Antelope Valley College

Cell and Molecular Biology Laboratory Manual

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The Metric System

Introduction

The metric system is a system of measurement that is based on multiples of 10. It is a system that has been internationally agreed upon. It is synonymous with the International System of Units (SI) and is used exclusively in almost every country in the world. The simplicity of the metric system lies in the fact that it is based on powers of ten. This makes converting between units of measure much simpler since there are no conversion values to memorize (it's just a matter of moving decimal places). In order to do metric system conversions, two things **do** have to be known, though. First, one must know the prefixes that are associated with the values at each level (for example, deci- is the prefix for the value 10^{-1} or 0.1). Second, one must also know in which direction the decimal will move in making conversions (which is simple if you understand that moving the decimal to the right makes a large number).

In the sciences, understanding math is crucial since it is what leads to the quantitative nature of science. Understanding the metric system is crucial because it is the system used in the sciences, worldwide. This gives everyone a chance to collaborate with people from all parts of the globe using a "universal language".

Materials

- Centimeter ruler
- Meter stick
- Electronic scale
- 200g calibration standards
- Graduated cylinders (10 & 100 mL)
- Beaker

Methods

A. Measuring length

1. Using the appropriate measuring tool, measure the height of your partner(s) in centimeters. Convert all measurements to meters, millimeters, micrometers, and nanometers. Express all measurements in scientific notation.
2. Using the appropriate measuring tool, measure the length of the right arm of your partner(s) in centimeters. You will measure the arm from under the arm pit to the longest fingertip. Convert all measurements to meters, millimeters, micrometers, and nanometers. Express all measurements in scientific notation.
3. Using the appropriate measuring tool, measure the length of the right index finger of your partner(s) in centimeters. You will measure the finger from the middle of the largest knuckle (metacorpophalangeal joint) to the fleshy tip of the finger. Convert all measurements to meters, millimeters, micrometers, and nanometers. Express all measurements in scientific notation.

B. Measuring mass

1. Record the mass of the calibration weight standards given to ensure proper scale calibration.
2. Using the electronic scale, measure each one of the various materials provided.

C. Measuring volume

1. Weigh the beaker that has been provided to you.
2. Using the graduated cylinders provided, measure out 5 mL of water into the beaker.
3. Weigh the (filled) beaker and determine the weight of the water.
4. Record all data collected.

Anthocyanidin Experiment

Introduction

Anthocyanins are colored water-soluble pigments. They are responsible for the colors, red, purple, and blue, are in fruits and vegetables. Berries, grapes, and some tropical fruits have high anthocyanins content. Red to purplish blue-colored leafy vegetables (like the cabbage used today), grains, and roots are the edible vegetables that contain a high level of anthocyanins. The colored anthocyanin pigments have been traditionally used as a natural food colorant. The color and stability of these pigments may be influenced by pH, light, and temperature. In acidic condition, anthocyanins appear as red, turn blue, and then eventually yellow, when the pH increases. Besides the use of anthocyanidins and anthocyanins as natural dyes, these colored pigments are potential pharmaceutical ingredients that give various beneficial health effects. Scientific studies, such as cell culture studies, animal models, and human clinical trials, show that anthocyanidins and anthocyanins possess antioxidative and antimicrobial activities and protect against various diseases.

Materials

- Red cabbage
- Graduated pipettes
- 0.1 M hydrochloric acid (pH=1)
- 0.1 M NaOH (pH=13)
- Test tubes
- Distilled water
- Blender

Methods

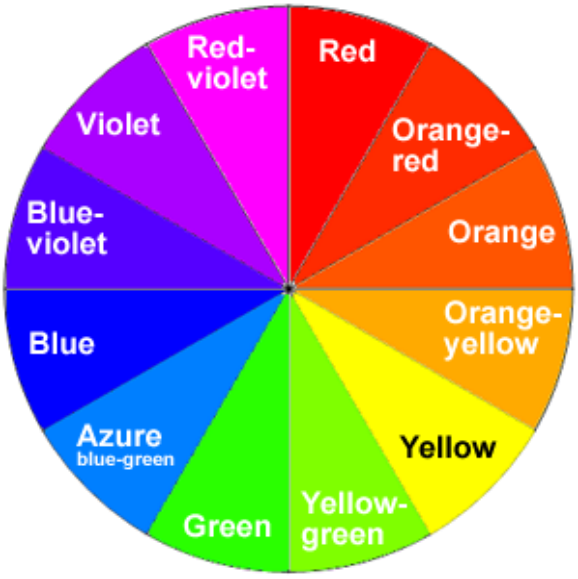
A. Anthocyanidin preparation (Instructor prep)

a. Take a few leaves of red cabbage and place in a blender.
b. Add a few milliliters of water (enough to cover the leaves).
c. Macerate the cabbage until a homogenous solution is obtained.
d. Filter the cabbage suspension through a piece of cheesecloth or gauze.

