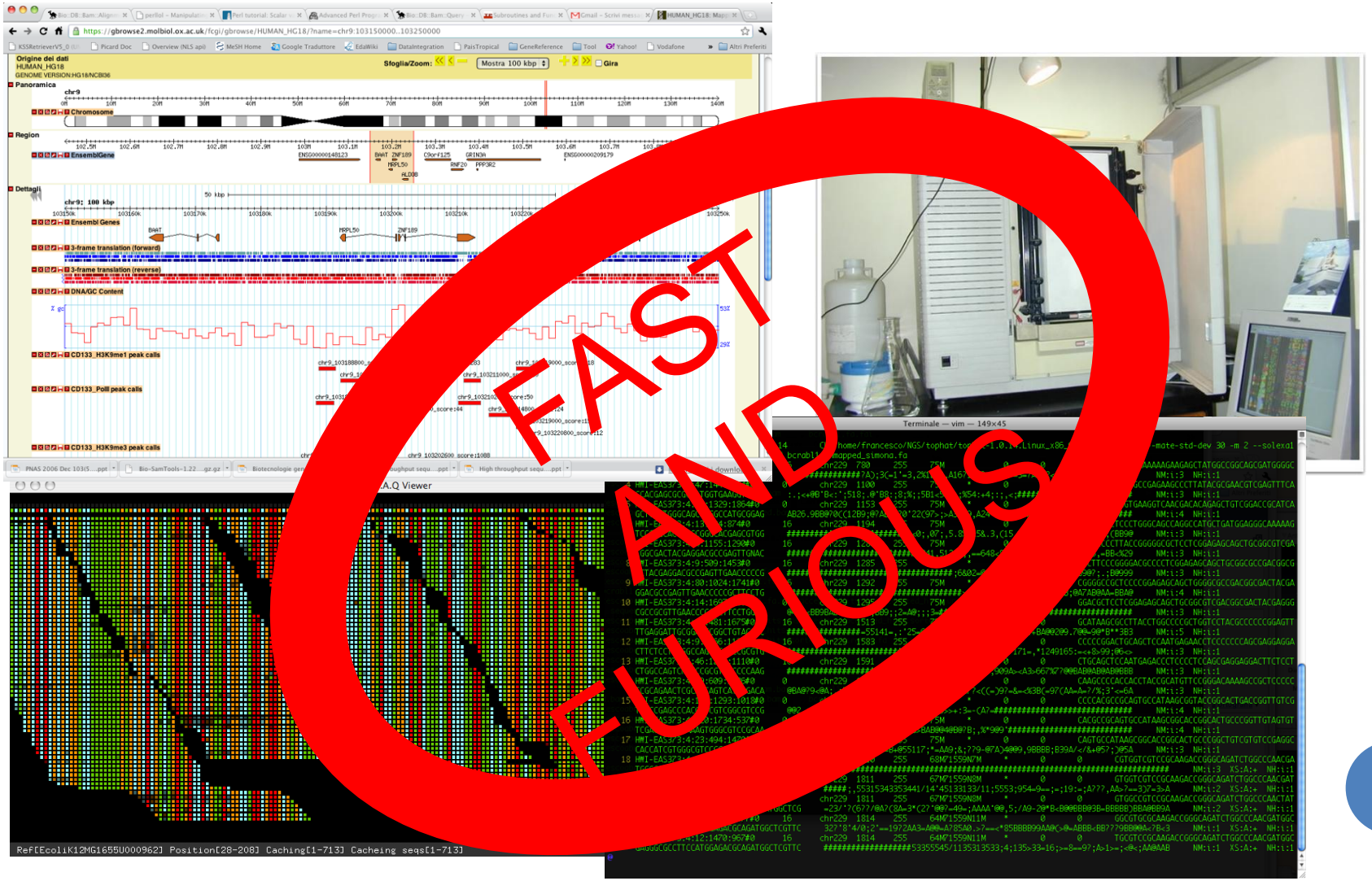
NGS Technology

DAUIN - Elisa Ficarra

Next Generation Sequencing



Nucleic acid thermodynamics
single 2 duplex

- **Hybridization** is the process of establishing a non-covalent, sequence-specific interaction between two or more complementary strands of nucleic acids (i.e. DNA/RNA) into a single hybrid, which in the case of two strands is referred to as a duplex

- **Annealing**, in genetics, means for DNA or RNA to pair by hydrogen bonds to a complementary sequence, forming a double-stranded polynucleotide. The term is often used to describe the binding of a DNA probe, or the binding of a primer to a DNA strand during a polymerase chain reaction (PCR). The term is also often used to describe the reformation (renaturation) of complementary strands that were separated by heat (thermally denatured)

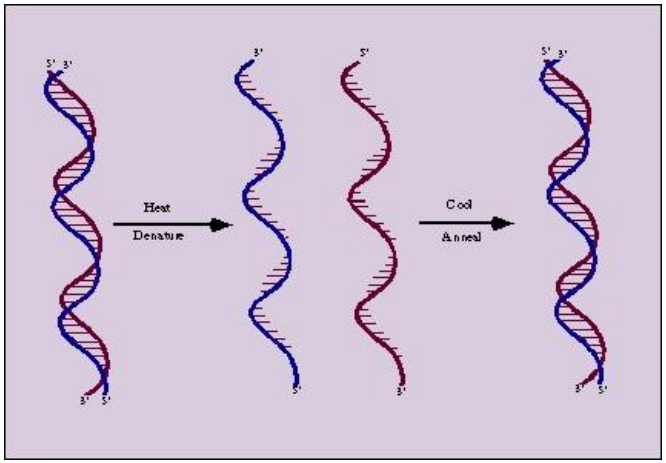
Nucleic acid thermodynamics

duplex 2 single

- **DNA denaturation**, also called **DNA melting**, is the process by which double-stranded deoxyribonucleic acid (DNA) unwinds and separates into single-stranded strands through the breaking of hydrogen bonding between the bases. Both terms are used to refer to the process as it occurs when a mixture is heated, although "denaturation" can also refer to the separation of DNA strands induced by chemicals

- For multiple copies of DNA molecules, the melting temperature (*Tm*) is defined as the temperature at which half of the DNA strands are in the double-helical state and half are in the random coil or single-stranded state.

