- Recombinant DNA cloning technology
- Applications of recombinant DNA technology
- Genomic analysis

DNA sequencing

1, Maxam-Gilbert sequencing

2, Sanger-Coulson sequencing

3, Next Generation Sequencing

Maxam-Gilbert sequencing (chemical cleavage method using double-stranded (ds) DNA)

1. Double-stranded DNA to be sequenced is labelled by attaching a radioactive phosphorus (32P) group to the 5' end.

2. Using dimethyl sulphoxide and heating to 90^{o}C, the two strands of the DNA are separated and purified

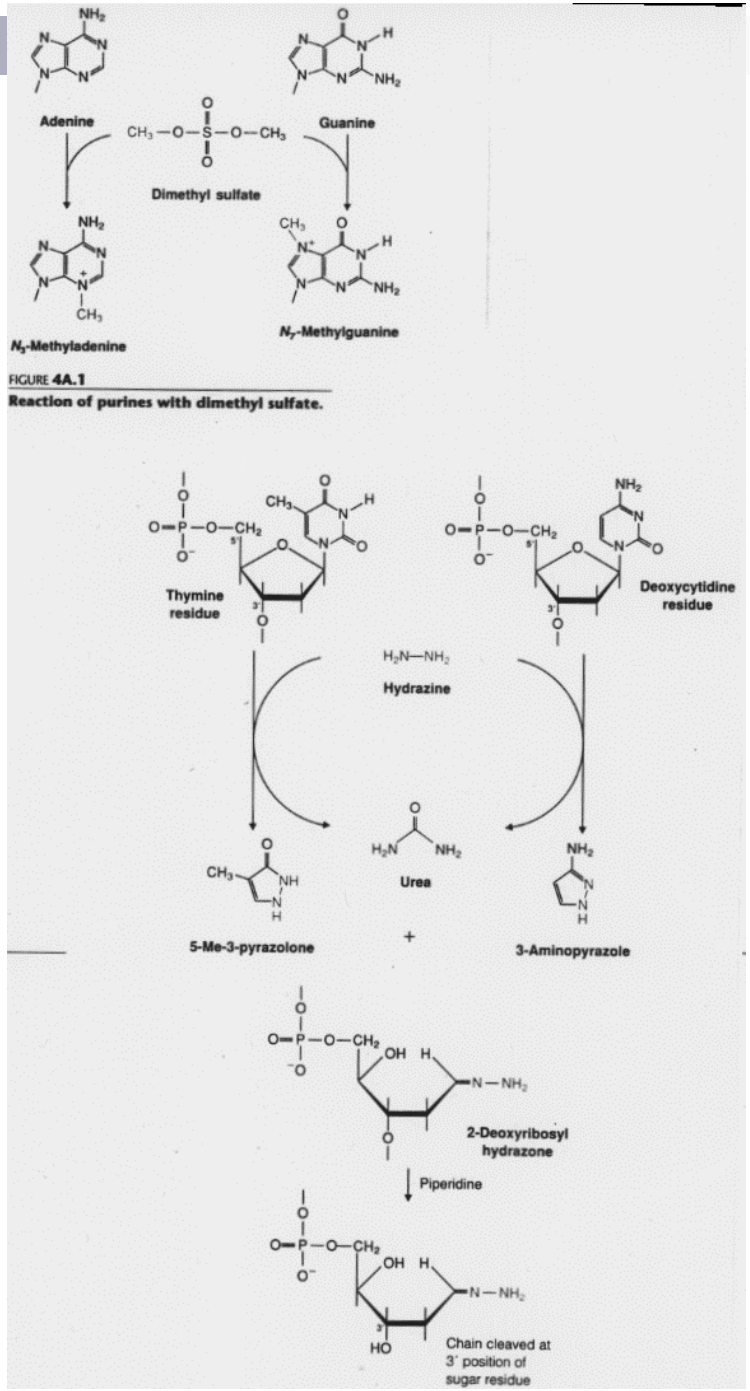
3. Single-stranded sample is split into separate samples and each is treated with one of the cleavage reagents.

4. If reactions have been arranged to give only one, or a few, cleavages per DNA molecule, a nested set of end-labelled DNA fragments of different lengths is produced.

5. The samples are run together on a sequencing gel which separates the fragments by electrophoresis depending on their size. DNA bands in the gel are visualized by autoradiography

6. The DNA sequence is read directly from the gel

The Maxam-Gilbert method of nucleotide sequence determination is based on preferential, base-specific methylation followed by chemical cleavage to generate a nested set of end- labeled derivatives.



Maxam-Gilbert sequencing

Base specificity	Chemical used for base alteration	Chemical used for altered base removal	Chemical used for strand cleavage
G	Dimethylsulphate	Piperidine	Piperidine
A+G	Acid	Acid	Piperidine
C+T	Hydrazine	Piperidine	Piperidine
C	Hydrazine + alkali	Piperidine	Piperidine
A>C	Alkali	Piperidine	Piperidine

Maxam-Gilbert sequencing

CHEMICAL CLEAVAGE OF A DNA SAMPLE AT C BASES

End-labelled DNA sample

^{32}P-A-p-T-p-T-p-G-p-C-p-G-p-C-p-T-p-G-p-C-p-A-p-C-p-G-p-C-p-T

^{32}P

^{32}P

^{32}P

^{32}P

^{32}P

End-labelled DNA fragments

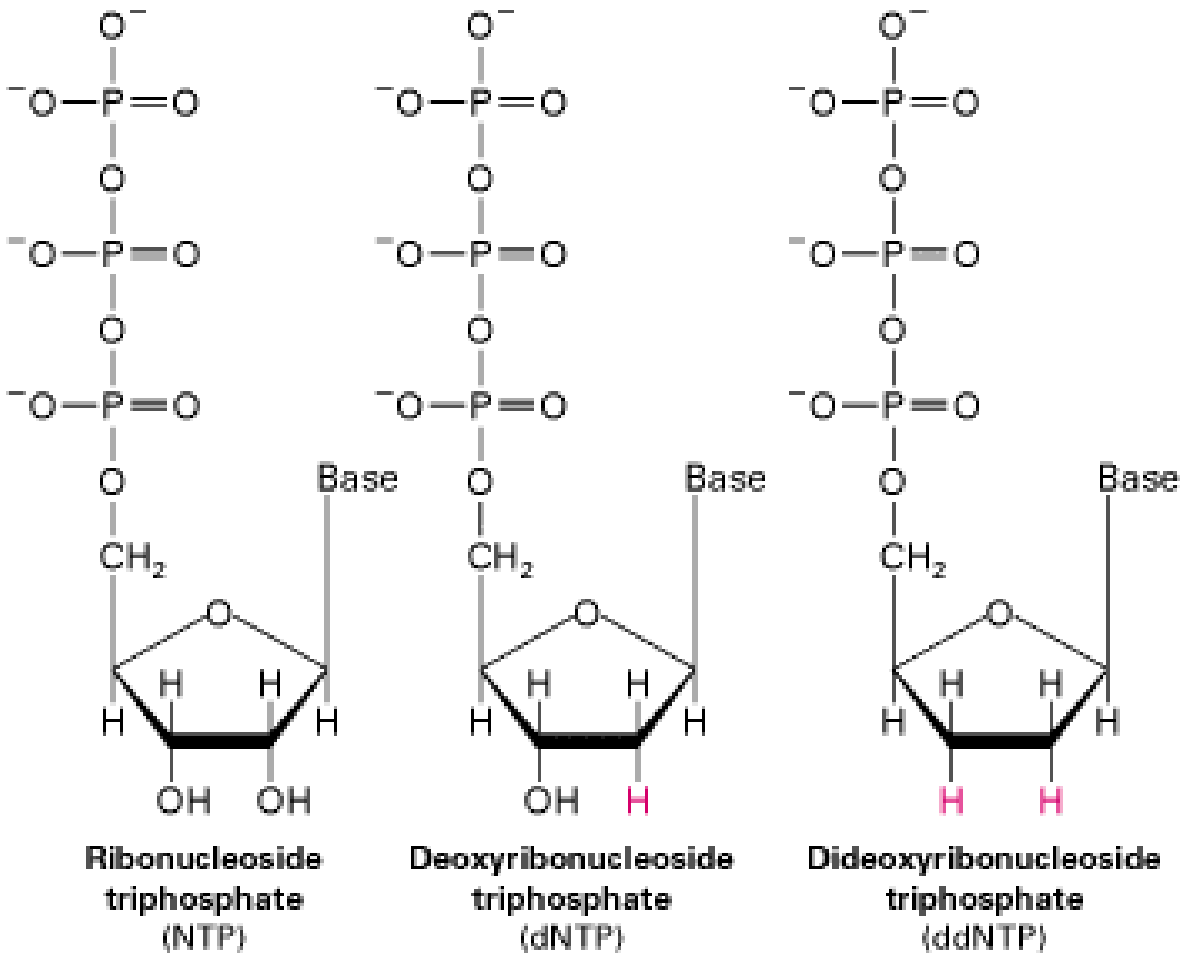
AUTORADIOGRAM OF SAMPLE
MAXAM-GILBERT SEQUENCING GEL

