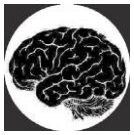
	SKILLS CENTER **STANDARD OPERATING PROCEDURE**	**A BIOFIZZ**  **PRODUCTON**
Protein Purification (IEX) **Module Hours: 5**	**Effective Date: 11/3/2023** **PRQs:** **Protein Expression**	**Revision 1.0** **Author: Zachary Hazlett, Oscar Cordova** **Checked by** **Editor: Carson Butcher**

Background

Biological systems are complex. It is difficult to study the structure and function of individual molecular components of these systems or how they might interact with other components of the system. To overcome this challenge, scientists have developed a variety of methods to isolate, or purify, protein of a single type. These purified proteins are obtained as homogeneous aqueous solutions of the pure protein often stored in a compatible buffering solution to maintain protein solubility and stability. Methods of protein purification vary greatly and depend on the biochemical properties of the protein of interest. In this series of SOPs ("Protein Purification 1-3"), we will provide training and instruction on purifying protein through (1) ion exchange, (2) size exclusion chromatography, and (3) affinity purification.

The choice of which protein purification method to use depends on the biochemical properties of the protein of interest, as well as the desired degree of purity. The biochemical characteristics of a protein that can be used to allow for separation from other molecular components include its size, charge, stability (precipitation or solubility under various conditions), presence of any affinity tags, etc. When one decides to purify a protein, the above conditions are important to consider, but even knowing all of them, there can still be variability in approach depending on the protein. If the protein of interest is one that has been purified before, it is recommended to study past protocols proven effective and use or modify them as needed to suit your needs.

Our protein of interest is 1GFP, which is composed of the fusion protein His6-GFP- TEV. His6 is an affinity tag made up of six consecutive histidine residues. This affinity tag is useful for affinity purification using nickel (Ni^{2+}) chelated resin. GFP is our protein target of interest, a highly stable fluorescent beta-barreled protein that was first isolated from the jellyfish *Aequorea Victoria*.[1] The Tobacco Etch Virus (TEV) protease site is a small peptide sequence that is useful for recognition and cleavage by the TEV protease. If the TEV site was between the His tag and the protein of interest, this site would allow for controlled removal of the His6 affinity tag post expression or purification. This is an approach many use, but is not used here, nor is it needed for our purposes. Together, this fusion protein product can be expressed, and purified by a number of different approaches, three of which are described in this series of SOPs.

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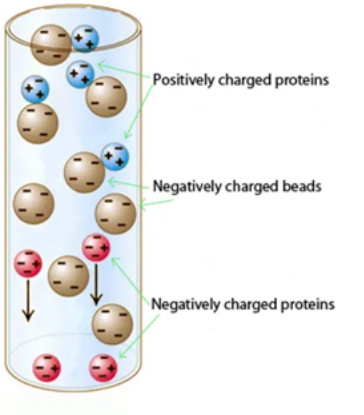

Figure 1. Ion exchange chromatography. Shown here is *cation* exchange chromatography where the negatively charged beads capture the positively charged (or cationic proteins). Tan spheres represent negatively charged ion exchange resin. Proteins with an overall positive charge (blue) will bind the negatively charged resin, where proteins with a net negative charge (red) will pass through.[4]

In this protocol, we describe purification of 1GFP via Ion Exchange chromatography. Ion Exchange Chromatography (IEX) separates proteins based on their charge. The primary characteristic that one considers when using IEX chromatography is the net charge of a single molecule of the protein to be purified. The value that gives us information about our protein's overall charge is the *isoelectric point* (pI). The isoelectric point is defined as the pH of a solution at which a particular molecule carries no net charge[2]. For example, our fusion protein 1GFP has pI=5.71 (Found using the amino acid sequence and an isoelectric point calculator[3]), meaning in a solution at pH=5.71, the 1GFP protein has a net charge of 0. Protein purification resin conjugated with either positively or negatively charged functional groups can be used to select for proteins of a specific pI within a solution. One must consider the pH of the buffering solution to determine the charge of the protein. If our fusion protein, 1GFP, was in a buffer at pH=7, we know that the protein will have a net negative charge as pH>pI. Knowing the protein has a negative charge at pH=7, one can

