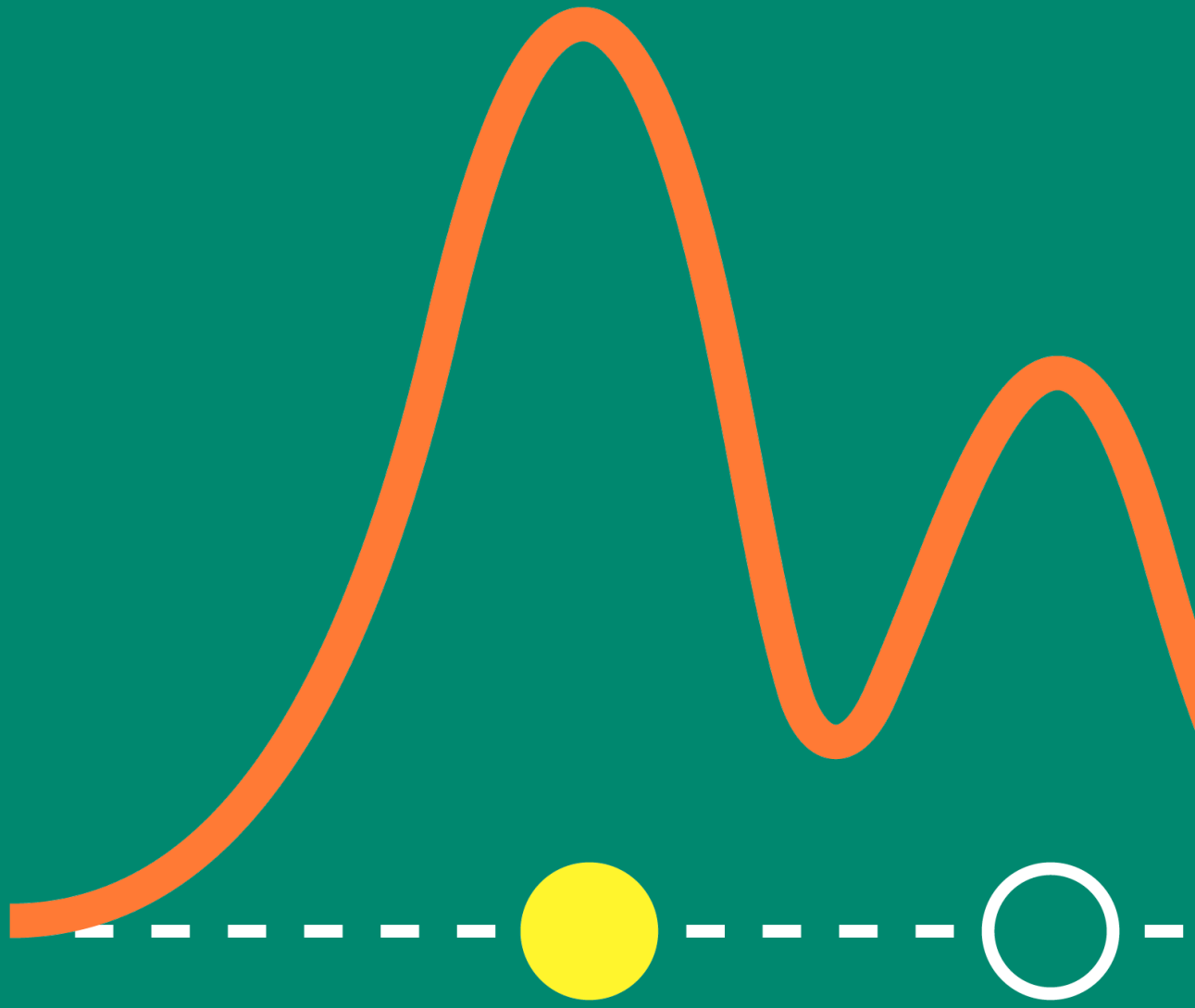
cytiva

Tips for successful ion exchange chromatography



Outline

- Introduction
- When is ion exchange chromatography (IEX) relevant to use?
- How to optimize protein binding/recovery
- How to optimize resolution/purity
- Reduce hands-on time with optimized system configuration
- How to select a chromatography resin/column
- Conclusions and useful tools

Introduction

What are the main chromatography techniques used for protein purification?

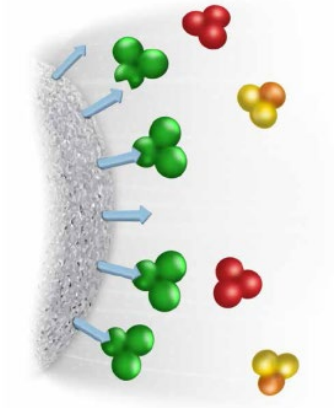
Size exclusion Chromatography

(SEC)

Size

Affinity Chromatography

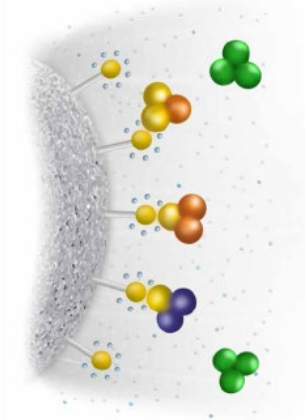
(AC)



Biorecognition

Hydrophobic interaction chromatography

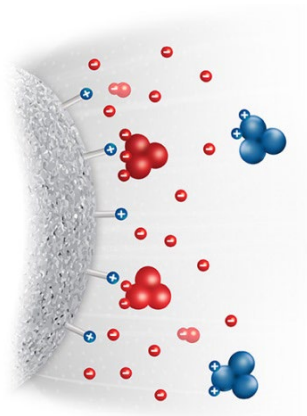
(HIC)



Hydrophobicity

Ion exchange Chromatography

(IEX)



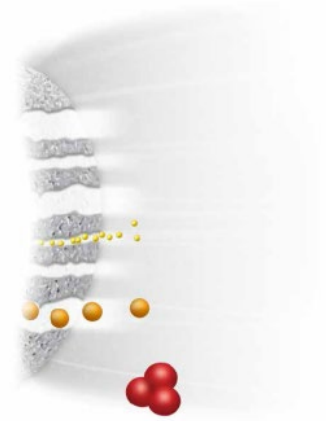
Charge

Chromatography techniques enable separation of proteins based on differences in specific properties

What are the main chromatography techniques used for protein purification?

Size exclusion Chromatography

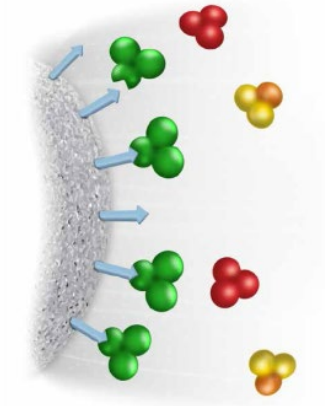
(SEC)



Size

Affinity Chromatography

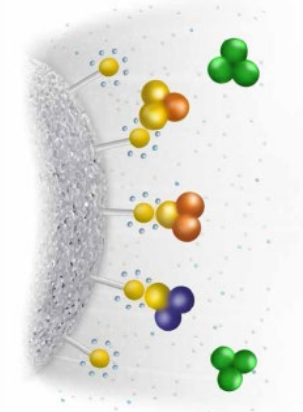
(AC)



Biorecognition

Hydrophobic interaction chromatography

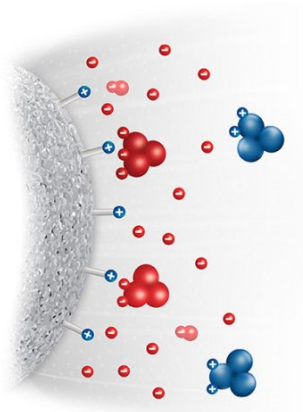
(HIC)



Hydrophobicity

Ion exchange Chromatography

(IEX)



Charge

IEX separates proteins with differences in net surface charge

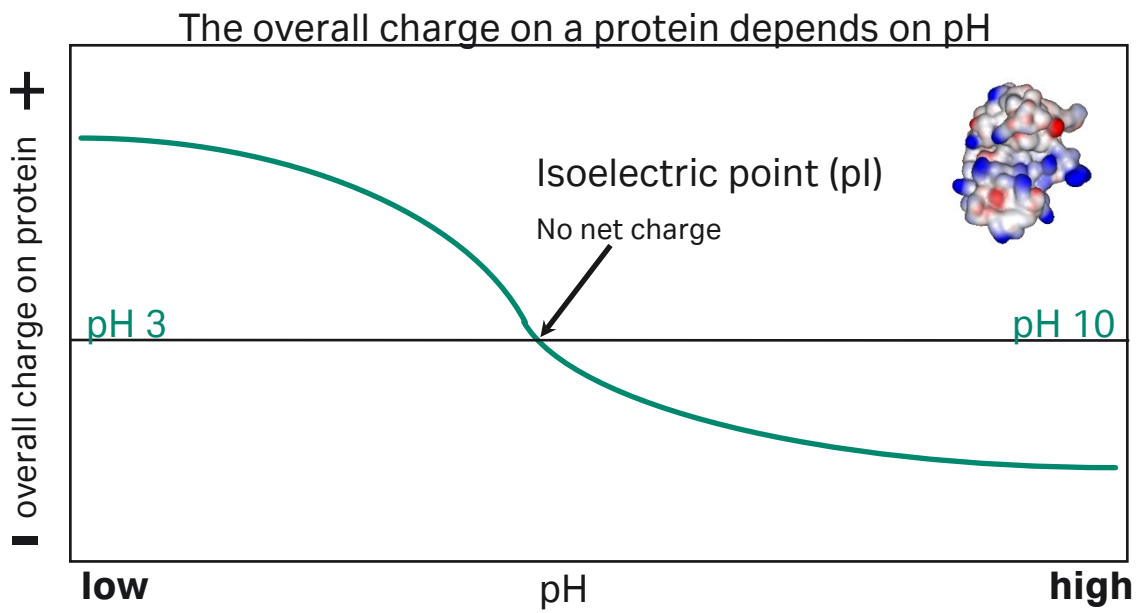
Why is IEX good for protein separation

Every protein has a specific overall net charge that will vary depending on the pH (see graph on the right):

- pI = pH at which a protein has no net charge
- Every protein has its own pI

This property allows proteins to interact to different degrees with an ion exchange resin

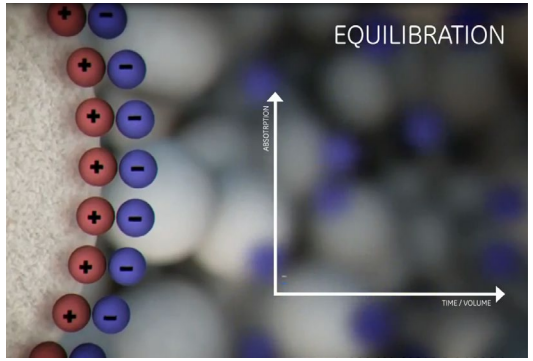
During a IEX run, different pH and ionic strength conditions are sequentially used to bind and recover the protein of interest



A protein's net surface charge is highly pH dependent: it can be utilized to separate proteins from each other

How does IEX work?

