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Cell Biology Lab Manual

BIOL 211

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Welcome

Dear Student,

Welcome to the Cell Biology Laboratory.

The course you're about to take is still a work in progress. Its contents have changed several times over the past 9 years – partially due to the availability of certain resources. As a result, some lab exercises may still change and protocols may have to be modified as we go along. Please keep this in mind as you prepare for your labs and understand that we may deviate a little from the schedule listed in this manual and your syllabus.

This manual and any handouts that might be posted online for you will form the laboratory manual for the course. They will contain the protocols for your exercises for each lab, as well as any relevant theoretical information, and definitions that you will need to perform your lab work effectively. Please be sure to bring this manual and any posted handouts to every 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. Prior preparation and understanding are important and will be periodically tested using quizzes (these are likely to occur near the start of the lab).

I hope that you enjoy the labs and I would appreciate any comments or suggestions that you have for future improvements (although, please keep in mind that I have limited resources and time).

I wish you much success in this semester,

R. Stefan Rusyniak

Lab 1: Introduction to the Cell Biology Lab

In this lab, we will be discussing some of the administrative details relevant to the lab part of the course. Please be sure you've looked at the syllabus and read through all of "Lab 1" in this manual **before** start of the lab.

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.
- Formulate an initial definition of a cell
- Describe some of the functions of cells and how these can be accomplished

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Pre-Lab Questions:

Read through the syllabus for the lab and answer the following:

- Is there a midterm in this course?
- How much are quizzes worth?
- Should you have a lab notebook?
- What are your TA's office hours?

Use any resources you think will be helpful to answer the following:

- What is an MSDS?
- Which MSDS section contains information on the toxicity of a chemical?
- Is your skin hydrophobic or hydrophilic?
- What does "aqueous" mean?
- What does the word "biohazard" mean?

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.

If we perform any DNA exercises, we will likely use ethidium bromide to visualize the DNA on a gel. There are two potential hazards here:

1. Ethidium bromide is a mutagen and may also be carcinogenic. Handle anything that contains ethidium bromide (ie. DNA gels) with care and dispose of the materials properly.
2. Ethidium bromide-stained gels are viewed using UV light. This can cause serious eye damage if proper eye protection is not used. We will likely be using an enclosed system to capture images of our gels – you should not be using the system unless properly trained.

Notebooks

A 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. 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. 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.

Exercises: Build a Cell

This is a thought exercise. It is meant to get you to think about cells in a way you probably haven't thought about them before. Consider the possibility of actually making an artificial cell in a lab – this is possible and some scientists are already working on this. Before you can do that however, you need to know a few things about cells.

1. What is a cell?
2. What are some of the things you will need to know about before trying to make one?

Homework 1:

Work as a **group** (4-5 students maximum) to answer the questions in the exercise above (no more than a page double-spaced) and submit them in the online assignment dropbox.

- Please be sure to indicate the members of your group in the submitted file

This is due in 2 weeks

Post-Lab Questions:

Take a look at the article here: http://goo.gl/JagXQM

Please think about these and answer them in your lab notebook:

> The article discusses the creation of a prokaryotic cell. How much more difficult would it be to create a synthetic eukaryote? Why?

Lab 2: Calculations of Preparation of Solutions

Today's lab 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:

- 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 website (https://urlzs.com/VTLRW) and read the first 2 sections dealing with homeostasis (they're very short). Based on that reading and what you know about diffusion and osmosis (from BIOL 101), answer these questions:
    - What is homeostasis?
    - Would it be okay to keep cells for an experiment in pure water? Explain.
    - Is water a good buffer? Explain.

(If you don't remember your CHEM 101 class, it may help to check this link to refresh your memory about pH and buffers).

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 along with the next one are meant to help you develop some of those basic lab skills that will make you more confident and capable when you do step into 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 several other labs later this semester – careless mistakes made today will affect you in a few weeks.

Units

In biological research labs, we tend to deal with very small amounts of chemicals. The units of measurement commonly used are milliliter (ml) and microliter (µl), and milligram (mg) and microgram(µg). These are very small amounts, thus even a small error in measurement could actually make a big difference. It is therefore very important that you learn to use all the tools properly and pay attention to details – this will help to minimize the chances of error in the solutions you prepare.

The metric system: The metric system is a base 10 system which has specific prefixes that are associated with certain values. Below are listed a few of these representing less than unit value:

value conversions	prefix	symbol	example for volumes	
10^{-3}	milli	m	milliliter (ml)	1L = 1000ml
10^{-6}	micro	µ	microliter (µl)	1ml = 1000µl
10^{-9}	nano	n	nanoliter (nl)	1µl = 1000nl

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 x 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 – this makes the calculations a little more complicated than they are for a percent 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 give 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.

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

Example 1: Make one liter of 1M NaCl

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 in the above case, but keep in mind that the information you are given at times may ask you to calculate a millimolar solution, and you may be asked for volumes in milliliters. 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

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 $MgCl_2$.

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 (you might have to round things off to 12.11g) of Tris and 10.165g $MgCl_2$ (or 10.17g). 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 – for example: a 5%NaCl solution would require 5g of NaCl to be dissolved in 100ml of water. Similarly, for a 10% sucrose solution, you would 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).

Try a Few Calculations:
How would you prepare the following solutions?

100ml of 0.1M Tris
50ml of 1M Glucose
100ml of 10mM Tris, 1mM EDTA, 5%Glucose
50ml of 0.9% NaCl

Molecular weights of commonly used reagents:

Tris	121.14 g/mol
EDTA	372.24 g/mol
NaCl	58.44 g/mol
Glucose	180.16 g/mol

Practical Aspects of Making Solutions

Today, you will be weighing out reagents in order to prepare stock solutions that you will be using for various labs 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). In order to make your solutions you will need to properly use three common pieces of laboratory equipment: a balance, a stir plate and a pH meter.

You will also need a beaker, a stir bar, a weigh boat (or weigh paper) and a spatula. Here are a few things to keep in mind as you work today:

- 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.

Here's a **general protocol** for preparing a solution:

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 double distilled water ("ddH_2O" or "ddw") or deionized water ("DIW"). The amount of water to add is approximately half of the total volume that you will be preparing.
    - In a laboratory, water can come in several levels of purity. Double distilled water is the best choice, deionized water is a close second and is often used if ddH_2O is not available.
    - DIW is used frequently because it is easier to obtain – many labs have special filters installed on some of their water taps (they usually have a white spout), whereas double distilled water (or Millipure water which goes through a special filter) requires special equipment and has to be stored in large water reservoirs.
    - 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 your beaker (it should already have a small amount of water in it – see 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.

    - 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.

6. 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.

7. Start the motor on the stir plate to initiate stirring. Don't turn it up too high for 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.

8. Maintain the stirring until the chemicals have completely dissolved.

9. 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.

Adjusting the pH of your Solution:

1. 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.

2. 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.

3. 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.

4. 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.

10. 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.

    - Employ 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.

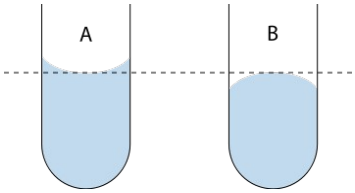
11. Hold the cylinder up to eye level and add water until the bottom of the meniscus is at the desired mark.

    - **Do not overshoot the volume**. If you add too much water, you will have to start over.

12. 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.



13. Pour the newly-made stock solution into a well-labeled storage vessel (usually a screw-capped bottle). The 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.

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

6. Rinse the pH probe/electrode with some DIW before placing it in your solution.
7. 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.
8. 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.

9. Add the acid or base drop-wise and let the solution equilibrate after each drop. Wait for the pH meter to stabilize.
10. When you get to the desired pH, you're finished.
11. Rinse the electrode well with DIW, and place it back in its storage solution.
12. Turn off the pH meter or set it to standby mode.
13. 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.

Exercises: 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.**

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 0.2M Succinate (succinic acid), pH 7.2
- 100ml of 66.6mM Na_2HPO_4, 33.4mM NaH_2PO_4, pH7.2
- 30ml of 1M D-Mannitol
- 30ml of 1.0M NaCl
- 30ml of 0.1M KCl
- 30ml of 0.1M of $MgCl_2$

Molecular Weights:

D-Mannitol	182.2 g/mol
NaCl	58.44 g/mol
KCl	74.55 g/mol
Na_2HPO_4	142.0 g/mol
$NaH_2PO_4 \cdot 2H_2O$	156.0 g/mol
$MgCl_2$	95.2 g/mol
Succinate	118.1 g/mol

2. Preparation of Solutions

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. Use the general protocol for making solutions to prepare the solutions from exercise 1.
    - 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.
2. Wash out your beakers and stir bars to reuse them as you make the solutions.
3. 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)
4. 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)
    - You can write with a sharpie directly on the Falcon tubes (use the white label area)

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 15 lbs/in^2). 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:

- We will be using the solutions you prepared today to make dilutions in the next lab. Some of the solutions have a specific pH already set. When we dilute them, their pH will not change. Use the Henderson-Hasselbalch equation to explain why.

Lab 3: Complex Solutions and Micropipetting

In today's lab you will continue to learn about the preparation of solutions. In this case, you will use the stock solutions you prepared in the previous lab to make some more complex solutions for use in later labs. Along the way, you will also be introduced to some of the tools you will need to be able to use properly in later labs – not just in this course, but in other courses and in many potential research positions.

Learning Objectives:

Students will:

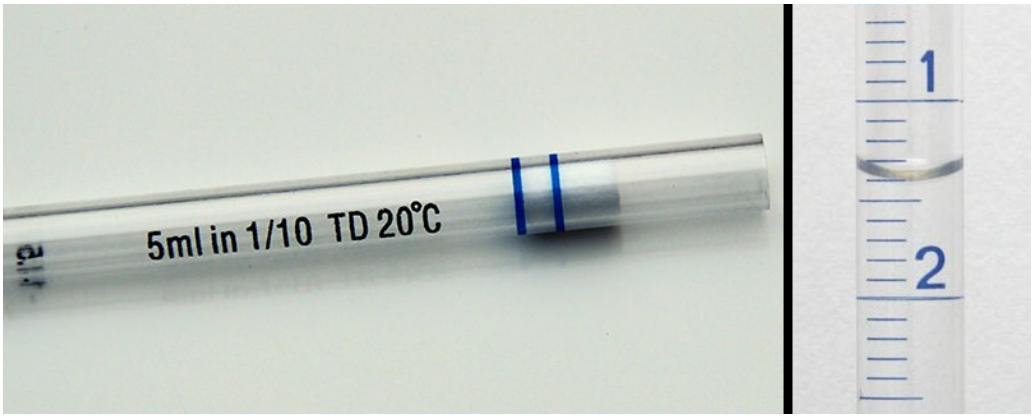
- Explain why the dilution of a buffer will not affect it's pH
- Perform the calculations to correctly prepare solutions using concentrated stocks
- Explain how a micropipettor works and how to use it properly
- Use volumetric tools with accuracy

Pre-Lab Questions:

Please view the videos linked here and here, and read the material on this webpage.

Also, feel free to use any other resources to help you answer the following:

- What is the difference between a serological pipette and a Mohr pipette?
- Find out what the "TD" and "TC" markings mean. Why might they be important?
- List all the pieces of information on the pipette on the left side of the image below.
- How much liquid is in this pipette? (hint: the answer isn't as simple as it looks)



- Make the calculations to prepare the solutions in Exercise 3 on page 30.

Volumetrics

As you found out last week, preparing solutions by weighing out reagents, dissolving them in the right amounts of solvent, and adjusting pH can be quite time-consuming – you wouldn't want to do it every time you needed to make a solution. For this reason, most shelves in research labs contain large bottles of "stock solutions". Stock solutions ("stocks") are simple, and usually fairly concentrated, solutions of components which are commonly needed in the preparation of a variety of more complex 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, and just need to find out the volume that you will need to use of the initial stock solution ($V_{initial}$).

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

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

$$V_i \times 95\% = 25ml \times 50\%$$

Rearranging: $V_i = (25ml \times 50\%) / 95\%$

$$V_i = 13.16ml$$

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.

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:

$$V_i \times 500mM = 200ml \times 50mM$$

Rearranging: $V_i = (200ml \times 50mM) / 500mM$

$$V_i = 20ml$$

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.

Try a Few Calculations:
How would you prepare the following solutions? *(don't look at the answers until you're done)*

1) 100ml of 0.1M Tris, 10mM EDTA , pH7.4
2) 50ml of 0.01M Tris, 0.025M EDTA, 0.1M NaCl, pH7.4
3) 100ml of 10mM Tris, 1mM EDTA, 5% Glucose, pH7.4
4) 10ml of 10mM Tris, 50mM KCl, 1.5mM $MgCl_2$, pH7.4

You have the following stock solutions:
1M Tris, pH 7.4
500mM EDTA, pH 7.4
1M NaCl
20% Glucose
0.1M KCl
0.1M $MgCl_2$

Answers:
1) 10ml of 1M Tris, 2ml of 0.5M EDTA
2) 0.5ml of 1M Tris, 2.5ml of 0.5M EDTA, 5ml of 1M NaCl
3) 1ml of 1M Tris, 0.2ml of 0.5M EDTA, 25ml of 20% glucose
4) 0.1ml of 1M Tris, 5ml of 0.1M KCl, 0.15ml of 0.1ml $MgCl_2$

Once you have made the calculations and know how much of each component to use in the new solution, you would add them all together and then bring the volume up to the "final volume" using distilled water. Please note that, if you are using buffers, the addition of the water would not affect the pH of the solutions because it would not change the ratio of acid to conjugate base in the buffer.

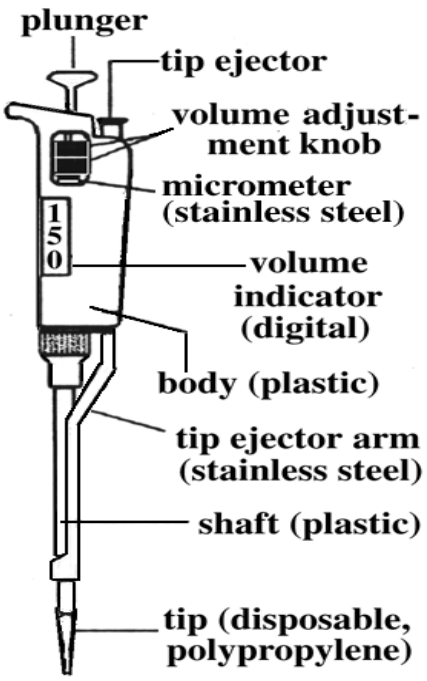
Introduction to Micropipettor Use

Now that you have practiced calculations and conversions, you may have noticed that some of the volumes can be quite small. You will thus need to use a tool which is capable of accurately measuring such small amounts – this is the micropipettor.

Micropipettors are very common in laboratories of all kinds, so learning how to use one properly will definitely help you in the coming years. It is one of the aims of this lab, that you will 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 and many others 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)



Source:
biology.hunter.cuny.edu/tech/TF-7.htm

- **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. It is not a pen. 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 disposable tip on it**.
- Use the appropriate micropipette tips for your istrument.

Types of Micropipettors

There are usually three micropipettors in a set - these are often referred to as a "P1000", "P200" and a "P20" (although there are other types as well). These names are based on the names given to the micropipettors produced by a company named "Gilson" - these were the first adjustable micropipettors on the market. Micropipettors are now produced by many different manufacturers, but the "Gilson names" tend to be applied to all of them for convenience.

The names are based on the maximum volume each of these instruments is designed to handle. You can usually tell which type of micropipettor you have by looking at the plunger or the body of your instrument – the Gilson brand micropipettors will have the name printed on the plunger, while other brands will just indicate their maximum and minimum volumes.

- 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, but you can use a P200 for volumes in that range)

Micropipettors: P20, P200, P1000.

Micropipette tips for a P1000, a P10 and a P20 or P200

You may also notice that your instrument has either blue or yellow colour-coding – this is to help you select the appropriate disposable micropipette tips. The blue tips are to be used with a P1000, while yellow tips are used with P200 and P20 micropipettors. A micropipettor like a P5 or a P10 would use a smaller tip (clear) – the yellow tips don't fit them.

Important

Never lay a micropipette down with a filled tip or hold it upside down or sideways. The liquid will not leak out if you hold it upright but it may enter the instrument if you hold it upside down, and contamination will result.

