Edvo-Kit #316

In Search of the Cholesterol Gene

Experiment Objective:

In this experiment students explore the concept of genetic disease and identify a mutation linked to high blood cholesterol levels. They will also perform and understand the biochemical principles and methodology involved in cholesterol assays.

See page 3 for storage instructions.

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

## Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Storage</th>
<th>Check (✓)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Cholesterol Standard Solution</td>
<td>Freezer</td>
<td></td>
</tr>
<tr>
<td>B Patient #1 Serum Sample</td>
<td>Freezer</td>
<td></td>
</tr>
<tr>
<td>C Patient #2 Serum Sample</td>
<td>Freezer</td>
<td></td>
</tr>
<tr>
<td>D Patient #3 Serum Sample</td>
<td>Freezer</td>
<td></td>
</tr>
<tr>
<td>E Normal DNA Sample</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>F FH Control</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>G Patient #1 DNA Sample</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>H Patient #2 DNA Sample</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>I Patient #3 DNA Sample</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>J DNA Standard Markers</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>K Cholesterol Oxidase Enzyme</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>L Potassium iodide concentrate</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>M Color enhancer, concentrate</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>N Color developer, concentrate</td>
<td>Refrigerator</td>
<td></td>
</tr>
</tbody>
</table>

## REAGENTS & SUPPLIES

*Store all components below at room temperature.*

<table>
<thead>
<tr>
<th>Component</th>
<th>Check (✓)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• UltraSpec-Agarose™</td>
<td></td>
</tr>
<tr>
<td>• Electrophoresis Buffer (50X)</td>
<td></td>
</tr>
<tr>
<td>• SYBR® Safe Stain</td>
<td></td>
</tr>
<tr>
<td>• Microcentrifuge Tubes (1.5 mL)</td>
<td></td>
</tr>
<tr>
<td>• Microcentrifuge Tubes (0.5 mL)</td>
<td></td>
</tr>
</tbody>
</table>
Experiment Requirements

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipettes with tips
- UV Transilluminator or Blue Light visualization system (Cat. #557 recommended)
- Water bath (65 °C)
- Incubation oven (80 °C)
- Microcentrifuge (optional)
- Microwave, hot plate or burner
- Assorted glassware (beakers, flasks and graduated cylinders)
- Spectrophotometer
- Hot gloves or beaker tongs
- Safety goggles and disposable laboratory gloves
- Plastic wrap
- Paper towels
- Forceps
- Distilled or deionized water
Background Information

Cholesterol is a complex lipid essential to all animal cells. Its primary function is as a stabilizing, structural component of cell plasma and organelle membranes. It is also a precursor for steroid hormones. Figure 1 shows the four ring backbone structure found in cholesterol and in steroids like testosterone and progesterone. Cholesterol is also a precursor for vitamin D and for bile salts, which facilitate the digestion of lipids in the intestine.

Cholesterol is synthesized in the liver and is absorbed from dietary sources. It is circulated in body fluids in spherical bodies known as lipoprotein particles. These lipoproteins are classified according to their density during centrifugation. Cholesterol is processed by the liver and packaged into particles known as very low-density lipoproteins, which are then processed in the circulation to form low-density lipoproteins (LDL) (Figure 2). Most circulating cholesterol is found in LDL. High-density lipoproteins (HDL) take up cholesterol from LDL and peripheral tissues and transport it back to the liver for repackaging or excretion (Figure 2). Because HDL take cholesterol out of the circulation, cholesterol found in these particles is often referred to as "good cholesterol" (HDL:H = healthy). Cholesterol found in LDL, in contrast, is the primary form found in circulation destined for the periphery and is therefore often termed "bad cholesterol" (LDL:L = lousy).

While cholesterol is essential for life, excess serum cholesterol can have serious negative consequences. The role of elevated blood cholesterol in cardiovascular disease is well established. Arterial cholesterol accumulation in occlusions known as "plaques" eventually leads to blood flow blockage, resulting in heart attack or stroke. Coronary heart disease is the leading cause of death in the United States and claims 512,000 lives every year. Stroke is the third leading cause of death at 150,000 per year. Over 30 billion dollars in medical costs are spent on stroke alone.

