Ion exchange chromatography

Anion (AIEC) – Cation (CIEC)
Ion exchange chromatography (IEC)

- Principles of IEC
- Main stages in Chromatography
- Resolution, efficiency, selectivity and capacity
- Determination of start conditions
- Parameters for absorption optimization
- Parameters for elution optimization
- Troubleshooting
- Examples
- Summary
What is ion exchange chromatography?

- IEC is a form of adsorption chromatography which separates molecules on the basis of their charge.

- Interactions of positively or negatively charged molecules “binding” to oppositely charged in the resin. This process is reversible via:
  - **Salt elution (Competitive)**: Increasing the ionic strength of the buffer by addition of salt elutes the bound molecules, in a selective way, producing a separation based on charge differences.
  - **pH elution (charge)**: By altering the pH of the mobile phase proteins become uncharged or oppositely charged and will elute according to their pl values.
Why use ion exchange?

- Useful at all stages of purification and at all scales.
- Controllable
- High selectivity
- High capacity
- Concentrating
- High recovery
Basis for selectivity

Some of the charged regions which will influence ion exchange

- Interaction between opposite charges
  - Charged groups on the proteins interact with charged groups on the ion exchanger. Different proteins have different charges and interact differently.

- Anion or cation exchange
  - Anion exchange binds negatively charged (anionic) proteins
  - Cation exchange binds positively charged (cationic) proteins
Main stages in Chromatography

- Equilibrate the gel and the sample to binding conditions
- Apply the sample
- Wash out contaminants
- Elute
- Wash and Regenerate column

https://www.youtube.com/watch?v=q3fMqgT1do8
Main stages in Chromatography

What happens in ion exchange?

Equilibration

Sample application and wash

Elution

Regeneration

anion exchanger bead
Effect of pH on charge

Low pH
Positive charge

R\text{NH}_3^+\text{COOH}

Hydrogen gained

High pH
Negative charge

R\text{NH}_3^+\text{COO}^-

Hydrogen lost

R\text{NH}_2\text{COO}^-

Low pH

R\text{NH}_3^+\text{COOH}

High pH

R\text{NH}_3^+\text{COO}^-

Hydrogen gained

R\text{NH}_2\text{COO}^-
Titration curves

The overall charge on a protein depends on pH

acid  isoelectric point  alkaline
excess positive charge  balanced positive and negative charge  excess negative charge

The overall charge on a protein depends on pH

pH
Controlling selectivity by pH

Charge change with pH: unique for each protein
Proteins that elutes together at one pH can be separated at other pH
Charged groups

- **Anion exchangers:** If the protein is most stable at a pH above its isoelectric point (pI)
  - Diethylaminoethyl (DEAE) \(-\text{OCH}_2\text{CH}_2\text{N}^+(\text{CH}_2\text{CH}_3)_2\) \(\text{Weak}\)
  - Quaternary aminoethyl (QAE) \(-\text{OCH}_2\text{CH}_2\text{N}^+(\text{C}_2\text{H}_5)_2\text{CH}_2\text{CHOHCH}_3\) \(\text{Strong}\)
  - Quaternary ammonium (Q) \(-\text{CH}_2\text{N}^+(\text{CH}_3)_3\) \(\text{Strong}\)

- **Cation exchangers:** If the protein is most stable at a pH below its isoelectric point (pI)
  - Carboxymethyl (CM) \(-\text{OCH}_2\text{COO}^-\) \(\text{Weak}\)
  - Sulphopropyl (SP) \(-\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-\) \(\text{Strong}\)
  - Methylsulphonate (S) \(-\text{CH}_2\text{SO}_3^-\) \(\text{Strong}\)

Either type of ion exchanger can be used if protein is stable at the pH we want to use.
Titration curves of ion exchangers

- Weak ion exchangers: charge varies with pH
- Strong ion exchangers: charge does not vary with pH
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Selection Guide — Ion Exchange Media

Selecting an anion or cation exchanger
Ion exchange separates proteins on the basis of differences in their net surface charge in relation to pH of the surroundings. The figure here illustrates how the net charge of a protein can vary with pH. Every protein has its own charge/pH relationship.

Intermediate purification
Remove bulk impurities
Sample condition: partially purified

Capture
Isolate, concentrate and stabilize target proteins
Sample condition: clarified or non-clarified

Polishing
Remove trace impurities or closely-related substances
Sample condition: almost pure

If isoelectric point (pI) of the target protein is known:
- select an anion exchanger (Q, DEAE, ANX) with a buffer pH above the pI.
- select a cation exchanger (S, SP, CM) with a buffer pH below the pI.