B. Acid/base dilutions

a. Prepare seven tubes with 5 ml each of the following acid/base solutions:
i. 0.1 M HCl (Stock solution)
ii. 0.001 M HCl
iii. 0.0001 M HCl
iv. Pure Water
v. 0.0001 M NaOH
vi. 0.001 M NaOH
vii. 0.1 M NaOH (stock solution)

b. Add 1-2 ml of the anthocyandin to each tube and record the results


Red
Orange-red
Orange
Orange-yellow
Yellow
Yellow-green
Green
Azure
blue-green
Blue
Blue-violet
Violet
Red-violet

Diffusion and Osmosis

Introduction

Diffusion is the primary mechanism for molecular movement. It may be defined as the random movement of molecules from a high concentration to a low concentration until equilibrium. This difference in molecular concentration is commonly referred to as the concentration gradient. Diffusion occurs under the molecules' own energy, therefore, this process does not require outside energy to take place. This process ends when all molecules are equally distributed. There are several factors that may affect the rate of diffusion and in this lab we will examine some of these factors.

Osmosis is a special case of diffusion where the substances that are diffusing across the membrane are solvent molecules (such as water) through a semi-permeable membrane (such as a plasma membrane). Again, the molecules (in this case, solvent molecules) will move from an area of high concentration to low concentration until equilibrium is reached. Therefore, the rate of osmosis is determined by the difference in water concentration on either side of the membrane.

Materials

- Diffusion and temperature
  - Beaker with hot water
  - Beaker with room temperature water
  - Beaker with cold water
  - Food coloring
- Diffusion and molecular size setup
  - Cellulose sac containing:
    - 5% glucose solution
    - 1% starch solution
  - Test tube with iodine
- Osmosis in cells
  - *Elodea* in water
  - Plastic pipettes
  - Forceps
  - Distilled water (in dropper bottle)
  - 5% sodium chloride (in dropper bottle)
- Egg osmosis experiment
  - Hypotonic, isotonic, and hypertonic solutions

Methods

A. Effect of temperature on diffusion

a. Place a drop of food coloring in each of the different beakers and record the amount of time it takes for each beaker to reach relative equilibrium.

B. Diffusion and molecular size

a. As a group, talk to the instructor regarding this demonstration.

C. Effect of osmosis on cells

a. Observe an *Elodea* leaf under two separate conditions: 5% NaCl and distilled water.

i. Record observations every 2 minutes for 10 minutes. Note any changes occurring with your observations. Draw cells from both leaves at t=0 and t=10 min.

b. Close to the end of the class period, observe the celery sticks that have been placed in salt water and distilled water. Note any differences between them and provide an explanation for any of these differences.

D. Egg osmosis experiment

a. As a group, talk to the instructor regarding this demonstration.

Survey of Kingdom Protista

Introduction

Kingdom Protista is a kingdom whose members are all grouped together by a negative characteristic- they are not animals, plants, nor fungi. Kingdom Protista is extremely diverse and each one can be characterized by features that are unique to certain protista (ex. type of body armor, type of motility, etc.).

Materials

- Toothpicks
- Protoslo
- Cultures
    - *Euglena*
    - *Amoeba proteus*
    - *Paramecium caudatum*
    - *Volvox*
    - *Spirogyra*
    - Hay infusion
    - Pond water
    - Termites
- Slides
    - *Euglena*
    - *Amoeba proteus*
    - *Paramecium caudatum*
    - *Chlamydomonas*
    - *Pandorina*
    - *Volvox*
    - *Spirogyra*
- Demonstrations
    - *Euglena*
    - Diatom cell wall
    - *Trypanosoma gambiense*
    - *Plasmodium falciparum*
- Coverslip

Methods

A. Observations

a. Observe all cultures and slides and record data (draw what you observe)

b. Observe demos and take note of what is happening in each one.

Euglena Growth Experiment

Introduction

Euglena is a genus that contains organisms that have the ability to feed in two ways. First, these organisms have the ability to employ the use of the chloroplasts in contains to undergo autotrophy. Second, the organisms have the ability to also function as heterotrophs by taking in material through their membrane (osmotrophy). In this experiment, we will see how different growth media will affect the growth of *Euglena*.

Materials

- *Euglena gracilis*
- 5 ml and 10 ml pipettes
- Disposable plastic pipettes
- Test tubes
- 100 ml graduated cylinders
- 10 ml graduated cylinders
- Distilled water
- 10x Klebs Solution
- Boiled rice
- Weighing boats
- 250 ml beakers
- 100 ml plastic vials

Methods

A. Culture preparation

a. Take stock Klebs solution and dilute down to 1x. The *Euglena* culture will be grown in 1x Klebs.

b. Two (2) separate drops of concentrated *Euglena* culture will be observed under the microscope.

c. Count the number of cells under **low-power magnification**.

d. Our goal is to dilute the initial culture to 5 cells/field.

B. Growth experiment

a. Cells will be grown in the following conditions:

i. Light only

ii. Light and rice

iii. Rice only (absent of any light)

b. Data will be collected over the course of several weeks.

c. After each week, ensure that the volume of the culture is still 50 ml. If it is not, adjust the volume with dI water.

d. Cell counts must be performed weekly during the length of this experiment. If necessary, dilute the culture 10-fold and then calculate the number of cells per field.

e. Plot this data on the graph paper provided with Time (in days) as the X-axis and *Euglena* cells/field as the Y-axis.

f. Determine the best fit line and provide the equation of the line in slope-intercept form.

