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ANALYSIS OF THE EVOLUTION OF TECHNOLOGIES FOR DETERMINING THE NUCLEOTIDE SEQUENCE OF A DNA MOLECULE

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Abstract. *Today, researchers and specialists in various fields are using this information to meet the demand for food by increasing the productivity of agricultural crops, in animal husbandry, in the direction of diagnostics, in the prediction and treatment of various complex diseases, in improving criminalistics, etc. In this article, we will analyze the principle of methods, their advantages and disadvantages from first generation sequencing (FGS) to third generation sequencing (TGS).*

Keywords: *information, generation sequencing, experimental method, researchers and specialists.*

DNA sequencing is an experimental method of determining the sequence of bases made up of nucleic acids (A, T, G and C) in a polynucleotide that encodes various proteins, functioning in the cell.

In the late 1970s, the discoveries of Sanger and Maxam-Gilbert caused revolutionary changes in the biological sciences, making sequencing a working tool for many researchers: biologists, medical doctors, criminalists and representatives of other fields. Over the last 40 years, DNA sequencing technologies have been equipped with new equipment that has led to significant advances in terms of instrumentation and sequencing methodology. Modern methods provide opportunities to expand the range of applications of sequencing, increase accuracy, improving productivity and reliability.

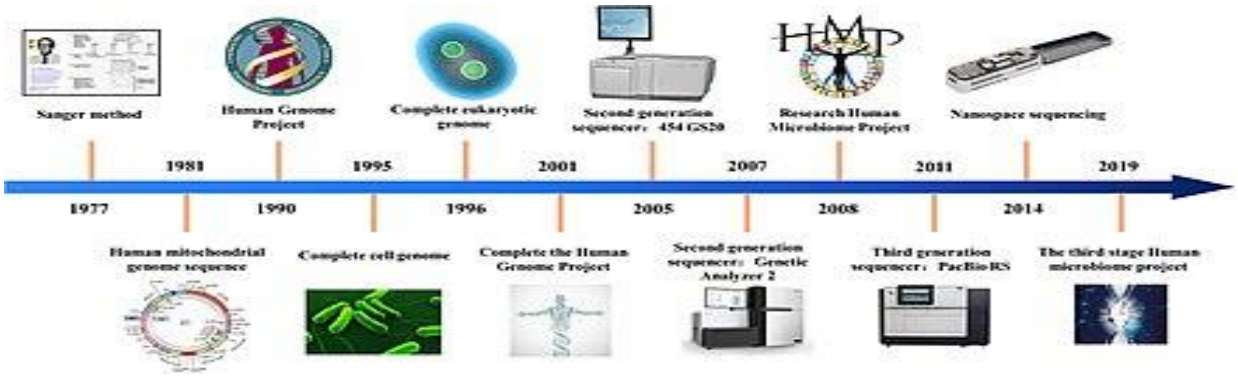

Figure 1. Evolution of DNA sequencing technologies

The popularization and ease of use of sequencing has led to an increase in the volume of our knowledge about various biological entities. The obtained information is filling a wide range of databases that can be used by researchers around the world.

Today, researchers and specialists in various fields are using this information to meet the demand for food by increasing the productivity of agricultural crops, in animal husbandry, in the

direction of diagnostics, in the prediction and treatment of various complex diseases, in improving criminalistics, etc.

Currently, three generations of sequencing technologies are distinguished.

The chemical degradation method proposed by Maksam and Gilbert, the deoxy-chain termination method by Sanger and his team in 1977, and the automatic sequencing technologies by fluorescent targeting developed in the 1990s formed the first generation of sequencing (FGS).

Second-generation sequencing (SGS) or next-generation sequencing (NGS) are high-throughput DNA sequencing technologies capable of sequencing millions or billions of DNA strands. In doing so, the sequencing process enables multiple sequencing of target regions and high-throughput properties.

Third generation sequencing (TGS) is characterized by the addition of single nucleotides, which provides long and precise sequencing results, and does not use amplification technologies. Single-cell sequencing also belongs to TGS technologies.

In this article, we will analyze the principle of methods, their advantages and disadvantages from first generation sequencing (FGS) to third generation sequencing (TGS).

FIRST GENERATION OF DNA SEQUENCING TECHNOLOGIES

The late 1970s and early 1980s were a very important period for the science of genetics and genomics. The discovery of the polymerase chain reaction (PCR) led to the creation of the first DNA sequencing technologies that could read the entire genome, realizing the possibilities of DNA amplification. First-generation sequencing methods such as Sanger and Maxam-Gilbert sequencing have dominated genomics for almost 40 years. They laid the foundation for future sequencing technologies.

Maxam - Gilbert sequencing

The principle

In 1976 A. Maksam and V. Gilbert created a method based on the specific chemical degradation of a DNA fragment targeted at one end [1]. The method of DNA sequencing by chemical degradation occurs through limited fragmentation of the targeted DNA fragment under the influence of special reagents.

A prerequisite for sequencing using this method is the presence of a single-end-targeted DNA fragment. Degradation products can be separated by high-voltage electrophoresis on a polyacrylamide gel, which can separate DNA fragments differing in length by one nucleotide, and subsequent autoradiography of the gel allows determining the nucleotide sequence of DNA.

The first step in carrying out a chemical degradation reaction is based on the limited modification of certain nucleotides with the help of various chemical agents. The concentration of the agent and the duration of its effect on the DNA molecule are selected in such a way that only one nucleotide is modified in each molecule.

Specific reactions of modification and quantitative degradation are carried out for each type of nucleotides or their combination. Thus, as a result of the reaction step, a mixture of molecules consisting of oligonucleotides differing in size by one nucleotide and carrying a target (usually radioactive) at one end is formed. In addition to the labeled molecules, unlabeled oligonucleotide fragments are also present in the reaction mixture, but they are not visible in the radioautography step.

Reaction products are separated in denaturing polyacrylamide gel in adjacent lanes, and after radioautography, stair-like DNA lines are visible on the X-ray film, and by reading them, it is possible to reconstruct the DNA nucleotide sequence.

