# Lab 9. Human Mitochondrial Analysis using PCR and Electrophoresis

## Prelab Assignment

*Before* coming to lab, read carefully the introduction and the procedures for each part of the experiment, and then answer the prelab questions at the end of this lab handout. Hand in the prelab assignment just *before* the start of your scheduled lab period. The following pages in your textbook should also be read as they are a good source of background information: PCR: pages 403-404; Electrophoresis: fig. 20.9 on page 405 in Biology, *9th ed by Campbell and Reece*

## Major Goals of this Experiment

To gain an understanding and hands-on experience of the principles and practice of each of the following:

- Isolate mitochondrial DNA (mtDNA) from human cheek cells and amplify two separate regions of the mtDNA using the polymerase chain reaction (PCR).
- Following PCR, use electrophoresis to separate and determine the size of the PCR amplified mtDNA fragments
- Compare DNA polymorphisms between individuals in the class
- Structure and function of mitochondria

## Overview of Experiment

In this experiment, students will examine their mtDNA (mitochondrial DNA) obtained from either human cheek cells or hair follicle cells. To do this, PCR is used to amplify two separate regions of the mitochondrial chromosome (see Fig. 3 on page 2). Amplification of these regions will result in two PCR products, each under 1200 base pairs in length. Following PCR, the amplified DNA fragments will then be subjected to gel electrophoresis and then staining with ethidium bromide for visualization.

This experiment has four modules:

I. Isolation of mtDNA from human cheek cells or hair follicles. (Pages 7 - 9)
II. Amplification of two regions of human mtDNA by PCR (Pages 9 - 11)
III. Separation of PCR amplified mtDNA regions by Electrophoresis (Pages 12 - 15)
IV. Staining of Gels and Visualization of the PCR amplified mtDNA regions (Page 16)

## Background Information

***Mitochondria*** (plural for mitochondrion) are the energy-producing organelles of the cell. Both plant and animal cells possess mitochondria. The number of mitochondria per cell varies depending on the cell type, ranging from only a few in skin cells to thousands in skeletal muscle cells.

Unlike other organelles, mitochondria have two separate membranes. The outer membrane is fairly porous, possessing a protein called ***porin***. The inner membrane, however, is highly impermeable to ions and is enriched in a rare, negatively charged phospholipid known as ***cardiolipin***. The inner membrane is highly convoluted, with infoldings called ***cristae*** (Fig. 1) that greatly increase the total membrane surface area. The inner membrane also contains the enzymes that catalyze cellular respiration, the process whereby energy is produced for the cell.

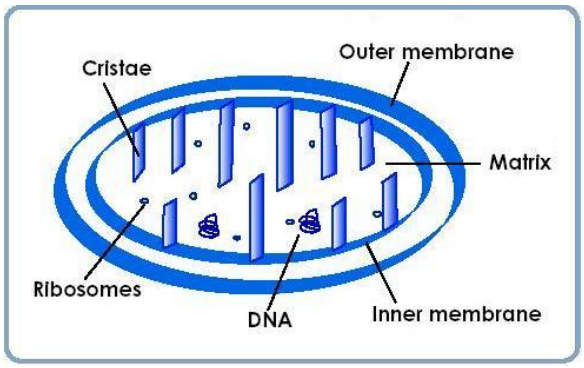



**Figure 1.** Structure of a mitochondrion

The space inside the inner membrane is known as the ***matrix***. Within the matrix and inner membrane, the chemical reactions that produce energy for the cell take place.

As shown in Fig. 2, sugars and fatty acids, broken down to two carbon units, enter a series of reactions known as the ***citric acid*** or ***Krebs cycle***. Sugars are broken down in the cytoplasm while fatty acids are broken down in the mitochondria by a process known as ***ß (beta) oxidation***. The citric acid cycle generates electrons that enter the ***electron transport chain***, a cluster of protein complexes that reside in the inner membrane of the mitochondria. In the final step of energy production, protons generated by the electron transport chain flow through a pump known as ***ATP synthase***, driving the production of ***ATP***, the primary energy containing molecule used in biological systems. This final energy-producing process is known as ***oxidative phosphorylation***.

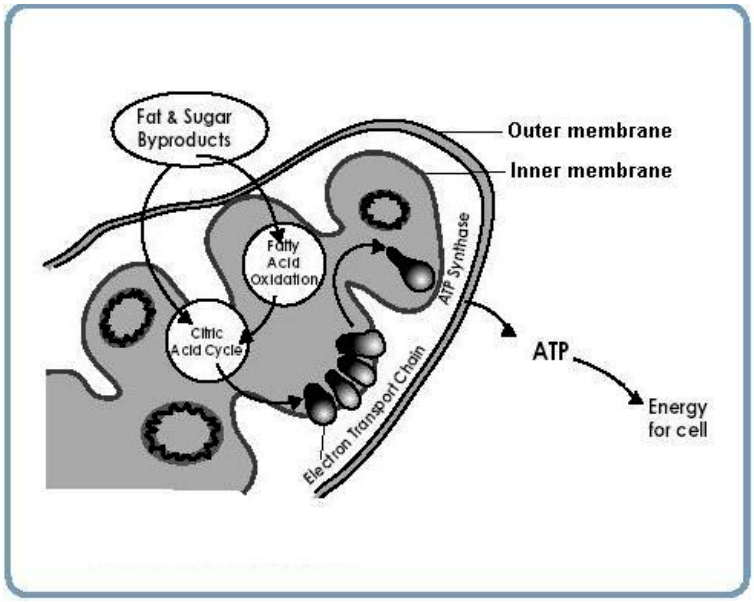



**Figure 2.** The mitochondrion is the site of metabolic pathways that convert the products of glycolysis (pyruvate and NADH) into a usable form of chemical energy, ATP, to that provides energy to power endergonic cellular processes.

The DNA present in the matrix is distinct from the DNA found in the cell's nucleus. ***Mitochondrial DNA*** (mtDNA) is contained in a single circular chromosome (Figure 3) that has been completely sequenced. The mitochondrial chromosome contains 16,569 base pairs of DNA and 37 genes. MtDNA encodes 13 polypeptides, all of which are subunits of the respiratory chain complex.

This is shown on the map in Figure 3, illustrating the locations of genes that encode proteins in complexes I and IV of the electron transport chain. As shown, mtDNA also encodes mitochondrial ribosomal RNA and the ATP synthase, in addition to cytochrome B, another constituent of the electron transport chain. MtDNA encodes only part of the electron transport chain; nuclear DNA encodes the remaining complex subunits. One peculiarity is that mitochondrial protein synthesis uses a slightly different genetic code than cytoplasmic translation.

