

Improvement of Biochemistry 2002 through experimental modification and organization
of course materials

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The Faculty of the Graduate School
At the University of Missouri

In Partial Fulfillment
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Master of Science

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

Improvement of Biochemistry 2002 through experimental modification and organization of course materials

presented by Michael Mitchell,

a candidate for the degree of master of sciences,

and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Brenda Peculis

Dr. Gerald Hazelbauer

Dr. David Emerich

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The Sophomore Biochemistry Lab, Biochem 2002, is an elective course in the Biochemistry Department. Over the past 3 years, this laboratory course was overseen by various instructors, Postdoctoral fellows, Teaching Assistants and Peer Learning Assistants. These individuals created, modified or adapted a variety of laboratory experiments and generated the starting material which became the foundation of this thesis.

The attached body of work was generated in partial completion of the requirements for the Masters degree in Biochemistry. I acknowledge the assistance of previous contributors and the ongoing support of several people who gave oversight during the development and implementation of this course development activity including: Jan Judy-March, Dr. Benda Peculis, Dr. Christopher Foote, Dr. David Emerich, Dr. Shuqun Zhang, Dr. Norma Houston, and Dr. Astrid Stromhaug.

This body of work was generated with the specific intent that the documents and materials described here would continue to be used by future Biochemistry Department faculty and appointed instructors of this course in future years. I give my permission for the Biochemistry Department to use these materials in teaching this class in future years.

TABLE OF CONTENTS

Acknowledgements.....	ii
Abstract.....	v
Lab 1: Safety, Pipetting, Solutions, and Dilutions	1
Lab 2: DNA Extraction and PCR	12
Lab 3: Agarose Gel, PCR Purification, and Restriction Digest	19
Lab 4: Gel Extraction, PCR Purification, and Ligation	25
Lab 5: Ligation Gel, Transformations, and Picking Colonies	29
Lab 6: Minipreps and Restriction Digest.....	34
Lab 7: Identification of Successful Transformants.....	36
Lab 8: PTP1 Purification by His-Tag chromatography and Quantification by Bradford Assay	37
Lab 9: SDS-PAGE and Dialysis.....	43
Lab 10:Effect of pH on Enzyme Activity	48
Vita	55

Abstract

The University of Missouri Biochemistry department offers a variety of courses, each designed with a specific purpose and scheduled to avoid overlap. Biochemistry 2002, sophomore lab, is currently an elective course meant to bridge the gap between the introductory Biochemistry 1090 offered to freshmen and Biochemistry 4270, a junior level class. In the past, the course has been burdened by inefficient experiments and lacking in both continuity and structure. My ultimate goal is to improve course coherence by redesigning experiments to increase classroom success rate, cataloging materials to facilitate lab setup, establishing realistic learning objectives, and generating new assignments to be compiled on the university H-drive. The course is subdivided into DNA Cloning and Enzyme Kinetics laboratories. The objective of the first seven weeks is to clone a tobacco acid phosphatase gene, NtPTP1, into the PET28a expression vector. The final four weeks introduces students to a protocol designed to purify the PTP1 protein from an E. coli cell lysate and analyze the activity of the enzyme at several pHs. Upon completion of the course, a solid framework of experiments and concepts has been established, all necessary materials have been catalogued, and a comprehensive list of potential issues has been outlined for future instructors. A new professor will no doubt find it imperative to adjust learning objectives to suit his/her specific teaching goals and personal experience; however, the preexisting foundation should minimize preparation time.

Name: _____
Lab day: M T W

Lab 1

Safety, pipetting, solutions, and dilutions

INTRODUCTION

Objectives

- To learn the operation and calibration of an air displacement pipette.
- To learn the proper use of the electronic balance and to learn the basics of solution preparation.
- To perform a serial dilution.

Safety

When you work in a lab you need to know:

- Where to find the emergency eye-wash and showers, and how they work.
- Where to find the fire extinguisher and First Aid kit.
- Take care when you handle chemicals and solutions.
- Inform your instructor or PLA about spills and broken glassware so proper action can be taken.

Eating, drinking, smoking etc. in the lab is absolutely forbidden. Everyone is required to wear closed-toe shoes and long pants. Special protective measures should be taken when working with hazardous chemicals (gloves).

We will let you know when you will be handling hazardous chemicals. If you believe that you have a health condition that puts you at risk, or believe yourself to be pregnant, please see your instructor in private to discuss the issue.

Equipment and lab drawer contents

You are assigned one lab drawer. It is your responsibility to keep your blue and yellow tip boxes filled. Tips will be set out in the lab.

It is also your responsibility to clean all glassware you have used and put it back in your drawer. Also, make sure your lab space is clean and tidy before you leave. Let us know if you break something or if something is missing so we can replace it.

Pipetting

In biochemistry, the ability to accurately and reproducibly measure and transfer small volumes of liquids is critical for obtaining useful results. In this experiment you will learn how to use air-displacement pipettes of different sizes and determine both their accuracy and precision.

We have four different air-displacement pipettes in the lab:

P-1000: measures volumes from 200 – 1000 μl

P-200: measures volumes from 40 – 200 μl

P-40: measures volumes from 5 – 40 μl

P-10: measures volumes from 0.5 – 10 μl

Make sure you use the correct pipette for the volume you need. Pipettes are usually the least accurate at the lower end of the range.

Disposable tips are always used. Yellow tips fit the smaller pipettes (those with a maximum range of 200 μl or less) and blue tip fit the large pipettes (with range from 200 μl to 1000 μl). When attaching the tip, make certain that the tip is the correct type for the pipette you are using, and that the tip is properly seated on the end of the pipette. Do not contaminate the tip by touching it.

Solutions

Concentration is the amount of solute in a solution and is generally expressed in terms of the amount of solute in a given volume of solution. The molarity (M) of a solution is the concentration expressed as the number of moles of solute per liter of solution (mol/L).

This is the same as the number of millimoles (mmol) of solute per milliliter (ml) of solution. Mass molecular weight and moles are related as follows:

$$\frac{\text{mass in grams}}{\text{molecular weight}} = \text{number of moles}$$

$$\text{volume (in L)} \times \text{molarity (in mol/L)} = \text{number of moles}$$

Here is an example of how you can use these relationships when making a solution. You want to prepare 1.0 L of a 1 M solution of sucrose. The molecular weight of sucrose is 342.3 g/mol. How many grams of sucrose do you need?

By using the relationships shown above we can derive the following formula:

$$\text{molecular weight} \times \text{molarity} \times \text{number of liters to be prepared} = \text{mass in grams of solute needed}$$
$$342.3 \text{ g/mol} \times 1 \text{ mol/L} \times 1 \text{ L} = 342.3 \text{ g sucrose.}$$

To prepare the solution you have to weigh 342.3 g sucrose, dissolve it in a little less than 1 L of distilled or de-ionized water and adjust the final volume to exactly 1.0 L.

Accuracy and precision

When you make measurements in science you want them to be both precise and accurate. *Accuracy* indicates how correct your measurement is; in other words, how close your experimental value ($V_{\text{experimental}}, V_e$) is to the accepted or true value (V_{accepted}, V_a). Scientists often express the error in a measurement by the absolute error (E) or the percent error (% E). The E is the difference between your experimental value and the accepted value:

$$\text{Absolute error (E)} = V_a - V_e.$$

$$\text{The percent error (\% E)} = \frac{V_a - V_e}{V_a} \times 100 \%$$

Precision is a measure of reproducibility (getting the same measurement each time). The precision of a set of measurements can be reported by the standard deviation (SD) of the mean. As you remember, the SD tells us how precise the mean is; in other words, how closely the data are clustered around the mean. The formula for standard deviation is shown below:

$$\text{Standard deviation, SD} = \sqrt{\frac{\sum_{i=1}^N (x_i - \bar{x})^2}{N - 1}}$$

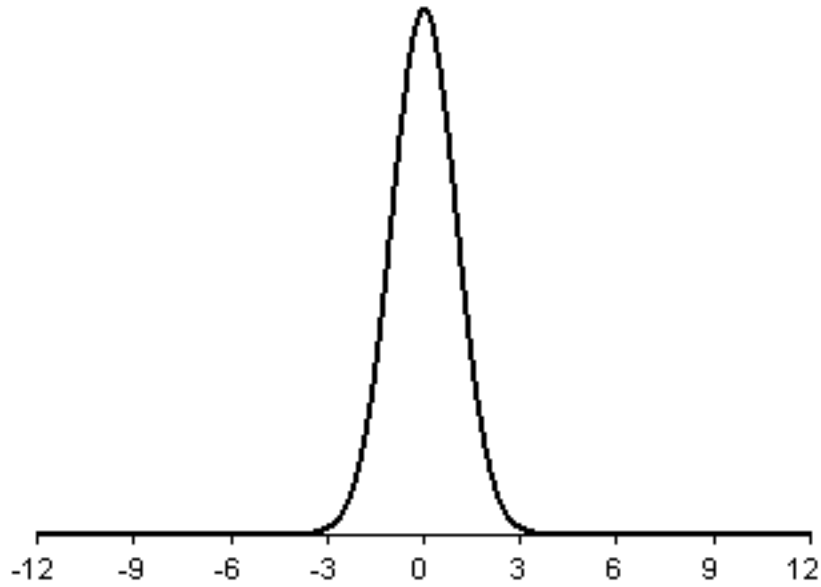
where x_i, \dots are the individual measurements, \bar{x} is the average (mean) of the measurements and N is the number of measurements.

WHAT IS STANDARD DEVIATION?

The standard deviation is a measure of how spread out your data is. Computation of the standard deviation is a bit tedious. The steps are:

1. Compute the mean for the data set.
2. Compute the deviation by subtracting the mean from each value.
3. Square each individual deviation.
4. Add up the squared deviations.
5. Divide by one less than the sample size.
6. Take the square root.

Suppose your data follows the classic bell shaped curve pattern. One conceptual way to think about the standard deviation is that it is a measure of how spread out the bell is. Shown below is a bell shaped curve with a standard deviation of 1. Notice how tightly concentrated the distribution is.



Shown below is a different bell shaped curve, one with a standard deviation of 2. Notice that the curve is wider, which implies that the data are less concentrated and more spread out.

Finally, a bell shaped curve with a standard deviation of 3 appears below. This curve shows the most spread.



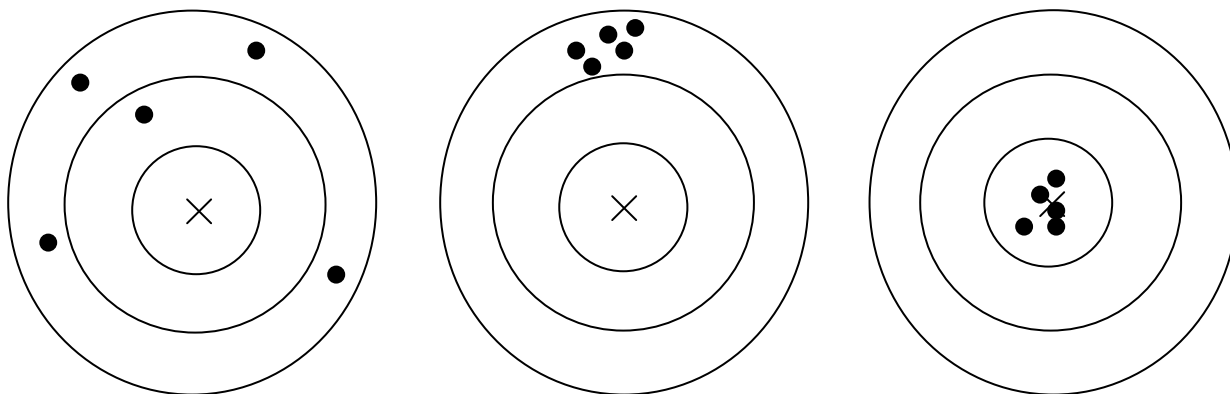
Example

Let's examine a standard deviation computation for data on PI max values in a sample of children with cystic fibrosis. **The seven values in this data set are 73, 58, 67, 93, 33, 18, and 147. The mean for this data set is 69.9.**

$(73-69.9)^2$	=	$(3.1)^2$	=	9.61
$(58-69.9)^2$	=	$(-11.9)^2$	=	141.61
$(67-69.9)^2$	=	$(-2.9)^2$	=	8.41
$(93-69.9)^2$	=	$(23.1)^2$	=	533.61
$(33-69.9)^2$	=	$(-36.9)^2$	=	1361.61
$(18-69.9)^2$	=	$(-51.9)^2$	=	2693.61
$(147-69.9)^2$	=	$(77.1)^2$	=	5944.41

For each data value, compute the squared deviation by subtracting the mean and then squaring the result. **The sum of these squared deviations is 10,692.87. Divide by 6 to get 1782.15. Take the square root of this value to get the standard deviation, 42.2.**

Let's look at throwing darts and trying to hit the "bull's eye" as an illustration of the two concepts accuracy and precision:



**Not precise
or accurate**

**Precise but
not accurate**

**Precise and
accurate**

(Source:<http://grandinetti.org/Teaching/Chem121/Lectures/MeasurementUncertainty/index.html>)

We can check the *accuracy* and the *precision* of a pipette by a process called calibration. When we calibrate the air-displacement pipettes we use the relationship between the volume and the weight of water which is as follows:

- a) the weight of 1 L water is 1000 g
- b) the weight of 1 mL water is 1 g
- c) the weight of 1 μ L water is 1 mg or 0.001 g

By weighing different volumes of water being dispensed by your pipettes you can determine the accuracy and precision of your pipettes.

PROCEDURE 1

How to use an air-displacement pipette

Take one pipette out of the rack. Try depressing the plunger. As the plunger depresses, you will feel a sudden increase in resistance. This is the first “stop”. If you continue pushing, you will reach the second “stop”.

Attach the correct tip to a pipette. Identify the following parts on the pipette:

1. Plunger
2. Tip ejector button
3. Tip ejector
4. Volume control knob
5. Volume window
6. Pipette tip

You can now practice using the pipette:

1. Depress the plunger to the first “stop”.
2. Place the tip into the liquid just below the surface of the solution.
3. Slowly and in a controlled manner, allow the plunger to move upward as solution is drawn into the tip. Do not allow air bubbles into the tip.
4. After the plunger is all the way up, hold the tip in the solution for several seconds to ensure the solution is no longer moving into the tip. This is particularly important if the solution is viscous.
5. Remove the tip from the solution and move the pipette to the receiving vessel.
6. Slowly depress the plunger to the first “stop” and then past the second “stop”. Allow time for the aliquot to drain from the tip. For small volumes the pipette should be rinsed by slowly pumping the solution in and out several times while the tip is in the solution.
7. Take pipette out of receiving vessel and dispose of the tip in the correct disposal container. Do this by pressing down on the ejector. Tips may not be discarded in regular trash; they must be discarded in the sharps (glass) trash.
8. When finished using the pipette, adjust setting to the highest volume and hang the pipette on the rack. Adjusting the setting preserves the spring as long as possible and hanging the pipette upright allows any liquid that may have been aspirated to drain out.

