GenoSensor Corporation

PCR-CRISPR Dx: Sickle Cell Detection Kit

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User's Manual

GenoSensor PCR-CRISPR Dx: Sickle Cell Detection Kit Manual

Table of Contents

Literature Citation
When describing a procedure for publication using these products, please refer to them as the *GenoSensor PCR-CRISPR Dx: Sickle Cell Detection Kit*.

Notes for Instructors

Kit Components and Storage Conditions:

Component	Storage
2X PCR Master Mix	-20ºC
5X CRISPR Mix	-20ºC
Sample A	-20ºC
Sample B	-20ºC
Sample C	-20ºC
Sample D	-20ºC
Negative Control	-20ºC
DNA ladder	-20ºC

Preparation for PCR and CRISPR Digest for up to 24 students (for 6 groups)

PCR preparation:

1. Set up thermal cycler and the PCR program (thermal cycling program in Exercise C).
2. Thaw 2X PCR Master Mix on ice. Don't thaw 5X CRISPR Mix until use. **Before opening tube**, spin 10 sec at 6,000 rpm or greater in a microcentrifuge. Vortex 10 seconds, then spin again for 10 seconds.
3. Label 6 microcentrifuge tubes "PCR" and aliquot 40 µL of 2X PCR Master Mix into six tubes. KEEP ON ICE.
4. Label 4 tubes per group (6 groups) each "A, B, C, and D" and aliquot 10µL of each DNA sample, store on ice.
5. In class, distribute 1 each "PCR" (40 uL) to every group.
6. Students will use 10 µL of 2X PCR Master Mix with 10 µL sample DNA for a final PCR volume of 20 µL.

CRISPR preparation:

1. Set heat block or water bath to 37ºC. For a heat block, it is recommended to add water or sand to ensure proper heat transfer. For a water bath, be sure tubes are tightly sealed and not fully submerged to avoid contamination.
2. Thaw 5x CRISPR Master Mix on ice. **Before opening tube**, spin 10 sec at 6,000 rpm or greater in a microcentrifuge. Vortex 10 seconds, then spin again for 10 seconds.
3. As described above, label 6 tubes "CRISPR" and aliquot 20 µL of 5X CRISPR Master Mix into each tube, store on ice.
4. Distribute "CRISPR" (20 uL) to each group, and add 5 µL of "CRISPR" to each DNA sample, final volume is 25 uL each reaction tube.

Electrophoresis

- Electrophoresis reagents are not provided in the kit. Please refer to the Additional Required Materials list, on page 4.
- Best results are obtained by adding DNA dye (e.g. Gel Red or Sybr Safe®) to molten agarose.
- For light sensitive DNA dyes, avoid exposing the agarose gel to light. It is best to store and run the gel in a dark room, or cover the gel with a box during gel polymerization and the whole electrophoresis process.
- DNA ladder supplied is enough to load 3 lanes with 10 µL each.

Shipping, Storage and Safety

Shipping and Storage

GenoSensor PCR-CRISPR Dx: Sickle Cell Detection kits are shipped at ambient temperature. Components should be stored at temperatures shown in the table above. At proper storage conditions, components are stable for 1 year from the date received. Expiration dates are also noted on product labels.

Safety Warnings and Precautions

This product is intended for research use only. It is not recommended or intended for the diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals. Consider all chemicals as potentially hazardous. Only persons trained in laboratory techniques who are and familiar with the principles of good laboratory practice should handle these products. Wear suitable protective clothing such as laboratory coats, safety glasses, and gloves. Exercise caution to avoid contact with skin or eyes: if contact should occur, wash immediately with water and follow your laboratory safety protocols. Material Safety Data Sheets for products are available upon request.

GenoSensor PCR-CRISPR Dx: Sickle Cell Detection Kit Overview

The GenoSensor PCR-CRISPR Dx: Sickle Cell Detection Kit introduces students to widely used techniques in DNA research and forensic analysis. The kit provides an educational experience with CRISPR, a modern biotechnology, by simulating the detection of sickle cell disease–associated genetic mutations. CRISPR is a powerful tool capable of identifying the single-nucleotide mutation responsible for sickle cell disease.

In this activity, four DNA samples are provided, and students perform simulated CRISPR-based experiments to determine which sample contains the disease-associated allele. The exercise also teaches students how to design CRISPR guide RNAs (gRNAs) and how to use the NCBI BLAST tool to evaluate the specificity of their designs.

Through this investigation, students learn key molecular biology concepts and techniques, including the principles of CRISPR, gRNA design, sequence analysis with BLAST, CRISPR-mediated DNA digestion, PCR, gel electrophoresis, and interpretation of experimental results from gel images.

