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Review Article

Biotechnology Section

Whole Genome Sequencing: Advantages of de Novo Hybrid Approach Utilising Illumina and Nanopore Technologies

JANAKIRAM BOBBILLAPATI[1], AS SMILINE GIRIJA[2], JEEVIGUNTA NAVEENA LAVANYA LATHA[3]

ABSTRACT

The groundwork for Next-Generation Sequencing (NGS) was laid by pioneering methods developed by Allan Maxam, Walter Gilbert and Frederick Sanger, a Nobel laureate. While the Sanger method remained standard for many years, technological advancements have led to the widespread adoption of NGS in genomics. NGS has revolutionised genetic diagnostics, significantly improving the speed and accuracy of disease detection. First-generation sequencing, which required labourious efforts to sequence small portions of Deoxyribonucleic Acid (DNA), has now evolved into advanced techniques capable of decoding entire genomes within a day. The introduction of second- and third-generation sequencing has further improved cost-efficiency, speed and precision. The vast amount of data produced through NGS requires sophisticated bioinformatics tools for analysis, which has accelerated advancements in NGS technology. This review provides an in-depth exploration of various NGS techniques, highlighting the advantages of a de novo hybrid approach utilising Illumina and Nanopore for bacterial genome sequencing.

Keywords: Bacterial genome, Deoxyribonucleic acid sequencing, Next-generation sequencing

INTRODUCTION

The discovery of the DNA double helix structure by Watson and Crick in 1953 marked a pivotal moment in genetics. This discovery, combined with advancements in sequencing techniques, culminated in the completion of the Human Genome Project in 2003, which successfully mapped the entire human genome [1]. The project aimed to decode the chemical structure of the 50,000 to 100,000 genes in the human genome, setting the stage for modern genomics. To manage the enormous data generated by such large-scale sequencing projects, researchers needed innovative methods that were faster, more cost-effective and highly accurate. The development of automated sequencing tools allowed for greater sequencing capacity while reducing instrument size and simplifying the entry of sequencing data into databases [2]. In the early stages, sequencing a complete gene was a formidable challenge. Researchers could only sequence small DNA fragments, but the advent of early RNA and DNA sequencing techniques transformed the field. The Maxam-Gilbert chemical degradation method, the first-generation sequencing approach, involved the chemical cleavage of DNA fragments, which were then separated by electrophoresis [3]. This method, though groundbreaking, was complex and hazardous.

Sanger sequencing, a simpler and more reliable technique, soon replaced Maxam-Gilbert sequencing. The automated version of Sanger sequencing, which utilised fluorescent dyes, became the dominant method for the Human Genome Project [4]. Due to its lower chemical requirements and toxicity, Sanger sequencing remains a valuable tool today. However, first-generation methods struggled with speed, cost and scalability, leading to the search for new approaches. The completion of the first human genome sequence fueled the demand for faster and less expensive sequencing methods. Massively parallel sequencing, or NGS, addressed this need by enabling millions of sequencing reactions to occur simultaneously. This method laid the foundation for a major technological shift in genomics [5].

NGS represents a significant advancement over Sanger sequencing. While sequencing the first human genome took years and cost billions, NGS can now sequence a genome in just a few days at a fraction of the cost. Second-generation sequencing methods have improved the speed and affordability of sequencing, while third-generation technologies offer longer read lengths and simpler, more rapid processing with minimal sample preparation. Third-generation single-molecule sequencing, in particular, has eliminated many intermediary steps, such as DNA amplification, further streamlining the process [6].

This review traces the development of DNA sequencing technologies from their origins to the present, focusing on NGS and the benefits of using a de novo hybrid approach that combines Illumina and Nanopore technologies for bacterial genome sequencing.

FIRST-GENERATION DNA SEQUENCING

First-generation DNA sequencing techniques encompass two primary methods: the Maxam-Gilbert (chemical degradation) method and the Sanger (chain termination) method [7]. Both techniques involve amplifying the template DNA, followed by separation through gel electrophoresis. Although pivotal in the sequencing of the human genome, these methods are now considered less efficient due to their higher costs and longer processing times compared to modern sequencing technologies.