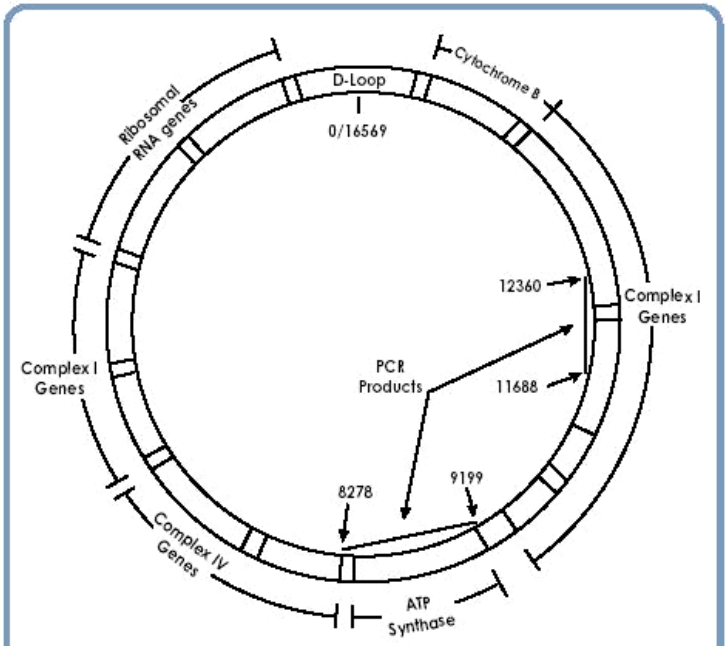



**Figure 3: Genetic map of mitochondrial DNA**

As all cells possess only one nucleus but several hundred or thousand mitochondria, mtDNA is present in great excess over nuclear DNA in most cells. This relative abundance of mtDNA is taken advantage of by forensic investigators after obtaining crime scene specimens that are degraded or otherwise insufficient for nuclear DNA PCR analysis. The D-loop (Fig.3) has a high degree of variability between individuals and can be sequenced to demonstrate variations. MtDNA typing, however, cannot be used to conclusively link suspects to crime scenes; rather, it is used to include or exclude suspects for further scrutiny.

During the past twenty years, an ever-increasing number of diseases have been shown to be due to ***mitochondrial dysfunction***. These disorders result when mitochondrial ATP generation is insufficient to meet energy needs in a particular tissue. Because muscle and nerve cells contain large numbers of mitochondria, these organ systems are most affected by mitochondrial dysfunction. Mitochondrial diseases may be due to mutations in mtDNA genes or mutations in nuclear genes that encode mitochondrial enzymes. Diseases caused by mtDNA mutations include the ***myopathies***, diseases that affect various muscles and ***encephalomyopathies***, which cause both muscular and neurological problems. ***Huntington's chorea***, a devastating disease that results in dementia and loss of motor control, is caused by defects in oxidative phosphorylation and has been mapped to a mutation in nuclear DNA encoding a non-mitochondrial protein. Other diseases such as ***Alzheimer's*** and ***Parkinson's disease*** involve mitochondrial abnormalities, although it is unclear how these abnormalities relate to disease pathology. Mitochondria also appear to play roles in aging and in programmed cell death, also known as ***apoptosis***.

Since mitochondria are present in the cytoplasm, they are inherited independently from the nucleus. A ***female egg cell*** possesses over 10,000 mitochondria, while a ***sperm cell*** has very few. Thus during fertilization, mitochondrial DNA is inherited almost exclusively from the mother. Although a small amount of paternal mtDNA is present in the fertilized egg, this DNA appears to be selectively destroyed by the newly fertilized egg. This pattern of inheritance of mtDNA is known as ***maternal inheritance***. Maternal inheritance is indicated when all offspring, male and female, of the mother are afflicted with a specific condition (Fig. 4).

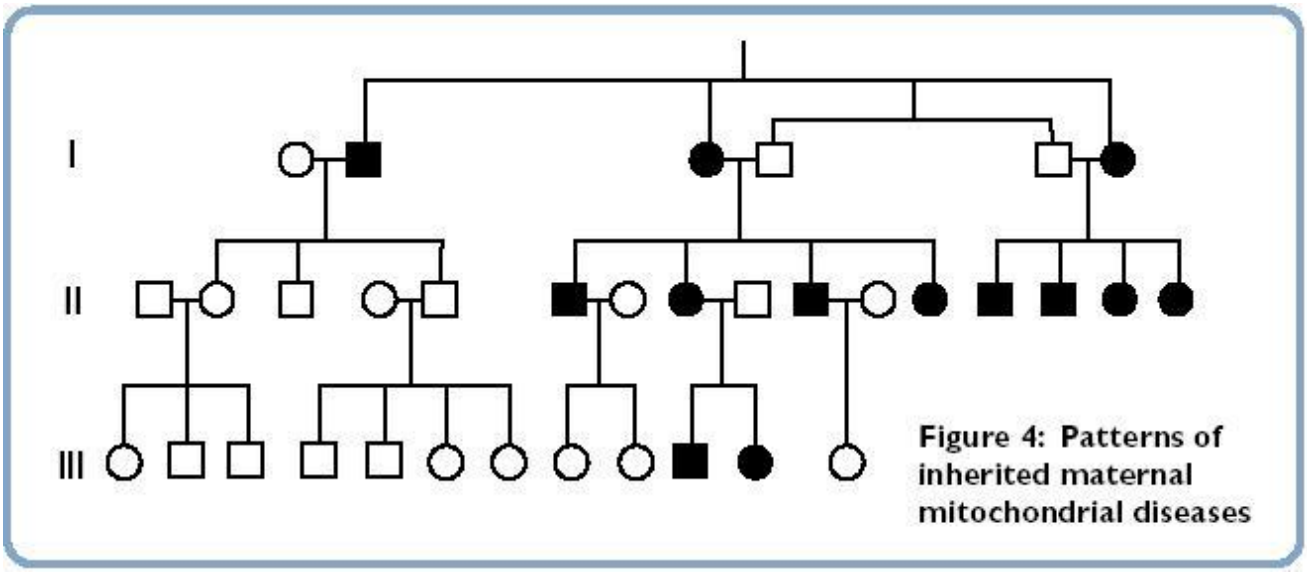



**Figure 4: Patterns of inherited maternal mitochondrial diseases**

The severity of any particular mitochondrial disorder is highly variable, depending on the number of mutated mitochondria inherited from the mother (Fig. 5).

