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

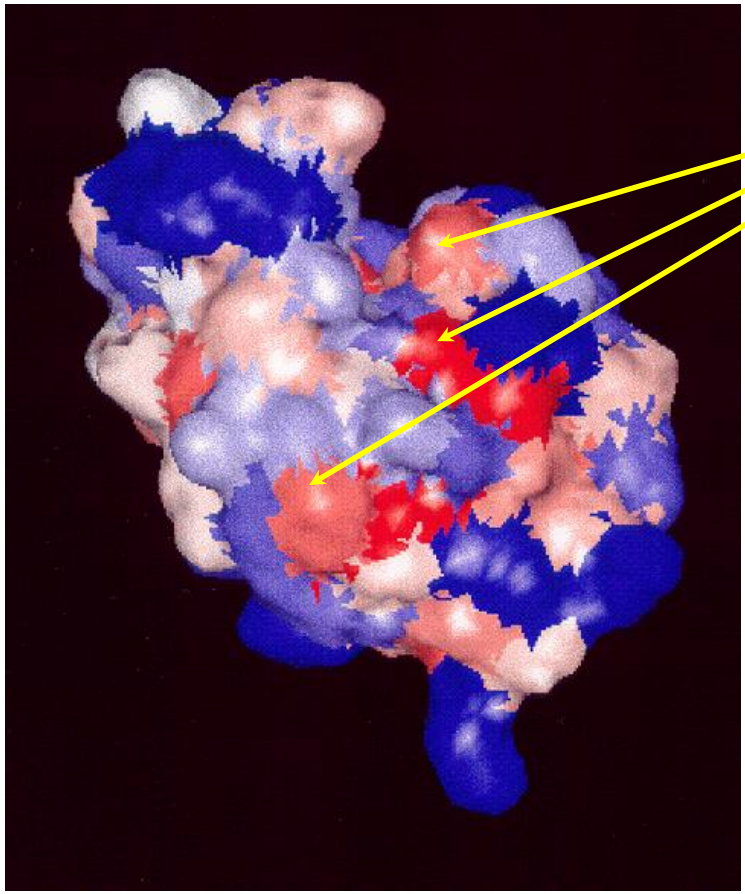
What is ion exchange chromatography?

- 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 pI 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



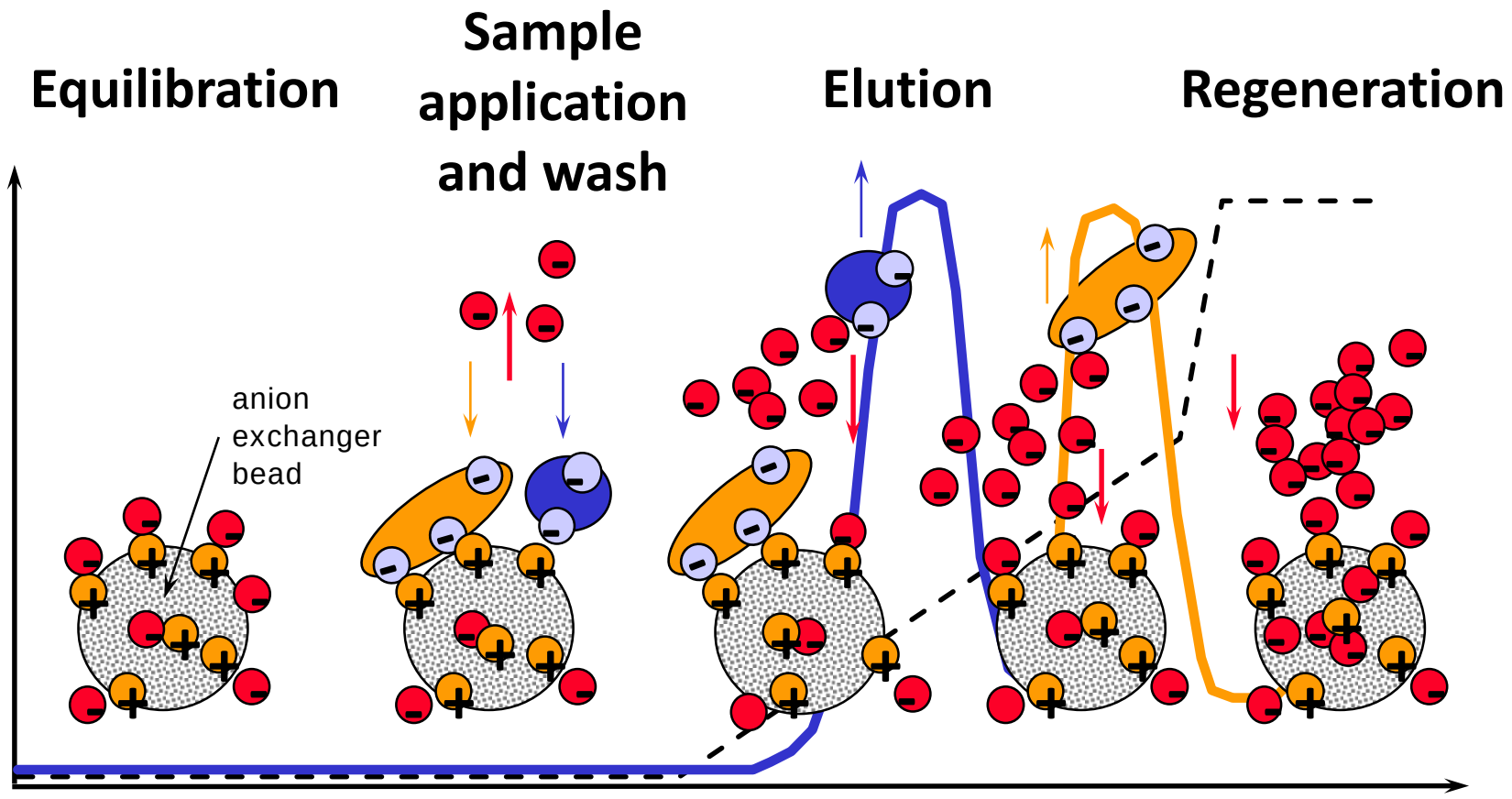
Some of the charged regions which will influence ion exchange

- 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

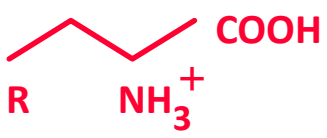
Main stages in Chromatography

- 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?



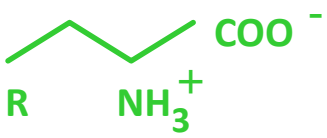
Effect of pH on charge



Low pH

Positive charge

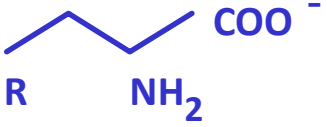
Hydrogen gained



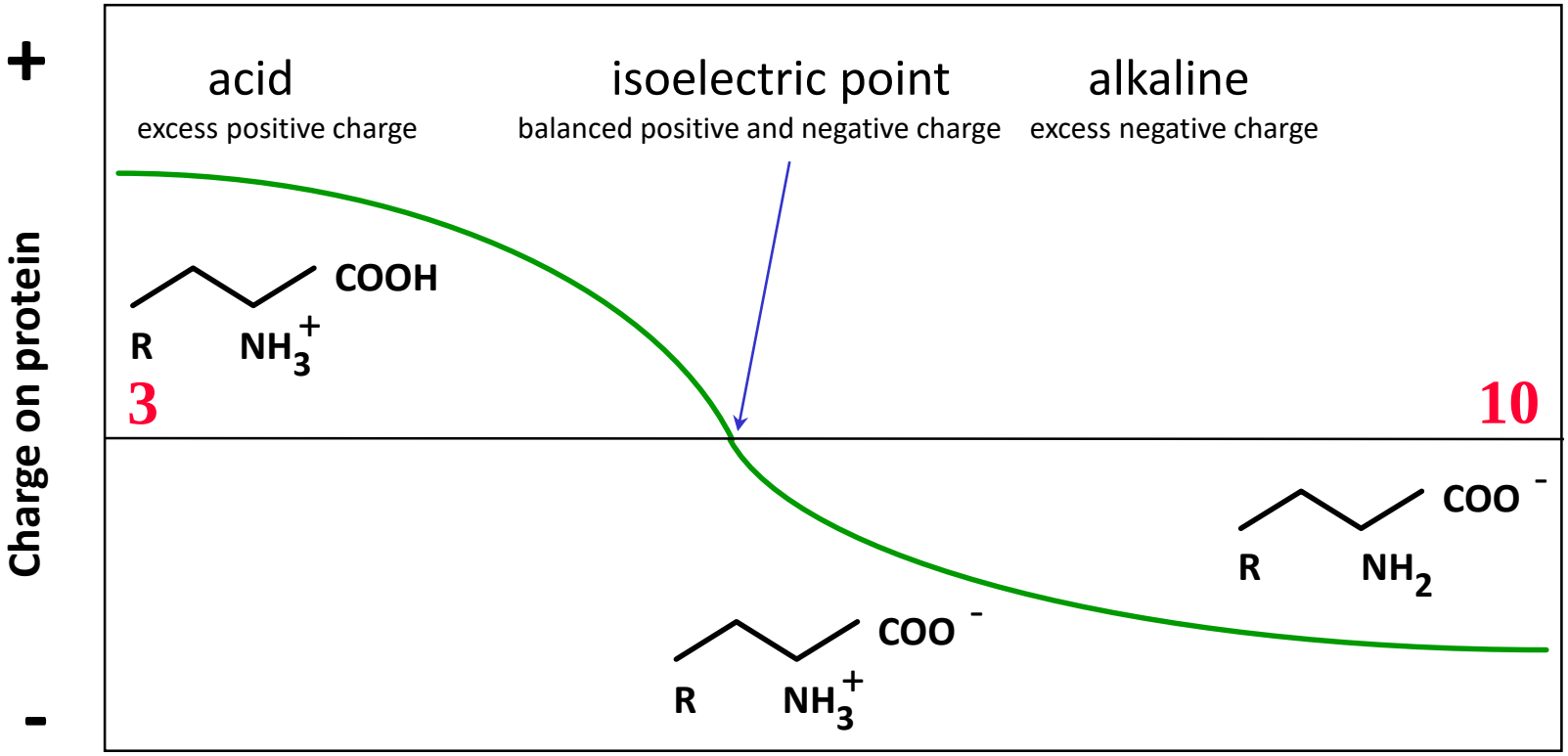
High pH

Negative charge

Hydrogen lost



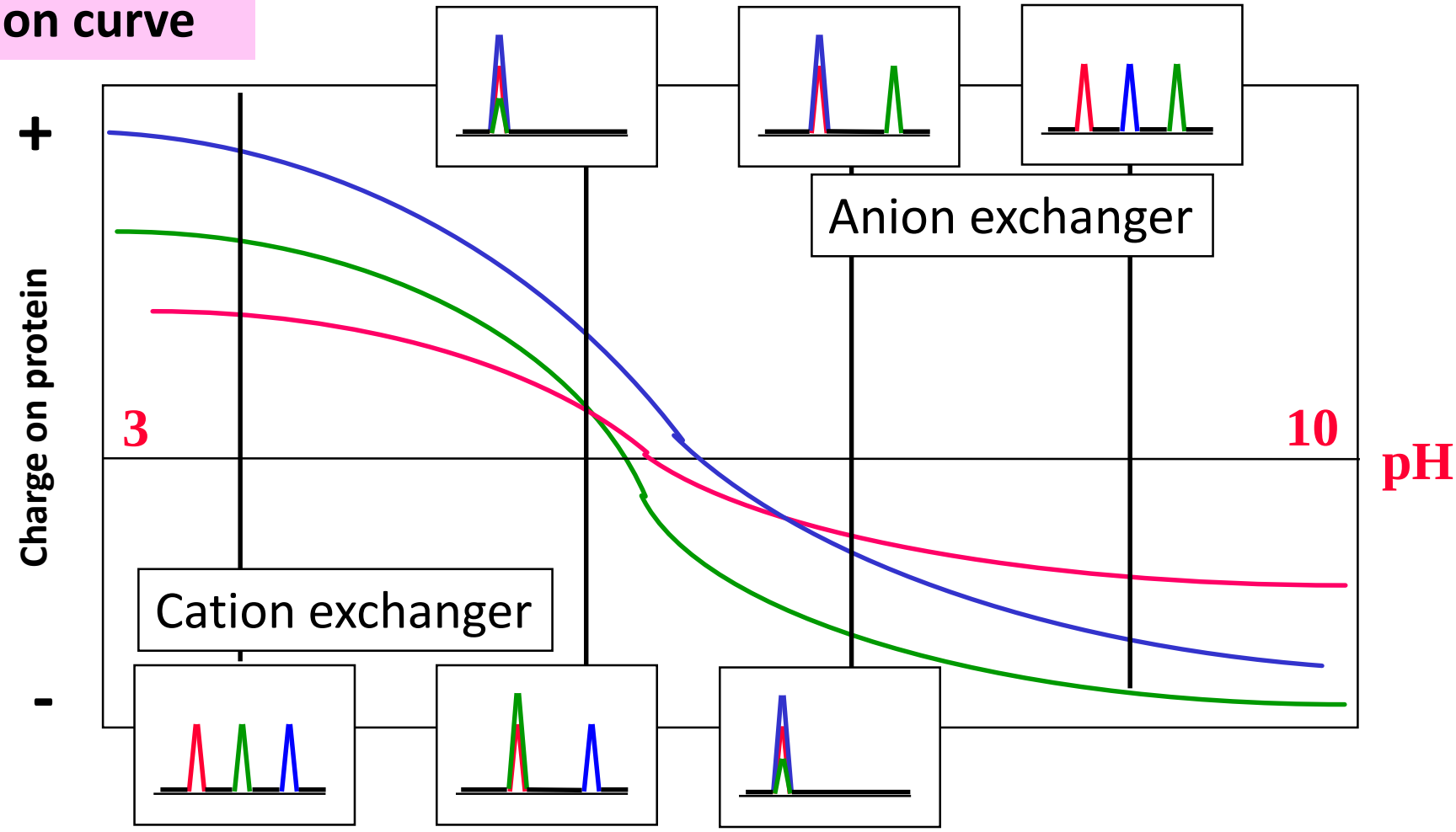
Titration curves



The overall charge on a protein depends on pH

Controlling selectivity by pH

Titration curve



Charge change with pH: unique for each protein

Proteins that elutes together at one pH can be separated at other pH

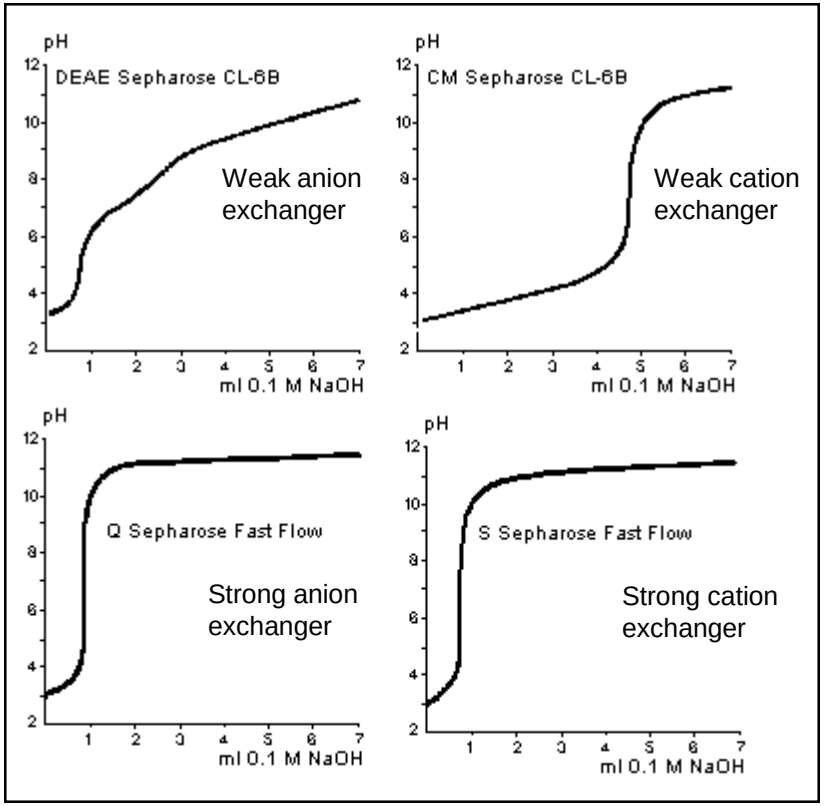
Charged groups

- Anion exchangers: If the protein is most stable at a pH above it pI
  - Diethylaminoethyl (DEAE) $-OCH_2CH_2N^+(CH_2CH_3)_2$ Weak
  - Quaternary aminoethyl (QAE) $-OCH_2CH_2N^+(C_2H_5)\ {}_2CH_2CHOHCH_3$ Strong
  - Quaternary ammonium (Q) $-CH_2N^+\ (CH_3)_3$ Strong

- Cation exchangers: If the protein is most stable at a pH below it pI
  - Carboxymethyl (CM) $-OCH_2COO^-$ Weak
  - Sulphopropyl (SP) $-CH_2CH_2CH_2SO_3^-$ Strong
  - Methylsulphonate (S) $-CH_2SO_3^-$ 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

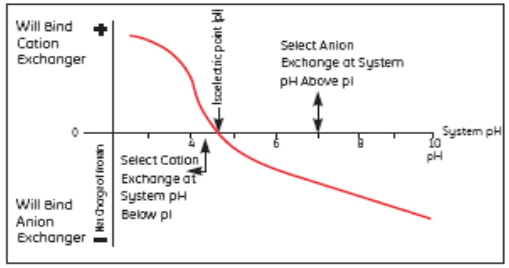
Ion exchange chromatography (IEC)

- 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

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.



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.

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

- **Highest resolution** µg/run
- **Highest resolution** mg/run
- **High resolution** High throughput Easy scale-up

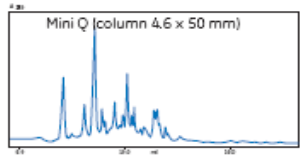
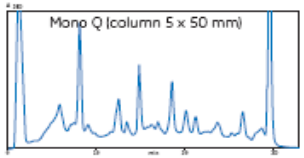
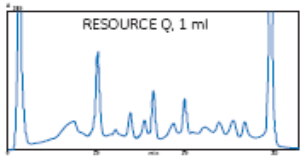
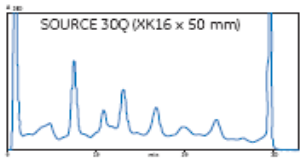
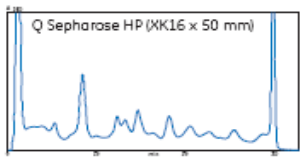
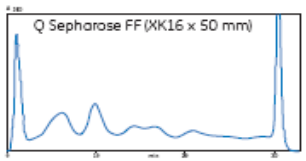
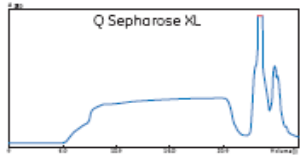
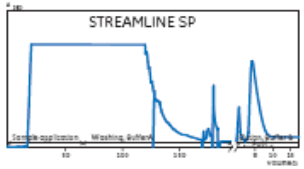
Intermediate purification
Remove bulk impurities
Sample condition: partially purified

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

Capture
Isolate, concentrate and stabilize target protein(s)
Sample condition: clarified or non-clarified

- 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

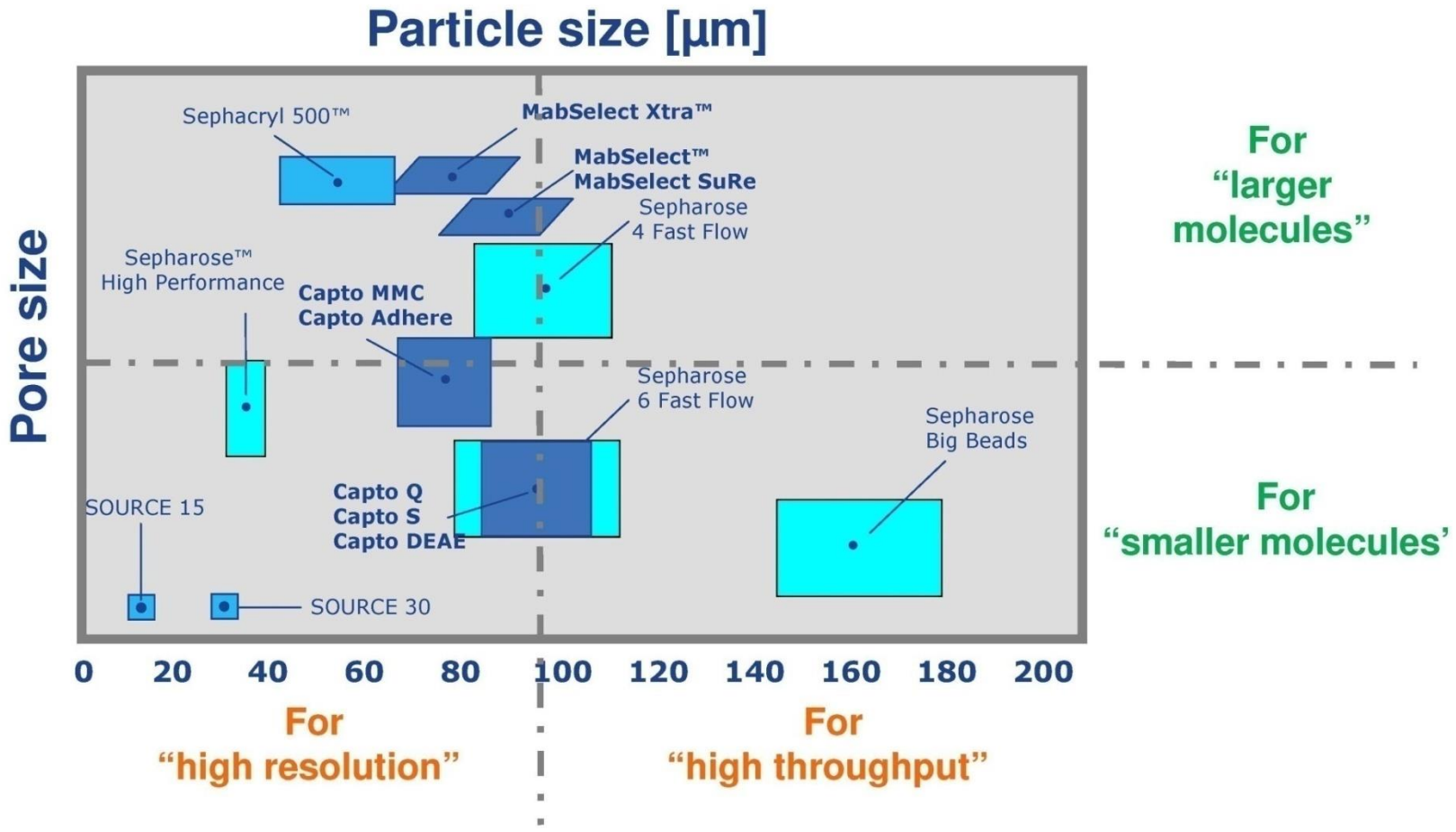
Start here

MiniBeads (Q or S)	Use for intermediate purification if column capacity is sufficient and no scale-up is required.	 Sample: Pancreatin Gradient elution
MonoBeads (Q or S)	Use for intermediate purification if column capacity is sufficient and no scale-up is required. Can be used for capture steps if sample is free from particulate matter.	 Sample: Pancreatin Gradient elution
SOURCE 15 (Q or S)	Use SOURCE 15 when resolution is top priority.	 Sample: Pancreatin Gradient elution
SOURCE 30 (Q or S)	Use SOURCE 30 when speed is top priority.	 Sample: Pancreatin Gradient elution
Sepharose High Performance (Q or SP)	Use HiTrap columns prepacked with Sepharose High Performance, Sepharose XL and Sepharose Fast Flow for media selection and pH scouting.	 Sample: Pancreatin Gradient elution
Sepharose Fast Flow (Q, SP, DEAE, CM, ANX)	Try weak ion exchangers such as DEAE, CM or ANX if the selectivity of Q or S is unsatisfactory.	 Sample: Pancreatin Gradient elution
Capto (Q, S, MMC)	Use high bed heights for increased productivity. Use MMC for high salt feed.	
Sepharose XL (Q or SP)	Use Sepharose Q XL virus licensed as an alternative to cesium chloride gradients for purification of viruses, including adenovirus, or viral vectors.	 Sample: Recombinant α-amylase Pilot scale: Gradient elution begins after 20 l
Sepharose Big Beads (Q or SP)	Use with step elution.	
STREAMLINE™ (Q XL, SP XL, SP, DEAE, HST)	Use STREAMLINE for direct capture from unclarified feed-stock. Use HST, a salt tolerant adsorbent, to minimize dilution and reduce process time.	 Sample: Recombinant antigen binding fragment Pilot scale: Step elution

