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Molecular Cell Biology Lab Manual

BIOL 310

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General Information

Dear Student,

Welcome to the Molecular Cell Biology Laboratory.

The lab you're about to take is a work in progress. Since I started teaching labs in this course, I noticed that there is a need for a study resource for our lab students. As a result, I have started adding some relevant theoretical background material to help students better understand the procedures they would be carrying out in the lab.

It is important that you read through each laboratory exercise and theory before coming to the lab. This will help you understand why you will be doing certain things and will make it more likely that you will successfully complete the exercises in the allotted time, it also helps to ensure that the labs are conducted safely.

This manual, and any handouts that will be posted online for you are also a work in progress. I hope that you will find them useful, and would appreciate any feedback, comments or suggestions that you have for future improvements.

I wish you much success in this semester,

R. Stefan Rusyniak

Lab Safety

Your concern for safety should begin even before starting your lab activity. Please make sure you **always read your lab manual / handouts**, and think about each laboratory assignment **before starting**. Doing so, will alert you to any chemicals and other potential hazards that you might encounter in the lab that day. It will also ensure that you've had a chance to think about how you will perform certain activities and why they must be performed in certain locations or in a certain order.

Always remember that you are not the only person working in a lab and your actions and knowledge (*or lack of knowledge*) can affect the safety of others. For this reason please familiarize yourself with the locations of safety equipment (safety shower, eye wash station, first-aid kit, fire extinguisher, and blanket) in the lab, as well as the location of the medical clinic office in case medical assistance is needed. Also, please make sure your books, bags and clothing are placed out of the way where they are unlikely to cause problems.

In order to protect yourself and others follow basic safety procedures in the labs. Some of our classes involve the use of harmful chemicals so please know and follow the following rules:

1. You **must wear a laboratory coat** for all laboratory work. You will not be allowed to work in the lab without one (and will be marked as absent).
2. **Eating, drinking, and chewing gum are prohibited** in the laboratory at all times.
3. Bulky clothes, abaya, long scarf, dangling jewelry and short skirts can be a hazard in lab. Please do not wear them to the lab or make sure they are secure and unlikely to cause problems.
4. **Confine long hair and scarf** when in the laboratory so that it will not catch on fire or come into contact with chemicals.
5. Wear shoes rather than sandals at all times in the laboratory. **Wearing open-toed shoes is hazardous** in a lab environment (you will be asked to leave the lab and marked as absent).
6. Special care is needed if you wear contact lenses since chemicals splashed in the eye may get under the lens and therefore be difficult to rinse.
7. Mascara has harmful effect on ocular lens (eyepiece) of microscope, and therefore should be avoided.
8. Do not use flammable liquids near open flames. Most organic liquids are flammable.
9. Smell chemicals carefully and only when instructed to do so. Waft odors towards your nose rather than sniffing directly.
10. **Wash hands thoroughly** with soap and water immediately after a spill and before leaving the lab.
11. Any broken glass should be removed from a work area and placed in the glass waste container. Biological waste should be disposed of in biohazardous waste containers.
12. Notify your lab instructor immediately if you are injured in any way.

For labs involving electrophoresis, high voltage power supplies can be a potential hazard. The gel tanks commonly used in teaching labs have electrodes that cannot carry current unless the lid is firmly in place, but there are a few things to remember.

1. Be sure there is sufficient buffer in the chamber to form a circuit between the anode and cathode.
2. When the electrophoresis is finished, shut off the power supply and only then, open the chamber.
3. If buffer spills from the chamber to the benchtop, turn off the power supply immediately and ask for assistance from the TA for clean-up so that no shock hazard will exist.

Work in a Molecular Biology laboratory often involves work with bacterial and/or yeast strains as well as recombinant DNA. Laboratory strains of microorganisms and DNA vector molecules are generally not harmful, and many have been modified such that they cannot live outside of the laboratory. Any scientist, however, should work under the assumption that the unexpected can happen. Therefore you should ensure that any risk of uncontrolled release, or harm to yourself and others is minimized.

1. Always wear gloves when handling host strains and recombinant DNA solutions.
2. Dispose of all solid waste containing bacteria, yeast, and recombinant DNA into the "BioHazard" bags so that they can be autoclaved.
3. Place liquids (medium) containing bacteria and recombinant DNA into a designated waste container (containing disinfectant) when you are finished with them.
4. Place sharps (glass Pasteur pipettes, razor blades) which have been in contact with bacteria or recombinant DNA into the appropriate plastic BioHazard waste containers.
5. Wash your hands well (with soap) before you leave the lab.

In our DNA labs, DNA will be detected in a gel by fluorescence of ethidium bromide, which intercalates between the DNA strands. There are two potential hazards here:

1. Do not look at the UV light box. The safety glasses will not block light in the UV range, nor will they protect your face. You risk serious eye damage by looking at a UV light source.
2. Ethidium bromide is a potent mutagen and may be carcinogenic. Handle it (and gels containing it) with care and dispose of them properly.

Waste handling

Waste is anything that is to be discarded. In terms of daily use, any contaminated materials will require actual removal from the laboratory or destruction. The overriding principle is that all contaminated or infectious materials should be decontaminated, autoclaved or incinerated after removal from the laboratory.

Disposal

Identification and separation system for infectious materials and their containers should be adopted. Categories in lab (SC118) include the following:

Category	Materials contain
Biohazard bags	Petri dishes, test tubes, Biological waste, gloves or autoclaved waste and similar. Solidified agar or agarose should be discarded in biohazard container, not in the sink.
Broken glassware box	For any broken glass.
Sharps containers	Needles, cutters, razor blades etc.
Small biohazard containers	Are provided on the bench for the contaminated equipment such as tips, Eppendorf tubes, etc.
White box near gel electrophoresis system	Ethidium bromide is a mutagenic substance that should be treated before disposal. Buffer/ tips/ agarose gel containing EtBr, acrylamide solid waste should treated in this container with 70% Ethanol or 10% Clorox before disposing in the biohazard container.
Organic reagents	Phenol, should be used in a fume hood and all organic waste should be disposed of in a labeled container, not in trash or sink

Decontamination

Autoclave:

Steam autoclaving is the preferred method for all decontamination processes. Materials for decontamination and disposal should be placed in containers, e.g. autoclavable plastic bags, which are color-coded according to whether the contents are to be autoclaved.

Disinfectant solutions:

In addition, the best way to disinfect the contaminated materials on the work bench, such as used flasks … etc. is by 70% Ethanol or 10% Clorox, which are available in the lab.

Clean-up

Ethidium Bromide

In case of small spills, clean the area with tissues soaked in 70% Ethanol. Dispose the tissues in the EtBr waste (white box near gel electrophoresis system).

Bacterial Culture

When working with bacteria, always clean the bench before and after this work with 70% Ethanol or 10% Clorox. After spilling material you have to clean immediately with dry paper towels and following this, the area of the spill should be disinfected with bench disinfectant.

Each day, before you leave your lab bench, clean off the bench surface. Remove matches and papers, and wipe down the surface with water and paper towels.

Chemicals/Reagents

Cleaning spills of chemicals (especially near balances):
Always clean spilled chemicals immediately. You should confine and contain the spill by using absorbent pillows.

Excess chemicals must be disposed of; they generally cannot be recycled. Therefore, do not take more of a chemical than is needed for an experiment. Do not contaminate stocks of chemicals (such as in bottles) by using dirty spatulas. Transfer a small amount to a clean paper filter disc to weigh out what is needed.

All chemicals should be disposed of in an approved manner. Do not pour any chemical down the sink unless specifically told to do so.

Notebooks

A proper laboratory notebook contains an accurate and detailed account of experimental procedures and results. It needs to be neat, well-organized and as complete as possible. The level of detail should allow another researcher to repeat your experiment and replicate your results.

It is important to keep an accurate and detailed laboratory notebook. Sometimes, a small detail may not seem important when you first see it, but it might be useful when you're trying to analyze the results – especially when they are not what you expected. Being able to go back through your procedures and observations in your notes can help you explain why your results are different from what you would expect. It might turn out that you skipped a step in the protocol, or that you made a mistake somewhere along the way (sometimes mistakes are good things – they lead to new discoveries).

The lab notebook is the property of the lab and not of the researcher who is writing in it. In a research setting, a laboratory notebook never leaves the lab. This is important, because many researchers working in that lab may need to refer to that notebook in order to perform their work.

For example, you might be a graduate student who is studying a particular gene. When you finish your project, you will need to be able to refer to your notebook to write your thesis. However, others may need to be able to use your notebook as well.

- Perhaps you made some modifications to a procedure that gave improved results – other people in your lab would benefit from having access to your notes on this procedure.
- Maybe one of your friends in the lab asked you to complete some part of an experiment for them, you would put your notes and observations in the notebook and your friend would later need to access them.
- Also, after you have completed your studies and left the lab, other graduate students might be asked to continue your research to find out more – these new grad students will benefit from having access to your notes (so they don't have to start from the beginning).

Advice on keeping a “proper laboratory notebook” varies depending on who you ask in a teaching lab, but there are a few things that are commonly done. Because some research can lead to financially rewarding discoveries and a laboratory notebook can be used as evidence of prior discovery in cases of disputes over intellectual property / patents, there are a few commonly agreed guidelines for keeping a notebook. Much of it has to do with ensuring that the contents of the book cannot be easily altered:

1. Notebooks should be permanently bound. This means that it should not be possible to remove (or add) pages from them without it being easily noticed.
2. All pages should be numbered. Again this will make it easier to notice if a page has been removed.
3. You should have a date on every page. This allows the identification of when an experiment was first performed or when a discovery was made.
4. You should write in pen and never use white-out or corrective tape. If you need to make corrections then just neatly cross out the incorrect material. A notebook that shows evidence of erasing or removal of data (use of corrective tape, etc.) is not trusted.

5. A laboratory notebook should have your name in it, and a contact number in case the book is found by someone who wishes to return it.

Because a laboratory notebook tends to contain the details of many experiments, it is also a good lab practice to include a Table of Contents on the first 1-2 pages. In the Table of Contents you should list the important experiments and items, and indicate on which pages those items can be found.

Things your TA would like to see in your notebooks:

The page numbers and dates should be in there as mentioned above. Ideally, for each lab, you should have a brief introduction – just a couple of sentences to summarize the purpose of the lab and the techniques used. This should be followed by a **flowchart** for that lab and any **calculations** that might be needed (get these done before the lab, so you don't have to spend time on them during the lab).

You should write your procedure and observations as you do the experiment or very shortly after you've done it – it should be completed before you leave the lab each day – your TA will be checking this **before** you can leave the lab. It is much better to have an accurate but somewhat messy description, than it is to have a less messy and less accurate one, written based on your memory a day or two after the lab was completed.

Lastly, any results/data that are posted for you after the lab should be added to the notebook with a brief explanation of what the result shows. You don't need to make any major conclusions about the results, but should write down information about the picture or graph that might be useful to understanding it later. Make sure you do this on a regular basis and not at the end of the semester – at that point you will likely have forgotten any important things relevant to the figure or graph.

Lab 1: Preparation of Solutions

In this lab, we will be discussing some of the administrative details relevant to the lab part of the course. It also introduces you to working with lab reagents and the preparation of solutions. This is important because you will be producing some of the solutions that you will use later this semester. In order to do this accurately, you will need to learn to make appropriate calculations first.

Please be sure to read through the introductory material and complete the calculations for the assigned solutions **before you come to class** – your TA will be asking students to put their calculations on the board.

Learning Objectives:

Students will:

- Demonstrate their knowledge of Lab Safety Rules by coming in dressed appropriately.
- Identify all pieces of safety equipment in their lab.
- Calculate the amounts of reagents needed for the preparation of solutions.
- Prepare stock solutions for use in future labs.
- Describe how a pH meter is able to measure the pH of an unknown solution.
- Demonstrate proper use and storage of pH meters.

Pre-Lab Questions:

- Go to the linked video and answer these questions:
  - https://youtu.be/vxAoGWUpdzE (please ignore the advertisement at the end)
  - What is a buffer?
  - Would it be okay to keep enzymes for an experiment in pure water? Explain.
- Use any resources you find helpful to answer the following
  - What is EDTA?
  - Why would we want to add EDTA to a DNA storage buffer?

Units and Measures

Most countries and virtually all labs around the world have adopted the metric system for their measurements. The metric system is a base 10 system which has specific prefixes that are associated with certain values. It is critical that you become familiar with these units are are able to easily and quickly convert between them. So please take some time to go through the following lists and at least make sure you can convert between the units in **bold** – these are used most often in Cell and Molecular Biology labs, so you will see them on a regular basis.

Size:

Molecular biology deals with things which are relatively small. The units of measurement typically used are the micrometer (especially in light microscopy) and the nanometer (in electron microscopy). For molecular measurements of atoms, the norm is the Angstrom. These units are defined within the following table:

Measure	Symbol	Relative Length	Exponential Notation
Meter	m	1	10^{0}
Decimeter	dm	0.1	10^{-1}
Centimeter	cm	0.01	10^{-2}
Millimeter	**mm**	0.001	10^{-3}
Micrometer or micron	**µm / µ**	0.000001	10^{-6}
Nanometer	**nm**	0.000000001	10^{-9}

From this table it is apparent that:

1000mm = 1 m
1000 µm = 1 mm

Volume:

Volumes are measured relative to a liter, with the most commonly used measurements, the milliliter and the microliter. The following table gives the relative volumes:

Measure	Symbol	Relative Volume	Exponential Notation
Liter	**L**	1	10^{0}
Millimeter	**ml**	0.001	10^{-3}
Microliter	**µl**	0.000001	10^{-6}

There are 1,000µl in 1ml.

Weight:

The most common measurements of weight are the gram, milligram and microgram.

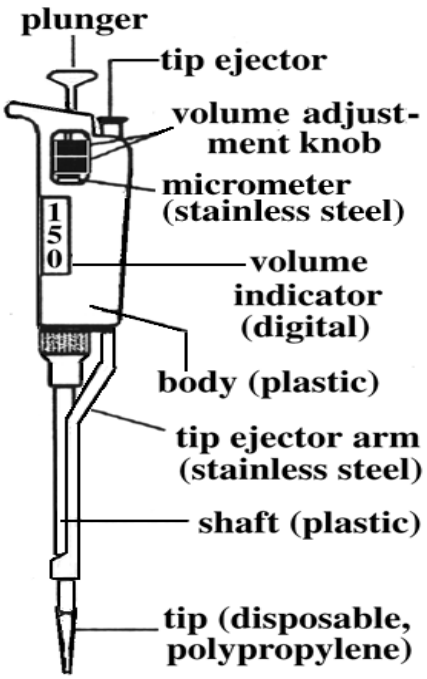
Measure	Symbol	Relative Weight	Exponential Notation
Kilogram	kg	1000	10^{3}
Gram	**g**	1	10^{0}
Milligram	**mg**	0.001	10^{-3}
Microgram	**µg**	0.000001	10^{-6}
Nanogram	**ng**	0.000000001	10^{-9}

Exercise 1: Micropipettors, Accuracy and Precision

Molecular biologists use micropipettors on a daily basis - we can not do our jobs without them. As budding molecular biologists, you must therefore become proficient in their use, and learn to accurately and reproducibly measure out small quantities of liquids. Your ability to obtain good quality results in our labs will depend on this.

Micropipettors are very expensive precision instruments that need to be handled with care. Improper/careless use of these instruments could result in a loss of accuracy and actual damage to the micropipettor. With this in mind here are some rules:

- **Never invert the micropipettor** (or hold it horizontally) – the liquid in the tip will flow into the barrel and contaminate it (in many cases it could also cause damage to the internal mechanism)
- **Do not allow the plunger snap back after pressing it**. This will cause damage to the internal mechanism, it also often causes liquids to be taken up too quickly and to be taken up into the barrel.
- **Do not play with the micropipettor** by pressing and releasing the plunger up and down very quickly. You are handling a precision instrument, rough treatment could damage the internal mechanism.
- **Never set the micropipettor outside its working range**. Doing so will damage the instrument.
- Never insert the barrel directly into liquid. **Only use the micropipettor after you've put a tip on it**.
- Use the appropriate micropipette tips for your instrument.



Types of Micropipettors

Obtain a micropipettor. Look at the plunger or the body of your instrument to determine it's maximum (and minimum) volume you will have one of the following:

- P20 - max vol 20µl, min vol 2µl (sometimes 1µl)
- P200 - max vol 200µl, min vol 20µl
- P1000 - max vol 1000µl, min vol 200µl (in some cases it's 100µl)

Please view this video for instructions on micropipettor use:

1. Pipetting Exercise

1. Set your micropipettor to its highest volume
2. Press down on the plunger knob until you feel some resistance – this is the "**first stop**"
3. Press down a bit harder until you feel resistance again – this is the "**second stop** "
4. Gently and slowly allow the plunger to return to its resting position
5. Repeat this a few more times until you have a good feel for it

6. Now, set your micropipettor to its lowest volume
7. Press down on the plunger knob until you get to the first stop
    - You will find that you will feel that first stop very quickly, it is very close to the resting position
8. Press down a bit harder until you feel resistance again - this is the second stop
9. Gently and slowly allow the plunger to return to its resting position
10. Repeat this a few more times until you have a good feel for it
11. This is important, it's very easy to miss the first stop in the low part of the micropipettor's range
    - Do not move on until you can reliably feel the first and second stops.

Preparation of Solutions

Most undergraduate biology and chemistry laboratories usually minimize the amount of time students spend preparing reagents/solutions and instead focus on introducing various techniques. Unfortunately, this prevents undergraduates from putting some of their 1st year knowledge and skills into practice and results in graduates who lack the very basic laboratory skills that everyone later assumes them to have. Such students are thus unprepared to function effectively in a real lab setting, whether it's as volunteers, undergraduate researchers or graduate students.

This lab is meant to help you develop some of those basic lab skills that will make you more confident and capable when you enter a research laboratory. In this lab, you will learn how to make your own solutions. It is important that you do this well because you will be using the solutions you prepare today in other labs later this semester – careless mistakes made today will affect you in a few weeks.

Solutions

As you may have discovered through your pre-lab preparation, the maintenance of cells and their components in their natural state may require solutions composed of multiple different ions (solutes) dissolved in a solvent (usually water). In fact, the study of cells and biological macromolecules requires the use of a variety of different solutions – each has a different purpose.

The solutes are dissolved in the solvent in very specific amounts – these are often determined by the optimal concentrations of that particular molecule or ion for the function of a cell or enzyme. There are two common ways in which the concentration of a solution can be indicated: as molarity or as a percent. Molarity is based on the number of moles of a solute in a liter of the solvent, while percent is based on the number of parts, either grams (for a solid solute) or milliliters (for a liquid solute) in 100ml of solvent.

When making solutions, it can be useful to remember that molecules can ionize in solution and thus a mole of a molecule like magnesium chloride ($MgCl_2$) will produce one mole of magnesium ions (Mg^{2+}), and two moles of chloride ions (Cl^-).

A **mole** is a unit of measure of the mass of 6.022×10^{23} molecules (Avogadro's number) of a substance. The number of grams in 1 mole of a substance is numerically equal to its molecular weight. The molecular weight is expressed in grams/mole and can be easily calculated with the help of a periodic table.

Molar Solutions

Molarity (M) is defined as the number of moles of a solute found in 1 liter of the solution. What causes students problems is that we do not actually measure things in moles, and we generally make a lot less than 1 liter of any solution.

In order to prepare a molar solution of any concentration you only need to know three things: the molecular weight of the solute, its final concentration, and the total volume of solution to be made. Once you know these three things, you can easily calculate how many grams of the substance to weigh out using the following formula:

molarity x molecular weight x volume

When doing the calculations for any solution, always remember to **write the units** of each component of the equation. In this case, the units would be as follows:

$$\frac{mole}{Liter} \times \frac{grams}{mole} \times Liters$$

This allows you to ensure that all the units match up – you should not be multiplying liters by milliliters. It also allows you to cancel out reciprocal terms and know the units of the final value.

$$\frac{\cancel{mole}}{\cancel{Liter}} \times \frac{grams}{\cancel{mole}} \times \cancel{Liters}$$

Examples

Example 1: Make one liter of 1M NaCl - A simple example

The molecular weight of NaCl is 58.44g/mole. Therefore, you need to weigh out 58.44grams of NaCl and dissolve it in water. After it has dissolved, adjust the final volume to one liter.

1mole/liter x 58.44grams/mole x 1 liter = 58.44grams

Notice that the units cancel out in the above case, but keep in mind that if the units are not the same, then you need to do some metric conversions to make them equivalent.

Example 2: Make 50ml of 20mM NaCl - Unit conversion needed

The first thing to note is that you will need to convert mM to M – this is a simple metric conversion: 20millimoles x 10^{-3} moles/millimole = 0.02moles, thus 20mM is the same as 0.02M. After that, you convert the 50ml to liters (0.05L) and plug the numbers into the formula. This gives 0.058g.

0.02moles/liter x 58.44g/mole x 0.05liters = 0.058grams

Therefore, to make the above solution, weigh out and dissolve 0.058g of NaCl in a small volume of water, then adjust the final volume to 50ml.

Example 3: Make 500ml of 0.2M Tris, 100mM $MgCl_2$ - More than one component

Both of these components would go **into the same beaker**. The molecular weight of Tris is 121.14g/mole and $MgCl_2$ is 203.3g/mole. For a solution like this, you need to calculate each of the amounts separately.

For the Tris:

0.2moles/liter x 121.14g/mole x 0.5liters (0.5L = 500ml) **= 12.114g**

For the $MgCl_2$:

0.1moles/liter x 203.3 g/mole x 0.5liters = 10.165g

Thus, to make this solution, you would weigh out 12.114g of Tris and 10.165g $MgCl_2$. Add both components to the same beaker and dissolve in water, then bring the final volume to 500ml.

Percent Solutions

The use of "percent" solutions is a little less common in Biological labs, but does happen from time to time. These percent solutions are usually expressed in one of two common forms: either as a "weight by volume (w/v)" solution or a "volume by volume (v/v)" solution. In each case, the percentage shows the number of grams (w/v) or milliliters (v/v) of solute in a final volume of 100ml. The type of solution is usually indicated when it's written down as follows:

5% NaCl (w/v), or 20% Glycerol (v/v), etc

In cases where it isn't indicated, the type is usually fairly obvious – for example 70% Ethanol. Ethanol is never a solid under regular lab conditions, so it must be a v/v type of percent.

In biology, the most common use of "percent" solutions is as (w/v). In practice, these are very easy solutions to prepare.

Examples

Example 1: Make a 5% NaCl solution

Weigh out 5g of NaCl and dissolve it in 100ml of water

Example 2: Make a 10% sucrose solution

Weigh out 10g of sucrose and add it to 100ml of water.

Unless specifically stated otherwise, solutions that don't indicate the type (ie. w/w) should be assumed to be (w/v)

Dilutions

Preparing solutions by weighing out reagents and dissolving them in the right amounts of solvent can be quite time-consuming. For this reason, most shelves in research labs contain large bottles of "stock solutions". Stock solutions ("stocks") are simple solutions which are commonly needed in a laboratory and are prepared as described above. The reason that they are useful is that they can also be easily and quickly diluted and mixed in many combinations to make many other solutions.

For example, having a 1M Tris stock solution and a 0.5M EDTA stock solution (and a few others) will allow you to make a variety of buffers commonly used in DNA purification, RNA purification, PCR, cell lysis, etc. All you need to know is which stock solutions you have available, the volume and composition of the solution you need to make, and the following formula:

$$V_{initial} \times C_{initial} \times = V_{final} \times C_{final}$$

where the "C" stands for concentration and the "V" stands for volume.

Usually you know 3 of the above 4 components of that equation, usually you just need to find out the volume of the initial stock solution ($V_{initial}$).

A common misconception students have is that you need to convert your volumes into liters just because the concentration is expressed in moles/liter. This is not true. What you need to ensure is that your initial concentration and final concentration have the same units, or that your initial volume and final volume have the same units.

Examples

Example 1: Make 25ml of 50% ethanol from a 95% ethanol stock.

Here, V_f = 25ml, C_f = 50%, and C_i = 95%, so:

x 95% = 25ml x 50%

Rearranging: V_i = (25ml x 50%) / 95%

V_i = 13.16ml

Example 2: Make 200ml of a 50mM $NaHCO_3$ solution from a 0.5M stock

Here, V_f = 200ml, C_f = 50mM, and C_i = 0.5M (0.5M = 500mM), so:

V_i x 500mM = 200ml x 50mM

Rearranging: V_i = (200ml x 50mM) / 500mM

V_i = 20ml

In the above example, one of the concentrations had to be converted to make the units of concentration equivalent. In this case I converted the initial concentration of 0.5M to 500mM, but I could also have converted the 50mM to 0.05M (C_f). It would not have affected the answer or the units of the answer.

Biological Buffers & pH Meters

A buffer is defined as a solution containing a mixture of a weak acid and its conjugate weak base that is capable of resisting substantial changes in pH upon the addition of small amounts of acidic or basic substances. In biology, buffering is required to maintain the integrity and function of macromolecules outside the cell (in vitro). This is because much of a macromolecule's 3D structure depends on weak molecular interactions like hydrogen bonding. These interactions can be disrupted by a lack of or an excess of H^+ ions in the solution, thus changing the shape of the molecule and likely affecting it's ability to function properly.

In practice, pH is measured and adjusted using a pH meter, which is a sensitive instrument capable of detecting very small electrical potentials. Because protons are charged ions, they can mobilize electrons in a conducting wire and thus induce an electrical potential. A pH meter measures the voltage between two electrodes, which are typically housed together in a single probe (this will be the part of the pH meter that you put into a solution – it is referred to as “the electrode” even though there are actually two electrodes in it).

Because the pH meter is actually a very sensitive Voltmeter and doesn't necessarily know anything about pH, it must be calibrated before it's used. The calibration allows us to “tell” the pH meter what a certain pH “looks like” - here, the pH electrode is immersed in a buffer with a known pH and the pH meter is adjusted to correspond to this pH value. This is often referred to as **standardization**.

Standardizing pH Meters:

1. Pull the electrode out of the storage solution and rinse it with some DIW (usually in a squirt bottle). Gently blot off excess water.
2. Immerse the electrode in a standard buffer. Stir or gently swirl the solution.
3. Set the meter to measure the pH. It should be close to the specifications of the standard, confirm if the instrument has listed the right pH (how you do this depends on the manufacturer of your instrument – check the instruction sheet near the pH meter).
4. You may need to repeat this with a few other standards.
5. Rinse the electrode, blot dry and then insert into your solution.