G	A+G	C+T	C	SEQUENCE (END)
		—	—	C (3')
—	—			G
	—			A
		—		T
		—		T
		—		T
		—	—	C
—	—			G
—	—			G
	—			A
		—		T
		—	—	C
	—			A
	—			A (5')

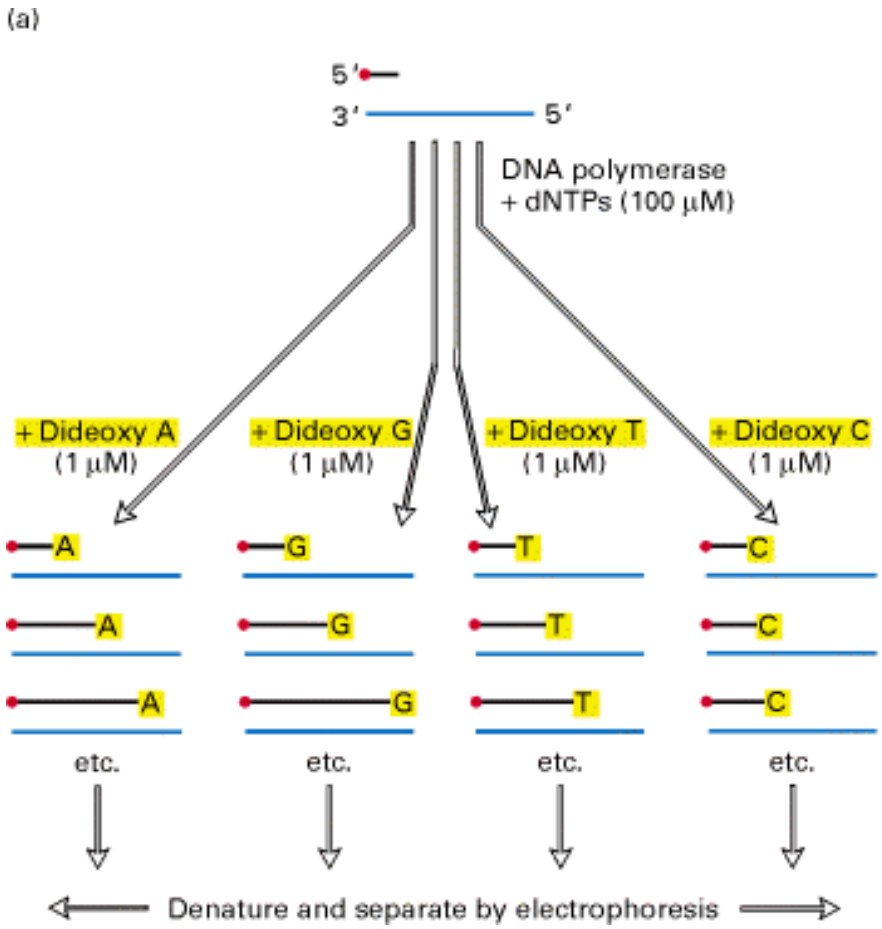
Sanger-Coulson sequencing (chain termination method using single-stranded (ss) DNA)

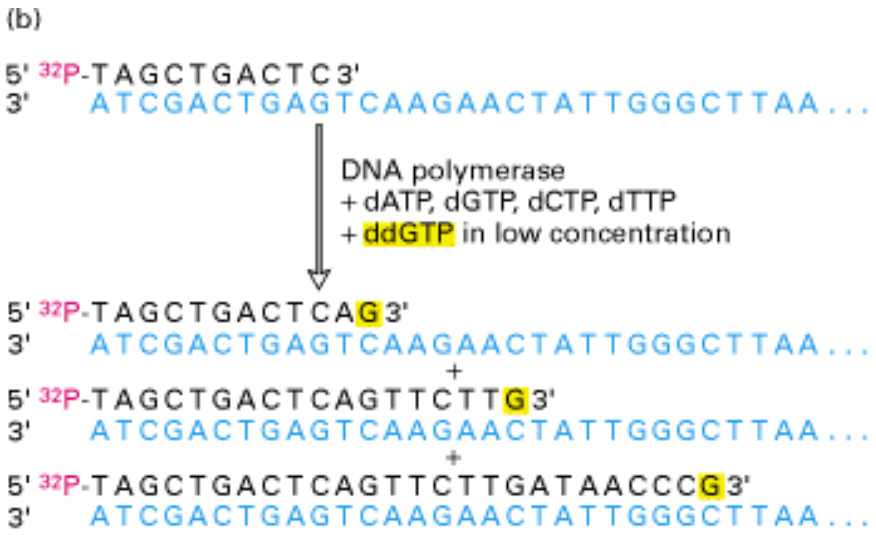
1. Sample DNA to be sequenced is clonded into M13 vector DNA to generate ssDNA.
2. A short oligonucleotide primer (usually chemically synthesized and sometimes labelled) is added to the ss recombinant DNA.
3. DNA polymerase is then added in the presence of: 4 normal nucleotides: d-ATP, d-CTP, d-GTP and d-TTP (one or more of which are labelled with **32**P) and a low concentration of 4 analogues of the normal nucleotides in separate incubation mixes.
4. Complementary strand synthesis occurs away from the primer.
5. Each of the 4 mixes is run together on a sequencing gel which separates the fragments by electrophoresis depending on their size. DNA bands in the gel are visualized by autoradiography.
6. The DNA sequence is read directly from the gel in a similar way to a Maxam-Gilbert sequencing gel.

Structures of ribonucleoside triphosphate (NTP), deoxyribonucleoside triphosphate (dNTP), and dideoxyribonucleoside triphosphate (ddNTP)



Sanger-Coulson sequencing

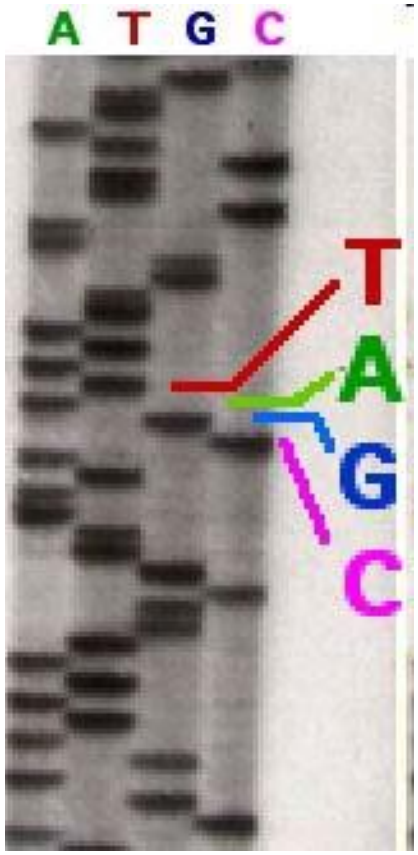




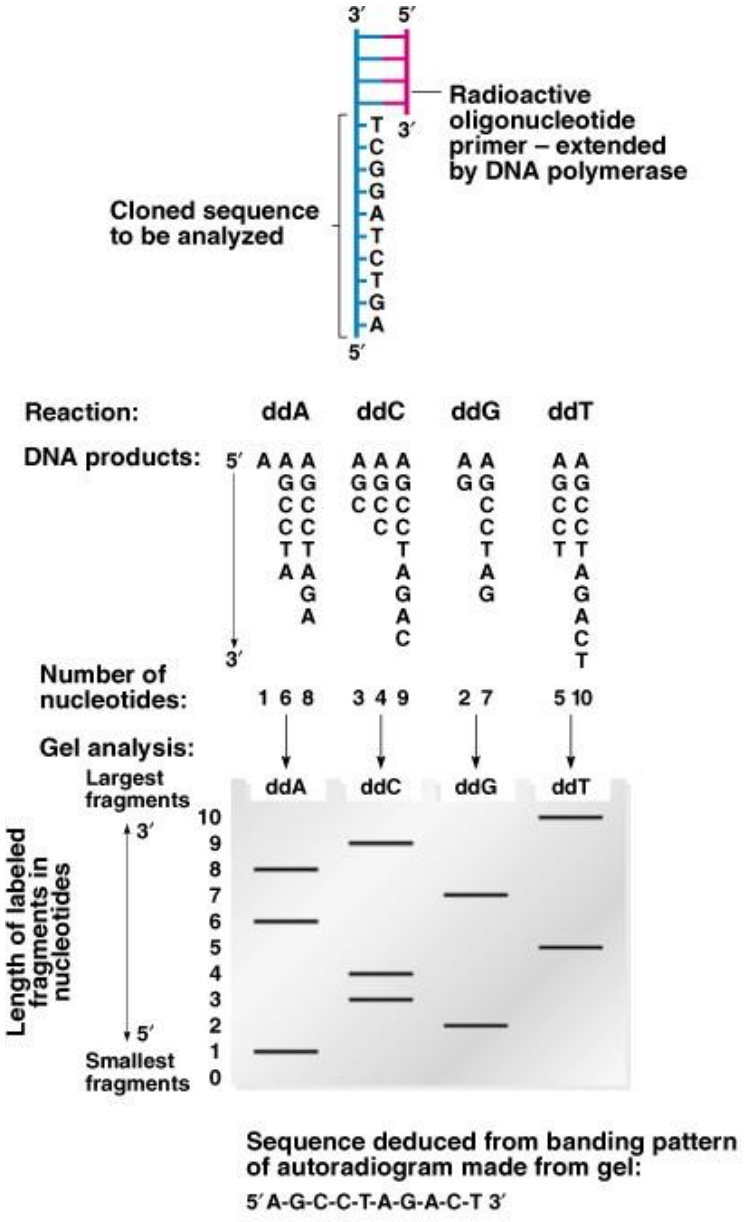
Sanger-Coulson sequencing

AUTORADIOGRAM OF SAMPLE
SANGER-COULSON SEQUENCING GEL

A	C	G	T	SEQUENCE (END)
			—	T (3')
	—			C
	—			C
		—		G
		—		G
			—	T
		—		G
—				A
	—			C
	—			C
		—		G
			—	T
	—			C
—				A (5')



