In Search of the Sickle Cell Gene

EXPERIMENT OBJECTIVE:

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

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment Components</td>
<td>3</td>
</tr>
<tr>
<td>Experiment Requirements</td>
<td>4</td>
</tr>
<tr>
<td>Background Information</td>
<td>5</td>
</tr>
<tr>
<td>Experiment Procedures</td>
<td></td>
</tr>
<tr>
<td>Experiment Overview and General Instructions</td>
<td>11</td>
</tr>
<tr>
<td>Module One: Agarose Gel Electrophoresis</td>
<td>12</td>
</tr>
<tr>
<td>Module Two: Southern Blot Transfer</td>
<td>14</td>
</tr>
<tr>
<td>Study Questions</td>
<td>18</td>
</tr>
<tr>
<td>Instructor’s Guidelines</td>
<td></td>
</tr>
<tr>
<td>Notes to the Instructor</td>
<td>20</td>
</tr>
<tr>
<td>Pre-Lab Preparations</td>
<td>22</td>
</tr>
<tr>
<td>Experiment Results and Analysis</td>
<td>23</td>
</tr>
<tr>
<td>Study Questions and Answers</td>
<td>24</td>
</tr>
<tr>
<td>Appendices</td>
<td></td>
</tr>
<tr>
<td>A Agarose Gel Preparation For Southern Blot Analysis</td>
<td>26</td>
</tr>
<tr>
<td>B Quantity Preparations for Agarose Gel Electrophoresis</td>
<td>27</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>STORAGE</th>
<th>CHECK (✓)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Samples for Electrophoresis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Sickle cell gene sample</td>
<td>-20° C Freezer</td>
<td>[ ]</td>
</tr>
<tr>
<td>B Sickle cell trait (carrier) sample</td>
<td>-20° C Freezer</td>
<td>[ ]</td>
</tr>
<tr>
<td>C Normal gene sample</td>
<td>-20° C Freezer</td>
<td>[ ]</td>
</tr>
<tr>
<td>D Mother’s DNA sample</td>
<td>-20° C Freezer</td>
<td>[ ]</td>
</tr>
<tr>
<td>E Child’s DNA sample</td>
<td>-20° C Freezer</td>
<td>[ ]</td>
</tr>
<tr>
<td>F Father’s DNA sample</td>
<td>-20° C Freezer</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Components for Membrane Transfer</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>• 5 Pre-cut Southern Blot Nylon Membranes (7 x 7 cm)</td>
<td>Room Temp.</td>
<td>[ ]</td>
</tr>
<tr>
<td>• 5 Pre-cut Blotting Filter Paper (7 x 7 cm)</td>
<td>Room Temp.</td>
<td>[ ]</td>
</tr>
<tr>
<td>• Blue-Blot DNA Stain™ Solution (10x)</td>
<td>Room Temp.</td>
<td>[ ]</td>
</tr>
<tr>
<td>• NaCl</td>
<td>Room Temp.</td>
<td>[ ]</td>
</tr>
<tr>
<td>• NaOH</td>
<td>Room Temp.</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Reagents &amp; Supplies</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>• UltraSpec-Agarose™ powder</td>
<td>Room Temp.</td>
<td>[ ]</td>
</tr>
<tr>
<td>• Concentrated electrophoresis buffer</td>
<td>Room Temp.</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

Before use, check all volumes of Standard DNA fragments and DNA samples for electrophoresis. Evaporation may have caused samples to become more concentrated.

If needed, tap tubes or centrifuge, then add distilled water to slightly above the 1.0 cm level and mix.

Approximate Volume Measurements

- 0.5 cm tube: 120 µl
- 4.1 cm

Experiment #315 contains enough reagents for 5 groups.

All 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.