Measuring the pH of Your Solution

1. Rinse the pH probe/electrode with some DIW before placing it in your solution.
2. Place the pH probe into your solution. The meter will display an initial pH reading, but it may fluctuate. Allow the electrode to equilibrate for about 10 seconds and the pH value should stabilize.
3. Turn on the stirrer and ensure that the stir bar is as far away from the electrode as possible. Allow the instrument to measure the pH.
    1. If the pH is too high, you need to add acid.
    2. If the pH is too low, you need to add base.
    3. If you overshoot the pH, do not try to "fix" it. Start over.
4. Add the acid or base drop-wise and let the solution equilibrate after each drop. Wait for the pH meter to stabilize.
5. When you get to the desired pH, you're finished.
6. Rinse the electrode well with DIW, and place it back in its storage solution.
7. Turn off the pH meter or set it to standby mode.
8. Wipe up any spills and ensure that the acids and bases used are capped and properly stored.

There are a few important things to remember about pH meters.

1. They are delicate instruments that need to be cared for and kept clean and dry.
2. The electrodes are very fragile. Don't touch them with anything other than a KimWipe™, and then only to blot off the excess liquid.
3. When used to measure pH in a beaker with a stir bar, be sure that the stir bar doesn't crash into the electrode.
4. The electrode bulb must remain wet, so work quickly, don't forget to rinse the electrode with distilled water before and after use, and always place it in its storage solution.

Exercise 2: Preparation of Stock Solutions

The main thing you will need to accomplish today is the preparation of the solutions shown below. Based on what you learned in the above sections and the provided molecular weight information, perform the necessary calculations to find out how many grams of each component you will need to make each of the listed solutions. Do this **before coming in to the lab.**

Please keep in mind that you are about to prepare stock solutions that you will be using in the lab later this semester. This means that you want to make sure you do not make any mistakes – a mistake at this point could end up affecting your results in a lab a few weeks from today (just like a real research lab).

1. Calculations

Calculate the amount of each reagent needed to prepare the solutions below – you should do this individually to make sure that you know how to do it. Compare your results to the other members of your group to ensure that everyone is calculating things properly.

- 100ml of 500 mM Tris-Cl, pH8
- 50ml of 100 mM EDTA-Na_2, pH8
- 50ml of 500 mM Glucose
- 50ml of 1.0M NaCl
- 50ml of 0.1M of $MgCl_2$
- 50ml of 0.1M $CaCl_2$
- 50ml of 5M Potassium Acetate

Molecular Weights

Tris	121.14 g/mol
Glucose	180.16 g/mol
EDTA	292.25 g/mol
NaCl	58.44 g/mol
$MgCl_2 \cdot 6H_2O$	203.3 g/mol
$CaCl_2 \bullet 2H_2O$	147.2 g/mol
Potassium Acetate	98.14 g/mol

2. Preparation of Solutions

Before you begin, please note that you may need to round off some of the numbers from your calculations because your balance may not be able to measure that level of accuracy. Check with your TA.

Although many chemicals are inexpensive, some are very expensive. Since you don't know which ones are the expensive ones, you should treat them all as very limited and expensive. Use only as much as you need – do not waste reagents.

Your hands can be a source of many contaminants which may at times affect your results. As a result, you should wear clean gloves when preparing stock solutions and try to work as cleanly as possible – you never know what that solution might be used for at a later time. Handle the spatula, the weigh paper and the stir bars only with a gloved hand.

Materials

- reagents
- 250ml Beakers (3)
- 100ml Graduated Cylinder (1)
- 50ml Falcon Tubes (4)
- 100ml bottles (2)
- Stir plate and magnetic stirrers (3)
- Scale and plastic weigh boats
- Plastic spoons
- pH meter, pH standards, waste beaker and squirt bottle
- Kimwipes
- 1M HCl, 0.1M HCl, 1M NaOH, 0.1M NaOH
- Droppers for acid and base solutions

Procedure

1. Have your calculations completed and be aware of the total volume of the stock solution you will be preparing. Also, make note of whether the solution has to be at a **specific pH**.

2. Obtain and label a beaker, and add approximately half of the total volume of water (ie. if you need to make 100ml, add only 50ml at the start).

    - Using pure water for your solutions instead of "tap water" ensures that you know that you have very good control over what chemicals are actually in your stock solutions. Normal tap water has a variety of ions dissolved in it and is not suitable for most lab uses.

3. Place a weigh boat (or a piece of weigh paper) on the balance and "tare" the balance.

    - Taring means that after the weigh boat has been placed on the balance, the balance is reset to measure zero.

4. Using a clean spatula, take a small amount of the chemical out of the stock bottle and place it in the weigh boat.

    - Avoid spilling chemicals on the balance or the bench top. If you do, be sure to clean up the mess. When cleaning the balance, **DO NOT press down on the pan!** Take the pan off carefully, wipe it clean and replace it carefully. Quite often there will be a small brush near the balance for this purpose.

    - If you find that you're taken out too much reagent, carefully scoop it up with your spatula and throw it away into a waste container. **DO NOT put chemicals back into their stock bottles**. Taking small amounts of reagent from the stock bottles will ensure that you don't waste chemicals.

5. Once you have finished weighing out the reagent, pour it into the beaker from step 2

    - It is a good idea to start with some water already in the beaker because some reagents are very fine powders, and if they are added to a beaker first, and water is added afterwards, they tend to form large clumps and are more difficult to dissolve.

6. If the solution you're preparing is supposed to have more than one component, then weigh out the next reagent in the same way (start at step 3) and **add it to the same beaker**.

    - In cases where you have multiple components, some students think that it would save time and resources to simply tare the balance again with the first reagent still in the weigh boat (to reset the weight to zero), and simply add the next

reagent until the appropriate amount has been weighed out. The one potential drawback to this is that if you add too much of the second reagent and have to remove it from the weigh boat, you may end up also removing some of the first reagent as well.

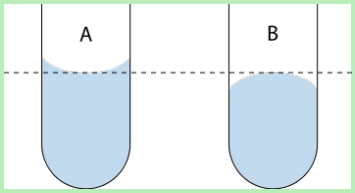
7. Gently, slide a stir bar in along the side of the beaker, and place the beaker on a stir plate. Make sure the stir bar is not too large – your TA will explain.
    - You might notice at this point that the volume in your beaker is higher than you intended. Do not worry yet, the stir bar displaces a certain volume but you will remove from the solution before setting the final volume.
8. Start the motor on the stir plate to initiate stirring. Don't turn it up too high or the stir bar may start bouncing and break the beaker. You want it to mix well but not violently.
    - Only one beaker per stir plate – it won't work if you try to put two beakers on there.
9. Maintain the stirring until the chemicals have completely dissolved.
10. Once the chemicals are dissolved, adjust the pH of the solution.

Go to the section on pH and standardization of the pH meter before performing any pH measurements.

11. After the chemicals have dissolved and the pH has been adjusted, turn off the stir plate and pour the contents of the beaker into a graduated cylinder.
    - Use a magnetic retriever ("magic wand") or another stir bar to prevent the stir bar from falling into the graduated cylinder. Your TA can demonstrate this.
12. Hold the cylinder up to eye level and add water until the bottom of the meniscus is at the desired mark.
    - **Do not overshoot the volume**. If you add too much water, you will have to start over.
13. Stretch some Parafilm™ over the top of the graduated cylinder and invert a few times to mix the solution evenly.

Meniscus

The meniscus is the curve in the upper surface of a liquid in a container. When making measurements, always align the flat portion of the meniscus with the gradations on the instrument.



14. Pour the newly-made stock solution into a well-labeled storage vessel (usually a screw-capped bottle). T**he label should include the solution's identity and concentration(s), and the date on which it was prepared.** Additionally, you should add your lab section and group name/number to help you identify your solutions.
15. Wash out your beakers and stir bars to reuse them as you make the solutions.
16. Once a solution is completed, transfer it into either a Falcon tube (if the total volume is 30ml) or into a glass bottle (if the solution is 60ml)

17. Label your tubes and bottles.
    - Put a piece of tape on the bottle and write your label on the tape
    - Use a sharpie (pen or pencil markings are likely to be removed during autoclaving)

Adjusting the pH of your Solution

- Once the pH meter is standardized, you should rinse the electrode with deionized water (DIW) over a waste beaker, wipe excess water with a KimWipe™, and then immerse it in your solution.

  CAUTION: Be sure that the stir bar does not come in contact with the electrode. Position the electrode such that only the tip is in the solution and the stir bar is as far away from it as possible to minimize the chances of contact.

- While stirring, slowly add acid or base drop by drop. Wait a few seconds between drops for the solution and thus the pH to equilibrate and be measured.
- Usually a few drops of concentrated acid or base (≈1M) is enough, and you may need to use dilute acid or base (≈0.1M) to make the final adjustment.
    - Some buffers are quite strong however and will resist changing until you've added a fairly large amount of acid or base – be patient.
    - **Don't overshoot the intended pH.** If you do, then you will have to start over.
- Once you've reached the intended pH, be sure to rinse the electrode well with DIW and replace it in the storage solution.
    - **Never let the pH electrode dry out** – it damages the mechanism and makes the electrode useless.

Once completed, the solutions will be sterilized using an autoclave. The autoclave is a device that operates at high temperatures (around 121°C) and at relatively high pressures (around 103kPa or 15psi). Treatment of your solutions and any laboratory tools for about 20 minutes under these conditions will kill all microorganisms, and ensure that your materials are sterile and ready for experiments.

It is important to remember that materials in the autoclave are in fact hotter than boiling water and under high pressure. These bottles have the potential to explode in some circumstances. For this reason, you must ensure that any bottles you send to the autoclave have loose caps. Among other things, this will ensure that the pressure of air inside the bottle has a chance to equalize with the pressure of air outside.

Also, once any solutions are returned to you from the autoclave, remember that they may be very hot (if they were autoclaved recently), and should be handled with extreme caution. You should always allow these materials cool down for a short time before handling them. Once they are cool, you must remember that the caps of your bottles were loose when you sent them for autoclaving, so you should make sure to tighten them before putting the solutions away.

Post-Lab Questions:

Please think about these and answer them in your notebook:

- Now that you have your stock solutions completed, you can make some preparations for the next lab. In Lab 2, you will need to use the following solutions:
    - Solution A: 25mM Tris-HCl, 50mM Glucose, 10mM EDTA-Na_2 ; pH: 8.0
    - Solution B: 0.2M NaOH, 1% SDS
    - Solution C: 5M Potassium Acetate / Glacial Acetic Acid
    - Solution D: Phenol:Chloroform:Isoamyl Alcohol (25:24:1)
    - Solution E: TE (10X): 100mM Tris-HCl, 10mM EDTA-Na_2, pH: 8.0

- In order to complete next week's lab efficiently, you should perform some calculations so that you can just start preparing the necessary solutions as soon as you get into the lab. Please use the dilution formula to determine how much of each of the components to use to prepare the following solutions (A, B and E):
    - 50ml of Solution A
    - 5ml of Solution B (you will have a 5M NaOH stock solution available)
    - 5ml of Solution E

Use $C_1 V_1 = C_2 V_2$

    - 50ml of Solution C will be prepared as follows:
        - 30ml of 5M Potassium Acetate
        - 5.75ml of Glacial Acetic Acid
        - 14.25ml of H_2O

    - Solution D will be provided for you.

Lab 2: Plasmid DNA Extraction

In today's lab you will extract a DNA plasmid from a bacterial culture. In this case, you will use the stock solutions you prepared in the previous lab to make the more complex solutions needed for this one. Along the way, you will also get some practice using some of the tools commonly used in various Biology disciplines. The skills you develop here will be useful to you, not just in this course, but in other courses and in many potential research positions.

Learning Objectives:

Students will:

- Explain the purpose of the main steps in a DNA extraction procedure
- Explain the importance of the various reagents being used in the DNA extraction procedure
- Determine the amount and purity of their DNA samples

Pre-Lab Questions:

Please view the video linked here, and read the material on this page. Also, feel free to use any other resources to help you answer the following:

- Explain the reasons why each of the 3 components are present in our Solution A.
- The author of the video does not mention using Phenol/Chloroform, but we will be. What does this solution actually do? Why are we using it?
- The author of the video made one fairly big mistake in his explanation. What was it?

Important Note:

This week's lab procedure is fairly long. If you come unprepared, you may not be able to complete the procedure before the lab ends. It's very important that:

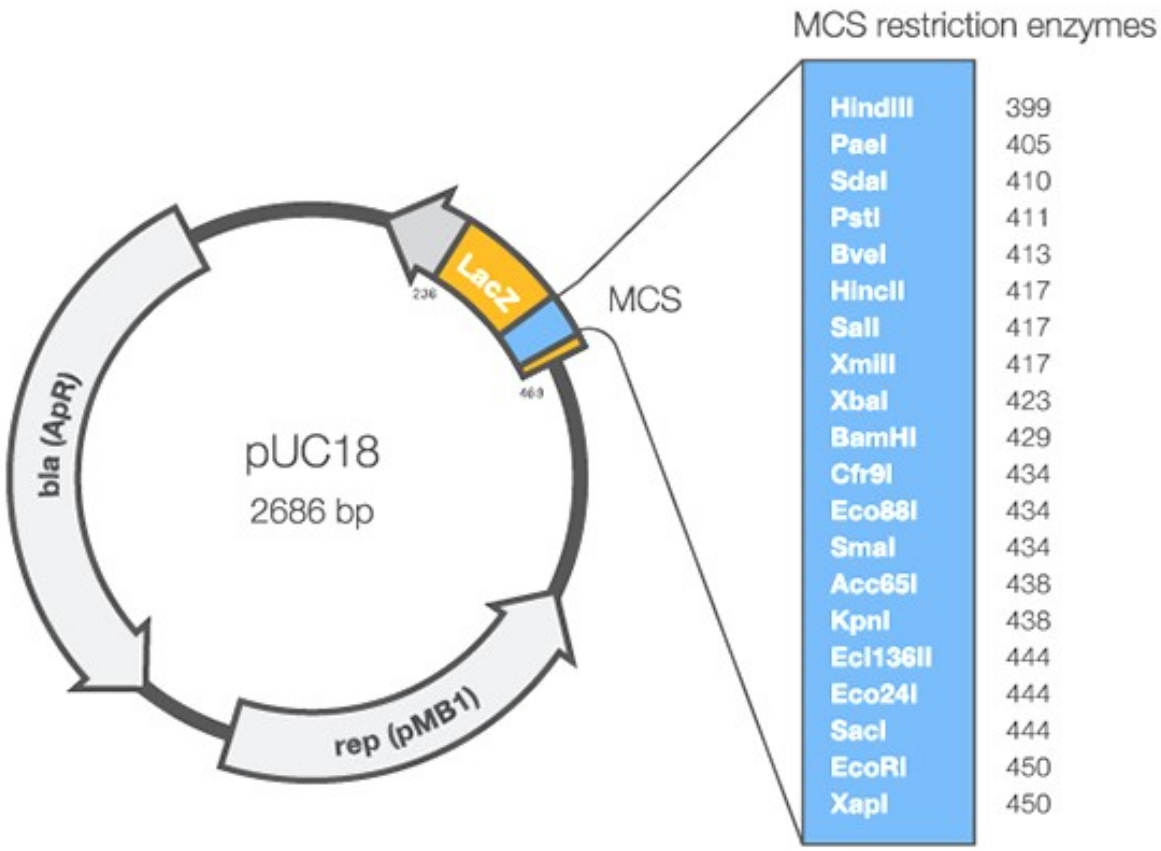
1. you come in with your **calculations completed**
2. you **check** what you calculated against what other students calculated
3. you **start making your solutions** as soon as you get into the lab

Introduction to Plasmid Isolation

The DNA you will extract today is an extrachromosomal DNA molecule that is commonly used in molecular biology. Plasmids are small, circular, naturally occurring molecules that have been modified for use in laboratories. In nature, these molecules often provide genes which increase the host cell's survival. For example, a plasmid might contain a gene which allows it to use a new macromolecule as a source of food.

These plasmid molecules are useful in the lab because they can be used by scientists to insert new genes into bacterial cells (host cells) like *E. coli* and allow them to study how the new gene affects the bacterium. Or they can be used to produce the protein encoded by the inserted gene. Most commonly, however, plasmids are inserted into bacteria to amplify (make many copies of) a particular DNA sequence.

A typical plasmid used for research purposes has been engineered to have the following **three important features**: an origin of replication, a selectable marker gene (ie. antibiotic resistance), and a multiple cloning site (MCS) where a gene of interest can be inserted.



As you will see later this semester, inserting plasmids into bacterial cells is very inefficient – only a small percentage of cells will actually take up a plasmid into their cytoplasm. Thus, we need some way of identifying those cells, and this is accomplished through the use of the "selectable marker gene". The selectable marker gene is usually a gene that provides the host cell with resistance to an antibiotic, and it is this resistance that allows researchers to "select" only the cells that contain the plasmid. Only the cells with a plasmid will be able to grow on the antibiotic and cells that don't have the plasmid will not grow. Thus we are "selecting" for a particular trait which "marks" these cells as having the plasmid that we want.

The origin of replication determines how many copies of the plasmid will be present in each cell. “High-copy-number” plasmids will replicate themselves until there are hundreds of copies in a single cell. This, along with the ability of cells like *E. coli* to double in number every 20-30min (ie. 1 million cells becomes 2 million cells in about 20min) means that a DNA sequence inserted into a plasmid can be significantly amplified very quickly and easily.

Once such a plasmid has been sufficiently amplified, scientists extract it from the bacteria and purify it. Today's lab will introduce you to one of the most common techniques of purifying plasmid DNA from *E. coli* – this is called the Alkaline Lysis Mini Prep.

Key Concepts Behind the Alkaline Lysis Procedure

The Alkaline Lysis procedure relies heavily on the small size of plasmids and the fact that they normally exist in a supercoiled conformation in the host cells. The supercoiling is important because it prevents the DNA strands from separating too much during the denaturation step – only short regions of the DNA will be able to separate because the physical strain introduced on the rest of the DNA double helix will prevent further separation. The small size is important because it will help keep the plasmids soluble while other macromolecules are precipitating out.

We won’t go into the details of what each of the steps does here (please check the webpage linked in the pre-lab exercise), but the key steps to this protocol are the addition of Solution B (NaOH/SDS) and Solution C (Acetate solution). Solution B is responsible for the cell lysis and denaturation of proteins and DNA, while Solution C promotes renaturation and precipitation of insoluble molecules.

The denaturation step affects plasmid and genomic DNA in similar ways – both of them will have their DNA strands separate, but during renaturation (after Solution C is added) the small size of the plasmid allows complementary regions to more easily find each other and stay soluble in the solution (please see this video: https://youtu.be/-UASLIUU6gI). The genomic DNA, due to its much larger size, is unable to efficiently renature and so starts to clump together and becomes insoluble.

Determining DNA Concentration & Purity

There are a few different methods for determining the concentration of DNA in your sample. Some require a UV spectrophotomter and some just rely on you having a gel and a DNA sample whose concentration you already know. In general, if you have the necessary tools, use the spectrophotometric methods – they’re more accurate and allow you to get some idea of your sample’s purity.

One of the most common ways to determine the concentration of RNA or DNA samples is by the use of a UV spectrophotometer. Both RNA and DNA absorb UV light very efficiently making it possible to detect and quantify either. In practical terms, the Absorbance of a sample is measured at 260nm and 280nm, and the absorbance at 260 can be used to find the concentration using this formula:

50µg/ml x A260 of the sample = concentration of your sample (µg/ml)

This formula is based on knowledge that 50µg/ml of pure DNA has an OD of 1. Similarly, 40µg/ml of pure RNA has an OD of 1

Purity at A260/A280 ratio

The A260/A280 ratio is used to determine the purity of the isolated nucleic acids. This is because 260nm is maximally absorbed by nucleic acids (DNA as well as RNA), while light at 280nm is well absorbed by things like peptide bonds (ie. protein contamination) as well as Phenol and other reagents that are commonly used in DNA extraction protocols.

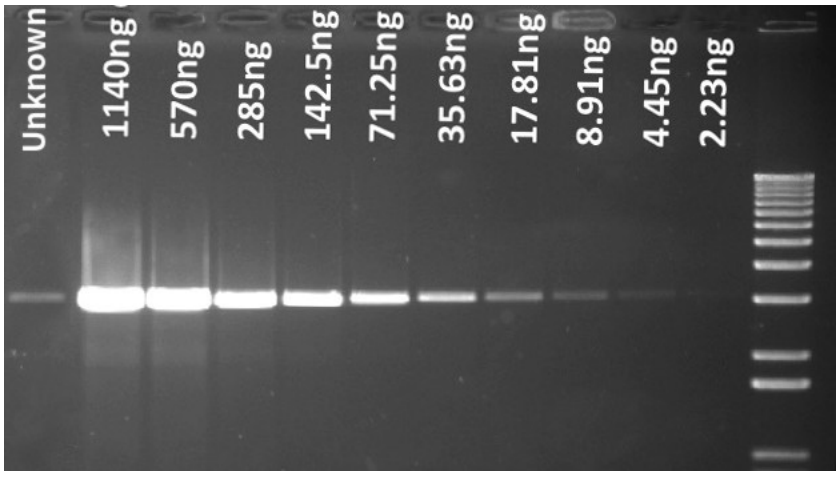
Because we're looking at the ratio of Nucleic Acids : Contaminants, the lower the A280 reading is, the higher the ratio, and the higher the purity the nucleic acids in your sample. Conversely, the more contaminants there are (high A280 reading), the lower the ratio will be. As a rule, a A260/A280 ratio in the range of 1.8-2.0 is considered pure. Usually pure DNA will give a ratio close to 1.8, while pure RNA will be closer to 2.0.

Purity at A260/A230 ratio

In some cases, researchers might also check the A260/A230 ratio. Using this ratio might be useful for those extracting from plants – carbohydrates (like cellulose) tend to absorb fairly strongly at 230nm. Also, a few extraction protocols use reagents (ie. TRIzol) which may be still present in the final sample and can be detected at 230nm. Knowing about such contaminants can be critical in cases where their presence could interfere with experiments you plan to do in the future (ie RT-PCR).

Gel Method

Alternatively, gel electrophoresis can be used to determine an approximate concentration of DNA in a sample. This is done by cutting the DNA with a restriction enzyme, running it on an agarose gel. The DNA is then visualized and the thickness/intensity of your sample can then be compared to a DNA standard (a sample of known concentration) as seen in the figure below.



While this method will tell you nothing about the purity of your sample, not all procedures require a DNA sample that is really clean. If your DNA sample will not be used for anything like that, running it out on a gel can be a simpler way of estimating your DNA sample concentration.

Exercises

In this lab, we will be using *E. coli* cells (a strain known as "TOP10"), which contain the pUC18 plasmid. The cultures you will be receiving were grown overnight at 37C and their growth was tested using spectrophotometry. Once the culture was determined to be at "mid-log" (OD600 = 1.0), it was harvested for this experiment.

Materials

- 3ml of *E. coli* cells (TOP10)
- Solution A: 50ml of 25mM Tris-HCl; 50mM Glucose; 10mM EDTA-Na2; pH8.0
- Solution B: 5ml of 0.2M NaOH; 1% SDS
- Solution C: 50ml of 5M Potassium Acetate/Acetic Acid
- Solution D: Phenol:Chloroform:Isoamyl Alcohol (25:24:1) - in the fumehood
- Solution E: 5ml of TE (10X): 100mM Tris-HCl ; pH: 8.0; 10mM EDTA-Na 2
- Solution F: TER: 1ml TE (1X) + 2µg RNase. - provided by lab tech
- 5ml of 100% Ethanol
- 5ml of 70% Ethanol
- micropipettors and tips
- sterile eppendorf tubes

1. Solution Preparation

1. Use the stock solutions prepared in the previous week to prepare solution A, B, C, and E.
2. As soon as you finish them, **place A, C and E on ice**, and leave B at room temp.

2. Alkaline Lysis Mini-prep

1. Add 1.5ml of culture to two eppendorf tubes
2. Centrifuge for 1min at maximum speed
3. For each tube, discard the supernatant, then invert the tubes over a paper towel and tap gently to remove any remaining drops of media
4. Repeat steps 1, 2 and 3 again to collect more cells (this will increase your yield)
5. Add 100µl of **ice-cold** Solution A to each tube and use a vortex to resuspend the pellets
    - The solution should become cloudy
6. Add 200µl of Solution B, and mix **by inverting** each tube quickly about 5 times and place the tubes on ice for 5min.
    - The solution should become clear
    - Do **NOT** vortex at this step! This is a very sensitive stage of the protocol. Vortexing here will break up the genomic DNA into small pieces and will cause it to contaminate your plasmid DNA.
7. Add 150µl of **ice-cold** Solution C and mix for 10sec using the vortex. Place on ice for 5min
    - You should see lots of white precipitate after vortexing – this is all the protein and genomic DNA along with the Potassium and SDS precipitating out of solution.
8. Centrifuge for 5min at maximum speed to pellet the precipitate.
9. Transfer 400µl of each supernatant into a new eppi tube.

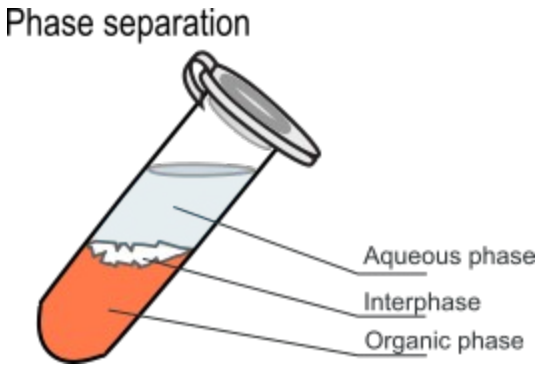
10. Add 400µl of Solution D, cap the tubes tightly, and shake them well for 1min

Caution:

- Solution D is corrosive, **please be very careful to not get it on your skin or spill it, and dispose of anything that touched it in a designated container in the fumehood.**

    - This step removes most proteins and some RNA

11. Centrifuge for 1min at maximum speed.
12. Take the tubes out of the centrifuge very carefully and keep them at the same angle at which they were in the rotor.
    - You should see something like the image on the right, but the difference in colour may not be as obvious.
    - Do not allow the layers to mix



13. Carefully transfer 350µl of the aqueous phase into fresh eppi tubes.
    - Use a P200 to do this because the tips are smaller and displace less liquid.
14. Centrifuge again for 1min at maximum speed. This step will help ensure that you separate out any of the organic phase that you may have transferred. You may see a small "bubble" of the organic phase at the bottom of the tube.
15. Carefully transfer 300µl of the aqueous phase into fresh eppi tubes.
16. Add 600µl (2 volumes) of absolute ethanol.
17. Mix by slowly inverting, and incubate for 2min at room temperature
18. Centrifuge for 5min at maximum speed, and discard the supernatant
19. Wash the pellet with 1ml 70% ethanol. Centrifuge for 1min and discard the supernatant
20. Invert the tubes and tap them on a paper towel to shake out as much of the ethanol as possible
21. Keep the eppendorf tubes open to **air dry the DNA** at room temperature for 10min to remove the remaining ethanol
    - this can be done in the fumehood with the sash nearly closed
    - You can check if the pellet is dry by checking if you can smell any ethanol.
22. While your DNA samples are drying, prepare 10ml of 1xTE from your 10x stock
23. Once the pellets are dry:
    1. add 50µl of Solution F to one of your tubes (tube 1) and incubate at 37C for 30min
    2. add 50µl of 1xTE to your second tube (tube 2)
24. Once done, these samples can be stored in the freezer at -20, or you can continue to exercise 3.

