Lecture 29: DNA sequence analysis methods-I

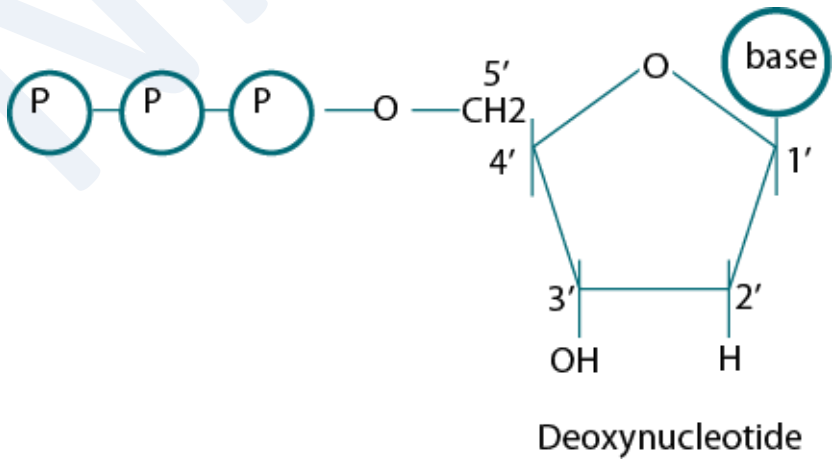
Introduction:

Before 1970's there was no direct method to determine the nucleotide sequence. In the mid of 1970's, two methods developed for the direct sequencing of DNA. These were the Sanger Coulson's chain termination method and Maxam Gilbert's chain termination method. For which they shared Nobel Prize in Chemistry (1980).

Sanger method:

Sanger et al. (1974) used the principle of DNA replication for the development of Dideoxy Sequencing method. For the coupling of nucleotides, the 3' hydroxyl group is needed. Sanger used this site to develop the chain termination reaction. He used dideoxyribose in which the hydroxyl group was missing at both 2' and 3' carbon places in the ribose sugar. These molecules terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide and the chain proliferation reaction irreversibly stops (Fig. 1).

The chain termination reaction depends on the synthesis of the second strand of the DNA so the template is needed in the single stranded form. Previously the template molecule is generally cloned into M13 vector to get a single stranded DNA and the second strand is enzymatically synthesized. Denaturation with alkali as NaOH, are also being applied to get single stranded DNA.



Deoxynucleotide

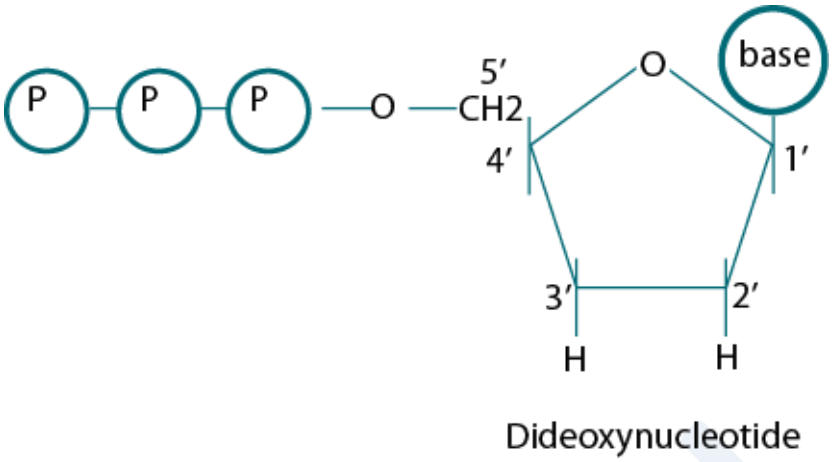


Figure 1: The structure of five carbon sugars deoxy and dideoxynucleotide.

Primer:

In order to perform the sequencing, the first step is the annealing of a gene specific or a universal oligonucleotide primer to the recombinant M13 vector or single stranded DNA. This primer acts as a starting point for the DNA polymerases enzymes for the complementary strand synthesis. Primer is generally radiolabelled to generate the autoradiograph. The label may also introduce into the new strand by adding radiolabelled deoxynucleotides e.g. S^{35} or P^{32} in the reaction mixture (Fig.2).

