

Activity # 4. Measurement of Enzyme Activity

Adapted from Experimental Molecular Biology Lab Manual,
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Week 1 – Protein Extraction, Concentration Measurement & Instrument Use

The first part of this experiment involves extracting all soluble proteins (including the enzyme acid phosphatase) from wheat germ and measuring the total protein concentration in the extract. The amount of protein in the sample will be determined by comparing it with a series of solutions with known protein concentrations.

Week 2 – Effect of Enzyme & Substrate Concentration on Enzyme Activity

During week 2, we will measure the amount of acid phosphatase enzyme in the crude extract based on its catalytic activity and will compare our wheat germ extract to a preparation of purified enzyme. Two different concentrations of each will be studied to determine the effect of enzyme concentration on activity, and to identify the optimal preparation to use for subsequent experiments. We will then determine how substrate concentration affects the reaction rate when using the same amount of enzyme. The goal for week 2 is to generate a large amount of data for analysis during week 3.

Week 3 – Analysis of Experimental Data Using Microsoft Excel

We will use the first half of the lab period to analyze, graph & interpret the data generated during weeks 1 & 2. The values for K_m , V_{max} and Specific Activity will be determined and their significance discussed.

Week 1 – Protein Extraction, Concentration Measurement & Instrument Use

Week 1 Learning Goals:

- To learn proper precautions for working with enzymes
- To learn how to properly select, set and use micropipettors to measure small volumes
- To learn the theory behind and practical use of a spectrophotometer
- To learn how to make serial dilutions and calculate concentrations after dilution
- To learn how to make and use a standard curve.

Background:

Proteins represent one of the most abundant dry mass components of cells, and they are without question the most diverse class of biomolecules in terms of their biological function. Although the fundamental structure of all polypeptides is similar in that they are linear polymers of amino acids, this common structure belies a great diversity in properties. The purification of a given protein relies on the specific chemical and physical properties of that protein, and thus purification procedures vary widely.

Purity refers to the increase in specific activity (rate of product formation/mg protein) as an enzyme becomes more highly concentrated or purified. Typically, when one wants to study an enzyme in detail, it should be obtained in as pure a form as possible; that is, with very few other proteins. Historically, this was accomplished by extracting all proteins from the cell, as we will do today, then separating the protein of interest from other proteins based on physical (size, charge, solubility) and sometimes functional properties (ability to bind to a specific molecule). More recently, people have begun to clone the genes for proteins of interest in such a way that a molecular tag or handle is attached to the protein to make purification much easier.

An important prerequisite for studying proteins is the ability to measure their concentration, often in dilute solutions using small volumes. The use of small volumes is important to save on cost, storage space, and to save the maximum amount of protein for subsequent experiments.

In this experiment, we will measure the concentration of total protein and the activity of the enzyme acid phosphatase in crude and purified preparations. The crude preparation will contain **thousands** of different proteins, and only a small percentage of the total protein will be acid phosphatase enzymes. Fortunately, the other proteins in the mixture will not interfere with our measurements. The purified preparation was purchased from Sigma Chemical Company, and should contain many fewer contaminating proteins.

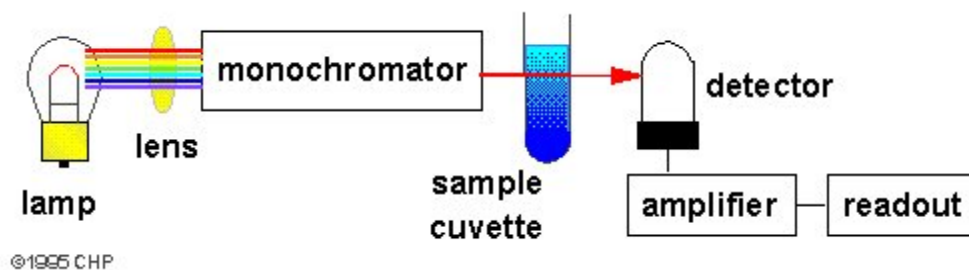
To measure the concentration of total protein in a sample, we will mix a small amount of the sample with a dye that changes its absorbance spectrum when bound to protein. The greatest change occurs at light wavelength of 595 nm. The absorbance of the solution will be measured using a spectrophotometer.

