GE Healthcare

WEBINAR

Tips for successful ion exchange chromatography

Questions and answers from live sessions

These questions and answers are from the live sessions of a Webinar presented by Marianne Carlsson on 17 May, 2017. After the presentation Marianne and a panel of R&D scientists participated in a live question and answer (Q&A) session. There was not enough time to answer all the questions that were asked. Marianne and the team have answered those questions, and we have compiled them in this document.

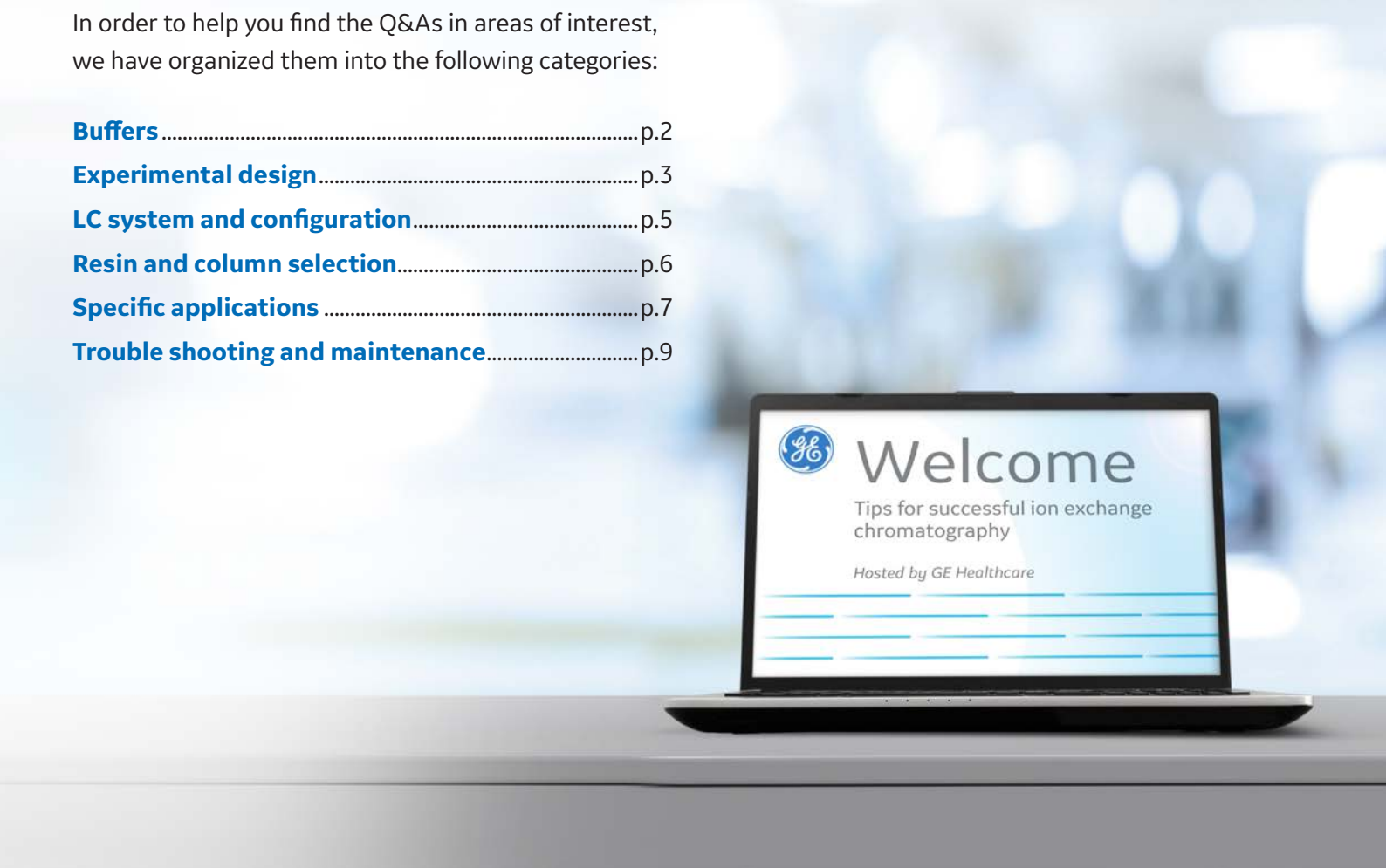
In order to help you find the Q&As in areas of interest, we have organized them into the following categories:

Do you have additional questions?

