Edvo-Kit #332

Mitochondrial DNA Analysis Using PCR

Experiment Objective:
In this experiment, students will isolate their genomic DNA and use the Polymerase Chain Reaction (PCR) to amplify two separate regions of the mitochondrial genome. Results are analyzed using agarose gel electrophoresis.

See page 3 for storage instructions.

IMPORTANT NOTE:
The PCR cycling conditions and electrophoresis buffer have changed. Please review the literature before performing the experiment.
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## Experiment Components

### Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Storage</th>
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<tbody>
<tr>
<td>PCR EdvoBeads™ PLUS</td>
<td>Room Temp.</td>
<td></td>
</tr>
<tr>
<td>Universal DNA Buffer</td>
<td>-20°C Freezer</td>
<td></td>
</tr>
<tr>
<td>TE Buffer</td>
<td>-20°C Freezer</td>
<td></td>
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<tr>
<td>Mitochondrial LyphoPrimer™ Mix</td>
<td>-20°C Freezer</td>
<td></td>
</tr>
<tr>
<td>LyphoControl™ (Complete PCR Control)</td>
<td>-20°C Freezer</td>
<td></td>
</tr>
<tr>
<td>EdvoQuick™ DNA ladder</td>
<td>-20°C Freezer</td>
<td></td>
</tr>
<tr>
<td>Proteinase K</td>
<td>-20°C Freezer</td>
<td></td>
</tr>
</tbody>
</table>

### Storage Check (√)

- Each PCR EdvoBead™ contains dNTP mixture, Taq DNA polymerase buffer, Taq DNA polymerase, MgCl₂, and reaction buffer.

### NOTE:

Components C and D are now supplied in our LyphoPrimer™ and LyphoControl™ form and require reconstitution prior to setting up PCR reactions.

## REAGENTS & SUPPLIES

Store all components below at room temperature.

### Component

<table>
<thead>
<tr>
<th>Component</th>
<th>Check (✓)</th>
</tr>
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<tbody>
<tr>
<td>UltraSpec-Agarose™</td>
<td></td>
</tr>
<tr>
<td>TBE Electrophoresis Buffer Powder</td>
<td></td>
</tr>
<tr>
<td>SYBR® Safe Stain</td>
<td></td>
</tr>
<tr>
<td>FlashBlue™ Stain</td>
<td></td>
</tr>
<tr>
<td>Snap-top Microcentrifuge Tubes</td>
<td></td>
</tr>
<tr>
<td>Screw-top Microcentrifuge Tubes (1.5 mL - use for boiling)</td>
<td></td>
</tr>
<tr>
<td>0.2 mL PCR tubes</td>
<td></td>
</tr>
<tr>
<td>Disposable plastic cups</td>
<td></td>
</tr>
<tr>
<td>Salt packets</td>
<td></td>
</tr>
<tr>
<td>15 mL conical tube</td>
<td></td>
</tr>
</tbody>
</table>

### NOTE:

- The PCR cycling conditions and electrophoresis buffer have changed. Please review the literature before performing the experiment.

- All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

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Requirements

- Thermal cycler (EDVOTEK Cat. #541 highly recommended)
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Balance
- Microcentrifuge
- Two Water baths for 55°C and 99°C Incubations (EDVOTEK Cat. #539 highly recommended)
- UV Transilluminator or Blue light visualization (EDVOTEK Cat. #557 or #558 highly recommended)
- White light visualization system (OPTIONAL - use if staining with FlashBlue™)
- UV safety goggles
- Automatic micropipettes (5-50 µL) with tips
- Microwave
- Pipet pump
- 250 mL flasks or beakers
- Hot gloves
- Safety goggles
- Disposable vinyl or latex laboratory gloves
- Ice buckets and ice
- Distilled or deionized water
- Drinking Water (if isolating DNA from cheek cells)
- Bleach solution
Background Information

MITOCHONDRIAL DNA ANALYSIS

Mitochondria (plural for mitochondrion) are the energy-producing organelles of the cell. Mitochondria are generally oblong or egg-shaped. 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 distinct membranes. A protein called porin is present in the outer membrane, making it permeable to ions and other molecules. In contrast, the inner membrane is enriched in a rare, negatively charged phospholipid known as cardiolipin, which helps make this membrane highly impermeable to ions. The inner membrane is highly convoluted, with infoldings called cristae (Figure 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.

The space enclosed by the inner membrane is known as the matrix. The chemical reactions that produce energy for the cell take place within the matrix and inner membrane. As shown in Figure 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 Beta oxidation (β-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, known as oxidative phosphorylation, protons generated by the electron transport chain flow through a pump known as ATP synthase. This electron flow drives the production of ATP, the primary energy-containing molecule used in biological systems.