Denaturation/Hybridization



Denaturation
Separating DNA into single strands

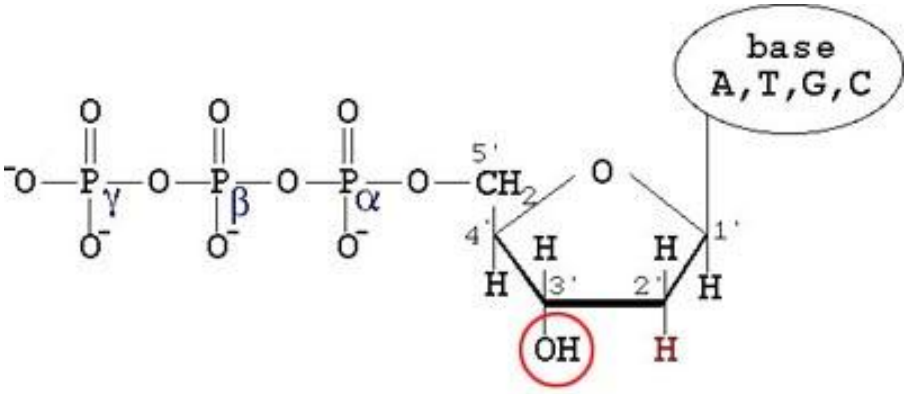
Hybridization / Annealing
Forming double-stranded DNA

Polymerase chain reaction (PCR)

- The **polymerase chain reaction** (**PCR**) is a technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

- The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA

Primers (dNTP)

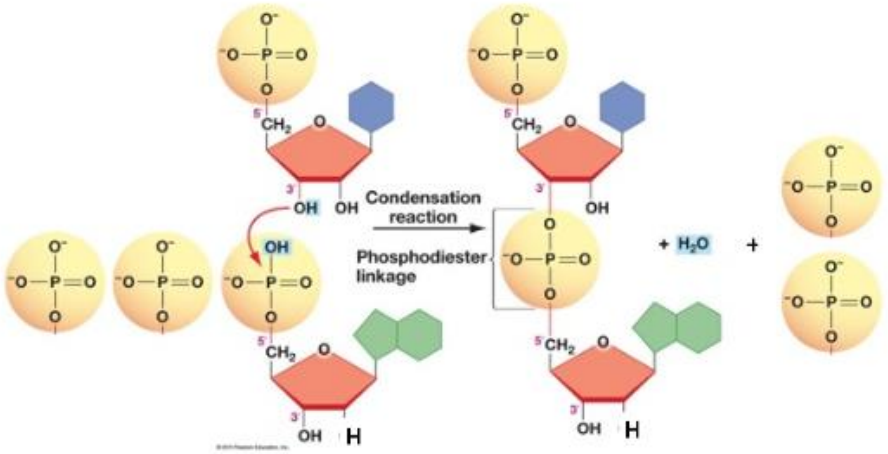


dNTP

deoxyribonucleotide triphosphate

Why triphosphate?