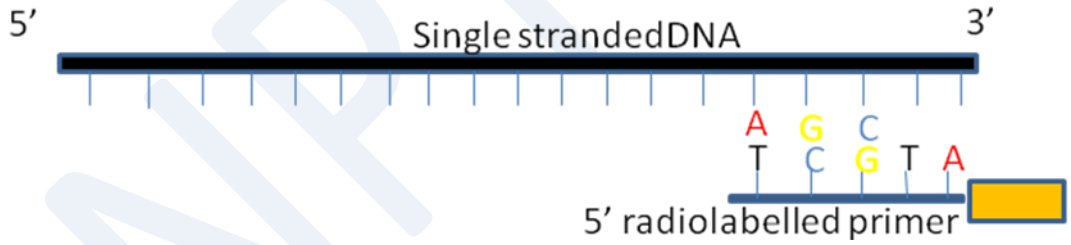


Figure 2: Annealing of primer to the Template DNA.

Enzyme:

To generate a DNA ladder, enzymes are being used that lacks 3'-5' exonuclease activity. Previously Klenow fragment was in use for this purpose and DNA synthesis was done at 37^0C which was the optimum temperature for DNA polymerase I. DNA polymerase adds either deoxynucleotide or the corresponding 2',3' dideoxynucleotide form at the extension step. Presently Sequenase, a modified bacteriophage T7 DNA polymerase has almost replaced the Klenow fragment. Sequenase has higher processivity and it generates bands with

similar intensity and a readable DNA sequence while Klenow has a lower processivity only upto 250 bp. Sequencing polymerases other than sequenases catalyze the incorporation of ddNTPs at only 0.02-1% of the rate of dNTPs but sequenase catalyses the incorporation of ddNTPs at 33% efficiency against its corresponding dNTP. The modified DNA polymerases are formulated in such a way so that they can pass easily through GC stretches and other difficult sequences easily and provide uniform peak heights.

Complementary Strand Synthesis:

The second strand synthesis is started by adding enzyme to a reaction mixture containing single strand DNA template with 5' radiolabelled DNA primers that are complementary to single stranded template, a mixture of dNTP with its dideoxydNTP form and the remaining three dNTPs. At a time four parallel reaction performed with different dideoxynucleotide i.e. dideoxyATP, dideoxyTTP, dideoyGTP, dideoxyCTP for each reaction. The concentration of dideoxyNTP should be only 1% of that particular dNTP to get a whole series of labelled strands ends up with that dideoxy NTP. The lengths of these strands are dependent on the location of the base relative to the 5' end (Fig. 3).