Dideoxy DNA sequencing of a theoretical DNA fragment



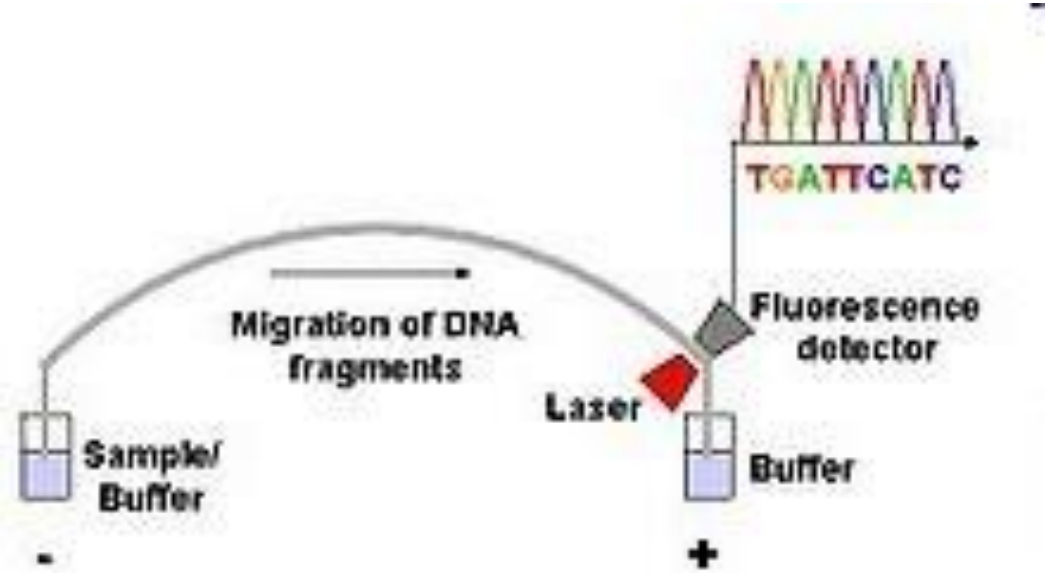
Dideoxy Sequencing of DNA

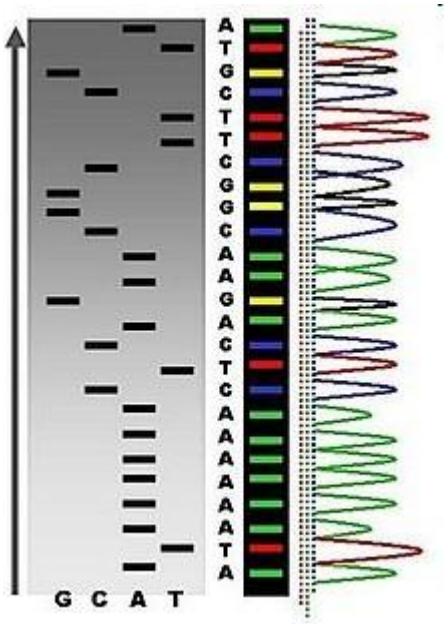
Movie

AUTOMATED DNA SEQUENCING

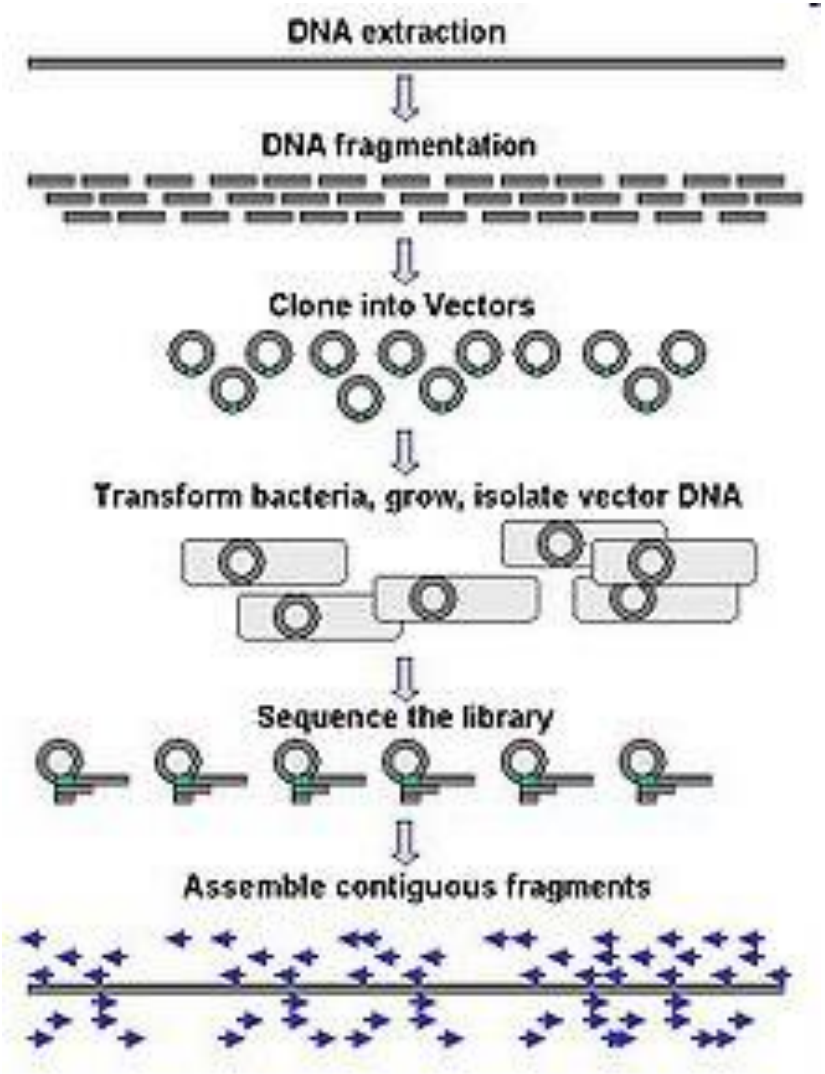
It is based on the Sanger-Coulson chain termination method but the 4 different dideoxy nucleotides (ddA, ddC, ddG and ddT) are **fluorescently** labelled.

4 different fluorophores are used, all 4 reactions can be run in the same tube.