If pI is unknown:
- test for selectivity using a strong ion exchanger, Q, S or SP. Strong ion exchangers maintain their charge over a wider pH range than weak ion exchangers and are suitable for most applications.

Highest resolution
μg/min

Highest resolution
mg/min

High resolution
High throughput
Easy scale-up

Easy scale-up
Broad choice of selectivity, including alternatives to Q or S ion exchange media

High volume through-put and high capacity
Easy scale-up

High binding capacity for selected proteins
Easy scale-up

Large scale, viscous samples

Industrial scale, filtration and capture in one step

Start here
Different base matrices (resins)
larger or smaller molecules – high capacity or high resolution
Materials use for base matrix (resin)
Good mechanical and chemical properties

Inorganic materials
Silica, Glass, Ceramic, Carbon etc

Bio-polymers
Agarose, Cellulose, Dextran, etc

Synthetic polymers
Polystyrene, Acrylamide, Methacrylate, etc

Mechanical properties and chemical stability are key issues to be considered
MERCK: Fractogel® Tentacle Chromatography Resin
Eshmuno® IEX Family of Chromatography Resin

✓ Synthetic methacrylate based polymeric beads
✓ Excellent pressure stability
✓ High flow rates
✓ M-type beads with a particle size of 40-90 μm
✓ S-type beads with a particle size in the range of 20-40 μm
✓ Tentacles are long, linear polymer chains that carry the functional ligands.
✓ Covalently attached to hydroxyl groups of the Fractogel® matrix.
✓ This configuration provides a high surface area for biomolecules to bind without steric hindrance
✓ Eshmuno® ion exchange resins carry an innovative tentacle structure that is able to bind target substances much more effectively
✓ Hydrophilic polyvinyl ether base matrix
✓ High binding capacity and excellent pressure-flow behavior
✓ High selectivity
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Determination of start conditions

- Literature references/known applications
- Isoelectric point: practical or theoretical (http://web.expasy.org/protparam/).
- Test tube method
- Scouting run at different pH
- pH stability range of target molecule
Determination of start conditions

Test tube method

1) Fill test tubes with 1 ml exchanger resin.

2) Equilibrate resins with buffers of different pH-values.

3) Add sample in buffers of different pH values.

Mix

4) Analyze the supernatants for protein of interest.

In this example, the sample is completely bound at pH 8.

Conclusion: use an anion exchanger, initial pH 8.
Determination of start conditions

Scouting different pH

pH scouting for the separation of pancreatin

Conditions:
A1: 0.05 M 1-methylpiperazine, 0.05 M Bis-Tris, 0.025 M Tris
A2: 0.1 M HCl
B1: Water
B2: 2 M NaCl
System: KTA™ explorer 100
Flow rate: 6 ml/min
Column: RESOURCE™ Q, 6 ml
Sample: 2 mg Pancreatin
Gradient: as depicted

Elution volume (ml)
Tips on choosing an ion exchange resin: Choose proper resin

- **Cation or Anion:**
  - pI charge characteristics of target and contaminants
  - pH stability of target
  - Empirical determination

- **According to purification step**

- **Sufficient capacity**

- **Scale-up potential**

- **Consistent, reliable supply**
Parameters for absorption optimization

Adjust start buffer conditions of the sample:

a) pH value  

b) buffer capacity  

c) salt concentration

- To ensure a proper pH value, the sample should be dissolved / equilibrated in buffer A, or adjusted. Very critical for large volume samples

- Buffering ion concentrations: 20–50mM are usually enough
Parameters for absorption optimization

- IEX is a binding technique, independent of sample volume
- Use the higher speed that do not affect considerably the dynamic capacity of the column
- Applied sample at the higher salt concentration that allow target binding and avoid binding of contaminants
- Sample loads can be increased if resolution is satisfactory or when using a step elution
- For good resolution in intermediate and polishing steps use around 20% of column capacity.
Parameters for optimization

Buffer components

- The buffer should have the same charge as the ligand: Tris HCl, Bis-Tris for AEIX; Acetate, MES, HEPES, phosphate for CEIX
- Counter-ion must be small: Na⁺ for CEIX and Cl⁻ for AEIX
- Type of salt affect elution strength:
  
