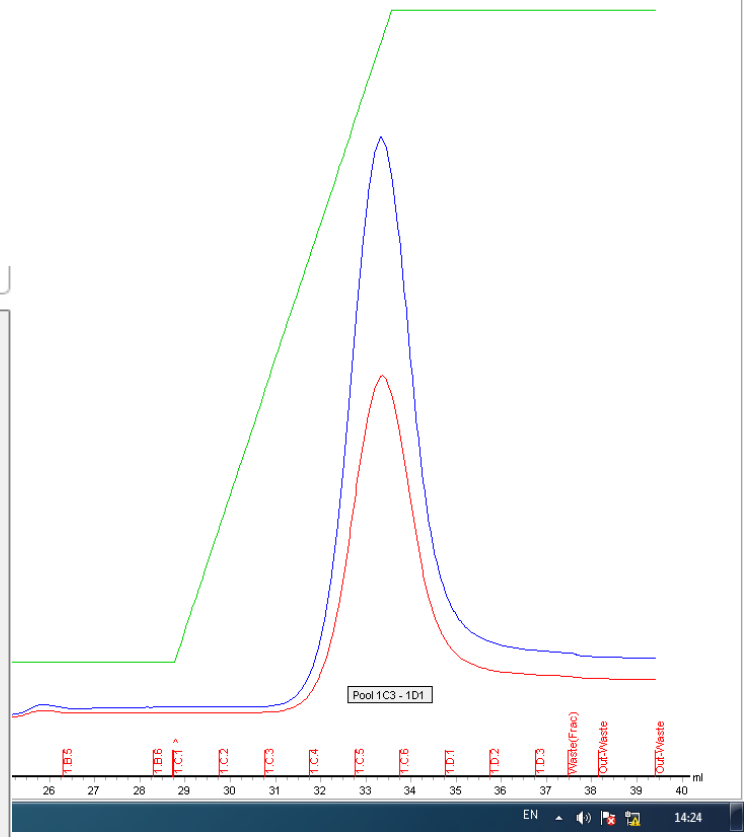
Method Editor



Evaluation

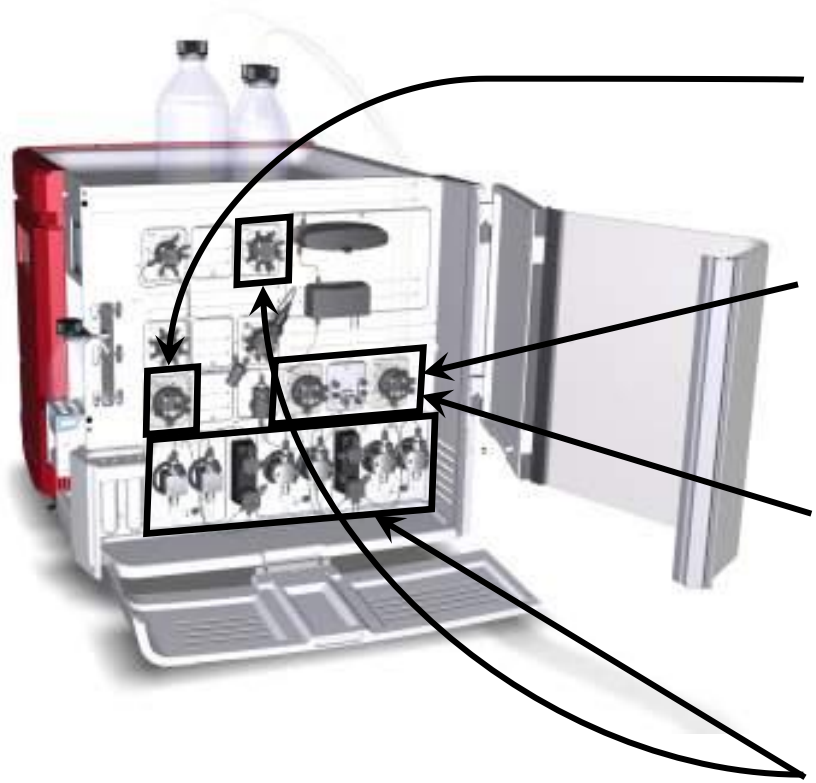


Parameters	Value	Unit
Technique	Affinity	
Column volume	0.962	ml
Column volume unit	ml	
Max pre-column pressure	0.5	MPa
Max delta column pressure	0.3	MPa
Pressure unit	MPa	
Default flow rate	1.0	ml/min
Max flow rate	4.0	ml/min
Default linear flow rate	155.91	cm/h
Max linear flow rate	623.63	cm/h
Min pH value (short term)	2	
Max pH value (short term)	14	
Min pH value (long term)	3	
Max pH value (long term)	12	



13.09 min Base SameAsMain
13.09 min Last tube filled Pause (Issued) (Processing)
13.10 min Last tube filled Pause (Completed)
13.10 min Fractionation Volume 8 ml tubes 2.0 {ml} Next tube (Issued) (Processing)
13.10 min Fractionation Volume 8 ml tubes 2.0 {ml} Next tube (Completed)
13.10 min End_Block (Issued) (Processing) (Completed)
13.10 min Block Gradient segment_(1) (Issued) (Processing) (Completed)
