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Pyrosequencing: Principles of A Next Generation Sequencing Technology

Priyanka Kumari[1], Jenny Priya Ekka[2], S K Tirkey[3], Ekhlaque Ahmad[4]

[1,2,3] Department of Genetics and Plant Breeding, Birsa Agricultural University, Ranchi-834006, Jharkhand, India;

[4]Zonal Research Station (Birsa Agricultural University), Chianki, Palamau-822102

***Corresponding author:** 22prinku147.pk@gmail.com*

Introduction: Pyrosequencing, is an appropriate detection technology which is also considered as a next generation sequencing method most probably centered on the principle of sequencing-by-synthesis that provides an advantage of quantitative real-time data to the researcher. Basically, a sequencing primer is hybridized to a single-stranded PCR amplicon which is used as a template and sequencing is based on the detection of the pyrophosphate (PPi) that is released during DNA synthesis (Ronaghi, 2001). It has been intensely utilized in characterizing single nucleotide polymorphisms (SNPs), insertion-deletions (indels), and several unknown sequence variants. In addition to this, it can considerately quantify frequencies of alleles and levels of DNA methylation at both CpG and non-CpG (CpN) sites. Pyrosequencing (Nyren and Skarpnack, 2001) with the PyroMark platform outperforms other sequence-based solutions by integrating detection and quantification of genetic variation into one powerful system for the analysis of targeted short DNA sequences. The essential features of this technique of DNA sequencing are as follows. It is based more particularly on four enzymes *i.e.* DNA polymerase, ATP sulfurylase, luciferase and apyrase. It also utilizes luciferin and adenosine 5-phosphate (APS) (Gharizadeh *et al.,* 2007).

Addition of deoxyribonucleotides during polymerase action releases pyrophosphate (PPi) residues and usually the klenow fragment of *E. coli* DNA polymerase serves as the polymerase enzyme which shows relatively slow polymerase lacking 3'-5' exonuclease function. The enzyme apyrase extracted from potato sequentially degrades the unincorporated nucleoside triphosphates to nucleoside diphosphates and finally to nucleosides. It degrades all the four dNTPs but the rate of degradation is slower than rate of dNTP incorporation by polymerase so that sufficient dNTPs are used for DNA synhesis. The enzyme ATP sulfurylase which converts adenosine 5'-phosphate (APS) into ATP by using the PPi released during DNA polymerase action is a recombinant version of the yeast enzyme (Karamohamed *et al.,* 1999). Here, the rate of ATP synthesis by this enzyme is much faster than ATP hydrolysis by apyrase so that adequate concentration of ATP is available for luciferase action. The enzyme luciferase acts on these ATPs to convert luciferin to oxyluciferin which generates visible light. The amounts of emitted light are probably proportional to the amount of ATP synthesized. PCR generated purified single-stranded DNA is used as a template for DNA polymerase. Pyrosequencing can be done either in solid-phase or in liquid phase. For solid phase system, the PCR uses biotinylated nucleotides in

the last cycle so that the PCR product is captured on streptavidin-coated magnetic beads followed by washing to yield double-stranded DNA. The biotinylated and non-biotinylated strands can then be seperated for sequencing to yield high quality data. Liquid-phase system is based on enzyme template preparation. It uses nucleotide degrading enzyme *i.e.* apyrase for the removal of dNTPs and exonuclease 1 for the removal of PCR primers. These enzymes are added to PCR product where the amplicon is double-stranded. The sequencing primer is then added after a period of incubation at room temperature or at 35^{0}C and the mixture is heated to inactivate the two enzymes. Finally cooling is done which allows annealing of the sequencing primer to pair with the template which is now ready for pyrosequencing.

Principles of Pyrosequencing

In pyrosequencing method, a reaction mixture is prepared which contains the DNA template, sequencing primer, adenosine 5'-phospahte (APS), luciferin and the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase. Then four dNTPs are added sequentially one after the other to this reaction mixture. The principle of sequence determination is discussed below:

A DNA segment is amplified and the strand which serves as the template for Pyrosequencing is biotinylated. From this, biotinylated single-stranded PCR amplicon is isolated after denaturation and hybridization is allowed with a sequencing primer. The biotinylated single-stranded template and hybridized primer are kept in incubation period with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase, as well as the substrates adenosine 5' phosphosulfate (APS) and luciferin. After the addition of first deoxyribonucleotide triphosphate (dNTP) to the reaction, it may or may not be incorporated at 3'end of primer depending on the complementarity. Addition of the dNTP to the sequencing primer is however catalysed by DNA polymerase if it is complementary to the base in the template strand. In each incorporation, pyrophosphate (PPi) is being released in a quantity equimolar to the amount of incorporated nucleotide.

