Genome Sequencing Technologies in combating COVID- 19 Pandemic

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Abstract: Genome sequencing is part of combating ever evolving COVID- 19 menace. For vaccine, drug and diagnostics development, monitoring mutations, virulence and host specificity evaluation, transmission tracking, tracing evolutionary paths genome sequence is essential. Sanger's sequencing, next generation sequencing, third generation sequencing and Meta genomic next generation sequencing technologies are presently available for genome sequencing.

Keywords: COVID-**19** Pandemic, Genome sequencing technologies, Next generation sequencing, Third generation sequencing

Introduction

COVID-19 pandemic started in December 2019 in Wuhan city of Hubei province of China spread to many countries disrupting normal life of billions of people, devastating economies and killing million people [1]. Severe Acute Respiratory Syndrome Corona virus 2 (SARS- Co2) that is responsible for the pandemic is evolving rapidly and attempts by several countries to contain are yet to be successful [2]. Several variants are identified from different countries. Both pharmaceutical and non-pharmaceutical intervention strategies are in use to combat COVID – 19 menace [3,4]. Whole genome sequence is required for vaccine, drug and diagnostics development, for monitoring mutations, to know virulence and host specificity, and for tracing evolutionary and transmission paths of virus. Under these circumstances sequencing genome of SARS – Co 2 virus may serve as one of the arsenal that can be used to combat COVID -19. In view of these genome sequencing technologies that are currently in use are presented in this article.

Double helical structure of DNA by Watson and Crick in 1953 initiated development of genome sequencing technologies worldwide which culminated with Human Genome Sequencing in 2000. Two groups Human Genome Consortium and Celera Genomics separately reported human genome sequence in 2001[5,6]. These groups adopted different approaches for human genome sequencing. A top down approach is followed by former group and shot gun approach by latter group. However both groups used Sanger's dideoxy chain termination method for sequencing.. Recently SARS –Co2 whole genome sequence from Nigeria and Bangladesh is determined by Sanger's sequencing [7,8]. Shot gun approach is also used for sequencing cereal genomes like rice and wheat which form major staple food of world population [9,10].

Sanger's dideoxy chain termination Method
Or
First generation genome sequencing

F.Sanger and his colleagues developed this method which is most commonly used to determine nucleotide sequence of DNA fragments upto 500 nucleotides long. It is first generation genome sequencing technology [11]. In this method daughter strands which are labelled at one end and differ in length by only one nucleotide are synthesized from DNA fragment to be sequenced. Then nucleotide sequence of original DNA fragment is established after separating truncated daughter strands by gel electrophoresis. 2', 3'- dideoxy nucleotide triphosphates (dd NTP s) are used to produce truncated daughter strands. These molecules in contrast to normal deoxy ribonucleotide tri phosphates (d NTP s) lack 3' hydroxyl group. DNA polymerase uses dd NTPs for polymerization like dNTP s. However due to lack of 3' hydroxyl incorporation of dd NTP cause chain termination. As a result a truncated daughter strand is formed.

1. It begins with denaturation of DNA fragment to generate a template strand for synthesis of copy of DNA strand to be sequenced.
2. A synthetic oligo deoxy nucleotide is used as primer
3. DNA polymerization is carried out using Klenow fragment of DNA polymerase –I which lacks exonuclease activity in presence of normal d NTP concentration and low concentration of one of four ddNTPs.
4. Four separate polymerization reactions are carried out each with low concentration of one of four ddNTPs and normal dNTPs.
5. In each reaction incorporation of ddNTP leads termination of polymerization. The reaction mixture contain mixture of pre maturely terminated (truncated) daughter strands each ending with one of the four dd NTPs.

6. The four reaction mixtures are subjected to electrophoresis on special poly acrylamide gels that separates single strand DNA molecules that differ in length by only one nucleotide.
7. In automated sequencing mechine a fluorescence detector is located at the end of gel. In automated sequencing dd NTPs tagged with four different fluorescent dyes corresponding to four different dd NTPs are used for polymerization.
8. Inclusion of four different color fluorescent tags allows each set of truncated daughter strands to be distinguished by their corresponding fluorescent color. For example all truncated fragments synthesized in presence of dd GTP tagged withYellow fluorescent dye end with G and give yellow fluorescence.
9. In automated sequencer a fluorescence detector is located at the end of gel. It distinguishes four fluorescent tags.
10. The sequence of DNA is determined from order in which different labelled fragments migrate past fluorescence detector.
11. DNA sequence is determined by manual methods also after separation on gel electrophoresis.
12. In manual methods the DNA chain is labelled by including radioactive dNTP in the reaction mixture. Chain termination occurs when dd NTP is incorporated. The reaction mixture contain many daughter fragments that are truncated.
13. The four reactions each containing one type of ddNTP and four dNTPs generate set of radiolabelled truncated daughter fragments.
14. By polyacrylamide gel electrophoresis separation of DNA fragments that are generated in four reactions is achieved.
15. Then autoradiography of gel is obtained from which sequence is read from smallest fragment upwards as shown in Fig. 1.
16. Using this method sequence of up to nine hundred bases may be read from the gel.
17. Until development of next generation sequencing i.e. up to 2006 it is the only most accurate sequencing method.
18. Now automated Sanger sequencing plat forms Seq Studio Genome Analyzer, 3500 and 3730 Genetic Analyzer are available.
19. Sanger's sequencing together with high through put technologies is used to sequence SARS- CoV-2 genome [12]