Resolution

Different base matrices (resins)

larger **or** smaller molecules – high capacity **or** high resolution

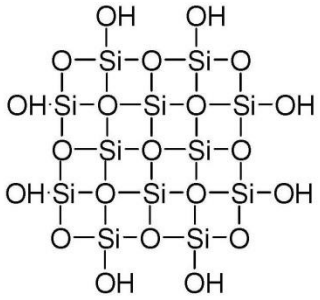


Materials use for base matrix (resin)

Good mechanical and chemical properties

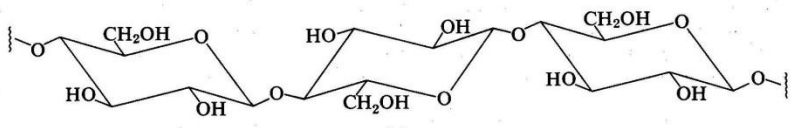
Inorganic materials

Silica, Glass, Ceramic, Carbon etc



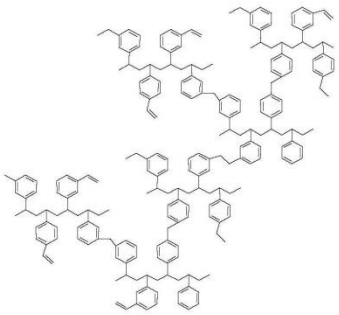
Bio-polymers

Agarose, Cellulose, Dextran, etc



Synthetic polymers

Polystyrene, Acrylamide, Methacrylate, etc

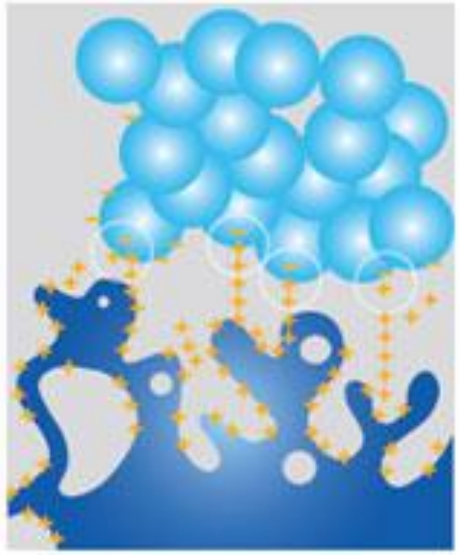


Mechanical properties and chemical stability are key issues to be considered

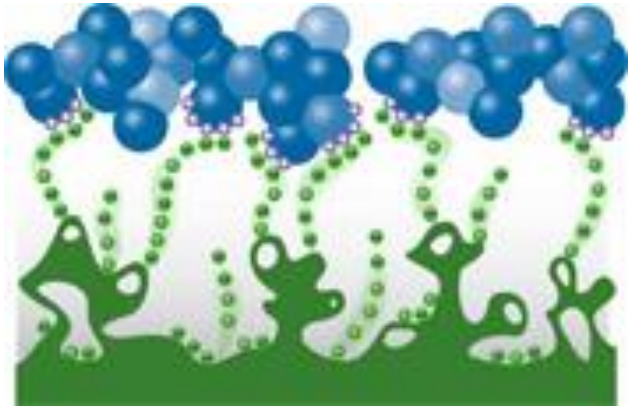
imagination at work

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



Ion exchange chromatography (IEC)

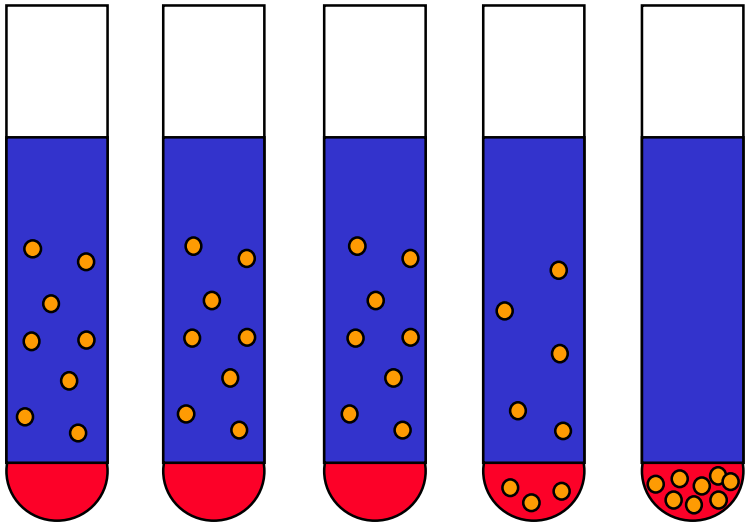
- Principles of IEC
- Main stages in Chromatography
- Resolution, efficiency, selectivity and capacity
- Determination of start conditions
- Parameters for absorption optimization
- Parameters for elution optimization
- Troubleshooting
- Examples
- Summary

Determination of start conditions

- Literature references/known applications
- Isoelectric point: practical or theoretical (//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



pH 6.0 6.5 7.0 7.5 8.0

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

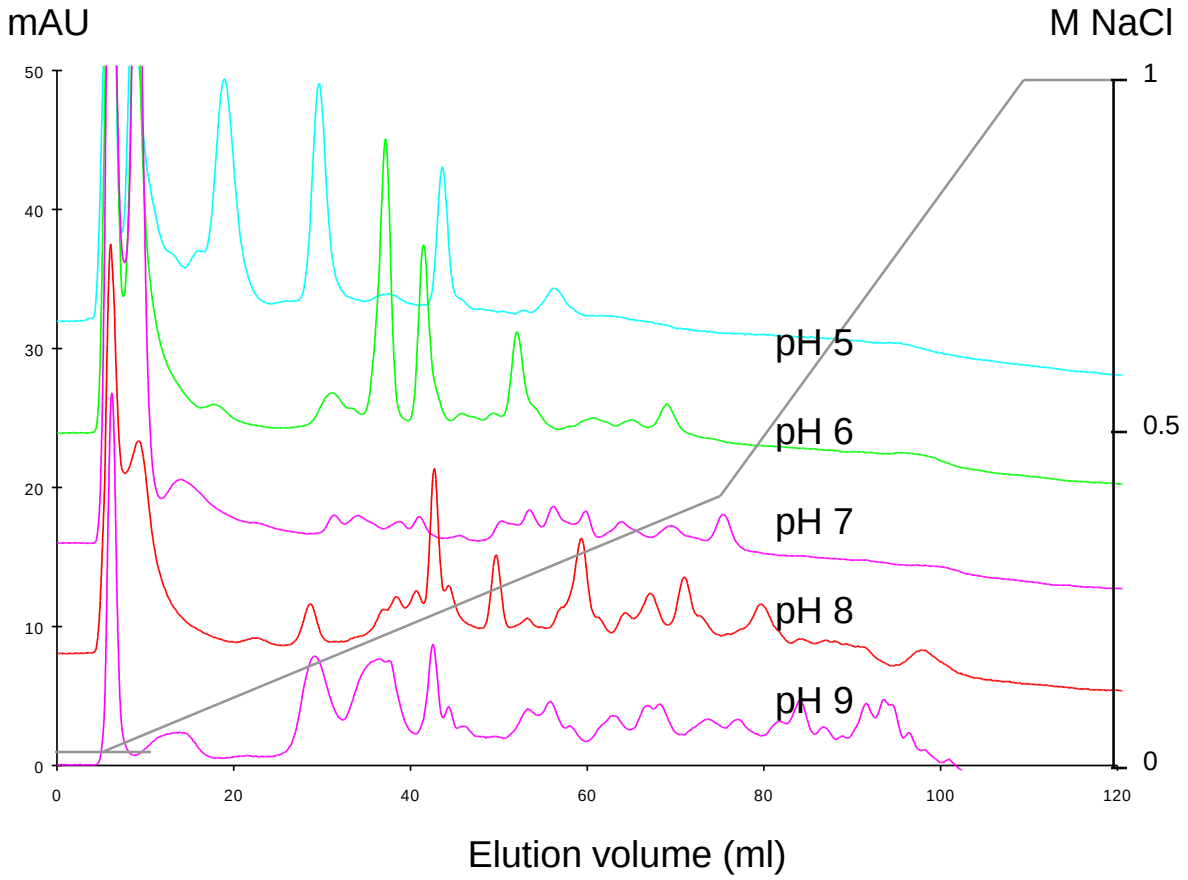
4) Analyze the supernatants for protein of interest.

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:	ÄKTA™*explorer* 100
Flow rate:	6 ml/min
Column:	RESOURCE™ Q, 6 ml
Sample:	2 mg Pancreatin
Gradient:	as depicted

Tips on choosing an ion exchange resin:

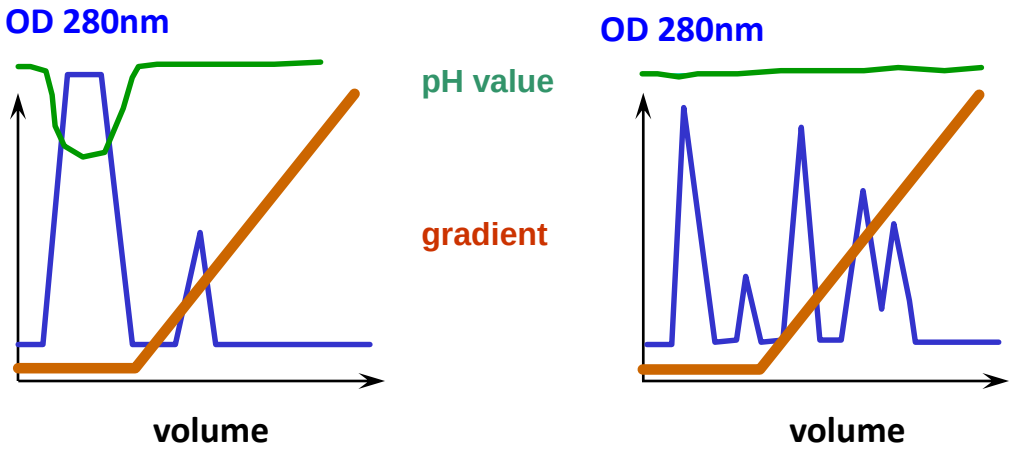
Choose proper resin

- 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



Dialysis

Dilution + buffer adjustment

Buffer exchange columns

Ultrafiltration (industry)

- 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 (SO_3^{-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)

pH interval	Substance
1.5-2.5	Maleic acid
2.38-3.38	Malonic acid
2.63-3.63	Citric acid
3.6-4.3	Lactic acid
3.8-4.3	Formic acid
4.3-4.8	Butanedioic acid
4.8-5.2	Acetic acid
5.0-6.0	Malonic acid
6.7-7.6	Phosphate
7.6-8.2	HEPES
8.2-8.7	BICINE

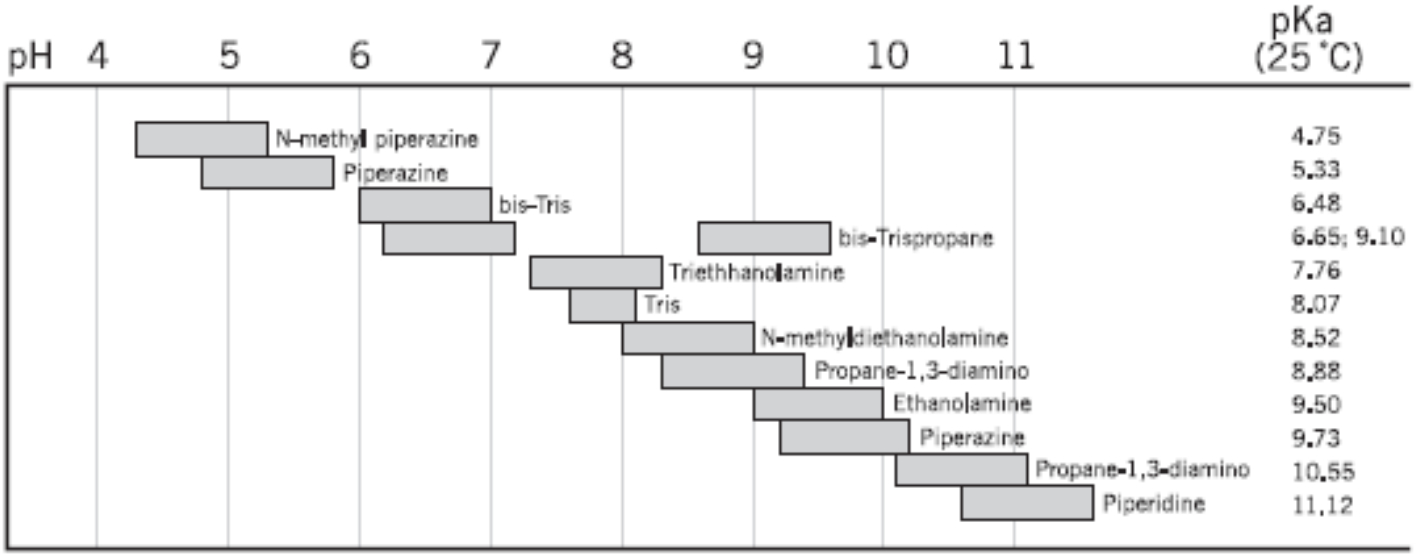
Cation Exchange

pH interval	Substance
4.5-5.0	N-methyl piperazine
5.0-6.0	Piperazine
5.5-6.0	L-histidine
5.8-6.4	bis-Tris
6.4-7.3	bis-Tris propane
7.3-7.7	Triethanolamine
7.6-8.0	Tris
8.0-8.5	N-methyl-diethanolamine
8.4-8.8	Diethanolamine
8.5-9.0	1,3-diamino-propane
9.0-9.5	Ethanolamine
9.5-9.8	Piperazine
9.8-10.3	1,3-diamino-propane
10.6-11.6	Piperadine
11.8-12.0	Phosphate

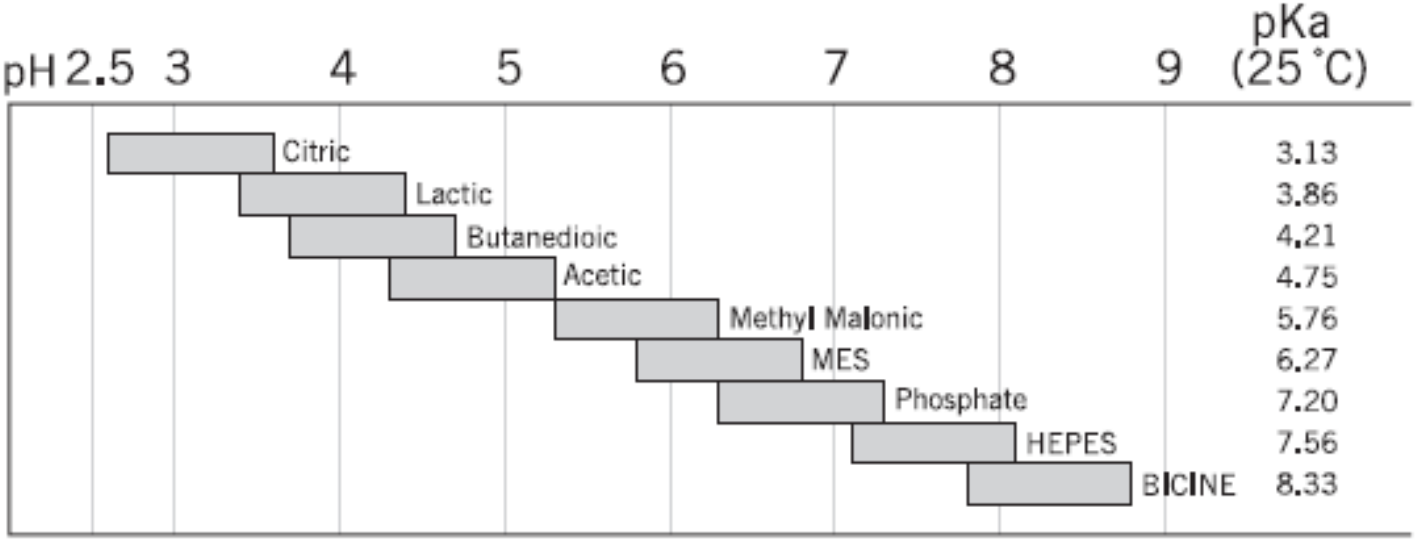
Anion Exchange

pH	Substance	Counter-ion
2.0	Formic acid	H^+
2.3-3.5	Pyridine/formic acid	$HCOO^-$
3.0-5.0	Trimethylamine/formic acid	$HCOO^-$
3.0-6.0	Pyridine/acetic acid	CH_3OO^-
4.0-6.0	Trimethylamine/acetic acid	CH_3COO^-
6.8-8.8	Trimethylamine/HCl	Cl^-
7.0-8.5	Ammonia/formic acid	$HCOO^-$
8.5-10.0	Ammonia/acid	CH_3COO^-
7.0-12.0	Trimethylamine/CO_2	CO_3^-
7.0-12.0	Triethylamine/CO_2	CO_3^-
7.9	Ammonium bicarbonate	HCO_3^-
8.0-9.5	Ammonium carbonate/ammonia	CO_3^-
8.5-10.5	Ethanolamine/HCl	Cl^-
8.9	Ammonium carbonate	CO_3^-

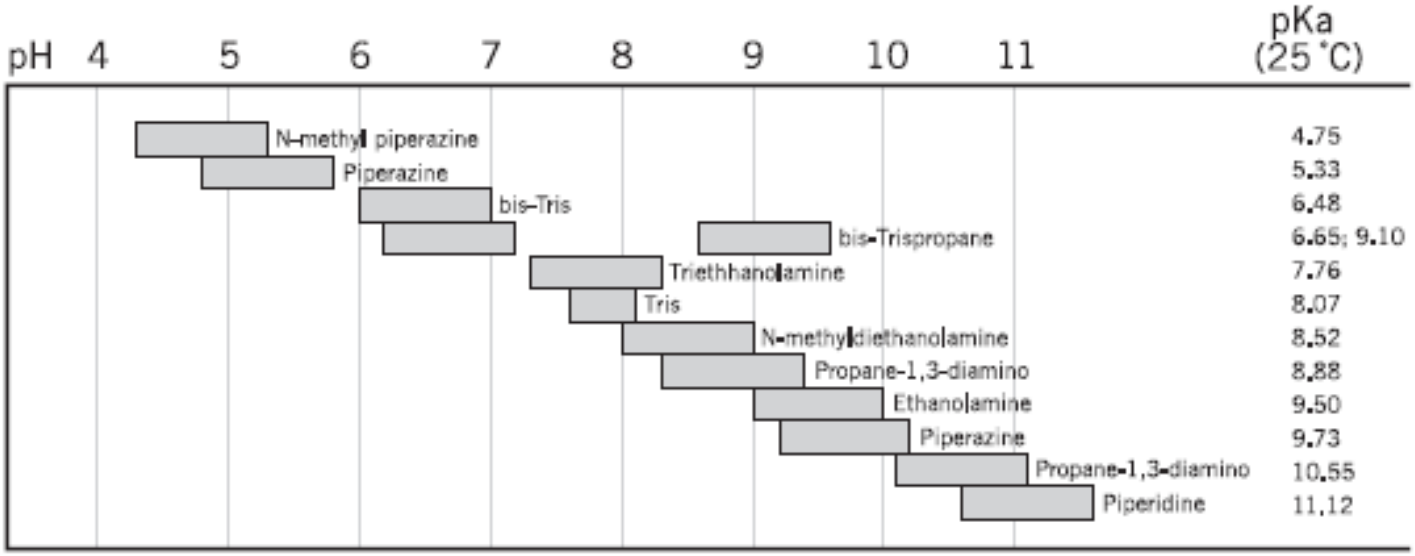
Volatile buffers



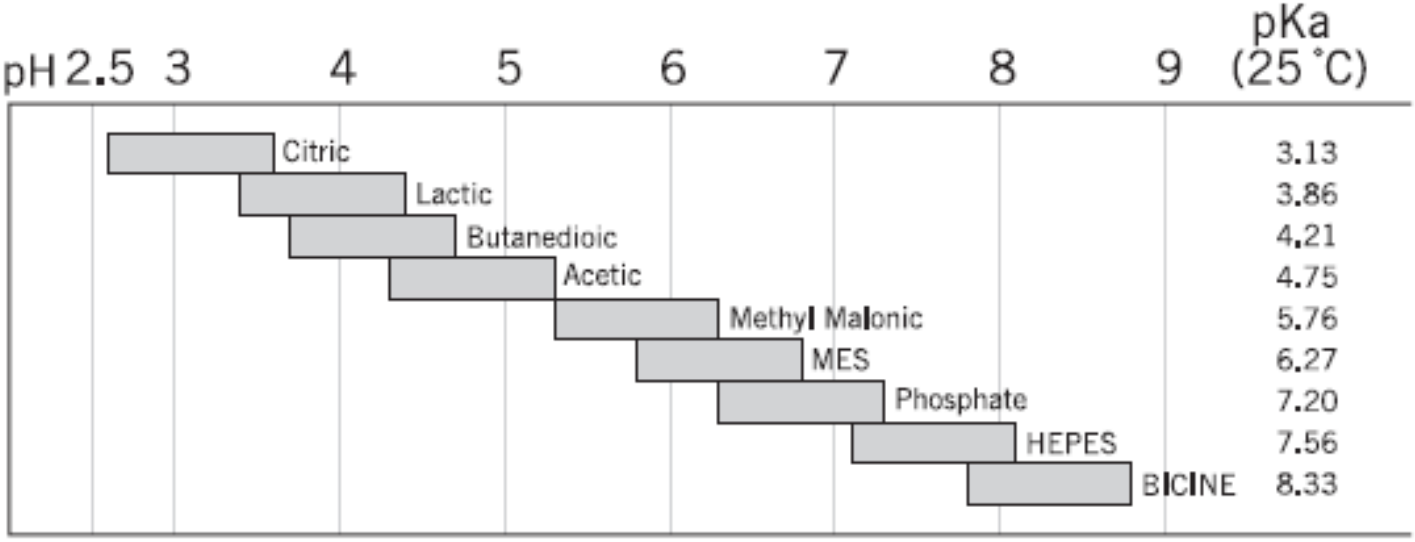
Recommeded buffers for anion exchange chromatography.



Recommeded buffers for cation exchange chromatography.



