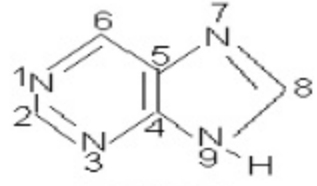
DNA SEQUENCING

SURENDER RAWAT
Msc. MICROBIAL BIOTECH
ROLL NO. 1784

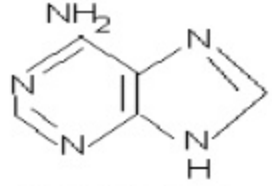
DNA SEQUENCING

- Determining the precise order of nucleotides within a DNA molecule.

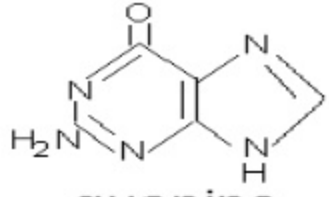
Purines



purine

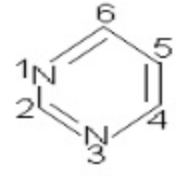


adenine

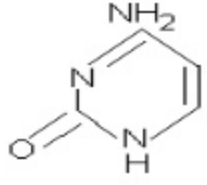


guanine

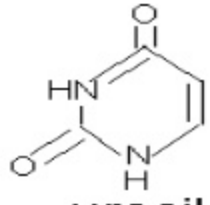
Pyrimidines



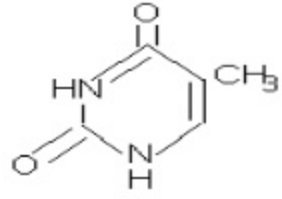
pyrimidine



cytosine



uracil



thymine

- Used to determine the sequence of individual genes, larger genetic regions, full chromosomes or entire genomes.
- The resulting sequences may be used by researchers in molecular biology or genetics to further scientific progress.

HISTORY OF DNA SEQUENCING

- 1972 – Earliest nucleotide sequencing – RNA sequencing of Bacteriophage MS2 by WALTER FIESERR
- Early sequencing was performed with tRNA through a technique developed by Richard Holley, who published the first structure of a tRNA in 1964.
- 1977 - DNA sequencing FREDRICK SANGER by Chain termination method
- Chemical degradation method by ALLAN MAXAM and WALTER GILBERT
- 1977 - First DNA genome t be sequenced of Bacteriophage ΦX174
- 1986 - LOREY and SMITH gave Semiautomated sequencing
- 1987 – Applied biosystems marketed Fully automated sequencing machines

- 1995 – CRAIG VENTER, HAMILTON SMITH and collegues published first complete genome sequence of *Haemophilus influenzae*

- 2003 – Human genome project

- 2ND Generation of DNA sequencing

- 3RD Generation of DNA sequencing

Determining the Sequence of DNA

- Methods:

1) Maxam and Gilbert chemical degradation method
2) Chain termination or Dideoxy method
   - Fredrick Sanger
3) Genome sequencing method
   - Shotgun sequencing
   - Clone contig approach
4) 2nd generation sequencing methods
   - Pyrosequencing
   - Nanopore sequencing
   - Illumina sequencing
   - Solid sequencing

SANGER SEQEUNCING

- Chain termination method of DNA sequencing.
- It involves following components:

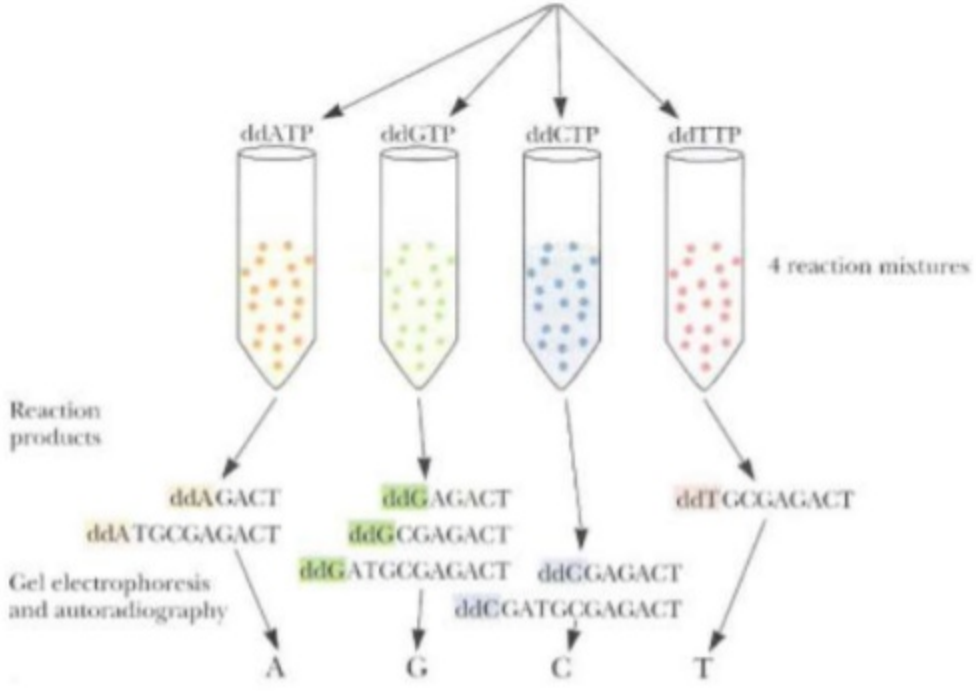
a) 1. Primer

b) 2. DNA template

c) 3. DNA polymerase

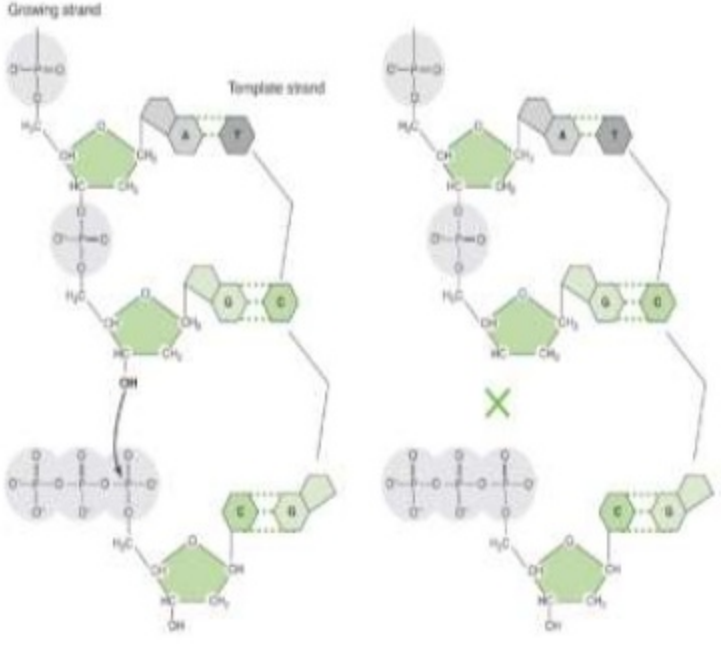
d) 4.. dNTPs(A,T,G,C)

e) 5. ddNTPs



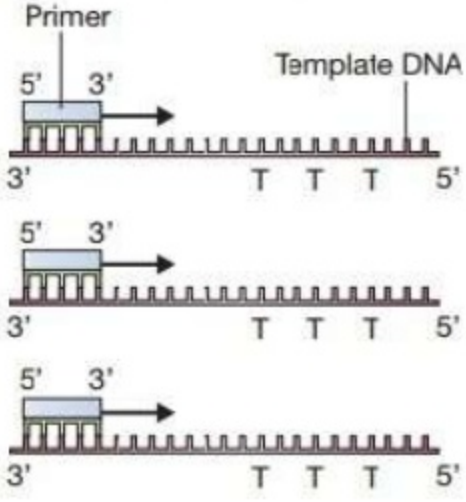
- 4 Steps:
    1. Denaturation
    2. Primer attachment and extension of bases
    3. Termination
    4. Poly acrylamide gel electrophoresis