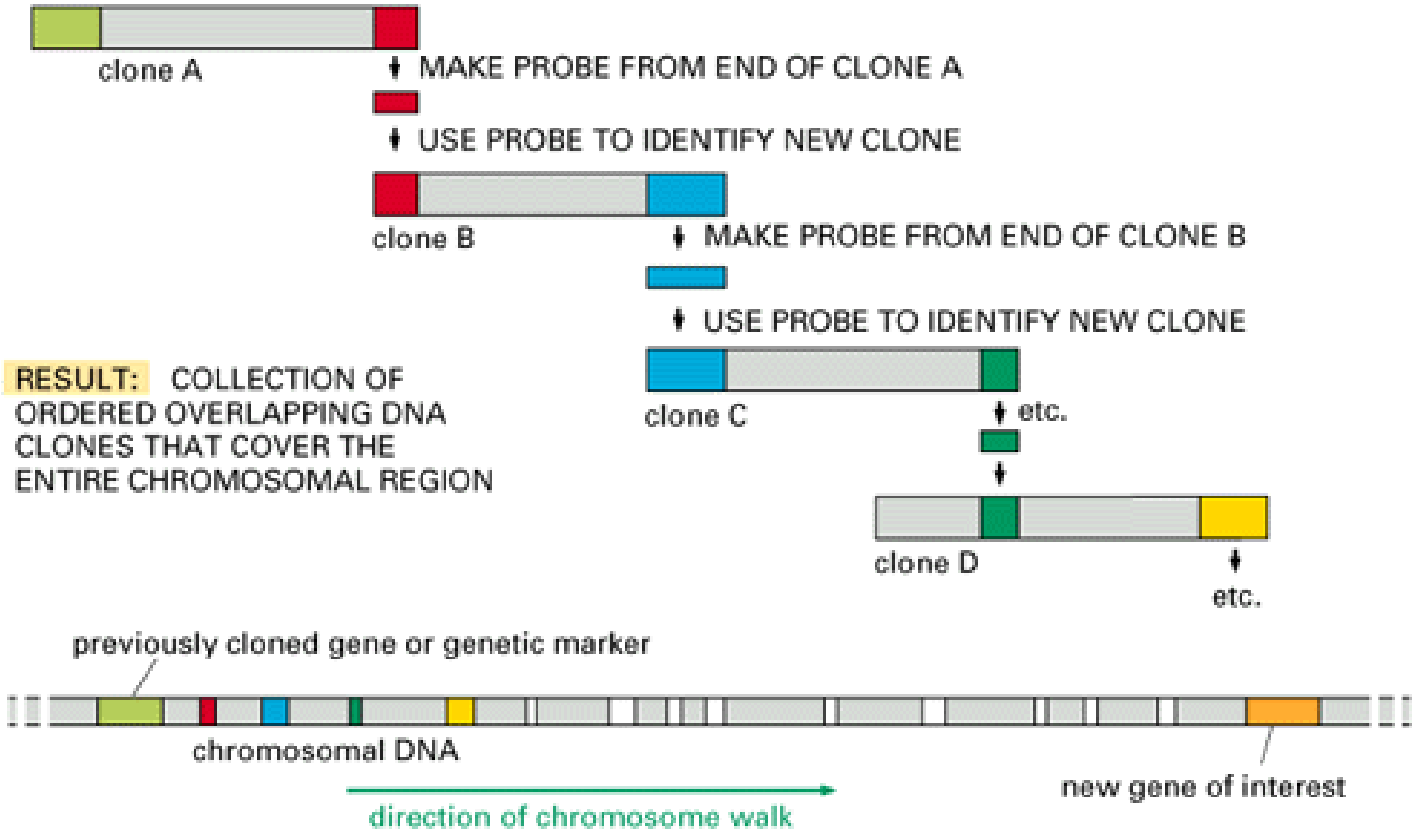




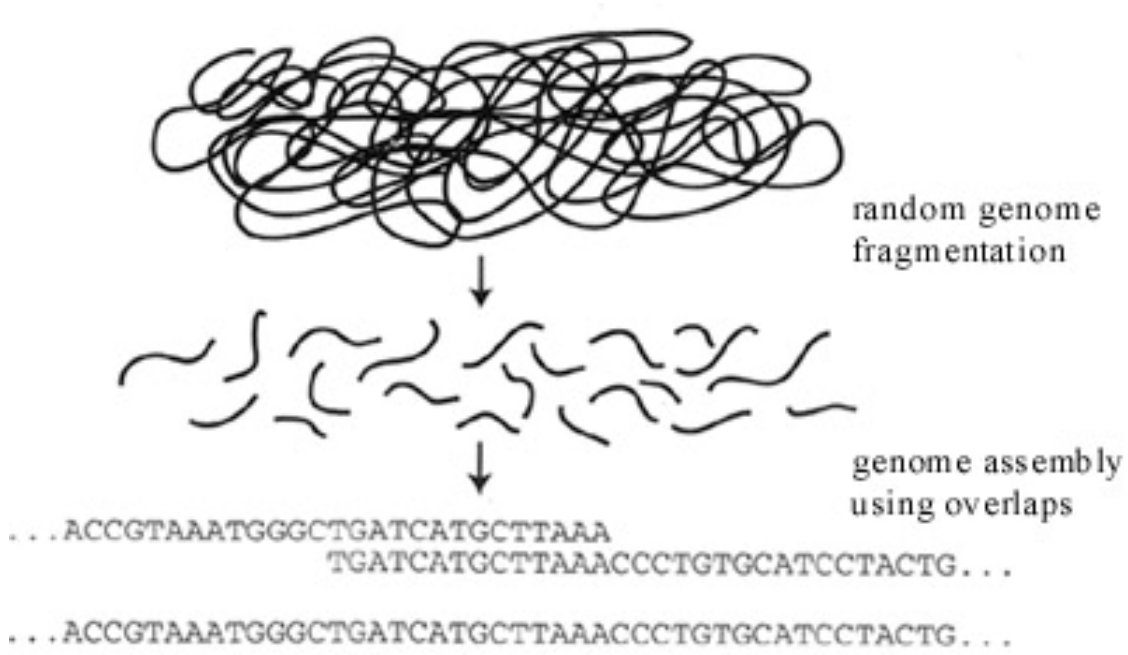
Genome Sequencing



Chromosome Walking



Genome **shotgun** sequencing



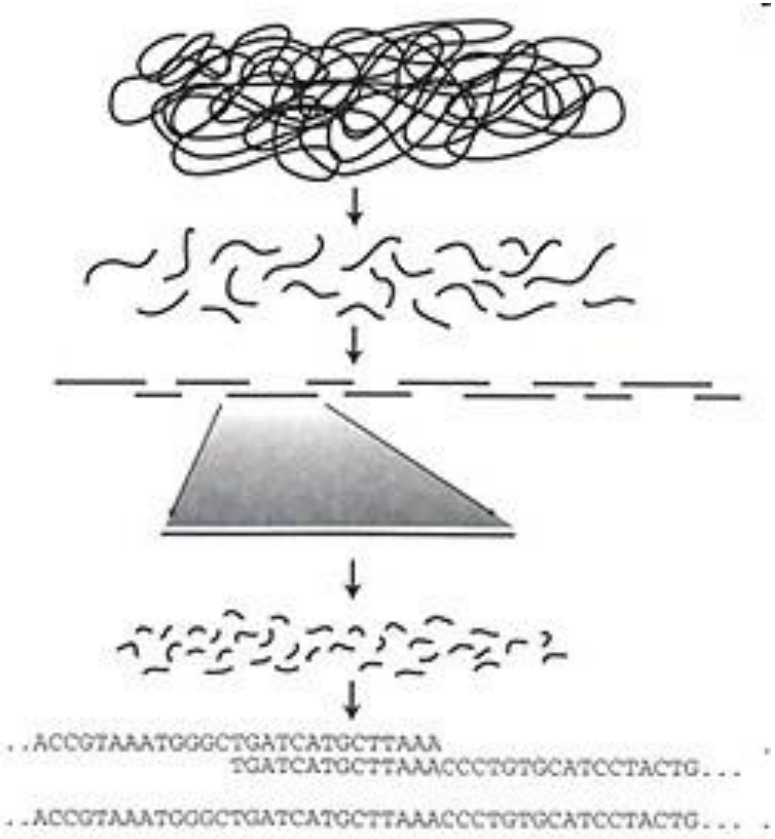
Strand	**Sequence**
Original	AGCATGCTGCAGTCATGCTTAGGCTA
First shotgun sequence	AGCATGCTGCAGTCATGCT------- -------------------TAGGCTA
Second shotgun sequence	AGCATG-------------------- ------CTGCAGTCATGCTTAGGCTA
Reconstruction	AGCATGCTGCAGTCATGCTTAGGCTA

Genome **shotgun** sequencing

HUMAN GENOME PROJECT (HGP) 1990

Hierarchical Shotgun

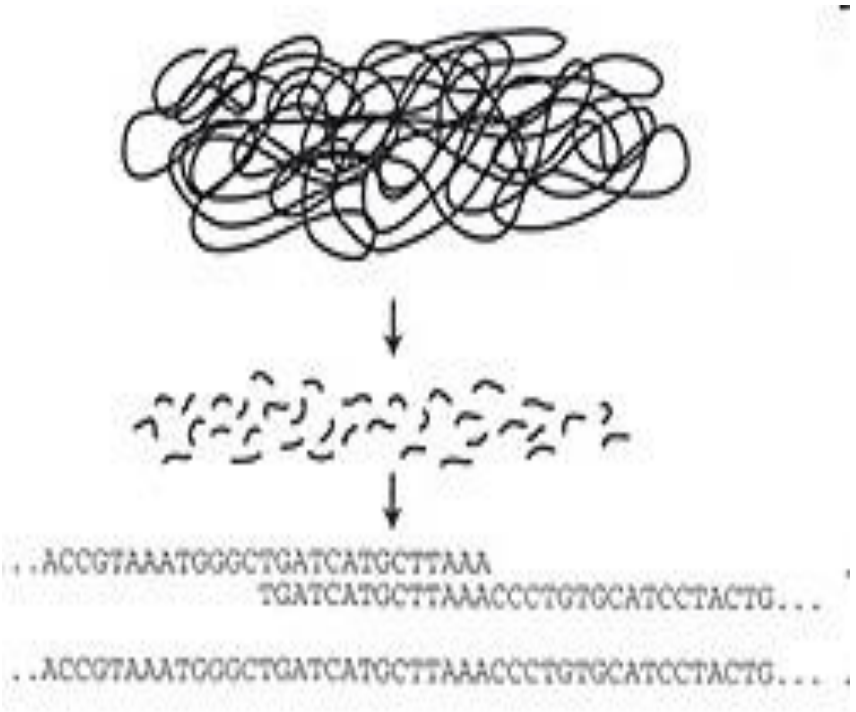
("map-based", "BAC-based", "clone-by-clone")



