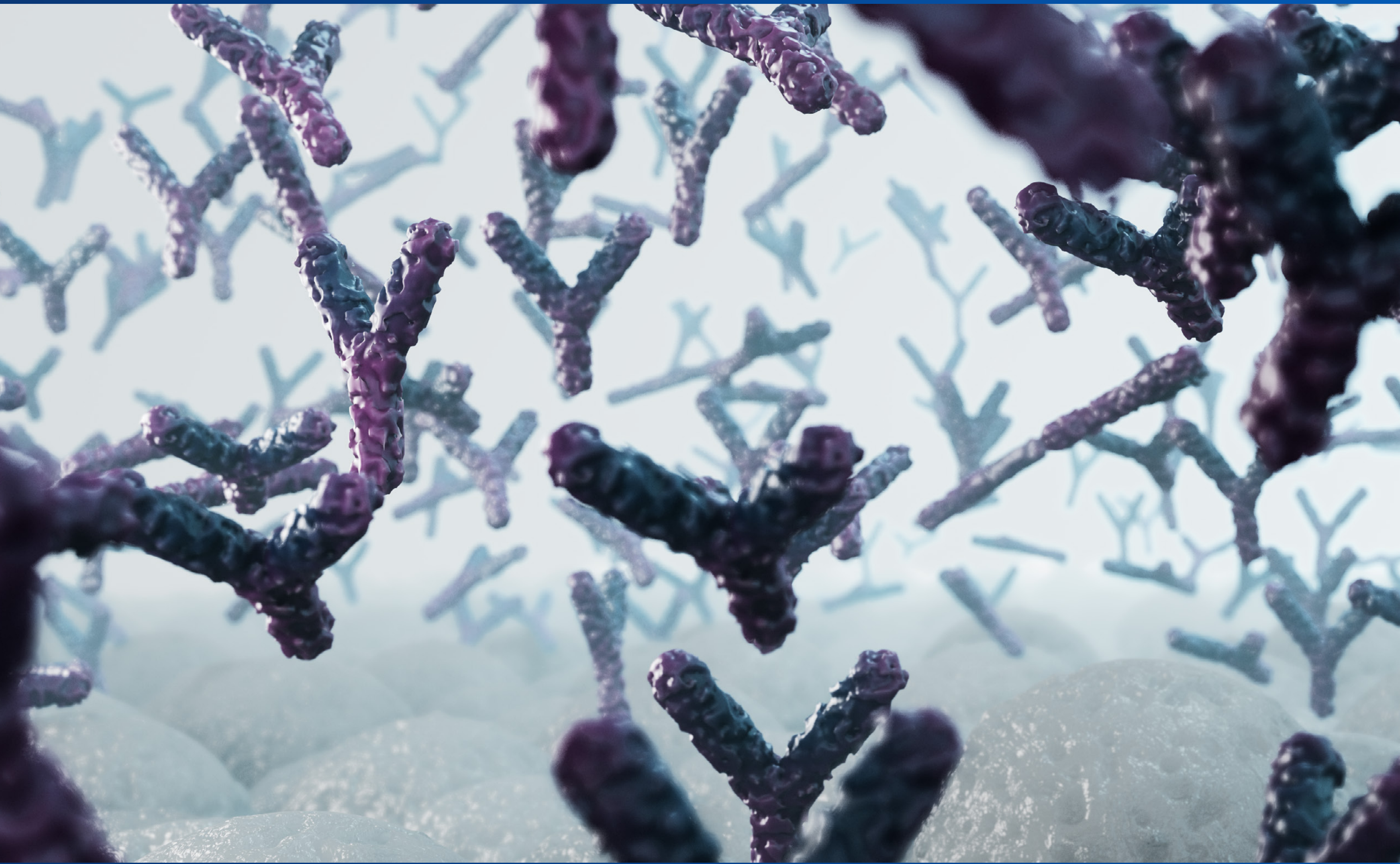
EDUCATIONAL GUIDE

Ion Exchange Chromatography



Contents

History of IEX	4
Understanding IEX	5
Manipulation of a Proteins Charge	6
Determination of pI	9
The Perfect Resin	9
Jetted Beads	10
IEX Ligands	12
IEX Useful at all Steps	13
Praesto IEX Beads	15
How to Perform Ion Exchange	16
Equilibration/Running Buffer	17
Sample Loading	19

Contents

Sample Stability 20

Binding Capacity of the Column 22

Wash and Elution 26

Effect of Column Length 28

Elution in Flow-through Mode 29

Tips and Tricks 30

History of IEX

Ion exchange (IEX) chromatography is a powerful technique that has been widely used for the separation and purification of proteins. IEX has been around thousands of years first being used to desalinate seawater. An early example comes from China (475-221 BC). Bamboo mats used for steaming rice, over time formed a thin outer layer, which functioned as an ion exchange for adsorbing salt and were used successfully for desalination.

The first modern ion exchange processes for water treatment were carried out using soil or minerals such as zeolites as stationary phases. In the 1940s and 1950s attempts were made to use IEX for the purification of proteins however, the hydrophobic nature of the resins prevented widespread use. A breakthrough in the mid-1950s by Sober and Peterson marked the beginning of the modern era of the IEX of proteins. They were able to synthesize ion exchange beads from cellulose, these beads were successfully used to fractionate plasma proteins. By the 1970s IEX was firmly established for the purification of protein including polyclonal antibodies. This provided a foundation for its application to monoclonal antibodies, and it evolved rapidly to become one of the dominant methods in the field.

IEX is consistently used for protein purification for several reasons, which include:

CHARGE PROPERTIES OF PROTEINS

Proteins have numerous ionizable groups, and their net charge can be predicted and manipulated based on the pH of the environment. This feature allows for selective binding of proteins to ion exchange resins under specific conditions, based on the isoelectric point (pI) of the proteins.

HIGH SELECTIVITY

This technique offers a high degree of specificity and selectivity because each protein has a unique distribution of charged amino acids and thus a unique isoelectric point at which the protein has no net charge. Adjusting the pH and ionic strength of the buffer can help fine-tune the chromatographic conditions to target specific proteins.

VERSATILITY

IEX can be used for both analytical and preparative purposes and successfully applied to a wide range of protein sizes, charges, and complexities. This makes it an indispensable tool that can be adapted for different purification strategies.

HIGH CAPACITY AND EFFICIENCY

Ion exchange resins typically have high protein binding capacities, allowing the purification of large amounts of protein using a relatively small volume of resin. The efficiency of separation is also generally very high, which is important for downstream processing.

COMPATIBILITY WITH BIOLOGICAL SAMPLES

IEX is normally conducted under conditions that are mild enough to maintain the biological activity of the proteins, which is preferential to other purification techniques that might denature the proteins, e.g., heat or organic solvents.

SCALABILITY

The process can be easily scaled from microgram quantities in a research lab to kilograms in industrial production without losing its effectiveness, which is crucial for the translation of laboratory methods to industrial applications.

COST-EFFECTIVENESS

Once established, ion exchange chromatographic protocols can be repeated many times with consistent results, and the resins used can normally be regenerated and reused, which makes the process more cost-effective for large-scale applications.

COMPLEMENTARITY WITH OTHER TECHNIQUES

IEX usually forms part of a multistep purification process and complements other chromatographic techniques such as size exclusion and affinity chromatography, thereby achieving a high level of purity.

Understanding IEX

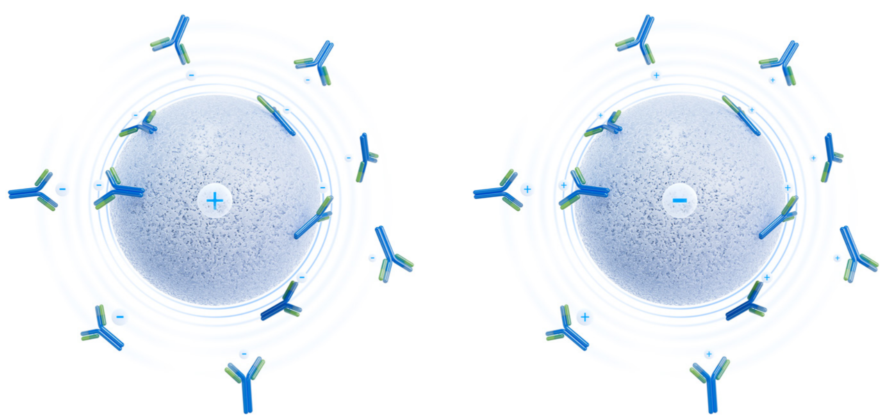
IEX chromatography involves the separation of molecules based on their charge.

IEX exploits the interaction between charged molecules in a sample and oppositely charged moieties in the stationary phase of the chromatography matrix. It is a form of adsorption chromatography. The stationary phase is composed of beads collectively called resin or matrix which have been derivatised with either positive or negative chemical ligands. These beads are packed into columns and sample pumped through. Figure 1 shows the 2 types of beads. Positively charged beads or anion exchangers bind and retard proteins with an overall negative charge. Positive molecules will be repelled and washed out of the column. Negatively charged or cation exchange beads bind to positively charged proteins with negative molecules being repelled and washed out of the column.

FIGURE 1

The 2 types of beads involved in IEX.

> To the left you can see a positively charged bead or anion exchanger which would bind to negatively charged proteins shown here using Y shaped antibody molecules, while to the right you can see a negatively charged or cation exchanger which would bind to positively charged proteins.