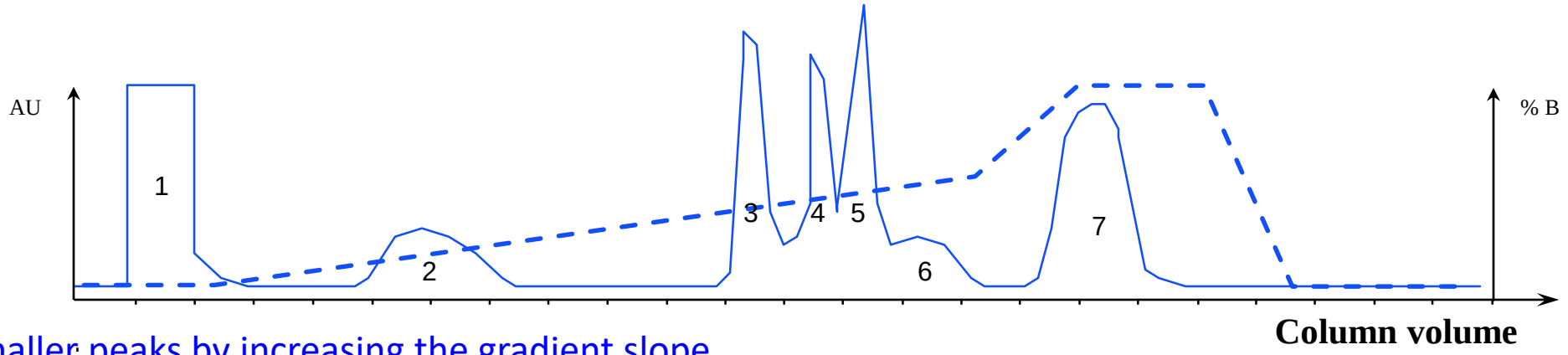
Recommeded buffers for anion exchange chromatography.



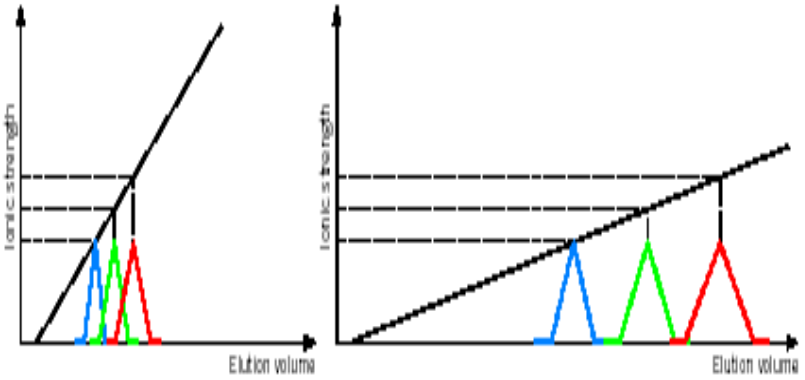
Recommeded buffers for cation exchange chromatography.

Parameters for elution optimization:

Continuous gradient elution



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



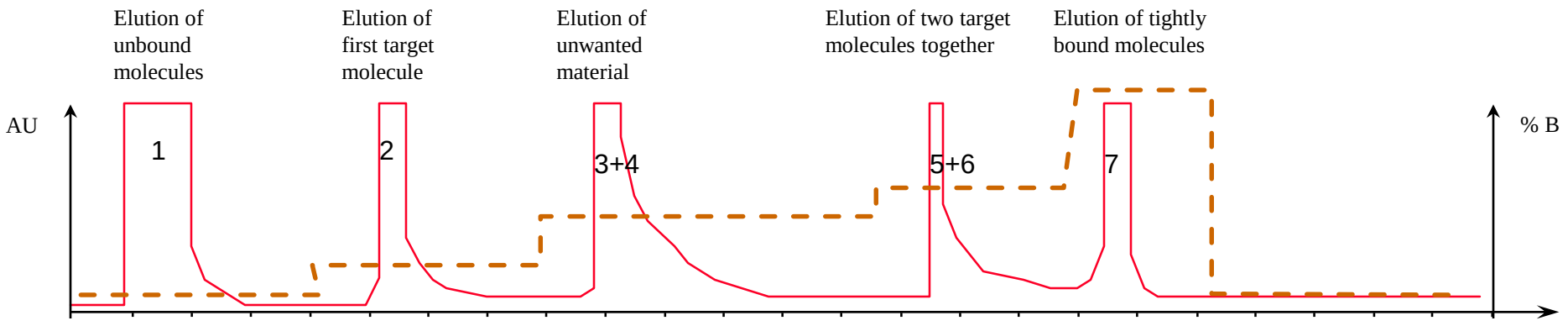
First choice during method development

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*



The ideal IEX separation for production

Parameters for elution optimization: **Step gradient elution (stepwise)**

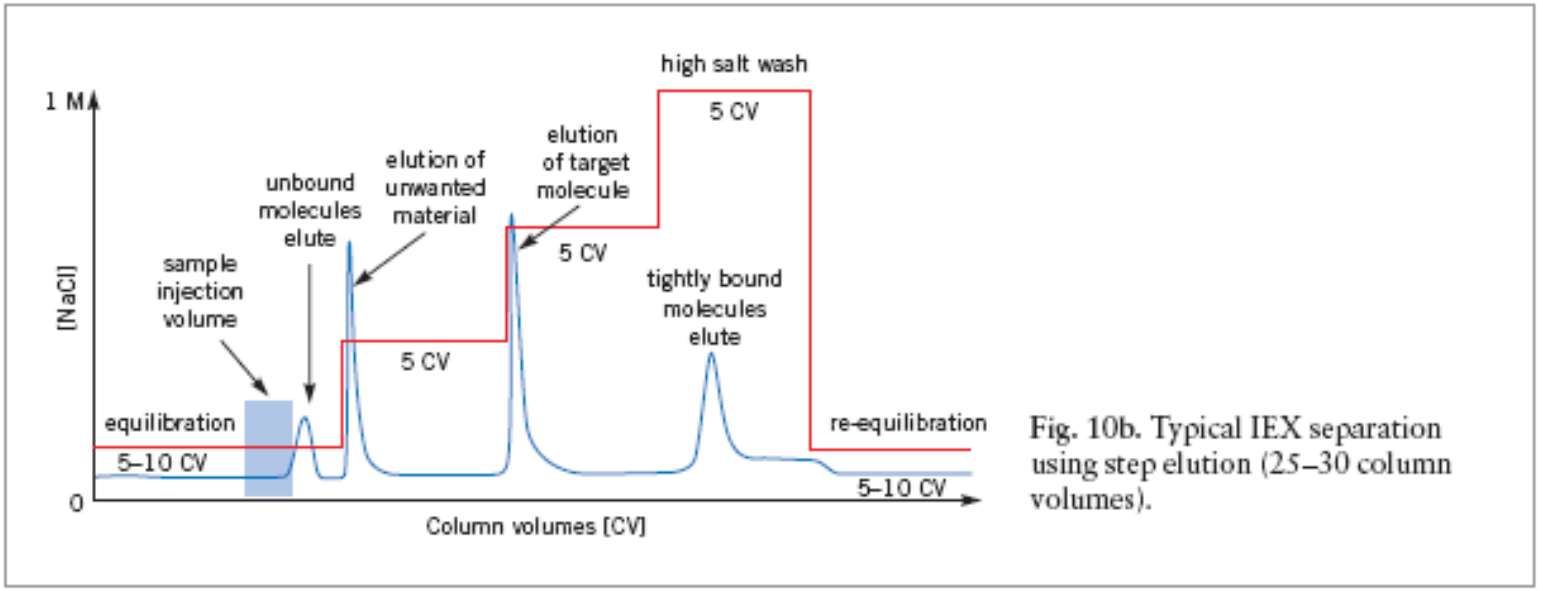


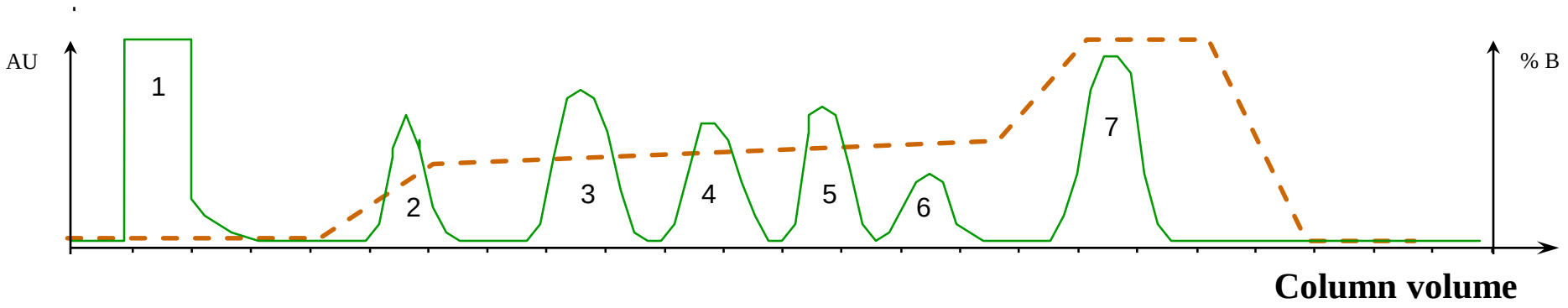
Fig. 10b. Typical IEX separation using step elution (25–30 column volumes).

- 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 <u>without eluting target</u>
- 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

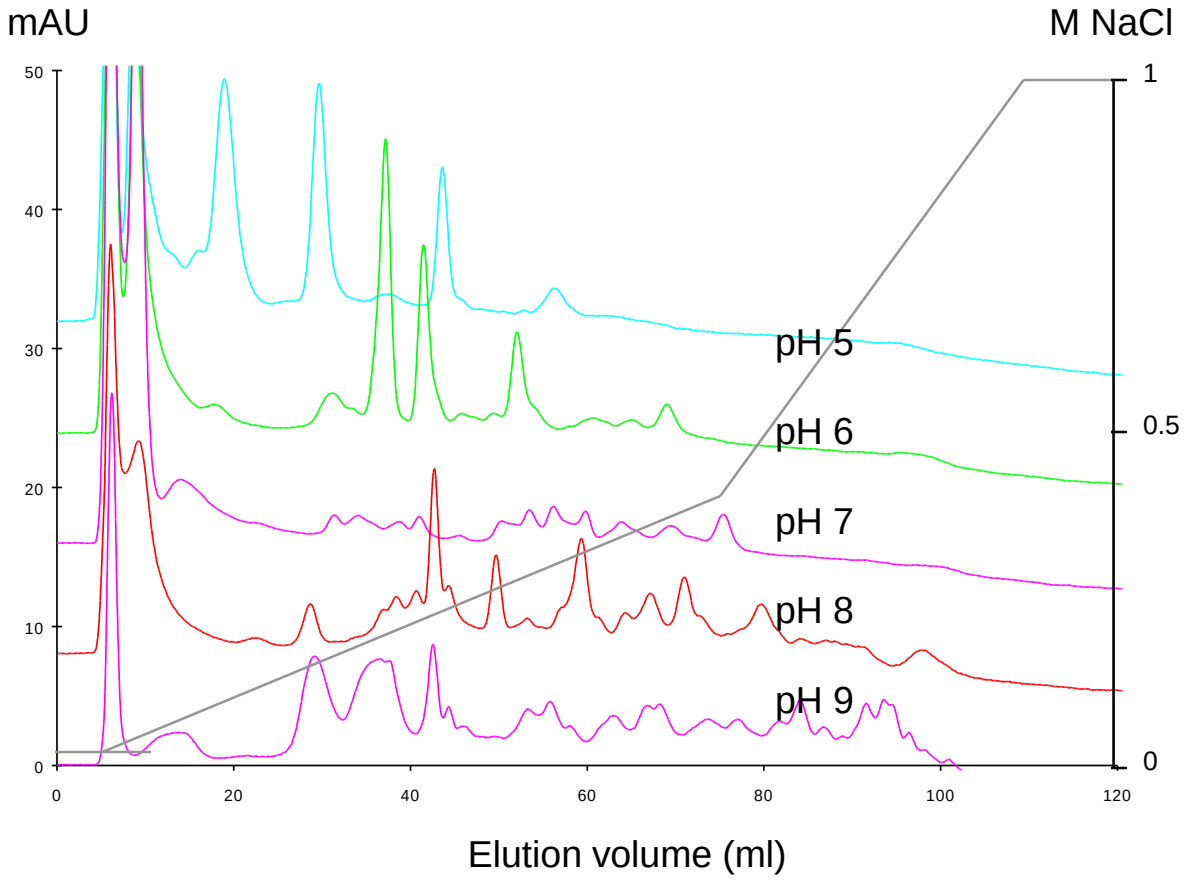
Cleaning: 51–100 % in 3–5 column volumes

pH working range: ± 1 pH units from the pI

CEIX: pH < protein pI **AEIX: pH > protein pI**

Parameters for elution optimization:

pH value

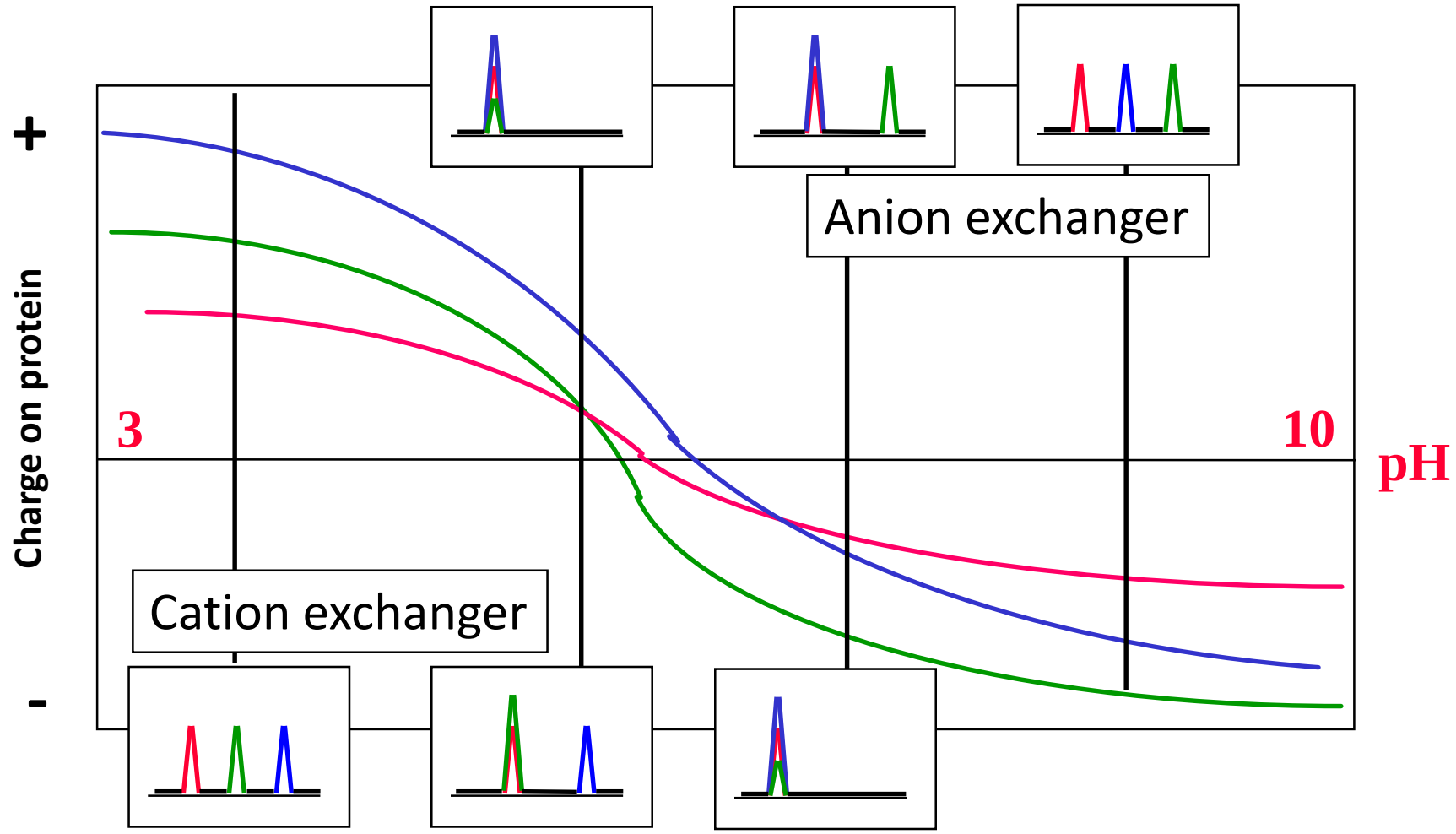


pH scouting for the separation of pancreatin

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:	ÄKTA™*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

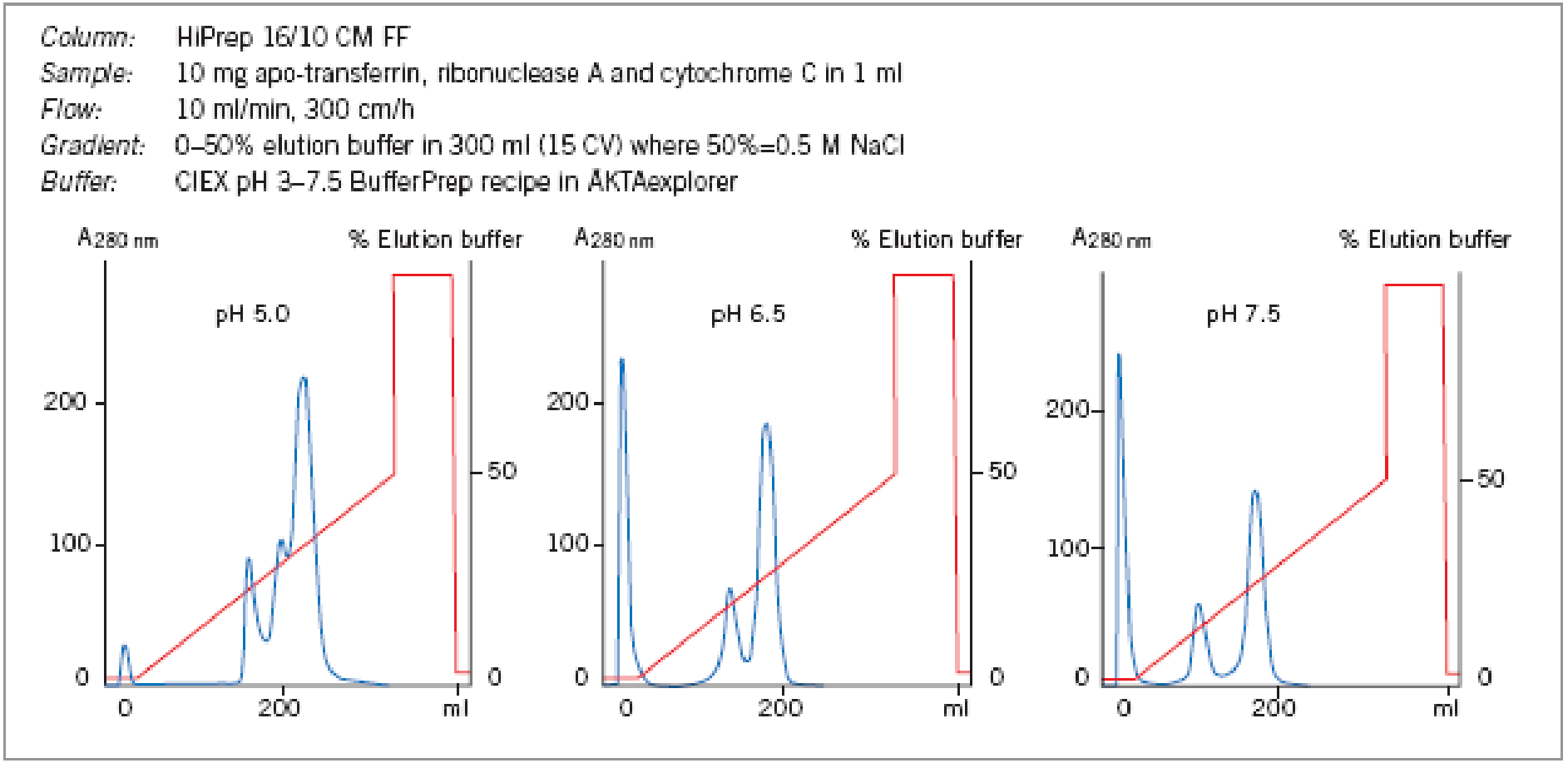


Fig. 61. Selecting optimal pH for separation of standard proteins on HiPrep 16/10 CM FF.

Controlling selectivity by pH

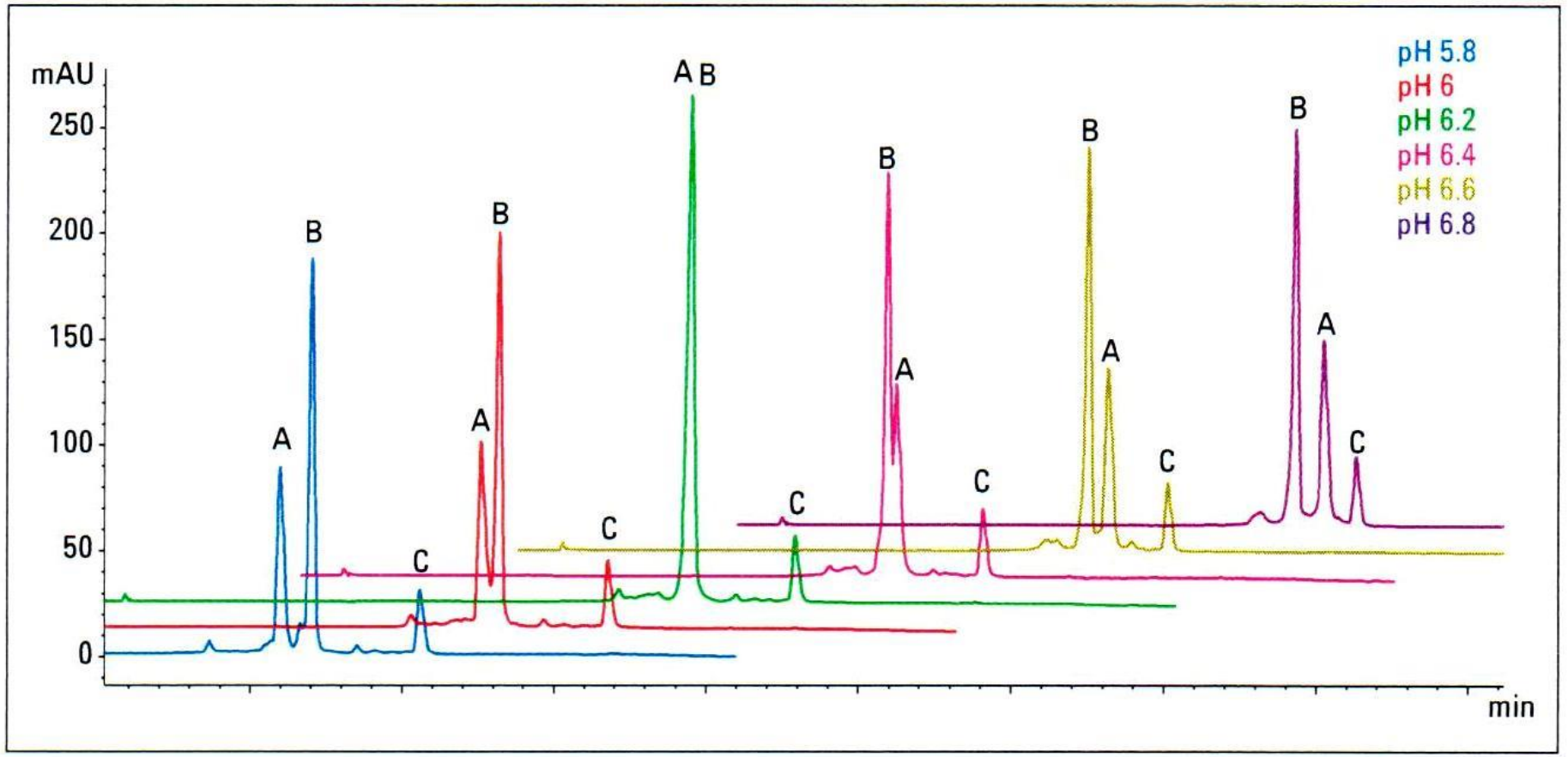


Figure 6
pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients.