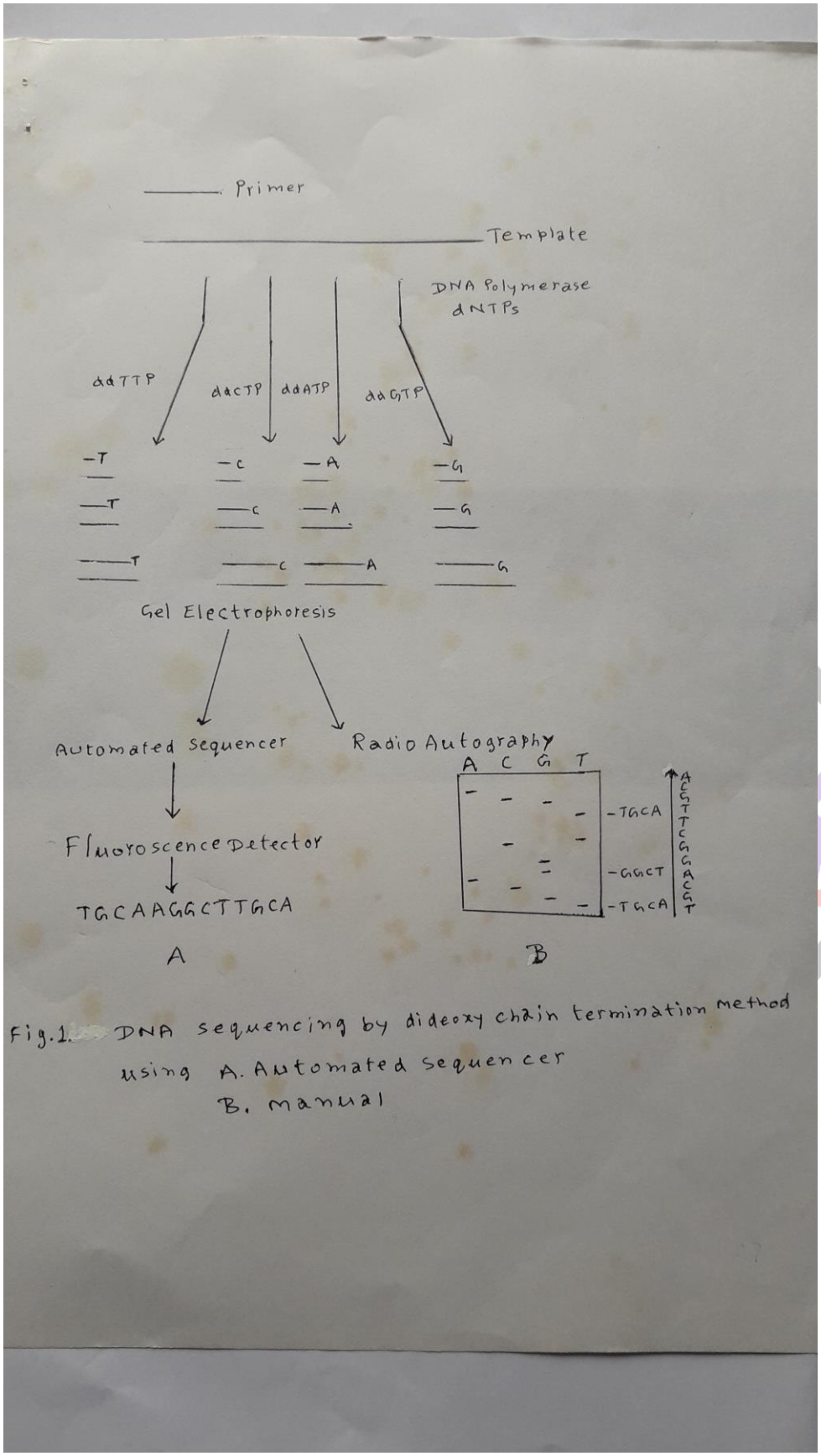


Fig.1. DNA sequencing by dideoxy chain termination method using A. Automated sequencer B. manual

