THIRD GENERATION SEQUENCING

SEQUENCING TECHNOLOGIES

- Sequencing technologies have revolutionized the field of genomics, enabling the decoding of DNA and RNA sequences.
- From first generation sequencing to advanced third generation techniques, each technology offers unique capabilities for genetic analysis.

Principles and Methods of First Generation Sequencing

Sanger Sequencing

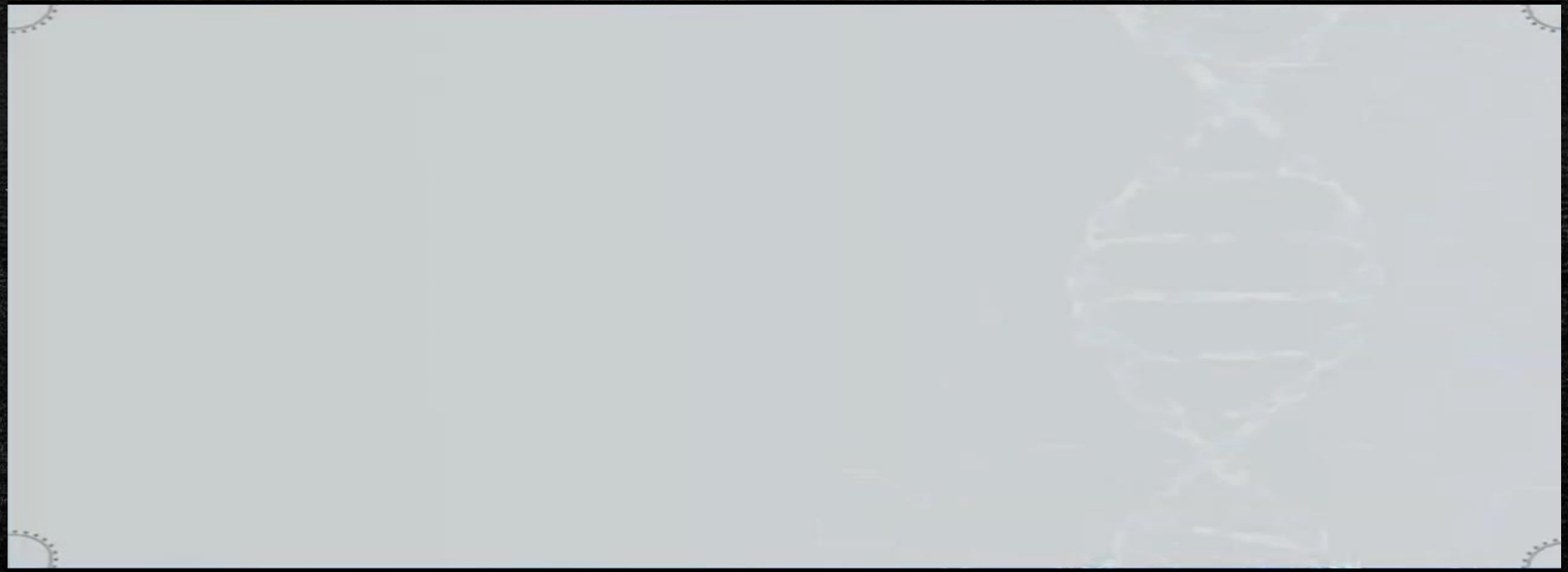
Sanger sequencing, developed by Frederick Sanger, relies on the termination of DNA synthesis using chain-terminating dideoxynucleotides.

This method involves separating the DNA fragments by size through gel electrophoresis to determine the sequence order.

Maxam-Gilbert Sequencing

This method, developed by Allan Maxam and Walter Gilbert, utilizes chemicals to break DNA at specific bases and subsequently allows the sequence to be deduced.

Sanger Sequencing



Advantages and Limitations of First Generation Sequencing

Advantages

- Long read lengths allow for sequencing of longer DNA fragments.
- High accuracy in base calling, resulting in reliable data.

Limitations

- Time-consuming and labor-intensive process.
- Lower throughput compared to newer sequencing technologies.

Second generation sequencing

- **Principles and methods:** Utilizes sequencing-by-synthesis, sequencing-by-ligation, or ion semiconductor sequencing technologies.
- **Advantages and limitations:** High throughput, cost-effective, but shorter read lengths and potential for higher error rates.
- **Applications:** Genomic analysis, metagenomics, transcriptomics, and targeted resequencing.