Feel free to continue the discussion about ion exchange chromatography on our Discussion Forum: proteins.gelifesciences.com/forum

Want to watch the Webinar again?

The Webinar can still be viewed on-demand and the presentation is available for download on protein.gelifesciences.com



Buffers

Q. Are there disadvantages for using Histidine buffer in AIEX or why you do not list this in the buffer list?

A. Histidine buffer can be used for anion exchange columns, having about the same buffer range as piperazine. However, the price might be considered a drawback, as well as the tendency of histidine to interact with metal ions. Normally a good buffer should not interact with other components.

Q. Does DTT have an effect on HiPrep™ Q FF column? We see a yellow color on the column.

A. 1–2 mM DTT is commonly used during separation on IEX columns. Equilibrate the column after the separation run with buffer without DTT. We have not detected the color change in the runs performed at our lab. A yellow color sometimes appear after extreme pH shifts or when the column needs cleaning.

Q. Does Imidazole affect anion exchange at neutral pH?

A. Imidazole is either uncharged or positively charged, so this buffer should be fine for anion exchange, just keep a low ionic strength during binding.

Q. How does polyvalent buffers influence the protein binding capability of an AIEX column?

A. Polyvalent buffers may compete strongly with proteins if ligands are of opposite charge to the buffer. So it is likely that polyvalent buffers such as phosphate may give a different selectivity which may be benficial or not.

Q. I have to include a certain amount of surfactant in the running buffers for membrane purification using SOURCE™ Q, any extra considerations I should have for better quality and quantity?

A. IEX resins such as SOURCE 15Q can be used for purification of membrane proteins, just as for water-soluble proteins, once the membrane proteins are solubilized in appropriate detergent. Detergent should be present in binding, washing and elution buffers during the purification. One should avoid using anionic detergents with anion exchange columns, and cationic detergents with cation exchange columns.

Q. Is a copy of the buffer list available?

A. The buffer tables shown in the presentation are available in the handbook Ion exchange chromatography–Principles and Methods available on gelifesciences.com.

Q. Is it possible to purify membrane proteins (buffer with detergent) by IEX?

A. Yes, it is possible to use non-ionic detergents for purification of membrane proteins. You can also use an ionic detergent as long as it does not compete with the binding of the target protein, which means that a detergent with the same charge as the IEX resin should be used.

Q. Why are different buffers recommended for CIEX and AIEX in the same pH range?

A. The general principle when choosing a buffering substance is a relevant pKa and also that the charged buffer species should not bind to the ligand, i.e. choose same charge as ligand. Otherwise you may experience local pH variances and unexpected pH-shifts that may ruin a separation.

Experimental design

Q. Can a cation exchange resin be used to separate protein isomers having pI between 5.6–6.15?

A. Yes, if the isomers differ in charge by this small amount, you should use a high resolution ion-exchange resin (e.g. MonoBeads™ columns) and apply low amounts of protein and run long gradients. However, you will probably need to do some optimization work to get a sufficient separation.

Q. Do you have any suggestion to purify samples with a low protein concentration? Is decreasing the binding flow rate to 1 mL/min in a 5 mL column a good strategy?

A. A low flow rate during sample application might be beneficial. Use a low ion strength (none or very low concentration of salt) during equilibration, sample application and wash. Also, a pH more than 1 pH unit away from the isoelectric point (pI) of the target protein can be used during the separation to promote stronger binding.

Q. How would you suggest testing column integrity for a preparation scale column?

A. We recommend to do a dynamic binding capacity test (e.g. at 10% breakthrough) with a known protein. Do that test when the column is new, to have a baseline of the binding capacity, and repeat the test after usage/column regeneration. If the column is insufficently cleaned, the binding capacity will be affected.