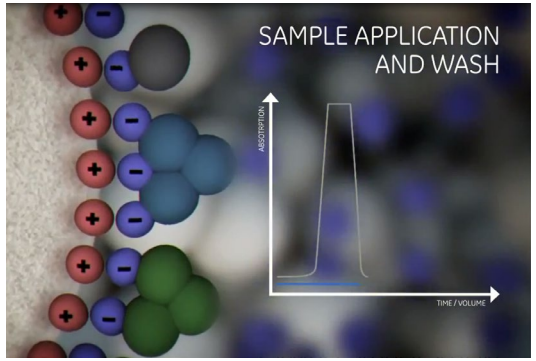
Equilibration



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.

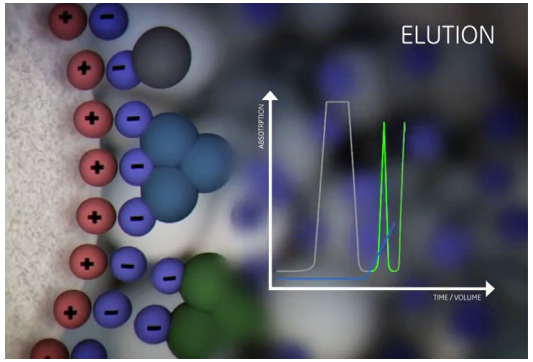
Sample application and wash



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.

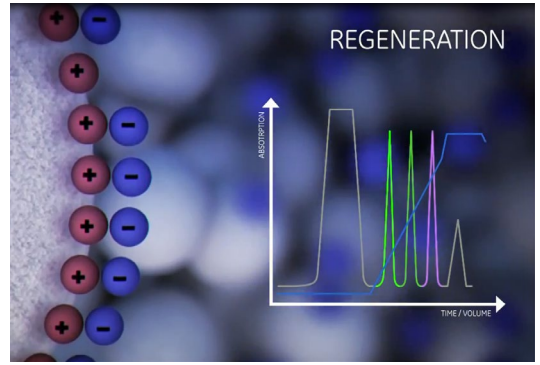
Elution



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.

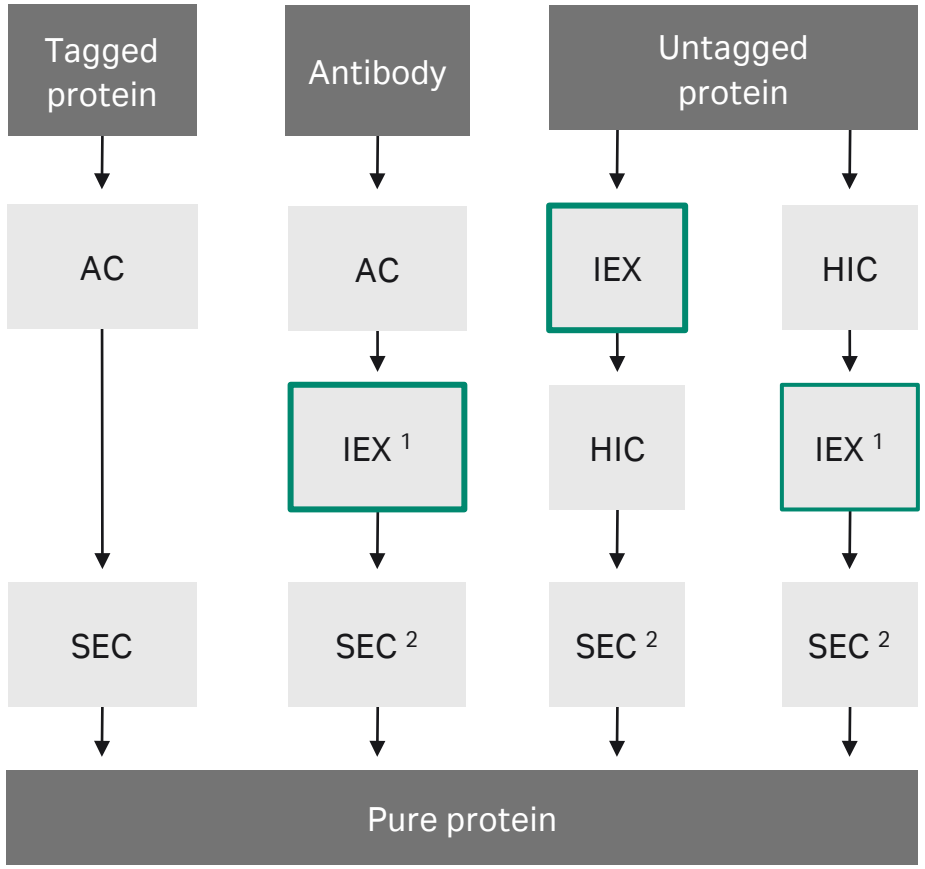
When is IEX relevant to use?

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

Capture:
Isolate, concentrate, stabilize

Intermediate purification:
Remove bulk impurities

Polishing:
Remove final contaminants



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

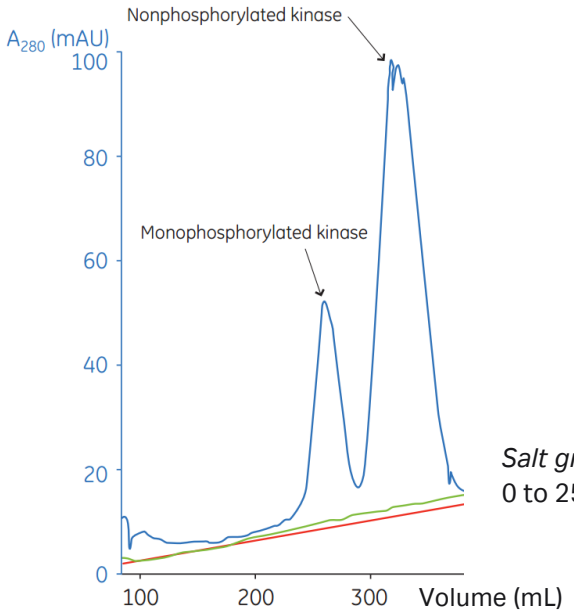
[2] SEC is not typically used as a polishing step in industrial applications, because scale-up is particularly challenging.

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.

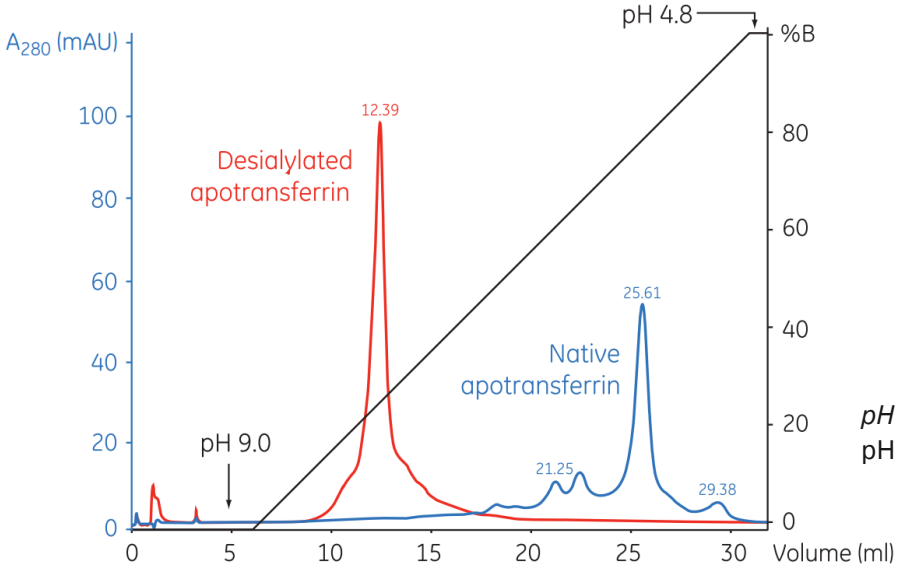


Salt gradient:
0 to 250 mM NaCl in 80 CV*

* CV = column volume

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.



pH gradient:
pH 9.0 to 4.8 in 25 CV

How to optimize protein binding/recovery

Did you recover less protein than expected?

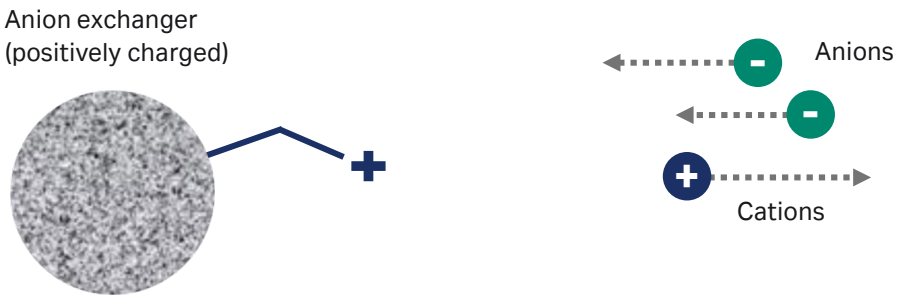
Maybe your protein did not bind properly to the resin.

Possible reasons for poor protein binding

- Incorrect ion exchanger
- Sample has wrong pH or incorrect buffer conditions
- Column is not equilibrated sufficiently in buffer
- Binding capacity of resin is exceeded
- Column is dirty

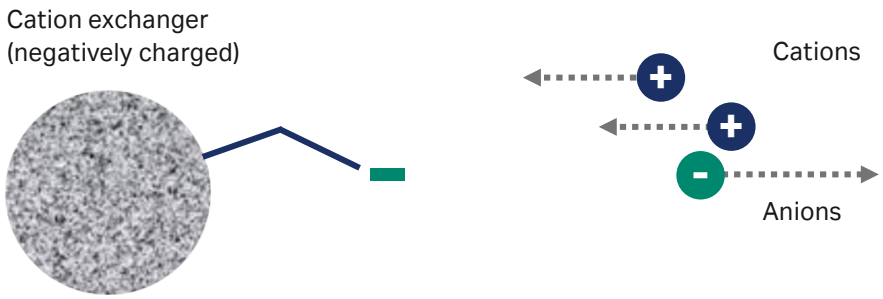
Different types of ion exchangers

Anion exchangers = bind anions



Most common ligands: Q (strong), DEAE (weak), ANX (weak)

Cation exchangers = bind cations



Most common ligands: S (strong), SP (strong), CM (weak)

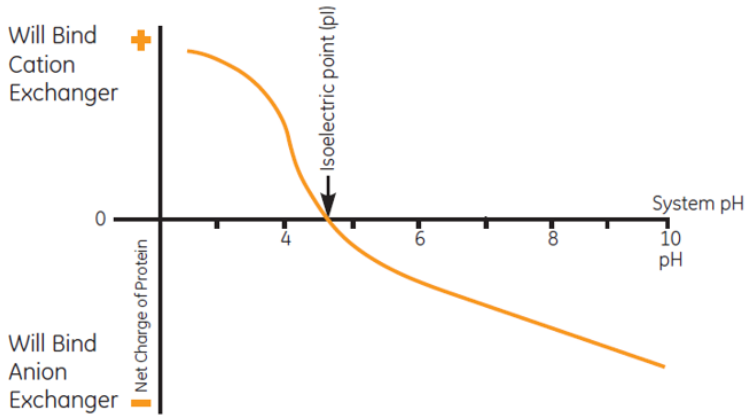
What does "strong" and "weak" mean?