Agilent

Parameters for elution optimization

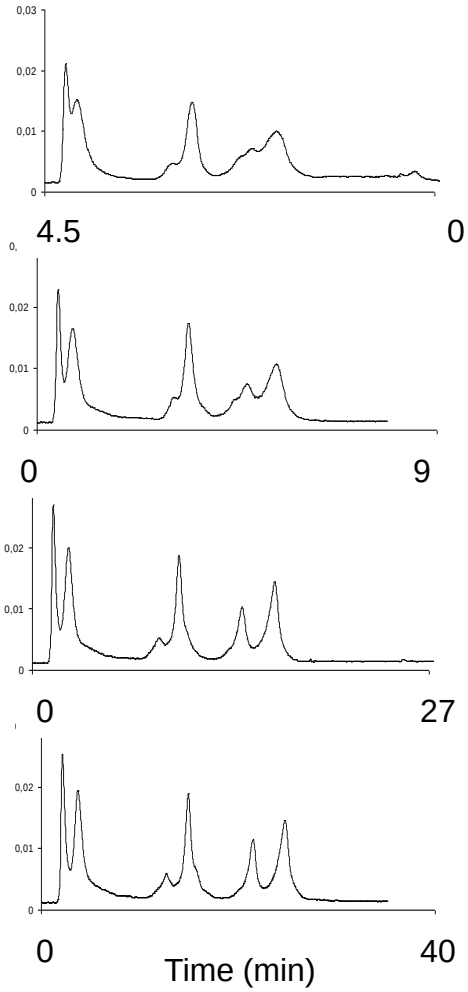
Flow rate

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)



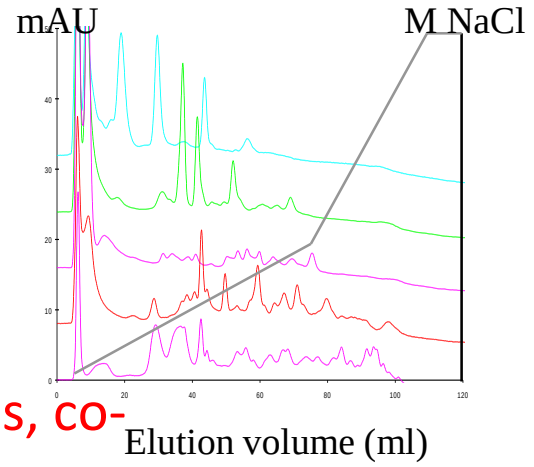
- Use high flow rate for high sample throughput high productivity
- Lower flow rate for maximum resolution
- **Select the highest flow rate that maintains resolution and minimizes separation time**

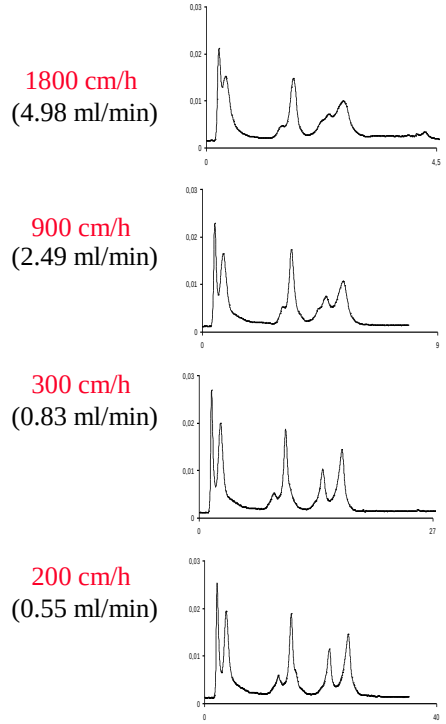
Conditions

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, co-factors, etc
- Effect of temperature
- Column length (volume)
- Type of ligand (strong, weak, mixed)
- Degree of substitution
- Particle size of matrix (efficiency)
- Supplier





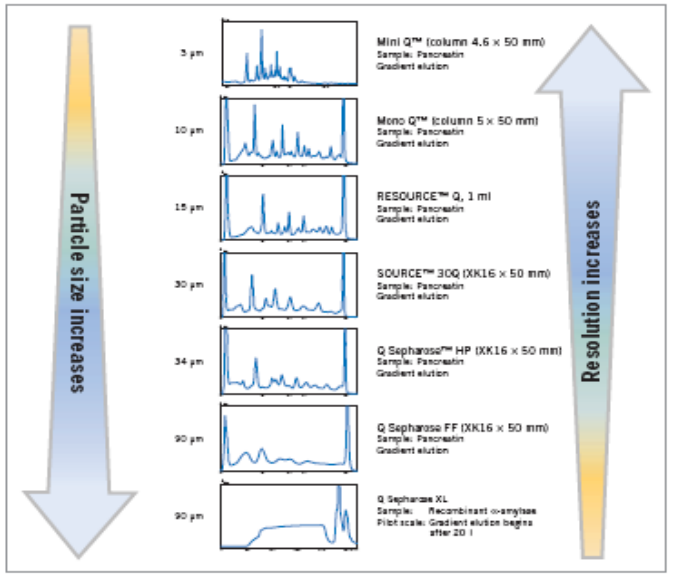


Fig. 7. The influence of particle size and selectivity on final resolution.

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

Ion exchange chromatography (IEC)

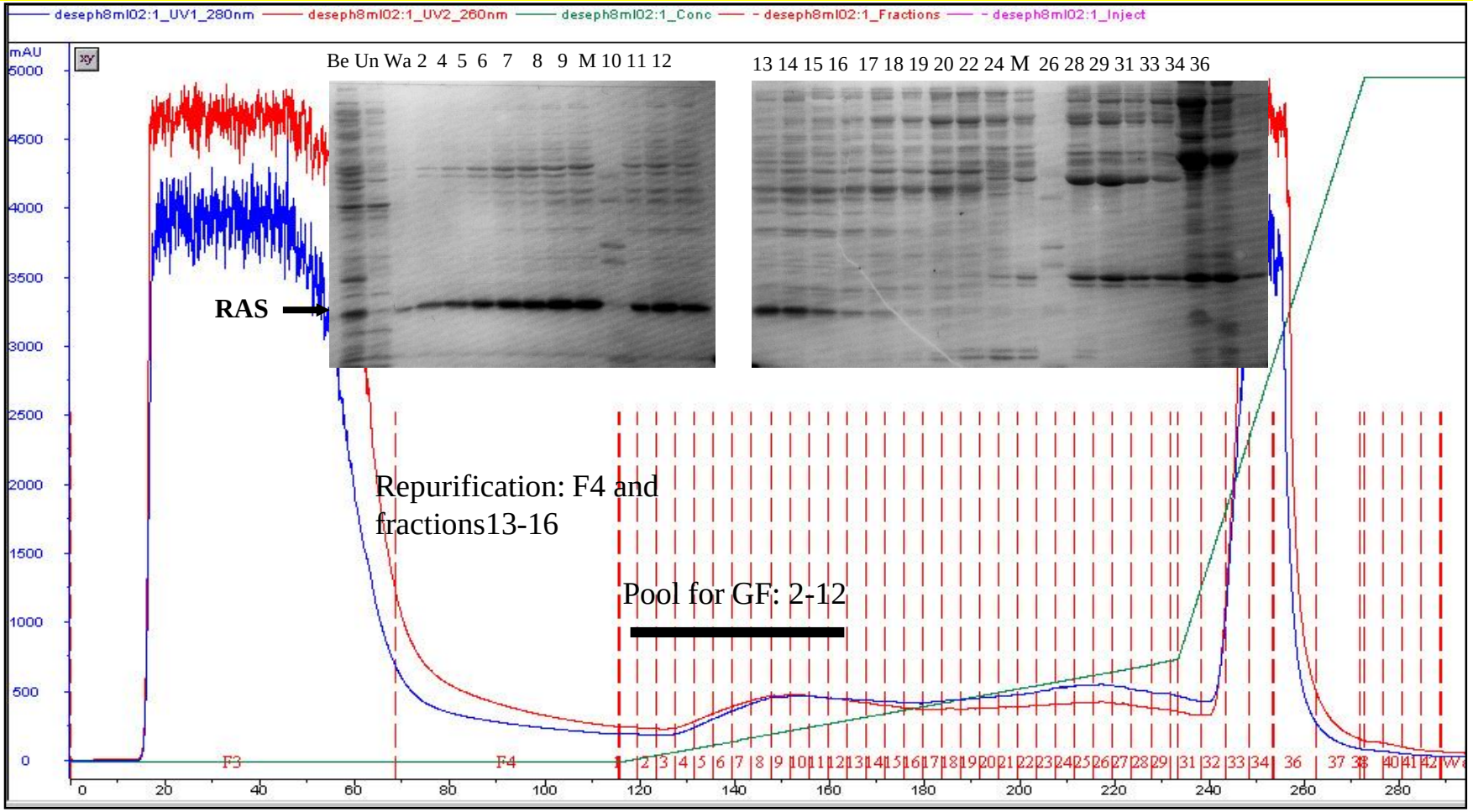
- Principles of IEC
- Main stages in Chromatography
- Resolution, efficiency, selectivity and capacity
- Determination of start conditions
- Parameters for absorption optimization
- Parameters for elution optimization
- Examples
- Troubleshooting
- Summary

AIEX – recProtein in *E.coli* cells

AEIX optimization

SEC as last step

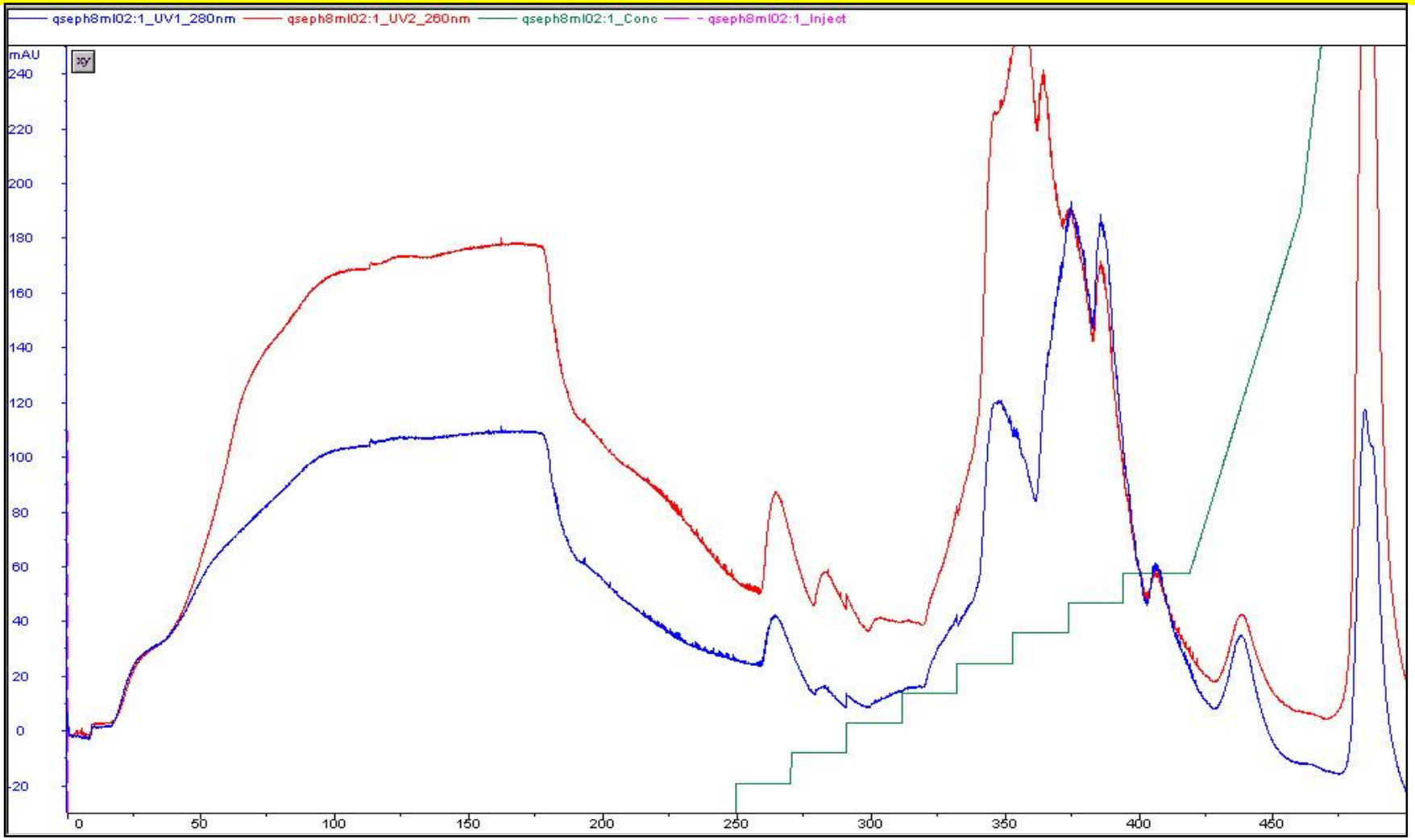
RAS - OPTIMIZATION 1st STEP



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

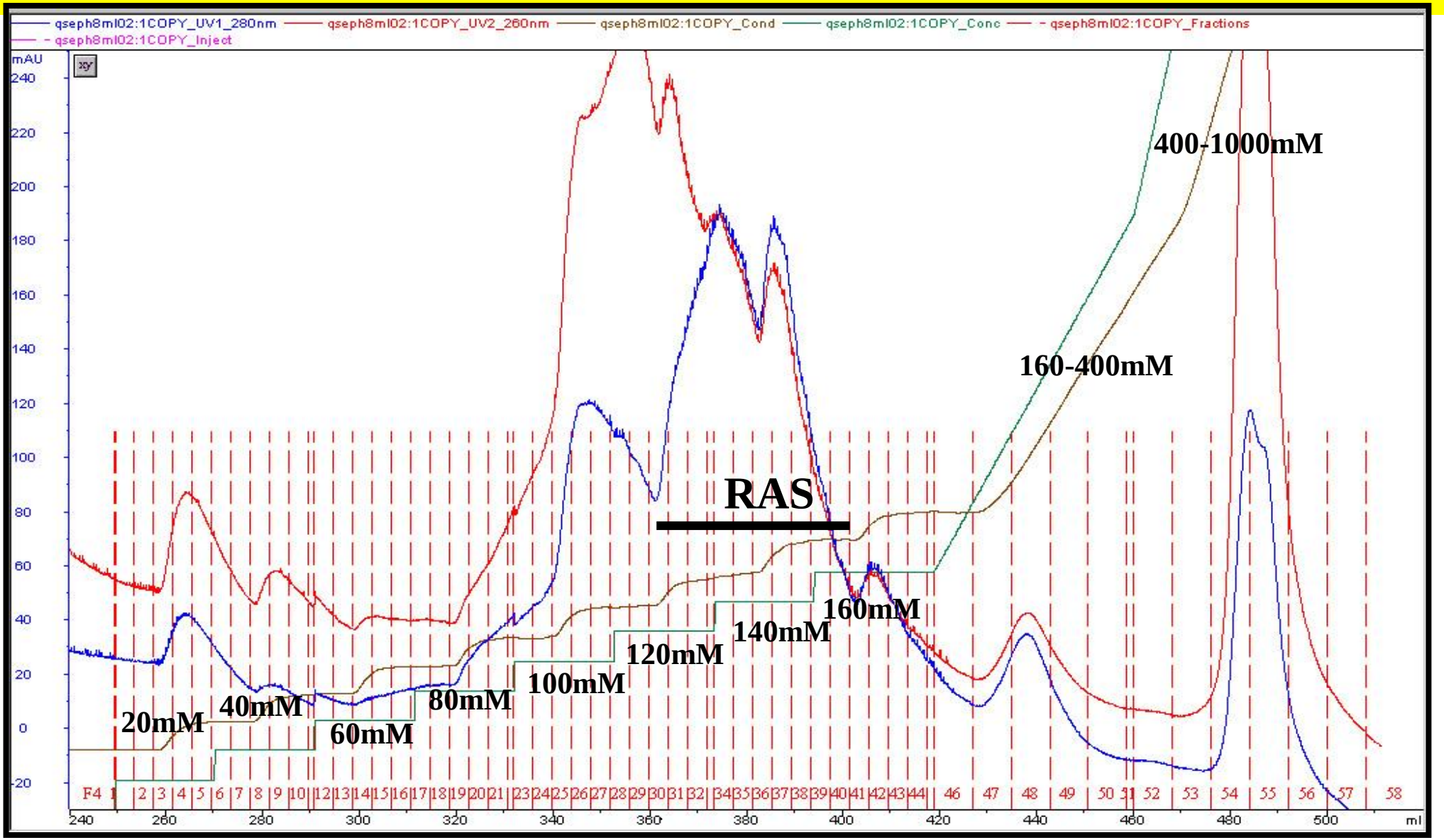
WASH: 5cv 0M NaCl ELUTION: gradient 15cv 0-0.15M NaCl + 5cv 0.15-1M NaCl

RAS - OPTIMIZATION 2nd STEP

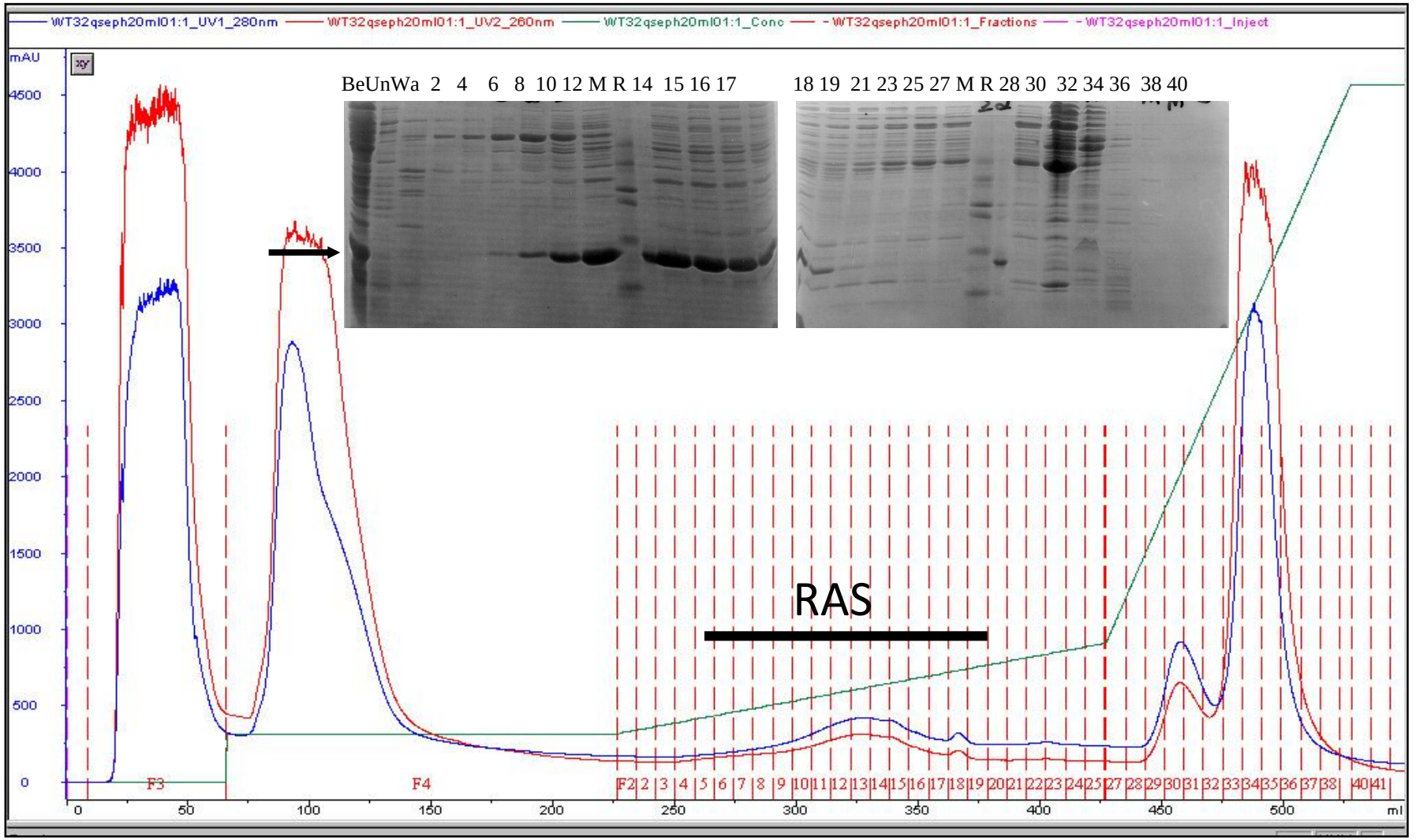


Step gradient from 20 to 160mM NaClpurification of RAS in Q-Sepharose FF column 100x10mm (~8ml) in 25mM TrisHCl pH7.9