The DNA present in the matrix is distinct from the DNA found in the cell’s nucleus. Mitochondrial DNA (mtDNA) was the first part of the human genome to be sequenced. The mitochondrial genome contains 16,569 base pairs of DNA that codes for 37 genes, as illustrated by Figure 3. MtDNA encodes 13 polypeptides, all of which are subunits of the electron transport chain. However, mtDNA does not encode the entire electron transport chain; for example, nuclear DNA encodes for Complex II and subunits in the other complexes. Additionally, mtDNA codes for cytochrome B (another constituent of the electron transport chain), and ATP synthase. One peculiarity of mitochondrial protein synthesis is that mitochondrial mRNA uses a slightly different genetic code than cytoplasmic translation. As
such, mtDNA also encodes mitochondrial-specific ribosomal RNA and transfer RNA. 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 (Figure 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 can be used to include or exclude suspects from 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, may be linked to defects in oxidative phosphorylation caused by damage to mitochondria in neuronal tissues. 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 (Figure 4). The severity of any particular mitochondrial disorder is highly variable, depending on the number of mutated mitochondria inherited from the mother (Figure 5).

To examine mitochondrial DNA, the Polymerase Chain Reaction (PCR) is usually employed. PCR was invented in 1984 by Dr. Kary Mullis at the Cetus
Corporation in California. 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. For this groundbreaking technology, Mullis was awarded the Nobel Prize in Chemistry in 1993.

Before performing PCR, template DNA is extracted from various biological sources. Because PCR is very sensitive, only a few copies of the gene are required. Nevertheless, freshly isolated DNA will provide better amplification results than older DNA specimens that may have become degraded. In order to amplify the specific DNA or target sequence, two primers (short & synthetic DNA molecules) are designed to correspond to the ends of the target sequence.

To perform PCR, the template DNA and a molar excess of primers are mixed with the four “free” deoxynucleotides (dATP, dCTP, dGTP, and dTTP), and a thermostable DNA polymerase. The most commonly used DNA polymerase is Taq DNA polymerase. This enzyme, originally purified from a bacterium that inhabits hot springs, is stable at very high temperatures. These components (template DNA, primers, the four deoxynucleotides, and Taq DNA polymerase) are mixed with a buffer that contains Mg²⁺, an essential cofactor for Taq polymerase. The PCR reaction mixture is subjected to sequential heating/cooling cycles at three different temperatures in a thermal cycler.

- In the first step, known as “denaturation”, the mixture is heated to near boiling (94° C - 96° C) to “un-zip” (or melt) the target DNA. The high temperature disrupts the hydrogen bonds between the two complementary DNA strands and causes their separation.
- In the second step, known as “annealing”, the reaction mixture is cooled to 45° C - 65° C, which allows the primers to base pair with the target DNA sequence.
- In the third step, known as “extension”, the temperature is raised to 72° C. This is the optimal temperature at which Taq polymerase can add nucleotides to the hybridized primers to synthesize the new complementary strands.

These three steps - denaturation, annealing, and extension - constitute one PCR “cycle” (Figure 6). Each PCR cycle doubles the amount of the target DNA in less than five minutes. In order to produce enough DNA for analysis, twenty to forty cycles may be required. To simplify this process, a specialized machine, called a “thermal cycler” or a “PCR machine”, was created to rapidly heat and cool the samples.

In this experiment, students will examine their mtDNA from their own cells. To do this, PCR is used to amplify two separate regions of the mitochondrial chromosome, as shown in Figure 3. Amplification of these regions will result in PCR products of 921 and 672 base pairs. Following PCR, the amplified DNA is analyzed using agarose gel electrophoresis.
Mitochondrial DNA Analysis Using PCR

Figure 6:
Polymerase Chain Reaction
EXPERIMENT OBJECTIVE

In this experiment, students will isolate their genomic DNA and use the Polymerase Chain Reaction (PCR) to amplify two separate regions of the mitochondrial genome. Results are analyzed using agarose gel electrophoresis.

LABORATORY SAFETY

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!

- Wear gloves and goggles while working in the laboratory.
- You will be using equipment that can be dangerous if used incorrectly, so use caution.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose.
- DO NOT DRAW SAMPLES INTO PIPETS WITH YOUR MOUTH - USE PIPET PUMPS.
- Always wash hands thoroughly with soap and water after working in the laboratory.
- Contaminated laboratory waste (saliva solution, cup, pipet, etc.) must be disinfected with 15% bleach solution prior to disposal. Be sure to properly dispose any biological samples according to your institutional guidelines.

LABORATORY NOTEBOOKS

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you will be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before Starting the Experiment:
- Carefully read the introduction and the protocol. Write a hypothesis that reflects the experiment.
- Predict the results of your experiment.

During the Experiment:
- Record your observations or photograph your results.

After the Experiment:
- Formulate an explanation from your results.
- Determine what could be changed if the experiment was repeated.
- Write a new hypothesis that would reflect these changes.
Module I-A: Isolation of DNA from Human Cheek Cells

1. **LABEL** a 1.5 mL screw top microcentrifuge tube and a cup with your initials.
   
   *NOTE: Saline solution MUST be used for cheek cell wash. Sports drinks will inhibit amplification of DNA by Polymerase Chain Reaction in Module II.*

2. **RINSE** your mouth vigorously for 60 seconds using 10 mL saline solution. **EXPEL** the solution into the cup.

3. **SWIRL** the cup gently to resuspend the cells. **TRANSFER** 1.5 mL of solution into the labeled tube.

