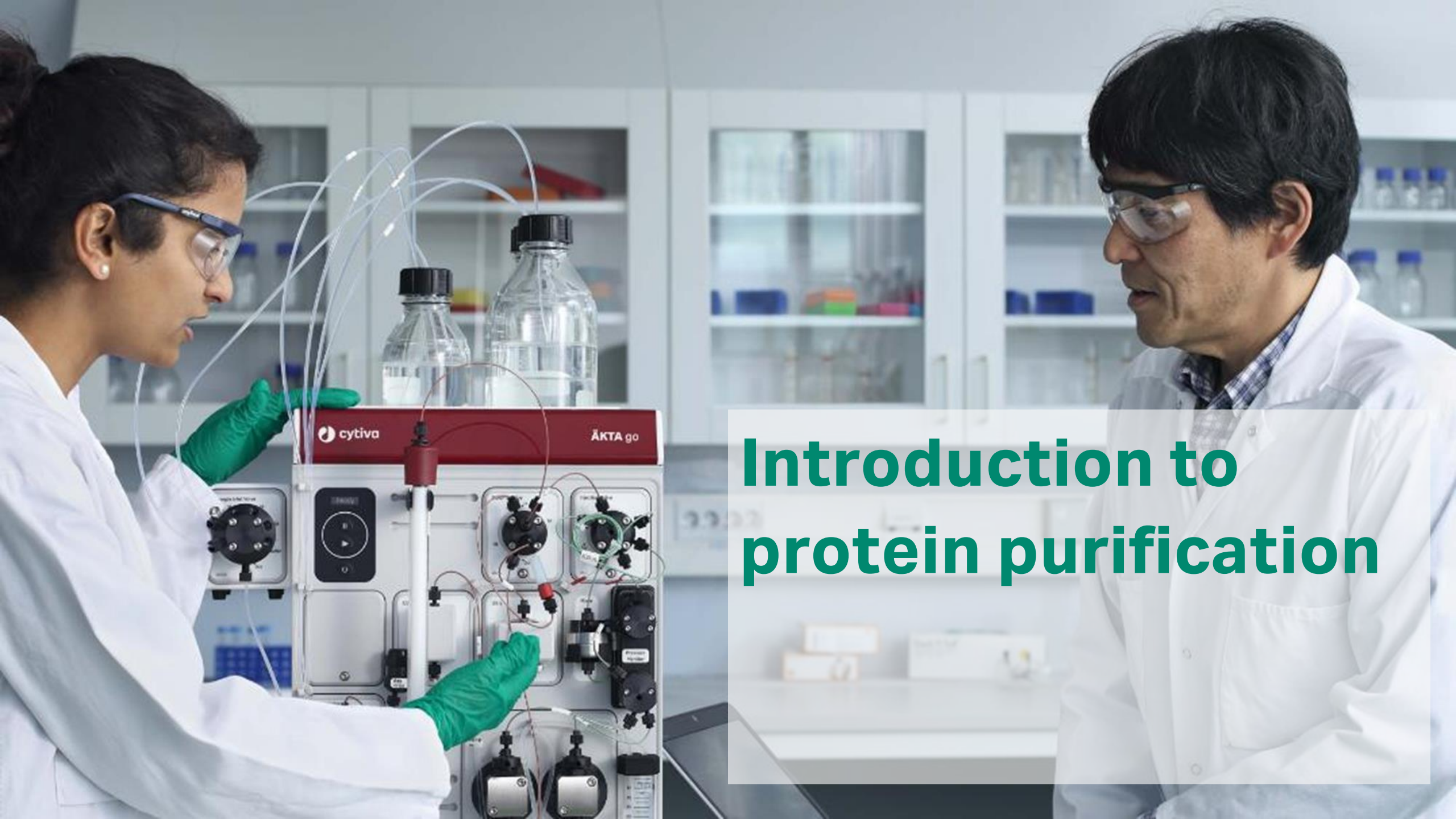

Introduction to protein purification


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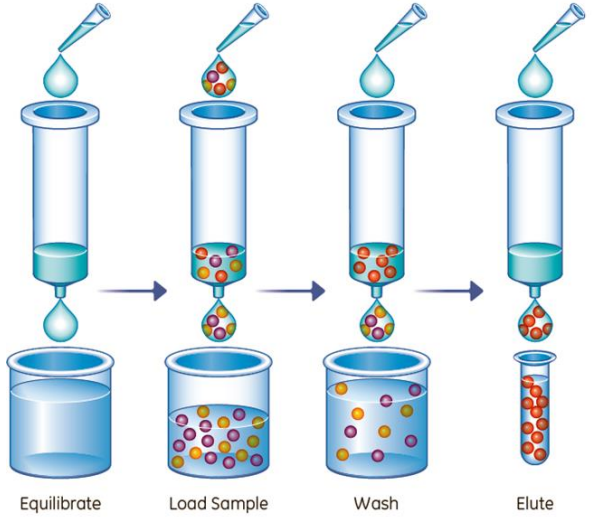
Liquid chromatography is used for protein purification

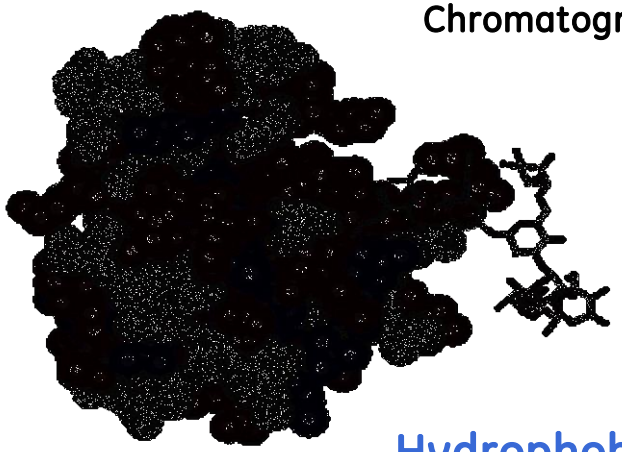
1959—Sephadex™, the world's first gel filtration medium



Each chromatography medium has its purpose

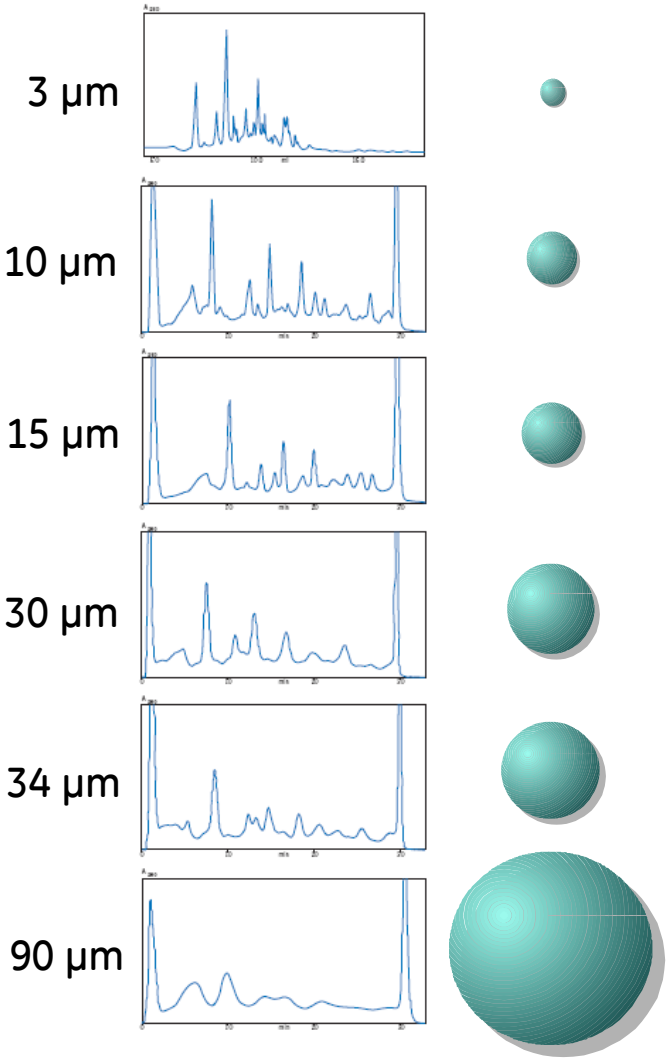
Size/globular volume
Gel Filtration (GF)

Net charge
Ion Exchange Chromatography (IEC)



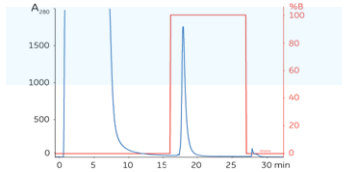
Biospecific affinity
Affinity Chromatography (AC)

Hydrophobicity
Hydrophobic interaction Chromatography (HIC)

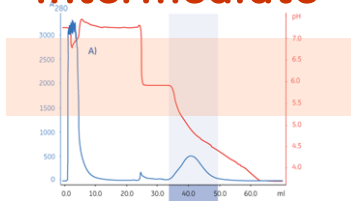


Application requirements

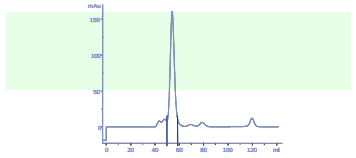
Capture



Intermediate



Polishing

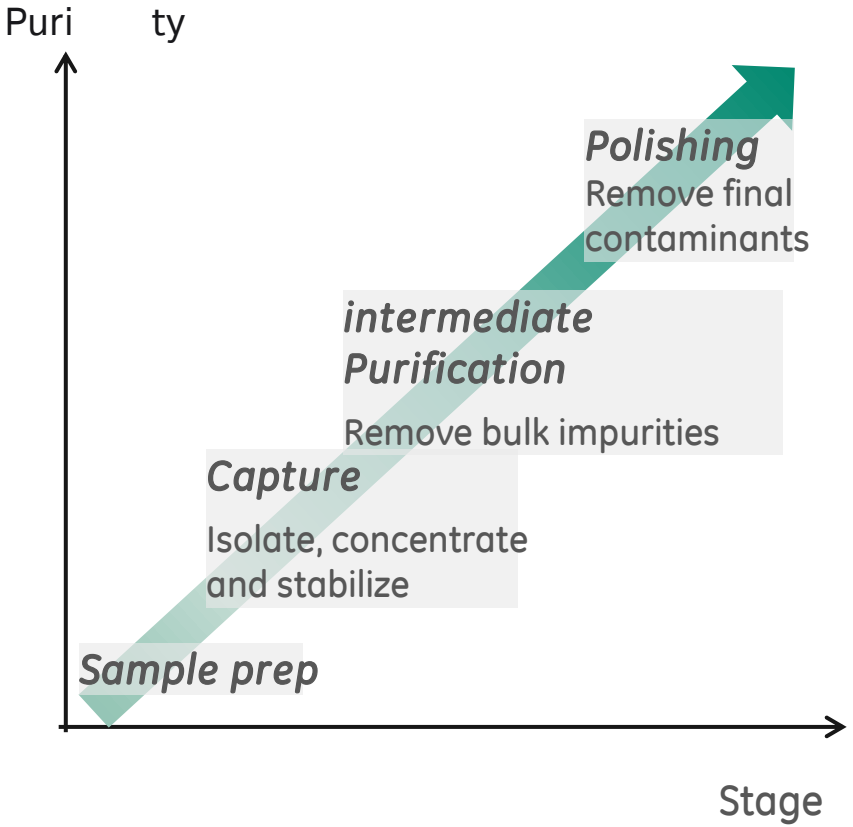


Media/Column
- Technique (*e.g.* Gel Filtration)
- Particle size
- Column format

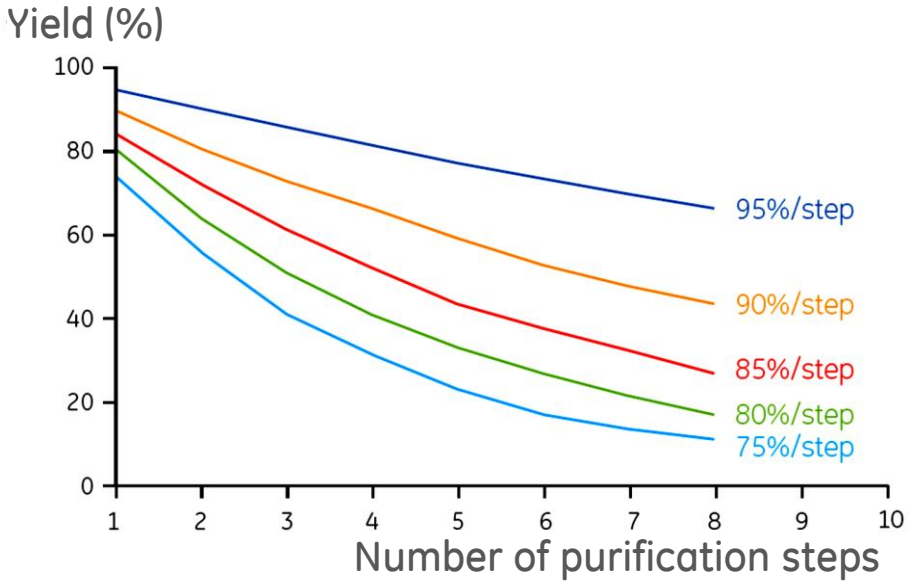
System
- Flow rate
- Dead volume
- Pressure

Introduction to CiPP purification strategy

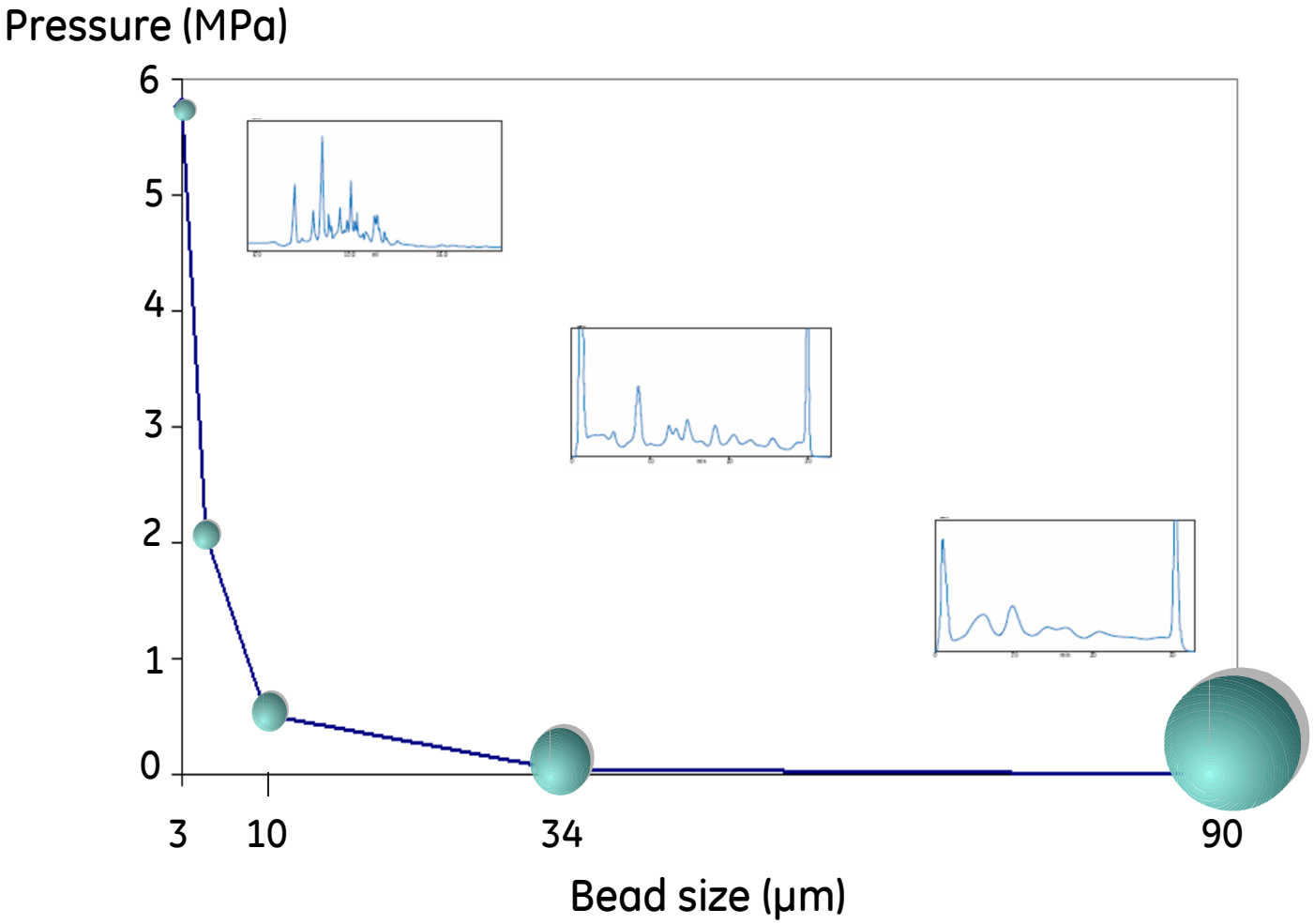
Purification strategy combining multiple steps



