

Introduction

This laboratory manual is designed to help students learn basic and advanced laboratory techniques in genetics, as well as general laboratory techniques used in all the sciences. The laboratory course is designed as follows: The first part involves the learning of many DNA analysis techniques by actually doing them in class. Students are responsible for reading the background information and knowing what a procedure accomplishes as well as how to accomplish the procedure. The second part of the lab course involves the designing of experimental protocols to answer questions pertaining to a specific problem. In other words the second part is experimental research.

This format places the responsibility for learning this material upon the student because this is the most effective method of teaching. I, as your instructor, will be there to explain and assist in you in learning of the techniques in the first part of this course. My role however changes in the second part of the course. I will become your "tech rep" technical advisor, biotechnology supplier, and lab assistant for all of your experimental needs. I will not however tell you what to do, so do not come to Lab totally unprepared and ask me "What do I do?" The proposed method of grading is outlined in your syllabus so I will not elaborate here except to say, your full engagement and participation will be required to do well in this laboratory in terms of a grade. Please make the commitment. Below are some guidelines for success you may want to refer to over the course of this semester. They are all equally important to your success.

- *Be prepared for laboratory by reading ahead and feel free to ask questions anytime you see me, in or out of class.
- *Write out all procedures and work out all calculations before coming to class.
- *For the second part of the course: Make a list of all materials, equipment and reagents you need and give them to me at least 1 week in advance so I may have them prepared and ready for you.
- *Know what you are doing and why you are doing it. Lab groups will be composed of no more than two individuals and you will evaluate each others performance as part of your grade. In other words do not depend upon your lab partner to know all the calculations and procedures; be aggressive, not passive in your pursuit of knowledge.
- * If you don't know or understand something, **ASK ME** no matter how trivial it seems.
- * The use outside sources of information is strongly encouraged. I have a very good Lab Literature Resource Center (**LLRC**) in LF 338 lab for you to use.
- * Keep a very complete detailed and legible record of all you do and all of your data. Your Lab notebook is evidence of your laboratory accomplishments which will essentially be the majority of your grade for the course. You may want to keep a back-up copy!
- * If you have any doubts about procedures, equipment or calculations **ASK ME!**
- * If you have any reason that you may be uncomfortable handling certain reagents (allergies etc.) or inability to perform certain procedures I will be your technician for that procedure no questions asked and no problem.

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Laboratory Rules and Safety Tips

As a general rule, most of the reagents and media we use in molecular biology are of limited toxicity. There are some, however, that **must** be handled with extra caution. The rules listed below apply to any reagent or media used in the lab.

1. Treat **all** reagents with respect and read **all** labels before using any reagent.
2. Never assume any substance is harmless; never touch, smell, or taste **any** solution or reagent.
3. Wear protective clothing, lab coats, safety glasses, and gloves when advised.
4. Know where the eyewash station, shower and sink are and know how to use them. If an accidental spill occurs take immediate action and report the incident.
5. Never aerosol (spray in air) any powdered or liquid reagents, not even the “harmless” ones.
6. If you have any known allergies or sensitivities to certain substances, make them known and have a lab colleague complete that portion of the experiment.
7. Don't Panic. Use common sense and don't play in the lab - it can be a dangerous place.
8. The breaking of these rules can result in immediate and irrevocable expulsion from the laboratory.

Partial List of Toxic Reagents

Phenol and Chloroform/Isoamyl alcohol mixtures. These are used extensively to extract protein from nucleic acids.

Concentrated Phenol, which we use, is very caustic and toxic to cells. The use of Phenol requires gloves, lab coat, and protective eyewear at all times. If it gets on unprotected skin, the skin will turn white. If contact is made, wash immediately with large amounts of water and soap.

Chloroform and all alcohols are generally highly flammable and toxic when taken internally or absorbed through the skin. wear protective gloves, eyewear, and lab coat when using these solvents.

Concentrated acids and bases are caustic (they burn holes in lots of things) and require full

protective gear. Concentrated acids should always be diluted in an exhausted safety hood. Concentrated acid is **always** added to water; **never** pour water into acid.

Ethidium Bromide is a chemical used in all DNA-agarose gel electrophoresis. It is a mutagenic agent (can cause mutations in DNA structure) and should always be handled with gloves and protective eyewear. Agarose gels containing this chemical must be handled with the same protective wear.

In this laboratory we will use bacterial cultures that are non-pathogenic, however, because we are a recombinant DNA laboratory, we are required to autoclave all cultures before we dispose of them. Any extraction lysates that may contain living cells must also be autoclaved before disposal.

UV Light Sources

UV (Ultraviolet) light is harmful to the retina. **Never** look into a UV light source without protective eyewear.

We will be using UV light sources to visualize DNA agarose gels on the UV transilluminator and to screen for fluorescence of bacterial colonies on plates, so protective eyewear is always required when using this equipment.

Special Note: If you are uncomfortable handling any reagents or performing a specific procedure--- please have a colleague or the instructor perform the procedure for you. There is absolutely no penalty for this.

Media

Most modern media are premixed and sold by commercial manufacturers. Difco is one of the largest media companies in the U.S. Much of our media and media components are supplied by Difco Co. Each medium has specific mixing and sterilization instructions on the label. Follow these instructions carefully.

Some media we make from components. Recipes are included in the laboratory exercises. Recipes generally require adding specific amounts of powdered reagents or mixtures (weighed in grams or milligrams) to pure water. These solutions are then mixed until dissolved, and then usually sterilized by autoclaving.

Sterilization & Disinfection – Autoclaving

An autoclave is basically a large, well regulated pressure cooker. The general rule of sterilization is that when any substance or item is heated to 121° C at 15 lbs of pressure per square inch for 15 minutes, it is considered sterile.

Sterilization is defined as the rendering of a body or material free from living cells and especially microorganisms usually by killing those present with heat. Autoclaving is the method most often used to sterilize solutions, containers, and media. It is also used to decontaminate bacterial cultures and other items before disposing of them.

Autoclaving can, however, break down most proteins and some chemical compounds. If solutions contain certain substances that are broken down by autoclaving, they must be sterilized by a special process called filter sterilization.

Filter Sterilization

Filter sterilization is the process of passing solutions through a solid membrane with a pore size (minute openings in the membrane) that will restrict passage of bacterial or other cellular contaminants, but will allow passage of the molecules of a compound in solution. A sterile filter and a sterile container must be used in filter sterilization.

Viral particles are not always excluded by certain filters with specific pore sizes. In fact, viruses were first discovered because of this characteristic, and were defined as particles that passed through filters. This is the way we sometimes separate bacteriophage from the bacterial culture that supports their replication. Two common filter sizes used are 0.45 micron and 0.22 micron pore size. These filters are very expensive and are therefore used only when autoclaving is not recommended.

Use of Open Flame

Exposure to open flame will also destroy all carbon based forms of life.

We generally flame inoculating loops and glass rods. Inoculating loops are flamed (until they glow red) before use and glass rods are dipped into ethanol and then flamed briefly before use.

Disinfection

Disinfection is not sterilization. This is usually done to eliminate large numbers of, but not all, organisms from a surface. Many agents are toxic to organisms and are used to disinfect items. Some of the best household disinfectants are chlorine-based chemicals, such as household bleach. Remember, however, that all chemicals that are toxic to bacterial cells are, to some extent, toxic to our own cells.

Some reagents we use are never sterilized because they are toxic in concentrated form and do not have the necessary components to support growth of any organism. Organic solvents such as alcohols and chloroform are in this category. Also included are reagents such as phenol and concentrated acids and bases.

Plate-Streaking Technique

- * Using a permanent marker or wax pencil, mark the bottom of each agar plate with your initials, the bacterial strain, and the date.
- * Hold inoculating loop like a pencil in the hand that you write with.
- * Flame the tip of the loop in a gas burner until it glows red hot. Once the tip glows, pass the rest of the wire through the flame. The entire wire portion of the inoculating loop should glow red.
- * Allow the loop to cool for 5-10 seconds. Do not allow loop to touch any surface, and don't blow on it to cool.
- * First, remove the lid from the culture plate and hold the lid face down.
- * Take freshly flamed loop and ground into a part of the agar with no colonies to cool.
- * Pick up a portion of the colony with the round part of the loop.
- * Place top back over culture plate.
- * Now, streak your labeled plates according to Figure on page.
- * After streaking, invert plates and place in a 37° C incubator overnight. Plates are inverted to prevent

Dilutions

Dilutions of bacterial cells and phage lysates are often necessary to obtain numbers of colonies or plaques that can be accurately counted. If too many cells are plated a lawn of bacteria or one confluent plaque is created on the surface of an agar plate. As you might imagine, this is useless for obtaining cell number counts or pure cultures from isolated colonies. *You must learn this process.*

If a bacterial culture is diluted 1×10^{-5} this means that the cells are diluted 1/100,000. To obtain this dilution you could mix 1 ml of cells into 99,999 mls of buffer or media. However we do not have any 100 liter flasks. Additionally if you are using expensive molecular biology grade reagents or growth medium this type of dilution would be prohibitively expensive. An alternative approach would be to perform a serial dilution.

Serial Dilution

A serial dilution may be accomplished as follows:

First make a 1/10 dilution; This may be done by adding 1 ml of culture to 9 mls of buffer or other fluid and mixing the diluted culture well.

Now remove 1 ml from this 1/10 dilution and add it to 9 mls of fluid and mix well. This solution is now diluted 1/100 or 1×10^{-2} . Remove 1 ml from this 1/100 dilution and place it in 9 mls and mix well. This tube is now diluted 1/1000 or 1×10^{-3} . Repeat this process two more times and you will have the desired dilution of 1×10^{-5} .

Note: this procedure uses only 50 mls of diluent as opposed to 100,000 mls.

You may also have figured out by now how to speed the process by making bigger dilutions.

Example 0.1 ml in 9.9 mls = 1/100 or 1×10^{-2} and 0.1 ml of this dilution into 9.9 mls of diluent = 1/10,000 or 1×10^{-4}

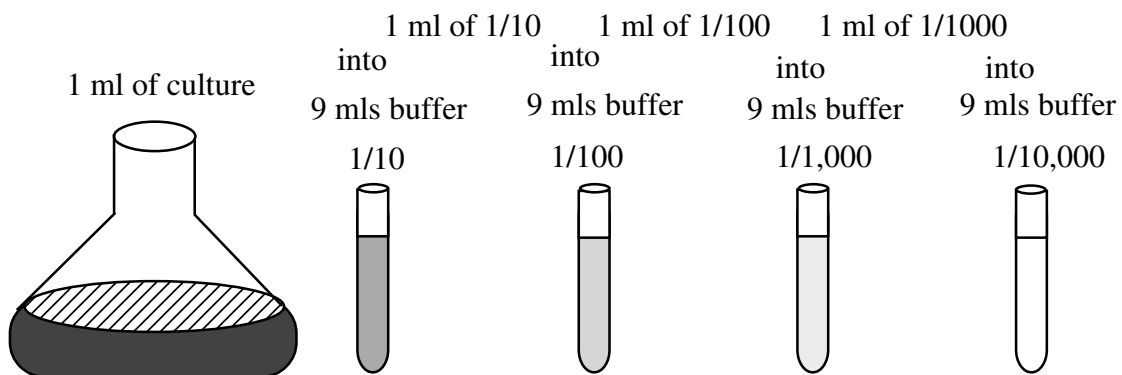
Additionally if your reagents are expensive you can use smaller volumes

0.1 ml in 0.9 mls = 1/10

We do not generally dilute smaller volumes that 0.1 ml. *Generally speaking* accuracy goes down as volume size goes down.

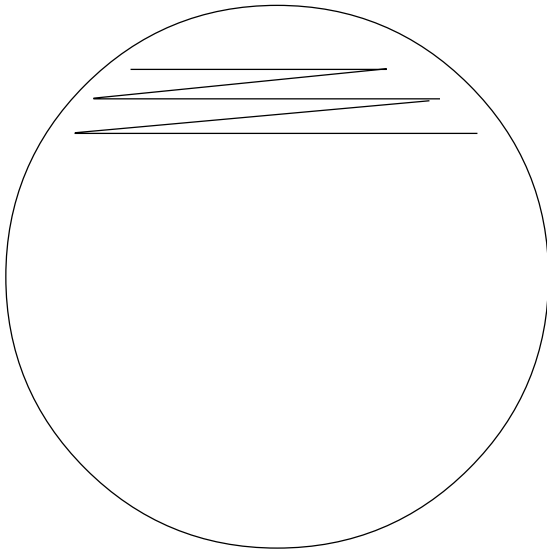
Serial Dilution

Figure 1

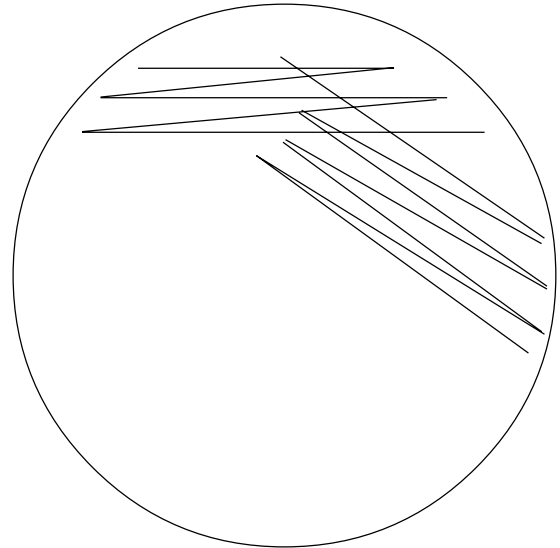


Streaking for isolated bacterial colonies onto agar

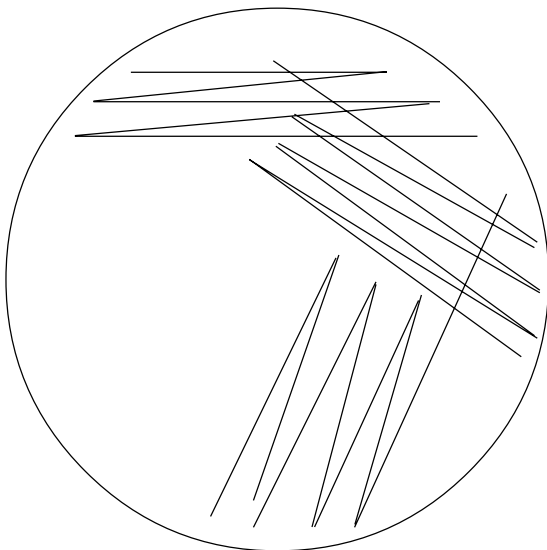
Figure 2



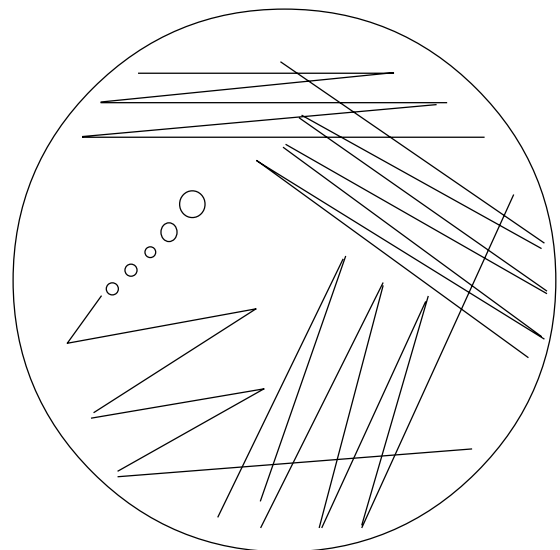
1. Streak the loop containing bacteria lightly across plate in the pattern shown above



2. Flame loop. Ground in agar on edge of plate to cool and lightly streak, pulling through the original streak



3. Flame loop and repeat process as shown.



4. Flame loop and complete final streak

Our objective is to obtain well isolated colonies along the final streaking pattern.

