Genomic DNA Isolation

a) Introduction

Genomic DNA is the DNA found in the chromosomes of the nucleus. It was first isolated (purified) from cells in the mid-1800s, but its role as the genetic molecule was not understood until almost 100 years later. In modern molecular biology laboratories, DNA is routinely isolated from cells as the first step in analyzing genes.

Obtaining purified DNA from cells at first seems difficult. The scientist must first release the DNA by breaking open the cell membrane and the nuclear membrane. Once the DNA is released from the nucleus, however, it can be destroyed by enzymes in the cytoplasm (called nuclease enzymes or DNase enzymes) that degrade DNA. The scientist must find ways to guard the DNA against attack by DNA-degrading enzymes from within the cell. Lastly, the released DNA must be separated from the proteins, lipids, and carbohydrates, amino acids, fatty acids, and other cellular molecules.

There are several different DNA isolation procedures. Which one is chosen depends on which species the DNA is being isolated from. The DNA isolation procedure for plant cells, for example, differs from the DNA isolation procedure from animal cells. There is not one "best" procedure because each species' cells have unique properties that require different methods to release the DNA.

Although there are many DNA isolation procedures, they all have some common features. The tissues (cells) are first homogenized, usually with a blender or a mortar and pestle. The liquid that comes out of the blender is called the "homogenate". The homogenate is usually filtered to remove any clumps of cells that the blender did not break apart. Next, different chemical substances that help in isolating the DNA are added to the homogenate, usually one at a time. Below is a list of some of these chemical substances and their roles in DNA isolation.

Isotonic buffer: In the early steps of the DNA isolation procedure, the tissue is homogenized by placing it in a blender. The researcher does not want the cells to break open during the blending step. Therefore, the tissue is homogenized in an isotonic buffer solution. The isotonic property of the solution prevents the cells from bursting due to osmosis, and the buffer prevents the cells from being damaged by pH changes. A common isotonic buffer used in DNA isolation is buffered sucrose.

EDTA: This is a common food additive and laboratory reagent. It binds to and removes positive ions such as magnesium (Mg^{2+}) and calcium (Ca^{2+}) ions. These ions strengthen membranes by neutralizing the negative charges on the phospholipids, so removing these ions weakens the cellular and nuclear membranes.

SDS detergent: Recall from your lab on lipids that detergents and soaps are molecules that break apart globules of lipids. Detergent molecules are attracted to both lipids and water, By binding to both lipid and water the detergent is able to

dissolve the membranes of the cell. In other words, detergents lyse (break open) cells membranes and nuclear membranes. The most common detergent used in biology laboratories (including today's DNA isolation lab) is called sodium dodecyl sulfate (SDS).

Sodium Chloride (NaCl, table salt): The detergents and the EDTA dissolve the cell's membranes, which releases the genomic DNA. But the released DNA is not pure. The cell's proteins are still present. To make matters worse, the proteins tend to form ionic bonds to the DNA, so they stick to the DNA and contaminate its purity. As a first step in separating the proteins from the DNA, a large amount of NaCl salt (which is Na^+ and Cl^- ions) is added. The Na^+ ions neutralize the negative charge on the DNA, whereas the Cl^- ions neutralize the positive charges on the proteins. With their charges neutralized, the proteins and DNA no longer form strong ionic bonds to one another and therefore can be easily separated.

Alcohol: After the NaCl has separated the proteins from the DNA, the final step in DNA isolation is usually to add ice cold alcohol to the mixture. The alcohol forms a separate layer on top of the cell homogenate. The DNA is not soluble in the alcohol layer, so it will form a precipitate (a mucus-like solid in the alcohol layer). The proteins, on the other hand, do not precipitate when the alcohol is added (they stay dissolved in the homogenate). The precipitated DNA can therefore be separated from the proteins by spooling it onto a rod, then the rod is used to transfer the spooled DNA into sterile water. The DNA has now been successfully isolated from the cell.

Although not "chemicals", there are two more features of most DNA isolations that help protect the DNA. The first is coldness: The DNA isolation procedure is usually done on ice to slow the action of cellular enzymes that that might degrade the DNA. The second is gentleness: DNA strands are extremely long and fragile. Therefore all steps should be done slowly and gently to avoid shearing the DNA strands.

b) Proper use of Micropipettes

Micropipettes are a common laboratory instrument in molecular biology laboratories. A micropipette is used to transfer small and exact volumes of liquids from one test tube to another.

