ÄKTA™ club web chat

GE Healthcare R&D experts' answers to your protein purification questions

We have gathered the questions and answers from our ÄKTA club web chat held on March 8, 2017. ÄKTA system users asked questions related to lab-scale protein purification and ÄKTA systems, which our GE experts answered during the live session. The expert panel included five scientists total, from our R&D team in Uppsala and from Scientific Support. ÄKTA club members submitted questions in writing. The questions were about chromatography techniques, troubleshooting tips and tricks, and the use of ÄKTA systems. In this document, we have included the questions that were answered, plus a few additional ones that we did not have time to answer during the web chat.

We look forward to continue discussing your questions live during our next web chat. Join ÄKTA club at gelifesciences.com/AKTAclub and select *I would like to receive occasional email updates from GE Healthcare* to get notified of upcoming web chats and other events.

* AC = Affinity chromatography

Do you have additional questions?

Feel free to continue the discussion about lab-scale protein purification and ÄKTA systems on our Discussion Forum at proteins.gelifesciences.com/forum

ÄKTA club

ÄKTA club is an online user community for ÄKTA system users enabling easy and quick access to trustable information about ÄKTA systems and protein purification. Connect to forums on ÄKTA club. Share your experience and results, engage in inspiring discussions, and learn from peers and GE experts.

ÄKTA systems

Q. I am seeing a pressure even when there is no flow. I opened the pump purge lines, and still there was no drop in pressure. Please suggest a solution.

A. Yes, always start your problem search up-flow, closest to the pump so that you can exclude the possibilities step-by-step. But this does sound like a problem with the pressure sensors. Have you tried to calibrate them? Note: When calibrating the sensors you will need to disconnect tubing to ensure atmospheric pressure.

Q. What's the best way of degassing the buffers, and does it make a difference? Has anybody looked into comparing degassed or non degassed buffers and their ability to generate air bubbles during the run?

A. It is always important to degas buffers, especially when working with high-pressure columns. This prevents air bubbles in pump heads or in the UV cell. On all ÄKTA systems, there is a special device called Flow Restrictor (FR-902) that helps to prevent air bubbles in the UV cell. I have never used non degassed buffers. But it really is better to spend 15–30 min to degas, which prevents air bubbles in pump heads or UV cells that would make your results very difficult to analyze.

Q. How do I know if the UV lamp is functioning fine – is there a quick test using a unique protein and one single column to run as an positive test to see the peaks?

A. For UV-900 the lamp has a very long life time. You can check the lamp intensity, and when it is below 20% you should contact us for a lamp replacement or change of internal optical fiber. If you are using another UV monitor please check in the troubleshooting section of the user manual for advice. You can also inject a sample and compare it to the absorbance signal that you get in a spectrophotometer.

Q. What is the best way to eliminate air loaded accidentally in the injection valve?

A. Air is always a hassle in LC. A good idea is to perform a pump wash (will go through the injection valve to waste). Check inlet tubings so that additional air does not enter. You could also release the outlet tubings.

Q. When loading a "dirty", i.e. partially precipitated, sample, would you remove the in-line filter to prevent a build-up of back pressure?

A. That is a good question! Partially precipitated samples could create blockage in filters, so pre-filtering of samples is sometimes neccessary. Yes, you could remove the inlet filters but sometimes we are just postponing the problems to other parts of the system. There are special "crude" columns available for certain applications; these columns handle "dirty" samples.

Q. How can delay volume be calculated?

A. The delay volume is either estimated from the sum of component volumes and the additional volumes from the capillaries (depending on ID and length) or it is measured. One way to measure the delay is to use an additional UV monitor at the capillary outlet (e.g., at the end of the fraction collector tubing) to measure the band broadening.

Q. I am fairly new to using ÄKTA, and we have a UPC 100. One of my questions is regarding sample loading: what's the main difference in using either a syringe, system pump or sample loop? I understand it depends on the volume of the sample, but why would you choose one over the other?

A. I appreciate this question. Things that affect the selected method are, for example, the injection volume itself (depending on column size in SEC applications) and the sample availability (we lose more sample in tubings if we are using the system/sample pump). If carryover is suspected or is a concern, a separate flow-path for sample application must be used (sample pump). Injecting large volumes using a pump is also advantageous because we can use the automatic pressure-flow control, which will eliminate uneccessary stops during sample loading.

Q. I have a question regrading the A_{280} scale of my ÄKTA runs. Sometimes, my runs start with a negative A_{280} value or this value keeps decreasing throughout the run. Therefore it becomes impossible to distinguish where the peak starts and where it ends. Or the size of the peak is hindered because of the slope in the line. Could it be because of a calibration issue of the UV monitor?

A. Background signal can sometimes be disturbing, I agree! Have you tried running without a column? UV calibration should not be needed; the method should use autozero for setting the UV signal to a baseline. A steady decrease of the signal could be due to some component in the buffer or something previously bound to the column.

Q. How do I load large amounts of sample on affinity columns on ÄKTA pure?

A. In binding mode and with large sample volumes I would prefer to use the sample pump or Superloop™, but this of course depends on the injected volume.

Q. Is refolding required for protein purified when using an ÄKTA system?

A. If the target protein must be solubilized (due to inclusion bodies or aggregates) during the purification process, then the protein must be refolded to regain its activity and structure.

Q. We have an ongoing discussion in our lab as to whether it is better to flush air out of the system using methanol or ethanol. Which would you use, or does it not make a difference?

A. There are data on dissolution of air in different solutions that include organic solvents. It is worthwhile to at least peek into this data to get a better understanding for the problems at specific conditions. The answer is more “it depends.”

Q. We have an ÄKTAprime system which we would like to keep using, however the software is outdated and not up to code with data integrity. Is there a version of UNICORN, or any other operating software, that is compatible with ÄKTAprime and up to date with data integrity requirements?