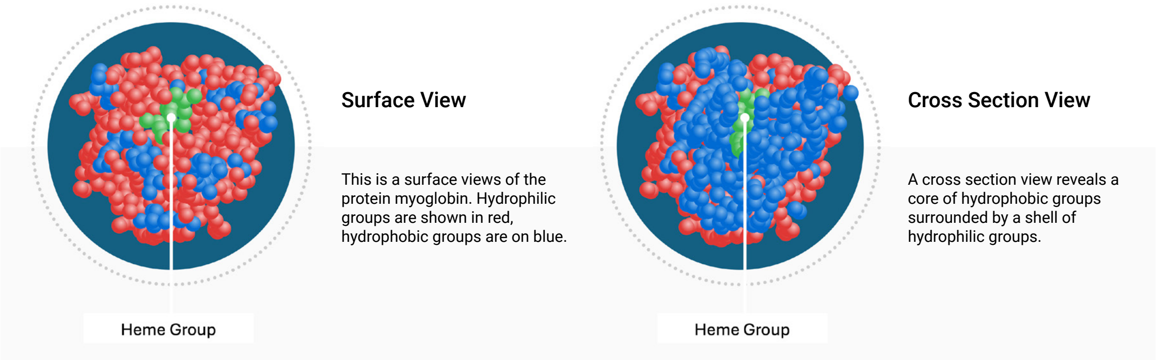
Anion Exchange Chromatography   Cation Exchange Chromatography

Most biomolecules consist of charged subunits, composed of amino acids or nucleotides, which allow them to interact to different degrees with an ion exchanger.

Lets think about this in context of proteins. A protein is a chain of amino acids all of which have different properties, some charged, some hydrophobic in nature. The shape of a protein is essential for function and is a direct result of how the chain of amino acids folds. For a soluble protein hydrophobic groups are mainly internal while ionic groups are external. You can see this in figure 2, the image on the right shows a cross sectional view of myoglobin, the core of the protein is dominated by hydrophobic residues (blue). In the image on the left, you can see a surface view of myoglobin. Here you can see many of the surface groups are charged (red). This surface charge and how this charge is distributed is utilised for IEX chromatography.

FIGURE 2

Myogoblin



The image on the right shows a cross sectional view of myoglobin, the core of the protein is dominated by hydrophobic residues (blue). The left image shows a surface view of myoglobin showing many of the surface groups are charged (red).

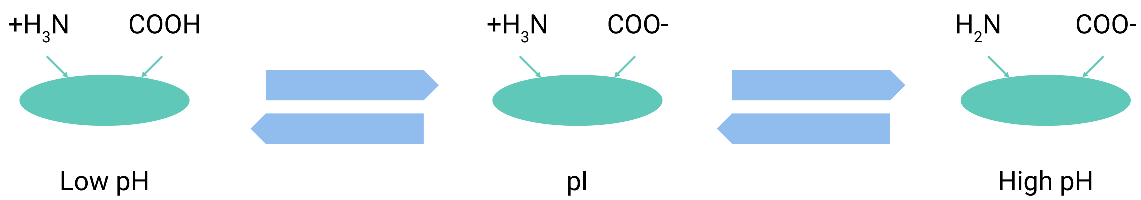
Manipulation of a Proteins Charge

pH is one of the most important factors in IEX as it determines both the charge on the IEX resin and the protein.

Proteins are amphoteric molecules called zwitterions which contain both positive and negative charges depending on the functional groups present in the molecule. They are affected by the pH of their surrounding environment and can become more positively or negatively charged due to the loss or gain of protons (H+).

Figure 3 shows the behaviour of the amino and carboxyl groups within a protein chain. As the pH decreases the amino group gains a hydrogen and thus carries a +ve charge. As the pH increases the carboxyl group loses a hydrogen and thus gains a negative charge. There is a pH between these 2 values where the sum of the positive charge equals the sum of the negative charge. Thus, the net charge is zero. This is called the Isoelectric point or pI.

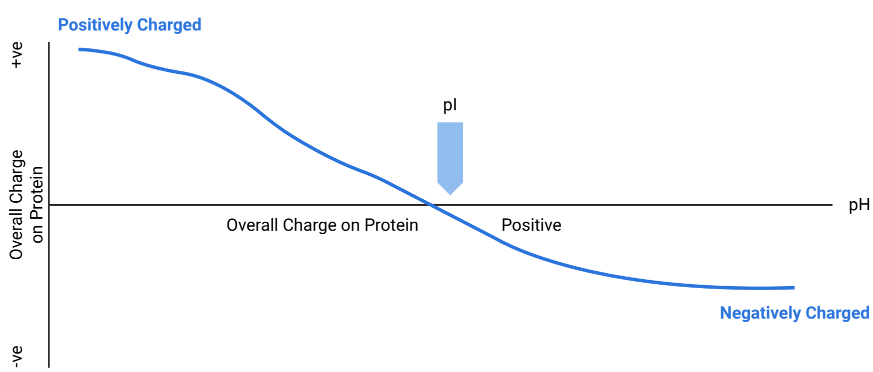
FIGURE 3



As the pH decreases the amino groups in an amino acid gain a hydrogen and thus carry a positive charge. As the pH increases the carboxyl group loses a hydrogen and thus gains a negative charge. There is a pH between these 2 values where the sum of the positive charges equals the sum of the negative charge. Thus, the net charge is zero. This is called the Isoelectric point or pI.

As a protein is titrated the net charge can be measured and plotted against pH resulting in a titration curve. As detailed in Figure 4. The pH value at which the protein has no net charge (pI) is equal to the intercept on the x-axis. At the pI, the protein is still charged, but the numbers of positive and negative charges are equal.

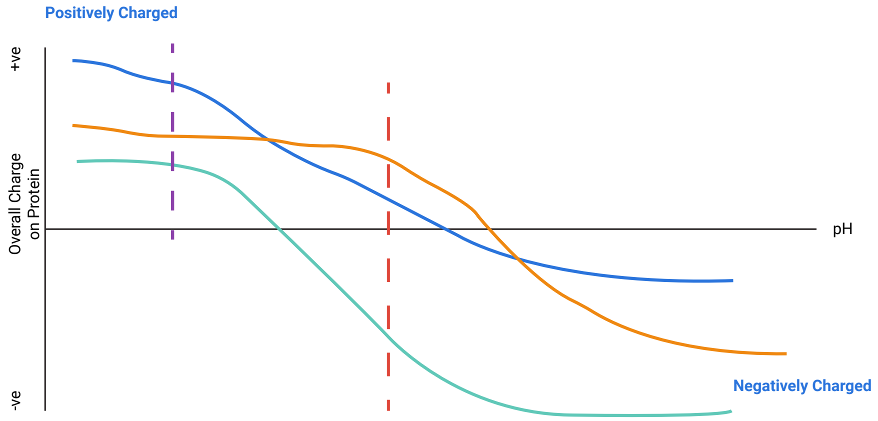
FIGURE 4



Theoretical titration curve of a protein, the net or overall charge on the protein is on the Y Axis while the pH is on the X Axis. The blue line shows change in net charge on the protein as the pH changes. The pH at which the protein has no net charge is the point at which the line crosses the x axis.

The difference in net charge is important in distinguishing the binding potential to an ion exchange matrix and the potential for separating the different proteins. As the pH of the solution moves further away from the pI the overall net charge is higher and results in stronger binding to an ion exchanger.

FIGURE 5



Theoretical titration curve of 3 proteins (orange, blue and green), the net or overall charge on the protein is on the Y Axis while the pH is on the X Axis. The orange, blue and green lines show the change in net charge on the orange, blue and green proteins as the pH changes. The pH at which the proteins have no net charge is the point at which the line crosses the x axis.

Since proteins differ in their amino acid composition, each protein has its own unique titration curve. The potential to separate proteins depends on the difference in net charge, the bigger the difference, the increased chance of separating them by ion exchange. This is shown in figure 5. 3 proteins orange, green and blue each have unique titration curves. If we ran cation IEX chromatography (negatively charged resin), equilibrating and loading the column using a low salt buffer with the pH indicated by the dashed purple line. All three proteins would carry positive charges and bind to the resin. During gradient elution (increasing salt concentration), the green protein would interact the weakest and elute first from the column early in the gradient (i.e. low salt concentration for elution) as it has the lowest overall positive charge. The blue protein would interact most strongly and elute last from the column as it has the highest overall positive charge. If we ran anion IEX chromatography (positively charged resin) using the same conditions just changing the pH of the buffers as indicated by the dashed red line the orange and blue proteins would carry positive charges and be repelled by resin and flow straight through the column during loading, while the green protein carrying a negative charge would bind and elute during the salt gradient. When considering the pH for your process it is normal to avoid the pI as no overall net charge can allow the protein to precipitate. Changing the pH is an extremely powerful way of controlling the selectivity in ion exchange.

