

SDS-PAGE ANALYSIS OF PROTEINS AND COMPUTER INTERFACED MICROSCOPY

Gel electrophoresis is a very powerful tool used to fractionate various macromolecules for analytical studies. Electrophoresis refers to the movement of charged molecules in an applied electric field. How fast a molecule moves is dependent upon the strength of the electric field, the net charge on the molecule, the size of the molecule, and the viscosity of the medium through which it travels.

Electrophoretic separations of biological macromolecules are usually performed in gels made of a porous insoluble material such as agarose or acrylamide. To separate proteins, polyacrylamide gels are often used, because acrylamide is inert and therefore does not interact appreciably with the samples. To make a polyacrylamide gel, acrylamide is reacted with a cross-linking reagent. The concentrations of both acrylamide and the cross-linking agent can be varied in order to create gels appropriate for separating proteins of various sizes.

The polyacrylamide gel serves as a molecular sieve that separates proteins based on their size. Smaller proteins migrate more quickly and, thus, further through the gel. In contrast, large molecules migrate only a short distance. In order to separate proteins on a gel solely according to size, all of the molecules must have the same charge-to-mass ratio. This can be accomplished by electrophoresing the samples under denaturing conditions that include treatment with a negatively charged detergent. This process is known as SDS-PAGE.

SDS-PAGE is the acronym for sodium dodecyl sulfate—polyacrylamide gel electrophoresis. SDS-PAGE is a simple and sensitive method used to fractionate proteins and provide an estimate of their denatured molecular weights. Protein samples are first boiled in a buffer containing sodium dodecyl sulfate (SDS), an anionic detergent, and a reducing agent such as β -mercaptoethanol, which breaks the disulfide bonds and disrupts the tertiary structure of the protein. The negatively charged SDS molecules bind to the hydrophobic amino acid residues on the interior of the protein, disrupting the noncovalent interactions. The protein unwinds. Because of the mutual repulsion of the negatively charged sulfate groups on the SDS anions, the protein takes on a linear conformation.

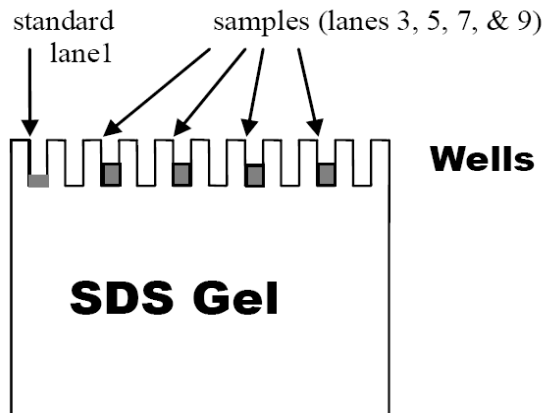
The denatured proteins are then loaded onto a polyacrylamide gel and electrophoresed. The negatively charged molecules migrate through the matrix toward the cathode at a velocity that is roughly inversely proportional to the logarithm of their molecular weight. Protein visualization is accomplished after electrophoresis by staining with a dye called Coomassie Blue that recognizes all proteins.

Since the denatured proteins migrate through the gel based on their size, it is possible to use SDS-PAGE to estimate the molecular weight of proteins on the gel. To do this, you must run standard proteins of known size on the same gel along with your protein samples. Molecular weight can then be estimated by visual comparison to the standards run or mathematically by preparing a standard curve of relative mobility (R_f) versus the logarithm of molecular weight. You will use SDS-PAGE to identify proteins according to molecular weight. Each person in your group will be given an unknown sample to load on the group's gel. Your task is to identify your sample and compare it to others based on its molecular weight and other observable SDS-PAGE characteristics: number of bands, their molecular weight and relative abundance. Each sample to be handed out is labeled by a gel letter and the lane number it is to be loaded into. Each gel will be labeled with a letter (A, B, C and so on) and has 10 lanes. Protein standards will always be

run in lane 1 on each gel. You will skip one lane between each sample. Your samples therefore will be loaded into lanes 3, 5, 7, and 9. For example you may be handed a sample labeled A5 that means you load your sample into gel A lane 5. See the image below.

