BIOCHEMISTRY LABORATORY
EXPERIMENTS

CHE 4350

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with original materials by Stephen Poole, Ph.D.
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WELCOME TO THE BIOCHEMISTRY LABORATORY!

This Biochemistry laboratory seeks to model work performed in a biochemical research laboratory. The course will guide you through basic lab techniques, investigations into DNA and enzyme kinetics, an intensive purification and characterization of an unreported protein, and will culminate in a formal research paper in the format of an article published in *Biochemistry*.

**Module 1** is concerned with basic lab skills. In these labs, we will learn to accurately and precisely measure small volume of liquid while avoiding sample contamination. We will also learn to compute and create buffer solutions for a variety of pH values.

**Module 2** is our first serious biochemical investigations, looking into the processes used to isolate, purify, amplify, and characterize DNA. We will isolate and purify DNA from a bacterial source, then use the polymerase chain reaction (PCR) to amplify a DNA region of interest to ascertain the nature of the DNA we purified. Finally, we will perform *in silico* studies of DNA cloning and design primers and choose restriction enzymes for incorporation of a human protein into a bacterial expression system.

**Module 3** is focused on enzyme kinetics, the measurement of the extent and mechanism by which enzymes catalyze biological reactions. We will investigate these processes by looking at the activity of acid phosphatases found in wheat germ, which catalyze the hydrolysis of monophosphoester bonds. We will also investigate the effect of enzyme inhibitors of these reactions.

**Module 4** will allow us to purify parvalbumin from the Rainbow trout (*Oncorhynchus mykiss*) using various fractionation techniques including homogenization, centrifugation, chromatography, and dialysis. We will characterize our products using biochemical methods including gel electrophoresis, UV-Vis spectroscopy, and fluorescence spectroscopy. Using modeling software on the computer, the structure and function of model, comparison parvalbumins will be investigated. The conserved cysteine, phenylalanine(s), tyrosine(s) and tryptophan residues found in other parvalbumins have all proved very useful in probing the structure and dynamics of this protein using biophysical techniques in past studies and will be probed in the present study to examine the structure and dynamics of this new parvalbumin. As a result of this project, we will determine how many parvalbumin isoforms are contained in the source, the molecular weight, the approximate number and type of aromatic residues, characteristic UV-Vis, fluorescence and ATR FT-IR spectra, and denaturation/renaturation properties of the apo- and native forms.

The emphasis of the lab is on learning to perform complex biochemical techniques, as well as on analyzing and interpreting data and using graphing programs. Lab instructions and report expectations are explained in the pages that follow.
Laboratory Safety

Rules for a Safe Lab Environment

Safety in the chemistry laboratory involves a cautious attitude and an awareness of potential hazards. Usually potential accidents can be anticipated and prevented. If safety precautions are followed, fewer accidents will occur. The number of laboratory accidents can be reduced if every student follows all of the directions given for the experiment and by the instructor. Special note should be taken of specific instructions that are given in an experiment to eliminate recognized potential hazards.

A. General Regulations

1. MSCD is not responsible for damage to personal effects.
2. Whenever students are performing authorized experiments in the laboratory, an instructor is expected to be present or a student needs explicit permission from the instructor to work in the laboratory in which case the student must have a partner present in the lab.
3. Any breakage of glassware or other breakable laboratory equipment is to be immediately reported to the laboratory instructor. DO NOT CLEAN UP THE BROKEN GLASS. Your laboratory instructor will clean up and dispose of all broken glassware and equipment.
4. Electronic devices, such as cellular phones and pagers, and any personal entertainment devices must be turned off prior to the beginning of the lab period. Failure to comply will result in dismissal.
5. No visitors should attend class without the prior consent of the instructor.
6. Failure to comply with laboratory rules and regulations will result in expulsion from the laboratory and referral to the Department Chair for further action.

B. Student Responsibility

1. LOCATE THE SAFETY EQUIPMENT. Find the eyewash, safety shower, fire extinguishers, fire blanket, first-aid kit and all exits that are to be used in an emergency.
2. PROTECT YOUR EYES. Eye protection (safety goggles) are to be worn at all times while working in the laboratory room. Failure to abide by this policy will result in expulsion from the lab and a grade of zero for the assigned lab experiment.
3. LONG HAIR NEEDS TO BE PULLED BACK.
4. SHOES WORN TO LAB MUST COVER YOUR FEET COMPLETELY. Since broken glass and spilled chemicals are all too common occurrences in lab, your feet will need more protection than that afforded by open-toed shoes or sandals. NO OPEN-TOED SHOES, NO CROCS.
5. Students must be dressed properly for lab. WEAR CLOTHES THAT WILL PROVIDE YOU WITH THE MAXIMUM PROTECTION AND COVERAGE AS POSSIBLE. OLD JEANS OR SLACKS ARE TO BE WORN TO THE LABORATORY. NO SHORTS. Shirts must cover to top of bottoms. Long skirts are allowed if they fall at the ankle.
6. FOOD AND DRINK ARE NOT ALLOWED IN THE LABORATORY ROOM.
7. DO NOT TASTE ANY CHEMICAL. To prevent the entry of any chemical substance into your mouth, it is best not to put any object in your mouth such as pens, pencils or fingers in the laboratory room. After lab is finished, hands should be washed with soap before leaving...
the laboratory room.

8. **DO NOT SMELL CHEMICALS DIRECTLY.** Use your hand to waft the odor to your nose if you are directed to note an odor in an experimental procedure.

9. When dealing with any biological material or chemical, take all necessary precautions to avoid skin contact, use adequate equipment and ventilation, and treat all samples with extreme care.

10. Wear gloves when working with the samples and chemicals.

**C. Housekeeping Rules**

1. REPORT ALL CHEMICAL SPILLS TO YOUR LABORATORY INSTRUCTOR. CLEAN UP ALL SOLID AND LIQUID SPILLS IMMEDIATELY.

2. **DO NOT POUR ANY CHEMICALS INTO THE SINK OR DISPOSE OF ANY CHEMICALS IN THE TRASH WITHOUT PRIOR AUTHORIZATION.**

3. BAKER SHOULD BE USED TO OBTAIN STOCK MATERIALS. If, when dispensing stock solutions you obtain too much, **DO NOT RETURN EXCESS STOCK SOLUTIONS BACK INTO THE STOCK SOLUTION CONTAINERS.** This will contaminate the stock solution.

4. **READ THE LABEL ON ALL STOCK SOLUTIONS AND CHEMICALS CAREFULLY.**

5. **DO NOT INSERT A DROPPER OR PIPET INTO A STOCK SOLUTION CONTAINER.** Pour a small amount of the stock solution into a beaker and then insert your dropper or pipet into the beaker.

6. **TAKE NO MORE OF A CHEMICAL THAN THE EXPERIMENTAL PROCEDURE REQUIRES.** Carefully read the procedure and determine the quantity of each stock solution and/or chemical you need. Obtain only that amount. If you take too much, share it with your neighbor. **NEVER RETURN THE EXCESS TO THE STOCK CHEMICAL BOTTLE.**

7. **DO NOT PUT PAPER OR SOLID WASTE INTO THE SINKS.**

8. Material Safety Data Sheets (MSDS) are available for all chemicals used in the laboratory.

**D. Accident and Emergency Procedures**

1. Each individual is to report any accident, no matter how small, to the laboratory instructor. If necessary, the laboratory instructor will give a written report of the incident to the Department Chair.

2. Should an incident occur and a staff or faculty member is not immediately available, contact the Health Center at Auraria at 303-556-2525 for assistance or call 911.

**E. Medical or Hospitalization Insurance Information**

If you are involved in an accident, all medical expenses will be your responsibility or your guardian's responsibility. If appropriate, please check with your guardians to see whether you are covered by medical insurance.
F. Contract

If you do not submit the signed contract to your Laboratory Instructor, you will not be allowed into the laboratory or be allowed to perform any laboratory work.

I, the undersigned, have read the discussion of good laboratory safety rules and practices presented in this laboratory manual. I recognize it is my responsibility to observe these practices and precautions while present in the laboratory. I understand if I do not comply with these regulations, I will be asked to leave the laboratory by my instructor and will receive a grade of ZERO for that experiment.

__________________________  _______________________
Signature of Student        Date

__________________________  _______________________
Print Full Name              Semester

__________________________  _______________________
Course                     Section

__________________________
Laboratory Instructor
Avoiding Contamination Issues: Standard Laboratory Practices

1. Clean, dedicated lab coats are worn when working with samples and preparing solutions.
2. Gloves should be worn when working with samples.
3. Gloves are changed whenever they may have become contaminated.
4. Biological waste should be double bagged in autoclave bags and taken directly to the autoclave.
5. Label all samples clearly with your name, date, and contents.
6. Sterilized microcentrifuge tubes and sterile aerosol resistant pipet tips are used when possible.
7. Pipet tips are changed between each sample. They do not need to be changed when aliquoting kit reagents, buffers, or other liquids repeatedly.
8. Equipment (centrifuges, pipettors, racks, etc) is cleaned as needed after each use.
9. Protein and reagents are stored in the freezer after use. Chemicals are stored as directed by instructor.
Module 1: Basic Biochemistry Laboratory Skills
1 Pipetting, preparing solutions and the use of computers in lab report preparation

Objective
To learn how to precisely use calibrated variable volume micropipettes, prepare solutions and create a lab report using computers.

Safety
Wear gloves, goggles, a lab coat, work in the hood and use extreme care when handling strong acids and bases. Report and clean up all spills immediately. Do not drop micropipettors.

Background
This “experiment” is meant to introduce you to a number of skills that are essential to success in the laboratory. These skills include the accurate use of pipettes, making solutions, and the use of computers to analyze data and prepare reports. As a scientist, you need to be able to accurately use calibrated variable volume micropipettors to add and transfer small amounts of reagents and samples precisely and accurately. This lab is a chance to test the accuracy of our tools and your understanding of how to appropriately set the micropipettors to precisely deliver the amount of liquid required. You also need to be able to accurately prepare solutions and dilutions.

Automatic pipettes are used to accurately transfer small liquid volumes. Glass pipettes are not highly accurate for volumes less than 1 milliliter (1 ml), but the automatic pipettes are both accurate (less than 1% error) and precise (less than 0.5%). These are continuously adjustable digital or rotary pipettes. Each pipette (Fig. 1.1) can be set to transfer any volume within its own volume range (Fig. 1.2) using specially designed tips (Fig. 1.3).
Fig. 1.1 Parts of the pipet. http://www.rainin.com/pdf/pipetman_manual.pdf

Fig. 1.2 Pipet tips. The tips can be purchased with or without the filter barrier (white disk). http://news.thomasnet.com/images/large/825/825855.jpg
First, set the volume to transfer (Fig. 1.3), then attach the disposable tip, depress the plunger to the first stop, immerse the tip in the sample, slowly draw up the sample with the tip completely immersed, pause for viscous samples, withdraw the tip, dispense the sample by pressing the plunger through the first stop to the second stop vertically, withdraw the pipet, release the tip into the trash (Fig. 1.4).

In this experiment, you will pipette water into a number of small beakers or weighboats. The amount of water dispensed, and therefore the accuracy with which you pipette, will be determined by weighing. 1.0000 mL of pure water weighs 1.0000 g. We can measure mass using a balance. The balance in our lab is precise to +/- 0.0001 g. The delivery of the
micropipettor can be examined and checked using the balance (e.g. 0.1000 mL should weigh 0.1000 g).

Finally, you will use spreadsheet software to analyze and graph the data you have collected in this experiment and write a lab report accurately describing accuracy and precision. Accuracy is how close a measured value is to the actual (true) value (even after averaging). Precision is how close the measured values are to each other (Fig. 1.5).

Fig. 1.5 Examples of Precision and Accuracy
https://www1.nga.mil/ProductsServices/PrecisePositioningTargeting/PublishingImages/accuracy.jpg

You will also prepare and dilute solutions of strong acids and bases. A 1 M solution is one mol of solute per liter of solvent. Dilutions can be prepared by ensuring that the number of moles of solute remain constant as the volume is increased: $M_1V_1 = M_2V_2$. This method is often used to prepare solutions from strong acids. Bases are typically prepared from the solid state. If you have solid NaOH pellets, you can prepare a solution by determining how many grams of solid are needed in a given volume using the desired molarity and molecular mass of the solid.

**Materials**
- various variable volume micropipettes (10 µL, 100 µL, 1000 µL) and tips
- various constant volume micropipettes (5 µL, 25 µL, 50 µL)
- beakers
- balance
- weigh boats
- water
- concentrated or partially-diluted HCl
- NaOH pellets
- volumetric flasks
Procedure: Numbers (measurements) should *always* be accompanied by units with the appropriate significant figures!

**Part A:**
1. a. Obtain some deionized water with a beaker. Pipet exactly 1.000 mL using a P1000 pipet. Obtain a weigh boat and place it on the balance and press tare. Pipet the water into the weighboat. Record the mass. Is it 1.0000 mL? Comment on the accuracy. If not, make adjustments to your technique until you are close to the expected value. b. Pipet 557 µL into the weigh boat. Record the mass. Comment on the accuracy.
2. Weight out 1000 µL of water ten times using a P1000 (or the maximum volume on another pipet) and record your results in a table. Comment on your precision.
3. Repeat the steps in #1. using at least 2 more different micropipettors (adjustable or fixed volume P10, P20, P100, P200) using the maximum volume and your choice of intermediate volume. Record the masses once each using the balance in a table.
4. Use a P1000 pipettor to dispense volumes of 0.200, 0.400, 0.600, 0.800 and 1.000 mL (or another pipet using similarly distanced volumes). Record the values used and the mass determined for each point. Graph your data.

**Part B:**
1. Prepare 50 mL (or 100 mL) of a 0.1 M HCl solution from concentrated (12 M) HCl or another available stock in a volumetric flask. Check and record the pH (ask instructor for assistance with pH meter if you are not familiar with the Vernier system.) The expected pH is 1.00. (pH = -log[H^+]) Comment on the pH if not 1.00.
2. Prepare 100 mL (or 50 mL) of a 0.00500 M NaOH solution using solid NaOH pellets in a volumetric flask. Check and record the pH. The expected pH is 11.699. Verify this number. Comment on the pH.

**Questions/Analysis**
1. Did the pipet deliver the mass/volume of water expected? If you needed to adjust your technique, was the delivery as expected? Why or why not? Was the pipet accurate?
2. Compute the average mass of the water delivered 10 times and the standard deviation. Show your work. Was the pipet precise?
3. Which of the pipettors that you used was the most accurate?
4. Using a computer, use a spreadsheet to make a plot of “volume dispensed” vs. the “Pipettor Setting” on a line graph as shown in the sample data. Calculate the “best” line (trendline) for this data using linear regression and add the “fit” to the line graph and include the R^2 value and line equation on the graph. Label your graph with a title (y vs. x), and x-axis and y-axis labels with units.
5. Show your calculations and comment on the expected and actual pH for your NaOH and HCl solutions.

**Graphing the data using Microsoft Excel:** Type your data into 2 columns, one for the x-axis data and one for the data to be plotted on the y-axis. Highlight all of the data using your mouse. If using Microsoft Excel, click on Insert, select Chart, select XY Scatter, next, and finish. Click anywhere on the chart and select Chart from the main menu again and add trendline, linear, click on options, then select in the boxes R^2 value and line equation on the chart. You can double-click with the left mouse button on any aspect of the chart to alter the presentation format.

**Sample Data (Table):**
*The density of water is 1.000 g/mL.*

<table>
<thead>
<tr>
<th>Actual Volume (mL)</th>
<th>Pipettor Setting (mL)</th>
</tr>
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<tbody>
<tr>
<td>0.1814</td>
<td>0.200</td>
</tr>
<tr>
<td>0.4004</td>
<td>0.400</td>
</tr>
<tr>
<td>0.5897</td>
<td>0.600</td>
</tr>
<tr>
<td>0.7998</td>
<td>0.800</td>
</tr>
<tr>
<td>1.0030</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Sample Graph:

**Beer's Law Plot**

\[ y = 1.0213x - 0.0179 \]

\[ R^2 = 0.9996 \]
2 Buffer calculations and preparation

**Objective**
To learn how to calculate reagents needed to prepare buffer solutions, prepare and check the pH of buffer solutions in the laboratory.

**Safety**
Do not ingest chemicals. Wash your hands prior to leaving lab. Clean up all spills immediately. Use the balances carefully and be careful not to spill chemicals in them. Do not move the balances.

**Background**
There are three definitions for acids and bases which you have covered in general chemistry. These include the Arrhenius (an acid is an H\(^+\) donor and a base is an OH\(^-\) donor), Bronsted-Lowry (an acid is an H\(^+\) donor and a base is an H\(^+\) acceptor, e.g. NH\(_3\)), and Lewis (an acid donates a share in an electron pair and a base accepts a share in an electron pair) definitions. Strong acids and bases ionize completely. Strong acids include HNO\(_3\), H\(_2\)SO\(_4\) (first proton only), HCl, HBr, HI, HClO\(_4\). Strong bases include LiOH, NaOH, RbOH, Mg(OH)\(_2\), Ca(OH)\(_2\), Sr(OH)\(_2\), and Ba(OH)\(_2\). Adding a strong acid or a strong base to a salt creates weak acids and weak bases, respectively.

Water is an example of a solvent and a weak acid/base “amphoteric” solution as it can perform either function. It forms 1 x 10\(^{-7}\) M H\(^+\) and OH\(^-\) in solution. This is the basis of the pH and pOH scales: by taking the -log of the H\(^+\) and OH\(^-\) concentrations, we get the pH and pOH (both 7 in pure water). Since the concentrations of H\(^+\) and OH\(^-\) are identical, the solution is neutral. Conversely, a solution is acidic if the [H\(^+\)] > [OH\(^-\)] and the pH < 7 and is basic if the [H\(^+\)] < [OH\(^-\)] and the pH > 7. The special equilibrium constant for water, K\(_w\), is equal to [H\(^+\)][OH\(^-\)] or 1 x 10\(^{-14}\). The pK\(_w\) can be determined by taking the -log of the K\(_w\) or 14. Thus, the pH + pOH = 14.

Weak acids and bases dissociate <10% forming an equilibrium of the product and reactants all present in the final solution. Combining these with a conjugate acid or base (common ion salt), strong acid, or strong base creates a buffer.

A pH buffer is a solution that resists large changes in pH due to small additions of acid (H\(^+\)) or base (OH\(^-\)) (Arrhenius definition). It contains a mixture of a conjugate acid (H\(^+\) donor) and a conjugate base (H\(^+\) acceptor); usually a weak acid and a salt of its conjugate base or a weak base and a salt of its conjugate acid. Common examples include a solution of acetic acid (weak acid) and sodium acetate (salt of its conjugate base); or a solution of ammonia (weak base) and ammonium chloride (salt of its conjugate acid). Both conjugate components (weak acid, “HA;” weak base, “A\(^-\)”) must be present in comparable concentrations (within a factor of 10) to have a buffer system: 0.1 < [A\(^-\)]/[HA]<10. The sum of the buffer concentration is the sum of the concentrations of the conjugate components: [HA] + [A\(^-\)] = concentration of common ion. Buffers work best at a pH near the pK of the ionizing group and the pKa is often used in solving pH problems with buffers. A useful equation for solving buffer problems is the Henderson-Hasselbalch equation:

\[
pH = pK_a + \log([A^-]/[HA])
\]
This lab is focused on practicing solving concentration and pH problems with strong and weak acids and bases, buffer solutions, and the effects of adding new components and disturbing the equilibrium and subsequently preparing buffer solution in the laboratory. The buffers prepared in this lab will be used for subsequent experiments involving chromatography, enzyme kinetics, electrophoresis, and protein characterization.

**Example problems and solutions:** Make sure you feel comfortable with these!

**Calculate the pH of 0.35 M acetic acid.** Weak acids and weak bases ionize < 5%. This is an equilibrium problem. Let x represent the number of moles or molarity ionized and the (initial concentration – x) to represent the amount of weak acid or base that remains in solution.

Ionization of Acetic acid: \[ HCH_3COO(aq) + HOH(l) \leftrightarrow CH_3COO^-(aq) + H_3O^+(aq) \]

Or: \[ HCH_3COO(aq) \leftrightarrow CH_3COO^-(aq) + H^+(aq) \]

\[
\begin{array}{c|c|c|c}
 & I & C & E \\
--- & --- & --- & --- \\
0.35 M & 0 & -x & 0.35-x \\
0 & +x & +x \\
\end{array}
\]

Forms \( H^+ \) so write \( Ka = Ka = [H^+][CH_3COO^-]/[HCH_3COO] = 1.8 \times 10^{-5} = [x][x]/[0.35-x] \)

If the value of the \( Ka \) is at least 100 times smaller than the concentration of the weak acid, ignore loss of x from 0.35 M and avoid solving the problem using the quadratic formula. \( x^2 = 6.3 \times 10^{-6} \), take square-root of both sides, \( x = 2.51 \times 10^{-3} \), solve for pH = - log[2.51 \times 10^{-3}] = 2.60

**Calculate the pH of 0.35 M sodium acetate.** This is an equilibrium problem of the pH of a salt of a weak acid.

Ionization of Acetate anion: \[ CH_3COO^-(aq) + HOH(l) \leftrightarrow CH_3COOH(aq) + OH^-(aq) \]

\[
\begin{array}{c|c|c|c}
 & I & C & E \\
--- & --- & --- & --- \\
0.35 M & 0 & -x & 0.35-x \\
0 & +x & +x \\
\end{array}
\]

Forms \( OH^- \) so find \( pK_b = 14- pK_a (4.74) \), so \( Kb = 10^{-pK_b} = 5.56 \times 10^{-10} \)

Then solve \( 5.56 \times 10^{-10} = [OH^-][CH_3COOH]/[CH_3COO^-] = [x][x]/[0.35-x] \)

\( x = [OH^-] \), solve for pOH then pH as in the previous example. You should find a pH of 9.14.

**Calculate the pH of a solution which is 0.35 M acetic acid and 0.35 M sodium acetate.** This is an equilibrium problem of the pH of a buffer which contains a weak acid or weak base and their salt which can product the conjugate base or conjugate acid, respectively. These have initial concentrations in the reactants and products.