Plating Bacteria

The word plate or plating in the context of molecular biology refers to a petri dish or plate that contains a solid medium that will support the growth of bacteria. Medium (plural media) refers to any mixtures of inorganic or organic compounds that will support the growth of a bacterial culture. Minimal medium is a mixture of inorganic salts with a carbon source (usually glucose) added. Rich media is not as well defined usually containing extracts of yeast or animal products that contain a variety of amino acids, sugars and other complex organic components. There are many different types of media that either select for or differentiate bacteria. A detailed discussion of media and its preparation will be addressed at a later date.

Often we will want to isolate and grow individual clones of cells. Theoretically, one bacterial cell will grow into one colony when isolated on solid medium. This cell is represented as one colony forming unit or **cfu**. All the bacterial cells from a single colony should be identical to one another. This would be true because the bacterial cell that forms the cfu reproduces itself asexually by binary fission with each daughter cell being an identical copy of the original. Since we always want to work with pure cultures or identical clones we must plate cells so they will grow into colonies that are distinct and well separated from each other. This physical separation of colonies allows us to manually pick cells up from a single colony for subculturing onto other media and for various other manipulations. Well isolated colonies are obtained using proper dilution and plating techniques. Serial dilution used to reduce the numbers of **cfus** has been detailed elsewhere and plating technique is performed as follows.

A culture of bacterial cells is diluted in an appropriate liquid buffer or medium. An aliquot of this liquid mixture is then transferred to the surface of an agar plate and spread evenly across the entire area using a bent glass rod referred to as a hockey stick. Usually 10 to 300 ul of diluted culture is pipetted onto the sterile agar surface of a plate and then evenly spread by a hockey stick that has been dipped in 95% ethanol and flamed briefly. Often this process may be facilitated by the use of a small turntable that is large enough to hold one petri dish. This small turntable spins the petri plate making spreading faster and more efficient.

Please remember these following tips about plating.

- *Plating is done aseptically, that is, using sterile technique!
- *The agar plate is sterile and all pipettes, pipette tips and spreading tools must be sterile also.
- *Glass or disposable pipettes are sterile from the metal can or plastic wrapper.
- *Plastic pipette tips are sterile from the plastic box
- *The glass rod or hockey stick is sterilized by flaming.

Proper flaming of your hockey stick!

Dip the bent portion of the glass rod into 95% ethanol and allow excess ethanol to run off. Then pass the glass rod through the flame igniting the alcohol. *Do not* hold the rod in the flame because this will cause the rod to overheat and kill the culture you are spreading. After the alcohol has burned off, lightly place the glass rod onto the surface of the agar and spread the liquid evenly. Once the liquid has been spread evenly across the surface of the agar replace the top of the dish and allow the fluid to soak into the agar. Invert the plate and incubate at the recommended temperature.

(Plates containing bacteria are inverted to avoid condensation from forming on the agar surface. Condensation on the agar will cause the colonies to run all over the agar and ruin the plate.

Cautions :

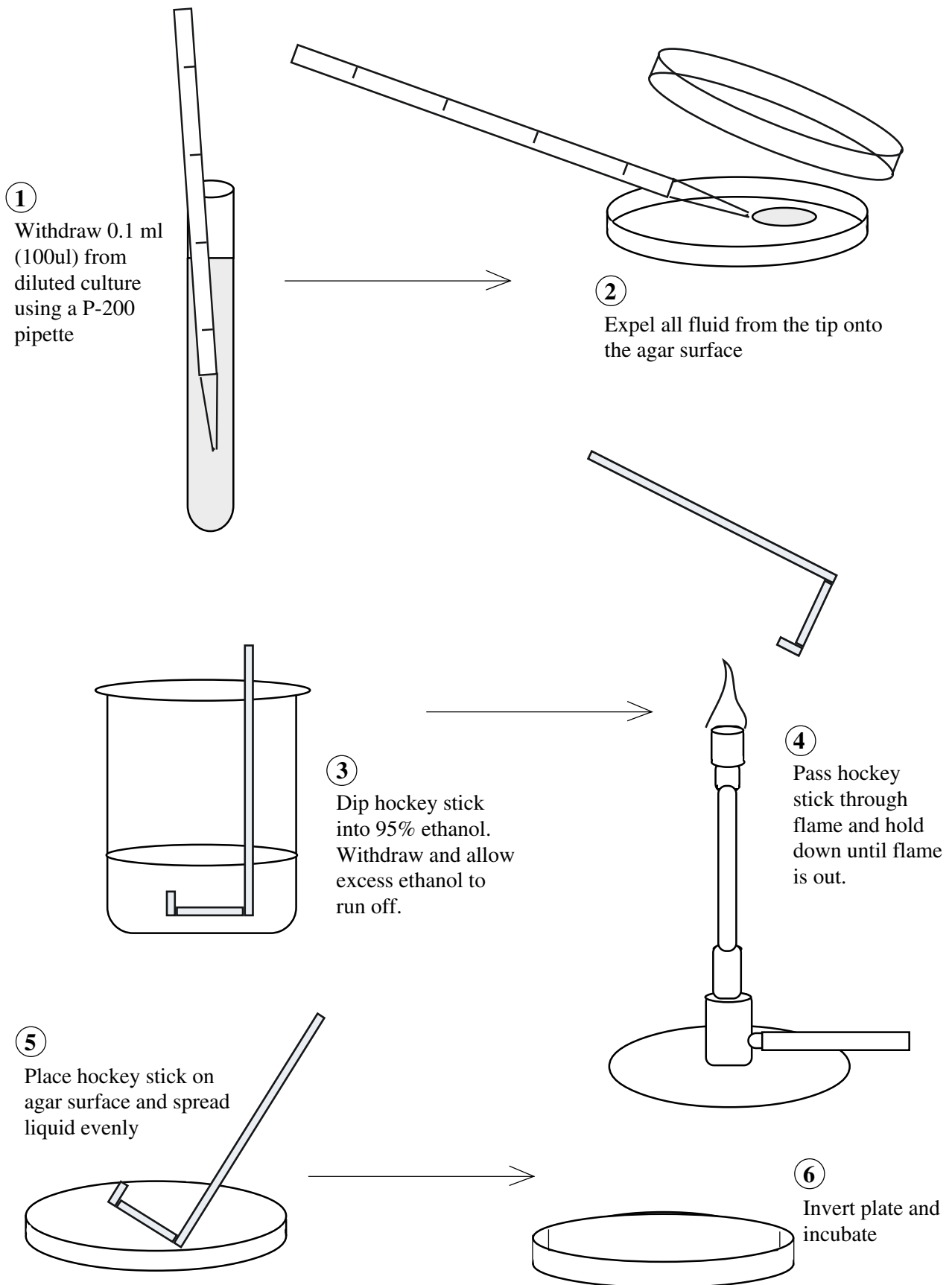
Keep the ethanol container ***away*** from any source of ***flame*** including your flaming glass rod.

Do not allow alcohol to run on to your fingers or anywhere that might flame: Flamed fingers are extremely ***painful!!!!!!!!!!***

Do not blow on the rod to cool or touch it to any surface except the agar surface.

Plating a liquid Bacterial Culture onto agar

Figure 3



Use of Sterile Pipettes

Pipettes directly from the wrapper are sterile.

Do not touch sterile pipets to any surface, if a pipette comes into contact with a surface, discard it in the waste and get a fresh, sterile one.

Using a pipette

Never mouth pipette solutions or cultures — This can be dangerous!

Many people flame their pipettes and the mouths of bottles or tubes containing sterile solutions, however if the outlined procedure is followed carefully and manipulations are done quickly, flaming will not be necessary. But do what works best for you.

*Light your gas burner

* Grasp the top of the pipette and pull it from can or wrapper. (Be careful not to touch the tip that will be placed in the sterile solution)

*Place the larger end of the pipette firmly into the white opening of the pipump.

*Submerge the tip of the pipette into a beaker full of water. Rotate the white wheel on the side of the unit until fluid is drawn up to the desired volume. Pull pipette out. Place into empty tube and expel water by rotating the wheel.

*Practice pipetting volumes with 1 ml, 5 ml, and 10 ml pipettes until you are comfortable performing the procedure.

Use Blue pipump for 1 ml pipettes and Green pipump for 5 ml and 10 ml pipettes.

Inoculation of sterile broth with a bacterial colony from a culture plate

* Label tube appropriately

* Flame your inoculating loop and touch red hot tip to area of sterile agar in plate to cool.

* Remove a portion of the colony from the plate.

* Remove cap and flame mouth of media tube.

* Place loop in medium and agitate to dislodge cells.

* Re-flame mouth of tube — replace cap.

* Incubate both tubes in 37° C shaking water bath overnight.

Use of Micropipets

* Notice the numbers on the side of the pipet

* Rotate the wheel and see how the numbers advance.

* Place the tip of a pipette into the top of a yellow pipet tip found inside a plastic box. Note: DO NOT touch these tips with anything — they are sterile.

* Press firmly to lodge tip into pipet.. DO NOT press too hard or the box will flip over.

* To check pipet tip placement, grasp the tip close to the pipette and check to see it is firmly attached.

* Depress white plunger gently until it stops; now, press harder. Notice that there are two stops. The first stop is used to withdraw fluid; the second stop is used to expel all fluid from the tip. Caution: DO NOT let the plunger go! This will cause fluid to be sucked up into the pipet and ruin it.

* Place just the end of the pipet tip into fluid after depressing to the first stop.

* Gently release thumb pressure to suction fluid into tip.

* Watch fluid go into tip.

* Angle tip against the edge of a tube or into parafilm.

* Depress plunger to second stop smoothly to expel fluid.

* Now, hold over empty waste beaker and press white button on side to eject tip into beaker.

* Practice your technique with both the P-20 and P-200.

Tips on the use of volumetric measurements:

Always use the measuring device with a volume closest to the volume you need. Example: 1 ul - a P 20 micropipetor; 4.5 mls - a 5 ml pipet; 150 mls - a 200 ml graduated cylinder.

Molar concentrations are always defined as grams/liter. That is, total volume in one liter, including the dissolved compound.

Percent solutions are grams/100 mls or, volume in mls/100 mls total.

When dissolving solutes in solution, never add reagent to a whole liter of solvent.

Don't try to mix in a graduated cylinder - it wasn't designed for mixing.

Add less than the desired volume of solvent in a beaker, add the reagent, dissolve, and pour the solution back into the appropriate size graduated cylinder. Bring the volume to the desired level by adding the solvent.

Use of a Balance

Electronic balances are very easy to use. Place a weigh boat or dish on the balance and push the tare bar. This automatically subtracts the weight of the boat and zeros the scale. Add the appropriate amount of reagent until the scale displays the correct number.

Remember: Do not exceed the capacity of the balance or the weigh boat. Do not forget to clean up spills immediately. Do not aerosol any powdered reagents. For smaller weights use the analytical balance (it has glass doors).

Use of the Centrifuge

Proper placement of the rotor and **Balancing of tubes in the rotor are essential for safe operation.** NEVER start a centrifuge before double checking the following:

* Rotor placement

* Tube placement and balancing

* Rotor cover on properly

Proper Balancing of Tubes in a Centrifuge

Tubes should always have the same volume or weight opposite each other in all rotors. If you wish to centrifuge 1,3,5 or an odd number of tubes, you must make a blank balance tube. All tubes that are opposite each other must have the same weight or volume in each tube.

To assure proper balance, start the rotor at slow speed first and increase to desired speed gradually.

Centrifuges are safe provided they are properly used and maintained. Until instructed otherwise, the instructor or teaching assistant must double check your tubes and rotor before you start any run on the centrifuge.