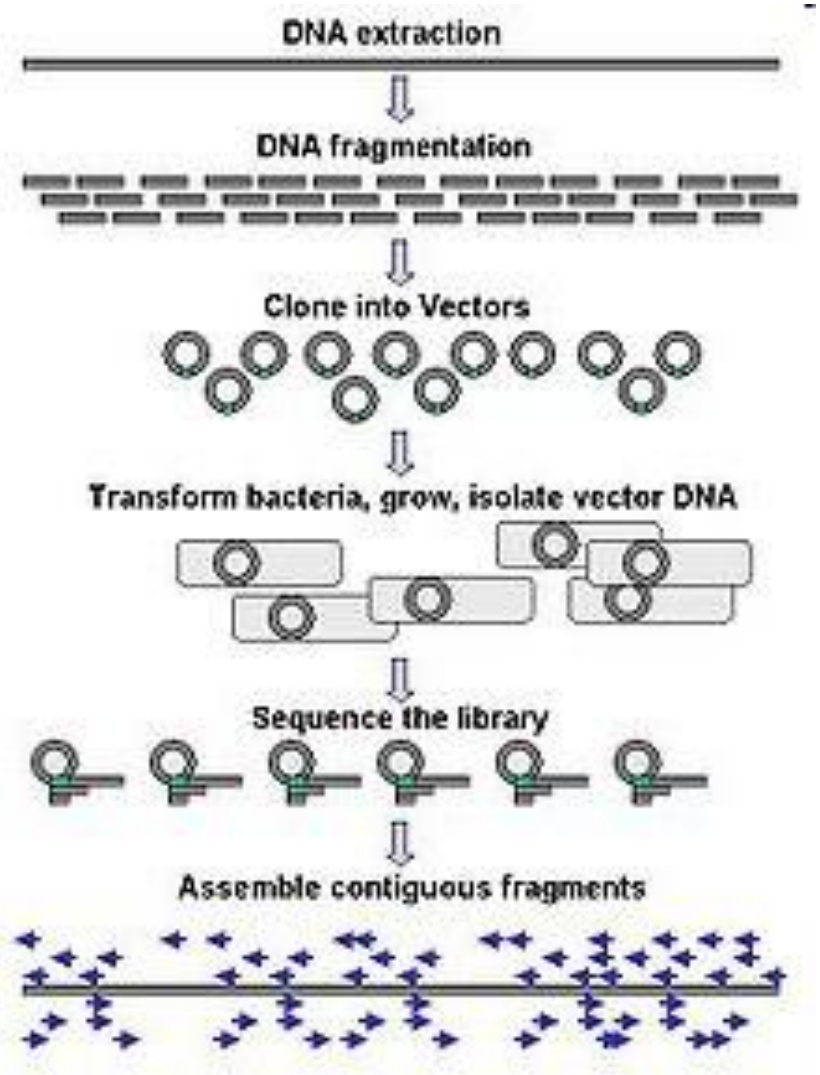
CELERA GENOMICS

1998

Whole Genome Shotgun



Next Generation Sequencing



1. Roche 454 pyrosequencing
2. Illumina (Solexa) sequencing
3. SOLiD sequencing

Roche 454 Sequencing,
based on sequencing-by-synthesis

Nucleotides are flowed sequentially in a fixed order across the PicoTiterPlate device during a sequencing run.

During the nucleotide flow, hundreds of thousands of beads each carrying millions of copies of a unique single-stranded DNA molecule are sequenced in parallel.

If a nucleotide complementary to the template strand is flowed into a well, the polymerase extends the existing DNA strand by adding nucelotide(s).

Addition of one (or more) nucleotide(s) results in a reaction that generates a light signal that is recorded by the CCD camera in the instrument.

The signal strength is proportional to the number of nucleotides incorporated in a single nucelotide flow.

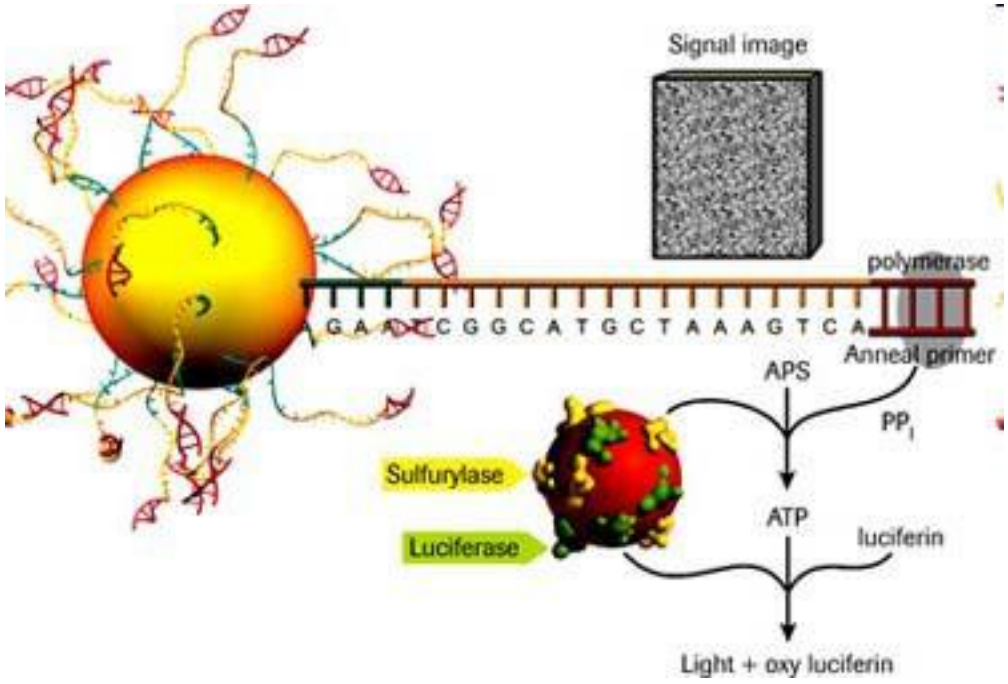
ssDNA template is hybridized to a sequencing primer and incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase, and with the substrates adenosine 5´ phosphosulfate (APS) and luciferin.

Addition of one of the four dNTPs, DNA polymerase incorporates the correct, complementary dNTPs onto the template. This incorporation releases pyrophosphate (PPi) stoichiometrically.

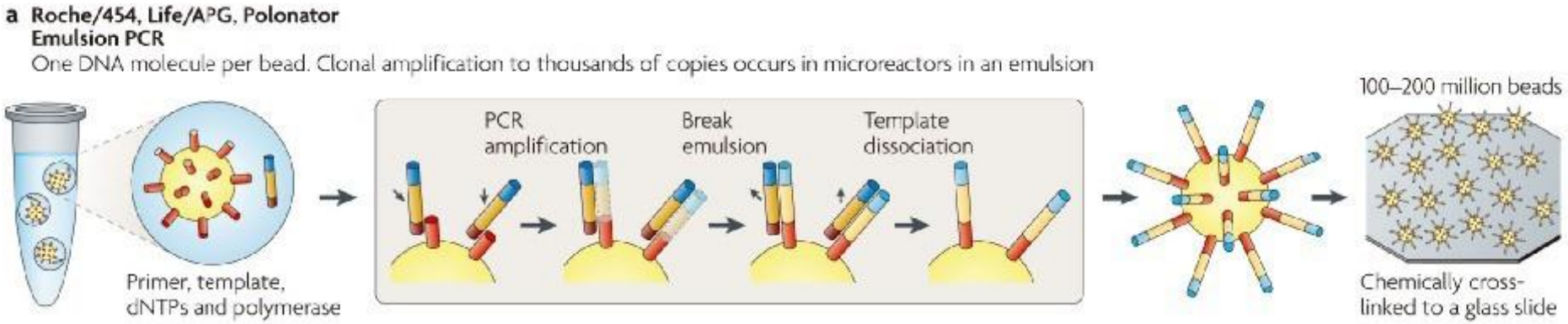
ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5´ phosphosulfate.

