# LABORATORY 4: SICKLE CELL ALLELES AND GEL ELECTROPHORESIS

## Objectives

A central goal of research in modern genetics is to identify the specific mutation(s) in DNA that are responsible for genetic diseases. Once the specific mutation is known, it becomes possible to perform a genetic test on a person's DNA to discover if they have the genetic disorder or are a carrier of a mutation that can be passed to the next generation. This laboratory project will focus on one example of this procedure – a genetic test for **sickle cell anemia**. This is a debilitating disease in which some of the patient's red blood cells distort into a sickle shape, reducing their ability to transport oxygen and causing a range of circulatory problems. The genetic test will employ a technique called **DNA gel electrophoresis**, in which DNA fragments are separated in a gel under the influence of an applied voltage. This technique has widespread use in many studies of DNA and genetic disorders.

This laboratory project has two parts:

**Part 1**: Performing a gel electrophoresis experiment.

**Part 2**: Interpreting the results of the genetic test.

## Preparation and Quiz

As preparation for this laboratory project and quiz you should carefully read the introduction, which describes both the molecular basis of sickle cell anemia and the technique of DNA gel electrophoresis. You should be familiar with the following concepts and terms:

- Hemoglobin and its subunits (α-globin and β-globin)
- Amino acid and DNA mutations in sickle hemoglobin (HbS)
- The evolutionary origin of the sickle cell mutation
- The inheritance of sickle cell anemia
- Restriction enzymes
- DNA gel electrophoresis
- Staining and visualizing DNA fragments in the gel

# INTRODUCTION

## Sickle Cell Anemia as a Molecular Disease

In the early twentieth century, physicians began to study a physiological disorder that caused patients to suffer from fatigue, aching joints, and heart problems. When blood from these patients was observed using the microscope, scientists saw that many of their red blood cells were distorted into a crescent shape. Because of these unusual cells, the disease was called **sickle cell anemia** (or sometimes simply sickle cell disease). These misshapen cells had a reduced ability to transport oxygen, producing severe anemia in the patient, and caused problems with circulation because of their tendency to aggregate in the capillaries. Several decades later, shortly after the end of World War II, scientists discovered that the unusual red blood cells were caused by a mutation in a protein named **hemoglobin**. This protein acts as the molecular transporter of oxygen from the lungs to the various tissues of the body, where the oxygen is then released.

Hemoglobin is a large protein consisting of four subunits, each of which contains a **heme** group that binds an oxygen molecule. There are two different types of subunits – called **α chains** and **β- chains** – which have small differences in the number and type of amino acids they contain. The complete hemoglobin protein contains two α-chains and two β-chains (subunits), often abbreviated as $\alpha_2\beta_2$. Each subunit type is produced by a different gene, with the gene for α-chain located on chromosome 16 and the gene for β-chain located on chromosome 11. The hemoglobin protein and its subunits are illustrated in *Figure 1* below.

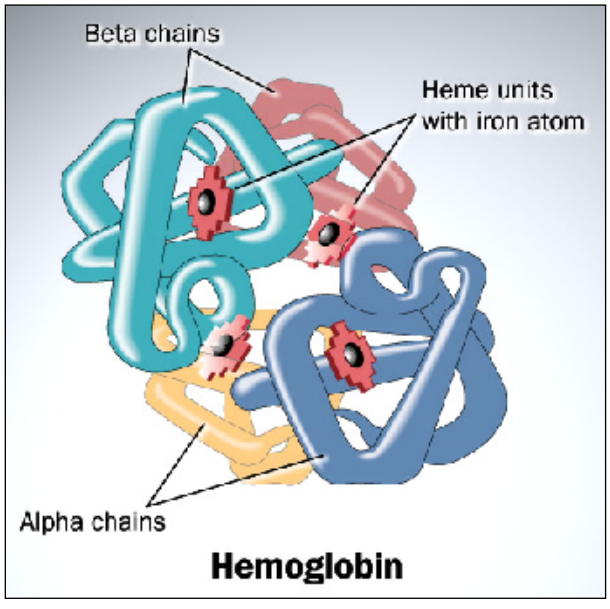



**Figure 1:** Hemoglobin and its four subunits.

The sequence of bases in the DNA of a gene determines the sequence of amino acids in its associated protein. A set of three DNA bases – called a **codon** – is required to specify one amino acid. After careful study of the mutated hemoglobin in sickle cell anemia patients, scientists discovered that the important difference was the change of one amino acid within the β-globin subunit. In the normal form of adult hemoglobin, called hemoglobin A (HbA), position 6 in the amino acid sequence of the β -globin chain is occupied by **glutamic acid**. This amino acid is specified by the 6$^{th}$ codon in the β-globin gene, which is the triplet of bases GAG. Patients with sickle cell anemia have the mutated form of hemoglobin (HbS), which has **valine** instead of glutamic acid at position 6 in the β-globin. The amino acid change is produced by the mutation of a single A → T base mutation in the β-globin, such that the 6$^{th}$ codon now reads GTG. A comparison of the amino acids and DNA bases in HbA compared to HbS is given in *Figure 2.*

