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1/29/2020 - Lab 1: Basic Techniques in a Laboratory

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Title:

Basic Techniques in a Laboratory

- **Part A - Light Microscope**
- **Part B - Micropipettes and Serological Pipettes**

Background:

Two very important lab techniques in which major biological and research advancement would not be possible are the use of microscopy and pipetting. There are various different types of microscopes: the light microscope use for this laboratory, scanning and transmission electron microscopes, fluorescence microscopes, and even more powerful (and more expensive) 3-dimensional x-ray and cryo-electron microscopes. The compound light microscope named for it compounding (multiplying power) lenses and use of visible light to illuminate specimens (Alberts etal 2014). We used the compound microscope with 10x ocular lens and 10x, 40x, and 100x lenses to look at 5 different specimens at various magnification levels. Pipetting is another useful tool as like microscopes there are many types of pipettes depending of the volume of solution required. They range from serological pipettes for volumes of 1 mL - 25 mL, to micropipettes for volumes of 1 - 1000 μL, and even specialized pipettes often termed enucleation pipettes are used for enucleating (removal of nucleus) from an oocyte and then inserting the nucleus from a somatic cell in a process called somatic cell nuclear transfer (SCNT) which has been used in very interesting studies of induced pluripotent stem cells (iPSCs). In this lab we tested our skills with both serological pipettes and micropipettes to pipette various volumes from 3 different range groups.

Hypothesis:

Part A - Light Microscope
As the size of cells under various the domains (Bacteria, Archaea, Eukarya) as well as size of virus particles vary in size from 100 nm for viruses to around 10 - 100 μm for animal and plant cells, I hypothesize that various objective lens magnifications will be the best for visualization depending on the specimen.

Part B - Pippetting
N/A

Protocol:

Part A - Light Microscope

<u>Prepared Slides</u>

Materials:

- Prepared slides for Erythrocyte cells, Elodea cells, and Escherichia coli cells

Procedures:

1. Obtain 3 prepared slides for erythrocyte cells, Elodea cells, and E. coli cells
2. Choosing one the slides first, place slide on microscope stage using clip slide in carefully not to break the slide and view under lowest objective lens magnification.
    a. Adjust light source as needed to improve visibility.
3. Once specimen is in focus at lowest magnification using only the coarse focus knob, adjust objective lens magnification and bring into focus only using the fine focus knob.
4. Move objective lens to maximum magnification and immerse specimen in oil if necessary.
5. Decide which objective lens was the best for viewing the specimen.
6. Sketch a visual of the specimen at that magnification along with the name of specimen and magnification level.
7. If present and visible indicate cell wall and nucleus.
8. Repeat steps 2-7 for each of the remaining 2 prepared slides.

<u>Preparation cheek and onion cells</u>

Materials:

- 2 glass microscope slides
- Iodine stain

- Methylene blue stain
- Cover slips
- Piece of onion
- Toothpick

Procedure - Onion cells:

1. Take a small piece of onion and snap it to unveil the translucent layer of epidermis.
2. Using forceps, remove a piece of the translucent layer of epidermis from the onion and place flat onto middle of microscope slide.
3. Place 1 drop of iodine stain covering entire tissue and lay coverslip flat on slide while avoiding creating any bubbles.
4. Remove any excess stain before placing under microscope.
5. View under microscope and analyze using procedures 2-7 above.

Procedure - Cheek cells:

1. Place 1 drop of DI water on microscope slide
2. Using a toothpick swab inside of cheek being careful not to pierce your cheek
3. Swirl toothpick around in DI water on slide
4. Add 1 drop methylene blue to liquid and lay coverslip flat over sample as above for onion cells
5. View under microscope and analyze using procedures 2-7 above.

Part B - Serological and Micropipettes Pipettes

Using Serological Pipettes

Materials:

- Six (6) 15 mL centrifuge tube
- Beaker of dyed water
- Different serological pipettes (1, 5, & 10 mL)

Procedures:

1. Label centrifuge tubes with volumes indicated in column 1 of Table 1 in results section below and place in rack in descending order.
2. Starting with 10 mL pipette, insert pipette end with cotton plug into the hole of the manual pipetting aid while twisting to secure.
3. Place tip of pipette into water and draw up liquid by scrolling down with the wheel.
4. Draw up specific amount in Table 1 below to where bottom of meniscus reaches volume. Use more than one draw if amount in table exceeds maximum volume of pipette being used.
5. Dispense water into designated centrifuge tube.
6. Record which pipette was used and mark filled to on centrifuge tube.
7. Repeat steps 2-6 for each volume in Table 1 below using the pipette that will give most accurate measurement.
8. When completed for all 6 tubes, compare tubes to standards at front of room.
    a. If one or more your centrifuge tubes have incorrect volumes, indicate in space below Table 1 why the reason might be.

Using Micropipettes

Materials:

- Beaker of dyed water
- Three (3) 1.5 mL centrifuge tubes
- Square parafilm
- Micropipettes (P-20, P-200, P-1000)

Procedures:

1) Choose correct pipette to use for each volume being measures. The table below is a guide. Practice with the P-1000 pipette.

Carolina, Edvotek		Pipetman, Gilson, Rainin	
P-1000	for 100-1000 µL	**P-1000**	for 200-1000 µL
P-100	for 10-100 µL	**P-200**	for 20-200 µL
P-10	for 1-10 µL	**P-20**	for 1-20 µL

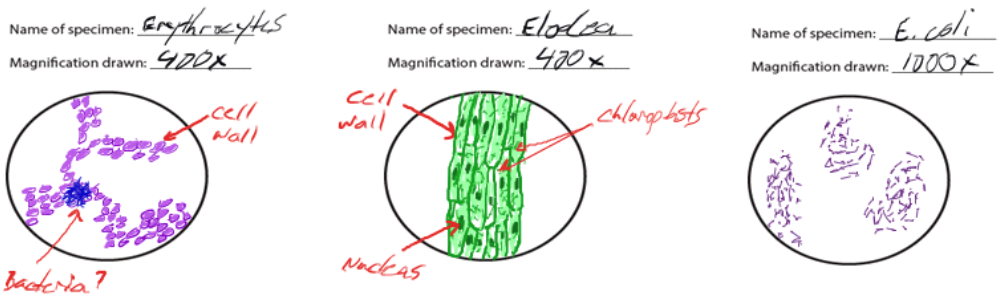
2) Adjust pipette to desired volume by either rotating plunger for Carolina and Edvotek or the volume knob for the others.
3) Check that volume readout on side of micropipette is correct.

4) Put on disposable pipette tip by pressing pipette down on tip in box is a little pressure.
5) Practice pipetting as described below:
    a. Press down lightly on plunger to the first resistance, this is the second position after the rest position.
    b. The three plunger positions are rest, first stop and second stop
6) Choose volume to practice with and adjust pipette to that volume.
    a. Press plunger to first stop using thumb or index finger.
    b. Put tip in water to draw up.
        i. Submerge tip just under surface. You can rest tip against side of contain to steady pipette.
        ii. If you submerge tip too far down under the surface, extra liquid may attach or associate to outside of tip and fall down into collection tube resulting in larger volume.
    c. Slowly release plunger back to rest stop make sure liquid is being drawn
        i. If you release plunger to quickly, this may result in liquid damaging pipette.
        ii. If liquid is viscous, liquid will not enter fast enough if releasing plunger too quickly.
        iii. Be care to keep pipette tip under surface of liquid to not draw up any air.
    d. Pull pipette out of tube.
        i. Check for air bubbles.
        ii. A bubble may result from pressing plunger to second stop
    e. Move pipette to empty microcentrifuge tube.
    f. Dispense liquid into tube by pressing on plunger to second stop
        i. Liquid should be dispensed by touching side of tube with pipette tip or into liquid if tube already has liquid in it.
    g. Keep plunger down as you remove pipette as to not draw up any liquid after dispensing
    h. Eject tip by pressing eject button
7) Now practice by measuring volumes using the P1000.
8) Label 3 microcentrifuge tubes with volumes **250, 620, and 900 µL** and place in small microcentrifuge rack.
9) Practice measuring each volume using correct micropipette techniques indicated in step 6. Use a separate pipette tip for each volume after dispensing into labeled microcentrifuge tube.
10) Compare water in tubes with standards on front desk repeating if measurements are off.
    a. Difficulty may arise due to pipette calibration or faulty use and storage of pipettes. If measurements are consistently off use a different pipette.
11) Next practice with colored water of following amounts: **250 µL, 620 µL, and 900 µL**.
12) Do this by:
    a. Using piece of parafilm paper, Gilson P-200 and P-20 micropipettes, and appropriate tip size.
        i. P-10s may require smaller size tips.
    b. Pick correct micropipette according to volume being measured and pipette colored water onto parafilm try to keep in 1 drop.
13) Compare size dots to each other, your lab partner, and instructor's.

Results:

Part A - Light Microscope

Prepared and unprepared specimens:



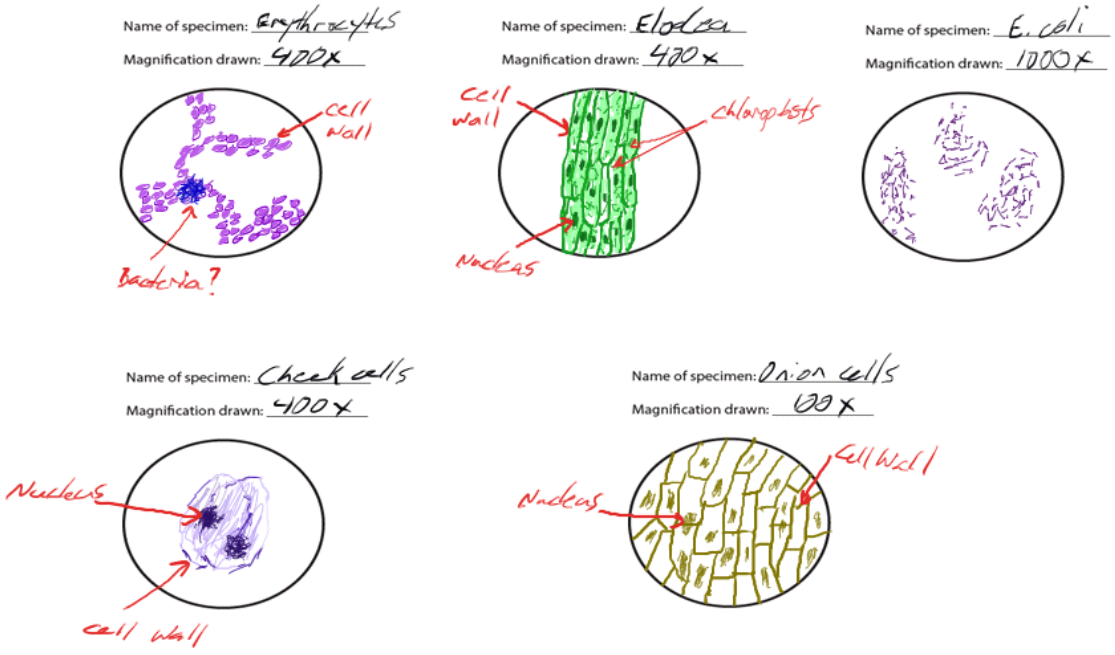


Figure 1. Results from Part A Light microscope. Images under microscope are labelled with name of specimen and magnification level. For the Elodea image drawn the structure took up entire view/circle under microscope similar to the onion cells but only some of the cells were drawn for simplicity. Similar sized cells expand the entire circle. There was a blue stain in various sections of the erythrocyte which may have been bacteria. Cheek cells show two overlapping cells. The cytoplasm of the onion cells was light yellow but shown in white for simplicity while the nucleus staining was blurry.

Part B - Pipetting

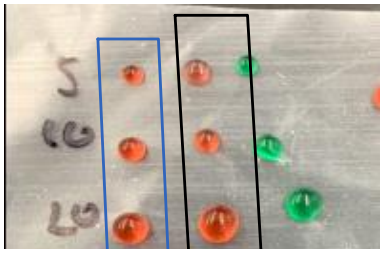
Volume to measure	Pipette used	Mark liquid is filled to (mL)	Appears accurate? (Yes/No)
14 mL	10 mL	14.2	No
12.2 mL	10 mL	12.4	No
6.8 mL	10 mL	6.9	No
3.7 mL	5 mL	3.7	Yes
2.1 mL	5 mL	2.0	No
0.7 mL	1 mL	0.7	Yes

Table 1. Results from procedures for Using Serological Pipettes

Reasons for inaccurate measurements: For the first 3 measurements I may have over compensated for the fact that when you pull out the serological pipettes the volume goes down so I may have brought up more than was need to compensate for that. For the last three it seems like I started to correct this.

Volume to measure	Pipette used	Dials used	Appears accurate? (Yes/No)
250 μL	P-1000	0,2,5	Yes
620 μL	P-1000	0,6,2	Yes
900 μL	P-1000	0,9,0	Yes
5 μL	P-20	0,5,0	Yes
10 μL	P-20	1,0,0	Yes
20 μL	P-20	2,0,0	Yes
60 μL	P-200	0,6,0	Yes

Table 2. Results from procedures for Using Micropipettes. The set of Gilson pipettes from the table in the procedures section was used for this experiment.



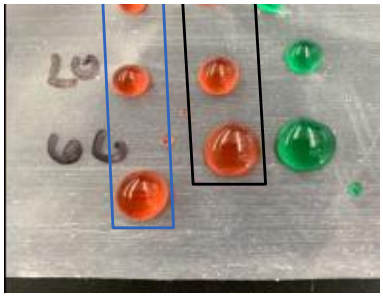


Figure 2. Measurements are in µL. The center column indicated by black perimeter is instructors column while the left column indicated by blue perimeter is my column and last column with green dye is my lab partner's. Note the two small red droplets of water were from a previous failure for 60 µL.

Discussion/Conclusion:

For the experiment for Part A: Light Microscope, we got some really good results in which we were able to see the cell wall and nucleus for most of the specimens. The two specimens we could not see the nucleoid (for bacteria) or nucleus (for eukaryotes) were E. coli and red bloods cells (RBCs) respectively. There are two reasons for this: first being E. coli cells need a higher magnification to see the DNA inside of the cells as bacteria cells are much smaller that eukaryotic cells and second being that well, RBCs do not have a nucleus, nor do they have DNA as their primary purpose is to be small and to carry as much oxygen as possible and so a nucleus would take up space otherwise usable by heme groups in hemoglobin to bind oxygen. E. coli cells were also too small to make out the cell wall or plasma membrane.

We were able see all specimens at the highest magnification level of 1000x but in order to get a better understanding of the entire cell structure we had to view the specimens at different magnifications levels. Therefore my hypothesis was correct that different objective lens magnification would be required to visualization the specimens the best. One issue we encountered with cheek cells and onion cells was the ability to only extract one layer of cells. We saw overlap of cells and the nucleus staining was not as a crisp as we would have liked. I also recall the nucleus staining being more prominent for the Elodea cells that what I am seeing online. This may have been because of the staining technique, or maybe I mistook deeper stained chloroplast or aggregate of chloroplasts for the nucleus. If I were to repeat this activity I would first want to prepare my own slide with my red blood cells with and without staining. I would also like to perform a Gram stain to visualize E. coli cells. I would have also repeated the cheek and onion cell preparation until I got a single layer and better stained nucleus.

For the experiment for Part B: Serological and Micropipettes, I performed much better with the micropipettes than with the serological pipettes. I had some difficulties with the size of serological pipettes, the pipette coming loose or bending around hole to the pipetting aid which may have caused some issues with the process but I don't think it made too much effect on the error in measurements I had gotten because I was still able to pipette to the correct lines and dispense all the liquid. I believe my reason for the three (3) measurement being over the correct volume of liquid due to over compensation for the fact the level moves down when the pipette is removed. For the 2.1 mL volume I was under and may have overcompensated correcting the initial overcompensation. This is highly unlikely but because I still thought I was pipetting the first 3 serological pipette volumes (14, 12.2, & 6.8 mL) correctly it may be that the measurements on either pipettes or centrifuge tubes were off, but again this is highly unlikely.

If I were to perform the serological pipette section of Part B again, I would rather use the motorized serological pipettes by Drummond Scientific that we used in my previous lab. In addition to a manual wheel and manual plunger you can press as switch to draw and dispense by holding a motorized button which gives the choice to perform serological pipetting manually or automatic.

Lastly, I was pretty comfortable using the micropipettes. As we were only able to visually confirm they amounts we pipetted were correct by comparing to the instructors, I would have like to have had our lab partner confirm the amount we pipetted was correct for all µL measuremnts. You can confirm this by repipetting from the microcentrifuge tubes (this is how we were graded by instructor in my last lab) with the corresonding volumes as well as repipetting from the parafilm. I have experience adding loading dye, plasmide DNA, and remaing µL of DI water on parafilm to load the gels faster for electrophoresis and you can tell you are correct when drawing up the total of 10 µL to load.

Resources:

1. Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M. C., Roberts, K., Walter, P. (2014). Essential cell biology.

2/5/2020 - Lab 2: Dilutions and the Bradford Protein Assay

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Title:

Dilutions and the Bradford Protein Assay

- **Part A - Concentrations and Dilutions**
- **Part B - Spectrophotometry**

Background:

The Bradford assay is an assay used in biotechnology to measure total protein with varying weight to volume concentrations. It utilized staining dyes that undergo measurable color change when bound to a chemical species, in our case protein. A very popular stain that is used for many protein analytical assays is Coomassie Blue G-250. This stain has 2 sulfonic acid groups (SO_3H) which have low pKa. When the pH of the solution is higher than their pKa they are usually deprotonated and carry negative charge. Therefore the dye targets basic amino acids (Arg, Lys, His) as well and hydrophobic interactions with aromatic amino acids (Phe, Trp, Tyr) because of the stacking interactions of the benzene rings.

