Basic Guide to Protein Chromatography/Purification

BASIC CHROMATOGRAPHY: The chromatography tutorial has already introduced you to the concepts of several chromatographic resins, the application of selecting a separation technique, the analysis of chromatography and the selection of the samples to pool and move onto the next step of purification. The information presented here is less about theory (there are several links for this on your laboratory webpage) and more about the practical aspects of purification. ***This is vital information you will not find in most textbooks.***

As mentioned in the introduction, there are six basic steps in purification:

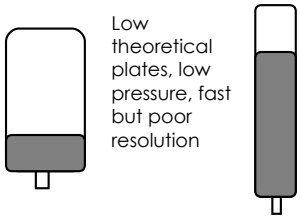


Fig. Short wide vs. tall thin column

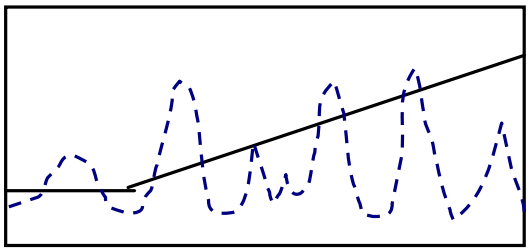
Step 1 - Design the Chromatography: This is the step that will make or break most of your efforts. Too little attention here will result in frustration when preparing solutions and running the column. Take the time to be thorough in this step. There are several components that need to be considered when designing your chromatographic purification: the type of separation technique, sample preparation, size of column, flow rate, buffers needed to bind proteins, wash unwanted proteins and elute the desired protein.
Before choosing a separation technique, you should review the purification strategy found in the tutorial and focus on the choices of purification methods. In short, you should try to avoid using a chromatography method that uses the same chemistry to separate compounds. In other words, you will typically get poor yield and purification from two ion exchange columns or two size exclusion columns in a row, one right after the other. You should also consider how the sample is eluted from the column. Will the final buffer be amenable to directly load onto the next column or will you have to prepare the pooled fractions by dialysis or concentrate them by ammonium sulfate fractionation before continuing on?

Once you've picked your purification resin, you need to determine the size of column, volume of resin, how to load the sample, flow rate (how fast to run the column), how much buffer to run through the column, how to elute the sample and the collection method. You will be given three or four different chromatography resins to chose from. In the protocol section on the class webpage, there are basic protocols to help with each step. You will be given the specifics on how to address the important questions listed above in these protocols. You should be aware of the advanced theory and practice of each chromatography. There are many excellent websites which have this information, your class website has a few. Chapter 11 in *Principles and Techniques of Biochemistry and Molecular Biology (6th Ed.)* has much of this key information for you to look over. However, there are a few basics that you should understand before looking through the protocols.

1) Column size – A short thick column will have less backpressure than a taller thin column (that is how much resistance the fluid has as it is pumped through the column). Too much backpressure and your tubing, connections and pump will fail. A wide short column will have a faster flow rate. If you are going to simply bind, wash, and elute with what is called a different wash (step or isocratic gradient) and not a gradual change from one buffer to another (gradient elution) then you might consider using a wider short column. **But you should also be aware that the eluted sample will be more dilute and less resolution (separation from contaminants).** If you are going to use a gradient elution or work with size exclusion resins, then you must have a taller thin column.

The specific amount of resin depends on the type of chromatography and the particular binding capacity of each resin. For resins that bind their analyate, the top 20% of the column should bind most of the protein, for SEC columns, the sample volume loaded should be no more than 3% of the bed volume (bed volume = the volume of resin in the column).

2) Flow Rate: For simple open columns, gravity will work just fine. If you are pumping buffers through the column, then the flow rate can vary. A fast flow rate may cause excess packing of the resin and the backpressure will build, causing the tubing and pump to leak and fail. At a high rate of flow, most peristaltic pumps (the kind with a tube stretched around a roller) will pulse back



Elution Volume

Gradient Elution

and forth, causing a dilution of the eluted proteins and mixing of unresolved proteins. Too slow and the proteins may lose activity waiting to elute. ***A simple rule of thumb is set the flow rate no faster than 2 or 3 times that of gravity for soft resins (agarose and sepharose) for 2-5 times that for more cross-linked resins.***

A 1 cm diameter column can easily be run at 0.5 to 2 ml per min. A 2.5 cm diameter column can handle a flow rate of up to 4 or 5 ml per min. Don't forget that if you flow the sample (analyate) too fast, you may get reduced binding kinetics to the resin, dilution of the analyate as it elutes or excessive rates can result in a poor elution & low yields.

