University of Lethbridge

Biochemistry 2000 Laboratory Manual

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Laboratory Information

The laboratory is worth **25% of your final grade** which is comprised of eight assignments (**21%**) and the keeping of a lab book (**4%**). There is one assignment for each laboratory exercise which may cover **all** elements of the laboratory exercises. This can include, but will not be limited to; data analysis and interpretation, data presentation, problem solving, conceptual understanding, and experimental design. Assignments will be graded as appropriate for the level of Biochemistry course and an understanding of concepts taught in prerequisite courses is assumed (e.g., scientific method and the basics of experimental design, construction of properly formatted graphs and tables, significant figures, SI units, proper citing and referencing of source material, etc.). It is strongly advised students review such concepts as needed and/or ask questions of their instructor, well in advance of any assignment due dates. Lab books will be collected at random throughout the semester and graded as per the guidelines found in **Appendix A**.

Whenever possible it would be wise to review the details of each assignment prior to starting to the exercise, assignments will generally be made available on Moodle before lab. All assignments are due one (1) week following completion of the exercise, PRIOR to the start of the next laboratory period (unless otherwise specified). All assignments are to be computer generated (hand-written work, in any form, is NOT acceptable) and only portable document files (.pdf) submitted to Moodle will be accepted. Be aware that plagiarism/AI detection services may be used to ensure the academic integrity of all work submitted (see the Student Code of Conduct).

Penalties for Late Assignments Any assignments handed in late will be subject to the following penalties;

Past specified due time but on the same calendar day	**10%**
1 calendar day late	**25%**
2 calendar days late	**50%**
3 or more calendar days late	**100%**

It is the students responsibility to ensure that the correct file for each assignment has been uploaded to Moodle to successfully. Please double check following your file submission!

Extensions (assignments only) Extensions will only be considered if a written application to your Laboratory Instructor, including work completed on the assignment to date, is made a minimum of 48 hours prior to the due date.

Regrading It is the student's responsibility to review all graded material and any concerns about grading must be brought to the lab instructor's attention within 7 days. Grade changes will not be made after that point. Note: Due to the nature of the rotating schedule of exercises, graded assignments will not be returned until after all students have submitted their assignment for a particular exercise.

Academic Offenses All students are expected to have read the Student Code of Conduct Policy, as referenced in the current academic Calendar, and should understand what constitutes an academic offense as well as the penalties associated with committing an academic offense. There is a zero tolerance policy for academic offenses committed within the Biochemistry 2000 labs. At the discretion of the laboratory coordinator, any graded work may be submitted to a plagiarism/AI detection services in addition to being compared to the work of other students. Protect your academic integrity, do not share your assignments with fellow students (particularly in an electronic form). *Having your work copied and submitted by another can result in penalties for all parties involved, including the original author of the work.*

Laboratory Performance Up to five (5) marks of your final grade out of 20 may be deducted for poor performance. Poor performance includes, but is not limited to the following;

- Lack of Preparedness. Students are expected to have read the laboratory exercise and any associated background reading or appendices prior to attending lab.
- Coming late to lab. The first portion of most labs is devoted to introduction to materials and equipment, to techniques used, and to outlining safety issues.
- Failure to take care of equipment. Instructions for the proper use of equipment used during the semester will be provided by your instructor and/or can be found in the lab manual.
- Failure to follow instructions from the lab instructor.
- Sloppiness and/or carelessness when carrying out experiments.
- Not bringing personal protective equipment (i.e., lab coat and safety glasses). These items may be borrowed once during the semester but students who need to borrow these items more than once during the semester will be penalized.
- If any student is asked to leave the lab due to creating an unsafe environment for either the individual in question or their fellow students will automatically forfeit the entire five (5) marks.

Absences and Illness

Students who are feeling unwell should NOT attend laboratory under any circumstance. Every reasonable attempt will be made to accommodate an *excusable absence* but there will not be an opportunity to make up missed lab exercises. Accommodations may include being provided with data to complete any relevant assignments, potentially attending another lab section later in the week (very limited opportunities), or pro-rating the laboratory grade to reflect a missed assignment. *Inexcusable absences* will result in a zero being assigned for the current assignment. The final determination of what constitutes an excusable or inexcusable absence is at the discretion of the lab coordinator.

Any student who has missed more than two (2) lab sessions, excusable or inexcusable, will receive a zero (0) for the lab portion of the course grade.

Please contact your lab instructor ASAP if you will miss a lab!!

Laboratory Exercises

Lab #	Exercise
1	Micropipet Calibration
2	Solution & Sample Preparation
3	Size Exclusion Chromatography (SEC)
4	Protein Quantification
5	Ion Exchange Chromatography (IEC)
6	SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE)
7	Homology Modeling
8	Experimental Design

Schedule

Date	Group A	Group B	Group C
Sept. 9	**FDOC - No Labs**	**FDOC - No Labs**	**FDOC - No Labs**
Sept. 14-15	Intro – Micropipet Calibration	Intro – Micropipet Calibration	Intro – Micropipet Calibration
Sept. 21-22	Solution & Sample Preparation	Size Exclusion Chrom.	Protein Quantification
Sept. 28-29	Protein Quantification	Solution & Sample Preparation	Size Exclusion Chrom.
Oct. 5-6	Size Exclusion Chrom.	Protein Quantification	Solution & Sample Preparation
Oct. 12-13	**No Labs – Thanksgiving**	**No Labs – Thanksgiving**	**No Labs – Thanksgiving**
Oct. 19-20	Ion Exchange Chrom.	SDS-PAGE	Homology Modeling
Oct. 26-27	Homology Modeling	Ion Exchange Chrom.	SDS-PAGE
Nov. 2-3	SDS-PAGE	Homology Modeling	Ion Exchange Chrom.
Nov. 9-10	**No Labs – Reading Week**	**No Labs – Reading Week**	**No Labs – Reading Week**
Nov. 16-17	Experimental Design	Experimental Design	Experimental Design
Nov. 23-24	Design Consult - Optional	Design Consult - Optional	Design Consult - Optional
Nov. 30 – Dec. 1	**No labs –** final assign. due	**No labs -** final assign. due	**No labs -** final assign. due

Safety

Students enrolled in laboratories in Chemistry & Biochemistry should be aware that there are risks of personal injury through accidents (fire, explosion, exposure to biohazardous materials, corrosive chemicals, fumes, cuts, etc). The guidelines outlined below are designed to:

a) minimize the risk of injury by emphasizing safety precautions and
b) clarify emergency procedures should an accident occur.

EMERGENCY NUMBERS:

City Emergency	911
Campus Emergency	2345
Campus Security	2603
Student Health Centre	2484

THE LABORATORY INSTRUCTOR MUST BE NOTIFIED AS SOON AS POSSIBLE AFTER THE INCIDENT OCCURS.

EMERGENCY EQUIPMENT:

Your lab instructor will indicate the location of the following items to you at the beginning of the first lab period.

- Closest emergency exit
- Closest emergency telephone and emergency phone numbers
- Closest fire alarm
- Fire extinguisher and explanation of use
- Safety showers and explanation of operation
- Eyewash facilities and explanation of operation
- First aid kit

GENERAL SAFETY REGULATIONS:

- **Eating and drinking is prohibited** in the laboratory. Keep pencils, fingers and other objects away from your mouth. These measures are to ensure your safety and prevent accidental ingestion of chemicals or microorganisms.
- **Personal protective wear is mandatory.** Lab coats, safety glasses and closed-toed shoes must be worn at all times during lab exercises which involve potential for chemical or biological spills.
- Contact lenses are not permitted in laboratories where chemicals or biological materials are handled.
- Coats, knapsacks, briefcases, etc. are to be hung on the hooks provided, stowed in the cupboards beneath the countertops, or placed along a side designated by your instructor. Take only the absolute essentials needed to complete the exercise* with you to your laboratory bench. (*e.g. Manual, pen or pencil)
- Mouth pipetting is NOT permitted; pipet pumps are provided and must be used.
- Always wash your hands prior to leaving the laboratory.
- Consult your instructor if you require additional supplies.
- Report any equipment problems to instructor immediately. Do NOT attempt to fix any of the equipment that malfunctions during the course of the lab.
- Use caution when handling chemical solutions. Consult the lab instructor for instruction regarding the clean-up of corrosive or toxic chemicals.