3. Determination of Concentration and Purity of DNA

Before storing your DNA samples (at -20C), you will determine their concentration and purity based on the information you were given in the introductory material to this lab. Because we will be using UV light to determine DNA concentration and purity, we will need to use special quartz cuvettes (plastic cuvettes absorb UV light) – please be sure handle the quartz cuvettes carefully in order to avoid introducing any scratches.

1. For each sample, prepare a 100-fold and a 1000-fold dilution with a total volume of 3ml
2. Using 1xTE as a blank, measure the absorbance of each of the diluted samples and enter the results I the table below
3. Calculate the DNA concentration (using the formula given in the background section) and the A260/A280 ratio to get determine the purity of your samples.

Data Table:

	Tube 1		DNA conc. (µg/ml)	A260/ A280	Tube 2		DNA conc. (µg/ml)	A260/ A280
	A260	A280			A260	A280		
1/100 dilution								
1/1000 dilution								

Post-Lab Questions:

Take a look at your results and answer the following in your notebook:

- What can you conclude about the purity of your samples (tube 1 and tube 2)?
- Did using solution F have much of an effect on the amount of nucleic acids you extracted? Did it make a big difference in terms of the purity values?
- There is a 10-fold difference in dilution factor between the two dilutions (1/100 and 1/1000) you performed for each sample. Is there also a 10-fold difference between the concentrations you've calculated? What does that tell you about the precision of your measurements?
- While the plasmid concentration you calculated for the table is in µg/ml, it can be more useful to express the concentration in µg/µl or ng/µl. Please try to make that conversion in your notes. What is the concentration of your plasmid samples in µg/µl?
- Write down the labels you put on your samples (this will help you identify them next week.

Lab 3: Restriction Digests

In this lab, we will use restriction enzymes to cut the plasmid DNA you purified last week. While the enzymatic reaction is taking place, we will discuss how such digests can be used to learn more about the DNA molecule you are studying through the process of restriction mapping. We will then separate DNA fragments by electrophoresis on an agarose gel in order to visualize it.

Learning Objectives:

Students will:

- Explain how restriction enzymes are used in molecular cloning
- Explain the importance of a recognition sequence in DNA
- Explain how gel electrophoresis works to separate DNA fragments
- Use molecular standards to determine the sizes of unknown DNA fragments

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Pre-Lab Questions:

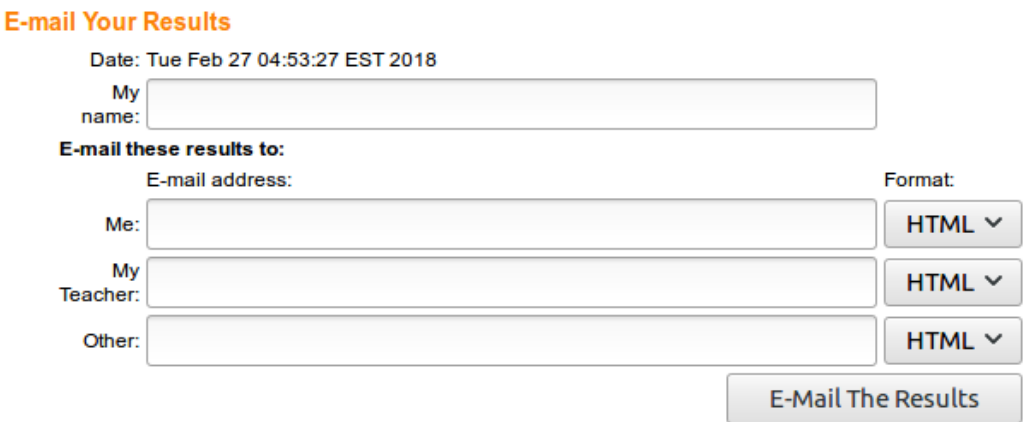
Complete the calculations and fill in the table on p. 39.

View the following video:

- https://www.youtube.com/watch?v=nfC689ElUVk
    - Near the end of the video, the presenter suggests a way to determine which plasmid contains the gene of interest. What is her suggestion? Why would it work? Under what conditions would it not work?

View the following video:

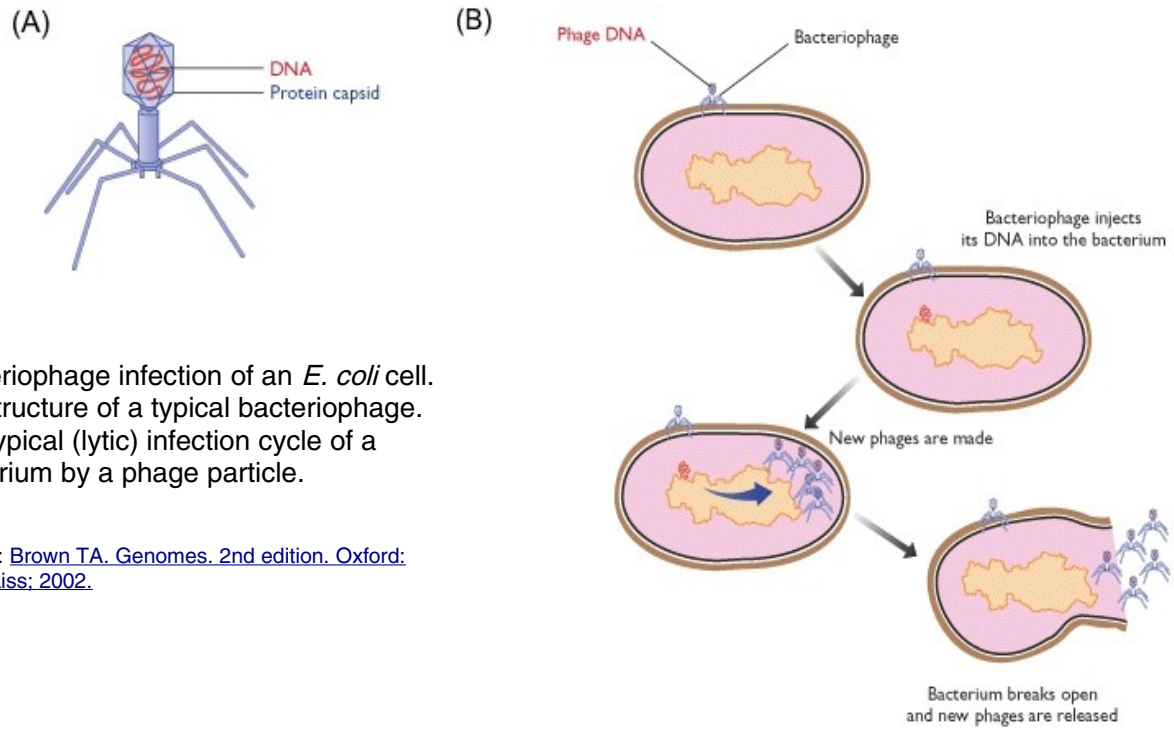
- https://youtu.be/pMKhOCi7X8w
- and complete the quiz here: Restriction Endonucleases
    - Fill out the form under the completed quiz to send your results to your TA



The Restriction Modification System

Like us, bacteria have to deal with viruses, and like us, they have developed a defense system that protects them from infection. A virus is a small infectious agent which can only replicate inside living cells. They do this by using their host cell's DNA/RNA and protein synthesis machinery to make copies of themselves. Viruses which infect bacteria are referred to as bacteriophages ("phages" for short).

A bacteriophage consists of an outer, protein shell, differentiated into a head and tail with "legs" for attaching to the bacterium, and an inner DNA core. When the bacteriophage binds to the bacterium, the phage DNA is injected into the bacterial cell. After a short period of time, the phage DNA takes over the metabolic mechanisms and resources of the cell in order to make and assemble new phage. The bacterium is then lysed and new phage are released.



Bacteriophage infection of an *E. coli* cell.
(A) Structure of a typical bacteriophage.
(B) Typical (lytic) infection cycle of a bacterium by a phage particle.

Source: Brown TA. Genomes. 2nd edition. Oxford: Wiley-Liss; 2002.

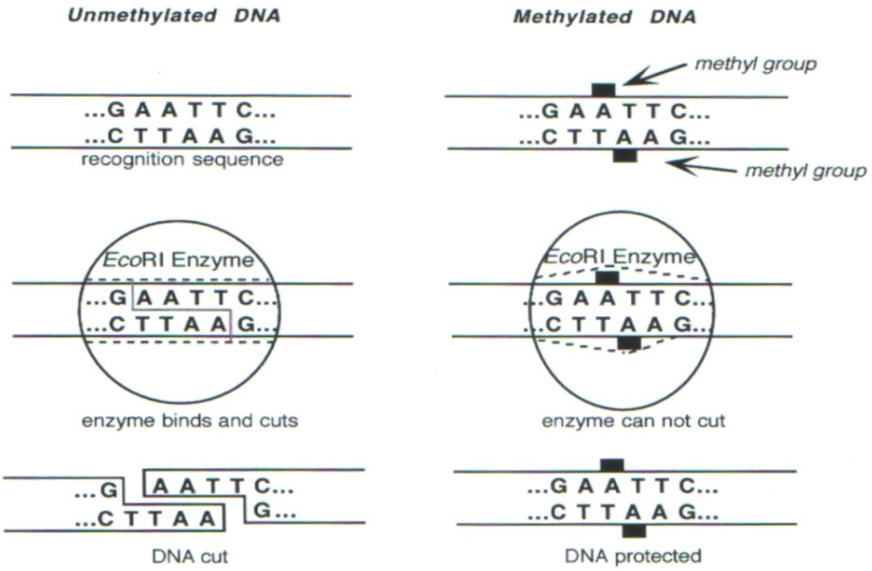
In response to this threat, bacteria have evolved the **Restriction Modification System** which is used to identify and destroy foreign DNA inside the bacterial cell (ie. DNA injected by a bacteriophage) before it can be used to take over the cell's replication machinery. This system uses enzymes which cut DNA at specific sequences – these are called **Restriction Endonucleases**.

Restriction Enzymes

Each restriction endonuclease cuts (restricts) viral DNA at specific sites. The endonuclease recognizes a sequence of four to six base pairs within the viral DNA molecule and, wherever that sequence occurs, cleaves the polynucleotide chain at that site. The enzyme binds at or near the recognition site and hydrolyzes the phosphodiester bonds of the DNA double helix . After cleavage, the two exposed ends each have a phosphate group at the 5' end and a hydroxyl group at the 3' end of the DNA chain.

Restriction endonucleases are potent enzymes for protecting bacteria from virus attack, but what prevents these enzymes from destroying the bacterium's own chromosomal and plasmid DNA?

For each restriction endonuclease produced by the bacterium, a companion enzyme called a **methylase** is also produced. Methylase adds a methyl group to the bacterial DNA sequences which would normally be recognized by the endonuclease. The methyl group prevents binding of the endonuclease to recognition sites on bacterial DNA, and thus the bacterial plasmid and chromosome are protected from restriction, but DNA that wasn't methylated (ie. foreign DNA) is not protected.



The EcoRI restriction-modification system. Left: binding of the EcoRI enzyme to the recognition site and subsequent cutting of DNA. Right: the methyl groups added to the recognition site by EcoRI methylase prevent proper binding of EcoRI and protects the DNA from cutting.

Source: Riggs C.D. (2004). BGYB12: Cell and Molecular Laboratory Manual . University of Toronto at Scarborough

The table below shows several examples of restriction endonucleases isolated from different bacteria as well as their DNA recognition sequences.

Species (strain)	Abbreviation	Recognition Sequence
Bacillus amyloliquefaciens H	**BamHI**	5' --- **G** GATCC --- 3' 3' --- **CCTAG** G --- 5'
Escherichia coli RY13	**EcoRI**	5' --- **G** AATTC --- 3' 3' --- **CTTAA** G --- 5'
Haemophilus aegyptius	**HaeII**	5' --- **PuGCGC** Py --- 3' 3' --- **Py** CGCGPu --- 5'
Haemophilus aegyptius	**HaeIII**	5' --- **GG** CC --- 3' 3' --- **CC** GG --- 5'
Haemophilus influenzae Rd	**HinDII**	5' --- **GTPy** puAC --- 3' 3' --- **CAPu** PyTG --- 5'
Haemophilus influenzae Rd	**HinDIII**	5' --- **A** AGCTT --- 3' 3' --- **TTCGA** A --- 5'
Providencia stuartii 164	**PstI**	5' --- **CTGCA** G --- 3' 3' --- **G** ACGTC --- 5'

C = cytosine, G = guanine, T = thymine, A = adenine, Py = any pyrimidine base (C/T), Pu = any purine base (A/G).

Modified from Micklos and Freyer, 1990.

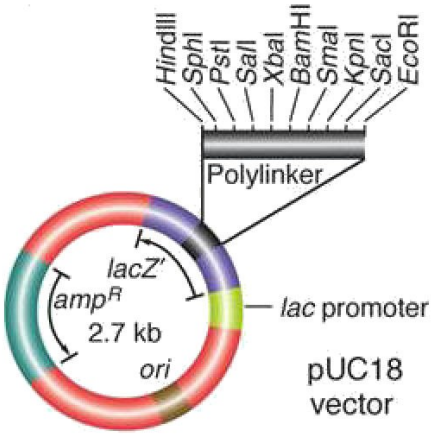
As you can see, some, such as HinDII and HaeIII cut straight across the DNA molecule to produce "blunt" ends, while others, such as HinDIII, make staggered cuts called "sticky" ends. The table also illustrates naming conventions for endonucleases. The first letter of the name comes from the genus of the bacterium, the second and third letters from the species name, the fourth letter, if present, from the strain designation, and the Roman numeral from the position of this enzyme in the list of discovered enzymes for this species.

So, the name HinDIII (pronounced "hin-dee-three") includes “H' for Haemophilus, “in” for influenzae, “D” for strain D, and “III” to indicate that this was the third endonuclease discovered for this species.

Although restriction endonucleases presumably evolved to protect bacteria from viruses, these enzymes will cleave double-stranded DNA from any source (assuming it's not methylated at the restriction sites). The endonucleases that produce "sticky" ends are particularly useful, because the overhanging ends they produce will undergo complementary base pairing with other fragments produced by the same enzyme, regardless of the source of the DNA.

For example, we can use a particular restriction endonuclease, like EcoRI, to cut up a piece of DNA from a mouse into fragments (called restriction fragments). These fragments can then be combined with a DNA vector (ie. a plasmid) to produce a recombinant DNA molecule.

Most plasmids used in labs have been engineered to ensure they have numerous different endonuclease recognition sequences (restriction sites). This allows us to insert foreign DNA cut with a variety of restriction enzymes. A representation of a plasmid molecule named pUC18 is shown below. This plasmid has several useful features, which you have likely learned about in lecture. In this case, we are interested in the region labeled as the “Polylinker” (also known as a Multiple Cloning Site - MCS).



Organization of the pUC18 plasmid with a general map of some of the more commonly-used restriction sites in the Multiple Cloning Site (polylinker).

Source: www.mun.ca

In the above example, the mouse DNA fragments could be inserted into the pUC18 vector by also restricting the vector DNA with the EcoRI enzyme, thus producing compatible ends. The “sticky” ends of the mouse DNA will be able to hydrogen bond with the “sticky” ends of the vector creating a “vector-insert hybrid” (recombinant vector) which can be ligated together using the **ligase** enzyme.

Buffers for Restriction Enzyme Digests

Setting up enzymatic reactions requires ensuring good conditions for the reaction to take place. This is accomplished through the use of reaction buffers. These buffers usually contain specific ions like Mg^{2+} (in the form of $MgCl_2$) or Ca^{2+} (in the form of $CaCl_2$) – this is because divalent cations are required by many enzymes as co-factors. The solutions are often buffered by Tris at pHs ranging from 7.5 to 8.5 and tend to also contain small amounts of NaCl.

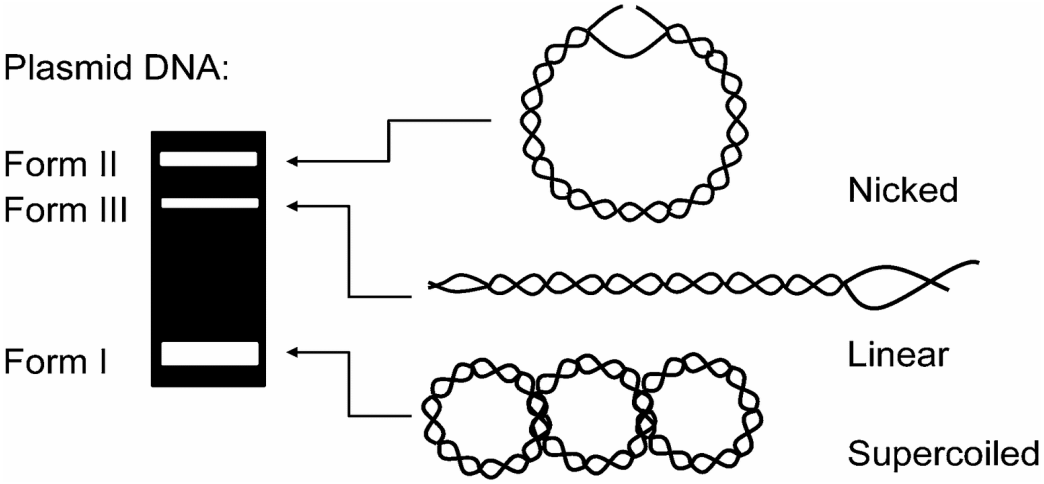
The buffer is usually provided separately for each enzyme and is often optimized for that enzyme, but sometimes we need to set up double or even triple digests (using two or three different endonucleases in the same reaction tube). In those cases, you may need to select a buffer that will allow all the enzymes to work relatively well (even if not optimally). Manufacturers often publish tables which indicate which buffers would work best for different enzymes.

Plasmid Conformations

Since we will be digesting a circular plasmid, it may be helpful to understand the forms that a plasmid DNA may take. Plasmids are small, circular DNA molecules, which tend to exist in a supercoiled conformation under normal circumstances. As described in the previous lab, supercoiling produces a lot of tension on the molecule and causes it to coil in on itself, resulting in a very compact appearance and higher density when compared with a circular (non-supercoiled) or linear molecule.

Plasmids are found in E. coli as supercoiled molecules, but during the purification process, “nicking” can occur. Nicking occurs when one strand of the double helix is cut while the other is left intact. Nicking can be caused by physical forces or be due to the action of nucleases (endonuclease activity), with the result being the relaxation of superhelical tension (for one end of the double helix is now free to rotate) and the formation of open circular DNA.

Sometimes, when two single-strand cuts are made near each other, or if a double-strand endonucleae is used, the supercoiled molecule goes to a linear form.



Plasmid conformations and their migration patterns on an agarose gel.

Source: Li & Grant (2016)

These differences in conformation are easily recognized after separation on an agarose gel.

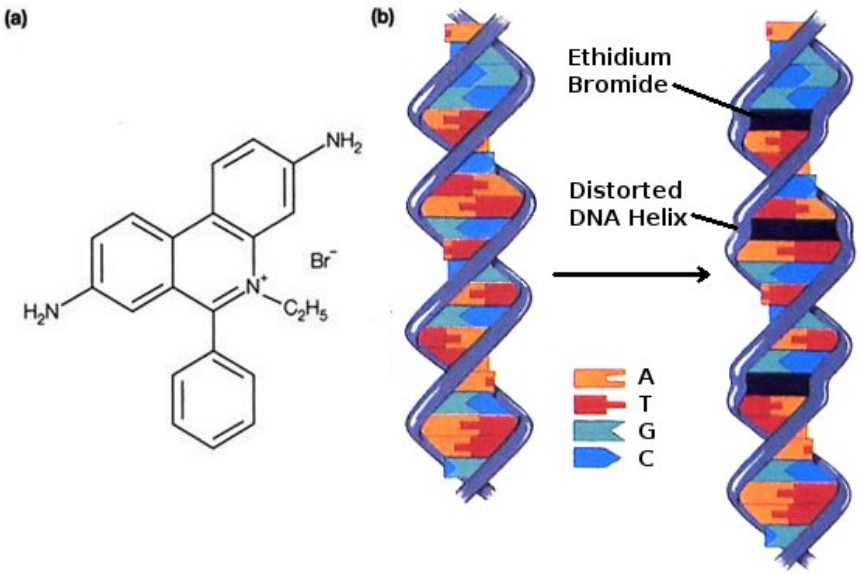
Agarose gels

The most common type of electrophoresis in molecular biology labs is agarose gel electrophoresis. Agarose is a derivative of agar; a long, neutral polysaccharide which is isolated from certain seaweeds. In the laboratory, agarose is dissolved in a buffer with the help of heating (it will not dissolve without heat). This is usually accomplished with the use of a microwave.

Ethidium bromide is often added to the agarose solution once it has cooled to approximately 50-60°C. Ethidium bromide is a small molecule that intercalates between the DNA base pairs and fluoresces orange upon excitation with ultraviolet (UV) light. Thus the addition of Ethidium Bromide (EtBr) to the gel will later allow easy visualization of DNA using UV light (DNA will glow orange against a black background).

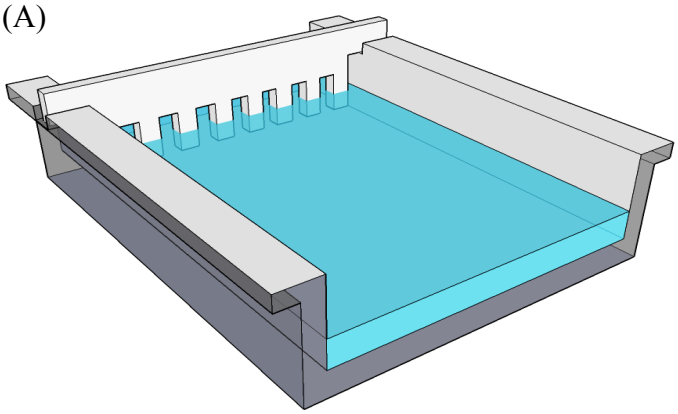
(a) Structure of ethidium bromide molecule. (b) Distortion of the DNA helix by the intercalation of ethidium bromide molecules.

Composite of images found at: sandwalk.blogspot.com and www.madsci.org

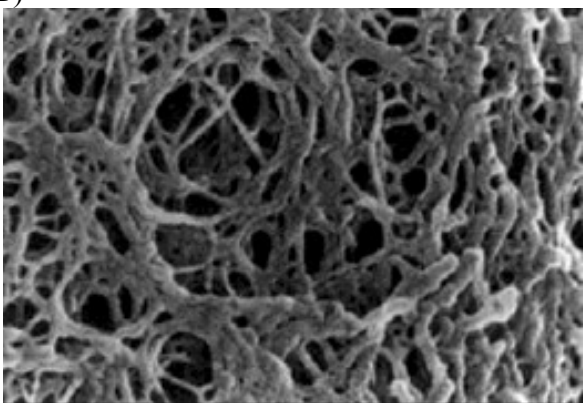


The hot solution is then poured into a plastic mold and a plastic "comb" is inserted into the solution in order to create "wells" in the gel once it is set. Once cooled, the gel forms a nearly transparent, solid matrix that has the consistency of gelatin. At a molecular level, the gel is now composed of many cross-linked agarose fibers which have small openings (pores) between them (see part B below). The size of these pores can be controlled by varying the concentration of the agarose in the gel solution.

(A)



(B)



(A) Schematic of an agarose gel poured into its mold with gel combs in place. (B) An electron micrograph of a gel matrix similar to what you might see in a solid agarose gel.

Source (a): commons.wikimedia.org Source (b): ocw.mit.edu

Gels of differing porosities can be made by adjusting the concentration of agarose – this will depend on the size of DNA you want to study on the gel. Resolution of small DNA fragments requires high percentage gels (>1%). With 2% agarose, double-stranded DNA's as small as 50 or 100 base pairs can be easily resolved (separated away from each other). Large DNA fragments separate best on low percentage gels (0.7-1%).

Resolution of Linear DNA on Agarose Gels

Agarose Gel % (w/v)	DNA size resolution
0.5	1kb – 30kb
0.7	800bp – 12kb
1.0	500bp – 10kb
1.2	400bp – 7kb
1.5	200bp – 3kb
2.0	50bp – 2kb

Souce: Promega Corporation

After the gel has set, the comb is removed and the gel is placed into an electrophoresis tank which is flooded with a weak salt solution. This solution will allow a current to flow when a voltage is applied. DNA samples can now be pipetted into the wells (small openings), which were formed when the comb was removed from the gel.

Since DNA has a negatively charged sugar-phosphate backbone, it will be attracted to the cathode (positive electrode) once an electrical current is applied to the gel. Large DNA molecules will travel slowly in the gel because they are impeded by the gel matrix. Smaller DNA molecules are able to pass through the pores in the gel matrix more easily due to their size, and therefore travel more quickly.

Because the size of a DNA fragment determines how fast it travels through the gel matrix, the smaller the DNA molecule, the farther it will travel over a period of time. We can thus use this migration information to help us determine the sizes of the DNA molecules on the gel. We do this by comparing the migration of DNA in our samples to the migration distances of a sample of DNA fragments of known sizes – a DNA standard, also known as a Molecular Marker.

By plotting the DNA fragment size of the molecular marker bands against distance migrated by the fragments on a semi-log plot, you should be able to obtain a graph with a fairly long linear section which will allow you to accurately determine the size of any other DNA fragment on that gel (assuming it falls within the linear range of your graph). Semi-log graph paper looks a little different from a regular grid – ask your TA about the proper way to use it (hint: using scientific notation can be very helpful here).

After you run your gel, you will visualize the DNA by viewing your gel on a UV light box. Any DNA on the gel will show up as a bright band on a dark background due to the ethidium bromide that has been "picked up" by the DNA as it migrated through the gel matrix.