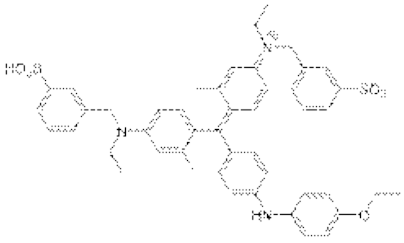


Figure 1. Chemical structure of Coomassie Blue G-250

We will be using the Bradford assay to create a linear standard curve for the Bovine Serum Albumin (BSA) protein. We will use a spectrophotometer to measure the calorimetric change (color change of Coomassie Blue and protein) at a wavelength of 595 nm, the maximum absorption peak of Coomassie Blue. We will do this at varying concentrations of BSA as a tool to compare the absorbance levels of an unknown sample.

Hypothesis:

Since Beer's law indicates an increase in protein concentration should result in a linear increase in the absorbance of the solution, I hypothesize if the unknown absorbance is between the average of two of the tubes then the derived concentration for the unknown concentration will also be between those two tubes.

Protocol:

Part A - Making Bovine Serum Albumin Dilutions and Running Bradford Assay

Bovine Serum Albumin Dilutions

Materials:

- 2 mg/mL (2%) bovine serum albumin (BSA) stock
- 8 1.5 mL microcentrifuge tubes
- Microcentrifuge stand
- 5 mL DI water
- Gilson micropipettes

Procedures:

1) Label 8 microcentrifuge tubes 1-7 and place in rack.
2) Add 100 µL of 2 mg/mL (2%) BSA to tube 1.
3) For remaining tubes 2-7 make dilutions according to table below. Record the BSA concentration for each tube using the formula below:

$$C_f = \frac{V_i x\, C_i}{V_f}$$

Number on tube	Volume (µL) and Source of BSA	µL of DI H_2O

2	300 of stock	100
3	200 of stock	200
4	200 of Tube 2	200
5	200 of Tube 3	200
6	200 of Tube 5	200
7	200 of Tube 6	200

4) Tube 8 of an unknown sample will be prepared for you by you instructor. Obtain this tube and place in rack.

Bradford Assay

Materials:

- 13.5 mL Coomassie Blue G-250 dye
- Nine (9) 3.5 mL cuvettes
- 7 tubes of serial dilution BSA from above
- 1 unknown tube BSA concentration from above
- 30 µL DI water
- Gilson micropipettes

Procedures:

1) Label cuvettes on lids 1-8
2) Pipette 30 µL of your prepared samples intro their respectively marked cuvettes
3) Add 1.5 mL of Coomassie Blue G-250 to each cuvette
4) Prepare a blank cuvette labelled "B" with 30 µL DI water and add 1.5 mL Coomassie Blue G-250
5) Cover tubes with lids securely and invert each tube 3 times to mix
6) Wait at least 5 minutes before examining with spectrophotometer

Part B: Spectroscopy

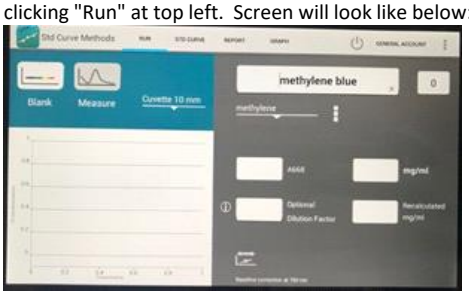
Materials:

- BSA standards 1-7 cuvettes from above
- Unknown sample from above
- DeNovix QFX fluorometer
- P-1000 pipette and tips
- Python Software

Procedures:

1) Making sure DeNovix QFX fluorometer is plugged in, turn on back switch until home screen display is showing.
2) Setup program by clicking on "Std Curve Methods" icon.
3) If method is not setup, set the method from the side sections:
    a. Change method name to "BSA".
    b. Set analysis nm to "595".
    c. Set units to "mg/mL".
    d. Set min nm to "300", max nm to "780", and baseline to "760".
    e. Click ok to configure making sure no cuvette is in while configuring.
4) Set up standard curve:
    a. Click "Std Curve" tab.
    b. Set concentrations for tubes 2-7 by clicking "Enter Stds" button.
5) Blanking Spectrophotometer
    a. Place blank cuvette "B" into spectrophotometer. The clear side of cuvette should face the light source.
    b. Click the "blank" icon so spectrophotometer can subtract out background absorbance.
6) Keep cuvettes clean from smudges using a Kim wipe.
7) Examine each solution in the spectrophotometer starting with tube 2.
    a. Place "Tube 2" cuvette standard into spectrophotometer.
    b. Click "Measure".
    c. Repeat 5 times to get average absorbance (Abs).
    d. Record the average absorbance at 595 nm.
    e. Repeat with each dilution cuvettes 3-7.
        i. For each new cuvette standard, click the Abs1 space next to respective concentration.
        ii. Remove Tube 1 average Abs from the standard curve if it makes the curve no longer linear.

8) Write down the R^2 value on top left of standard curve graph.
9) Measure the absorbance of the unknown sample in Tube 8 now in cuvette 8 by clicking "Run" at top left. Screen will look like below:



   a. Place unknown cuvette 8 in cuvette holder and hit "Measure" to determine absorbance and record the absorbance value.
   b. The program will use your standard curve to calculate the concentration of the unknown sample. Record this concentration value.

10) Using Python instead of excel, plot the absorption at 595nm for standard solution (Tube 2-7).
   a. Make sure to add linear regression line, linear regression equation, and R^2 value to plot.
   b. Record standard curve equation (linear regression equation) and the R^2 value.
   c. If the R^2 value is < 0.95 then you like have pipetting errors and concentrations are incorrect.
   d. Using your standard curve equation, calculate your unknown concentration using equation below and record it.

$$x = \frac{(y - b)}{m}$$

   e. Determine percent error of unknown concentration (experimental) from the expected (theoretical) total protein concentration provided by your lab instructor using the equation below and record it. Successful experiments should have less than 5% error.

$$Percent\ Error\ (\%) = \frac{|Experimental - Theoretical|}{Theoretical}\ x\ 100$$

Results:

Part A - Making Bovine Serum Albumin Dilutions and Running Bradford Assay

Bovine Serum Albumin Dilutions

Number on tube	Volume (µL) and Source of BSA	µL of DI H_2O	Calculated BSA Concentration (mg/mL)
2	300 of stock	100	$\frac{300\mu L \times 2\frac{mg}{mL}}{100\mu L + 300\mu L} = \frac{600\mu L\frac{mg}{mL}}{400\mu L} = 1.5mg/ml$
3	200 of stock	200	1.0 mg/mL
4	200 of Tube 2	200	0.75 mg/mL
5	200 of Tube 3	200	0.50 mg/mL
6	200 of Tube 5	200	0.25 mg/mL
7	200 of Tube 6	200	0.125 mg/mL

Table 1. Concentrations after performing serial dilutions of BSA.

Part B - Spectroscopy

Tube #	Concentration of BSA (mg/mL)	Average Absorbance (at 595 nm)
1	2.0 mg/mL	1.278
2	1.5 mg/mL	1.219
3	1.0 mg/mL	0.893
4	0.75 mg/mL	0.788
5	0.50 mg/mL	.0541
6	0.25 mg/mL	0.288

7	0.125 mg/mL	0.154

Table 2.

DeNovix Standard Curve R^2 value without Tube # 1 = __0.9780_____

A_{595} for Unknown Tube #8 = _____I need this from Lab Partner_(1.086?)_____

DeNovix Standard Curve Protein Concentration of Unknown Tube #8 = __1.258_____

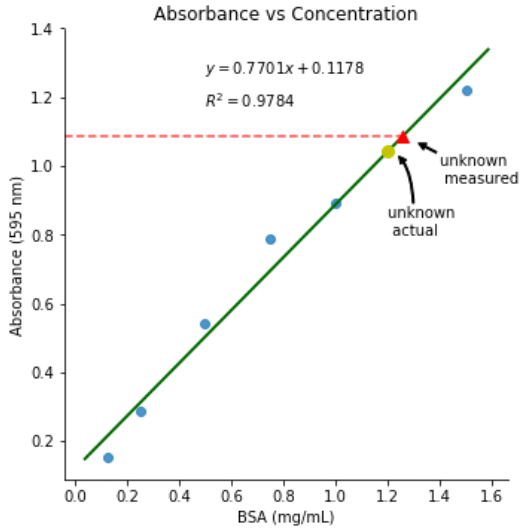


Figure 2. Linear regression curve for A_{595} absorbance with respect to concentration of BSA (mg/ML). Graph was created in Python using Seaborn and Matplotlib packages.

Standard curve: ____A_{595} = .7701 x Concentration + .1178__

R^2 value: _____0.9784_____

Unknown Protein Concentration Tube #8 (mg/mL)___$x = \frac{(y-b)}{m} = \frac{(\mathbf{1.086}-.1178)}{.7701} =$ **1.258 mg/mL**_____

Percent Error for Unknown Tube #8: $Percent\ Error\ (\%) = \frac{|Experimental - Theoretical|}{Theoretical}\ x\ 100 = \frac{|1.258 - 1.2|}{1.2}\ x\ 100 =$ ___**4.83%**_____

Discussion/Conclusion:

Having only had a 4.83% error, we have concluded that our standard curve was fairly accurate and our hypothesis was correct that having an absorbance level at 595 nm (1.086 for the unknown) between two serial dilutions' (1.0 mg/mL & 1.5 nm/mL) absorbance levels, the concentration of the unknown solution concentration was calculated to be between the concentrations of the two known concentrations.

The small percent error along with the concentration calculated to be between the two known concentrations as well as the graphical representation (see Figure 2.) support the theory of the Lambert-Beer law with respect to a linear increase in absorbance as the concentration of solution increases.

The percentage error may have to do with pipetting errors, which can always be a reason for lack of perfect accuracy, but I also noticed that some of the experimental points we further away from the regression line and so after initially have the first serial dilution of 0.75 mg/mL pipetted incorrectly this can also influence future experimental points in concordance with the linear curve. Next time I use a spectrophotometer I may do more than 3 measurements before recording an average absorbance as molecules can be moving in solution.

2/12/2020 - Lab 3: Introduction to Gel Electrophoresis

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Title:

Gel Electrophoresis

Background:

Gel electrophoresis is an extraordinary powerful tool in biological research and experimentation. It provides information about DNA for which we cannot obtain from viewing under a microscope as we cannot resolve specific DNA bases under the microscope to quantify the DNA. Gel electrophoresis can be used to separate many type of molecules in addition to DNA by using a porous gel that can be made with a buffer, usually Tris-Acetate-EDTA (TAE), with the polysaccharide agarose for larger molecules and DNA fragments or for smaller molecules with polyacrylamide. We apply electrical current from negative to positive for DNA and RNA, where we load the DNA and RNA at near the negative electrode so it will repel the molecules toward the positive electrode. We can then view the gels after running under UV light to get an estimate of the fragment lengths of our samples.

For the gel in our experiment we will be a adding fluorescent stain, SYBR Green, so that the bands of DNA can be viewed under UV light. The gels will also be ran with two dye mixes that contain the molecules in Table 1 and a column with DNA standards (ladder) that have a set of known DNA fragment lengths. We use different tracking dyes depending on what we predict will be our smallest and largest DNA fragments so we can run the gel for as long as we can to obtain the most resolution between bands without losing any DNA fragments in our unknown sample.

Table 1. Base pair equivalence of marker dyes (1.2% gel)		
Dyes	**Color**	**Base Pair Equivalence**
Xylene Cyanol	Blue-Green	2,800
Bromophenol Blue	Purple-Blue	250
Orange-G	Orange	70
Blue dye (from 6x purple)	Blue	4,000
Pink/red dye (from 6x purple)	Pink/Red	300

Table 1. Tracking dyes of different colors are used to be able to track how far the DNA run in the gels.

Our objective is compare the location and distance migrated of the unknown fragment using the distances migrated and lengths for the known fragments in our DNA standard. We can then approximate, or even better, use a standard curve to estimate the length of our unknown DNA fragment.

Hypothesis:

Part A - Gel Electrophoresis
As migrating bands of DNA are separated by length while running gel electrophoresis the distances traveled are inversely proportional to the fragment size, I hypothesize that if the unknown fragment has migrated between two bands for the DNA standards then the unknown fragment will be shorter than the top Standard band and longer than the bottom Standard band.

Protocol:

Part A - Gel Electrophoresis

Materials:

- 1.2% agarose/TAE (agarose g / TAE mL) in 55°C water bath
- TAE (Tris-acetate-EDTA) buffer
- P-20 and P-200 micropipettes with tips
- Casting tray with 15 well comb and blocks
- Electrophoresis gel box and power supply
- Dye mixture tubes
- 6x loading dye
- 1 kb ladder

- Unknown DNA tube
- SYBR Safe staining dye (ratio 1:10,000 SYBR:gel)

Procedures:

1. Put on gloves!
2. Pouring agarose gel:
    a. The 1.2% agarose gel/TAE buffer is already prepared at sitting in 55°C water bath.
        i. SYBR has already been added at ratio 1:10,000.
    b. Prepare gel in casting tray:
        i. Add block to each end of gel.
        ii. Insert gel comb at marking.
        iii. Note: methods for gel electrophoresis systems differ for various manufacturers.
    c. Carefully pour agarose in gel tray making sure not to overfill or cause bubbles.
    d. Wait 15-20 minutes for gel to set.
3. Collect samples to be loaded into the gel: 1 kb ladder, unknown DNA sample, and two (2) dyes: Dye Mix and 6x Purple Dye.
    a. See Table 2. for fragment number and size of 1 kb ladder used to create standard curve.
    b. The unknown DNA sample and 1kb ladder have been pre-loaded with loading dye. (Why would the DNA sample be pre-loaded with dye? Wouldn't the SYBR make is visible when viewing under UV?)

Table 2: 1 kb molecular weight marker fragments and their sizes.

Fragment # (largest to smallest)	Fragment Length (kb)
1	10 kb
2	8 kb
3	6 kb
4	5 kb
5	**4 kb**
6	**3 kb**
7	**2 kb**
8	**1.5 kb**
9	**1.0 kb**
10	**0.5 kb**

4. After gel has solidified, place gel in gel box.
    a. Remove tape from edges.
    b. Make sure the wells are facing to the side with negative charge.
5. Add TAE buffer until gel is fully submerged about 1 cm above the gel.
6. Using Table 3. as a guide load you gels:
    a. Both you and your lab partner will load two wells each.
    b. Skip well 1 and leave gap between Dye Mix and other samples.
    c. Add 10 µL of each sample to respective wells.
    d. Record gel loading detail.

Table 3: Gel loading details	
Sample Well #	**Sample**
2	Dye Mix A (Xylene cyanol, Bromophenol blue, Orange G mix)
4	6x Purple Dye (Blue and red/pink)
5	Unknown DNA sample (Tube U)
6	1 kb Ladder

7. Put tip of pipette into wells of gel making sure not to puncture sides or bottom of wells.
8. Press carefully on pipette plunge making sure sample is going to the well.
9. Press plunger to second stop to load entire sample in well.
10. Remove pipette tip in the biohazard waste.
11. Repeat process for all samples using a new pipette tip for each sample.
12. Connect cords for gel lid to power supply.
    a. Make sure red matches red and black matches black.
    b. Make sure that the red color (+ charge) is facing away from wells so DNA

runs to Red.

13. Set power supply to 125 V for 30-40 minutes and record the specific time ran.
14. Monitor position of three dyes (Dye Mix, 6x Purple Dye, 1 kb ladder) as it is running. Run until the bromophenol blue (purple dye color) in Dye Mix A and DNA has migrated about 2/3 of the gel making sure bromophenol blue does not run off the gel.
15. While wearing gloves remove casting tray from gel box and slide gel out at a low angle on plastic wrap.
16. Place gel carefully on UV imager and take photograph of gel with the imager.

Part B - Analyzing the data

Materials:

- Excel Software
- Image J Software
- Gel photograph from Part A

Procedures:

1. Upload photograph on lab computer and analyze with ImageJ software.
2. Using ImageJ set the scale to cm for the length of the entire gel and measure and record the distances migrated for all the bands in the DNA ladder as well as the unknown DNA sample.
3. Use Excel to create a more accurate standard curve.
    a. Enter in values for DNA migration length in (cm) and DNA fragment lengths in kb.
    b. Create log transformation of DNA fragment lengths in (kb)
    c. Plot the independent variable log transformed DNA fragment lengths on y-axis and DNA migration length on x-axis.
    d. Add trendline, equation, and R^2 value to chart.
    e. Use equation to solve for DNA fragment length of unknown DNA sample.
    f. Reverse the log transformation of the log DNA fragment length.
4. Based on the expected sizes of you unknown bands(s) from your instructor calculate the percent error of DNA fragment size.
5. In results include:
    a. Table for Step 2 with caption.
    b. Include image of gel with caption.
        i. Label wells.
        ii. Try to identify dye in the dye mixtures if UV shadow is present and label dyes.
        iii. Label the molecular weight markers on standard curve.
    c. Include standard curve graph with caption and proper axis labels.
        i. Include standard curve equation and R^2 value.
        ii. Include calculations and results to determine unknown DNA fragment size.

Results:

Part A - Gel Electrophoresis

Table 3.