- Contain and wipe up any spills immediately and notify your lab instructor (see SPILLS below). Heed any special instructions outlined in the lab manual, those given by the instructor or those written on reagent bottles.
- Long hair must be restrained to prevent it from being caught in equipments, Bunsen burners, chemicals, etc.
- Dispose of broken glass, microscopes slides, coverslips, and glass pipets in the specially marked white and blue boxes. There will be NO disposal of glassware in the wastepaper baskets.
- You are responsible for leaving your lab bench clean and tidy. Glassware must be thoroughly rinsed and placed on paper toweling to dry.

SPILLS:

- Spill of SOLUTION/CHEMICAL: While wearing gloves, wipe up the spill using paper towels and a sponge as indicated by the lab instructor.

- Spill of ACID/BASE/TOXIN: Contact instructor immediately. DO NOT TOUCH.

- BACTERIA SPILLS: If necessary, remove any contaminated clothing. Prevent anyone from going near the spill. Cover the spill with 10% bleach and leave for 10 minutes before wiping up. Discard paper towels in biohazard bag. Discard contaminated broken glass in designated biohazard sharps container.

DISPOSAL:

- Broken glass, microscope slides, coverslips and Pasteur pipets are placed in the **upright white 'broken glass' cardboard boxes**. NO PAPER, CHEMICAL, BIOLOGICAL OR BACTERIAL WASTE MATERIALS should be placed in this container

- Petri plates, microfuge tubes, pipet tips should be placed in the **orange biohazard bags**. The material in this bag will be autoclaved prior to disposal.

- Bacterial cultures in tubes or flasks should be placed in **marked trays** for autoclaving.

- Liquid chemicals should be disposed of as indicated by the instructor. DO NOT dispose of residual solution in the regent bottles. In case of any uncertainty in disposal please consult the lab instructor.

- Slides of bacteria should be placed in the trays filled with 10% bleach that are located at the ends of the laboratory benches.

HEALTH CONCERNS:

Students who have allergies, are pregnant, or who may have other health concerns should inform their lab instructor so that appropriate precautions may be taken where necessary.

Laboratory 1 – Micropipet Calibration

Introduction:

In any laboratory experience, precise and accurate measurements are essential for obtaining meaningful data. In the field of biochemistry, the macromolecules of interest are almost exclusively dissolved in a solvent system (generally aqueous). Consequently, virtually all experiments will require the precise and accurate transfer of solutions. The primary tool for delivering small volumes of a solution is the micropipet.

In order to track the status of the micropipets that will be utilized in the laboratory, a calibration curve will be generated for each micropipettor at the beginning and the end of the semester.

Reagents & Equipment:

- d^2H_2O (8x 20 mL)
- thermometer
- micropipets and tips (8 sets)
- microcentrifuge tubes (1.5 mL)
- gloves (all sizes)
- analytical balance (8)

Protocol:

Each student will initially perform this protocol for only ONE micropipet of the set of three. Once complete, students will regroup for further instruction. Following this, each student will repeat the protocol on the <u>same micropipet used prior to instruction</u>. Once complete, work with your lab partner to complete the protocol on the third micropipettor in the set.

(1) Record the temperature in the room, select a micropipettor and record its number.

(2) Set the micropipettor to the desired volume (see Table 1).

(3) Fit a clean micropipet tip snuggly to the micropipet barrel.

(4) Place a clean microcentrifuge tube on the balance and once the reading has stabilized tare the balance.

(5) Remove the microcentrifuge tube from the balance and carefully transfer the indicated volume of d^2H_2O to the microcentrifuge tube.

(6) Transfer the microcentrifuge tube to the balance and record the mass.

(7) Repeat steps 1-5 <u>*in triplicate for each*</u> of volumes listed in Table 1 (you can add the subsequent aliquots to the same tube in order to reduce waste).

Table 1. Volumes of d^2H_2O to be used for the calibration of each specified micropipet. Each volume listed is to be measured in triplicate.

Micropipet	**Volumes**				
1 – 10 µL	1.00	2.00	4.00	7.00	10.00
10 – 100 µL	10.0	20.0	40.0	70.0	100.0
100 – 1000 µL	100	200	400	700	1000

Laboratory 2 – Crystallization of Lysozyme

An Exercise in Solution Preparation

Introduction:

Proteins, like many materials, are able to form crystals, a solid with a high degree of internal order. Chemists frequently use the crystallization of small molecules as a method of purification, as impurities will typically remain in solution as the crystals form. While proteins are not typically purified in this manner, the ability to crystallize a given protein is fundamental for X-ray crystallography. X-ray crystallography is used to determine the arrangement of atoms within a crystal based upon the diffraction pattern produced when x-rays strike the crystal. This technique has greatly aided our understanding of many proteins by determining their three-dimension structure at high resolutions.

The crystallization of proteins is inherently more difficult than for smaller molecules, such as NaCl or SiO_2, due to a their size, irregular surfaces, and overall stability. The primary method utilized to crystallize proteins is by vapour diffusion which involves placing a small drop of highly purified protein in a sealed system with a reservoir. The composition of the reservoir is specific to the protein to be crystallized but typically contains a buffer to maintain pH and a precipitant (a compound that aids with protein precipitation). The reservoir solution is mixed with the protein drop prior to sealing the system, and as water vapourizes from the drop to rejoin the reservoir, the concentration of the protein and precipitant in the drop is slowly increased (Figure 1). It is this gradual increase in concentration of the protein and precipitant that is favourable for crystal formation.

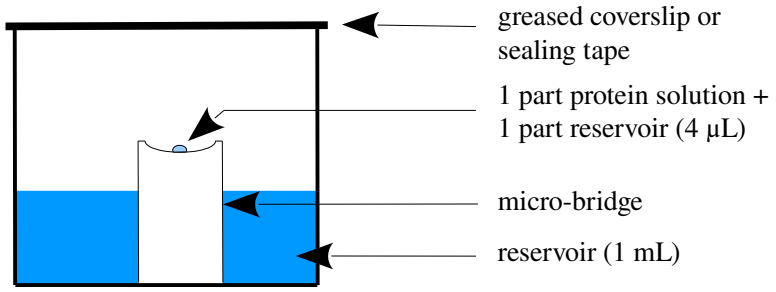

Figure 1: Typical setup for a sitting drop vapour diffusion experiment.

Determining the composition of the reservoir solution to crystal a protein for the first time is generally a very tedious and time consuming task. Fortunately, the protein you will be working with in this exercise, lysozyme, has a well documented set of conditions for its crystallization. The preparation of reagent and sample solutions from both solid and liquid sources is a fundamental component of all biochemical investigations. While solution preparation is generally straightforward, individuals that are new to laboratory sciences are often confused by the variety of concentration measurements that are commonly utilized.

The SI units of molarity (M) are the preferred unit of concentration measurement but additionally, the units of % weight per volume (w/v) and % volume per volume (v/v) are commonly encountered in the life sciences. In this laboratory exercise, you will prepare several solutions using both standard and non-standard concentration measurements frequently encountered in biochemistry and subsequently use these solutions to grow crystals of the protein lysozyme.

Reagents & Equipment:

- $NaCl_{(s)}$
- Ethylene $Glycol_{(l)}$ (M_w=62.07g/mol)
- 0.500 M $NaCH_3CO_2$ (pH 4.8)
- 50 mg/mL Lysozyme (in d^2H_2O)
- microcentrifuge tubes
- analytical balance
  - w/ weigh paper & spatulas
- crystallization plates (sitting drop)
- sealing tape
- razor blades & scissors

Protocol:

This exercise is to be completed individually. Record the actual values for masses, volumes, etc. to create the various solutions as these will be needed for the assignment.

Preparation of Stock Solutions

(1) Prepare approximately 1 mL of 30.0% w/v NaCl from solid NaCl in a microcentrifuge tube.

(2) Prepare approximately 1 mL of 4.0 M NaCl from solid NaCl in a microcentrifuge tube.

(3) Prepare 1 mL of 25.0% v/v ethylene glycol in d^2H_2O in a microcentrifuge tube.

