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MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH
خميس مليانة -جامعة جيلالي بونعامة
UNIVERSITY OF DJILALI BOUNAAMA-KHEMIS MILIANA
كلية علوم الطبيعة والحياه و علوم الأرض
FACULTY OF NATURE AND LIFE SCIENCES AND EARTH SCIENCES
قسم العلوم البيولوجية
DEPARTMENT OF BIOLOGICAL SCIENCES

MOLECULAR BIOLOGY AND GENITIC ENGINEERING MODULE
INTENDED FOR STUDENTS OF THE MICROPBIOLOGY DEGREE

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Introduction:

- According to Michel Morange , molecular biology is "the set of techniques and discoveries which have enabled the molecular analysis of the most intimate processes of living things, those which ensure their sustainability and reproduction."

I. Expression of genetic information

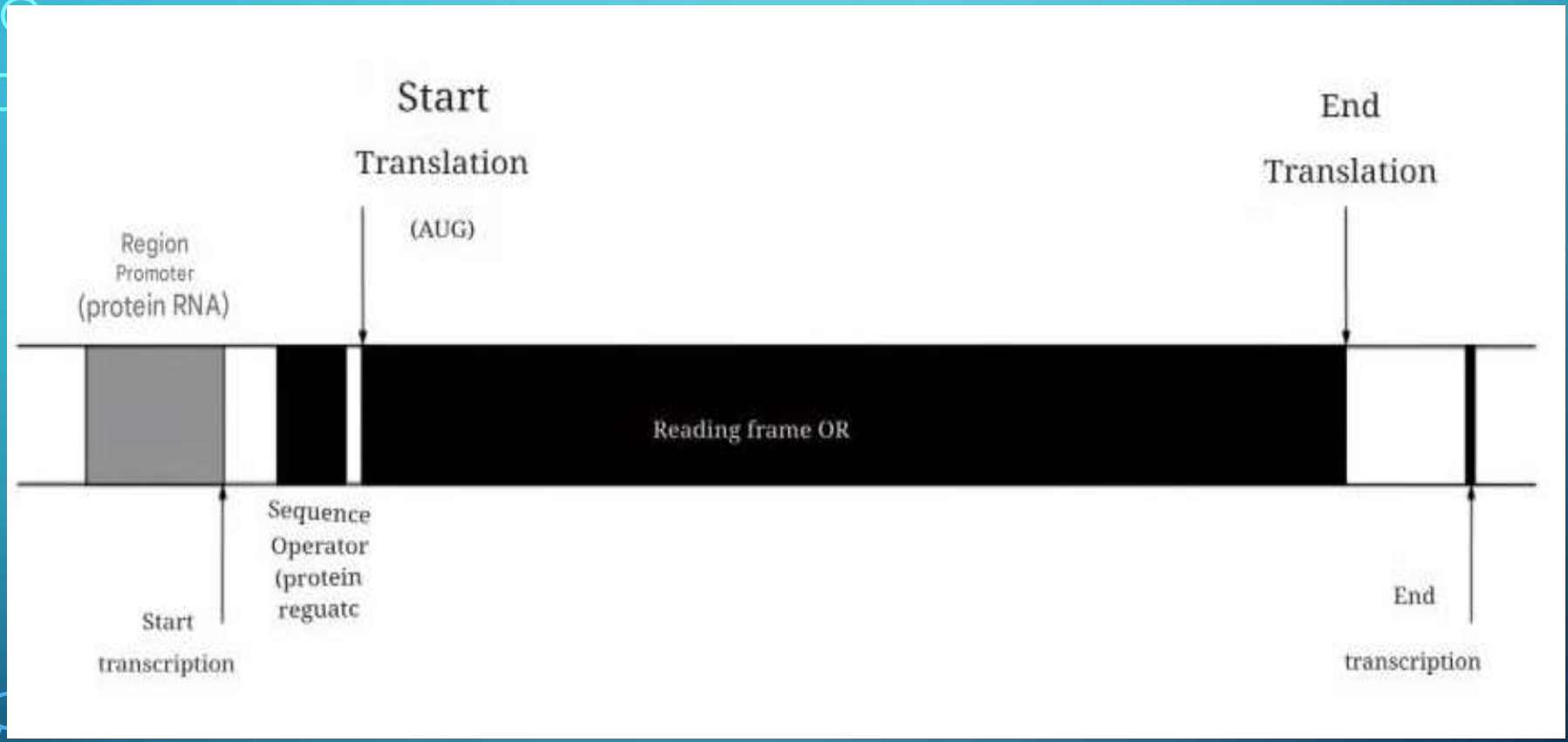
1. Protein synthesis

- Protein synthesis involves two steps: transcription and translation.
- Bacteria, along with archaea, form the group of prokaryotes.
- The genetic material of bacteria is present in the cytoplasm along with plasmids.
- A gene contains in its nucleotide sequence the information allowing the synthesis of a polypeptide, characterized by its amino acid sequence.

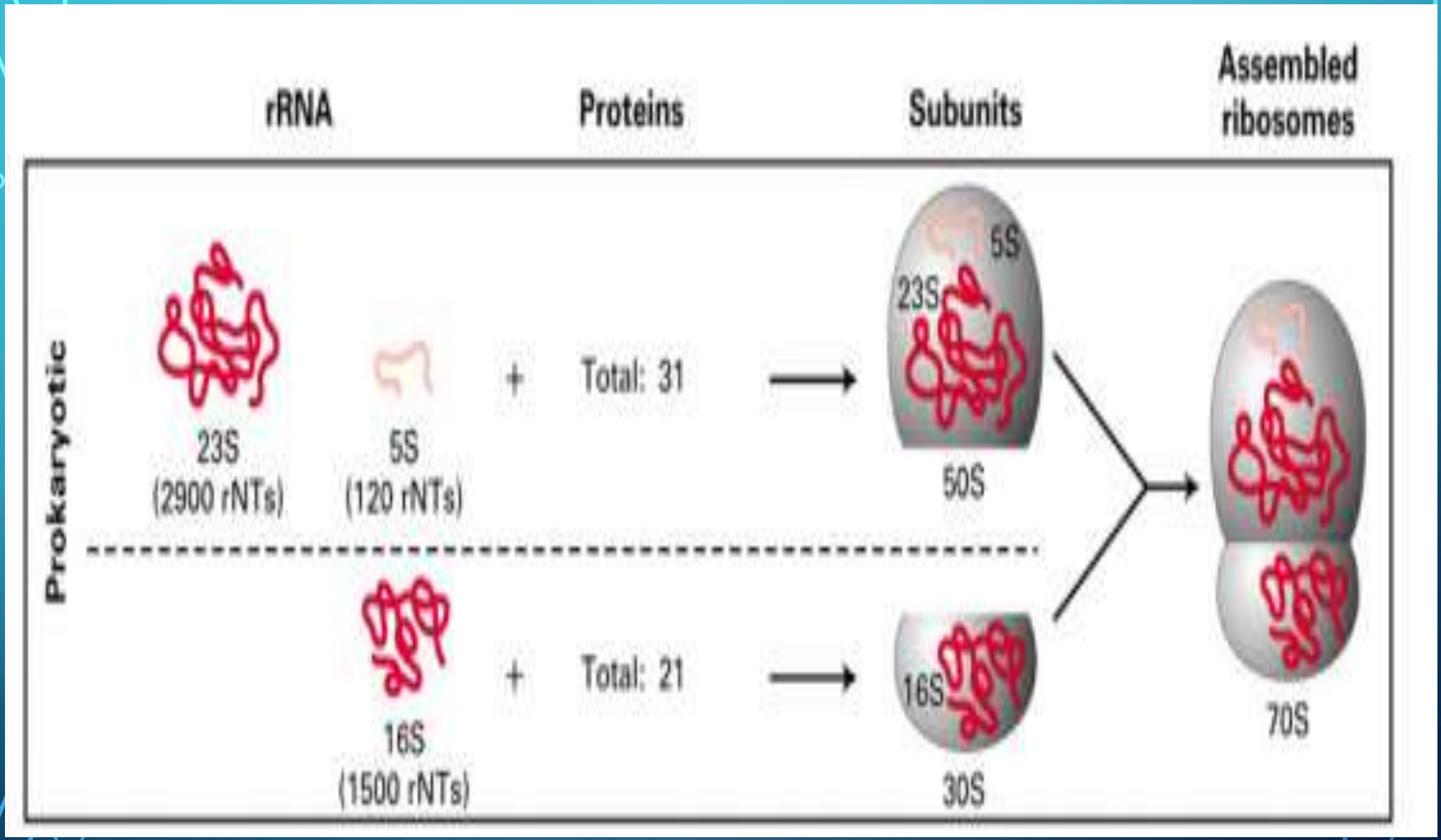
- Bacterial genes do not have introns.
- The different genes coding for proteins of the same cellular mechanism can be grouped together in the form of an operon.
- An operon is a set of genes that are transcribed together, in the form of a single mRNA that carries several ribosome binding sites, which will allow the translation of the different proteins encoded by this mRNA. This is called polycistronic mRNA.

Gene structure:

- Simple structure.
- Start and end of the regions to be transcribed and translated.
- Short sequences of punctuation.
- The regions upstream and downstream of the ORF (Open Reading Frame) are called the 5’ and 3’ untranslated regions (5‘UTR and 3‘UTR), respectively; UTR = UnTranslated Region).


Start
Translation
(AUG)
End
Translation
Region
Promoter
(protein RNA)
Reading frame OR
Sequence
Operator
(protein
reguatc
Start
transcription
End
transcription

- **Transcription:**
  - For each gene, only one strand of DNA is transcribed. RNA synthesis is catalyzed by RNA polymerase (5'->3').
  - >90% of genes possess specific termination signals called intrinsic terminators.
  - 1) inclusion of a palindrome (5’-CGGATG | CATCCG-3’) and
  - 2) six U's following the palindrome.


rRNA
Proteins
Subunits
Assembled ribosomes
Prokaryotic
23S
(2900 rNTs)
5S
(120 rNTs)
+
Total: 31
5S
23S
50S
16S
(1500 rNTs)
+
Total: 21
16S
30S
70S

- rRNA is the main constituent of ribosomes (ribonucleoprotein complex) used for the translation of genetic information coded on an mRNA.
- In prokaryotes **the** large 50S ribosomal subunit contains the following rRNAs:
  - 23S rRNA;
  - 5S rRNA.
- The small 30S subunit contains only 16 rRNA with at least 21 proteins.

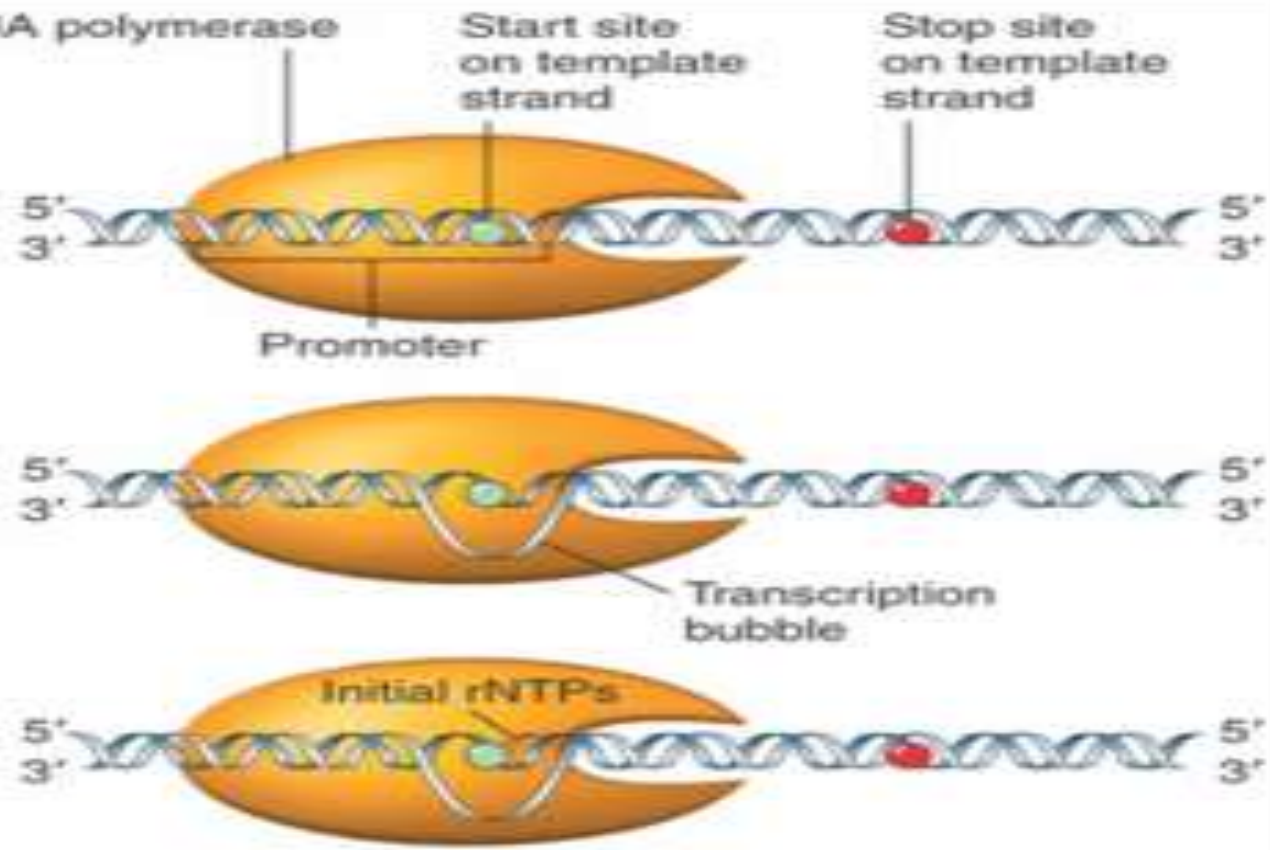
- Transcription is the process of copying DNA into mRNA. It is carried out using RNA polymerase.
- It is a DNA-dependent, multimeric protein possessing α, β, β' and σ subunits. It exists in two forms: the core enzyme (α2ββ') and the holoenzyme (α2ββ'σ). RNA polymerases do not require a primer and do not possess exonuclease activity .
- In *E. coli* , a single RNA polymerase catalyzes the synthesis of all RNAs in the cell.

