EDVOTEK®

THE BIOTECHNOLOGY EDUCATION COMPANY®

Edvo-Kit #
106

Edvo-Kit #106

Principles of DNA Sequencing

Experiment Objective:

The objective of this experiment is to develop an understanding of DNA sequencing and analysis. This is a dry lab which contains autoradiographs from an actual DNA sequencing experiment.

See page 3 for storage instructions.

1.800.EDVOTEK • Fax 202.370.1501 • www.edvotek.com • info@edvotek.com

Table of Contents

Experiment Components & Requirements

Experiment can be stored at room temperature.

COMPONENTS	**Check (✓)**
• 5 Autoradiographs	❑

REQUIREMENTS

• White Light Box	❑

A white light box is recommended. The EDVOTEK White Light Gel Visualization System (Cat. # 552) is well-suited for this lab. An autoradiograph may also be placed on an overhead projector and shown to the whole class.

Background Information

DNA SEQUENCING

The basic unit of all living organisms, from bacteria to humans, is the cell. Contained within each cell is a molecule called deoxyribonucleic acid (or DNA). Today, we know that DNA is the blueprint used to build an organism – our genetic makeup, or genotype, controls our observable characteristics, or phenotype. The directions encoded in our genes control everything from growth and development to cell-type specification, neuronal function, and metabolism.

A strand of DNA is composed of building blocks known as deoxynucleotides (dNTPs) that are linked together into a long chain (Figure 1). Each dNTP comprises three basic parts: a phosphate group, a deoxyribose sugar, and a nitrogen-containing base. While the phosphate group and deoxyribose sugars are constant, the specific bases determine the identity of the dNTP: adenine (A), cytosine (C), guanine (G), or thymine (T). The order of these nucleotides in the chain gives rise to genes, each with a unique sequence. In addition, the 3′ hydroxyl group on the sugar of one nucleotide forms a covalent bond with the 5′ phosphate group of its neighbor, resulting in DNA strands with a distinct polarity. This polarity ensures that the strand of DNA is read in the correct direction during gene transcription.

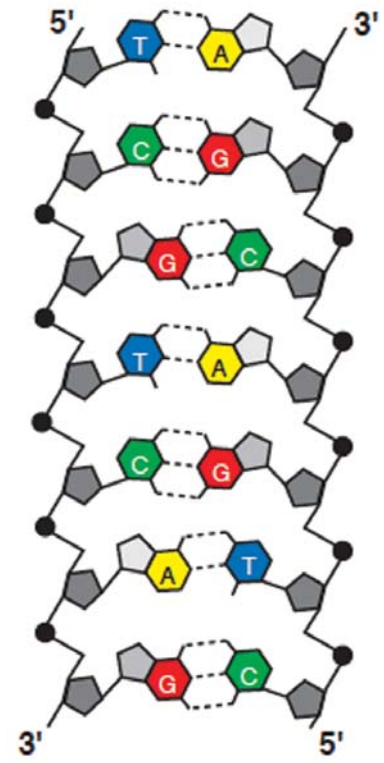


Figure 1: Structure of DNA

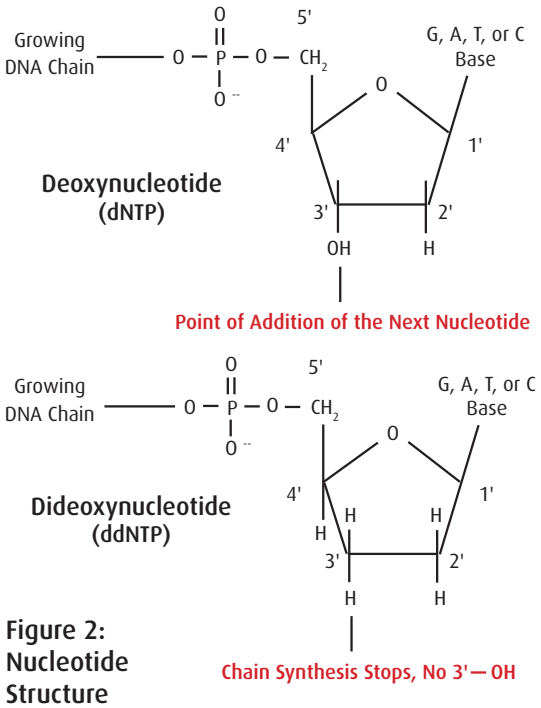


Figure 2: Nucleotide Structure

DNA sequencing is the process of determining the nucleotide order for a DNA segment of interest. As recently as the 1970s, scientists were unable to sequence even the most simple of genomes. This all changed in 1977 when Fred Sanger and his team exploited the natural process of DNA replication to allow scientists to sequence DNA accurately. This method, now known as "Sanger Sequencing", resulted in the 1980 Nobel Prize in Chemistry and is still in use today.