(4) Prepare 1 mL of 2.0 M ethylene glycol ($\rho_{ethylene\ glycol}$=1.115 g/mL) in microcentrifuge tube.

You will be provided a 0.5 M acetate buffer at pH 4.8 in a stock bottle and a 50 mg/mL (highly concentrated) solution of lysozyme.

Preparation of Reservoir Solutions

(5) Using your freshly created stock solutions (above), prepare the four reservoir solutions listed in Table 3 in separate, clean microcentrifuge tubes.

Table 3. Reagent concentrations for each of the four solutions to be used for the crystallization of lysozyme.

Solution #1	**Solution #2**	**Solution #3**	**Solution #4**
8.00% NaCl	10.0% NaCl	1.40 M NaCl	1.60 M NaCl
0.20% Ethylene Glycol	0.010 M Ethylene Glycol	1.00% Ethylene Glycol	0.040 M Ethylene Glycol
50.0 mM acetate	20.0 mM acetate	40.0 mM acetate	30.0 mM acetate
d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O
The final volume of each solution in its reservoir should be 0.5 mL.			

Crystallization of Lysozyme by Vapour Diffusion

(6) To set up your vapour diffusion experiment, transfer the prepared reservoir solution to the appropriate reservoir well of the crystallization plate. This is the bottom of the well surrounding the central post (see figure 1).

> **Note:** Please set up your 4 trials in a *column* (top to bottom) of the plate and NOT in a row (left to right), this allows for more efficient use of the plate and easier to identify an individual's trials.

(7) Transfer 10 µL of the reservoir solution from the well to the top of the central post. Add 10 µL of 50 mg/mL lysozyme, found in the deli fridge, to the reservoir solution aliquot on the central post. Try not to introduce any bubbles.

(8) Repeat steps 6 & 7 for the remaining reservoir solutions for a total of four separate crystallization experiments.

(9) Return the lysozyme solution to the appropriate rack in the deli fridge.

(10) Seal the column of crystallization experiments using transparent crystallization sealing tape. Use caution when sealing the plate so as not to disturb the crystallization experiments but ensure that each well is completely air tight.

> **Note:** The sealing tape is the perfect width to seal two columns at the same time. If you are setting up your trials at the same time as another student, use adjacent columns to simplify the sealing of the wells.

(11) Label the column with the solution numbers and your initials, **do not write directly on the plate** but rather on the sealing tape (do not obstruct the view of the well with your label). Give the crystallization plate to your instructor.

Laboratory 3 – Size Exclusion Chromatography

Introduction:

The separation and purification of biological macromolecules present a number of daunting problems. In particular, biological macromolecules have similar physiochemical properties, are prone to chemical modification and easily denature. The most common and powerful technique used to purify macromolecules is chromatography. Chromatographic methods applied to protein purification involve an inert solid matrix that exerts a retarding force upon macromolecules in solution that are flowing past the matrix. The type of retarding force depends upon matrix being utilized and can result in separations based upon size, ionic charge, polarity or any combination of these properties.

In this laboratory exercise you will utilize a technique referred to as size exclusion chromatopgraphy (SEC; also referred to as gel filtration, gel permeation, or molecular sieve chromatography). SEC utilizes a column packed with an inert, porous matrix (beads) and a mixture of proteins, when applied to the top of the column, is separated based upon their size, and to some extent their shape. Larger molecules within the mixture are excluded from the pores within the matrix and as a result flow faster through the column than smaller molecules. Smaller molecules, which are able to fit inside the pores, get 'side-tracked' and as a result flow slower through the column (Figure 2). The smaller the molecule, the more easily it will be side-tracked resulting in an increased volume of buffer needed to elute these small molecules from the column.

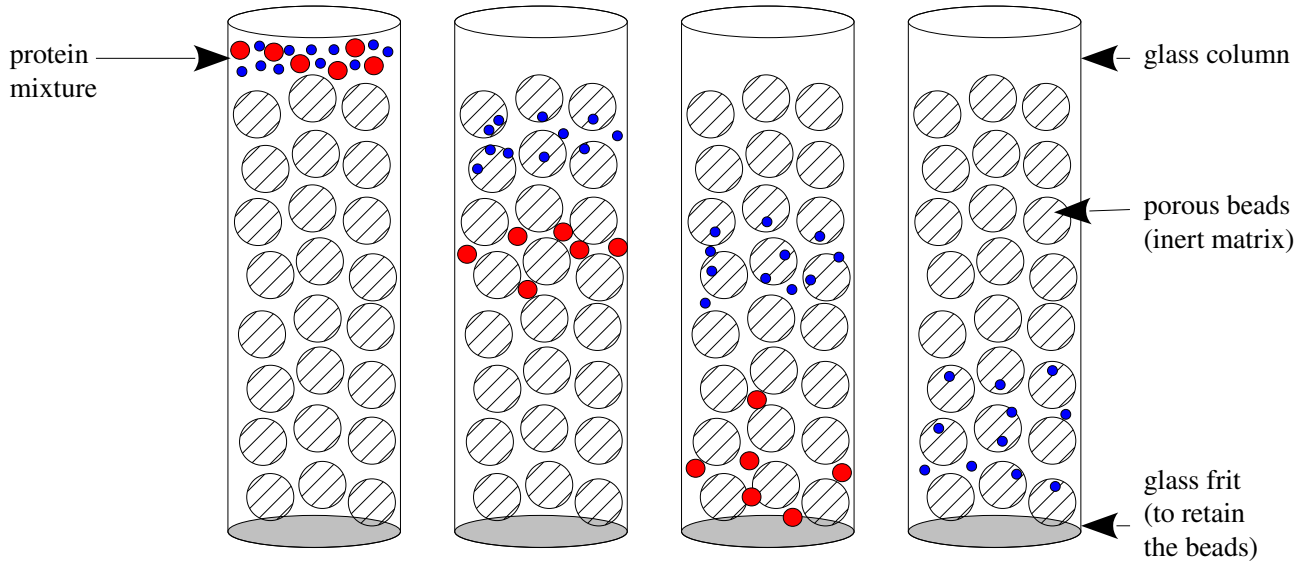

Figure 2: Schematic of how a mixture of small proteins (blue circles) is separated from larger proteins (red circles) by an SEC column. As buffer flows through the column, the smaller proteins are retained within the porous beads while the larger proteins elute from the column later (left to right).

There is a wide variety of SEC resins available with varying pore sizes, the selection of resin to be used depends upon the nature of the proteins to be separated. Any proteins larger than the pore size of the beads (the **exclusion limit**) will elute from the column together in the **void volume** (**V_o**). Proteins

below the exclusion limit will elute from the column, largest to smallest, at specific **elution volumes** (**V_e**). The **total column volume** of any given column (**V_T**) is defined based on the V_e of a very small molecule (e.g. a salt) and represents the volume of the column available, both inside and outside the matrix, to such a small molecule. It should be noted that the V_e of a protein is specific to a particular column. To report a value that is independent of the specific column parameters, a K_{av} is typically calculated:

$$K_{av} = \frac{(V_e - V_o)}{(V_T - V_o)}$$

The objective of this lab will be to separate catalase, cytochrome c, and potassium chromate from a mixed sample utilizing SEC. Table 4 provides some of the key characteristics of these compounds that may be useful for completion of this exercise.

Table 4. Properties of compounds to be separated by SEC.

Compound	**Appearance**	**M_r**	**A_{max} (nm)**
catalase	Light brown	240,000	280
cytochrome c	Dark red	13,000	410
potassium chromate	yellow	150	280 & 410

Reagents & Equipment:

- running buffer
  - 50 mM Tris pH 8.0
  - 100 mM NaCl
  - 0.1 mM EDTA
- storage buffer w/ 20% EtOH
- sephadex G-50 medium (50% slurry)
  - pore size: 1,500 – 30,000
- protein sample (pre-mixed)
- chromatography column and stand
- waste beaker
- 1.0 and 5.0 mL disposable pipet w/ pump
- microcentrifuge tubes and rack
- UV spectrophotometer w/ semi-micro cuv.

