Experiment (9): Sanger Sequencing

Aim:

- To determine the order of the nucleotides in a given DNA sample.

Introduction:

The term DNA sequencing refers to methods for of determining the precise order of nucleotide bases (As, Ts, Cs, and Gs) in a molecule of DNA. Knowledge of DNA sequences has become necessary numerous applied fields such as medical diagnosis, biotechnology and forensic biology. A sequencing can be done by different methods including: Maxam – Gilbert sequencing (chemical degradation method), **Sanger sequencing** (dideoxy chain-termination method) and high- throughput sequencing technologies. The most commonly used method is the dideoxy chain termination method developed by Sanger and co-workers in 1975 owing to its relative easy and reliability (**Figure.1**).

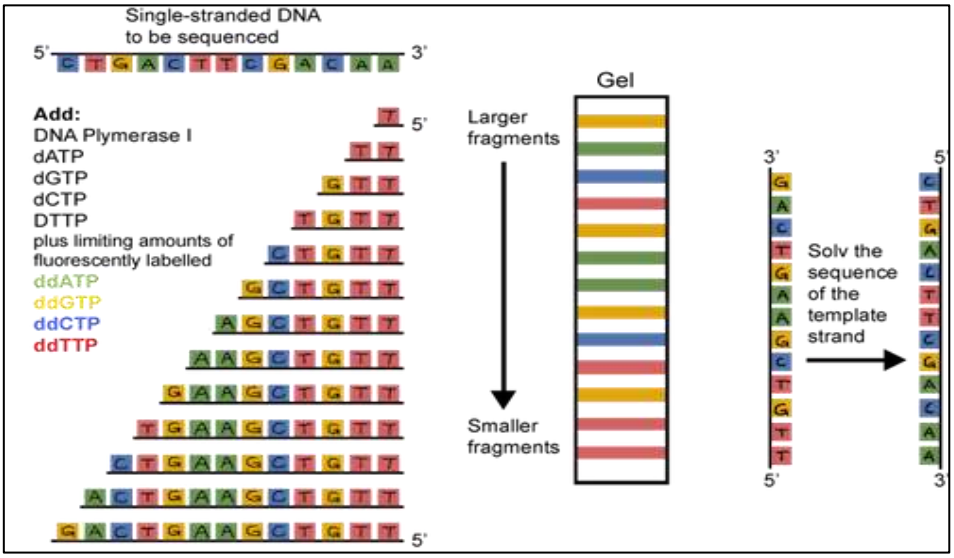
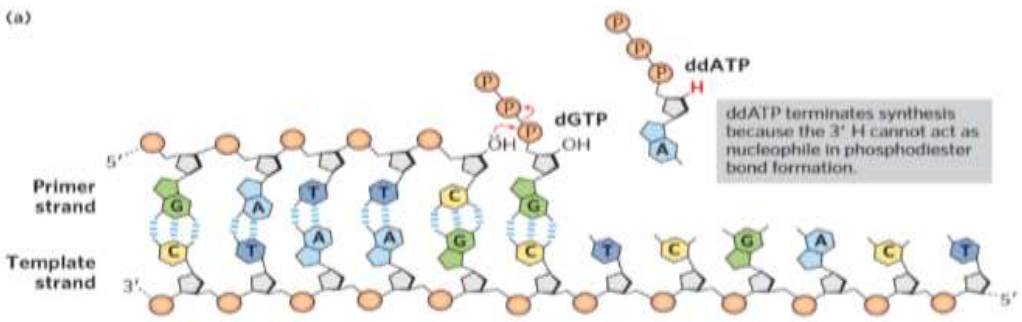


Figure.1. Chain Termination DNA Sequencing.

PAUSE AND THINK ➔ Why the reaction terminated by the ddNTPs (dideoxynucleosides) and cannot be continued?



Principle of automated Sanger method:

In this method, **cycle sequencing**, the **dideoxynucleosides** -not the primers- are tagged with different colored fluorescent dyes, thus all four reactions occur in the same tube and are separated in the same lane on the gel. As each labelled DNA fragment passes through the bottom of the gel, a laser reader detect the fluorescence of each fragment (blue, green, red or yellow) and compiles the data into an image (**Figure.2**).

This method makes use of the mechanism of DNA synthesis by DNA polymerases. It requires the enzymatic synthesis of a DNA strand complementary to the strand under analysis, using **ddNTPs** tagged with fluorescence dye (different color for each nucleotide). In the reaction catalyzed by DNA polymerase, the 3'-hydroxyl group of the primer reacts with an incoming deoxynucleoside triphosphate (dNTP) to form a new phosphodiester bond. The identity of the added deoxynucleotide is determined by its complementarity, through base pairing, to a base in the template strand. In the Sanger sequencing reaction, nucleotide analogs called dideoxynucleoside triphosphates (ddNTPs) interrupt DNA synthesis because they lack the 3'-hydroxyl group needed for the next step. For instance, the addition of ddCTP to an otherwise normal reaction system causes some of the synthesized strands to be prematurely terminated at the position where dC would normally be added, opposite a template dG, and the same for the others nucleotides. This results in different colored DNA fragments, which can be separated by size in an electrophoretic gel in a capillary tube. All fragments of a given length migrate through the capillary gel together in a single band, and the color associated with each band is detected with a laser beam. The DNA sequence is read by identifying the color sequences in the bands as they pass the detector. The amount of fluorescence in each band is represented as a peak in the computer output.