Principles and Use of the Spectronic 20D (Spec-20) (figures from



<http://www.chemistry.adelaide.edu.au/external/soc-rel/content/uv-vis.htm>)

The Spec-20 contains a lamp to generate white light and a filter (monochromator) that allows only a certain wavelength of light to pass through. This wavelength of light then passes through the sample compartment. The sample is placed into a cuvette which is then placed into the sample compartment. For the Spec-20, we use 13 x 100mm borosilicate glass test tubes, however, some spectrophotometers use square cuvettes, or cuvettes made of quartz which allows UV light to pass through more readily. When there is nothing in the sample compartment, a gate blocks the light path into the sample compartment. As a cuvette is placed into the sample compartment, a lever opens the gate, allowing light to pass through the sample to a detector. The detector measures the amount of light that is transmitted through the sample.



If a cuvette contains pure water, what percent of light would be transmitted through the sample? _____. How much would be absorbed? _____

What is the relationship between transmittance and absorbance?

To determine the percent of light being transmitted through a sample, it is necessary to know the amount of light entering and leaving the sample. Because it is not practical to measure the light entering a sample (how could this be done?) and the amount of light absorbed by the glass tube, or the solvent that the sample is dissolved in, one must define 0% transmittance and 100% transmittance on the

spectrophotometer. 0% transmittance will be set as the amount of light reaching the detector when the sample compartment gate blocks the light path (no sample inserted) and 100% transmittance will be defined as the amount light passing through a tube of water.

Beer's law according to Dr Dave Franz..."The deeper the mug, the darker the brew, the less light gets to you."

There is a direct (linear) relationship between the concentration of protein, the length of the light path and the absorbance of light. Because we will consistently use tubes with the same diameter, the distance of the light path will not be a variable. We will use a set of standards (i.e. solutions with known protein concentrations) to determine the relationship between protein concentration and absorbance and prepare a **standard curve**. Solutions containing a higher concentration of protein will appear darker and have a higher absorbance. We can then interpolate on this graph to correlate the absorbance of our crude and purified protein preparations with protein concentrations, and thus determine the concentration of protein in these samples.

General Procedure for Zeroing the Spectrophotometer

NOTE: Each individual student needs to learn how to do this, without written instructions, for the lab practical exam—so make sure each group member has a chance to practice today.

1. Use the left front knob to turn on the spectrophotometer and allow the lamp to warm up for at least 15 minutes
2. Use the top right knob to set the **wavelength** to the appropriate value.
3. Use the left front knob to set **0% transmittance** (with no tube in the sample holder – this keeps a panel in a position to block the light path).
4. Add 3 mL dH₂O to a 13 x 100 tube, wipe the outside of the tube with a kimwipe, and insert the tube into the sample holder on the top left of the instrument. Use the right front knob to set **transmittance** to 100%.
***** It is important to have at least 2.5 mL of sample in the tube due to the height at which the light passes through the sample**
5. Press the **mode** button to switch to **Absorbance**. The display should read 0.000. The spectrophotometer is now ready for use.

Dilution calculations

The spectrophotometer has a limited effective range of measurement and some of the samples will have protein concentration higher than this range. Therefore, we must dilute the samples to accurately measure the protein concentration. When making dilutions of this sort, it is easiest to work with factors of 10. Work through the following example:

If your original sample contains 12 mg protein/mL of solution, and you add 0.1 mL of your protein solution to 0.9 mL of water, what will be the concentration in the diluted sample?

How much protein is in 0.1 mL of your original solution? _____

What is the final volume of your new diluted solution? _____

Since concentration = amount/volume, the concentration is _____.

Another way to solve problem is to think about the dilution factor. If you are diluting the sample by a factor of 10 (a 10-fold dilution; going from 0.1 mL to 1.0 mL), the concentration of the dilution will one tenth that of the original sample. The diluted solution, in this case, might be referred to as a 0.1x or a 10^{-1} dilution. If some of the 0.1x dilution were diluted 10-fold into another tube, this would create a 0.01x or 10^{-2} dilution. The preparation of samples in which one dilution is used to make another, which is then used to make another is referred to as “making **serial dilutions**”

A third way to solve dilution problems is to use the formula:

$V_1C_1=V_2C_2$ where V_1 and C_1 are the volume and concentration of the original sample and V_2 and C_2 are the volume and concentration of the diluted samples. In our original example. ... $(0.1 \text{ mL}) (12 \text{ mg/mL}) = (1.0 \text{ mL}) C_2$

Solving for C_2 we get $C_2 = 1.2 \text{ mg/mL}$

This last method is most useful when determining how much of a stock solution to add to a mixed solution that you are preparing (solve for V_1), and when the dilution factors are not multiples of 10.