Post Genomic DNA Sequencing Methods

Until completion of human genome project by international groups DNA sequencing involved preparation of multiple copies of DNA to be sequenced either by expression in E. Coli after cloning with plasmid or preparation of amplified PCR product. Then sequence of a copy of DNA is determined by Sangers technique involving electrophoretic separation on gels and processing of fluorescent signal. Due to realization of limitations of Sangers protocol like need for automation , large laboratories , polymers for

separation , less number of samples which can be analyzed, users and developers of DNA sequencing techniques initiated efforts to develop techniques without gels , avoiding cloning and more samples or millions can be sequenced in parallel in post genomic period. These novel DNA sequencing methods provide high speed through put, reduces cost, lab requirements and time. Sequencing by Sangers technique that takes several years can be now completed in a matter of week and at very low cost. They are called as **next generation DNA sequencing methods** or **next generation parallel DNA sequencing methods (ngs) .** Progression and genetic epidemiology of SARS – CoV-2 are studied by using next generation sequencing [13 ,14]..

They are of two types

A. Second generation DNA sequencing techniques (SGST)

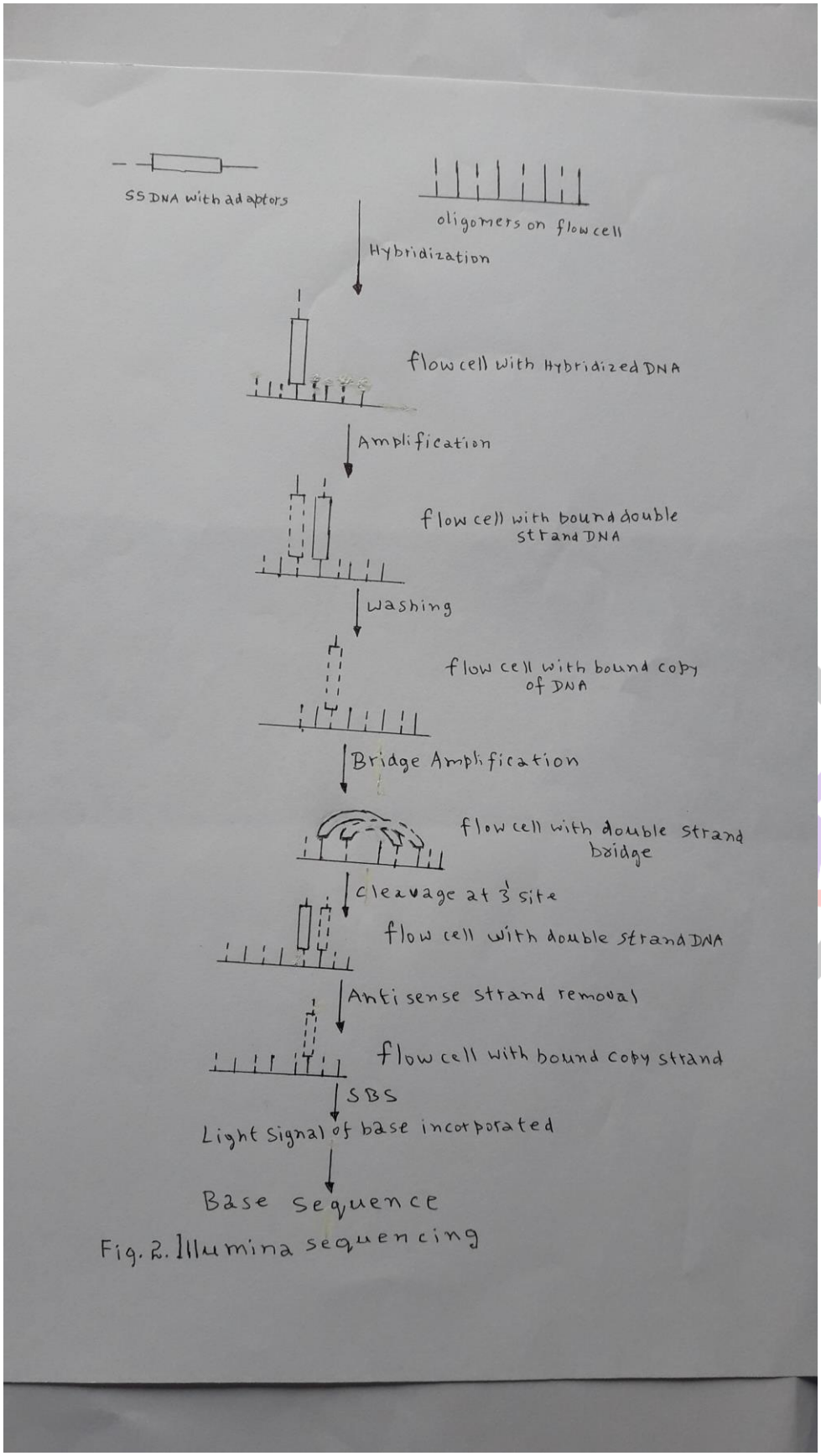
B. Third generation DNA sequencing techniques (TGST)