Determination of pI

It is easy to work out the pI for an amino acid, it is the average pKa value. Every amino acid has 2 pKa values, one for the amino group which is around 10 and one for the carboxyl group which is around 3. Amino acids with an ionic side chain will have a 3rd pKa. In theory, to determine the pI of a protein you take the average value for all the amino acids it contains. There are three problems with this theory. The first is that the pKa for amino acids can change when in a protein structure. A good example of this is Aspartate 70 and Histidine 31 in the enzyme Lysozyme, the individual amino acids have an average pKa of 3.5-4 and 6.8. When folded in to the structure of lysozyme a salt bridge forms between the pair altering their pka to 0.5 and 9.8 respectively. The second problem is that some charged amino acids can be buried within he structure and thus wont affect the surface charge. This means that a theoretical pI generated on a PC based on the amino acid composition can be misleading and it is always best practice to generate a pI experimentally, one method would be to use an isoelectric focusing gel (IEF). There is one final complication is the distribution of charge may not be even over the proteins surface, there can be several zones of charge. This is why proteins can bind to an IEX column at their pI when the net charge is zero. Proteins that are unfolded (denatured) or have a difference in folding state can have different isolectric points due to different amino acids being brought to the surface. Post translational modifications such as glycosylation can also affect pI. Both of these can be exploited during purification.

The Perfect Resin

Chromatography resins can be made of various materials including cross-linked dextran, agarose, agarose/dextran, polystyrene, silica, divinyl benzene, glass or polyacrylamide. Resin material needs to be macro porous to give a large surface area and rapid kinetics and should be inert resulting in low non-specific protein interactions.

Being chemically resistant is important to allow cleaning, sanitization and re-use of the resin. Coming in a range of bead sizes gives flexibility when designing the process. Good quality control in manufacture gives batch to batch reproducibility to ensure the purification remains robust from lot to lot of resin. It is also important that the resin enables efficient mass transfer, this will allow sample to access the beads quickly without compromising structural integrity of the resin.

As the diameter of the column is increased the packed bed receives less support from the column wall. Most support is lost when going from 5 to 10cm diameter, but the support will continue to decrease with minimal incremental loss after 30cm diameter. As a result of this loss of support the flow rate may need to be decreased with wider column diameters. To negate this effect rigid resin, designed for production should be used, these allow direct scale up with maintained flow rates.

Agarose is an ideal chromatography material as it is very hydrophilic resulting in very little none-specific binding to the resin beads and is chemically and physically strong.

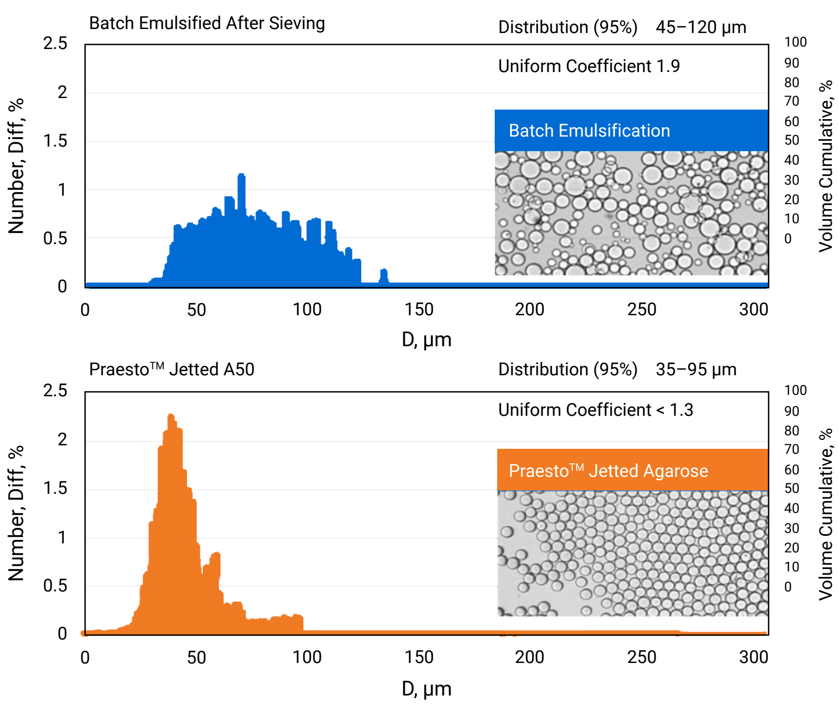
Resins designed for production are called bioprocess resins, they have the properties needed to run in large diameter columns, are available at scale and have the regulatory support information needed. Finally the manufacturer of the resin needs to be able to guarantee security of supply, have a business continuity program, have manufacturing knowledge and support for biopharmaceutical industry.

Jetted Beads

The method of production of a resin bead will affect the properties of the chromatography column. The traditional method of agarose bead production is batch emulsification, this involves dissolving the agarose in solvent, stirring to reduce the particle size followed by extensive sieving of the resultant beads to narrow the size range distribution.

Purolite use a proprietary method of agarose bead production called Jetting, which produces beads with a much narrower size range distribution. This process uses no solvents to dissolve the agarose, does not involve the extensive sieving process which results in a greater yields and less waste.

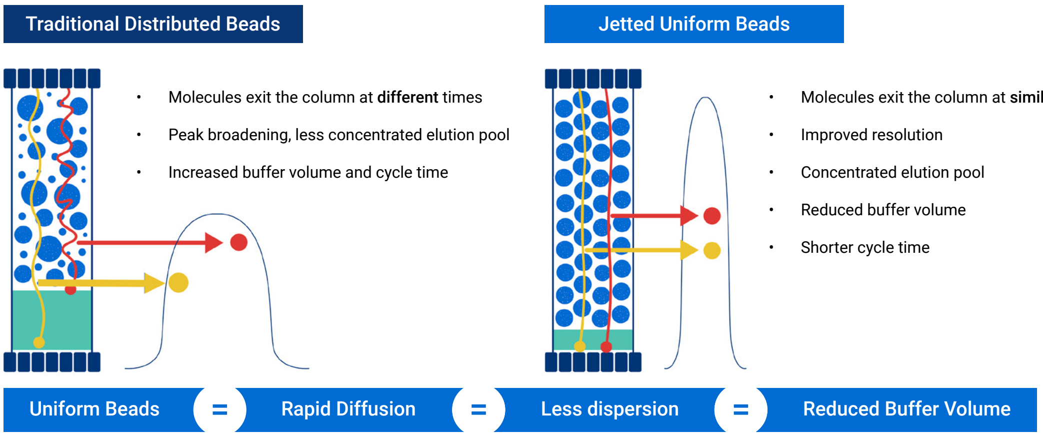
FIGURE 6



The top panel shows the average bead size distribution for a 95μm resin produced by batch emulsification. A 95μm bead will have on average 45–120μm range when produced this way. A microscope image of the beads are shown. The lower panel shows the same information for a 50μm resin produced by Jetting, you can clearly see the narrower size distribution range resulting from Jetting both in the graph and microscope image.

The narrower bead distribution gives a resin that is easy to pack in to columns, resulting in a homogenous packed bed with less opportunity to have significant voids or channeling. The path of the target molecule or buffer solutes is very similar with less potential for mixing/dispersion, resulting in greater efficiency and sharp elution peaks. It also means rapid buffer transitions which can facilitate optimization of washes and reduced buffer consumption and cycle times. The features combined result in higher resolution, especially with gradient elution, less band broadening so sharper more concentrated peaks and high capacity with faster mass transfer. The fast mass transfer and diffusion kinetics can translate to efficient optimization of the non-load wash steps resulting in a reduction of overall buffer use, time savings and productivity gains. These properties are key to SMART cycling.

FIGURE 7



The left-hand panel shows the properties of a column packed with beads made the traditional way using batch emulsification. The right-hand panel shows the properties of a column packed with Jetted beads.

Differences in bead composition, bead size and pore size can give rise to differences in resin compressibility, chemical resistance, permeability, available surface area, and mass transfer properties. For example, increasing the pore sizes can increase mass transfer (i.e. transfer of target to the ligand), and improve flow properties, however if you increase the pore size while maintaining the bead dimensions the surface area will decrease, meaning the binding capacity will decrease. This can be compensated for by increasing the ligand density. The ligand density used for ion exchange resins is normally higher than that used for other chromatography techniques. Variation in the density (or ionic capacity) can impact the performance, which is why it is important the resin supplier has tight controls over the specification and release of their resin post manufacture.

Selection of the resin type is application specific and depends on the best compromise between capacity, product purity, and throughput as determined by the resin flow-characteristic.

IEX Ligands

Ion exchange ligands are the charged groups added to the resin beads. Ion exchangers are usually classified as weak and strong. Examples of strong ligands are Q (Quaternary amine, positive charge) and SP (Sulfopropyl, negative charge). Examples of weak ion exchange ligands are DEAE (Diethylaminoethyl, positive charge) and CM (Carboxymethyl, negative charge).

FIGURE 8

Anion Exchangers

Strong:	Quaternary Amine (Q) (Q) $-CH_2N^+(CH_3)_3$
Weak:	Diethylaminoethyl (DEAE) $OCH_2CH_2N^+(CH_2CH_3)_2$

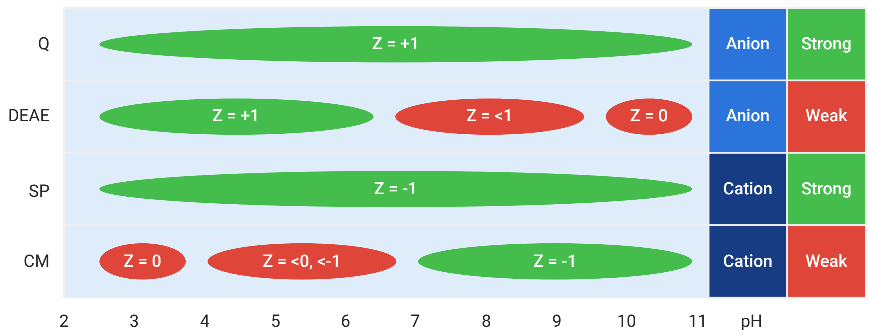
Cation Exchangers

Strong:	Sulfopropyl (SP) $CH_2CH_2CH_2SO_3^-$ Sulfonate (S) $CH_2SO_3^-$
Weak:	Carboxymethyl OCH_2COO^-

Examples of strong and weak ion exchange ligands that can be attached to chromatography beads to give ion exchange resins.