Caution:

- **Ethidium bromide is a mutagen and probably a carcinogen**. Your TA will handle the chemical but you are required to wear gloves when handling the gel. The gel must be disposed of in the bag marked "ethidium bromide waste".
- **The UV light box emits light which could cause damage to the retina of the eye**. Make sure the plastic cover (plastic blocks UV light) is closed over the light source before turning on the lamp, or make sure that you always wear a full face shield when viewing a gel on a UV light box.

Exercises

In the first exercise, you will be cutting your plasmid DNA with different restriction enzymes. In the second exercise, we will be using electrophoresis to help us to determine if the plasmid you purified is of the expected size.

Before you do the digests, however, you will need to calculate how much of each of your samples to use. You will want to load 1µg of DNA per well, so the first thing you should do, is determine the volumes you will need to use in order to load similar amounts of DNA into each reaction. **Please complete this part of the exercise before coming in to the lab.**

Determine how much (in µl) of each DNA sample you will need for the reactions. Use the table below to help you with the calculations.

	Amount required (µg)	Conc. of sample (µg/µl)	Volume needed (µl)
Plasmid 1 ("TE")	1		
Plasmid 2 ("TER")	1		

- **Conc. of sample** – the concentration you determined **from the last lab**. Please fill this in based on your results.
- **Volume needed** – Calculated by taking the amount required and dividing it by the sample concentration

1. Setting up a Restriction Digest

Once you have your calculations and have checked them with your lab partners, please set up your reactions as indicated in the table below. Each group will be given a different enzyme and buffer. Please make a note of which one you are using.

Materials

- Your DNA samples
- SmaI (S) and 10X SmaI Buffer (SB)
- BamHI (B) and 10X BamHI Buffer (BB)
- Sterile water (DIW)
- Ice bucket with ice
- Eppendorf tubes
- Small beaker
- Micropipettors and tips
- Microfuge
- 37°C Waterbath
- 65°C Waterbath

Important:

Once thawed, **place your DNA samples on ice**. Keeping your samples cold ensures that any DNases that might be present in the samples are not able to degrade your DNA. Allowing your samples to remain at room temperature for prolonged periods of time will increase the chances that the DNA, that you worked so hard to purify in the previous lab, will be destroyed before you've had a chance to do anything useful with it.

Procedure:

1. Add some **cold** tap-water to a beaker and place your DNA samples into it to thaw. The samples will likely be defrosted in less than 1min using this method.

2. Obtain four epitubes, label them, and place them on the ice.

3. Use the volumes you calculated above to fill out the amounts of plasmid DNA to add in each of the tubes in the table below. Then determine how much water (DIW) you will need to use to obtain a total volume of 20µl in each reaction tube.

    - The volume of water you will use will depend on the volume of DNA you will be adding and on whether the tube will be receiving an endonuclease (our negative controls will not get endonucleases).

	Plasmid 1		Plasmid 2	
Tube/Label	p1-E	p1-Neg	p2-E	p2-Neg
DIW				
Plasmid				
BSA	2µl	2µl	2µl	2µl
10X Buffer	2µl	2µl	2µl	2µl
Enzyme	2µl	-	2µl	-
Total Vol.	20µl	20µl	20µl	20µl

4. Put the above components into each tube, making sure to keep them on ice as you do this.

    - Be sure to add each component to the tubes in the order indicated in the table. In any reaction, the enzyme should always be the last component added – the reaction begins as soon as it's added.

5. Spin down your samples for 30sec at maximum speed. This will collect all of the components of the reaction at the bottom of the tube.

6. Place your tubes at 37°C for 10min.

7. After 10min, place your tubes into the 65°C water bath for 10min to inactivate the enzymes.

2. Agarose Gel Electrophoresis

Materials

- Your digested DNA samples
- Ice bucket with ice
- 0.81% Agarose gel
- Electrophoresis apparatus
- 5X Gel Loading Dye (LD)
- Eppendorf tubes
- Micropipettors and tips
- Microfuge

Procedure

1. Remove your samples from the waterbath and immediately put them on ice for 1min.
2. Wipe the water from the tubes and place them in a microfuge. Remember to balance your tubes.
3. Spin down your samples for 30sec at maximum speed.
    - After incubating at 65°C, there will be some condensation along the sides of your tubes. A brief spin in a microfuge will collect all that condensation back at the bottom of the eppitube.
4. Add 5ul of loading dye (LD) to each tube – your samples and the standards. The total volume in each tube will now be 25µl.

Loading Dye

The loading dye actually has several functions. As a result, it's not just composed of a dye, but also a few other components.

One of the main components is a dye mixture – usually Bromophenol Blue (BΦB) and another dye. These dyes migrate at different rates through a gel and allow us to get an idea of how far different DNA fragments have migrated without having to use UV light.

Another component is EDTA, which ensures that any enzymatic reactions are stopped before the DNA samples are loaded on a gel.

Lastly, the loading dye also contains a fairly high concentration of glycerol or sucrose. This makes the dye (and also your sample) denser than the electrophoresis buffer and allows it to sink to the bottom of the well on a gel.

5. Spin down your samples for 30sec at maximum speed if needed.
6. Load your samples on the gel as follows:

Lane	1	2	3	4	5
Tube	MM*	p1-E	p1-Neg	p2-E	p2-Neg

*MM – Molecular Marker – Your TA will load this

7. Once all the groups have loaded, set the voltage to 100 Volts, and run the gel for 20-30 min. Or until the loading dye (Bromophenol Blue) has sufficiently moved away from the sample loading wells. A picture will be posted for you on Blackboard.

Post-Lab Questions:

Take a look at your gel results as posted online – print a copy for your notebook. Use your notes about your loading pattern so assign the appropriate labels to your lanes, and determine the sizes of the molecular marker bands (please see the relevant "Special Topic" section below to help you with this).

Write a brief analysis of your gel results. In your analysis, be sure to answer the following:

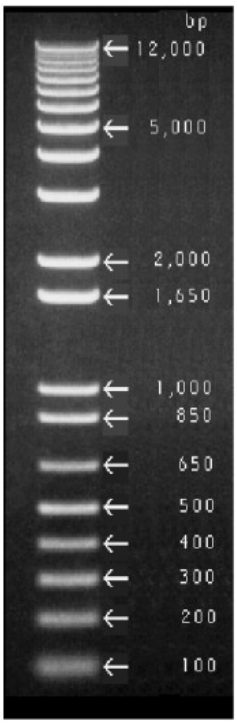
1. Are there any visible differences between P1-E and P2-E?
2. Based on what you did in the previous lab, is there a difference between the contents of the two plasmid samples?
3. Are there any differences between the digested and undigested plasmid samples? What could account for these differences?
4. In theory, you digested 1µg of your plasmid sample, but it was probably less because your samples were likely not pure. Please take a look at the DNA samples on p.26 of this manual and try to estimate the actual amount (in ng) that you loaded. Then divide that number by the volume of the plasmid sample that you added to the digest (ie. the "volume needed" on p.39). This should give you a more accurate concentration of your plasmid samples. You will need to know these numbers in the future, so write them down in your notes.

Special Topic: Determination of DNA Fragment Sizes

Any time a scientist does any electrophoresis, a molecular size standard ("molecular marker") is run along with the experimental samples. This is true regardless of what type of gel is being run (ie. DNA gel, protein gel or RNA gel). These molecular markers are used to help researchers determine the sizes of their molecules of interest by comparing the migration of their molecules through a gel to the migration of molecules in the "marker".

A molecular marker contains a mixture of DNA (or protein or RNA) molecules of known sizes. For DNA gels, these standards can be made by performing appropriate restriction enzyme digests on a DNA molecule with a known restriction map. For example Lambda DNA cut with HinDIII is commonly used as a DNA molecular marker. Molecular markers can also be purchased commercially from a manufacturer who will provide information about the sizes of all the DNA fragments in their DNA standard.

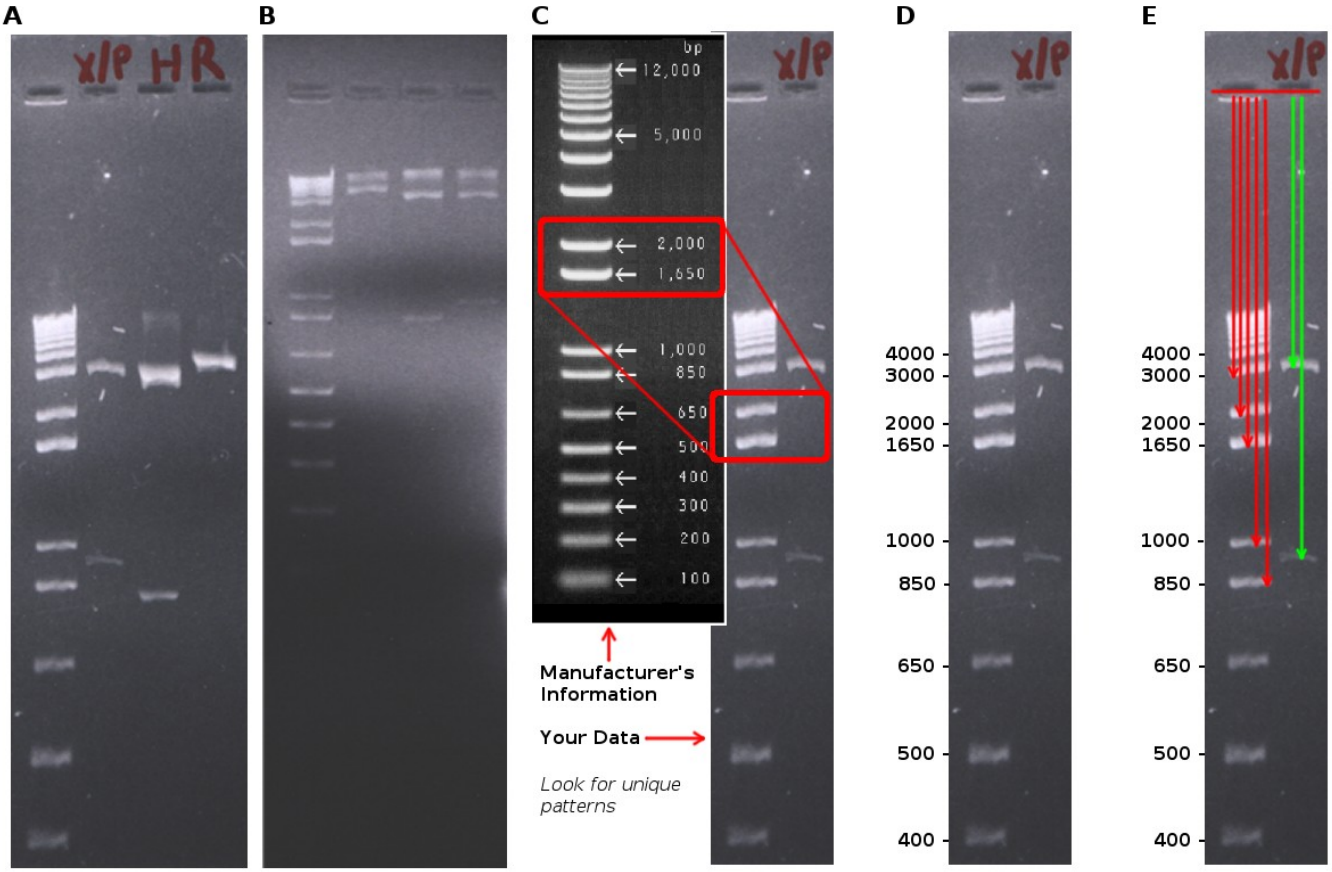
There is a great variety of molecular markers that are available, and they are selected based on the range of sizes of DNA fragments that they contain. A molecular marker like the "1kb Plus DNA Ladder" from a manufacturer called "Life Technologies" (formerly "Invitrogen") is commonly used because it can be used to accurately determine sizes of DNA fragments between 100bp and 12 000bp. The information provided by the manufacturer about this product is shown below.



1 Kb Plus DNA Ladder
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

It is important to understand how to use the information presented in this image – most students do not do it correctly. For various reasons, the molecular marker that is on your gel will look a little different than the one in the picture form the manufacturer (see part A and part B in the figure on the next page). When looking at molecular markers on your gels, you should note any distinctive patterns; all molecular markers have some distinctive feature that will help you assign sizes to the bands in the molecular marker on your gel.

For example, when you look at the figure on the left, notice that some bands are brighter than others, some are close together, some are far apart. One distinctive feature I would point out in the molecular marker in this image, is the pair of bands at 1.65kb and 2kb which form a very distinctive doublet. Try to identify them on the two example gels in the figure on the next page (A and B) – they both have the same molecular marker, but were run for different amounts of time. Identifying those two bands on your gels will help you to correctly assign sizes to the remaining molecular marker bands.

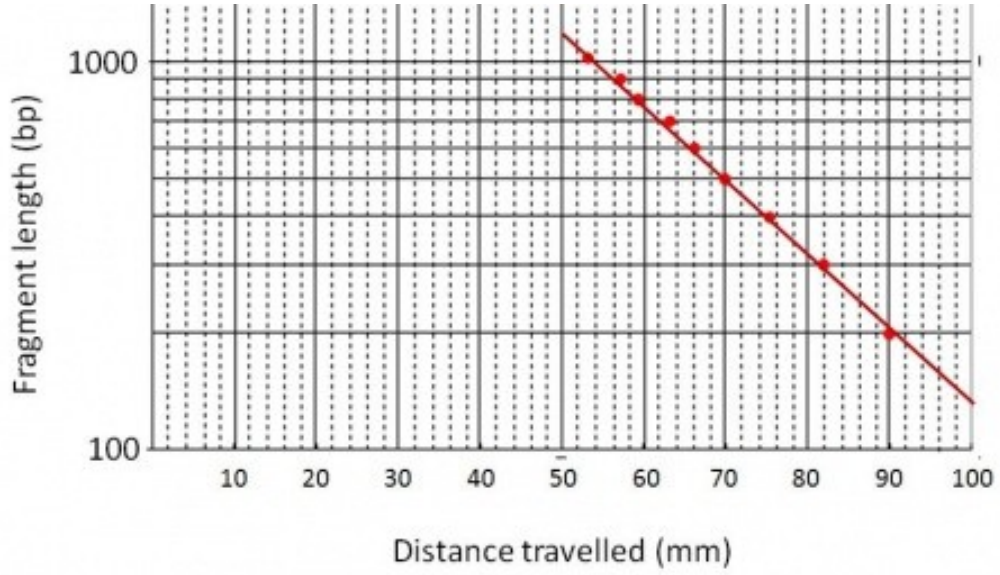


Assignment of sizes to molecular marker bands on gels in order to determine the sizes of the DNA bands in the experimental digests. (A) and (B) represent two DNA gels which were run for different amounts of time. The gel in (A) was run for a very long time and thus some of the small bands of the molecular marker ran off the bottom of the gel and are no longer visible. The gel in (B) was run for a very short time and the large DNA bands of the molecular marker have not been able to separate from each other. (C) A comparison of the picture of a molecular marker provided by the manufacturer with the same molecular marker seen on the experimental gel (corresponding bands are indicated by the red boxes). (D) The experimental gel with the sizes assigned to the molecular marker bands. Notice that not all of the marker bands that are visible on the manufacturer's picture in (C) are visible on this gel. (E) The determination of the sizes of the DNA bands from an XhoI/PstI digest. The red line across the top of the gel is used to help measure the migration distance of each band. The migration distance of each of the bands in the molecular marker is determined first (represented by the red arrows) and graphed. The graph is then used to determine the sizes of the bands in the second lane based on their migration distance (represented by the green arrows) from the red line.

A mistake often made by students when assigning sizes to the bands of their molecular markers is that they use the manufacturer's information to start assigning sizes to the bands on their gels from the bottom. This is a mistake, because not all of the small bands will be visible on a gel – some may have run off the gel before electrophoresis was stopped [(A) – 100, 200 and 300bp bands are missing] or may be too difficult to actually see on the gel [(B) – 100bp band is not visible and 200bp band is very faint].

For this reason, you should always search for a characteristic feature of your molecular marker as described on the previous page and assign the sizes based on that. Once you've assigned the sizes to the molecular marker on your gel, you can determine the size of any other DNA band on that gel.

You do this by drawing a straight line across the top of your gel (across the wells is preferable) as shown in part (E) in the above figure, and measuring the distance migrated by each of the bands of the molecular marker. You should then plot the distance migrated by the marker fragments versus their size on a semi-log graph paper.



Determination of the sizes of unknown DNA fragments by plotting the distance migrated by molecular standards on a gel in relation to their sizes on a semi-log graph paper.

Source: bcrc.bio.umass.edu

By measuring the migrated distance of the DNA bands in the experimental samples and correlating this to the plot of the standards, one can determine the length of the restriction fragments. The reason that semi-log paper must be used is that it allows us to draw this plot as a straight line. If you tried this on a regular grid, you would have to draw a curve – it is much more difficult to accurately intrapolate data from a curve.

Lab 4: PCR Amplification

Over the next few labs, we will be trying to produce a DNA fragment of interest (cry1A gene), clone it into a vector, and use that to transform some bacterial cells. We will then extract DNA from some transformed cells and attempt to identify cells that actually contain our DNA of interest.

This lab will focus on generating the initial DNA fragment through PCR.

Learning Objectives:

Students will:

- Explain the process of PCR (ie. what happens at each stage)
- Explain the requirement for a thermostable DNA Polymerase
- Determine Annealing temperature of their primers
- Discuss the importance of the different components of the PCR mixture

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Pre-Lab Questions:

Please watch the videos about PCR below and answer the following questions.

- https://youtu.be/matsiHSuoOw
- https://youtu.be/3XPAp6dgl14
    - What do you think would happen to primer binding to the template if the annealing temperature is too high?
    - How would PCR be affected if we have primers that have very different annealing temperatures?
    - Why do we add $MgCl_2$ to the reaction mix?

Polymerase Chain Reaction (PCR):

PCR, is a technique which allows the synthesis and massive amplification of specific regions of a DNA template. It uses the same basic DNA replication mechanism as a cell, but performs multiple DNA replication cycles in-vitro. In essence, it is a cell-free DNA replication system.

Dr. Kary Mullis (a chemist) is credited with developing the technique in 1983. While the use of a DNA polymerase to produce new DNA was not new, Dr. Mullis introduced the idea of using sequence-specific primers to ensure that the DNA synthesis would start and stop at specific points along a single strand of DNA, and the idea of performing numerous cycles of this DNA replication. This breakthrough idea allowed scientists to target specific DNA regions for amplification by the DNA polymerase.

Dr. Mullis and his colleagues were able to also improve upon the idea through the use of a thermostable DNA polymerase (isolated from a heat-loving bacterium – *Thermus aquaticus*) – *Taq* DNA Polymerase. This made PCR much easier.

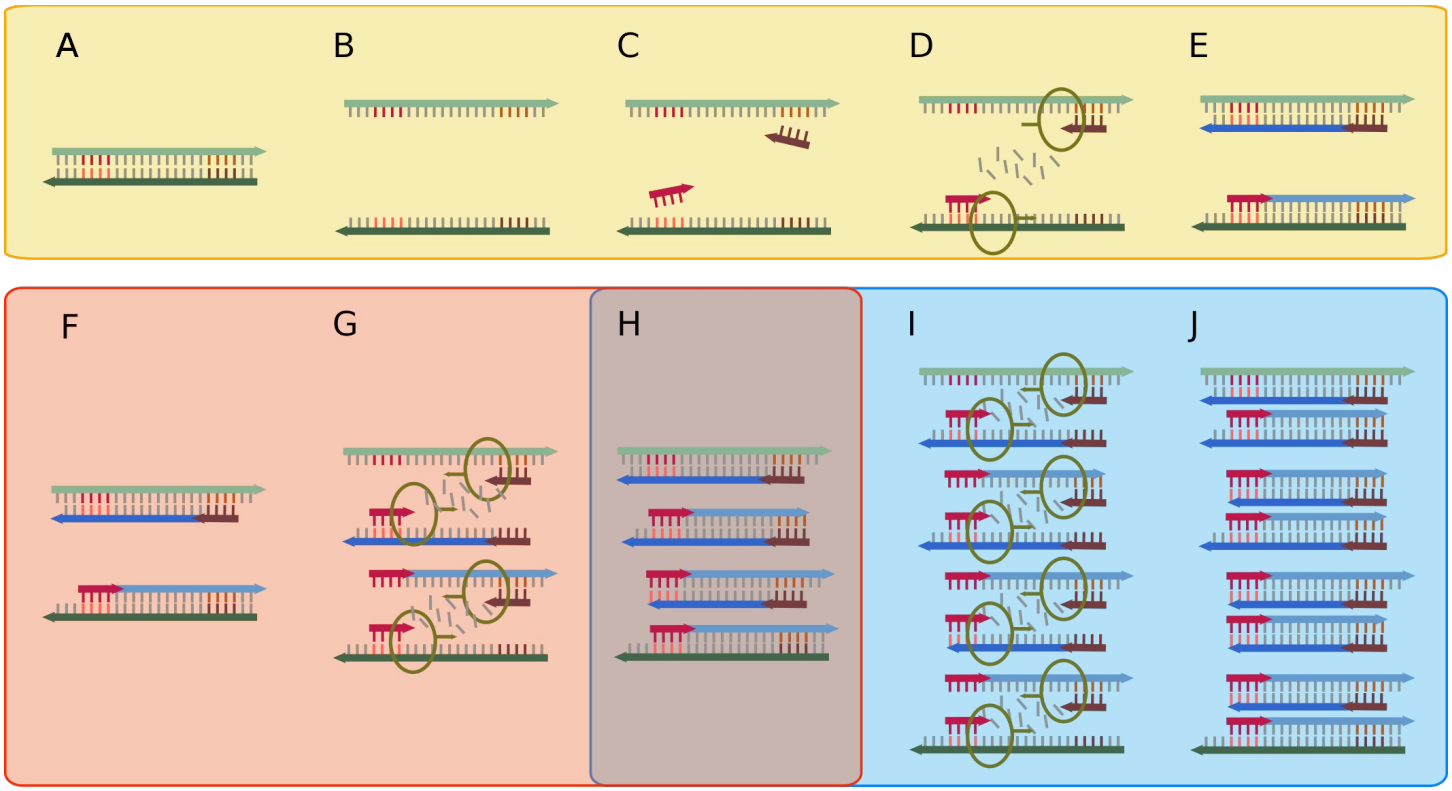

Figure 1. Three cycles of PCR. Cycle 1 (yellow) starts with a dsDNA template (A), which is **Denatured** (B) by heating to separate the strands. Primers are then allowed to **Anneal** (C) after which *Taq* Polymerase is able to bind to the DNA (D) and **Elongate** the strands to produce two copies of the gene of interest (E). These copies(F) are then used in the Cycle 2 (orange), where they go through the same set of steps [only Elongation (G) is shown here] to produce 4 copies of the gene of interest (H), which then go directly into Cycle 3 (blue) to produce 8 copies (J).

Source: this figure was modified from: https://commons.wikimedia.org/wiki/File:PCR-Schema-v2.svg

This is because, in order for PCR primers to bind to the DNA template, the double-stranded DNA must first be denatured into individual strands by using high temperatures. Prior to the use of *Taq* Polymerase, scientists would have to sit patiently next to a few different water baths and add fresh DNA polymerase after each DNA denaturation step. The introduction of the *Taq* DNA polymerase allowed for the automation of the whole procedure.

Taq DNA polymerase can withstand repeated DNA denaturation (heating to 94°C). Once the template DNA is denatured, PCR primers are able to bind to the template (at a lower temperature), and then the *Taq* polymerase can elongate (at 72°C – its optimum temperature) the new DNA strand starting at the primers.

The proper binding of the primers is critical to the success of the reaction – especially the 3' end of the primers. If the annealing temperature is too low, the primers may bind non-specifically; if the temperature is too high, they may not be able to bind to the template at all.

Even though the *Taq* polymerase is in the reaction mixture from the start of the experiment, it can't produce a new DNA strand (elongation) until the 3' ends of the primers are attached to the template. This is why PCR primers are designed in pairs – it allows the computer programs to ensure that the T_m of the primers (and thus the annealing temperate) will be very similar.

Excercises

1. Amplification of Genes of Interest using PCR

The aim of this exercise is to amplify a DNA fragment [gene *cry1A*] encoding a protein [delta-endotoxin: Bioinsecticide] of *B. thuringiensis*. You will be setting up three different reactions: one will be a negative control without a template, one will be a negative control with DNA from a different organism (non-specific template), and the third will be with the *B. thuringiensis* DNA which was used to design the PCR primers. Doing these reactions should allow us to demonstrate that our primers are specific to their template.

Materials (per group):

- *B. thuringiensis* DNA (DNA1)
- non-specific DNA (DNA2)
- PCR Primers (Fwd) and (Rev)
- 5X *Taq* polymerase buffer
- *Taq* DNA polymerase
- sterile water
- micropippettes and tips
- Sterile PCR tubes
- dNTP mix
- 25mM $MgCl_2$
- 10X TE

Procedure:

1. Obtain three PCR tubes, label them, and place them in the ice bucket.

2. Put the following components into each tube, making sure to keep them cold.
    - Be sure to add them in the order indicated – ie. the enzyme should be added last.

	PCR Reaction Mixes		
Tube/Label	noDNA	DNA1	DNA2
DIW	2µl	-	-
gDNA	-	2µl	2µl
5X Buffer	5µl	5µl	5µl
dNTP Mixture	10µl	10µl	10µl
25mM $MgCl_2$	2µl	2µl	2µl
Forward Primer	2µl	2µl	2µl
Reverse Primer	2µl	2µl	2µl
Taq Polymerase	2µl	2µl	2µl
Total Vol.	25µl	25µl	25µl

3. Place the PCR tubes inside open eppitubes and place them in a microfuge.

4. Spin the contents of the tubes down to the bottom.

5. Place the PCR tubes onto a thermocycler and start the following program:

First cycle: 3 min at 94°C
Then,
30 cycles:
- 45s of denaturation at 94°C
- 45s of primer annealing at 47°C (the temperature is primer-specific)
- 90s of elongation at 72°C

Then,
- hold at 4°C (this will keep the samples at 4°C until the technician collects them)

Post-Lab Questions:

Why is the first denaturation step longer than the other denaturation steps?

Why is the binding of the 3' end of the primer to the template more important than the binding of the 5' end?

Once you've seen your PCR results, try to determine the size of the PCR fragment. Please use the graph method described in the last lab.

Lab 5: Bioinformatics

In this lab, we will be meeting in a computer lab and you will be performing some basic sequence analysis on a genomic DNA sequence that will be provided for you by the TA. The analysis you will be performing will simulate the sort of analysis you would have to do before doing the experiments in the next few labs of this course.