A. PrimeView™ 5.31 allows the user of ÄKTAprime or ÄKTAprime plus to view the monitor signals from the chromatographic run in real time on the computer screen. The logbook pane displays all actions during a separation run (e.g., method start and end, base instruction, method instructions, and manual instructions such as Pause or Hold). A result file is automatically generated at the end of a run and contains a complete record of the run, including method information, system settings, curve data, and a run log. For additional functionality you might want to consider the ÄKTA start system or ÄKTA pure system. Both are UNICORN™ controlled. Please contact your local GE representative for more information.

Q. I’m using the ÄKTA start system for fractionation of sucrose gradients, in which I want to detect polysomal fractions. I see an increase for my 280 nm-signal, but no typical polysomal absorbance pattern. What could be the reason for that?

A. This is a tricky question, because polysome profiles depend on the lack of RNA degradation and the need to preserve both the RNA and protein complexes. Check the literature for the best advice on maintaining an RNA degradation-free environment (e.g., ice, RNase inhibitors and protease inhibitors). Using sucrose gradients increases complexity even further, and there are thorough descriptions in literature on how to handle them. But sometimes it could also be related to the system, because tubings can get blocked or you might introduce bubbles into the gradient during the process.

Q. I recently saw a wave fluctuation of both conductivity and flow in my gel filtration using my ÄKTAxpress system. I first thought it could be a temperature difference because of the fans in the cooling fridge it is standing in, as I have seen the conductivity fluctuate like that before. But now the flow was also fluctuating. What could be the cause – a leak in the pump?

A. I would suggest that you contact your local GE representative to get this case filed for tech support. More information is needed in order to be able to give you a correct answer on how to proceed.

AC – Antibody purification

Q. When neutralizing eluates from a protein G column using citrate buffer, does it make a difference whether Tris-HCl is added to the fraction after it is collected or placed in the empty collection tubes before collecting? I've heard that adding the base to the empty tubes will cause drastic pH changes in the fractions, so it is better to do it afterwards (drops of protein into a strong base vs. a few microliters of strong base into 1 mL of protein).

A. It is a question of kinetics. If you have a few microliters of basic buffer in the tube, the drops of proteins that will fall into the tube will allow a better mix of the neutralizing buffer in the fraction. It is also a question of time. If the neutralization is very quick and happens as early as possible (during the collection), this prevents denaturation/aggregation of the proteins.

Q. We are packing a Millipore VL32 160cm column with MabSelect Sure™ LX resin. We calculated our slurry ratio to be about 79%. So we packed 222 mL of slurry into the column, and let the resin settle. We then flow packed and compressed the resin to a 20 cm bed height and 160 mL volume. Our first asymmetry test gave a result of 1.57. Our manager hoped to get that down a little bit, so we performed a conditioning run with our chromatography method and buffers but without protein. Our second asymmetry result was worse at 1.8. We are using 2 M NaCl solution + low concentration of sodium phosphate for testing. This study is being performed on an ÄKTA avant 150 system.

A. It is good that you are using NaCl for testing, because acetone can have some interactions with the Mabselect Sure family of resins.

I do not think that phosphate should affect the asymmetry, but you should try to test without it. If you have a tailing you might need to start with a lower settling flow rate or increase the compression flow rate to get a harder packed bed. See the answers to "What are some things we can try to improve the asymmetry result of the column that we packed?" for more suggestions.

With ÄKTA avant 150 you have bigger volumes in the system compared to ÄKTA pure, for example. Perhaps your 32 mm diameter column is too narrow for the ÄKTA avant 150 system, which is better suited to columns with a broad diameter, such as GE's 50 mm diameter HiScale™ columns.

AC – Tagged protein purification

Q. I have a his tag protein I am trying to purify. It's around 24 kDa. On SDS-PAGE and gel filtration column it looks like the right size. However, it's inactive in my enzyme assays. Is there any other common impurity that can copurify around that size?

A. Impurities that interact with your target protein might have been copurified, because there are histidine-rich proteins that can also bind to IMAC resin. You can try to further optimize the method by increasing the imidazole concentration in the wash step, or add a small concentration of reducing agent or detergent to break interactions that occur. You can try to check if it is the correct protein by running a Western blot using an anti-his antibody. If you cannot see a band, then it is not your protein. If you are measuring the activity directly after the IMAC purification you might need to exchange the buffer, because the IMAC buffer might interfere with the assay. Another possibility is perhaps your protein is not active/stable in the buffers you are using.

Q. Is the column for his-tagged protein purification compatible with ÄKTAprime?

A. Yes, all our affinity columns for his-tagged proteins (i.e., HisTrap™, HiTrap™ TALON®, and HisPrep™) are low-pressure columns. They are all compatible with ÄKTAprime, ÄKTAprime plus, and ÄKTA start system.

Q. My His-tagged protein is in inclusion bodies. What should I do to keep it soluble?

A. The induction of expression should be slower. Decrease temperature from 37°C to 25°C or lower, and decrease the concentration of inducer. If your protein remains in inclusion bodies, you can try to work from them. Concentrate inclusion bodies with centrifugation and try to do matrix-assisted refolding experiments. For example, solubilize inclusion bodies with chaotropic agents with or without detergents. Desalt the sample, and then bind the solubilized proteins on HisTrap columns. Inject a refolding buffer, and wait for 30 min before eluting with increased imidazole concentration. There are many documents on our Web site describing such experiments.

Q. Can a 5 mL his column still be used after the expiry date?

A. We cannot give any guarantee about the good/full functionality of the columns after the expiry date. If they have been properly stored and look fine you can use them only for internal tests but not for publishing results.

Q. The binding of urea-solublized inclusion body to the His-Tag column is very poor. How can I solve such a problem?

A. After solubilization, did you try to decrease the urea concentration using desalting columns (HiTrap or HiPrep™ Desalting)? After desalting the urea concentration should be low, which would be expected to improve the binding.

Q. What kind of feedback do you receive (if you receive any at all) from your customers regarding the efficiency of the interaction between the His-tag and the Ni^{2+}-NTA (IMAC) resin? I mean the position of the His-tag C- terminus or N-terminus. Or can you suggest a linker between the affinity tag and the protein which usually provides an optimized interaction between the recombinant protein and the resin?