Most micropipettes transfer microliter volumes of liquids. A microliter is 1/1000 of a milliliter or 1/1,000,000 of a liter! It is a volume of water about the size of a head of a pin. There are three micropipettes you will use this semester, each with a different range of ul that it transfers. The p20 micropipette can transfer 1 – 20 microliters (ul) of liquid. The p200 micropipette can transfer 20 – 200 ul of liquid. The p1000 micropipette can transfer 100 – 1000 ul of liquid.

Your instructor will demonstrate the proper use of the micropipette, but review the list of steps below before using the instrument.

1) Always place a disposable tip on the micropipette. The instrument will be damaged if you pipette liquids directly into the barrel of the

pipette itself.
2) Set the micropipette for the exact volume you wish to transfer. This is done by turning the plunger until the proper volume (in microliters) is displayed in the window.

3) To draw a sample into the micropipette, press the plunger down to the first stop BEFORE you put the tip into the liquid, THEN place pipette tip fully into the liquid. Slowly release the plunger to draw the sample upward into the tip. Don't pull the tip out of the liquid until the plunger is fully released or you will get air bubbles in the tip. If done correctly, this will draw the correct volume of sample into the tip. After you remove the tip from the liquid, inspect to be sure that there are no bubbles in the tip.

4) To deliver the sample into a test tube, place the tip into the BOTTOM of the tube that will receive the sample. To deliver the sample, press the plunger down to the second stop. Keep the plunger pressed all the way down until after you have removed the tip from the tube, or you will withdraw some of your sample back into the micropipette.

5) Eject the tip into the biohazard waste.

c) Isolating Genomic DNA

1) Obtain the following items:
- a) a 500 ml beaker with ice
- b) One small disposable glass test tube
- c) a microcentrifuge tube with NaCl solution
- d) a 5 ml serological pipette (with pipetter device).
- e) a 1000 ul micropipette with several disposable tips
- f) a wax pencil
- g) a microcentrifuge tube with ETDA solution
- h) a microcentrifuge tube with SDS solution

2) Go to the front of the room and obtain a 15 mL test tube with calf thymus cells in sucrose buffer. *Note how these cells were prepared: The cells were obtained from thymus tissue that was homogenized in a blender with isotonic sucrose buffer. Blending separates cells from each other. Most of the separated cells are intact but blending breaks open some of the cells, releasing their nuclei. Afterward homogenizing, the homogenate was filtered through cheesecloth to remove any unhomogenized pieces of tissue.*

3) Resuspend the cells by using the 5 mL pipette to gently pipet the cells in the test tube up and down several times until the solution is homogenous (no clumps or pellets of cells). Place the test tube on ice in your 500 ml beaker.

4) Ask your instructor to come by and take a small sample of your cells. Your instructor will transfer one drop of your resuspended cells/nuclei onto a microscope slide. Your instructor will then add a drop of methylene blue stain (which stains DNA a deep shade of blue) to the cells on the slide, and then cover the cells with a cover slip. Later, you will observe this "Intact cells/Intact nuclei" slide under the microscope.

5) Using your micropipette, add 1 mL of EDTA solution to the nuclei in your test tube. (Note: 1 mL = 1000 uL. Use the p1000 micropipette). Mix gently by capping the tube and inverting it several times. Let the tube incubate on ice for five minutes.

6) Put a fresh tip on your p1000 micropipette, then add 200 uL of 10% SDS solution. Mix gently by inversion then return the tube to the ice (you do not need to wait 5 minutes after adding the SDS). Together, the EDTA and the SDS lyse open the nuclear membrane to release the genomic DNA.

7) Again ask your instructor to come by and take a small sample of your cells. Your instructor will transfer one drop of your lysed cells/nuclei to a microscope slide. Then your instrcutor will add a drop of methylene blue stain to the slide, and then cover the sample with a cover slip. Later you will observe this "lysed nuclei" slide under the microscope.

8) Put a fresh tip on your p1000 micropipette, then add 500 uL of NaCl solution to the lysed nuclei in your test tube. Mix gently by inversion. Return the tube to ice.

9) From the freezer, obtain a plastic test tube containing 5 mL of ice-cold ethyl alcohol. The alcohol is less dense than the lysed nuclei solution in your test tube and therefore if you pour the alcohol slowly and gently enough into the tube, it will form a separate layer that floats on top of the DNA solution. Tilt the test tube with the lysed nuclei to a 45 degree angle. Very slowly and very gently, trickle the alcohol down the inside of the test tube. Remember, your goal is to **not** let the liquid layers mix.