Advantages and Limitations of Second Generation Sequencing

Advantages

- High throughput, enabling the sequencing of large amounts of DNA in a relatively short time.
- Cost-effective, making it accessible for various research and clinical applications.
- Enhanced accuracy, resulting in more reliable sequencing data.

Limitations

- Shorter read lengths compared to third-generation sequencing platforms.
- Potential for sequencing bias and errors due to amplification during library preparation.
- Challenging for sequencing long repetitive regions or resolving complex structural variations.

THIRD GENERATION SEQUENCING

- Third generation sequencing is also known as long – read sequencing
- In NGS, DNA is fragmented into tiny bits, amplified and then sequenced.
- But, third-generation methods will not break or amplify DNA; instead, they sequence a single DNA molecule directly.

PACBIO SEQUENCING

- Single molecule, real- time sequencing developed by Pacific BioScience offers longer read lengths than the second-generation sequencing (SGS) technologies.
- PacBio sequencing captures sequencing information during the replication process of the target DNA molecule. The template, called a SMRTbell, is a closed, single stranded circular DNA that is created by lighting hairpin adaptors to both ends of a target double- stranded DNA (dsDNA) molecule.

FEATURES...

- Dose not requires PCR amplification, can easily cover high-GC and high-repeat regions, and is more accurate in quantifying low-frequency mutation.
- Average read length iss 8-15 kb and upto 40-70 kb.
- Time-effective at the rate of 10 nt per second.
- High accuracy: the consensus accuracy of PacBio SMRT sequencing can be greater than 99.999%.
- PacBio sequencing is a method for real-time sequencing and dose not require a pause between read steps.

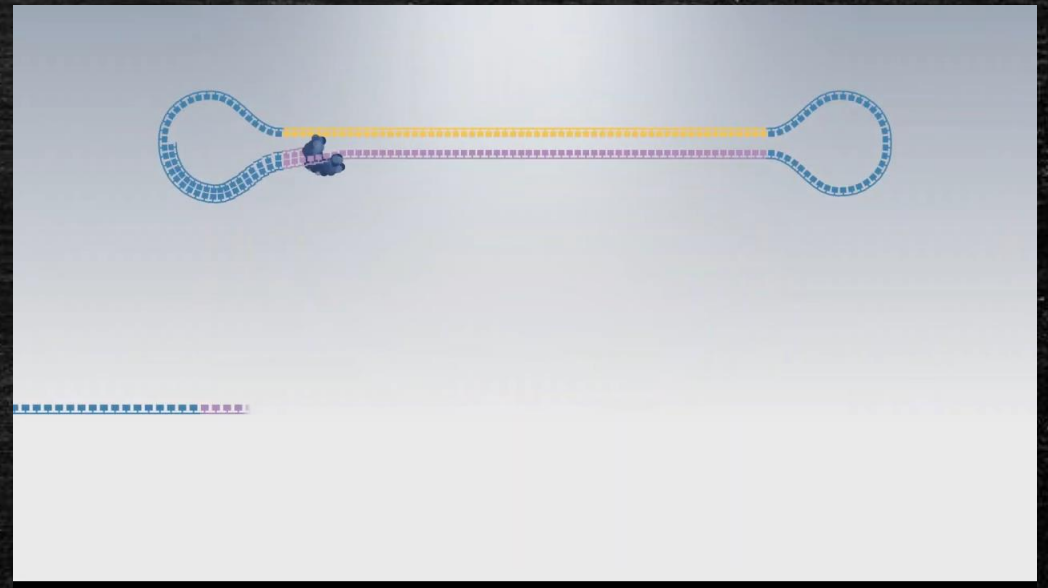
PacBio-SMRT

PacBio-SMRT

Two Squencing modes

1. Circular Consensus Sequencing (CCS)

2. Continuous Long Read (CLR) sequencing



Application of SMRT

- Advantages….
- Closes gaps and copletes genomes due to longers reads.
- Identifies non-SNP SVs
- Identifies full-legth transcripts isoforms without need for a reference genome.
- Detects novel isoforms and fusion events.