Recipe

A. 10X Modified Klebs Solution

Potassium nitrate……. 0.25 g
Magnesium sulfate……...0.25 g
Potassium phosphate, monobasic…….0.25 g
Calcium nitrate…….0.01 g
Bacto Tryptone…….0.01 g
Distilled water……1000 ml

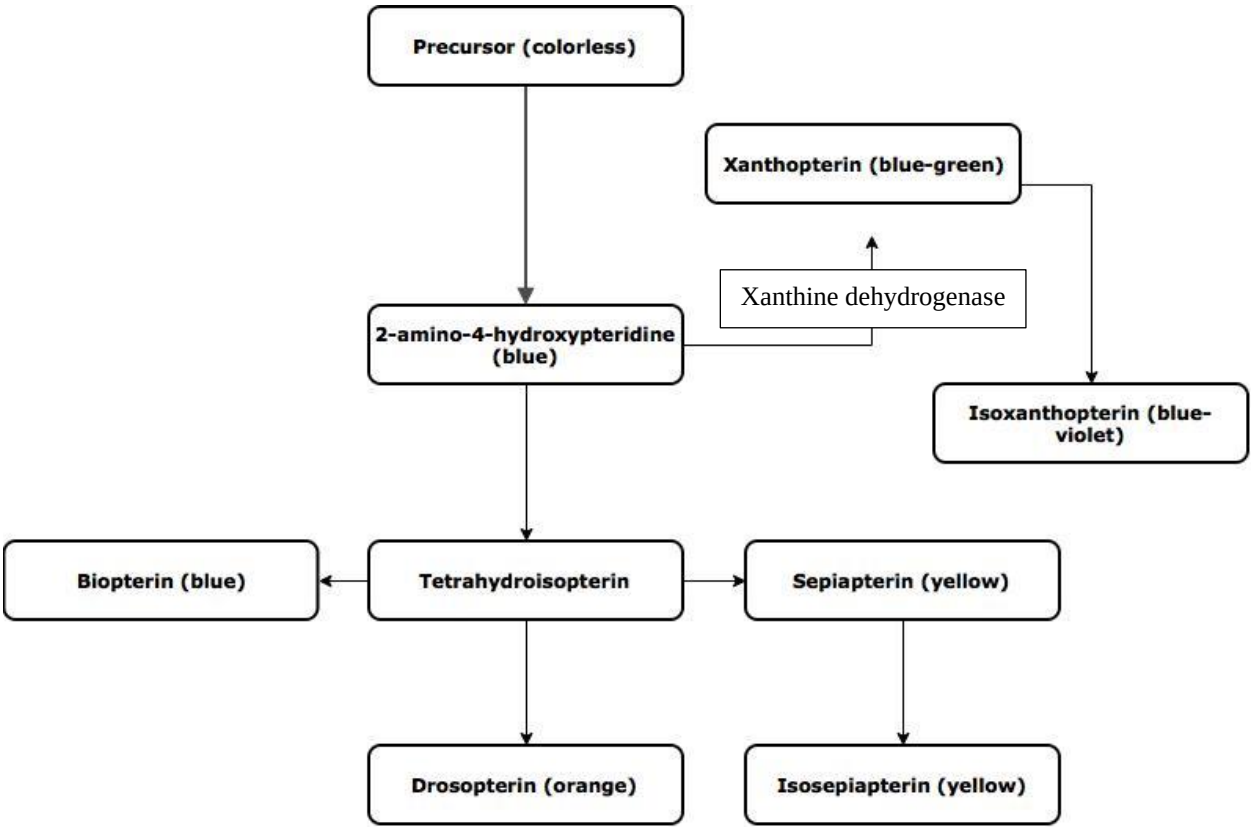
Drosophila Eye Pigment Chromatography Lab

Introduction

In this lab, you will study the biochemical pathway that produces the pigments that give *Drosophila* their characteristic eye color. The strains of fruit flies we will work with are

- Wild type - red eyes
- Sepia mutant - brown eyes
- White mutant - white eyes

The biochemical pathway that produces the various eye pigments in wild type flies is below. Names of chemical intermediates are given; numbers refer to enzymes that catalyze the conversion of one intermediate to another. Eye color mutants are defective in one enzyme in the pathway that you will try to deduce from your results.



You need to determine which pigments are present (or missing) in the mutants and in wild type flies. We will use paper chromatography to separate the eye pigments.

Chromatography is used to separate mixtures of substances into their components. All forms of chromatography work on the same principle. They all have a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different

components travel at different rates. In paper chromatography, the stationary phase is a very uniform absorbent paper (filter paper). The mobile phase is a suitable liquid solvent or mixture of solvents.

Materials

- *Drosophila melanogaster* fruit flies (6 of each eye color)
- Fly Nap
- Anesthetic wand
- Large paper towel
- Glass rod
- Needle/sharp edge

Methods

A. Anesthetizing flies

1. Using the anesthetic wand, dip the absorbent tip into the Fly Nap.
2. Make sure to remove any excess liquid from the tip.
3. Carefully remove the plug of the culture vial containing flies and put the soaked wand in.
4. Leave the wand in for about 3-4 minutes.
5. Remove the wand once the majority of the flies are anesthetized.

B. Fly selection

1. Choose six flies of each of the different eye colors.
2. Using a stereomicroscope, decapitate the fly heads and set them aside.
3. Be cautious in ensuring that you have the correct eye colors grouped together.