  Sulfate (SO₃⁻²)150mM  similar to  Chloride (Cl⁻¹)350mM
- Buffer contaminants may produce extra peaks. Highly purified buffer. Blank run
- Only **additives without charge**: sugars, alcohol, urea, non-ionic or zwitterionic detergents (Important to avoid aggregation problems)
- Add additives **only** if necessary.
## Choice of Buffer

*According to: GE Healthcare (Amersham-Biosciences – Pharmacia)*

<table>
<thead>
<tr>
<th>pH interval</th>
<th>Substance</th>
<th>pH</th>
<th>Substance</th>
<th>Counter-ion</th>
</tr>
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<tbody>
<tr>
<td>2.0</td>
<td>Formic acid</td>
<td></td>
<td></td>
<td>H⁺</td>
</tr>
<tr>
<td>2.3-3.5</td>
<td>Pyridine/formic acid</td>
<td>HCOO⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0-5.0</td>
<td>Trimethylamine/formic acid</td>
<td>HCOO⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0-6.0</td>
<td>Pyridine/acetic acid</td>
<td>CH₃OO⁻</td>
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<tr>
<td>4.0-6.0</td>
<td>Trimethylamine/acetic acid</td>
<td>CH₃COO⁻</td>
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<td></td>
</tr>
<tr>
<td>6.8-8.8</td>
<td>Trimethylamine/HCl</td>
<td>Cl⁻</td>
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<tr>
<td>7.0-8.5</td>
<td>Ammonia/formic acid</td>
<td>HCOO⁻</td>
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<tr>
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<td>Ammonia/acid</td>
<td>CH₃COO⁻</td>
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<td>Ammonium bicarbonate</td>
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<td>7.0-12.0</td>
<td>Triethylamine/CO₂</td>
<td>CO₃⁻</td>
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<tr>
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<td>Ammonium carbonate</td>
<td>HCOO⁻</td>
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<tr>
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<td>Ammonium carbonate/ammonia</td>
<td>CO₃⁻</td>
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<tr>
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<td>Ethanolamine/HCl</td>
<td>Cl⁻</td>
<td></td>
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<tr>
<td>8.9</td>
<td>Ammonium carbonate</td>
<td>CO₃⁻</td>
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</tr>
</tbody>
</table>

### Cation Exchange

<table>
<thead>
<tr>
<th>pH interval</th>
<th>Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5-2.5</td>
<td>Maleic acid</td>
</tr>
<tr>
<td>2.38-3.38</td>
<td>Malonic acid</td>
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<tr>
<td>2.63-3.63</td>
<td>Citric acid</td>
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<tr>
<td>3.6-4.3</td>
<td>Lactic acid</td>
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<tr>
<td>3.8-4.3</td>
<td>Formic acid</td>
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<tr>
<td>4.3-4.8</td>
<td>Butanedioic acid</td>
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<tr>
<td>4.8-5.2</td>
<td>Acetic acid</td>
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<td>5.0-6.0</td>
<td>Maleic acid</td>
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<td>6.7-7.6</td>
<td>Phosphate</td>
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<td>N-methyl-diethanolamine</td>
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<td>8.4-8.8</td>
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<td>9.0-9.5</td>
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<td>9.5-9.8</td>
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<td>1,3-diaminopropane</td>
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<td>10.6-11.6</td>
<td>Piperidine</td>
</tr>
<tr>
<td>11.8-12.0</td>
<td>Phosphate</td>
</tr>
</tbody>
</table>

### Anion Exchange

### Volatile buffers
Recommended buffers for anion exchange chromatography.

Recommended buffers for cation exchange chromatography.
Recommended buffers for anion exchange chromatography.

Recommended buffers for cation exchange chromatography.
Parameters for elution optimization:

Continuous gradient elution

- Smaller peaks by increasing the gradient slope.
- Give faster separations and sharper peaks, but peaks will be eluted closer (together).
- Higher selectivity by decreasing the gradient slope.
- But separation times will be longer and there will be greater peak broadening (volume).

First choice during method development

Use results to optimize separation
Parameters for elution optimization:

**Step gradient elution (stepwise)**

- High resolution and small peak volumes.
- But: Substances can elute together.
- Peaks tend to have sharp fronts and pronounced tailing.
- Reduces the total number of column volumes used for a separation.
- This *speeds up separation times and reduces buffer consumption*.