Correlation between cardiovascular disease and elevated blood cholesterol, serum cholesterol levels are now determined routinely, both in clinical laboratory tests and, more recently in home tests. Elevated levels indicate the need for cholesterol reduction either by diet, or, if necessary, medication. To measure blood cholesterol levels, a chemical and an enzymatic assay have been used. The chemical method, which is quite laborious, has largely been replaced by the enzymatic method, which is quite rapid and simple.
In this assay, outlined in Fig. 3, cholesterol esterase is used to reduce cholesterol ester to cholesterol. Cholesterol is then oxidized by cholesterol oxidase to produce cholestenone plus hydrogen peroxide. The hydrogen peroxide then acts as a substrate for peroxidase to produce water plus oxygen, which reacts with the detection reagents to form a color compound. By using density gradient centrifugation, the fractions of total cholesterol contained in LDL ("bad") or HDL ("good") can be determined. Total cholesterol levels greater than 200 mg/100 mL or LDL levels greater than 130 mg/100 mL are considered high risk factors for the development of cardiovascular disease. The levels of another lipid, known as triglyceride, which has also been associated with increased risk of cardiovascular disease, are also usually determined.

As mentioned above, most circulating cholesterol is found in LDL. Animal cells take up LDL from the circulation by a specific LDL receptor. Persons with a condition known as familial hypercholesterolemia (FH) possess mutations in the gene for the LDL receptor and are unable to efficiently remove LDL from the circulation, resulting in elevated blood cholesterol levels. Patients who are heterozygous for this mutation still have one functional gene and therefore possess half the normal amount of LDL receptors as unaffected individuals. Patients, who are homozygous for the mutation, however, completely lack the LDL receptor and therefore possess extremely high blood cholesterol levels (often greater than 600 mg/100 mL). These patients, if untreated, usually die in childhood of coronary artery disease.

FH can result from hundreds of different mutations in the LDL receptor. Many mutations may be detected by a technique known as restriction fragment length polymorphism (RFLP) analysis. In this technique, DNA is digested with a specific restriction enzyme. A probe is then hybridized to a specific region of DNA. An RFLP is defined as a variation in the restriction pattern in the region in diseased vs. healthy individuals. In RFLP analysis, a specific region of DNA, usually within or near a disease-causing gene, is first amplified using the Polymerase Chain Reaction (PCR).

ABOUT POLYMERASE CHAIN REACTION

PCR has two important advantages. The first is sensitivity, which allows for DNA fingerprinting identification using much smaller amounts of DNA since PCR amplifies DNA. The second advantage is the speed of PCR analysis, which allows critical questions to be answered more quickly as compared to Southern Blot analysis.

PCR amplification requires the use of a thermostable DNA polymerase, such as Taq polymerase. Purified from a bacterium known as Thermus Aquaticus that inhabits hot springs, Taq polymerase is commonly used in PCR because it remains stable at near-boiling temperatures. Also included in the PCR reaction are the four deoxynucleotides (dATP, dCTP, dGTP, and dTTP) and two synthetic oligonucleotides, typically 15-30 base pairs in length, known as "primers". These components, together with the DNA to be amplified, are incubated in an appropriate buffer that contains Mg2+. The primers are designed to correspond to the start and end of the DNA to be amplified, known as the "target".

The PCR reaction mixture (which contains the DNA polymerase, buffer, deoxynucleotides, primers, and template) is subjected to sequential heating/cooling cycles at three different temperatures (Figure 4).
**Figure 4: Polymerase Chain Reaction.**

- **Denature**: 94°C
- **Anneal**: 40°C - 65°C
- **Extension**: 72°C
• In the first step, the enzyme reaction is heated to near boiling (92° - 96°C) to denature or "melt" the DNA. This step, known as "denaturation" disrupts the hydrogen bonds between the two complimentary DNA strands and causes their separation.

• In the second PCR step, the mixture is cooled to a temperature that is typically in the range of 45° - 65°. In this step, known as "annealing", the primers, present in great excess to the template, bind to the separated DNA strands.

• In the third PCR step, known as "extension", the temperature is raised to an intermediate value, usually 72°C. At this temperature the Taq polymerase is maximally active and adds nucleotides to the primers to synthesize the new complimentary strands.

Following PCR of the marker region, the amplified DNA is digested with a specific restriction enzyme. If the patient possesses a mutation in the LDL receptor gene, the digestion pattern will differ from the pattern obtained from unaffected individuals.

In this experiment, simulated DNA samples from three patients will be analyzed for the FH mutation. Each sample has previously been amplified and digested with a restriction enzyme and is ready to be separated by electrophoresis on an agarose gel. From the DNA pattern after staining the gel, one can determine whether the patients are normal (one DNA band), heterozygous (three DNA bands), or homozygous (two DNA bands) (Figure 5). You will also perform a cholesterol assay on serum samples from each of the three patients. Using a spectrometer, you will determine the total cholesterol in each sample and calculate the cholesterol concentration of each patient at the time the samples were collected.
EXPERIMENT OBJECTIVE:

In this experiment students explore the concept of genetic disease and identify a mutation linked to high blood cholesterol levels. They will also perform and understand the biochemical principles and methodology involved in cholesterol assays.