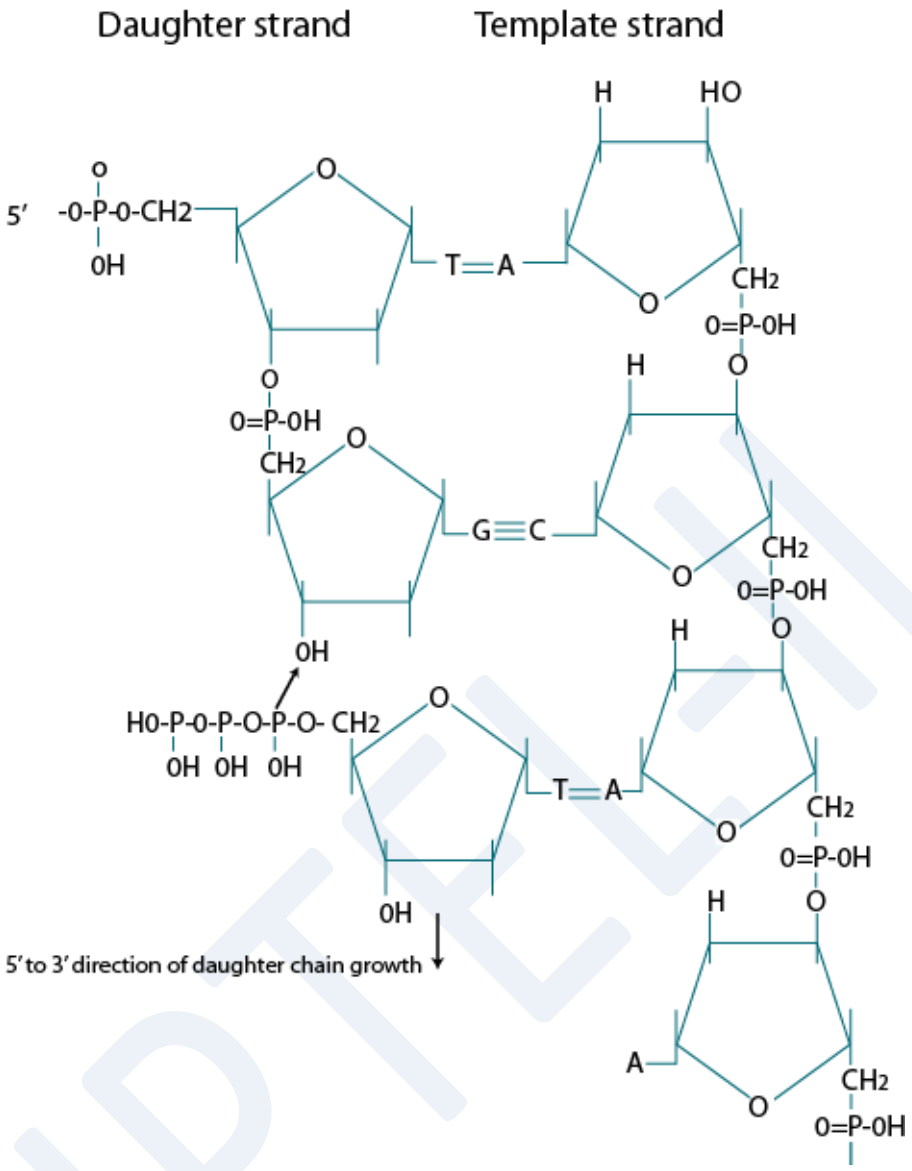


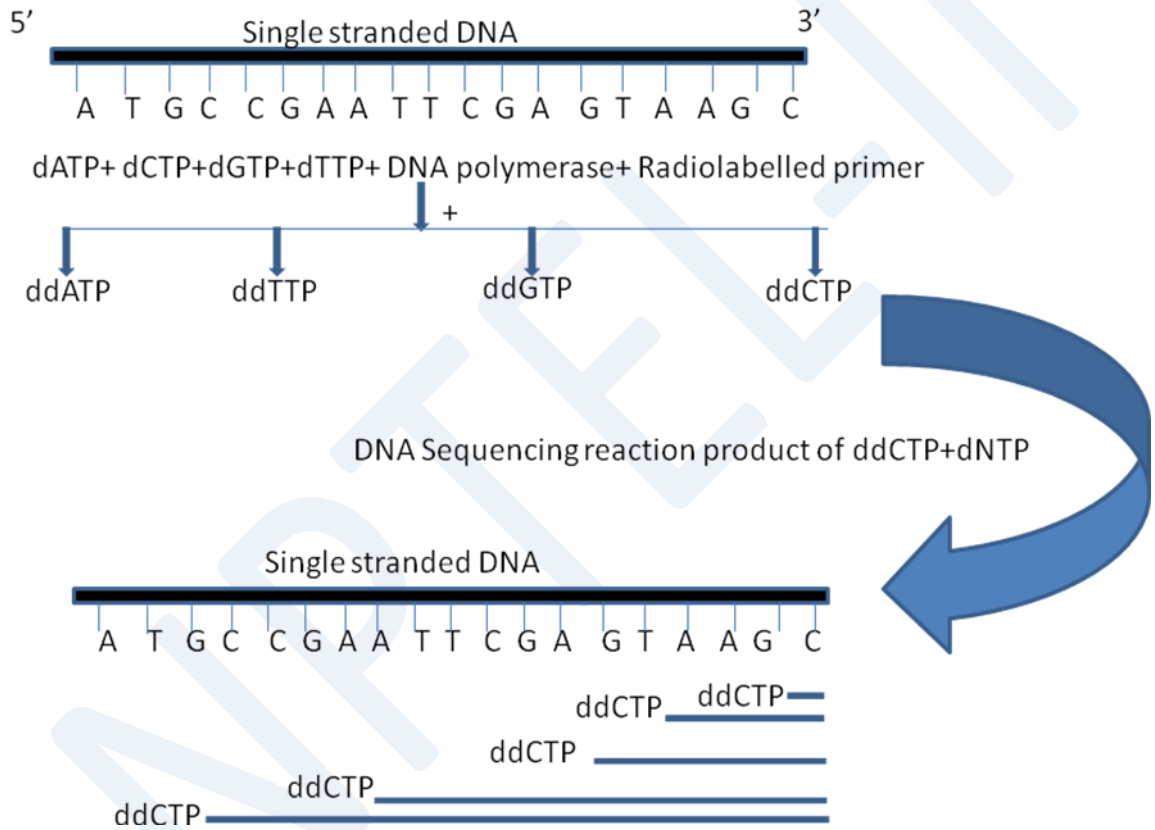
Figure 3: Growing Daughter strand with reference to the template nucleotide.