Protocol:

Work as pairs to complete this exercise

Pouring and Packing the Column

(1) Obtain a clean column, drain and discard the storage buffer in the column, and with the stopcock closed, add approximately 5 mL of running buffer.

(2) Add approximately 50 mL of the supplied sephadex G-50 slurry (i.e., just fill the column to the top directly from the supplied flask of resin slurry, as the column holds approximately 50 mL). Be sure to completely re-suspend the slurry immediately prior to pouring the column and pour the column in one smooth motion.

(3) Check the column to ensure that no air bubbles are trapped and once satisfied begin packing the sephadex G-50 by opening the stopcock and allowing the buffer to drain off the bottom of the

column. Continue to pack the column with an additional 2 column volumes of running buffer (50 – 60 mL) by continuously topping off the column with buffer. Be gentle when adding the buffer so as not to disturb the top of the resin bed as it forms.

Do not let the column run dry at any point during this exercise!

(4) Towards the end of the above step the resin will form a distinct, visible layer from the buffer. At this point float a small piece of filter paper to act as a 'shock absorber' when loading the protein sample in the next section.

> **Note:** Make sure to cut the filter paper to size **prior** to packing the column and ensure the cut diameter of the filter paper is smaller than the inner diameter of the glass column (NOT the yellow plastic fittings on the column).

(5) Crudely *estimate* the total column volume using the supplied calibrated column or the calibration marks drawn onto the columns for you. This value will be needed to complete the steps below.

Loading and Running the Protein Sample

(6) Drain the column until the meniscus of the buffer just disappears into the matrix.

(7) Gently layer 500 µL of the protein sample onto the top of the matrix using a 1.0 mL serological pipet. Do not to disturb to the matrix while loading your sample.

(8) Drain the column until the meniscus of the protein sample just disappears into the matrix.

(9) *Carefully and quickly* top off the column with running buffer using the 10.0 mL serological pipet to very gently layer buffer on top of the resin. Once again, do not disturb the top of the matrix.

(10) Open the stopcock and begin collecting the fractions, with a volume no greater than 1.0 mL, in microcentrifuge tubes, until the yellow colour has been completely eluted form the column. Continue to add running buffer to the top of the column while collecting the fractions. This should require no more than two column volumes of running buffer (50 – 60 mL).

(11) Obtain a 96-well, *UV transparent* plate from your instructor. Load 100 µL of each fraction collected to an individual well of the plate as directed by your instructor (the order in which the wells are loaded is important).

(12) Use the plate reader (a fancy UV spectrophotometer) to measure the absorbance at 280 and 410 nm for each fraction collected. Your instructor will help you with the operation of the plate reader and with the saving of your data (the data will be posted to Moodle for completion of the assignment).

(13) Determine the fractions with the highest absorbance reading corresponding to each of the three compounds in your sample, see Table 4 (i.e., you should identify three (3) separate fractions). You will use these three fractions in exercise #6 (SDS-PAGE).

(14) For each of the three fractions identified above, transfer 50 µL to a new microcentrifuge tube and add 50 µL of 2x reducing sample buffer (2x RSB). Mix well, properly label the three tubes, and store them in the -20 ºC freezer as directed by your instructor.

Column Clean-up

(15) Run an additional two column volumes of storage buffer (provided) through the column.

(16) Add storage buffer to the column leaving a 5 – 10 mL air gap and seal the column. Remove the column from the stand and rock it back and forth until the Sephadex G-50 is completely re-suspended and pour the resin *back into the original flask provided* (this may need to be repeated to fully drain the resin from the column). Rinse the column thoroughly with distilled water.

Do not discard the resin down the sink or into the garbage!

(17) Store the rinsed column with approximately 10 mL of storage buffer.

(18) Clean and return all glassware and supplies used.

Laboratory 4 – Protein Quantification

Introduction:

An essential component of all molecular studies in chemistry and biochemistry is the ability to accurately and reliably measure concentration. Protein (and nucleic acid) concentrations are most commonly quantified using spectroscopic methods as these methods are rapid, accurate and inexpensive. The goal of this laboratory exercise is to become familiar with several different spectroscopic methods for quantifying protein concentration.

The simplest spectroscopic methods for quantifying protein concentrations rely upon the association of protein and a ligand that produces a shift in absorbance maximum in the visible spectrum. The magnitude of absorption is directly proportional to the concentration of protein and typically requires a standard curve from which protein concentration can be interpolated. As an example of these methods, students will utilize the Bradford method (based upon the dye Coomassie brilliant blue G-250) to determine the concentration of an unknown protein sample.

Coomassie brilliant blue G-250 interacts with ariginine, lysine, and aromatic residues (Trp, Tyr, Phe, and His) exposed on the surface of a protein. The absorbance maximum of the unbound dye shifts from 470 nm to 595 nm when bound to the surface of a protein. It is this absorbance shift that allows for its use in the quantification of proteins. Furthermore, the dye is unaffected by many common reagents used within a biochemistry laboratory with one notable exception, detergents. High concentrations of detergents will interact with Coomassie brilliant blue G-250, resulting in an overestimation of the protein concentration being determined.

Alternative spectroscopic methods for determining protein concentration make use of the intrinsic UV absorbance of most proteins. Once again, the absorbance is directly proportional to the concentration of the protein. While these methods are extremely simple, they require knowledge of a protein and solution specific constant (extinction coefficient) to determine accurate concentrations.

The objective of this exercise will be to 1) determine the concentration of an unknown protein sample using the Bradford assay and 2) calculate the extinction coefficient for this protein using this concentration value (Bradford assay) and the data from an A_{280} assay.

Reagents & Equipment:

- Unknown protein sample
- Standard protein sample (1.0 mg/mL)
- Protein buffer
  - 10 mM KH_2PO_4 (pH 6.9), 100 mM NaCl
- Bradford Reagent
- Microcentrifuge tubes & racks
- UV/Visible Cuvettes
- Micropipettors
- Kimwipes

Protocol:

Work as pairs to complete this exercise

Ensure all reagents needed are obtained from the fridge and have a chance to equilibrate to room temperature BEFORE beginning the assay.

Bradford Assay

(1) Obtain 0.6 mL of the Standard protein sample (1.0 mg/mL).

(2) Obtain 1.5 mL KH_2PO_4 buffer.

(3) Create the dilutions of the Standard protein solution as outlined in Table 6, **in duplicate**.

Table 6. Dilutions of the Standard protein solution to be used for the Bradford assay.

Standard Curve Samples	Standard Protein (µL)	Protein Buffer (µL)
0.0 mg/mL	0	100
0.2 mg/mL	20	80
0.4 mg/mL	40	60
0.6 mg/mL	60	40
0.8 mg/mL	80	20
1.0 mg/mL	100	0

(4) Obtain 150 µL of the Unknown protein sample

(5) Dilute the Unknown protein sample according to Table 7.

Table 7. Dilutions of the Unknown protein solution to be used for the Bradford assay.

Unknown Samples	Unknown Protein (µL)	Protein Buffer (µL)
A	10	90
B	30	70
C	90	10

(6) Obtain 15 mL of Bradford reagent

(7) Add 0.90 mL of Bradford reagent to each Standard and Unknown sample.

(8) Incubate all samples at room temperature for 5 minutes.

(9) Blank the spectrophotometer at 595 nm using the 0.0 mg/mL Standard Curve Sample.

> **Note:** Do not use a quartz cuvette for the Bradford assay as the Bradford reagent will stain the quartz, wrecking the cuvette. Only use the disposable, semi-micro (1 mL) cuvettes for the Bradford assay.

(10) Record the absorbance of each Standard and Unknown sample at 595 nm.

A_{280} Assay

Utilize the small volume (100 µL) quartz cuvette for the A_{280} assay. Your instructor will help you with setting this up and how to use this cuvette properly.

(1) Blank the spectrophotometer with 100 µL of the protein buffer (10 mM KH_2PO_4 [pH 6.9], 100 mM NaCl)

(2) Add 2 µL of your protein sample directly to the cuvette using the 'reverse pipetting technique'.