SANGER'S METHOD

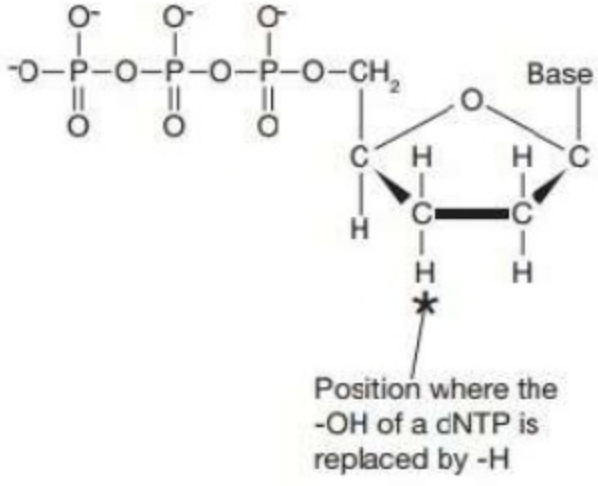


Copyright © 2007 F.A. Davis Company www.fadavis.com

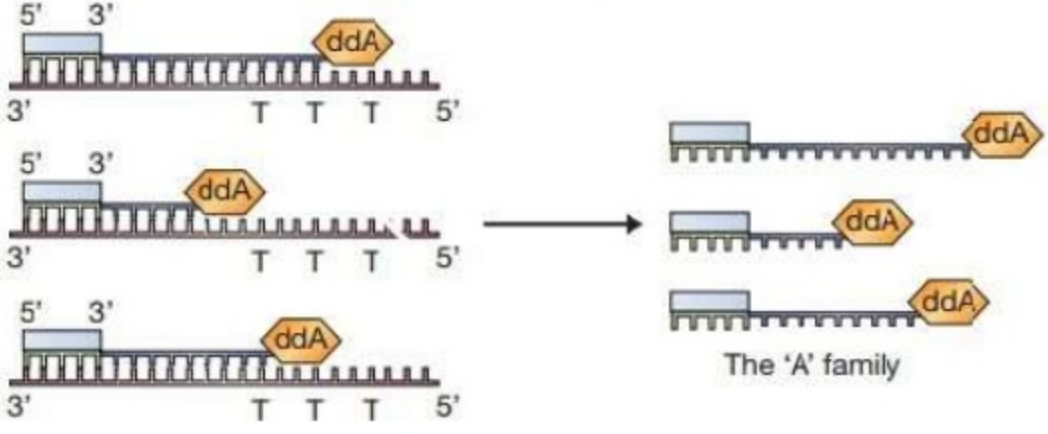
(a) Initiation of strand synthesis



(b) A dideoxynucleotide



(c) Strand synthesis terminates when a ddNTP is added



Chain Termination (Sanger) Sequencing

ddATP + four dNTPs

ddA
dAdGdCdTdGdCdCdCdG

ddCTP + four dNTPs

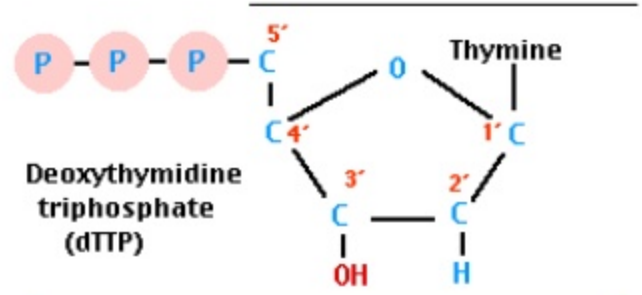
dAdGddC
dAdGdCdTdGddC
dAdGdCdTdGdCddC
dAdGdCdTdGdCdCddC