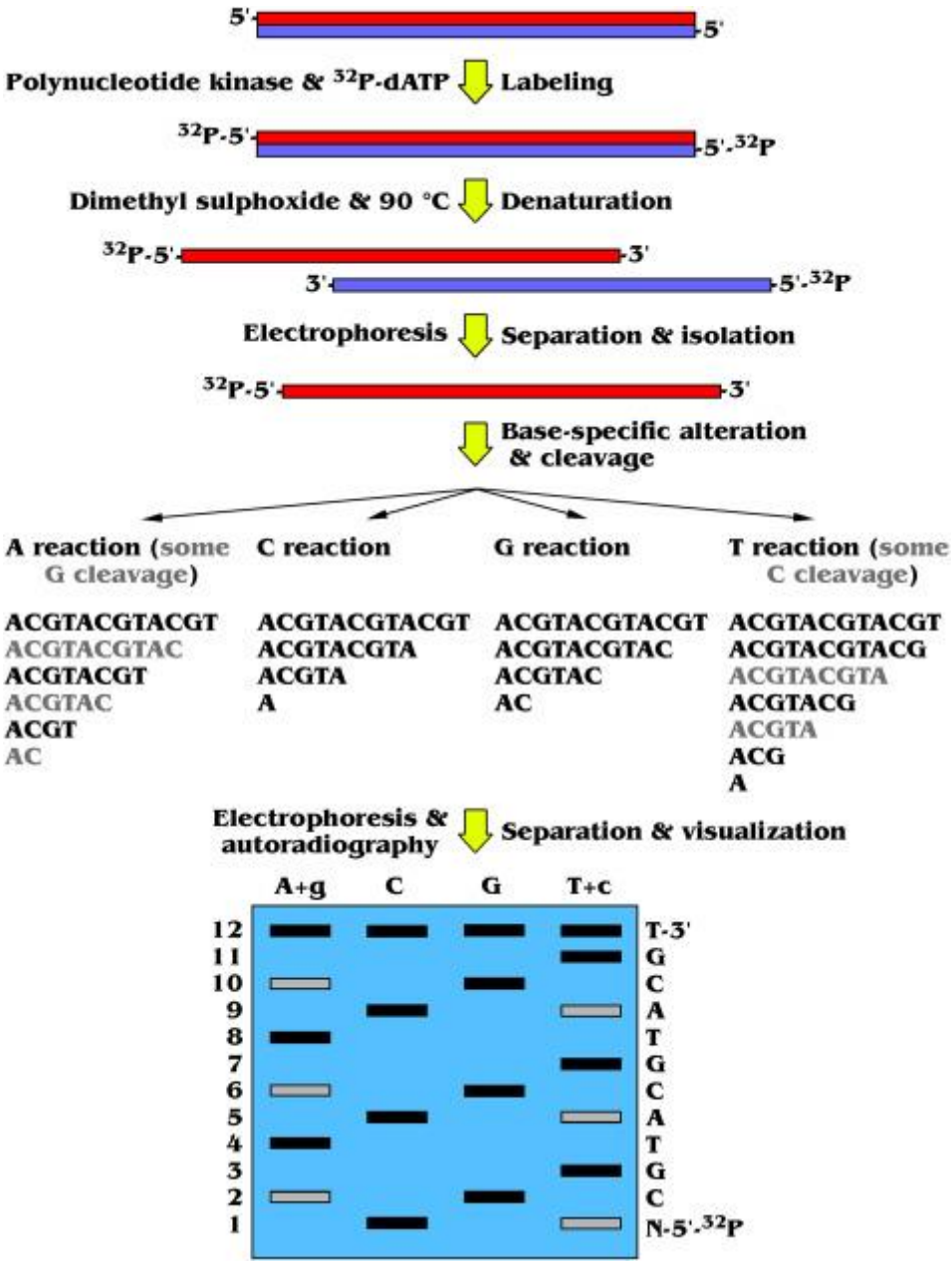

Figure 2. Maxam-Gilbert sequencing, based on the specific cleavage of the DNA strand, in which DNA fragments of different sizes are obtained.

Advantages and disadvantages

The main convenience of Maxam-Gilbert sequencing is that the DNA template used in this method can be single-stranded or double-stranded.

The reason the Maxam–Gilbert method was dominant over the Sanger method for a time was that the Sanger method required cloning of single-stranded DNA for each read head. Maxam-Gilbert method can also be used for DNA-protein and epigenetic modifications of DNA.

The limitations of the Maxam-Gilbert sequencing method were the use of harmful chemicals and X-rays and radioactive targets. The difficulty of expanding and using these

methods, as well as the use of a strong neurotoxin-hydrazine, made the method useless and practically insignificant in the future development of technologies.

Sanger sequencing method.

The history

Sanger sequencing [2-3] is considered one of the first methods of DNA sequencing.

Together with his colleagues, Frederick Sanger pioneered sequencing technologies first for insulin, then for RNA, and finally for DNA. His research led to the creation of the Sanger chain break sequencing method in 1977.

The technology was commercialized by Applied Biosystems. This method is considered to be the method implemented by Sanger sequencers in many laboratories of the world when sequencing the complete DNA of a person within the framework of the "Human Genome" project.

The chemical reaction is performed in four separate reaction tubes, each containing template DNA, primers, DNA polymerase, and four dNTPs, one of which is radiolabeled. The DNA sample is separated into four separate sequencing reactions, each containing four standard deoxynucleotides (dATP, dGTP, dCTP, and dTTP) and DNA polymerase.

Only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP) is added to each reaction, and the other added nucleotides are normal nucleotides. After denaturation and primer annealing, DNA polymerase begins adding dNTPs to the newly synthesized DNA strand, and the reaction stops if ddNTPs are added. This condition causes DNA strands of different sizes to accumulate separately in the four reaction tubes. The reaction product is then placed in separate wells of the polyacrylamide gel. A radioactive spot indicates DNA fragments with ddNTPs in a specific position. The nucleotide sequence is read from bottom to top on the separating gel, and the sequence of nucleotides in the different lanes of the terminator creates a nucleotide sequence template.

