Universidade Federal de Santa Catarina
Centro de Ciências Biológicas
Programa de Pós Graduação em Biotecnologia e Biociências

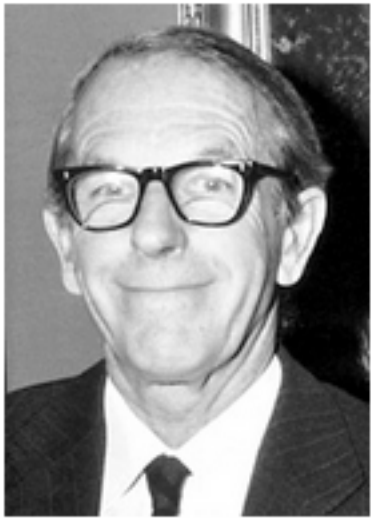
SEQUENCIAMENTO DE DNA

Patrícia H. Stoco
Edmundo C. Grisard

Sequenciamento DNA: 1ª geração

1977

Walter Gilbert

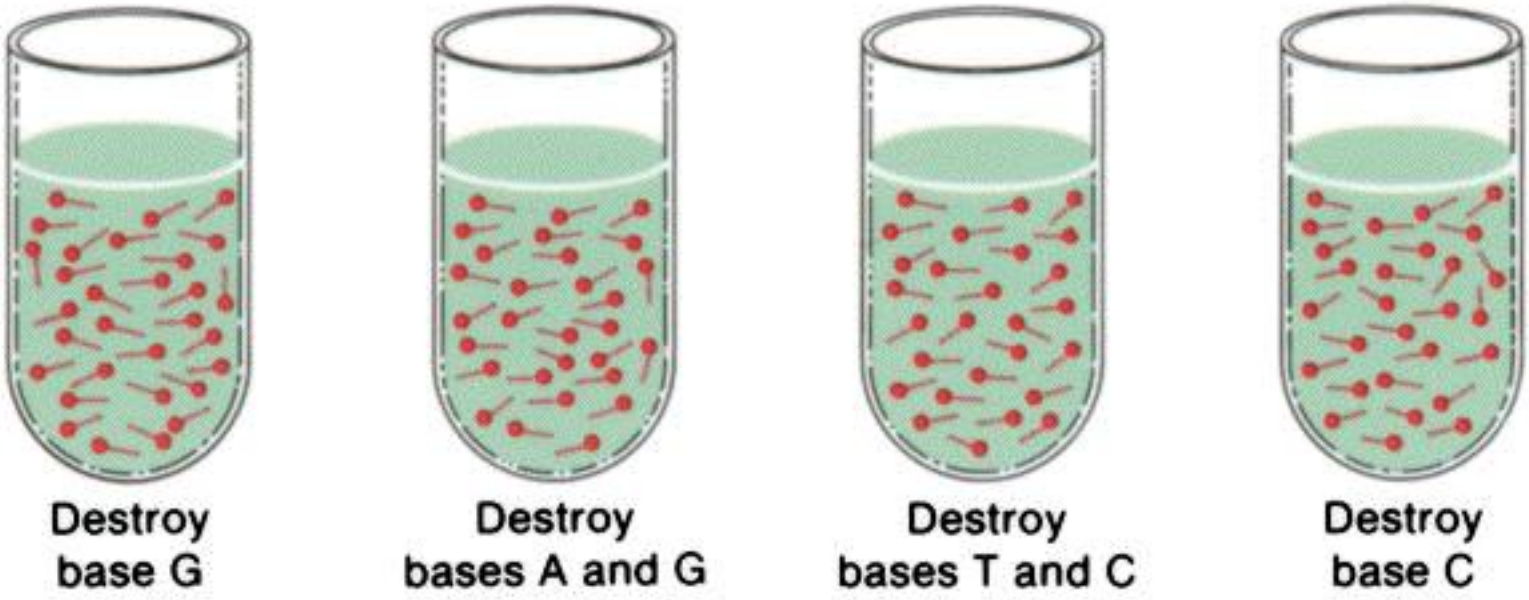


Frederick Sanger

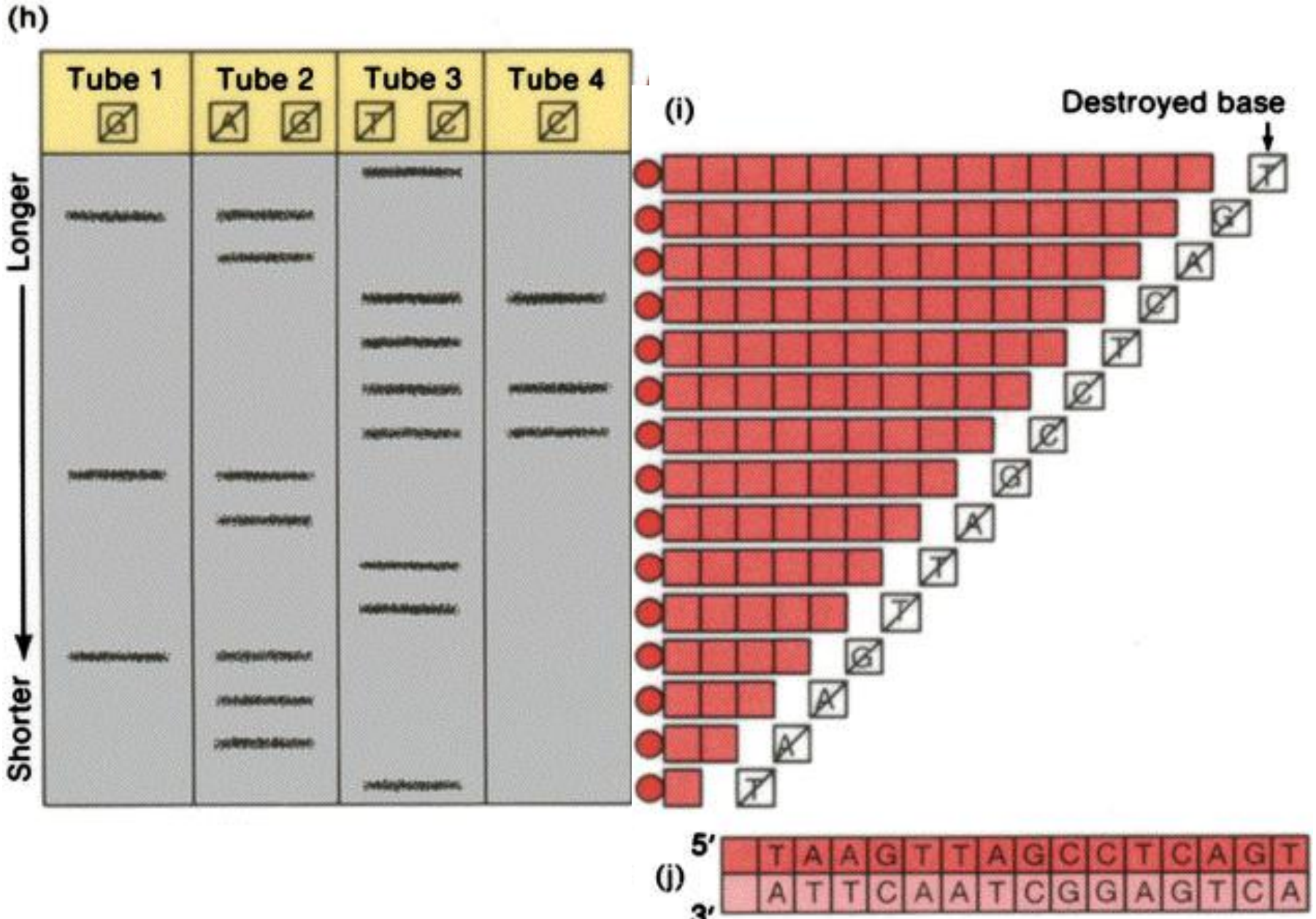
Degradação química do DNA

Sequenciamento enzimático
Dideoxi
Término de cadeia

Sequenciamento DNA: Maxam-Gilbert



Especificidade de bases	*Químico usado para alteração de bases*	*Químico usado para remoção de bases*	*Químico usado para a clivagem da fita*
G	Dimetilsulfato	Piperidina	Piperidina
A+G	Ácido	Ácido	Piperidina
C+T	Hidrazina	Piperidina	Piperidina
C	Hidrazina + álcali	Piperidina	Piperidina


(h)
Tube 1
Tube 2
Tube 3
Tube 4
Longer
Shorter
(i)
Destroyed base
(j)
5′
3′
T A A G T T A G C C T C A G T
A T T C A A T C G G A G T C A

Sequenciamento DNA pelo método Sanger

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5463–5467, December 1977
Biochemistry

DNA sequencing with chain-terminating inhibitors

(DNA polymerase/nucleotide sequences/bacteriophage ϕX174)

F. SANGER, S. NICKLEN, AND A. R. COULSON

Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England

Contributed by F. Sanger, October 3, 1977

ABSTRACT **A new method for determining nucleotide sequences in DNA is described. It is similar to the "plus and minus" method [Sanger, F. & Coulson, A. R. (1975) *J. Mol. Biol.* 94, 441–448] but makes use of the 2′,3′-dideoxy and arabinonucleoside analogues of the normal deoxynucleoside triphosphates, which act as specific chain-terminating inhibitors of DNA polymerase. The technique has been applied to the DNA of bacteriophage ϕX174 and is more rapid and more accurate than either the plus or the minus method.**

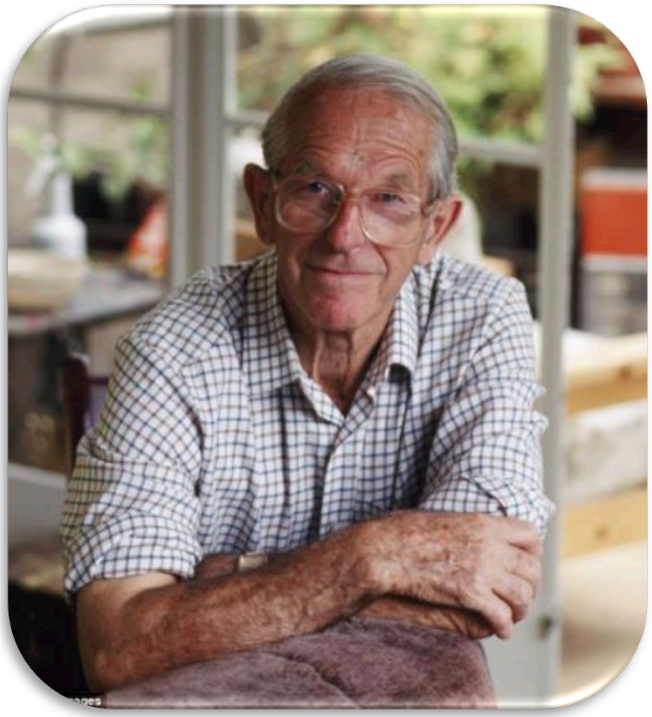
The "plus and minus" method (1) is a relatively rapid and simple technique that has made possible the determination of the sequence of the genome of bacteriophage ϕX174 (2). It depends on the use of DNA polymerase to transcribe specific regions of the DNA under controlled conditions. Although the method is considerably more rapid and simple than other available techniques, neither the "plus" nor the "minus" method is completely accurate, and in order to establish a sequence both must be used together, and sometimes confirma-

a stereoisomer of ribose in which the 3′-hydroxyl group is oriented in *trans* position with respect to the 2′-hydroxyl group. The arabinosyl (ara) nucleotides act as chain terminating inhibitors of *Escherichia coli* DNA polymerase I in a manner comparable to ddT (4), although synthesized chains ending in 3′ araC can be further extended by some mammalian DNA polymerases (5). In order to obtain a suitable pattern of bands from which an extensive sequence can be read it is necessary to have a ratio of terminating triphosphate to normal triphosphate such that only partial incorporation of the terminator occurs. For the dideoxy derivatives this ratio is about 100, and for the arabinosyl derivatives about 5000.

METHODS

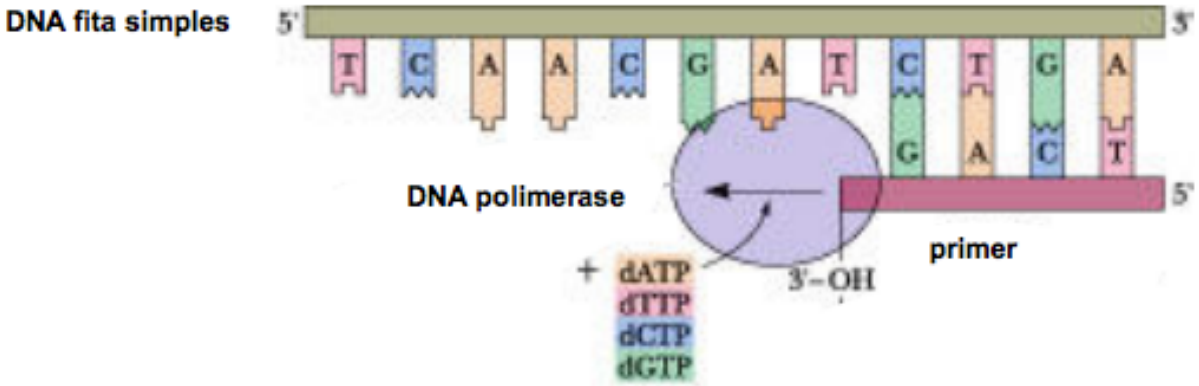
Preparation of the Triphosphate Analogues. The preparation of ddTTP has been described (6, 7), and the material is now commercially available. ddA has been prepared by

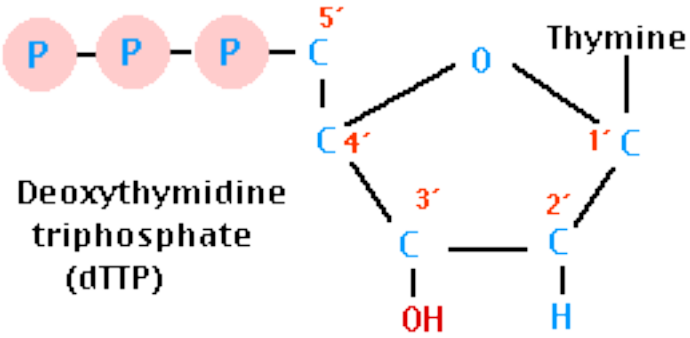
Frederick Sanger
13 Aug 1918 – 19 Nov 2013

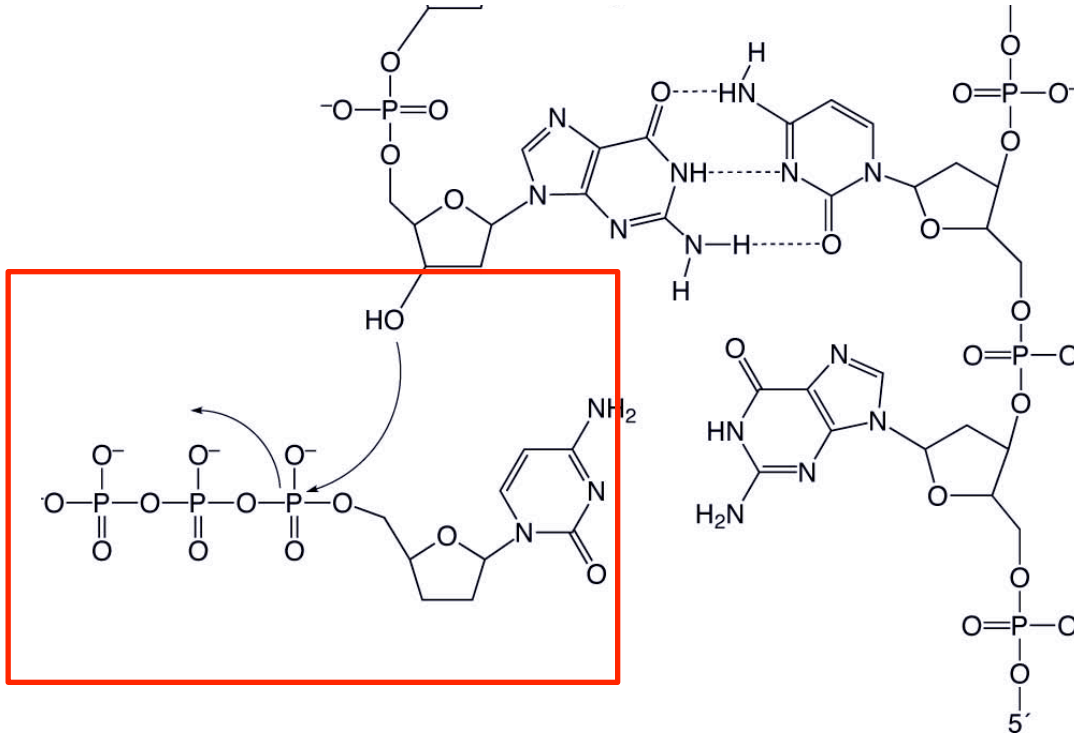


- Prêmio Nobel Prize de Química em **1958** e **1980**
- Publicou o método de sequenciamento em 1977