INITIATION

1 Polymerase binds to promoter sequence in duplex DNA. "Closed complex"

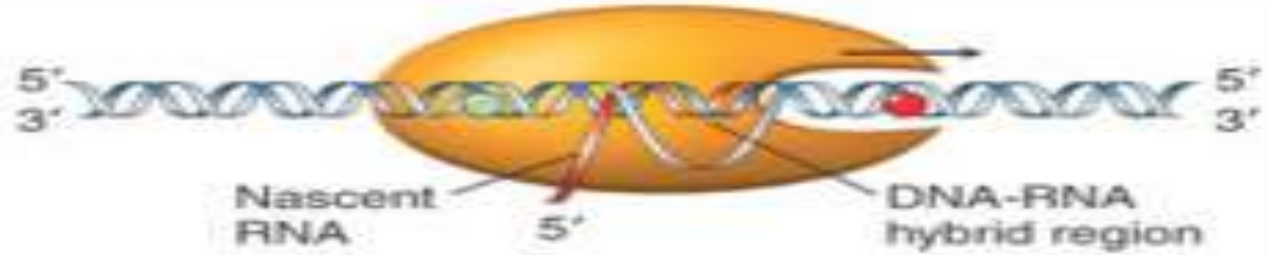
2 Polymerase melts duplex DNA near transcription start site, forming a transcription bubble. "Open complex"

3 Polymerase catalyzes phosphodiester linkage of two initial rNTPs.



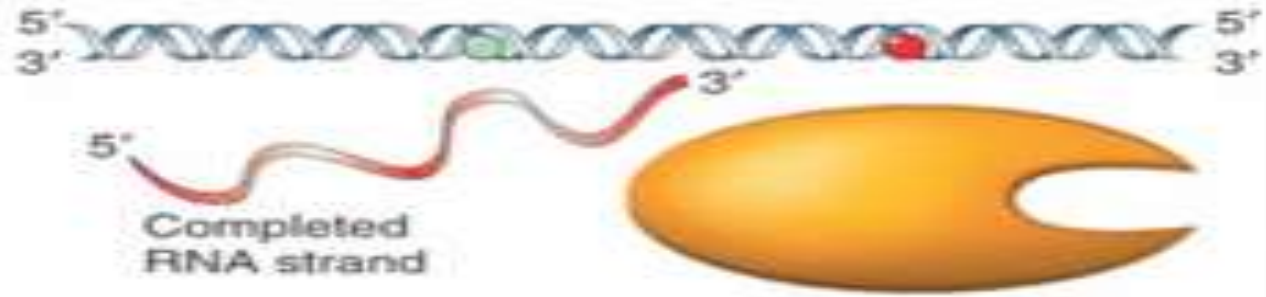
ELONGATION

4 Polymerase advances 3′ → 5′ down template strand, melting duplex DNA and adding rNTPs to growing RNA.



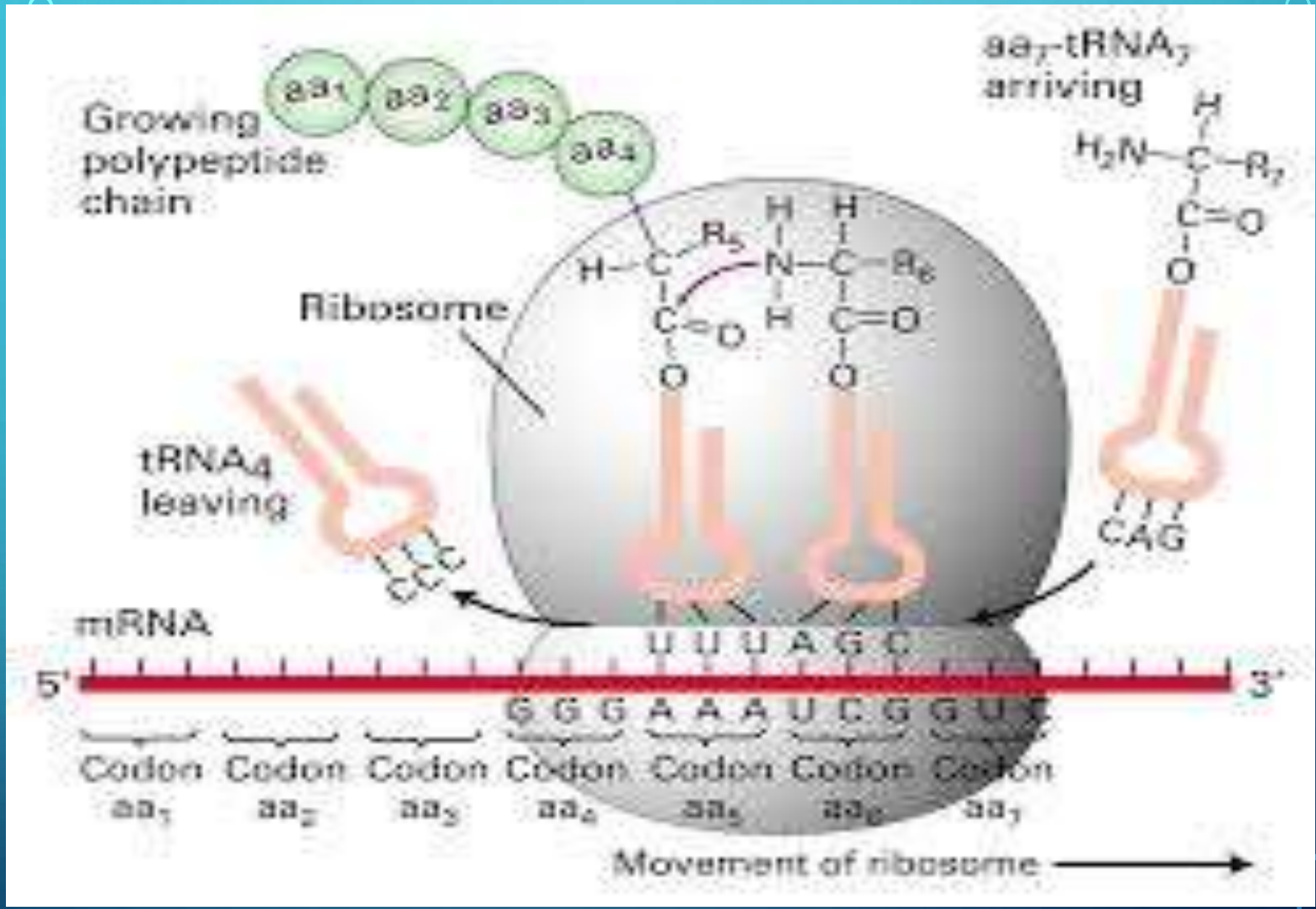
TERMINATION

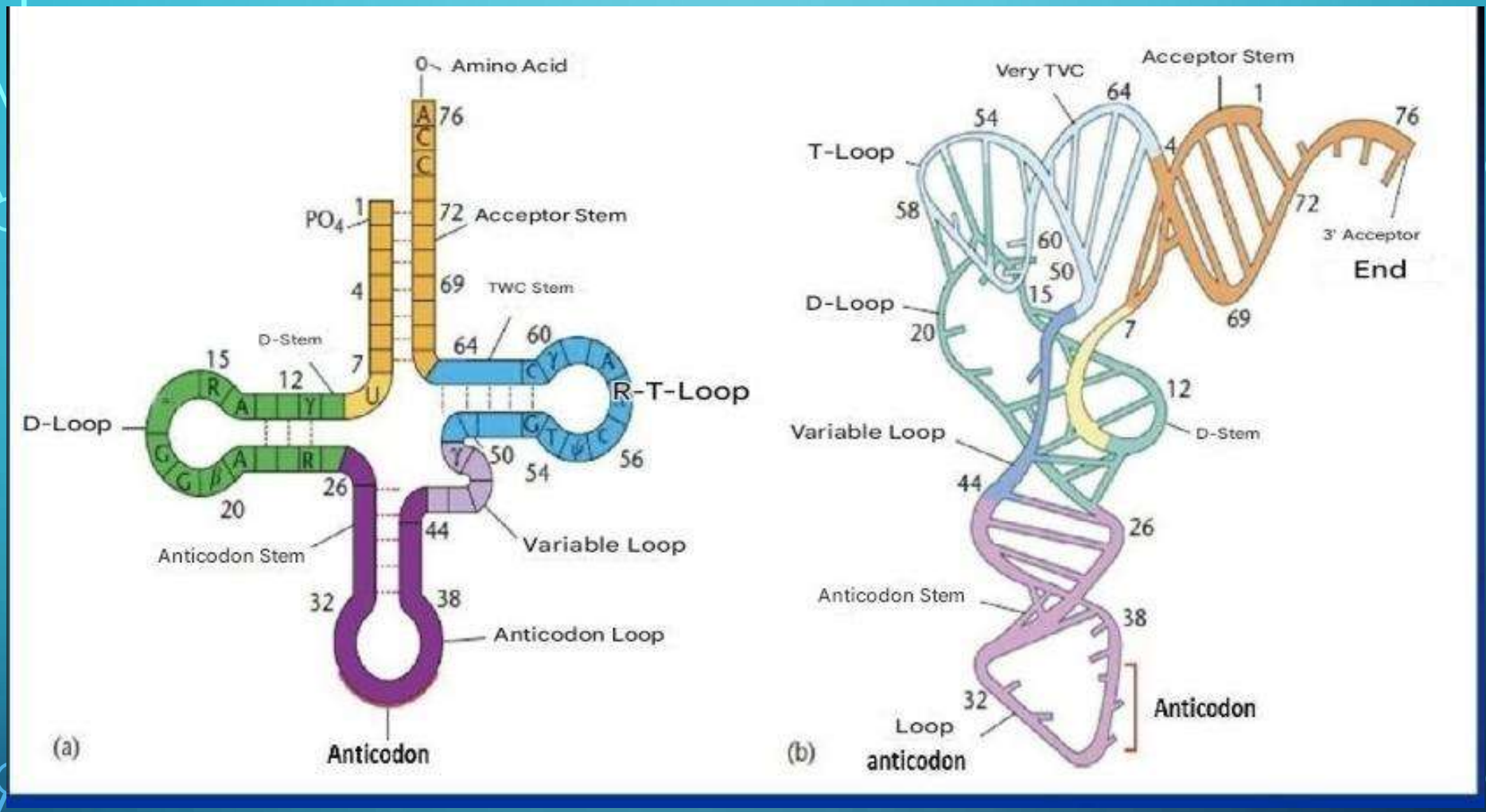
5 At transcription stop site, polymerase releases completed RNA and dissociates from DNA.



Translation :

- mRNA is translated into protein by the combined action of transfer RNA and the ribosome, which consists of many proteins and two main ribosomal RNA molecules (pairing between tRNA anticodons and complementary codons in mRNA).
- The formation of a peptide bond between the N-amino group of the incoming aa-tRNA and the carboxy - terminal C on the nascent protein chain is catalyzed by one of the tRNAs