Second Generation DNA sequencing Technology Systems (SGSTS)

The basic principle of second generation DNA sequencing technology devices is sequencing of millions of copies of DNA in parallel without gels. The DNA samples are attached to solid support or beads or DNA chips in micro reactors or miniature Microsystems. In micro reactor sequencing by synthesis approach is used to sequence sample DNA. Addition and detection of incorporated base in growing DNA chain in each cycle is monitored by light sensitive charge coupled devise (CCD) camera. Sequence data are acquired by processing images with special software. Depending on devise or manufacturer one or more of these steps may be modified. Many SGST are available in the market at present. They are, Illumina Genome sequencers, 454 Genome Sequencer, Nano Ball sequencer and ION torrent sequencer etc...

Illumina Genome sequencer

1. This device works by bridge amplification (BA) and sequencing by synthesis (SBS) approach and takes place in flow cell.
2. Sample DNAs are fragmented and separated. To the single strand fragments adaptors are ligated.
3. They are amplified after loading into a flow cell.
4. The flow cell is a glass containing several lanes. Each lane is a channel with oligo nucleotide chains attached to inside surface which serve as primers for copying by polymerase.
5. The oligo nucleotide sequence is complementary to ligated adaptors. This allows hybridization of DNA fragment with complementary oligo nucleotide chain on the surface of flow cell lanes.
6. Now polymerization is carried out and this is followed by washing to remove original fragment. The newly formed strand only remains bound to flow cell.
7. All newly formed single strands undergo bridge amplification and clustering. Flow cell attached strands are used to generate clusters by amplification
8. The bridge amplification of illumina generates high number of clusters. The number of cluster formation depends on amount of loaded sample.
9. The new fragment forms bridge by hybridizing to complementary adjacent oligo nucleotide chain and it is amplified and hence the name bridge amplification.
10. Cleaving at 3'cleavage site of oligonucleotide chains leads to formation of double strand straight chain.
11. Now all the anti-sense strands are removed by using cleavable site of the surface oligo nucleotide strand and newly formed strands remains attached to flow cell.
12. Then sequencing reactions of amplified bound fragments are carried out with four reversible terminator nucleotides (3'-OH blocked) each labelled with different fluorescent dye.
13. After incorporation into growing strand the terminator nucleotide as well as its position is detected and identified via fluorescent dye .
14. This system generates at least 1.5 Gb per minute.
15. iSeq 100 , Mini Seq , Mi Seq , Next Seq 550 , 2000 and Nova Seq 6000 are Illumina sequencing platforms currently in use.
16. Illumina Mi Seq and Illumina Next Seq 500 are used for sequencing of SARS- CoV -2 by two different methods [15, 16] . Illumina MiSeq is used for complete genome sequence of SARS –C0V -2 strain isolated from China
[17] and Nepal
[18].Complete genome of SARS-CoV -2 sequence from Bangladesh is obtained from Illumina iSeq 100 platform
[19]. Using Illumina Next Seq complete genome sequence of SARS-CoV-2 isolated from Northern Germany is obtained
[20]. 17.Illumina MiSeq is used to sequence whole genome of SARS-CoV-2 prepared by employing three different Illumina Protocols for quick and accuracy during pandemic in South Africa
[21]. various steps of Illumina Sequencing are shown in Fig.2



The 454 Genome Sequencer

1.It has through put of 700 Mb per run time of 23 hours.
2.In this GS system single strand DNA (SS DNA) fragments to be sequenced are ligated with adaptors at both ends whose length is 454 bases hence the name.
3.The adaptors cause binding of DNA fragment to bead at one end.
4.A sequence primer is hybridized to adaptor at position that is immediately next to start of unknown sequence.

5.The DNA fragments are amplified by emulsion PCR to about one million copies.
6.These amplified single molecules are sequenced en mass.
7.The beads containing amplified DNA are randomly deposited to micro fabricated array of pico litre scale wells.
8. In each cell single bead provides fixed location for sequencing and monitoring. Further during sequencing one side of array function as flow cell for introducing and removing sequencing reagents where as other side is bonded to fibre optic bundle for CCD based light signal detection.
9. Sequencing is performed by **pyrosequencing** method that involves sequencing by synthesis approach.
10 . Each cycle of pyrosequencing involves addition of one of the four nucleotide species , DNA polymerase followed by adenosine phosphosulphate (APS).
11.Pyrophosphate released on incorporation of nucleotide by polymerase is detected as emitted light.
12..Sulfurylase combines γ- pyrophosphate and APS to generate ATP. Then light is produced at well by luciferase using ATP and luciferin which is detected by CCD camera.
13.Sequence data is generated by processing of images acquired with software that is specifically developed [22].
14. This plat form is used for Dengue Viral Genome sequencing [23]. In other research areas also it is used [24] but its utilization for SARS – CoV – 2 Genome sequencing is yet to begin.