C. Preparing Paper Chromatography

1. Take a piece of rectangular filter paper.
2. Draw a horizontal line with a pencil about 1.5-2 cm from the bottom.
3. Place 6 decapitated fly heads of one eye color on the left side of the paper.
4. Repeat this for the other fly eye colors, placing them in the middle and right side of the line, respectively.
5. Crush the heads to allow the eye pigments to penetrate the paper.
6. Allow the paper to dry and then make a tall cylinder out of the paper by folding and stapling it.
7. Carefully place the cylinder in the solvent and allow it to develop.
8. Once the solvent front is 1 cm from the top, remove the cylinder.
9. Unravel the cylinder and place it on a paper towel to dry.
10. View the paper chromatogram under black light to view the pigments.

Enzymes

Introduction

Enzymes are essential to the vital reactions that living organisms deal with on a day to day basis. Without enzymes, the metabolic reactions that are necessary for life may take a long time to occur. A catalyst is a compound that can speed up the rate at which a chemical reaction occurs. An enzyme is a protein that is a biological catalyst. Enzymes affect the reaction rate by putting stress on the bonds in the substrate thereby lowering the activation energy required for the reaction to go forward. This lower activation energy allows the substrate to reach a transition state much faster leading to eventual products. Enzymes are specific to their substrate and are released unchanged when the reaction is over. Since enzymes are proteins, they are subject to the same environmental pressures that may lead to denaturation and this will result in a non-functioning enzyme.

There are two models that are used to describe how the enzyme-substrate complex forms. The lock and key model is the one that is more often talked about in biology. In this model, the enzyme has a specific substrate that "fits" into it, the same way a particular lock has a particular key. The second model that has been used is the induced fit model which states that the enzyme binds to the substrate and a strong fit is *created*.

Materials

- X-gal (5-bromo-4-chloro-4-indolyl-β-D-galactopyranoside
- Lactaid tablets
- 3% hydrogen peroxide
- Yeast
- DI water
- Distilled water (in dropper bottle)

Methods

A. Catalase

a. Test for the presence of catalase in living tissue by adding each one to 3 ml of hydrogen peroxide.

b. Record the results of each reaction noting the degree of the reaction (i.e. += weak, ++= strong, +++=very strong).

B. Effect of temperature on enzymes

a. Label 5 microcentrifuge tubes with the following temperatures:

i. 0°C
ii. 25°C
iii. 37°C
iv. 75°C
v. 100°C

b. Aliquot 100 µl lactaid in each tube.

c. Place the tubes in the dry bath in their respective temperatures.

d. Allow the enzyme to incubate for 15 minutes

e. Aliquot 300 µl X-gal in each tube.
f. Allow the mixture to develop for 15 minutes
g. Place 200 µl of each tube into a separate well of the first column of a 96-well plate. For example, put the 0°C condition in A1, the next condition in B1, etc.
h. Place in the microplate reader and conduct readings at the appropriate wavelength.
i. Record all results obtained.
j. Using the graph paper provided, plot an enzyme activity vs. temperature graph.

C. Effect of pH on enzymes

a. Label 5 microcentrifuge tubes with the following pH values:
  i. pH=0
  ii. pH=3
  iii. pH=7
  iv. pH=10
b. Aliquot 100 µl lactaid in each tube.
c. Allow the enzyme to incubate for 15 minutes
d. Aliquot 300 µl X-gal in each tube.
e. Allow the mixture to develop for 15 minutes
f. Place 200 µl of each tube into a separate well of the first column of a 96-well plate. For example, put the 0°C condition in A1, the next condition in B1, etc.
g. Place in the microplate reader and conduct readings at the appropriate wavelength.
h. Record all results obtained.
i. Using the graph paper provided, plot an enzyme activity vs. pH graph.

Absorption Spectra of Photosynthetic Pigments

Introduction

In order for light to be used as energy for photosynthesis to occur, pigments are needed to absorb light. Chloroplasts are plastids that may contain a mixture of several pigments. In many cases, this mixture will include chlorophyll a, chlorophyll b, carotene, and xanthophyll. This mixture of pigments can be separated by paper chromatography.

Materials

- Spectrophotometer and five (5) cuvettes
- Ethanol
- Chlorophyll extract
- 50 ml beakers
- Glass stirring rods
- 100% acetone
- Large filter paper square
- Chromatography running solvent (92% petroleum ether & 8% acetone)

Methods

A. Pigment preparation

a. Take the large filter paper and draw a line 1 cm from the bottom with a **pencil**.
b. Using a capillary tube, take up some of the chlorophyll extract and "line" the pencil mark.
c. Allow this line to dry and repeat this three to four (3-4) times.
d. Once dry, roll the filter paper and place it in a beaker containing running solvent.
e. Allow the solvent front to reach the top of the filter paper.
f. Cut each resulting pigment band and extract the pigment by putting these bands in the appropriately labeled beaker containing 7 ml of acetone.
g. The extract is now ready for spectrophotometric analysis.

B. Spectrophotometry

a. For each extracted pigment, spectrophotometer readings will be conducted from 350 nm to 700 nm in 10 nm increments.
b. Make sure to use the blank each time that the wavelength is changed.
c. Plot the resulting data for each pigment on the graph paper provided, using different colors to indicate each pigment.