General Procedure for Use of Micropipettes:

To measure small volumes accurately, we will use **micropipettes**. These will be used frequently in this course and many subsequent biology courses, so it is essential that you learn how to use them properly now.

A “set” consists of three pipettes that together can accurately measure and dispense volumes from 0.5 μL to 1000 μL (1 mL).

- Volumes between 0.5 μL and 20 μL are measured using the smallest micropipette, the **I-20**. The yellow digit on the bottom of the setting display indicates tenths of a microliter, e.g. setting of “0 - 5 - 3” corresponds to 5.3 μL .
- Some sets have **I-100**'s and some have **I-200**'s for the intermediate volumes between 20 μL and 100 μL or 200 μL . The 3 digits on the display show hundreds, tens and ones; e.g. “0 – 7 – 5” corresponds to 75 μL .
- The largest micropipette, the **I-1000**, is used for volumes between 100 and 1000 μL . The yellow digit at the top of the setting display indicates thousands of μL ; e.g. “0 – 8 – 0” corresponds to 800 μL (or 0.80 mL).

Your lab instructor will demonstrate the use of the pipettes.

1. **Set dial to desired volume.** Do not try to turn volume setting dial beyond the maximum volume for the pipettor. This is how students most frequently damage the pipettors.
2. **Attach a pipette tip.** The small yellow or white tips are used on the I-20, I-100 and I-200's; the large blue tips are used with the I-1000 pipettors. Be sure tip is attached securely to pipettor, liquid will drip out if seal is not tight.
3. **Press plunger down to 1st stop. Place tip into liquid**
4. **Slowly release plunger so that liquid is drawn into pipette tip.** *Always hold pipettors with the shaft pointing down, especially when liquid is in the tip.* If liquid does get into the shaft, please tell your instructor immediately, so that it can be cleaned out. Damage and corrosion can occur if salt solutions dry inside pipettor.
5. **Dispense liquid into desired tube by pushing down plunger to the second stop. Remove tip from liquid before releasing plunger,** otherwise you will draw liquid back into tip.
6. **Eject used tips into coffee cans at your bench.**

Practice using the pipettors to measure the following volumes of water; 2 μL , 12 μL , 32 μL , 100 μL , 350 μL , 900 μL . **How could you tell whether your measurements were accurate?** (Hint: the density of water is 1 gram per mL, and we have balances) How is accuracy different from precision?

Procedure – for groups of 4

* Note, because some enzymes can be denatured and lose their activity easily, it is good practice to **always** keep enzyme preparations on ice (0-4°C). When pipetting solutions of enzymes, care must be taken to prevent the introduction of **bubbles**. The large air/liquid interface surface can also denature proteins.

A. Extraction Wheat Germ Protein and Preparation of Dilutions

1. Add 10 ml of ice-cold, distilled H₂O (dH₂O) to 2.5 g of wheat germ in a screw cap centrifuge tube. Place tube in crushed ice, mix intermittently for 10 minutes.
2. Centrifuge at 6000 x g for 10 minutes in large centrifuge. **IMPORTANT:** Be sure tubes are balanced to within 0.1g before starting the centrifuge.
3. Decant supernatant into a clean screw cap tube, labeled 1xE, and store on ice. Discard pellet and clean out centrifuge tube.
4. Prepare a 10-fold dilution of the extract by mixing 1 mL of crude extract with 9 mL of dH₂O in a small screw cap tube and labeling it 0.1xE. Invert several times to mix, store on ice.
5. Prepare a 100-fold dilution of the extract by mixing 1 mL of 0.1xE with 9 mL of dH₂O in a small screw cap tube and labeling it 0.01xE. Invert several times to mix, store on ice.

If the concentration of protein in the original crude extract (1xE) was 10 mg/mL, what would be the concentration in the 0.1xE tube? _____

The 0.01xE tube? _____

If the concentration of protein in the original crude extract (1xE) was 0.5 mg/mL, what would be the concentration in the 0.1xE tube? _____

The 0.01xE tube? _____

B. Total Protein Determination

To measure the relative purity, or specific activity of enzyme preparations, it is necessary to know the total concentration of protein in the sample. This measurement is made with a simple colorimetric assay (test) in which a dye is added to a diluted protein sample. When the dye binds protein, it absorbs light at 595 nm. Samples with higher protein concentrations absorb more light than those with lower concentrations. A standard curve must be generated to correlate specific absorbances with actual protein concentrations. We will use Bovine Serum Albumin (BSA) solutions at known protein concentrations as our standard. Where does this protein come from?