13.10 min Base SameAsMain
13.10 min Gradient 5.0 {%B} 0.00 {CV} (Issued) (Processing)
13.11 min Gradient 5.0 {%B} 0.00 {CV} (Completed)
13.11 min System wash 15 {ml} Outlet valve (Issued) (Processing)
13.11 min Continue 04/08/2014 11:16:49 +03:00 (Issued) (System)
13.11 min Continue 04/08/2014 11:16:49 +03:00 (Completed) (System)
13.11 min System wash 15 {ml} Outlet valve (Completed)

Scouting runs for method optimization



Sample conditions

Wash conditions

Elution conditions

Entire process

Factors (X´s):

Load pH
Load conductivity
Load concentratior
Mass load
Wash volume
Wash pH
Wash conductivity
Elution pH
Gradient elution
Step elution level
Cut OD
Elution Additive
Media type
Column size
Bed Height
Flow rate
Residence time

Responses (Y´s):

External data:

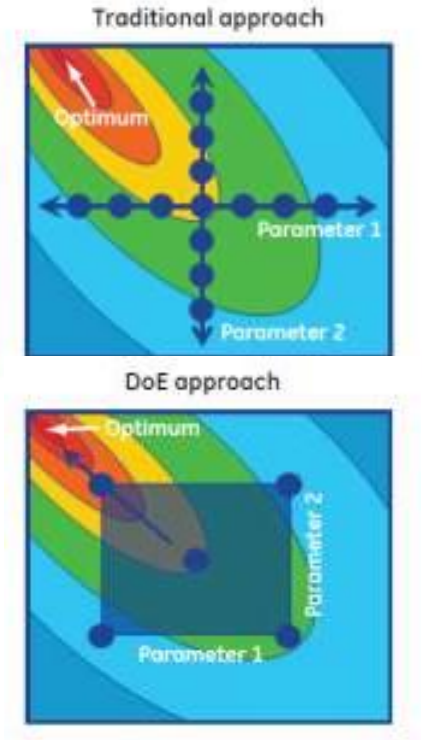
Capacity
DBC (Frontal analysis)
Yield
Purity/Selectivity
Molecular weight
Activity
HCP
DNA
Aggregates
Protein A

Peak Data:

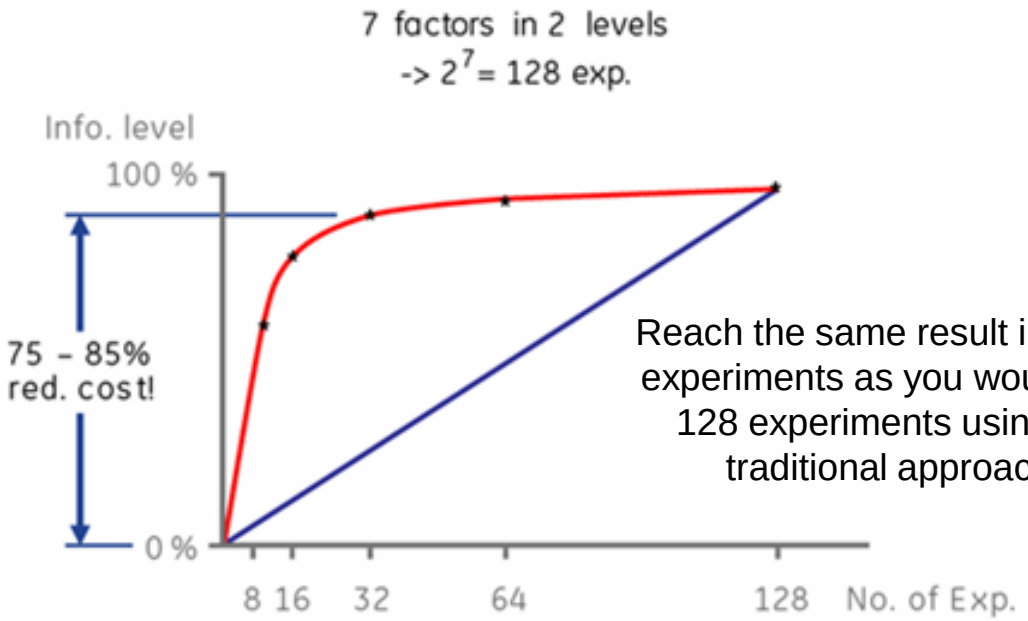
Area
Concentration
Amount
Resolution
Asymmetry
Plates per meter

DoE: Design of Experiment

A tool for easy method optimization



DoE measures the effect of each factor individually and in combination, allowing detection of interaction to give you more precise results quicker

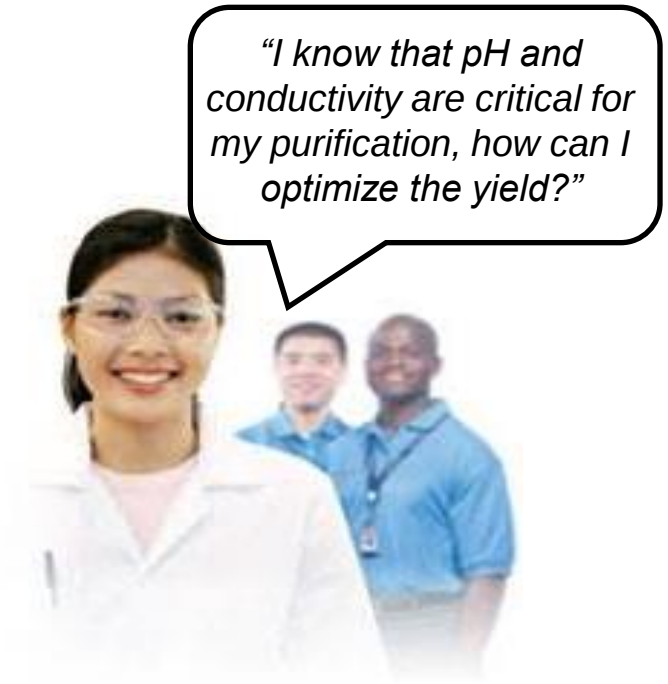


Reach the same result in 16-32 experiments as you would with 128 experiments using the traditional approach!

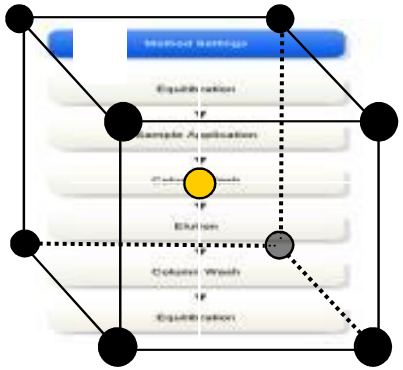
UNICORN™ 6 allows the maximum amount of information to be obtained from a minimum number of experiments with DoE - now an integrated part of UNICORN 6!