Learning Objectives:

Students will:

- Describe the basics of how a sequence similarity search works
- Explain the difference between the GenBank and RefSeq databases
- Perform a basic interpretation of a BLAST result
- Identify various pieces of useful information in a GenBank Entry
- Perform a virtual restriction digest
- Design PCR primers using online tools

Pre-Lab Questions:

Please read the following brief page and answer the following questions:

- http://www.bbc.co.uk/guides/z8yk87h
  - What is a database?
  - Why are databases useful?

Please watch the video on in the link below and answer the following questions:

- https://youtu.be/c-f1H07D_70
  - Why do we normally use computers to help us design PCR primers?
  - What is a GC clamp?
  - What is a primer dimer?
  - Once you've designed the primers, it's always a good idea to BLAST them against the target organism's genome. Why?
  - Why should you also BLAST your primers against the human genome?

Bioinformatics

Bioinformatics is the subdiscipline of Biology that applies a computer's ability to find patterns in large amounts of data to help Biologists do data analysis. Bioinformatics arose because of the need to compare and analyze an increasing number of DNA and protein sequences. This sort of information is very simple (sequences of A-s, T-s, C-s and G-s) but can be very laborious to deal with by a single person directly. Thus, with a constantly increasing amount of sequence information, scientists began generating computer programs that could take over the very tedious job of direct sequence analysis.

These programs are based on very simple but numerous sets of rules which allow them to search through millions of sequences in a very short time to find ones which match the criteria set by the researcher using them.

A Quick Review of the Relevant Molecular Biology

Bioinformatic analysis works because sequence information determines biological function – this is the essence of the Central Dogma of Molecular Biology. Recall that the Central Dogma states that DNA is transcribed to mRNA, mRNA is translated to protein, and proteins perform the biological functions of the cell.

Thus, knowledge of the DNA sequence of a gene can allow us to predict the RNA sequence and the protein sequence, and therefore the function of that gene. We can make these predictions because biologists have deciphered the genetic code.

Each amino acid of a protein is encoded by a triplet code where 3 nucleotides code for 1 amino acid. Because DNA uses 4 different nucleotides and there are 3 nucleotides in a codon, there are 64 combinations (4^3) of nucleotides.

These 64 different codons can be used to encode the 20 commonly used amino acids. Because there are so many possible codons and so few amino acids, there is degeneracy (redundancy) in the code.

One important consequence of this degeneracy is seen in the figure on the right. Many amino acids are encoded by very similar codons. In fact, in many cases, the third nucleotide can be any of the four possible nucleotides and the amino acid is still the same. Thus, a certain amount of variation in the third base is tolerable and will not affect the resulting protein or its function.

First base	Second base: U	Second base: C	Second base: A	Second base: G	Third base
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

The genetic code. The codons are written 5' to 3', as they appear in the mRNA.

Source: www.nature.com

Therefore, the knowledge of DNA sequence and of the genetic code enables us to determine the sequence of any protein encoded by that DNA.

Is this useful?

Yes it is. Proteins are linear polymers of amino acids, and it is the interactions between the functional groups of these amino acids that facilitates the folding of the protein into its final three dimensional structure.

Many years of research by numerous scientists has given us the ability to predict secondary and tertiary structural features from the amino acid sequence. We now have numerous simple rules for protein folding that have been turned into computer algorithms, which can be used to help us analyze our sequences. Thus, if you know the amino acid sequence, you can predict the 3D structures.

Scientists have also been able to correlate many of the secondary structures, and combinations of these secondary structures, with specific functions. These regions of a protein are known as domains, and have been identified in numerous different proteins.

Based on what you've just read, it is clear that the identification of such functional domains from only sequence information is possible. This is useful because a particular functional domain (ie. DNA-binding domain) may be found in a variety of proteins. Also, a single protein can have several different domains. Thus knowing which domains are likely to be found in a protein encoded by a particular sequence will help us make some predictions about the potential functions of our protein of interest.

Ultimately, we want to find out what a protein product of a gene does. So doing some bioinformatic analysis of a particular sequence can give you a lot of information about it. This will help you make up hypotheses about your sequence and will help to guide the direction of your research and the types of experiments you might want to perform on your actual proteins.

Important: Bioinformatic sequence analysis by itself is just a starting point for research. Any predictions made through such means **need** to be confirmed through actual experiments.

Sequence Analysis

There is a wide variety of bioinformatic tools. One of the most commonly used tools, and often one of the first to be used, is **BLAST** (Basic Local Alignment and Search Tool). This program takes your sequence and compares it to every other sequence present in the world-wide databases. It scans your sequence for similarity to the billions of sequences out there, and returns the sequences that are most similar to yours.

As it searches for similarities, it checks for conservative substitutions (ones that we know which substitutions are least likely to affect the function of a protein domain) and ranks the matches based on their overall similarity. A match in BLAST could help identify possible functions of your sequence, possible members of a gene family within an organism, or possible related genes in other species (orthologs). The BLAST algorithm does it's best to help scientists find matches with biological significance.

A simple online search will also uncover a wide variety of other online tools which will allow you to do some very basic DNA sequence analysis and manipulation, perform virtual restriction digests, find open reading frames, identify introns in genomic sequences, translate the DNA sequence into a protein sequence, identify regulatory elements in genes, design PCR primers, etc.

Similarly, there are numerous online software tools available for analysis of protein sequences. With these you can perform multiple sequence alignment, predict secondary structure, predict 3D

structure, find transmembrane domains and perform hydrophobicity plots, identify sites of post-translational modifications, etc.

All this from a simple DNA sequence!

Exercises

You will be provided with a genomic DNA sequence for analysis. Below, you will find some general instructions for this exercise. Your TA will walk you through the details of the software to use for your analysis.

1. Using BLAST to Identify a cDNA Sequence of Interest

Before the start of the lab, ask one person in your group to email your TA to request a sequence, then make sure everyone in your group gets it. You will be sent a genomic DNA sequence.

Procedure:

Use your browser to go to the NCBI BLAST tool. You can just search for it or click this link.

1. Paste your sequence into the BLAST text field, and perform a search to try to identify any known genes that are similar to yours. This may give you a clue as to the function of your gene of interest.
    - Note which part of the sequence has the similarity (write down where the similarity starts and ends).
2. Try to find a matching sequence that belongs to an mRNA (cDNA) and go to that GenBank entry. Try to select the best/longest possible sequence in the database.
    - Write down the **Accession Number** here: _____
    - How long is the DNA sequence? _____ bp
    - Name of protein/enzyme: _____
3. Look at the information in the database entry and try to find features of interest in the DNA sequence(ie. start and stop codon). Knowing this will be helpful in later experiments when you're trying to amplify the full coding region of your gene.
    - Location of START codon: _____
    - Location of STOP codon: _____
4. Try to find a GenBank entry for the genomic DNA of your gene as well. This will give you some idea of the structure of the gene (ie. number and locations of exons, etc)
    - How many exons does your gene have: _____
    - Does your sequence have more than one possible coding “variant”? ____
        - How do the variants differ? _____

          _____

          _____

2. Restriction Analysis of DNA Sequence

Use your browser to find a piece of software that can perform virtual restriction digests. A tool called "Webcutter" is a fairly good one, but there are others around as well. One version of "Webcutter" can be found here.

1. Enter the full length of your mRNA (technically it's a cDNA) sequence and try to identify any enzymes which will cut the sequence before the START codon as well as any that will cut after the STOP codon (ie. in the untranslated regions).
    - In Webcutter, you can specify an ORF (Open Reading Frame) based on the information you obtained from GenBank earlier – ie. START and STOP codon locations
2. Once you've identified these restriction sites:
    1. Check to make sure there **aren't** any of these sites within the coding region
    2. Check the MCS of pUC18 to see if any of the same restriction enzymes also cut the MCS.
        - The aim of this is to see if we will be able to use these enzymes to clone the target DNA into our plasmid vector.
3. Write down the names of these enzymes: _____
    - Ideally, they should be two different enzymes, this will allow us to use directional cloning.
        - Position of restriction site for first enzyme: _____
        - Position of restriction site for second enzyme: _____

3. Protein Sequence Analysis

Use your browser to find the ExPASy server – it contains a large selection of tools used for analysis of protein sequences (there are not many tool to do other things as well, but most people still initially go there for protein sequence analysis). You can access it directly from here.

1. Go back to your GenBank entry for your DNA sequence and find the protein sequence. Copy that sequence somewhere to make it easily accessible.
2. Go to the ExPASy website – there are a lot of tools there, so use the search box to help you find relevant ones.
3. Find out the pI and molecular weight of your protein: _____
4. Find a tool to help you identify secondary structures of your protein sequence.
5. Find out whether your protein has any trans-membrane domains.

Assignment 1

You will work on this as a group and submit only one hard copy.

This assignment involves you designing some PCR primers which would help you clone a cDNA of your gene of interest into the pUC18 vector. We will use some of the research you've started in the in-class exercise with your DNA sequence.

Once you have found the boundaries of your gene of interest (start and stop codons), you will design some PCR primers, which will bind to either side of the coding region – ie. they will bind to the untranslated regions (UTRs) of your cDNA sequence.

1. Go to the GenBank entry for your cDNA and look for a link that says "Pick Primers"
2. You want primers that will allow you to amplify the whole coding region.
    - Use the information in the database entry to help you figure out:
        - The length of your coding region – this will be the minimum size of your product. The "maximum" length will be the full length of the mRNA/cDNA
        - The beginning and end of the 5' UTR
        - The beginning and end of the 3' UTR
            - These will allow you to indicate where you want the software to look for primer sequences
    - Criteria for PCR primer design:
        - the primers should be between 16 and 21 nucleotides in length.
        - They should have a melting temperature between 55°C and 65°C
        - The primers are designed in pairs to make sure that their melting temperatures are similar (within 2°C of each other), and that they're are unlikely to anneal to each other.
        - The 3' end of each primer should ideally end in a G or a C, and should have at least 2 of the last 3 nucleotides as a C or a G. This is called a GC-clamp and it ensures that the 3' end binds very strongly to the template strand. This is something that is "nice to have" in your primers, but isn't absolutely necessary for this assignment.
        - Single-nucleotide stretches should be avoided (ie. you should not have "AAAAAA" or "GGGGG" in your primer).
    - A useful thing to know:
        - Will your primers be able to bind to human DNA?
3. Run your mRNA/cDNA sequence through WebCutter again, but this time identify any enzymes that **do not cut** your target DNA, and **do cut** the pUC18 plasmid in the MCS.
    - Pick two of those enzymes
    - Add a recognition sequence for one of the enzymes to the 5' end of one of the primers

- Add a recognition sequence for the other enzyme to the 5' end of the other primer
- Recalculate the T_m of your primer pair.

4. ~~Once you have found an appropriate set of primers, you will BLAST the primer sequences against the databases to see if it's possible for them to amplify any sequences besides the DNA sequence of interest.~~
    - ~~This is especially important for human DNA. This way, if you manage to contaminate the sample with your own DNA, you won't be amplifying unwanted genes.~~
    - NCBI's primer design software seems to now automatically check for other potential targets. It's also currently not possible to simply run a couple of primers through the regular BLAST search – they will be too short to generate any results in the search.

What to Submit:

1. A printout of the GenBank entry for your **cDNA**
    - highlight the 5' and 3' UTRs
    - indicate where your primers will bind in these regions
2. A list of enzymes that do not cut the cDNA
3. The sequences of your modified forward and reverse primers
    - highlight the added restriction sites
    - indicate which restriction enzymes would cut the sites
4. The new melting temperatures for each of the primers
5. Answer to the following question:
    - What could you do to lower the melting temperature of these modified primers?

Lab 6: DNA Ligation

In today's lab, we will be generating some recombinant DNA, by ligating our purified plasmids with the PCR product from Lab 3.

In order to set up the experiment, you will need to take a look at the gel data from the previous lab to see if you have sufficient DNA and if it was cut by the SmaI enzyme.

Learning Objectives:

Students will:

- Describe the function of DNA ligase
- Explain why using DNA with “sticky ends” is preferable to using “blunt ends”
- Explain why using directional cloning is better than cloning using a single restriction enzyme
- Describe some of the potential problems with directional cloning
- Explain the importance of temperature in a ligation reaction

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Pre-Lab Questions:

Take a look at the agarose gel pictures from the last lab to determine if your plasmid DNA samples are as concentrated as you expected (ie. the DNA bands are clearly visible on the gel), and that they were linearized by the restriction enzymes we used.

– If the gels show that the plasmid DNA was not cut, then you probably didn’t add enzyme, or you added too much.

– If the gels didn’t show any DNA bands, then you didn’t load enough DNA into your digests. You will need to make some adjustments to the digests in this lab.

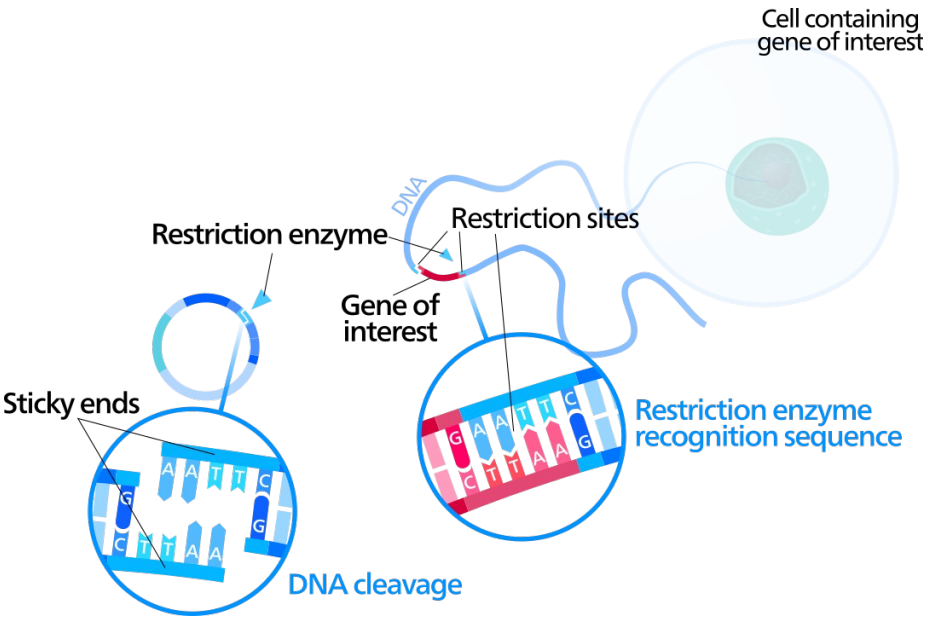
Read the introductory materials for this lab and answer the following questions:

- Why do vector dimers from directional cloning pose a problem in interpreting results after transformation?
  (it may help to think about what you will be looking for on a plate after the transformation)
- What is the melting temperature of a sticky end produced by an EcoRI enzyme?
  (you may want to think back to what you learned about PCR primers)

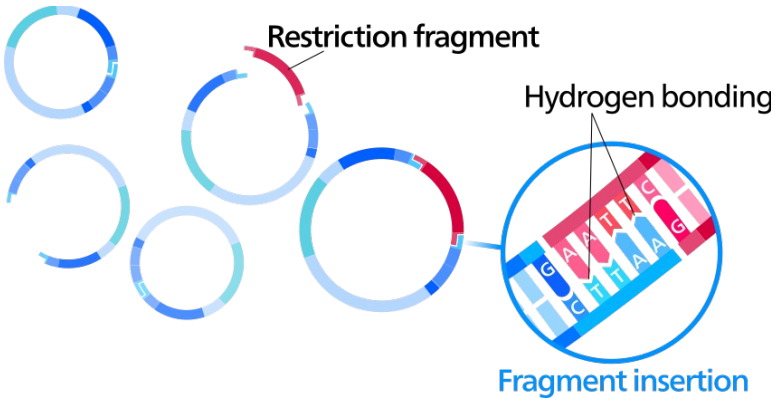
The Basics of Gene Cloning

Gene cloning refers to the insertion of foreign DNA into a DNA vector, which results in a recombinant DNA molecule (sometimes known as a chimeric molecule). This method is commonly used to help amplify, store, or express a gene of interest.

In practical terms, a gene of interest is first isolated from the original organism, this can be done by producing a DNA library from an organism and isolating the gene of interest from that library, or by amplifying the gene of interest by PCR (much more common these days). In either case, the target DNA is then prepared for insertion into a vector – this is usually done by digesting the DNA with a specific enzyme (or pair of enzymes).

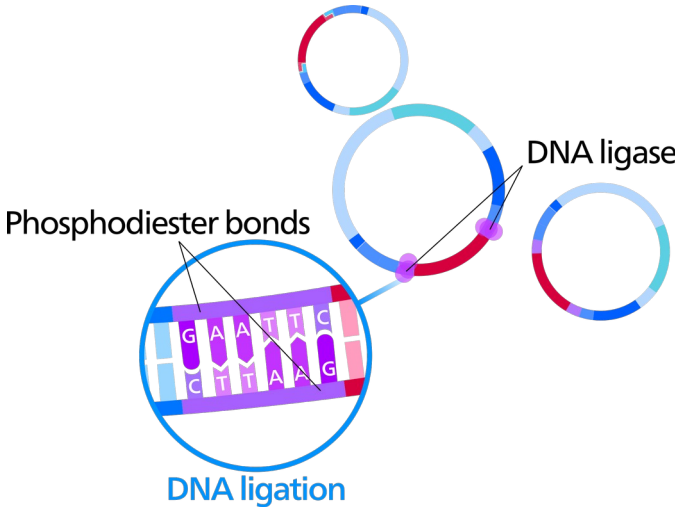


The vector DNA (ie. a plasmid) is cut with the same restriction enzyme(s) to generate free ends that are compatible (ie. can hydrogen bond) with the foreign DNA molecule. They are then placed into the same tube and allowed to bond to each other by having their "sticky ends" interact (assuming you used restriction enzymes that generate sticky ends).



The presence of sticky ends makes the whole process much more efficient, DNA ends produced by "blunt end cutters" are not able to make a stable connection with other DNA ends and have to rely on the random event of the two blunt ends being in just the right position when a DNA Ligase binds to them.

Once the insert and vector molecules have had their compatible ends hydrogen-bond with each other, a DNA Ligase enzyme can come along and seal the gap between the DNA backbones of the two molecules. DNA Ligases, like the one used in this lab (T4 DNA Ligase), use the 5'-Phosphate of one nucleotide, and the 3'-OH of another nucleotide to form a phosphodiester bond, thus forming a covalent (permanent) bond between two DNA molecules to form one recombinant molecule.



This molecule is then used to transform some host cells, which will be able to amplify the gene and could be used to produce a protein encoded by the DNA inserted into the vector.

Practical Aspects of Ligation Reactions

There are several factors which can affect the efficiency of a ligation reaction – most have to do with how the DNA molecules, that you wish to ligate, are prepared.

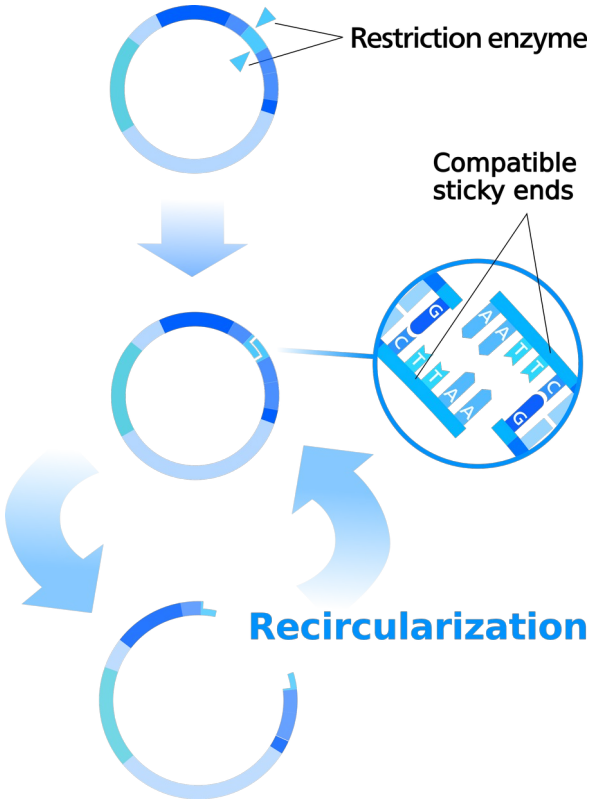
1. Vector and Insert prepared using a single restriction enzyme.

   - **Problem:** when the vector is opened using a single enzyme, it has self-compatible ends. These ends can easily self-anneal and recircularize the vector without taking up an insert molecule. The chances of this are very high because the two "free ends" are always close to each other – they're both on the same DNA molecule.

     This results in the production of many non-recombinant plasmids at the end of the ligation reaction.

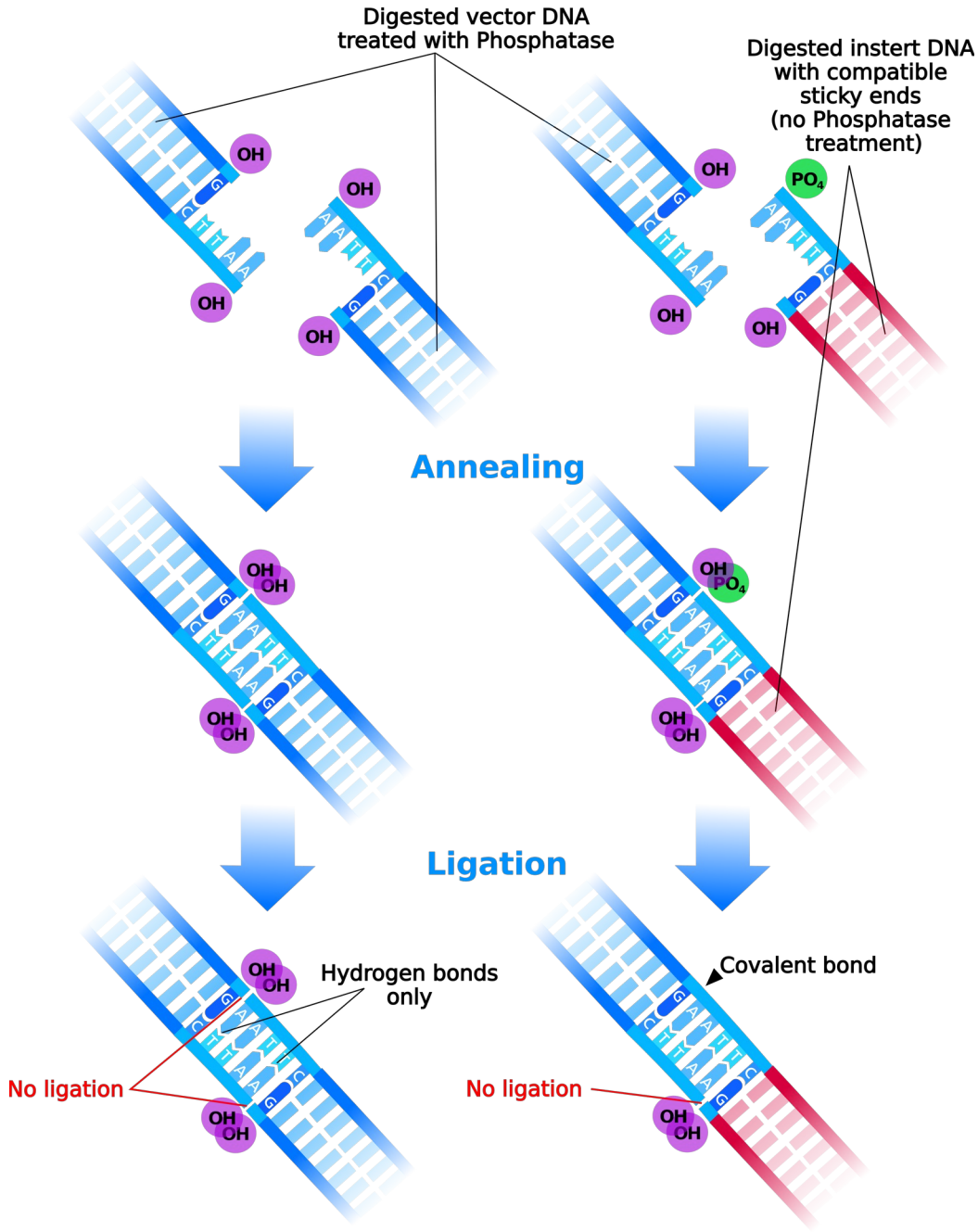
   - **Solution #1:** the cut vector DNA can be treated with an enzyme called a Phosphatase (Calf intestinal Phosphatase [CIP] or Shrimp Alkaline Phosphatase [SAP] are commonly used), which converts the 5' Phosphate groups to OH groups.

     This means that DNA ligase will not be able to make a phosphodiester bond – a 5' Phosphate group is necessary for that



reaction – and it increases the chances of producing a recombinant plasmid because the insert DNA would not be treated with CIP and would have 5' Phosphate groups.

Thus, when a vector combines with an insert, it will result in a ligation (a strong covalent bond), while the reannealing of the vector would only be stabilized by weak hydrogen bonds.



Please note: as can be seen from the above diagram, the ligation of a phosphatase-treated vector and insert produces DNA that is not completely sealed – it has a 'nick' in one of the strands. This 'nick' is later repaired by the the host cell which takes up this recombinant vector during transformation.

- **Solution #2:** cut the vector with two different restriction enzymes, do the same with the insert – this is called directional cloning. (see blow)

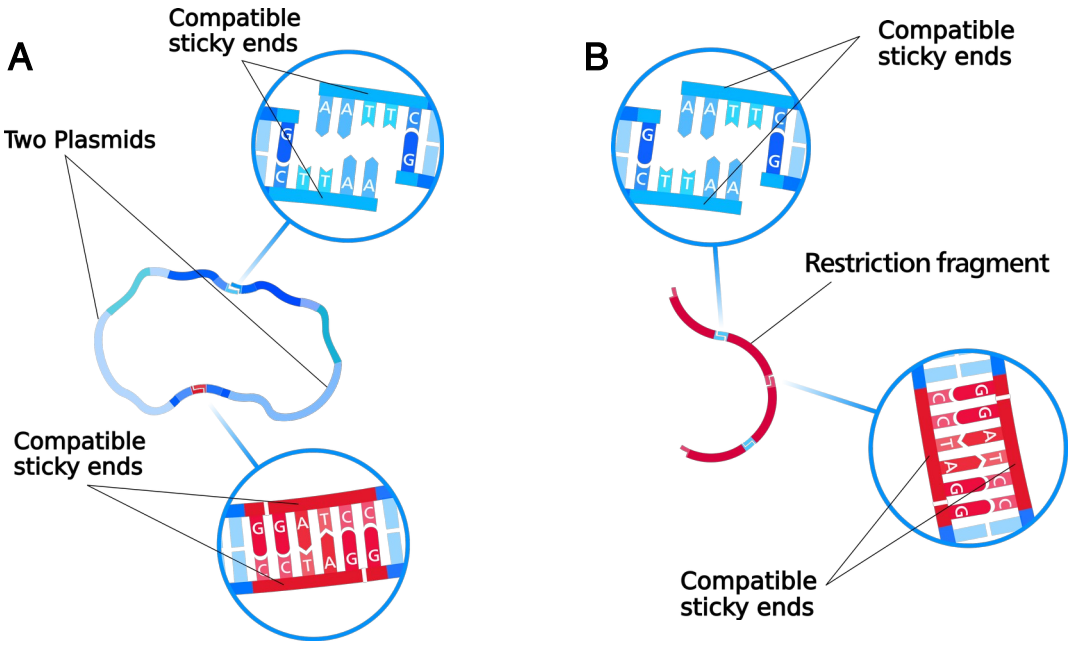
2. Vector and Insert prepared using a double digest.