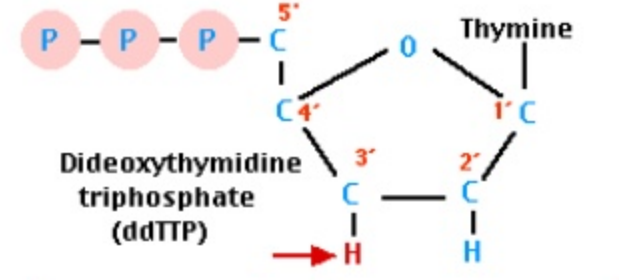
ddGTP + four dNTPs

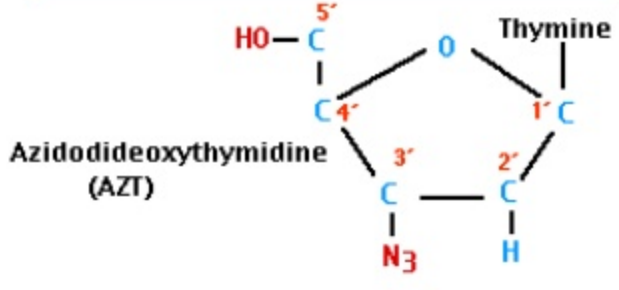
dAddG
dAdGdCdTddG
dAdGdCdTdGdCdCdCddG

ddTTP + four dNTPs

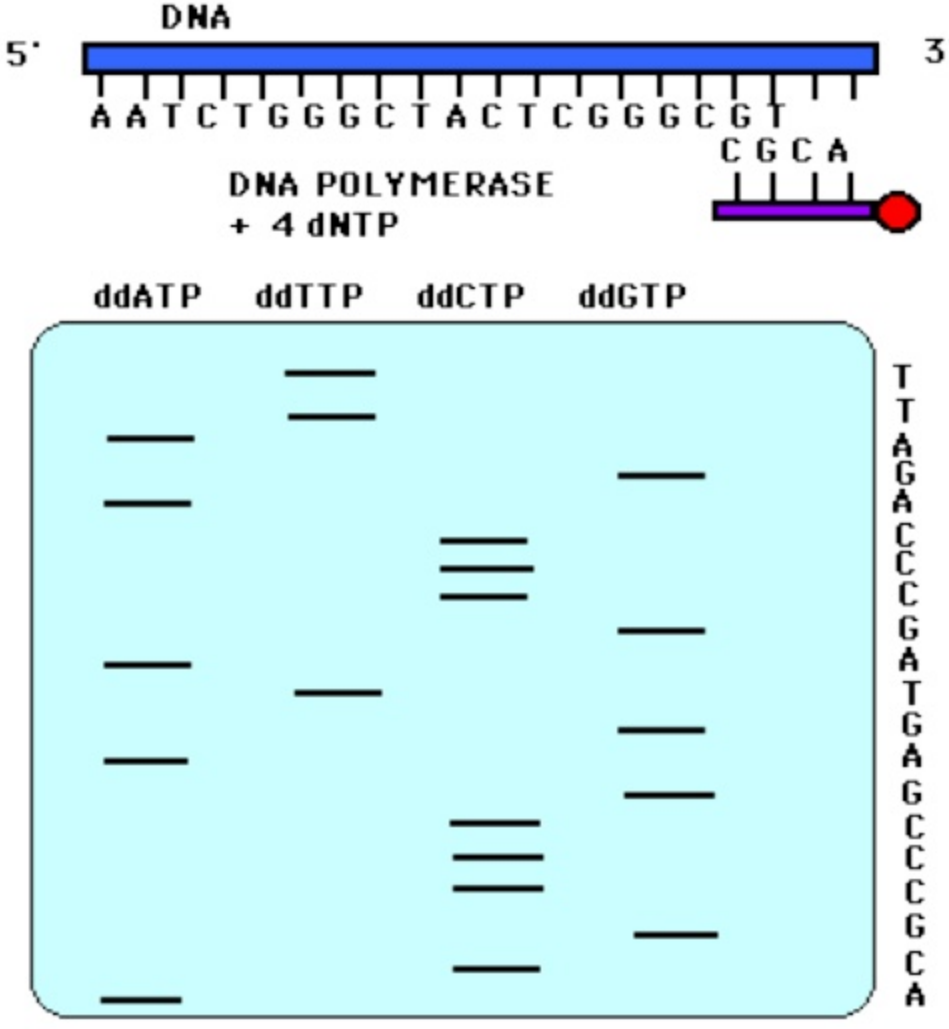
dAdGdCddT
dAdGdCdTdGdCdCdCdG

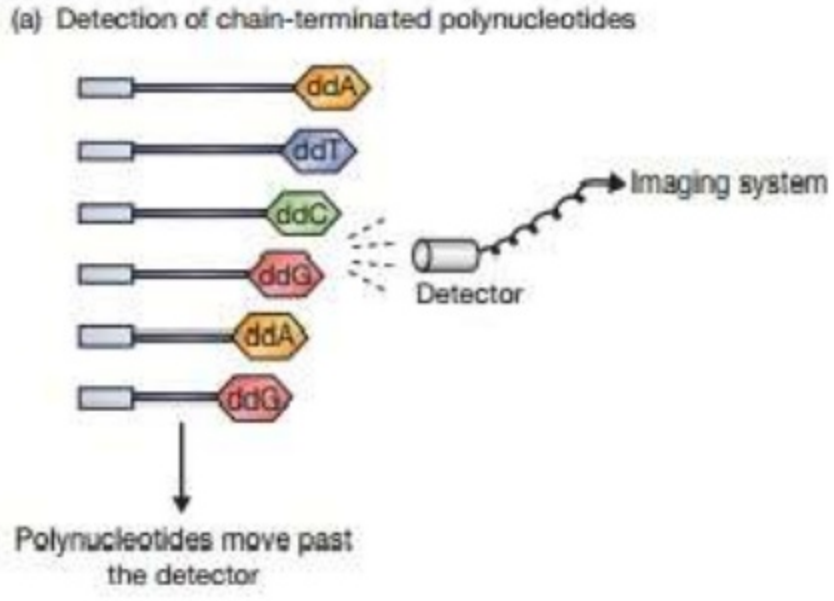


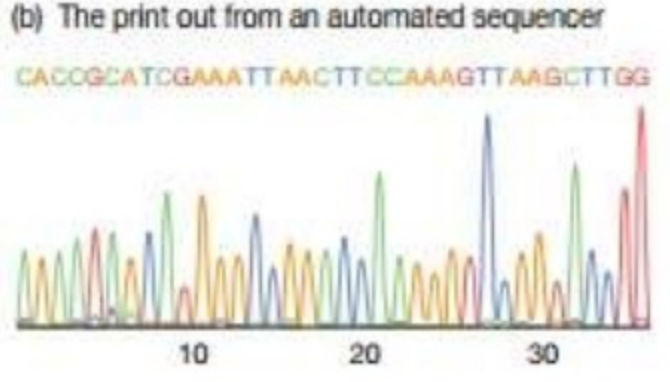




Determination of nucleotide sequence







SANGER'S METHOD

- Not all polymerases can be used as they have mixed activity of polymerizing and degrading.
- Both exonuclease activities are detrimental.
- Klenow fragment was used in orignal method but it has low processivity.
- So Sequenase from bacteriophage T7 was uesd with high processivity and no exonuclease added.
- Method requires ss DNA. So it is obtained by
  - Denaturation with alkali or boiling
  - DNA can be cloned in phagemid containg M13 ori and can take up DNA fragments of 10kb

PYROSEQUENCING

- Pyrosequencing is the second important type of DNA sequencing methodology in use today.

- The addition of a DNTP is accompanied by release of a molecule of pyrophosphate.