Photosynthesis

Introduction

As you may recall, photosynthesis is the process by which photosynthetic organisms capture the light energy from the sun and convert it to the energy found in the chemical bonds of carbohydrates. In the light-dependent part of this process, water is split by Photosystem II to produce molecular oxygen and hydrogen ions. These hydrogen ions help to provide the proton gradient that is necessary for the chemiosmosis that leads to ATP synthase generating ATP. In the light-independent process, carbon dioxide is used to create glucose.

In order to calculate the number of molecules of oxygen produced in this experiment, a few considerations must be made. First, you must keep in mind that when performing calculations on gases, standard temperature and pressure (STP) needs to be taken into account. The standard temperature is 0°C (273 K) and the standard pressure is about 1 atm of pressure. So, at 1 STP, 1 mole of gas occupies 22.4L of volume.

Materials

- 8-10 cm piece of *Elodea*
- Large test tubes (2)
- 0.2% $NaHCO_3$ solution
- Battery jar filled with water (used as a heat filter)
- Light source
- "Oxygen-measuring device"

Methods

A. Preparation

a. Fill the battery jar with tap water and place the lamp behind it.
b. Turn on the lamp and wait about 5 minutes for the elements to come to equilibrium
c. Away from the light source, place an 8-10 cm piece of *Elodea* in one of the large test tubes and fill the test tube with bicarbonate solution.
d. Place the "oxygen-measuring device" on top of the tube.
e. Using the syringe, adjust the level of the pipet to read "0".

B. Photosynthesis

a. As photosynthesis occurs, oxygen will build up in the tube pushing bicarbonate solution out through the pipet end of the device.
b. Record the amount of fluid pushed out after 15, 30, 45, and 60 minutes.
c. Using the information provided, calculate the amount of sugar produced in your experiment.
d. Plot a graph with the X-axis as time (in minutes) and the Y-axis as amount of oxygen produced.

Cellular Respiration

Introduction

Cellular respiration refers to the processes involved by which cells will take organic material and ultimately convert the chemical bonds found in the organic material into those found in adenosine triphosphate (ATP). All living cells must carry out cellular respiration. In some cases, cellular respiration can be aerobic and in others, it may be anaerobic. In most cases, aerobic cellular respiration is what is described.

Cellular respiration is thought of as occurring in three stages: Glycolysis, Krebs Cycle, and the Electron Transport Chain. Glycolysis occurs in the cytoplasm of all cells, while the other processes occur in the mitochondria of eukaryotic cells and in the cytoplasm and membrane of prokaryotic cells. As organisms respire, oxygen is consumed since it is the ultimate electron acceptor in the electron transport chain of aerobic organisms and carbon dioxide is released as a consequence of the several decarboxylation events that occur during the Krebs Cycle.

Materials

- LabQuest
- Oxygen sensor
- Carbon dioxide sensor
- Mealworms
- Nalgene bottle (BioChamber)

Methods

A. Cellular respiration

a. Each table will choose one of the six experiments listed below. Each condition will be studied **once.** In all the experiments oxygen and carbon dioxide will be recorded.

i. Observing the cellular respiration of five mealworms at room temperature
ii. Observing the cellular respiration of ten mealworms at room temperature
iii. Observing the cellular respiration of five mealworms in the refrigerator
iv. Observing the cellular respiration of ten mealworms in the refrigerator
v. Observing the cellular respiration of five mealworms at 45°C
vi. Observing the cellular respiration of ten mealworms at 45°C

B. Equipment setup

a. **Chamber setup**

i. Place mealworms in the respiration chamber
ii. Attach the oxygen sensor to the top of the chamber (please see the photo below)
iii. Attach the carbon dioxide sensor to the neck of the chamber (the carbon dioxide gas is heavier and will settle, making it easier for the "lower" sensor to detect the gas)

b. **LabQuest setup**

i. Connect the sensors to any of the ports on the LabQuest
ii. The software will auto-detect both sensors and you will start to see data.

iii. After 90 seconds (the time it takes for the sensor to warm-up), the carbon dioxide reading should start to increase. Once it does, begin data collection (press the green "play" button)
iv. Allow the experiment to run for 15 minutes.
v. Remove the sensors, return the mealworms to the appropriate container, and rinse out the BioChamber.

C. Analysis

a. To find the respiration rate, you have to find the slope of the both graphs.
b. To find the slope of the carbon dioxide graph, click on:
    i. Analyze, Curve Fit, CO_2
    ii. From the pull-down menu, choose linear and record the slope on the data table.
c. To find the slope of the oxygen graph, repeat the same process except choose O_2 after you choose Curve Fit.
d. Make conclusions based on the information obtained.

Collect Data button

Transformation of Chemically-Competent *E. coli*

Introduction

When researchers successfully identify a protein of interest, one of the first objectives is to isolate the gene that encodes the protein. Once that is achieved, the gene is inserted into a plasmid so that the gene can be cloned, as additional copies of the gene will be needed for ongoing studies. Later, cloned genes are inserted into plasmids that have been engineered specifically for protein expression in bacteria or other suitable organisms. Plasmids used in these cases are known as *expression vectors*. The *gfp* gene used in our experiments was inserted into an expression vector named pGLO. These expression vectors are then used to transform cells.