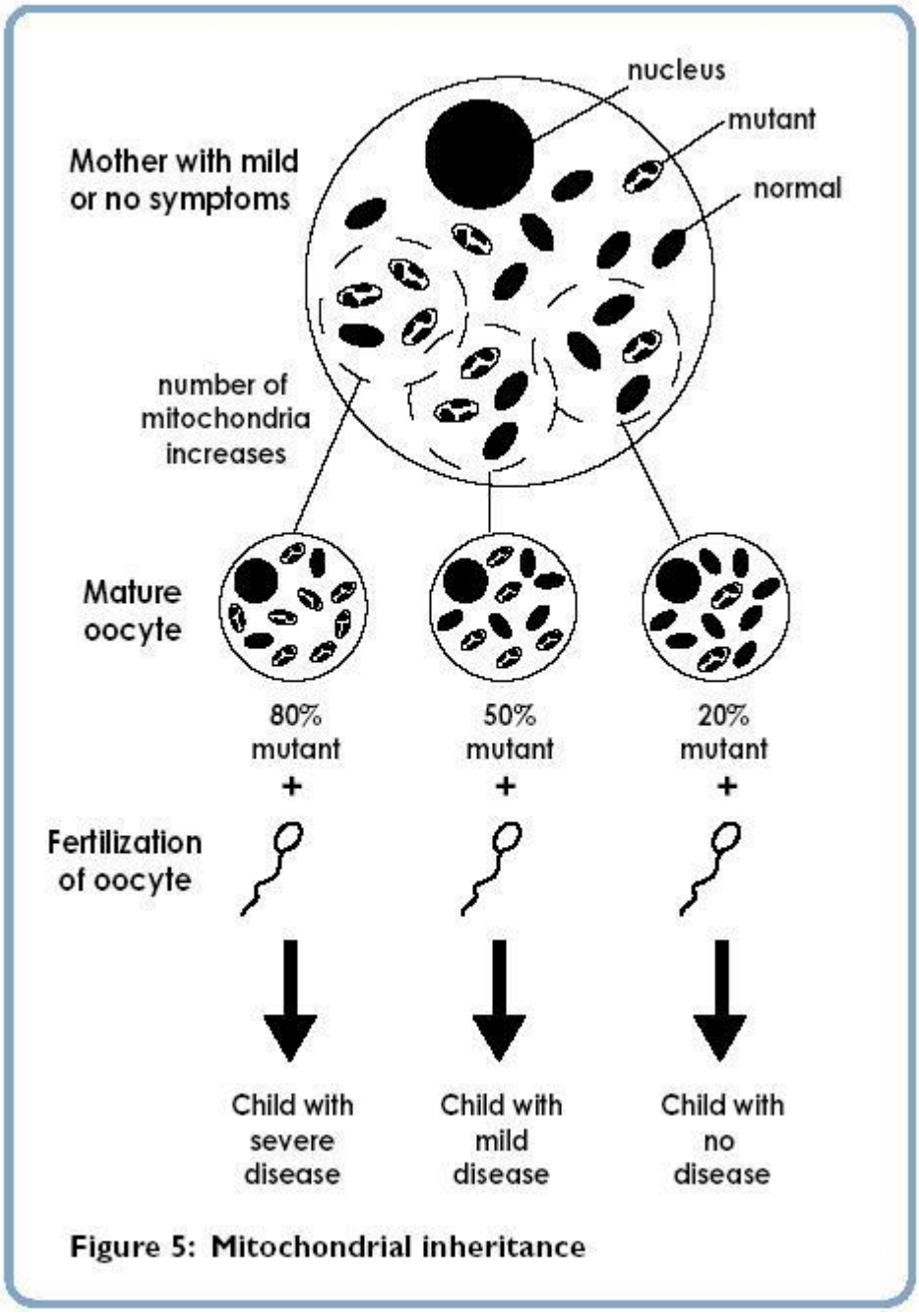



**Figure 5: Mitochondrial inheritance**

To examine mitochondrial DNA, the ***polymerase chain reaction*** (PCR) is usually employed. PCR, invented in 1984, has gained widespread use and its inventor, ***Kary Mullis***, was awarded a Nobel Prize in 1994. The enormous utility of the PCR method is based on its ease of use and its ability to allow the amplification of small DNA fragments. The essence of the PCR method (Fig. 6) is the use of an enzyme known as **Taq polymerase**. This enzyme, purified from a bacterium isolated from hot springs, is stable at very high temperatures.

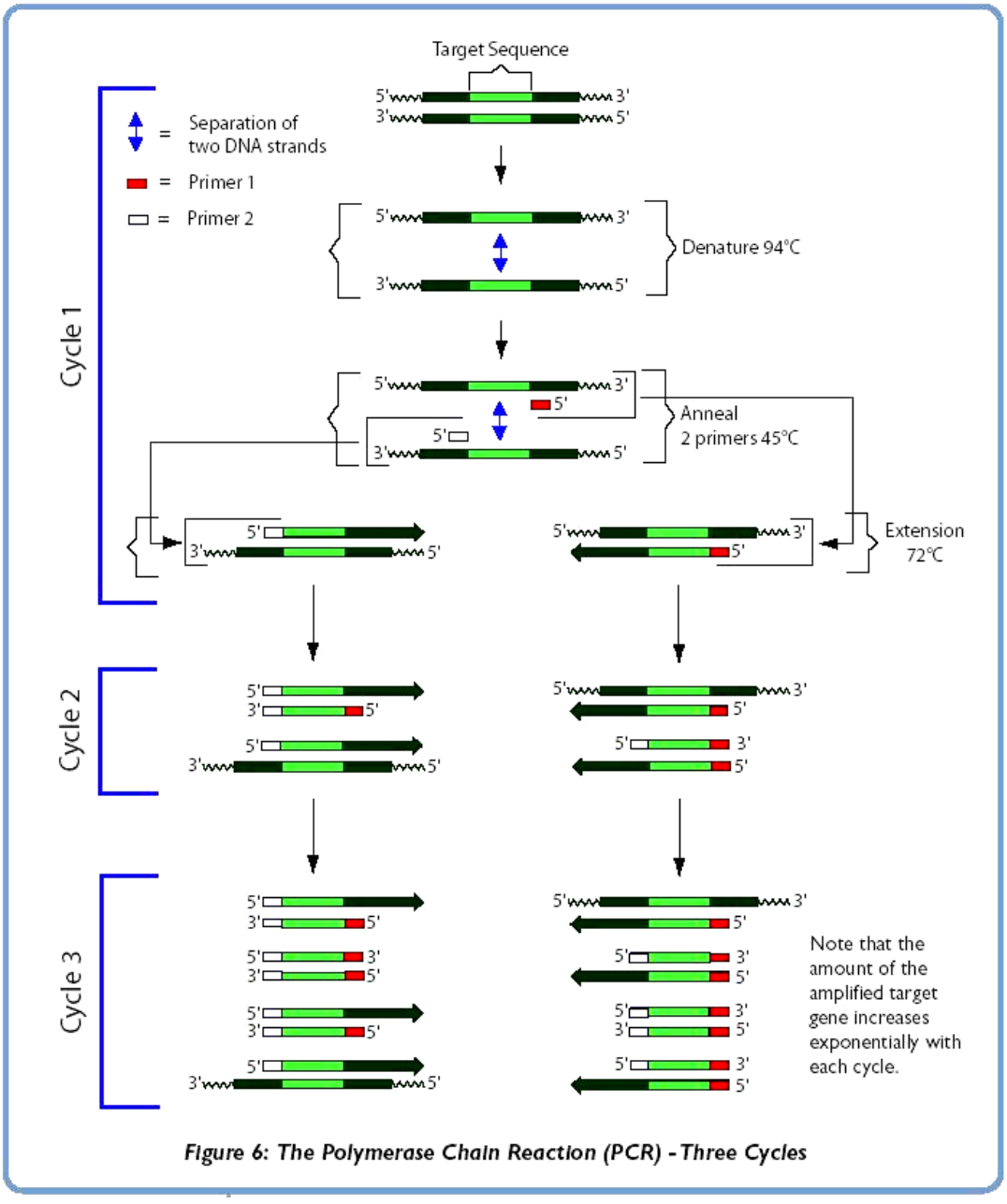



***Figure 6: The Polymerase Chain Reaction (PCR) - Three Cycles***

In the ***first step of PCR***, known as denaturation, the DNA complimentary strands are separated at 94 °C, while the polymerase remains stable. In the ***second step***, known as annealing, the sample is cooled to a temperature in the range of 42°C to 65°C to allow hybridization of small (15-30 nucleotides) synthetic oligonucleotides, known as "***primer***s", to the target to be amplified. In the ***third step***, known as extension, the temperature is raised to 72°C and the DNA polymerase then adds nucleotides to the primers to complete each new complementary strand of the target. These three steps constitute one "cycle". This process is typically repeated for 25 to 50 cycles, amplifying the target exponentially (Fig. 6). PCR is performed in an instrument known as a thermal cycler, which is programmed to heat the sample at the designated temperature for each step and then rapidly change temperatures for the following step.

## Overview of the Procedure

**Important Note!!** To get an overview of this laboratory activity and to use your lab time efficiently read the following procedures *before* attending lab. If you and your group members are not familiar with these procedures before coming to lab you will have great difficulty completing this exercise during the lab period.