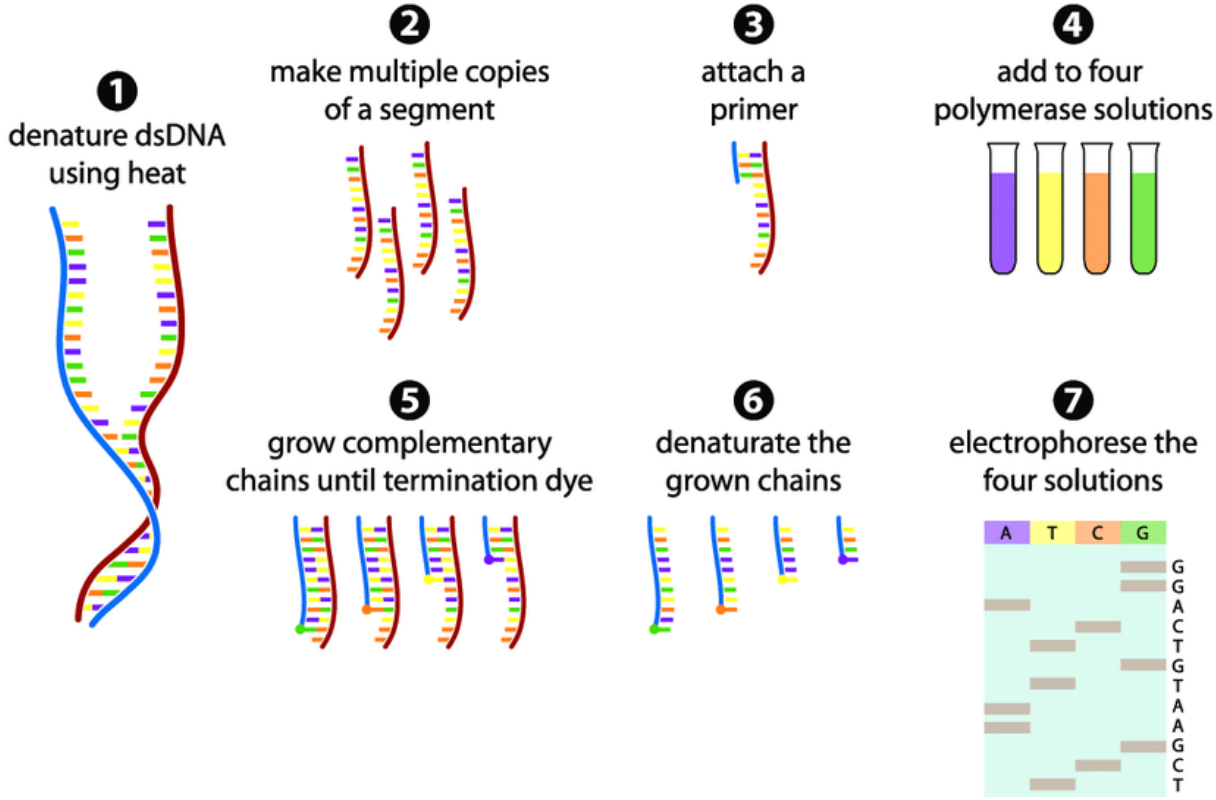

Figure 3. Basic scheme of Sanger sequencing

Advantages and disadvantages

Sanger sequencing has helped researchers identify the primary cause of mutations and genetic diseases. This method is considered the most advanced in sequencing short tandem repeats and isolated genes. However, the biggest disadvantage is that it takes a long time due to the low conductivity. The method can process only short sequences of DNA (300-1000 nucleotide pairs) at a time.

Automated DNA Sequencing

The principle

The characteristics of this sequencing method are similar to Sanger sequencing [4], but the process is automated and the reactions take place in one test tube, all four dideoxynucleotide triphosphates stained with four different fluorescent dyes emitting light of a certain wavelength. Data on sequences are collected and analyzed by computer.

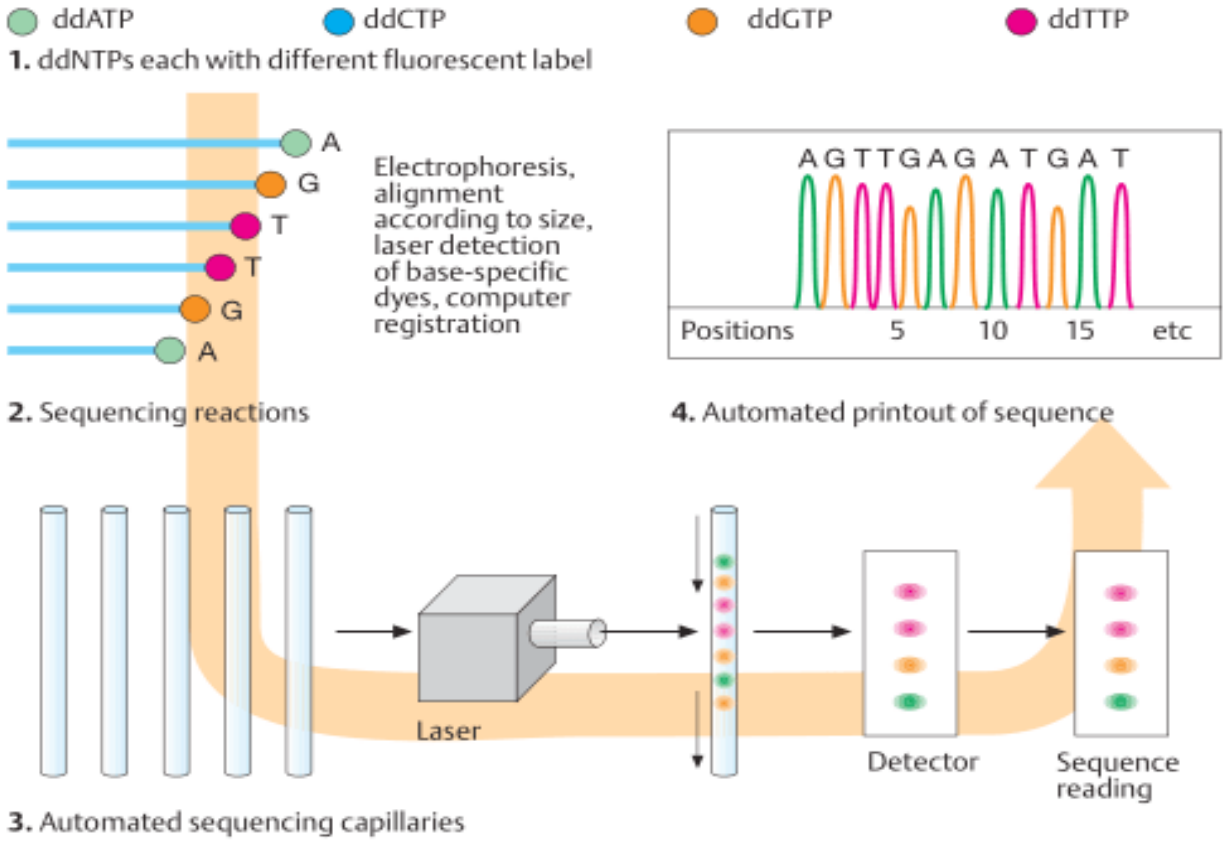

Figure 4. Automated DNA sequencing.

a — capillary electrophoresis system; b — laser recording of fluorescent markers; c — DNA sequence electrophorogram [5]

Advantages and disadvantages

Automated DNA sequencers are expensive and difficult to sequence repetitive parts of sequences.

Despite the advent of next-generation sequencers, automated Sanger sequencing is still considered the "gold standard" due to read length and accuracy. However, this sequencing method is slow and expensive for mass application.

Pyrosequencing

The principle

According to this technology [6], solutions of four types of nucleotides are added sequentially during sequencing of DNA fragments and washed after each reaction. When the next

nucleotide is added to the strand complementary to the previously prepared single-stranded DNA matrix, a light signal is recorded due to the binding of the pyrophosphate luciferase enzyme. During the reaction, the complementary strand of DNA is restored, and the sequence of nucleotides is determined by the peaks of the light signal in the pyrogram, the intensity of which is proportional to the amount of one type of nucleotide added to the DNA strand from the next reaction mixture.