Maxam-Gilbert Method

Developed in the late 1970s by Allan Maxam and Walter Gilbert, the Maxam-Gilbert method is based on the chemical degradation of DNA. The process begins with the conversion of DNA into a single strand. An enzyme called alkaline phosphatase removes the phosphate group from the DNA, and polynucleotide kinase attaches a radioactive phosphate group (P32) to the 5' end of the single-stranded DNA. The DNA is then amplified using the Polymerase Chain Reaction (PCR) to generate millions of copies. Specific chemicals are used to cleave the DNA at particular bases (A, G, T, and C), with separate reactions conducted for each base in four different tubes [Table/Fig-1a].

The resulting fragments are separated by gel electrophoresis, which sorts the DNA fragments by size based on their movement through the gel under an electric current. Since DNA carries a negative charge, shorter fragments travel further down the gel. The

radioactive labels are detected by placing the gel onto X-ray film, where the radiation from the labeled fragments darkens the film. The DNA sequence is then determined by reading the band pattern from bottom to top, corresponding to shorter and longer fragments, respectively [1].

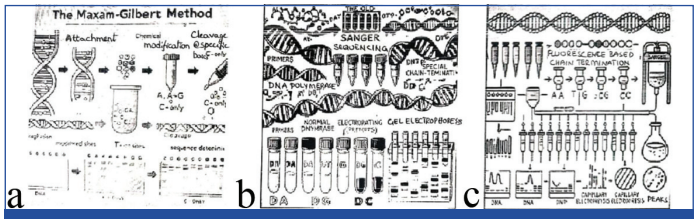
The Maxam-Gilbert method uses specific chemicals for DNA cleavage: dimethyl sulfate for guanine, hydrazine for cytosine, formic acid for both adenine and guanine, and hydrazine in high salt to differentiate between thymine and cytosine. Despite being revolutionary in its time, the method is rarely used today due to several limitations, including the hazardous nature of the chemicals, difficulty in accurately sequencing long fragments (greater than 500 base pairs), and the potential for errors during DNA cleavage.

Sanger Method

Introduced by Frederick Sanger in 1977, the Sanger method relies on the controlled termination of DNA chain elongation [8]. This process involves the use of DNA polymerase and special nucleotides called dideoxynucleoside triphosphates (ddNTPs), which lack the 3' hydroxyl group needed for further elongation. When a ddNTP is incorporated into the growing DNA strand, it prevents the addition of further nucleotides, thus terminating the chain [Table/Fig-1b].

Each ddNTP corresponds to one of the four DNA bases, requiring four separate reactions, one for each base. These reactions contain the template DNA, primer, DNA polymerase, a mix of regular deoxynucleotide triphosphates (dNTPs), and a lower concentration of ddNTPs. As a result, DNA fragments of varying lengths are generated, which are then separated by gel electrophoresis.

Technological advancements have since improved the Sanger method. Fluorescently labeled ddNTPs are now used, allowing all four reactions to be carried out in a single tube. The fluorescent labels are base-specific, enabling simultaneous detection of the final base added to each fragment during capillary electrophoresis. When the labeled DNA fragments pass through the capillary, a laser excites the fluorescent dyes, and a Charge-Coupled Device (CCD) detects the emitted light. Software is used to analyse the resulting sequence data [Table/Fig-1c].



[Table/Fig-1]: First-generation sequencing methods: (a) Maxam–Gilbert sequencing method; (b) Old Sanger method; (c) New Sanger method.

