Bi10, Spring 2017

INTRODUCTION.
Bi 10 is a laboratory course designed to introduce you to some basic techniques and concepts used in modern cell and molecular biology. We will be following a project lab format in this course. The experiments that you are doing will build upon each other from week to week, just like they do in authentic laboratory research. We hope that this format exposes you to the anticipation, excitement, and occasional disappointment that accompany "live" laboratory research in biology. For technical reasons, the course is divided into two parts. In the first, a gene that encodes a specific protein will amplified by PCR, cloned into an expression vector, and the construct analyzed by digestion with restriction enzymes and agarose gel electrophoresis. The second part makes use of a similarly-produced construct where the gene product is an enzyme. This gene has been cloned into an expression vector, which will allow it to be over-expressed in bacteria. The resultant enzyme will be purified and its activity assayed. This set of experiments recapitulates many of the manipulations that researchers on the cutting edge perform as they attempt to elucidate the functions of novel proteins identified by a variety of methods including genetic analysis and genome sequencing projects.

As the course proceeds, we want you to concentrate on:

(i) becoming proficient in the execution of basic techniques
(ii) understanding the concepts underlying the techniques you will be using
(iii) understanding the importance of controls for interpreting the results of experiments

In order to pass Bi 10 you must carry out all nine lab exercises and describe them in two written lab reports.

GRADING.
Your grade will be determined by your performance in four activities:(1) general laboratory activities, (2) maintenance of a coherent lab notebook, (3) answers to prelab questions, and (4) written reports.

General laboratory performance. You will be graded at each session for your: (1) punctuality, (2) preparation for the lab, (3) effort in the lab, (4) how well you keep your workplace in order, and (5) responsibility in carrying out your tasks.

(1) Punctuality (5 pts). Class begins at 2:00 PM and ends at 4:55 PM. Check in with your TA when you arrive and when you leave. **A point** will be deducted for every 2 min you are late, up to 5 points.

Students who would like to start a little earlier than 14:00 should make arrangements with the staff.

(2) Preparation (5 pts). Students are encouraged to read through the exercise before coming to class. The better prepared you are, the less time it takes to finish your work and you will make fewer mistakes. Before coming to class, write a few lines in your notebook about what you hope to accomplish and how you will proceed. Show your summary to your TA when you arrive.

(3) Effort (5 pts) No penalties will be given for failure to achieve the correct result. You will be penalized, however, for careless errors in technique; e.g. labeling tubes incorrectly, not following directions, discarding important samples, etc.

(4) Order (5 pts) Remember that someone will be using your lab bench the next day. Leave it the way you would like to find it. If we have to clean up after you, you will lose points.

(5) Responsibility (5 pts). Responsibility includes failure to take out plates, inoculate cultures on time, turn off an instrument after use, etc.

Lab notebooks. Maintaining a good notebook is key to becoming a successful experimental scientist and is required for the course. Your notebook should be well organized and provide a clear description of your experiments. You will be given a standard bound "composition book" for a lab notebook. Save page 1 as the Title page. Save pages 2 and 3 for a Table of Contents and fill them in as you go. Start writing your lab notes on Page 4. Add pictures, tables, etc. as necessary. Use as many pages as necessary for each session, single-sided only. Be generous with space.

For each session, write a brief description of what you hope to accomplish and how you will proceed. Leave space to record and discuss the results when they become available. Do not rewrite procedures that are already written out in the Lab Manual, but simply refer to the relevant page numbers. When collecting data, write directly in your notebook. Be sure to record when you deviate from the procedures given in the Lab Manual. Later on, you can make tables or graphs. This will all be useful when you have to write your reports.

Notebooks will be collected and inspected twice during the course, in weeks #5 and #9. Up to 25 pts will be awarded each time for a total of 50 pts for the course.

Prelabs. Prelab questions will be posted on the Bi 10 website prior to each week's lab session. Please download and print out the prelab form, fill in the answers, and return it to the lockbox in room 347K on your lab day. Handwritten prelabs are not appreciated! The prelab questions will be due at the start of each lab section and an answer key will be posted the following week. 5 points will be deducted if the prelab is handed in late the same week. No points will be awarded once the answer key is posted. Each prelab is worth 25 pts, for a total of 225 pts for the course.
Note: **If your lab session falls on Ditch Day, your prelab will be due the following work day.**

Each week, one of the TAs is responsible for writing and grading a Bi 10 prelab. The same TA will also be available to answer questions about the prelab on Monday, in room 347KRK, right after the Bi 10 discussion session (4pm-5pm).

Lab Reports. There will be two lab reports due during the course: a midterm report covering weeks 2-5 (due on your lab day, May 9, 10, or 12), and one covering weeks 6-9 (due June 9, at noon for seniors; June 16, at noon, for everyone else.) Guidelines for preparing an acceptable lab report are described on pages 5-9. Do not hand in your lab reports late, as this will adversely affect your grade. Late lab reports will be penalized as follows: 1-3 days late, 75% credit max; 3-10 days late, 50% credit max; 10-17 days late, 25% credit max.

50% of your grade will reflect the overall quality of your lab reports. Use only your own independently collected data. If one of your experiments does not work well, you will still receive credit for a lab report that analyzes carefully what went wrong and what might have made the experiment work better. More detailed instructions will be posted later on the B1 10 website. The TAs will hold a Q&A session concerning the Midterm Report before the reports are due. The time and place for the Q/A session will be posted on the Bi 10 website under “Announcements”.

Distribution of Points:

225 pts (25 pts/wk for 9 wks):	general lab performance
50 pts (checked 2 times):	notebooks
225 pts (25 pts/wk for 9 wks):	prelab questions
250 pts:	first lab report
250 pts:	second lab report
1000 pts:	TOTAL

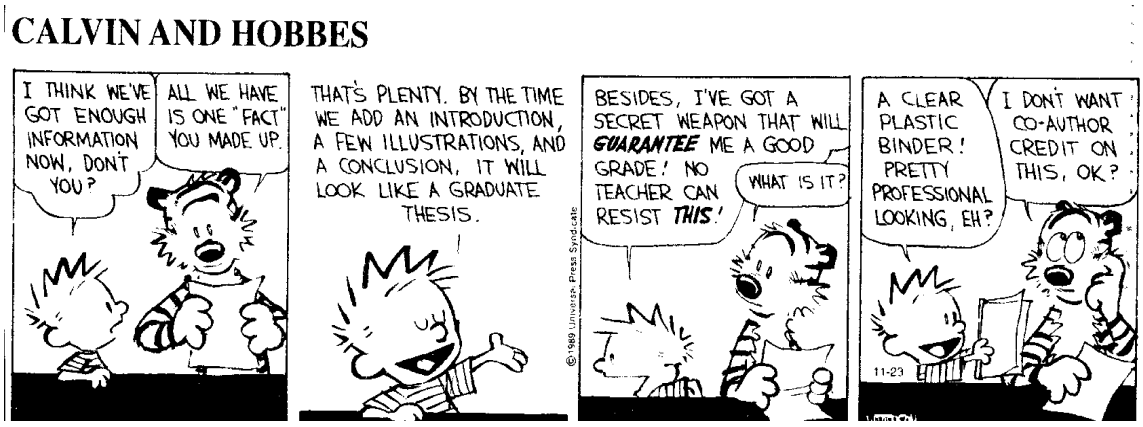
Extra points. 5 extra points will be awarded for carrying out some exercises successfully. These include:

Week #1: a bacterial streak that produces at least 10 well-isolated colonies.
Week #2: obtaining PCR product.
Week #3: obtaining green colonies.
Week #6: getting the correct value for the protein unknown.

If one of these lab sessions falls on Ditch Day, no extra points will be given for that exercise.

ATTENDANCE POLICY.
Since the labs in this course build upon one another from week to week, attendance is mandatory. If you are absent because of illness, you will need a doctor's note. If you must be absent for any other reason, you must obtain **prior** permission from Dr. Bertani. Unexcused absences will result in a grade of F. All excused absences will need to be made up prior to the start of the following week's lab period.

COLLABORATION POLICY.
Although Bi 10 is letter-graded, there are no exams, so it is important that we have some idea of your individual abilities. In general, we follow the rules of the honor system set out by the BOC committee. Although discussion with other students about prelabs or reports is encouraged **before** writing, the actual writing should take place without further collaboration. Looking at another student's finished prelab or report is forbidden.



Guidelines for Preparing the Lab Reports

General information:

- Each student is to hand in his or her own original report. You may consult your classmates in thinking about the data, but graphs, figures, text, and other aspects of the finished report should be yours alone (even if it didn't work).

- Do not copy or paraphrase the handouts (or anything else, for that matter). That is plagiarism and will not be accepted!

- The report should have a Title and include sections called Introduction, Materials and Methods, Results and Discussion. The Results and Discussion sections may be combined, if you wish.

- In writing the report, present your data and analysis in a logical, rather than a chronological, order. The individual experiments in the lab manual are arranged to allow for the time constraints of the course and do not necessarily reflect the way one would carry out the project under less constrained conditions. No one cares **when** you did an experiment, rather what you did and how it contributes to the project as a whole.

- Do not show all of your calculations, only your final result.

- **DO NOT** e-mail your report; hand in a **hard** copy.

Formatting (15 points total):

- The report should be prepared with a word processor, with the exception of drawings, which may be hand written (**5 points**).

- The text should be 12 pt font, double-spaced, with your Name, Section, and the Date on the header of each page; page number at the bottom (**5 points**).

- The report should be written in good English, using the passive voice. Do not use "I" (**5 points**).

Sections: The content of each section is described below.

Title (**5 points**)

A description of your project in **one short sentence**, without the details of the procedures used.

Introduction (**30 points**; **maximum of 1 page**)

Example of the usefulness of protein fusions (5 points)

A brief description of the steps you followed, pointing out the reason for each step and the advantages of the procedures you used, without giving details of the procedures. (The details of the procedures should be put in Methods section). (20 points)

Brief statement on project's success or failure (5 points)

Materials and Methods (25 points; maximum of 1 page):

List each procedure. Describe briefly its purpose and refer to the pages in lab manual for quantitative details. (20 points)

Add any changes or additions that you made to the procedures, either deliberately or accidently. (5 points)

Results and Discussion (165 points):

The results and discussion may be written as a combined section, divided into three parts. The content of the parts will be described in more detail for each report. Each part should contain:

A brief introduction describing the purpose of the experiment(s) and the methods that were used. Refer to the Methods section for details of the procedures. (5 points)

A presentation of the data. Use graphs, tables and diagrams whenever possible, rather than writing a verbal summary of your data. Tables and Figures should be numbered, titled, contain a figure legend, and further labeled (sizes for molecular weight ladders, arrows pointing to important components for later discussion). (20 points)

A discussion of the data. Discuss the data at each step without repeating the details of the experiments. Explain your logic in drawing your conclusions. What do the data suggest? What were your expected results? Did the data conform to your expectations and if not, why not? (30 points)

Final summing up (10 points)

Overall analysis of project. (5 points)
Suggest improvements. (5 points)

2017 Prelab Discussion Schedule for Bi10

Lecture	Date	Topics
1.	4/03	Polymerase Chain Reaction
2.	4/10	Agarose gel electrophoresis
3.	4/17	Bacterial transformation;
4.	4/24	Purification of plasmid DNA from E. coli.
5.	5/01	Analytical Gels
6.	5/08	Inducible promoters and Bradford assay.
7.	5/15	Affinity purification.
8.	5/22	SDS-polyacrylamide gel electrophoresis
9.	5/29	Memorial Day (no discussion)
10.	6/05	Enzyme assays

Discussions will be held in 119 Kerckhoff at 3:00 PM.

2017 Lab Schedule for Bi10

Week	Dates	Topic
#1	4/04, 05, 07	Microbiolgical procedures and use of the pipettor. Start PCR
#2	4/11, 12, 14	Characterization and digestion of PCR product.
#3	4/18, 19, 21	Assembly of a recombinant plasmid encoding a maltose-binding protein (MBP)-GFP hybrid protein.
#4	4/25, 26, 28	Small-scale preparation and restriction enzyme digestion of plasmid DNAs encoding MBP-GFP.
#5	5/02, 03, 05	Analysis of plasmid DNA restriction digest by agarose gel electrophoresis.
#6	5/09, 10, 12	Expression of MBP-β-galactosidase in *E. coli*. Quantitative determination of protein.
#7	5/16, 17, 19	Affinity purification of MBP-β–galactosidase.
#8	5/23, 24, 26	Evaluation of MBP-galactosidase purification by SDS-PAGE and quantitative protein determination.
#9	5/30,31;6/02	Determination of kinetic properties of MBP-β-galactosidase.
#10	6/06,07,09	Report writing (Optional).

Laboratory sessions will be held Tuesdays, Wednesdays, and Fridays from 2-5 PM in 347 KRK.

LAB RULES FOR YOUR SAFETY

Wash your hands well before leaving the lab.

Wear shoes, sneakers, or boots that cover your entire foot. Do not wear open sandals that leave your feet vulnerable to chemical spills etc.

Don't have books or personal items on your lab bench while you are doing lab work. Avoid paper towels and other clutter that can catch fire easily.