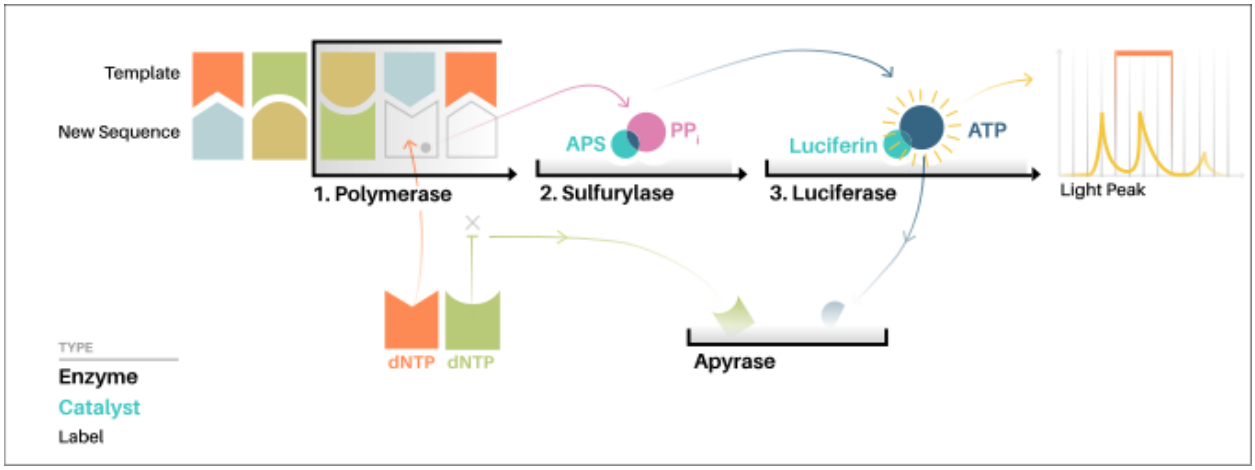

Figure 5. Scheme of the principle of pyrosequencing.

454 Life Sciences' modification was based on the simultaneous parallel preparation of hundreds of thousands of DNA fragments for sequencing and the use of emulsion PCR.

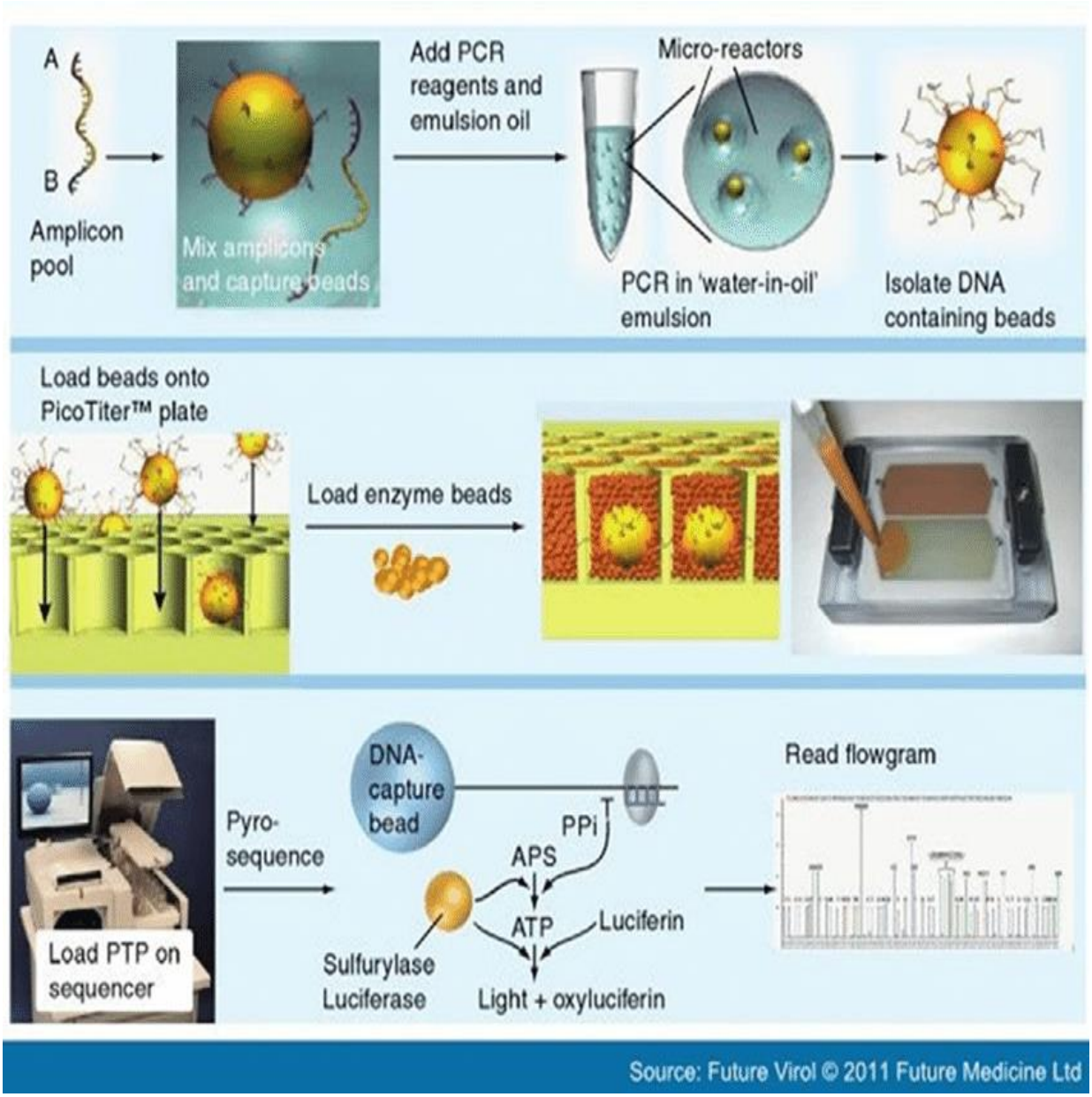

Figure 6. Roche 454 [7] mass pyrosequencing.

After the preliminary stages, each of the four different nucleotides mixed with other reagents of pyrosequencing is sent in turn to a fluid chamber containing a base consisting of several billion wells filled with microspheres (one well - one microsphere). If the type of nucleotides complementary to the repairing single-stranded DNA matrix falls into the well, the polymerase binds them, and as a result of the cascade of reactions, pyrophosphates are released and the overall light signal in the well is increased.

Advantages and disadvantages

The main advantage of pyrosequencing is that it saves time, so the process can be carried out in real time.

The method is more cost-effective than dideoxynucleotide chain termination sequencing and facilitates the phasing of haplotypes and the identification of the structure of genetic variations covering tens of kilobases of the genome matrix. However, the main drawback of the method is significant frameshift errors, which lead to systematic errors in the reading process. Also, using

this method, sequencing of long fragments consisting of the same nucleotide is not error-prone. Only relatively short regions can be determined by the method.