Conversions and Formulas

Commonly Used Volumetric Measurements

liters = l

milliliters = ml

microliters = ul

1 liter = 1,000 mls = 1,000,000 ul

1 ul = .001 mls = .00001 liters

Commonly Used Concentrations of Chemical Compounds

1 Molar = molecular weight or Formula weight of compound in

1 liter total volume

Example! On jar of NaOH (sodium hydroxide) pellets

Formula wt = 40 40 grams in 800 mls of water dissolve; bring volume up to 1 liter in volumetric cylinder = 1 molar NaOH

1 Molar = 1M

1 millimolar = 1mM

1 micromolar = 1uM

1 M solution = 1000 millimolar = 1,000,000 micromolar

Solutions made as per cent %

50% v/v (volume to volume) = 50 mls compound in 100 mls water total

50% w/v (weight to volume) = 50 grams compound in 100 mls of water total

Note: water = solvent and compound = solute

solvent may be any liquid the solute is soluble in. If solvent is not specified; water is used.

Common weight measurements

1 gram = 1 g.

1 milligram = 1 mg.

1 microgram = 1 ug.

1g. = 1000 mg. = 1,000,000 ug.

Students must be able to make these conversions with ease.

Equipment you will be using and will be tested on as to function and name

Analytical balance
Balance - scale
Beaker
Centrifuge
Centrifuge (rubber adaptor)
Centrifuge rotor
CRT Camera
Eppendorf tube - microfuge tube
Erhlinmeyer flask
Eyewash station
Fire extinguisher
Flammable cabinet
-20° Freezer
-70° Freezer
Gas burner - bunsen burner
Gas burner striker
Gel electrophoresis apparatus-horizontal
Glass cover slips
Glass slides
Glassine papers
Graduated cylinder
Hockey stick - Bent glass rod
Hoods- Safety and Laminar flow
Ice buckets
Inoculating loop
Kimwipes
Klettometer - Klett
Lenspaper
Magnetic stirring bar
Media - media components
Microfuge - Eppendorf
Micropipetor - Pipetman
Microscope
Oakridge tube
Pasteur pipette
Petri dish - plate
Pipette
Pipette pump - Pipump
Pipette tips
Plate turntable - spreader
Power supply
Refrigerator
Rotator - rotator platform
Shaking incubator
Spatula
Spectrophotometer
Speedvac - evaporator
Stationary incubator
Stir bar retriever
Stirplate -Hotplate
Test tube
Label tape
Transilluminator
Tube racks
U.V. Goggles - Safety glasses
U.V. Light source
Vacuum pump
Vortex
Water bath
Weigh boat

Genetics Lab Exercise 1

Agarose Gel Electrophoresis of DNA an Introduction

What is gel electrophoresis?

Gel electrophoresis is a process used to move biological molecules in an electric field. Phoresis is defined as the transmission of a substance from the Greek meaning "to carry". The electrical field in electrophoresis carries charged molecules toward the negatively or positively charged pole depending upon the net charge at a given pH of the entire molecule. In agarose gel electrophoresis the agarose which forms a gelatin like substance is the matrix in which we suspend the substance to be separated. The agarose acts like a molecular sieve separating molecules by size. The smaller molecules travel through the small pores of the agarose faster than larger molecules. Another type of matrix used in electrophoresis is polyacrylamide. This polymeric substance acts as a very small sieve to separate smaller molecules such as proteins. Gel electrophoresis can separate molecules by charge and size. However when a molecule has a uniform charge as nucleic acids do, the molecules are separated by size alone.

How do molecules become charged?

The greater the net charge the faster the molecule will move in the electric field. The net charge of a protein molecule depends upon the numbers of the positively and/or negatively charged amino acids and the pH of the buffer in which it is suspended. Remember pH is defined as the negative log of the hydrogen ion concentration thus the lower the pH the greater the number of free protons and the higher the pH the fewer the number of free protons. In acidic solution the acidic or negatively charged amino acids are fully protonated and are uncharged. As pH increases toward the basic part of the scale (8 or above) the protons are not available to add on to the acidic residues and the amino acid is negatively charged. The same thing occurs in reverse for the basic amino acid residues on a protein suspended in buffers that are acid ($\text{pH} < 7$).

DNA is very different from proteins because it has a uniform negative charge at pH's of 8.0 and above. As you know, the Nucleic acids (RNA and DNA) are composed of nucleotides which are monomeric units consisting of a 5 carbon sugar, a nitrogenous purine or pyrimidine base and a phosphate group. It is the phosphate group which gives nucleic acid its negative charge because it acts as an acid. In basic solutions of pH 8-9 the phosphate gives up protons causing each nucleotide to have a negative charge. Since nucleic acids are composed of repeating nucleotides there is one negative charge per nucleotide residue. Therefore nucleic acids have an equal charge to mass ratio or a uniform negative charge. For example a polynucleotide 100 nucleotide bases long has 100 negative charges while a polynucleotide 200 nucleotide bases long has 200 negative charges. Thus nucleic acids separate by size alone during electrophoresis because their charge distribution is constant.

This is not true for proteins as you can see by reviewing the structures of the 20 basic amino acids. In order to separate proteins by size one must coat proteins with a negatively charged detergent called Sodium Dodecyl Sulfate or **SDS** which gives them an artificial, uniform negative charge.

What are buffers and why are they so important in electrophoresis?

Buffers are critical in all of biochemistry. Buffers are substances that control pH fluctuation by the absorption of protons from solutions that are too acidic and by the release protons into solutions which are too basic. If your blood was not buffered you would quickly become nonfunctional. Why? All anabolic and catabolic metabolism in organisms is catalyzed by enzymes which are either protein or nucleic acid in nature. These molecules must be dissolved in solution to be operational and the activity of these molecules is very sensitive to the pH or proton concentration of the solution in which they are dissolved. As I mentioned previously the pH of a solution can determine the charge of proteins. As you probably already know the charged portions of protein enzymes are generally located at the active site of the of the enzyme (where chemical activity occurs). If you think about this you will understand the importance of controlling any fluctuation of pH in biological systems.

What is the purpose of the agarose gel buffer TBE and the DNA sample buffer? TBE stands for Tris, Borate and EDTA which are the individual components of the buffer. Tris is a buffering agent which acts as an excellent buffer at pH's between 8 and 9, Borate is Boric acid which also acts as a buffering agent and brings the pH of the Tris solution to approximately 8.8. EDTA is a chelator of magnesium ions (a chelator absorbs free ions from solution) which absorbs the Mg⁺⁺ ions which are essential cofactors for DNase enzymes. Therefore the inclusion EDTA in this buffer prevents degradation of the DNA from unwanted DNases which are ubiquitous in nature.

Many buffers used in biochemistry contain Tris and EDTA at various concentrations. Of course the higher the concentration of either component the more effectively it controls pH or chelates free Mg⁺⁺ ions. Some commonly used buffers and their uses are listed below.

DNA sample buffer is the blue colored buffer added to DNA samples before they are loaded and electrophoresed in the agarose gel. While there are several formulations for DNA sample buffer (referred to as blue juice) the sample buffer we use contains two blue dyes, xylene cyanol, an aqua blue color, and bromphenol blue, a purple blue color, which comigrate with DNA fragments of 2800 and 250 base pairs respectively. These dyes allow one to approximate the distance DNA has moved in a gel and are called tracking dyes. DNA sample buffer also contains Tris, EDTA (**TE Buffer**) and glycerol. The glycerol when mixed with an aqueous solution makes the fluid more dense than water which allows the DNA sample to flow through the TBE electrophoresis buffer and sink into the well of the agarose gel.

A partial list of common buffers or solutions used in Biotechnology Research

Buffer	Components	Use
TBE	Tris, Borate, EDTA	DNA Gel Electrophoresis Buffer
TAE	Tris, Acetate, EDTA	DNA Gel Electrophoresis Buffer
TE	Tris, EDTA	Resuspending of DNA
Tris Gly	Tris, Glycine	Protein Gel Electrophoresis Buffer

Glucose Tris EDTA	Same	Plasmid extraction Buffer
Acetate Buffer	K Acetate, Glacial acetic acid	Plasmid extraction Buffer
Na-Acetate	Sodium Acetate	DNA Precipitation
Z Buffer	Na Phosphate, β ME	β -galactosidase assay Buffer
CaCl ₂	Calcium Chloride	Prep of competent <i>E. coli</i> DNA
Restrict.enz Buff	Various components	DNA Restriction digest

Exercise on Agarose Gel Electrophoresis of DNA

Skills to be learned in this exercise:

Preparation of agarose gel, Preparation of electrophoresis buffer, Preparation of DNA samples, Loading DNA into agarose gel, Use of the power source and a horizontal gel electrophoresis apparatus; Use of micropipets, Use of microfuge and microfuge tubes, Use of UV transilluminator to visualize DNA, Use of a DNA standard.

Preparation of a 1% Agarose Gel

*First determine how much volume of agarose you will need. This can be done by putting together the gel tray apparatus (**as shown by instructor**) and seeing how much water is necessary to fill the tray to an appropriate level using a graduated cylinder

Record amount in notebook for reference.

*Add the amount of 0.5 X TBE buffer into a flask 5 or more times greater in volume than the desired quantity of agarose. (Note: TBE buffer is generally kept as a 10X concentration and is diluted 1:20 in water to obtain a 0.5 X final concentration of buffer.) Add agarose to a 1% final wt/v concentration. Example: 1 gram of agarose in 100 mls TBE = 1% agarose wt/v. Do not make 100 mls for one gel. Calculate the correct ratio for the volume you need (**as shown by instructor**).

Record procedure in notebook for reference

*Swirl briefly place magnetic stir bar in flask and place onto hot plate until the solution boils and is perfectly clear, containing no particulate matter.

Watch this closely so noboiling over occurs.

*Using a hot pad glove, remove flask containing agarose from hotplate (do not agitate or it will boil over). If any undissolved agarose can be seen place back on hot plate until solution is clear.

*Carefully place on lab bench to cool to approximately 55C. "Cheek warm" (**as shown by instructor**)

* Put together your minigel casting tray by closing both ends of the tray with label tape and insert comb or (**as shown by instructor**).

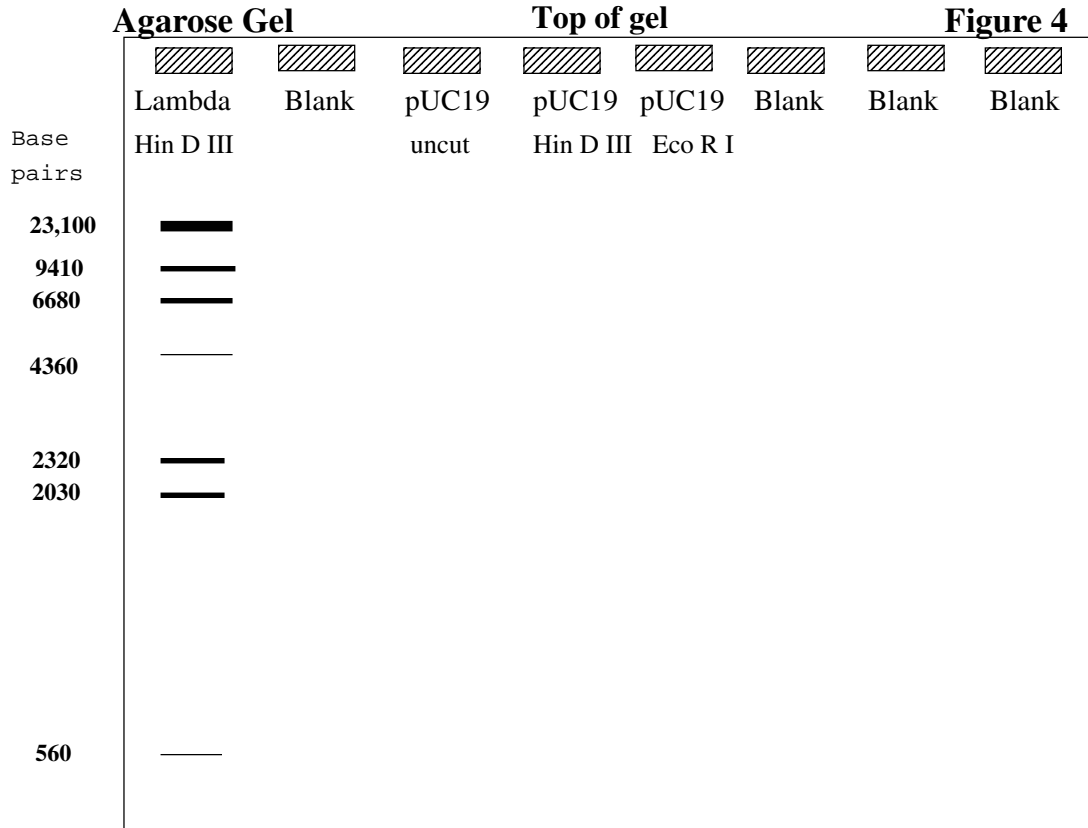
*While the container containing the agarose is still liquid, but cool enough to touch, put on gloves and safety glasses and add 1 ul of Ethidium Bromide (EtBr) solution, mix gently by swirling and pour the molten agar into the gel casting tray . **No bubbles please.**

Caution: do not touch this solution or these gels without gloves! Ethidium Bromide binds DNA readily and is highly mutagenic

*Allow the gel to cool on the lab bench. When it is solid it becomes opaque.

*Wearing gloves remove the gel comb, remove the tape carefully from the gel tray ends; place the casting tray into the electrophoresis apparatus and fill with 0.5X TBE buffer and load the DNA samples as described in the exercise as described below

Note: before loading, draw a picture of your gel on paper and label the wells as illustrated below



Preparation of DNA samples

You will find tube racks and autoclaved microfuge tubes in your team kits in LF338.

* Lambda (λ) DNA will be used always on every DNA gel you will run in this laboratory. (λ) is a standard DNA obtained from the lambda phage, a virus which commonly infects *E. coli*. (λ) DNA is often digested with the Restriction enzyme HinD III. This is almost a universal DNA standard used in Biotechnology. More information can be obtained from the **Lab Literature Resource Center (LLRC)** located in the LF 338 Genetics/ Molecular Biology Lab.

