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Syllabus

Course Title and Number: Biochemistry Lab CHEM 3131
Instructor: Dr. Ricardo A. Bernal
Teaching Assistants: Bianka Holguin or Alex Rodriguez

TA Office Hours: _____________________________________________________________

Email Address: rbernal@utep.edu, baholguin3@miners.utep.edu, arodriguez109@miners.utep.edu

TA Office Location: CCSB G.0910 (ring doorbell otherwise no one will hear the knocking)

TA Office Phone Number: 747-8432 or 747-5587(lab)

Textbooks

No Textbook required for this lab. The lab manual will be provided free as a PDF file. You’re welcome...

Required: Must be purchased by the student
- Safety goggles
- Lab notebook/binder to insert lab reports and to keep lab notes
- Lab Coat
- Sharpie for labeling things

Course Information and web-site
The material related to this course (e.g., syllabus, reading material, slides, etc.) will be available through Blackboard. The instructor will upgrade the information every week, therefore you are encouraged to consult the information available on course website (Blackboard) on a weekly basis. You are expected to utilize the internet to get information about details not contained in the lab manual. The lab manual in certain instances will be brief by design and for this purpose.

Prerequisites
It is recommended that students take CHEM 3330/3332 (Introductory Biochemistry) either before or concurrently with this lab.

Course Description
The course will focus on modern biochemistry techniques used in most modern laboratories. It is anticipated that everyone taking this course will learn not only the theory behind the techniques but will also get hands on experience in doing them. The goal for the semester is to clone and purify a recombinant protein while learning all the techniques along the way.

Grading:
There will be a laboratory report due for each laboratory at the beginning of the next lab. Reports will be considered late if not turned in within the first 10 minutes of class. Late reports will automatically have 10 points deducted per day for that report and no report will be accepted after 2 days.

A straight average of all the lab reports will determine the 90% of your overall grade. There will be attendance regularly. Attendance will make up 10% of your final grade. NO grades will be dropped and only excused absences (official University recognized) will be allowed for missed labs or reports. There will be no “extra credit” or additional assignments given at the end of the semester so please do not come begging to have your grade bumped up for no reason. You are in complete control over your grade so please try hard from the first day of class to the last. Absolutely no lab report re-do’s allowed.

As a rule, there are no grades of incomplete given in this lab. If you have missed a significant amount of work or labs you should drop the class. This is due to the fact that it is nearly impossible to make up a missed lab.
**Lab Reports**

Requirements for lab reports.

1. This will be due within the first 10 minutes of the following lab.
2. You are required to work on your lab reports individually (no copying others work)
3. Each laboratory module will require the creation of a laboratory report that contains the following sections;
   a. An appropriate Title
   b. Abstract/Summary of entire lab report (no more than 200 words)
   c. Introduction – Gives a brief statement of about what is known about the project (aka background), why this is important and the approach taken.
   d. Materials & Methods – This is an important section that describes in detail the methods you used.
   e. Results – This section presents your results as obtained in your experiments during the lab. You should present all your results in a professional manner using images, diagrams, plots and graphs, etc. as appropriate to present a clear impression of what was done. Note that even negative results are important. Present your results here but Do Not Analyze Results (save that for Discussion section).
   f. Discussion – Here you should discuss the results obtained in the previous section. Compare results to others and provide an analysis of what they might mean.
   g. Conclusions – What is the major outcome of this experiment? No more than 2-3 sentences.
   h. References Cited – Give credit where credit is due. Make sure you cite everything you read that others did.
4. It is highly encouraged that you take photographs (with cell phones) of experimental results so that you can include these in your reports.
5. Some data analysis may require you to use statistical methods in spreadsheets such as Excel. UTEP My Apps (my.apps.utep.edu) gives you access to this software.

**Withdrawal Policy:**

The last day for you to withdraw from any course with an automatic "W" is **listed in the current academic calendar.** Please note that it is the student's responsibility to officially withdraw from a course. We will not administratively drop anyone after the deadline.

**Class Attendance:**

Lab attendance is required. Attendance will be routinely taken. Students are responsible for attending lab regularly and knowing what takes place during the lab. This includes not only the material covered in the class, but also all announcements, handouts, changes in the syllabus, etc. If you must miss a lab, you need to make a special effort to learn what occurred during your absence.

It is expected that the material be read over before the topic is presented in class. With this background, the lectures and the lab will prove to be more meaningful. Unexcused absences will count against your in your final grade!!!!

**Disability:**

If you have or suspect a disability and need accommodations you should contact Disabled Student Services Office (DSSO) at 747-5148 or at dss@utep.edu or come by Room 106 Union East Building.

Since the schedule for each laboratory is very tight, you are expected to come to each lab prepared. You should read each lab before coming to class and familiarize yourselves with any calculations that will be required and procedures that you will need to master that day. If your absence is unavoidable, it is advised that you attend a different section so you don't miss class.
Lab Safety Rules

1. Always wear eye protection in the lab.
2. A lab coat MUST be worn in the lab always.
3. Don't Eat or Drink in Lab
4. Only authorized personnel are to be allowed into the lab (don't bring your friends).
5. DO NOT BE WASTEFUL with reagents and supplies.
6. Dress appropriately (Closed toe shoes and long trousers must be worn in the lab. Sandals and shorts are not allowed.)
7. Clean up after yourself. Wash all your glassware and clean (disinfect) your work area.
8. Identify the Safety Equipment in case you need it in the future.
9. Don't Casually Dispose of Chemicals Down the Drain. There are special containers for everything.
10. Long hair must be tied back when using open flames.
11. Always wash your hands before leaving lab.
12. Excess reagents are never to be returned to stock bottles.
13. Always pour strong acids/bases into water and not the other way around. If you pour water into acid, the heat of reaction will cause the water to explode into steam, sometimes violently, and the acid will splatter.
14. If chemicals come into contact with your skin or eyes, flush immediately with copious amounts of water and consult and report the incident your TA.
15. Do not place backpacks or other personal items on the lab benches.
16. Keep the lab CLEAN (it should be spotless).
17. Treat all equipment with care!!
18. Before using an instrument, make sure you are trained properly.
20. Be as careful about the safety of others as for yourself. Think before you act.

Emergency Number 911 (City of El Paso Police, Fire, EMS)
UTEP Emergency Number 5611 (Campus Police)
Poison Control Center 1-800-POISON-1 (1-800-764-7661)
Emergency Spill Response Coordinator EH&S Main Phone No. 747-7124
Facilities Services 7116
Commonly used Biochemical Standards

1.0 \text{A}_{260} \text{ unit ds DNA} = 50 \mu g/mL = 0.15 mM \text{ (in nucleotides)}

The average molecular weight of an amino acid is 110 Da

1 mL water = 1 g/cm$^3$

Standard Conditions
- $T = 25^\circ C = 298K$
- $P = 1 \text{ atm}$
- Water 55.6 M
- H$^+$ conc. = $10^{-7}$ M (pH=7.0)

How to make a solution from a concentrated Stock Solution
- $M_1V_1 = M_2V_2$ where $M=$concentration and $V=$volume (make sure units match)

Common Units
- molarity (M, mM, μM, nM, pM, etc.)
- normality ($N$, whose use is discouraged by IUPAC)
- weight/volume (g/L, mg/mL, μg/mL, μg/μL, etc.)
- percent weight or volume (%)
- dilution factor (1000x, 10x, 1x, etc.)

Common Terms and Notations
- ATP  adenosine 5'- triphosphate
- DNA  deoxyribonucleic acid
- mM  millimolar, $10^{-3}$ Molar, unit of concentration
- ADP  adenosine 5'- diphosphate
- kDa  kilodalton
- μM  micromolar, $10^{-6}$ Molar, unit of concentration
- E. coli  \textit{Escherichia coli}
- Apo  nucleotide-free, unbound state
- \textit{in vivo}  within the living
- EM  electron microscopy
- PCR  polymerase chain reaction
- EDTA  ethylene-diamine-tetraacetic acid
- HPLC  high-performance liquid chromatography
- pI  isoelectric point
- SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
- OD600  optical density, 600 nanometer wavelength
- DTT  dithiothreitol, Cleland’s reagent
- mg/ml  milligrams/milliliter, $10^{-3}$g/$10^{-3}$L, unit of concentration
- PDB  protein databank
- \textit{in vitro}  within the test tube
- 2xTY  yeast, tryptone and salt-based growth medium
- IPTG  isopropyl β-D-1-thiogalactoside
- μM  micromolar, $10^{-6}$ Molar, unit of concentration
- HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- DNase  deoxyribonucleic acid hydrolase
- SEC  size-exclusion chromatography
- μg  microgram, $10^{-6}$ gram, unit of weight
- BCA  bicinchoninic acid
Laboratory #1, Basic Pipetting and Buffer Dilutions

Objectives

1. To learn how to properly measure solution volumes using serological pipets and micro-pipettors.
2. Learn to measure solutions of varying viscosity accurately.
3. Learn to prepare the following solutions from stock solutions.
   a. 50 mL of TE Buffer (for DNA) containing
      i. 10 mM Tris-HCL pH 8.0
      ii. 1 mM EDTA pH 8.0
   b. 500 mL TAE buffer for DNA gels

Experimental procedure

1) You will work in groups of two so select your partners. Do not start on anything until given instructions by the TA.

2) First, check that you have the needed supplies on your workbench. If not, contact your TA.
   a. Serological Pipets Pipettes (one 5 mL and one 10 mL)
   b. One Serological Pipet bulb
   c. One set of Micropipettors (P20, P200 & P1000)
   d. One box each of P1000 and P200 tips
   e. One 50 mL conical tubes (with purple cap)
   f. One microcentrifuge tube

3) Your TA will give you instructions on the operation of the Micropipettors and serological pipet bulbs. Please make notes on their proper use and ask plenty of questions.

4) You will now figure out how to pipet the following volumes for a dry run. Ask TA if unsure
   a. 539 μl
   b. 233 μl
   c. 143 μl
   d. 27 μl

5) Weigh your microcentrifuge tube and write down the value on a piece of paper (2 significant figures) or on the tube itself with a sharpie.

6) You will now pipet the following into the pre-weighed microcentrifuge tube to create a mixture (you will mix solutions A through D in the microcentrifuge tube).
   a. 539 μl of solution labeled A (water)
   b. 233 μl of solution labeled B (glycerol)
   c. 143 μl of solution labeled C (ethanol)
   d. 27 μl of solution labeled D (acetonitrile)
7) When you are done, close the cap tightly and re-measure the weight. Now subtract this weight from the initial weight determined earlier. The difference will be the weight of the solution you just made.

8) Now you need to calculate how much of each component you need to make the 50 mL of TE. You will need to use the serological pipet and pipet bulb for this solution (for water). Please prepare 50 mL of TE in a 50 mL conical tube for use in next weeks’ lab.

   - Make 50 mL of TE Buffer (buffer for DNA next week) containing a **final** concentration of
     - 10 mM Tris-HCL pH 8.0
     - 1 mM EDTA pH 8.0

   - The **Stock** solutions given to you have the following concentrations.
     - 1 M Tris pH 7.5
     - 0.5 M EDTA pH 7.5

9) You need to also prepare 500 mL of the 1 X TAE buffer for next week.