- Limitations….
- One of the major problem is that the flow cell is not as high throughput when compared to the illumina platform.
- Another problem is that the sequencing error rate produced using PACBIO SMRT is still high compared to the illimina platform

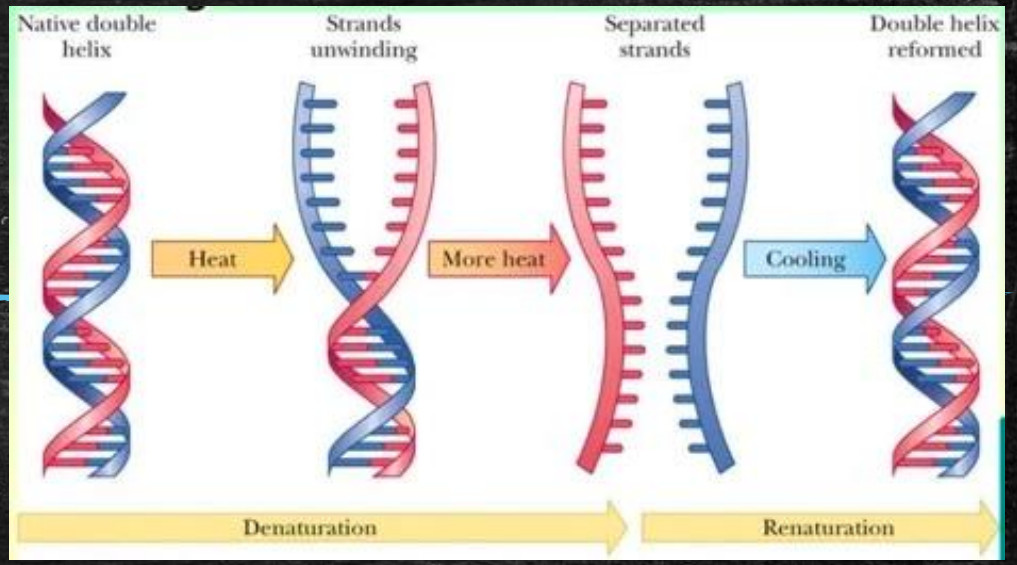
Nanopore Sequencing

1. Nanopore sequencing is a unique, scalable technology that enables direct, real-time analysis of long DNA or RNA fragments.

1. It works by monitoring changes to an electrical current as nucleic acids are passed through a protein nanopore.

1. The resulting signal is decoded to provide the specific DNA or RNA sequence.

ONT - Steps



Sample Preparation -

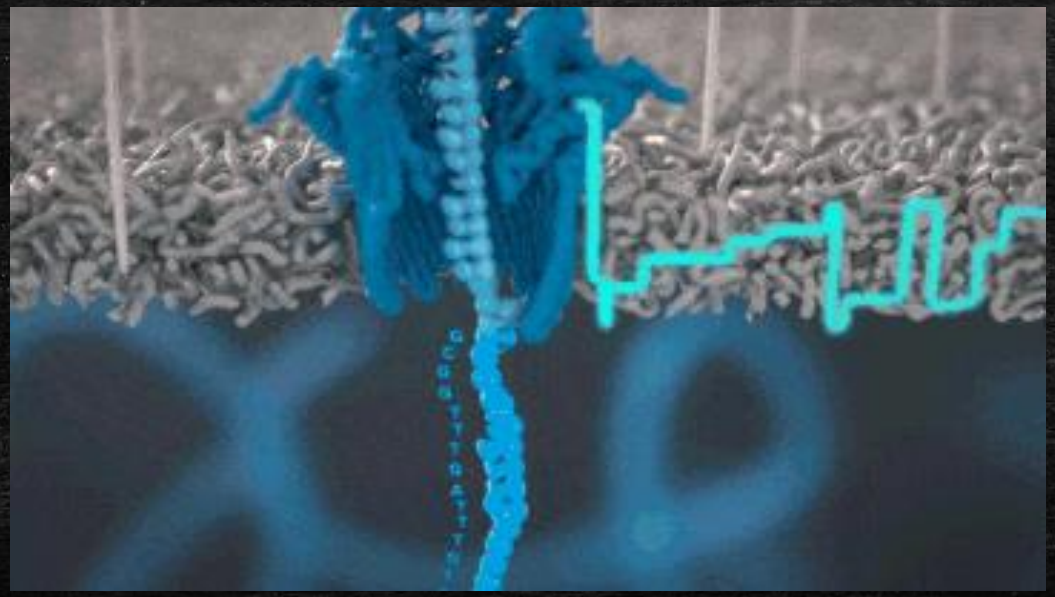
1. Convert dsDNA to ssDNA.
2. The reverse transcription step to convert RNA into cDNA is not needed.

Library Preparation -

1. Adapter Ligation - Community - Ligation sequencing gDNA - Native Barcoding Kit 24 V14 (SQK-NBD114.24) - MinION - Adapter ligation and clean-up. (n.d.). Oxford Nanopore Technologies. https://community.nanoporetech.com/docs/prepare/library_prep_protocols/ligation-sequencing-gdna-native-barcoding-v14-sqk-nbd114-24/v/nbe_9169_v114_revq_15sep2022/adapter-ligation-and-clean-up?devices=minion
2. Motor Protein.