By using two different restriction enzymes, we generate incompatible sticky ends so the vector will not be able self-anneal. Using the same restriction enzymes to cut the insert DNA as well, will generate sticky ends that will be compatible with the vector and **increase the chance** of annealing and ligation between a vector and insert.

Using this method greatly increases ligation of insert DNA into vector DNA, but it isn't perfect. There is still potential for some problems with this ligation.

- **Problem:** cutting vector DNA using two different enzymes will prevent the vector from **self**-annealing, but does not prevent it from binding to a second vector molecule to form a '**dimer**' (Diagram A). Similarly, insert DNA can also link to other insert DNA fragments using the compatible ends (see Diagram B below) to form what is known as a **concatemer** (a long continuous DNA molecule, which is composed of two or more copies of the same DNA sequence). This can happen in the case of a single digest as well.

  In this case, DNA ligase would form covalent bonds to seal the breaks formed by the restriction enzymes.

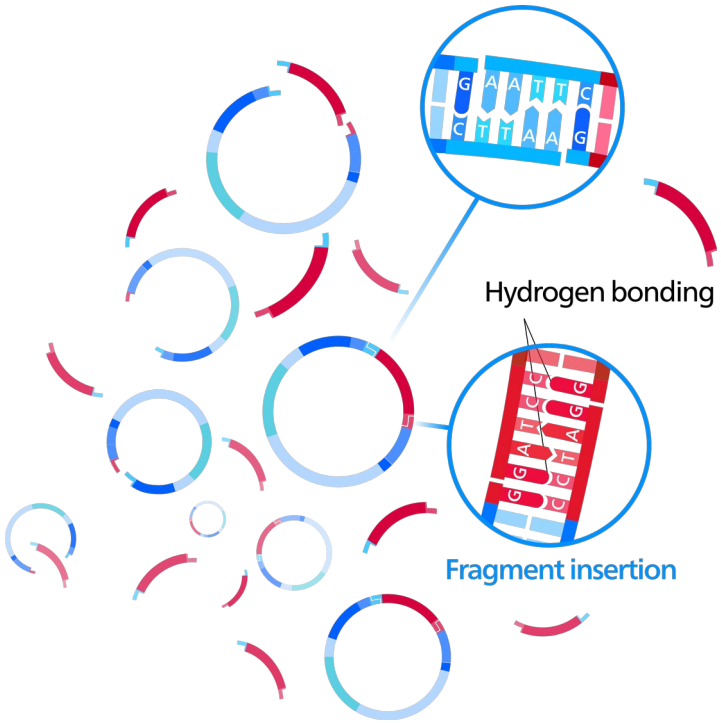


The linking of multiple copies of insert DNA isn't a really a big problem. It is not likely to cause any problems with data analysis, it will simply make fewer copies of the insert DNA for ligation into a vector. But the formation of vector dimers, could potentially cause some problems for data analysis.

  - *Can you think of any reasons why the formation of vector dimers could produce confusing results after transformation with such a molecule?*

- **Solution:** While it is possible to simply treat the vector molecule with CIP before adding the insert DNA and the DNA ligase, research has shown that there is a simpler solution. The molar ratio of vector DNA and insert DNA can be set in a way that increases the probability that an insert DNA will anneal to a vector molecule, instead of a vector molecule annealing to a vector molecule.

You can achieve this by simply ensuring that there are more insert DNA molecules in the ligation reaction than there are vector molecules. In fact, it seems that ensuring a ratio of 3:1 of moles of insert DNA to vector DNA seems to produce the optimal amount of recombinant DNA production.



All images in this section were modified from:
https://commons.wikimedia.org/wiki/File:Gene_cloning.svg

This ensures that there are three times as many free ends belonging to the insert DNA available, and thus they are three times more likely to anneal to a vector than the compatible end of another vector molecule.

- The amount of insert DNA needed for a reaction, in order to obtain the appropriate molar ratio can be calculated using the following formula:

$$\frac{insert\ amount\,(ng)}{insert\ length\,(bp)} = 3 \times \frac{vector\ amount\,(ng)}{vector\ length\,(bp)}$$

The vector amount is generally decided upon first, and so the formula can be rewritten to solve for the amount of insert:

$$insert\ amount\,(ng) = 3 \times \frac{vector\ amount\,(ng) \times insert\ length\,(bp)}{vector\ length\,(bp)}$$

3. Temperature of the ligation reaction.

- Ligations tend to be done at a fairly low temperature – generally around 15-16°C. At this temperature, a ligation reaction is generally allowed to continue for a few hours, or simply left overnight. The length of the reaction can be made shorter (~20-30min) by performing the reaction at room temperature (~21°C).

In this case, the recommended reaction temperature has nothing to do with the optimal temperature for the enzyme – the T4 Ligase, which is commonly used in labs, works optimally at 37°C. The reason for the lower temperature (some researchers actually prefer to do their ligations at 4°C) is that it stabilizes the annealing of the sticky ends. Since the sticky ends tend to be very short (~4 unpaired nucleotides), it requires very little energy (ie. relatively low temperatures) to break their hydrogen bonding to a complementary strand. Thus, keeping the reaction temperature low stabilizes those weak interactions between the DNA molecules, and allows the DNA ligase to ligate them together more easily.

4. Age of the ligation buffer.

   - **Problem:** The ligation buffer contains ATP, which is necessary for the DNA ligase to function. Unfortunately, ATP degrades over time as it undergoes multiple freeze-thaw cycles – a common situation in labs that may need to do ligations on different days (ie. teaching labs with multiple sections). Thus, sometimes, no matter how well you did everything else, a ligation simply may not work because the ligation buffer does not have enough ATP – this is not something that can be checked in any other way.

     To be clear, this is not a common problem, and it doesn't happen after 3 or 4 freeze-thaw cycles. It takes frequent use and many such cycles to exhaust all the ATP in a buffer.

   - **Solution:** Some labs try to avoid this issue by some preventative measures. When they purchase DNA ligase, they simply take the ligase buffer that comes with the enzyme, and aliquot individual tubes of 5, 10 or 20µl for storage at -20°C. Whenever they need to set up some ligations, they simply thaw one of these tubes for use, while the other aliquots remain safely frozen. This way, each tube of ligase buffer is thawed only once and should thus have the maximum possible ATP.

Exercises

1. Setting up the Digests

Please take a look at the digest data from last week, and determine which of the two plasmid samples gave the better results. Use that sample to set up one restriction digest with SmaI and one with BamHI. Please note that we don't need to do the negative controls this time, so you will be preparing only two epi tubes.

Materials

- Your best DNA sample
- SmaI (S) and BamHI (B)
- 10X Multi-Core Buffer
- Sterile water (DIW)
- Ice bucket with ice
- Eppendorf tubes
- Small beaker
- Micropipettors and tips
- Microfuge
- 37°C Waterbath
- 65°C Waterbath

Procedure:

1. Obtain four epitubes, label them, and place them on the ice.
2. Use the same amount of plasmid DNA as you used to load the last agarose gel. (unless the amount was too low to detect on the gel – ask your TA for help in those cases)

	Digests	
Tube/Label	P-Sma	P-Bam
DIW		
Plasmid		
BSA	2µl	2µl
10X Buffer	2µl	2µl
Enzyme	2µl	2µl
Total Vol.	20µl	20µl

3. Put the above components into each tube, making sure to keep them on ice as you do this.
    - Make sure the enzyme is the last component added.
4. Spin down your samples for 30sec at maximum speed. This will collect all of the components of the reaction at the bottom of the tube.
5. Place your tubes at 37°C for 10min.
6. After 10min, place your tubes into the 65°C water bath for 10min to inactivate the enzymes.
7. Take your tubes out of the water bath and put them immediately into ice for 1min.
8. Spin down your samples for 30sec in a microfuge.

2. Setting up a Ligation Reaction

In this part, you will set up two ligation reactions and incubate them at room temperature.

Materials (per group):

- Vector DNA and PCR sample #2
- T4 DNA Ligase
- 10x DNA Ligase Buffer
- beaker with ice
- distilled water
- P20 and P200 micropipettors
- Boxes pipette tips
- Epi tubes
- Microcentrifuge
-

Procedure:

1. Obtain two epi tubes, label them, and place it in the ice.
2. Use the table below to help you add components into the tubes.

Component	P-Sma-R	P-Bam-R
DIW	_____µl	_____µl
Plasmid DNA	5µl	5µl
Insert DNA	_____µl	_____µl
10X Buffer	2µl	2µl
T4 DNA Ligase	1µl	1µl
Total Vol.	20µl	20µl

- Your TA will let you know how much insert DNA to use
- Keep the contents of the tube cold until you're ready to start the reaction
- The enzyme should be added last

3. Place the epi tubes in a microfuge and spin down for 30sec.
4. Incubate your tube on your bench (at room temperature) for 30min.
5. Leave your samples at -20°C until the next lab.

Post-Lab Questions:

1. How much plasmid DNA (in ug) did you digest in part 1?
2. How much of that plasmid DNA did you put into the ligation mixture?

These numbers will be important later...

3. Based on what you've read in the intro to this lab and on your procedure, which of the ligation samples is likely to generate recombinant plasmids?
4. What could we do to increase our ligation efficiency?

Lab 7: Restriction Mapping

This week's lab is a little different. In this case, we won't be using any tools except for pens, paper, and our brains. We will be solving biological puzzles.

Learning Objectives:

Students will:

- Understand the role of restriction mapping in genome sequencing
- Understand how a restriction map can be used to verify the identity of a cloned gene without having to sequence it
- Use an understanding of how restriction enzymes work to generate restriction maps for other groups to practice
- Solve restriction maps based on provided DNA digest data

Pre-Lab Questions:

Please view the short video linked below. It gives a brief overview of the process of sequencing of the human genome. In a couple of places, it makes a reference to today's topic.

- https://youtu.be/AhsIF-cmoQQ
    - At approx. 1:35, the narration mentions specific "fingerprints" at the ends of large DNA fragments that would then be used to help scientists put the final sequences together.
    - At approx. 2:45, the video mentions a "mapping step" in the method used by the Human Genome Consortium.

Why do you think the "fingerprints" (restriction maps) were so important to the genome sequencing project?

Restriction Mapping Strategy

Hundreds of restriction enzymes are available to researchers. Each enzyme recognizes a specific (usually short) nucleotide sequence and such restriction sites are distributed fairly randomly throughout a particular DNA region. Since each restriction enzyme is very specific and only cuts DNA at specific locations, it will cut a particular DNA region in the same, very specific pattern every time it is used.

A restriction map is generated to identify where restriction enzymes would cut a particular DNA molecule. Since the order and location of such restriction sites is dependent on the DNA sequence, a precise restriction map can be unique to each specific DNA fragment and can be used to identify it. This can be done to any DNA without knowing its sequence.

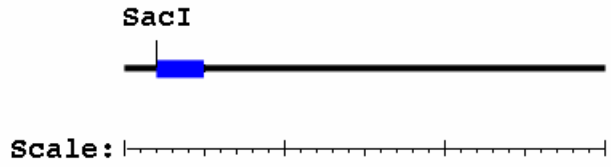
The map of restriction sites in a particular region of DNA can (and has been in the past) also used as an easily identifiable landmark on a longer piece of DNA – for example, restriction maps can be used to help arrange DNA sequences in the proper order when doing genome sequencing.

Restriction mapping involves digesting DNA with several restriction enzymes, alone and in combination with each other. The DNA fragments that are generated in each case are then separated on an agarose gel by electrophoresis. The sizes of the cut DNA fragments are then determined by comparing them to a molecular weight standard ("DNA ladder"), and the data is used to arrange the fragments into the appropriate order.

This last part can be quite challenging (and fun if you like puzzles). Let's take a look at some examples below:

Example 1:

Let's start off with a simple, linear piece of DNA...



In this case, we know that it is 3kb in length. And to make things easier, we know that it has a **SacI** restriction site 0.2kb from the origin (don't worry about the blue region for now).

Now, let's take that same DNA fragment and cut it with **EcoRI**., and run that out on an agarose gel to see the sizes of the fragments that are generated by this enzyme. For this example, let's say the gel shows 2 fragments – they are 1.3kb and 1.7kb in length. But the gel can only tell you about the sizes, it does not tell you the order in which the fragments occur.

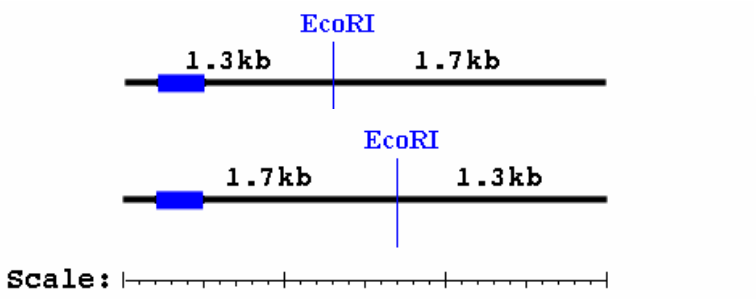
Possibility 1:

Possibility 2:

So, if you want to generate a restriction map for this DNA fragment, there are two ways in which the EcoRI site could be mapped. See below:

Possibility 1:

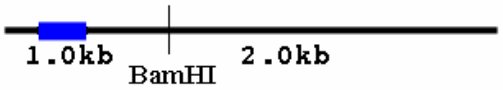
Possibility 2:



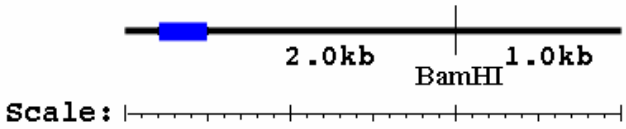
As you can see, there are two equally likely ways that this can be plotted, and we can't know which is correct – this known as an **ambiguous site**. We can resolve the ambiguity by doing a double digest with EcoRI and another enzyme.

You could try using **BamHI** as the other enzyme. When you do a digest with BamHI alone, you notice that it cuts the DNA into two pieces, 1kb and 2kb in length. Which can be mapped like this:

Possibility 1:

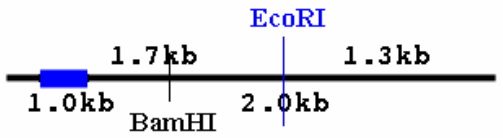


Possibility 2:

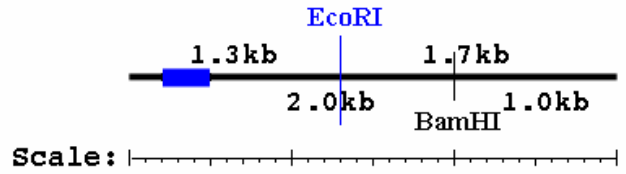


Unfortunately, since the location of the BamHI restriction site is also ambiguous, it may not be the best enzyme to use in a double digest with EcoRI to resolve the ambiguity in its location. In fact, if the double digest yields fragments 0.7kb, 1.0kb and 1.3kb you would still be left with two possible combinations:

Possibility 1:



Possibility 2:

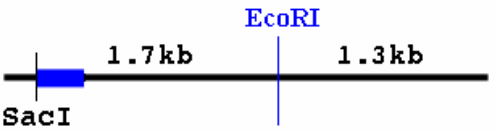


So, BamHI is not a good example of an enzyme that would help us resolve the ambiguity in the location of the EcoRI site. It would be much better to use a site whose location you already know, thus SacI would be more likely to generate an informative double digest with EcoRI.

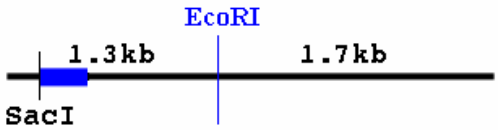
Because we already know that the SacI restriction site is 0.2kb from the origin, we can use it to "anchor" our restriction map - we can plot the EcoRI site RELATIVE to the SacI site. And once we know the position of EcoRI, then we can plot BamHI.

So, lets say that we did a double digest of **EcoRI/SacI** and the digest generated 3 fragments: 0.2kb, 1.5kb and 1.3kb. First, let's look at these fragments and compare them to EcoRI by itself (1.3kb and 1.7kb). You should quickly notice that one of the fragments was preserved (1.3kb), while the other was further cut into 1.5kb and 0.2kb fragments. Drawing a restriction map based on this should be relatively simple, but you can always try to simply superimpose the two individual restriction maps.

Possibility 1:

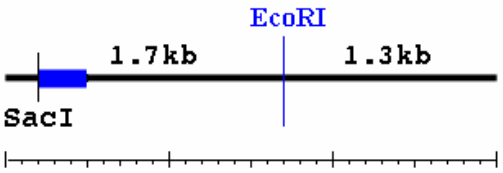


Possibility 2:



There is only one way to map this, Possibility #1 above is the only one that fits the data. Possibility #2 is rejected because it doesn't (the fragments generated by that DNA map would be: 0.2, 1.1 and 1.7).

Based on the above map, you should now be able to determine the position of BamHI. Recall that the double digest with **EcoRI** and **BamHI** yields fragments 0.7kb, 1.0kb and 1.3kb (ignore the SacI site). Where would you put the BamHI site on the map below?



For the purposes of this example, we can also assume that a double digest of **SacI** and **BamHI** would generate the following fragments: 0.2kb, 0.8kb and 2kb.

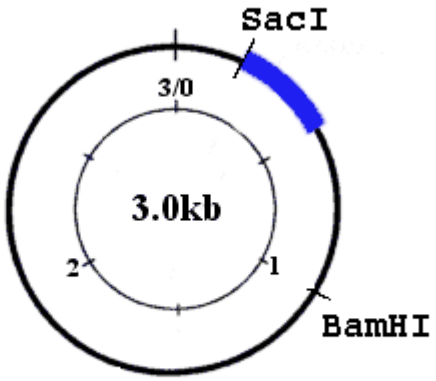
Where do you think the BamHI site is located?

Congratulations, you have just mapped two different ambiguous restriction enzyme sites, with the help of an anchor point.

Example 2:

Let's try doing this again, but this time with a circular piece of DNA (like a plasmid). It is important to note that circular molecules don't have any free ends, so it can be difficult to know how to measure them; the measurements are usually taken from the origin of replication.

Thus, when we describe the plasmid below as having a SacI site at 0.2kb and a BamHI site at 1.0kb, these positions are being mapped relative to the origin (and therefore the BamHI site is 0.8kb away from SacI in the clockwise direction). Furthermore single digest with either one of those enzymes only yields 3.0kb DNA fragments on a gel. This means that the plasmid is 3kb in size and becomes linearized by each enzyme – thus a cut at one restriction site produces only one linear DNA fragment.



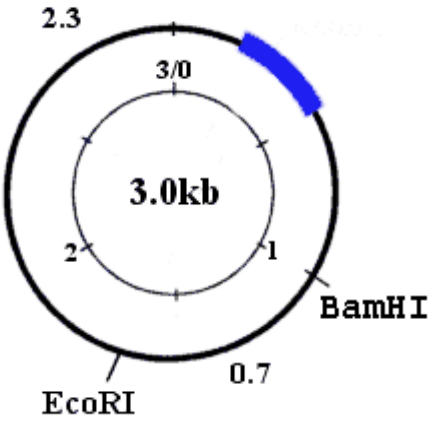
Now, let's pretend that we've set up some digests in the lab with this plasmid in order to determine it's restriction map. Here is the data that we might obtain from the gel:

Enzyme(s) used:	Sizes of DNA fragments:
SacI	3kb
BamHI	3kb
EcoRI	3kb
BamHI / EcoRI	0.7kb and 2.3kb
EcoRI / SacI	1.5kb and 1.5kb

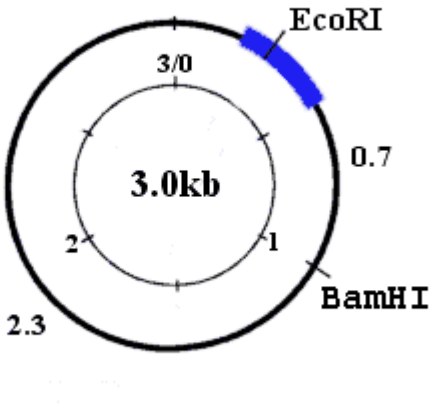
Thus, when we cut this plasmid with EcoRI, we got a single 3kb fragment. That means that EcoRI has only one restriction site on the plasmid. Without any more information however, we cannot determine it's exact location - it could be anywhere (that's 3000 possible positions!).

A double digest with one of the known enzymes (like BamHI) should help us determine the position of EcoRI. The BamHI/EcoRI digest yields two fragments, 0.7kb and 2.3kb in length.

Here are the two ways that this can be mapped:



Possibility 1



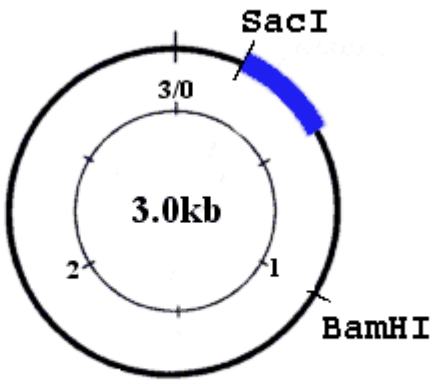
Possibility 2

Clearly, the above digest didn't establish an exact location, but it did narrow the possibilities down to two (it was 3000 possibilities before this, so that's progress).

Now, let's use a double digest of EcoRI and SacI to see if that helps pinpoint the location of the EcoRI site.

The EcoRI/SacI digest yields two 1.5kb fragments, this definitely helps. This is because there is only one way to map that digest. There can be no ambiguity about the location of the site - the EcoRI site is 1.5kb away from the SacI site (in the clockwise and counterclockwise directions).

Fill in the EcoRI site on the map below:



This plasmid map may be starting to look a little familiar. This is because if we were to "open" this plasmid at the origin (3/0 point) and plot it as a line it would look just like the map from Example 1 There is however, an important difference between Example 1 and Example 2.

When you compare the restriction mapping data for both examples, you will notice that in linear DNA, 1 restriction site produces 2 DNA fragments, but in circular DNA, 1 restriction site results in 1 DNA fragment. The main rules of restriction mapping remain the same – use known restriction sites as anchor points to help you map unknown ones.

Exercises

We will now use the restriction map we have generated above to continue adding sites for other enzymes.

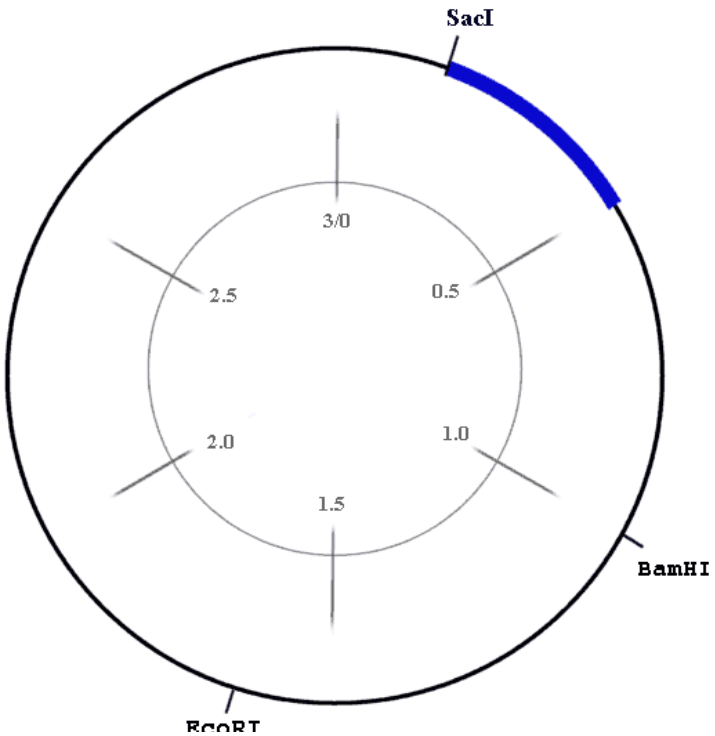
1. Practice Problem 1

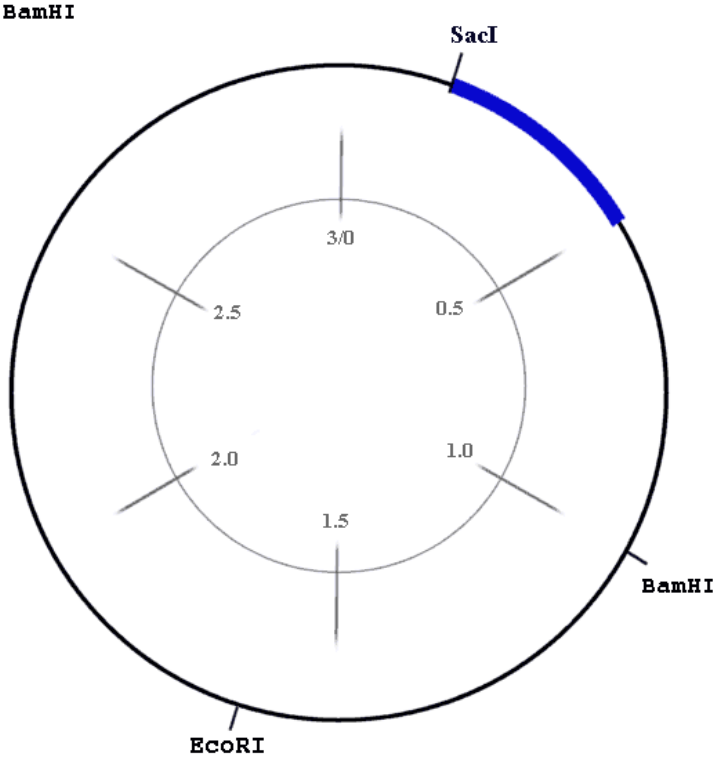
Please use the following data to map the restriction sites for HinDIII and EcoRV restriction enzymes on the above plasmid. Please remember that in all of the digests shown below, we are cutting the same DNA molecule – the locations of the restriction sites can't change from digest to digest.

