BHARATHIDASAN UNIVERSITY

Tiruchirappalli- 620024,
Tamil Nadu, India

Programme : M.Sc., Biochemistry

Course Title : GENETIC ENGINEERING

Course Code : BC302CR

Unit – III

MOLECULAR TECHNIQUES

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GENETICENGINEERING

MOLECULAR TECHNIQUES-

PCR, DNA SEQUENCING- SANGER SEQUENCING AND MAXAM- GILBERT SEQUENCING

POLYMERASE CHAIN REACTION

•PCR was invented by Kary Mullis in 1983 and patented in 1985.

•Mullis was awarded the Nobel Prize in Chemistry in 1993 for inventing PCR.

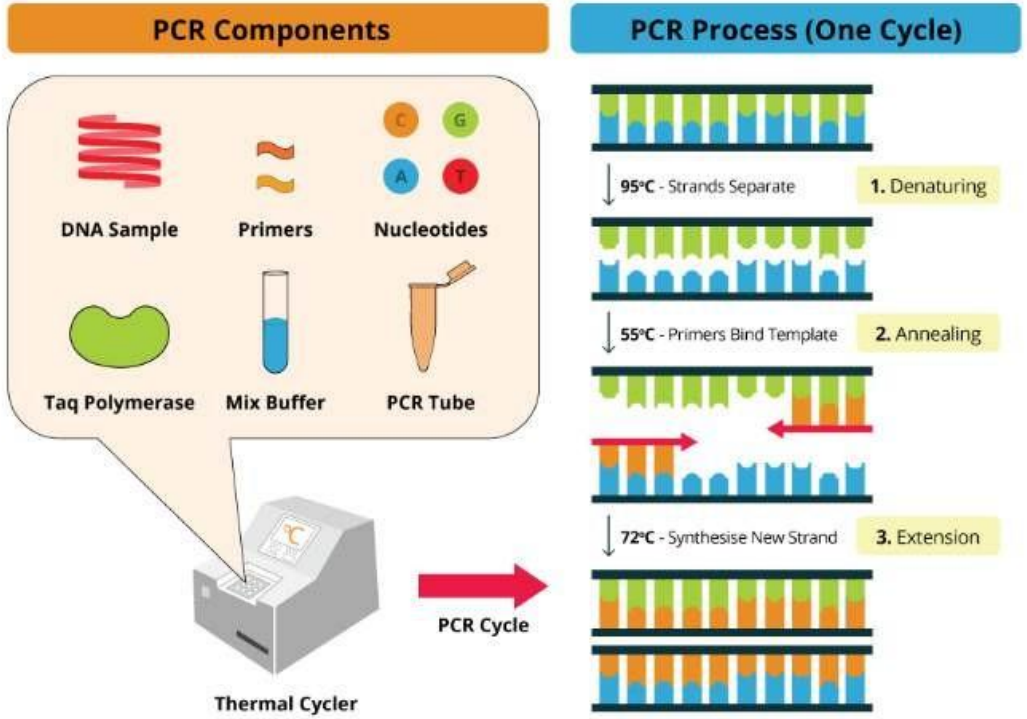
•The heat-resistant enzymes used in PCR were discovered in the 1960s from a microbe living in Yellowstone's hot springs

•PCR is an in vitro technique that replicates specific DNA sequences using DNA polymerase.

•It involves three main steps repeated in cycles: denaturation, annealing, and extension.

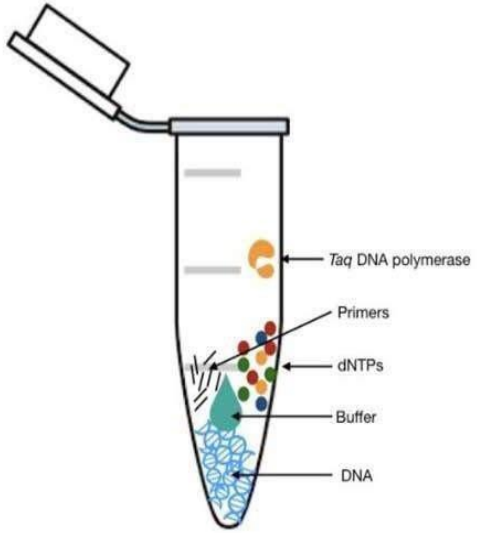
•PCR can generate billions of copies of a target DNA sequence from minute amounts of template DNA.

PRINCIPLE



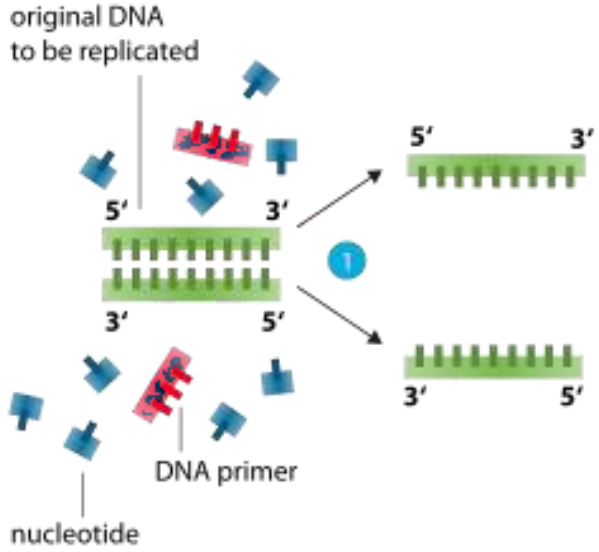
- It is a technique for obtaining large amounts of a specific DNA sequence from a DNA sample.
- This amplification is based on the replication of a double-stranded DNA template.
- It is broken down into three phases:
  - a denaturation phase,
  - a hybridization phase with primers [annealing], and
  - an elongation phase.
- The products of each synthesis step serve as a template for the following steps, thus exponential amplification is achieved

- The polymerase chain reaction is carried out in a reaction mixture which comprise
  - The DNA extract (template DNA),
  - Taq polymerase,
  - the primers, and
  - the four deoxyribonucleoside triphosphates (dNTPs) in excess in a buffer solution.



- The tubes containing the mixture reaction are subjected to repetitive temperature cycles several tens of times in the heating block of a **thermal cycler** (apparatus which has an enclosure where the sample tubes are deposited and in which the temperature can vary, very quickly and precisely, from 0 to 100°C by Peltier effect).
- The apparatus allows the programming of the duration and the succession of the cycles of temperature steps.
- Each cycle includes three periods of a few tens of seconds.

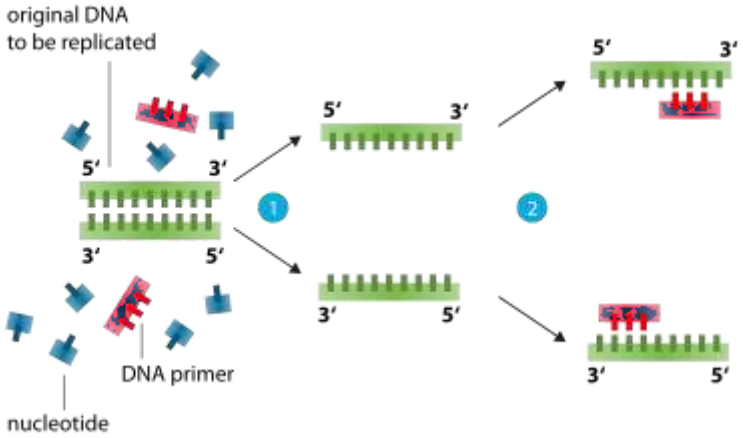
- The process of the PCR is subdivided into three stages as follows:
    1. **Denaturation**
    2. **Annealing**
    3. **Extension**



1. The Denaturation:

- It is the separation of the two strands of DNA, obtained by raising the temperature.
- The first period is carried out at a temperature of **94°C**, called the denaturation temperature.
- At this temperature, the matrix DNA[template], which serves as matrix during the replication, is denatured.
    - The hydrogen bonds cannot be maintained at a temperature higher than 80°C and the double-stranded DNA is denatured into single-stranded DNA (single-stranded DNA).

PRINCIPLE:

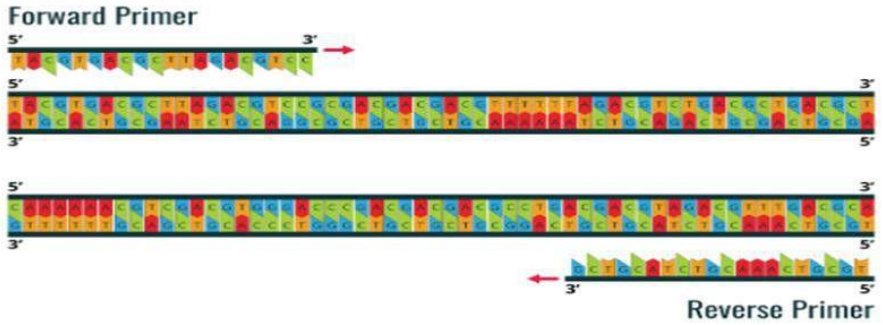


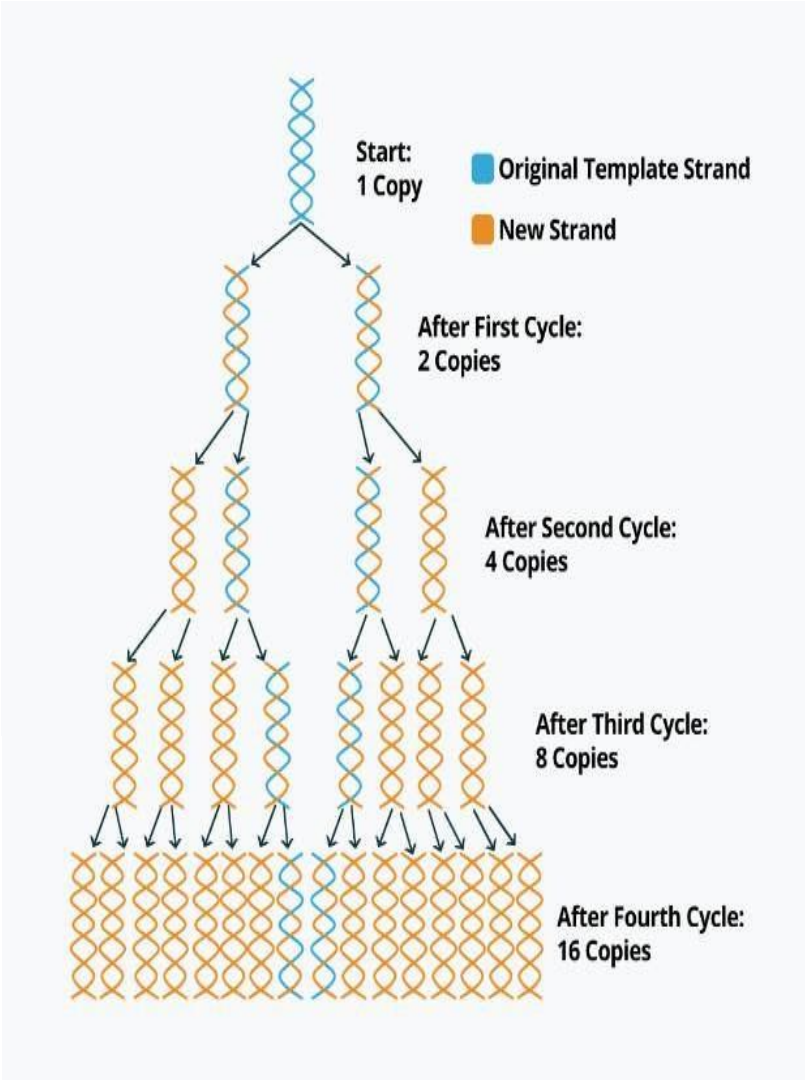
2. Annealing:

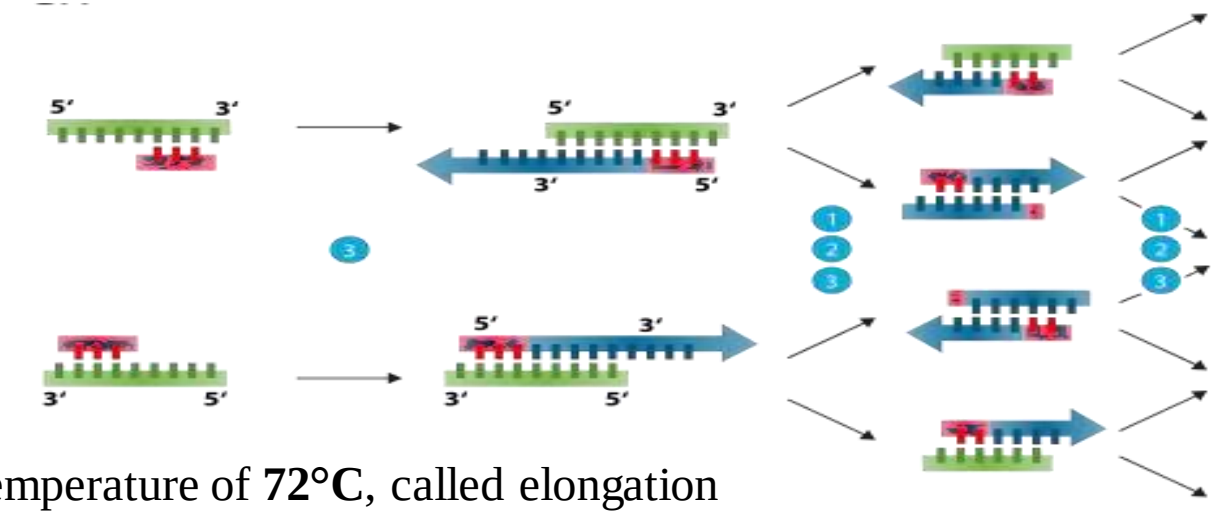
- The second step is hybridization/annealing.
- It is carried out at a temperature generally between **40 and 70°C**, called primer hybridization temperature.
- Decreasing the temperature allows the hydrogen bonds to reform and thus the complementary strands to hybridize.
- The primers, short single-strand sequences complementary to regions that flank the DNA to be amplified, hybridize more easily than long strand template DNA.
- The higher the hybridization temperature, the more selective the hybridization, the more specific it is.

PRIMERS:

- PCR utilizes the DNA polymerase enzyme, which naturally catalyzes the synthesis of DNA sequences.
- All DNA polymerases synthesize DNA in the 5'→3' direction.
- In order to start the synthesis process, it is necessary to have a pair of chemically synthesized **oligonucleotide primers made of DNA** (as the primers are synthesized by cells).
- These two primers are designed to flank the DNA fragment, which will be amplified.



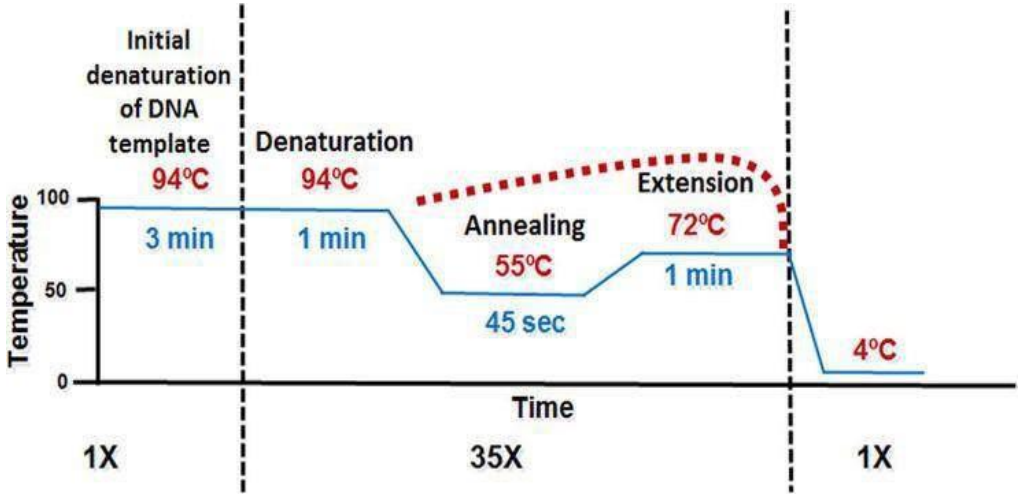




3. Elongation

- The third period is carried out at a temperature of **72°C**, called elongation temperature.
- It is the synthesis of the complementary strand.
- At 72°C, Taq polymerase binds to primed single-stranded DNAs and catalyzes replication using the deoxyribonucleoside triphosphates present in the reaction mixture.
- The regions of the template DNA downstream of the primers are thus selectively synthesized.

- In the next cycle, the fragments synthesized in the previous cycle are in turn matrix and after a few cycles, the predominant species corresponds to the DNA sequence between the regions where the primers hybridize.
- It takes 20–40 cycles to synthesize an analyzable amount of DNA (about 0.1 μg).
- Each cycle theoretically doubles the amount of DNA present in the previous cycle.
- It is recommended to add a final cycle of elongation at 72°C, especially when the sequence of interest is large (greater than 1 kilobase), at a rate of 2 minutes per kilobase.
- PCR makes it possible to amplify sequences whose size is less than 6 kilobases.
- The PCR reaction is extremely rapid, it lasts only a few hours (2–3 hours for a PCR of 30 cycles).



Taq polymerase

DNA polymerase allows replication.

A DNA polymerase purified or cloned from of an extremophilic bacterium, *Thermus aquaticus*, which lives in hot springs and resists temperatures above 100°C.

This polymerase (Taq polymerase) has the characteristic remarkable to withstand temperatures of around 100°C, which are usually sufficient to denature most proteins.

Thermus aquaticus finds its temperature of comfort at 72°C, optimum temperature for the activity of its polymerase

Taq DNA Polymerase

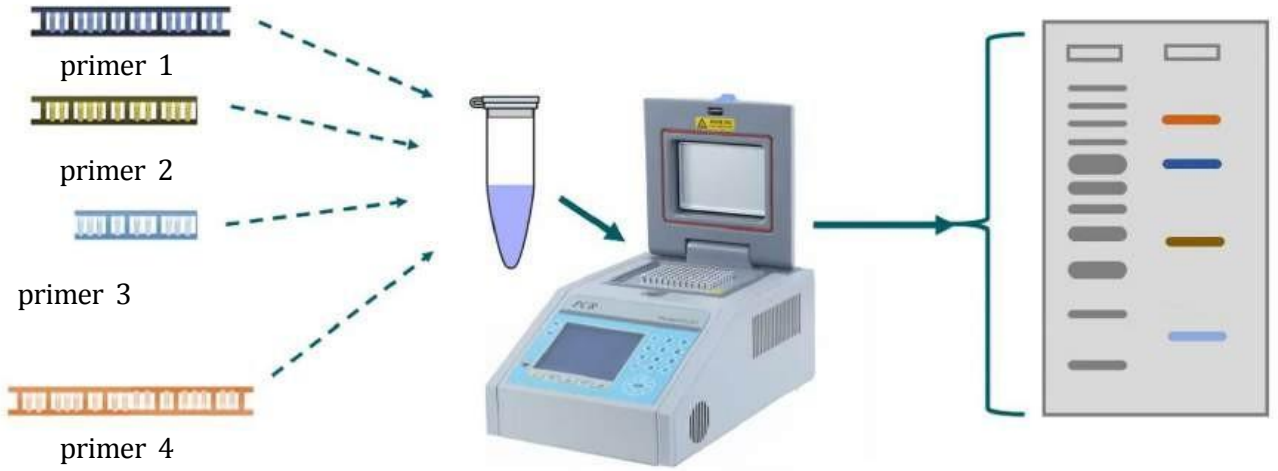
Initialization	96^0C	5 minutes
Denaturation	96^0C	30 seconds
Annealing	68^0C	30 seconds
Elongation	72^0C	45 seconds

Numbers of Cycles: The number of cycles required for amplification depends on the number of copies of template DNA present at the beginning of the reaction and the efficiency of primer extension and amplification. The completion of each cycle doubles the number of target DNA molecules. A 30 cycle amplification process generally amplifies the target between 100,000-10,000,000 fold. Subsequently target sequences from 100-2000 bp in length are most commonly used for PCR amplification.

TYPES OF PCR:

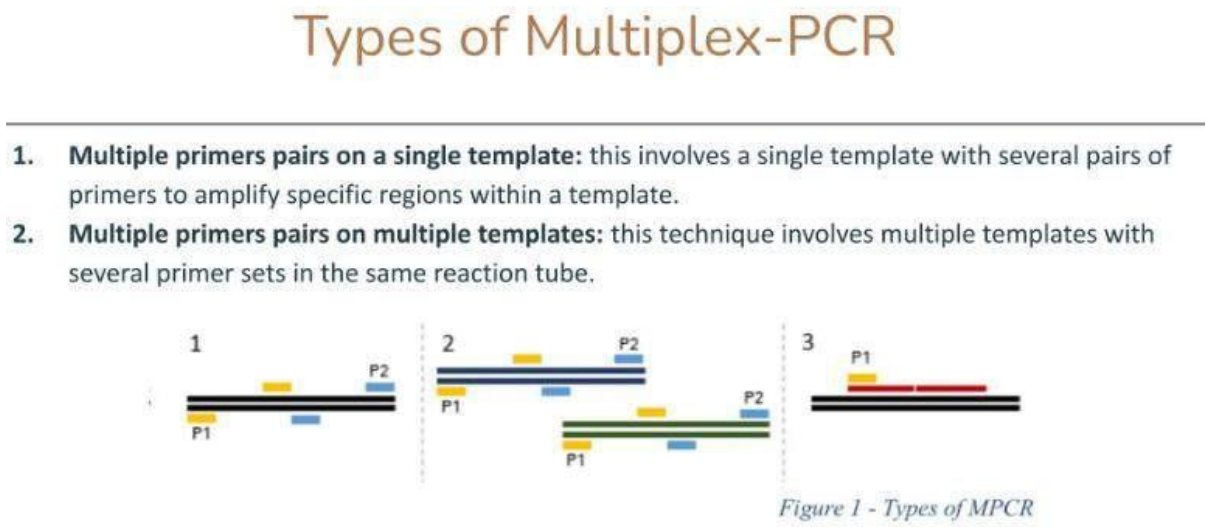
- Conventional (Qualitative)PCR.
- Multiplex PCR
- Nested PCR
- Reverse transcriptase-PCR
- qRT-PCR
- Methylation-specific PCR.

1. MULTIPLEX PCR:



- Multiplexing is a term that refers to multiple samples being processed at the same time, usually to save time and money.
- Multiplex PCR is a technique whereby PCR is used to amplify several different DNA sequences simultaneously.
- It is a type of target enrichment approach.
- It was **first described in 1988** as a method to detect deletion mutations in the dystrophin gene – the largest known human gene.
- The dystrophin complex connects the cytoskeleton of muscle cells with the extracellular matrix, which consists of proteins and other molecules outside of the cell.