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The ideal IEX separation for production.
Parameters for elution optimization:

- **Step gradient elution (stepwise)**

- Conditions are chosen to maximize binding of the target proteins and minimize binding of contaminants during sample application: increase capacity for the target protein.

- Wash at highest salt concentration that maximize elution of contaminants without eluting target.

- Elute target protein using conditions that minimize elution of unwanted contaminants.

- Use highest flow-rate that do not compromise resolution.

- Advantage of step elution when used at larger scale: lower buffer consumption, less time, higher HT.
Parameters for elution optimization: 

Complex gradient elution

- Choose either highest selectivity or smallest peak volumes
- Offers maximum flexibility in terms of combining resolution with speed
- Long, shallow gradients when you need maximum separation between peaks
- Short, steep gradients where resolution is good enough

Aim: reduced separation time, minimal volume and maintained resolution
First trial, general conditions

Binding buffer: 20-50 mM

Elution buffer: 20-50 mM + 1 M NaCl

Gradient: 0–50 % B in 20 column volumes

Cleaning: 51–100 % in 3–5 column volumes

pH working range: ± 1 pH units from the pl

**CEIX:** pH < protein pl  **AEIX:** pH > protein pl
Parameters for elution optimization:

**pH value**

**pH scouting for the separation of pancreatin**

Conditions:
- A1: 0.05 M 1-methylpiperazine, 0.05 M Bis-Tris, 0.025 M Tris
- A2: 0.1 M HCl
- B1: Water
- B2: 2 M NaCl

System: KTA™ explorer 100
Flow rate: 6 ml/min
Column: RESOURCE™ Q, 6 ml
Sample: 2 mg Pancreatin
Gradient: as depicted
Controlling selectivity by pH

Each protein has its own unique net charge when changing pH buffer

Visualized as a titration curve
Controlling selectivity by pH

*Column:* HiPrep 16/10 CM FF
*Sample:* 10 mg apo-transferrin, ribonuclease A and cytochrome C in 1 ml
*Flow:* 10 ml/min, 300 cm/h
*Gradient:* 0–50% elution buffer in 300 ml (15 CV) where 50% = 0.5 M NaCl
*Buffer:* CIEX pH 3–7.5 BufferPrep recipe in AKTAexplorer

Fig. 61. Selecting optimal pH for separation of standard proteins on HiPrep 16/10 CM FF.
Controlling selectivity by pH

Figure 6
pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients.

Agilent
Parameters for elution optimization

Flow rate

- **Use high flow rate for high sample throughput and high productivity**

  - **1800 cm/h** (4.98 ml/min)
  - **900 cm/h** (2.49 ml/min)
  - **300 cm/h** (0.83 ml/min)
  - **200 cm/h** (0.55 ml/min)

- **Lower flow rate for maximum resolution**

  - **Select the highest flow rate that maintains resolution and minimizes separation time**

**Conditions**

- **Sample:**
  - Myoglobin: 0.11 mg/ml
  - Conalbumin: 0.34 mg/ml
  - Transferrin: 0.29 mg/ml
  - b-Lactoglobulin B and A: 0.51 mg/ml
- **Volume:** 100 µl
- **Buffer A:** 10 mM piperazine pH 6.0
- **Buffer B:** 10 mM piperazine + 1 M NaCl pH 6.0
- **Gradient:** 0 % B for 3 ml
  - 0-40 % B for 17 ml
- **Column:** 100 x 4.6 mm ID
- **Detection:** UV 280 nm
- **System:** FPLC
Critical Factors in IEX resolution

- Shape and volume of the gradient
- Effect of flow rate on resolution
- Effect of pH
- Effect of different salts
- Use of additives (detergents, ligands, cofactors, etc)
- Effect of temperature
- Column length (volume)
- Type of ligand (strong, weak, mixed)
- Degree of substitution
- Particle size of matrix (efficiency)
- Supplier

Fig. 7: The influence of particle size and elution rate on final resolution.
First trial, general conditions

Binding buffer: 20-50 mM

Elution buffer: 20-50 mM + 1 M NaCl

Gradient: 0–50 % B in 20 column volumes

Cleaning: 51–100 % in 3–5 column volumes

pH working range: ± 1 pH units from the pI

Lower pH than the protein pI when using CEIX

Higher pH than the protein pI when using AEIX
CRITICAL FACTORS

Selecting media

- Type of ligand
- Degree of substitution
- Type of Matrix: Particle size

Selecting adsorption condition

- Effect of salt concentration
- Effect of pH
- Column Volume
- Effect of temperature
- Effect of different salts