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.
- Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose.
- DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
- Always wash hands thoroughly with soap and water after working in the laboratory.

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’ll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.
Module I: Agarose Gel Electrophoresis

1. **DILUTE** concentrated (50X) buffer with distilled water to create 1X buffer (see Table A).
2. **MIX** agarose powder with 1X buffer in a 250 mL flask (see Table A).
3. **DISSOLVE** 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).
4. **COOL** agarose to 60 °C with careful swirling to promote even dissipation of heat.
5. While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps, **PLACE** the well template (comb) in the appropriate notch. **NOTE:** This experiment requires 6 wells.
6. Before casting the gel, **ADD diluted SYBR® Safe** to the molten agarose and swirl to mix (see Table A).
7. **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.
8. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

### Table A

| Size of Gel Casting tray | Concentrated Buffer (50X) | Distilled Water | Amount of Agarose | TOTAL Volume | Diluted SYBR® Safe
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>7 x 7 cm</td>
<td>0.6 mL</td>
<td>29.4 mL</td>
<td>0.24 g</td>
<td>30 mL</td>
<td>30 µL</td>
</tr>
<tr>
<td>10 x 7 cm*</td>
<td>0.9 mL</td>
<td>44.1 mL</td>
<td>0.36 g</td>
<td>45 mL</td>
<td>45 µL</td>
</tr>
</tbody>
</table>

* Recommended gel volume for the EDGE™ Integrated Electrophoresis System.
Module I: Agarose Gel Electrophoresis, continued

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

10. LOAD 18-20 µL of each sample into the well in the order indicated by Table 1.

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

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

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

OPTIONAL STOPPING POINT:
Solidified gels can be stored in the refrigerator for up to 2 weeks. Place 1-2 mL of electrophoresis buffer in a sealable bag with the gels to prevent them from drying out. Excessive buffer will cause SYBR® Safe to diffuse out of the gels. Do not freeze gels.

TABLE 1: Gel Loading

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA standard markers (DNA mkr)</td>
</tr>
<tr>
<td>2</td>
<td>Normal Control DNA sample (Neg)</td>
</tr>
<tr>
<td>3</td>
<td>FH control (Pos)</td>
</tr>
<tr>
<td>4</td>
<td>Patient #1 DNA sample (DNA 1)</td>
</tr>
<tr>
<td>5</td>
<td>Patient #2 DNA sample (DNA 2)</td>
</tr>
<tr>
<td>6</td>
<td>Patient #3 DNA sample (DNA 3)</td>
</tr>
</tbody>
</table>

**TABLE B: 1x Electrophoresis Buffer (Chamber Buffer)**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Total Volume Required</th>
<th>Dilution 50x Conc. Buffer + Distilled Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDGE™</td>
<td>150 mL</td>
<td>3 mL 147 mL</td>
</tr>
<tr>
<td>M12</td>
<td>400 mL</td>
<td>8 mL 392 mL</td>
</tr>
<tr>
<td>M36</td>
<td>1000 mL</td>
<td>20 mL 980 mL</td>
</tr>
</tbody>
</table>

**TABLE C: Time and Voltage Guidelines (0.8% Agarose Gel)**

<table>
<thead>
<tr>
<th>Electrophoresis Model</th>
<th>EDGE™</th>
<th>M12 &amp; M36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volts</td>
<td>Min/Max (minutes)</td>
<td>Min/Max (minutes)</td>
</tr>
<tr>
<td>150</td>
<td>10/20</td>
<td>20/35</td>
</tr>
<tr>
<td>125</td>
<td>N/A</td>
<td>30/45</td>
</tr>
<tr>
<td>100</td>
<td>15/25</td>
<td>40/60</td>
</tr>
</tbody>
</table>

Reminder:
Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.
Module I: Agarose Gel Electrophoresis, continued

14. SLIDE gel off the casting tray onto the viewing surface of the transilluminator.
15. TURN the unit on. DNA should appear as bright green bands on a dark background.
   PHOTOGRAPH results.
16. TURN the unit off. 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 Two: Cholesterol Assay of Patients’ Serum Samples