Protein recovery plotted against the number of purification steps



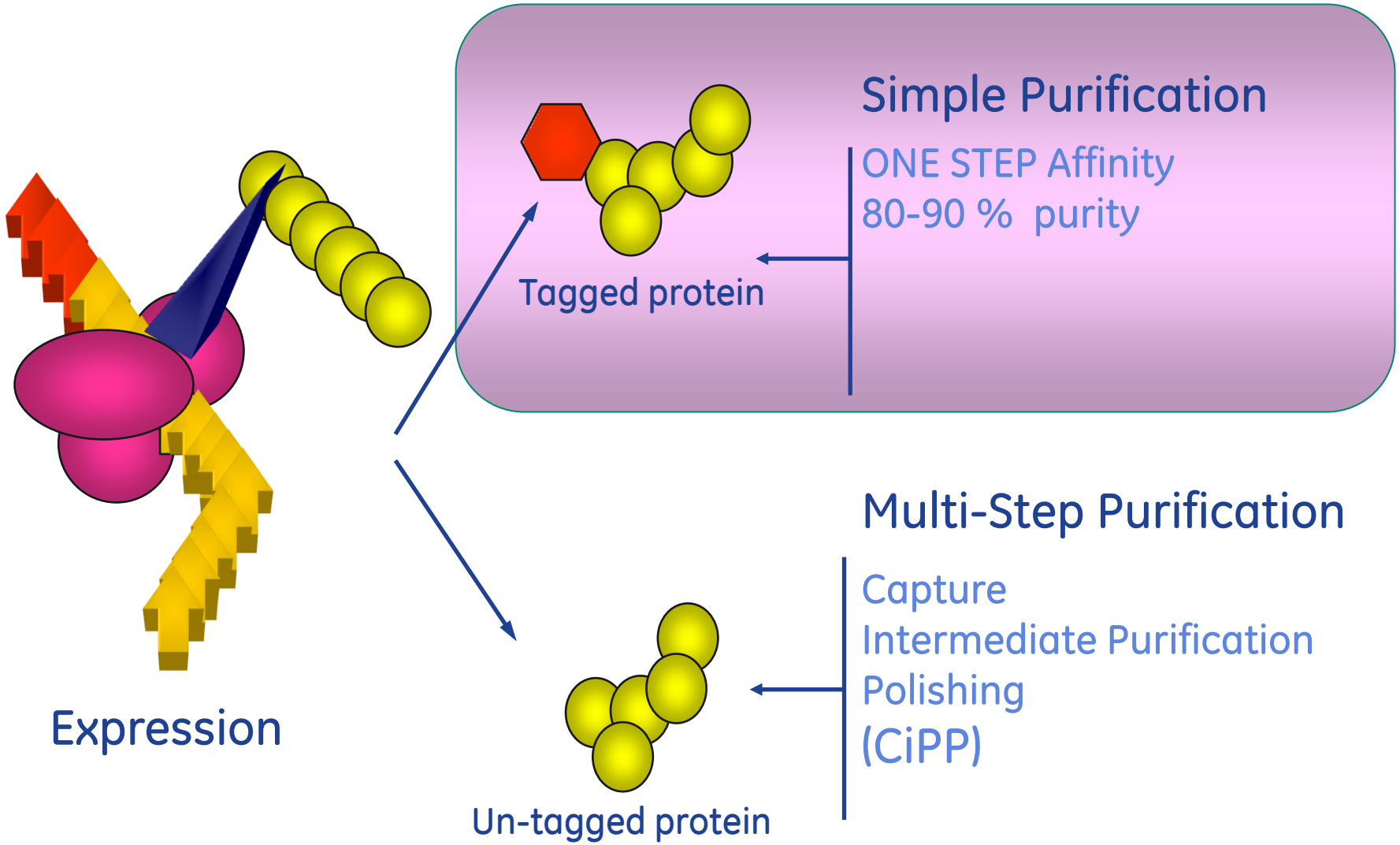
Small beads give high back-pressure



Three phase strategy Ranking of chromatography techniques

Technique	Capture	Intermediate	Polishing	Considerations
GF		★	★★★	limited sample volume limited flow rate range
IEX	★★★	★★★	★★★	
HIC	★★	★★★	★	
AC	★★★	★★★	★★★	protein ligand is sensitive to harsh cleaning conditions
RPC		★	★★★	use of organic solvents, loss of biological activity

Protein purification strategy

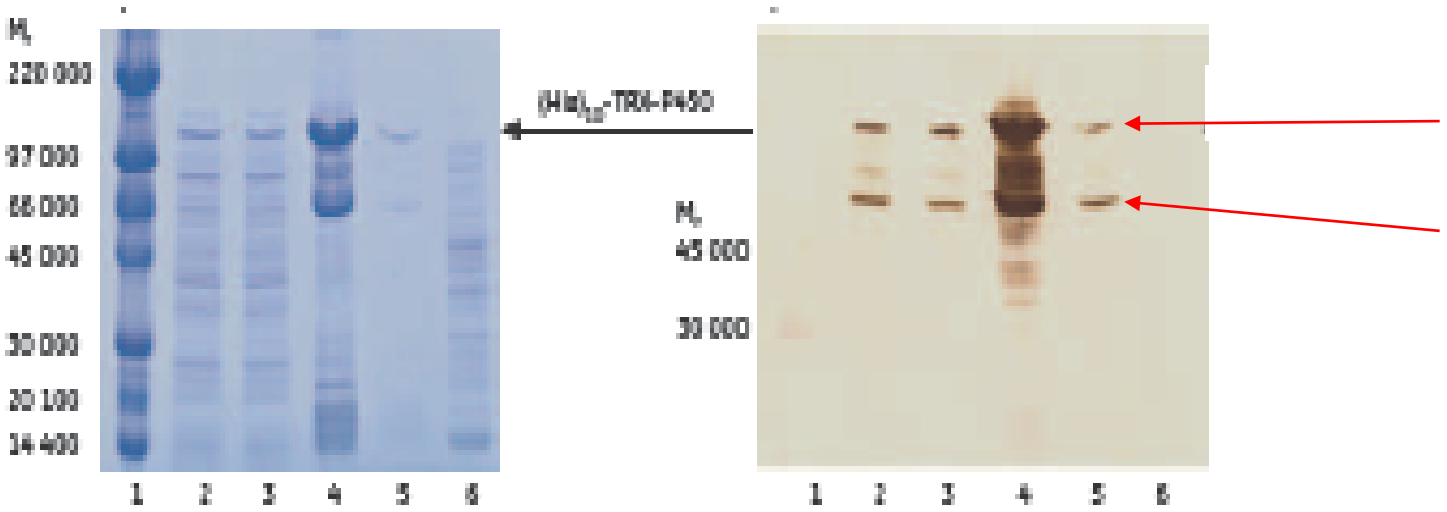


Expression systems

	Bacteria	Yeast	Insect cells	Mammalian cells
Inclusion bodies	+/-	(+)/-	-	-
Secretion	+/-	+	+	+
PTM or Post-translational modification	-	+	+	+
Proteolytic cleavage	+/-	+/-	-	-

+ = Yes **- = No**

Analytical approach and protein degradation



Full length protein

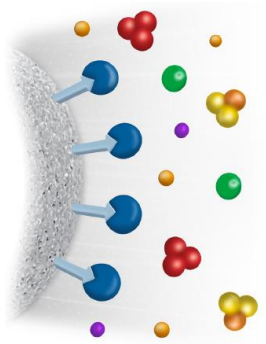
Truncated forms with the tag

SDS-PAGE

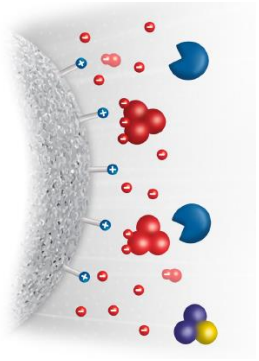
Western blot

The principles of chromatography techniques

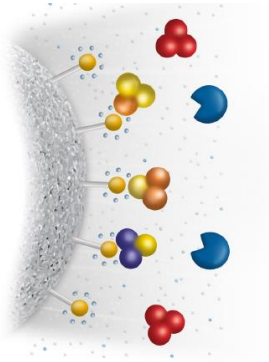
Affinity Chromatography (AC)



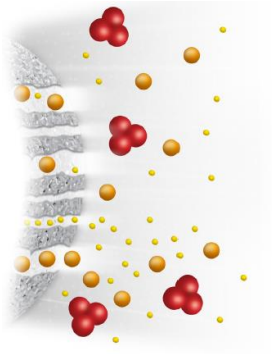
Ion exchange Chromatography (IEX)



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

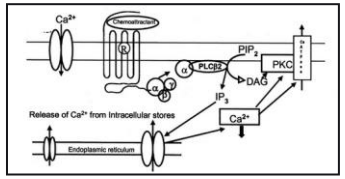
Protein purification is diverse

Basic purification strategy is the same

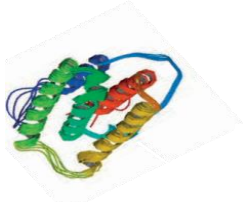
Several types of target protein

- Affinity tagged
- Non-tagged
- From natural source
- Recombinant
- Soluble protein
- Membrane protein
- Protein complex

Different objectives



Functional studies



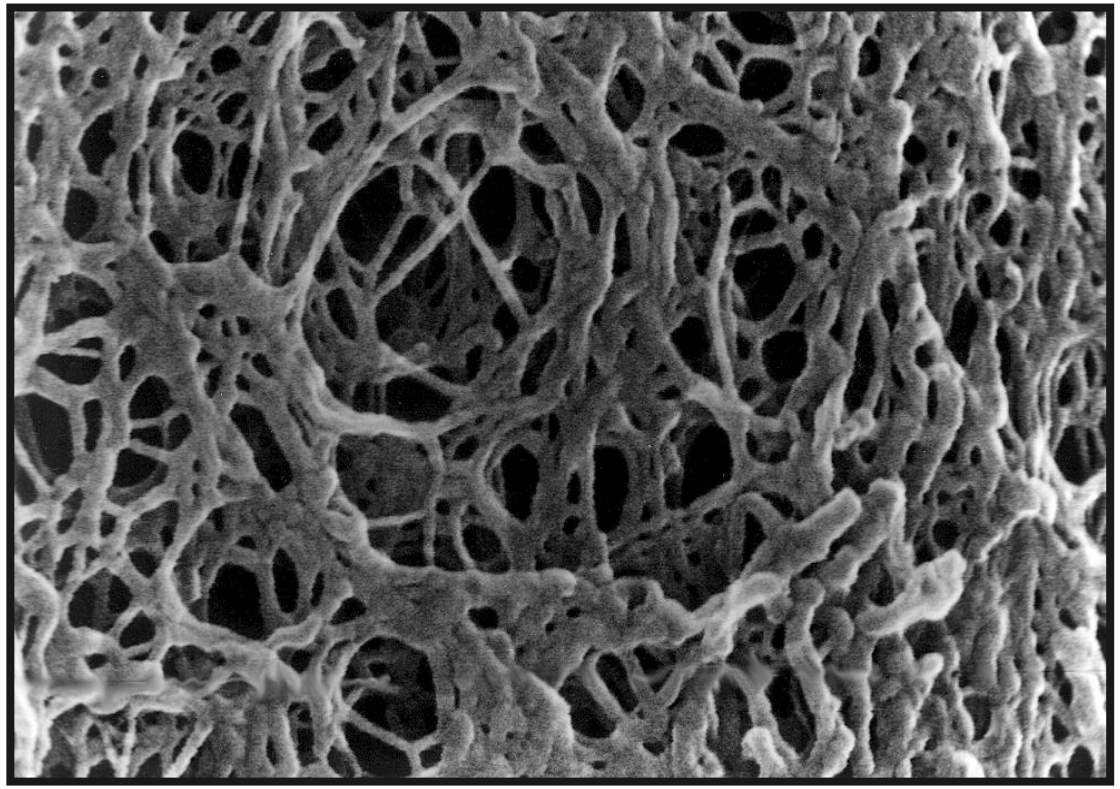
Structural studies

Use as biochemical reagent

Several scales

Various equipment

Gel structure



AGAROSE

A good gel for gel filtration contains about

95% water

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Introduction to Affinity Chromatography (AC)

Achieve desired purity with ease in routine protein purification

Choosing ligands

Mono-specific ligands

- Has affinity for a single substance such as:

- Antigen ⟷ Antibody
- Hormone ⟷ Receptor

- Example: MBP, GST etc...
- Usually home-made affinity media
- Elution protocols based on general principles

Group-specific ligands

- Has affinity for a group of structurally/functionally similar substances:

- Lectins ⟷ Glycoproteins
- Protein G ⟷ IgG antibodies
- Dye-stuffs ⟷ Enzymes

- Often ready-made affinity media
- Standard, tested elution protocols

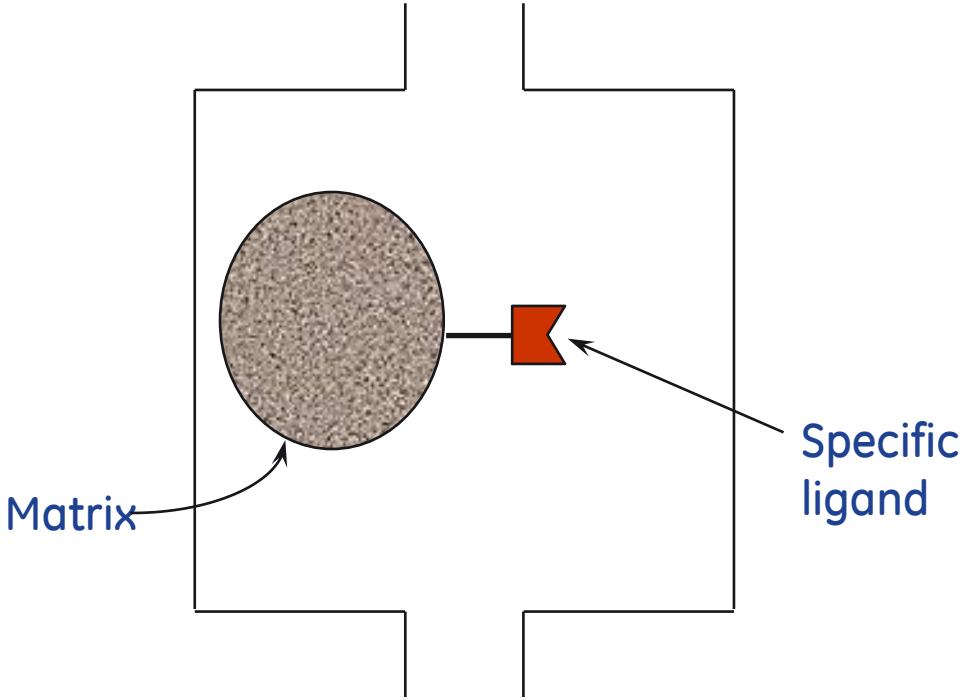
Group-specific ligands

Ligand	Specificity
Protein A	Fc region of IgG
Protein G	Fc region of IgG
Concanavalin A	Glucopyranosyl and mannopyranosyl groups
Cibacron™ Blue	Broad range of enzymes, serum albumin
Lysine	Plasminogen, ribosomal RNA
Benzamidine	Serine proteases
Calmodulin	Proteins regulated by calmodulin
Heparin	Coagulation factors, lipoproteins, lipases, hormones, steroid receptors, protein synthesis factors, nucleic acid-binding enzymes
Metal ions (e.g. Ni^{2+})	Proteins and peptides which contain histidine