Selecting elution conditions

- Shape and volume of the gradient
- Effect of flow rate on resolution
- Effect of pH
- Type and column length
- Use of additives

Unexpected results

- Poor resolution
- Elution too early or too late
- Precipitation of protein
- Poor recovery
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AIX – recProtein in *E. coli* cells

AEIX optimization
SEC as last step
500ml culture after lysis and sonication. DE-Sepharose FF 100x10mm (~8ml) in 50mM TrisHCl pH8.0 buffer + additives.

WASH: 5cv 0M NaCl  ELUTION: gradient 15cv 0-0.15M NaCl + 5cv 0.15-1M NaCl
RAS - OPTIMIZATION 2nd STEP

Step gradient from 20 to 160mM NaCl purification of RAS in Q-Sepharose FF column 100x10mm (~8ml) in 25mM TrisHCl pH7.9
RAS - OPTIMIZATION 2nd STEP
RAS - 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
60 OD280nm (8ml) RAS after Q-Sepharose. Load Sephacryl S100 920x26cm - Flow 2.5ml/min - Pool RAS after GF: 36.8 OD280nm
CIXE – recProtein in medium insect cells

Recombinant protein from Insect cells. pI: ~9.5. No tag
- Two subunits connected by a.a. bridge
- CEIX optimization
- Two CEIX at different pH - Different suppliers
Sample: Load 90 ml supernatant
Cation Exchange EMD-SO3 (M) 3 ml
Buffer A: 20 mM NaPO4 pH 7.0
Buffer B: A + 1 M NaCl

PAGE-SDS Coomasie staining

Western
Load 150ml crude supernatant
cation exchange EMD-SO3 (M) 2.2ml
Buffer A: 20mM NaPO4 pH7.0
Buffer B: A + 1M NaCl

Load x 2 150ml sup GS3 on 2.2ml Fractogel SO3(M) ~2.2ml

15cv 0%B + 15cv 20%B + 4cv 25%B + 5cv 30%B + 7cv 45%B + 4cv 50%B + 6cv 75%B + 8cv 100%B

Pool: 16-18

PAGE-SDS Coomassie staining
Load after Fract SO3
Cation Exchange Mono S 1ml
Buffer A: 20mM NaPO4/Citrate pH6.0 + 0.15M NaCl
Buffer B: 20mM NaPO4/Citrate pH6.0 + 1M NaCl

Load x 2 150ml sup GS3 on 2.2ml Fractogel SO3(M) ~2.2ml

15cv 0%B + 15cv 20%B + 4cv 25%B + 5cv 30%B + 7cv 45%B + 4cv 50%B + 6cv 75%B + 8cv 100%B

Pool: 16-18
Sample: Crude Supern (Ins. cells) + 130mMNaCl+ 1mMEDTA + 0.5mMBenzam. + 0.8mM PMSF
Column: Fractogel EMD SO3 (M) 10x1cm=7.8ml First CEIX pH 7.0
Eluent A: 20mM NaPO4 pH 7.0 - Eluent B: 20mM NaPO4 pH 7.0 + 1M NaCl
Sample: Pool after first CEIX - Dilution 1:5 with A after First CEIX
First CEIX: Res 15S 100x10mm ~7.8ml  **Second CEIX pH 6.0**
Use a more resolutive column and from a different supplier. # pH since impurities that elute together at pH 7.0 can be separated at #pH)
Eluent A: 20mM MES pH6.0 - Eluent A: 20mM MES pH6.0 + 1M NaCl

**Analytical GF of the main peak, shows an homogeneous peak without any aggregation**
Protein concentration with Centriplus 30000 to 3.3 OD280nm/ml

Analytical Superose 12 anal column 300x10mm

PAGE-SDS Coomasie staining

3ug BSA

1ul 3.3 OD280nm/ml

Load 10ul 3.2OD280nm/ml

4ml 0.047OD280/ml concentr. (30000) X 80 to 55 ul 3.2OD280/ml

Superose 12 ana. 300x10mm - 20mM Citr./PO4 pH 6.0 + 0.35M NaCl

158 67 43 32 13.7 kDa

Coomasie staining

3ug BSA
Similar variant of last protein (full-length)