Why do you think it is commonly used as a protein standard?

One must also consider that if the concentration of protein is too high (>750 μ g/mL), the absorbance will be outside of the effective range of the spectrophotometer. Therefore, we must measure the absorbance in more dilute solutions to calculate the protein concentration in the original tube.

1. Label ten 13 x 100mm tubes as follows: 0, 50, 100, 250, 500, 750, 1xP, 1xE, 0.1xE, 0.01xE
2. Add 2.34 mL dH₂O to each tube. Then add 60 μ L of the appropriate BSA protein standard (provided to you in 1.5 mL microfuge tubes--**Be sure to use a new pipette tip for each dilution or extract!**) to the 0, 50, 100, 250, 500, and 750 tubes. Add 60 μ L of the phosphatase enzyme stock to the 1x P tube, add 60 μ L of the wheat germ extract or the dilutions to the appropriate tubes. Vortex each of the tubes to mix.
3. Add 600 μ L Bio-Rad protein assay dye to each tube, vortex, and allow to stand for 5 minutes at room temperature.
4. The appropriate wavelength, 0% transmittance and 100% transmittance must be set on the spectrophotometer prior to use.
 - a. Use the left front knob to turn on the spectrophotometer and allow the bulb to warm up for at least 15 minutes
 - b. Use the top right knob to set the **wavelength** to 595 nm
 - c. Use the left front knob to set **0% transmittance** (with no tube in the sample holder – this keeps a panel in a position to block the light path).
 - d. Add 3 mL dH₂O to a 13 x 100 tube, wipe the outside of the tube with a kimwipe, and insert the tube into the sample holder on the top left of the instrument. Use the right front knob to set **transmittance** to 100%.
 - e. Press the **mode** button to switch to **Absorbance**. The display should read 0.000. The spectrophotometer is now ready.

5. Remove the water tube from the spectrophotometer, then measure the absorbance of each of your 10 tubes and record your data in Table 1 below. Remember to wipe the outside of each tube clean before you insert it.

Table 1. Determination of Protein Concentration.

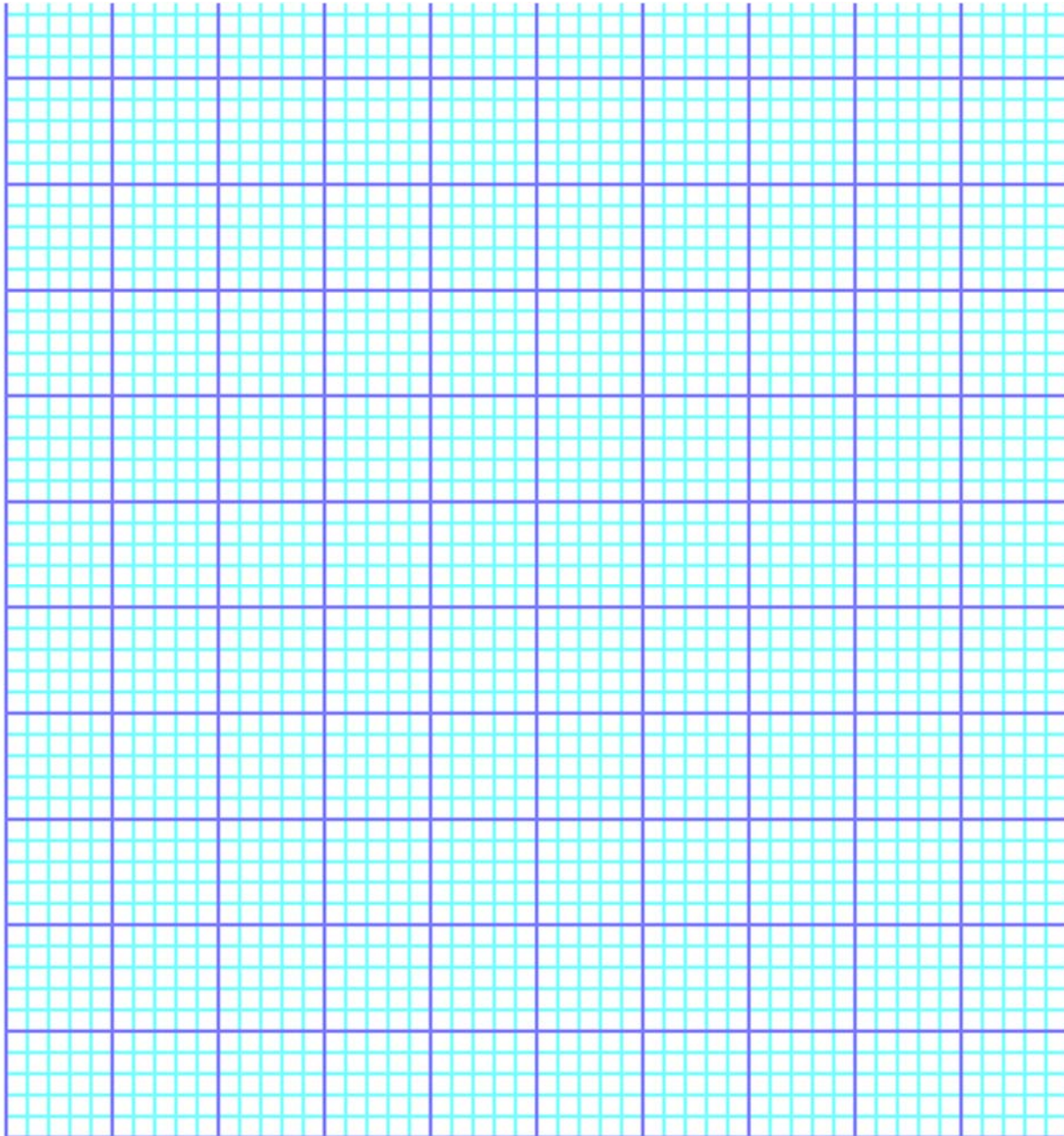
Sample	A595	[Protein] ($\mu\text{g/mL}$)
BSA - 0		0
BSA - 50		50
BSA - 100		100
BSA - 250		250
BSA - 500		500
BSA - 750		750
1x P		
1x E		
0.1x E		
0.01x E		