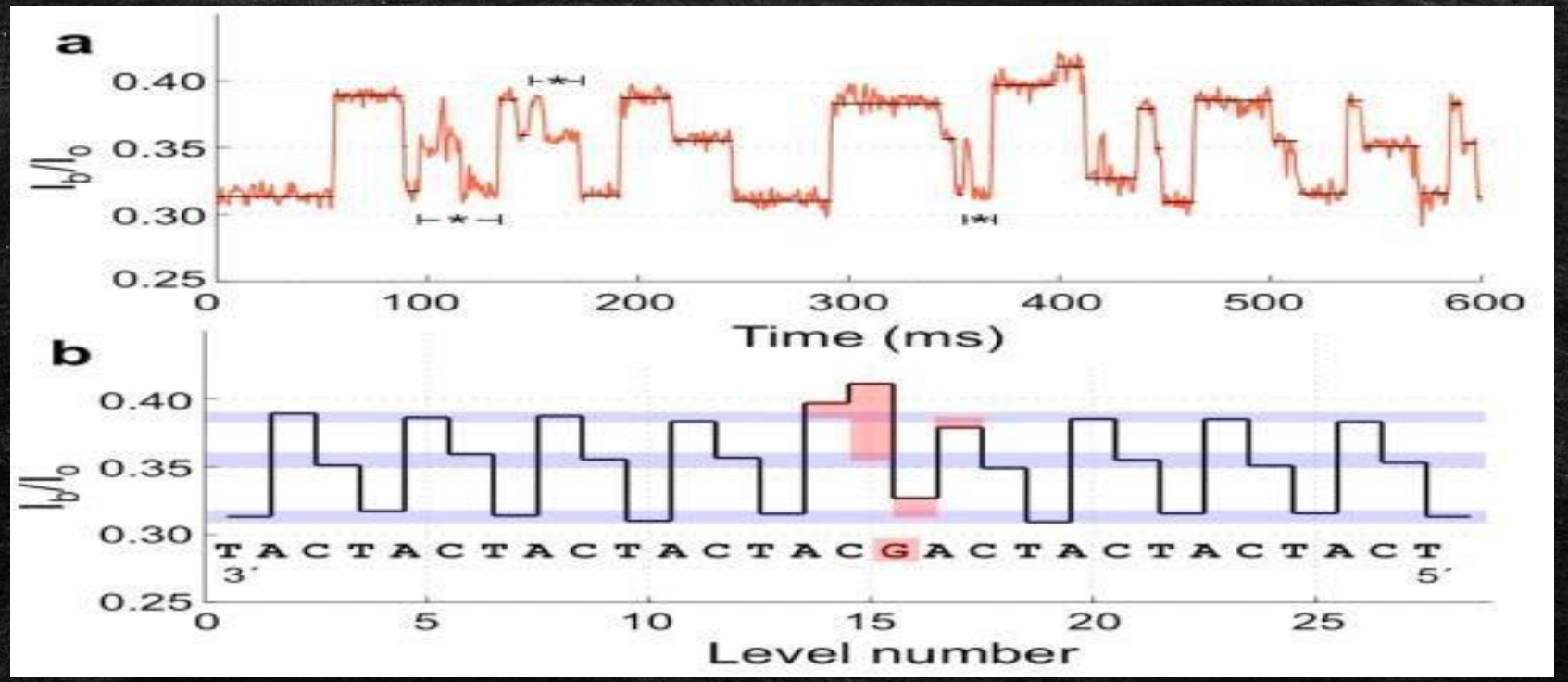
Principle Involved

- The principle of operation of the nanopore sequence technique is the analysis of the DNA strand directly as the molecule is drawn through a tiny pore suspended in a membrane. Changes in electrical current, or tunneling current, are used to read off the chain of bases.