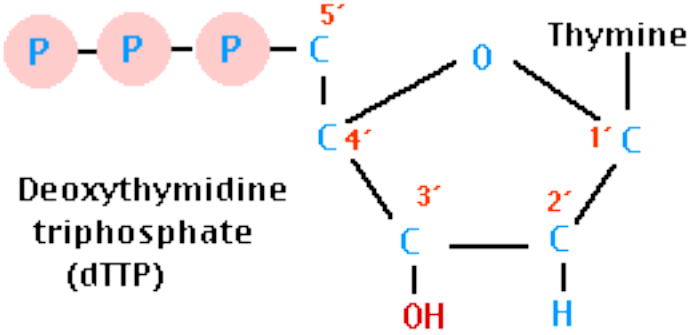
Sequenciamento DNA: Sanger

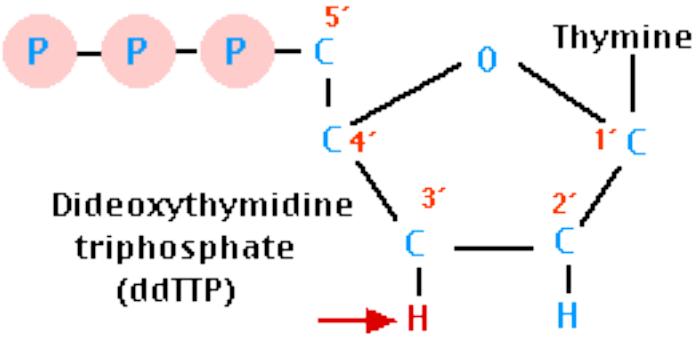


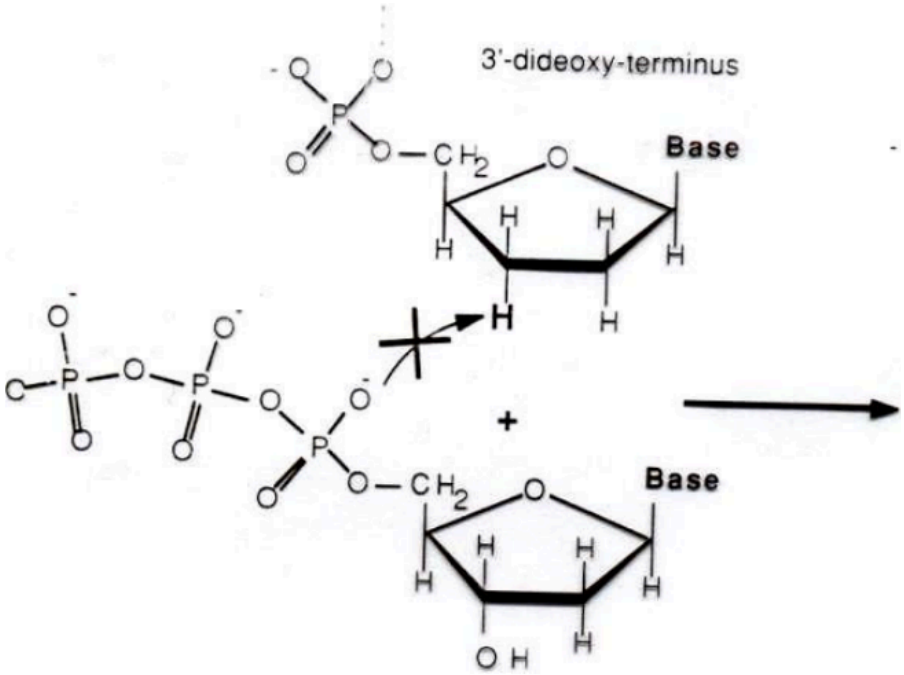


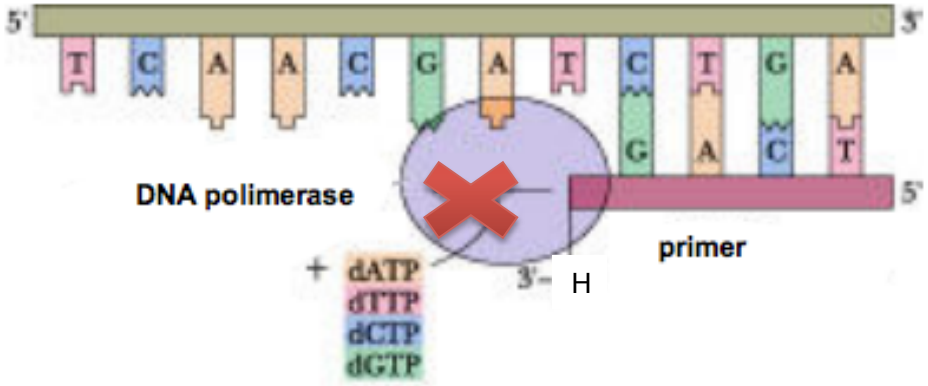


Sequenciamento DNA: Sanger

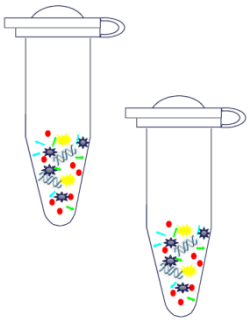








PCR convencional



Amostra (cDNA; gDNA)

Iniciadores (S e AS)

dNTPs (dATP, dTTP, dCTP, dGTP)

Enzima DNA Polimerase

Tampão da enzima

Mg^{2+} ($MgCl_2$ ou $MgSO_4$)

Reação de Sequenciamento

Amostra (cDNA; DNA)

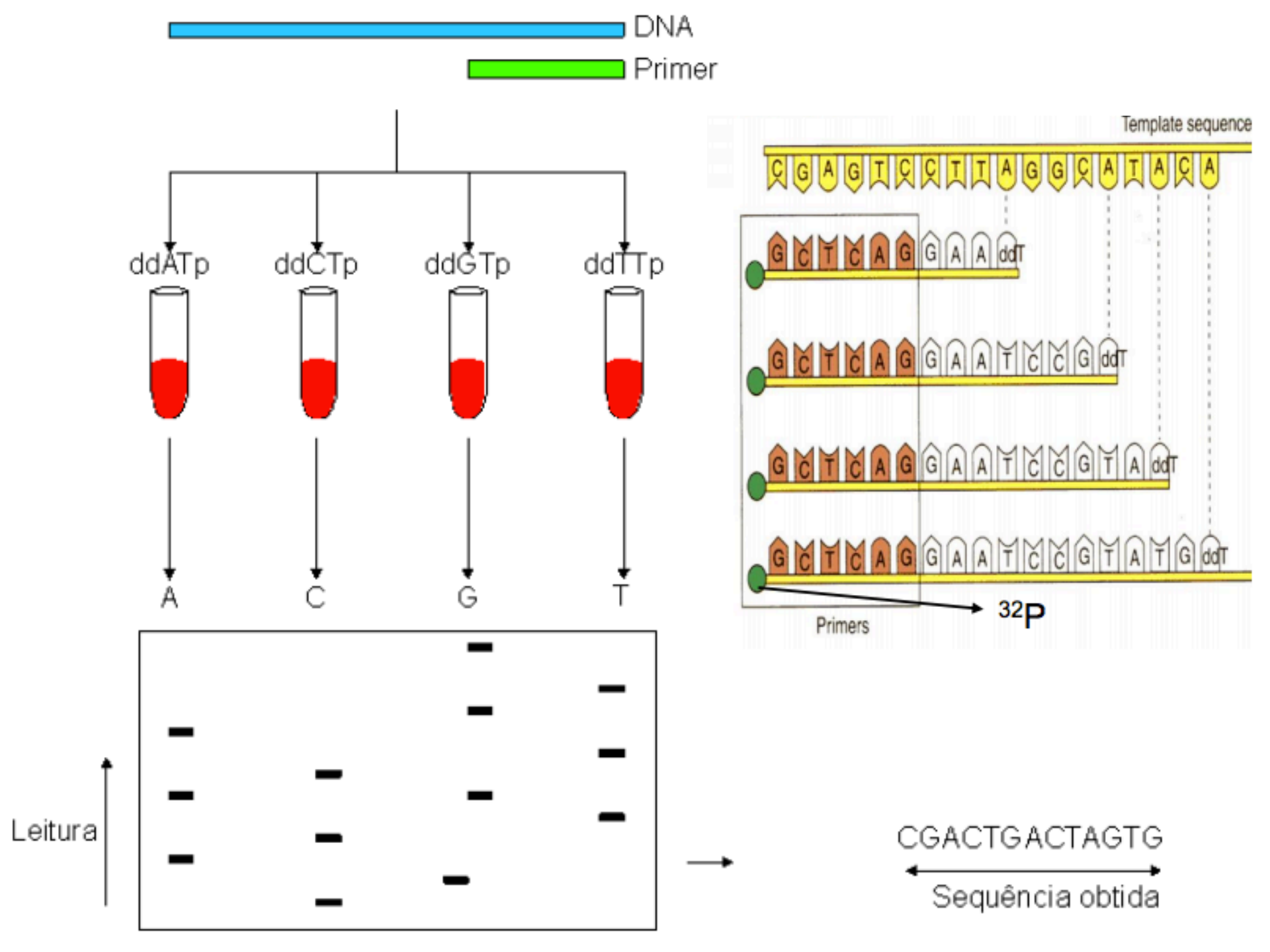
dNTPs (dATP, dTTP, dCTP, dGTP)

Tampão da enzima

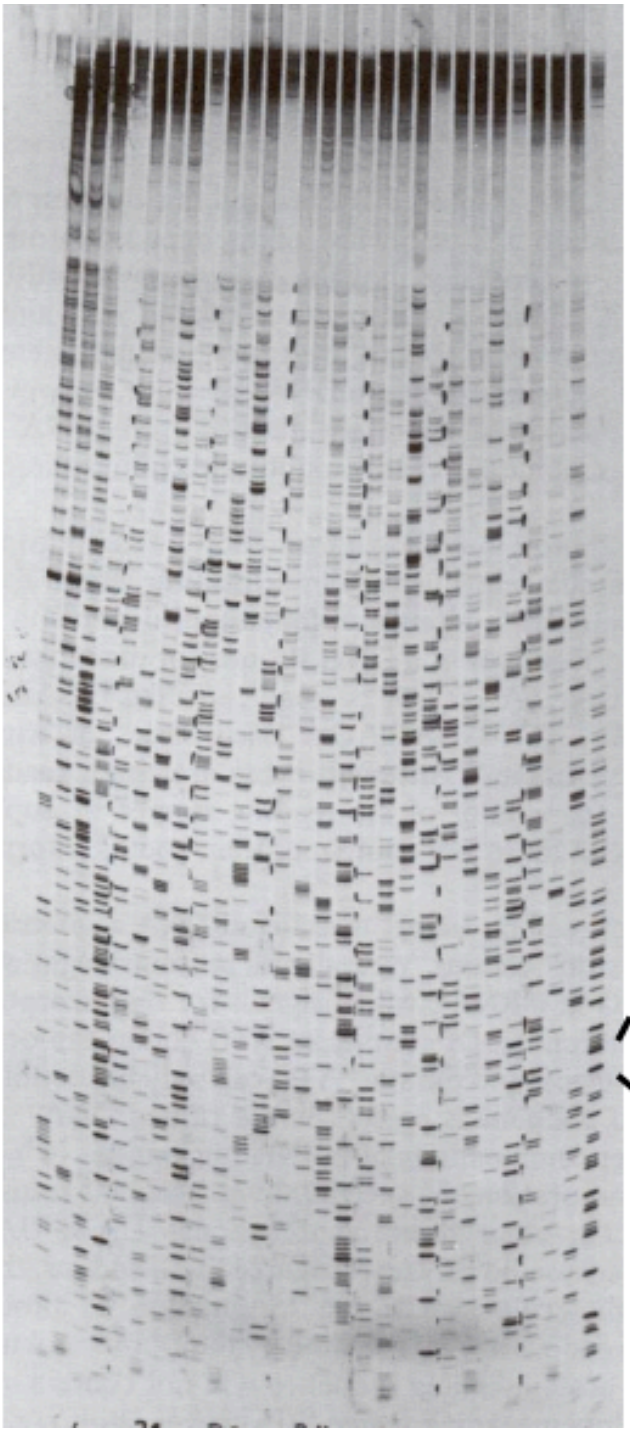
Enzima DNA Polimerase

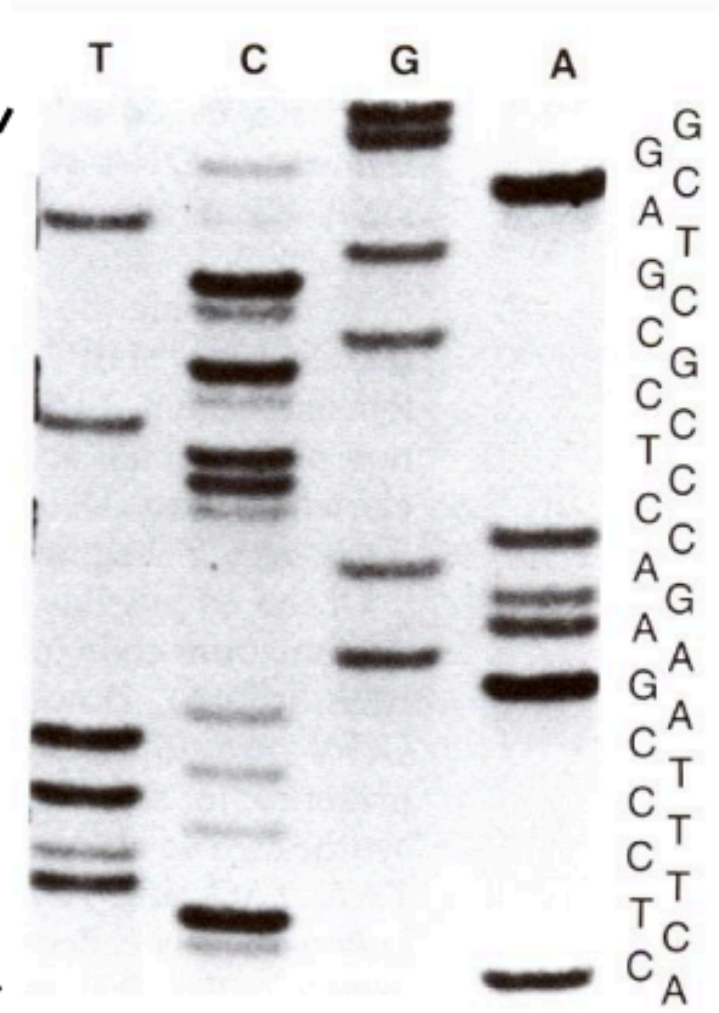
Iniciador (S ou AS)

ddNTPs (ddATP, ddTTP, ddCTP, ddGTP)

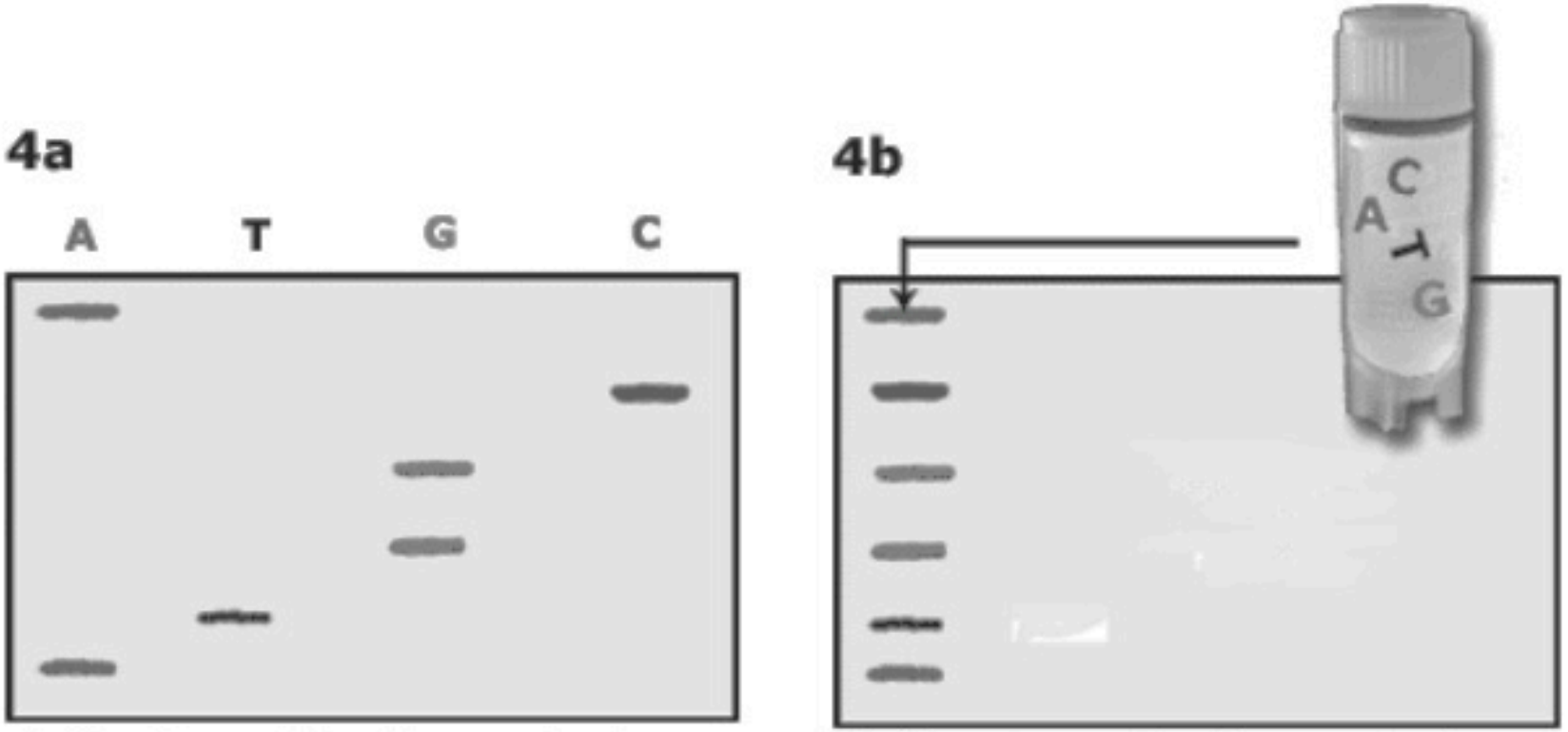

DNA
Primer
ddATp
ddCTp
ddGTp
ddTTp
A
C
G
T
Template sequence
C G A G T C C T T A G G C A T A C A
G C T C A G G A A ddT
G C T C A G G A A T C C G ddT
G C T C A G G A A T C C G T A ddT
G C T C A G G A A T C C G T A T G ddT
Primers
32P
Leitura
CGACTGACTAGTG
Sequência obtida