SECOND GENERATION OF DNA SEQUENCING TECHNOLOGIES

Sanger sequencing has been used for nearly 30 years. Cost and time inefficiencies in carrying out this process became evident as a major problem. The next wave of sequencing technologies, known as second-generation sequencing, emerged in the mid-2000s and focused on the goals of reducing throughput, increasing speed, and being electrophoresis-free.

Sequencing by Illumina/Solexa synthesis

The principle

(SBS) Sequencing platform (SBS) using Illumina/Solexa synthesis is based on the reversible termination method (reversible termination) [8-9].

Illumina sequencing by synthesis consists of four steps: sample preparation, cluster generation, sequencing, and data analysis.

Preparation of libraries. The DNA chain is randomly separated into fragments (200-600 n.j) using the transposase enzyme. Then the adapters (P5 / P7) are connected to the 5′ and 3′ ends.

As an alternative, six-nucleotide indices are introduced, which create a unique barcode for a sample that allows different samples to be sequenced simultaneously. Adapter-linked fragments are amplified by PCR and then purified.

Cluster generation. The sample is immersed in a cell that is washed and composed of two types of oligosides that complement the sequence of P5 / P7 adapters of DNA fragments.

Each hybridized DNA fragment is attached to a complementary oligoside, and the DNA polymerase enzyme creates a complementary strand. The double-stranded DNA is denatured and the original template is washed away, while the new fragment covalently bound to the washed cell is trapped.

The ssDNA (single-stranded DNA) with the adjacent complementary primer forms a bridge with the help of hybridization and is elongated with the help of polymerase, resulting in a double-stranded DNA bridge.

The dsDNA bridge is denatured, and the end result is the formation of two strands of SsDNA that are covalently attached to the washed cell.

The amplification cycle is repeated many times. Analogously, each fragment is amplified into separate clonal clusters by bridged amplification, leaving a cluster of identical DNA sequences.

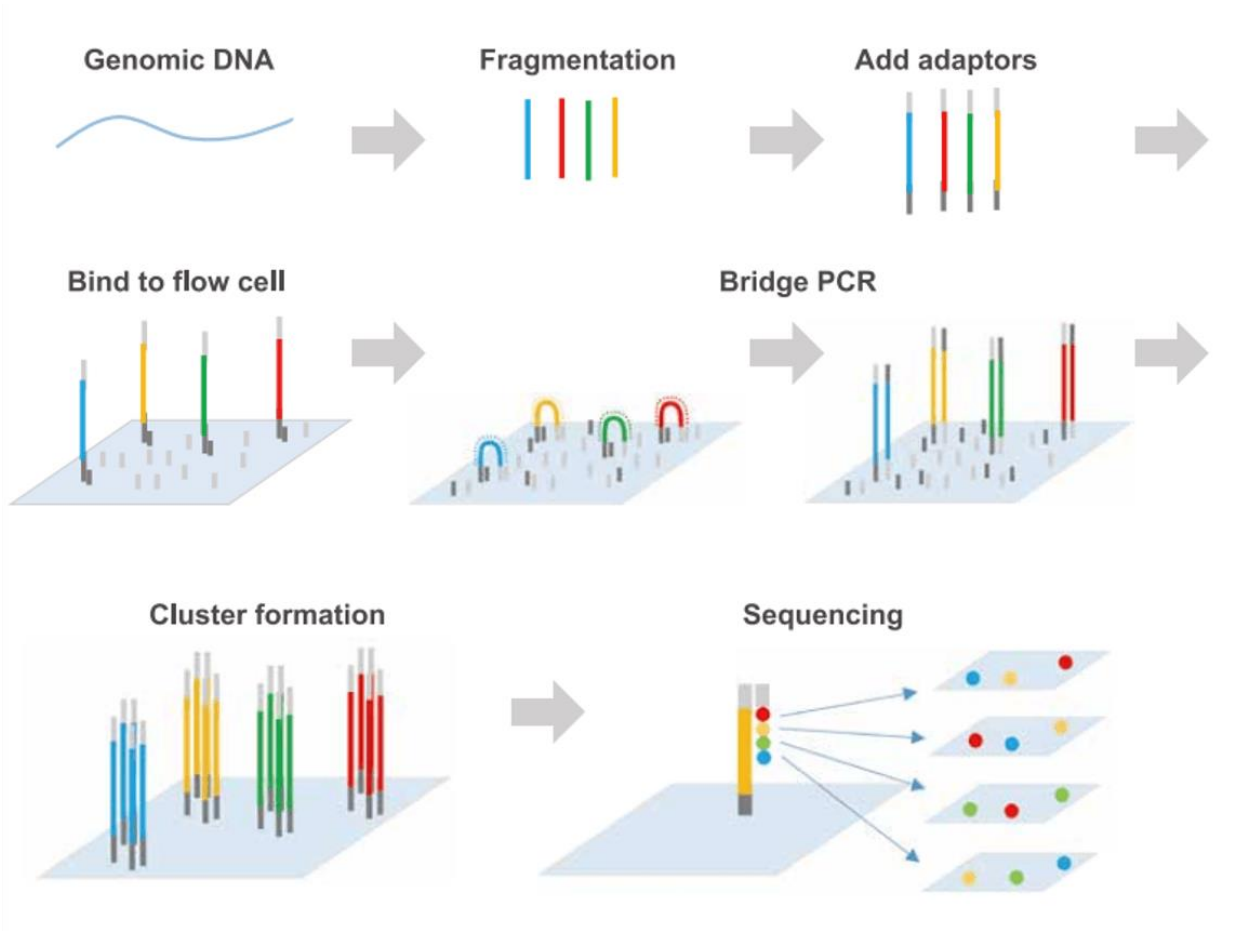

Figure 7. Scheme of SBS sequencing [24]

Sequencing. After clonal amplification, the *reverse* ssDNA is isolated and washed, leaving the forward ssDNA attached to the washed cell.

The primer anneals to the forward strand and begins adding fluorescently labeled ddNTPs. Only one base pair is joined per attempt using a reversible terminator that prevents multiple joins at once.

When the base is added and the fluorophore is excited by the laser, the emission is recorded. The fluorophore is cleaved, and the terminator is removed. The cycle is repeated until the entire chain is read, thus determining the sequencing order.

Data analysis. The Illumina sequencing platform identifies sequences of 50-300 nucleotides on average. This is much shorter than the capabilities of Sanger sequencing. Sequencing on NGS platforms results in extremely large amounts of data, which requires high-throughput computing techniques.

The data processing consists of the following main steps: sequence filtering and error correction, generation and integration, and analysis of the results.