MULTIPLEX PCR:

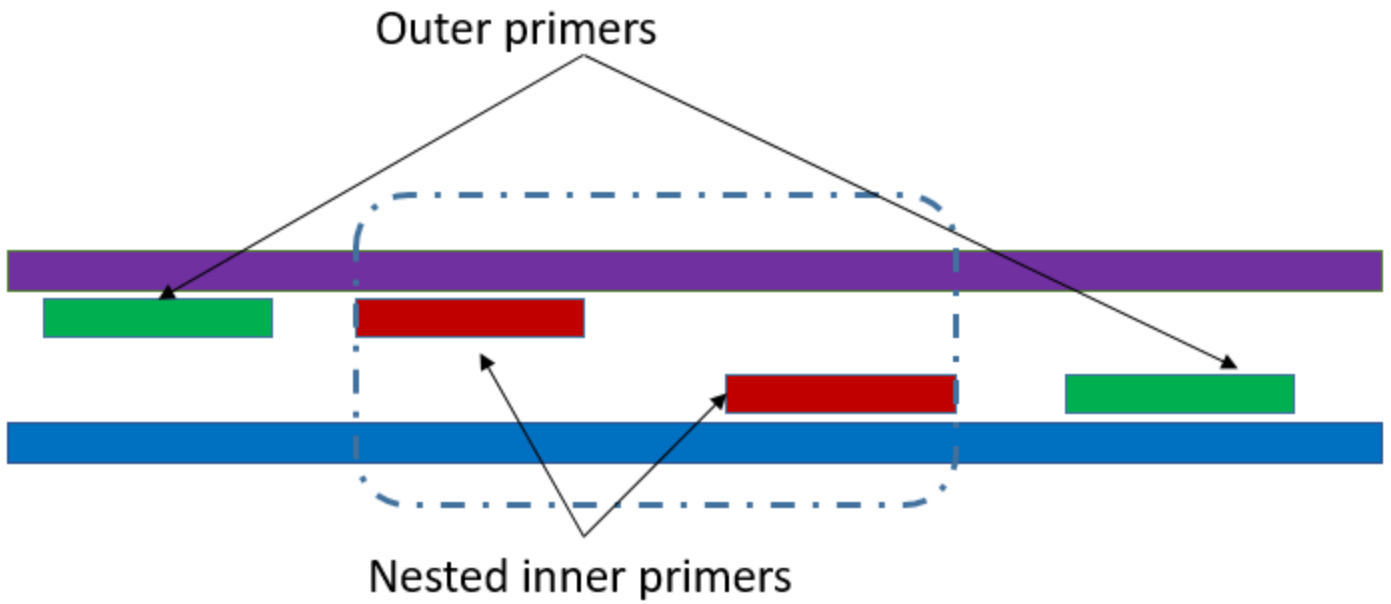


APPLICATIONS OF MULTIPLEX PCR

- Pathogen detection: Multiplex PCR is a valuable tool for identifying bacteria, viruses, fungi, and parasites that cause infectious diseases.
- Forensic identity testing: Multiplex PCR is used for forensic identity testing, such as STR typing.
- Gene expression and deletion analysis: Multiplex PCR can be used to analyze gene expression and deletion

2.NESTED-PCR

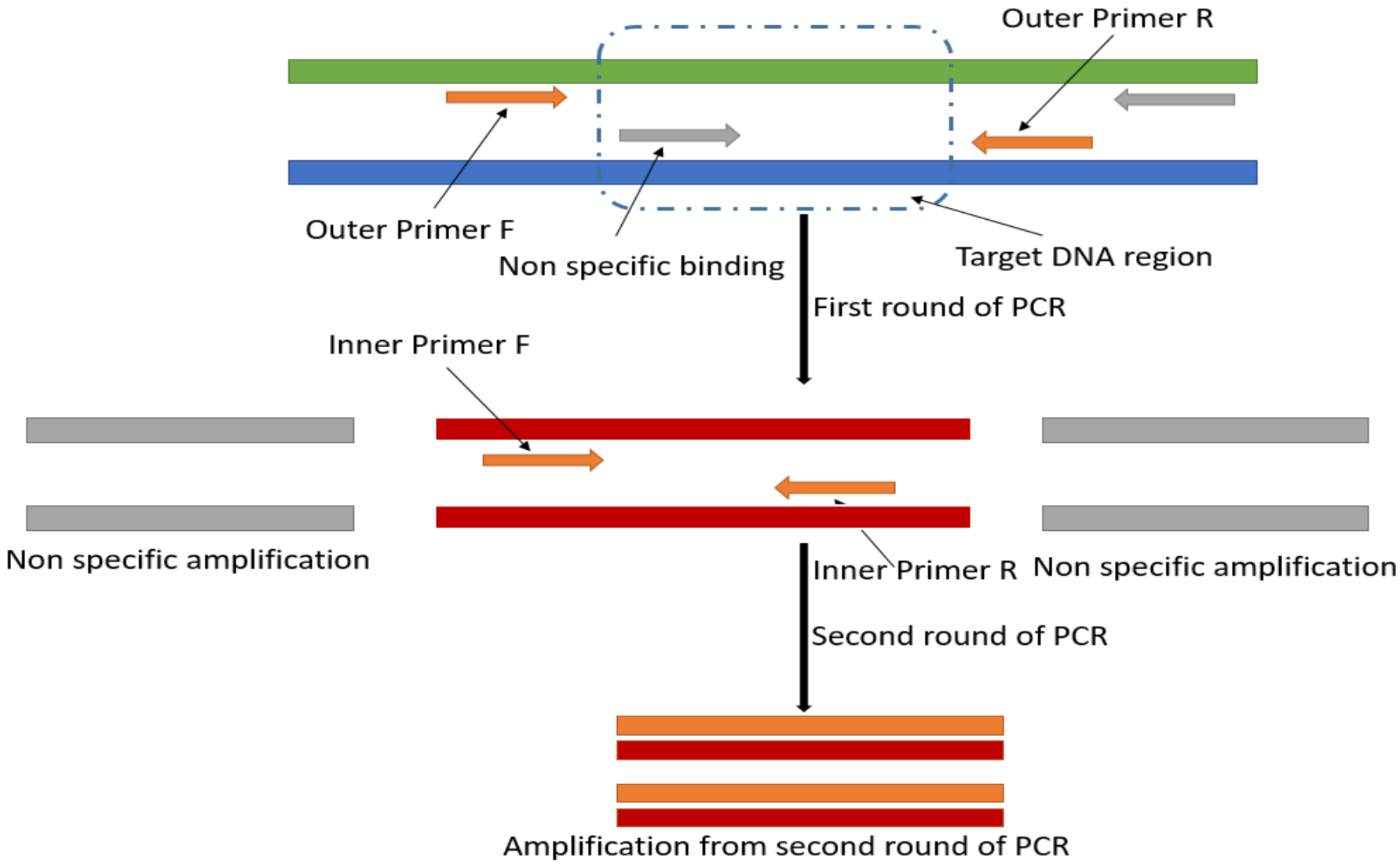
- Nested polymerase chain reaction (Nested PCR) is a kind of polymerase chain reaction which reduce non-specific binding in products with the help of the two sets of primer.
- Any additional reagent, chemical or instrumentation besides conventional PCR reactions is not required.
- Nested PCR uses perform two round of PCR with two sets of primers.
- The products of second round of PCR are the target gene fragments



NESTED-PCR:

- The principle of the nested PCR experiment is to design two pairs of primers based on the DNA template sequence, and use the external primers to perform standard amplification of target DNA for 15-30 cycles.
- After the first round of amplification, a small portion of the initial amplification product is diluted 100-1000 times and added to the second round of the amplification system as a template, using internal primers (bound to the interior of the first round of PCR products) 30 cycles of amplification.
- The amplified fragments of the second round of PCR were shorter than the first round. Compared to conventional PCR technique, the use of two sets of primers increases the specificity of the amplification because there are fewer target sequences that are complementary to both sets of primers.
- If the first amplification produces an incorrect fragment, the probability of paired amplification of the internal primer and the incorrect fragment is extremely low, thereby increasing the specificity and sensitivity of the PCR amplification reaction.

NESTED-PCR:


Outer Primer R
Outer Primer F
Non specific binding
Target DNA region
First round of PCR
Inner Primer F
Non specific amplification
Inner Primer R
Non specific amplification
Second round of PCR
Amplification from second round of PCR

3. REVERSE TRANSCRIPTASE-PCR

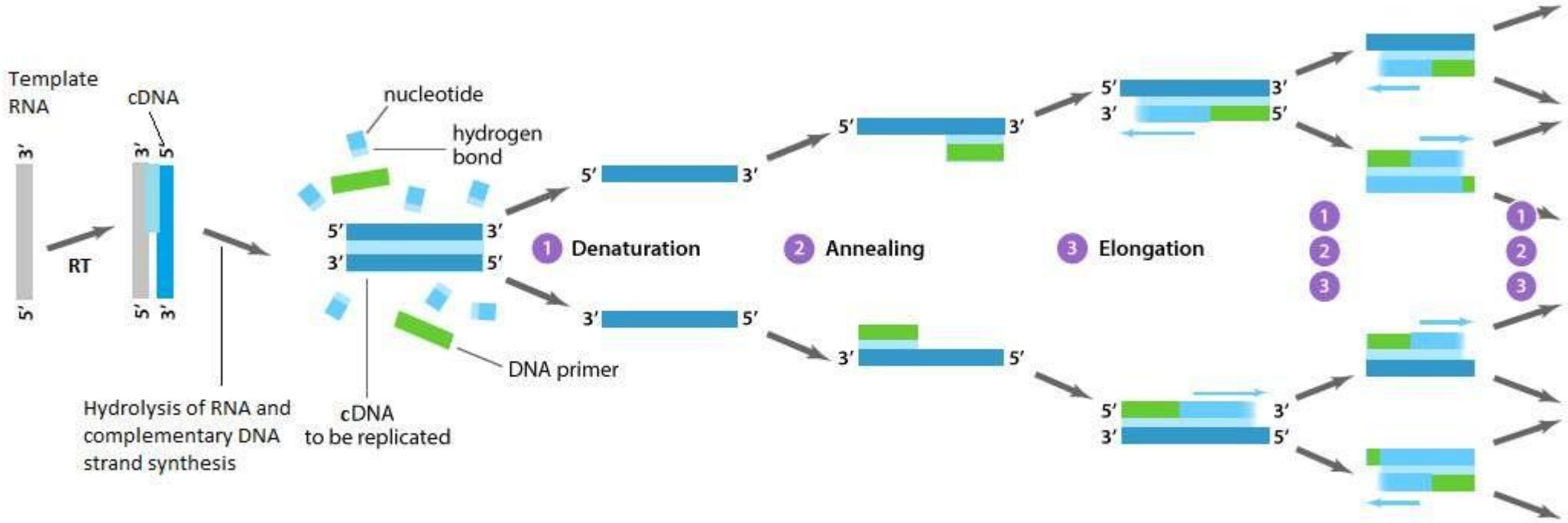
- Reverse transcriptase PCR, instead of DNA, uses mRNA as the starting template.
- the principle of converting the RNA template to a complementary DNA (cDNA) using the reverse transcriptase enzyme. This cDNA then undergoes exponential amplification using PCR to form multiple copies, which are then used for downstream analysis.
- In a typical PCR, DNA is the template, and the enzyme used is Taq polymerase.
- In order to amplify RNA, reverse transcription needs to be carried out since RNA is not an efficient template for Taq polymerase.
- Hence, the modified version of PCR has an extra step of RNA being converted to DNA, then PCR is carried out.

3. REVERSE TRANSCRIPTASE-PCR

The RT-PCR process:

- After RNA isolation is performed, the RNA is extracted.
- An aliquot of extracted RNA sample is added to a reaction mixture. The reaction mixture consists of nucleotides, reverse transcriptase, and primers specific for the gene of interest.
- Primers will anneal to the extracted RNA if the target is present.
- Reverse transcriptase performs its function of synthesizing the complementary DNA (cDNA) strand.
- The RNA/DNA hybrid now undergoes the steps of PCR:
- **Denaturation**: At 95^{o} C the RNA/DNA strands denature.
- **Annealing**: Primers anneal to the newly formed cDNA
- **Extension**: Extending from the primer, the polymerase synthesizes a new DNA strand by replication.
- Multiple cycles in the thermal cycler increase the number of copies of DNA as PCR products.

3.REVERSE TRANSCRIPTASE-PCR



REVERSE TRANSCRIPTION PCR

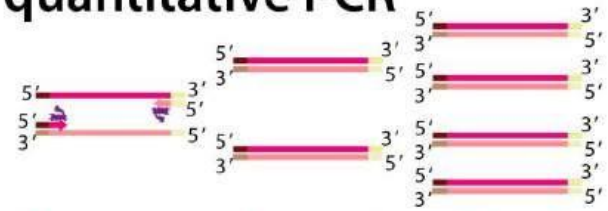
4. qRT-PCR

- Quantitative PCR can 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 in proportion to the amplification of the PCR product, cycle after cycle.
- It requires a thermal cycler coupled to an optical reading system that measures fluorescence emission.
- A nucleotide probe is synthesized so that it can hybridize selectively to the DNA of interest between the sequences where the primers hybridize.
- The probe is labeled on the 5′ end with a fluorochrome signal (e.G., 6-carboxyfluorescein), and on the 3′ end with a quencher (e.G., 6-carboxy-tetramethyl rhodamine).
- This probe must show temperature hybridization (tm) greater than that of the primers so that it hybridizes 100% during the elongation phase (critical parameter)

4. qRT-PCR

qPCR counts how many copies of a sequence there are

quantitative PCR



makes lots of copies of a tested-for region in the starting DNA & produces a fluorescent signal corresponding to how many are being made

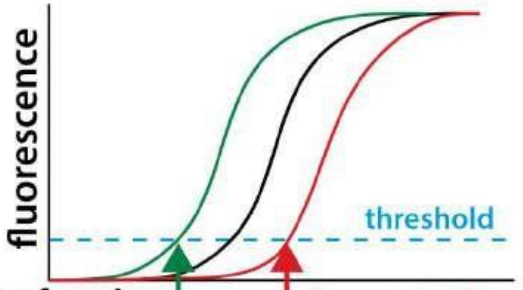
the more copies you start with, the faster the fluorescence rises, so the fewer cycles are required to pass the threshold (the lower the Cq)

where's the signal come from?

dsDNA (double-stranded DNA) binding dyes bind & fluoresce

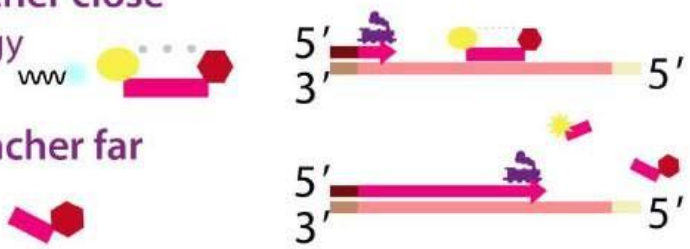
or

reporter probes bind in the amplified region and get cleaved in the next cycle



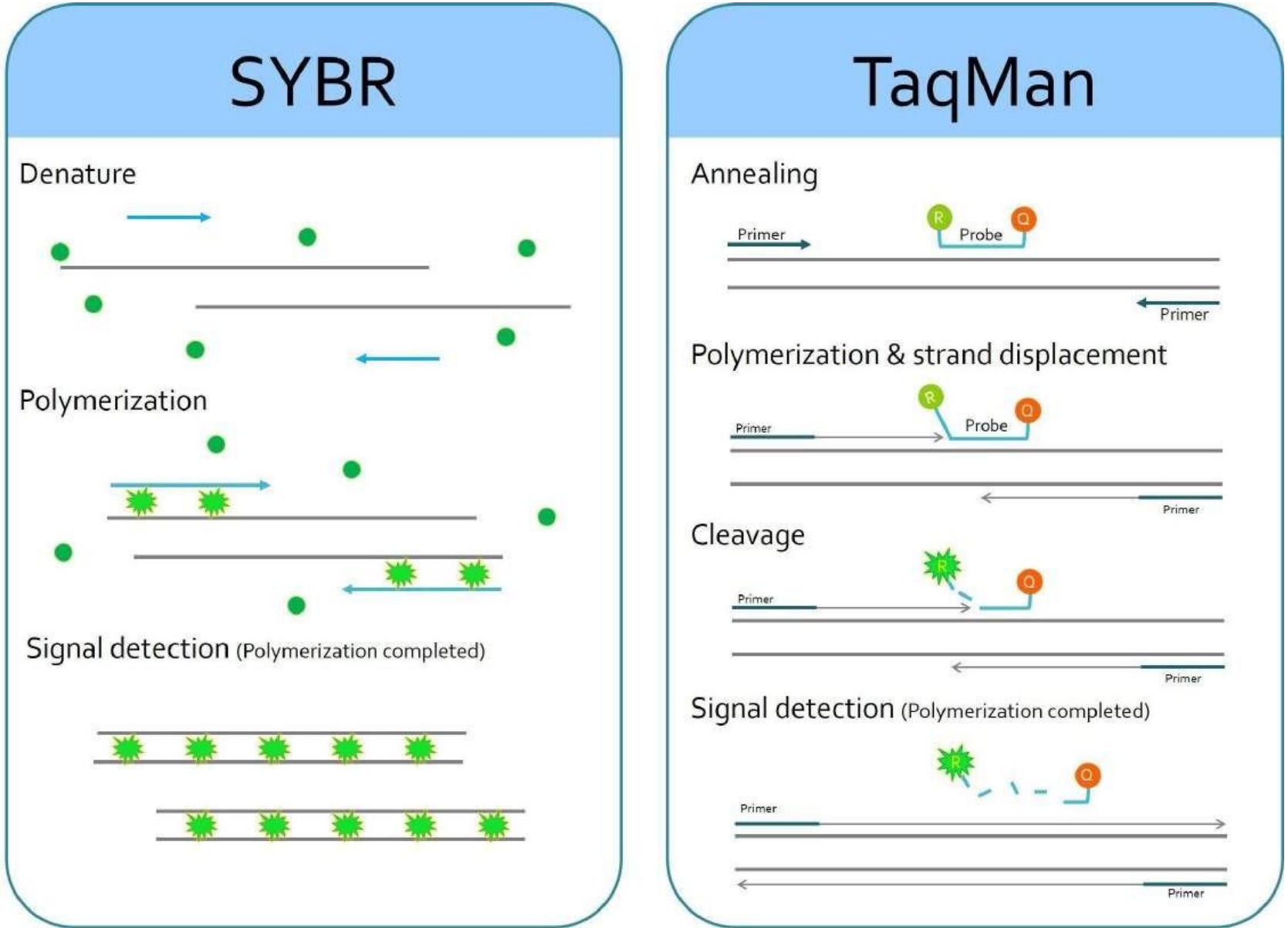
fluorophore & quencher close

FRET (non-radiative energy transfer) hides signal



fluorophore & quencher far

4. qRT-PCR


SYBR
Denature
Polymerization
Signal detection (Polymerization completed)
TaqMan
Annealing
Primer
R
Probe
Q
Primer
Polymerization & strand displacement
Primer
R
Probe
Q
Primer
Cleavage
Primer
R
Q
Primer
Signal detection (Polymerization completed)
R
Q
Primer
Primer

4. qRT-PCR

There are many different markers used in Real Time PCR but the most common of them include:

1. Taqman probe.
2. SYBR Green.

Taqman Probe

•It is a hydrolysis probe which bear a reporter dye, often fluorescein (FAM) at its 5' end and a quencher tetramethylrhodamine (TAMRA), attached to the 3' end of the oligonucleotide.

•Unders normal conditions, the probe remain coiled on itself bringing the fluorescence dye near the quencher, which inhibits or quenches of fluorescent signal of the dye.

•The oligonucleotide of the Taqpolymerase has a homologous region with the target gene and thus when the target sequence is present in the mixture, it bind with the sample DNA.

•As the taqpolymerase start to sunthesize new DNA strand in the extension stage, it causes degradation of the probe by 5' end nuclease activity and the fluorescein is separated from the quencher as a result of which fluorescence signal is generated.

•As this procedure continues, in each cycle the number of signal molecule increases, causing the increase in fluorescence which is positively related with the amplification of the target.

4. qRT-PCR

SYBR Green

•This is a dye that emits prominent fluorescent signal when it binds at the minor groove of DNA, nonspecifically.

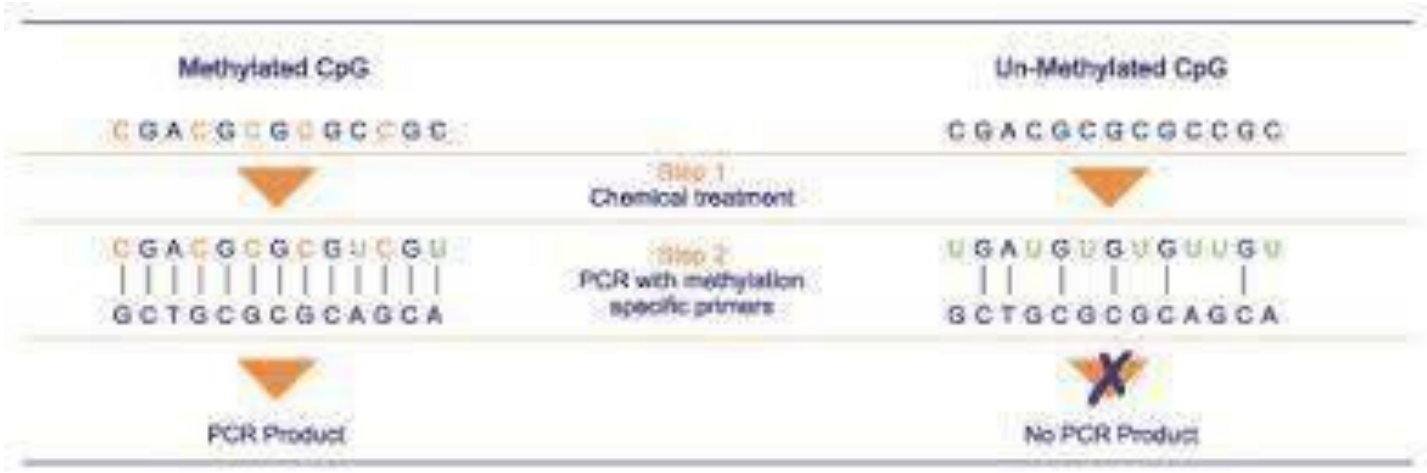
•Other fluorescent dyes like Ethidium Bromide or Acridine Orange can also be used but SYBR Green is better used for its higher signal intensity.

•SYBR Green is more preferred than the Taqman Probe as it can provide information about each cycle of amplification as well as about the melting temperature which is not obtained from the Taqman probe.

•However, its disadvantage is the lack of specificity as compared to Taqman Probe.

5. METHYLATION-SPECIFIC PCR

- Used to identify patterns of DNA methylation at cytosine guanine islands (C&G islands) in genomic DNA. CpG islands, are concerned in regulation of gene expression in mammalian cells.
- Target DNA is first treated with sodium bisulfite,which converts unmethylated cytosine bases to uracil, which is complementary to adenosine in PCR primers.
- Two amplifications are then carried out on the bisulfite-treated DNA:
  - One primer set anneals to DNA with cytosine(corresponding to methylated cytosine),
  - The other set anneals to DNA with uracil(corresponding to unmethylated cytosine).
  - MSP used in quantitative PCR provides quantitative information about the methylation state of a given CpG island.



APPLICATIONS OF PCR:

Forensic Science

•*DNA Profiling*: PCR is essential for analyzing DNA samples in criminal investigations, allowing for the identification of individuals from minute biological samples, such as hair or blood.

•*Paternity Testing*: It is used to establish biological relationships by comparing DNA profiles.

Research and Genetics

•*Gene Expression Studies*: PCR, particularly reverse transcription PCR (RT-PCR), is utilized to analyze gene expression levels by converting mRNA into cDNA for amplification.

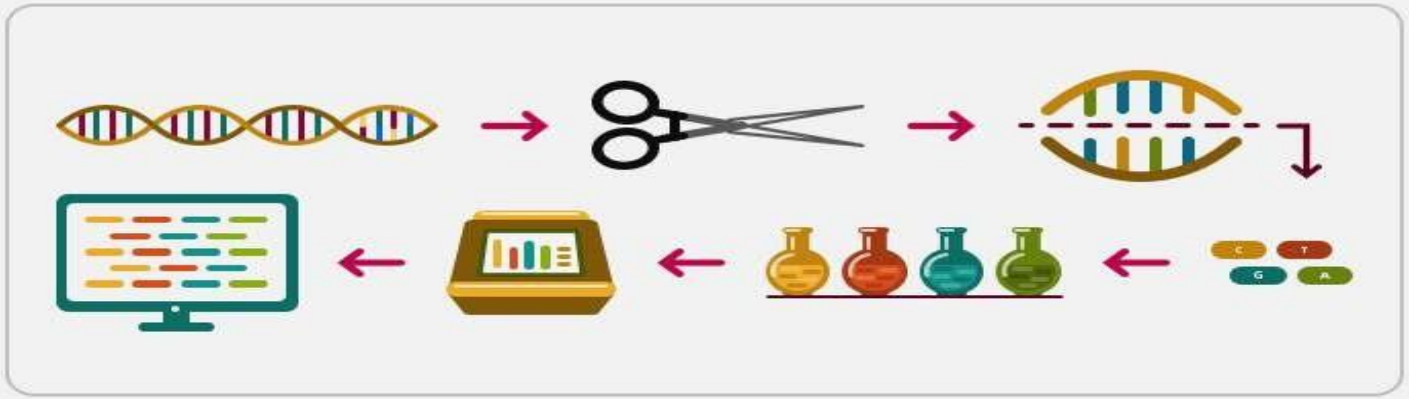
•*Phylogenetic Analysis*: It aids in studying evolutionary relationships by amplifying DNA from various organisms, including ancient specimens.