Auto-radiogramas de um gel de seqüenciamento de DNA





Sequenciamento
Manual X Automático



Reação de Sequenciamento

Amostra (cDNA; DNA)

dNTPs (dATP, dTTP, dCTP, dGTP)

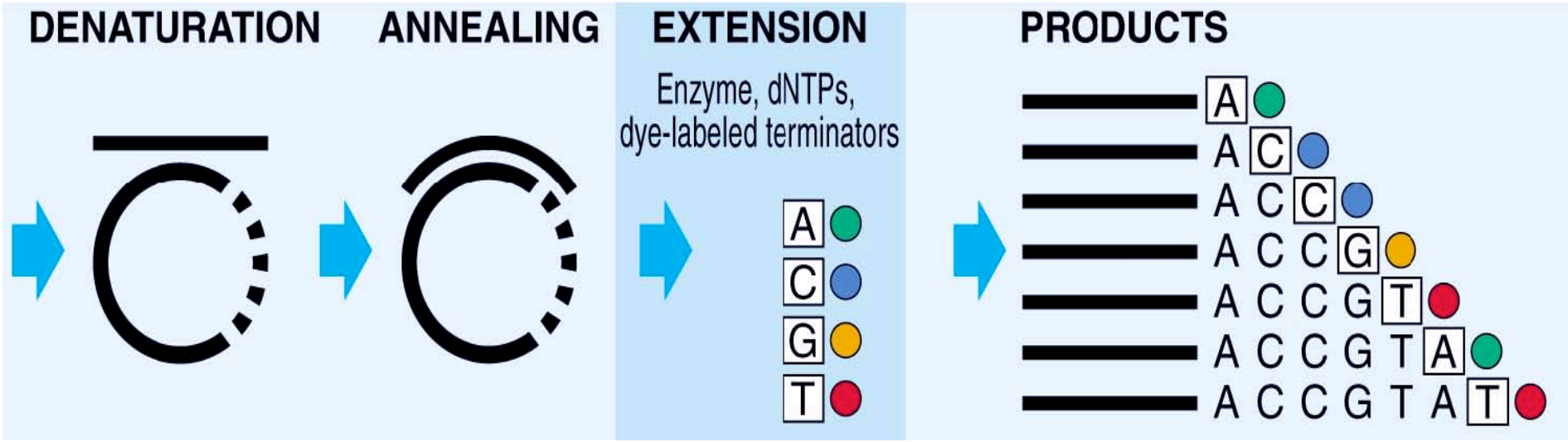
Tampão da enzima

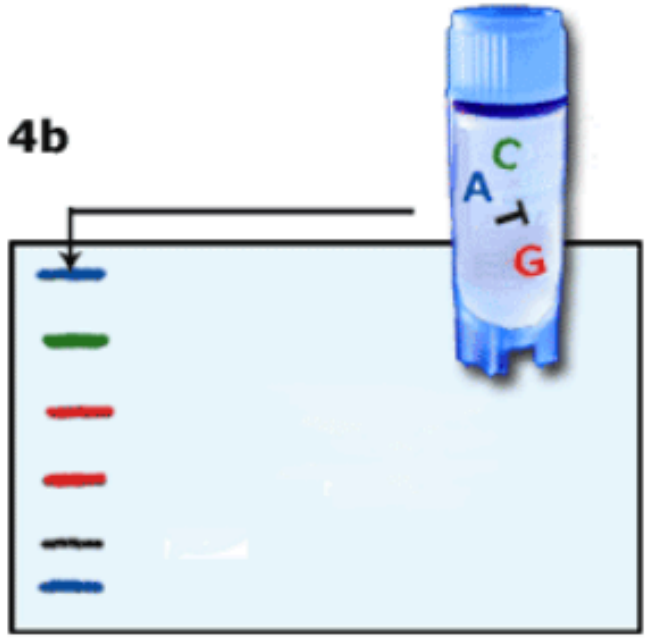
Enzima DNA Polimerase

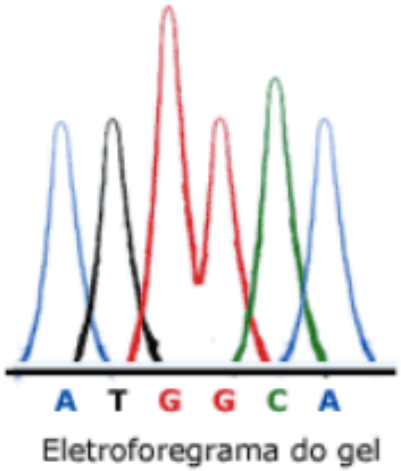
Iniciador (S ou AS)

ddNTPs (ddATP, ddTTP, ddCTP, ddGTP)

