GE Healthcare

First ÄKTA™ club web chat

Your protein purification questions discussed with GE Healthcare R&D scientists

Our first ÄKTA club web chat was held on September 28, 2016. 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 scientists from our R&D team in Uppsala and from Scientific Support. Questions, about chromatography techniques, troubleshooting tips and tricks, and the use of ÄKTA systems, were submitted in writing by ÄKTA club members. 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 to get notified of upcoming web chats and other events.

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 platform 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.

Meet our GE experts and read their answers to your questions

Dr. Tuomo Frigård, Scientist, GE R&D

At GE Healthcare since 2003, Tuomo has been involved with developing ÄKTA chromatography systems and UNICORN™ software. He authored the 'Design of Experiments in Protein Production and Purification' handbook. Tuomo also leads ÄKTA system and DoE training courses and lectures for academic and industrial participants in Asia, Europe, and USA.

Q. What is the minimum height of the column to check the dynamic binding capacity of resin, for instance Capto™ S? Can a prepacked column be used for this? If not, why can't it be?

A. Of course, the minimum height depends on the packing efficiency. In our labs we are running columns down to 0.5 cm bed heights for this purpose. Usually we do not use prepacked columns, but we will add this suggestion to our wish list.

Q. How do I calculate the DBC if breakthrough happens?

A. There is a UNICORN extension that can be downloaded at the ÄKTA club webpage for calculating DBC at breakthrough.

Q. What's the best way to detect my protein of interest if it can't be seen by ÄKTA system UV detector?

A. Detection of a protein of interest also depends on selecting the appropriate cell length (10 mm is the longest). But if you are trying to detect trace levels of proteins some other assay must be used. Other in-line techniques are of course interesting depending on the nature of the protein. For example, if you are interested in protein aggregates a light scattering detector might come in handy.

Q. What would be the effect on the resin if we do not store it at the recommended temperature, for example 4°C, due to some limitation like no cold room in a GMP environment? How can we check the functionality and efficiency?

A. Of course, the recommended column storage temperatures are based on the assumption that we always want to work with tools that are fully functional and performing at their best. However, certain resins containing ligands that could be susceptible to breakdown for different reasons should be treated with more care. Using a product that has not been stored according to recommendations does not hinder its use but should be tested with buffers in column performance studies and prior to running precious samples. We cannot guarantee functionality if storage recommendations have not been followed. Procedures for column performance studies are available in UNICORN.

Q. When purifying protein with a GFP tag, will this leave a residue in the flow path and skew absorbance measurements?

A. We have purified loads of GFP in the lab, and we have not seen this. If you believe your absorbance measurements are skewed, check if appropriate CIP-system procedures have been used.

Q. How can I purify a glycoprotein hormone? Do you have any standard procedure like for mAb? What total product recovery is normal?

A. I'm not aware of any standard procedures for purifying these proteins, but we know that affinity chromatography is often used as an initial step. We would have to scout users in our labs to see if there are some expected recovery rates.

Q. Can design of experiments be used in our old ÄKTAexplorer system (UNICORN 5)?

A. No, ÄKTAexplorer systems running UNICORN 5 or upgraded to UNICORN 6.4 do not support DoE. To use DoE with ÄKTAexplorer you will have to run a DoE software externally, which of course will have a negative effect on the ease-of-use and level of automation. ÄKTA avant and ÄKTA pure can be used with the UNICORN DoE module.

Q. How should we do the step elution if we have two peaks during the gradient elution?

A. If you know where in the gradient the peaks are eluted, you can make a two-step elution, changing the salt concentration after the first peak has eluted.

Q. Can ÄKTA pure and ÄKTA avant be set up to have 20 or more sample inlets?

A. Yes, it is possible to achieve this. You will have to combine using additional sample inlet valves and extra valves. You will also need to modify the UNICORN method so that it works according to expectations.

Q. Do you offer any troubleshooting course so that we can learn to troubleshoot ourselves instead of calling an engineer to our site?

A. Currently, we offer courses in North America that discuss troubleshooting tools to identify and resolve common problems. More information can be found here: https://promo.gelifesciences.com/gl/K15176/reg.html?extcmp=K15176-GL-pubfly-sales.

For trainings in other regions, we recommend that you ask for availability on: https://proteins.gelifesciences.com/discussion-forum/ask-an-expert.

Troubleshooting information is also included in the user manual for each ÄKTA system.

Q. I'm considering an ÄKTA system for protein purification, but my HPLC system works fine. What are the benefits to using an ÄKTA system over my HPLC?