No eating or drinking in the lab.

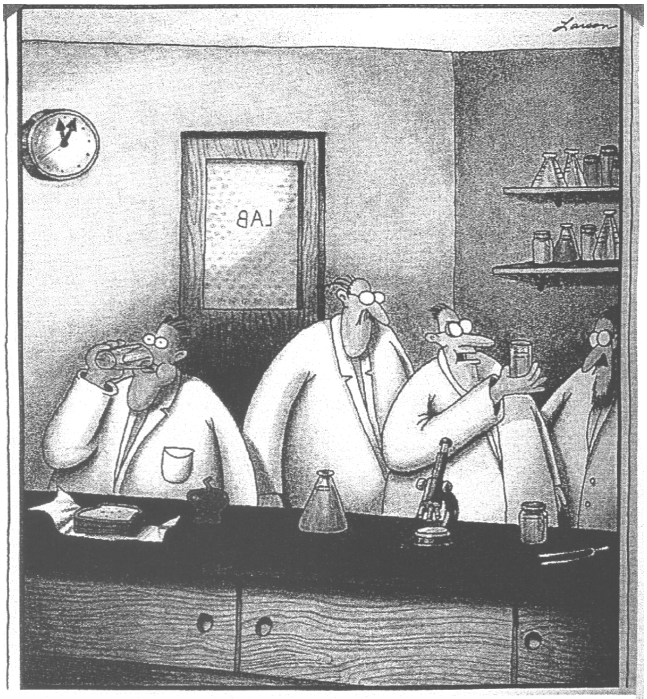
Do not pipet by mouth.

Tie back long hair, flowing sleeves, etc., so that they will not fall into cultures.

Let the instructor know about any spills, cuts or burns immediately.

Wear gloves when working with hazardous substances.

Electrophoresis involves high voltage. Never touch the apparatus while it is turned on. Disable the apparatus by turning off the power supply **and** disconnecting the leads, to be doubly sure that there is no power going to it before you touch it.



WASTE DISPOSAL

We are required by law to dispose of all chemical and biological waste generated during the course in specific containers. Ideally, nothing dangerous should go down the drain into the public water supply.

You will be provided with a zip-lock bag into which you should discard all microfuge tubes (liquid included), pipet tips, empty Falcon tubes, etc. At the end of each session, zip up your bag and discard it. For the first 6 weeks of the course, we will be using antibiotic-resistant bacteria, so the zip-lock bags should be discarded in the **large autoclavable bag**. After that, they can be discarded in the regular wastebaskets.

Glass culture tubes should be first drained into the biological waste container in the hood, rinsed with tap water, and then discarded into the plastic trays on the cart.

Falcon tubes should be emptied into the same container before being placed in the zip-lock bag.

Long plastic pipets, which are contaminate with bacteria, should be discarded directly into the autoclavable bag. Otherwise, they may be discarded in the regular trash.

Plates containing bacteria should be discarded into the autoclavable bag.

Gels contaminated with EtBr can be discarded directly into the EtBr waste container.

Tips contaminated with EtBr will be collected up in a special bag.

Solutions used during the last 3 weeks of the course can for the most part be poured down the drain, with the exception of Bradford reagent which must be poured into the red solvent container. Glass reaction tubes containing Bradford should first be emptied into this container and then discarded into the special containers for broken glass.

Wash solutions, running buffers, destain, Z-buffer etc, as well as unused media and left-over buffer from agarose gels may be poured down the drain.

Before leaving, wipe down your bench with 409, so the next class can start with clean benches.

Remember-- you will lose points if you do not dispose of your waste properly!

Week #1: Microbiological procedures and use of the pipettor.

Before beginning, prepare your notebook according to the instructions on p.2 of the lab manual and show it to your TA for comments.

Objective : In this week's lab you will first learn some microbiological techniques and use them to explore your personal and environmental microbiota. Then, you can familiarize yourself with the use of an automated pipettor. Accurate pipetting and good sterile technique are essential for the successful completion of the experiments in this course. When you feel that you are proficient with the pipettor, you set up the reactions for the PCR (polymerase chain reaction) exercise, which will be continued at the next session.

Part I. Exploring the microbiota

Bacteria can be found in the air, on all exposed surfaces, and on the skin. If a sterile LB plate is left open for 1-2 hrs or you rub your fingers on the agar surface, bacterial colonies will appear on the plate after 1-3 days. In your lab exercises, you will be using a strain of *E. coli*. The normal habitat of *E. coli* is the human gut and it does not do well in other environments. If you find *E. coli* on your plates, it means you are not washing your hands very well. In fact, that is what they look for in restaurants when they are checking for cleanliness. There are lots of other bacteria, however, that live in the soil and are perfectly happy blowing about the lab.

Materials:

On your bench:	6 LB agar plates (color-code, one blue streak). 1 LB+amp plate (color-code one blue + one orange streak). LB Broth in Falcon tubes Distilled H2O 1.7 ml microfuge tubes (carton; green label) Loops Wooden inoculator sticks
On the table:	LB plates with colonies. Glycerol and Triton X-100 solutions. Sterile, capped test tubes

1. Start by exploring the microbes in your environment. LB is a standard growth medium for bacteria and can be used in either liquid (broth) or solid (agar plate) form. Take one of the LB agar plates and, using a magic marker, label the bottom of the plate with your initials and the day of your lab section. Remove the lid, and set the plate open on your bench. As a negative control, take a second LB plate, label it, and place it next to the first, except leave the lid on. Any microorganisms that are present on dust particles floating

about in the air can settle on the exposed LB agar surface and begin to multiply. This will take some time, so leave the plate open to the air while you perform the rest of today's exercises. At the **very end** of the session, close the plate and place it in the cardboard box at the end of the room. This box will be left at room temperature.

2. Next, you can examine your own personal microbiota and ask the following questions: Are there bacteria on my hands? Are they susceptible to antibiotics? Can they be removed by washing my hands?

   To investigate the bacteria on your hands, take another LB agar plate and also the LB amp plate. Using a magic marker, divide each plate in two halves by drawing a line down the middle of the back side of the plate. Mark one half "before" and the other half "after". Moisten your fingers with tap water and rub them over the surface of the "before" half of both plates.

   Now, wash your hands with the liquid soap provided at the sinks. Wash thoroughly, especially under rings or fingernails where bacteria might hide and dry your hands with a paper towel. Then, repeat the process of rubbing your fingers on the “after” half of each plate. This is your test of the effectivness of washing your hands. Put the lids back on the plates and let them stand on your bench top until the liquid soaks in.

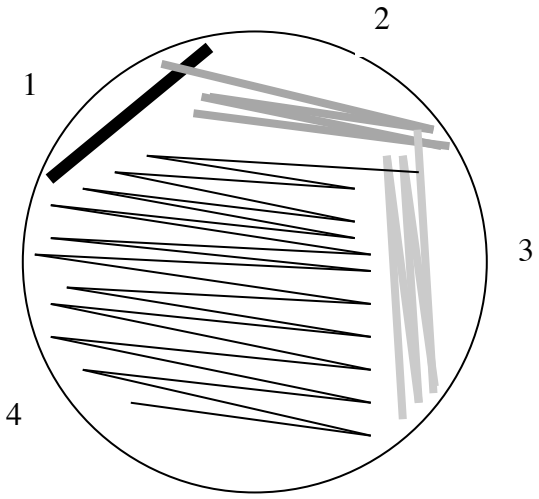
   How about your internal microbiota? Take a fresh LB plate, label, and either touch your tongue to the agar surface or spit a small amount of fluid from your mouth onto the surface of the agar. Spread the liquid over the surface of the plate with a plastic loop. Cover the plate, and let it stand on your bench top until the liquid soaks in. Incubate all the plates from step 2 in the 37°C incubator.

Part II. Sterile techniques.

To protect our strain from other contaminants, we use sterile techniques. All media, test tubes, pipet tips etc. are sterilized either by autoclaving at 15 psi or by filtering through sterilizing membranes. Then, we try to keep things sterile by not leaving bottles, plates or pipet tip boxes open to the air and by not touching anything, like the tip of a pipet, inoculation stick, toothpick, etc. that will later come in contact with the sterile media.

1. Using the two remaining LB plates, practice streaking out bacteria using sterile plastic loops. The technique of single colony isolation is invaluable for the genetic purification of a strain or for purifying it away from other contaminating bacteria. Obtain a plate containing bacterial colonies from the table. A colony originates from a single bacterium that has produced so many progeny that the clump has become visible to the naked eye. The object of this exercise is to dilute out the bacteria so much that they are cleanly separated from each other and produce individual colonies. Since even a small swatch of bacteria on the end of a loop can contain 10-100 million cells, you will need to **change loops each time** you streak.

Streak according to this pattern:



(i.) Scoop up a single bacterial colony with a loop and draw the bacteria in a line near the edge of the plate..

(ii.) Discard the loop, rotate the plate slightly and, using a fresh loop, make several rapid back-and-forth movements, drawing the material from a small area of the first streak into an unused part of the plate.

(iii.) Again, discard the loop, rotate the plate slightly and, using a fresh loop, make several rapid back-and-forth movements, drawing the material from the second streak into an unused part of the plate.

(iv.) Continue the process one more time, drawing material from the third streak into the fourth. Plan to cover the entire surface of the plate.

Repeat with a second colony, using the second plate. **Do not streak 4 colonies once each.**

Make sure that all of your plates are labeled with magic marker on the bottom with your initials and the day of your lab section. Turn over the plates and place them lid-side down in the 37°C incubators. The plates will be removed for you tomorrow. You will examine them all at the next session.

2. You will now practice sterile manipulation of liquid solutions and preparation of bacterial cultures. You will find some LB broth (this is the same growth medium as is in the plates, but without the agar solidifying agent) on your bench. Using a plastic 10 ml pipet and a pipet pump, transfer 2 ml of LB to each of 4 sterile, capped test tubes. The TAs will demonstrate how to pipet sterile solutions. Using sterile inoculation sticks,

inoculate 2 of the tubes with bacterial colonies. For the remaining 2 tubes, perform mock inoculations (stick, but no colony) as negative controls. **Do not** leave the sticks in the test tubes. Label the tubes to indicate your name, lab section, and whether or not they have been inoculated with bacteria. Place the tubes in the 37°C shaker incubator. Tomorrow they will be removed for you and placed in a fridge until the next session.

Part III. Use of the Automated Pipettor.

1. You should have a set of the 'pipettors' listed below:

   P10: Used to pipet very small volumes from 0.5-10 µl. Uses a colorless ultra-fine tip. Dial increases (as read from top to bottom) from 001, which represents 0.1 µl, to 100, which represents 10.0 µl.

   P20: A P20 can be used to pipet from 0-20 µl - however, between 0-10 µl, it is preferable to use a P10. From top to bottom, the dial reads from 001, which equals 0.1 µl, to 200, which equals 20 µl. The P20 is used with a yellow tip.

   P200: A P200 is used to pipet from 0-200 µl. From top to bottom, the dial reads from 001, which equals 1 µl, to 200, which equals 200 µl. Between 0-20 µl, it is best to use a P20 instead of a P200. The P200 uses the same yellow tip as the P20.

   P1000: A P1000 can be used to pipet between 10-1000 µl (1.0 ml). The dial reads, from top to bottom, 001, which equals 10 µl, to 100, which equals 1.0 ml. In the low range from 0-200 µl, it is preferable to use a P200. The P1000 uses a blue tip.

   P10 ml: A P10 ml can be used to pipet between 1 and 10 ml. The dial reads from 0.1 ml to 10 ml. In the low range, from 0.1 to 1 ml, it is preferable to use the P1000. The P10 ml uses extra large tips found in the tall ice cream carton on your bench.

2. All pipettors have a plunger which, when depressed with your thumb, can stop at either of two points. To use a pipettor, dial in the volume you wish to pipet and attach the appropriate tip to the base of the pipettor barrel. Make sure that the tip is seated firmly on the barrel. Depress the plunger of the pipettor to the first stop, and then insert the tip into the liquid that you wish to pipet. Now, allow the spring loaded plunger to return slowly to its original position. As the plunger moves upward, liquid is sucked up into the tip attached to the pipettor. Do not allow the plunger to snap back too quickly, as this will almost certainly lead to errors in pipetting. Also, do not remove the tip from the solution that you are pipetting until the plunger has reached its starting position. If you remove the tip from the liquid while the plunger is still rising, air will be sucked up into the tip, resulting in a pipetting error. When you are pipetting, do not insert the tip too deeply into the liquid you are pipetting. A few millimeters is usually

sufficient. When you are using a pipettor, **always keep your eye on the pipet tip**. Always take a quick look at the tip after pipetting to make sure that it has been properly loaded. To dispense a liquid sample from a pipettor tip, simply press the plunger down to the first stop, wait for the liquid to drain from the tip, and then depress the plunger to the second stop to expel any liquid remaining in the tip. Practice pipetting water for several minutes with each pipettor until you can quickly and confidently fill and unload the pipettor tip. Practice dispensing droplets of different volumes, especially the smaller volumes like 1-10 µl, onto a piece of Parafilm. Notice what they look like. Pipet five 10 µl volumes into a microfuge tube with a P10 and then check if the volume is 50 µl by drawing the whole contents of the tube up into a P200 set for 50 µl.