This experiment is a simulation. THIS EXPERIMENT DOES NOT CONTAIN HUMAN DNA. None of the experiment components are derived from human sources.
Requirements

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipets with tips
- Balance
- UV Transilluminator
- Waterbath (65° C)
- 80° C incubation oven
- Microcentrifuge (optional)
- Microwave, hot plate or burner
- Assorted glassware (beakers, flasks and graduated cylinders)
- 250 ml flasks or beakers
- Hot gloves or beaker tongs
- Safety goggles and disposable laboratory gloves
- Plastic wrap
- Paper towels
- Forceps
- Small plastic trays for soaking gels
  (clean, recycled lids from micropipet tips work well)
- Distilled or deionized water
- Concentrated HCl
The Sickle Cell Gene

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 BRCA 1 and II genes have been identified as markers with 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 homozygous with respect to that trait. If an individual has one dominant and one recessive, the individual is heterozygous for the particular trait.

Mendelian inheritance can be demonstrated with a 2 x 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 the mutation of a single nucleotide. This results in a
The Sickle Cell Gene

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 unoxygcnated form of hemoglobin 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.

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%. 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 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 adult hemoglobin A (Hb A) in which an amino acid substitution occurs in the β globin chain. 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 which ushered molecular medicine. These procedures are tedious and difficult. It should be noted that this predates Polymerase Chain Reaction (PCR) and DNA Sequencing.

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 of hemoglobin. This change introduces an amino acid with a polar (neutral) side chain valine instead of the acidic glutamic acid (negative) residue and changes the property of the hemoglobin molecule. This substitution also changes

Figure 2: Effect of the specific point mutation (A –> T) results in the altered amino acid code.
The Sickle Cell Gene

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, fetal DNA from cells can be obtained by amniocentesis and 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 converts 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. The restriction enzyme digests can then be analyzed by electrophoresis and Southern blotting using the appropriate probe for the β-globin gene. Alternatively, DNA Sequencing can be used to determine the conversion of A → T that is the basis of the sickle cell trait and sickle cell anemia.

Other blood diseases such as 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 on sequence of discovery or hematologic features.

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 Sickle Cell Gene

Figure 3: Polymerase Chain Reaction
The Sickle Cell Gene

The PCR reaction mixture (which contains the DNA polymerase, buffer, deoxy-nucleotides, primers, and template) is subjected to sequential heating/cooling cycles at three different temperatures (Figure 3).

- 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° C. 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.

ABOUT SOUTHERN BLOTS

Analysis of complex DNA is facilitated by Southern blot analysis. After electrophoresis, the gel is sequentially treated in HCl and NaOH. The HCl treatment introduces apurinic sites in DNA which makes phosphodiester bonds at these sites labile and introduces nicks in double-stranded DNA. These Apurinic sites result when the purine base is removed, as in adenine residue from the A=T base pair. The NaOH treatment disrupts the the interstrand hydrogen bonds between the base pairs. The sequential acid and base treatments therefore result in the formation of small fragments from large DNA fragments. This facilitates the transfer of DNA fragments onto the nylon membrane. This procedure causes the double stranded restriction fragments to be converted into single stranded form. A replica of the electrophoretic pattern of DNA fragments in the gel is made by transferring (blotting) to a membrane of treated nylon. This is done by placing the nylon membrane on the gel after electrophoresis and transferring the fragments to the membrane by capillary action or by electrotransfer. The DNA becomes permanently adsorbed to the membrane, which can be manipulated much more easily than the gel. At this point the DNA is not visible on the nylon membrane.

Analysis of the transferred DNA is often done by hybridization with a DNA probe. Currently, non-isotopic detection systems are employed to detect DNA bound to the membrane. These probes are chemically synthesized and can be easily labelled by fluorescent tags or by radioisotopes.
The Sickle Cell Gene

A solution containing the single-stranded probe is incubated with the membrane containing the transferred, single-stranded DNA fragments. Under the proper conditions, the probe will only base pair (hybridize) to those fragments containing the complementary repeated sequences. The membrane is then washed to remove excess probe and is exposed to a sheet of x-ray film. Only those DNA fragments that have hybridized to the probe will reveal their positions on the film because the localized areas of radioactivity cause exposure of the x-ray film. This process is known as autoradiography. The hybridized fragments appear as discrete bands (fingerprint) on the film and are in the same relative positions as they were in the agarose gel after electrophoresis. The reason that well-defined bands can be visualized is because only a small fraction of the hundreds of thousands of fragments present contain sequences complementary to the probe.