Ionization of Acetic acid: \[ HCH_3COO(aq) \leftrightarrow CH_3COO^-(aq) + H^+(aq) \]

\[
\begin{array}{c|c|c|c}
 & I & C & E \\
--- & --- & --- & --- \\
0.35 M & 0.35 M & 0 & 0 \\
0.35-x & +x & +x & +x \\
\end{array}
\]

“HA” “A”

Solve for the pH using the Henderson-Hasselbalch equation: \( pH = pK_a + log [A^-/HA] \), \( pH = 4.74 + log (0.35/0.35) \), \( pH = 4.74 + 0 \), \( pH = 4.74 \)

**Calculate the pH of a solution resulting from initial concentrations of 0.35 M acetic acid and 0.20 M NaOH.** The equilibrium is changed by the addition of a strong acid or strong base. Determine how much, if any weak acids or weak bases are left and if there is excess strong acid or strong base. If the strong acid or strong base is completely consumed, use the Henderson-Hasselbalch equation to compute the pH. If the strong acid or strong base is completely consumed, use the Henderson-Hasselbalch equation to compute the pH. If the strong acid or strong base is completely consumed, use the Henderson-Hasselbalch equation to compute the pH. If the strong acid or strong base is completely consumed, use the Henderson-Hasselbalch equation to compute the pH. If the strong acid or strong base is completely consumed, use the Henderson-Hasselbalch equation to compute the pH.
base is in excess (not completely neutralized), compute the pH using \( \text{pH} = -\log[H^+] \) or \( \text{pOH} = -\log[OH^-] \) and 14-\( \text{pOH} \) = pH. The strong bases (e.g. NaOH) will react with weak acids (e.g. HCH$_3$COO) and the conjugate bases (e.g. CH$_3$COO$^-$) will react with strong acids (e.g. HCl).

Example: HCH$_3$COO(aq) + NaOH $\leftrightarrow$ CH$_3$COO$^-$ (aq) + H$^+$ (aq)

Alternatively, solve the system of equations \( \text{pH} = \text{pKa} + \log ([A^-]/[HA]) \) and \([A^-] + [HA] = \text{concentration of common ion} \). Here, common ion = 0.35 M, \( \text{pKa} = 4.74 \), and the common ion is initially in the acid ([A-]) form. So [A-] was 0.35 M, but the added base will turn it into [A-] = 0.15 M and [HA] = 0.2 M. Then, use Henderson-Hasselbalch: \( \text{pH} = 4.74 + \log (0.15/0.2) = 4.62 \).

The last step is the “reality check”, which is perhaps the most important step. Above, we saw that 0.35 M acetic acid has a pH of 2.60. To that solution, we added base (NaOH). Our computed pH was higher than 2.60, which is consistent with the effects of adding base. Always reality check your answers!

Prepare a 100. mL of 0.20 M acetate buffer, pH 4.40 from sodium acetate trihydrate (NaC$_2$H$_3$O$_2$·3H$_2$O, MW = 136 g/mol), available 0.500 M HCl and 0.500 M NaOH. Calculate how much salt(s) or solution and how much HCl or NaOH to add to prepare each of the following buffer solutions. Use the pKa closest to the desired pH given for your calculations. Then write a recipe for each.

In preparing buffers, first determine the number of moles of the common ion you have (e.g. acetate). Determine total moles of common ion: 0.20 mol/L * 0.100 L = 0.0200 mol acetate which is equal to the total acetate to be used for the production of weak acid and the rest that remains as salt so HA + A$^-$ = 0.0200 mol and A$^-$ = 0.0200 mol – HA

Then determine the mass of solid needed from the source given: 0.0200 mol acetate * 136 g/mol NaC$_2$H$_3$O$_2$·3H$_2$O = 2.72 g NaC$_2$H$_3$O$_2$·3H$_2$O

Then determine the ratio of [A$^-$]/[HA] using the Henderson-Hasselbalch equation remembering that the A$^-$ = 0.0200 mol - HA:

\[ 4.40 = 4.74 + \log \frac{[(0.0200 - HA)/HA]}{HA} \]

so \( 0.457^*HA = 0.0200- HA \), add HA to both sides: \( 1.457HA = 0.0200 \text{ mol, HA = 0.0137 mol and A}^- = 0.0063 \text{ mol} \)

Determine if you need to add HCl or NaOH to create the buffer. In this case, you weighed out the solid salt, sodium acetate trihydrate so you need to create some acetic acid. To do this you must add some HCl to the salt. How much? HA = 0.0137 mol so 0.0137 mol * 1 L/0.500 mol HCl = 0.0274 L HCl = 27.4 mL HCl

So the final recipe: Weight out 2.72 g NaC$_2$H$_3$O$_2$·3H$_2$O on an analytical balance, add to a 100.0 mL volumetric flask, dissolve in ~25 mL deionized water to dissolve the solid, add
27.4 mL of 0.500 M HCl, add more deionized water to the line on the volumetric flask and then check the pH.

Finally, reality check. You are adding acetate, which should have a high pH. So you need to add a strong acid (HCl) to reduce the pH. If you add more strong acid than the weak base, the pH will not be in the buffering range (and will get very low), so you need to add fewer moles of HCl than the total moles of common ion. Everything checks out!

**Materials**

- various variable volume micropipettes (10 µL, 100 µL, 1000 µL)
- various constant volume micropipettes (5 µL, 25 µL, 50 µL)
- volumetric flasks
- balance
- weigh boats
- deionized water and water bottles
- salts (Tris base, sodium acetate, phosphoric acid or phosphate salts)
- acids and bases (0.1 M and 1.0 M and 12 M HCl & 0.1 M and 1.0 M NaOH)
- tips
- Erlenmeyer flasks
- pH meters

**Procedure:**

**Part A.: Complete the pH of the following buffer problems.** (Consult your Biochemistry textbook or a General Chemistry textbook as necessary to review pH calculations.)

**Useful equations and information:**

\[
\text{pH} = -\log[H^+], \quad 10^{-\text{pH}} = [H^+], \quad \text{pOH} = -\log[OH^-], \quad \text{pK}_a = -\log K_a, \quad \text{pK}_b = -\log K_b, \quad \text{pK}_w = -\log K_w, \quad \text{pH} = \text{pK}_a + \log \frac{[A^-]}{[HA]}, \quad \text{in neutral water: } [H^+] = [OH^-] = 1 \times 10^{-7} \text{ M}, \quad K_w = 1 \times 10^{-14} \text{ M}, \quad \text{pH} + \text{pOH} = \text{pK}_w = 14, \quad K_a \times K_b = K_w
\]

**pKa values:**
- phosphate: 1.8, 6.90, 12.5
- acetate: 4.74
- tris: 8.00
- aspartate: 2.00, 3.90, 10.0
- lysine: 2.20, 9.20, 10.8

1. pH of 0.45 M HNO₃
2. [H⁺] of solution pH = 3.23
3. pH of 0.45 M KOH
4. pH of 0.45 M acetic acid
5. pH of 0.45 M sodium acetate
6. pH of a solution which is 0.45 M acetic acid and 0.45 M sodium acetate
7. pH of a solution resulting from initial concentrations of 0.45 M acetic acid and 0.20 M NaOH
8. pH of a solution resulting from initial concentrations of 0.45 M sodium acetate and 0.20 M HCl
9. Prepare a 100. mL of 0.45 M acetate buffer, pH 4.75 from sodium acetate trihydrate (NaC₂H₃O₂·3H₂O), 0.500 M HCl and 0.500 M NaOH
10. Prepare 500. mL of 0.15 M Tris buffer, pH 8.25 from Tris monohydrochloride (Tris-HCl) (C₄H₁₁NO₃ClH), 0.400 M HCl and 0.350 M NaOH
Part B.: Circle the acidic protons or add the basic ionizable proton(s) in each of the following molecules.

[Images of molecules: Acetic acid, Tris, Phosphoric acid, Aspartic acid, Lysine]

Part C.: Preparing the Buffer Solution
1. Prepare 100. mL of 0.100 M phosphate buffer, pH 7.70 from solid KH$_2$PO$_4$ and available (0.1 M and 1.0 M) HCl and NaOH solutions. Show all of your work in your lab report.
2. Prepare 100. mL of 0.100 M phosphate buffer, pH 6.95 from solid KH$_2$PO$_4$ and solid K$_2$HPO$_4$. Show all of your work in your lab report.

Part D.: Checking the pH using a pH meter
1. The pH meter should be standardized with pH 4, 7, and 10 standards prior to use. Your instructor will demonstrate the use of the Vernier pH meter.
2. Check the pH values for your 2 buffers. The pH should be within 0.1 pH units of the theoretical value. If it is not, try preparing the buffers again.

Part E.: Effects of Adding Acid or Base
1. To 9 mL of water, add 1 mL 0.1 M HCl. Record the pH.
2. To 9 mL of water, add 1 mL of 0.1 M NaOH. Record the pH.
3. To 9 mL of each of your two buffers, add 1 mL 0.1 M HCl. Record the pH for each.
4. To 9 mL of each of your two buffers, add 1 mL 0.1 M NaOH. Record the pH for each.

Part F.: Effects of Dilution
1. To 1 mL of each of your two buffers, add 9 mL water. (Note: this is a 1:10 dilution.) Record the pH.
2. Add 1 mL 0.1 M HCl to your diluted buffers. Record the pH for each.
3. Add 1 mL 0.1 M NaOH to your diluted buffers. Record the pH for each.

Questions/Analysis
Calculate the expected pH values for the measurements in Part E. and Part F. Are the values what you expected? Explain your answer.
Module 2: Biochemistry of DNA
**3 Isolation of Plasmid DNA from Bacterial Culture**

**Objective**
To learn how to isolate and purify plasmid DNA from a bacterial pellet, as well as to learn how to characterize the quantity and purify of the plasmid DNA obtained.

**Safety**
Do not ingest chemicals. Wash your hands prior to leaving lab. Clean up all spills immediately. Use the vortex and microcentrifuge carefully and be careful not to spill chemicals in them. Lysis solution is caustic and may burn skin.

**Background**
Bacterial cultures are often used to generate large quantities of DNA. To do so, a small circular double-stranded DNA called a plasmid is introduced into the bacterial cell. Once there, the bacteria will make copies of the plasmid and as the bacteria divides and grows, each bacterial cell will retain copies of the plasmid. In this way, growing up a large culture of bacterial cells allows the generation of many millions of copies of a given plasmid.

Double-stranded DNA is two long polyanion chains held together by hydrogen bonds between the bases. DNA stays in solution because it is solvated by the strongly polar water molecules. One of the most useful techniques in nucleic acid biochemistry is selective precipitation from solution by alcohols. By adding 2 to 2.5 volumes of ethanol (EtOH) the solvent changes from 100% H$_2$O to only 30% H$_2$O and 70% of the less polar EtOH molecules. Under these conditions, DNA molecules have a tendency to associate with each other rather than with the solvent molecules, clumping together and precipitating out of solution. However, these tendency is countered by the electrostatic repulsion from the many negatively charged phosphate groups of different chains. Thus, to be able to precipitate out there must be enough cations, such as Na$^+$ or Li$^+$ ions, present to neutralize the negatively charged phosphates. The concentration of these cations must be ~0.3 – 0.4 M. NaCl is not very soluble in ethanol and will precipitate out at these concentrations, so it is rarely used for these purposes. However, sodium acetate is relatively soluble in EtOH and will remain in solution when the DNA precipitates out.

After precipitation, the purified DNA can be resuspended in water. The DNA concentration can then be determined spectrophorometrically by measuring its absorbance at 260 nm, the wavelength where the DNA bases absorb light.

**Materials**
- Various variable volume micropipettors (20 µL, 100 µL, 200 µL, 1000 µL) and tips
- Ice
- 1.5 mL microcentrifuge tubes
- Lysis solution
  - 10 mM Tris (pH to 7.5)
  - 1 mM EDTA (pH to 8.0 to dissolve)
  - 100 mM sodium hydroxide
  - 0.5% sodium dodecyl sulfate
- 3 M Sodium acetate, pH 5.2
- Pre-chilled (at -20 degrees C) 100 % ethanol
• 70 % Ethanol
• Distilled water
• Overnight bacterial culture
• Vortex mixer
• Microcentrifuge

Procedure
Part A. Isolation of Plasmid DNA
1. Transfer 1.5 ml of overnight bacterial culture into a clean microcentrifuge tube. Centrifuge the tube (tightly capped!) for 30-60 seconds in a microcentrifuge. You should see the bacterial cells pellet at the bottom of the tube.
2. Carefully decant the supernatant. The final volume of bacterial cells remaining in the tube should be ~ 50 to 100 ul. Some liquid may remain in the tube, which is fine.
3. Cap the microcentrifuge tube and vortex at high speed for 5 seconds to fully resuspend the bacteria pellet.
4. Add 350 ul of Lysis solution and cap tightly.
5. Vortex the solution for 5 seconds to mix. You should see the solution become significantly more viscous (like slime) as the genomic DNA is released during cell lysis. Do not allow the tube to sit for more than 5 minutes before proceeding to the next step, as degradation of the bacterial genomic DNA may occur, obscuring your plasmid results.
6. Add 150 ul of sodium acetate to the solution, then cap tightly and vortex for 5 seconds.
7. Transfer the tube to a microcentrifuge and spin at max speed for 3 minutes. After spinning, the tube should have a clear supernatant and a white pellet of cellular debris.
8. Carefully use a pipet to transfer the supernatant (but none of the debris-- it is better to err on the side of caution and leave some supernatant!) to a fresh microcentrifuge tube.
9. Add 900 ul of pre-chilled 100 % ethanol. Ethanol must be ice cold to precipitate DNA!
10. Transfer to a microcentrifuge and spin at max speed for 3 minutes. After spinning, the tube should have a clear supernatant and a white pellet of cellular debris.
11. Carefully use a pipet to transfer the supernatant (but none of the debris-- it is better to err on the side of caution and leave some supernatant!) to a fresh microcentrifuge tube.
12. Carefully decant supernatant, being careful not to disrupt the pellet, and add 1 ml of 70 % ethanol.
13. Transfer to a microcentrifuge and spin at max speed for 5 minutes.
14. Carefully decant the 70% ethanol supernatant.
15. Transfer the microcentrifuge tube containing the pellet into a rack and leave the tube open to air dry for 15 minutes. Cover the tube loosely with a Kim Wipe to prevent debris from falling into the tube.
16. Resuspend the pellet in 30 ul of distilled water.

Part B. Quantification of Plasmid DNA
1. Plasmid DNA is now ready for estimation of DNA concentration and purity.
2. Accompany your lab instructor to the biology lab, where we will use a NanoDrop spectrophotometer to characterize our samples.
3. Carefully clean the NanoDrop sample pedestal as instructed.
4. Pipet 1.5 ul of plasmid sample to the sample pedestal and gently close the sample arm.
5. Measure DNA concentration using the NanoDrop program. Record the absorbance values at 260 nm and 280 nm.
6. Using the relationship 1 Abs = 50 ng/ul DNA, calculate the concentration and total yield of this DNA.
of plasmid DNA.
7. Evaluate the ratio A260/A280. A value of greater than 1.7 indicates essentially pure DNA, while lower values indicate protein contamination.

Question/Analysis
Tabulate and record your A260, A280, DNA concentration, and A260/A280 ratio. Do you think that you successfully extracted plasmid DNA? Why do you think the lysis solution destroyed protein but not DNA?
4 PCR and Agarose Gel Electrophoresis of DNA

Objective
To learn how PCR (Polymerase Chain Reaction) amplifies DNA. To learn to pour an agarose gel and use electrophoresis to determine the molecular weight and purity of the amplified DNA from your extracted DNA sample.

Safety
Do not touch the gel while the power supply is on. Danger: potential electrocution. Wash your hands before leaving lab.

Background
Early biochemistry efforts were often hampered by the very small quantities of DNA that could be feasibly attained. Beginning in the 1970s, scientists began to experiment with polymerases, enzymes that would replicate (copy) a sequence of DNA. They remained essentially curiosities until 1983, when Kary Mullis developed the general principles of the polymerase chain reaction: a method to rapidly generate essentially unlimited copies of a DNA of interest. This capability revolutionized modern biochemistry and molecular biology, and PCR is used in almost every aspect of modern biochemical efforts, from cloning and genetic engineering, to forensics, to basic biochemical studies.

The core principle of PCR is the process of thermal cycling—controlling the temperature of a solution of DNA, polymerase, and the raw components of more DNA, in order to control how the enzymatic process proceeds (Fig. 4.1). The first step is denaturation, where the solution of double-stranded DNA is heated to ~95 °C, which causes the two strands of the DNA to separate from each other. Next is annealing, where the temperature is lowered to ~65 °C to allow short DNA sequences that are complementary to the ends of the DNA segment of interest (called primers) to form double-stranded interactions with the DNA. Next is elongation, where the temperature is raised to ~72 °C, the optimal level for the DNA polymerase to recognize the small double-stranded regions composed of primer bound to DNA, then enzymatically attach additional DNA bases, extending the primer until it is the full length of the sequence. Lastly, this whole process is repeated (cycled) for an additional 20-30 rounds. As each cycle doubles the quantity of DNA, PCR can rapidly expand a few molecules of DNA in billions of copies in an hour. In this experiment, we will be amplifying plasmid DNA from the material isolated in the previous lab.

In the mid-1930’s Arne Tiselius found that if ions of similar charge were placed in solution between two oppositely charged electrodes, the smaller ions of the same charge move faster toward the electrode of opposite charge faster than the larger ions. If ions of different charge are placed in solution, the more highly charged ions migrate faster than the lower charged ions. This makes electrophoresis a good technique for separating substances (Fig. 4.2). Modern techniques use a gel on a piece of plastic, glass or paper. It is easy to stain, analyze, and even to re-extract the protein sample.

Agarose is a polysaccharide isolated from marine algae. It is used in the laboratory as a matrix support for gels in electrophoresis. It is derived from seaweed and is a low sulfate polysaccharide with pores in the size range of 2000 Å or 200 nm diameter. It can be poured in a mold and it will harden to a gel the consistency of a soft or hard Jell-o depending upon
the amount of agarose used. DNA is negatively charged due to the phosphate linkages in the backbone. Negatively-charged molecules migrate toward a positive pole (red) (Fig. 4.1). Because the DNA has to migrate through the gel substance, the shorter, lower molecular weight DNA will migrate further than longer, higher molecular weight DNA in a given time because they can better form a ball and tumble through the matrix (Ogsten sieving) or linearly snake through the matrix (Reptation theory). The distance the DNA will migrate from the well is related to the size and structure (single-stranded, double-stranded, supercoiled) and the degree of complexing of the agarose matrix (concentration). The size of DNA can be calculated using nucleotide standards of known molecular weight run next to the DNA of unknown molecular weight (Fig. 4.2). The distance the unknown DNA has migrated can be used to estimate its molecular weight graphically by comparison with the distance migrated by the known standard oligonucleotides. Agarose gel is non-toxic and requires only agarose boiled in buffer (e.g. TBE). The gel is easy to pour and solidifies upon cooling within 10-15 minutes.

You will be visualizing your DNA in the gel by use of SYBR Green I, a small molecule dye that intercalates (binds inside) double-stranded DNA. When SYBR Green is bound to DNA, its spectral properties are changed so that it absorbs medium-range ultraviolet and blue light and fluoresces green. Thus, if the gel is soaked in an SYBR Green solution, only the SYBR Green that is bound to DNA will glow when placed in a UV light box, allowing convenient visualization. Note that SYBR Green will bind to your DNA as well, and this binding can disrupt the normal process of DNA replication, making SYBR Green a possible carcinogen. Always exercise caution when using DNA stains.

Figure 4.1  Summary of the polymerase chain reaction (PCR) amplification of DNA.  http://www.odec.ca/projects/2005/anna5m0/public_html/pcr.png
Fig. 4.2 Sample gel demonstrating the use of the MW standard to determine DNA size and varying concentration on band intensity.

http://www.quantabio.com/img/performance/4%20kb%20PCR%20Gel.jpg

ng of human genomic DNA

Materials
• Various variable volume micropipettors (20 µL, 100 µL, 200 µL, 1000 µL) and tips
• Ice
• Agarose
• Gel boxes and combs
• DNA molecular weight standards
• Gloves
• Gel light box and digital photography system
• Hot plate and water bath
• Microwave
• 125 mL Erlenmeyer flask
• 1.0 -1.5 mL microcentrifuge tubes
• Power supply
• SYBR Green stain (caution: possible carcinogen!)
• Coomassie Blue 200 mg/mL for making loading buffer
• 1x TBE buffer (10.9 g Tris base, 5.57 g Boric acid, 2 mL of 0.5M EDTA, water to 1 L)
• 0.5M EDTA stock solution
• Loading buffer (1xTBE buffer with 25% glycerol and 1 µL 200 mg/mL Coomassie Blue)
• (Purified DNA (from experiment 3))
• (Thin-wall 0.2 mL plastic PCR tubes)
• (10X DNA polymerase buffer)
• (10 mM solution of dNTPs)
• (Forward DNA primer)
• (Reverse DNA primer)
• (Taq DNA Polymerase)
Procedure

Part A: Polymerase Chain Reaction (PCR) of genomic DNA

Due to time constraints, the PCR process of Part A will be performed for you. However, you remain responsible for understanding the steps of PCR.

1. Place PCR tubes on ice, to prevent enzymatic activity before cycling.
2. Each reaction will be 50 uL, add:
   - 2 uL of purified DNA (from experiment 3)
   - 5 uL of 10X polymerase buffer
   - 1 uL of dNTP mix
   - 2.5 uL of Forward DNA primer
   - 2.5 uL of Reverse DNA primer
   - 0.25 uL of Taq DNA polymerase (1 unit) – add last
   - 32.75 uL of sterile water
3. Seal the PCR tubes and place into the PCR thermocycler machine.
4. Run the PCR program:
   - Step 1: Initial denaturation for 2 minutes at 95 °C
   - Step 2: Denature for 1 minute at 95 °C
   - Step 3: Anneal primers for 30 seconds at 55 °C
   - Step 4: Extend DNA for 2 minutes at 72 °C
   - Step 5: Repeat steps 2 through 4 for 25 cycles
   - Step 6: Final extension for 10 minutes at 72 °C
5. Allow PCR tubes to cool, remove from thermocycler and place on ice. Pool PCR tubes into a clean 1.5 mL plastic microcentrifuge tube and label.