Sequenciamento DNA: Sanger

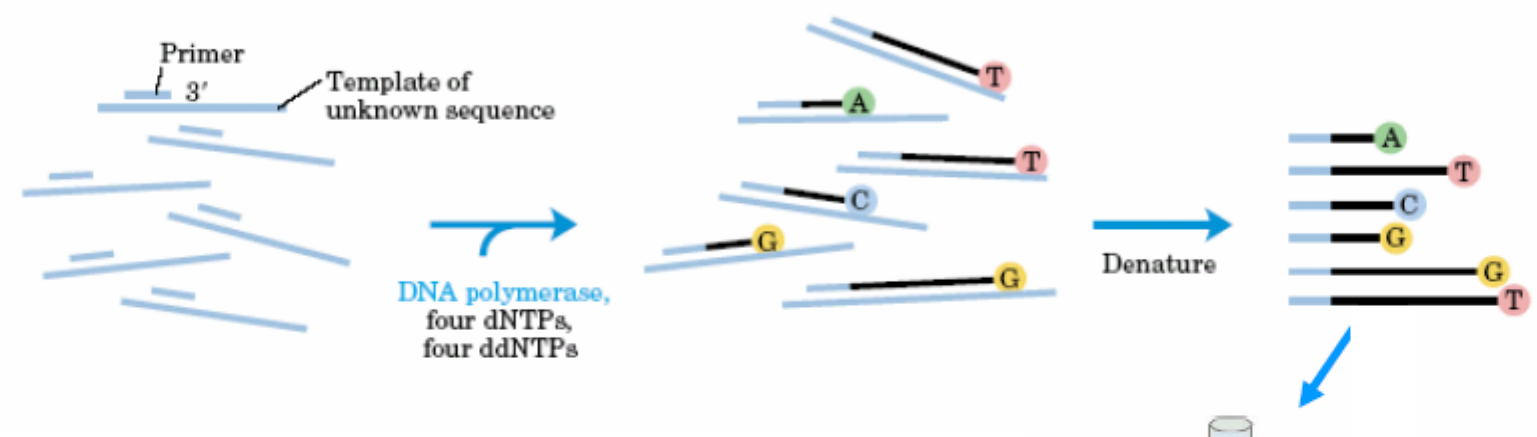


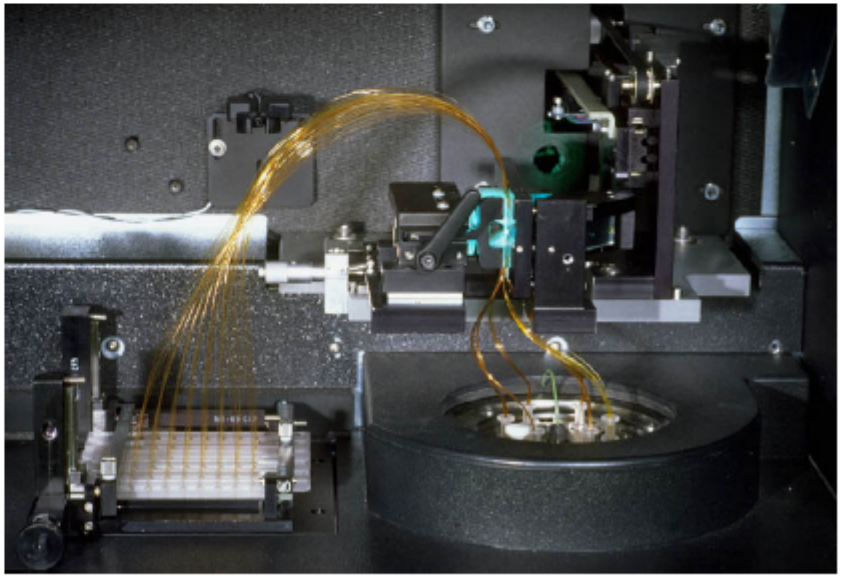


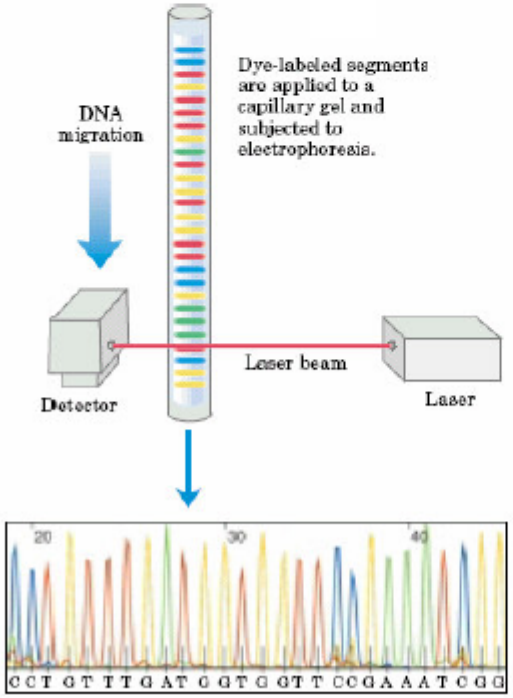


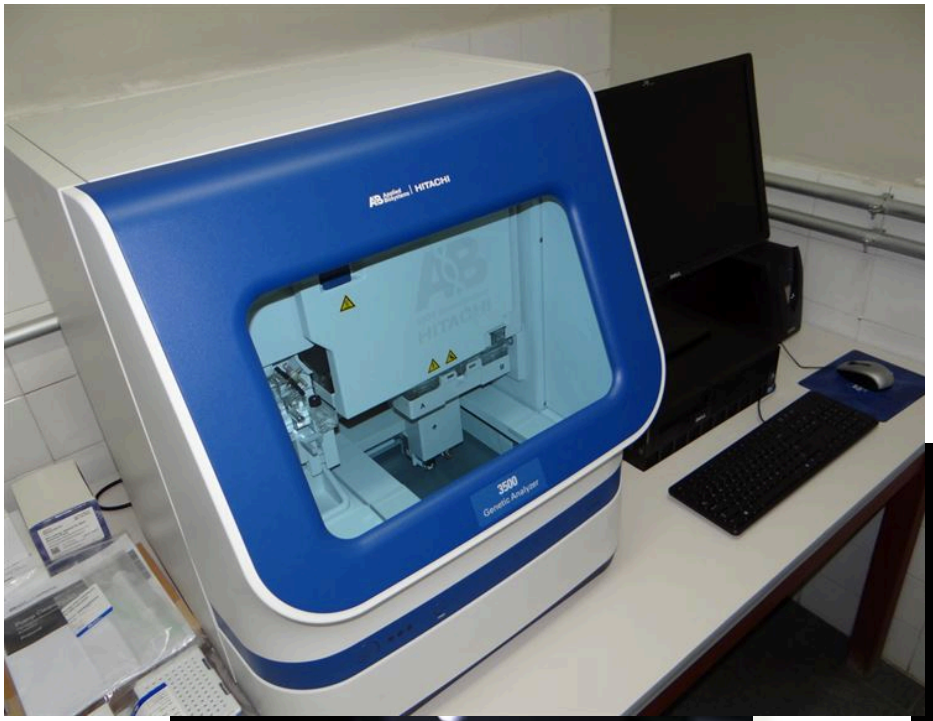
Eletroforegrama do gel

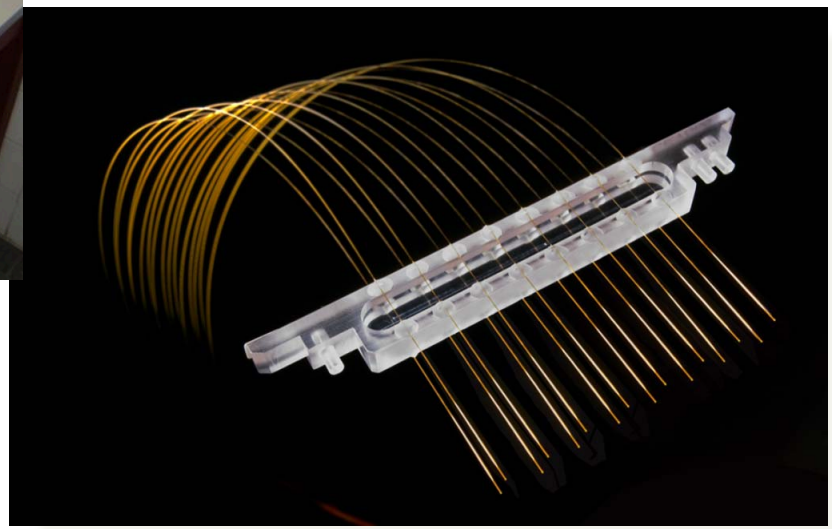
Sequenciamento em capilar

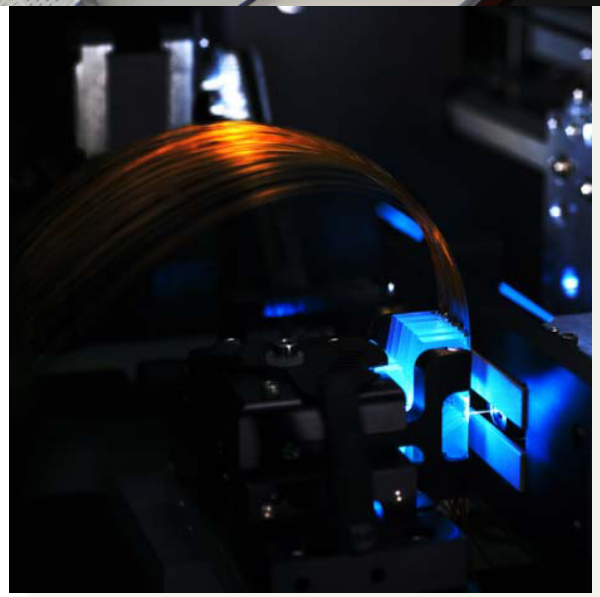






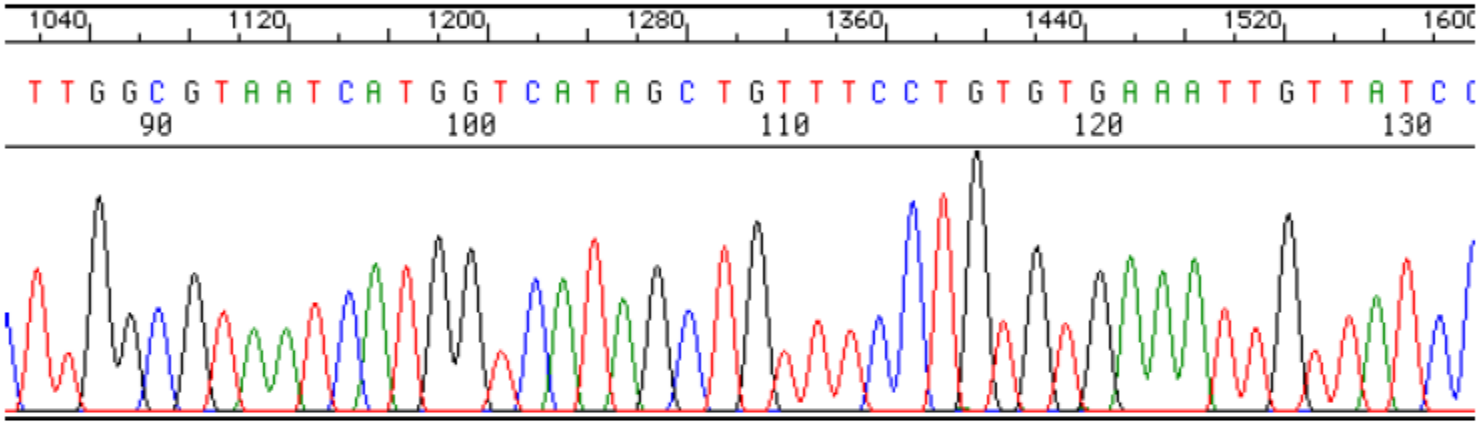

HITACHI
3500
Genetic Analyzer





Sequenciamento DNA: Sanger

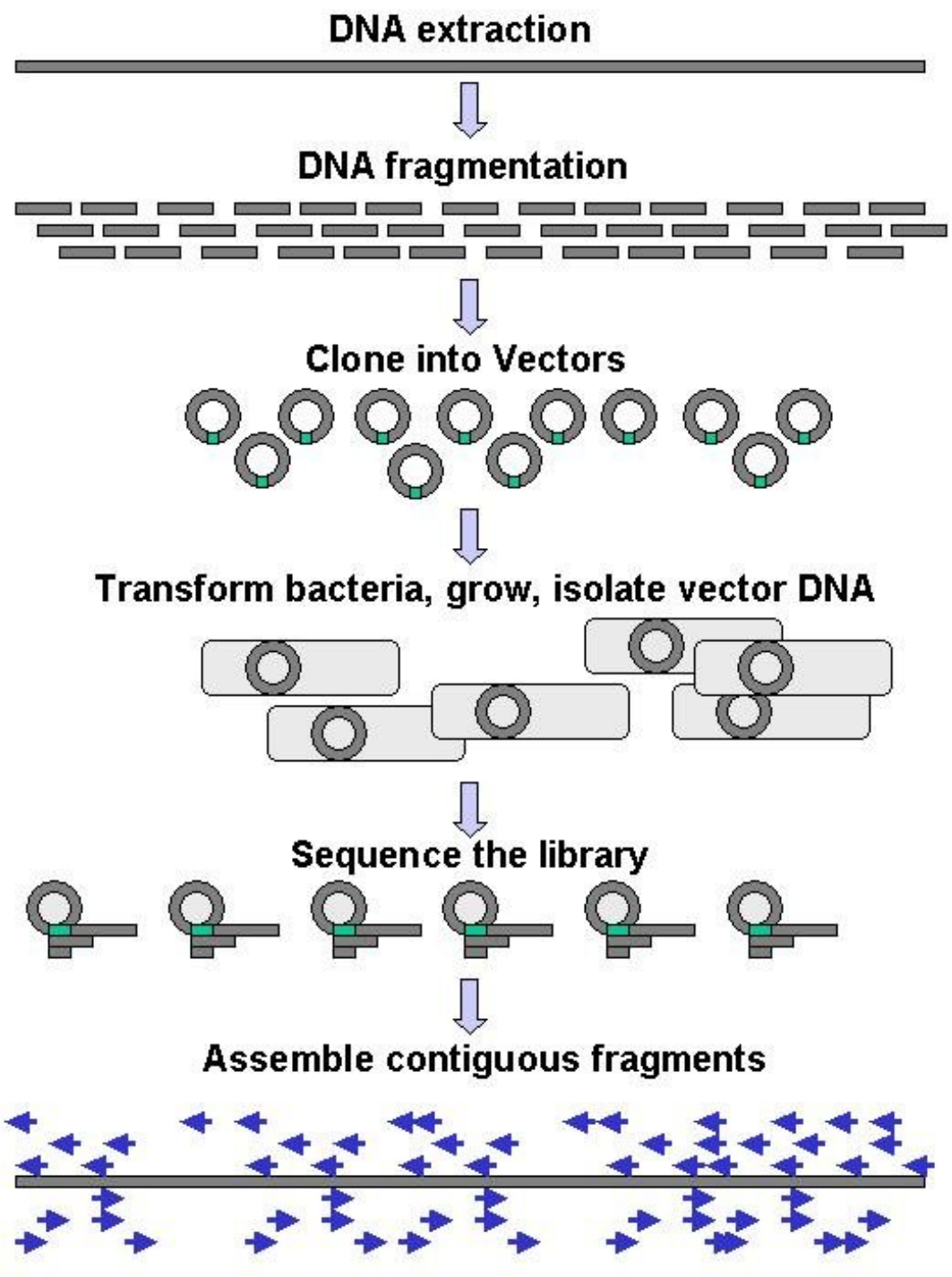
SANGER → Revolução → ERA GENÔMICA



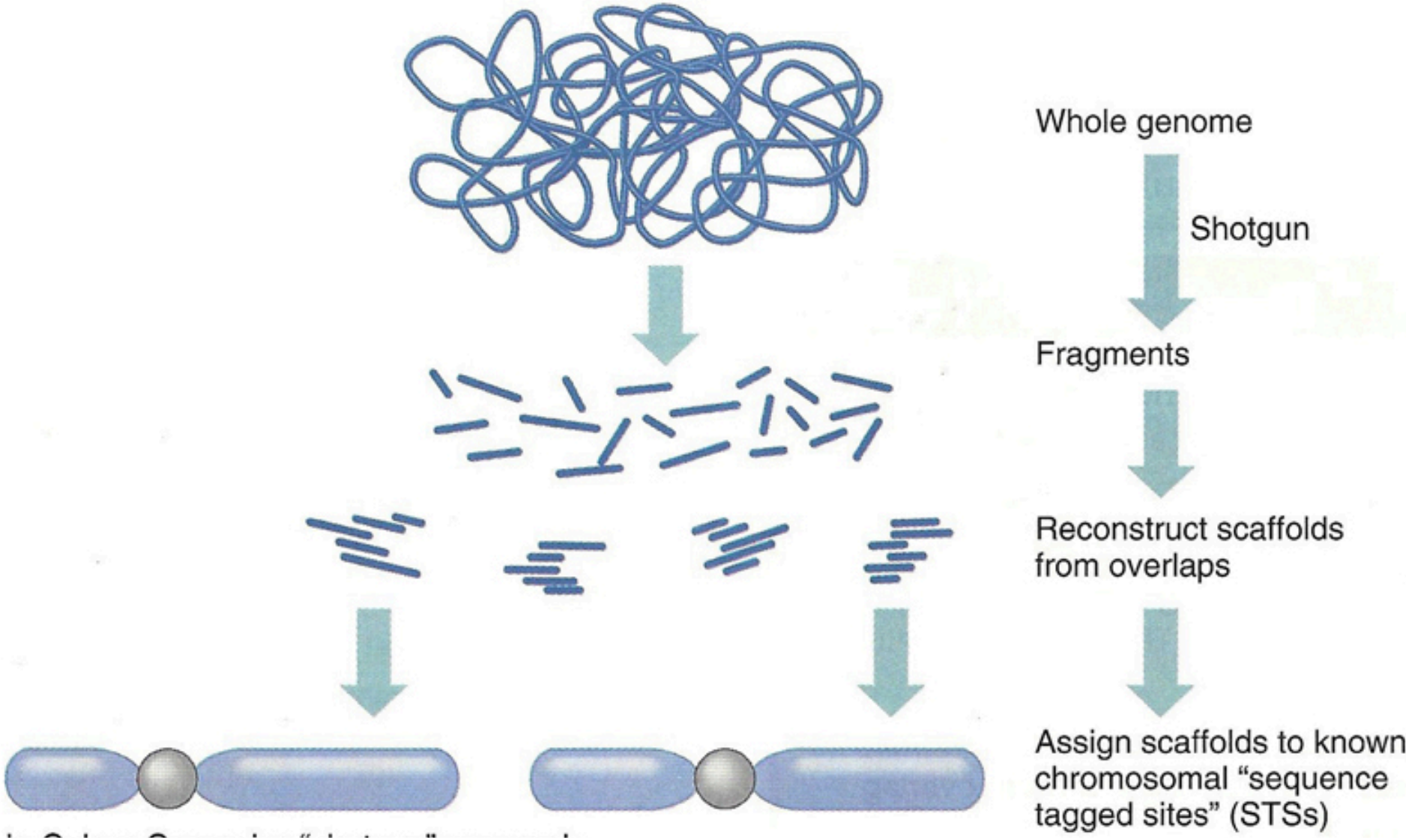
	Run Time	Read Length	Reads / Run	Total nucleotides sequenced	Cost / MB
Capillary Sequencing (ABI3730xl)	20m-3h	400-900 bp	96 or 386	1.9-84 Kb	$2400

Como sequenciar um genoma com esta metodologia?

Genoma bactéria→ >600 Kb – precisaria fragmentar em ~1000 partes


DNA extraction
DNA fragmentation
Clone into Vectors
Transform bacteria, grow, isolate vector DNA
Sequence the library
Assemble contiguous fragments

Biblioteca Genômica de *Shotgun*

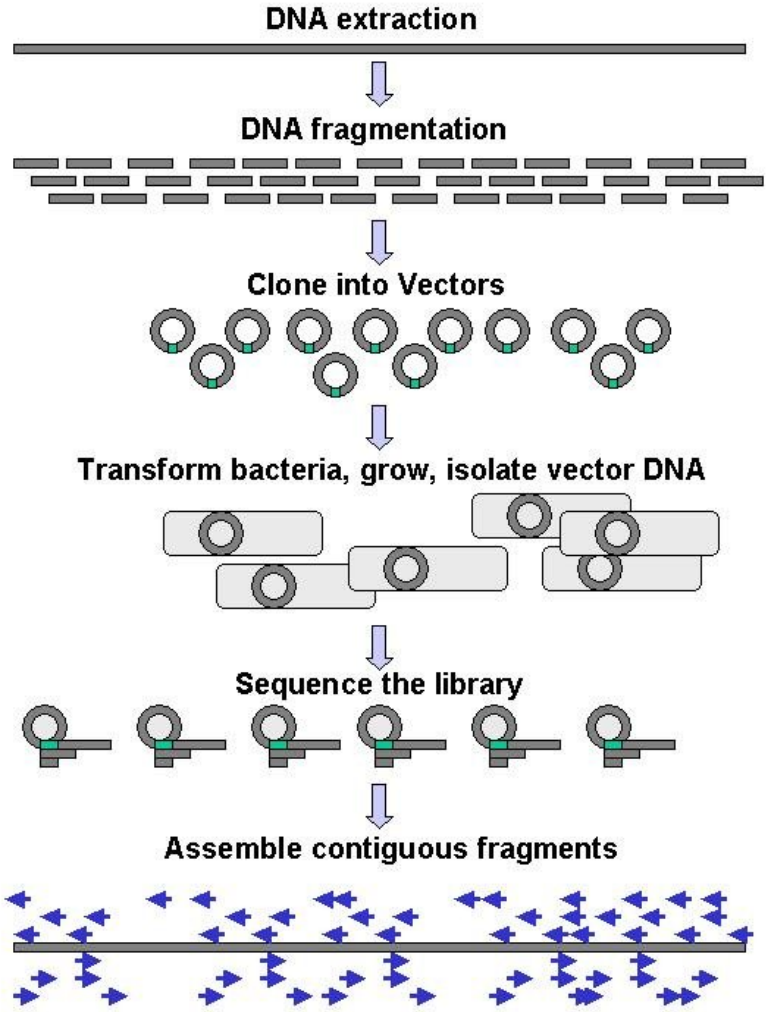


Problemas com a técnica de Sanger

- Erros ou não detecção dos primeiros 15-40 pb
- Dependente de enzima
- Excesso de background
- Preparação das amostras
  - PCR kit, primers, passos de purificação, clonagem
- Única reação pode sequenciar 300-800 pb
  - Pouco eficiente para sequenciar genomas

Necessidade de novas estratégias de sequenciamento

- Rápidas
- Menor custo
- Maior acurácia
- Maior capacidade de leitura
- Atender as pesquisas no genoma humano



2ª Geração de Sequenciadores

NGS – Next Generation Sequencing

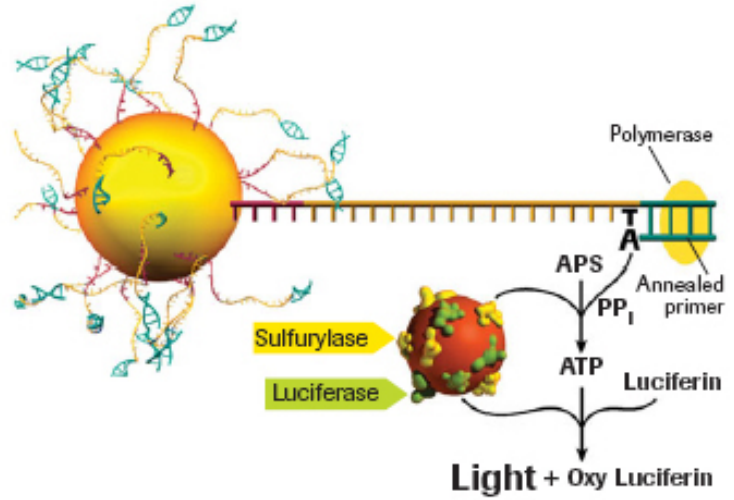
- Fim da era de tecnologias baseadas em eletroforese

- Alta capacidade de geração de dados → genomas em única corrida.

- Métodos alternativos à clonagem tradicional

Pirosequenciamento

Roche (454) GS FLX sequencer

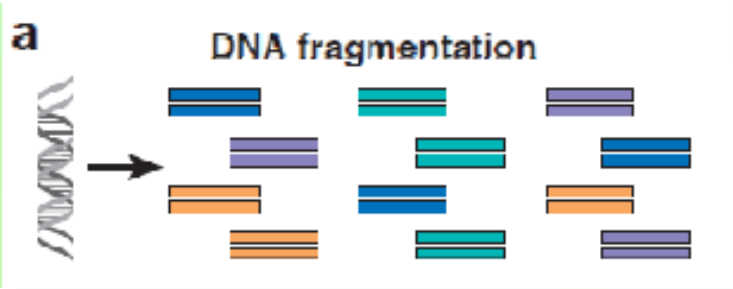


"Massively parallel amplification"

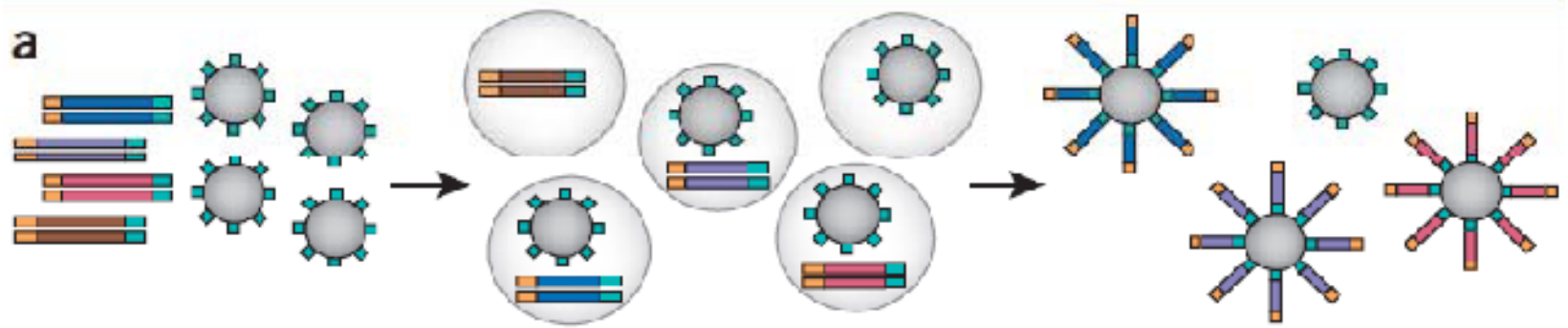
- Emulsão de água em óleo

- DNA polymerase
- ATP sulfurylase
- Luciferase
- Apyrase
- Substrates: adenosine 5' phosphosulfate (APS) e luciferina

Pirosequenciamento



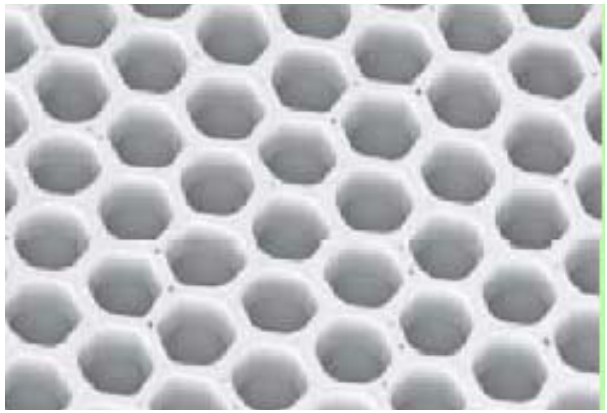
- Fragmentação do DNA
- Ligação à adaptadores
- Captura em esferas