- Strong ion exchangers → the ion exchanger is fully charged over a broad pH range
- Weak ion exchangers → the charge of the ion exchanger varies with pH

Tip!
Start with a strong ion exchanger. If the selectivity is not good enough, try a weak ion exchanger.

Ensure protein binding by using the most appropriate ion exchanger

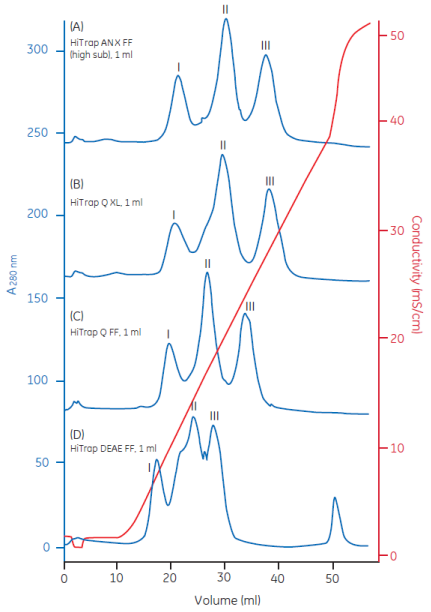
If pI of your protein is known

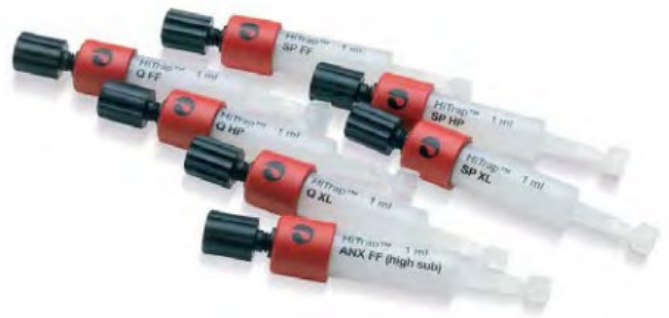


- Select an anion exchanger (Q, DEAE, ANX) with a buffer pH above pI
- Select a cation exchanger (S, SP, CM) with a buffer pH below pI

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



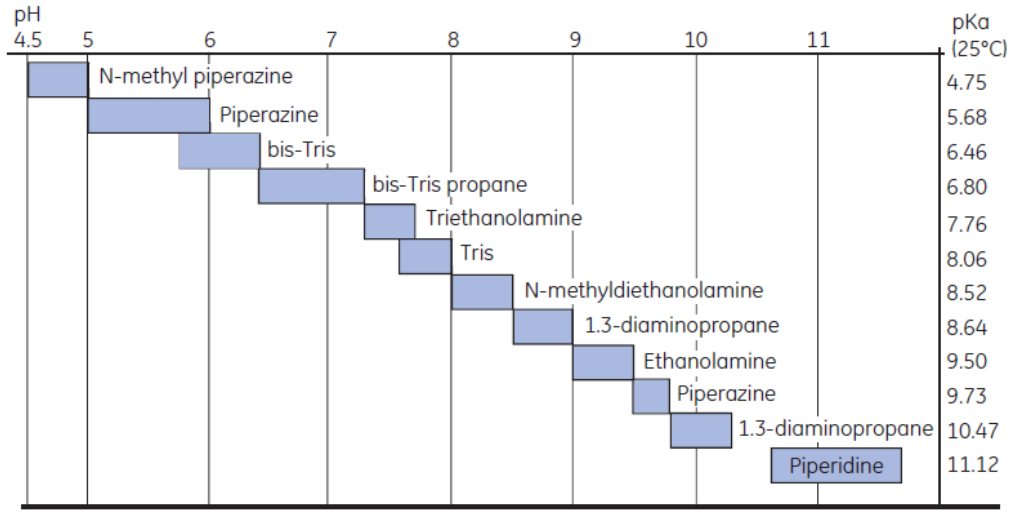


HiTrap™ IEX Selection kit

Ensure protein binding by using appropriate buffer substances

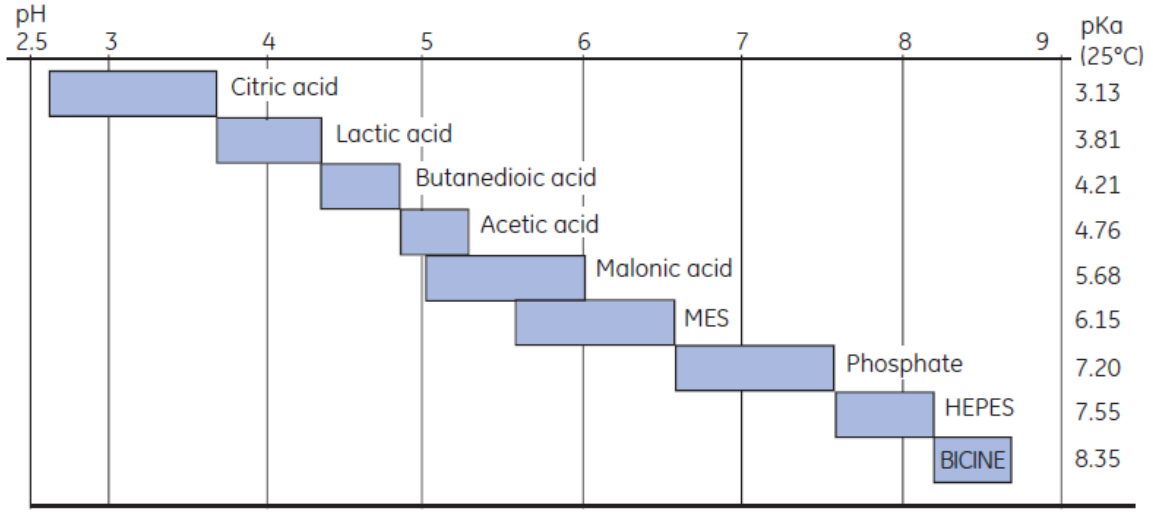
Buffer choice depends on protein pI and type of IEX

Buffers for anion exchange chromatography



- Choose buffer 0.5-1 pH units **above** pI

Buffers for cation exchange chromatography

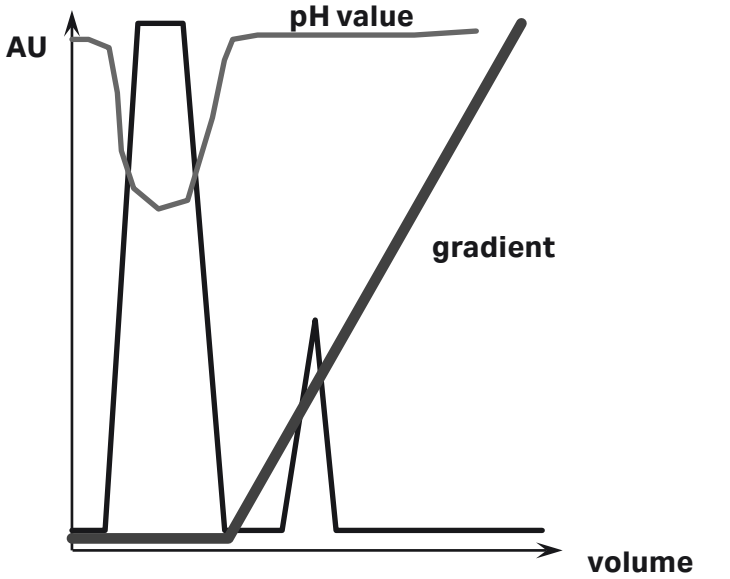


- Choose buffer 0.5-1 pH units **below** pI

✓ Consider stability window of protein (often unstable around pH=pI)
✓ Ensure that your column is sufficiently equilibrated in buffer
✓ Choose a buffering ion with same charge as the resin to prevent it from taking part in the ion exchange process

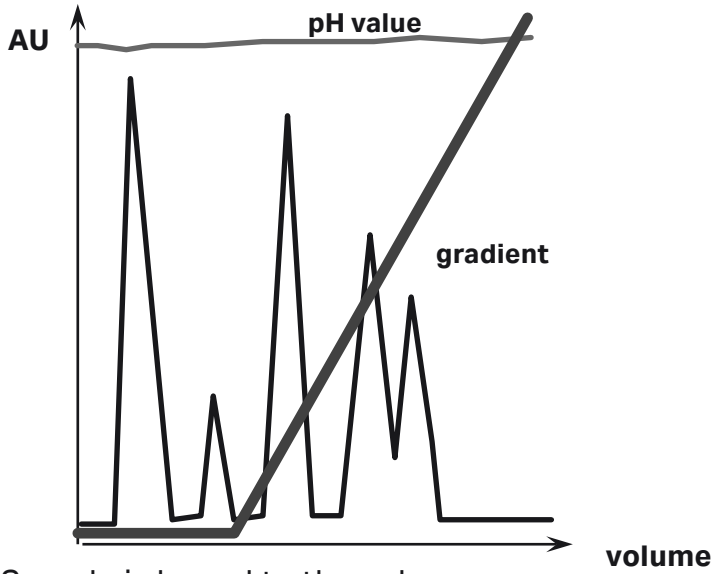
Ensure protein binding by using the correct sample pH

Sample was not adjusted to the starting buffer pH



A large amount of sample is not bound to the column and is washed out

Sample was adjusted to the starting buffer pH



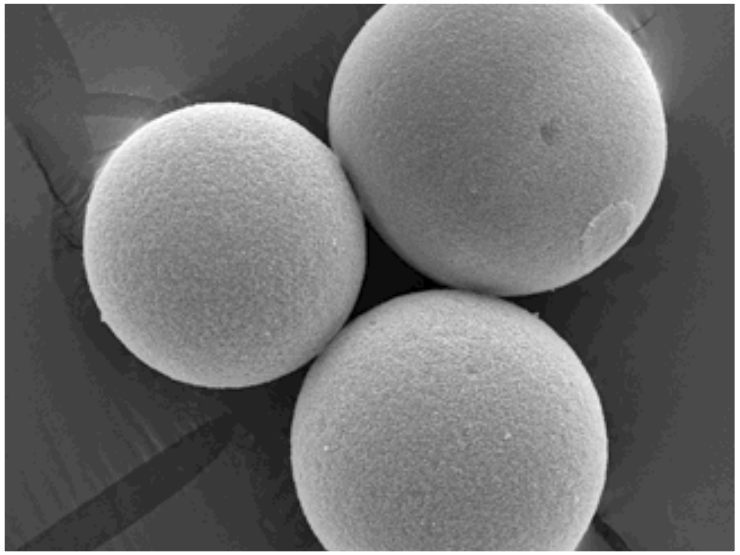
Sample is bound to the column

Tip! For maximized binding, transfer the sample in binding buffer prior to application to the columns. Especially important with large sample volumes.