Day 1 Activities:
- Module I: Isolation of mtDNA from human cheek cells. (Pages 7 - 9)
- Module II: Amplification of two regions of Mitochondrial DNA by PCR *(Pages 9 - 11)*

Day 2 Activities:
- Module III: Separation of PCR amplified mtDNA regions by Electrophoresis (Pages 12 - 15)

Day 3 Activities:
- Module IV: Stain Gels and Visualization of the PCR amplified mtDNA regions (Page 16)
- Size Determination of the of the PCR amplified mtDNA regions—done at home (Page 17)

## Laboratory Safety


1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Special caution is required when handling human cheek cells. Swabs used for harvesting cheek cells should be soaked in a 15% bleach solution after the cells are suspended in the buffer. Swabs can be disposed in solid waste after soaking in the bleach solution.
3. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
4. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
5. Exercise caution when using any electrical equipment in the laboratory.
6. Although electrical current from the power source is automatically disrupted when the cover is removed from the apparatus, first turn off the power, and then unplug the power source before disconnecting the leads and removing the cover.
7. Turn off power and unplug the equipment when not in use.
8. EDVOTEK injection-molded electrophoresis units do not have glued junctions that can develop potential leaks. However, in the unlikely event that a leak develops in any electrophoresis apparatus you are using, IMMEDIATELY SHUT OFF POWER. Do not use the apparatus.
9. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the lab.

## Day 1 Module I. Isolation of mtDNA from Human Cheek Cells

**WARNING!**

- It is critical that there is a sufficient volume of cells to obtain enough DNA to yield positive DNA fingerprinting results. To maximize success, carefully read and follow all experimental instructions.
- **Use only screw-cap tubes when boiling for DNA isolation. Do not use snap-top tubes when boiling.**

### Materials for Module I

One Per Student (unless indicated otherwise)

- 10 mL Saline in plastic cup
- 1.5 mL screw cap microtest tube (one per student)
- 300 µL Lysis solution in a 1.5 screw-top tube (***shared between two students***)
- Boiling water bath (*one per table*)
- Float for 1.5 mL microtest tubes
- 0.5 mL microcentrifuge tube.
- Small fingerbowl with ice
- One disposable transfer pipette

Classroom set

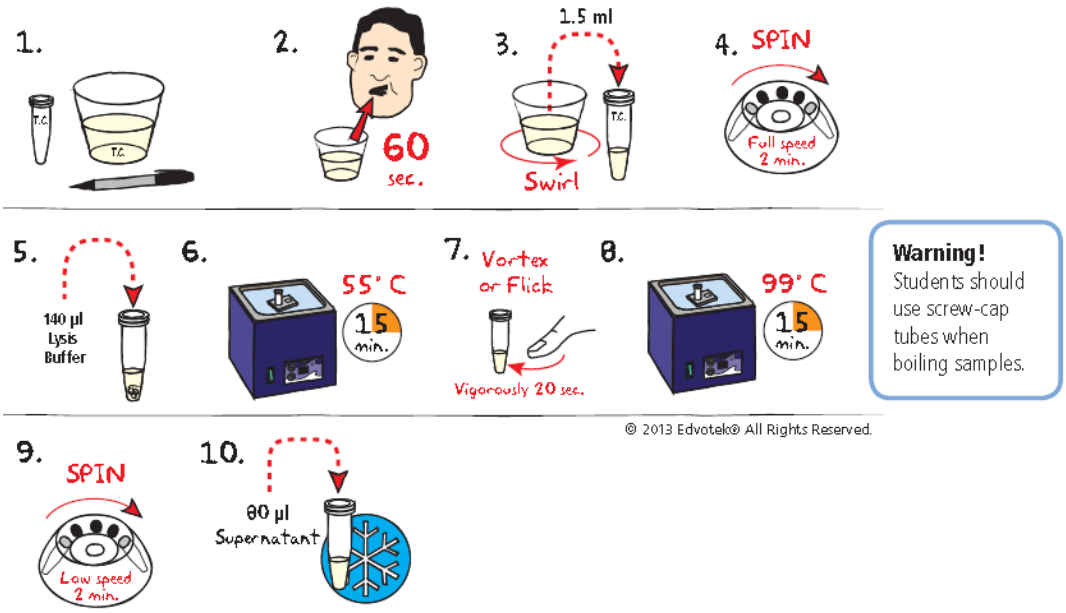
- Micropipettes set at **140 µL**
- Micropipettes set at **80 µL**
- Water bath set at **55**°C
- Vortex
- Microcentrifuge
- Disposable transfer pipettes
- Latex gloves
- Ice

***Boiling water bath*** (for step 8 on the next page)

SET UP as soon as possible the **boiling water bath** on a hotplate using a 600 or 1000 mL beaker half full with DI water—one water bath per table!

**Go to the next page for the procedure for module I**

## **Procedure for Module I** (Work individually in teams of two)




1. LABEL a ***1.5 mL screw top microcentrifuge tube*** and ***plastic cup*** with you initials and group #.
2. Place ***10 mL of saline*** in the plastic cup and RINSE your mouth vigorously for <u>*60 seconds*</u> with the saline solution. EXPEL the solution from your mouth into the cup.
3. SWIRL the cup gently to resuspend the cells. Use a transfer pipette to TRANSFER ***1.5 mL*** of the solution into the labeled <u>*1.5 mL screw top tube*</u>.
4. CENTRIFUGE the cell suspension for **2 minutes** at full speed to pellet the cells. POUR off the supernatant (liquid), but DO NOT DISTURB THE PELLET!
    - Repeat steps 3 and 4 twice
    - <u>*IMPORTANT*</u>!!! If the pellet of cells is not large enough, repeat steps 3 & 4 until the pellet is at least the size of a match head.
5. For every team of two students, OBTAIN from the instructor <u>*one*</u> ***1.5 mL Screw-top tube*** containing ***300 µL lysis solution***. MIX the lysis solution by vortexing or pipetting up and down.

    <u>*The lysis solution contains*</u>:

    a) 25 millimolar Tris-HCl pH 8.0 buffer (helps to maintain a constant pH)

    b) 50 µg/ml proteinase K (digests proteins found in the solution)
6. CAP tightly the tube containing the cheek cells and lysis solution and PLACE in a ***55°C water bath for 15 minutes***. (Use the thermostatically controlled water bath is in the back of the room.)
7. REMOVE the tube from the water bath and ***VORTEX*** the tube for 15 seconds.
8. Incubate the tube in a float and place in <u>*gently*</u> ***boiling water for 15 minutes***.
    - Boiling is required to obtain cell and mitochondrial lysis.
    - Boiling does <u>*not*</u> degrade the DNA

## Module I. Isolation of mtDNA from Human Cheek Cells (cont.)

9. REMOVE the tube containing the cellular lysate from water and CENTRIFUGE for 2 minutes at low speed (~6000 rpm)
10. Carefully REMOVE ***80 µl of supernatant*** (it contains your DNA!!) and TRANSFER it to a clean, labeled ***0.5 ml microcentrifuge tube***. Transfer the DNA (supernatant) to the new 0.5 ml microcentrifuge tube very carefully as it will be used for the PCR reaction.
11. Place the tube on ice.
12. Proceed with steps as outlined in Module II: Amplification of two mitochondrial DNA regions.