*Each group will have a tube of sample DNA buffer which we call Blue juice (abbreviated as BJ). This contains the 6X DNA sample buffer which will be added to all DNA samples to be loaded on agarose gel. Blue juice makes the DNA sample dense enough to

fall into the well in the agarose gel made by the comb and contains two different dyes which allows us to track the DNA during electrophoresis.

*Students should load the digested DNA samples into agarose gels as supplied and demonstrated by the instructor. **Be sure to outline the process**, because you will be required to perform it on your own, in the experimental second half of the laboratory.

Loading DNA into Agarose Gels and Electrophoresis of the DNA Gel

* The solid gels will be placed in the horizontal gel electrophoresis apparatus which should be filled with 0.5X TBE to just cover the gels. The samples of DNA will be loaded through the electrophoresis buffer (**as shown by instructor**). After loading, place the cover over the gel apparatus box, plug the red and black leads into the power source (red to red and black to black).

*Gels should be run between 80 and 100 volts until the Bromphenol Blue (purple-blue dye) is near the end of the gel. This will take between 30-60 minutes

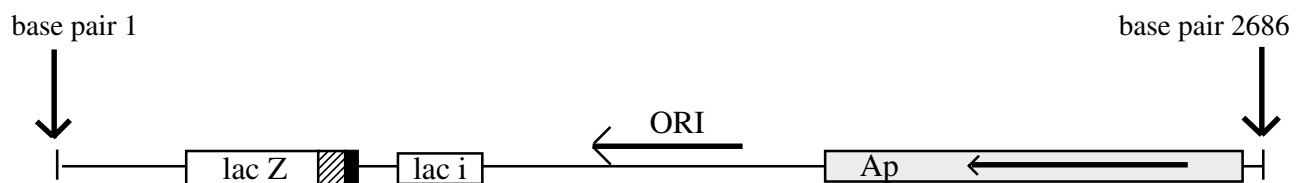
(Note: Be sure your apparatus always has red electrode at the bottom of the gel)

*Wearing gloves and Safety glasses remove the gel, place on transilluminator and photograph (**as shown by instructor**). Print the picture and place in results section of your lab notebook.

Below I have included a diagram and explanation of a common cloning plasmid vector called pUC 19. This is one of the plasmids you will be working with this semester and it is very important for you to understand both its structure and function. This plasmid DNA is obtained commercially and its entire DNA sequence is known. It consists of pieces of DNA from several different sources and some of the DNA contained in it has been synthetically constructed for a specific purpose (e.g. MCS).

*More information can be obtained from the **Lab Literature Resource Center (LLRC)**

Plasmid map of a typical cloning vector pUC19 - Figure 5

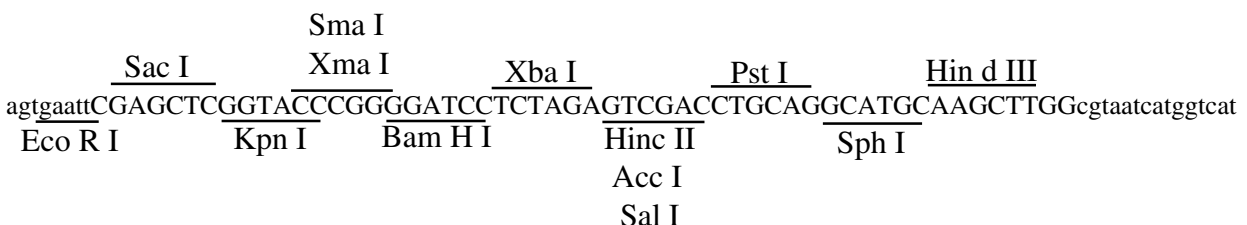


lac z = gene that codes for beta-galactosidase enzyme

lac i = gene that code for galactoside permease enzyme

ORI = DNA responsible for ability of the plasmid to self-replicate: (Origin of replication)

■ small black box = MCS or multiple cloning site which is illustrated below as DNA sequence



Genetics Lab Exercise 2

Restriction Analysis of DNA : Plasmid versus Chromosome

Introduction

Although all DNA (2-deoxyribonucleic acid) has the same polymeric structure, which consists of nucleotide monomers, types of DNA can vary greatly in their structures. The two types of DNA we will be working with today both come from a prokaryotic (bacterial) source.

The genomic DNA or bacterial chromosome of *E. coli* is approximately 4.0×10^6 base pairs. While this is a very small when compared to any eukaryotic genome it is enormous when compared to most plasmid DNA which generally range in size between 2,000 and 20,000 base pairs. Plasmid DNA is extrachromosomal circular DNA which replicates independently from the bacterial chromosome (autonomous replication).

Plasmid DNA is a naturally occurring form of DNA in prokaryotes which first evolved to provide resistance to toxic environmental agents such as heavy metals and naturally occurring antibiotic agents. Generally speaking plasmids replicate in what we term multiple copies per bacterial cell. This is in contrast to chromosomal DNA which in normally dividing bacteria is present as one copy per cell. Some plasmids can have a copy number as high as 200 copies per cell in some organisms. This makes plasmids an ideal way to amplify the amount of small pieces of DNA using DNA cloning methodologies. For example if you need lots of DNA to study you could cut open a plasmid insert your piece of DNA place it back into a bacterial cell and harvest the progeny of that cell. If each cell produces 50 copies of the plasmid, then you have also 50 copies per cell of the cloned DNA and thus a 50 fold amplification of your DNA of interest per bacterial cell. In addition to the use of plasmids for the amplification of DNA, they are invaluable for the transfer of DNA sequences which can impart unique characteristics through the transfer of genes to many different types of organisms.

In this laboratory we will use enzymes that are specific to prokaryotes, called restriction enzymes, to digest both chromosomal and plasmid DNA from prokaryotes. Restriction enzymes are produced by bacteria as a defense against the integration of foreign DNA. For instance the restriction enzyme Eco RI from *E. coli* will digest or cut any DNA having the sequence 5' GAATTC 3' and therefore restrict the integration of this DNA into its genome. However *E. coli* will not digest its own DNA with Eco R I because it methylates it with methylation enzymes in a specific pattern so that it can distinguish its own DNA from foreign DNA. Scientists have discovered, isolated and cloned hundreds of different restriction enzymes from a multitude of different bacterial species.

These restriction enzymes are used extensively in the genetic manipulation of organisms. Today we will digest our pUC 19 plasmid with different restriction enzymes. Most of the restriction enzymes used in biotechnology applications are called Type II Restriction enzymes. These enzymes recognize a specific DNA sequence and cut within this sequence. In contrast Type I enzymes recognize a specific DNA sequence but cut the DNA at a distant location and Type III restriction enzymes require ATP to digest DNA. Type II restriction

enzymes are much more useful to digest DNA easily and with predictable results, therefore Type II Restriction enzymes are widely used for molecular biology research.

DNA Restriction

In this exercise you will perform a digest on ~1 ug of plasmid and chromosomal DNA with 2 different restriction enzymes (**as outlined by instructor**) in separate tubes. You will also perform a digest of a control DNA from phage lambda, a well known bacterial virus which infects *E. coli* cultures. The lambda DNA sequence is known and when digested with Hind III gives DNA fragments of predictable sizes, as illustrated on the gel picture in Figure 4.

All of these DNA digestions will be run on a horizontal agarose gel apparatus which separates the DNA fragments by size using electrical current. Each team will perform the following procedure:

Restriction Enzyme Digestion

- * Label all eppendorf tubes with the DNA type and restriction enzyme to be used
- * Place 1 ug of either plasmid, chromosomal or Lambda DNA provided (**as outlined by instructor**) into appropriately labelled tube.

Please remember 1 type of DNA per tube only.

- * Determine the final volume of your digest. Usually we use 15-20 uls total volume for DNA digestions

*Add enough 10X digestion buffer of the appropriate type for Eco R I or Hin D III digestion to each appropriately labelled tube to bring the final concentration to 1X

* Bring volume up to desired total volume with pure sterile dd H₂O .

* Balance the tubes in the Eppendorf centrifuge and spin for ~ 5 seconds. (Hint use the pulse button)

* Remove tubes from centrifuge and add 1 ul of Eco R I or Hin D III or appropriate enzyme to each labelled tube. (**Caution: use a fresh pipet tip for each tube and keep enzymes on ice at all times!**)

The following is an example of a restriction enzyme digest, assuming a DNA concentration of 0.5 ug /ul and final volume of 20 ul

Add 2 ul DNA to eppendorf tube

Add 2.0 ul 10X restriction enzyme buffer

Add 15 ul pure sterile dd H₂O

Add 1 ul BSA (Bovine serum albumin)

Always add about 1 ul of each enzyme for a complete enzyme digest (Spin 1 second)

* Place tubes in 37° water bath from 30 minutes to one hour

* Remove your tubes from the water bath, add 3 ul of blue juice to each tube, balance the tubes in the Eppendorf centrifuge and spin for ~ 5 seconds.

* Most of each sample should be loaded into the wells of the agarose gels as previously described.

Electrophoresis of the DNA Gel

* The gels should then be placed in the horizontal gel electrophoresis apparatus which should be filled with 0.5X TBE to just cover the gels. Place the cover over the gel apparatus box, plug the red and black leads into the power source (red to red and black to black).

*Gels should be run as in previous exercise

(Note: Be sure your apparatus always has red electrode at the bottom of the gel)

*Wearing gloves and Safety glasses remove the gel, place on transilluminator and photograph as demonstrated by instructor.

Gel Electrophoresis and Fragment Size Calculation

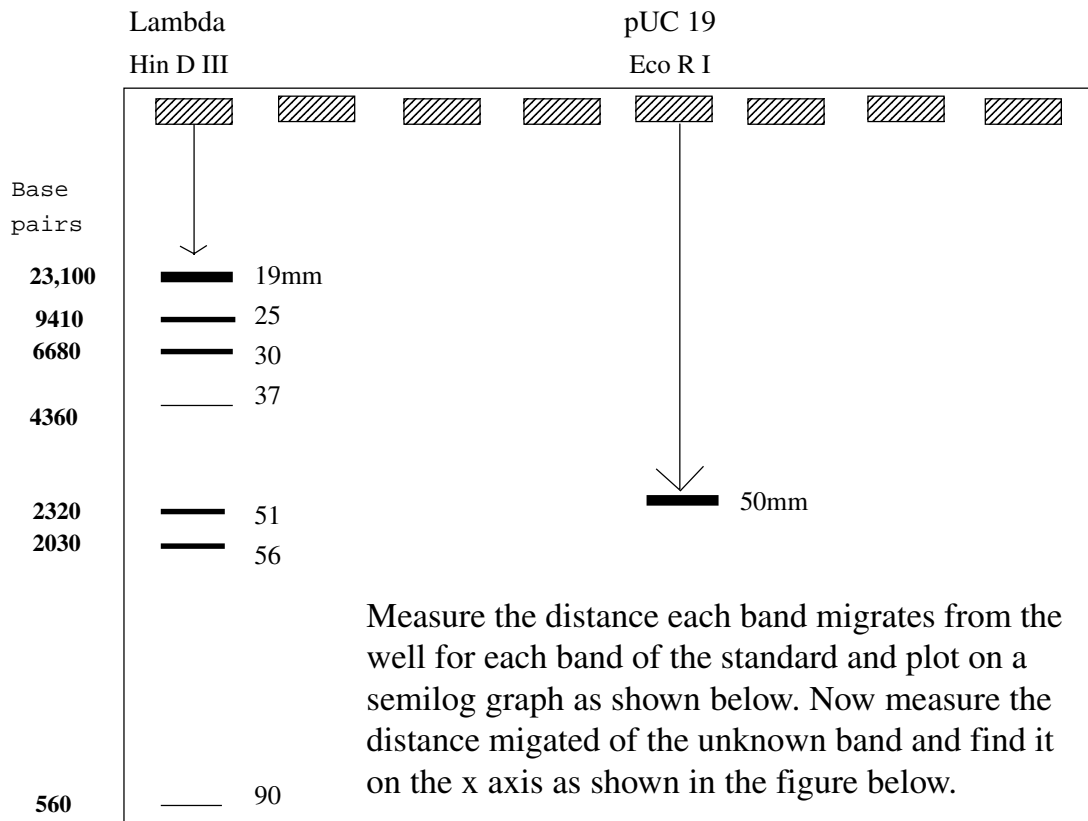
*In this exercise students will calculate the size of their DNA plasmids by using a standard curve based upon the distance Lambda DNA fragments generated by HindIII restriction will migrate in a 1% agarose gel. This is accomplished by following the procedure .

* Measure the distance in mm from the well containing the Lambda DNA digested with HindIII to each band of DNA visible in the photograph. (*Lambda digested with HindIII is almost the universal standard in Molecular Biology!*)

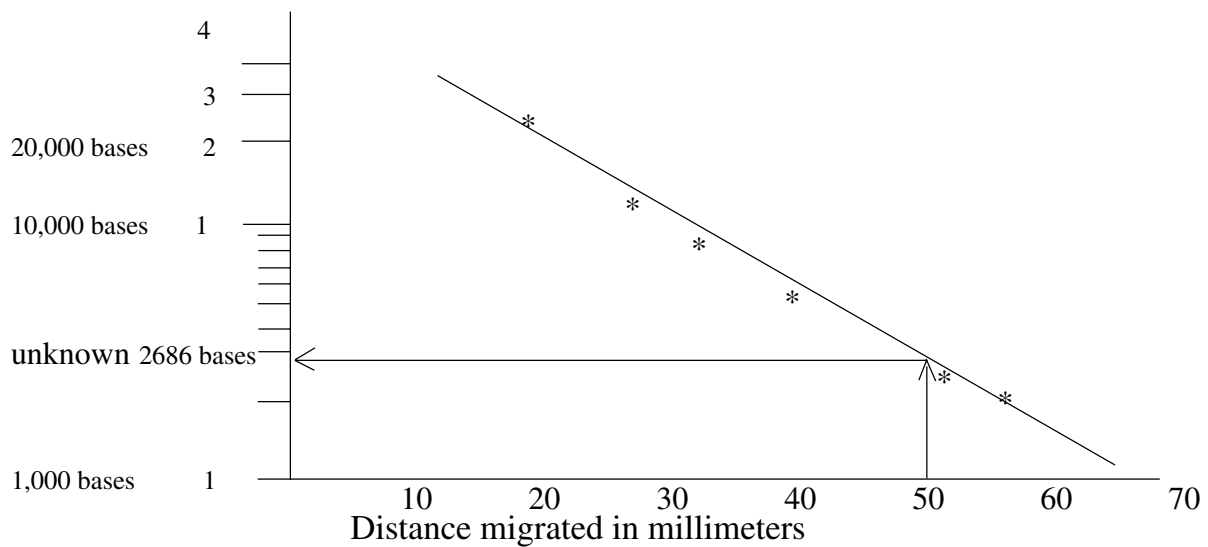
*Plot the size of the DNA fragments on the y axis on semilog graph paper and the distance in mm on the x axis. Then draw a line through the points. This represents your standard curve

*Measure the distance from the wells to each DNA band for each unknown sample. Then find the corresponding distance on your graph and read the size of the fragment in base pairs from the curve.