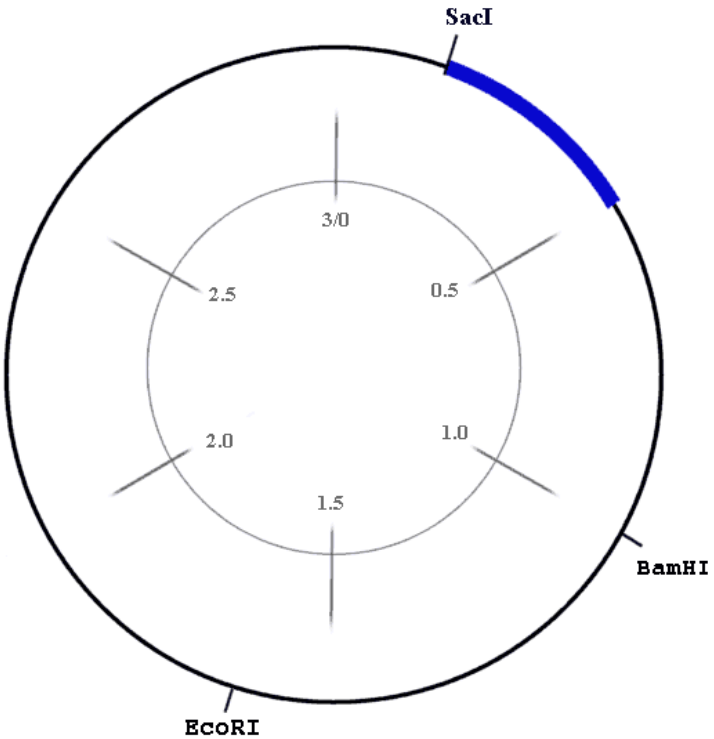
Enzyme(s) used:	Sizes of DNA fragments:
HinDIII	1.5kb and 1.5kb
HinDIII / BamHI	0.5kb, 1.0kb and 1.5kb
HinDIII / EcoRI	0.3kb, 1.2kb and 1.5kb
EcoRV	3kb
EcoRV / SacI	0.2kb and 2.8kb
EcoRV / BamHI	0.6kb and 2.4kb

How many HinDIII sites are there on this plasmid? Explain?

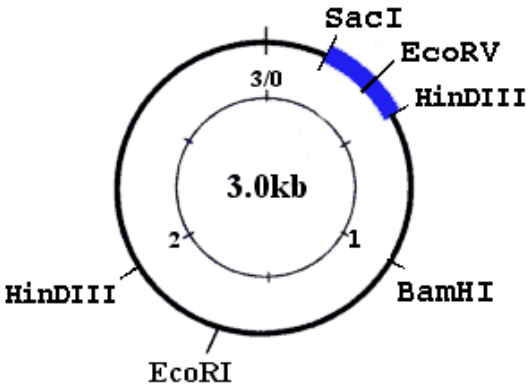
There are some maps you can use for this on the next page.


SacI
3/0
0.5
1.0
1.5
2.0
2.5
BamHI
EcoRI


SacI
3/0
0.5
1.0
1.5
2.0
2.5
BamHI
EcoRI

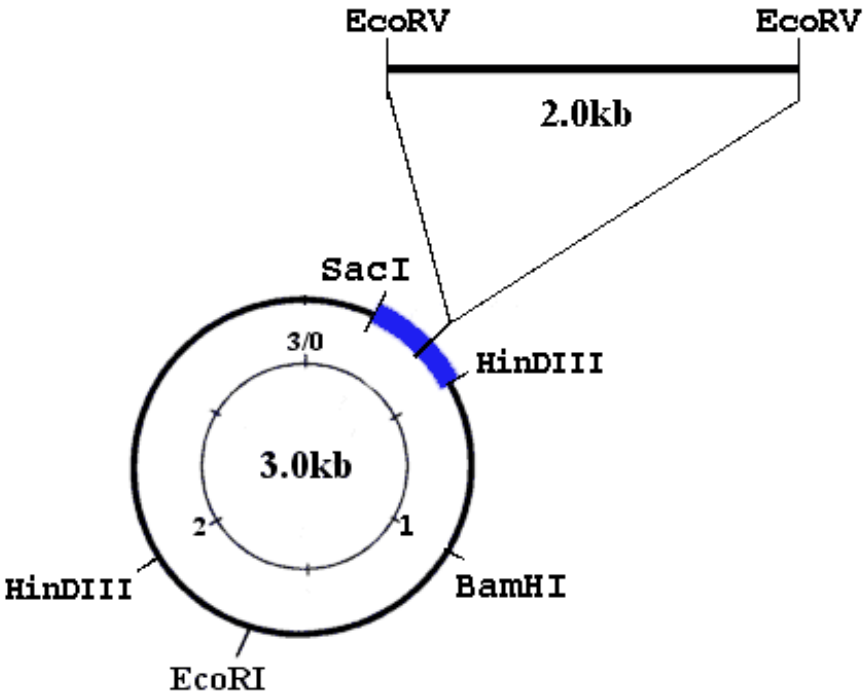

SacI
3/0
0.5
1.0
1.5
2.0
2.5
BamHI
EcoRI

Hopefully, at this point, your map looks like this:



2. Practice Problem 2

Now that we have all the restriction sites on the plasmid mapped, we're no longer interested in the plasmid itself. This time we're going to insert a piece of DNA (referred to as “the insert”) into the EcoRV site of the Multiple Cloning Site (MCS) - yes, that's the blue region.



This diagram introduces some new information. First of all, it shows a 2.0kb insert. Inserts are often drawn as triangles coming out of the MCS – this is just a diagram. In reality, the insert actually becomes part of the circular DNA, but is drawn this way to enable us to differentiate between the known plasmid and the unknown insert.

Another new thing that is introduced here, is the insertion of an “EcoRV fragment” into the MCS of the plasmid. It is important to understand that initially, there is only one EcoRV site in the MCS (as in the top figure). It is cut to produce two sticky ends, which are used to insert a 2.0kb DNA fragment (the insert) with a complementary EcoRV sticky end on each side - thus generating two new EcoRV sites bracketing the insert.

The last thing to note in the diagram, is that you will now be dealing with 5.0kb of DNA (3.0kb from the plasmid and 2.0kb from the insert). Your restriction maps will need to take this into account (your fragments should now add up to 5.0kb). However, at this point, we know everything there is to know about the plasmid DNA, and any new restriction sites that you will be mapping **must be on the insert DNA**.

So, let's map a few sites on the insert. Here is some restriction data for this 5kb recombinant plasmid.

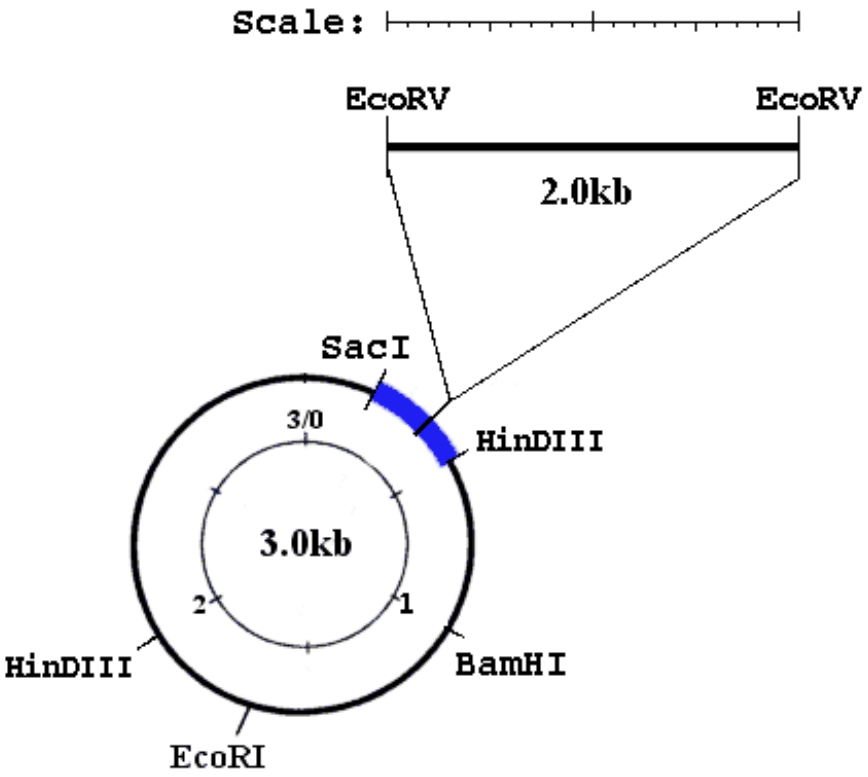
Enzyme(s) used:	Sizes of DNA fragments:
EcoRV	2kb and 3kb
BamHI	5kb
EcoRI	1.8kb and 3.2kb
EcoRI / BamHI	0.7kb, 1.1kb and 3.2kb
HinDIII	0.8kb, 1.5kb and 2.7kb
PvuII	5kb
PvuII / EcoRI	1.1kb, 1.8kb and 2.1kb

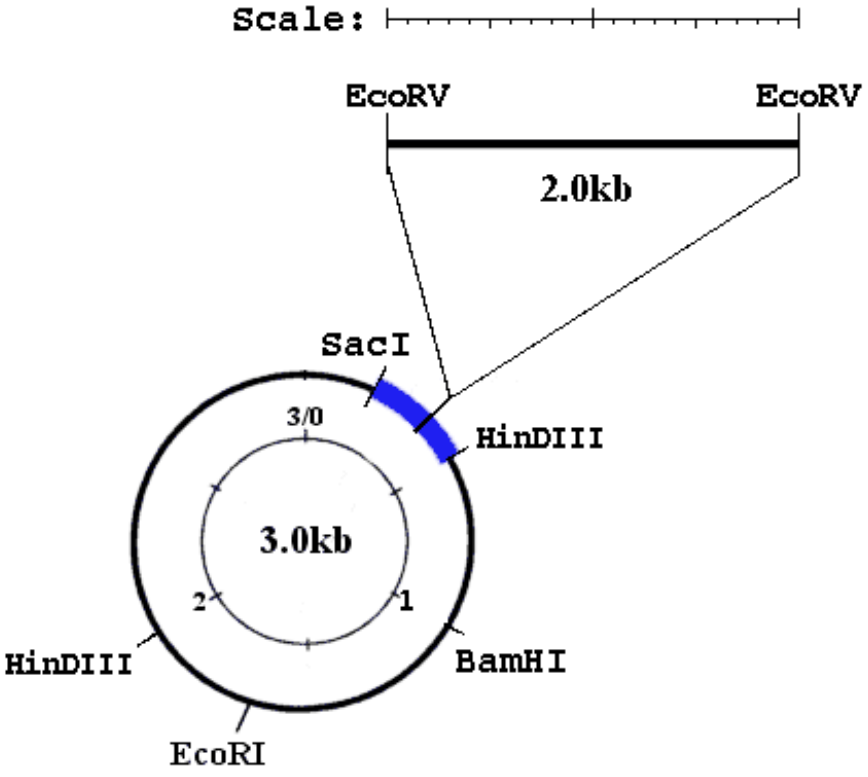
Remember, we know the locations of all the sites on the plasmid.
Your job is to determine the locations of any new sites on the insert.

How many HamHI sites are there on the insert DNA? Explain?

How many EcoRI sites are there on the insert? Explain?

Please use the diagram below to help you plot the restriction sites onto the insert DNA.

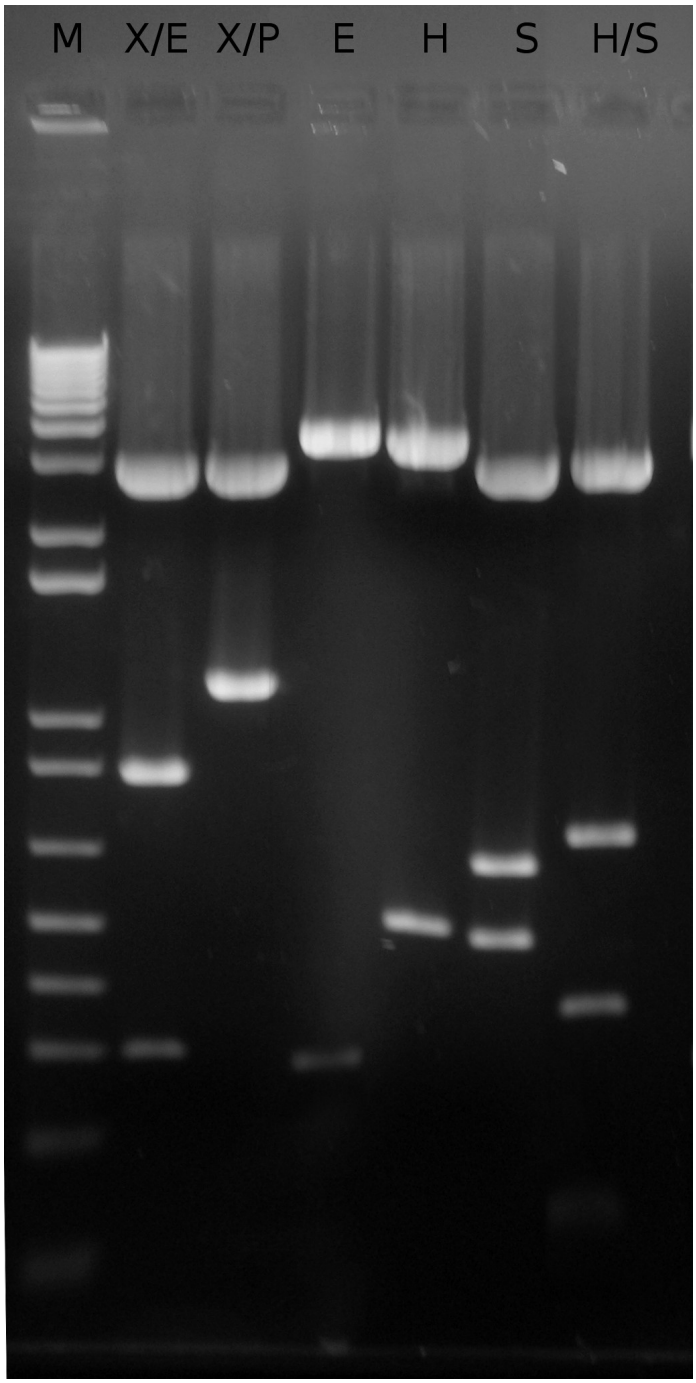




Assignment 2

<u>You will work on this as a group and submit only one hard copy.</u>

The DNA shown on the gel below was digested with several different enzymes (just as you have done in an earlier lab). In all cases, it's the exact same DNA plasmid



This gel represents digests of a recombinant plasmid with the following enzymes and combinations of enzymes:

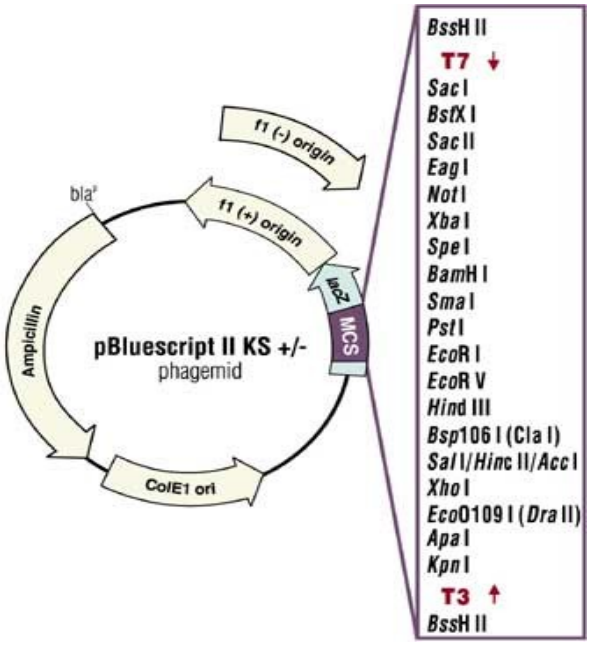
- **X/E** – XbaI and EcoRI
- **X/P** – XbaI and PstI
- **E** – EcoRI
- **H** – HinDIII
- **S** – SacI
- **H/S** – HinDIII and SacI

"M" is the 1Kb Plus DNA Ladder described on p.43.

Please use the instructions in that part of the manual to help you determine the fragment sizes

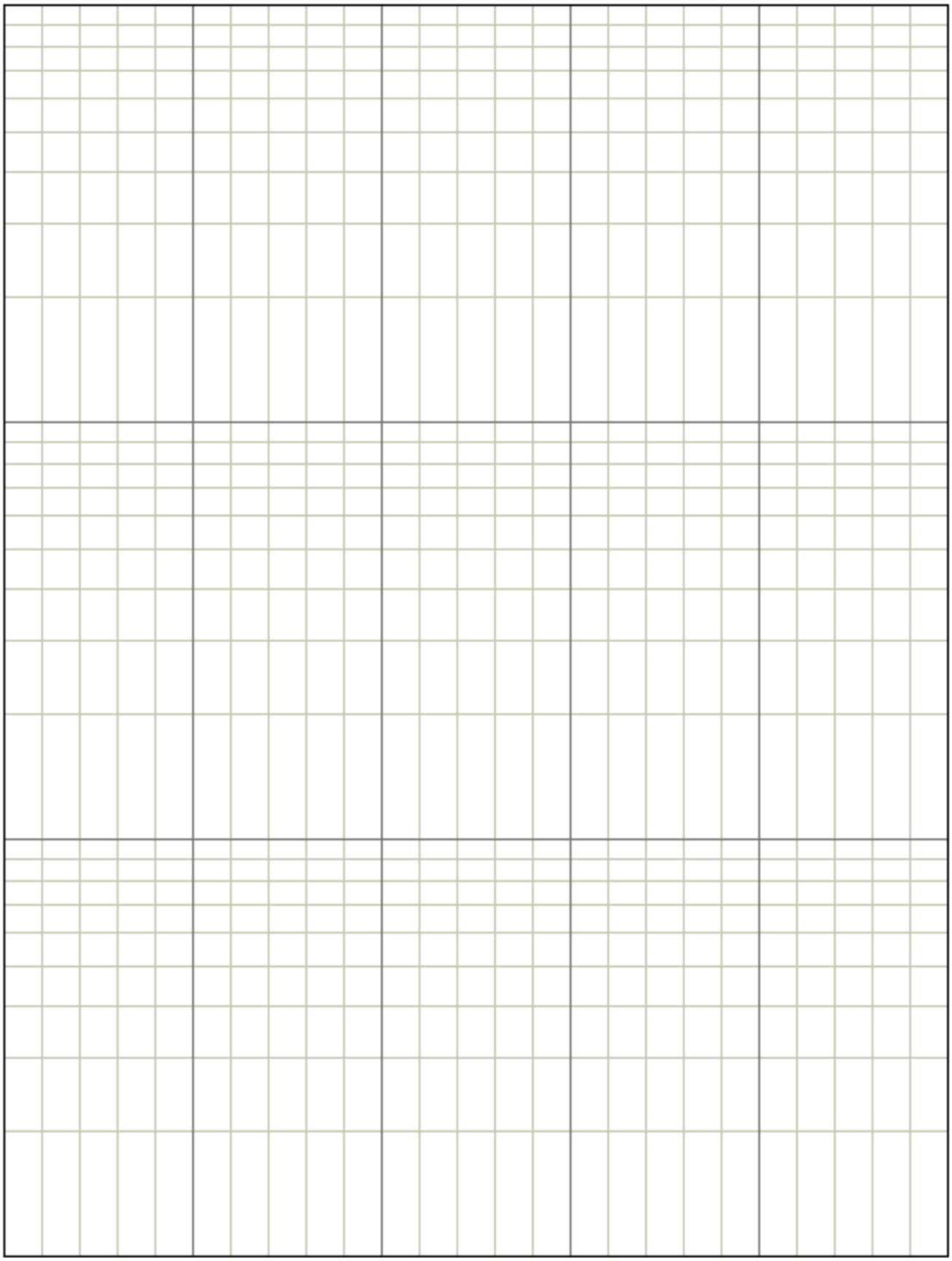
The DNA being digested is a recombinant pBluescript II plasmid (shown below) containing an XbaI/PstI fragment. This means that a DNA fragment containing an XbaI site on one end and a PstI site on the other end was inserted into the plasmid's MCS at those specific restriction sites.

The box to the right of the plasmid in the image shows the order of all the sites in the MCS of this plasmid. Use it to identify any sites that are relevant to this digest and keep track of their locations relative the the XbaI and PstI sites – this will tell you on which side of the insert they are. For the purposes of restriction mapping, restriction sites that are near one another in the MCS can be considered to be in the same position (ie. the EcoRI, EcoRV and HinDII sites can be considered to be in the same position for mapping purposes because of how close they are)



For this assignment, you must complete the following tasks:

1. Assign sizes to the bands of the molecular marker in the gel image above.
2. Draw a graph of size vs. migration distance of the molecular marker bands.
    - Use the semi-log paper provided on the next page
3. Use the graph to determine the sizes of the DNA fragments in each of the digests. You can round the sizes off to the nearest 0.1kbp.
4. Use the fragment size data to generate a restriction map of the insert DNA.
    - Hint: the size of the **insert DNA** can be determined from the XbaI/PstI digest.



Lab 8: Transformation

In today's lab, you will treat *E. coli* cells to make them competent to take up DNA, and then conduct a chemical transformation using your plasmids from the previous labs. The transformed cells will then be plated out on different types of media to see differences in growth patterns.

Learning Objectives:

Students will:

- Produce chemically competent cells for transformation
- Explain the current understanding of how chemical transformation works
- Explain the need for a recovery step after the heat shock
- Explain why a selectable marker gene is necessary on a plasmid vector
- Explain reasons for the different growth patterns on LB and LB+Amp plates
- Determine the transformation efficiency based on their experimental results

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Pre-Lab Questions:

Please read through the slides here:

- www.cengage.com/biology/discipline_content/animations/recomb-DNA-tutorial/index.html

… and also watch the animation linked below:

- https://youtu.be/dSh-jztoYyA

Answer the following questions:

  - if cells in a colony contain only a recircularized plasmid (ie. no insert DNA), what colour would you expect them to be when grown on media containing X-gal?
  - Why would the colour be different if the cells contain a recombinant plasmid?

Transformation

The term "transformation" originates from experiments carried out by Griffith(1928)[1] as well as Avery, MacLeod and McCarthy (1944)[2], which showed that DNA from a virulent bacteria could be used to convert ("transform") a non-virulent bacterial strain into a virulent one. At the time, they were trying to figure out which type of macromolecule was responsible for carrying genes, but the terminology has stayed with us and is now used to describe a common procedure in molecular cloning.

Transformation refers to the process of inserting DNA molecules (usually recombinant DNA) into bacterial cells where they can be propagated (cloned) and possibly used to express particular proteins. Two of the most common methods of transforming bacteria are: Chemical Transformation and Electroporation. *An analogous process of inserting foreign DNA into eukaryotic cells is called "Transfection"*.

Chemical Transformation

The most common transformation technique is Chemical Transformation, also known as the Heat-Shock Method, and it relies on the ability of "competent cells" to take up DNA when briefly exposed to a high temperature.

The first key to the success of this procedure is the the presence of "competent cells." These are bacterial cells which have been treated for a relatively long time with $CaCl_2$ at low temperatures. While we do not yet fully understand why this method works, it is believed that the calcium ions interact with the negatively charged phosphates in the phospholipids of the cell membrane and create a relatively rigid structure at low temperatures. The sugar-phosphate backbone of the DNA also interacts with the $CaCl_2$ and causes the precipitation of the plasmids onto the membranes.

The second component of this method is a brief "heat shock." The competent cells and precipitated plasmid DNA are placed into a 42°C waterbath for 30-90sec (this depends on the bacterial strain being used) and then immediately placed back on ice. This **sudden** change in temperature is thought to generate temporary holes in the membrane (recall that the membrane is fairly rigid due to the Ca^{++} coating), which allow plasmid DNA to enter cells.

Chemical transformation is relatively inefficient - only about 10^6-10^8 cells may take up a plasmid when 1µg of supercoiled DNA (that's a lot of plasmid) is added to them. And the efficiency is lower if the cells are not very "competent" (ie. their preparation did not follow the ideal procedure), or the DNA is large, or not supercoiled. Thus, Chemical Transformation is used with relatively small plasmids, and when high transformation efficiency is not necessary.

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1 Griffith, F. (1928). The Significance of Pneumococcal Types. *The Journal of Hygiene*, *27* (2), 113–159.

2 Avery O.T., MacLeod, C.M., McCarty, M. (1944). "Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types: Induction of Transformation by a Deoxyribonucleic Acid Fraction Isolated from Pneumococcus Type III". *Journal of Experimental Medicine. 79* (2): 137–158.

Electroporation

This method is quicker, more efficient (~10^{10} colonies/µg of DNA), and allows transformation with larger DNA molecules. But it does require specialized equipment, specialized "electro-competent" cells, and for the DNA to be very, very "clean" (ie. no contamination from salts, etc.). Having any contaminating ions will result in the electrical current killing the cells and a loss of that sample.

In this procedure, cells are mixed with the DNA sample, and placed in a specialized cuvette between two electrodes with different electrical potentials. A current is applied, which is thought to cause transient openings in the membranes of the bacterial cells. It also helps to move the negatively charged DNA through the sample (like in electrophoresis) and into the cells through the pores that have been opened.

Exercises

1. Preparation of Competent Cells

Making cells competent is fairly easy. In the traditional procedure, bacteria are grown to mid-log, the media is removed, they are resuspended in cold $CaCl_2$, and incubated on ice for about 20 minutes. The cells are then collected, and resuspended in fresh $CaCl_2$ and refrigerated overnight; this is called “seasoning” and tends to increase transformation efficiency.

In our case, we will be working within the time limits imposed on us in a teaching lab, so we will not be attempting to produce highly competent cells. The procedure below will produce some cells that will be capable of taking up plasmids, but the transformation efficiency will not be as high as it would be if we followed the classical procedure.

Materials:

- 0.1M $CaCl_2$ (you’ve prepared this)
- mid-log *E. coli* cell culture (Top10 strain)
- ice bucket with ice
- microfuge
- eppi tubes
- micropipettors and tips

Procedure:

1. Place all solutions and tubes on ice as soon as you get into the lab.
    - Add some tap water to your ice buckets to help chill your samples more efficiently
2. Take 3 sterile epi tubes and transfer 1ml of bacteria from the culture tube into each of them
3. Spin the cells down in a microfuge for 30 seconds to pellet the bacteria.
4. Discard supernatant and place the tubes back on ice.
5. Add 500µl of the sterile, cold $CaCl_2$ solution into each tube.
6. Resuspend the cells by **gently** pipetting with a P1000 and leave on ice for 2min
    - The tip of a P1000 has a larger opening than the smaller yellow tips, so resuspending the cells with this tip will be less stressful on the cells.

