3-D Molecular Designs
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Biotechnology Kit©

PCR as a Medical Tool Sickle Cell Anemia

PCR: Can copies find mutations?

PCR, **Polymerase Chain Reaction (PCR)** can be used by scientists to detect small differences between two sets of DNA. Through this activity, we will connect what we have already learned about PCR and how it works, to how scientists and physicians are using it in the field to diagnose diseases and to suggest treatment protocols using those diagnoses.

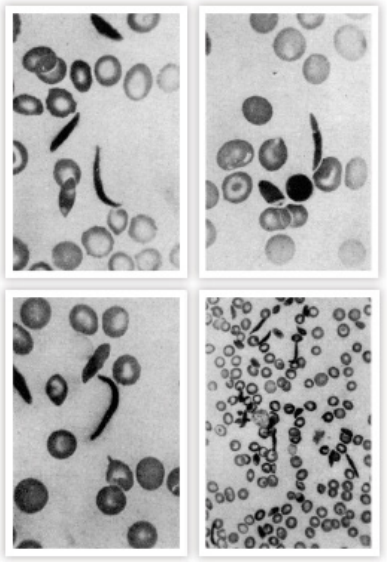
Before you begin though: In the space below, write down everything you remember about PCR. Some things you might want to think about might be what components were used in thereaction tubes, to even how the process worked.

Goal: To expand your working knowledge of PCR to model how it is used in the field to detect point mutations in the genome and diseases that can result from them, like Sickle Cell Anemia.

Introduction

Your DNA holds the key to understanding diseases that can ravage your body. From mutations that have been shown to be an indicator for an increased likelihood to develop breast cancer, to others that can determine if we are more likely to be susceptible to diseases like Huntingtons or Alzheimer's, looking inside of your DNA can be like peering into a crystal ball and uncovering our future.

The disease that you will be looking at is **Sickle Cell Anemia**. This disease is caused by a **single point mutation** in the hemoglobin beta (**β-globin**) gene found on chromosome 11. Although various forms of anemias were recognized by historical practitioners as early as the 6th century BC, sickle cell anemia was not identified until 1904 when Ernest Irons, a medical resident from the Chicago Presberterian Hospital, examined Walter Noel and produced the first ever picture of Noel's blood. In 1910, James Herrick, Irons' supervisor, included the pictures in a paper he wrote for the Yale Journal of Biology and Medicine.



Looking at Herrick's picture (above right), answer the following questions.

- What physiological differences do you see in the blood cells?

_____

- What is the purpose of the traditional shape of the red blood cells? How potentially could the altered shape of sickle shaped red blood cells affect this purpose?

_____

_____

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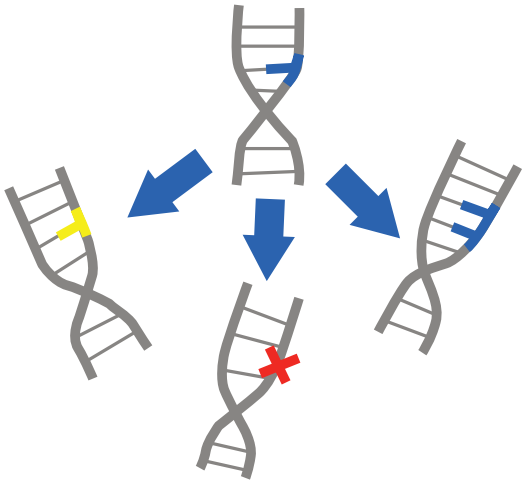
Mutations

Mutations occur when there are changes to the genetic information encoded on chromosomes. These changes can occur in either **somatic** or **germline** cells. By changing the sequence of DNA bases that encode the genes, the resulting mRNA and polypeptide chain will also be changed. Since amino acids code for the sequence (therefore structure) proteins, any change in the amino acid sequence can potentially change the structure of the protein.

There are many different kinds of mutations that can occur.

- **Deletion** (a base is lost)
- **Insertion** (an extra base is inserted)
  - Deletion and insertion may cause what's called a **frameshift**, meaning the reading "frame"changes, changing the amino acid sequence. This will not happen if it is in a multiple of three.
- **Substitution** (one base is substituted for another)
  - If a substitution ***changes*** the amino acid, it's called a **missense** mutation.
  - If a substitution ***does not change*** the amino acid, it's called a **silent** mutation.
  - If a substitution ***changes the amino acid to a "stop,"*** it's called a **nonsense** mutation.