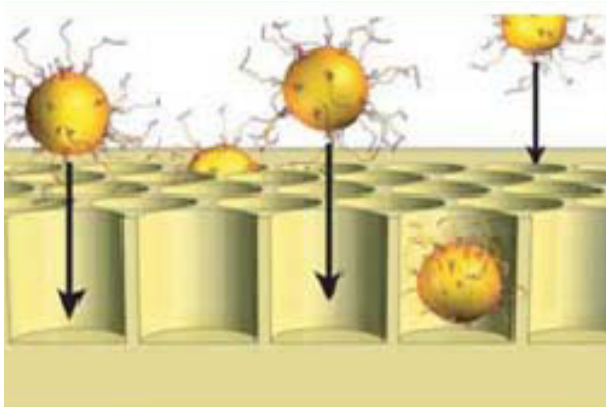
1 fragmento por esfera

Adaptadores

Pirosequenciamento



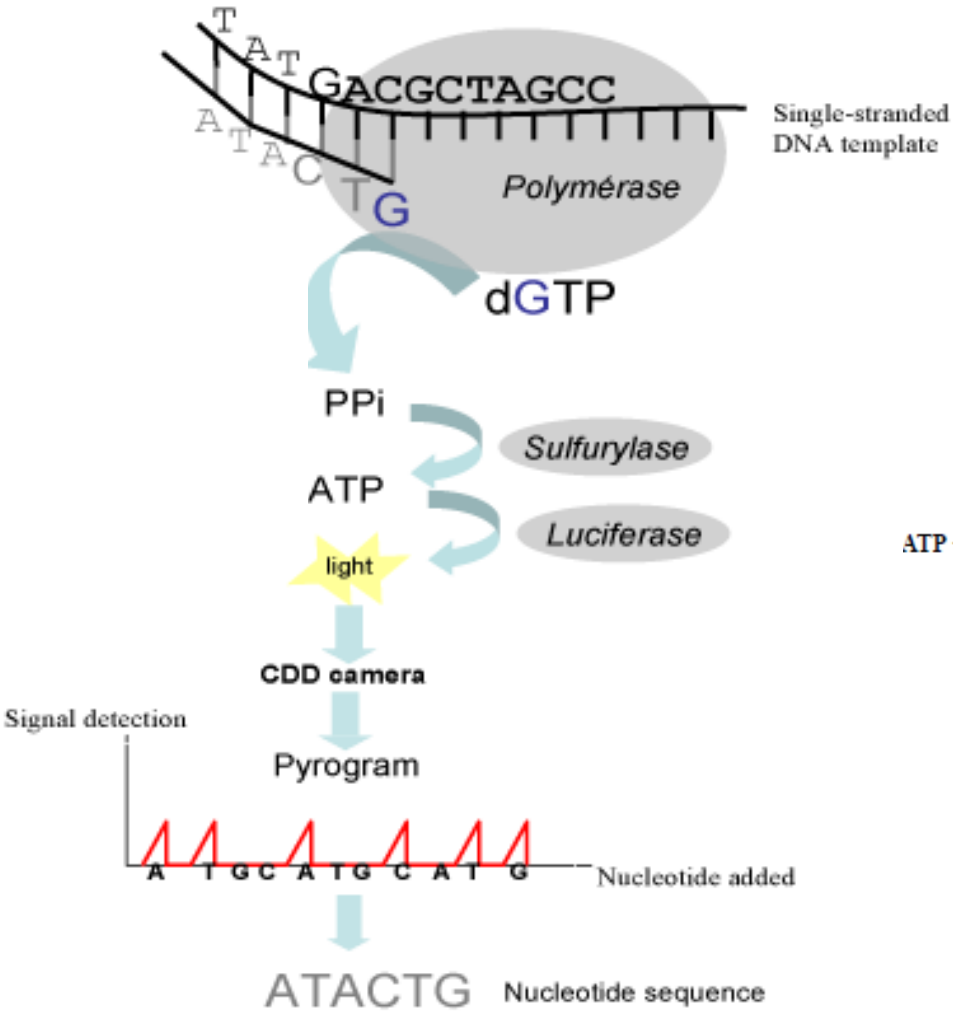
Lâmina óptica de 44 um de diâmetro



Uma esfera por orifício

Pirosequenciamento

Cada base é adicionada separadamente

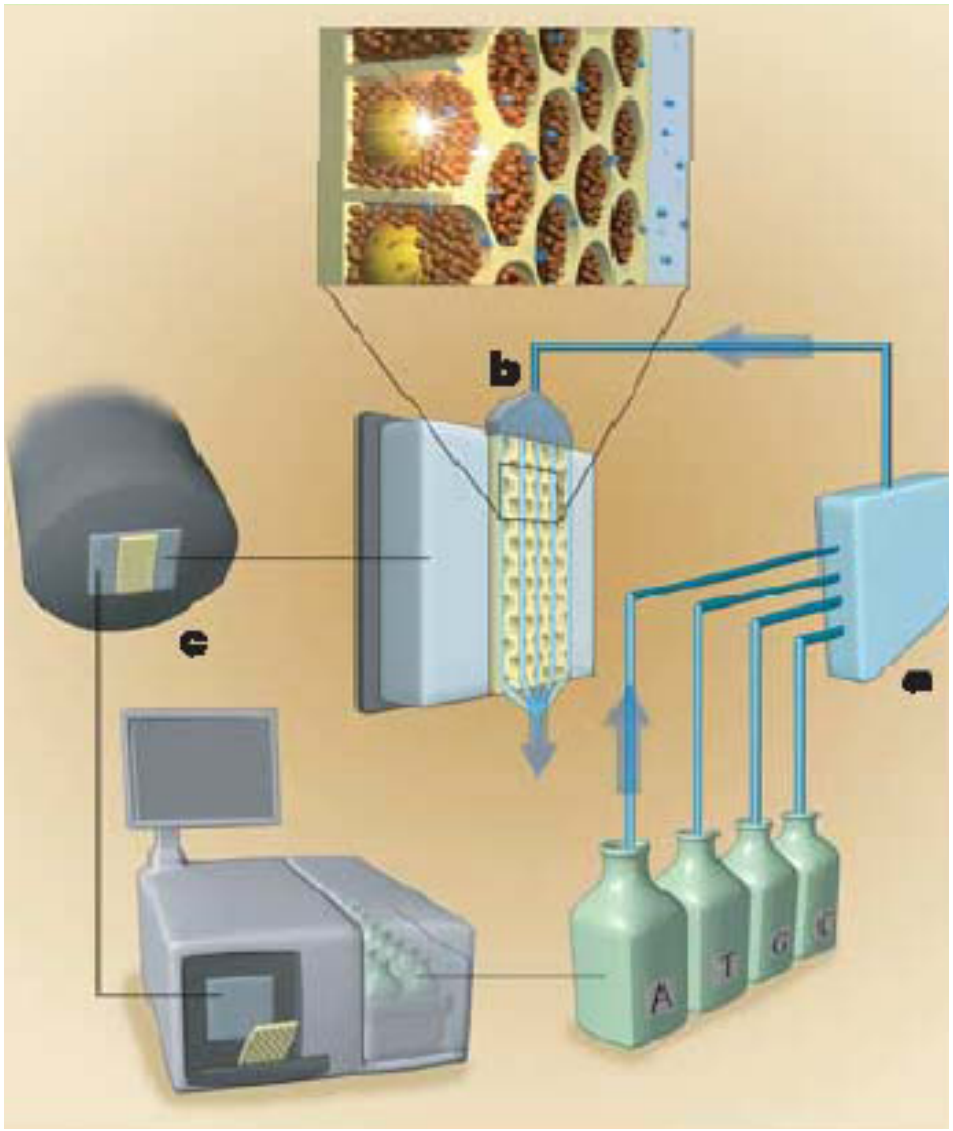


$$DNA_n + \text{Nucleotídeo} \xrightarrow{\text{Polimerase}} DNA_{n+1} + \mathbf{PPi}$$

$$\mathbf{PPi} + APS \xrightarrow{\text{ATP Sulforilase}} SO_4 + \mathbf{ATP}$$

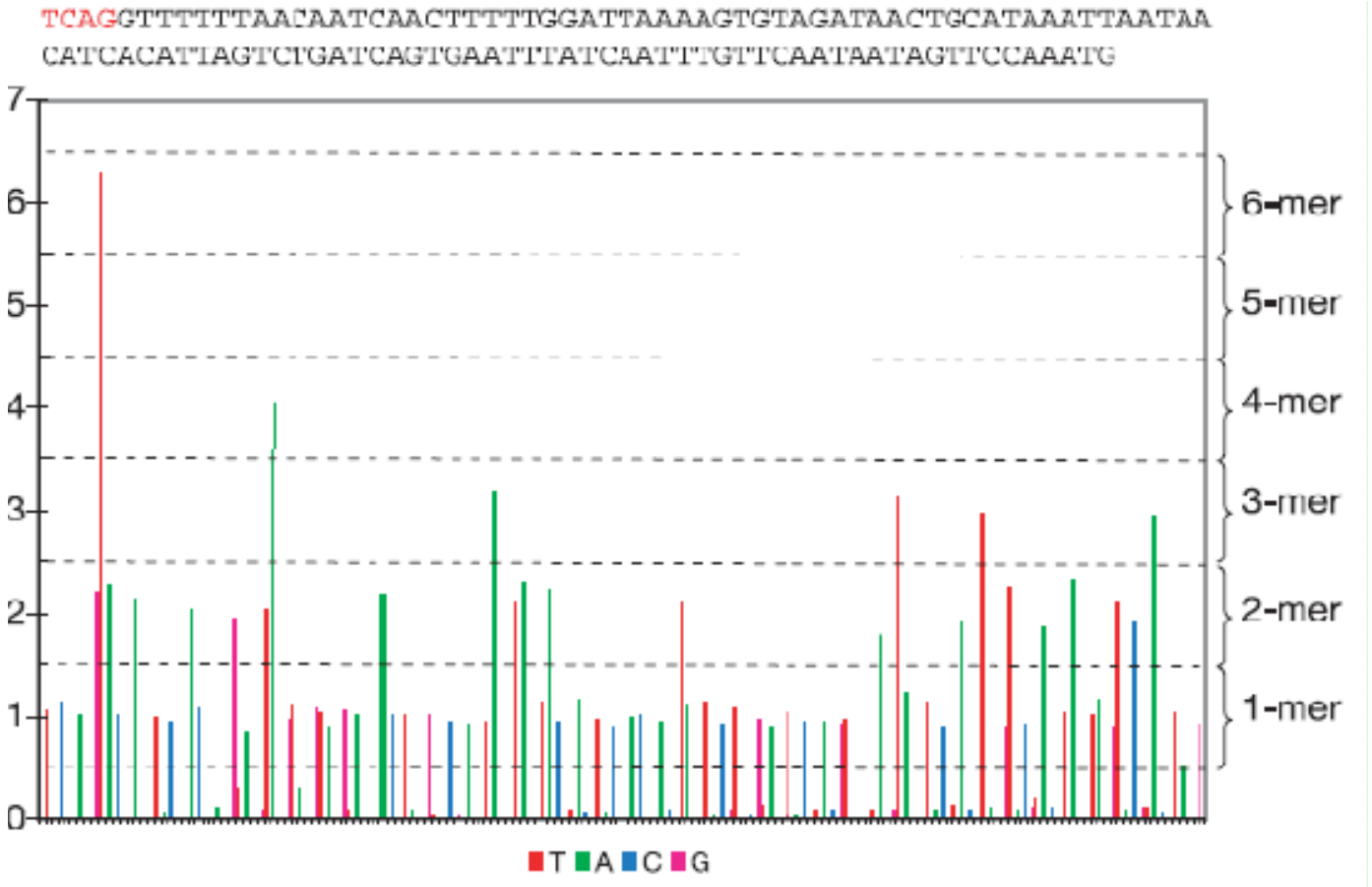
$$\mathbf{ATP} + \text{Luciferina} + O_2 \xrightarrow{\text{Luciferase}} AMP + PPi + \text{Oxiluciferina} + CO_2 + \mathbf{Luz}$$

Pirosequenciamento

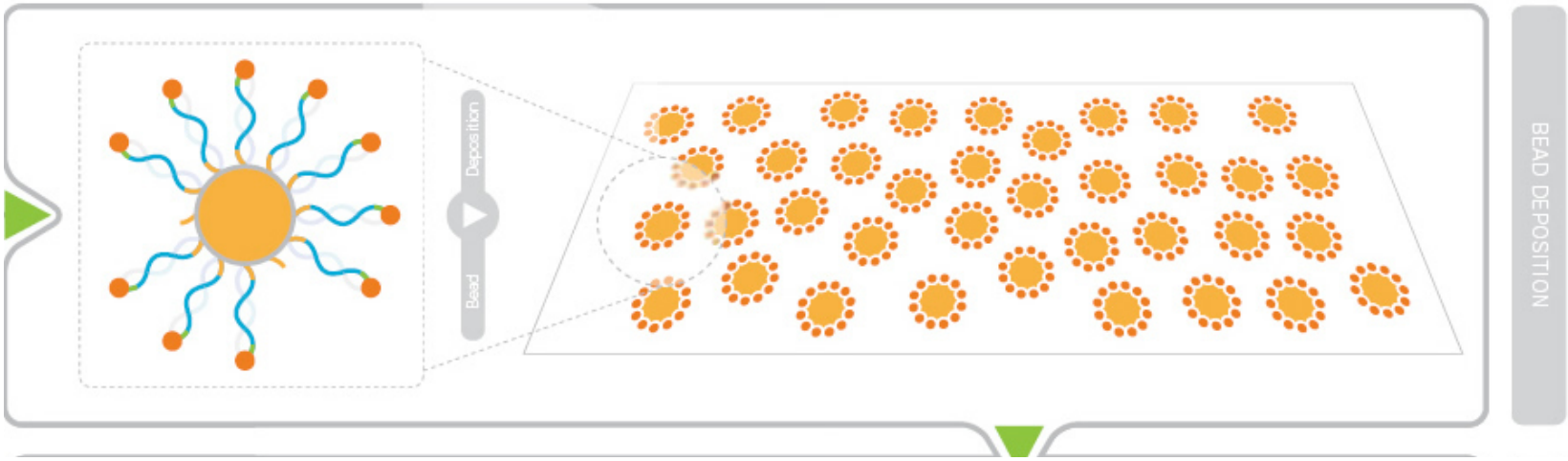


Pirosequenciamento

Análise dos resultados

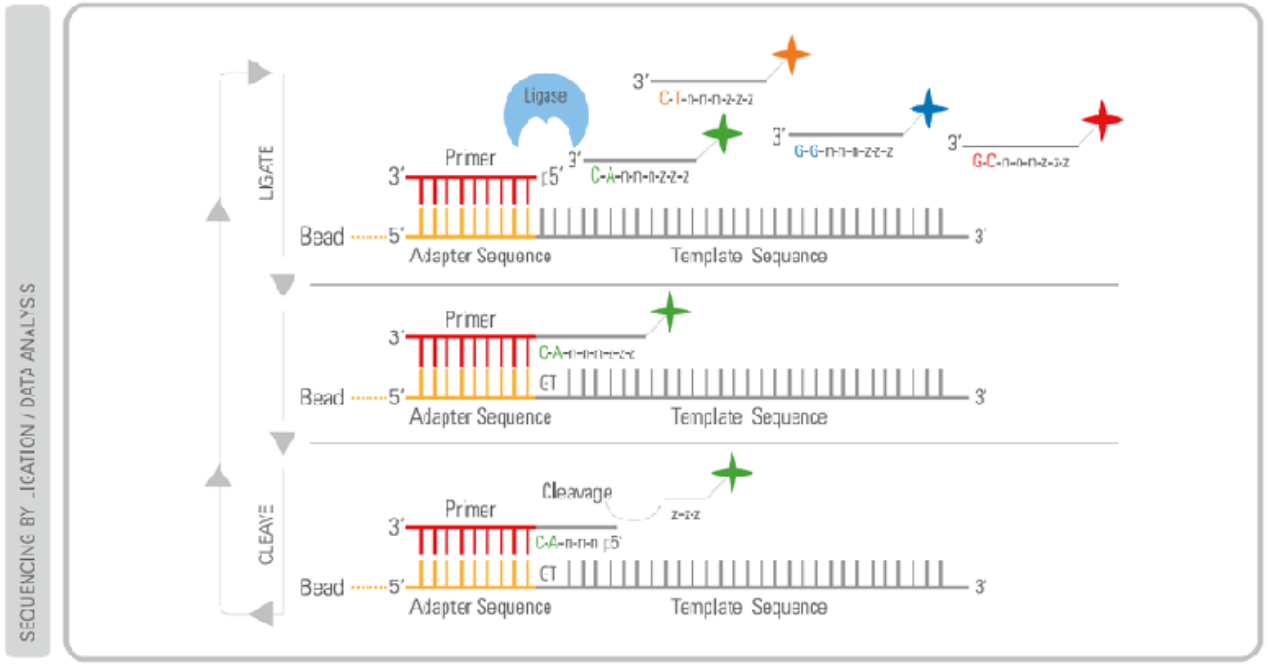


https://www.youtube.com/watch?v=bNKEhOGvcaI

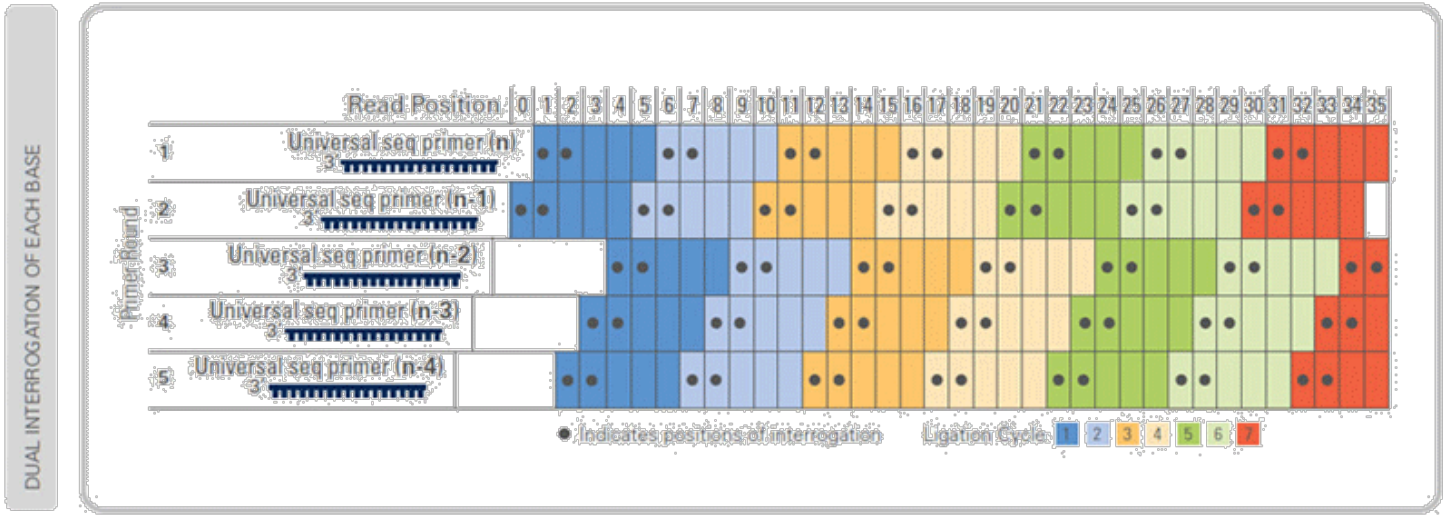


Procedimento

1. Anela *primer adaptador*
2. Adiciona o 1º grupo de primers (8nt)
3. Clivagem dos últimos 3nt
4. Emissão de fluorescência
5. Remoção do fluoróforo
6. Um pentanucleotideo fica ligado
7. Repete o procedimento (7x)
8. Desnatura o DNA
9. Utiliza um novo primer adaptador (comprimento n-1)