choose to use a positively charged IEX resin (cation exchange chromatography) or negatively charged IEX resin (anion exchange chromatography). One can plan their purification strategy to involve binding the protein to the resin and isolating it from the other components, or binding components of opposite charge to the protein of interest and letting the protein of interest pass the column and flow through to the collection tubes (Fig. 1). Either approach can be used to select for your protein and achieve greater amounts of protein purity.

Figure 2. Our FPLC station

Ion exchange chromatography is most often accomplished using the above mentioned charged resin pre-packed into a cylindrical glass columns of a defined volume. Examples of these columns include Mono Q™ (anion exchange) columns[5] and the Mono S™ (cation exchange) columns[6]. Each with an entry and exit valve, these columns are connected to a machine that allows one to control the purification of proteins across these ion exchange columns. These machines are known as **high-performance liquid chromatography (HPLC)** and also known as Fast-Protein liquid chromatography (FPLC) purification systems (Fig. 2). Aside from the HPLC purification columns, Figure 2 shows a diagram of the additional primary components of a HPLC

system. These include the buffering solvent or solvents, a pump to control flow speed of the sample and buffer solutions, a small port before the HPLC column to allow for injection of the protein sample to be purified, a detector for monitoring separation of molecular components (usually in the form of a UV spectrophotometer), a fractionator (Turntable shown in Fig. 2) used to collect defined small volume aliquots of fractionated sample, and finally the waste container to collect the components not needed. The HPLC is connected to a computer that serves as the primary interface for operating the HPLC as well as collecting post column absorption data and the location of collected fractions across that data. As we will see more in later SOPs within this series, the HPLC serves as a highly versatile tool for controlled purification of liquid soluble proteins.

Given the quantity of components of HPLC protein purification and the information required to operate these types of systems, this skill can take time to master. There are many small details to consider and questions you will generate as you familiarize yourself with the HPLC protein purification system. Please use your available resources (proctors, AKTA user manuals, etc) to ensure you are properly prepared before attempting to purify your protein of interest.

After one successfully purifies their protein across a particular chromatography column, it is common practice to run a series of samples of your collected fractions over SDS-PAGE to determine which samples are the are the purest and which you desire to collect, pool and store as your final product. After completion of the above, it is important to restore the HPLC system back to its default state with the proper wash cycles and filling of the lines with the proper storage buffer.

1. Purpose

The purpose of this SOP is to instruct students on the background and procedures necessary for protein purification via ion exchange chromatography.

2. Scope

This procedure applies to qualified skills center users.

3. Responsibility

3.1. It is the responsibility of the user to understand and perform the procedure described in this document.
3.2. It is the responsibility of the user performing the procedure to fully document any deviations from the written procedure.
3.3. It is the responsibility of the user to become trained on the procedure.

4. Definitions

4.1 Chromatography - The process used to separate components of a soluble mixture. The mixture to be purified is composed of the mobile phase, which passes through the purification chromatography resin, and a stationary phase, which is captured or bound to the purification resin. Immobilized proteins of the stationary phase can be eluted using chemical components that stimulate the unbinding of protein from resin. Eluted protein is collected and stored as the purified final product.

4.2 Isoelectric point – The pH at which a particular protein carries a net charge of zero. Useful for determining buffer pH's to use to control the charge state of a target protein.