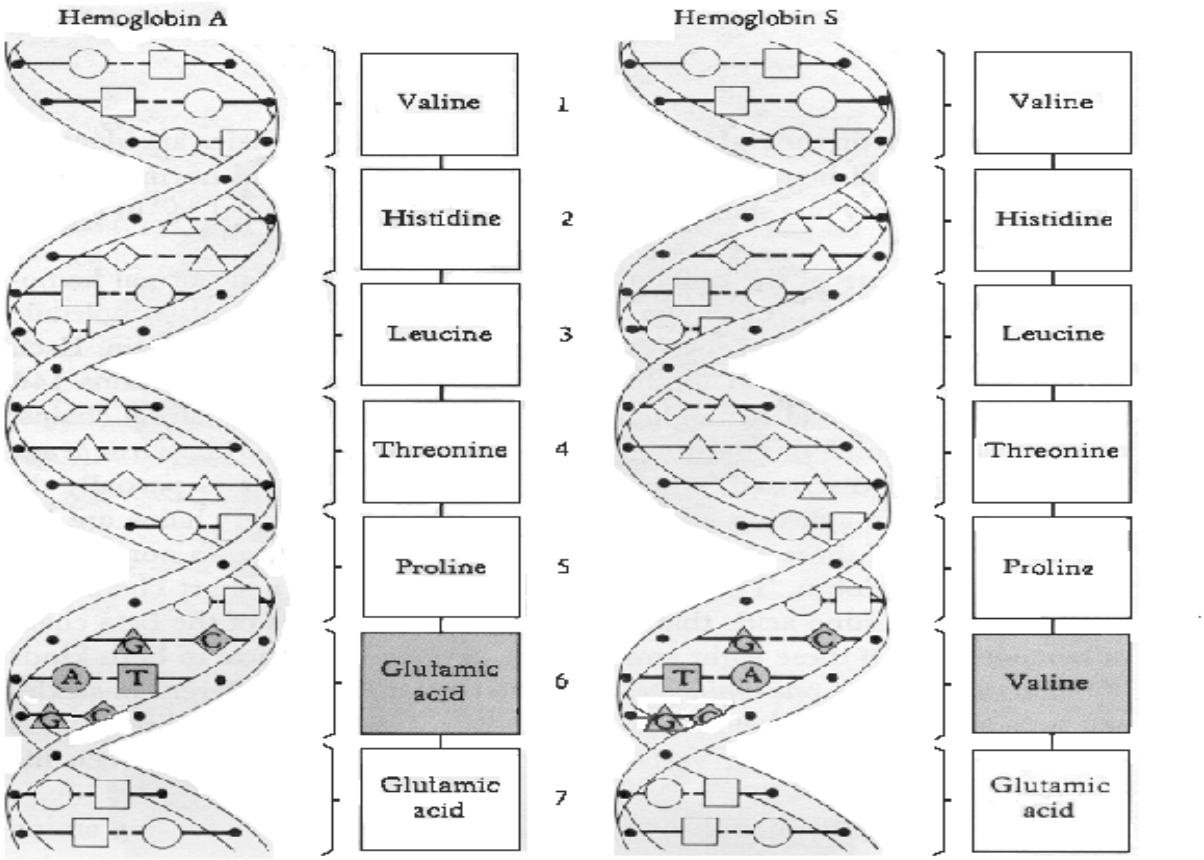



**Figure 2:** Comparison of DNA and amino acid sequences for Hemoglobin A and Hemoglobin S. The single amino acid substitution of valine for glutamic acid occurs at position 6.

Since the β-globin subunit has a total length of 146 amino acids, a single change may not seem very important. However, glutamic acid and valine have different chemical compositions that affect their solubility in water. The sidechain of glutamic acid is polar and dissolves easily in water, whereas the sidechain of valine is oily and is not soluble in water. To avoid exposing the valine sidechain to the aqueous environment of the cell, binding occurs between hemoglobin

proteins in their **unoxygenated form** to produce long fibers. These fibers distort the cell into its characteristic sickle shape which reduces the ability of hemoglobin to bind oxygen in the red blood cells.

In the United States, sickle cell anemia is of special interest since it is estimated that 8% of African Americans are unaffected carriers (heterozygotes) of the sickle cell mutation. In some regions of Central Africa, the frequency of carriers can rise as high as 10%. Furthermore, the mutated allele has also been found in populations ranging from southern Greece to northern India. Scientists began to ask themselves why this genetic mutation has remained so common and so widespread in the world's population. The clue to this puzzle arose from the observation that the highest incidence of sickle cell anemia occurs in regions of the world where malaria is also very prevalent. The parasite that causes malaria spends part of its life cycle in red blood cells and has greater difficulty entering sickled cells compared to those that are normal. Having one copy of the mutated allele generates enough sickled cells in the blood to provide preferential resistance against malaria infection. However, the number of sickle cells remains low enough to avoid any circulatory problems associated with sickle cell anemia. Having one normal allele and one mutated allele therefore gives individuals protection against malaria without causing them to exhibit any negative effects from the genetic disease. In evolutionary terms, this type of genotype is known as the **heterozygote advantage**. The mutated allele persists in the population because, in some circumstances, it has a beneficial effect.