3. There are two special circumstances which make using a pipettor a bit tricky. The first is viscous solutions. When working with enzymes, one often has to pipet solutions containing high concentrations of glycerol, which is commonly used to stabilize enzyme preparations. Since glycerol is viscous and flows slowly, there will be a significant delay between the movement of the plunger and the movement of liquid into or out of the tip. There will be 50% solutions of glycerol for you to practice with. Try pipetting glycerol with each pipettor to see how it compares to pipetting water. A second situation you will encounter is solutions that contain either high concentrations of protein or detergent. Both of these substances behave as surfactants, and lower the surface tension of the solution. This is manifested by a tendency of the solution to "creep" into an empty tip (especially a pre-wetted one), and to create bubbles during the filling or unloading of a pipet tip. There will be solutions of Triton X-100 in the lab; this is a commonly used non-ionic detergent. Try pipetting this detergent with each pipettor to see how it compares to pipetting water.

4. Whenever you are using an automated pipettor, it is **important to change tips between each pipetting operation.** Failure to change tips can result in either inaccurate dilution or even worse, cross-contamination of solutions that you are pipetting. You do not have to change tips, however, if you are pipetting aliquots of the **same solution** into many tubes.

Part IV. Setting up the PCR reactions:

Materials:

On the table:

"P" = a mixture of the forward and reverse oligo primers, GFPF and GFPR.
"T" = the template DNA
"N" = the dNTP (deoxynucleoside triphosphate) mix
"B" = the buffer for the Taq enzyme.

Taq = Taq enzyme
200 µl PCR tubes (ice cream carton; **yellow** label)

Once you feel confident of your pipetting ability, fetch some ice and set up the following PCR reactions on ice (ice buckets are at the end of the room).

Place tubes, "P", "T", "N", and "B" on ice. There should be distilled water already on your bench. The Taq enzyme will be shared with your bench partner.

"P", "T", "N", and "B" are the components of the PCR reaction. They are described in more detail in the Appendix. PCR will be the subject of next week's lecture, but right now just concentrate on pipetting the right things in the right tubes.

Prepare your three PCR reactions on ice in the **special 200µl PCR reaction tubes** following the instructions in the table given below.

> *The first reaction lacks the PCR oligo primers, the second reaction contains both oligos but lacks template DNA, and the third reaction contains all of the components required for successful PCR. The first two reactions are controls that are typically used in a PCR experiment to ensure that amplification depends on both PCR oligos and DNA template.*

Make sure that all solutions are **completely** thawed before using them. Add the components **in the order given, starting with distilled water**. Pipet each component directly **into the water** and mix by stirring with the pipet tip. Tubes should be cooled on ice before adding enzyme. Be especially careful to mix in the enzyme thoroughly. Change tips every time!

	Reaction #		
Component:	1	2	3
distilled water	70 µl	70 µl	60 µl
"T"(2.5 ng/µl template DNA)	10 µl	-	10 µl
"P" (10 µM oligo mix)	-	10 µl	10 µl
"N" (10X dNTPs)	10 µl	10 µl	10 µl
"B" (10X PCR reaction buffer)	10 µl	10 µl	10 µl
Taq DNA polymerase	0.5 µl	0.5 µl	0.5 µl

When your reactions are ready, place them in the Thermal Cycler, currently on "Icebucket". Make sure that your tubes are clearly labeled with your initials. When all reactions are ready, the TAs will start the Thermal Cycler. The details of the PCR program are given in

the Appendix. The reactions will be collected later on when the cycle has finished and given to you at the beginning of Week #2.

Check out with your TA.

Clean up:

Pour leftover LB down the drain.

Place gloves, loops, inoculation sticks, parafilm, **empty** Falcon tubes, T, P, N, and B, in your ziplock bag and discard into the large, autoclavable bag.

Leave the sterile water on your bench.

Return any leftover Taq polymerase to your TA.

Return plates with colonies, glycerol, and Triton X-100 to the table.

Wipe off your bench with 409.

Week #2: Purification of GFP Coding Sequences Produced by the Polymerase Chain Reaction.

Objective: At the end of last week's exercise, you set up PCR reactions to produce large amounts of the gene encoding green fluorescent protein (GFP). This week you will use gel electrophoresis to confirm that PCR product was obtained. In addition, the product will be purified and digested with restriction enzymes in preparation for cloning. The availability of large amounts of custom-synthesized GFP DNA will facilitate construction of a recombinant plasmid that, when induced, expresses GFP fused to the maltose-binding protein (MBP) in *E. coli*.

The template DNA (see appendix) consists of a GFP gene that has already been cloned into a plasmid, so you will actually be **subcloning** the gene into an expression vector. Otherwise, you would have to use jellyfish genomic DNA as template, a much more difficult procedure. Details of the PCR product are given in the Appendix. Notice that the oligo primers are constructed so as to add restriction sites to the PCR product. You should be able to see both the restriction sites and the start codon (ATG) of the GFP gene in the primers.

A picture of your gel should be included as a figure in your first report.

Materials:

On your bench:	125 ml graduated Erlenmeyer flask
	6X dark blue loading dye (bromphenol blue)
	PB buffer
	PE buffer
	Qiaquick purple PCR purification column
	Distilled H_2O
On the table:	1 kb molecular weight ladder = 1 kb
	EcoRI buffer = B
	EcoRI enzyme = EcoRI
At each end of the room:	agarose powder and balance.
	TAE running buffer
Ethidium Bromide Station:	10 µl tips
	Ethiduim bromide solution= EtBr
	Ethidium bromide tip discard bag

Experimental Protocol:

1. Begin by pouring a 1% agarose gel.

   a. Weigh out 0.5 grams of agarose and transfer the powder to a 125 ml Erlenmeyer flask. Using the graduations on the flask, fill to 50 ml with 1x TAE buffer.

   TAE stands for Tris-acetate plus EDTA. Tris-acetate is a buffer that maintains proper pH control during gel electrophoresis. EDTA is a chelator of divalent ions. EDTA is often used in experiments involving DNA, since DNA-degrading enzymes (DNases) require the divalent ion Mg^{2+} for activity.

   b. To melt the agarose, heat the suspension in a microwave until it begins to boil. This may take less than a minute. Gently swirl the flask from time to time. **Be extra cautious, since super heated solutions of agarose have a tendency to boil explosively. Wear protective mittens when you handle the hot flask and keep it away from your face!!**. Continue heating and swirling the agarose solution until it is **completely** clear and free of translucent agarose particles.

   c. Let the flask stand on your bench for 5-10 minutes to allow the agarose to cool a bit. . (A quicker method to cool the agarose is to hold the flask for a few minutes under running tap water, swirling the contents gently all the time so that the agar does not begin to solidify on the sides.) Cool until you can put your hand on the flask without feeling too much pain. Then, add 2.5 µl of a 10 mg/ml stock of the DNA-staining dye ethidium bromide. **Be careful - ethidium bromide is a mutagen**. Use the EtBr and tips at the EtBr station. Do not take the EtBr to your bench! Discard your tip in the plastic bag provided. Swirl the flask to disperse the dye. Set up your gel box, taking care that the rubber gaskets of the tray are aligned in the grooves and fit snugly against the box to avoid leaks. (The gaskets slide more easily if they are moistened with distilled water). Pour the agarose + ethidium bromide into the gel tray and place a comb with 10 slots in the end position. (While you are waiting for the gel to harden, proceed to step 2.)

   Upon cooling, solutions of agarose solidify to yield matrices with fairly regular pore sizes. The physical characteristics of these matrices render them well-suited for fractionating nucleic acids by electorphoresis. Ethidium bromide is a planar molecule that intercalates between the stacked base pairs of a DNA double helix. Upon irradiation with UV light, the ethidium bromide fluoresces an orange-pinkish color, making it easy to locate the DNA fragments within the agarose gel matrix.

   d. Once the gel hardens, which will take 20-30 minutes, remove the comb, pull up the gel tray, rotate it 90° and place it back in the gel box with the wells at the negative (black) end. Add enough 1X TAE buffer to just cover the gel.

2. Obtain your 3 PCR reactions from the TAs. While the reactions are thawing, label three 1.7 ml microfuge tubes #1, #2. and #3. Pipet 10 µl of each reaction into the appropriate tube and mix it with 1 µl of blue gel-loading dye. Close the tubes #1, #2, and #3 tightly and set aside. The rest of reaction #3 will be purified further.

> *DNA gel loading dye has 3 important components. First, it contains blue dye, which makes it easy to see what you are doing when you are loading the gel. The dye, which migrates equivalently to a DNA fragment of ~300-500 bp, also serves as a migration standard. Second, it contains EDTA, which quenches most enzymatic reactions involving DNA, since most enzymes that modify DNA require Mg^{2+} as a cofactor. Third, it contains the polymer Ficoll, which makes it dense. It needs to be denser than water, since it needs to sink into the wells of the gel upon loading.*

3. The remainder of reaction #3 will be purified to separate the PCR product from excess oligonucleotides, deoxynucleoside triphosphates, and Taq DNA polymerase.

a. Pipet the remainder of reaction #3 into a fresh 1.7 ml microfuge tube and add 5 volumes (5 x 90µl = 450 µl) of buffer PB to it. Close the tube and mix thoroughly by vortexing.
b. Carefully pour or pipet the mixture onto a purple Qiaquick PCR purification column. Collect the last drops in the PCR tube with a yellow pipet tip and add those to the column.
c. Centrifuge the column for 60 seconds in a microfuge. This will force the DNA solution to pass through the DNA-binding membrane at the base of the column.
d. Discard the column flow-through from the collection tube and then re-insert the column into the same tube.
e. Add 750 µl of buffer PE (wash buffer) to the column, and force the buffer through the membrane by centrifuging the column for 60 seconds in a microfuge.

   This wash step removes residual oligos, dNTPs, and enzyme, which do not stick tightly to the DNA-binding membrane

f. Discard the column flow-through, re-insert the column in the collection tube, and centrifuge the column unit again for 60 seconds in a microfuge to ensure that the last traces of the PE wash buffer have been removed from the column membrane.
g. Insert the column into a **clean** 1.7 µl microfuge tube and discard the collection tube. Elute the DNA from the column by adding 30 µl of distilled water to the top surface of the column membrane. Add the water so that it falls on the center of the column membrane. Be careful not to puncture the membrane with the pipet tip.
h. Let the column stand for at least 60 seconds and then centrifuge for 60 seconds in a microfuge to force the water through the column. The small volume of water at the base of the microfuge tube should contain your purified GFP PCR product.

DNA binds to the column membrane via hydrophobic interactions. Since hydrophobic interactions are greatly strengthened by high concentrations of salt, the DNA sample is diluted with a salty solution (buffer PB) before being loaded on the membrane. After washing with ethanol (buffer PE), the membrane is washed with a low–salt solution (i.e. water), which greatly weakens its hydrophobically-driven interaction with DNA, resulting in elution of the DNA.

4. When your PCR product has been purified, mix 5 µl of the purified DNA with 4 µl sterile distilled water and 1 µl blue loading dye (=#4).

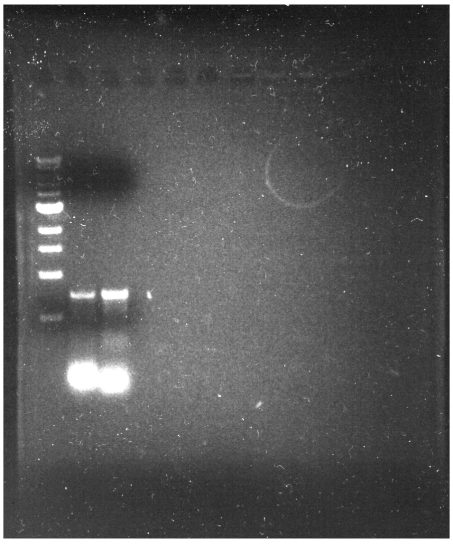
Also mix 1 µl of 1 kb molecular weight ladder with 8 µl water and 1 µl loading dye (=#5).

5. Then load all 5 samples onto your gel in the following order:

#1 = 10 µl PCR rxn 1
#2 = 10 µl PCR rxn 2
#3 = 10 µl PCR rxn 3 (rxn #3, before purification)
#4 = 10 µl purified PCR rxn 3
#5 = 10 µl 1 kb MW ladder

Once you have finished loading the gel, electrophorese the samples at ~110-120 volts for ~30 min. (While you are waiting, proceed to step 6).

When the dye has run about half way down the gel, turn off the power and photograph the gel under UV light to visualize the DNA fragments. Take 2 pictures – one for your lab notebook and one for the instructor. Do all samples have PCR products? Use the picture of the molecular weight markers given in the Appendix to estimate the sizes of the PCR products. Does this agree with the expected size? Below is a picture of the lower part of the 1 kb ladder (the 0.5 kb band is obscured by the dye) and the PCR product (controls not included).