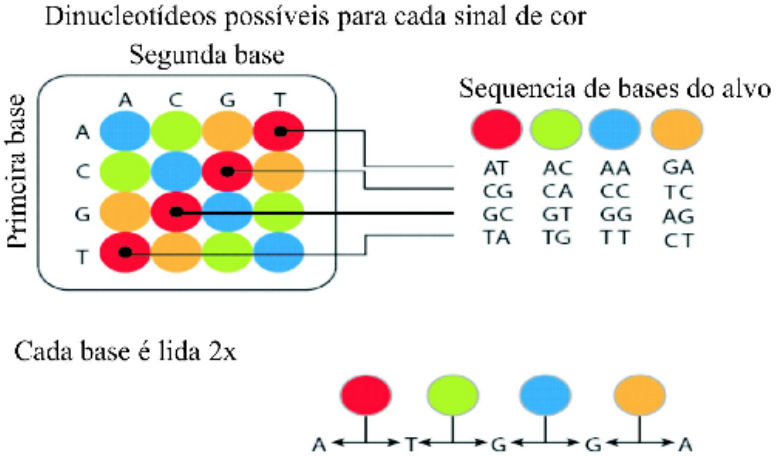


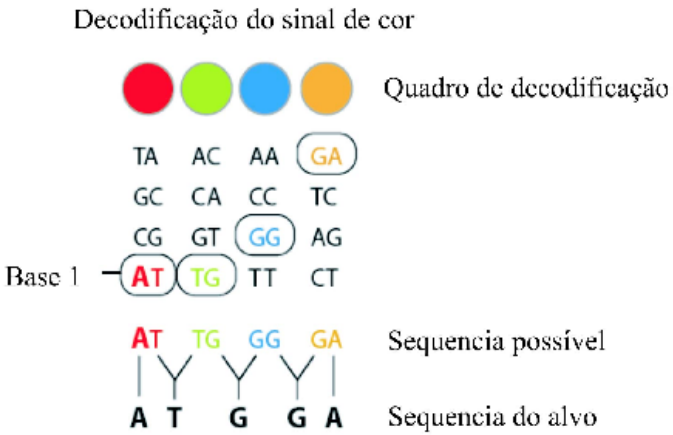
ABI Solid



- A fluorescência indica duas bases

- Desvantagem
  - 35pb





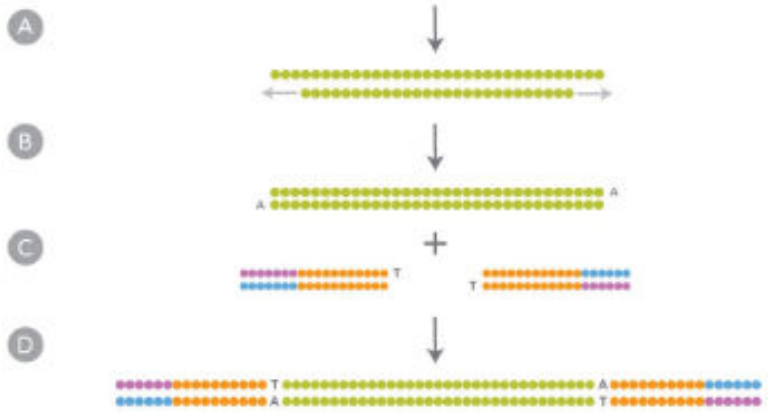
http://www.dnatube.com/video/27908/Solid-DNA-sequencing-animation

Illumina (Solexa)

Simple, Automated Workflow

1 Library Prep

6 hours | 3 hours hands-on time



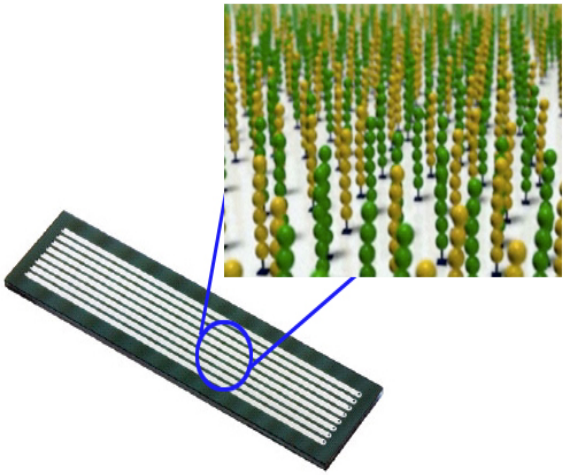
A Fragment DNA

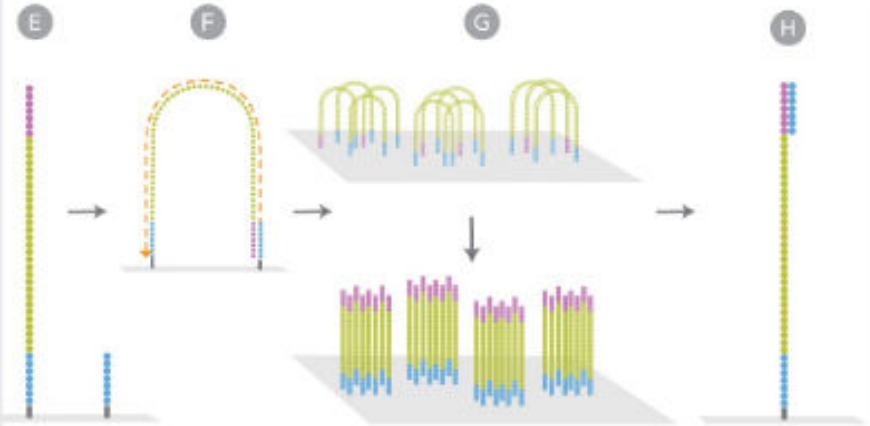
B Repair ends/ Add A overhang

C Ligate adapters

D Select ligated DNA

2 Cluster Generation





E Attach DNA to flow cell

F Perform bridge amplification

G Generate clusters

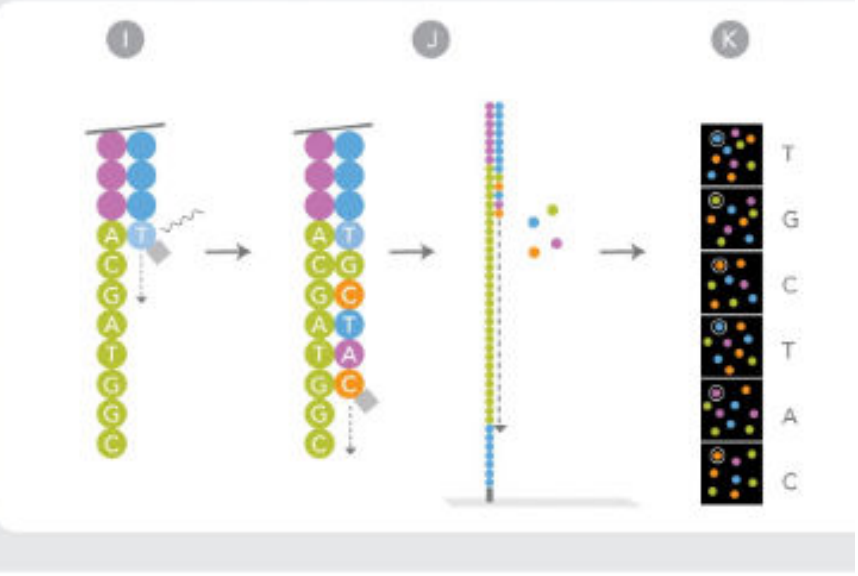
H Anneal sequencing primer

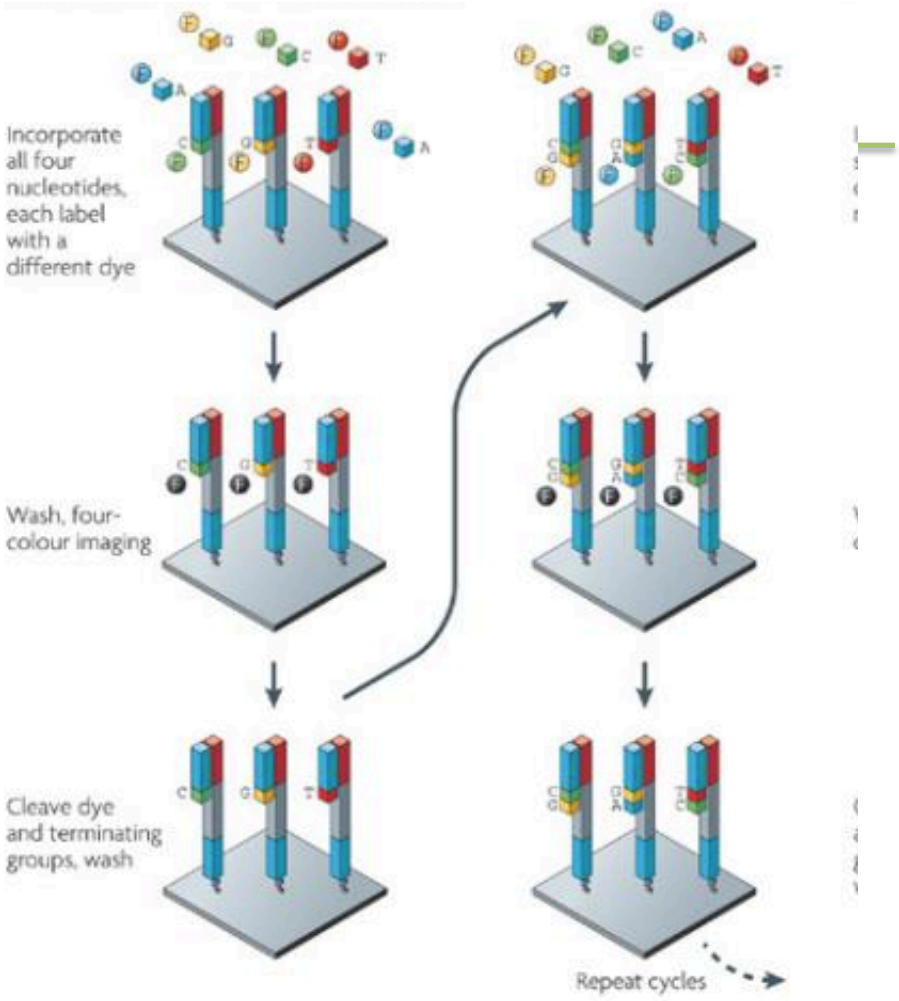
Illumina

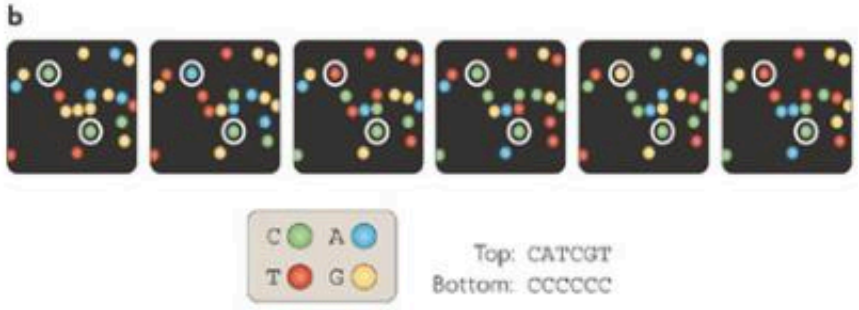
3 Sequencing

2–3 days (single-read)
4–6 days (paired-end)

30 min. hands-on time (1–8 Samples)

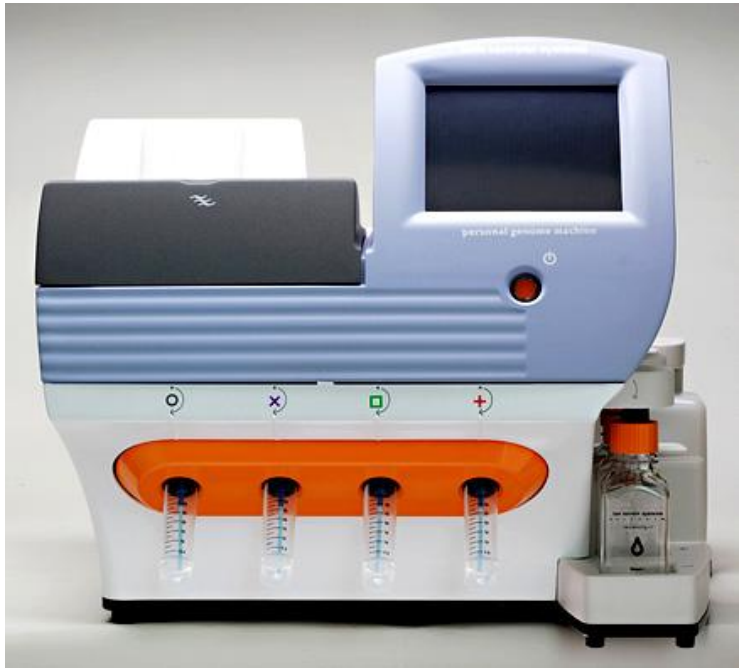


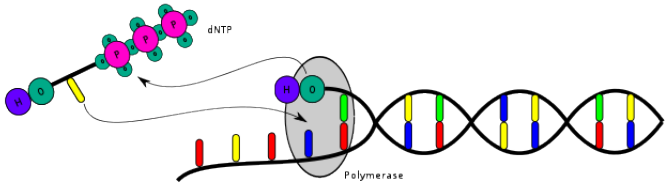




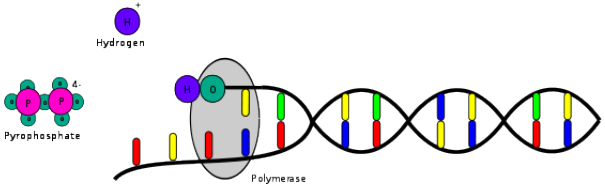
https://www.youtube.com/watch?v=womKfikWlxM