5. Materials/Equipment

5.1 Ice bucket + ice
5.2 Harvested bacterial pellet of cells post induced expression of 1GFP
    5.2.1 Obtained from the end of the protein expression SOP
5.3 Freshly prepared Lysis Buffer, Make lysis buffer if not available
    5.3.1 Liquid TritonX-100
    5.3.2 Powdered Sodium Phosphate Monobasic, Monohydrate (MW=137.99 Da)
    5.3.3 Powdered Sodium Phosphate Dibasic, Anhydrous (MW=141.96 Da)
5.4 Buffer A and Buffer B (See recipes below)
    5.4.1 Powdered Bis-Tris (MW=209.2418 Da)
    5.4.2 Powdered NaCL (MW=58.44 Da)
    5.4.2 500 ml graduated Beaker
    5.4.3 Stir bar (that fits base of graduated cylinder) + stir plate
    5.4.4 pH meter
    5.4.5 Concentrated HCL
    5.4.6 Glass filter sterilizer
    5.4.7 2x Clean autoclaved 500 ml buffer bottles

6. Recipes

6.1 500 ml Lysis Buffer: 10 mM NaCl, 1.0% TritonX-100, 50 mM Phosphate, pH 7.4

Measure 400 ml dH2O in clean 500 ml graduate beaker
Add .292 g of NaCl
Add 5 mL of 100% TritonX-100
Add 3.55 g of Sodium Phosphate Dibasic, Anhydrous
Add 3.45 g of Sodium Phosphate Monobasic, Monohydrate
Bring up to total volume of 500 ml after dry reagents are dissolved
Adjust desired pH using concentrated HCl/NaOH

6.2 500 ml Buffer A: 20 mM bis-Tris buffer, pH 8.5.

Measure 400 ml dH2O in clean 500 ml graduated beaker
Add 2.0924 g powdered Bis-Tris
Mix with stir bar on stir plate till dissolved
Starting pH will be ≥ 8.5. Measure pH and if needed, adjust to desired pH using concentrated HCL/NaOH to final pH = 8.5.
Add dH20 to final volume of 500 ml.
Filter Sterilize by Vacuum Filtration

REF: “How to Filter Buffers (Vacuum Filtration)” Training video→https://www.youtube.com/watch?v=Haw14QOFxL8
NOTE: Instructor mentions turning on “water.” This is just the valve that provides vacuum suction from the tube connected to the filtering flask. The same filter can be used for both buffers, but please filter buffer A before buffer B to prevent salt contamination

Add filter sterilized buffer to clean autoclaved 500 ml buffer bottle
Label with buffer, pH, date made, your name.
Can store at room temperature with other buffers.

6.3 500 ml Buffer B: 20 mM bis-Tris buffer, 0.5M NaCl, pH 8.5

Follow the above directions for buffer A, but add 14.61 g NaCl to powdered bis-tris before dissolving each in 400 ml dH20.

7. Procedures (Adapted from Van Dyke M., 2021[10])

7.1 Obtain bacterial pellet of harvested cells post induced expression of 1GFP.

7.1.1 Thaw slowly on ice and briefly with gloved hands to warm.

7.1.2 If you do not have a pellet of cells containing expressed protein, please stop and start at the protein expression SOP.

7.2 Measure approximate volume of thawed cells. Transfer all into 2 ml microcentrifuge tubes.

7.2.1 Save 100 µl aliquot of freeze/thawed sample (F/T). Label and store for later.

7.3 Resuspend the cell pellet in TX-100 Lysis buffer by pipetting or vortexing briefly until the suspension is homogenous:

7.3.1Use 0.025 - 0.075 mL of Tx-100 Lysis buffer for every 1 UOD600 harvested. To calculate the UOD600, multiply the volume harvested by the OD600 reading.

-For example, a 5 mL culture harvested at OD600 1 gives 5 mL x 1.0 = 5 UOD600. In this example, 0.125 – 0.375 mL Lysis Reagent is required to lyse efficiently.

-If harvested cells are weighed, use 10 mL of lysis buffer per 1 gram of cells.

7.3.2 Incubate the resuspended cells at room temperature for 10 - 20 min with gentle shaking, gentle rotation, or swirling. Lysis is usually visible with a clearance of the suspension.