6. While the gel is running, examine your plates and tubes from last week. Record your observations from part I.

How many and how many different kinds of organisms have grown up on the plates?

Are the organisms on all plates the same or do they differ? How? Why?

Did anything grow on the LB + amp plate?

Was washing your hands effective?

Did you get single colonies from your streak? If not, ask your TA for another plate and try again. Show your streak to the instructor to qualify for extra points.

Were your inoculations as expected?

Also, set up your EcoRI digestions. Mix together the following components in a 1.7 ml microfuge tube that is labeled with your name. Add buffer and enzyme directly to PCR product.

25 µl of purified PCR product
3 µl 10x EcoRI buffer
2 µl EcoRI restriction enzyme

Once all of the components have been added, gently vortex the tube and collect the contents at the bottom of the tube by centrifugation for 2-3 sec in a microfuge. **Close the tube tightly** and incubate in the 37°C water bath. Your tube will be transferred from the 37°C bath to the freezer later tonight.

If you did not get any PCR product, you can share another student's digest at the next session.

Clean up.

Rinse E-flasks with tap water and place in gray trays on the cart by the door to be washed.

Pour electrophoresis buffer down the drain, rinse gel box with distilled water, and leave on your bench.

Discard gels in special EtBr container.

Return MW ladder, EcoRI enzyme and buffer to ice buckets.

Leave the blue loading dye, PB, PE, and distilled water on your bench

Place used microfuge tubes, columns, tips etc. in your zip-lock bag, seal, and discard in regular trash.

Discard plates in autoclavable bag.

Empty your glass test tubes into the "Biological Waste" container, rinse them with tap water, and place them in the gray trays to be washed.

Swab your bench with 409.

Week #3: Assembly of a recombinant plasmid encoding a maltose-binding protein-GFP hybrid protein.

Objective: The purpose of this week's lab is to construct a recombinant plasmid that encodes a maltose-binding protein-GFP fusion protein. This is accomplished by ligating purified, Eco RI-digested, GFP DNA to plasmid DNA encoding the maltose-binding protein (MBP). The products of the ligation reaction will then be transformed into competent *E. coli* cells to recover recombinant molecules.

Materials.

On your bench:
- PB buffer
- PE buffer
- Qiaquick purple PCR purification column
- Distilled water
- LB broth
- 4 LB + amp plates.
- Sterile spreaders

On the table:
- RI-digested, phosphatased pMAL vector = V
- Control plasmid DNA = pMAL
- 2x Quick ligase buffer = QLB
- Quick ligase = QL
- Competent DH5a cells (screw-cap vials).
- Dry-ice ethanol bath

Experimental Protocol:

Ligation:

1. At the end of last week's lab period you set up a restriction digest of your amplified DNA segment. Following several hours incubation at 37°C, these digests were stored in a freezer. Recover your digest from the TAs, thaw the tube, and centrifuge briefly. Before you can ligate this fragment to a plasmid vector, the restriction enzyme must be removed to prevent it from recleaving molecules that are joined during the ligation reaction. Purify the GPF DNA fragment using a purple Qiagen PCR purification column and the same procedure that you did last week.

   a. Add 5 volumes (5 x 30 µl = 150 µl) of buffer PB to the Eco RI digest. Close the tube and mix thoroughly by vortexing.
   b. Carefully pipet the mixture onto a purple Qiaquick PCR purification column.

c. Centrifuge the column for 60 seconds in a microfuge. This will force the DNA solution to pass through the DNA-binding membrane at the base of the column.

d. Discard the column flow-through liquid and reinsert the column in the collection tube.

e. Add 750 µl of buffer PE (wash buffer) to the column, and force the buffer through the membrane by centrifuging the column for 60 seconds in a microfuge.

f. Discard the column flow-through liquid, reinsert the column in the collection tube, and centrifuge the column unit again for 60 seconds to ensure that the last traces of the PE wash buffer have been removed from the column membrane.

g. Insert the column into a **clean** microfuge tube and discard the collection tube. Elute the DNA from the column by adding 30 µl of distilled water to the top surface of the column membrane. Add the water so that it falls on the center of the column membrane. Be careful not to puncture the membrane with the pipet tip.

h. Let the column stand for at least 60 seconds and then centrifuge for 60 seconds in a microfuge to force the water through the column. The small volume of water at the base of the microfuge tube should contain purified Eco RI-digested GFP DNA.

2. After purification is complete, set up ligation reactions between your EcoRI-digested PCR product (insert) and the plasmid vector DNA (V) that will be given to you. For the ligation you will use "Quick Ligase". With "Quick Ligase" the ligation takes only five minutes instead of 1 hr as is usually the case for T4 ligase.

a. Set up the ligation reactions as described in the table below, in the order indicated. Remember to mix in the enzyme very thoroughly.

	Reaction #:	
component	1	2
distilled water	9 µl	0µl
insert (GFP fragment)	0 µl	9µl
pMAL vector DNA	1 µl	1 µl

In a ligation reaction, the plasmid DNA is commonly referred to as the "vector" and the DNA fragment being joined to it is referred to as the "insert." The vector DNA that you are using has been linearized with EcoRI, dephosphorylated with alkaline phosphatase, and purified on an agarose gel. The alkaline phosphatase removes the phosphate groups from the 5' ends of the cleaved plasmid, thereby preventing the plasmid from recircularizing during the ligation reaction. Notice that reaction 1 has no

insert. This is the most basic control for ligation reactions. It provides an estimate of both the degree to which the purified vector is contaminated by uncleaved supercoiled plasmid, and the efficiency of the dephosphorylation reaction. Contaminating supercoiled plasmid or phosphorylated vector can give rise to a large number of bacterial transformants containing insert-free vector, making it difficult to identify recombinant plasmids.

b. Add 10 µl 2X ligation buffer to each reaction.

c. Add 1 µl of Quick Ligase to each and mix thoroughly with a pipet tip.

d. Centrifuge briefly and incubate 5 min at room temperature.

e. Chill on ice.

Transformation:

1. You will be provided with a tube containing 500 µl of frozen competent bacteria. Thaw the tube on ice, flick to resuspend the cells, and pipet four 100 µl aliquots into **prechilled** 1.7 ml microfuge tubes. Immediately place the remaining 100 µl in the dry ice-ethanol bath. **It is crucial that the cells not warm to more than 4°C during these manipulations, as this will destroy their competency.**

2. Also obtain some control plasmid (pMAL) DNA. This is the vector plasmid before it was digested with RI and dephosphorylated. It has also been diluted 1000X. Its original concentration was 100 µg/ml.

3. Set up four transformation reactions: one for each ligation, one for the plasmid control, and one for a minus DNA control.

Set up the transformations as indicated below:

	Transformation #			
Component	1	2	3	4
competent cells	100 µl	100 µl	100 µl	100 µl
DNA	10 µl ligat'n1	10 µl ligat'n2	2 µl pMAL	none

Although bacteria normally have an impenetrable cell wall and cell membrane, they can be induced to take up plasmid DNA molecules by

treating the bacteria with calcium chloride. Calcium chloride-treated bacteria, which are referred to as competent, can be stored for up to several months in a -80°C freezer. When performing a transformation with a new batch of competent cells, it is essential to perform the minus DNA and supercoiled plasmid controls. The minus DNA control will indicate whether your competent cells have become contaminated by antibiotic-resistant bacteria or by plasmid DNA containing an antibiotic-resistance gene. The supercoiled plasmid control will reveal how well your competent cells are performing. Depending upon the bacterial strain and the method of transformation, one can typically obtain between one million and one billion transformed bacterial colonies from 1 μg of plasmid DNA.

Incubate the transformation reactions for 20 minutes on ice.

4. Heat shock the cells to induce them to take up plasmid DNA by incubating each reaction at 37°C for exactly 45 seconds, and then place the tubes on ice for 2 min.

5. Add 900 μl of LB medium (antibiotic-free) to each reaction. Close the tubes tightly and tape them horizontally to the platform of the 37°C shaking incubator. Shake for at least 30 minutes.

LB medium is a commonly-used "rich" medium used for growing E.coli. It contains all of the metabolites that are necessary to sustain growth of wild-type strains of E.coli. It is used for growing bacteria in liquid cultures, and can also be solidified with agar to make petri plates. During the incubation in LB, the bacteria will resume growth, and the cells that have taken up plasmid DNA will begin to express the antibiotic-resistance gene present on the plasmid.

6. After 30 minutes incubation at 37°C, microfuge the tubes for 2 min at 8000 rpm, pour off the supernatant and resuspend cells in the 100 μl or so of LB that remains at the bottom of the tube. Pipet the entire contents of each tube onto an individual LB + amp plate and spread it over the surface of the plate with a sterile spreader. Mark the plates with your initials and the day of your lab section. Place the plates upside down in a 37°C incubator. They will be collected after about 16 hr incubation and stored at 4°C.

Please note: You will have to come in the afternoon before next week's lab to inoculate transformants for plasmid DNA preps!! Tuesday section can come in either before (2:30-3 PM) or after (4-4:30 PM) the discussion session on Monday. The other sections can come any time class is running (2-5 PM) on Tuesday or Wednesday. There will be TAs to help you.

CLEAN UP:

Leave PB, PE, and distilled water on your bench.

Return Quick Ligase to the ice bucket.

Place all remaining microfuge tubes, columns, empty Falcon tubes, spreaders, etc, in your zip-lock bag. Seal tightly and place in the large autoclavable bag.

Swab your bench with 409.

Week #4: Small-Scale Preparation of Plasmid DNA

Objective: The PCR product, which has been digested with EcoRI, can be inserted into the EcoRI site on plasmid DNA in either of two orientations. The purpose of this week's lab will be to identify a recombinant plasmid containing the GFP gene inserted in the correct orientation at the 3' end of the maltose-binding protein (MBP) gene. This will be accomplished by isolating plasmid DNA from each of four different bacterial colonies and analyzing the isolated DNA by digesting it with restriction enzymes and examining the resulting bands on an agarose gel. A diagram of the construct can be found in the Appendix.

Experimental Protocol:

Part I

This step is to be done the afternoon before your lab section!!

Get your plates from the box and scan them with one of the hand-held UV light monitors that will be available. Observe the colonies BRIEFLY (a few seconds) as UV can damage the bacteria. What do you expect to see? What do you see? Count the colonies on all the plates **without** destroying them. Record both fluorescent and non-fluorescent colonies. Decide which colonies you will be using for minipreps. Make a table of your data to include in your first report.

Obtain four sterile, capped test tubes each containing 6 ml of LB amp. Using a sterile inoculation stick, inoculate each tube with a single colony of bacteria. Inoculate two of the tubes with a bacterial colony that fluoresces green. Inoculate the remaining two tubes with bacterial colonies that do not fluoresce. Label your tubes #1-4 (1,2 for green colonies, 3,4 for uncolored), and make sure that they are marked with your initials. Place all four tubes in the 37°C. shaker. Put your plate#2 back in the box and discard the rest in the autoclavable bag.

> *Green fluorescent protein absorbs ultraviolet light, and the energized protein returns to its ground state by emitting a photon whose wavelength your eyes perceive as green. The hand-held UV light is used to excite GFP molecules contained in the bacterial cells. Colonies that fluoresce green are those that presumably took up correctly ligated MBP-GFP plasmids. Those colonies that are uncolored presumably took up incorrectly ligated plasmid molecules. Be careful to shine the UV light upon the colonies BRIEFLY (only a few seconds), because UV light is mutagenic and bactericidal.*

Part II:

To isolate plasmid DNA, we will use a Qiagen Spin Miniprep Kit. This is faster and more convenient than the older method which uses phenol and chloroform. With the Qiagen Kit, the DNA is first bound to a silica-gel membrane at high salt concentration and then eluted from the membrane with water. Otherwise, many of the steps are the same.

Materials

On your bench:	LB amp plate
	P2 buffer
	N3 buffer
	PE buffer
	Distilled water
	Qiaquick blue spin prep columns
On table:	P1 buffer (return to ice bucket immediately after using.)
	EcoRI (RI)and NcoI (N)enzymes
	Enzyme buffer (B)

1. At the start of the lab period, remove your cultures from the shaker. Divide an LB+ampicillin plate into four sectors and transfer a small droplet (~5 µl) of each culture onto an individual sector. Mark the plate with your initials and the day of your lab section, and place the plate in the 37°C incubator. These plates will provide a check to see if you picked the right colonies. They will be stored at 4°C until the next session.

2. In order to have as much DNA as possible, collect bacteria from the entire culture by repeated centrifugations, as follows: pipet 1.5 ml (2 x 750 µl) of each bacterial culture into a single 1.7 ml microfuge tube. Label. Cap and microfuge at 10,000 rpm for 2 minutes. Discard the overlying supernatant liquid ("sup") and pipet in 1.5 ml more of the same culture onto the pellet of cells. Centrifuge again 2 minutes. Repeat this procedure once more, for a total of 4.5 ml.

3. After the last centrifugation, pour off the culture supernatant, and remove as much of the remaining liquid as possible with a pipet tip.