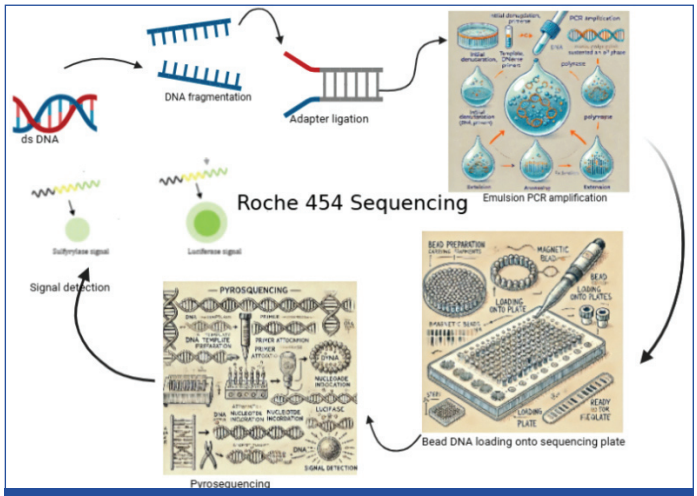
Despite its age, the Sanger method remains widely used due to its high accuracy and reduced toxicity compared to the Maxam-Gilbert method, which involves hazardous chemicals and radioactivity. Automated versions of the Sanger method continue to be a standard in DNA sequencing, providing complementary sequences of target DNA with high precision.

SECOND-GENERATION SEQUENCING

Second-generation sequencing technologies revolutionised genome sequencing by offering fast, cost-effective solutions using a range of platforms, including Roche 454, Illumina, SOLiD, and Ion Torrent. These platforms enable high-throughput sequencing, making it possible to sequence entire genome quickly [9].

Roche 454 Method

The Roche/454 platform uses a method called pyrosequencing, which was introduced by Nyrén et al., in 1997. This technique relies on emulsion PCR and detects pyrophosphate release during DNA synthesis. The DNA fragments, typically between 400 and 600 base pairs long, are tagged with adapter sequences to create a fragment library [10]. During the process, each DNA molecule is attached to a bead in a well of a PicoTiter Plate (PTP) with 100,000 wells, where clonal amplification occurs [Table/Fig-2].

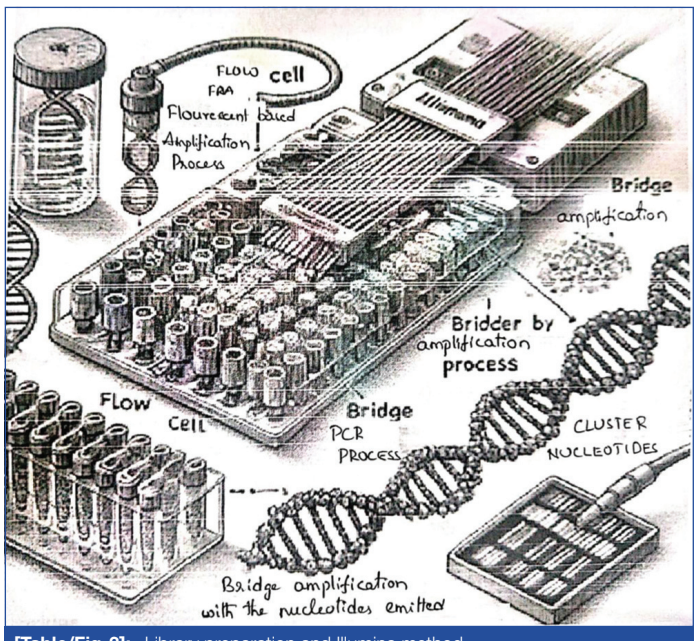


[Table/Fig-2]: Roche 454 sequencing method illustrating key steps from DNA fragmentation to sequence detection through pyrosequencing chemistry. Each step is clearly labeled to aid in understanding this sequencing technique.

During sequencing, nucleotides are added one by one, and the incorporation of each nucleotide triggers a cascade of reactions that produce light. The intensity of the light is recorded, allowing for sequence determination [11]. While pyrosequencing enables the parallel processing of many DNA fragments, its reliance on expensive reagents and enzyme-based detection systems makes it less commonly used today [12].