RAS - OPTIMIZATION 2nd STEP



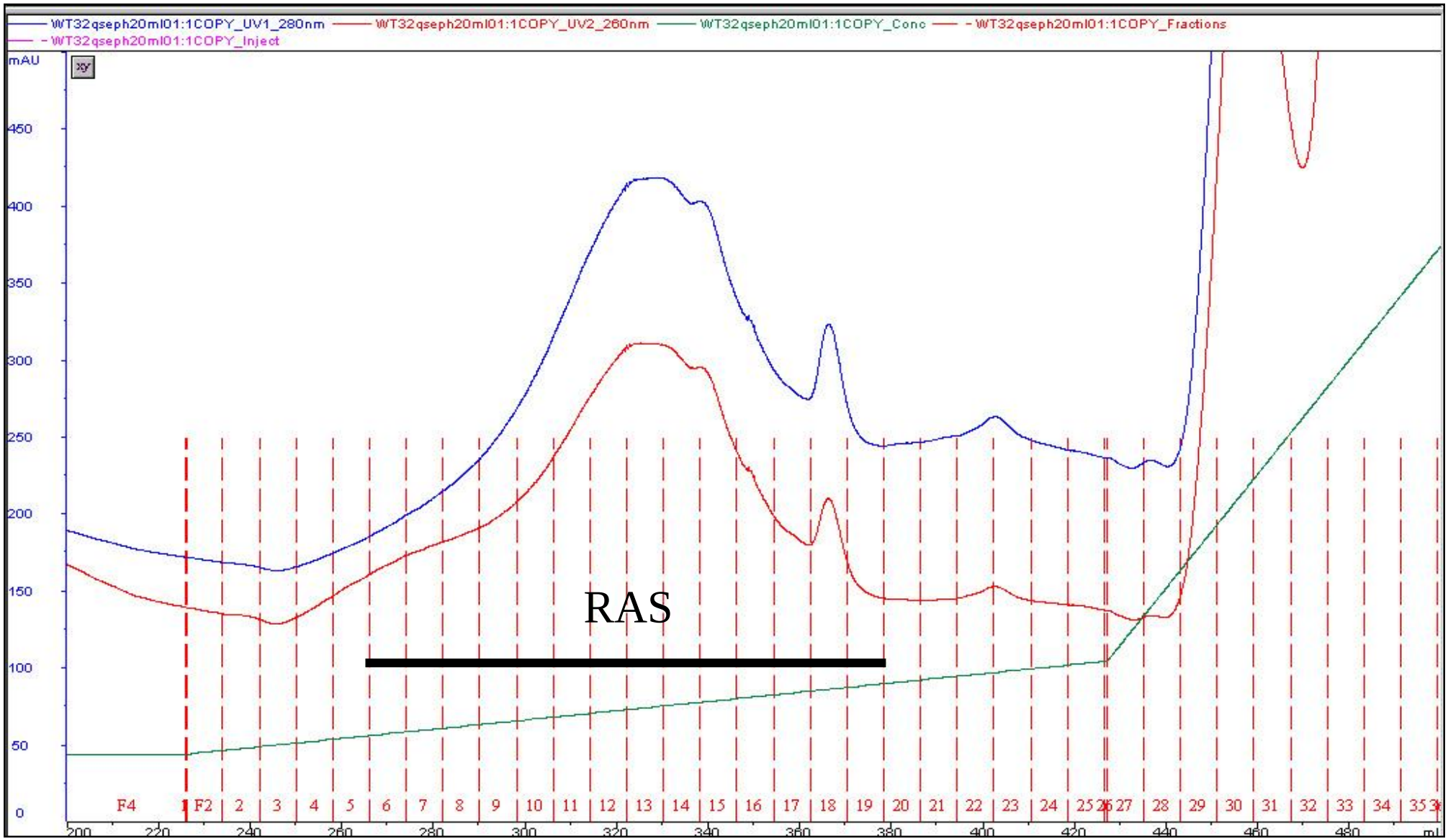
RAS - CAPTURE - Anion Exchange



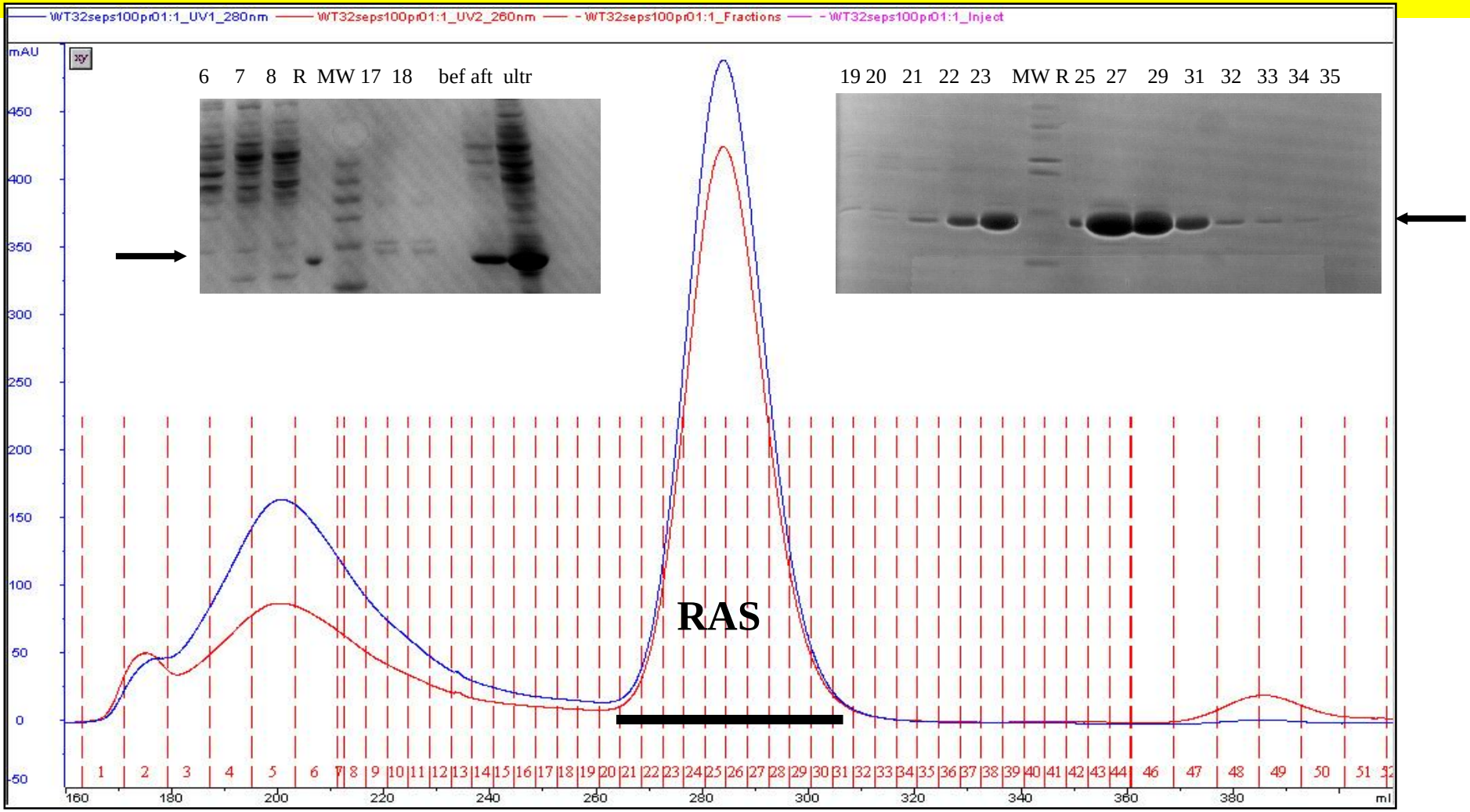
500ml culture after lysis and sonication. Q-Sepharose FF 100x16mm (~20ml) in 25mM TrisHCl pH8.0 buffer + additives

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

RAS - CAPTURE - Anion Exchange



RAS - 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

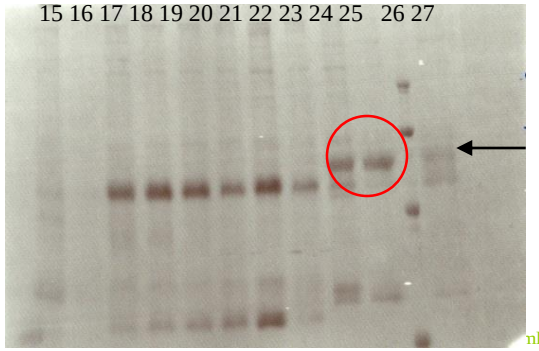
CIEX – recProtein in medium insect cells

Recombinat protein from Insect cells. pI: ~9.5. No tag
Two subunits connected by a.a. bridge

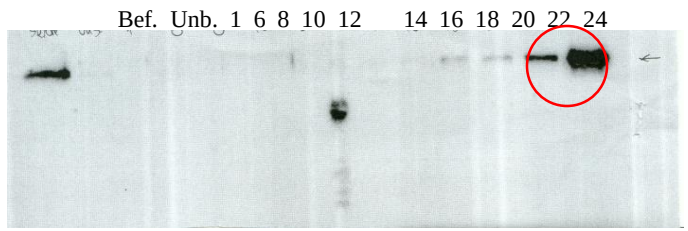
CEIX optimization

Two CEIX at different pH - Different suppliers

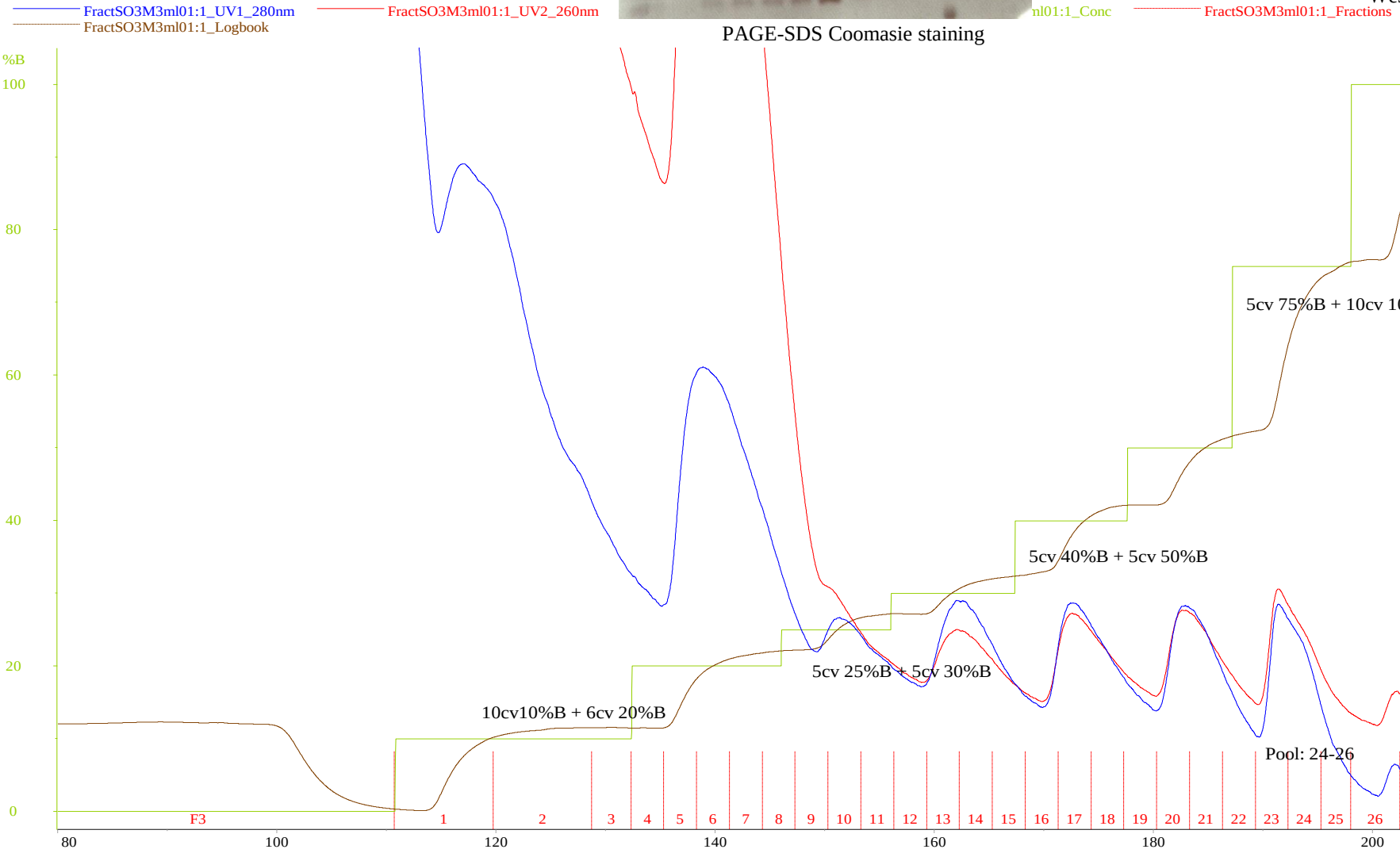
Sample: Load 90ml supernatant
Cation Exchange EMD-SO3 (M) 3ml
Buffer A: 20mM NaPO4 pH7.0
Buffer B: A + 1M NaCl



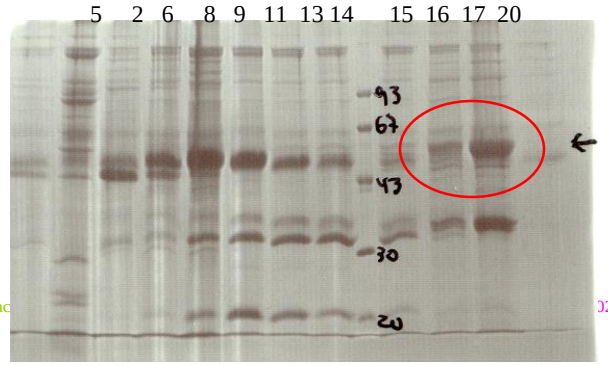
PAGE-SDS Coomasie staining



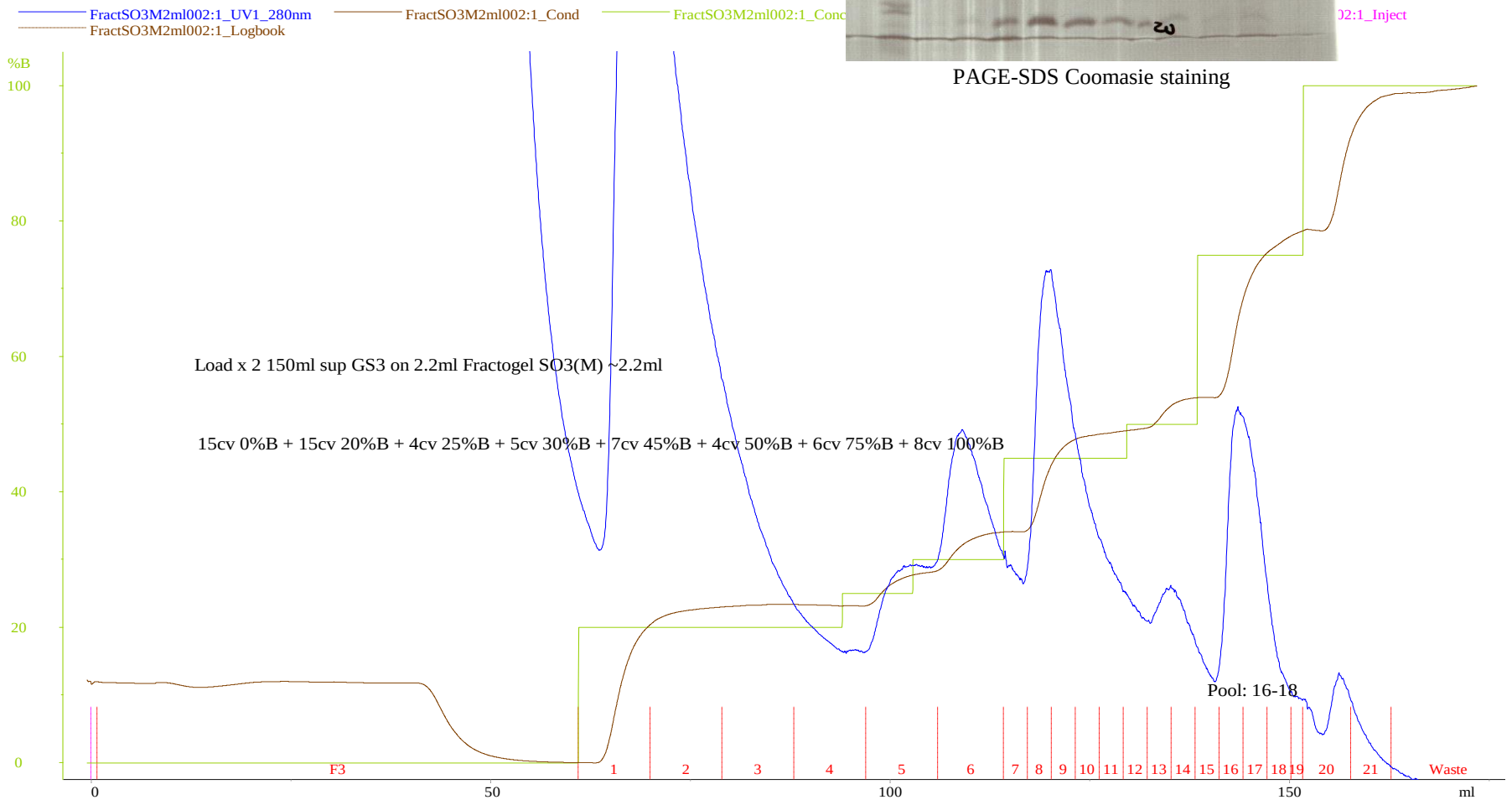
Western



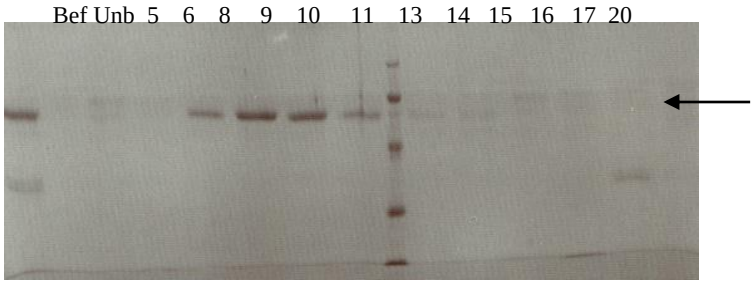
Load 150ml crude supernatant
Cation Exchange EMD-SO3 (M) 2.2ml
Buffer A: 20mM NaPO4 pH7.0
Buffer B: A + 1M NaCl



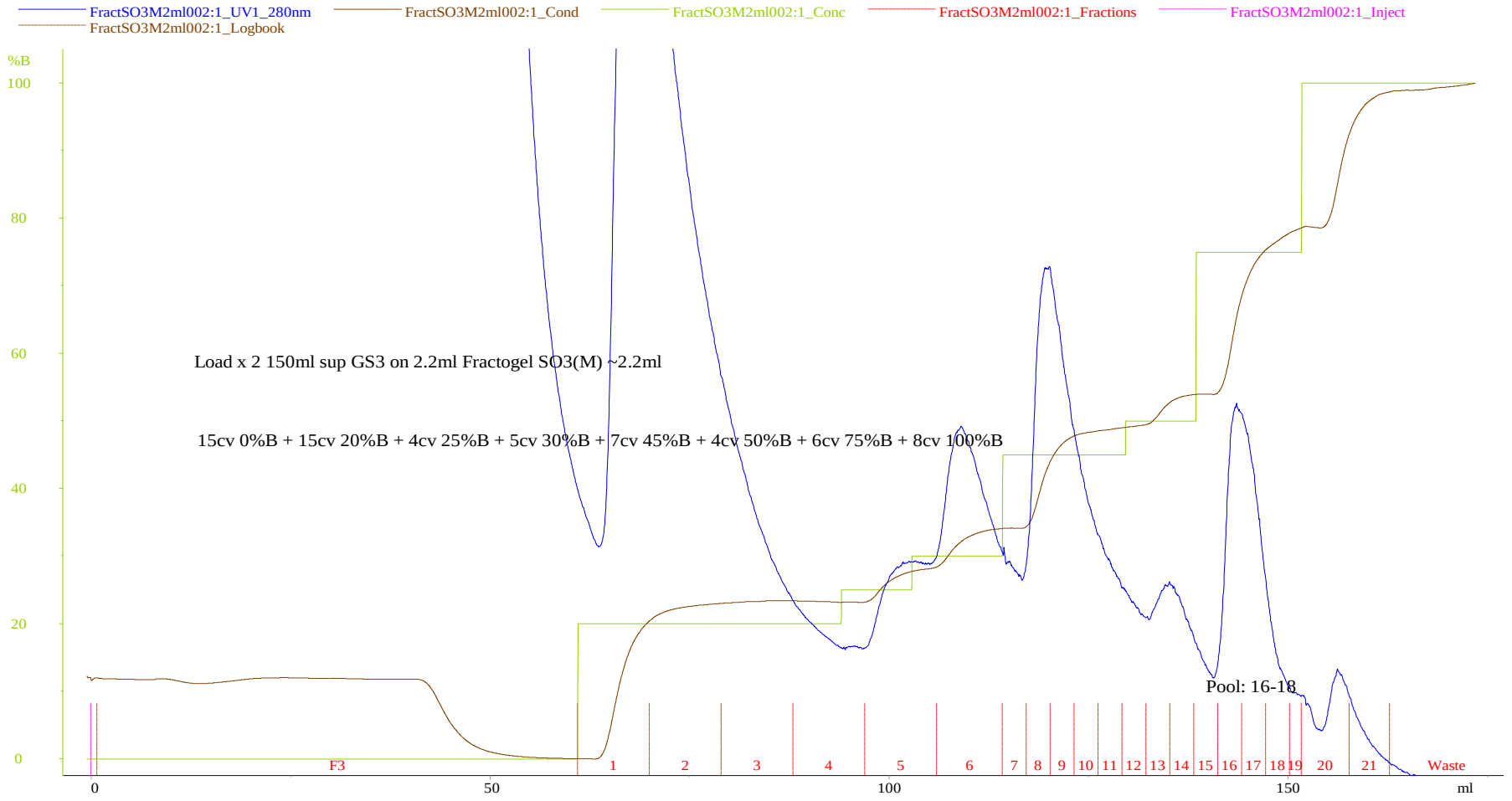
PAGE-SDS Coomasie staining



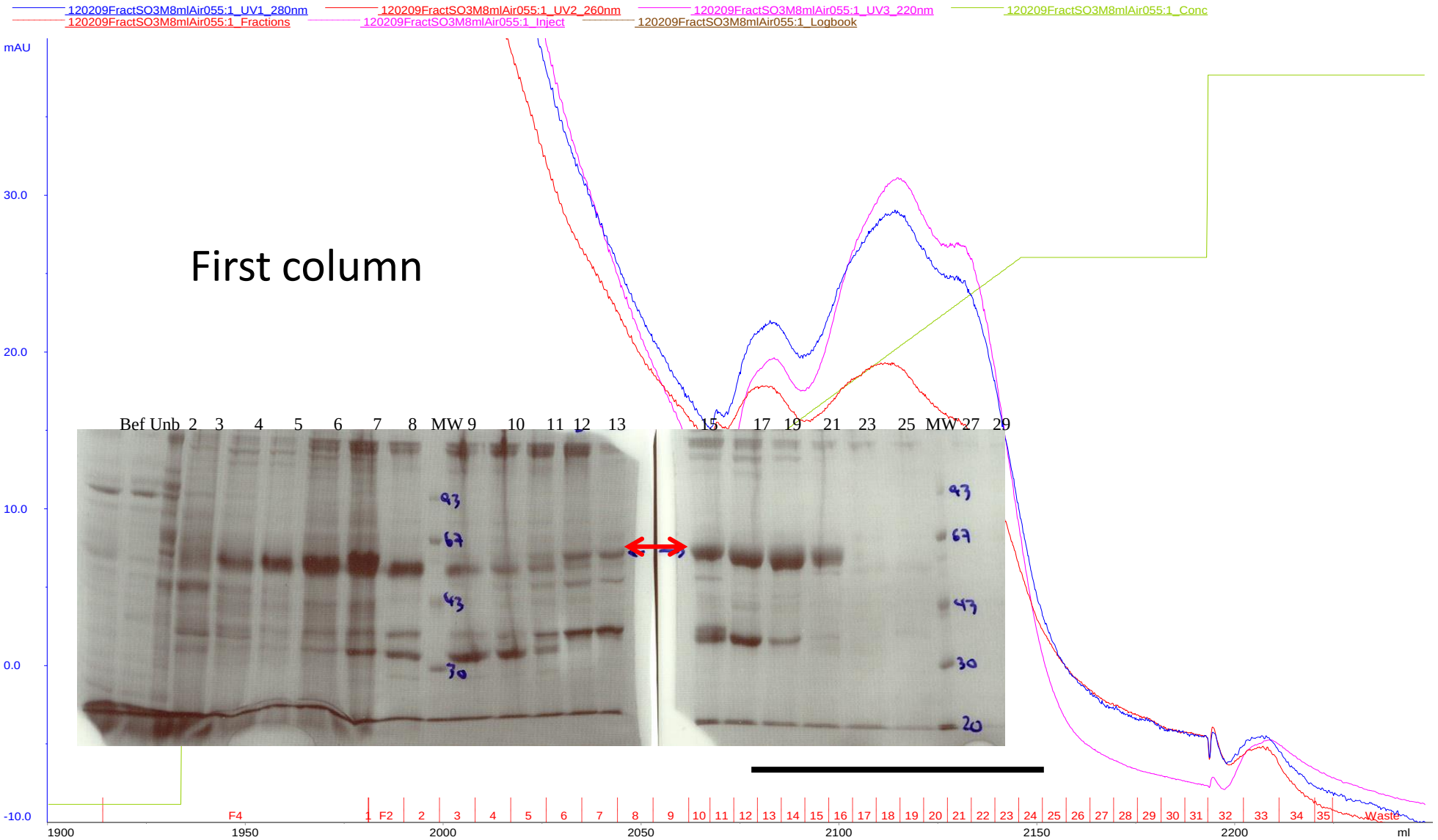
Load after Fract SO3
Cation Exchange Mono S 1ml
Buffer A: 20mM NaPO4/Citrate pH6.0 + 0.15M NaCl
Buffer B: 20mM NaPO4/Citrate pH6.0 + 1M NaCl