The labels weak and strong refers to the pKa valves of their charged groups, it does not mean the strength at which they will bind proteins. The number of charged groups is called the z valve, this is important as it shows the pH at which the ion exchanger will be fully charged. Figure 9 shows the z values vs pH for strong and weak ion exchangers. For anion exchangers with a full charge the Z value equals +1, for cation exchangers with a full charge the Z value will equal -1. Strong ion exchange ligands have full charge over a wide pH range, this ensures the ion exchanger has maximum capacity regardless of pH. Therefore, experiments are more predictable and controllable. Strong ion exchangers can't act as buffers, so they are faster and easier to equilibrate. This is the reason it is recommended to start with strong ion exchangers. Weak ion exchangers are not fully charged over the whole pH range, and as such would be second choice, however they can give alternate selectivity which may be useful with tricky purifications.

FIGURE 9



Z charge for strong and weak ion exchange ligands over a range of pHs.

IEX Useful at All Steps

A purification procedure can be considered as 3 steps. The first being 'capture', speed is vital here as you need to isolate your target molecule from a potentially harmful environment as fast as possible. Capacity is also important at this stage as you will have the highest protein concentration. The aim is to isolate concentrate and stabilise your protein. Capture steps are typically carried out in bind and elute mode, which isolates the target molecule from bulk contaminants concentrating and stabilizing the protein.

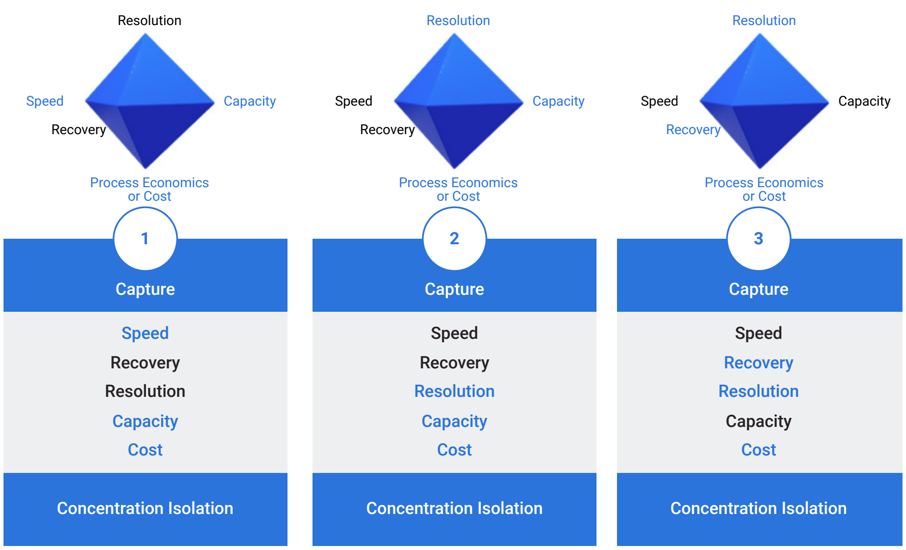
Larger resin beads (90–50µm) are normally used in the capture step. This step has the largest volume of feed (sample) and impurities and is often more viscous. At this early stage in the process the column will be exposed to extensive fouling and clogging due to the crude nature of the feed. Fouling and clogging can cause serious problems with high back pressure and reduced flow rates and may even completely block the flow through the column. Extensive cleaning, repacking, and replacing of resin will also give increased costs. To prevent this, a large particle size should be used with this kind of feed material. Large particle size means that higher flow rates can be used without excessive increase in back pressure. High flow rate means high throughput.

The second step intermediate purification' removes the bulk of the impurities, capacity is still a concern however, speed now becomes less important with resolution taking centre stage. A smaller bead at this stage may give an increase in dynamic binding capacity due to an increase in surface area and higher resolution, due faster mass transfer. The optimal bead size at this stage will depend on the nature of the feed (how clean it is), the extent of the separation problem and the scale of work, typically it will be between 35–90µm.

The final 'polishing' step separates out molecules closely related to your target; thus resolution is still essential. Polishing also removes trace contaminants which can impact the safety of the product, for example leached affinity ligand, endotoxin and host cell DNA. Capacity takes a back seat now with recovery and purity being more important. Bead sizes range from 35–65μm. The costs associated with using small beads (price, back pressure) will be less pronounced here since the scale of work has been reduced at this stage. Fouling and clogging of columns is also less of a problem since the sample will be cleaner. Polishing also removes trace contaminants which can impact the safety of the product, leached affinity ligand, endotoxin and host cell DNA.

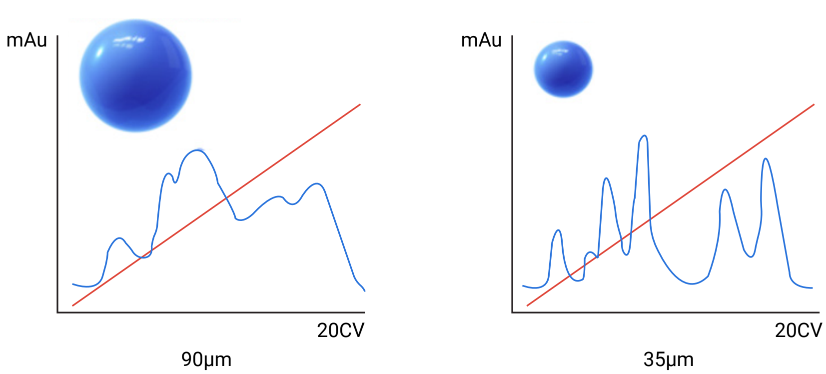
FIGURE 10

3 Step Purification Scheme



Capture, Intermediate Purification and Polishing. The performance parameters affecting each step are shown in the grey area and visually on the diamond. The aim of each step is shown in the lower light green box.

FIGURE 11



Both chromatograms show separation using an ion exchange column, the running conditions and sample are the same for the 2 runs except for the bead size of chromatography resin. The left hand uses 90um beads and the right 35um. The smaller bead gives increased resolution due to faster mass transfer and higher efficiency.

Praesto IEX Beads

Praesto Jetted beads have an advanced high flow, highly cross-linked agarose base matrix and are manufactured with a porosity and pore structure ideal for high performance protein chromatography. Praesto Q is a strong anion exchanger with a quaternary ammonium functional group. Praesto SP has a sulfopropyl functional group and is a strong cation exchanger. Strong ion exchange ligands maintain charge and subsequent capacity over a wide pH range.

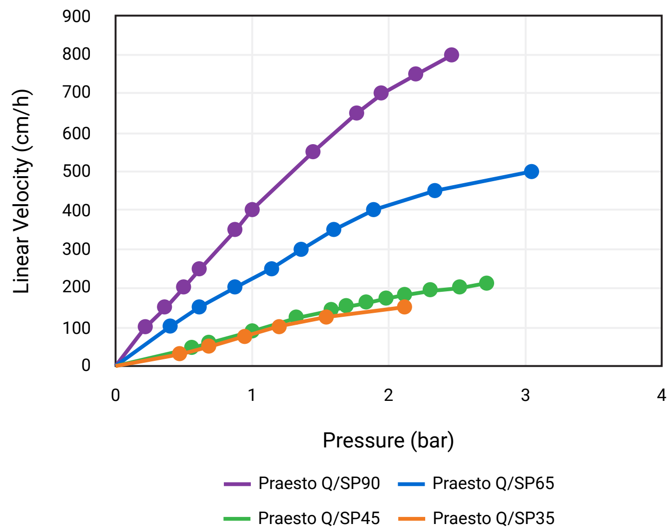
Purolite have 4 size beads for IEX: 90, 65, 45 and 35µm. Figure 13. The larger beads give the ability to flow raster with reduced pressure and are excellent for a capture step, the smaller beads have faster mass transfer and higher efficiency thus give improved resolution. Smaller beads will give higher capacity than larger beads, typically due to increased surface area, however the pressure will increase and thus the flow decreases. The porosity and ligand density is the same on all the bead sizes which means you will get the same selectivity on all 4. Why is this an advantage? Timesaving in your optimisation work is the key benefit, as all the beads have the same selectivity you know if you increase bead sizes you can flow faster and if you decrease you can get better resolution with no re-optimization for selectivity required. We have the strong cation ligand SP and strong anion ligand Q on all 4 bead sizes.

FIGURE 12

Purolite have 4 size beads for IEX: 90, 65, 45 and 35µm. The larger beads give the ability to flow raster with reduced pressure and are excellent for a capture step, the smaller beads have faster mass transfer and so give improved resolution. The porosity and ligand density is the same on all the bead sizes which means you will get similar selectivity on all 4. Figure 14 shows the pressure flow of the 4 bead sizes in a 20cm bed height column. The larger beads give the ability to flow raster with reduced pressure and are excellent for a capture step, the smaller beads have faster mass transfer and so give improved resolution.