Sickle Cell Anemia is a product of a point mutation. Given the DNA data for both normal (**wild type**) and sickle cell (**mutant**) human hemoglobin-beta gene, complete/identify the resulting the mRNA and amino acid sequence.

Normal hemoglobin DNA **C A C G T G G A C T G A G G A C T C C T C**

Normal hemoglobin mRNA _____

Normal hemoglobin AA sequence
Using the AA chart on the last page, write the three letter abbreviation for the AA that the mRNA codon will code for. _____

Sickle cell hemoglobin DNA **C A C G T G G A C T G A G G A C A C C T C**

Sickle cell hemoglobin mRNA _____

Sickle cell hemoglobin AA sequence
Using the AA chart on the last page, write the three letter abbreviation for the AA that the mRNA codon will code for. _____

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PCR: DNA's Mutation Sleuth?

For this activity, you will model how PCR can be utilized to detect the single nucleotide polymorphism (SNP), or point mutation, present in sickle cell anemia. Before we go on, read the excerpt of an introduction from a paper that utilizes this type of PCR detection to identify the SNP that is present.

Looking at both your work on the previous page, as well as the image to the right, answer the following questions.

- What was the original DNA sequence of the affected codon sequence that was changed?

  _____

- What is the DNA sequence of the mutated codon? How did the codon change?

  _____

- What is the amino acid that is produced in normal/ wild type hemoglobin? What type of amino acid is it? (hydrophobic, hydrophilic, acid, base)

  _____

- What amino acid is produced by the mutant codon? What type of amino acid is it? (hydrophobic, hydrophilic, acid, base)

  _____

- What was the original DNA sequence of the affected codon sequence that was changed?

  _____

  _____

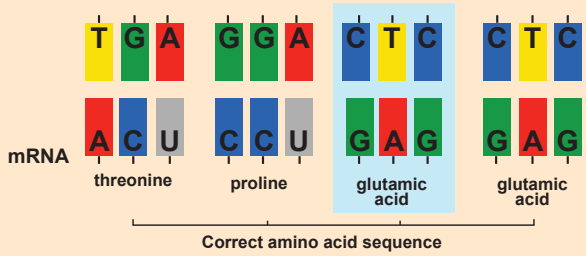
  _____

  _____

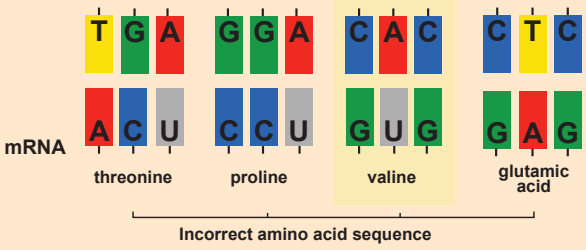
  _____

  _____

Normal DNA sequence (HbA)



Mutated DNA sequence (HbS)



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PCR has also been adapted for the detection of well-characterized SNPs using allele-specific amplification (ASA); oligonucleotides complementary to a given DNA sequence except for a mismatch at their 3′-hydroxyl residue will not function as primers in the PCR under appropriate conditions. A typical ASA test consists of two complementary reactions, each containing a common primer, an allele-specific primer and *Taq* DNA polymerase lacking 3′→5′ proofreading activity. The first reaction contains a primer specific for the normal (or wild type) DNA sequence and refractory to amplification from mutant DNA at a given locus. Similarly, the second reaction contains a mutant-specific primer unable to amplify wild-type DNA. Molecular conformation is achieved by analysis of the resulting PCR amplicon profiles. A normal individual will generate product in the first reaction only; a heterozygote amplifies products in both reactions; and a homozygous mutant individual does so only in the mutant-specific reaction. Internal control amplification is necessary, providing a positive control for the PCR test (9).

More recently, single tube adaptations have been introduced for known SNP detection in a two-allele system including competitive oligonucleotide priming(10), multi-colored fluorescent oligo labelling systems (2), tetra-primer PCR (11) and overlapping PCR strategies (12,13). The latter both use bi-directional primer arrangements in which common 'outer' primers define the size of each allele-specific fragment allowing simple identification using electrophoretic methods. Maintaining the integrity of all ASA techniques, particularly for diagnostic application, requires the optimization of many experimental parameters. The literature suggests that a magnesium and oligonucleotide titration is sufficient to obtain discriminatory conditions. Generally, further mismatches are incorporated at the 3′-end of the allele-specific primer to weaken hydrogen bonding between the primer and template, increasing the likelihood of discrimination (14). However, these conventional approaches are simplistic considering the complex component interactions in a PCR reaction and prove time consuming and expensive. This has limited the applicability of PCR-based SNP diagnosis for routine clinical application. In particular, bi-directional systems require significant optimization due the effects of primer competition caused by multiple primer sets in the PCR amplification.