During Sanger Sequencing, an enzymatic reaction is performed to create a new DNA sequence that is complementary to the target or template DNA. This reaction requires DNA polymerase (the enzyme that will create DNA molecules by assembling nucleotides), a short DNA primer that serves as a starting point for sequencing, and two types of nucleotides – normal dNTPs and a specialized base analog called a dideoxynucleotide (ddNTP). The ddNTPs lack a hydroxyl-group at the 3′ carbon of the ribose sugar, which prevents new nucleotides from being added to the DNA strand after a ddNTP has been incorporated (Figure 2).

Background Information

DNA synthesis is initiated where the primer is annealed to the template. The polymerase then adds nucleotides to the strand in an order that complements the original single stranded template, always pairing C with G and A with T. This process continues to construct a DNA fragment until a ddNTP is incorporated, at which point no other nucleotide can be added. The initial ddNTP concentration in the reaction is kept relatively low so that they are incorporated into a growing DNA strand randomly and infrequently. The result is the generation of millions of DNA fragments with different end points (Figure 3).

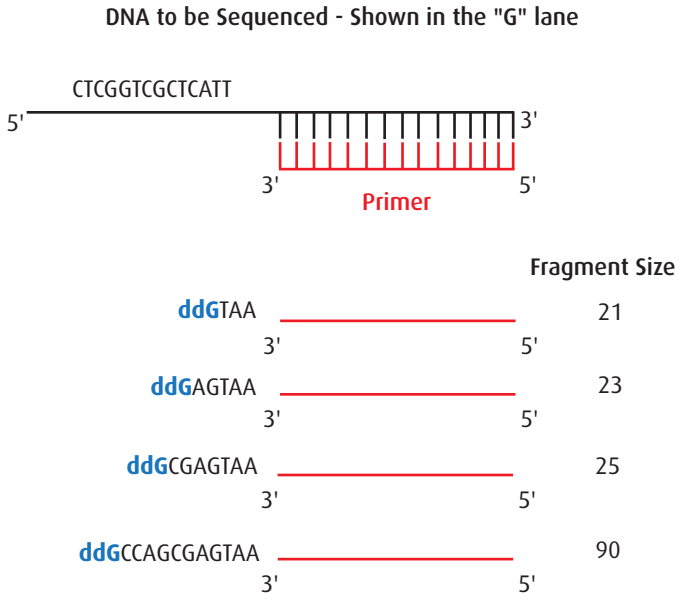


Figure 3: Random Incorporation of ddNTPs

During a sequencing experiment four separate enzymatic reactions are performed, one for each ddNTP. For example, the "G" reaction contains dideoxyGTP, while the "C" reaction contains dideoxyCTP. As the sequencing experiment progresses the ddNTPs will be randomly added to terminate the growing chain. Since the reaction contains only one type of ddNTP, each reaction will contain a population of molecules with the same 5′ start site and with the same ddNTP base at the 3′ end. However, since the ddNTP is inserted randomly the reaction will contain nucleotide fragments of different sizes, depending on when the ddNTP was incorporated (Figure 3).

The next step in a sequencing reaction involves using polyacrylamide gel electrophoresis to analyze the sequencing fragments. First, the sequencing reactions are added into depressions (or "wells") within a polyacrylamide gel and an electrical current is passed through the gel. Because the sugar-phosphate backbone of DNA has a strong negative charge, the current drives the DNA through the gel towards the positive electrode. At first glance, a polyacrylamide gel appears to be a solid at room temperature. However, on the molecular level, the gel contains small channels through which the DNA can pass. Small DNA fragments move through these holes easily, but large DNA fragments have a more difficult time squeezing through the tunnels. Because molecules of different sizes travel at different speeds, they become separated and form discrete "bands" within the gel. This technique is precise enough to distinguish fragments that differ in size by a single nucleotide. When used after sequencing, gel electrophoresis of the four ddNTP reactions can reveal the DNA's sequence, with each fragment corresponding to a different nucleotide position.

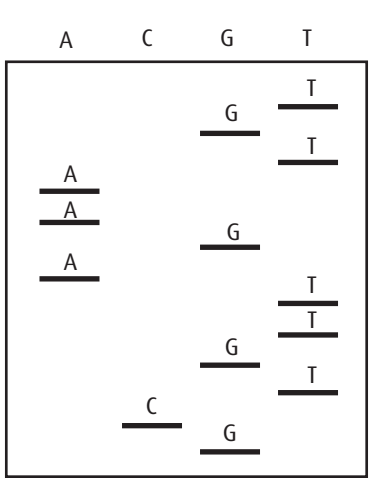
While polyacrylamide gel electrophoresis can separate the DNA fragments, the DNA bands are initially clear and colorless. To visualize the DNA, scientists add radioactive dATP into the four reactions, allowing it to incorporate into the DNA strands. After the electrophoresis is completed, the radiolabeled DNA can be visualized by autoradiography. The polyacrylamide gel is placed into contact with a sheet of x-ray film, allowing the DNA fragments to create a dark exposure band on the film. Since the smallest fragments move through the gel faster than the larger fragments, the sequence is read from the bottom of the gel to the top. For example, the simulated autoradiograph (or "autorad") in Figure 4 would result from Sanger sequencing analysis.