Sample Well #	Sample
2	Dye Mix A
4	6x Purple Dye
5	Unknown DNA sample (Tube U)
6	HindIII digestion of Lambda DNA Ladder

Time gel ran: 38 minutes

Part B: Analyzing the data

As the 1kb ladder was really a restriction digest ladder for HindIII digestion of Lambda DNA below we should obtain bands with length in Figure

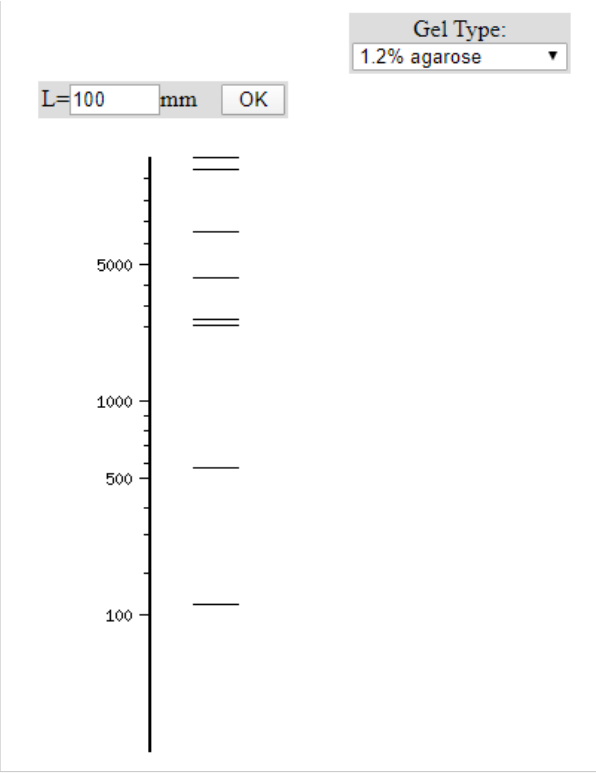

Figure 1. Image of HindIII digest for Lambda DNA. Image was created using NEBcutter V2.0 from New England Biolabs.

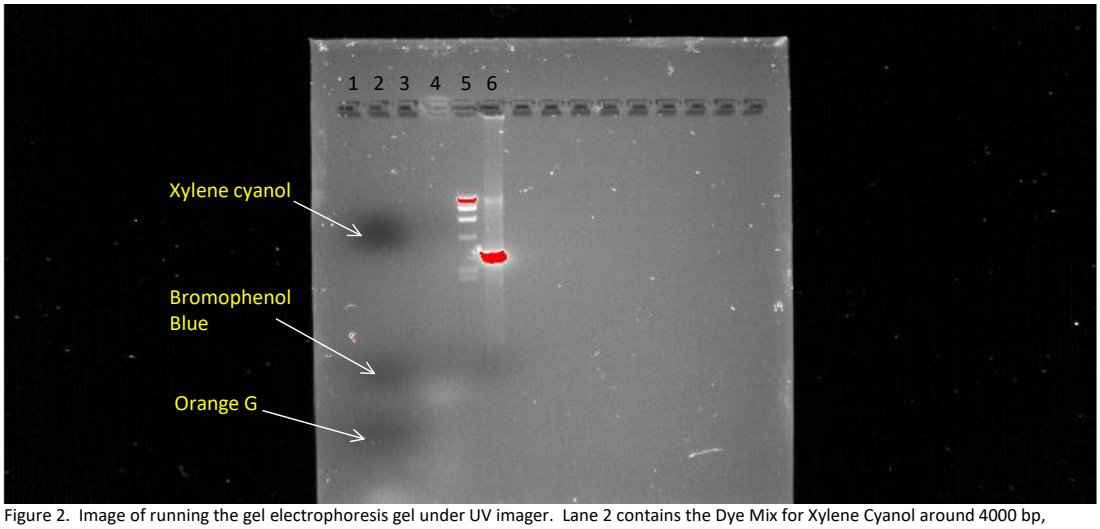

Figure 2. Image of running the gel electrophoresis gel under UV imager. Lane 2 contains the Dye Mix for Xylene Cyanol around 4000 bp, Bromophenol Blue (cannot visualize from ladder) and Orange G (cannot visualize from ladder). Lane 3 was skipped as was lane 1. Lane 4 contains 6x purple loading dye and shadows cannot be seen on gel. Lane 5 contains the HinDIII digestion of Lambda DNA Ladder. Note the first red band for largest fragment was not used in the data. Lane 6 contains the unknown DNA fragment.

Length of gel in cm: 10 cm

Fragment	**DNA migration length (cm)**
23130	**1.944**
9416	**2.183**
6557	**2.441**
4361	**2.837**
2322	**3.552**
2027	**3.75**
Unknown	**3.413**

Table 4. Placeholder for caption.

Unknown DNA fragment length (KB): _____3.413 cm_____

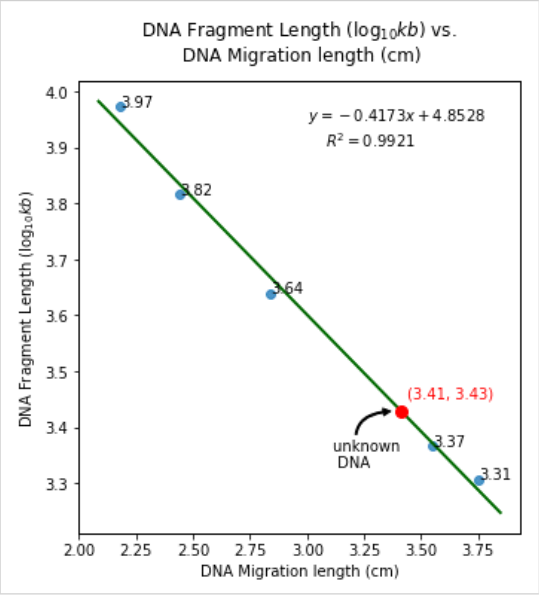

Figure 3. Standard log curve for DNA fragment length. The log10 was taken for each fragment length and graphed against the distance migrated on the x-axis. Plot was done in Python with Seaborn and Matplotlib packages.

Equation: __log(DNA Length) = -0.4173 (Migration Length) + 4.8528__

R^2: _____0.9921___

log(DNA Length) = -0.4173 (Migration Length) + 4.8528
log(DNA Length) = -0.4173 * 3.413 + 4.8528
log(DNA Length) = 3.4285

Log transformed DNA size: __3.4285_____

$10^{\log(\text{DNA Length})}$ = bp size
$10^{3.4285}$ = 2682.60 bp

Unknown DNA fragment length (KB): ___2682.60 bp or 2.6826 kb_____

(*Percentage error not needed as no way to clarify)

Discussion/Conclusion:

We discovered while viewing our gels in the UV imager, that the DNA kb standards were not the sample marked as 1 kb standards. Instead it was a restriction digest of Lambda DNA with HinDIII. Therefore we have different measurements to use in our standard curve. Regardless, after throwing out the largest fragment from this digest, the standard curve for log(kb) vs migration length gave us an R^2 value close to 1 (.9921). In terms of the R^2 value the DNA fragments migrated as expected. We were able to make our shadows for the Dye Mix A for Xylene cyanol, Bromophenol blue, and Orange G in the second lane. However we were not able to see in the image the shadows for the Purple 6x dye in the picture taken from under the UV light. These tend to be fainter when viewing under UV but we were able to see them on the gel.

Using our standard curve with the log(base pair) length on the y-axis and DNA migration length on the x-axis, were used the distance travelled for our unknown DNA fragment of 3.43 centimeters to plug into the standard curve and then performed log transformation which resulted in an estimate of the unknown fragment length to be 2682.60 bps. We were not able to perform the percentage error as we had been instructed by our teacher that she could not verify the length to be 3000 bps. Comparing the shadows that were visible on the UV image of our gel, we can see that first shadow from the wells, the one for Xylene cyanol was the closest and this would be best one alone to compare to estimate the length of our unknown DNA fragment length.

If I were to perform this experiment again I would first obviously use the 1 kb DNA standard ladder but I would also load more than one well for the unknown DNA for multiple samples of the unknown DNA so that we can compare there were no issues in the pre-experiment of isolating these fragments. It was probably created using some restriction digest or DNase to make the cut to a particular length and there can be errors or nicks in the DNA to throw off the results. Also depending on where the DNA is supercoiled, circular or linear, it can migrate faster than if it were not super coiled. Linear runs faster than circular but not faster than supercoiled.

Resources:

1. Gel Electrophoresis Analysis:LabDirections. 2015. Retrieved from https://www.lachsa.net/ourpages/auto/2015/1/27/48704779/Electrophoresis%20AnalysisLAB.pdf

2/12-26/2020 - Lab 4: Comparative Proteomics Protein Profiling and Western blot

Table of Contents

Wednesday, January 29, 2020 2:49 PM

Title:

Comparative Proteomics Protein Profiling and Western blot

Background:

Gel electrophoresis used to separate proteins as well as DNA. In this procedure we will use sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). The mass, shape, and charge of a protein affect it's mobility through the gel and not all proteins carry the same net charge or are structurally folded the same way. We are interested in creating a protein profile for 5 different fish species: Cod, Salmon, Trout, Catfish, and Flounder so that we can compare their profile alignments and confirm their evolutionary relationship. For this lab in order to separate the fish proteins from the muscle of the 5 different species by mass only, we add an SDS buffer to the protein solution to mask the charges of the proteins with a negatively charged coat as well as denature the proteins so they become linear and so the net charge densities even and shapes are relatively linear. We will also be adding Dithiothreitol (DTT), a reducing agent to break the disulfide bonds of the cysteine residues in the proteins facilitate denaturation to the proteins primary structure. After running the gel using an electrical is completed we will stain the gel with Coomassie Blue in order to be seen and resolved on the gel as well as to transfer and visualized using a Western blot.

Western Blot is a powerful tool that is used to analyze level of protein expression, and specifically target certain kinds of proteins or an individual protein or proteins in a given sample. Tagging proteins can be accomplished with the use of different antibodies that specifically bind to an epitope section of the protein we wish to tag. After applying the current in the Trans Blot Turbo the negatively charged proteins stained with Coomassie Blue will migrate toward and be blotted on the cellulose membrane. We then will add blocking buffer prior to tagging our proteins of interest. Two proteins we will be tagging are myosin light chain 1 (MLC1) and myosin light chain 2 (MLC2). We will add a primary anti-myosin antibody as well as a secondary antibody with and enzyme which will undergo a color producing reaction with Horseradish Peroxidase (HRP) substrate to visualize the tagged MLC1 and MLC2 of our fish proteins for comparison to see if they align or differ from each in the evolution tree in Figure 1 based on how closely MLC1 and MLC2 are in molecular weight and primary structure.

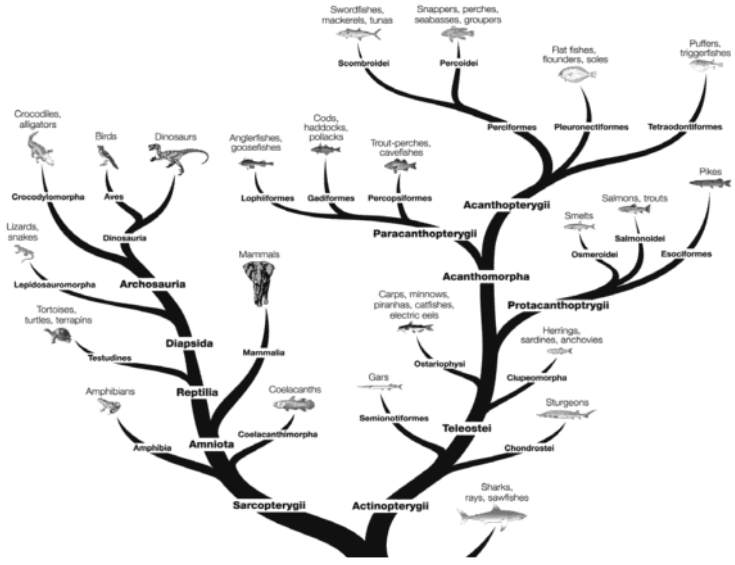

Figure 1. Evolutionary tree showing where reptiles and mammals broke off from fish and where different fish species break off from each other. Image taken from Bio-rad Comparative Proteomics Kit I: Protein Profiler Module[1]

Hypothesis:

Part B: Electrophoresis
As organisms with higher protein sequence alignment (i.e. similar primary sequence) are indicative of closely genetic evolution, I hypothesize that since Salmon and Trout are closer in the evolutionary tree shown in Figure 1 above, they will have the most similar protein profiles.

Part C: Western Blot
As deviations of a particular homologous protein in a set of species, in the case of this lab myosin light chain 1 (MLC1) and myosin light chain 2 (MLC2), generates different phenotypic attributes of that species, I hypothesize that fish with closely aligned bands representing MLC1 and MLC2 will be closer in evolution and the fish with bands for MLC1 and MLC2 that are further away from each other will be further from each other in evolution and display different phenotypes.

Protocol:

Part A - Preparation of Fish Tissue

Materials:
- 5 fish samples - Salmon, trout, catfish, flounder, cod
- Scissors
- Ten (10) 1.5 mL microcentrifuge tubes
- P-200 and P-1000 micropipettes with tips
- Laemmil buffer w/ 70 mM Dithiothreitol (DTT)

Procedures:
1. Put on gloves!
2. Label 2 sets of five (5) 1.5 mL microcentrifuge tubes (2 for each fish).
3. Obtain square chunks of each fish sample approximately 0.25 cm^3 from the instructor.
4. Place each fish chunk in it respective microcentrifuge tube with 250 µL Laemmil buffer containing 70 mM DTT.
5. Cap tubes and flick each tube 15 times.
6. Incubate tubes for 5 minutes at room temperature.
7. Transfer 50 µL from the buffer while excluding the fish tissue to the second tube for each fish changing pipette tips each time.
8. Place tubes in 95°C water bath for 5 minutes to deature proteins.
9. Take samples to instructor for storage at -20°C.

Part B - Electrophoresis and Start of Western Blot

Materials Electrophoresis:
- 5 fish proteins from last week - Salmon, trout, catfish, flounder, cod
- 4-20% Mini-PROTEAN TGX gels
- 1x tris-glycine-SDS (TGS) buffer
- Block heater plate set to 95°C
- 6 Mini-PROTEAN tetra cell vertical gel electrophoresis units
- Power supply for gel electrophoresis units
- Precision Plus Protein Kaleidoscope standards
- Actin and myosin standard
- P20 micropipettes with tips
- Bio–Safe™ Coomassie Stain
- DI water
- Large weigh boats

Materials Western Blot:
- DI water
- Blocker buffer (5% dry block in wash buffer)
- Trans Blot Turbo unit
- Trans Blot Turbo transfer pack (0.2 µm nitrocellulose)
- Test tube as WB roller
- Large weigh boats
- Primary antibody (anti-myosin)
- Secondary antibody (goat anti-mouse HRP)
- Horseradish Peroxidase (HRP) color detection substrate
- Wash buffer
- Liquid waste container
- ImageJ software
- Excel software
- Ruler

Procedures SDS-PAGE Electrophoresis:

1. Setup vertical gel boxes.
2. Assemble pre-cast polyacrylamide gel cassettes:
    a. Open pouch with cassette and remove tape from gel bottom.
    b. Remove green comb carefully.
    c. Insert gel cassette with gel in electrophoresis chamber with shorter plate facing electrodes.
3. Add 1x TGS buffer first to cassette and then to main chamber fully submerging the larger back plate of gel cassette with buffer.
4. Rinse each well of gel with TGS buffer using micropipette.
5. Load samples and standards according to Table 1. below:

Table 1. Samples to Load in SDS-PAGE

Well #	Sample	Volume
1	None	Empty
2	None	Empty
3	Pre-Stained Protein Standards	5 µL
4	Cod	5 µL
5	Salmon	5 µL
6	Trout	5 µL
7	Catfish	5 µL
8	Flounder	5 µL
9	Actin & Myosin Standard	5 µL
10	None	Empty

6. Connect gel box to power supply matching positive and negative electrodes.
7. Set voltage on power supply to 200V and turn power on.
8. Run gel for 21 minutes at 200V.
9. Once gel is finished running turn off power and remove polyacrylamide from gel box.
10. Laying the short plate of cassette facing up pull apart gel plates carefully.
11. Transfer gel to large weigh boat with DI water to allow gel to detach.
12. Drain liquid and refill with DI water and rest for 5 minutes.
13. Repeat step 12 for second rinse.
14. Drain DI water and add 50-100 mL Bio-Safe Coomassie stain.
15. Place weigh boat and gel on shaker table for 1 hour at low speed.
16. Discard stain and replace with DI water to de-stain overnight.
17. Take of picture of gel and measure length in mm.
18. Using ImageJ set the scale to cm for the length of the entire gel and measure and record the distances migrated for all the bands in the Precision Plus Protein Kaleidoscope Prestained Standards <=50 kDa as well as all the fish samples and Actin & Myosin standards.
19. Create a log standard curve and use the curve to calculate the molecular masses of fish samples and Actin & Myosin standards.

Procedures Western Blot:

Part 1 -

1. Using the Trans-Blot Turbo mini-transfer packs, the top(-) stack will go on top with the (-) electrode while the bottom(+) stack with the nitrocellulose will go under the gel with the (+) electrode.
2. Make the blotting sandwich as indicated in picture below.
    a. Added the bottom(+) blotting papers down first with the nitrocellulose on the bottom(+) cassette and use blot roller to remove bubbles.
    b. Add the gel and use blot roller again.
    c. Add the top(+) blotting paper and use blot roller one more time before adding top(-) cassette lid and lock cassette lid.