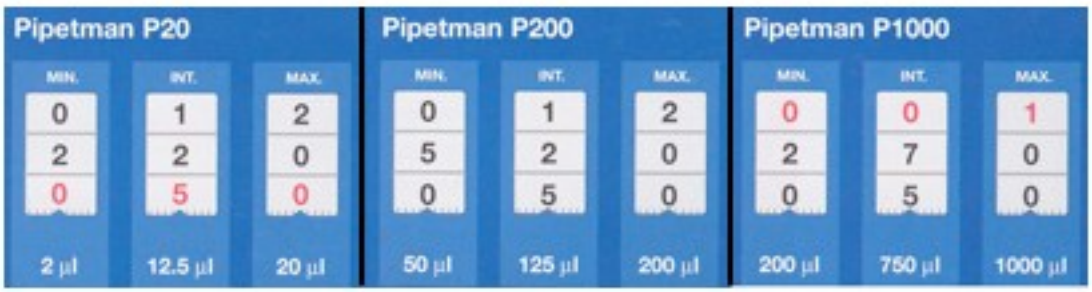
Exercises

In the first two exercises you will practice setting the volume on the micropipettes. Your TA will likely lead you through this first part. Please follow the instructions below and ask questions if you're unsure about anything. Each person in your group should set at least one micropipettor and have it checked by other group members and/or your instructor.

Look at the top of the micropipette to identify its measuring range. Remember that the highest value listed on the top is the largest volume you can measure on that pipette.

- On a 100 to 1000µl micropipette, the largest measurable volume is 1000µl
- On a 20-200 micropipette, it is 200µl. Likewise, the smaller value in the range is the smallest measurable volume
- On a 2-20µl micropipette, the smallest measurable volume is 2µl

Look at the volume indicator window and try to determine the volume your instrument is currently set to. Think about how you would set a micropipette to 0.45ml, or 0.15ml, or 0.015ml. Which micropipette would you use in each case? What are these volumes when converted to µl? You should practice doing that kind of conversion in your head, it will be useful when working in a lab.



Volume indicators of Gilson instruments at different volumes.

Source: *http://bio305lab.wikidot.com/appendix:pipettes#toc7*

Materials

- 2 coloured solutions
- distilled water
- small beakers or flasks (4)
- 1.5ml micro-centrifuge tubes
- Micro-centrifuge tube rack
- set of 3 micropipettes
- box of 20-200µl tips
- box of 100 – 1000µl tips

1. Micropipetting Practice

Part 1

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

Part 2:

1. Set your micropipettor to its highest volume (never set it outside it's working range)
2. Put on a pipette tip (by gently tapping it on – if you do it too hard, the tip will be difficult to remove later)
3. Depress the plunger knob to the first stop
4. Insert the tip into the liquid to be measured (just underneath the surface of the liquid)
5. In this case, you can use any solution you have available
6. Gently let the plunger return to the resting position
7. Withdraw the tip from the sample solution.
8. Hold the micropipettor vertically (do not hold it at an angle) and take a look at the liquid inside the tip
9. Are there any air bubbles in the liquid? (the air above the liquid does not count)
10. if you see air at the bottom of the tip then your technique needs work
11. Place the tip against the wall of the receiving vessel
12. Slowly depress the plunger to the first stop
13. Depress it further to the second stop
14. Remove the tip from the vessel
15. Gently let the plunger return to the resting position
16. Press ejector button to remove the tip into a waste container
17. Repeat this exercise after setting the instrument to the lowest volume in its working range

The Micropipetting Cycle

The figure below shows the complete pipetting cycle and the position of the plunger for each step.

1. With a tip in place on the shaft, press down on the plunger knob to the first stop (A).

2. Immerse the tip into the liquid to be measured (go only about 5mm below the surface), and gently let the plunger return to the resting position (B).

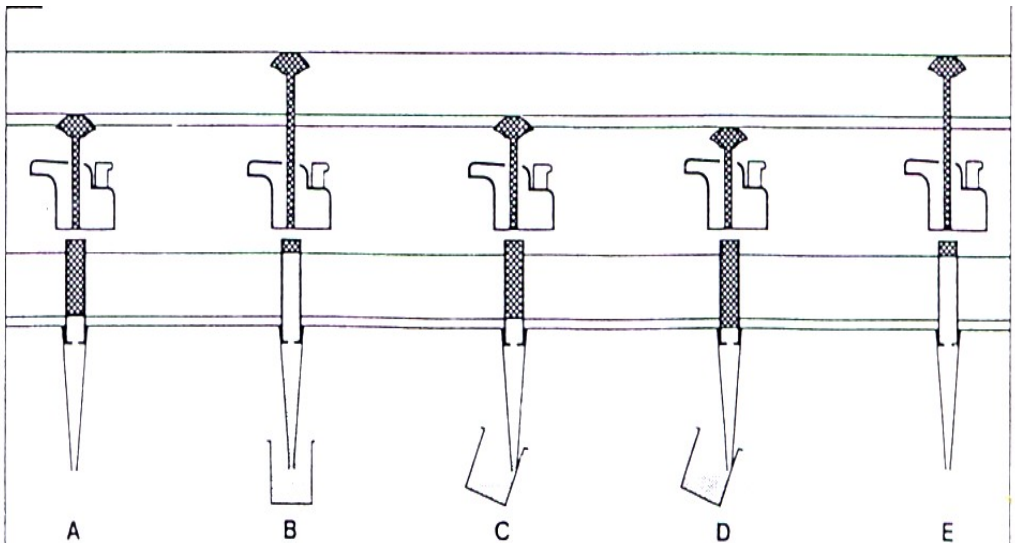
3. Withdraw the tip from the sample solution.

4. Place the tip against the wall of the receiving vessel and slowly depress the plunger to the first stop (C).

5. Wait one second, then depress it further to the second stop (D).

6. Gently let the plunger return to the resting position (E).

7. Remove the tip by ejecting it into a waste container.



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

2. Preparation of Solutions with Small Volumes

Part 1

1. Have a graduated 1.5ml micro-centrifuge tube in a rack ready to hold the liquid you measure in the next steps. Micro-centrifuge tubes are often called Eppendorf tubes or eppi tubes. Eppendorf is a popular brand of lab ware.

2. You will be pipetting 600µl of colored sugar solution. The color helps you see how much you are measuring. Choose the correct size micropipette and set it to 600µl.

3. Place a tip on the end of the pipette. Do not touch the tip with your hands. Leave it in the box and push the end of the micropipette firmly into the tip.

    - The smaller tips fit both the 2-20 and the 20-200µl micropipettes. They are often yellow or clear. The larger tips are for the 200-1000µl micropipette and are sometimes blue.

4. Press down to the first stop. Submerge the end of the tip just under the surface of the liquid. You may rest the tip against the side of the container just under the water line to steady it. If you submerge more than just the end of the tip, liquid will collect on the sides of the tip and drip into the collection tube when you deliver it. This will result in a larger volume of liquid than was desired.

5. Slowly release the plunger. If you release the plunger too quickly, the liquid may splash up into the micropipette and contaminate it. If you are pipetting viscous (thick) liquids, such as the sugar solution you are using, and you release too quickly, the liquid won't enter the tip fast enough and your measurement will be inaccurate.

    - Sometimes this happens with thin liquids as well, so you should always pipette slowly. Be careful not to remove the tip from the liquid before it is filled with the desired volume or you will get an air bubble in the tip and less liquid than was desired. If you released the plunger slowly and kept the tip in the liquid but you still got a bubble, you probably pushed the plunger down to the second stop instead of the first. Practice the stops again.

6. Without removing the tip from the beaker, dispense the liquid by pushing the plunger slowly down to the first stop. Try not to make any bubbles. Repeat step 4. Drawing up the liquid twice (in labs it is called "pipetting up and down") can improve the accuracy of the measurement.

7. Dispense the liquid into 1.5ml microcentrifuge tube and be sure it is near the 0.6ml mark (600µl = 0.6ml). This is just a check to make sure you used the correct micropipette and set it correctly. Show the instructor your tube.

8. Discard the tip in a waste beaker by pressing the eject button. You may want to practice this technique a few times, as it is a very important skill to master

Part 2

In this part, you should work as a group. You are going to measure different colors and amounts of water into 10 micro-centrifuge tubes. Being careful not to contaminate all of the sterile tubes, you will remove 10 microcentrifuge tubes from their container. Your TA may demonstrate this for you. You will then label the lids and place them into an eppitube rack. Always label the tubes on their lids so that it can be read without removing the tube from the rack, and orient the tubes in the same direction so that you won't confuse letters like "H" and "I" and numbers like "6" and "9". Also, only use a permanent marker, such as a Sharpie, that will not erase or bleed if it gets wet. If your tubes are to be stored or spun in a micro-centrifuge etc., then it is good practice to label your tubes with a group name, or your name, and the date of the experiment.

1. Obtain 10 eppi tubes.
2. Open all of the lids of the tubes so they are ready to receive the solutions.
    - Before you begin measuring, think of what will be the most efficient and least wasteful way of dispensing the liquids.
        - If several of the tubes contain the same liquid, you can measure them all out before you change tips, as long as you do not touch the tip to the inside of a tube containing some other solution. Even water can be a contaminant if it changes the concentration of a given solution.
        - You may also want to first fill all of the tubes that have the same measure of liquid so you don't have to change the setting too often.
        - Sometimes it matters which ingredient is added first or last, as is the case when diluting acids and bases, or when adding enzymes.
3. Measure the following amounts into the indicated tubes.

Tube #	Contents	Tube #	Contents
1	5µl Solution 1	7	100µl Solution 2 20µl Solution 1
2	10µl Solution 1		
3	100µl Solution 1	8	500µl Solution 2 20µl Solution 1
4	1000µl Solution 1		
5	5µl Solution 2 20µl Solution 1	9	1000µl Solution 2 20µl Solution 1
6	20µl Solution 2 20µl Solution 1	10	500µl Solution 2 500µl Solution 1

4. Mix the contents by pipetting up and down several times. DO NOT pipet so vigorously that you make bubbles.
    - This can degrade some sensitive solutions such as enzymes, and can also contaminate the micropipette.
    - You may want to close the tubes as they are filled or move them back one row to avoid accidentally filling the same tube twice.
5. Put the tubes in a microcentrifuge in a "balanced configuration". Call the instructor to check the balance of your tubes before spinning.

- Tubes must be balanced or the rotor of the centrifuge can become damaged because of the very high forces being exerted on it. This can cause damage to the instrument and possibly serious injury to you.

6. Spin the tubes at high speed for about 30 seconds to "spin down" the liquid so it is all in the bottom of the tube.

7. In cases like this, the actual speed and amount of time do not matter too much. You're simply trying to apply enough force to the samples to push all the liquid to the bottom of the tube.

8. Remove your samples from the centrifuge.

9. Check the accuracy of your measurements by setting a micropipette to the total volume in the tube and slowly withdrawing all of the solution from each tube.

    - Your pipetting was accurate if you leave no solution behind and have no air bubble in your tip.

    - The amounts in tubes 1 and 2 are so small that if any is clinging to the side you won't be able to draw it up. You would especially want to centrifuge these samples.

Also have your instructor check your tubes before discarding them, as he or she may wish to watch you draw up the amount to check accuracy.

3. Preparation of Solutions for Future Labs

In this part of the lab, you will be using the stock solutions you made last week to prepare the solutions you will be using in an upcoming lab.

Materials

- Stock solutions from last week
- 1M D-Mannitol
- distilled water
- set of 3 micropipettes
- box of 20-200µl tips
- box of 100 – 1000µl tips
- test tube rack for Falcon tubes
- 50ml Falcon tubes (5)
- 1ml, 2ml and 5ml pipettes (4-5)
- pipette aid (2)
- 50ml graduated cylinder

1. Prepare the following solutions:

- 20ml of **Grinding Buffer**
  0.4M Mannitol, 20mM Phosphate, pH 7.2, 1mM EDTA
- 20ml of **Assay Buffer**:
  0.4M Mannitol, 20mM Phosphate, pH 7.2, 5mM $MgCl_2$, 10mM KCl

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Homework 2:

Based on our discussion of Scientific Literature today, Your job is to find 2 other review articles (**not** ones we looked at in class) about apoptosis. Over the course of this week, you should **discuss** them with other girls in the class – help each other figure them out (some of the articles can get very technical).

Work **individually** to answer the questions on the form on the next page (p.33) and submit them in the online assignment dropbox.

This is due in 2 weeks

Post-Lab Questions:

Please do a little research, think about these, and answer them in your notebook:

- What does EDTA do? Why is it used in biological buffers?

The isotonic buffer will be used in a future lab to store organelles that will be collected from lysed cells, while the the assay buffer will be used when setting up enzymatic reactions with these organelles.

Why do these buffers only differ by the presence of EDTA? Why would you not want EDTA in a reaction buffer?

Name: _____ Student ID: _____

Title of Article 1:
Citation for Article 1 in CBE/CSE format:
Title of Article 2:
Citation for Article 2 in CBE/CSE format:
1. List three things you learned from the articles that you didn't know before.
2. Which of the two articles did you like better? Why?

Lab 4: Microscopy Basics

In this part of the course, we will learn some of the ways in which microscopy is used in the study of cells. We will discuss some aspects of sample preparation for microscopy, you will prepare some slides, and you will learn the basics of how to set up and use a compound microscope. Later this term, you will also learn how to use microscopes to perform viable cell counts on a cell culture, and you will learn about fluorescent microscopy. In today's lab, we will focus on the basics.

Learning Objectives:

Students will:

- Explain the difference between magnification and resolution
- Describe the functions of the various components of a compound light microscope
- Set up their microscopes to have appropriate IPD and diopter adjustments
- Describe the major steps in tissue processing and explain their purposes
- Prepare a stained slide

Pre-Lab Questions:

Read and complete the exercises on this page:

- https://h5p.org/node/312895

  Take a screenshot of your answers on this site and submit it on Blackboard to your TA **before** the start of the lab.

Microscopy

Much of what biologists wish to study is not observable by our unaided senses. For this reason, many fundamental questions about the nature of living things simply could not be answered by early scientists. As a result, many tools have been developed over time to extend our ability to observe nature. One such invention was the microscope.

Microscopes of various designs have been around for quite some time, but modern microscopy is generally traced back to the mid-17th century, when Anton von Leeowenhoek was the first to report the existence of microorganisms, and Robert Hooke coined the term "cell" to describe the small compartments he observed in cork with his microscope. Von Leeowenhoek went on to describe a variety of different cell types and made many improvements to the optics of his microscopes to enhance their resolution.

Magnification vs Resolution

Increased resolution and magnification are two crucial properties of microscopes, with resolution being the more important of the two. Magnification is the process of making an object appear larger than it really is, while resolution refers to the amount of detail that is discernible in the resultant image (Figure 1).

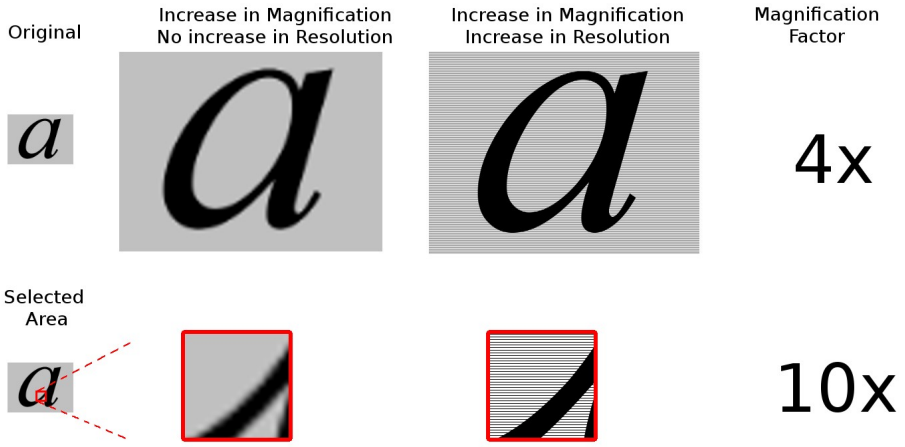


Figure 1. An illustration of the differences between magnification and resolution. The letter "a" was printed on a white background with very thin, black lines. Because the lines are very close together, they make the background appear gray. If the image is magnified without increasing resolution, no new detail is seen in the image (the background is gray). If resolution is increased along with magnification, then more detail is visible (thin lines in the background).

The lens system of a typical modern student microscope is designed to provide a resolution of about 0.5 micrometers. Careful matching of a light source and a precision lens system allows light microscopes in research labs to reach a resolution of 0.2 micrometers. This means that structures that are 0.2um apart can be seen as separate from one another.

For a compound light microscope, the specimen being examined is placed on a glass slide and viewed using light that is transmitted through the specimen. Light emitted by the light source is focused onto the specimen and projected onto the objective lens by the condenser lens assembly. The objective lens then magnifies the image and conducts it to the ocular lens. The objective and ocular lenses are responsible magnifying the specimen, but it is the

quality of the light and its focusing by the condenser assembly that determines the resolution and contrast of the image.

Parts of a Compound Light Microscope

1. **Ocular lenses** (eyepieces). One of these usually has an adjustment ring for astigmatism, so please find it and use it to help you avoid eye strain. The binocular scopes can also be adjusted for interpupillary distance (the distance between the pupils of your eyes). Adjusting both of these features will help you customize the microscope for your comfort.

2. **Objective turret or nose piece.** Allows the changing of objective lenses to change the magnification. Be careful when swinging in the longer objective lenses – make sure they don't hit your specimen.