Cleaning a dirty column can restore the binding capacity

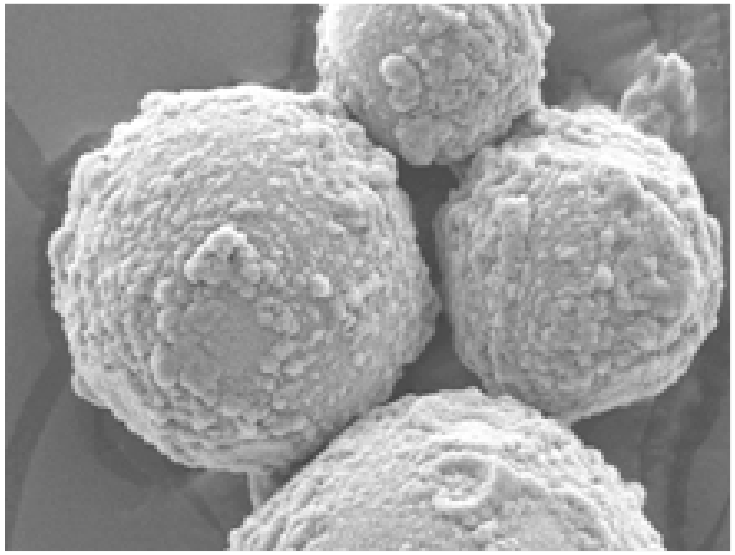
Fresh column

Capacity:
62 mg ovalbumin/mL (100%)



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

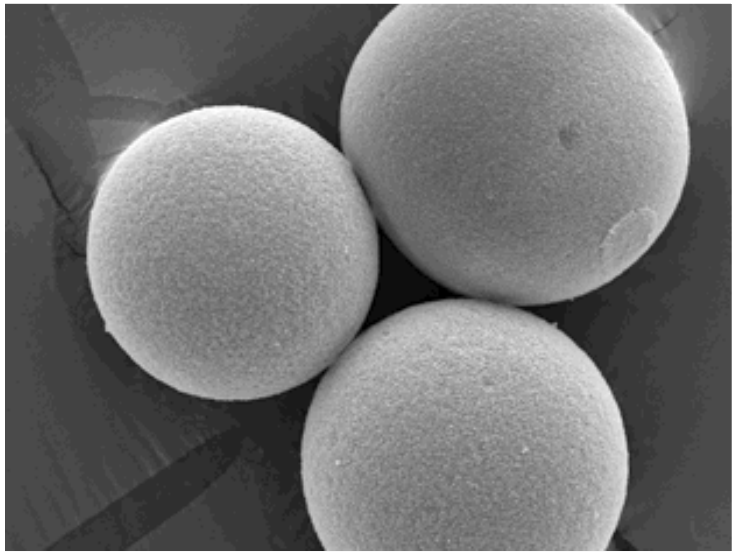
Capacity:
48 mg ovalbumin/mL (77%)



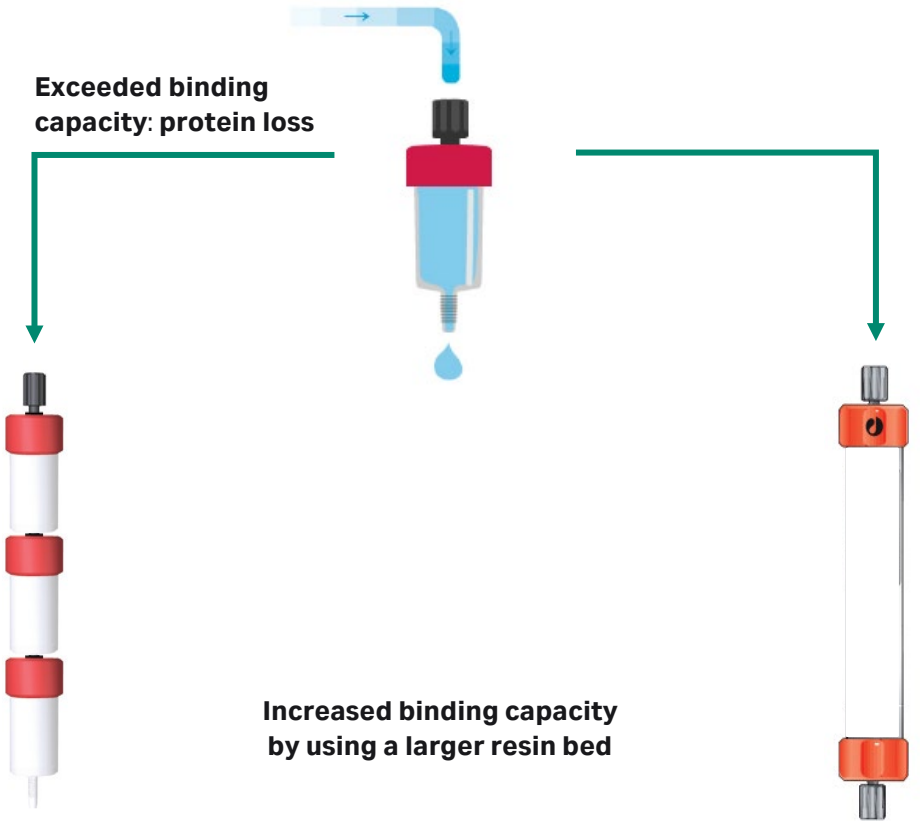
Column: HiTrap™ Q HP 1 mL
Sample: Ovalbumin

After cleaning

Capacity:
59 mg ovalbumin/mL (95%)



Ensure protein binding by using a correct amount of resin



Three HiTrap columns in series

HiPrep 20 mL column

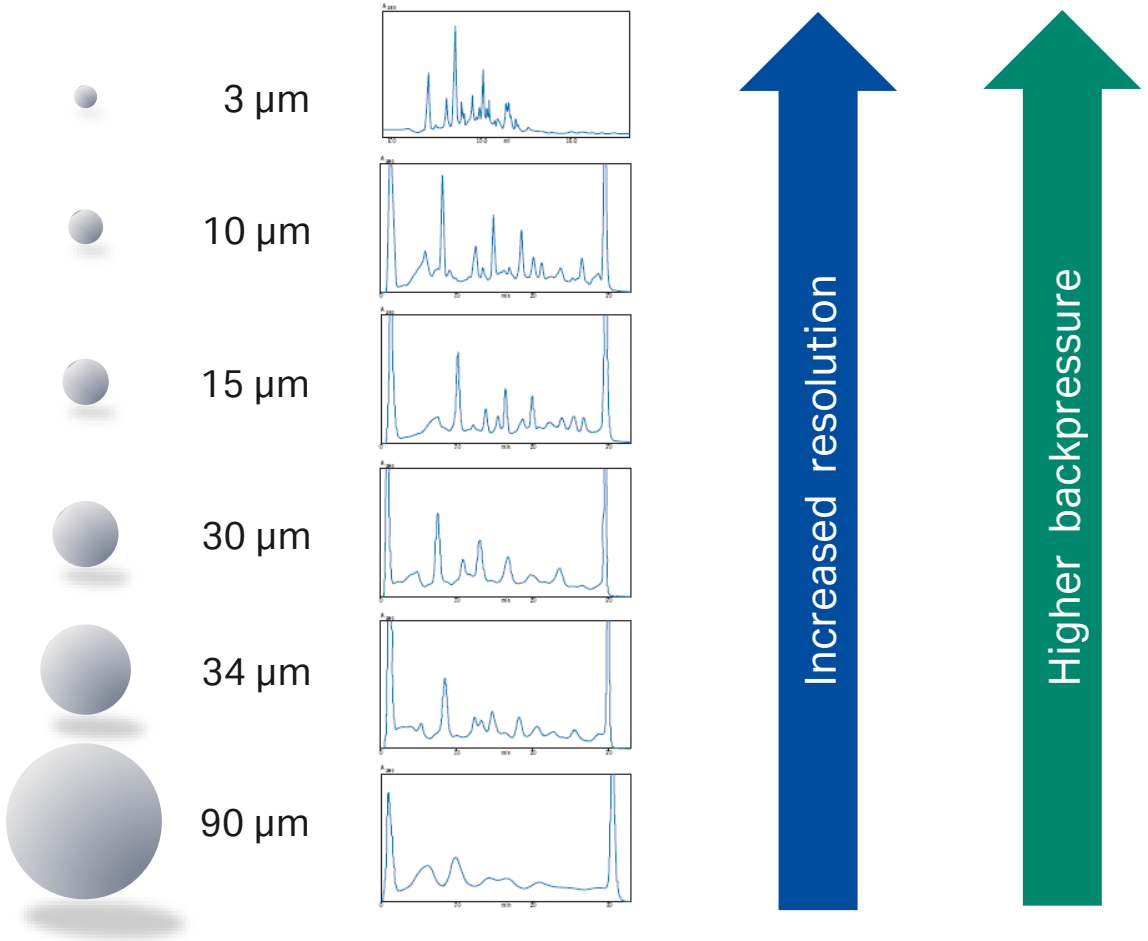
If binding capacity is exceeded, you might need a larger amount of resin to purify your protein sample.

Two possible options:

- Use a larger column, for example, HiPrep™ format
- If using a HiTrap™ column, connect up to three columns in series

How to optimize resolution/purity

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.