Ion Torrent

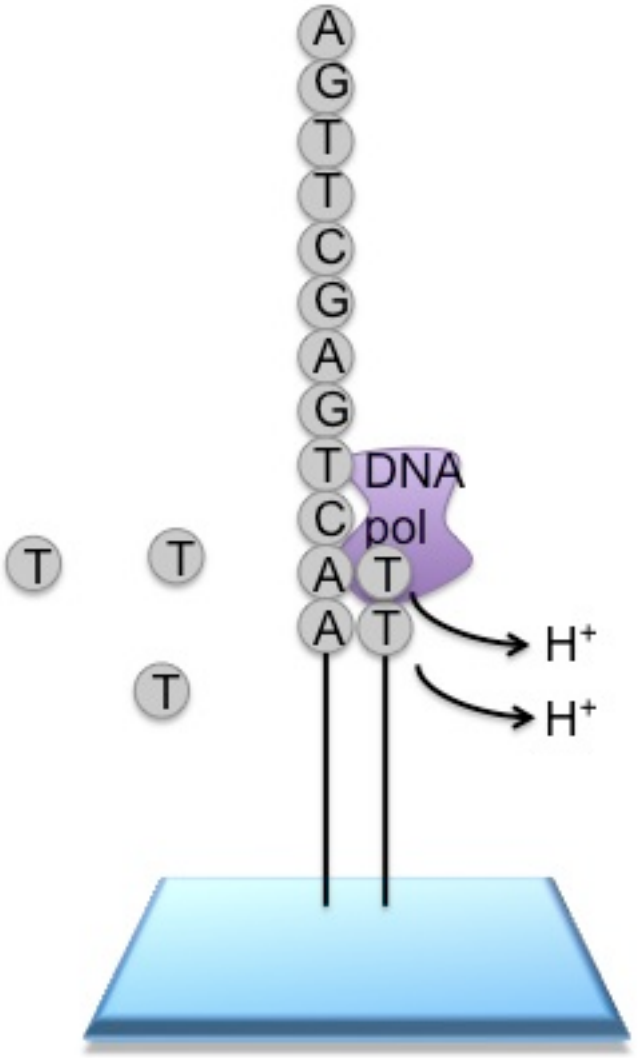




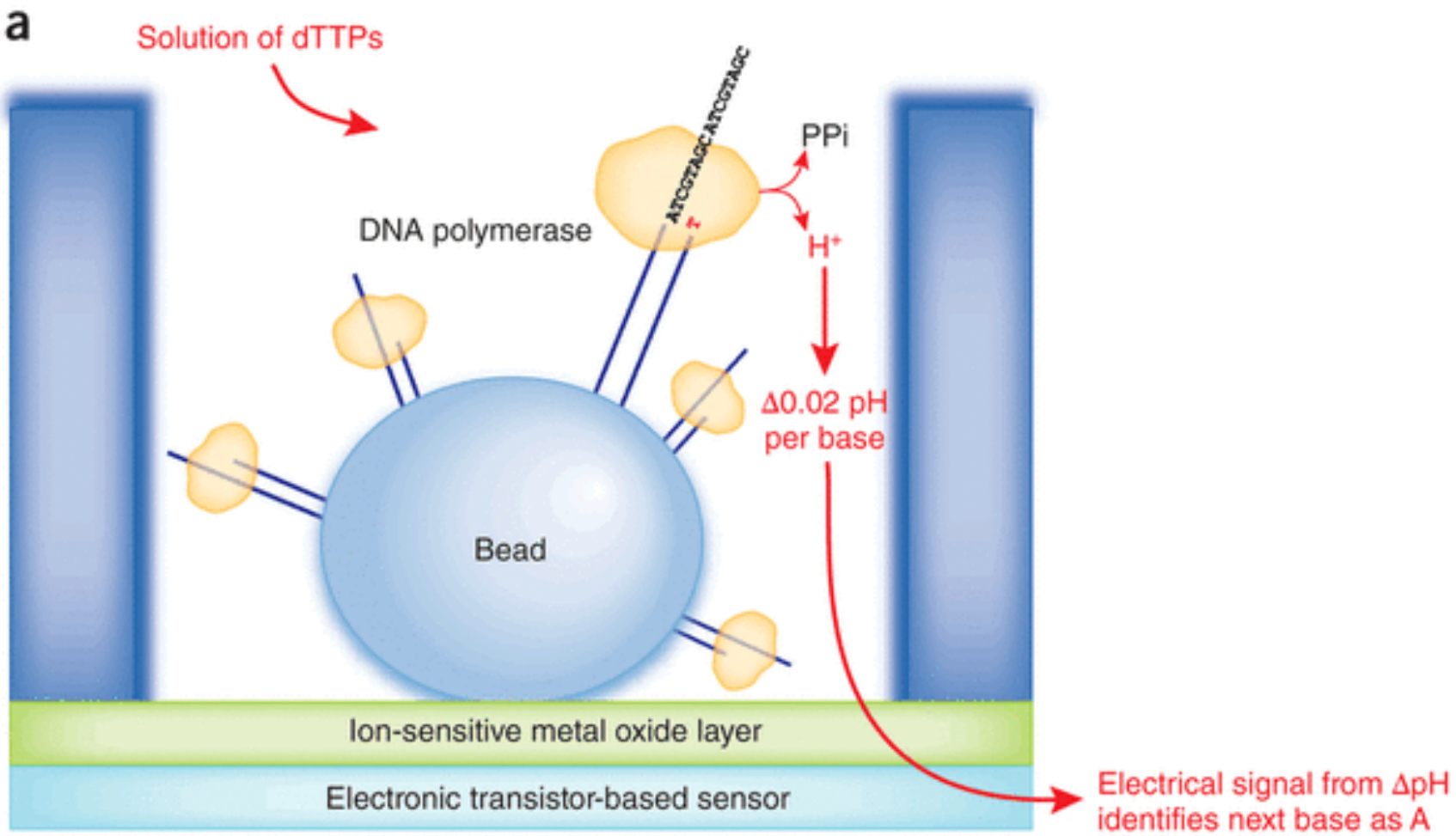
Polymerase integrates a nucleotide.



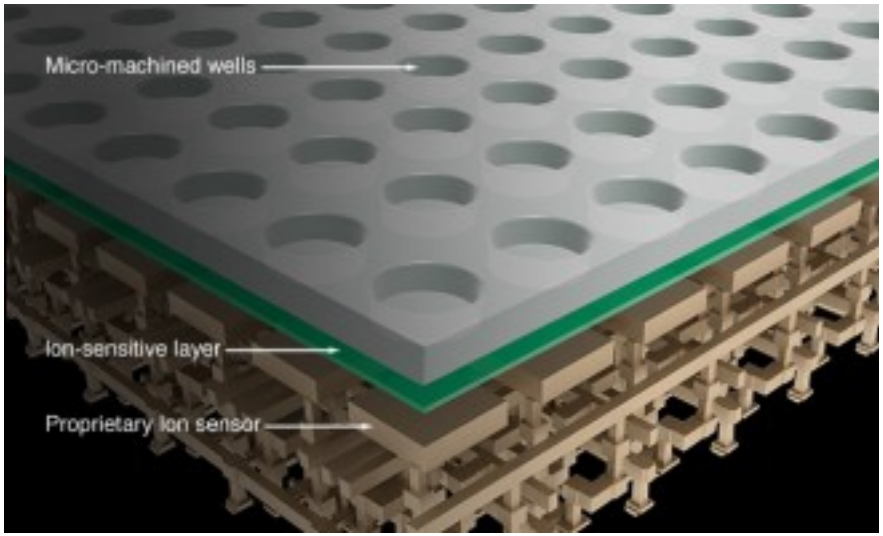
Hydrogen and pyrophosphate are released.

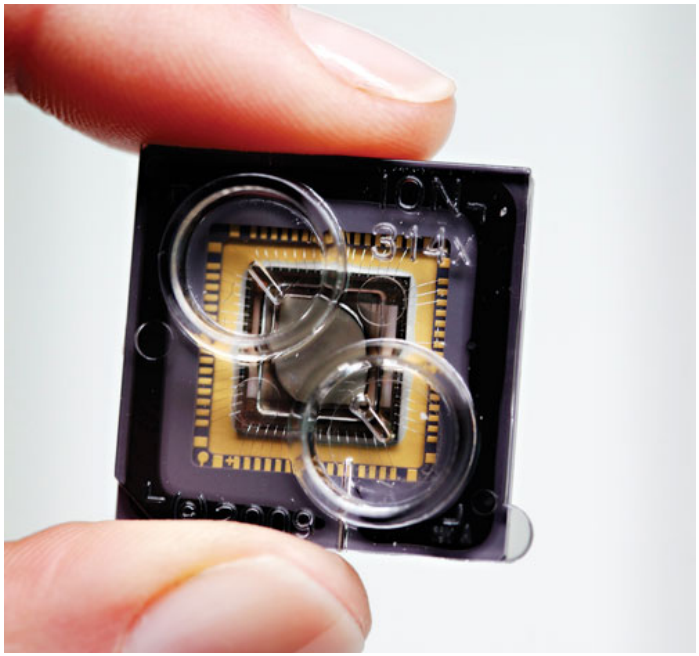


Ion Torrent


a
Solution of dTTPs
PPi
DNA polymerase
H+
Δ0.02 pH
per base
Bead
Ion-sensitive metal oxide layer
Electronic transistor-based sensor
Electrical signal from ΔpH
identifies next base as A

Ion Torrent





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

Genômica: NGS – Next Generation Sequencing

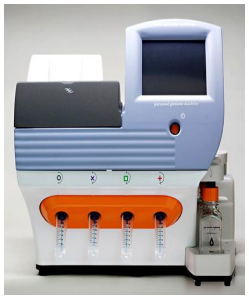
ABI 3730xl

Roche/454 FLX

Illumina/Solexa GA

ABI SOLiD

Ion Torrent



	ABI 3730xl	Roche/454 FLX	Illumina/Solexa	ABI SOLiD	Ion Torrent
Método	Sanger	Pirosequenciamento	Sequenciamento por Síntese	Sequenciamento por Ligação	Sequenciamento por semicondutor
Dados/run	**290 Kb**	**~300 Mb**	**~7 Gb**	**> 15 Gb**	**> 1Gb**
Tempo/run	**1 hora**	**5 horas**	**3-7 dias**	**10 dias**	**4 hora e meia**
Tamanho	**~500 - 800 pb**	**~200 - 500 pb**	**~35-100 pb**	**~25 - 35 pb**	**~200 pb**
Custo/run	**$48**	**$6.800**	**$9.300**	**$11.000**	**$ 600.00**

3ª Geração de Sequenciadores

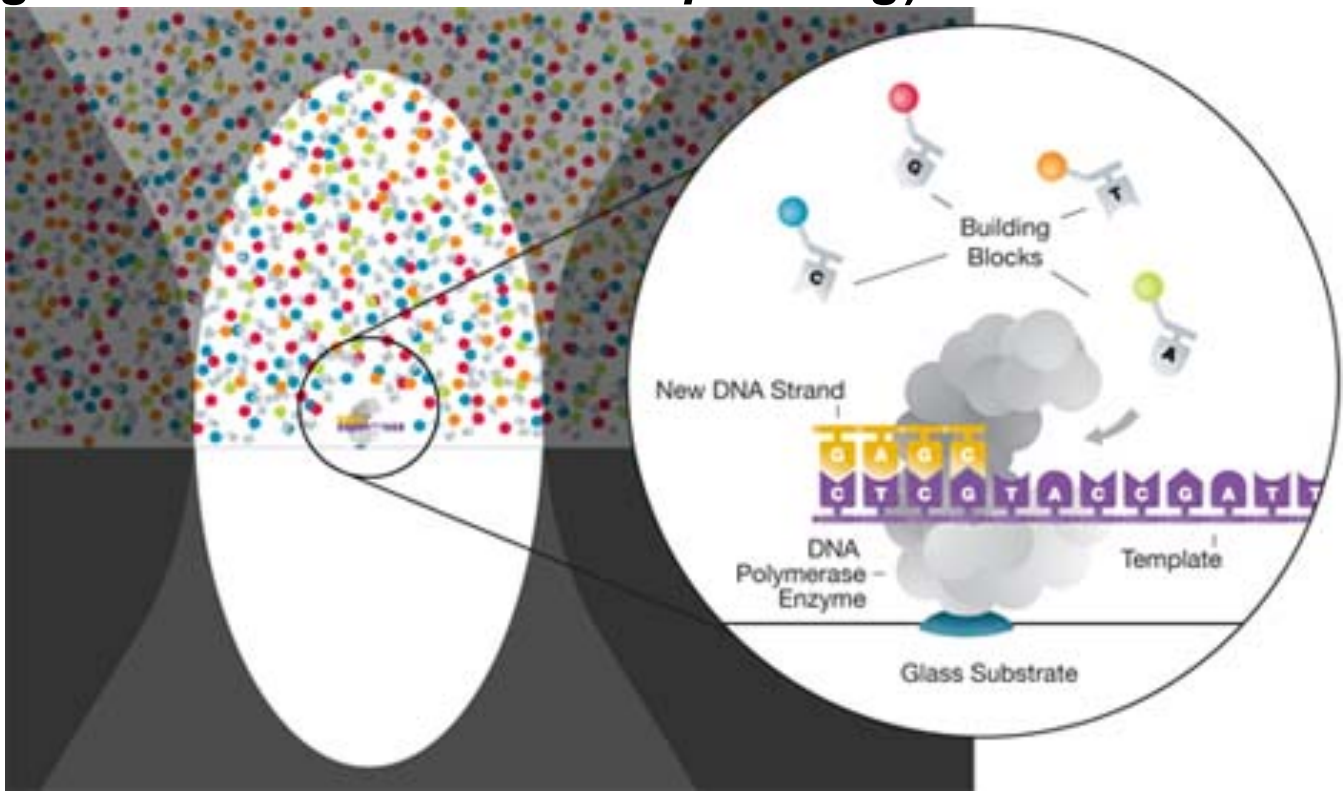
• Capacidade de sequenciamento de uma única molécula de DNA. Sem necessidade de amplificação.

• Era da nanotecnologia

• Altíssima capacidade de geração de dados → genoma humano em única corrida.

Pacific Biosciences SMRT sequencing
(*Single Molecule Real Time Sequencing*)

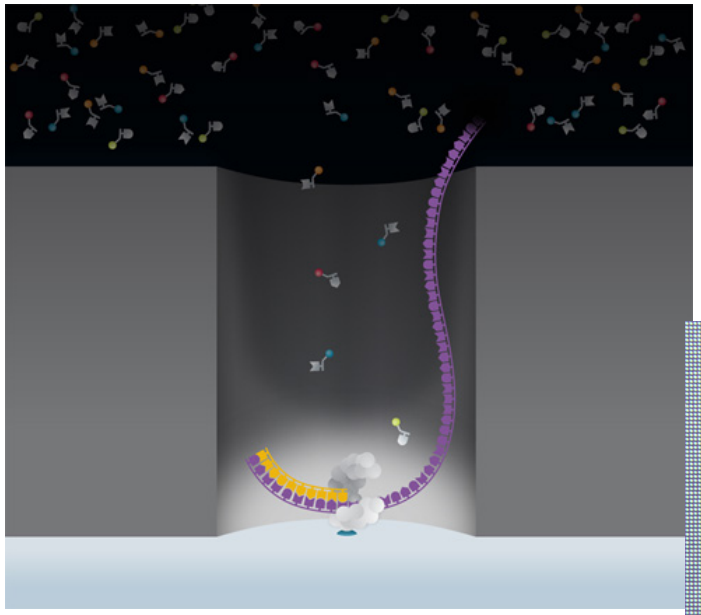
PacBio RS II



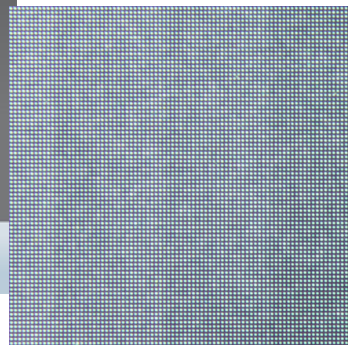
- **Sequenciamento de uma única molécula de DNA**
- **Sem necessidade de amplificação**
- **Altíssima capacidade de geração de dados:**
  - **Genoma humano em única corrida**

Janela de observação em nano-escala (ZMW)

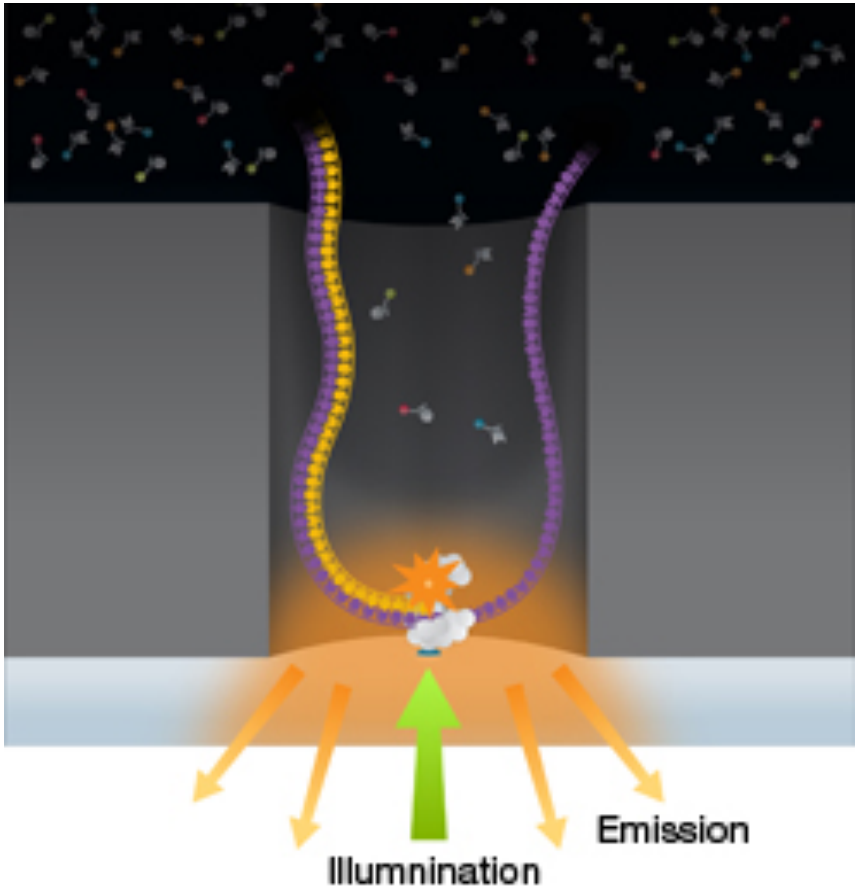
- **DNA polimerase adere a uma base transparente de nanotubos cilindricos de metal.**
- **Uma molécula de DNA se liga a polimerase com bases fosfoligadas**
- **Fluorescência liberada quando o fosfato é clivado.**
- **A luz é detectada e transformada em dado de sequência**