Part B: Agarose gel electrophoresis

You will begin here in lab.

1. Prepare gel and running TBE buffer as indicated in the materials for 1 L of 1X buffer.
2. Prepare a 2 % gel using 1.0 g of agarose for 50 mL of 1x TBE.
3. Heat the agarose-buffer solution for 30-60 seconds in the microwave to dissolve the agarose. Do not over heat - you will have to wait longer for the agarose to cool and for the gel to solidify. Allow the gel material to cool to the touch; do not allow to overcool as the high gel percentage gels are goopy.
4. Assemble the gel mold in the gel box. Tape the ends of a gel mold or use rubber ends to make a square in the gel box to prevent the agarose from spilling. Pour the agarose into the gel mold, add a 10 or 14-well comb to one end of the gel, and wait until the gel solidifies (~10-15 minutes).
5. Remove the tape, if necessary. Arrange the wells to position near the negative electrode (black).
6. Pour 1x TBE buffer over the wells to cover the gel and remove the comb.
7. As we do not know the extent to which the PCR reaction was successful (what quantity of amplified DNA was generated), we will load varying amounts of amplified product and compare to a reference sample to ascertain concentration. Prepare a set of 6 amplified product samples, with a total volume of 15 uL each.
Each sample should contain 5 μL of loading dye, and either 1, 2, 4, 6, 8, or 10 μL of amplified sample. The remaining volume should be composed of water. Show your calculations.

8. To the wells, pipet your 6 samples (15 μL), each in a different well. Be sure to record what sample you placed where! Do not load more than 20 μL per well.
9. Pipet the known molecular weight DNA standard markers into a well (15 μL).
10. Connect the black electrode with the black wire and the red electrode with the red wires to the power source.
11. Plug in the power source.
12. Run for 1 hour. Use no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel). This value will vary depending on the gel apparatus you are using—consult your instructor (~125-150 V for large gels). Run until the dye front is 2 cm from the end of the gel or until time permits.
14. DO NOT touch the gel while the power is on.
15. Turn off power. Then disconnect all power cords.

**Part C: Visualizing the DNA Bands with SYBR Green**

1. Remove the gel from the box.
2. Prepare a solution of SYBR Green by putting 5 μL of SYBR Green stock solution into 100 mL of 1xTBE in a plastic box. Reminder: SYBR Green is mildly toxic. Wear gloves as all times and notify the instructor if there is a spill.
3. Carefully slide your gel into the SYBR Green solution, being careful not to splash, and place the plastic box on the rotator to incubate for 15 minutes.
4. Carefully remove your gel from the SYBR Green solution and transfer into a plastic box with 100 mL of 1xTBE.
5. Analyze the gel using a UV light box in the Biology department (we will visit together). The DNA will be bound with the SYBR Green and should appear as bands or lines.
6. Take a photograph with the digital camera using the SYBR filter.

**Question/Analysis**

Insert the picture of your gel into the data section of your report. From the photograph of your gel, estimate the size and homogeneity/purity of your amplified DNA samples.

Do you think your PCR captured a single sequence?
5 Using *ab initio* methods to construct a DNA sequence for protein cloning

**Objective**
To learn how to use the online webservers NCBI to retrieve a protein sequence, Reverse Translate to reverse translate the protein to DNA, IDT DNA tools to predict the complementary DNA strand, melting temperature and secondary structure of the DNA, and NE Biolabs website to look up the sequences cleaved by restriction enzymes and add that site to the DNA in order to insert the DNA into a plasmid.

**Safety**
No special safety precautions. This is a computer-based *in silico* lab.

**Background**
In this laboratory, we will use modern computational tools to obtain a primary sequence of a protein of interest and manipulate it to determine the most likely DNA sequence. We will determine the DNA complementary strand and determine the melting temperature and secondary structure of the DNA fragment. Then, for a plasmid of interest, we will look up the DNA sequences of the restriction enzymes in the coding region and add that sequence to the DNA fragment of interest to prepare it to inserted into the plasmid.

Specifically, we will use NCBI to obtain the protein sequence, and Reverse Translate to reverse translate the protein to DNA. We will use the IDT DNA tools to predict the complementary DNA strand and melting temperature and secondary structure of the DNA. We will use NE Biolabs website to look up the sequences cleaved by restriction enzymes and add that piece of DNA to the DNA for the protein of interest in order to insert the DNA into a plasmid.

Your isozymes of metabolic proteins and enzymes, blood type, and ability to metabolize nutrients from lipids, proteins, and carbohydrates are all inherited characteristics. These traits are the result of genes inherited from your parents. Genes are specific segments of molecules called DNA. Humans have 23 pairs of chromosomes, one maternal and one paternal. If the DNA from one human cell was stretched end to end, it would be 7 feet long! In 1958, Francis Crick proposed a relationship between DNA, RNA and proteins known as the “Central Dogma of Molecular Biology”. It states: 1) DNA directs the making of its own copy (i.e., replication); 2) genetic information is transferred from DNA to RNA (i.e., transcription); 3) RNA directs the transfer of genetic information to the amino acid chain during protein synthesis (i.e., translation). This dogma holds true for all animal and plant cells as well as those of bacteria, protists, and fungi. However, research has shown that in some viruses, the viral RNA directs the synthesis of DNA and RNA.

RNA (ribonucleic acid) and DNA (deoxyribonucleic acid) belong to a class of compounds called nucleic acids. Nucleic acids are polymers of monomer units called nucleotides. Nucleotides, when hydrolyzed, yield a nitrogen-containing base, a five- carbon sugar, and a phosphate group. There are two classes of nitrogen-containing bases found in nucleotides: pyrimidines and purines. Pyrimidines are single ring heterocyclic amines and purines are double ring heterocyclic amines. The bases uracil (U), thymine (T), and cytosine (C) are classified as pyrimidines, and adenine (A) and guanine (G) as purines. The base uracil is only found in RNA and the base thymine is found only in DNA.

In 1953, James Watson and Francis Crick proposed a three-dimensional structure for DNA.
to explain its chemical and physical properties. Their model of DNA consisted of two helical polynucleotide chains coiled around the same axis, forming a double helix. The hydrophilic sugars and negatively charged phosphate groups of the individual nucleotides are located on the outside of the helix; the hydrophobic bases are inside. The nucleotides making up each strand of DNA are connected by phosphodiester bonds between the phosphate group and the deoxyribose sugar. This forms the backbone of the DNA strand from which the nitrogen-containing bases extend. The bases of one strand of DNA will complementary pair with nitrogen-containing bases on the other strand through hydrogen bonding. The hydrogen bonding in DNA is very specific. The structure of adenine permits it to hydrogen bond only with thymine, and cytosine will bond only with guanine. As a result of this hydrogen bonding, the two strands of DNA are not identical, but complementary: where thymine appears on one strand, adenine will appear on the other (Fig. 5.1). However, the H-bonds are not identical: two H-bonds are formed between adenine and thymine and three H-bonds are formed between guanine and cytosine. DNA will automatically H-bond with its complementary strand (no chaperone is needed). Since the H-bonds are non-covalent interactions, they can be broken by heating the strands; this is called the melting temperature. DNA can also form secondary structures (e.g. hairpins) with itself due to self-complementarity. The amount of heat that is needed to melt these structures is typically given as the Gibbs free energy. This value can be related to the temperature using the thermodynamic relationship $\Delta G = \Delta H - T\Delta S$.

**Fig. 5.1 Nucleotide base complementarity in DNA.**


In this laboratory, we will perform a series of computational tools to prepare to clone a gene. Many times, an investigator wants to produce the protein encoded by a piece of DNA. For this purpose, expression plasmids or vectors have been constructed. Plasmids (Appendix: pET-15b vector) are bacterial viruses and can be used to insert DNA into bacterial cells using a method called transformation. Other plasmids can be used to express the protein in eukaryotic cells. The plasmid will be amplified by the bacteria using DNA polymerase.
DNA ligase can be used to catalyze covalent bonds between neighboring DNA bases. Once inside the cell, the promoter will attract the RNA polymerase (transcribes DNA to RNA) and lead to the production of an mRNA. The cell's ribosomes will translate the RNA into a protein. This allows investigators to produce large amounts of the protein, produce a tagged protein, or produce a range of protein concentrations if the promoter can be regulated. The tag can be a protein (e.g. GST) or a small peptide (6x His) that can be used to purify the protein from lysed bacterial cells (Fig. 5.2).


A very popular way to regulate the amount and the timing of protein expression is to use an inducible promoter. An inducible promoter is not always active the way constitutive promoters are (e.g. viral promoters). Some inducible promoters are activated by physical means (e.g. heat shock promoter). Others are activated by chemical such as IPTG or Tetracycline (Tet). IPTG is a classic example of a compound added to cells to activate a promoter. It is often used to activate the lacZ gene when cloning a new fragment of DNA and using blue/white selection. IPTG can be added to the cells to activate the downstream gene or removed to inactivate the gene.
Tet is an antibiotic and can be used to create two beneficial enhancements to inducible promoters. One enhancement is create an inducible on or off promoter. The promoter can be constitutively activated until Tet is added or constitutively inactivated until Tet is added; this is the Tet on/off promoter. The second enhancement is the ability to regulate the strength of the promoter. The more Tet added, the stronger the effect so that the expression vector can be modulated in the same manner as the volume using a radio dial.

**Materials**
- computer with an internet connection
- Biochemistry textbook

**Procedure:** Numbers (measurements) should always be accompanied by units!

**Part A.: NCBI to retrieve the protein primary sequence**
2. Turn the “All Databases” dropdown menu to “Protein” and search for: 1TJB (This is an excised EF-hand segment from Troponin C.) Select one isoform, and record the “GI” accession number.
3. Click on the link for the protein, then scroll down to ORIGIN and select the 1-letter protein code sequence and paste it to a text file. (Notepad works well for this.)

**Part B.: Reverse Translate to convert the protein to DNA**
2. Scroll down to DNA -> Protein
3. Select Reverse Translate
4. Paste your protein sequence using the fasta format omitting the numbers and using a > carrot, a name, then skip a line and insert the sequence.
   ```
   >name
   MTHREFYPA
   ```
   Use the defaults for *E. coli*. Paste your result of the most likely codons and consensus codons into the test file. Why do these differ?
5. Convert the DNA most likely codon sequence to RNA by hand. (Hint: How do DNA and RNA differ?)

**Part C.: IDT tools to create the DNA complement using the most likely codons**
2. On the dropdown “SciTools” menu, select OligoAnalyzer
3. Paste the most likely codon DNA sequence to the box.
4. Click Analyze. Copy and paste the results to the text file. What is the sequence of the complementary strand? What is the melting temperature? What is the meaning of the melting temperature for a DNA molecule? What is the GC-content?
5. Now click Hairpin. Save your results in the text file. What is the meaning of the ∆G mean given for the secondary structure?
6. Click Self-Dimer. Save your results in the text file.

**Part D.: Cloning into a plasmid**
1. Using the given plasmid map for pET-15b, select one or more restriction enzymes to clone in the gene of interest in the cloning site with *NdeI*, *BamHI*, or *XhoI*. State which
one(s) you chose.
2. What is the purification system of the protein grown in this plasmid?
3. How is the protein expression upregulated or induced?

Part E.: Adding ends that will stick to a complementary DNA if cut with a restriction enzyme
1. Go to http://www.neb.com/
2. Under NEB Tools, select “NEB Cutter”
3. Paste in your DNA sequence from Part B, and hit “submit”. The display will show restriction enzymes that cut your DNA. You can also select “0 cutters” to display enzymes that do NOT cut your insert.
4. Given the pET-15b plasmid map, prepare your DNA to be spliced into it in the coding region after the promoter and His-tag (restriction site). Determine the DNA sequence that will be cleaved by the restriction enzyme you choose by searching for the restriction enzyme of interest.
5. Add this sequence to the end(s) of the DNA from Part C. Which enzyme is used to catalyze the formation of the covalent bonds between the DNA construct and the plasmid?

Questions/Analysis
Answer the questions in the procedure.
MODULE 3: Enzyme Kinetics
6 Enzyme Kinetics of Wheat Germ Acid Phosphatase

Objective
To learn the key equations and graphing methods for explaining and examining enzyme activity. To learn methods for spectrophotometrically measuring the kinetics of an enzymatic reaction.

Safety
Be careful not to spill solutions in the spectrophotometer. Clean up all spills immediately.

Background
Enzymatic catalysis is the rate enhancement of a chemical reaction that occurs in the presence of an enzyme. This catalysis is essential for life, due to the very slow rates of uncatalyzed reactions in the cell. The study of this rate enhancement due to catalysis is known as enzyme kinetics. Like other catalysts, enzymes do not alter the equilibrium of a reaction, but act on the reaction rate. In order to measure this rate enhancement, we can observe the rate of product formation of a given reaction under conditions of varying amount of enzyme added. These measurements of different initial reaction rates allow the calculation of kinetic rate constants that describe the degree and nature of enzymatic catalysis.

In this experiment, we will study the kinetic properties of an acid phosphatase from wheat germ. The general purpose of these experiments is to give you a feel for “real life” kinetics and how they relate to the theoretical studies you may have learned about in lecture.

Phosphatases are a large group of enzymes that catalyze the hydrolysis of phosphomonoesters with the consequent release of inorganic phosphate (Figure 6.1). These enzymes are ubiquitous in nature. Some are highly specific for certain substrate molecules while others have very broad substrate specificities (act on many different substrates). This latter group can be classified as either acid or alkaline phosphatases based on the pH at which they function optimally.

Figure 6.1. The reaction catalyzed by a generic phosphatase.

\[
R-O-P-O^- + H_2O \rightarrow R-OH + P_i
\]

Plant seeds are particularly rich in acid phosphatases. The precise function of these enzymes is unknown but it is likely that they are involved in releasing phosphate from internal stores during germination.

In our experiment, we will provide a synthetic substrate, para-nitrophenyl phosphate. This substrate is useful because the product of its hydrolysis, para-nitrophenol, absorbs light at 405 nm when put into an alkaline solution (Figure 6.2). Using this property, we can track the extent of phosphomonoester hydrolysis—the activity of our enzymes.
Figure 6.2. The formation of p-nitrophenol.

\[
\text{O}^\text{N} = \text{D} \text{O}^\text{P} \text{O}^- + \text{H}_2\text{O} \rightarrow \text{O}^\text{N} = \text{D} \text{O} \text{O}^\text{P} \text{I}^- + \text{P}_1 \]

Materials
- 0.15 M Ethylenediamine – 0.10 M Citrate buffer, pH 5.0
- 25 mM and 2.5 mM Disodium p-nitrophenyl phosphate, pH 4.8 (NPP). Keep cold (in 0.10 M Citrate buffer, pH 4.8)
- 0.10 M NaOH
- 2 mg/ml crude Wheat Germ acid phosphatase in 0.1% Triton X-100
- 0.10 M Citrate buffer, pH 4.8
- 0.1% Bovine Serum Albumin
- Glass test tubes
- Plastic spectrophotometer cuvettes
- Various variable volume micropipettors (20 µL, 100 µL, 200 µL, 1000 µL) and tips
- Ice
- 37°C water bath

Procedure:
Part A: Optimization of Enzyme Concentration
The purpose of this section of the experiment is to find an enzyme concentration that will give a constant rate of product formation as well as produce enough product so that we can easily detect its accumulation spectrophotometrically. If the enzyme is too concentrated, enough substrate will be converted to product during the course of the reaction that the substrate concentration will change appreciably, and the reaction velocity will slow down. This would violate one of the assumptions of Michaelis-Menten kinetics: that you are measuring the initial velocity ($v_0$) of the reaction. However, if the enzyme is too dilute, so little product will be formed that it would be difficult to detect above background in the spectrophotometer. Thus, the determination of a “working dilution” that gives a constant and measurable product formation is crucial for this and the following experiments.

1. You will have to try a series of dilutions of the crude wheat germ acid phosphatase to get a constant rate of product formation. Suggested dilutions to try initially are 1/10, 1/40, and 1/100. You will be taking 5 time points for each dilution, and you will be doing each reaction in duplicate to increase the accuracy of your results. For each dilution that you will be testing, label two glass test tubes for your reaction mixes (i.e., for your 1/10 dilution, you will need 2 glass tubes labeled “1/10 A” and “1/10 B”).

2. Add 0.5 ml of Ethylenediamine-citrate buffer, pH 5.0 to each “reaction” tube.

3. Prepare your “Stop tubes”. You will be taking 5 time points from each reaction mix: 0, 5, 10, 15, and 20 minute time points. Thus, for each reaction mix, label 5 test tubes (e.g.,
“1/10 A-0 min”, “1/10 A-5 min”, etc; similarly for “1/10 B-0 min” and all the remaining reactions). Add 1.8 ml of 0.10 M NaOH to each “stop” tube.

4. To each “reaction” tube, add 0.5 ml of 2.5 mM NPP.

5. Incubate the “reaction” tubes at 37° C for 2 minutes in a water bath to allow the mixture to come to thermal equilibrium.

6. Make your serial dilutions of the crude wheat germ acid phosphatase. Suggested dilutions are 1/10, 1/40, and 1/100. However, you may have to dilute more to get the right working dilution, depending on the wheat germ acid phosphatase stock. Once you decide on a dilution, you will need 10 ml to carry out the remainder of the reactions. Thus, make 15 ml of each dilution (to have some extra) and dilute into ice-cold 0.1% BSA. Make sure to use new pipette tips for every manipulation! Store your dilutions on ice.

7. For the following steps, timing is crucial! It is suggested that you write out the timing and what you’ll be doing before you begin. Have a P200 set to 200 ul ready to take the time points.

For each reaction, you will start the reaction by adding 250 ul of diluted enzyme to the appropriate “reaction” tube at 37° C. Mix, and immediately remove 200 ul of the reaction to the appropriate 0 min stop tube and mix. Take additional 200 ul time points 5, 10, 15, and 20 minutes later.

You obviously can’t add stuff to and remove aliquots from two or more reactions simultaneously! Instead, do your manipulations at discrete timed intervals, such as at 15 second intervals. For example, at t=0, start reaction 1/10A and take the 0 min time point. At time t=15 seconds, start reaction 1/10B and take its 0 min time point. At time t=30 seconds, start reaction 1/40A, and so on. At time t=5 min, take the 5 min time point for reaction 1/10A. At time t=5 min, 15 sec take the 5 min time point for 1/10B, etc. This takes forethought, planning, and concentration. In the midst of furious manipulations you don’t want to be distracted by something else. This is why everything should be labeled and planned to the second beforehand.

8. After all the time points are collected, determine the absorbance A_{405} for each stop tube. Use the Spec-20s provided as the spectrophotometers, and use the 0 minute time points as the blanks for later time points. You should be able to simply pour directly from the stop tubes into a cuvette. Re-use the plastic cuvettes! Once you have taken a reading, pour out the sample, rinse the cuvette, and pour in the next sample. You only need 1-2 cuvettes for the entire experiment. One partner can rinse one cuvette while the other partner can take a reading with the other cuvette.

9. Determine the change in absorbance (ΔA_{405}) per minute for each of the time points from 5 min to 20 min. Do this for all dilutions you have tested.

10. Plot the ΔA_{405}/min versus time for each dilution.

11. Does the result for any dilution give a straight horizontal line (i.e., slope = 0 = constant reaction velocity)? If not, repeat steps 1-10 for another dilution until you find an optimal “working dilution”.

12. You will use your optimal working dilution for all subsequent enzyme kinetics experiments.
Part B: Data Collection for Determination of the Michaelis Constant

In this experiment, you will determine the $K_m$ for p-nitrophenyl phosphate as a substrate for the wheat germ phosphatase reaction. As you know, from the definition of $K_m$ it is the substrate concentration at which the velocity of the enzymatic reaction is one-half the maximum velocity. In order to determine the $K_m$ of the reaction, then, we must determine the reaction velocity at various substrate concentrations. In your data analysis, you will use these results to calculate the $K_m$ and $V_{max}$, and compare and contrast the results you obtain using different methods (Michaelis-Menten, Lineweaver-Burke, direct linear).

1. Prepare “reaction” tubes in duplicate using 0.02, 0.04, 0.08, 0.20, and 0.35 ml of 25 mM NPP. There should be 10 tubes in total. Add 0.4 ml of 0.10 M Citrate buffer, pH 4.8 to each tube. Add enough $H_2O$ to each tube to bring the volume up to 0.8 ml.

2. Prepare a corresponding set of “stop” tubes. For each reaction you will be taking three time points: 0, 10, and 20 minutes. Thus, you will need 30 appropriately labeled stop tubes, each containing 1.8 ml of 0.10 M NaOH.

3. Incubate the “reaction” tubes at 37 C for 3 minutes.

4. Because you have so many tubes, it is essential that you keep track of time as you do the following manipulations. Below is a suggested format for a table for you to set up in your lab notebook to help you keep track of timings. For each reaction tube, you will add enzyme, immediately take a 200 ul aliquot for a zero time point, and then take additional aliquots at 10 minutes and 20 minutes. We suggest the following procedure: At time $t=0$, add enzyme to tube 1, mix, take a zero-time aliquot. At time $t=30$ seconds, add enzyme to tube 2, mix, and take an aliquot. At time $t=1$ minute, add enzyme to tube 3, etc., until enzyme has been added to all tubes. At time $t=10$ minutes, take the 10 minute time point for tube 1. At time $t=10.5$ minutes, take the time point for tube 2, etc., continuing for all 10 tubes. We suggest that one person watches a timer and keeps track of the tubes, while the other partner does the actual aliquoting.

Suggested table set-up:

<table>
<thead>
<tr>
<th>Time</th>
<th>Rxn Tube</th>
<th>Stop Tube</th>
<th>$A_{405}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.02A</td>
<td>0.02A-0min</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.02B</td>
<td>0.02B-0min</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.04A</td>
<td>0.04A-0min</td>
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<td>1.5</td>
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<tr>
<td>10.5</td>
<td>0.02B</td>
<td>0.02B-10min</td>
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<td>etc...</td>
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<td>0.02A</td>
<td>0.02A-20min</td>
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<td>20.5</td>
<td>0.02B</td>
<td>0.02B-20 min</td>
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</tr>
<tr>
<td>etc...</td>
<td>etc...</td>
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<td></td>
</tr>
</tbody>
</table>

5. When ready to start (as discussed in step 4), add 0.2 ml of your working dilution of enzyme to the first tube, mix, and immediately remove a 0.2 ml aliquot and add to the appropriate NaOH-containing stop tube. Continue at your defined intervals.