Illumina Method

Illumina's sequencing technology, first commercialised in 2007 with the Solexa Genome Analyser, uses a method known as bridge amplification. Single-stranded DNA is amplified on a flow cell to form clusters, and each cluster is sequenced using reversible dye terminators. Fluorescently labeled nucleotides are added one at a time, with a different dye for each base (A, T, C, G). After each nucleotide is incorporated, a laser excites the dye, and the emitted fluorescence is detected to determine the base [Table/Fig-3]. The process repeats, allowing the entire sequence to be read [13].



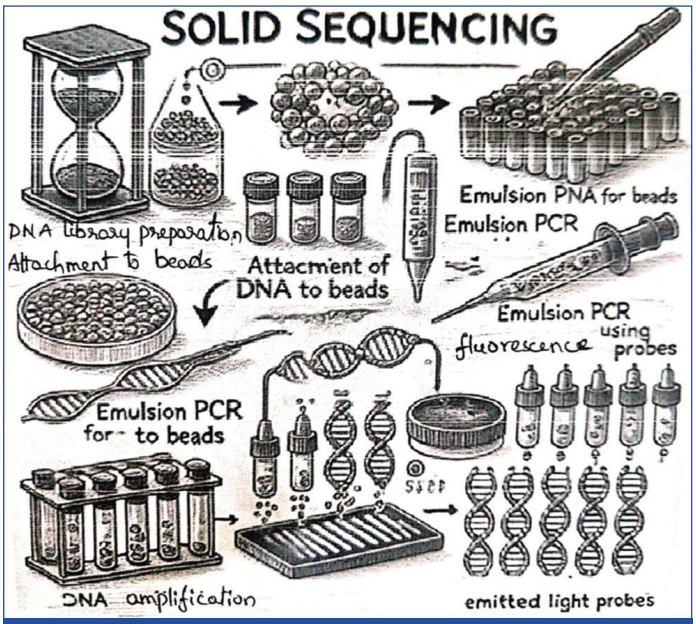
[Table/Fig-3]: Library preparation and Illumina method.

The Illumina HiSeq platform can generate over 50 Gb of data and 1.6 billion paired-end reads in about 10 days, while the MiSeq

platform is designed for shorter, rapid experiments, producing 1.5 Gb of data in just one day [14]. Compared to older methods like Sanger sequencing, Illumina offers a much faster, more efficient solution with a low error rate of 0.1% [15]. It is also more cost-effective due to its use of DNA polymerase rather than enzyme-intensive approaches like pyrosequencing.

SOLiD Method

The SOLiD (Sequencing by Oligonucleotide Ligation and Detection) method, acquired by Applied Biosystems in 2006, is a ligation-based platform. It uses emulsion PCR, like the Roche 454 method, to attach DNA fragments to beads. After amplification, these beads are immobilised on a glass flow cell, where DNA ligase incorporates two-base coded probes into the growing DNA chain [Table/Fig-4]. Each probe emits a fluorescent signal upon binding, which is detected to reveal the sequence [16].



[Table/Fig-4]: SOLiD sequencing method.

The SOLiD platform offers high accuracy, with an error rate of 0.15%, and can generate up to 30 Gb of data in a single run. However, its shorter read lengths limit its application for certain sequencing tasks. Despite this, it is widely used for Whole-Genome Sequencing (WGS), targeted region sequencing, and gene expression analysis [17].