A. The opinions in the literature vary regarding recommendations. Linkers with Gly and Ser are often used. His6 is normally sufficient, but His10 is sometimes used when it is suspected that the tag is not fully exposed (as for some membrane protein-detergent complexes, but possibly also for some non-membrane proteins). The best position regarding good surface exposure of the tag, C- or N-terminal, varies from protein to protein. If you want to have a protease linker sequence for tag removal close to the tag, check the cleavage pattern of the protease – an N-terminal tag position will usually leave fewer (or no) non-natural amino acids on the target after cleavage. If the tag is N-terminal, one also has the opportunity to include an extra segment at the very N-terminus that improves the transcription start or translation start. If premature transcription or translation stop is a problem, a C-terminal tag positioning will prevent his-tagged truncates from forming *in vivo*, which sometimes can be a great advantage.

Q. What would be the best column to use if you work with membrane proteins and need to isolate proteins that could be purified only in detergents like digitonin?

A. First, you have to choose if you want the membrane protein (MP) affinity-tagged. In most cases tagging will facilitate purification very significantly, and we know that most purified MPs are today his-tagged, often with a long spacer and a 10-his-tag to make the tag come out from the detergent part of the MP-detergent complex. For IMAC of his-tagged MPs, we recommend Ni Sepharose HP (e.g., HisTrap HP columns). IMAC will work in detergents, but the problem might be (as mentioned) the exposure of the his-tag, and/or a low expression level, which is typical for most MPs.

Q. I want to get the cell membrane fraction using His-tag. I will express my target gene with His-sequence on HEK cells and break cells by sonication. Do you think I can get the membrane fraction by HisTrap?

A. You do not get a membrane fraction with IMAC, but I think you refer to purification of a his-tagged membrane protein (MP) that is present in the membrane fraction. I know that today most MPs are his-tagged for purification, often with a long spacer and a 10-his-tag to make the tag come out from the detergent part of the MP-detergent complex. For IMAC of his-tagged MPs, we recommend Ni Sepharose HP resin (e.g., HisTrap HP columns). Also, I advise having a look in the literature, and study how successful MP purification experts work (e.g., those people that have obtained 3-D structures). For example, you must learn how to selectively isolate membranes before detergent-solubilization (to get rid of soluble proteins), and how to select the best detergent (often a compromise between solubilization efficiency and MP stability). Some experts do not bother to isolate the membrane fraction; instead, they treat the whole lysate with excess detergent. A starting source for literature studies is GE Healthcare's handbook Purifying Challenging Proteins, which is available as a pdf on our web site.

Q. Do you have any suggestions on alternative elution buffers that avoid imidazole?

A. This is a difficult question, because imidazole is very efficient. Decreasing the pH should give elution (pH ~ 4–5), although not as efficiently as imidazole, at least not with Ni (possibly more efficient with Cu and Zn [?]). Also, some proteins will not like low pH. Elution with EDTA is possible. It will also strip off the metal ions (unless you use Ni Sepharose excel), but the EDTA-metal ion complexes can be removed fairly easily from the eluted protein by buffer exchange. Having to reload the column with new metal ions might have some advantages, because the EDTA and reloading procedure will clean the column for the next use. But note that stronger cleaning with NaOH or other agents might also be needed in some cases. With various degrees of success, ammonium salts have been used at high concentration. Histidine has been used historically for elution in IMAC.

Q. I use ÄKTA start to purify a heterologous His-tagged protein recovered from the inclusion bodies from *E. coli*. This means that the buffers applied to the system contain high concentration (8M) of urea, both to load the protein mixture onto a HisTrap column as well as to elute the purified protein with imidazole. The challenge in my experiment resides in the refolding step, since I have tried various dialysis protocols and dilution-concentration procedures for buffer exchange, but the protein of interest has not remained in solution after any of these techniques. Would there be any inconvenience in eluting the purified protein from the HisTrap column using a buffer that is different from that in which the inclusion bodies were first denatured and loaded onto the column? That is, performing the purification and buffer exchange steps all at once and in the same column. I have not found anyone who has done this before and so I wonder if there is any potential risk of damaging the column.

A. Indeed, on-column refolding on an IMAC column (or other column that binds the protein) can be done, and I think it is used routinely by some folding specialists. The principle is to refold the protein while bound (by decreasing, at a slow rate, the concentration of denaturant, i.e., urea), thus preventing the biggest problem in conventional refolding, namely that the partially refolded molecules aggregate hydrophobically. Because each protein is bound individually, aggregation during folding cannot be a dominating process in on-column refolding. One could argue that having an attachment point could restrict the molecular mobility and thus be negative to the refolding process. I do not know if that is the case with some proteins. After the decreasing-denaturant gradient is finished, imidazole-elution is done, often gradient-wise because contaminants also might have been bound. IMAC columns are quite robust and can be recharged with new metal ions if some are lost, so I do not think that the column can be functionally harmed as long as chelating substances are not applied. Also, be a bit cautious with reducing agents (use TCEP if possible instead of DTT/DTE/beta-ME). Thus, if the refolding involves disulfide formation with red/ox substances present, special protocols might have to be developed. I could imagine that there are literature protocols with application of disulfide isomerase or other "folding helpers" into the column. Examples of on-column refolding should be in the literature, and there is an example and references in our handbook Purifying Challenging Proteins (can be found as pdf on GE Healthcare Web site).

Q. I have expression of my His-tagged target protein, as seen by SDS-PAGE of the carefully clarified *E. coli* extract. The M_r seems to be as expected, but I do not get any binding to the IMAC column. Do you have any recommendations on how to get binding to the IMAC column?

A. Is the pH of the extract too low? IMAC is often run at pH 7–8; histidines will be protonated and nonbinding at lower pH values. Have you confirmed (by Western blotting or otherwise) that the his-tag indeed is there? (The his-tag is too small to have its presence verified only from the M_r on SDS-PAGE.) If the his-tag is not on the target protein the problem could be an incorrect plasmid sequence, premature translation stop, protease cleavage, or other possibilities.