6. Incubate the reaction tubes at 37 C, and at time $t=10$ minutes and $t=20$ minutes, take a 0.2 ml aliquot from the first reaction tube and add to the appropriate stop tube.
Do this at the appropriate intervals for all tubes.
7. Using the zero time points as blanks, determine the A405 for each reaction. Re-use the plastic cuvettes from Part A of the experiment! As always, record your data in your lab notebook.
8. To determine the Michaelis constant from your data, a fair amount of data analysis is necessary (Part C).

**Part C: Data Analysis of Michaelis Data to determine Km**
1. Determine the substrate (NPP) concentration in millimoles / liter in each of the 5 different reactions mixes.
2. Using Beer’s Law, calculate the amount of product (p-nitrophenol) formed at each time point. Remember that your sample was diluted 1/10 when you stopped the reaction, and the assay volume was 1 ml.

   Remember that Beer’s Law is \( A = \varepsilon c L \), where \( A \) is absorbance, \( L \) is the pathlength of the cuvette (1 cm for us), \( c \) is the concentration of product that you are trying to find, and \( \varepsilon \) is the molar extinction coefficient, which for our product is \( 1.88 \times 10^4 \text{M}^{-1}\text{cm}^{-1} \) at 405 nm.
3. Using these product concentrations, you should be able to calculate the reaction velocity in micromoles of p-nitrophenol produced per ml per minute at each substrate concentration.

   It is easiest to do these calculations in excel, but you must print them out and tape them into your lab notebook.
4. Download the Excel template for Enzyme Kinetics available on the course website at [http://bonhamchemistry.com](http://bonhamchemistry.com)
   - Due to program limitations, this sheet will only work on Windows computers.
     - If you do not have one available, it will run on the windows lab computer or on computers in the student computer labs.
   - Excel must have the “Solver” add-in installed (“Tools” menu → “Add-Ins” → Checkmark next to “Solver”.
   - Solver must be referenced. If you meet the above two requirements and the sheet is still not working, try this: In Excel, hit Alt-F11 to call up the Visual Basic Editor, and then go to “Tools” → “References”, make sure the “Solver” box is checkmarked. Then, close the Visual Basic Editor.
5. Open the Excel template on a Windows computer. The file has several different worksheets set up with Instructions, Data Entry, and Analysis.
6. Click on the “Data Entry” tab. Enter your raw Abs 405 data and your calculated reaction velocities. **You MUST enter the data in the correct boxes to correspond to your measurements.**
7. Once all the data are entered, click on the “Hyperbolic Curve Fit” tab. In this worksheet, click on the “Curve Fit” button at the top. **If this gives an error, see step 4.**

   Excel will do a least-squares fit of the Michaelis-Menten equation to your data by rapidly varying Vmax and Km. The fitted Vmax and Km values (the apparent Vmax and Km) will appear in the boxes. Be aware that for inhibitor studies, only a simple hyperbolic fit will be done, not a fit to any particular inhibition model.
8. Scroll down to see the experimental data, the calculated velocities for each [S], the Residuals (the Residual is the difference between the calculated velocity and the measured velocity at each substrate concentration), and the Residuals Squared (used
for the least-squares non-linear regression). Scroll down even further to see a plot of the residuals vs the [S], which will give you an idea of how well your data fit the model. How well did your data fit Michaelis-Menten kinetics, based on these residuals?

9. Now click the “Hyperbolic Plots” tab. On this worksheet, your original data have been plotted as a scatter plot. In addition, the non-linear regression of the best fit to the Michaelis-Menten equation is plotted as a line.

10. Now click on the “LB curve fit” tab. Here is your data transformed to 1/[S] and 1/v values for a Lineweaver-Burk plot. Click on the “LB Plots” tab to see these data plotted. Also shown is a line derived by using the Vmax and Km values derived from the best fit of the hyperbolic.

11. Now click on the “LB linear regression” tab. This is a Lineweaver-Burk plot of the same data points, but now the plotted line is a linear regression of these data points fitted to the lineeweaver-Burk transformation of your substrate and velocity data. How do the Km and Vmax values from the Lineweaver-Burk transformation compare with the values determined by the hyperbolic plot? Why or why not is there a difference, and which would you rely on more (and why)?

Questions/Analysis
Why was NaOH needed in the stop tubes? What role does BSA play in the enzyme dilutions? What was your correct dilution for your “working dilution”? What was the Km for p-nitrophenyl phosphate? Answer the questions posed in Part C as well.
7 Inhibition of Wheat Germ Acid Phosphatase Activity

Objective
To learn how to determine the inhibition constant (Ki) for various inhibitors of enzyme reactions.

Safety
Be careful not to spill solutions in the spectrophotometer. Clean up all spills immediately.

Background
In this experiment, we will extend our studies of the enzyme kinetics of wheat germ acid phosphatases to examine the effect of potential inhibitors of these enzymes. Inhibitors are molecules that bind to enzymes and decrease their catalytic activity. There are a variety of mechanisms by which this binding and subsequent inhibition happen. Since decreasing an enzyme’s activity can correct regulatory imbalances or kill hostile micro-organisms, the majority of drugs that humans take are enzyme inhibitors. There are also many naturally occurring enzymatic inhibitors that allow fine regulation and metabolic control within the cell.

There are two broad classes of inhibition: reversible and irreversible. Irreversible inhibitors form covalent modifications of enzyme, causing permanent damage to the ability of the enzyme to function. Reversible inhibitors, on the other hand, can bind and unbind, allowing temporary modification of enzyme activity. There are four major classes of reversible inhibition, organized by how the inhibitor interacts with the enzyme and the substrate. Competitive inhibitors cannot bind to the enzyme at the same time as the substrate, causing a change in apparent Km but leaving Vmax unchanged. Non-competitive inhibition is when the binding of the inhibitor does not affect substrate binding, causing a change in Vmax but not in Km. Uncompetitive inhibition is when the inhibitor only binds the substrate-enzyme complex, resulting in decreases in both apparent Km and Vmax. Lastly, mixed inhibition is a catch-all for situations where the binding of the inhibitor affects the binding of the substrate, or the inhibitor has different affinities for the free enzyme and the enzyme-substrate complex. It typically affects Km and Vmax as well.

Here, we will test the effects of inorganic phosphate and fluoride ions as inhibitors of wheat germ acid phosphatase.

Materials
• 25 mM and 2.5 mM Disodium p-nitrophenyl phosphate, pH 4.8 (NPP). Keep cold (in 0.10 M Citrate buffer, pH 4.8)
• 0.10 M NaOH
• 2 mg/ml crude Wheat Germ acid phosphatase in 0.1% Triton X-100
• 0.10 M Citrate buffer, pH 4.8
• 0.1% Bovine Serum Albumin
• 0.05 M Sodium Phosphate, pH 4.8
• 0.10 M Sodium Fluoride
• Glass test tubes
• Plastic spectrophotometer cuvettes
Various variable volume micropipettors (20 µL, 100 µL, 200 µL, 1000 µL) and tips
Ice
37°C water bath

Procedure:
Part A: Data Collection for Determination of the Inhibition Constants
In this experiment, you will determine the Ki for inhibition by inorganic phosphate or by fluoride in the wheat germ phosphatase reaction. This will be done by re-doing your Michaelis constant determination from last week, but incorporating potential inhibitors to the reaction mixes.

1. Prepare “reaction” tubes in duplicate using 0.02, 0.04, 0.08, 0.20, and 0.35 ml of 25 mM NPP. However, also add either 40 ul of 50 mM sodium phosphate, pH 4.8 or 100 ul of 100 mM sodium fluoride to each tube (depending on which inhibitor you are investigating). There should be 10 tubes in total. Add 0.4 ml of 0.10 M Citrate buffer, pH 4.8 to each tube. Add enough H2O to each tube to bring the volume up to 0.8 ml.

2. Prepare a corresponding set of “stop” tubes. For each reaction you will be taking three time points: 0, 10, and 20 minutes. Thus, you will need 30 appropriately labeled stop tubes, each containing 1.8 ml of 0.10 M NaOH.

3. Incubate the “reaction” tubes at 37 C for 3 minutes.

4. Because you have so many tubes, it is essential that you keep track of time as you do the following manipulations. Below is a suggested format for a table for you to set up in your lab notebook to help you keep track of timings. For each reaction tube, you will add enzyme, immediately take a 200 ul aliquot for a zero time point, and then take additional aliquots at 10 minutes and 20 minutes. We suggest the following procedure: At time t=0, add enzyme to tube 1, mix, take a zero-time aliquot. At time t=30 seconds, add enzyme to tube 2, mix, and take an aliquot. At time t=1 minute, add enzyme to tube 3, etc., until enzyme has been added to all tubes. At time t=10 minutes, take the 10 minute time point for tube 1. At time t=10.5 minutes, take the time point for tube 2, etc., continuing for all 10 tubes. We suggest that one person watches a timer and keeps track of the tubes, while the other partner does the actual aliquoting.

Suggested table set-up:

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<td>0.04A-0min</td>
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</table>

5. When ready to start (as discussed in step 4), add 0.2 ml of your working dilution of enzyme to the first tube, mix, and immediately remove a 0.2 ml aliquot and add to
the appropriate NaOH-containing stop tube. Continue at your defined intervals.
6. Incubate the reaction tubes at 37°C, and at time t=10 minutes and t=20 minutes, take a 0.2 ml aliquot from the first reaction tube and add to the appropriate stop tube. Do this at the appropriate intervals for all tubes.
7. Using the zero time points as blanks, determine the A405 for each reaction. Re-use the plastic cuvettes from Part A of the experiment! As always, record your data in your lab notebook.
8. To determine the inhibition constant from your data, a fair amount of data analysis is necessary (Part B).

**Part B: Data Analysis of Michaelis Data to determine Ki**

1. Determine the substrate (NPP) concentration in millimoles / liter in each of the 5 different reactions mixes.
2. Using Beer’s Law, calculate the amount of product (p-nitrophenol) formed at each time point. Remember that your sample was diluted 1/10 when you stopped the reaction, and the assay volume was 1 ml.
   Remember that Beer’s Law is \( A = \varepsilon \cdot c \cdot L \), where \( A \) is absorbance, \( L \) is the pathlength of the cuvette (1 cm for us), \( c \) is the concentration of product that you are trying to find, and \( \varepsilon \) is the molar extinction coefficient, which for our product is \( 1.88 \times 10^4 \text{ M}^{-1}\text{cm}^{-1} \) at 405 nm.
3. Using these product concentrations, you should be able to calculate the reaction velocity in micromoles of p-nitrophenol produced per ml per minute at each substrate concentration.
   It is easiest to do these calculations in excel, but you must print them out and tape them into your lab notebook.
4. Use the Excel Kinetics Template file, as described in Part C of Experiment 6. Make sure to enter your data in the correct boxes, and answer all questions found in Part C of Experiment 6 in regards to your inhibitors.
5. Based on the changes in Km and Vmax between data from Experiment 6 and your inhibitor data, predict what type of inhibitor (“Competitive”, “Uncompetitive”, “Non-Competitive”, or “Mixed”) you expect Phosphate and Fluoride to function as.

**Questions/Analysis**

Why would you suspect that inorganic phosphate would inhibit the reaction? What were your inhibitions constants for inorganic phosphate and fluoride? What type of inhibitors were inorganic phosphate and fluoride?
MODULE 4 Purification and Characterization of Parvalbumin protein
Parvalbumins constitute a class of low-molecular weight (typically, MW = 12,000), acidic proteins (pI 3.9-4.9) which have the ability to bind two calcium ions (using the "EF-hand" motif) with high affinity. Parvalbumin constitutes one of the 32 subfamilies within the EF-hand superfamily of calcium-binding proteins all sharing the common helix-loop-helix (EF-hand) motif. Structurally, each of the proteins in this EF-hand superfamily consists of varying numbers of EF-hand “helix-loop-helix” motifs. Specifically, the EF-hand motif consists of an α helix, a connecting loop in which the Ca$^{2+}$ is bound, and a final α helix positioned roughly perpendicular to the first. Parvalbumins contain three EF-hands and typically bind two calcium ions. Extensive research on this subject has produced a large amount of structural data for both the α and β forms from both biophysical experiments as well as the structural determination methods of x-ray crystallography and nuclear magnetic resonance spectroscopy. For years the apo (calcium-free) parvalbumin eluded structural determination, but a structure was finally solved in 2008. Binding to the calcium (or other) metal ion induces significant conformational changes influencing both the structure and function in proteins. The number of known metal binding proteins numbers in the thousands.

There are two distinct lineages of parvalbumins, α and β, that are present in lower and higher vertebrates. The two lineages differ in a number of characteristics, including isoelectric points, sequence characteristics, metal ion binding affinities, structure, cell- type specific expression, chromosomal localization, and physiological functions. Parvalbumins are particularly abundant in the white muscle tissue of lower invertebrates, however, they are found in various tissues of all eukaryotic species, including humans, and are highly polymorphic in the lower vertebrates. For example, five isoforms have been found in carp muscle, four in frog muscle, and three in silver hake muscle, whereas only a single isoform has been found in muscles from the chicken, rat, and rabbit.

Observations have led researchers to conclude that parvalbumin is a muscle relaxation protein found in mammalian skeletal muscle involved in muscle relaxation and contraction, calcium buffering, and signal transduction and facilitates Ca$^{2+}$ transport from the myofibrils to the sarcoplasmic reticulum. Other roles proposed for parvalbumins include involvement in the triggering of gene expression, in cell division, in processes influencing cell shape and motility and in immune system development. Parvalbumin has been shown to potently modulate short-term synaptic plasticity in mice. It is particularly significant that the parvalbumin-induced relaxation rate was found to be highly correlated to the Mg$^{2+}$ off rate from the protein. Calcium is bound by a number of cellular proteins but is widely used as a neurotransmitter in the cell cycle and a second messenger rather than for structural support. Calcium is used as a second messenger and is taken up by proteins such as calmodulin, the S100 proteins, calcineurin, calbindin, troponin C, and parvalbumin, to name a few. A parvalbumin deficiency from fast-twitch muscles of mice, otherwise expressing parvalbumin in high levels, slowed down the speed of twitch relaxation while the maximum force was not affected. In 2009, parvalbumin was reported to be the major allergen in fish. In spite of continued research, the function of parvalbumin continues to be disputed and only further research will finally elucidate the physiological function of this abundant protein.
Purification Scheme: Parvalbumin from *Onchorhyncus mykiss*

1. Obtain Rainbow Trout
2. Fillet & Dice Fish
3. Extract with chilled deionized water
4. Homogenize in Blender
5. Centrifuge to pellet insoluble portion
6. Re-extract pellet with chilled deionized water
7. Store pellet (aqueous insoluble, lipids) at -20 °C
8. Add ammonium sulfate (AS) to 70% to precipitate unwanted proteins
9. Discard 0-70% ammonium sulfate protein pellet
10. Filter proteins soluble in 70% AS through glass wool to remove solids
11. Add AS to 100% to precipitate all proteins, including parvalbumin
12. Dialyze 100% AS proteins in 0.05 M NH$_4$HCO$_3$ to remove AS salt
13. Concentrate dialyzed proteins
14. Filter 70-100% proteins through glass wool to remove solids; concentrate
15. Separate proteins by size using gel filtration chromatography (G-75)
16. Discard high molecular weight proteins
17. Concentrate low molecular weight parvalbumin proteins
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<tr>
<td>Separate low molecular weight proteins by charge (pI) using DEAE anion exchange column to yield isoform(s)</td>
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<td>Determine structure-function relationships in parvalbumin by modeling known structures in DeepView</td>
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<tr>
<td>Characterize parvalbumin protein by UV-Vis spectroscopy, fluorescence spectroscopy, gel electrophoresis (MW, purity)</td>
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8 Extraction and homogenization of white meat from fish

Objective
To learn how to homogenize and fractionate a tissue mixture using a series of water fractionations and centrifugations. To learn how to isolate and prepare protein samples for subsequent analysis. To learn how to separate protein solutions into fractions via salting out.

Safety
Do not ingest the fish. Wash your hands prior to leaving lab. Clean up all spills immediately. Use the balances carefully and be careful not to spill chemicals in them. Do not move balances. Alcohol wipe the lab benches before leaving lab.

Background
In order to study DNA and proteins it is crucial for biochemists to obtain a sample which contains only the molecule(s) of interest. The source may be a naturally-produced bacterial, animal or plant source or a genetically-engineered bacterial or plant source. In the production of proteins for commercial purposes, typically a large amount of protein is needed at high purity.

A purification scheme for protein might consist of a combination of the following steps: a) preparing a crude cell extract, b) ammonium sulfate precipitation, c) ion exchange chromatographic separation, and d) affinity chromatographic separation. This particular sequence of steps is not always applicable which often means that a unique purification protocol has to be developed for each new substance you wish to isolate. The most important rule is that the steps should complement each other and that the degree of purity should increase with each step. The number of steps included the protocol depends on the state of the starting fraction and on how pure you want your substance. To get a good recovery of the substance (e.g. minimize loss) it is desirable that each step is as specific as possible. To check purity and yield you may use absorbance measurements, various types of electrophoresis, and preferably also some kind of activity measurement for enzymes. The column chromatography part may be performed in many different ways ranging from manual methods to methods employing pumps and computers (e.g. FPLC or HPLC). In this lab, we will initially run columns manually, however, routine purifications on this system can be reconfigured to monitor absorbance or fluorescence in real-time using a FPLC (Fast Protein Liquid Chromatography) or HPLC (High Performance Liquid Chromatography) which is a programmable system with powerful pumps and an attached UV-Vis detector. The focus of this experiment will be a. preparing a crude cell extract.

In many respects, the biochemical purification of DNA samples can be a simpler process. Nature has selected DNA as a very robust molecule that is resistant to chemical modification. This protects the genetic heritage of each organism, but also enables scientists to use harsher treatments to quickly degrade and remove all non-nucleic acid material. A purification scheme for DNA might consist of a) preparing a crude cell extract, b) treatment with concentrated enzymes to degrade non-DNA, c) salt and ethanol precipitation, and d) polymerase chain reaction (PCR) amplification of the DNA of interest. Here, we focus on steps a through c.

The muscle of the Rainbow trout will be chopped into 1-cm³ squares and suspended in an ice-cold water solution. For the protein extraction, a blender will be used to pulverize the
proteins at a high-speed and the resulting soup (containing the proteins, DNA, fats, and tissue material) will be separated using centrifugation and subsequently clarified using a mat of glass wool or several layers of cheese cloth. The supernatant will be a white-colored liquid that should be clear or only slightly cloudy containing the water-soluble proteins. In a subsequent lab, the different proteins will be separated using an ammonium sulfate precipitation by a “salting-out” technique. The concentration of the proteins will be determined using UV-vis spectroscopy in a later lab. A small amount (~1 mL) of precipitate should be saved for analysis in future labs.

For the DNA extraction, a sample of the pulverized tissue soup (containing the proteins, DNA, fats, and other tissue material) will be treated with Proteinase K, an enzyme that breaks down and degrades proteins. The resulting protein-free solution will then be purified by washing the solution through a silica membrane that binds only nucleic acids (such as DNA). This nucleic acid material, composed of DNA and RNA in solution, will be saved for future analysis.

The centrifuge will be programmed in revolutions per minute (RPM). To calculate the g-force or relative centrifugal force for a specific rotor from the RPM of the centrifuge, use: [http://www.beckman.com/resourcecenter/labresources/centrifuges/rotorcalc.asp](http://www.beckman.com/resourcecenter/labresources/centrifuges/rotorcalc.asp) and your measurement of the inner radius of the JA-14 rotor to the center of the sample in the centrifuge tube or the nomogram below (Fig. 8.1).

**Fig. 8.1 Nomogram for determining relative centrifugal force or g-force.**
[http://aquaticpath.umd.edu/nomogram.html](http://aquaticpath.umd.edu/nomogram.html)
Lastly, the proteins will be separated using an ammonium sulfate precipitation by a “salting-out” technique. To explain “salting-out”, we must note that the solubility of proteins varies according to the ionic strength and hence according to the salt concentration of the solution. Two distinct effects are observed. At low concentrations of salt, the solubility of the protein increases with salt concentration. This phenomenon is called 'salting-in' (Fig. 8.2). However, as the salt concentration (ionic strength) is increased still further, the solubility of the protein begins to decrease. At sufficiently high ionic strength the protein solubility will have decreased to the point where the protein will be almost completely precipitated from solution - an effect called 'salting-out'. The theoretical basis of salting-out is complex but one factor is probably the competition between the protein and salt ions for available water molecules for solvation. At high salt concentrations, insufficient water molecules are available for full solvation of the protein so that protein-protein interactions become predominant over protein-water interactions, and due to density, precipitation occurs.

**Fig. 8.2 Salting-in and salting-out.**

Since proteins differ markedly in their solubilities at high ionic strength, salting-out is a very useful procedure to assist in the purification of a given protein or enzyme. Indeed, enzyme purification schemes almost invariably include such a step. In practice, ammonium sulfate is the salt commonly used since it is highly water-soluble, relatively cheap and available at high purity. Furthermore, it has no adverse effects upon enzyme activity and thus protein conformation.

Great care must be taken to ensure that the salt concentration of the whole solution increases uniformly without the occurrence of local high concentrations which could precipitate the protein of interest along with the undesired proteins. Therefore the solution is stirred continuously as small aliquots of crushed, solid ammonium sulfate (or preferably saturated ammonium sulfate solution) are added. After each addition, the ammonium sulfate is allowed to disperse fully before the next addition. Once the required ammonium sulfate concentration is reached, incubation at 0 - 4°C is continued for a brief period to allow protein precipitation to occur, and the precipitated protein is then recovered by centrifugation.

An ammonium sulfate concentration is chosen which will precipitate the maximum proportion of undesired protein whilst leaving most of the desired protein or enzyme still in solution. After the unwanted, precipitated protein is removed by centrifugation, the
ammonium sulfate concentration of the remaining solution is increased to a value that will precipitate most of the desired protein while leaving the maximum amount of residual protein contaminants still in solution. The precipitated protein is recovered by centrifugation and dissolved in fresh buffer for the next stage of purification. Residual ammonium sulfate will be present in this enzyme solution and may need to be removed by gel filtration or dialysis before the next purification step can be attempted.