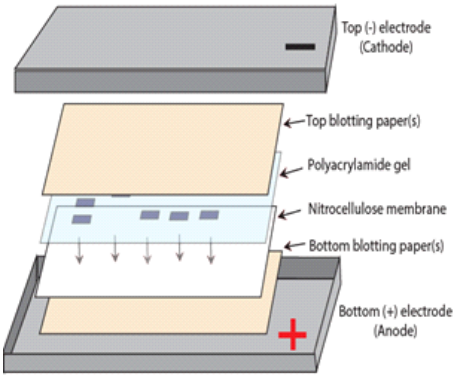

Figure 2. Cartoon model for Trans Blot Turbo cassettes to be inserted to perform the transfer of the proteins from the Electrophoresis gel to the cellular membrane.

3. With dial facing up insert the cassette into Trans Blot Turbo to interlock.
4. Select "LIST" and then "Mini-TGX" transfer protocol.
    a. Run mini gel at 25V, 2.5 A for 3 minutes.
5. Press the corresponding *:RUN button depending on location bay in Trans Blot Turbo .
6. Remove cassette after transfer is complete and unlock cassette.
7. Disassemble blotting sandwich by peel membrane off gel. Check for colored bands.
8. Place membrane in staining tray with blocking solution and cover tray with plastic wrap.
9. Store membrane at 4° C.

Part 2 -

1. Obtain membrane from 4° C freezer.
2. Add 10 mL anti-myosin primary antibody to tray and rock for 20 minutes.
3. Pour off anti-myosin primary antibody and rinse with 50 mL wash buffer and pour off.
4. Add 50 mL wash buffer again and rock for 3 minutes.
5. Pour off wash buffer and add 10 mL second antibody and place on rocker for 15 minutes at setting high enough to cover membrane.
6. Rinse with 50 mL wash buffer.
7. Add 50 mL wash buffer and place on rocker for 3 minutes.
8. Pour off wash buffer and add 10 mL horse radish peroxidase (HRP). Place on rocker for 10 minutes.
9. Once bands develop discard HRP and rinse twice with DI water.
10. Pat dry membrane with paper towel and let air dry for 1 hour.
11. Take photo of dried membrane.
12. Using ImageJ, measure the distances migrated for both myosin light chain 1 (MLC1) and myosin light chain 2 (MLC2) for all the fish samples and Actin & Myosin standards and using the standard curve from SDS PAGE calculate the molecular weights.

Results:

Part A - Preparation

No results to present.

Part B - Electrophoresis and Start of Western Blot

Time gel ran: 21 minutes
Gel length: 7.1 cm

The gels that we ran, as indicated by the instructor, were not the best batch of gels. As you can see below in Figure 3. the bands for each of the lanes are not too straight and dip down in the middle. Therefore the class as a group will be using an image of a gel from a previous version of this specific lab shown in Figure 4. below.

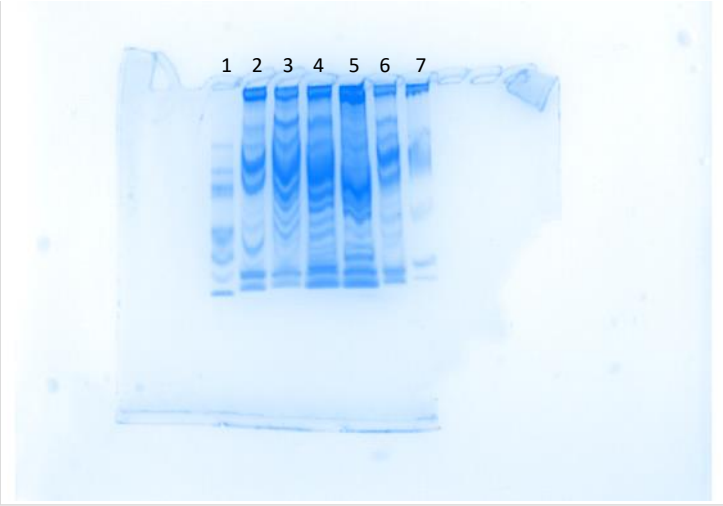

Figure 3. Image of gel we ran for this lab from running the electrophoresis on 4-20% Mini-PROTEAN TGX gels. Gel was ran for 21 minutes at 200V. Gel was then stained with 50 mL of Bio-Safe Coomassie Stain and placed on shaker for 1 hour and then de-stained with DI water for 1 hour. There may have been issue with these gels that we ran as the bands ran wrong. 1: Protein Standards, 2: Cod, 3: Salmon, 4: Trout, 5: Catfish, 6: Flounder, 7: Actin & Myosin standards.

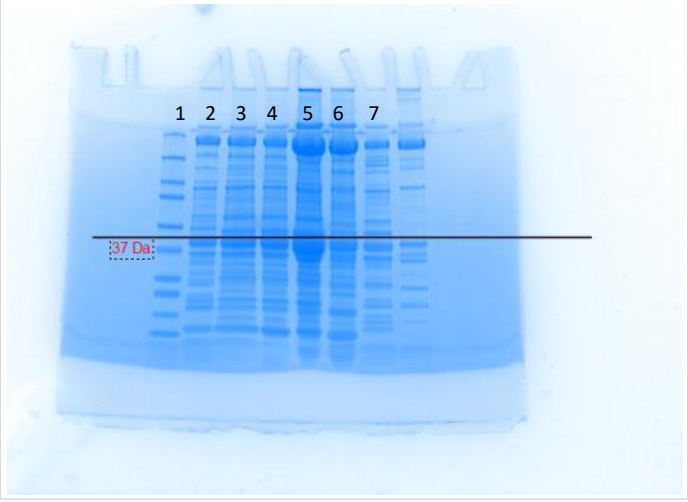

Figure 4. Image of Gel ran from a previous lab with better results. We used this gel for the measurements of the bands for all the columns. We started all measurements measuring from the blank line at the 50 kDA standard for each fish. 1: Protein Standards, 2: Cod, 3: Salmon, 4: Trout, 5: Catfish, 6: Flounder, 7: Actin & Myosin standards.

Length of Gel (bottom of loading well to bottom of gel**): _____7.1 cm_____**

When taking the measurements of the migration distances for the protein standards as well as the fish samples we measured from the bottom of the respective well to the bottom of the bands. All measurements taken using ImageJ software were completed in centimeters (cm).

Protein Standards from 10 –50 kDa

Precision Plus Protein Kaleidoscope Prestained Standards Molecular Mass (kDa)	Logarithmic Transformation of Molecular Mass	Distance Migrated (cm)
250	2.40	0.962
150	2.18	1.424
100	2	2.001
75	1.88	2.309
50	1.70	2.982
37	1.57	3.444
25	1.40	4.021
20	1.30	4.387

15	1.18	4.753
10	1.0	5.137

Table 2. Molecular weight and log transformed molecular weight of the Precision Plus Protein Kaleidoscope Prestained Standards along with the distances migrated for each of the standard bands. This table was used to create the standard curve and standard curve equation in Figure 4.

When taking the measurements of the migration distances for fish samples on the first go around the distances were a little bit off so we went again and remeasured for two of the samples, salmon and trout. The protein bands we started to measure at are indicated in Figure 4. just below the black line and just above the 37 kDa protein standard in lane 1.

Samples and Action/Myosin Standards 50 kDa and under

	Distances Migrated (cm)										
	Band 1 (just below 50 kDa)	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8	Band 9	Band 10	Band 11
Cod	3.290	3.463	3.646	3.916	4.012	4.098	4.320	4.454	4.608	4.781	5.099
Salmon	3.311	3.467	3.66	3.852	3.97	4.067	4.239	4.425	4.592	4.804	5.060
Trout	3.327	3.501	3.676	3.888	4.005	4.082	4.237	4.469	4.623	4.856	5.126
Catfish	3.361	3.496	3.592	3.707	3.932	4.13	4.336	4.47	4.721	4.913	5.259
Flounder	3.361	3.54	3.637	3.72	3.964	4.143	4.149	4.535	4.714	4.881	5.016
Actin & Myosin STDs	3.29	3.47	3.617	3.867	4.175	4.535	4.849				

Table 3. Distances migrated in centimeters for each of the 5 different fish samples as well as the actin and myosin standards. The bands for the starting measure points we just over the 37 kDa protein standard and below the 50 kDa protein standard.

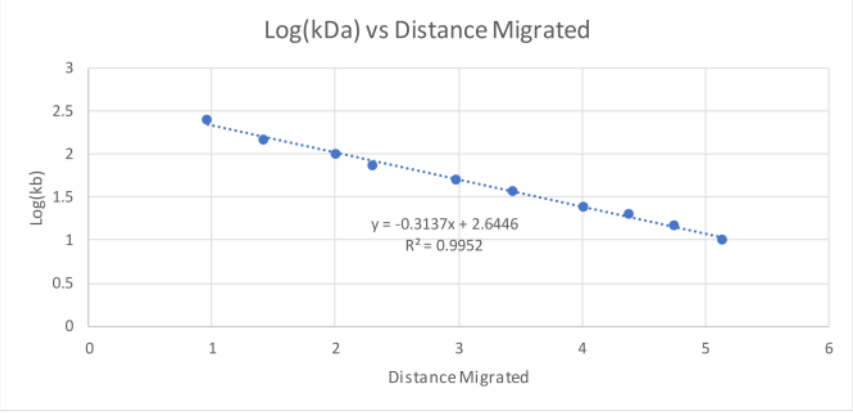

Figure 4. Standard curve for molecular weights of the Precision Plus Protein Kaleidoscope Pre-stained Standards. Molecular weights are on a $\log_{10}$ scale on the y-axis and distances migrated. First point is for 50 kDa molecular weight protein while last point is for 10 kDa molecular weight protein.

Standard Curve Equation (y=mx+b): $\log_{10}$(Mol weight) = -0.3137(Distance Migrated) + 2.6446

R^2: 0.9952

Our accuracy of our standard curve was pretty high as indicated by the R^2 value of 0.99. We can also see visually that the large proteins migrated less than the smaller proteins and so we have a negative slope.

Conversion migrated weights based on inverse $\log_{10}$(Mol weight)

	Molecular Weight (kDa)										
	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8	Band 9	Band 10	Band 11
Cod	40.976	36.162	31.685	26.071	24.324	22.859	19.472	17.676	15.815	13.957	11.093
Salmon	40.359	36.058	31.366	27.304	25.073	23.377	20.586	17.894	15.769	13.727	11.618
Trout	39.895	35.183	31.006	26.603	24.447	23.125	20.675	17.485	15.645	13.221	10.879

Catfish	38.927	35.311	32.945	30.319	25.771	22.337	19.428	17.473	14.575	12.688	9.882
Flounder	38.927	34.206	31.891	30.036	25.182	22.128	18.128	16.671	14.649	12.985	11.778
Actin & Myosin STDs	40.796	35.980	32.355	27.010	21.622	16.671	13.288				

Table 4. This is the inverse $\log_{10}$ transformation from Table 3. This represents the actual molecular weights of the bands on the gel in kDa.

When we created a graph to portray the weights for each of the bands for all 5 fish we see that in Figure 5. that the salmon and trout lines (see figure for details) lined up the closest than the other fish did to each other. The cod and catfish may look like the second two closest species and flounder did not really align much with any species from the graph representation below. We will quantify this below however to complement the visual.

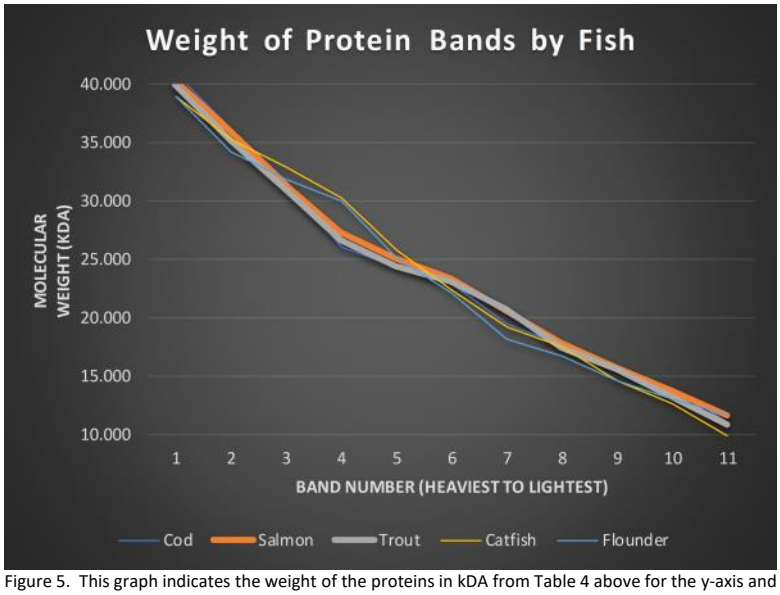

Figure 5. This graph indicates the weight of the proteins in kDA from Table 4 above for the y-axis and the protein band number from the gels on the x-axis. As you can see the two lines that follow the closest are orange and gray for Salmon and Trout respectively. The cod and catfish lines, darker blue and orangish-yellow respectively, were the second two relatively closest.

Molecular Mass Protein marking

Distance Migrated (cm)	Protein Molecular Mass (kDa)	Cod	Salmon	Trout	Catfish	Flounder
3.29-3.31	40.5-41	X				
3.31-3.33	39.8-40.5		X	X		
3.36-3.39	38-39				X	X
3.43-3.47	36.1-37.1	X				
3.47-3.5	35.1-36.1		X		X	
3.51-3.55	34-35					X
3.59-3.63	32-33				X	
3.63-3.68	31-32	X	X	X		X
3.68-3.72	30-31				X	X
3.82-3.87	27-28		X			
3.87-3.92	26-27	X		X		
3.92-3.96	25.2-26				X	X
3.97-4.02	24.1-25.1	X	X	X		
4.03-4.09	23-24		X	X		
4.09-4.15	22-23	X			X	X
4.22-4.28	20-21		X	X		
4.28-4.35	19-20	X			X	
4.35-4.43	18-19					X
4.43-4.51	17-18	X	X	X	X	
4.51-4.59	16-17					X
4.59-4.68	15-16	X	X	X		
4.68-4.78	14-15				X	X

4.78-4.88	13-14	X	X	X		
4.88-4.99	12-13				X	X
4.99-5.11	11-12	X	X			X
5.12-5.27	9.8-10.9			X	X	

Table 5. This table reflects where each of the bands falls on a range for the distance migrated and the Molecular weight in kDa. Please note that the ranges for the distance migrated and weight are both increasing so they are reversed with respect to the hyphen. i.e. for distance migrated of 3.29-3.31 cm and protein mass range of 40.5-41 kDA, the 3.29 cm lower bound of range refers to the 41 kDA upper bound of range. We decided to keep the ranges always increasing for simplicity.

By analyzing the common marked rows in Table 5. we were able to quantify the common proteins across each species in Table 6. below. As salmon and trout are closer together in the evolutionary tree, our hypothesis that two species that share a closer common evolutionary ancestor would have similar protein migration profiles was correct.

Common Proteins Count

	Cod	Salmon	Trout	Catfish	Flounder
Cod	11	6	6	3	3
Salmon	6	11	8	2	2
Trout	6	8	11	1	1
Catfish	3	2	1	11	6
Flounder	3	2	1	6	11

Table 6. This table represent a quantification of the common marked rows from Table 5.

Part B - Western Blot

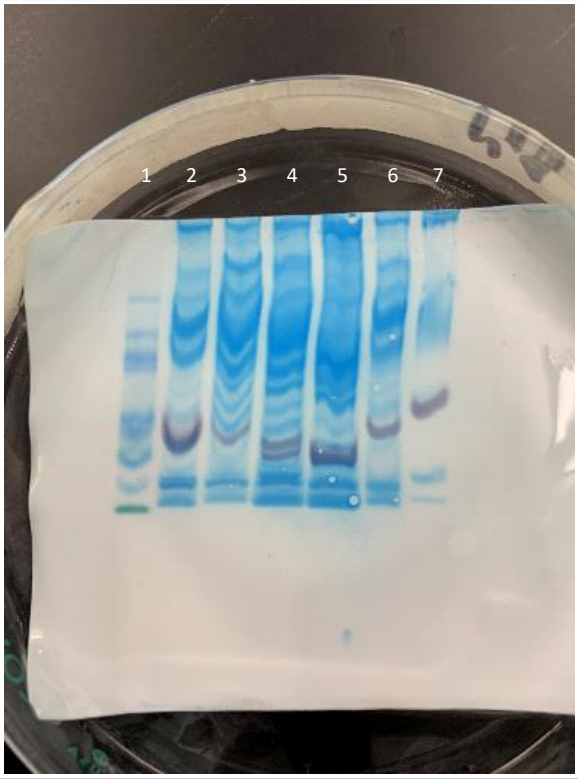

Figure 6. Western blot membrane from our gel transfer tagged with antibodies for each of the myosin light chain proteins. Cellulose membrane was treated with 10 mL anti-myosin primary antibody for 20 minutes followed by 50 mL rinse with DI water. Membrane was then treated with 10 mL of secondary antibody followed by 50 mL rinse with DI water and additional 3 minutes wash of DI water on shake. Lastly membrane was treated with add 10 mL horse radish peroxidase (HRP). 1: Protein Standards, 2: Cod, 3: Salmon, 4: Trout, 5: Catfish, 6: Flounder, 7: Actin & Myosin standards.

As we did not have a valid gel for our Western Blot transfer we were not able to measure the distances migrated on the cellulose membrane with the tagged antibodies for myosin light chain 1 and 2. Therefore we will not be completing the tables below.

