

**CHM-4304L
BIOCHEMISTRY LAB**

LABORATORY MANUAL

**By
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**Florida International University
Department of Chemistry
Revised, spring 2009**

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**CHEM4304L Spring 2008
BIOCHEMISTRY LABORATORY**

Syllabus

Instructor: Dr. Fenfei Leng
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Office Hours T: 11:00 AM-12:00 PM

Scheduled Class Time: Tuesday or Wednesday 2:00-4:50 PM HLS275

Course Description:

This course is designed for advanced undergraduate students at chemistry department of Florida International University. Our aim is to provide the basic training in biochemical laboratory for our students.

Textbook:

Fundamental Laboratory Approaches for Biochemistry and Biotechnology by Alexander J. Ninfa and David P. Ballou.

Grading:

Your grade will depend on your experimental results, your lab reports, and your performance in each class. Your preparation of experiments, understanding of each experiment, and answers to the instructor's questions in the class will also contribute to your final grade.

The instructor's expectations for students during the semester: The students will be responsible for all materials covered in each lab and assigned in the book and lab reports. The students are expected to read the appropriate materials in the text before each laboratory. The key to success is hard work.

Learning Outcomes: The overall goal of this course is for students to understand the basic principles of biochemistry laboratories. To this end, the following major learning outcomes shall apply:

Students are expected to understand basic techniques in biochemistry.

Laboratory Procedure

A necessary prerequisite for professional performance in the laboratory is preparation. Since our schedules are very tight for every experiment, you have to make sure you have read the experimental and instrumental instructions before coming to the class. Please bring an outline how to make your solutions and perform your experiments to the class.

We have included a schedule in this manual. **You are expected to take all classes. Otherwise, you will be advised to drop the class.** However, some special emergency circumstances, for example illness when accompanied with a signed letter from a medical doctor stating explicitly that you are not able to take the class on that day for your own health reasons, will be considered. If you are not able to attend the class, please inform the instructor immediately so that an alternative can be arranged. **Since you are registering a formal class at FIU, it is your benefit to arrive prior to 2:00 PM. You are allowed to be late for two times. After the third time late, you will be advised to drop this class. You will not allow attending the class after 2:30 PM. please come to the class on time!**

All experimental results should be documented in your laboratory notebook, which will provide a solid basis for writing your lab report. The original data sheets must accompany your reports.

Important rules

- 1. Please clean the lab after each class. Your instructor will advise you for saving or disposing your solutions.**
- 2. Keep your work area clean. Your grades may be deducted if you keep littering your bench.**
- 3. You must have a lab notebook for writing everything in black or blue ink (no pencils). When correcting your writing, scratch the error with a single line and write the correct one above or below the line.**
- 4. Always wear a lab coat.**
- 5. Read the operating instructions for every instrument before you use it. If you have any question, please ask your instructor!**
- 6. Pay attention to the due date for each experiment. You will be penalized for late submitting your lab report.**
- 7. You should finish your own lab report. Do not copy old lab reports, or copy your classmate's report. Plagiarism is illegal and unethical at FIU. Any evidence of plagiarism in this class will result in your failure in the class. Further disciplinary actions will be taken upon your instructor's decision.**

Laboratory Reports

You are required for submitting a lab report for each experiment. Reports are due after one week from the date you have performed your experiment. There will be a 10% grade deduction upon your late submission. I recommend you use a word processor such as Microsoft Word for typing your reports; however, you can neatly handwrite your lab reports. I also strongly recommend you use a graphic program to plot your graphs, in which all axes should be labeled and the proper units are displayed.

Your lab reports should consist of the following sections:

1. The title of the laboratory report: this includes the title of your report, the course name and number, and your name and the date.
2. Abstract section: the abstract will be a brief description of your experiment and the results, and should be kept under 200 words.
3. Introduction section: Introduction should be a succinct statement of the state of knowledge of the project at the time of its inception, the purpose of the research, and the approach taken. The basis of the project and the major conclusion may also be included
4. Materials and Methods: this section should describe the methods in detail, and where you obtained your materials.
5. Results section: this section presents your results that were obtained in your experiments. Your results are often shown as graphs and tables. Calculations and errors are required to be shown and explained.
6. Discussion: in this section, you should discuss your findings and compare your results with the previous one if available. You can discuss the theory that supports your results or is against your conclusion. Finally, you can suggest possible improvements and present your major conclusion.
7. References cited: include any references you have cited as follows:
Samul, R. and Leng, F. (2007) *J. Mol. Biol.* 374, 925-935.