In this experiment, two parents will be tested to determine if they are carriers of the sickle cell trait. In this hypothetical case, the parents have concerns about the possibility that their child is a carrier of the sickle cell gene. They have decided to determine the hemoglobin status of their child. In this experiment, simulated DNA digests will be separated by gel electrophoresis, followed by a Southern Blot, and then analyzed.
**Experiment Overview and General Instructions**

**EXPERIMENT OBJECTIVE:**

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

**BRIEF DESCRIPTION OF THIS EXPERIMENT:**

Mutations in DNA can be inherited from one or both parents. Many germ-line 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, after which you will perform a Southern blot. You will then analyze the results of simulated DNA from hypothetical parents and offspring.

**LABORATORY SAFETY**

1. Wear gloves and goggles routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

**LABORATORY NOTEBOOK RECORDINGS:**

Record experimental results in your laboratory notebook or on a separate worksheet. Before starting the Experiment, write a hypothesis that reflects the experiment and predict experimental outcomes. During the Experiment, record (draw) your observations, or photograph the results. Following the Experiment, formulate an explanation from the results and determine what could be changed in the experiment if the experiment were repeated. Write a hypothesis that would reflect this change.
Module One: Agarose Gel Electrophoresis

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

AGAROSE GEL REQUIREMENTS FOR THIS EXPERIMENT

- Recommended gel size: 7 x 7 cm
- Number of sample wells required: 6
- Agarose gel concentration: 0.8%

PREPARING THE AGAROSE GEL

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

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

3. To a 250 ml flask or beaker, add agarose powder and buffer as indicated in the Reference Tables (Appendix A) provided by your instructor. Swirl the mixture to disperse clumps of agarose powder.

4. With a marking pen, indicate the level of the solution volume on the outside of the flask.

5. Heat the mixture using a microwave oven or burner to dissolve the agarose powder.

6. Cool the agarose solution to 60° C with careful swirling to promote even dissipation of heat. If detectable evaporation has occurred, add distilled water to bring the solution up to the original volume marked in step 4.

After the gel is cooled to 60° C:

7. Place the bed on a level surface and pour the cooled agarose solution into the bed.

8. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes.

9. After the gel is solidified, be careful not to damage or tear the wells while removing the rubber dams or tape and comb(s) from the gel bed.

10. Place the gel (on its bed) into the electrophoresis chamber, properly oriented, centered and level on the platform.

11. Fill the electrophoresis apparatus chamber with the appropriate amount of diluted (1x) electrophoresis buffer (refer to Table B on the instruction sheet from the Appendix provided by your instructor).
Module One: Agarose Gel Electrophoresis

LOADING THE SAMPLES

12. Make sure the gel is completely submerged under buffer before loading the samples and conducting electrophoresis. Load 18-20 µl of each DNA sample.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A Sickle cell gene sample</td>
</tr>
<tr>
<td>2</td>
<td>B Sickle cell trait (carrier) sample</td>
</tr>
<tr>
<td>3</td>
<td>C Normal gene sample</td>
</tr>
<tr>
<td>4</td>
<td>D Mother’s DNA sample</td>
</tr>
<tr>
<td>5</td>
<td>E Child’s DNA sample</td>
</tr>
<tr>
<td>6</td>
<td>F Father’s DNA sample</td>
</tr>
</tbody>
</table>

RUNNING THE GEL

13. After the DNA samples are loaded, properly orient the cover and carefully snap it onto the electrode terminals.

14. Insert the plugs of the black and red wires into the corresponding inputs of the power source.

15. Set the power source at the required voltage and conduct electrophoresis for the length of time determined by your instructor.

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

17. After the electrophoresis is completed, disconnect the power and remove the gel from the bed for Southern blot analysis.

Reminders:

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.