In order to produce more plasmids, the insertion of plasmid DNA into bacterial cells is necessary. In general, bacteria do not naturally take up DNA from their environment. In order to improve the probability that the bacteria will internalize the plasmid DNA, it is necessary to make the cells "competent" to take in the plasmid DNA. Competent cells are significantly more fragile than normal bacteria. Vortexing the cells, heating the cells above 42°C or to 42°C for prolonged periods, or exposure of the cells to any of a number of other even mildly abusive treatments may kill them. If done correctly, the cells will break open long enough to take in any plasmid DNA that is in the tube. The cells are then allowed to "heal". This process is called transformation.

Materials

- 42°C water bath
- Ice Bucket
- Foam test tube rack
- $CaCl_2$-competent *E. coli* HB101 cells
- pGLO plasmid
- LB broth
- LB agar plates with and without anibiotics
- Sterile loops

Methods

A. Preparation

a. Label two (2) microcentrifuge tubes as "+DNA" and "–DNA", respectively.
b. Also label the tubes with your group number or initials.
c. Gather or locate materials needed for the experiment (pipettes, ice bucket, hot water bath, etc.)
d. Ensure that the growth media is labeled **properly**. You should have four agar plates labeled: LB/amp +DNA, LB/amp/ara +DNA, LB/amp –DNA, and LB –DNA.
e. Make note of the pGLO DNA solution shown under UV light.

B. Transformation

a. Aseptically transfer 250 µl of $CaCl_2$ into each labeled test tube.
b. Place both tubes in the foam test tube rack provided, on ice.
c. Aseptically transfer a single colony of *E. coli* and mix into the $CaCl_2$ solution in the +DNA tube, ensuring a homogenous mixture.

d. Repeat the previous step for the –DNA tube.
e. Obtain 5 µl of plasmid (pGLO) DNA to the +DNA tube, mix gently, and return the tube to the ice bucket.
f. Incubate the tubes on ice for ten (10) minutes.
g. Transfer the test tube rack holding your two (2) test tubes into the 42°C water bath and incubate for **EXACTLY** 50 seconds. This **heat shock** step is extremely time-sensitive and must be performed very accurately.
h. Immediately put the test tube rack back on ice for two (2) minutes.
i. Transfer 250 µl LB broth to the +DNA tube and mix gently.
j. Repeat this step for the –DNA tube, using a new pipette.
k. Incubate both tubes at room temperature for ten (10) minutes.
l. Transfer 100 µl of each culture tube onto the appropriate labeled agar plate.
m. Using a sterile loop, perform a "lawn" streak of the bacterial culture.
n. Allow the plates to sit, lid side up, for a few minutes before inverting the plates.
o. Tape together the plates and incubate them, lid side down, at 37°C. These plates will be incubated for 24 hours.

Isolation of Green Fluorescent Protein from transformed *E. coli*

Introduction

Transformed cells are allowed to express the protein and then lysed (broken open) to release the newly synthesized protein from the cell. The protein is isolated, from the other cytoplasmic proteins, purified, and tested for activity. You have already completed much of the work that parallels this drug discovery scenario. The bacterial cells that have been growing in the LB/amp/ara medium have been expressing green fluorescent protein and are now ready to be lysed and the Green Fluorescent Protein (GFP) purified using column chromatography.

To purify the GFP, the bacterial lysate you prepared will be loaded onto a hydrophobic interaction column (HIC). Recall that GFP contains many hydrophobic amino acids making this protein much more hydrophobic than most other bacterial proteins. In the first step of this process, you will pass the supernatant containing the bacterial proteins and GFP over an HIC in a salty buffer. The salt causes the three-dimensional structure of proteins to actually change so that the hydrophobic regions of the protein move to the exterior of the protein and the hydrophilic regions move to the interior of the protein.

When your sample is loaded onto the column (containing hydrophobic beads) in the salty buffer, the hydrophobic proteins should stick to the beads. The more hydrophobic the proteins, the tighter they will stick. The more hydrophilic the proteins, the less they will stick. As the salt concentration is decreased, the three-dimensional structure of proteins change again so that the hydrophobic regions of the proteins move back into the interior and the hydrophilic ("water-loving") regions move to the exterior.

Materials

- Microcentrifuge tube
- Transformed culture from previous experiment
- HIC Chromatography Kit
    - HICs
    - TE buffer
    - Lysozyme
    - Equilibrium buffer
    - Binding buffer
    - Wash buffer
- Test tubes
- UV light

Methods

A. Bacterial concentration

a. Obtain a microcentrifuge tube and label it with a "+" and initials.

b. Choose the transformed culture that has the greatest green fluorescence.

c. Ensure that the tube is well-mixed before pipetting 2 ml of the culture into your labeled microcentrifuge tube.

d. Concentrate the bacteria by centrifugation for 5 minutes at maximum speed.

e. Pour out the supernatant and observe the bacterial pellet by UV light.

f. Add 250 µl of TE buffer to the microcentrifuge tube and carefully resuspend the bacterial pellet.