For the energy required to for the phosphodiester bond



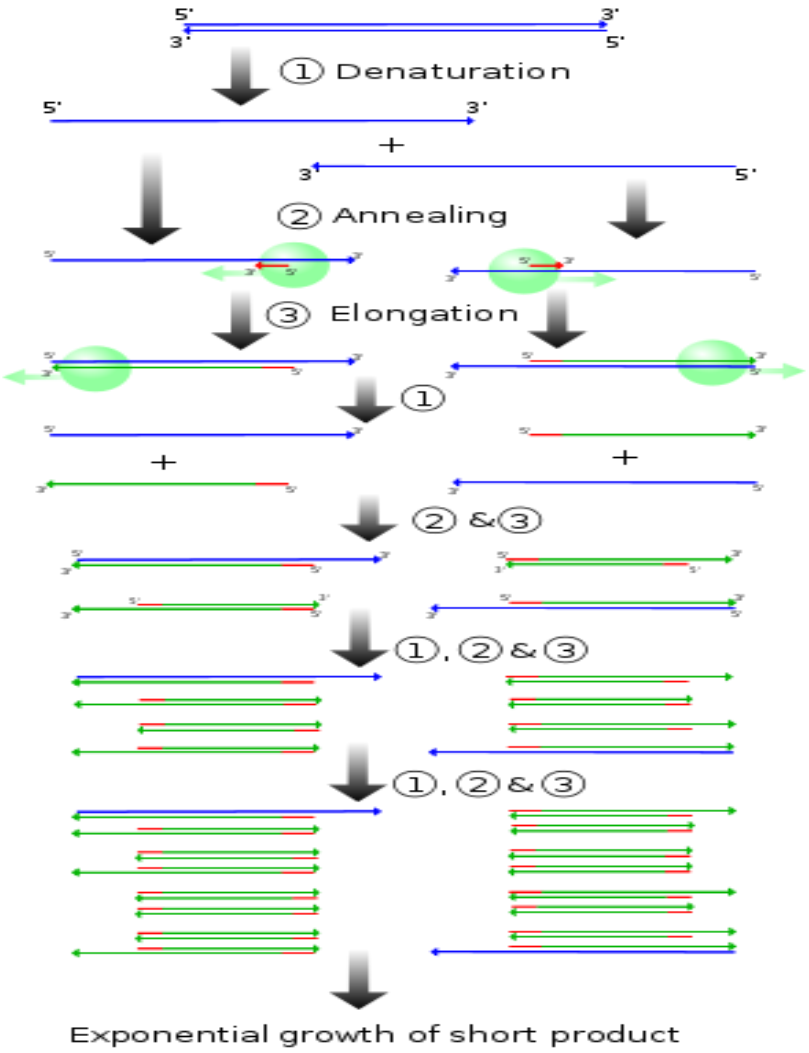


Figure: Schematic drawing of the PCR cycle.

(1) Denaturing at 94–96 °C.

(2) Annealing at ~65 °C

(3) Elongation at 72 °C. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding bases that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the bases with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. Exponential growing

Four cycles are shown here. The **blue lines** represent the DNA template to which primers (**red arrows**) anneal. These are extended by the DNA polymerase (light green circles), to give shorter DNA products (**green lines**), which themselves are used as templates as PCR progresses.

PCR video

- https://www.dnalc.org/resources/animations/pcr.html

Core ideas NGS

- The library is constructed by a novel way of doing PCR, where the fragments are seperated by physico-chemical means (emulsion PCR or bridge PCR).
- Very many fragments are sequenced in parallel in a flow cell, observed by a microscope with CCD camera.

Principle of Pyrosequencing

(http://www.biotagebio.com/DynPage.aspx?id=7454)

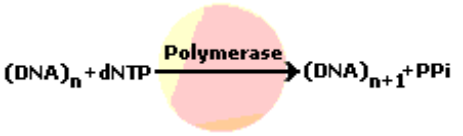
Pyrosequencing™ is sequencing by synthesis, a simple to use technique for accurate and consistent analysis of DNA sequences (SEE next slide to have a picture of the whole process)

Step 1

A sequencing primer is hybridized to a single stranded, PCR amplified: a DNA template (called *primer*) is incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5´ phosphosulfate (APS) and luciferin.

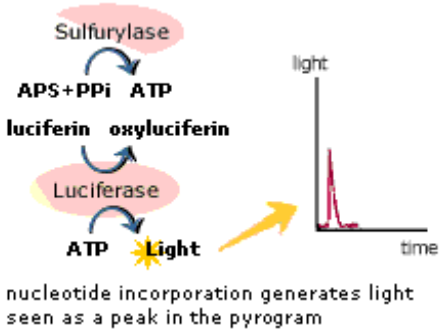
Step 2

The first of four deoxynucleotide triphosphates (dNTP, i.e. *nucleotide* / *base*) is added to the reaction. DNA polymerase catalyzes the incorporation of the nucleotide into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.



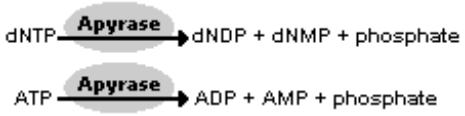
Step 3

ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5´ phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a pyrogram™. Each light signal is proportional to the number of nucleotides incorporated.



Step 4

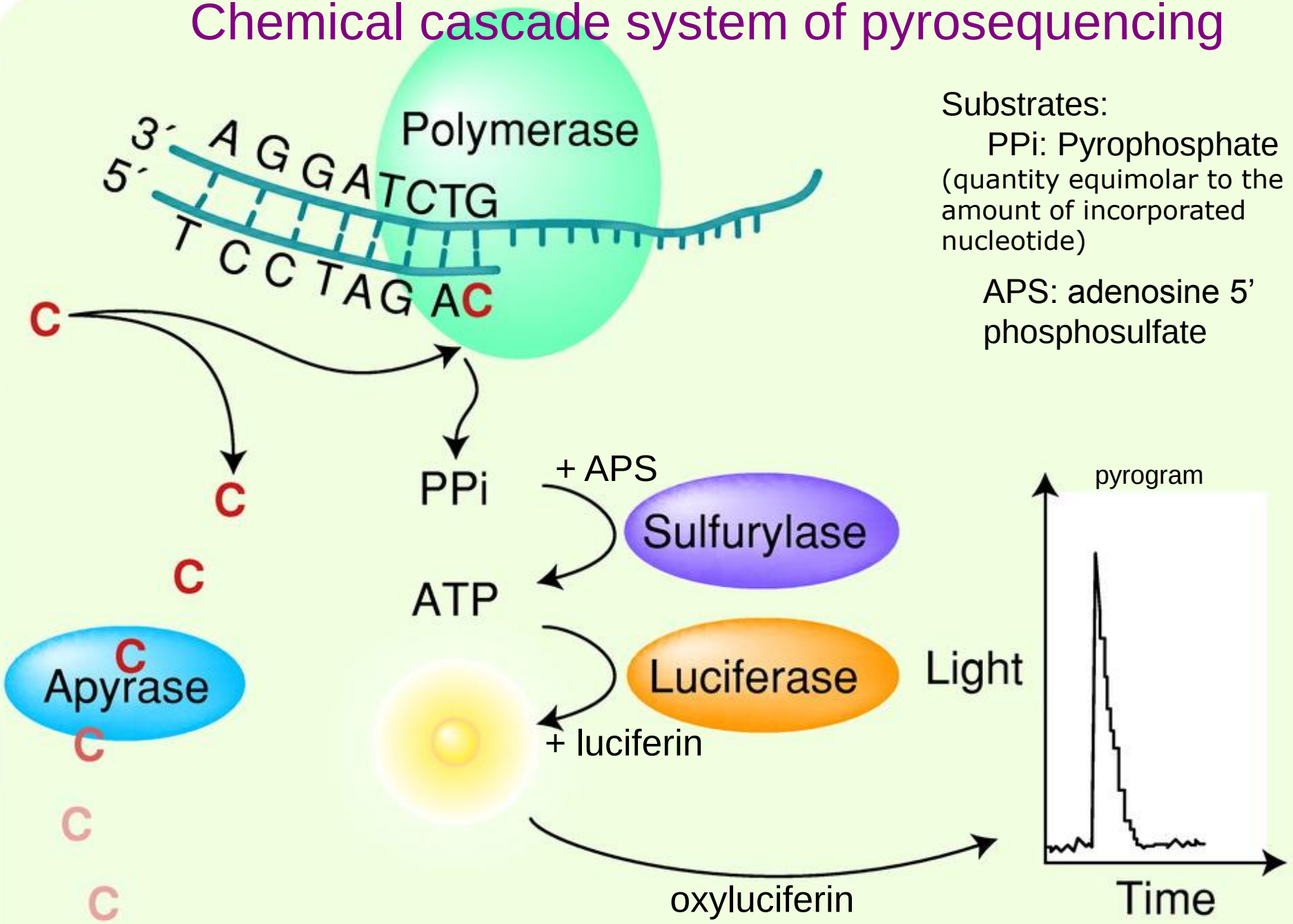
Apyrase, a nucleotide degrading enzyme, continuously degrades unincorporated dNTPs and excess ATP. When degradation is complete, another dNTP is added.