Principle Involved

https://nanoporetech.com/applications/dna-nanopore-sequencing



Results

Nanopore Sequencing

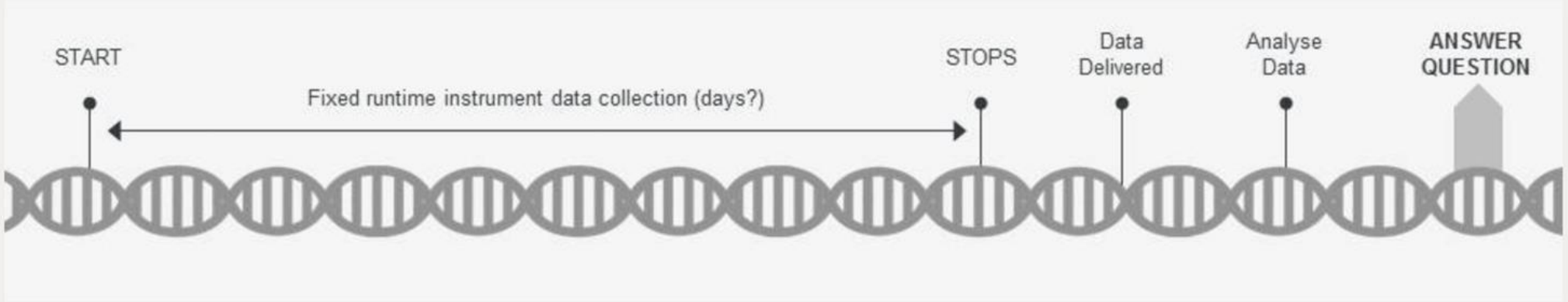
Advantages

- Long-read sequencing: over 2Mb read lengths have been achieved.
- Direct RNA sequencing - Traditional RNA sequencing approaches require the conversion of RNA to cDNA, which can introduce bias from reverse transcription or amplification.
- Nanopore sequencing is the only sequencing technology that offers real-time analysis (for rapid insights), in fully scalable formats, can analyse native DNA or RNA, and sequence any length of fragment to achieve short to ultra-long read lengths.

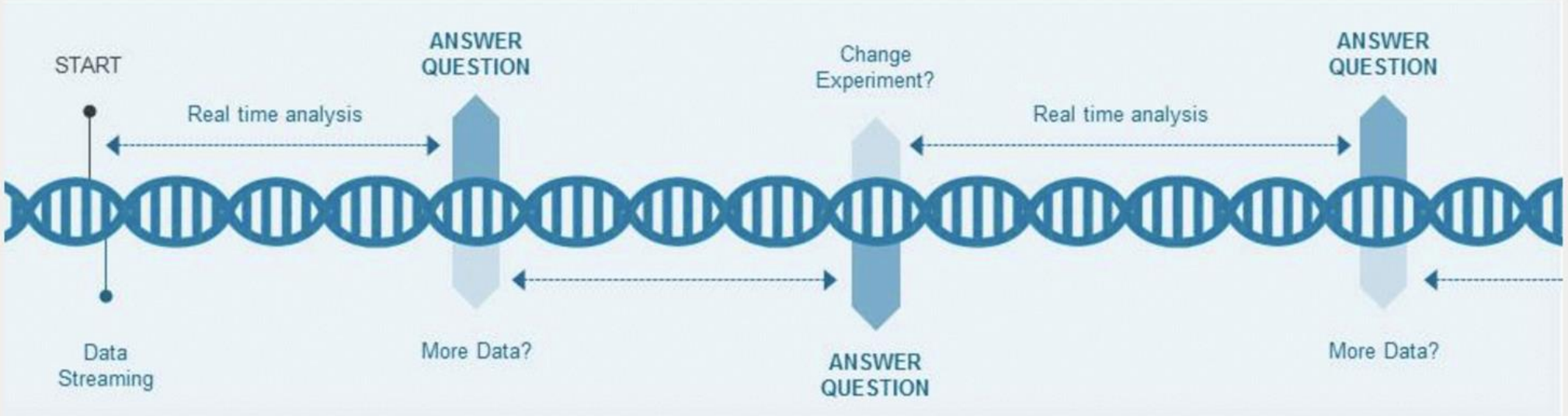
Limitations

- It tends to be error prone; error rates in nanopore sequencing can be as high as 15%.
- Its an expensive method.

Traditional workflow



Run until



Reference

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- PacBio Sequel II Sequencing, https://genomics.umn.edu/service/pacbio-sequel-sequencing#:~:text=Based%20on%20well%2Destablished%20Single,and%20isoform%20detection%2C%20and%20epigenetic
- **The nanopore sequencing workflow. (2021, November 5). Oxford Nanopore Technologies. https://nanoporetech.com/how-it-works/nanopore-sequencing-workflow**
- **Wang, Y., Zhao, Y., Bollas, A., Wang, Y., & Au, K. F. (2021, November 1). Nanopore sequencing technology, bioinformatics and applications. Nature Biotechnology. https://doi.org/10.1038/s41587-021-01108-x**
- **Pacbio sequencing, https://youtu.be/_lD8JyAbwEo?si=KYyigpeZKdtk9xy4**