Kit Components and Storage Conditions (For 6 groups)

Component	Amount (24 rxns)	Storage
2X CRISPR Master Mix	240 µL	-20ºC
5X CRISPR Mix	120 µL	-20ºC
Sample A	60 µL (6 rxns)	-20ºC
Sample B	60 µL (6 rxns)	-20ºC
Sample C	60 µL (6 rxns)	-20ºC
Sample D	60 µL (6 rxns)	-20ºC
Negative Control	60 µL	-20ºC
DNA ladder	30 µL	-20ºC

Additional Required Materials

- Thermocycler
- Heat Block or (heat plate, Beaker with de-ionized water; water bath, Tube floater; Thermometer)
- Ice
- Microcentrifuge
- Microcentrifuge tubes (30)
- Vortexer
- Micropipettes (p10, p100)
- Pipette tips
- Tube Racks
- Electrophoresis equipment (gel box & power source)
- Electrophoresis supplies: agarose, TBE, DNA loading buffer, running buffer, gel dye (i.e., SYBR® safe, Gel Red)
- UV light box or “Gel Doc” equipment and program
- Permanent marker

Student Guide

Objective Overview

By completing this activity, students will:

1. Understand how changes in DNA can lead to disease development.
2. Investigate molecular biology techniques used in modern DNA technology, including the principles of CRISPR, DNA digestion, guide RNA (gRNA) design, the BLAST sequence analysis tool, PCR, and gel electrophoresis.
3. Develop an understanding of gel electrophoresis procedures and learn how to analyze and interpret experimental data.

Science and Technology Background

Every second of every day, millions of red blood cells race through your body. They travel along vast networks of blood vessels, delivering oxygen to your brain, muscles, heart, and every other organ that keeps you alive. Most of the time, this process happens smoothly and silently, without us ever noticing.

For people with sickle cell disease, however, this journey can be painful, dangerous, and sometimes life-threatening.

Sickle cell disease (SCD) is a genetic disorder that affects the shape and function of red blood cells. Instead of being round, soft, and flexible, many red blood cells become stiff and curved, resembling a farmer's sickle (Fig 1). These abnormal cells do not move easily through small blood vessels.

Fig 1. Comparison of normal and sickle-shaped red blood cells. Healthy red blood cells are round, flexible, and easily move through small blood vessels. In sickle cell disease, red blood cells become rigid and crescent-shaped, causing blockages that reduce oxygen delivery and lead to pain and organ damage.

Symptoms of SCD usually begin around 5–6 months of age. Sickled cells can block blood flow, reduce oxygen delivery, and cause severe pain episodes, swelling in the hands and feet, frequent infections, dizziness, and damage to major organs. Over time, the liver, heart, kidneys, lungs, gallbladder, eyes, bones, and joints can all be affected. Before effective treatments were available, many people with SCD did not survive into adulthood.

What makes SCD especially fascinating to scientists and students is its remarkably simple genetic cause. The disease results from a single change in DNA. This tiny alteration reduces how well hemoglobin—the oxygen-carrying protein in red blood cells—binds oxygen and causes red blood cells to change shape into rigid, sickle-like forms.

In this activity, you will explore how scientists use a powerful modern technology called CRISPR to detect this small but critical genetic change. You will see how biology,

genetics, and technology come together to help diagnose disease—and how understanding DNA is shaping the future of medicine.

Genes, DNA, and Hemoglobin

Genes are specific segments of DNA that contain instructions for making proteins. These proteins influence inherited traits such as eye color, blood type, and susceptibility to certain diseases. Genetics is the scientific study of how genes are passed from parents to offspring and how they vary among individuals.

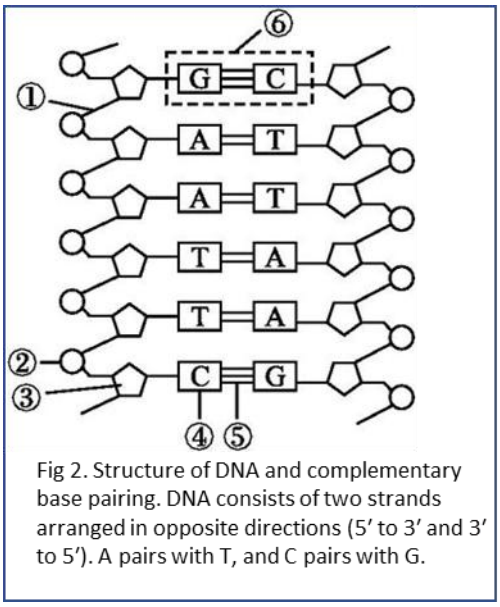


Fig 2. Structure of DNA and complementary base pairing. DNA consists of two strands arranged in opposite directions (5′ to 3′ and 3′ to 5′). A pairs with T, and C pairs with G.

DNA (deoxyribonucleic acid) is a long polymer composed of repeating units called nucleotides. Each nucleotide contains a sugar (deoxyribose), a phosphate group, and a nitrogenous base. The sugar-phosphate backbone provides structural support for the DNA molecule.

There are two classes of nitrogenous bases: Purines: Adenine (A) and Guanine (G), which contain two rings, and Pyrimidines: Thymine (T) and Cytosine (C), which contain a single ring.

These bases form complementary pairs across two DNA strands: A pairs with T, and G pairs with C (Fig. 2). The paired strands twist together to form the double-helix structure, often compared to a spiral staircase.

Each individual has a unique sequence of A, T, G, and C within their DNA. While human DNA sequences are highly similar overall, small differences can be used to distinguish individuals or identify genetic mutations.

DNA consists of two complementary strands arranged in opposite orientations: the sense (coding) strand and the antisense strand. The sequence of bases encodes genetic information.