Also, the target protein could have formed soluble aggregates in the extract, thus preventing most or all IMAC binding. The presence of aggregates cannot be detected by SDS-PAGE, because aggregates will be dissociated by the SDS (and the reducing agent) when analyzing the extract and the flowthrough, leading to misinterpretation of the SDS-PAGE.

Sometimes not getting eluted protein can be interpreted as meaning there was no binding, but this is not always true. In cases where you see binding (i.e., the applied target protein is not in the flowthrough) but get no elution, the problem can be irreversible precipitation of the target protein in the column due to too high concentration in the desorbed protein zone. Investigate the latter by applying far lower amount of protein in a test.

Q. I have seen or heard a lot about imidazole of inferior quality. How would you know imidazole is of high/good quality - is there any particular thing that we have to check, or is ACS grade good enough? Also, can imidazole be autoclaved? And what's the stock concentration that you normally suggest?

A. Pure imidazole will not have any, or very low A_{280} absorbance. If it has A_{280} absorbance, there are impurities. The impurities give a slightly yellow-colored imidazole powder. We have seen several imidazole brands named "ultrapure" or similar, which are clearly yellow! We buy our imidazole from German Merck (Merck KGaA, Darmstadt) – they call it "Imidazole buffer substance". We have no information on autoclaving. The stock can be 2.0 M imidazole-HCl, pH 7.4. Lots of HCl will be needed. If the HCl is added too quickly, temperature will rise, and then it must be cooled to room temperature before the final pH adjustment.

Q. I am trying to purify 6xHis tagged proteins produced in CHO cells. I have tried different resins (cobalt Talon Superflow™, HisTrap excel, HisTrap crude, His Mag Sepharose excel bead...) with no imidazole in the sample or washing buffer, but most of my protein is washed away in the flow though. Do you have any ideas (besides changing the location of the tag)?

A. Is the pH of the extract too low? IMAC is often run at pH 7–8; histidines will be protonated and nonbinding at lower pH values. Have you confirmed (by Western blotting or otherwise) that the his-tag indeed is there? (The his-tag is too small to have its presence verified only from the M_r on SDS-PAGE.) If the his-tag is not on the target protein the problem could be an incorrect plasmid sequence, premature translation stop, protease cleavage, or other possibilities.

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Is this a case of secretion from the CHO cells? In such cases we recommend Ni Sepharose excel if it turns out that the ordinary Ni Sepharose loses too many Ni ions due to chelating substances in the cell culture supernatant. Some cell culture media have extreme levels of chelators that very severely compete with the his-tags for IMAC binding (although the chelators do not cause Ni loss from Ni Sepharose excel). To check this, take a part of the cell culture supernatant and do a buffer exchange to conventional IMAC binding buffer, to get rid of any problematic chelators.

Q. I am working with a 20 kDa protein. I always get impurities of higher molecular weights while purifying using a Nickel column and imidazole gradient. Do you have any suggestions?

A. Your protein might co-purify with a chaperone protein. A way to get rid of the chaperones is to add detergent and/or reducing agents before sonicating the cells. The chaperone GroEL can be dissociated from the protein by washing with buffer containing ATP (5 mM ATP, 20 mM $MgCl_2$).

Q. I am trying to remove two proteins from a reaction mixture via affinity capture. Currently both have *a strep* tag and are removed in one go with *Strep*-Tactin™ columns, however this is leading to inefficient removal. I was thinking of trying two types of affinity in conjunction, in a single chromatography (i.e., one column after another) to reduce running time and costs. Have any of the panelists performed this kind of one-step, double affinity capture before, and can they comment on the efficiency and difficulties that might be associated with it?

A. Yes, double-tagging is known from the literature, often with histidine-tag (his-tag) as one of the tags. I would imagine most people use two separate steps: Collection of the eluate from the first column, then adjustment/buffer exchange to get the proper buffer for binding to the second column. However, two columns in tandem should in principle be possible because the presence of imidazole, up to a considerable concentration, is regarded compatible with strep tag binding to Strep-Tactin. See related examples in this poster associated with ÄKTAxpress: https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314774443672/litdoc28930532_20161014162116.pdf

Then there is TAP, tandem-affinity purification, that in its original form (Rigaut *et al.*, *Nature Biotechnology*, 1999) was designed to avoid dissociating protein complexes during purification by using especially mild binding and elution conditions, and uses the tags calmodulin binding peptide and protein A. The resins are Calmodulin Sepharose™ and IgG Sepharose. A protease cleavage site is situated between the two tags, and one of the tricks is to desorb from the first column (protein A tag binding to IgG Sepharose) by means of TEV protease cleavage. Then the eluate is allowed to directly enter the second column (Calmodulin Sepharose), where the desorption subsequently is done with EDTA because the binding there is calcium-dependent. At the moment, we do not have any direct TAP experience. There are several variants of TAP tagging and TAP purification – see the literature. Some variants might fit your requirements, even if you do not work with complexes.

Q. When running nickel-affinity purification, the A_{280} reading increases linearly with increasing imidazole concentration, resulting in significant background. Is there any way to correct for this using the software?

A. One of the most useful ways to deal with this is to do a blank run and subtract that from your sample run during analysis.

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AC – General

Q. Can I load the sample (200–400 mL) through the buffer inlet valve on an affinity column?

A. Yes, I see no problem with that as long as you clean the inlets afterwards. You will have a minor loss of sample in the inlet tubing, but it sounds like sample availability is not a problem.

Q. In our lab we are having some problems using VIIISelect for purifying factor VIII. Although we followed the recommended instruction protocol, we systematically obtained low recoveries (less than 40%). Since no fVIII is present in the flowthrough fraction we assume the problem is in elution buffer. Is there a new protocol for that?