10) Obtain a wooden rod (from a cotton swab) to collect the DNA. Gently bend the last cm (about half an inch) of the rod to form a hook. The goal is to spool the DNA onto the hook at the end of the rod. You should gently pull DNA from the lower layer into the

upper alcohol layer. Try to spool the DNA without mixing the alcohol layer with the lower layer. Some tips for spooling are:

• Dip the hook slightly into the lower layer then pull it up quickly into the alcohol layer. You should see some mucus-like DNA follow the hook up in the alcohol layer. Twirl the hook to spool the DNA onto the rod. Repeat the dip-and-swirl several times until the hook contains a large glob of DNA. The more, the better!

• Do not dip or twirl so hard that the layers intermix. The idea is to keep pulling the DNA up from the lower layer into the upper layer. This may take 5 minutes, but keep doing it until you have a big glob of DNA.

11) When you have spooled as much DNA on the rod as possible, add 6 mL of de-ionized water to the glass test tube. With a marker, label the tube “Genomic DNA”.

12) Transfer the DNA on the rod into the water in the Genomic DNA tube. The DNA may not totally dissolve but you should help some of it dissolve by shaking and flicking the bottom of the tube.

13) Clean up

a) The glass 500 mL beaker should be washed and returned to where you obtained it.

d) The empty plastic alcohol tube should be left on the front desk.

c) You can discard into the trash the plastic test tube with the homogenate, the disposable pipette tips, the serological pipette, the wooden rod, the small microcentrifuge plastic tubes, and all other materials.

d) Confirming that DNA was isolated

When biologists isolate DNA (as you have done today), the last step is usually to test the isolated DNA to confirm that it is DNA and to see how pure the isolated DNA is.

The chemical diphenylamine can be used to confirm that the isolated substance is indeed DNA. Diphenylamine is clear but it turns blue-gray when it is boiled in the presence of DNA.

When it is confirmed that DNA was isolated, the purity of the DNA is usually tested by measuring the DNA's absorbance at two different wavelegnths of light (260 nanometers and 280 nanometers). If the DNA is pure, the ratio of the two absorbances listed above (the "260/280 ratio") is 1.8 - 2.0. If the 260/280 ratio is below 1.8 or above 2.0, that indicates that the DNA is not pure.

For time and safety reasons, you will not perform either of these two tests on your DNA. In other words, you will not do the diphenylamine test or the 260/280 absorbance ratio test.

However, your instructor will write on the whiteboard the results of a diphenylamine test (with positive and negative controls) and the results of a 260/280 absorbance test. Use the results on the whiteboard to complete the results tables on the next page.

Diphenylamine results table

Assume that your instructor added diphenylamine to three test tubes. These three test tubes contain (a) the DNA that you isolated today, (b) pure water, and (c) pure DNA. After adding the diphenylamine, the test tubes were put into a boiling water bath. The diphenylamine in all three test tubes was clear before being put into the boiling waterbath. Using the information that your instructor has written on the whiteboard, fill in the results table below.

Tube:	Color before boiling:	Color after boiling:
Your genomic DNA	_____	_____
Negative control (pure water)	_____	_____
Positive control (pure DNA)	_____	_____

Based on the above results table, you did/didn't (←circle one word) isolate DNA.

260/280 absorbance results table

Assume that your instructor measured the DNA's absorbance of light at two different wavelengths (260 and 280 nanometers). Using the information that your instructor has written on the whiteboard, fill in the results table below.

	Absorbance at 260 nm:	Absorbance at 280 nm:	Ratio of absorbances (260 abs/280 abs)
Your genomic DNA	_____	_____	_____

Based on the above results table, your DNA was/wasn't (←circle one word) pure DNA.

e) Viewing the cell samples under the microscope

1) Recall that your instructor sampled your Intact cells/Intact nuclei before you lysed the cells and nuclei. Your instructor added methylene blue stain then made a microscope slide of the intact cells and nuclei. At the back of the room a micoscope for viewing this slide has been set up, using the 40X objective lens (so 400X total magnification).

View the Intact cells/Intact nuclei slide.

2) On the slide, look for a tiny tiny dark spot at the tip of the pointer. That tiny dark spot is a nucleus.

In the space below, sketch the slide and label the nucleus. Next to your sketch, estimate the size of the nucleus in micrometers (μm). To do this estimation, first look up the field of view size (from the results table in your Microscopy lab) to get the diameter of the field of view at 400X. Next, convert that field of view diameter from mm to μm (there are 1000 μm per mm). Lastly, estimate the size of the nucleus by comparing the nucleus size to the size of the field of view.

3) Also recall that your instructor made a slide from a sample of your lysed nuclei. At the back of the room there is a microscope for viewing the lysed nuclei slide. The microscope has been set up using the 4X objective lens (so 40X total magnification).