Reverse pipeting technique

This technique of pipetting is used when transferring volumes of viscous liquids or to minimize the risk of introducing air bubbles into a solution. For this exercise, the introduction of even the smallest (microscopic) air bubble can have a significant impact on your A_{280} readings and render the data completely unusable. The reverse pipetting technique is carried out as follows:

1. Set the micropipettor to the desired volume to be transferred.
2. Depress the plunger to the **second stop**.
3. Place the pipettor tip into the solution to be transferred and slowly release the plunger. You will now have a volume in the tip larger than the set volume.
4. To dispense the solution, steadily press the plunger to to the **first stop only**. Discard any remaining liquid with the tip.

(3) Mix your sample using a 10-100 µL micropipet set to 50 µL. Take care not to introduce bubbles into the cuvette.

(4) Record the absorbance

(5) Add an additional 2 µL of the protein sample as described above. Mix as before and record the absorbance.

(6) Repeat Step 5 until you have a total of five absorbance readings.

Laboratory 5 – Ion Exchange Chromatography

Introduction:

Ion exchange chromatography (IEC) is used to separate proteins, and other biomolecules, based upon charge differences between the different molecules. The charged groups of a protein of interest can be reversibly bound, through ionic interactions, to an ion exchange matrix carrying the opposing charge. Proteins (and other molecules) which are neutral or have the same charge as the ion exchange matrix being utilized can then be washed away leaving the protein of interest bound to the matrix. The bound protein can then be eluted from the matrix by altering the pH or increasing the ionic strength of the buffer.

There are two general categories of ion exchange chromatography, anion exchange and cation exchange. Anion exchange chromatography uses an immobile matrix (e.g. sephadex or sepharose) which has a covalently attached positively charged functional group (e.g. diethylaminoethyl [DEAE]) and will therefore bind negatively charged ions (anions). Conversely, cation exchange chromatography utilizes negatively charged functional groups (e.g. carboxymethyl [CM]) to bind positively charged ions (cations). The choice between a cation or anion exchanger, and the type of matrix to use, depends upon the characteristics of the specific protein to be purified. Of particular importance is the isoelectric point (pI) of the protein and the range of pH values for which the protein is structurally stable.

This exercise will utilize a weak cation exchanger, CM-sepharose, in an attempt to isolate individual proteins from a mixture. The term weak does not refer to the attraction strength between the functional group of the resin and the protein of interest. Instead, weak ion (cation or anion) exchangers have a narrower pH range over which the functional group remains charged whereas strong ion exchangers are those with functional groups that remain charged over a much wider pH range (typically 2-12).

Reagents & Equipment:

- loading buffer
  - 50 mM Tris pH 8.0
  - 100 mM NaCl
  - 0.1 mM EDTA
- elution buffer (stock)
  - 50 mM Tris pH 8.0
  - 2.5 M NaCl
  - 0.1 mM EDTA
- storage buffer
  - 50 mM Tris pH 8.0
  - 100 mM NaCl
  - 0.1 mM EDTA
  - 20% ethanol
- protein sample
  - 2.5 mg/mL lysozyme
  - 2.5 mg/mL catalase
- carboxymethyl sepharose column
  - ~1 mL column volume
- microcentrifuge tubes and rack
- UV spectrophotometer
- graduated cylinder (10 mL)
- 1.0 mL serological pipet
- 20 mg/mL lysozyme (standard)
- 20 mg/mL catalase (standard)
- *Micrococcus luteus* in TSB (48 hr)
- uninoculated TSB
- glass slides
- 10% (v/v) H_2O_2

Note: Students need to prepare 10 mL each of the elution buffers with 0.2, 0.3, and 0.5 M NaCl using the provided loading and stock elution buffer (2.5 M NaCl), *in 15 mL falcon tubes*.

Calculations should be done in advance to save time.

Protocol:

Work as pairs to complete this exercise

Applying and Eluting the Protein Sample

(1) Drain any storage buffer present and equilibrate the column with 3 column volumes of loading buffer. Columns have been prepacked for you.

(2) Once the meniscus has dropped to the top of the resin, use a 1.0 mL serological pipet to gently load the protein sample to the top of the resin bed.

(3) Drain the sample into the resin while collecting the "flow through" into a single microcentrifuge tube.

(4) Wash any unbound sample with 3 column volumes of loading buffer. Collect 1 mL fractions as the meniscus drops to the top of the resin bed.

(5) Begin to elute any bound protein by adding 3 column volumes of the 0.2 M NaCl elution buffer and collecting 1 mL fractions. Repeat this step for each of the remaining elution buffers from lowest to highest NaCl concentration (0.3, 0.5, and 2.5 M).

(6) Store the column in loading buffer containing 20% ethanol.

UV Absorbance of collected fractions

(7) Blank the UV spectrophotometer using the loading buffer and record the absorbance at 280 nm for each fraction collected.

(8) Identify the fraction with the highest (and significant) absorbance for each of the elution buffers used in step 5.

(9) Identify the contents of these fractions by carrying out the activity assays for catalase and lysozyme, as described below.

Catalase Activity Assay

Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide (H_2O_2) into H_2O and O_2. This reaction can be easily monitored by looking for the production of O_2 which will result in the formation of visible bubbles. This assay is to be carried out on all the fractions identified in step 8 *with the appropriate positive and negative controls* (20 mg/mL catalase will be provided).

(1) Spot out 10 µL of each solution to be tested onto a glass slide. What would be an appropriate negative control for this assay?

(2) Add 10 µL of 10% H_2O_2 to each sample and observe if any bubbles form.

Lysozyme Activity Assay

Lysozyme catalyzes the hydrolysis of the 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-gluosamine in the peptidoglycan of bacterial cell walls, which can result in cell lysis. *Micrococcus luteus*, a small, Gram-positive bacterium, is particularly sensitive to lysis by lysozyme, thereby making it an ideal candidate for use in this assay. Cell lysis can be followed spectrophotometrically at 600 nm, a wavelength commonly used to monitor bacterial cell culture density. As with the catalase activity assay, this assay should also be performed with the appropriate positive and negative controls (20 mg/mL lysozyme will be provided) in addition to the fractions identified in step 8.

(1) Dilute the provided *M. luteus* culture with tryptic soy broth (TSB) to a final volume of 10.0 mL as directed by your instructor (approximately a 1:20 dilution is typically needed). MIX WELL and check that the absorbance at 600nm of the bacterial culture to ensure is within the range of the equipment being used; if not, continue to dilute the culture.

(2) Aliquot 1.0 mL of the diluted culture into 8 cuvettes so you have sufficient aliquots for every sample to be tested (do not forget the positive and negative controls!).

(3) Load all 8 cuvettes to the carousal, pay attention to ensure the cuvettes are oriented correctly, and record the initial absorbance at 600 nm of each cuvette.

(4) Add 20 μL of the fraction/control to be tested, separately, to the 1.0 mL aliquot of diluted culture.

(5) Record the absorbance at approximately 5 minutes for a 30-45 minute period or until a clear trend showing lysozyme activity is present.

> **Note**: Bacterial cultures will settle if allowed to stand undisturbed, be sure to mix the contents of the cuvette immediately prior to recording the absorbance. Do not cross contaminate your samples!

Laboratory 6 – SDS-Polyacrylamide Gel Electrophoresis

Introduction:

Perhaps the most common biochemical method utilized to detect the presence of proteins is sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). The method is rapid, inexpensive, sensitive and separates proteins and protein subunits based upon their size.

Electrophoresis is a technique that utilizes an electric field to force oppositely charged ions (*ie.* proteins) through a porous, cross-linked gel (polyacrylamide) matrix. SDS is an anionic detergent that denatures or unfolds almost all proteins by binding to the protein in a constant ratio of ~1 SDS molecule for every 2 residues. As a result, in the presence of SDS, proteins adopt an extended conformation, have a constant negative charge density and may be quantitatively separated based upon their size.

The SEC fractions collected earlier in the semester (exercise 3) will be analyzed by SDS-PAGE and compared to a set of known proteins. The migration distance of any unknown protein can be compared to the known proteins, by constructing a standard curve, to determine their relative molecular mass.