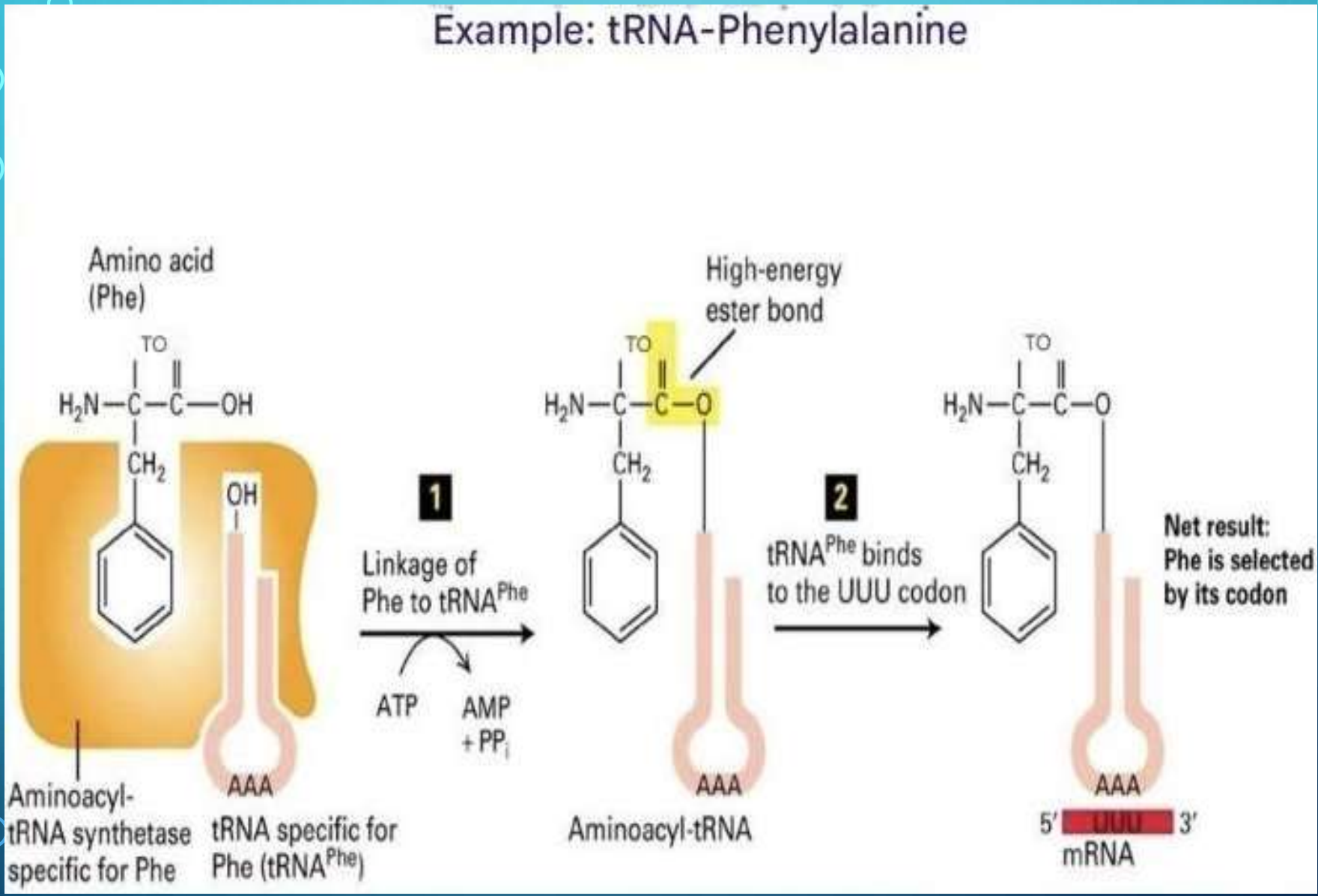

Growing polypeptide chain
aa1
aa2
aa3
aa4
aa7-tRNA7 arriving
Ribosome
tRNA4 leaving
CAG
mRNA
5'
3'
U U U A G C
G G G A A A U C G G U C
Codon aa1
Codon aa2
Codon aa3
Codon aa4
Codon aa5
Codon aa6
Codon aa7
Movement of ribosome



Structure of a transfer RNA (tRNA): (**a**) Diagram of the secondary structure of a tRNA schematically represented in the shape of a clover. Universally conserved bases are indicated by their position number. (**b**) Three-dimensional L-shaped structure adopted by tRNA when the D stem folds onto the TΨC stem

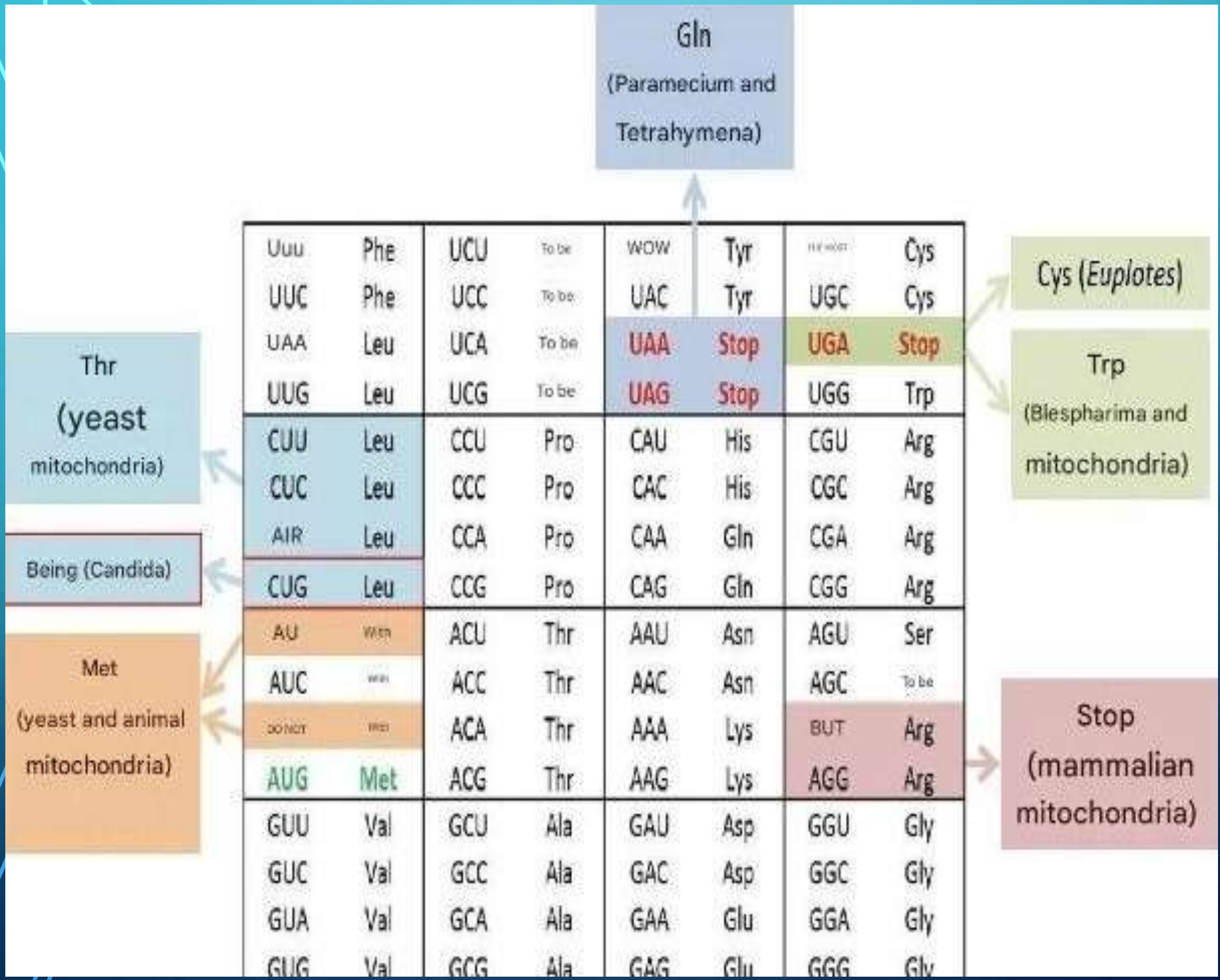
- Translation corresponds to the decoding of information carried by mRNA into proteins, using the genetic code.
- Translation takes place at the level of ribosomes by tRNAs charged with the corresponding AA. The fixation of tRNAs is done by molecular recognition between the triplet of nucleotides of the mRNA (codon) and the triplet of the tRNA (anti-codon). We can distinguish 3 successive stages: initiation, elongation and termination.

Example: tRNA-Phenylalanine



- All tRNAs fold into a four-paired stem, three-loop conformation. The CCA sequence at the 3' end is also present in all tRNAs.
- The binding of Aa to the 3' A produces an aminoacyl-tRNA . Some of the residues A, C, G and U are modified in most tRNAs (box) dihydrouridine (D) is almost always present in the D loop: similarly ribothymidine (T) and pseudouridine (Ψ) are almost always present in the T loop Ψ CG

- The two-step decoding process for translating nucleic acid sequences in mRNA into amino acid sequences in proteins: an aminoacyl-tRNA synthetase first couples a specific amino acid via an energy-rich ester bond to the 2' or 3' hydroxyl of the terminal adenosine in the corresponding
- A three-base sequence of tRNA (anticodon) then pairs with a codon in the mRNA specifying the attached amino acid


Gln
(Paramecium and
Tetrahymena)
Thr
(yeast
mitochondria)
Being (Candida)
Met
(yeast and animal
mitochondria)
Cys (Euplotes)
Trp
(Blespharima and
mitochondria)
Stop
(mammalian
mitochondria)
Uuu Phe
UUC Phe
UAA Leu
UUG Leu
CUU Leu
CUC Leu
AIR Leu
CUG Leu
AU
AUC
AUG Met
GUU Val
GUC Val
GUA Val
GUG Val
UCU To be
UCC To be
UCA To be
UCG To be
CCU Pro
CCC Pro
CCA Pro
CCG Pro
ACU Thr
ACC Thr
ACA Thr
ACG Thr
GCU Ala
GCC Ala
GCA Ala
GCG Ala
WOW Tyr
UAC Tyr
UAA Stop
UAG Stop
CAU His
CAC His
CAA Gln
CAG Gln
AAU Asn
AAC Asn
AAA Lys
AAG Lys
GAU Asp
GAC Asp
GAA Glu
GAG Glu
UGC Cys
UGA Stop
UGG Trp
CGU Arg
CGC Arg
CGA Arg
CGG Arg
AGU Ser
AGC To be
BUT Arg
AGG Arg
GGU Gly
GGC Gly
GGA Gly
GGG Gly

- The genetic code is the set of rules that allow genetic information to be transcribed and translated into RNA, then into proteins.
- The code of bacteria is identical to that of all living beings, according to the principle of the universality of the genetic code.

II. Regulation of gene expression:

- Regulation of gene expression= To respond to changing conditions in the immediate environment.
- Genetic regulation is a way for the cell to develop mechanisms that allow it to repress genes that code for unnecessary proteins and activate them when they become needed.
- Two modes of regulation of the expression of a target gene by a regulatory molecule:

1- in a positive way: the interaction triggers the transcription of the gene.

2- in a negative way: the interaction prevents the transcription of the gene.

Regulation of transcription from the *E. coli* *lac operon* :

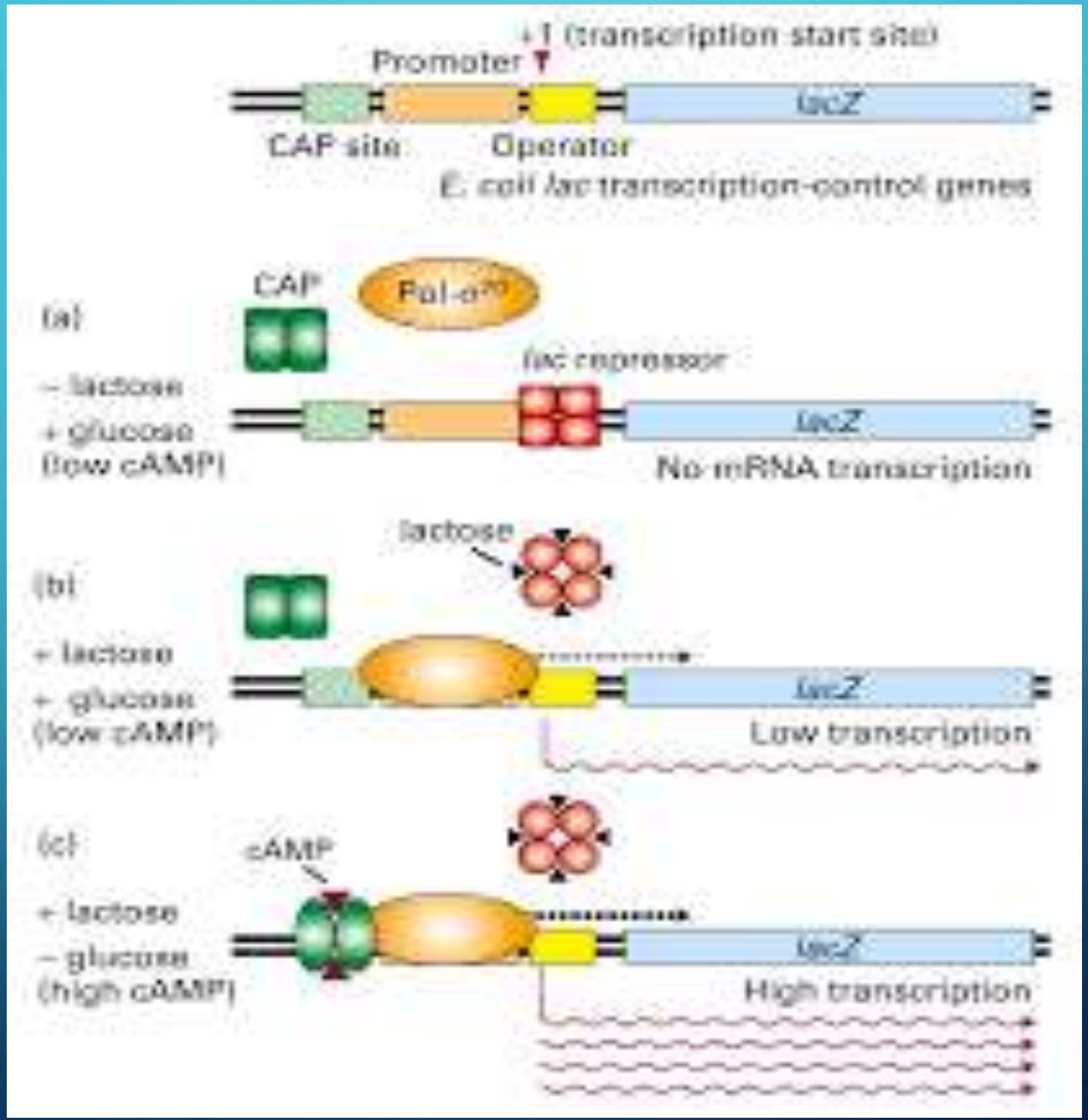
- bp (base pair) transcription control region includes three protein binding regions:

-the CAP site (catabolite Activator protein), which binds the catabolite activator protein, the lac promoter to which the RNA polymerase complex binds, and the lac operator, which binds the lac repressor.