Various steps of 454 Genome Sequencer are shown in Fig.3.

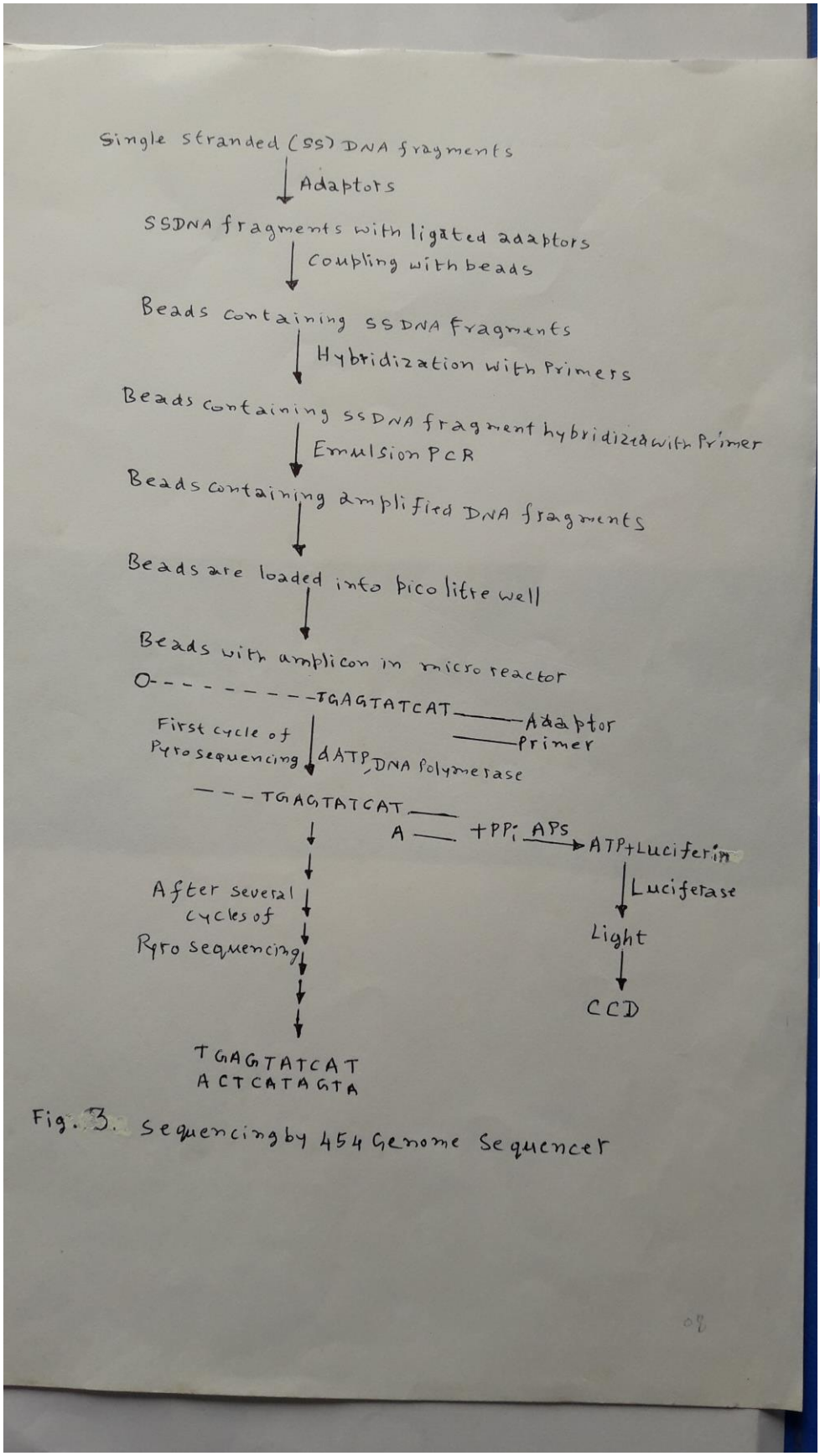
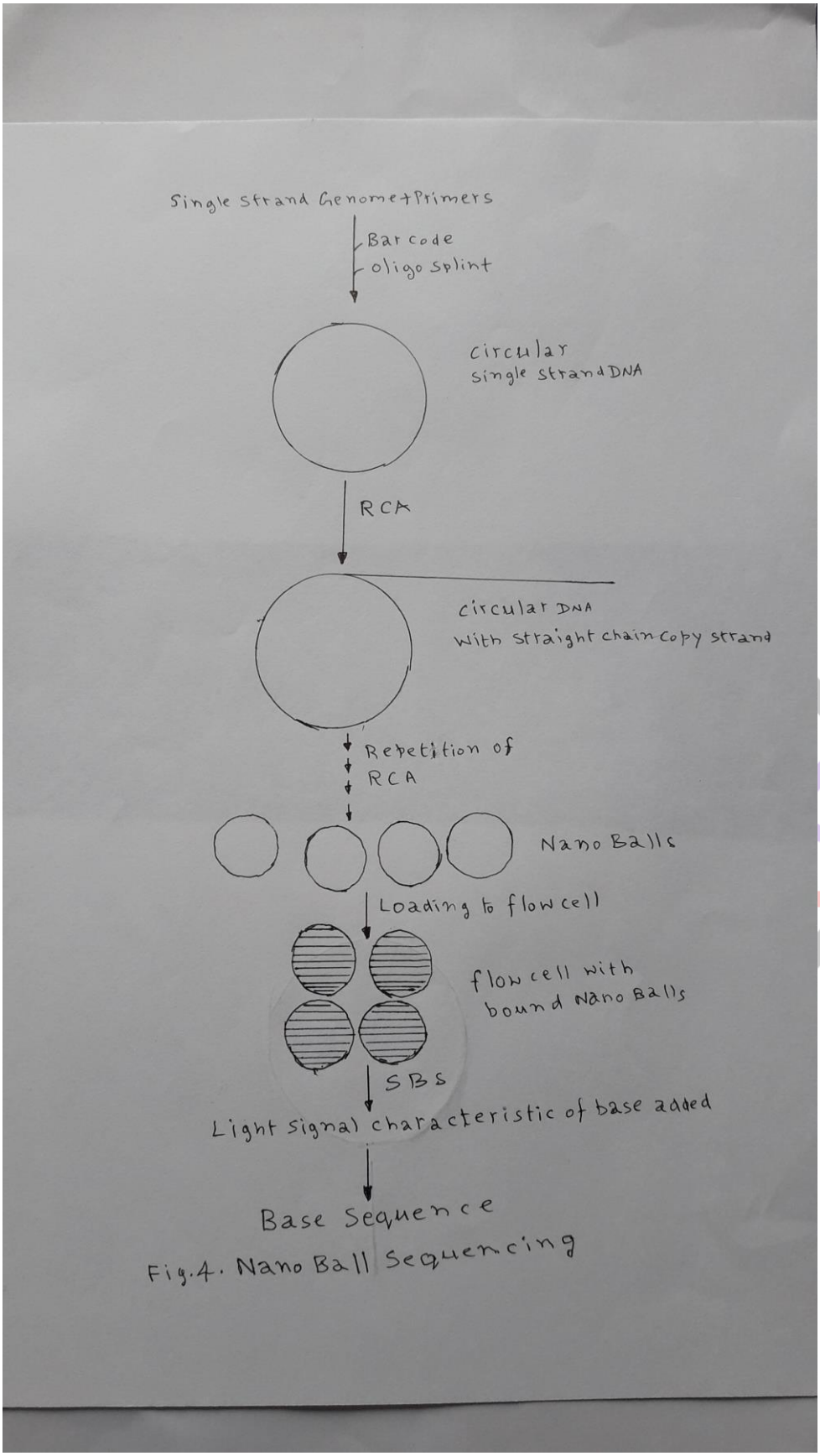