Example of Agarose Gel and Graph used to Calculate DNA Fragment Sizes - Figure 6



Lambda DNA Fragments graphed on semilog graph paper



Notes on restriction enzymes:

- * Restriction enzymes are very sensitive to heat so they are always stored at -20°C or kept on ice
- * Restriction enzymes are *very* expensive.
- * Never remove a restriction enzyme from the freezer without having ice available.
- * Always use a fresh pipette tip when pipetting restriction enzymes

Study Questions for Genetics Laboratory 2

1. Where does λ Lambda DNA come from and what is it used for in Biotechnology?

2. What are Type I and Type III enzymes and why are they seldom used in?

3. Where do restriction enzymes come from and what is their function in nature?

4. What are plasmids and what uses do they have in biotechnology?

Genetics Lab Exercise 3

Molecular Cloning

Introduction

Molecular cloning refers to the process of inserting a DNA fragment into a carrier DNA molecule called a vector, which is then placed into a recipient cell (usually *E. coli*) for amplification of the DNA vector via cell growth and division. Vectors include plasmids, bacteriophage DNA and combinations of the two called phagemids. Bacteriophage are viruses that infect bacteria which may have double stranded circular genomic DNA similar to plasmid DNA. There are many types of bacteriophage (phage) and combinations of phage and plasmid DNA used for the transfer of DNA from one organism to another.

The process of DNA cloning involves restriction digestion of the vector with one or more restriction enzymes. These must be unique restriction site/s in the vector so that the circular DNA becomes 1 linear fragment. Any greater number of linear vector fragments greatly reduces the chances that the DNA to be cloned will insert into the vector at the correct location. The DNA fragment to be inserted into the vector must have ends that are complementary to the digested ends of the DNA vector so that they may anneal together (**See Figure 8 pg. 34**). This can be accomplished by digesting the vector and insert DNA with the same enzyme, such as EcoRI, and allowing them to anneal together. The backbone of the two DNA fragments is joined together by the addition of DNA ligase and an appropriate buffer containing ATP to the DNA vector and insert mixture. DNA ligase catalyzes the formation of phosphodiester bonds between the two DNA fragments and covalently bonds or (ligates) the two DNA molecules together.

There are many ways to clone DNA but we will use an example of cloning with pUC19 a very convenient plasmid vector used for cloning. pUC 19 has several features which makes it an excellent cloning vector. It has a high copy number in *E. coli* ~ 50 plasmids per cell and it contains a gene which encodes a β -lactamase enzyme allowing the cells that take up the plasmid to grow in the presence of the penicillin derived drug, Ampicillin.

This plasmid also has a *lac Z* gene from *E. coli* which encodes β -galactosidase, an enzyme whose activity is easily measured by a color indicator in the agar medium. The production of the β -galactosidase enzyme by the cells, catalyzes the breakdown of the artificial substrate called X-gal and causes the *E. coli* colonies to turn blue.

This β -galactosidase enzyme also indicates if a DNA fragment has been cloned into a vector. The multiple cloning site (MCS) (see Figure 8) (a small piece of DNA containing overlapping restriction enzyme recognition sites) lies within the *lac Z* gene sequence. If a DNA fragment is successfully cloned into the MCS, the *lacZ* gene sequence will be interrupted by insertional activation. This means β -galactosidase enzyme will not be produced and the colonies will remain white. This is a very simple and reliable way to distinguish between colonies that contain a cloned fragment in pUC 19 and those colonies that have taken up the plasmid with no DNA fragment insert.

The use of the Lac Operon of *E. coli* in Molecular Cloning

A brief explanation of the bacterial operon regulation of lactose metabolism

Introduction

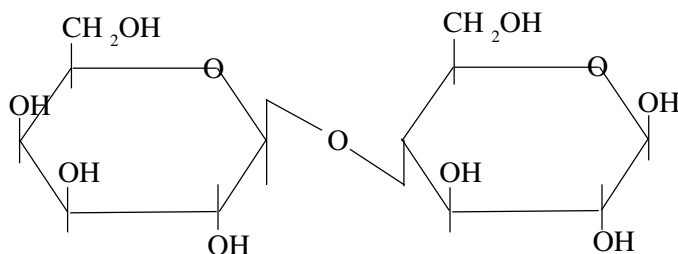
The Lac operon is one of the best understood mechanisms of genetic regulation in prokaryotic organisms. The operon system is a highly efficient system which allows bacteria to switch genes on and off rapidly in response to a change in environmental conditions. An operon is a cluster of genes all relating to the same specific function under the regulation of one promoter and operator. A **promoter** is a region of DNA, preceding a gene or genes, which is responsible for the binding of the RNA polymerase to the correct location on the DNA molecule to allow for the transcription of those genes into mRNA before they can be translated into functional proteins. An **operator** is a region of DNA that lies between the promoter DNA and the 5' end of a structural gene DNA sequence whose function is either to block the movement of the RNA polymerase to prevent transcription and therefore prevent translation or to allow RNA polymerase to proceed its transcription of the structural genes so that they may be translated into functional proteins. The operator control region is essential to the conservation of energy by bacteria by preventing transcription and translation of unnecessary genes and gene products. Because nucleic acid polymerization and protein synthesis require most of the energy manufactured by the cell, the tight regulation of transcription and translation is very important to the survival of bacteria.

There are two basic types of operons which regulate these processes; the inducible operon and the repressible operon. The inducible operon regulates the formation of gene products involved in the usage of different substrates as energy sources such as the formation of glucose from lactose by gene products of the Lac operon. The inducible operon in its natural state is off. This means that the cell produces a gene product from a repressor gene called a repressor protein. The lac repressor protein binds the operator DNA region of the Lac operon and blocks RNA polymerase from moving onto the structural genes keeping the Lac operon turned off. Repressible operons such as the Trp operon, which is a cluster of genes responsible for the biosynthesis of the amino acid tryptophan, in their natural state are on. Because bacteria must synthesize all of their amino acids unless they are readily available in the environment, essential "housekeeping functions" such as the biosynthetic pathway operons must be on unless the bacteria are exposed to the product of its operon. If an *E. coli* somehow comes into a ready supply of tryptophan it can save energy by shutting off the tryp operon and using the available tryptophan.

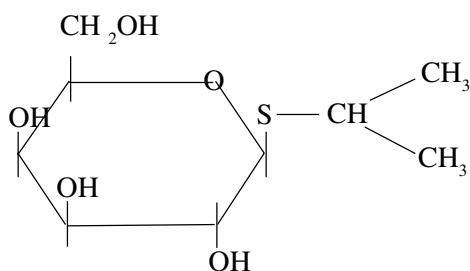
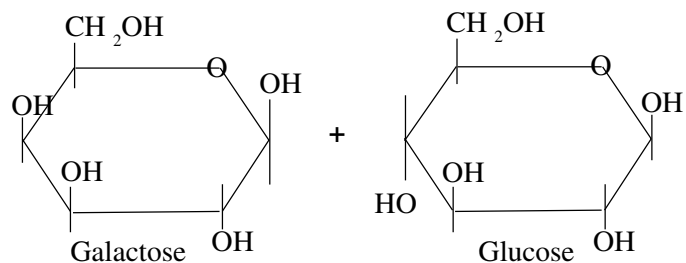
The inducible Lac operon is composed of three structural genes, the *lac Z* gene product encodes the enzyme β -galactosidase which catalyzes the hydrolysis of lactose into 1 glucose and 1 galactose. This disaccharide therefore contains the equivalent energy of 2 glucoses per 1 lactose molecule. The two other structural genes of this operon include *lac Y* which produces β -galactoside permease, a substance that facilitates the uptake of lactose and *lac A* which produces a transacetylase having some function in lactose utilization.

When lactose is made available to *E. coli* the lactose diffuses into the cell and binds to the lac repressor protein. This results in the loss of the repressor protein from the lac operator DNA, which effectively turns the lac operon transcription on. Therefore in the presence of its substrate, lactose can now be metabolized in the cell via the production of the gene products of the Lac operon.

As I referred to earlier, the production of the lacZ gene product, β -galactosidase, is used to determine if a DNA fragment has been inserted into a plasmid such as pUC 19. This works as follows: the MCS (multiple cloning site) the region in pUC 19 which contains unique restriction sites for cloning, has been placed between the promoter DNA and the lacZ gene. Normally the plasmid when stimulated by the presence of lactose substrate or a lactose imitator substrate such as IPTG (known as a gratuitous inducer) produces β -galactosidase. When the colony of *E. coli* containing pUC19 plasmid grows on an agar plate containing an artificial substrate such as **X-gal** the colony turns blue because the β -galactosidase enzyme catalyzes the cleavage of the substrate which results in a blue color. When a DNA fragment is cloned into the MCS, the promoter is physically separated from the lacZ gene, no transcription of lacZ occurs, no β -galactosidase is produced, no break down of substrate occurs and no blue color of the colony results. Thus the presence of a white colony containing a blue/white lacZ cloning vector, such as pUC 19, in the presence of IPTG and X-gal substrates in the agar plate, indicates the insertion of a cloned fragment of DNA into the cloning vector (plasmid DNA). This is very useful to the biotechnologist and saves much time and work when screening for DNA clones. Below are the structural formulas for lactose, galactose glucose and IPTG.



Lactose a β - galactoside



Isopropylthiogalactoside (IPTG)

Restriction enzyme digest of pUC 19

*Students will perform a digest of plasmid vector DNA (**as outlined by instructor**) to generate a vector in which to clone a DNA fragment

* Students will perform a digest of chromosomal DNA with the same enzyme/s (**as outlined by instructor**) to generate chromosomal DNA fragments to insert (**clone**) into the cut plasmid vector

Please always remember to:

****Label all tubes and containers before you start**

****Write out protocol before you start**

****Perform all calculations and check them with a colleague before you start**

****Have all materials located and available for use before you start**

*Add correct amount of DNA (**as outlined by instructor**)

* Add 10 X digestion buffer for appropriate enzymes to each labelled tube

* Add pure sterile water up to total volume.

* Balance the tubes in the Eppendorf centrifuge and spin for ~ 1 second. (Hint use the pulse button)

* Remove tubes from centrifuge and add 1 ul restriction enzyme to each appropriately labelled tube. (**Caution: use a fresh pipet tip for each tube and keep enzymes on ice at all times!**)

NOTE: Enzymes will be located on ice or in the freezer in room LF338

* Briefly spin (~ 1 second) **BALANCED** tubes in the Eppendorf centrifuge.

* Remove tubes and incubate in 37°C water bath and allow digestion to proceed for 30 to 60 minutes.

* Remove your tubes from the water bath.

NOTE: While the DNA digestion is proceeding please :

Make up your agarose gel , Write data in notebook, Do calculations, Plan ahead etc.

In other words use your time wisely.

Purification and Concentration of DNA digests

To inactivate the restriction enzyme and to stop the digestion activity students will extract each tube containing enzyme with a mixture of 50:1 chloroform and isoamyl alcohol. To reconcentrate the DNA students will then perform a Sodium Acetate / Ethanol DNA precipitation reaction as described below.

The rationale for these procedures is as follows. Proteins are extracted by the hydrophobic Chloroform solvent due to the amino acids of proteins solubilizing between the aqueous and hydrophobic phases when chloroform and an aqueous solution are mixed together. The hydrophobic amino acid residues solubilize in the chloroform phase while the hydrophilic

amino acid residues solubilize in the aqueous (water based) phase. This immobilizes the protein and the upper aqueous phase, containing only your purified DNA, may then be drawn off the top with a small pipette and placed into a clean tube. The precipitation reaction involves the drawing of the hydration spheres of water away from the DNA molecule causing the DNA to precipitate out of solution. The salt and ethanol in the precipitation reaction readily absorb the water away from the DNA. The DNA is then vacuum dried to remove all traces of Ethanol (which may inhibit the next enzymatic reaction) and the DNA is then resuspended in pure water and used for the DNA ligation reaction.

Chloroform Extraction of DNA

Note: please wear Gloves and Safety glasses and a Lab Coat for the extraction

* Bring the volume of the tubes containing the DNA restriction digests up to 100 ul by adding by adding ~ 85 ul of TE Buffer.

* Add 100 ul of Chloroform to each DNA restriction digest tube and vortex for 10 seconds mixing well.

* Place into an Eppendorf Centrifuge and centrifuge for 1 minute at high speed

* Carefully withdraw the top layer containing the aqueous phase using a pasteur pipette and place into a clean labelled tube. **Keep the upper phase** (aqueous phase). **It contains your plasmid DNA**

Ethanol concentration of DNA and resuspension of plasmid DNA

Ethanol concentration of DNA and resuspension of plasmid DNA

* Add 1/10 th volume 3M Sodium Acetate (10 ul) to your tube containing the 100 ul upper aqueous phase which has your plasmid DNA. - Vortex 1 second.