10) **You must answer the questions on the next page and turn them in instead of the lab report.**

11) Please return all supplies and equipment to your TA.

12) Finally, you should wipe down your bench with ethanol provided.
Lab Assignment
Since this is a short lab; In lieu of a formal laboratory report, you must answer the following questions which are due at the beginning of the next lab period (within the first 10 minutes). (please use only the space provided)

1. The weight of the solution in the microcentrifuge tube has some experimental error in it. Calculate what the solution in the microcentrifuge should have weighed and compare this to your experimental value.

2. Where does the error come from?

3. What is the appropriate pipettor to use for measuring out 205 μl and why? What would be an alternative way to measure out this same value and perhaps with more precision?

4. What formula do you use to calculate how to make a buffer from a more concentrated stock solution? For example, if you want to make 30 mL of a 25 mM MOPS buffer from a 1.5 M stock solution. How can you calculate how much of the stock solution to add?

5. How many micrograms in a gram? How many nanoliters in a microliter?

6. What is TE buffer used for?

7. What is the purpose of EDTA in TE buffer?

8. What is TAE used for?
Laboratory #2, Polymerase Chain Reaction

Objectives

1. To learn how to perform Polymerase Chain Reaction (PCR) for the amplification of DNA for future recombinant protein production.
2. To analyze the results of PCR using DNA gel electrophoresis.

Introduction

This semester, you will be cloning β-galactosidase. Part of the cloning procedure is to first amplify the gene of interest so that you can have a sufficient quantity to manipulate it further (purification, digestion of restriction sites, etc.). PCR is very important and a key component of modern molecular biology. For your lab report, you need to find the DNA sequence for β-galactosidase online. You also should design the forward and reverse primers to be used in the PCR reaction. For today’s experiment, you will be given the PCR primers and the DNA template but you need to find those sequences yourself for the report.

Experimental procedure (Protocol begins on page 13)

- The above named β-galactosidase primers were designed and purchased from IDT.
- TA made 100 µL of a 10 µM stock of each for you as indicated below.

<table>
<thead>
<tr>
<th>Primer #</th>
<th>Concentration of Stock DNA</th>
<th>Volume of DNA</th>
<th>Volume of water</th>
<th>Final concentration of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 µM</td>
<td>10 µL</td>
<td>90 µL</td>
<td>10 µM</td>
</tr>
<tr>
<td>2</td>
<td>100 µM</td>
<td>10 µL</td>
<td>90 µL</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

New England Biolabs Protocol

The following protocol was taken from New England BioLabs website
https://www.neb.com/protocols/2012/08/29/protocol-for-q5-high-fidelity-2x-master-mix-m0492

Please note that protocols with Q5 High-Fidelity DNA Polymerase may differ from protocols with other polymerases. Conditions recommended below should be used for optimal performance.

Reaction Setup:
We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed prior to use. See table below
<table>
<thead>
<tr>
<th>Component</th>
<th>25 µl Reaction</th>
<th>50 µl Reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q5 High-Fidelity 2X Master Mix</td>
<td>12.5 µl</td>
<td>25 µl</td>
<td>1X</td>
</tr>
<tr>
<td>10 µM Forward Primer</td>
<td>1.25 µl</td>
<td>2.5 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>10 µM Reverse Primer</td>
<td>1.25 µl</td>
<td>2.5 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>variable</td>
<td>variable</td>
<td>&lt; 1,000 ng</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>to 25 µl</td>
<td>to 50 µl</td>
<td></td>
</tr>
</tbody>
</table>

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Transfer PCR tubes to a PCR machine and begin thermocycling.

Thermocycling Conditions for a Routine PCR:

<table>
<thead>
<tr>
<th>STEP</th>
<th>TEMP</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>98°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>25–35 Cycles</td>
<td>98°C</td>
<td>5–10 seconds</td>
</tr>
<tr>
<td></td>
<td>*50–72°C</td>
<td>10–30 seconds</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>20–30 seconds/kb</td>
</tr>
<tr>
<td>Hold</td>
<td>4–10°C</td>
<td></td>
</tr>
</tbody>
</table>

*Use of the NEB Tm Calculator is highly recommended.

General Guidelines:

Template:
Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

<table>
<thead>
<tr>
<th>DNA</th>
<th>AMOUNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Genomic</td>
<td>1 ng–1 µg</td>
</tr>
<tr>
<td>Plasmid or Viral</td>
<td>1 pg–1 ng</td>
</tr>
</tbody>
</table>

1. Primers:
Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 can be used to design or analyze primers. The best results are typically seen when using each primer at a final concentration of 0.5 µM in the reaction.
2. Mg++ and additives:
The Q5 High-Fidelity Master Mix contains 2.0 mM Mg++ when used at a 1X concentration. This is optimal for most PCR products generated with this master mix.

3. Deoxynucleotides:
The final concentration of dNTPs is 200 μM of each deoxynucleotide in the 1X Q5 High-Fidelity Master Mix. Q5 High-Fidelity DNA Polymerase cannot incorporate dUTP and is not recommended for use with uracil-containing primers or templates.

4. Q5 High-Fidelity DNA Polymerase concentration:
The concentration of Q5 High-Fidelity DNA Polymerase in the Q5 High-Fidelity 2X Master Mix has been optimized for best results under a wide range of conditions.

5. Denaturation:
An initial denaturation of 30 seconds at 98°C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 3 minutes) for templates that require it.

During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10 second denaturation at 98°C is recommended for most templates.

6. Annealing:
Optimal annealing temperatures for Q5 High-Fidelity DNA Polymerase tend to be higher than for other PCR polymerases. The NEB Tm Calculator should be used to determine the annealing temperature when using this enzyme. Typically use a 10–30 second annealing step at 3°C above the Tm of the lower Tm primer. A temperature gradient can also be used to optimize the annealing temperature for each primer pair. For high Tm primer pairs, two-step cycling without a separate annealing step can be used (see note 10).

7. Extension:
The recommended extension temperature is 72°C. Extension times are generally 20–30 seconds per kb for complex, genomic samples, but can be reduced to 10 seconds per kb for simple templates (plasmid, E. coli, etc.) or complex templates < 1 kb. Extension time can be increased to 40 seconds per kb for cDNA or long, complex templates, if necessary. A final extension of 2 minutes at 72°C is recommended.

8. Cycle number:
Generally, 25–35 cycles yield sufficient product. For genomic amplicons, 30-35 cycles are recommended.

9. 2-step PCR:
When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol (combining annealing and extension into one step) is possible.

10. Amplification of long products:
When amplifying products > 6 kb, it is often helpful to increase the extension time to 40–50 seconds/kb.

11. PCR product:
The PCR products generated using Q5 High-Fidelity 2X Master Mix have blunt ends. If cloning is the next step, then blunt-end cloning is recommended. If T/A-cloning is preferred, the DNA should be purified prior to A-addition, as Q5 High-Fidelity DNA Polymerase will degrade any overhangs generated.
Protocol for Class

1. Get a thin walled PCR tube from TA and label it with your group name.

2. Set up PCR reactions as follows (added in order). Total volume is 50 µL

   **Reaction:**
   1. 19.0 µl deionized and nuclease free H₂O
   2. 25.0 µl Q5 2X Master Mix
   3. 2.5 µl 10 µM Forward Primer
   4. 2.5 µl 10 µM Reverse Primer
   5. 1.0 µl Template

   **PCR Reaction Conditions:**
   __seconds at __°C
   __seconds at __°C
   __seconds at __°C
   __seconds at __°C
   __minutes at __°C
   Indefinite hold at 4°C

3. **Analysis of PCR products on a 0.7% Agarose Gel (per group per gel)**
   a. Prepare 35 mL of 0.7% agarose in 1X TAE buffer (0.26 g in 35 mL TAE)
   i. Weigh out 0.26 g of agarose using a weigh boat and place it in 35 mL of 1X TAE in a 100 mL bottle.
   ii. Loosen the cap a lot and then microwave it until it just begins to boil.
   iii. You can use wet folded paper towels to grab the bottle (not from cap) without burning yourself. Remove from microwave and swirl it to mix (do **not** create bubbles). Be careful not to burn yourself !!!!!!!!
   iv. Microwave again followed by mixing until all agarose has dissolved. Make sure there are no little translucent grains remaining. Keep microwaving as needed.
   v. While the agarose cools, put tape on gel tray to create dams for when you pour the agarose.
   vi. Place the comb into the slot of the tray so that you are ready to pour the agarose.
   vii. When agarose cools to the touch (when you can hold the bottle without it burning you) add 5 µl of SybrSafe DNA stain to the agarose then pour enough into the gel casting tray to create wells a few millimeters deep.
   viii. Wait until it cools before you move it. The agarose solidifies when there is a color change and it looks opaque instead of clear.

   b. In a separate microcentrifuge tube, add 5µL of your **PCR product** + 5µL DNA Loading Buffer. Mix gently by tapping it with finger then load it all onto the agarose gel as listed below. Make a note of where your sample is located in the gel.
1. Markers
2. PCR Reaction

c. Run the gel at 120 Volts for about 30 minutes or until the dye front is nearing the bottom of the gel.
d. Carefully take the gel along with the gel tray to the blue-light box to observe your results (you probably need to turn off the room lights).
e. Take a picture of the gel for your lab report (remember to label all lanes and molecular weight markers).

4. Please return all supplies and equipment to your TA.
5. Finally, you should wipe down your bench with 70% ethanol provided.
6. For lab report you need to find the sequence of β-galactosidase so that you can design the PCR primers. We gave you the primers to use in today's experiment but you still need to design them as if you had to order them from a company.

   b. Search for the nucleotide sequence by typing "β-galactosidase" in the search area.

7. You can look at YouTube videos of PCR and primer design to get a better understanding of what we are trying to do.

Instructions for Lab Report

Abstract- Summarize what you did for the day and the purpose of the experiments.

Intro- Describe in detail how the PCR reaction works, what it is used for, what the primers are for and how they are incorporated, what the template is for (plasmid), and why the polymerase and free nucleotides are needed (Q5 High-Fidelity 2X Master Mix contains the polymerase and free nucleotides). You will also explain how DNA agarose gels are used as an analytical technique in deciding the correct size of your amplified gene.

Materials and Methods- List all the materials used for this lab, this includes the primers, template etc. the thermocycler and all the items required for the DNA gel. In addition, you must include the thermocycling conditions for the PCR reaction; a template to help guide you is on page 11. Once you have researched how the PCR reaction works you will understand how to set up your conditions; the most important variables you must choose are the annealing temperature which will be based on your primer melting temperature and the extension time which is dependent on the length of your sequence. Once you have designed your primers go to this website tmcalculator.neb.com/#1/ to obtain the Tm of your primers (this is the temperature you will use to anneal your primers during the reaction which will be between *50-72°C). Once you have opened the Tm website make sure to select Q5
High Fidelity 2X Master Mix under Polymerase/Kit and enter 10000 for the primer concentration. Then enter the primers you have designed, and the calculator will give you the annealing temperature at the right-hand corner. For the extension time simply multiply 20-30 seconds per kilobase (one kilobase equals 1000 bases) I have provided the sequence to you on a word document, make sure you know the exact length of this sequence. A more detailed guide to help with setup of your conditions is on page 12.