3) Buffer Selection: Buffer composition – Buffers: You MUST include a weak acid base (buffer) in each solution. The pH should be within one pH unit of the pKa for a buffer to be effective. For most uses, a 10 – 25 mM concentration of buffers will do just fine. If you add a metal chelator such as EDTA to maintain stability of the protein, appropriate concentrations run from 0.1 to 2 mM. Reducing Agents: Most purifications require that a reducing agent be added to keep the protein native. Dithiothreitol is typically used at a 0.1 to 1 mM concentration. Use this fresh. The DTT will quickly be oxidized by the dissolved oxygen in water and thus will only last for a few hours. 1M DTT is typically stored in a small aliquot in the freezer and used each time you run a purification process. Protease Inhibitors are also commonly used. Simple metal chelators like EDTA are used at a 0.1 – 5 mM concentration to inhibit metaloproteases. EDTA inhibits proteases by binding with the metal that is necessary for the activity. EDTA is stable at room temp. and is used from a stock solution. Phenylmethylsulfonyl fluoride (PMSF) is very unstable in aqueous solutions. PMSF is relatively inexpensive and routinely used by many laboratories. A100 mM stock solution is made in isopropanol and stored in the dark at -20 degrees Celsius. It must be warmed to 37 degrees in order to get into solution. Use a 1 mM solution; since it is only stable for two hours or so, it must be used immediately. There are other cocktails of inhibitors that inhibit a wide range of proteases. If the protein you are purifying is a target for proteases, you should look into using a mixture of protease inhibitors.

4) Loading: Make certain you do not have interfering compounds in your buffer. You must inspect the components of your pooled samples to see whether each component will interfere with the purification of your protein. Common problems can include the use of EDTA (a metal chelator) when loading onto a Nickel column. EDTA will compete with the His-tagged protein to bind to the metal on the resin. Another common problem encountered in purification is the salt concentration. Ion exchange resins do not bind many proteins if the salt concentration is 50 mM or greater (NaCl or KCl). Hydrophobic resins will not bind unless there is more than 200 mM salt in the buffer.

Column equilibria: Each time you use or pour a column, the resin should be washed from proteins that may still be bound from a prior use. For most purifications, a 1M NaCl in your buffer will suffice. SEC chromatography does not need this step. Check the elution conditions for each column you use and wash two or three column volumes of this through the column prior to use. At this point your resin will not bind your protein unless the salts and other components are removed and the column equilibrated by loading an equilibration buffer. ***Therefore, after washing your column with a high salt wash, you must wash 5 to 10 column volumes of equilibria/loading buffer through the column prior to loading the column.***

Wash and Elution Volumes: Loading a column – Once you've initially pumped your sample onto the column, it is important to realize that the material still in the tube, pump or in the column needs to be washed through the column. Washing more of the equilibrium buffer through the column is important to remove unbound protein before eluting the protein. Typically a two to five column volume of equilibrium buffer will do just fine. Washing a column – After loading you may want to wash off weakly bound proteins or use a buffer with a sub-optimal concentration of ligand, salt or pH to remove other proteins, leaving your protein on the column. To do this, simply stop the column;

switch the tube into a new beaker or flask containing your wash buffer and wash the column with 3 to 5 column volumes of the new buffer. By this time nearly all the protein that will elute has eluted. Eluting the targeted protein from the resin - Most of a protein eluted in an isocratic gradient will come off in two or three column volumes or less for a gradient. It is a general rule of thumb to use 8-10 column volumes of elution buffer when using isocratic washes. When using a gradient, a steep gradient (a large change in buffer concentration or short volume of gradient {1-5 column volumes for the total gradient}) will result in a tight concentrated elution of your protein, but the resolution from contaminating proteins will be less than ideal. Using a larger, more shallow gradient (one with 8 to 10 column volumes of buffer for the total gradient) will result in a much better resolution but will also dilute your protein. If the dilution effect is significant your protein may be too dilute to detect. A 5 to 10 column volume gradient is a good starting point.

5) Collection Method (picking a fraction size): When to collect a fraction or not… This is a typical and challenging question to answer. The more fractions, the more test tubes you will have to assay to find your protein. However, on the other hand, the more fractions, then the more likely you will be able to isolate your protein from other contaminant proteins.

First, if you know that your protein is NOT in a given wash, then it is wise to collect that wash as a single fraction. The same goes for the flow-through fraction (this is the material that does not bind to the column during a load step). Each of these steps could easily be collected in one beaker for each step and analyzed as one fraction. Alternatively, you could also collect fractions from the beginning to the end of a purification process.