Step 5

Addition of dNTPs is performed one at a time. It should be noted that deoxyadenosine alpha-thio triphosphate (dATPαS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peak in the pyrogram.

Chemical cascade system of pyrosequencing

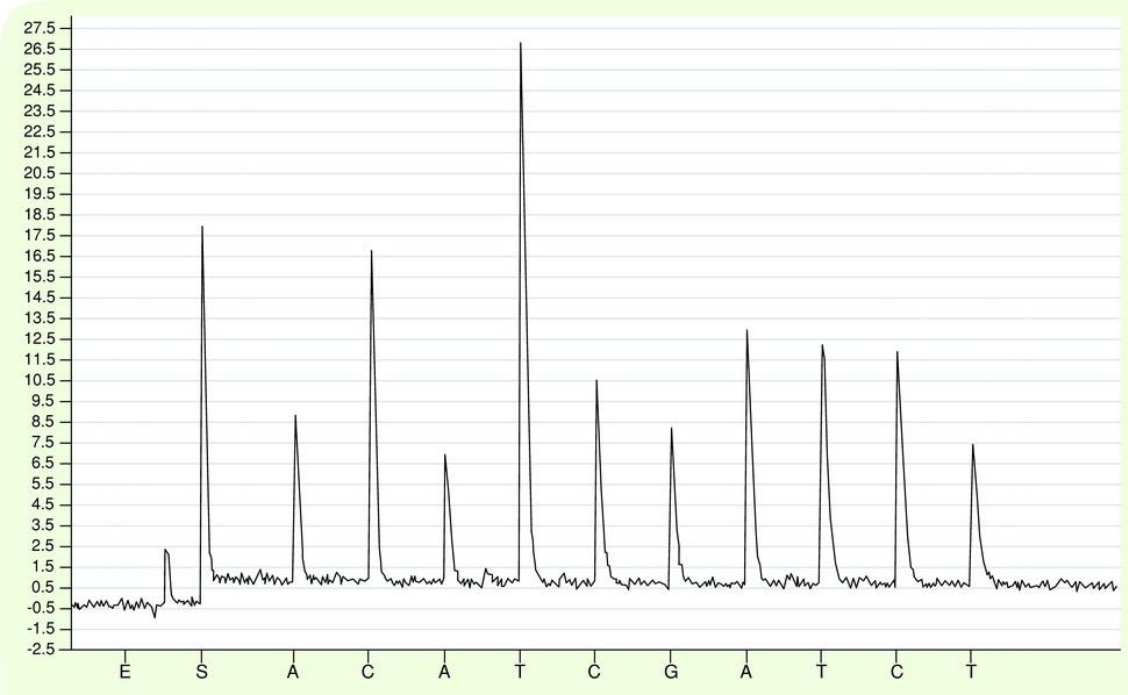


Substrates:

PPi: Pyrophosphate (quantity equimolar to the amount of incorporated nucleotide)

APS: adenosine 5' phosphosulfate

Pyrogram indicating peaks attained from pyrosequencing



The y-axis indicates the level of fluorescence emitted by the incorporation of a nucleotide during pyrosequencing.
The x-axis shows the order in which each nucleotide is added.
A half peak represents incorporation of a nucleotide while a double peak represents the incorporation of two nucleotides