A. Is it possible that you have lost some fVIII during washes before elution? I guess you have so far used the recommended elution buffers: 20 mM histidine, 20 mM calcium chloride, 1.5 M sodium chloride, 0.02% Tween 80 dissolved in 50% ethylene glycol at pH 6.5 (given in the typical protocol in the Data file for VIIISelect). We have little or no direct experience with other elution protocols, but it would be wise to test increasing the concentrations of all the ingredients, one by one, and in combination. Other agents that might help with desorption in affinity chromatography in general are propylene glycol, arginine and MgCl2; the two latter are often used at concentrations of 1 M or higher. As for the recommended elution buffer, combinations of the three mentioned agents should be tested.

IEX – Ion exchange chromatography

Q. I have a question regarding DEAE resin. I am looking for a DEAE resin with pore size of ~ 1000 Å without sacrificing flow rate and functionality. I am trying to capture a 900 kDa protein on DEAE. Or will diffusion of that size protein in 5 min residence time require a bigger pore size?

A. The pore sizes for our agarose beads vary, so we cannot give you a fixed number. The pore sizes are not as large as 1000 Å and the pore size depends on how much agarose we have in the beads. I think you can use our DEAE Sepharose Fast Flow resin for a 900 kDa protein, but that is something you need to try yourself.

Q. What are the differences between strong and weak ion exchangers?

A. The main difference is how much the charge of the matrix varies with pH. For strong ion exchangers the charge is almost constant over a wide pH range. This allows you to see only the pH effect on your sample without interference from a change in the matrix's charge. So strong or weak does not refer to the strength of protein binding or to the binding capacity of the matrix.

Q. Would you please give an example when HIC will be more advantageous than IEX?

A. There are no general guidelines on when proteins will act better with IEX or HIC. To get a definite answer you need to try both. However, if you know that you have a more hydrophobic and salt tolerant protein, HIC is a good choice. If you have a problem binding your protein because it has a pI close to the pI of contaminants that you want to remove, then HIC can be a good alternative. And if the protein is pH sensitive, HIC could be an option because it is based on hydrophobicity.

Q. Why is acetone better than 1 or 2 M NaCl to measure A_s on IEX?

A. It depends on the application. We usually recommend acetone for IEX, because Na or Cl ions can interact with the resin.

Q. I am currently using a combination of CIEX and NTA columns to purify a translocation protein, with a C-terminal 12 his-tag. I am thinking about running the CIEX then following up with the NTA column (mainly because NTA followed by CIEX has given mixed results). Will the presence of the his-tag change either the buffer conditions required to isolate the protein in a cation exchange column or the protein's ability to bind to the cation exchanger in general?

A. Because histidine has a pI of 7.59, it depends on the pI of your protein. If it is not close to 7.59, then the his-tag might have an effect. But I cannot say how much, based on the information provided. How much the tag will affect the binding and elution conditions using cation exchange (CIEX) can only be known by testing, because it also depends on how the histidines are positioned in the 3-D structure of the protein.

SEC – Size exclusion chromatography

Q. What's the difference between Sephacryl and Superdex columns? I see that Superdex columns are expensive – why? Why would you choose Superdex columns when the separation range is more or less similar in some cases?

A. Superdex™ prep grade and Sephacryl™ columns have different performance, where Superdex pg are the high performance columns. Both the resins and the column hardware are different. Superdex pg resins have an average particle size of ~ 34 µm while Sephacryl have ~ 50 µm. This is the main reason that Superdex pg columns will give much higher resolution compared to Sephacryl columns. Because Superdex pg also have higher pressure tolerance, you can run at higher flow rate compared to Sephacryl. The recommended flow rate is twice as high on Superdex pg columns. Maximum pressure over the packed bed is 0.3 MPa for Superdex pg and 0.15 MPa for Sephacryl columns. The column hardware in HiLoad Superdex pg is our XK glass columns, which can be opened and repacked if needed. HiPrep Sephacryl columns are plastic (polypropylene) and cannot be opened. These are the reasons why HiLoad Superdex pg columns are more expensive than HiPrep Sephacryl columns.

Q. How do I best manage pressure and temperature using GE's SEC "Increase" columns?

A. The flow rates from our instructions are given at 20°C to 25°C. If you are working at 4°C, flow rates should be divided by 2. And if the column contains 20% ethanol in water as a storage solution, divide the flow rate by 2 again.

Q. I purified a protein using an antibody column. When I load 100 µL of the "pure" protein into a Superdex 200 10/300 GL I see a major peak eluting around 8 mL. The peak is still there (though reduced) when running a serum sample. Any ideas about what it could be? I thought it could be antibody eluting from the column.

A. On a Superdex 200 10/300 GL column 8 mL is very close to the dead volume of the column. There are two possible reasons for these results. One possibility is that the peak contains very high molecular weight proteins in the form of aggregates. If you are working with IgM the size is larger than 600 000. Another possibility is that your target molecule is not passing into the pores of the resin due to high charge. In that case, you might need to optimize your buffer conditions using, for example, higher salt concentrations or ionic strengths.

Q. What kind of column should I use to work in a purification range between 50 kDa to 1 MDa?

A. If you want to cover the whole M_r range, we recommend Superose™ 6 pg or Superose 6 Increase. However, if you want to separate proteins in specific ranges that are lower than M_r 600 000, then Superdex 200 can be used. Superdex 200 Increase columns have a molecular weight fractionation range of approximately 10 000 to 600 000. The fractionation range for Superose 6 Increase is M_r ~ 5000 to ~ 5 000 000. If you have volumes less than 250 µL the format 10/300 is recommended. For volumes between 0.5 and 3 mL, use the HiLoad™ 16/600 format. For volumes between 1.6 and 12 mL, use the HiLoad 26/600 format.

Q. We want to resolve multimers of a unique protein. It has forms at 100, 200, 300, and 400 kDa, with 300 kDa being the dominant form. Which would be the best column to separate only the 300 kDa form?