Results- Although you would have needed to design your primers to determine the annealing temperature for your thermocycling conditions in the Methods section please put your forward and reverse primers in this section both written in the 5 prime to 3 prime direction. DNA polymerases require a primer to synthesize DNA because they cannot add nucleotides to single stranded DNA, with this being said the forward primer must be complementary to the reverse strand (3'-5') in order for the polymerase to synthesize the forward strand (5'-3') because it needs a "prime" in order to begin synthesis of a strand. Conversely, the reverse primer must be complementary to the forward strand to synthesize the reverse strand. The forward primer will bind the 3-prime end of the reverse strand and the reverse primer will bind the 3 prime end of the forward strand. Keep in mind the reverse primer is exactly how it sounds, its direction is reversed and so it is more difficult to make because you are essentially working backwards when designing it, please watch lots of videos on how to design primers or schedule a time to meet with me so you can get this right! (Disregard the instruction to include the restriction enzyme sequences with your primers, you will add these sequences to your primers for the second lab report after we have completed the restriction enzyme digest.) Also include the DNA gel I have posted on blackboard in this section, you will all use the same gel picture since everyone obtains the same results.

Discussion- Discuss why or why not the DNA gel confirms the correct amplified gene.

Conclusion- Explain the general idea of this technique.

Cite at least two references including any videos you watched.

Rubric

• 10 points for each section (total of 70 points)
• 10 points for the thermocycling conditions (materials and methods section)
• 10 points for designing primers correctly (results section)
• 10 points for analyzing the gel and explaining why or why not the size indicates that the correct gene was amplified (discussion section)
Laboratory #3, PCR Product Purification and Restriction Enzyme Digest.

Objectives

1. To learn how to purify DNA.
2. To learn how to use restriction enzymes to create “sticky ends” for subsequent ligation reactions where the gene of interest is inserted into a plasmid.
3. To analyze the results of Restriction Enzyme digests using DNA gel electrophoresis.

Introduction

Nucleic acids have the property of being able to selectively bind to silica. An excellent source of information about the process of purifying DNA using silica is the following website. http://bitesizebio.com/13516/how-dna-extraction-rna-miniprep-kits-work. After purification of your nucleic acid, your purified DNA (either plasmid or PCR product) is ready for restriction enzyme digestion. Restriction enzymes are used because they leave overhangs in one of the strands which can be used to ligate to another strand that had also been digested with the same restriction enzyme. This overhang adds specificity to the ligation so that the gene of interest is inserted into a plasmid in a specific direction only.

Experimental procedure

Spin Column Purification of PCR product


Monarch® PCR & DNA Cleanup Kit (5 μg) Protocol (NEB #T1030)

General Guidelines:

Input amount of DNA to be purified should not exceed the binding capacity of the column (5 μg). A starting sample volume of 20–100 μl is recommended. For smaller samples, TE can be used to adjust the volume to the recommended volume range. Centrifugation should be carried out at 16,000 x g in a standard laboratory microcentrifuge at room temperature.

Buffer Preparation:

Always keep all buffer bottles tightly closed when not in use.

All centrifugation steps should be carried out at 16,000 x g. (~13K RPM in a typical microcentrifuge). This ensures all traces of buffer are eluted at each step.

1. Dilute sample with DNA Cleanup Binding Buffer according to the table below. Mix well by pipetting up and down or flicking the tube. Do not vortex. A starting sample volume of 20–100 μl is recommended. For smaller samples, TE can be used to adjust the volume. For diluted samples larger than 800 μl, load a portion of the sample, proceed with Step 2, and then repeat as necessary.
### SAMPLE TYPE | RATIO OF BINDING BUFFER: SAMPLE | EXAMPLE
---|---|---
dsDNA > 2 kb (plasmids, gDNA) | 2:1 | 200 μl:100 μl
dsDNA < 2 kb (some amplicons, fragments) | 2:1 use this ratio!! | 200 μl:100 μl
ssDNA (cDNA, M13) | 7:1 | 700 μl:100 μl

2. You will do the 2:1 ratio for your PCR and Vector RE Digestions

3. Insert column into collection tube and load sample onto column and close the cap. Spin for 1 minute, then discard flow-through.

4. Re-insert column into collection tube. Add 200 μl DNA Wash Buffer and spin for 1 minute. Discarding flow-through is optional.

5. Repeat wash (Step 3).

6. Transfer column to a clean 1.5 mL microfuge tube. Use care to ensure that the tip of the column does not come into contact with the flow-through. If in doubt, re-spin for 1 minute to ensure traces of salt and ethanol are not carried over to next step.

7. Add 35 μl of 1 mM Tris Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.

   **Note:** Typical elution volumes are 6–20 μl. Nuclease-free water (pH 7–8.5) can also be used to elute the DNA. Yield may slightly increase if a larger volume of DNA Elution Buffer is used, but the DNA will be less concentrated. For larger size DNA (≥ 10 kb), heating the elution buffer to 50°C prior to use can improve yield.

   Care should be used to ensure the elution buffer is delivered onto the matrix and not the wall of the column to maximize elution efficiency.

Normally, you would determine the concentration of the purified DNA spectrophotometrically by checking the absorbance of the DNA at a wavelength of 260 nm. However, to save time you can just digest your entire 35 μl in the restriction enzyme digest in the next step.

### Restriction Enzyme Digest of PCR Product to create overhang ends

**1. Restriction Enzyme Digest of PCR Reaction Product**

<table>
<thead>
<tr>
<th>Amount</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μL</td>
<td>DNA 6 μg/μl purified PCR product</td>
</tr>
<tr>
<td>5 μL</td>
<td>10X Buffer</td>
</tr>
<tr>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>1 μL</td>
<td>Enzyme #1 HIND III of 1:10 dilution in 1x Buffer</td>
</tr>
<tr>
<td>1 μL</td>
<td>Enzyme #2 BamHI of 1:10 dilution in 1x Buffer</td>
</tr>
<tr>
<td>50 μL</td>
<td>Total Volume</td>
</tr>
</tbody>
</table>
2. Plasmid Vector

<table>
<thead>
<tr>
<th>Amount</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>_______</td>
<td>DNA 1.0 µg/µl vector</td>
</tr>
<tr>
<td>5 µL</td>
<td>10X Buffer</td>
</tr>
<tr>
<td>_______</td>
<td>Water</td>
</tr>
<tr>
<td>1 µL</td>
<td>Enzyme #1 HIND III of 1:10 dilution in 1x Buffer</td>
</tr>
<tr>
<td>1 µL</td>
<td>Enzyme #2 Bam HI of 1:10 dilution in 1x Buffer</td>
</tr>
<tr>
<td>50 µL</td>
<td>Total Volume</td>
</tr>
</tbody>
</table>

- Incubate the RE reactions at 37 degrees for ~20 minutes or as long as it take to prepare gel.
- Purify the rest of your RE Digested DNA with the columns as you did after the PCR reaction to get it ready for next week.
- Prepare a 0.7% agarose gel as described in previous lab and run it. Don’t forget to add the SybrSafe stain.
- Load the gel as follows.
  - Lane 1 = 5 µL Molecular Weight standard
  - Lane 2 = 5 µL RE Digested Purified DNA + 5 µL Loading Buffer
- Observe on transilluminator and take a picture with your phone for your report.
- Please return all supplies and equipment to your TA.
- Finally, you should wipe down your bench with ethanol provided

**Instructions for Lab Report**

Additional lab report instructions on page 5 of lab manual

Abstract- Summarize what you did for the day and the purpose of the experiments.

Intro- Explain why we need to purify our amplified DNA before we continue with subsequent reactions. Describe how restriction enzymes work and what the purpose is for using them. State which restriction enzymes we used and what sequences they recognize.

Materials and Methods- List all the materials used for this lab.

Results- Last week you designed your forward and reverse primers, for this lab report you will now add the restriction enzyme sequences to your primers. The sequence for HINDIII (5’-AAGCTT-3’) goes on the forward primer and the sequence for BamHI (5’-GGATCC-3’) goes on the reverse primer. As you research how restriction enzymes work you will notice that in order for a restriction enzyme to be able to cut the sequence it recognizes there must be flanking nucleotides it can bind to. Because restriction enzymes go on the very ends of the gene the other side will be empty and the enzyme would not have anything to hold on to, so you must add extra sequences on the 5 prime ends of your primers to ensure the enzyme will be able to bind efficiently and carry out its cutting. I have attached a document on blackboard containing a list of the recommended flanking bases to add next to your restriction enzyme sequences. The document contains a list of many restriction enzymes and their recommended flanking bases but only pay attention to HINDIII and BamHI (I have highlighted them for you). Now you have successfully designed the actual primers you would use for the PCR reaction we performed in the beginning (the primers you used during the PCR reaction did contain all the necessary
sequences as they were designed before). If you designed your primers incorrectly the last time, please take this opportunity to make them correctly.

Discussion- Explain why additional flanking bases are required.

Conclusion- Explain the general idea of this technique.

Cite at least two references including any videos you watched.

Rubric

• 10 points for each section (total of 70 points)
• 10 points for adding restriction enzyme sequences correctly
• 10 points for adding additional flanking nucleotides correctly
• 10 points for overall cohesiveness
Laboratory #4, Ligation and Bacterial Transformation

Objectives

1. To learn how to quantitate DNA spectrophotometrically
2. To learn how to set up a reaction to ligate two pieces of DNA together.
3. To learn how to transform competent bacteria with plasmid DNA

Introduction

Molecular biology techniques including cloning have revolutionized scientific research by giving scientists the ability to manipulate genetic material. For this reason, cloning is often referred to as genetic engineering. After amplification of a gene of interest with PCR and restriction enzyme digestion to create overhanging end segments (sticky ends), the gene of interest can be ligated into the plasmid (vector) of interest. Just as with restriction site digestion, the ligation reaction involves the use of an enzyme taken from a bacteriophage called T4. In this experiment, you will ligate the DNA you produced previously into the plasmid digested last week and will then introduce this plasmid into bacteria. This process is called transformation. Bacteria are treated so they will take the plasmid after heat shocking the cells. Transformation involves mixing competent bacteria (CaCl₂ pre-treated cells) with plasmid DNA and then selecting bacteria containing the plasmid using agar plates that contain an antibiotic.

Experimental procedure

1. Determine the concentration of the recovered DNA by diluting your sample 1:100 in water (10 μl of your RE digested DNA and 990 μl water).
2. Place the diluted sample in a cuvette. Do not touch the bottom portion of the cuvette because this is where the readings are taken and fingerprints affect the absorbance reading.
3. Read the A₂₆₀nm and A₂₈₀nm measurements and record the results below.