Adsorbtion chromatography

1. **Equilibration**
2. Sample application
3. Binding and washing
4. Desorption and elution

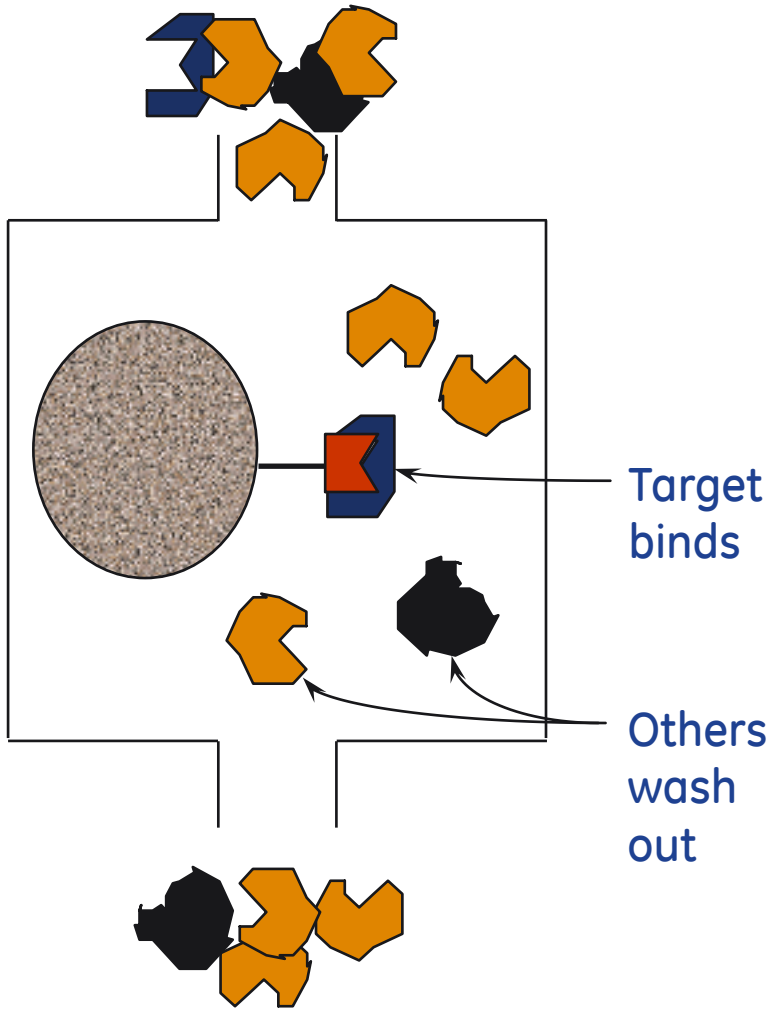
Equilibrate the column and the sample to binding conditions.



Adsorbtion chromatography

1. Equilibration
2. **Sample application**
3. **Binding and washing**
4. Desorption and elution

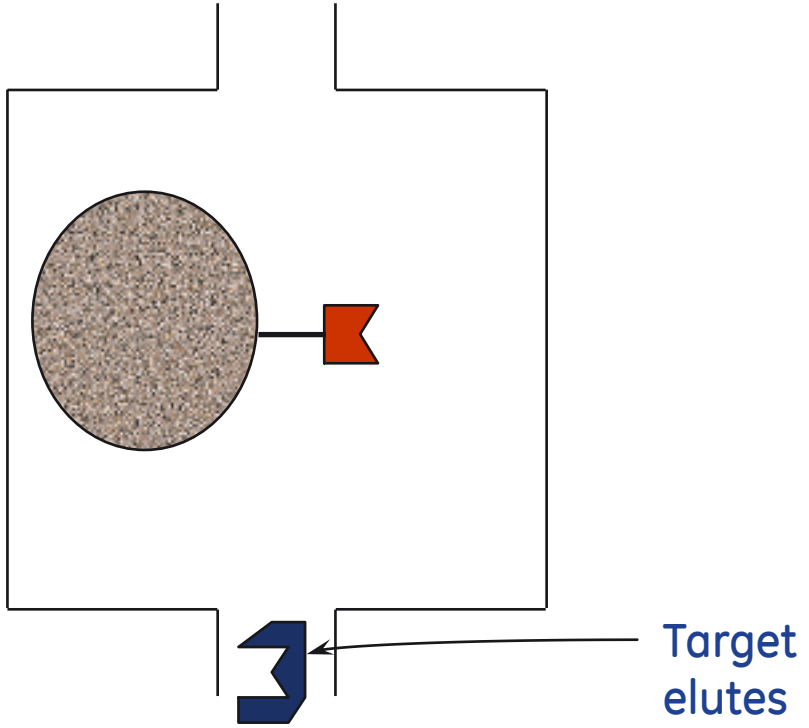
Apply sample under binding conditions.



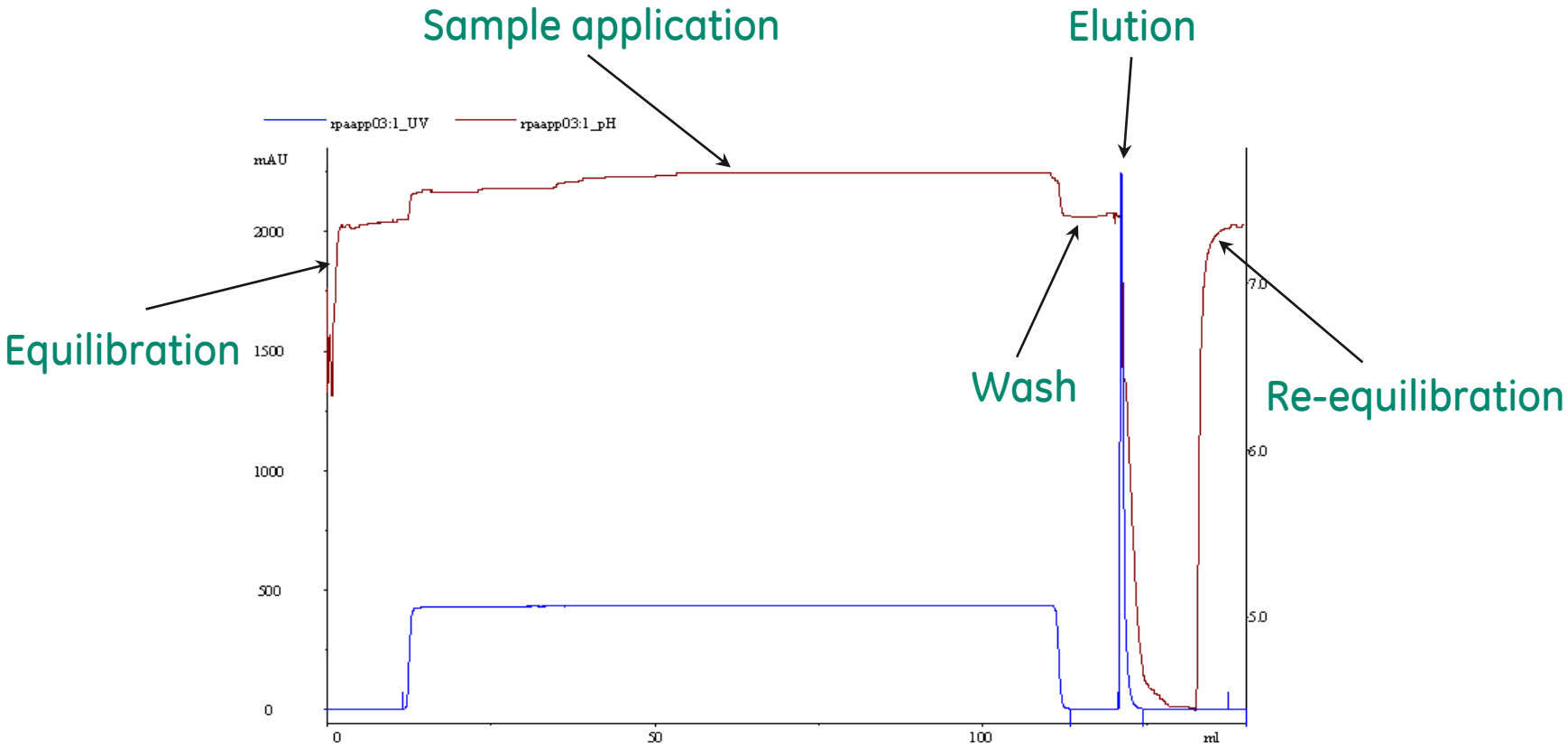
Adsorbtion chromatography

1. Equilibration
2. Sample application
3. Binding and washing
4. **Desorption and elution**

Change the eluent to elute the target.

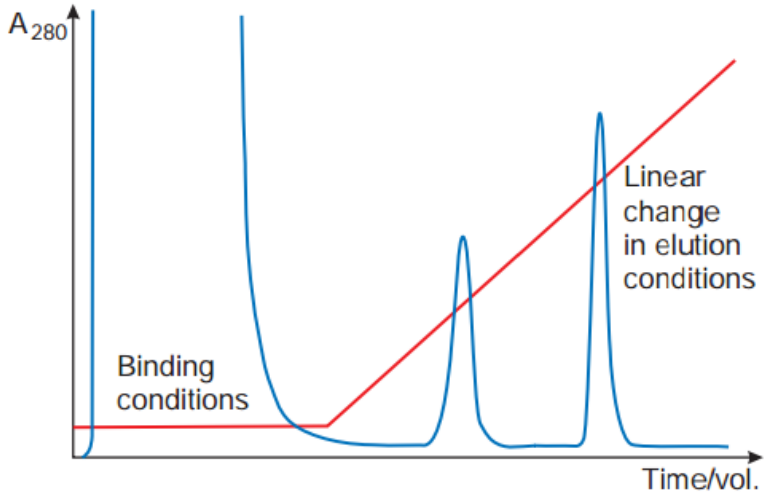


The main stages in affinity chromatography

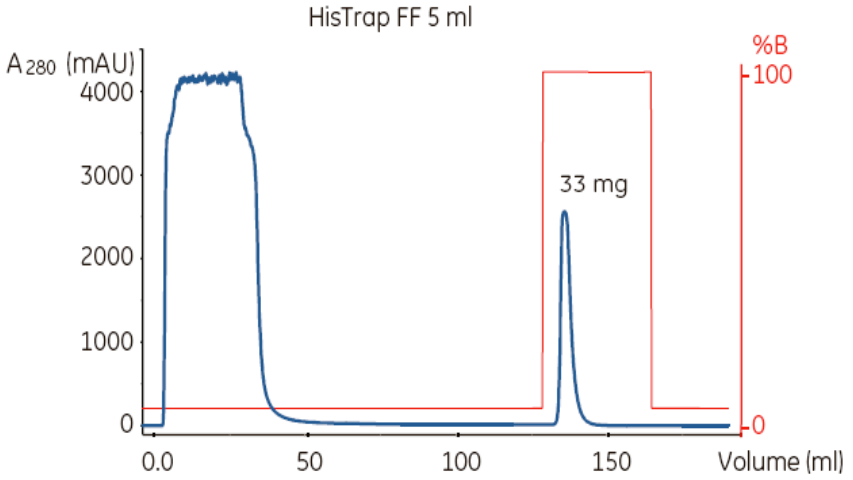


Elution – step or gradient elution

- During development and optimization of affinity purification, use a gradient elution to scan for the optimal binding and/or elution conditions



Gradient elution



Step elution

Some practical details for affinity

Column volume:

- Choose the column volume according to amount of target and capacity of the chromatography medium, dilute

The sample if it is too viscous or too concentrate

Column length:

- Between 2cm up to 20 cm. Increase the internal diameter to make scale-up, keep the length.

Equipment:

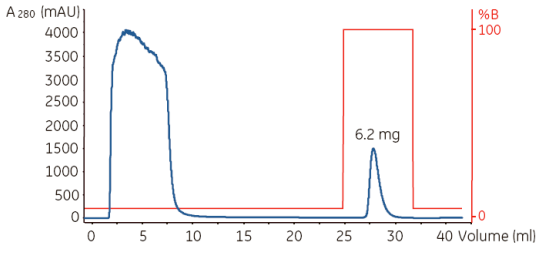
- Classically low pressure technic, use beads with size of 34µm up to 90µm. No needs of specific system, syringe is ok.

Sample:

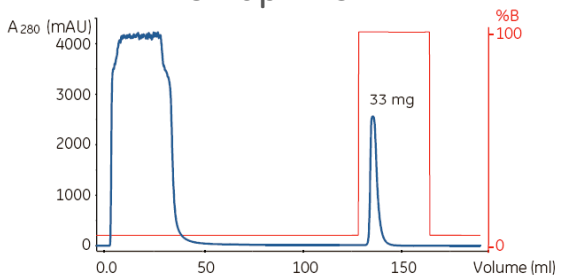
Filtered or centrifuged. Use dessalting to exchange buffer if needed. For strong affinity increase the flow rate and decrease with weak affinity

Classical column type for affinity

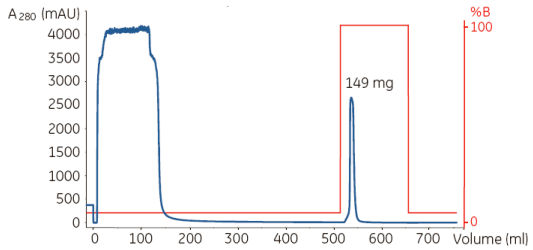
HisTrap™ FF 1 ml

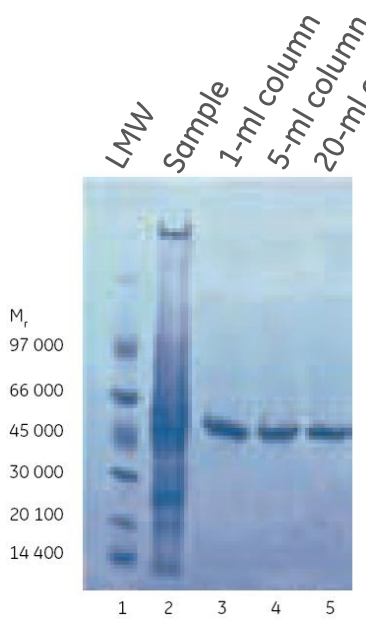


HisTrap FF 5 ml



HisPrep™ FF 16/10 (20 ml)