Electrophoresis example: 1. Load the gel.

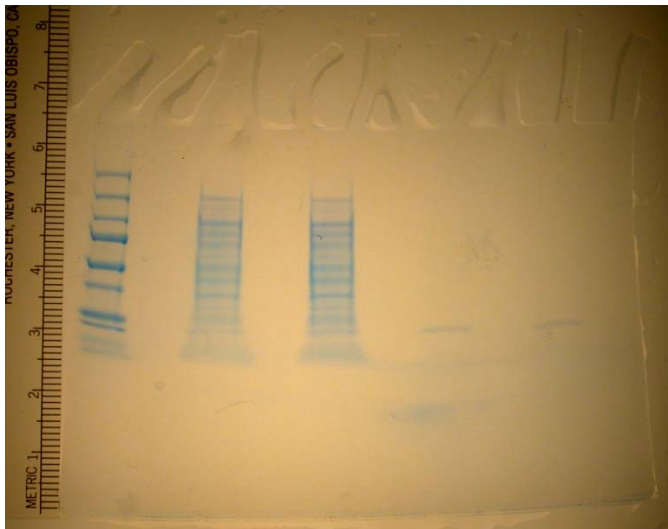
Sample wells, where the standard or other samples are loaded, are filled in to represent a full well.



2. Run the gel....



3. Stain and destain the gel, protein bands become visible.



Procedure — Wear gloves throughout the following procedures

- ❑ 1) Your TA will give you a sample. Notice they are blue, they have sample buffer already added to them. Heat your sample for 5 minutes in the lab's hot water bath.
- ❑ 2) The Mini-Protean electrophoresis apparatus that you will use to run your gel is on the counter top and contains two precast gels. Each group will load their samples into one gel (a total of four samples per gel) Familiarize yourself with the apparatus; your lab instructor will

instruct you how to load your samples onto the gel. You will be able to practice with a practice gel and sample buffer before you load your actual sample onto your gel.

- ❑ 3) Your instructor will load 10µl of standard onto your gel, and each member of your group will load their sample onto the group's gel, using the instructions previously mentioned. Record in your notebook your gel and sample number (ie B7, D3, etc)
- ❑ 4) Before you put the cover on the gel rig, notice that you will need to match the red ringed electrode with the red cable and the black ringed electrode with black cable. Do your best not to disturb the loaded samples as you put the cover on the rig.
- ❑ 5) Make sure the power switch on the power supply is in the "off" position. Connect the color coded leads to the power supply red to red and black to black. Turn the power supply on and slowly turn the voltage up to 120 V. Do not move the gel rig while it is running.
- ❑ 6) Your instructor loaded a protein standard that is dyed and can be seen migrating as you run your gel. Observe the gel periodically to see the various protein bands separate, it also shows you if the gel is running properly.
- ❑ 7) Electrophorese the samples until the bromophenol blue dye front or the smallest band of the pre-stained standard has traveled to the very bottom of the gel (120 V for approximately 1 hour 15 minutes.)

During the running and staining of your gel (steps 9 and 11), perform Part II of this lab, "Extraction of Mitochondria", which will be needed for next week's lab.