In addition to its role as an extremely useful and universally applicable purification step, ammonium sulfate purification is often employed again at later stages of purification simply to concentrate the protein from dilute solution after procedures such as gel filtration.

**Fig. 8.3 Properties of Saturated Ammonium Sulfate Solutions (AS is 3.93 M at 4 °C)**

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<th>Temperature (°C)</th>
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<td>Weight Percentage</td>
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<td>Moles of AS in 1000 mL solution</td>
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<td>Moles of AS in 1000 g of water</td>
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<td>Grams of AS in 1000 mL of water</td>
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<td>Grams of AS into 1000 mL of water</td>
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**Fig. 8.4 Fractionation with Solid Ammonium Sulfate**

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<th>Initial Concentration of Ammonium Sulfate (% saturation at 0 °C)</th>
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The cold protein extract will be extracted with 70% AS precipitation. Your instructor will perform a final 100% ammonium sulfate precipitation to concentrate the 70% AS protein containing the parvalbumin protein of interest. Ammonium sulfate may be added in as a saturated solution or as a pure solid. Solid ammonium sulfate will have to be added to the most concentrated samples to saturate it completely. Adding ammonium sulfate solution necessarily dilutes the protein concentration, so solid salt is preferable when preparing
the higher saturation levels. The solid salt must be added slowly to the cold solution with very gentle shaking to avoid irreversible denaturation. Any precipitate that forms is collected by centrifugation at 12,000 RPM for 20 minutes (or as long as it takes to clarify the solution).

**Materials**
- fish protein source
- centrifuge tubes (250 mL)
- deionized water and water bottles
- graduated cylinders
- centrifuge rotor (cold room)
- vortex mixer
- microcentrifuge tubes
- glass wool or cheesecloth
- blender
- knives/scalpels
- Beckman J-21C centrifuge
- ice in buckets
- beakers
- balance
- salt (ammonium sulfate)

**Procedure:**
**Part A.: Homogenization**
1. Thaw source Rainbow trout if frozen. Mass the fish and record your data.
2. Cut back skin and fat layer.
3. Dice muscle into 1 cm³ squares carefully using a knife.
4. Place cubed muscle in a clean beaker on ice.
5. Mass and record the total tissue isolated (100-200 grams is sufficient).
6. Add an equal volume of cold deionized water (from the refrigerator); record the volume.
7. Using a blender, pulse-homogenize the mixture until a pasty consistency (light pick color) is reached.
8. This resulting solution will be used for part B.

**Part B.: Protein Extraction**
1. Divide the remaining homogenate into equal mass sections (mass with balance to within 1 g) in the 250 mL centrifuge tubes and centrifuge for 20 minutes at 12,000 RPM at 4 ºC in a Beckman J-21C centrifuge using a JA-14 rotor. Three groups should centrifuge their samples together to save time.
2. Decant and save the supernatant.
3. Re-extract the tissue with an equal volume of cold deionized water.
4. Divide the homogenate into equal mass sections in centrifuge tubes and centrifuge for 20 minutes at 12,000 RPM at 4 ºC in a Beckman J-21C centrifuge using a JA-14 rotor.
5. Again, decant and save the supernatant.
6. Pool the supernatants from the two centrifugations. Record the total volume of the supernatant by measuring the volume in a graduate cylinder.
7. Pour the supernatant through unpacked glass wool in a glass funnel to remove any
unwanted solids and lipid material.
8. Remove 1 mL of supernatant and 1 mL of pellet into a 1.5 mL plastic microcentrifuge tube and store in the freezer (-20 °C) for a subsequent lab for analysis. Label the tubes well indicating the contents and your groups’ initials.

**Part C: Salt Fractionation of Proteins**
1. Saturate the supernatant with ammonium sulfate (AS) using either solid AS measured on the balance or by measuring the saturated AS solution (in fridge/cold room) in a graduated cylinder to reach 70% concentration. Slowly add the AS to the supernatant in a beaker slowly stirred on a stir plate.
   Calculating the AS to add: If you have 20 mL of supernatant and you add 10 mL of sat. AS: 10 mL AS/30 mL total = 33% saturated
2. Mass your two centrifuge tubes.
3. Centrifuge the AS precipitations (balance centrifuge tubes to within 1 g) at 12,000 RPM for 20 minutes.
4. Record the volume of the supernatant using a graduated cylinder.
5. Record the mass of the centrifuge tube with the precipitate and determine the mass of precipitate. Remove 1 mL of supernatant and precipitate and save in separate 1.5 mL plastic microcentrifuge tubes and store in the freezer for a subsequent lab for analysis. Discard the rest of the precipitate.
6. Pour the 70% supernatant through glass wool in a glass funnel to remove any unwanted solids and lipid material.
7. Remove 1 mL of 70% supernatant into a 1.5 mL plastic microcentrifuge tube and store in the freezer for a subsequent lab for analysis.
8. Saturate the rest of the supernatant to 100% AS and allow your solution to sit overnight at 4 °C (we’ll let it sit all week). At 100% AS, parvalbumin and all other acidic proteins will be forced to precipitate and the AS will concentrate the solution.
9. Next Week: Centrifuge the 100% AS salt saturated mixture at 12,000 RPM for 30 minutes at 4 °C. Dissolve the pellet (containing the parvalbumin) in a minimal amount of cold water (try 1 mL, add up to 5 mL) to remove it from the tube. The extract is now ready for dialysis.

**Questions/Analysis**
Tabulate the recorded values including the mass of the fish, mass of the white muscle, volume of the total water used for extraction, volume of the total supernatant recovered, and volume used for DNA extraction. Do not round units from the balance; appropriately report the volumes with the correct significant figures for the measuring tool employed.
Determine the relative centrifugal force (xg) value for 12,000 RPM at 4 °C in Beckman J-21C centrifuge using the JA-14 rotor and your measurements of the radial distance used from [http://www.beckman.com/resourcecenter/labresources/centrifuges/rotorcalc.asp](http://www.beckman.com/resourcecenter/labresources/centrifuges/rotorcalc.asp) State the fraction containing the parvalbumin protein at the completion of this lab. Determine the % of protein recovered by mass by dividing the mass of white meat by the mass of the total fish and multiplying by 100%. Tabulate the volume or mass of the ammonium sulfate added, %AS, and volume of supernatant and mass of the precipitate. Which fraction contained the parvalbumin protein at the completion of the lab?
9 Buffer exchange by dialysis

Objective
To learn how to use dialysis tubing to exchange buffers, concentrate protein, and remove ammonium sulfate.

Safety
Be careful not to puncture the dialysis tubing. Prepare the tubing in the hood by boiling in acetic acid. Clean up all spills immediately. Wear gloves while handling the dialysis tubing.

Background
At this point your protein is in a solution with a high (100%) concentration of ammonium sulfate (AS) salt. After a protein has been precipitated by ammonium sulfate and redissolved in buffer or water at a much greater protein concentration than before precipitation, the solution will contain a lot of residual ammonium sulfate which was bound to the protein. One way to remove this excess salt is to dialyze the protein against a buffer low in salt concentration. The excess and ammonium sulfate salt must be removed because it will interfere with the process of the gel filtration chromatography, the next purification step. In addition, a different buffer is needed for the gel filtration chromatography step. We will perform this method again prior to the anion exchange chromatography step as well to change the buffer for that column. There are several methods that can be used for desalting, concentration, and/or buffer exchange. We will use dialysis, because it is relatively simple to set up and not time-consuming in person-hours and our sample size is small.

Dialysis tubing is a semi-permeable membrane available in a wide range of size dimensions and pore sizes (molecular weight cut-offs). Our procedure uses dialysis tubing with a 6,000-8,000 Dalton molecular weight cut-off as the parvalbumin is expected to be 12 kDa, larger than the pores of the tubing so it should remain in the tubing throughout the process. Our extract (~1-5 mL) is placed inside the tubing and the ends are sealed off. We will use knots and clamps. The tubing is then suspended in a large volume of the desired buffer solution (e.g. 2 L or more). Thus, the protein amounts to about 0.25% of the buffer total. The pores in the membrane allow molecules that are smaller than the pores to move freely across the membrane down the concentration gradient. Therefore, the ammonium sulfate ions will cross out of the tubing into the buffer until an equilibrium concentration is reached inside and outside of the dialysis tubing. Eventually, the concentration of ammonium sulfate is equal inside and outside of the tubing. However since the volume outside the tubing is much greater than inside, and this outside volume is replaced with fresh buffer for a total of 3 exchanges, over time most of the salt will leave the tubing (Figs. 9.1 and 9.2). Larger molecules (such as most proteins and those higher than the molecular weight cut-off) are retained within the membrane. Since most buffer components are small molecules and can therefore pass through the pores, dialysis is also used as a method for changing buffers. It can be used to clear the ammonium sulfate and exchange to a new buffer simultaneously.
Other methods for desalting and buffer exchange include gel filtration chromatography because it separates molecules on the basis of size. Smaller molecules can move inside the gel beads and therefore progress more slowly through the column than larger molecules, such as proteins, which remain outside of the gel beads. If the column has been equilibrated with the new buffer, the protein will elute in the new buffer, leaving
the salts from the old buffer in the column. Other methods of concentrating proteins include using Millipore Centricon spin filter columns and lyophilization.

**Materials**  
- 6,000-8,000 MW cut-off dialysis tubing from Spectra/Por  
- 2 L graduated cylinders  
- stir plates  
- stir bars  
- clamps  
- parafilm  
- pipets & tips  
- 3% acetic acid

**Procedure:**  
**Part A: Dialysis of AS fractions**  
1. Record the volume of your 100% AS precipitate. Determine how much dialysis tubing you will need to hold your sample and cut a piece that has an extra full-length. Always handle dialysis tubing with gloves.  
2. Wash your dialysis tubing. Place the tubing in a beaker with water. Place the beaker in the cold and stir for at least 2 hours. Boil tubing in 2 liters of 3% acetic acid for approximately 5 minutes. Then rinse tubing and boil in 2 liters of deionized water for approximately 5 minutes. Rinse the dialysis tubing again with water. This will remove impurities, such as antifungal and antibacterial agents with which the tubing has been treated. Never let your dialysis tubing dry out once it has been wetted.  
3. Prepare 2 L of 0.05 M ammonium bicarbonate buffer, pH = 7.8 from solid ammonium bicarbonate.  
3. When washing and preparations are completed, remove the water carefully from the tubing. Knot the dialysis tubing at one end. Clasp the bottom of the tubing with a dialysis clasp. Test the tubing by pouring water in to check for punctures.  
4. Carefully pipette your sample into the dialysis tubing, being careful not to puncture the tubing. Also, don't spill your sample! Make a knot in the top, clasp the top, and, if desired, attach a string to the top of the tubing.  
5. Place the tubing in a beaker or graduated cylinder containing approximately 2000 ml of 0.05 M ammonium bicarbonate buffer, pH = 7.8 (to be used for the G-75 column in the next step) and tape the string to the side of the container.  
6. Place the beaker or flask on a stir plate and stir at least two hours (we’ll leave our samples overnight) in the cold room or fridge.  
7. Change the buffer after allowing at least another two hours of stirring. Repeat this process for a total of 3 times over at least a 24 hour period. Ideally, you should not take longer than 2 days to accomplish three changes; our protein will sit until next week.  
8. Next Week: Carefully remove the protein from the tubing with a pipette and squeeze out as much as possible of whatever is left into a centrifuge tube.  
9. Centrifuge the recovered protein at 10,000 RPM for 10 minutes to remove any insoluble material and carefully separate the supernatant from the pellet. Alternatively, filter the protein through glass wool or cheesecloth to rid impurities.  
10. Record the volume of the supernatant from centrifugation or filtrate. The supernatant/filtrate will be stored in the refrigerator for future labs.
Questions/Analysis
Make a table of the initial and final recovered volumes of 100% AS protein before and after dialysis. Comment on your observations about the volumes.
Why did the protein remain in the dialysis tubing?
What was the purpose of dialysis?

References
The Biotechnology Project Website,
http://matcmadison.edu/biotech/resources/proteins/labManual/chapter_4/section4_3.htm
10 Separation of blue dextran and protein with gel filtration chromatography

Objective
To learn how to pour a column for chromatography and separate proteins or buffers using gel filtration chromatography.

Safety
Be careful not to let the top of the column (column bed) run dry. Do not ingest the chemicals. Wash your hands before leaving lab.

Background
Gel filtration separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. The system contains two phases, one stationary and one mobile. The stationary phase usually consists of a cross-linked polysaccharide which forms porous beads. The mobile phase normally consists of a buffer. The separation depends on the ability of molecules to enter the pores of the stationary phase. Smaller molecules can diffuse into the beads and move more slowly down the column. Molecules are therefore eluted in order of decreasing molecular size. By varying the degree of cross-linking, the gels are optimized for different molecular weight ranges (Fig. 10.1).

Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree of separation between peaks). Consequently, a significant advantage of gel filtration is that conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation. Gel filtration is well suited for biomolecules that may be sensitive to changes in pH, concentration of metal ions or co-factors and harsh environmental conditions. Separations can be performed in the presence of essential ions or cofactors, detergents, urea, guanidine hydrochloride, at high or low ionic strength, at 37 °C or in the cold room/fridge according to the requirements of the experiment. Purified proteins can be collected in any chosen buffer. A buffer and pH that are compatible with protein stability and activity should be selected. Use a buffer concentration that is sufficient to maintain buffering capacity and constant pH. Gel filtration media come in various sizes and so a medium with a suitable fractionation range for your sample should be selected (your protein should fall in the middle of this range). The gel material we will use is G-75, a cross-linked dextran. G-75 separates molecules in the 3-80 kDa range (Fig. 10.2). 1 gram of this material will swell to 12-15 mL. The bead diameter is 40-120 µm. The columns we will use have a volume of ~50 mL. The buffer we will use is NH₄HCO₃ (pH 7.8).
Fig. 10.1 Column chromatography. Using G-75 Sephadex for size fractionation, the high molecular weight proteins (red) will elute before the low molecular weight proteins (green). http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=mboc4&part=A1618

Fig. 10.2 Gel filtration media
http://www6.gelifesciences.com/aptrix/uppt01077.nsf/Content/Products?OpenDocument&ModuleId=166177

TECHNICAL SPECIFICATIONS

<table>
<thead>
<tr>
<th>Sephadex</th>
<th>Sephadex</th>
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</thead>
<tbody>
<tr>
<td>Composition</td>
<td>cross-linked dextran</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle size</td>
<td>dry, min. 85% volume share between 40-120 µm</td>
<td>dry, min. 90% volume share between 100-300 µm, wet (in 0.15 M NaCl), 75-510 µm</td>
<td>dry, min. 90% volume share between 50-150 µm</td>
<td>dry, min. 80% volume share between 20-80 µm, wet (in 0.15 M NaCl), 35-210 µm</td>
<td>dry, min. 80% volume share between 20-50 µm, wet (in 0.15 M NaCl), 20-125 µm</td>
<td>dry, min. 80% volume share between 40-120 µm, wet (in 0.15 M NaCl), 35-310 µm</td>
<td>dry, min. 80% volume share between 20-50 µm, wet (in 0.15 M NaCl), 20-155 µm</td>
<td></td>
</tr>
<tr>
<td>Fractionation range, globular proteins</td>
<td>up to 700</td>
<td>1.5 × 10³</td>
<td>1.5 × 10³ - 3 × 10⁴</td>
<td>3 × 10⁴ - 7 × 10⁶</td>
<td></td>
<td></td>
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<tr>
<td>pH stability (operational)</td>
<td>2-13</td>
<td>2-10</td>
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<tr>
<td>CIP stability (short term)</td>
<td>2-13</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Pressure/flow spec</td>
<td>24-49 cm/h, pressure drop cm H₂O/bed height = 2, bed height 30 cm, 2.6 cm i.d.</td>
<td>20-47 cm/h, pressure drop cm H₂O/bed height = 2, bed height 30 cm, 2.6 cm i.d.</td>
<td>min 400 cm/h, pressure drop cm H₂O/bed height=3, bed height 30 cm, column 5 cm i.d.</td>
<td>Uₘₐₓ min 150 cm/h, bed height 10 cm, column 5 cm i.d.</td>
<td>min 60 cm/h, pressure drop cm H₂O/bed height=15, bed height 10 cm, column 5 cm i.d.</td>
<td>Uₘₐₓ min 90 cm/h, bed height 10 cm, column 5 cm i.d.</td>
<td>Uₘₐₓ min 11 cm/h, bed height 10 cm, column 5 cm i.d.</td>
<td></td>
</tr>
</tbody>
</table>
A few parameters for column chromatography are listed below. For group separations sample volumes up to 30% of the total column volume can be applied. For high resolution fractionation a sample volume from 0.5–4% of the total column volume is recommended, depending on the type of medium used. For most applications the sample volume should not exceed 2% to achieve maximum resolution. Depending on the nature of the specific sample, it may be possible to load larger sample volumes, particularly if the peaks of interest are well resolved. This can only be determined by experimentation. For analytical separations and separations of complex samples, start with a sample volume of 0.5% of the total column volume. Sample volumes less than 0.5% do not normally improve resolution. If you are unsure what your separation profile will look like, it is best to first perform an analytical separation and scale up to a preparative separation once it is well-established that your protein elutes in a unique region of the profile. To increase the capacity of a gel filtration separation samples can be concentrated. However concentrations above 70 mg/mL protein should be avoided as viscosity effects may interfere with the separation. A sample G-75 column chromatography separation profile from fractionating the 70-100% ammonium sulfate Rainbow trout proteins is shown in Fig.10.3; 2 mL of protein solution was added (4% of column volume).

Fig. 10.3 A sample G-75 column chromatography separation profile from fractionating the 70-100% ammonium sulfate Rainbow trout proteins.
You may also determine the native molecular weight of a protein by this method since there is a linear correlation between the elution volume of proteins and the logarithms of their molecular weights (MW) if known molecular weight standard proteins are run on the same column under the same conditions.

The result from a gel filtration experiment is often plotted as the variation of substances eluted as a function of the elution volume, $V_e$ (see figure below). $V_e$ is however not the only parameter needed to describe the behaviour of a substance since this also is determined by the total volume of the column and from how it was packed.

By analogy with other types of partition chromatography the elution of a solute may be characterized by a distribution coefficient ($K_d$). $K_d$ is calculated for a given molecular type and represents the fraction of the stationary phase that is available for the substance. In practice $K_d$ is difficult to determine and it is usually replaced by $K_{av}$ since there is a constant relationship between $K_{av}$: $K_d$. $K_{av}$ is obtained from

\[
V_t = V_o + V_p
\]

$V_{total} > V_T$, because the gel occupies some space.

\[
K_{av} = (V_e-V_0)/(V_t-V_0); \quad V_t-V_0 = V_p
\]

$K_d = partition \ or \ distribution \ coefficient = (V_e-V_0)/V_p$

The total volume of the column ($V_t$) is simply calculated from $p \times r^2 \times h$ and the void volume ($V_0$) is determined by passing a large substance that does not interact with the beads (like blue dextran, Appendix) through the column.

where

- $V_e = \text{elution volume}$
- $V_t = \text{total column volume}$
- $V_0 = \text{void volume}$
- $V_p = \text{pore volume}$

Gel filtration chromatography can be used for purification and it may also be used to estimate the native (non-denatured) molecular weight of a protein. A plot of $K_d$ or $K_{av}$ versus log $M_r$ (molecular weight) should yield a straight line (Fig. 10.4). The molecular weight of the “high molecular weight” protein can then be determined from its elution volume, together with this plot.
Fig. 10.4 A plot of log MW vs. Kav for 70-100% ammonium sulfate Rainbow trout proteins and blue dextran from the G-75 column separation.

![Graph showing Kav versus log MW](image)

y = -0.225x + 0.7429
\[ R^2 = 1 \]

**Materials**
- Ammonium bicarbonate salt
- 6 plastic columns
- Tygon tubing
- ring stands and clamps
- 5 Erlenmeyer flasks as reservoirs
- 5-250 mL beakers
- 50 g G-75 Sephadex in ammonium bicarbonate, pH 7.8
- Blue dextran solution (2 mg/mL in ammonium bicarbonate buffer) (a column void volume marker with the molecular weight of 2 million Daltons)
- 5 graduated cylinders
- 100% AS protein mixture (at least 3 mg/mL concentration recommended)
- numbered test tubes in racks

**Procedure:**

**Part A: Preparing the G-75 Beads**
1. Prepare 2 L of 0.050 M ammonium bicarbonate buffer, pH 7.8 buffer from the solid salt (1x buffer).
2. Heat 150 mL of the Sephadex G-75 slurry (in 0.05 M ammonium bicarbonate buffer, pH = 7.8) to near boiling in an Erlenmeyer flask. Allow to cool.
3. Remove the clear supernatant and floating fine particles by aspiration.

**Part B: Pouring the G-75 Column**
1. Transfer about 100 mL of swollen G-75 resin from the large G-75 stock beaker to a small beaker (~250 mL).
2. Add an approximately equal volume of 0.05 M ammonium bicarbonate (NH_4HCO_3) buffer, pH = 7.8.
3. Set up a plastic column on a ring stand with 2 clamps. Close the clamp at the bottom of the column. Make sure the spigot is closed.
4. Fill the column with 0.05 M ammonium bicarbonate buffer about 1/3 of the way up from the bottom. Make sure the buffer is not flowing out of the bottom of your column.
5. Using a glass stirring rod, stir the G-75 slurry in your beaker.
6. Pour the slurry into your column (fill to the top).
7. Let the G-75 beads settle to the bottom of your column. Before the column completely settles, add more beads.
8. As clear buffer forms at the top of the column (4 cm), remove it with a Pasteur pipette and pipette bulb and discard it to a waste beaker.
9. Again, using a glass stirring rod, stir the G-75 slurry in your beaker and pour the slurry into your column (fill to the top).
10. When you have formed your “chromatographic bed” of approximately 4 cm, start your column flowing (about 1 drop every 5 seconds) to make more space.
11. Continue to remove clear buffer from the top of the column with the pipette and replenish with additional G-75 slurry. Continue this process until the column is packed with resin (leave 2-3 cm at the top of the column as space for the sample to be applied).
12. After the column is poured, run buffer through the column for about 10 minutes (or 2-3 column volumes). You can use a pipette for this process; but be sure that the bed does not go dry!
13. Set up a siphon with tygon tubing from a reservoir with buffer to continue this process. Secure the buffer reservoir on a clamp above the column.
14. Mark the top of the column bed with a Sharpie marker or grease pen. You will need a measurement of column volume for your calculations. You may calculate this number using the volume of a cylinder or measure the total column volume with water in the column after it is emptied at the end of the experiment.