	Distance Migrated (cm)	
	MLC1	**MLC2**
Cod	_____	_____
Salmon	_____	_____
Trout	_____	_____
Catfish	_____	_____
Flounder	_____	_____
Actin/Myosin STD	_____	_____

	Molecular Weight (kDa)	Molecular Weight (kDa)
	MLC1	**MLC2**
Cod	_____	_____
Salmon	_____	_____
Trout	_____	_____
Fish Species D	_____	_____
Fish Species E	_____	_____
Actin/Myosin STD	_____	_____

Discussion/Conclusion:

Since we were not to make the measurements for the protein profiles on our individual gels due to some error in the gel preparation by Bio-rad, the entire class made measurements on a gel previously ran for this particular lab. According to the results for the number of proteins shared in Table 6., Salmon and Trout has the closest protein profiles. As they are the closest in the evolutions tree our hypothesis was correct that they would have the closest protein profiles. This makes sense as these species are not only closer in the evolutionary tree but as described in Post Lab question 4A, when we were directed to look up the fish in the database fishbase.net I noticed a phenotypical resemblance between both salmon and trout and that these species are mostly in fresh water. Additionally, evidence that the protein profiles are similar for Salmon and Trout according to our anti-myosin antibody tagging in Figure 6. (lanes 3 & 4) and that of the best groups results of anti-myosin antibody tagging, we can conclude that even though the bands are bent, the bottom of the only band in lane 3 for Salmon matches up to the bottom of the top band in lane 4 for Trout. This provides evidence that the myosin light chain protein is very similar in molecular weight and length in Salmon and Trout.

The two species that had the most dissimilar profiles were Trout compared to both Catfish and Flounder. I would have expected Trout to be very different from Flounder based the facts that they look very different phenotypically and they are in completely different environments, freshwater lakes and the bottom of saltwater sea and ocean floors respectively. However, based on the anti-myosin tagging in Figure 6., it looks like the myosin light chain bands for both proteins are pretty close to each other in distance migrated in lanes 4 and 5 for Trout and Catfish respectively. I can attribute this to the fact that myosin light chains are evolutionarily conserved protein in muscle not only across fish species but mammals as well. I would expect a conserved orthologous protein like myosin light chain to be pretty close in size and shape in different fish species as they all basically share the same muscle movements to swim, breath, eat and survive.

On the cellulose membrane from the Western blot we were able to see tagged bands for myosin light chain 1/2 that were either very thick and mostly likely includes both chains 1 and 2 or two bands for both Trout and Catfish. The only species we did not see two bands or a thick band for myosin light chains 1 and 2 was for the Salmon species. I can only assume that there may not have been a sufficient

amount of myosin light chain either in the preparation stages of the muscle proteins, or the Western Blot membrane transfer stage for Salmon.

Resources:

1. Bio-Rad Laboratories, Inc. *Comparative Proteomics Kit I: Protein Profiler Module*. (2006). Retrieved from http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10004530.pdf

3/4/2020 - Lab 5: Kinetic Characterization of Trypsin

Wednesday, January 29, 2020 2:49 PM

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Title:

- **Part A - Enzyme Reaction Kinetics**
- **Part B - Effect of Temperature on Enzyme Activity**

Background:

Polymer macromolecules from food need to be broken down until smaller molecules to be transported across membranes so they can be used by cells for energy and to perform necessary function, replication as well as programmed cell death. Digestive system uses enzymes to chemically break down large macromolecules into smaller molecules. One example of these enzymes is Trypsin. Trypsin is a serine protease and being a protease it is able to break down and hydrolyze long chain polypeptide proteins into their amino acid monomers to be used by the cell. Trypsin is not always in its active form and is stored as a precursor, trypsinogen, in the pancreas before entering the intestines where it is activated by another enzyme where it is able to recognize and cleave peptide bonds of a substrate on the C-terminal side of positively charged amino acids (see Figure 1).

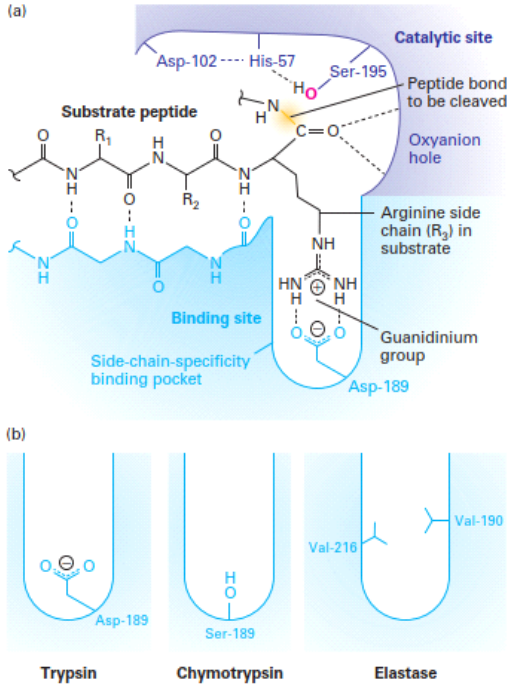


Figure 1. Image taken from "Molecular Cell Biology: 8th Edition" by Lodish, etal.[1] a) shows the active site for serine proteases. The substrate peptide fits into the active site of the protease which include an aspartate, histidine, and serine residues as well an enzyme specific side chain binding pocket. b) the different residues of the enzyme specific binding pockets for trypsin, chymotrypsin, and elastase. Trypsin is able to specifically recognize and cleave C-terminal side of positively charged amino acids because of the

The substrate we will use in our lab to analyze the enzyme kinetics of trypsin is the artificial substrate benzoyl-dL-arginine p-nitroaniline (BAPNA). We will use one of the resulting products of trypsin cleavage of BAPNA, p-nitroaniline, which has a yellow pigment that can be measured over time using a spectrophotometer at an absorbance level 410 nm. We will measure the rate at which p-nitroanaline is synthesized until the leveling off point for a variety of initial substrate concentrations of BAPNA to plot a curve of substrate concentration vs the initial rate of reaction which will be measured as absorbency at 410 nm. The maximum absorbency level will be the V_{max} for trypsin and the substrate concentration that reaches 1/2 V_{max} and half the enzyme's active sites are occupied will be its K_m. We will be performing reactions at two different temperatures, room temperature and another chosen temperature and completing plots for reaction rate vs substrate concentration as well as double reciprocal Lineweaver-Burke plot to compare the kinetics of trypsin at different temperatures.

Hypothesis:

As the optimal temperature for trypsin activity is human body temperature, I predict that when increasing the temperature for the trypsin reaction from room temperature to body temperature, the rate of reaction V_o will increase.

Protocol:

Part A - Enzyme Reaction Kinetics

Materials:

- Sample of benzoyl-dL-arginine p-nitroaniline (BAPNA) at varying concentrations:
  - 0.75 mM, 1.0 mM, 1.5 mM, 1.75 mM, 2.0 mM, 2.5 mM, or 3.0 mM
- Tris buffer
- 1 mM HCl
- Trypsin solution $\left(\frac{1.0\ mg\ trypsin}{1\ mL * 1\ mM\ HCl}\right)$
- Parafilm
- Cuvettes
- Denovix QFX Fluorometer
- Excel Software

Procedures:

1. Setup Denovix QFX Fluorometer for "Kinetic" option and change wavelength to 410 nm.
2. Make sure to uncheck "Set Heater Temperature"
3. Set interval and duration for stage 1 and 2.
4. Obtain group sample of benzoyl-dL-arginine p-nitroaniline (BAPNA) and record concentration marked on tube.
5. Prepare blank cuvette with:
   a. 2 mL of 1.5 mM BAPNA
   b. 0.9 mL Tris buffer (Tris-HCl + $CaCl_2$)
   c. 0.1 mL 1 mM HCl
6. Invert cuvette to mix components.
7. Run the blank on the Denovix QFX Fluorometer.
8. Prepare trypsin reaction in cuvette 2 with:
   a. 2 mL of 1.5 mM BAPNA
   b. 0.9 mL Tris buffer
9. Quickly add 0.1 mL trypsin $\left(\frac{1.0\ mg\ trypsin}{1\ mL * 1\ mM\ HCl}\right)$.
10. Seal and invert cuvette 2.
11. Cap cuvette with lid and invert.
12. Insert cuvette into Denovix QFX Fluorometer as quickly as possible.
13. Records A410 nm for each time measured.
14. Run 10 minutes or until measurement start to level off.
15. Calculate V_o in Excel with measurements.
    a. Add column for minutes and column for amount p-nitroaniline formed
    b. Convert seconds to minutes
    c. Create graph with time on x-axis and p-nitroaniline on y-axis.
    d. Keeping only the linear data points add trendline, equation and R^2 values.
    e. Record the slope 'm' or V_o for your BAPNA concentration as well as for all the other groups.
16. Construct Lineweaver-Burke Plot to calculate Km and Vmax by calculating and graphing the double reciprocal for 1/[BAPNA] and $1/V_o$.
    a. Use equation below to solve for Vmax and Km:

$$\frac{1}{Vo} = \frac{Km}{Vmax} * \frac{1}{[Substrate]} + \frac{1}{Vmax}$$

Part B - Effect of Temperature on Enzyme Activity

Materials:

- Same as Part A
- Water baths/incubators/ice

Procedures:

1. Select the trypsin sample from the 37°C incubator/water bath.
2. Using same concentrations as Part A, or if doing 0.75 mM concentration use higher concentration. Record concentration.
3. Place BAPNA, 1 mM HCl, and trypsin in chosen temperature above for at least 15 minutes.
4. Prepare blank cuvette as in steps 5-7 of Part A. above and blank Denovix QFX Fluorometer.
5. Prepare 2nd cuvette as in steps 8-11 of Part A above and records A410 as in step 12 above.
6. Continue to run for 10 minutes or until measurements level off.
7. Calculate V_o as in Part A above and record and compare with that of room temperature.

Results:

Part A - Enzyme Reaction Kinetics

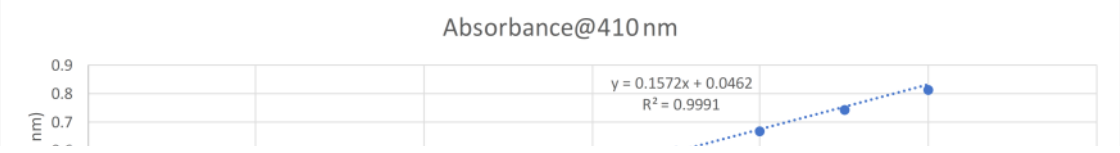
Concentration of BAPNA: _____1.5_____ (mM)

Temperature of solution at room temperature: _____22.4° C_____

Trypsin reaction at room temperature:

Time	Absorbance@410 nm	Time	Absorbance@410 nm
0 seconds (0 min)	0.036	2.5 minutes	0.448
10 seconds (0.167 min)	0.065	3.0 minutes	0.526
20 seconds (0.333 min)	0.092	3.5 minutes	0.602
30 seconds (0.5 min)	0.129	4.0 minutes	0.673
40 seconds (0.667 min)	0.146	4.5 minutes	0.747
50 seconds (0.833 min)	0.174	5.0 minutes	0.818
60 seconds (1 min)	0.202	5.5 minutes	0.89
70 seconds (1.167 min)	0.229	6.0 minutes	0.959
80 seconds (1.333 min)	0.259	6.5 minutes	1.029
90 seconds (1.5 min)	0.292	7.0 minutes	1.093
100 seconds (1.667 min)	0.312	7.5 minutes	1.159
110 seconds (1.833 min)	0.34	8.0 minutes	1.216
120 seconds (2 minutes)	0.368	8.5 minutes	1.28
		9.0 minutes	1.337
		9.5 minutes	1.392
		10.0 minutes	1.445

Table 1: Measuring the absorbency of product of trypsin reaction at room temperature. The absorbance at 410 nm measures how much product, p-nitroaniline, has been produced by the reaction of trypsin on BAPNA. Note the time highlighted in yellow marks where the absorbency started to level off.



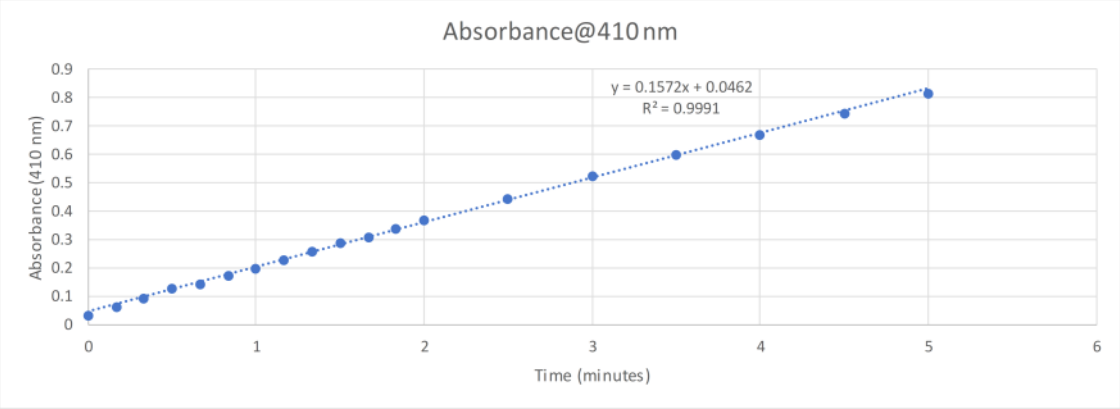


Figure 2. This graph represents the numerical values in Table 1. for the readings from the catalysis of trypsin at room temperature. The results Go up to 5 minutes where the rate of catalysis was no longer linear. The slope of the line represents the rate of catalysis for substrate concentration of 1.5 mM BAPNA.

Initial rate of reaction (V_o) at room temperature: ___0.1572_____

Group Names	BAPNA Concentration	V_o
		(p-nitroaniline/minute/0.1 mg trypsin)
	0.75	0.1324
	1	0.1508
	1.5	0.1572
	1.75	0.1599
	2	0.1631
	2.5	0.108
	3	0.126

Table 2. The various initial rates of trypsin catalysis for varying concentrations of substrate BAPNA. Note the yellow highlight is the cutoff where the results below we withheld from the downstream analysis for V_{max} and K_m.

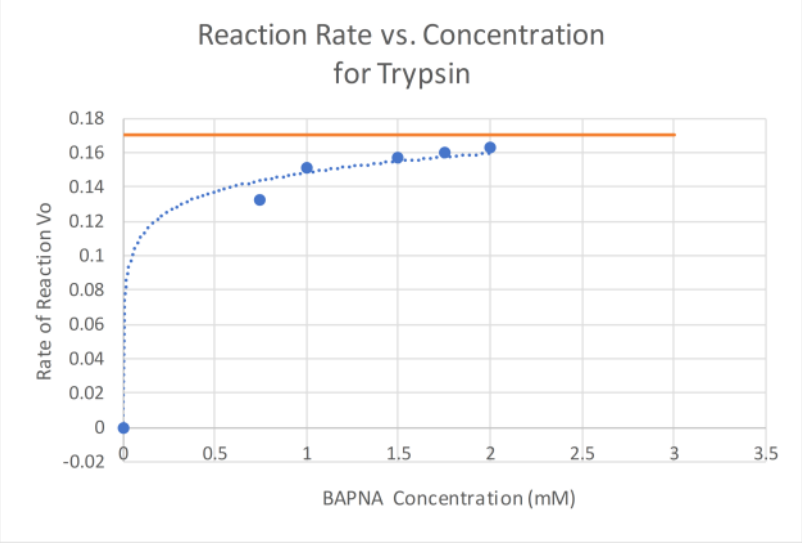


Figure 3. The plot of the various concentrations of substrate BAPNA and the initial rates of catalysis from Table 2. Note the line should be fit better than logarithmic fit from Excel. It is too steep in the beginning.

1/ [BAPNA] (mM^{-1})	$1/V_o$
1.33	7.55
1.00	6.63
0.67	6.36
0.57	6.25

0.50	6.13
0.40	9.26
0.33	7.94

Table 3. This table represents the reciprocal of each of the columns from Table 2.