When collecting fractions, the volume collected for each fraction depends on the number of tubes you have in your fraction collector and the volume of your wash and elutions. Again, it is important to consider how many fractions you can practically collect and measure while maintaining reasonable purity. For larger columns (>10 ml column volume), a starting point is to collect fractions 1/5th to 1/10th the size of the column. For a 1-2 ml column, I recommend using 0.5 ml fractions. For a 2-10 ml column, fractions of 1 or 2 ml would suffice.

Use the purification check-sheet in the protocols section of your laboratory web page to help you through this section. Each type of chromatography resin will have a short protocol and guide found in the protocol section of the laboratory web page.

Step 2 - Prepare the column and buffers: Now that you've planned your chromatography, you need to make the buffers and pour the columns. Prepare at least 25% more buffers than you calculate you need. The handout for each type of chromatography has specific suggestions on buffers and preparing the resins. All stock solutions are found in the laboratory. Use these items to prepare your buffers. Store buffers in the cold room across the hall. Plan to make buffers one day and run the column on a different day.

Packing the column - Columns should be packed in one step or pour to avoid distinct layers of the resin.

- Pour enough resin in a small beaker to use and let settle.
- Decant the clear buffer from the beads and add enough equilibria buffer to make a 50% slurry.
- Attach the glass or plastic column to a stand, cap the bottom of the column with a small closure or turn the stopcock to the off position. Fill the column 1/3 with buffer.
- Resuspend the beads by swirling and pour the beads into the column.
- Once the beads have settled open the bottom of the column to drain excess buffer. Cap when the buffer is just to the top of the resin.
- If using a pump, fix the flow adaptor to the top of the column, being careful not to introduce air into the tube or column. If using a gravity fed column, you are now ready to equilibrate.
- ***DO NOT LET THE COLUMN RUN DRY.*** This will result in cracking and the buffer and proteins will flow around and not through your column. If the column does run dry, empty and repour. If this happens during a run when your samples are already loaded, gently use a glass pipette and stir the beads with a minimal amount of equilibria buffer.

Important note: *Do not use mechanical methods to empty columns or transfer chromatography beads. Using glass rods or scoops will result in crushed beads causing particulate matter to interfere with column flow and purification. Use buffer or water to mix the container or column to resuspend any beads. Alternatively, a slight pressure of air introduced to the bottom of the column (using the lab air or a syringe) will usually push the resin out of a column.*

Step 3 - Load and run the chromatography: This may take longer than you think. Plan to spend at least 30 min gathering materials and getting the column and pump set up. Do NOT start until your sample is ready to load and the buffers are all prepared.

Calculate how long the column will run. Plan an appropriate time to start and stop the column. Remember other people will be using the equipment so sign up for a time and be polite by keeping to your schedule. Make certain to collect your fractions right away and set them aside for later assay!

TIPS FOR RUNNING A SUCCESSFUL CHROMATOGRAPHY:

- If your sample is not clear and has precipitate in it, centrifuge the sample in the swinging bucket rotor found in the cold room for 10 – 15 min.
- When using the pump to flow buffer through the column, make certain all tubes are placed in the bottom of your buffer beaker. Tape may be needed to ensure the hose doesn't slip.
- Practice a few ml run with the pump/column/fraction collector to ensure things are set up right before loading on your fraction.
- Plan to stick around and watch the load and flow. ALWAYS watch the first few fractions go through the fraction collector. NEVER assume it will just work right.
- Label ALL tubes.
- Store fractions covered with tinfoil or saran wrap in the cold room. Don't forget to label with your name and lab section.
- Save 300 µl of the lysate and each pooled sample you take along the way. FREEZE these samples in well labeled microfuge tubes. Record the volumes of each pool for later assay.

Save fractions and run assay as soon as possible! Your laboratory notebook should have all the observations and settings from this chromatography. You should be able to give this notebook to anyone in the lab and expect them to repeat the experiment without additional instructions from you.

Step 4 - Analyze the fractions for Enzyme and total protein: In the purification tutorial, you clicked on a button to run an assay to determine which fractions had your enzyme. You could also look at the chromatograph to see where all of the protein eluted from the column. Conduct a protein assay on EACH fraction to determine the total amount of protein in the fractions. When you are finished, you will prepare a chromatograph using two Y axes (one for protein concentration in mg/ml and the other for Enzyme acivity). From this graph you will determine which fractions to save.

Step 5 - Pool fractions (save portion): This is a critical step. If you save all of the fractions to get a high yield, the result is a low purification step. However, take just the fractions with the highest enzyme activity, and you may not achieve a reasonable yield. In a perfect world, you would run an SDS PAGE gel and a Western blot each time, BUT this take lots of time and money. Instead, you have to use your judgment based on experience.