B. Bacterial cell lysis

- **a.** Add approximately 50 µl (1 drop) of lysozyme to the bacterial suspension. Mix the contents well and observe under UV light once again.
- **b.** Place the microcentrifuge tube in the freezer for at least 20 minutes. During this time, the HICs can be prepared.
- **c.** After the bacterial suspension has frozen, remove the tube from the freezer and thaw in hand.
- **d.** Separate the insoluble bacterial debris using centrifugation for 10 minutes at maximum speed.
- **e.** Immediately remove the tube from the centrifuge and examine the tube under UV light.
- **f.** Transfer 250 µl of the supernatant into a new microcentrifuge tube labeled "+".
- **g.** Add 250 µl of binding buffer to the supernatant

C. Preparation of column

- **a.** Remove the cap and snap off the bottom of the prefilled HIC.
- **b.** Allow the buffer to drain from the column (this should take between 3-5 minutes).
- **c.** Add 2 ml of equilibrium buffer to the top of the buffer.
- **d.** Drain the buffer to the 1 ml mark (on the column) and cap the top and bottom of the column.
- **e.** Label three (3) collection tubes (1, 2, 3, respectively). And place them in the test tube rack.

D. Protein isolation using HIC chromatography

- **a.** Take the cap off of the top and bottom of your column and place it in test tube "1".
- **b.** Allow the remaining buffer to drain into the test tube.
- **c.** Carefully load 250 µl from the "+" microcentrifuge tube onto the top of the column, ensuring that the solution touches the inner wall of the column.
- **d.** After the column stops dripping, observe the column under UV light
- **e.** Transfer the column to test tube "2".
- **f.** Add 250 µl of wash buffer to the column and allow it to completely drain.
- **g.** Observe the column under UV light.
- **h.** Transfer the column to test tube "3".
- **i.** Add 750 µl of TE buffer to the column and allow it to completely drain. View the draining process under UV light.

Restriction Enzyme Digestion

Introduction

Restriction enzymes are extremely important tools in biotechnology. Restriction enzymes cleave DNA only at specific sequences. Different bacteria contain a large variety of restriction enzymes, and enzymes with specificity for large numbers of sequences are commercially available. In order to decide which restriction enzymes to use for a particular experiment, you will need to know the DNA sequence of the gene of interest as well as the plasmid map of the vector you are using. There are a number of resources to aid in the process of obtaining sequence information, including the NCBI website.

In order to prepare for construction of a new plasmid, we will first verify the identity of the plasmid we are using. We will do this using a technique called restriction mapping. Here we will employ the use of a plasmid map or other resources to determine what restriction enzymes to use and predict the size of the resulting fragment(s). Restriction enzymes must cleave both strands of the double stranded DNA. They can do this in a number of ways. Some restriction enzymes cleave both strands at the same location, resulting in a "blunt" end. Other restriction enzymes cleave at different locations on the different strands, leaving short "overhangs" of single stranded DNA also known as "sticky ends". These staggered end cuts are desired over their blunt end counterparts because it may facilitate ligation of foreign segments of DNA into the plasmid vector. In order to prepare for ligation, then, the plasmid *and* the gene of interest (foreign DNA) must be digested with *two* different restriction enzymes.

A potential problem with using multiple restriction enzymes is that each one is active in specific buffers; the best buffer for one enzyme may not be the best one for another enzyme. However, recently, there have been companies that have engineered buffers that can host a wider range of restriction enzymes. Traditionally, however, it is necessary to run one reaction, deactivate the enzyme or perform a DNA purification, and then run the other reaction. In other cases, there may be a complementary pair of enzymes that share a buffer and a double digest may be performed. Restriction enzymes should be kept on ice at all times, and solutions containing them should be buffered at the proper pH. The concentration of a restriction enzyme is usually expressed in **enzyme units per volume**. One unit of restriction enzyme is defined as the amount of enzyme needed to digest 1 μg of DNA in 1 hour.

Materials

- DI water
- Lambda DNA/EcoRI + HindIII Marker
- Lambda DNA
- Plasmid DNA
- FastDigest Restriction Enzymes
    - EcoRI
    - HindIII
- FastDigest Buffer
    - Proprietary buffer from Thermo Scientfic
        - Universal buffer with optimal pH, glycerol concentration, and salt concentration
    - Supports 100% activity of all FastDigest restriction enzymes

- No star activity

Methods

A. Restriction enzyme digest

a. Digest the plasmid DNA with EcoRI, plasmid DNA with HindIII, Lambda with EcoRI and Lambda with HindIII.

b. Set up **each** digestion as follows:

Water	15 µL
10X Restriction Enzyme Buffer	2 µL
DNA	2 µL
FastDigest enzyme	1 µL

c. Mix all components well and spin down.

d. Incubate the reaction for 5 minutes at 37°C.

e. View results of restriction enzyme digest using gel electrophoresis or place tubes in -20°C for future analysis.

Gel Electrophoresis

Introduction

The purpose of agarose gels is to allow for the estimation of DNA fragment size. This can be useful for verifying that a fragment of the correct size was produced in a polymerase chain reaction or to assess the size of restriction enzyme digestion fragments. It is also useful to run plasmid DNA on a gel in order to estimate both the concentration of the DNA and the quality of the preparation (*i.e.* to look for possible contaminants or excessive fragmentation of the plasmid DNA).