PROCEDURE 2

Calibration of air displacement pipettes

Get a P-200 (range 40 – 200 μl) and a P-10 (range 10 – 1 μl).

You also need to use an electronic balance with an accuracy of 0.001 g. Why?

Choose one pipette and place the appropriate tip on the pipette. Set the pipette to its lower limit (40 μl or 1 μl).

1. Place a beaker of water beside the balance.
2. Place a weighing boat on the balance and tare the balance to zero by pressing the tare button once.
3. Transfer the designated volume of water to the weighing boat. Record the weight of the water in table 1 in the lab report.
4. Repeat this procedure two times more so you have three values. Set the pipette to the intermediate volume, and repeat 3-4. Record data in table 1.
5. Set the pipette to the upper limit volume, and repeat 3-4. Record data in table 1.
6. Select the other pipette and repeat step 2-6.

Calibration of air displacement pipettes

Table 1: Data for accuracy and precision

	P10			P200		
	Lowest volume	Intermediate volume	Highest volume	Lowest volume	Intermediate volume	Highest volume
	1 μl		10 μl	40 μl		200 μl
Mass in grams (g)						
Mean (g)						
Standard Deviation (SD)						
Absolute Error (E)						
Percent Error (%E)						

1. Calculate the mean mass in grams for each volume. Show one calculation below.
2. Calculate the SD for each mean. Show one calculation below.
3. Calculate the absolute error (E) for each volume. Show one calculation below.
4. Calculate the percent error (% E) for each volume. Show one calculation below.

Questions:

1. In your opinion, which pipette was on the whole the most accurate? Explain your reasoning.
2. In your opinion, which pipette was on the whole the most precise? Explain your reasoning.

Procedure 3

Make a serial dilution using colored water.

You have in front of you a .01mg/mL sample with a MW of 100g/mol. Using the equipment provide create a 1nM solution. Show all calculations. *Hint: 1nM is 10^{-9} molar*

When you have completed this exercise, have an instructor or PLA check your work.

Equipment identification

You will be only one drawer at your work space. Check off the items in your drawer on the list below. You will share these items with students in other sections so it is your responsibility to put things back and keep everything clean. Let the instructors help you identify the items if you are not sure what they are.

Lab drawer arrangement:

Biochem 2002 Laboratory Drawer Checklist

Personal Drawer # _____

Beaker set: 1 each of 600 ml____, 400 ml____, 250 ml____, 150 ml_____

2 Erlenmeyer large flasks (200, 250 or 300 ml)_____

Graduated cylinder set 1 each: 50 ml____ 25 ml_____

1 Funnel plastic_____

1 watch glass _____

1 Amber bulb_____

1 stirring rod_____

100 ml volumetric flask with lid_____

1 scraper_____

1 box 1 ml pipette tips-blue
(filled)____

1 box 200 ul tips-yellow
(filled)____

1 green pi-pump lg _____

1 blue pi-pump sm _____

Student sign: _____

TA or Prof:_____

Name: _____
Lab day: M T W

Lab 2

DNA extraction and PCR

INTRODUCTION

Objectives

The isolation of high molecular weight chromosomal DNA is often the first step in molecular cloning (genetic engineering). Today we will start the cloning of the NtPTP1 gene which codes for a protein tyrosine phosphatase. Today's objectives are:

- Extract DNA from E. coli.
- Determine the amount and purity of the extracted DNA.
- Use the extracted DNA as a template to amplify the NtPTP1 gene by Polymerase Chain Reaction (PCR).

DNA extraction

The NtPTP1 gene codes for a phosphotyrosine phosphatase in tobacco plants. The original gene contained introns, so the mRNA was isolated from the organism and turned into an "intron free" cDNA and amplified by a process known as reverse transcriptase PCR. The gene was cloned into the PET28a vector using the restriction enzymes NdeI and XhoI. The newly formed vector was then transformed into E.coli and frozen. If the gene had not contained introns we could have isolated the genomic DNA of the tobacco plant by lysing the cells and performing a phenol chloroform extraction; however because the gene was previously cloned, we will be using the Qiagen miniprep kit to extract the vector DNA from E. coli. This will give us a high starting concentration of our gene and your PCR should yield a lot of product.

A closer look at the DNA extraction procedure

The purpose of the first section of today's lab is to isolate plasmid DNA from a strain of E. coli, and quantify it using the Tecan. We will be using a kit from Qiagen which doesn't require a phenol chloroform extraction step. A major consideration in DNA isolation procedures is the inhibition or inactivation of DNases, which can hydrolyze DNA. The buffer in which the cells are resuspended (P1) should have a slightly basic pH (8 or greater), which is above the optimum of most DNases. EDTA is also included in the resuspension buffer to remove divalent cations (such as Mg^{2+} and Ca^{2+}), which are required by DNases. DNase activity is further controlled by keeping cells and reagents cold. P2 is a lysis buffer that disrupts the cellular membranes of E.coli releasing DNA into the solution. N3 is a precipitation buffer that makes cellular debris insoluble. When you spin the mixture the membranes will pellet in the bottom of the tube leaving the

DNA in the supernatant. PB and PE are both wash buffers that will “clean” your DNA after it binds to the spin column.

The procedure consists of three basic steps:

- Preparation and clearing of a bacterial lysate
- Adsorption of DNA onto the QIAprep membrane
- Washing and elution of plasmid DNA

Centrifugation notes

- All centrifugation steps are carried out at 13,000 rpm (~17,900 x g) in a conventional, table-top microcentrifuge.

Polymerase Chain Reaction (PCR) Step by Step

PCR is today a common and often indispensable technique used in medical and biological research labs for a variety of applications including [DNA cloning](#).

In PCR a [DNA polymerase](#) is used to amplify a piece of [DNA](#) by [in vitro enzymatic replication](#). As PCR progresses, the DNA generated is used as a template for replication. This sets in motion a [chain reaction](#) in which the DNA template is [exponentially](#) amplified generating millions or more copies of the DNA piece.

PCR involves a repetitive series of cycles, each of which consists of template denaturation, primer annealing, and extension of the annealed primer by Taq DNA polymerase. Following DNA sample preparation, the template DNA, oligonucleotide primers, thermostable DNA polymerase (Taq), the four deoxynucleotides (A, T, G, C), and reaction buffer are mixed in a single micro test tube. The tube is placed into a thermal cycler. The rapid heating and cooling of this thermal block is called *temperature cycling* or *thermal cycling*.

The first step of the PCR temperature cycling procedure involves heating the sample to 94°C. At this high temperature, the template strands separate (denature). This is called the *denaturation step*.

The thermal cycler then rapidly cools to 50 - 68°C to allow the primers to anneal to the separated template strands. This is called the *annealing step*. The two original template strands may reanneal to each other or compete with the primers for the primers' complementary binding sites. However, the primers are added in excess such that the primers actually out-compete the original DNA strands for the primers' complementary binding sites.

Last, the thermal cycler heats the sample to 72°C for Taq DNA polymerase to extend the primers and make complete copies of each template DNA strand. This is called the *extension step*. Taq polymerase works most efficiently at this temperature. Two new copies of each complementary strand are created. There are now two sets of double-stranded DNA (dsDNA). These two sets of dsDNA can now be used for another cycle and subsequent strand synthesis. At this stage, a complete temperature cycle (thermal cycle) has been completed.

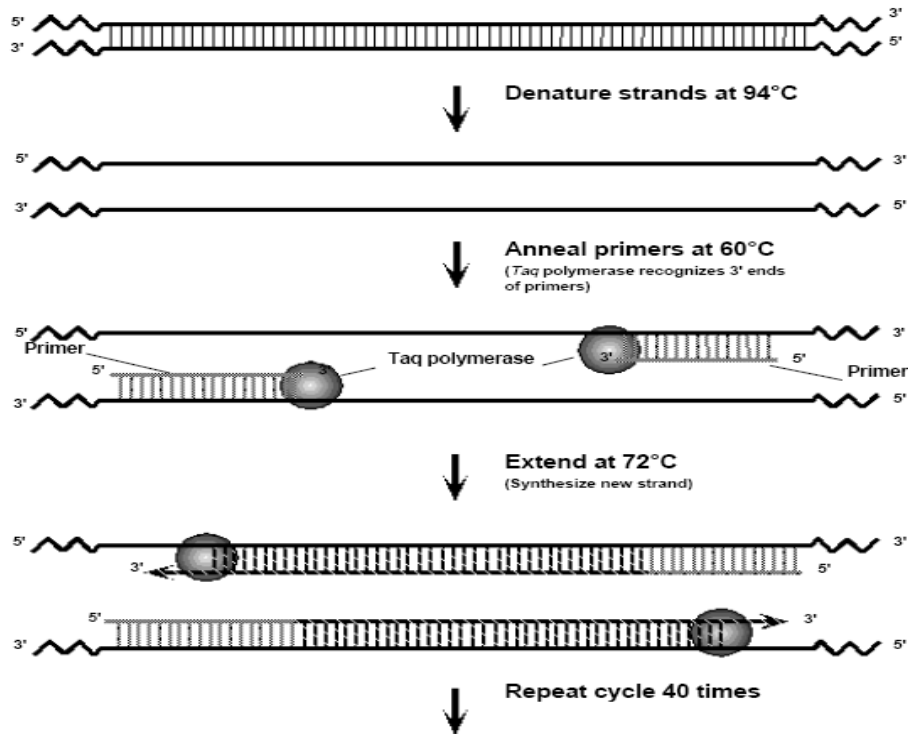


Fig. 4. A complete cycle of PCR.

Usually, thermal cycling continues for about 40 cycles. After each thermal cycle, the number of template strands doubles, resulting in an exponential increase in the number of template DNA strands. After 40 cycles there will be 1.1×10^{12} more copies of the original number of template DNA molecules. PCR generates DNA of a precise length and sequence. On the first cycle, the two primers anneal to the original genomic template DNA strands at opposite ends and on opposite strands. After the first complete temperature cycle, two new strands are generated that are shorter than the original template strands but still longer than the length of the DNA that the researcher wants to amplify. It isn't until the third thermal cycle that fragments of the precise length are

generated.

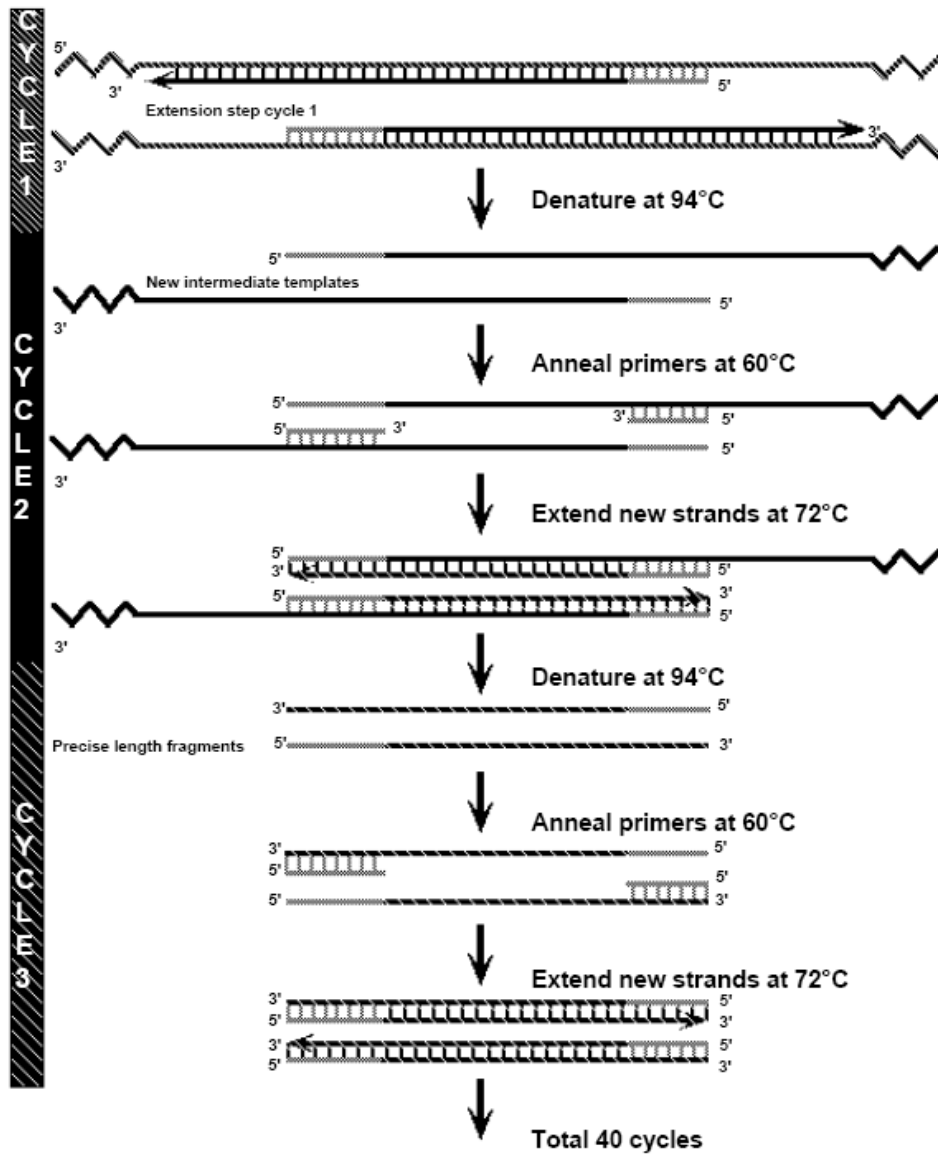


Fig. 5. Generation of precise-length fragments.

It is the template strands of the precise length that are amplified exponentially (X^n , where X = the number of original template strands and n = the number of cycles). There is always one set of original long-template DNA molecules which is never fully duplicated. After each thermal cycle, two intermediate-length strands are produced, but because they can only be generated from the original template strands, the intermediate strands are not exponentially amplified. It is the precise-length strands generated from the intermediate strands that amplify exponentially at each cycle. Therefore, if 20 thermal cycles were conducted from one double-stranded DNA molecule, there would be 1 set of original

genomic template DNA strands, 20 sets of intermediate template strands, and 1,048,576 sets of precise-length template strands. After 40 cycles, there would be 1 set of original genomic template DNA strands, 40 sets of intermediate template strands, and 1.1×10^{12} sets of precise-length template strands (Figure 6).