After connecting the apparatus to the D.C. power source, check that current is flowing properly - you should see bubbles forming on the two electrodes.

Electrophoresis can be completed in 15-20 minutes under optimal conditions. For Time and Voltage recommendations, refer to Table C (Appendix A).
Module Two: Southern Blot Analysis

Quick Reference:
The depurination procedure must be brief (no longer than 8 minutes). Prolonged exposure to HCl completely depurinates DNA strands. Subsequent treatment with a denaturation solution would fragment the depurinated DNA molecules into very short oligonucleotides, which are poor targets for probe-based detection.

During this procedure, the bromophenol blue tracking dye in the gel will change color. After 8 minutes, the dye will be greenish to slightly yellow in color.

OVERVIEW

In this module, you will transfer the DNA fragments from the agarose gel, to a nylon membrane. After the transfer, the membrane will be baked for a short time to fix the DNA to the membrane.

DEPURINATION AND DENATURATION
(Approximately 1 hour)

The HCl treatment introduces apurinic sites in DNA which makes phosphodiester bonds at these sites (obtained upon hydrolysis of purine bases) labile and introduces nicks in double-stranded DNA. Apurinic sites result when the purine base is removed, such as an adenine residue from the A/T base pair. The NaOH treatment disrupts the the interstrand hydrogen bonds between the base pairs. The sequential acid and base treatments result in the formation of small fragments from larger DNA fragments. This procedure causes the double stranded restriction fragments to be converted (melt) into single stranded form and that facilitates the transfer of DNA fragments onto the nylon membrane.

1. After electrophoresis, depurinate the agarose gel by placing it in a tray containing 200 ml of 0.25 M HCl. Leave at room temperature for 8 minutes. Make sure gel is immersed in the liquid.
   Stop depurination if the dye becomes completely yellow before 8 minutes.
2. Carefully discard the HCl solution; do not reuse the solution.
3. Rinse the agarose gel with several changes of 200 ml distilled water.
4. Soak the agarose gel for 15 minutes in 200 ml of DNA Denaturation Solution (0.5 M NaOH/0.6 M NaCl). Make sure gel is immersed in the liquid.
5. Periodically shake the tray to immerse the gel, which will float because of the density of the solution.
6. Discard the solution.
7. In a second 200 ml of DNA Denaturation Solution (0.5 M NaOH/0.6 M NaCl), continue soaking the agarose gel for 15 minutes. Do not discard the DNA Denaturation Solution. Save the solution to wet the nylon membrane in Step 12.
Module Two: Southern Blot Analysis

SETTING UP THE SOUTHERN BLOT TRANSFER
(Approximately 30 minutes)

After the second denaturation of the gel, set up the Southern Transfer:

8. Place a sheet of plastic wrap (such as Saran Wrap) on a flat level lab bench.

9. Remove the gel from the tray and place it (well side down) directly onto the plastic wrap. Inverting the gel places the smooth surface on top for contact with the membrane.

10. Wearing gloves and using forceps and scissors, trim the nylon membrane to the size of the gel.

11. Carefully pick up a membrane at the edges with clean forceps.

12. Slightly bend the membrane in the middle and slowly wet the membrane (from the middle out) in the DNA Denaturation Solution contained in the tray from step 9.

13. Release the membrane and gently submerge it for 5 minutes until it is thoroughly saturated with DNA Denaturation Solution.

14. Use forceps to remove the saturated membrane from the DNA Denaturation Solution and place it on top of the inverted agarose gel.

15. Trim the white blotting filter paper to the same size as the gel and the membrane.

16. Place the white filter paper on top of the membrane.

17. Roll a 5 or 10 ml pipet across the filter paper to remove air bubbles.

18. Carefully place a stack of paper towels approximately 4 - 5 cm thick on top of the filter paper.

19. Place an empty tray on top of the paper towels. Put a small object, such as an empty 400 ml beaker, inside the tray for weight.