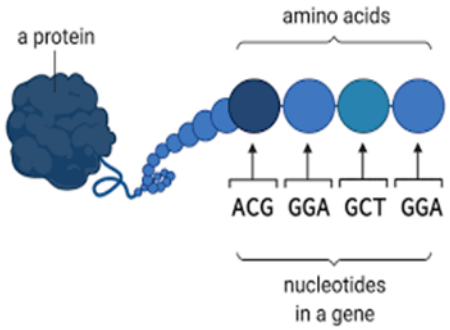


Fig 3. Genes are segments of DNA that provide instructions for building proteins. Changes in DNA sequence can alter the amino acid sequence of a protein, potentially affecting its structure and function.

A gene provides the instructions for making a specific protein (Fig 3). Proteins perform many essential cellular functions, including building structures, sending signals, carrying oxygen, and defending against disease. The gene responsible for producing part of hemoglobin is called the *HBB* gene (Hemoglobin Subunit Beta).

One of the most striking facts about sickle cell disease is that it is caused by a change in just **one DNA letter** within the *HBB* gene. This single nucleotide change results in the replacement of one amino acid in hemoglobin. Although small, this change alters hemoglobin's structure and behavior.

Under low-oxygen conditions, the altered hemoglobin molecules stick together. This causes red blood cells to become stiff and sickle-shaped, leading to the symptoms of the disease.

Before modern genetic technologies existed, doctors diagnosed sickle cell disease by examining blood samples under a microscope and using chemical tests to analyze hemoglobin.

Today, advances in molecular biology allow scientists to diagnose SCD by directly examining DNA.

One of the most important scientific breakthroughs of the 21st century is **CRISPR**, a technology originally discovered in bacteria.

About CRISPR

Bacteria are constantly attacked by viruses. To defend themselves, they evolved a system that allows them to capture fragments of viral DNA, store those fragments as a genetic memory, and recognize and destroy the virus if it attacks again. This system is called CRISPR, which stands for Clustered Regularly Interspaced Short Palindromic Repeats**.**

When a virus infects a bacterial cell, the bacterium saves a small piece of the virus's DNA and inserts it into its own genome. This stored sequence is later used to produce a short RNA molecule called a **guide RNA (gRNA)**. The guide RNA helps the bacterial defense system recognize matching viral DNA through complementary base pairing.

When the same virus attacks again, the guide RNA binds to the viral DNA, and a CRISPR-associated enzyme destroys it. Scientists realized they could adapt this natural system for research, diagnostics, and medicine.

CRISPR has two main components, 1) Cas enzyme – a protein that binds to and cuts DNA, often described as molecular scissors, and 2) Guide RNA (gRNA) – a short RNA sequence that matches a specific DNA target and directs the Cas enzyme.

Together, the CRISPR-Cas complex scans DNA inside a cell. If it encounters a sequence that perfectly matches the guide RNA, the Cas enzyme cuts the DNA at that location.

To cut DNA accurately, the Cas enzyme also requires a short nearby sequence called a **PAM** (protospacer adjacent motif, Fig 4)**.** Different Cas enzymes recognize different PAM sequences. The commonly used Cas9 enzyme from Streptococcus pyogenes recognizes the PAM sequence **5′-NGG-3′**, where "N" can be any nucleotide.

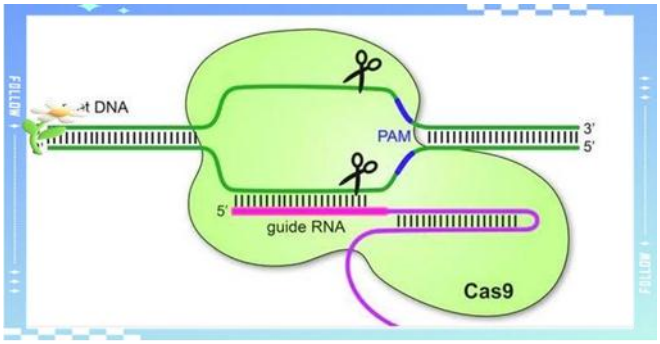


Fig 4. The CRISPR-Cas9 system consists of a guide RNA (gRNA) and the Cas9 enzyme. Cas9 requires a short DNA sequence known as a protospacer adjacent motif (PAM), and the gRNA directs Cas9 to a specific DNA sequence through base pairing, allowing Cas9 to bind and cut the target DNA.

This remarkable precision allows CRISPR to target specific DNA sequences among billions of letters, making older bacterial defense systems seem crude by comparison.

The ability of CRISPR to precisely target DNA has transformed genetics, molecular biology, and synthetic biology. Researchers can sequence a gene, design a guide RNA to target it, and introduce CRISPR into cells to modify DNA.

One common use of CRISPR is to disrupt genes. When CRISPR cuts DNA, the cell attempts to repair the break. This repair process often introduces small insertions or deletions that disrupt gene function, creating loss-of-function mutations**.**

CRISPR is already being used to improve crops by increasing disease resistance, stress tolerance, and yield. In this experiment, however, you will use CRISPR for a different purpose: diagnosis.

You will design guide RNAs targeting the *HBB* gene, evaluate their specificity using BLAST, and test whether the most specific guides successfully recruit Cas to cut the *HBB* gene in a simulated system.

Although CRISPR is often associated with gene editing, it can also be used in a safer and simpler way—to detect DNA sequences.

If a target DNA sequence is present, CRISPR binds to it. If even a single DNA letter is different, binding may fail. This extraordinary precision allows CRISPR to detect single-letter mutations, exactly the type of mutation responsible for sickle cell disease.