A rule of thumb is to save 70% of the peak. If there is extensive tailing (non-gausian curves), then you may need to adjust.

SAVE 0.3 ml OF YOUR POOLED FRACTION. RECORD THE VOLUME OF YOU POOLED FRACTION IN YOUR NOTEBOOK. You do not need to save samples from the other factions.

Wash your test tubes and prepare for the next chromatography.

Step 7 - Prepare pooled samples for next chromatography: At this point, you should plan which chromatography to use next. Now is the time for you to determine if your sample needs to be concentrated (ammonium sulfate precipitation or ultra filtration) or dialyzed to change buffer and/or desalt your sample. Then repeat your chromatography steps as above for the next chromatography. Save all of your final pooled sample and store.

STORAGE OF PURIFIED PROTEINS: Now that you have spent so much time and hard work into your protein, the last thing you want to do is forget about storing the protein to maintain activity. Long term storage of proteins (> 24 hours) at extreme pH or pH near the pI or with buffers with imidazole will cause your protein to denature and … well … we will not even think about it…

For short term storage (up to 24 hrs), most proteins can be kept at 4°C. Keep the sample covered! Bacteria and mold will easily contaminate an open protein solution.

For long term storage after purification (>24 hours), it is important to freeze your protein. To stabilize your protein, you should add enough glycerol to make the solution 10% glycerol. You

may want to add DTT at a final concentration of 0.1 mM. Alternatively, longer storage can be done by precipitating the protein with ammonium sulfate and storing at 4°C. This is extremely stable. Another long-term storage solution is to freeze-dry or lyophilize the sample and store at 4°C.

LOGISTICS & IMPORTANT INFORMATION:

Updates and ordering materials – Timely information and notices will be found on the webpage and/or the blackboard at the front of the laboratory. ***It is YOUR responsibility to check both.***

Make sure that you write any chemicals or buffers that are running low on the chalkboard. Don't wait until a container is empty.

As soon as you finish a chromatography run, transfer your test tubes to a test tube rack and return the parts to your table for the next group.

Plan for 1-2 hours to prepare buffers for each chromatography, 2-3 hours to run a column chromatography and 2 hours to analyze each run.

Buffers provided and location – Stock buffers will be located at the table and cabinet at the front of the classroom. Please indicate any needed buffers on the blackboard when the buffer is nearing empty.

Stock buffers that will be provided for you will be:

- 1M Tris-Cl pH 8.0 (make pH adjustments of your buffers)
- 1M Potassium Phosphate Buffer pH 7.0. NOT THE SAME AS PBS
- 10 X Phosphate Buffered Saline (PBS)
- 0.5 M EDTA
- PMSF

Where to find chemicals and materials –

- Dry chemicals such as NaCl, ammonium sulfate and Imidazole will be located in the cabinet at the front of the classroom.
- BSA for protein assays – 20°C freezer on the top shelf.
- Bradford Assay Reagent – 1X ready to use Bradford reagent will be in the re-pipettor in the east hood. 5X concentrated Bradford reagent will be in the refrigerator. Only use this when there is no remaining 1X. Prepare 500 ml 1X at a time.
- Black 96 well plates for enzyme or fluorescence assays will be on the table at the front of the classroom. Wash and return when finished.

Storage in the cold room/box – There is a labeled shelf in the cold room for each laboratory section. You can only use this space for storing your fractions and samples. Do not store your materials in the fridge. There is not enough room.

ALL BUFFERS MUST BE LABELED WITH THE FOLLOWING:

- Name of buffer and be specific
- Components of buffer
- Your name and date
- Which lab section Tues or Thurs

For example:

DEAE Gradient II Buffer (10 mM Tris-Cl pH 8.5, 0.1 mM EDTA, 300 mM NaCl) Provost 1/Oct/06 Tues Lab Section.

Protein Purification

Guide to Protein Purification Ed M. P. Deutscher Methods in Enzymology Vol 182 Academic Press (one of my favorites for many years)

Principles and Techniques of Biochemistry and Molecular Biology 6th Ed. K. Wilson & J. Walker Ed. Cambridge University Press (A great source for theory and advance information on chromatography and most things biochemical)

Modern Experimental Biochemistry 3rd Ed. R. Boyer Benjamin Cummings (has good introductions plus nice experiments)

LAB NOTEBOOK:

Accurately record the procedures conducted during lab. This will include:

- Observations of the experiment
- Planning and calculations for each chromatography. Include volumes of buffers prepared and key info for each column.
- Data and calculations for quantifying your protein.