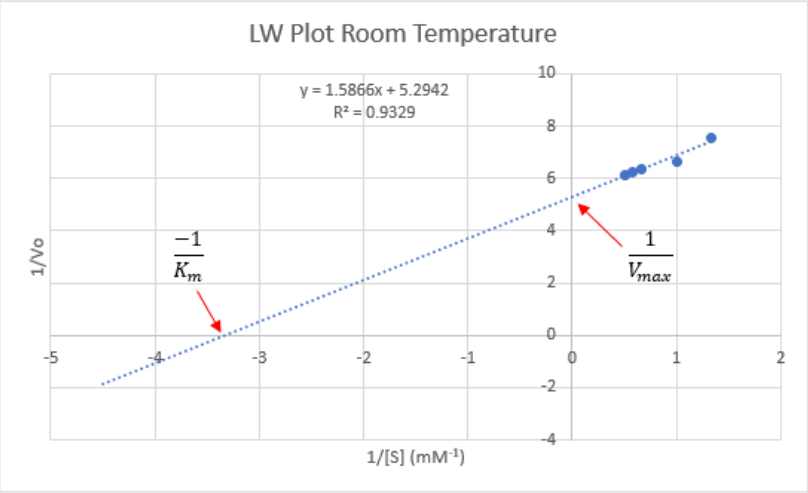


Figure 4. The Lineweaver-Burke plot from the data in Table 3 for the reciprocal of the concentrations of substrate BABNA and reciprocal for the initial rate of reactions for the catalysis of trypsin. Note the y-intercept represents the reciprocal of V_{max} and the x-intercept represents the negative reciprocal of K_m.

$$\frac{1}{Vo} = \frac{Km}{Vmax} * \frac{1}{[Substrate]} + \frac{1}{Vmax}$$

$$\frac{1}{Vo} = y \quad , \quad \frac{1}{[S]} = x$$

$$\frac{Km}{Vmax} = 1.5866\ (slope)$$

When x = 0, $\frac{1}{Vo} = y = \frac{1}{Vmax} = y - intercept$:

$$\frac{1}{Vmax} = 5.2942\ \ (y - intercept)$$

$$Vmax = \frac{1}{5.2942} = 0.1889$$

When y = 0, $0 = \frac{Km}{Vmax} * \frac{1}{[S]} + \frac{1}{Vmax} = x - intercept$:

Need to find x-intercept where $x = \frac{1}{[S]}$

$$0 = \frac{Km}{Vmax} * \frac{1}{[S]} + \frac{1}{Vmax}$$

$$-\frac{1}{Vmax} = \frac{Km}{Vmax} * \frac{1}{[S]} \quad \text{(divide by } \frac{Km}{Vmax}\text{)}$$

$$-\frac{1}{Km} = \frac{1}{[S]} = x - intercept \quad \text{(because y = 0)}$$

$$0 = 1.5866x + 5.2942$$

$$-5.294 = 1.5866x$$

$$-\frac{5.294}{1.5866} = x = -3.3368 = x - intercept \quad \text{(because y = 0)}$$

Now solve for K using x-intercept

$$-\frac{1}{Km} = x - intercept = -3.3368$$

$-3.3368 * Km = -1$

$$Km = \frac{-1}{-3.3368} = 0.2997$$

Vmax: _____0.1889_____

Km: _____0.2977 mM_____

Part B - Effect of Temperature on Enzyme Activity

Concentration of BAPNA: ___1.5 mM_____ (mM) <-make sure to include in your procedures

Temperature tested: _____37 C_____

Trypsin reaction: Change in Temperature

Time	Absorbance@410 nm	Time	Absorbance@410 nm
0 seconds (0 min)	0.041	2.5 minutes	0.33
10 seconds (0.167 min)	0.064	3.0 minutes	0.378
20 seconds (0.333 min)	0.084	3.5 minutes	0.425
30 seconds (0.5 min)	0.103	4.0 minutes	0.469
40 seconds (0.667 min)	0.122	4.5 minutes	0.513
50 seconds (0.833 min)	0.141	5.0 minutes	0.556
60 seconds (1 min)	0.161	5.5 minutes	0.595
70 seconds (1.167 min)	0.181	6.0 minutes	0.631
80 seconds (1.333 min)	0.2	6.5 minutes	0.663
90 seconds (1.5 min)	0.21	7.0 minutes	0.69
100 seconds (1.667 min)	0.23	7.5 minutes	0.727
110 seconds (1.833 min)	0.254	8.0 minutes	0.756
120 seconds (2 minutes)	0.273	8.5 minutes	0.78
		9.0 minutes	0.803
		9.5 minutes	0.829
		10.0 minutes	0.854

Table 4. Measuring the absorbency of product of trypsin reaction at 37°C. The absorbance at 410 nm measures how much product, p-nitroaniline, has been produced by the reaction of trypsin on BAPNA.

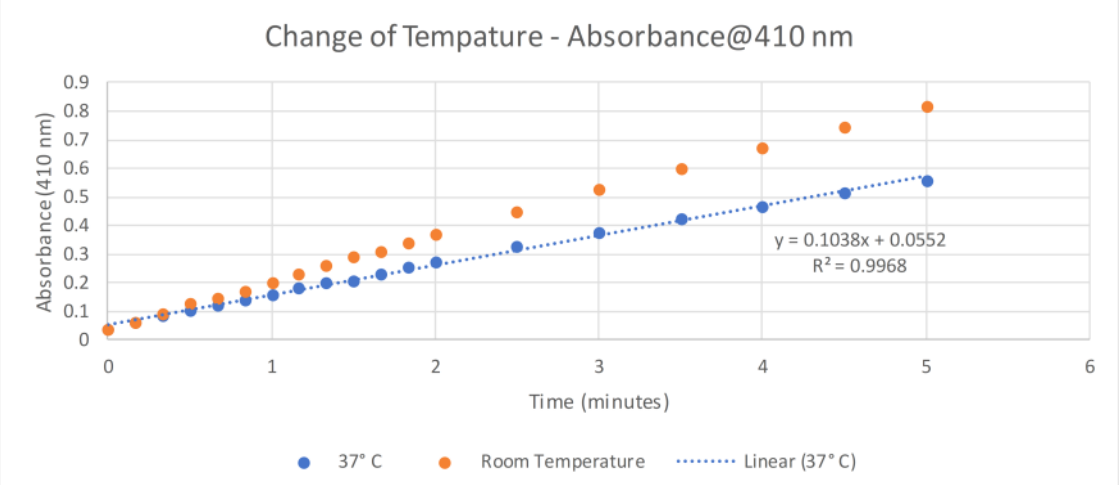


Figure 5. This figure represents the rates of product absorbance from the numerical values from Table 4 for trypsin at body temperature. The slope blue line represents the initial rate of reaction of trypsin with BAPNA substrate concentration of 1.5 mM. The results from the catalysis of trypsin at rooms temperature with same substrate concentrations are superimposed on orange.

Initial rate of reaction (V_o) at new temperature: ___.1038_____

Discussion/Conclusion:

The results we observed when we changed the temperature for the reaction of trypsin were not as expected. Trypsin is an enzyme in the intestines and pancreas of mammals which is primarily kept at around 37°C. Trypsin should work best at this temperature and it's rate of catalysis should be maximum at body temperature. However, we saw a decrease in the rate of catalysis, indicated by the slope of the blue line at body temperature compared to the higher slope of room temperature in orange. I can only assume that this result occurred because the trypsin either did not mix well enough with BAPNA, we may have forgot to set the temperature of the Denovix QFX Fluorometer to 37°C, the pH/salt content o of the buffer for the trypsin may not have been exactly 8.2 for our sample at body temperature, or the concentration of trypsin at 37°C could have been different than 1.0 mg/mL of 0.001 M HCl. Any of these errors/differences or a combination of any could have influenced why we saw a drop in rate of catalysis when we should have observed an increase in catalysis.

Resources:

1. Lodish, H., Berk, A., Kaiser, C., Krieger, M., Bretscher, A., Ploegh, H., Amon, A. and Martin, K. (2016). Molecular Cell Biology. New York: W.H. Freeman and Company.

3/11/2020 - Lab 6: Gel Filtration Chromatography

Wednesday, January 29, 2020 2:49 PM

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Title:

- **Gel Filtration Chromatography**

Background:

Gel filtration chromatography is another laboratory method that similar to gel electrophoresis is used for separating molecules such as DNA, proteins, and carbohydrate polymers by size. To separate the molecules we use a column that contains carbohydrate polymer beads and add water to the beads so they swell and contain different pore sizes. As the pore sizes are different sizes, the molecules can enter only if they are smaller than size of the pore. This means that smaller molecules can enter more beads that larger molecules and take longer to elute from the column. In this lab we will be using proteins standards with known weights to determine the molecular weight of rabbit hemoglobin which will be unknown at the start. In order to standardize the volumes that are eluted from the column and minimize the variation for each of the known protein standards as well as the unknown rabbit hemoglobin, we will also be using a polysaccharide, Blue Dextran, which will completely avoid entering any of the beads because of its size. Using the known molecular weights for 3 protein standards, Horse Cytochrome C, Horse Myoglobin, and Bovine Serum Albumin + bromothymol blue dye, we will created a standard curve with these weights and their standardized elution volumes (V_e) to void volume (V_o) ratio we will then be able to calculate the weight of Rabbit Hemoglobin based on its elution volume ratio.

Hypothesis:

As smaller molecules take long to elute because they spend more time in the beads of the column than larger molecules, and since the expected molecular weight of Rabbit Hemoglobin is around 4,000 daltons less than BSA/bromothymol blue, I hypothesize that elution volume (Ve) of Rabbit Hemoglobin will be slightly more than the elution volume for BSA/bromothymol blue.

Protocol:

Materials:

- Ring stand
- 9 mL chromatography column
- 20 mL reservoir
- Porous disk
- Column cap
- Sephadex G-100 (hydrated)
- 0.5 mL microcentrifuge tubes
- Transfer pipettes
- Micropipettes with tips
- Small break for waste buffer
- Wash buffer
- 10 mL graduated cylinder
- Ruler
- Horse Cytochrome C
- Horse Myoglobin
- Blue Dextran
- Bovine Serum Albumin (BSA)/bromothymol blue
- Rabbit Hemoglobin
- Excel software

Procedures:

1. The assembling of chromatographic columns and bead packing process will be completed by the instructor.

2. Separate the standards:
   Obtain two standard sample tubes for the following labelled samples to run on one column:
   1. Horse Myoglobin and Blue Dextran
   2. Horse Cytochrome c and BSA/bromothymol blue

Molecule	Chemical Nature	Color	Molecular Weight*
Horse Cytochrome C	Protein	Orange	12,000
Horse Myoglobin	Protein	Brown	17,000
Bovine Serum Albumin + bromothymol blue dye	Protein	Blue	68,624 P = 68,000 Dye = 624
Rabbit Hemoglobin	Protein	Red	TBD**
Blue Dextran	Polysaccharide	Blue	2,000,000

   a. Remove cap from bottom of column to drain.
   b. Replace cap and remove column reservoir.
   c. Transfer 150 µL of one of the samples onto the column at the upper bed surface.
   d. After letting sample drain into gel bed, add 200 µL wash buffer.
   e. Add reservoir back to column and continue to add wash buffer until column and reservoir are filled.
   f. Placing graduated cylinder under column, remove the cap at bottom of column to collect the buffer.
   g. Observe the separation of the three substances holding a white piece of paper behind the column.
   h. Make sure to keep adding buffer so that the top of the gel bed is covered.
   i. As soon as first color substance Blue Dextran (or BSA/bromothymol blue) begins to exit the bottom of column record the elution volume of buffer.
   j. As soon as second colored substance Horse Myoglobin (or Horse Cytochrome c) begins to exits record its elution volume.
   k. Continually add buffer until all colored substance elutes out.
   l. Place cap back on bottom of column after running clear.
   m. Rinse and drain graduated cylinder.
   n. Repeat steps c-m with second sample.
3. Separating Rabbit Hemoglobin:
   a. After buffer is running clear and column is capped, add 100 µL Rabbit Hemoglobin onto column as in step c above. Repeat steps d-m above.
   b. Record the elution volume.
   c. Repeat k above until running clear.
   d. Rinse graduated cylinder.
   e. Calculate Ve/Vo for each of the standards and Rabbit Hemoglobin using Vo from Blue Dextran.
4. Determine Molecular Weight for Rabbit Hemoglobin:
   a. Using Excel software create standard curve using the 3 protein standards.
   b. Make the y-axis the log transformed weight in daltons and the x-axis the Ve/Vo amount.
   c. Add trendline and record equation.
   d. Use equation to calculate the log molecular weight for Rabbit Hemoglobin and then transform back to daltons.
   e. Record the molecular weight for Rabbit Hemoglobin and then calculate late the amount of amino acids by dividing by 120 daltons.
   f. Calculate the percent error for the expected molecular weight of Rabbit Hemoglobin.

Results:

Elution Volumes (Ve) in mL:

Blue dextran	2.2 mL	BSA/bromothymol blue	2.5 mL
Horse Myoglobin	3.8 mL	Horse Cytochrome C	4.5 mL

Table 1.

Elution Volumes (Ve) in mL:

Rabbit Hemoglobin	2.2 mL

Molecule	Molecular Weight	Log MW	Ve (mL)	Ve/Vo
Horse Cytochrome C	12,000	4.0792	4.5	4.5/2.2 = 2.0455
Horse Myoglobin	17,000	4.2304	3.8	3.8/2.2 = 1.7273
BSA/bromothymol blue	68,000	4.8325	2.5	2.5/2.2 = 1.1364
Rabbit Hemoglobin	*See table 3	*See table 3	2.2	2.2/2.2 = 1

Table 2.

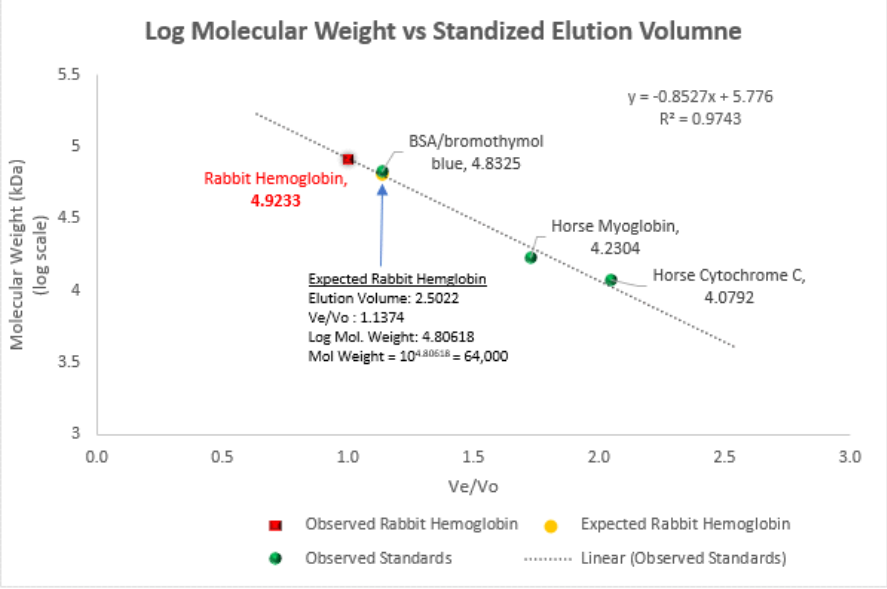

Figure 1. This chart represents the data from the experiment. The three standards from the experiment are in green in which the regression line was created. The observed value from the elution volume in the experiment for Rabbit Hemoglobin is indicated by the red square point. The yellow circular point shows where the expected standardized elution volume (Ve/Vo) should have been according to the regression line from the experiment as the expected molecular weight for Rabbit Hemoglobin provided by the instructor.

Calculation for Molecular Weight for Rabbit Hemoglobin:
Elution Volume - 2.2 mL
Ve/Vo - 1.0
Log Mol. Weight = -0.8527(Ve/Vo) + 5.776 = -0.8527(1.0) + 5.776 = 4.9233
Mol Weight = $10^{4.9233}$ = 83,810.8

Linear Regression Equation	Log Molecular Weight = -0.8527(Ve/Vo) + 5.776
Rabbit Hemoglobin (M.W.) *	83,810.8
Rabbit Hemoglobin (# of amino acids) **	83,810.8/120 = 698.4233

* Observed from Gel Filtration
** The average amino acid residue in a protein is 120 daltons[1]

The expected molecular weight for rabbit hemoglobin = 64,000 da

$$Percent\ Error = \frac{|Experimental\ MW - Expected\ MW|}{Expected\ MW} x\ 100\%$$

$$Percent\ Error = \frac{|83{,}810.8 - 64{,}000|}{64{,}000} x\ 100\%$$

$$\boldsymbol{Percent\ Error = 30.95\%}$$

Discussion/Conclusion:

As you can from the graphical representation in the figure 1 above with respect to the observed and expected molecular weight, the observed standardized elution volume was less than expected meaning that it was faster for the observed Rabbit Hemoglobin to elute and is the reason for the observed larger molecular weight. According to our standard curve we should have seen a greater standardized elution volume of 1.137 meaning that it should have taken a little longer for Rabbit Hemoglobin to elute indicative of the smaller expected weight. I believe a reason for this error, in which we observed lesser elution volume/faster elution, is that we noticed the beads started sinking from the top of column down as we added our samples into the reservoir, and so the Rabbit Hemoglobin sample which was added last, started at a lower level and therefore eluted faster than it should have and provides explanation for the over calculated molecular weight of around 130.95% of the expected weight.

Bonus/Extra

I also completed a sequence analysis using Biopython[2] and abstracted that fasta sequences from NCBI's protein database for the alpha subunit[3] and beta subunit[4]. I found that the tetramer (α2, β2) contains 572 total amino acids in total and according to the molecular mass from Biopython's class a total mass of 63,441.87 daltons for an average of 109.76 daltons (63,441.87/572) which aligns with average molecular weight of 110 daltons from a few resources [5,6,7]. I also found the 5 most common amino acid counts are [('L', 70), ('V', 56), ('A', 56), ('K', 48), ('S', 42)]. The reason biochemists use 110 daltons is to account for the weight of loss of water from the formation of the peptide bonds. Analysis script can be found at https://github.com/BJWiley233/Practical-Computer-Concepts-Files/blob/master/Python/Bioinformatics/lab_6_rabbit_HG.py

Resources:

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4/8/2020 - Lab 7: Erythrocyte Permeability and Transport Properties

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Wednesday, January 29, 2020 2:49 PM

Title:

- **Erythrocyte Permeability and Transport Properties**

Background:

The lipid bilayer of the plasma membrane and membrane organelles is semipermeable, where small non-polar molecules are able to easily cross the bilayer, small uncharged molecules such as water can diffuse more slowly, and larger molecules and ions cannot diffuse easily. Diffusion is the ability for movement of molecule from area of higher concentration of the molecule to lower concentration of the molecule. When concentrations of molecules inside and outside the cells produce a steep gradient from inside the cell to outside the cell, this can influence the diffusion of water across the membrane in a process called osmosis. We will be looking at the diffusion of water in three different environments where the concentration of a saline solution consisting NaCl will be either isotonic to blood serum in which the concentration of NaCl will be the same in the solution and our sample of heparinized sheep's blood, hypotonic in which the concentration of NaCl will be less in solution outside the cells compared to the blood causing water to flow in to higher NaCl concentration, and hypertonic solution with higher concentration of NaCl outside the blood in which water will flow out. We will also experiment with other solutes including small non-polar molecules, polar molecules, and two substances that disrupt plasma membranes. We will observe the time it takes for the cells in the heparinized sheep's blood to hemolyze which is proportional to the rate of water or lipid soluble molecule that diffuse across the membrane into the blood cells. Hemolysis is the process of the cell lysing or bursting due to increased water diffusion through the membrane into the cell.