DNA sequencing allows us to understand the kind of genetic information that a segment of DNA carries. For example, in sections of DNA that code for a protein the nucleotide order determines amino acid order. This in turn

Background Information

determines the type of protein produced by a cell. Because of this, sequence information can be used to analyze genes for changes, know as mutations. Sequencing has become essential for many different scientific disciplines. For example, molecular biologists and geneticists use sequencing information to explore how our genes affect cellular behavior. Similarly, evolutionary biologists can use sequence information to provide clues about similarities and differences between species. Finally, doctors and researchers use DNA sequencing to diagnose harmful mutations and provide counseling and preventative care for diseases.

Figure 4: Simulated Sequencing Gel



Deduced Sequence: GCTGTTAGAATGT

In the laboratory, scientists identify mutations by comparing their sequence information to a reference sequence that represents the "wild type", or established, sequence of the gene (summarized in Figure 5). One common type of mutation is called a substitution mutation. In a substitution mutation one base pair nucleotide is replaced by another base pair nucleotide – for example a T replaces a G. Many times, the mutation is silent, meaning that the mutation is not harmful to the organism. When the sequence is in a coding region such a change can alter the protein product by changing one amino acid in the protein sequence (missense) or by creating a new stop signal that ends the creation of that protein (nonsense).

Two other types of mutations are insertion and deletion mutations. An insertion mutation changes the number of DNA bases by adding a piece of DNA, whereas a deletion mutation changes the number of DNA bases by removing a piece of DNA. These insertions or deletions, known collectively as "indels", can be as small as one base pair or as large as an entire chromosome region. Even a one base pair indel can cause dramatic changes when it occurs in a protein-coding region. For example, if an insertion occurs and the number of inserted nucleotides is not a multiple of three then every amino acid following the insertion will be different. The same is true for deletions. This type of change, where the grouping of the nucleotides is changed, is known as a frame shift mutation.

In this experiment, you will read the autoradiograph of DNA sequenced from the bacteria virus M13. The nucleotide sequence is then compared with a reference sequence to identify any mutations.

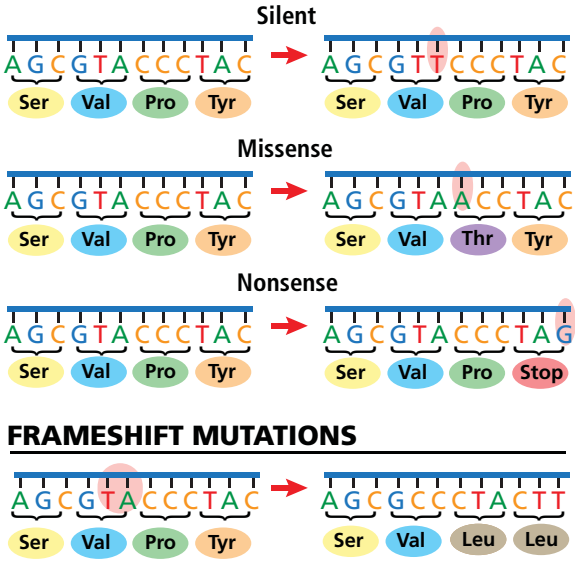


Figure 5: Substitution and Frameshift Mutations

Experiment Procedures

EXPERIMENT OBJECTIVE:

The objective of this experiment is to develop an understanding of DNA sequencing and analysis. This is a dry lab which contains autoradiographs from an actual DNA sequencing experiment.

EXPERIMENT PROCEDURE:

1. Obtain the sample autoradiograph and place it on a light box to enhance visualization.
2. The sequencing reactions have all been loaded in order: G-A-T-C.
3. Begin analysis of the DNA sequence at the bottom of the autoradiograph with the circled band, which is an A.
4. Compare the deduced sequence to the wild type sequence shown in Figure 6.
5. Identify the location of the mutant nucleotide. What was the mutation? Is there more than one mutation?

5'-AGCTTGGCTGCAGGTCGACGGATCCCCGGGAATTCGTAATCATGGT-3'

Figure 6: Wild Type Reference Sequence

Study Questions

1. Why is DNA important? What is it made of?
2. What is DNA Sequencing?
3. Why are Sanger sequencing experiments divided into four reactions?
4. Name and describe five reagents needed to carry out Sanger sequencing.

Please refer to the kit insert for the Answers to Study Questions