4. **CENTRIFUGE** the cell suspension for 2 minutes at full speed to pellet the cells. **POUR** off the supernatant, but **DO NOT DISTURB THE CELL PELLET**! **REPEAT** steps 3 and 4 once more.

5. **RESUSPEND** the cheek cells in 140 µL lysis buffer by pipetting up and down or by vortexing vigorously.

6. **CAP** the tube and **PLACE** in a water bath float. **INCUBATE** the sample in a 55°C water bath for 5 minutes.

7. **FLICK** or **Vortex** for 20 seconds.

8. **INCUBATE** the sample in a 99°C water bath for 5 minutes. *NOTE: Students MUST use screw-cap tubes when boiling DNA isolation samples.*

9. **CENTRIFUGE** the cellular lysate for 2 minutes at full speed.

10. **TRANSFER** 80 µL of the supernatant to a clean, labeled microcentrifuge tube. **PLACE** tube in ice.

11. **PROCEED** to Module II: Amplification of the Mitochondrial Regions.

**OPTIONAL STOPPING POINT:**

The extracted DNA may be stored at -20°C for amplification at a later time.
Module I-B: Isolation of DNA from Human Hair

1. LABEL a 1.5 mL screw top microcentrifuge tube with your initials.
2. Using tweezers, GRASP 2-3 hair shafts at the base and PULL quickly. COLLECT at least 5 hairs that include the root and the sheath (a sticky barrel-shaped layer of cells that encircles the root end of the hair).
3. Using a clean scalpel or scissors, TRIM away any extra hair from the root (leave about 1 cm in length from the root). TRANSFER the roots to the labeled tube using forceps.
4. CAP the tube and CENTRIFUGE the sample for 10 seconds at full speed to collect the roots at the bottom of the tube.
5. ADD 140 µL lysis buffer to the tube. For best results, completely IMMERSE the follicles in the solution.
6. CAP the tube and PLACE it in a water bath float. INCUBATE the sample in a 55° C water bath for 15 min.
7. MIX the sample by vortexing or flicking the tube vigorously for 20 seconds.
8. CENTRIFUGE the sample for 10 seconds at full speed to collect the roots at the bottom of the tube.
9. INCUBATE the sample at 55° C for an additional 15 min.
10. MOVE the sample to a 99° C water bath. INCUBATE for 10 min. Be sure to use screw-cap tubes when boiling samples.
11. MIX the sample by vortexing or flicking the tube vigorously for 20 seconds.
12. CENTRIFUGE the cellular lysate for 2 min. at low speed (6000 rpm).
13. TRANSFER 80 µL of the supernatant to a clean, labeled microcentrifuge tube. PLACE tube in ice.
14. PROCEED to Module II: Amplification of the Mitochondrial Regions.

OPTIONAL STOPPING POINT:
The supernatant may be stored at -20° C for amplification at a later time.

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Warning!
Students should use screw-cap tubes when boiling samples.

IMPORTANT:
For best results, harvest hairs from the scalp. The root structure from these hairs will be thicker and will yield more DNA than those from the eyebrow.

Steps 7 & 11: If a vortex is not available, mix samples by flicking the tube vigorously for 20 seconds.

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Module II: Amplification of the Mitochondrial Regions

1. **OBTAIN** the red extracted DNA from Module I.
2. **LABEL** a fresh 0.2 mL PCR tube with your initials.
3. **ADD** 20 µL D1S80 primer mix (yellow), 5 µL extracted DNA (red), and a PCR EdvoBead™ PLUS.
4. **MIX** the PCR sample. Make sure the PCR EdvoBead™ PLUS is completely dissolved. If mixed correctly, the final solution will be light orange.
5. **CENTRIFUGE** the sample for a few seconds to collect the sample at the bottom of the tube.
6. **AMPLIFY** the DNA using PCR.
   **PCR cycling conditions:**
   - Initial denaturation 94° C for 1 minute
   - 94° C for 30 seconds
   - 55° C for 30 seconds
   - 72° C for 2 minutes
   - Final Extension 72° C for 1 minute
   25 cycles
7. After PCR, **PLACE** the tubes on ice. **PROCEED** to Module III: Separation of PCR Products by Electrophoresis.

**OPTIONAL STOPPING POINT:**
The PCR samples may be stored at -20° C for electrophoresis at a later time.
Module III: Separation of PCR Products by Electrophoresis

PREPARING THE AGAROSE GEL WITH SYBR® SAFE STAIN

1. **MIX** the agarose powder with 1X TBE buffer in a 250 mL flask (see Table A).
2. **DISSOLVE** the agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
3. **COOL** agarose to 60°C by carefully swirling the flask to promote even dissipation of heat.
4. While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the comb in the appropriate notch.
5. Before casting the gel, **ADD** SYBR® Safe concentrate to the cooled molten agarose and swirl the flask to mix (see Table A).
6. **POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
7. **REMOVE** the end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

**OPTIONAL STOPPING POINT:**
Gels can be stored overnight submerged in electrophoresis buffer, in the fridge, and protected from light.

**IMPORTANT:**
7 x 7 cm gels are recommended. Each gel can be shared by 4-5 students. Place well-former template (comb) in the first set of notches.