Because of this, CRISPR has enormous potential as a fast, accurate, and powerful diagnostic tool for genetic diseases.

About Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a powerful molecular biology technique used to **amplify**, or produce millions to billions of copies, of a specific DNA segment. Often described as molecular photocopying, PCR allows scientists to start with a very small amount of DNA and generate enough material for detailed analysis.

In clinical and research settings, the amount of DNA available from patient samples is often limited. PCR therefore plays a critical role in modern diagnostics by enabling detection of genetic material even when only trace amounts are present. When combined with emerging technologies such as CRISPR, PCR-based workflows have become highly sensitive and precise tools for genetic disease detection.

PCR was invented in 1983 by American biochemist Kary Mullis, who was awarded the Nobel Prize in Chemistry in 1993 for this breakthrough discovery. A key factor in PCR's success is the use of **Taq polymerase**, an enzyme isolated from the heat-tolerant bacterium *Thermus aquaticus*. Unlike many enzymes, Taq polymerase remains stable at the high temperatures required during the PCR process, making repeated cycles of DNA amplification possible.

PCR is performed in an instrument called a **thermal cycler**, which repeatedly changes temperature to allow specific steps in DNA replication to occur:

1. Denaturation (approximately 94–98°C):
   The double-stranded DNA is heated so that the two strands separate into single strands.
2. Annealing (approximately 50–65°C):
   The temperature is lowered to allow short DNA sequences called **primers** to bind to their complementary target regions on the template DNA.
3. Extension (approximately 68–72°C):
   DNA polymerase extends the primers by adding nucleotides, synthesizing a new DNA strand complementary to the template.

Each cycle approximately doubles the amount of DNA present. After about 30 cycles, a single DNA fragment can be amplified into more than one billion copies.

A standard PCR reaction mixture (often called a master mix) typically contains: Primers**:** Short, custom-designed DNA fragments that define the region to be amplified. DNA Polymerase**:** An enzyme (commonly Taq polymerase) that synthesizes new DNA strands. Nucleotides (dNTPs)**:** The building blocks (A, T, C, and G) used to construct new DNA. Buffer and Magnesium Ions: Provide the optimal chemical environment required for enzyme activity. A researcher needs to add specific DNA template or the DNA of interest to master mix for the PCR reaction.

By enabling the amplification of extremely small amounts of DNA, PCR transforms what was once a “needle in a haystack” problem into a detectable and measurable signal. This capability has made PCR one of the most influential technologies in modern biology.

PCR is an essential tool across many fields of science, medicine, and forensic analysis. In this exercise is the detection of sickle cell disease.

Exercise A: Design of Guide RNA (gRNA)

The objective of this exercise is to design guide RNAs.

Task 1: Write the Complementary Antisense Strand

Below is a small segment of the *HBB* gene sequence. DNA sense (coding) strand sequence is usually presented and written in the 5′ to 3′ direction. So the sequence below is a sense strand and from 5’ to 3’ (left to right).

Based on the pairing information from the Background, write the complementary antisense strand first in the 3′ to 5′ direction.

Then write the complementary antisense strand in the 5’ to 3′ direction.

Complementary strand:

5’- CCA_____ -3’

Task 2: Identification of PAM Sites

To design a guide RNA (gRNA), you must first identify PAM (protospacer adjacent motif) sites within the target DNA sequence. In this exercise, assume the use of Cas9 from *Streptococcus pyogenes*, which recognizes the PAM sequence **5′-NGG-3′**, where “N” can be any nucleotide.

Cas9 binds and cuts DNA **immediately upstream (in the 5′ direction)** of a PAM sequence. Therefore, Cas9 will bind near any of the following sequences:

- AGG
- TGG
- CGG
- GGG

Because Cas9 can target either DNA strand, both the sense and complementary antisense strands must be examined for PAM sites.

Example gRNA

One gRNA is shown below as an example. The PAM sequence (AGG**,** yellow) is highlighted and is located on the sense strand. The corresponding target sequence for gRNA is the **20 nucleotides (nt)** immediately upstream (5′ direction) of the PAM site and is underlined.

5’- AGACACCATG GTGCATCTGA CTCCTGAGGA GAAGTCTGCC GTTACTGCCC TGTGG -3’

Question: How many PAM sites have you identified on both sense and complementary strands?

Task 3: Write gRNA Sequences

Now, we know gRNA sequence is 20 nt immediately upstream of the PAM site. Record at least three gRNA sequences (written 5’ to 3’, label sense or antisense):

Design Name	Target Sequence	PAM Sequence	Strand
gRNA A	5’-CATGGTGCATCTGACTCCTG-3’	AGG	Sense
gRNA B			
gRNA C			
gRNA D			

Question: How many possible gRNA sequences can you see?

Sickle Cell Mutation

In patients with sickle cell disease, a single nucleotide substitution occurs in the above sequence: **A** → **T**, changing the codon **GAG** → **GTG** (bolded below).

Wild-type (healthy) allele:
5′-AGACACCATGGTGCATCTGACTCCT**GAG**GAGAAGTCTGCCGTTACTGCCCTGTGG-3′

Mutant (sickle cell) allele:
5′-AGACACCATGGTGCATCTGACTCCT**GTG**GAGAAGTCTGCCGTTACTGCCCTGTGG-3′

This mutation results in an amino acid change from glutamic acid (Glu) to valine (Val), altering the structure and function of hemoglobin and causing red blood cells to adopt a sickle shape.