Part C: Running the G-75 Column and Collecting the Fractions with Blue Dextran
1. After you have washed your column for at least 10 minutes with buffer, turn off the valve at the bottom of the column and remove the siphon.
2. Remove buffer to just above the surface of the bed. (BE CAREFUL NOT TO LET THE TOP OF THE COLUMN RUN DRY!)
3. Gently overlay 0.5 mL (or 0.5% of the geometric CV) of the 2 mg/mL Blue Dextran solution by applying the solution to the sides of the column (not directly to the bed) SLOWLY. The elution volume for Blue Dextran is equal to the column void volume (Vo). The rate of solubilization of the Blue Dextran may be increased by heating the buffer to 50°C before adding the Blue Dextran.
4. Immediately after applying the Blue Dextran solution, open the valve at the bottom of the column and begin collecting the effluent in test tubes.
5. After the Blue Dextran has totally entered the column, GENTLY layer buffer on top of the column.
6. Connect a reservoir of buffer on the top of the column and collect the effluent until the Blue Dextran starts to elute.
7. Measure the collected volume in a graduated cylinder (your void volume) and dispose.
8. After the Blue Dextran is completely eluted, continue to wash the column with 50 mL of buffer; then turn off the column flow.
9. The elution of Blue Dextran can also be conveniently monitored at 620 nm. Always calculate the void volume from the buffer prior to the first eluted (blue) peak from Blue Dextran.
Part D: Running the G-75 Column and Collecting the Fractions with Parvalbumin

1. You are now going to repeat the above process (Part C.) with your parvalbumin protein mixture (< 70 mg/mL) from the ammonium sulfate precipitation.
2. Remove the buffer from the top of the column bed.
3. Gently apply your protein mixture (0.5 mL-2 mL or 0.1%-4% but <30 % Vc) to the top of the column sides.
4. To get good resolution, the sample size should not exceed 2% of the geometric column volume, Vc. (Vc= r^2 × π × l where r is radius and l is column length).
5. Allow the protein to enter the column.
6. Open the valve and begin collecting 3.0 mL fractions in test tubes.
7. Gently apply buffer to the column bed and set up the siphon.
8. Allow approximately 200 mL of buffer to run through the column.
9. Measure the absorbance of your fractions using the Agilent 5493 UV-vis spectrophotometer at 260 nm and 280 nm. Save the absorbances and spectra. Use this to determine V_e, the elution volume for the high molecular weight (HMW) and low molecular weight (LMW) protein. Estimate the V_e by totaling the volumes in the tubes that precede these two peaks. Ask your instructor for assistance with this task. A sample of 0.5% of V_c will be diluted 5 to 15-fold during the run.
10. Save 1 mL of high molecular weight and low molecular protein for analysis later in 1.5 mL plastic microcentrifuge tubes.
11. Pool the fractions which constitute each of the peaks (after consulting with your instructor) and concentrate the low molecular weight protein by lyophilization. The buffer will sublime in the following decomposition reaction: NH_4HCO_3(s) → NH_3(g) + CO_2(g) + H_2O(g). The protein solid will be buffer-free and will no longer smell of ammonia. Alternatively, dialyze the low molecular weight protein against 0.015 M HCl-piperazine buffer, pH 5.70 in the cold room.
12. Dispose of the rest of the fractions and tubes.
13. Wash the column with 3 column volumes (150 mL) of 1x ammonium bicarbonate buffer.
14. Store the column in buffer at the temperature which it was poured.

Questions/Analysis
After how much buffer did the blue dextran (void volume), high molecular weight (elution volume 1), and low molecular weight (elution volume 2) elute? Plot an “elution profile” of the absorbance at 280 nm vs. fraction number. In what order did the molecules elute? Which substance(s) bound or did not bind to the G-75 resin? Calculate the Kav values for the standard proteins using the equation: Kav = (Ve – Vo)/(Vc – Vo) where Vo = column void volume, Ve = elution volume, and Vc = geometric column volume. Determine Ve (from the elution profile) and Kav (from the equation) for the each protein peak. Make a table of your elution/void volumes, known MW, log MW, and Kav values. Using the molecular weights of the protein and polysaccharide (parvalbumin, 12 kDa and blue dextran, 2000 kDa), construct a plot of Kav vs. log molecular weight either on semilogarithmic paper or with a calculation program and comment on the plot and compute the likely MW of the “high molecular weight” protein that eluted from the G-75 using the slope of the line.
11 Separation of low molecular weight protein isoforms using anion exchange chromatography

Objective
To learn how separate protein by charge using anion exchange chromatography.

Safety
Be careful not to let the top of the column (column bed) run dry. Do not ingest the chemicals. Wash your hands before leaving lab.

Background
The ability to reversibly bind molecules to immobilized charged groups is used in ion exchange chromatography (IEC) of which anion exchange chromatography is one example. Which type of charged group one selects (e.g. positive or negative) depends on the net charge of the protein which in turn depends on the pH. IEC is maybe the most commonly used technique today for the separation of macromolecules and is almost always included as one of the steps in the purification protocol. The experiment may be divided into four different parts.

1. Equilibration of the ion exchanger in a buffer in such a way that the molecule(s) of interest will bind in a desirable way (regeneration step).
2. a) Application of the sample. Solute molecules carrying the appropriate charge are bound reversibly to the gel.
   b) Unbound substances are washed out with the starting buffer (low ionic strength).
3. Elution with a salt gradient of increasing ionic strength (e.g. 0-2 M NaCl). This gradually increases the ionic strength and the molecules are eluted. The solute molecules are released from the column in the order of the strengths of their binding i.e. the weakly bound molecules elute first.
4. Substances that are very tightly bound are washed out with a concentrated salt solution and the column is regenerated to the starting conditions.

The buffer we will employ in this lab is 0.015 M piperazine-HCl, pH 5.70 (Fig. 11.1). A sample elution profile from anion exchange separation is given in Fig. 11.2.

Fig. 11.1 Piperazine

![Piperazine](attachment:fig111.png)
Fig. 11.2 A sample DEAE column chromatography elution profile for parvalbumin from the Silver Hake. Note the elution of three isoforms, A-C.

In this lab, anion exchange chromatography will be used to purify various parvalbumins from the low molecular weight pool by isoelectric point. The DEAE medium (diethylaminoethyl cellulose) (Fig. 11.3) is positively charged (amino group) and the proteins are negatively charged at pH 5.70. The most strongly held will be most acidic (lowest pI) and elute last.

Fig. 11.3 DEAE


Materials
• 6 L 0.015 M HCl-piperazine buffer, pH 5.70
• 6 plastic columns
• Tygon tubing
• ring stands and clamps
• 10- Erlenmeyer flasks as reservoirs
• 5- 250 mL beakers
• 50 g regenerated DEAE in 0.015 M HCl-piperazine buffer, pH 5.70
• 5 graduated cylinders
• Dialyzed/concentrated low molecular weight protein mixture from G-75 (3 mg/mL concentration recommended)
• numbered test tubes in racks
• 0.99 meq/g DEAE cellulose

Procedure:
Part A: Preparing the DEAE Anion Exchange Beads using Sigma’s instructions (Appendix) (Instructor)

1. Suspend the resin in 5 column volumes (CV) of distilled water and allow to settle for 30-45 minutes.

2. Measure the volume of the settled resin. This is the CV for the washing solution
volumes.
3. Suspend the resin in 2 CV of 0.1 M NaOH containing 0.5 M NaCl for 10 min. (not
   longer than 30 min.) and pour the slurry into a Buchner funnel (3 CV) while
   applying a GENTLE suction, and allowing 1 CV buffer flow in 5 min. Continue
   pouring in slurry until all of the resin is added to the funnel. Continue washing
   with 2 more CV of 0.1 M NaOH-0.5 M NaCl.
4. Repeat step 3. with deionized or distilled water rather than 0.1 M NaOH-0.5 M
   NaCl.
5. Repeat step 3. with 0.1 M HCl rather than 0.1 M NaOH-0.5 M NaCl.
6. Repeat step 3. with deionized or distilled water rather than 0.1 M NaOH-0.5
   M NaCl.
7. Continue washing with 5-10 CV of deionized or distilled water until the pH
   of the effluent is ideally pH 5 or greater (pH can be slightly lower).
8. Filter resin with 5 CV of water.
9. Resuspend resin with 2 CV of 10x buffer (0.15 M HCl-piperazine, pH 5.70) and
   filter.
10. Resuspend resin with 5 CV of 1 x buffer (0.015 M HCl-piperazine, pH 5.70) and
    filter.
11. Resuspend resin with 2 CV of 1 x buffer (0.015 M HCl-piperazine, pH 5.70) and
    filter. The pH should be within 0.15 units of 1 x buffer. If not, repeat this step.

Part B: Pouring the DEAE Column
1. Transfer about 100 mL of swollen DEAE resin from the large DEAE stock beaker
   to a small beaker (~250 mL).
2. Add an approximately equal volume of 0.015 M HCl-piperazine buffer, pH 5.70.
3. Set up a plastic column on a ring stand with 2 clamps. Close the clamp at the
   bottom of the column. Make sure the spigot is closed.
4. Fill the column with 0.015 M HCl-piperazine buffer, pH 5.70 buffer about 1/3 of
   the way up from the bottom. Make sure the buffer is not flowing out of the bottom
   of your column.
5. Using a glass stirring rod, stir the DEAE slurry in your beaker.
6. Pour the slurry into your column (fill to the top).
7. Let the DEAE beads settle to the bottom of your column.
8. As clear buffer forms at the top of the column (4 cm), remove it with a Pasteur
   pipette and pipette bulb and discard it to a waste beaker.
9. Again, using a glass stirring rod, stir the DEAE slurry in your beaker and pour the
   slurry into your column (fill to the top).
10. When you have formed your “chromatographic bed” of approximately 4 cm, start
    your column flowing (about 1 drop every 5 seconds).
11. Continue to remove clear buffer from the top of the column with the pipette and
    replenish with additional DEAE slurry. Continue this process until the column is
    packed with resin (leave 2-3 cm at the top of the column as space for the sample
    to be applied).
12. After the column is poured, run buffer through the column for about 10 minutes
    (or 2-3 column volumes). You can use a pipette for this process; but be sure that
    the bed does not go dry!
13. Set up a siphon with Tygon tubing from a reservoir with buffer to continue this
    process. Secure the buffer reservoir on a clamp above the column.
Part C: Running the DEAE Column with Low Molecular Weight Proteins from the G-75 Column and Collecting the Fractions

1. After you have washed your column for at least 10 minutes with buffer, turn off the valve at the bottom of the column and remove the siphon.
2. Remove buffer to just above the surface of the bed. (BE CAREFUL NOT TO LET THE TOP OF THE COLUMN RUN DRY!)
3. Gently overlay 0.5-2 mL of the low molecular weight, parvalbumin-containing fraction in 0.015 M HCl-piperazine buffer, pH 5.70 by applying the solution to the sides of the column (not directly to the bed) SLOWLY. A concentrated sample will work best.
4. Immediately after applying the protein solution, open the valve at the bottom of the column and begin collecting the effluent in test tubes.
5. After the protein has totally entered the column, GENTLY layer buffer on top of the column.
15. Connect a reservoir of buffer on the top of the column to set up the siphon. This time, you need a second (distal) reservoir containing 0.015 M HCl-piperazine buffer, pH 5.70 and 0.2 M NaCl, running into the first (proximal) reservoir with piperazine buffer, to create a 0-0.2 M salt gradient to elute the proteins. The buffers can be contained in Erlenmeyer flasks and connected by Tygon tubing.
16. Open the valve and begin collecting 3.0 mL fractions in test tubes.
17. Allow approximately 200 mL of total buffer to run through the column. All proteins should elute by four column volumes at the most (~70 tubes).
18. Measure the absorbance of your fractions using the Agilent 8453 UV-vis spectrophotometer at 260 and 280 nm and save your data. One group can use the fluorimeter for this purpose exciting at 260 nm and emitting at 290 nm.
19. Consult with your instructor about which tubes should be included in the fractions. Pool the fractions which constitute each of the peaks or isoforms and store them in the freezer in separate tubes. Discard the rest of the tubes.

Questions/Analysis
Plot an “elution profile” of the absorbance at 280 nm vs. fraction number.
In what order did the molecules elute?
Which substance(s) bound or did not bind to the ion exchange resin?
Which of the eluted proteins is the most/least acidic?
Imagine that you had used a buffer at pH 3.0 when you did the ion exchange experiment. How would that influence the elution pattern of the proteins?
Could these molecules have been separated by some other methods? Suggest one.
12 Modeling protein structure-function relationships and engineering mutants in parvalbumin

Objective
To learn how to use a free-ware computer program, DeepView, to visualize parvalbumin protein structures from the Protein DataBank and interpret structure-function relationships.

Safety
No special safety concerns. This is a computer lab.

Background
For this laboratory, we will use the free-ware computational modeling program, DeepView, formerly known as Swiss pdb Viewer (spdbv). You can download it at home via: http://swissmodel.expasy.org/spdbv/

In this lab, we will use spdbv to view and model parvalbumin protein structures in silico, but this program can also be used to view other biological macromolecules, including DNA, carbohydrates and lipids. Protein structures are calculated from data from NMR (nuclear magnetic resonance), x-ray crystallography, or cryo-electron microscopy experiments. From these experiments, the x, y, and z coordinates for all the atoms are calculated. The coordinates are generally published in the form of a scientific research paper and deposited into a public databank called the Protein Databank (http://www.rcsb.org/pdb/). The structures available to look at today include parvalbumin proteins including apo- and native α and β parvalbumins and calcium-ion binding site mutants. The first parvalbumin structure was solved almost 20 years ago and reported in 1990. All were all downloaded from the Protein Databank. Structures of many other Ca^{2+}-loaded parvalbumins, fragments, and other calcium-ion binding proteins have been solved by x-ray crystallography and NMR spectroscopy. Two structures were solved for the apo form of parvalbumin by NMR spectroscopy only recently (2007 & 2008), significant since the apo form has eluded structure determination for nearly 20 years.

Subset of parvalbumin structures:
1bu3.pdb: β parvalbumin from silver hake
1rk9.pdb: α parvalbumin
1b8i.pdb: D51A/E101D/F102W Triple Mutant of β carp parvalbumin
1s3p.pdb: rat α-parvalbumin S55D/E59D mutant
1xvj.pdb: rat α-parvalbumin D94S/G98E mutant
2jww.pdb: apo (calcium free) rat α-parvalbumin
2nln.pdb: apo (calcium free) rat β-parvalbumin

Swiss-PdbViewer (DeepView) is an application that provides a user friendly interface that can be used to analyze several proteins at the same time. The proteins can be superimposed in order to deduce structural alignments and compare their active sites or any other relevant parts. Amino acid mutations, H-bonds, angles and distances between atoms are easy to evaluate. DeepView can also be used to look at surfaces of the molecules or view the molecules in space-filling form.
DeepView also has an interface to Swiss-Model, an automated homology modeling server. Working with these two programs, it is possible to thread a protein primary amino acid sequence (such as the parvalbumin we are currently investigating once we have the sequence) onto a 3D template to make a potential model based on sequence or structural similarity. (This is the focus of Laboratory 15). The program can also analyze missing loops and sidechain packing. DeepView reads electron density maps from x-ray crystallography data sets. It also uses a popular energy minimization package (GROMOS) for energy minimization calculations to find the lowest energy conformation for sidechain atoms (or mutations) in vacuo (much faster than computing in solvent).

The average EF-hand is 29 amino acid residues in length. It is suspected that parvalbumin, containing three EF-hands, arose from intra-gene duplication of this EF-hand domain. In the EF-hand, six of 12 sequential residues (1, 3, 5, 7, 9 and 12) in the Ca$^{2+}$ ion binding loop region between the two $\alpha$-helices are responsible for cation binding. The cation is coordinated by the oxygen atoms at the axial positions x, y, z, -y, -x, -z in a Cartesian coordinate system (Kretsinger and Nockolds, 1973). The residues form a pentagonal bipyramid requiring seven ligands. The first five ligands contribute one ligand to the binding site. In all EF-hand proteins, there is a bidentate carboxyl group at -z, consistent with a geometry best described as a "skewed pentagonal bipyramid" whose axis is x to -x. In parvalbumins, the EF-hands are labeled AB, CD, and EF, consecutively. In parvalbumin, the -x position is filled by glutamic acid that contributes two carboxylate oxygen ligands to the binding site. The ligands mainly consist of negatively charged aspartic and glutamic acid side chain oxygen atoms, though some of the residues are typically main chain carbonyl oxygen atoms or an oxygen atom from a ligating water molecule. In most of the EF-hand proteins, but not the CD site of parvalbumins, there is a coordinating water molecule at -x. In parvalbumins, the ninth ligand in the CD EF-hand is a conserved glutamic acid residue, whereas in the EF EF-hand, a solvent water molecule forming a hydrogen bond with a glycine forms the ninth ligand at -x.

Molecular mechanics calculations on the calcium bound and free forms of parvalbumin and isolated EF-hand fragments provide interesting insights on the energetic changes in the EF-hand upon calcium release. In this lab, we will view the structures of known parvalbumins and mutants and then probe the results of mutations at the CD and EF calcium-ion binding sites in the whole protein (and in isolated, cleaved EF-hands, time-permitting).

Materials
• computer with an internet connection
• downloaded program and structure files
  • DeepView at http://spdbv.vital-it.ch/
  • Structure files at http://www.rcsb.org/
• Biochemistry textbook

Procedure:
Part A: Visualization of Protein Structures for Determining Protein Structure-Function Relationships: An Introduction to DeepView
(SHORT CUT to highlight entire column in control panel window = Shift+Click)
1. Double left click on the spdbv/DeepView icon to start the program. An excellent tutorial that extends beyond the scope of this lab is available online at http://spdbv.vital-it.ch/TheMolecularLevel/SPVTut/

2. Click the red X box to close the “About Swiss-Pdb Viewer” window.

3. Download structure files from the Protein Databank (http://www.rcsb.org/pdb/home/home.do). Click on File- Open pdb file to open one of the files (e.g. 1BU3.pdb). This will allow you to view a pdb structure. Close any loading progress windows.

4. Open 1BU3.pdb first from the Biochemistry pdb folder on the Desktop. You will see a molecule appear in the window. Click close to close the general communication window. Then close the load window. You are looking at the carbon trace (bonds) of the primary structure folded into the tertiary structure. The red +’s are water molecules (red for oxygen). Oxygen atoms are red, hydrogen is light blue, carbon is white, sulfur is yellow, and nitrogen is dark blue. The color can help you to determine if you are viewing backbone or sidechain or which sidechain.

5. Click window – Control panel and Window- Layers infos to turn on these windows. All of the windows are independently mobile and can be rearranged on the screen. If you scroll down on the right in the control panel, you can see the components of the structure. The three-letter names represent the names of amino acids with and CA (Look in Text for Blue highlighted words) represents the metal ion, calcium, non-protein cofactor part of the structure.

6. In the Control Panel, clicking in the show column will turn an amino residue on or off for viewing. To turn all atoms on/off at the same time, hold shift and click in the desired column. Click on the side column to control just the sidechain. Labl turns on the sidechain labels, dot-dot v turns on an electrostatic surface, and ribn turns on a ribbon structure. Try them out as below.

7. Click on label to label the residues.

8. Click on v to show an electrostatic surface.

9. Click on ribbon to view the protein in a ribbon depiction.

10. Click on ribbon color to control the color (BS box).

11. Click on BS to change what you are manipulating- backbone, backbone and sidechains, sidechains, ribbon, label, or surface.

12. Clicking in vis of Layers Infos turns the molecule display on or off. Clicking on mov allows the molecule to move or not move.

13. Clicking on the other options (H, C, O etc.) turns these displays on and off.

14. Now, let’s move to the main icon line. From left to right: left clicking the sidechain box (top-left of the main window) centers the molecule, clicking on the hand moves the molecule vertically and horizontally, clicking on the box zooms, and clicking on the circular arrow rotates the molecule. The molecule is viewed as in 3D.

15. Clicking on the 1.5A box allows you to pick two atoms with the mouse (left click) and measure the distance between them.

16. To visualize the molecule in 3D, shift-click on the dot-dot v column in the Control Panel and then select from the pull-down menus Display-Use Open G/L rendering and Render in solid 3-D.

Part B: Questions about the structures
1. Display the 1BU3.pdb molecule with the ribbon on to evaluate the structure and geometry in the protein.
   a. What type of secondary structure does this protein have? (e.g. alpha, beta, loops)
   b. Rotate the molecule. How are the helices (A, B, C, D, E, and F) related geometrically to each other?
2. Select Color- Accessibility to view the surface and core of the protein.
   a. What does this do to the protein? Where is this protein likely to be in the cell? (e.g. cytosol or membrane)
   b. What types of sidechains project from the surface of parvalbumin? (list some by residue name and number)
   c. What types of side chains are located on the inner faces of these helices and contribute to the core of the protein structure? (list some by residue name and number)
3. How long is the molecule?
   a. Clicking on Leu41 box in the main panel along the top of the program identifies whichever atom you pick and which sidechain it belongs to.
   b. Then select the 1.5 Å button on the main window and pick two atoms far apart consecutively for the measurement.
4. Determine the ligands for the two calcium ions and distance of each to the calcium ions.
   a. Determine the ligands that bind each calcium ion.
   b. Then select the 1.5 Å button on the main window, pick a calcium ion, then pick the second calcium ion to measure the distance between the two calcium ions.
5. Scroll down the Control panel and locate all of the Phe, Trp, and Tyr residues. Identify if they are on the surface or buried.
6. Use tools H-bonds to compute the number of hydrogen bonds.
   a. How many do you see? (e.g. give number or too many to count)
   b. Which sidechains are involved in the hydrogen bonding? (give an example of the atoms) If you toggle them on and off in the Layers infos window, they may be easier to see.
7. Close this structure by going to File –Close.
8. Compare the 1BU3.pdb molecule to an apo form of parvalbumin (e.g. 2NLN.pdb) by doing steps 1.-7. for that protein.
9. Continue to compare structures of mutant proteins and proteins of the two lineages as time permits.