Remember that DNA is a negatively charged molecule due to their phosphate backbone. Agarose forms a low-density matrix and is therefore suited to running the large DNA molecules. Various concentrations of agarose can be used to separate different sized DNA: 0.75% for DNA > 3 kb, 2% or 3% for 50 – 400 base pairs (bp), and 1% for DNA between these ranges. In our lab, however, we will run 0.7% agarose gels. Agarose gels are usually run in either TBE (Tris-borate-EDTA) or TAE (Tris-acetate- EDTA) buffer (as it is in our lab). The agarose comprises a very small percentage of the gel while the remainder is buffer and because of this, the gel is merely submerged in the buffer, with the electrical current running through both the buffer and the gel. This is why agarose gel electrophoresis is also known as "submarine electrophoresis".

In order to correctly determine the size of the resulting DNA/fragments, it is necessary to also load and run molecular weight size marker/ladder. These "ladders" can be DNA, RNA, or protein (depending on the electrophoresis application used) and, in the case of DNA at least, some type of DNA that is digested with a restriction enzyme or combination of enzymes. The molecular weight of the nucleic acid/protein is inversely proportional to the migration rate through some type of gel matrix (again, depending on the application). Since the ladder provides fragments of different sizes, they can be used to estimate the size of the results of gel electrophoresis.

Materials

- Agarose
- 50X TAE buffer
- Gel electrophoresis chamber
- Power supply
- Gel casting material
    - Gel tray
    - Rubber dams
    - Plastic comb

Methods

A. Agarose gel preparation

a. Make 60 ml of 1% agarose

i. Make 1L of 1X TAE buffer using 50X stock
ii. Dissolve agarose in 1X TAE buffer
iii. Heat agarose and buffer in microwave for 1 minute
iv. Use hot gloves to remove flask from microwave and begin to cool

b. Pour warm agarose into gel mold (with rubber blocks and comb installed)

c. When the gel is solidified, remove the rubber blocks. The gel is now ready to be placed in the gel electrophoresis chamber.

B. Ladder/Marker Preparation

a. Heat the ladder for 5 minutes at 65°C and cool on ice for 3 minutes. Prepare the DNA ladder as follows:

Water	14 µL
6X DNA Loading Dye	3 µL
DNA ladder	3 µL

C. Gel electrophoresis

a. Prepare the samples as follows:
i. Use 1 µL of sample + 3 µL of 6X DNA Loading Dye + 16 µL of water

b. The following is an example of the possible lane assignments:
i. Plasmid DNA (undigested)
ii. Lambda DNA (undigested)
iii. Plasmid DNA EcoRI
iv. Plasmid DNA HindIII
v. Lambda DNA EcoRI/HindIII ladder
vi. Lambda DNA EcoRI
vii. Lambda DNA HindIII
viii. Lambda DNA EcoRI/HindIII ladder

c. Run the gel at ~80 V for about 90 minutes.

d. Stain the gel accordingly.

Bradford Protein Assay

Introduction

There are several ways to determine protein concentration. The Bradford assay is a rapid and accurate way to measure protein concentration using spectrophotometric analysis. Typically, in this assay, Coomassie Brilliant Blue is the dye used. It exists in three forms: a blue anionic form, a green neutral form, and a red cationic. When protein binds to Coomassie, it stabilizes the blue form. When Coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465nm to 595nm with a color change from brown to blue. Therefore, as protein concentration increases, blue color increases. Protein concentrations are estimated by reference to absorbances obtained for a series of standard protein dilutions, which are assayed alongside the unknown samples. The ThermoScientific Coomassie Plus Kit is a fast Coomassie-binding, colorimetric method for determining total protein concentration. This method is a modification of the Bradford method and results in significantly less protein-to-protein variation than is observed with other Bradford-type Coomassie formulations.

Materials

- Bovine Serum Albumin (BSA)
- Sterile water
- Test tube
- Spectrophotometer (Spec 20) or plate reader
- Coomassie Plus Reagent

Methods

1. Pipette 50 µl of each standard or unknown sample into appropriately labeled test tubes.
2. Add 1.5 mL of the Coomassie Plus Reagent to each tube and mix well.
3. For the most consistent results, incubate samples for 10 minutes at room temperature.
4. With the spectrophotometer set to 595 nm, zero the instrument on a cuvette filled only with water. Then, measure the absorbance of all the samples.
5. Prepare a standard curve by plotting the measurement for each BSA standard vs. its concentration in µg/ml. When preparing the standard curve, use a point-to-point as opposed to a linear fit line. Use the standard curve to determine the protein concentration of each unknown sample.

Vial	Volume of Diluent	Volume and Source of BSA Final BSA Concentration	Final BSA Concentration
A	0	300 µl of Stock	2000 µg/mL
B	125 µl	375 µl of Stock	1500 µg/mL
C	325 µl	325 µl of Stock	1000 µg/mL
D	175 µl	175 µl of vial B dilution	750 µg/mL
E	325 µl	325 µl of vial C dilution	500 µg/mL
F	325 µl	325 µl of vial E dilution	250 µg/mL
G	325 µl	325 µl of vial F dilution	125 µg/mL
H	400 µl	100 µl of vial G dilution	25 µg/mL
I	400 µl	0	0 µg/mL