Common Used Biochemical Data

Nucleic Acid Data

Average weight of a DNA base pair (sodium salt) = 650 daltons

1.0 A_{260} unit ds DNA = 50 $\mu\text{g/ml}$ = 0.15 mM (in nucleotides)

1.0 A_{260} unit ss DNA = 33 $\mu\text{g/ml}$ = 0.10 mM (in nucleotides)

1.0 A_{260} unit ss RNA = 33 $\mu\text{g/ml}$ = 0.10 mM (in nucleotides)

MW of a ds DNA molecule = (# of base pairs) X (650 daltons/ base pair)

1 μg of 1000 bp DNA = 1.52 pmol = 9.1×10^{11} molecules

1 μg of pBR322 DNA = 0.35 pmol = 2.1×10^{11} molecules

1 μg of l DNA = 0.03 pmol = 1.8×10^{10} molecules

Agarose gel resolution

% Gel	Optimum Resolution for linear DNA (kb)
0.5	30 to 1.0
0.7	12 to 0.8
1.0	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

Polyacrylamide gel resolution

% Gel	Optimum Resolution for linear DNA (bp)
3.5	1000-2000
5.0	80-500
8.0	60-400
12.0	40-200
15.0	25-150
20.0	6-100

Linear Range of Separation for protein (SDS-PAGE) (kD)

15	10-43
12	12-60
10	20-80
7.5	36-94
5.0	57-212

Tentative Laboratory Schedule

Date	Lab
January 6 or 7, 2009	Organization Meeting
January 13 or 14, 2009	Lab 1: Basics: pipetting, buffers, and dilutions.
January 20 or 21, 2009	Lab 2: Purification of plasmid DNA from <i>E. coli</i> cells and determination of their concentration.
January 27 or 28, 2009	Lab 3: Digestion of plasmid DNA by restriction enzymes and DNA electrophoresis.
February 3 or 4, 2009	No class
February 10 or 11, 2009	Lab 4: Polymerase Chain Reaction Technology.
February 17 or 18, 2009	Lab 5: Determination of protein concentrations.
February 24 or 25, 2009	Lab 6: Protein electrophoresis.
March 3 or 4, 2009	No Class
March 10 or 11, 2009	No Class
March 17 or 18, 2009	Spring Break
March 24 or 25, 2009	Lab 7: Ligand binding: optical titrations.
March 31 or April 1, 2009	Lab 8: Enzyme kinetics.

Biological Chemistry Lab 1

Basics: pipetting, buffers, and dilutions

(Chapter 1)

Objectives

1. To learn how to use different types of pipettes (serological, Mohr, volumetric, and Pasteur pipettes) and micropipettor (pipetman);
2. To make the following solutions:
 - a) 100 mM Tris-HCl (pH 8.0) (100 ml)
 - b) 100 mM EDTA (pH 8.0) (50 ml)
 - c) TE (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0))
 - d) 5 x TBE (0.45 M Tris-Borate and 10 mM EDTA) (10.8 g Tris base, 5.5 g boric acid, and 20 ml of 100 mM EDTA) (200 ml)
3. To review Henderson-Hasselbalch equation:
$$\text{pH} = \text{pK}_a' + \log \frac{[\text{R}^-]}{[\text{RH}]}$$
4. To learn how to dilute a solution for biochemistry. Diluting 100 mM Tris-HCl (pH 8.0) into 10 mM and 1 mM solutions, and measure their pH; Using micropipettor to dilute 100 mM Tris-HCl (pH 8.0).
5. To learn how to write a Laboratory Report (Title/Abstract/Introduction/Materials and Methods/Results/Discussion).

Introduction

There are two parts to this laboratory. The first part involves the use of micropipettors and the second part involves the use of pH meters. Please read pages 14 to 23 in the textbook before the laboratory session. Pages 47-48 of the textbook will be helpful when it comes to writing up the laboratory report.

Experimental procedure

1) Pipettes

Micropipettors

The first portion of this lab involves becoming familiar with the use of micropipettors. Micropipettors accurately measure volumes ranging from less than 1 microliter to greater than 1 milliliter, the types that you will use measure from 0.1-2.5 microliters (P-2.5), 1-20 microliters (P-20), 20-200 microliters (P-200) and 100-1000 microliters (P-1000). The disposable tips that fit on the end of the micropipettors are sometimes colored yellow and blue. The yellow tips (which are white for our experiments but are sometimes colored yellow) are for volumes of 1-200 microliters and fit on the ends of the P-20 and P-200 micropipettors but not the P-1000 micropipettor. The blue tips (which are white for our experiments but are sometimes colored blue) are for volumes of 100-1000 microliters and fit on the end of the P-1000 micropipettor.

The following steps describe the use of the micropipettor. Adjust the micropipettor volume to the required setting. Press the tip on firmly with a slight twisting motion to ensure an airtight seal. The plunger on the top of the micropipettor is then depressed to the first point of resistance and while holding the micropipettor vertical the tip is placed just below the surface of the appropriate solution (1-2 mm for yellow and 2-4 mm for blue). The plunger is then slowly released and the solution is sucked up into the pipet tip, wait 2 seconds to ensure that the full volume has been drawn up into the tip. Once the plunger has been released the tip is removed from the solution (any excess liquid should be wiped from the side of the tip but keep the cloth away from the end) and transferred to the receptacle where the tip is placed on the side of the receptacle and the plunger is once again depressed to the point of first resistance, wait, then continue on past the point of first resistance (overshoot). With the plunger fully depressed withdraw the micropipettor from the vessel allowing the tip to slide along the wall of the vessel. Allow the plunger to return to the top position. Normally the tip would then be ejected by pressing the tip ejector button to prevent cross contamination of samples but for our purposes in this exercise it should only be necessary a few times. Attention should be given to the speed and smoothness during depression and release of the button, pressure on the button at the first stop, immersion

depths (if the depth is incorrect bubbles may get into the sample), and minimal angle from the vertical axis.

