A single nucleotide change in the DNA sequence of an important gene can affect health and disease. A large number of genetic diseases are identified where such changes have been correlated to the changes in a single nucleotide. More recently, mutations in oncogenes and tumor suppressor genes such as p53, have been associated with lung, colon and breast cancer. Other mutations in genes such as the BRCA1 and II genes have been identified as specific markers with good potential as diagnostic tools for breast cancer.

Human genetics follows the basic findings of the Augustine monk, Gregor Mendel, who studied plant genetics in the mid-1800’s. Mendelian genetics, which predicts traits inherited by offspring, is based on the inheritance of two alleles, or forms of the gene. These two alleles are inherited one from each parent. Alleles, and corresponding traits, can be either dominant or recessive. When a dominant allele is inherited, the trait coded by that allele will be apparent in the offspring. The presence of a dominant allele will, in effect, mask the trait coded by the recessive allele. To observe a recessive trait, it is required that both parental alleles be the recessive type. If both alleles are the same type, either both recessive or both dominant, the individual is said to be homozygous with respect to that trait. If an individual has one dominant and one recessive, the individual is said to be heterozygous for that trait.

Mendelian inheritance can be demonstrated with a $2 \times 2$ matrix, as shown in Figure 1. Parental alleles are placed on the sides of the matrix, and the genotype (what is genetically inherited) and phenotype (the way we look) of the offspring can be predicted. By convention, the dominant allele is denoted by an uppercase letter and the recessive allele by a lowercase letter. For example, assuming both parents each carry one dominant allele and one recessive allele, we can predict that $3/4$ of their children will have the dominant phenotype and $1/4$ of their children will have the recessive phenotype. Genotypically, $1/4$ of the children will carry two dominant alleles; $1/2$ of the children will carry one dominant and one recessive allele, and $1/4$ will carry two recessive alleles. These estimates would be observed if there are a large number of offspring from two parents, as in the case of insects or plants.

Hemoglobin, which is present in red blood cells, is the carrier of oxygen to cells in the body. In capillaries carbon dioxide, which is a by product of metabolism, enters red cells and is converted to carbonic
acid. The acidic pH reduces the affinity of oxygen binding to hemoglobin resulting in the release of oxygen in cells. Likewise when the bound carbon dioxide is released from red cells in the lungs there is an increase in pH which favors the binding of oxygen to hemoglobin. In individuals who suffer from certain blood diseases such as sickle cell anemia, the binding and subsequent transport of oxygen is compromised due to a single nucleotide mutation. This results in a deficiency of oxygen and carbon dioxide exchange in the patient. In sickle cell anemia patients, the substitution of the polar side chain (Glu) with a nonpolar hydrophobic side chain (Val) results in the polymerization of the unoxygennated form and subsequent precipitation of such polymers in red blood cells. The precipitation gives red blood cells a sickle shape due to the lack of diffusion through capillaries.

Blood disease such as sickle cell anemia and β-thalassemias are attributed to various point mutations or other translational product aberrations. Almost 400 different hemoglobin (Hb) variants of known structure have been identified. The early recognized variants were historically assigned alphabetical initials based sequence of discovery or hematologic features.

In the United States, sickle cell anemia is of special interest since it is estimated that 8% of African Americans are carriers of the sickle trait. Therefore, pregnancies at risk of an offspring suffering from sickle cell anemia is 8% x 8%, which equals 0.64 or 3.75%. It is of interest to note that heterozygous individuals for Hb S have a high resistance to the malaria parasite, part of whose life cycle is spent in red blood cells. Historically, sickle cell anemia has provided a selective advantage in some regions of the world such as parts of Africa. This can also explain the reason for the high frequency of this homozygous gene amongst African Americans.

Hemoglobin is made up of two α chains and two β chains. The gene where the α is located is on the short arm of chromosome 16, while the β-globin gene cluster is on the short arm of chromosome 11. In addition to the adult form of Hb encoded within the β Hb cluster are the Hb forms that substitute for the adult β Hb during the various stages of development. Hemoglobin S (Hb S) is the variant form of the normal adult hemoglobin A (Hb A) in which an amino acid substitution occurs in the B polypeptide. The amino acid substitution is that of Valine (Val) in Hb S for the glutamic acid (Glu) normal Hb A hemoglobin (Figure 2). This significant finding was reported in 1957 by Vernon Ingram who was able to determine this structural change using peptide mapping analysis. These procedures are tedious and difficult. It should be noted that this predates recombinant DNA technology.

The single base mutation is an A to T in the triplet codon of the amino acid residue number 6 from the amino acid end in the beta chain. This
change introduces an amino acid with a polar (neutral) side chain valine instead of the acidic (negative) residue and changes the property of the hemoglobin molecule. This substitution changes the electrophoretic mobility of Hb S compared to Hb A. At slightly basic pH, such as 8.4, Hb S will be relatively more positive than Hb A and therefore will travel slower towards the positive (anode) electrode. This change in mobility is used as a diagnostic test of the presence of Hb S.