-in the absence of lactose, very little lac mRNA is produced because the lac repressor binds to the operator, thus inhibiting the initiation of transcription by RNA polymerase

-in the presence of glucose and lactose, the lac repressor binds lactose and dissociates from the operator, allowing RNA polymerase to initiate transcription at a low rate

-Maximum transcription of the lac operon occurs in the presence of lactose and the absence of glucose. In this situation, cAMP increases in response to the low glucose concentration and forms the CAP- cAMP complex , which binds to the CAP site, where it interacts with RNA polymerase to stimulate the rate of transcription initiation.


+1 (transcription start site)
Promoter
lacZ
CAP site
Operator
E. coli lac transcription-control genes
CAP
Pol-σ70
(a)
lac repressor
– lactose
+ glucose
(low cAMP)
lacZ
No mRNA transcription
lactose
(b)
+ lactose
+ glucose
(low cAMP)
lacZ
Low transcription
(c)
cAMP
+ lactose
– glucose
(high cAMP)
lacZ
High transcription

III. BASIC TECHNIQUES OF MOLECULAR BIOLOGY

1. Extraction of nucleic acids

- Regardless of the sample, whether a swab or pure bacterial culture, the DNA must first be extracted before proceeding with molecular tests.
- The extraction yield reflects optimal purification of bacterial DNA while avoiding destruction of this DNA.

- Extraction therefore relies on a compromise between **aggressive means** of extracting DNA from a complex mixture including the biological constituents of tissues and bacteria and **preserving** the integrity of this targeted DNA.
- The extraction yield will be related to both the type of tissue sampled and the types of bacteria present.
- The extraction takes place in two phases:

- lysis of the collected tissue;
- purification of DNA from the lysis mixture;

- Several types of lysis can be used alone or in combination:

- mechanical lysis by grinding using beads, Potter, ultrasound;

- thermal lysis in a bain-marie in boiling water or in a microwave;

- Nucleic acid purification methods are usually combinations of two or more of the following techniques: extraction/precipitation, chromatography, centrifugation.

- chemical lysis using sodium dodecyl sulfate (SDS) or Chele ×® (chelating agents);

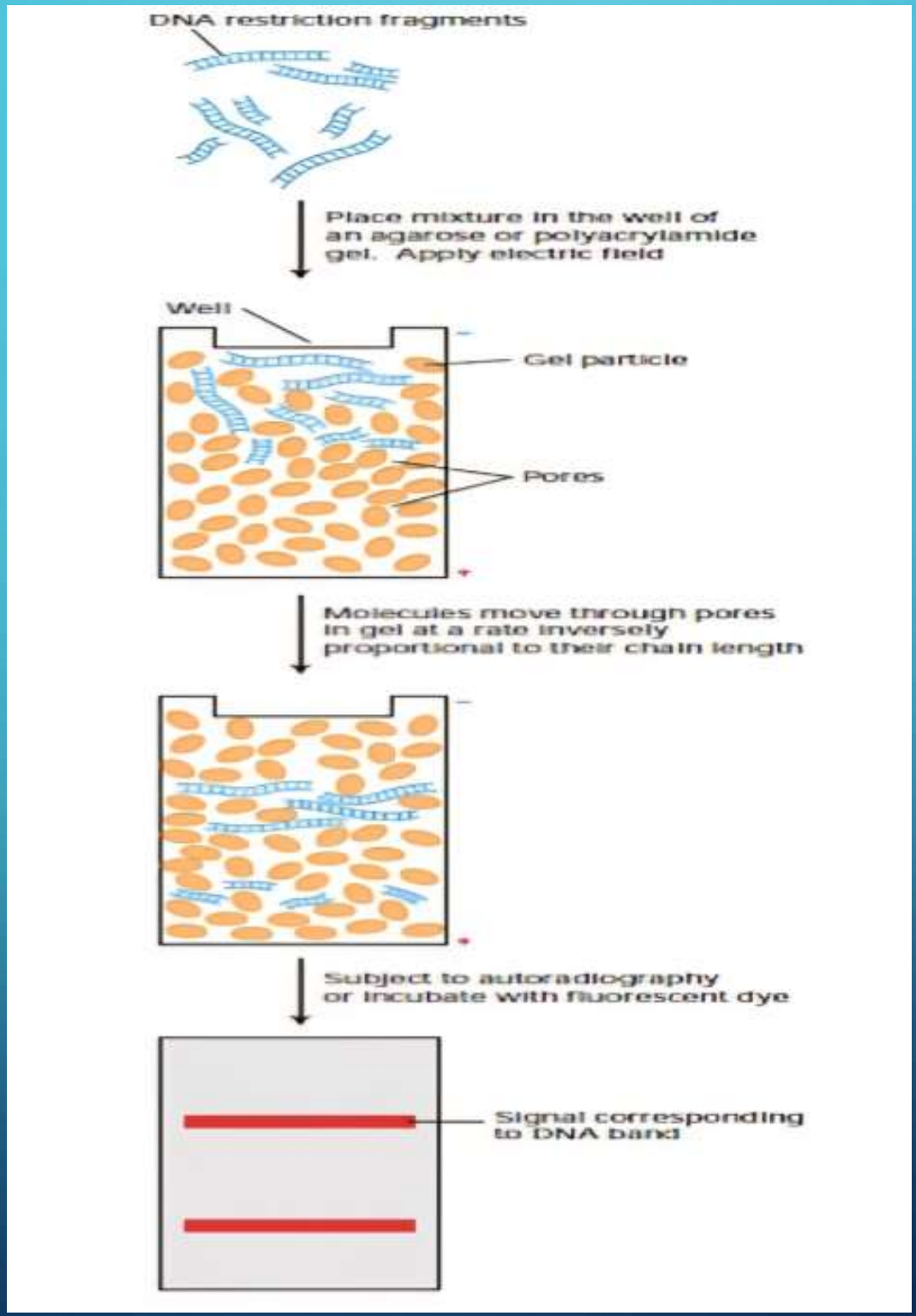
- enzymatic lysis using proteinase K, lysozyme.

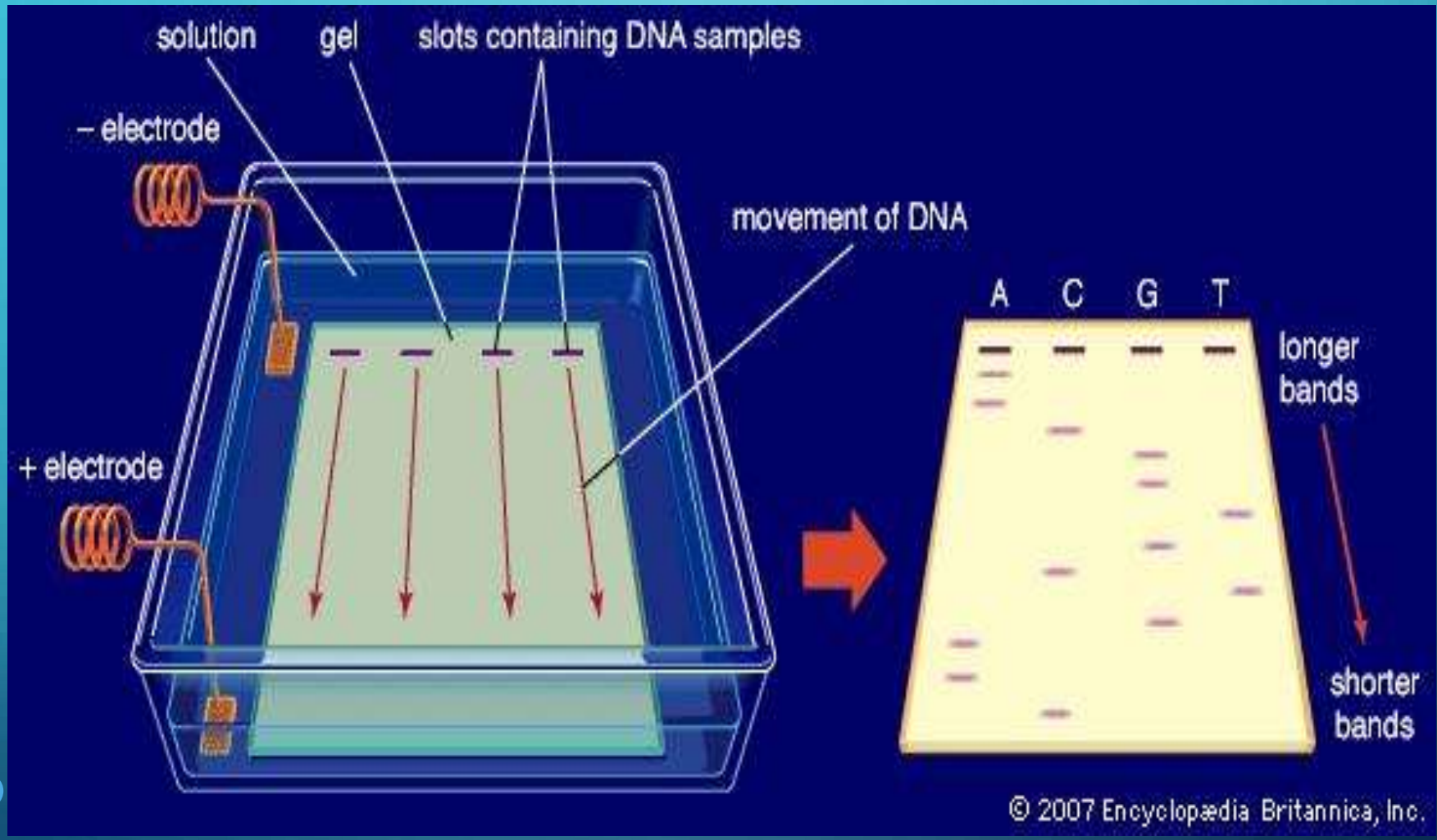
Mechanical lysis by grinding and enzymatic lysis by proteinase K are the most used.

2. Separation of nucleic acids by gel electrophoresis:

- Separation of DNA molecules according to their size.
- Gel preparation (a few mm):
- Pour a solution of molten agarose or acrylamide between two glass plates.
- Polymerization of agar or acrylamide into polyacrylamide (network).
- large DNA fragments = in agarose gels, small ones in polyacrylamide gels.
- the mixture of DNA fragments loads into a trough at the top of the gel,

- apply a potential difference between two ends of the gel;
- The DNA fragments migrate towards the positive pole, to form bands visible after autoradiography (radioisotopes), or by staining using a fluorescent substance.


DNA restriction fragments
Place mixture in the well of an agarose or polyacrylamide gel. Apply electric field
Well
Gel particle
Pores
Molecules move through pores in gel at a rate inversely proportional to their chain length
Subject to autoradiography or incubate with fluorescent dye
Signal corresponding to DNA band


solution
gel
slots containing DNA samples
– electrode
movement of DNA
+ electrode
A
C
G
T
longer bands
shorter bands
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3. Pulsed-field gel electrophoresis separates large DNA molecules

- Electrophoresis gels only resolve DNA fragments of 20 kb or less;
- Pulsed-field gel electrophoresis allows the separation of larger DNA.
- Method: Large DNAs placed in an electric field; migrate parallel to the direction of the field and stretch in length;
- When the current is cut off: the molecules shrink into a random ball.
- Long molecules shrink less than small ones in the new field.
- This repeated reversal of the field direction eventually pushes the large DNA molecules further and further apart.