4. Resuspend each pellet of cells in 250 µl of P1 buffer. Make sure that the cell pellet is **completely** resuspended. This can be accomplished either by vigorously vortexing the tube, or by pipetting the suspension up and down with a P200 pipettor or both.

> *P1 buffer contains RNase. Since RNA and DNA share many physical properties (eg. insolubility in alcohol in the presence of salt), and there is such a great excess of RNA relative to DNA in cells, RNA typically contaminates most DNA preparations. Contaminating RNA can be readily and specifically eliminated by digestion with the hydrolytic enzyme RNase.*

5. When the pellets are **thoroughly** resuspended, add 250 µl of buffer P2 to each and mix gently by inverting the tube several times. Do not vortex, as this will result in the shearing of genomic DNA. Let the lysis reaction stand on the bench for a maximum of 5 minutes. You should see some clearing of the suspension.

> *Buffer P2 contains sodium hydroxide and sodium dodecylsulfate (SDS). SDS is a detergent, and it will lyse the cells by solubilizing their membranes. Thus, the cloudy suspension should become clear. Once the cells are lysed, the sodium hydroxide will disrupt the base-pairing of double-stranded DNA, converting it to single-stranded DNA (which is often referred to as 'denatured' DNA).*

6. Add 350 µl of buffer N3 to each tube and invert the tube immediately, but gently, 4-6 times. A sticky white precipitate will form.

> *Buffer N3 contains sodium acetate. The sodium acetate neutralizes the sodium hydroxide. The closed circular plasmid molecules will quickly renature, whereas the large linear fragments of genomic DNA aggregate into an insoluble mass. This is so because the linear single-stranded fragments of the bacterial chromosome diffuse away from each other, and thus cannot effectively find each other when conditions permissive for base-pairing are restored. The single DNA strands of the closed circular plasmid molecules, however, remain topologically linked and thus can readily reanneal upon neutralization of the sodium hydroxide.*

7. Centrifuge the samples **at room temperature** for 10 minutes in a microfuge at top speed. During centrifugation, label 4 blue QIAprep spin columns plus collection tubes. When the centrifugation is finished, pour the supernatants onto the columns. Drain the microfuge tube well, but be careful that no white precipitate is carried onto the column.

> *The aggregated genomic DNA forms an insoluble pellet, whereas the reannealed closed circular plasmid DNA remains in solution.*

8. Centrifuge the columns plus collection tubes for 1 minute at top speed. Discard each flow-through from the collection tube. The collection tubes are to be used again.

9. Wash each column by adding 750 µl of buffer PE to each and centrifuging 1 minute again.

> *Buffer PE contains ethanol which will remove the salts and precipitate the DNA fragments. In the presence of high concentrations of salt ions (in this case, Na^+ $acetate^-$), DNA is insoluble in alcohol. Alcohol precipitation is a simple method that is commonly used to concentrate DNA or remove alcohol-soluble impurities.*

10. Discard flow-throughs and centrifuge each column for an additional minute to remove residual wash buffer.

> *This is an important step. Otherwise, the DNA may not elute properly. In addition, residual ethanol may inhibit subsequent enzyme reactions.*

11. Discard the collection tubes and place each QIAprep column in a **clean 1.7 ml microfuge tube**. Label ! Elute the DNA by adding 40 µl of distilled water to the center of each column. Wait 1 minute and then centrifuge 1 minute. Your DNA preps are at the bottom of the microfuge tubes. Place these tubes on ice.

Part III.

Now that you have completed the small-scale isolation of plasmid DNA, you will digest the DNA with restriction enzymes. Next time you will analyze the products of the digest by agarose gel electrophoresis. The enzymes used will be EcoRI and NcoI. Based on the expected structure of the MBP-GFP recombinant plasmid (see the diagram in the Appendix), you should be able to predict the sizes of the DNA fragments generated by these restriction enzymes. The sizes of the DNA fragments detected on the agarose gel will be estimated by comparison with a set of fragments of known size (a molecular weight ladder).

1. Prepare a set of 4 EcoRI digestions by adding 17 µl of each DNA prep to a microfuge tube. Label. To each of these tubes add 2 µl of enzyme buffer and 1 µl of enzyme EcoRI.

2. Prepare a set of 4 NcoI digestions as above, but add 2 µl of enzyme buffer and 1 µl enzyme NcoI to each tube, instead of EcoRI.

3. Mark the tubes with your initials, and place them in the 37°C water bath. After an hour, your tubes will be removed from the bath and stored in the freezer until next week.

CLEAN UP.

Leave P2, N3, PE, and distilled water on your bench.

Return P1 to ice bucket.

Empty glass test tubes into the container under the hood, rinse empty tubes with tap water, and place in the gray trays to be washed.

Place microfuge tubes, columns, etc, in your zip-lock bag. Seal tightly and place in the large autoclavable bag.

Swab your bench with 409.

Week #5: Analysis of restriction digests by agarose gel electrophoresis.

Objective: In this exercise you will use agarose gel electrophoresis to examine the restriction digests of the DNA minipreps that you prepared last week. By examining the pattern of DNA fragments produced upon digestion, it should be possible to identify a recombinant plasmid that contains the GFP gene (in the correct orientation!) located downstream of the *MBP* gene. A picture of your gel and a table of the sizes of the bands observed should be included in your first report.

This week's lab will complete the first part of the course. Your first lab report should include Part III of Week #1 and everything in Weeks #2-5.

Materials.

On your bench: 125 ml graduated Erlenmeyer flask
6X light blue loading dye (Xylene cyanol)
Distilled H_2O

On the table: Ethiduim bromide solution = EtBr
1 kb molecular weight ladder = 1 kb

At each end of the room: agarose powder and balance.
TAE buffer

Experimental Protocol:

Part I.

Start by preparing an agarose gel.

a. Weigh out 0.5 grams of agarose and transfer the powder to a 125 ml Erlenmeyer flask. Using the graduations on the flask, fill to 50 ml with 1x TAE buffer.

b. To melt the agarose, heat the suspension in a microwave until it begins to boil. This may take less than a minute. Gently swirl the flask from time to time. **Be extra cautious, since super heated solutions of agarose have a tendency to boil explosively. Wear protective mittens when you handle the hot flask and keep it away from your face!!**. Continue heating and swirling the agarose solution until it is **completely** clear and free of translucent agarose particles.

c. Let the flask stand on your bench for about five minutes to allow the agarose to cool a bit, and then add 2.5 µl of a 10 mg/ml stock of the DNA-staining dye ethidium bromide. **Be careful - ethidium bromide is a mutagen**. Use the EtBr at the table. Do not take it to your bench! Discard the tip in the plastic bag provided.. Swirl the

flask to disperse the dye. (A quicker method to cool the agarose is to hold the flask for a few minutes under running tap water, swirling the contents gently all the time so that the agar does not begin to solidify on the sides. Cool until you can put your hand on the flask without feeling too much pain.) Set up your gel mold, taking care that the rubber gaskets are aligned in the groove and fit snugly between the mold and the box to avoid leaks. (The gaskets slide more easily if they are moistened with distilled water). Pour the agarose + ethidium bromide into the gel mold and place a comb with 10 slots in the end position. (While you are waiting for the gel to harden, proceed to step 2.)

d. Once the gel hardens, which will take 20-30 minutes, remove the comb, remove the gel mold, rotate it 90^{o} and place it back in the gel box with the wells at the negative (black) end. Add enough 1X TAE buffer to cover the gel.

Part II.

1. After last week's lab period, your restriction digests were removed from the 37° bath and transferred to the freezer by the TA's. Reclaim your digests now, and thaw the frozen samples. Centrifuge briefly to draw all the droplets down into the tubes.

2. To each tube, add 2 µl of DNA gel loading dye. Mix the dye with your sample by stirring with a pipet tip or tapping the side of the tube gently with your finger.

   Xylene cyanol has been used for this loading buffer because bromphenol blue obscures one of the digestion products.

3. Also prepare DNA molecular weight standards. Add 2 µl standards to 18 µl distilled water and then add 4 µl of loading dye to that.

4. Next, load all samples on the agarose gel. Load as much as you can. Divide the molecular weight standards between 2 lanes, #1 and # 10. The loading order should be as follows:

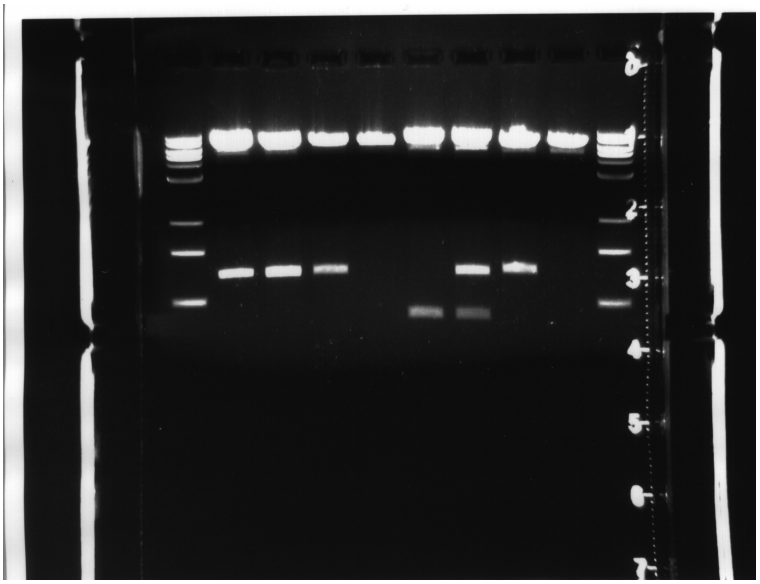
Lane 1	DNA molecular weight standards (1/2)
Lane 2	mini prep #1 digested with EcoRI
Lane 3	" #2 "
Lane 4	" #3 "
Lane 5	" #4 "
Lane 6	mini prep #1 digested with NcoI
Lane 7	" #2 "
Lane 8	" #3 "
Lane 9	" #4 "
Lane 10	DNA molecular weight standards (1/2)

5. Hook the gel up to the power supply, and set the power to 110-120 volts. .

6. When the dye front has moved about 1.5-2 cm, turn off the power supply and photograph the gel under UV light.

Consult the diagram of the construct in the Appendix. What size EcoRI and NcoI fragments should the correct plasmids contain? Identify a transformant that has the appropriate fragments. Does this check with the appearance of your colonies?

This is a picture of a typical gel (courtesy of Sunny Chun, 2007):



Do you see a construct with the correct structure?

CLEAN UP.

Rinse E-flasks with tap water and place in gray trays on the cart by the door to be washed.

Pour electrophoresis buffer down the drain, rinse gel box with distilled water, and leave on your bench.

Discard gels in special EtBr container.

Return MW ladder to ice bucket.

Leave the blue loading dye and distilled water on your bench

Place used microfuge tubes, columns, tips etc. in your zip-lock bag, seal, and discard in regular trash.

Discard plates in autoclavable bag.

Swab your bench with 409.

Special Note: Please bring a calculator to the next session.

Week #6: Synthesis of MBP-β galactosidase in E. coli and Introduction to Quantitative Determination of Protein

Objective: This week we are starting a new set of experiments to induce the synthesis of a fusion protein in a culture of growing bacterial cells. The fusion is between the maltose-binding protein (MBP) and the enzyme β galactosidase. This construct was made in the same way as the GFP-MBP construct that you just made in the first part of the course. In addition, you will learn how to make a standard curve for quantitative protein determination. You will be using quantitative protein assays in three weeks to monitor the purification of the MBP-β galactosidase hybrid protein.

Materials:

On your bench:
- Cultures of pMAL and β-GAL
- LB + amp (50 μg/ml ampicillin) in Falcon tubes.
- Two sterile, capped Erlenmeyer flasks
- IPTG (200 mM) = "I".
- TNE
- Sterile wooden sticks
- Ethanol-insoluble marker pen

Experimental Protocol:

Part I.

1. 10 ml cultures of the pMAL vector (pMAL) and the vector containing the MBP-β galactosidase hybrid protein (β-GAL), as well as two 40 ml aliquots of LB amp are on your bench. Pour each tube of LB amp into a capped Erlenmeyer flask. Pour the pMAL culture into one flask and the β-GAL culture into the other. Label each flask with the name of the strain and your initials. Keep the 50 ml conical Falcon tubes to be reused in step 5.

2. To initiate synthesis of the MBP and MBP-β-galactosidase proteins, add 50 μl of 200 mM IPTG to each flask.

> *The synthesis of MBP in these cells is normally repressed by the action of Lac repressor. Lac repressor binds to DNA segments in the transcriptional promoter upstream of the MBP gene, and blocks the initiation of mRNA synthesis by RNA polymerase. Isopropylthiogalactopyranoside (IPTG) binds tightly to Lac repressor, and prevents it from binding to its target sequences in the MBP promoter. This relieves the transcriptional repression of the MBP gene, resulting in the synthesis of copious amounts of MBP (and, consequently, MBP-β galactosidase) mRNA and protein.*

3. Place the flasks in the 37°C environmental shaker for 1.5 hours to allow the cells to synthesize and accumulate MBP and MBP-β galactosidase. While you are waiting, proceed to Part II.

