ADVANCED MOLECULAR BIOLOGY
BIOCHEM-602 3(3+0)

Block 1: Unit 3

NEXT GENERATION SEQUENCING (PYRO SEQUENCING)

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LECTURE NO-9 TO 10

Objective-

- **Introduction**
- **Classification of Next Generation Sequencing**
- **Introduction of Pyro-Sequencing**
- **Mechanism of Pyro-sequencing**
- **Application of pyro-sequencing**

Next Generation Sequencing (NGS)

- Next Generation Sequencing (NGS) is a powerful platform that has enabled the sequencing of thousands to millions of DNA molecules simultaneously.
- Next-generation sequencing (NGS), also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern sequencing technologies.
- The high demand for low-cost sequencing has driven the development of high-throughput sequencing which produce thousands or millions of sequences at once.

- They are intended to lower the cost of DNA sequencing beyond what is possible with standard dye-terminator methods.
- Thus, these recent technologies allow us to sequence DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, and as such have revolutionized the study of genomics and molecular biology.
- Classified to different generations, NGS has led to overcome the limitations of conventional DNA sequencing methods and has found usage in a wide range of molecular biology applications.

Classification of NGS

The generations it is classified into include:

First Generation

1. Sanger Sequencing

Second Generation Sequencing

1. Pyrosequencing
2. Sequencing by Reversible Terminator Chemistry
3. Sequencing by Ligation

Third Generation Sequencing

1. Single Molecule Fluorescent Sequencing
2. Single Molecule Real Time Sequencing
3. Semiconductor Sequencing
4. Nanopore Sequencing

- **Fourth Generation Sequencing**

1. Aims conducting genomic analysis directly in the cell.

SECOND GENERATION SEQUENCING (PYROSEQUENCING)

- A parallelized version of pyrosequencing was developed by 454 Life Sciences, which has since been acquired by Roche Diagnostics.
- The method amplifies DNA inside water droplets in an oil solution (emulsion PCR), with each droplet containing a single DNA template attached to a single primer-coated bead that then forms a clonal colony.
- The sequencing machine contains many picolitre-volume wells each containing a single bead and sequencing enzymes.

- Pyrosequencing uses luciferase to generate light for detection of the individual nucleotides added to the nascent DNA, and the combined data are used to generate sequence read-outs.
- This technology provides intermediate read length and price per base compared to Sanger sequencing on one end and Solexa and SOLiD on the other.

Process of Pyrosequencing

Pyrosequencing occurs in six major steps:

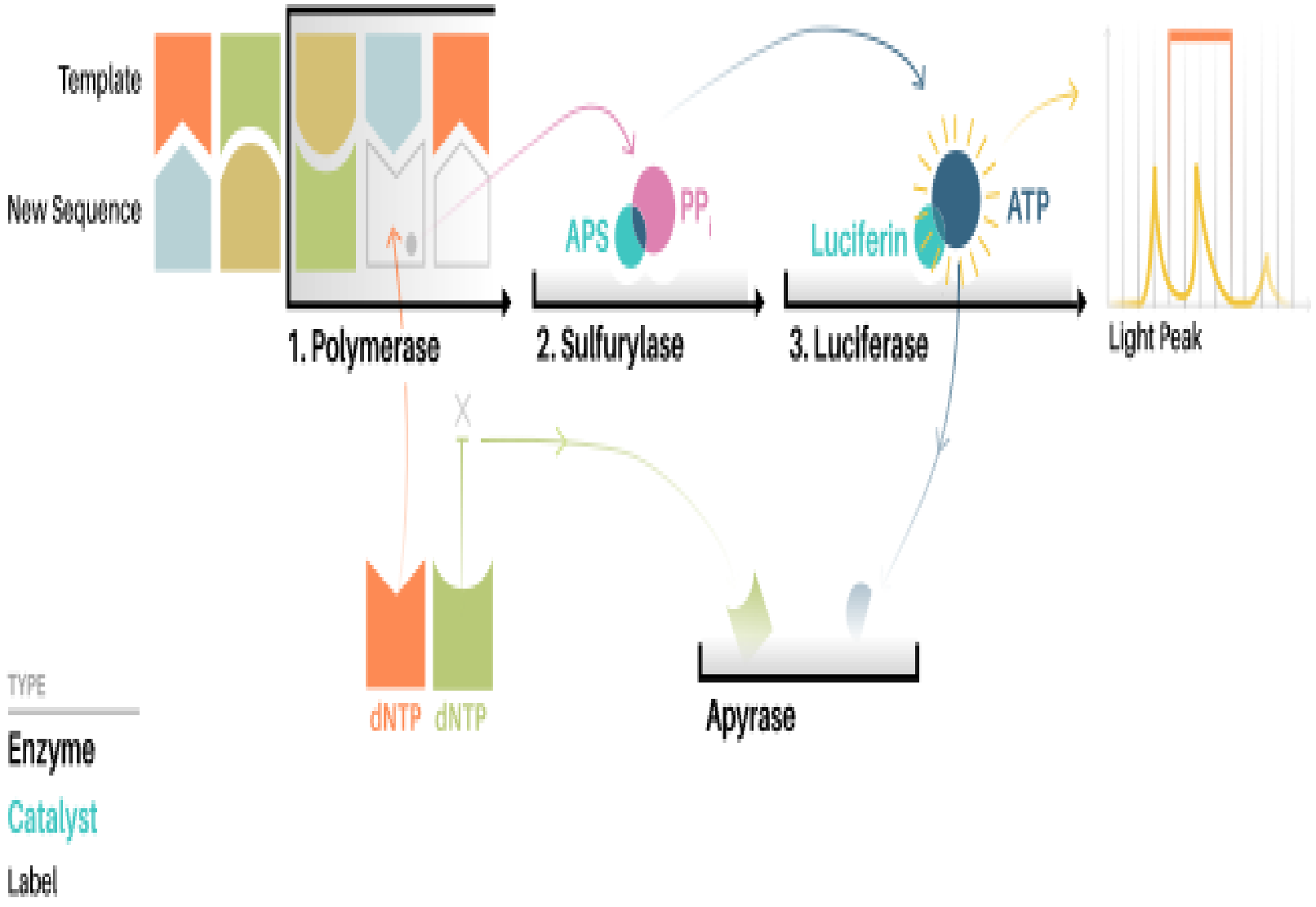
1. The DNA that is to be sequenced is broken up into fragments of around 100 base pairs of single-strand DNA.
2. A polymerase chain reaction (PCR) is run to create millions of identical copies of each DNA fragment, which are split across thousands of wells, with just one type of DNA fragment per well.
3. The DNA fragments are incubated with DNA polymerase, ATP sulfurylase, and apyrase enzymes, and adenosine 5’ phosphosulfate and luciferin substrates.

4. One of the four types of nucleotides that make up DNA are added to the wells, which begin to be incorporated onto the single-strand DNA template by DNA polymerase at the 3’ end, releasing pyrophosphate.ATP sulfurylase then converts pyrophosphate to adenosine triphosphate (ATP) in the presence of adenosine 5’ phosphosulfate. ATP then takes part in the luciferase-mediated conversion of luciferin to oxyluciferin. This process emits light proportionately to the amount of ATP taking part in the conversion, which is picked up by a detector.
5. Unused nucleotides and ATP degrade to apyrase, allowing the reaction to start again with another nucleotide. This process is repeated, adding each nucleotide one after the other until the synthesis is complete.

6. A detector picks up the intensity of light emitted by the process, which is then used to infer the number and type of nucleotides added. For example, if three cytosine nucleotides are added sequentially to the same fragment of DNA, the light emitted will be three times more intense than fragments of DNA with only one cytosine nucleotide added.

If no light is emitted upon the addition of cytosine, then the next complimentary base in the single-strand DNA template must be one of the other three nucleotides.

It should be noted that of the four nucleotides added during pyrosequencing, deoxyadenosine triphosphate (A) is replaced with deoxyadenosine α-thio triphosphate to avoid a false signal from early reaction with luciferase.


Template
New Sequence
1. Polymerase
APS
PPi
2. Sulfurylase
Luciferin
ATP
3. Luciferase
Light Peak
X
dNTP
dNTP
Apyrase
TYPE
Enzyme
Catalyst
Label

Applications of pyrosequencing

1. Pyrosequencing is used to reveal the genetic code of a section of DNA. It is also able to detect single nucleotide polymorphisms, insertion-deletions or other sequence variations, in addition to being able to quantify DNA methylation and allele frequency.
2. For example, genome regions associated with a genetic disorder can be narrowed down further to identify specific genes and variations within them, or an inherited negative response to certain drugs can be checked for before the drug is prescribed.
3. The method of drug resistance in a new strain of bacteria can be determined by its genetic change compared to an ancestor, or epigenetic methylation of DNA in a cancer cell can be detected in order to determine the best course of treatment.