Hypothesis:

Saline:

Because water will diffuse to the area that has a higher solute concentration, I predict that when placing the cells in a hypotonic solution of 0.077 M NaCl (hypotonic to the cell), this will produce the most rapid rate of hemolysis as there is a higher concentration of NaCl inside the cell causing water to flow in.

Lipid Soluble:

Because higher octanol-water partitioning coefficient indicates higher non-polarity and permeability, I predict that 2-proponal, with its higher K_{ow}, will have the highest rate of hemolysis for this group as the water will flow into the cells with the increased concentration of 2-propanol solute inside the cell.

Polar Molecules:

As polar molecules cannot diffuse through the lipid bilayer and require facilitated diffusion through specific pores, I predict that whatever pore size is most prevalent with respect to the size of the polar molecules, then that molecule will lead to the most rapid hemolysis. As we do not know which pore size is most prevalent we cannot know which one of the three pass through the bilayer through facilitated diffusion the greatest/fastest.

Agents that act directly on the membrane:

Because SDS is mixed with another agent, DTT, that helps to disrupt the membrane, I predict SDS with DTT will cause hemolysis faster than Triton-X.

Protocol:

Materials:

- See Lab Manual

Procedures:
- See Lab Manual

Results:

In the treatments for hypotonic, isotonic, and hypertonic solutions, the hypotonic solution of 0.077 M NaCl caused the blood to hemolyze faster and was noticed immediately as the image was seen in 1 second for a relative rate of hemolysis of 1/sec. The appearance of these cells under the microscope were like those of hemolyzed cell fragments. In both the isotonic solution at 0.154 M NaCl and hypertonic tonic solution at 0.513 M NaCl hemolysis was not notice within the 10 minutes and so both relative rates of hemolysis were less than 0.00166/sec. The appearance of the cells in the isotonic solution were intact and normal looking erythrocytes under the microscope and the appearance of the cells in the hypertonic solution were crenated or shriveled.

In the experiment section for small non-polar lipid soluble molecules, all molecules induced hemolysis and cell fragments were visible under the microscope except for 0.8 M glycerol which had intact normal cells. The 0.8 M n-propanol hemolyzed fastest with the image first being seen at 1 second, 0.8 M ethanol second fastest at 5 seconds, 0.8 M propylene glycol third fastest at 20 seconds, and 0.8 M ethylene glycol fourth fastest at 41 seconds for image being seen respectively. The relative rates for hemolysis for 0.8 M n-propanol, 0.8 M ethanol, 0.8 M propylene glycol, and 0.8 M ethylene glycol were 1/sec, 0.2/sec, 0.05/sec, and 0.024/sec respectively.

For the detergents the 2% Triton-X hemolyzed at 3 times the rate of 1% SDS + 10 nM DDT with the image being seen immediately at 1 second for 2% Triton-X and image being seen at 3 seconds for the latter. The relative rates of hemolysis of were 1/sec for the former and 0.33/sec for the latter. Cell fragments were noticed for each of the two detergents under the microscope. Finally the experiment section for polar molecules of different sizes, the 0.3 urea solution caused hemolysis fastest with the image being seen at 5 seconds. It took 93 seconds for the image to be seen for 0.3 M thiourea despite thiourea being not much larger of a molecule than urea. Both urea solutions showed cell fragments under the microscope. Glucose did not have any impact on hemolysis as the image was not able to be seen after 10 minutes of adding the sheep's blood to the glucose solution.

Treatment	Log Kow	Image First Seen – Time (sec)	Relative Rate (Sec -1)	Appearance (Intact, Crenated, Ghost, Cell Fragments Only)
DI H20	-	1	1	cell fragments
0.077 M NaCl	-	9	0.111	cell fragments
0.154 M NaCl	-	>600	<0.00166	intact, normal
0.513 M NaCl	-	>600	<0.00166	intact, crenated
0.8 M n-propanol	0.13	1	1	cell fragments
0.8 M ethanol	0.03	5	0.2	cell fragments
0.8 M propylene glycol	-0.92	20	0.05	cell fragments
0.8 M ethylene glycol	-1.36	41	0.024	cell fragments
0.8 M glycerol	-1.76	>600	<0.00166	intact, normal
2% Triton-X	-	1	1	cell fragments
1% SDS + 10 mM DDT	-	3	0.33	cell fragments
	Mol. Weight (g/mol)			
0.3 M urea	60	5	0.2	cell fragments
0.3 M thiourea	76	93	0.011	cell fragments
0.3 M glucose	180	>600	<0.00166	intact, normal

Table 1. The green highlighted lines refer to the lines I was correct in my hypothesis that led to hemolysis faster.
The red highlighted lines also led to hemolysis faster in which my hypothesis was wrong.

Discussion/Conclusion:

My hypothesis for incorrect for the larger polar molecules as my understanding of the mechanisms were incorrect. These results from the prior lab make sense now that I have reviewed again both lab documents (Word and PowerPoint). I was incorrect in assuming in the instructions from the Word document where it indicated "Larger polar molecules need to undergo facilitated diffusion by passing through channel proteins of specific pore sizes", where I had assumed that all three of these polar molecules were considered larger and that they can only diffuse through a pore specific to their size and that smaller large polar molecules such as urea would not be able to diffused through a pore that was larger than its size such as a pore the size of a glucose molecule. I think both of my assumptions were incorrect based on the results from the previous lab. Urea is considered a smaller large polar molecule and secondly a molecule that is smaller than the size of the pore can still diffuse through a membrane protein pore that is larger than its size. I had confused the terms selectivity filter from chapter 12: "Ion Channels Are Ion-Selective and Gated" and pore size to be the same thing when this is incorrect. After reading

slide 12 from the PowerPoint (ironic same chapter same slide) that urea would most likely not need a channel protein and can diffuse faster through the lipid bilayer or pass through transport protein with pore equal to its size or larger. Therefore urea being smaller than thiourea and glucose, it does make sense that adding bloods cells to urea would cause hemolysis to occur faster as urea can pass through the plasma membrane the fastest.

Adding blood cells to the hypotonic saline solution of .077 M NaCl induced hemolysis faster than the isotonic and hypertonic solution in concordance with the fact that with less solute outside the cell than inside which causes a concentration gradient of higher concentration of water outside the cell than inside cell and causing water to flow in. My hypothesis was correct here. My hypothesis was also correct for small nonpolar molecules, as molecules with higher octanol solubility compared to water solubility are able to permeate the plasma membrane faster causing a shift in the concentration of solute to being higher inside the cell causing hypotonic conditions and water to flow into the cell causing hemolysis faster. The results of the rate of hemolysis were directly correlated with the molecules's Log K_{ow}. The higher the K_{ow}, the faster the rate of hemolysis.

Triton-X caused faster disruption of the plasma membrane and induced hemolysis three times faster than SDS mixed with DTT. My hypothesis was incorrect. I did not take into account the factor that disruption and inducing hemolysis might be affected by whether the detergent were non-ionic, ionic, or polar. As Triton-X is non-polar, I would attribute this to the reason why the blood cells hemolyzed faster. If non-polar or non-ionic molecules can diffuse faster than polar and ionic molecules, I would assume or expect that non-ionic detergents can disrupt the plasma membrane faster than ionic detergents and this is why we saw the results of Triton-X causing hemolysis three times faster.

4/15/2020 - Lab 9: Clinical Diagnosis of Diabetes using ELISA

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Wednesday, January 29, 2020 2:49 PM

Title:

- **Clinical Diagnosis of Diabetes using ELISA**

Background:

Diabetes is classified by a person consistently having increased or higher blood sugar levels than the normal healthy amount of 75-100 mg glucose/dL of blood. There are two classification of diabetes; type I diabetes is primarily diagnosed in younger population and children and is caused by destruction of beta cells in the pancreas and decreased or completely depleted production of insulin which is a protein hormone that is secreted by the pancreas to signal cells to take up glucose from the blood to be utilized for energy conversion in cellular respiration. The other type of diabetes is type II diabetes and is caused by unhealthy nutrition and lack of exercise in which insulin is produced but the signaling between the insulin receptors on cell's membrane and the glucose transporters that regulate the influx of glucose becomes disrupted.

When a person begins to experience symptoms associated with those whom have been diagnosed with diabetes such as rapid weight loss and tiredness in juvenile diabetes type I as well as excessive thirst and urination which is are also symptoms of type II diabetes, a physician will first take a urine analysis to measure the glucose levels and glucose levels in the urine are directly correlated with glucose levels in the blood. In our lab today we will be measuring the amount of glucose in the urine of 3 samples as well as samples for negative control (no diabetes) and positive control (has diabetes). As glucose is a reducing sugar we will use the Benedict's test for estimating the amount of glucose in the urine. If the substrate $CuSO_4$ is reduced, the color of the solution will change color from green with lower levels but still increased glucose levels to bright red for extremely high levels of glucose. If levels are high in the urine and therefore the blood indicating diabetes, this can also be followed up by performing an enzyme-linked immunosorbent assay (ELISA) to determine the type of diabetes based on whether the assay detects mature insulin is present and therefore the ability for the patient's pancreas to produce insulin. The ELISA is a very interesting tool to detect proteins. It is similar to immunoblotting in that we use antibodies specific to the protein/antigen we are interested in, which in our case is the c-peptide of mature insulin. The difference with the ELISA is that both primary and secondary antibodies are specific to the antigen and the primary antibody is added first to a microarray plate, while in immunoblotting the protein/antigen is blotted first on a nitrocellulose membrane.

Hypothesis:

Patient 1: Patient 1 is a 50-year-old male of average weight, who considers himself very active. He has expressed symptoms of excessive urination. His last physical indicated he had high blood pressure.

Considering Patient 1 is very active and only experiences one of the symptoms, excessive urination, and as high blood pressure is usually a symptom of high salt/fluid in blood although high sugar in blood could have similar effect, I hypothesize this gentleman does not have diabetes and the frequent urination could be an effect of drinking lots of fluids during work outs.

Patient 2: Patient 2 is a 12-year-old female who is under weight. She has shown symptoms of excessive thirst and dramatic weight loss. Teachers have reported to her parents that she often falls asleep in class.

As Patient 2 has multiple symptoms of diabetes such as excessive thirst, rapids weight loss, and fatigue, and considering she is 12 years of age, I hypothesize this patient may have type I (1) diabetes as type I diabetes is consistent with juveniles.

Patient 3: Patient 3 is a 50-year-old male who is overweight and who lives a mostly sedentary lifestyle. He has expressed symptoms of excessive urination and his last physical indicated borderline high blood sugar levels.

As Patient 2 has a poor diet and is overweight along with displaying two symptoms of diabetes, having excessive urination and recently has had borderline high blood sugar levels, I hypothesize this patient was able to utilize his insulin when young, but now at an older age is not able to utilize his insulin because of resistance and defective signaling indicating he might have type II (2) diabetes.

Protocol:

Materials:

- See Lab Manual

Procedures:

- See Lab Manual

Results:

Results from test using Benedicts Solution.

According results below in Figure 1. The sample for patient 1, after adding Benedict's solution, the reagent in Benedicts solution was not reduced by the sample as observed by the blue color not changing indicating a blood glucose level of 0 mg/dL. The sample for patient 2 drastically reduced the reagent in Benedict's solution shown by the brick red color indicating a blood glucose level of > 2000 mg/dL. Patient 3's sample still reduced the Benedict's solution, albeit not as much at patient two shown by the bright orange color indicating a glucose blood level of around 500-1000 mg/dL. This patient is most likely diabetic with such high blood glucose levels.

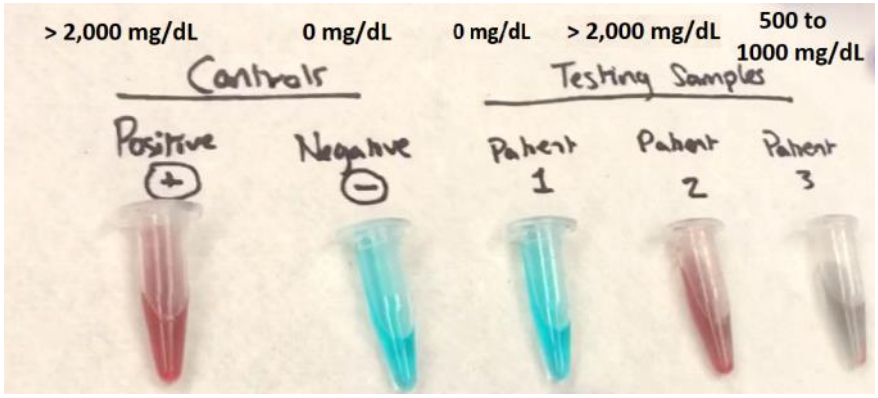

Figure 1. Benedict's test results for each of the three patients along with negative control for no diabetes and positive control for diabetes. Positive tests for diabetes in the patients are indicated by how red the solutions become which is directly proportion to the amount of reducing sugar in the sample. The estimated blood glucose levels are indicated above each sample.

According to the results of the ELISA test, the positive tagging green tagging for having c-peptide insulin for patient 1 is confirming patient 1 is able to produce insulin by having substantial amounts of c-peptide cleaved to produce mature insulin. Patient 2 does not produce any c-peptide based on the clear color on the microplate and is therefore not able to produce any mature insulin at all. Patient 3 had a positive tagging for c-peptide in the ELISA test which shows that although Patient 3 can produce mature insulin, albeit at a reduced amount with respect to the positive control and patient 1, is not able to utilize the insulin correctly in concordance with the high blood glucose levels in the Benedict's test and most likely has type II (2) diabetes.

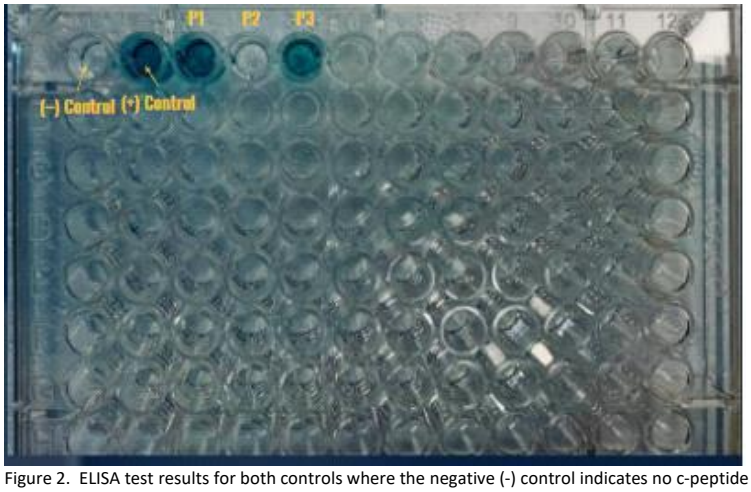

Figure 2. ELISA test results for both controls where the negative (-) control indicates no c-peptide aligning with the positive control above for the Benedict's test, while the positive (+) control indicates presence of c-peptide aligning with the negative control above for Benedict's test. Patient's 1, 2, and 3 are indicated by P1, P2, and P3 respectively.

Discussion/Conclusion:

The first patient seemed at first the hardest to diagnose as he had one symptom of diabetes with the excessive urination and a symptom of high blood pressure that could be overlapping with hypertension and diabetes diagnoses. I really had to make my best assessment based on the fact that his weight was normal and because of his very active lifestyle that maybe the frequent urination was due to staying well hydrated. Patient 1's urinalysis was negative for diabetes like the negative control with healthy level of blood glucose of 0 mg/dL after 12 hours of fasting. The positive tagging for c-peptide insulin confirms patient 1 does not have diabetes in concordance with his Benedict's test. This might not have been the right path to the correct diagnosis of not having diabetes as proved by the negative result of patient 1's urine analysis in combination with positive ELISA test for being able to produce mature insulin. In my assumption based on the case study, I believe patient 1 was closer to the realm of not having diabetes than having diabetes which ended up being correct.

The other two patients seemed easier to diagnose, of which patient 2 seemed the easiest. She showed all of the symptoms of juvenile diabetes including excessive weight loss and thirst along with the symptom of fatigue. Her urinalysis was positive for extremely high amounts of glucose, greater than 2000 mg/dL, shown by the high reduction and bright red color of the Benedict's solution. In addition to having high blood glucose it was indicated by the negative ELISA test for the inability to produce any mature c-peptide insulin. Given both the high blood glucose and lack of ability to produce any mature insulin, the hypothesis that patient 2 had type I juvenile diabetes was correct. This was not surprising as her history is very clear from her symptoms as well as her age that she had high probability of being positive for type I diabetes.

The third patient was also a good candidate for diabetes and given his age, it was pretty clear that it was not type I or he would have experienced severe symptoms earlier in his lifetime. He was also not severely underweight and his lifestyle and symptoms were sure tell signs of type II diabetes. This hypothesis was also correct since he had high levels of glucose from the urinalysis in the 500-1000 mg/dL range as indicated by the light orange color in the reduced Benedict's solution. In his follow up test he was still able to produce insulin so it was not lack of insulin that was the cause for his high blood glucose but some malfunction between the insulin pathway and glucose importer/import pathway indicating his prognosis for type II diabetes.

4/22/2020 - Lab 10: pGLO E. coli Biotransformation

Wednesday, January 29, 2020 2:49 PM

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Title:

- **pGLO E. coli Biotransformation**

Background:

Genetic transformation is an extremely valuable tool today in the field of Biotechnology. It is defined as changing/modifying the genetic content of an organism by inserting special DNA vehicles called plasmids or vectors which have genetic material for genes that can express certain genes, or activate or repress other genes depending on the location the organisms takes up the DNA plasmids. One application of genetic transformation is transforming bacteria with human insulin and growing mass amounts of it to be able to purify and use in the treatment of type I diabetes which we just covered in our previous lab.