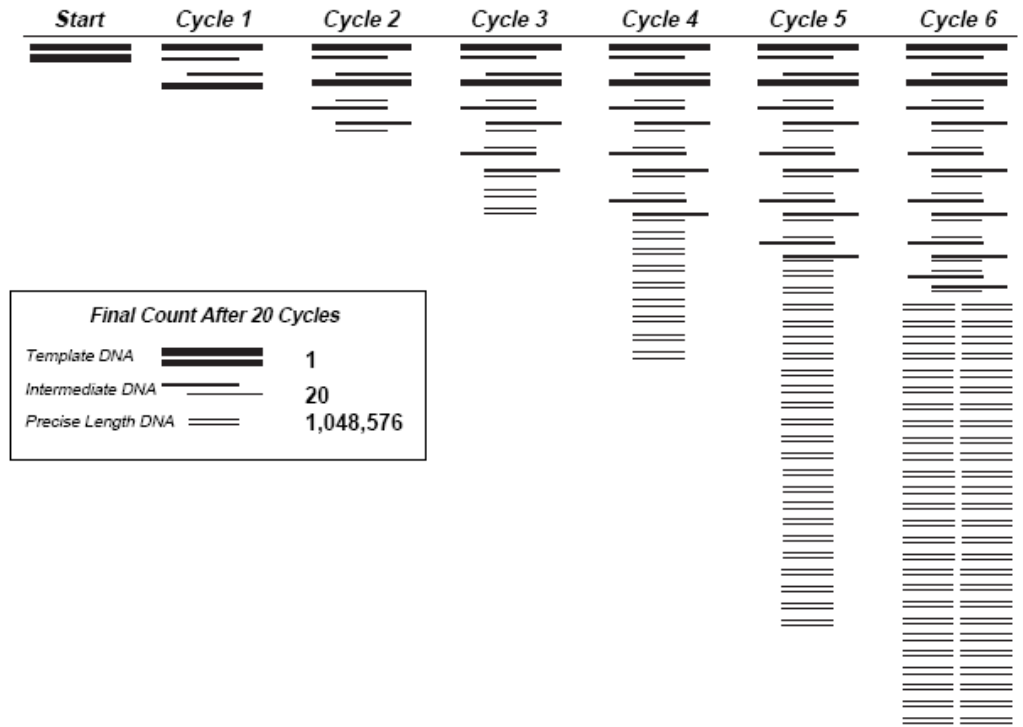


Fig. 6. Schematic of PCR amplification of DNA fragments.

Protocols

DNA Extraction: Obtain a 1.5 mL tube of E. coli NtPTP1/PET28a from the instructor, and spin at 8000 rpm for 3-5 minutes. Pour off the supernatant and, with a pipet, remove as much of the residual supernatant as possible without disturbing the pellet. For the next steps refer to *Qiagen MiniPrep Kit Protocol Step 2*. Note: in the elution step (Step 10), use 50 μ L of dH₂O and let sit for 10 minutes before centrifugation. This will increase your final DNA yield. dH₂O, or deionized water, is purified water that we use for experiments. It comes from the white tap in the sink or has been autoclaved (super-heated and pressurized) and put in jars at your bench. Do not use the regular faucet for experiments.

DNA Quantification:

Tecan Infinite M200 Instructions

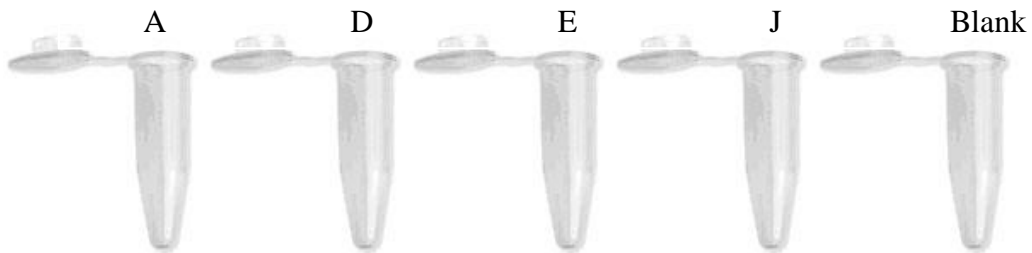
- 1) Turn on the computer and allow it to start up, do NOT turn on the Tecan unit yet.
- 2) Open the “i-control” software. An icon for the program should be on the desktop.
- 3) Immediately after launching the program, turn on the plate reader (power switch is located on the back of the unit, above the power cord)
- 4) The software should automatically recognize the unit. If you are prompted to select an instrument, select Infinite M200 and make sure the box for “reconnect to selected instrument at next start up” is checked.

NOTE: If the unit is not displayed then power cycle the unit, cancel the instrument selection dialog box, and click on either the USB icon or navigate to “Instrument... >> Connect to instrument” to bring up the prompt again. The instrument should now be listed. If you are still having trouble, ask an instructor.

- 5) You will then be prompted to open a template. Select “Default script” and click ok.
- 6) Select the applications tab at the bottom left part of the window
- 7) Double click *Nucleic acid quantification*, NanoQuant Plate Tecan 16 Flat Black should be automatically selected
- 8) Select the “Individual Blanking” box to the right of the plate on the screen and make sure dsDNA is selected from the pull down box underneath.
- 9) Select the wells you intend to use for the assay and load 1 μ L of dH₂O to each. Every well used must be blanked beforehand.
- 10) Click “Start Blanking” and the plate transport will move out for the plate. Put the plate on the transport and select OK.
- 11) After a successful blank the screen will change to a green color. Select the wells in which samples are loaded (by dragging) and click the Start button on the top left of the screen.
- 12) The plate transport will eject, but click OK (if all necessary samples are loaded) and wait for measurement to complete. An excel spreadsheet will be automatically created.
- 13) Record your DNA concentration

PCR:

- Each .2 mL PCR tube will contain
 - 2 μL ($\sim 100\text{ng}$) DNA from steps 1 and 2
 - 2.5 μL of each 10 mM primer (for and rev)
 - .5 μL Taq Polymerase
 - 15.3 μL of epicentre master mix (A, D, E, or J)
 - dH₂O to make final volume 30 μL (Dependent on DNA conc.)
- There will be a total of 5 samples, one with each epicentre master mix and a blank sample that has no DNA. Note: For the blank, use the D Epicentre mix. The blank is necessary to provide a negative control and demonstrate what happens when the experiment doesn't work.



- Create a “master mix” of primer, Taq, and dH₂O which contains enough material for 6 samples and aliquot appropriate amount into your 5 PCR tubes. **A master mix is a mixture of like components of an experiment so that they be pipetted quickly and precisely. It will help reduce disparity between samples.** Note: make sure to try to keep any sample with enzyme on ice until you are ready to place it in the PCR.
- Add a different epicentre mix to each sample and make sure to label your tubes accordingly so that you can recognize them next class period.
- Add 1-2 μL of your DNA sample (depending on your final DNA conc.) and place your samples in the appropriate PCR machine.
 - For this experiment, half the class will use a 50°C annealing temperature and the other half will use 60°C. This should demonstrate the effect of temperature on PCR efficiency.
 - The PCR cycler is programmed as follows:
 - 95°C for 2 minutes
 - 95°C for 30 seconds
 - 50°C or 60°C for 30 seconds
 - 72°C for 1.5 minutes
 - 72°C for 10 minutes

	}	1 cycle
	}	40 cycles
	}	1 cycle
 - The instructor will place the samples in the -20°C freezer after the protocol is finished for next week's lab.

Name: _____
Lab session: M T W

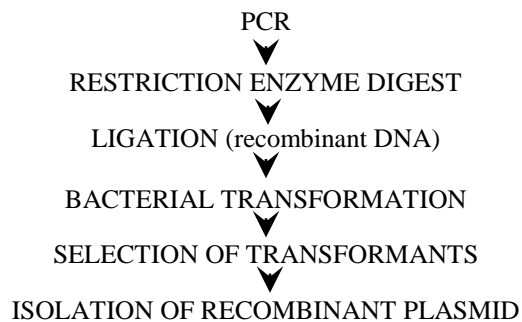
Lab 3

Agarose Gel Electrophoresis, PCR Purification, and Restriction Enzyme Digest

INTRODUCTION

Objectives

In last week's lab Polymerase Chain Reaction (PCR) was used to amplify the NtPTP1 gene from *E. coli* DNA. In the next 3 laboratories this gene will be cloned into a vector and subsequently transformed into *E. coli*. A flow chart of the cloning procedures/methods we will use is shown below.



In this laboratory you will identify the set of PCR parameters that correctly amplified your gene, purify the product, and digest the amplified DNA fragment (NtPTP1 gene) and an expression vector (pET 28) with restriction enzymes to generate complementary “sticky” ends. This will enable us to insert the gene into the vector (create recombinant DNA) which we are doing in the next lab period by a process called ligation. After the restriction enzyme cutting you will separate the digested products by size on an agarose gel to check that your cutting has been successful.

Agarose gel electrophoresis

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA or RNA molecules by charge and size. Nucleic acid is negatively charged at neutral pH because of the presence of phosphate groups. When these negatively charged molecules are placed in an electrical field between two electrodes, they all migrate toward the positive electrode.

Separation of fragment by size can be achieved because smaller fragments move farther than longer fragments in the agarose matrix in a given amount of time. Increasing

the agarose concentration of the gel reduces migration speed and enables separation of smaller DNA molecules. The higher the voltage, the faster DNA migrates. Voltage is limited though, by the fact that it heats and ultimately causes the gel to melt.

Before we load our sample on to the gel we add loading buffer to our samples. The loading buffer visualizes our sample with a dye and sediments it into the gel well. We also need to add a dye that can bind DNA so the DNA bands can be visualized after the gel electrophoresis is completed. The most common dye used to visualize DNA in an agarose gel is ethidium bromide (EtBr). It fluoresces under UV light when intercalated into DNA or RNA (absorbs invisible UV light and transmits the energy as visible orange light). A disadvantage with EtBr is that it is a carcinogen but alternative non-toxic dyes are available. The dye we will be using is the non-toxic SYBRsafe DNA gel stain from Invitrogen. This dye also intercalates DNA but it fluoresces when illuminated with blue light. The gel can then be photographed. The DNA can also be cut out of the gel and dissolved to retrieve the purified DNA

You will only be running ~10% of your PCR on the gel. After the gel has run you will be able determine which of your PCR reactions worked, which didn't, and which samples you will be purifying and using for the restriction digest.

PCR Purification

We will be using a PCR purification kit from Qiagen which is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions

What is cloning?

The methods used to accomplish DNA cloning are referred to as “recombinant DNA technology” or “genetic engineering”.

DNA cloning generally involves:

- 1) Separating a specific gene or DNA segment from a larger chromosome. This is usually done by cleaving the chromosome with a restriction enzyme to obtain the fragment of interest or by amplifying the specific region of DNA by PCR.
- 2) Ligation of the DNA segment into a cloning vector (plasmids or viral DNAs) to form recombinant DNA.
- 3) Moving the recombinant DNA into a host cell that will provide the enzymatic machinery for replication (transformation).
- 4) Selecting and identifying host cells that contain recombinant DNA.

Please click on the link below to see and excellent animation of the cloning steps:

<http://www.sumanasinc.com/webcontent/animations/content/plasmidcloning.html>

Restriction enzyme digest

Different species of bacteria make different restriction nucleases, which protect them from bacteriophages (viruses that infect bacteria) by degrading incoming DNA.

Restriction enzymes cleave double stranded DNA by hydrolyzing the backbone of DNA between deoxyribose and phosphate groups. This leaves a phosphate group on the 5` ends and a hydroxyl on the 3` end of both strands. A few restriction enzymes will cleave single stranded DNA, although usually at low efficiency.

Each nuclease recognizes a specific sequence of four to eight nucleotides in DNA called recognition sequences. These sequences in the genome of bacteria themselves are protected by methylation at an A or C residue: the sequences in foreign DNA are generally not methylated and so are cleaved by restriction nucleases.

Large numbers of restriction nucleases have been purified from various species of bacteria and are catalogued according to their restriction sites. Some restriction enzymes make staggered cuts on the two DNA strands, leaving two to four nucleotides of one strand unpaired at each resulting end. These unpaired strands are called “sticky ends”, because they can base pair with each other. Other restriction enzymes cleave both strands of DNA at the opposing phosphodiester bonds, leaving no unpaired bases on the ends, often called “blunt ends”.

Each of the primers we used in the PCR reaction was designed to contain a restriction enzyme cutting site. The forward primer contained a Nde I cutting site and the reverse primer a Sac I cutting site.

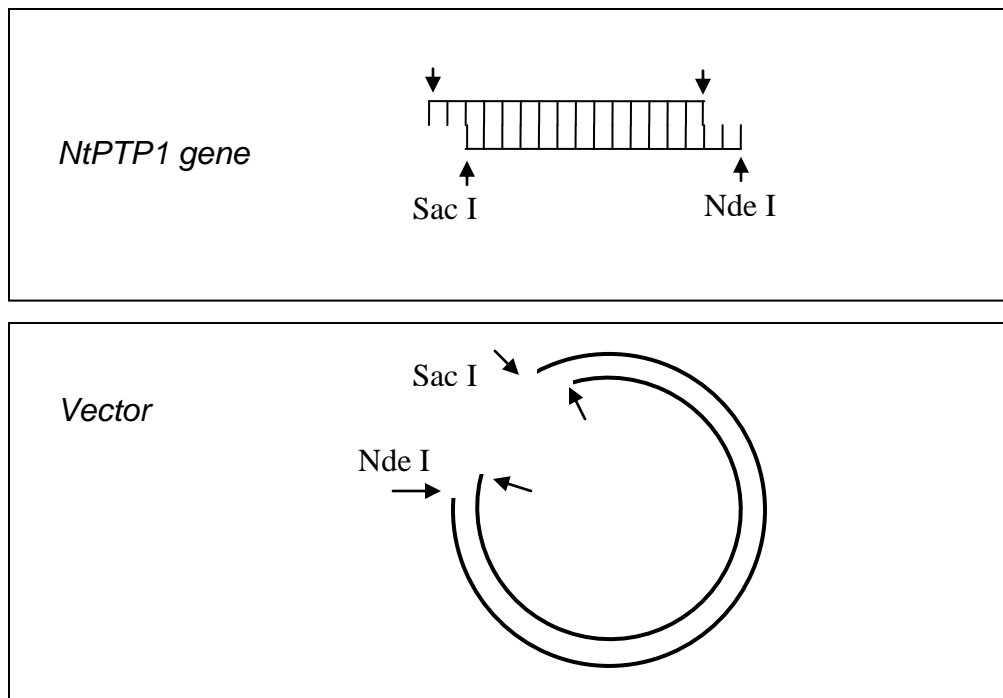
Forward NtPTP1 primer:

5' – AGA TCG **CAT ATG** GCT GCC GCC GGT AAT CCC TT – 3'
 Nde I
 cutting site

Reverse NtPTP1 primer:

5' – TAA AGT **GAG CTC** TGG AGT CTT CTC TCC ATT GTC AT – 3'
 Sac I
 cutting site

This means that our amplified DNA fragment (NtPTP1 gene) contains these restriction enzyme cutting sites at each end. The expression vector we are using also contains cutting sites for these enzymes in the polylinker (see page 11). We will digest both the DNA fragments and the pET28 vector with Sac I and Nde I to make complementary “sticky ends” as shown below.