# A Genetic Test for Sickle Cell Anemia

## Inheriting the Sickle Cell Mutation

Equipped with knowledge of the specific DNA mutation that causes sickle cell anemia, scientists have been able to create a genetic test for the disease. Consider the scenario of an African-American couple who are expecting a child and are seeking genetic counseling about the possibility of their future son or daughter being born with sickle cell anemia. This disorder exhibits **autosomal recessive inheritance**, requiring the child to inherit two copies of the recessive allele to show symptoms of the disease. The inheritance characteristics can be most easily considered using a Punnett square like the one presented in *Figure 3*. HbA represents the normal (dominant) allele for hemoglobin and HbS represents the mutated (recessive) allele. As shown in the figure, a child with sickle cell anemia (genotype HbS HbS) can be born to parents who show no symptoms of the disorder but are both carriers (genotype HbA HbS). The probability of the child inheriting both mutated alleles from two carrier parents is one in four, or 25%.

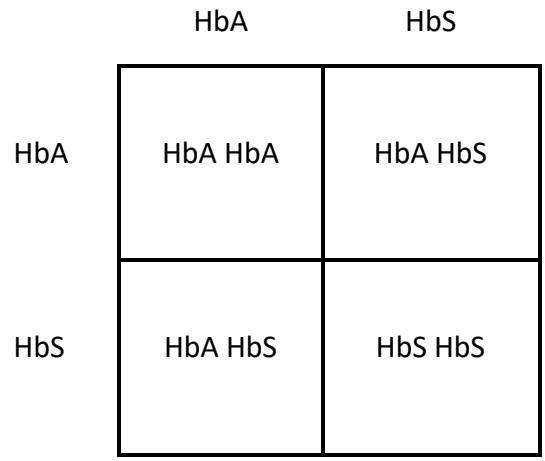



**Figure 3:** A Punnett Square showing the possible genotypes of a child born to parents who are both heterozygote carriers of the mutated gene (HbA HbS). The child will develop sickle cell anemia only if he or she inherits two copies of the mutated recessive allele (HbS HbS).

The first step in genetic testing for sickle cell anemia is to therefore determine the genotype of the parents, since an affected child can be born only if both parents have at least one copy of the mutated HbS allele. If both parents are determined to be heterozygote carriers, we can say that the couple has a 1-in-4 chance of having a child with the disease. The element of uncertainty can be removed, however, by a direct genetic test on the developing fetus. Cells from the fetus can be obtained by **amniocentesis,** where a long needle is used to penetrate the abdomen and withdraw fluid from the amniotic sac surrounding the fetus. These cells can be cultured to grow them in sufficient number in order to extract the DNA for analysis. Alternatively, a more recent technique called the **polymerase chain reaction (PCR)** can generate millions of copies of a specific region of DNA without requiring cell cultures.

## Restriction Enzymes

Genetic tests aim to provide information about the presence of mutations that are linked to disease. We have learned that the mutation in the HbS protein arises from a single base mutation within the 3 billion bases that constitute the human genome. How is it possible to detect such a small change as a single base mutation? In theory, we could determine the complete sequence of bases in a person's gene for β-globin, but that process would be difficult, expensive, and time-consuming.

Scientists developed an indirect method of testing for mutations that uses one of the central tools of genetic engineering – **restriction enzymes**. These enzymes are obtained from bacteria or viruses and normally serve to protect these organisms against foreign invaders. They function by recognizing specific sequences of DNA and cutting the DNA strand at this region.

Different restriction enzymes recognize different regions of DNA. *Table 1* lists a selection of enzymes together with their recognition sequences (written, according to convention, in the 5' → 3' direction).

**Table 1**: Restriction Enzymes and their DNA Recognition Sequences.

| Restriction Enzyme | DNA recognition sequence |
|---|---|
| *HaeIII* | 5' – G G C C – 3' |
| *EcoRI* | 5' – G A A T T C – 3' |
| *HindIII* | 5' – A A G C T T – 3' |
| *MstII* | 5' – C C T N A G G – 3' |

The enzyme names in *Table 1* are based on a specific convention. The first three letters refer to the organism from which the enzyme was isolated, a letter refers to the strain of the organism, and a Roman numeral indicates one out of several different restriction enzymes that have been isolated from the same organism. For example, the restriction enzyme "*Eco*RI" comes from *E. Coli*, strain RY13, and was the first enzyme to be obtained from this organism. Note also that some enzymes have a short DNA recognition sequence (e.g., *HaeIII*), some enzymes recognize longer DNA sequences (e.g., *EcoRI* and *HindIII*), and still others can tolerate any base somewhere in their recognition sequence (e.g., *MstII*, where "N" means any base).