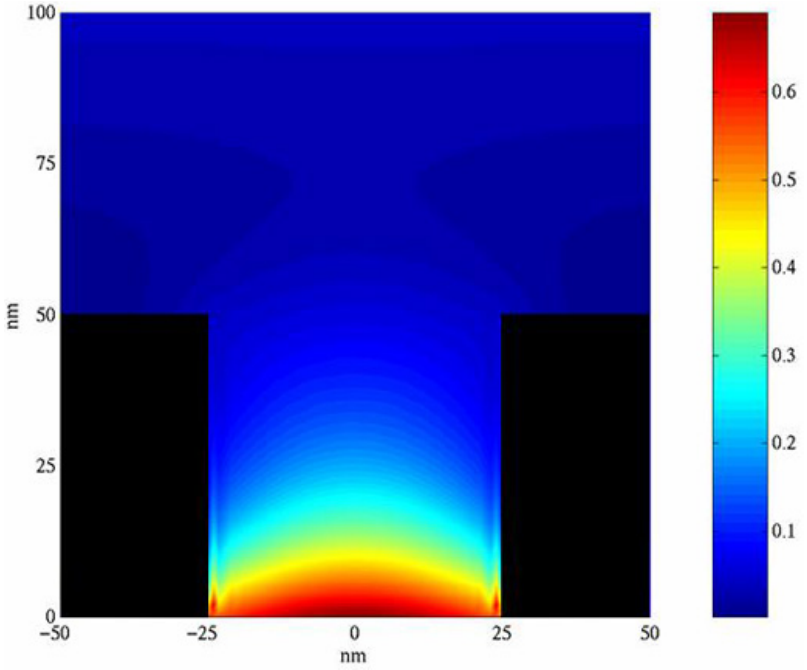


20.000 X mais rápido que o método de Sanger



Placa com milhares de cilindros em nano-escala

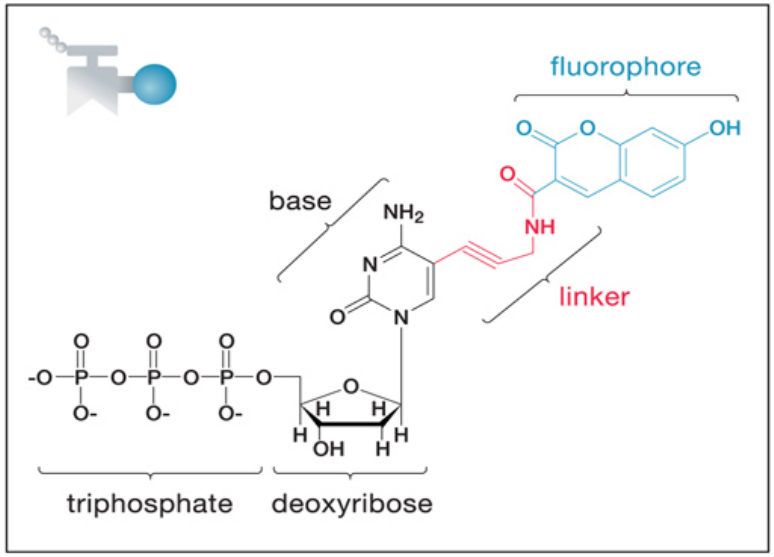




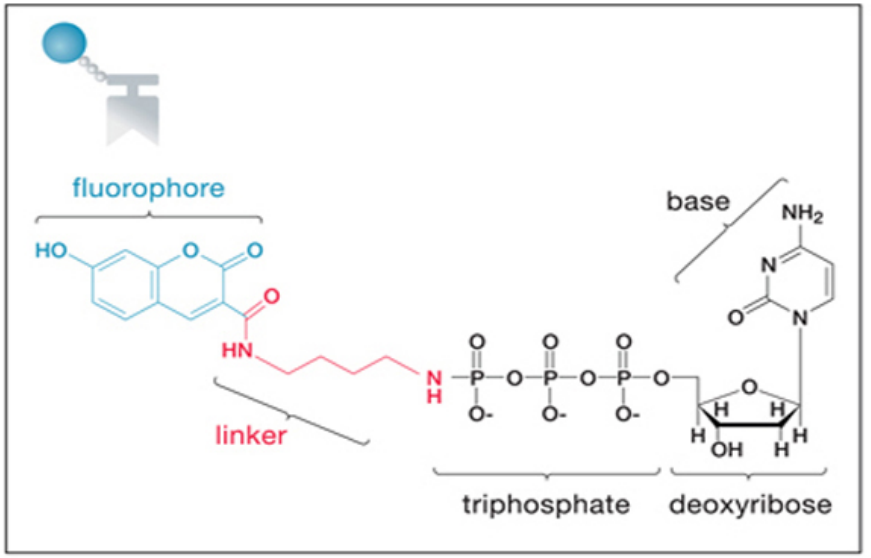
Sinal fluorescente é detectado de uma janela com interferência mínima de luz

https://www.youtube.com/watch?v=v8p4ph2MAvI

Fluoróforos diferenciados

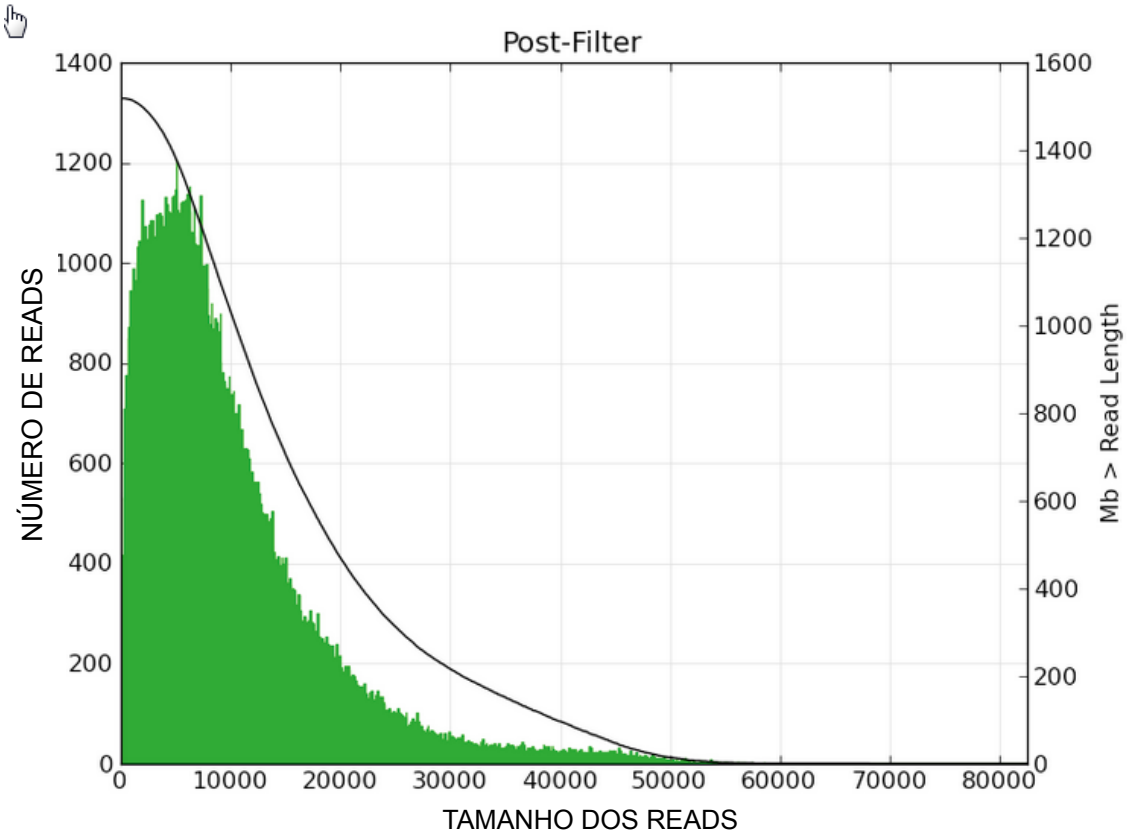


Fluoróforo traditional ligado a base nitrogenada



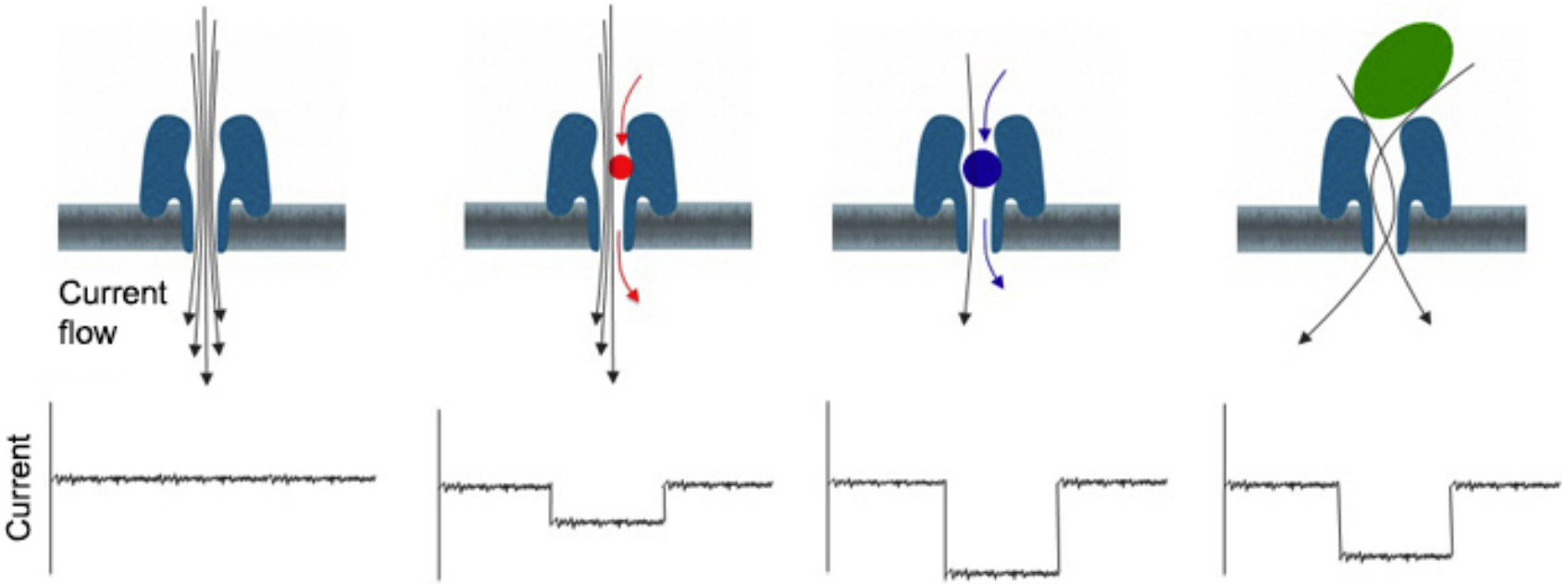
Fluoróforo Fosfo-ligado (SMRT)

Sequenciamento SMRT



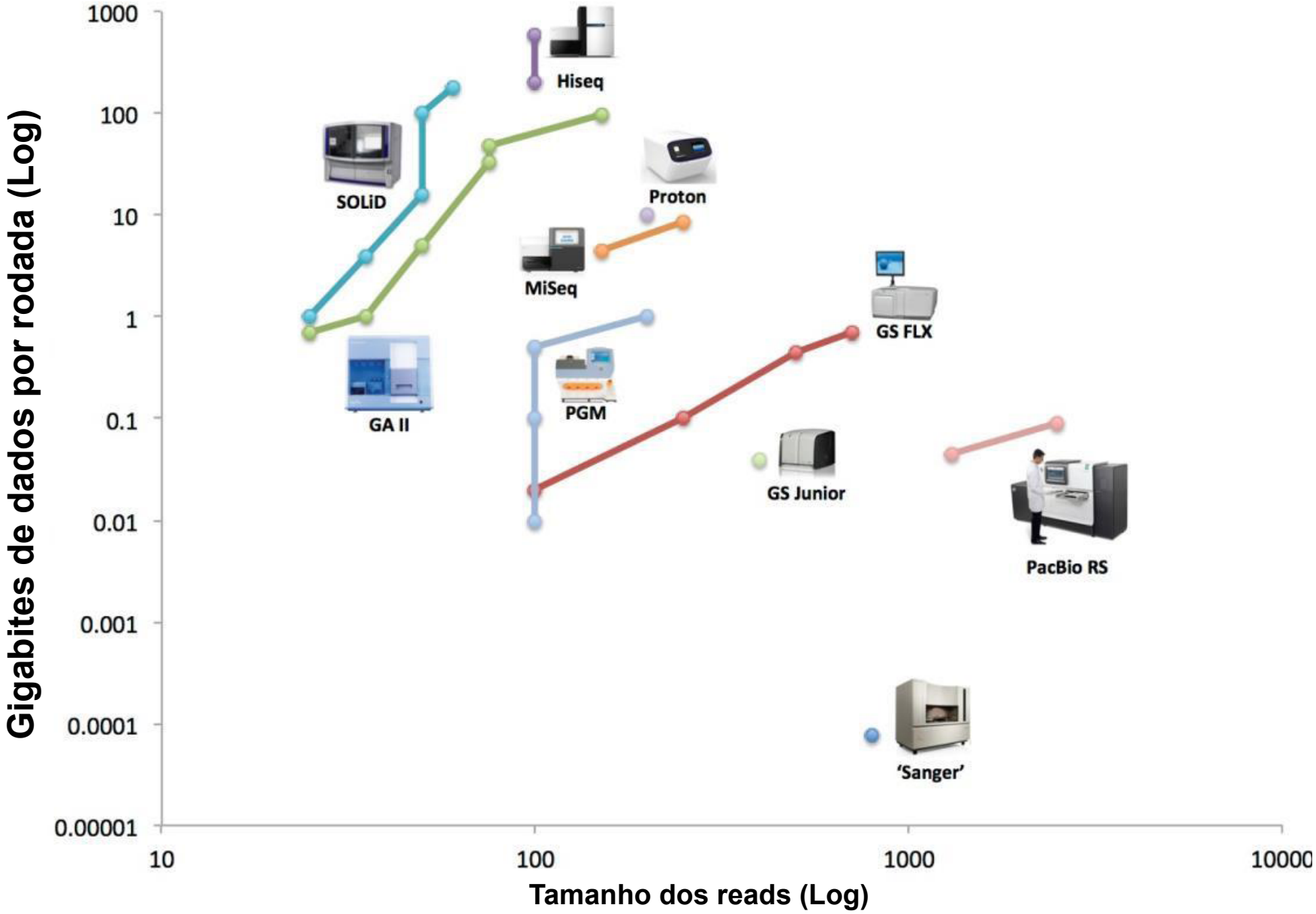
- **Sequencia longas moléculas de DNA (MÉDIA >10,000 pb)**
- **Até 10 bases por segundo**
- **Limitado pela velocidade de detecção, não pela polimerase.**

Oxford Nanopore Technologie - Minion

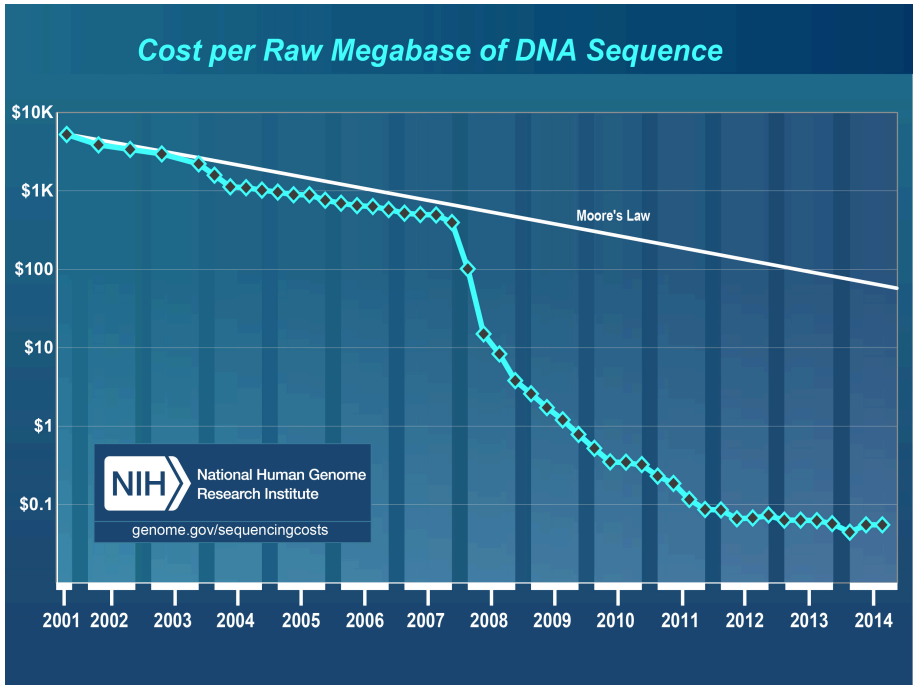


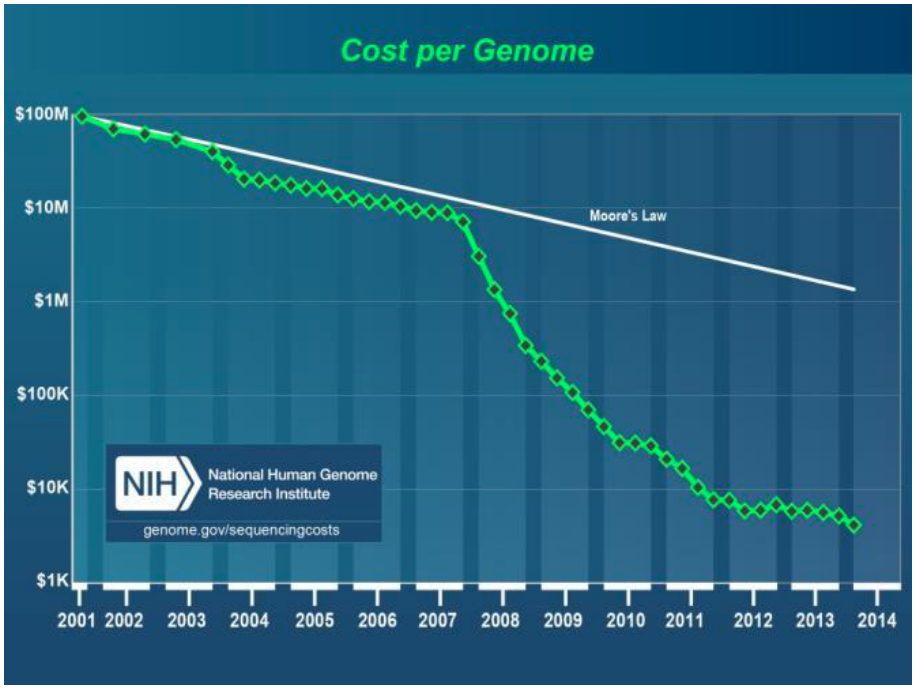
https://www.youtube.com/watch?v=3UHw22hBpAk

Evolução do sequenciamento



Custo de sequenciamento





Genomas sequenciados / Em sequenciamento

Genomes Online Database