Please try the above procedure a few times with each of the micropipettors using water as the solution to gain practice. Once the technique has been mastered, take your solution of water over to the balances and pipet out five times each of the following volumes in any order (30 times in all). Change the tip only when you change the volume.

- i) 5.0 microliters with the 1-20 microliter micropipetter
- ii) 20.0 microliters with the 1-20 microliter micropipetter
- iii) 20.0 microliters with the 20-200 microliter micropipetter
- iv) 100.0 microliters with the 20-200 microliter micropipetter
- v) 100.0 microliters with the 100-1000 microliter micropipetter
- vi) 1000.0 microliters

Record the weight of water added each time. *A statistical analysis for mean and standard deviation should then be performed on each set of results. In addition, you should indicate the relative error and the relative standard deviation. Also, indicate which micropipetter would be best for 20 microliters and 100 microliters.*

Volumetric and serological pipets

Volumetric pipets are calibrated to deliver a specific volume of solution at a certain temperature and have a single line with no scale. With a rubber bulb (DO NOT USE YOUR MOUTH) draw liquid to a level 2-3 cm above the line, wipe the tip with a kimwipe (not necessary for your practices), touch the tip of the pipet on the inside wall of the container from which it was filled. Release liquid until the meniscus is directly on the fill line. Transfer the pipet to the inside of the second container and release the liquid, when the flow stops wait 5-10 seconds then touch the tip to the side of the vesicle. The pipet should then be removed from the container. The pipet is TD (“to deliver”) and therefore any remaining liquid should not be blown out of the pipet.

Fractional volumes are transferred with graduated pipets, one example being a serological pipet. The procedure is the same as the volumetric pipet except that the solution starts at the desired amount and the pipet is blown out at the end.

Please try the above procedure a number of times with each of the pipets using water as the solution to gain practice. Once the technique has been mastered, take your solution of water over to the balances and pipet out five times:

- vii) 10.000 mL with the volumetric pipet
- viii) 10.000 mL with the serological pipet

Record the weight of water added each time. *A statistical analysis for mean and standard deviation should then be performed on each set of results. In addition, you should indicate the relative error and the relative standard deviation. Also, indicate which pipet would be best for 10 mL.*

2) Buffers

How to Make a Buffer

To prepare a buffer solution based upon an acid such as acetic acid or phosphoric acid add a strong base such as sodium hydroxide to a solution of the acidic form of the buffer until the desired pH is reached. To prepare a buffer solution based upon a base such as Tris or Imidazole add a strong acid such as HCl to a solution of the basic form of the buffer until the desired pH is reached. For preparation of a Tris buffer solution, the amount of Tris necessary to generate a given volume of buffer solution at a certain concentration is calculated i.e. 1 L of a 100 mM buffer solution would require 0.1 moles of Tris. The calculated amount of Tris is then dissolved in distilled water, the volume of water used should be about 80% of the given volume of buffer solution, i.e. 800 mL of water for the example above. A pH meter is then placed in the solution and the pH of the stirred solution is adjusted to the desired value with acid, usually 1 N or concentrated HCl for Tris. The solution is then topped off with distilled water to the final volume, 1 L for the example above. If small quantities of buffer solution are to be used it is sometimes desirable to make a bit extra due to the increased ease of making the solution, standard pH meters work best with a volume of greater than 10 mL. In addition, when making concentrated buffer

solutions, especially for Tris, make sure the solution is close to room temperature before topping off with distilled water.

i) Please make the following solutions:

a) 100 mM Tris-HCl (pH 8.0) (100 ml)

b) 100 mM EDTA (pH 8.0) (50 ml)

c) TE (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)) (100 mL)

d) 5 x TBE (0.45 M Tris-Borate and 10 mM EDTA) (10.8 g Tris base, 5.5 g boric acid, and 20 ml of 100 mM EDTA) (200 mL)

Before the lab calculate how much Tris (MW 121.1), and EDTA (MW 372.2) you will need.

ii) The Henderson-Hasselbalch equation describes how the ratio of acid to conjugate base alters the pH of the solution.

$$\text{pH} = \text{pK}_a' + \log \frac{[\text{R}^-]}{[\text{RH}]}$$

Several practical issues can be derived from the equation. As the pH approach the pK_a of the buffer it requires more and more acid or base to alter the pH. As you move away from the pK_a of the buffer it requires less and less acid or base to alter the pH. The best buffer has a pK_a value similar to the pH of the solution. **In your laboratory report**, calculate the change in pH when 5 mL of a 1 M HCl solution is added to 1 L of a 100 mM Tris solution at pH 8.10, $\text{pK}_a = 8.10$. Also, calculate the change in pH when 5 mL of 1 M HCl is added to 1L of a 100 mM Tris solution at pH 7.10, $\text{pK}_a = 8.10$. One drawback with Tris is that it changes its pH with temperature so be careful. A 50 mM pH 8.0 solution of Tris at 25 °C is pH 8.58 at 5 °C.

iii) The Henderson-Hasselbalch equation also suggests that as you dilute the buffer the pH will not change. Try diluting 100 mM Tris-HCl (pH 8.0) into 10 mM and 1 mM solutions, and measure their pH; Using micropipetter to dilute 100 mM Tris-HCl (pH 8.0). Remember $M_1V_1 = M_2V_2$. **Before the lab** calculate the necessary volume of water required to dilute 10 mL of a 100 mM Tris-HCl solution to a concentration of 10 mM Tris-HCl and to dilute 1 mL of a 100 mM Tris-HCl solution to a concentration of 1 mM Tris-HCl.