Reagents & Equipment:

- 5% stacking gel premix
  - 5% (w/v) acrylamide/bis-acrylamide
  - 126 mM Tris-HCl pH 6.8
  - 1% (w/v) SDS
- 15% resolving gel premix
  - 15% (w/v) acrylamide/bis-acrylamide
  - 375 mM Tris-HCl pH 8.8
  - 1% (w/v) SDS
    - effective separation range: 20-100 kDa
- 100 mg/mL ammonium persulfate (APS)
- TEMED
- 2 x reducing sample buffer (2x RSB)
  - 120 mM Tris-HCl pH 6.8
  - 4.0% (w/v) SDS
  - 20% (v/v) glycerol
  - 0.01% (w/v)bromophenol blue
  - 143 mM 2-mercaptoethanol
- running buffer
  - 0.5 M Tris-HCl
  - 1.0 M glycine
  - SDS
- stain solution
  - 10% v/v acetic acid
  - 40% v/v methanol
  - 0.1% coomassie brilliant blue
- destain solution
  - 10% v/v Acetic Acid
  - 40% v/v methanol
- 70% (v/v) ethanol
- PAGE apparatus
  - short and spacer plates
  - casting frame, stand and combs
  - electrode assembly and power supply
  - clamping frame and mini tank
- heating block (95 ºC)
- platform shaker
- micropipettes and tips
- microcentrifuge tubes
- graduated cylinder (10 mL)
- pasteur pipets and bulb
- glass scintillation vials

Note: Obtain the aliquots of the stacking and resolving premix and allow these to equlibrate to room temperature prior to adding the APS and temed.

Protocol:

Work as pairs to complete this exercise

(1) Ensure the short and long plates are clean. If there are any spots or streaks on the glass plates clean them thoroughly with soap and water and rinse well with distilled water. Once clean, handle the plates by the edges *only.*

(2) Rinse the glass plates with 70% ethanol and use a lint free tissue (i.e., kimwipe) to wipe them dry.

(3) Place the short plate on top of the glass spacers glued to the sides of the long plate plate to create a 0.75 mm gap (this is where the gel will be cast). Slide both plates into the green casting frame on the lab bench. Ensure the plates are evenly aligned within the casting frame and both plates are flush with the lab bench.

(4) Lock the casting frame and ensure the plates are held together securely. Flip the assembly upside down and check that the bottom of the plates are perfectly aligned with each other.

(5) Place the grey rubber mat on the transparent casting stand and insert the casting frame with the plates.

(6) Using a Pasteur pipet, fill the space between the plates with distilled water and check for leakage. If there is any leakage, repeat the above steps. If the leakage persists, placing 2 or 3 layers of paper towel or kimwipes underneath the grey rubber mat (NOT between the rubber mat and the plates) usually adds sufficient pressure to prevent any further leakage.

(7) Remove the water by inverting casting frame (carefully) over the sink and rinse away any remaining water with 70% ethanol.

(8) Obtain the SDS-PAGE premix solutions for both stacking and resolving gels.

(9) In a glass scintillation vial, add 25 µL 100 mg/mL APS and 5 µL TEMED *to* 5.0 mL of the resolving gel premix and mix with a gentle swirling motion.

(10) Using a pasteur pipet, transfer the resolving gel solution to the space between the plates. Leave sufficient space for the stacking gel and combs (~ 2 cm)

(11) Add a layer of 70% ethanol to the top of the resolving gel (3-5 mm) and allow to the resolving gel to polymerize. To check if polymerization is complete, examine the unused portion of the resolving gel in the scintillation vial.

(12) Once the stacking gel has polymerized *and* you are ready to cast the stacking gel, pour off the ethanol overlay added in the previous step.

(13) In a second scintilliation vial, add 15 µL 100 mg/mL APS and 5 µL TEMED *to* 2.5 mL of the stacking gel premix and mix with a gentle swirling motion.

(14) Using a pasteur pipet, transfer the stacking gel solution to the space between the plates until completely filled. Carefully insert a clean comb and allow to the stacking gel to polymerize.

(15) When polymerization is complete, carefully remove the comb and then remove the gel sandwich

(plates and gel) from the casting stand and frame.

(16) Using two gel sandwiches assemble the electrode assembly with the short plates facing each other; place it in the clamping frame and then place both in the mini tank. If only one gel is being used, use the buffer dam in place of the second gel.

(17) Fill the inner chamber with running buffer until the level is above the short plate and fill the outer chamber until the level is at least touching the bottom of the gel sandwich.

(18) Carefully rinse out each of the wells with running buffer using a micropipettor.

(19) Heat your samples at 95 ºC for 10 minutes in a heat block. This can be performed in advance, storing the samples at room temperature when complete.

(20) Load 25 µL of each sample and 10 µL of the markers (aka standard proteins) into separate wells using a micropipettor. Be sure to record, in your lab book, where each sample was loaded.

(21) When the samples are loaded, place the lid on the mini tank and run the gel at 200 V until the dye front reaches the bottom of the gel, typically about 45 min..

(22) When the gel has finished running, disassemble the electrode assembly and remove the short plate.

(23) Mark the gel for future identification and carefully transfer to the staining solution.

(24) Incubate the gel in the staining solution on the orbital shaker for 10 minutes before transferring to the destaining solution on the orbital shaker.

(25) Bands should be faintly visible after 10 – 20 minutes of destaining. However, destaining will be carried out overnight before digital images of the gels are obtained (either by digital camera or a flatbed scanner).

Laboratory 7 – Homology Modeling

Introduction:

Antibiotic resistant bacteria are becoming more and more prevalent and pose a major health risk. One bacterial species particularly notorious for demonstrating antibiotic resistance is *Pseudomonas aeruginosa*. This opportunistic human pathogen poses a particular threat to humans since strains of this species have developed multidrug resistance. One mechanism of antibiotic resistance employed by *P. aeruginosa* is the use of efflux pumps. Efflux pumps are transmembrane proteins that are capable of translocating a target molecule from the cytosolic side of the membrane to the extracellular matrix. In the case of antibiotic resistance, efflux pumps lower the cellular concentration of antibiotics. One way to combat the contribution of efflux pumps to antibiotic resistance in *P. aeruginosa* is to design inhibitors of the efflux pumps. Alternatively, one could target the machinery responsible for inserting the pumps into the cellular membrane.

The mechanism for inserting transmembrane proteins into their target membrane is conserved across all domains of life. In this process, a ribonuclear protein, known as the signal recognition particle (SRP), binds to a signal peptide at the N-terminus of the target protein as it is being translated on the ribosome. In bacteria, the SRP consists of a protein (Ffh) and a non-coding RNA (4.5S RNA). After binding the signal peptide cargo, the SRP binds to a receptor on the target membrane and passes the protein to a membrane channel. As the protein is translated by the ribosome, the membrane channel embeds the transmembrane regions of the protein into the membrane.

By developing inhibitors to a protein essential to the localization of the efflux pumps, such as Ffh in *P. aeruginosa,* one could inhibit proper localization of efflux pumps that confer drug resistance. Since the development of novel drugs is greatly assisted by three-dimensional models of the drug target, a good first step in designing an inhibitor of Ffh would be to generate such a model for Ffh from *P. aeruginosa*. Ideally, one would crystallize the protein of interest and solve its structure by X-ray diffraction techniques but this is a labour intensive and time consuming endeavor. However, structures for homologous proteins may have already been solved by others and these can be used to predict the model of the protein of interest. Since the X-ray crystal structure for Ffh from the archeal species *Sulfolobus solfataricus* has already been solved (Janda et al., 2010), this model can used as a template for predicting the three-dimensional structure of Ffh from *P. aeruginosa* using homology modeling.

References

Janda, CY, Li, J, Oubridge, C, Hernández, H, Robinson, CV, and Nagai, K. **2010** Recognition of a signal peptide by the signal recognition particle. Nature, 465 (27) 507-510.

Reagents & Equipment:

- Computer with;
  - web-browser and an internet connection
  - Swiss-PDB Viewer
- Ffh sequence for *S. Solfataricus*
  - S_solfataricus_Ffh.txt
- Ffh 3-D coordinates for *S. Solfataricus*
  - 3KL4.pdb

****Disclaimer**** Do NOT attempt to start this exercise early without consulting your laboratory instructor as the online servers and tools are frequently updated. If needed, an updated protocol and assignment questions will be posted to Moodle to reflect any changes or updates made between the lab manual's publication and the exercise date.