If you are unfamiliar with agarose gel prep and electrophoresis, detailed instructions and helpful resources are available at www.edvotek.com.

<table>
<thead>
<tr>
<th>Table A</th>
<th>Individual 2.0% UltraSpec-Agarose™ Gel with SYBR® Safe Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of Gel Casting tray</td>
<td>1X TBE Buffer + Amt of Agarose = TOTAL Volume</td>
</tr>
<tr>
<td>7 x 7 cm</td>
<td>25 mL</td>
</tr>
<tr>
<td>7 x 14 cm</td>
<td>50 mL</td>
</tr>
</tbody>
</table>
Module III: Separation of PCR Products by Electrophoresis, continued

RUNNING THE GEL

8. PLACE the gel (on the tray) into an electrophoresis chamber. COVER the gel with 1X TBE electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.

9. Using Table 1 as a guide, LOAD the entire sample (25 µL) into the wells in consecutive order.

10. CHECK that the gel is properly oriented, then PLACE safety cover onto the chamber. Remember, the DNA samples will migrate toward the positive (red) electrode.

11. CONNECT the leads to the power source and PERFORM electrophoresis (See Table C for time and voltage guidelines).

12. After electrophoresis is complete, REMOVE the gel and casting tray from the electrophoresis chamber.

OPTIONAL STOPPING POINT:
Gels can be stored for several days. Protect from light, refrigerate, and keep hydrated by storing each gel in a water-tight plastic bag with a small amount of electrophoresis buffer.

Table 1: Sample Table

<table>
<thead>
<tr>
<th>Lane</th>
<th>Recommended</th>
<th>Sample Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EdvoQuick™ DNA Ladder</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Student #1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Student #2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Student #3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Student #4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Control DNA*</td>
<td></td>
</tr>
</tbody>
</table>

* Optional, or additional student sample.

Table B: 1x TBE Electrophoresis Buffer (Chamber Buffer)

<table>
<thead>
<tr>
<th>EDVOTEK Model #</th>
<th>Total Volume Required</th>
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<tbody>
<tr>
<td>M6+ &amp; M12 (new)</td>
<td>300 mL</td>
</tr>
<tr>
<td>M12 (classic)</td>
<td>400 mL</td>
</tr>
<tr>
<td>M36</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Table C: Time and Voltage Guidelines (2.0% Agarose Gels)

<table>
<thead>
<tr>
<th>Volts</th>
<th>Time: 7 x 7 cm gel</th>
<th>Time: 7 x 14 cm gel</th>
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</thead>
<tbody>
<tr>
<td>125</td>
<td>30 min.</td>
<td>60 min.</td>
</tr>
<tr>
<td>70</td>
<td>60 min.</td>
<td>120 min.</td>
</tr>
<tr>
<td>50</td>
<td>90 min.</td>
<td>150 min.</td>
</tr>
</tbody>
</table>
Module III: Separation of PCR Products by Electrophoresis, continued

13. SLIDE the gel off the casting tray onto the viewing surface of the transilluminator and turn the unit on. ADJUST the brightness to the desired level to maximize band visualization. DNA should appear as bright green bands on a dark background.

14. PHOTOGRAPH the results.

15. REMOVE and DISPOSE of the gel and CLEAN the transilluminator surfaces with distilled water.

VISUALIZING THE SYBR® GEL

Be sure to wear UV goggles if using a UV transilluminator.
Module IV: Staining Agarose Gel with FlashBlue™ Stain (OPTIONAL)

FlashBlue™ Stain is a simple and effective visible DNA stain that can be used as an alternative, or in addition to, UV-reactive DNA stains like SYBR® Safe. **IF staining with both SYBR® Safe and Flash Blue, you must examine and record the SYBR® Safe bands before beginning the FlashBlue™ Staining.**

1. DILUTE 10 mL of 10X concentrated FlashBlue™ with 90 mL of distilled water in a flask. MIX well.
2. REMOVE the agarose gel and casting tray from the electrophoresis chamber. SLIDE the gel off the casting tray into a small, clean gel-staining tray.
3. COVER the gel with the 1X FlashBlue™ stain solution. STAIN the gel for 2-3 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 3 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
4. POUR the 1X FlashBlue™ back into the flask (the stain can be reused). COVER the gel with warm water (40-45° C). Gently RINSE the gel for 20-30 seconds. POUR off the water.
5. COVER the gel with clean, warm water (40-45° C). DESTAIN for 5-15 minutes with gentle shaking (longer periods will yield better results). DNA bands will start to appear after 5 minutes of destaining. Changing the water frequently will accelerate destaining.
6. Carefully REMOVE the gel from the destaining liquid. VISUALIZE results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

**ALTERNATIVE FLASHBLUE™ STAINING PROTOCOL:**

1. DILUTE 1 mL of 10X FlashBlue™ stain with 499 mL distilled water.
2. COVER the gel with diluted FlashBlue™ stain.
3. SOAK the gel in the staining liquid for at least three hours. For best results, stain gels overnight.
4. Carefully REMOVE the gel from the staining liquid. VISUALIZE results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.
Study Questions

1. What are the three energy-producing sets of chemical reactions that take place inside the mitochondrion?

3. How are mitochondria different from other organelles inside the cell?

3. Is it possible for a child to be healthy if his/her father is affected with a mitochondrial disease? From an unaffected mother? Why or why not? What might be some symptoms of such a disease?