<table>
<thead>
<tr>
<th>Vector (Plasmid)</th>
<th>Insert (PCR Product)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance 260 nm</td>
<td></td>
</tr>
<tr>
<td>Absorbance 280 nm</td>
<td></td>
</tr>
</tbody>
</table>

Concentration = OD x 50 µg/mL x dilution factor = XXX µg/mL

Example

OD₂₆₀ = 0.041 and you did a 1:20 dilution, then

0.041 x 50 x 20 = 41µg/mL or 0.041µg/µl → 2.632 µg total yield

Concentration = OD x 50µg/mL x dilution factor = µg/mL

PCR Product = OD = ________ x 50 x ________ = ______µg/mL or ______ng/µl

Vector = OD = ________ x 50 x ________ = ______µg/mL or ______ng/µl
Now that you know the DNA concentrations you can set up the Ligation Reaction

Ligation Reaction

In order to set up a reaction where you have the same number of molecule ends to ligate together, you first need to calculate the molar concentration of each

Calculation of mole ends;

Insert = \[ \frac{2 \times (\text{ng DNA})}{(\text{3075 base pairs}) \times (650 \text{ Daltons} / \text{bp})} = \text{nmole} \]

Vector = \[ \frac{2 \times (\text{ng DNA})}{(\text{base pairs}) \times (650 \text{ Daltons} / \text{bp})} = \text{nmole} \]

Adjust the amounts to get a vector to insert ratio of 1:3

Go to into the NEB website and use their calculator to save time. However, for the lab report, you need to calculate the above values for the table below manually.

http://nebiocalculator.neb.com/#/ligation

1. β-galactosidase insert / vector Setup

<table>
<thead>
<tr>
<th>Amount</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>_______ µL</td>
<td>_________ ng Insert DNA</td>
</tr>
<tr>
<td>_______ µL</td>
<td>_________ ng Vector DNA</td>
</tr>
<tr>
<td>2 µL</td>
<td>10X Reaction Buffer</td>
</tr>
<tr>
<td>_______ µL</td>
<td>water</td>
</tr>
<tr>
<td>1 µL</td>
<td>T4 DNA Ligase (straight, no dilution)  ↗ add last</td>
</tr>
<tr>
<td>_______ µL</td>
<td>Total Volume</td>
</tr>
</tbody>
</table>

2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. For cohesive (sticky) ends, incubate at 16°C overnight or **room temperature for 20 minutes**.
4. Heat inactivate at 65°C for 10 minutes and store for next week -20 degrees.
   The transformation needs to be done the following week.
Transformation Protocol for BL21(DE3) Competent Cells
Adapted from https://www.neb.com/protocols/1/01/01/transformation-protocol-for-bl21-de3-competent-cells-c2527

1. Chill on ice and transform 1-5 μl of the reaction into 50 μl competent cells.
2. Thaw a tube of BL21(DE3) Competent E. coli cells on ice for 10 minutes. It is critical that the tube is never removed from the ice or warmed up!
3. Add 1–5 μl containing 1 pg–100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4–5 times to mix cells and DNA. Do not vortex.
4. Keep the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at exactly 42°C for exactly 10 seconds. Do not mix.
6. IMMEDIATELY place on ice for exactly 5 minutes. Do not mix.
7. Pipette 950 μl of room temperature SOC media into the mixture.
8. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
9. Warm two LB + 100 μg/mL Ampicillin plates to 37°C.
10. Mix the cells thoroughly by flicking the tube and inverting.
11. Spread 50 & 100 μl of cells onto each selection plate with a sterile spreader and incubate overnight at 37°C.

Transformation Protocol Variables

Thawing: Cells are best thawed on ice and DNA added as soon as the last bit of ice in the tube disappears. Cells can also be thawed by hand, but warming above 0°C will decrease the transformation efficiency.

Incubation of DNA with Cells on Ice: For maximum transformation efficiency, cells and DNA should be incubated together on ice for 30 minutes. Expect a 2-fold loss in transformation efficiency for every 10 minutes you shorten this step.

Heat Shock: Both the temperature and the timing of the heat shock step are important and specific to the transformation volume and vessel. Using the transformation tube provided, 10 seconds at 42°C is optimal.

Outgrowth: Outgrowth at 37°C for 1 hour is best for cell recovery and for expression of antibiotic resistance. Expect a 2-fold loss in transformation efficiency for every 15 minutes you shorten this step. SOC gives 2-fold higher transformation efficiency than LB medium; and incubation with shaking or rotating the tube gives 2-fold higher transformation efficiency than incubation without shaking.

Plating: Selection plates can be used warm or cold, wet or dry without significantly affecting the transformation efficiency. However, warm, dry plates are easier to spread and allow for the most rapid colony formation.

- Please return all supplies and equipment to your TA.
- Finally, you should wipe down your bench with ethanol provided
- The lab report will be combined with Lab 5 and so it will be turned in after next weeks lab.
Laboratory #5, Protein Expression and Cell Lysis

Objectives

1. To learn how to express recombinant proteins in bacteria.
2. To learn how to lyse bacteria to extract your protein of interest.

Introduction

In molecular biology (genetic engineering), it is critical to be able to induce a cell (typically bacteria) to express or produce a protein from a gene that was introduced into that cell via a vector (carrier). In our case, it is a plasmid carrying the gene for β-galactosidase. That plasmid also contains a selectable marker typically in the form of antibiotic resistance. Any cell that gets a plasmid will become resistant to that antibiotic and all cells that did not get the plasmid will be killed off. The plasmid also contains the gene of interest under the control of an inducible promotor. In our case, the promotor is part of the lactose operon and is responsible for the induction of β-galactosidase when the cell detects the presence of lactose. In other words, lactose induces the production of β-galactosidase (an enzyme that degrades lactose). Instead of adding lactose to the bacterial culture to induce production of our protein, we add a non-hydrolyzable lactose analog so that its concentration remains constant. After protein production, the cells are harvested by centrifugation and then lysed. Once lysed, the cellular debris is removed before the protein is purified from the crude extract. Cell lysis is accomplished using lysozyme, an enzyme that digests the peptidoglycan layer of the cell wall. The cells are then placed in a hypotonic solution so that the cells burst.

Experimental procedure

To save time, your TA selected a colony from your plates and inoculated a liquid culture in 250 mL of media called 2XTY (see recipe below). This must be done early in the morning because it takes the cells about 8 hours to grow to log phage and to an Optical Density (OD) of 0.6 at 600 nm. You will begin todays experiment at Cell Lysis step below.

Prepare cells and Induce protein production:

1. Prepare a 50 mL of 2XTY containing 100 µg/mL ampicillin in a baffled flask for increased aeration.

<table>
<thead>
<tr>
<th>2XTY</th>
<th>For 1 Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>16 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>10 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Add 2 drops of 10 M NaOH to adjust the pH to 7.0</td>
<td></td>
</tr>
<tr>
<td>Autoclave for 20 minutes at 121.1°C</td>
<td></td>
</tr>
</tbody>
</table>

2. Pick a single isolated colony from last week’s plate and inoculate the media.
3. Incubate the culture at 37 °C with shaking at 220 rpm for ~ 8 hours until the optical density at 600 nm (OD<sub>600</sub>) is between 0.6-0.8.
4. Induce protein production after the OD<sub>600</sub> has reached the indicated OD by adding 1 M IPTG to a final concentration of 1mM. This is just a simple 1:1000 dilution.
5. Protein induction is to proceed for 2 hours at a reduced temperature of 25 °C. Keep flask shaking 220 rpm for proper aeration.

Cell Lysis

1. After induction, the TA will give you a total of about 4ml of cells. Spin down cells at 6000xg for 30 minutes at 4 °C.
2. Remove the supernatant (old spent media) with a Pipetman and discard.
3. Resuspend the pellet/cells in a final volume of 1 mL Lysis Buffer for each 50 mL original culture.

<table>
<thead>
<tr>
<th>Lysis Buffer</th>
<th>For 1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM HEPES pH 7.5</td>
<td>10 mL of 1 M</td>
</tr>
<tr>
<td>50 mM EDTA</td>
<td>100 mL of 0.5 M</td>
</tr>
<tr>
<td>0.02 % Sodium Azide</td>
<td>1 mL of 20% Stock</td>
</tr>
</tbody>
</table>

4. Add 10 μl of Lysozyme solution to the resuspended cells and gently mix end-over-end every minute for 30 minutes at 4 °C.
5. Freeze at -20 °C until frozen.
6. Thaw the tube by gently mixing end-over-end every minute at room temperature until thawed.
7. Repeat step 4-5 for a total of 3 cycles of freeze/thaw.
8. Once sample has become thick and extremely viscous then the cells are lysed. Otherwise you need to repeat freeze/thaw until sample has become viscous.
9. Add 10 μl of DNase I solution and 60 mM MgCl₂.
10. Gently mix end-over-end every minute for 30 minutes at 4 °C. Sample should go from viscous to liquid again as DNA is degraded.
11. Transfer sample to two 2ml microcentrifuge tubes and place them in a high-speed microcentrifuge and spin down at maximum speed for 30 minutes.
12. Carefully pipet the supernatant into a new microcentrifuge tube. Discard cellular debris pellet.
13. Freeze supernatant containing protein of interest at -80 °C for purification next week.
14. Please return all supplies and equipment to your TA.
15. Finally, you should wipe down your bench with the ethanol provided.
Instructions for Lab Report

Additional lab report instructions on page 5 of lab manual

Abstract- Summarize what you did for the day and the purpose of the experiments.

Intro-Describe the ligation reaction and all the components needed. Explain what transformation is and why the plates must contain an antibiotic.

Materials and Methods- List all the materials used for this lab. Explain the method used for obtaining the volumes you pipetted for the insert and for the vector DNA. I know you did this part in lab and used a website to do most of the calculations but you should be able to understand where the numbers come from based on the molar ratio required for the reaction and the concentrations of the insert and vector DNA.

Results- Include the picture of your plate, circle any colonies you would potentially screen (to check for the presence of your gene).

Discussion- Discuss your results. If you got colonies your transformation was successful. If you didn’t get colonies discuss possible problems during the transformation protocol. Remember a successful transformation doesn’t always mean a successful ligation, please discuss why this is the case. If you got a bacterial lawn explain why you would not be able to screen any colonies for the presence of your gene.

Conclusion- Explain the general idea of these techniques.

Cite at least two references including any videos you watched.