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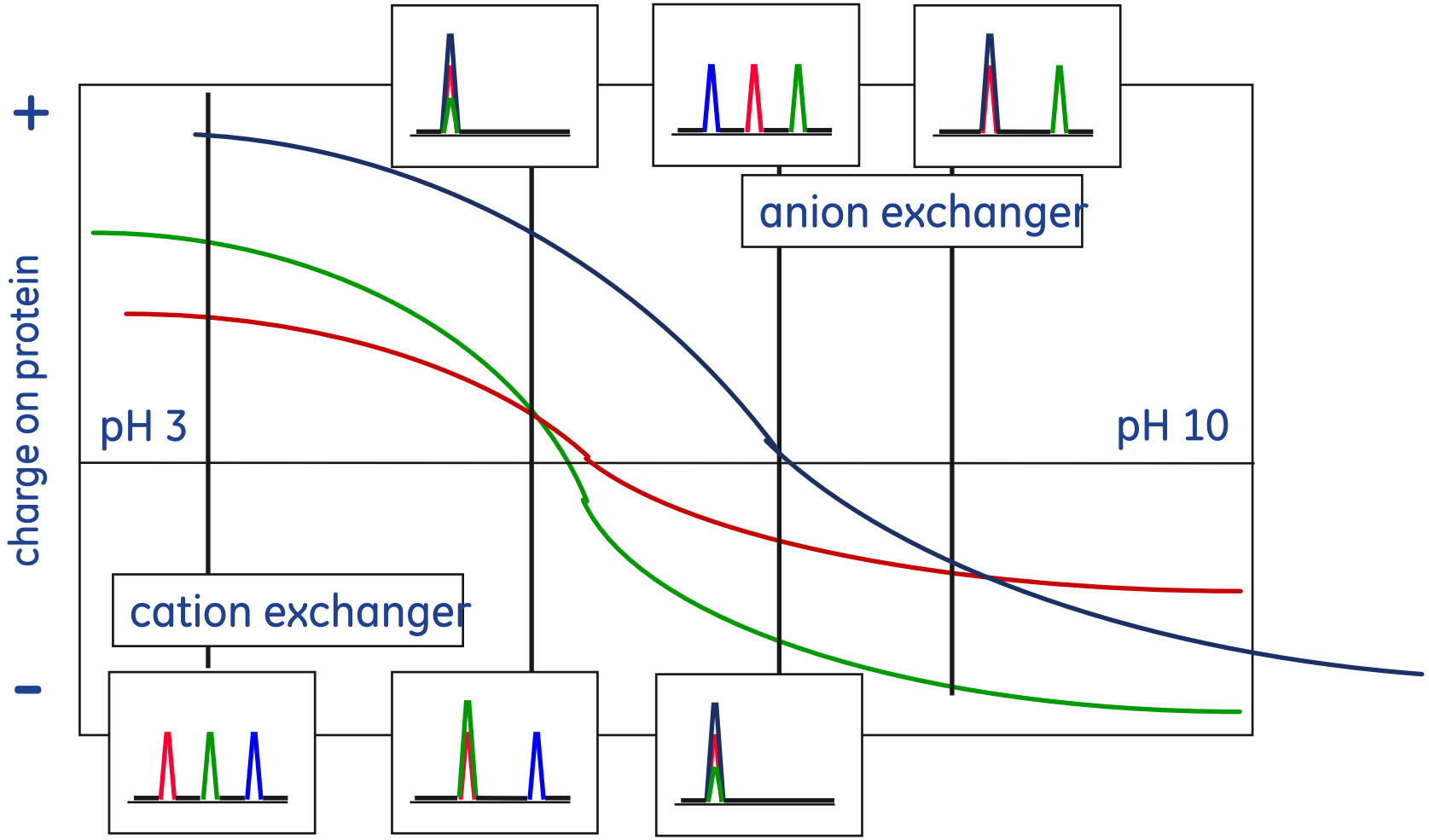
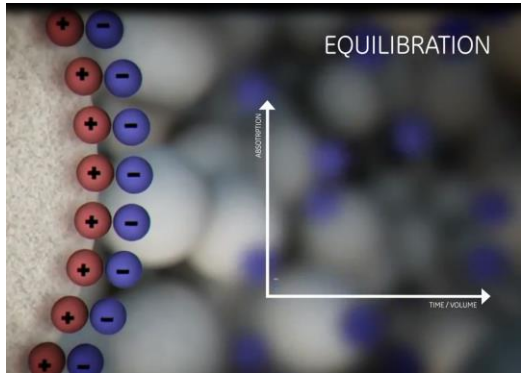
Introduction to ion exchange Chromatography (AC)

Controlling selectivity by pH



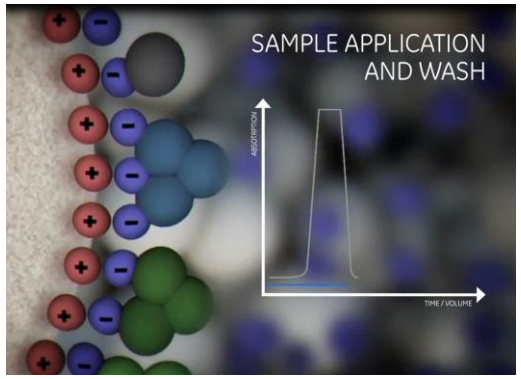
How does IEX work?

Equilibration



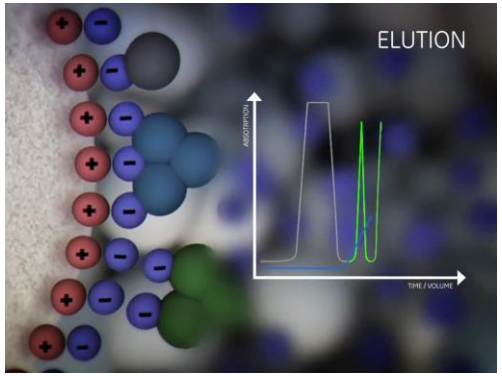
- **The first step is the equilibration of the stationary phase to the desired start conditions.**
- When equilibration is reached, all stationary phase charged groups are associated with exchangeable counter ions such as chloride or sodium.

Sample application and wash



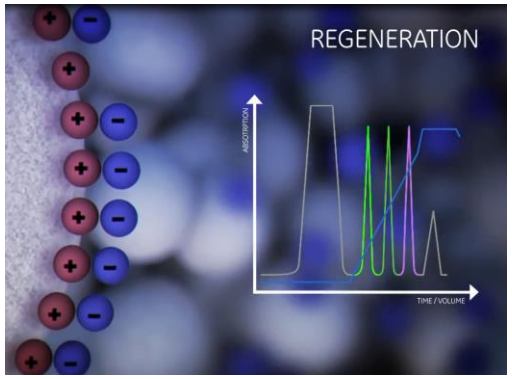
- **The goal in this step is to bind the target molecules and wash out all unbound material**
- The sample buffer should have the same pH and ionic strength as the starting buffer in order to bind all appropriately charged proteins.

Elution



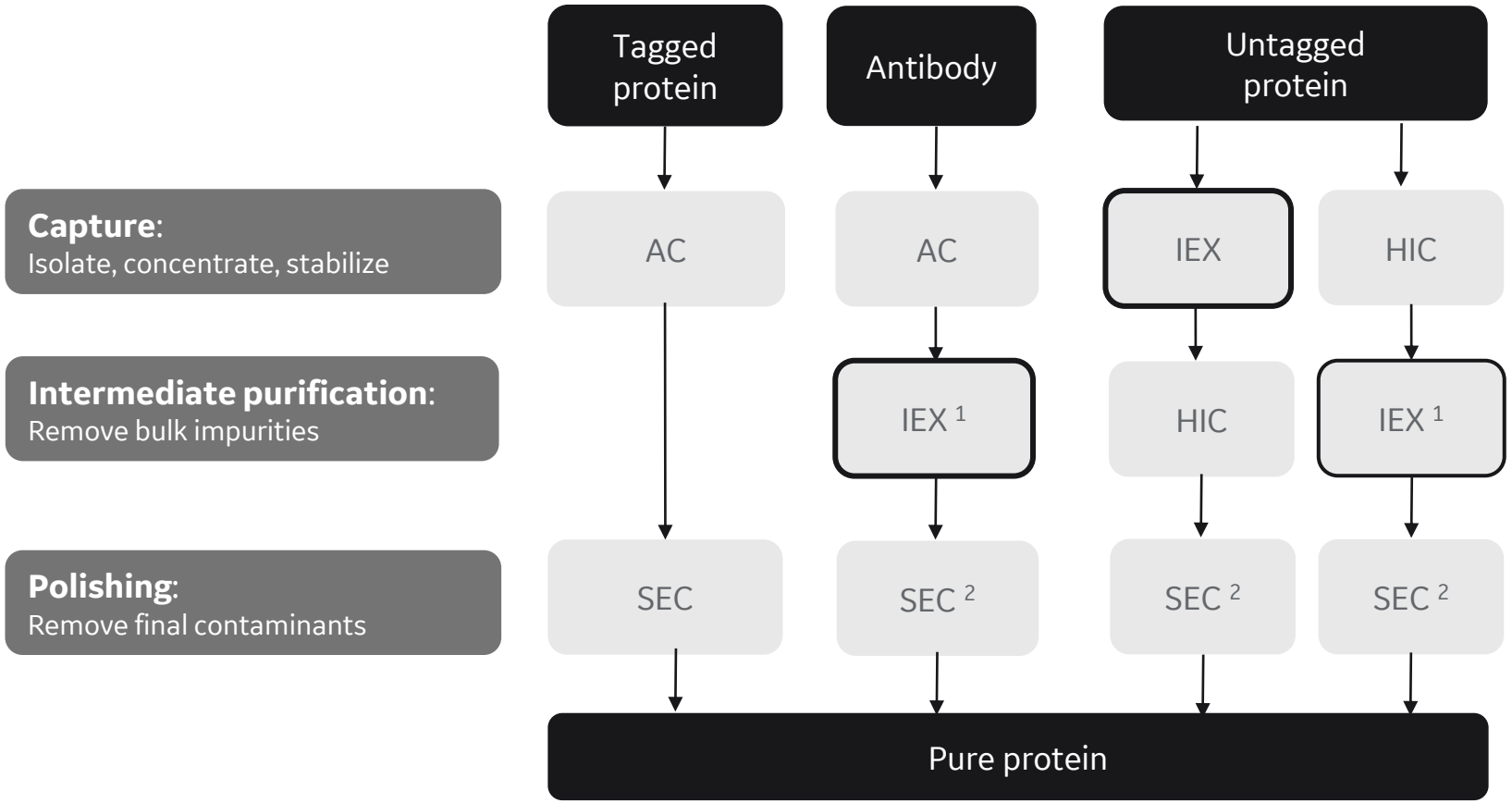
- **Biomolecules are released from the ionic exchanger by a change in the buffer composition**.
- A common elution method is to increase the ionic strength with sodium chloride or another simple salt in order to desorb the bound proteins. Proteins are desorbed relative to their number of charged groups on their surface.

Regeneration



- **The final step, regeneration, removes all molecules still bound.**
- This ensures that the full capacity of the stationary phase is available for the next run.

IEX can be used in various stages of the protein purification protocol

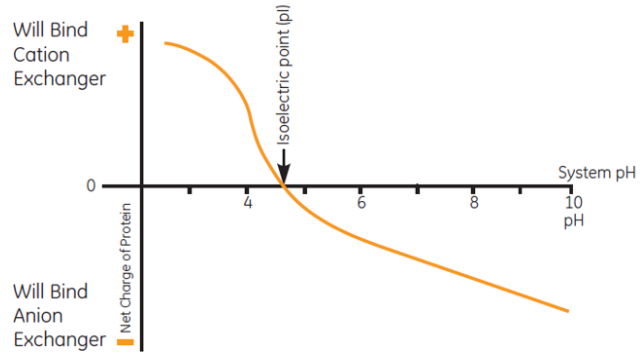


[1] Use of IEX as an intermediate step is not systematic and will depend on the level of purity needed.

[2] SEC is not typically used as a polishing step in industrial applications, because scale-up is particularly challenging.

Ensure protein binding by using the most appropriate ion exchanger

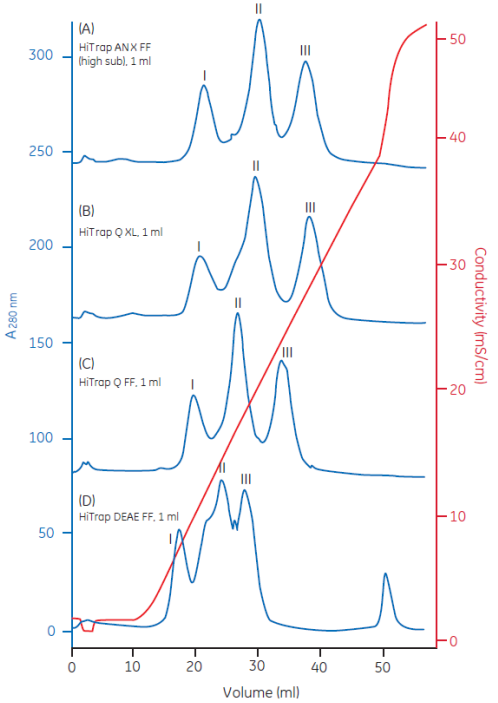
If pI of your protein is known

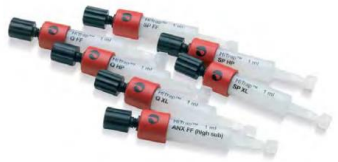


- Select an anion exchanger (Q, DEAE, ANX) with a buffer pH above pI
- Select a cation exchanger (S, SP, CM) with a buffer pH below pI
- Column size from 2cm to 20 cm

If pI of your protein is unknown

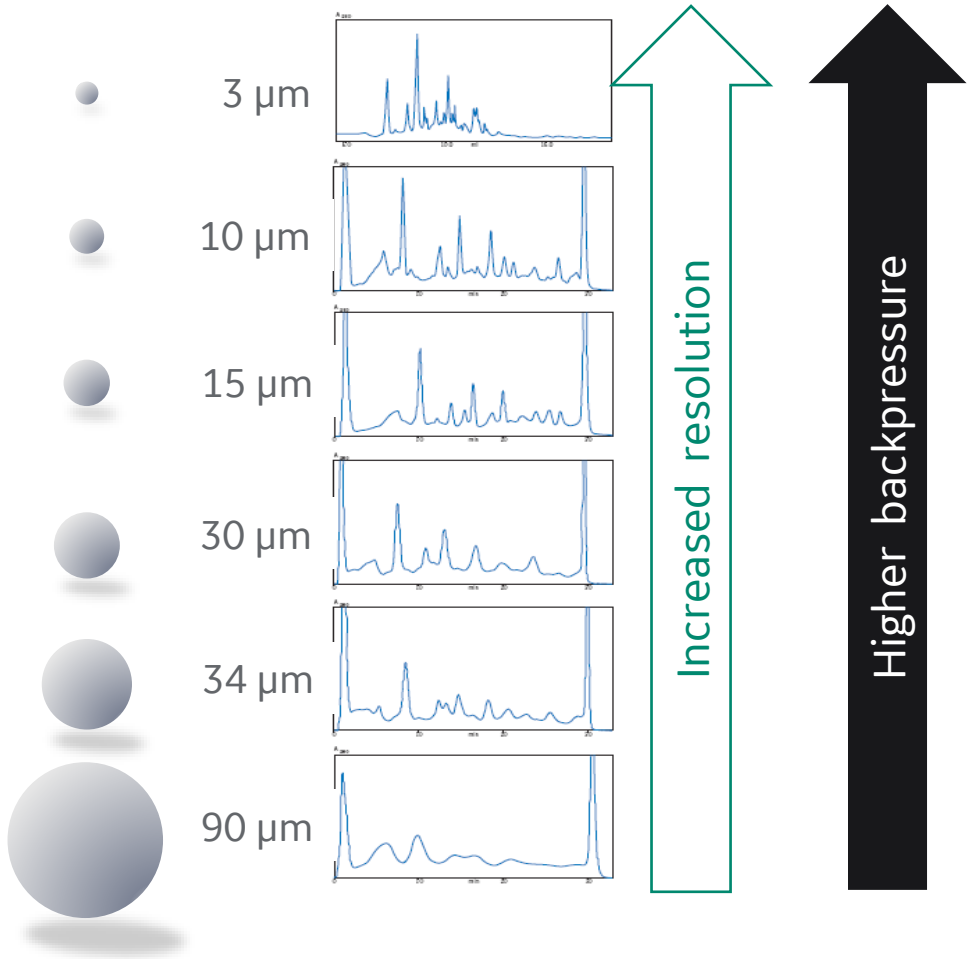
- Start by using a strong anion exchanger (Q)
- Use IEX selection kits for screening of the most appropriate ion exchanger





HiTrap™ IEX Selection kit

Smaller bead size delivers increased resolution but higher back pressure



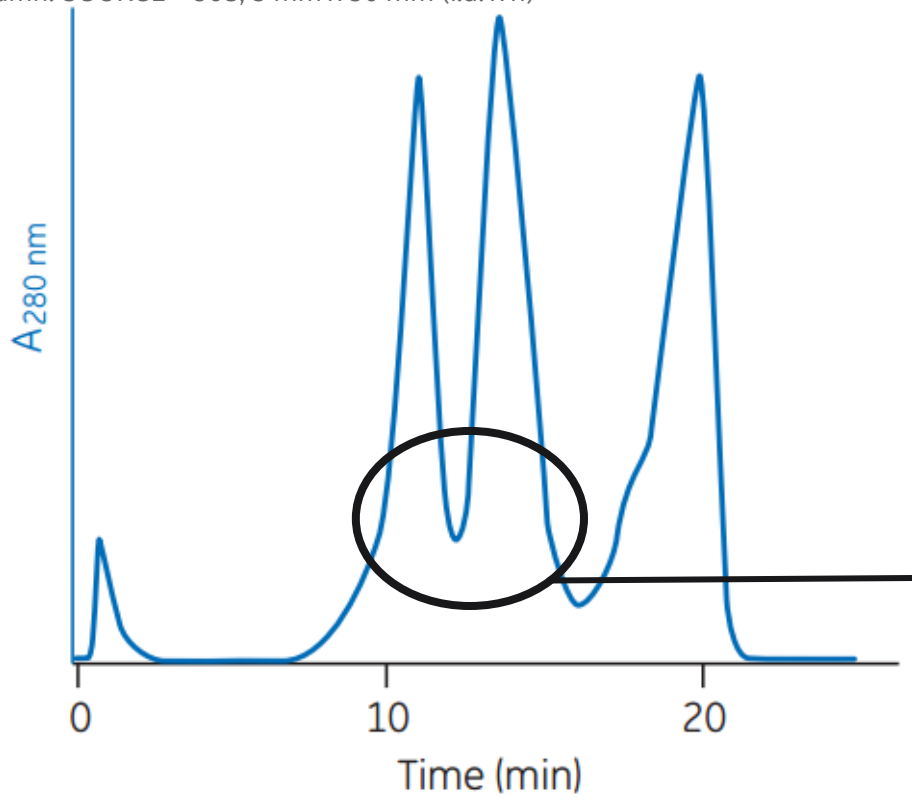
High resolution gives high purity.