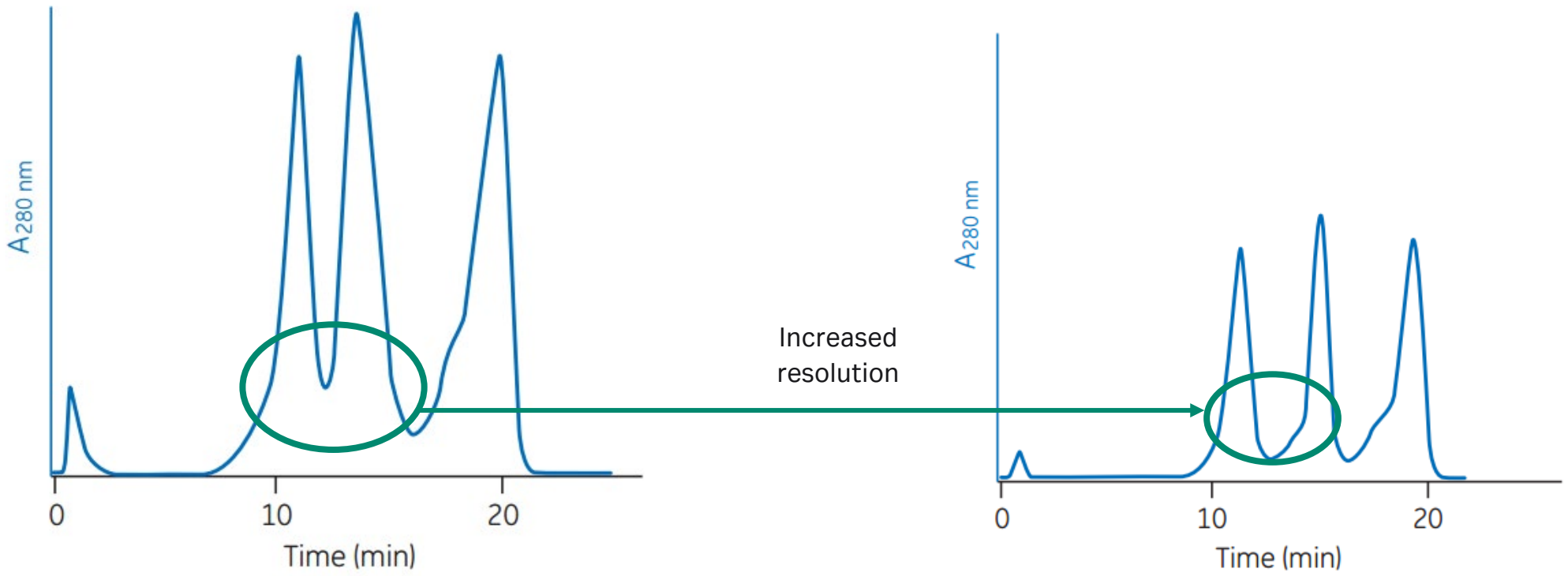
Decreasing the sample load will increase resolution

Sample load: 10 mg

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

Sample load: 1 mg

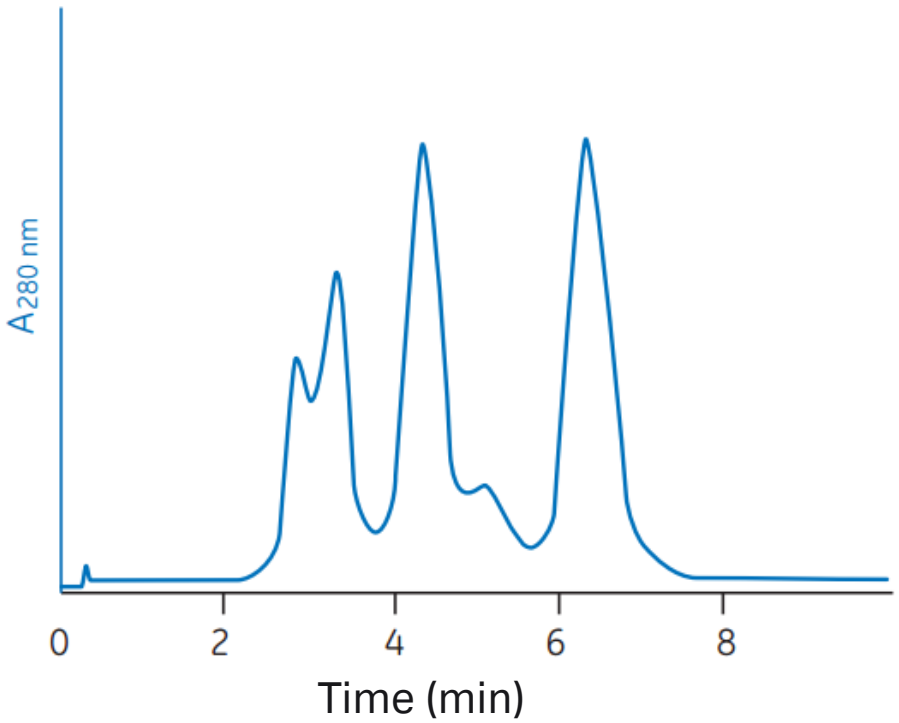
Column: SOURCE 30S, 5 mm × 50 mm (i.d. × h)



Decreasing the flow rate will increase resolution

Flow rate: 13 mL/min

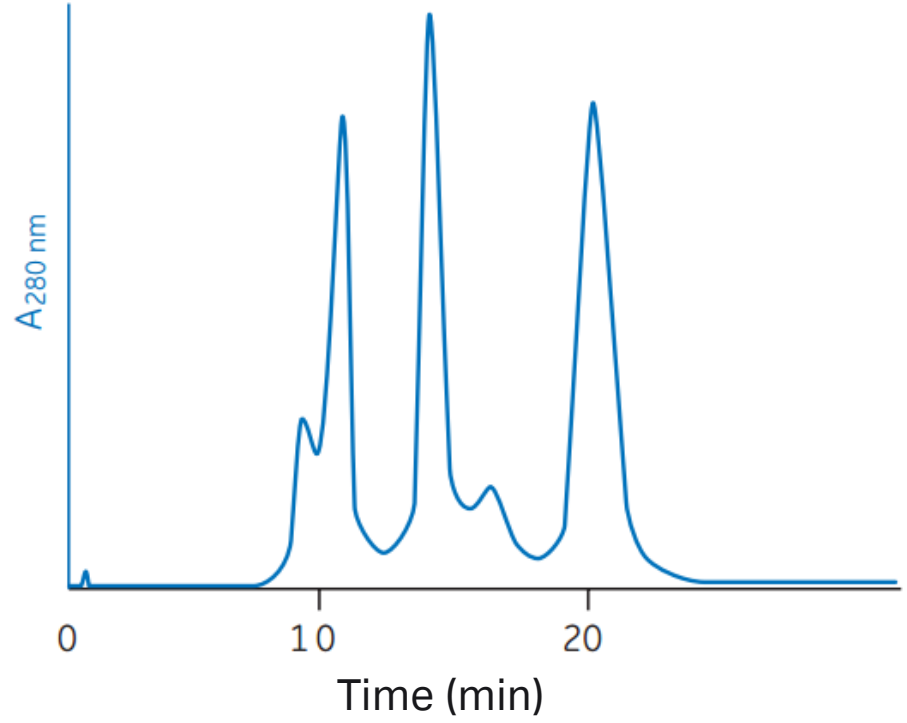
Column: SOURCE™ 30Q, 10 mm × 50 mm (i.d. × h)



Increased resolution

Flow rate: 4 mL/min

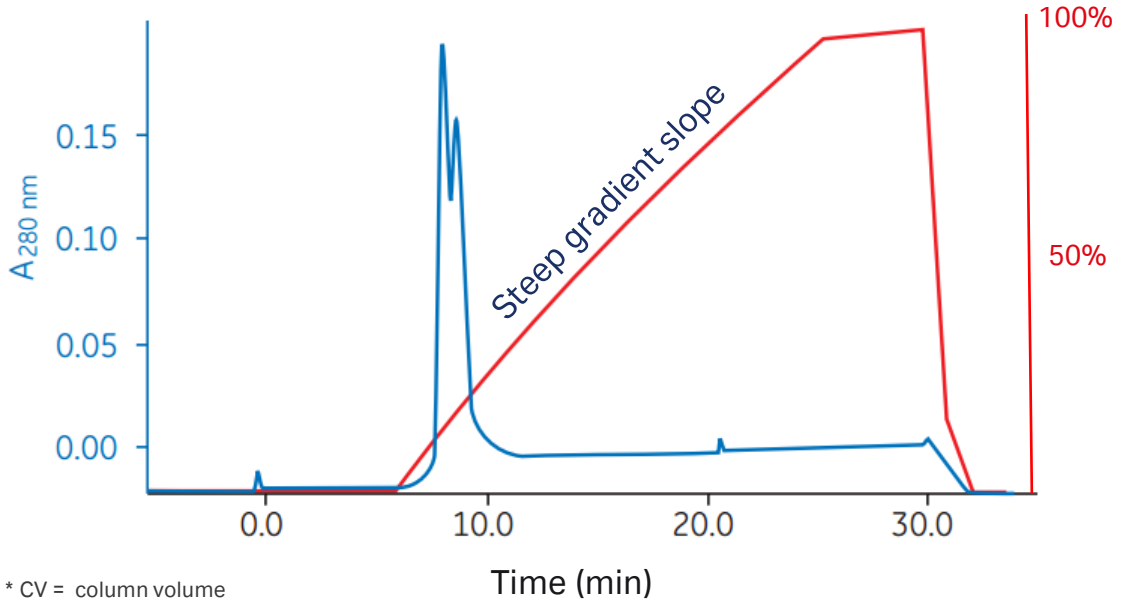
Column: SOURCE 30Q, 10 mm × 50 mm (i.d. × h)



A more shallow gradient will increase resolution

Steep gradient slope

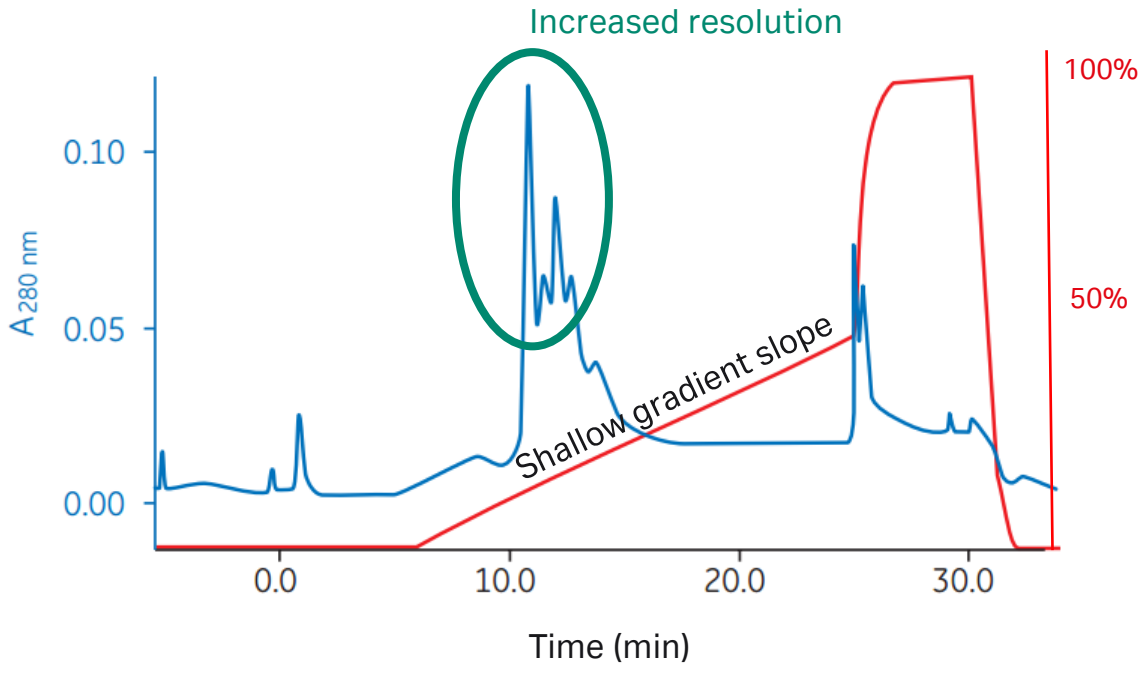
Gradient: 0% to 100% elution buffer in 20 CV*