Advantages and disadvantages

The main advantage of SBS technologies is that up to 96 samples can be sequenced in one cycle using standard reagents. Also, SBS technology has a clear advantage in sequencing homopolymer sequences compared to 454 or Ion Torrent, as it allows adding one nucleotide per reaction.

Another advantage of the technology is that it can read paired-end DNA sequences. The main limitation of SBS technology is the short read length. This is especially evident when reading the sequences de novo.

Switching errors due to increased background noise at each cycle, and GC drift in bridged amplification also limit the capabilities of this technology. The high cost of this technology is also one of its disadvantages.

Ligase sequencing: ABI/Solid

The principle

The method consists in creating genome libraries, ligation and conducting sequencing reactions.

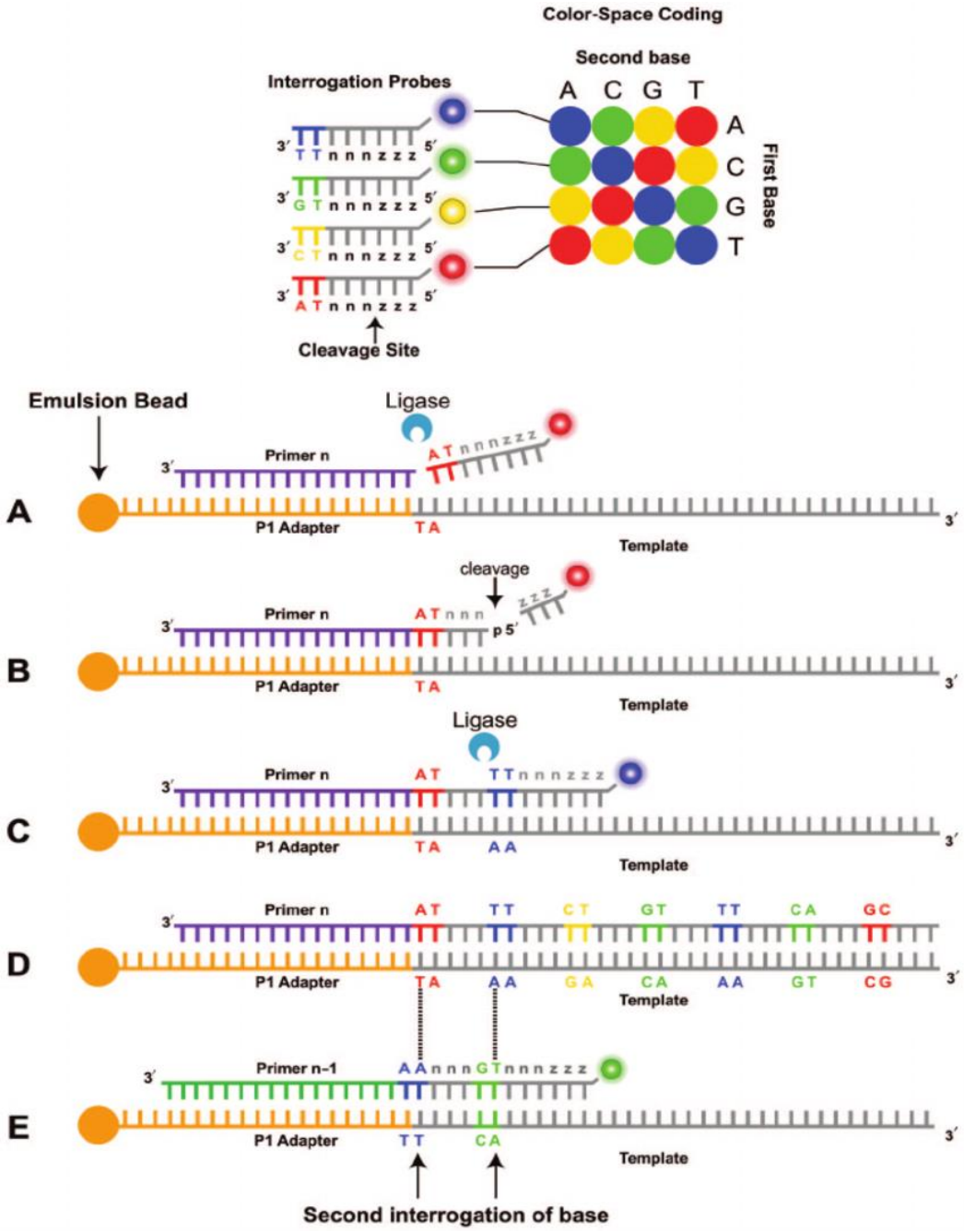

Figure 8. The working principle of ABI/SOLID technology.

This sequencing technology uses DNA ligase enzyme instead of DNA polymerase for sequencing. The genome is randomly fragmented, then magnetic beads for clonal amplification

are attached to the adapter molecule in such a way that only one DNA fragment is present on the surface of the magnetic bead. Emulsion PCR is performed for DNA fragments trapped by the sphere.

In this method, after conducting emulsion PCR, each microsphere holds about a million copies of the original DNA fragment on its surface. The DNA fragments are then denatured and modified at the 3' end, allowing billions of microspheres coated with DNA fragments to covalently bind to the substrate. Next, cyclic ligase sequencing is performed.

A primer is attached to a single-stranded DNA template and eight-stranded synthesized fluorescently labeled oligonucleotide probes are added. After one of the probes is complementary to the first five bases of the primer in the single-stranded DNA template, the ligase connects it to the primer.

In this case, the target is recognized and excluded together with the last three universal nucleotides of the probe. After that, hybridization with another probe complementary to the next five bases occurs, and the process continues until the end of the DNA matrix.

Then, the primer that is free from the created complementary strand is added and moved to a new place with a difference of one nucleotide from the place where the previous primer sat, and it is returned until the second link of the connection method is completed.

To determine the complete sequence of the sequenced fragment, this procedure requires five primers that differ by one nucleotide in their cross-linking position.

Advantages and disadvantages

The advantage of ABI/SOLiD sequencing technology is that it is the only NGS sequencing method in which each nucleotide is read twice, which increases the accuracy of sequencing.

The main limitation of ABI/SOLiD Sequencing technology is the difficulty of sequencing palindromic sequences, the method records them as different random sequences.

Ionic semiconductor

Ion Torrent sequencing

The Ion Torrent platform is the first technology that does not use optical connectors. [10]. In this technology, the light works on a linker, a receiver in the middle. In this method, a semiconductor recording system is used for sequence sequencing. The method is based on the recording of hydrogen ions released as a by-product of nucleotides added to the template chain during polymerization.

Enriched DNA capture beads are inserted into the microcells on the chip. The microwells containing the template DNA molecule being sequenced are filled with a type of deoxyribonucleotide triphosphates (dNTPs).

If the inserted dNTP is complementary to the initial nucleotide of the template, it is added to the growing complementary strand. This condition causes the release of hydrogen ions, causing the ISFET ion-coupler to activate, thus indicating that a reaction has occurred.