Cloning vectors

Most cloning vectors are plasmids or bacteriophages. Plasmids are circular DNA molecules that can replicate separately from the host chromosome. Many different plasmid vectors suitable for cloning have been developed by modifying naturally occurring plasmids. Typically a cloning vector will contain (see below):

- A) an *origin* of replication (Ori)
- B) a *selectable genetic marker* (usually an antibiotic resistance gene)
- C) a *polylinker* with restriction enzyme cutting sites. The polylinker is where the foreign DNA can be inserted.

Expression vectors

Expression vectors are one type of cloning vectors. Expression vectors are used when you are interested in studying the protein product of the cloned gene, rather than the gene itself. Expression vectors contain the *transcription* and *translation signals* needed for the regulated protein expression of a cloned gene (see below). The pET28vector we are using in this laboratory is an expression vector which is especially useful for expressing large amounts of the cloned protein in bacterial cells.

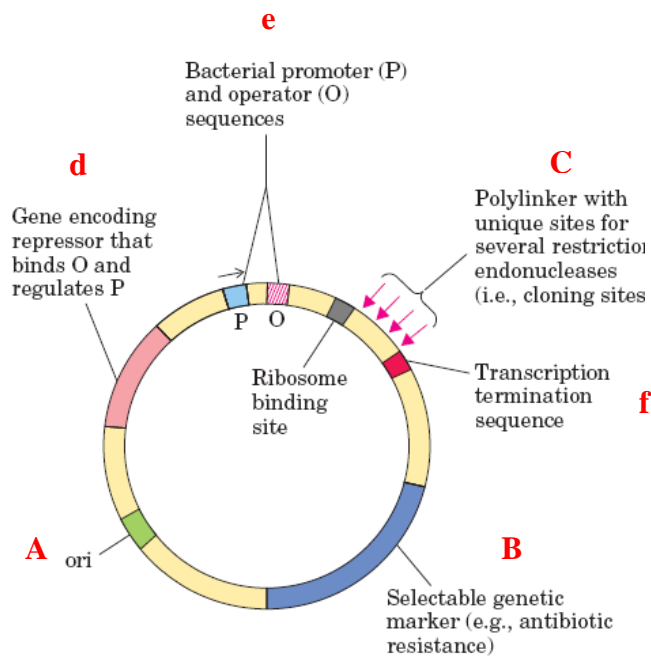


Figure 1.

pET-28a-c(+) Vectors

TB074 12/98

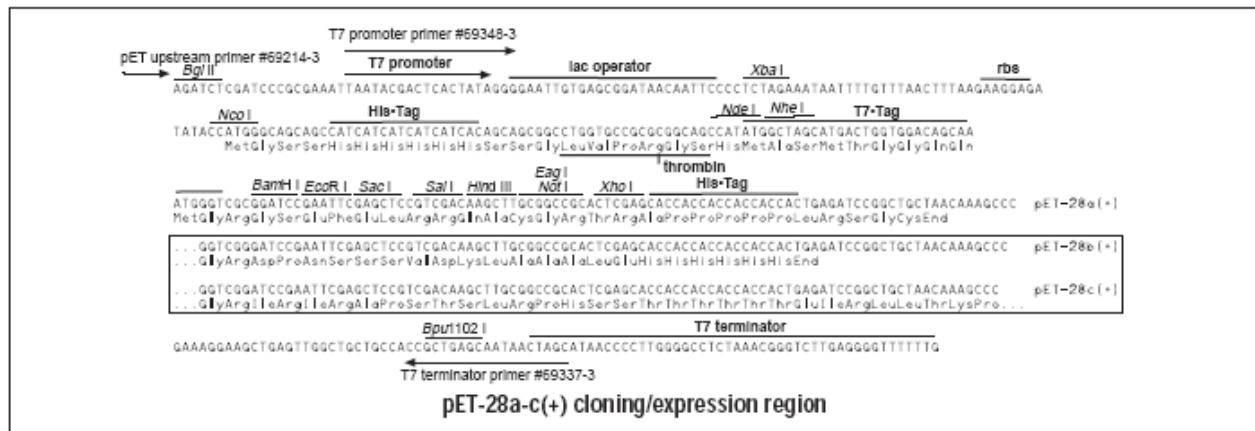
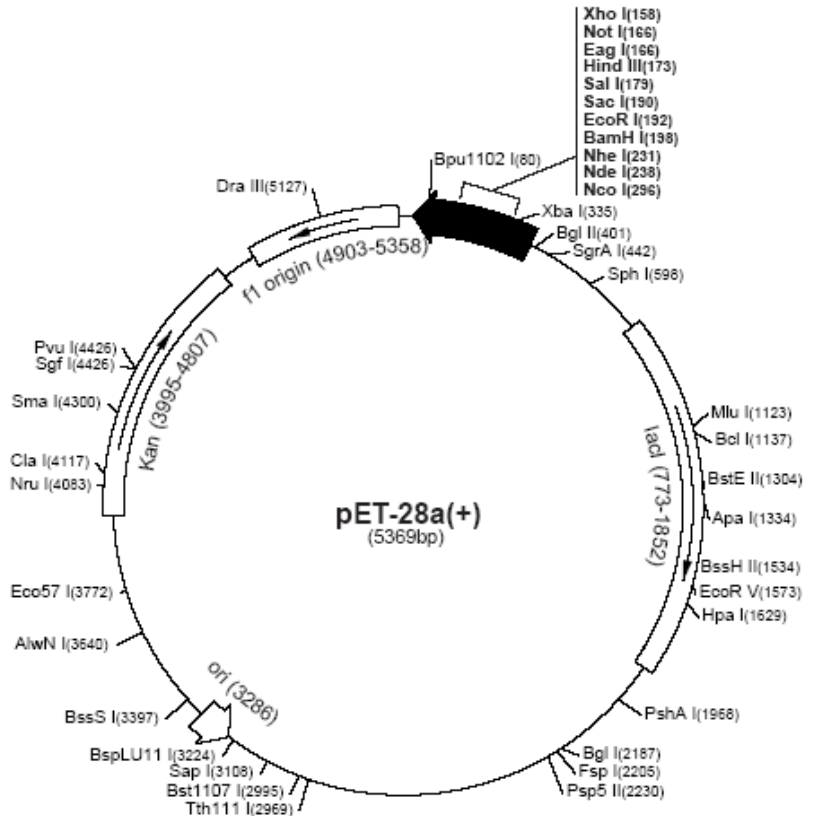
	Cat. No.
pET-28a DNA	69864-3
pET-28b DNA	69865-3
pET-28c DNA	69866-3

The pET-28a-c(+) vectors carry an N-terminal His*Tag*/thrombin/T7*Tag* configuration plus an optional C-terminal His*Tag sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3).

pET-28a(+) sequence landmarks

T7 promoter	370-386
T7 transcription start	369
His*Tag coding sequence	270-287
T7*Tag coding sequence	207-239
Multiple cloning sites (<i>Bam</i> H I - <i>Xho</i> I)	158-203
His*Tag coding sequence	140-157
T7 terminator	26-72
<i>lac</i> I coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.



Protocols

Agarose Gel Electrophoresis

0.7% agarose gels will already be poured in the essence of time. Collect the 5 PCR samples (from last week) from the instructor and begin making a master mix of dH₂O and 6X loading dye (enough for 6 samples). Aliquot the appropriate amount of master mix to each sample and tube add 10% of the PCR rxn. Each new sample tube should contain:

3 μ L of PCR rxn (A,D,E,J, or Blank)
3.3 μ L 6X loading dye
13.7 μ L dH₂O
20 μ L total

There should be 5 tubes, each with a different PCR sample. Turn the gel so that it runs black to red and fill the gel rig to the “fill line” with TBE buffer. Once submerged CAREFULLY remove the comb. Obtain the 1kb DNA ladder from the instructor and begin loading the gel, making note of the order of the samples.

When the samples are loaded, slide the top of the gel rig into place, turn on the power supply and set to 60V. Hit run. After the gel has finished the instructor will demonstrate how to image the gel, and interpret the results.

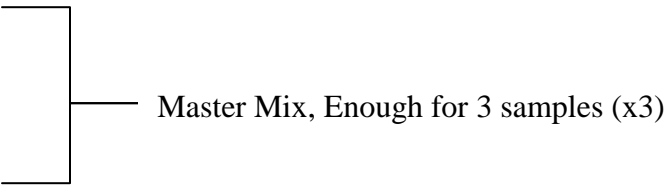
PCR Purification

After the gel has been imaged it should be clear which samples worked, and which didn't. Mix the successful reactions and using the protocol for the Qiagen PCR purification kit, begin to purify the DNA (starting with Step 1). Remember to balance the centrifuge with another sample, preferably another group's to minimize wait time. For the elution step (Step 8), use 35 μ L of dH₂O, add it to the spin column, and wait 5-10 minutes before centrifuging to maximize DNA yield.

Restriction Digest

Create a new master mix for 3 samples, **DO NOT ADD YOUR cDNA PRODUCT**, assuming that each individual digest will have the following:

Per Tube

- 35 μ L cDNA product
 - 5 μ L 10x NEB4
 - 0.5 μ L 100x BSA
 - 2 μ L NdeI
 - 2 μ L SacI
 - 5.5 μ L dH₂O
 - 50 μ L Total
- 
- Master Mix, Enough for 3 samples (x3)

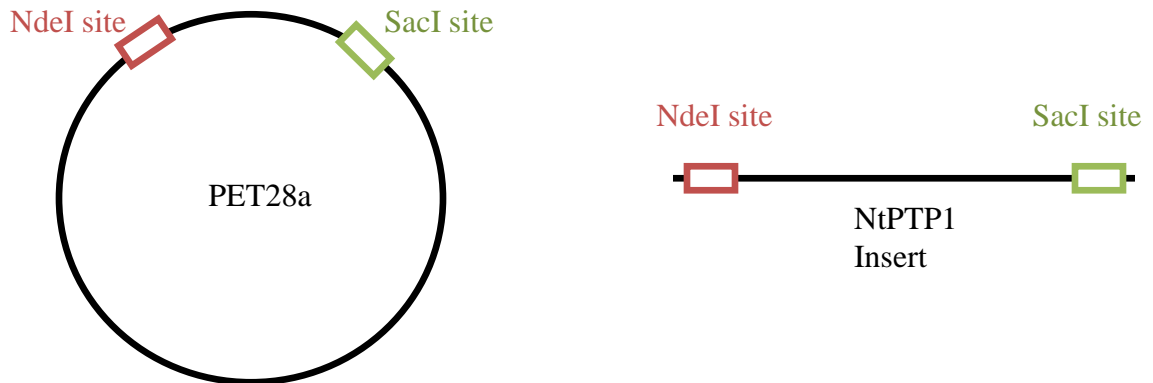
Aliquot 15 μ L of master mix into 2 separate tubes. To one add 35 μ L of purified PCR and To the other add 6 μ g of PET-28 diluted to 35 μ L. Label the samples and place them in the 37°C water bath.

Name: _____
Lab session: M T W

Lab 4 Gel Extraction, PCR Purification, and Ligation

INTRODUCTION

The purpose of today's lab is to purify the DNA from the digested PET28a vector and NtPTP1 insert from the restriction enzymes used in last week's lab, and ligate them together. The ligation will be done by mixing the two in the presence of ATP and T4 DNA ligase. During the ligation hydrogen bonds will form between the "sticky ends" on the DNA fragment and vector, and then the ligase will repair the phosphate backbone, creating a circular recombinant plasmid. In the case of the PET vector, the DNA was cleaved to yield two different sized strands, both of which have an NdeI a SacI cut site. These two strands have the ability to re-ligate and form false colonies during a transformation. These strands will be separated on a 1% agarose gel and purified from the gel using a Qiagen gel extraction kit. The NtPTP1 digestion will only need to be PCR purified (Just like in Week 3), because DNA is being purified from protein rather than DNA from DNA.



First, a 1% agarose gel must be prepared. A 1% gel is 1 gram of agarose per 100mL of buffer, but the mini gel rigs only hold ~50mL. Set up the gel at the start of class, and there will be a lecture while they are running. When the gel has run for ~30-45 minutes, excise the band and start the extraction/purification protocols. After the DNA is purified quantify the DNA on the Tecan, and set up a ligation using 50ng of vector and a 3-fold molar ratio of insert (explained in class). After the ligation is set up, the instructor will check notebooks.

Procedure 1

Agarose gel electrophoresis

1. Two groups will prepare a gel together. Set up the gel tank and the gel loading tray. Follow the instructor's directions. Find the comb that will be used to make the wells in the gel.
2. Prepare 50 ml of a 1.0 % agarose gel. First, calculate how much agarose is needed.
3. Weigh out the appropriate amount of agarose in weighing paper and add it to a 125 ml Erlenmeyer flask. Add 50 ml of SYBRsafe DNA gel stain solution.
4. Put the Erlenmeyer flask in the microwave and heat for 45 sec. Take it out with a mitten, swirl gently and check to see if agarose is dissolved. If not, microwave another 15 sec. and swirl again. **DO NOT POINT IT AT YOURSELF**
5. Allow the solution to cool slightly, and then pour it into the the rig (if the agarose is too hot it will create waves in the gel that will hurt the resolution). With a small pipet tip remove any bubbles from the gel and insert the comb.
6. Let the gel set for about 20 min, and place the gel in the appropriate orientation with the wells near the negative (black) electrode. Add 1X TBE buffer to the fill line and carefully remove the comb.

The restriction digests should be 50 μ L. 6X loading dye has been provided. Calculate the amount necessary for a 50 μ L sample and add it directly to the **PET28 digest only**. There should be close to 60uL of sample and if pipetted carefully it should all be able to fit into one well. Be careful not to bump the gel rig if any sample has already been loaded. After loading the samples, put on the gel rig cover and run at 120V for ~45 minutes.