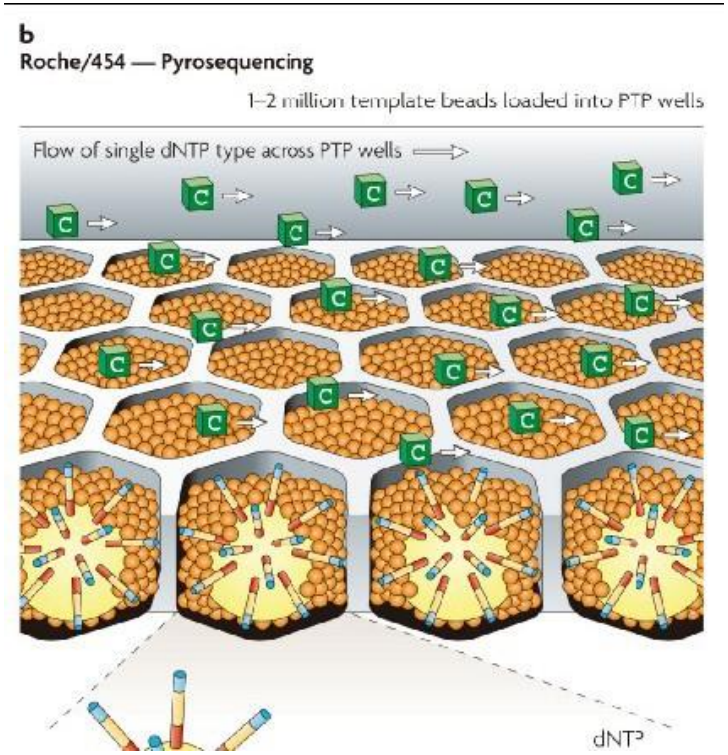
This ATP acts as fuel to the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a camera.

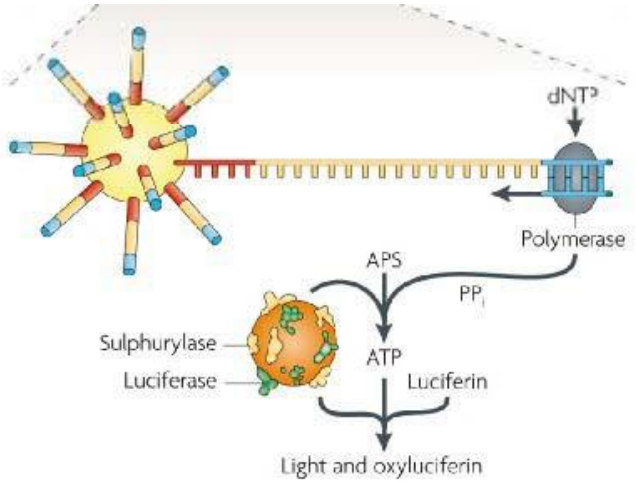
Unincorporated nucleotides and ATP are degraded by the apyrase, and the reaction can restart with another nucleotide.

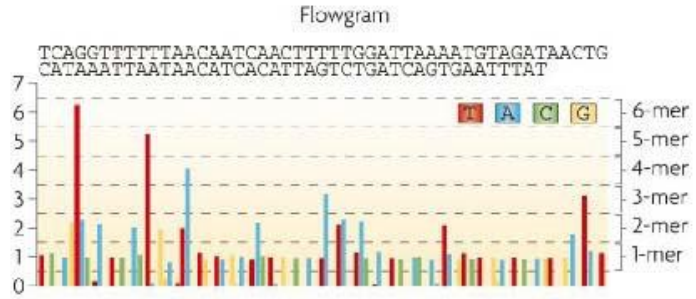


Roche 454 Sequencing





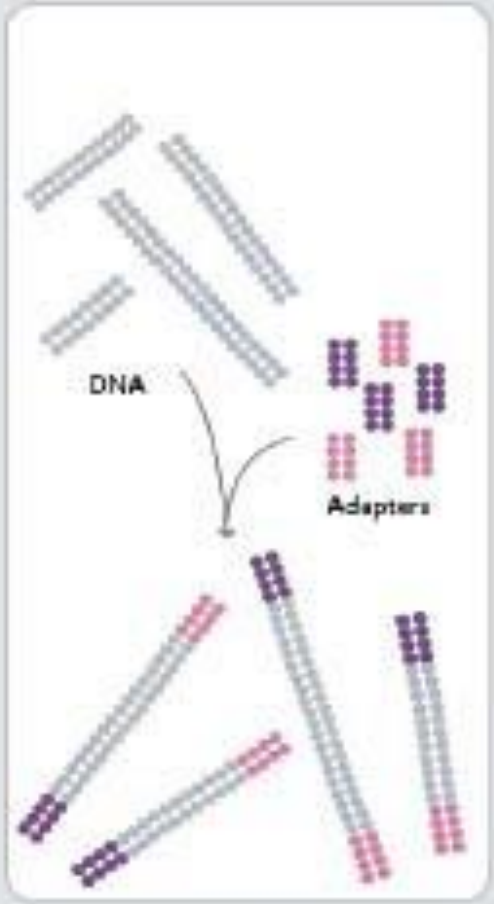




Illumina Solexa

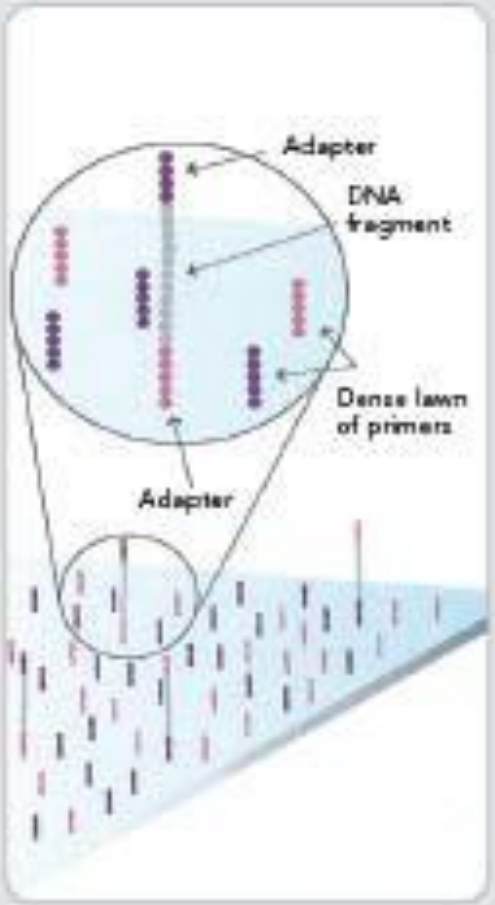
Illumina Solexa

1. PREPARE GENOMIC DNA SAMPLE



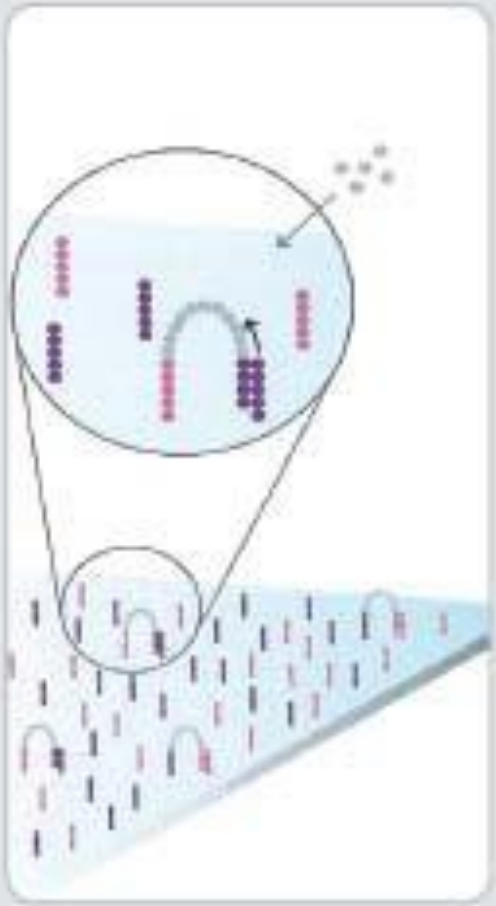
Randomly fragment genomic DNA and ligate adapters to both ends of the fragments.