	Wildtype (healthy)	Mutant (SCD)
Nucleotide	A	T
Codon	GAG	GTG
Amino acid	Glu	Val

The gRNA must be located 20 nucleotides upstream of a PAM site. To ensure that the gRNA spans the mutation site for diagnostic purposes, only two gRNAs—one on each allele—are suitable.

Question: Identify and write the sequences of the two gRNAs that cover the mutation site.

gRNA	Target Sequence	PAM Sequence	Strand
gRNA 1 (wildtype)			
gRNA 2 (mutant)			

Question: If we want to cut mutant (sickle cell) allele and the Cas9 only cuts perfect match, which sequence will you choose?

gRNA	Target Sequence	PAM Sequence	Strand
gRNA 2 (mutant)	5'-GTAACGGCAGACTTC TC**CAC**-3'	AGG	complementary

Did you answer it correctly?

Exercise B: Determining gRNA Specificity Using BLAST

The objective of this exercise is to examine the specificity of guide RNAs.

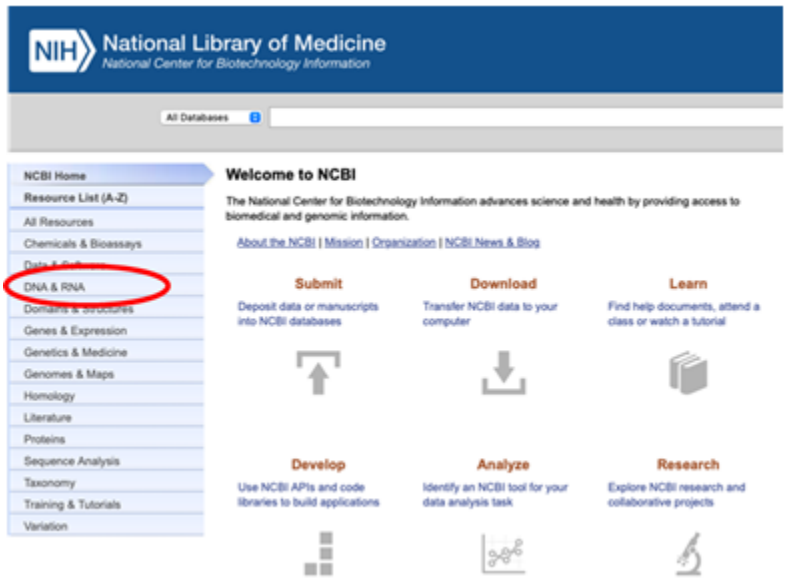
In this exercise, you will use the Basic Local Alignment Search Tool (BLAST) to evaluate the specificity of the gRNA identified from **Exercise A**.

BLAST identifies regions of similarity between a query sequence (your gRNA target) and sequences stored in the GenBank database, which contains nearly all known DNA sequences. Matches between the query and database sequences are called hits.

This exercise uses the free BLAST service provided by the National Center for Biotechnology Information (NCBI).

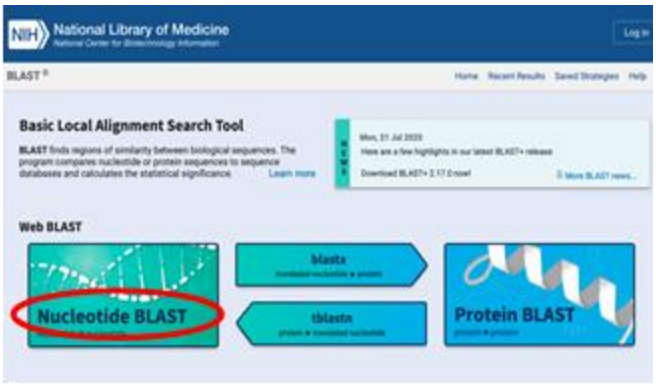
BLAST Procedure

1. Open a web browser and navigate to the NCBI website (www.ncbi.nlm.nih.gov).
2. Select **"DNA & RNA"** from the left-hand toolbar.