In our lab today we will be genetically transforming E. coli with a specific DNA plasmid called pGLO. Looking at Figure 1. below we see that there is a lot going on in only 5 kb of DNA but of particular importance the Green-Fluorescent-Protein (GFP). This is a protein found in the jellyfish *Aequorea victoria* and fluoresces green when excited by UV light. This will help us in being able to select the bacteria which took up the pGLO plasmid. First however since, bacteria plasma membranes are only semipermeable and the negative charge of the DNA backbone is repelled by the polar head groups of the plasma membrane, we need to mask the charge with positive cations from dissociation of $CaCl_2$ so that the plasmid DNA can diffuse into the bacteria cell.

There are two other important genes and promoter within the plasmid pGLO. The first is the promoter araBAD which is a bidirectional promoter and in the presence of the sugar, arabinose, the protein AraC, a gene that is transcribed from the bidirectional promoter araBAD, changes conformation in a way to recruits RNA Polymerase to the promoter to turn on transcription and expression of GFP so the cells will fluoresce under UV light. The second important gene is AmpR which stands for ampicillin resistant/resistance and we also use this to select for successful transformation by plating the bacteria on agar plates with ampicillin. Only the bacteria that take up the plasmid will be resistant to the ampicillin on the agar and be able to grow while the cells that do not take up the plasmid will not be able to grow as they are not naturally resistant to ampicillin.

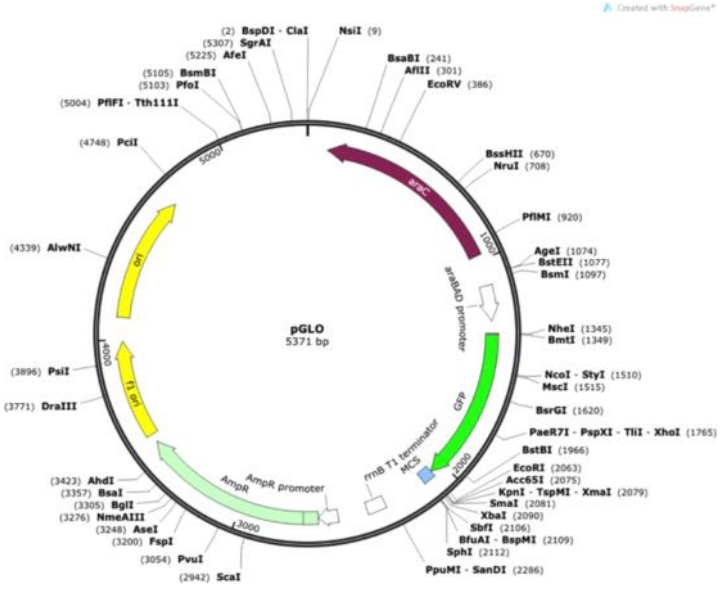

Figure 1. Although not indicated in the figure, the AmpR at around 3kb is the B-lactamase gene and the AmpR promoter is the B-lactamase promoter.[1]

Hypothesis:

10A: Transformation of E.coli with pGLO plasmid containing GFP.

As E. coli cells are not naturally resistant to ampicillin I hypothesize that only the E. coli cells we added the plasmid with the ampicillin resistant gene to, i.e. pGLO positive tubes, will be able to grow on the

agar plates with ampicillin and that no bacteria colonies will grow from the pGLO negative tube on the negative control plate with ampicillin.

10B: Data Collection and SDS-PAGE analysis

PART A: Data Analysis for Transformation

In addition, as GFP expression is only induced in the presence of arabinose, I hypothesize that the all the bacteria colonies that will grow on the agar with ampicillin and arabinose will show green under UV light and only a few (basal transcription levels) if not none of colonies will slightly fluoresce green on ampicillin alone without arabinose.

PART B: SDS-PAGE

As adding heat will fully denature the GFP protein and change the conformation of key nearby residues to the three (3) residues of the chromophore (Ser-Tyr-Gly), I hypothesize that only the lane "Green/no heat" lane 4 will illuminate green when placing the UV light over the gel while running. In addition "Green/no heat" lane 4 should have two bands, one around the 50 kDa molecular weight of the ladder for the initially correctly folded GFP protein and one around the 25kDa molecular weight of the ladder for initially partially folded, in which these two bands would illuminate green if the gel is placed under UV light.

Protocol:

Materials:

- See Lab Manual

Procedures:

- See Lab Manual

Results:

Group 1

Figure 1. Bacteria growth for pGLO- with no plasmid grown on LB agar without any ampicillin. Note there are a few colonies for this positive control.

Figure 2. Bacteria growth for pGLO- with no plasmid grown on LB agar with ampicillin. Note there is no bacteria growth on this negative control.

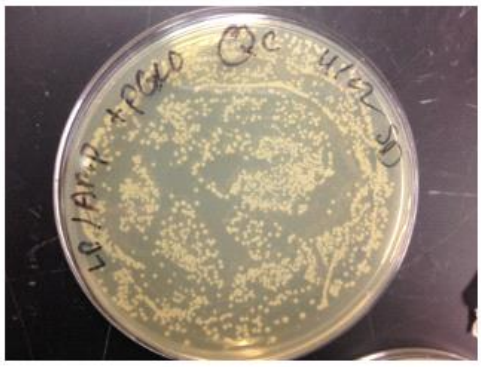

Figure 3. Bacteria growth for pGLO+ with plasmid grown on LB agar with ampicillin but without any arabinose.

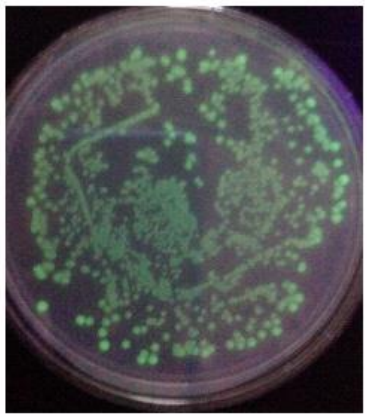

Figure 4. Bacteria growth for pGLO+ with plasmid grown on LB agar with ampicillin and arabinose.

For group 1 in Figure 1. above there was only one colony that grew for the positive control which is surprising. If there was no ampicillin plated then there should have been more than one or two colonies that grew. It should look more like Figure 3 except the only difference is no ampicillin resistant gene with no ampicillin plated vs. ampicillin resistance gene with ampicillin plated. In either case bacteria should grow. This makes me wonder if they only incubated pGLO+ plates at 37°C in step 12 of Lab 10A.

Total amount of pGLO DNA used initially with 10uL added = **0.15 ug**

Volume of transformation solution spread onto the plate = 100 uL

Total volume of solution = **600 uL**

Total amount of DNA (µg) = concentration of DNA (µg/µL) x volume of DNA added (µL)

Total amount of pGLO DNA: .015 µg/µL * 10 µL = 0.15 µg

$$Fraction\ of\ DNA\ used = \frac{Volume\ spread\ on\ LB/amp/ara\ plate\ (\mu L)}{Total\ sample\ volume\ in\ test\ tube\ (\mu L)}$$

Fraction of pGLO DNA used: 100/600 = 0.1667

$$Amount\ of\ pGLO\ DNA\ spread\ in\ \mu g = Total\ amount\ of\ DNA\ (\mu g) x\ fraction\ of\ DNA\ (\mu g)$$

Amount of pGLO DNA spread on plate (µg): 0.15 µg * 0.1667 = 0.025 µg

$$Transformation\ efficiency = \frac{Total\ number\ of\ \boldsymbol{fluoresent\ colonies}\ on\ LB/amp/ara\ plate}{Amount\ of\ DNA\ spread\ on\ the\ agar\ plate\ (in\ \mu g)}$$

$$Transformation\ efficiency = \frac{636}{0.025\ \mu g} =$$ 25,440 or **25.44x10^3 transformants/µg DNA**

The transformation efficiency was very high for group 1 at more than 3 times the expected 7.0 x 10^3 transformants per microgram of DNA.

Group 2

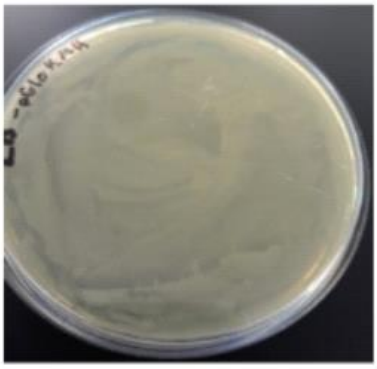

Figure 5. Bacteria growth for pGLO- with no plasmid grown on LB agar without any ampicillin. Note there may be a few satellite colonies near the top but no colonies of E. coli grew.

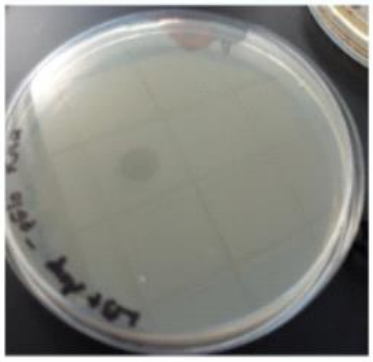

Figure 6. Bacteria growth for pGLO- with no plasmid grown on LB agar with ampicillin. Note there is no E.coli growth on this negative control but maybe one satellite colony.

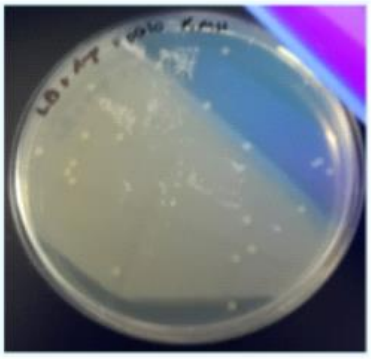

Figure 7. Bacteria growth for pGLO+ with plasmid grown on LB agar with ampicillin but without any arabinose.

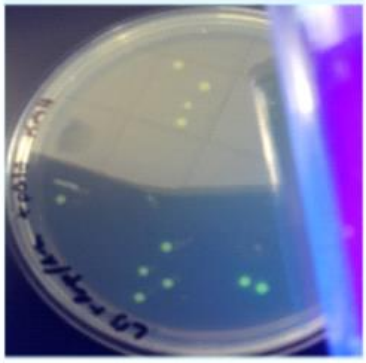

Figure 8. Bacteria growth for pGLO+ with plasmid grown on LB agar with ampicillin and arabinose.

$Transformation\ efficiency = \mathbf{480\ transformants/\mu g\ DNA}$

SDS-PAGE Gel after staining with Coomassie Blue

According to the SDS-PAGE image in Figure 9. the only lane to show visual GFP expression was lane 2 for the Green with no heat added sample. The fluorescence was just under the 75 kDa standard marker. This was the Green transformed E. coli with arabinose induced expression of GFP which was not added to the 95°C water bath. Lanes 1, 3, and 4 did not show any GFP protein expression.

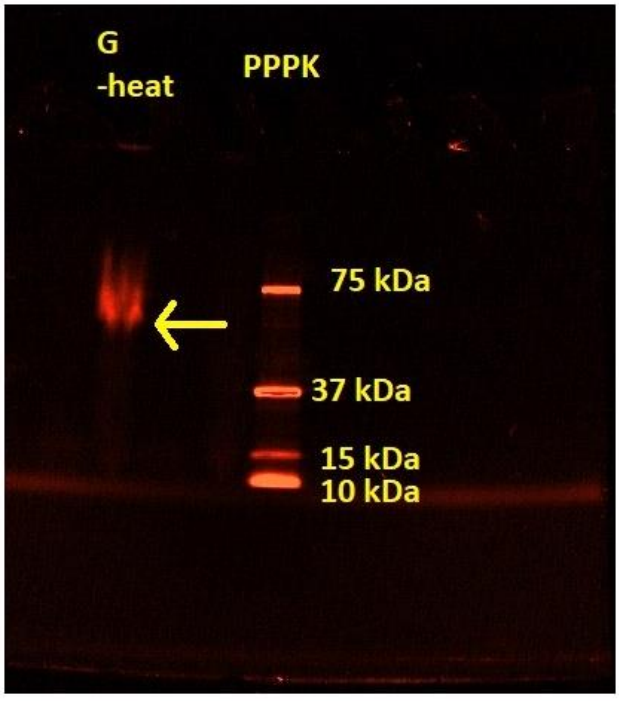

Figure 9. Visual of SDS-PAGE under UV light before staining with Coomassie Blue. The lane G -heat (Green, no heat) is for the pGLO+ transformed E. coli induced with arabinose as was positive for GFP in first part of lab. This is only lane that showed fluorescence for the GFP protein. According to the Precision Plus protein Kaleidoscope Standard (PPPK) and the space between the 75 kDa standard and 37 kDA standard this fluorescent band for Green, no heat closer to the upper end of 40-60 kDa for partially denatured GFP.

SDS-PAGE Gel after staining with Coomassie Blue

After staining the SDS-PAGE with Coomassie Blue the White bacteria with and without heat added showed lighter and less frequent bands in comparison the Green bacteria with an without heat indicated in Figure 10. The Green bacteria without heat had bands at around 60-65 kDa as well as 27 kDa as did the Green bacteria with heat added at 27 kDa.

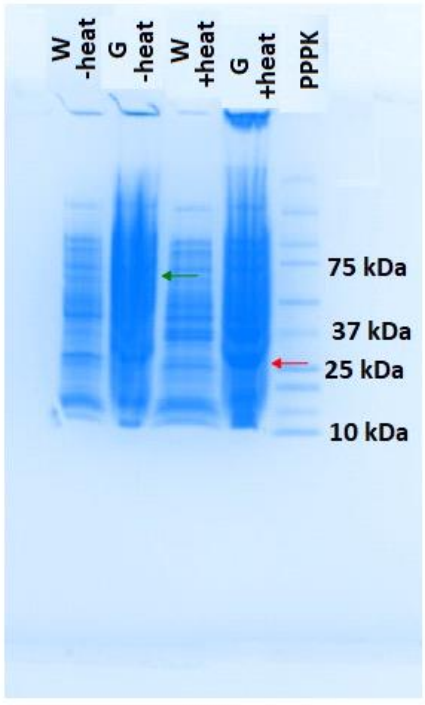

Figure 10. Visual of SDS-PAGE after staining with Coomassie Blue. The green arrow under lane G -heat for the Green, no heat points to the partially denatured GFP protein that shows fluorescence corresponding to the G -heat in Figure 9. above. This is around 60-65 kDa. The red arrow indicates the fully denatured GFP protein at around 27 kDa. Note that lane 2 for Green, no heat also has a band for this 27 kDa fully denatured GFP.

Discussion/Conclusion:

First thing I wanted to point out is that in my hypothesis for predicting the fluorescent bands for the Green, no heat lane in the SDS-PAGE, I don't know why I thought there would be a fluorescent band at the fully denatured GFP protein at 27 kDa. That was obviously wrong since there would be no surrounding residues to resonate the electron energy to promote fluorescence in the chromophore when excited by UV light.

The transformation efficiency was much higher for group 1 and is visually noticeable in the difference between plates in figures 3/4 and 7/8. Utilizing the entire recovery time of incubation for 60 minutes at 37°C after heat shock allowed the E. coli cells to recover from the added $CaCl_2$ to make the cells competent as well as time to express both the ampicillin resistance gene if the cells took up the plasmid and expression of GFP induced by the arabinose that was plated. 37°C is optimal temperature for proteins like RNA Pol II and AraC. This environment gave ample time to express both the AmpR as well as GFP genes. If group 2 removed the plates before the 1 hour incubation this could have affected expression of ampicillin resistance in some of the cells that did take up the plasmid and so without the expression they would not survive on agar plates coated with ampicillin.

There could be more reasons for why the bacteria growth and transformation efficiency for group 2 was insufficient. First off there could have been errors in the process for making cells competent. Prior to transformation the E. coli growth phase needs to be at the peak growth to increase transformation efficiency. This usually requires incubating or adding to a shaker at 37°C until the OD600 reading in a spectrophotometer reaches 0.4. This reading could have been lower for group 2's E. coli sample than the optimal growth for group 1's E. coli.

Usually the $CaCl_2$ solution has to be prepared by measuring the solid or hydrated form purchased from lab with DI water. There could have been errors here in preparing the $CaCl_2$ solution. Group 2 might have not have picked a large starter colony or suspended the E. coli in the $CaCl_2$ efficiently. In my other lab we also centrifuged the E. coli to remove the LB supernatant before adding $MgCl_2$ directly to the bacteria, then centrifuging again and removing supernatant, and then repeating with $CaCl_2$.

This wouldn't help however is group 2 was also not efficient with keeping the samples on ice directly before and after heat shock during the actual transformation process. It is very important to keep on ice. With respect to the actual transformation process, group 2 may have not mixed the 10 µL of plasmid with the competent cells by pipetting the added plasmid up and down. They also might have heat shocked before the 10 minutes of ice incubation. As indicated previously, group 2 most likely did not incubate for the 60 minutes at the optimal growth conditions before adding the samples to the agar plates and so the transformed E. coli, even with the ampicillin gene, would not have expressed the protein for the ampicillin resistance. Lastly with respect to the preparation of the agar plates and adding the transformed and non-transformed samples, group 2 might have not added all 100 µL of sample to each plate. We also used spreaders in my other lab to spread the transformed bacteria around the plate. This would be helpful so that all the nutrients of the agar for bacterial growth could be optimized but lack of doing this is unlikely as seen with group 2's plate in figure 8 showed some transformed bacteria all around the plate.

Resources:

1. Deutch, C.E. The American Biology Teacher, Vol. 81 No. 1, January 2019; (pp. 52-55) DOI: 10.1525/abt.2019.81.1.52