Integrated experimental design for quicker results

DoE could also be: ”Design of Experience”

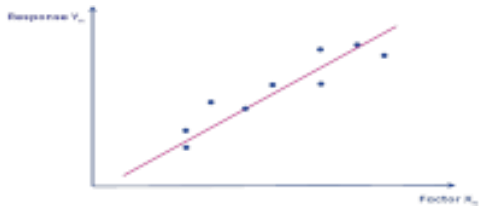


DoE method scheme

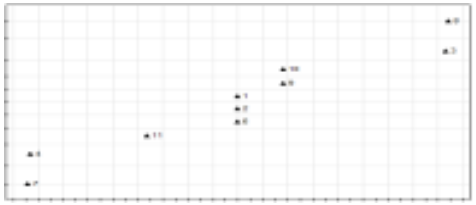


Scouting run

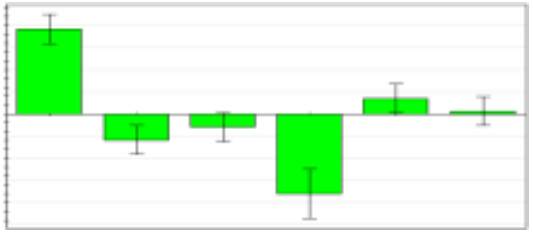
Model creation



Model validation



Decision using model



Literature

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http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/AMERSHAMHandbookProtPurific%20%281%29.pdf

- GE-HEALTHCARE Strategies for Protein Purification Handbook
http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/GE_StratProtPurificHanbook.pdf

- GE-HEALTHCARE Protein purification Applications
http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/AMERSHAM_ProtPurifApplicGuide.pdf

- GE-HEALTHCARE Protein and Peptide Purification - Technique selection guide
http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/AMERSHAM_ProteinPurificGuide.pdf

- GE-HEALTHCARE Purifying Challenging Proteins: Membrane proteins, Multiprotein complexes and Inclusion bodies http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/AMERSHAM_PurifChallengingProt.pdf

- GE-HEALTHCARE ÄKTAdesign Purification Method handbook. ÄKTAexplorer™ or ÄKTApurifier™ chromatography
http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/GE_AKTAdesignPurification%20%281%29.pdf

- GE-HEALTHCARE Purification of tagged proteins
http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/GE_TagProtPurificat.pdf

- TOSOH: General Principles of Chromatography
http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/T0SOH_PrinciplesChromatPoster.pdf

- Mario Lebendiker Website http://wolfson.huji.ac.il/purification/index.html

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- GE-HEALTHCARE ÄKTAdesign Purification Method handbook. ÄKTAexplorer™ or ÄKTApurifier™chromatography
http://wolfson.huji.ac.il/purification/Course92632_2014/Purification%20Strategy/GE_AKTAdesignPurification%20%281%29.pdf

- GE-HEALTHCARE Packing of Gel Filtration column (movie) http://wolfson.huji.ac.il/purification/Purification_Protocols.html

- GE-HEALTHCARE Packing of IEX, Affinity or HIC column (movie) http://wolfson.huji.ac.il/purification/Purification_Protocols.html

- GE-HEALTHCARE Column evaluation (movie) http://wolfson.huji.ac.il/purification/Purification_Protocols.html

- MERCK_MILLIPORE Protein Concentration and Diafiltration by Tangential Flow Filtration
http://wolfson.huji.ac.il/purification/PDF/dialysis/MILLIPORE_TFF.pdf

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- Recombinant protein expression in Escherichia coli: advances and challenges - **G.Rosano et. al.**- *Frontiers in microbiology 2014 | Volume5 | Article 172*
- Challenges and opportunities in the purification of recombinant tagged proteins - **A.Pina et. al.** - *Biotechnol Adv (2013), http://dx.doi.org/10.1016/j.biotechadv.2013.12.001*
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- Protein production and purification – **Core Facilities Europe** – *(2008) - NATURE METHODS | VOL.5 NO.2 | FEBRUARY 2008 | 135*