PCR Purification

If the instructor finishes and dye front hasn't moved far enough, start the PCR purification of NtPTP1 digest starting with Step 1. The Qiagen PCR Purification kit from week 3 will be used and once again elute (Step 8) with 35 μ L of dH₂O (after allowing it to sit on the column for 5-10 minutes).

Gel Extraction.

Once the gel has finished running, tell the instructor and he will demonstrate how to find and excise the band. Try and cut off as much excess gel as possible and assume it weighs

333 mg. Follow the Qiagen gel extraction protocol and elute with 50uL of dH₂O in Step 12. Proceed to DNA Quantification.

Procedure 2

DNA Quantification: The Instructor will help with the operation.

Tecan Infinite M200 Instructions

- 14) Turn on the computer and allow it to start up, do NOT turn on the Tecan unit yet.
- 15) Open the “i-control” software. An icon for the program should be on the desktop.
- 16) Immediately after launching the program, turn on the plate reader (power switch is located on the back of the unit, above the power cord)
- 17) The software should automatically recognize the unit. If you are prompted to select an instrument, select Infinite M200 and make sure the box for “reconnect to selected instrument at next start up” is checked.

NOTE: If the unit is not displayed then power cycle the unit, cancel the instrument selection dialog box, and click on either the USB icon or navigate to “Instrument... >> Connect to instrument” to bring up the prompt again. The instrument should now be listed. If you are still having trouble, ask an instructor.

- 18) You will then be prompted to open a template. Select “Default script” and click ok.
- 19) Select the applications tab at the bottom left part of the window
- 20) Double click *Nucleic acid quantification*, NanoQuant Plate Tecan 16 Flat Black should be automatically selected
- 21) Select the “Individual Blanking” box to the right of the plate on the screen and make sure dsDNA is selected from the pull down box underneath.
- 22) Select the wells you intend to use for the assay and load 1 µL of dH₂O to each. Every well used must be blanked beforehand.
- 23) Click “Start Blanking” and the plate transport will move out for the plate. Put the plate on the transport and select OK.
- 24) After a successful blank the screen will change to a green color. Select the wells in which samples are loaded (by dragging) and click the Start button on the top left of the screen.
- 25) The plate transport will eject, but click OK (if all necessary samples are loaded) and wait for measurement to complete. An excel spreadsheet will be automatically created.
- 26) Record your DNA concentration

Ligation

Add each of the following to a 1.5 mL eppendorf tube:

50-100 ng digested PET vector

3-fold molar ratio of insert to vector
4 μL 5X ligation buffer
Add dH₂O to equal a total of 20 μL
1 μL Quick Ligase
21 μL total

Label the ligation so that it can be recognized next week, and leave it at the front of the class.

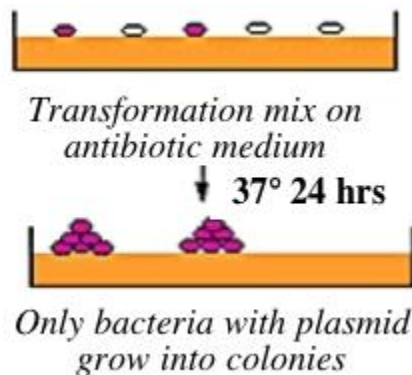
Name: _____
Lab session: M T W

Lab 5 Ligation Gel, Transformations, and Picking Colonies

INTRODUCTION

The purpose of today's lab is to determine whether the ligation, set up at the end of last week's lab, was successful, and then to transform the ligated vector/insert into competent bacterial cells. Half of the ligation will be loaded onto an agarose gel to run at 120V and the other half will be transformed into "competent" bacterial cells. "Competent" bacterial cells have been made permeable to foreign DNA by treating the cells with calcium chloride. This treatment pokes holes in the bacterial cell wall. During transformation a single recombinant plasmid enters a single bacterium. Once inside the bacteria the recombinant plasmid can be replicated.

The recombinant plasmid contains a Kanamycin resistance gene. Kanamycin directly interacts with the 30S subunit of the bacterial ribosome. It induces mistranslation and interferes with ribosomal translocation, directly inhibiting protein synthesis. Thus, a transformed bacterium will grow on agar medium containing Kanamycin while untransformed cells will die.



Perform the transformation at the same time the gels are running. Often times, even if the gel isn't sensitive enough to detect a successful ligation there will still be enough ligated DNA for the transformation to work.

The class will have to come in later in the week to pick colonies for minipreps so that they have time to grow before the next lab period.

Procedure 1 (This step has been done for you)

Preparing LB agar plates.

LB media is a generic rich media suitable for growing many aerotolerant species of bacteria, including *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus* or *Staphylococcus epidermidis* and different yeasts including *Saccharomyces* and *Candida* species. Many kinds of molds will also grow (*Aspergillus* or *Penicillium*, for instance).

Preparing the LB agar media. (This step has been done for you)

1. 10 g tryptone: tryptic digest of proteins to release small peptides and amino acids that bacteria can use for food and/or protein synthesis.
2. 5 g yeast extract: source of additional proteins, vitamins and minerals; some sugars present, necessary for growth.
3. 10 g NaCl (table salt)
4. 1 L distilled water. Fill flask to be autoclaved only half full with medium.
5. 15 g agar (the agar is added last and directly into the flask being autoclaved).

- To melt the agar and to sterilize the medium, we need to autoclave it. After autoclaving, gently swirl the flask while holding it in water-proof oven or heat-proof gloves. This action is necessary to insure even distribution of the agar in the media; else it often remains denser near the bottom. The media can then be stored at room temperature.

Melting the LB agar and pouring plates.

- Melt the 150 ml of solidified LB media in the microwave. Set the microwave on power level 10 and heat for 7 minutes, taking the bottle out every minute to swirl. Don't agitate the solution too much, to prevent the formation of bubbles near the surface. Stock Kanamycin has been prepared (50mg/mL = 1000x) and must be added to the agar. 1uL of Kanamycin/ 1mL of agar. Do not add the Kanamycin until the agar has cooled substantially.

- Get your plates ready. Lift the lid of the plates and pour the plates about 1/2 full, approximately 25 mL of media per plate. Plates poured generally solidify within 1/2 hour at room temperature.

Storing your plates.

- Stack your solidified plates right-side up and slide the original bag in which the empty Petri plates came. Turn the plates over so the opening of the bag is on top - the plates should now be facing agar-side up, exactly as how they should be stored. Tape the bag shut and label it with the type of media and date poured. Store plates in this position at 4°C in a refrigerator. If antibiotics have been added, cover the stack of plates in tin foil to prevent light-inactivation of the antibiotic.

Standard LB plates will keep for a few weeks or up to a month in this state. Be certain the bag is sealed to prevent loss of moisture.

Procedure 2

Agarose gel electrophoresis

7. Set up the gel tank and the gel loading tray. Follow the instructor's directions. Find the comb that will be used to make the wells in the gel.
8. Prepare 35 ml of a 0.7 % agarose gel. First you have to calculate how much agarose you need.
9. Weigh out the appropriate amount of agarose in weighing paper and add it to a 125 ml Erlenmeyer flask. Add 35 ml of SYBRsafe DNA gel stain solution.
10. Put the Erlenmeyer flask in the microwave and heat for 45 sec. Take it out with a mitten, swirl gently and check to see if agarose is dissolved. If not, microwave another 15 sec. and swirl again.
11. Allow the solution to cool slightly, and then pour it into the the rig (if your agarose is too hot it will create waves in your gel that will decrease the resolution). With a small pipet tip remove any bubbles from the gel and insert the comb.
12. Let the gel set for about 20 min, and place the gel in the appropriate orientation with the wells near the negative (black) electrode. Add 1X TBE buffer to the fill line and carefully remove the comb.

Preparing your solutions

Each group should only need 4 of the 6 wells. The samples will include: a 1kb ladder (5-10 μL), the ligation, digested PET28a, and digested insert. Each sample should be $\sim 20 \mu\text{L}$ (with the exception of the prestained ladder), and should contain 3.5uL of 6X loading dye. Examples:

10 μL of ligation
3.3 μL 6X loading dye
6.7 μL dH₂O
20 μL total

1 μL digested insert
3.3 μL 6X loading dye
15.7 μL dH₂O
20 μL total

2 μL digested vector
3.3 μL 6X loading dye
14.7 μL dH₂O
20 μL total

These volumes are based on anticipated concentrations of your DNA. If you have especially low concentrations of vector or insert, be sure to add at least 100 ng to each tube.

After loading the samples run the gel at 120V for about an hour. While the gel is running set up the transformations.

Procedure 3 Transformations

Obtain competent cells from the instructor. They are incredibly sensitive so remember to keep them on ice until ready to work with them. Also, typically each group should do a positive control using only undigested PET28a vector to show that the transformation worked. This step verifies that if the transformation failed it was because of the ligation rather than the transformation step; however, only the instructor will be doing a positive control as an example because it would be troublesome, expensive, and double the amount of plates for each to do their own.

1. Take 10 μ L of your ligation and add it to DH5 α (competent) cells (Transfer the reaction to a 1.5 mL eppendorf tube considering some of the subsequent steps will require you to do so anyway.)
2. Let the mixture sit on ice for 20 minutes.
3. Heat shock at 42°C for EXACTLY 90 seconds
4. Cool on ice for 30 seconds
5. Add 1mL LB media (or YETM)
6. Heat at 37°C (water bath) for +30 minutes
7. Spin at 3000rpm for 3-5 minutes (be considerate of others waiting for the microcentrifuge)
8. Remove all but ~75-100 μ L of the supernatant with a pipet
9. Resuspend the pellet in the residual LB media (be careful not to pipet too vigorously)
10. Add BSA to 1X to each of the tubes (starting concentration is 100X)
11. Add the reaction to the plate and use an inoculating loop or sterilized (by Bunsen burner) spreader to spread your cells evenly over the surface of the agar. There are spinners in the back of the room.
12. **Label the plates, so that they can be recognized when colonies are picked**, and place them in the 37°C incubator.

The instructor will take them out of the incubator the next day and place them in the 4°C freezer so that colonies can be selected on a date decided upon in class.

Picking Colonies

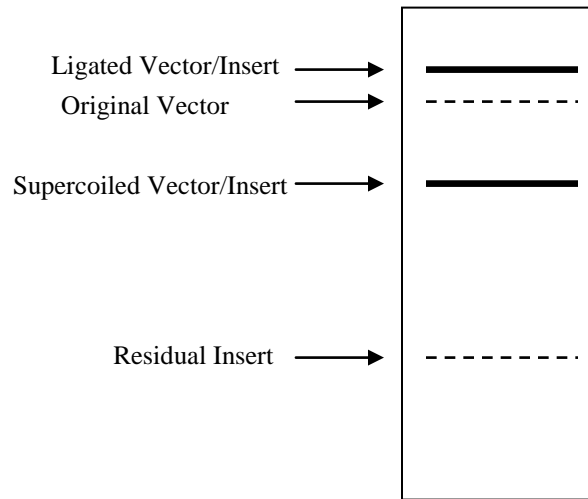
1. Fill 6 miniprep tubes with 3 mL of LB + Kanamycin
2. Use an autoclaved toothpick to select a colony by scraping it off of the agar media
3. Place that toothpick (without touching the walls of the tube) into the 3 mL of LB + Kan that you poured into a tube and put on the cap
4. Repeat 5 more times (using different tubes) and give them to your instructor who will put them in the 37°C shaker overnight. **Make sure to label the tubes.**

Name: _____
Lab session: M T W

Lab 6

Miniprep and Restriction Digest of Transformants

Today will be a short lab. Both of the procedures for today have been done in previous weeks. Last week ligations were analyzed on a gel and half was transformed into DH5 α cells. Six colonies were picked and grown in 3mLs of LB+Kanamycin. These colonies should contain vector + insert, however there are a few mechanisms, such as inefficient digestion, by which false colonies may have formed.



Today, DNA will be extracted from half of the E.coli sample grown up last week, and then a restriction digest will be performed on the purified DNA. Next week the resultant digest will run on an agarose gel to determine if the plasmid DNA actually has the insert (NtPTP1 gene). Normally one would pick upwards of 15-20 colonies just to make sure there is a significant sampling of the transformation, but due to a lack of time, space, and solution each group only picked 6. Samples were placed in the 37°C shaker overnight and then in the 4°C refrigerator. Collect the samples at the beginning of class and pour about 1-1.5 mLs of each into labeled tubes.

Normally, after determining which of the minipreps was successful, one would add the rest of their sample to a larger volume of media and grow up cells (containing your plasmid) to perform Maxipreps. Maxipreps allow scientists to purify large amounts of DNA from 1-2 liter cultures of E. coli cells. This DNA would be used as a stock solution for transformations into E.coli cells that are designed to express protein at high levels. These cells would then be pelleted by centrifugation, lysed, and the protein purified for other uses (such as the kinetic assays that will be done later in the semester). The class will be using a stock cell lysate prepared by the instructor. Therefore, once the restriction digests are set up, the remainder of the miniprep solution can be discarded.

Procedure

Take the 6 samples from last week and spin them at 6-8000g for 3-5 minutes. Remove the supernatant with a pipet then follow the protocol for the QIAprep Spin Miniprep kit that was posted on blackboard (starting with Step 2). Remember to elute with 35uL of dH₂O.

There will be 6 restriction digests so master mix will need to be prepared for 7 samples. Each digest will contain:

- 2 μ L 10X NEB4
- 0.2 μ L 100X BSA
- 0.8 μ L NdeI
- 0.8 μ L SacI
- 6.2 μ L dH₂O
- 10 μ L of the E.coli DNA (samples 1-6)
- 20 μ L total

Add everything but the extracted DNA to the master mix and calculate how much of the master mix to add to each tube. Add the DNA into the appropriate sample tubes and place the digests into the 37°C water bath. These will run overnight and be put in the -20°C freezer for next week.

QUIZ TIME!

Name: _____
Lab day: M T W

Lab 7

Identification of Successful Transformants

INTRODUCTION

Last week DNA was extracted from E. coli culture preps that may or may not contain vector + insert DNA. Restriction digests were set up, with NdeI and SacI enzymes, for each of the 6 colonies that were selected in Lab 5. The digests ran overnight in the 37°C water bath and were placed in the -20°C freezer. As mentioned last week there are several ways to produce “false positive” colonies that have been transformed with an empty plasmid. The purpose of today’s lab is to identify which of the colonies contain the cloned insert. After the identification of successful transformants one would typically grow up a large quantity of the correct E.coli sample, extract the DNA, and transform it into E. coli cells that are specially designed to produce protein.