•*Cloning and DNA Sequencing*: PCR simplifies the cloning process by amplifying specific DNA fragments, making it easier to prepare samples for sequencing

DNA SEQUENCING:

GENOMIC SEQUENCING

What is Genomic Sequencing?



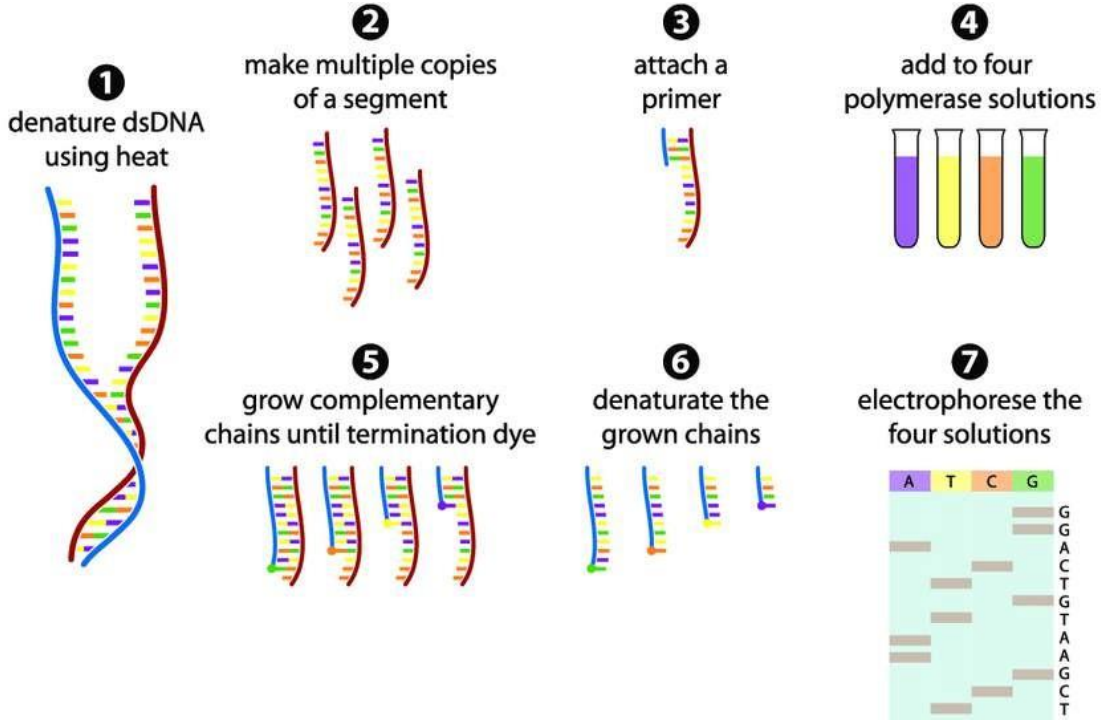
GENOMIC SEQUENCING

Genomic sequencing refers to methods of determining the entire DNA sequence of an organism's genome. In simpler terms, it determines the order of As, Ts, Cs and Gs that make up an organism's DNA. A genomic sequence is depicted by a very long line of these letters arranged in a specific order.

Definition	Determines the order of nucleotides in DNA, identifying the sequence of adenine, guanine, cytosine, and thymine.
Importance	Essential for biological research, medical diagnosis, biotechnology, forensic biology, virology, and systematics.
Applications	Diagnosing diseases, characterizing antibody repertoire, guiding patient treatment, and organism identification.

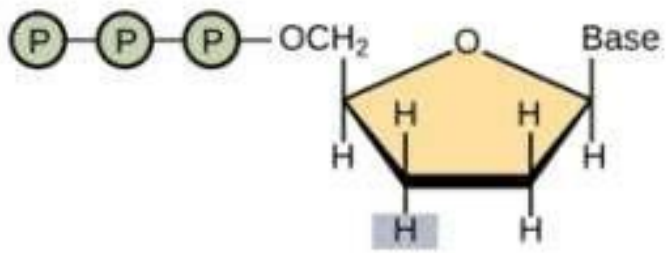
SANGER'S DNA SEQUENCING:

- Sanger sequencing = chain termination method
- Developed by **Frederick Sanger and his colleagues in 1977.**
- Designed for determining the sequence of nucleotide bases in a piece of DNA
- Sanger sequencing was used in the Human Genome Project to determine the sequences of relatively small fragments of human DNA (900 bp or less).
- These fragments were used to assemble larger DNA fragments and, eventually, entire chromosomes.

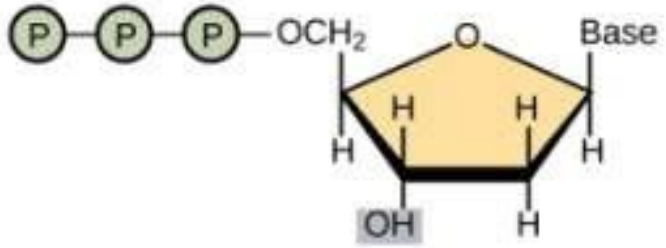


SANGER'S DNA SEQUENCING:

- A DNA primer complementary to the template DNA (the DNA to be sequenced) is used to be a starting point for DNA synthesis.
- In the presence of the four deoxynucleotide triphosphates (dNTPs: A, G, C, and T), the polymerase extends the primer by adding the complementary dNTP to the template DNA strand.
- To determine which nucleotide is incorporated into the chain of nucleotides, four dideoxynucleotide triphosphates (ddNTPs: ddATP, ddGTP, ddCTP, and ddTTP) labeled with a distinct fluorescent dye are used to terminate the synthesis reaction.
- Compared to dNTPs, ddNTPs has an oxygen atom removed from the ribonucleotide, hence cannot form a link with the next nucleotide.
- Following synthesis, the reaction products are loaded into four lanes of a single gel depending on the diverse chain-terminating nucleotide and subjected to gel electrophoresis.
- According to their sizes, the sequence of the DNA is thus determined.



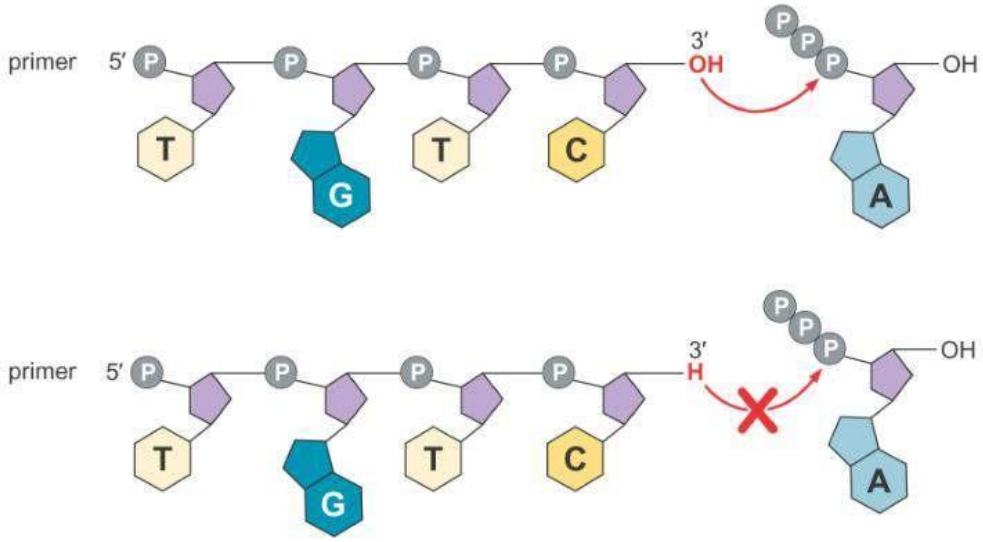
Dideoxynucleotide (ddNTP)



Deoxynucleotide (dNTP)

SANGER'S DNA SEQUENCING:

- Sanger introduced a specific factor, denoted as ddNTP. Though ddNTP can be utilized by DNA polymerase as a substrate for DNA strand synthesis similarly to dNTP, it lacks an oxygen atom at the 3′ end.
- Consequently, it is unable to form a phosphodiester bond with the adjacent deoxyribonucleotide, which ultimately inhibits DNA synthesis at the point of ddNTP incorporation.



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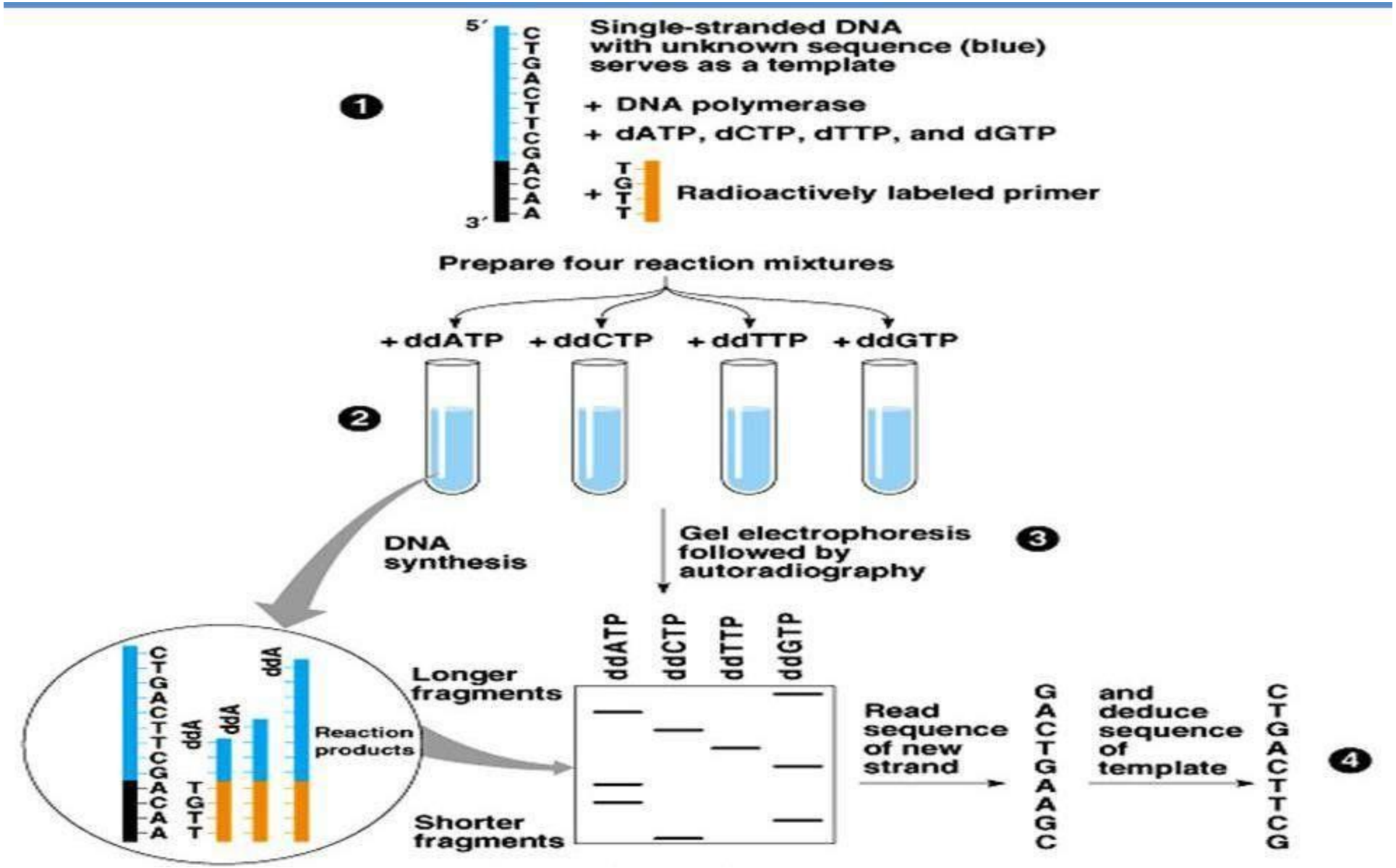

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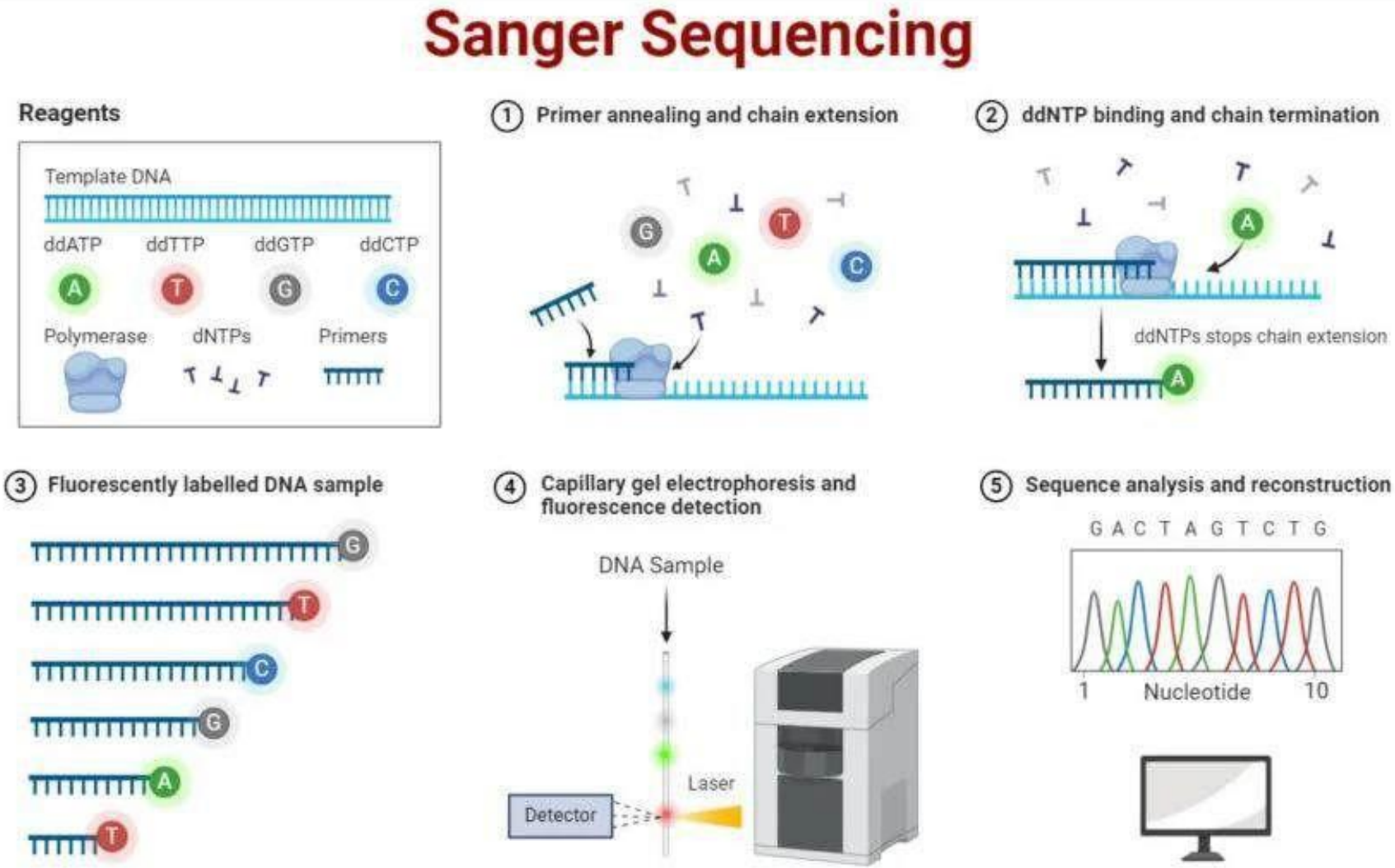


SANGER'S DNA SEQUENCING:

APPLICATION:

•Sanger sequencing is used **in medical diagnosis** to identify genes associated with different diseases. It is useful for targeted sequencing of specific regions of the genome making it a useful tool in clinical diagnostics and genetic testing.

•Sanger sequencing can be used **to identify new species** by comparing them with gene sequences of already known species. It is also useful to study the evolutionary history of many species.

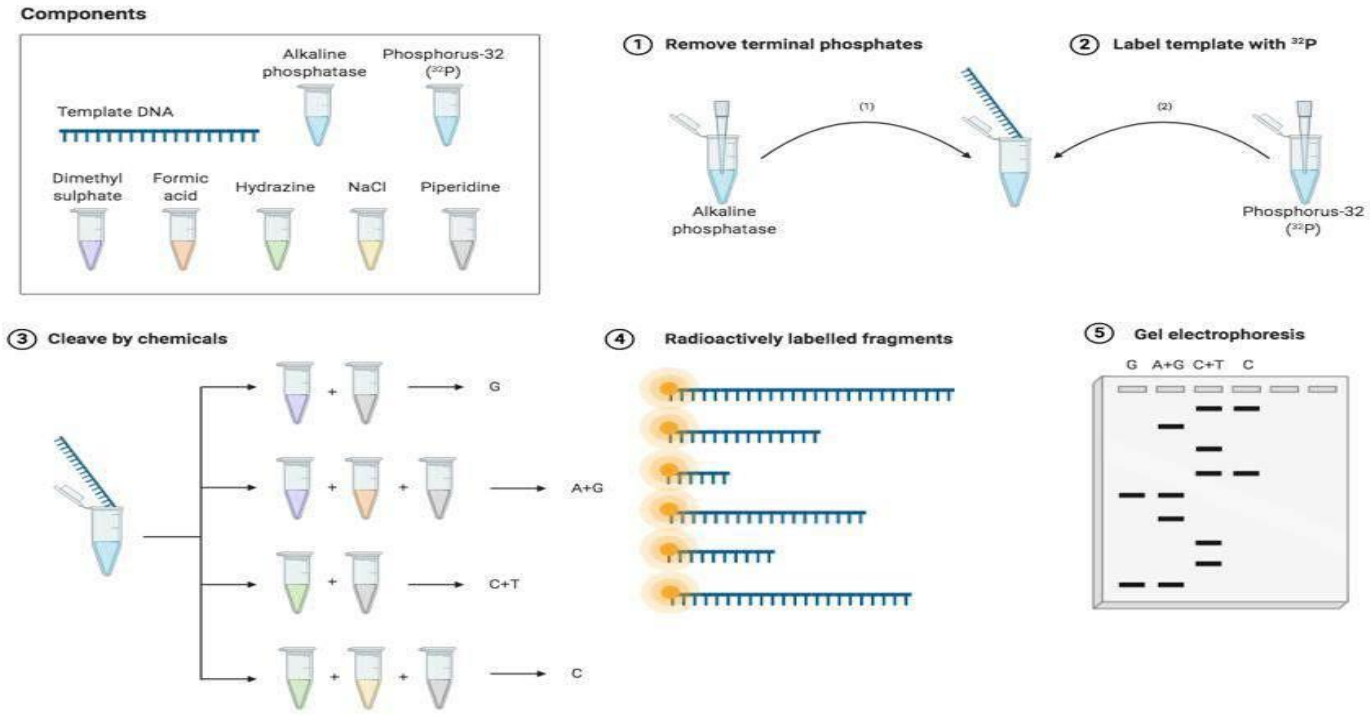
•**In forensic science**, sanger sequencing is useful in personal identification and DNA fingerprinting. This helps us analyze DNA evidence in criminal cases.

•Sanger sequencing is also useful **in agriculture** to identify different breeds of crops and livestock. It can be used in the conservation of these species.

•Sanger sequencing also finds applications in newer technologies including single-cell sequencing and synthetic biology.

MAXAM-GILBERT DNA SEQUENCING:

- Maxam-gilbert sequencing = Chemical sequencing
- In **1976-1977, Allan Maxam and Walter Gilbert** developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases.
- Although Maxam and Gilbert published their chemical sequencing method two years after the ground-breaking paper of Sanger and Coulson on plus-minus sequencing



MAXAM-GILBERT DNA SEQUENCING: PRINCIPLE

- A strand of source DNA is labeled at one end with 32P. The two strands of DNA are then separated.
- The labeled DNA is distributed into four samples (in separate tubes).
- Each sample is subjected to treatment with a chemical that specifically destroys one(G, C) or two bases (A + G, T + C) in the DNA.
- Thus, the DNA strands are partially digested in four samples at sites G, A + G, T + C and C.
- This results in the formation of a series of labeled fragments of varying lengths.
- The actual length of the fragment depends on the site at which the base is destroyed from the labeled end.
- Thus for instance, if there are C residues at positions 4, 7, and10 away from the labeled end, then the treatment of DNA that specifically destroys C will give labeled pieces of length 3, 6 and 9 bases.
- The labeled DNA fragments obtained in the four tubes are subjected to electrophoresis side by side and they are detected by autoradiograph.
- The sequence of the bases in the DNA can be constructed from the bands on the electrophoresis.

MAXAM-GILBERT DNA SEQUENCING:

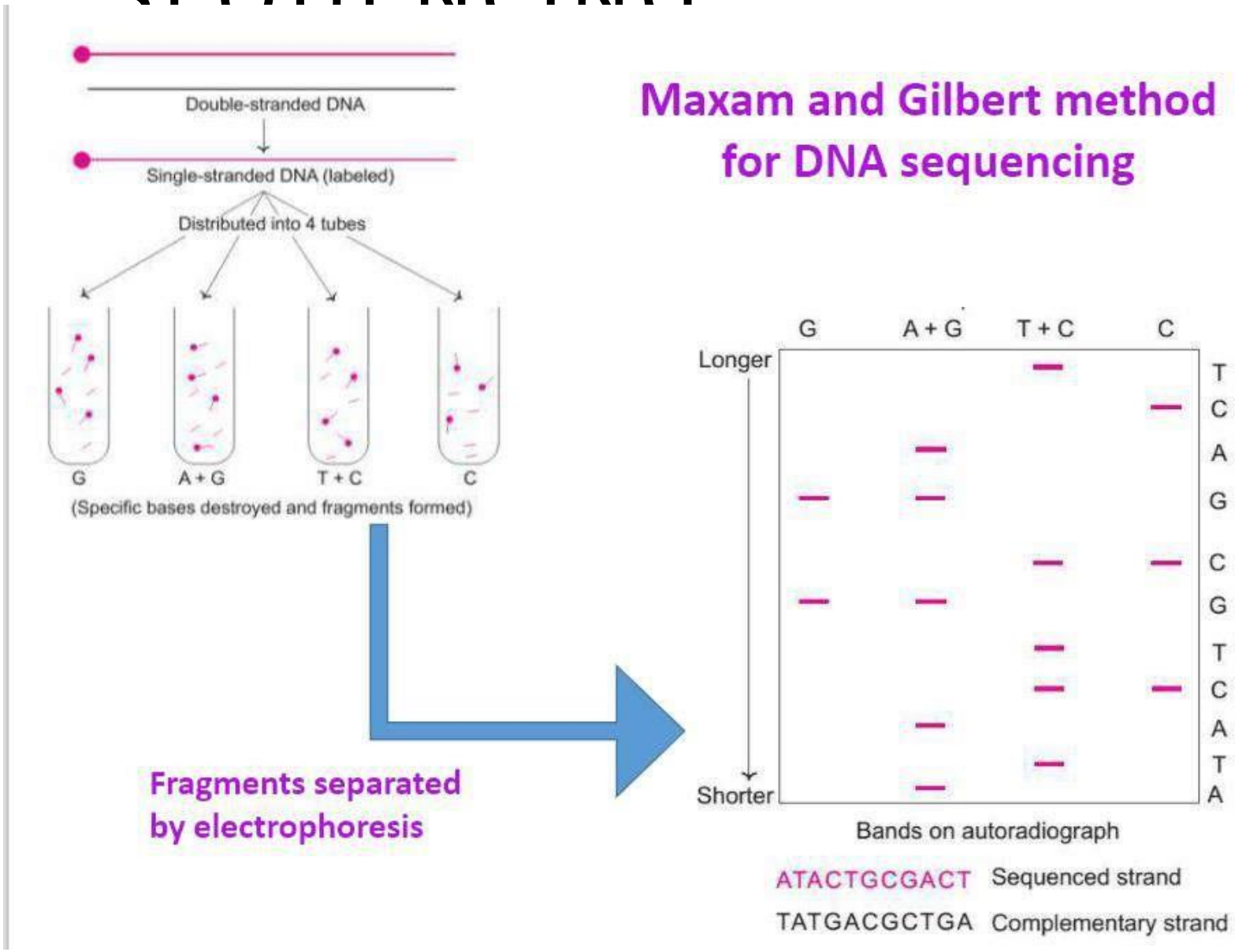
- The chemical reactions in Maxam Gilbert method involve a two-step chemical degradation process using piperidine and two chemicals that selectively attack purines and pyrimidines.
- Purines will react with dimethyl sulfate (DMS), and pyrimidines will react with hydrazine.
- These reactions will break the glycoside bond between the ribose sugar and the base and displace the base.
- Piperidine will then catalyze phosphodiester bond cleavage where the base has been displaced.