* CV = column volume

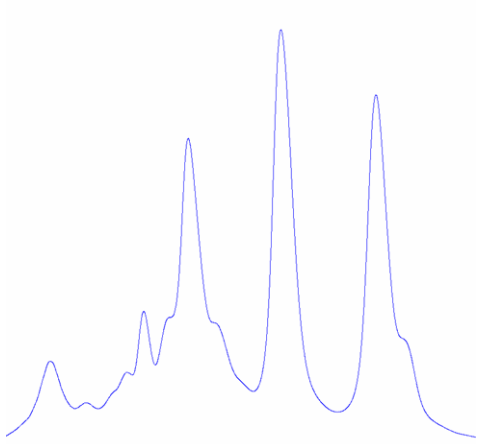
Shallow gradient slope

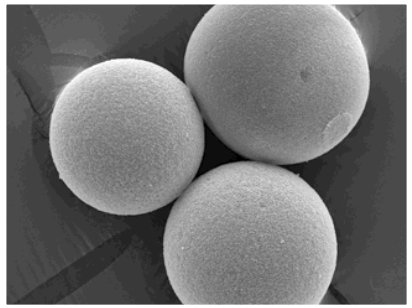
Gradient: 0% to 40% elution buffer in 20 CV



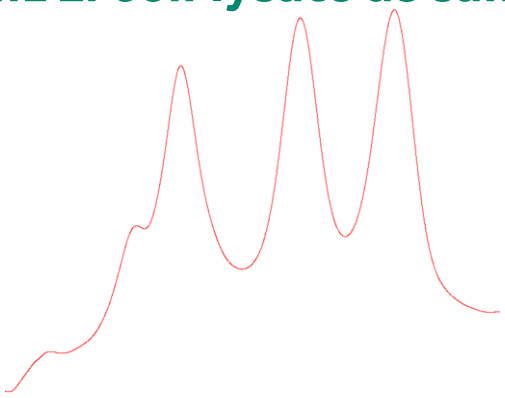
Cleaning a dirty column can restore the resolution

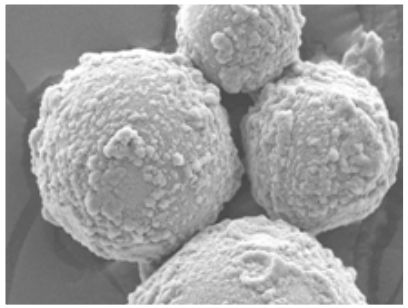
Fresh column



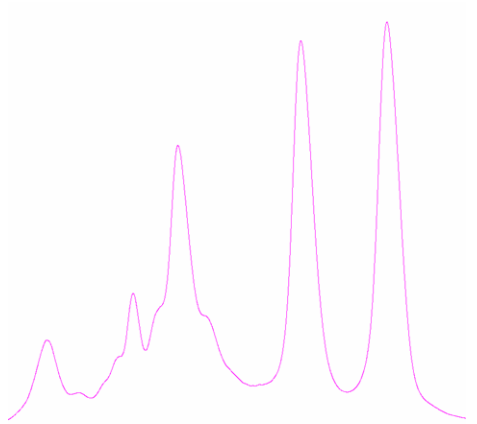


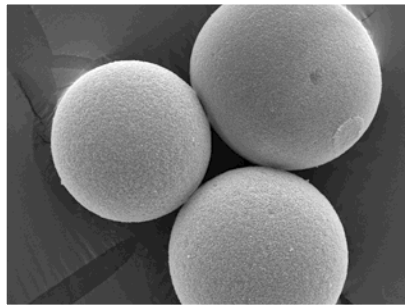
After a purification step using 30 mL *E. coli* lysate as sample





After cleaning

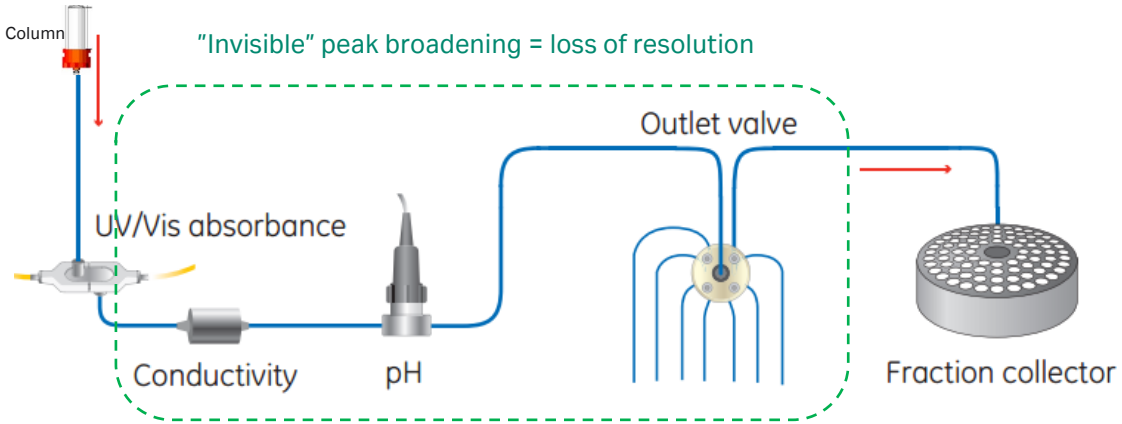


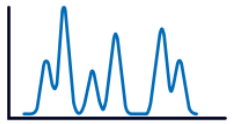


Column: HiTrap™ Q HP 1 mL
Sample: Standard proteins

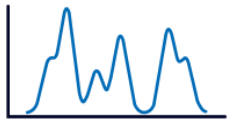
Prevent resolution loss with minimized volumes after column

What happens after the UV detector?



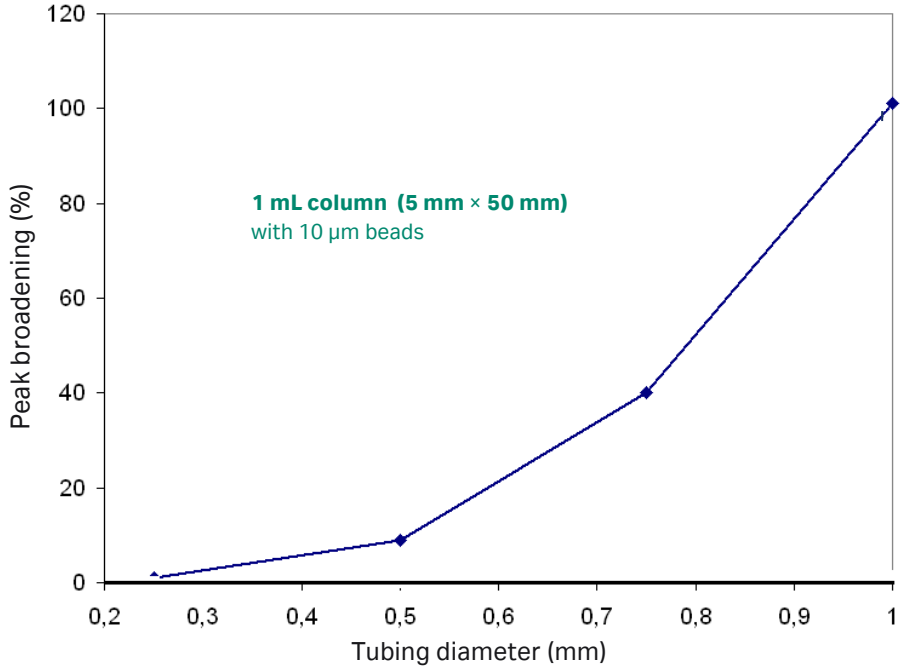


This is the peak separation at the UV/Vis absorbance detector. It is also what is displayed on the chromatogram.



This is the actual peak separation caused by the "invisible" peak broadening.

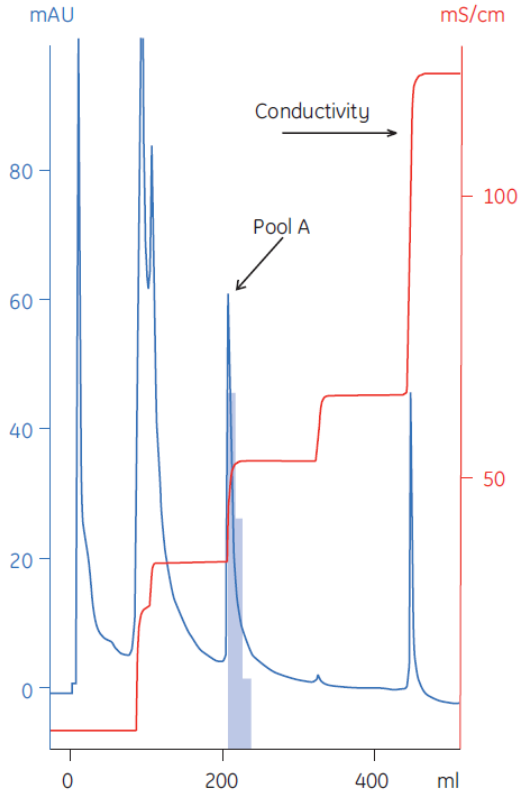
Effect of tubing diameter on peak broadening



Minimize volumes after column by decreasing tubing diameter and minimizing length

Addition of a size exclusion chromatography step (SEC) can improve purity

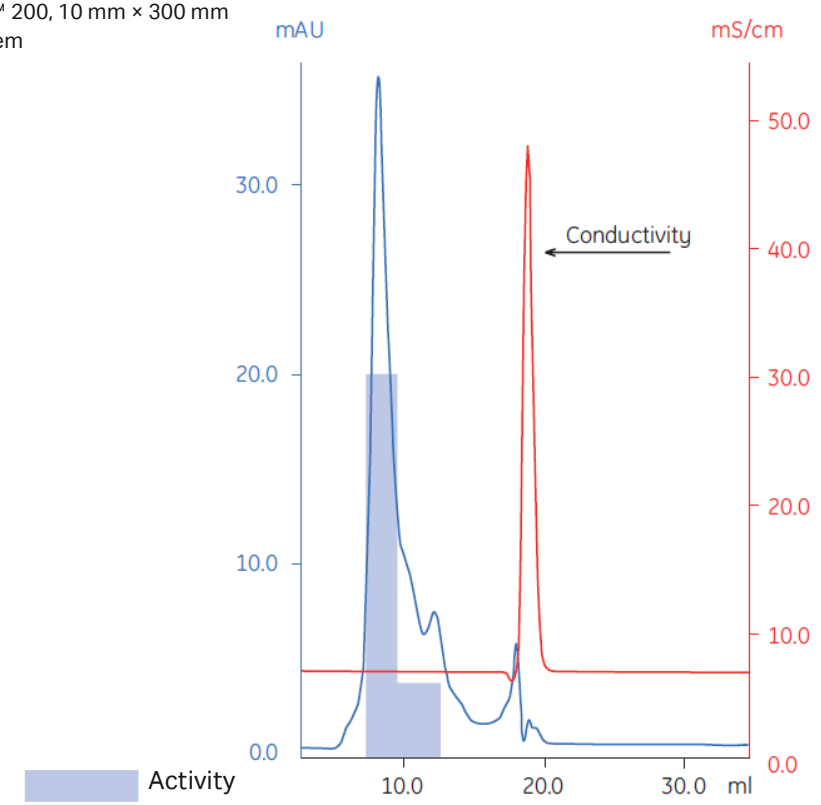
1st step: IEX



Column: HiPrep™ 16/10 Q XL
Sample: Extracted mitochondrial membrane protein
System: ÄKTA™ system