4. DETECTION, IDENTIFICATION AND CHARACTERIZATION OF NUCLEIC ACIDS

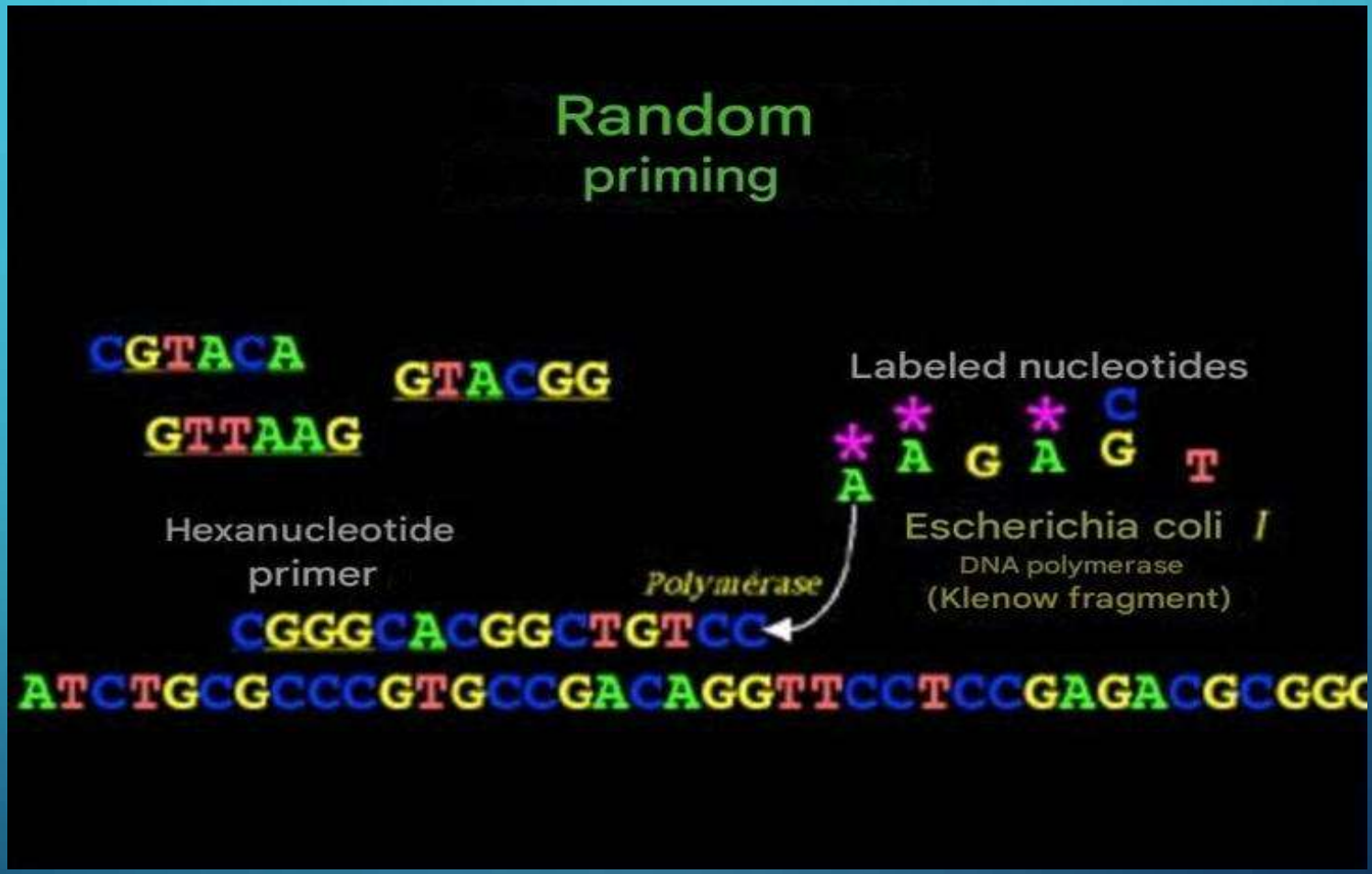
4. 1. Labeling and tracking of nucleic acids:

- Marking: in all techniques that use a probe (hybridization, northern , etc.).
- **"hot"** marking : radioactive isotopes (p32)
- markings : molecules with fluorescent, luminescent properties.

a. Double-stranded probes:

Random Priming :

- Southern and Northern Blot: The two strands of DNA in the probe are first separated by heating and then cooling. Then, a mixture of synthetic oligonucleotides is added. These oligonucleotides will hybridize with the probe. These fixed oligonucleotides will serve as primers for the DNA fragment.


Random
priming
CGTACA
GTACGG
GTTAAG
Labeled nucleotides
Hexanucleotide
primer
Polymérase
Escherichia coli I
DNA polymerase
(Klenow fragment)
CGGGCACGGCTGTCC
ATCTGCGCCCGTGCCGACAGGTTCCTCCGAGACGCGGC

- -3' marking: marking at the ends.
- *with DNA pol .: Klenow fragment , T4 DNA pol ., Taq pol ., reverse transcriptase.
- *with an exonuclease
- *with terminal transferase
- DNA can be labeled at its 5” end using a kinase (T4 polynucleotide kinase extracted from *E. coli* infected with bacteriophage T4).
- - Nick Translation Marking: Marking inside uses two enzymes:
- * DNAse I to generate some single-stranded breaks in the fragment of interest
- *DNA pol.I to degrade DNA in the 5'-3' direction at these breaks and repolymerize in the presence of a hot (radioactive) nucleotide.
- The deoxynucleoside triphosphates used are labeled in the alpha position with 32P.

4.2. Cold markings:

- **Fluorescence:**

-Absorption of light energy by a molecule (fluorochrome);

-Transition to the state of excited molecule;

- Partial relaxation with heat loss through exchange with the surrounding environment

-Return to the ground state by light emission = fluorescence spectrum

- **Colorimetry:**

Dyes: biotin, fluorescein

Example: The target DNA is fixed on a membrane and the probes are biotinylated . Detection is carried out by adding a streptavidin -HRP complex (Horse Radish peroxidase or Horseradish Peroxidase), the enzyme that allows the oxidation of a chromogen. The signal is measured by colorimetry

Chemiluminescence:

- Chemiluminescence occurs during a chemical reaction when excess energy is released as light.
- Many reactions produce this phenomenon and each emits light of a specific wavelength, and of intensity proportional to the quantity of reactant molecules present.

4.3. DNA Denaturation and Molecular Hybridization:

- **Denaturation of DNA:**
  - Slowly increasing the temperature (up to 94°C) of a DNA solution breaks the hydrogen bonds between the bases and separates the two strands: this is called denaturation.
  - It can be monitored by measuring the absorption of UV light (optical density) at 260 nm.

- The temperature that causes half of the DNA molecules to denature is called the melting temperature or Tm . temperature).

- It is possible to directly measure the melting temperature of double-stranded DNA by measuring the increase in absorbance of the solution at 260 nm as a function of temperature.
- Tm=2x(A+T)+4x(G+C)

From N = 20, we correct with a multiplier proportional to the length beyond this figure: 1 + [(N-20)/20].

Tm = 2 x (A+ T) + 4 x (G + C) x (1+[(N-20)/20]

Renaturation or hybridization:

- Slowly cooling the denatured DNA solution causes the complementary strands to reassociate to form a double helix: this is renaturation or hybridization. This reassociation can also occur between DNA and RNA, resulting in DNA/RNA hybrids.
- Molecular hybridization refers to the association that can take place between two single-stranded nucleic acids of complementary sequences and which leads to the formation of a double strand or duplex.
- The formation and stability of duplexes depends on many factors in addition to base composition: duplex length, sequence complexity.

Nucleotide probes

- A nucleotide probe is a segment of nucleotides that allows the specific search for a fragment of nucleic acid that one wishes to study. This probe-fragment reaction corresponds to a molecular hybridization reaction.
- A nucleotide probe can be either a single-stranded DNA or RNA sequence. Its size varies greatly: an oligonucleotide of 20 to 30 nucleotides or, on the contrary, several hundred nucleotides. The probe is complementary and antiparallel to the fragment sought.

4.4. DNA SEQUENCING

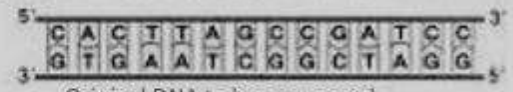
- Sequencing consists of determining the linear sequence of nucleotides in a DNA fragment
- or, more generally, of a genome. Its history begins in 1977 when Maxam and Gilbert
- develop a technique based on the radioactive labeling of fragments and their selective cutting
- by chemical degradation. In parallel, Sanger takes a completely different approach.

Sanger sequencing:

- In the same year, Sanger based his sequencing technique on an enzymatic synthesis of DNA fragments after their amplification by cloning. He uses the property of DNA polymerases to synthesize a strand complementary to a template strand in the presence of dNTPs .
- He added fluorescently labeled ddNTPs to this medium ; these act as random elongation terminators in the reaction. By migrating the fragments of different sizes obtained on a polyacrylamido gel , he was able to read their sequence.

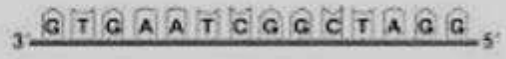
- Since then, Sanger sequencing has been automated: the first machines using polyacrylamide gels were quickly supplanted by capillary sequencers. This technology, mainly represented by the 3230 DNA Analyzer from Applied Biosystems (now Life Technologies) since its commercialization in 2002, is now considered the first generation of high-throughput sequencing and has helped decipher the human genome.
- We use labeled primers (*dye primers*) or labeled nucleotides (*dye terminators*).

1 Isolated unknown DNA fragment

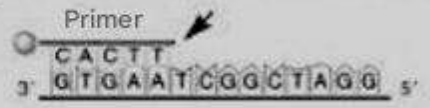


Original DNA to be sequenced.

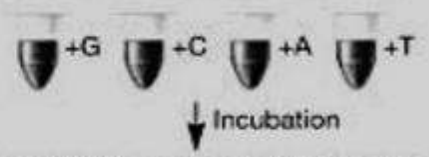
2 The DNA is denatured to give a single-stranded template.



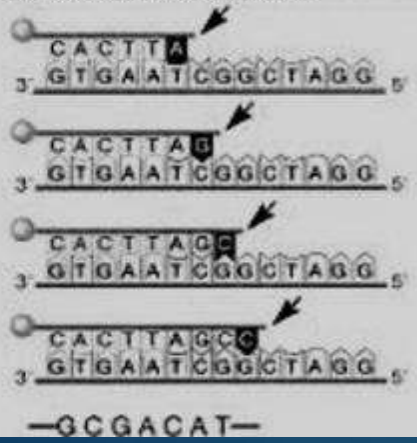
3 A specific labeled primer molecule hybridizes with the DNA strand,



4 DNA polymerase and a mixture of the normal nucleotides (dATP, dGTP, dCTP, and dTTP) are added. The ddA, ddG, ddC, and ddT are introduced into separate reaction tubes with the normal nucleotides. The dinucleotides are labeled with types of tracers that allow their visualization.

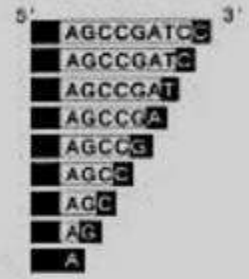


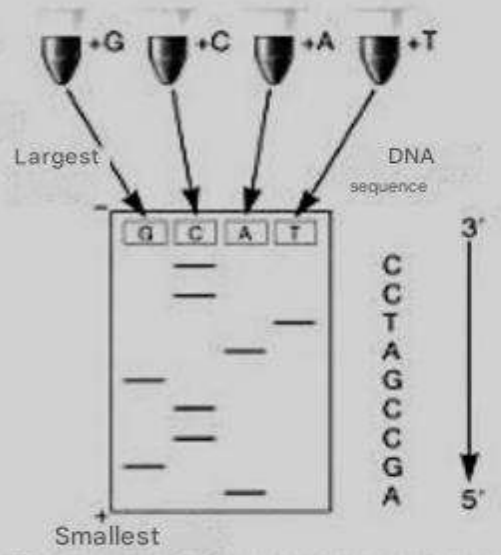
5 The newly replicated strands terminate at the site of the addition of a ddnucleotide.



(a) —G C G A C A T—

6 Schematic representation of all possible positions of a labeled nucleotide on the fragment.





7 Migration of the reaction mixtures on a gel, in four different lanes, separates their fragments by size and nucleotide type. Reading from bottom to top, base by base, gives the correct sequence

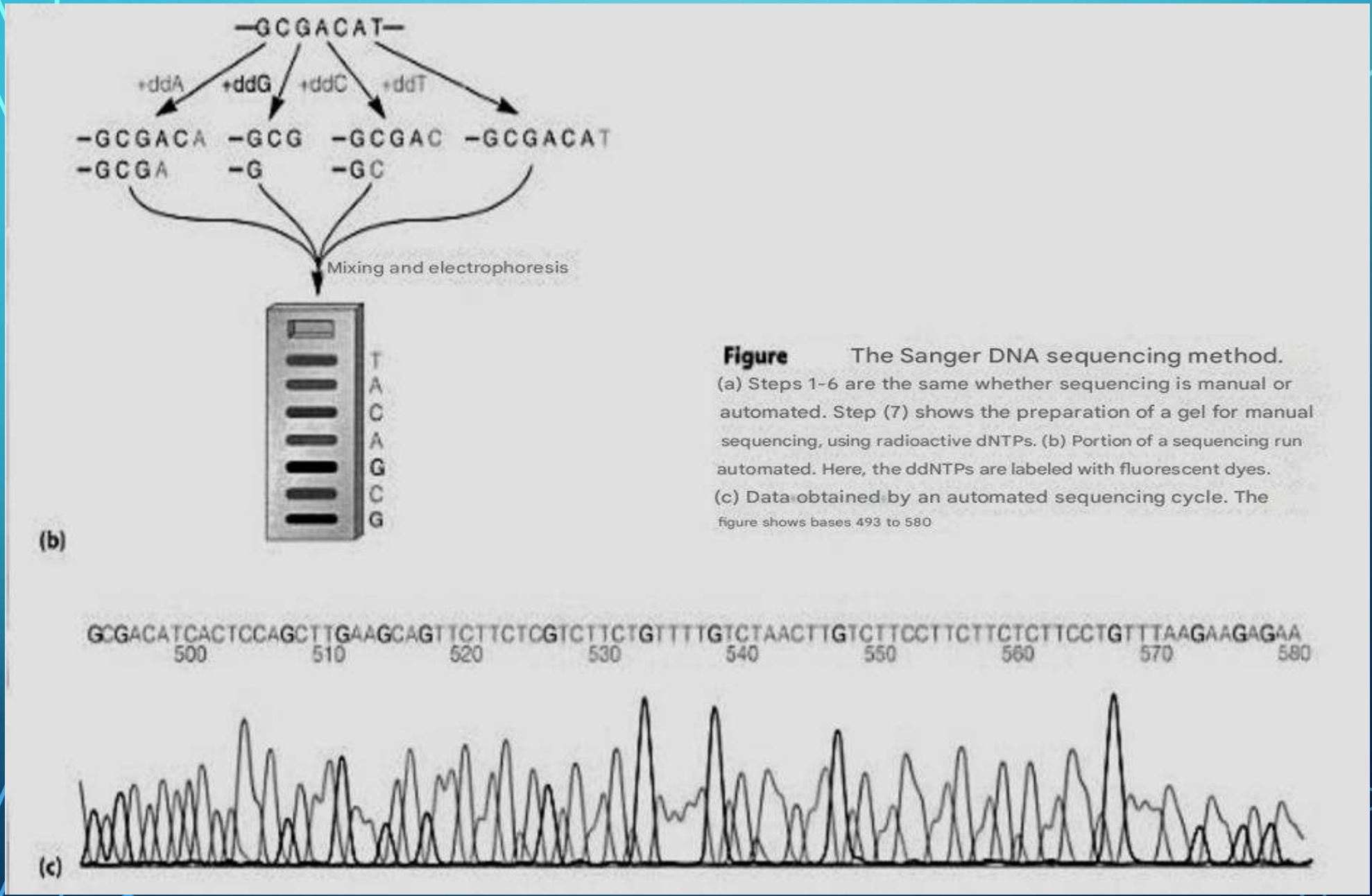


Figure The Sanger DNA sequencing method. (a) Steps 1-6 are the same whether sequencing is manual or automated. Step (7) shows the preparation of a gel for manual sequencing, using radioactive dNTPs. (b) Portion of a sequencing run automated. Here, the ddNTPs are labeled with fluorescent dyes. (c) Data obtained by an automated sequencing cycle. The figure shows bases 493 to 580

4.5. PCR (POLYMERASE CHAIN REACTION)

- **Amplification of bacterial DNA:**

➢ Amplification is a molecular biology technique widely used in microbiology. It allows, from a single nucleic acid molecule, to obtain sufficiently large quantities of target material in order to be able to analyze it using different techniques .