A. Preparative protein purification systems such as ÄKTA systems are salt-tolerant, low-flow/pressure chromatography systems where the pumps and internal plumbing, flexibility, and automation capabilities are optimized for protein purifications. In addition, and maybe even more importantly, these conditions are more likely to maintain the integrity of your protein. High pressure conditions used in HPLC may be detrimental to the protein structure.

Dr. Jon Lundqvist, Scientist, GE R&D

Jon has more than 20 years of experience in protein purification, carbohydrate fractionation, resin development, and methodology development. His skill set includes size exclusion chromatography, affinity chromatography, glycosylated proteins, and enzymology.

Q. We are involved in protein purification (34–128 kDa) and use ÄKTA start. We use affinity chromatography to first isolate the proteins of interest. But before we proceed to the next step, gel filtration (S-200 HR), almost 60 percent is lost. We checked to see if it was in the flow through or washed out as unbound filtrate, but nothing showed that it had escaped. What advice will you give me?

A. One possibility could be that the protein still is bound to the resin or has been lost in sample preparation. Depending on which affinity resin you are using, you might need to change the elution conditions by increasing the concentration of the elution agent (for example, the concentration of imidazole in IMAC). A rougher way to check whether something is bound to the resin is to CIP the column using 0.5 M NaOH or analyze the beads with SDS-PAGE.

Q. We were using Superdex™ 200 10/300 GL columns and have now replaced them with Superdex 200 Increase 10/300 GL. The runs with Superdex 200 Increase 10/300 GL gave strange results: More peaks were detected using the same sample consisting of a pure protein and also the HMW Calibration Kit from GE. We injected the same protein batches on Superdex and Superdex increase columns. The results from the old Superdex column are okay (one peak). The results from Superdex Increase column show several peaks. Could it be an issue with packing of the gel?

A. The resolution on Superdex 200 Increase is significantly higher compared to Superdex 200, and it is not surprising that more peaks will be separated. The application for the proteins in the calibration kit is as molecular weight (MW) markers. They are not 100% pure, and they might also contain aggregated forms. The main peak should be used as the MW marker. So it is not surprising that there is more than one peak in the kit and that more peaks were separated on the Superdex 200 Increase column.

Q. My histidine-tagged (his-tagged) protein seems to disappear when I try to run IMAC purification. With anti-his antibodies in Western blotting analyses, my protein seems present in the *E. coli* extract and in the material applied to the IMAC column. But both the column flowthrough and the elution contain only very small amounts. What is going on?

A. The solubility limit of the protein could be passed when the protein is enriched on the column (i.e., it precipitates in the IMAC column when desorbed at elution). As a test to find out if this is the problem, try applying less material to the IMAC column, in order to keep down the protein concentration in the zone of eluted material. For the same purpose, use gradient elution if you now use step elution, or use a shallower gradient if you now use gradient elution.

Q. With the ÄKTA start system and the UNICORN software, am I able to make my own cleaning method? Then, I would only have to select one method rather than having to wash pump A then select another method to wash pump B, and so on for all other parts that need washing.

A. Yes, you can set up a method where it is possible to use two different eluents for pumping through the system. It should not matter whether it is a purification method or a cleaning method.

Q. I'm working with interleukin 34 that has a his-tag. So after binding to a Ni-IMAC column, we eluted with imidazole. Then the protein was kept for overnight dialysis with PBS. But unfortunately, our protein completely precipitated. What should we do now? Do you have any suggestions to purify IL-34?

A. Based on the information, you should try to purify the protein faster by using an IMAC column followed by a desalting column. It is always a risk to keep a protein overnight at room temperature. One of the reasons it might precipitate is that it is hydrophobic in its character. So if you still would prefer to perform dialysis instead of using a desalting column, I would recommend adding 10% glycerol to stabilize the protein and keeping it at a colder temperature.

Q. Can I use an ÄKTA start system with the XK 16/20 column flowing at 1.5 mL/min and 0.05 MPa?

A. The limits for ÄKTA start are a pressure range of 0–0.5 MPa and a flow rate of 0.5–5 mL/min. So it should be possible, even though it is not the optimal system to use.

Q. Do you have any data you can share on the effects of flow rates, pressures, and exposure time of the proteins for increasing efficiency of binding 6-his tagged proteins to IMAC resins?