3. **Objective lens.** These lenses are often sold as a matched set and are parfocal. This means that once you get your specimen in focus with one of these lenses, you should only require the use of the fine focus knob to bring it back into focus after changing the lenses to a different magnification.

4. **Coarse focus adjustment knob.** Used bring things into partial focus, especially at lower magnifications. Avoid using this knob at high magnification.

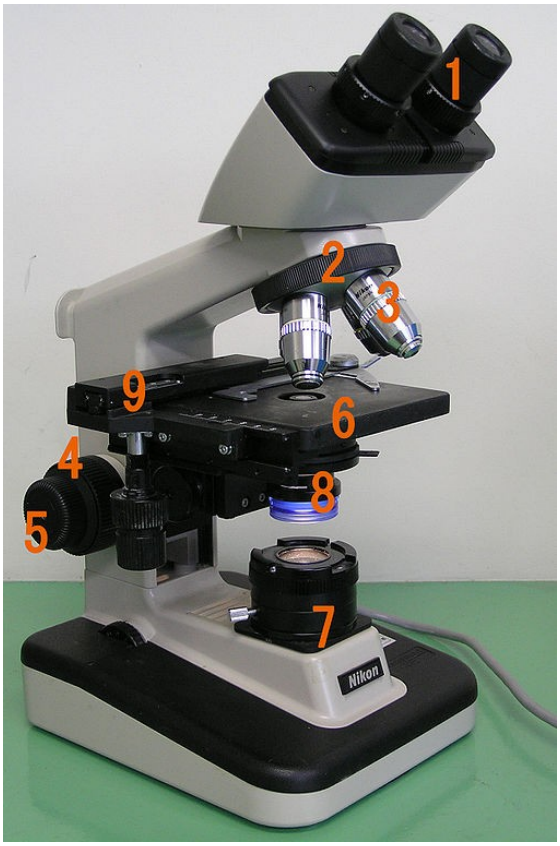


Figure 2: Parts of a compound microscope. (Image taken from Wikimedia Commons)

5. **Fine focus adjustment knob.** Used to bring specimen into better focus. Should be the only adjustment needed at higher magnifications.

6. **Stage with slide-clip and location markings (9).** The clip is used to hold the slides securely in place as you move it around on the stage with the stage adjustment knobs. The location markings allow you to note down the specific locations of points of interest on your slides in case you need to find them again later.

7. **Light source (lamp housing) with a field-iris diaphragm.** The amount of light being emitted can usually be controlled through a separate control knob, and should never be set high (to protect your eyes). The top of the structure usually has an adjustment ring to control the opening of the field-iris diaphragm.

8. **Condenser lens assembly.** It usually includes the diaphragm lenses, condenser-iris diaphragm, and condenser centering controls. The position of the condenser lens assembly is controlled by a condenser focusing knob located near the fine and coarse-focusing knobs. You will be using the condenser lens assembly when calibrating the microscope. You are also likely to periodically adjust the condenser-iris diaphragm opening while viewing slides to help increase or decrease contrast of the image in your field of view.

Exercises

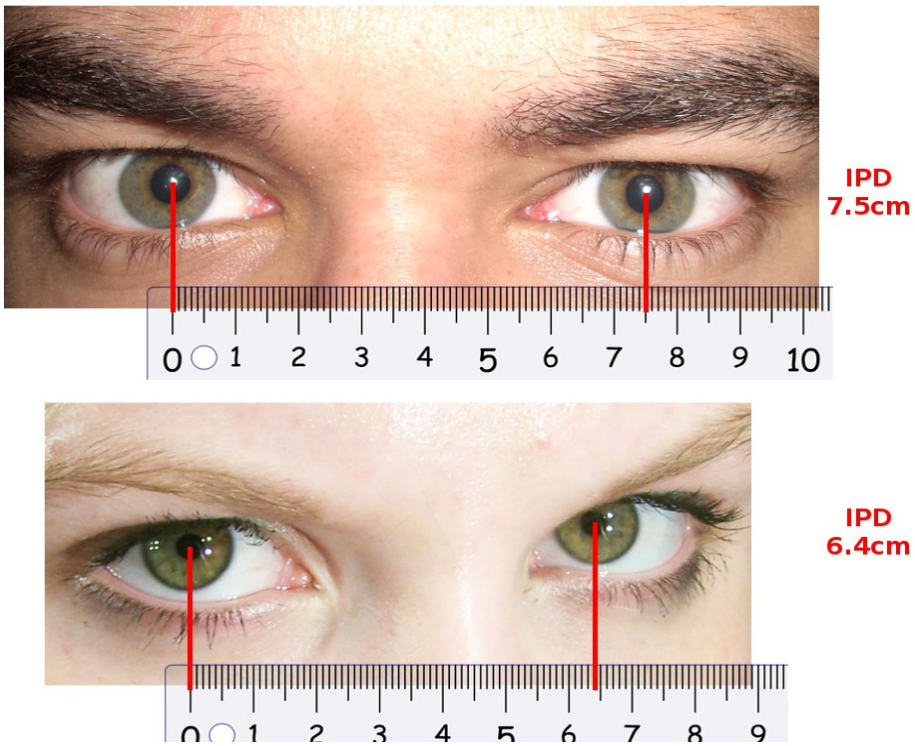
In the first two exercises, you will learn how to customize a typical microscope to your eyes. You will determine your interpupillary distance (IPD) and diopter setting. Knowing these will allow you to quickly set any microscope you might use in the future to the most optimal settings for your eyes.

Materials

- Microscope
- a prepared slide

1. Adjusting binocular eyepieces for Interpupillary Distance (IPD).

The interpupillary distance is the distance between the centers of your two pupils and is unique to each person. During your first session with a new microscope, you should determine the correct setting for your eyes and set the microscope for that distance. During subsequent sessions, you should set the microscope to that setting.



If you look through the eyepieces and see two images, the interpupillary distance is not correct. To correct it, slide the eyepieces closer together or farther apart until the two fields merge to form a single circle of light. The interpupillary distance is now correct for you.

Procedure

1. Examine the ocular eyepieces on your compound microscope and note how they move closer and farther apart.
2. Place a specimen on the stage, look down one ocular with one eye, and focus the specimen. Do not worry whether you are using one or both eyes, or one or both oculars at this point.

3. Slowly move the two oculars closer together or farther apart until you can see the specimen with both eyes through both oculars. Instead of seeing two separate fields of view – one with each eye or overlapping fields of view, you should now see only one uniform field of view if you have adjusted the two eyepieces to the correct IPD for your eyes.
4. Record your IPD from the scale located near the ocular lenses of your microscope so you can quickly set up your microscope in future labs.

My Interpupillary Distance (IPD) is _____.

2. Adjusting for vision differences between your eyes.

Those of you who wear glasses, may be familiar with the idea that each of your eyes can have different abilities to focus on objects (ie. you may need a different prescription lens for each eye). This isn't only true for people who wear glasses – even people who don't wear glasses may have some differences between their eyes. With modern microscopes, it is possible to adjust for such difference between your eyes. We do this by adjusting the "diopter" setting on one of the ocular lenses (the one that looks larger/thicker).

Procedure

1. With a slide on your microscope, locate the specimen under low power (4X or 10X) and then find a portion you wish to focus on.
2. Look at the specimen with only your right eye and get the slide into focus using the coarse and fine focus knobs. The left eyepiece is usually adjustable to compensate for refractive differences between your two eyes. Look for a diopter adjustment ring on the left eyepiece.
3. Close your right eye and look through the left ocular lens. Do not touch the focusing knobs. Adjust the focus for your left eye using only the diopter dial on the ocular lens.
4. Now you should be able to see the specimen in sharp focus for both eyes.
5. There is usually a scale under the diopter adjustment ring. Check to see if you can see any numbers on there and if they are positive or negative and record them for future reference.

My diopter setting is _____.

Sample Preparation for Microscopy

Microscopic study of unicellular organisms is relatively easy – the whole organism can be placed on a slide and studied. This is not the case with multicellular organisms – many are too large to fit on a slide and too large to allow light to pass through them. In those cases, we must remove a small cell/tissue sample from the organism in order to observe it. The problem with this is that the removal of those cells from their natural environment will cause some physical changes to their appearance and eventually their death. Thus a method was developed that preserves the appearance of such cells/tissues so that they appear the same as they would have in the body of the organism from which they were taken.

Thus, the purpose of all the steps in slide preparation is to preserve the cells and tissues and show them in as close to their natural state as possible while making their details easily distinguishable under the microscope.

During this process, the tissue sample is placed into a chemical fixative. This preserves the tissue in its "natural state" and prevents the activation of processes that might affect its appearance. This sample will later be thinly sectioned to allow light to pass through it more easily, and stained to increase the contrast between the structures on the slide and allow the detection of various details.

The above is a very simplified overview of permanent slide preparation. There are several other methods for producing slides, but this is the most common. The actual process is very prolonged and involves the following major steps: Fixation, Dehydration, Clearing, Embedding, Sectioning and Staining

Fixation

Fixation is the initial and most crucial step of the slide preparation process. It ensures that the sample will be preserved in the condition it was in at the time that it was extracted. One of the main functions of the immediate use of a fixative is to terminate any cellular function in the specimen. This accomplishes a few things:

- It ensures that degradative processes associated with cell stress and cell death are stopped before damage to the cells can occur.
- Any microorganisms that may otherwise have caused tissue damage will be killed.
- It denatures and cross-links proteins to one another which leads to tissue hardening and increased physical strength.

Once the tissue is fixed, it will need to be infiltrated with wax before it can be sectioned. The wax will make the tissue sample hard enough to cut into the very thin sections needed for microscopy (4-6µm). The problem with this is that wax needs to enter the cells and tissues, and fill the spaces that are normally occupied by water. Since water and wax do not mix, water needs to be completely removed from the tissues before wax can be applied. This is why the next two steps are performed.

Dehydration and Clearing

The replacement of water with something that is miscible with wax is a two stage process. First the water is replaced by ethanol (dehydration), and then the ethanol is replaced by an organic solvent like xylene or toluene (clearing). The ethanol is used because it is miscible with both the water and the organic solvents. This process is gradual so that the tissue is not damaged or distorted. At the end of this, the sample should be ready for infiltration by wax.

Infiltration and Embedding

Infiltration is the process of replacing the clearing agent inside the cells and tissues with an embedding material – usually melted paraffin wax.

Embedding involves carefully placing and orienting the infiltrated tissue into a mold containing solidifying paraffin wax. This mold will then be used to allow the wax around the specimen to set and form a solid support (known as a block) that can be easily held and manipulated by the apparatus used for sectioning.

Sectioning

Sections for light microscopy are usually cut using a precision tool called a microtome, which allows us to cut sections of the tissue that are extremely thin (generally 4-10µm in thickness). This ensures that we will be looking at only one or two layers of cells under the microscope, which will make it easier to interpret what we see.

Staining

Aside for a few pigment-producing cells, most cells are transparent. Also, sectioned tissues are too thin to absorb or deflect enough light to be clearly visible. For this reason, tissue needs to be stained.

Most stains interact with their targets through a charge-charge interaction. Such stains are frequently subdivided into acidic and basic stains. An acidic stain has a chemical functional group that is anionic and can interact with positively charged cell components on a slide. Such components are often referred to as being acidophilic. A basic stain has a cationic functional group and can bind to acidic cellular components – these components are referred to as basophilic.

Exercises

3. Slide Staining

Today, you will not be working through all the steps outlined above. Instead, you will be working with slides that already contained sectioned tissue. The tissue sections on your slides are still embedded in wax. Wax will not allow your stain to penetrate and bind to anything, so it must first be removed and replaced with something that will allow your stains to penetrate easily. Since your stains were made with ethanol, we simply need to replace the wax with ethanol.

Pass the slides in the various solutions provided in the following order to obtain a tissue section stained with H&E:

Materials

- Coplin jars
- Xylene x2
- 100% Ethanol x3
- 95% Ethanol x2
- 90% Ethanol x2
- 80% Ethanol x2
- 70% Ethanol x2
- 50% Ethanol x2
- Slides with tissue sections
- Forceps

Procedure

1. Obtain a slide with sectioned tissue
2. Label your slides (there is usually a “frosted” portion on one end where you can write your labels **in pencil**)

Move slides from Coplin jar to Coplin jar in the following sequence:

3. Xylene for 5min
4. Xylene for 5min
5. 100% Alcohol for 2min

6. 95% Alcohol for 2min
7. 90% Alcohol for 2min
8. 80% Alcohol for 2min
9. 70% Alcohol for 2min
10. 50% Alcohol for 2min

11. Stain in Hematoxylin for 10-15min
12. DIp in Distilled water for ~5-10sec

13. 50% Alcohol for 2min
14. 70% Alcohol for 2min
15. 80% Alcohol for 2min
16. 90% Alcohol for 2min

17. Stain in Eosin for 2-3min
18. Dip in Distilled water for ~5-10sec

19. 95% Alcohol for 2min
20. 100% Alcohol (fresh ethanol) for 2-5min
21. 100% Alcohol (fresh ethanol) for 2-5min
22. Xylene for 2min
23. Put a small drop of Canada balsam onto the coverslip.
24. Place the cover slip on the stained tissue section.
25. Gently press the coverslip down.

Place slide on a slide warming plate for 2 days to dry.

4. Preparation of a Blood Smear Using the Leishman's Stain

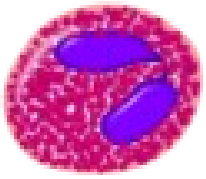
A blood smear (or blood film) is a frequently used diagnostic procedure in the health field. It allows medical doctors and pathologists to microscopically examine the various blood cells and quickly diagnose disorders that tend to show up in the blood. The blood smear is so common because it is quick and easy to prepare and can yield some important information about the health of a patient.

Your blood contains a mixture of cells. A majority of them are erythrocytes (red blood cells), while a small proportion (about 1-2%) are commonly known as "white blood cells" (leukocytes). The leukocytes are subdivided into several cell types, and tend to be found in the bloodstream in fairly consistent proportions. Noticing abnormal levels of one cell type or another can give doctors clues about the health problems that a patient might be presenting.

The most common of the leukocytes is the **Neutrophil**. This cell is highly phagocytic and is frequently used for defence during bacterial infections. It makes up about 50-60% of all leukocytes in your bloodstream. It is fairly easy to identify because of it's multilobed nucleus (the nucleus is elongated and very irregular in shape).

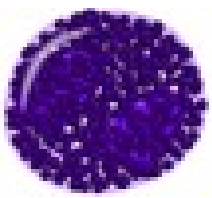
Another leukocyte you're likely to frequently (30-40%) see on a blood smear is the **Lymphocyte**. Lymphocytes are the cells involved in fighting viral infections and producing antibodies. These cells are relatively small (about the same size as a red blood cell) and show very little cytoplasm surrouding a round and fairly intensely-stained nucleus.

Monocytes make up about 8-10% of the white blood cells in your bloodstream. These cells are also very highly phagocytic, but they tend to migrate out of the bloodstream and differentiate into other, more specialized phagocytic cell types. Monocytes are large cells, characterized by a large, pale, and slightly indented nucleus ("kidney-shaped").



Eosinophils are the cells involved in allergic reactions and in fighting parasitic infections. They make up about 2-5% of the leukocytes in the bloodstream and are characterized by very intensely staining granules and a bi-lobed nucleus (the nucleus is divided into two sections which are connected to each other). The granules of an eosinophil stain with a dye called Eosin and look pink or red on most slides.

The least common (<1%) of the leukocytes is the **Basophil**. These cells also appear to be involved in allergic reactions to some extent, but it is very unusual to see many of them in a blood smear. In fact, seeing many basophils in a patient's slide probably indicates some very serious/fatal health problems. Basophils also contain large granules, but these are basophilic. Stains that bind to these granules tend to be blue or purple. For this reason, it is often very difficult to see the bi-lobed nucleus of a basophil (it's the same colour as the granules).



(Images taken from Wikimedia Commons)

In this exercise you will be producing two blood smears for viewing under a microscope.

Materials

- Leishman's stain
- Glass slides
- Large coverslips (if available)
- Water, pH 6.8
- Running water (from a tap)
- Blood sample

Procedure

The Blood Smear

1. Place a small drop of blood in the middle of one end of a slide, near the labelled end (usually frosted).
2. Use the side of another slide as a spreader. Place the edge of this "spreader" against your slide and hold it at an angle (~30-40°) pointing away from the drop of blood.
3. Slide the "spreader" towards the drop of blood until it touches the edge of the droplet. The blood is likely to then spread sideways along the edge of the "spreader" by capillary action. You can slightly wiggle the "spreader" to help the blood migrate sideways.
4. Once the blood has spread from one edge of the slide to the other edge, pull the "spreader" down the length of the slide to "pull" the blood and spread it along the slide. You should do this in one smooth motion. Ideally, the smear should cover a majority of the slide and should end in a feathered/rounded edge.