Part C: Molecular mechanics calculations to evaluate parvalbumin mutants
1. For the following calculations, use as your starting structure the “A” chain (only chain in this structure) of 1BU3 solvated (or unsolvated) by the crystallographic water molecules. As the parameters for the GROMOS forcefield as implemented in DeepView do not differentiate between similarly charged metals, experiments in which the metal ion is varied cannot be executed. GROMOS also computes in a vacuum so the presence of the water is arbitrary.
2. Treat the protonation state of ionizable groups at neutral pH (7).
3. Select Preferences-Energy minimization preferences. Enter 100 steps of steepest descents and 100 steps of conjugate gradients.
4. For the native protein, select all of the residues in the Control window by holding the left mouse button down and scrolling down the column.
5. Select Tools-Energy minimization. When the report is printed on the screen, scroll to the bottom and record the total energy in kJ/mol.
6. Then mutate the desired amino acid residue using the mutate button in the main icons. Mutating is a method of testing if activity or calcium binding is knocked-out. Bound calcium should be found within 2-3 angstroms of a carboxylate ligand. Alternatively, delete the calcium ions in the text file (open in textedit) to delete to create the apo form.
7. Repeat steps 4.-5.
8. Compare the results of additional single (and multiple, if desired) point mutations using the “mutate” option. Select the most stable rotamer for each mutation. Reject mutants that were unable to be in a suitable orientation, that is to say “facing” the ligand, or had a distance greater than 4.00 angstroms.
9. Repeat for a total of 3 mutations.

Questions/Analysis
Compare the two (or more) parvalbumin structures and mutants you evaluated by creating a table to answer the questions in the procedure and repeated below. What types of sidechains project from the surface of parvalbumin? What types of side chains are located on the inner faces of these helices and contribute to the core of the protein structure? Where in the cell would you expect to find this protein? Where are the Trp, Tyr, and Phe aromatic residues located? How many of each are there in the structure(s) you looked at? How are the helices A, B, C, D, E, and F geometrically related to each other? What is the distance between the Ca\(^{2+}\) ions? What are the calcium ion binding ligands (by residue name and number) in each structure at the six coordinating positions? List the calcium to ligand distances for each. Tabulate the wild type and mutant energies by mutant. Provide an analysis of your results. Compare and contrast the structures of the apo and calcium-bound parvalbumin structures.

References

The Protein DataBank, rscb.org
13 Protein characterization and quantification by UV-vis spectroscopy

Objective
To learn how to characterize and quantitatively determine the concentrations of the initial protein from the homogenate, dialysis, G-75 and DEAE fractions from the purification scheme using UV-vis spectroscopy.

Safety
Be careful not to spill solutions in the UV-vis spectrophotometer. Handle the quartz cuvette carefully. Clean up all spills immediately.

Background
Both proteins and DNA have substantial absorbance in the UV region of the electromagnetic spectrum. UV absorbance is a commonly used method for determining concentrations and purity of protein or DNA although other methods are more sensitive. The Bradford method of determining protein concentration involves the production of standards and comparison of the standards and unknown protein solution with Coomassie Brilliant Blue G. Other methods of determining protein concentration include: modified Lowry, Biuret, and the Bicinchoninic acid methods.

Even so, proteins can be quantified quickly using UV-vis spectroscopy. Light has a dual nature; meaning it has both wave and particle-like characteristics. As a wave, light has an associated wavelength ($\lambda$) and frequency ($\nu$). These two properties are related to each other by the following mathematical relationship:

$$c = \lambda \nu,$$

where $c$ is the speed of light in a vacuum ($2.998 \times 10^8$ m/s) and $\lambda$ has units of meters (m) and $\nu$ has units of 1/seconds.

A particle of light is called a photon. A photon has a certain quantized amount of energy (E) associated with it. This relationship is given by the following mathematical equation:

$$E = h \nu \quad \text{or} \quad E = \frac{hc}{\lambda},$$

The symbol, $h$, is Planck's constant which has a value of $6.626 \times 10^{-34}$ J*s.

Spectrophotometry is based on the interaction of matter with radiation (Fig. 13.1). A beam of radiation is composed of a series of waves, each having a specific wavelength.

According to quantum theory, a molecule or atom may not possess or absorb any arbitrary quantity of energy. Rather, matter can only exist in certain permitted states of energy. If a molecule or atom absorbs energy in the form of radiation, it will be raised to a higher energy level. Each photon absorbed has a specific wavelength associated with it. When a beam of polychromatic radiation or radiation having many wavelengths is passed through a sample, two things happen. Some energy is absorbed and some of the energy will pass through the sample without being absorbed. An irradiated sample will absorb energy whose wavelengths correspond with appropriate or allowed molecular or atomic energy transitions. Energy whose photon's wavelengths are not permitted will simply be transmitted.
An instrument called a spectrophotometer makes absorbance measurements (Fig. 13.1). Light emanating from the spectrophotometer's lamp passes through an entrance slit and is dispersed by a diffraction grating. The spectrum of light produced by the diffraction grating falls on a dark screen having a slit. Only the portion of the spectrum which falls on the slit will go through and into the sample. Any part of the spectrum can be projected onto the slit by simply adjusting the diffraction grating. The light reaching, but not being absorbed by the sample, passes through to the phototube. The phototube measures the intensity of this light electronically. The phototube is capable of converting radiant energy into electrical current. The current is then used to operate a meter that indicates the amount of light the sample absorbs.

Two scales can express the amount of light absorbed by the sample: absorbance (A) and percentage of light transmitted (%T). The ratio of radiant power transmitted (P) by a sample to the radiant power incident (P₀) on the sample is called the transmittance, T:

\[ T = \frac{P}{P_0} \]

Absorbance and percent transmittance are inversely related. Percent transmittance (%T) can be converted to absorbance by the following mathematical relationship:

\[ A = 2 - \log(\%T) \quad \text{or} \quad A = -\log T \]

Every molecular and atomic species has a characteristic absorption spectrum. An absorption spectrum is obtained by plotting absorbance (A) as a function of wavelength (λ) (Fig. 13.2). An absorption spectrum is a chemical fingerprint and can be qualitatively used to identify molecular and atomic species present in a sample. Fig. 13.2 demonstrates the varying fingerprint absorbance spectra for amino acids phenylalanine, tyrosine, and tryptophan.
The spectral characteristics in the 240-300 nm range for a peptide or protein is related to its tryptophan (W), phenylalanine (F), tyrosine (Y) and cysteine (C) amino acid composition. Phenylalanine contributes predominantly to the 240-265 nm region (187.5 nm, 205 nm, 242 nm, 252 nm, 257 nm major peak, 263 nm, 267 nm) of the spectrum and the $\varepsilon = 195 \text{ M}^{-1}\text{cm}^{-1}$ at 257 nm. The absorption maxima for tryptophan is 280 nm with $\varepsilon = 5500 \text{ M}^{-1}\text{cm}^{-1}$ and this wavelength that for tyrosine is 274 nm with $\varepsilon = 1490 \text{ M}^{-1}\text{cm}^{-1}$ at this wavelength ($\varepsilon = 2340 \text{ M}^{-1}\text{cm}^{-1}$, 293 nm). Fig. 13.3 provides a summary of this information.

The spectral properties of tryptophan, a strong chromophore, can be used to gather structural information about the native protein and the conformational change that takes place in the transition from the native to apo state. A comparison of amino acid sequences of various parvalbumins reveals that at position 102 there is an invariant aromatic residue (phenylalanine or tryptophan), implying an important structural or functional role for this amino acid.
Fig. 13.3 Absorbance maxima for common protein chromophores
http://sbio.uct.ac.za/Sbio/documentation/ProbingProteinStructurebySpectroscopy.php

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>$\lambda_{\text{max}}$</th>
<th>Molar extinction coefficient ($\varepsilon$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>280</td>
<td>5600</td>
</tr>
<tr>
<td></td>
<td>219</td>
<td>47000</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>274</td>
<td>1400</td>
</tr>
<tr>
<td></td>
<td>222</td>
<td>8000</td>
</tr>
<tr>
<td></td>
<td>193</td>
<td>48000</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>257</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>206</td>
<td>9300</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>60000</td>
</tr>
<tr>
<td>Histidine</td>
<td>211</td>
<td>5900</td>
</tr>
<tr>
<td>Cysteine</td>
<td>250</td>
<td>300</td>
</tr>
</tbody>
</table>

In this laboratory experiment, you will determine characterize your extracted separated proteins from the homogenate, G-75 (Fig. 13.4), and DEAE column (Fig. 13.5) by UV-vis spectrophotometry. You will record an absorption spectrum plot for your extracted proteins from your DEAE column over a range of 240-300 nm. You may use this technique to characterize SDS or urea denatured proteins also.

Fig 13.4 Absorbance versus wavelength plot for 70-100% ammonium sulfate precipitated dialyzed protein from the Rainbow trout: UV-Vis spectra of high molecular weight proteins (top) and low molecular weight proteins (bottom) from G-75 column chromatography separation.
Absorption spectroscopy also has a quantitative application and we will use it in this lab to determine the concentration of the extracted protein solutions. If radiation of one wavelength (typically the $\lambda$ where absorbance is maximum represented by $\lambda_{\text{max}}$) is passed through a solution, then the quantity of light absorbed will be proportional to the concentration of the absorbing species in that solution. As the concentration of the solution increases, the amount of light absorbed will increase. The relationship between concentration ($c$) and absorbance ($A$) is linear and is known as Beer's Law. Beer's Law is given as:

$$A = abc$$  \hspace{1cm} \text{Equation 1}

The symbols $a$ and $b$ represent the solution's absorptivity coefficient or constant ($a$) and the path length that the light travels through the solution ($b$). Beer’s Law states that molar absorptivity is constant (and the absorbance is proportional to concentration) for a given substance dissolved in a given solute and measured at a given wavelength. For this reason, molar absorptivities are called molar absorption coefficients ($\varepsilon$) or molar extinction coefficients. Because transmittance and absorbance are unitless, the units for molar absorptivity must cancel with units of measure in concentration and light path.
Standard laboratory spectrophotometers are fitted for use with 1 cm-width sample cuvettes; hence, the path length is generally assumed to be equal to one. Absorbance does not have units. When \( b \) is in centimeters and \( c \) is in moles per liter, \( a \) or \( \varepsilon \) has the units \( L \text{ mol}^{-1} \text{cm}^{-1} \). The value of the molar absorptivity constant is unique for each absorbing species. Its value can be derived using Beer's Law or from a Beer's Law calibration curve. The molar absorptivity constant is the slope of the line in a Beer's Law calibration curve.

Beer's Law calibration curves are made experimentally by preparing a series of solutions, each with a known concentration of the absorbing species in an appropriate solvent. These solutions are referred to as standards (e.g. BSA). The absorbance of each solution is measured at the same wavelength and a calibration curve showing the relationship between the absorbance and the concentration of each standard is prepared. A typical Beer’s Law calibration curve is shown in Fig. 13.6. This calibration curve was prepared by plotting \( A \) (absorbance at \( \lambda_{\text{max}} \)) versus the concentration of the standard solutions. This plot can be used to determine the unknown concentration of a solution by measuring the absorbance of the solution at the same wavelength used to measure the absorbance of the standards. For example, if \( A \) (absorbance) of the unknown were found to be equal to 0.60, the concentration according to the calibration curve would be \( \sim 0.6 \text{ mg/mL} \). Note that the line passes through the origin.

**Fig. 13.6 Beer's Law calibration curve for Human albumin**

![Beer's Law Plot](image)

The molar absorption coefficient of a peptide or protein is related to its tryptophan (W), tyrosine (Y) and cysteine (C) amino acid composition. At 280 nm, this value is approximated by the weighted sum of the 280 nm molar absorption coefficients of these three constituent amino acids, as described in the following equation which can be used if the numbers of the amino acids are known:

\[
\varepsilon = (nW \times 5500) + (nY \times 1490) + (nC \times 125) \quad \text{Equation 2}
\]
where \( n \) is the number of each residue and the stated values are the amino acid molar absorptivities at 280 nm. Dividing the measured absorbance of a peptide or protein solution by the calculated or known molar extinction coefficient yields the molar concentration of the peptide or protein solution. The peptide or protein amino acid composition must be known to calculate the molar extinction coefficient. However, using the absorbance at 260 nm and 280 nm, the ratio of the absorbances can be determined to assay the protein quality and get a quick measurement of concentration. Even minor differences in buffer type, ionic strength and pH affects absorptivity values at least slightly. Most protein preparations, even those of equal purity, differ slightly in conformation and extent of modifications, such as oxidation, and these also affect absorptivity. Therefore, the best extinction coefficient value is one that is determined empirically using a solution of the study protein of known concentration dissolved in the same buffer as the sample. Correct measurement is only possible when the protein is free of buffers and other molecules that absorb at these wavelengths and readings are at values between 0.1 and 1 absorption units. Protein preparations should be vortexed shortly and diluted accordingly using 10 mM Tris-HCl or water.

For calculating the concentration of proteins of unknown sequence with possible nucleic acid contamination quickly, use the following formula:

\[
\text{Concentration (mg/ml)} = (1.55 \times A_{280}) - (0.76 \times A_{260}) \quad \text{Equation 3}
\]

Total protein yield (mg) = protein concentration (mg/mL) x sample volume (mL)

A standard procedure of measuring protein quality is the determination of the absorption quotient (Q) of readings at \( A_{260nm} \) and \( A_{280nm} \):

\[
Q = \frac{(A_{260nm} - A_{320nm})}{(A_{280nm} - A_{320nm})} \quad \text{Equation 4}
\]

For a pure protein preparation, Q, the 260 nm/280 nm ratio, is 0.6.

In this laboratory experiment, you will determine the concentration of your extracted proteins and obtain a UV fingerprint of the protein by spectrophotometry. First, prepare varying concentration Bovine or Human Serum Albumin protein solutions in order to gathering absorbance data to construct a Beer’s Law calibration curve by plotting the A (absorbance) versus wavelength (\( \lambda \)) data at 260 nm and 280 nm. The molar absorptivity coefficient for BSA is 43,824 M\(^{-1}\)cm\(^{-1}\). From the A260 nm and A280 nm values for your extracted proteins, determine the calculation using Equation 1 (Beer’s Law) and Equation 3. Use Equation 4 to calculation the quality of the isolated protein for the homogenate, G-75 fractions, and DEAE fractions.

If time permits, UV-vis spectroscopy can also be used to determine the number of the various residues by evaluating serial dilutions of the isolated protein from a DEAE fraction by recording the absorbance at the \( \lambda_{\text{max}} \) (Fig. 13.7). The extrapolated x-axis intercept gives the number of tyrosines per protein molecule.
Fig. 13.7 Determination of number of tyrosine residues per protein molecule
http://sbio.uct.ac.za/Sbio/documentation/ProbingProteinStructurebySpectroscopy.php

Materials
- various variable volume micropipettes (10 µL, 100 µL, 1000 µL) and tips
- UV-vis spectrophotometer and quartz cuvette
- stock protein solution and homogenate and purified protein from the homogenate, dialysis, G-75 and DEAE purifications
- test tubes
- deionized water
- known concentration Bovine or Human Serum Albumin (BSA) protein to prepare serial dilutions
- parafilm

Procedure:
A: Initial Set-up of the Spectrophotometer
1. The spectrophotometer needs to warm up for at least 10-30 minutes prior to any use. We will use the Agilent 8453 UV-Vis spectrophotometer located in the Biochemistry laboratory. If the spectrophotometer has not been turned on when you enter the laboratory, turn it on. Do this by pushing the power button to the spectrophotometer and then turning on the computer. Load the cagbootp server. Turn on the lamp. Open the UV software (Start-programs-UV-Vis chemstations-instrument 1 (online2)). Setup the software to read the UV absorbance at 260 nm, 280 nm and 320 nm and display an absorption spectrum between 240-300 nm.
2. The spectrophotometer must be calibrated with a blank (buffer) prior to the analysis of your protein solutions. Your instructor will explain in detail how to carry out this setup and calibration prior to use.
Part B: Preparation of Solutions for Absorption Spectrum Plot and Beer's Law Plot

1. In a microcentrifuge tube or small test tube, obtain ~3 mg of the BSA (record exactly what you measure on the balance) and dilute with 3.000 mL of deionized water to prepare a ~1 mg/mL standard.

2. Obtain six more test tubes. Dilute the BSA serially to make standards to the following concentrations: 0 mg/mL (deionized water only), 0.000010 mg/mL, 0.00010 mg/mL, 0.010 mg/mL, 0.10 mg/mL, 1.0 mg/mL, using \( c_1V_1 = c_2V_2 \).

(Make a table of your dilutions.) Using micropipettors, pipet the appropriate amount of BSA into the corresponding tube to create a final volume of 3 mL of the appropriate concentration.

3. Dilute each of the samples with deionized water to 3 mL final volume. Cap each tube with parafilm and mix well.

4. For the blank (0 mg/mL), obtain an unknown in a microcentrifuge tube and add deionized water to the 3 mL final volume. Cover tube and mix well.

Part C: Beer's Law Plot and Determination of Unknown's Concentration

1. Set the spectrophotometer to read absorbances at 260 nm, 280 nm, and 320 nm (should be zero). Put distilled water in the quartz cuvette and place the cuvette in the sample holder in front of the laser. Blank the instrument using the distilled water.

2. Remove the blank solution from the sample holder and replace it with the most dilute DNA sample solution. Record its absorbance at 260 nm, 280 nm and 320 nm. Continue to read the absorbances from all of the standards from least to most concentrated.

3. Read the concentration of your unknowns. If the unknown’s absorbance value does not fall within an absorbance range of 0.1-1.0 or the range of your standard solutions, dilute the unknown appropriately so it will be in that range.

4. Prepare a Beer’s Law calibration curve by plotting the absorbances for the standard solutions versus their concentration (mg/mL).

5. Determine the concentration of the extracted protein samples (unknown concentrations) from this Beer’s Law calibration curve.

6. Add a trendline, line equation, and \( R^2 \) value on the graph and use the points on the line to calculate the concentration of the unknown. \( \text{Slope}=\Delta y/\Delta x= y_2-x_1/x_2-x_1 \)

Part D: Absorption Spectrum Plot

1. Measure and record the absorbance spectrum of your extracted protein fractions from the homogenate, dialysis, and G-75 and DEAE column separations by scanning over the wavelength range from 240 nm to 300 nm by taking readings at 1 nm intervals.

2. Save as .csv files.

Questions/Analysis

1. Create a Beer’s Law plot for your BSA dilution. Be sure to use a scatter plot and include the trendline and line equation.

2. Calculate the concentration of your extracted protein samples using Beer’s Law and a graphical method from the BSA standards (Equation 1) and using the given equations (Equation 3). Make a table of your values. Compare the values.

3. Give an absorption plot (Absorbance versus wavelength) for your extracted proteins (homogenate, dialysis, G-75 and DEAE fractions). Which amino acids are likely found in each sample? Justify your answer.
4. Calculate the quality of your extracted proteins using the absorbance values at 260 nm and 280 nm using Equation 4. Tabulate and interpret your values.

5. From the absorbance data, calculate and tabulate the concentration (in mg protein/mL of extract) of your homogenate, post-dialysis solution, G-75 and DEAE fraction. Be sure to account for any dilution (e.g. 1:3000 you may have done,) and then calculate total mg recovered by multiplying by the total volume of each sample. From the concentration determination above and with the knowledge of the total mass of the fish and knowledge of the mass of fish muscle used, calculate the total protein (extracted) for each sample per gram of fish muscle (in units of mg protein/g fish muscle) and total fish (mg protein/fish). Tabulate protein mg/mL, total volume (mL), percent yield (mg recovered/total mg fish x 100%) and purification factor.

References
14 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) to determine molecular weight and purity of the recovered proteins

Objective
To learn how to run SDS-PAGE and use electrophoresis to determine the molecular weight and purity of the purified protein

Safety
Do not touch the gel while the power supply is on. Danger: potential electrocution. Wash your hands before leaving lab. Acrylamide is a neurotoxin- wear gloves at all times and immediately wash if exposed to unpolymerized (liquid) acrylamide.

Background
As discussed in experiment 6, if ions of similar charge are placed in solution between two oppositely charged electrodes, the smaller ions of the same charge move faster toward the electrode of opposite charge faster than the larger ions. If ions of different charge are placed in solution, the more highly charged ions migrate faster than the lower charged ions. This process, called electrophoresis, is a good technique for separating substances. Modern techniques use a gel on a piece of plastic, glass or paper. It is easy to stain, analyze, and even to re-extract the protein sample.

Proteins may be positively or negatively charged (although parvalbumins are negatively charged). Negatively-charged, acidic proteins migrate toward a positive pole (red) (Fig. 14.1). Because the protein has to migrate through the gel substance, the lower molecular weight proteins will migrate further than higher molecular weight proteins in a given time because they can better form a ball and tumble through the matrix (Ogsten sieving) or linearly snake through the matrix (Reptation theory). The distance the protein will migrate from the well is related to the size and structure (folded, molten globule, or unfolded) and the degree of complexing of the gel matrix (concentration). The size of a protein can be calculated using protein standards of known molecular weight run next to the protein of unknown molecular weight (Fig. 14.1). A calibration curve can be made by plotting the log of the molecular weight of the standards (Protein ladder) against the distance each has migrated (Fig. 14.2). The distance the unknown protein has migrated can be used to determine its molecular weight graphically using a regression line created with the known standard proteins.