➢ Currently, the PCR technique is the most used to amplify bacterial DNA.

Historical:

- PCR was developed in 1985 by Kary Mullis , the technique experienced considerable growth following the commercialization (around 1988) of a DNA polymerase resistant to high temperatures (Taq polymerase)(*Thermus aquaticus*), which allows automation of the technique.

Definition:

Polymerase Chain Reaction (PCR) is a targeted *in vitro replication technique* .

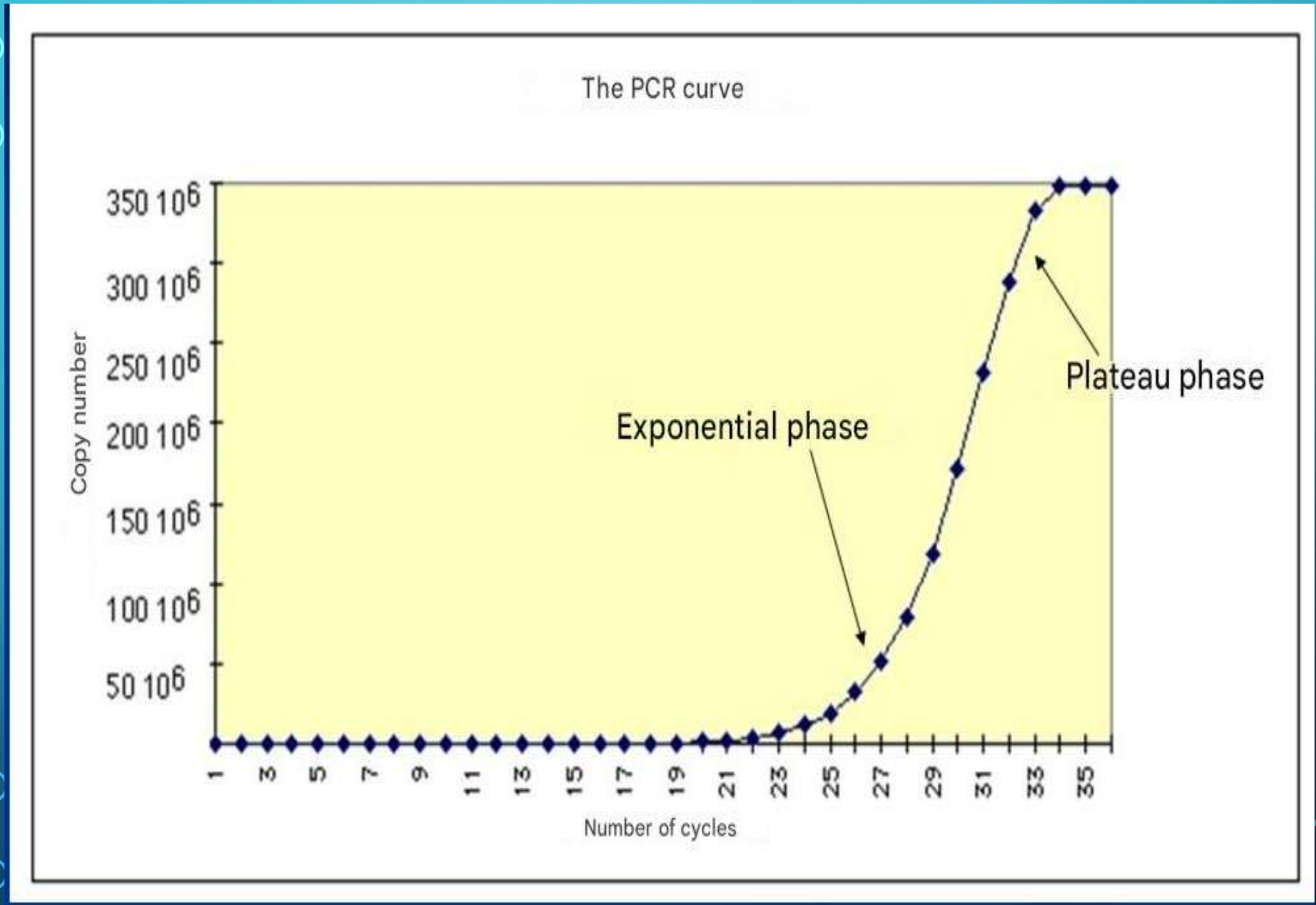
It allows large quantities of a specific DNA fragment of defined length to be obtained from a complex and sparse sample. The order of magnitude to be used is that of a million copies in a few hours.

Principles of PCR

PCR is based mainly on three elements:

- heating double-stranded DNA to at least 90°C allowing it to be separated into two single strands (step of denaturation);
- after heating and separation of the double-stranded DNA into single strands, gradual cooling allowing a hybridization between complementary sequences (step primer hybridization);
- the property of copying a target strand from a primer complementary hybridized to the strand using DNA polymerases, thermostable enzymes (elongation step).

- In a tube, the target DNA, two primers in sufficient quantity, deoxyribonucleotide triphosphates (dNTPs) and DNA polymerase will be brought into contact, all in a buffer ensuring a stable pH (Tris(**trishydroxymethylaminomethane**) , HCl at basic pH 8.5 to 9) containing divalent Mg2+ cations, essential cofactors for the polymerization reaction.
- The tubes containing the reaction mixture are subjected to temperature cycles repeated several dozen times in the heating block of a thermal cycler .


The PCR curve
Copy number
350 10^6
300 10^6
250 10^6
200 10^6
150 10^6
100 10^6
50 10^6
Exponential phase
Plateau phase
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
Number of cycles

Denaturation of the matrix:

- During denaturation, the double strand opens to form single-stranded DNA.
- The two complementary chains are separated by an increase in temperature. This is called denaturation.
- To achieve DNA denaturation, the temperature is usually increased to around 93–96 °C. In this way, strong H-bonds are broken and the number of unpaired bases increases.
- double-stranded DNA has become single-stranded DNA.

Primer hybridization:

- Hybridization, or rehybridization , of DNA strands takes place at a lower temperature (usually 55–65 °C). Once the temperature is lowered, the two complementary single-stranded DNA chains will reform into a double-stranded DNA molecule . During this phase, the primers move freely and ionic bonds are constantly formed and broken between the single-stranded primer and the single-stranded template.

Primer Extension:

- The primers are extended over the target sequence using DNA polymerase in the presence of dNTPs , resulting in duplication of the starting target material (T ° = Taq DNA polymerase is 72 °C).
- The bases are coupled to the primer on the 3' side.
- The duration of the primer extension steps can be increased if the region of DNA to be amplified is long; however, for the majority of PCR reactions, an extension time of 1 minute is sufficient to achieve complete extension.

Amplification, as the final copy number of the target sequence, is expressed by the following equation:

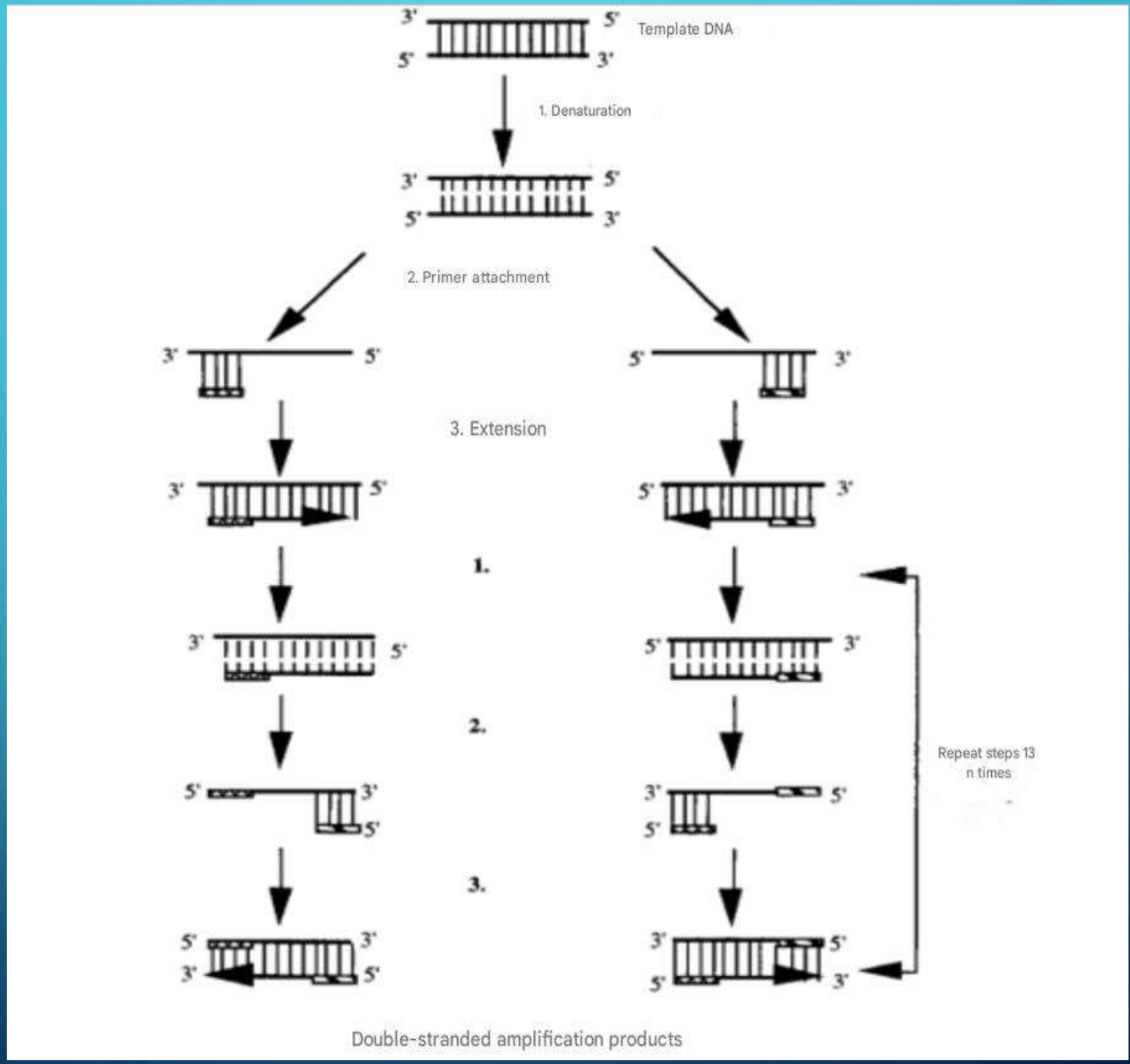
$$(2^{n}-2n)x$$

N= number of cycles,

2n= the first product obtained after the first cycle and the second product obtained after the second cycle,

X = the number of copies of the original matrix.

After 20 PCR cycles, there will be a 2-fold amplification ، assuming 100% efficiency during each cycle.