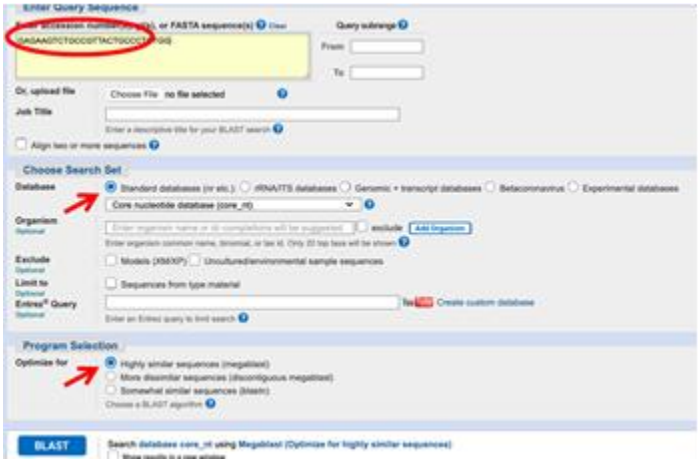


3. Scroll to the **Tools** section and click **"Basic Local Alignment Search Tool (BLAST)"**.

4. Select **“Nucleotide BLAST”**.



5. Paste your gRNA target sequence into the query box.

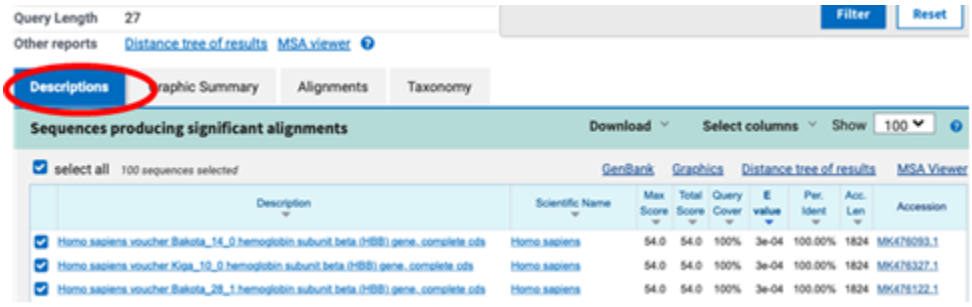


6. Ensure that **“Standard databases”** and **“Highly similar sequences (megablast)”** are selected.
7. Click **“BLAST”** to run the search.

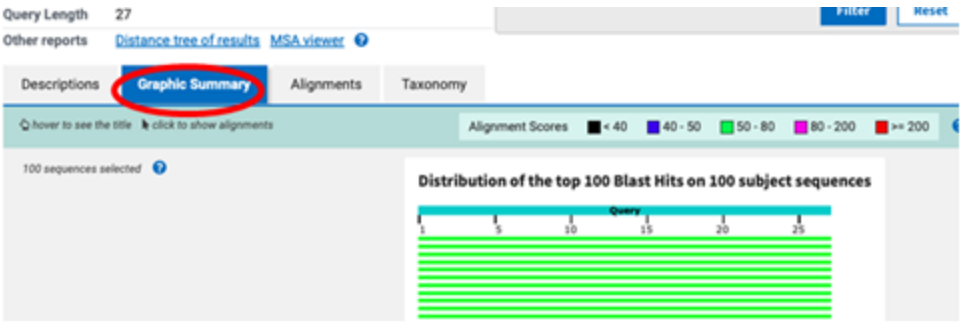
BLAST Output

The BLAST results include:

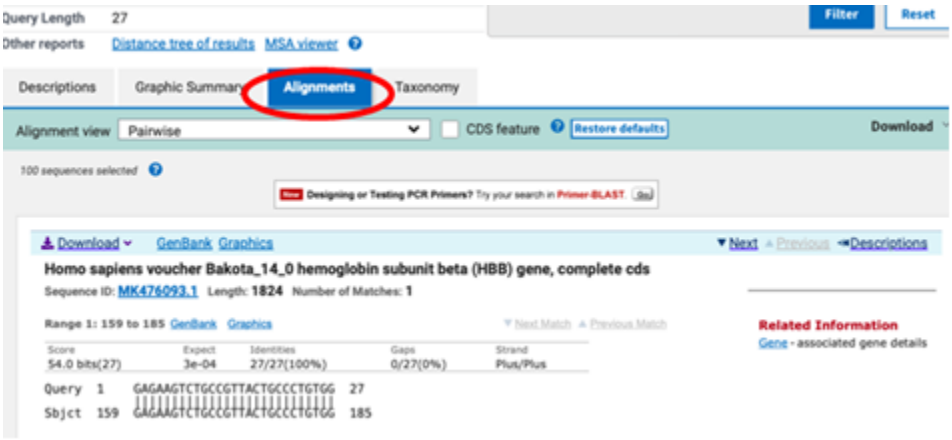
- **Search Summary:** Overview of BLAST parameters
- **Filter Results:** Option to restrict matches to specific organisms
- **Descriptions:** List of sequences with significant similarity to the query



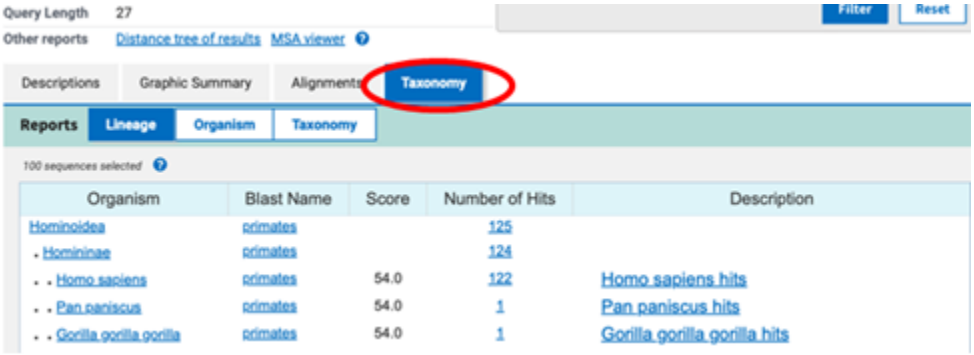
- **Graphic Summary:** Visual representation of alignments, with red indicating the highest alignment scores



- **Alignments:** Detailed sequence alignments, including identity percentage, gaps, and orientation



- **Taxonomy:** List of organisms containing matching sequences



The top hit should correspond to the human *HBB* gene (Homo sapiens). Multiple database entries may appear, but they should all align with the same gene.