A. Superdex 200 Increase is probably the best choice, but it also depends on the sample volume. For small volumes (less than 500 µL), Superdex 200 Increase 10/300 GL will be the best column to use. Using two columns in series will provide the highest resolution. For preparative runs of larger sample volumes, Superdex 200 resin is available in HiLoad columns.

Q. I am trying to use SEC to get pure h-albumin from a commercial HSA. What column can serve the experiment? When I use a Superdex 200 column (16/600), I have a broad HSA peak.

A. If you want to have sharper peaks I would recommend Superdex 200 Increase 10/300. It has smaller beads. You can also try the Superdex 75 10/300, which has a molecular weight range up to ~ 70 000. However, you should only load volumes up to 250 µL to keep a high resolution. If you want to continue to use the Superdex 200 pg 16/600 column, you should lower the injection volume (max 2 mL on the 16/600 for optimized resolution). Furthermore, the system also has a big effect on the resolution. Try to minimize the volumes of the tubings. Large diameters and long lengths affect the resolution in a negative way.

Q. We have discovered some older Superdex 26/60 columns in our store room that are technically out of date (expired 2016). But they have never been used. What would you say is the likelihood that we can still use them with confidence, and how would you test these particular columns?

A. We cannot guarantee that it would work, but it probably will. If you are uncertain try to test the column using a HMW kit and also perform a performance test by injecting 200 µL of 2% acetone in 20% ethanol. You can find the procedure in the instruction for the column.

Q. I can see a gap at the top of my Tricorn™ Superdex 200 10/300 GL when I put it on flow rate. What should I do?

A. First you should check if the pressure is okay. If it is too high, then you need to clean the column and lower the flow rate. We recommend cleaning it at least after 10 runs. It should be cleaned using recommended cleaning procedures, which you can find in the instruction. As a last procedure you should change the top filter if the pressure still is too high.

Q. Which columns would you suggest for purification of peptides?

A. For SEC I recommened Superdex Peptide 10/300 or Superdex 30 pg columns. Reversed phase columns are also commonly used for separation of peptides.

Q. When I store an XK column packed with Superdex 200 prep grade resin in a 20% ethanol solution, the following day I can observe a liquid gap. This forces me to repack the resin. Is there any way to avoid this situation?

A. If you are adding the 20% ethanol with a flow rate that is too high, there is a risk for a gap. Always check that the pressure is not exceeding the maximum pressure for the column. Use a lower flow rate for 20% ethanol when you see that the pressure is increasing.

Q. For mid-range antibody production from hybridomas and transfected cell lines (typically 50–200 mg) what is the best resin to use for size exclusion to remove aggregates? We are a research institute and currently pack our own Superdex 200 prep grade columns of various sizes, from 20–500 mL.

A. For smaller scales (sample volumes < 250 µL) I would recommend Superdex 200 Increase 10/300 columns. They are well suited for separation of antibodies and their aggregates. For larger sample volumes, HiLoad 16/600 (sample volumes 0.53 mL) and 26/600 (sample volumes 1.6–12 mL) packed with Superdex 200 prep grade would be prefered.

Q. I often run into the problem of having too large sample volumes for my SEC polishing. For example, the combined pool from IMAC is often too large for my 16x600 mm Superdex pg column.

A. Apart from getting a larger column, you could consider A) concentrating the sample by ultrafiltration to decrease the volume, B) using repeated SEC runs with application of portions of a large sample, C) discarding the front and tail of the IMAC peak(s), harvesting only the center.

There are various single-use devices for centrifugal ultrafiltration that take sample volumes from < 1 mL up to 60 or 100 mL. The device can also be topped up with even more sample as soon as the first centrifugation has been done, and two or more devices can of course be used for the same sample. For repeated SEC runs: If one run takes 2 h on the column, including re-equilibration, repeated sample applications allows for 4 runs in one day (8 h).

Q. I am working with membrane proteins, so my buffers contain detergent. During a gel filtration (I use Superdex 200 10/300), are the MW obtained from standard curve (made using soluble proteins) reliable? I need to add an ion exchange step of a membrane protein negatively charged (pI is around 5.9 and buffer pH is 8) in a buffer with DDM (nonionic detergent). What should I be particularly aware of when I plan this step? Could I be rid of the free detergent micelles? Will the detergent interfere with this purification step (influencing the protein pI, for example, or interacting with the column)?

A. The MW of integral membrane proteins (MPs) cannot be determined in gel filtrations calibrated with soluble proteins, because they are present as MP-detergent complexes with a size that of course is larger than the size of the polypeptide (i.e., the protein without any bound detergent). The elution position depends on the MW of the complex, while the elution position of a soluble, globular protein depends on the MW of the polypeptide. The amount of detergent bound to each MP molecule varies

between different MPs, but a very rough rule of thumb is that 50% of the MW of the MP-detergent complex is detergent and 50% is polypeptide. I have not seen any gel filtrations published where the calibration has been done with membrane proteins in a straightforward manner (especially because MPs are not very stable in detergent solution). But the problem is dicussed in the literature, for example Kunji, E. R. S. *et al. Methods* **46**:62–72 (2008) and Le Maire, M. *et al. Analytical Biochemistry* **154:**525–535 (1986). (When reviewing the literature, note that "integral" membrane protein means that it is embedded in the membrane bilayer, and not merely associated with the membrane surface.)

For the IEX, you should be aware that the pI (calculated from the sequence I guess) might not be the actual one because some charges of the protein, especially charges located at the surface of the membrane bilayer, might be sterically hidden by the bound DDM. So you should also consider other pH values if pH 8 does not work. In fact, if the extramembraneous parts of the MP are very small, the bound DMM could sterically prevent all electrostatic interactions with the resin. I do not think that the DDM, neither free monomer DDM nor DDM micelles, will interact with the column. If you want complete DDM binding to the MP (saturation), the DDM should be in excess, and I think that most people say that having excess also means that some detergent micelles are present. However, complete DDM binding might not be needed for some MPs. They can be solubilized in a stable way also with slightly lower concentrations of DDM, but it can take a lot of work to find out if that is possible with your MP. I guess most people purify their MPs in excess detergent. Some people consider an excess to be a concentration above the CMC, but the concept and data of CMC is based on pure detergent and not a mixture of detergent, membrane lipids and MPs; often, concentrations of the two latter are unknown. So it is not quite clear which total detergent concentration to aim for. There is some advice and practical examples of MP purifications in the GE Healthcare handbook Purifying Challenging Proteins, available as downloadable pdf on our Web site. You should also study published examples of successful MP purifications, which are frequently found in papers on MP crystal structures.