Template DNA
1. Denaturation
2. Primer attachment
3. Extension
1.
2.
3.
Repeat steps 13
n times
Double-stranded amplification products

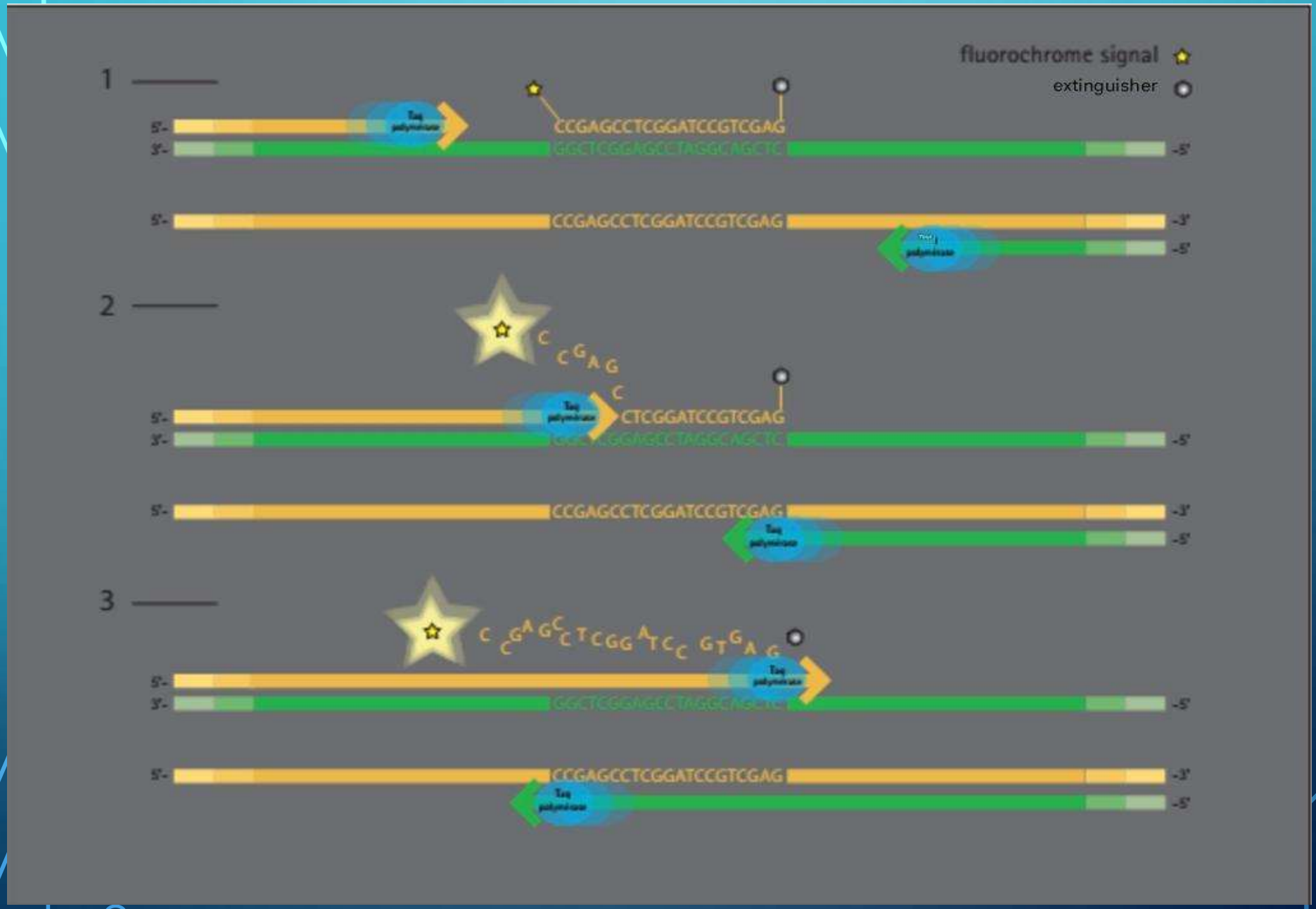
4.6. RT-PCR (Reverse Transcriptase PCR):

- cDNA (complementary DNA) copies . This reaction is catalyzed by the reverse transcriptase of retroviruses, which synthesizes a DNA chain from an RNA template.
- The single-stranded cDNAs are then replicated by DNA polymerase during a first temperature cycle. Further cycles are repeated to amplify the cDNAs. double-stranded in large quantities.

4.7. Quantitative real-time PCR:

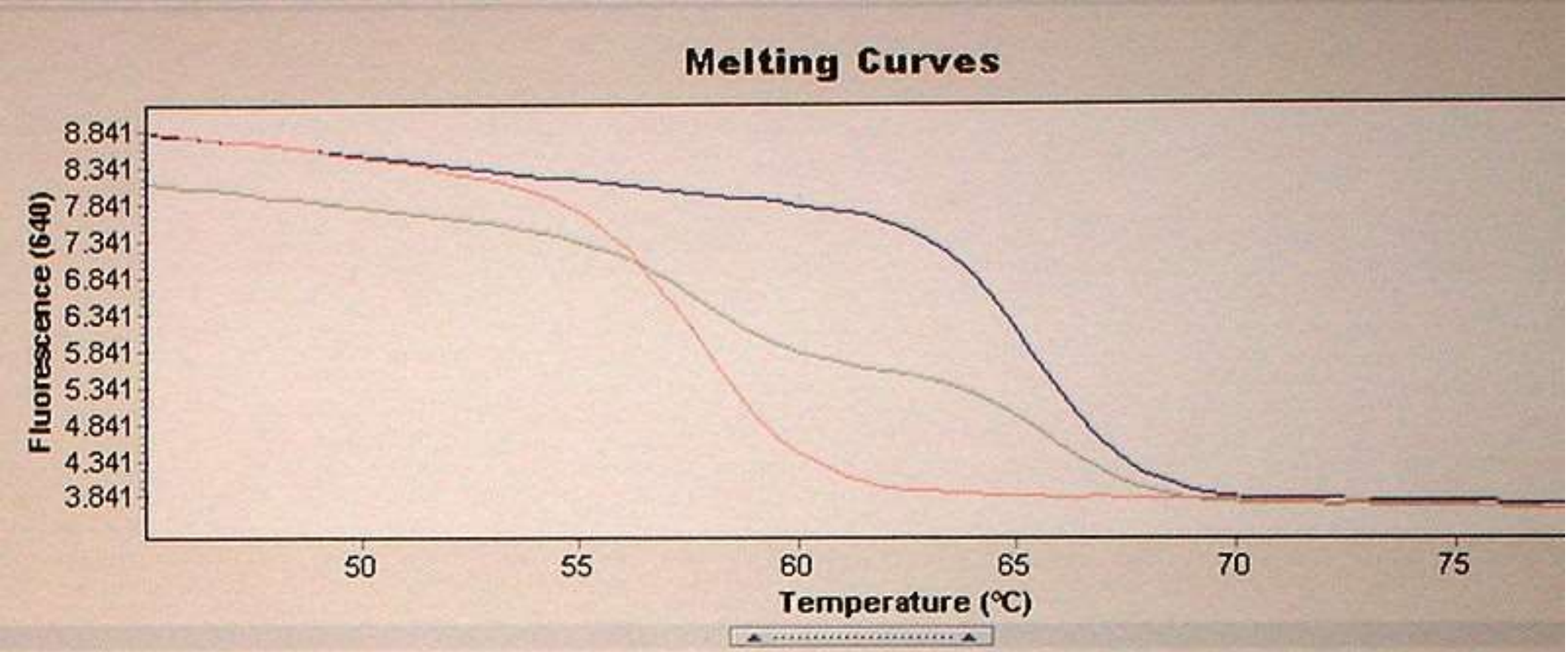
- Quantitative PCR is used to determine the level of specific DNA or RNA in a biological sample. The method is based on the detection of a fluorescent signal that is produced proportionally to the amplification of the PCR product, cycle after cycle. It requires a thermal cycler coupled with an optical reading system that measures fluorescence emission.
- A nucleotide probe is synthesized so that it can selectively hybridize to the DNA of interest, between the sequences where the primers hybridize.

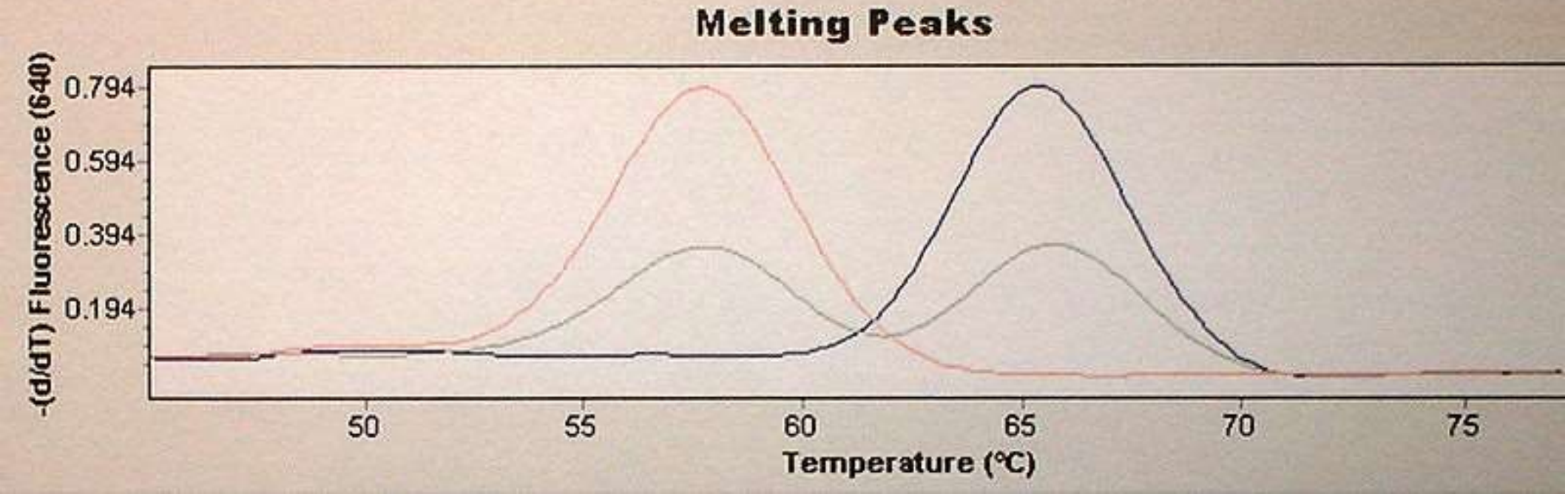
- The probe is labeled on the 5' end by a signal fluorochrome (e.g. 6-carboxyfluorescein), and on the 3' end by a quencher fluorochrome (e.g. 6-carboxy-tetramethyl-rhodamine).
- This probe must show a higher hybridization temperature (Tm) than the primers so that it hybridizes 100% during the elongation phase (critical parameter).
- As long as both fluorochromes remain present at the probe, the quencher prevents the signal from fluorescing. In fact, the proximity of the quencher and the signal induces an absence of fluorescence emission.
- During the elongation phase, Taq polymerase degrades the probe and releases the signal fluorochrome .
- The rate of fluorescence then released is proportional to the quantity of PCR products generated at each cycle.


fluorochrome signal
extinguisher
1
2
3
5'-
3'-
CCGAGCCTCGGATCCGTCGAG
CTCGGATCCGTCGAG
Taq polymerase

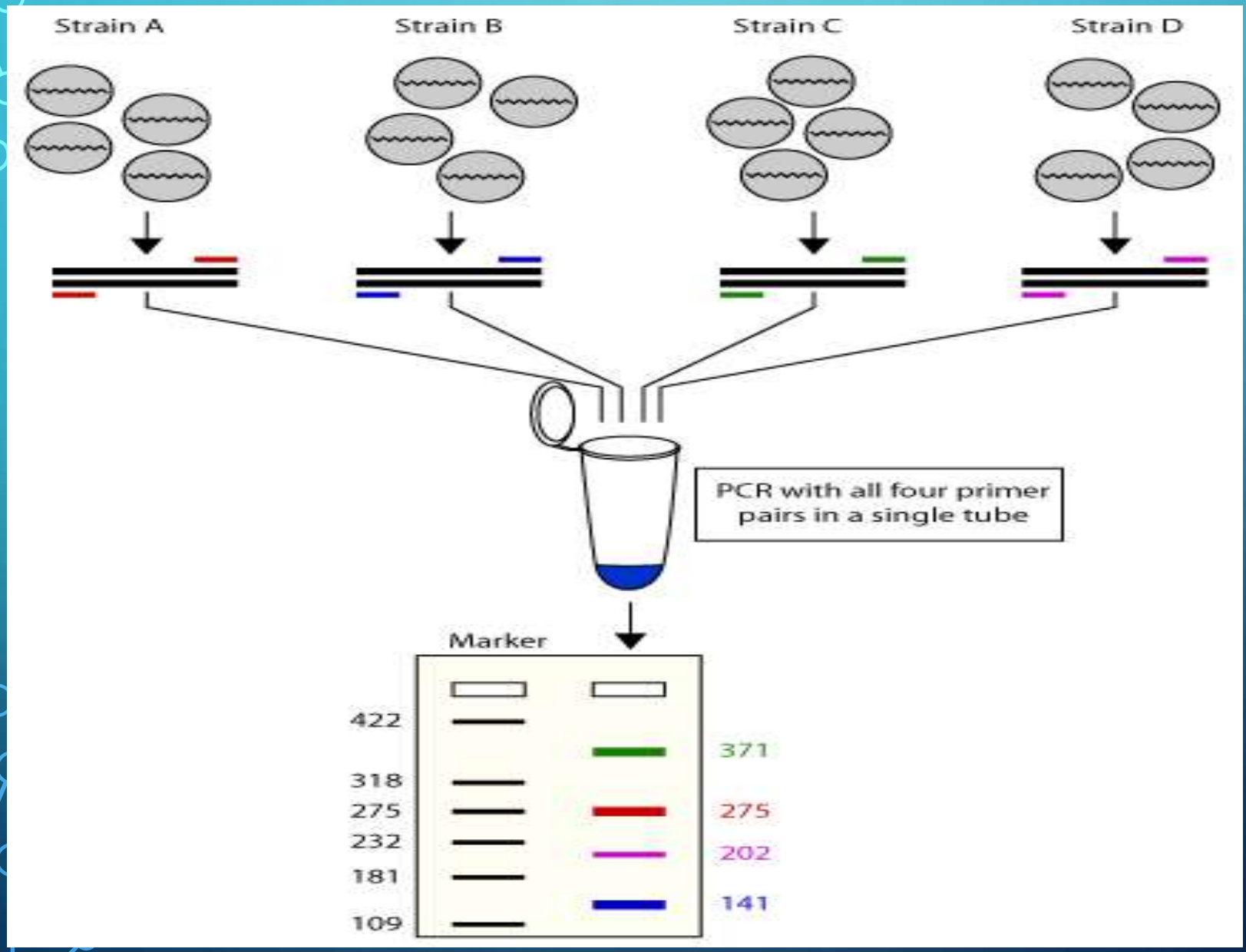
- A **melting curve** in real-time PCR is an additional programmed step at the end of amplification cycles.