2nd step: SEC

Column: Superdex™ 200, 10 mm × 300 mm
System: ÄKTA system



Reduce hands-on time with optimized system configuration

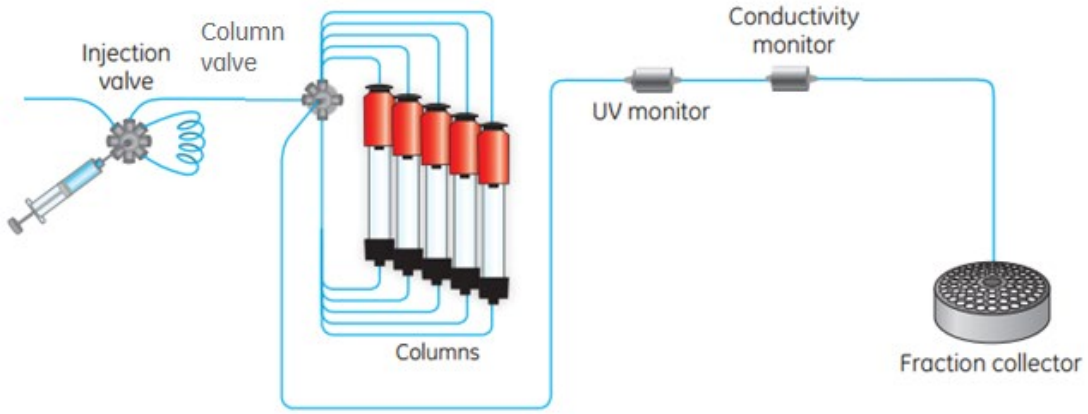
Reduce hands-on time — column screening automation

Traditional screening of columns/resins

- Each column needs to be manually changed between each run

Automated column/resin screening

- Several columns can be connected to the system from the start:
  - up to five columns with ÄKTA™ pure
  - up to ten columns with ÄKTA avant
- The column selection valve enables automatic switch from one column to the next one
- Minimizes manual intervention
- Reduces further the risk of introducing air into the column



Column selection valves enables column screening on ÄKTA pure and ÄKTA avant systems

Reduce hands-on time — online buffer preparation for automated pH screening

Traditional pH screening

A detailed screening would be very time consuming:

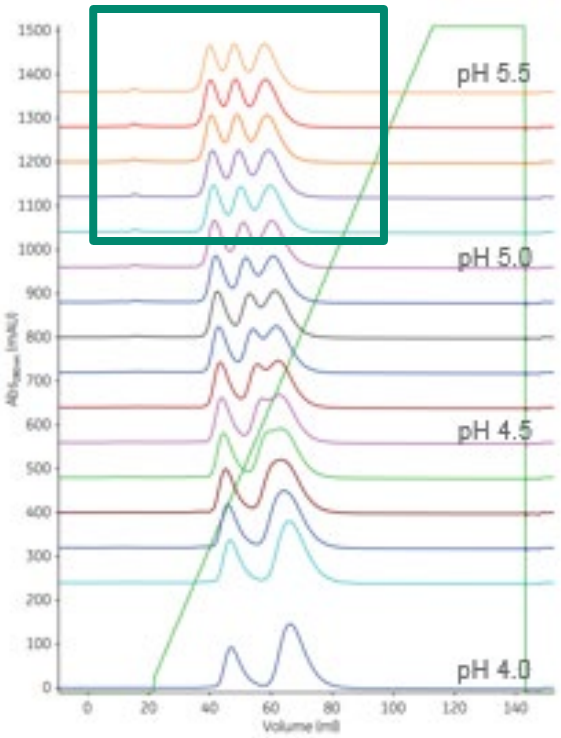
- Each pH buffer needs to be prepared, including manual titration

Automated pH screening with BufferPro* functionality

Enables an accurate, detailed pH screening:

- Prepare four stock solutions. Buffers are automatically prepared inline
- Predefined or user-defined buffer recipes

*available on ÄKTA avant system



Example of automated pH screening with BufferPro in ÄKTA™ avant system with HiScreen™ Capto™ S :

- 16 runs in 0.1 pH steps were automatically performed
- Results show that the best separation was at pH > 5.0

Reduce hands-on time — two-step purification* automation

Non-automated protocol

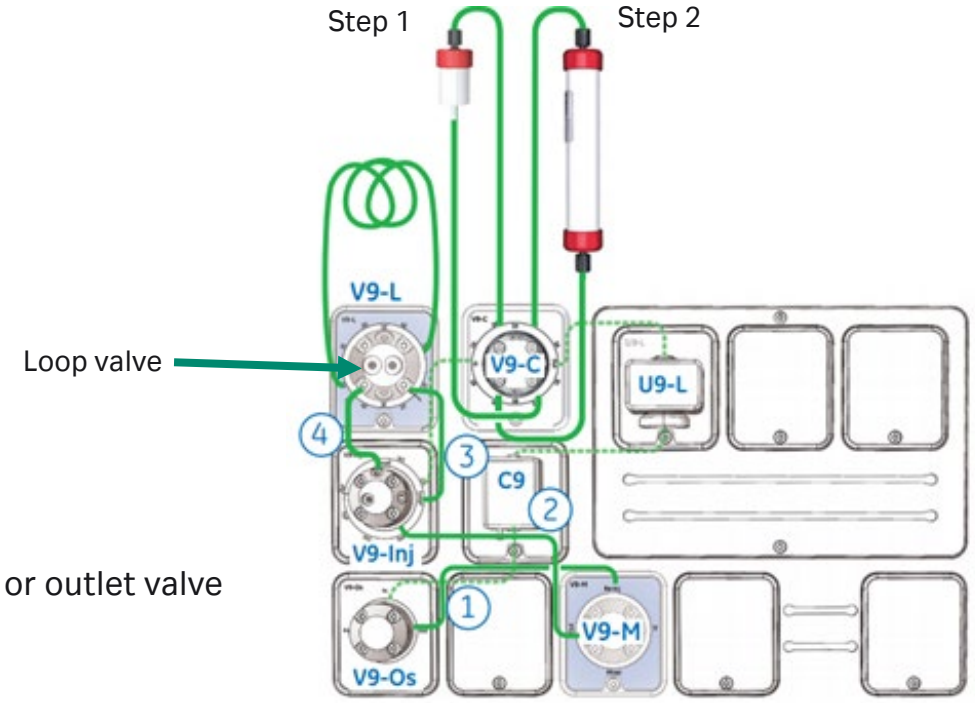
Transition from step 1 to step 2 is done manually:

- Collect the eluted material from first step
- Switch to the second column (using a column valve or manually)
- Purge pumps with buffer for the second step
- Re-inject sample to the second column

Automated protocol

- Transition from step 1 to step 2 is automated with the use of an injection-, loop-, or outlet valve
- Both columns are connected from the start

*also called 2-D purification

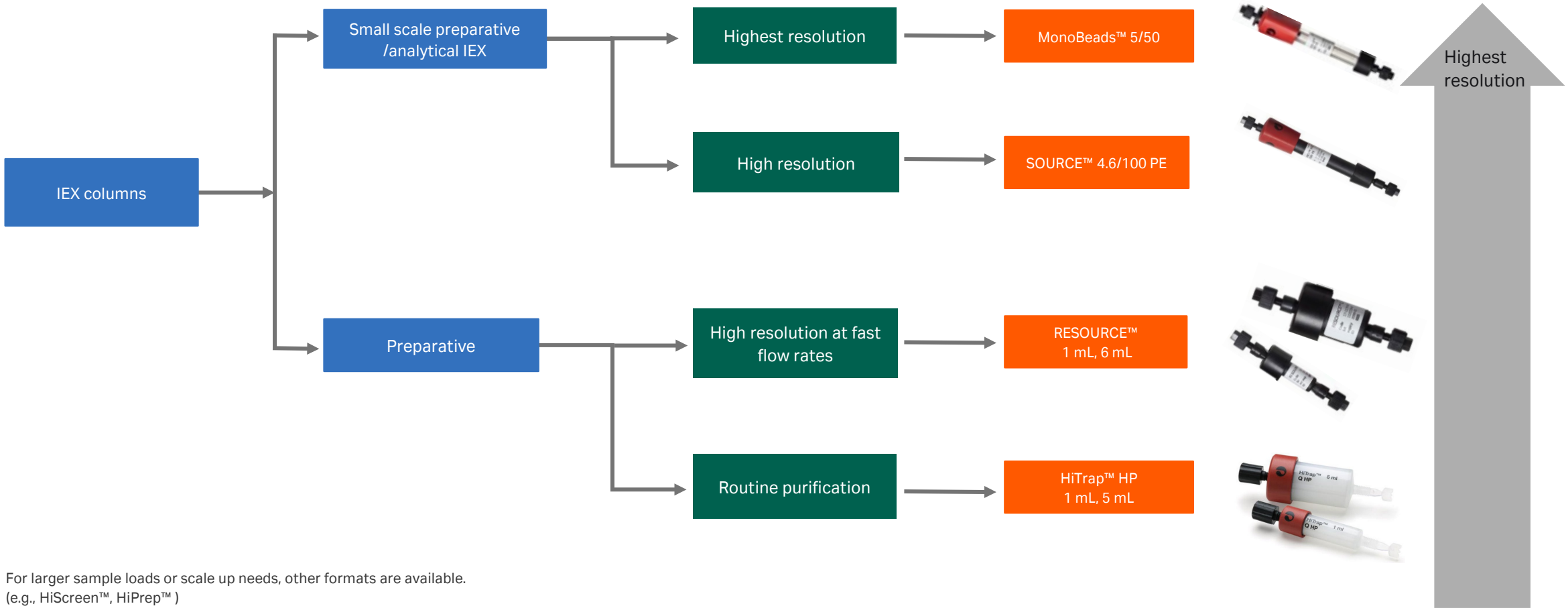


ÄKTA pure configured for automated 2-step purification

ÄKTA™ pure and ÄKTA avant enable flexible configuration for automated two-step applications