- ❑ 8) After electrophoresis, get your group's labeled stain box, be sure the box label matches your gel letter and half fill it with deionized water. (For example: Group B use the B box for staining.) Remove one glass plate from the gel, you will see a thin delicate gel supported by the second glass plate — carefully move it into the stain box and rinse in the water for 2-3 minutes, then carefully hold down the gel (with a gloved finger) and pour off the water well. Now add stain.
- ❑ 9) Add enough Biosafe Coomassie blue stain to the box to cover the gel by 5 mm, approximately 60ml should do it. Be sure to cover the gel with the stain, so it does not float on the top. Let it sit for at least 1 hour and gently shake the box to mix the stain from time to time. During the staining of your gel, continue to perform Part II of this lab, "Computer Interfaced Microscopy".
- ❑ 10) Your gels will be destained, photographed and posted on the course website in no more than 2 days; destaining takes a while so we will do this for you. Your gel will resemble the gel photo in the previous text.
- ❑ 11) Photographs of the gels will be in folders labeled by lab meeting time: Tues am, Tues pm, Wed pm and Thurs am. Within each lab's folder will be photos of each group's gel, labeled by group letter and the names recorded on the gel box's attached paper.

- 12) Print out a picture and paste it into your notebook. If you have a computer and are online at home, you can get your image off of the Blackboard Vista course web site, and also download your own version of ImageJ as described in the appendix. Then carry out your measurements as described in “Results”. You can download an image anywhere that has internet access, like in the general use labs in our library, or a public library, just bring a disk with you to save your gel image onto and print it out. You can always go to the cell lab (outside of scheduled lab times) and use ImageJ there but it is not necessary.

For your Lab Notebook and Lab Report:

Results: Determining the Molecular Weight

Complete the analysis of the provided gel image on the computers in class. Take your time and learn the procedure as you will need to know how to use ImageJ for analyzing your SDS-PAGE results. Follow the instructions below (you will repeat this process using your own gels outside of lab). Enter your results in your note book for the TA to check before you leave and as a reference for later on. For analysis of the gel you ran today, after the gels have destained, are photographed and posted on the course website you will repeat this protocol outside of class. The gels should be online one to two days after your lab-all will be up by Friday afternoon.) Using the data from your own gels answer the questions below. Your TA will check these results next time you come to lab.

In your notebook do the following and answer the questions below:

1. Label the standard and your sample on your group's gel.
2. Is there a protein band present in your sample lane? Do you have more than one?
3. In order to determine the molecular weights of your protein(s), use the lane with the protein standards to construct a standard curve of the relationship between molecular weight and the distance each polypeptide migrates in the gel:
 - a. For each band in the standard lane, measure the distance in cm **from the bottom of the well to the middle of the band**. You could use a ruler to measure this manually but today you will be taking these measurements using ImageJ software (see the procedure below). To measure the distance using ImageJ, follow this protocol:
 - 1) Use the computers in lab and the **ImageJ** software to open and measure the image of your gel. (Or get the image from the Blackboard Vista web site, and download **ImageJ** yourself: see Appendix)
 - 2) Click on the line tool in ImageJ menu. Draw a long line along the centimeter ruler on the side of the image of your gel, starting at 0 on the ruler and stopping at a whole cm position, using most of the visible ruler for more precise calibration (e.g. 7 cm).
 - 3) Go to the ImageJ menu bar, select **Analyze**, then select **Set Scale**.
 - 4) It will give you the pixels that make up the line you drew. Fill in the **Known Distance** as whatever whole cm you measured (in our example, 7 cm), the **Units** as cm, and select **Global**. Keep pixel aspect ratio at 1.0.
 - 5) Now you can measure and record the migration of each protein band that makes up the standard and then measure the proteins of interest from your samples. For each band, draw a straight line **from the bottom of the well to the middle of the band**, then click on **Analyze** and select **Measure**. The length of the line will be recorded in