Too high back pressure can cause column bed compression, column leakage, and breakage of system components.

Decreasing the sample load will increase resolution

Sample load: 10 mg

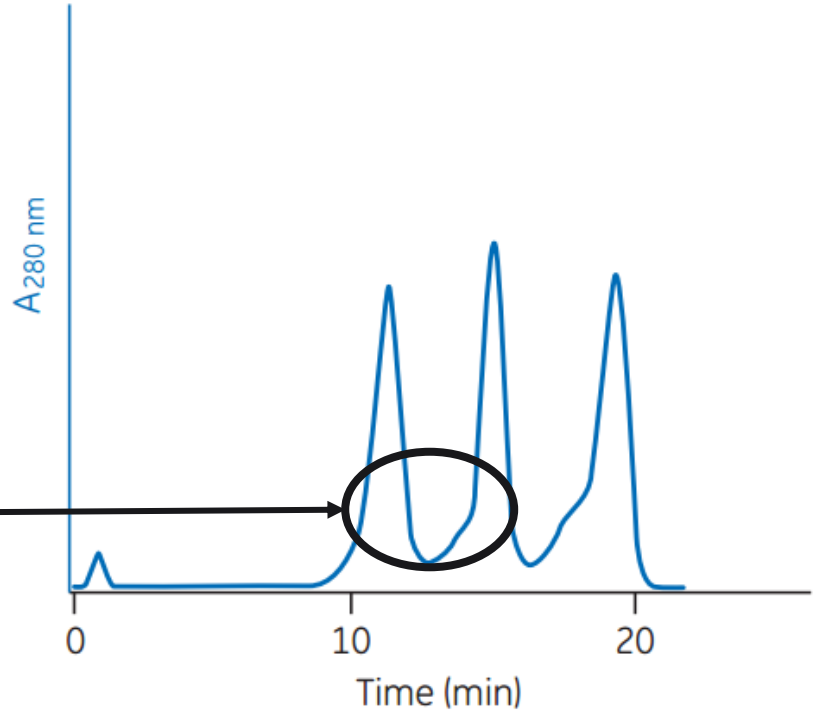
Column: SOURCE™ 30S, 5 mm x 50 mm (i.d. x h)



Increased resolution

Sample load: 1 mg

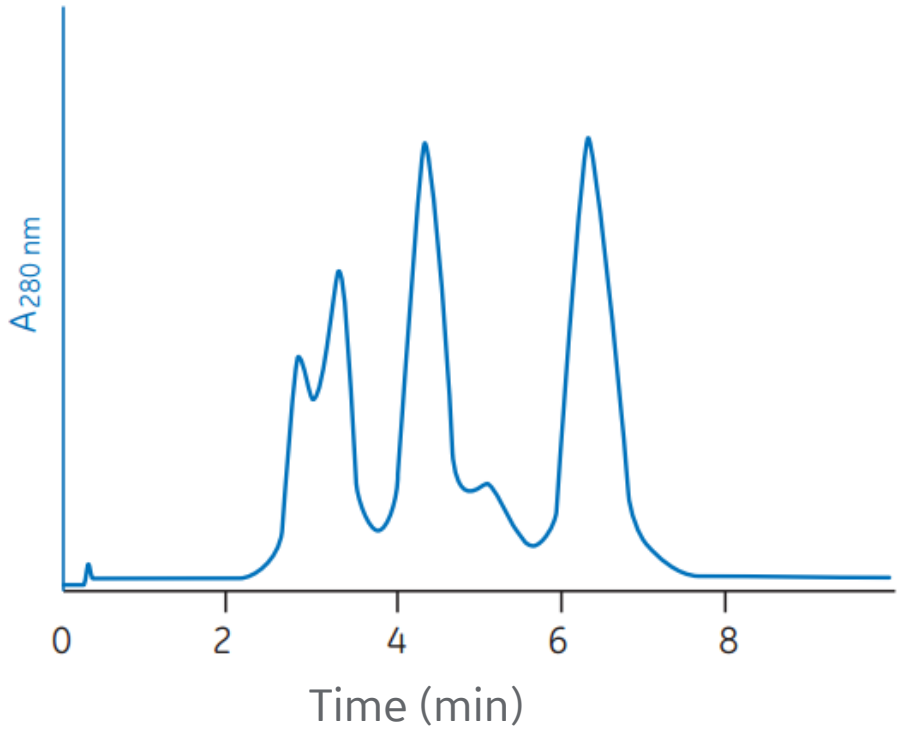
Column: SOURCE 30S, 5 mm x 50 mm (i.d. x h)



Decreasing the flow rate will increase resolution

Flow rate: 13 mL/min

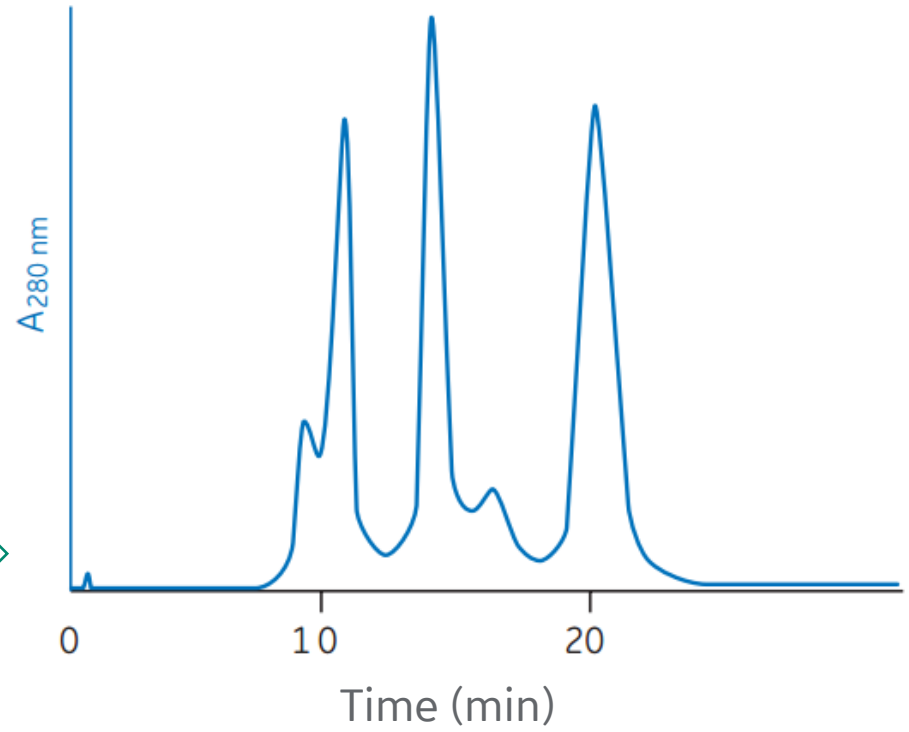
Column: SOURCE™ 30Q, 10 mm x 50 mm (i.d. x h)



Increased resolution

Flow rate: 4 mL/min

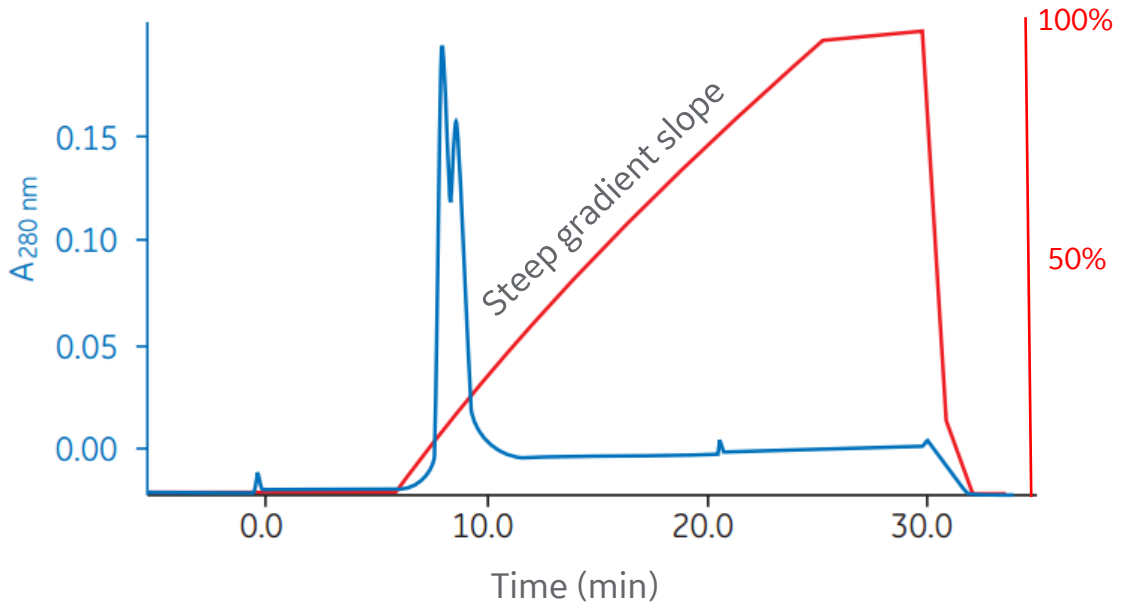
Column: SOURCE 30Q, 10 mm x 50 mm (i.d. x h)



A more shallow gradient will increase resolution

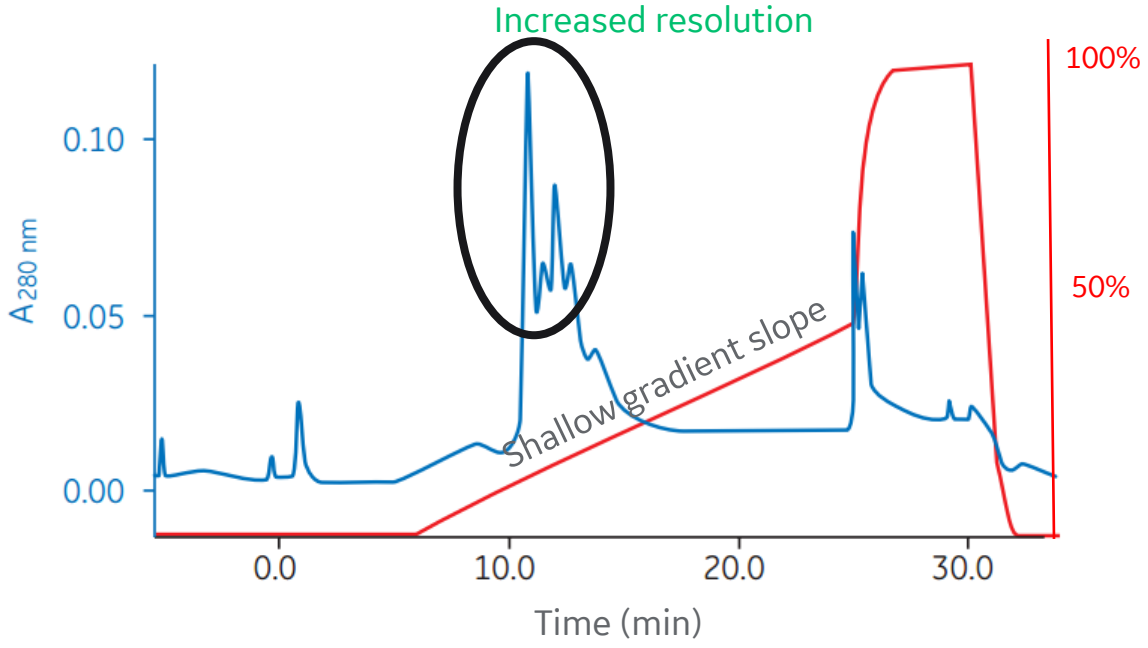
Steep gradient slope

Gradient: 0% to 100 % elution buffer in 20 CV*



Shallow gradient slope

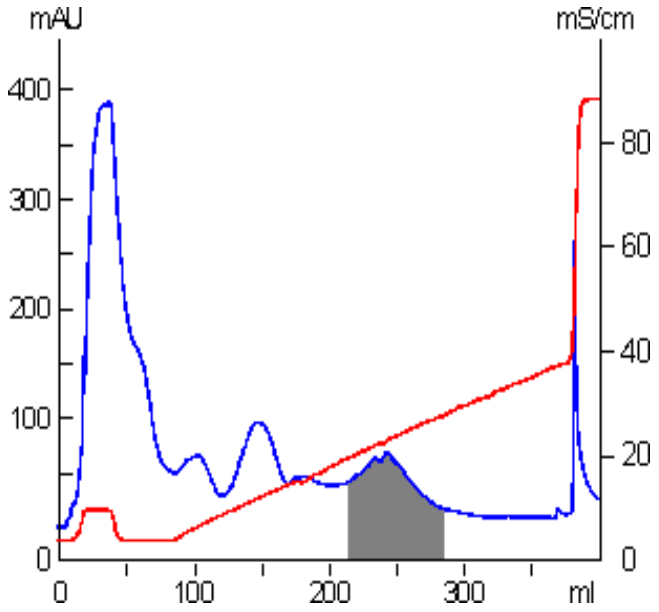
Gradient: 0% to 40% elution buffer in 20 CV



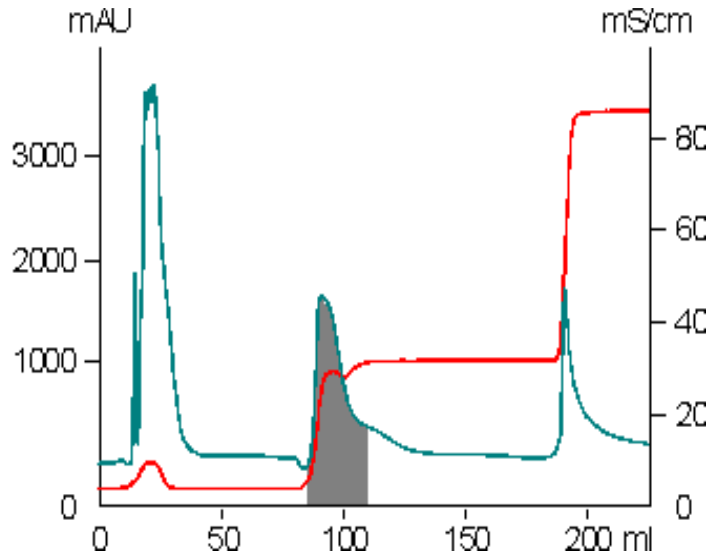
* CV = column volume

Step gradient for capture

linear gradient, starting point



optimised step elution



HiPrep™ 16/10 Q XL: rec DAOCS, *E. coli*

IEX can also be used for purity and heterogeneity analysis

Isoforms can be identified and separated using high-resolution ion exchange chromatography.