If a single nucleotide repeat occurs in the sequence of the template strand, multiple dNTPs are added in one cycle. This in turn causes an increase in the amount of hydrogen ions and a proportionally higher electrical signal.

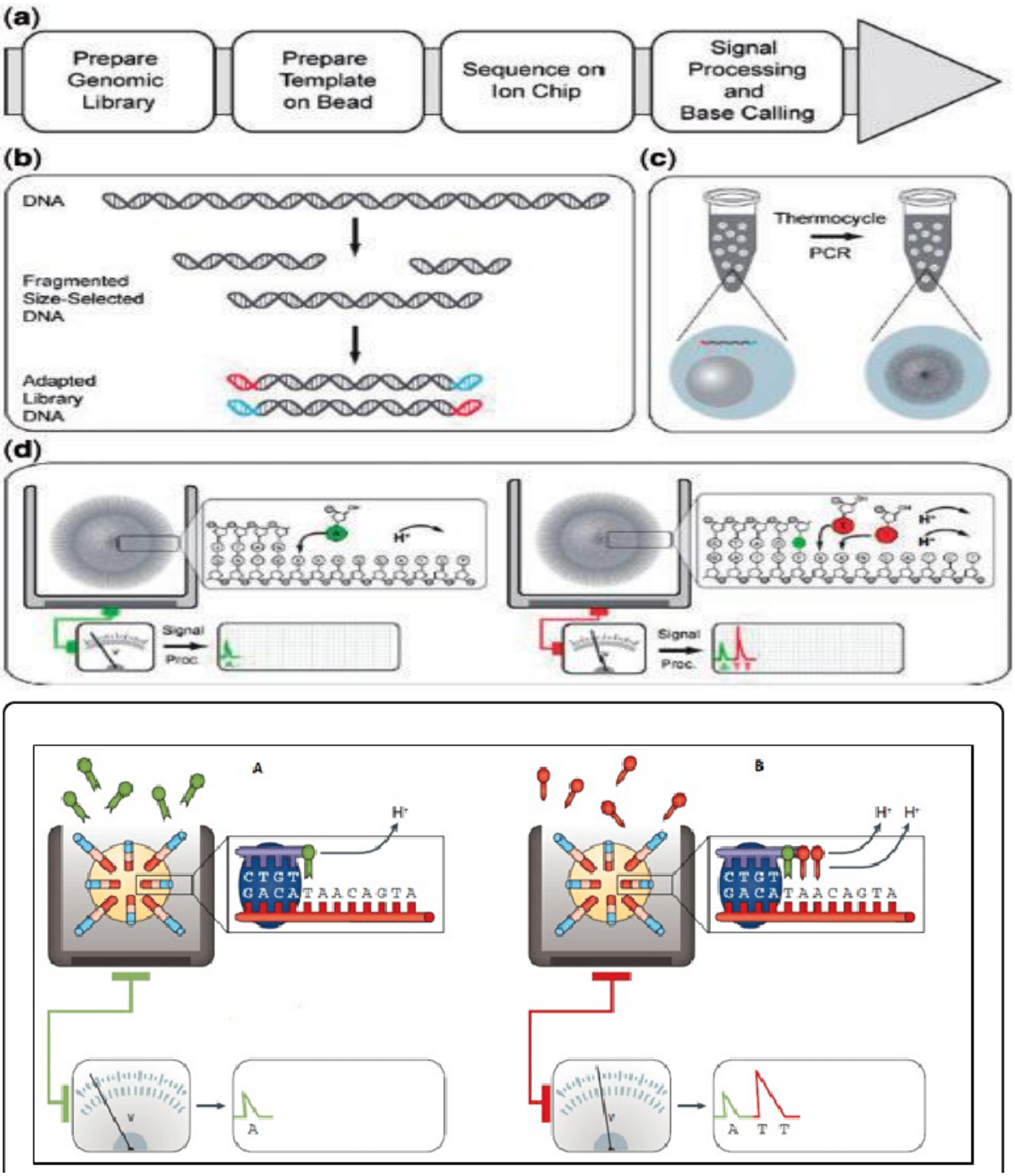

Figure 9. Working principle of Ion Torrent sequencing

The preparation of DNA samples in this method does not differ from the ligase sequencing described above. A preliminary emulsion PCR is necessary to amplify the signal indicating a change in the pH value.

The variation of pH in each individual well on the chip gives millions of copies of the initial DNA fragments attached to the surface of the microsphere. For this, the prepared DNA library is denatured and distributed to microelectronic chips holding an ion-sensitive surface (one well-one microsphere) with millions of microwells connected to hooks.

Then, the reaction PCR mixture containing only one of the four nucleotides is sequentially added, and the change of the pH indicator in the wells, which represents the joining of these nucleotides to the complementary strand of DNA, is recorded.

Advantages and disadvantages

The main advantage of Ion Torrent sequencing is that it uses simple chemistry in the sequencing process and requires a small sample size for analysis. Because of this, the sequencing speed is high and the operating costs are low.

Disadvantages are the occurrence of single nucleotide insertions and deletions during sequencing.

To address this issue, Life Technologies has released an updated version of its Ion Reporter product. Another disadvantage of the system is the short length of reads compared to Sanger sequencing or pyrosequencing..

THIRD GENERATION OF SEQUENCING METHODS

Although second-generation sequencing technologies allow the sequencing of multiple genomes at low cost, the analysis of large structural changes and de novo sequencing pose a challenge for these methods. The next step in DNA sequencing is to avoid DNA amplification and provide longer reads in a single attempt. However, such technologies are still at research stage.

Single molecule sequencing

Single-molecule real-time sequencing (SMRT) is the third generation of DNA sequencing. This method is used to read relatively long stretches of DNA sequences in real time.

The principle

SMRT-sequencing technology is based on monitoring the synthesis of a new strand by DNA polymerase in a DNA matrix in real time [11].

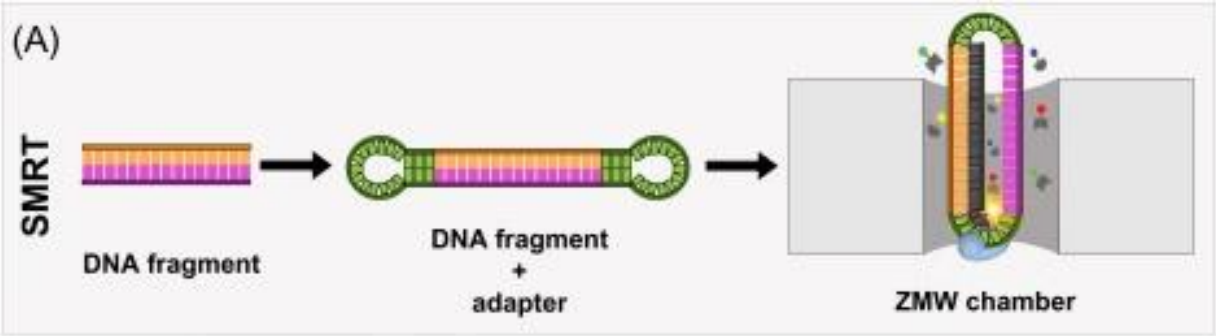

Figure 10. Working principle of Single Molecule Real Time Sequencing (SMRT) Technology

SMRT technology relies on two main innovations: zero-mode waveguides (ZMW) and presence of fluorophores attached to the terminal phosphate group of nucleotides. ZMW waveguides bring light only to the lower part of the cell, where the complex (matrix for DNA polymerase-synthesis) is immobilized.