How does the use of restriction enzymes help us to design a genetic test? Consider, for example, the restriction enzyme called *HaeIII* (from the influenza virus) that recognizes and cuts the DNA sequence 5' – G G C C – 3'. Suppose we add this enzyme to a tube with a DNA sample that contains this particular sequence of bases once somewhere along its length. The enzyme will recognize and cut the sequence to give two pieces of DNA, as illustrated in *Figure 4.*

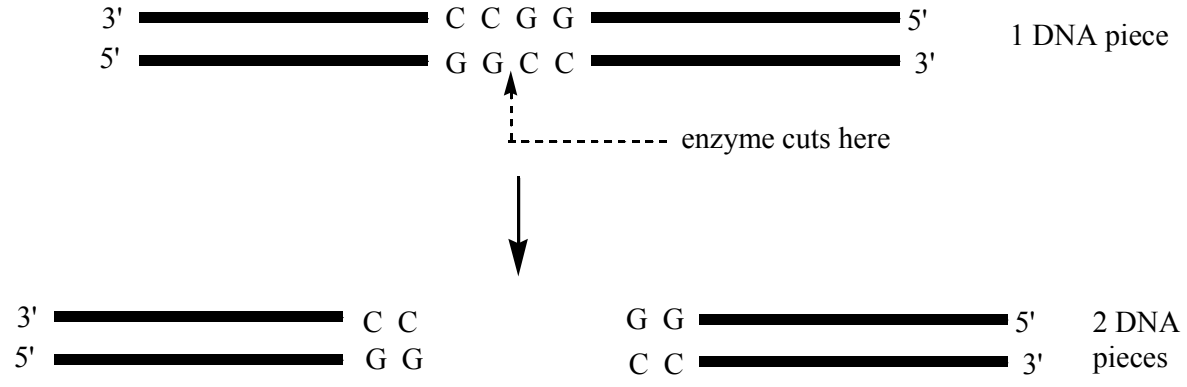



**Figure 4:** The HaeIII restriction enzyme will recognize and cut the sequence 5' – G G C C – 3' in a region of DNA.

Suppose a mutation occurs in the sequence that the restriction enzyme usually recognizes – i.e., the sequence mutates from 5' – G G **C** C – 3' to 5' – G G **G** C – 3'. The sequence that the restriction enzyme recognizes is no longer present due to the mutation, so *the enzyme will not cut the mutated DNA strand.* The DNA region is preserved, as seen *Figure 5*.

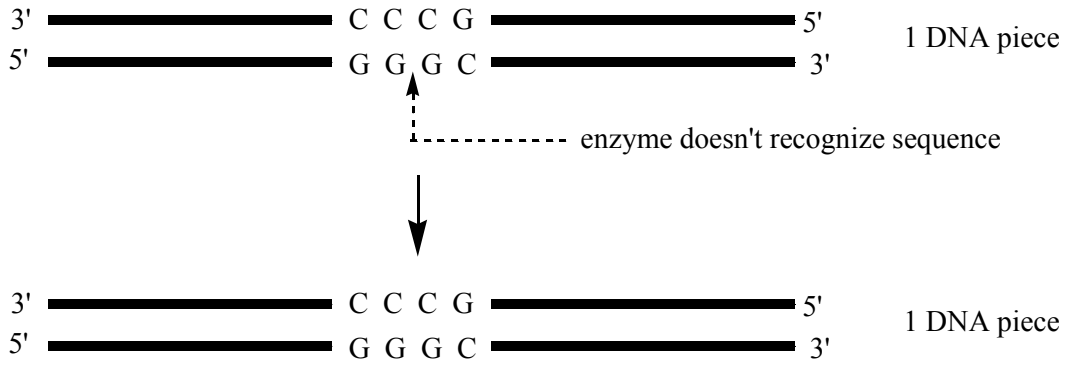
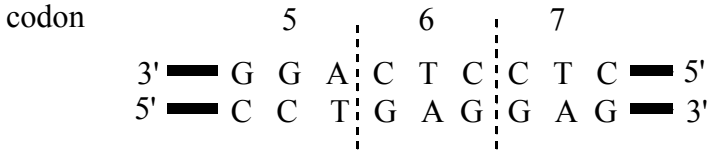
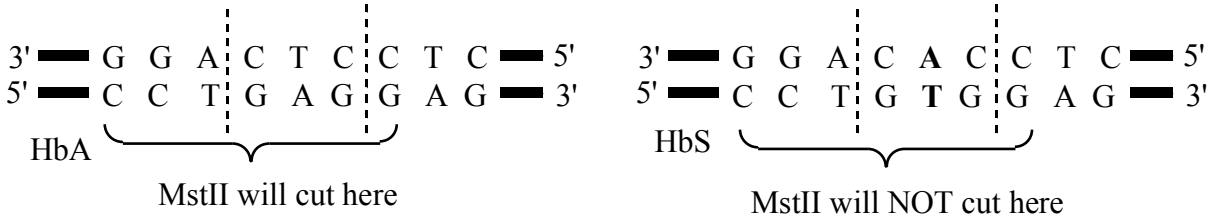



**Figure 5:** If a C → G mutation occurs in the recognition sequence for the HaeIII enzyme, the enzyme will not cut this region of DNA.