- Reaction mixture contains

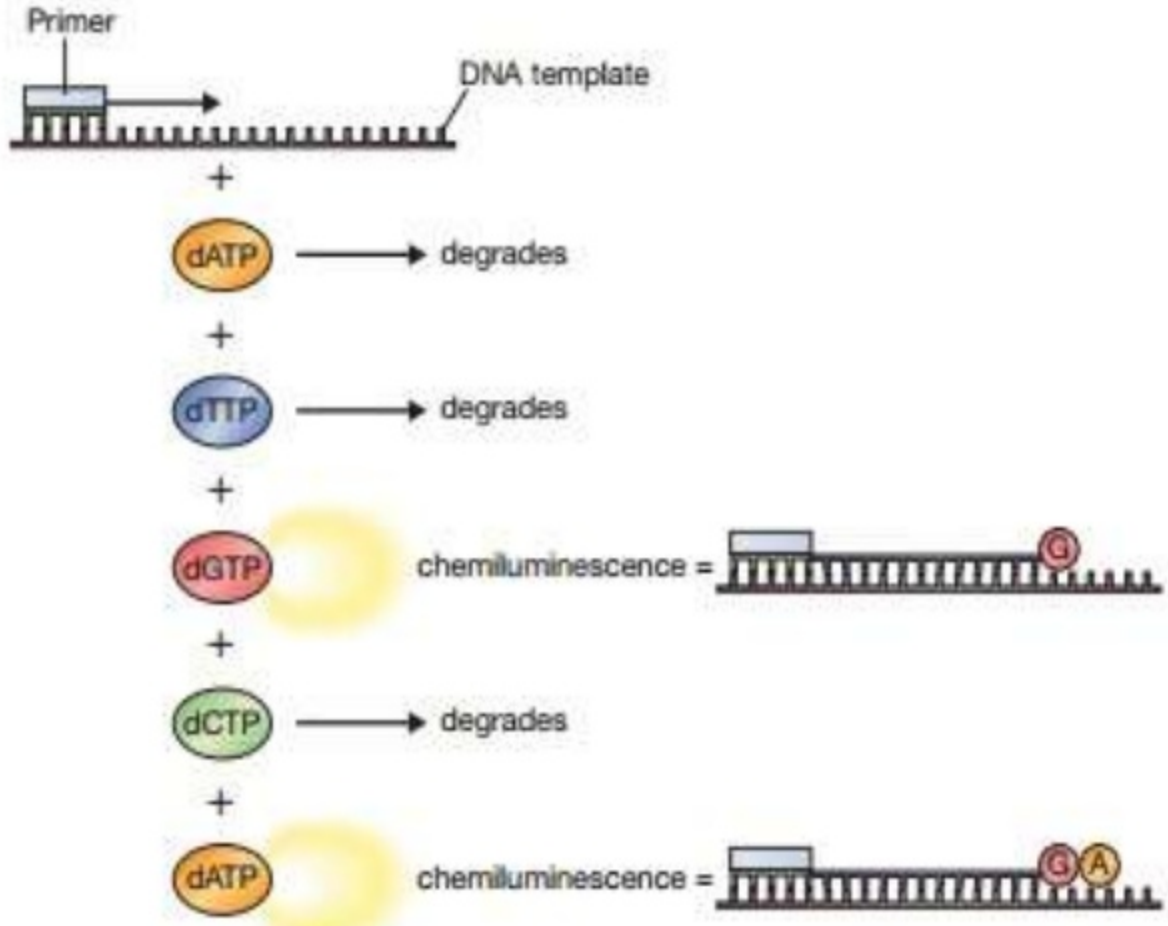
❖ DNA sample to be sequenced
❖ Primers
❖ Deoxynucleotides
❖ DNA polymerase
❖ Sulfurylase

- The release of pyrophosphate is converted by the enzyme sulfurylase into a flash of chemiluminescence which is easily automated.

PYROSEQUENCING

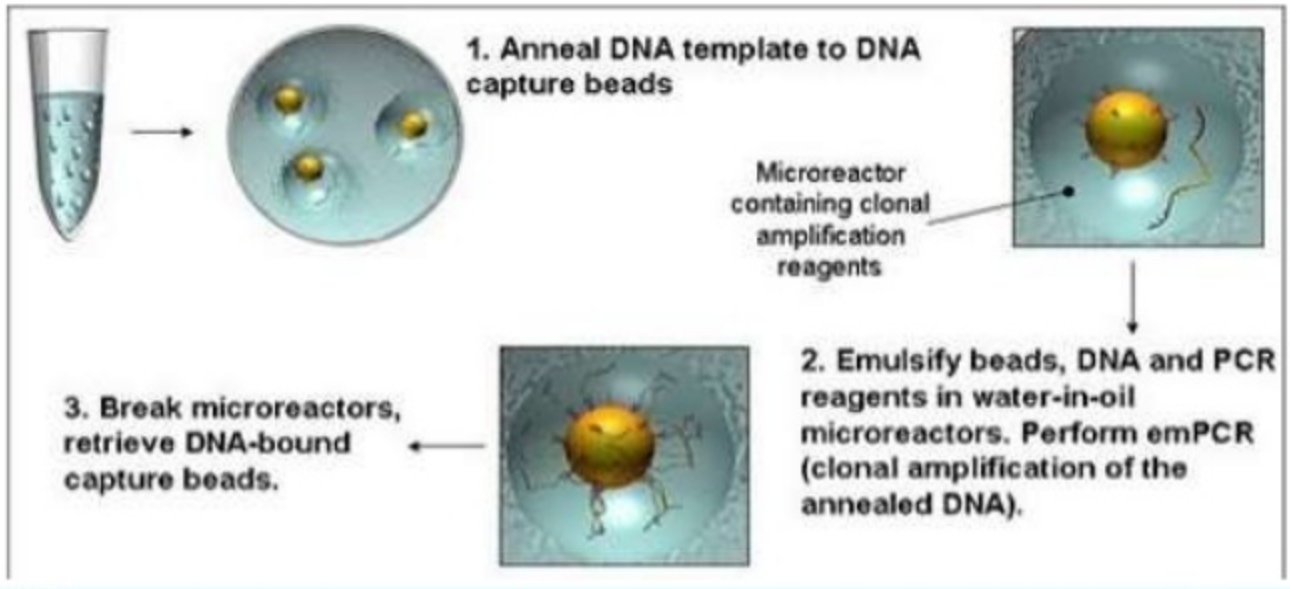
- **Advantages:**
  - Accurate
  - Parallel processing
  - Easily automated
  - Eliminates the need for labeled primers and nucleotides
  - No need for gel electrophoresis