Instructions for Calibrating and Using the pH Meters (the instruction of the new pH meters may be different from this instruction)

Press the mode function until the display reads pH.

Press the Calibration function

Press the right side arrow next to the display (Because you want the two point calibration)

Take the electrode out of the water and dry with a Kim-Wipe.

Immerse the electrode in the pH 7 buffer and press the read function.

Press the read function a second time to fix the pH at 7.

When the display reads “read” take the electrode out of the pH 7 buffer, rinse with deionized water, dry with a Kim-Wipe and immerse it in the pH 4 buffer solution. Press the read function and wait till the pH gets as close to four as possible.

At this point, press read again to fix it at four and wait till the pH meter beeps (the display should read a number between 53 and 59.)

Remove the electrode, rinse with deionized water and dry with a Kim-Wipe. Immerse it in the solution to be tested. Press the read function once to obtain the reading.

Check that the decimal point of the displayed pH is blinking throughout the titration pH readings.

If it is not, the pH readings have been locked and you are reading the same pH throughout. To get out of that mode, press the read function one more time and it should start blinking.

Please write a lab report.

Biological Chemistry Lab 2

Purification of plasmid pBR322 DNA from *E. coli* cells and estimates its concentration from the gel

Objectives

To learn how to purify plasmid DNA from *E. coli* cells by using a commercial kit.

Introduction

The purification of plasmid DNA is an important part of molecular biology. In Chapter 11 of your textbook the use of plasmid DNA is discussed. Experiment 11-4 describes an alternate procedure for the purification of plasmid DNA. In your laboratory report discuss the advantages of your procedure? In molecular biology the use of purchased kits is very popular. Kits in general provide step-by-step procedures, are simple to use, and require minimal effort and thought on the part of the practitioner. In this experiment, we will use a Qiagen kit to purify plasmid pBR322. You can find out more about the kit at the company's website: <http://www1.qiagen.com/default.aspx>. Please pay special attention to pages 22-24 which you should read before the lab. A 1% agarose gel will then be run to determine whether you have obtained your plasmid or not. In the next lab, you will learn how to prepare the 1% agarose gel.

Materials:

1. *E. coli* cells containing plasmids pBR322;
2. Buffer P1, P2, N3, PE and EB from Qiagen (a biotech company);
3. QIAprep Spin Columns;
4. Buffer TE;

Experiment Procedure:

1. Centrifuge *E. coli* cells for 2-3 minutes at 13,000 rpm. A pellet will be formed in the bottom of your microfuge tubes;
2. Resuspend pelleted bacterial cells in 250 μ l of buffer P1;
3. Add 250 μ l of P2 and gently invert the tube 4-6 times to mix;
Mix gently by inverting the tube. Do not vortex. Finish that step within 5 min.
4. Add 350 μ l of buffer N3 and invert the tube immediately but gently 4-6 times;
5. Centrifuge for 10 min at 13,000 rpm;
6. Apply the supernatants from step 4 to the QIAprep column by pipetting;
7. Centrifuge 1 min. Discard the flow-through;
8. Wash QIAprep spin column by adding 0.5 ml of buffer PB and centrifuging 1 min. Discard the flow-through;
9. Wash QIAprep spin column by adding 0.75 ml of buffer PE and centrifuging 1 min.
10. Discard the flow-through, and centrifuge for an additional 2 min to remove residual wash buffer;
11. Place QIA prep column in a clean 1.5 ml microfuge tube. To elute DNA, add 50 μ l of buffer EB (10 mM Tris-Cl, pH 8.5), let stand for 2 min, and centrifuge for 2 min.
12. Run a 1% agarose gel to determine whether you have gotten your plasmids.
13. Estimate the DNA concentration from the intensity of the EB fluorescence in your gel (compare with the DNA marker, lambda DNA HindIII digest).
14. Save your plasmid DNA pBR322 for the next laboratory.
15. Write a lab report.

Biological Chemistry Lab 3

Digestion of plasmid DNA pBR322 by restriction enzymes and DNA electrophoresis

Objectives

The objective of this laboratory is to digest plasmid DNA pBR322 purified in lab 2 and analyze it by agarose gel electrophoresis.

Introduction

The digestion of plasmid DNA is an important tool of molecular biology. In Chapter 11 of your textbook the use of plasmid DNA is discussed.

Experimental procedures

1) Preparation of buffer

You will prepare some 5 x TBE buffer. The 5 x refers to the concentration being five times that of the standard concentration used.

5 x TBE contains 0.45 M Tris-Borate and 10 mM EDTA (10.8 g of Tris base, 5.5 g of boric acid, and 0.744 g of EDTA in 200 mL). The pH should be 8.2 and is adjusted with Tris or boric acid as necessary.