This use of restriction enzymes gives us an *indirect* way of testing for genetic mutations. In devising a genetic test for sickle cell anemia, we need to select a restriction enzyme that can distinguish between the normal β-globin gene (called HbA) and the mutated sickle-cell gene (called HbS). The two DNA strands for codons 5, 6 and 7 in the β-globin gene are shown below.




In *Table 1* there is a restriction enzyme called *MstII* that recognizes and cuts DNA when its sequence is 5' – C C T N A G G – 3'. The letter "N" is used to mean that the enzyme accepts "any" base (A, T, G, or C). This recognition sequence is found in the region of the β-globin gene (HbA) that extends over the 5th, 6th, and part of the 7th codons. But in HbS, the middle base of the 6th codon mutates from T → A (in the upper DNA strand) and A → T in the lower DNA strand. This changes the DNA sequence that is usually recognized by the *MstII* restriction enzyme; it now reads 5' – C C T N **T** G G – 3'. Consequently, the enzyme will no longer recognize and cut the DNA sequence in this region.




So now we have the basis of a genetic test for sickle cell anemia:

- If the gene is normal (HbA), then the DNA will be cut by the *MstII* restriction enzyme to give two pieces.
- If the gene is mutated (HbS), the DNA will NOT be cut by the MstII restriction enzyme and the DNA will remain as one long piece.

## DNA Gel Electrophoresis

After the restriction enzymes have digested the DNA into fragments, we need a way to separate the DNA fragments and identify them according to their size. This is accomplished using a technique called **gel electrophoresis**. The gel is made of **agarose**, which polymerizes to form a solid matrix with microscopic pores. The agarose gel is placed in an electrophoresis chamber containing a solution of **buffer** that uses a specific mixture of ions to maintain a constant pH.

The samples are loaded, using a pipette, into wells at the top of the gel. The DNA is then induced to move by applying a **voltage** so that an electric current passes through the electrophoresis chamber. Since the chemical structure of DNA contains **phosphate groups**, the DNA fragments possess a negative charge when placed in solution near neutral pH (this is the reason for inclusion of the word "acid" in the name deoxyribonucleic acid). Since the DNA is negatively charged, it will migrate towards the positive electrode. The arrangement of a gel electrophoresis experiment is shown in *Figure 6* on the following page.

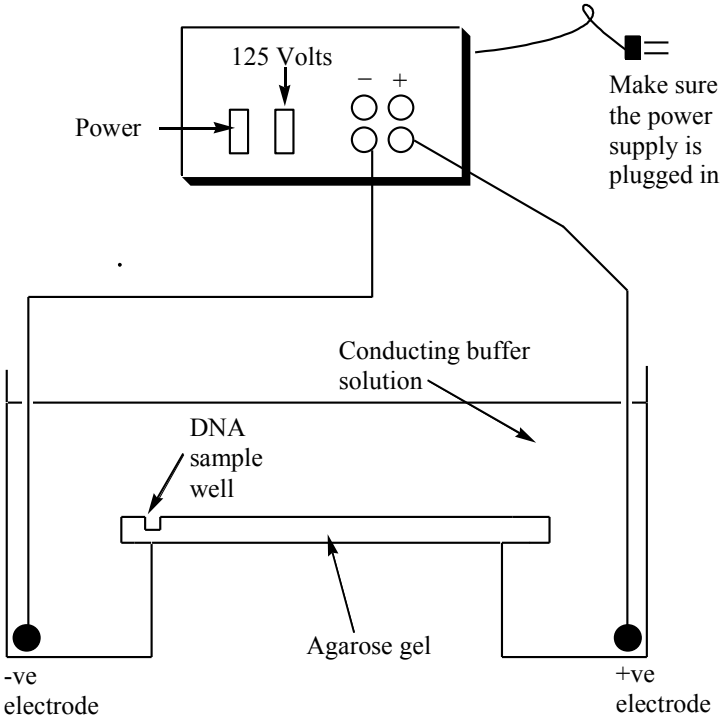



**Figure 6.** Equipment for performing a DNA gel electrophoresis experiment.

When the DNA fragments move through the gel they must maneuver through the microscopic pores within the polymerized agarose. Smaller fragments are able to move through the pores more easily than larger fragments and therefore migrate through the gel at a faster rate. After a fixed amount of time, the smaller fragments will have traveled further through the gel towards the positive electrode. Gel electrophoresis therefore enables us to separate DNA fragments according to their size.