PAGE-SDS Coomasie staining



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 pH7.0 - Eluent B: 20mM NaPO4 pH7.0 + 1M NaCl

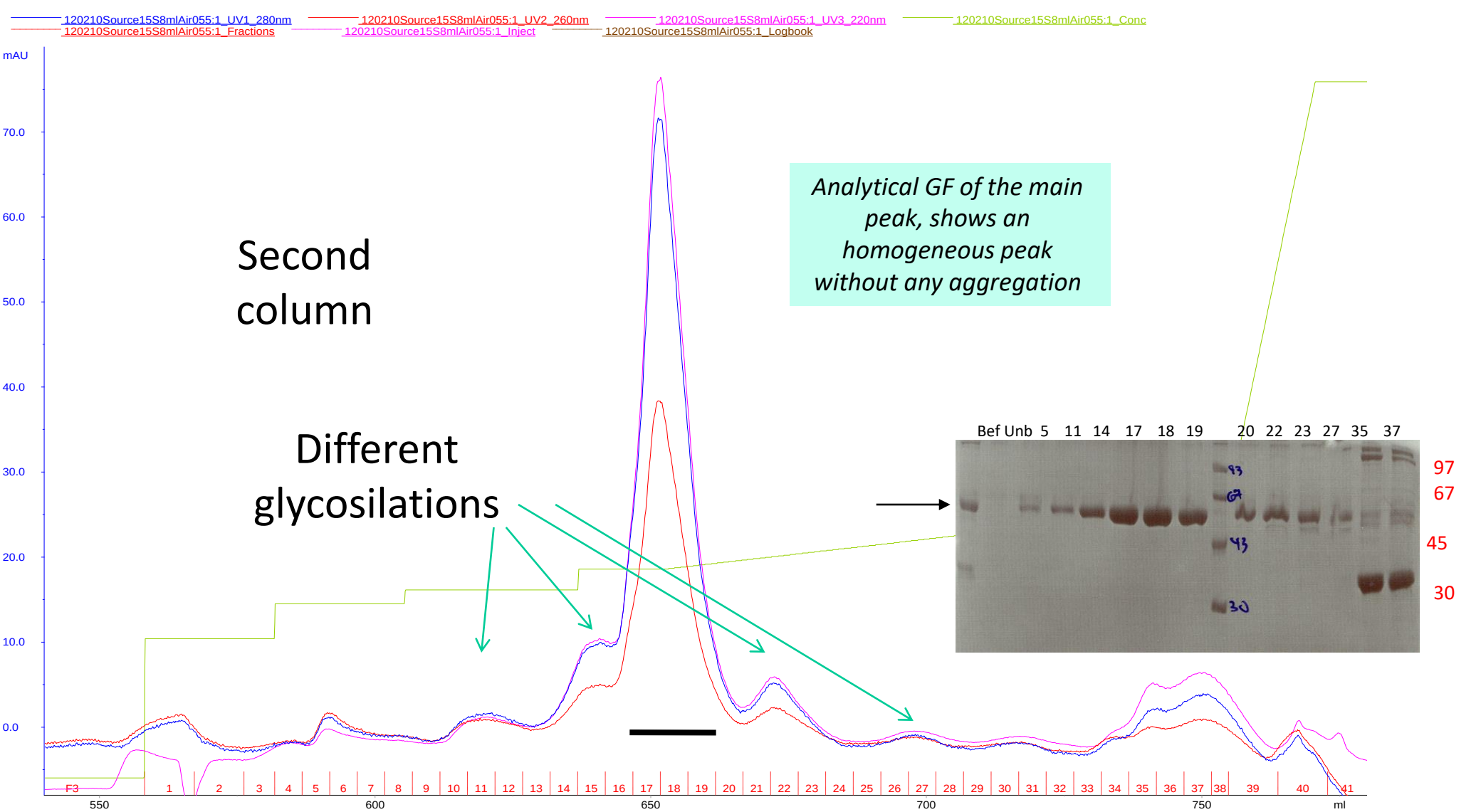


Sample: Pool after first CEIX - Dilution 1:5 with A after First CEIX

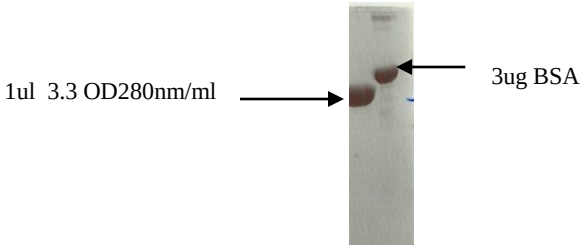
First CEIX: Res 15S 100x10mm ~7.8ml **Second CEIX pH 6.0**

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)

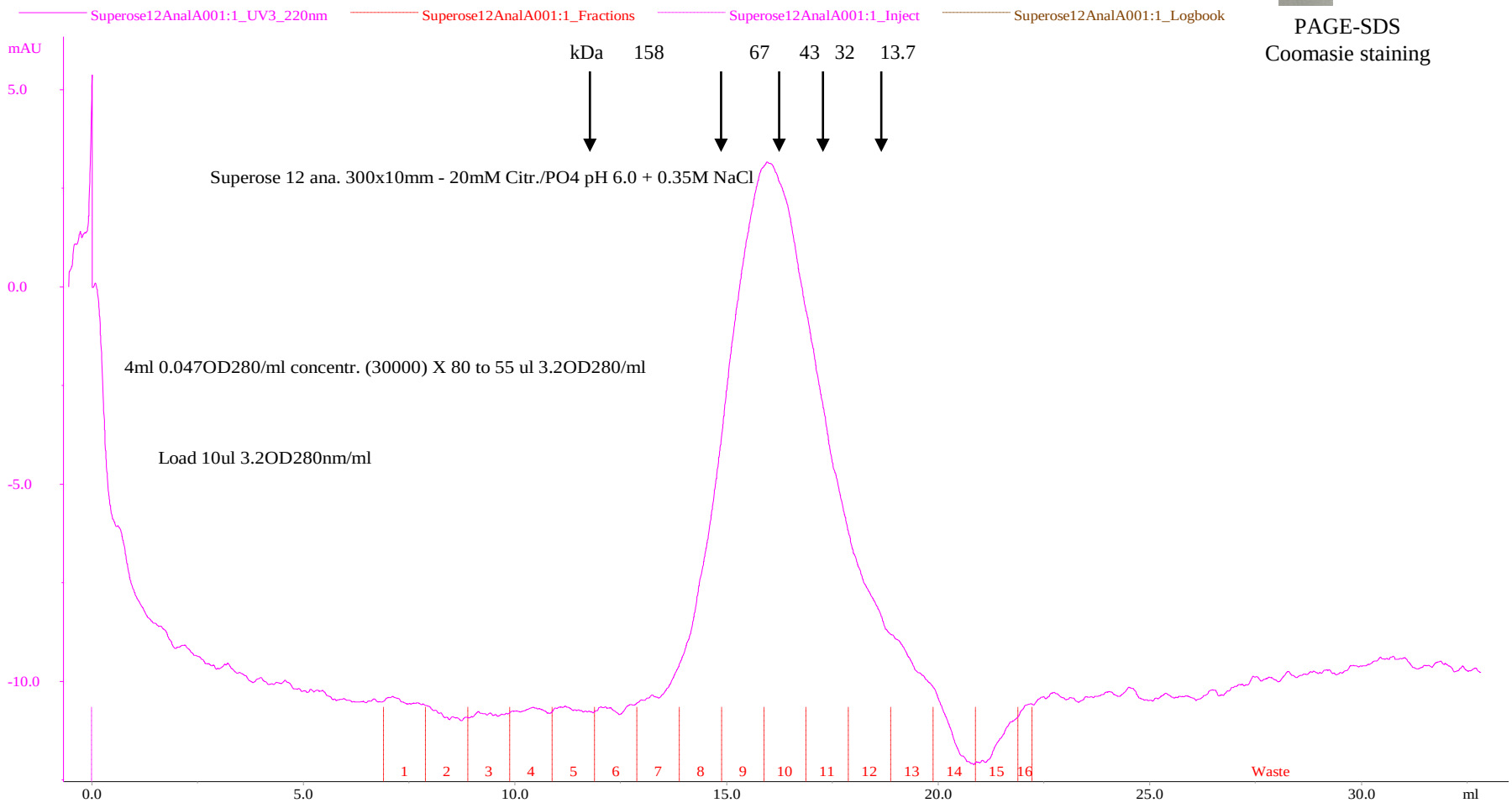
Eluent A: 20mM MES pH6.0 - Eluent A: 20mM MES pH6.0 + 1M NaCl



Protein concentration with Centriplus 30000 to 3.3 OD280nm/ml
Analytical Superose 12 anal column 300x10mm



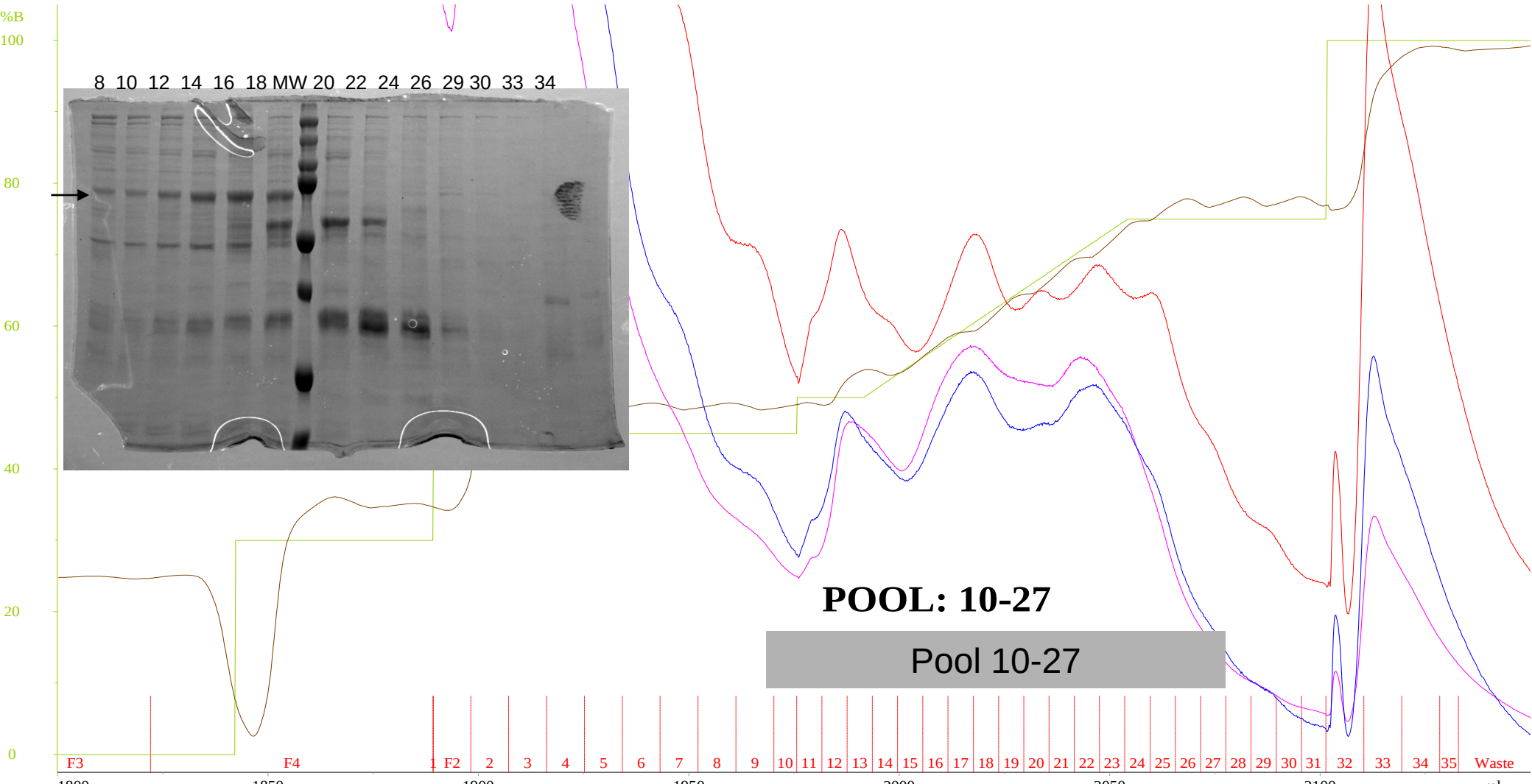
PAGE-SDS
Coomasie staining



Similar variant of last protein (full-length)

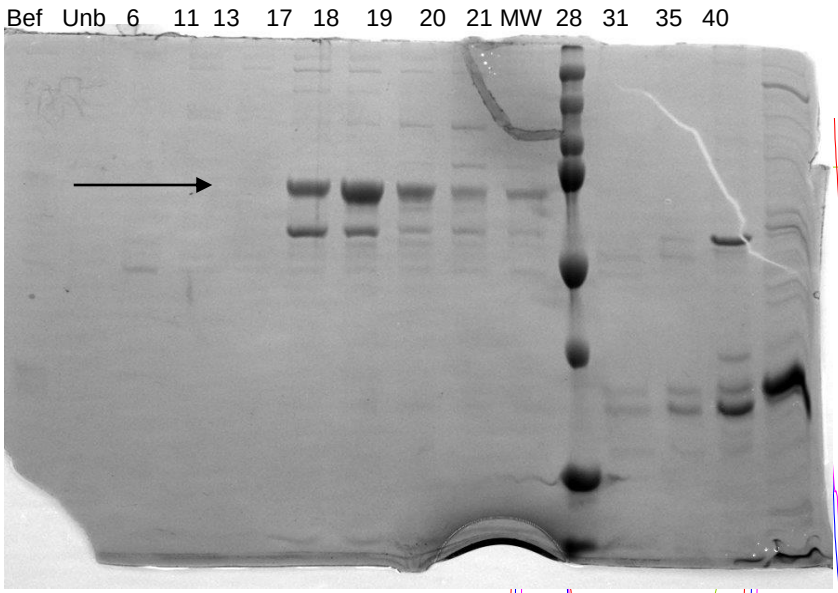
1st column (capture): Fractogel EMD SO_3 (M) 10x1cm ~7.8 ml

Buffer A: 20mM $NaPO_4$ pH 7.0 - Bufer B: A + 1M NaCL



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

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



16-21

%B
100
80
60
40
20
0

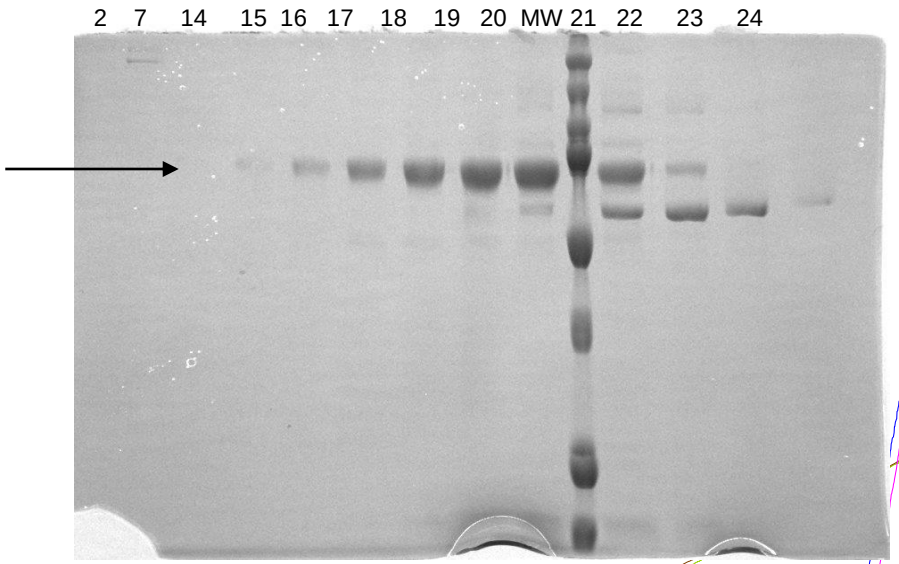
Pool 16-21

F3 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 Waste

550 600 650 700 750 ml

3rd column (polish): Fractogel EMD SO_3 (M) 20x0.5cm ~4.0 ml

Buffer A: 20mM $NaPO_4$ pH 7.0 - Bufer B: A + 1M NaCL



%B
100
80
60
40
20
0

Load dil. 1:3 + 2cv 35%B + 3cv 45%B + 5cv 45-70%B + 1cv 70%B + 1cv 755B + 2cv 80%B + 0.5cv

Pool 15-20 for Crystal and 21-22 for repurification

Pool 15-20

F3 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

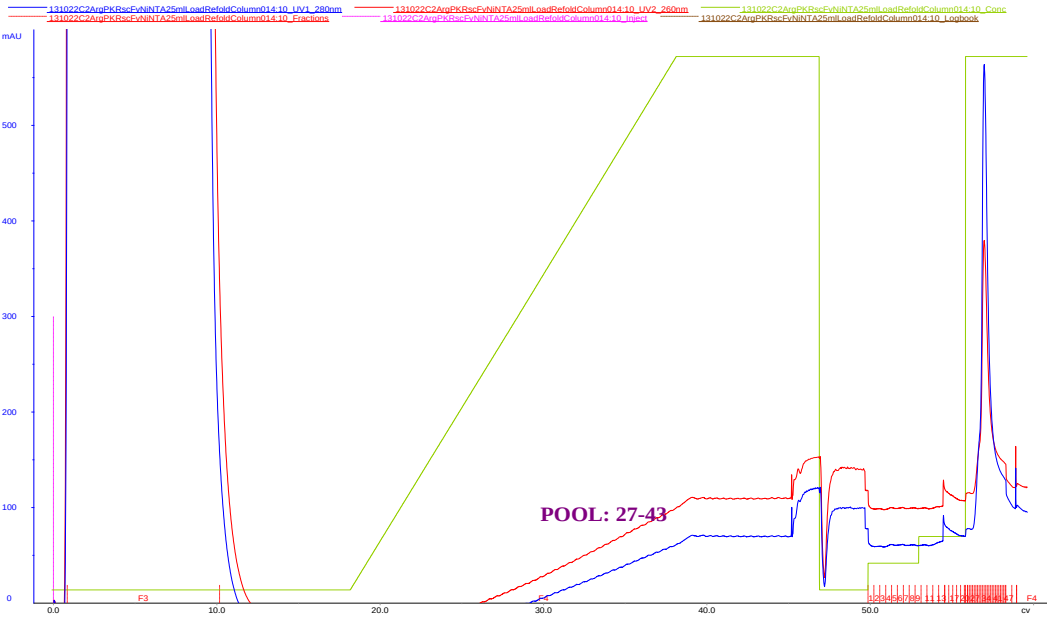
90.0 100.0 110.0 120.0 130.0 140.0 150.0 160.0 ml

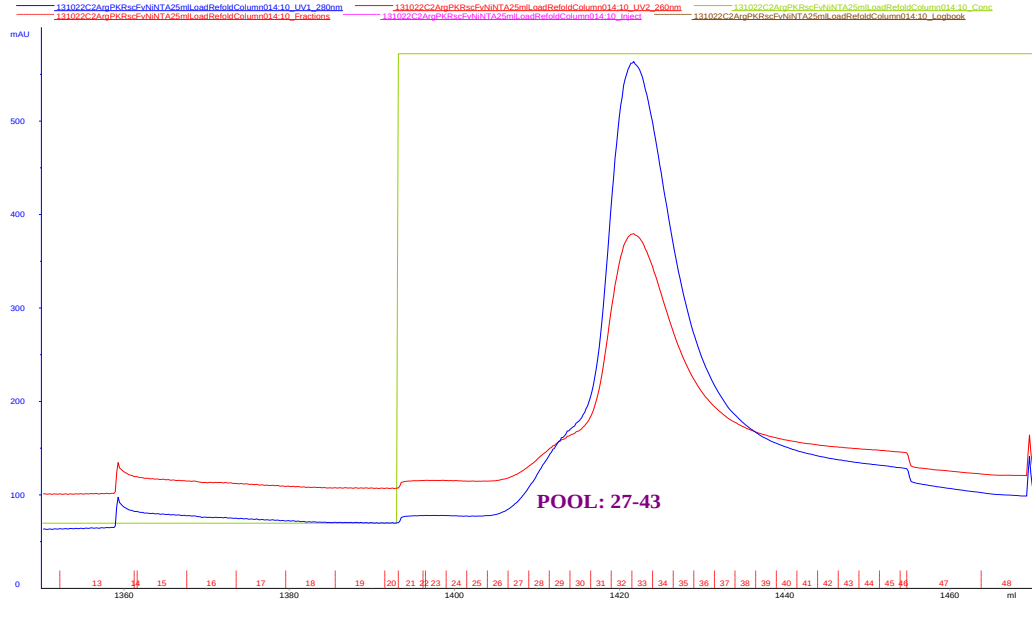
Purification of rec Chimeric Protein with two domains: one for recognition a second a toxic RNA binding protein

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

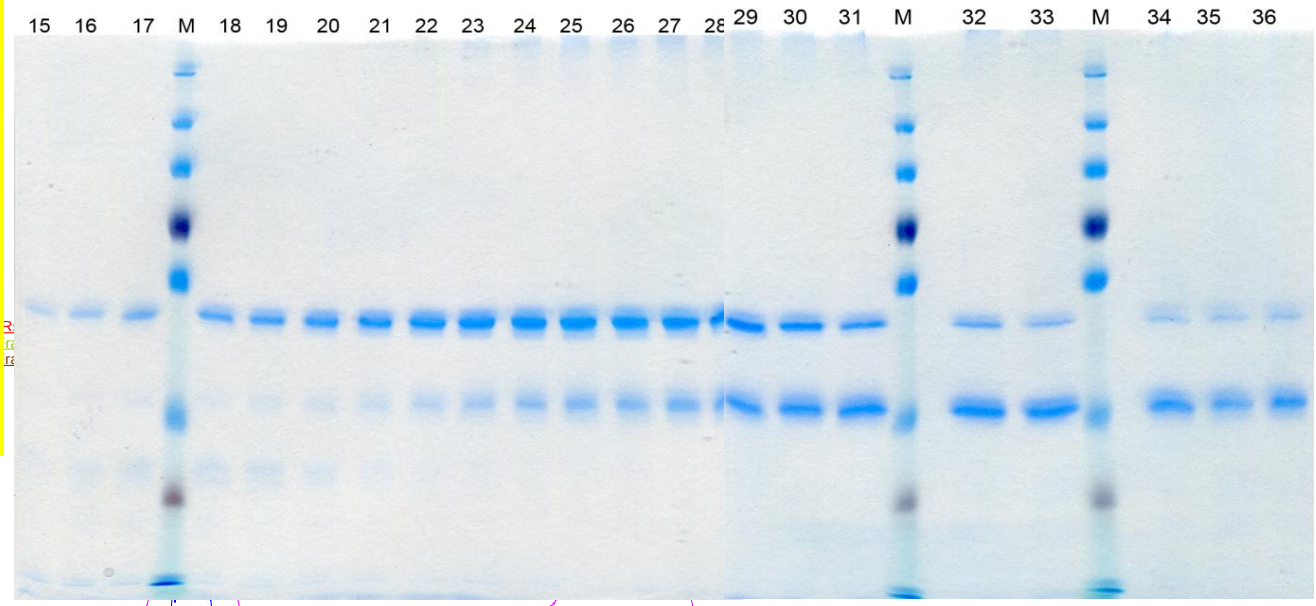
B1: 20mMHEPES pH7.5 + 0.5MNaCl + 10%Glyc + 500mMImid + 0.5%Tween 80