With the advent of biotechnology, parental or fetal DNA from cells obtained from amniocentesis can now be analyzed with a high degree of accuracy. DNA from a few cells can provide sufficient DNA to amplify using Polymerase Chain Reaction (PCR). Alternative methods can include growing cells in culture to yield sufficient DNA for analysis. The basis of the test is the recognition by restriction enzymes of specific palindromic sequences in DNA. In the normal β globin gene, the sequence of nucleotides that specifies amino acids 5, 6 and 7 (Pro-Glu-Glu) are CCT-GAG-GAG. The point mutation in codon 6 converting the A to T changing the sequence CCT-GTG-GAG. The palindrome recognition site of the restriction enzyme Mst II is CCTNAGG, where N can be any of the four nucleotides. Close examination of the sequence shows that Mst II will recognize the normal β globin CCT-GAG-G where N is a G, but not the mutated form.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Normal Hemoglobin</th>
<th>Sickle Cell Hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Valine</td>
<td>Valine</td>
</tr>
<tr>
<td>2</td>
<td>Histidine</td>
<td>Histidine</td>
</tr>
<tr>
<td>3</td>
<td>Leucin</td>
<td>Leucin</td>
</tr>
<tr>
<td>4</td>
<td>Threonin</td>
<td>Threonin</td>
</tr>
<tr>
<td>5</td>
<td>Glutamic acid</td>
<td>Proline</td>
</tr>
<tr>
<td>6</td>
<td>The only structural difference is one change in the sequence of each beta chain.</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>7</td>
<td>Glutamic acid</td>
<td>Valine</td>
</tr>
<tr>
<td>8</td>
<td>Lysine</td>
<td>Lysine</td>
</tr>
</tbody>
</table>

Figure 2
EXPERIMENT OBJECTIVE

In this experiment, you will learn an important application of biotechnology to biomedical diagnosis, as it is related to sickle cell anemia.

LABORATORY SAFETY

Gloves and safety goggles should be worn routinely as good laboratory practice.

Agarose Gel Preparation

Mutations in DNA can be inherited from one or both parents. Many germline genetic diseases are passed on from one generation to the next in Mendelian genetics. Thus if one parent is a carrier of a gene mutation that causes a genetic disease while the other does not, the offspring could be a carrier of the gene in one chromosome while the other chromosome will carry the normal gene. Such individuals could be carriers of the trait for the disease but usually do not manifest clinical traits.

In this experiment, you will separate DNA samples by electrophoresis. You will then analyze the results of simulated DNA from hypothetical parents and offspring.
Sample Delivery and Practice Gel Loading

An automatic micropipet is used to deliver accurate, reproducible volumes of sample. For gels to be stained with Methylene Blue Plustm or DNA Blue InstaStain™, load the sample well with 35 - 38 microliters of sample. Check with your instructor regarding the amount of sample you should be delivering.

With the EDVOTEK system, an alternative sample delivery method with disposable micropipets can be used. Transfer pipets are not precise, and because their volumes can not be accurately controlled, significant sample waste can occur. Delivery of small sample volumes with transfer pipets can be facilitated by gently squeezing the pipet stem, instead of the bulb.

PRACTICE GEL LOADING

If you are unfamiliar with loading samples in agarose gels, it is recommended that you practice sample delivery techniques before conducting the experiment. EDVOTEK electrophoresis experiments contain a tube of practice gel loading solution. Casting of a separate practice gel is highly recommended. One suggested activity is outlined below:

1. Cast a gel with the maximum number of wells and place it under buffer in an electrophoresis apparatus chamber.

   Alternatively, your teacher may have cut the gel in sections between the rows of wells. Place a gel section with wells into a small, shallow tray and submerge under water.

2. Practice delivering the practice solution to the sample wells. Take care not to damage or puncture the wells with the pipet tip.

3. If you need more practice, remove the practice gel loading solution by squirting buffer into the wells with a transfer pipet.

4. Replace the practice gel with a fresh gel for the actual experiment. The practice gel loading solution is diluted in the buffer and will not interfere with the experiment.
Conducting Agarose Gel Electrophoresis

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.

Have a waterbath or beaker of water warmed to 65°C for heating the tubes containing DNA fragments before gel loading. At 65°C, non-specific aggregation due to sticky ends generated by restriction enzyme digestions will melt. This will result in sharp individual DNA bands upon separation by agarose gel electrophoresis.

LOADING DNA SAMPLES

1. Heat the DNA samples A-F for two minutes at 65°C. Allow the samples to cool for a few minutes.

2. Load each sample in tubes A - F into the wells in consecutive order. The amount of sample that should be loaded is 35-38 µl.