Today will be a short lab. Samples will be run on a 0.7% agarose gel and there will be a review to reinforce the concepts of each of the previous labs for the midterm exam next week.

Procedure

Each group has 6 digest samples from last week. First, prepare 35 mL of 0.7% agarose in SYBR safe DNA gel stain solution. Microwave for 45-60 seconds until all the agarose dissolves, wait a few minutes for it to cool, pour the gel, and add a 10 well comb (remember to remove bubbles with a small pipette tip). Let the gel solidify, place it in its correct orientation (black to red), fill the rig with 0.5X TBE, remove the comb, and load the samples.

Sample preparation:

- Each of the digests should be 20 µL. Add the appropriate amount of 6X loading dye to each of the samples and they are ready to load.
- There also need to be two control samples: 1. Digested vector (which will be provided), and 2. Digested insert which should be leftover in the class box. Prepare each so that there is ~100ng of DNA, the appropriate amount of 6X loading dye, and dH₂O to 20 µL.
- Remember to add the 1kb ladder to one of the empty lanes.

Load the samples and run the gel at 120V. There will be a review during this time. When the gel is finished running, the instructor will image it across the hall.

Save the image of the gel, put one in your notebook and another in your final draft of your DNA report.

Name: _____
Lab session: M T W

Lab 8

PTP1 Purification by His-Tag chromatography and Bradford Assay

INTRODUCTION

INTRODUCTION

The objective for this lab is to purify the enzyme Protein Tyrosine Phosphatase 1 (PTP1) from *E.coli* by His-affinity chromatography. After purification, the eluted fractions will be quantified by Bradford assay. The fractions with the highest concentration of protein will then be mixed and dialyzed to remove the imidazole and salts present in the buffer. This is necessary for subsequent accurate protein quantification and enzyme activity assays.

Protein purification

Purification of proteins is a stepwise process. In a series of independent steps, the various physiochemical properties of the protein of interest are used to separate it from other substances in the extract. Some common purification procedures and the protein characteristics they depend on are shown below:

Protein Characteristic	Purification Procedure
Solubility	Salting out
Ionic charge	Ion exchange chromatography Electrophoresis Isoelectric focusing
Polarity	Hydrophobic interaction chromatography
Size	Gel filtration chromatography SDS-PAGE
Binding Specificity	Affinity chromatography

Affinity chromatography will be used to purify the His tagged PTP from the *E.coli* lysate. The principle behind affinity chromatography purification is that when a mixture of protein is passed through a chromatographic matrix, the desired protein (expressed with a suitable affinity tag) binds the immobilized matrix ligand, but other proteins are washed through the column with the buffer. The desired protein can then be recovered in highly purified form by changing the elution conditions to release the protein from the matrix.

The six consecutive His residues that are added to the PTP protein function as metal ion-binding sites that allows the recombinant protein to bind to the Ni resin (an agarose

matrix with attached Ni²⁺ charges). A commercially available Ni²⁺ resin will be used to bind and immobilize the protein PTP1 from a mixture of *E. coli* proteins. After washing the column to remove unbound proteins the bound protein is then eluted by an appropriate method. Imidazole will be present in the elution buffer to elute PTP. Imidazole will strongly compete with the poly-Histidine tag for binding the Ni²⁺ resin. Since the affinity of Imidazole is stronger than that of the poly histidine tag the enzyme will be released from the matrix, that is, it will be eluted.

Protein quantification by the Bradford dye method

The Bradford dye method is a frequently used protein assay. The method is based on the proportional binding of the dye Coomassie to proteins. The dye used, Coomassie Brilliant Blue G-250, is red when free in solution and blue when bound to protein. Within the linear range of the assay, the more protein present, the more Coomassie binds. Furthermore, the assay is colorimetric; as the protein concentration increases, the color of the test sample becomes darker. The protein-dye complex has a high molar absorptivity and thus makes this assay useful over a range of 1-60 µg protein. The protein concentration of a test sample is determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in this assay. Although different protein standards can be used, we have chosen the most widely used protein as our standard - Bovine Serum Albumin (BSA). This assay method has the advantages of being rapid, simple to perform and very sensitive.

This assay depends on non-specific, non-covalent binding of the dye to the protein(s). The Bradford dye reagent reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. Obviously, the assay is less accurate for very basic or very acidic proteins. Unfortunately, the assay is susceptible to interference by a number of common compounds (e.g., Tris, strong base, sucrose, beta-mercaptoethanol, glycerol, EDTA, detergents). Not all of these interferences can be corrected for by the use of appropriate blanks. Thus, this assay is unsuitable in many instances. Also, many investigators feel the response of the dye binding to be more variable, and somewhat less reliable than other methods.

Bradford Assay

Low [Protein] ← → High [Protein]



Procedure

NTA Column

Each group has a column that has been set up with a 300uL resin bed volume (referred to as column volume or CV). The resin is specially designed to bind to the His-tag on the NtPTP1 protein. The resin (blue) is stored in 20% ethanol (EtOH). BL21 cells, containing the NtPTP1 insert, were grown up in 2L of LB+Kan, pelleted, lysed, and diluted with lysis buffer to 50mL. Each group will be given 2mL of this sample to run on their column.

- Let the EtOH drain from the column.
- Equilibrate the column by adding 10 CV (~3 mL) of lysis buffer and letting it flow through the column. **From this point on do not let the resin dry out and pipette the solutions slowly so as not to disturb the resin.**
- Pass sample over column and collect flow through in 15 mL conical tube.
- Wash column with 10 CV of lysis buffer and collect in 15 mL conical tube
- Wash column with 10 CV of wash buffer and collect in 15 mL conical tube
- Elute column with 10 CV elution buffer and collect ~0.5 mL fractions in labeled microcentrifuge tubes
- Transfer ~1 mL of the collected samples (Flow Thru, Lysis Wash, and Wash Wash) to microcentrifuge tubes so that they are easier to store. Label and save all of the samples in the class box.
- When finished with the column add 4 mL of ethanol to the column and let half flow through
- Cap the column on both sides with parafilm when.

- Give the column to your instructor who will put it in the 4°C refrigerator

BSA Standard

One partner can do this while the other tends to the column. The Bradford Assay measures protein concentration. In order to accurately quantify protein concentration it is necessary to create a standard relates protein concentration to absorbance. BSA is often used as a convention because it is stable, inexpensive, and readily accessible. The BSA that will be used has a stock concentration of 10mg/mL. The assay is supposedly linear up to 1 mg/mL, so prepare 8 samples with varying concentrations of BSA from 0-1mg/mL. Each group will only need 10 µL of each stock per assay, but a new standard will be used in Lab 10, so create stocks with a final volume between 30-50 µL.

- BSA standard samples should have the following concentrations: 1, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0 mg/mL BSA.

Serial dilutions must be performed. Do not simply dilute the stock 100 fold to obtain a 0.1mg/mL concentration. This creates a large opportunity for pipette error to skew results. Show all calculations in your notebook and label your tubes VERY clearly. These will be important in Lab 10.

Bradford Assay

BSA stock solutions were prepared with concentrations from 0-1mg/mL. These solutions will be used a standard in order to calculate the protein concentration in each of the eluted fractions. The fractions with the highest concentration of protein will subsequently be mixed and dialyzed to remove the salt and Imidazole. Bradford reagent binds to proteins and has a maximum absorbance at 595 nm. It comes in a 5X stock which has been diluted to 1X and given to each group. Each group should have 8 BSA samples as well as 9-10 column fractions including: flow thru, lysis buffer wash, wash buffer wash, and 6-7 elution fractions. **KEEP THEM ON ICE!**

- Prepare and label a microcentrifuge tube for each of your 17-18 samples.
- Add 200 µL of 1X Bradford reagent to each tube
- Add 10 µL of sample to each tube (only 1 sample/tube) and mix as quickly as possible. After the sample is mixed a blue color should be noticeable in tubes that contain high concentrations of protein.
- Wait for 5 minutes and add 175 µL of each sample to a 96 well microtiter plate. Make sure to remember the order in which the wells are filled.
- Measure the absorbance of the samples at 595nm and record your results. (The instructor may print the results on a spreadsheet)
- After the samples have been labeled and measured give them to the instructor to save for next week.

Tecan Infinite M200 Instructions

- 27) Turn on the computer and allow it to start up, turn on the Tecan unit using the switch on the back of the unit above the power cord.
- 28) Open the “i-control” software. An icon for the program should be on the desktop.
- 29) The software should automatically recognize the unit. If you are prompted to select an instrument, select Infinite M200 and make sure the box for “reconnect to selected instrument at next start up” is checked.

NOTE: If the unit is not displayed then power cycle the unit, cancel the instrument selection dialog box, and click on either the USB icon or navigate to “Instrument... >> Connect to instrument” to bring up the prompt again. The instrument should now be listed. If you are still having trouble, ask an instructor.

- 30) You will then be prompted to open a template. Click Cancel
- 31) Select the Grenier 96 well Transparent Plate
- 32) Drag “Absorbance” from the left hand menu to the area under the picture of the plate
- 33) Change the wavelength to 595 nm, and select the area of the plate that you will be reading by dragging with the mouse
- 34) Click the Move Plate Out (red arrow) button and place your plate with A1 at the top left of the tray holder.
- 35) Click the Move Plate In (green arrow) button and when the tray is inside the machine click Start.
- 36) You will be prompted to save the script. Click Save and Overwrite. Wait for the machine to finish so that you can access your Excel file, then have your instructor print a copy.

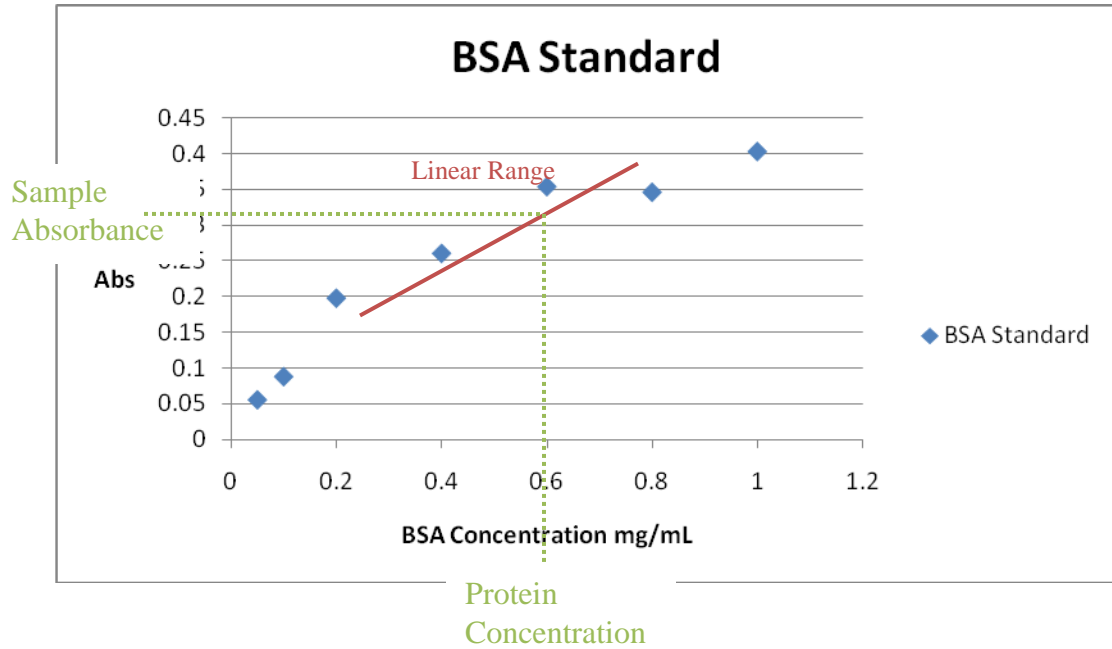
Graphing

Each group will prepare a graph of their BSA standard curve with graphing paper provided to you by the instructor.

- Subtract the blank from each of the samples and plot the data (protein concentration on the x-axis and absorbance on the y-axis).
- Disregarding obvious outliers (samples that have atypical absorbance values), draw a best fit line on your graph paper selecting only the samples that give a linear curve (usually from 0.2-0.6mg/mL).
- Either determine the equation of the line using the standard $y = mx + b$ formula, or determine the concentration of each of the eluted fractions by comparing its absorbance to that of the standard (**remember to subtract the blank from each of these samples as well**).
- Record the concentration of each of the elution fractions.

Example:

This is what the BSA Standard should look like. Select the values which give an approximate linear range and draw a best fit line. Match the position on the line to the absorbance and determine the protein concentration of each sample.



Next week, the purified protein will be dialyzed, and aliquots of the samples will be run on an SDS-PAGE.

Name: _____
Lab session: M T W

Lab 9 SDS-PAGE and Dialysis

INTRODUCTION

INTRODUCTION

In last week's lab PTP1 protein was purified by His-Affinity chromatography. A BSA standard curve was also created in order to quantify the elution fractions by Bradford. Today, a small aliquot of each of the samples will be loaded on an SDS-PAGE (Polyacrylamide gel electrophoresis) to analyze which solutions contain the PTP1 protein. The samples that contain protein will then be combined and dialyzed to remove the salt and Imidazole from the column elution.

SDS-PAGE

SDS-PAGE separates proteins on the basis of size and can determine the purity of eluted protein in comparison to other eluted fractions.

SDS (sodium dodecyl sulfate, $C_{12}H_{25}NaO_4S$) has a central role in SDS-PAGE. All protein samples are incubated with SDS and heated to $95^{\circ}C$ before they are loaded on the gel. This anionic detergent wraps around the backbone of proteins and coats proteins uniformly with negative charges (figure 1A). SDS binds to most proteins in amounts that are roughly proportional to the molecular weight of the protein (1.4 g of SDS binds to 1 g of protein). The addition of β mercaptoethanol to the protein samples is also necessary to prevent the formation of disulfide bridges between cysteine residues in proteins (figure 1B).

Figure 1A.