A. The yields obtained using different flow rates are of course protein dependent. We have tested one protein that is a low to medium binder to Ni Sepharose™ HP. The different flow rates we used were 1, 2, and 4 mL/min on a HisTrap™ HP 1 mL column. The back pressure was still below the pressure limit of 0.5 MPa. The load of protein was approximate 20% of the dynamic capacity. The results we obtained showed only a minor loss of protein running at higher flow rate. We had a yield of 80%–85% at 1 mL/min and between 60% and 70% at the higher flow rates.

Q. Will stacking IMAC columns increase their capacity to bind his-tagged proteins? I want to get rid of his-tagged proteins from my protein of interest. In this case, if I stack several columns together, will I get better purity?

A. The capacity will be increased, but it will not improve the purity of native histidine-containing proteins unless you overload the columns with histidine-tagged protein. What you can do is to optimize the imidazole concentration in the sample and wash so only the histidine-tagged protein binds to the resin. We have not tried this kind of experiment, so it would be interesting to see if it works.

Q. Can gel filtration columns be equilibrated overnight, taken off the ÄKTA system the next morning, and the GF run performed in the afternoon? (I am asking because I need to do a his-tag purification in the morning).

A. Yes, it is possible. It is not a problem to store the column for a couple of hours.

Q. What can produce high pressure on a RESOURCE™ Q column during washing with H_2O?

A. The H_20 itself does not increase the pressure. If you want to equilibrate a new or a stored column, it probably contains 20% ethanol, which increases the pressure. If that is the case, you need to lower the flow rate until you have removed the ethanol (which can be seen by the pressure and conductivity).

Q. The recommendation for CIP is 1 M NaOH overnight. But we have to use a lot of water to reduce the pH back to normal. Do you have any other method for CIP?

A. It depends on what kind of system you have. If you are using an ÄKTA system you can set up a method for cleaning the system. Then, you do not need so much water to remove the NaOH, probably less than 100 mL.

Q. How often do we need to buy a new his-tag column if we use it for bacteria lysates?

A. It depends on whether you are using the same sample on the column and how crude the sample is. Normally we say that you can reuse one column 5–8 times when loading the same sample, then the column should be cleaned. When using different kinds of samples, you need to clean the column after each time or use a new one.

Dr. Lars C. Andersson, Senior Scientist, GE R&D

For 25 years Lars has been working with protein purification, resin development, and methodology development, especially affinity chromatography. His experience includes purification of integral membrane proteins (IMP).

Q. I am working on a protein which has three cysteines, all of them pegylated with 10 or 20 kDa PEG. I tried to separate the pegylated protein from nonpegylated by using GF, IEX, HIC, and nothing worked. RP-HPLC is not an option because the protein is precipitating. Please suggest any other alternatives.

A. The Cys modification by PEG couplings may not be complete, so it is important to prevent formation of -SS- bonds between molecules that still have their Cys residues. Thus, reduce with DTT, and run the SEC with DTT in the eluent (e.g., 1–5 mM). For SEC, one must first select the resin that has the most suitable M_r separation range for the molecules in question. For this demanding separation problem, high-resolution SEC is needed. Although you do not mention the M_r of the protein, it seems like the separation range of one of our highest-resolution SEC resins, in our Superdex 200 Increase or Superose™ 6 Increase columns, could be suitable. One must remember that the PEG chains are not compactly folded like the polypeptide chain is, which affects the expected size of PEG-protein molecules. SEC optimization is crucial to get a good resolution. Very important factors are flow rate, sample volume, and dead volumes (i.e., extra-column volumes). Check out tips on SEC optimization in the instructions for the two types of columns I mentioned and in our SEC handbook, both found as PDFs on our Web site at gelifesciences.com. Finally, is it possible to use only one size range of PEG molecules (i.e., either the 10 or the 20 kDa range) to get less size heterogeneity at the end?

Q. Can you give suggestions to optimize Ni-affinity purification of membrane proteins solubilized in detergents? Varying the imidazole concentration during binding, washing, and elution is straightforward. But how would you optimize the buffers and pH?

A. In fact, I think the choice of detergent is crucial, because above all, the solubilized protein must be stable and prevented from aggregating in the detergent. Aggregates will not bind in IMAC. A detergent that gives efficient membrane solubilization (high yield) may not be optimal for stability. The usual IMAC conditions should be used (i.e., pH 7–8), but possibly with lower NaCl concentration than the typical 500 mM. Instead, try 150 mM NaCl to minimize hydrophobic interactions. Phosphate buffer is preferred in most cases. Tris or HEPES is also possible, although Tris may show some competition due to Ni affinity. Also, for membrane proteins, it is popular to use a 10-his-tag rather than a 6-his-tag, along with a spacer to get the tag out of the detergent torus around the membrane protein.