FIGURE 13

Packed bed pressure flow curves for Praesto Q/SP90 (20 cm ID), Praesto Q/SP65 (20 cm ID), Praesto Q/SP45 (20 cm ID) and Praesto Q/SP35 (20 cm ID) at a 20 cm bed height in 0.1 M NaCl at 20 °C.

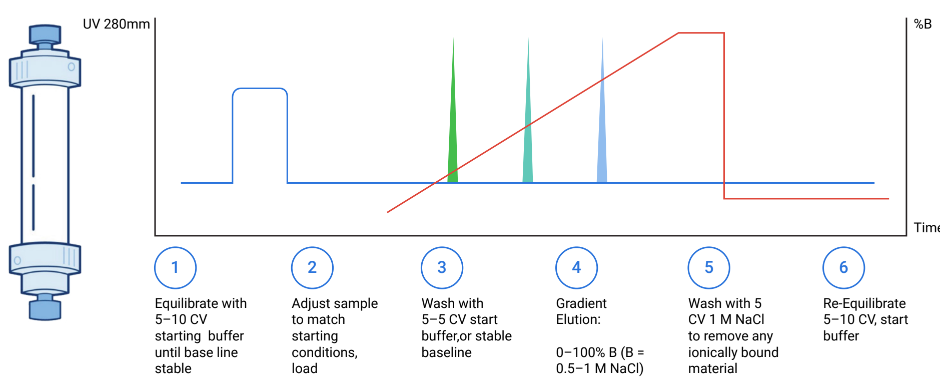


How to Perform Ion Exchange

Figure 14 shows a typical ion exchange run. The column is first equilibrated using a start buffer which contains low salt for 5–10 column volumes or until UV pH and conductivity baselines are stable. The sample is loaded. It is volume independent, as it is an absorption technique. The loading limit is dictated by the specific binding capacity of the resin. The sample should be adjusted to match the starting pH and ionic strength of the equilibration buffer prior to loading. Once loaded the column is washed with 5–10 column volumes of equilibration buffer or until the baseline, is stable i.e. when all unbound material has washed through the column. Elution is achieved using a gradient of 10–20 column volumes with an increasing ionic strength up to 1.0 M, typically using NaCl. The column is washed with the high salt buffer to elute any remaining ionically bound material before re-equilibration with 5–10 column volumes of start buffer or until pH and conductivity reach the required values.

FIGURE 14

The steps in an IEX run.



Equilibration/Running Buffer

As we have discussed pH is essential for ion exchange, thus the buffer chosen is critical to success, as it must be able to maintain the chosen pH. Figure 16 and 17 show the recommended buffers for anion and cation exchange. The usable pH range is approx. 0.5 units above and below the pKa for value of the buffer. Looking at Tris the Pka is 8.07 — so the recommended range of pH for this solution is 7.6-8.6. pKa can varies with temperature. For Tris the variation is about -0.028/degree — thus a tris buffer prepared at room temp has about 0.05 units lower pH in a cold room. The pH will also change when adding salt to a buffer, due the large increase in ionic strength, for this reason the solution should be at the temperature of use and contain the final salt concentration prior to adjusting the pH.

FIGURE 15

Buffers for anion exchange chromatography.

pH Interval	Substance	Conc. (mM)	Counterion	pKa (25 °C)	d(pKa)/dT (°C)
4.3–5.3	N-Methylpiperazine	20	Cl^-	4.75	-0.015
4.8–5.8	Piperazine	20	Cl^- or $HCOO^-$	5.33	-0.015
5.5–6.5	L-Histidine	20	Cl^-	6.04	
6.0–7.0	bis-Tris	20	Cl^-	6.48	-0.017
6.2–7.2; 8.6–9.6	bis-Tris Propane	20	Cl^-	6.65; 9.10	
7.3–8.3	Triethanolamine	20	Cl^- or CH_3COO^-	7.75	-0.020
7.6–8.6	Tris	20	Cl^-	8.07	-0.028
8.0–9.0	N-Methyldiethanolamine	20	SO_4^{2-}	8.52	-0.028
8.0–9.0	N-Methyldiethanolamine	50	Cl^- or CH_3COO^-	8.52	-0.028
8.4–9.4	Diethanolamine	20 at pH 8.4 50 at pH 8.8	Cl^-	8.88	-0.025
8.4–9.4	Propane 1,3-Diamino	20	Cl^-	8.88	-0.031
9.0–10.0	Ethanolamine	20	Cl^-	9.50	-0.029
9.2–10.2	Piperazine	20	Cl^-	9.73	-0.026
10.0–11.0	Propane 1,3-Diamino	20	Cl^-	10.55	-0.026
10.6–11.6	Piperidine	20	Cl^-	11.12	-0.031

[1] Ref: *Handbook of chemistry and physics, 83rd edition, CRC, 2002 to 2003.*

FIGURE 16

Buffers for cation exchange chromatography

pH Interval	Substance	Conc. (mM)	Counterion	pKa (25 °C)	d(pKa)/dT (°C)
1.4–2.4	Maleic Acid	20	Na^+	1.92	
2.6–3.6	Methylmalonic Acid	20	Na^+ or Li^+	3.07	
2.6–3.6	Citric Acid	20	Na^+	3.13	-0.0024
3.3–4.3	Lactic Acid	50	Na^+	3.86	
3.3–4.3	Formic Acid	50	Na^+ or Li^+	3.75	-0.0002
3.7–4.7; 5.1–6.1	Succinic Acid	50	Na^+	4.27; 5.64	-0.0018
4.3–5.3	Acetic Acid	50	Na^+ or Li^+	4.75	-0.0002
5.2–6.2	Methylmalonic Acid	50	Na^+ or Li^+	5.76	
5.6–6.6	MES	50	Na^+ or Li^+	6.27	-0.0110
6.7–7.7	Phosphate	50	Na^+	7.20	-0.0028
7.0–8.0	HEPES	50	Na^+ or Li^+	7.56	-0.0140
7.8–8.8	BICINE	50	Na^+	8.33	-0.0180

[1] Ref: *Handbook of chemistry and physics, 83rd edition, CRC, 2002 to 2003.*

An initial buffer concentration between 20 and 50 mM is recommended to give good buffering capacity. Higher buffer concentrations in the starting buffer can sometimes inhibit binding.

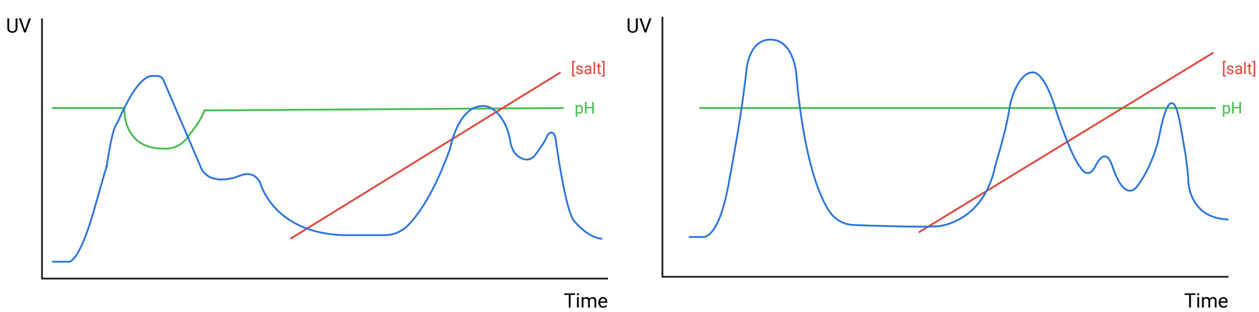
As you can see from the buffer tables figure 15 and 16 anionic buffers should be selected for cation exchange and cationic buffers for anion exchange. This ensures that the buffer and resin have the same charge. If this is not adhered to the buffer will bind to the ion exchanger depleting the solution and its buffering capacity. Upon elution the buffering ion will elute and create a ghost peak. An exception to this rule is seen in the frequent use of phosphate buffers with anion exchange separations. Phosphate buffers must be carefully prepared to ensure reproducibility between batches. When IEX is scaled up, economic issues, and others such as availability, environmental and disposal concerns must also be considered.

For anion exchange chromatography, a starting pH is selected, which is just slightly above the value where the target protein is bound. Choosing a higher pH will increase the strength of binding but will also allow more impurities to bind, thus reducing the available capacity for the component of interest. The converse is true for cation exchange. A starting pH is selected, which is just slightly below the value where the target protein is bound. Increasing the strength of binding may help resolve early eluting components in the subsequent salt gradient. Decreasing the strength of binding may help resolve components which elute later in the gradient. This is valid for both types of ion exchangers.

Sample Loading

It is important to adjust the pH and salt concentration of the sample prior to loading on the column. Figure 17 shows possible consequences: components of the sample that should have bound to the column may be washed out during sample load and initial column wash.

FIGURE 17



Both chromatograms show purification by ion exchange, in the left-hand chromatogram the sample pH was not adjusted to match that of the starting buffer — you can see the dip in pH as the sample loads and a large amount of sample is not bound to the column during loading. The right-hand chromatogram shows the performance of the column when the sample was adjusted before loading.

A similar result may be observed if the salt concentration of the sample is not adjusted to match the start conditions. If the salt concentration of the sample is at or above the salt concentration necessary for elution, the sample will not bind.