	Base removal	cleavage
G	DMS	Piperidine
A+G	DMS + Formic acid	Piperidine
C+T	Hydrazine	Piperidine
C	Hydrazine + 1.5M NaCl	Piperidine

MAXAM-GILBERT DNA SEQUENCING:

- Dimethyl sulfate (DMS) alone will selectively cleave guanine nucleotides, while DMS along with formic acid will cleave both guanine and adenine nucleotides. Similarly, hydrazine alone will cleave both thymine and cytosine nucleotides, whereas hydrazine and 1.5M NaCl will selectively cleave cytosine nucleotides.
- Thus, a series of labelled fragments are generated from the radiolabeled end to each molecule's first 'cut'.
- The fragments in the four reactions are electrophoresed side by side in denaturing acrylamide gels for size separation.
- To visualize the fragments, the gel is exposed to X-ray film for autoradiography, yielding a series of dark bands each showing the location of identical radiolabeled DNA molecules.
- From presence and absence of certain fragments the sequence may be inferred

MAXAM-GILBERT DNA SEQUENCING:



MAXAM-GILBERT DNA SEQUENCING:

ADVANTAGES:

- Directly read purified DNA
- No premature termination due to DNA sequencing, so no problem with polymerase to synthesize DNA.
- Used sequence heterogeneous DNA as well as homopolymeric sequences
- Used to analyse DNA protein interaction i.e footprinting
- Used to analyse epigenic modification and nucleic acid structure to DNA.

DISADVANTAGES:

- Not widely used.
- Use of toxic chemicals and extensive use of radioactive isotopes, highly poisonous and unstable.
- Cannot read more than 500bp.
- Setup is quite complex, technically complexity
- It is difficult to make Maxam Gilbert based DNA sequencing kit.

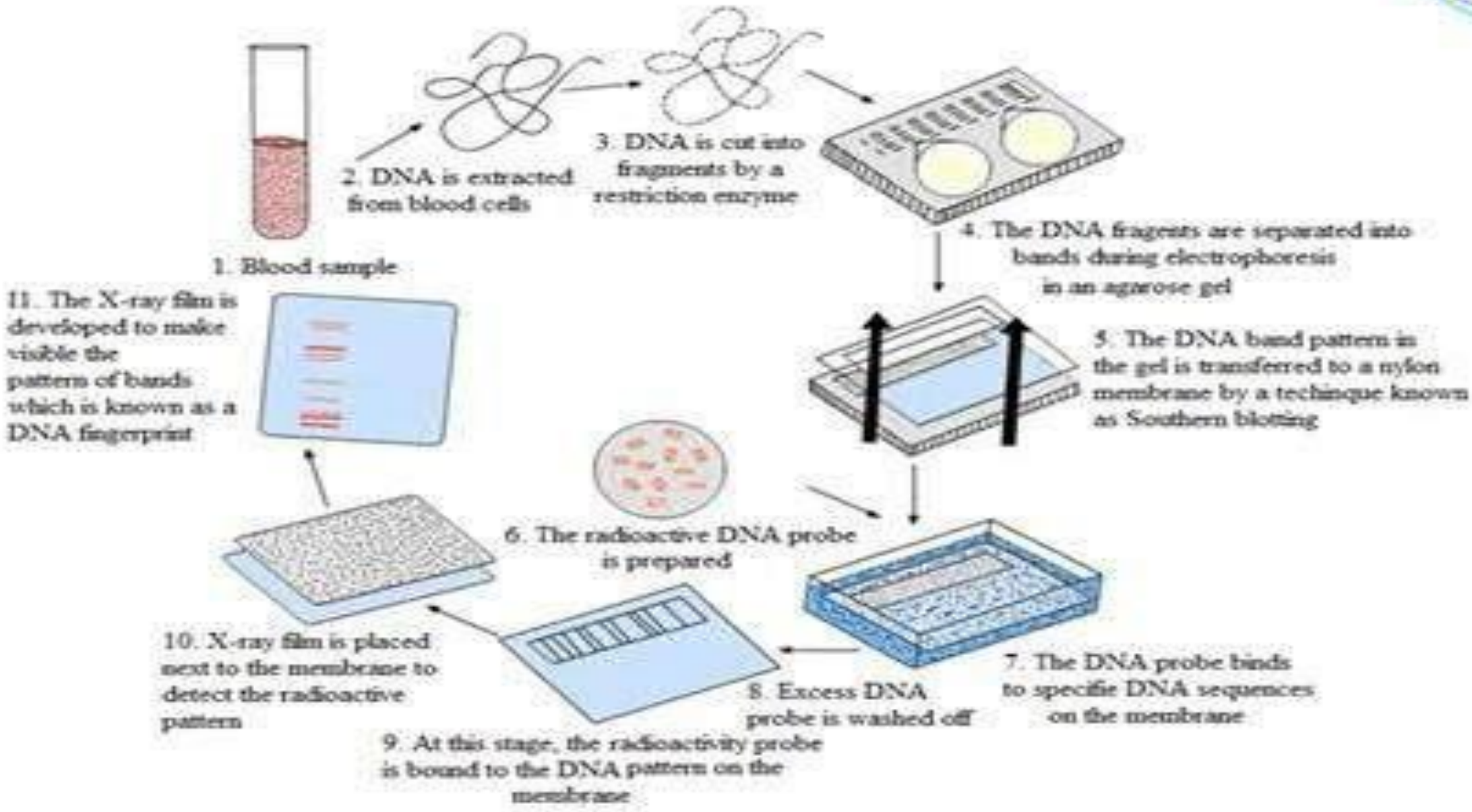
MAXAM-GILBERT DNA SEQUENCING: APPLICATIONS

- DNA Footprinting:
  - Used to study protein-DNA interactions by identifying protected regions on DNA.
- Structural Studies:
  - Analyzes DNA structure, including modifications and conformational changes.
- Small-Scale Sequencing:
  - Applicable for sequencing small amounts of DNA in research settings.

DNA FINGERPRINTING

DNA FINGERPRINTING:

- DNA fingerprinting, also known as DNA profiling, is a technique used to identify individuals based on their unique DNA patterns.
- The process involves analyzing specific regions of the genome that are known to vary greatly among individuals.
- This technique was invented by Alec Jeffreys in 1984.


1. Blood sample
2. DNA is extracted from blood cells
3. DNA is cut into fragments by a restriction enzyme
4. The DNA fragents are separated into bands during electrophoresis in an agarose gel
5. The DNA band pattern in the gel is transferred to a nylon membrane by a technique known as Southern blotting
6. The radioactive DNA probe is prepared
7. The DNA probe binds to specific DNA sequences on the membrane
8. Excess DNA probe is washed off
9. At this stage, the radioactivity probe is bound to the DNA pattern on the membrane
10. X-ray film is placed next to the membrane to detect the radioactive pattern
11. The X-ray film is developed to make visible the pattern of bands which is known as a DNA fingerprint

STEPS

- **Sample Collection**:

DNA can be obtained from various sources, such as blood, hair, saliva, or other biological materials.

- **DNA Extraction**:

The DNA is isolated from the collected cells using chemical processes.

- **DNA Amplification**:

Certain regions of the DNA are amplified using a technique called polymerase chain reaction (PCR). These regions are often selected because they are highly variable among individuals.

Separation and Analysis:

The amplified DNA is then separated using gel electrophoresis or other methods. This separation allows the different DNA fragments to be visualized and compared.

Comparison:

The resulting DNA profile is compared to other DNA profiles. Because the probability of two people having the same DNA profile is extremely low (except for identical twins), this comparison can provide a high level of certainty about a person's identity.

APPLICATIONS:

- **Forensic Science:** Identifies suspects or victims in criminal cases by matching DNA found at crime scenes.
- **Paternity Testing:** Determines biological relationships, such as confirming paternity.
- **Medical Diagnostics:** Helps in diagnosing genetic disorders and tailoring treatments based on genetic profiles.
- **Ancestry and Genealogy:** Assists in tracing family history and genetic heritage.
- **Wildlife Conservation:** Tracks endangered species and combats poaching by identifying individuals.
- **Personal Identification**: Used in cases like missing persons or verifying identity in various legal and security contexts.

RAPD:

- RAPD is a molecular biology technique used to generate random DNA markers by amplifying random segments of the genome with short, arbitrary primers during PCR (Polymerase Chain Reaction).
- This method produces a unique pattern of DNA fragments, which can be used to assess genetic diversity, verify genetic modifications, and track the inheritance of traits in genetically engineered organisms.

PRINCIPLE

- **Random Primers**: RAPD uses short, random primers (typically 10 nucleotides long) to amplify DNA segments. These primers bind to random sites on the genome, creating a variety of DNA fragments of different sizes.
- **Polymerase Chain Reaction (PCR)**: The technique employs PCR to amplify these random DNA segments. During PCR, the DNA template is denatured, and the primers anneal to the template DNA, followed by extension of the DNA strand by DNA polymerase.
- **Polymorphic Patterns**: The result of this random amplification is a set of DNA fragments with varying lengths, which reflect the genetic differences among individuals or species. These differences are due to the presence or absence of certain DNA sequences in the genome, leading to polymorphic patterns.
- **Gel Electrophoresis**: After amplification, the DNA fragments are separated by size using gel electrophoresis. The pattern of bands on the gel can be compared between samples to determine genetic similarity or diversity

METHODS

- **Sample Preparation:** DNA is extracted from the sample (e.g., plants, animals, microorganisms).
- **Polymerase Chain Reaction (PCR):** The DNA is subjected to PCR using short, random primers (usually 10 nucleotides long). Unlike other PCR methods that use specific primers, RAPD uses these random primers to amplify segments of the genome.
- **Amplification:** The PCR process generates a series of DNA fragments of varying lengths, depending on the presence of complementary sequences in the genome.
- **Gel Electrophoresis:** The PCR products are separated using gel electrophoresis. This process creates a pattern of bands that represent the different amplified fragments.
- **Analysis:** The banding patterns are analyzed to identify genetic variations between samples. These patterns are compared to determine genetic relationships or variations.

APPLICATION:

- **Genetic Diversity Studies:** Assessing genetic variation within and between populations.
- **Species Identification:** Differentiating between closely related species or strains.
- **Marker Discovery**: Identifying genetic markers linked to specific traits or diseases.
- **Genetic Mapping:** Constructing genetic maps by linking banding patterns to known genetic traits.

RACE:(Rapid Amplification of cDNA Ends)

- **RACE (Rapid Amplification of cDNA Ends)** is a technique used to obtain the complete sequence of RNA transcripts, specifically the 5' and 3' ends of mRNA.
- This method is crucial for studying gene expression and understanding gene structure.

PRINCIPLE:

- The RACE (Rapid Amplification of cDNA Ends) principle is used to obtain the full-length sequence of cDNA ends, especially for studying gene expression. There are two main types of RACE:

✓ **5' RACE**: Identifies the 5' end of a transcript. It involves reverse transcribing RNA into cDNA with a specific primer and then using a gene-specific primer to amplify the 5' end.

✓ **3' RACE**: Determines the 3' end of a transcript. This method uses a poly-A tail or a specific primer to reverse transcribe RNA into cDNA and amplify the 3' end.

METHODS:

- ***5' RACE***

- **Reverse Transcription:** RNA is reverse-transcribed into cDNA using a primer specific to the transcript of interest.
- **Addition of a Homopolymeric Tail:** A template-independent polymerase adds a tail (e.g., dC tail) to the cDNA.
- **PCR Amplification:** Using a gene-specific primer and a primer complementary to the tail, PCR amplifies the 5' end of the cDNA.
- **Sequencing:** The amplified product is sequenced to determine the 5' end sequence.

- ***3' RACE:***

- ***Reverse Transcription:*** RNA is reverse-transcribed into cDNA using a primer complementary to the poly-A tail or a specific 3' primer.
- ***PCR Amplification:*** PCR is performed with a gene-specific primer and a primer that binds to the tail or known 3' sequence.
- ***Sequencing:*** The amplified product is sequenced to determine the 3' end sequence.

AFLP AND RFLP ANALYSIS, PEDIGREE ANALYSIS

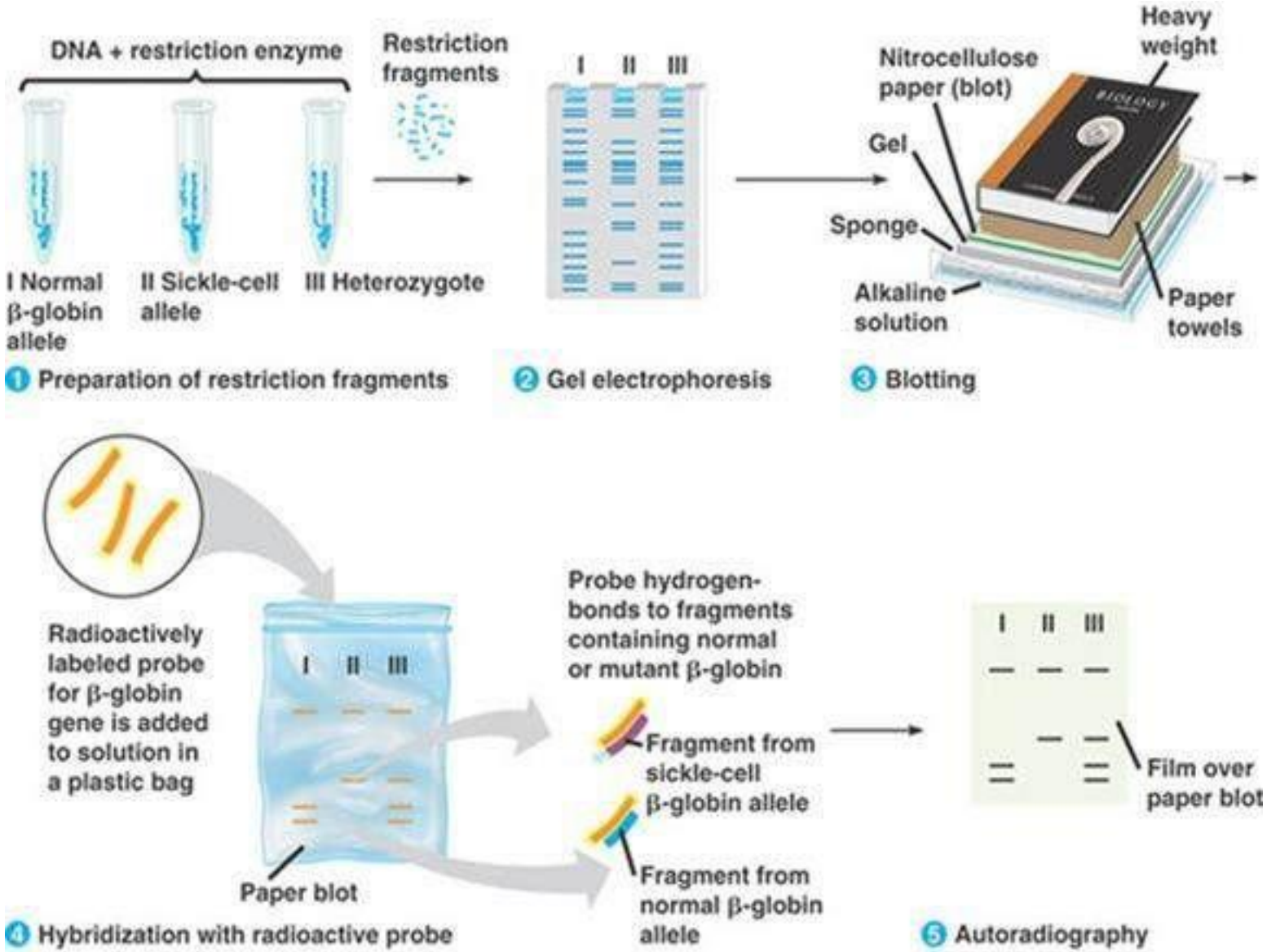
- RFLP is a molecular technique used to detect differences in DNA sequences.It relies on variations in the length of restriction enzyme-digested DNA fragments.
- Developed in the 1980s, it was one of the first DNA fingerprinting techniques.

Protocol

- Step 1: DNA ExtractionExtract DNA from the sample.
- Step 2: Restriction Enzyme DigestionDNA is cut at specific sites by restriction enzymes.
- Step 3: Gel ElectrophoresisDigested DNA fragments are separated by size using gel electrophoresis.
- Step 4: Southern BlottingDNA fragments are transferred to a membrane.
- Step 5: Hybridization with ProbesRadioactively or fluorescently labeled DNA probes are used to bind to complementary sequences.
- Step 6: DetectionFragments are visualized, revealing polymorphic patterns.

Mechanism of RFLP

- Variations in DNA sequences between individuals (mutations, insertions, deletions) cause changes in the restriction enzyme cut sites.
- These variations lead to different fragment lengths after digestion, resulting in unique patterns for each individual.


DNA + restriction enzyme
Restriction fragments
I Normal β-globin allele
II Sickle-cell allele
III Heterozygote
I II III
Nitrocellulose paper (blot)
Heavy weight
Gel
Sponge
Alkaline solution
Paper towels
1 Preparation of restriction fragments
2 Gel electrophoresis
3 Blotting
Radioactively labeled probe for β-globin gene is added to solution in a plastic bag
Paper blot
Probe hydrogen-bonds to fragments containing normal or mutant β-globin
Fragment from sickle-cell β-globin allele
Fragment from normal β-globin allele
Film over paper blot
4 Hybridization with radioactive probe
5 Autoradiography

Application

- Genetic Mapping: RFLP is used to create genetic linkage maps in organisms.
- DNA Fingerprinting: Used in forensics and paternity testing to distinguish between individuals based on their unique DNA patterns.
- Disease Diagnosis: Helps in identifying genetic mutations linked to diseases like sickle cell anemia.
- Plant and Animal Breeding: RFLP markers are used to track desirable traits.
- Phylogenetics: Determines evolutionary relationships between species.

Advantages of RFLP

- High accuracy and reliability in detecting DNA sequence variations.
- Can distinguish between homozygous and heterozygous individuals.Provides co-dominant markers, which offer detailed genetic information.

Limitations of RFLP

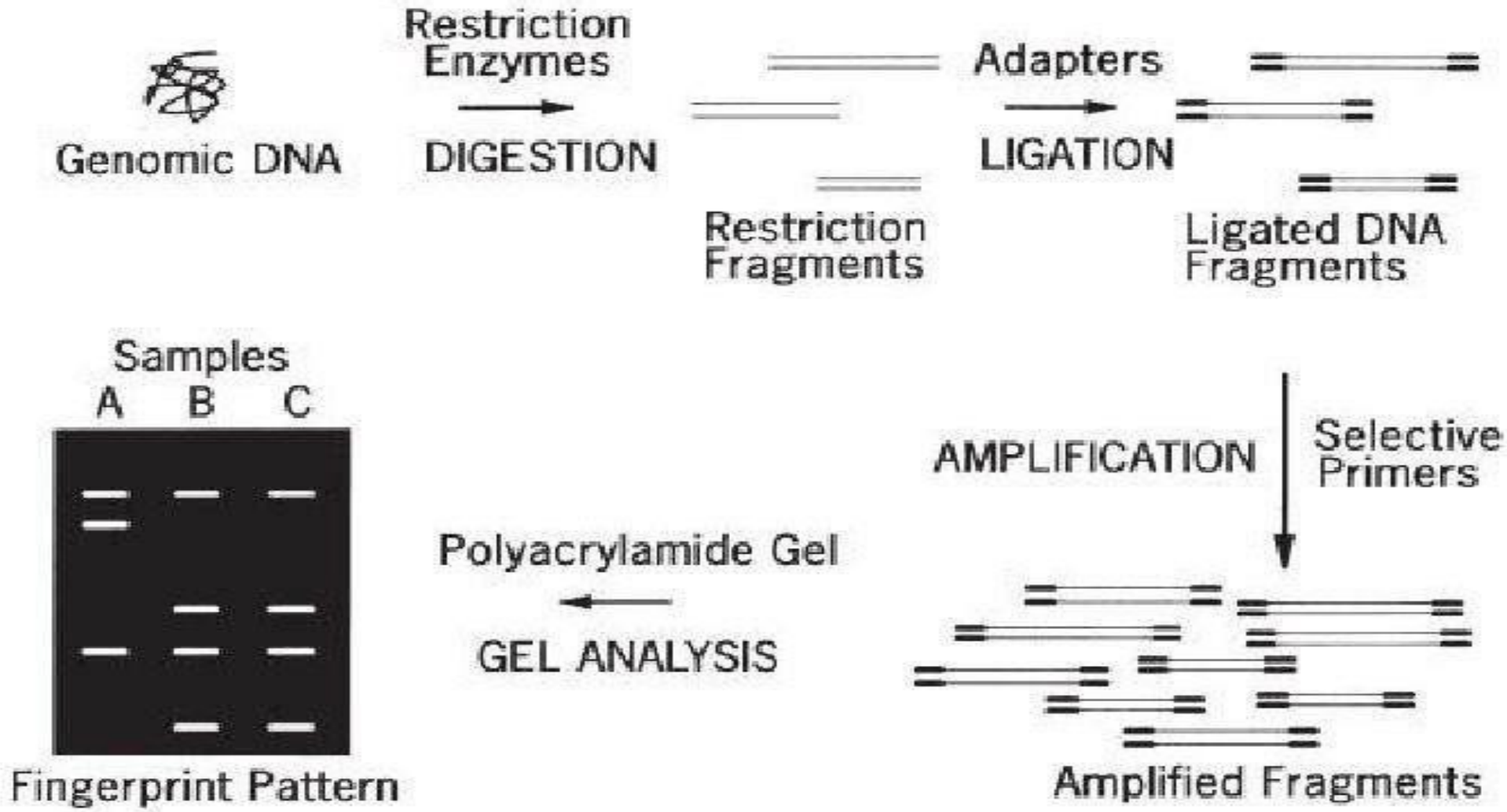
- Requires a large amount of high-quality DNA.
- Labor-intensive and time-consuming compared to more modern techniques.
- Limited ability to detect smaller polymorphisms, such as single nucleotide polymorphisms (SNPs).
- Not suitable for high-throughput analysis.

AFLP

- AFLP (Amplified Fragment Length Polymorphism) is a widely used molecular technique for DNA fingerprinting and genetic diversity studies.
- AFLP is a PCR-based method used for detecting DNA polymorphisms.It combines the techniques of restriction digestion and selective amplification.
- Widely used in phylogenetic studies, population genetics, and DNA fingerprinting.

Protocol

- Step 1: DNA ExtractionDNA is isolated from the organism of interest.
- Step 2: Restriction Enzyme DigestionDNA is digested using two restriction enzymes (commonly EcoRI and MseI).
- Step 3: Ligation of AdaptorsSynthetic oligonucleotide adaptors are ligated to the ends of restriction fragments.
- Step 4: Selective AmplificationPrimers complementary to the adaptors and adjacent nucleotides are used for PCR amplification.
- Step 5: Gel or Capillary ElectrophoresisAmplified fragments are separated by size.


Genomic DNA
Restriction Enzymes
DIGESTION
Restriction Fragments
Adapters
LIGATION
Ligated DNA Fragments
AMPLIFICATION
Selective Primers
Amplified Fragments
Polyacrylamide Gel
GEL ANALYSIS
Samples
A B C
Fingerprint Pattern

Application

- Genetic Diversity Analysis: AFLP can identify variations at multiple loci.
- Phylogenetics: Used to infer evolutionary relationships among species.
- Marker-Assisted Breeding: AFLP markers are used in crop improvement programs.
- Conservation Genetics: Helps in assessing the genetic structure of endangered species.DNA Fingerprinting: Important in forensic and paternity testing.