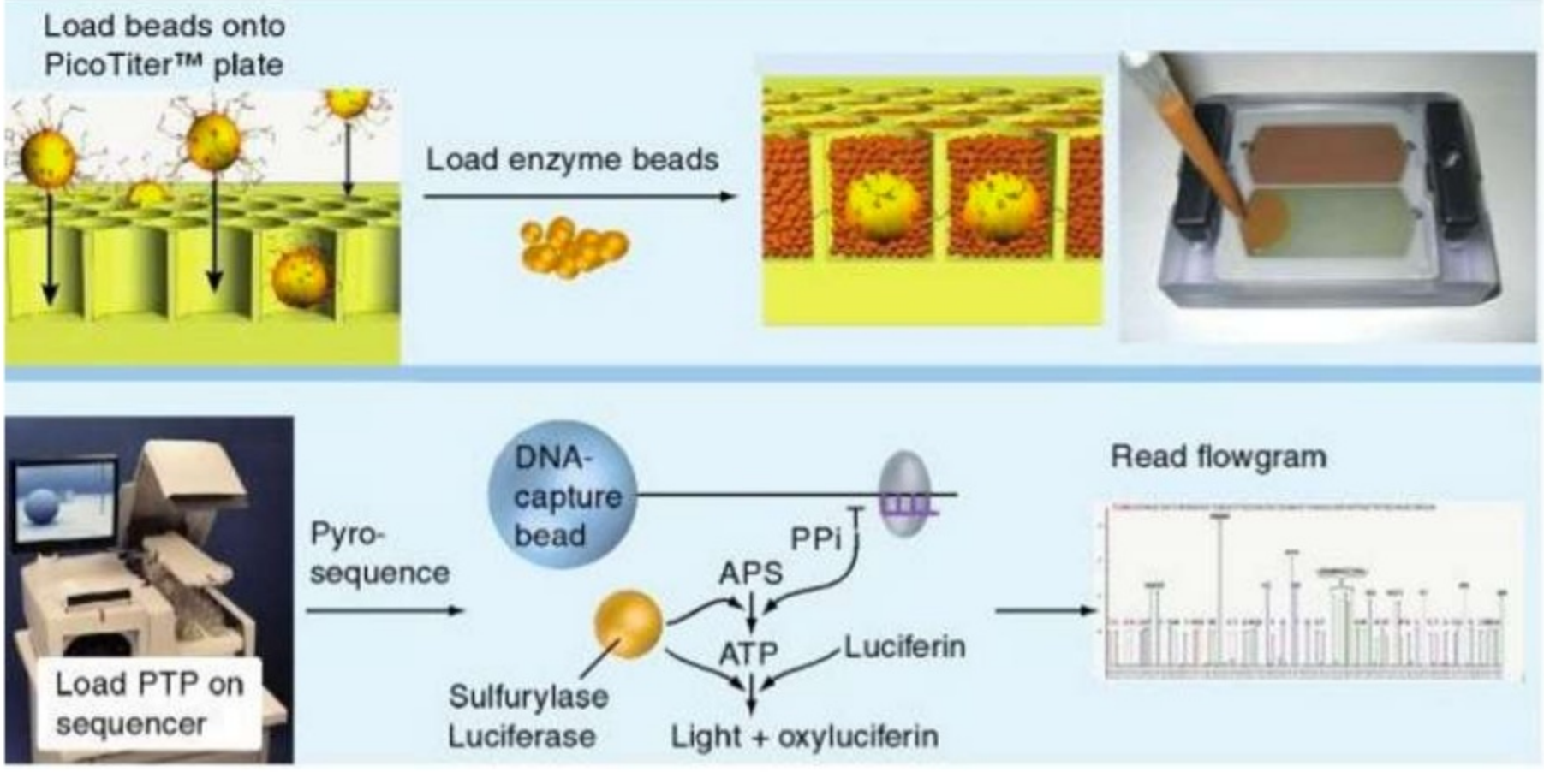
- **DISADVANTAGES**
  - Smaller sequences
  - Nonlinear light response after more than 5-6 identical nucleotides



MASSIVELY PARALLEL PYROSEQUENCING

- The DNA is broken down into fragments between 300 to 500bp
- Each fragment is ligated with a pair of adaptor
    - To attach to the beads
    - Provide annealing sites for the primers for performing PCR
- Adaptors are attached to beads by biotin-streptavidin linkage
- Just one fragment becomes attached to one bead
- Each DNA fragment is now amplified using
- PCR is carried out in a oil emulsion, each bead residing within own droplet in the emulsion
- Each droplet contains all the reagents for PCR and is physically seprated from all the other droplets by the barrier provided by the oil components in the emulsion.
- After PCR, the droplets are transferred on wells on plastic strip and pyrosequencing reactions are carried out


1. Anneal DNA template to DNA capture beads
Microreactor containing clonal amplification reagents
2. Emulsify beads, DNA and PCR reagents in water-in-oil microreactors. Perform emPCR (clonal amplification of the annealed DNA).
3. Break microreactors, retrieve DNA-bound capture beads.


