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) a 15 ml plastic test tube (in a rack)
- c) Two glass microscope slides (and cover slips)
- d) Three small disposable glass test tubes
- 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
- i) a microcentrifuge tube with NaCl solution
- h) a 5 ml serological pipette (with pipetter device).

2) Thymus tissue will be the source of the genomic DNA in today's experiment. In the front of the room is a beaker containing homogenized thymus. It was homogenized in a blender with isotonic sucrose buffer. Blending separates cells from each other. Most cells are intact but blending breaks open some of the cells, releasing their nuclei. Afterwards, the homogenate was filtered through cheesecloth to remove any unhomogenized pieces of tissue.

Transfer 8 ml of the filtrate (the homogenized tissue that passed through the filter) into your 15 ml plastic test tube.

3) Write your group's name on the test tube and place it in the centrifuge on the front desk. Before you start a centrifuge, always be sure that the centrifuge is balanced. This means that each tube must have a tube of equal weight opposite it in the rotor.

Centrifuge your 8 ml sample of the filtrate for 5 minutes at maximum speed to pellet the nuclei.

4) When your sample is done centrifuging, pour off the supernatant (it can be discarded down the drain). Using your serological pipette, add 4 ml of cold sucrose buffer (on the front desk) to the pellet of cells and nuclei. Resuspend the pellet by pipetting the contents of the test tube (the pellet and the buffer) up and down several times until it is homogenous. Place the test tube on ice in your 500 ml beaker.

5) Label a microscope slide "Intact nuclei". Transfer one drop of the resuspended cells/nuclei to the microscope slide. Add a drop of methylene blue stain (which stains DNA a deep shade of blue) to the cells on the slide and cover it with a cover slip. Put the slide aside for now (you will observe it under the microscope later).

6) 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.

7) 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.

8) Label one microscope slide "Lysed nuclei". Transfer one drop of the lysed nuclei to the microscope slide. Add a drop of methylene blue stain to the lysed nuclei on the slide. Gently mix the methylene blue with the lysed nuclei on the slide, then cover it with a cover slip. Put the slide aside for now.

9) 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.

10) 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.

11) 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.

12) When you have spooled as much DNA on the rod as possible, add 6 ml of de-ionized water to each of the three empty large glass test tubes. With a marker, label one tube "Genomic DNA", mark another tube "negative control", and mark the last tube "positive control". Also write your group's name in large letters on these tubes.

13) 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. Add 1 ml of control DNA (your instructor has this on ice) to the positive control tube.

d) Viewing the samples under the microscope

1) Get out your compound microscope. As always, start out on the lowest magnification (40X, which is obtained using the 4X objective lens).

2) Observe the stained intact nuclei under the microscope at 40X, 100X and 400X total magnifications. The methylene blue stain will color the DNA blue. In the space below, sketch and label some nuclei at 400X. Next to your sketch, estimate the size of the nucleus.

3) Observe the lysed nuclei slide under the microscope at 40X (lowest power) . In the space below, sketch and label the uncoiled genomic DNA (called "chromatin").

e) Confirming that DNA was isolated

To be certain that the substance you isolated is DNA, you will use a chemical test that detects deoxyribonucleic acids. The chemical diphenylamine turns blue-gray in the presence of DNA.

1) In the fume hood is a bottle of diphenylamine. **Warning:** The diphenylamine solution is a strong acid and also carcinogenic. All tubes and bottles with diphenylamine must remain in the fume hood. Wear gloves when pipetting it and clean up any spills that occur.

2) Using the disposable pipette in the hood, transfer 3 ml of diphenylamine into each of the three large test tubes (Genomic DNA, negative control, and positive control).

3) Place the three tubes into the boiling water bath in the fume hood for 10 minutes.

4) After 10 minutes, inspect the tubes. Record your results in the table below.

Tube:	Color before boiling:	Color after boiling:
Genomic DNA	_____	_____
Negative control	_____	_____
Positive control	_____	_____

5) Clean up:

a) When you are done boiling your tubes in the hood, leave them in the hood but transfer them from the boiling water bath into a test tube rack in the hood. The technician will clean these tubes.

b) Wash off the 2 microscope slides. Be sure to remove the writing on the slides. Rinse them with de-ionized water then dry them. Put the slides back in the slide box that you obtained them from. (Cover slips can go into the glass disposal box, NOT the trash).

c) The glass 500 ml beaker should be washed and returned.

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.

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 did you use to view the cells under the microscope?

7) When you viewed the first slide under the microscope, the DNA was inside/outside (circle one word) of the nucleus. When you viewed the second slide under the microscope, the DNA was inside/outside (circle one word) of the nucleus.

8) When you used diphenylamine to confirm that you isolated DNA, you also included a tubes called the control tubes. What was in the positive control tube? What did the positive control prove about your experiment? What was in the negative control tube? What did the negative control prove about your experiment?

9) Define these terms:

Genomic DNA:

Homogenate:

Lysis:

Filtrate:

10) Describe the method you used to add the alcohol to the homogenate.

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

12) 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?

13) 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?

14) In today's exercise you used chemicals such as SDS detergent, EDTA, and salt to isolate DNA from the other molecules in the cell (like lipids and proteins). Do you think you isolated RNA along with the DNA? Why or why not? To answer this question, you should consider: a) Do you expect to find any RNA in cells? b) Chemically, how similar are DNA and RNA? Do they have more features in common or more features that differ?