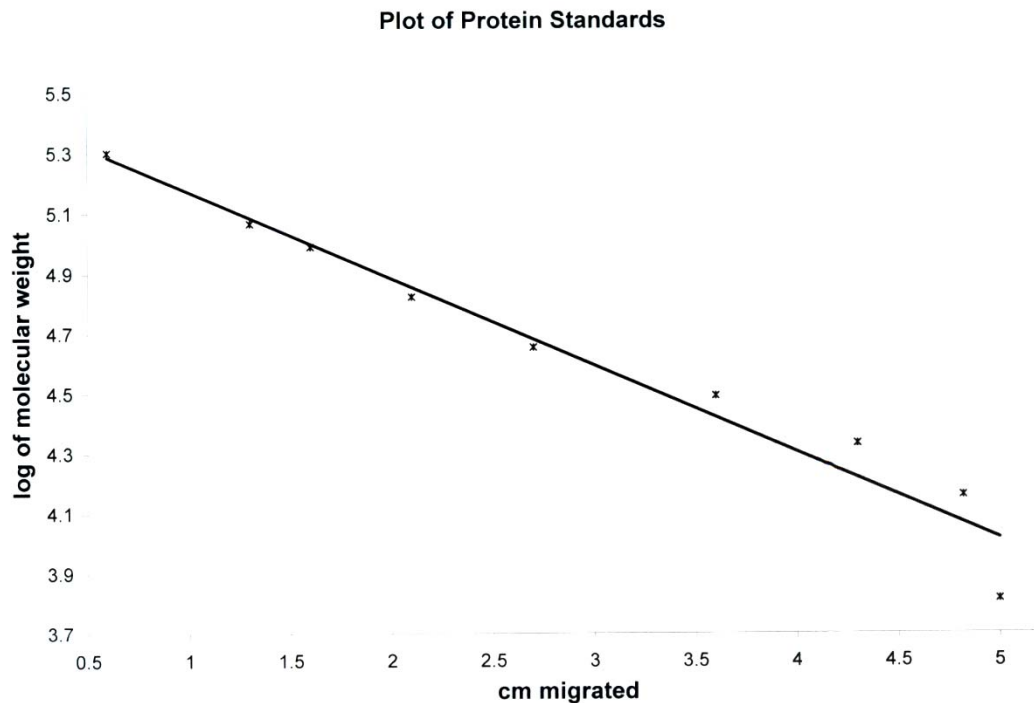
a separate **Results** window. Copy the measured distances into the chart below. Be consistent in your measurements.

- Double check as you measure each, you should be able to estimate the distance in centimeters before you draw the line. Is the computer's measure similar to what you expect? It should be similar – if not, double check/repeat the calibration by going to **Analyze**, and selecting **Set Scale**.

6) Use a calculator to determine the log of each molecular weight value and record them in the chart below.

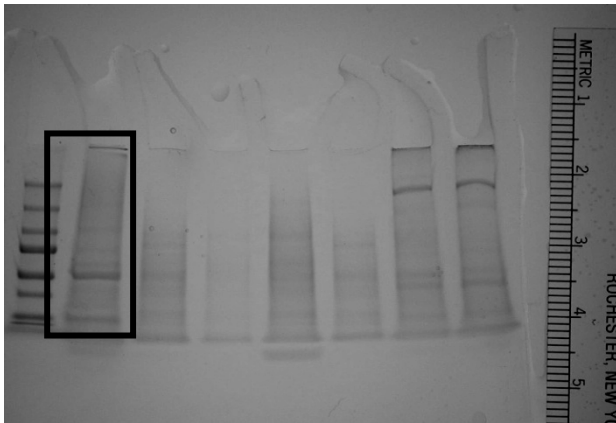
Standard	Molecular weight In kDaltons (kDa)	Log of molecular weight	Distance from bottom of well (cm)
1	250		
2	150		
3	100		
4	75		
5	50		
6	37		
7	25		
8	20		
9	15		
10	10		

b. Using Excel, plot the distance traveled on the X axis vs. the log of molecular weight on the Y axis. Draw a trend line connecting the data points. Your graph should somewhat resemble the graph below-though it is a different standard. Record the equation of the line that your graph produces.



c. To determine the molecular weight of any other polypeptide run on the same gel, measure the distance that the band migrated. You can then read the molecular weight directly from the standard curve or use the equation of the line to calculate log of molecular weight. You can then use the inverse log function on your calculator to determine molecular weight.