2. ATTACH DNA TO SURFACE



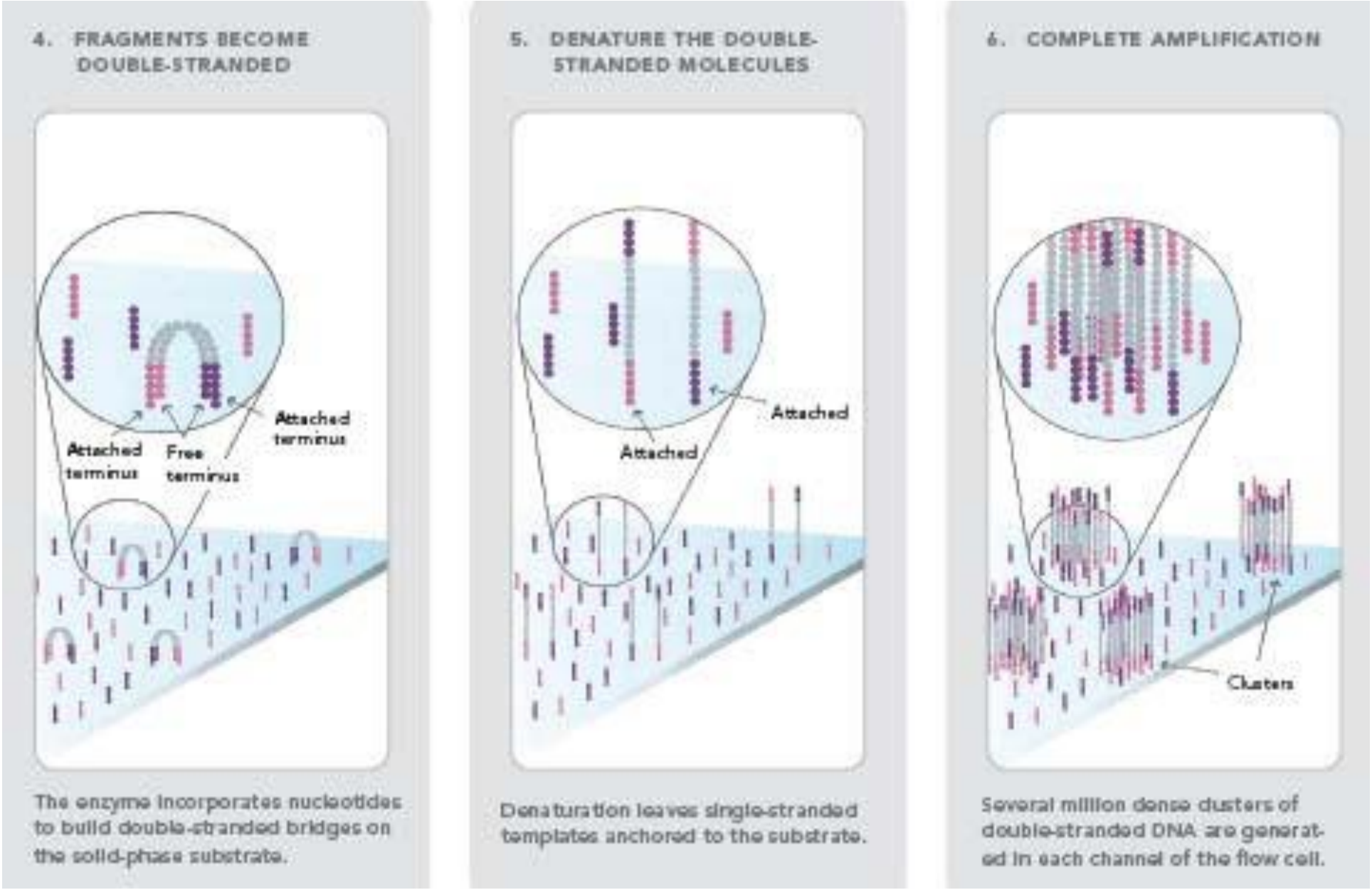
Bind single-stranded fragments randomly to the inside surface of the flow cell channels.

3. BRIDGE AMPLIFICATION



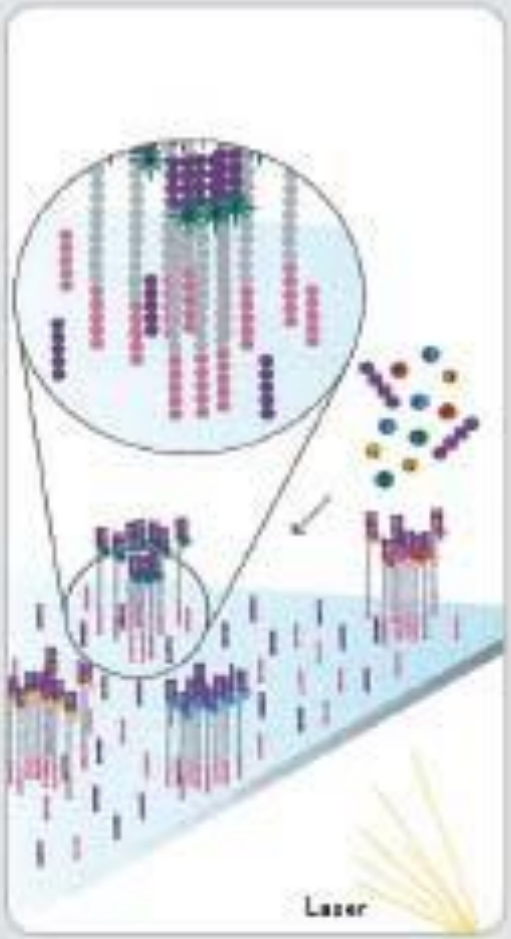
Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.

Illumina Solexa



Illumina Solexa

7. DETERMINE FIRST BASE

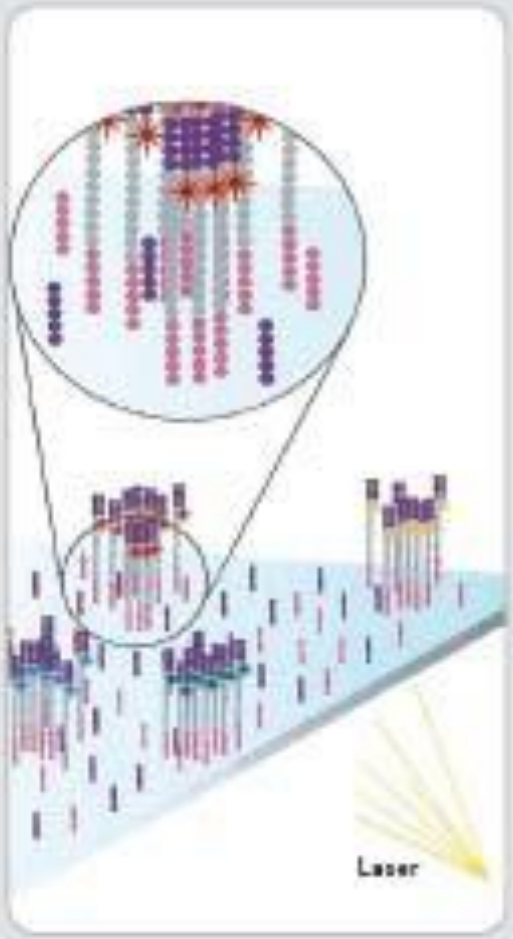


The first sequencing cycle begins by adding four labeled reversible terminators, primers, and DNA polymerase.

8. IMAGE FIRST BASE

After laser excitation, the emitted fluorescence from each cluster is captured and the first base is identified.

9. DETERMINE SECOND BASE



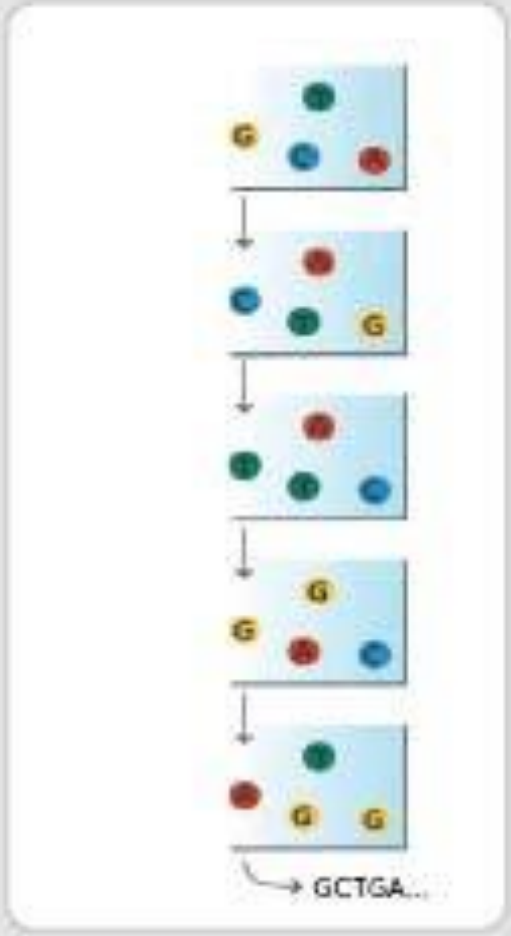
The next cycle repeats the incorporation of four labeled reversible terminators, primers, and DNA polymerase.

Illumina Solexa

10. IMAGE SECOND CHEMISTRY CYCLE

After laser excitation, the image is captured as before, and the identity of the second base is recorded.