7.3.3 Save 150 µl aliquot of soluble sample. Label (“L/S”). Store on ice.

7.3.4 Dilute one of the 150 ul aliquots with 1.35 mL of Buffer A to be purified by IEX purification to make a total of 1.5 mL of sample in solution. Vortex Briefly.

7.4 Purify protein via ion exchange chromatography.

7.4.1 Prepare appropriate buffers. See recipes for preparation of buffers A & B above.

7.4.2 Be sure to familiarize yourself with the primary components of our lab's HPLC (Fig. 2b).
7.4.2 Familiarize yourself with the IEX column you will use → Hi Trap Q HP, 1 ml (https://www.cytivalifesciences.com/en/us/shop/chromatography/prepacked-columns/ion-exchange/hitrap-q-hp-anion-exchange-chromatography-column-p-00607)

7.4.2.1 Document the following: Type of IEX column, volume of column, max pressure limit, recommended pressure limit, max flow rate, recommended flow rate.

7.4.3 Make sure to always be aware of the flow path of buffers through the solution.
7.4.4 The column should already be connected to the FPLC and stored in EtOH. Check to ensure it is connected. Make note of the valves the column is connected to. We will tell the computer to direct the pathflow out these valves and across our column soon.
7.4.5 Prepare HPLC, column, and fractionator

7.4.5.1 Pump wash both pumps A & B.

-Make sure to open the wash valve and plug the top of the mixer avoid washing the column with high flowrate buffer

7.4.5.2 Set high pressure alarm, set flow speed, and select the pump that will pump water through the system.
7.4.5.3 Set UV detector to 280 nm (for protein) and 255 nm (for detecting potential nucleic acid contaminants).
7.4.5.3 Flow 3 column volumes (CVs) of water through your column. This will displace the EtOH it was stored in.
7.4.5.4 Remove tubing leads from water (weighted ends of HPLC tubing currently in water flasks; Should be labelled "A" and "B" for the pump they correspond to). No need to rinse leads as they were previously in water. Place "A" and "B" tubing lead into low salt buffer and high salt buffer, respectively.
7.4.5.5 Pump wash with buffer solutions. This prepares your pumps with the buffer that will soon pass over your column.

-Remember to open the wash valve and plug the top of the mixer during wash steps to avoid washing the column with high flowrate buffer

7.4.5.6 Wash column as in previous step with high salt buffer, then the same afterward with low salt buffer. Make sure that the baseline UV reading is flat after low salt buffer wash. This is your baseline. Presence of your protein eluting from the column will be observed with noticeably large 280 nm peak.

7.4.5.7 Make sure fractionator is in place.

-Place the collector on top of the first tube

7.4.5.8 Load fractionator with appropriate collection tubes.

If collecting up to 1 ml samples, you can fill fractionator with full sized collection tubes with 1.5 ml capless epi tubes sitting within each large tube.

7.4.5.9 Fill rack with 65 collection tubes.

7.4.5.10 Lastly, place the waste output tubing into a large clean empty flask to collect waste.

7.4.6 Prepare protein sample

7.4.6.1 Visually inspect protein sample. If you observe large quantities of precipitate, centrifuge briefly (high speed, 5 min, 4ºC), collect supernatant and transfer to new tube.

7.4.6.3 Take note of the location, and volume of the sample loop (See figure 4), the looping of tubing sized to hold your protein sample prior to its injection onto the column. Make sure the volume of your sample loop is greater than, or equal to the total volume of your sample. If the sample loop is smaller than the total volume of your sample, exchange it for an appropriately sized sample loop.

7.4.6.3 Select a glass syringe with a volume large enough to hold your entire sample.

7.4.6.4 Carefully draw up the filtered sample into a glass syringe. Carefully displace any air bubbles.

NOTE: Introducing air bubbles into the HPLC and connected columns has the potential to disrupt the viability of the column and

proper functioning of the HPLC. Please exercise caution to prevent this. Contact a proctor if you have any concerns regarding this.