1st column (capture): Fractogel EMD SO₃ (M) 10x1cm ~7.8 ml

Buffer A: 20mM NaPO₄ pH 7.0 - Buffer B: A + 1M NaCl

Pool: 10-27
2nd column (interm purific): Source 15S 10x1cm ~7.8 ml

Buffer A: 20mM MES pH 6.0 - Buffer B: A + 1M NaCL

16-21

[Graph and Image]
3rd column (polish): Fractogel EMD SO₃ (M) 20x0.5cm ~4.0 ml

Buffer A: 20mM NaPO₄ pH 7.0 - Buffer B: A + 1M NaCL

Load dil. 1:3 + 2cv 35%B + 3cv 45%B + 5cv 45-70%B + 1cv 70%B + 1cv 75%B + 2cv 80%B + 0.5cv 100%B

Pool 15-20 for Crystal and 21-22 for repurification
Purification of rec Chimeric Protein with two domains: one for recognition a second a toxic RNA binding protein

Elimination of RNA interferents with urea, refolding on-column and final CEIX purification: large and resolutive column
Purification of a Chimera protein: scFV anti receptor + RNA binding protein.

4M Urea lysis to separate RNA from protein, ON refolding, and Imidazol elution (+ additives)

Column: NiNTA 4.8 x 2.6 ~25ml Lysis buffer A11

A18 + A11: 20mMHEPES pH7.5 + 0.5MNaCl + 10mMImid + 10%Glyc + 4Murea
A12 + B2: 20mMHEPES pH7.5 + 0.5MNaCl + 10mMImid +10%Glyc + 0.5%Tween 80
B1: 20mMHEPES pH7.5 + 0.5MNaCl + 10%Glyc + 500mMImid + 0.5%Tween 80
FractogSO3M ~23ml 30x1cm

Dilution buffer: A without NaCl+0.05%Tween 80
Buffer A11&A18: 30mM MES pH 6.0 +10%
Glycerol +100mMNaCl + 0.001%Tween80
Buffer B1: 30mM HEPES pH 7.8 10%Glycerol + 2MNaCl + 0.001%Tween80

Gradient: 4cv 9%B +
1cv 20%B + 1cv
25%B +1cv 30%B
+1.5cv 35%B +4.5cv
35-45%B + 2cv 45%B
+ 1cv 50%B + 1cv
55%B + 2cv 55-100%B

POOL I: 18-27 (highly pure) ~1.8mg
POOL II: 13-17 + 28-38 for repurification

4cv 9%B + 1cv 20, 25, 30%B + 1.5cv 35%B + 4.5cv 35-45%B + 2cv 45%B + 1cv 50, 55, 55-100%B + 2cv 100%B
FractogSO3M ~23ml 30x1cm
Re-purification of some fractions
Reproducing irreproducible results
Case Study in Heterogeneous Glycosylation

What happen when you use non-credible suppliers
Column: Hi Trap SP HP 1 ml
Buffer A: 25mM NaAc pH 5.0
Buffer B: A + 1M NaCl
pI: ~ 6.0
Secreted HEK cells
Glycosilated

Active Protein

Non-active Protein
Non-Active  |  Active  
250  |  15  |  20  |  25  |  37  |  50  |  75  |  250  |  150  |  100  |  75  |  50  |  37  |  25  |  20  |  15  |  100  |  75  |  50  |  37  |  25  |  20  |  15  |  10

Protein Staining

Western Blot (specific Ab)
Adeno Associated Viruses (AAVs) purification

Separation of empty from full capsids by AEIX
AAV9 crude sample on AIEX (Q HP 5ml)

Abs. at 280nm
Conductivity

Impurities: proteins and DNA

Empty capsids + proteins

Full AAV particles

Time (sec)

Elution buffers:
1. 0.5M NH₄Ac in Tris pH9
2. 1M NaCl (for final wash)

Elution buffers:

Tem pictures of empty capsids from unbound peak

Tem pictures of full AAV (first peak)
Troubleshooting - I

- Some of the protein do not bind or elutes before starting salt gradient
  - Increase column volume
  - Reduce ionic strength of sample by desalting, or dilution with start buffer.
  - Increase buffer pH (for anion exch), or decrease buffer pH (for cation exch).
  - Consider possibility of protein precipitation or aggregation: use advices for prone to aggregate proteins (like use of detergents, additives, low temp, work quickly, change buffers conditions, etc. or improve expression system)

- Protein(s) of interest eluting in more than one peak of the gradient
  - Consider possibility of protein precipitation or aggregation, # oligomeric concentrations, complexes, # post-translational modifications, etc
Protein(s) of interest eluting late in gradient

Proteins are binding too strongly. Increase ionic strength of gradient. Decrease buffer pH for anion exchanger, or increase buffer pH for a cation exchanger.