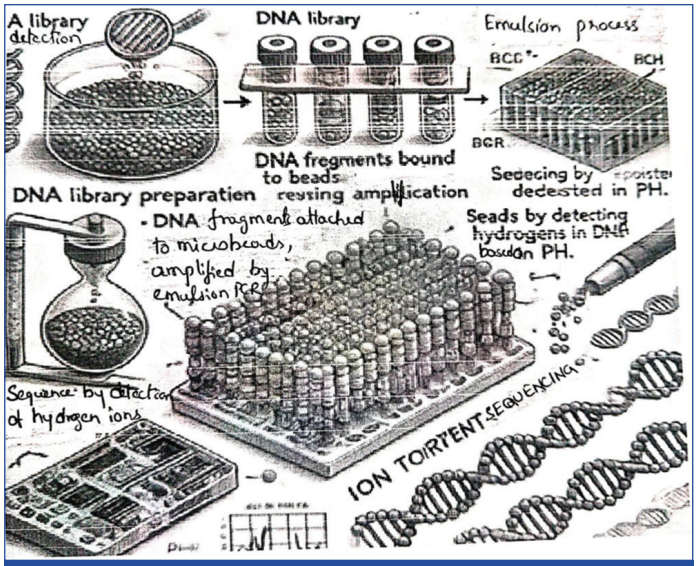
Ion Torrent Method

Ion Torrent, introduced as a more accessible and affordable sequencing method, employs semiconductor technology to detect nucleotide incorporation [18]. In this system, DNA fragments are attached to microbeads, amplified by emulsion PCR, and placed into wells on a microchip. As nucleotides are added to the growing DNA chain, hydrogen ions are released, causing a pH change. This change is detected by an ion-sensitive semiconductor, which converts it into an electrical signal [Table/Fig-5].

Unlike optical-based methods, Ion Torrent directly measures the chemical changes during sequencing. Its strengths include longer read lengths, flexible workflows, and faster sequencing times. However, it struggles with accurately reading homopolymer regions, where long stretches of the same nucleotide occur. Despite this limitation, its speed and lower costs make it an attractive option for various applications, including small RNA analysis and WGS [15,19].

THIRD-GENERATION DNA SEQUENCING

With the growing application of DNA sequencing, continuous advancements in technology have become essential. Although first- and second-generation methods transformed DNA sequencing, challenges remain in improving speed, cost and accuracy. To



[Table/Fig-5]: Ion Torrent sequencing and ion reporter.

address these challenges, third-generation sequencing methods have been developed, offering advantages such as longer read lengths, lower costs and faster processing. A significant innovation in these methods is that they sequence single, unfragmented DNA molecules in real-time without the need for amplification. Third-generation sequencing is primarily divided into two prominent techniques: Pacific Biosciences (PacBio) and Oxford Nanopore Technology (ONT).

Pacific Biosciences (PacBio) Method

Launched in 2010, the PacBio method is based on Single-Molecule Real-Time (SMRT) sequencing, which allows for the sequencing of long DNA fragments by replicating natural DNA synthesis. One of the main benefits of this technique is its relatively short sample preparation time. The method involves attaching hairpin-shaped sequences, called “SMRTbell adapters,” to both ends of a double-stranded DNA molecule, creating a circular structure. This circularisation reduces sequencing errors by enabling multiple passes through the same sequence [Table/Fig-6a].

In SMRT sequencing, the SMRTbell-adapted DNA is introduced to polymerases that are immobilised at the base of a chip using Zero-Mode Waveguide (ZMW) technology [20]. Each chip contains thousands of picoliter-sized wells, each holding a single polymerase molecule. As nucleotides labeled with distinct fluorescent markers are incorporated into the growing DNA strand, a unique color signal corresponding to each base (A, T, C, or G) is emitted [21]. The signal is detected in real time as the polymerase cleaves the fluorescent dye after incorporation. ZMW technology ensures that only the bottom of each well is illuminated, preventing interference from adjacent wells.

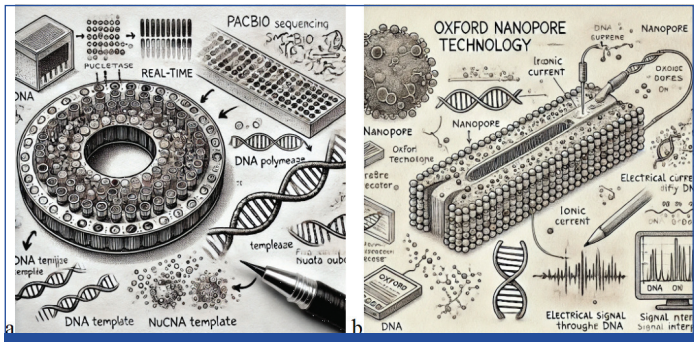
In addition to DNA sequencing, the PacBio method can detect structural variations and epigenetic modifications, such as methylation. Although SMRT sequencing has a higher error rate compared to other methods, this issue is mitigated by performing multiple reads of the same sequence to improve accuracy [5]. The sequencing duration depends on the length of the DNA but generally ranges from 30 minutes to four hours.