4. If a crime scene sample is too degraded for normal DNA profiling, are any further analyses possible? If so, what assay(s) could be performed?
# Instructor's Guide

## OVERVIEW OF INSTRUCTOR’S PRELAB PREPARATION:

This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

<table>
<thead>
<tr>
<th>Preparation For:</th>
<th>What to do:</th>
<th>When:</th>
<th>Time Required:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Module I:</strong> Isolation of DNA from Hair or Cheek Cells</td>
<td>Prepare and aliquot various reagents (saline).</td>
<td>Up to one day before performing the experiment.</td>
<td>30 min.</td>
</tr>
<tr>
<td></td>
<td>Prepare Lysis buffer.</td>
<td>IMPORTANT: Prepare Lysis buffer no more than 30 min. before performing experiment.</td>
<td>5-10 min.</td>
</tr>
<tr>
<td></td>
<td>Equilibrate water baths at 55 °C and boiling.</td>
<td>Anytime before performing the experiment.</td>
<td>5 min.</td>
</tr>
<tr>
<td><strong>Module II:</strong> Amplification of the Mitochondrial Regions</td>
<td>Prepare and aliquot various reagents (Primer, DNA template, ladder, etc.).</td>
<td>One day to 30 min. before performing the experiment.</td>
<td>30 min.</td>
</tr>
<tr>
<td></td>
<td>Program Thermal Cycler.</td>
<td>Anytime before performing the experiment.</td>
<td>15 min.</td>
</tr>
<tr>
<td><strong>Module III:</strong> Separation of PCR Products by Electrophoresis</td>
<td>Prepare TBE buffer and dilute SYBR® Safe Stain.</td>
<td>Up to one week before performing the experiment.</td>
<td>45 min.</td>
</tr>
<tr>
<td></td>
<td>Prepare molten agarose and pour gel.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Module IV:</strong> Staining Agarose Gels with FlashBlue™ (OPTIONAL)</td>
<td>Prepare staining components.</td>
<td>Up to 10 min. before the class period.</td>
<td>10 min.</td>
</tr>
</tbody>
</table>

![Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.](image)

**NOTE:**
The PCR cycling conditions may have changed. Before running the experiment, confirm that the program matches the settings below:

- Initial denaturation 94° C for 5 minutes
- 94° C for 30 seconds
- 68° C for 30 seconds
- 72° C for 60 seconds
- 32 cycles
- Final Extension 72° C for 4 minutes
Pre-Lab Preparations

MODULE I: ISOLATION OF DNA

NOTE: For Module I-A, Saline solution MUST be used for cheek cell wash. Sports drinks will inhibit amplification of DNA by Polymerase Chain Reaction in Module II. If you have used sports drinks for the cheek cell wash, please DISCARD the samples and REPEAT the DNA extraction with saline solution.

DISINFECTING LABORATORY MATERIALS: Contaminated laboratory waste (saliva solution, cup, pipette, etc.) must be disinfected with 15% bleach solution prior to disposal. Be sure to properly dispose of any biological samples according to your institutional guidelines.

Prepare the Saline Solution (For Module I-A ONLY):

1. To prepare the saline solution, dissolve all 8 salt packets (~4 g) in 500 mL of drinking water. Cap and invert bottle to mix.

2. Aliquot 10 mL of saline solution per cup. Distribute one cup per student.

Prepare the Lysis Buffer (For Module I-A or I-B):
Prepare no more than 30 min. before starting the experiment.

1. Add 100 µl of Universal DNA Buffer (A) to the tube of Proteinase K and allow the sample to hydrate for several minutes. After the sample is hydrated, pipette up and down several times to thoroughly mix the material.

2. Transfer the entire amount of the rehydrated Proteinase K solution to a 15 mL conical tube containing an additional 4 mL of Universal DNA Buffer (A).

3. Invert the tube several times to mix. Label this tube “Lysis Buffer”. At this point, the Lysis Buffer can no longer be stored. It should be used as soon as possible.

NOTE: The Lysis buffer should be light red and free of any undissolved clumps.

4. Aliquot 300 µl of Lysis Buffer into 13 labeled microcentrifuge tubes.

5. Distribute one tube of “Lysis Buffer” to each student pair.

FOR MODULE I-A
Each student should receive:
- One cup containing 10 mL of saline solution
- One screw-cap tube
- One microcentrifuge tube

Reagents to be Shared by Two Students:
- 300 µl Lysis buffer
- 15% bleach solution

FOR MODULE I-B
Each student should receive:
- One screw-cap tube
- One microcentrifuge tube

Reagents to be Shared by Two Students:
- 300 µl Lysis buffer

Warning !!
Remind students to only use screw-cap tubes when boiling their DNA samples. The snap-top tubes can potentially pop open and cause injury.
Pre-Lab Preparations

MODULE II: AMPLIFICATION THE MITOCHONDRIAL REGIONS

This kit features the NEW EDVOTEK® LyphoControl™ and LyphoPrimer™. The reagents are also color coded so that a correctly assembled PCR reaction should appear orange in color.