Pyrosequencing and NGS Library videos

- https://www.youtube.com/watch?v=nFfgWGFe0aA
- http://www.youtube.com/watch?v=bFNjxKHP8Jc

https://www.youtube.com/watch?v=JNqXgLKOzKU

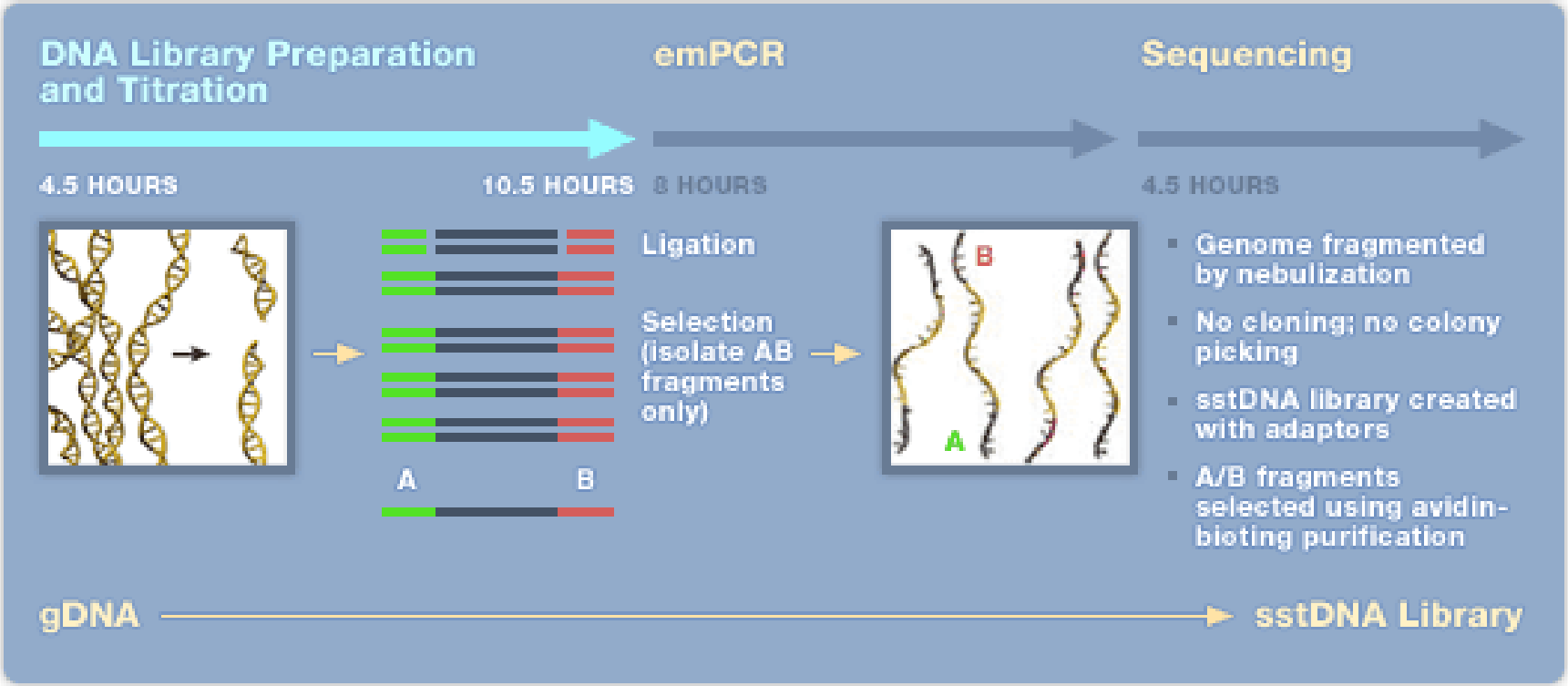
https://www.youtube.com/watch?v=jFCD8Q6qSTM

DNA Library preparation

- Most sequencing approaches use an *in vitro* cloning step to amplify individual DNA molecules
- that is because their molecular detection methods are not sensitive enough for single molecule sequencing

FIGURE 7

1. DNA Library preparation



Preparation of the DNA library consists of a few simple steps:

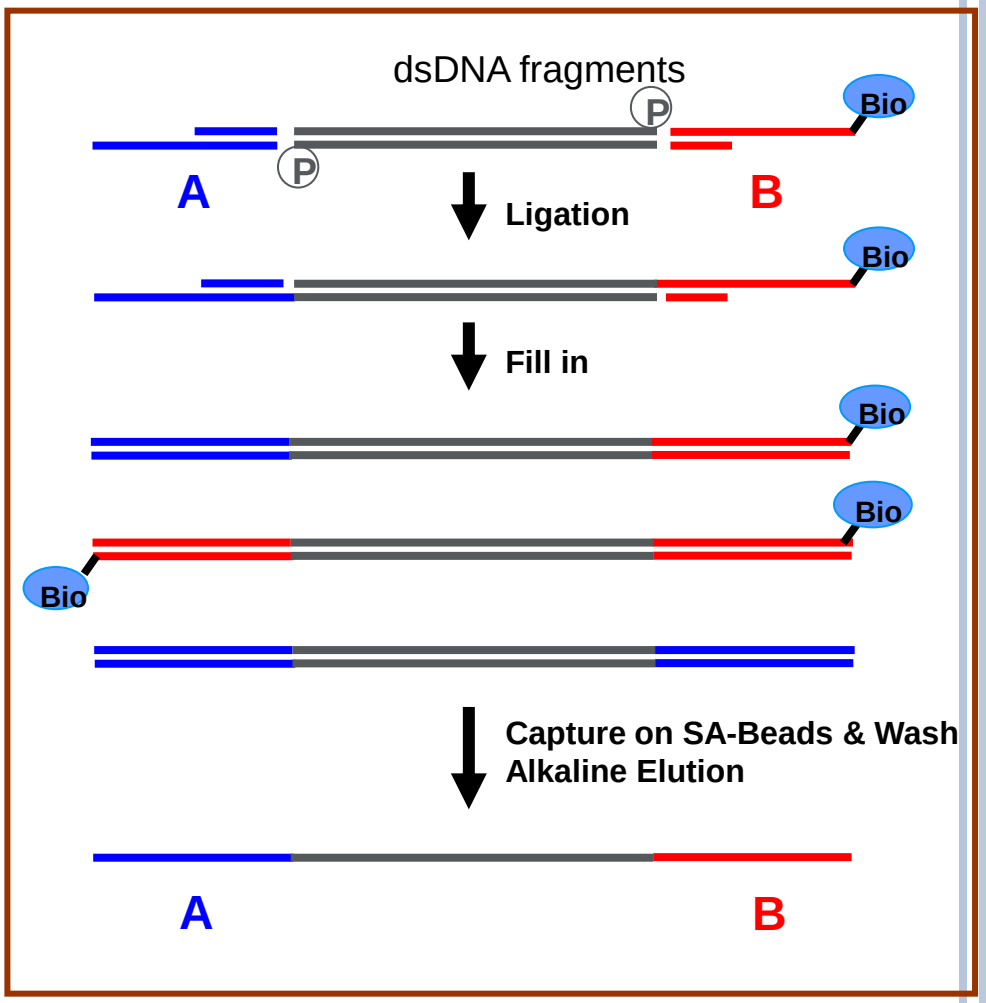
•Genomic DNA is fractionated into smaller fragments (300-500 base pairs) that are subsequently phosphorylated (addition of a phosphate (PO4) group; phosphorylation activates or deactivates many protein enzymes).