4. After 1.5 hours, pour each culture back into the 50 ml Falcon tubes that were set aside in step 2. Harvest the cells by centrifugation for 10 min at 4,000 rpm in the Sorvall tabletop centrifuge.

5. Discard the supernatants, add 5 ml of TNE (50 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA) to each cell pellet, and disrupt each pellet with a sterile wooden stick. Disperse the cells into suspension by either vortexing or pipetting up and down with a 5 ml pipet. Adjust the suspension to 25 ml volume by adding 20 ml of TNE and centrifuge again for 5 min at 4,000 rpm. This step will wash away remaining traces of the growth medium.

6. Discard the supernatants and add 1 ml of TNE to each cell pellet. Disrupt each pellet with a wooden stick and then disperse the cells into suspension by either vortexing or pipetting up and down with a P1000 pipetman. Once the cells are in suspension, transfer the suspension to a microfuge tube and cap the lid tightly. Label the tubes with your name and the protein being expressed (MBP or MBP-β galactosidase) using an **alcohol-insoluble** marker, and quick freeze them by placing them in the dry ice-ethanol bath. The preps will be stored at -80°C until next week's lab period. Rinse out your Erlenmeyer flasks with tap water and discard them in the containers on the cart by the door.

Part II:

Materials:

On your bench:	Protein standard (5 mg/ml bovine gamma globulin) = "S"
	Unknown protein = "U"
	Bradford reagent (Brown liquid)
	Reagent tubes

The Bradford Assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The Bradford method for protein determination has several advantages over the commonly used Lowry method, including: (i) it is faster; (ii) it requires fewer reagents, (iii) it is not affected by many compounds that interfere with the Lowry assay

1. Before you do anything else, turn on the Spec 20 to allow it to warm up

2. Prepare a standard curve (absorbance vs concentration in µg/ml) for the Bradford protein assay as follows: pipet 5.0 ml of Bradford reagent into each of 10 13 x 100 mm Borax dispo glass reagent tubes. (Do not use the cuvette as a reagent tube!) Next, add 5 mg/ml protein standard ("S") to these tubes according to the scheme shown below.

When adding the protein standard, observe the following: (i) try not to switch pipettors, as this may result in an inflection point in your standard curve, although you must use a fresh tip each time. (ii) do not froth the samples as this will skew the response of the dye. Mix by covering the top of the tube with parafilm and inverting twice. Mix each tube i**mmediately** after adding the protein.

Warning: do not confuse µl and µg (how many µl of 5 mg/ml protein standard to get 25 µg?).

Tube #	protein standard
1, 2	0 µg (blank)
3, 4	25 µg
5, 6	50 µg
7, 8	75 µg
9, 10	100 µg

In addition to your standard curve, set up several more tubes containing 5 ml of Bradford reagent to determine the concentration of an unknown protein ("U") that will be given to you. Prepare 2 or 3 different concentrations of this protein.

3. Incubate all of your samples at least 10 min before reading their absorbance. To read the absorbance of your samples:

a. With the lid down and no tube in the sample compartment, set the wavelength to 595 nm and turn the knob on the left side of the Spec-20's front panel until the % Transmission equals 0.

b. Pour the contents from tube 1 (blank) into a Spec-20 cuvette, wipe the outside of the cuvette with a tissue to remove finger prints and liquid, insert the cuvette into the sample compartment, and close the lid. The cuvette has a white orientation marker on its side. Insert the cuvette such that the orientation marker lines up with the mark on the side of the cuvette port. Turn the knob on the right side of the Spec-20's front panel until % Transmission equals 100.

c. Remove the cuvette from the sample compartment, drain the sample back into its original tube, dab the lip of the cuvette with a tissue to remove excess liquid, and pour the contents of tube #3 into the cuvette. Change mode from transmission to absorbance. Wipe the outside of the cuvette with a tissue to remove finger prints, liquid, etc, insert the cuvette into the sample compartment, close the lid, and record the absorbance value. You may need to leave the cuvette in the sample holder for several seconds before taking your reading to allow the absorbance value to stabilize.

d. Repeat step c for tubes 5, 7, and 9, followed by your unknowns. Once you have completed your unknowns, read samples 2, 4, 6, 8, and 10.

4. Using the linear regression function on a calculator, determine the concentration of protein in your unknown. Some instructions for calculating linear regression are given in the Appendix. Your linear regression curve will be a figure in your second report.

5. When you have determined the concentration of the unknown protein, tell your TA, who will write it down on your performance sheet. You will receive 5 extra points if you come within 20% of the correct value.

CLEAN UP:

Pour used Bradford Reagent into the red solvent can.

Discard **empty** glass tubes in the broken glass container.

Give unused Bradford Reagent back to your TA.

Place unused "S" and "U" back on ice.

Do not discard your cuvette! Leave it on your bench.

Place reaction tubes, IPTG, and all tubes that contained bacteria in your zip-lock bag and discard in the autoclavable bag.

Bradford Reagent is particularly messy, so swab your bench with 409 before leaving.

Remember to turn off your Spectronic!

Week #7: Affinity Purification of MBP-β galactosidase

Objective: The purpose of this week's lab is to purify the MBP-β galactosidase fusion protein from crude bacterial lysates by exploiting the powerful technique of affinity chromatography. In parallel, you will purify MBP alone, which will be used as a negative control for the enzyme assays that you will be doing in 2 weeks.

Materials:

On your bench:	Lysozyme (10 mg/ml) = "L"
	TritonX-100 detergent (20%) = "Tx"
	DNase (1 mg/ml) = "Dn"
	MgCl2 (1 M) = "Mg"
	Amylose resin = white solid in Falcon tube
	Two empty columns + caps
	Four 15 ml Falcon tubes
	TNE (Tris-NaCl-EDTA) buffer
	TNE/N (TNE + extra salt)
	TNE maltose (10 mM) = "TNE-M"
	Bradford reagent
	ONPG reagent
	Microtiter plate
	Ethanol-insoluble marker pen
On the table:	Dry ice-ethanol bath

Experimental Protocol:

First you will prepare **lysates** of your two cell suspensions.

1. Obtain the frozen cell suspensions that you prepared last week. Thaw cell suspensions by placing tubes in a bath of room temperature water.

2. To each tube of cell suspension, add 20 µl of 10 mg/ml lysozyme. Mix in thoroughly with the pipet tip. Incubate 15 min at room temperature. While you are waiting, set up and begin to wash your columns, as in steps 8 and 9.

> *Lysozyme is a hydrolytic enzyme that degrades the polysaccharides that comprise the bacterial cell wall. It is necessary to remove the cell wall to release the contents of the bacterial cells. Cell wall-less bacteria are referred to as 'spheroplasts'*

3. Add 25 µl 20% Triton X-100 to each tube. Mix in thoroughly with a pipet tip. Incubate at room temperature for 5 min.

Triton X-100 is a nonionic detergent that solubilizes the lipids of the bacterial plasma membrane, thereby allowing the cytoplasm to leak out from the spheroplasts.

4. Add 10 µl of 1 M $MgCl_2$ and 10 µl of 1 mg/ml DNase solution to each tube. Mix in thoroughly with a pipet tip. Incubate at room temperature for 5 minutes. From this point onwards, keep your cell lysates on ice whenever possible. Protein extracts are labile and don't like to sit around at room temperature!

DNase requires Mg++ for activity. The DNase will help reduce the viscosity of the lysate.

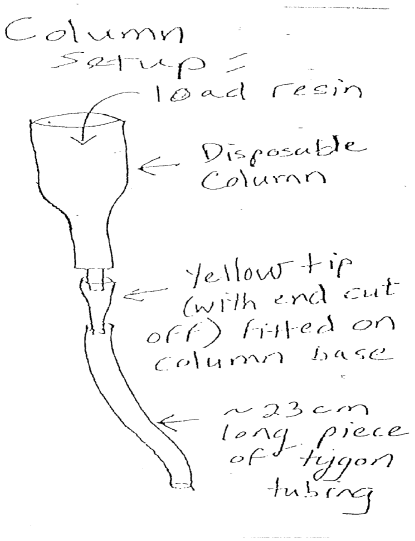
5. Dilute the lysates by filling each tube to the top with cold TNE/N, mix, and centrifuge your samples in a microfuge for 10 min at 4°C.

6. **QUICKLY** decant the supernatant fractions into graduated 15 ml Falcon tubes. Dilute each lysate further by adding 0.5 ml of cold TNE/N.

The centrifugation step removes unbroken cells and insoluble debris from the cell lysates. The functions of the various components in the lysis buffer are as follows. Tris: pH control; EDTA: chelates divalent cations, which serve as cofactors for some proteases; NaCl: blocks non-specific "sticking" of proteins to affinity matirx. The affinity matrix is composed of polysaccharide, which is very hydrophilic. Many hydrophilic proteins can bind weakly via ionic interactions to the polysaccharide matrix. These ionic interactions can be competed by including moderate concentrations of monovalent ions (e.g. Na^+, Cl^-), thereby improving the specificity of the affinity purification.

7. If your diluted lysate appears clear and free of debris, proceed to step 8 (or 11 if your columns are ready). If it still has particulate material in it, you will have to either recentrifuge your lysate or filter it . Your TA will show you how to filter.

8. Prepare two 1.0 ml columns of amylose resin by transferring 2.0 ml of a 50% slurry of amylose resin into each of two BioRad dispo columns set up as diagrammed below:



9. Equilibrate the columns by running 5.0 ml of TNE/N through the resin beds. (Using a 1 ml pipettor and a blue tip, add ~1.0 ml of TNE/N to the top of each bed,. Once all of the buffer has entered the resin apply a second 1.0 ml aliquot of TNE/N. Repeat 3 more times.) If you are not ready to use your column right away, keep it wet by continuing to add buffer or fill it with buffer and cap it.

10. Important point: Although the chromatography step will be conducted at room temperature for convenience's sake, keep in mind that crude lysates typically are not very happy on your bench top, and prefer the cozy confines of an ice bucket. Thus, keep your extract on ice before applying it to the column, keep the column wash and elution buffers on ice, and place the column flow-throughs, washes, and eluates on ice immediately after you finish collecting them.

11. Label one of the columns “MBP”, and label the second column “β-GAL”. **BEFORE** loading the columns, transfer a 400 µl aliquot of each lysate to a labeled microfuge tube on ice (label,: L/MBP, L/β-GAL; L stands for column **L**oad).

    Important: Use the ethanol-insoluble marker pen for these and all other collection tubes.

    Apply the remainder of the pMAL lysate to the column labeled 'MBP' and the remainder of the β-GAL lysate to the column labeled 'β-GAL'. Apply the lysate in stages as was done when you equilibrated the column with TNE/N (i.e., in 1 ml amounts). **DO NOT MIX UP THE TWO LYSATES!!!.**

Collect the column flow-throughs in separate 15 ml Falcon tubes (label: MBP FT, β-GAL FT).

The basis for this chromatography step is that maltose binding protein binds very tightly to amylose. The columns you are using are composed of tiny polysaccharide beads that have amylose covalently coupled to their surface. When you run the bacterial lysates through the columns, the MBP and MBP-βgal in the lysates binds tightly to amylose, thereby retaining these proteins β–GAL the column. After washing the column free of all unbound cytoplasmic proteins, the MBP and MBP-βgal are eluted from the beads with free maltose.

12. Washes: Wash each column 3 times with 1 ml TNE/N, and collect each wash in a microfuge tube (label MBP-W1, MBP-W2, MBP-W3 β–GAL-W1, β-GAL-W2, β-GAL-W3). Continue washing with TNE/N until a **total** of 5 ml has passed through each column. Then, wash each column with an additional 1 ml of TNE (no extra NaCl). The last 2 TNE/N washes and the TNE wash can be collected in a beaker and discarded.

13. Elution: Elute each column with 4 applications of 0.7 ml of TNE -M. Collect each elution separately in a microfuge tube (label: MBP-E1, MBP-E2. β-GAL- E1, β-GAL-E2...etc).

MBP binds tightly to maltose. When maltose is added to the column, it competes with the immobilized amylose for binding to MBP and MBP- β-gal*. Since the maltose in solution is at a much higher concentration than the amylose on the resin, the MBP and MBP-* β-gal *are effectively dislodged from the column.*

14. Quickly estimate the amount of protein in the elution fractions as follows. Pipet 100 µl of Bradford reagent into each of 20 wells of a microtiter plate positioned on a sheet of white paper. Add 10 µl of each fraction (L, FT, W1–W3, E1–E4) from the MBP- β–GAL purification to wells #2-10 (well #1 is used as a blank). Mix by stirring with the pipet tip. Assess and record the intensity of the blue color (e.g. +++ = dark blue, ++ = medium blue etc.). Repeat for the MBP samples (use wells 12-20). This simple assay provides a quick and dirty estimate of the amount of protein in each fraction.

15. To test for β-galactosidase activity in each of the MBP-β–GAL fractions, repeat this procedure using ONPG reagent instead of Bradford reagent.

ONPG is a chromogenic substrate for enzyme activity. You will use it for more quantitative measurements at the next session.