Q. How can I find out the isoelectric point (pI) of my protein?

A. It is easy to calculate the theoretical isoelectric point of your protein once you have the sequence of the protein. One can use the tools publicly available, such as Expasy “Compute pI/MW” tool (web.expasy.org/compute_pi/)

In the rare case that you do not have the sequence of your protein, you might need to determine the pI by experimentally running a titration curve like the one shown in the presentation.

Q. How does sample volume effect resolution on IEX?

A. The volume itself does not have an effect on resolution. It is actually the amount of load material applied that can have an impact on resolution as was shown in the Webinar.

Q. How do I best evaluate the efficiency of column regeneration (the restoring of column binding capacity)?

A. We recommend to do a dynamic binding capacity test (e.g. at 10% breakthrough) with a known protein. Do that test when the column is new, to have a baseline of the binding capacity, and repeat the test after usage/column regeneration. If the column is insufficently cleaned, the binding capacity will be affected.

Q. If my equilibration buffer is 2 pH units higher than the pI of my protein, and the sample is 1 pH units higher than the pI of my protein, is it a problem that they are not exactly the same since they are both higher than the pI of the protein?

A. If your sample is in a buffer with pH that is 1 pH unit above the pI of your protein and you are using an anion exchanger, your protein should bind to the resin, so it is not a problem.

Q. Is there a problem if my buffer is 3 pH units above my protein pI?

A. It is no problem to use a buffer that is 3 pH units above the protein’s pI, as long as the protein is stable at that pH. The protein will bind to anion exchanger at that pH.

Q. How would you suggest testing the column integrity of a large column, how to know when the binding capacity has lowered or what it was intitially?

A. We recommend to do a dynamic binding capacity test (e.g. at 10% breakthrough) with a known protein. Do that test when the column is new, to have a baseline of the binding capacity, and repeat the test after usage/column regeneration. If the column is insufficently cleaned, the binding capacity will be affected.

Q. Our lab does not have an ÄKTA™. How can I perform manual purification of proteins using ion exchange chromatography?

A. HiTrap™ 1 mL IEX columns can be used manually together with a syringe. After equilibration, sample application and wash, elution is done gradually by increasing the salt concentration. If you have no way to form a gradient, you can do this using steps of different salt concentrations. Keep the steps small to simulate a linear gradient. (e.g. 50 mM, 100 mM , 150 mM, etc). Ensure that you collect the eluate from each salt step. Once the column is eluted, you can measure the 280 nm absorbance of the fractions one at a time with a spectrophotometer to obtain the chromatogram. SDS-PAGE can be used to see where your protein eluted.

Q. What is a reasonable load amount for screening on a 1 mL HiTrap™ IEX column to minimize sample consumption, without sacrificing information?

A. It's a difficult question. It depends on the nature of the protein, the extinction coefficient, detector parameters etc. A rule of thumb is that the peak should be at least 5 times the noise to provide a credible value. This must be empirically tested in each case.

Q. What should I do if I am experiencing protein precipitation at lower or no salt concentrations?

A. If you want to purify your protein using IEX and your protein precipitates in low salt, you might consider using multimodal ion exchangers, such as Capto™ MMC and Capto MMC ImpRes. In addition to electrostatic interactions, the ligand structure of multimodal ion exchanger provides for additional interaction modes such as hydrophobic interaction and hydrogen bonding. They provide high binding capacity even at high salt concentration, e.g. Capto MMC has a DBC of > 45 mg BSA/mL medium at a conductivity of 30 mS/cm.

Q. When running theoretical plates, what is the recommended flow rate? Production flow rate is 25 L/min. Currenty running TP at 5L/min to get a good resolution. I would like to speed up the flow rate.

A. Based on the information given in the question, it's impossible to give a good answer. But we recommend studying the Handbook Ion Exchange Chromatography Principles and Methods (11000421 AC), Appendix 3, for guidance on how to measure the column efficiency and plate number. The handbook can be downloaded from: proteins.gelifesciences.com

Q. When the buffer pH is optimized, will the protein bind more strongly with strong IEX than on a weak one, or will the binding force be similar?