1. From your instructor, obtain tubes marked "std", "Serum 1", "Serum 2", "Serum 3", and "assay solution".
2. Slowly pipet 20 µL of each patient sample into 1.5 mL tubes. Label each tube.
3. Prepare cholesterol standards from the tube marked "std". The cholesterol standard solution is supplied at a concentration of 5.0 µg/µL.
4. Calculate and record (in a table as shown below) the volume of standard solution needed to prepare standards of 0, 20, 40, 60, 80, and 100 µg.
5. Pipet the appropriate volumes into 1.5 mL tubes and label each tube accordingly.
6. Add 1.0 mL assay solution to each tube, including the serum samples (20 µL volumes) from the three patients.
7. Incubate all the samples (standards plus patients') for 15 minutes in a 37 °C oven or water bath. Save unused assay solution for step 10.
8. Remove samples from the oven or water bath.
9. If using a Spec-20 spectrophotometer, transfer each sample to a larger glass tube or cuvette. Add 2 ml of distilled water to each tube and mix. If using another spectrometer follow the manufacturer’s instructions for sample preparation.

<table>
<thead>
<tr>
<th>Cholesterol Standards</th>
<th>Patient Samples</th>
<th>Absorbance at 500 nm (y-axis)</th>
<th>Total Cholesterol in sample (x-axis)</th>
<th>Cholesterol Concentration (mg/100 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µL</td>
<td></td>
<td>0</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>__ µL</td>
<td></td>
<td>20</td>
<td>20</td>
<td>500</td>
</tr>
<tr>
<td>__ µL</td>
<td></td>
<td>40</td>
<td>40</td>
<td>500</td>
</tr>
<tr>
<td>__ µL</td>
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<td>60</td>
<td>60</td>
<td>500</td>
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<tr>
<td>__ µL</td>
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<td>80</td>
<td>80</td>
<td>500</td>
</tr>
<tr>
<td>__ µL</td>
<td></td>
<td>100</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>Serum 1 (20 µL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 2 (20 µL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 3 (20 µL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Module II: Cholesterol Assay of Patients' Serum Samples, continued

10. Blank a spectrophotometer, set to a wavelength of 500 nm, with unused assay solution.

11. Read and record the absorbance of each sample.

12. Using column 4 (total cholesterol in sample) as x values and column 3 (absorbance) as y values, either:
   a. Use graph paper and plot the x and y values. Draw a straight line that best fits the data. From the best-fit line and the absorbance (y) values for each unknown, determine total cholesterol (x) values for each patient sample. Enter the values in the table.

   OR:

   b. Using a calculator or computer, perform linear regression of the x and y values. Based on the absorbance values, determine the total cholesterol (x) values for each patient sample. Enter the values in the table.

13. Complete the table by calculating and recording the cholesterol concentration of the serum from each patient, expressed as mg / 100 ml (remember the serum volume obtained from each patient was 20 µl).

Example: If the 20 µl sample contains 50 µg cholesterol, the cholesterol concentration is:

\[
\begin{align*}
50 \text{ µg} / 20 \text{ µl} & = 2.5 \text{ µg} / \mu l \\
2.5 \text{ µg} / \mu l & = x \text{ µg} / 100 \mu l \\
x & = (2.5 \text{ µg} / \mu l)(100 \mu l) \\
& = 250 \text{ µg}
\end{align*}
\]

Therefore the concentration is:

250 µg / 100 µl or 250 mg / 100 ml
Study Questions

1. What is familial hypercholesterolemia (FH)? If both parents of a child are heterozygous for FH, what is the probability that the child will be mildly afflicted with the disease? What are the chances that the child will be severely afflicted?

2. What is RFLP analysis? How can RFLPs be used to screen for a genetic disease?

3. Describe the differences between LDL and HDL. Does it make any difference (to one's health) whether cholesterol is found in LDL or HDL?

4. Why is it important to avoid having high blood cholesterol levels? What are some possible causes of high cholesterol levels?