## Visualizing the DNA Samples

Since small pieces of DNA cannot be seen directly, we need a technique to allow us to visualize the final position of the DNA fragments in the gel. After the electrophoresis is completed, the agarose gels are transferred to a plastic bag and then stained with a fluorescent dye. After the dye has bound to the DNA fragments, it is illuminated with ultraviolet light using a device called a **transilluminator**. The UV light emitted from the transilluminator induces the dye to fluoresce. The DNA fragments are revealed as white bands where the fluorescent light from the dye has been recorded on the film. Since more dye binds to larger DNA fragments than to smaller fragments, the large fragments are easily observable while the smaller fragments often appear quite faint.

# EXPERIMENTAL PROCEDURE

## PART 1: GEL ELECTROPHORESIS

### Practice Gels

The success of the gel electrophoresis experiment relies on your ability to accurately dispense the entire DNA sample into the wells of the gel as in the figure below. In order to give you practice with pipetting, we have provided **practice gels** and **loading dye**. **(Note: The practice gels are far thicker and firmer than the actual gels loaded into the bed.)**

1. You will be using a pipette with an adjustable volume (see **Figure 8**). Set the pipette to a volume of **15 µl**. **Add a plastic tip to the end of the pipette for collecting the sample.**

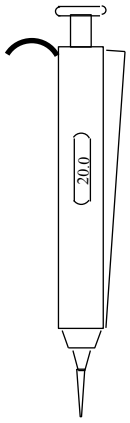



**Figure 8**: An adjustable pipette used for small volumes (e.g., 15 µl).

2. Practice pipetting the loading dye sample until you can routinely obtain a **dark blue** solution in the wells of the gel as seen in **Figure 9**.

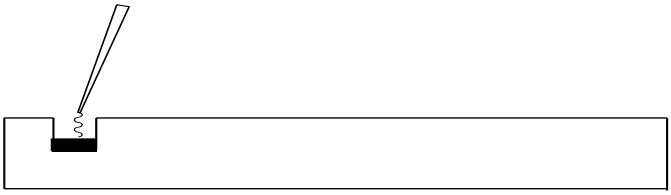


**Figure 9.** Dispensing the samples into the practice gel.

**SHOW YOUR PRACTICE GEL TO YOUR LABORATORY INSTRUCTOR BEFORE PROCEEDING TO LOAD YOUR ACTUAL DNA SAMPLES.**

## Loading the DNA Samples

1. Before loading the samples, check that the wells in your gel are closer to the **negative** (black) terminal of the electrophoresis chamber. Since the phosphate groups in DNA give it a negative charge when placed in solution, the DNA fragments will migrate towards the positive (red) terminal under the influence of an electric field.

2. You are provided with a strip of wells containing six DNA samples. The wells containing samples are labeled A through F. These samples contain 25 µl of liquid, which is a **very small volume**. The sample will be at the bottom of the well as shown in Figure 10. If any liquid is sticking to the side of the well, tap the strip on the table to collect the entire sample at the bottom.


**Figure 10:** Strip containing DNA samples

3. Use the pipette to load the 6 samples from **left-to-right** into the wells of the gel according to Figure 11. **You must be very accurate with your pipetting and use a <u>new plastic tip</u> for each sample!! Make sure your pipette is set to <u>15 µl</u>.**