Advantages of AFLP

- High reproducibility and resolution.
- Can analyze large numbers of loci without prior sequence knowledge.
- Works well with small amounts of DNA.
- Applicable to any organism.

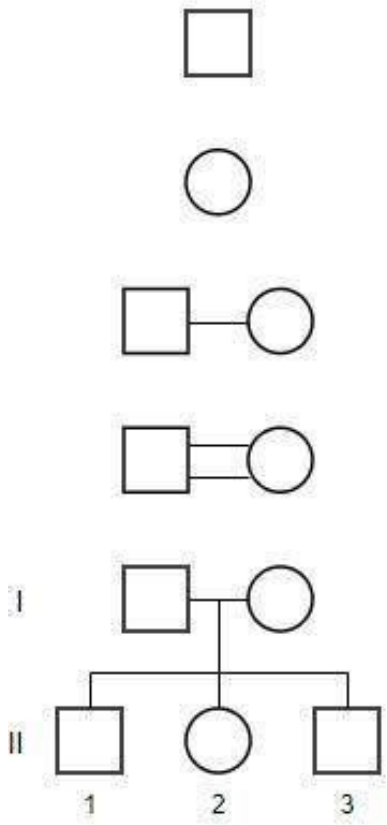
Limitations of AFLP

- Technically demanding and requires optimization.
- Results may sometimes be challenging to interpret due to dominance and fragment homoplasy.
- Lower throughput compared to modern sequencing technologies like NGS.

Pedigree Analysis

- Definition: Pedigree analysis is a diagrammatic representation of a family tree that tracks the inheritance of specific genetic traits.
- It helps to determine the mode of inheritance (dominant, recessive, X-linked, etc.) of genetic conditions or traits.
- Commonly used in medical genetics, genetic counseling, and breeding programs.

Pedigree analysis symbols



Male

Female

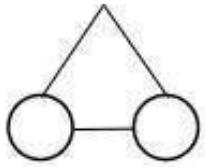
Marriage

Consanguineous marriage

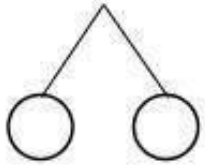
Roman numerals symbolize generations.

Arabic numbers symbolize individuals within a given generation.

Birth order, within each group of offspring, is drawn left to right, first-born to last-born.



Identical twins



Fraternal twins females

Affected male and female

Affected by history female and male

Carrier

Deceased female and male

Unknown sex

Common Modes of Inheritance

Autosomal Dominant

- Trait appears in every generation.Affected individuals have at least one affected parent.Example: Huntington's disease.

Autosomal Recessive

- Skips generations.Two unaffected carriers can have affected offspring.Example: Cystic fibrosis, sickle cell anemia.

X-linked Recessive

- More common in males.Females are typically carriers.Example: Hemophilia, color blindness.

X-linked Dominant

- Affected fathers pass the trait to all daughters but not sons.Example: Fragile X syndrome

GERMPLASAM

Germplasm maintenance is crucial in agriculture and plant breeding as it involves preserving the genetic material of plants to ensure their continued availability and potential use.

What is Germplasm?

Germplasm refers to the genetic material (seeds, tissues, or cells) of plants that is used for breeding, research, and conservation. It includes the genetic diversity of a plant species or variety.

Objectives of Germplasm maintenance

Preservation of Genetic Diversity: Ensuring that a wide range of genetic material is kept intact to avoid loss of genetic variation.

Agricultural Improvement:
Providing genetic resources for breeding programs to develop new varieties with desirable traits.

Research and Development: Supporting scientific studies and innovations in plant science

Methods of Germplasm Maintenance
Seed Storage:

Short-term Storage: Seeds are kept in controlled conditions with low humidity and temperature.

Long-term Storage: Seeds are stored in seed banks under conditions that slow down aging, often at temperatures below freezing.

Tissue Culture:
In Vitro Culture: Plant tissues or cells are cultured in a nutrient medium. This method helps preserve plant genetic material without needing large amounts of space.

Field Maintenance:
Living Collections: Plants are grown in field plots or botanical gardens to maintain and observe their traits over time. This is often used for species that do not store well as seeds.

•**Cryopreservation:**

•**Freezing:** Plant tissues, particularly seeds or germplasm in the form of embryos or cells, are frozen at very low temperatures using liquid nitrogen to halt metabolic processes and preserve viability.

•**Backup Systems:**

•**Multiple Storage Locations:** Germplasm is often stored in multiple locations to safeguard against potential losses due to disasters or other unforeseen events

Challenges in Germplasm Maintenance

Genetic Drift: Changes in genetic composition over time, especially in small populations.

Viability Loss: Decrease in seed viability or tissue culture performance over time.

Resource Intensive: Requires significant resources for storage, management, and monitoring.

Importance for the Future

Maintaining germplasm is essential for food security, agricultural sustainability, and biodiversity conservation.

It provides a reservoir of genetic material that can be used to adapt to changing environmental conditions, combat pests and diseases, and improve crop yields.

GENETIC COUNSELLING

- Genetic counseling is a healthcare service that provides information, support, and guidance to individuals or families about genetic conditions and their potential impact.
- It helps people make informed decisions about managing or mitigating genetic risks.
- Genetic counseling is particularly useful in contexts like prenatal care, family planning, managing inherited diseases, and understanding the implications of genetic test results.

What is Genetic Counselling?

Genetic counseling is a skill-based communication process that employs scientific knowledge regarding principles of human genetics and genomics, psychological aspects of genetic disease, and communication expertise to support individuals and their families to understand and cope with genetic diagnoses.

History

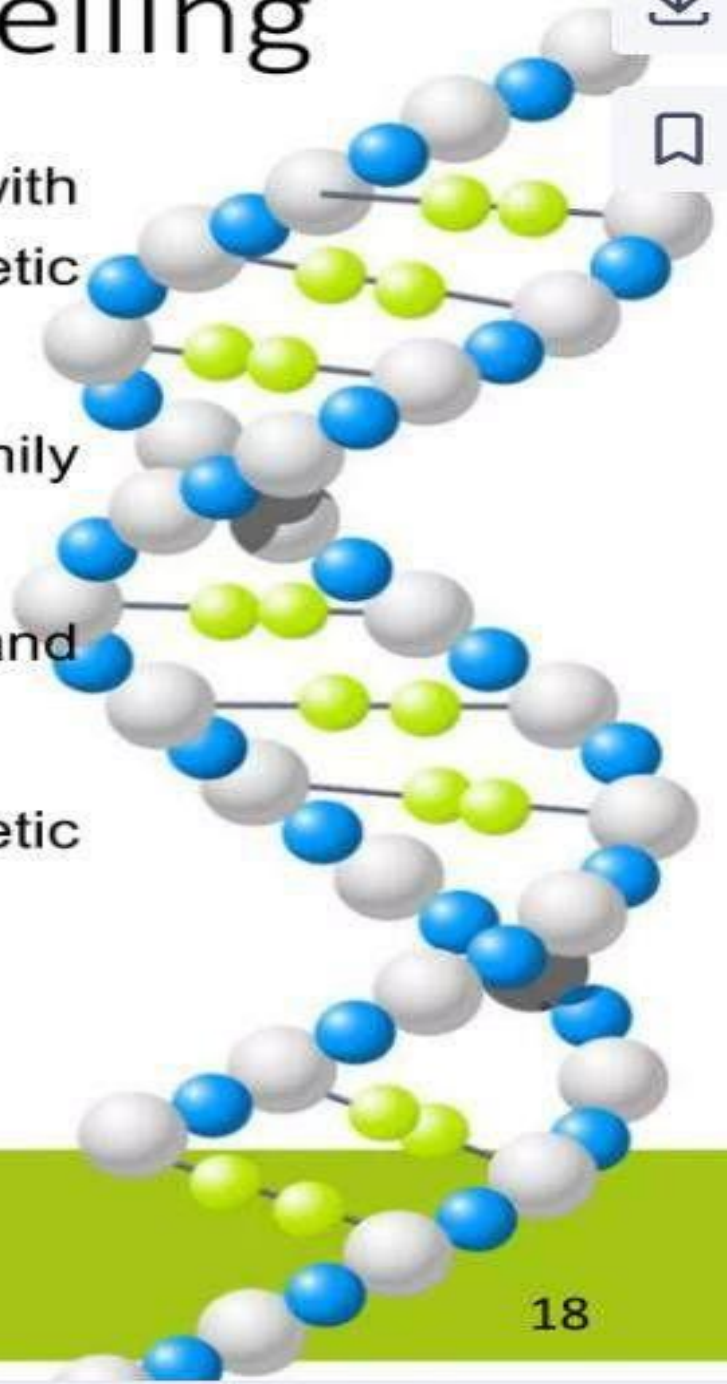
- In the **1950s**, **Sheldon Reed** proposed the idea of a medical social worker and promoted the term **genetic counseling**.

- The first program for graduate education in genetic counseling to establish a new health-care provider, the genetic counselor, was initiated at **Sarah Lawrence College** in **Bronxville**, **New York**, in **1969**.

Aims of genetic counselling

- The genetic counseling aims to provide the family with complete and accurate information about genetic disorders.

1. Promoting informed decisions by involved family members
2. Clarifying the family's options available treatment and prognosis
3. Explaining alternatives to reduce the risk of genetic disorders
4. Decreasing the incidence of genetic disorders
5. Reducing the impact of the disorders



Reasons for Genetic Counselling

Based on your personal and family health history, your doctor can refer you for genetic counseling. There are different stages in your life when you might be referred for genetic counseling:

Indications for genetic counselling

- Congenital malformations
- Pregnancy loss
- Mental retardation
- Neurodegenerative disorders
- Muscular dystrophies and congenital myopathies
- Inborn error of metabolism
- Disorders of sexual development
- Skeletal dysplasia
- Familial cancer or cancer prone diseases
- Exposure to know or suspected teratogen
- Consanguineous marriage
- Advanced maternal age
- Positive screening for a genetic disorder

GENETIC COUNSELLING

They are of 2 types:

1. Prospective
2. Retrospective

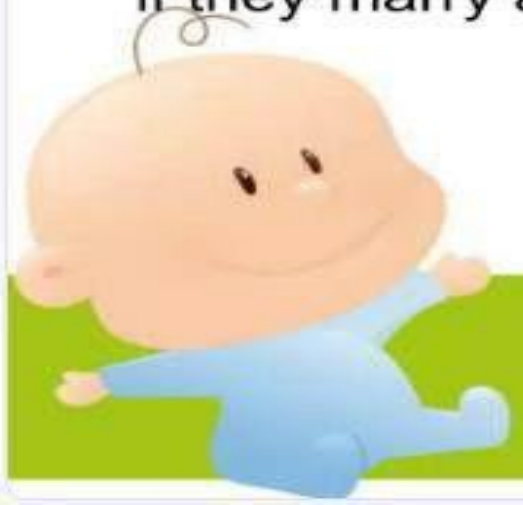
1. Prospective genetic counselling

This allows for the true prevention of disease.

This approach requires

Identifying heterozygous individuals for any particular defect by screening

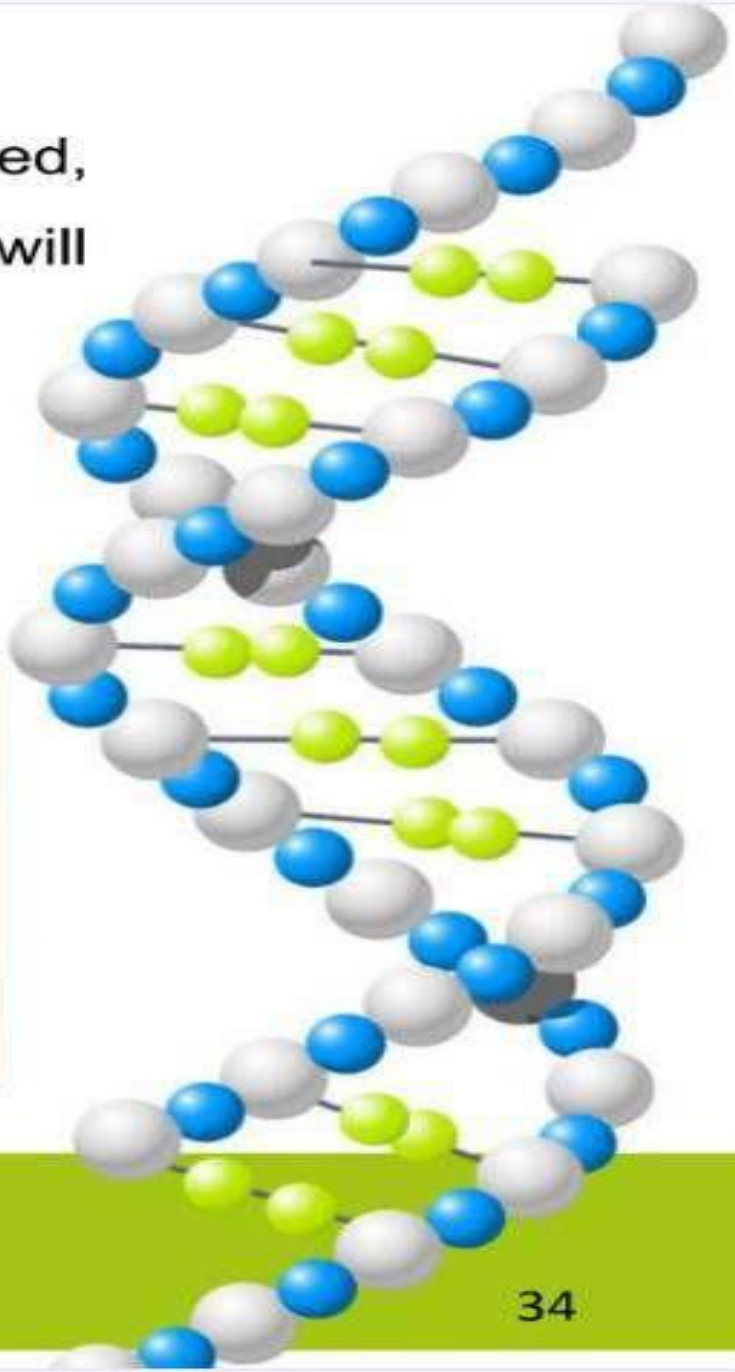
Explaining to them the risk of their having affected children if they marry another heterozygote for the same gene.



If heterozygous marriage can be prevented or reduced, the prospects of giving birth to affected children will diminish.

EX: Sickle cell anemia

Thalassemia



2. Retrospective genetic counselling:

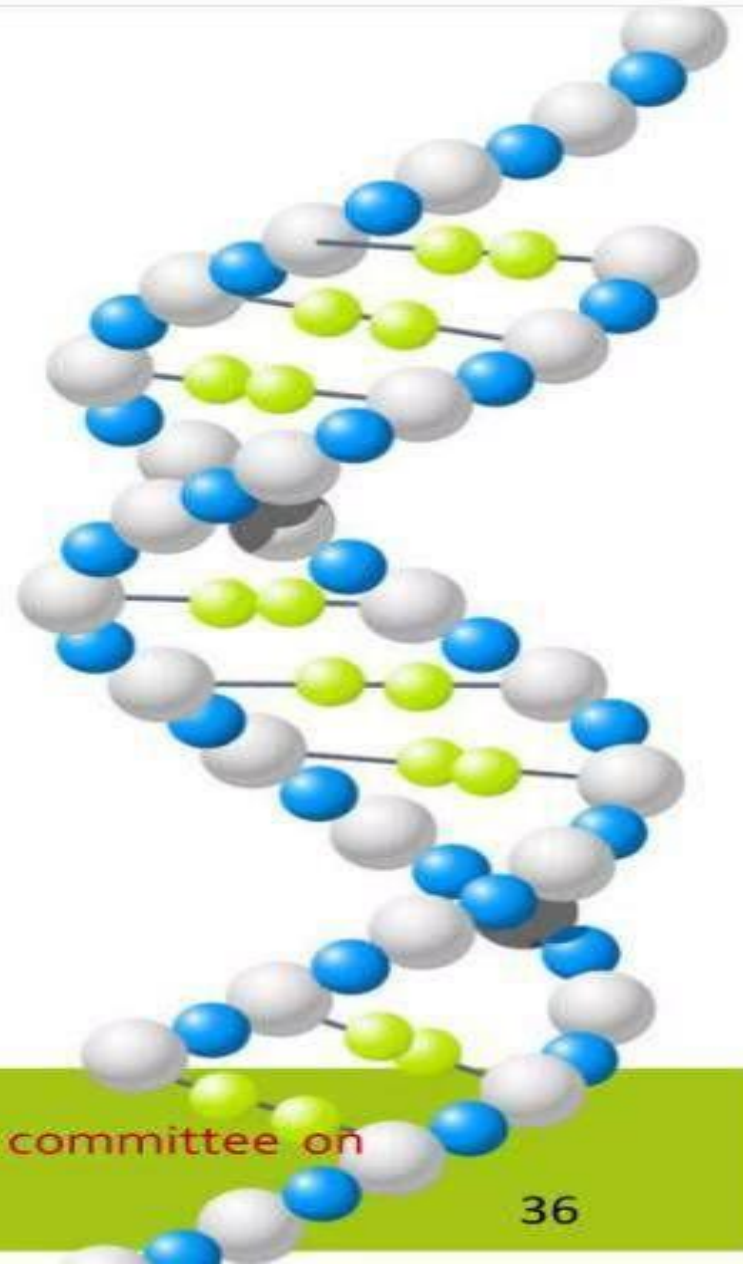
Most genetic counselling at present is retrospective, i.e, the hereditary disorder has already occurred within the family .

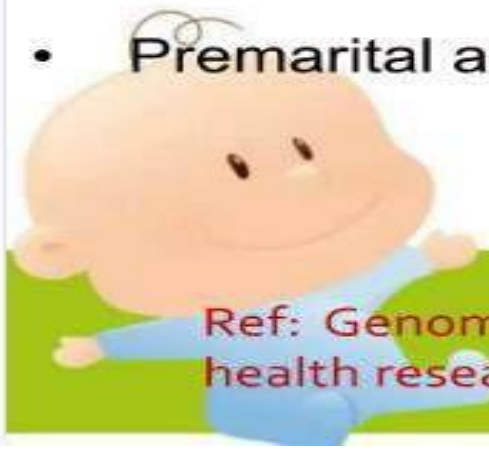
The methods which could be suggested under retrospective genetic counselling are:

1. Contraception
2. Pregnancy termination.

A survey carried out by the WHO showed that genetic advice was chiefly sought in connection with congenital abnormalities

- Mental retardation
- Psychiatric illness
- Inborn errors of metabolism
- Premarital advice





Ref: Genomics and world health: Report of the advisory committee on health research, Geneva, WHO (2002).

KEY ASPECTS OF GENETIC COUNSELING

•**Assessment of Risk**:
•Counselors evaluate family and medical histories to determine the likelihood of genetic conditions being inherited or passed on.
•**Education**:
•They explain how genes, inheritance patterns, and conditions work.
•Provide information about genetic testing options and their implications.
•**Emotional Support**:
•Counselors address concerns and emotional responses associated with the possibility or diagnosis of a genetic condition.
•**Decision-Making**:
•They guide individuals and families in making decisions regarding testing, treatments, lifestyle adjustments, or reproductive options.

Who Might Seek Genetic Counseling?

- Individuals with a family history of genetic disorders (e.g., cystic fibrosis, sickle cell anemia).
- Couples planning a family who are concerned about passing on inherited conditions.
- Pregnant women at risk of having children with genetic abnormalities (e.g., due to advanced maternal age or abnormal prenatal test results).
- Individuals diagnosed with genetic conditions seeking management strategies.
- People considering genetic testing for predisposition to conditions like cancer or cardiovascular diseases.

- **The Role of a Genetic Counselor:**
- Genetic counselors are healthcare professionals with specialized training in medical genetics and counseling. They act as a bridge between complex genetic information and the practical, emotional, and ethical implications it may have for patients and their families.

APPLICATIONS OF GENETIC COUNSELING

- Genetic counselors work with people concerned about the risk of an inherited disease or condition. These people represent several different populations
- Prenatal Genetic Counseling
- There are several different reasons a person or couple may seek prenatal genetic counseling. If a woman is of age 35 or older and pregnant, then there is an increased chance that her fetus may have a change in the number of chromosomes present. Changes in chromosome number may lead to mental retardation and birth defects

DNA FOOTPRINTING

DNA footprinting is a molecular biology technique used to identify the specific sequence of DNA that interacts with a DNA-binding protein, such as transcription factors or other regulatory proteins. This method helps to map protein-DNA interactions, revealing the binding sites and their role in regulating gene expression.

HOW DNA FOOTPRINTING WORKS

The basic principle involves:

1. **Binding**: A protein is allowed to bind to a specific segment of DNA.
2. **Digestion**: The DNA-protein complex is treated with a nuclease (e.g., DNase I) or a chemical agent that cleaves the DNA at exposed regions.
    1. The bound protein protects the DNA in the binding region from being cleaved.
3. **Analysis**: The resulting DNA fragments are analyzed on a sequencing gel to determine the exact region protected by the protein.

STEPS OF DNA FOOTPRINTING

•**Preparation of Labeled DNA**

•A specific DNA fragment is labeled at one end, typically with a radioactive or fluorescent tag.

•**Protein Binding**:

•The DNA fragment is incubated with the DNA-binding protein of interest.

•**Cleavage of DNA**:

•DNase I (or another cleaving agent) is added in limited amounts to ensure only one cut per DNA molecule.

•The protein-bound region is protected from cleavage.

•**Separation of Fragments**:

•The DNA fragments are denatured and separated by size using polyacrylamide gel electrophoresis.

Visualization:

1. The pattern of DNA fragments is visualized (e.g., using autoradiography or fluorescence imaging).
2. A "footprint" appears as a gap in the ladder of DNA fragments, indicating where the protein protected the DNA from cleavage.

APPLICATIONS OF DNA FOOTPRINTING

1. **Mapping Protein Binding Sites**:
    1. Identifies specific sequences where transcription factors or other regulatory proteins bind.
2. **Studying Gene Regulation**:
    1. Helps understand how proteins regulate transcription by interacting with promoters, enhancers, or other regulatory elements.
3. **Drug Discovery**:
    1. Used to identify how drugs or small molecules interact with DNA-binding proteins, which is crucial for designing targeted therapies.
4. **Comparative Analysis**:
    1. Assists in comparing binding sites under different conditions (e.g., varying cellular environments or mutant proteins).

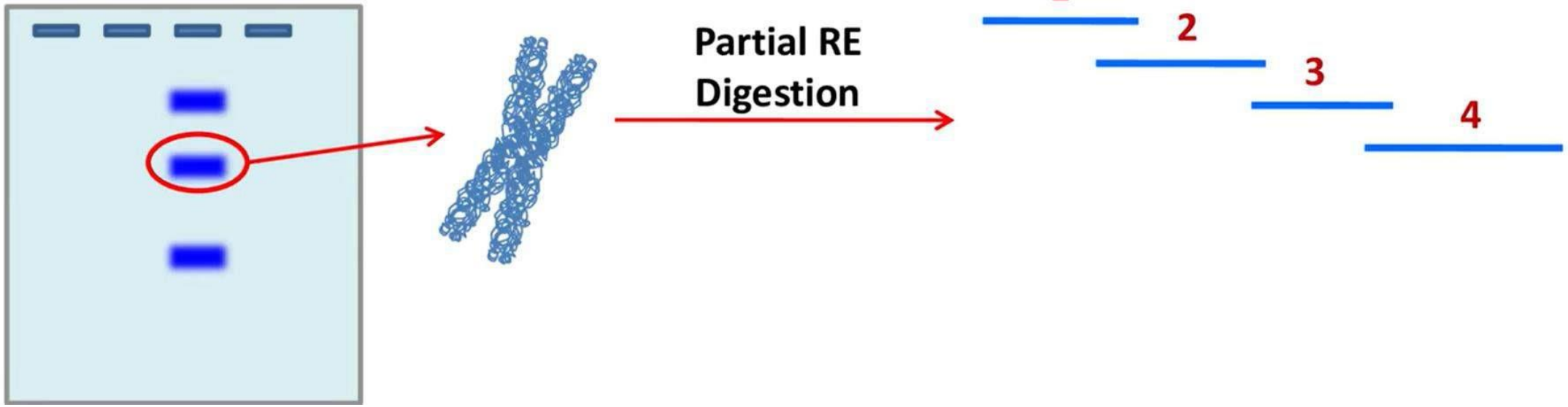
- **Limitations**
- Requires high-purity DNA and proteins.
- Sensitive to experimental conditions like enzyme concentration and incubation times.
- Provides limited information about dynamic or transient interactions.