1. Using your graph, estimate the molecular weight(s) of the band(s) in your sample lane. Indicate the most abundant and the relative abundance of the others. The most abundant is the darkest band. **Note:** if you do not see a band in your lane, contact your TA and they will assign a different sample to you.
2. Describe your sample in as much detail as possible in terms of molecular weight of the bands and their relative abundance. For example, if you refer to the image below and your sample is



the one in the rectangle. You could describe it as having multiple bands, but 3 distinct. The most abundant (the darkest and thickest) is approximately 48kD, the others are smaller, close to 21 and 31 kD. There are at least two more faint bands (not abundant and difficult to see) with greater molecular weights of around 66 and 97kD.

3. Out of all the gels run in your lab section, which lanes also contain your type of protein? Record their sample numbers.
4. Look at all of the gels run by your lab section. How many different types of protein samples in total can you distinguish?

Part II. Extraction of Mitochondria

To understand the biochemical properties of cellular compartments such as the endoplasmic reticulum, Golgi complex, mitochondria, or nucleus, scientists had to develop protocols to purify individual organelles from cells. More than 50 years ago, scientists discovered that by centrifuging cell lysates in the appropriate buffer and relative centrifugal force (RCF), dense organelles, such as nuclei, can be separated from less dense organelles, such as mitochondria. Similarly, mitochondria can be separated from vesicles and cytosol by centrifuging at a different RCF. Today you will utilize this technique (known as differential centrifugation), to generate different cellular fractions that are enriched for nuclei, mitochondria, cytoplasm, and vesicles. You will then freeze these fractions and use them next week.

Once scientists had the techniques to separate cellular compartments, they could then study the behaviors and functions of different organelles. These types of studies allowed for discoveries such as how the TCA cycle occurs in mitochondria and how glycolysis occurs in the cytosol. Next week you will thaw your isolated mitochondria fractions and assay the activity of succinate dehydrogenase, an enzyme that functions in TCA cycle that occurs in the mitochondria. You will also test for inhibition of this activity.

Protocol for Differential Centrifugation for Isolating Subcellular Fractions

1. Remove several (5-6) rosettes from a head of cauliflower. Using a clean razor blade, slice off the outer 3–5 mm of each rosette, until approximately 40 g of tissue have been accumulated.
2. Place half of the sliced cauliflower into a mortar. Add 9 g of cold sea sand and 20 ml of cold mannitol isolation buffer. Grind the tissue vigorously for one minute. Add more of the cauliflower and grind until you have added it all and the mixture becomes a smooth paste. (about 2-3 minutes)
3. Add an additional 20 ml of cold mannitol isolation buffer to the cell paste and grind well for 2 minutes more. The cauliflower tissue should look more translucent than white.
4. Transfer the thick slurry to a funnel lined with four layers of cheesecloth supported by a flask and collect the liquid into the flask. Wash the mortar with 5 ml of cold mannitol isolation buffer and pour the wash through the cheesecloth as well. You should carefully squeeze out the remaining liquid from the mash.
5. Transfer the filtrate to a cold 50 ml centrifuge tube. Squeeze out all of the juice into the tube. Put 1-2 large drops on a clean glass slide labeled “H” for homogenate and cover it with a coverslip. **IMPORTANT NOTICE:** as you perform steps 6 and 8, be sure to have the instructor check the balancing of all tubes and have him/her stand by as you start up the centrifuge. In this experiment you are using high-speed centrifugation and it is therefore extremely important that the tubes be accurately balanced, all tubes must weigh the same amount, and the centrifuge started up properly.
6. Be sure that your tube and its contents weigh exactly the same as everyone else's, this should be ~44g. Centrifuge the tube for 10 minutes at 2,500 rpm in a refrigerated centrifuge set at 4 °C.