7.4.7 Load protein sample onto sample loop (Read all prior to execution)

7.4.6.5 Before injecting, screw syringe into injection port, resting on the injection port syringe holder.

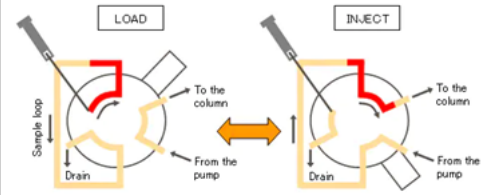

Figure 4. The two changing flow paths of the sample loop. When the system is set to "LOAD," sample can be loaded into the sample loop. When the system is set to "INJECT," it is ready for buffer to pass through the loop and inject the sample into the downstream components of the system (Source: Hitachi. Configuration of HPLC System.)[11]

7.4.6.6 The injection valve should be preset to "LOAD." Make sure to check this setting in the manual injector settings. THEN you are free to load your sample into the super loop. Leave syringe connected to injection valve and resting on syringe holder.

7.4.8 Double check everything prior to run.

7.6.8.1 Ensure proper preparation of HPLC and buffers, full fractionator to collect samples, and empty flask to collect waste.

7.4.9 Run pre-prepared **HiTrap Q HP IEX protein purification run**

7.6.9.1 Select pre-prepared purification protocol.

7.6.9.2 Make sure high pressure alarms match those you have been using.

7.6.9.3 Select "Run."

7.6.9.4 Monitor the beginning stages of HPLC protein purification. Make sure the samples begin to be dispensed into the fractionator collection tubes, and that no pressure alarms go off.

7.4.10 AFTER RUN IS COMPLETE, observe the UV absorption data for your run. You should have a strong peak at 280 nm indicative of your purified protein. You will likely have other smaller peaks/larger peaks that come from non-target protein being passed through the UV detector. Ensure that peaks from other

absorbances do not correlate with your protein's 280 nm peak. If there are, then you might still have contamination of other molecules in your purified protein sample. You can confirm the presence of GFP protein by visually inspecting the elution tube. A faint green color shall be apparent. Other protein purification steps can be taken to further purify your protein if needed.

7.4.10.1 Cover your fraction collector loosely with seran wrap.

7.4.10.1 Print a copy of the UV absorbance data.

7.4.10.2 Select a sampling of fractions that capture the beginning, middle, and end of prominent peaks, as well as a few others within each peak. Mark these on your printed UV data sheet.

7.4.10.3 Gather enough 0.5 ml epi tubes to capture all peaks you would like to test for protein.

7.4.10.4 Fill each with 2 µl 6X SDS-PAGE running buffer (dark blue). Label these tubes with the corresponding fraction numbers you will test for protein.

7.4.10.5 Add 10µl of each sample you've selected into the corresponding tube containing SDS-PAGE running buffer. Gently mix via pipetting.

7.4.10.6 Recover fractionator containing samples. Temporarily store in 4ºC fridge. Can be stored overnight.

7.4.11 Along with an appropriate SDS-PAGE ladder, and all labelled pre-purification samples, run all samples over SDS-PAGE and stain with Coomassie.

Consult SDS-PAGE SOP for details if needed.

7.4.11.1 After gel is de-stained, observe bands that you believe to be corresponding to the molecular weight of your protein of interest. Document notes.

7.4.12 Pool all samples corresponding to the most-pure fractions of your protein of interest. Dispose of the rest.

7.4.12.1 Measure concentration of purified protein sample via UV VIS spectrophotometer.

Consult proctors for help if needed.

7.4.12.2 Measure your purified protein's UV Vis *Spectra* (Y-axis: Absorption, X-axis: Wavelength). Compare spectra to your observations

about the observed protein's color. Why do you think the sample is green?

7.4.12.2 Label sample of pure protein with your name, date, the protein, the concentration of protein, and the contents and pH of the buffer the protein is in.