Oxford Nanopore Technology (ONT) Method

Oxford Nanopore Technology (ONT) represents another third-generation sequencing approach, developed by Oxford Nanopore Technologies. Initially conceived in 1990, ONT has only recently become commercially viable. Unlike other methods, ONT does not depend on enzymes, amplification, or fluorescently labeled nucleotides. Instead, it utilises an artificial nanopore embedded in a membrane through which an electric current is applied [22].

This nanopore is often derived from the protein alpha-hemolysin, sourced from *Staphylococcus aureus*.

Before sequencing, an adapter sequence with a poly-A tail is attached to the DNA, and a motor protein transports the DNA through the nanopore. As the single-stranded DNA passes through the pore, different bases (A, T, C, G) cause distinct disruptions in the ionic current that flows through the membrane [Table/Fig-6b]. These disruptions are detected and analysed by an algorithm, allowing the sequence of the DNA to be determined [23].



[Table/Fig-6]: Third generation sequencing methods: (a) the basic principle of Pac Biosequencing method; (b) the Oxford nanopore technology method.

One of the main strengths of ONT is its ability to provide rapid sequencing results, as it does not require secondary signals like fluorescence or pH changes. Additionally, because no enzymes are involved, sequencing can be carried out at lower temperatures, and the absence of amplification reduces preparation time. ONT is also versatile, extending its applications beyond DNA sequencing to include the characterisation of RNA, proteins, peptides, polymers, and other macromolecules [24]. However, a major challenge with ONT is the complexity of the bioinformatics algorithms needed to process the data it generates. Both PacBio and ONT have greatly expanded the capabilities of DNA sequencing, enabling faster, more efficient analysis of genomes with fewer limitations compared to earlier methods.

Advantages of NGS Techniques over Conventional Methods

NGS has become a leading technology in genetic analysis due to its ability to offer high throughput, cost efficiency, speed, versatility and accuracy, along with the capacity to detect rare genetic variants. These characteristics have propelled NGS beyond traditional sequencing methods, fueling advancements in genomics and its clinical applications. NGS has transformed the field by allowing the simultaneous sequencing of numerous DNA fragments, making it possible to sequence entire genomes quickly. This represents a significant improvement over traditional techniques like Sanger sequencing, which can only process one fragment at a time, making it inefficient for large-scale projects [25,26].

Cost efficiency: One of the key benefits of NGS is its reduced cost per base pair. By generating vast amounts of sequencing data in a single run, NGS minimises the expenses related to labour, reagents, and equipment. This cost efficiency has democratised access to genomics research, allowing more institutions to engage in high-level genetic studies [4,27].

Speed: NGS provides rapid results, making it particularly valuable in both research and clinical settings. Whereas traditional sequencing methods could take weeks or months, NGS can deliver outcomes within days or weeks, which is crucial in urgent situations like disease outbreaks or critical medical diagnoses [14,28].

Data resolution: The precision of NGS is another of its strengths, offering high-resolution data that can identify genetic variants, mutations, and structural changes with exceptional accuracy. This fine resolution is essential for exploring complex genetic landscapes and understanding the genetic basis of various diseases [7,13].

Versatility: NGS is a highly versatile tool that can be applied to a wide range of sequencing tasks, including WGS, targeted gene panels, and RNA sequencing. In contrast, traditional sequencing methods are often less adaptable and more specialised, limiting their application across diverse research areas [20,22].