2) Preparation of 1% agarose gel

The 1% refers to the fact that the mixture contains 1 g of agarose per 100 mL. Please make up 50 mL containing 1% agarose and 1 x TBE. Think about this **before the lab**. You will need to calculate the appropriate amount of agarose, 5 x TBE, and water to use. This mixture is heated in a microwave oven to form a homogeneous solution and then cooled to 60 or 70 °C. Pour the gel into a gel tray with comb and wait 30 minutes for it to harden. Once the gel becomes hard you can run the gel in a gel apparatus. The percent agarose can be adjusted depending on the

size of the DNA. Small pieces of DNA work best with higher percentages of agarose. For very small pieces of DNA acrylamide gels are used, wait for the next lab.

3) Digestion of plasmid pBR322 (the same one you purified last lab) using restriction endonucleases. Restriction endonucleases cleave the DNA in very specific locations. Each restriction enzyme is supplied with one of about 4 buffers. If only one restriction enzyme is used then the optimum buffer is selected. In general restriction enzymes are used at 37 °C. See http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/default.asp for more information.

Digestion of pBR322 by Hind III.

- 5 µl of pBR322 (you purified in lab 2)
- 2 µl of Hind III
- 2 µl of 10 x Buffer (**NE buffer 2**)
- 11 µl of H₂O

Mix them well, and incubate at 37 °C for 30 min.

Digestion of pBR322 by Hinc II

- 5 µl of pBR322
- 2 µl of Hinc II
- 2 µl of 10 x Buffer (**NE buffer 3**)
- 11 µl of H₂O

Mix them well, and incubate at 37 °C for 30 min.

4) After the digestion the gels can be loaded and run.

- 7.5 µl reaction mixture
- 2 µl of Dye which contains glycerol to make the sample more dense than water

10.5 μ l of TE buffer (see lab #1)

5) Running of the gels will take some time but the results will be emailed to you. After the gels are run they will be stained with ethidium bromide, which fluoresces only when it is intercalated into DNA. Ethidium bromide thus allows you to detect the location of the DNA on the gel. Ethidium bromide in water is shaken with the gel for 30 minutes. Ethidium bromide is also a mutagen so be careful when you work with it or gels stained with it. The gel will also contain a lane with lambda DNA digested by Hind III. This gives linear DNA of 564; 2027; 2322; 4361; 6557; 9416; and 23130 bp (base pairs).

6) Analyze the data and plot log of the DNA sizes versus the migration distance of the band.

7) Write a lab report.

Biological Chemistry Lab 4

Polymerase Chain Reaction (PCR) Technology

Objectives

To understand polymerase chain reaction (PCR) technology and analyze the PCR product by polyacrylamide gel electrophoresis.

Introduction

The amplification of DNA in defined regions by PCR is very important in molecular biology. The use of PCR is described in Chapter 12 of your textbook. You will be amplifying a small region of DNA (the mouse *hmga2* gene, ~330 bp) using the two primers below. In your laboratory report you should discuss what the primers do, how they work, and why it is important to use a thermally stable polymerase such as Taq.

Materials:

1. 10 x PCR buffer containing Mg^{2+} ;
2. Template DNA pMGM1;
3. Primers:
 - 5'-CATATGAGCGCACGCGGTGAGGG-3' forward primer
 - 5'-CTCGAGCTAATCCTCCTCTGCGGA-3' reverse primer;
4. dNTP;
5. Taq DNA polymerase.

Experimental Procedure:

1. Set up a 50 μ l PCR reaction contain the following components:

- 5 μ l 10 x PCR buffer;
- 4 μ l 25 mM $MgCl_2$;
- 1 μ l template DNA (10 ng);
- 1 μ l dNTP (10 nmol);
- 1 μ l forward primer (20 pmol);
- 1 μ l reverse primer (20 pmol);
- 0.5 μ l Taq DNA polymerase;
- 36.5 μ l H_2O ;

The PCR reaction is usually set up in a thin walled microcentrifuge tube. The thin wall allows the transfer of heat from the heat block (thermal cycler) to the liquid inside to occur rapidly. The PCR reaction contains each of the four deoxynucleoside triphosphates (dNTPs: dGTP, dATP, dTTP, dCTP), $MgCl_2$ (required by the DNA polymerase; usually 2 mM), the DNA polymerase (Taq), template DNA (the DNA that is being amplified), primers (define the region to be amplified), and a buffer. The DNA polymerase Taq comes from a thermophile, an organism that likes hot temperatures, even warmer than those in Florida.

2. Your instructor will put your samples to a thermal cycler to amplify your DNA. We will use the following program:

- Denaturation: 96 $^{\circ}C$, 15 s
- Annealing: 55 $^{\circ}C$, 30 s
- Extension: 72 $^{\circ}C$, 90 s

18 to 20 cycles will be run; then a final extension at 72 $^{\circ}C$ for 5 min will be performed.

(The thermal cycler rapidly changes temperature so that the DNA alternately denatures, anneals, and then extends. This cycle of denaturation, annealing, and extension is repeated 18 to 20 times).

3. Run a 6% polyacrylamide gel to analyze your sample.

Polyacrylamide gels are used to analyze shorter pieces of DNA and have been used to sequence DNA. Polyacrylamide gels are made by polymerizing acrylamide with the crosslinking agent bisacrylamide. Acrylamide purchased for molecular biological application, normally already contains the bisacrylamide, although in this case you will need to add it. **Please note that acrylamide and bisacrylamide are toxic.** Polyacrylamide is much more benign. The polymerization reaction is a radical reaction initiated by ammonium persulfate and TEMED.