7.4.16 In many applications, proteins can be concentrated for their specific use if needed after this final step.

7.4.13 Clean up

7.4.13.1 Wash HPLC

7.4.13.1.1 Set pressure alarms for all below. Run 3+ CVs of high salt, then low salt buffer across column.

7.4.13.1.2 Remove weighted buffer tubing leads from buffers. Rinse ends of leads and tubing with DI from a squirt bottle over an unused beaker.

7.4.13.1.2 Return leads to DI water flask.

7.4.13.1.3 Run water through the column.

7.4.13.1.4 Lower flow speed (as EtOH is more viscous than water) and run EtOH through system and through the column. The column can be stored in EtOH.

7.4.13.2 Rinse Super loop

7.4.13.2.1 Rinse glass syringe with clean DI water three times

7.6.13.2.2 Using this glass syringe or other clean syringe, rinse super loop with three or more super loop volumes with DI water.

7.4.13.3 Empty fraction collector and dispose of waste

7.6.13.3.1 0.5 ml capless epi tubes used to collect fractions that now do not contain your protein of interest can be disposed of.

7.6.13.3.2 Any larger fraction collection tubes can be rinsed a few times with DI water.

7.4.13.4 Dispose of any unused buffers.

7.4.13.5 Clean up any remaining used space in the lab.

8. Troubleshooting

8.1 There are many places for complications in purifying protein via a HPLC machine. Please read through notes carefully prior to executing protocol. Contact available proctor for help as needed.

9. **References**

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10. MODULE METHODS TASK

1. Starting at the post-expression bacterial pellet, purify 1GFP via IEX chromatography.
2. Describe how, specifically, Ion-exchange chromatography can be used to purify our protein of interest.
3. What are the variables we must consider when choosing this as our protein purification method?
4. Display your results of the UV absorbance (A280) over time/volume from your purification run. Clearly label which peaks you believe to be corresponding to your protein of interest. Label which fractions you used to test for the presence of your protein.
5. Display your results from your SDS-PAGE gel run from all collected samples. Identify which bands correspond to your protein of interest, and why you believe those bands are your desired protein.
6. Attach a photo of your final purified protein product containing the tube labelled with your name, date, protein, protein concentration, and buffer information.
7. Do you have any evidence that your protein sample is not completely pure? If so, what evidence does this consist of? What steps might you take next to further purify your protein of interest? What is a drawback of subjecting your protein to an entirely separate purification step or steps?
8. Please provide us with any feedback you wish to offer: What you liked about this training module and what you feel would be helpful to change.

11. **1GFP Information**

1. Plasmid vector: pET His6 GFP TEV LIC cloning vector
2. Gene of interest: His6 GFP TEV (1GFP)
3. Gene length: 801 bp
4. Gene sequence:

5'-
ATGGGTTCTTCTCACCATCACCATCACCATGGTTCTTCTGTGAGCAAGGGCGAG
GAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAA
ACGGCCACAAGTTCAGCGTGCGCGGCGAGGGCGAGGGCGATGCCACCAACG

GCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTG
GCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACC
CCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTAC
GTCCAGGAGCGCACCATCTCCTTCAAGGACGACGGCACCTACAAGACCCGCG
CCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGG
CATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACT
TCAACAGCCACAACGTCTATATCACGGCCGACAAGCAGAAGAACGGCATCAAG
GCGAACTTCAAGATCCGCCACAACGTCGAGGACGGCAGCGTGCAGCTCGCCG
ACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGA
CAACCACTACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGC
GCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGG
CATGGACGAGCTGTACAAGGGGATCGAGGAAAACCTGTACTTCCAATCCAATA
TTGGAAGTGGA**TAA**
-3’

5. Peptide sequence:

N-term
MGSSHHHHHHGSSVSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNG
KLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQ
ERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNV
YITADKQKNGIKANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQS
KLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKGIEENLYFQSNIGSG
C-term

6. Protein Parameters (ProtParam)

Expasy: https://web.expasy.org/cgi-bin/protparam/protparam
Number of amino acids: 266
Molecular weight: 29.752 kDa
Theoretical Isoelectric point: pI = 5.71