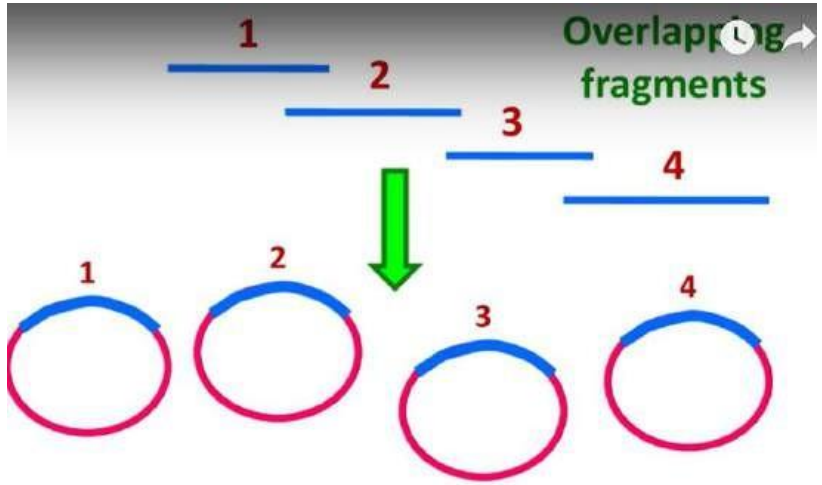
CHROMOSOME WALKING,CHROMOSOME JUMPING AND MUTAGENICITY TEST – AMES TEST

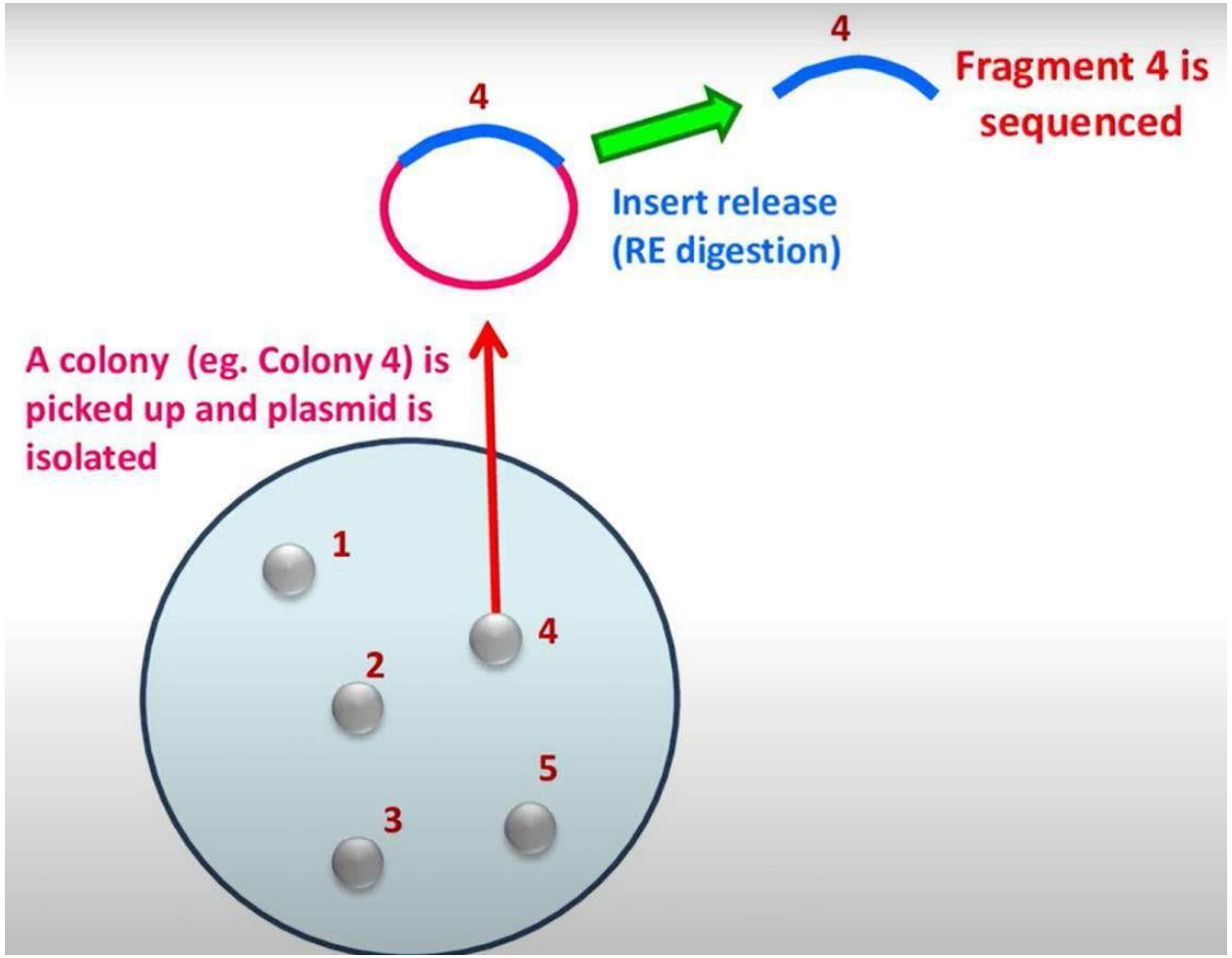
CHROMOSOME WALKING

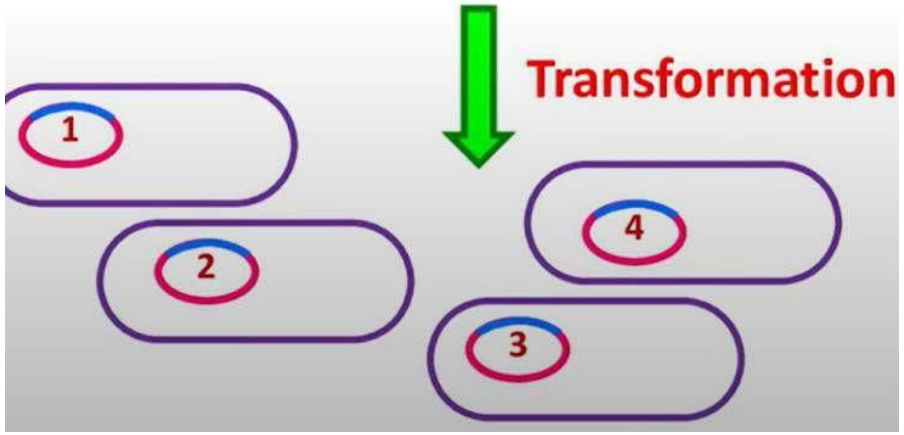
- Chromosome walking is a molecular biology technique used to identify and clone DNA sequences that are adjacent to a known gene or marker.
- This method is particularly useful for exploring genomic regions where the exact location of a gene is unknown, allowing researchers to map and sequence long stretches of DNA by utilizing overlapping fragments

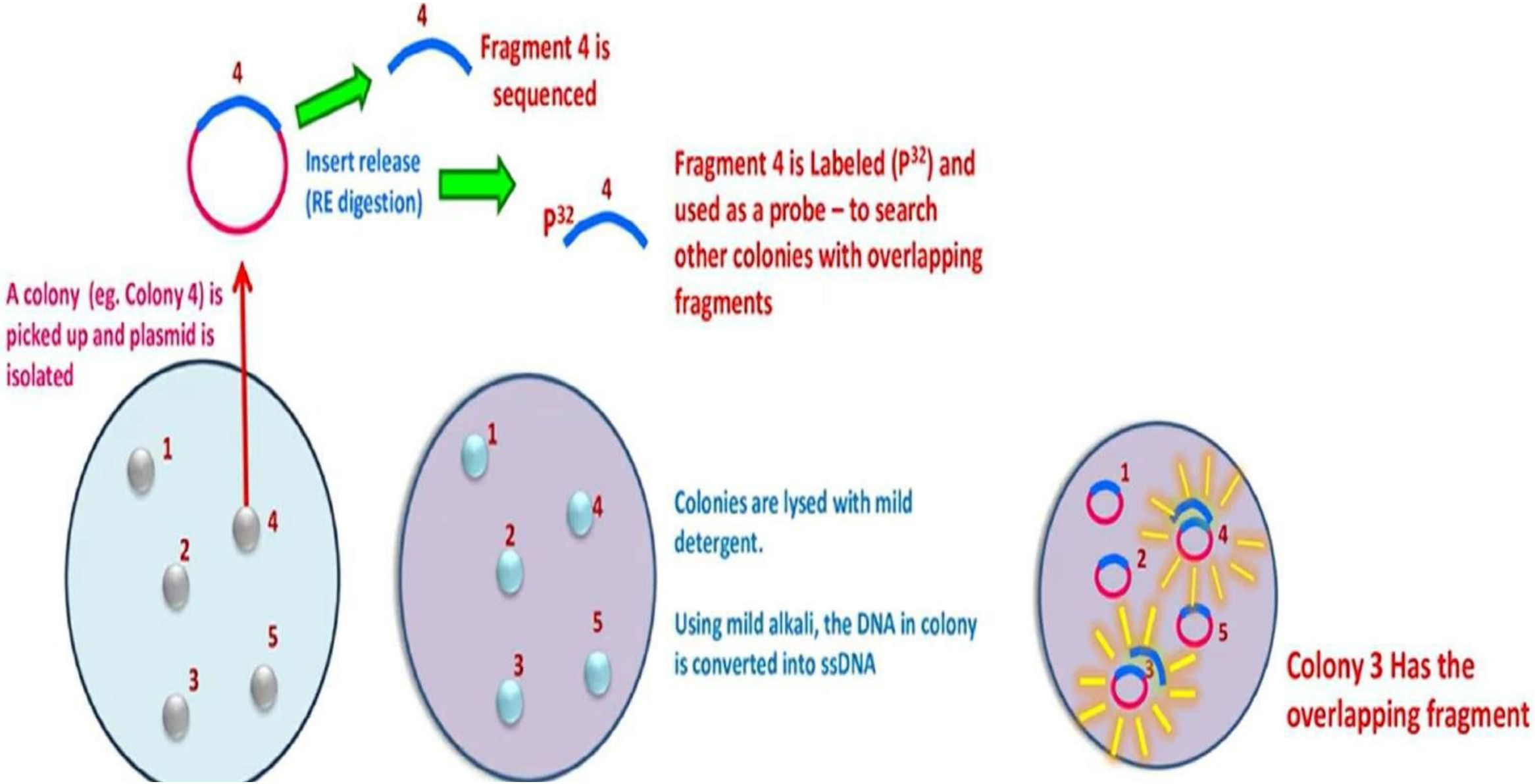
Chromosomes are isolated using pulsed field gel electrophoresis

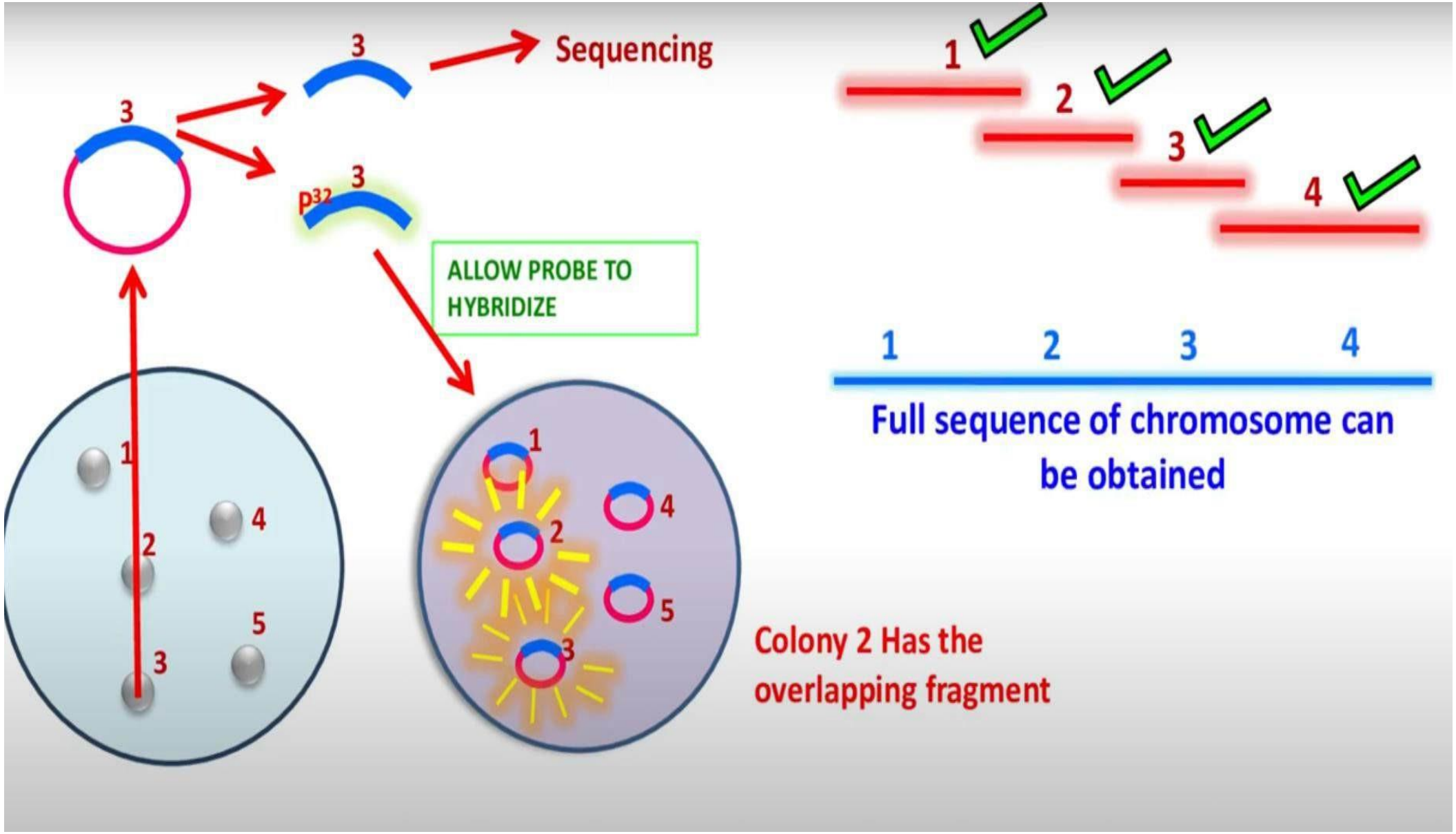



1
2
3
4
Overlapping fragments
1
2
3
4
The fragments are ligated in plasmid


4
4
Fragment 4 is sequenced
Insert release (RE digestion)
A colony (eg. Colony 4) is picked up and plasmid is isolated
1
2
3
4
5


Transformation
1
2
3
4


4
4
Fragment 4 is sequenced
Insert release (RE digestion)
P32
4
Fragment 4 is Labeled (P32) and used as a probe – to search other colonies with overlapping fragments
A colony (eg. Colony 4) is picked up and plasmid is isolated
1
2
3
4
5
Colonies are lysed with mild detergent.
Using mild alkali, the DNA in colony is converted into ssDNA
Colony 3 Has the overlapping fragment


3
Sequencing
3
P32
3
ALLOW PROBE TO HYBRIDIZE
1
2
3
4
5
1
2
3
4
5
1
2
3
4
1
2
3
4
Full sequence of chromosome can be obtained
Colony 2 Has the overlapping fragment

METHOD

1. **Isolation of Chromosomal DNA**: Using pulsed-field gel electrophoresis. This allows for the separation of large DNA fragments.
2. **Partial Digestion**: The isolated DNA is partially digested with restriction enzymes, resulting in overlapping fragments. This partial digestion is crucial as it ensures that the fragments retain regions of overlap, which are essential for subsequent steps.
3. **Ligation into Vectors**: The resulting DNA fragments are ligated into plasmid vectors. These vectors can replicate within host cells, allowing for the amplification of the inserted DNA.

4. **Transformation and Colony Formation**: The ligated plasmids are introduced into competent bacterial cells (often *E. coli*), which are then plated to form colonies. Each colony represents a population of bacteria containing a different DNA insert.

5. **Screening for Overlapping Fragments**: A colony is randomly selected, and the plasmid containing the DNA insert is isolated. This insert is then used as a probe to screen other colonies for overlapping fragments.

6. **Hybridization:** The colonies are transferred to a membrane, and the DNA is treated to create single-stranded DNA. The probe is allowed to hybridize with the single-stranded DNA on the membrane, identifying colonies that contain overlapping sequences.

7. **Sequencing**: Once overlapping fragments are identified, the plasmid from these colonies is isolated, and the insert is sequenced. This process can be repeated multiple times to walk along the chromosome, progressively revealing more of the genetic sequence.

APPLICATIONS

- Gene Discovery: Chromosome walking is instrumental in discovering new genes, especially in complex genomes where genes are closely linked.
- Mapping Genomes: It aids in the physical mapping of genomes, which is essential for understanding **genetic organization and function.**
- Studying Genetic Disorders: The technique can be used to locate genes associated with genetic diseases, providing insights into their function and potential therapeutic targets.

CHROMOSOME

- Chromosome jumping is a molecular biology technique designed to clone large segments of DNA that are separated by significant distances on a chromosome.
- This method is particularly useful for identifying and isolating genes that are located far apart from known sequences, overcoming the limitations of traditional cloning methods such as chromosome walking.

METHODS

1. **Genomic DNA Isolation**: The process begins with the isolation of genomic DNA from the organism of interest.

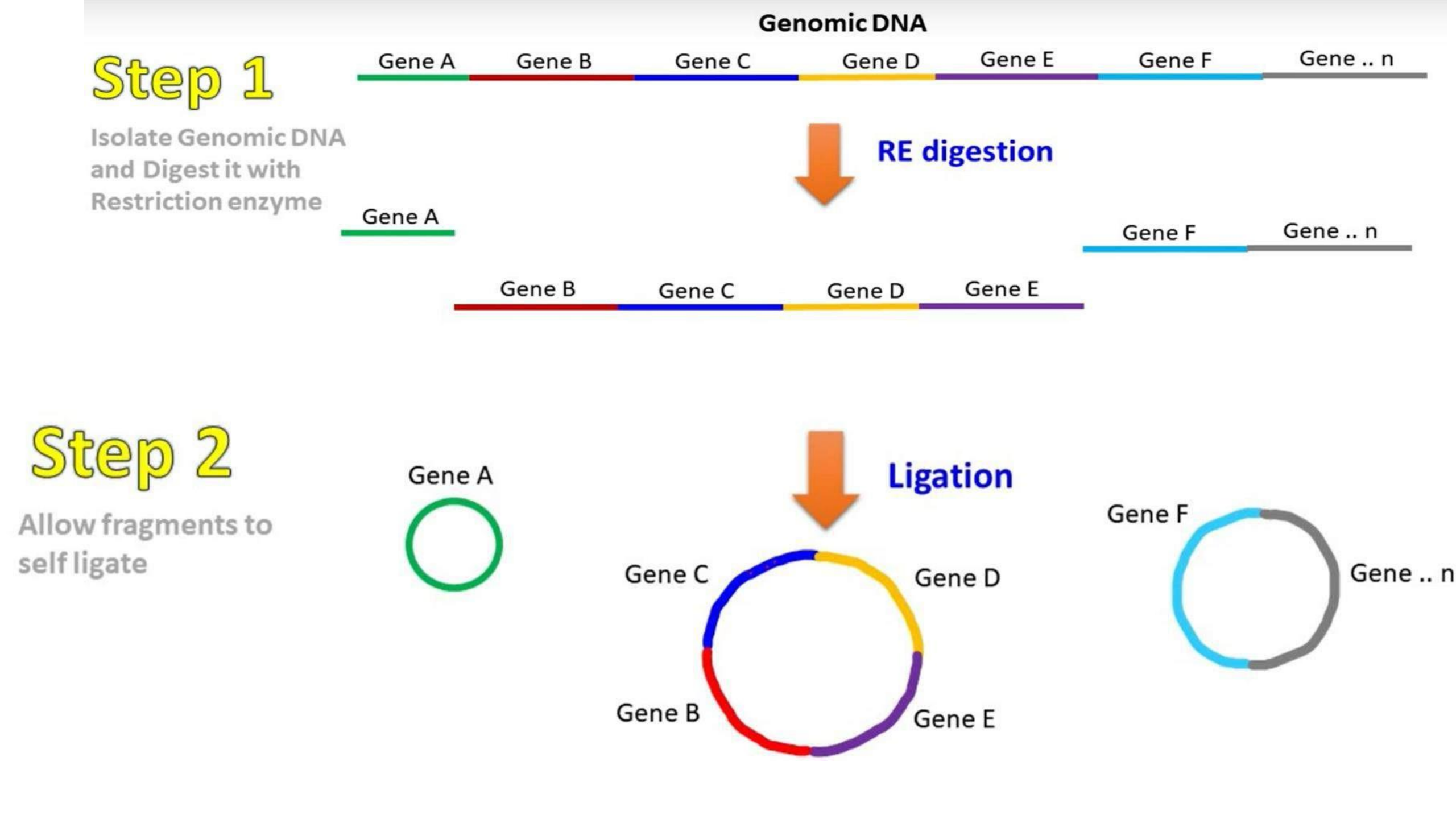
2. **Partial Digestion**: The isolated DNA is partially digested using a rare-cutting restriction enzyme (e.g., NotI). This results in large DNA fragments, typically ranging from 100 to 150 kilobases.

3. **Circularization**: The resulting fragments are circularized through self-ligation, forming circular DNA molecules. This step is crucial as it allows for the manipulation of the DNA fragments while retaining the necessary sequences.

5. **Cloning into Vectors**: The remaining fragments, which now contain sequences from the original DNA that are far apart, are ligated into a suitable cloning vector (commonly a plasmid).

6. **Transformation and Screening**: The recombinant plasmids are introduced into competent bacterial cells (e.g., *E. coli*), which are then plated to form colonies. Each colony contains a different DNA insert. A probe with a known sequence is used to screen these colonies to identify those containing the desired DNA fragments.

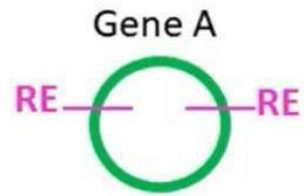
7. **Isolation and Sequencing**: Colonies that hybridize with the probe are selected, and the plasmid DNA is isolated. The insert can then be sequenced to identify the genes or genetic elements of interest.