Polyacrylamide gel electrophoresis (PAGE) is an excellent and commonly used method to analyze protein fractions. Polyacrylamide gel is formed by co-polymerization of acrylamide (carcinogen) and a cross-linking monomer N,N'- methylene. To polymerize the gel, a system consisting of ammonium persulfate (initiator) and tetramethylene ethylene diamine (TEMED) is added. Both the concentrations of the initiator and crosslinker must be carefully measured and the initiator must be fresh to propagate the reaction quickly- but controllably. The concentration of the monomers may be varied to give gels of different density, usually gels with 10-20% acrylamide are used. Typically, polyacrylamide gels are formed in a two-layer system: a large running (or separation) gel of the desired density for separation of proteins, and a thin stacking gel on top to allow samples in the well to quickly migrate through until they contact the interface between the two gels. This quick migration to the contact serves to give very sharp, clear bands. Staining in situ can be performed
using Coomassie Blue. It may be used to estimate the molecular weight of protein subunits, protein purity and homogeneity, and isoelectric point.

In general, fractionation by gel electrophoresis is based on differences in size, shape and net charge of macromolecules. Systems where you separate proteins under native conditions cannot distinguish between these effects and therefore proteins of different sizes may have the same mobility in native gels. In SDS-PAGE this problem is overcome by the introduction of an anionic detergent SDS (sodium dodecyl sulfate) which binds strongly to most proteins. When hot SDS is added to a protein all non-covalent bonds are disrupted and the proteins acquire a negative net charge. A concurrent treatment with a disulfide reducing agent such as β-mercaptoethanol or DTT (dithiothreitol) further breaks down the macromolecules into their subunits. The electrophoretic mobility of the molecules is now considered to be a function of their sizes i.e. the migration of the SDS-treated proteins towards the anode is inversely proportional to the logarithms of their molecular weights, or more simply expressed: Small proteins migrate faster through the gel (Fig 14.3).

Fig. 14.1 Sample gel demonstrating the use of the MW standard to determine protein size and varying concentration on band intensity.  
Fig. 14.2 Plot of Log MW (kDa) of the molecular weight standard versus Distance traveled (mm) for the molecular weight standard for the gel in Fig. 11.2. Using the line equation, the size of bovine erythrocyte carbonic anhydrase can be estimated (28 kDa).

![Graph of Log MW (kDa) vs. Distance travelled (mm)](image)

Fig. 14.3 Sodium Dodecyl Sulfate (SDS), an anion detergent, denatures proteins and provides a uniform electric charge dependent only on size.

http://talon.niagara.edu/~391s08/giacomini/images/sds_page.png

Diagram showing the effect of SDS on protein denaturation and mobility in a gel. Mercaptoethanol denatures protein, SDS binds protein stochiometrically.
Figure 14.4 Discontinuous PAGE setup, illustrating the use of a thin stacking gel and a thick running (or separation) gel.

Materials
- Various variable volume micropipettors (20 µL, 100 µL, 200 µL, 1000 µL) and tips
- Purified protein (25-50 µg recommended)
- Ice
- 30% acrylamide/bis-acrylamide solution (caution: unpolymerized acrylamide is a neurotoxin!)
- Ammonium Persulfate solution (10% solution)
- TEMED
- 0.5M Tris, pH 6.8
- 1.5M Tris, pH 8.8
- 10% SDS solution
- Butanol
- Water
- Gel boxes and combs
- Molecular weight and/or isoelectric point standards: MW for ovalbumin: 69293; MW for bovine albumin: 42922 (stock: 0.04 g/4 mL or 100 µg/10 µL)
- Gloves
- Gel light box and digital photography system
- Hot plate and water bath
- 125 mL Erlenmeyer flask
- 1.0 -1.5 mL microcentrifuge tubes
- Power supply
- Coomassie Blue stain (500 mL 1x TBL buffer + 0.0125 g Coomassie Blue)
- 200 mg/mL Coomassie Blue to add to samples
- 1x Tris-Glycine-SDS running buffer (3.03 g Tris base, 14.4 g Glycine, 1g SDS for 1 L)
- Loading buffer (Tris-SDS-EDTA buffer with 25% glycerol and 1 µL 200 mg/mL Coomassie Blue)
- 1 M EDTA
- Molecular weight marker (116.0 kDa beta-galactosidase, 66.2 kDa BSA, 45.0 kDa ovalbumin, 35.0 kDa lactate dehydrogenase, 25.0 kDa restriction endonuclease, 18.4
kDa beta-lactoglobulin, 14.4 kDa lysozyme)

Procedure

Part A: Preparing the polyacrylamide gel
1. Prepare running Tris-Glycine buffer as indicated in the materials for 1 L of 1X buffer.
2. Set up the PAGE gel apparatus, locking front and back plates into the frame.
3. In a 15 mL plastic centrifuge tube, combine all components of running gel except TEMED, and mix gently by inversion:
   - 2.6 mL water
   - 3.2 mL 30% acrylamide solution
   - 2.0 mL 1.5M Tris, pH 8.8
   - 80 uL 10% SDS
   - 80 uL 10% ammonium persulfate
4. Ensure that gel plates are sealed in the gel apparatus, and set a pipettor to 1 mL volume and attach a tip. Add 8 uL TEMED to the gel mixture, and quickly but gently mix twice by inversion.
5. Immediately begin carefully adding running gel to the space between the two gel plates using the 1 mL pipettor, filling to the fill line.
6. Pipet 1 – 2 mL of butanol to the top of the gel, forming a solid layer of butanol above the gel (polymerization is inhibited by oxygen, and the butanol isolates the gel from the atmosphere).
7. Wait for polymerization to complete, using the remaining gel solution in the 15 mL plastic centrifuge tube to judge; typically 15 to 20 minutes.
8. Remove butanol by carefully lifting gel apparatus and turning upside down—ensure that the gel plates are firmly attached first. Next, use a Kim wipe to gently reach into the space between the plates to remove remaining butanol.
9. In a fresh 15 mL plastic centrifuge tube, combine all components of stacking gel except TEMED, and mix gently by inversion:
   - 2.6 mL water
   - 1 mL 30% acrylamide solution
   - 1.25 mL 0.5M Tris, pH 6.8
   - 50 uL 10% SDS
   - 50 uL 10% ammonium persulfate
10. Add 5 uL of TEMED to the gel mixture and quickly but gently mix twice by inversion.
11. Immediately begin carefully adding stacking gel to the space between the plates using the 1 mL pipettor, filling the the top of the plates.
12. Insert gel comb into the top of the plates, proceed slowly to ensure fit and avoid splashing toxic acrylamide.
13. Wait for polymerization to complete, using the remaining gel solution in the 15 mL plastic centrifuge tube to judge, typically 15 to 20 minutes.

Part B: SDS-polyacrylamide gel electrophoresis
1. Transfer the PAGE gel and plates from the pouring apparatus to the gel running box.
2. Pour 1x Tris-Glycine buffer with Coomassie Blue stain (500 mL buffer +
0.0125 g Coomassie Blue) over the wells to cover the gel and remove the comb.

3. To the purified protein (10 µL) add loading dye (5 µL) and 1 µL of 200 mg/mL of Coomassie Blue for the agarose gel electrophoresis.

4. Add DTT (0.5 µL of 1M) if you desire reduced protein. Boil your samples for 10 minutes before proceeding if you desire denatured protein.

5. To the wells, pipet your samples (homogenate, dialysate, HMW, LMW, DEAE peaks) (15 µL), each in a different well. Be sure to record what sample you placed where! Do not load more than 20 µL per well.

6. Pipet the known molecular weight protein standard markers into a well (15 µL).

7. Connect the black electrode with the black wire and the red electrode with the red wires to the power source.

8. Plug in the power source.

9. Run for 1-2 hours. Use no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel). This value will vary depending on the gel apparatus you are using- consult your instructor (~125-150 V for large gels). Run until the dye front is 2 cm from the end of the gel or until time permits.

10. DO NOT touch the gel while the power is on.

11. Turn off power. Then disconnect all power cords.

Part C: Visualizing the Protein Bands with Coomassie Blue

1. Remove the gel from the box and rinse the gel in warm (40-55 ºC) tap water for 3 rinses of 5 minutes each to remove Coomassie blue and buffers/salts from the gel surface. (Note: Use of boiling water to rinse the gel will melt the gel!)

2. Analyze the gel visually or using a white light box. The protein will be bound with the blue stain and should appear as bands or lines. The detection limit is 1 µg protein.

3. If desired, you may dry the gel using a gel dryer and keep the original gel.

4. Alternatively, you may take a photograph with the digital camera. (To do this, we will take a field trip to the Biology Department.) The settings for Coomassie Blue Agarose Gel Photos in the Biology Department are: Aperture (top setting): 5.6; Depth of Field (Middle Setting): halfway between 20 & 12.5; Distance scale (Bottom setting): a little after 1.

Question/Analysis

Insert the picture of your gel into the data section of your report. From the photograph of your gel, estimate the molecular weight and homogeneity/purity of your protein samples. What do you observe when you compare the original homogenate to the dialysis and G-75 fractions to the DEAE fractions? Plot the log \(M_r\) (in kDa) versus the distance the marker (known molecular weight standard) proteins migrated in the gel. That plot should give a straight line (Fig. 14.2). Use this plot to estimate the molecular weights of the G-75 and DEAE (unknown) proteins using the slope. Suggest a better method to determine the molecular weight of a protein if this one fails to yield a mass of approximately 12 kDa for parvalbumin.
15 Protein characterization by fluorescence spectroscopy

Objective
To learn how to characterize protein spectral characteristics using fluorescence spectroscopy.

Safety
Be careful not to spill solutions in the fluorescence spectrophotometer. Clean up all spills immediately.

Background
Every molecular and atomic species has a characteristic absorption spectrum (a ground state electron is promoted to an excited state). In addition to absorbing light, some molecules also fluoresce, meaning that they emit light (rather than just heat) of a longer wavelength after undergoing an energetic transition (from a higher excited state to one of lower energy when the electron relaxes to the ground state) (Fig. 15.1).

Fig. 15.1 Fluorescence energy emission of an electron
http://chemistry.rutgers.edu/grad/chem585/lecture2.html

An emission spectrum is obtained by plotting fluorescence intensity (relative units) as a function of wavelength (λ) (Fig. 15.2). A fluorescence spectrum is a chemical fingerprint and can be qualitatively used to identify molecular and atomic species present in a sample. It is more sensitive than absorbance alone and can detect nanomolar quantities of molecules (absorbance can detect micromolar quantities).
Each fluorescent molecule has a quantum yield, the ability of it to absorb light. The fluorescence quantum yield gives the efficiency of the fluorescence process. It is defined as the ratio of the number of photons emitted to the number of photons absorbed.

\[ \Phi = \frac{\# \text{ photons emitted}}{\# \text{ photons absorbed}} \]

The maximum fluorescence quantum yield is 1.0 (100%); every photon absorbed results in a photon emitted. Compounds with quantum yields of 0.10 (10%) are still considered quite fluorescent. Fluorescent molecules also undergo excited state decay loss of energy in addition to fluorescent emission. These are caused by mechanisms other than photon emission and are therefore often called "non-radiative rates", which can include: dynamic collisional quenching, resonance energy transfer, internal conversion and intersystem crossing.

Proteins and DNA both fluoresce as their chemical molecules contain pi-conjugated electron systems. They absorb light in the UV region and emit light in the visible region of the electromagnetic spectrum. The amino acid tryptophan is an important intrinsic fluorescent probe for proteins which can be used to estimate the nature of microenvironment of the residue: one can see if it is buried within the protein or exposed on the protein’s surface. When performing experiments with denaturants, surfactants or other amphiphilic molecules, the microenvironment of the tryptophan might change. The absorbance maximum for tryptophan is 280 nm. For example, if a protein containing a single tryptophan in its 'hydrophobic' core (e.g. Trp-102 in parvalbumin, \( \lambda_{\text{max em}} = 308 \) nm) is denatured with increasing temperature, a red-shift emission spectrum will appear (\( \lambda_{\text{max em}} = 350 \) nm) indicating the tryptophan is completely solvent exposed. This is due to the exposure of the tryptophan to an aqueous environment as opposed to a hydrophobic protein interior. In contrast, the addition of a surfactant to a protein which contains a tryptophan which is exposed to the aqueous solvent will cause a blue shifted emission.
spectrum (back to $\lambda_{\text{max em}} =$308 nm) if the tryptophan is embedded in the surfactant vesicle or micelle or buried in an apolar core. Intermediate $\lambda_{\text{max em}}$ values of 315-330 nm indicate that the tryptophan is interacting with neighboring polar groups or partially exposed on the surface of the protein. The quantum yield is 0.20. Proteins that lack tryptophan may be coupled to a fluorophore. The tyrosine absorption maximum is 274 nm and the emission maxima occurs at 303 to 304 nm if buried and its width is 34 nm. The quantum yield of tyrosine is 0.14 at room temperature. Red-shifted values indicate that the environment is partially or even fully exposed to the solvent. Freezing shifts the emission to 298 or 299 nm. Due to the reduced quantum yield of tyrosine compared to tryptophan, it will only be traceable if the protein does not contain tryptophan. Likewise, phenylalanine will also fluoresce but only be detected if tryptophan and tyrosine are absent as its quantum yield is 0.038 to 0.045 in water. The spectral maxima are 257 nm for absorption and 282 nm for emission; the emission is red-shifted 5-nm in basic conditions.

In the spectrofluorimeter, the light from an excitation source passes through a filter or monochromator, and strikes the sample. A proportion of the incident light is absorbed by the sample, and some of the molecules in the sample fluoresce. The fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second filter or monochromator and reaches a detector, which is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector (180 degree angle) (Fig. 15.3). Xe allows for excitation from 200-800 nm. Filters and/or monochromators may be used in fluorimeters. A monochromator transmits light of an adjustable wavelength with an adjustable tolerance using a diffraction grating. The monochromator can then be adjusted to select which wavelengths to transmit. For allowing anisotropy measurements the addition of two polarization filters are necessary: One after the excitation monochromator or filter, and one before the emission monochromator or filter.

**Fig. 15.3** A schematic of a fluorometer with 90° geometry utilizing a Xe light source

In this laboratory, we will characterize the DEAE protein peaks using fluorescence spectroscopy to determine their excitation and emission maxima. From this data, you can predict whether Trp, Tyr and/or Phe amino acids are present in the protein.

**Materials**
- various variable volume micropipettes (10 µL, 100 µL, 1000 µL) and tips
- PE LS-50B luminescence spectrophotometer and quartz cuvette or 96-well plate
- pooled protein solutions from anion exchange chromatography
- deionized water or buffer
- heating block for denaturation
- EDTA to make apo-protein or apo-protein
- DTT to reduce dimers
- 0.100 M CaCl₂ for binding constant determination

**Procedure:**

**A: Initial set-up of the fluorimeter and preliminary data collection**
1. Start the computer.
2. Turn on the lamp. It needs to warm up for 30 minutes.
3. Click on the FL Win Lab software.
4. Set the absorbance to scan from 250-290 nm and emission to detect from 300-350 nm with a 5 nm slit width and 5 nm/scan. Select the 290 nm cut-off emission filter.
5. Set the absorbance to scan from 240-260 nm and emission to detect from 270-300 nm with a 5 nm slit width and 5 nm/scan. Select the appropriate emission filter.
6. From the above scans, determine if Trp, Tyr and/or Phe is present.
7. Save the spectra .sp3 files.
8. Replot the spectra as 3D contour plots.
9. Export these spectra to paint and save a .jpg for inclusion as data in your lab report.

**B: Collecting data on your purified parvalbumin protein**
1. Record qualitative spectra for all of your peaks from the DEAE anion exchange column chromatography, DTT reduced protein, heat-denatured protein, and EDTA-chelated apo-protein.
2. Save the spectra .sp3 files.
3. Replot the spectra as 3D contour plots.
4. Export these spectra to paint and save a .jpg for inclusion as data in your lab report.

**C: Collecting data on your purified parvalbumin protein**
1. Titrate 4.000 mL of apo-protein with 0.5-1.0 µL increments of calcium from a 0.100 M solution recording data at the excitation and emission maxima.
2. Save the spectra .sp3 files.
3. Replot the spectra as 3D contour plots.
4. Export these spectra to paint and save a .jpg for inclusion as data in your lab report.
Questions/Analysis
Tabulate the excitation and emission maxima for your parvalbumin isoforms and the qualitative experiments (heating, adding DTT, adding EDTA) on parvalbumin. Based on the $\lambda_{\text{max \ em}}$ of the protein, describe the expected environment for tryptophan or tyrosine or phenylalanine in the parvalbumin. How does the fluorescence spectra of the perturbed proteins compare to the native proteins (e.g. red or blue shifted)?

To analyze the titration of calcium to the apo-parvalbumin, normalize the data by dividing the highest fluorescence intensity value ($F_0$) by itself to equal 1.000. Divide all of the other collected fluorescence intensities at 350 nm by the highest number ($F_0$) to yield the normalized fluorescence values ($F$). Convert the concentration of calcium ions to micromolar. Consider the effects of dilution on the initial peptide were considered in determining the overall calcium concentration in the cuvette using $M_1V_1 = M_2V_2$. Compute $F_0/F$ by dividing the initial fluorescence intensity ($F_0$) by the normalized fluorescence values ($F$). Graph the $F_0/F$ vs. micromolar calcium concentration using a scatter plot.

The binding constant can be determined using the Stern-Volmer equation: $F_0/F = 1 + K_{SV}[Q]$. In the Stern-Volmer model, $F_0$ is the fluorescence intensity of the sample before the addition of quencher; $F$ is the fluorescence intensity at a specific concentration of the quencher, $Q$; $K_{SV}$ is the Stern-Volmer quenching constant; and the y-intercept is expected to be 1. The value of $K_{SV}$ can be calculated from the slope of a straight line, fitted to the data points and is equivalent to a binding constant under conditions in which the metal and peptide are at similar concentrations.
Use of Perkin-Elmer LS-50B

by Mike Furnell

Using a Perkin-Elmer LS-50B with software loaded on a Compaq NT machine, the following is the process for acquiring fluorescence spectroscopy data.

1. Start the computer and warm up the lamp in the spectrophotometer for about 30 minutes.
2. Click on the FLWinLab software icon.
3. Set up the software and verify appropriate settings.
   - Menu - Application
   - Well Plate Reader
   - Set to read well A1 only by clicking on the A1 button
   - Stat View Results Tab
   - Instrument File Menu
   - Setup Method
   - Filename
   - Filename.sp (filename is limited to 8 characters)
   - Clicked on the far right button under the main menu at the top of the screen – This activate the mouse-over function that gave information about each button as the mouse was placed over the button – this was needed to find the 3D button
   - Using the mouse, the mouse pointer was slowly scanned over the various buttons to find the 3D plot button
   - When it was found The 3D plot button was clicked to activate the 3D plot mode

LS50B Setup
- EX Mono Fields on dialog box

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<th>Value (nm)</th>
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<tr>
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</table>

4. Under the Instrument drop down on the Application/Scan screen the Run option was selected to run the program for 3D scan plots of the proteins.
5. After running the 3D plot, under the main applications menu
   - 3D □ File □ open was executed and the file name was found for the 3d plot

6. Click on View □ Format □ select False Color Map with contours
7. The mouse was placed in the center contour of the contour map in the center of the white area to display the Intensity, and excitation levels for this map.
8. Click on Edit □ Copy
9. On the main windows screen a Right mouse button click on the lower left hand button on the screen produced the windows file system.
10. Under accessories, the ‘Paint’ program was found and started by clicking on the program option
11. Under the paint program, Edit $\rightarrow$ Paste was selected and the color graph and the contour map in memory (From item 8 above) was pasted into the paint program
12. Under the main menu, File $\rightarrow$ Save As was selected to save the file as a bitmap so that it could be retrieved for reporting purposes.
16 Final formal research lab report in the style of *Biochemistry*

An end-of-semester paper is required from you as part of your upper-division biochemistry laboratory experience. This report should be written in the format of a formal scientific research article complete with a Title Page, Abbreviations and Textual Footnotes, Abstract, Introduction (no heading), Experimental Procedures (Materials and Methods), Results, Discussion, Acknowledgment, References, Tables, Structures, Charts, Schemes, Figure Legends, and Figures. Write the paper (regular article) based on author guidelines (http://pubs.acs.org/page/bichaw/submission/authors.html) for the ACS journal *Biochemistry* (http://pubs.acs.org/journal/bichaw). Note that the format is different than what is required for most (if not all) class lab reports. Include only material from the protein purification and characterization laboratories; omit material from the pipetting DNA cloning, protein modeling, and buffer labs.

Abstracts should be mini-papers of 250 words or less stating the overall objective, overall methods, specific results and one sentence conclusion.

The introduction should cite at least 10 peer-review primary references from the literature of related studies in the field that should justify your current, further study. A good starting point for locating papers is Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/). SciFinder (in the library) is also an excellent search mechanism. Always reference ideas, statistics and facts if they are not your own. Failure to do so is plagiarism. Use the *Biochemistry* citation style for articles, journals, books, webpages, etc. that you use using their superscript numbering style for the references cited in the order used.

The experimental procedures should be written in the third person, past tense, and using complete sentences. Do not break sections by week; omit text such as “last week,” “next week,” and so forth. When tabulating masses of the total protein and supernatant masses and volumes, do not report masses of beakers and tubes, etc. in the paper. Report the species used with both common name and formal *Genus species*. Include name, concentration, and pH of all reagents and buffers, as applicable. Write all relevant procedures; you may break this into titled sections. Remember space is expensive. State everything as concisely as possible.

Your results should be compiled and organized in MS Excel or an alternative graphing program (copied from previous reports and labeled Figure 1, Figure 2, Figure 3, etc. or Table 1, Table 2, Table 3, etc. at the end of the report after the references). UV-vis graphs should be redrawn from ASCII files in an appropriate graphing program. You should also include any work that did not produce results and explain why. Report all other non-tabulated data in the results text in complete sentences. Equations used for calculations should be given in text (e.g. Equation 1, etc.); sample calculations can be omitted. Report calculated results only.

In your discussion you should discuss in words all results and major conclusions to all experiments (labs).

In the conclusion, you should describe the future directions of the research project.
including any directions that we have together discussed in addition to ideas that you have after reading other scientific articles in the field.

Spell and grammar check. Have a peer proofread for you. The report will be graded on accuracy, setting the problem in context and using at least 10 references, using correct chemical vocabulary and third person past tense, methods description, organization, analysis of data, completeness of methods and data, validity of conclusions, correctness of figures and tables, following *Biochemistry* format, and timeliness. Refer to the rubric from grading points/weights.
APPENDIX