Load beads onto PicoTiter™ plate
Load enzyme beads
Load PTP on sequencer
Pyro-sequence
DNA-capture bead
PPi
APS
ATP
Luciferin
Sulfurylase
Luciferase
Light + oxyluciferin
Read flowgram

SHOTGUN SEQUENCING

• **Shotgun sequencing**, also known as **shotgun cloning**, is a method used for sequencing long DNA strands or the whole genome.

•In shotgun sequencing, DNA is broken up randomly into numerous small segments and overlapping regions are identified between all the individual sequences that are generated.

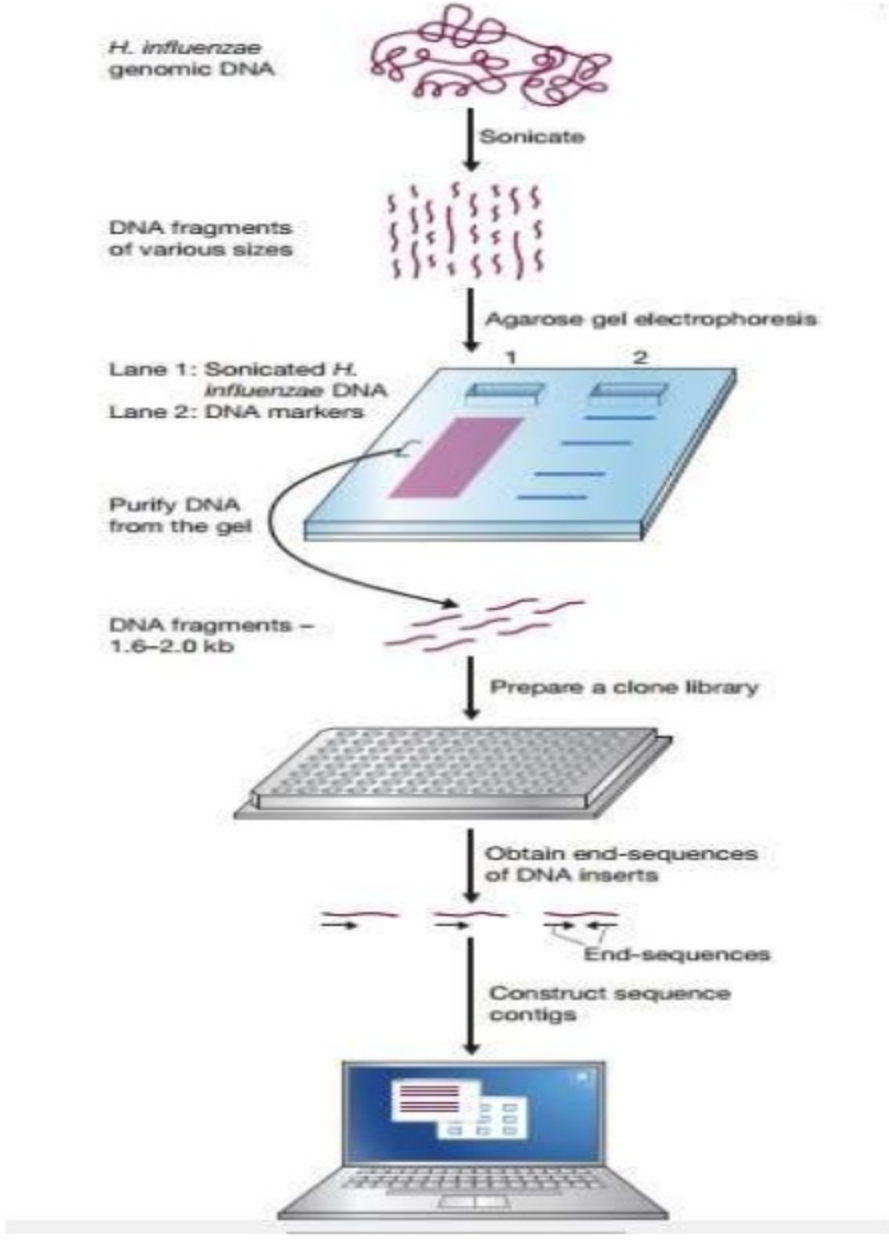
• Multiple overlapping reads for the target DNA are obtained by performing several rounds of this fragmentation and sequencing.

•Computer programs then use the overlapping ends of different reads to assemble them into a continuous sequence.