Figure 3: Two peripheral blood smears suitable for characterization of cellular blood elements. Left smear is unstained, right smear is stained with Wright-Giemsa stain. (Image taken from Wikimedia Commons)

The Leishman's Stain

1. Allow the slides to air dry.
2. Put 4-8 drops of Leishman's stain on the slide (should be enough stain to cover the surface of the slide but not spill over the sides) and incubate at room temperature for 3 min. Leishman's stain includes methanol which will act as a fixative and preserve the cells.
3. Add an equal amount of buffered water, pH 6.8 (if you added 4 drops of stain, then add 4 drops of water). Add water slowly and mix it in with the stain by sucking the stain up and down with a plastic pipette.
4. Incubate the slides with the diluted stain for another 12 min. It is common, at this stage, to see some "filmy residue" on the slides – this is normal and can be ignored.
5. Wash off the stain under a slowly-running tap. Then put the slide down again, flood it with buffered water, pH 6.8 and incubate for one more minute.
6. Air dry the slide and mount with a coverslip if available.

Lab 5: Cell Counting and Viability

In today's lab, we will be using Haemacytometers to count cells and to determine the health of a cell culture by using a dye exclusion assay. This is a very commonly used set of tools in cell biology labs – in many cases you can't start your experiment until you've done a proper cell count and checked cell viability.

Learning Objectives:

Students will:

- Explain why checking cell concentration and the viability of a sample is important
- Explain how the dye exclusion assay works
- Properly set up a haemacytometer for use
- Perform an accurate cell count and determine the concentration of a cell sample

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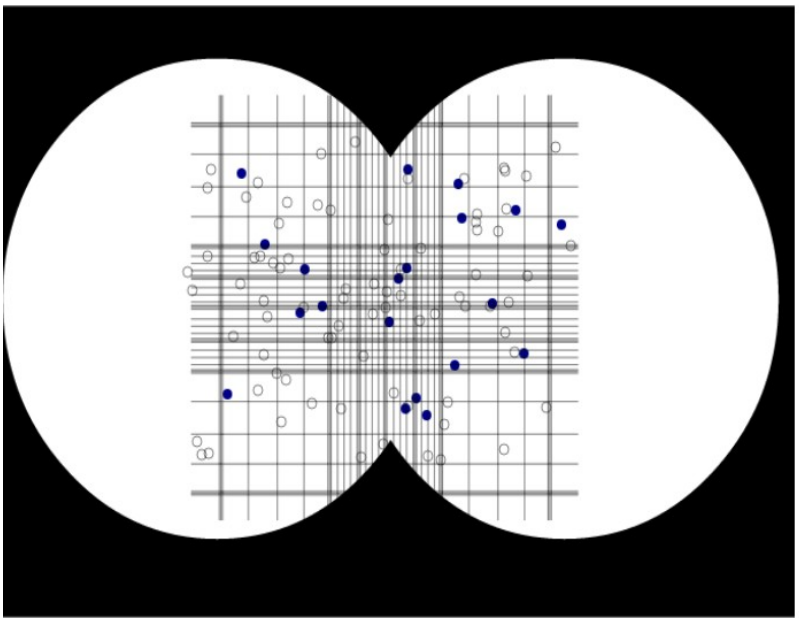
Pre-Lab Questions:

Watch the video linked below:

- youtu.be/WWS9sZbGj6A

Go to the virtual lab at this link, click on binocular view and change the "No. of Squares Counted" to 5. Perform a cell count – fill in the table on the right – take a screenshot and submit it on Blackboard to your TA **before** the lab.



TRINOCULAR VIEW

Select Lens

10X

Original volume of sample1 ml

No: of squares counted

4 5

	Viable cell count	Dead cell count
Square1	0	0
Square2	0	0
Square3	0	0
Square4	0	0
Total		

Percentage cell viability

Total Cells: 0

The Haemacytometer

Cell counting is done in a Haemacytometer, which is basically a specialized microscope slide that has raised edges ("shoulders") to support a coverslip, and a set of lines ("Grid") etched into it to divide up the sample and make it easier to determine the cell concentration.

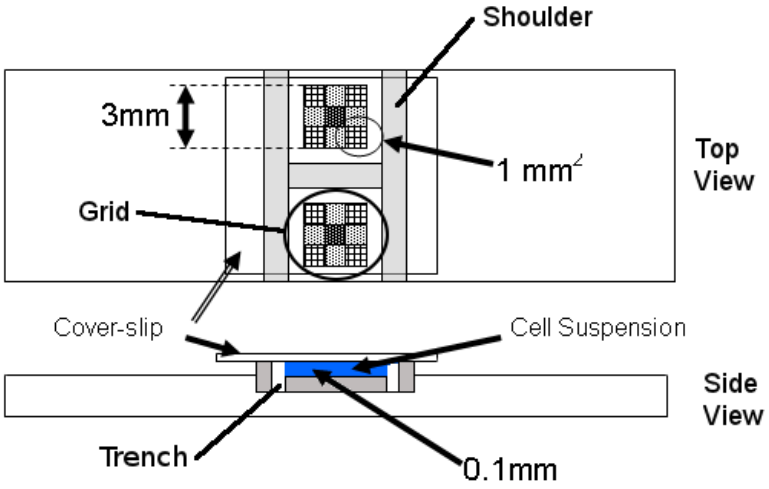


Diagram of a typical Haemacytometer, shown from the top and from the side. The dimensions of the counting chamber allow us to accurately determine the cell concentration of the applied sample.

Because the hemacytometer has an exact volume under the coverslip, one can determine the concentration (cells/ml). In general, cells in one of the large corner squares (like the one labeled as "1 mm^2" in the above diagram) of the chamber are counted. Because the dimensions of that square are 0.1cm x 0.1cm (equivalent to 1mm^2) and the depth of the chamber is 0.01cm, the volume of liquid under that one large square is 0.0001cm^3 or 0.0001ml (because 1cm^3 = 1ml). Thus, once the cells in the above square have been counted, the concentration is easily determined by dividing the number of cells by the volume of that area of the chamber. The cell concentration of the original cell suspension will be the same as that of the chamber – except for any dilutions made.

Because the cells in the counted sample are randomly distributed, it is important to ensure that the result of the count is confirmed by counts of other areas of the counting chamber. For this reason, all four of the corner squares are counted. This helps to ensure that any bias in terms of the distribution of the cells in the counting chamber is removed from the final number.

Exercises

One of the challenges faced by cell biologists is that cells are generally very small and it is very difficult (especially in cell culture) to visually differentiate between living and dead cells. However, the success of many experimental manipulations depends on starting with a healthy and abundant cell population. For this reason, biologists will often perform viable cell counts on their cell samples before continuing with their experiments.

Since we know that cells use their membranes to control the flow of molecules into and out of the cell, we can use this ability to help us make that distinction. Dead cells do not control the flow of molecules across their membrane and will allow the accumulation of a variety of dyes like trypan blue inside their cytoplasm (and appear blue under the microscope). This is in contrast to living

cells , which will exert control over what will enter and will exclude such dyes or actively pump them out if they do manage to enter (and appear white). Thus, living cells can be distinguished from dead cells and a percentage of viable cells in a sample can be calculated.

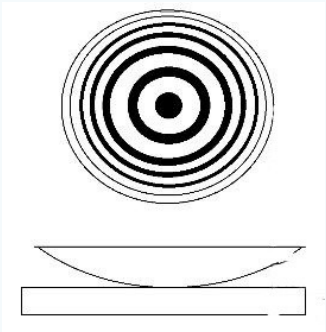
The purpose of today's experiment is to give you some practice using a counting chamber and for you to determine concentration (cells/ml) of a cell culture, as well as to determine the percentage of living cells in the provided sample.

Materials (per group):

- Eppendorf / Microfuge tubes
- Micropipettor (P20)
- Haemocytometer / counting chamber
- Cell Suspension
- 0.4% Trypan Blue Stain
- Microscope

Newton's Rings

A set of alternating light and dark circles, caused by interference of light waves passing through a convex lens, placed curved side down, and reflecting off a flat piece of glass.



1. Preparing the Haemacytometer

Procedure:

1. Clean the Haemacytometer with 70% ethanol.
2. Wipe it clean with a Kimwipe
3. Moisten the “shoulders” of the haemacytometer with a small amount of water (use a micropipettor for this)
4. Cover the chamber with a coverslip

You may need to apply gentle pressure to the coverslip on the “shoulders” of the hemacytometer until “Newton's rings” are visible. This ensures the appropriate distance between the coverslip and the bottom of the counting chamber.

2. Cell Preparation

5. Obtain the cell suspension and mix gently before taking a sample for analysis.
6. Transfer 20µl of cells into an Eppendorf tube
7. Add 20µl of 0.4% Trypan Blue stain
8. Mix gently again.

You now have a 2x dilution of cells – you will need to keep that in mind when calculating the actual cell concentration of your original sample.

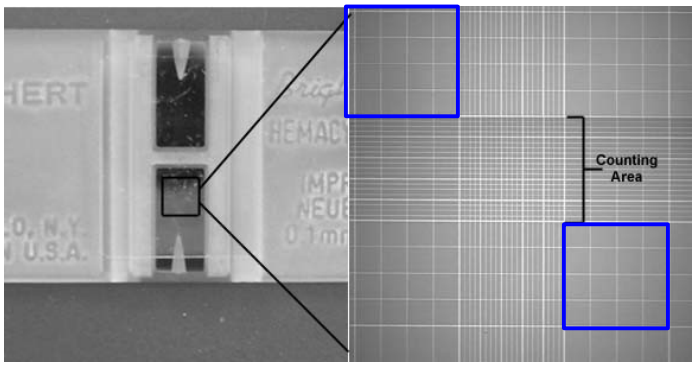
3. Using the Heamacytometer to Obtain a Cell Count

9. Take 10µl of the stained cells (don't forget to make sure they're well mixed)
10. Slowly apply a small amount of the sample at the edge of the coverslip of the counting chamber and allow the liquid to be drawn under the coverslip by capillary action. The release of the initial pressure you applied to the coverslip to generate the “Newton's

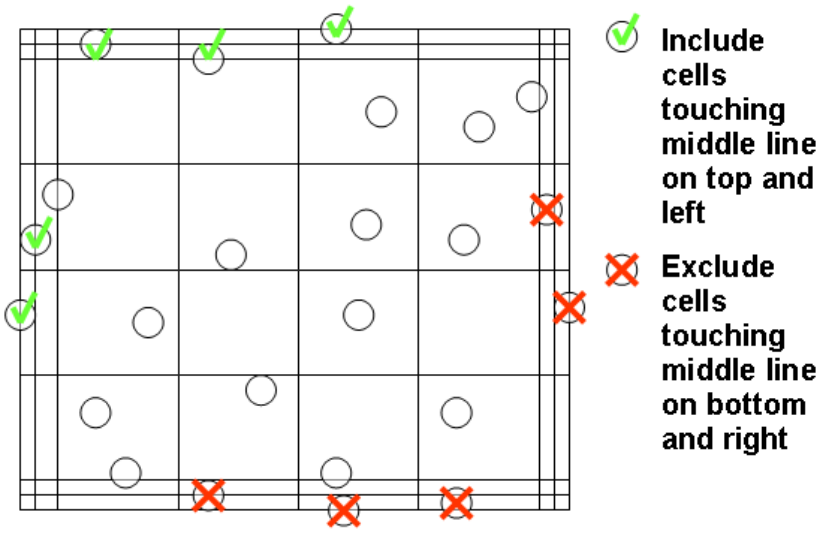
rings" will also help to pull the sample in – it probably won't take the full 10µl to fill the chamber.

(do not over-fill the chamber – the cell suspension should not spill into the "trench")

11. Place the Haemacytometer under a compound microscope and set the objective lens to 10x
12. Find the central counting area of the Haemacytometer, then identify the four larger areas at the corners. Each of these has a volume of **10^{-4}ml (0.1µl)**.



13. Count the cells in 5 of the **large** squares (like the ones outlined in blue above)
14. Begin counting the cells in one of the large corner squares. To ensure accuracy and consistency in the counts, please use the figure below to guide your counting.



The corner squares are outlined by a triple line. The above system will help you decide when to count cells that are on the boundary of the square. In general, if a cell is on the outer boundary of the square and it is touching only the outer line, it is not counted.

If the cell number in the large square is much more than 50-60 cells, then go back to step 1 and take a smaller sample of the cell culture. For example, you could take 40µl of the cell culture and 160µl of the Trypan blue stain to make a 5x dilution, or 20µl of the cells and 180µl of the stain for a 10x dilution.

If the cell number in the large square in lower than 5-10 cells then the original suspension should be centrifuged to pellet the cells and resuspended in a lower volume of buffer. At that point you should repeat the protocol from step 1.

15. Be sure to keep track of the living and dead cells so that you know the total cell count and a viable cell count.
16. Count the cells in the remaining four squares. Determine the total cell concentration of the original sample (living and dead cells together).

Counting Chamber #1	Square 1		Square 2		Square 3		Square 4		Square 5	
	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Cell Count										
Volume	0.1µl		0.1µl		0.1µl		0.1µl		0.1µl	
Conc. in Epi tube										

17. Count the cells in the five large squares of the second counting chamber. This will help you confirm the accuracy of your counts from the first chamber – the numbers should be relatively similar. Counting the second chamber will also make the math a bit easier later.

Counting Chamber #2	Square 1		Square 2		Square 3		Square 4		Square 5	
	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Cell Count										
Volume	0.1µl		0.1µl		0.1µl		0.1µl		0.1µl	
Conc. in Epi tube										

18. Determine the total number of living cells and the total number of cells (living and dead cells together).

Total Number of Live Cells	Total Number of Cells

19. Since the volume under one of the large squares is 0.1µl, then adding up the cells counted in all 10 large squares will give you the number of cells in 1µl (10 x 0.1µl).

    Thus you have a concentration in cells/µl of the diluted solution

20. To obtain the concentration in the original cell culture, you still need to convert it to cells/ml and multiply by the dilution factor.

    (live cell # in 10 counting areas) x (1000) x (2)

Using a "live cell #" here instead of a "total cell #" is more useful, can you think of why?

21. Determine the Viability of the cell culture. This is the percentage of the cells that are alive in your culture.

(total live cells)/(total cells) x 100%

Post-Lab Questions:

Please do a little reading about growth curves, and answer the following questions in your notebook:

- Why do we care about cell viability when looking at cell culture samples before an experiment?
- If you found that your sample had a viability of 62%, would you use it for further experiments? Explain.

Think about why we count cells in more than one square and answer the following:

- How would you calculate the cell concentration if you saw the following results?

Counting Chamber #1	Square 1		Square 2		Square 3		Square 4		Square 5	
	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Cell Count	21	3	23	1	14	2	10	1	13	0

Counting Chamber #2	Square 1		Square 2		Square 3		Square 4		Square 5	
	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Cell Count	22	1	23	2	20	1	18	3	24	2

Lab 6: Cellular Fractionation

In this week's lab, we will be focusing on breaking open cells and collecting the various organelles. This is the start of a series of connected lab experiments, so be sure to keep all your samples at the end of every lab and don't throw anything away unless you are absolutely certain that you won't need it.

Learning Objectives:

Students will:

- Describe different cell lysis methods
- Explain the difference between differential centrifugation and density gradient centrifugation
- Explain the importance of the composition of each of the solutions being used in the protocol

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Pre-Lab Questions:

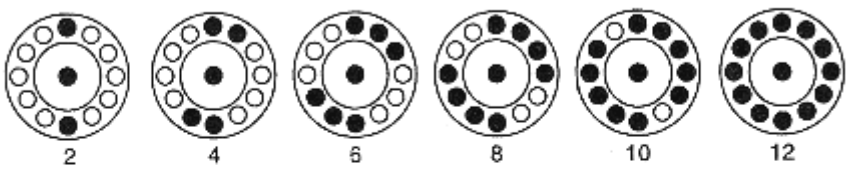
Take a look at the following videos to understand what a "g" means. Please note how many "g-s" the the astronauts are normally exposed to and what that means in terms of body weight.

- https://youtu.be/fAqa982j1a0
- https://youtu.be/Hgz7kJJSksM

In this video an airforce pilot gives you some idea of how uncomfortable such g-forces can be for the human body.

- https://youtu.be/kY7Id7TpfgU

Now, consider a situation where you put two samples into a centrifuge (in a balanced configuration). The samples differ in weight by only 1 gram. If you were to centrifuge these samples at 10,000g-s, what would be the difference in the weight of those samples at that speed?



"Balanced configurations" for different numbers of tubes in a centrifuge rotor.

Studying the Organelles of a Eukaryotic Cell

One need only look at drawings of a "typical plant and animal cell" in any 1st year Biology textbook to get a glimpse of the challenge awaiting anyone wishing to perform a detailed study some small component of one of these cells. A eukaryotic cell is a very complex mixture of various types of macromolecules, some of which are found in the cytosol while others are enclosed within a variety of organelles.