View the lysed nuclei slide.

4) In the space below, sketch and label some uncoiled genomic DNA (also called “chromatin” or "free genomic DNA") at 40X. Estimate the size of one piece of free genomic DNA (in μm) using the same method that you used to estimate the size of the nucleus.

f) Review Questions

To answer some of these questions you may have to review the lecture notes on nucleic acids (from the beginning of the semester).

1) Briefly explain the function of each of the following chemicals and items in our DNA isolation. * = also explain chemically how it performs its function.

a) Diphenylamine

b) Ethyl alcohol

c) Sodium chloride*

d) SDS (detergent)*

f) Stirring rod with hook

g) EDTA*

h) Isotonic sucrose buffer

2) To purify DNA, it must be separated from what other common cellular macromolecules?

3) What enzymes must the DNA be protected from? Why don't these enzymes harm the DNA in living cells?

4) Why are DNA isolation procedures usually done on ice?

5) Why is it important that all mixing steps be done gently when isolating genomic DNA?

6) What chemical stain was used to view the cells under the microscope?

7) Your instructor added diphenylamine to the glass test tube that contained your isolated DNA to confirm that you isolated DNA. In this confirmation test, your instructor included two additional tubes called the control tubes.

a) What was in the positive control tube? _____

b) What was in the negative control tube? _____

8) Your instructor tested your DNA's absorbance of light to see how pure your DNA was.

a) At what wavelengths of light did your instructor measure the DNA's absorbance?

b) To gauge the purity of the DNA, your calculated the _____ of the two absorbances.

9) If a DNA sample has a 260/280 ratio of _____ to _____ then the DNA sample is considered pure.

10) Define these terms as they were defined in this handout and in the pre-lab lecture:

a) Genomic DNA:

b) Homogenate:

c) Lysis:

d) Filtrate:

11) Describe the method you used to add alcohol to the genomic DNA.

12) What are the 3 molecular components of a DNA nucleotide?

13) Genomic DNA is made up of two complementary DNA strands wrapped together in a double helix shape. The complementary bases of the two DNA strands are joined to each other by what type of bonds?

14) List the molecular difference(s) between RNA and DNA. In other words, how does an RNA strand differ from a DNA strand at the molecular level?

f) Review Question answers

1)

a) Stains DNA.

b) Precipitates the DNA.

c) Separates DNA from proteins by neutralizing the opposite ionic charges that attract DNA and proteins to each other.

d) Dissolves the cell's nuclear membrane and cell membrane by its ability to be attracted to water and membrane lipids.

f) Spools the precipitated DNA.

g) Weakens cell membranes by removing positive ions that stabilize cell membranes.

h) Protects the cells from bursting due to osmosis during the early steps of the procedure.

2) Proteins, membrane lipids, carbohydrates, amino acids, fatty acids, and other cellular molecules.

3) DNase enzymes, which are enzymes that degrade (destroy) DNA molecules. In intact cells, these enzymes do not degrade the genomic DNA because the genomic DNA is located in the nucleus and the DNase enzymes are located in the cytoplasm.

4) To protect the DNA from DNA-degrading enzymes. Cold temperatures slow down enzymes, including DNase and other DNA-destroying enzymes.

5) All genomic DNA isolation steps should be done slowly and gently to avoid shearing the DNA strands because genomic DNA strands are extremely long and fragile.

6) Methylene blue

7)

a) Pure DNA

b) Water

8)

a) 260 nanometers and 280 nanometers

b) Ratio

9) 1.8 - 2.0

10) Define these terms as they were defined in this handout and in the pre-lab lecture:

a) The DNA found in the nucleus. In other words, the chromosomal DNA

b) A liquid that contains an even mixture of substances. In today's experiment, the homogenate was the liquid of broken open cells that was produced by blending a tissue sample in a blender.

c) Breaking open a cell.

d) The liquid that passes through a filter. In today's experiment, the filtrate was the part of the homogenate that passed through the cheese cloth filter.

11) The alcohol was added slowly and gently to the DNA solution to keep the alcohol layer from mixing with the DNA layer.

12) A phospate functional group, a deoxyribose sugar, and a nitrogenous base. The nitrogenous bases of a DNA nucleotide can be either G, A, T, C.

13) Hydrogen bonds

14) RNA nucleotides have ribose sugar instead of deoxyribose sugar.

The nitrogenous bases of a RNA nucleotide can be either G, A, U, C, instead of G, A, T, and C.

RNA molecules are single stranded instead of double stranded