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%Glycero + 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

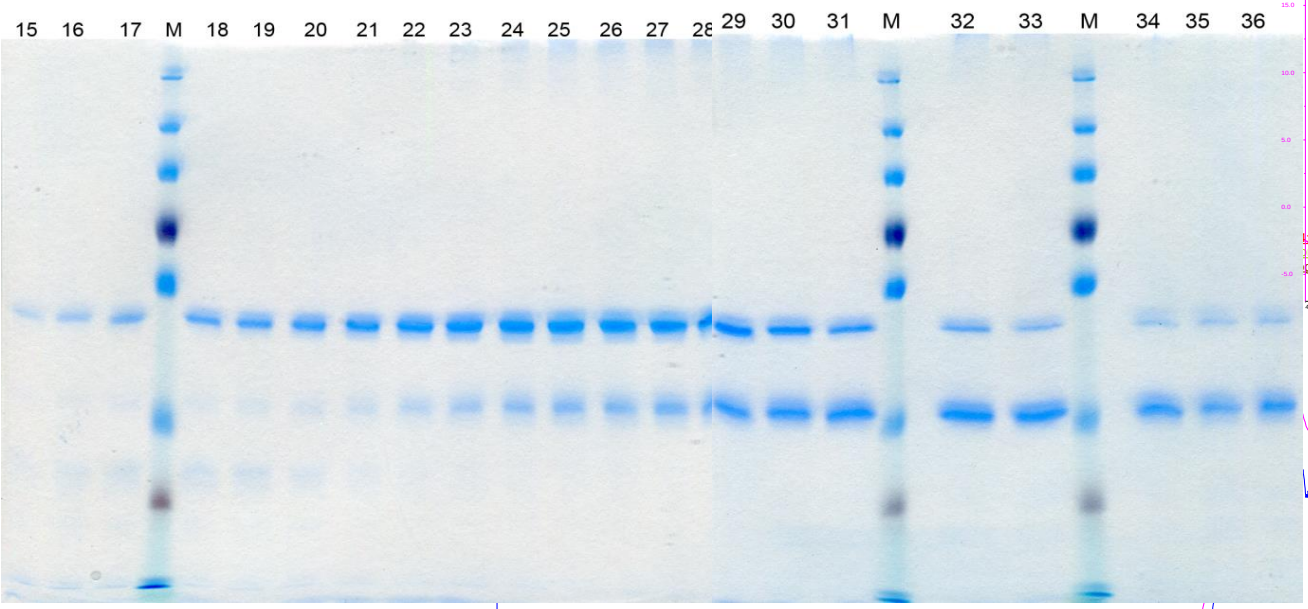
40.0 30.0 20.0 10.0 0.0 -10.0

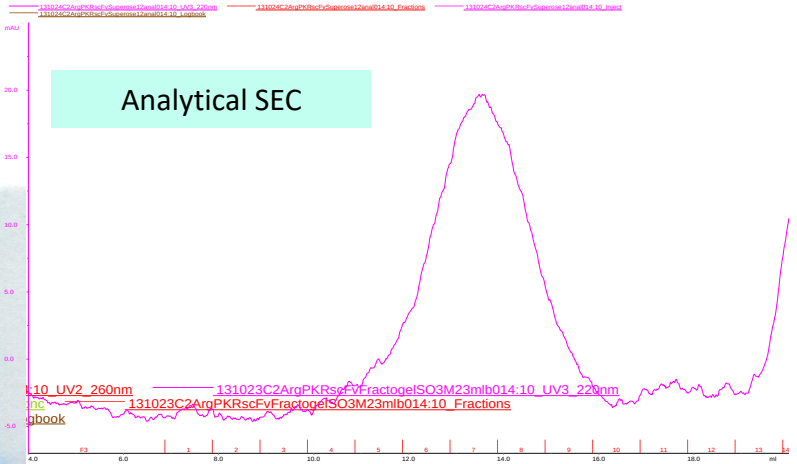
1 2 3 4 5 6 7 8 9 10 11 12 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 62 63 64 65 66 67 68 69 70

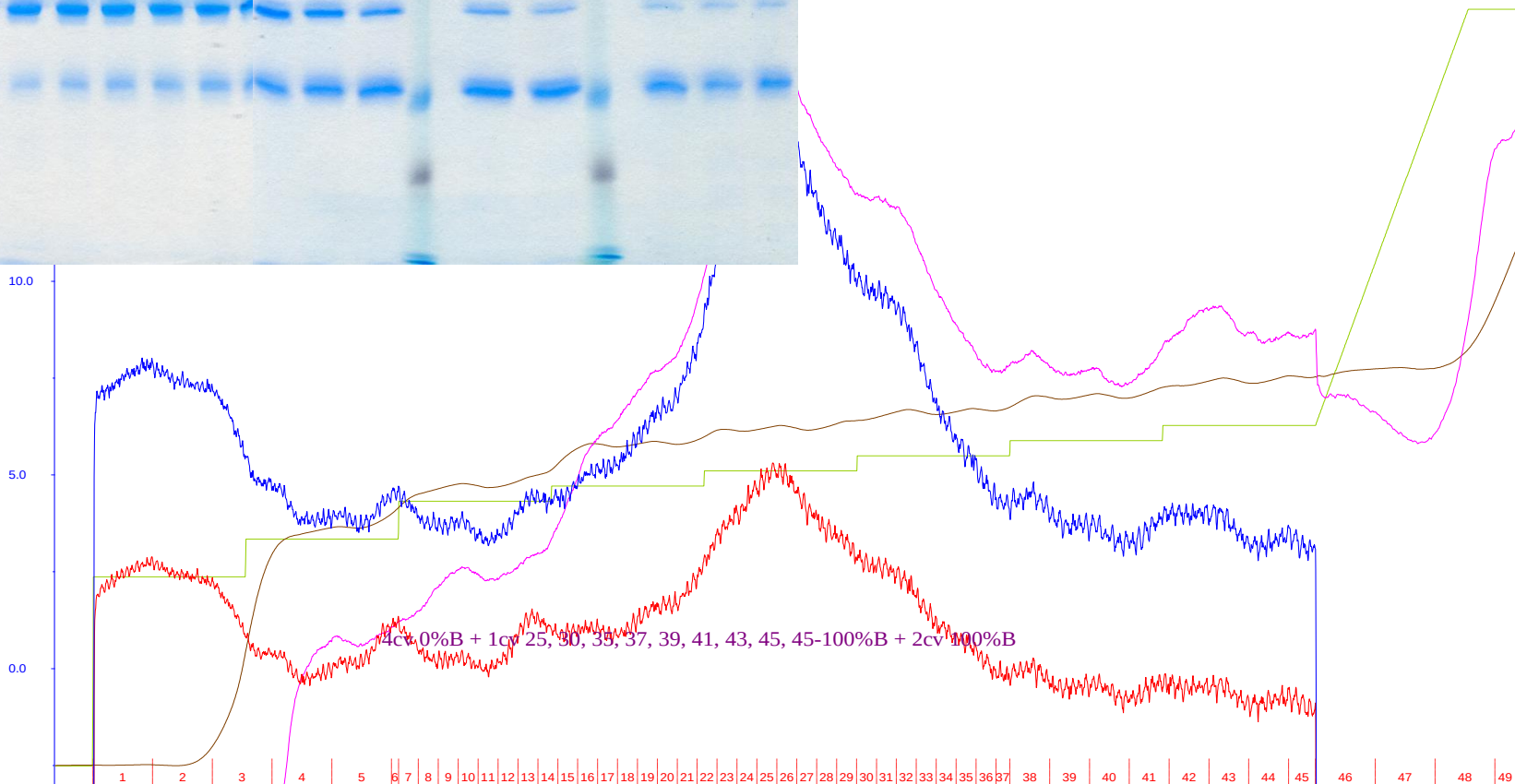
200 250 300 350 400 450 ml

FractogSO3M ~23ml 30x1cm

Re-purification of some fractiions



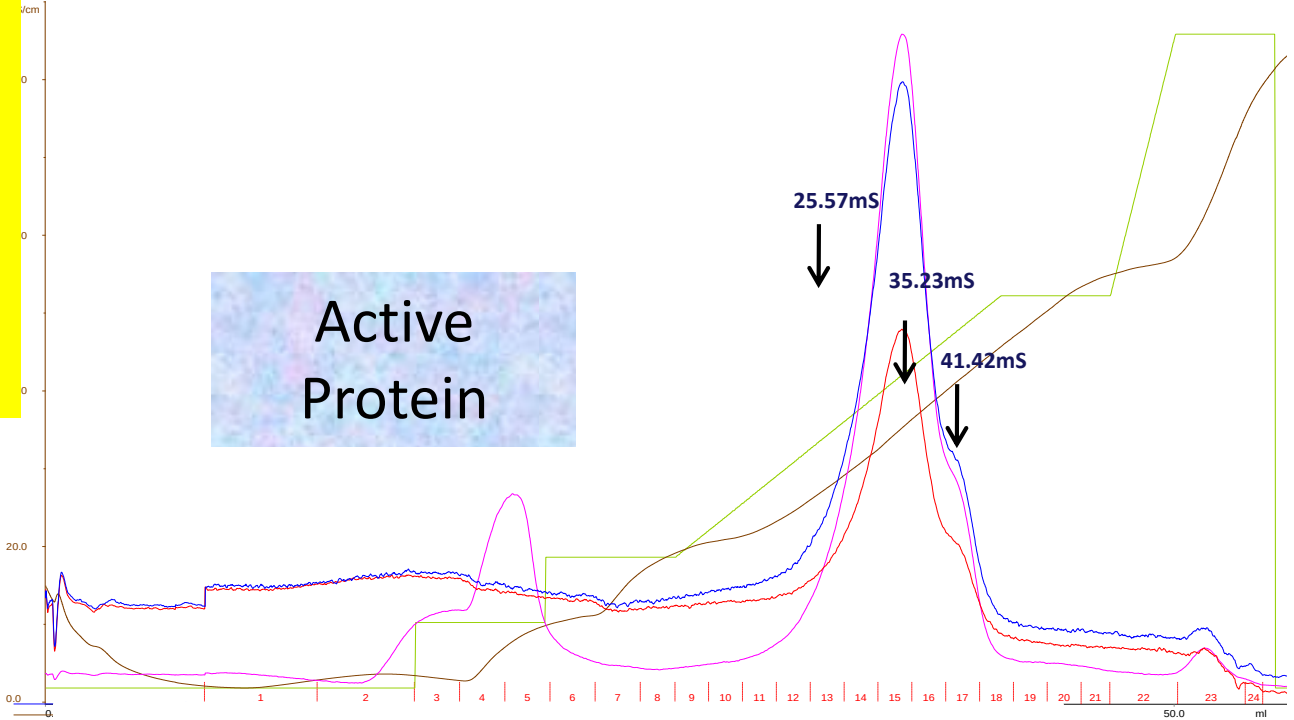


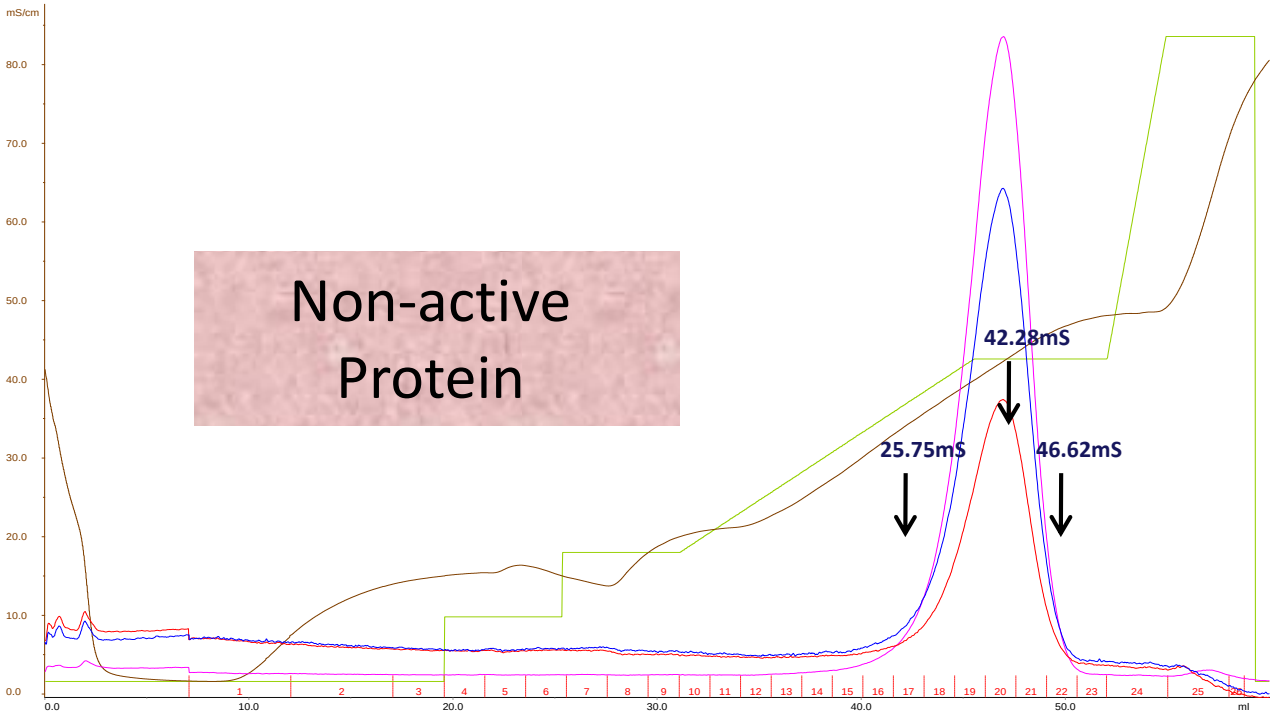


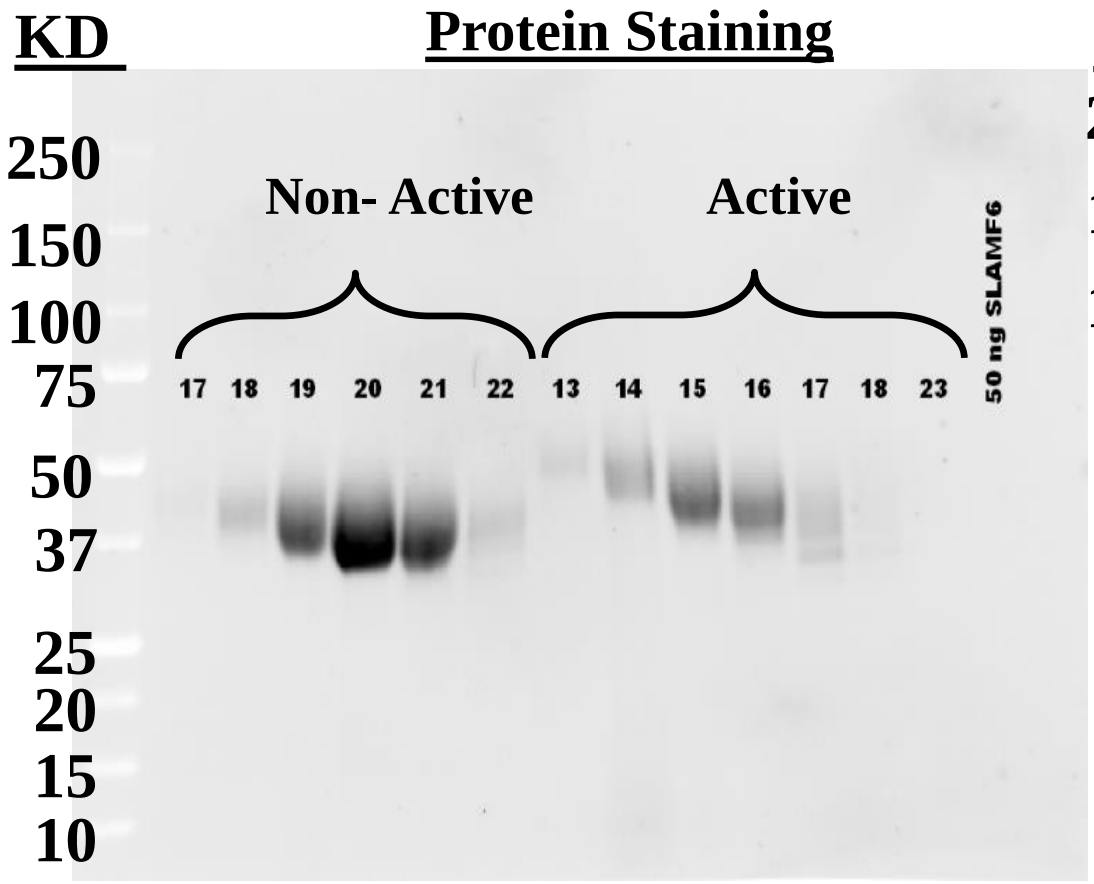
Reproducing irreproducible results
Case Study in Heterogeneus Glycosylation

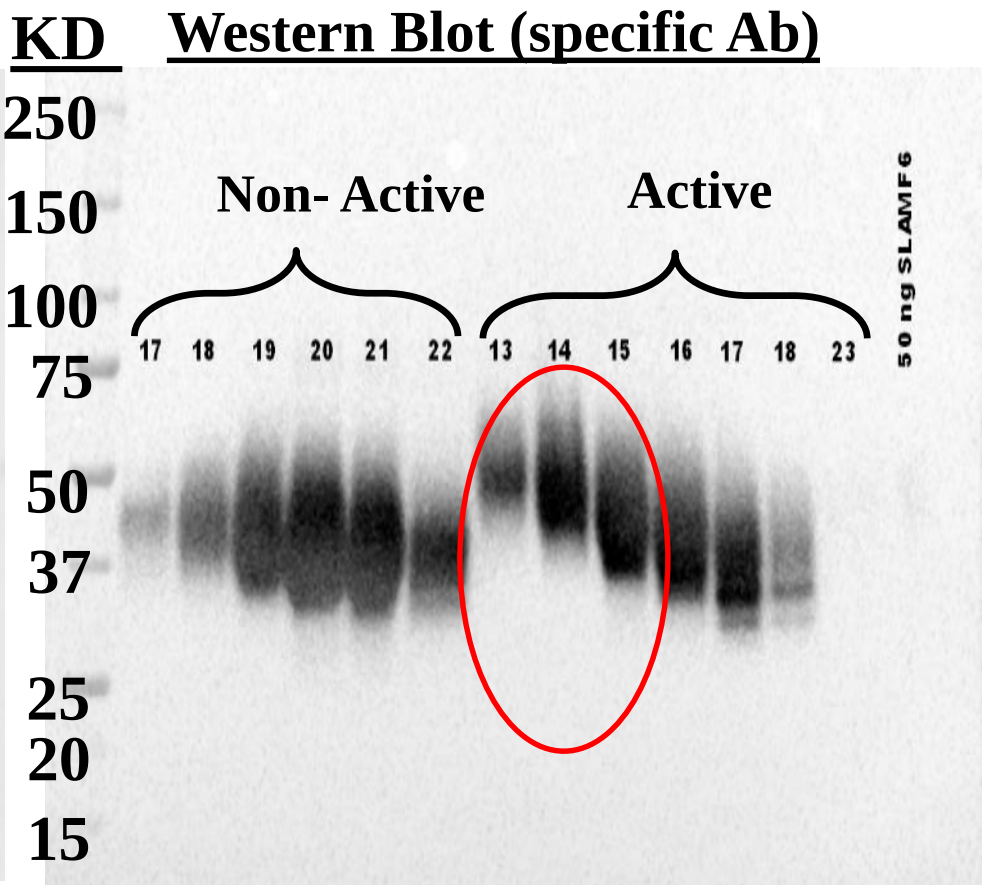
What happen when you use non-credible suppliers

Column: Hi Trap SP HP 1ml
Buffer A: 25mMNaAc pH 5.0
Buffer B: A + 1MNaCl
pI: ~ 6.0
Secreted HEK cells
Glycosilated



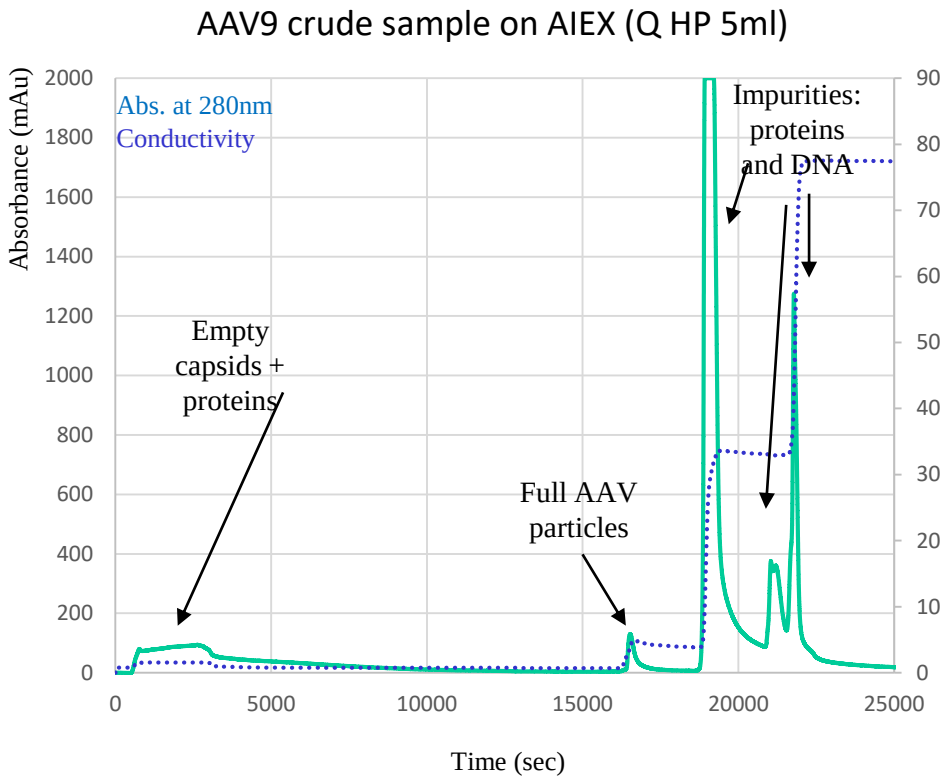



KD
Protein Staining
250
150
100
75
50
37
25
20
15
10
Non- Active
Active
17 18 19 20 21 22 13 14 15 16 17 18 23
50 ng SLAMF6