RUNNING THE GEL

1. After the samples are loaded, carefully snap the cover down onto the electrode terminals.
   Make sure that the negative and positive indicators on the cover and apparatus chamber are properly oriented.

2. 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).

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

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

5. Allow the tracking dye to migrate 3.5 to 4 centimeters from the wells for adequate separation of the DNA bands.

Table C: Time and Voltage

<table>
<thead>
<tr>
<th>Volts</th>
<th>Recommended Time</th>
<th>Optimal</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>60 min</td>
<td>2.0 hrs</td>
</tr>
<tr>
<td>70</td>
<td>45 min</td>
<td>1.5 hrs</td>
</tr>
<tr>
<td>125</td>
<td>30 min</td>
<td>45 min</td>
</tr>
</tbody>
</table>

* The EDVOTEK Model #M8 should not be run at higher than 70 volts.
Conducting Agarose Gel
Electrophoresis, continued

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

7. Remove the gel on its bed. Place your hands on each end of the gel to prevent it from slipping off the bed.

8. Transfer the gel from the bed for DNA Visualization with DNA Blue InstaStain™ or Methylene Blue Plus™.
DNA BLUE INSTASTAIN™

EDVOTEK Series 100 electrophoresis experiments now feature a new proprietary staining method for staining DNA separated on agarose gels. Based on state-of-the-art technology, DNA Blue InstaStain™ is safe, quick, and minimizes the mess of conventional DNA staining with blue stains.

Staining with DNA Blue InstaStain™

1. After electrophoresis is completed, place the gel on a flat surface. Moisten the gel with several drops of electrophoresis buffer.

2. Wearing gloves, place the blue side of the DNA Blue InstaStain sheet on the well-moistened gel.

3. Firmly run your fingers over the entire surface of the DNA InstaStain. Do this several times.

4. Place the gel and DNA Blue InstaStain on a piece of plastic wrap. Then put the gel casting tray and a small empty beaker on top.

   *This will ensure that the InstaStain sheet maintains good contact with the gel surface.*

   Allow the DNA Blue InstaStain™ to sit for 15 minutes.
Destaining and Visualization of DNA

5. After 15 minutes, remove the sheet of DNA Blue InstaStain and transfer the gel to a large weigh boat or small plastic container.

6. Conduct destaining with distilled water that has been warmed to 37°C.
   - First destain: submerge the gel under a small amount of 37°C distilled water for 10 minutes with occasional agitation.
   - Second and third destain: submerge the gel under a small amount of 37°C distilled water for another 10 minutes with occasional agitation.

7. After the first destain, the larger DNA bands will be visible as dark blue bands against a lighter blue background. When completely destained, the dark blue DNA bands will become clearer and the entire background will become uniformly light blue in color.

8. Carefully remove the gel from the destain solution and examine the gel on a Visible Light Gel Visualization System. To optimize visibility, use the amber filter provided with EDVOTEK equipment.

9. If the gel is too light and bands are difficult to see, repeat the staining and destaining procedures.

Storage and Disposal of Gel

- A gel stained with DNA Blue InstaStain™ may be stored in the refrigerator for several weeks. Place the gel in a sealable plastic bag with destaining liquid.

   DO NOT FREEZE AGAROSE GELS.

- Stained gels which are not kept can be discarded in solid waste disposal.
TRADITIONAL LIQUID STAINING WITH METHYLENE BLUE PLUS™

1. Remove each gel from its bed and totally submerge the gel(s) in one tray containing 600 ml of diluted Methylene Blue Plus™ stain.
   Do not stain gel(s) in the electrophoresis apparatus.

2. Stain gel(s) for a minimum of 30 minutes, with occasional agitation.

3. Conduct destaining twice in 600 ml of distilled water that has been warmed to 37°C.
   • First destain: completely submerge the gel(s) in 600 ml of 37°C distilled water for 15 minutes with occasional agitation. Then discard the destaining solution.
   • Second and third destain: completely submerge the gel(s) in 600 ml of 37°C distilled water for another 15 minutes with occasional agitation.

Bands will start to become clearly visible after the second destain. You may also leave the gel(s) in destain overnight.

4. Carefully remove the gel from the destain solution and examine on a Visible Light Gel Visualization System. To optimize visibility, use the amber filter provided with EDVOTEK equipment.

5. If the gel is too light and bands are difficult to see, repeat the staining and destaining procedures.

Storage and Disposal of Gel

- Gels stained with Methylene Blue Plus™ may be stored in the refrigerator for several weeks. Place the gel in a sealable plastic bag with destaining liquid.

  DO NOT FREEZE AGAROSE GELS.

- Stained gels which are not kept can be discarded in solid waste disposal.
Study Questions

1. Describe the mechanism of the blood disease sickle cell anemia and how it affects its victims.

2. How many polypeptides are contained in hemoglobin?

3. What is the point mutation that causes sickle cell anemia? Where is it located?

4. Explain the methods for detecting sickle cell in patients?