Q. Have you analyzed the amount of Ni-ions that will leak from HisTrap columns? How about excel columns, do they retain 100 percent of Ni-ions?

A. Metal ion leakage depends heavily on conditions, sample type and volume, eluents and eluent volumes, so figures are difficult to give. No IMAC resin is really completely without leakage at all conditions. But for Ni Sepharose excel, the Ni binding is really very strong, much stronger than for other IMAC resins. Any metal ion leakage at purification can be further minimized, if necessary, by first running blank run(s) with your elution conditions, and then equilibrating with binding buffer before using it to purify your protein.

Q. What is the optimal length of the his-tag in your experience? Are there any amino acids which should not be close to the his-tag?

A. For non-membrane proteins, a 6-his-tag is mostly used and normally sufficient. For membrane proteins, where the tag needs to reach out from the detergent torus around the protein, His10 and a longish spacer sequence is often used. As with other tags, glycines are often used before the tag. I have not seen any hard data that certain amino acids should be avoided.

Q. If the ÄKTA system has to be placed at room temperature due to space limitations, what can we do to protect proteins during purification (especially when purifying aggregation-prone proteins using gel filtration, which takes a long time)?

A. One should investigate whether there are conditions (pH, buffer, ionic strength, additives) that counteract the aggregation. It should be ruled out that the aggregation is not oxidative (i.e., due to disulfide formation). If cooling is absolutely needed, “cold cabinets” are available, which are like large, standing refrigerators with sufficient space for a chromatography system inside.

Our HiLoad™ Superdex SEC columns and the XK-series of empty columns are equipped with a cooling mantle outside of the column tube. To cool other columns, tubing could be connected to a cooling water bath and the tubing wound around the column. Such a method seems very complicated and nonoptimal, but it is in principle possible.

Q. What is the best column for his-tag purification from bacterial lysates? When I compared HisTrap FF and excel columns, FF columns seemed to bind my fusion protein better. With the excel columns the fusion protein tended to wash out in the sample application waste when using a start buffer with 20 mM imidazole. What 5 mL column shall I use to elute my protein? It is an aggregation-prone protein, so I do not want to dilute or concentrate it. Can step elution with less dilute samples compare to linear elution? What can I do if the sample contains 0.5 mM EDTA? My protein of interest does not contain a his-tag, but I want to run it through his columns to get rid of the fragments that contain a his-tag and get rid of the TEV enzyme.

A. Indeed, the his-tag affinity is generally lower with Ni Sepharose excel than with the regular Ni Sepharose (HisTrap FF and HP). That is why we recommend the regular resin, except in cases with too much loss of Ni ions from the regular resin. Ni Sepharose excel should not be used with 20 mM imidazole at binding (and possibly not 20 mM at wash either) – see the instructions.

If you need to have a certain protein concentration at elution (not too high, not too low), you could make trials with different lengths of the elution gradient. Then, compare those results to step elution

(which normally gives the least dilution), to hopefully find the right conditions for your required concentration. Also, the concentration in the eluate will of course depend on the protein amount and sample amount you load. If you do not use Ni Sepharose excel, the 0.5 mM EDTA should be removed by buffer exchange before IMAC, unless you see in trials that it is possible to have the 0.5 mM EDTA and still get the desired result with respect to removal of his-tagged fragments and his-tagged tobacco etch virus (TEV) protease.

Q. Which gel filtration column should I use for polyglutamine proteins that are aggregation-prone and form dimers? The protein sizes are ~ 13 kDa, 10 kDa, and 9 kDa. Currently we have HiLoad Superdex 75 16/600 (28989333). Is this column suitable for all three proteins?

A. Yes, Superdex 75 has the suitable separation range, M_r ~ 3000–70 000. So you should be able to detect and separate the dimers and possibly also larger oligomers of your 13, 10, and 9 kDa proteins. Note that the resolution of the new Superdex 75 Increase columns is far higher than for Superdex 75 prep grade resin in HiLoad columns. However, the former resin is only available up to a 10×300 mm column size. That column size could be used for your analysis to get ideas about how to run the larger preparative HiLoad column. See the SEC Handbook and instructions for Superdex 75 Increase for information about optimization, which is always important in SEC.

Q. My new target protein, which is not expected to be a membrane protein, seems impossible to keep in solution during expression. I always find it in the pellet when I clarify the *E. coli* extract by centrifugation. Do you have any tips for me on how to move on?