Phosphorylated form vs non-phosphorylated form

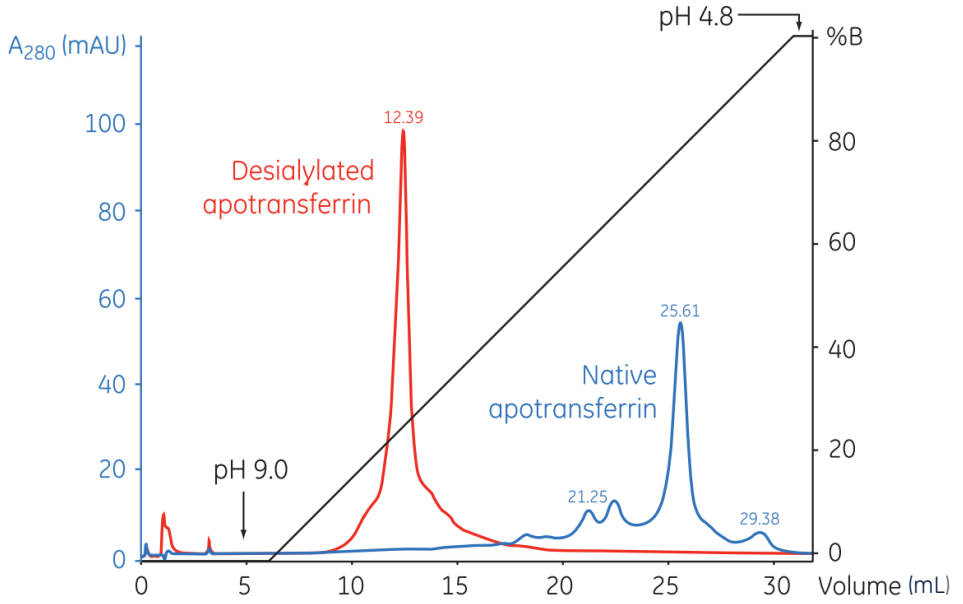
Separation of non-phosphorylated and mono-phosphorylated kinase (ZAP-70) by cation exchange chromatography with Mono S™ resin.

Salt gradient: 0 to 250 mM NaCl in 80 CV*

Volume (mL)

Native form vs desialylated form

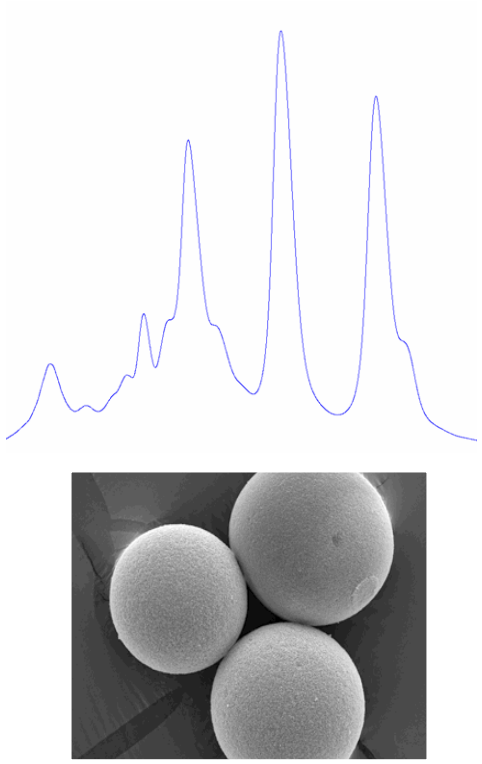
Analysis of desialylated and native apotransferrin by anion exchange chromatography in a pH gradient, with Mono Q™ resin. Overlay of two runs.



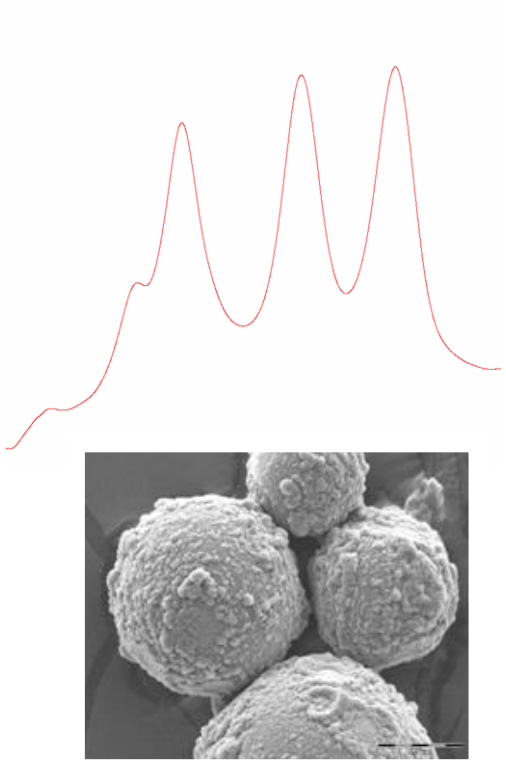
* CV = column volume

Cleaning a dirty column can restore the resolution

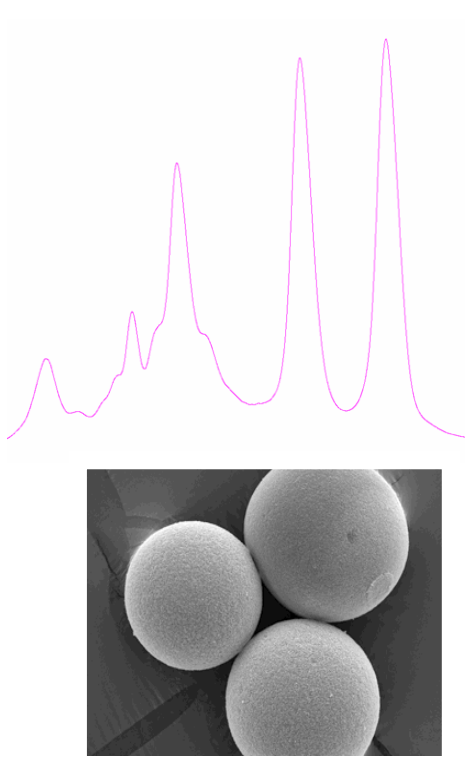
1. Fresh column



After a purification step using 30 mL *E. coli* lysate as sample



After cleaning



Column: HiTrap™ Q HP 1 mL
Sample: Standard proteins

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Introduction to Size Exclusion Chromatography (SEC)

Terms and explanations

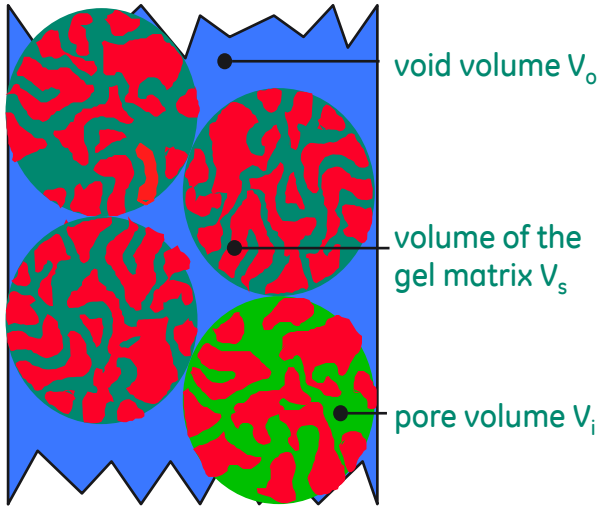
V_o = void volume

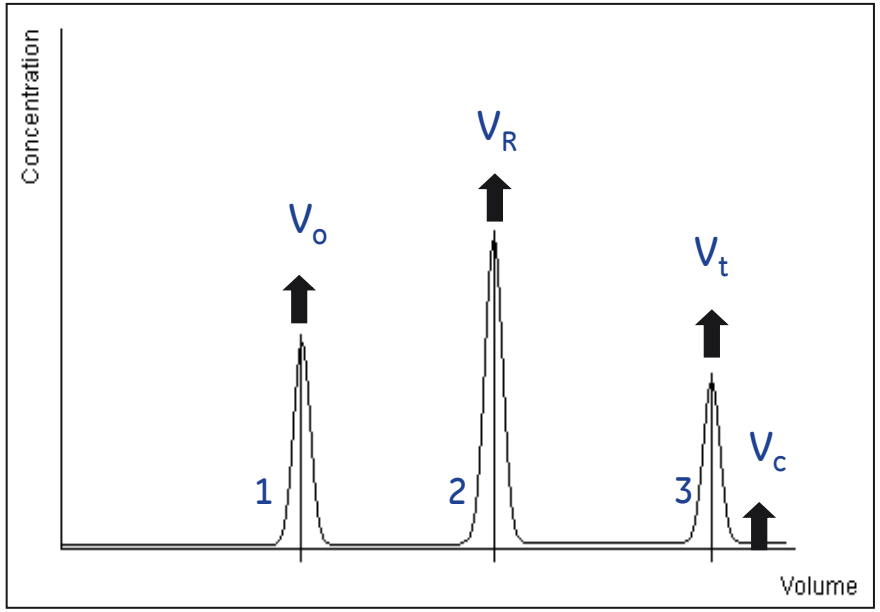
V_R = retention (elution volume)

V_t = total liquid volume

V_i = pore volume = V_c - V_s – V_o

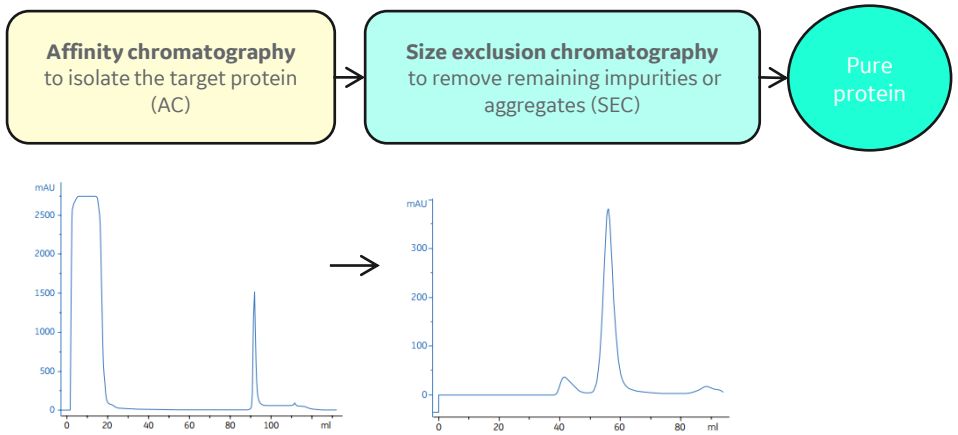
V_c = geometric column volume

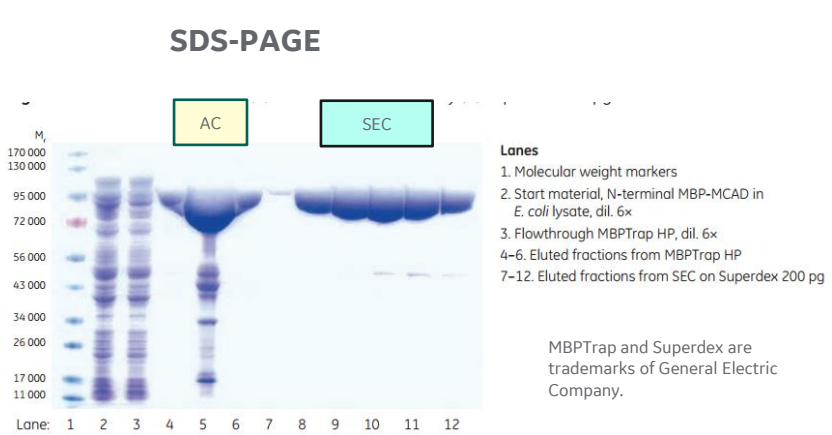




SEC can be used for preparative purposes…

- SEC is usually used as a last polishing step to obtain pure target molecules in sufficient amount





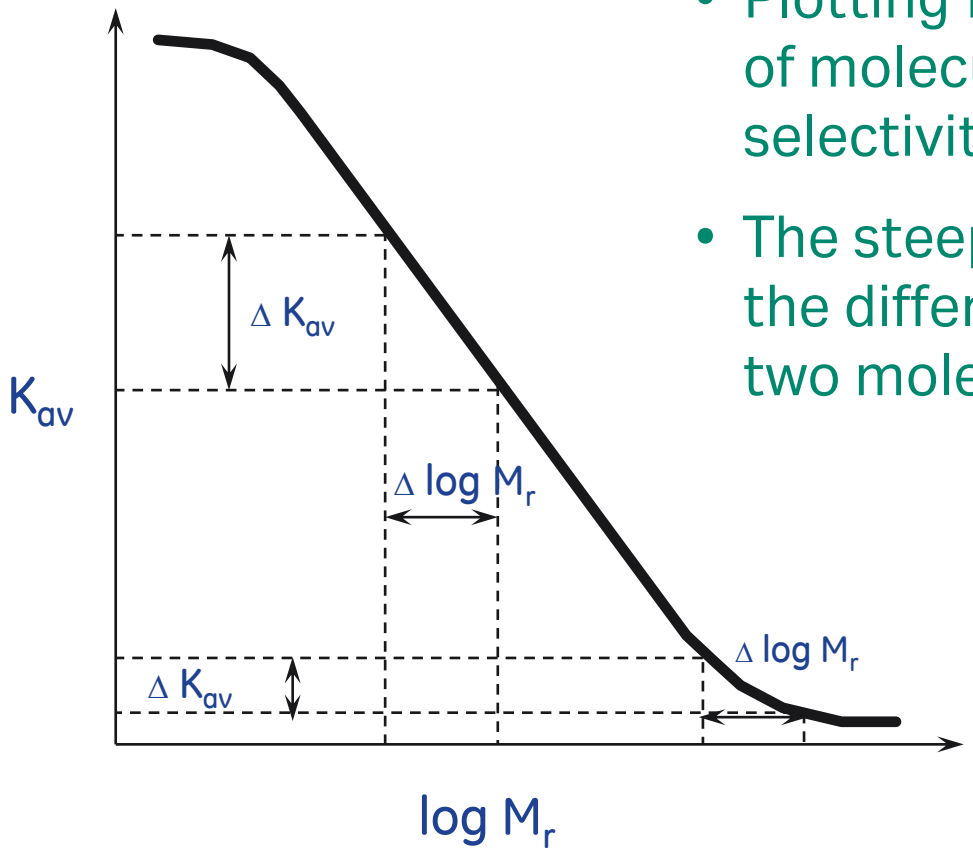
MBPTrap and Superdex are trademarks of General Electric Company.

The coefficient Kav

$$K_{av} = \frac{V_R - V_0}{V_c - V_0}$$

K_{av} is easy to get and it is more useful in practice

Selectivity curves



- Plotting K_{av} versus the logarithm of molecular mass yields the selectivity curve of the matrix.
- The steeper this curve, the greater the difference in elution volume for two molecules of different sizes.

Column size depends on sample volume

Desalting/Buffer exchange

- Sample volume up to 30 % of the total column volume can be applied
- Length of the column is not so important

High resolution fractionation

- Sample volume of 0.5 % to 4 % of the total column volume can be applied
- Column length required: 30-100 cm
  - For rapid purity check, 15 cm length can be good enough

What is the sample volume?