Q. When running a gel filtration column in water and then changing the bottle with buffer, is it necessary to do a system wash step?

A. System wash or pump wash is always good when changing liquids during a run. The difference between these are that the pump wash sends the liquid via the injection valve to waste, whereas the system wash sends the liquid to waste via the outlet valve.

Q. We saw a gap on top of our S-200 column that we then ran in reverse flow order. Now it cannot be run even at very slow flow rate without going over pressure. Can it be fixed? Just to clarify, the red filter bit didn't move and the gap was between this and the resin.

A. I suspect filter blockage. Try changing the adapter filters. Is this maybe a column packing issue? If the column was not purchased prepacked, were the packing instructions followed? I am addressing several questions here, and maybe you could contact your local GE representative to discuss this in more detail.

Protein analysis

Q. My collected fractions are sometimes not concentrated enough to allow me to see proteins in SDS-PAGE. Is it possible to concentrate them? I cannot load a higher volume in each lane of my gel.

A. You can perform a TCA/acetone precipitation on a portion of each collected fraction. For example, you can precipitate all your proteins from 200 µL of a fraction to a final volume of 20 µL. And then put these 20 µL + 20 µL SDS buffer in your well.

Q. I need to measure the Cibacron blue in Capto™ Blue eluates. The available literature suggests the spectrophotometric method using standard addition. Is there any other method for this purpose?

A. I would say that the spectrophotometric approach would give high sensitivity only if absorptivity (Epsilon in Beer-Lambert's Law) of Cibacron blue is very high. I do not have any idea if an ELISA approch is possible for a more sensitive quantitation.

Q. Do I usually need SDS-PAGE to analyze purity? Isn't it sufficient to see "nice and symmetric peaks" at IMAC and at the SEC polishing that follows?

A. It is generally not possible to say that a chromatography peak corresponds to a pure component, if the fractions from the peak have not been analyzed. In terms of resolution, versatility, and relative ease, nothing beats SDS-PAGE for purity analysis and for semi-quantitative info on target protein recovery (e.g., comparison with known amounts of target protein). Information on possible losses of target protein into flowthrough or wash is also obtained, if those samples are analyzed. Combining SDS-PAGE with Western blotting (using target-specific antibody) can also give confirmation of protein identity. It is seldom seen in SDS-PAGE that two components are present in a single band, if the dilution is sufficient to get a thin band.

UNICORN software

Q. Is there a software program you can recommend to prepare chromatograms for publications/dissertations? Copy/paste directly from UNICORN into Word and other programs results in low resolution images.

A. Have you tried the copy/paste function in UNICORN 7.0 or later? We have improved the image quality in the evaluation module of UNICORN 7.0.

Q. When I run different columns but similar samples and want to compare the results, the overlay looks very strange since the CV are different. Is there a way I can easily compare the results?

A. In UNICORN 7.0 Evaluation and later you can compare results with CV on the x-axis. In this way you can compare runs performed on different column sizes.

Q. In my lab we often run similar methods, with small changes, over and over again. Is there any way that I can avoid changing the methods in between runs?

A. I would suggest that you define the changes you do as variables in the method and present a variable list when starting the method. Then you can change the parameters just before start of the run without the need to re-program the method each time.

Q. We have UNICORN 5.31 running. Can we upgrade to UNICORN 7.0, for running UPC 100? If yes, how do I go about it – is it a seamless upgrade or do I have to buy the software?

A. ÄKTA purifier UPC 100 is supported by UNICORN 7. You can import methods and results into UNICORN 7 if you like. You will need to buy the software license for UNICORN 7.

Q. I use UNICORN version 7 and the evaluation module. My result is not integrated when I open it. Why?

A. In general, runs generated on previous UNICORN version will not be auto integrated but can be integrated with a single click when they are opened. Another possibility is that the auto integration function has been turned off in Tools:Options.

Q. The current software which we have (UNICORN 5.31) does not take into consideration the presence of the sample pump 960 in the diagram of the flow path. Is there a fix for that?

A. The flow scheme presentation is determined by the strategy used. I do not have full insight into all existing strategy versions, but if you use a late version of the strategy and it is not supported, then I am afraid this is the case.

Q. Is there a version of PrimeView or UNICORN that is compatible with the ÄKTAprime but is up to date with all data integrity requirements?

A. There is, unfortunately, no version of PrimeView or UNICORN that is compatible with ÄKTAprime and uses the database to support the data integrity requirements. If you require data integrity, we recommend using another system such as ÄKTA pure with UNICORN 7.

Q. Is it possible to open more than one result for comparisons by UNICORN 6.3?

A. Curve overlays are available in UNICORN 6.3, and you can stack results. For even more ways to compare results try the new evaluation module in UNICORN 7.

Miscellaneous questions

Q. What is the difference between selectivity and resolution?

A. Resolution is the ability of a packed column to separate two solutes (e.g., proteins). The solutes are monitored using detectors, most commonly UV. Selectivity is the relative retention of two solutes (e.g., proteins) in a column. Selectivity is related to the distance between two peaks.

Q. Which is the most efficient reducing agent, DTT or DTE (or TCEP)?

A. Formation of a 6-membered ring structure (cyclic disulfide) is part of the driving force for the redox reaction of DTT and DTE. I have seen in the literature that DTT should be slightly more efficient, because the ring structure formed upon oxidation of DTT is more stable than the ring formed from DTE. This has to do with the lower steric interference of the two –OH groups on the DTT ring (trans position of the –OH groups) compared to the DTE ring (cis position). TCEP is more efficient than DTT/DTE and is more stable. TCEP is also considered to be fully compatible with IMAC, because it does not contain chelating –SH groups.