DNA Sequence Analysis by Autoradiograph:

After the completion of each reaction, Polyacrylamide gel electrophoresis (PAGE) is performed. Each reaction mix is loaded in a separate lane. The reaction condition should be carefully controlled to separate the strands that differ just by a single nucleotide. PAGE is done in denaturating condition in presence of urea or less frequent formamide. Urea and formamide lowers the melting point of DNA molecule, denatures DNA by disrupting the H bond and the newly synthesized strand separates from the template strand.

Electrophoresis is carried out at high voltage to prevent the renaturation of DNA due to high heat generation in gel.

After complete run, the gel is transferred on nitrocellulose filter and autoradiography is performed so the only bands having the 5' radiolabelled molecule will be visible as bands. In PAGE the shortest fragment moves faster so the bottom most molecule is the first dideoxynucleotide which stopped the chain elongation by its incorporation and thus that should be the first sequenced nucleotide. So by this bottom up approach in all the lanes we can get a combined DNA sequence of the query (Fig .4).



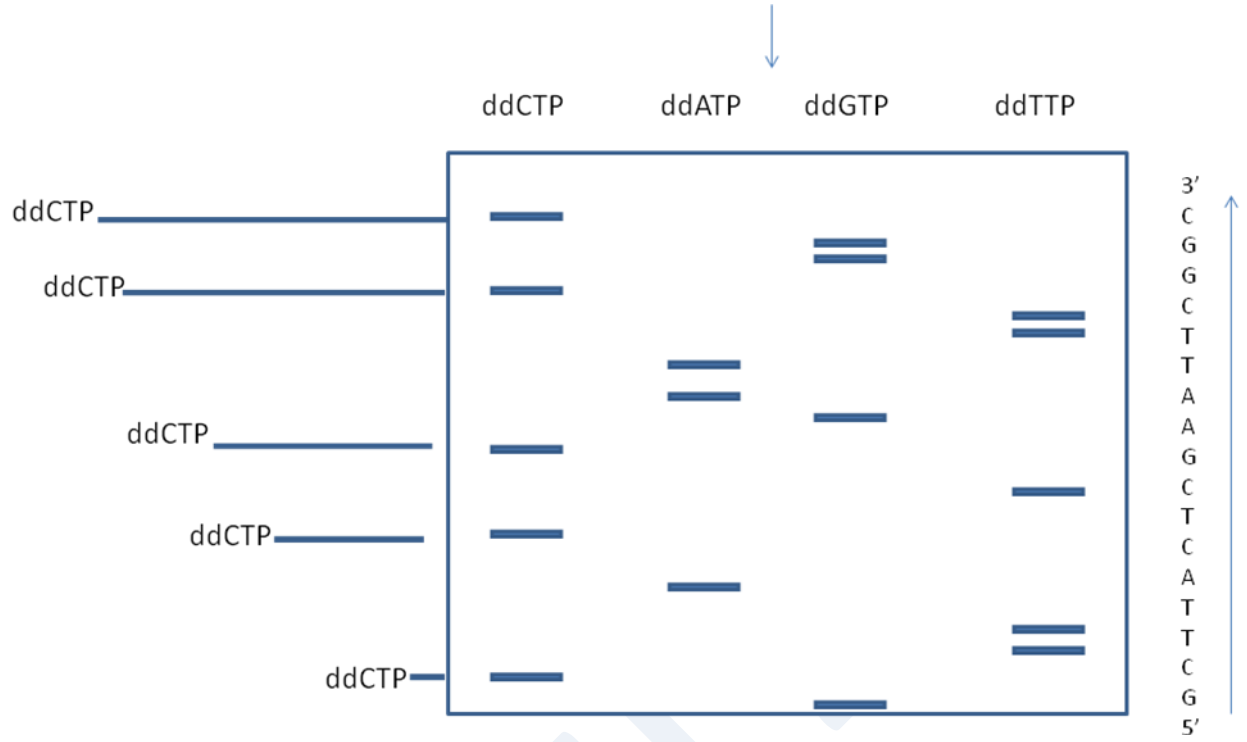


Figure 4: A schematic diagram of Sanger DNA sequencing method with an autoradiograph. The sequence is in the 5' to 3' direction and is the complementary of query.