A. If this is not a "simple" protein-solubility problem, perhaps the target protein is being adsorbed to cell debris particles at the non-physiological conditions of lysis. Or the protein could be functionally bound (e.g., to the cell wall or cell membrane fragments). In such cases, you could try changing the pH and ionic strength of the lysis buffer or adding some detergent. Consider adding a solubility tag to the construct. If everything fails, you could consider making another construct and express only the interesting domain/fragment of the protein of interest, hoping that the solubility properties will turn out to be less problematic.

Q. After affinity purification of my protein and several early trials of IEX-polishing, purity checks using SDS-PAGE always show two lower-M_r co-purified bands, along with a band that is of the apparent correct M_r. What can be done?

A. This could be a case of proteolytic formation (*in vivo* or during lysis and extraction) of truncated form(s) of the target protein with an intact affinity tag. Truncation can also be caused by transcription or translation problems. Size separation by SEC will very likely improve the purity, even if it turns out that the extra bands are not truncated forms. The exception is when the SDS-PAGE pattern does not reflect the sizes of the native proteins in solution (e.g., when a PAGE band is the monomer of a protein that in fact is natively dimeric).

Another explanation could be that the two extra bands in fact are components that are in complex with the target protein during purification, which become detached due to the SDS, heating, and reduction done for PAGE.

Q. I have a protein that I am pegylating, and I am trying to separate the native protein from the mono- and di-pegylated forms. I have tried SEC but have been unable to separate these forms. I came across MadCap SP and Q. Will this help me to separate those two species?

A. SEC never has the size-resolution of, for example, SDS-PAGE. SEC often has to be optimized to give the best results. See the tips in the SEC handbook and the optimization tips in the instructions for Superdex 75 Increase or Superdex 200 Increase. The handbook and instructions are available on our Life Sciences Web site. For optimal resolution, high-resolution resins are of course needed in addition to optimization. The two resins that I just mentioned are our highest resolution SEC resins.

I am not familiar with MadCap, but our MacroCap™ ion exchangers are indeed developed for PEG-proteins and other large proteins. Please review the instructions and Data files for those resins, which can be found on our Web site. Of course, the best chance to get the separation you need in IEX is to use a shallow elution gradient.

Q. Whenever I do buffer exchange for my protein (e.g., from his column elution buffer to PBS), about 20%–40% of protein will precipitate during dialysis or on the desalting column. How can I fix the problem? Is it OK to keep my protein in imidazole buffer?

A. In principle, imidazole does not need to be removed if it does not cause any problems in downstream use. If you need to remove the imidazole, there are many possibilities for buffer exchange – see the handbook 'Strategies for Protein Purification' on our Web site. The buffer exchange should be done without delay. Try counteracting aggregation by modifying the conditions during buffer exchange, for example by changing the pH, salt concentration, or temperature. If it is possible that the protein is aggregating due to formation of -SS-bonds (i.e., oxidation), you could add a reducing agent. You could also try adding a detergent if this does not disturb downstream use. If the protein has a biologic cofactor, you might consider stabilization by having the cofactor present. Last, consider adding a solubility tag to the construct, if possible.

Q. When purifying from inclusion bodies, will use of urea burden the columns? Do we need to change columns more often when using urea in buffers? How often do we need to rigorously clean FF or excel columns? When do we need to recharge the FF columns with $NiSO_4$? How often do we need to change the columns?

A. I believe you are referring to IMAC resins. Urea will not affect the chemical stability of IMAC ligands. Use of 8 M urea in IMAC is considered to only slightly decrease the affinity between metal ion and his-tag, compared to non-denaturing conditions. Often, a higher purity can be reached when using urea with IMAC, because the unwanted binding of non-tagged proteins often is decreased. Because 8 M urea is a very good solubilizer, the burden on IMAC columns (e.g., in the form of accumulation of debris and aggregates on the column) is decreased when purifying in urea. But note that urea-solubilized samples normally will need centrifugation or filtration before IMAC even though they might look clear, because the refractive index of the urea can make the sample look more clear than it in fact is. So columns generally can be cleaned less often when you use urea.

How often an IMAC column needs to be cleaned and how often it needs recharging depends on many factors, such as the types of sample, how much the samples are diluted, the sample volumes, how well clarified the samples are, and the tendency of the samples to strip off metal ions. A HisTrap FF crude column will generally need less cleaning than other HisTrap columns do, because it is designed for unclarified samples. You could wait to clean the column until you see an increased back pressure or an unexplained decrease in purity. If you are very careful or use the same column for purification of more than one protein, you might choose to clean and recharge after every column use.