Desalting

Product	Max sample volume
HiTrap™ Desalting* (5 ml)	1.5 ml
HiPrep™ Desalting* (53 ml)	15 ml

*Possible to connect several columns to increase sample volume

Small volume buffer exchange

Product	Format	Max sample volume
PD-10 desalting columns	Gravity column	2.5 ml
PD MidiTrap™ G-25	Gravity column	1 ml
PD MiniTrap™ G-25	Gravity column	0.5 ml
PD SpinTrap™ G-25	Spin column	130 µl
PD MultiTrap™ G-25	96-well filter plate	130 µl

Fractionation

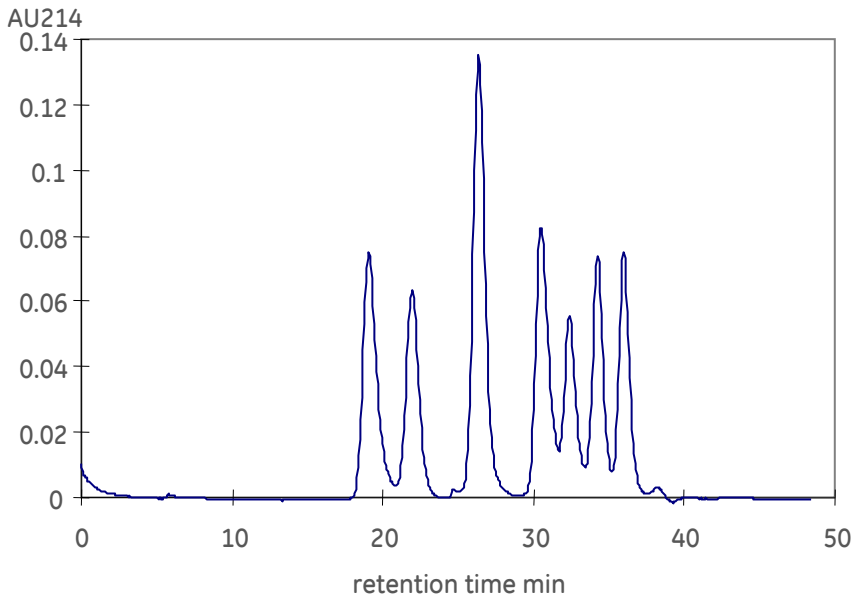
Column dimensions	Max sample volume
Analytical scale (µl)**	
10/300	250 µl
5/150	50 µl
3.2/30	25 µl
Preparative scale (ml)***	
16/60	5 ml
26/60	13 ml

**Tricorn™ and Precision columns
***HiLoad™ and HiPrep columns

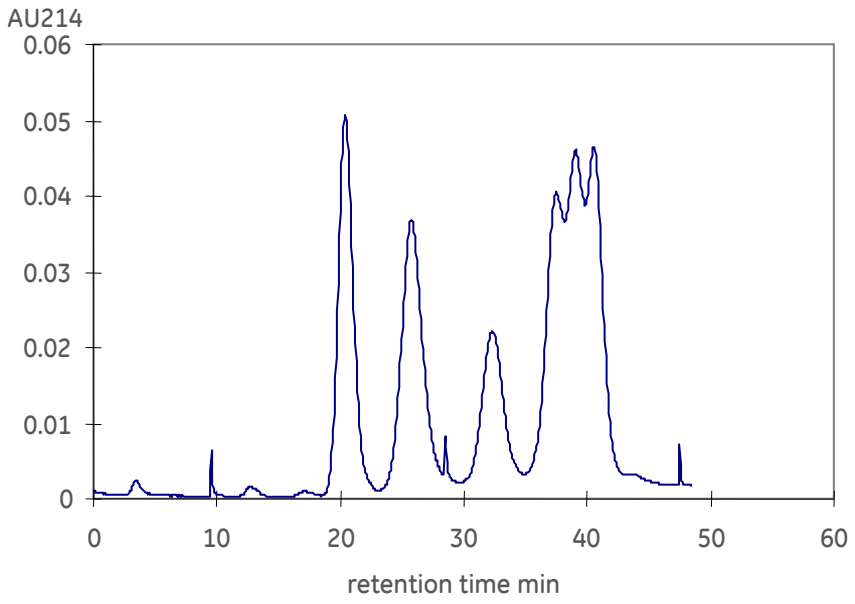
Anything larger, purchase media, empty column and pack yourself

Peak width depends on particle size

- Superdex™ Peptide 13-15 µm

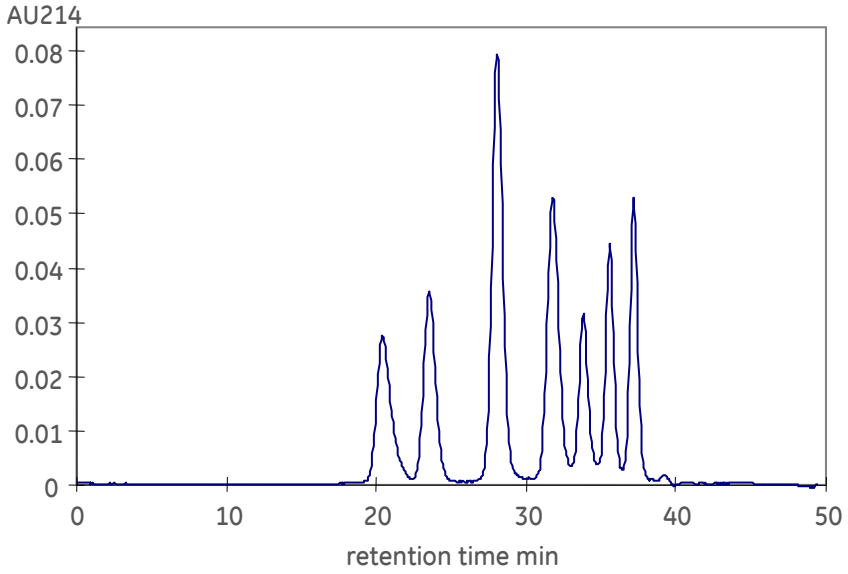


- Superdex 30 prep grade 24-44 µm

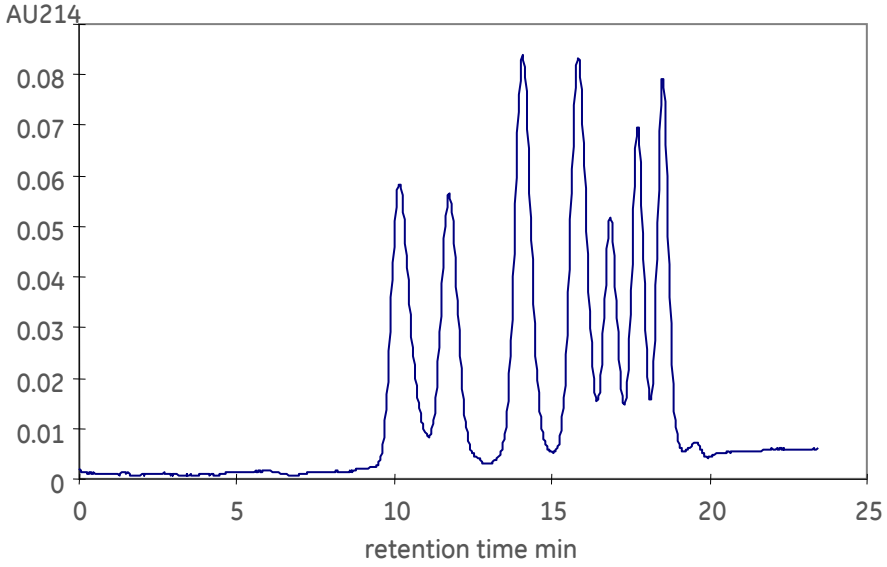


Resolution depends on column length

2 x Superdex™ Peptide column
10 x 300 mm i.d x bed height

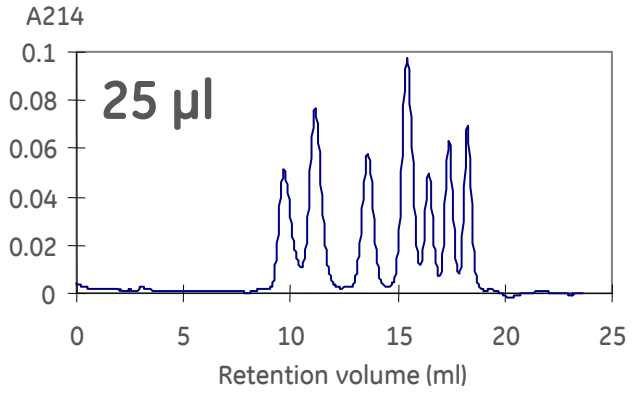


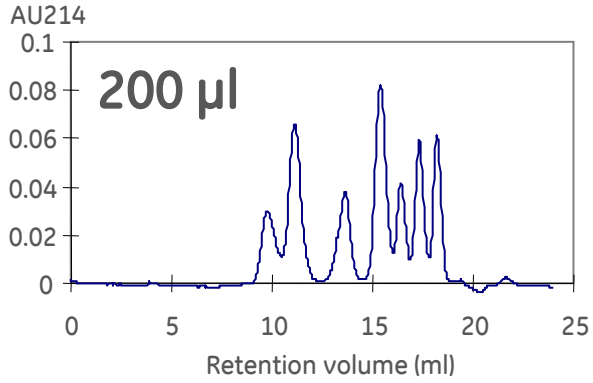
1 x Superdex Peptide column
10 x 300 mm i.d x bed height

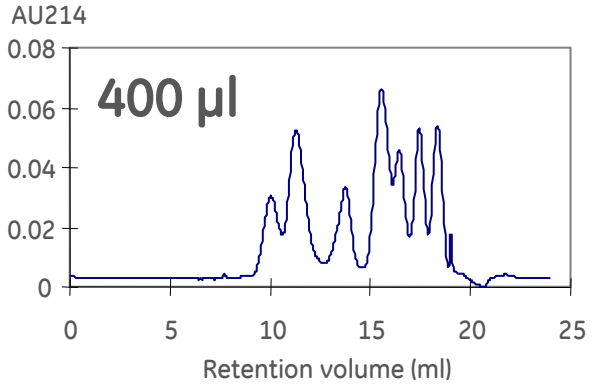


Resolution depends on sample volume

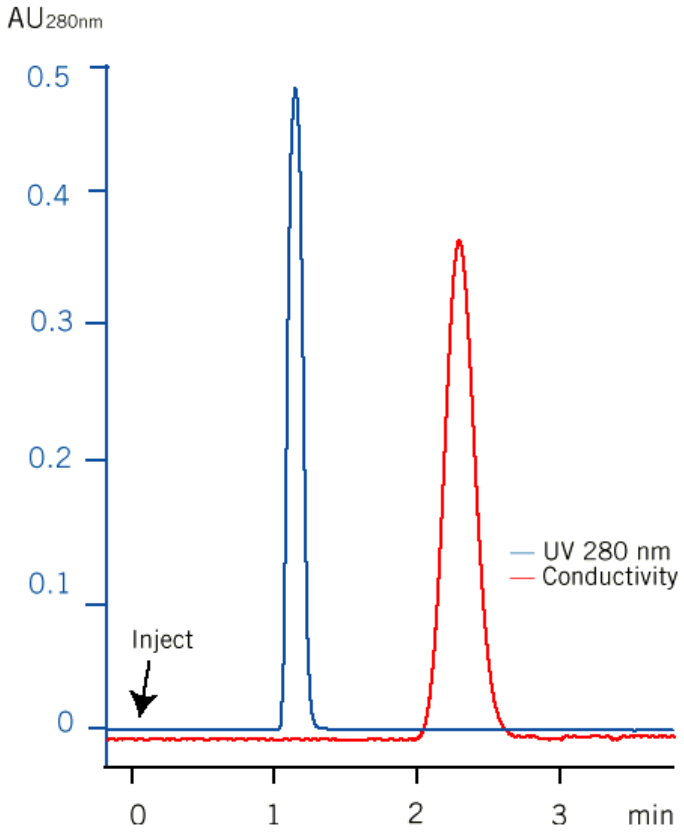
- Superdex™ Peptide 10 x 300 mm column





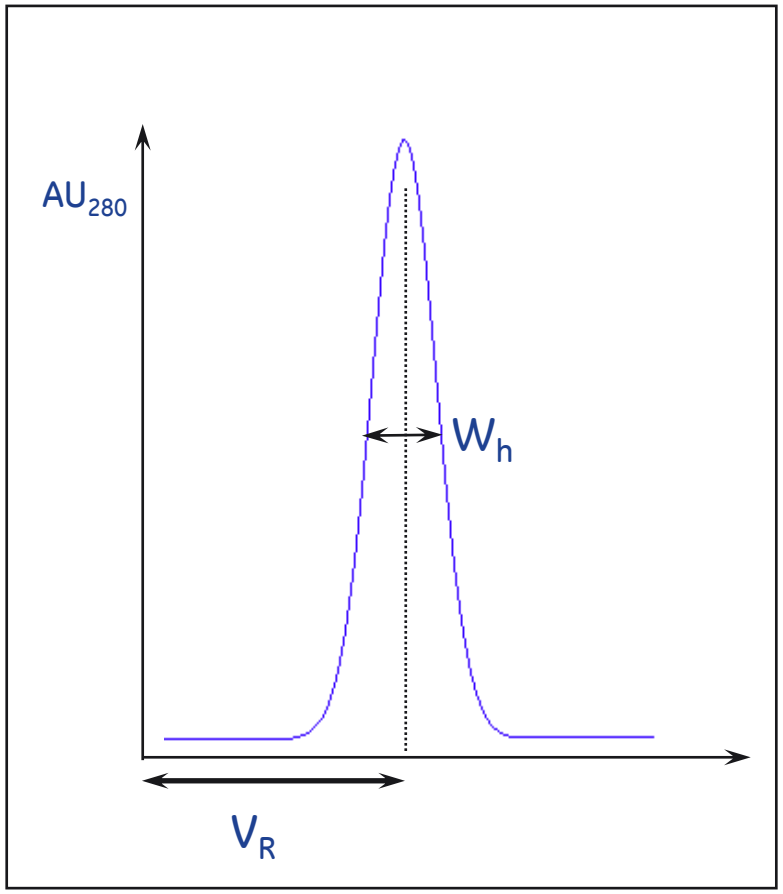


Buffer exchange using HiPrep 26/10 Desalting Column



Sample: BSA dissolved in 50 mM piperazine, 0.5 M sodium chloride, pH 6.2
Column: HiPrep™ 26/10 Desalting
Buffer: 20 mM sodium phosphate, 0.15 M sodium chloride, pH 7.0
System: ÄKTAprime™, 20 ml/min

Efficiency



$$N/_m = 5.54 \left(\frac{V_R}{W_h}\right)^2 x \frac{1000}{L}$$

- $N/_m$ = Number of theoretical plates per meter
- V_R = Peak retention volume
- W_h = Peak with at half peak height
- L = Bed height mm
- V_R and W_h are in same units
- Test: 1% solution of acetone about 0.5% of column volume, at 280 nm. Alternatively use 2 M NaCl and conductivity monitor.

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Introduction to micro Size Exclusion Chromatography (SEC)

Superdex™ 200 Increase

Three column formats to match your needs

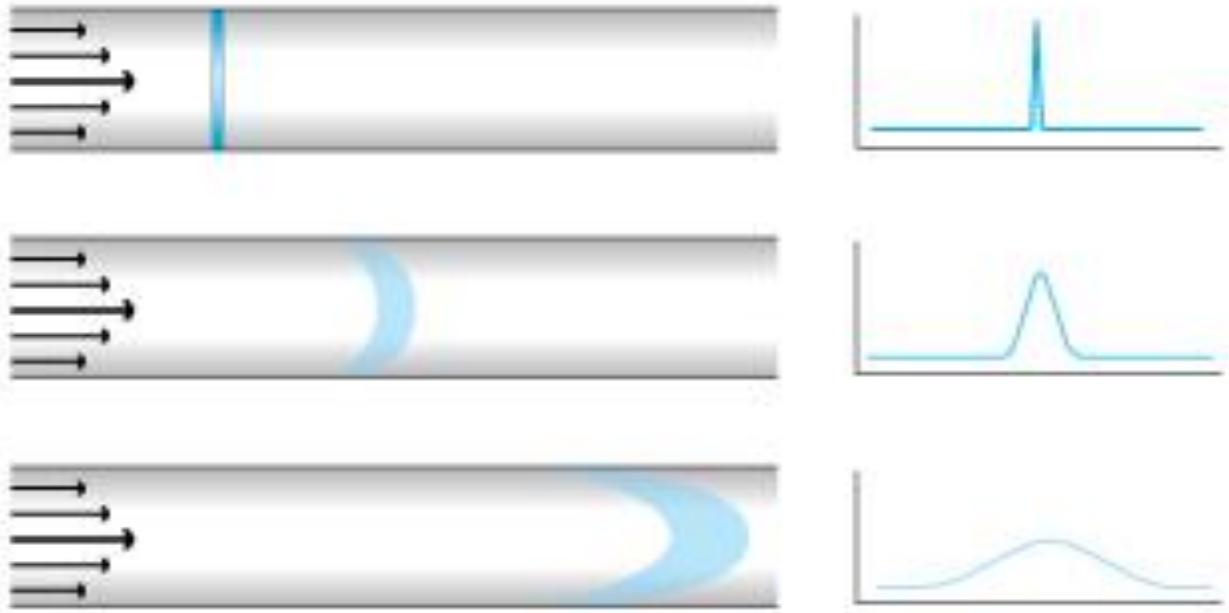
- **Superdex 200 Increase 10/300 GL**
- Small-scale preparative runs (mg)
- High resolution analysis (25 – 500 µl)

- **Superdex 200 Increase 5/150 GL**
- Purity check
- Rapid screening
- Small sample and buffer consumption (4 - 50 µl)

- **Superdex 200 Increase 3.2/300**
- High resolution analysis (4 - 50 µl)
- Micro scale preparative runs (µg
- Small sample and buffer consumption

Peak Broadening in tubing

- The peak broadening in connecting tubings is mostly coming from the laminar flow profile



- Liquid flows faster in the middle of a tubing, the larger the inner diameter and the longer the tubing the broader the peak becomes