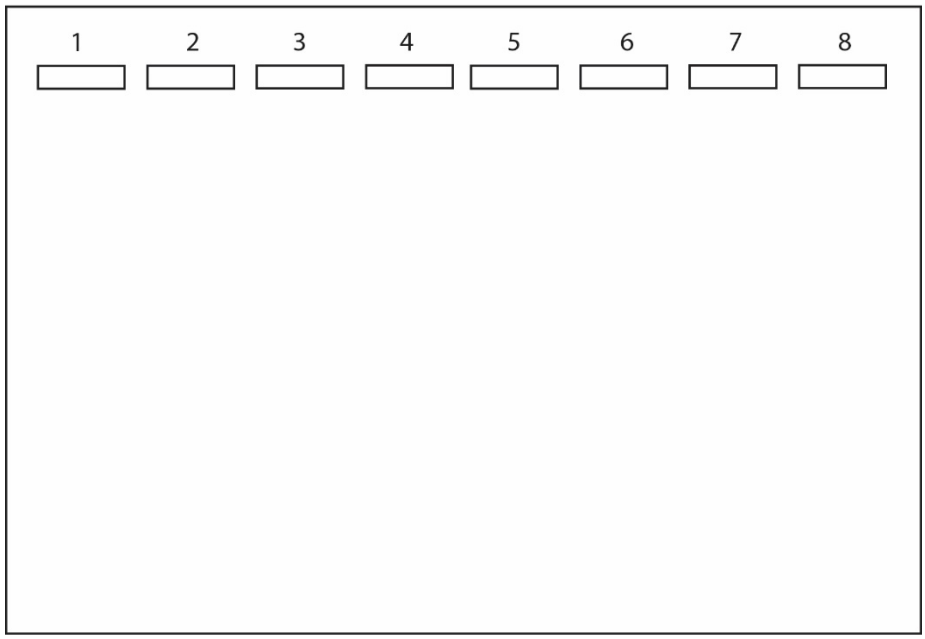



**Figure 11.** The gel will look like Figure 11, with wells 1-8 across the top.

| Lane | Sample |
|---|---|
| 1 | Sickle cell gene sample |
| 2 | DNA sample from a sickle cell carrier |
| 3 | Normal gene sample |
| 4 | Mother's DNA sample |
| 5 | Child's DNA sample |
| 6 | Father's DNA sample |

**(Do not load any samples into lanes 7 and 8.)**

You will notice that the samples in the tube have a blue color. This color arises from the loading dye that is mixed with the actual fragments of DNA. One reason for adding the dye is to make it easier to see the samples when you load them into the wells of the gel. The other reason is that the loading dye has a negative charge like the DNA samples, so it also migrates towards the positive electrode. The dye moves at the same rate as very small DNA fragments. By following the progression of the dye through the gel during the electrophoresis experiment we can monitor the location of the smallest DNA fragments in the gel.

## Running the Gel

1. Check your gel to make sure that it is properly positioned in the gel bed in the center of the electrophoresis chamber. If it has slipped during the pipetting procedure, **SLOWLY AND CAREFULLY** use the tip of the pipette to nudge it back into position. You must perform this maneuver delicately to avoid losing the DNA sample from your wells.
2. Carefully place the lid on the top of the electrophoresis chamber. Make sure that you have **CORRECTLY MATCHED** the positive (red) and negative (black) terminals of the lid and the chamber. If you switch the terminals, the DNA will migrate towards the top of the gel and will be lost into the buffer solution.
3. Plug in the power supply and switch it on with the voltage set at **175 V**. To check that electric current is flowing through the buffer, look for small bubbles that form at the electrode inside the electrophoresis chamber. These bubbles arise from electrolysis of the water in the chamber.

4. It is important to achieve good separation between the DNA fragments, so the gel should run for **30 minutes**. During the electrophoresis experiment you should periodically check on the position of the loading dye in the gel.
5. While the experiment is running you will study a blood smear slide and answer the "practice questions" on the Data Sheets.

**NOTE:** The exercises for using the microscope to study the blood smear slide are given in **Part 2** of the experimental procedures.

## Viewing the Gel

**NOTE: WEAR GLOVES FOR THIS PART OF THE EXPERIMENT.**

1. When your lab instructor informs you to stop the electrophoresis, TURN OFF AND UNPLUG THE POWER SUPPLY. Carefully remove the lid of the electrophoresis chamber.
2. Carefully remove the plastic gel bed, which holds the gel, and place it on the blue transilluminator. The gels have been pre-stained so you should see the **fluorescent orange bands** scattered down the gel against the UV illuminated background**.** Take a digital photograph of the gel, and then fill in the chart provided in the next section.

# PART 2: SICKLE CELL ANEMIA BLOOD SMEAR SLIDE

As described in the introduction, a consequence of the sickle-cell mutation is to generate long fibers of the hemoglobin protein that distort the structure of the red blood cell. Not all cells are distorted in this manner, but a sufficient number are affected to produce serious physiological problems with oxygen transport and the flow of blood through narrow capillaries. In this exercise you will use the microscope to study a blood smear from a sickle cell patient and identify the distorted red blood cells.

## Observing the Sickle Cell Slide

1. Locate the red blood cells under the 40X objective lens on both the "Human Blood" slide and the "Sickle Cell Anemia" slide.
2. You will notice that most of the red blood cells are their usual circular shape. However, on the "Sickle Cell Anemia" slide, if you look closely and move the slide on the stage you will notice some red blood cells that are distorted into a sickle shape.
3. Turn to the data sheets and answer the questions about this slide.

# COVER SHEET

# LABORATORY 4: SICKLE CELL ALLELES AND GEL ELECTROPHORESIS

**HUMAN GENETICS – CORE-UA 303 – Professor Fitch**

Name: _____ Date: _____

Lab Partner Name(s): _____

_____

Laboratory Instructor: _____ Section: _____

# CLEAN-UP CHECKLIST

It is essential that you clean up properly after the experiments are complete.

☐ Dispose of <u>all</u> used pipette tips in the trash can.

☐ Dispose of the stained gels and any items that they came into contact with.