Depending on the type of cell being studied, the number and types of organelles and macromolecules can differ very widely, and having a method for obtaining a sample that is enriched for the component of interest can be very useful. The isolation and/or identification of a component of interest is at the heart of many of the techniques you will learn about in your biology labs in the coming years. The techniques you will learn in today's lab are a very common starting point and can lead to the purification of a wide variety of cellular components.

Homogenization

The initial step in the isolation of anything from the inside of a cell, involves breaking it open and releasing its contents so that they can be separated from each other. This process of lysing the cells results in a cell suspension known as a **homogenate**. The lysis can be accomplished using a variety of methods which can be classified as either physical methods (ie. grinding, dounce homogenization, sonication, etc), or chemical methods (ie. solubilization using a mild detergent), or enzymatic methods (especially useful in breaking down cell walls of plant cells). Because the aim of homogenization is the preservation of the component of interest in its natural state, the solutions used in the procedure are usually buffered at a pH similar to that of the cell, and isotonic (usually using sucrose or mannitol) to prevent the lysis of the organelles in the homogenization solution.

Cellular Fractionation

Once you've obtained a homogenate, the next step is the separation of the cellular components from each other based on their properties. This can be accomplished in a variety of ways, but two common ones are **differential centrifugation** and **density gradient centrifugation**.

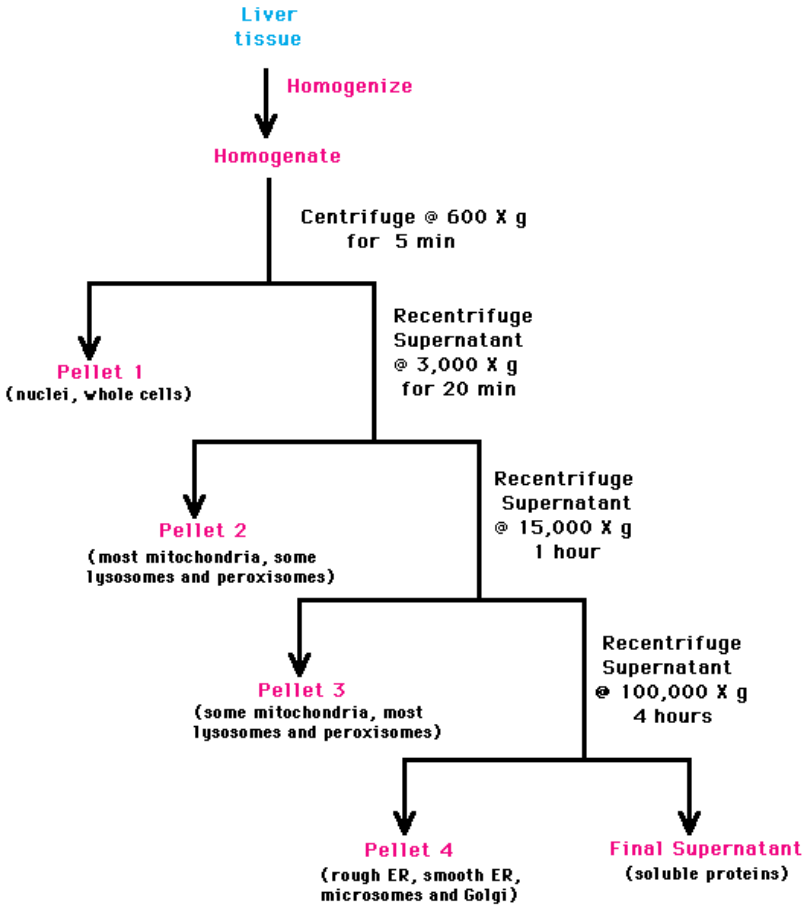
The purpose of differential centrifugation is the separation of cellular organelles based on their size, shape and density. These particles move through a solution in response to the force being exerted by a centrifuge. How quickly they move depends on their size and weight – heavier particles will sediment more quickly than lighter particles. Thus, by using different amounts of force (centrifugation speeds), we can selectively separate the heavier organelles from the lighter organelles.

In general, the centrifugation is first performed for a short time at a low speed. This allows for the sedimentation of the largest and heaviest cell components into a **pellet** at the bottom of the sample tube, while all other components remain in solution (the **supernatant**). Once the pellet and supernatant are separated, the supernatant can be centrifuged again for a longer time and at a faster speed to pellet smaller and smaller organelles. This can be repeated many times and with various centrifugation speeds and times to obtain a pellet containing a particular organelle. Each of the samples generated in this way is often referred to as a **fraction**.

One of the limitations of this technique is that it does not produce pure samples of your organelle of interest. This is because there could be several organelles that have the same size, so they would sediment together. Density gradient centrifugation can be used to further separate these organelles based on their density. In this technique, the forces generated by the centrifuge

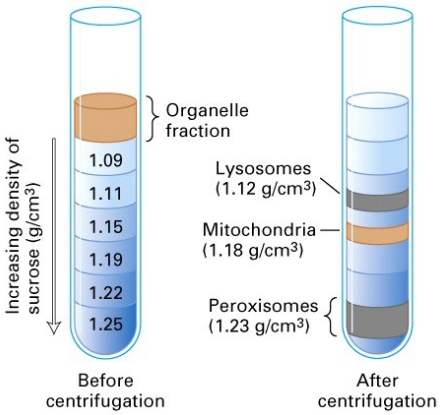
will cause your cell extract to migrate through a solution that has varying densities. This is because the centrifuge tube is prepared with a solution that is very dense at the bottom of the tube, has an intermediate density in the middle and has a very low density at the top. The organelles in the mixture will migrate through this gradient until they reach a point where the solution is denser than the organelle; this is where they will stop migrating. Thus denser organelles will travel farther than less dense organelles.

A sample flowchart for a differential centrifugation is shown below:



Source: http://www.biotechspace.site90.com/wp-content/uploads/2012/10/11.gif

Density Gradient Centrifugation:



Source: http://www.pha.jhu.edu/~ghzheng/old/webct/note1_5.files/F05-24.jpg

Exercises

Today you will be performing a differential centrifugation to isolate different organelles from plant cells. Because plants have cell walls, this protocol can sometimes include an incubation with enzymes or chemicals that would break down some of the cellulose and allow us to more easily break open the cells. In our case, we will simply use physical methods to break open the cells.

You will use a mortar and pestle to grind the broccoli tissue into a fine slurry. You will be doing this in a small amount of the Grinding Buffer you prepared in the previous lab. This buffer will maintain the pH of the solution and keep macromolecules like proteins and enzymes from denaturing, the mannitol in the solution will maintain the osmolarity of the solution and prevent the released organelles from lysing.

Once the homogenization is complete, you will perform several cycles of centrifugation, collect the different fractions and store them for next week's lab.

It is important to remember to keep your samples cold for as much of the lab as possible – the cold temperatures will help preserve the contents of your tubes and minimize degradation.

Materials (per group):

- broccoli
- clean (autoclaved) grinding sand
- 20ml of cold Grinding Buffer (0.4M Mannitol; 10mM Phosphate Buffer, pH 7.2, 1mM EDTA)
- cheese cloth
- ice in an ice bucket
- mortar and pestle on the ice
- funnel
- 4 centrifuge tubes
- 10 test tubes
- test tube rack
- balance
- weigh boat
- Pasteur pipettes / droppers
- plastic spoon
- centrifuge
- Parafilm
- Eppendorf tubes

1. Homogenization

Procedure:

1. Label the first centrifuge tube “A” and place it on ice
2. Use a knife or razor blade to remove ~10 g of the outer 2-3 mm of the broccoli surface.
3. Place the plant tissue in a cold mortar (on ice), add some grinding sand and **10ml** of the grinding buffer.
4. Use the pestle to grind the broccoli into a smooth slurry.
5. Place a funnel over the centrifuge tube
6. Fold the cheesecloth to give it at least 4 layers and spread it over the funnel

Use the Sand

Use the sand to add roughness to your slurry and help you crush the tissue more efficiently. Don't worry about adding what may seem like a lot, most of it will be filtered out in the cheesecloth, and the rest will be pelleted down in the first centrifugation.

7. Pour/scrape the homogenized plant tissue into the cheesecloth and gently press down on it to try to get as much liquid out of the homogenized tissue as possible into the centrifuge tube (tube "A")
8. Keep this tube on ice for the next part – this is your homogenate
9. Transfer 0.6ml of the homogenate into an Eppendorf tube labeled "H" and leave it on ice until you've collected all of the other samples.

2. Differential Centrifugation – Generation of Cell Fractions

In a teaching laboratory, we are limited by the time we have to complete an experiment, so our protocol will differ from what you might do in a research lab (and thus will be a little less efficient).

The initial centrifugation step is done at a very low speed and is meant to remove any whole cells and cellulose from the solution. It will also likely pellet the nuclei – we could try using a slower speed and do more steps, but we're reducing the number of steps to accommodate the amount of time we have for the lab. The second spin should pellet down most of the mitochondria into the pellet and leave smaller organelles and proteins in the supernatant.

10. Balance your tube "A" with another tube "A" or a "balance tube" for centrifugation.
    - Place the tubes in the centrifuge such that they are directly opposite one another in the rotor
11. Centrifuge your samples at 600 RCF for 5min (your TA will operate the centrifuge)
12. We will be doing the next spin in a centrifuge that can only accommodate eppitubes, so label a few eppi tubes (tube B1, B2, B3, etc) and place them on ice
13. Recover your tube (A) after the centrifugation and **gently** place it on ice
14. Carefully transfer the supernatant to the tubes labeled as "B". Depending on the volume you have, you may only need two or more epi tubes. If using more than one tube (ie. if you have more than 1ml of liquid), then pipette equal amounts into each tube.
15. Remove any remaining liquid from tube "A" – we want it to only contain the pellet.

The next spin will be at very high speed, so we need to ensure that the tubes have the same volumes in order to balance them properly. If you have only one tube "B", then please use an equal volume of the Grinding Buffer to make a balance tube for the tube "B".

16. Centrifuge your samples (tubes "B") at 15000 RCF for 30min
17. Label a test tube "C" (this will be for the supernatants) and and a test tube "B" and place them on ice.
18. Carefully transfer the supernatants from your eppi tubes to tube "C". Try not to take up any of the pellet.
19. Gently resuspend the pellets in tubes "B" in 0.2ml of Grinding Buffer and transfer them all into test tube "B".
20. Label samples with your names and store them for the next week's laboratory.
21. Samples (A, B, C, H) will be stored at -20ºC for the next week's laboratory.

Post-Lab Questions:

In the next few labs, you will be trying to test for the activity of enzymes that might be present in one of your fractions.

- Why did we save the “H” tube? What do you think we might want to use it for?

Lab 7: Protein Quantitation

Today's lab deals with determining the concentration of protein in the samples you collected in the previous lab. Additionally, you will attempt to view your fractions under the microscope to determine if there are differences between them that can be detected visually.

Learning Objectives:

Students will:

- Explain how light can be used to determine the concentration of components in a solution
- Describe how a spectrophotometer works
- Use a standard curve to estimate concentrations of their solutions
- Describe how the Beer/Lambert Law can be used to determine concentrations
- Explain the advantages of testing several different concentrations of the same component
- Explain how “enrichment” is different from “concentration”
- Prepare a wet-mount

Pre-Lab Questions:

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

- https://youtu.be/qbCZbP6_j48

How is Absorbance related to Transmittance?

How can we use a graph to help us identify the concentration of a solution?

Determination of Sample Contents

You now have a few fractions, each containing different cellular components. In the next lab, you will be testing these fractions for the presence of your organelle of interest (we will be looking for mitochondria). This will be accomplished by detecting the presence of a specific enzyme – a "**marker enzyme.**"

One of the problems with your fractions from last week is that they are the result of only differential centrifugation. This technique does not yield purified organelles, but can produce a few fractions with your organelle of interest. It would therefore be useful to find out which fraction contains the most of that organelle – this is **enrichment**.

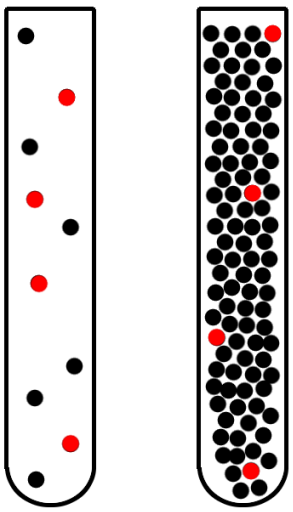
What is an "assay"?

You will see the word "assay" showing up frequently in biological laboratories. You can think of it as simply meaning "a test for the presence of something in a sample".

The fractions you isolated in the last lab have different compositions. Some fractions may have many other organelles along with your organelle of interest, while others will have very few other organelles and mostly your organelle of interest. Finding out which of the fractions has the most of your organelle of interest is what you will do using a **marker enzyme assay.** This is an experiment that helps you identify which fraction has your organelle of interest by finding out which one has a molecule/enzyme that is unique to your organelle. In our case, it will also help us find out which fraction has the most of our organelle of interest, by showing us which has the most of the marker enzyme.

This may seem simple, but it is complicated by the possibility that each of your fractions may contain a different concentration of total protein.

Example:



You have two samples. Your marker enzyme might make up 40% of all the proteins in fraction 1, and only 4% of all proteins in fraction 2. So your organelle is enriched in fraction 1 – unfortunately, you can't just see this, that's what the marker enzyme assay is supposed to be able to tell you.

The process of fractionation might produce samples that have very different concentrations (as shown on the left), so it's possible that fraction 1 is also 10x more dilute than fraction 2. If that's the case, then it would be very difficult to actually see the difference in enrichment if we did the test using equal volumes of each fraction.

If we did this, the assay might show us that each sample has only "four molecules" of the marker enzyme (in red), and you might then conclude that they have equal amount of enrichment. The problem is that using equal volumes here would not allow for a fair comparison of the samples.

It is therefore useful to know the protein concentration of your samples before testing them. This ensures that the assay can be performed under conditions where the total amount of protein used from each fraction is approximately equal, and the only difference will be how much of "marker enzyme" there is relative to the total protein in each fraction.

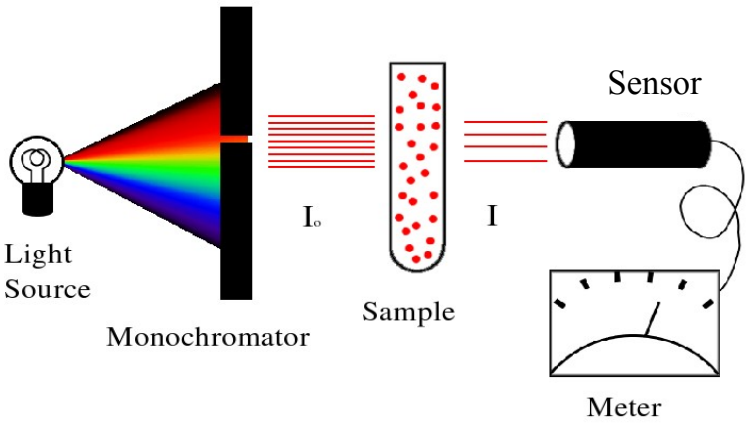
In this lab, we will use a **Bradford assay** to determine how much protein is in your samples. The Bradford assay uses a dye that is capable of binding to basic and aromatic amino acids in a protein. This binding causes the dye to change colour to blue and absorb light at a wavelength of 595nm. The higher the concentration of protein, the more blue colour is developed and the more light is absorbed. This is because more basic and aromatic amino acids are available to bind the dye.

This change in colour can be measured using a **spectrophotometer**, which can relate the absorption of light due to the colour change to the concentration of the protein.

Spectrophotometry

A spectrophotometer consists of five basic parts. A lamp emits a wide variety of wavelengths of the light spectrum (including UV light in some spectrophotometers). The light is directed through a monochromator, which is used to permit only the selected wavelength of light to pass through to the sample. The light then passes through a cuvette (special sample tube). A light sensor (photomultiplier) is used to detect and measure any light that passes through the cuvette, and to send the measurement to a device which displays the output.

The cuvette can contain one of two things, it can contain a reference standard or a sample. The reference standard, or **blank**, contains all of the components of the sample except the one being measured. The incident light coming from the monochromator passes through the standard, and the transmitted light is measured by the photomultiplier. Despite the fact that the blank may contain light-absorbing molecules, the spectrophotometer is set to an absorbance value of zero (equivalent to 100% light transmission). The blank is then removed and replaced by the sample, and any increase in light absorbance relative to the blank is measured and related to the concentration of the material being measured in the sample.



For most spectrophotometric measurements, the sample will obey the Beer/Lambert law, which states that absorbance (A) is directly proportional to solute concentration (c) and to the length of the light path through the sample (l). The Beer/Lambert equation is thus:

$$A = \alpha\, c\, l$$

α - is the absorption coefficient

c - is the concentration

l - is the light pathlength (equivalent to the cuvette width – usually 1cm)

The term α is the absorption coefficient, and is determined from the slope of the line on a graph of absorbance plotted against concentration. This can be easily obtained if you perform spectrophotometric measurements using pure solutions of known concentration of the molecule of interest. A standard curve can be constructed from such data and from that graph, the amount of an unknown sample can be easily determined. You can read it from the graph, or you can determine the absorption coefficient and then measure the absorbance of the unknown solution, and then plug in the values into a rearranged Beer/Lambert equation, solving for c.