•Short Adaptors (A and B) are then ligated onto the ends of the fragments. These adaptors provide priming sequences for both amplification and sequencing of the sample-library fragments. Adaptor B contains a 5'-biotin tag that enables immobilization of the library onto streptavidin coated beads (in fact, Biotin binds very tightly to the tetrameric protein avidin (also streptavidin) creating one of the strongest known protein-ligand interactions, approaching the covalent bond in strength; biotin-binding is resistant to extremes of pH, temperature, organic solvents, denaturants, detergents and proteolytic enzymes).

Streptavidin beads

Non-phosphorylated **A and B adaptors are ligated to the ends of phosphorylated, double-stranded genomic DNA fragments**. The A and B adaptors differ in both nucleotide sequence and the presence of a 5' biotin tag on the B adaptor.

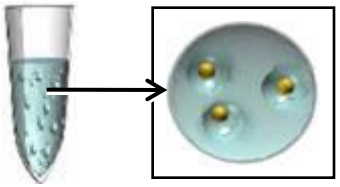
The library fragment are filled in by the strand-displacement activity of DNA polymerase.



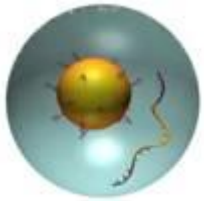
Fragments are then bound to Streptavidin beads. That is, **unbound material (composed of homozygous A/A adaptor sets, which lack biotin) is washed away. The immobilized fragments are then denatured; both strands of the B/B fragments remain immobilized through the biotinylated B adaptor, while A/B fragments are washed free and used in subsequent sequencing steps.** Replicate library preparations were observed to yield coverage of the genome.

2. Clonal amplification during emPCR

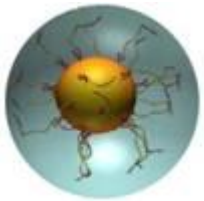
Emulsion-based clonal amplification



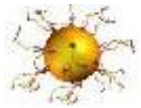
Anneal sstDNA (single strand DNA) to an excess of DNA Capture Beads



Emulsify beads and PCR reagents in water-in-oil microreactors



Clonal amplification occurs inside microreactors



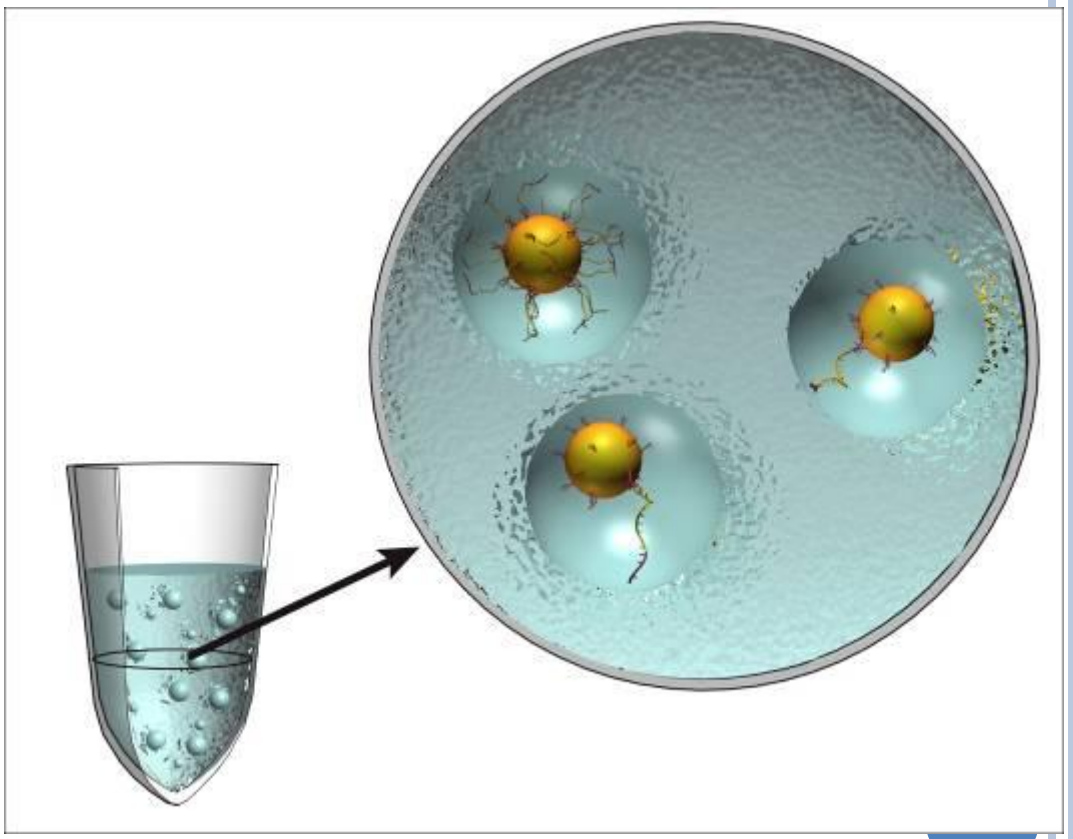
Break microreactors, enrich for DNA-positive beads

