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Next-Generation Sequencing in the Future Diagnosis of Molecular Biology

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ABSTRACT

Next-generation sequencing is an innovative method of recognizing genetic mutations and changes in the sequencing of DNA or RNA. Next-generation sequencing generates large amounts of sequence data faster than the traditional Sanger sequencing approach. It is performed in various steps, including DNA fragmentation, Gene Library, Sequencing, and Data analysis. Second-generation sequencing includes Pyrosequencing and Ion torrent sequencing available for clinical diagnosis. Nanopore sequencing is the fourth hybrid NGS and does not require PCR amplification. NGS is widely used to identify inherited genetic disorders, novel cancer mutations, drug resistance, HLA typing, and sequencing of meta-genomics. It is concluded that NGS has changed the landscape in clinical settings and research studies. Using this technique, a lot of complex sequencing is reachable that was not attainable with traditional methods. Implementing NGS in clinical settings will enhance healthcare outcomes and improve patient care.

Keywords: Next generation sequencing, genome sequencing, Pyrosequencing, Ion torrent

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Introduction

Next-generation sequencing is an innovative method of recognizing genetic mutations and changes in the sequencing of DNA or RNA. Next-generation sequencing (NGS) generates large amounts of sequence data faster than the traditional Sanger sequencing approach. This technique can rapidly analyze entire genomes or different DNA and RNA size sequences by employing a wide range of chemical procedures, matrices, and computational biology tools. DNA sequencing is performed in various steps which include DNA fragmentation, Gene Library, Sequencing, and Data analysis (1). Next-generation techniques revolutionized genomic discovery and helped us to understand molecular patterns, recognition, diagnosis, and treatment options. The Next-generation sequencing (NGS) instruments can produce billions of nucleotides in a very short amount of time, all at a low cost (2).

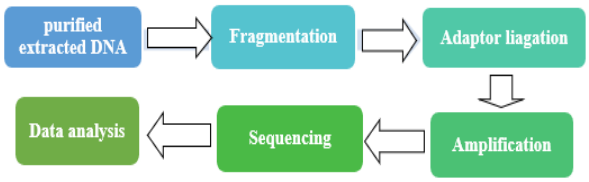

Fig. 1: Workflow of Next-generation sequencing

DNA Fragmentation

Targeted DNA is cleaved into several small segments using different methods like sonication, and enzymatic digestion. The required short segments are extracted employing different methods such as Hybridizations, Capture Assay, and Amplicon Assay.

Genomic library

The organism's genetic material is fragmented into smaller segments and each piece is cloned into a unique vector and inserted in a unique microbial cell. The collection of recombinant vectors represents the whole genome library. This library is useful in the segregation and analysis of specific gene expressions and functions. The first step in DNA sequencing procedures is to join the short oligonucleotide sequences (which are called adapters) to the 3′ and 5′ ends of the broken DNA. The fragmented DNA is connected to solid substrates (such as beads or flow cells) on one end of these 20–40 base pair adapters, which have known sequences, while the other end anneals to a primer to start the polymerase chain reaction (PCR). The process involves creating multiple copies of each DNA fragment using a technique called polymerase chain reaction (PCR). These copies are then sequenced simultaneously in large numbers at the same time, a method known as massively parallel sequencing (1).

Pyrosequencing

The DNA fragment is attached to small plastic beads, and then Visible light is created when a series of enzymatic processes are carried out to produce almost one million copies of DNA fragments by PCR amplification in an oil-water emulsion connected to each plastic bead.

The PCR products undergo denaturation, after which each bead is carefully placed into small wells of picolitre volumes. Within these tiny compartments, three critical enzymatic reactions occur. In the initial stage, DNA synthesis takes place, facilitated by the addition of a primer and a single kind of unlabeled deoxynucleotide triphosphate (dNTP)—such as dATP, dGTP, dCTP, or dTTP—under the action of DNA polymerase. As each nucleotide is incorporated into the elongated DNA strand, pyrophosphate (PPi) is released as a byproduct. Following this, the enzyme ATP sulfurylase catalyzes the conversion of the released PPi into ATP, a crucial energy currency that drives various cellular processes. This reaction sequence is vital for amplifying and providing real-time insights into the sequencing process (3).

Ion Torrent sequencing

In Ion Torrent sequencing, a semiconductor chip, intricately designed with millions of tiny wells, substitutes for processing extensive sequencing information derived from biochemical cues. This advanced method employs Ion Torrent sequencing, initiating the fragmentation of DNA into pieces ranging from 200 to 1500 bases in length. Each fragment is then ligated to specialized adapters, essential for the subsequent steps. The next phase involves the binding of these DNA fragments to beads coated with complementary sequences, ensuring a precise attachment. Emulsion PCR (emPCR) is then employed to amplify the DNA fragments on each bead, leading to the replication of specific DNA sequences. As this process continues, beads are carefully introduced to the chip, where each one is allowed to occupy a distinct well among the millions available. This flow of beads ensures that each well contains a unique DNA fragment, setting the stage for accurate sequencing and analysis. After the sequencing reagents are added to the wells, a signal is generated by the release of a hydrogen ion when the correct nucleotide is incorporated. This changes the pH of the solution which can be detected as a voltage change by an ion sensor, functioning similarly to a pH meter. This incorporation is immediately converted into a voltage signal, which is recorded directly, greatly speeding up the process. One of the main advantages of this system is that it does not require a camera, light source, or scanner. (4)

Nanopore sequencing

Nanopore sequencing works by creating holes through which DNA or RNA molecules pass utilizing transmembrane proteins embedded in a lipid membrane. Alpha hemolysin and Mycobacterium smegmatis porin A (MspA) are two proteins used to form holes. When motor proteins are added, such as phi29, a highly processive DNA polymerase that moves DNA forward upon nucleotide addition, the velocity of DNA transit through the pores is regulated and controlled. That enables unwinding and "ratcheting" of the DNA nucleotides via the nanopore for detection. Furthermore, various accessory proteins are required to assist in the process. These include a DNA helicase, exonuclease I, or oligonucleotides that bind DNA strands. Tens of thousands of nucleotides of DNA can flow through the pores at a steady constant speed without any interruption.