## Module II. PCR Amplification of Two Mitochondrial DNA Regions (Day 1)

### Materials for Module II

One per Student

- 0.5 mL microcentrifuge tube from step 10 of module I (contains mtDNA)
- 0.2 mL microcentrifuge tube containing PCR reaction pellet™

Shared by two students

- 50 µL Mitochondrial Primer Mix

Classroom set

- Micropipettes set at 20.0 µL
- Micropipettes set at 5.0 µL
- Mitochondrial primer mix
- Vortex
- Microcentrifuge
- PCR Cycler
- Latex gloves
- Ice
- 10x Gel Loading Solution

**Go to the next page for the procedure for module II**

## **Module II Procedure** (Work individually in teams of two)


### Preparation for the PCR Reaction

1. OBTAIN a ***0.2 mL PCR tube*** containing the ***PCR reaction pellet™*** (one tube per student).
   - Use a permanent marker to ***label the 0.2 mL PCR tube*** containing the PCR reaction pellet™ with your initials.
   - The ***PCR reaction pellet™*** contains the *Taq* DNA polymerase, $Mg^{2+}$, four dXTPs (i.e. A, T, G and C deoxynucleotides) and the PCR reaction buffer.
2. TAP the reaction tube to assure the PCR reaction pellet™ is at the bottom of the tube. ADD the following to the pellet and then VORTEX to mix the contents:
   - ***20.0 µL mitochondrial primer mix***
   - ***5.0 µL*** **Cheek cell** DNA (supernatant)
3. After VORTEXING, quick spin the tube in a microcentrifuge to collect the entire sample at the bottom of the tube. *Make sure the PCR reaction pellet™ is completely dissolved*.
4. Place your PCR tube into the programmed thermal cycler—***be sure the cap is closed firmly and labeled with your initials***.

### Polymerase Chain Reaction Cycling

5. Each student should place his/her PCR tube in the programmed thermal cycler. The cycler is programmed as follows:

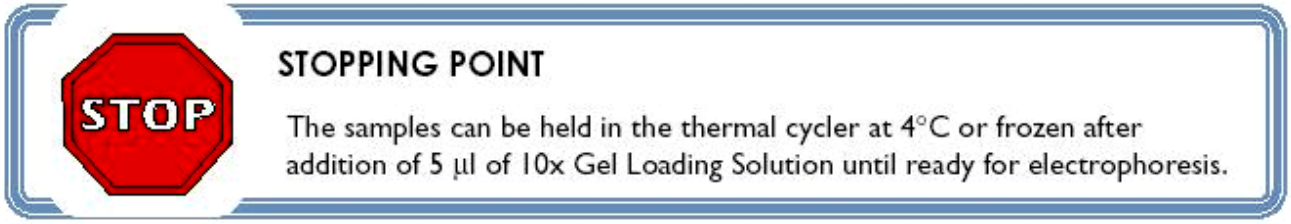
| Initial Denaturation | 25 cycles at | Final Extension |
|---|---|---|
| 94°C for 4 min. | 94°C for 1 min.<br>55°C for 1 min.<br>72°C for 2 min. | 72°C for 5 min |

6. The Biology Lab Technician will monitor the PCR process. At the completion of the last PCR cycle she will add 5µL 10x Gel Loading Solution to each tube and store the tubes in the refrigerator overnight.

## Module II. PCR Amplification of two Mitochondrial DNA Regions (cont.)

**Control Reaction:** (prepared by the *instructor* for the entire class)

1. Obtain a ***0.2 mL PCR tube*** containing the ***PCR reaction pellet™***. If the PCR reaction pellet is not in a 0.2 mL PCR tube, carefully transfer the PCR reaction pellet to one To prepare the PCR Control reaction, add the following to the pellet in a 0.2 mL PCR tube:
    - **20.0 µL** mitochondrial primer mix
    - **5.0 µL** Control DNA
2. Gently mix the control tube and quickly spin it in a microcentrifuge to collect the entire sample at the bottom of the tube. Make sure the PCR reaction pellet™ is completely dissolved.
3. Ensure that the cap is closed firmly and labeled "C" for control. Now place the control tube into the programmed thermal cycle and proceed to step 6, above.



# Module III. Agarose Gel Preparation and Electrophoresis (Day 2)

If using Edvotek electrophoresis units, a 7 x 14 cm gel is recommended for this experiment to achieve better resolution of the PCR products.


## Materials for Module III

One Per Student

- 0.5 mL PCR tube from step 9 of module II containing amplified mtDNA regions and
- 5µL 10x Gel Loading Solution

Classroom set

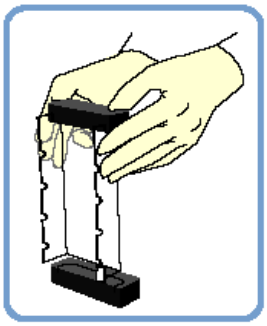
- Microcentrifuge tube containing 30 µL of the 200-DNA base pair ladder (one tube per gel)
- 1.0% Agarose gel at 55 $^{o}$C (prepared in advance by the biology lab technician)
- Latex gloves
- Electrophoresis apparatus (7 x 14 cm is preferred for better resolution)
- Electrophoresis power source
- Water bath set at 50 $^{o}$C
- 10x Gel Loading Solution
- EDVOTEK (50x) Electrophoresis Buffer
- 200 base-pair DNA Ladder (i.e. DNA fragments of 200, 400, 600, 800, 1000 and 1200 bp)
- Micropipettes set at 30.0 µL
- Rubbermaid Food Containers for storing gels

### Preparing the Gel Beds (Casting Trays)

1. Close off the open ends of a clean and dry gel bed (casting tray) by either using rubber dams or by raising the Plexiglas dams of the casting tray

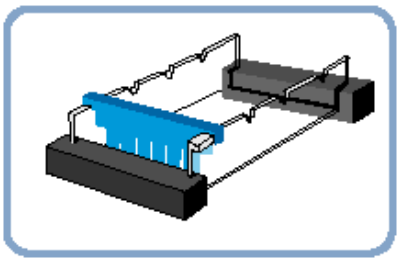
    - ***Using rubber dams***: Place a rubber dam on each end of the bed. Make sure the rubber dam fits firmly in contact with the sides and bottom of the bed.

    - ***Using Plexiglas dams:*** loosen the plastic screws at each end, raise the Plexiglas dams and gently tighten the screws to hold the dams in place.



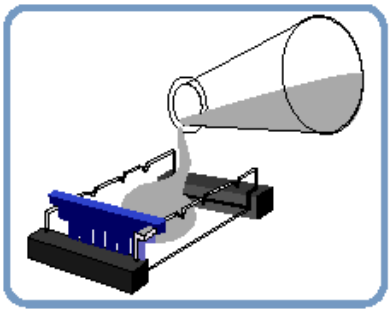
**Inserting Rubber Dams**

2. Place a well-former template (comb) in the first set of notches at the end of the bed. Make sure the comb sits firmly and evenly across the bed.



# Module III. Agarose Gel Preparation and Electrophoresis (Day 2, cont.)

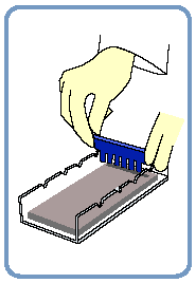
## Casting the Agarose Gel



3. Pour the cooled agarose solution into the bed. Make sure the bed is on a ***level surface***.
4. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately ***20 minutes***.

## Preparing the Gel for Electrophoresis

5. After the gel is completely solidified after 20 to 30 minutes of cooling, carefully and slowly remove the rubber dams or lower the Plexiglas ends.
   - Be especially careful not to damage or tear the gel wells when removing the rubber dams. A thin plastic knife, spatula or pipet tip can be inserted between the gel and the dams to break possible surface tension.



6. Remove the comb by slowly pulling straight up. Do this carefully and evenly to prevent tearing the sample wells.
7. Place the gel (still on its bed) into the electrophoresis chamber, properly oriented, centered and level on the platform.