DNA Library Preparation → emPCR → Sequencing

4.5 h and 10.5 h | 8 h | 7.5 h

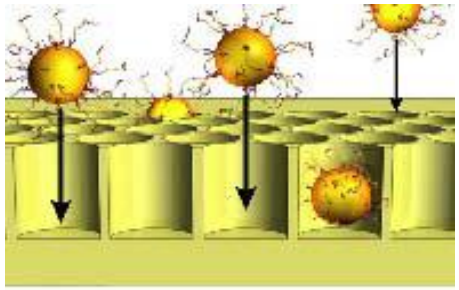
2. Clonal amplification during emPCR

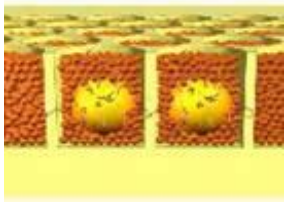
- 1 starting effective fragment per microreactor
- ~10^6 microreactors per ml
- All processed in parallel



3. Sequencing - Bead loading on PicoTiterPlates

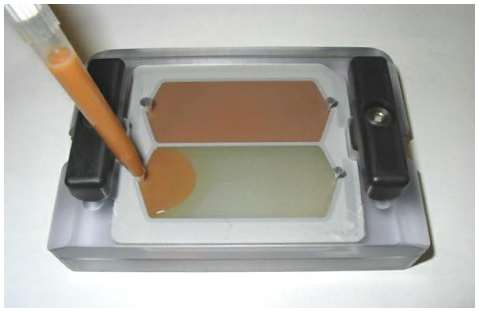
Depositing DNA beads into the PicoTiterPlate device

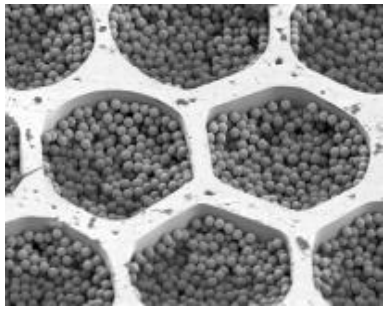


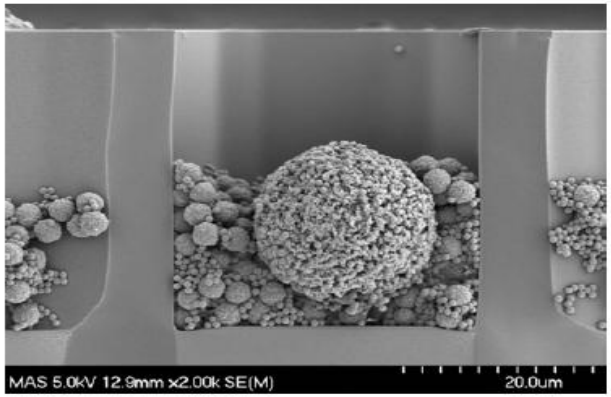


- Well diameter: average of 44 µm
- 400,000 reads obtained in parallel
- A single clonally amplified sstDNA bead is deposited per well

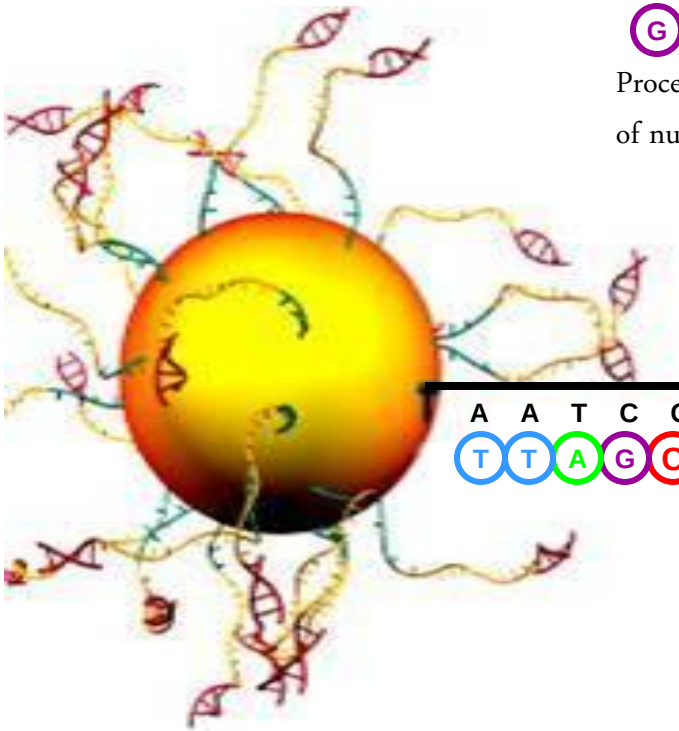
Amplified sstDNA library beads → **Quality filtered bases**