Ni Sepharose excel rarely needs any recharging, because the Ni-ions are so strongly bound that they cannot be removed with EDTA treatment.

Dr. Lena Nyholm, Scientist, GE R&D

Lena brings her background in both analytical chemistry and biochemistry to GE Healthcare, where she has been employed for 16 years. For the last 5 years Lena has been product owner for UNICORN control software in the R&D department. Lena is also knowledgeable about our lab systems.

Q. Can I run UNICORN with Windows® 10?

A. Yes, UNICORN 7.0.2 is compatible with Windows 10.

Q. Is the S2 inlet valve easy to install on ÄKTA avant?

A. Yes, all modules in the ÄKTA avant system are installed with a single screw and a cable. The sample inlet valve can be installed in any of the empty positions.

Q. Is UNICORN 7.0.2 compatible with my older ÄKTA systems from 2008 and 2003?

A. UNICORN 7.0.2 is compatible with the following systems: ÄKTA avant, ÄKTA pure, ÄKTAexplorer, ÄKTApurifier, ÄKTAprocess™, UniFlux™, WAVE 25 Bioreactor™, and ÄKTApilot™.

Q. I often perform very similar runs and only do small changes between my runs. To change the method each time is tedious. I would like to know if there is any better way to do this.

A. I suggest that you define all parameters that you might want to change as variables. Then, select to display the variable page in the start protocol. In this way you can change the value of the parameters when you start the method. If you want to vary parameters automatically, you can set up a scouting run where selected parameters are varied.

Q. Programming UNICORN is quite difficult for me. Can you give us some quick tips?

A. I believe you are using UNICORN 5. UNICORN 7, which is used in ÄKTA avant and ÄKTA pure, has a very different Method editor. In UNICORN 7 it is a lot easier to make your own methods, and you can also find templates based on the chromatography technique that you want to use.

Q. We are looking into the possibility of buying an ÄKTA pure system, but we also have two ÄKTAexplorer systems. Do we have any possibility to connect the old systems with the new one and run the same software?

A. Yes, ÄKTAexplorer and ÄKTA pure are both supported by UNICORN 7 software.

Q. What versions of Microsoft® SQL servers are supported by UNICORN 7?

A. UNICORN 7.0.2 has been verified on the following servers: SQL Server 2008 R2, 32-bit and 64-bit, running on Windows 7 Professional with SP1 installed, 32-bit and 64-bit; SQL Server 2012 with SP2 installed, 64-bit, running on Windows Server 2012 R2; SQL Server 2014, 64-bit, running on Windows Server 2012 R2.

Q. Why does UNICORN 6.4 stop working if you try to use a method from another user account?

A. If two different users need to be able to use the same method, you must make sure that the method is saved in a folder to which both users have access.

Q. We have an ÄKTA pure 25 system with UNICORN 6.4. We set up a separate login for each user, but we would like to share some common methods. I have written a method, but I do not know how I should save it so that all the different users (with different logins) can see and use it.

A. You save the method in a folder to which all users have access. Then the different users can log in and access the method.

Q. From the chromatogram, can we predict the concentration of the product, maybe from the UV absorbance given? Or do we still have to check via UV-spectrophotometer/Protein A-HPLC?

A. In UNICORN 7 you can calculate the amount and concentration in peaks or fractions if you know the extinction coefficient for your protein.

Q. Where can I find the most recent computer specs to run UNICORN 7.0.2? The Web site file is vague.

A. An installation guide including computer hardware requirements for UNICORN 7.0.2 can be found here: https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1460991695340/litdoc29201410_20161016035104.pdf

Q. Using the ÄKTA pure system and the new UNICORN 7 software, we have noticed that some of the changes have taken away the ability to select specific starting points for fraction collection positioning and the ability to add new vessels or update positioning during a run. Is GE planning to do firmware or software updates in the near future to return these options to the system?

A. We are aware that the possibility to define a starting position when fractionating is an appreciated feature. Thus, we are looking into how and in which version of UNICORN it can be implemented.

Jens Widehammar, Senior Research Engineer, GE R&D

Jens has 10 years of experience with lab-scale chromatography applications, including several years in lab-scale column packing at GE Healthcare. For the past 2 years Jens has been a product owner for UNICORN control software, with the responsibility for capturing user needs to guide further software development.