Preparation of the Primer Mix

1. Thaw the TE buffer (B). Mix well before using.
2. Before preparing the primer mix, make sure the solid material is at the bottom of tube of LyphoPrimer™ Mix (C). If not, centrifuge the tube at full speed for 20 seconds or tap the tube on the lab bench.
3. Dilute the LyphoPrimer™ by adding 1 mL of TE Buffer to the tube. Cap and mix well and place on ice. The solution should be clear and yellow in color, and no solid pieces should remain.
4. Pipette 20 µL of the diluted primer mix per tube. Label these tubes “Primer Mix”. Distribute one tube per student.

Preparation of the PCR Control Mix

NOTE: This kit contains enough Control DNA for 6 reactions. We strongly recommend running all 6 control reactions to ensure the PCR was successful.

1. Ensure that the lyophilized solid is at the bottom of the LyphoControl™ tube (D). If not, centrifuge the tube at max speed for 10 seconds.
2. Add 160 µL of TE Buffer (B) to the tube containing the LyphoControl™ Control DNA (D). Pipet up and down to mix.
3. Dispense 25 µL of the diluted Control DNA for each control reaction. NOTE: The LyphoControl™ already contains all necessary PCR components and does not need a PCR EdvoBead™ PLUS. Once diluted, the LyphoControl™ is ready to be amplified by PCR alongside student samples, if there is room in the thermal cycler, or can be run prior to the student experiment and stored at -20°C until needed. One 25 µL LyphoControl™ reaction should be run on every student gel to ensure the PCR was successful.

Additional Materials

- Distribute one fresh 0.2 mL PCR tube and one PCR EdvoBead™ PLUS per student.

PCR Amplification

The Thermal cycler should be programmed as outlined in Module II in the Student’s Experimental Procedure.

- Accurate temperatures and cycle times are critical. A pre-run for one cycle (takes approximately 3 to 5 min.) is recommended to check that the thermal cycler is properly programmed.
- For thermal cyclers that do not have a heated lid, it is necessary to place a layer of wax or mineral oil above the PCR reactions in the microcentrifuge tubes to prevent evaporation. Visit www.edvotek.com for more information.
Pre-Lab Preparations

MODULE III: SEPARATION OF PCR PRODUCTS BY ELECTROPHORESIS

Preparation of TBE Electrophoresis Buffer:

For this experiment, we recommend preparing the 1X TBE Electrophoresis Buffer in bulk for sharing by the class. Unused diluted buffer can be used at a later time. See Appendix B for instructions.

Preparation of Agarose Gels:

This experiment requires one 2.0% agarose gel per 4-5 students. A 7 x 7 cm gel is recommended. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

SYBR® Safe Stain Preparation

Prepare diluted SYBR® Safe by adding 250 µL of 1X TBE buffer to the tube of SYBR® Safe and tapping the tube several times to mix. Diluted SYBR® Safe will be used during agarose gel preparation.

Individual Gel Preparation

Each student group can be responsible for casting its own individual gel prior to conducting the experiment (see Module III in the Student’s Experimental Procedure). Students will need 1X TBE buffer and agarose powder. In addition, each 7 x 7 cm gel will need 25 µL of diluted SYBR® Safe Stain.

Batch Gel Preparation

To save time, a larger quantity of agarose solution can be prepared for sharing by the class (see Appendix B).

Preparing Gels in Advance

Gels may be prepared ahead and stored for later use. Solidified gels can be stored for up to 1 week in the refrigerator in water-tight bags with a small amount of buffer to prevent drying. We recommend adding 2 mL of buffer to the bag; excess buffer can lead to diffusion of SYBR® Safe out of the gels.

Do not store gels at -20º C as freezing will destroy them.

Gels that have been removed from their trays for storage should be “anchored” back to the tray with a few drops of molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

Additional Materials

Each 2.0% gel should be loaded with the EdvoQuick™ DNA ladder and PCR reactions from 4 or 5 students.

- Pipette 30 µL of the EdvoQuick™ DNA ladder (E) into labeled microcentrifuge tubes and distribute one tube of EdvoQuick™ DNA ladder per gel.

NOTE:

Accurate pipetting is critical for good experiment results. This experiment is designed for students who have had previous experience with micropipetting techniques and agarose gel electrophoresis.

If students do not know how to use micropipettes, we recommended performing Cat. #5-44, Micropipetting Basics or Cat. #5-43, DNA DuraGel™ prior to conducting this advanced level experiment.

FOR MODULE III

Each Group should receive:

- 1X TBE Buffer
- UltraSpec-Agarose™ Powder
- Tube of SYBR® Safe (25 µL)
- EdvoQuick™ DNA ladder (30 µL)

NOTE:

QuickGuide instructions and guidelines for casting various agarose gels can be found on our website www.edvotek.com/quick-guides

Cat. #557

TruBlu™ LED Transilluminator

The all-new TruBlu™ LED Transilluminator utilizes blue light to view DNA gels stained with SYBR® Safe, thus eliminating the need for UV light or ethidium bromide. The spacious viewing area fits multiple agarose gels. And the high intensity control and orange lid ensure superior visualization.