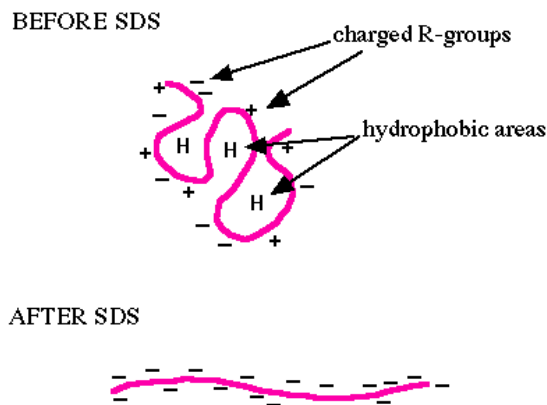
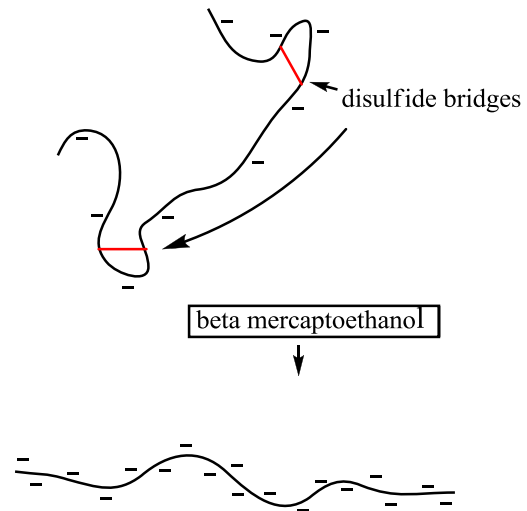


Figure 1B.



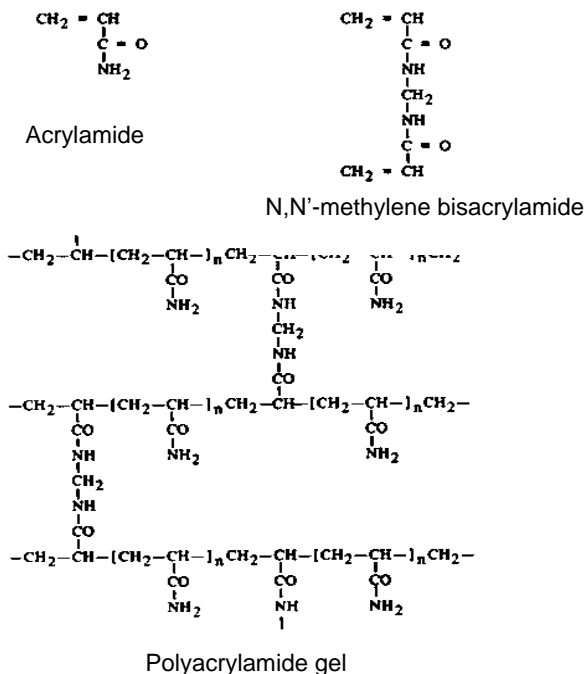
Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE: SDS-PAGE is a method for separating proteins that are linearized and uniformly charged by SDS. Under these conditions the native form of the protein is destroyed and the parameters of shape and charge become unimportant (See figure 1A and B). Separation is achieved solely on the basis of molecular weight. One thing one has to bear in mind is that two different proteins with different native shape but same molecular weight will migrate the same distance when linearized and charged by SDS, and can therefore not be separated by this method. Procedures for SDS-PAGE generally involve 1) making a gel (or using a commercially available pre-cast gel) and assembling the gel apparatus, 2) mixing protein samples with sample buffer containing SDS and heat the mixture at high temperature, 3) loading samples and running the electrophoresis, 4) staining the separated proteins.

Native PAGE: In native PAGE (which is performed in the absence of SDS) proteins remain intact in regards to their native structure and function. Different proteins migrate differently due to the differences in their secondary, tertiary, or quaternary structure, overall charge, and molecular weight. Two proteins with the same molecular weight may migrate differently in the gel matrix due to differences in structure and overall charge.

The polyacrylamide gel matrix.

The gel matrix is a porous matrix of cross-linked polyacrylamide. The formation of this gel matrix involves the reaction between acrylamide and N,N'-methylenebisacrylamide (called "bis"). The polyacrylamide gel forms when a dissolved mixture of acrylamide and the 'bis' cross linker polymerizes into long, covalently linked chains.



The concentration of acrylamide can be varied to alter the 'pore' size through which proteins migrate under the influence of an electric field. High concentration of polyacrylamide results in smaller 'pores' and only small proteins can migrate effectively through the gel. Very large polypeptides cannot penetrate far into the gel and thus their corresponding bands may be too compressed for resolution.

You should strive to use an acrylamide gel that will give maximum resolution in the size range of the protein that you are studying. You can change this range by changing the concentration of the acrylamide used in making the gel. A typical gel of 7% acrylamide composition nicely separates polypeptides with molecular mass between 45 and 200 kDa. Polypeptides below the cutoff of around 45 kDa do not resolve. A denser gel, say 14%, usually resolves all of the smallest polypeptides in a mix. It would be useless for resolving bands much above 60 kDa, though. To analyze the entire profile of a fraction that contains heavy and light polypeptides, one should usually run two gels. A rule of thumb is to use an acrylamide concentration that will place the protein of interest at the center of the gel.

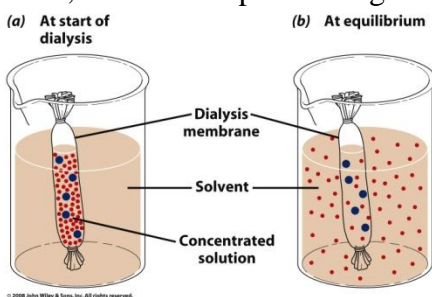
Visualizing proteins by staining.

After the separation, the gel is colorless and the protein bands are not visible. Only the bromophenol blue dye in the gel front will be visible. PageBlue™ protein staining solution can be used to visualize the proteins and standard protein markers. PageBlue™ contains Coomassie Brilliant Blue G-250 dye. This dye preferentially stains proteins without significant staining of the gel matrix, so the destaining time is short. The stain does not contain toxic methanol or acetic acid, so used materials can be washed down the sink. There are several other methods for visualizing proteins in a gel that differ in their sensitivity and ease of use. Coomassie Blue is the most widely used dye but not the most sensitive. Silver staining is a very sensitive method but more time consuming. Another technique is to incorporate

radioactivity into proteins, which are then visualized using X-ray film applied to the radioactive gel. This method is very sensitive, but hazardous.

Dialysis

The elution buffer you used in the His-affinity purification contains Imidazole and salts. These components need to be removed before performing SDS gel electrophoresis and Western blotting. Dialysis is a way to remove salts and other undesired molecule from your sample. The protein solution is placed in a dialysis bag which has very small pores. The dialysis bag filled with the protein solution is then placed in large volume of 100 mM NaCl, 30mM Tris buffer, 4 mM β -mercaptoethanol, and pH'd to 7.2. The bag is left in dialysis for 16 to 24 hours. This allows the small molecules inside the bag to equilibrate with the solution outside the bag, but the proteins are retained inside the dialysis bag. The dialysis tubing has a pore size of 12000-14000 Daltons. The size of the enzyme we are purifying is about 55000 Daltons, so it cannot pass through these pores.



Procedure

SDS-PAGE

Bio-Rad's Mini-Protean TGX Precast 7.5% gels will be used to analyze last week's samples. Refer to the Quick Start Guide from Blackboard for setting up the gel. The following came from the detailed kit protocol on Bio-Rad's website in regards to sample preparation:

3.5 Sample Preparation

The appropriate concentration of sample depends on the load volume and the detection method used. (See Section 6 for approximate stain sensitivities). Add 50 μ l 2-mercaptoethanol per 950 μ l of sample buffer for a final concentration of 5% 2-mercaptoethanol, or 710 mM.

As an alternative, DTT may be used at a final concentration of 350 mM (54 mg/ml). Dilute 1 part sample with at least 1 part sample buffer with added reductant. Heat the mixture at 95°C for 5 min.

Coomassie Brilliant Blue gel stain has sensitivity near 100 ng, therefore 500 ng of protein should be visible. This of course is based on the calculated concentrations from last week's graph. If the samples don't contain protein (such as the later elution fractions) simply add the same volume as other similar fractions.

- 500 ng protein

- 10 μ L laemmli buffer + BME
- dH₂O to 20 μ L

After your samples are mixed, heat them for 5 minutes at 95°C to completely denature the proteins. Assemble the gel boxes according to the Bio-rad Quick Start Guide and in class demonstration, load the samples, and run at 100V until the dye reaches the end of the gel. While the gels are running, set up dialysis. When they finish, stain and image them.

Dialysis

Mix together the eluted fractions that contain considerable amounts of protein. Note: this **does not** mean flow thru, LW, or WW samples.

- Cut an appropriate length of dialysis tubing for your sample. Remember to leave enough room to fold and clip the ends.
- Soak the tubing in dialysis buffer for 1-2 minutes, until it is completely saturated.
- Fold and clip one end of your tube so that you can load your sample. **Make sure you hear a click when you put on the clip.**
- Load your sample as demonstrated and place a clip on the other side of the bag.
- Place your dialysis in the 4L dialysis buffer. This will be placed in the 4°C refrigerator overnight.

Staining and Imaging

Break apart the gel as shown in the Bio-Rad Quick Start Guide, and gently place the gel in a plastic container. Follow the PageBlue™ protein staining solution protocol from blackboard. A rocker for the gels is in the back of the room. When finished, scan the gel and print/save the results. Remember to label the lanes of the gel.

Name: _____
Lab session: M T W

Lab 10

Effect of pH on Enzyme Activity

INTRODUCTION

INTRODUCTION

In the previous lab the PTP1 protein was dialyzed to remove excess salt from the NTA column purification. In this lab, the activity of the enzyme will be determined as well as the general activity of proteins extracted from wheat germ, at different pHs. The crude protein extract will contain numerous enzymes. The Bradford assay will again be used to determine the concentration of protein in the wheat germ extract, and a p-Nitrophenolate assay will be used to measure the *enzyme activity* of acid phosphatase. Then calculate *specific activity* and *total activity* of acid phosphatase in the wheat germ extract.

Preparing a crude extract from Wheat Germ.

Extraction of enzymes from biological materials requires disrupting the cells by some method. In this experiment enzymes and other material can be extracted from wheat germ by suspending the wheat germ in a hypotonic medium (e.g., distilled water). The undesired debris in the extraction mixture can be removed by filtration and/or centrifugation. All extraction procedures should be done at 4°C in order to minimize the danger of destroying the enzyme.

Measuring protein concentration by the Bradford method.

To review, the Bradford method is based on the proportional binding of the dye Coomassie to proteins. The dye used, Coomassie Brilliant Blue G-250, is red when free in solution and blue when bound to protein. The Bradford assay depends on non-specific, non-covalent binding of the dye to the protein(s). The Coomassie dye reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. Obviously, the assay is less accurate for basic or acidic proteins.

Within the linear range of the assay, the more protein present, the more Coomassie binds. Furthermore, the assay is colorimetric; as the protein concentration increases, the color of the test sample becomes darker. The protein-dye complex has a high molar absorptivity and thus makes this assay useful over a range of 1-60 µg protein.

Unfortunately, the assay is susceptible to interference by a number of common compounds (e.g., Tris, strong base, sucrose, β-mercaptoethanol, glycerol, EDTA, detergents). Not all of these interferences can be corrected for by the use of appropriate blanks. Thus, this assay is unsuitable in many instances. Also, many investigators feel

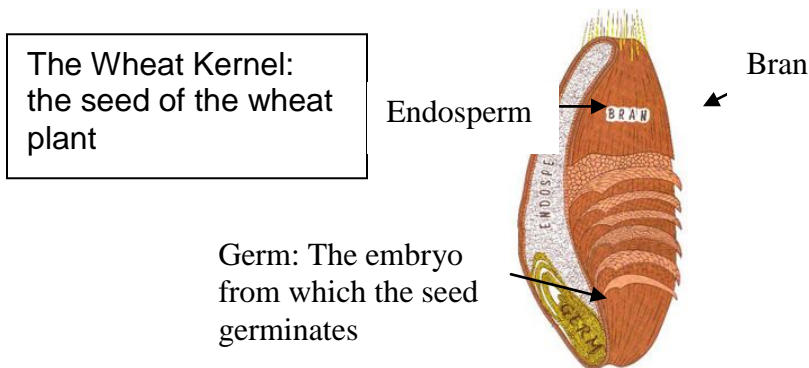
the response of the dye binding to be more variable, and somewhat less reliable than other methods. Finally, the dye-protein complex binds to the cuvettes, necessitating special cleaning.

The protein concentration of a test sample (a sample of unknown protein concentration) can be determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in this assay. Although different protein standards can be used, we will use the most widely used protein as our standard - Bovine Serum Albumin (BSA).

Acid phosphatase

A phosphatase is an enzyme that hydrolyzes phosphate ester bonds. Both acid and alkaline phosphatases exist in living cells and are enzymatically active at the pH indicated by its name. The specificity of acid phosphatase is the phosphoester bond; it is therefore active against a wide variety of phosphorylated substrates. Although both diester and monoester phosphatases exist in the plant, the procedure used will assay only for the monoester phosphatases.

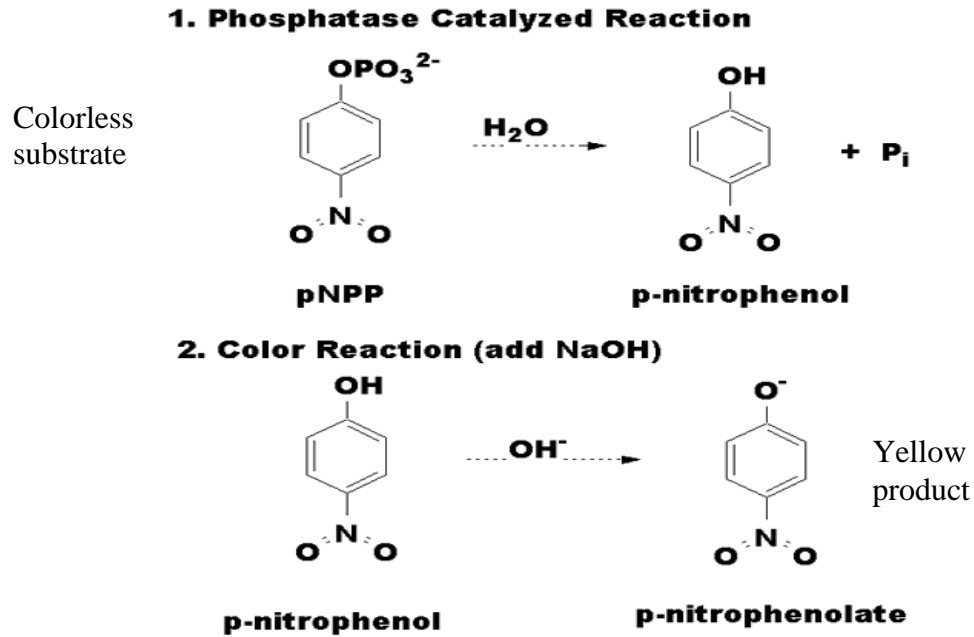
The physiological function of acid phosphatase in wheat germ is to provide inorganic phosphate to the growing wheat seedling during germination. Many different phosphate esters of sugars and substrates are stored in the wheat seed and these need to be hydrolyzed during germination, which makes the carbohydrates available as an energy source and the phosphate to be used as building blocks in making new cells (new RNA and DNA all need phosphate in their backbones).