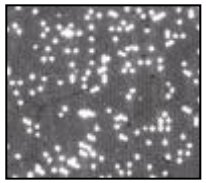


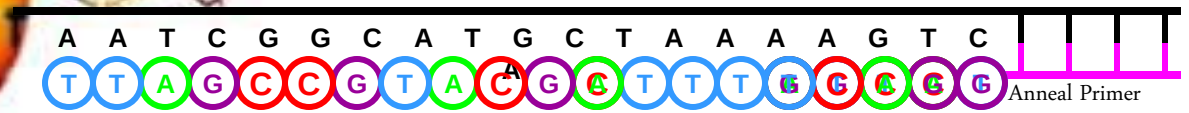
GS Technology
Sequencing-by-synthesis

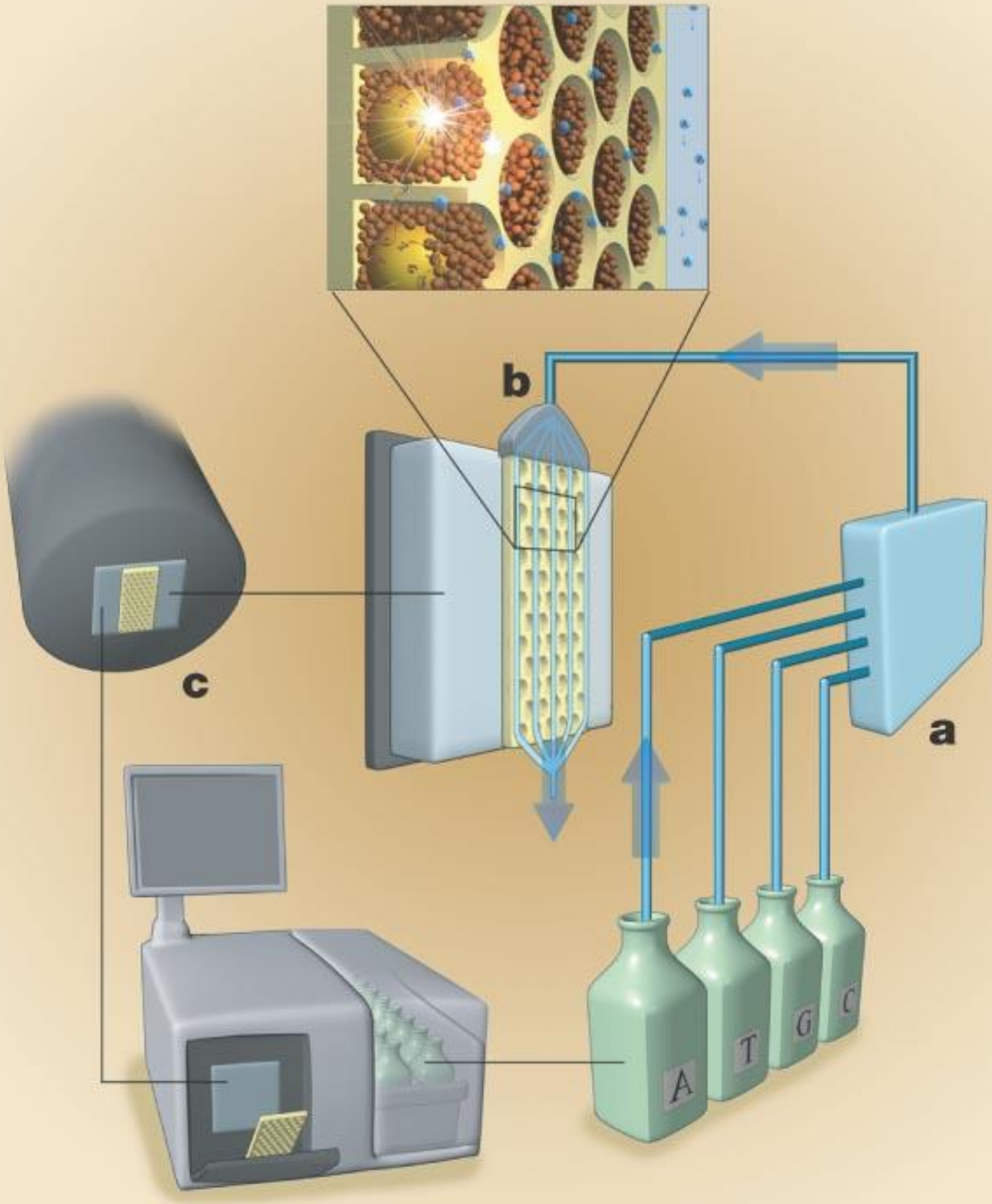


Repeated dNTP flow sequence:

Process continues until user-defined number of nucleotide flow cycles are completed.







The sequencing instrument consists of the following major subsystems:

a fluidic assembly (**a**), a flow chamber that includes the well-containing fibre-optic slide (**b**), a CCD camera-based imaging assembly (**c**), and a computer that provides the necessary user interface and instrument control.

Paired end sequencing

- The two ends of the fragments get different adapters.
- Hence, one can sequence from one end with one primer, then repeat to get the other end with the other primer.
- This yields “mate pairs” of reads, separated by a known distance.
- For large distances, “circularisation” might be needed.

Paired ends

- Paired-end sequencing is useful
  - to find micro-indels
  - to find copy-number variations
  - for assembly tasks
  - to look for splice variants and gene fusions