Q. I'm purifying a protein from refolding of bacterial inclusion bodies. I have used a self-packed Ni excel column on ÄKTApurifier with a linear gradient of 0–500 mM imidazole. The yield is good with minimal need for dialysis, but the purity is not as good as what I had with Co^{2+} after extensive dialysis. Should I: 1) switch to a prepacked 5 mL Ni excel column? 2) try a different gradient/step elution? What would you recommend?

A. I would modify the gradient on the column you already have. Try to see when your target protein is eluting, and improve the method in steps. Also, it is often helpful to add a small amount of imidazole (max. 5 mM) in the wash buffers.

Q. I still have contamination after the IMAC purification. How should I remove the impurities?

A. There are many options. But you could re-run the IMAC purification (after buffer exchange into loading buffer, using a desalting column) with some imidazole in the wash steps. Use a flat gradient to elute weak binding proteins before the target protein. Other possibilities are to run other techniques such as SEC to separate based on size or ion exchange to separate based on charge.

Q. How can we incorporate a few of the same columns into a method for UNICORN 6.4, for example two or more 5 mL HisTrap columns?

A. It is a bit unclear if you want to perform a scouting or if there is a need to have columns in series (which gives CV=10 mL). In the former case the process is straightforward by selecting the column as a scouting variable. In the latter case you can create a custom column and save the data under a selected column name. Be aware about flow and pressure limits that will differ from the recommendations for individual columns.

You can also connect many columns to the column valve and run them one by one. Then, you would have the ability to select the specific column that is used for each run. The column type can then be selected in the method to get pressure limits and also recommend flow rates for this column type.

Q. We are about to set up a UNICORN installation, and we would like to have some tips on how to set up the users.

A. UNICORN 7 provides a lot of possibilities. It could be very useful for all users to use the same login as the Windows login. Then, different access groups could be created, for example Project 2 and Project 3, and users given access to the projects they are working on. That way it will be possible to see which user has done what, while still allowing different project members to easily share the data.

Q. Would the loop valve kit allow me to automate several runs?

A. Yes, with this valve you will have the possibility to pre-fill up to five loops with sample and then automatically perform five runs.

Q. Is there any method for finding out if my RESOURCE Q column was dried?

A. The best option is to try using the column. But in my experience RESOURCE Q is a very reliable column, and even if has dried re-equilibration most often restores functionality.

Q. I would like to add a second sample inlet valve to ÄKTA avant 25. Will that give me 14 sample inlets?

A. Yes, that will give you 14 sample inlets.

Q. We cleaned the column with 2 M NaCl and 1 M NaOH. When removing the NaOH from the column we had an increased pressure. Why is this the case?

A. It is not strange that the back pressure increases when running 1 M NaOH. This effect would be due to it loosening possible impurities from the beads rather than to the solution's viscosity. Be aware that it takes at least 1.5 CV of buffer through the column to remove the NaOH used for cleaning.

Q. I would like to add an extra sample inlet valve V9-S2 to an ÄKTA avant 25 system. Can the extra 7 sample inlets from V9-S2 be controlled or programmed by UNICORN instead of manually?

A. Yes, the valve can be programmed directly from UNICORN. Add the extra valve in System Properties from the Admin module. Then, the ability to program the methods will be activated in the Method editor.

Dr. Dominique Dutaud, Senior Scientist, GE Scientific Support

Dominique joined GE Healthcare in 1999 after receiving a PhD for his work on proteasome purification and characterization. Over the past 15 years he has been focusing on chromatography in Scientific Support, where he uses his deep knowledge to help our users design or optimize protein purification protocols. Based in France, Dominique also leads customer trainings on chromatography techniques such as size exclusion, affinity chromatography, ion exchange, and hydrophobic interactions.

Q. What would you suggest as the best alternative to Mono S if this column is not available?

A. The best alternative to Mono S™ columns will be the RESOURCE S columns available in 1 mL or 6 mL. The bead size is 15 µm instead of 10 µm with Mono S.

Q. I have a question about a his-tagged system. I have a tagged protein that I cannot get to bind to a HisTrap excel column (5 mL). It just flows through with no binding at all. Under the same buffer conditions this protein will stick to HisTrap crude (5 mL). This makes no sense to me, can you explain?

A. Indeed, the his-tag affinity is generally lower with Ni Sepharose excel than with the regular Ni Sepharose (HisTrap FF, HisTrap FF crude, HisTrap HP, and IMAC Sepharose). That is why we recommend the regular resins, except in cases with too much loss of Ni ions from the regular resin. Also, you cannot use the same buffer conditions at binding for the two resins with respect to imidazole. Binding buffer for regular Ni Sepharose normally has 20–40 mM imidazole, but we recommend no imidazole for binding to Ni Sepharose excel.