- check that there is only one amplified PCR product.
- If the curve is polymodal, the results should be interpreted with caution or the experiment should be repeated with other, more specific primers.
- If the curve is unimodal , the calculation of its primary derivative gives a bell-shaped curve whose abscissa of the maximum is the Tm of the PCR product (amplicon).


Melting Curves
Fluorescence (640)
8.841
8.341
7.841
7.341
6.841
6.341
5.841
5.341
4.841
4.341
3.841
50
55
60
65
70
75
Temperature (°C)


Melting Peaks
-(d/dT) Fluorescence (640)
0.794
0.594
0.394
0.194
50
55
60
65
70
75
Temperature (°C)

4.8. Multiplex PCR



4.9. Detection methods:

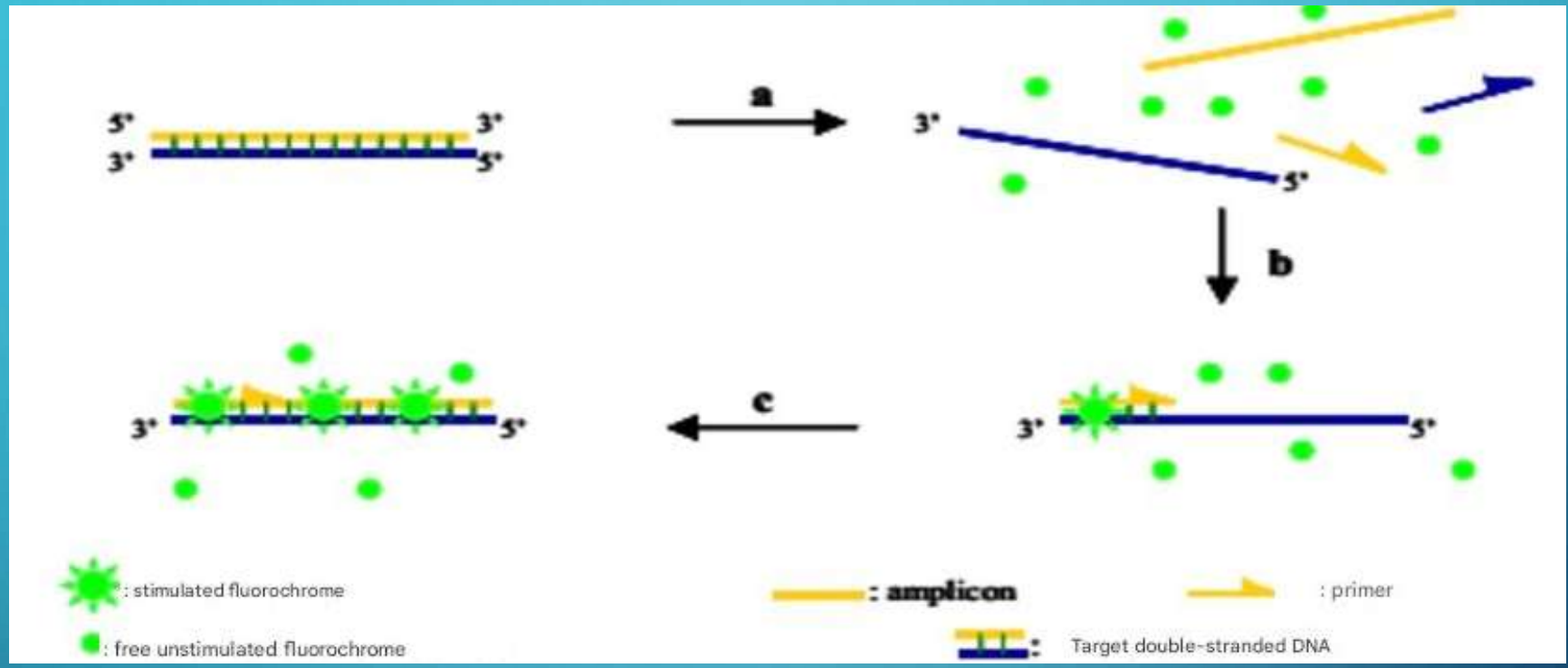


Fig: *Agents binding to double-stranded DNA.* (a) During denaturation, free SYBR Green I exhibits little fluorescence. (b) At the pairing temperature, a few molecules bind to the nascent double strand of DNA resulting in fluorescence emission upon excitation. (c) During the polymerization phase, more and more molecules bind to the nascent strand and the increase in fluorescence can be followed in real time.

4.10. Probes:

➢ Taqman or probe hydrolysis:

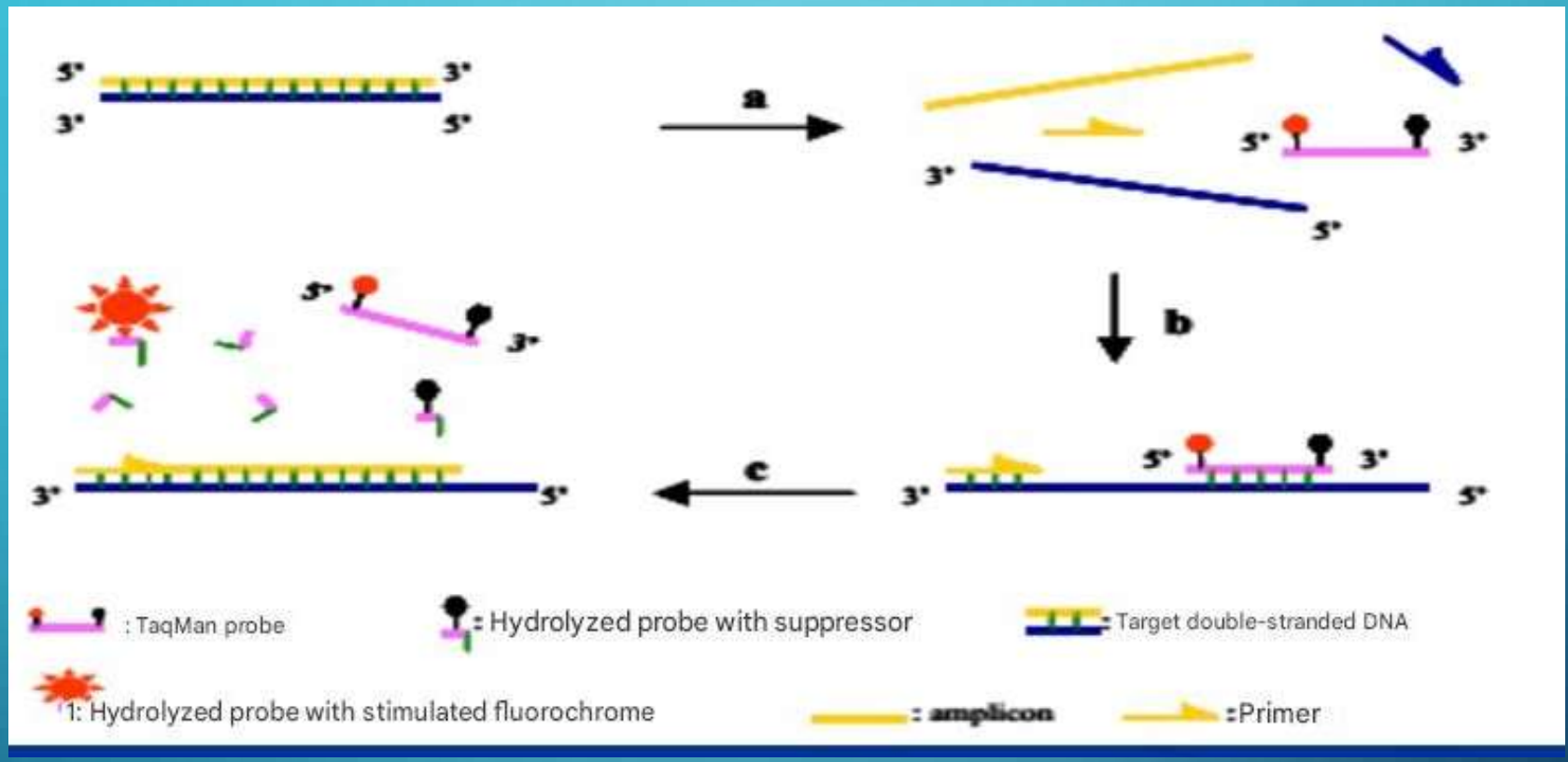


Fig: ***Hydrolysis of probes (Hydrolysis probes: Taqman assay)*** **(a) During the denaturation step, the probe is free in solution. (b) At the annealing temperature, the probe and primers hybridize to their respective target sequences and the proximity of the fluorochromes allows inhibition of fluorescence. Polymerization begins. (c) The polymerase displaces and hydrolyzes the probe. The emitting fluorochrome is released from the suppressor environment, allowing fluorescence emission.**

4.11. HybProbes (FRET) or hybridization of 2 probes

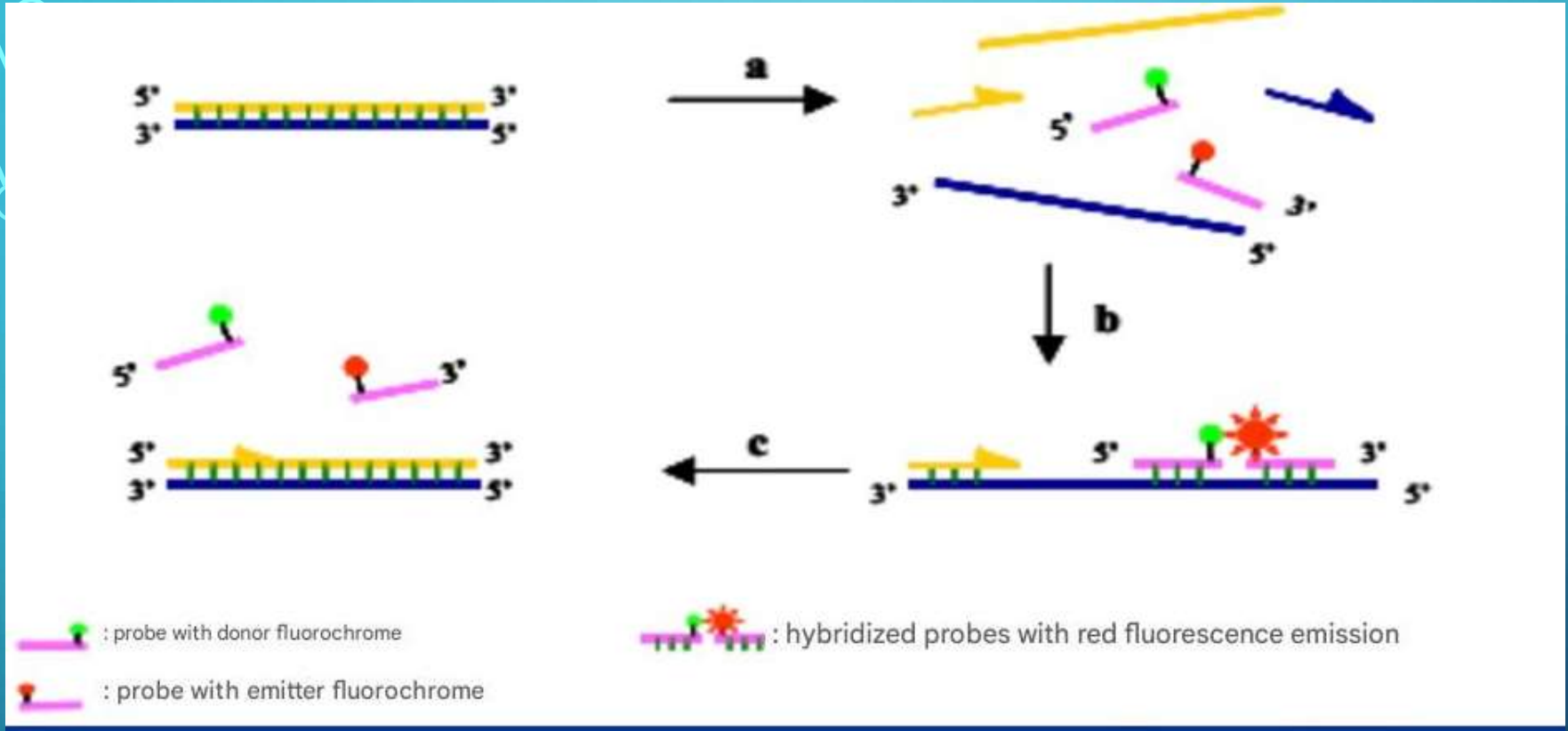


Fig.: *Hybridization of 2 probes* (a) During the denaturation step, the two probes remain separate and in solution. (b) At the temperature pairing, the probes hybridize to their respective target sequences and the proximity of the fluorochromes allows the emission of red fluorescence by the FRET principle. (c) The probes return free to solution.