5. What is a statin?
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></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Agarose Gel Electrophoresis</strong></td>
<td>Prepare diluted electrophoresis buffer.</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 gels (OPTIONAL) or aliquot agarose, buffer, and SYBR® Safe.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aliquot DNA.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Module II:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cholesterol Assay of Patient’s Serum Samples</strong></td>
<td>Aliquot standard and patient serum samples.</td>
<td>Day of Module II.</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>Prepare Cholesterol Assay Solution.</td>
<td>No more than 30 min. before Module II.</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>Set up spectrometer.</td>
<td>Any time before Module II.</td>
<td>Varies</td>
</tr>
</tbody>
</table>

**Technical Support**
1.800.EDVOTEK
Mon.- Fri. 8 AM to 5:30 PM EST

Please Have the Following Info:
• Product Number & Description
• Lot Number on Box
• Order/Purchase Order #
Pre-Lab Preparations - Module I

Prepare SYBR® Safe Stain:
1. Prepare 1x Electrophoresis Buffer by combining 20 µL of 50X Concentrated Buffer with 980 µL of distilled water.
2. Add 450 µL of the 1X buffer from step 1 to the tube of SYBR® Safe and mix by tapping the tube several times. The SYBR® Safe Stain is now ready to be used during agarose gel preparation.

Preparation of Agarose Gels:
This experiment requires one 0.8% agarose gel for each group. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow 30-40 minutes for this procedure.

• 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 50X electrophoresis buffer, distilled water, agarose powder, and diluted SYBR® Safe Stain (see Table A on page 12 for volumes).

• Batch Gel Preparation
   To save time, a larger quantity of agarose solution can be prepared for sharing by the class. See Appendix B for instructions.

• 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. Gels with SYBR® Safe must be stored in the dark away from light.

   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:
1. Label five 0.5 ml tubes “DNA mkr” (J). Dispense 45 µl of DNA standard marker into each tube. Each tube is for two groups.
2. Label five 0.5 ml tubes “neg”. Dispense 45 µl of Normal DNA Sample (E) into each tube (for two groups).
3. Label five 0.5 ml tubes “pos”. Dispense 45 µl of the FH control (F) into each tube (for two groups).
4. Label five 0.5 ml tubes for each of the three Patient DNA Samples “DNA1”, “DNA2”, and “DNA3”. Dispense 45 µl of the Patient DNA samples into each of the tubes (for two groups).

Visualization and Photodocumentation:
Gels are ready to visualize immediately after electrophoresis is completed. If you wish to photograph your results, there are many different photodocumentation systems available, including digital systems that are interfaced directly with computers. Specific instructions will vary depending upon the type of photodocumentation system you are using.
Pre-Lab Preparations - Module II

1. Remove the cholesterol oxidase, patient serum samples, and cholesterol standard solution from the freezer for thawing. The components can take up to an hour to thaw.

2. Label five 0.5 ml tubes "std". Dispense 130 μl cholesterol standard solution (A) per tube. Each tube will be shared by two groups.

3. Label five 0.5 ml tubes for each of the three Patient Serum Samples “Serum1”, “Serum2”, and “Serum3”. Dispense 45 μl of the Patient Serum samples into each of the tubes. Each tube will be shared by two groups.

4. Prepare the Cholesterol Assay Solution. This solution will be shared by the class.
   *NOTE: Prepare this solution no more than 30 minutes before module II.*
   a. Add 50 ml of the thawed cholesterol oxidase enzyme (K) to a clean 100 ml beaker.
   b. Add 10 ml potassium iodide solution (L).
   c. Add 10 ml of the enhancer solution (M).
   d. Add 10 ml of the developer solution (N).
   e. Add 20 ml distilled water.
   f. Mix well and label the beaker "Assay Solution".
   g. Store in the dark on ice or in the refrigerator.

5. Set up spectrometer. If your spectrometer uses cuvettes clean or procure these.

6. Hand out nine 1.5 mL microcentrifuge tubes to each group.
Experiment Results and Analysis

**MODULE I: AGAROSE GEL ELECTROPHORESIS**

Genetic Analysis of Patients’ DNA Samples

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA standard markers (DNA mkr)</td>
<td>-----</td>
</tr>
<tr>
<td>2</td>
<td>Normal Control DNA sample (Neg)</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>FH control (Pos)</td>
<td>Heterozygous for FH mutation</td>
</tr>
<tr>
<td>4</td>
<td>Patient #1 DNA sample (DNA 1)</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>Patient #2 DNA sample (DNA 2)</td>
<td>Heterozygous for FH mutation</td>
</tr>
<tr>
<td>6</td>
<td>Patient #3 DNA sample (DNA 3)</td>
<td>Homozygous for FH mutation</td>
</tr>
</tbody>
</table>

The idealized schematic shows the relative positions of the bands, but the results are not depicted to scale.

**MODULE II: CHOLESTEROL ASSAY OF PATIENTS’ SERUM SAMPLES**

The pipetting accuracy, enzyme lot, and the spectrophotometer will affect cholesterol determinations. Typical results are presented below. Your results may vary.