8. Fill the electrophoresis apparatus chamber with the required volume of diluted buffer for the specific unit you are using (see guidelines in Table B).
   - For DNA analysis, the same EDVOTEK (50x) Electrophoresis Buffer is used for preparing both the agarose gel buffer and the chamber buffer. The formula for diluting EDVOTEK (50x concentrated buffer is 1 volume of buffer concentrate to every 49 volumes of distilled or deionized water.

The electrophoresis (chamber) buffer recommended is Tris-acetate-EDTA (20 mM tris, 6 mM sodium acetate, 1 mM disodium ethylenediamine tetraacetic acid) pH 7.8. Prepare the buffer as required for your electrophoresis apparatus.

**Table B** Dilution of Electrophoresis (Chamber) Buffer

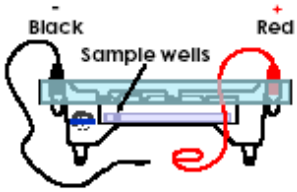
| EDVOTEK Model # | Concentrated Buffer (50x) (ml) | + | Distilled Water (ml) | = | Total Volume (ml) |
|---|---|---|---|---|---|
| M6+ | 6 | | 294 | | 300 |
| M12 | 8 | | 392 | | 400 |
| M36 (blue) | 10 | | 490 | | 500 |
| M36 (clear) | 20 | | 980 | | 1000 |

9. Make sure the gel is completely covered with buffer.
10. Proceed to loading the samples and conducting electrophoresis.

# Module III. Agarose Gel Preparation and Electrophoresis (Day 2, cont.)

**Reminder:**

During electrophoresis, the DNA samples migrate through the agarose gel towards the positive electrode. Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

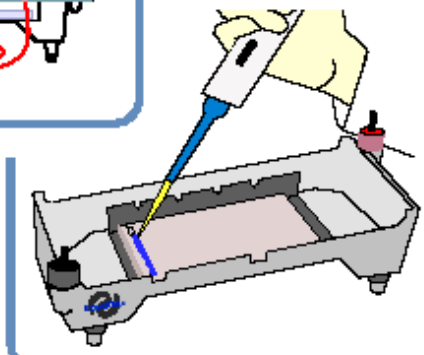



## LOADING THE SAMPLES

This experiment requires a 1.0% agarose gel. Have a water bath or beaker of water warmed to 50°C for heating tubes containing DNA fragments before gel loading.

## LOADING DNA SAMPLES

1. Heat the 200 bp DNA ladder and PCR samples for two minutes at 50°C. Allow the samples to cool for a few minutes.



2. Load Samples

   Load entire volume (30 µl) of the samples in the following sequence.

| Lane | |
|---|---|
| 1 | 200 bp DNA ladder |
| 2 | Control (optional) |
| 3 | Student #1 |
| 4 | Student #2 |
| 5 | Student #3 |
| 6 | Student #4 |

3. Record the position of your sample in the gel for easy identification after staining.

***Go to running the gel on the next page!***

# Module III. Agarose Gel Preparation and Electrophoresis (Day 2, cont.)

## RUNNING THE GEL

4. After the DNA samples are loaded, carefully snap the cover down onto the electrode terminals.

   Make sure that the negative and positive color-coded indicators on the cover and apparatus chamber are properly oriented.

5. Insert the plug of the black wire into the black input of the power source (negative input). Insert the plug of the red wire into the red input of the power source (positive input).

6. Set the power source at the required voltage and conduct electrophoresis for the length of time determined by your instructor. General guidelines are presented in Table C.

**Table C** Time and Voltage (1.0% - 7 x 14 cm gel)

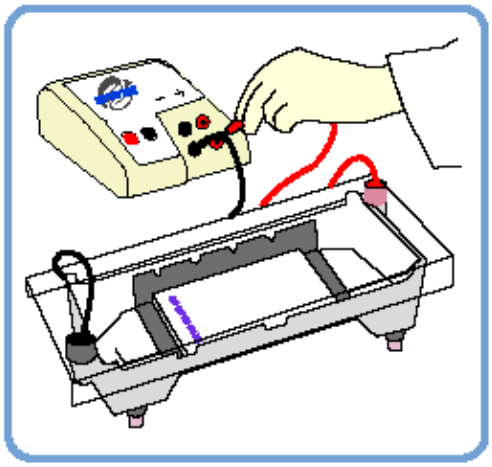
| Volts | Recommended Time Minimum | Maximum |
|---|---|---|
| 125 | 55 min | 1 hr 15 min |
| 70 | 2 hrs 15 min | 3 hrs |
| 50 | 3 hrs 25 min | 5 hrs |

7. Check to see that current is flowing properly - you should see bubbles forming on the two platinum electrodes.

8. Allow the tracking dye to migrate 4.5 cm (small gel) or 6-7 cm (large gel) from the wells for adequate separation of the DNA bands. Terminate the electrophoresis before the tracking dye moves off the end of the gel.

9. After the electrophoresis is completed, turn off the power, unplug the power source, disconnect the leads and remove the cover.

10. Remove the gel from the bed for staining with InstaStain® Ethidium Bromide.




**Label a Rubbermaid food storage container for easy identification. Place the gel in the container with a small amount of buffer to prevent the gel from drying out, cover and store in the refrigerator overnight.**

# *Module IV. Staining Gel with InstaStain® Ethidium Bromide and DNA Fragment Visualization (Day 3)*

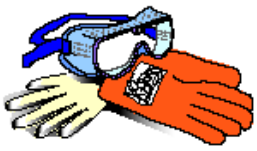
## Materials for Module IV

One Per Gel

- Gel from Module III
- One InstaStain® Ethidium Bromide card
- Casting Tray
- Two empty 100 mL beakers
- Eyedropper

Classroom set

- Plastic Wrap
- Latex gloves
- Disposal bag for used InstaStain® Ethidium Bromide cards
- UV Transilluminator
- Polaroid camera and film



Wear gloves and safety goggles

www.edvotek.com

Visit our web site for an animated demonstration of InstaStain® EtBr.

### STAINING WITH INSTASTAIN® ETBR

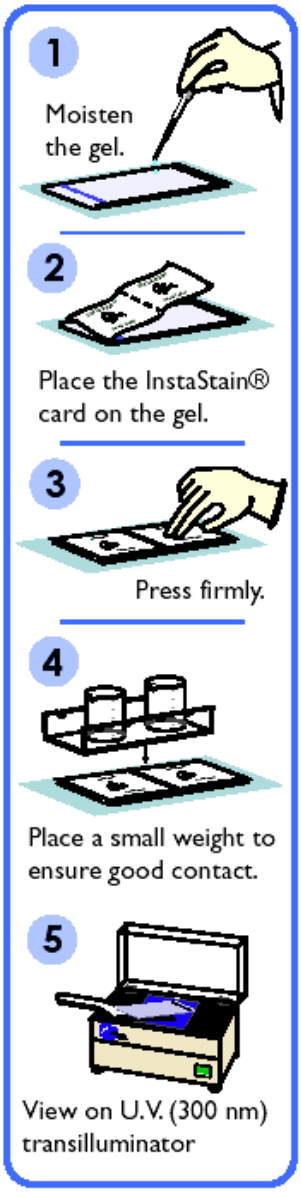
1. After electrophoresis, place the gel on a piece of plastic wrap on a flat surface. Moisten the gel with a few drops of electrophoresis buffer.
2. Wearing gloves, remove the clear plastic protective sheet, and place the unprinted side of the InstaStain® EtBr card on the gel.
3. Firmly run your fingers over the entire surface of the InstaStain® EtBr. Do this several times.
4. Place the gel casting tray and a small empty beaker on top to ensure that the InstaStain® card maintains direct contact with the gel surface.