Fig. 3. Sequencing by 454 Genome Sequencer

Nano Ball Genome Sequencer

1.This type of genome sequencing involve**s amplification** of genome into Nano Balls . Then sequence of genome is obtained from Nano Balls by synthesis approach.
2. Single strand Genome (DNA) to be sequenced is obtained by denaturation of original genome.
3. Next single strand genome is converted to circular genome. Bar code and primers are attached prior to circularization. Oligo splint aids circularization.

4. The circularized genome is repeatedly amplified to produce single strand massive genome by employing Rolling Circle Amplification (RCA) process.
5. It involves copying of genome by polymerase using primers leading to formation of new circular copy which is hybridized to native genome. The newly formed copy of genome dissociates as second cycle of circular amplification begins. Repetition of this process several times generates massive single strand genome .
6. The single strand massive genome then forms what are known as Nano Balls.
7. These Nano balls are loaded into flow cell. The flow cell contains numerous patterned array of spots (wells).
8. These spots are positively charged and hence a single Nano ball which bears negative charge binds.
9. Now sequencing of Nano ball is carried out in each well (spot) by synthesis approach. When nucleotide is incorporated as dictated by base pairing rule it is identified by detecting light emited..
10. Simultaneous synthesis at each Nano ball generate high accuracy sequence data.
11. MGISEQ-T7,DNBSEQ- G50, DNBSEQ –G400 and DNBSEQ –T7 are available sequencing platforms.
12.DNBSEQ has been used to sequence SARS-CoV-2 genome from different sources[25,26,27].
Variou steps of Nano Ball Genome sequencing are shown in Fig. 4