20. Allow the blot transfer to progress 3-4 hours or overnight.

OPTIONAL STOPPING POINT
Module Two: Southern Blot Analysis

After incubating for 3-4 hours (or overnight):

21. Gently remove the tray, beaker, and all the paper towels.

22. Wearing rinsed gloves and using forceps, flip the stack (gel - nylon membrane - filter paper) over to lie on the filter paper.

23. Using a blue ink pen, draw through the six sample wells and trace their positions on the nylon membrane.

24. Using forceps, remove the gel from the membrane.

   Note the thickness and consistency of the now dehydrated gel. The gel can be discarded since all further processing takes place with the nylon membrane.

25. Lay the membrane on a dry paper towel with the DNA side up (the side which was in direct contact with the gel). The bromophenol blue tracking dye will be visible on the membrane.

26. Using a blue ink pen, label the DNA side of the membrane with your lab group number or initials.

27. For optimal results, completely dry and fix the DNA to the membrane:
   - place the membrane between two small sheets of filter paper
   - place into an 80°C oven for 30 minutes

OPTIONAL STOPPING POINT

The dried membrane can be stored at room temperature, away from moisture, and between two sheets of Whatman filter paper until you are ready to continue with the non-isotopic detection procedure.
Module Two: Southern Blot Analysis

NON-ISOTOPIC DETECTION OF DNA

During this procedure you will visualize the DNA on the membrane. Blue Blot DNA Stain™ is a non-isotopic reagent, developed by EDVOTEK for classroom use, that eliminates all the associated hazards of working with radioactive isotopes or chemicals used in non-isotopic labeling.

28. Place the membrane with the DNA side up in 60 ml of dilute BlueBlot™ solution. If necessary, use a ziploc bag to completely submerge the membrane.

29. Soak the membrane at room temperature for 10 to 15 minutes.

30. Remove the membrane with forceps and rinse in 200 ml of distilled water.

31. Replenish the distilled water once, or until the membrane is destained and DNA bands are clearly visible.
Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

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

2. If there are approximately 600,000 African American births each year in the United States, what would be the frequency of risk of sickle cell births? What number of these births would be carriers of the disease? How many babies would actually have the disease?

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

4. Briefly explain the DNA-based detection of sickle cell anemia in patients.
Instructor’s Guide

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances. If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

NATIONAL CONTENT AND SKILL STANDARDS

By performing this experiment, students will learn to load samples and run agarose gel electrophoresis. Analysis of the experiments will provide students the means to transform an abstract concept into a concrete explanation. Please visit our website for specific content and skill standards for various experiments.

EDUCATIONAL RESOURCES

The EDVOTEK web site provides several suggestions and reminders for conducting electrophoresis, as well as answers to frequently asked electrophoresis questions.
Notes to the Instructor:

MICROPIPETTING BASICS AND PRACTICE GEL LOADING

Accurate pipetting is critical for maximizing successful experiment results. EDVOTEK Series 300 experiments are designed for students who have had previous experience with agarose gel electrophoresis and micropipeting techniques. If your students are unfamiliar with using micropipets, EDVOTEK highly recommends that students perform Experiment # S-44, Micropipetting Basics, or other Series 100 or 200 electrophoresis experiment prior to conducting this advanced level experiment.

APPROXIMATE TIME REQUIREMENTS

- **Pre-lab preparations**
  Pre-lab preparations and dispensing of biologicals and reagents take approximately 1-2 hours.

- **Agarose Gel preparation**
  Whether you choose to prepare the gel(s) in advance or have the students prepare their own, allow approximately 30-40 minutes for this procedure. Generally, 20 minutes of this time is required for gel solidification. See section “Options for Preparing Agarose Gels” below.

- **Conducting Electrophoresis**
  The approximate time for electrophoresis will vary from 15 minutes to 2 hours. Generally, the higher the voltage applied, the faster the samples migrate. However, depending upon the apparatus configuration and the distance between the two electrodes, individual electrophoresis units will separate DNA at different rates. Follow manufacturer’s recommendations. Time and Voltage recommendations for EDVOTEK equipment are outlined in Table C.