Q. What product do you suggest if we want to purify a his-tagged protein expressed and secreted in a mammalian cell line?

A. Ni Sepharose excel has been designed for such an application, especially if traditional Ni Sepharose shows very low or no binding linked to Ni^{2+} ion leakage. Mammalian cell culture media may contain some EDTA-like reagents that remove Ni^{2+} ions from the matrix.

Q. Which metal affinity column(s) and reducing agents can be used for IMAC under reducing conditions?

A. IMAC is always very sensitive to reducing agents such as DTT. DTT could be used up to 5 mM final concentration. Sometimes, the resin in a column turns from blue to brown. This color is linked to the reduced ion (Ni^{2+} -> Ni), which produces a dark color. Most of the time, only some of the Ni^{2+} ions are reduced, so the column still shows binding capacity because there are enough Ni^{2+} ions for binding.

Q. I'm working with IL-34 that has no tag for purification. We tried to purify it with Blue Sepharose but couldn't identify our protein of interest band on the SDS-PAGE gel. Could you suggest purification strategies? It's expressed in mammalian cell lines.

A. You could try an affinity strategy by coupling antibodies against IL-34 on NHS-Activated Sepharose. Another option is to capture IL-34 using ion exchange, testing both an anionic and a cationic exchanger.

Q. I need to scale up my SEC purification step since the SEC seems powerful in the small-scale trials I have done for purification of my protein. I know that SEC suffers from the limitations of sample volume. What do you suggest?

A. SEC scale-up is not as straightforward as for binding techniques like ion exchange or affinity. One obstacle may be that some SEC resins (like Superdex Increase and Superose Increase resins) cannot be packed and used in columns larger than 10×300 mm. In such cases, separate optimization must be done when going from the small-scale resin to the larger-scale resin (and for convenience that optimization can perhaps first be done in columns smaller than the intended full scale.) Besides the obvious of using a larger SEC column for scale-up, you should also consider that the sample volume limitations of SEC can be partly overcome by A) concentrating the sample by ultrafiltration to decrease the volume and B) using repeated SEC runs with application of portions of a large sample. There are products for centrifugal ultrafiltration that take sample volumes from < 1 mL up to 60 or 100 mL. The product can also be topped up with even more volume as soon as the first centrifugation has been done, and two or more devices can of course be used for the same sample. There are also hollow fiber cartridges for cross-flow ultrafiltration. Note that for some proteins, there is a risk of aggregation or precipitation when concentrating. For repeated runs, let's say that a single SEC run with low flow takes four hours on a large column, including re-equilibration. With repeated sample applications (e.g., overnight) four times as much sample can be purified in 16 hours (four runs).

Q. Can I scale up my purification from Superdex 200 Increase 10/300 column to HiLoad Superdex 200 prep grade?

A. Yes, this could be an option. HiLoad Superdex is a larger column, so the injected volume of sample will be higher. But Superdex 200 prep grade will not give the same resolution. This scale-up should be optimized for experimental conditions.

Q. Is there a method, tip, column, or other product that can be used on an ÄKTA system (e.g., ÄKTAexplorer) for removing endotoxin from an antibody preparation (monoclonal or polyclonal)?

A. Our validated protocol for removing endotoxin is based on NaOH, which is used for cleaning the system. But we cannot guarantee 100% efficiency for ÄKTAexplorer, because it is not a BioProcess system, and there could be some dead volumes in connections or valves that might not get cleaned. Only BioProcess systems can be sanitized very efficiently. In your case perhaps your resins could be sanitized with NaOH if they are stable at very high pH.

Q. Cleaning my resins with NaOH could help to prevent endotoxin contamination of my sample during purification on an ÄKTA system. But when I've already contaminated my sample is there a procedure or quick tip that can polish the dirty preparation (of purified antibody)?

A. Endotoxin could be removed by a multimodal resin such as Capto adhere. This step should be optimized for binding endotoxin.

Q. We tried to purify IL-34 by binding to hCSFR1 that also has a mouse IgG_3-Fc tag. For batch binding we added an IL-34 harvest and purified hCSFR1 to MabSelect SuRe™ resin. We ran out the elutions on an SDS-PAGE gel but we couldn't find the IL-34 band and only found the hCSFR1 band.

A. An option could be to couple hCSFR1 to NHS-Activated Sepharose Fast Flow for capturing IL-34.

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