* To the same tube containing your salt and plasmid mixture (now 110 ul) add~ 2 volumes (200 ul) of 95% pure ethanol. Mix well by inversion and centrifuge for 5 to 10 minutes at high speed.

* Remove the tube from the centrifuge trying **not** to remix the contents and pour the ethanol off into a waste beaker.

* With out turning the tube back over (top side down) gently tap the mouth of the tube onto a kimwipe drawing excess ethanol off.

* Place the open tube into the vacuum centrifuge (make sure each tube has a balance).

* After all tubes are placed in the vacuum centrifuge the vacuum pump and centrifuge will be run for 10 minutes to evaporate the last traces of ethanol.

* Remove your tubes and add 5 ul of TE buffer or pure sterile ddH₂O to each tube to reconstitute the DNA.

Be sure to vortex and mix the tube very well to resuspend the DNA pellet.

* Place all tubes into the eppendorf centrifuge and hit the pulse button for 1 second to draw the liquid to the bottom of the tube.

Your DNA plasmid vector and insert DNA are now ready to be combined together in a DNA ligation reaction mixture.

Preparing DNA Ligation Reactions

DNA Ligation reactions involve the "sticking together" of ends of DNA by using a naturally occurring DNA Ligase enzyme used in many cellular processes. We purchase this enzyme as a purified protein from a biotechnology supply company. Normally we use a protein found in a T4 bacteriophage a common virus of *E. coli* bacteria. Please refer to the **LLRC** for additional information. To perform a ligation reaction you will mix two types of DNA both cut with the same restriction enzyme/s in a ligation buffer provided with the enzyme. You will then add the enzyme and incubate at the appropriate temperature from 10° C - 37° C. One type of DNA must be a plasmid vector which is designed to carry the DNA fragment and have the ability to replicate within a host cell (such as *E. coli*). The plasmid vectors we often use (such as pUC 19) have several remarkable features for easy manipulation. They are easily extracted out of bacteria because they are very small in comparison to the bacterial chromosome. Plasmid vectors often indicate when a foreign DNA fragment is present by insertional inactivation of an enzyme, which is easily detected by a color change of the bacterial colony, on agar plates containing the appropriate indicator. Vectors all have a selectable marker to indicate their presence in the bacterial cell (such as the ability to grow on medium containing antibiotic). Most vectors also replicate autonomously in high copy number, amplifying the number of copies of the cloned DNA fragment along with plasmid replication.

* You will perform the ligation reaction as outlined in class by your instructor

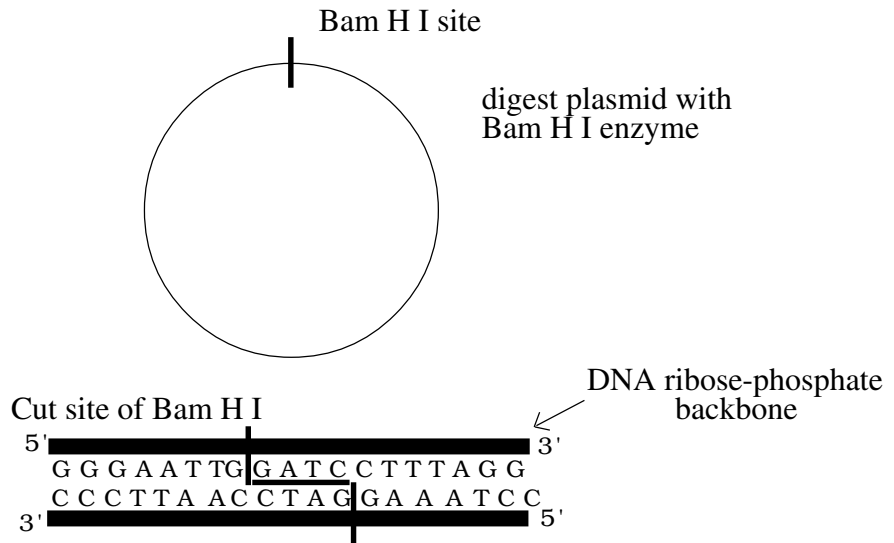
The protocol is as follows

- * Add digested plasmid to the appropriately labelled tube and add DNA to be cloned into the plasmid in the same tube
- * To the tube now add 1/10th volume of 10 X Ligation Buffer
- * Bring the total volume of the ligation reaction mixture to the desired amount by adding sterile nanopure water
- * Mix by vortexing 1 second, place tube into the eppendorf centrifuge and hit the pulse button for 1 second to draw the liquid to the bottom of the tube.
- * Add **only 1 ul** of DNA Ligase enzyme by submerging the pipette tip into the ligation mixture and expelling the enzyme into the mixture. Mix gently by rotating the pipette tip in the ligation solution.
- * Incubate tubes in specially prepared 16 ° water bath overnight. Tubes may then be placed into the refrigerator for the next lab

Note: Always store your remaining DNA digests in the -20 degree freezer for later use

Study Questions for Laboratory 4

1. Define what cloning means in the context of molecular biology and its uses.
2. What is a vector?
3. Why is pUC 19 a useful cloning vector? Describe all its features.
4. Explain how the Salt/ethanol precipitation of DNA works.



Bam H I digest of vector DNA generates sticky or overhanging or complementary base pair ends



Sau 3A I digest of insert DNA generates sticky or overhanging complementary base pair ends



Complementary base pairs hydrogen bond together



DNA ligase seals breaks in DNA backbone by reforming covalent bonds

Genetics Lab Exercise 4

Preparation and Transformation of Competent *E. coli* Cells

Introduction

In this laboratory students will prepare and transform competent *E. coli* cells for plasmid transformation. Competent bacteria are able to take up DNA molecules and then replicate this DNA during bacterial growth. Bacterial cultures will be grown to the appropriate density and be ready for centrifuging at the beginning of laboratory. Treatment of growing *E. coli* cells with ice cold CaCl_2 permeabilizes the outer membrane and cell wall so that DNA molecules are readily absorbed into the cell. Cells should be handled very gently during and after CaCl_2 treatment because the treatment makes their cell wall and membrane fragile.

Any warming of the culture once the CaCl_2 treatment has begun will greatly decrease the transformation efficiency. Transformation efficiency is defined as the number of the total cells that will take up DNA. An essential step in the transformation of *E. coli* via the CaCl_2 method is the heat shock incubation. After the plasmid DNA is mixed with the CaCl_2 treated cells and incubated on ice for 30 - 60 minutes the cells are transferred from the ice to a 42°C water bath for 60 seconds. This step is known as a heat shock and greatly facilitates the cellular uptake of DNA.

After heat shock, cells are transferred to a culture tube containing LB broth with no antibiotic and incubated at 37°C with aeration in a shaking incubator for 30 - 60 minutes. This step is essential to allow the *E. coli* cells to recover from the CaCl_2 treatment and to allow them time to produce enzyme β -lactamase from their newly acquired plasmid DNA. The β -lactamase will allow cells that have taken up and expressed the plasmid DNA to grow in the presence of Ampicillin while the untransformed cells will not grow in the presence of Ampicillin.

The cultures are removed from the shaking incubator and spread or plated onto LB agar containing Ampicillin at a concentration of 50 to 100 $\mu\text{g}/\text{ml}$. One very important step in this type of experiment is the inclusion of a negative control. Often cultures can become contaminated with yeast or bacteria which may grow in the presence of Ampicillin naturally. The negative control, which involves treating one vial of cells just as the plasmid transformed cells but without addition of plasmid DNA, ensures that the presence of colonies on the experimental plates did not arise from contamination.

Preparation of Competent *E. coli* (CaCl_2 competence protocol)

* *E. coli* cells will be grown, by the instructor, in liquid (Luria Broth) L.B. medium at 37°C with aeration in a shaking incubator to mid exponential phase (Approximately 50-60 Klett units or 0.6 Spec Units at OD^{600})

Note: Once harvested by centrifugation cells must be kept ice cold at all times

-
- * Withdraw 10 mls of bacterial culture and place into a sterile Oakridge tube
 - * Place cells on crushed ice immediately after removal from incubator (Allow cells 5 minutes to cool down)
 - * Harvest cells by centrifugation at **7000** rpms for 10 minutes in Beckman rotor **JA20** or equivalent held at 4°C.

Note: cells are fragile from this point .

You will see a round small circular pellet on one side toward the bottom of your Oakridge tube. This pellet will stick to the tube when you pour off the LB liquid culture. The LB should be transparent after the cells have been pelleted.

- * Pour supernatant off of the pellet (LB broth) into waste beaker for decontamination at a later time.
- * Keep bacterial pellet on ice
- * Add 10 mls (or an equal volume) of ice cold 0.05 M (50mM) CaCl₂ to 10 mls cells.
- * Resuspend cells in the CaCl₂ buffer by gently sucking the fluid up and down in with a 5 ml pipette until clumps of cells are no longer visible
- * Allow cells to incubate on ice for 20 minutes.
- * Harvest cells by centrifugation at **7000** rpms for 10 minutes in Beckman rotor **JA20** or equivalent held at 4°C.
- * Decant supernatant into waste beaker (pour off CaCl liquid) and retain cell pellet.
- * Resuspend cell pellet gently in 1 ml of CaCl₂, then follow one of two procedures below depending upon your research plans.

Procedure 1. - Freezing and storage at -70°C of competent *E.coli* cells for later use

Before freezing add sterile pure glycerol to cells resuspended in CaCl as above to a final concentration of 25% to 30% glycerol. Swirl gently while cells remain chilled to completely mix cells with glycerol. Place 100ul aliquots of cell mixture into pre-chilled and pre-labelled eppendorf tubes. Freeze tube in super cold dry ice ethanol bath and place in labelled freezer box into the -70° freezer.

Procedure 2. - Use of competent *E. coli* cells immediately after preparation.

Cells which have been resuspended in cold CaCl are aliquoted into pre-chilled and pre-labelled eppendorf tubes and kept on ice for up to 18 hours. These cells may be transformed at any time but increase in competency when kept on ice upto 18 hours after resuspension.

Transformation of Competent Bacteria

The term **transformation** refers to the uptake and expression of a DNA molecule by a bacterial cell. In this exercise we will transform plasmid DNA into the bacteria *E. coli*.

Plasmid Transformation of *E. coli* as demonstrated or outlined by instructor

Note: Keep cells cold at all times

Basic protocol for *E. coli* transformation

**Label all cell tubes with appropriate information, plasmid type, ligation type, date or as a negative control*

* Add from 1 to 5 ul of your DNA ligation preparation to one tube of competent *E. coli* cells.

* Add nothing to one tube of competent cells but treat it exactly as the experimental tube

Note: Treat all negative and positive control tubes the same in all respects except for the addition of DNA.

* Mix cells very gently by tapping with finger and incubate on ice for 30 minutes

* Remove cells from ice and heat shock for 1 minute at 42°C in water bath

* Place all tubes of cells on ice 1 minute and dilute cells 1/10 by adding all 100 ul (0.1ml) of cells to 0.9 mls of appropriately labelled tubes of LB broth

* Place the tubes containing the cells in the LB medium in the 37°C shaking incubator at ~ 200 rpm for 30 to 60 minutes

* Remove tubes of cells from incubator and then plate 0.2mls (200 uls), cells directly onto pre-labelled LB Amp agar plates (L.B. medium containing 50 ug/ml Ampicillin and agar as a solidifying agent) from each tube of cells.

* Allow the surface of the plates to dry, by absorption of the fluid into the agar, and place inverted in 37° C stationary incubator overnight.

*Plates should be checked for colony growth and color or fluorescence the following day and refrigerated until the next laboratory.

Study Questions for Laboratory 4:

1. Why do we plate our *E. coli* cells onto agar medium containing Ampicillin after transformation.
2. Describe why cells that take up pUC 19 grow in the presence of Ampicillin.
3. What is a DNA transformation?

Genetics Lab Exercise 5

Plasmid DNA Extraction

Introduction

The basic procedure for the DNA extraction from a cellular lysate relies upon the same principles regardless of the type of DNA being extracted (plasmid or chromosome) or the type of organism used. A lysate is composed of all internal contents of a cell including the cytoplasm all of the solubilized proteins and nucleic acids as well as all of the insoluble cell contents including cell wall, cell membrane ribosomes and other cellular organelles. There is one major difference between the extraction of DNA from the Bacteria and Fungi and Plant cells and the extraction of DNA from Animal cells. The Bacteria, Fungi and Plants all have cell walls that first must be cracked open before cell lysis can occur. Animal cells however are surrounded by a plasma membrane which is much easier to disrupt without disturbing the cell cytoplasmic contents. Also prokaryotic cells are different from eukaryotic cells due to the absence of the nucleus and other membrane bound organelles. In this way it makes it somewhat easier to extract DNA away from other cytoplasmic contents in prokaryotes.

The first step in the isolation of plasmid DNA from bacteria involves the cracking open of the cell wall. Fortunately this is relatively simple because almost all eubacteria have a cell wall made of peptidoglycan. Peptidoglycan is a polymeric carbohydrate made up of two different sugar residues (N-acetyl-glucosamine and N-acetyl-Muramic acid) which are repeated over and over to form long chains. Peptidoglycan is degraded by an enzyme called lysozyme which catalyzes the splitting of the covalent bond between the N-acetyl-glucosamine and N-acetyl-muramic acid sugar residues causing the cell wall to become perforated. Once this is accomplished the addition of a strong ionic detergent such as SDS, which is dissolved in a very basic solution of NaOH, causes the final dissolution of the cell wall and also solubilizes the cell membranes. The cell lysate at this point becomes clarified and is therefore referred to as a cleared lysate. The cleared lysate at this point has a very basic pH, contains a strong detergent and has all of its large macromolecular substances in solution.