   Allow the InstaStain® EtBr card to stain the gel for 10-15 minutes.
5. After 10-15 minutes, remove the InstaStain® EtBr card. Transfer the gel to a ultraviolet (300 nm) transilluminator for viewing. Be sure to wear UV protective goggles.

### DISPOSAL OF INSTASTAIN

Disposal of InstaStain® cards and gels should follow institutional guidelines for chemical waste.

**Caution:** Ethidium Bromide is a listed mutagen.




**Additional Notes About Staining**

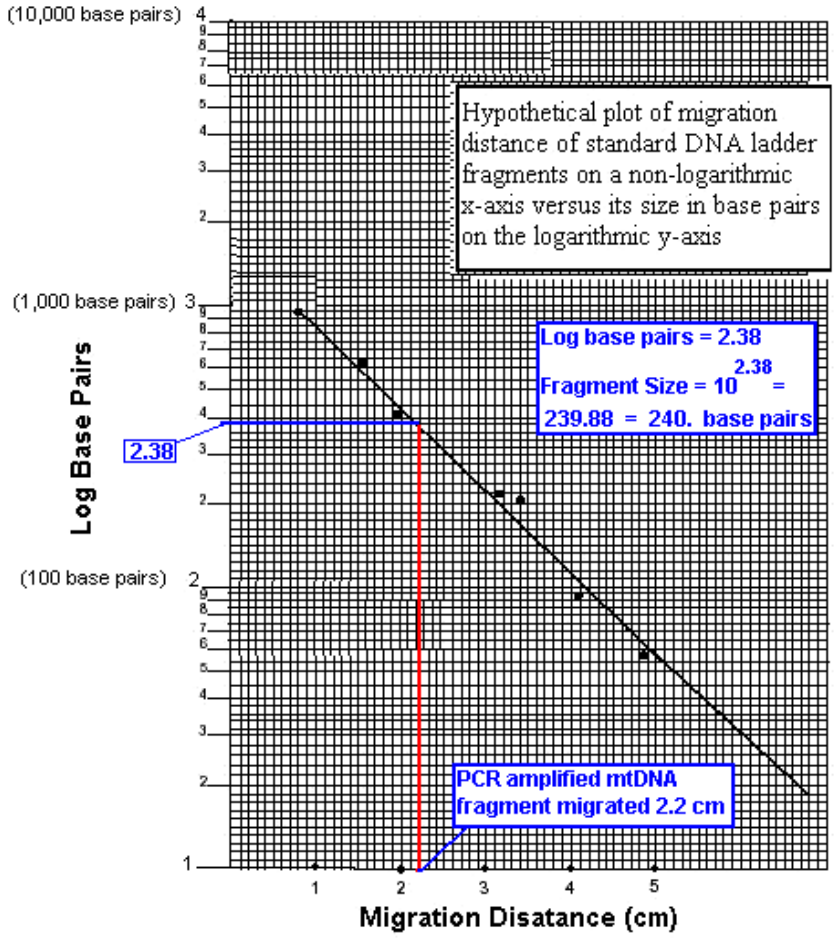
- If bands appear faint, or if you are not using EDVOTEK UltraSpec-Agarose™, gels may take longer to stain with InstaStain® EtBr. Repeat staining and increase the staining time an additional 10-15 minutes.

6. The instructor will take a Polaroid picture of the gel on the UV transilluminator and will provide copies of the picture for all group members.

## Determination of the Size of the PCR Amplified mtDNA Regions (Done outside of class)

The approximate size of the PCR amplified mtDNA regions can be determined by comparing the distance these fragments migrated in the gel to that of the standard 200 base pair DNA ladder. However, in order to compare polymorphisms of the two mtDNA regions that were amplified, a more accurate size must be determined for each fragment.

7. Measure the distance traveled in the agarose gel by each of the standard DNA ladder fragments.
   - For each fragment, measure distance traveled from the *lower edge* of the sample well to the *lower edge* of each band on the gel. Record the distance traveled in centimeters (to the nearest millimeter) on the *report sheet in Table 1*.
8. Label the semi-log graph paper on page 21 of the report sheet as follows:
   - Label the non-logarithmic x-axis "Migration Distance (cm)."
   - Label the logarithmic y-axis "Log Base Pairs." Assume the first cycle on the y-axis represents 0 – 100 base pairs, the second cycle 100 – 1000 base pairs, and the third cycle represents 1000 – 10,000 base pries.
   - Choose your scales for each axis so that the data points are well spread out and the resulting curve occupies as much of the graph as possible.




9. For each standard DNA ladder fragment, plot the measured migration distance on the x-axis versus it size on the y-axis.
10. Draw the best average ***line of best fit***. The line should have approximately equal numbers of points scattered on each side of the line. Some points may be right on the line, others above or below it.
11. Extrapolate the size of the two PCR amplified mtDNA fragments. Show your work on your graph.

## *Batch Gel Preparation: Prepared in Advance by the Biology Lab Technician*

To save time, the agarose gel solution can be prepared in a batch for sharing by the class. Any unused prepared agarose can be saved and remelted for gel casting at a later time. For a batch (400 ml) preparation of 1.0% agarose gel:

1. Use a 500 ml flask to prepare the diluted gel buffer.
   - Add 8.0 ml of buffer concentrate
   - Add 392 ml of distilled water.
2. Pour 4.0 grams of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.

   Note: UltraSpec-Agarose™ kit components are often labeled with the amount the bottle/package contains. If the amount of agarose is not specified or if the bottle's plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.

Make sure that the agarose solution is completely clear of "clumps". Distortion of electrophoresis DNA band patterns will result if the gel is not correctly prepared.

3. With a marking pen, indicate the level of solution volume on the outside of the flask.
4. Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
5. Cool the agarose solution to 55°C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume (400 ml) as marked on the flask in step 3.

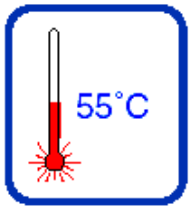



**Table D** Batch Preparation of 1.0% UltraSpec-Agarose™

| Amt of Agarose (gm) | + | Concentrated Buffer (50x) (ml) | + | Distilled Water (ml) | = | Total Volume (ml) |
|---|---|---|---|---|---|---|
| 4.0 | | 8.0 | | 392 | | 400 |

6. Dispense the required volume of cooled agarose solution for casting the gels. The volume required is dependent upon the size of the gel bed (refer to Table A for individual gel casting guidelines).
7. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Then proceed with preparing the gel for electrophoresis.

**Lab 9 Report Sheet** **Name** _____
***Mitochondrial DNA Analysis*** **Group Number** _____ **Date** _____
**Biol 211**