Protein(s) of interest eluting too early in gradient

Proteins are not binding strongly. Decrease ionic strength of gradient. Increase buffer pH for anion exchanger, or decrease buffer pH for a cation exchanger.
Proteins(s) of interest not sufficiently resolved

Change gradient, use more resolutive columns, change pH buffer, reduce flow-rate, increase column volume, change salt type

Low recovery of activity, but normal recovery of protein.

Protein may be unstable or inactive in the buffer. Determine the pH and salt stability of the protein.

Protein yield lower than expected.

Protein may have been degraded by proteases. Or adsorbed to filter. Or sample precipitates. Or hydrophobic (sticky) protein.
CIP (Cleaning in Place) Protocols

According to: GE Healthcare (Amersham-Biosciences – Pharmacia)

Remove ionically bound proteins by washing the column with 0.5 bed volumes of a 2 M NaCl solution, contact time 10-15 minutes, reversed flow direction.

Remove precipitated proteins, hydrophobically bound proteins and lipoproteins by washing the column with 1 M NaOH solution at a linear flow rate, contact time 1-2 hours, reversed flow direction. Wash with at least 3 bed volumes of starting buffer.

Remove strongly hydrophobically bound proteins, lipoproteins and lipids by washing the column with 4 bed volumes of up to 70% ethanol or 30% isopropanol, reversed flow direction. Apply increasing gradients to avoid air bubble formation when using high concentrations of organic solvents.

Alternatively, wash the column with 2 bed volumes of detergent in a basic or acidic solution. Use, for example, 0.1-0.5% non-ionic detergent in 0.1 M acetic acid. Wash at a low linear flow rate, contact time 1-2 hours, reversed flow direction. After treatment with detergent always remove residual detergent by washing with 5 bed volumes of 70% ethanol.
Charge interactions of biomolecules to the resin

Complementary to GF, HIC, affinity

Elution by increasing ionic strength (most of the cases) or change in pH (difficult to reproduce)

Binding and elution conditions must to be established to increase capacity and resolution

Fast binding

Many variables to change (**selectivity**): pH, salts, additives, column length, supplier, flow-rate (for large beads), others

A technique with very high resolution potential. Optimal for every stage: from production to analytics

Use: capture, target purification, separation of aggregates, PTModifications, DNA and endotoxin removal

Scale-up is easy, predictable, controllable

Limitation: pH and conductivity binding (possible use of MMC or salt tolerant resins)

Only additives without charge (zwiterionic or non-ionic detergents, Urea, neutral a.a., etc)

Over-crowding on top of the column can trigger aggregation
A Novel pH Gradient Separation Platform for Monoclonal Antibody (MAb) Charge-Variant Analysis

Shanhua Lin et al., Thermo Fisher Scientific

✓ pH gradient consisted of piperazine, imidazole, and tris, covering a pH range of 6 to 9.5
✓ MAbPac SCX-10, 10 µm, 4 × 250 mm column
✓ THERMO multicomponent zwitterionic buffer buffers: 0% B (pH 5.6) to 100% B (pH 10.2)

A : 20 mM MES and 60 mM NaCl (pH 5.6), and B:20 mM MES and 300 mM NaCl (pH 5.6)

pH gradient, 0% B (pH 5.6) to 100% B (pH 10.2)
Comparision of pH Gradient and salt gradient

Shanhua Lin et al., Thermo Fisher Scientific

**BSA**

- **pH Gradient**
  - Column: ProPac SAX-10, 4 mm i.d. × 250 mm
  - Mobile Phase: A: 20 mM Piperazine + 20 mM triethanolamine + 20 mM bis-tris propane + 20 mM N-methylpiperazine, pH = 3.7 (titrated with HCl)
  - B: 20 mM Piperazine + 20 mM triethanolamine + 20 mM bis-tris propane + 20 mM N-methylpiperazine, pH = 9.7 (titrated with HCl)
  - Gradient: 0–100% B in 15 min
  - Flow Rate: 1.00 mL/min
  - Detection: UV at 280 nm