Theory

Laminar flow in tubing

Back pressure

$$\Delta P = \frac{128\, L\, Q\, \eta}{\pi\, d^4}$$

ΔP = back pressure

L = length of tubing

Q = volumetric flow rate

η = viscosity of buffer

d = inner diameter of tubing

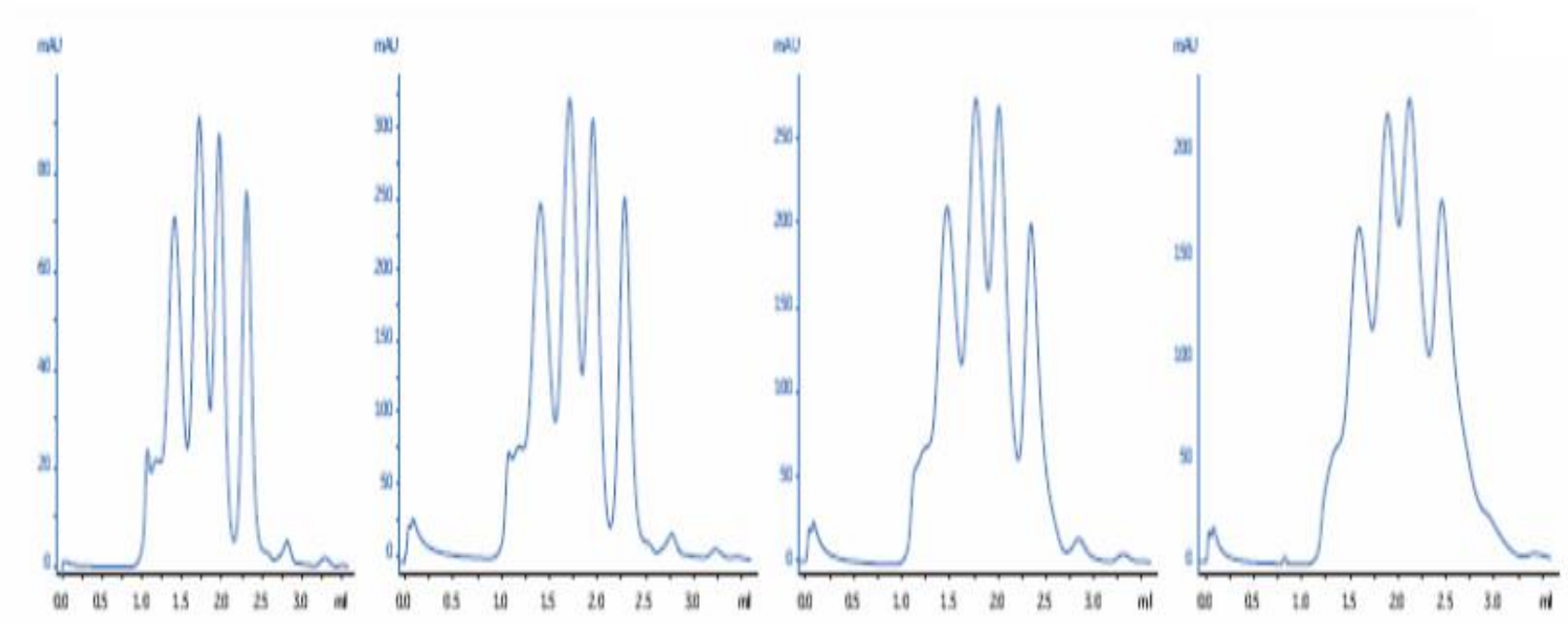
Peak broadening

$$\sigma^2 = \frac{\pi\, L\, Q\, d^4}{384\, D_m}$$

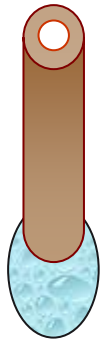
σ^2 = peak broadening

D_m = diffusion coefficient

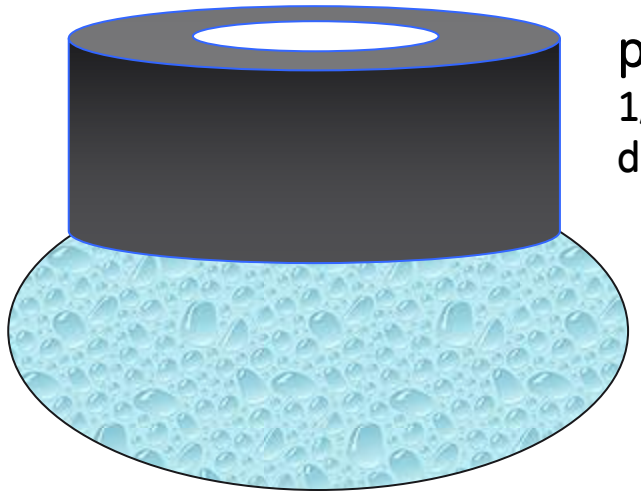
Effect of system dead volume on resolution



Importance of external diameter of tubing for micro-collection



Fused silica
200 µm o.d.
drop size 4 µl

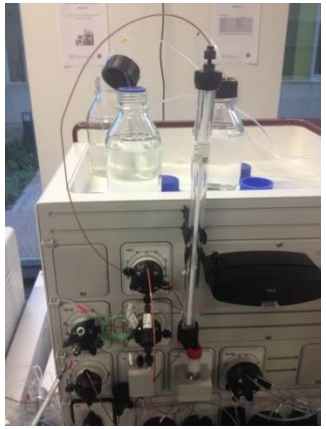


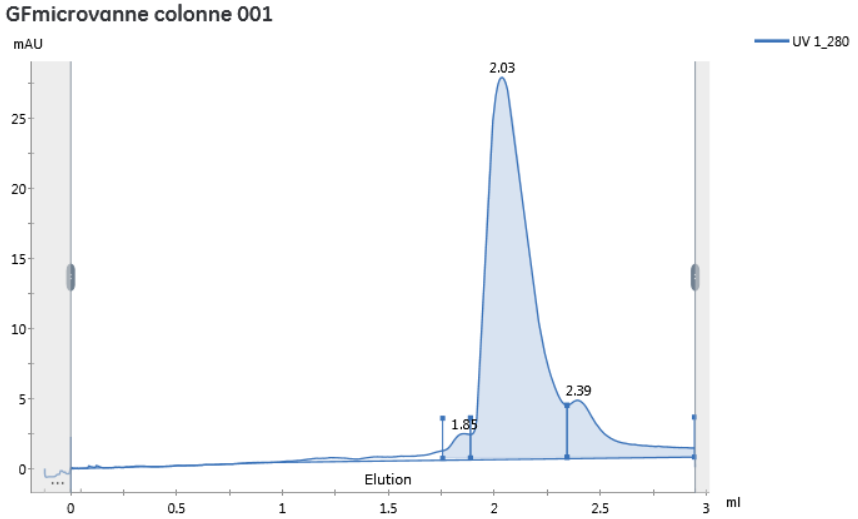
peek capillary
1/16" o.d.
drop size 50 µl

Test Results

Classical Configuration

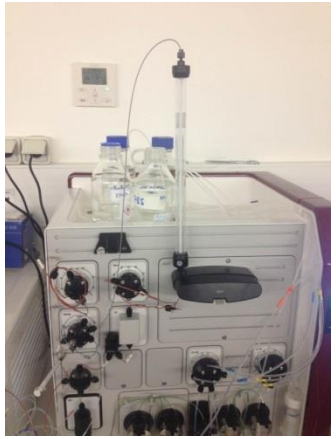
46µg
Cytochrome C

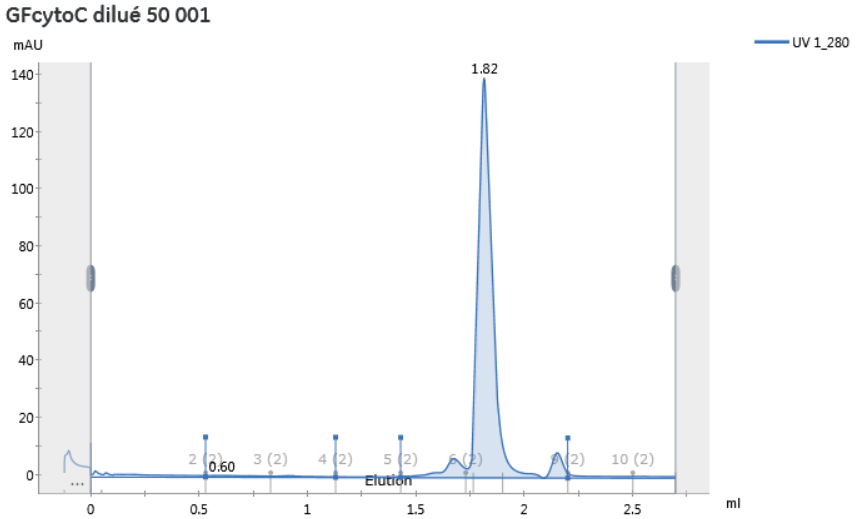




Micro Configuration

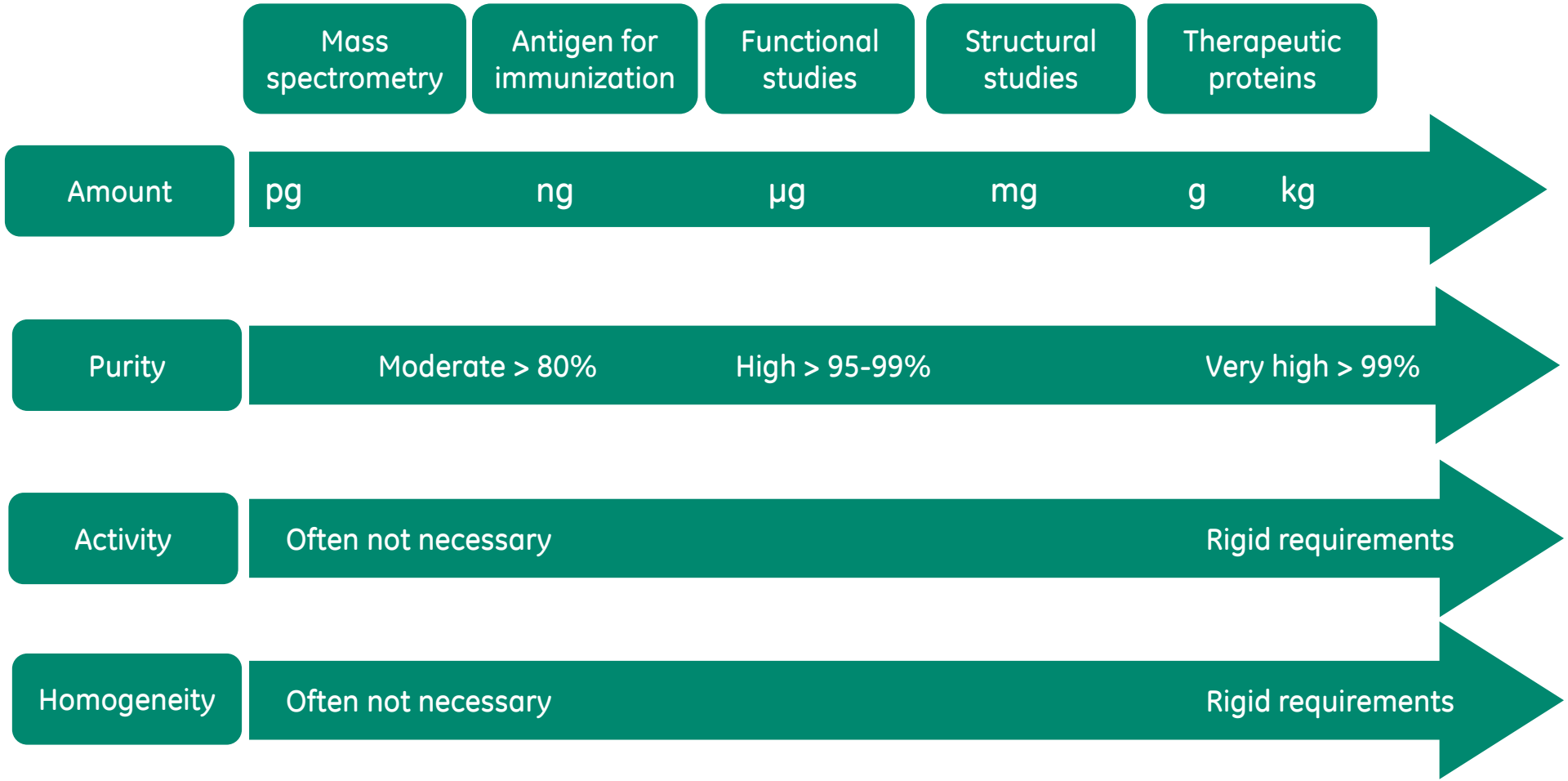
16µg
Cytochrome C



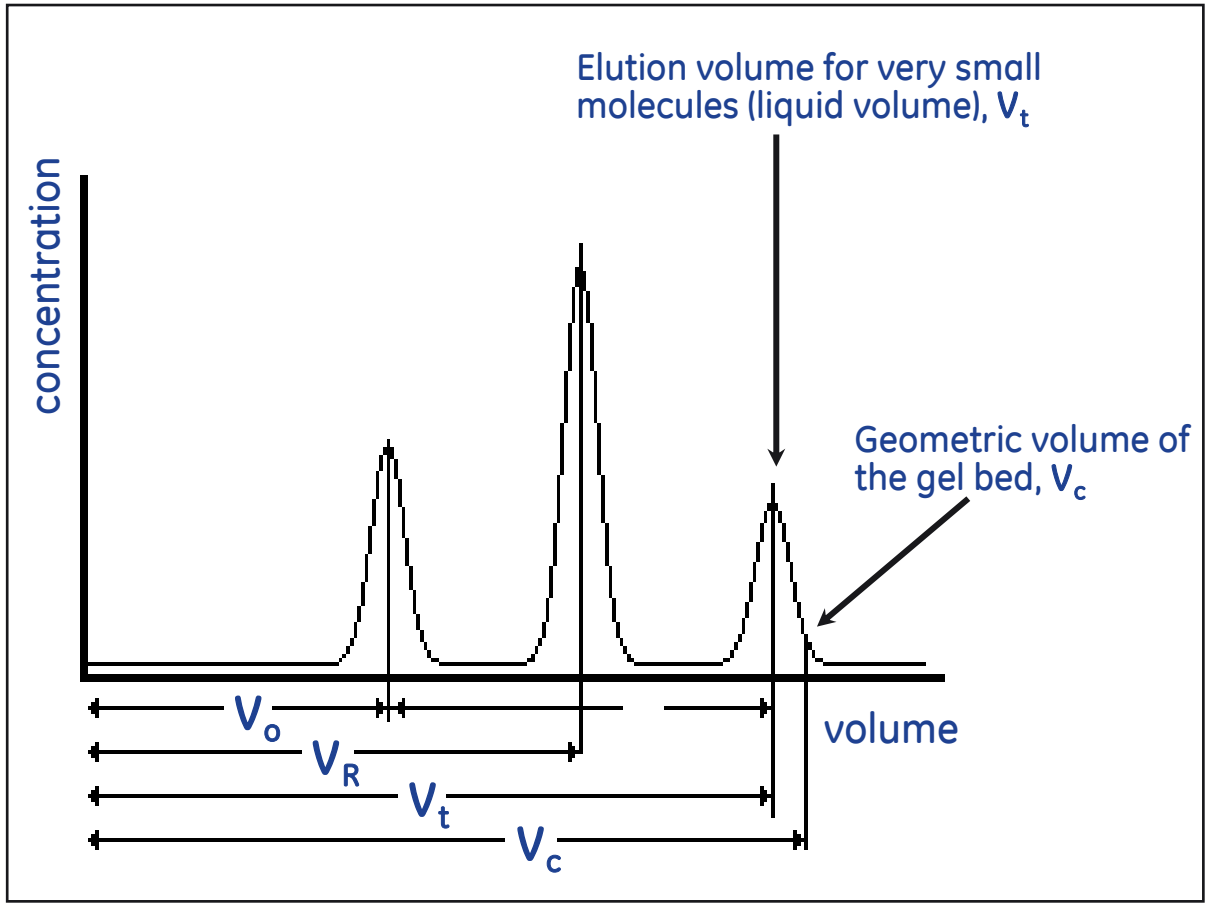


Thank you

Defining the purification objectives



Vt and Vc


Elution volume for very small molecules (liquid volume), V_t
concentration
Geometric volume of the gel bed, V_c
V_o
V_R
V_t
V_c
volume