Now that you are familiar with the target gene used to diagnose sickle cell disease using CRISPR, proceed to the next exercise to simulate a laboratory experiment.

Exercise C: DNA Amplification and Digestion

The objective of this exercise is to perform experiments using PCR and CRISPR techniques.

Scenario

In a hospital setting, DNA samples from four patients—Sample A, Sample B, Sample C, and Sample D—must be tested for sickle cell disease.

PCR is performed to amplify specific *HBB* gene region from genomic DNA. Then a disease-specific gRNA (from Exercise B) is used for CRISPR experiment. Cas9 will cut the DNA only if the gRNA perfectly matches the sickle cell (mutant) allele. DNA from healthy individuals, which contains a mismatched sequence, will **not** be cut (Fig 5).

You will analyze the gel results to determine which samples carry the sickle cell mutation.

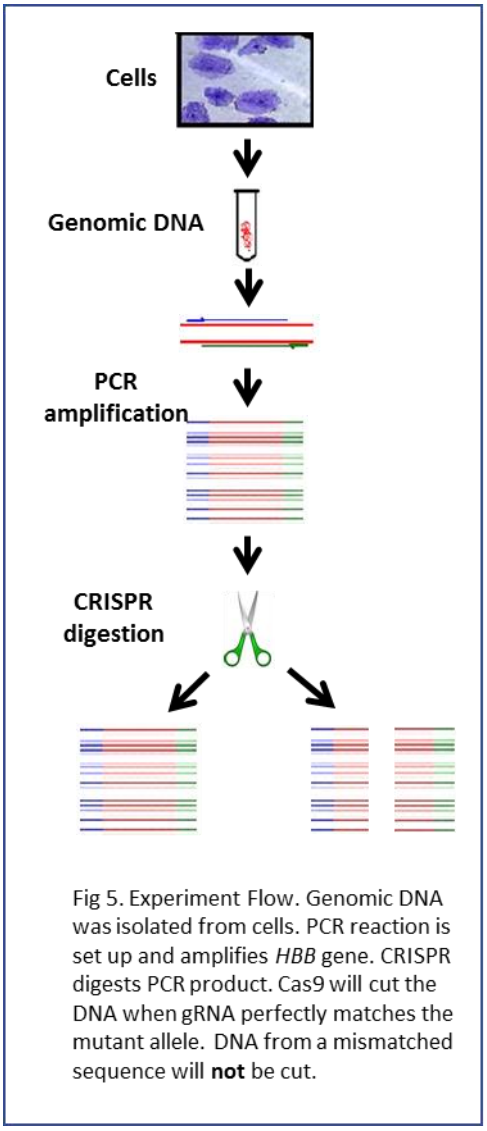


Fig 5. Experiment Flow. Genomic DNA was isolated from cells. PCR reaction is set up and amplifies *HBB* gene. CRISPR digests PCR product. Cas9 will cut the DNA when gRNA perfectly matches the mutant allele. DNA from a mismatched sequence will **not** be cut.

Lab Setting

Materials are enough for 24 reactions (4 students per group and 6 groups).

Reagent Preparation

Refer to “Notes for Instructors – Preparation for Restriction Digest” on Page 2.

Pre-Experiment Observations

1. Describe the DNA samples (physical properties: color, viscosity, etc.). Can you see the DNA?

2. Is there any observable difference between the samples of DNA?

3. Describe the appearance of the 2X PCR Master Mix and 5X CRISPR Mix. Can you see the enzymes?

PCR Amplification Protocol

Keep the 2X PCR Master Mix and all samples on ice when not in use.

1. *Wear gloves and handle solutions carefully.* Spin the "MM" tube for 10sec, vortex for 10sec, then spin for another 10sec.
2. **Using a NEW TIP for each sample**, pipette 10 µL of the 2X PCR Master Mix, (containing Taq DNA polymerase, nucleotides, primers, and PCR reaction buffer) into the sample tubes "A, B, C, and D," already containing 10 µL of each DNA.

	2X CRISPR Master Mix	Sample A	Sample B	Sample C	Sample D	Total Volume
Tube A	10 µL	10 µL				20 µL
Tube B	10 µL		10 µL			20 µL
Tube C	10 µL			10 µL		20 µL
Tube D	10 µL				10 µL	20 µL

3. Pipette up and down carefully to mix well. Tightly cap each tube. Alternatively, mix the components by gently flicking the tubes with your finger. Arrange the tubes in a microcentrifuge and spin for 5 seconds to force all liquid to the bottom of the tubes. (Be sure the tubes are in a BALANCED arrangement in the rotor).
   NOTE**:** If the teacher did not pre-aliquot samples, add the DNA samples THEN the master mix (enzyme) to your microcentrifuge tubes, changing tips each time.
4. Store the samples on ice until they are ready to be loaded into the thermal cycler.

PCR parameters

Program the thermal cycler as follows:

1. 94ºC – 30 seconds
2. 94ºC denaturing – 20 seconds}
3. 58ºC annealing – 20 seconds} **repeat steps 2, 3, & 4 for 35 cycles**
4. 68ºC extension – 30 seconds}
5. 68ºC – 5 minutes
6. 4ºC – finished / hold

STOPPING POINT – For classes with shorter time periods, the PCR samples should be stored at 4ºC until the next lab period.