DO NOT VORTEX!

7. Spin the cells down in a microfuge for 30 seconds to pellet the bacteria.

8. Discard supernatant and place the tubes back on ice.
9. Add 500µl of the sterile, cold $CaCl_2$ solution into each tube.
10. Resuspend the cells by **gently** pipetting with a P1000 and leave on ice for 10min
    - After this incubation, you can use these cells for transformation, or store them at -80°C for future use.

2. Transformation of Competent Cells

Once the cells are competent, you will transform them by the Heat-Shock method. The key to this method is that the changes in temperature are very sudden – make sure you have the ice bucket with you next to the waterbath.

Materials (per group):

- Your DNA plasmid sample (P-Sma-R)
- Your DNA plasmid sample (P-Bam-R)
- Competent *E. coli* cells (Top10 strain)
- ice bucket with ice
- 37°C and 42°C waterbaths
- LB (Luria Broth)
- micropipettors and tips

Procedure:

1. Obtain 3 epi tubes of competent cells and label them "+S", "+B", and "-"
2. Add 5µl of the "P-Sma-R" plasmid to the cell suspension in the tube marked "+S ".
3. Add 5µl of the "P-Bam-R" plasmid to the cell suspension in the tube marked "+B".
4. Do not add anything (or add sterile water) to the tube labeled: " - "
5. Mix each tube gently by tapping lightly with your finger.
6. Leave the samples on ice for 20 minutes.

Now for the heat shock. **It is important that the cells are shocked**.

7. Take your ice bucket to the 42°C water bath, and place the tubes into the water for 45sec.
    - Since the incubation is short, just use your hands to hold the tubes in the water.
    - Do not shake or tap the tube, just hold it in place for 45 seconds.
8. **Immediately** after the heat-shock, return the tubes back to the ice for 2min.
9. Add 500µl of LB to each tube. Place in the 37°C water bath for 30 minutes to allow the cells to recover from the "rough treatment".

3. Plating of Transformed Cells

After the "recovery" step, you will be plating your cells out on some LB agar plates. You will treat the "+S" sample and the negative control the same way – you will plate out each culture on plates that do not have antibiotic, and on plates that do have antibiotic to see the effect of having a plasmid.

You will also plate out your "+S" and "+B" cultures on plates that has some IPTG and X-gal, to see the effect that these two components will have on the transformed cells. Please think about what you expect from your ligations from the previous lab in order to try to predict your results in this lab.

Materials (per group):

- Transformed *E. coli* cells
- 2 LB Agar plates
- 2 LB+Amp Agar plates
- 2 LB+Amp+IPTG+X-gal plates
- Spreader, ethanol and burner
- micropipettors and tips

Procedure:

1. Spread 300µl of "+S" and "-" cultures on an LB and an LB + Ampicillin plate.
2. Spread 300µl of "+S" and "+B" on the LB + Ampicillin plates containing IPTG and X-gal. This does not have to be done with the negative control.
3. Allow the liquid to be absorbed by the plates for 5min and then place them upside down in the 37°C incubator overnight.
4. Clean up your lab bench and dispose of materials properly.

You will need to complete this procedure on the next day – you may need to check the fridge for your samples.

5. Make observations about the bacterial growth you see on all the plates. Count the colonies when possible – especially in the LB+Amp plates.

Post-Lab Questions:

Please look at your plates and answer the following:

- What did you observe on the LB plates? Why are they different from the results on the LB+Amp plates? Explain.
- What did you observe on the LB+Amp+IPTG+X-gal plate? Explain.
- Are the numbers of colonies on th LB+Amp and LB+Amp+IPTG+X-gal plates similar for the " + " culture?
- How many µg of your DNA plasmid did you use for transformation?
- What was the transformation efficiency of your experiment?

$$\textit{Transformation Efficiency} = \frac{\#\text{ of colonies on the LB+Amp plate}}{\mu g\ of\ plasmid\ DNA\ used} * \frac{volume\ plated}{\text{total volume in the eppi tube}}$$

Lab 9: Colony PCR

In this week's lab, we will be selecting white bacterial colonies from our transformation experiment and confirming that they have our insert of interest by Colony PCR.

It is possible that some of the white colonies from last week's transformation may be false positives or satellite colonies (your TA will explain), so confirming that cells in a white colony actually have your recombinant plasmid is very important.

Learning Objectives:

Students will:

- Explain the growth characteristics they see on their plates from last week
- Identify colonies containing recombinant and non-recombinant plasmids
- Explain what a satellite colony is
-

Pre-Lab Questions:

Take a look at the following video and answer the questions below the link:

- https://youtu.be/h0yRrDWtdA4
- In what way is the PCR protocol different from what you did in the previous PCR lab?
- Why is it still okay to do it that way?

The aim of the practical is to do the screening of the recombinant transformants obtained during the last practical, using the blue-white screening method based on the expression of lacZ gene encoding b-galactosidase. The white colonies on your plates should correspond to colonies containing recombinant plasmids (please see the discussion of ligation in an earlier lab for more details), and the blue ones correspond to non-recombinant transformants. But, it is possible to have false positives in this type of simple screening, so the identity of the "putative positives" needs to be confirmed.

We will be doing this using the Colony PCR technique. This form of PCR will allow us to quickly confirm the presence of the cloned insert using the primers Lep2A-Lep2B. If we have amplification of a fragment of 0.9 kb we will confirm genotypically the success of the cloning.

The main difference that you might notice between this PCR and the one you've already done in the past is that this one uses cells from the colonies directly taken from the surface of the Petri dish. There won't be a DNA extraction procedure to purify the plasmid.

Excercises

1. Amplification of Insert DNA using Colony PCR

You will be picking 2 white colonies and 1 blue colony, and setting up four different PCR reactions: one will be a positive control with DNA from your previous PCR, and one for each colony.

Since all of your samples will contain the exact same PCR components in the same amounts, it would be more efficient to set up something called a "master mix". A master mix simplifies the preparation of multiple PCR reactions by having you prepare one tube that contains everything you need to add to each PCR tube in a larger volume. So that later, instead of adding each primer, and each buffer, and each dNTP mix to each tube individually, you just add one volume of the master mix solution and the PCR tube is ready.

To set up a master mix, you consider the number of total samples that you need to run, and then you prepare one epi tube where you will mix the right amounts of all the PCR components. If you have a larger number of samples to run, it's a good idea to make a little bit more than you might need – for example: if you have to run 4 reactions (like today), calculate the amounts that will be added to your master mix as if you were setting up 5 reactions. In this way, you can make sure you don't run out of the mixture due to minor pipetting errors. Also, because this allows you to work with larger volumes, it decreases the chances of pipetting errors.

Materials (per group):

- PCR product from lab 3 (DNA1)
- Your plates from last week
- PCR Primers (Fwd) and (Rev)
- 5X *Taq* polymerase buffer
- *Taq* DNA polymerase
- sterile water
- micropippettes and tips
- Sterile PCR tubes
- dNTP mix
- 25mM $MgCl_2$
- 1X TE

Procedure:

Setting up the Master Mix.

1. Since we're setting up 4 reactions, we will make enough master mix for 5 reactions. Take a look at the table below for amounts:

	Components needed for just one tube	Components needed for 5 PCR reactions
5X Buffer	5µl	25µl
dNTP Mixture	10µl	50µl
25mM $MgCl_2$	1µl	5µl
Forward Primer	1.5µl	7.5µl
Reverse Primer	1.5µl	7.5µl

2. Obtain an epi tube and add all of the components in the column for "5 reactions"
    - This is your "Master Mix"
    - It doesn't contain the Taq Polymerase – you will add that separately to make sure the reaction only starts when you want it to start.

Preparation of DNA samples

1. Obtain 3 epi tubes and label them "wCol1", "wCol2" and "bCol" and add 200µl of TE to each one.
2. Pick a white colony from your "+S" culture on LB/Amp/IPTG/X-gal plate and transfer that cell mass into the TE in the "wCol1" tube.
3. Pick a second white colony from your "+S" culture on LB/Amp/IPTG/X-gal plate and transfer that cell mass into the TE in the "wCol2" tube.
4. Pick a blue colony from your "+B" culture on LB/Amp/IPTG/X-gal plate and transfer that cell mass into the TE in the "bCol" tube.
5. Place these three tubes into a heating block at 95C for 10min
6. Centrifuge the samples at maximum speed for 5min – the supernatant will contain the plasmid DNA

PCR Setup

7. Obtain 4 PCR tubes, label them as shown in the table below, and place them in the ice bucket.
8. Your previous PCR product (from Lab 3) will act as your positive control. You will be adding that as your "Template" to the "+" tube.

9. The centrifuged samples from the section above will be the “templates” for the remaining samples.

10. Put the following components into each tube, making sure to keep them cold.
    - Be sure to add them in the order indicated – ie. the enzyme should be added last.

	PCR Reaction Contents			
Tube/Label	**+**	**wCol1**	**wCol2**	**bCol**
Template	5µl	5µl	5µl	5µl
Master Mix	19µl	19µl	19µl	19µl
***Taq* Polymerase**	1µl	1µl	1µl	1µl
Total Vol.	25µl	25µl	25µl	25µl

11. Place the PCR tubes inside open eppitubes and place them in a microfuge.

12. Spin the contents of the tubes down to the bottom.

13. Place the PCR tubes onto a thermocycler and start the following program:

First cycle: 3 min at 94°C
Then,
30 cycles:
- 45s of denaturation at 94°C
- 45s of primer annealing at 47°C (the temperature is primer-specific)
- 90s of elongation at 72°C
Then,
- hold at 4°C (this will keep the samples at 4°C until the technician collects them)

Post-Lab Questions:

5. Once the reactions are completed, we will run them on a gel. What do you think you are likely to see for each of your samples on the gel?

6. If one of your white colonies was a “false positive”, what will you see on the gel?

Lab 10: Gene Regulation

In today's lab, we will be studying gene regulation. We will do this using a relatively simple system called the lac operon in *E. coli* bacteria.

Learning Objectives:

Students will:

- Explain the logic behind controlling gene expression in a cell
- Describe the way in which the lac operon is controlled
- Explain the difference between how the operon responds to IPTG and Lactose
- Explain why there is a need to use minimal media when using Lactose as an inducer

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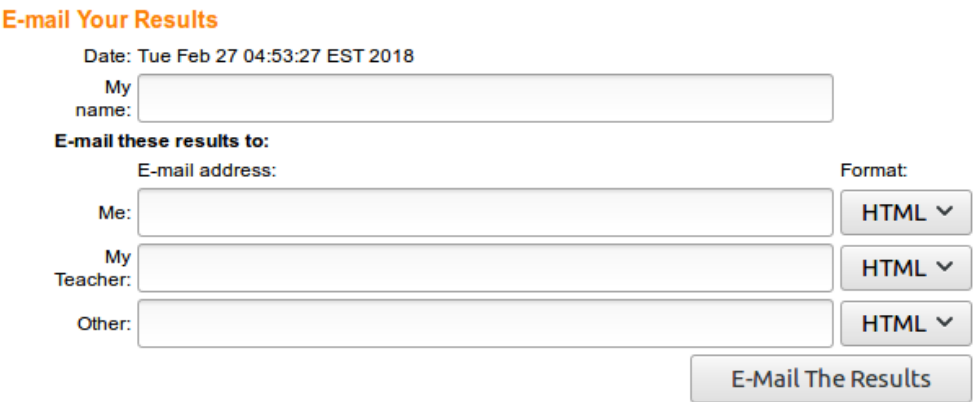
Pre-Lab Questions:

Please take a look at the following video to help you visualize how transcription happens:

- https://youtu.be/-AnsJlLjbz8

Go to the following link:

- The Lac Operon
  - View this video https://youtu.be/AVuj0q4mKa8 and complete the quiz.
  - Fill out the form under the completed quiz to send your results to your TA



Gene Regulation

Each one of your cells has a genome composed of $3x10^9$ base pairs, which is believed to contain between 19,000 and 20,000 genes (protein-coding regions). But not all of those genes are transcribed and translated into proteins at the same time. Some genes are activated at different times during development (ie. fetal hemoglobin [γ-globin] gene vs. the "adult" hemoglobin [β-globin] gene), some are activated only in certain specific cell types (ie. gamma-enolase [NSE] in neurons only), and some are activated in response to specific conditions (ie. heat shock protein 70 [HSP70] in stress response). This means that cells have a way of activating specific genes at specific times.

This is known as **Gene Regulation**. The term "gene regulation" refers to the mechanisms which are involved in causing a gene to be expressed, or not expressed, as a functional protein. This is often accomplished "at the DNA level" and can involve chemical modification of the DNA, changes in levels of DNA packaging, and binding of various proteins to various DNA regions to regulate transcription. Control over the activity of a gene can also occur at the level of the mRNA as well as protein.

The Lac Operon

Much of what science knows about gene regulation has come from research on the lac operon in *E. coli*. The lac operon is a region of DNA containing genes involved in the metabolism of lactose. Because transcription and translation require energy, cells only turn on genes like the ones in the lac operon when they are needed - when the only source of energy is in the form of lactose (ie. glucose is not available).This allows the cell to be efficient in how it uses its resources.

Please watch the animation dealing with the lac operon in the pre-lab exercise to get an understanding of how the lac operon works under normal conditions.

Exercises

In this lab, we will be using a modified (non-wildtype) *E. coli* cell to help us study gene regulation. Many laboratory *E. coli* strains have been engineered to have certain genes in their genomes "knocked out". This often includes the LacZ α-subunit sequence, which results in an inability to produce a functional β-galactosidase enzyme.

The effect of this mutation is reversed by the presence of a plasmid like pUC18, which contains a good copy of the LacZ α-subunit . Therefore, when the cell contains the plasmid, it is capable of producing all of the necessary subunits to express of a functionally active β-galactosidase. This is called **complementation** and is the basis of what is known as the Blue-White Reporting System.

In molecular biology labs, we often use this system to help us identify plasmids carrying recombinant DNA – the LacZ α-subunit is disrupted by inserted DNA and thus is not functional. Therefore, when cells containing a mixture of plasmids (recombinant and non-recombinant) are plated out on media containing a chromogenic chemical called X-Gal, the expression of the functional enzyme will result in the breakdown of X-Gal into a blue product (ie. colonies will look blue), while the expression of a non-functional enzyme (ie. in cells containing a recombinant plasmid) will not cause the break down X-Gal and result in white colonies.

Since the lacZ gene on the pUC18 plasmid is under the control of the lac promoter, the expression of a functionally active β-galactosidase depends on the presence of a molecule which can activate its expression - an inducer.

In this lab, we will be testing the effectiveness of two different inducers – Lactose and IPTG – in activating the lac operon in our cells.

1. Effect of Inducer on the Lac Operon

We will be using two different types of plates – the typical LB/agar plates that are commonly used to grow E. coli as well as M9/agar plates. M9 media is a minimal media which is meant to support the growth of cells, but provides only the most basic necessities for growth and minimal glucose.

The plates you will be given (LB and M9) already have X-gal on them, you will need to add the IPTG or Lactose to the appropriate plates so that you can make a comparison.

Materials (per group):

- 3 plates of LB/Amp/X-gal
- 3 plates of M9/Amp/X-gal
- 2ml of bacterial culture (with plasmid)
- 1ml of 1mM IPTG
- 1ml of 1mM Lactose
- spreaders for plating out inducers
- 70% ethanol
- incinerators

Procedure:

1. Obtain 3 plates of LB/Amp/X-gal
2. Plate out 300µl of IPTG on one plate
3. Plate out 300µl of Lactose on a second plate
4. Do not add anything to the last plate – this will be your negative control
5. Obtain 3 plates of M9/Amp/X-gal
6. Plate out 300µl of IPTG on one plate
7. Plate out 300µl of Lactose on a second plate
8. Do not add anything to the last plate – this will be your negative control
9. Allow the solutions to soak into the plates for about 10-15min before plating the cells
10. Plate out 300µl of bacterial culture on each plate.
11. Incubate the plates overnight at 37°C
12. Come back the next day to view the plates and make observations.
13. Check the plates again a day or two later to see if there are any changes

Post-Lab Questions:

1. What did you observe on the LB plates? Is there a difference between IPTG and Lactose as inducers? Why do you think that is?
2. Was there a difference between your observations on the LB plates vs. M9 plates? Would you expect a difference? Why?

Lab 11: SDS-Page

In today's lab, we will be dealing with protein expression and producing samples for SDS-PAGE electrophoresis.

Learning Objectives:

Students will:

- Identify important features of an expression vector
- Explain the importance of denaturation in SDS-PAGE
- Describe how SDS-PAGE works

Pre-Lab Questions:

View the video linked below and answer the following questions:

- https://youtu.be/On_ZotdZexI
    - What does SDS do in the denaturing buffer?
    - What would be the effect of not adding β-mercaptoethanol (β-ME) to your protein samples?
    - Why do we use a discontinuous gel system? What is its main advantage?

Expression Vectors

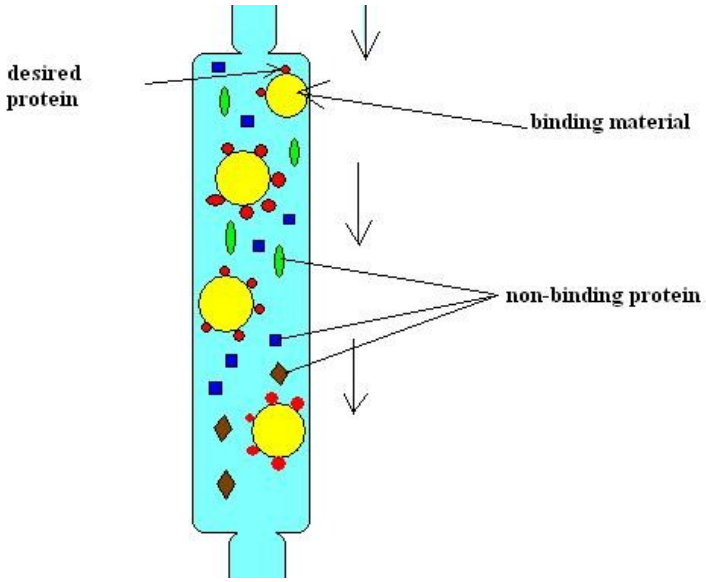
In a previous lab, you cloned a gene into a vector. One of the reasons to do this, is to produce a protein. Many vectors have been specifically designed for the expression of recombinant proteins - some are meant for use in bacteria, while others are used in eukaryotic host cells. One of the reasons that we may not want to use *E. coli* as our host, is that not all eukaryotic proteins can be properly produced in prokaryotes. In those cases, eukaryotic expression systems are used. In this lab, we will focus on the bacterial protein expression system.

One of the first considerations when choosing an expression vector is the promoter that is present upstream of the MCS.

As you've learned in the Gene Regulation Lab, cells are able to regulate the activation of their promoters. This type of control is equally important to an expression vector. Thus, the promoters found there must be capable of being turned on or off. This is critical because, some of the proteins you may wish to express could be toxic to the host cell (sometimes at high levels, and sometimes even in small amounts), thus you will want to grow the host cells to a high cell number before activating expression. In this way, even if the protein is toxic to the cells, you will be able to get at least some protein production from the cells before they're killed.

The ideal vector will contain a strong promoter (ie. lac or tac promoters) in order to facilitate a high level of gene expression. The control elements, which can be used to control expression from these promoters are often also located on the vector, but sometimes they are present within the host genome.

Just as with any other type of vector, the origin of replication is also an important factor. Using high-copy-number plasmids will allow your recombinant plasmid to amplify itself within the host. This increases the number of copies of your gene of interest in the host (high gene dosage), and thus increases the level of protein production when the vector is induced.



Another important feature of an expression vector is the inclusion of a mechanism for purifying the expressed protein. This often takes the form of a 'tag' which can bind to a chromatography column and allows the removal of all other proteins, leaving only pure protein of interest.

Commonly used 'tags' include GST (Glutathione-S-Transferase - a small protein with an affinity for glutathione) and Histidine-affinity tag (a short sequence encoding a region containing many Histidine residues, which have an affinity for Ni++ ions). Such tags are frequently engineered into the vector along with a "cleavage" site, which can be recognized and cut by an enzyme (protease) after the protein has been purified.

Practical Aspects of Cloning into Expression Vectors

As you've already seen, there are a few different ways of getting foreign DNA ligated into a plasmid vector. In the case of expression vectors, the orientation of the foreign DNA sequence relative to the promoter is very important, so **directional cloning** is often employed. Additionally, we must also ensure that the DNA is cloned '**in-frame**' relative to the promoter or the protein tag (in cases where a **fusion protein** is being generated) - the Start-codon has to be in the right position. This will affect your choice of vectors and restriction sites.

Exercises

1. Induction of Protein Expression

This part has already been done for you by the lab technician because there isn't enough time in the lab to do the induction and to run a protein gel.

Materials (per group):

- *E. coli* containing recombinant vector
- 1M IPTG stock solution
- Micropipettors (P200 & P1000) and tips
- 37°C incubator with shaker

Procedure:

1. Grow *E. coli* cultures until they reach an OD600 between 0.6 and 0.8
2. Add enough IPTG to the bacterial culture to reach a final concentration of 0.5mM
3. Add an equivalent amount of water to a second bacterial sample
4. Incubate cells for 4hrs at 37°C before harvesting them for the next part of the lab

2. Separation of Proteins by SDS-PAGE

SDS-PAGE is commonly used to separate proteins in a sample based on their size. In order to obtain a good separation, proteins are first treated with SDS, 2-mercaptoethanol, and heat to allow them to fully linearize.

Materials (per group):

- Sample of induced cells
- Sample of uninduced cells (control)
- 5X Denaturing Buffer (0.25M Tris pH 6.8, 10% SDS, 40% Glycerol, 20% β-mercaptoethanol, 0.01% Bromophenol Blue)
- ice in a beaker
- 95°C water bath or heating block
- epi tubes
- Microfuge
- Micropipettors (P20 and P200) and tips
- SDS-PAGE Gel Apparatus
- 12% SDS-PAGE Gel (each group will load 6 lanes)

Procedure:

1. Label two epi tubes as "Ind" (induced) and "un" (uninduced)
2. Obtain your bacterial samples
3. Transfer 500µl of induced culture into the epi tube labeled "ind"

4. Transfer 500µl of the uninduced culture into the epi tube labeled “un”
5. Balance the tubes in a microfuge and spin at max for 1min.
6. Carefully remove the supernatant
7. Add 80µl of water and 20µl of Denaturing Buffer to each tube
8. Resuspend the peliet by pipetting.
    (a) Set the micropipettor to a lower volume – you can try setting it to half of the volume in the tube
    (b) Press the plunger to the first “stop” and **then** put the pipette tip into the liquid
    (c) Slowly release the plunger and make sure that the pipette tip is **always** under the surface of the liquid.
    (d) Expel the liquid from the tip, but only to the first “stop”. As you do this, make sure to aim the tip at the pellet.
    (e) Slowly take up liquid again as in step “(c)”, and expel the liquid again.
    (f) This should be done carefully and relatively slowly to avoid generating bubbles.
    (g) Do this until the solution appears uniform and no pellet is visible.

9. Place the resuspended samples into a boiling water bath or a heating block at 95°C for 5min to fully denature the proteins.
10. Place the sample into some ice-water in your beaker. This will cause condensation of water in your epi tubes and till help prevent water loss when you open the tube.
11. Wipe the outside of the tubes and spin the samples in a microfuge at max for ~30sec to collect all the liquid at the bottom of the tubes.
12. Load 25ul of each sample on the gel. (Your TA will demonstrate)
    - Load 2 lanes of each sample to make sure each member of your group gets a chance to load.
13. Run the gel at 130V for about 2hrs.

3. Visualization of Proteins on a Gel

Depending on how much time is left in the lab, this may be done for you, or we may try to do the short version of the protocol ourselves.

Materials (per group):

- Coomassie Blue stain (0.1% Coomassie R250, 10% acetic acid, 40% methanol)
- Destain Solution (30% methanol, 10% acetic acid)
- DIW
- Kimwipes
- Plastic dish/tray (microwaveable)
- 12% SDS-PAGE Gel with proteins
- Gel knife / painter’s spatula

Procedure:

1. Disassemble the electrophoresis system
2. Carefully separate the gel plates (your TA will demonstrate). The gel will stick to one side or the other.

3. Carefully pull the gel off the plate and place it into a container with water and gently rock for about 2min.
4. Pour away the water. You can gently press on the gel with your finger to keep it from sliding out of the plastic dish.
5. Add enough of the stain solution to cover the gel by about 0.5cm.
6. Incubate the gel in the stain for 1hr (to overnight if necessary)
    - This step can be modified as follows:

    a. Place the gel in stain into a microwave, and heat on high for about 1min
    b. Keep an eye on the tray and stop the microwave when you start to see the solution just begin to boil
    c. Open the microwave door slowly, a small crack at first, to allow the steam to escape. Methanol (a component of the stain) should not be inhaled – it is hazardous.
    d. Remove the container from the microwave and gently rock with the stain for 5-10min to allow it to penetrate the gel.

7. Pour the Coomassie stain into a separate container (it can be reused).
8. Rinse the gel briefly with DIW.
9. Remove the water and replace it with “Destain solution”, and place a couple of Kim-wipes folded flat over the gel. Make sure there is about 0.5cm of solution above the surface of the gel.
10. Incubate with the Destain solution for 1hr (to overnight if necessary)
    - This step can be modified as follows:

    a. Place the gel in destain solution into a microwave, and heat on high for about 1min
    b. Keep an eye on the tray and stop the microwave when you start to see the solution just begin to boil
    c. Open the microwave door slowly, a small crack at first, to allow the steam to escape. Methanol (a component of the stain) should not be inhaled – it is hazardous.
    d. Remove the container from the microwave and gently rock with the solution for 5-10min to allow it to penetrate the gel.
    e. The kimwipes should be able to absorb unbound protein away from the gel.

11. You can change out the Kim-wipe if you have excessive stain left.
12. The gel should be clear with dark purple protein bands.

Post-Lab Questions:

1. What did you observe on the gel? Is there a difference between induced and uninduced protein samples? Why do you think that is?
2. Gels used for sequencing are also made of polyacrylamide. Why do you think that is? (hint: think about the size of an enzyme compared to the size of a plasmid)
3. Do you think SDS or BME would be added to a polyacrylamide gel used for DNA sequencing? Why/why not?