ION Torrent Next Generation Sequencer (ITNGS)

1. This sequencer works by ion sensing. A semiconductor chip is employed to conversion signals to electrical signal. Ion torrent chip consist of flow compartment and solid state p^{H} sensor micro arrayed wells. Hence the name ION Torrent sequencing [28].
2. Sequencing by synthesis (sbs) approach is used for sequencing but labeled nucleotides are not used. Oil emulsion PCR (em PCR) is used for amplification of sample DNA.
3. DNA to be sequenced is fragmented and each fragment is ligated with two different adaptor molecules.

4. DNA fragments are added to acrylamide beads and amplified by emulsion PCR (e mPCR).
5. Sequence templates are generated on the bead or sphere. An oil water emulsion is created to partition reaction vesicle. Each part of reaction vesicle contains one bead, one library molecule and amplification reagents.
6. Two primers that are complementary to the ligated adaptors are present. Among two primers one is bound to the bead and other is present in solution.
7. This allows binding of fragments containing two different adaptors while not allowing fragments containing two adaptors of same type. As result of this all the library molecules orient uniformly on the bead.
8. Millions Of copies are produced from sequence library molecules during emPCR that are bound to beads for sequencing.
9. Semiconductor chip contains numerous tiny wells. Single bead is placed in each well along with library molecule, primer and polymerase.
10. Now sequencing is carried out by adding four different nucleotides and incorporation of a nucleotide is monitored by signals arising from sensor.
11. Addition of deoxy nucleotide by polymerase to growing strand releases H^+ ion and pyrophosphate.
12. Release of protons changes p H which is converted to voltage signal by pH sensitive field effect transistor (pHFET).
13. Sequence is obtained from signals generated that takes place when a base is incorporated using base calling algorithm.
14. Ion Proton system + Ion 318 chip , Ion PGM (Personal Genomics Mechine) system + PI chip, Ion Gene Studio S5 System + Ion 540 chip , 550chips and ion Genexus are some of the platforms available at present. They have minimum through put of 2 Gb per day.
15. Ion Torrent system Ion Xpress Plus Fragment Library Kit + Ion 318 v2 chip are used for SARS-CoV – 2 genome sequencing of first COVID -19 patient from Mexico [29]. Ion Torrent S5 system is used to sequence genome of SARS –C o V- 2 from COVID- 19 patients of Fujian province of China [30].
16. SARS-CoV- 2 genome of first case of COVID – 19 patient from Italy is sequenced by using Ion Torrent S5 system [31].

Third Generation Sequencing Technologies (TGST)

The available SGST depends on manipulated DNA that was obtained after amplification either by expression or PCR , original DNA or natural DNA are not used. Each step of DNA manipulation causes artifacts and inaccuracies in DNA measurements. TGST analyze unmanipulated original DNA molecule in massively parallel manner. Nano pore genome sequencing and Single Molecule real time DNA Sequencing (SMRTDS) technologies are emerging TGST.

Nanopore genome sequencing (NPGS)

1. It is nanotechnology based third generation sequencing. An engineered mutant protein is used as nanopore.
2. The protein is embedded in synthetic membrane. The potential of this membrane is maintained by solution
3. Prior to sequencing adaptors are ligated at both the ends of single strand genome. These adaptors facilitates binding of molecular motor or processing enzyme and strand to nano pore [32].
4. An alternative way of sequencing double stranded molecule is preparing single strand molecule by covalent attachment of template strand and complementary strand with hair pin adaptor.
5. During sequencing single strand is translocated through the nanopore by molecular motor upon capturing.
6. Further the processing enzyme allows unidirectional displacement of single nucleotide of the strand in millisecond time scale.
7. When molecule pass through the nanopore a sensor detects potential changes caused by change of nucleotide in the pore. Sequence is obtained by monitoring potential differences.
8. In the case of double strand molecule information provided by template and complementary strands is combined to deduce high quality sequence information.
9. Native DNA and RNA can be used. It does not require labeled nucleotides, amplification by PCR.
10. Further this device is not based on synthesis approach and it is portable and this makes it suitable for use during outbreaks.
11. Since DNA and RNA is used directly base modifications also can be identified. This is not feasible with many other sequencers.
12. When is used directly for sequencing then it is referred as Direct RNA Nano pore Sequencing [27].
13. Sequencing is rapid about 400 bases per second and one pore reads one nuclec acid at a time. This system is capable of producing very long sequence reads upto 800,000 bases length.
14. MinION Mk 1B, Mk1C; Grid ION Mk1;Nova Seq 6000; Prometh ION 24 ,28 are currently available nanopore sequencing platforms.
15 Min ION is used to sequence SARS-CoV-2 of nasopharyngeal swabs of COVID-19 patients [33]. For accurate and comprehensive detection of detection of SARS –CoV-2 and other respiratory viruses .Nanopore targeted sequencing is carried out by using MinION [34].
16. FLOWMIN 106 D of Oxford Nanopore Technologies is used for sequencing SARS – COV-2 genome of Columbian COVID patient [35]. To confirm re infection of COVID -19 in patients from Mumbai, India whole genome sequence analysis of SARS – CoV-2 is performed by using MinIONMk1B platform of Oxford Nanopore Technologies [36].