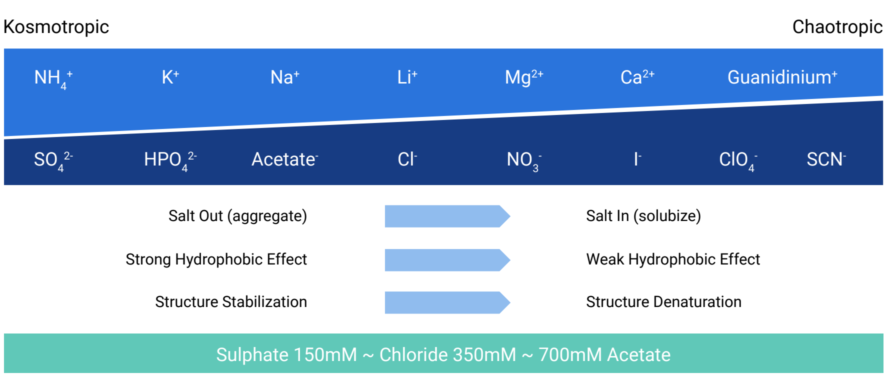
Buffer exchange (dialysis, diafiltration or desalting) is commonly used to adjust the sample buffer prior to loading on the column. Sample dilution is often an easy, effective way to decrease the ionic strength of a sample.

The sample buffer condition is vital for sample volumes greater than the column volume, as the column will be re-equilibrated in the sample buffer during sample application. For small sample volumes (less than 10% column volume) the buffering capacity of the mobile phase can compensate for small variations in the sample buffer.

Sample Stability

It is important to know the limits of pH and salt concentration under which your protein is stable as this will define the conditions viable for your purification. There are many ways to do this, the simplest is to measure the degree of precipitation at 600 nm using a range of conditions. From this you will see a window of operation where your protein remains in solution. Figure 18 Shows the Hofmeister series which is a classification of ions in order of their lyotropic properties, which is the ability to salt out or salt in proteins. The salts to the left of the figure have greater salting out strength that those on the right. If you find your protein is salt sensitive, you can use a stronger salt (one further to the left in the series) at a lower concentration this will give the same power for elution whilst taking into consideration that the target molecule is sensitive to higher salt concentrations. Similar elution strengths are given by 150 mM sulphate SO3(2-) and 350 mM chloride (Cl(-)).

FIGURE 18



The Hofmeister series or lyotropic series is a classification of ions in order of their lyotropic properties, which is the ability to salt out or salt in proteins. The salts to the left of the figure have greater salting out strength that those on the right.

The initial pH for your IEX test run must fall within the window of operation. If you are unable to run an IEF gel to experimentally determine the pI or you know you have a complex molecule that has uneven charge distribution, then a simple method to determine the pH value at which the target protein binds to an ion exchanger is shown in figure 19. A small amount of resin is added to several wells in a 96 well plate. Buffers with different pHs are added to each well along with sample. The plates are shaken on an orbital shaker for 30 min and then spun to pellet the resin. The supernatant is analysed for sample. Screening can also be carried out using small volumes of resin in a tip based format such as IMCS (read more here). The pH at which the target molecule is no longer in the supernatant can be used as the initial binding pH.

FIGURE 19

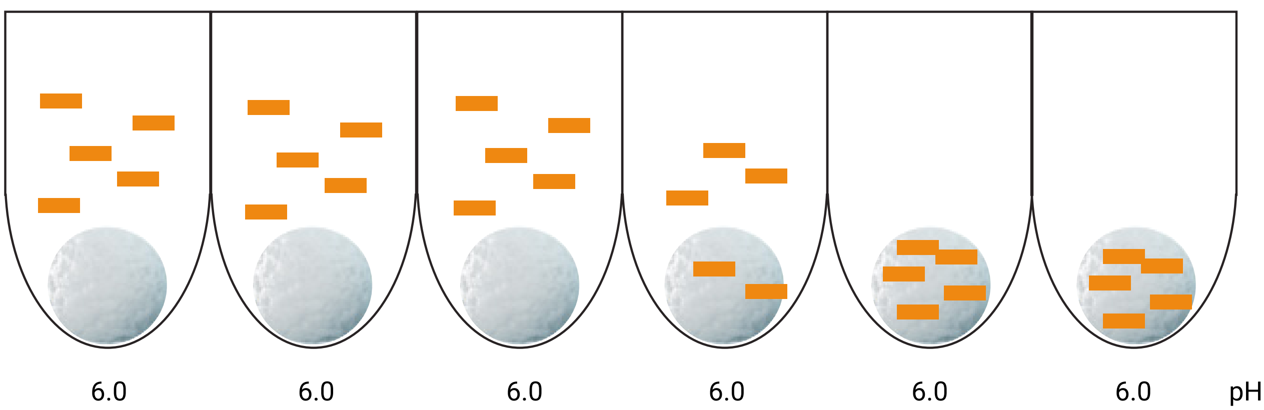


Plate method to determine the best pH for binding. 50ul of Praesto Q65 anion exchange resin is added to several wells in a 96 deep well plate. 1ml of buffer with different pHs is added to each well along with sample. The plates are shaken on an orbital shaker for 30 min and then spun to pellet the resin. The supernatant is analysed for the target protein. In this example the protein binds completely at pH 8 and above.

Assessment of these experiments can by achieved by a specific assay for your target, SDS PAGE (assuming your target protein is overexpressed in a high enough level compared with background expression level) or western blot if you have access to an antibody for your target molecule. Remember using 280nm absorption will not only show your target, but the total protein in solution. If the target can't easily be detected and the pI is unknown a good, general starting point would be pH 8.0 with a strong anion exchanger and pH 5 with a strong cation exchanger both eluted with a linear salt gradient from 0-1M NaCl.

Binding Capacity of the Column

There are 3 types of capacity quoted for IEX columns. The total ionic capacity describes the number of charged groups on the ion exchanger. It is given as mmol/ml resin and is a specification set by the resin manufacturer.

The static binding capacity (SBC) is determined by saturating a resin with sample and then measuring the amount bound.

The dynamic capacity (DBC) is the most useful and relevant measurement as it includes the effect of flow rate. The resin is packed into a column and run under defined conditions. At very low flow it will approach the SBC as there is sufficient time for saturation of the ion exchanger, at higher flow the DBC is lower than the SBC. Typical values are shown in figure 20.

The amount of protein that can be bound to a column depends on the size of the protein and the porosity of the ion exchanger, pH and ionic strength in the buffer and sample. Smaller pores equate to less binding as proteins can't access the space due to stearic hinderance, the size of the protein also influences binding for the same reason, very large proteins can't access the pores and thus only bind on the surface of the bead, hence the capacity is very low.

FIGURE 20

	Praesto SP				Praesto Q			
Matrix	Cross-linked agarose							
Functional Group	$CH_2CH_2CH_2SO_3^-$				$CH_2N^+(CH_3)_3$			
Ionic Capacity, mmol/mL resin	0.11–0.18 (H^+)/mL medium	0.11–0.18 (H^+)/mL medium	0.11–0.18 (H^+)/mL medium	0.11–0.18 (H^+)/mL medium	0.11–0.18 (Cl^-)/mL medium	0.09–0.18 (Cl^-)/mL medium	0.09–0.18 (Cl^-)/mL medium	0.09–0.18 (Cl^-)/mL medium
Average Particle Size (d_{50v}), µm	35	45	65	90	35	45	65	90
Flow Velocity+	Up to 120 cm/h (30 x 20 cm)	Up to 200 cm/h (20 x 20 cm)	Up to 350 cm/h (20 x 20 cm)	Up to 550 cm/h (20 x 20 cm)	Up to 120 cm/h (30 x 20 cm)	Up to 200 cm/h (20 x 20 cm)	Up to 350 cm/h (20 x 20 cm)	Up to 550 cm/h (20 x 20 cm)
Binding Capacity, mg/mL Resin at a 6-minute Residence Time	≥ 90 mg IgG*	≥ 80 mg IgG*	≥ 70 mg IgG*	≥ 50 mg IgG*	≥ 80 mg BSA**	≥ 70 mg BSA**	≥ 60 mg BSA**	≥ 50 mg BSA**
Operating pH Stability	Short Term	3–14			2–14			
	Long Term	4–12			2–12			
Working Temperature	4–30 °C				4–30 °C			
Chemical Stability	All commonly used aqueous buffers, 1 M NaOH, 8 M urea, 6 M guanidine, 30% isopropanol, 70% ethanol				All commonly used aqueous buffers, 1 M NaOH, 8 M urea, 6 M guanidine, 30% isopropanol, 70% ethanol			
Avoid	Oxidising agents, cationic detergents				Oxidising agents, anionic detergents			
Storage	20% ethanol, 0.2 M sodium acetate, 4–30 °C				20% ethanol at 4–30 °C			

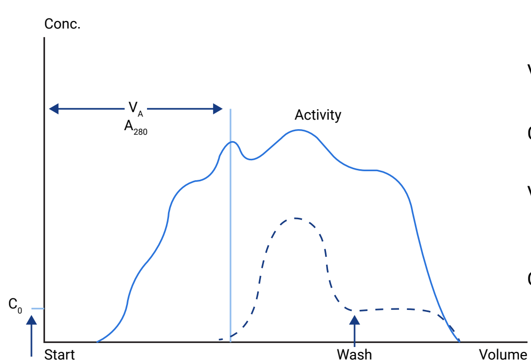
* Dynamic binding capacity — 10% breakthrough at 6 minutes residence time, 50 mM sodium acetate, pH 4.7 in a 1 x 3 cm column format.
** Dynamic binding capacity — 10% breakthrough at 6 minutes residence time, 50 mM Tris-base, pH 8.5 in a 1 x 3 cm column format.
+ Pressure flow determined using 0.1 M NaCl at 20 °C.