Features:

- 14.5 x 18 cm viewing area
- Blue light intensity control
- Orange contrast lid
- Durable steel casing
- Made in the USA

INSTRUCTOR’S GUIDE

EDVO-Kit #332         Mitochondrial DNA Analysis Using PCR

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Pre-Lab Preparations

**MODULE IV: STAINING AGAROSE GELS WITH FLASHBLUE™ (OPTIONAL)**

FlashBlue™ can be used as an alternative or in addition to SYBR® Safe in this experiment. If only staining with FlashBlue™, you can omit SYBR® Safe from the gel preparation. However, FlashBlue™ is less sensitive than SYBR® Safe and will take a longer time to obtain results. Alternatively, gels can be visualized first with SYBR® Safe and then with FlashBlue™.

Agarose gels can be stained with diluted FlashBlue™ for 5 minutes and destained for only 20 minutes. For the best results, leave the gel in liquid overnight. This will allow the stained gel to develop in the destaining solution, resulting in dark blue DNA bands that contrast with a uniformly light blue background. A white light box (Cat. #552) is recommended for visualizing gels stained with FlashBlue™.

- Stained gels may be stored in destaining liquid for several weeks if they are refrigerated, although the bands may fade with time. If this happens, re-stain the gel.
- Destained gels should be discarded in the garbage and destaining solutions should be disposed of down the drain.

**Photodocumentation of DNA (Optional)**

Once the gels are stained, you may wish to photograph your results. There are many different photodocumentation systems available, including digital systems that interface directly with computers. Specific instructions will vary depending upon the type of photodocumentation system you are using.
Experiment Results and Analysis

Student's PCR products will show two bands with lengths of 672 and 921 base pairs. The smaller fragment corresponds to DNA from two Complex I genes, whereas the larger fragment corresponds to the ATP synthase gene.

Note – Depending on the PCR conditions used, a diffuse, small-molecular weight band, known as a “primer dimer”, may be present below the 200 bp marker. This is a PCR artifact and can be ignored. Other minor bands may also appear due to nonspecific primer binding and the subsequent amplification of these sequences.

Includes EDVOTEK’s All-NEW EdvoQuick™ DNA Ladder

- Better separation
- Easier band measurements
- No unused bands

EdvoQuick™ DNA ladder sizes: 2640, 1400, 1100, 700, 600, 400, 200
Please refer to the kit insert for the Answers to Study Questions
Appendices

A  EDVOTEK® Troubleshooting Guide
B  Bulk Preparation of TBE Electrophoresis Buffer and Agarose Gels

Safety Data Sheets can be found on our website:
www.edvotek.com/safety-data-sheets
# Appendix A

## EDVOTEK® Troubleshooting Guides

### DNA EXTRACTION

<table>
<thead>
<tr>
<th>PROBLEM:</th>
<th>CAUSE:</th>
<th>ANSWER:</th>
</tr>
</thead>
<tbody>
<tr>
<td>There is no cell pellet after centrifuging the cheek cell suspension.</td>
<td>Not enough cheek cells in suspension.</td>
<td>Mouth must be vigorously rinsed for at least 60 sec. to harvest loose cheek cells. Pool additional suspension and repeat centrifuge step until a cell pellet is seen.</td>
</tr>
<tr>
<td></td>
<td>Sample not centrifuged fast enough.</td>
<td>Spin cells at maximum speed (17,000 x g) for 2 min. If your centrifuge does not reach this speed, spin at highest available speed for 4 min.</td>
</tr>
<tr>
<td>I was not able to extract DNA from hair.</td>
<td>Not enough hairs used for extraction.</td>
<td>Use at least five hairs for the DNA extraction.</td>
</tr>
<tr>
<td></td>
<td>No follicle was present on hair shaft.</td>
<td>The best place to collect hairs for this experiment is the head. Pick hair follicles which have a bulbous base (sheath cells).</td>
</tr>
<tr>
<td>Poor DNA Extraction.</td>
<td>Samples not mixed well enough during extraction.</td>
<td>In addition to flicking the tube, vortex or pipet up and down to mix the sample.</td>
</tr>
<tr>
<td></td>
<td>Proteinase K inactive because it was prepared too far in advance.</td>
<td>Prepare Proteinase K within one hour of use.</td>
</tr>
<tr>
<td></td>
<td>Water baths not at proper temperature.</td>
<td>Use a thermometer to confirm water bath set point.</td>
</tr>
<tr>
<td></td>
<td>Not enough DNA.</td>
<td>Try cheek cell extraction. Final DNA concentrations are usually higher.</td>
</tr>
<tr>
<td>The extracted DNA is very cloudy.</td>
<td>Cellular debris from pellet transferred to tube.</td>
<td>Centrifuge the sample again and move supernatant to a fresh tube. Do not touch the pellet.</td>
</tr>
<tr>
<td></td>
<td>Cellular debris not separated from supernatant.</td>
<td>Centrifuge the sample again. If possible, centrifuge at a higher speed. Move cleared supernatant to a fresh tube.</td>
</tr>
</tbody>
</table>
## PCR AND ELECTROPHORESIS