About UltraSpec-Agarose™

DNA electrophoresis experiments from EDVOTEK feature UltraSpec-Agarose™, which is particularly well-suited for separating DNA molecules in the range of 500 to 30,000 base pairs. Gels cast with UltraSpec-Agarose™ are sturdier and more resistant to breakage than conventional agarose. Its enhanced resolving power and translucent quality results in greater visual clarity and definition of separated DNA fragments after staining.
OPTIONS FOR PREPARING AGAROSE GELS

This experiment is designed for DNA staining after electrophoresis with InstaStain® Ethidium Bromide. There are several options for preparing agarose gels for the experiment.

1. Individual Gel Casting:
   Each student lab group can be responsible for casting their own individual gel prior to conducting the experiment.

2. Preparing Gels in Advance:
   Gels may be prepared ahead and stored for later use. Solidified gels can be stored under buffer in the refrigerator for up to 2 weeks.

   Do not store gels at -20° C. Freezing will destroy the gels.

   Gels that have been removed from their trays for storage, should be "anchored" back to the tray with a few drops of hot, molten agarose before placing the gels into the apparatus for electrophoresis. This will prevent the gels from sliding around in the trays and the chambers.

3. Batch Gel Preparation:
   A batch of agarose gel can be prepared for sharing by the class. To save time, a larger quantity of UltraSpec-Agarose can be prepared for sharing by the class. See instructions for "Batch Gel Preparation".

GEL CONCENTRATION AND VOLUME

The agarose gel concentration required for this experiment is 0.8% weight by volume.
Pre-Lab Preparations

GENERAL PREPARATIONS

1. For each gel to be processed for Southern blotting, gather the following items:
   - 1 piece of pre-cut nylon membrane (7 x 7 cm)
   - 1 piece of pre-cut blotting filter paper (7 x 7 cm)
   - 20 paper towels (should be large enough to cover the gel)

2. On day two of the Southern Blot transfer, warm an 80° C oven.

PREPARATION OF SOLUTIONS FOR THE SOUTHERN BLOT TRANSFER (FOR 5 BLOTS)

1. Prepare 1.0 liter of approximately 0.25 N HCl. Mix together:
   - 21 ml Concentrated HCl (12 N)
   - 979 ml Distilled/deionized water

2. Prepare 2.0 liters of the alkaline/salt DNA denaturation solution, 0.5 M NaOH/0.6 M NaCl.
   - 1.8 L Distilled or deionized water
   - 40.0 g NaOH pellets
   - 70.0 g NaCl

   Add NaOH and NaCl to the water. Use a magnetic stir plate to dissolve. Add distilled water to a final volume of 2.0 liters.

PREPARATION OF BLUE-BLOT™ DNA STAINING SOLUTION

On the day the blots are to be stained for visualization, prepare Blue-Blot™ DNA stain by mixing the following:

   - 30 ml BlueBlot™ Stain (10x concentrate)
   - 270 ml Distilled or deionized water
Experiment Results and Analysis

Actual results will yield broader bands of varying intensities. The idealized schematic shows the relative positions of the bands, but the results are not depicted to scale.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A Sickle cell gene sample</td>
</tr>
<tr>
<td>2</td>
<td>B Sickle cell trait (carrier) sample</td>
</tr>
<tr>
<td>3</td>
<td>C Normal gene sample</td>
</tr>
<tr>
<td>4</td>
<td>D Mother’s DNA sample</td>
</tr>
<tr>
<td>5</td>
<td>E Child’s DNA sample</td>
</tr>
<tr>
<td>6</td>
<td>F Father’s DNA sample</td>
</tr>
</tbody>
</table>

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 1997-2014 EDVOTEK, Inc., all rights reserved. 315.140702
Please refer to the kit insert for the Answers to Study Questions
Appendices