<table>
<thead>
<tr>
<th>Cholesterol Standards</th>
<th>Patient Samples</th>
<th>Absorbance at 500 nm (y-axis)</th>
<th>Total Cholesterol in sample (x-axis) (µg)</th>
<th>Cholesterol Concentration (mg/100 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µL</td>
<td>serum 1 (20 µL)</td>
<td>0.570</td>
<td>35.2</td>
<td>176</td>
</tr>
<tr>
<td>4 µL</td>
<td>serum 2 (20 µL)</td>
<td>1.10</td>
<td>70.0</td>
<td>350</td>
</tr>
<tr>
<td>8 µL</td>
<td>serum 3 (20 µL)</td>
<td>1.60</td>
<td>103</td>
<td>515</td>
</tr>
<tr>
<td>12 µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Please refer to the kit insert for the Answers to Study Questions
Appendices

A  EDVOTEK® Troubleshooting Guide
B  Bulk Preparation of Agarose Gels

Safety Data Sheets:
Now available for your convenient download on www.edvotek.com/safety-data-sheets
# Appendix A

**EDVOTEK® Troubleshooting Guides**

<table>
<thead>
<tr>
<th>PROBLEM:</th>
<th>CAUSE:</th>
<th>ANSWER:</th>
</tr>
</thead>
<tbody>
<tr>
<td>There is only a small amount of SYBR® Safe in my tube.</td>
<td>SYBR® Safe is a concentrate that is diluted before use.</td>
<td>Centrifuge or tap the tube to move the contents to the bottom of the tube. Dilute the SYBR® Safe before use as outlined on page 17.</td>
</tr>
<tr>
<td>Bands are not visible on the gel.</td>
<td>The gel was not prepared properly.</td>
<td>Ensure that the electrophoresis buffer was correctly diluted.</td>
</tr>
<tr>
<td></td>
<td>Malfunctioning electrophoresis unit or power source.</td>
<td>Contact the manufacturer of the electrophoresis unit or power source.</td>
</tr>
<tr>
<td>After staining the gel, the DNA bands are faint.</td>
<td>The gel was not stained properly.</td>
<td>Ensure that diluted SYBR® Safe was added to the gel.</td>
</tr>
<tr>
<td></td>
<td>The gel was not stained for a sufficient period of time with FlashBlue™.</td>
<td>Repeat staining protocol.</td>
</tr>
<tr>
<td></td>
<td>The background of gel is too dark after staining with FlashBlue™.</td>
<td>Destain the gel for 5-10 minutes in distilled water.</td>
</tr>
<tr>
<td>DNA bands were not resolved.</td>
<td>Tracking dye should migrate at least 3.5 cm from the wells to ensure adequate separation (if using a 7x7 cm tray).</td>
<td>Be sure to run the gel at least 3.5 cm before visualizing the DNA.</td>
</tr>
<tr>
<td>There is no separation between DNA bands, even though the tracking dye ran the appropriate distance.</td>
<td>The wrong percent gel was used for electrophoretic separation.</td>
<td>Be sure to prepare the correct percent agarose gel. This experiment requires a 0.8% agarose gel.</td>
</tr>
</tbody>
</table>
Appendix B

Bulk Preparation of Electrophoresis Buffer and Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

### Bulk Electrophoresis Buffer

Quantity (bulk) preparation for 3 liters of 1x electrophoresis buffer is outlined in Table D.

### Batch Agarose Gels (0.8%)

For quantity (batch) preparation of 0.8% agarose gels, see Table E.

1. Use a 1 L flask or bottle to prepare the diluted gel buffer.
2. Pour 3.6 grams 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. Add the entire tube of diluted SYBR® Safe stain (see page 17) to the cooled agarose and mix well.
7. Dispense the required volume of cooled agarose solution for casting each gel. Measure 30 mL for a 7 x 7 cm tray and 45 mL for a 10 x 7 cm tray.
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.

### Table D

<table>
<thead>
<tr>
<th>50x Conc. Buffer</th>
<th>Distilled Water</th>
<th>Total Volume Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mL</td>
<td>2940 mL</td>
<td>3000 mL (3 L)</td>
</tr>
</tbody>
</table>

### Table E

<table>
<thead>
<tr>
<th>Amount of Agarose</th>
<th>Concentrated Buffer (50x)</th>
<th>Distilled Water</th>
<th>Total Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6 g</td>
<td>9 mL</td>
<td>441 mL</td>
<td>450 mL</td>
</tr>
</tbody>
</table>