Exercises

The protocol you will follow should be capable of detecting protein concentrations as low as 0.1mg/ml in your samples. You will determine the concentrations of your samples by comparing the measurements you get for your samples to a set of standards. The standards are simply samples of known protein concentration which are assayed using the same protocol as your samples.

Once the absorbance readings for the standards (known protein concentrations) are known, they are plotted on a graph to generate a "standard curve". Standard curves (they're usually straight lines) allow us to use information about known samples to determine something (like the concentration) about an unknown sample.

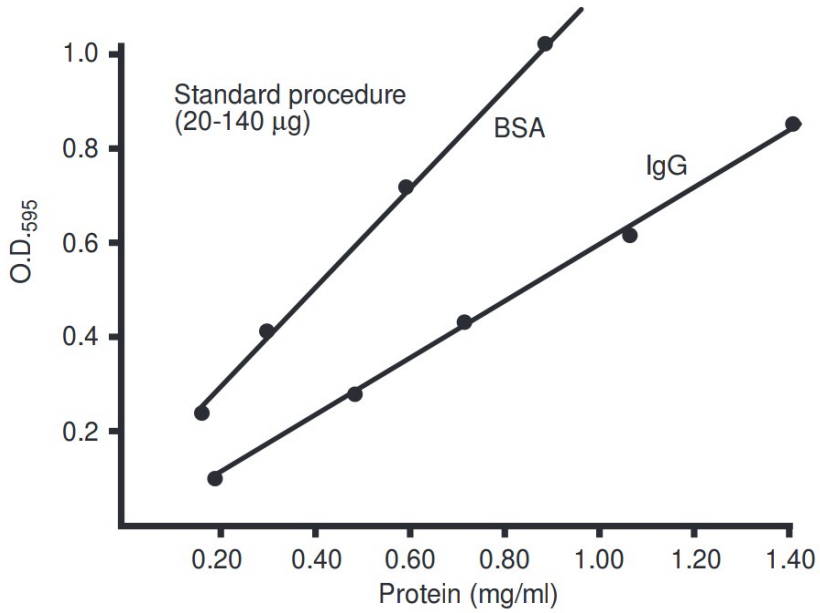


Fig. 1. Typical standard curve for the Bio-Rad Protein Assay, bovine gamma globulin (standard I), bovine serum albumin (standard II). $O.D._{595}$ corrected for blank - 200-1,400 µg/ml x 0.1 ml = 20-140 µg protein.

Source: Bio-Rad Technical Bulletin for the Bradford Reagent

Under normal circumstances, in a typical research lab, you would generate your own standards and the data necessary to draw a standard curve – a graph of absorbance vs concentration. In our case, however, we will be using a pre-drawn curve in order to help us get through our lab more quickly - your TA will provide one in class or you will use the one below.

Materials (per group):

- Cuvettes
- Spectrophotometer
- 20ml of Bradford Assay Buffer
- 1.5ml of 1M NaOH
- Ice bucket with ice for samples
- Eppendorf tubes and test tubes
- Micropipettors (P200 and P1000)
- PBS

1. Quantitation of Sample Protein Contents

Procedure:

Because you all started with different amounts of starting material, and the homogenization was not exactly the same for everyone, it is impossible to know the concentration of your samples ahead of time. For this reason, it is possible that the protocol below may need to be repeated in order to obtain a sample reading that will fall within the range of your standards.

If the absorbance value for a sample is very low, you will need to perform the Bradford reaction again using more of your sample. On the other hand, if an absorbance value is too high, you will need to repeat the reaction again using less material or a diluted sample. Your TA will advise you. To be usable, your absorbance readings must fall within the range of the standards on the graph.

1. Gently mix each cell fraction before taking a sample for analysis.
2. For each fraction, produce a 1:10 dilution (10µl of sample + 90µl of PBS) and a 1:100 dilution (10µl of sample + 990µl of PBS) in separate Eppendorf tubes.

You should now have twelve tubes containing different dilutions of your fractions.

3. For each sample, transfer 100µl of the sample into a new labeled eppi tube.
4. Add 1ml of Bradford assay buffer to each tube.
5. Cap and vortex the tubes
6. Look to see if there is a colour change.
7. Allow the reaction with the assay buffer to occur for at least 5min.
8. Transfer each reaction mixture into a cuvette and measure absorbance at 595nm
9. Fill in the absorbance values in the first row of the table below.

Please note, this protocol may have to be modified during the lab depending on the reagents we have available.

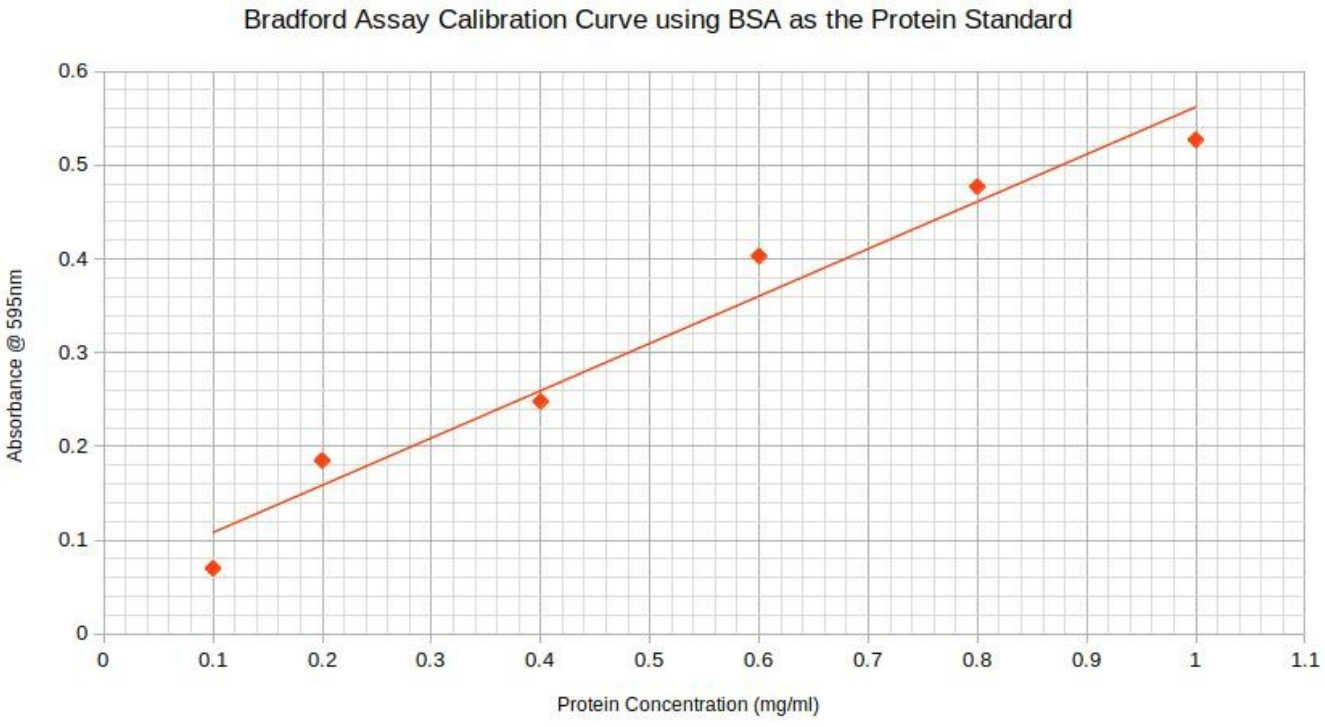
	Sample A			Sample B			Sample C			Sample H		
	Original	1/10 dilution	1/100 dilution	Original	1/10 dilution	1/100 dilution	Original	1/10 dilution	1/100 dilution	Original	1/10 dilution	1/100 dilution
Abs_{595}												
[Protein]												
Dilution Factor	1	10	100	1	10	100	1	10	100	1	10	100
[Undil. Sample]												

10. Check the absorbance values of your unknown samples. At least one of them for each of your unknowns should be in the range of the standards in the graph (your TA will explain what that means).

    Check with your TA if this is not the case – you may need to repeat the test with a different dilution of your unknown sample. Use the table below if needed.

Abs_{595}												
[Protein]												
Dilution Factor												
[Undil. Sample]												

11. Use the graph below to help you determine the concentration of your samples.



Lab 8: Marker Enzyme Assay for SDH

In today's lab, you will perform a marker enzyme assay.

Learning Objectives:

Students will:

- Explain the idea of a “Marker Enzyme”
- Describe the process involved in detecting mitochondria using SDH
- Identify the negative and positive controls in their experiment and explain why they are needed
- Explain why measuring Transmittance in the marker enzyme assay might make it easier to interpret the results

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Pre-Lab Questions:

Please take a look at the following video.

- https://youtu.be/3Tn-7JcZJuQ

After watching it, please try to do some research to find the answers to the questions below

- What is Catalase?
- Where is it found in a cell?
- How could we use the above information and video in a lab?

Marker Enzyme Assay – Testing for Presence of Mitochondria

Once you've obtained test tubes containing fractionated cell components, you want to verify that your organelle of interest is present and identify the fraction that has it. Most organelles are colourless and difficult to identify visually, so we often employ a **marker enzyme assay** to verify their presence in our test tubes. This type of assay checks for the presence of a macromolecule (usually an enzyme) that is exclusively present in a particular organelle – thus, if the "marker enzyme" is present, then our organelle of interest is also present. In this case we will be testing for the presence of the enzyme Succinate Dehydrogenase.

Succinate Dehydrogenase (SDH)

Much of the energy derived from cellular respiration is generated in the Mitochondria. The enzymes associated with the Kreb's cycle (cytric acid cycle) and the electron transport chain are found here (and in no other organelle). Thus testing for the presence of such enzymes in fractionated samples is likely to reveal which one contains mitochondria.

The Kreb's cycle is a pathway involving eight enzymes. One of the best studied enzymes in that pathway is Succinate Dehydrogenase (SDH), which catalyzes the oxidation of succinate into fumarate:

$$\text{Succinate} + \text{FAD} \rightarrow \text{Fumarate} + \text{FADH}_2$$

In this reaction, two hydrogen atoms and two electrons are transferred from succinate to FAD, a prosthetic group that is tightly attached to succinate dehydrogenase. The two electrons from the reduced SDH-$FADH_2$ complex are then transferred to coenzyme Q (ubiquinone), a component of the electron transport chain.

Because SDH is bound to the inner membrane, it is easily isolated along with the mitochondria by the technique of differential centrifugation (even if some of the organelles are damaged and have released the contents of their matrix).

Exercises

The activity of SDH can be measured by monitoring the reduction of an artificial electron acceptor. To use an artificial electron acceptor, the normal path of electrons through the mitochondrial electron transport chain must be blocked. This is accomplished by adding either sodium azide or potassium cyanide to the reaction mixture.

Caution

Azide and Cyanide are just as poisonous to you as they are to the cauliflower cells. They can stop the electron transport system in the mitochondria of your cells as well. Please wear gloves.

These are cellular respiration poisons that act by inhibiting the transfer of electrons from Cytochrome a_3 to oxygen, thus preventing electrons transfer from $FADH_2$ to Coenzyme Q. Instead, the electrons from SDH-$FADH_2$ can be picked up by an artificial electron acceptor, such as the dye 2,6-dichlorophenolindophenol (DCIP). The reduction of DCIP can be quantified using a spectrophotometer, since the oxidized form of the dye is blue and the reduced form is colorless. This reaction can be summarized as:

$$\underset{}{\text{SDH-FADH}_2} + \underset{\textit{blue}}{\text{DCIP}_{\text{oxidized}}} \rightarrow \underset{}{\text{SDH-FAD}} + \underset{\textit{colourless}}{\text{DCIP}_{\text{reduced}}} + 2\text{H}^+$$

The change in absorbance can be detected 600 nm, and can be used to determine the extent of the reaction over time. Note that a reduction of DCIP will lead to a decrease in absorption. Thus, samples that contain SDH (and therefore mitochondria) will lose colour (become less blue)

Materials (per group):

- Samples from the previous lab
- 20 ml of Assay Buffer
- 0.04M Sodium Azide or Potassium Cyanide
- 0.2M Sodium Succinate pH 7.2
- 0.05% Dichlorophenolindophenol (DCIP)
- ice in an ice bucket
- 14 test tubes
- test tube rack
- spectrophotometry cuvettes
- spectrophotometer
- Micropipettors (P200 and P1000)
- Parafilm
- Eppendorf tubes

1. Measurement of Succinate Dehydrogenase Activity

In order to be able to compare your results to one another, you want to make sure that you're testing the same amount of each sample. This way any differences in the results of the marker enzyme assay can be attributed to the amount of the enzyme of interest in the sample. Thus, the first thing you will do is prepare sample solutions of equal (or as close as possible) concentration.

1. Calculate the amount of each of your original fractions to use in order to make solutions at a concentration of 1mg/ml of protein.

Use the formula $c_1v_1 = c_2v_2$

Fraction	Original Concentration	Final Concentration	Final Volume	Amount of fraction to use	Amount of Isotonic Buffer
H		1mg/ml	1ml		
B		1mg/ml	1ml		
C		1mg/ml	1ml		
D		1mg/ml	1ml		

2. Based on the above calculations, prepare the solutions in Eppendorf tubes.
3. Label 10 test tubes (1, 2, 3, 4, 5... etc) and prepare them as outlined in table 1

Table 1. Sample preparation for Succinate Dehydrogenase enzyme assay.

			Tube A		Tube B		Tube C		Tube H	
Tube #	1	2	3	4	5	6	7	8	9	10
Assay Buffer (ml)	1.6	1.4	1.4	1.2	1.4	1.2	1.4	1.2	1.4	1.2
Azide or Cyanide (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Succinate (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
DCIP (ml)	0	0.2	0	0.2	0	0.2	0	0.2	0	0.2
Cellular suspension (ml)	0	0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

4. Parafilm all tubes carefully
5. Mix the contents of each tube by inverting 2-3 times
6. Place tubes in a 37ºC water bath for 15min
7. Turn on the spectrophotometer and set it to 600nm
8. Obtain 2 cuvettes per group

9. Use the odd-numbered samples to blank the spectrophotometer for the even-numbered samples

   (ie. Tube 1 is the blank for tube 2, tube 3 is the blank for tube 4, etc.)

10. Measure the absorbance of each of the samples and record it below:

			Tube B		Tube C		Tube D		Tube H	
Tube #	1	2	3	4	5	6	7	8	9	10
Abs_{600}	0		0		0		0		0	

Post-Lab Questions:

Did you achieve enrichment of mitochondria in any of your tubes?

Why did we include sample “H”?

Take a look at the picture of your gel. Are there any differences between the different lanes? Try to explain them.

Lab 9: Gel Electrophoresis

In today's lab, you will perform two types of gel elelctrophoresis. You will run some DNA on an agarose gel and will be running your protein samples from the previous lab on a polyacrylamide gel to view protein profiles of the different fractions.

Learning Objectives:

Students will:

- Explain how electrophoresis is used to separate molecules based on their size
- Explain how and why DNA molecules migrate in an electrical field
- Describe some of the hazards involved in both types of electrophoresis in this lab
- Describe the composition of DNA loading dye and explain why the different components are there.
- Explain the purpose of adding Denaturing Buffer to protein samples and heating them before loading them on an SDS-PAGE gel

---

Pre-Lab Questions:

Please use your results from the Protein Quantitation (Bradford Assay) lab to help you **complete the calculations on the table on page 74**.

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?

Electrophoresis refers to the movement of charged molecules in an electric field – ie. molecules will migrate towards the positive or the negative electrode depending on their charge. In gel electrophoresis, we apply an electrical current to a sample of charged biological molecules and force them to migrate to their preferred electrode through a dense matrix (a gel), this allows us to separate the molecules based on their different abilities to move through that matrix – ie. their size.

Proteins, DNA molecules and RNA molecules are often separated from each other in this way, but the composition of the gel matrix tends to differ depending on the type of molecule being applied to the gel.

In each case, there is a wide variety of methods, so what follows below is a very brief overview of the most common methods for each macromolecule.

Protein samples are usually run on a polyacrylamide gel, this is because polyacrylamide forms a very dense matrix which allows for the separation of relatively small molecules like proteins (note: very short DNA molecules, like those generated during DNA sequencing, are also separated on polyacrylamide gels for this reason).

In order to separate proteins based purely on their length, they must first be denatured. This is why protein samples are treated with SDS (unfolds the molecule and gives it a uniform negative charge) and β-mercaptoethanol (reduces disulfive bonds within a peptide and between separate peptides, like enzyme subunits), and heated before being applied to the gel. Also, the gel will contain SDS, hence the term "SDS-PAGE" (SDS **P**oly**A**crylamide **G**el **E**lectrophoresis).