The next step involves the extraction of the DNA from the rest of these cytoplasmic and cellular contents. In the extraction of plasmid we capitalize upon the size difference between the plasmid DNA and the chromosomal DNA to selectively precipitate the large macromolecular nucleic acids (chromosome) and other macromolecular cellular components (cell wall, cell membrane, proteins and ribosomes) away from the plasmid DNA and small RNA molecules. In order to facilitate this process we add a solution containing a 3M potassium acetate salt solution suspended in acetic acid. The salt absorbs the water molecules disrupting the spheres of hydration which are responsible for the solubilization of the macromolecular cellular components and the acid in this solution reacts with the basic NaOH and neutralizes this solution. A white precipitate forms upon

addition of the acidic salt solution which can be separated from the liquid phase containing the plasmid by centrifugation allowing the transfer of the clarified solution to another container.

This clarified solution now contains the plasmid DNA, small solubilized proteins and some small cellular RNAs (tRNA and rRNA). These smaller molecules can be recovered by the addition of 95% ethanol to a final concentration of 70%. At a 70% concentration of ethanol and a 3M salt concentration plasmid DNA readily precipitates out of solution. Remember the precipitation reaction involves the drawing of the hydration spheres of water away from the DNA molecule causing the plasmid DNA to precipitate out of solution. The salt and ethanol in the precipitation reaction readily absorb the water away from the DNA.

At this point you have in your precipitate, not only plasmid DNA but also some protein and RNA molecules. If very pure DNA is needed there are further steps that may be performed such as RNase treatment to digest the RNA for removal by further precipitation and phenol/chloroform extraction to eliminate protein contaminants. In addition many newer protocols include the use of chromatographic separation of the DNA for further purification. This procedure involves the absorption of the negatively charged DNA onto a positively charged matrix made up of tiny beads. Once the DNA is attached, the matrix is washed to remove further impurities and then the DNA is eluted off of the matrix by disruption of the positive and negative charge interaction using a salt solution of high ionic strength.

NOTE : In addition to this protocol you will also extract the same plasmid using a kit called " Perfect Prep" purchased from 5 to 3' a biotech supply company.

Minipreparation or Rapid Plasmid Isolation Procedure

- * Cultures from transformed colonies will be grown overnight in 2 mls of selective medium (LB + 50 ug/ml Ampicillin) in sterile 18 mm test tubes at 37° C and 250 rpms in the shaking incubator.
- * Pipette 1.5 mls of bacterial culture into eppendorf tubes and centrifuge cells in the Eppendorf centrifuge at maximum speed for 60 seconds to pellet cells.
- * Resuspend pellet thoroughly in 100 ul Lysozyme buffer, (**Solution I**).

Note: Incomplete suspension of the cell culture will greatly reduce your plasmid yield.

- * Add a tiny bit of lysozyme on the end of a toothpick to the suspended cells and swirl toothpick in the tube to mix.
- * Incubate at RT° for 20 minutes.
- * Add 200 ul of lytic mix (**Solution II**) and MIX GENTLY by inversion.

Note: use freshly prepared lytic mix and treat the mixture gently from this point on. Rough handling will shear the chromosomal DNA into small pieces which will then contaminate your plasmid by co-precipitating with the plasmid.

- * Place on ice 5 minutes.

-
- * Add 150 ul of Potassium Acetate Buffer (**Solution III**) MIX GENTLY by inversion.
 - * Place in ice water for 10 minutes to precipitate large cellular constituents such as cell wall, membrane proteins and chromosomal DNA.
 - * Spin in the Eppendorf centrifuge 5 minutes
 - * Decant supernatant to clean eppendorf tube by pouring or by transferring with a pasteur pipette
 - * Fill tube with RT° 95% EtOH-mix by inversion, and centrifuge 5 minutes
 - * Pour off ethanol, keep pellet, and dry pellet under vacuum in the Vacuum centrifuge evaporator for 10 minutes.

Note: It is important to remove all ethanol because it inhibits the activity of enzymes.

- * Add 30 ul of sterile nanopure water to the DNA pellet and mix well by vortexing for 1 minute to reconstitute the plasmid DNA
- * Place the plasmid in a **well-labelled tube** at -20° C for long term storage.

Buffers for Alkaline Lysis DNA Extraction

Solution I - 100 mls of Lysozyme buffer

- 50mM glucose (1.8 mls of 50% glucose)
- 25mM Tris HCl (2.5 mls of 1 M Tris, pH 7.6)
- 10 mM EDTA (2 mls of 0.5 M EDTA, pH 8)
- 94 mls of ddH₂O

Solution II - 10 mls of Lytic mix (make fresh each time)

- 0.2 N NaOH final concentration (from 10 M Stock) 100 uls 10 M NaOH
- 1% SDS (1ml 10% SDS)
- 8.9 mls ddH₂O

Solution III - Potassium acetate buffer

- 5 M K Acetate 60 ml
- Glacial acetic acid 11.5 ml
- H₂O 28.5 ml

Note: Solution III will always be used cold, store at 4° C or on ice

Genetics Lab Exercise 6

DNA Quantitation by UV Absorption and Gel Electrophoresis Spectrophotometric Determination of DNA or RNA

Introduction

One of the most commonly used methods to determine the quantity of nucleic acids in a suspension depends upon the chemical characteristic of nucleic acids which allows them to absorb ultraviolet (UV) light strongly in the wavelengths between 254-260 nm. Nucleic acids absorb in this range because of the nitrogenous base, pyrimidine and purine ring structures. Because nucleic acid absorbs UV light proportional to its concentration, it is easily quantitated using this method.

It has been previously calculated through the use of standard concentrations of nucleic acids that 50 ug of DNA will give an absorption value of 1.0 at 260 nm and that 40 ug of RNA will give an absorption value of 1.0 at 260 nm. One failing of this quantitative method is the possible contamination of the DNA preparation with RNA and/or organic solvents containing ring structures such as phenol and chloroform. For this reason critical DNA quantitations should be performed upon highly purified DNA preparations only. However using UV absorption spectroscopy it is possible to determine if a DNA preparation has protein contamination. When a DNA quantitation is performed by measuring the absorption of a solution at 260 nm one also measures the absorption of the solution at 280 nm. The rationale for this procedure stems from the ability of protein to absorb UV light in the 260 to 280 nm range. Proteins absorb in this range due to the presence of the ring structures on the amino acid residues phenylalanine, tryptophan and tyrosine. However pure nucleic acid absorbs two times more strongly at 260 nm than at 280 nm Therefore if you measure your nucleic acid at both 260 and 280 nm pure DNA should have a 260/280 ratio of 2:1. If the ratio is smaller than this your DNA has some contaminating substance and should be quantitated by some other method for accuracy.

1. Dilute plasmid extract from previous laboratory by adding 10 ul of extracted DNA to 1 ml water in a glass test tube and mix well
2. Place 1 ml of your diluted plasmid in one quartz cuvette and 1 ml of water in the other.
3. The instructor will then demonstrate the use of the UV spectrophotometer to the class.
4. Students will do a spectrophotometric reading at both the ultraviolet wavelengths of 260 nm and 280 nm.

In order for your Nucleic Acid determination to be accurate, you must measure the ratio of the Optical Density (OD) at 260 nm versus 280 nm.

Some proteins also absorb in these wavelengths therefore when the ratio at OD_{260} / OD_{280} is 2 or the absorbance at 260 nm is twice that at 280 nm the protein has been extracted and the

preparation contains relatively pure nucleic acid.

If RNA is present in your preparation this will add to your OD 260 and subsequent quantitation will be inaccurate since this method does not discriminate between RNA and DNA.

DNA Quantitation

The number that you read from the spectrophotometer is the Optical Density or OD. Use the following equation to quantitate the amount of DNA or RNA in this mixture.

$$\frac{\text{OD}^{260} \text{ 1.0}}{50 \text{ ug DNA}} = \frac{\text{OD}^{260} \text{ reading}}{\text{X ug DNA}}$$

Example: your solution has an $\text{OD}^{260} = 0.2$

$$\frac{\text{OD}^{260} \text{ 1.0}}{50 \text{ ug}} = \frac{0.2}{\text{X ug}}$$

$$\text{X} = 50 \text{ ug} \times 0.2 \text{ divided by } 1 = 10 \text{ ug of DNA}$$

Note: Please read the following on the use of quartz cuvettes

- * Each quartz cuvette costs approximately \$100.00
- * Quartz cuvettes are matched. If one is broken the others are useless.
- * Quartz cuvettes scratch and chip very easily
- * Rinse repeatedly with pure deionized water to clean
- * Replace cuvettes in case after use and do not dry with regular toweling (use Kimwipes)

The best way to determine how much of your preparation is DNA is to run it on an agarose gel in the presence of EtBr and *LOOK* at the band on the UV transilluminator.

DNA Restriction

In this exercise you will perform a digest on 5 ul of each plasmid DNA extract with the restriction enzymes Eco R I and Hin D III to verify the cloning of the appropriate DNA fragment. Digestion with both enzymes should cut out any fragments inserted into the Multiple Cloning Site (MCS) of pUC 19.

All of these DNA digestions will be run on a horizontal agarose gel apparatus and the size of the DNA fragments will be calculated using the procedure as on pages 25 and 26.

Each team will perform the following procedure **(as outlined by the instructor)**

Restriction Enzyme Digestion

- * Label eppendorf tubes
- * Add ~ 1ug of cloned plasmid extract from previous extract
- Please remember 1 type of DNA per tube only.*
- * Add 1/10th volume of appropriate 10X restriction digestion buffer .
- * Add pure sterile water to each tube to bring the total volume up to 15 or 20 ul.
- * Balance the tubes in the Eppendorf centrifuge and spin for ~ 1 second. (Hint use the pulse button)
- * Remove tubes from centrifuge and add 1 ul of the appropriate enzyme or enzyme mixture to each tube. (**Caution: use a fresh pipet tip for each tube and keep enzymes on ice at all times!**)
- * Balance the tubes in the Eppendorf centrifuge and spin for ~ 1 second using the pulse button.
- * Remove tubes and incubate in 37°C water bath and allow digestion to proceed for 30 to 60 minutes .

NOTE: While the DNA digestion is proceeding please make up your agarose gel as previously described.

- * Remove your tubes from the water bath, add ~ 3 ul of blue juice to each tube and spin for ~ 1 second using the pulse button.
- * Most of each sample should be loaded into the wells of the agarose gels as previously done.

Electrophoresis of the DNA Gel

- * The gels should then be placed in the horizontal gel electrophoresis apparatus which should be filled with 0.5X TBE to just cover the gels. Place the cover over the gel box, plug the red and black leads into the power source (red to red and black to black).
- * Gels should be run as described previously.

(Note: Be sure your apparatus always has red electrode at the bottom of the gel)

- * Wearing gloves and Safety glasses remove the gel, place on transilluminator and photograph as demonstrated by instructor. Use it to calculate the sizes of the DNA fragments.

Genetics Lab Exercise 7 - Southern Blot Hybridization and Analysis

Introduction

In 1975 E. Southern published a technique which he developed that virtually revolutionized molecular biology. The technique allows scientists to determine if specific sequences may be found in any genomic DNA and more recently has contributed to the development of DNA fingerprinting. The technique works like this: Restriction digested genomic or plasmid DNA is run on a horizontal agarose gel which results in rather indistinct banding patterns. The DNA in this gel is denatured to single strand the DNA and is then transferred to a highly purified paper matrix by capillary attraction, vacuum or electrophoretic transfer. The DNA is permanently attached to the paper or blot by baking at 80 C or by UV irradiation that crosslinks the DNA to the blot. The paper is then bathed with a solution containing a labelled fragment of DNA or RNA that is complementary to the DNA sequence of interest. The fragments of nucleic acid (called probes) originally were labeled with a phosphorus 32 isotope (P^{32}) incorporated into a nucleotide such as dATP or UTP. These radioactive blots may then be exposed to X-ray film. Complementary DNA (probes) will stick to the matching DNA sequences on the blot and appear as distinct dark bands on the X-ray film. More recently non-isotopic method for labeling and detecting complementary DNA or RNA sequences have been developed. In the next couple of laboratories we will be using some of these newer methodologies to confirm the presence of specific cloned DNA into a plasmid vector. **(Instructor will outline all protocols in class)**

General protocol

- * Digest DNA of interest with appropriate enzymes.
- * Add blue juice DNA dye
- * Load onto a 1.0% agarose gel:
- * Run on agarose gel
- * Photograph and label picture with DNA destinations and enzymes used for digestion.

Denaturing the gel

- * Soak gel in **0.25 M HCl** gently rotating until dark blue dye turns yellow ~ 5 minutes. **Do not overdo this step** or DNA will be overly depurinated and unable to bind probe. Place gel immediately into alkaline buffer after this soak is done.
- * Place gel into bath of **0.5 M NaOH, 1M NaCl** and gently rotate for 20 minutes at room temperature.
- * Neutralize gel in a bath of **0.5M Tris-HCl pH 7.4, 3M NaCl** for 30 minutes at room temperature gently rotating. **Capillary transfer of DNA to Tropilon-plus positively charged nylon membrane.**

Note: when handling filter paper, gel or blot **always** wear gloves **your hands will contaminate** and ruin the paper.