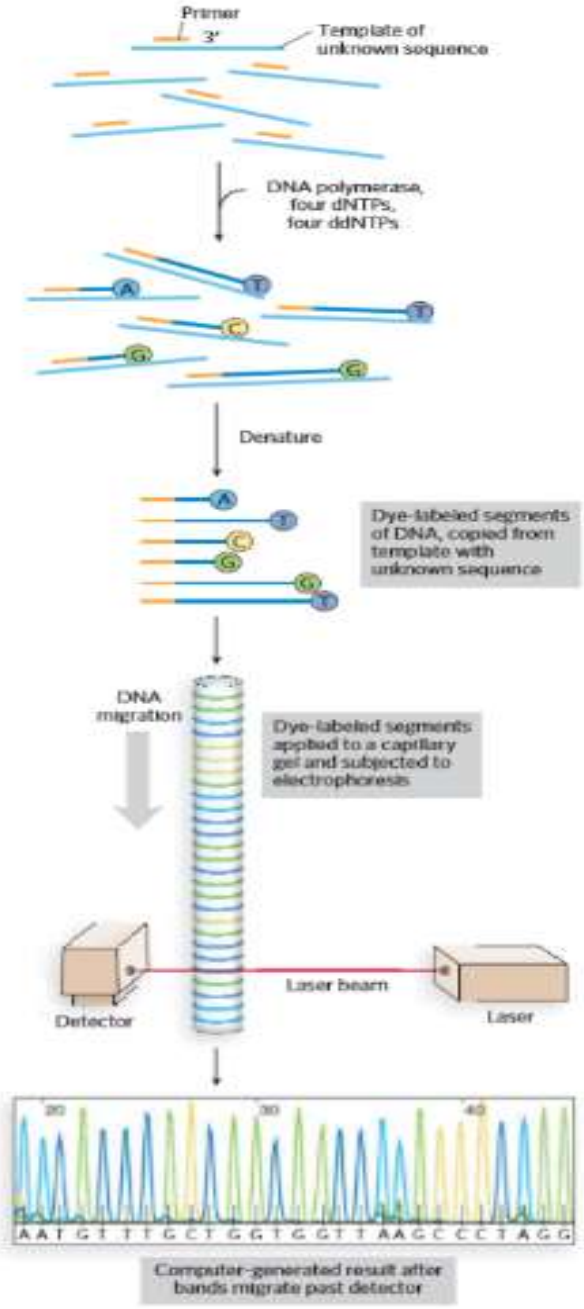


Figure.2. Automation of DNA-sequencing reactions.

Sanger sequencing performing steps:

1. PCR amplification.
2. Purification of PCR product: removal of unwanted primers and dNTPs from PCR product mixture.
3. Sequencing reaction.
4. Post reaction clean-up: The post sequencing reaction product needs to be purified for removal of excess dye terminators and unused primer by using ethanol precipitation protocol.
5. Capillary electrophoresis.
6. Data analysis

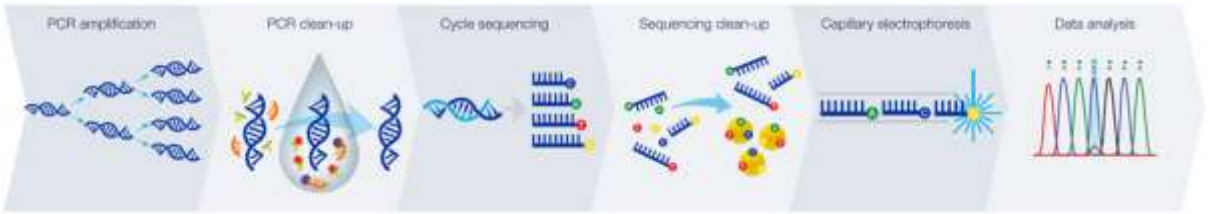


Figure.3. Sanger sequencing workflow.

Sanger sequencing application:

Sanger sequencing supports a wide range of DNA sequencing applications including:

1. Single nucleotide polymorphism (SNP) detection.
2. Single-strand conformation polymorphism (SSCP).
3. Mutations detections.

References:

1. Cox M, Doudna J, O'Donnell M. Molecular Biology genes to proteins. p.226. (2012).
2. http://www.thermofisher.com/sa/en/home/life-science/sequencing/sanger-sequencing/sanger-sequencing-workflow.html
3. Munshi A. DNA sequencing –methods and applications. InTech, 2012.