Waterfall, C. M. and Cobb, B. D. (2001). Single tube genotyping of sickle cell anemia using PCR-based SNP analysis. *Nucleic Acid Res.* 29(23): e119.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC96713/

After reading the passage, answer the following questions:

- Knowing what you know about PCR and sickle cell anemia, how would you design primers to detect the disease?

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- If you want to visualize your results using gel electrophoresis, how would you account for this in your experimental design?

_____

_____

_____

_____

Modeling Time

You are now going to begin modeling how to utilize PCR to detect a disease. You will be given an unknown sample of dsDNA from a patient β-globin gene. Your goal will be to determine if the sickle cell gene is present on that section of DNA or not.

Before we begin, think about two things:

- What materials (both cellular/nucleotide-based and lab-based) were needed to perform PCR?

_____

- In the space below, sketch out how PCR "looked" when modeled.

To perform this activity, we are going to utilize special primers that will tell us if the section of target DNA you are working with contains the wild type β-globin or the mutation based on the length of the short, PCR product that they produce. Before we begin, assemble **one set of each of these primers**.

Primer Set A	**Primer Set B**
Primer 1: 5' CTGA 3'	Primer 1: 5' ACTC 3'
Primer 2: 5' GCAG 3'	Primer 2: 5' TCCA 3'

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Looking at your dsDNA, which of the primer sets fits with your sample? *Remember, both of the primers have to fit for the set to work.*

_____

Now that you know which primer set you will be utilizing, prepare **seven sets** of the primers. Keep each primer in **two separate piles** on your desk.

You will be completing three rounds of PCR, just like you did when we first used this kit to model PCR. The steps for PCR are:

1. **Denature** the double stranded DNA (dsDNA). By separating the strands, you are representing the thermocycler heating up to **95°C** and breaking the hydrogen bonds that hold together the base pairs of the target DNA.
2. **Anneal** the primers. **This time, only use the primers that you determined would work with your specific target DNA**. Using the grey primers, Primer 1 and Primer 2, find a complementary binding site on the target DNA. These primers provide a 3' starting point so that the polymerase can elongate and add free nucleotides (dNTPs). The thermocycler will reduce the temperature to **55°C** for annealing.
3. **Extend** the sequence by adding free nucleotides (dNTPs) to the 3' end of Primer 1 and Primer 2. Add bases along the template DNA until you reach the end of the strand. The thermocycler increases the temperature during this phase to **72°C**.
4. Complete three cycles of PCR. Be sure to place cycle pins in each of the grey primers as you progress through the cycles to keep track of each one.

Reflection Time

Determining a single base pair difference is quite hard to do. The way that these primers are designed is so that they exhibit a difference in the length of the small PCR product that they produce after the thirty cycles of PCR. Remember, you may only have a small number of short fragments now, but each time the sequences go through the PCR process, the number of those short PCR products will increase, and you will get a clear band present when you run the results on an agar gel.

The wild type format of target DNA will react with its respective primers to produce a short PCR product that is 16bp in length, while the mutant will react with its primers to produce a short PCR product that is 11bp in length.

- Which sample number do you have? _____
- Looking at your results, did the sample of target DNA that you were provided contain the wild type or the mutant allele? How do you know?

_____

_____

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Only one transcript of DNA represents a concept called a **polymorphism**, or one option that your genes can express. However, humans have a **diploid** number of chromosomes - therefore, you have two copies of each type of gene. Sickle cell anemia is known as an **autosomal recessive** trait. With this type of trait, individuals must have two copies of the gene to present with symptoms. If you have only one, you are said to be a carrier of the disease, but you would not exhibit red blood cells that are misformed.

Looking at both your results and your partner table results, answer the following questions:

- What table are you partnered with? *AND* What sample number did they have?

_____

- If your two samples represented a small section the diploid, homologous chromosome 11 present in your patient, what would your patient be? How do you know?

Homozygous Dominant ***Heterozygous*** ***Homozygous Recessive***

_____

_____

Now, think about how a gel with you and your partner table would look. Also, think back to other labs that we have done with electrophoresis. What other samples might you want to put into the lanes on the gel to confirm your results? Draw what your gel would look like in the space to the right and explain why/how you came to that determination below.

_____

_____

_____

_____