A. Strong ion exchange resins are charged over a broad pH range. Weak ion exchange resins are charged within a narrower pH range. But the binding force is also dependent on which type of ligand and the number of charges it carries at a certain pH, more than if it's a strong or weak ion exchanger.

LC system and configuration

Q. Any tips on what procedure should be used when changing buffers during method development?

A. This is a question that can be answered in several different ways because it also depends on your objective. Here are a few tips:

- Use different inlet valves for different buffer solutions.
- Equip the system with additional valves.
- Use BufferPro, if you are working with the ÄKTA avant system, for buffer preparation.
- Increase efficiency using the integrated DoE functionality in UNICORN™ software: This function will automate an experimental series of runs using a defined number of experiments, help to identify significant parameters and aid you in optimizing your process.
- Automate your runs using scouting protocols.
- Run the process using quarternary gradients effectively by selecting appropriate mixing ratios and utilizing the Q-valve.

Q. Can column selection valves be purchased separately?

A. Column selection valve, V9-C or V9H-C, with integrated bypass and reverse-flow functions, can connect to five columns for automatic column switching. Connection of multiple columns minimizes manual intervention and reduces the risk of introducing air into the column. The column selection valve can be added to ÄKTA pure systems. With the ÄKTA avant system one column selection valve is included and a second one can be added as an option.

Q. Why is BufferPro not available on the ÄKTA pure. It was on the old purifier systems?

A. We appreciate your comment and we do understand that these needs may come over time when using the different chromatography systems. The predecessor of BufferPro, called BufferPrep, was included with the ÄKTApurifier system. The function to prepare buffer in-line was further developed and launched with the ÄKTA avant system as BufferPro. ÄKTA avant is our more advanced system mainly for process development and this is where this feature would be of most value to the users.

Q. You mentioned that BufferPro recipes can be user generated. Two GE applications scientists have told me that you can only use the predefined recipes. Is the user-defined option new?

A. Sometimes, due to information gaps, we may misunderstand each other. The BufferPro functionality enables you to create your own recipes using the BufferPro algorithm for buffer calculations. This algorithm uses common equations for calculating the mixing ratio for the buffer components for a set buffer concentration and pH.

Resin and column selection

Q. Can you separate two proteins with very similar pI on a Q Sepharose™ HP column?

A. Yes you can, although you may not achieve baseline separation. However, the selectivity/resolution is not only dependent on the pI of the proteins, but also the size, tertiary structure etc. To achieve even better separation power, you might need a high resolution media, such as the Mono Q™ or Mono S™.

Q. Is it possible to purify polysaccharide through ion exchange chromatography and which resin will be suitable for this type of separation?

A. Charged carbohydrates can be separated by using DEAE or Q functionalized resins for acidic (most common) carbohydrates. SP or CM functionalized ion exchangers can be used for basic carbohydrates. Examples in literature, show frequent use of DEAE resins for carboxylated carbohydrates and Q resins for sulfo and phosphoylated carbohydrates. Elution is commonly achieved by a shift in pH or by using a salt gradient.

Q. I am a postgrad student working on the purification of different human hair keratin subtypes, which possess pI range from 4.53–4.86 (type I keratin) and 5.23–7.61 (type II keratin). Do you think strong anion exchanger will work in resolving such similar pI value?

A. Yes, that might work very well, but you might need a high resolution anion exchanger such as Mono Q.

Q. The weak anion exchangers can give in theory a better resolution than strong anion exchangers. However, the GE products are manufactured to withstand lower pressure, bigger bead sizes and in plastic column shells. Hence, the strong anion exchangers (e.g. Mono Q 5/50GL columns) are delivered in glass column shells with high-pressure resins etc. and are still giving better resolution due to how the resin was manufactured (see GEE brochures with standard protein mixes loaded). So why should I use a weak anion exchanger?

A. A weak ion exchange resin give you a pH-dependent selectivity in contrast to strong ion exchange resins. They are frequently used if you separate your proteins with a pH-gradient.