Please make 50 mL of a 30% acrylamide/bis-acrylamide solution in water. The weight ratio is 29 parts acrylamide: 1 part bisacrylamide. Once everything has dissolved filter the solution and then store it at 4°C in a brown glass bottle.

The 10% Ammonium Persulfate (APS) solution will be prepared fresh for you.

Stock solutions: 30% acrylamide, 10% Ammonium Persulfate (APS), 5 x TBE, and TEMED;
60 ml of 6% polyacrylamide gel:

35.5 ml of H₂O
12 ml of 30% acrylamide
12 ml of 5 x TBE
480 µl of APS
20 µl of TEMED

First assemble the gel caster, and then add the 6% gel mix to gel caster. After add the comb to the gel, you should wait until gel is fully polymerized (about 30 to 60 min). The gel can then be loaded. The running buffer is 1 x TBE.

2 µl reaction mixture
2 µl dye
16 µl TE buffer (see first lab)

The gels are then visualized using ethidium bromide and UV light.

Please write a lab report

Biological Chemistry Lab 5

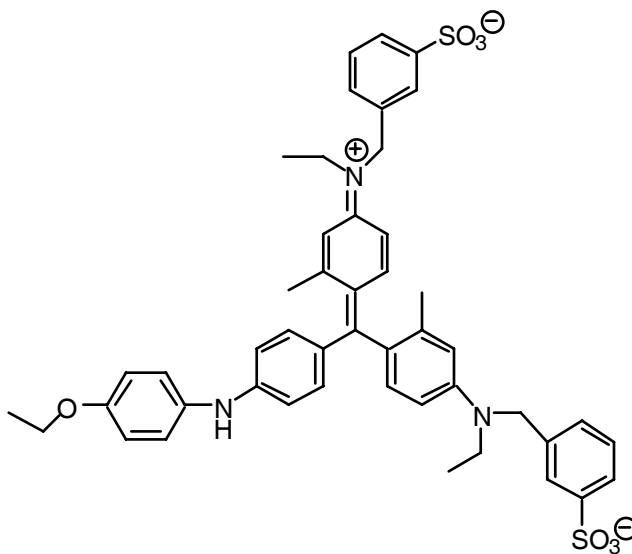
Determination of protein concentration

Objectives

To determine the concentration of an unknown protein.

Introduction

The first step to characterize a new protein is to determine its concentration. There are several popular assays for determination of protein concentration. In this laboratory, we will use one commonly used method, the Bradford assay to determine an unknown protein's concentration. The Bradford Assay involves calibrating the Bradford protein assay using the protein bovine gamma globulin (sometimes bovine serum albumin or lysozyme is also used). Each of you will then determine the protein concentration of your protein solution. **BE CAREFUL** the Coomassie Brilliant Blue G will bind just as well to your proteinaceous skin or lab coat and it is not easy to get off. The solution is also quite acidic. The UV spectroscopy requires an extinction coefficient to be determined.



Coomassie Brilliant Blue

(Bradford Reagent for protein determination may be used to determine the concentration of proteins. The protein-dye complex causes a shift in the dye absorption maximum from 465 to 596 nm. The amount of absorption produced is proportional to the protein concentration. Sigma Bradford Reagent requires no dilution and is suitable for both standard and micro assays. The analysis gives linear response from 1 μg to 140 μg based on Bovine Serum Albumin (BSA). *We will use IgG instead of BSA*).

Materials:

1. Diluted protein assay dye reagent from Bio-Rad (Coomassie Brilliant Blue G-250 dye, Phosphoric Acid, and methanol). This solution also is called as Bradford solution;
2. 0.25, 0.5, 0.75, 1.0, and 1.5 mg/ml of bovine gamma globulin (IgG);
3. TE buffer.
4. two protein samples with unknown concentration.

Experimental Procedure

1. Pipet 40 μl of 0.25, 0.5, 0.75, 1.0, and 1.5 mg/ml IgG into five different test tubes, and add 2.0 ml of diluted Bradford solution; Gently mix these solutions well.
2. Incubate at room temperature for at least 5-60 min, and measure absorbance at 595 nm as quick as possible using **plastic curves**.
3. The above measurements will be served as your standards, and you should generate a standard curve (Plot the absorbance vs. concentration of the standard protein solutions).
4. Add 40 μl of your samples to tested tubes, and add 2.0 ml of diluted Bradford solution. Incubate at room temperature for at least 5 min, and measure absorbance at 595 nm; From the standard curve, you should get the concentrations of your protein (RNase H);

NOTES:

1. The assay is relatively reliable in the presence of interfering substances (see reference #1.) Results are minimally affected by the presence of detergents (SDS, Triton X-100), buffer salts - and are probably not affected by guanidium chloride.

2. Bradford also reports that the dye reagent tends to stain quartz cuvettes, so glass or plastic cells should be used.

Please write a lab report.

Biological Chemistry Lab 6

Protein Electrophoresis (SDS-PAGE)

Objectives

To be familiar with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins and determine molecular weight of RNase H you purified.