16. Tabulate your data as a table for your second report.

17. Transfer 1.0 ml of each flow-through to an appropriately labeled microfuge tube. Quick freeze your fractions: L, FT, W1, W2, E1, E2, E3, E4 for both MBP and MBP-β–GAL in the dry ice-ethanol bath provided. They will be stored in the –80^{o}C freezer until next session.

CLEAN UP:

Return leftover TNE, TNE/N, TNE-M, Bradford reagent and ONPG reagent to your TA.

Remove the tubing and yellow adaptor piece from your columns and leave the tubing and adaptor on your bench.

Place used microfuge tubes, Falcon tubes, columns, leftover resin, and the micro-titer plate in your ziplock bag. Seal the bag tightly and throw in the **regular trash**.

Swab your bench with 409.

Week #8: Protein assay and SDS-PAGE analysis of MBP- β-galactosidase purification.

Objective: The success of a protein purification procedure is typically monitored by performing SDS-PAGE analysis, protein assays, and enzyme assays of the various fractions generated during the course of the purification. This week, we will employ both protein assays and SDS-PAGE to assess the effectiveness of the affinity purifications that you performed last week.

Experimental Protocol:

Part 1:

Materials:

On your bench: 4X SDS-PAGE sample buffer = 4X
Molecular weight standard = MW
Gel box with 2 gels mounted and containing SDS-PAGE running buffer.
650 µl microfuge tubes (carton; red label)
Special long pipet tips
Plastic box for staining (to be shared with partner).

Back of room: Coomassie blue stain

1. Obtain the cardboard box containing your frozen samples from one of the TAs. Thaw all of your samples by incubating them in a room- temperature water bath until just before the last traces of ice melt. Remove the tubes from the water bath, tap them with your finger a few times to mix the contents, and immediately place the tubes on ice.

2. Label fourteen empty 650 µl (PCR) microfuge tubes: L/MBP, FT/MBP W1/MBP, W2/MBP, E1/MBP, E2/MBP, E3/MBP; L/β–GAL, FT/β- GAL, W1/β-GAL, W2/β-GAL, E1/β-GAL, E2/β-GAL, E3/β-GAL. Add 5 µl of 4x SDS-PAGE sample buffer to each. Transfer 15 µl from each of your fractions to the appropriately labeled tube containing 4x SDS-PAGE sample buffer. Tap the tube several times with your finger to mix the contents, and cap the tube tightly.

   This scheme assumes that the protein was eluted in fraction 2 or 3. If this is not the case, use your best elution fraction, as well as the ones before and after it.

3. Transfer the dye-containing tubes to the Thermal Cycler set at 95°C and incubate for 3 minutes. Remove the samples from the heat source and allow them to cool in a rack

for approximately 2 minutes.

SDS-PAGE sample buffer contains the detergent sodium dodecylsulfate. When proteins are heated in the presence of SDS, the proteins unfold and become coated with molecules of SDS. SDS binds to proteins in a constant ratio of 1.6 g SDS/1 g protein. SDS-denatured proteins resemble flexible rods with a constant density of negative charge. When applied to a matrix in an electric field, these rods will migrate away from the cathode at velocities that are inversely proportional to their length. Hence, an SDS-polyacrylamide gel will fractionate proteins based on their size, with small proteins migrating rapidly and large proteins migrating slowly. SDS-PAGE buffer also contains the following components: (i) a reducing agent (ß-mercaptoethanol or dithiothreitol) to disrupt inter- and intra-chain disulfide bonds; (ii) glycerol, which makes the sample denser than water so that it sinks to the bottom of the gel well upon loading; (iii) bromophenol blue, which makes it easy to see the sample as you load it, and serves as a marker for the ion front during the electrophoresis; and (iv) Tris, for pH control

4. The TAs will have set up gel boxes containing SDS-polyacrylamide gels for you. Please note that the gel boxes are two-sided. You and your bench partner will share a single gel box.

5. Load your samples into the wells of the gel in the order given below. To load the gel, use a P10 pipettor and special long tips. Also load the denatured molecular weight standards provided.

The molecular weight standards contain a series of proteins of known molecular weight. By comparing the migration of your purified proteins with the molecular weight standards, you will be able to estimate the molecular weights of MBP and MBP-β-GAL.

Lane 1 5 µl molecular weight ladder
Lane 2 MBP load, 10 µl
Lane 3 MBP flow through, 10 µl
Lane 4 MBP wash 1, 10 µl
Lane 5 MBP wash 2, 10 µl
Lane 6 MBP elution 1, 10 µl
Lane 7 MBP elution 2, 10 µl
Lane 8 MBP elution 3, 10 µl
Lane 9 MBP-β-GAL load, 10 µl
Lane 10 MBP-β–GAL flow through, 10 µl
Lane 11 MBP-β-GAL wash 1, 10 µl
Lane 12 MBP-β–GAL wash 2, 10 µl
Lane 13 MBP-β-GAL elution 1, 10 µl
Lane 14 MBP-β-GAL elution 2, 10 µl
Lane 15 MBP-β–GAL elution 3, 10 µl

6. Once you and your partner have finished loading your gels, hook the gel box up to a power supply. Be sure to attach the cathode (black) terminal to the black terminal on the power supply. Likewise, the red terminal on the gel box should attach to the red terminal on the power supply. Run the gel at 170-180 volts for about 1 hr.

7. While the gel is running, go to Part II and begin your protein assays.

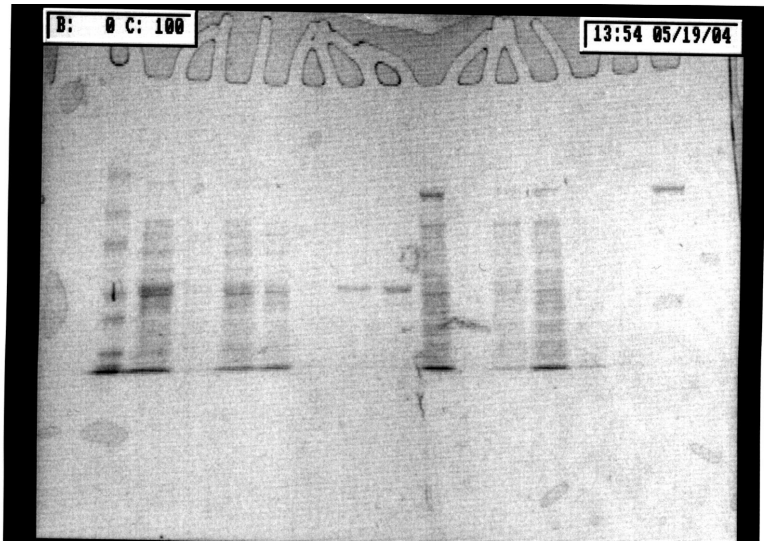
8. When the gels have finished running, remove your gel from the gel box, pry apart the plates that sandwich your gel, cut off the bottom right-hand corner of the gel with a razor blade or spatula to provide an orientation marker, and transfer the gel to a plastic box. The TAs will demonstrate how to do this.

9. Add enough Coomassie blue stain to completely cover the gel.

   Coomassie blue stain binds tightly to protein, and thus can be used to reveal the positions of individual polypeptides within the SDS-polyacrylamide gel.

10. Put the plastic box on an orbital shaker and allow it to stain until the next lab session, when it will be ready to be photographed.

This is a typical protein gel showing both MBP and the MBP-β-GAL fusion protein:



Part II.

Materials:

On your bench: Protein standard = "S"
Bradford reagent (Brown liquid)

On the table: Dry ice-ethanol bath

While your gel is running, you will begin to assay the protein fractions from last week's purification for their content of protein using the Bradford technique that you practiced a few weeks ago. These data should appear in a figure in your final report. In addition, make a table of the amount of protein in each of your fractions L, E2 etc. This will be useful for next week's lab and should also be included in your report.

1. Before you do anything else, turn on the Spec 20 to allow it to warm up

2. Prepare a standard curve for the Bradford protein assay as follows: add 5.0 ml of Bradford reagent to each of five 13 x 100 mm dispo test tubes. Next, add 5 mg/ml protein standard (bovine gamma globulin) to these tubes according to the scheme shown below. When adding the protein standard, observe the following: (i) try not to switch pipettors, as this may result in an inflection point in your standard curve, but use a fresh tip each time; (ii) do not froth the samples as this will skew the response of the dye. Mix by covering the top of the tube with parafilm and inverting twice. Mix each tube i**mmediately** after adding the protein.

Warning: do not confuse µl and µg (how many µl of 5 mg/ml protein standard to get 25 µg?).

Tube #	protein standard
1	0 µg (blank)
2	25 µg
3	50 µg
4	75 µg
5	100 µg

3. Once you've set up your standard curve, add 5 ml of Bradford reagent to 5 more tubes. Measure the protein concentration in the MBP-L fraction (column load) and in the E fraction (column eluate) that had the most protein according to the quick screen that you carried out at the last session. Also prepare samples for the MBP-β–gal-L fraction and for two of the E fractions that had the most protein. Most probably those will be E2 and E3, but consult your microtiter plate, just in case. To get a reading that will fall within the absorbance range circumscribed by your standard curve, we recommend that you start with 10 µl from the L fractions and 50 µl from the E fractions. As you are preparing your samples, make an eyeball comparison with the standard curve to judge if they are going to

yield reasonable measurements. If some samples have too little or too much protein to get an accurate reading, set up another tube of Bradford reagent and add either less or more sample, as appropriate. You may even have to dilute the protein in TNE before taking a sample (i.e., add 10 µl of protein to 90 µl TNE and use 10 µl of the dilution for the sample.)

4. Allow all of your samples to incubate at least 10 min before reading their absorbance. To read the absorbance of your samples:

   a. With the lid down and no tube in the sample compartment, set the wavelength to 595 nm and turn the knob on the left side of the Spec-20's front panel until the % Transmission equals 0.

   b. Pour the contents from tube 1 (blank) into a Spec-20 cuvette, wipe the outside of the cuvette with a tissue to remove finger prints and liquid, insert the cuvette into the sample compartment, and close the lid. The cuvette has a white orientation marker on its side. Insert the cuvette such that the orientation marker lines up with the mark on the side of the cuvette port. Turn the knob on the right side of the Spec-20's front panel until % Transmission equals 100.

   c. Remove the cuvette from the sample compartment, drain the sample back into its original tube, dab the lip of the cuvette with a tissue to remove excess liquid, and pour the contents of tube #2 into the cuvette. Wipe the outside of the cuvette with a tissue to remove finger prints, liquid, etc, insert the cuvette into the sample compartment, close the lid, and record the absorbance value. You may need to leave the cuvette in the sample holder for several seconds before taking your reading to allow the absorbance value to stabilize.

   d. Repeat step c for tubes 3, 4, and 5, followed by your unknowns.

5. When you have finished, quick freeze samples L and the best samples E (E2 or E3?) in the dry ice-ethanol bath and place them in the box. You will need these for the next session. All others can be discarded.

CLEAN UP:

1. Place microfuge tubes, gel frames, etc. in ziplock bag and discard in regular trash.
2. Pour running buffer down the drain, rinse box with **distilled water** and leave on your bench.
3. Empty glass reaction tubes and discard them in the broken glass container. Leave your cuvette on your bench.
4. Pour used Bradford into the container in the sink.
5. Give unused Bradford Reagent and protein standard back to your TA.
6. Bradford reagent is particularly messy, so wipe off your bench with 409 before leaving

Remember to turn off your Spectronic!

Week #9: Assay ß-galactosidase activity of purified MBP-ß-galactosidase chimera

Objective: The enzyme ß-galactosidase cleaves the chromogenic substrate o-nitrophenyl-ß-D-galactoside (ONPG) to produce the colored product o-nitrophenol (ONP). In this week's lab, you will use this assay to determine the effectiveness of the affinity purification of your MBP- ß-galactosidase fusion protein and to the measure the specific activity of the purified protein. In addition, you will study the kinetics of ONPG hydrolysis and also determine the effect of various treatments on ß-galactosidase activity.

Preparation: Before the lab period begins, you should prepare a table showing the amount of protein in each of your fractions "L", "E2" etc for both MBP and MBP- ß-galactosidase.

Materials:

On your bench:

Z buffer = "Z"
ONPG (wrapped in aluminium foil)
Na_2CO_3 = "SC"

On the table:

Sterile 50 ml Falcon tubes for part III
Samples of glucose, SDS, etc. for part IV.
More dispo reaction tubes.

On the bench under the windows:

Heat blocks at different temperatures

Experimental Protocol:

The first question we want to ask is: **how effective was the purification**? To answer this compare the enzyme activity per mg protein in the load and the eluate. This information will be included in your report.

Obtain from your TA the frozen aliquots of MBP and MBP-ß- gal that you purified two weeks ago and that you decided last week to save for the enzyme assay. Thaw the tubes by placing them in a water bath at room temperature. When the tubes are thawed, place them on ice. Before you get underway with the enzyme assay, turn on the spectrophotometer to allow the lamp to warm up.