Q. What is the difference between HP, XL, and FF?

A. Sepharose High Performance (HP) is a 34 µm bead resin and Sepharose Fast Flow (FF) is a 90 µm bead resin. Sepharose XL is a variant of Sepharose FF and is only used as a base matrix for ion exchange. It is a 90 µm beaded resin that has long chains of dextran coupled to the agarose matrix. These chains increase the exposure of the charged groups (Q or SP) resulting in higher loading capacities than the corresponding Sepharose FF resins.

Q. Would you please elaborate on your antibody purification columns and techniques?

A. Antibody purification usually begins with an affinity chromatography to remove the bulk impurities. The affinity chromatography can use Protein A, Protein G or Protein L resin. The partially purified antibodies can be subjected to an ion exchanger to remove aggregates or other minor impurities.

Specific applications

Q. Can anion exchange be used to remove aggregates?

A. Ion exchange can be used to separate monomers from aggregates. Although monomers and aggregates are chemically similar, aggregates usually have a greater surface charge than monomers and thus, the two can be separated on high resolution ion exchangers.

Q. Can I use ion exchange for preparation of solubilized inclusion bodies? I´ve been trying to solubilize proteins from *E. coli* inclusion bodies using several methods. The last one that I´ve tried was 6M Guanidine. However, it seems that it did not help. I would like to know if I should use 8M Urea in order to obtain better results?

A. Yes, you can solubilize the inclusion bodies in a low salt buffer solution using 8 M urea. Also, you could try solubilizing the inclusion bodies with an ionic detergent, matching the charge of the IEX resin for example by the use of N-Lauroyl sarcosine. Keep ionic strength low during binding.

Q. Can IEX be used for highly polar peptides?

A. Yes, provided that the appropriate buffers can be used for the solubility of the peptides as well as binding/elution conditions.

Q. How does glycosylation affect the theoretical pI of the protein and binding to the IEX column?

A. Glycosylation can affect the pI of a protein when they contain charged monosaccharides, such as sialic acids or sulphated monosaccharides. For example, sialic acids in the glycan will decrease the pI of the protein and make the glycoprotein elute later in a salt gradient on an anion exchanger. Even with non-charged glycan, glycosylation can affect the behaviour of glycoprotein on an ion exchanger compared to the de-glycosylated protein. The glycan chains might shield part of the charged surface causing the protein to appear less charged, although the effect is not as large as with charged carbohydrates.

Q. My antibody sample contains aggregates, how can I remove them chromatographically?

A. Size exclusion chromatography (SEC) separates molecules on the basis of differences in size, so it is well-suited to separate antibody monomers from aggregates if your sample volume is small, since scale-up of SEC is particularly challenging (as described in the presentation).

IEX is also used to separate antibody monomers from aggregates.

Another effective way of removing aggregates is to use Capto adhere, which is an anion exchanger with multimodal functionality, including hydrophobic interaction. Removal of aggregate contaminants is achieved in flowthrough mode under conditions that allow the antibodies to pass directly through the column while the contaminants are adsorbed.

Q. We are planning for a two-step purification procedure for our his-tagged target protein: a first IMAC step followed by ion exchange chromatography to separate charged variants. Would it be possible to proceed directly with the eluted sample from the IMAC column to the IEX column?

A. It is not recommended to apply the sample eluted from the IMAC column directly to the IEX column. The sample eluted from IMAC contains both high concentration of imidazole and high concentration of salt, usually 500 mM NaCl. In order to get efficient binding to the ion exchange column the sample should contain very low salt concentration, or no salt at all. Adding the sample eluted from the IMAC column to the IEX column will most probably result in obtaining the target protein directly in the flow through. Except for this, the presence of imidazole in the sample will also disturb the equilibrated buffer conditions in the column.

Thus, it is strongly recommended to perform a buffer exchange of the sample before proceeding to the next step. The exchange is performed to the buffer used as binding buffer in the IEX separation.

Q. We saw a very high OD260 in the beginning of the salt gradient, using high pH buffer plus DTT. Is it possible that it is DNA? Without DNA that peaks at the end of gradient?