***NOTE**: Check with your lab instructor that your bench is <u>suitably clean</u> before leaving the lab.*

**<u>Complete clean-up procedures</u> are worth <u>5 points</u> towards your lab score.**

**Lab Instructor Initials _____**

| **GRADING** | |
|---|---|
| Attendance: | _____ / 5 |
| Quiz: | _____ / 10 |
| Lab Project: | _____ / 30 |
| Clean Up: | _____ / 5 |
| Total: | _____ / 50 |

***For TA use only***

# DATA SHEETS

**Question 1:** Consider a couple in genetic counseling who are expecting a child. The man has sickle cell anemia but the woman is unsure whether or not she is a carrier for the mutation. Suppose that you are providing advice to this couple as their counselor.

**(a)** If the woman is **not** a carrier, what can you tell the couple about the probability of their child being born with sickle cell anemia? Explain.

**(b)** If the woman **is** a carrier, use a Punnett square to show the possible genotypes that a child could have. In this case, what would you tell the couple about the probability of their child being born with sickle cell anemia?

**Question 2**: Consider a hypothetical single-base mutation in the β-globin gene of hemoglobin. The DNA sequences for the normal and mutated allele are listed below:

Normal allele: 5' – C T A A C C T T C G – 3'

Mutated allele: 5' – C T A A G C T T C G – 3'

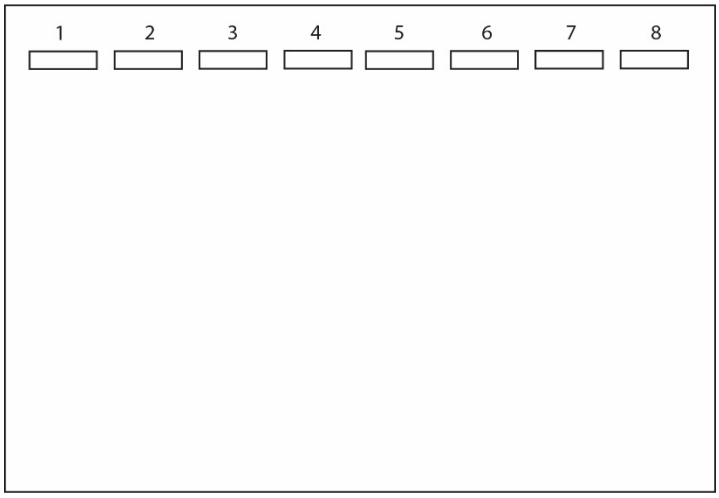
**(a)** What specific mutation has occurred in the DNA sequence?

**(b)** If you wanted to develop a genetic test for this mutation, which restriction enzyme would you select from Table 1 in the experiment introduction? Explain your choice.

**Question 3**: Suppose that you were given the following 4 samples with known lengths of DNA. The sizes of the DNA pieces are given in base pairs (bp).

**Sample 1:** 2,000 bp **Sample** 2: 500 bp **Sample 3:** 8,000 bp **Sample 4:** 5,500 bp

The diagram below shows a schematic picture of a gel and the samples that are loaded into each well. Draw the relative positions of the DNA bands that would be observed after performing an electrophoresis experiment and staining the gel with ethidium bromide dye.

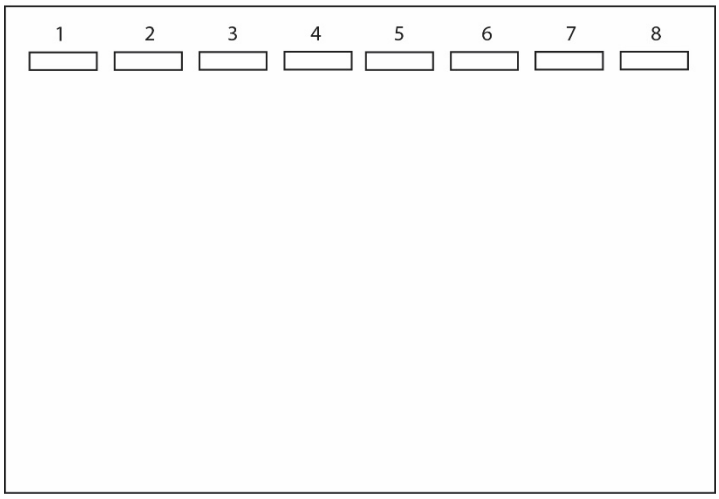



# SICKLE CELL ANEMIA BLOOD SMEAR SLIDE

**Question 4:** In the space below, use the colored pencils to draw the shape of a regular red blood cell and a distorted sickle cell. Try to capture as accurately as possible the shape of these two cells.

# GEL ELECTROPHORESIS

**Question 5**: In the space below, draw the DNA band patterns from your gel photograph. In the space above each lane, label what DNA sample was used.




**Question 6**: **(a)** Based on the results of this genetic test, what are the genotypes of the mother, child, and father (use the symbols HbA and HbS).

Mother:

Father:

Child:

**(b)** If you were the couple's genetic counselor, how would you use the results of the test to explain whether or not their child will be born with sickle cell anemia? Could the child pass on a mutated gene to his or her own children?