11. SEQUENCING OVER MULTIPLE CHEMISTRY CYCLES

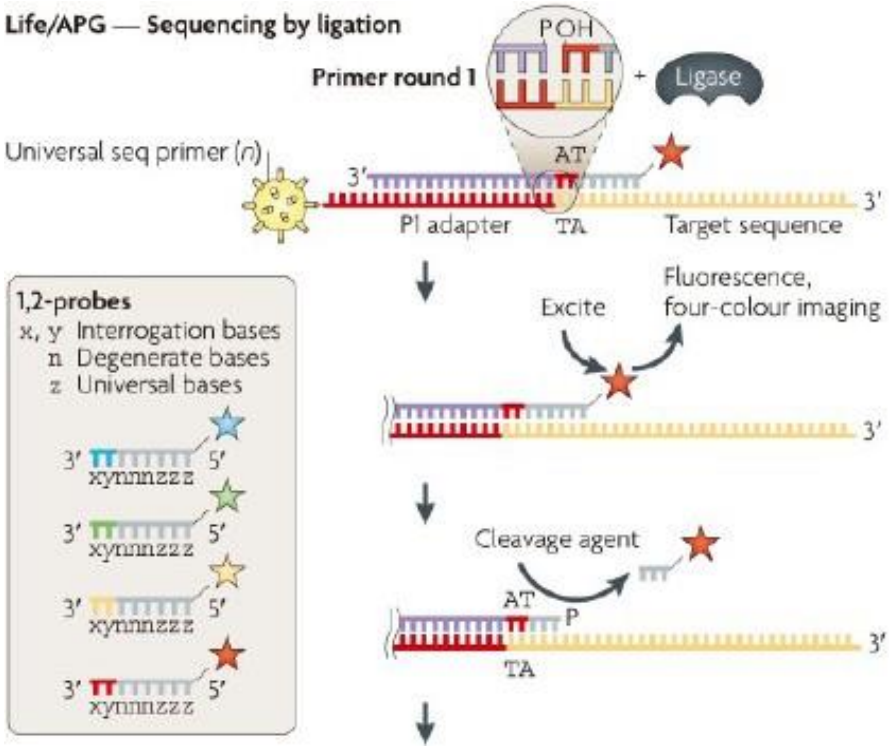


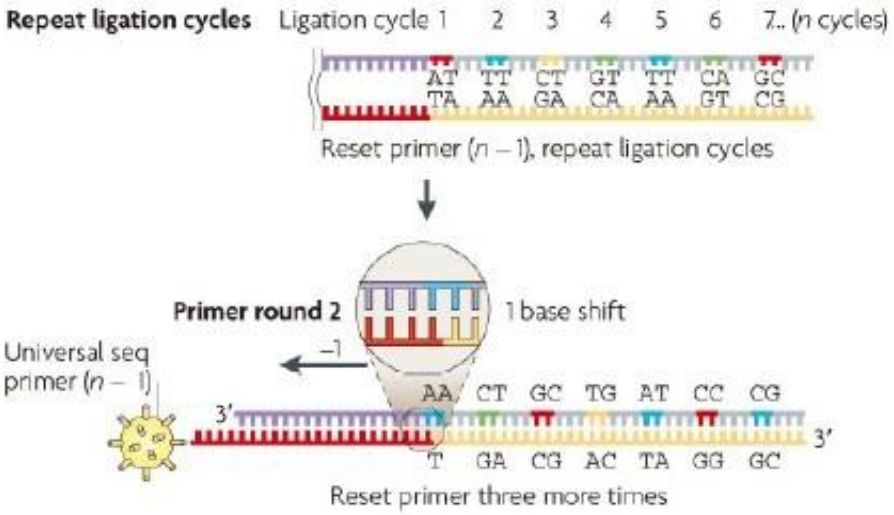
The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time.

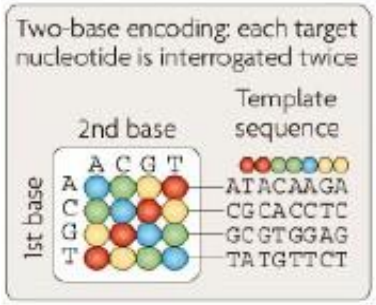
12. ALIGN DATA

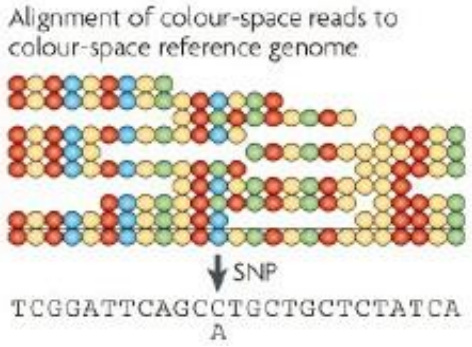
The data are aligned and compared to a reference, and sequencing differences are identified.

Life/APG's SOLiD







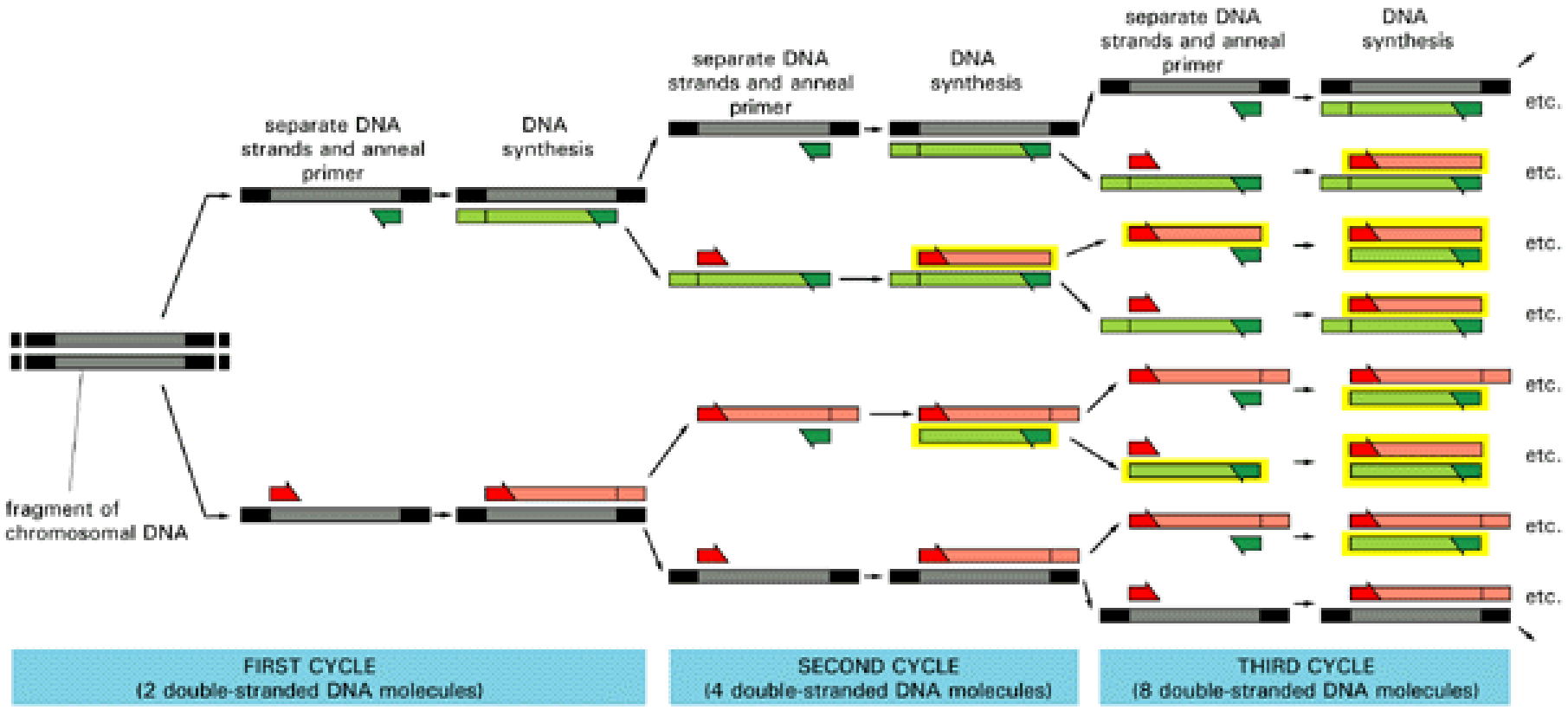


Third Generation Sequencing

DNA sequencing methods currently under development include labeling the DNA polymerase, reading the sequence as a DNA strand transits through nanopores, and microscopy-based techniques, such as AFM or transmission electron microscopy that are used to identify the positions of individual nucleotides within long DNA fragments (>5,000 bp) by nucleotide labeling with heavier elements (e.g., halogens) for visual detection and recording.

Polymerase Chain Reaction (PCR)

polymerase chain reaction (PCR)



polymerase chain reaction (PCR)

movie

polymerase chain reaction (PCR)

Diagnostic Applications of PCR

- detecting pathogens using genome-specific primer pairs
- screening specific genes for unknown mutations
- genotyping using known STS markers

Subcloning DNA targets using PCR

- T/A Cloning
- Restriction Site Addition
- Blunt-end Ligation

PCR-mediated in vitro mutagenesis

Generation of DNA probes

Amplification of differentially-expressed gene sequences

- Differential display reverse transcriptase PCR (DDRT-PCR)
- Suppression subtraction hybridization (SSH)
- Amplification of cell-specific transcripts using RT-PCR