A Agarose Gel Preparation For Southern Blot Analysis

B Quantity Preparations for Agarose Gel Electrophoresis

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

0.8% Agarose Gel Electrophoresis Reference Tables for Southern Blot Analysis

If preparing a 0.8% gel with concentrated (50x) buffer, use Table A.1

If preparing a 0.8% gel with diluted (1x) buffer, use Table A.2

Table A.1

<table>
<thead>
<tr>
<th>Size of Gel (cm)</th>
<th>Amt of Agarose (g)</th>
<th>Concentrated Buffer (50x) (ml)</th>
<th>Distilled Water (ml)</th>
<th>Total Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 x 7</td>
<td>0.23</td>
<td>0.6</td>
<td>29.4</td>
<td>30</td>
</tr>
<tr>
<td>7 x 14</td>
<td>0.46</td>
<td>1.2</td>
<td>58.8</td>
<td>60</td>
</tr>
</tbody>
</table>

* 0.77 UltraSpec-Agarose™ gel percentage rounded up to 0.8%

For DNA analysis, the recommended electrophoresis buffer is Tris-acetate-EDTA, pH 7.8. The formula for diluting EDVOTEK (50x) concentrated buffer is one volume of buffer concentrate to every 49 volumes of distilled or deionized water. Prepare buffer as required for your electrophoresis unit.

Table B

<table>
<thead>
<tr>
<th>EDVOTEK Model #</th>
<th>Total Volume Required (ml)</th>
<th>Dilution 50x Conc. Buffer (ml)</th>
<th>Distilled Water (ml)</th>
<th>Total Volume Required (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6+</td>
<td>300</td>
<td>6</td>
<td>294</td>
<td>300</td>
</tr>
<tr>
<td>M12</td>
<td>400</td>
<td>8</td>
<td>392</td>
<td>400</td>
</tr>
<tr>
<td>M36</td>
<td>1000</td>
<td>20</td>
<td>980</td>
<td>1000</td>
</tr>
</tbody>
</table>

Table C

<table>
<thead>
<tr>
<th>EDVOTEK Electrophoresis Model</th>
<th>M6+</th>
<th>M12 &amp; M36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volts</td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>150</td>
<td>10 / 15 minutes</td>
<td>20 / 30 minutes</td>
</tr>
<tr>
<td>125</td>
<td>15 / 20 minutes</td>
<td>35 / 45 minutes</td>
</tr>
<tr>
<td>70</td>
<td>35 / 45 minutes</td>
<td>60 / 90 minutes</td>
</tr>
<tr>
<td>50</td>
<td>50 / 80 minutes</td>
<td>95 / 130 minutes</td>
</tr>
</tbody>
</table>

Time and Voltage recommendations for EDVOTEK equipment are outlined in Table C.1 for 0.8% agarose gels. The time for electrophoresis will vary from approximately 15 minutes to 2 hours depending upon various factors. Conduct the electrophoresis for the length of time determined by your instructor.
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.

1. Use a 500 ml flask to prepare the diluted gel buffer
2. Pour 3.0 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. Dispense the required volume of cooled agarose solution for casting each gel. The volume required is dependent upon the size of the gel bed and DNA staining method which will be used. Refer to Appendix A or B for guidelines.
7. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Then proceed with preparing the gel for electrophoresis.

### Table D

**Bulk Preparation of Electrophoresis Buffer**

<table>
<thead>
<tr>
<th>Concentrated Buffer (50x) (ml)</th>
<th>Distilled Water (ml)</th>
<th>Total Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>2,940</td>
<td>3,000 (3 L)</td>
</tr>
</tbody>
</table>

### Table E.1

**Batch Preparation of 0.8% UltraSpec-Agarose™**

<table>
<thead>
<tr>
<th>Amt of Agarose (g)</th>
<th>50x Conc. Buffer (ml)</th>
<th>Distilled Water (ml)</th>
<th>Diluted Buffer (1x) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 g</td>
<td>7.5 ml</td>
<td>382.5 ml</td>
<td>390 ml</td>
</tr>
</tbody>
</table>

Note: The UltraSpec-Agarose™ kit component is often labeled with the amount it contains. In many cases, the entire contents of the bottle is 3.0 grams. Please read the label carefully. If the amount of agarose is not specified or if the bottle’s plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.