Introduction

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used in biochemistry and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein folding, posttranslational modifications and other factors).

The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non-disulfide-linked tertiary structures and dithiothreitol (DTT) or 2-mercaptoethanol (beta-Mercaptoethanol/BME) which reduces the disulfide bonds, and applies a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in folding, as differences in folding patterns would cause some proteins to better fit through the gel matrix than others. Adding SDS solves this problem, as it linearizes the proteins so that they may be separated strictly by length (primary structure, or number of amino acids). The SDS binds to the protein in a ratio of approximately 1.4 g SDS per 1.0 g protein (although binding ratios can vary from 1.1-2.2 g SDS/g protein), giving an approximately uniform mass:charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. A tracking dye may be added to the protein solution to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run.

The denatured proteins are subsequently applied to one end of a layer of polyacrylamide gel submerged in a suitable buffer. An electric current is applied across the gel, causing the negatively-charged proteins to migrate across the gel. Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty. After a set amount of time (usually a few hours—though this depends on the voltage applied across the gel; higher voltages run faster but tend to produce somewhat poorer resolution), the proteins will have differentially migrated based on their size; smaller proteins will have traveled farther down the gel, while larger ones will have remained closer to the point of origin. Thus proteins may be separated roughly according to size (and therefore, molecular weight). Following electrophoresis, the gel may be stained (most commonly with Coomassie Brilliant Blue or silver stain), allowing visualisation of the separated proteins, or processed further (e.g. Western blot). After staining, different proteins will appear as distinct bands within the gel. It is common to run "marker proteins" of known molecular weight in a separate lane in the gel, in order to calibrate the gel and determine the weight of unknown proteins by comparing the distance traveled relative to the marker.

Gel electrophoresis is usually the first choice as an assay of protein purity due to its reliability and ease. The presence of SDS and the denaturing step causes proteins to be separated solely based on size.

Materials:

1. 30% Acrylamide/bis (29:1) solution;
2. 4 x Resolving gel buffer: 1.5 M Tris-HCl (pH 8.8), 0.4% SDS;
3. 4 x Stacking gel buffer: 0.5 M Tris-HCl (pH 6.8), 0.4% SDS;
4. 1 x Electrophoresis buffer: 25 mM Tris-HCl (pH 8.3), 250 mM glycine, 0.1% SDS.
5. 3 x protein loading buffer: 0.19 M Tris-HCl (pH 8.0), 3% glycerol, 6% SDS, 300 mM DTT, and 0.25% bromophenol blue;
6. 10% Ammonium persulfate (APS);
7. TEMED;
8. Gel staining buffer: 1 g of Coomassie Brilliant Blue dissolved in 400 ml of solution containing 45% methanol, 45% H₂O, and 10% acetic acid;

9. Destaining buffer: 25 % methanol, 10% acetic acid, and 65% H₂O;
10. Gel Drying Buffer: 40% ethanol, 4% glycerol, and 56% H₂O.
11. Protein samples.
12. Protein standards.

Experimental Procedure:

1. Prepare 40 ml of 15% resolving gel solution as following:

- 9.68 ml of H₂O
- 20 ml of 30% Acrylamide/bis solution
- 10 ml of 4 x resolving gel buffer
- 300 µl of 10% APS
- 20 µl of TEMED

First assemble the gel caster, and add the 15% gel mix to gel caster. Overlay the solution gently with water or 1-butanol saturated with H₂O, and allow to be polymerized. You should see the gel-H₂O interface disappear and then reappear when the gel is polymerized;

2. Prepare 20 ml of 5% stacking gel solution as following:

- 11.5 ml of H₂O
- 3.3 ml of 30% Acrylamide/bis solution
- 5 ml of 4 x stacking gel buffer
- 200 µl of 10% APS
- 10 µl of TEMED

Add the gel mix to the gel caster, and insert the comb to the gel solution; you should wait until gel is fully polymerized. Once the gel is polymerized, the comb can be removed gently. Then assemble the gel into the electrophoresis apparatus.

3. Load your samples:

Mix two volumes of your samples with 1 volume of 3 x sample loading buffer, and heat the samples over 90 °C for 5 min, and load 20 µl of your samples to each well of the gel. Also add the protein standards in two lanes on each gel.

4. Run the gels:

Apply power at 100-150 V until the dye has reached the top of the resolving gel, and then increase the power into 200 V. When the dye reaches the bottom of the resolving gel, turn off the power supplier. Disassemble the gel apparatus, and stain the gel for about one hour at Gel staining buffer. Destain your gel at the destaining buffer until the protein bands are clearly seen in the gel, and dry the gel if it is possible.

5. Determination of molecular weight of the protein provided in your class:

Measure the migration of proteins and the dye, and calculate the mobility of each protein by using the following equation. Plot the mobility versus log value of molecular weight of the stand proteins, and then determine the molecular weight of the protein sample:

Mobility = (Distance of the protein migrated)/ Distance of the dye migrated)

Please write a lab report

Biological Chemistry Lab 7

Ligand Binding: Optical titrations to determine the DNA-binding constant of ethidium bromide

Objectives

To study ethidium binding to herring sperm DNA by visible spectroscopy, and extract the binding parameters from the experimental data.