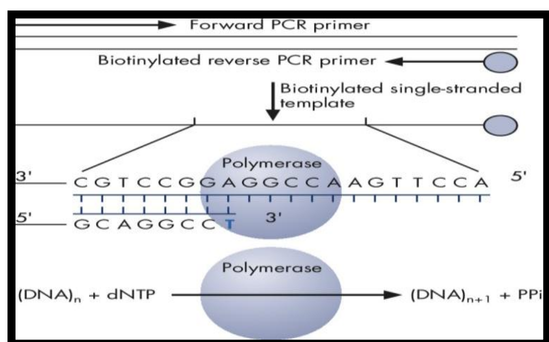
The enzyme ATP sulfurylase utilizes the PPi residues to convert APS molecules into ATP. The produced ATP is used in the conversion of luciferin to oxyluciferin mediated by luciferase generating visible light which are proportional to the amount of ATP produced. CCD sensors are installed for detecting the light produced in the luciferase-catalyzed reaction and can be observed as a peak in the pyrogram. The height of each peak or light signal obtained is directly proportional to the number of nucleotides incorporated.

Nevertheless, the enzyme apyrase degrades unincorporated nucleotides and ATP continuously. As a result, the light production due to ATP stops and the free dNTP molecules get hydrolysed. Another nucleotide can then be sequentially added after the completion of degradation.

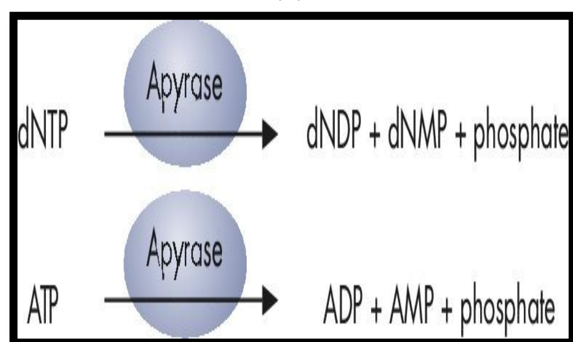
As a substitute for natural deoxyadenosine triphosphate (dATP), usually deoxyadenosine alfa-thio triphosphate (dATPαS) is used that are not recognized by luciferase enzyme as well as efficiently used by DNA polymerase.

Various reactions catalysed by the four enzymes used in pyrosequencing

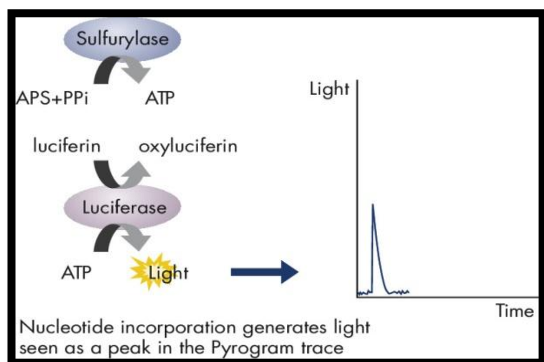
1. Release PPi by DNA Polymerase Action
2. ATP synthesis by ATP Sulfurylase
3. Luciferase uses ATP to generate visible light
4. Pyrase action degrades dNTPs



(a)



(b)



(c)

Fig 1. Diagrammatic representation of the pyrosequencing technique of detection. (a) Roles of all the four enzymes used are depicted (a) DNA polymerase (b) apyrase (c) sulfurylase and luciferase

Advantages

The technique of pyrosequencing required very small quantities of DNA; even one picomole of DNA generates enough light to be detected by using charge-coupled device (CCD) camera. It is very rapid such that overall reaction from polymerization to light detection takes only3-4 sec. at room temperature. It also has accuracy of 99% in SNP genotyping with automated systems. However, the entire process including reading of pyrograms has been automated and it is highly cost competitive in nature utilizing standard pyrosequencing techniques.

Limitations

It can be used for sequencing relatively short segments of DNA and sequences beyond 300-500 nucleotides are unusual. Therefore sequences containing a large amount of repetitive DNA are difficult to be sequenced by this method. Moreover, lack of proof-reading activity limits the accuracy of this technique.

Conclusion

The technique of pyrosequencing is a method of real time detection of DNA synthesis through bioluminescence. It has a wider range of applications in the field of genomic studies such as SNP genotyping, gene identification, microbial genotyping, multiplex sequencing, clone checking and detection of mutation. Moreover, it can be further improved in its accuracy as well as in minimizing the volumes of reagents utilized which would be more promising in the future.

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