CRISPR Digest Protocol

Keep the master mix, all samples and reaction mixtures on ice when not in use.

1. Using a NEW pipette tip for each sample, pipette 5 µL of the 5X CRISPR Mix, which contains the Cas9 enzyme along with the gRNA and digest buffer, into each of the four DNA sample tubes labeled "A, B, C, and D" (already containing 20 µL of previous PCR reaction).

	Finished PCR Reaction	5X CRISPR Mix	Total Volume
Tube A	20 µL	5 µL	25 µL
Tube B	20 µL	5 µL	25 µL
Tube C	20 µL	5 µL	25 µL
Tube D	20 µL	5 µL	25 µL

2. Carefully pipette the mixture up and down to mix thoroughly. Tightly cap each tube. Alternatively, mix the components by gently flicking the tubes with your finger. Arrange the tubes in a microcentrifuge machine and spin for 5 seconds to force all liquid to the bottom of the tubes. (Be sure the tubes are in a BALANCED arrangement in the rotor).
3. Incubate the tubes at 37°C for 30 minutes in a water bath or heat block.
4. **STOPPING POINT**-If there is no time to continue, store samples @ 4°C until following lab period.

Questions. While waiting for the samples to be amplified and digested, answer the following questions

1. After combining the 2x PCR Master Mix and later 5X CRISPR Mix with the DNA samples, was there any visible change or any sign of reactivity?

2. Was there any evidence indicating that your samples of DNA were cleaved or altered in any way by the addition of the CRISPR mix? Explain.

3. In the absence of any visible evidence of change, is it still possible that the DNA samples were cleaved? Explain.

4. After the incubation period, are there any visible clues that Cas9 enzyme altered the DNA in any of the tubes? Explain.

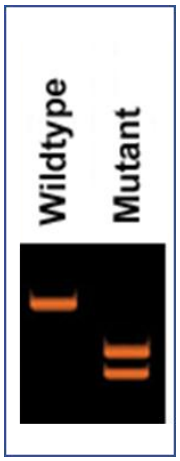
Exercise D. Gel Electrophoresis and Result Interpretation

The objective of this exercise is to identify which sample contains the *HBB* mutation by running a gel and examining the DNA band patterns.

General procedure, detailed directions given by instructor

1. Prepare 1% agarose.
2. For staining, use a DNA dye which is added directly to the molten agarose. For light sensitive dyes, keep the gel in the dark during gelation. This can be done by performing in a dark room or placing a box over the gel.
3. Set up electrophoresis apparatus and pour in the 1% molten agarose with DNA dye for gelation.
4. Load previously incubated sample to agarose gel. No loading buffer is needed as it is already included in the Master Mix. Use at least 10 µL of sample product to visualize results on the agarose gel. If gel well volume will accommodate more than 10 µL, a higher volume is preferred.
5. Record which wells hold which samples.
6. Run at ~120V for 20 – 30 minutes and stop before loading dye runs off of gel. Depending on the DNA dye used, caution may need to be taken to reduce exposure of gel to light.
7. Visualize under UV light exposure and record the results manually or by photography.
8. Compare the bands. The DNA ladder can be used as a band size reference.

Result Interpretation



Because a disease-specific gRNA is used in this assay, the healthy allele (wildtype) will not be recognized or cut by Cas9 and will therefore remain as a single, intact (longer) DNA band on the gel. In contrast, the sickle cell (mutant) allele will be cleaved by Cas9, producing two shorter DNA fragments (see gel image to the right).

Examine the four patient samples and compare their banding patterns to the DNA ladder loaded alongside the gel. Record your observations. Based on these results, determine which patient sample(s) correspond to individuals with sickle cell disease.

	Sample A	Sample B	Sample C	Sample D
Health (wildtype)				
SCD (mutant)				

Questions. While waiting for your samples to electrophorese, answer these questions with your group members.

1. The electrophoresis apparatus creates an electrical field with positive and negative poles at the ends of the gel. DNA molecules are negatively charged. To which electrode pole of the electrophoresis field would you expect DNA to migrate?

2. After the DNA samples are loaded into the sample wells, they are "forced" to move through the gel matrix at different speeds. What size fragments (large vs. small) would you expect to move toward the opposite end of the gel and travel a longer distance most quickly? Explain.

3. What do you see moving through the gel? (Tricky question).

Troubleshooting

Problem	Possible causes	Solutions
Incomplete or no digestion of DNA	2X CRISPR Master Mix not properly prepared	It's vital that the Master Mix be properly thawed, spun down and vortexed before use to ensure the enzyme and all components are properly mixed
	Heat block/Water bath/Heating source temperature incorrect	Be sure the heat source used for incubation has stabilized at 37°C
	Incubation time too short	Shorter times should work, but if you're having trouble, increase the incubation times
Weak bands/faint signal	DNA Dye degradation during preparation	Light sensitive dyes should be kept in the dark during gel preparation. Prepare in dark room or place a box over the electrophoresis apparatus during gelation and electrophoresis
	Expired, contaminated or degraded DNA dye	Verify that the DNA dye has not degraded in storage, been contaminated or expired

Technical Service

For more information or technical assistance, please call, write, fax, or email.

GenoSensor Corporation
4665 S. Ash Avenue
Suite G-18
Tempe, Arizona 85282
Email: tech_service@GenoSensorcorp.com
Web: www.genosensoreducation.com

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