- **Salt Gradient**
  - Column: ProPac SAX-10, 4 mm i.d. × 250 mm
  - Mobile Phase: A: 20 mM TRIS, pH 8.5
  - B: Same as A + 0.5 M NaCl
  - Gradient: 0–100% B in 15 min
  - Flow Rate: 1.00 mL/min
  - Detection: UV at 280 nm

**Ovalbumin**
Chromatograms of IgG monoclonal antibody at different ionic strengths.

Chromatogram of IgG monoclonal antibody pH 7.0 to 8.0 (0 to 20 minutes), 30 mM

Chromatogram of IgG monoclonal antibody pH 6.5 to 7.5 (0 to 20 minutes), 50 mM.
Figure 1. pH scouting using rituximab biosimilar from pH 6.2 to 7.2 on an Agilent 1260 Infinity II Bio-inert LC.

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Figure 3. Direct overlay of the charge variant profiles of rituximab innovator and biosimilar on an Agilent 1260 Infinity II Bio-inert LC.
Buffer systems for pH gradient IEC


<table>
<thead>
<tr>
<th>Substance</th>
<th>pKa₁</th>
<th>pKa₂</th>
<th>Conc.[mM]</th>
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<tbody>
<tr>
<td>AEC buffer pH 10.5–3.5</td>
<td></td>
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<td></td>
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<tr>
<td>Methylamine</td>
<td>10.75</td>
<td>-</td>
<td>9.8</td>
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<tr>
<td>1,2-Ethanediamine</td>
<td>9.93</td>
<td>6.99</td>
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<tr>
<td>1-Methylpiperazine</td>
<td>9.16</td>
<td>4.78</td>
<td>6.4</td>
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<tr>
<td>1,4-Dimethylpiperazine</td>
<td>8.15</td>
<td>4.04</td>
<td>13.7</td>
</tr>
<tr>
<td>Bis-tris</td>
<td>6.22</td>
<td>-</td>
<td>5.8</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>5.67</td>
<td>-</td>
<td>7.7</td>
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Min ionic str. = 8.5 mM/max ionic str. = 104.3 mM

<table>
<thead>
<tr>
<th>CEC buffer pH 4.0–11.0</th>
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<tbody>
<tr>
<td>CAPS</td>
<td>10.50</td>
<td>-</td>
<td>15.6</td>
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<tr>
<td>CHES</td>
<td>9.39</td>
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<tr>
<td>TAPS</td>
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<td>-</td>
<td>4.6</td>
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<tr>
<td>HEPPSO</td>
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<td>-</td>
<td>9.9</td>
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<tr>
<td>MOPSO</td>
<td>6.90</td>
<td>-</td>
<td>8.7</td>
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<tr>
<td>MES</td>
<td>6.10</td>
<td>-</td>
<td>11.0</td>
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<tr>
<td>Acetate</td>
<td>4.76</td>
<td>-</td>
<td>13.0</td>
</tr>
<tr>
<td>Formate</td>
<td>3.75</td>
<td>-</td>
<td>9.9</td>
</tr>
</tbody>
</table>

Min ionic str. = 8.3 mM/Max ionic str. = 89 mM
In this technical article, the group highlight the importance of screening different resin types and load conditions during process development, not only with regard to binding capacity and selectivity but also with regard to possible undesirable effects of resins of different suppliers on protein structure.

They show that a monoclonal antibody exhibits a two- or three-peak elution behavior CEX (cation exchange); the first two from monomers (according to light scattering), and the third peak is an aggregate that increases if the protein is held in the bound state for increasing lengths of time.

They claim that the two peaks belong to a bi-modal pore size distribution, with weaker binding occurring in the larger pores and stronger binding in the small pores. Moreover, they show that the protein bound to the weak sites remains stable while the protein bound to the strong sites undergoes kinetically limited conformational changes that result in a destabilized surface species and final aggregation. And this aggregation does not occur on CEX resins of other suppliers with similar particle size but with a more homogeneous structure, or when arginine (a known protein aggregation inhibitor) is added to the run.

By re-injecting one of the peaks, they obtain again the dual monomeric peak, demonstrating that this behavior is not related to charge variants and other isoforms.
Literature for IEC

- Kastner M. Protein Liquid Chromatography. Amsterdam: Elsevier Science; 2005