Fluorescently labeled nucleotides allow monitoring of immobilized complexes as DNA–polymerase synthesizes the DNA chain. The principle behind Pacific Biosystems differs dramatically from other sequencing methods based on SMRT . It uses a single molecule for recording, so no amplification process is needed to prepare a library of amplicons.

Fluorescently labeled nucleotides are inserted into the well, and when it binds to single-stranded DNA bound to immobilized DNA polymerase, the fluorescently labeled nucleotide is separated from the nucleotide.

The released fluorophore is recorded. Matrix sequencing is performed in real-time monitoring mode.

Advantages and disadvantages

Real-time single-molecule sequencing (SMRT) allows obtaining very long reads (on average from 20,000 to 60,000 nucleotides), which helps to solve a number of problems that arise when working with short-length fragments, facilitating further analysis.

Free from the initial amplification of the DNA being studied by PCR. This method provides a very high speed of sequencing (theoretically, it is limited only by the speed of DNA polymerase). The method has a high level of sensitivity and specificity and can detect inclusions with a probability of 0.1% in mixed samples.

The main drawback of SMRT-sequencing is the high level of error, which sometimes reaches 15%.

The weak point of the technology is the dependence on the immobilization of DNA-polymerase/SMRTbell-matrix complexes, leading to the predominance of short DNA fragments.

Nanopore sequencing

A major leap forward in nanopore sequencing technologies occurred in 2001 with the discovery of solid nanopores. In 2005, Oxford Nanopore Technologies was established. It is considered the first company to offer commercial sequencers based on nanopore technology.

The principle

The working principle of ONT sequencers is based on measuring the electrical current generated by the nucleic acids passing through the nanopores [12].

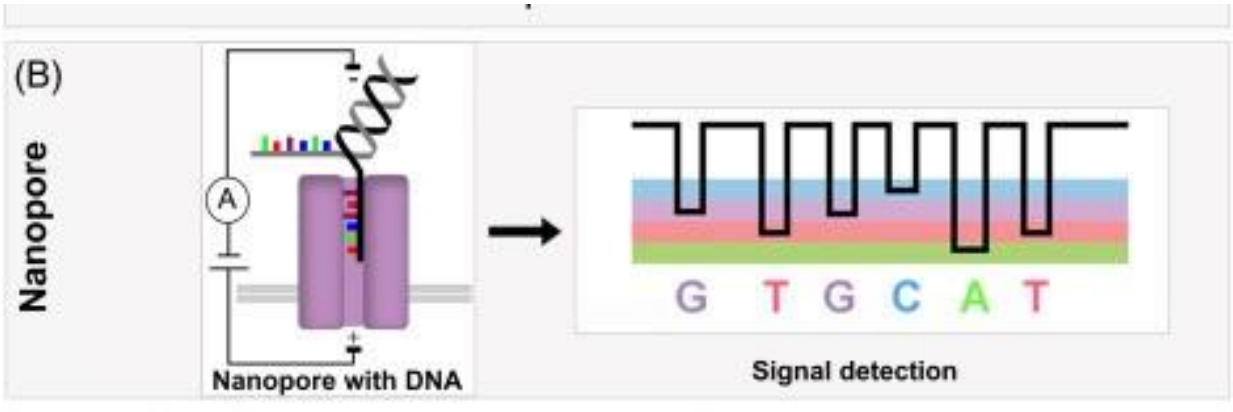

Figure 11. The working principle of nanopore sequencing.

Detection takes place in a chamber separated by a nanoporous membrane. A voltage is applied to the chamber that forces the DNA or RNA to move through the pore. During the passage of molecules, the cross-section of the pore decreases, and as a result, the current decreases. In this way, it is possible to determine which type of nucleotide is passing by measuring the current in a given time interval.

Advantages and disadvantages

Among known sequencing methods, this method is superior in terms of low cost and ease of use, high sensitivity, large read length (tens of thousands of nucleotide pairs), compactness, rapid analysis, and the ability to display results in real time. Among the disadvantages, it is possible to consider the low quality of reading, the failure of biological probes over time. In addition, it was determined that the environment affects the speed and quality of reading.

CONCLUSIONS AND SUGGESTIONS

After the chemical chain termination method introduced by Maxam and Gilbert in 1977, the Sanger method, discovered in the same year, revolutionized biology. These techniques led to the sequencing of larger genomes, culminating in the Human Genome Project. As a next step, large-scale sequencing projects can be shown as an example used to study human variation. However, for such large-scale projects, the Sanger method was too expensive and time-consuming. In 2004, the National Human Genome Research Institute (NHGRI) launched a program to reduce the cost of reading a complete genome to $1,000 over 10 years.

This led to the development of fast and inexpensive NGS technologies capable of multiplying many millions of reactions in one cycle. The main advantage of NGS technologies was to avoid bacterial cloning of DNA fragments and electrophoretic separation of sequence products. Currently, Illumina's NGS technologies are leading the global market. Due to its low cost, NGS technology has made genome sequencing possible for small laboratories. The direction with the goal ‘Human Genome – cheaper than 1000 dollars’ was implemented a few years ago. Currently, NGS technologies are the mainstay of biology and are widely used in clinical and agronomic research.

While NGS technologies are highly productive, their major drawback is short read lengths. Because genomes contain many repetitive sequences that are slightly larger than NGS-readable lengths, the inaccurate result gathering and breaks are observed. Because of this, many genomes are divided into hundreds and thousands of contigs. Furthermore, large structural variations (SVs) are difficult to find and characterize, while single nucleotide variations (SNVs) and short indices can be accurately detected using short reads.

This is an important issue because most medical samples are SV in nature. Branches with extreme GC percentages are also inefficiently amplified by PCR.

After NGS came TGS technologies. In 2014, Oxford Nanopore Technologies (ONT) proposed a nanopore sequencing method. PCR amplification is not used in this method.

However, third-generation sequencing technologies have a number of drawbacks. An example is the high error rate of nanopore-based sequencing. To solve these problems, world laboratories and companies continue research.

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