DNA SEQUENCING:

• Compare and contrast the chemical (Maxam/Gilbert) and chain termination (Sanger) sequencing methods.
• List the components and molecular reactions that occur in chain termination sequencing.
• Discuss the advantages of dye primer and dye terminator sequencing.
• Derive a text DNA sequence from raw sequencing data.
• Describe examples of alternative sequencing methods, such as bisulfite sequencing and pyrosequencing.
UNIT-IV DNA SEQUENCING

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Sequencing Methods

- Maxam/Gilbert chemical sequencing
- Sanger chain termination sequencing
- Pyrosequencing
- Array sequencing
Maxam-Gilbert sequencing is performed by chain breakage at specific nucleotides.
Maxam-Gilbert Sequencing

Sequencing gels are read from **bottom to top** (5′ to 3′).
Chain Termination (Sanger) Sequencing

- A modified DNA replication reaction.
- Growing chains are terminated by dideoxynucleotides.
Chain Termination (Sanger) Sequencing

The 3′-OH group necessary for formation of the phosphodiester bond is missing in ddNTPs.
Chain Termination (Sanger) Sequencing

- A sequencing reaction mix includes labeled primer and template.

- Dideoxynucleotides are added separately to each of the four tubes.
Chain Termination (Sanger) Sequencing

- **ddATP +**
  - dNTPs: four dNTPs
  - ddA: dAdGdCdTdGdCdCdCdG

- **ddCTP +**
  - dNTPs: four dNTPs
  - ddC: dAdGdCdTdGddC
  - dAdGdCdTdGdCdCdCddC

- **ddGTP +**
  - dNTPs: four dNTPs
  - ddG: dAdGddC
  - dAdGdCdTdGdCdCdCddC

- **ddTTP +**
  - dNTPs: four dNTPs
  - ddT: dAdGdCdTdGdCdCdCdG
  - dAdGdCdTdGdCdCdCdG
Chain Termination (Sanger) Sequencing

- With addition of enzyme (DNA polymerase), the primer is extended until a ddNTP is encountered.
- The chain will end with the incorporation of the ddNTP.
- With the proper dNTP:ddNTP ratio, the chain will terminate throughout the length of the template.
- All terminated chains will end in the ddNTP added to that reaction.
Chain Termination (Sanger) Sequencing

• The collection of fragments is a sequencing ladder.
• The resulting terminated chains are resolved by electrophoresis.
• Fragments from each of the four tubes are placed in four separate gel lanes.
Sequencing gels are read from bottom to top (5′ to 3′).
Cycle Sequencing

• Cycle sequencing is chain termination sequencing performed in a thermal cycler.
• Cycle sequencing requires a heat-stable DNA polymerase.
Fluorescent Dyes

• Fluorescent dyes are multicyclic molecules that absorb and emit fluorescent light at specific wavelengths.

• Examples are fluorescein and rhodamine derivatives.

• For sequencing applications, these molecules can be covalently attached to nucleotides.
Fluorescent Dyes

- In **dye primer** sequencing, the primer contains fluorescent dye–conjugated nucleotides, labeling the sequencing ladder at the 5′ ends of the chains.

- In **dye terminator** sequencing, the fluorescent dye molecules are covalently attached to the dideoxynucleotides, labeling the sequencing ladder at the 3′ ends of the chains.
Dye Terminator Sequencing

• A distinct dye or “color” is used for each of the four ddNTP.

• Since the terminating nucleotides can be distinguished by color, all four reactions can be performed in a single tube.

The fragments are distinguished by size and “color.”
Dye Terminator Sequencing

The DNA ladder is resolved in one gel lane or in a capillary.
Dye Terminator Sequencing

- The DNA ladder is read on an **electropherogram**.
Automated Sequencing

- Dye primer or dye terminator sequencing on capillary instruments.
- Sequence analysis software provides analyzed sequence in text and electropherogram form.
- Peak patterns reflect mutations or sequence changes.
Alternative Sequencing Methods:

**Pyrosequencing**

- Pyrosequencing is based on the generation of light signal through release of pyrophosphate (PPI) on nucleotide addition.
  
  - \( \text{DNA}_n + \text{dNTP} \rightarrow \text{DNA}_n+1 + \text{PP}_i \)

- PPI is used to generate ATP from adenosine phosphosulfate (APS).
  
  - \( \text{APS} + \text{PP}_i \rightarrow \text{ATP} \)

- ATP and luciferase generate light by conversion of luciferin to oxyluciferin.
Alternative Sequencing Methods:

Pyrosequencing

- Each nucleotide is added in turn.
- Only one of four will generate a light signal.
- The remaining nucleotides are removed enzymatically.
- The light signal is recorded on a pyrogram.
Alternative Sequencing Methods:

**Bisulfite Sequencing**

- Bisulfite sequencing is used to detect *methylation* in DNA.
- Bisulfite deaminates *cytosine*, making uracil.
- Methylated cytosine is not changed by bisulfite treatment.
- The bisulfite-treated template is then sequenced.
Alternative Sequencing Methods:
Bisulfite Sequencing
The sequence of treated and untreated templates is compared.

Methylated sequence: $\text{GTC}^{\text{Me}}\text{GGC}^{\text{Me}}\text{GATCTATC}^{\text{Me}}\text{GTGCA}...$

Treated sequence: $\text{GTC}^{\text{Me}}\text{GGC}^{\text{Me}}\text{GATUTATC}^{\text{Me}}\text{GTGUA}...$

DNA Sequence:
(Untreated) reference: ...$\text{GTGGCCGATCTATCGTGCA}...$
Treated sequence: ...$\text{GTGGCGATUTATCGTGUA}...$

This sequence indicates that these Cs are methylated.
Summary

- Genetic information is stored in the order or sequence of nucleotides in DNA.
- Chain termination sequencing is the standard method for the determination of nucleotide sequence.
- Dideoxy-chain termination sequencing has been facilitated by the development of cycle sequencing and the use of fluorescent dye detection.
- Alternative methods are used for special applications, such as pyrosequencing (for resequencing and polymorphism detection) or bisulfite sequencing (to analyze methylated DNA).