Initially, long dsDNA molecules form a stable attachment to a processive enzyme like phi29 polymerase. Single strands of DNA enter nanopores when the complex encounters them, and DNA polymerase translocation and synthesis control the rate at which the DNA moves through the pore. The

DNA undergoes a continuous and processive "ratcheting" movement facilitated by a specialized processive enzyme. As the enzyme interacts with DNA, it creates a pathway causing a temporary disruption in the applied electrical current whenever a nucleotide progresses through. This phenomenon is known as a current disruption event, and each nucleotide generates a unique electrical signal that can be identified. Because this recording proceeds in real-time, it is theoretically possible for hundreds of kilobases (kb) of DNA to traverse each nanopore and be analyzed. Currently, readings of around 10 kb are considered impressive, but the vision is to extend the segments of DNA and modify our understanding of genetic material at groundbreaking scales. Nanopore sequencing enables PCR-free cDNA sequencing and direct RNA sequencing (1).

Application for Next-generation sequencing

Inherited Genetic Diseases

Multiple genes are associated with multifactorial diseases like diabetes, infertility, late growth and development, and puberty-related disorders. Whole genome sequencing and whole exome sequencing play a vital role in the evaluation of multiple genes and their variants that contribute to this complex disease. By analyzing the entire genome of that patient, researchers identify the protein-coding region that causes the onset and progression of conditions. This allowed the individual approach to the treatment and management strategies.

Identification of novel cancer mutation by NGS

NGS successfully identify the novel mutation of a variety of cancers including small cell lung carcinoma, chronic lymphocytic leukemia, acute myelogenous leukemia, prostate cancer, renal cell carcinoma, and bladder carcinoma (5).

HLA associated disease

The HLA gene is involved in more than 100 multifactorial diseases mainly autoimmune and inflammatory diseases (psoriasis, Rheumatoid arthritis, type diabetes, systemic lupus erythematous SLE) and infectious diseases such as leprosy tuberculosis hepatitis, and other malignancies. It's been challenging to identify the genetic mutation of HLA loci. Next-generation sequencing by gene mapping enhances to determine the unambiguous and unreported alleles. For example, a new association of HLA DPB1 and BTNL2 genes was reported by NGS (6).

Whole genomic sequencing

It is used for the whole genome sequence of rare genetic diseases whose specific mutation is not known and cannot be available for diagnosis by other molecular techniques. This assay helps to investigate dozens and hundreds of genes in cancer patients of myeloid leukemia or carcinoma to find rare allelic mutations by deep sequencing techniques, enabling a more comprehensive study to know the genetic variations contributing to the disease.

Infectious Diseases

The diagnosis of the exact etiological agent in microbial infections is important for proper precision medicine. The valuable information obtained through NGS on microbial identification and drug resistance genotyping, e.g., in MTB, HIV, and SARS-CoV-2 is crucial for the welfare of public health. This data has proven a pivotal role in disease surveillance, public health epidemiological studies, policymaking, disease containment, and rapid therapeutic interventions.

Detection of Drug Resistance

Next-generation sequencing is widely used for analyzing HIV-1 genotypic variation, particularly in drug resistance. This advanced sequencing method enables the determination of the specific mutation that affects the antiretroviral therapies.

Others

DNA Methylation Profiling (important in the regulation and modification of gene and cellular processes) and Histone Modification Analysis (it comprises various chemical changes including acetylation, phosphorylation, and methylation serve as critical epigenetic marks that regulate the shaping of chromatin structure and gene expression (7).

Advantages of Next-Generation sequencing over traditional techniques

1. NGS is a particularly effective tool with the increasing demands for testing multiple gene markers with fewer inputs of nucleic acids.
2. NGS enables parallel sequencing by significantly increasing the speed and efficiency of a genetic disorder
3. NGS is now a more economical choice for sequencing whole genomes or large gene panels.
4. NGS significantly increases the turnaround time for rapid sequencing of clinical isolates, beneficial for timely results and treatment strategies
5. It covers comprehensive genetic variation such as deletion, insertion, and substitutions of complex diseases (8).

Conclusion and future outcomes

Next-generation sequencing has changed the landscape in clinical settings and research studies. Using this technique, a lot of complex sequencing is obtainable that was not attainable with traditional methods. High throughput and scalability enable researchers to analyze the entire sequencing of the genome and targeted sequences in a precise way and contribute to major developments in infectious disease diagnosis, cancer genomics, and personalized medicine. Despite significant achievements in the field, various challenges remain in data analysis and interpretation. These obstacles can be overcome by continuous innovation in bioinformatics tools and the establishment of standardization. Implementing NGS in clinical settings will enhance healthcare outcomes and improve patient care.

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