Start by diluting your enzyme preps as follows:

1. Pipet 90 µl of TNE buffer into each of 4 microfuge tubes. Add 10 µl of undiluted MBP-ß- gal "L" to one of the tubes and mix well. This is a 10-fold dilution of the MBP-ß- gal protein. Label 10^{-1} L.

2. Pipet 10 µl of 10^{-1} L into another of the microfuge tubes and mix well. This is a 100-fold dilution of the MBP-ß- gal protein. Label 10^{-2} L.

3. Repeat the dilutions using your best MBP-ß- gal "E" fraction to obtain 10-fold and 100-fold dilutions of that protein. Label 10^{-1} E and 10^{-2} E.

Keep all dilutions on ice, as you will be using them more than once.

Then set up preliminary ß-galactosidase enzyme assays using these dilutions as follows:

1. Pipet 2.4 ml of Z-buffer into each of 7 disposable reaction tubes. Add 10 µl of TNE to the first (this will be your blank).

2. Add 10 µl of undiluted "L" to the second, 10 µl of "10^{-1} L" to the third, and 10 µl of "10^{-2} L" to the fourth.

3. Repeat for the last 3 tubes – 5, 6 and 7, - using undiluted, "10^{-1} E", and "10^{-2} E" fractions.

4. Initiate the reaction by adding 0.6 ml ONPG to each tube. Vortex each briefly to mix. After 10 min incubation at room temperature, add 1.5 ml of 1 M sodium carbonate stop buffer to each. Vortex.

Since the incubations are so brief, you should stagger the initiation of the reactions so that each incubation is of identical duration. The way to do this as is follows. Set up all your tubes with Z buffer and protein. Start your timer and initiate the first reaction by adding substrate solution (ONPG) to the tube. When the timer reaches 30 sec, add substrate to tube #2; at 60 sec, add substrate to tube #3 etc. When the timer reaches 10 min, add stop buffer to tube #1; at 10 min 30 sec, add stop buffer to tube #2 etc.

ONPG is a colorless substance that contains a bond that is sensitive to cleavage by ß-galactosidase. Upon treatment with ß-galactosidase, ONPG is cleaved to yield two compounds: galactose, which is colorless, and o-nitrophenol, which is bright yellow. The amount of o-nitrophenol produced can be easily evaluated using a spectrophotometer. The linear range for the enzyme assay falls approximately in the range of absorbance from 0.100 - 0.800. The sodium carbonate terminates the enzyme reaction by overwhelming the pH control provided by the sodium phosphate in "Z" buffer. Sodium carbonate is very basic and increases the pH to a level where the ß–galactosidase enzyme is no longer active.

Once all of the reactions have been terminated, measure their absorbance at 420 nm and record the values in your notebook. Make sure that the samples are well mixed before you read the absorbance values. You should have at least one reading for each within the 0.10-0.80 range.

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> *Average the data you have just collected to calculate the **specific activities of the proteins.** The specific activity of an enzyme is usually measured in terms of units/mg protein. (You measured the protein in the previous session using the Bradford reagent). A unit is defined as the amount of prep that will convert one nmol/min of substrate under standard conditions of temperature, pH, ionic strength, etc. (Pure ß-galactosidase has an activity of about 3 x 10^5 units/mg.) 0.0045 absorbance units at 420 nm correspond to 1 nmol of o-nitrophenol(ONP)/ml. These values for the specific activity of your enzyme preparation should be included in your lab report.*

II. Run a control to test whether the ONPG cleavage you are observing is due to ß-galactosidase activity, as opposed to MBP or a contaminant of your amylose column eluate, by preparing a reaction containing a volume of MBP alone (from the best MBP "E" fraction) equivalent to the highest amount of MBP-ß-gal that you have used. This is a crucial control in experiments involving heterologous protein expression!

III. Enzyme kinetics. From the previous assay, you can see that the amount of enzyme activity observed is directly proportional to the amount of enzyme present. You will now perform an experiment to test whether the amount of substrate cleaved is directly proportional to the duration of the enzymatic reaction.

Set up enzyme reactions containing an amount of enzyme sufficient to produce an absorbance reading of ~0.750 after 10 min. of incubation. The reaction should be scaled up 6 fold (for six time points):

> 14.4 ml Z buffer
> (amount of enzyme that generates A_{420} of 0.75 in 10 min) x 6
> [3.6 ml substrate] <u>to be added only when you are ready to start !!</u>

Also, prepare 6 tubes marked 0, 1, 2, 5, 7.5, and 10. Each tube should contain 1.5 ml of 1 M sodium carbonate stop buffer.

a. Initiate the reaction by adding 3.6 ml substrate.

b. At 0, 1, 2, 5, 7.5, and 10 minutes following the addition of substrate, transfer 3 ml to an appropriately marked tube containing 1.5 ml of 1 M sodium carbonate. Mix well.

If you are short of enzyme, decrease the number of time points and/or reduce the final reading from 0.75 to something more attainable.

IV. Independent Investigations. Now that you have examined the cleavage of ONPG by ß-galactosidase as a function of time and enzyme concentration, devise your own experiments to investigate the effect of three different treatments on enzyme activity. Several reagents (SDS, lactose, Triton X-100, glucose, sucrose) will be available for you to test. Try adding 50 µl of any of these reagents to an enzyme reaction. Make sure that you set up appropriate control reactions. Is there any effect on activity? You can also test the effect of heating your enzyme preparation to different temperatures. Tabulate your results and include them in your report.

Remember to take a picture of your SDS-PAGE gel before leaving. Mark the pink reference band.

Remember to bring your laptop, notes and pictures covering weeks 6-9 to the study session next week.

CLEAN UP:

Empty contents of glass tubes down the drain and place empty tubes in the broken glass containers.

Leave the cuvette on your bench.

Give unused Z-buffer and ONPG back to your TA.

Place other items in your zip-lock bag and discard in regular trash.

Turn off your “Spec”.

Discard gels in the regular trash.

Rinse the plastic box with distilled water and place it in the gray trays to be washed.

Swab your bench with 409.

Session #10. (Optional)

The last session may be used for make-ups or for working on your Bi 10 final report, which will cover weeks 6-9 of the course. You can make a draft version to be finished later or print out and hand in your final version today. The TAs will answer any questions you have concerning formatting, analysis of the data, etc.

Final reports should be submitted by June 16.

Please place your finished reports in the lockbox in room 347 KRK.

Session #9 a. Ditch Day Alternative.

In the event that you lose a lab session because of Ditch Day, follow this protocol for the last lab session. In addition, remember to take a picture of one of the SDS-PAGE gels in room 313K to analyze for your Final report.

Part I. Protein assay of MBP-β galactosidase purification.

Objective: The success of a protein purification procedure is typically monitored by performing SDS-PAGE analysis, protein assays, and enzyme assays of the various fractions generated during the course of the purification. This week, we will employ only enzyme assays to assess the effectiveness of the affinity purifications that you performed last week. The SDS-PAGE analysis will be omitted. For your report, take a picture of one of the backup gels available and analyze that instead.

Materials:

On your bench: Protein standard = "S"
Bradford reagent (Brown liquid)

Experimental protocol.

Begin by assaying the protein fractions from last week's purification for their content of protein using the Bradford technique that you practiced a few weeks ago. Make a table of the amount of protein in each of your fractions L, E2 etc. This will be useful for Part II of this session and should also be included in your final report.

1. Before you do anything else, turn on the Spec 20 to allow it to warm up

2. Prepare a standard curve for the Bradford protein assay as follows: add 5.0 ml of Bradford reagent to each of 5 13 x 100 mm dispo test tubes. Next, add 5 mg/ml protein standard (bovine gamma globulin) to these tubes according to the scheme shown below. When adding the protein standard, observe the following: (i) try not to switch pipettmen, as this may result in an inflection point in your standard curve, but use a fresh tip each time; (ii) do not froth the samples as this will skew the response of the dye. Mix by covering the top of the tube with parafilm and inverting twice. Mix each tube i**mmediately** after adding the protein.

Warning: do not confuse µl and µg (how many µl of 5 mg/ml protein standard to get 25 µg?).

Tube #	protein standard
1	0 µg (blank)
2	25 µg
3	50 µg
4	75 µg
5	100 µg

3. Once you've set up your standard curve, add 5 ml of Bradford reagent to 5 more tubes. Measure the protein concentration in the MBP-L fraction (column load) and in the E fraction (column eluate) that had the most protein according to the quick screen that you carried out at the last session. Also prepare samples for the MBP-β–gal-L fraction and for two of the E fractions that had the most protein. Most probably those will be E2 and E3, but consult your microtiter plate, just in case. To get a reading that will fall within the absorbance range circumscribed by your standard curve, we recommend that you start with 10 µl from the L fractions and 50 µl from the E fractions. As you are preparing your samples, make an eyeball comparison with the standard curve to judge if they are going to yield reasonable measurements. If some samples have too little or too much protein to get an accurate reading, set up another tube of Bradford reagent and add either less or more sample, as appropriate. You may even have to dilute the protein in TNE before taking a sample (i.e., add 10 µl of protein to 90 µl TNE and use 10 µl of the dilution for the sample.)

4. Allow all of your samples to incubate at least 10 min before reading their absorbance. To read the absorbance of your samples:

a. With the lid down and no tube in the sample compartment, set the wavelength to 595 nm and turn the knob on the left side of the Spec-20's front panel until the % Transmission equals 0.

b. Pour the contents from tube 1 (blank) into a Spec-20 cuvette, wipe the outside of the cuvette with a tissue to remove finger prints and liquid, insert the cuvette into the sample compartment, and close the lid. The cuvette has a white orientation marker on its side. Insert the cuvette such that the orientation marker lines up with the mark on the side of the cuvette port. Turn the knob on the right side of the Spec-20's front panel until % Transmission equals 100.

c. Remove the cuvette from the sample compartment, drain the sample back into its original tube, dab the lip of the cuvette with a tissue to remove excess liquid, and pour the contents of tube #2 into the cuvette. Change mode from transmission to absorbance. Wipe the outside of the cuvette with a tissue to remove finger prints, liquid, etc, insert the cuvette into the sample compartment, close the lid, and record the absorbance value. You may need to leave the cuvette in the sample holder for several seconds before taking your reading to allow the absorbance value to stabilize.

d. Repeat step c for tubes 3, 4, and 5, followed by your unknowns.

Part II. Assay ß-galactosidase activity of purified MBP-ß-galactosidase chimera

Objective: The enzyme ß-galactosidase cleaves the chromogenic substrate o-nitrophenyl-ß-D-galactoside (ONPG) to produce the colored product o-nitrophenol (ONP). In this week's lab, you will use this assay to determine the effectiveness of the affinity purification of your MBP- ß-galactosidase fusion protein and to the measure the specific activity of the purified protein. In addition, you will study the kinetics of ONPG hydrolysis and also determine the effect of various treatments on ß-galactosidase activity.

Materials:

On your bench:

Z buffer = "Z"
ONPG (wrapped in aluminium foil)
Na_2CO_3 = "SC"

On the table:

Sterile 50 ml Falcon tubes for part III.
Samples of glucose, SDS, etc. for part IV.
More dispo reaction tubes

Experimental Protocol:

I. The first question we want to ask is: **how effective was the purification**? To answer this compare the enzyme activity per mg protein in the load and the eluate. This information will be included in your final report.

Start by diluting your enzyme preps as follows:

1. Pipet 90 µl of TNE buffer into each of 4 microfuge tubes. Add 10 µl of undiluted MBP-ß- gal "L" to one of the tubes and mix well. This is a 10-fold dilution of the MBP-ß- gal protein. Label 10^{-1} L.

2. Pipet 10 µl of 10^{-1} L into another of the microfuge tubes and mix well. This is a 100-fold dilution of the MBP-ß- gal protein. Label 10^{-2} L.

3. Repeat the dilutions using your best MBP-ß- gal "E" fraction to obtain 10-fold and 100-fold dilutions of that protein. Label 10^{-1} E and 10^{-2} E.

Keep all dilutions on ice, as you will be using them more than once.

Then set up preliminary ß-galactosidase enzyme assays using these dilutions as follows:

1. Pipet 2.4 ml of Z-buffer into each of 7 disposable reaction tubes. Add 10 µl of TNE to the first (this will be your blank).

2. Add 10 µl of undiluted "L" to the second, 10 µl of "10^{-1} L" to the third, and 10 µl of "10^{-2} L" to the fourth.

3. Repeat for the last 3 tubes – 5, 6 and 7, - using undiluted, "10^{-1} E", and "10^{-2} E" fractions.

4. Initiate the reaction by adding 0.6 ml ONPG to each tube. Vortex each briefly to mix. After 10 min incubation at room temperature, add 1.5 ml of 1 M sodium carbonate stop buffer to each. Vortex.

Since the incubations are so brief, you should stagger the initiation of the reactions so that each incubation is of identical duration. The way to do this as is follows. Set up all your tubes with Z buffer and protein. Start your timer and initiate the first reaction by adding substrate solution (ONPG) to the tube. When the timer reaches 30 sec, add substrate to tube #2; at 60 sec, add substrate to tube #3 etc. When the timer reaches 10 min, add stop buffer to tube #1; at 10 min 30 sec, add stop buffer to tube #2 etc.

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