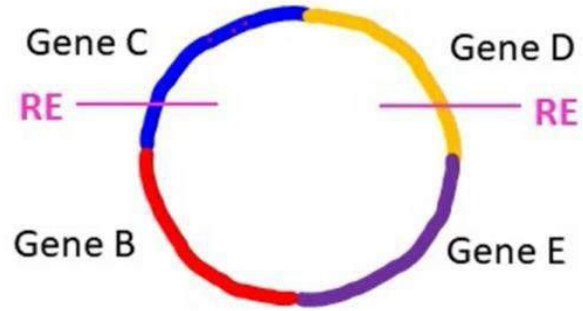

Genomic DNA
Gene A
Gene B
Gene C
Gene D
Gene E
Gene F
Gene .. n
Step 1
Isolate Genomic DNA and Digest it with Restriction enzyme
RE digestion
Gene A
Gene F
Gene .. n
Gene B
Gene C
Gene D
Gene E
Step 2
Allow fragments to self ligate
Gene A
Ligation
Gene C
Gene D
Gene B
Gene E
Gene F
Gene .. n

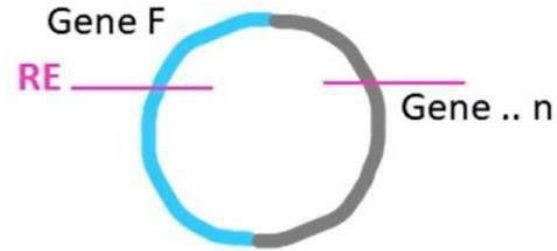
Chromosome Jumping

Step 3

Second RE digestion



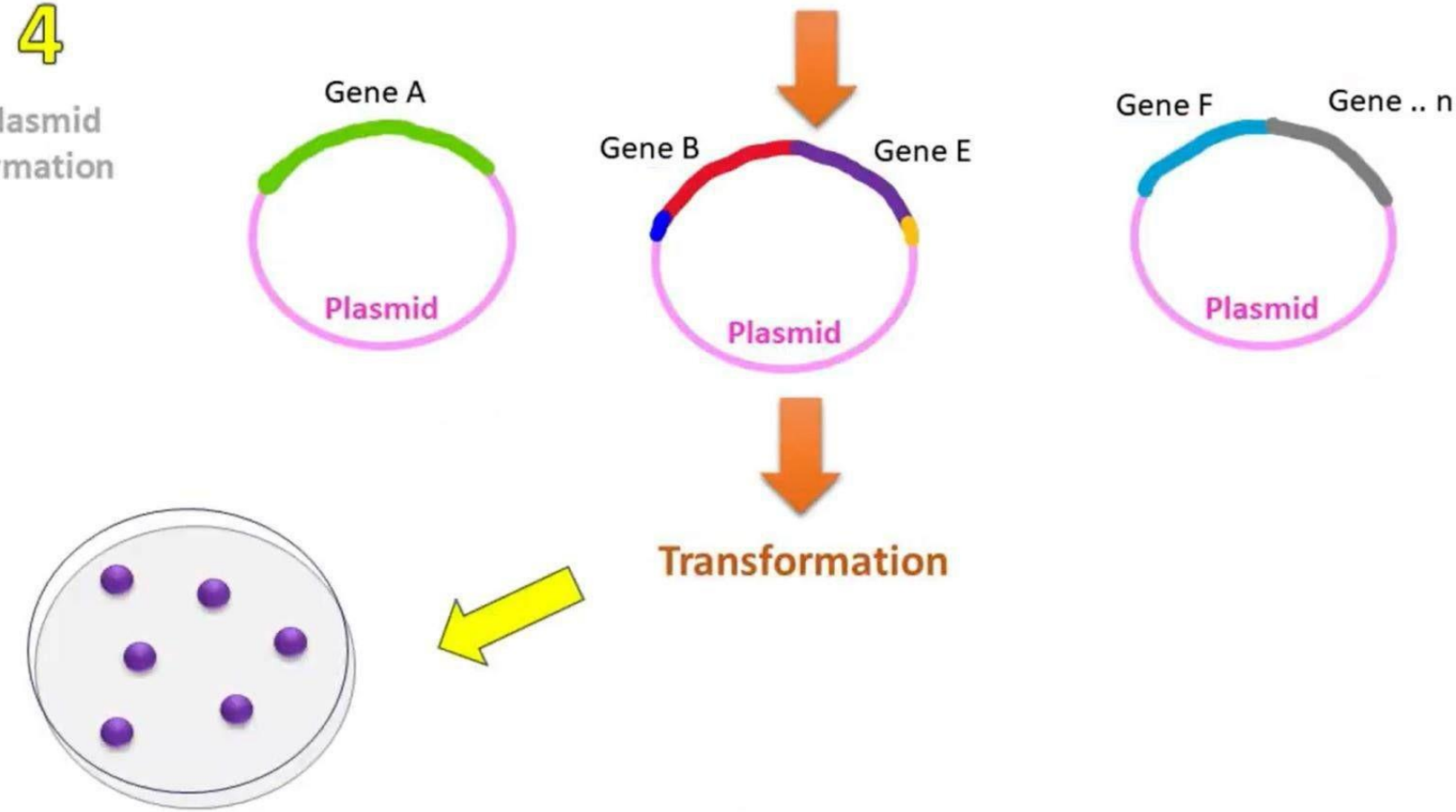


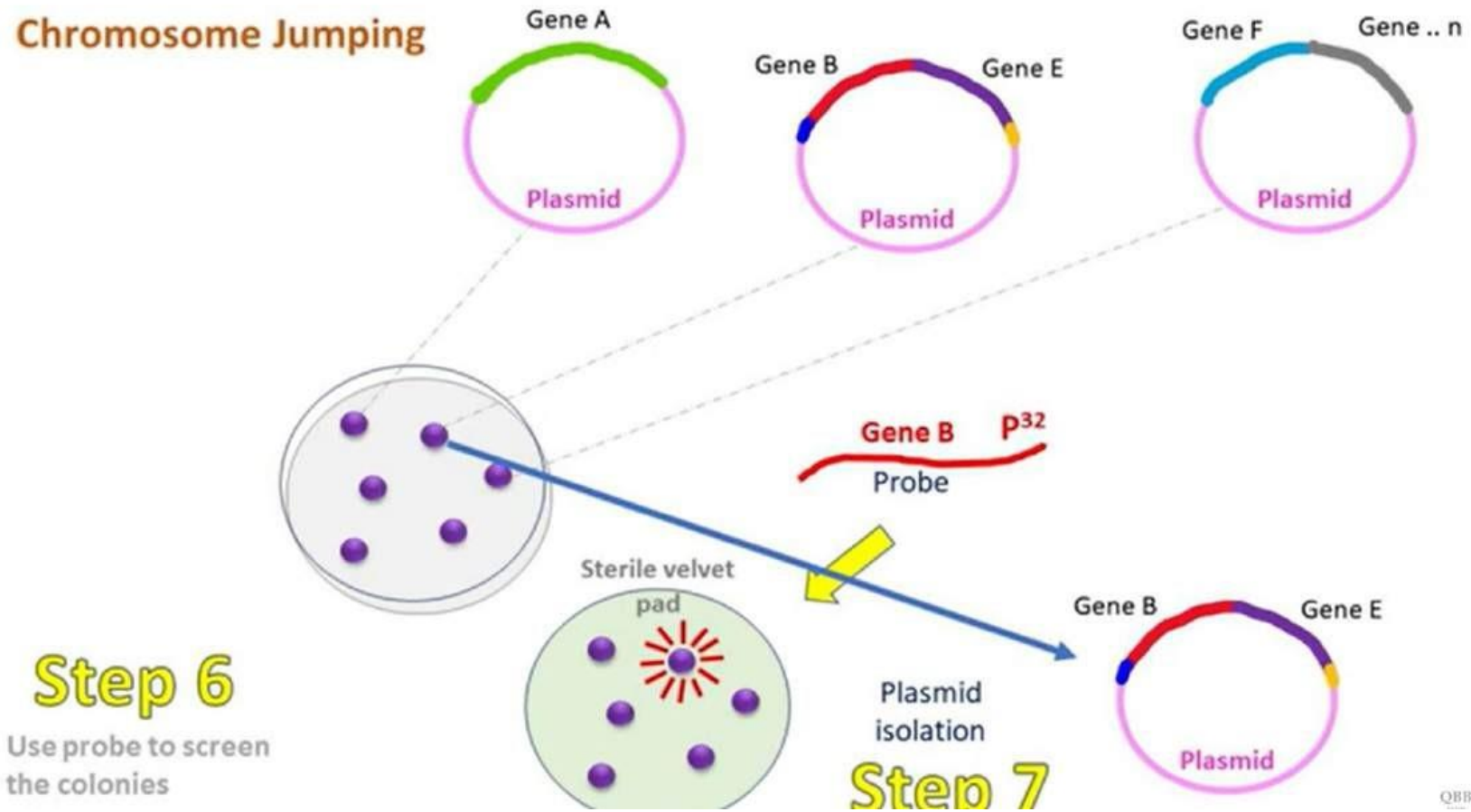


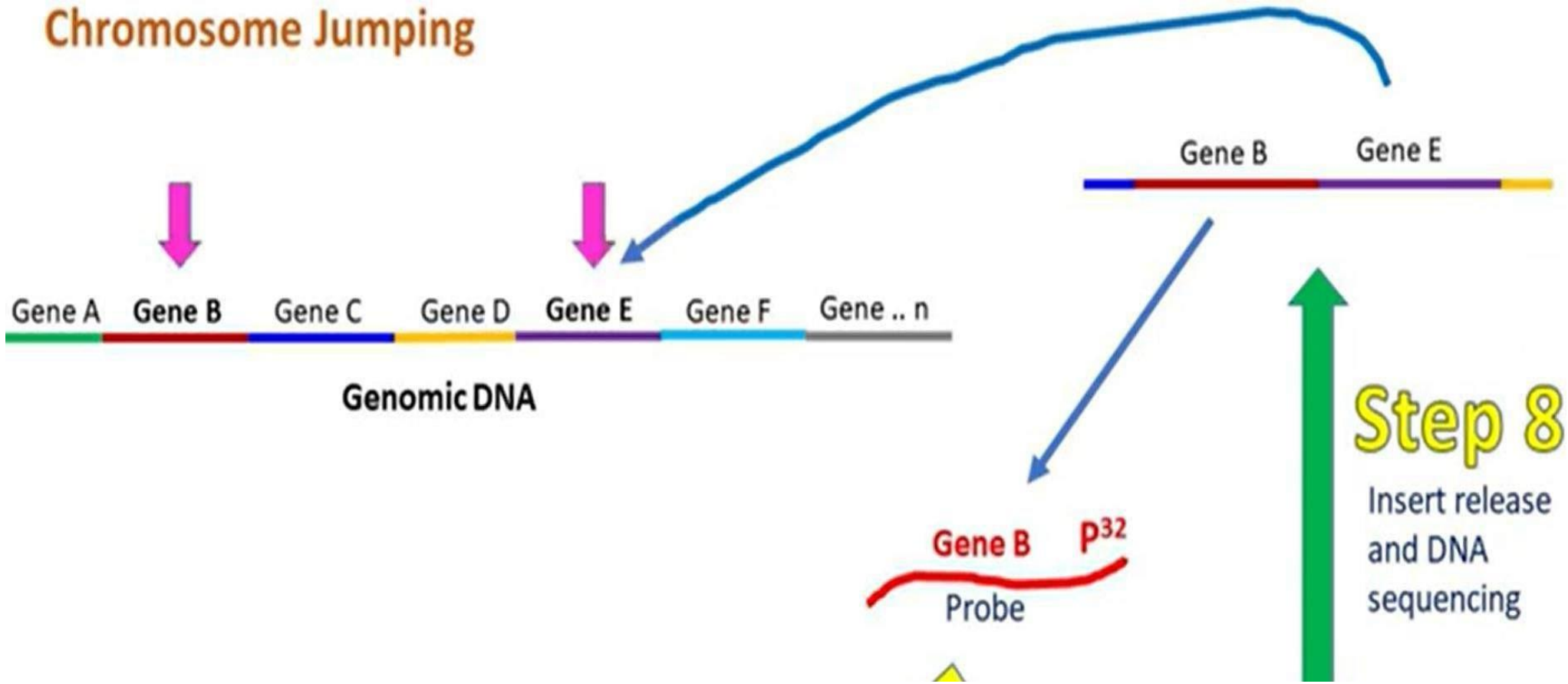
RE digestion

Step 4

Ligation in plasmid
and transformation




Chromosome Jumping
Gene A
Plasmid
Gene B
Gene E
Plasmid
Gene F
Gene .. n
Plasmid
Gene B
P32
Probe
Sterile velvet pad
Step 6
Use probe to screen the colonies
Plasmid isolation
Step 7
Gene B
Gene E
Plasmid
QBB

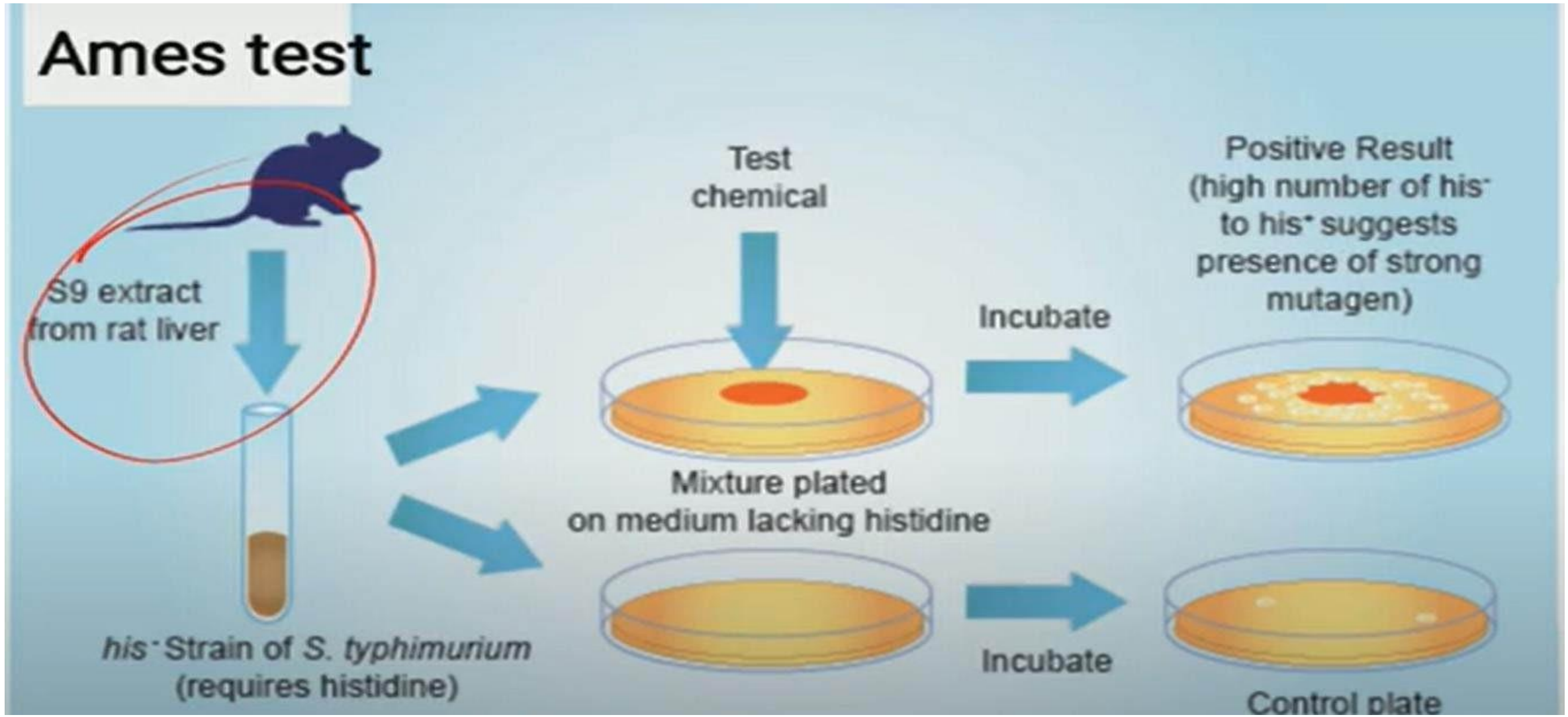

Chromosome Jumping
Gene A
Gene B
Gene C
Gene D
Gene E
Gene F
Gene .. n
Genomic DNA
Gene B
Gene E
Gene B
P32
Probe
Step 8
Insert release and DNA sequencing

MUTAGENICITY

- A **mutagenicity test** is used to determine whether a substance can cause genetic mutations. Mutations are changes in the DNA sequence, which can lead to harmful effects, such as cancer, birth defects, or other genetic disorders.
- Mutagenicity testing is crucial in assessing the safety of chemicals, pharmaceuticals, and environmental agents. It helps identify substances that might cause changes in genetic material, which can be passed down to future generations or lead to serious health problems.

- Common mutagenicity tests include:

1. **Ames test:** Uses bacteria to detect mutations.
2. **Micronucleus test:** Assesses chromosomal damage in cells.
3. **Chromosomal aberration test:** Identifies structural changes in chromosomes.


Ames test
S9 extract
from rat liver
Test
chemical
Positive Result
(high number of his⁻
to his⁺ suggests
presence of strong
mutagen)
Incubate
Mixture plated
on medium lacking histidine
his⁻ Strain of S. typhimurium
(requires histidine)
Incubate
Control plate

INTRODUCTION

- The Ames test is a widely utilized method for assessing the mutagenic potential of chemical compounds, developed by Bruce Ames in the 1970s.
- This biological assay primarily employs strains of the bacterium *Salmonella typhimurium*, which are auxotrophic mutants requiring histidine for growth due to specific mutations in their histidine synthesis genes.
- The test is designed to determine whether a chemical can induce mutations that restore the bacteria's ability to synthesize histidine, allowing them to grow on a histidine-deficient medium.

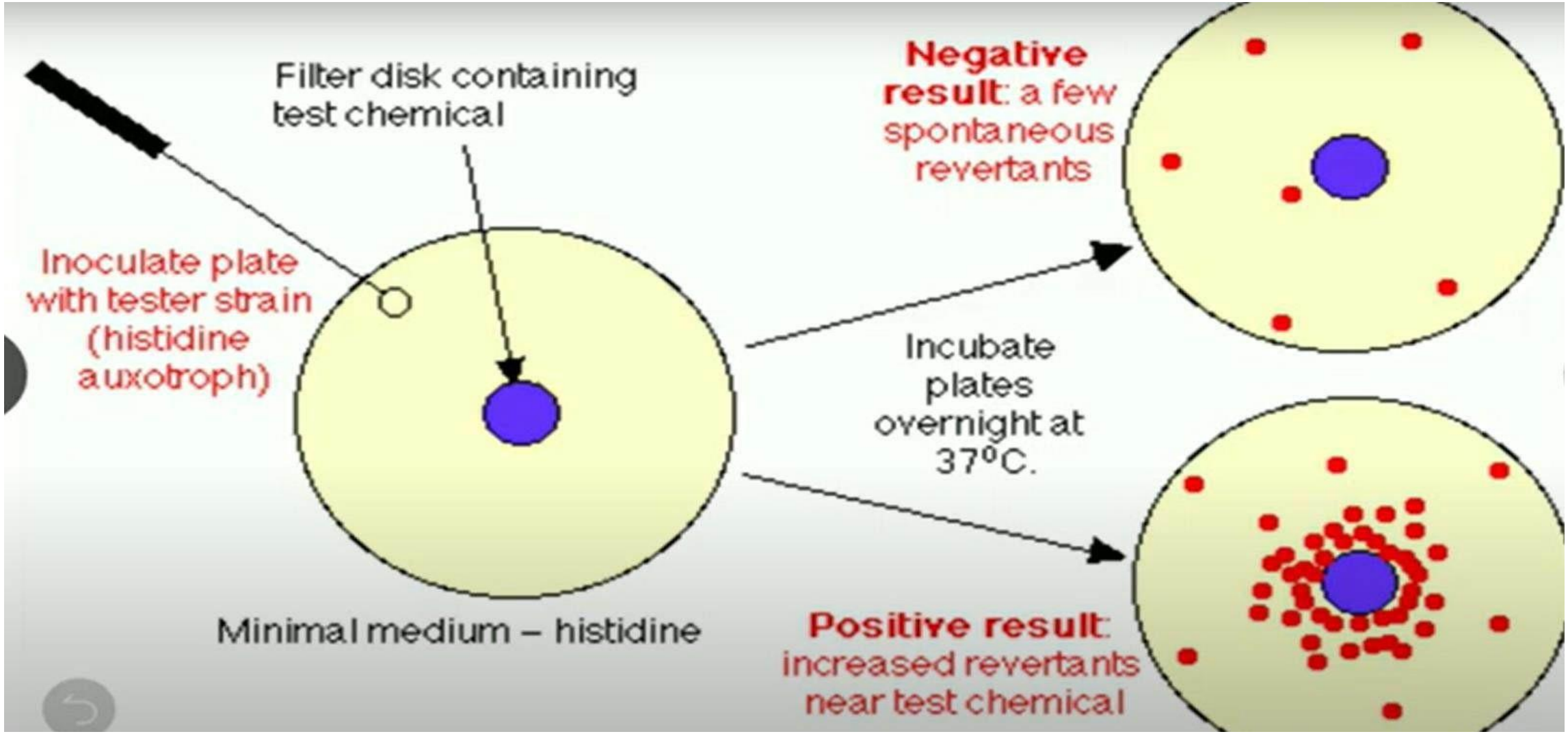
P R O C E D U R E

- Use of Mouse Liver in the Ames Test
- S9 Mix Preparation

1. The S9 mix is a crucial component in the Ames test when assessing the mutagenicity of compounds that may need metabolic activation. This mix is prepared from the liver of mice (or rats) that have been induced with certain chemicals to enhance the activity of liver enzymes. The S9 fraction contains various enzymes, including cytochrome P450, which are responsible for the metabolic conversion of pro-mutagens into their active forms.Collection: Mouse liver is harvested and homogenized.
2. Centrifugation: The homogenate is centrifuged to separate the supernatant, which contains the enzymes (the S9 fraction).
3. Storage: The S9 mix can be frozen for later use in the Ames test

Testing Procedure

1. Bacterial Strains: The test employs *Salmonella typhimurium* strains that are histidine auxotrophs, meaning they cannot synthesize histidine and require it for growth.
2. Exposure: The test compound is mixed with the S9 fraction and the bacterial strains. This combination allows for the metabolic activation of the compound.
3. Plating: The mixture is then plated on agar lacking histidine. Only bacteria that have undergone reverse mutations (restoring their ability to synthesize histidine) will grow.
4. Analysis: After incubation, the number of colonies that form is counted. An increase in revertant colonies compared to control plates indicates mutagenicity.


Filter disk containing test chemical
Inoculate plate with tester strain (histidine auxotroph)
Minimal medium – histidine
Incubate plates overnight at 37°C.
Negative result: a few spontaneous revertants
Positive result: increased revertants near test chemical

MARKERS LINKED TO DRUG &DISEASE RESISTANT GENES CRISPR-CAS-9

Markers linked to drug resistant genes

- Drug resistance markers are specific genetic sequences or mutations that can confer resistance to certain drugs.
- These markers are often found within genes that encode proteins involved in drug metabolism, transport, or target interactions.

Mechanism

Altered Drug Metabolism: Some markers can lead to changes in how the body metabolizes a drug, making it less effective.

Reduced Drug Uptake: Other markers might prevent the drug from entering the target cells or organisms.

Modified Drug Targets: In some cases, mutations in the genes that encode drug targets can make those targets less susceptible to the drug's effects.

Bacteria

Methicillin-resistant Staphylococcus aureus (MRSA)

Marker: mecA gene

Mechanism: The mecA gene encodes a protein that alters the target site of methicillin and other beta-lactam antibiotics, making them ineffective. MRSA strains are resistant to methicillin and often other antibiotics, complicating treatment options.

Detection: PCR (polymerase chain reaction) can be used to detect the mecA gene in clinical samples.

Virus

Human Immunodeficiency Virus (HIV):

Markers: Mutations in the reverse transcriptase gene (e.g., M184V, K103N)

Mechanism: These mutations confer resistance to specific antiretroviral drugs. For example, M184V is associated with resistance to lamivudine and emtricitabine, while K103N confers resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs).

Detection: Genotypic resistance testing is performed on viral RNA to identify these mutations.

Fungi

Candida species

Marker: ERG11 gene

Mechanism: The ERG11 gene encodes the enzyme lanosterol 14α-demethylase, a target of azole antifungals. Mutations in this gene can lead to reduced drug binding and resistance to azoles like fluconazole.

Detection: Molecular assays or sequencing of the ERG11 gene can reveal mutations linked to azole resistance.

Parasites

Plasmodium species (causing malaria)

Markers: pfcrt and pfmdr1 genes

Mechanism: Mutations in pfcrt are associated with chloroquine resistance, while alterations in pfmdr1 are linked to resistance to multiple antimalarial drugs, including mefloquine and artemisinin derivatives. These genes affect drug transport and accumulation in the parasite.

Detection: Genotyping or sequencing of the pfcrt and pfmdr1 genes can help identify resistant strains.

Markers linked to disease resistant genes

- Markers linked to disease-resistant genes are specific genetic variations or mutations that confer resistance to certain diseases or infections.
- These markers are used in various fields, including agriculture, medicine, and genetics
- Examples: Plant disease resistance,Animal disease resistance ,Human disease
- resistance, Marker assisted Selection

Plant Disease Resistance

R-Genes (Resistance Genes)

Mechanism: R-genes typically encode proteins that recognize specific pathogen effectors.