Single Molecule Real Time (SMRT) Genome Sequencer

This type of sequencing does not require clonal amplification and directly sequences native genome or DNA. It is based on sequencing by synthesis approach. But blocker is not bound to deoxy ribose. So incorporation of nucleotide by polymerse is detected continuously by camera from fluorescence emitted in real time about 75 frames per second and hence name real time sequencing..
1. Sample DNA is isolated from cell is fragmented and library of DNA molecule is generated.

2. Both the ends of DNA are ligated with hair pin adaptors forming a closed single strand circle. Further the adaptors serve as primers for DNA polymerase during sequencing. This referred as SMRT bell [37].
3. Next SMRT bell is loaded to SMRT flow cell which diffuses into nano sized sequencing well called as zero-mode wave guide (ZMW) which contains an immobilized polymerse at bottom. An SMRT flow cell contains about 150000 wells. New Sequel platform contains millions of wells.
4. It can analyze millions of DNA fragments simultaneously generates sequence through put in Giga bases range about 15-30Gb per flow cell.
5. Polymerase in each well can bind either of the hair pin adaptors and initiates synthesis. Now four differently labeled nucleotides are added to well. The fluorescence dye is attached to phosphate group.
6. The sequencing process involves cyclically incorporating each of four fluorescently labelled nucleotides with polymerase. It begins with binding of labeled nucleotide to active site of polymerase associated with template strand and a light signal characteristic of the nucleotide is produced which is captured by camera. This is followed by cleavage of pyrophosphate linked dye that diffuses out of the well [38].
7. Next nucleotide get associated with template strand bound polymerase and light signal is produced corresponding to the base attached.
8. Sequence information is generated by processing light signals acquired. Sequence processes in all wells are recorded by movie of light signals and light signals corresponding to each well is sequence of bases.
9. Sequel system +1M SMRT cell and Sequel II system + 8 M SMRT cell are some of the platforms for SMRT genome sequencing.
10. Long read sequencing of SARS-CoV -2 by Sequel system + single SMRT cell is carried out to characterize viral transcriptome [39]. Sequel I system and SMRT cell are used for Whole genome sequencing of a 2019 novel corona virus (SARS-CoV-2) isolated in Vietnam [40].

Meta Genomic Next Generation Sequencing (mNGS)

It is an extension of next generation sequencing. In clinical management of diseases identification of viral genes in body fluids by diagnostic methods may not be of prognostic use because other known or unknown infections can increase mortality and morbidity. Hence rapid identification of pathogenic agents from infected body fluid compartments is essential. Metagenomic next generation sequencing is used to identify all pathogenic agents simultaneously from samples. It is sequencing of total DNA or RNA or genomes of samples. Further most of the next generation sequencing methods are rapid takes from less than 6 hours to maximum 16 hours or 24 to 72 hours. However methodology and sample types varies depending on clinical condition [41]. Long read Oxford Nanopore third generation meta genomic next generation sequencing of nasopharyngeal swab from COVID-19 patient is carried out to identify SARS-CoV -2 and other infecting pathogens[42]. Rapid Meta genomic net generation sequencing on Ilumina I Seq 100 plat form is used for characterization of a imported case of COVID -19 in Combodia [43]. Corona virus from pneumonia cases of Wuhan outbreak in 2019 are identified by using RNA based mNGS approach on Illumina Miseq plat form [44].

Conclusion

Sequencing rapidly mutating Severe Acute Respiratory Syndrome Corona Virus -2 (SARS-CoV-2) Genome is essential to contain Covid -19 pandemic. Globally Sanger's first generation sequencing, second generation Illumina sequencing, 454 genome sequencing, Nano Ball sequencing, Ion Torrent genome sequencing ; third generation NanoPore sequencing , Single Molecule Real Time (SMRT) sequencing and meta genomic next generation sequencing (mNGS) technologies are used to sequence genome of SARS-CoV-2.

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