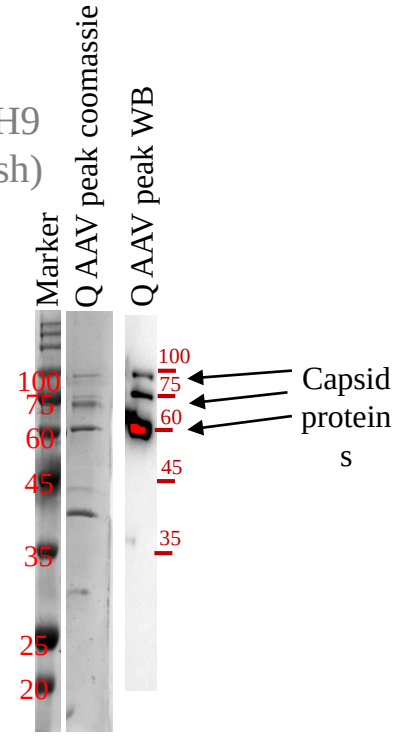

KD
Western Blot (specific Ab)
250
150
100
75
50
37
25
20
15
Non- Active
Active
17 18 19 20 21 22 13 14 15 16 17 18 23
50 ng SLAMF6

Adeno Associated Viruses (AAVs) purification

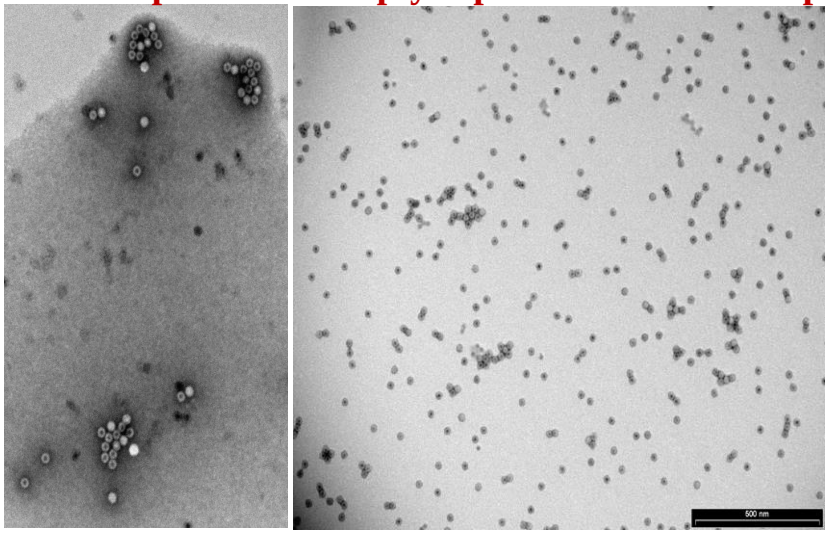
Separation of empty from full capsids by AEIX



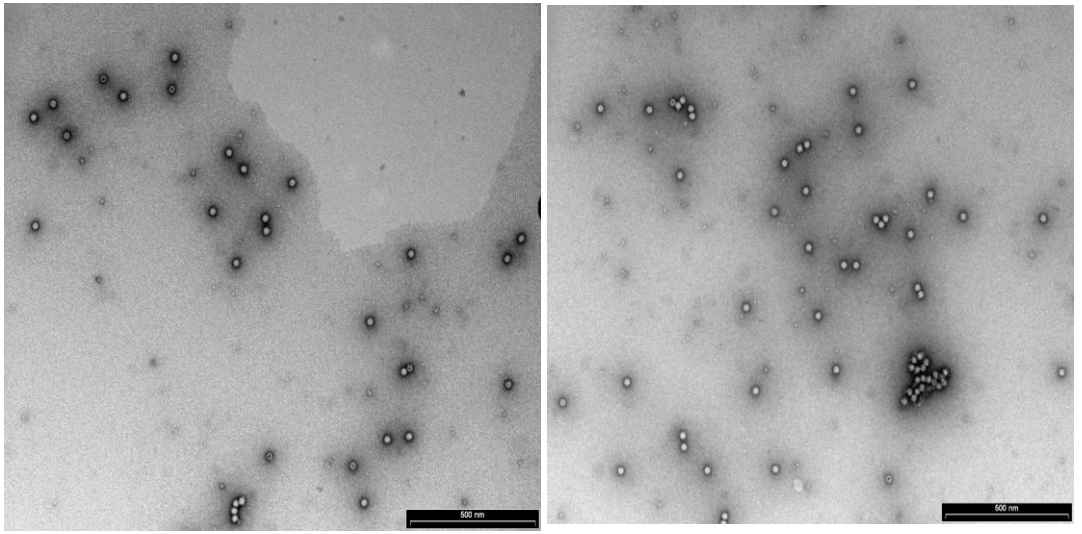
Elution buffers:
1- 0.5M NH_4Ac in Tris pH9
2- 1M NaCl (for final wash)



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

Troubleshooting - II

- **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.

Troubleshooting - III

- **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 10-15minutes, 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.

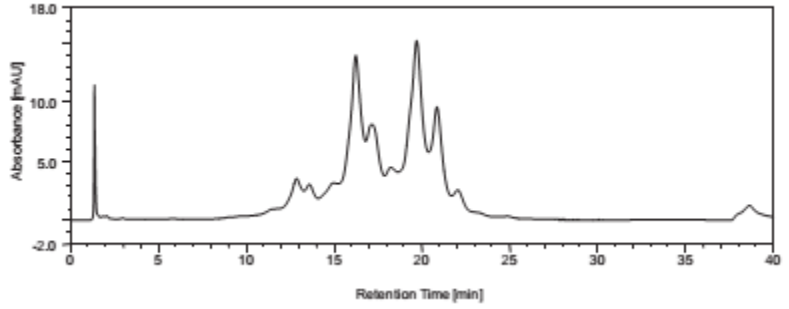
Summary

- 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, 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

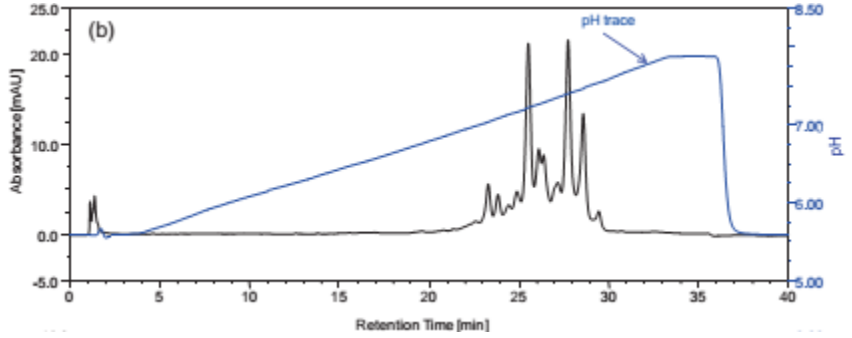
A Novel pH Gradient Separation Platform for Monoclonal Antibody (MAb) Charge-Variant Analysis

Shanhua Lin et al., Thermo Fisher Scientific

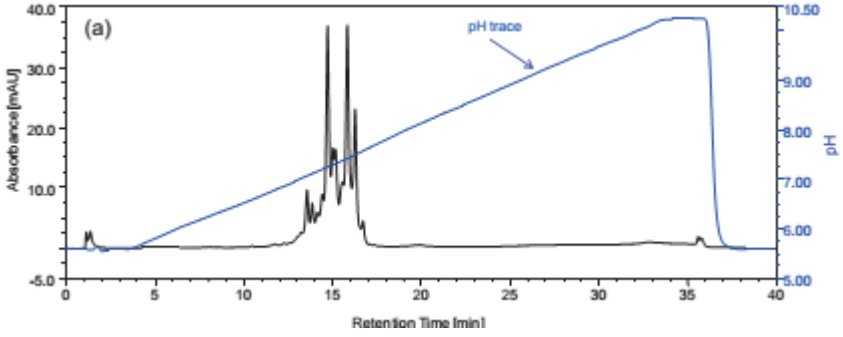
- ✓ pH gradient consisted of piperazine, imidazole, and tris, covering a pH range of 6 to 9.5
- ✓MAbPac SCX-10, 10 µm, 4 × 250 mm column
- ✓THERMO multicomponent zwitterionic buffer buffers: 0% B (pH 5.6) to 100% B (pH 10.2)



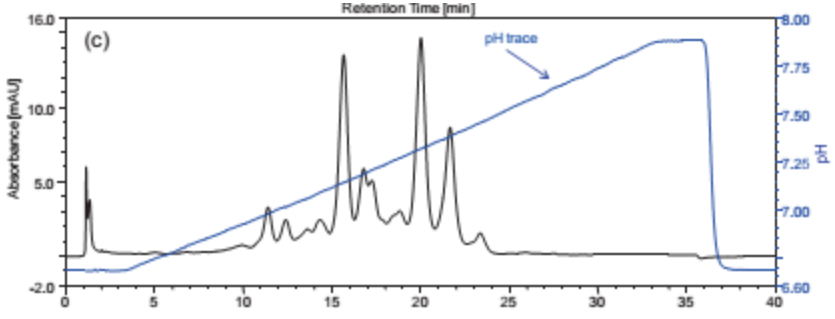
A : 20 mM MES and 60 mM NaCl (pH 5.6), and
B:20 mM MES and 300 mM NaCl (pH 5.6)



pH gradient, 0% B (pH 5.6) to 50% B (pH 7.9)



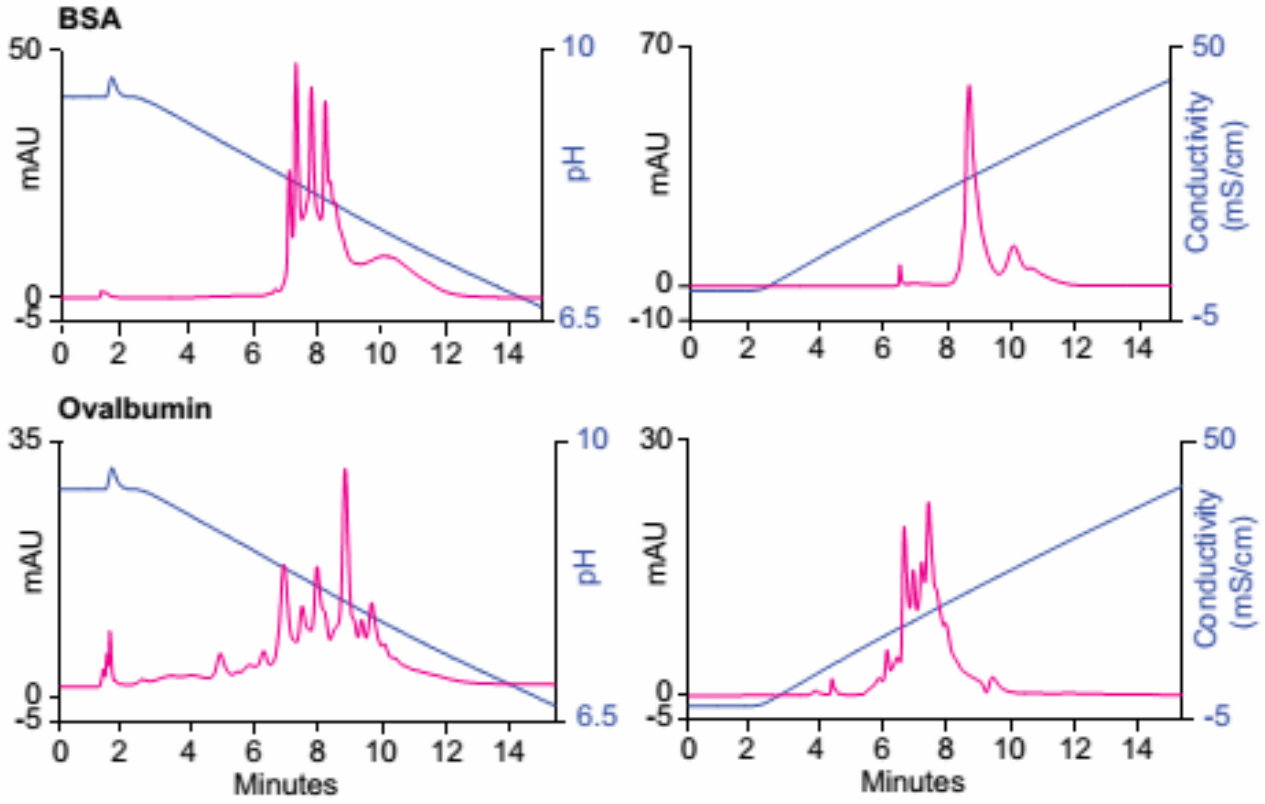
pH gradient, 0% B (pH 5.6) to 100% B (pH 10.2)



pH gradient, 25% B (pH 6.75) to 50% B (pH 7.9)

Comparision of pH Gradient and salt gradient

Shanhua Lin et al., Thermo Fisher Scientific



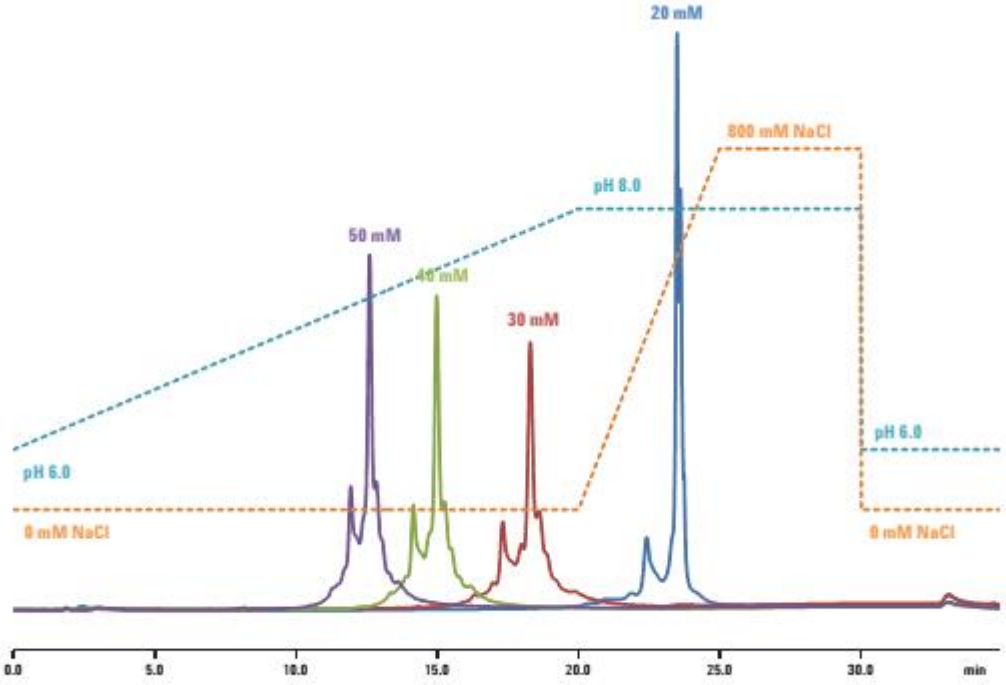
pH Gradient

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

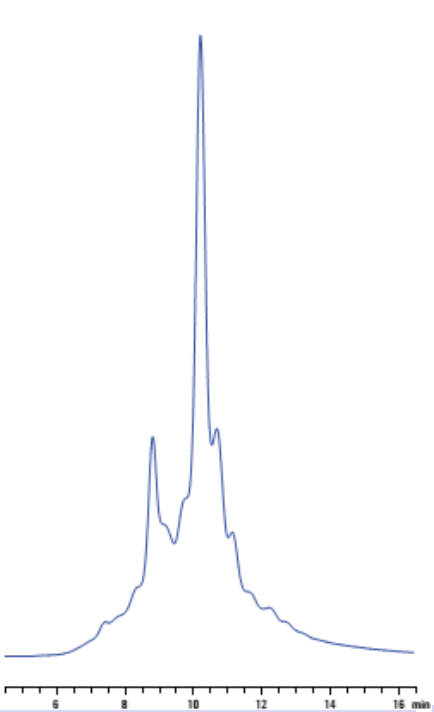
Salt Gradient

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

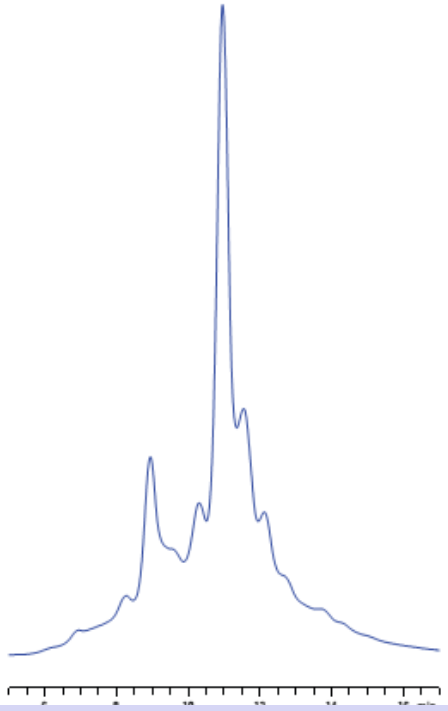
Thermo SCIENTIFIC



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.

Agilent Technologies

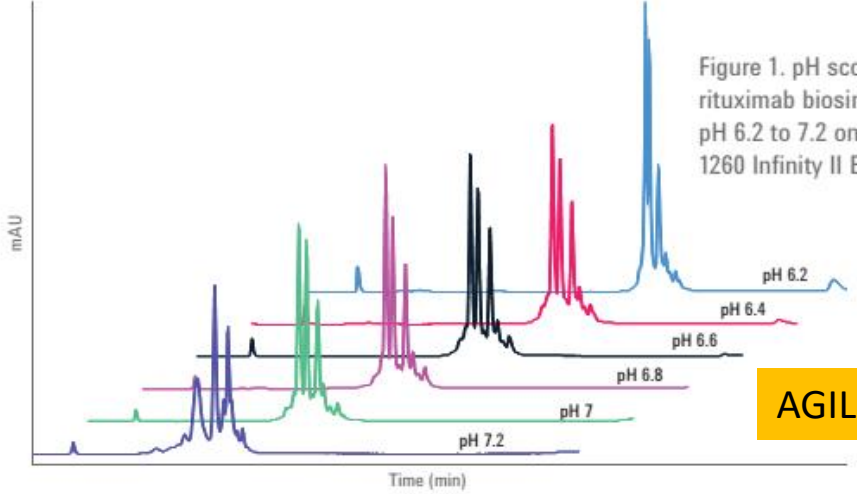


Figure 1. pH scouting using rituximab biosimilar from pH 6.2 to 7.2 on an Agilent 1260 Infinity II Bio-inert LC.

AGILENT

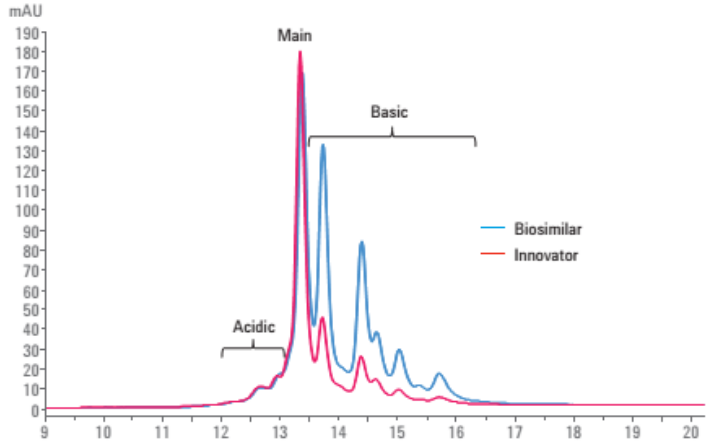


Figure 3. Direct overlay of the charge variant profiles of rituximab innovator and biosimilar on an Agilent 1260 Infinity II Bio-inert LC.

Buffer systems for pH gradient IEC

F. Kröner, J. Hubbuch / J. Chromatogr. A 1285 (2013) 78– 87

Substance	pKa_1	pKa_2	Conc.[mM]
AEC buffer pH 10.5–3.5			
Methylamine	10.75	–	9.8
1,2-Ethanediamine	9.93	6.99	9.1
1-Methylpiperazine	9.16	4.78	6.4
1,4-Dimethylpiperazine	8.15	4.04	13.7
Bis–tris	6.22	–	5.8
Hydroxylamine	5.67	–	7.7
Min ionic str. = 8.5 mM/max ionic str. = 104.3 mM			
CEC buffer pH 4.0–11.0			
CAPS	10.50	–	15.6
CHES	9.39	–	9.4
TAPS	8.44	–	4.6
HEPPSO	8.04	–	9.9
MOPSO	6.90	–	8.7
MES	6.10	–	11.0
Acetate	4.76	–	13.0
Formate	3.75	–	9.9
Min ionic str. = 8.3 mM/Max ionic str. = 89 mM			

Surface induced three-peak elution behavior of a monoclonal antibody during cation exchange chromatography

Guo J. et al. *J Chromatogr A* 2016 Nov 25; 1474:85-94 - **Giorgio Carta** - University of Virginia, Charlottesville, VA, USA

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

- **GE Healthcare. Ion Exchange Chromatography - Principles and Methods Handbook**
  https://cdn.gelifesciences.com/dmm3bwsv3/AssetStream.aspx?mediaformatid=10061&destinationid=10016&assetid=13101
- Jungbauer, A and Hahn, R. Ion-Exchange Chromatography - Methods in Enzymology, Volume 463- ISSN 0076-6879, DOI: 10.1016/S0076-6879(09)63022-6 (2009)
- Cummins PM, Dowling O, O'Connor BF. Ion-Exchange Chromatography: Basic Principles and Application to the Partial Purification of Soluble Mammalian Prolyl Oligopeptides. In: Walls D, Loughran ST. (ed.) Protein Chromatography Methods and Protocols. New York: Springer; 2011. p215-228.
- Kastner M. Protein Liquid Chromatography. Amsterdam: Elsevier Science; 2005
- Amartely, H., Avraham, O., Friedler, A., Livnah, O. & Lebendiker, M. Coupling Multi Angle Light Scattering to Ion Exchange chromatography (IEX-MALS) for protein characterization. *Scientific Reports* 8 (1), 6907, doi:10.1038/s41598-018-25246-6 (2018).
- Li, K. *et al*. High-resolution separation of homogeneous chito oligomers series from 2-mers to 7-mers by ion-exchange chromatography. *J. Sep. Sci.* **36**, 1275–1282 (2013).
- Pabst, T. M. *et al*. Separation of protein charge variants with induced pH gradients using anion exchange chromatographic columns. *Biotechnol. Prog.* **24**, 1096–1106 (2008).
- Quan, C. *et al*. A study in glycation of a therapeutic recombinant humanized monoclonal antibody: Where it is, how it got there, and how it affects charge-based behavior. *Anal. Biochem.* **373**, 179–191 (2008).
- Farnan, D. & Moreno, G. T. Multiproduct High-Resolution Monoclonal Antibody Charge Variant Separations by pH Gradient Ion-Exchange Chromatography. *Anal. Chem.* **81**, 8846–8857 (2009).
- Rea, J. C., Moreno, G. T., Lou, Y. & Farnan, D. Validation of a pH gradient-based ion exchange chromatography method for high resolution monoclonal antibody charge variant separations. *J. Pharm. Biomed. Anal.* **54**, 317–323 (2011).
- Cummins, P. M., Rochfort, K. D. & O'Connor, B. F. Ion-Exchange Chromatography: Basic Principles and Application. *Methods Mol. Biol.* **1485**, 209–223 (2017)