Rubric

- 10 points for each section (total of 70 points)
- 10 points for discussing why the transformation is independent of the ligation of our gene
- 20 points for overall cohesiveness
Laboratory #6, Protein Purification using Column Chromatography

Objectives

1. To learn about column chromatography for protein purification
2. To learn about the different types of chromatography columns available
3. To analyze column chromatography results

Introduction

Column chromatography is a very old, classical, and powerful technique for the purification of proteins that is still very useful today. Columns are generally made of glass or plastic that can withstand high pressures and are in the shape of a long cylinder that can be filled with an appropriate matrix or stationary phase. The matrix of a column is generally in the form of a microscopic bead that resembles a powder. The beads can be made to have very specific characteristics such that they interact with proteins in various ways. The microscopic beads can be used to pack a column when suspended into an aqueous liquid (generally water). Because the beads are spherical, they can pack together quite tightly but leave empty space between them the way a container full of ping-pong balls would pack. This way liquid can still pass over the beads and through the matrix. As the liquid passes through the matrix, proteins in the liquid can interact with the beads or perhaps even bind to the beads. Column chromatography of various types is available which exploits different characteristics of a protein to purify it. These different types include;

a. Size Exclusion Chromatography (SEC) – This technique separates proteins based on their size and shape exclusively. The column matrix contains beads that have microscopic pores in them. The pore size can be controlled very precisely and can be of various sizes. As proteins pass through the matrix, smaller proteins can enter the small pores on the bead surface whereas larger proteins cannot enter the pores. The result is that larger proteins run past the beads faster while smaller proteins are retarded by all their interactions with the beads. The result is that proteins are separated by their size with large proteins eluting first and small proteins eluting later.

b. Ion Exchange Chromatography (IEC) – Ion exchange chromatography exploits the charges on the surface of proteins to purify them. Some proteins have a net negative charge and others have a net positive charge on their surface. The strength of the charge also varies from protein to protein depending on the total number of charges. To exploit this characteristic, one only needs to have a matrix where the micro-beads are charged. Anion exchange chromatography uses positively charged beads to bind negatively charged proteins whereas cation exchange chromatography uses negatively charged beads to bind positively charged proteins. The strength of the interactions can be controlled by making the beads either weakly charged or strongly charged to change the characteristics of the column required. As proteins are passed through the column matrix, they will either bind to the column or pass through the column depending on their surface charges. The proteins can then be washed by passing buffer through the column to remove any unbound proteins. The proteins can then be eluted by running a gradient of NaCl through the column from low to
high concentration. Because NaCl completely ionizes in solution, the ions can be used to compete with the proteins for the charged binding sites on the matrix beads which in turn helps elute the proteins from the column.

c. Hydrophobic Interaction Chromatography (HIC) – In this category of column chromatography, the micro-beads of the column are functionalized with a hydrophobic molecule. The molecule chosen depends on the strength of the hydrophobicity required. For example, for weak hydrophobic interactions the beads can be functionalized with methyl groups and for strong interactions one can add a phenyl group. Proteins are passed through the column under very high salt conditions to encourage hydrophobic interactions between proteins and the matrix. To elute the proteins from the column you use a reverse gradient that starts at high salt concentration and drops to low concentration. Typically, this starts at 1-2 molar ammonium sulfate and then gradually drops to 0 molar ammonium sulfate. The idea is that at high salt you change the activity of water such that all the water molecules are used up trying to solvate the salt and so they are unavailable to interact with the proteins. Proteins are forced to interact with the column since there is so little water molecules available. As salt is removed, more and more water becomes available to solvate proteins and so the proteins no longer have to interact with the matrix and can actually become soluble in the water (which they prefer).

d. Isoelectric Focusing (IEF) – This is a less commonly used technique but it involves exploiting the surface charges on the surface of the proteins just like in ion-exchange chromatography with a major difference. In IEF, a pH gradient is established inside a gel matrix using charged molecules called ampholytes (zwitterions). With the pH gradient established in the column, proteins pass through the gel until they reach the pH that matches their isoelectric point (where their net charge is zero). At that point, they stop migrating and “focus” at that location. Isoelectric focusing can resolve proteins that differ in as little as 0.01 pH units.

e. Affinity Chromatography – This type of protein purification includes 2 major classes. One uses antibodies and a column with affinity for the Fc portion of the antibody. The second makes use of tags that can be cloned into the protein to create a fusion protein. The tag can then be used to fish out the protein of interest using a column with specificity for the tag. Examples include

a. Histidine tag – 6-10 histidines in a row added to the N or C terminus of a protein. Histidine has affinity to nickel ions and so these proteins will bind to a nickel-NTA column.
b. GST tag – Glutathione S transferase has very high affinity for glutathione and so creating a Glutathione S transferase fusion with our protein of interest will allow us to purify our protein by running it through a glutathione column. One purified, the GST tag can be removed using a protease which can cleave at a protease cleavage site engineered between the fusion proteins.
c. There are many other tags such as maltose binding protein (MBP), chitin binding protein (CBP), FLAG-tag, and an infinite number of others.
Most modern column chromatography is now computer controlled and proteins are monitored using UV absorption as they elute from the column. In today’s lab, we will use an AKTA Start chromatography system. The results of the protein purification will be analyzed next week.

**Experimental procedure**

**Column Chromatography**

1. Your TA has thawed out your crude extract from the -80 °C freezer from last week and you will purify the protein on a column.
2. Before loading the sample on the column, we must spin down insoluble debris on micro-centrifuge at max speed for 5 minutes.
3. Dilute the sample 20-fold in Chromatography Buffer A.
4. Load the total volume onto a High Trap Q HP 5 ml Column (a strong anion exchange column).
5. The chromatography run is completely automated (apart from sample loading) and has been programmed to wash the column with a large volume of Chromatography Buffer A so that any unbound proteins are completely removed.
6. The computer will monitor the UV₂₈₀nm and the conductivity of the buffer coming out of the column. This data is recorded and graphed on the screen for you.
7. β-galactosidase will elute at a specific conductivity that you need to figure out after you determine which peak has it when you run the gel next week.
8. Save the fractions by capping the tubes of interest and make sure to label the tubes.
9. Please return all supplies and equipment to your TA.
10. Finally, you should wipe down your bench with the ethanol provided.

<table>
<thead>
<tr>
<th>Chromatography Buffer A</th>
<th>For 500 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM HEPES</td>
<td>5 ml of 1 M</td>
</tr>
<tr>
<td>5 mM EDTA</td>
<td>5 ml of 0.5 M</td>
</tr>
<tr>
<td>No NaCl</td>
<td>0 ml of 5 M</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chromatography Buffer B</th>
<th>For 500 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM HEPES Buffer</td>
<td>5 ml of 1 M</td>
</tr>
<tr>
<td>5 mM EDTA</td>
<td>5 ml of 0.5 M</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>100 ml of 5 M</td>
</tr>
</tbody>
</table>
While you wait for the Chromatography run to finish, please work on the following questions.

1. Predict the elution sequence of the listed compounds on anion exchange, cation exchange and hydrophobic interaction chromatography at pH 7.0.

   Arg                                      Cys-Lys-Arg-Gly
   Glu-Lys                              Leu-Phe-Ala
   Phosphorylated Tyr            Trp-Val-Phe

**Hydrophobic Interaction Column**

1. ________________
2. ________________
3. ________________
4. ________________
5. ________________
6. ________________

**Cation Exchange Column**

1. ________________
2. ________________
3. ________________
4. ________________
5. ________________
6. ________________

**Anion Exchange Column**

1. ________________
2. ________________
3. ________________
4. ________________
5. ________________
6. ________________
2. What type of column would you choose if your protein has the following characteristics?

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Column Type to Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>The protein is rich in glutamate and has a molecular weight of 30 kDa</td>
<td></td>
</tr>
<tr>
<td>The protein is rich in tryptophan and has a molecular weight of 20 kDa</td>
<td></td>
</tr>
<tr>
<td>The protein is rich in lysine and has a molecular weight of 50 kDa</td>
<td></td>
</tr>
<tr>
<td>The protein has a molecular weight of 150 kDa and is isoelectric at pH 7</td>
<td></td>
</tr>
<tr>
<td>The protein is rich in cysteine and has a molecular weight of 30 kDa</td>
<td></td>
</tr>
</tbody>
</table>

3. You find that your protein sample loses activity during storage but looks fine on an SDS-PAGE. What can you do about this? Circle all the correct answers
   a) Add many more additional purification steps
   b) Use a protease inhibitor during purification steps
   c) Perform each step as quickly as possible, in a cold-room
   d) Be careful not to damage your protein during purification
   e) All of the above

4. Your protein is large and has all amino acids represented in its sequence. The isoelectric point is exactly at the pH which you normally use to purify proteins (pH 7.5). Figure out a strategy to be able to use ion exchange chromatography with this protein.
Instructions for Lab Report

Additional lab report instructions on page 5 of lab manual

Abstract- Summarize what you did for the day and the purpose of the experiments.

Intro- Describe the method for extracting protein from cells. Explain the purpose of using lysozyme and DNase/MgCl₂. Explain why proteins must be purified. Describe liquid chromatography and the two types of column chromatography we used to purify beta-gal. We used anion exchange chromatography in class and I ran a size exclusion column for you.

Materials and Methods- List all the materials used for this lab. List all the steps (in order) from pelleting the cells to acquiring purified protein.

Results- Include the chromatograms for the ion exchange run and the size exclusion run. Complete the homework on pages 30 and 31 of the lab manual, you can paste the homework onto this section of your lab report or turn in a hard copy. The lab manual is available on black board for you.

Discussion- Discuss your results. What do the chromatograms say about the characteristics of your protein beta-galactosidase? (charge and size)

Conclusion- Explain the general idea of these techniques.

Cite at least two references including any videos you watched.

Rubric

- 10 points for each section (total of 70 points)
- 5 points for listing the method steps in order
- 5 points for answering the homework questions correctly
- 10 points for interpreting the chromatograms correctly (in the discussion)
- 10 points for overall cohesiveness
Laboratory #7, SDS-PAGE for Analytical Protein Gels

Objectives

1. To learn how to analyze proteins using acrylamide gels and electrophoresis
2. To learn how an SDS polyacrylamide gel works
3. To analyze purified proteins from last week on an SDS-PAGE

Introduction

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is one of the most common analytical techniques used in the biochemistry laboratory. This technique separates proteins exclusively on their size and can be used to estimate not only protein purity but also its apparent molecular weight. The method is based on the separation of proteins using the gel matrix (polymerized acrylamide) as a molecular sieve where the proteins are pulled through the sieve by an electrical current. The separation is dependent on the fact that proteins must first be completely denatured by treating the proteins with the ionic detergent SDS, a strong reducing agent to break disulfide bonds (Dithiothreitol or β-mercaptoethanol) before boiling the sample for at least 5 minutes. SDS, is a very strong detergent with the formula CH₃(CH₂)₁₁SO₄Na. The 12-carbon tail inserts itself into the protein and denatures it while the sulfate group imparts a strong negative charge to the protein. The result is that the protein is completely linearized and is uniformly negatively charged. Because the protein is now completely negatively charged, when an electrical field is applied, it will run towards the opposite charge (anode). When exposed to the electric field, the SDS denatured proteins will migrate towards the anode (positively charged electrode) and separated by the molecular sieving effect based solely on size. After the visualization by a staining the proteins, the size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder (marker).
Experimental procedure

Setting up the apparatus

1. Today's lab will be including the use of precast gels and so you will not have to make your own gel. First, get the following items from your TA
   a. Precast 4-20% gel
   b. Protein sample
   c. Electrophoresis apparatus
   d. Sample buffer
   e. Running buffer
   f. P20 Pipettor and tips
2. You need to prepare your protein for electrophoresis. You need to take your protein and add sample buffer to it. Your TA will tell you the amounts to use.
3. Heat your sample to 100 °C for 5 minutes and let it cool on your bench until needed.
4. Next you need to assemble your electrophoresis apparatus.
   a. Remove the gel from the storage pouch and prepare it for assembly
   b. GENTLY remove the comb: Position thumb on the indentation (middle of comb) and remove the comb by pulling straight upward in one smooth motion.
   c. Remove the tape: Pull gently to remove the green tape from the bottom of the cassette. If necessary, use the opening key or comb to help remove the tape at the corners.
   d. Rinse the wells: Use a syringe, wash bottle, or disposable transfer pipet to rinse the wells with running buffer. Leave buffer in the wells to prevent drying.