A. DNA and other nucleic acids may bind strongly to positively charged anion exchange resins. Larger DNA fragments may need about 0.7 M NaCl for elution, but up to 2 M NaCl may be needed to remove bound DNA from an anion exchange resin. Smaller fragments may co-elute with proteins.

Trouble shooting and maintenance

Q. In HP resin like Q Sepharose HP sometime a shoulder occur in front of main peak. Analytical IEX- or RP-HPLC indicates no differences between shoulder and main peak. Is there something known about this behavior?

A. Not much is known about this. We have sometimes seen that refolded proteins may give rise to several peaks, probably due to conformation differences, but that should be seen also in analytical IEX or RP-HPLC.

Q. Can I clean my HiTrap™ Q HP column often by using NaOH?

A. Yes, you can clean your HiTrap Q HP with NaOH often as long as the column is returned back to neutral pH after the cleaning. After NaOH cleaning it is recommended to wash the column with water followed by your equilibration buffer to lower pH to return back to normal (water is less efficient for rapid change of pH).

Q. How can we know that the material is completely cleaned? It is practically impossible to measure binding capacity after every cleaning?

A. The back pressure of the column is a strong indication of the performance, the pressure should be back to normal after a successful cleaning. Also, a small mix of some stable test proteins can be used to give a sensitive check of resolution. Note that the protein mix should be tested immediately on a new column to get a reference for future tests.

Q. How often do we have to clean the column?

A. The number of times a column should be cleaned depends very much on the sample. For example, if an *E. coli* sample is used you might need to clean the column with NaOH after every run or at least quite often. On the other hand, if the ion exchange column is used for polishing and the sample is quite pure, a high salt wash after the separation might be sufficient, and NaOH-cleaning required more rarely.

An indication that cleaning is absolutely necessary is that the back pressure of the column has increased, or if there is a visible coloring of the resin. Some columns can be cleaned in reverse mode, but in that case it is advised in the instructions for the specific column.

This is an example of a typical cleaning of IEX columns:

1. Wash with at least 2 column volumes (CV) of 2 M NaCl.
2. Wash with at least 4 CV of 1 M NaOH.
3. Wash with at least 2 CV of 2 M NaCl.
4. Rinse with at least 2 CV of distilled water.
5. Wash with 5 CV of start buffer until eluent pH and conductivity have reached the required values.

If NaOH-cleaning is not sufficient other substances can be tested, such as acetic acid or isopropanol. See also answer in question titled: What cleaning agents/denaturants would you recommend for cleaning columns (i.e. HiTrap) besides NaOH?, on page 12.

Additional information regarding cleaning is available in IEX handbook Appendix 10 which can be downloaded for free on proteins.gelifesciences.com.

Q. How do I remove bound DNA during protein purification? My protein has a DNA binding domain and has a pI of 6. It is overexpressed in *E. coli* and his tagged. After Ni column, I found there is a lot of DNA in my sample based on 280/260. On the SDS-PAGE, there are a lot of contamination bands. I suspect that the proteins are binding to the same DNA and getting co-purified by the Ni column. What can I do to release the DNA from my target protein?

A. Usually high salt concentrations are needed, e.g. 0.5–2 M NaCl, to dissociate DNA from DNA binding protein.

Q. I observed yellow colorization and backpressure increase after using my SP Sepharose HP with *E. coli* fermented material. I used the detailed cleaning protocol but it did not remove the yellow color. I was able to remove it after a 1-week, non-stop wash with 1N sodium hydroxide which reduced the binding capacity drastically. Can you suggest any other reagents?

A. A yellow colorization of resin after applying a complex cell extract may come from many different contaminants. Cleaning with 1 M NaOH with the reversed flow direction is the best method if the contaminants are hydrophobic. Residence time is dependent on the contaminant, but 1 week seems too long. For very hydrophobic proteins you may also try using 70% ethanol or 30% iso-propanol with reversed flow direction.

Q. I would like to know how often I can clean the column after using it? And should I wash it after every run? What cleaning agents/denaturants would you recommend for cleaning columns (i.e. HiTrap) besides NaOH?