Introduction

DNA is the main genetic information carrier for most organisms. Ligand-DNA interaction is critical for many biological processes. Because of its importance, we may use this property to design different drugs. For example, one strategy to treat cancer patients is to bind a small chemical to DNA molecule to block these biological processes such as transcription, DNA replication and recombination. Among them, DNA intercalators are very potent to certain cancers. These aromatic compounds intercalate into DNA base pairs and therefore block DNA replication and transcription. The thermodynamic driving force for intercalation is due to the system minimizing the number of interactions between the hydrophobic drug and water by forcing the drug into a region where no water is found, i.e., the stacked bases of DNA.

In this experiment you will determine the binding constant of a dye, ethidium bromide, toward herring testes DNA using absorption spectroscopy. The method involves titrating DNA into a constant concentration of ethidium bromide. By varying the DNA concentration over six orders of magnitude, it is possible to have almost totally unbound DNA and DNA which is saturated with ethidium. With the value of the molar extinction of bound and unbound ethidium at a particular wavelength and the total concentrations of DNA and ethidium in solution, the concentration of bound ethidium can be calculated from absorbance measurements. This information and the expressions below will allow calculation of the binding constant of ethidium toward DNA.

Materials

1. Herring sperm DNA solution (1.84 mM);
2. 3 mM Ethidium;
3. 10 x TE;
4. H₂O.

Experimental Procedure

1. Prepare 1 ml of 90 μM Ethidium in TE buffer:

100 μl of 10 x TE, 30 μl of 3 mM Ethidium, 870 μl of H₂O

2. Centrifuge your sample for a few seconds, and your instructor will put your samples to a Vis spectrophotometer to measure the absorbance at 480 nm, and then add 4 μl of your DNA stock solution to the EB solution until the absorbance of your solution at 480 nm is not changed.
3. Calculate your C_{bound}, C_{free} and r according your absorbance and extinction coefficients (ε_{480nm}), and determine the binding constant (K), binding site size (n) from a Scatchard plot (r/C_{free} vs r).

$$\begin{aligned}\mathbf{Abs} &= \epsilon_{\text{free}} * C_{\text{free}} + \epsilon_{\text{bound}} * C_{\text{bound}} \\ &= \epsilon_{\text{free}} * (C_{\text{total}} - C_{\text{bound}}) + \epsilon_{\text{bound}} * C_{\text{bound}} \\ &= \epsilon_{\text{free}} * C_{\text{total}} + (\epsilon_{\text{bound}} - \epsilon_{\text{free}}) * C_{\text{bound}}\end{aligned}$$

$$\mathbf{r} = C_{\text{bound}} / [\text{DNA}]$$

$$\mathbf{r} / C_{\text{free}} = k(n-r)$$

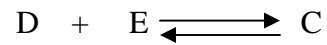
Free ethidium

$$\epsilon_{480\text{nm}} = 5600 \text{ cm}^{-1}\text{M}^{-1}$$

DNA-bound ethidium

$$\epsilon_{480\text{nm}} = 2497 \text{ cm}^{-1}\text{M}^{-1}$$

Scatchard equation may be derived according to the following equilibrium:



D = DNA

E = Ethidium bromide

C = Complex

$$K = \frac{[\text{C}]}{[\text{D}] [\text{E}]} \quad (1)$$

Known information:

Total DNA concentration = [DNA]

Total ethidium bromide concentration = C_{total}

Please write a lab report

Biological Chemistry Lab 8

Enzyme Kinetics (Chapter 8)

Objectives

To determine the Michaelis constant (K_m) and the maximal velocity (V_{max}) of alkaline phosphatase.

Introductions

Enzyme kinetics is the study of the rates of chemical reactions catalysed by enzymes. The study of an enzyme's kinetics provides insights into the catalytic mechanism of this enzyme, its role in metabolism, how its activity is controlled in the cell and how drugs and poisons can inhibit its activity. In this laboratory, we will determine the Michaelis constant and the maximal velocity of alkaline phosphatase.

Materials

1. 0.4 mM PNPP (p-nitrophenylphosphate) in 0.2 M Tris-HCl (pH 8.0);
2. 0.2 M Tris-HCl (pH 8.0);
3. 300 mU/ml Alkaline phosphatase.

Experimental Procedure

1. Prepare the following solutions and mix well:

	1	2	3	4	5
0.4 mM PNPP	0.1 ml	0.3 ml	0.5 ml	0.8 ml	1.3 ml
0.2 M Tris-HCl (pH 8.0)	2.7 ml	2.5 ml	2.3 ml	2.0 ml	1.5 ml

2. Adjust the spectrophotometer to zero absorbance with the first sample, and then add 0.2 ml of enzyme to the same tube. Mix quickly and measure the increase in the absorbance at 410 nm at 20 s intervals for at least 3 minutes.
3. Measure the absorbance change of four other samples in an identical method.
4. Determine the initial velocity of the reaction (v) for each sample by plotting the change of the absorbance versus time (slope of the linear line for the initial several points).
5. Plot v versus $[S]$ (concentration of the substrate), and present the graph.
6. plot $1/v$ versus $1/[S]$, and determine K_m and V_{max} (Lineweaver-Burk plot).

Please write a lab report

DNA Markers