Shows the general characteristics of Praesto ion exchangers. Praesto SP and Praesto Q are compatible with all ranges of temperature, pH, chemical and physical conditions typically used in biopharmaceutical processes. The physical and chemical stability allows cleaning with sodium hydroxide, resulting in a very long, functional lifetime.

A breakthrough study allows calculation of the DBC, figure 21. This is useful to accurately predict how much resin you will need for your purification. It becomes essential if you are going to scale up for manufacturing as a too large a column will result in unnecessary costs in resin, buffer and processing time.

To carry out this study a column is overloaded with sample, fractions are taken throughout the loading and the point at which you can see 5% breakthrough of your target is noted. The chromatogram shows how this would look for ion exchange where you have a lot of other proteins in the feed. The dotted line shows the target. On the Y axis you can see concentration of target, Co is the concentration of target in the feed. VA is volume applied until the concentration of target in the flow through has reach 5% of Co. Qb (or DBC) 5% is calculated by VA x Co divided by the Vc or total bed volume. The results are in mg/ml.

FIGURE 21



Vc = Total column volume

C_0 = Concentration of target solute in feed

V_A = Volume applied until the conc. of target solute in effluent has reached 5% of C_0

$$Q_{A,\,5\%} = \frac{V_A \times C_0}{V_c} \quad \text{(mg/ml gel)}$$

A breakthrough study to determine the DBC. The chromatogram shows the experiment needed. A column is overloaded with sample (solid line total protein UV), fractions are collected and analysed for target (dashed line) as loading progresses. In the example shown the volume of sample loading required to see 5% breakthrough is noted (VA). Using the formula shown in the green box the DBC or QB can be calculated in mg/ml.

The binding capacity of the column will change depending on the flow rate, the faster the flow the lower the binding capacity. The contact or residence time (RT) on the column is calculated by dividing the bed height by the linear flow (cm/hr) and multiplying by 60, figure 22.

FIGURE 22

The formula required to convert linear to volumetric flow, volumetric to linear and determination of the residence time.

Linear Flow Rate = cm/hr
Volumetric Flow Rate = ml/min

ml/min (volumetric) to cm/hr (linear):
[Volumetric/Cross Sectional Area] x 60

cm/hr (linear) to ml/min (volumetric):
Cross Sectional Area x [Linear Flow Rate/60]

Residence Time (min) = [bed height (cm)/ linear flow (cm/h)] x 60

Linear Flow

- Linear flow is maintained when scaling up while volumetric flow increases
- Scaling up does not take longer if you maintain bed height and increase width
- Used to calculate residence time

Optimal residence times will depend on:

- Speed of Mass Transfer (i.e. size of bead, access to ligands and surface area)
- Size of the target molecule (diffusion, steric hindrance)
- Capacity v yield v productivity

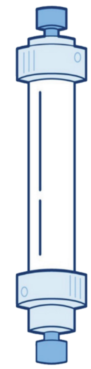
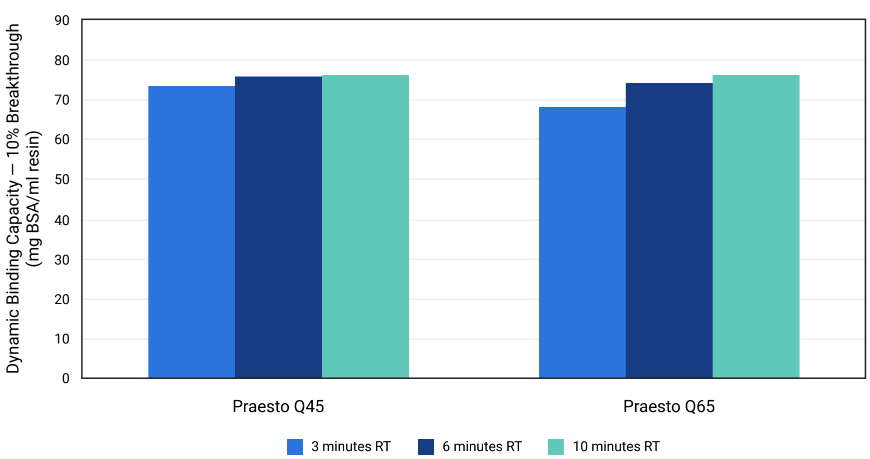


Figure 23 shows the DBC for BSA at 3 different residence times on 2 or our IEX resins, Praesto Q45 and Praesto Q65, with the numbers denoting bead size in µm. As you can see the residence time used will have an effect on the DBC and should be optimised as part of the process development. It is affected by speed of mass transfer this is dependent on the size of the bead, surface area, access to the ligand and running conditions. The size of the target molecule will also have an effect due to diffusion and stearic hinderance. Ultimately the decision as to which RT to use is a balance between capacity, yield and productivity. It is worth remembering that the RT may become restricted by bed height and column diameter as the process scales up thus it is essential to consider the end scale and manufacturing capabilities of your facility.

FIGURE 23



The dynamic binding capacity of Praesto Q45 and Q65. The columns were equilibrated with 5 CV, 50 mM Tris pH 8.0. The sample (BSA) in the same buffer was loaded and the column washed with 5CV Tris pH 8.0. Elution was performed using a drop in pH and an increase in salt achieved using 20mM sodium acetate and 1M NaCl. The RT (residence time) reflects the contact time of the sample to the column, shorter residence times corresponds to faster flow rates. The experiments were run 3 times for each resin at 3, 6 and 10 minutes RT.

Wash and Elution

After sample loading it is important that the column is washed back to UV baseline, normally around 3 CV. The simplest way to do this is using the equilibration buffer. Once you have a good understanding of where your contaminant and target molecules elute the wash buffer can be optimised such that all of the contaminants that bind more weakly than your target are forced into the flow-through or unbound fraction by adjusting the salt concentration or pH of loading. This will ensure you have maximum column capacity available for your target to bind.

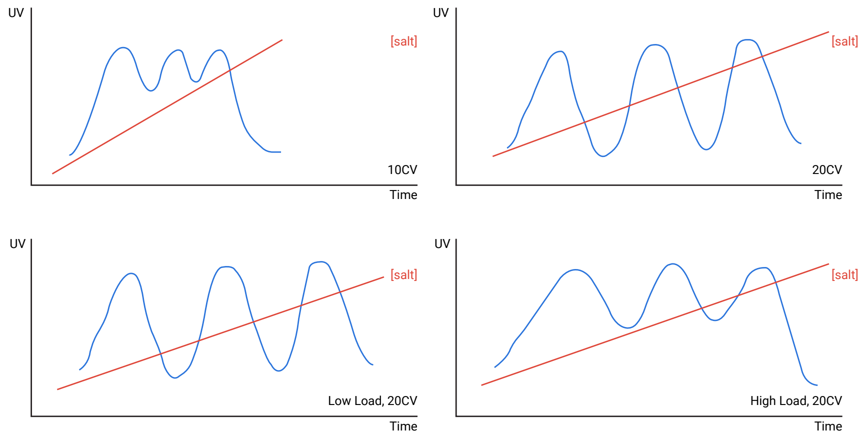
A linear gradient is ideal when investigating and optimising your purification. Gradients of increasing salt concentration are easy to produce. A gradient is generated by gradually mixing the start buffer having low salt and the final buffer having a salt concentration of 0.5 to 1.0 M. The flow rate is kept constant during mixing which is achieved using a chromatography system or manual gradient maker. An initial gradient volume equal to 20 column volumes is recommended. A longer gradient will increase the separation of molecules, however longer gradients also result in more dilute peaks.

Sodium chloride is the most used salt for elution. It is inexpensive, readily available, and normally does not contain impurities which interfere with the purification. It is important to check that your salt and buffer components are compatible. Compounds having anions such as sulphide (S2-), hydroxide (OH-), carbonate (CO3-), and phosphate (PO4-) can be insoluble in water. A precipitate will form if a solution containing one of these anions is added to a solution containing a metal cation such as Fe2+, Cu2+, or Al3+. Always test your buffer components for solubility at low and high salt prior to chromatography.

Remember that the elution strengths of different salts are quite different because of the charge state of the ion or position in the Hoffmeister series Figure 18. Similar elution strengths are given by 150 mM sulphate SO3(2-) and 350 mM chloride (Cl(-)). Sulphate can be very useful if the target molecule is unstable at higher concentrations of salt.