A. The number of times a column should be cleaned depends very much on the sample. For example, if an *E. coli* sample is used it might be needed to clean the column with NaOH after every run or at least quite often. On the other hand, if the ion exchange column is used for polishing and the sample is quite pure, a high salt wash after the separation might be sufficient, and NaOH-cleaning needed more rarely.

An indication that cleaning is absolutely necessary is that the back pressure of the column has increased, or if there are visible coloring of the resin. Some columns can be cleaned in reverse mode, but in that case it is advised in the instructions for the specific column.

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If NaOH-cleaning is not sufficient other substances can be tested, such as acetic acid or isopropanol. See also answer in question titled: What cleaning agents/denaturants would you recommend for cleaning columns (i.e. HiTrap) besides NaOH?, on page 12.

Additional information regarding cleaning is available in IEX handbook Appendix 10 which can be downloaded for free on proteins.gelifesciences.com

Q. In general what do you advice for cleaning Q and S columns?

A. See answers in question titled: I would like to know how often I can clean the column after using it? on page 10 and question titled: What cleaning agents/denaturants would you recommend for cleaning columns (i.e. HiTrap) besides NaOH?, on page 12.

Q. The cleaning protocols in the GE-manuals (e.g. MonoQ 5/50 GL column) are quite mild and when talking to GE representatives they told me that it can withstand higher NaOH concentrations for much longer times. Where do I find the information showing what the column/resin can really withstand e.g. to clean it from bacterial endotoxins?

A. The cleaning procedures for e.g. Mono Q 5/50 are given in the column instruction and Data file, but also in the Handbook – Ion Exchange Chromatography Principles and Methods, all available on the web. The cleaning procedures includes what the columns are tested for, in the case of Mono Q this is divided into two steps: regular cleaning and more rigorous cleaning, the latter can not be considered as particularly mild.

Q. We have Mono Q that is 20 years old. Can we still use it?

A. The Mono Q resin is known to be very robust with a long lifetime, but the exact lifetime is difficult to predict, much depending on the column usage, storage conditions etc. I suggest you do a function test of the column with a known protein or protein mixture to evaluate the separation and binding/elution characteristics

Q. We have recently encountered problems with low yields of target protein. We have also noted somewhat higher back pressure on our anion exchange column. Any suggestions on what to do?

A. Problems with low yield and tendency of higher back pressure may be associated with precipitates of the sample in the column. The sample should have the same conditions (pH and ionic strength) as the equilibration buffer to avoid a sudden change during binding. Perform a buffer exchange of the sample, or dilute 10–20 times with equilibration buffer. It is important that the sample can tolerate both the start buffer and the elution buffer. A simple test can be performed with small amounts of sample and buffers in test tubes to look for precipitations.

If the sample gets turbid in combination with the equilibration buffer it may help to add 1–2% glycerol or urea.

If precipitation occurs in combination with the elution buffer it might be necessary to change the conditions for the separation. For example, if a salt gradient is used it might work better to use a pH-gradient for elution. It may also help to lower the load of sample. A change from a anion exchange to a cation exchange column can also be considered.

Note, if you are going to use your anion exchange column again, be sure to clean it according to the instructions before the next run.

Q. What cleaning agents/denaturants would you recommend for cleaning columns (i.e. HiTrap) besides NaOH?

A. For precipitations in the column it can be tested to wash with denaturing agents, like urea or guanidine hydrochloride, alternatively a pepsin wash over night (1 mg pepsin/mL in 500 mM NaCl, 100 mM acetic acid). For extended cleaning a wash with 4 CV 70% ethanol or 30% isopropanol can be performed.

It is important to use a low flow rate during cleaning (about half of the flow rate used for separations). Also, rinse the column with water between different solutions and buffers to avoid any precipitations. Always end the cleaning with equilibration with start buffer to obtain the required pH.

Check for solvent compatibility in the instructions supplied with the specific column.

Q. Which are the right or better conditions to keep/store an anion HiTrap 5 mL column if you don't need to use it for some months?

A. Always store the column in the solution that the column was delivered in, in most cases 20% ethanol. It is also good to keep the column in a cold room or in a refrigerator when not in use.

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