Approximate migration of proteins on various percentage gels.
e. Set the electrode assembly to the open position on a clean, flat surface (A).
f. Place the gel cassettes into the electrode assembly. Use the buffer dam (included with the cell) to complete the assembly.
   i. Place the first cassette with the short plate facing inward and so the gel rests at a 30° angle away from the center of the electrode assembly. Make sure the electrode assembly remains balanced and does not tip over.
   ii. Place the second gel or buffer dam on the other side of the electrode assembly, again by resting the gel on the supports. The gels rest at 30° angles, one on either side of the electrode assembly, tilting away from the center of the frame (B).
g. Gently push both gels toward each other, making sure that they rest firmly and squarely against the green gasket that is built into the electrode assembly. Align the short plates to ensure the edge sits just below the notch at the top of the green gasket (C).
h. While gently squeezing the gel cassettes (or cassette and buffer dam) against the green gaskets (maintaining constant pressure and with both gels in place), slide the green arms of the clamping frame one at a time over the gels, locking them into place (D,E).
i. The wing clamps of the electrode assembly lift each gel cassette up against the notch in the green gasket, forming a seal. Check again that the short plates sit just below the notch at the top of the green gasket (C).
j. Place the electrophoresis module into the tank (F) and fill the buffer chambers with 1x running buffer:
   i. Fill the inner buffer chamber completely to the top
   ii. Add most if not all the remaining buffer to the outer buffer chamber
5. Load samples and run the gels at a constant voltage of 200V.
6. Stop the run when the dye front reaches 0.5 cm from the bottom of the gel.

Removing and Staining the gel
1. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.
2. Remove the lid from the tank and remove the gels from the cell. Pour off and discard the running buffer.
3. To open the cassette, align the arrow on the opening lever with the arrows marked on the cassette and insert the lever between the cassette plates at indicated locations. Apply downward pressure to break each seal. Do not twist the lever.

4. Pull the two plates apart from the top of the cassette, and gently remove the gel by teasing it with the pipet tip and drop it into the staining solution.
   a. Do not touch the gel with your hands because you will contaminate it!
   b. Stain the gel for a minimum of 1 hour.
   c. De-stain overnight or until desired intensity is achieved.

5. Please return all supplies and equipment to your TA.

6. Finally, you should wipe down your bench with the ethanol provided.

COOMASSIE BLUE STAINING
Detection of protein bands in a gel by Coomassie blue staining depends on nonspecific binding of a dye, Coomassie brilliant blue R, to proteins. The detection limit is 0.3 to 1 µg/protein band. In this procedure, proteins separated in a polyacrylamide gel are precipitated using a fixing solution containing methanol/acetic acid. The location of the precipitated proteins is then detected using Coomassie blue (which turns the entire gel blue). After de-staining, the blue protein bands appear against a clear background. The gel can then be stored in acetic acid or water, photographed, or dried to maintain a permanent record.

**Coomassie blue staining solution**
50% (v/v) methanol
0.05% (w/v) Coomassie brilliant blue R-250
10% (v/v) acetic acid
40% H₂O

**Fixing solution for Coomassie blue**
50% (v/v) ethanol
10% (v/v) acetic acid
40% H₂O
Store at room temperature

**Methanol/acetic acid de-staining solution**
40% (v/v) ethanol
5% (v/v) acetic acid
Store at room temperature
Instructions for Lab Report

Additional lab report instructions on page 5 of lab manual

Abstract- Summarize what you did for the day and the purpose of the experiments.
Intro- Explain in detail how SDS-PAGE works and what it is used for.
Materials and Methods- List all the materials used for this lab.
Results- Include a picture of the gel we ran.
Discussion- Discuss your results. Explain why the protein is or isn’t the correct size based on the protein ladder. State whether your protein sample is pure or not.
Conclusion- Explain the general idea of this technique.
Cite at least two references including any videos you watched.

Rubric
• 10 points for each section (total of 70 points)
• 10 points for explaining if you have the correct protein or not
• 20 points for overall cohesiveness
Laboratory #8, Protein Quantitation Methods (BCA Assay)

Objectives

1. To learn how to determine the concentration of an unknown protein.
2. To perfect pipetting skills
3. To analyze the results of the concentration assay using an EXCEL spreadsheet.

Introduction

The bicinchoninic acid (BCA) assay was developed by Paul Smith at the Pierce Chemical Company in 1985 and is used to determine the total protein concentration in a solution. Protein is determined by a color change from light green to purple depending on the amount of protein present. There are two reactions that occur in the assay that result in the color change. First, the peptide bonds of a protein will reduce Cu$^{2+}$ ions to Cu$^{+}$ ions at an elevated temperature (between 40-60 °C). The amount of Cu$^{2+}$ ions reduced is directly proportional to the amount of protein present. In the Second reaction, bicinchoninic acid will chelate the resulting Cu$^{+}$ ions which produces the purple colored complex BCA-Cu$^{+}$. The purple color that is developed can be quantitated using absorption of light at 562 nm in a spectrophotometer. The unknown concentration of a protein must be compared to a known concentration of a control protein and so a standard curve must first be generated. The absorption of the unknown protein is then compared to the standard curve to estimate the concentration of the unknown. The control protein that is usually chosen is bovine serum albumin because it is a very stable and well known protein.

Experimental procedure

1. You will be working as usual in groups of three and you will need the following items from your TA.
   a. BSA standard
   b. Reagent A and B for the BCA assay
   c. Cuvettes for the spectrophotometer
   d. A portable PASCO wireless spectrophotometer
      i. Download the software from the following website (for phone laptop or tablet)
   e. Pipettors and tips
   f. 15 microcentrifuge tubes and a tube rack
   g. A bottle of deionized water
   h. One 50 ml conical tube.
      i. Two spectrophotometer cuvettes (one for standards and one for unknowns)
2. Label your microcentrifuge tubes A-K and 1-4 as indicated in the Standards and Unknowns table below.
3. Prepare a set of protein standards of known concentration by adding everything as listed in the “Standards” table below and in the order listed here.
   a. First pipet out the diluent (diH$_2$O) into all the tubes.
   b. Second, pipet the BSA standard (or unknown protein) into appropriate tubes.
   c. Finally, prepare and pipette the working reagent.
4. For “Unknowns”, use 1 µl for tubes 1-2 and 2 µl for tubes 3-4 instead of the BSA standard.
5. Mix 180 µl Reagent B with 8.8 ml Reagent A (1:50 solution immediately clears to a light green color). Shake vigorously to mix very well.
6. Add the working reagent, cap the tubes and mix well.
7. Incubate at 60°C for 15-30 minutes.
8. After incubation, cool the tubes to room temperature, transfer to 1.5 mL disposable cuvettes and read the absorbance at 562 nm vs. a water (or air) reference.
9. For unknowns, use 1-2 microliters (or more if you expect it to be dilute).

### Standards

<table>
<thead>
<tr>
<th>Tube #</th>
<th>2 mg/ml BSA Stock</th>
<th>Diluent</th>
<th>Working Reagent</th>
<th>(A_{526}) (Y-axis)</th>
<th>Protein (µg) (X-axis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0 µl</td>
<td>500 µl</td>
<td>500 µl</td>
<td>0</td>
<td>0 µg</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>499</td>
<td>500 µl</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>498</td>
<td>500 µl</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>497</td>
<td>500 µl</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>496</td>
<td>500 µl</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>495</td>
<td>500 µl</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>G</td>
<td>6</td>
<td>494</td>
<td>500 µl</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>H</td>
<td>7</td>
<td>493</td>
<td>500 µl</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>I</td>
<td>8</td>
<td>492</td>
<td>500 µl</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>J</td>
<td>9</td>
<td>491</td>
<td>500 µl</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>K</td>
<td>10</td>
<td>490</td>
<td>500 µl</td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

### Unknowns

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Sample Description</th>
<th>Sample Amount</th>
<th>Diluent (Bring up to 1 mL)</th>
<th>Working Reagent</th>
<th>(A_{526})</th>
<th>Resulting Concentration µg/sample amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>1 µl</td>
<td>499 µl</td>
<td>500 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Duplicate of tube 1</em></td>
<td>1 µl</td>
<td>499 µl</td>
<td>500 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>2 µl</td>
<td>498 µl</td>
<td>500 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>Duplicate of tube 3</em></td>
<td>2 µl</td>
<td>498 µl</td>
<td>500 µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

10. To analyze the results, download the excel spreadsheet from blackboard and input all of your absorbance values. The spreadsheet automatically creates a plot of your standard curve using linear regression to generate a best fit line through your data. It will also automatically calculate your unknown protein concentration by automatically averaging duplicates and adjusts for the amount of protein added.
11. Make sure you print out the spreadsheet results (entire page) for your lab report.
12. Please return all supplies and equipment to your TA.
13. Finally, you should wipe down your bench with the ethanol provided.
Pasco Wireless Spectrophotometer Instructions for Use


   a. Connecting the Wireless Spectrometer to a Tablet or Computer via Bluetooth
      i. Turn on the spectrometer, and briefly hold the ON/OFF button on the back. After all three status LEDs blink in sequence, release the ON/OFF button. The Bluetooth Status LED will blink blue.
      ii. Pair /connect through Bluetooth to your computing device. This step will depend on your computing device and operating system. The Bluetooth Status LED will shine blue.
      iii. Start the Spectrometry software. (Refer to the Spectrometry Software User Guide at the PASCO web page: www.pasco.com/spectrometer under Resources.)
      iv. If the Wireless Spectrometer is not found, select Choose Wireless Device.
      v. In the Menu, select the Wireless Spectrometer with the Device ID # that matches the Device ID # on the bottom of the device.

   b. Connecting the Wireless Spectrometer to a Computer with a USB Cable
      i. Connect the small end of the included USB into the USB port on the back of the Spectrometer
      ii. Connect the other end of the USB cable to a USB port on the computer, or into a USB hub connected to the computer.
      iii. Turn on the Spectrometer, and briefly press and hold the ON/OFF button on the side. After all three status LEDs blink in sequence, release the ON/OFF button.
      iv. The USB Status LED will shine green. (NOTE: The Bluetooth Status LED will slowly blink blue. The Battery Status LED may also blink to indicate that the battery is charging through the USB cable.)

2. Calibration

   a. Once you plug the spectrometer into your computer using the USB cable or wirelessly associate the spectrometer using Bluetooth, then open the spectrometry application.
   b. Select ANALYZE SOLUTION from the menu at the top of the screen.
   c. Select CALIBRATE DARK from the Menu at the bottom of the screen. The Spectrometer will turn off all its lights and perform the calibration. A check mark will appear when the calibration is finished.
   d. Put distilled water into a cuvette. This should be the same distilled water that was used as a diluent for the solutions being analyzed. Always handle the cuvette by the ridged sides. Wipe off any fingerprints using a lint free wipe. Place the cuvette into the spectrometer so that the ridged sides are facing the violet and
green light icons and clear sides face the white light and absorbance spectrum icons.

- Select CALIBRATE REFERENCE from the menu at the bottom of the screen. A check mark will appear when the calibration is complete.