When a pathogen attacks, these proteins trigger a defense response, often involving localized cell death and the activation of various defense pathways

Examples:

Rps Genes in Soybeans

R Genes

Molecular Markers

Markers such as SSRs, SNPs, and SNP chips are used to identify R-genes linked to resistance traits.

Applications: Marker-assisted selection (MAS) enables breeders to select plants with desirable traits more efficiently, accelerating the development of disease-resistant varieties.

Animal resistance genes

Genetic Markers in Livestock

Cattle:MHC GENES

Function: Major Histocompatibility Complex (MHC) genes play a crucial role in the immune response. Specific alleles can enhance resistance to diseases like bovine tuberculosis and mastitis.

Marker Use: Genotyping MHC genes can guide breeding for improved disease resistance.

Sheep

- **Scrapie Resistance**:
  - **Markers**: Variants in the **PRNP gene** (e.g., A136R and R154Q) are associated with resistance to scrapie, a transmissible spongiform encephalopathy.
  - **Application**: Breeding programs use these markers to select resistant breeding stock.

Human Disease Resistance Markers

CCR5-Δ32 and HIV Resistance

Gene/Marker: CCR5-Δ32

Function: The CCR5 gene encodes the CCR5 protein, a chemokine receptor found on the surface of T cells, macrophages, and dendritic cells. This receptor is a critical entry point for HIV-1, enabling the virus to infect immune cells.

Sickle Cell Trait (HbS) and Malaria Resistance

- **Gene/Marker: HBB Gene (Hemoglobin Beta) - HbS Allele**
- **Function**: The HBB gene encodes the beta-globin subunit of hemoglobin. The HbS allele results from a single nucleotide mutation (GAG to GTG) leading to the substitution of valine for glutamic acid at the sixth position of the beta-globin protein.

Human Disease Resistance Markers

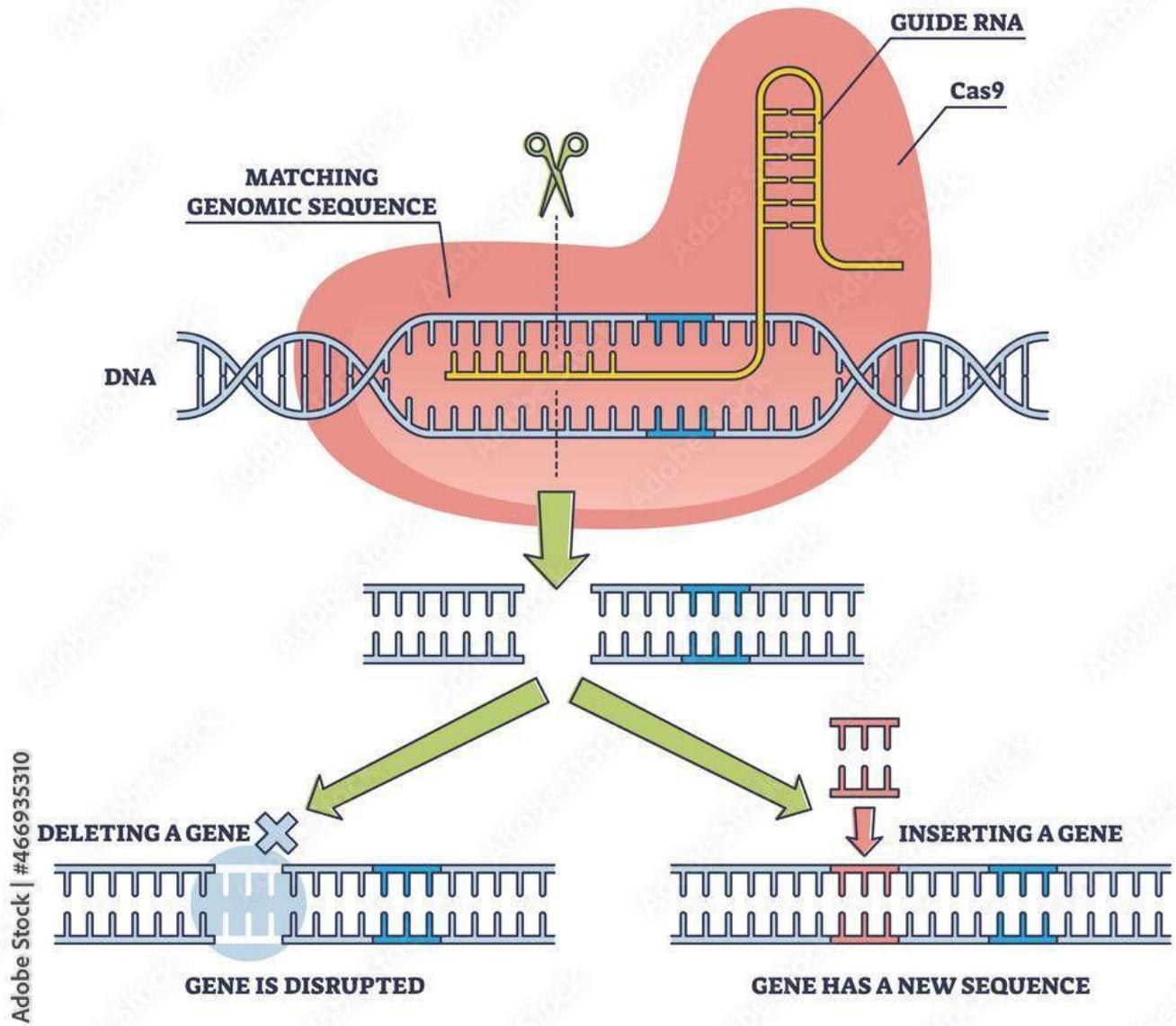
HLA (Human Leukocyte Antigen) System and Immune Response

- **Gene/Marker**: **HLA Genes (Especially HLA-B, HLA-A, HLA-DR)**
- **Function**: HLA genes encode proteins essential for the immune system to recognize and present foreign antigens. They play a pivotal role in adaptive immunity by presenting peptide fragments to T cells.

CRISPR CAS 9

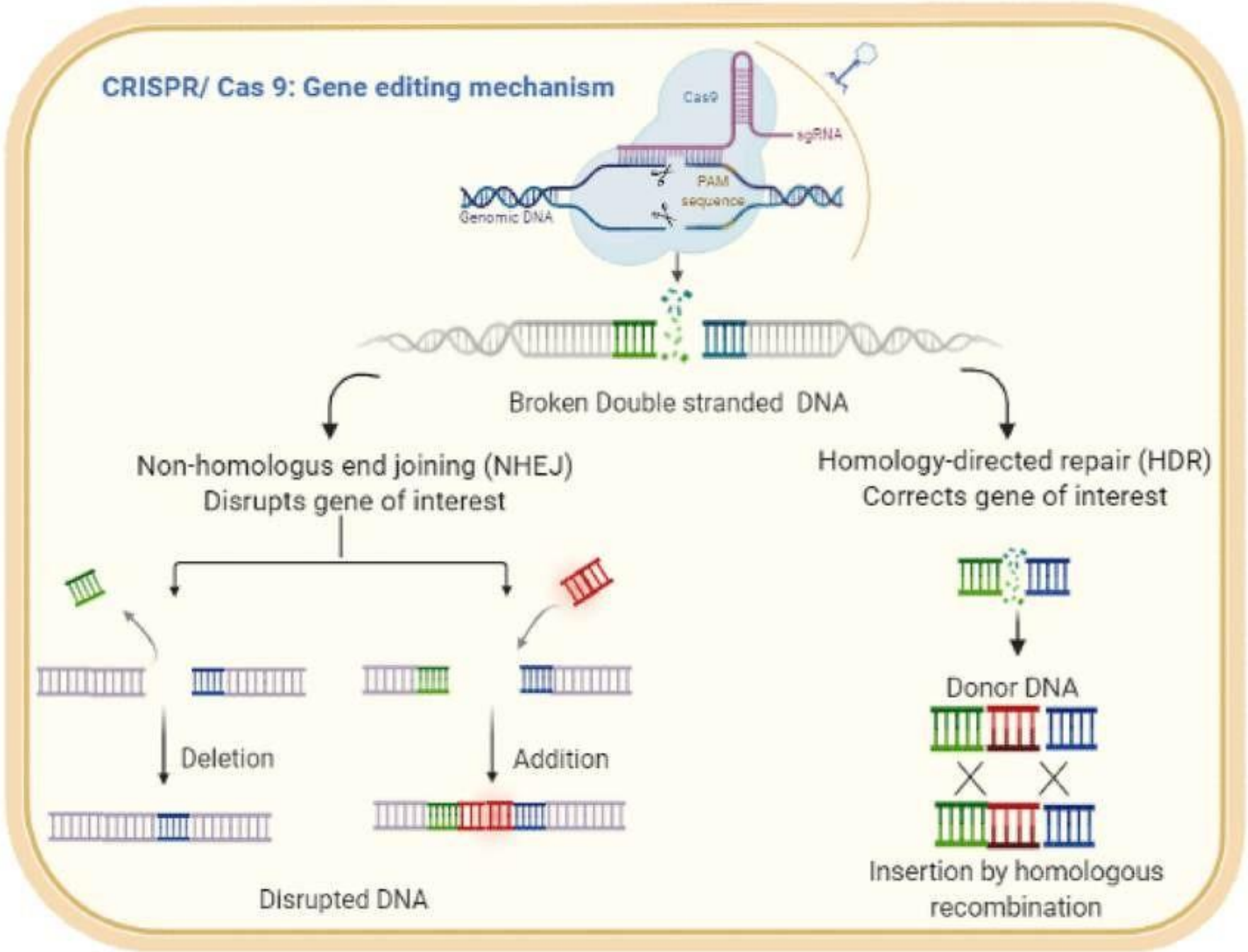
- The term "CRISPR" stands for **Clustered Regularly Interspaced Short Palindromic Repeats**,
- CAS 9 stands for CRISPR Associated proteins.
- CRISPR sequences are segments of prokaryotic DNA containing short repetitions of base sequences.
- Each repetition is followed by short segments of "spacer DNA" derived from previous exposures to viruses (bacteriophages). These spacer sequences serve as a genetic memory, allowing bacteria to recognize and defend against future invasions by the same viruses.

CRISPR



Cas 9

- **Cas9** is an RNA-guided DNA endonuclease enzyme associated with the **CRISPR** (Clustered Regularly Interspaced Short Palindromic Repeats) adaptive immune system found in *Streptococcus pyogenes* bacteria.
- In the CRISPR-Cas9 system, Cas9 acts as molecular scissors that can precisely cut DNA at specific locations, enabling targeted gene editing.


CRISPR/ Cas 9: Gene editing mechanism
Cas9
sgRNA
PAM sequence
Genomic DNA
Broken Double stranded DNA
Non-homologus end joining (NHEJ)
Disrupts gene of interest
Homology-directed repair (HDR)
Corrects gene of interest
Deletion
Addition
Disrupted DNA
Donor DNA
Insertion by homologous recombination

ANTISENSE TECHNOLOGY AND ITS APPLICATION MICROARRAY TECHNOLOGY:GENOMIC AND cDNA arrays

ANTISENSE

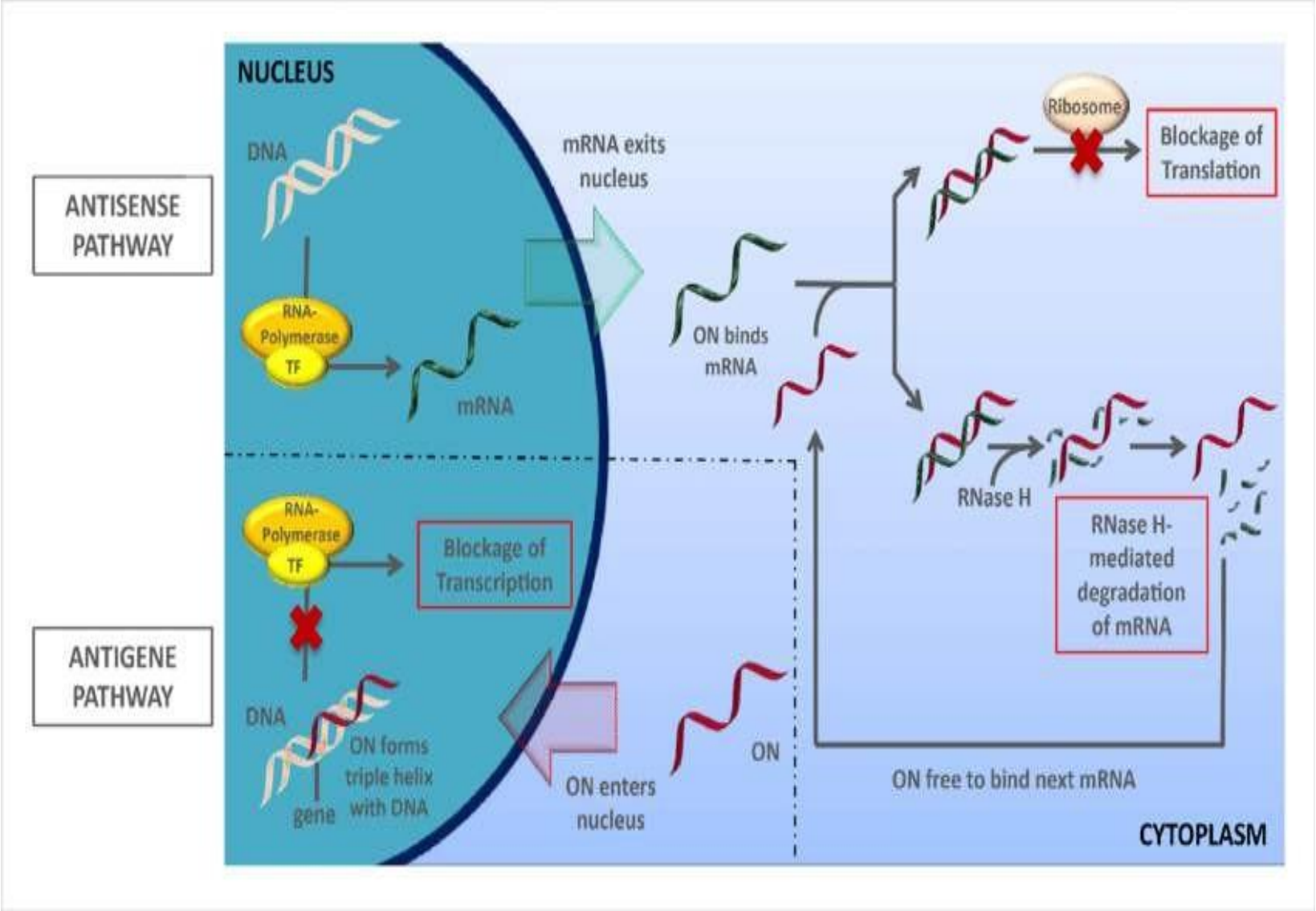
- Antisense DNA technology is a method to inhibit or downregulate the production of a target protein by using antisense DNA or RNA molecules.
- It should be perfectly complementary to the target nucleotide sequence present in the cell.

There are two possible mechanisms for an antisense effect.

- The method that relies on targeting of the mRNA is called the antisense strategy.
- When the double-stranded DNA or genes situated in the nucleus are targeted, the approach is called the antigene strategy.

translation of the

In the antigene approach oligonucleotides sequence selectively genomic, double-stranded DNA and interfere with transcription and the DNA processing machinery via triple helix formation.



- The introduction of antisense oligonucleotides can help determine the role of a specific gene in a specific physiological process.
- Perhaps the most widely discussed application of antisense technology lies in its applications to gene therapy.
- In this case, a variety of vectors is used to introduce antisense-encoding genes into a large number of cells in a patient or animal to produce long-term inhibition of a protein.
- For example, an antisense oligonucleotide inhibitor of an apo-B protein is used to treat Familial Homozygous Hypercholesterolemia.
- ASO is a molecular drug that inhibits gene expression by specifically binding to the target gene DNA or mRNA, and regulates it at the gene level.

Applications of antisense RNA in plants

- In plants, antisense RNAs are mainly used in the inhibition of fruit maturation, virus resistance, flower coloration, starch synthesis, male sterility, and fertility.

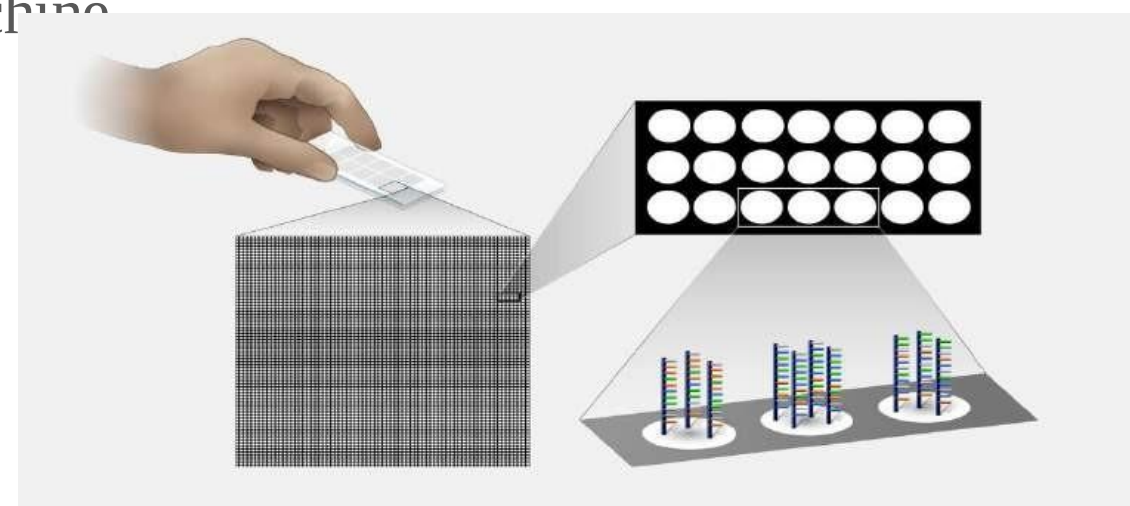
Antisense in viral infection:

- Antisense oligonucleotides are selective and highly specific and binds with targeted viral mRNA and down regulate the expression of viral proteins, thus inhibit the virus replication.
- Vitravene (fomivirsen) is the first antiviral Antisense drugs used to treat cytomegalovirus retinitis.

Antisense in cancer:

- The antisense oligonucleotides are currently investigated to treat various cancers in humans or for the deactivation of oncogenes.
- For the first time an antisense oligonucleotide in combination with cisplatin was approved to treat bladder cancer.

- Microarray technology is a general laboratory approach that involves binding an array of thousands to millions of known nucleic acid fragments to a solid surface, referred to as a “chip.”
- The chip is then bathed with DNA or RNA isolated from a study sample (such as cells or tissue).
- Complementary base pairing between the sample and the chip-immobilized fragments produces light through fluorescence that can be detected using a specialized machine

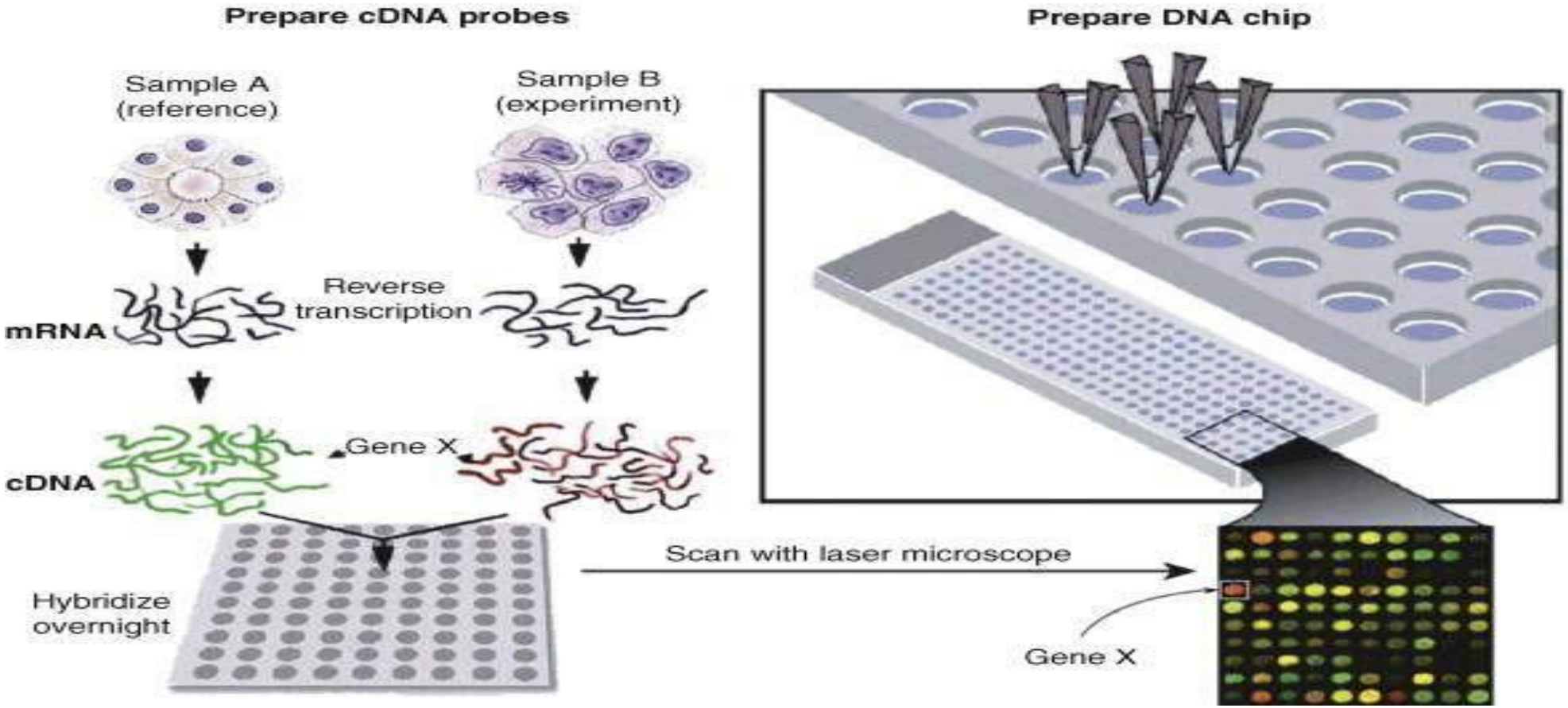


GENOMIC MICROARRAY

- A **DNA microarray** (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface.
- The core principle behind microarrays is hybridization between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs.

cDNA MICROARRAYS

- cDNA microarray is the method to use cDNA clones fixed on a glass slide in an ordered two-dimensional matrix as probes.
- The transcription levels of each gene can be calculated by the intensity of hybridized targets (RNA, cDNA), which are derived from tissues of interest and labeled with florescent dye.
- Sample RNA and a reference RNA are differentially labeled with fluorescent Cy5 or Cy3 dyes, respectively, using reverse transcriptase.
- The subsequent cDNAs are hybridized to the arrays overnight.
- The slides are washed and scanned with a fluorescence laser scanner.
- The relative abundant of the transcripts in the samples can be determined by the red/green ratio on each spotted array element.



Principle of cDNA microarrays. PCR products are printed onto glass slides to produce high density cDNA microarrays. RNA is extracted from experimental samples and reference samples, and differentially labeled with Cy5 and Cy3, respectively, by reverse transcriptase. The subsequent cDNA probes are mixed and hybridized to cDNA microarray overnight. The slides are washed and scanned with fluorescence laser scanner. The relative red/green ratio of gene X indicates the relative abundance of gene X in experimental samples versus reference.

Why is cDNA used in microarray instead of mRNA?

- cDNA is used in microarray instead of mRNA since mRNA is degraded easily, it is necessary to convert it into a more stable cDNA form.

Applications

- Use of DNA microarrays for gene expression and discovery
- Use of DNA microarrays for predicting various biochemical pathways
- Use of DNA microarrays for drug discovery and development

❑REFERENCE :

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- *DNA Cloning : a Practical Approach, DM Glover and BD Hames, IRL Press*
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https://en.wikipedia.org/wiki/File:From_spit_to_DNA-sample.webm

THANK YOU