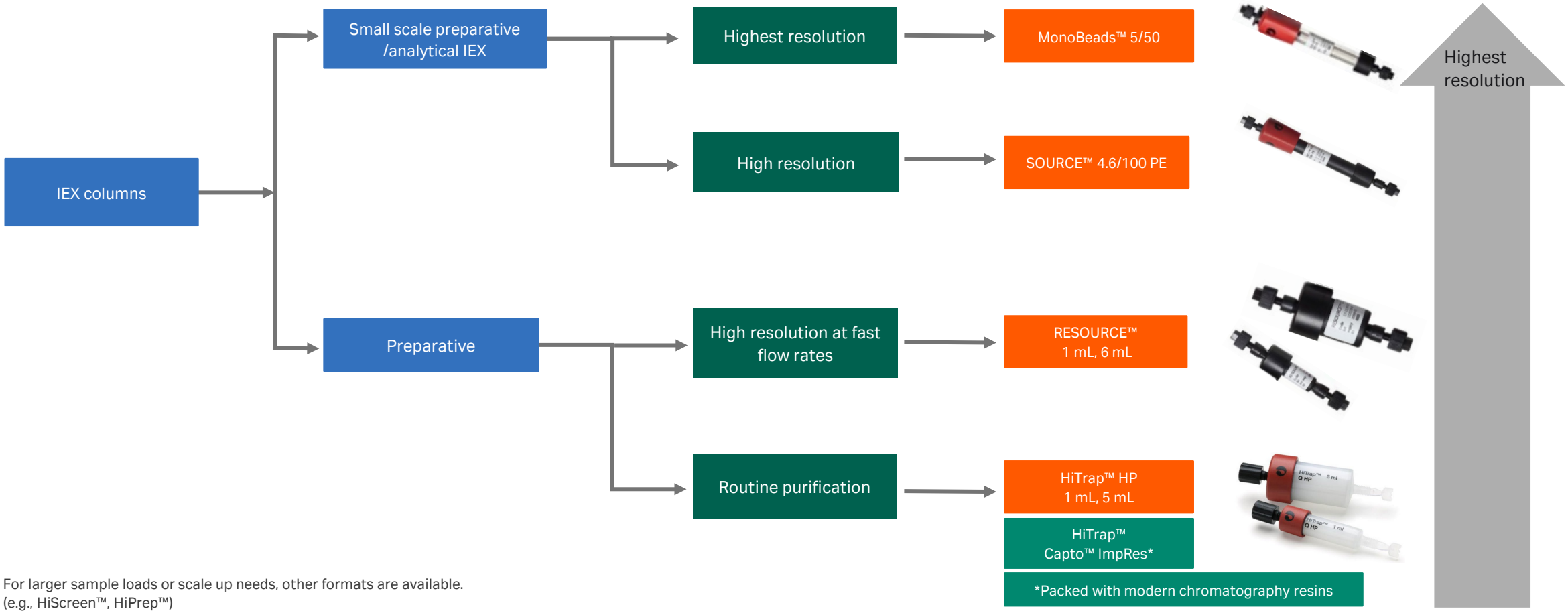
How to select a chromatography resin/column

Key IEX chromatography columns used in basic research



For larger sample loads or scale up needs, other formats are available.
(e.g., HiScreen™, HiPrep™)

Key IEX chromatography columns used in basic research



For larger sample loads or scale up needs, other formats are available.
(e.g., HiScreen™, HiPrep™)

Modern IEX resins

In order to meet growing demands from industry, Cytiva has developed resins that deliver improved pressure/flow properties at large manufacturing scales.

The majority of these resins are also packed in HiTrap™ format for use in research scale and PD* for purifying small protein samples.

Example: HiTrap Capto™ ImpRes columns

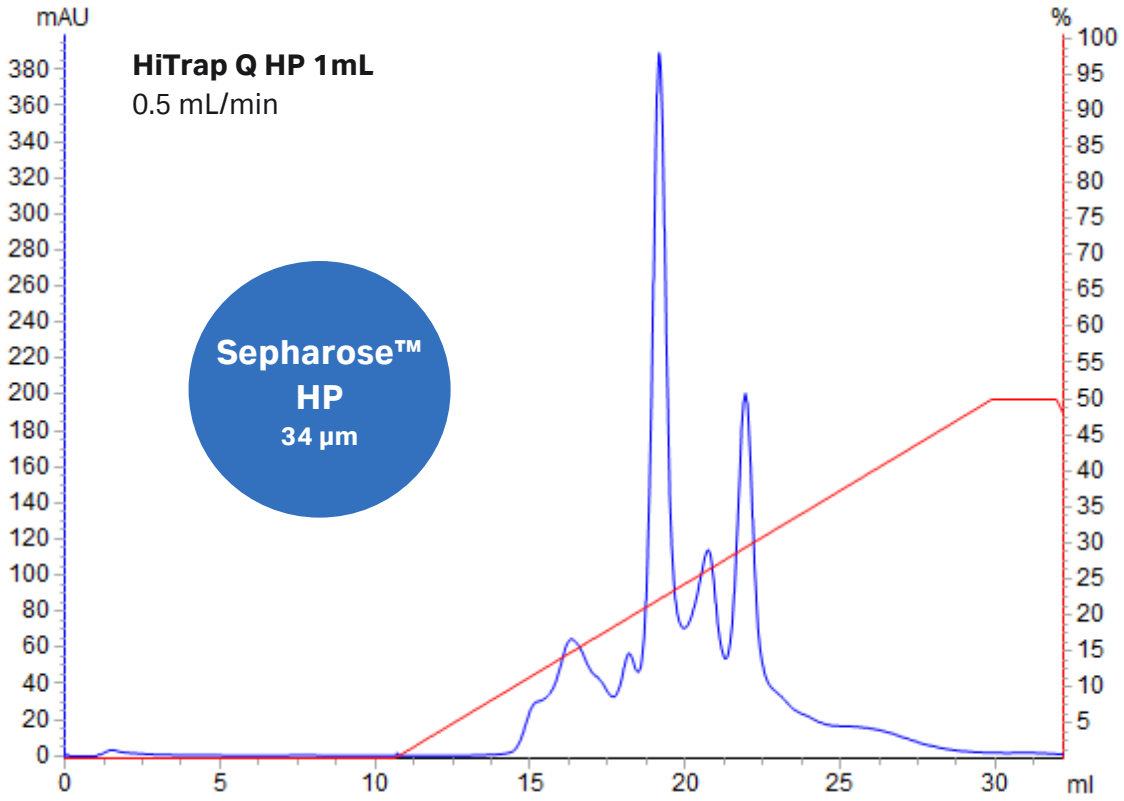
HiTrap column packed with modern resin Capto ImpRes
(HiTrap Capto SP ImpRes)

* PD = process development

Comparing HiTrap HP vs HiTrap Capto ImpRes

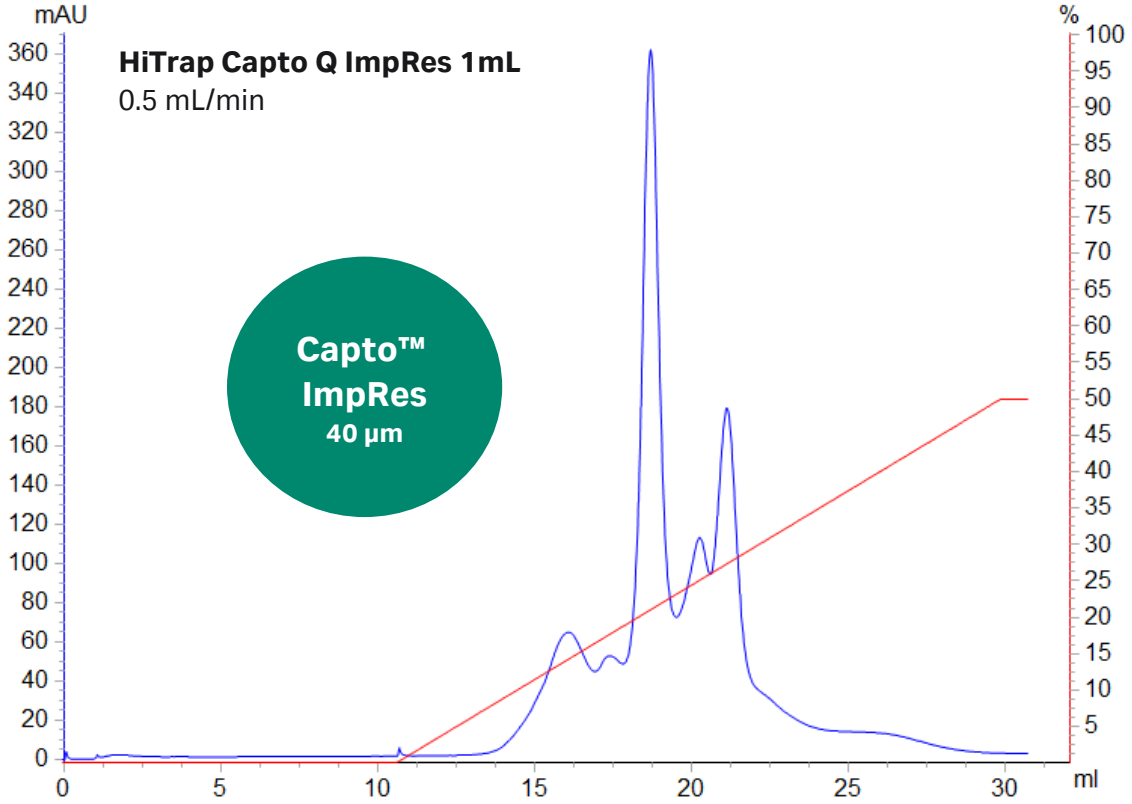
Similar resolution is achieved with the same protocol

HiTrap™ HP column



Sample: 0.5mL Conalbumin (0.3 mg/mL), α-lactoalbumin (0.4 mg/mL), soybean trypsin inhibitor (0.8 mg/mL)

HiTrap Capto™ ImpRes column



HiTrap Capto ImpRes — packed with modern IEX resin

Delivers similar resolution as HiTrap HP
Limited needs for protocol optimization

Is a newer generation resin
that is already commonly used in industry for process development and drug manufacturing

Available with strong ion exchangers
Strong anion exchanger: HiTrap™ Capto™ **Q** ImpRes
Strong cation exchanger: HiTrap Capto **SP** ImpRes

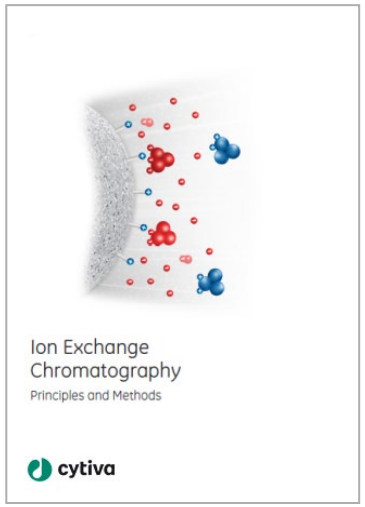
Conclusions and useful tools

Conclusions

IEX can be used in various stages of the protein purification protocol. It is also an excellent tool for purity and heterogeneity analysis.

- To increase **resolution** (purity :
  - Reduce flow rate and/or sample load
  - Use a shallow gradient
  - Consider the bead size and system tubing dimensions
- To increase protein **recovery**:
  - Ensure that an appropriate ion exchanger is used and that sample and buffer conditions are correct
  - Clean your column regularly
  - Do not exceed the binding capacity of the column
- Reduce hands-on time with optimized system configuration
- Select the appropriate chromatography column depending on your needs

Useful tools



Ion exchange chromatography handbook
cytiva.com/ProteinHandbooks

Purify App column and resin interactive selection tool
cytiva.com/purify

Thank you

For local office contact information, visit cytiva.com/contact

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