3. Finding the 562 nm Wavelength to Analyze
   
   a. Place the cuvette into the spectrometer as you did in the calibration.
   b. Select the red RECORD circle at the bottom left of the screen to start analyzing the solution. (It changes into a square while data is being collected.)
   c. Select the red STOP RECORDING square to stop data collection.
   d. Select SCALE TO FIT to rescale your data.
   e. Use the ADD COORDINATE to locate the 562 nm wavelength to analyze on the curve.
   f. A small hand will replace your cursor. Move it to the box that has appeared on the graph and drag the box slowly toward the curve. As you get near the curve an arrow will appear that indicates a specific wavelength on the curve. Releasing the box will snap the box to the point on the curve the arrow is pointing to.
   g. Drag the box along the curve to find the 562 nm wavelength to analyze.

4. Record the absorbance value in the table above and repeat the procedure for all unknown and known solutions you prepared.
5. Please return all supplies and equipment to your TA.
6. Finally, you should wipe down your bench with the ethanol provided.

**Instructions for Lab Report**

Additional lab report instructions on page 5 of lab manual

**Abstract** - Summarize what you did for the day and the purpose of the experiments.

**Intro** - Describe exactly how the BCA assay works.

**Materials and Methods** - List all the materials used for this lab.

**Results** - Include the results obtained from the BCA calculator, including the graph and the concentration of your protein.

**Discussion** - Discuss your results. Explain if the concentration given can be trusted based on the protein standard curve. Explain where the error could have come from if your standard curve is not good.

**Conclusion** - Explain the general idea of this technique.

Cite at least two references including any videos you watched.

**Rubric**
- 10 points for each section (total of 70 points)
- 10 points for including the BCA calculator results
- 10 points for discussing the standard curve and concentration given
- 10 points for overall cohesiveness.
Laboratory #9, β-Galactosidase Activity Assay

Objectives

1. To learn how to check that a protein you purified is biologically active.
2. To learn about the proper conditions that are required to get biological activity.
3. To analyze and interpret the results of the activity assay

Introduction

Enzymes are the biological version of catalysts in that they accelerate the rate of a biological reaction by reducing the activation energy barrier that is required for the reaction to proceed. Just like small molecule catalysts, enzymes are not consumed in the reaction and are available to be reused repeatedly in many reactions. Enzymes like most other proteins are globular in shape and can also be denatured which will render them inactive. To ensure that you are working with a native and fully active protein, you can check for biological activity after the enzyme has been purified. To check for activity, you must know certain characteristics about the enzyme to create a suitable activity assay. First you must know what the natural substrate is for this enzyme. You can utilize either the natural substrate or a derivative the closely mimics the natural substrate but gives you an easy way to detect the product whereas the natural substrate cannot be detected. For example, in today’s lab, we will be using ortho-Nitrophenyl-β-galactoside (ONPG) instead of the natural substrate for β-Galactosidase in *E. coli*, lactose. Cleavage of the disaccharide lactose results in one D-galactose and one D-glucose. Both cleavage products are colorless and difficult to detect as lactose cleavage products. However, ONPG (is colorless) cleavage produces D-galactose and O-nitrophenyl. The O-nitrophenyl as a bright yellow color that can easily be detected by a spectrophotometer at a wavelength of 420nm.

If you can detect the appearance of yellow ONP and the absence of yellow color in the negative control, then you can be confident that your enzyme, in this case β-Galactosidase, is active. Keep in mind that different enzyme purifications will have different levels of activity, indicating varying levels of native/denatured enzyme. Some preparations will be bad while others will be excellent. This assay will only tell you if there is active enzyme present in your purified protein and not the quality of the preparation. Next week, we will be doing kinetics which is much more complex but is also more qualitative.
Experimental procedure

We will again be using the PASCO spectrophotometers for this week’s lab. Please refer to the set up and use in last week’s lab write-up (Section labeled “Pasco Wireless Spectrophotometer Instructions for Use”).

1. You will be working as usual in groups of three and you will need the following items from your TA.
   a. A portable PASCO wireless spectrophotometer
   b. You will need an aliquot of
      i. Purified β-Galactosidase enzyme (0.08 mg/ml)
      ii. ONPG (1 mg/ml (3.3mM)) supplied as a 10 X stock
      iii. Reaction buffer (10mM HEPES pH 7.5, 50 mM NaCl, 5 mM EDTA, 10mM MgCl₂)
   c. Pipettors and tips
   d. 2 microcentrifuge tubes and a tube rack
      i. Label these - control, and experimental
   e. Two spectrophotometer cuvettes (one each for negative control and experimental).
      i. Label these - control and experimental. Remember to label these on the rough side and **not** the side with ☐ symbol.

2. First, please connect your spectrophotometer to your computer. You will not have time to set this up after you mix your reaction components so do it first.
   a. It is going to be critical that you begin collecting data as soon as you mix your components because the reactions occur remarkably fast (*E. coli* β-Galactosidase can carry out as many as 400 reactions per second at room temperature). If you don’t start collecting data immediately, you will miss the reaction!
   b. You will be using the timed run for your spectrophotometer readings. Set it up to collected data for 10-15 minutes with 10 second intervals.
   c. In the software, go to Analyze solution. Collect the dark reference and a light reference (just use air). Then go to TIME and start recording when ready.

3. You will do your negative control first (it will also serve as practice).
   a. Add 950 μl Reaction buffer to a labeled cuvette.
   b. Add 0 μl Purified β-Galactosidase enzyme and mix well.
   c. Place cuvette in spectrophotometer and begin reading as soon as you add the ONPG in step d below.
   d. Add 50 μl ONPG (10 mg/ml) supplied as a 10 X stock and mix well by pipetting up and down several times.
   e. Once the run has started, do NOT touch the spectrophotometer. Even bumping it will give you altered readings.
   f. Once the run is finished hit the stop record button and export the file to a csv file which you can open with excel. Please create a plot of your data for your report.

4. Next you will do your experimental reading.
a. Add 948 μl Reaction buffer to a labeled cuvette.
b. Add 2 μl Purified β-Galactosidase enzyme and mix well.
c. Place cuvette in spectrophotometer and begin reading as soon as you add the ONPG in step d below.
d. Add 50 μl ONPG (10 mg/ml) supplied as a 20 X stock and mix well by pipetting up and down several times to mix very well. Mixing is critical!!!
e. Once the run has started, do NOT touch the spectrophotometer and do not take out the cuvette. Even bumping it will give you altered readings.
f. Once the run is finished hit the stop record button and export the file to a csv file which you can open with excel. Please create a plot of your data for your report.

5. Please return all supplies and equipment to your TA.
6. Finally, you should wipe down your bench with the ethanol provided.

Instructions for Lab Report

Abstract- Summarize what you did for the day and the purpose of the experiments.

Intro- Describe what the purpose of performing an activity assay is. Explain exactly how the assay works. Why do we use the analog ortho-nitrophenyl-beta-galactosidase instead of beta-galactosidases’ natural substrate?

Materials and Methods- List all the materials used for this lab.

Results- Plot your negative control and the experimental reading on the same graph. Make sure to plot only time versus absorbance.

Discussion- Discuss your results. Is your protein active? Explain why it is necessary to perform a negative control.

Conclusion- Explain the general idea of this technique.

Cite at least two references including any videos you watched.

Rubric

- 10 points for each section (total of 70 points)
- 10 points for including both data plotted on one graph
- 10 points for correctly explaining all the elements of the assay (introduction)
- 10 points for overall cohesiveness
Laboratory #10, β-Galactosidase Enzyme Kinetics

Objectives

1. To learn how to determine the Km and Vmax and type of enzyme inhibition present.
2. To learn about reaction rates and enzyme inhibitors.
3. To analyze and interpret complex experimental kinetics data using an excel spreadsheet.

Introduction

This week’s lab is an extension of last week where you looked at the activity assay to answer the simple question “Is my protein active”. This week you will be asking “How active is my protein compared to other enzymes and what is the effect of an inhibitor”. Kinetics data is calculated differently in that you keep the enzyme concentration constant while you modify the substrate concentration. You then determine the initial velocity (V₀) of the reaction at those different substrate concentrations. The initial velocities are then plotted versus substrate concentration to calculate the Kᵣ and Vmax. To calculate the V₀ you simply calculate the slope of the line when you plot the absorbance vs. time. For V₀ only use the time from zero to about 5-10 minutes when the velocity is still linear.

Experimental procedure

The first part of the set-up this week is identical to last week.

1. You will be working as usual in groups of three and you will need the following items from your TA.

   a. A portable PASCO wireless spectrophotometer
   b. You will need an aliquot of
      i. Purified β-Galactosidase enzyme (0.08 mg/ml)
      ii. ONPG (10 mg/ml (33mM)) supplied as a 10 X stock
      iii. Reaction buffer (10mM HEPES pH 7.5, 50 mM NaCl, 5 mM EDTA, 10mM MgCl₂)
      iv. IPTG (1 mg/ml)
   c. Pipettors and tips
   d. At least 6 microcentrifuge tubes.
   e. Spectrophotometer cuvettes (one each for negative control and each experimental run).
      i. Label these (-) control and Tube # X. Remember to label these on the rough side and not the side with  symbol.

2. First, please connect your spectrophotometer to your computer. You will not have time to set this up after you mix your reaction components so do it first.

   a. It is going to be critical that you begin collecting data as soon as you mix your components because the reactions occur remarkably fast (E. coli β-Galactosidase can carry out as many as 400 reactions per second at room temperature). If you don’t start collecting data immediately, you will miss the reaction!
b. You will be using the timed run for your spectrophotometer readings. Set it up to collected data for 10-15 minutes with 10 second intervals.

c. In the software, go to Analyze solution. Collect the dark reference and a light reference (just use air). Then go to TIME and start recording when ready.

3. To calculate initial velocities ($V_o$), each group will run 6 reactions as listed in the table below (if there are not enough groups then only do the first sets of reactions listed).

   a. Results from the entire class will be pooled together to calculate the kinetics values. Be sure to ask the TA about which reactions you are responsible for before you start so that you do not replicate data that someone else is also doing.

      i. Add the reaction buffer to the cuvette first
      ii. Next add the β-Galactosidase enzyme and mix well
      iii. The substrate will initiate the reaction and so you must do this as quickly as possible.

         1. Place the cuvette in the spectrophotometer
         2. Take the appropriate amount of ONPG and put it into a microcentrifuge tube (NOT the cuvette with the enzyme!).
         3. Add the inhibitor to the ONPG and mix it well.
         4. Now pipet the ONPG/inhibitor mixture with a P200 pipettor set to 200 μl and quickly mix it into the reaction mixture by pipetting up and down three times. DO NOT MAKE BUBBLES/FOAM
         5. Collect data for about 10 minutes and do not touch the cuvette or spectrophotometer because it may influence your results.
         6. After you stop the recording, export your data as a tube#.csv file (which can be read by excel).
         7. Move on to the next tube and proceed as above.

Experimental setup table is on the next page
Experimental Reaction Setup

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Reaction Buffer (µl)</th>
<th>β−Galactosidase (µl)</th>
<th>ONPG (µl)</th>
<th>Inhibitor (IPTG) (µl)</th>
<th>Resulting $V_o$</th>
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4. For data analysis, you need to plot your data in excel and then calculate a best fit line through the data (I would recommend that you use linear regression). The slope of that line is your initial velocity. We will need an initial velocity data point from each one of the experimental runs in the tables above to calculate the kinetics data.