•The shotgun approach was first used successfully with the bacterium *Haemophilus influenzae.*

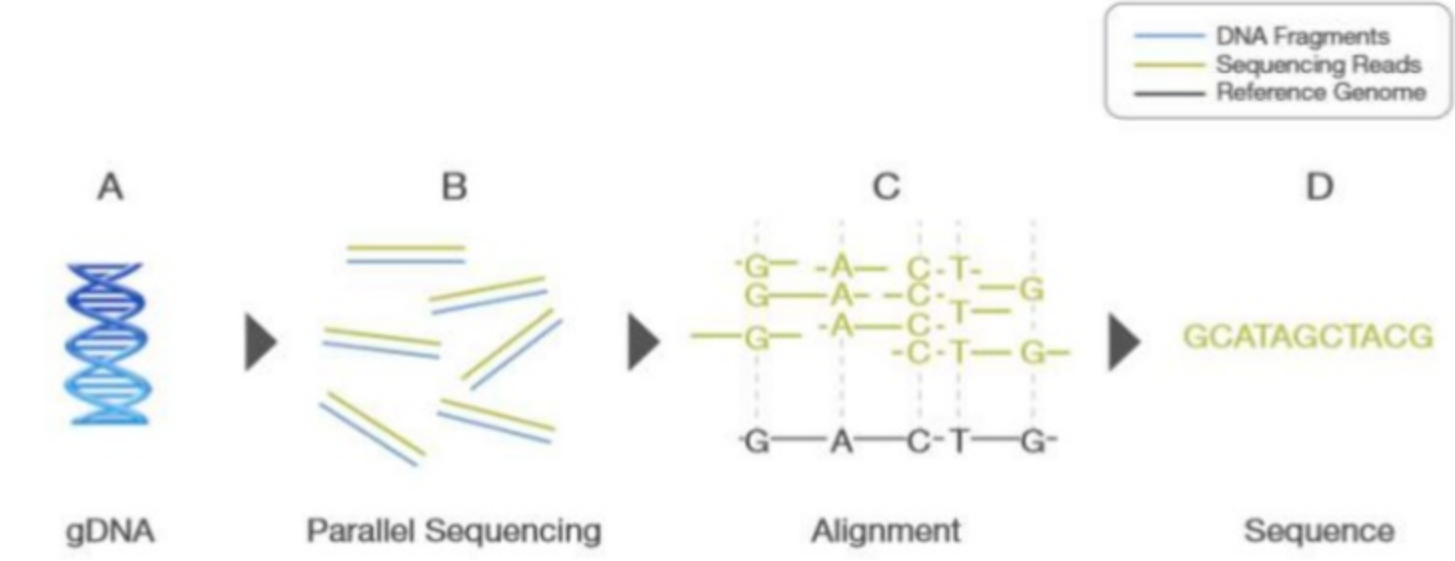
•Craig venter used this method to map the Human genome project in 2001.

Shotgun sequencing



NEXT GENERATION SEQUENCING

- The concept behind NGS – the bases of small fragments of DNAare sequentially identifed as signals emitted as eachfragment is resynthesized from a dna template strand
- NGS extends this process across millions of reactions in a massively parallel fashion rather than being limited to a single or a few dna fragments



A. Extracted gDNA.
B. gDNA is fragmented into a library of small segments that are each sequenced in parallel.
C. Individual sequence reads are reassembled by aligning to a reference genome.
D. The whole-genome sequence is derived from the consensus of aligned reads.

Next Generation Sequencing

Different platforms

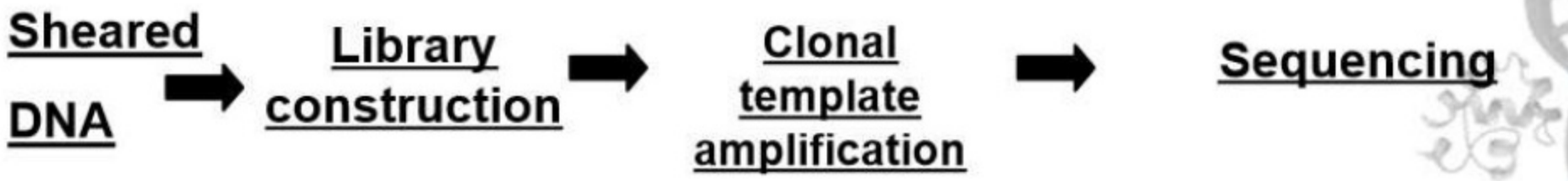
Andy Vierstraete,
Department of Biology,
Ghent University. June 2012

- 454 Sequencing / Roche
  - GS Junior System
  - GS FLX+ System
- Illumina (Solexa)
  - HiSeq System
  - Genome analyzer IIx
  - MySeq
- Applied Biosystems - Life Technologies
  - SOLiD 5500 System
  - SOLiD 5500xl System
- Ion Torrent - Life Technologies
  - Personal Genome Machine (PGM)
  - Proton
- Helicos
  - Helicos Genetic Analysis System
- Pacific Biosciences
  - PacBio RS
- Oxford Nanopore Technologies
  - GridION System
  - MinION

Next Generation Sequencing
Amplified Single Molecule Sequencing

Third Generation Sequencing,
Next Next Generation Sequencing,
Single Molecule Sequencing

Differentiating Next Gen technologies



Illumina	Library Construction	Clonal amplification via bridge amplification	Massively parallel sequencing-by-synthesis of DNA clusters
454	Library Construction	Clonal amplification with emulsionPCR and enrichment	Massively parallel pyrosequencing of bead bound DNA templates
SOLiD	Library Construction	Clonal amplification with emulsionPCR and enrichment	Massively parallel ligation-based sequencing of bead bound DNA templates

Illumina sequencing