7. Using a 10 ml serological pipette, remove the post-nuclear supernatant without disrupting the pellet and put into a clean, cold, 50 ml centrifuge tube. Resuspend the remaining pellet, which contains the “nuclear fraction” in 5ml mannitol isolation buffer. Put 1-2 large drops on clean glass slide labeled “N” for nuclei pellet and cover with a coverslip.
8. Be sure that the tube with the supernatant from the last spin and its contents each weigh exactly the same as everyone else’s, this should be **40g, remove any excess liquid so that it is**. Centrifuge the supernatant at 15,000 rpm for 30 minutes at 4°C.
9. Remove the post-mitochondrial supernatant from the mitochondrial pellet. Put 1-2 large drops on clean glass slide labeled “MS” for post-mitochondrial supernatant and cover with a coverslip. Transfer 10ml of the supernatant into a 15ml plastic tube in which it will be frozen to be used next week. Be sure to label the tube with your group’s identification and that it is the supernatant. (MS) Add 2.5 ml of 50% glycerol, cap tube properly and mix gently but thoroughly by inverting the tube several times. Keep on ice, in the instructor’s ice bucket to be frozen for use next week.
10. Add 10ml of cold assay buffer to the mitochondrial pellet remaining in the tube. Thoroughly resuspend the pellet in the buffer (mix using a pipette to disperse the clumps by pumping the liquid) **weight and record it on the last page of this lab**. Transfer into the plastic tubes in which it will be frozen for next week. Be sure to label the tube with your group’s identification and that it is the mitochondria pellet. (MP) Save 1ml of your mitochondria pellet in a microfuge tube labeled “MP” in your ice bucket to look at today. To the 15ml “MP” tube to be frozen, add 2.5 ml of 50% glycerol, cap tube properly and mix gently but thoroughly by inverting the tube several times. Keep on ice, in the instructor’s ice bucket to be frozen for use next week.
11. You will now examine the cell fractions of your mitochondria preparation by comparing the three slides you prepared using a phase-contrast microscope at the 40x and 100x. Note the concentration and appearance of each fraction and note on the last page. You will create dilutions of the last fraction to view and to estimate a count of mitochondria.
12. Using the 1ml sample you saved in the microfuge tube, make a serial dilution of **MP** (mitochondrial pellet) to view under the microscope and to facilitate counting the mitochondria. You’ll need three spec tubes, make a 1/10, 1/100, 1/1000 serial dilution following the chart below.

Dilution factor	MP or dilution of	Buffer (ml)	Total volume	Mitochondria count /field of view x 25	Mitochondrial per ml (average count x 500)
1	--	--	--	----	
1/10	0.5ml MP	4.5ml	5ml	----	
1/100	0.5ml 1/10	4.5ml	5ml		
1/1000	0.5ml 1/100	4.5ml	5ml		

13. Add one drop of Janus Green B stain to each dilution tube. Cap and invert each dilution to mix well. Use one slide and label 3 areas 1/10, 1/100 and 1/1000 then using a micropipette transfer a 2 microliter drop of each solution onto the slide in the appropriate

place, let the drops dry. Draw a circle carefully around each with a lab marker as they do. You will assume that they probably will take up about 25x the space that the field of view using 100x allows you to count.