<table>
<thead>
<tr>
<th>PROBLEM:</th>
<th>CAUSE:</th>
<th>ANSWER:</th>
</tr>
</thead>
<tbody>
<tr>
<td>There is very little liquid left in tube after PCR.</td>
<td>Sample has evaporated.</td>
<td>Make sure the heated lid reaches the appropriate temperature. If your thermal cycler does not have a heated lid, overlay the PCR reaction with wax or mineral oil. Make sure students close the lid of the PCR tube properly.</td>
</tr>
<tr>
<td>Pipetting error.</td>
<td>Students should pipette 20 µL primer mix and 5 µL extracted DNA into the 0.2 mL tube.</td>
<td></td>
</tr>
<tr>
<td>The ladder, control DNA, and student PCR products are not visible on the gel.</td>
<td>The gel was not prepared properly.</td>
<td>Ensure that the electrophoresis buffer was correctly diluted. Gels of higher concentration (&gt; 0.8%) require special attention when melting the agarose. Make sure that the solution is completely clear of &quot;clumps&quot; and glassy granules before pouring gels. The proper buffer was not used for gel preparation. Make sure to use 1x Electrophoresis Buffer.</td>
</tr>
<tr>
<td>The gel was not stained properly.</td>
<td>Ensure that SYBR® Safe Stain was added to the gel. Repeat Staining.</td>
<td></td>
</tr>
<tr>
<td>Malfunctioning electrophoresis unit or power source.</td>
<td>Contact the manufacturer of the electrophoresis unit or power source.</td>
<td></td>
</tr>
<tr>
<td>After staining the gel, the DNA bands are faint.</td>
<td>The gel was not stained for a sufficient period of time.</td>
<td>Repeat staining protocol. Submerge the gel in distilled or deionized water. Allow the gel to soak for 5 minutes.</td>
</tr>
<tr>
<td>After staining the gel, the gel background is very dark.</td>
<td>The gel needs to be destained longer.</td>
<td></td>
</tr>
<tr>
<td>After staining, the ladder and control PCR products are visible on the gel but some student samples are not present.</td>
<td>Student DNA sample was not concentrated enough.</td>
<td>Poor DNA extraction. Repeat Module I (Isolation of DNA from Human Cheek Cells).</td>
</tr>
<tr>
<td>Student DNA sample was degraded.</td>
<td>If DNA is not used right after extraction, store sample at -20°C.</td>
<td></td>
</tr>
<tr>
<td>Wrong volumes of DNA and primer added to PCR reaction.</td>
<td>Practice using micropipettes.</td>
<td></td>
</tr>
<tr>
<td>Some student samples have more/less amplification than others.</td>
<td>Concentration of DNA varies by sample.</td>
<td>There is an inherent variability in the extraction process.</td>
</tr>
<tr>
<td>Low molecular weight band in PCR samples.</td>
<td>Primer dimer.</td>
<td>Low concentration of extracted DNA in PCR reaction.</td>
</tr>
<tr>
<td>DNA bands were not resolved.</td>
<td>To ensure adequate separation, make sure the tracking dye migrates at least 3.5 cm on 7 x 7 cm gels and 6 cm on 7 x 14 cm gels.</td>
<td>Be sure to run the gel the appropriate distance before staining and visualizing the DNA.</td>
</tr>
<tr>
<td>DNA bands fade when gels are kept at 4°C.</td>
<td>DNA stained with FlashBlue™ may fade with time.</td>
<td>Re-stain the gel with FlashBlue™.</td>
</tr>
</tbody>
</table>
Appendix B

Bulk Preparation of TBE Electrophoresis Buffer and Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities that the whole class can share. Leftover diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

**BULK 1X TBE ELECTROPHORESIS BUFFER**

For this experiment, we recommend preparing the 1X TBE Electrophoresis Buffer in bulk for sharing by the class. Unused diluted buffer can be used at a later time.

1. Measure 3.7 L of distilled or deionized water and place in a large vessel. *(NOTE: If using purchased water in a gallon jug, remove and discard 80 mL water.)*
2. Add the entire amount of TBE Electrophoresis Buffer powder to the vessel and mix well.
3. Label the vessel as "1X TBE Electrophoresis Buffer".
4. Use within 60 days of preparation.

**BATCH AGAROSE GELS (2.0%)**

Bulk preparation of 2.0% agarose gel is outlined in Table D.

1. Measure 250 mL of 1X TBE Electrophoresis Buffer and pour into a 500 mL flask.
2. Pour 5.0 g of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.
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 60°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 as marked on the flask in step 3.
6. If staining with SYBR® Safe, add the entire volume of diluted SYBR® Safe from page 21 to the cooled agarose and mix well.
7. Dispense the required volume of cooled agarose solution for casting each gel. Measure 25 mL for each 7 x 7 cm gel, 50 mL for each 7 x 14 cm gel. For this experiment, 7 x 7 cm gels are recommended.
8. 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. Alternatively, gels can be stored in water-tight plastic bags with 2 mL of 1X electrophoresis buffer for up to 1 week in the refrigerator.