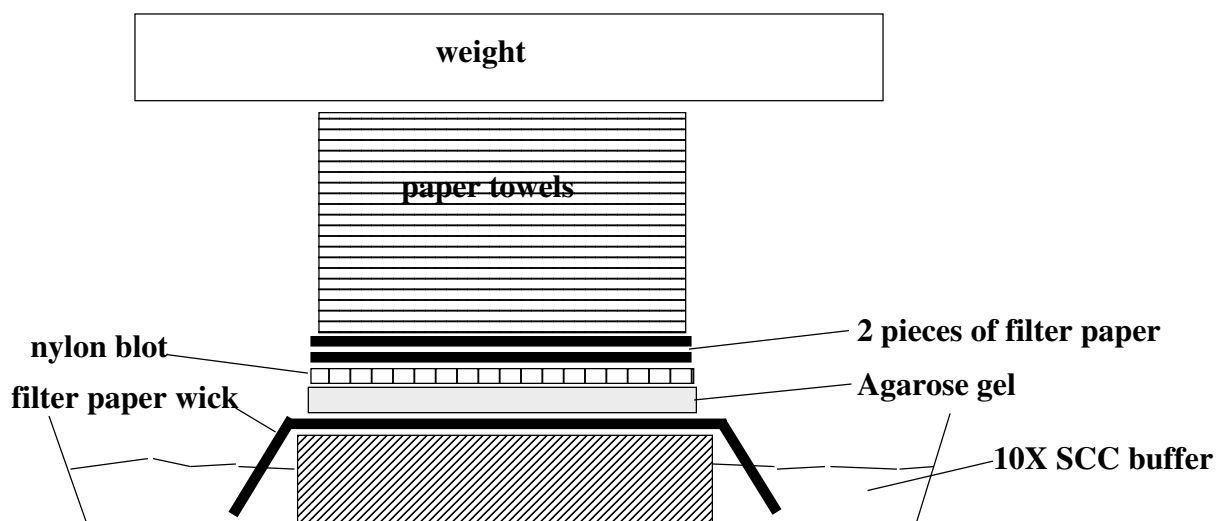
- * Cut a wick of whatman filter paper slightly wider than the gel and long enough to overlap the stage of the horizontal gel apparatus and dip into the transfer buffer.
- * Cut paper towels, a very large amount (at least a 4 inch stack) and two pieces of whatman filter paper to the exact size of the gel.

* Cut one piece of positively charged nylon paper to the exact size of the gel. Do this on clean filter paper allow **nothing to come into contact with this paper** it costs a small fortune and can be rendered useless very easily.

Capillary transfer blot apparatus

- * Fill the gel chamber or large baking dish with transfer buffer 10X SCC.
- * Fold your whatman filter paper wick around the edges of your stage, place into dish and allow ends of filter paper wick to dip into transfer buffer 10X SCC.
- * Turn gel over and place on saturated filter paper, that is bottom of gel up. Make sure there are no air bubbles between gel and wetted filter paper.
- * Wet the positively charged nylon blot paper in deionized water completely and place on top of gel. Be sure no air bubbles are trapped between membrane and nylon paper. Also handle nylon by edges only with scrupulously clean flat forceps if possible.
- * Place two pieces of wetted whatman filter paper on top of nylon blot making sure no air bubbles are trapped
- * Place a stack of dry paper towels on top of the filter paper and place a board with weight on top of the paper towels
- * Allow to transfer overnight, carefully remove membrane, dry on saran wrap for 10 minutes at 37 C, label L and R sides at top of membrane DNA side up with a #2 pencil and expose blot for two minutes on the UV transilluminator DNA side down. Store in clean whatman Filter paper for later hybridization.

Diagram of capillary transfer apparatus - Figure 10



Hybridization Analysis of DNA

In this laboratory exercise we will probe for plasmid DNA using a photobiotin labelled DNA probe. The labeling of the probe will be accomplished by your instructor but a copy of the procedure will be included in this laboratory. The procedure is taken directly from the BRL-Gibco procedure furnished with every purchase of photobiotin. In addition the Biotin detection system is the Southern-Light™ from Tropix Corporation Bedford MA. The procedure for the hybridization protocol has been taken from the instruction manual from Tropix with a few modifications.

Southern Hybridization Protocol

- *Rinse membrane in 2X SSC pH 7.5 for 10 minutes with agitation
- *Prehybridize in Hybridization Buffer for 1 hour at 45° C
- *Dilute 5µL biotinylated probe (heated to 80-85° C) in 25 mL fresh Hybridization Buffer (preheated to 55° C) and add to membrane. Incubate overnight at 55° C
- *After incubation decant hybridization solution and treat as follows:

Detection of Probe by Southern-Light™ Detection Kit

- *Wash 2 X 5 min at room temp. in 2X SSC, 1.0% SDS (1 ml/cm²)

- *Wash 2 X 15 min at 55° C in 0.1X SSC, 1.0% SDS

- *Wash 2 X 5 min at room temp in 1X SSC

- *Wash 2 X 5 min in Blocking Buffer (0.5 ml/cm²)

- * Incubate for 10 min in Blocking Buffer (1.0 ml/cm²)

- *Dilute AVIDx-AP conjugate 1:5000 in Blocking Buffer. Use 2.0 ul of conjugate in 10 ml Blocking Buffer per 100 cm²(0.1ml/cm²)

- *Incubate blot for 20 min at room temp. at 200 rpm

- *Wash 1X5 min in Blocking Buffer (0.5 ml/cm²)

- *Wash 3X5 min in Wash Buffer (1 ml/cm²)

- *Wash 2X2 min in Assay Buffer (0.5 ml/cm²)

- *Dilute 50 ul Chemiluminescent Substrate Solution in 5 ml Assay Buffer and add to blot
- *Agitate by hand for 5 min., drain excess substrate solution from blot.

* Place in plastic development folder, tape shut and place in autoradiograph cassette.

Exposure and developing the autoradiograph on X-ray film

Do in the DARK!!!!

*Place one sheet of film on top of the blot in total darkness and secure autoradiograph cassette. Put film away and lights may be turned on.

Do in the DARK!!!! or under safelight conditions

*Expose film for 5-30 minutes remove and develop in GBX Developer diluted 1 to 5 in water for 3 minutes then transfer to GBX Fixative diluted 1 to 5 and fix for 2-5 minutes.

*Turn on light look at blot to determine different exposure time; if perfect **celebrate!!!!**

Solutions

Hybridization Buffer (50 mL)

1mM EDTA	0.25 ml 0.2M EDTA
7% SDS	17.5 ml of 20% SDS
0.25M Disodium Phosphate, pH 7.2	25ml 0.5M Disodium Phosphate, pH 7.2

Stringency Wash Buffers (200 ml)

2X SSC, 1.0% SDS	16 ml 25X SSC, 10 ml 20% SDS
0.1X SSC, 1.0% SDS	0.8 ml 25X SSC, 10ml 20% SDS
1X SSC	8 ml 25X SSC

Blocking Buffer (300 ml)

0.2% I-Block Reagent	0.6g I-Block Reagent
1X PBS	30 ml 10X PBS
0.5% SDS	7.5 ml of 20% SDS

Add PBS to 200 ml of dH₂O. Add I-Block reagent and heat to 70 C on hot plate for 5 min. Do not boil. Add SDS and dH₂O to 300 ml.

Wash Buffer (500 ml)

1X PBS	50 ml 10X PBS
0.5% SDS	12.5 ml 20% SDS

Assay Buffer (250 ml)

0.1 M Diethanolamine	2.4 ml or 2.6 g DEA
1mM MgCl ₂	0.05 g MgCl ₂

Dissolve DEA in 200 ml of dH₂O and adjust pH to 10.0 with HCl. Add MgCl₂. Bring up volume.

Individual Student Research Projects: Part Two of Genetics K323 Laboratory

In this part of your genetics laboratory experience, you are given the opportunity to carry out your own research project. However due to the time constraints of laboratory and the numbers of students in class this research is limited to using either *E. coli* bacteria and/ or an extremely halophilic (salt-loving) archaeobacteria *Halobacterium salinarum*. You may use any techniques learned in class or available in my laboratory.

The object of this research project is to allow students to gain real world, hands-on experience with individualized scientific research. All students are required to:

- * Write a brief research proposal based upon literature research
- * Design all aspects of an experimental scientific project
- * Perform the experimental protocols necessary to advance their research
- * Develop better technical skills using the equipment and procedures in the area of biotechnology
- * Design, prepare and present a poster presentation of their research to the Genetics class

Following are some suggestions for some Genetics projects students have done in the past. Please feel free to design your own project, but discuss it with me so we can see if it is feasible.

- * Determine the lowest concentration of salt which will support the growth of our halophile. Then experiment to see if you can gradually condition the halophile to an even lower concentration of salt. Continue from there. Check DNA fingerprint?
- * See if you can clone DNA from the Halophile into an *E. coli* vector designed to look for promoter DNA. Then clone into *E. coli* bacteria and look for gene expression.
- * Determine if different types and concentrations of salts will support the growth of our halophile. Then experiment to see if you can gradually condition the halophile to different salts. Continue from there. Check DNA fingerprint?
- * Try to PCR DNA from the Halophile chromosome using *E. coli* primers. Generate DNA fingerprints of different Halophiles or Halophiles which have been mutagenized.
- * Determine how much deliberate mutagenesis Halophiles can take or screen for characteristics to change, including salt requirements. Do ribotype to confirm absence of contaminants.

Almost every experiment will require a chromosome extraction from the Halophile. On the next page you will find this information and MUCH MORE about the research project.

Extraction, Characterization, Cloning and Expression of Chromosomal DNA from Halophilic Bacteria *Halobacterium salinarium* - A class experiment

As a previous laboratory states the basic procedure for the DNA extraction from a cellular lysate relies upon the same principles regardless of the type of DNA being extracted (plasmid or chromosome) or the type of organism used. A lysate is composed of all internal contents of a cell including the cytoplasm all of the solubilized proteins and nucleic acids, as well as all of the insoluble cell contents including cell wall, cell membrane ribosomes and other cellular organelles. There is one major difference between the extraction of DNA from the Bacteria and Fungi and Plant cells and the extraction of DNA from Animal cells. The Bacteria, Fungi and Plants all have cell walls that first must be cracked open before cell lysis can occur.

Bacterial cell wall, as stated above, is incredibly strong when subjected to shearing forces. Because of this many devices have been invented to crack open bacterial cell walls for instance mechanically by the French Press and by implosion using a Parr Nitrogen Bomb apparatus. However overwhelming osmotic differences between the inside and the outside of cell walls can in some cases exert forces strong enough to explode cell walls. While the cytoplasmic contents of the eubacteria (modern bacteria) are not very saline on the inside a group of archaebacteria (ancient bacteria) have evolved which have learned to tolerate extreme environments such as the halophilic (salt-loving) archaebacteria. The name Halophile aptly describes this group of bacteria. An aqueous solution which is not saturated with salt can not support the growth or survival of these extreme halophilic archaebacteria. Even though high salt concentrations have long been used as a preservative to inhibit the growth of eubacteria, halophilic archaebacteria require high levels of salt. When these halophiles are placed into low salt solutions, osmosis provides the force to rupture cell walls. Water rushes across the cell wall and membrane into the cytoplasmic contents of the cell, the hydrostatic pressure builds and the cell literally explodes. This makes it very simple to open the cell walls of these bacteria.

However I have many questions about DNA from halophilic organisms such as: How does the DNA inside the cell tolerate high salt or low salt conditions? Is it sequestered in the cytoplasm? Could DNA from Halophilic archaebacterial organisms be expressed in a Eubacteria? Does the lack of high saline in the DNA extraction alter the DNA so it can not function under low saline conditions? I just don't KNOW and its driving me crazy!

I know, why don't you guys FIND OUT!

It should be easy to adapt a chromosomal DNA extraction for these bacteria, so you will be the researcher. Your job is to extract the DNA using one of the chromosomal DNA extractions provided or do some literature research (Search Halophiles on the WWW) on what is known about the manipulation of halophilic bacterial DNA. Then design experiments to answer some of these interesting questions using the techniques learned in the first part of this class using the DNA of a specific Halophile, *Halobacterium salinarium*. After determining if the DNA is easily manipulated, you may choose to clone random

fragments of the *H. salinarium* chromosome into a cloning vector, which is very similar to pUC19. If a piece of DNA containing a promoter or promoter like sequence works in this vector pEGFP it will produce a green fluorescent protein (GFP) which fluoresces under a UV light source.

The map of all plasmids will be provided and each team will come up with all of their own experiments and protocols. Remember this independent lab project is the major portion of your grade. I want you all to be as creative as you can. You may alter the chromosome extracts and even digestion procedures as you like but please only alter one variable at a time and always carefully document the exact change. This is called controlled scientific experimentation.

What are you responsible for? Everything! The planning and writing up of all of your protocols, the gathering of the appropriate materials, (I am your biotechnology supplier and it's all free of charge) the execution of the experiments and analysis of your data. Consider me your technical support, lab assistant and biotechnology supplier. All of my literature and protocols, manuals and resources are at your disposal as well as my considerable years of experience and technical expertise. Please just don't come up and ask me "What do I do?" Come to me with a plan based upon what you have learned in lab and ask for my comments or input. Remember first you must ask the questions and perhaps propose a hypothesis. For instance you make the assumption that this DNA will behave exactly the same as any other DNA. Plan your experiments accordingly.

Your grade will not necessarily be based on your success but your trial experimentations and **COMPLETE and TOTAL Laboratory Documentation** of all of your research efforts. For example if you come to me and say "My plasmid extract didn't work, what should I do?" I will ask for your notebook with the details of the exact procedure you performed, a picture of your agarose gel, the quantitation numbers from your quantitation and the tube of your remaining extract. If you don't even get beyond the first step of your project this does not mean you won't get a good grade, but it does mean that you better show me that you have put in considerable effort, even though the experiments didn't work.

Scientific experimentation doesn't always work, actually there are usually more experimental failures than successes, however many of the protocols you are using are tried and true, the real variable in this project is the DNA of the Halophile.

It will be in your best interest to do all of your planning and analyzing of results outside of the lab time because you will need every minute of lab time to do your experiments. To get a very good outcome as measured by me in your development as an experimental scientist and as a creative thinker you must commit yourself to this laboratory course. This will not be a lab course in which everyone will make a good grade just because they show up. You may have to completely alter your thinking about laboratory science and maybe even science in general. I am very excited about this project and it can offer you great personal satisfaction and rewards if you choose to participate whole heartedly. I will be supplying information on an overview of the whole project before we begin in class.

Appendix of Protocols and Plasmid Maps