Like Proteins, **RNA** molecules tend to fold and form a variety of "secondary structures" (ie. they're not linear), and thus need to be denatured before electrophoresis. For this reason, loading dyes and gels used for RNA electrophoresis tend to contain some form of a denaturant (ie. fornaldehyde). This is especially important for use in Northern Blotting – the RNA **needs** to be fully unfolded and all the bases need to be available for binding to a probe molecule. Unlike proteins, however, in most cases RNA is run on an agarose gel (containing formaldehyde in this case – this is known as "denaturing agarose electrophoresis").

Lastly, **DNA** is probably the simplest biological macromolecule to use in electrophoresis. Most extracted DNA samples tend to contain purified DNA which has had most proteins (including histones) removed. Such DNA molecules are thus usually relatively linear (except for intact plasmids or circular chromosomes) and do not require any additional (ie. denaturation) steps to prepare them for electrophoresis.

Additionally, most of the DNA molecules in a typical lab are relatively large (when compared to proteins) in size and can thus be separated in an agarose gel – the larger pore size of the agarose matrix allows for the separation of larger molecules like DNA and RNA molecules, but wouldn't allow for good separation of most protein preparations.

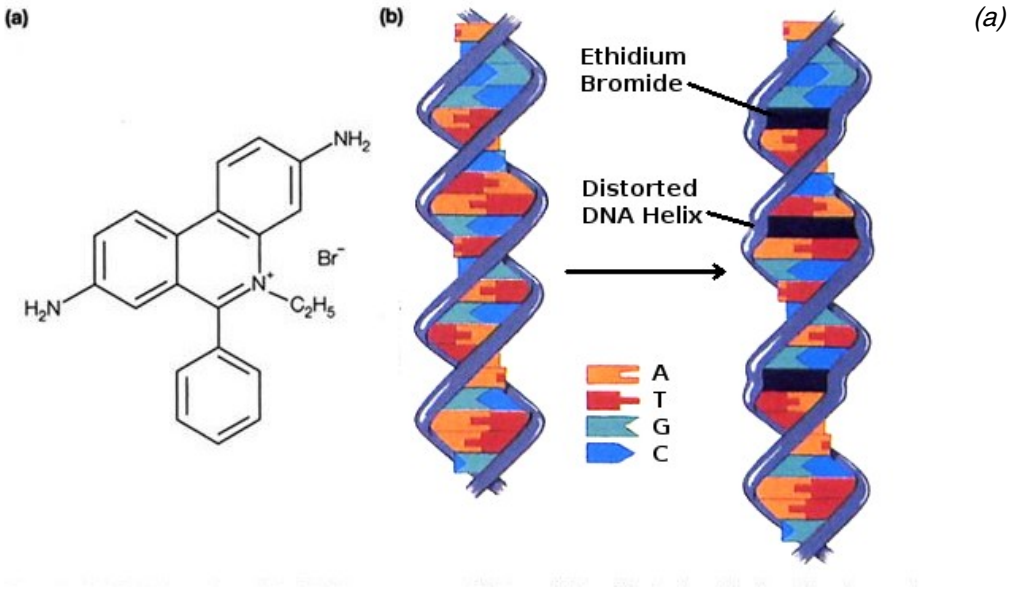
One of the strengths of agarose as a matrix material is that it's much easier and safer to prepare – the preparation of polyacrylamide gels involves working with the unpolymerized acrylamide which is a potent neurotoxin, while agarose is a harmless polysaccaride that just needs to be heated and then allowed to cool to polymerize.

Agarose gels

The most common type of electrophoresis in 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 heat (it will not

dissolve without heat), 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).



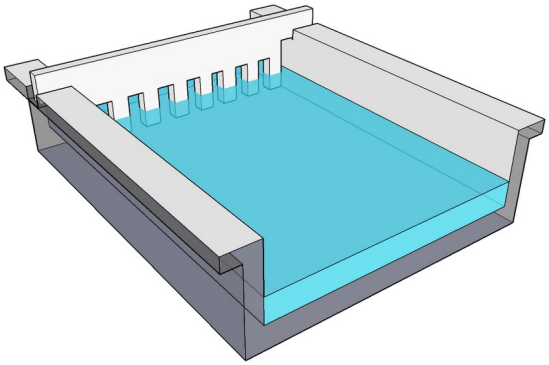
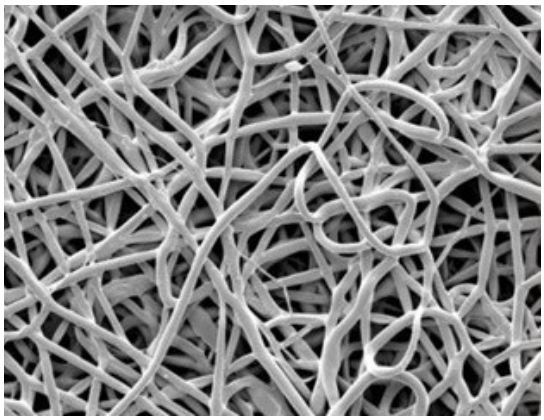
Structure of ethidium bromide molecule. (b) Distortion of the DNA helix by the intercalation of ethidium bromide molecules.

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

The warm agarose 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.

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%).

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.

(A)  (B) 

(A) Schematic of an agarose gel poured into its mold with a gel comb 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): www.biomech.ethz.ch

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.

In order to find the sizes of DNA fragments, you would generate a graph of the size of the molecular marker bands against distance migrated by the fragments on a semi-log plot. From this you would be able to accurately determine the size of any other DNA fragment on that gel (assuming it falls within the linear range of your graph). In most cases, however, you can simply estimate the sizes of your DNA molecules by visually comparing their migration to the migration of the standards.

After running the gel, you will visualize your DNA samples 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.

There are two safety notes associated with this procedure.

1. 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”.
2. The second caution relates to the use of the UV light source. The light box emits light in the UV range (260-360nm), and this causes damage to the retina of the eye. In our case the UV light box is enclosed, and we use digital camera to view our gels. However, in labs that have a more traditional set-up, you will need to ensure that you always wear a full face shield when viewing a gel on a UV light box.

Exercises

1. Separation of DNA Fragments by Agarose Electrophoresis

Materials (per group):

- DNA samples
- 1.2% agarose gel
- 0.5X TBE Buffer for the gel tank
- Agarose gel tank
- Power Supply
- Loading dye
- P20 and P200 micropipettors
- Boxes of 20µl and 200µl tips
- Epi tubes
- Microcentrifuge

Your first task today, is to load an agarose gel. You will need to thaw the DNA samples you generated last week. Once you've loaded your samples on the gel, you will be able to start with part 2 of your lab.

1. Transfer 20ul of each sample into a new microfuge tube and add 5ul of Loading Dye.
2. Load the 25ul of sample onto a 1.2% agarose gel and run the gel at 100V for 20-30min.
3. A picture of your gel will be posted on Blackboard

2. Determination of Protein Profiles Through SDS-PAGE

SDS-PAGE is commonly used in many experiments in cell and molecular biology to view protein profiles of collected samples. Proteins are visualized placing a finished protein gel in a non-specific protein stain such as Coomassie Brilliant Blue, this stain attaches to the proteins separated on the gel and makes them visible at blue bands on a clear background.

Most research labs make their own gels, but this can be a hazardous task because acrylamide (the monomer form of the molecule that is used to prepare the gel) is a neurotoxin. Thus, great care must be exercised in handling it.

In our case, the gel will have been prepared for you and your task will be to simply load your samples onto it and to run the gel.

Materials (per group):

- 5X Denaturing Buffer (0.25M Tris pH 6.8,
- 10% SDS, 40% Glycerol, 20% β-mercaptoethanol, 0.01% Bromophenol Blue
- ice in an ice bucket
- 95°C water bath or heating block
- 4 eppi tubes
- Microfuge
- Micropipettors (P20 and P200) and tips
- SDS-PAGE Gel Apparatus
- 12% SDS-PAGE Gel

1. Fill in the table below to determine how much of each component to add. The “[protein]” is the concentration you determined from the last lab.
2. The total volume of each sample (for the calculations in this table) is 20µl.

Sample	Original protein concentration	Volume needed for 50µg protein	DIW to bring volume to 20µl
A			
B			
C			
H			

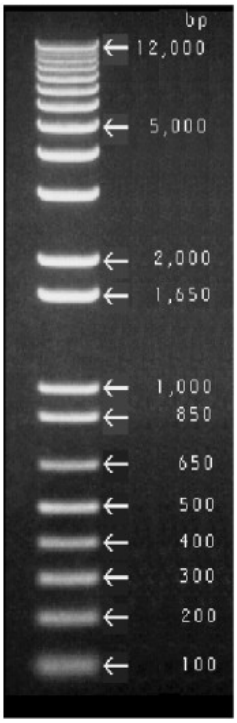
3. Prepare the samples as calculated. Then, add 5ul of 5x DB.
4. Once your samples are ready, place them in the 95°C block heater or boiling water bath for five minutes.
5. Collect your samples carefully. Wipe away any excess liquid, then pulse spin them in the microfuge to recover all the liquid to the bottom of the tube.
6. Load the samples.
7. Run the gel at 100V for about 1-2hrs.
8. The gel will be stained for you and a picture of the results will be posted on Blackboard.

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.

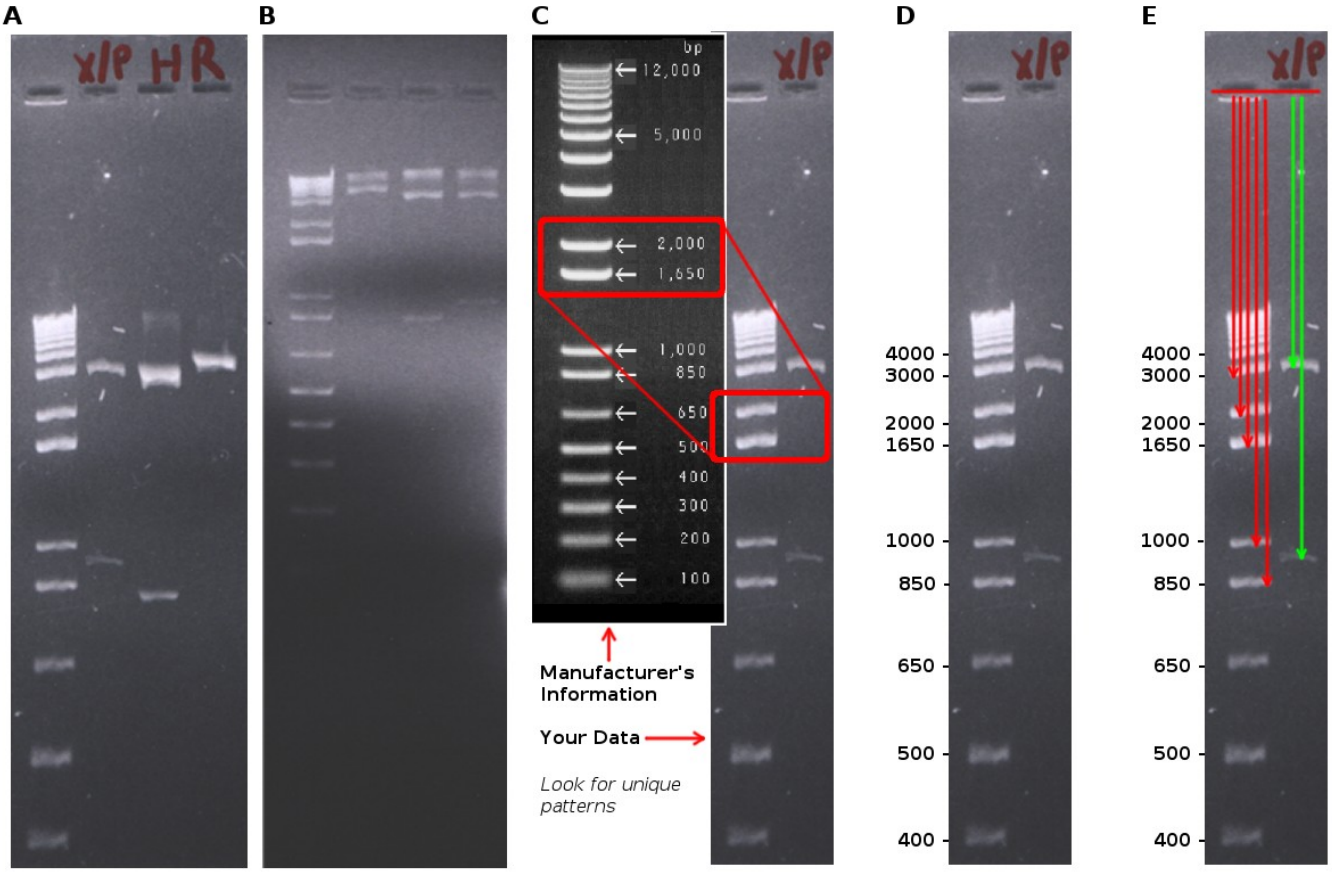
There is a great variety of molecular markers that are available, and they are selected based on the range of sizes of DNA fragments that they contain. A molecular marker like the "1kb Plus DNA Ladder" from a manufacturer called "Life Technologies" (formerly "Invitrogen") is commonly used because it 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.

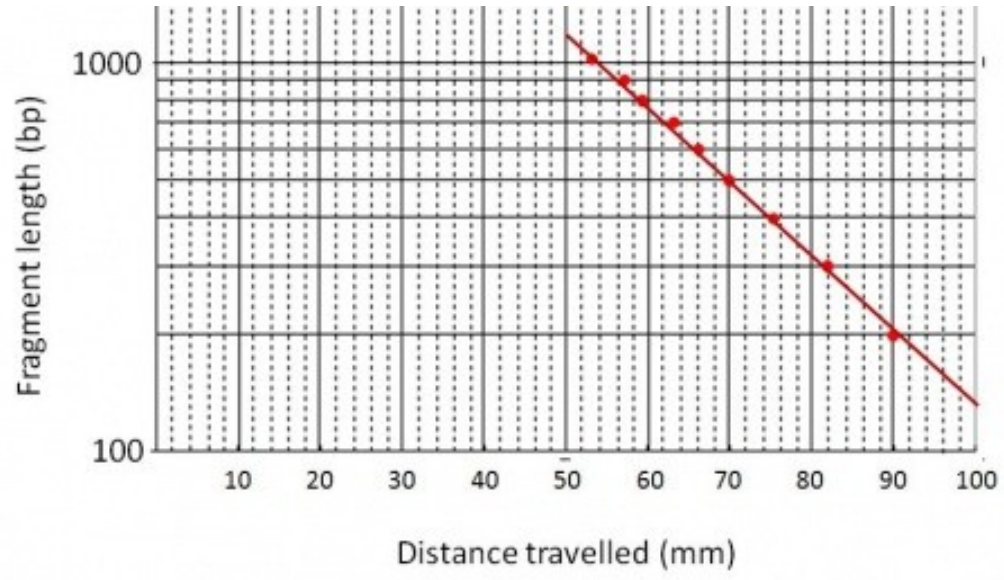


Assignment of sizes to molecular marker bands on gels in order to determine the sizes of the DNA bands in the experimental digests. (A) and (B) represent two DNA gels which were run for different amounts of time. The gel in (A) was run for a very long time and thus some of the small bands of the molecular 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.