Protocol:

Work individually to complete this exercise

(1) Identify the amino acid sequence for the protein Ffh from *P. aeruginosa.*

(a) Go to the Uniprot Protein sequence database at www.uniprot.org.

(b) Download the sequence, in FASTA format, for the signal recognition particle protein (Ffh) from *P. aeruginosa* (strain ATCC 15692). Ensure your search is worded carefully and be sure to select the correct sequence, as requested!!

(2) Generate a sequence alignment for the amino acid sequences of the Ffh proteins from *P. aeruginosa* and *S. solfataricus*.

(a) Go to the Clustal Omega online server at https://www.ebi.ac.uk/jdispatcher/msa/clustalo.

(b) Paste both Ffh sequences from *P. aeruginosa* (from the previous step) and *S. solfataricus* (provided on Moodle) into the window provided. Change the names of the two sequences by editing the string preceded by the ">" to something **less than 10 characters** in length (no spaces).

Note: Paste the *P. aeruginosa* sequence first, followed by the *S. solfataricus* sequence. Reversing the order will not impact the sequence alignment but it will cause errors when you generate your homology model in the following step (3).

(c) Ensure that the "Output Format" is set "ClustalW with character counts".

(d) Click "Submit" to run the alignment. You do not have to enter an e-mail address.

(e) The computed alignment will be displayed on the screen in Clustal Omega format. This can be saved as a text file by clicking on the "Download" link. You may wish to change the file extension to ".txt" or you will need to specify the program to open the file depending on your computer's operating system.

(f) Under the aligned sequences you will find characters indicating invariant residues (*), conserved substitutions (:), and semi-conserved substitutions (.).

(3) Generate a homolgy model for the *P. aeruginosa* Ffh protein based on the structure for Ffh from *S. solfataricus*.

(a) Go to the Swiss-Model structure homolgy-modeling server at http://swissmodel.expasy.org.

(b) Under "Modeling" drop down menu (top right of the screen), select "Alignment Mode" and then select "Target-Template Alignment" under the "Supported Inputs" section.

(c) Enter your e-mail address and a suitable project title in the spaces provided. This step is not required but will allow you to access your project for several days from any computer which may prove to be quite useful.

(d) Cut and paste the Clustal Omega alignment from step 2 into the space provided, ensure you include the header "CLUSTAL O (1.2.4) multiple sequence alignment" when doing so. Swiss-Model will automatically identify the target and the template sequence.

Target sequence – This is the *P. aeruginosa* sequence that has no experimentally determined 3-D structure (i.e., the one Swiss-Model will attempt model a structure for us). In our case the *P. aeruginosa* ffh sequence should be identified as the "Target"

Template sequence – This is the *S. solfataricus* sequence that has an experimentally determined 3-D structure from x-ray diffraction and Swiss-Model will use this structure to model the target sequence. The coordinate file will automatically be retrieved from the database (3kl4.1.a) and this sequence will labelled accordingly.

(e) After checking to ensure that Swiss-Model has interpreted your sequence alignment correctly, select the "Build Model" option. This may take some time depending on how busy the server is (*hours or days, do not leave this until the day the assignment is due!*), if an email address was entered you will receive notification when your job is complete.

Errors – If any errors are reported at this stage, take a screen shot and get a hold of your instructor.

(f) The homology modeling output will be displayed on the screen when completed.

Save a copy of the "Modelling Report" from the homology modeling generation, available from the drop down "Display Files" menu on the left. This file contains important information about how your homology model was generated and you may need to refer to it later.

(g) Download your homology model as a .pdb file.

(4) Visualize the generated homology model and the template structure using Swiss-PDB viewer (aka DeepView) by unzipping the file provided on Moodle (SPDBV_4.10_PC.zip) and run the Windows executable file. Alternatively this is free software that can be readily downloaded with a quick internet search and run on any Windows, Linux, or most Mac operating systems (Note: The latest versions of MacOS DOES NOT allow Swiss-PDB viewer to run. There is no known work around for this issue, if this affects you will have to find another computer to complete this portion of the exercise).

(a) Open the crystal structure for Ffh from *S. solfataricus* (3kl4.pdb provided on Moodle) and the homology model you generated for Ffh from *P. aeruginosa* in Swiss-PDB viewer.

Note: The 3kl4.pdb coordinate file provided on Moodle is the same coordinate file that Swiss-Model used to generate your model.

(b) Set the following display options to provide pretty pictures.

i. Under the “Prefs” drop-down menu click on “Ribbons...” and check the “Render as Solid Ribbon” box.

ii. Under the “Display” drop-down menu click the “Render in 3D” option.

iii. Under the “Color” drop-down menu select the “act on Backbone + Sidechains by:” and then select “act on Ribbon”.

iv. Under the “Wind” drop-down menu click the “Control Panel” selection. This will cause a window to pop up on the side of your screen that displays each amino acid in the structure (if not already present).

(c) Display 1) the crystal structure of *Ffh* from *S. solfataricus* (chain A in the 3KL4.pdb file); 2) the crystal structure of the SRP substrate (chain B in the 3KL4.pdb file); and 3) the generated homology model for *P. aeruginosa* as ribbons, each in a different colour.

i. Highlight the desired residues in the left column of the control panel.

ii. Display the highlighted residues as ribbons by clicking on the column header “ribn” in the control panel. Checkmarks, shown as ‘v’ should appear in the “ribn” column for the highlighted residues indicating they are visible.

iii. Colour the ribbons by clicking on the “col” column header in the control panel and selecting the desired colour.

iv. Hide the backbone and sidechains by deselecting all residues and clicking the “show” and “side” column headers in the control panel.

(5) Structure Analysis.

Note: For this section you will find it useful to make use of all the information you have gathered to this point. Additionally, use the various settings in Swiss-PDB viewer to help answer the question in this section (e.g., show side chains of specific residues, make only certain residues visible, etc.). Swiss-PDB viewer has a lot of options, do hesitate to experiment with some of them.

(a) Amino acids 310-329, *P. aeruginosa* numbering, were not resolved in the X-ray crystal structure, likely due to flexibility of this region within the crystal during x-ray diffraction collection..

(b) Amino acids 410-430, *P. aeruginosa* numbering, form a helix, ₃M4, that interacts with the bound signal peptide.

(6) Final thoughts.

Laboratory 8 – Experimental Design

Introduction:

You have just finished your undergraduate degree in biochemistry and have started graduate school at the prestigious Institute of Biomolecular Science (aka I. BS), an institute devoted solely to teaching and research in the fields of biochemistry and molecular biology. As part of your graduate program you are assigned a teaching assistantship and will be responsible for the delivery of senior undergraduate labs in biochemistry. Two days prior to the start of labs you still have not heard from the lab coordinator and decide to head down to his office to determine the exact nature of your duties. Upon arriving at his office you find a note on his door, addressed to you, explaining that he has left on vacation for three months but everything you need can be found in the undergraduate laboratory.

You find the laboratory, which bears an uncanny resemblance to the very room you took your own undergraduate biochemistry labs in, and after reviewing the lab manual and the coordinator's own lab book, you realize you have a serious problem. The undergraduate students are about to begin the characterization of four different enzymes, including the determination of their respective kinetic parameters. However, the coordinator, in his rush to leave for vacation, opted to mix the entire supply of all four enzymes into a single solution to save preparation time. Realizing the disastrous effects this would have upon the student's data, you decide this must be corrected. You scour the department looking for a fresh supply of the enzymes in order to properly prepare individual aliquots, and while finding every imaginable chemical, reagent, and buffer (even the coordinator's supply of milk and sugar for his coffee in the laboratory refrigerator), you are unable to find the enzymes needed. All hope is not lost, as you realize the Biochemistry 2000 labs you took as an undergraduate have given you the knowledge and experience needed to isolate the four needed enzymes from each other.