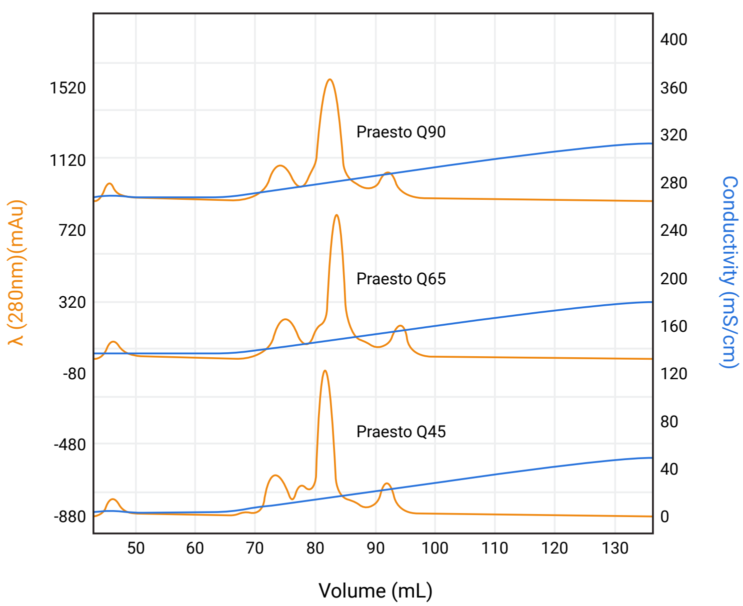
The gradient length in combination with loading on the column will affect the resolution. Figure 24 shows how both can impact the chromatography. Shorter gradients will result in peaks eluting closer together, often overlapping. With longer gradients the peaks move further apart and become more dilute (elute in a larger volume). Overloading the column will also decrease the resolution. As mentioned previously bead size will also impact resolution as shown in figure 25, reducing the dead diameter increases the resolution due to faster mass transfer and higher efficiency.

FIGURE 24



The top chromatograms show the same run, just differing in gradient length, increasing length increases the separation and elution volume. The bottom chromatograms show the same run just differing in the quantity loaded. Overloading the column will decrease the resolution.

FIGURE 25



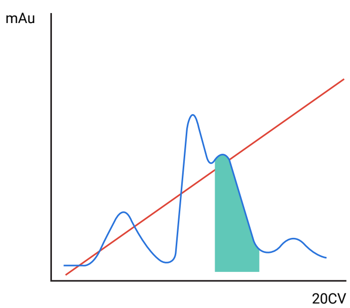
Resolution profiles for Praesto Q45, Praesto Q65, and Praesto Q90 upon application of a protein mixture containing 0.3 mg/mL apo-transferrin, 0.4 mg/mL α-lactalbumins, 0.6 mg/mL soybean trypsin inhibitor.

Effect of Column Length

A long bed height and smaller bead size means an increased number of theoretical plates (N), i.e. increased column efficiency. Learn more with our guide on scaling protein purification. Longer bed lengths are not required for affinity chromatography due to the extremely high selectivity. In gradient techniques, the bed height needed will depend on the differences in retention of the components to be separated. If the sample contains components which are similar then a longer bed hight can be an advantage. In the beginning of the gradient, when the components move down the column with different velocities, the bed height is crucial to achieve separation. However, from the point in the gradient where none of the components to be separated bind to the column extra bed height will have no positive effect on resolution. The shortest bed height that gives acceptable resolution should be used to give the shortest possible separation time.

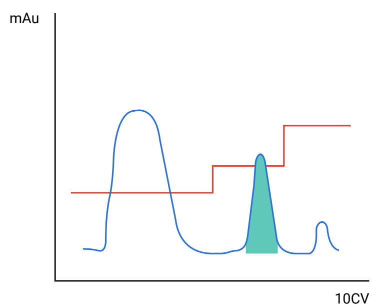
A gradient is an excellent tool to investigate the binding behaviour of the sample, if the purification is to remain at research scale a gradient can be used as a tool for the routine purification of your target. When a process is scaled up consideration must be given to the kit available, process costs and time for the purification. Large scale chromatography skids with a mixer are capable of creating a gradient however they can't be created with the same accuracy as a lab scale chromatography system. Gradients also take time and result in dilute peaks. For these reasons as you scale up your purification elution is normally carried out using steps which are determined from the gradient results.

FIGURE 26



Linear Gradient

- Defining points of elution
- R and D
- Wide peaks
- Time and buffer high



Adapted Step

- Concentrated peaks
- Split joiny peaks
- Less buffer, less time
- Better at scale

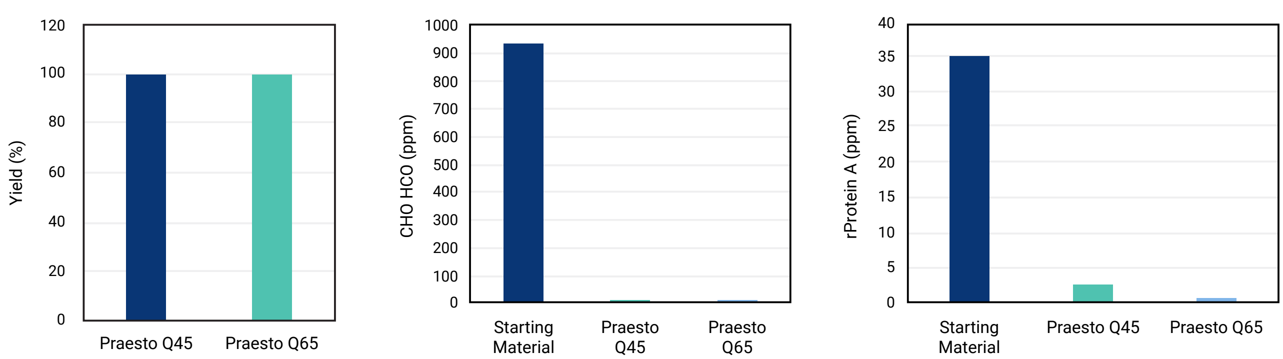
The chromatograms above show the same purification run, repeated twice keeping all factors constant except for how the eluting solution is applied, in this case NaCl. The chromatogram on the left uses a gradient elution, 0–500mM NaCl over 20 CV, the chromatogram on the right loads using 160mM, elutes using 250mM and strips with 500mM.

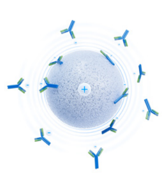
Elution in Flow-through Mode

Thus far we have discussed running IEX in bind and elute mode. It is also very useful in flow through mode, where your target molecule flows through the column and the contaminants bind. This is especially useful during the polishing stages of your purification process as there will be a great deal less contaminants compared to the level of your target. Thus, in flow through mode you can have a much smaller column as you need a lot less capacity to bind the small quantity of contaminants. This results in less resin and buffer costs and faster purification times.

The pH and salt concentration needs to be optimised to allow the target protein to flow through, while maximising the binding of contaminants. Optimisations such as this are often achieved using high through put methods such as Robocolumns or IMCS tips. Both of these devices contain a small amount of resin and be tested in parallel with a range of salts, salt concentrations and pH. The advantage of these small devices are the number of experiments that can be run at the same time and the small volume of sample required to complete the screen. Optimal conditions defined by the screen are then tested on a small chromatography column before scaling up. Figure 27 shows the successful implementation of anion exchange using both Praesto Q45 and Q65 as a flow through polishing step in the purification of a monoclonal antibody. You can see the yield or recovery after flowing through the column is high, however yield is not the only consideration, contamination clearance is vital to this step. As you can see clearance of contaminating host cell proteins and leached protein A ligand from the initial capture step is very effectively achieved.

FIGURE 27





	Starting CHO HCP (ppm)	Eluate — CHO HCP (ppm)
Praesto Q45	969.70	2.07
Praesto Q65	885.21	1.58
Praesto Q90	952.01	1.88

	Eluate: rProtein A (ppm)
Starting Material — Praesto Jetted A50	35
Praesto Q45	27
Praesto Q65	0.8
Praesto Q90	1

Anion exchange using Praesto Q45 and Q65 as a flow through polishing step in the purification of a monoclonal antibody. The graph on the left shows the yield of the purification, is extremely high. The centre graph shows the clearance of host cell protein contaminants (CHO HCP), while the graph to the right shows the clearance of leached protein A ligand.

Tips and Tricks

- Screening is a great way to determine which resin is best for your purification. However, if you are short on time you can use your pI as a guide. Is your protein more stable above the pI use anion exchange, while if it is more stable below cation exchange would be your first port of call.
- Na+ Cl- are the most common counter ions. Use more effective eluting salts, such as sodium sulphate, to elute biomolecules that are unstable in higher salt concentrations.
- Sulphate 150mM has about the same strength as Chloride 350mM.
- A prolonged residence time on the column can make proteins difficult to elute therefore avoid very slow flow rates or batch binding for long periods of time.
- For salt sensitive proteins the ionic strength to be used for loading should be close to the strength for desorption.
- Anion exchange for capture is not ideal as there will be a lot of DNA present which binds very tightly to the resin, reducing capacity for your target.
- 20–50mM buffer salt concentration is normally enough to maintain the pH during the run as long as the pKa of the buffer does not differ by more than 0.5 pH units from the working pH
- pH elution is an option for proteins which are very salt intolerant.
- Non-ionic detergents can be useful additives for less soluble proteins. If you need to use a charged detergent ensure it has the same charge as your ion exchange resin and keep the concentration as low as possible to prevent micelle formation. Detergents will absorb at 280nm, it is advisable to perform a blank run prior to loading your sample.
- Chaotropic reagents can be used to maintain the solubility of proteins. Urea is compatible with ion exchange chromatography, if using it should be freshly prepared as it can carbamylate on storage. Guanidine HCl is not compatible with IEX and should be avoided.
- Organic modifiers can be used to improve stability and solubility, one of the most common is glycerol. Be aware that the viscosity of the solution will increase and may result in the requirement for the flow rate to be reduced to avoid pressure problems.
- EDTA will bind to AEX columns, eluting early in a salt gradient. It should be avoided if possible.

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