## Results

1. Use tape to attach a copy of your group's gel in the space below:

## Size Determination of the PCR Amplified mtDNA Fragments

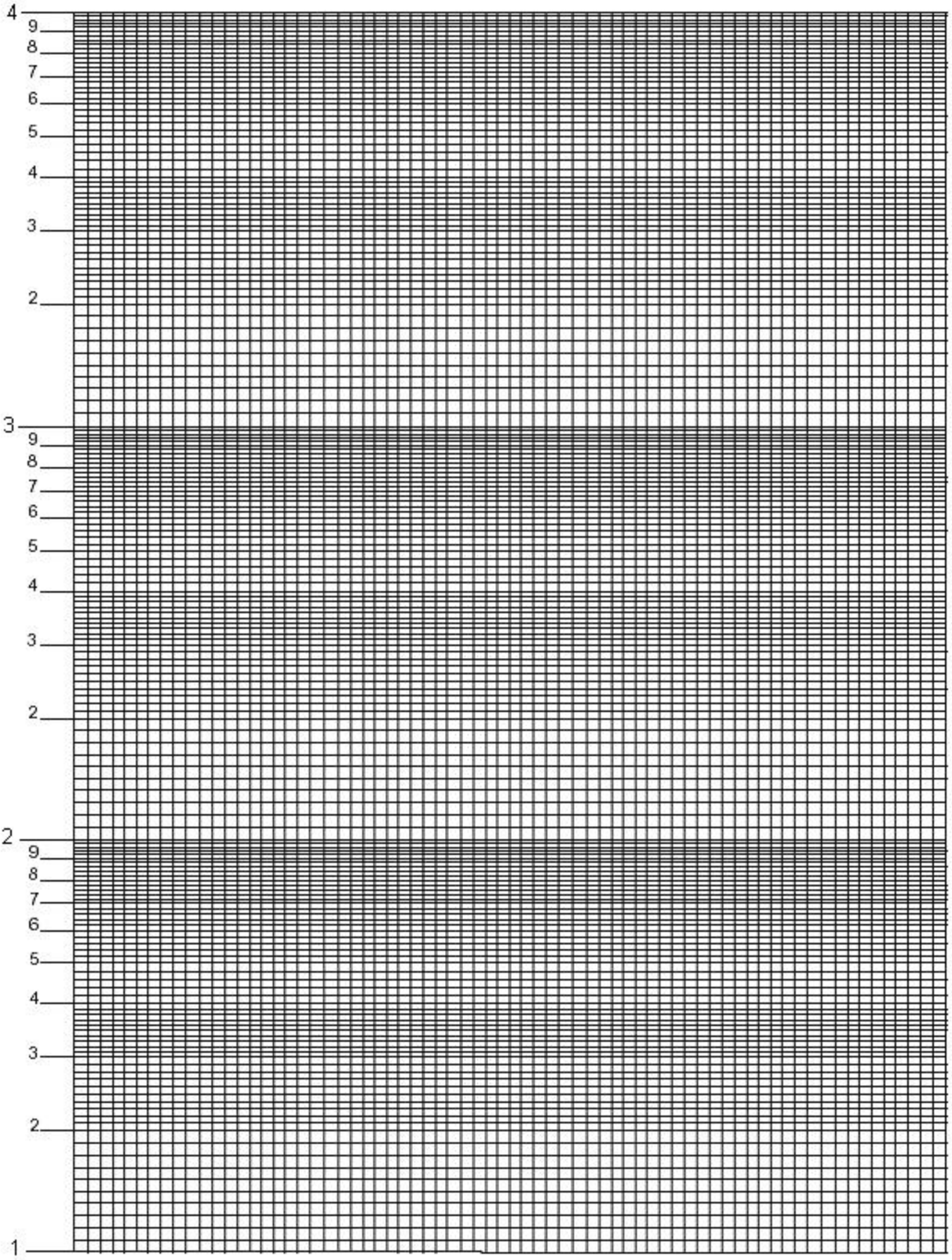
2. Neatly record in the table below the distance traveled (in cm) by each of the standard DNA fragments and the RT-PCR amplified fragment—show how you made these measurements on the copy of your group's gel, above. See step 7 on page 17 for details on how to measure the distance traveled. Calculate and enter in the table the log the size of each standard DNA fragment.

**Table 1.** Distance traveled by Standard DNA fragments and the RT-PCR amplified fragment in the agarose gel.

| Standard DNA Fragment No. | Size of DNA Fragment (Base pairs) | Log (base pairs) | Distance traveled in Gel (cm) |
|---|---|---|---|
| 1 | 200 | | |
| 2 | 400 | | |
| 3 | 600 | | |
| 4 | 800 | | |
| 5 | 1000 | | |
| 6 | 1200 | | |
| | | PCR Amplified mtDNA fragment #1 | |
| | | PCR Amplified mtDNA fragment #2 | |

3. Plot the appropriate data from table 1 on the semi-log graph paper on the following page (or use Excel to plot the data). See page 17 for details on how to determine the size of the PCR amplified mtDNA fragments. Give the graph an appropriate/informative title and label each axis fully.

Title of Graph:



4. What is the size of your PCR amplified mtDNA fragment(s)? Show your work on the graph on the previous page and record your results in the table under #5, below.
   - If you used *Excel* to plot the data, show your work below using the equation of the trendline from the *Excel* plot.

5. Examine the map of human mitochondrial DNA, figure 3 on page 2. What would you expect to be the size of each of the two PCR amplified mtDNA regions? *Show/explain how you arrived at each answer.*

| **PCR amplified mtDNA region** | **Length of Fragment** (base pairs) | |
|---|---|---|
| | Expected Length | Actual Length of your fragments (from #4, above) |
| Shorter fragment | | |
| Longer fragment | | |

Work:

6. Examine the data in the table above in #5, above. Account for any differences in the expected and actual lengths of the PCR amplified mtDNA regions.

**Class Data**

| **Student** | **Length of PCR amplified Mt DNA region** (Base pairs) | |
|---|---|---|
| | Shorter Fragment | Longer Fragment |
| 1. | | |
| 2. | | |
| 3. | | |
| 4. | | |
| 5. | | |
| 6. | | |
| 7. | | |
| 8. | | |
| 9. | | |
| 10. | | |
| 11. | | |
| 12. | | |
| 13. | | |
| 14. | | |
| 15. | | |
| 16. | | |
| 17. | | |
| 18. | | |
| 19. | | |
| 20. | | |
| 21. | | |
| 22. | | |
| 23. | | |
| 24. | | |

7. Examine the class data, above. Account for any differences the lengths of the PCR amplified mtDNA regions. Is there evidence for DNA polymorphisms between different individuals in the class? Explain and use specific numerical data from the class data, above, to support your response.

**Lab 9: Prelab Questions** **Name** _____
**Mitochondrial DNA Analysis** **Group Number** _____ **Date** _____
**Biol 211**

**Note:** Read the introduction and procedure for this lab activity *before* attempting to answer the questions that follow! Hand in this assignment just *before* the start of your scheduled lab period. The following pages in your textbook should also be read as they are a good source of background information: PCR: pages 403-404; Electrophoresis: fig. 20.9 on page 405 in Biology, *8th ed by Campbell and Reece*

**Mitochondrial Diseases**

1. What might be some symptoms of a mitochondrial disease? *Explain.*

2. Is it possible for a child to be healthy if the child's father is severely affected with a mitochondrial disease? Explain why or why not.

3. Is it possible for a child to have a mitochondrial disease if the child's Mother is phenotypically unaffected by the disease? Explain why or why not.

4. Is it possible for a child to be severely affected by a mitochondrial disease if the mother is severely affected by the disease? Explain why or why not.

5. Is it possible for a child to have only mild symptoms of a mitochondrial disease if the mother is severely affected by the disease? Explain why or why not.

**Polymerase Chain Reaction**

6. Why are two different primers required to amplify a specific region of DNA using PCR?

7. Explain how PCR results in the amplification (cloning) of a specific region of DNA. Your explanation should include the roles of each of the following: denaturation, annealing, extension, DNA primers, *Taq* Polymerase, repeated cycling of temperatures: 94$^{o}$C to 55$^{o}$C to 72 $^{o}$C and back to 94 $^{o}$C. <u>In addition to an explanation, include a *labeled diagram* of that illustrates the process going through ***3 cycles***</u>.

**Electrophoresis**

8. Explain how electrophoresis separates DNA fragments.

9. Should the sample wells face (i.e. be closest to) the negative electrode (cathode) or the positive electrode (anode) when separating DNA fragments by gel electrophoresis? Explain.