Accuracy: NGS is recognised for its high accuracy, often with error rates comparable to or better than those of traditional methods. Its ability to perform deep sequencing—reading each fragment multiple times—ensures a high level of precision. This accuracy is especially critical when detecting genetic variations linked to diseases, where even minor changes in the DNA sequence can have significant implications [10,15].

Detection of Rare Genetic Variants

One of the standout features of NGS is its ability to identify rare genetic variants. This capability is particularly important in conditions such as certain cancers, where rare mutations may be pivotal in understanding the disease. The deep sequencing capability of NGS allows for the detection of these variants, which might otherwise go unnoticed by traditional methods. Additionally, this feature is valuable in population genetics and evolutionary studies, providing insights into genetic diversity and adaptation [23,24].

Combining Long- and Short-Read Sequencing (SRS) for Hybrid Approaches

The WGS has become an essential tool in addressing challenges such as antibiotic resistance across species [27]. SRS technologies like Illumina are known for their high accuracy and have been widely used to track microbial transmission and genetic relationships in outbreaks (https://www.illumina.com/). Techniques such as whole-genome SNP analysis and core genome Multi-Locus Sequence Typing (cgMLST) have proven effective in studying bacterial genomes [29]. However, SRS technologies face challenges when sequencing complex regions, including large structural variations and repetitive sequences, which are crucial for understanding drug resistance and virulence.

To overcome these limitations, Long-Read Sequencing (LRS) technologies such as ONT and Pacific Biosciences (PacBio) have been introduced. These technologies provide better resolution for complex genomic regions, offering deeper insights into genetic rearrangements and large insertions or deletions. ONT, in particular, stands out for its portability, affordability and ability to perform WGS rapidly. By integrating both SRS and LRS in a hybrid approach, researchers can achieve more accurate and complete genome assemblies. SRS is typically used for de novo assembly, while LRS fills in ambiguous regions, providing a complementary and robust sequencing strategy.

Advantages of Oxford Nanopore and Illumina Technologies in De Novo Assembly

Oxford Nanopore and Illumina technologies offer complementary strengths in de novo genome assembly. Illumina's SRS is renowned for its accuracy, making it ideal for capturing fine-scale genetic details. During Illumina's library preparation, DNA is fragmented, and adapters are added, allowing millions of short reads to be sequenced in parallel. This high-throughput capability is particularly effective in detecting small genetic variants and mutations.

In contrast, ONT produces long reads that can span complex genomic regions such as repetitive sequences and structural variations, which are difficult for short-read technologies to resolve. ONT's library preparation is simple, involving minimal fragmentation and ligation, enabling LRS without the need for amplification. In de novo assembly, these long reads help close gaps between contigs generated by Illumina, resulting in more complete and contiguous genome assemblies.

By combining the detailed accuracy of Illumina with ONT's ability to sequence complex genomic regions, researchers can produce more comprehensive and precise genome assemblies. This synergy between short- and long-read technologies enhances the overall quality and resolution of genomic data, offering a powerful tool for genomics research.

CONCLUSION(S)

The complementary strengths of Oxford Nanopore and Illumina in hybrid sequencing approaches overcome the limitations inherent in individual technologies. This synergy enables accurate, comprehensive, and efficient sequencing, supporting applications in clinical diagnostics, environmental studies, and de novo genome assembly. By integrating these advanced methods, researchers can achieve greater precision and resolution, paving the way for novel discoveries and impactful scientific breakthroughs.

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PARTICULARS OF CONTRIBUTORS:
1. Research Scholar, Department of Microbiology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India; Clinical Microbiologist, National Reference Laboratory Trust Lab Diagnostics Pvt. Ltd., Hyderabad, Telangana, India.
2. Professor, Department of Microbiology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India.
3. Associate Professor, Department of Biosciences and Biotechnology, Krishna University, Machilipatnam, Andhra Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Jeevigunta Naveena Lavanya Latha,
Associate Professor, Department of Biosciences and Biotechnology, Krishna University, Rudravaram, Machilipatnam, Andhra Pradesh, India.
E-mail: jnlavanyalatha@yahoo.co.in

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