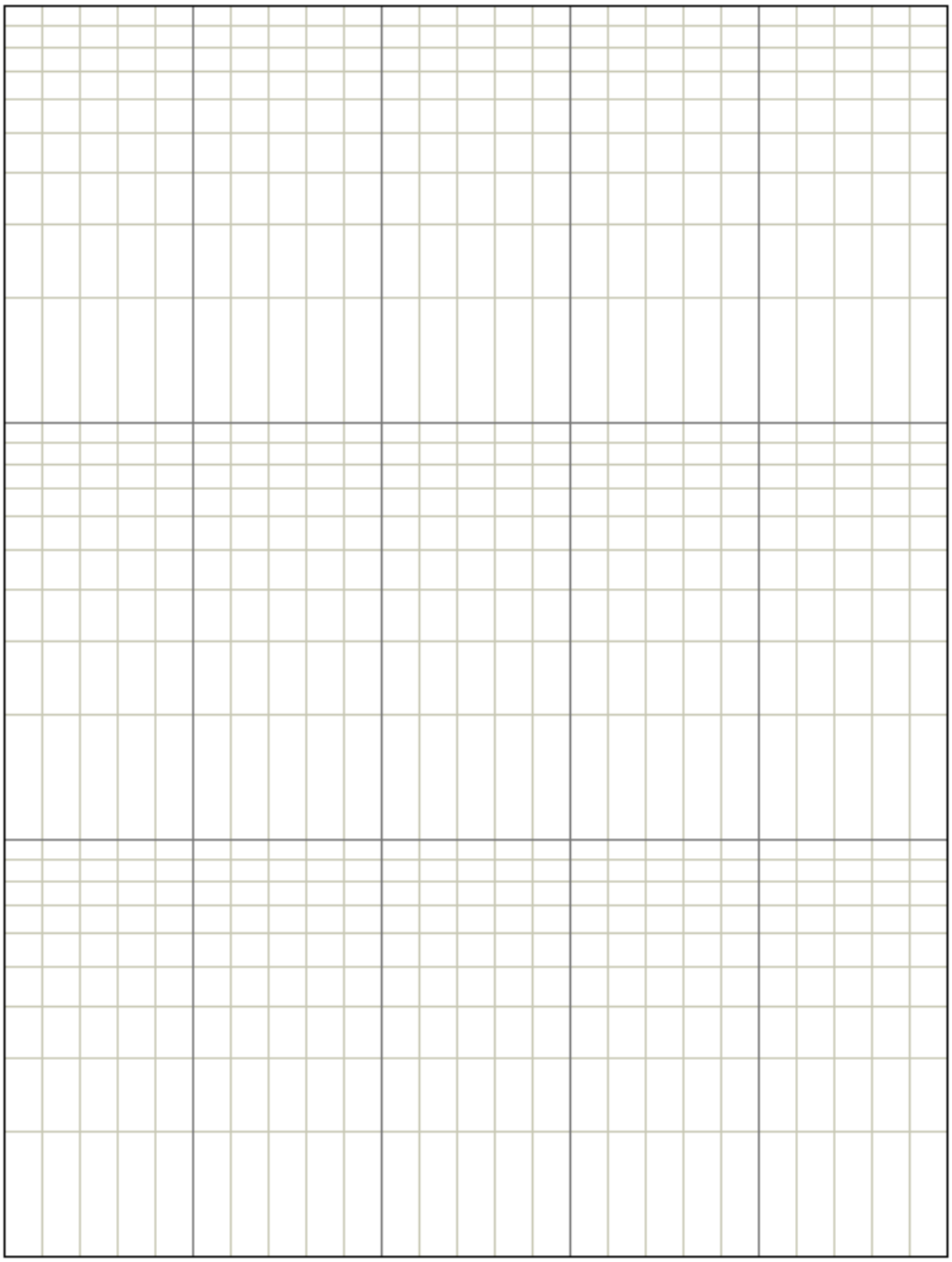
Please note that the same method can be used to determine the sizes of proteins on a polyacrylamide gel but the sizes would be expressed in Daltons (DA) or kilodaltons (kDa).

Homework 3:

Work **as a grouop** and use the information above to assign sizes to the molecular marker bands on the DNA gel we ran this week, and use it to determine the size of at least one of the DNA bands in the sample lanes. Please be sure **everyone** in the group knows how to do this.

Draw your graph on the paper supplied on the next page, clearly indicate the unknown sizes you are measuring and submit it in the online assignment dropbox.

This is due in next week



The unknown band(s) is/are in lane: _____

The size of the band(s) is /are ; _____

Lab 10: Western Blotting

In today's lab, you will use the Western Blotting technique to transfer proteins from a gel to a solid support (nitrocellulose membrane), and use antibodies to find a specific target protein on the blot.

Learning Objectives:

Students will:

- Describe the set up of a Western Transfer
- Describe how antibodies are used to detect their target
- Discuss the difference between what you can see on a stained gel and what you see on a western blot result

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Pre-Lab Questions:

Please take a look at the following video.

-

Important note.

This section will likely be added later this semester. This will depend on the availability of the necessary equipment and materials. It these are not available, we will do the DNA extraction lab in the next page.

Lab 10: DNA Extraction from Apoptotic Cells

Over the next few labs, we will be looking at how a process like apoptosis can be studied. Today's lab deals with extracting DNA from a cell sample. Once extracted, we will run this DNA sample on a gel to check for one of the characteristic signs of apoptosis.

Please note that someone from your group will need to come into the lab **tomorrow** to complete your experiment.

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
- Explain the reasons for using Proteinase and RNase in the protocol and give reasons for the order in which they are used

---

Pre-Lab Questions:

Please watch the video below and then compare what is demonstrated there to the protocol we will be using in our lab.

- https://youtu.be/DaaRrR-ZHP4

Answer the following question and submit it on Blackboard **before** the start of the lab.

- In what way is the protocol in the video similar to our protocol?

Collection of DNA for analysis

There are many different methods for DNA extraction and purification, however they all rely on the same basic principles and mostly are modifications of the same basic method. In order to extract and purify DNA, you must:

1. lyse the target cells while maintaining the DNA in a soluble state
2. remove the cellular debris (the insoluble materials like membrane fragments)
3. separate the DNA from protein and RNA
4. precipitate the DNA and resuspend it in an appropriate buffer

Step 1. Cell Lysis

This can be accomplished using a variety of different ways, but it generally involves a buffer – usually Phosphate or Tris (to maintain a physiological pH and prevent DNA denaturation), a mild detergent like SDS or Triton-X (to solubilize the membranes and start to denature some of the proteins), and EDTA which is a cation chelator (to prevent degradation of DNA by DNases found inside the lysed cells).

Step 2. Removal of Cell Debris

This involves centrifugation. Insoluble components will pellet down, while the soluble DNA will remain in the supernatant. Because genomic DNA is very long, it is important to treat the samples fairly gently – rough mixing can result in the shearing (breaking) of the DNA into small pieces.

Step 3. Removal of Contaminating Protein and RNA

There are several ways of doing this. The classical procedure involves the use of organic solvents like Phenol and Choloroform to take advantage of the differential solubility of DNA and protein in aqueous and organic phases (you've probably done a similar experiment in Organic Chemistry lab). This works because many of the contaminating proteins will be soluble in the organic phase while DNA preferentially will remain in the aqueous phase. Thus, by mixing an aqueous solution of DNA and protein with an organic solvent, you can remove most of the protein and get an aqueous solution of nearly pure DNA.

RNA is generally removed through the enzymatic breakdown by RNase, which is an enzyme that specifically targets RNA molecules and leaves DNA intact. At this point, it is important to ensure that no DNases are introduced into the solution.

Step 4. DNA Precipitation

This step involves removing water from the DNA backbone. This is accomplished by increasing the ionic strength of a solution by the addition of a salt and the addition of a high concentration of an alcohol (isopropanol or ethanol). These two components will attract lots of water to themselves and thus pull much of it away from the DNA. Once the DNA backbone has had water removed from it, it loses solubility and starts to precipitate. This precipitated DNA can then be collected and resuspended in a solution of your choice (usually pure water or a solution of Tris and EDTA)

Excercises

For our lab, we will use a modified procedure based on a protocol found in Singh (2000). Here is the relevant section of the Materials and Methods section:

> *Cells were incubated at 37°C in an incubator for 24 h, centrifuged at 500g for 5 min, and suspended in 20ml of PBS. Cells were then lysed by the addition of 100ml of lysing solution having 0.25 M NaCl, 0.1% sodium lauroyl sarcosine, 10 mM EDTA, 1 mM Tris, (pH 7.5) at room temperature for 30 min. Samples in microfuge tubes were centrifuged at 11,500g for 15 min to pellet cellular debris. Supernatants, which consisted mainly of lysed apoptotic cells, were incubated first with 10mg/ml of RNAse A (Amresco, Solon, OH) for 30 min at 37°C, and then with 1 mg/ml of proteinase K (Amresco) for 30 min at 37°C.*
>
> Singh, NP (2000). A Simple Method for Accurate Estimation of Apoptotic Cells. *Experimental Cell Research, 256*(1), 328–337.

Materials (per group):

- Sample of induced cells
- Sample of uninduced cells (control)
- Lysis Buffer (250 mM NaCl, 1 mM Tris, 10 mM EDTA, 0.5% SDS, pH 7.5)
- PBS
- 1ml of 8M Ammonium Acetate
- 3ml of 100% Ethanol
- 3ml of 70% Ethanol
- 200ul TE (10mM Tris, 1mM EDTA, pH 8)
- Eppendorf / Microfuge tubes
- Micropipettor (P200)
- RNase A
- Proteinase K

1. Cell Lysis, and Removal of Protein and RNA

You will be working with cells that were chemically treated to induce apoptosis and with a control sample that was not induced. Based on the information provided in the above section of the Materials and Methods, we will perform our protocol as follows:

Procedure:

1. Transfer approx. 1.5-2 x10^6 cells to a microfuge tube.
2. Spin the cells down at 500g for 5min (repeat at a higher speed if necessary).
3. Remove the supernatant.
4. Add 100ul of PBS and resuspend the pellet by flicking and tapping the tubes.
5. Add 400ul of Lysis Buffer, mix gently and incubate at room temperature for 30min.
6. Centrifuge the samples at 12000g for 15min.
7. Transfer the supernatants to fresh tubes.

Is the supernatant viscous?

Viscosity (“stickyness”) indicates the presence of nucleic acids.

Based on the volumes you just used, calculate the volumes of RNase and Proteinase that you will need to use (please check the concentrations of the stock solutions). You will want a final concentration of approximately 10mg/ml of RNase and 1mg/ml of Proteinase.

8. Add _____ ul of RNase to the cell extract and place the tube at 37C for 10min.
9. Add _____ ul of Proteinase to the cell extract and return the tube to 37C for 10min.

2. DNA Precipitation

As you perform this part, look for evidence of a white precipitate (that's the DNA). In solution, the ammonium acetate will ionize. The positive charge on the ammonium will interact with the sugar-phosphate backbone of the nucleic acids. This disrupts it's interaction with water and makes the molecule far less hydrophilic and therefore less soluble in water. The ethanol will also help to "dehydrate" the DNA by making the total concentration of water much lower.

1. Spin down the contents of the epi tubes. You should have 300ul of each cell lysate.
2. Add 0.5 volumes (150ul) of Ammonium Acetate and mix the tube contents.
3. Add ~2.5 volumes (1000ul) of cold 100% Ethanol.
4. Incubate the tubes in the freezer (-20C) overnight.

This protocol will need to be completed tomorrow.

Your group should designate someone who can come at some point tomorrow and complete it. The remaining protocol should not take more than 1hr to complete (~45min)

Day 2

Please make sure that your tubes are balanced properly – doing this with a member of another group would be best, but you can prepare a "balance" tube with an equivalent volume if noone else is doing their centrifugation.

5. Centrifuge the samples for 30min at maximum speed.
6. Remove the supernatant (you can usually just pour it off, the pellet should remain attached to the bottom of the tube)
7. Add 1ml of 70% Ethanol, and invert once.

   This is a short step to wash the pellet. It helps to solubilize some of the contaminating molecules and salts, it also helps to rehydrate the DNA a little bit. Do not try to resuspend the pellet yet, and do not leave the Ethanol in there too long.

8. Centrifuge the samples at maximum speed for 1min.
9. Remove the ethanol. Invert the tube and tap it on a paper towel to remove any visible droplets.
10. Air dry the pellet of DNA for at least 10min.

    Even if you don't see the DNA pellet, it's probably there – DNA pellets tend to be very small and colourless. To speed up the drying process, you can place the epi tubes in a rack and place it just inside a fume hood, then close the sash to leave a small gap that allows air to still flow into the fume hood. The rapid flow of air over the top of the epi tubes will help to draw out the ethanol and leave only water.

11. Resuspend the pellet in 50ul of TE and leave it in the freezer for the next lab.

Post-Lab Questions:

Why did you use Proteinase **after** the RNase?

Lab 11: Fluorescence Microscopy

In today's lab, we will be doing a test to find out if the cells in our sample are undergoing apoptosis. We will check the nuclei of the cells for fragmentation using fluorescence microscopy.

Learning Objectives:

Students will:

- Define the term “fluorescence”
- Describe the pathway of light in a fluoresce microscope
- Explain how “tags” are used to help highlight various cellular components

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Pre-Lab Questions:

View the video linked below and answer the following questions:

- https://www.jove.com/science-education/5040/introduction-to-fluorescence-microscopy

What is the Stokes Shift?

What is a fluorophore?

How can different fluorophores be used to visualize different cell components?

Fluorescence Microscopy

Fluorescence microscopy has quickly established itself as a powerful tool in the study of cell biology. It improves upon compound light microscopy by allowing the highlighting of specific molecules and/or structures in a cell with a very high level of contrast. It accomplishes this through the use of fluorescent dyes, fluorescently tagged antibodies, and/or expression of genetically modified, fluorescently tagged proteins by the cell of interest.

Fluorescence is defined as the nearly simultaneous emission of light by a molecule which has absorbed light at a shorter wavelength. A similar phenomenon, but one where the emission of light occurs after the excitation light is removed, is called **phosphorescence**.

Both of these are explained by the basic principle of chemistry which states that atoms can absorb energy (for example light radiation) by sending their electrons to higher-energy orbitals. The subsequent return of these electrons to their normal, lower-energy orbitals results in the release of energy (usually in the form of photons of light). In the process of this conversion, some of the initial energy is lost (due to entropy) and thus the light energy emitted in the process is at a lower energy level (longer wavelength) than the initial excitatory energy (shorter wavelength).

Scientists have been able to harness this property of certain molecules (known as "fluorophores") by linking them to stains and antibodies, and applying them to their microscopy samples to highlight structures and molecules of interest.

Fluorescence microscopes utilize high-energy wavelengths of light (usually in the UV range) to activate these fluorophores in the dyes on their slides. The fluorescent stains then emit light at a lower energy wavelength which can be viewed/detected by the observer. Because different fluorophores are activated by different excitatory wavelengths, the fluorescent microscope must have a way to specifically select them. This is accomplished by the use of different **excitation filters**, which block all other light and only permit the desired wavelength to pass through them.

When the excitation wavelength of light strikes the sample and activates the fluorescent molecules, a longer wavelength of light is emitted into the objective lens. Before this emitted light can be detected, however, it passes through an **emission filter** in order to block any other light (ie. any reflected "excitation" light). This ensures that the resulting image will only show the location of the structures stained by the fluorophore being activated at that time. Because all other light is being blocked by the excitation and emission filters, the areas of the slide surrounding the highlighted structures will be black; this results in excellent contrast (a clear delineation of the structure of interest from everything else).

Fluorescent Dyes and Tags

A variety of fluorescent dyes have been developed for fluorescence microscopy. The choice of fluorescent dye is frequently based on its ability to bind to a specific cell component of interest. This is based on the same interactions as regular light microscopy dyes.

In cases where a fluorescent dye for a specific component does not exist, scientists can generate antibodies which can attach to that component. A fluorophore can then be chemically attached to such antibodies and used as a molecular tag. This fluorescent antibody is then used to specifically bind to the target molecules in a cell and report/show their presence.

Since the background colour is black and only the tagged components are visible in fluorescence microscopy, it is important to be able to show the presence of cells on a slide (in

case the molecule or organelle of interest is absent). For this reason, stains that bind to DNA (nuclei) are very commonly used in fluorescence microscopy.

Probably the most commonly used stain is **DAPI** (4',6-diamidino-2-phenylindole). It is a fluorescent dye with an optimal excitation wavelength of 358nm and an emission wavelength of 461nm, giving it a blue colour. DAPI binds to AT-rich regions of double stranded DNA, within the minor groove of the double helix, and is very specific to DNA (there is usually no staining in the cytoplasm). This stain is generally used with dead/fixed cells because it has difficulty passing through an intact cell membrane (living cells will not take it up).

Another common nuclear stain is the **Hoechst 33342** dye. It has an excitation wavelength of 360nm and an emission wavelength of 460nm giving it a colour similar to DAPI staining. Also, similarly to DAPI, Hoechst 33342 dye binds in the minor groove of AT-rich regions of DNA. Unlike DAPI however, it is able to enter living cells and can also be used to stain the nuclei of cells undergoing early phases of apoptosis.

Exercise

1. Visualization of Changes in Nuclear Morphology

In today's protocol, we will be using a kit containing the Hoechst 33342 dye to stain the nuclei of cells treated with a chemical that induces apoptosis. You will produce some temporary slides and view them using a fluorescence microscope.

Materials (per group):

- Sample of induced cells
- Sample of uninduced cells (control)
- PBS
  (10mM PO_4^{3-}, 137mM NaCl, 2.7mM KCl)
- Micropipettors (P200 & P1000) and tips
- Slides and coverslips
- NucBule fluorescent staining kit from Life Technologies

Procedure:

1. Transfer approx. 1 x10^6 cells to a microfuge tube.
2. Spin the cells down at 700g for 5min.
   a. Repeat this step if necessary to collect more cells
3. Remove the supernatant.
4. Resusped the pellet in 1ml of PBS by flicking and tapping the tubes.
   Do this very gently, DO NOT pipette up and down to resuspend.

In order to stain the nuclei, we will be following the protocol provided with the kit

5. Transfer 100ul of each cell suspension into new epitubes.
6. Spin the cells down again at 700g for 5min.
7. Remove 700ul of the supernatant
8. Resuspend the cells in the remaining supernatant
9. Transfer 20ul of each cell suspension onto individual slides and cover with coverslips
10. View slides using a Fluorescence Microscope