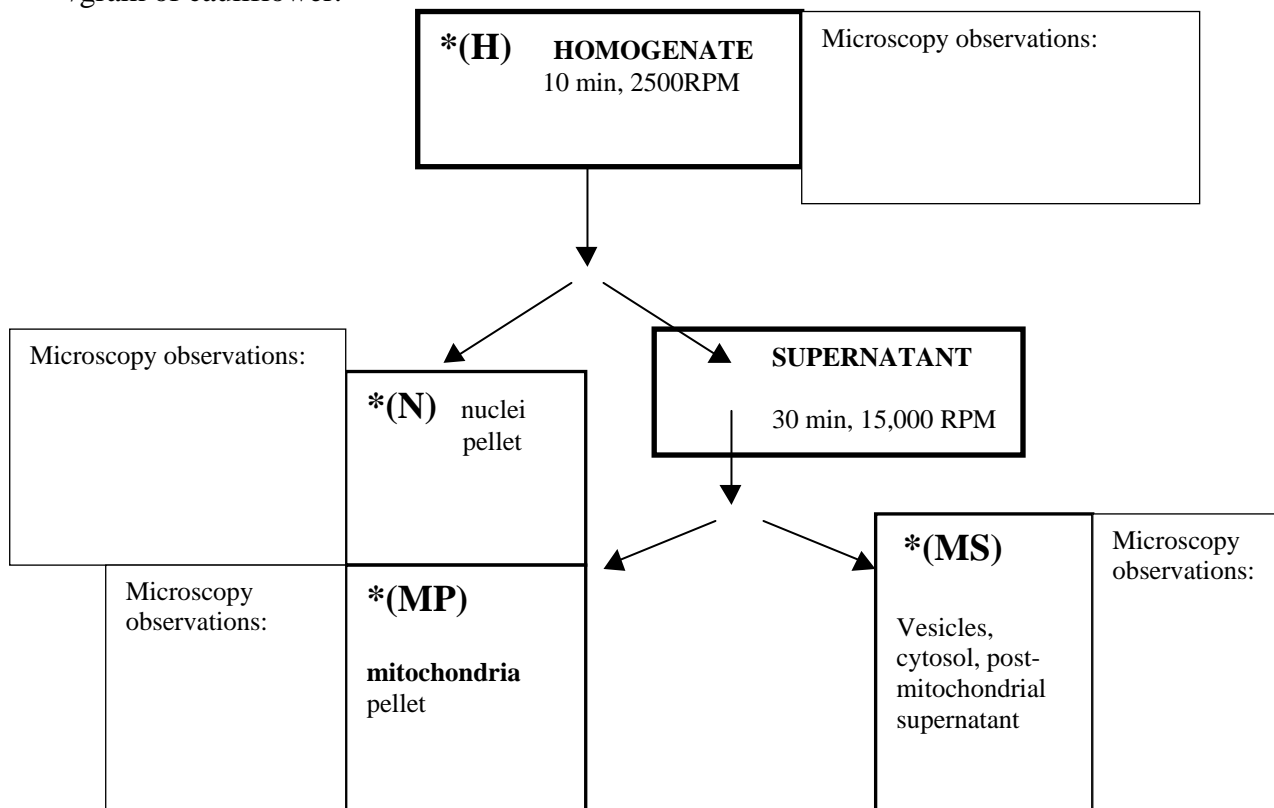
14. Put a cover slip on top of the slide and focus with 40x on the edge of the circle you drew around the 1/10 drop, when that is in focus you will be able to fine focus to see a lot of tiny dots, the mitochondria. Increase to 100x and fine focus. They will be either blue or red, depending on how much oxygen is present. Compare the three dilutions, choose the one you feel is easiest to count and count the mitochondria in one field of view.

15. **Calculate a crude estimate** of the total number of mitochondria in 1ml of your stock **MP**. Assume the space of your field of view is approximately one 25th of the total area that the dried up 2µl drop takes up, to estimate the mitochondria in 2µl you must multiply by 25 per 2µl, then by 1000 µl/ml, finally by the inverse of your dilution factor.

For example, if you counted 10 mitochondria using the 1/1000 dilution the stock solution would be estimated to have: $10 \text{ mitochondria} \times 25/2\mu\text{l} \times 1000\mu\text{l/ml} \times 1000 = 1.25 \times 10^8 \text{ mitochondria/ml}$.

16. Calculate the average mitochondria in one gram of your cauliflower sample.

For example: using the result from the example above and all other steps in this protocol:
 $10\text{ml MP resuspension}/40\text{g cauliflower} \times 1.25 \times 10^8 \text{ mitochondria/ml} = 3.125 \times 10^7 \text{ mitochondria/gram of cauliflower}$.



Crude estimate of mitochondria in 1ml of **MP** stock: _____

Crude estimate of the mitochondria in one gram of cauliflower used _____