6. Create a graph of [protein] vs. Absorbance using the data obtained **with the samples of known concentration (BSA samples)**. Do not plot data points for the 1XP, 1XE, 0.1XE, or 0.01X E—yet!

Tips/reminders for graphing

- Independent variable is placed on the X-axis
What is the independent variable for this experiment? _____
- Dependent variable is placed on the Y-axis
What is the dependent variable for this experiment? _____
- Scale must be consistent
- Always include title, axis labels and units

7. Use this graph to determine the protein concentration based on the absorbance of the 1xP sample and whichever crude extract dilution has an absorbance reading within the range observed with the 0-750 $\mu\text{g}/\text{mL}$ samples. Use the concentration of crude extract dilution that was in range to calculate the concentration of protein in the original crude extract.



Week 1 Summary Questions

- 1 mL = _____ μL 0.75 ml = _____ μL
0.1 mL = _____ μL 0.005 mL = _____ μL
200 μL = _____ mL 80 μL = _____ mL
 $7 \times 10^{-5} \text{ L}$ = _____ mL = _____ μL
 $4.76 \times 10^{-2} \text{ L}$ = _____ mL = _____ μL
 $2.34 \text{ mL} + 60 \mu\text{L} + 600 \mu\text{L} =$ _____
2. How could you tell if your pipettor is measuring accurately?
3. How could you tell if your pipettor is measuring precisely?
4. How are absorbance and percent transmittance related to each other?
5. How are absorbance and concentration related to each other?
6. How can you determine the amount of protein in an unknown sample?
7. What are 5 characteristics of a good scientific graph?
8. Why is it important to keep enzyme preparations on ice as much as possible?

9. If the [protein] of the 0.1xE sample was 320 $\mu\text{g}/\text{mL}$, then the concentration in 0.01xE would be _____ and the concentration of 1xE would be _____.
10. In the example above, what would be the [protein] concentration in the actual 0.1xE protein assay tube that was put in the spectrophotometer?
11. How much of a 1M Tris buffer stock solution should be used to make 250 mL of solution containing 25 mM Tris buffer?
12. When 30 μL of a sample is added to 2.97 mL of water to make dilution A, this is a _____-fold or 10 _____ dilution.
13. When 30 μL of a dilution A above is added to 2.97 mL of water to make dilution B, the total is a _____-fold or 10 _____ dilution relative to the original sample.