Q. What is the best way to eliminate bacterial DNA contaminants during protein purification?

A. Instead of removing DNA, sonication and DNase treatment will only break down DNA into fragments, thus decreasing viscosity. But that might not help from having problems with DNA fragments ending up as a final contaminant, if that is what you have experienced. If those treatments do not help, an alternative is to use a DNA/RNA precipitation method on the bacterial extract (e.g., polythyleneimine, spermine, and even ammonium sufate). A good starting point to search the literature/web for details and protocols on DNA precipitation is DeWalt, B. *et al.* Compaction Agent Clarification of Microbial Lysates. *Protein Expr Purif.* **28:**220-223 (2003). DNA precipitation methods might co-precipitate some of the target protein, although the DeWalt paper claimed low loss of protein.

Another option is IEX: Because DNA is multicharged, bacterial DNA should show a relatively high affinity for anion exchangers, provided that the ionic strength is not too high. In favorable cases, the AIEX conditions can be arranged so that either the target protein does not bind or the target can be eluted at far lower salt concentrations than the DNA can.

Q. Sometimes I have some scale problems when I run IMAC. The A_{280} scale starts with negative values or it decreases throughout the run. An autozero solves the problem sometimes but not always. Could it be an issue due to calibration of the UV monitor?

A. The problem could be that the autozero is made too early in the method, directly (within millisec) at the method start when the flow and the UV signal(s) are not yet stable. In a test run, make manual autozeros a couple of times after 1.5–2 column volumes (CV) of the column equilibration and/or just at the end of the column equilibration and see if that helps (equilibration should be at least 5 CV – check that UV, conductivity, and pH all are stable at the end of equilibration). Also, use a separate spectrophotometer with water as reference to check if your buffers have an abnormal absorbance. Imidazole of inferior quality has A_{280} absorbance, which can complicate abs curves and also can be a problem at method start and equilibration if you have imidazole in the equilibration buffer.

A rather common problem seen, which possibly also can give zeroing- and baseline-problems, is that 100% elution buffer (imidazole) is left in the tubings before the column, because a pump B wash was done at an improper time. Do the pump washes to introduce the buffers separately before the method start, and pump equlibration buffer through the tubings before attaching the column, to safely get rid of the B-buffer from the pump wash.

Although seldom seen, try to exclude UV lamp problems (e.g., by observing the UV signal stability with just flowing buffer or water over some hours, no column).

Q. How can I wash and solubilize the inclusion bodies from *E. coli* when I have a hydrophobic protein of interest?

A. Maybe you refer to the fact that all proteins are hydrophobic if they are in an unfolded state, thus aggregating hydrophobically if no denaturant is present (like urea) and the concentration is too high. But if you mean that your protein will be hydrophobic also after it has folded to the native structure, I would say that one possibility is to go for on-column refolding. In such a procedure, the folding is accomplished by first getting binding to a resin in the presence of denaturant, and then lowering the concentration of denaturant while the protein is still bound to the resin (e.g., an IMAC or IEX column). After the refolding has been accomplished (note that some proteins are almost impossible to refold, whether on-column or in solution), elution from the IMAC or IEX column is done. At that point, for a hydrophobic protein, some measures can be taken to avoid aggregation (e.g., having a suitable detergent or a proper percentage of organic solvent present in the elution buffer).

Regarding on-column refolding and refolding in general (including washing and solubilization), as a starting point for getting more detailed information from the literature/web, please see examples in our handbook called Purifying Challenging Proteins, available as pdf at the GE Healthcare Web site.

If you specifically refer to washing of inclusion bodies and solubilization of them with denaturants, I cannot see that those procedures will differ between “normal” proteins and other proteins that will be hydrophobic after the refolding.

Q. I have two broad peaks eluting one after the other over about 3 mL fractions, with my protein of interest appearing in all. Is there an ideal way to concentrate the elution into one larger peak?

A. A spin column with correct molecular weight cutoff might be a good idea. But it also sounds like your target is co-eluting with the impurities. Have you tried some other “orthogonal” technique as a second purification step?

Q. What can cause an asymmetry value that is unacceptable?

A. Factors related to the chromatography system can affect the peak asymmetry factor (As). If there is too much system volume in tubing and valves, the peaks will be broader. Minimize the length and diameter of tubing, and use sample and column valves with small volumes. However, in most cases when an asymmetry factor is unacceptable, it is due to the packing of the column. If you can see that the peak has a tail or is fronting (i.e., spreading out in the beginning of the peak), you need to repack the column. For repacking suggestions see the answers to "What are some things we can try to improve the asymmetry result of the column that we packed?"

Q. What are some things we can try to improve the asymmetry result of the column that we packed?

A. The slurry concentration and packing flow rate/pressure influence the packing results. Here are some suggestions:

- If you adjust the slurry, keep the other parameters as before. When As is too high, a lower slurry concentration (e.g., 65%) could be tried.
- If you adjust compression, keep the slurry concentration the same. When As is too high, additional compression might be helpful.
- High slurry concentration (80%) generally gives unstable beds, so if possible avoid that.
- Perform a two-step packing method. Start with settling the bed at a low flow rate. Then increase the flow rate and compress the bed to the desired compression or bed height. If the As of a twostep packing method is high (As - 3), it is usually a good approach to decrease the settling flow rate, increase the compression flow rate, or decrease the slurry concentration. Choose one of the three, evaluate the results, and adjust if needed.

Q. I'm seeing a noticeably significant decrease in conductance during protein elution peaks (i.e. when UV absorbance is the highest during elution). Is this normal, or do I need to change the running parameters? It has occurred in an NTA (IMAC) column and multiple CIEX columns.

A. Indeed, it is normal that high protein concentration will tend to decrease the conductivity. This is due to protein-volume exclusion of the small ions that give most of the conductivity, and to the low mobility and thus low conductivity contribution of protein.

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