A model system for studying acid phosphatase *in vitro*

It is often possible to study an enzyme *in vitro* using a model system. In this system the substrate used is different than the *in vivo* substrate, but since the enzyme is non-specific, it will catalyze the breakdown of the artificial substrate. Some reasons for using a model system include ease of obtaining and handling the substrate and measuring the product of the reaction.

The figure below shows the reaction catalyzed by wheat germ acid phosphatase with p-nitrophenylphosphate (pNPP) as the substrate:



Under alkaline conditions, the p-nitrophenol (pNP) is converted into p-nitrophenolate which is yellow. Using a spectrophotometer, the concentration of the pNP product of the phosphatase-catalyzed reaction can be measured by the increase in absorbance due to the yellow color of the immediately generated p-nitrophenolate. We can therefore use the amount of product formed as a relative measure of the amount of enzyme present in a solution.

Enzyme activity

The *Enzyme activity* of an enzyme can be defined as the amount of product formed per unit time per assay volume ($\mu\text{moles}/\text{min}/\text{ml}$). Activity can also be expressed in Units (U), one enzyme unit being the amount of enzyme causing formation of 1 μmol of product per minute

The *specific activity* of an enzyme is the enzyme activity per milligram of protein ($\mu\text{moles}/\text{min}/\text{mg}$). Specific activity is frequently used to measure the purity of an enzyme during protein purification. After each purification step, the enzyme activity and the total amount of protein in the preparation can be measured. The ratio of the two gives the specific activity. Enzyme activity and total protein generally decrease with each purification step, but the loss of total protein is usually much larger than the loss of enzyme activity. Therefore specific activity usually increases for each purification step. A protein is generally considered pure when further purification steps fail to increase the specific activity further and when only a single protein species can be detected (for example by SDS-PAGE). Below is a table which shows that the specific activity of an enzyme increases for each purification step.

TABLE 3-5 A Purification Table for a Hypothetical Enzyme

Procedure or step	Fraction volume (ml)	Total protein (mg)	Activity (units)	Specific activity (units/mg)
1. Crude cellular extract	1,400	10,000	100,000	10
2. Precipitation with ammonium sulfate	280	3,000	96,000	32
3. Ion-exchange chromatography	90	400	80,000	200
4. Size-exclusion chromatography	80	100	60,000	600
5. Affinity chromatography	6	3	45,000	15,000

Note: All data represent the status of the sample after the designated procedure has been carried out. Activity and specific activity are defined on page 94.

The *purification factor* can be calculated by dividing the specific activity of the purified sample by the specific activity of the unpurified starting material.

Procedure:

Bradford Assay

The BSA stock solutions that you used in Lab 8 should still be in the freezer. A new BSA standard curve must be created for each new Bradford to account for differences in solutions and assay time.

Create the following dilutions of your extract (These do not need to be serial dilutions): Undiluted, 1/2, 1/5, 1/10, 1/20, 1/50, 1/100. Make sure to write down all of the calculations. In microcentrifuge tubes, add 200 μL of 1X Bradford reagent for each sample (There should be 16 samples **including the dialyzed bacterial enzyme**). Add 10 μL of each sample to a tube containing Bradford Reagent. Mix and wait at least 5 minutes. Pipette 200 μL of each sample into a different well of a transparent 96 well plate and measure at 595 nm.

Subtract the blank from each sample of the BSA standard and plot on graph paper concentration vs. absorbance. Draw a best fit line for the samples that give a linear range. Subtract the blank from the dilution absorbencies and identify the samples that fall within the linear range on the BSA standard curve. Select one and that will be the enzyme concentration used for the kinetics assays.

p-Nitrophenol Standard curve

1) Use the standard solution of p-nitrophenol (0.25 $\mu\text{moles/ml}$ in 0.1 N NaOH) to prepare a series of tubes containing 0, 0.05, 0.10, 0.15, 0.20, 0.25 μmoles p-nitrophenol per tube. Bring each tube to a total volume of 100 μL with water. Do these assays in duplicate (set 1 and set 2). Complete Table 5 before coming to laboratory.

Table 1. Dilution table for p-nitrophenol (pNP) standard curve

Dilution Table						
p-NP (mM)	0	0.05	0.10	0.15	0.20	0.25
p-NP per tube(μL)			40 μL			

Water per tube(μL)			60 μL			
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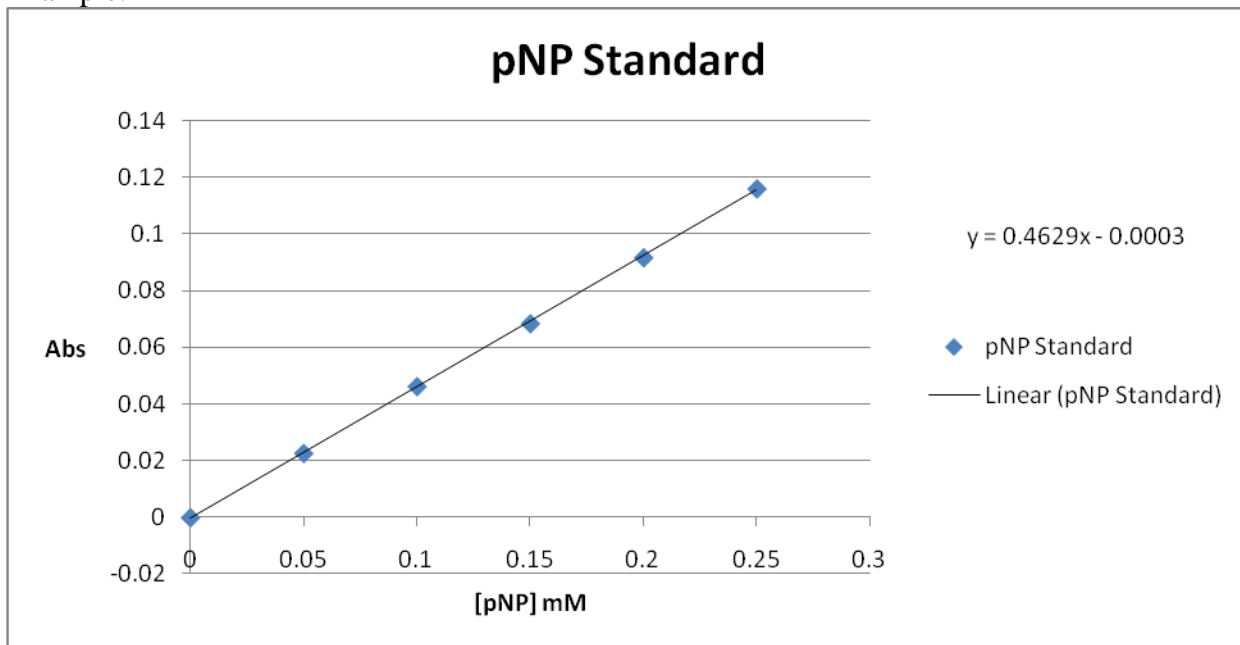
2) Add 500 μL of 0.1 N NaOH to each tube and mix well. Add 200 μL of each sample to a separate well of a 96 well plate and read absorbance at 410 nm. The blank should contain water and NaOH (use the tube with no pNP as blank)

Table 2. Absorbance values of p-nitrophenol (pNP) standards

Absorbance at 410 nm						
p-NP (μmoles)	0	0.05	0.10	0.15	0.20	0.25
Set 1						
Set 2						

3) Plot absorbance at 410 nm versus amount of pNP (in mM) for the standard curve. One standard curve can be prepared and used for all subsequent assays. This is a dilution curve and should be linear. It should pass through zero. Show the curve and data to the instructor. If the standard is non-linear or the data is widely scattered the standard must be repeated.

Example:



Acid phosphatase activity in wheat germ extract

In this section, the pNP assay will be performed to determine the amount of pNP product made. From this the activity of the acid phosphatase can be determined. Each group will have 18 samples. 12 will contain the wheat germ extracted enzyme and 6 will contain the dialyzed bacterial enzyme. The optimum pH for the bacterial PTP1 is 7, which is made with Tris and pH'd to 7 with HCl. Each group will be analyzing the activity of their wheat germ enzyme at pH 4.8 (the enzyme's optimum pH) and one other pH that will be assigned in class. After the results are collected, the class will share data in order to create a pH vs. activity curve. Each sample will have a different substrate concentration. The

undiluted substrate is 1 mM and the buffers are premade by the instructors. These results will be used to create individual kinetics curves for each group.

In this assay, **buffer and substrate (pNPP) are mixed in a reaction tube first** and then enzyme is added last to start the reaction. After mixing, reactions are incubated for 20 minutes at 37°C and then terminated by the addition of NaOH solution. The addition of base (NaOH) at the end of the reaction serves two purposes. It converts the nitrophenol into the yellow nitrophenoxide. The NaOH also stops the reaction by denaturing the enzyme. This is how to set up the assay:

Citrate or Tris Buffer	50 µL
Substrate (pNPP)	25 µL
Diluted enzyme (wheat germ extract or bacterial enzyme)*	25 µL
NaOH**	500 µL

* add when ready to start reaction.

** add when ready to stop reaction

After 20 minutes of incubation, add 500 µL of .1M NaOH and mix the sample. Take 200 µL of each sample, add it to a well in a 96 well plate, and read the absorbance at 410 nm. Using the p-nitrophenol standard curve, determine the amount (in µmoles) of p-nitrophenol formed. Calculate enzyme activity, specific enzyme activity and total enzyme activity.

Negative controls are important in the acid phosphatase assay. One negative control is a blank in which buffer and substrate are mixed and water is substituted for enzyme. This controls for any absorbance that might be produced by spontaneous breakdown of the substrate. Another important negative control consists of enzyme and buffer with water substituted for substrate. This controls for any light absorbance by the enzyme preparation itself. This is the blank used in the assay.

Table 3. Absorbance values of p-nitrophenol (pNP) standards

Absorbance at 410 nm						
Substrate	0.0	0.2	0.4	0.6	0.8	1.0
W.G. pH - 4.8						
W.G. pH -						
Bacterial pH - 7						

Graphing The Results

This lab will have the largest results section of any lab this semester. Graph the data on graph paper in class. Then take the data home and use Excel to analyze it electronically.

You will be preparing 4 graphs in class which are to be shown to the instructor before you are allowed to leave. These graphs will count as Prelab 10:

- BSA Standard [BSA] vs Abs

- pNP Standard [pNP] vs Abs
- pH vs. Velocity
- $1/[S]$ vs $1/V$ Wheat Germ

Next week there will be a lecture to discuss kinetics, after which we will discuss the results we expect to be in your final lab reports. If you know how to use Excel take your data home and create the following graphs. Bring your computer with you next week so that we can help you fix any mistakes. If you aren't familiar with Excel try to analyze your data with a partner, but we will be there to help you next week. You will need to produce the following 6 graphs:

- BSA Standard
- pNP Standard
- pH vs. Velocity
- Wheat Germ- Substrate Concentration vs Velocity
- $1/[S]$ vs $1/V$ Wheat Germ
- PTP1- Substrate Concentration vs Velocity
- $1/[S]$ vs $1/V$ PTP1

Remember V stands for velocity which is a measure of enzyme activity, or product formation per second. The units for V are mM/second unless you intend to account for the reaction volume which was 0.1 mL.

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Education

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Master of Science in Biochemistry; May 2011
GPA to present: 3.60

Bachelor of Science in Biochemistry; May 2009
Minors in Biology and Chemistry
GPA: 3.419/4.0

Work Experience/Research

University of Missouri

Graduate Student Researcher

(06/09-05/11)

Characterization of PGAM5 Protein for elucidation of oxidative stress pathways involved in cancer initiation and metastasis

- Independently obtained fluency in a wide array of experimental techniques and adapted various protocols for use under specific conditions
- Analyzed and corrected numerous technical problems on a daily basis
- Efficiently communicated complex scientific data to colleagues and supervisors as well as untrained undergraduate researchers
- Coordinated and organized collaborations between a variety of specialized faculty
- Catalogued various samples and reagents for individual as well as general laboratory use
- Developed proficiency with an extensive variety of computer programs and applications
- Acquired an in depth understanding of various writing styles associated with research articles, proposals, and reviews

Undergraduate Research Assistant

(11/07-05/09)

Preparation of Technetium-99 Radio-labeled Peptides for use in Melanoma imaging

- Organized and maintained efficient laboratory working conditions
- Assisted in the synthesis of radio-labeled peptides

Mizzou Rec Complex

Preventative Maintenance

(12/06-05/07)

- Organized and catalogued equipment
 - Identified and corrected problems within the complex, without guidance from supervisors
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Teaching Experience

University of Missouri

Teaching Assistant for Undergraduate Biochemistry

(08/10-12/10)

- Scheduled and conducted weekly review sections outside of mandatory class period
- Counseled students and clarified difficult in-class concepts and exercises
- Communicated complex ideas and procedures to students with varying levels of understanding
- Evaluated student knowledge and designed examination questions to reinforce learning objectives
- Assembled and presented two, one hour lectures to a 180 person class, receiving compliments on relevance of content

Laboratory Instructor for Topics in Biochemistry

(01/11-05/11)

- Independently designed new course curriculum as well as making drastic changes to learning objectives, the syllabus, and overall course structure. Goal is that course becomes departmental requirement.
- Evaluated previous semester's experiments and troubleshoot broken protocols
- Created write-ups and PowerPoint presentations to assist students' assimilation of the material
- Coordinated and delegated responsibilities to several undergraduate laboratory assistants
- Compiled notes and materials lists for future instructors
- Redesigned pre-labs, quizzes, and exams to reinforce major concepts

Teaching Assistant for undergraduate non-majors biochemistry course

(01/11-05/11)

- Met independently with students to discuss disparities between lecture and assigned coursework
- Set up and independently instructed laboratory portion of the class
- Worked in conjunction with undergraduate learning assistants to establish learning objectives and improve laboratory conditions
- Conveyed the importance of a basic scientific understanding to the development of a personal world view

Service

Greek Week Blood Drive 2006

(April 2006)

- Volunteered as collection bag distributor

Rockin' Against Multiple Sclerosis 2006

(March 2006)

- Performed various household repairs, such as repainting wheelchair scuffs and installing kick-plates, for a woman with multiple sclerosis
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