5. The lab for next week will include a tutorial on the data analysis for Km and Vmax calculations. Therefore, you will not need to turn in a lab report until the end of the next lab. In other words, we are combining the lab report from this week with next week’s lab.
   a. If you want to get ahead for next week, you can download and try out the excel spreadsheet called “Lineweaver_Burk_Template.xlsx” from Blackboard.
   b. Make sure you bring a laptop with excel with you next week!

6. Please return all supplies and equipment to your TA.

7. Finally, you should wipe down your bench with the ethanol provided.
Laboratory #11, Kinetics Calculations, continuation from last week.

Objectives
1. To learn how to calculate the initial velocity from the absorption data collected last week.
2. To learn how to calculate the Km and Vmax using excel
3. To analyze the results and determine the effect of the inhibitor IPTG.

Introduction
This week’s lab is a continuation of last week. We will use the data collected last week to calculate the kinetics values Km and Vmax. This is a relatively simple idea but it is data heavy which can lead to some confusion. Therefore, a spreadsheet has been created for you to help guide you along the data analysis pathway. You’ll be walked through the data analysis in class.

Experimental procedure
1. There is no wet lab this week. You need a laptop computer (or take very good notes) that has excel installed on it so you can follow along as everything is explained to you.
2. Please download the spreadsheet named “Lineweaver_Burk_Template.xlsx” from Blackboard.
3. There are two spreadsheets in the excel file. The first one is for calculating the Vo and the second brings all the data from the entire class to plot a Lineweaver-Burk plot.
   DO NOT MAKE CHANGES TO ANYTHING OTHER THAN THE YELLOW CELLS!
4. First, use your data from last week to calculate the six Vo values for your group.
   a. Open the tube-1.csv file in excel for your first reaction from last week.
   b. Copy the absorbance values and paste them into the yellow column on the Vo calculation sheet.
   c. Use only the linear part at the beginning of the reaction. You will therefore have to adjust the formula to use fewer cells. Adjust the formula by putting the cursor on cell F23. Then modify the values underlined here [INDEX(LINEST(C2:C14,A2:A14),1)]. Leave the rest of the formula alone!
   d. The slope of the line is equal to the initial velocity Vo and shows up automatically at F23.
   e. Enter the Vo value you get in the table below and then repeat the same procedure for your other reactions until you have calculated all the Vo values for your group.
5. Once all the Vo values have been determined, switch to the Lineweaver-Burk spreadsheet by clicking on the second tab at the bottom of the Excel program window.
6. Now fill in the yellow cells with the initial velocity Vo values you just calculated. Fill in all others with the appropriate Vo values from all the other groups in you class.
7. The Lineweaver-Burk plot is plotted automatically. The Km and Vmax values are also calculated automatically and show up in the cells L22 and L23.
8. You can also determine the type of IPTG inhibition by checking the Lineweaver-Burk plot and how the lines behave relative to each other.
<table>
<thead>
<tr>
<th>Tube #</th>
<th>Reaction Buffer (μl)</th>
<th>β−Galactosidase (μl)</th>
<th>ONPG (μl)</th>
<th>Inhibitor (IPTG) (μl)</th>
<th>Resulting $V_o$</th>
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Appendix
List of supplies for TAs

Laboratory #1 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. Spray bottle with Ethanol
2. A bag of 50 mL conical tubes (2 for each group)
3. 5 mL serological pipets (6)
4. 10 mL serological pipets. (6)
5. 6 pipet bulbs.
6. Waste containers for tips etc. (6)
7. 50 mL conical tube racks (6)
8. 1.5 mL tubes racks (6)
9. Microcentrifuge tubes (1 per group)
10. 6 sets of micro pipettors (P1000, P200, P20). 7th set of micro pipettors is for TA
11. 6 sets of micropipettor tips (P1000 and P200) Preferably the ART tips to avoid pipet contamination.
12. 6 each 25 mL of 1 M Tris stock solutions (for all lab sections)
13. 6 each 25 mL of 0.5M EDTA stock solutions (for all lab sections)
14. 6 each 25 mL of 10X TAE stock solutions (for all lab sections)
15. 6 sets of microfuge tubes with 1 mL water (labeled A)
16. 6 sets of microfuge tubes with 1 mL glycerol (labeled B)
17. 6 sets of microfuge tubes with 1 mL ethanol (labeled C)
18. 6 sets of microfuge tubes with 1 mL acetonitrile (labeled D)
19. You will use balance already in analytical labs

Laboratory #2 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. Spray bottle with Ethanol
2. Thermocycler
3. Microwave oven
4. Power supply for gel chambers
5. PCR tubes
6. 6 ice buckets with ice
7. PCR Master Premix
8. 6 sets of each of forward and reverse Primer
9. 6 sets of ladder for gel
10. powder agarose for gel
11. Waste containers for tips etc. (6)
12. 1.5 mL tubes racks (6)
13. Microcentrifuge tubes
14. 6 sets of micro pipettors (P1000, P200, P20). 7th set of micro pipettors is for TA
15. 6 sets of micropipettor tips (P1000 and P200) Preferably the ART tips to avoid pipet contamination.
16. P10 micro pipettors (2) and tips.
Laboratory #3 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. Spray bottle with Ethanol
2. UV Spectrophotometer (use the one in analytical lab)
3. 3 microcentrifuges (2 new Eppendorf and one old BioRad)
4. Spin column kits for DNA purification (including spin columns and all associated buffers)
5. Waste containers for tips etc. (6)
6. 1.5 mL tubes racks (6)
7. 37°C heat block
8. Microcentrifuge tubes
9. 6 sets of micro pipettors (P1000, P200, P20). 7th set of micro pipettors is for TA
10. 6 sets of micropipettor tips (P1000 and P200) Preferably the ART tips to avoid pipet contamination.
11. P10 micro pipettors (2) and tips.

Laboratory #4 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. Spray bottle with Ethanol
2. Waste containers for tips etc. (6)
3. UV Spectrophotometer (use the one in analytical lab)
4. 3 microcentrifuges (2 new Eppendorf and one old BioRad)
5. 65°C heat block
6. 6 ice buckets with ice
7. 37°C incubator (already in that room)
8. 50 mL conical tube racks (6)
9. 1.5 mL tubes racks (6)
10. Bag of 1.5 mL Eppendorf tubes
11. 6 sets of micro pipettors (P1000, P200, P20). 7th set of micro pipettors is for TA
12. 6 sets of micropipettor tips (P1000 and P200) Preferably the ART tips to avoid pipet contamination.
13. Methacrylate 1.5 mL cuvettes
14. 42-degree water bath
15. 12 LB + 50µg/mL Kanamycin plates per lab section
16. 6 sets of SOC media
17. 6 sets of bacteria speaders.
18. 10X buffer for ligation reaction
19. T4 DNA ligase (6 microliters per lab section)
20. Gas burner and cell spreader for plating cells
Laboratory #5 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

3. Lysis Buffer

<table>
<thead>
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<th>Lysis Buffer</th>
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<tr>
<td>10 mM HEPES pH 7.5</td>
<td>10 mL of 1 M</td>
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<tr>
<td>50 mM EDTA</td>
<td>100 mL of 0.5 M</td>
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<tr>
<td>0.02 % Sodium Azide</td>
<td>1 mL of 20% Stock</td>
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</table>

4. Lysozyme and DNAse solutions
5. Liquid nitrogen for cell freezing
6. 1.5 mL tubes racks (6)
7. Bag of 1.5 mL Eppendorf tubes
8. 6 ice buckets with ice
9. 6 sets of micro pipettors (P1000, P200, P20). 7th set of micro pipettors is for TA
10. 6 sets of micropipettor tips (P1000 and P200). Preferably the ART tips to avoid pipet contamination.
11. 3 microcentrifuges (2 new Eppendorf and one old BioRad)
12. Spray bottle with Ethanol
13. Waste containers for tips etc. (6)

Laboratory #6 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. AKTA Start Chromatography system
2. Chromatography buffers A and B

<table>
<thead>
<tr>
<th>Chromatography Buffer A</th>
<th>For 500 mL</th>
<th>Chromatography Buffer B</th>
<th>For 500 mL</th>
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</thead>
<tbody>
<tr>
<td>10 mM Hepes</td>
<td>5 ml of 1 M</td>
<td>10 mM HEPES Buffer</td>
<td>5 ml of 1 M</td>
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<tr>
<td>5 mM EDTA</td>
<td>5 ml of 0.5 M</td>
<td>5 mM EDTA</td>
<td>5 ml of 0.5 M</td>
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<tr>
<td>No NaCl</td>
<td>0 ml of 5 M</td>
<td>1 M NaCl</td>
<td>100 ml of 5 M</td>
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</table>

3. Student protein samples from previous week
4. Ice bucket with ice
5. Spray bottle with Ethanol
6. Waste containers for tips etc. (6)
Laboratory #7 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. Precast 4-20% gels (6, one for each group)
2. Green clamp to pry open the precast gels after they are run
3. Protein sample (6, one for each group)
4. Electrophoresis apparatus (3, 2 groups per apparatus)
5. Sample buffer (6, one for each group)
6. Running buffer (6, one for each group)
7. P20 Pipettor and tips
8. Electrophoresis tank and dam (6, one for each group)
9. Heat block set to 98 °C
10. Spray bottle with Ethanol
11. Waste containers for tips etc. (6)

Laboratory #8 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. Spray bottle with Ethanol
2. Waste containers for tips etc. (6)
3. BSA standard (aliquoted for each of 6 groups per section).
4. Reagent A and B for the BCA assay (aliquoted for each of 6 groups per section)
5. Cuvettes for the spectrophotometer (2 for each of 6 groups per section)
6. A portable PASCO wireless spectrophotometer (6)
7. Pipettors and tips (6 sets including 6 P10 pipettors)
8. 15 microcentrifuge tubes and a tube rack (6 sets for each of 6 groups)
9. A bottle of deionized water (one per group, 6 total per section)
10. One 50 ml conical tube (6 total per section)

Laboratory #9 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. Spray bottle with Ethanol
2. Waste containers for tips etc. (6)
3. Portable PASCO wireless spectrophotometers
4. Purified β-Galactosidase enzyme (0.08 mg/ml)
5. ONPG (1 mg/ml (3.3mM)) supplied as a 10 X stock
6. Reaction buffer (10mM HEPES pH 7.5, 50 mM NaCl, 5 mM EDTA, 10mM MgCl₂)
7. Pipettors and tips
8. Microcentrifuge tubes and tube racks
9. Spectrophotometer cuvettes (one each for negative control and experimental).
Laboratory #10 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. Spray bottle with Ethanol
2. Waste containers for tips etc. (6)
3. Portable PASCO wireless spectrophotometers
4. Purified β-Galactosidase enzyme (0.08 mg/ml)
5. ONPG (10 mg/ml (33mM)) supplied as a 10 X stock
6. IPTG (10 mM, as inhibitor)
7. Reaction buffer (10mM HEPES pH 7.5, 50 mM NaCl, 5 mM EDTA, 10mM MgCl₂)
8. Pipettors and tips
9. Microcentrifuge tubes and tube racks
10. Spectrophotometer cuvettes (one box).

Laboratory #11 Required Supplies and Equipment

For TA: Supplies Needed per lab section of 18 students. 6 groups of 3

1. Nothing other than a projector and a computer to explain the spreadsheet to students.