For this exercise, you are to design an experimental protocol to purify the four enzymes; alpha-amylase (Uniprot ID: P0C1B3), catalase (Uniprot ID: P00432), chymosin (Uniprot ID: P09873) and lysozyme (Uniprot ID: P00698), from the single solution created by the lab coordinator. As you currently only have access to the undergraduate laboratory at I. BS, your choice of equipment and supplies is limited to what is found within this space (an identical collection as to what is found in the current biochemistry teaching laboratory) but as mentioned you have access to virtually any chemical, reagent and buffer you can imagine. When designing your protocol, keep in mind the practical limitations observed for each of the techniques performed this semester (as indicated by your own data sets), feel free to modify your methods to adjust for these limitations. Do not forget to incorporate adequate controls as appropriate.

Further details the specific information required for the assignment will be provided when you are scheduled to perform this exercise.

Appendix A: Preparation of a Lab Book

Your lab book provides you with a detailed record of your experiments performed. This record proves invaluable when preparing manuscripts for publication, or, more immediately, when preparing lab reports or assignments.

NOTE: Keeping of a lab book is required for Biochemistry 2000 laboratories but it is a good idea to keep track of your work within the laboratory portion of this course.

Choice of Lab Book

Standard black lab books can be purchased from the book store but these are not required for this course. The only required features are;

1. Pages are non-removable (no spiral bindings)
2. **All pages** must be numbered in the top outer corner
   - page numbers may be hand-written on EVERY page in INK

In General

- all entries must be made in blue or black ink
- date EVERY entry
- **never** remove a page or use white-out
  - if an entry needs to be deleted, strike out the entry with a single straight line (the deleted entry **must** be readable)
- keep up to date, a lab book is meant to be filled out as the experiments are carried out and NOT after the fact
- record anything that may be useful to you when preparing your lab reports
- leave plenty of space throughout the lab book to add comments after the fact

Table of Contents

Designate the first 2 pages as the Table of Contents

- record descriptive information and pages numbers as you go
- every lab exercise should be listed

Lab Entries

For each lab session be sure to include the following;

1. Objective
   - state the purpose of the work you are about to carry out
   - keep it brief, no more than a sentence or two
2. Method Summary
   - do not rewrite the protocol from the lab manual

- highlight any specific changes to the lab protocol
- include times and dates for when work was performed
- record product names and manufacturers used
  - enzymes, chemicals, equipment (micropipettors, baths)
- include incubation conditions for cultures and reactions

3. Observations & Results
   - record any observations, this goes beyond number results
   - include **diagrams**, **gel pictures**, and any other form of raw data
   - include calculations as appropriate
4. Conclusions
   - did you achieve your objective? Why or why not?
   - use your results to support your conclusions
   - if you created a new strain or DNA/protein construct, you should provide it with a name and a description

Supply List

You may find it helpful to reserve the last page or two of the lab book to be used as a supply list for materials utilized throughout the experiments. This can be particularly useful for bacterial strains, DNA, and enzymes utilized. Listing details such as the supplier, growth conditions, concentrations, etc. can save you a lot of time searching for this information at a later date.

Appendix B: Units

International System of Units

The International System of Units (SI) is the predominant system of measurement with official adoption by nearly every country in the world and it is the prevalent system used in science and technology. All students should already be familiar with this system which is summarized in Tables 1-3.

Table 1. SI base units.

Base Property	Name	Unit
Amount of Substance	mole	mol
Electric Current	ampere	A
Length	meter	m
Luminous Intensity	candela	cd
Mass*	kilogram	kg
Temperature	kelvin	K
Time	second	s

* gram is not the base SI unit of mass.

Table 2. SI derived units, not exhaustive.

Derived Property	Name	Unit	SI units
Concentration*	molarity	M	mol/m^3
Energy	joule	J	N m
Electric Charge	coulomb	C	S A
Electric Potential	volt	V	W/A
Electric Resistance	ohm	Ω	V/A
Frequency	hertz	Hz	s-1
Force	newton	N	$kg\ m/s^2$
Power	watt	W	J/s
Pressure	pascal	Pa	N/m^2
Temperature**	Celsius	C	K

* a 1 M solution is defined as 1 mol/L (or 1 molar)
** a degree Celcius (°C) is equivalent to 1 K.

Table 3. SI prefixes and their associated values.

Prefix	Symbol	Factor	Name
exa	E	10^{18}	quintillion
peta	P	10^{15}	quadrillion
tera	T	10^{12}	trillion
giga	G	10^{9}	billion
mega	M	10^{6}	million
kilo	k	10^{3}	thousand
hecto	h	10^{2}	hundred
deka	da	10^{1}	ten
		10^{0}	one
deci	d	10^{-1}	tenth
centi	c	10^{-2}	hundredth
milli	m	10^{-3}	thousandth
micro	μ	10^{-6}	millionth
nano	n	10^{-9}	billionth
pico	p	10^{-12}	trillionth
femto	f	10^{-15}	quadrillionth
atto	a	10^{-18}	quintillionth

Non-Standard Units

Angstrom (Å) – 10^{-10} m or 0.1 nm. Commonly used to describe the sizes of atoms, bond lengths, crystallographic dimensions, and wavelengths of visible light.

Percent concentration – commonly used in the life sciences for ease of calculations and convenience. The two most common ways of using percent concentration are;

1) **Percent weight/volume (% w/v)** being defined as grams of solute per 100 mL of solution, or

2) **Percent volume/volume (% v/v)** being defined as mL of solute per 100 mL of solution.

Enzyme/catalytic activity – There is a derived SI unit for enzyme activity, a **katal (kat)**, which is defined as the activity of enzyme needed to convert one mole of substrate per second (mol/sec). However a katal is not a practical unit for most biological systems as molar quantities of substrates are simply not encountered.

A more practical measurement is commonly used, an **enzyme unit (U)**, which is defined as the enzyme activity needed to convert one micromole of substrate per minute (μmol/min). This is a more reasonable measure of substrate quantities typically found in most biological systems and better reflects the time scale at which most enzyme assays are carried out, in minutes and not seconds. Strictly speaking this does not utilize the SI base unit of time (seconds) and therefore is not regarded as being part of the SI system.

Unfortunately there are numerous circumstances where the above units are impractical, if not impossible to use. As a result there are many examples of commonly used and varying methods to measure enzyme activity. A few examples are provided below.

- Restriction enzyme activity – 1 unit of activity is defined as the ability to digest 1 μg of DNA in a 50 μL reaction in 60 min (there are variations of this).
- Lysozyme activity – The amount of enzyme needed to decrease the absorbance of a bacterial culture (typically a *Micrococcus* sp.) by 0.001 per minute is defined as a unit of activity.
- DNA/RNA polymerase activity – A unit here is usually defined as the amount of enzyme needed to incorporate 10 nmol of dNTPs into an acid insoluble material in 30 min at 75 °C.

Absorbance and **Transmittance** of light – Passing light of a specific wavelength through a sample is an effective and practical way to determine the concentration of a variety of substances of in solution. This technique is widely used within biochemistry (and yes, in other fields too), so much so that the teaching lab itself has nearly $100,000 worth of spectrophotometry equipment and related supplies. This involves either measuring the amount of light that passes through a sample (**transmittance**) or by how much light is absorbed by the sample (**absorbance**).

1) **Transmittance (T)** is the fraction of light that passes through a sample, the transmitted light (I), as compared to the original amount of light used, the incident light (I_O). Where $T = I/I_O$ and is most commonly expressed as a percentage where $\%T = I/I_O \times 100\%$.

2) **Absorbance (A)**, commonly referred to as **optical density (OD)**, is essentially the opposite of transmittance where the amount of light absorbed by a sample is measured. Unlike transmittance, this is not measured on a linear scale but rather a logarithmic one and is calculated as $A = \log(10)\ I_O/I$.

It is worth noting that both transmittance and absorbance, being calculated as a fraction of I and I_O, are unitless dimensions. Having said this, is is not uncommon to use arbitrary "absorbance units" or AU when reporting absorbance measurements. Absorbance is more commonly used within biochemistry and it is important to report the wavelength of light used along with the absorbance values. For example if reporting an absorbance value of 0.451 when using light with a wavelength of 280 nm this would be reported at $A_{280} = 0.451$.

The above explanation of select units is based on feedback from students over the years. If there is a unit or system of measurement that is not clear to you, let me (Q) know and I will consider adding it to this appendix.