Clinical Laboratory Science Department
College of Applied Medical Sciences

Polymerase Chain Reaction (PCR): Types & Applications

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What is PCR?

Polymerase chain reaction is an in vitro amplification of **target DNA** whose sequence is known or which lies between two regions of known sequence via repetitive thermal cycles (30-50 cycles), i.e., alternate heating and cooling aiming at duplication of target each cycle until reaching millions or billions of its initial count.

Why "Polymerase"?

- It is called "polymerase" because the only enzyme used in this reaction is DNA polymerase (Thermostable).

Why "Chain"?

- It is called "chain" because the products of the first reaction become substrates of the following one, and so on.

The "Reaction" Components

1)Target DNA - contains the target sequence to be amplified after extraction outside its cell.

2) DNA Polymerase- the enzyme that synthesizes new strands of DNA complementary to the target sequence. There are different types of these enzymes, but the first and most commonly used is *Taq* DNA polymerase (from *Thermus aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA.

3) Primers- short pieces of single strand DNA (18- 30 bases) that are complementary to the target sequence. For each target gene at least a pair of primers needed.

4) Nucleotides- (dNTPs or deoxynucleotide triphosphates, which are essentially "building blocks" for new DNA strands.

5) Buffer solution – maintains pH and ionic strength of the reaction solution to be suitable for the enzyme activity.

6) Mg^{++} ions - cofactor of the enzyme.

What is before and after PCR?

- Extraction of Nucleic acid
- Amplification of the target sequence
- Detection of PCR product (Amplicon)
- After PCR manipulations (sequencing, Southern blot, Restriction enzyme analysis and gel electrophoresis, in which running of amplified DNA out on an agarose gel and stain with a dye which makes it visible, (the brighter the visible band, the more copies of target have created.)

Types of PCR

A.Standard PCR, DNA

B.Reverse Transcription-PCR, RNA

C.Real-Time PCR – RTQ-PCR (DNA or RNA)

The Standard Polymerase Chain Reaction

PCR tube

Thermal Cycler

A. Standard PCR

- Basic PCR (uniplex)
- Multiplex PCR
- Nested PCR
- Hot start PCR
- Restriction fragment length (RFLP-PCR)
- Amplification refractory mutation system PCR (ARMS-PCR)

Basic (Uniplex) PCR

What are the steps of PCR?

- Denaturation: separation of DNA (DNA melting) into 2 single strands by heating usually at 94-96 °C. This disrupts the hydrogen bonds between complementary bases. The time needed for this step is usually seconds.

- **Annealing:** giving the chance for the specific primer to anneal to its single-stranded DNA complementary sequence on the target by decreasing the temperature to 55-60 °C for 20–40 seconds. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should only bind to a perfectly complementary part of the template.

- Typically the annealing temperature is about 3–5 °C below the melting temperature (Tm) of the primers used. It is very vital to determine the annealing temperature in PCR. This is because in PCR, efficiency and specificity are affected by the annealing temperature. An incorrect annealing temperature will cause an error in the test.

- **Extension/Elongation step:** This begins by raising temperature to 72 °C. which is needed for action of *Thermus aquaticus (Taq)* polymerase enzyme, the thermostable Polymerase commonly used for this purpose. However different enzymes can be used other than *Taq* polymerase e.g. *Pfu* pol and *Vent* pol. Each has its own optimum temperature of activity.

- The polymerase binds to the primer-template hybrid and begins DNA formation. The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding nucleotides (dNTPs) that are complementary to the template in 5' to 3' direction.

steps of PCR

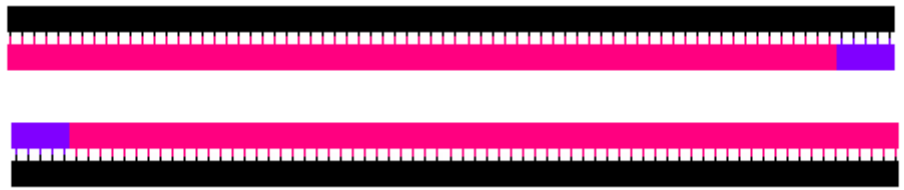
Denature (heat from 94°C- 96°C)

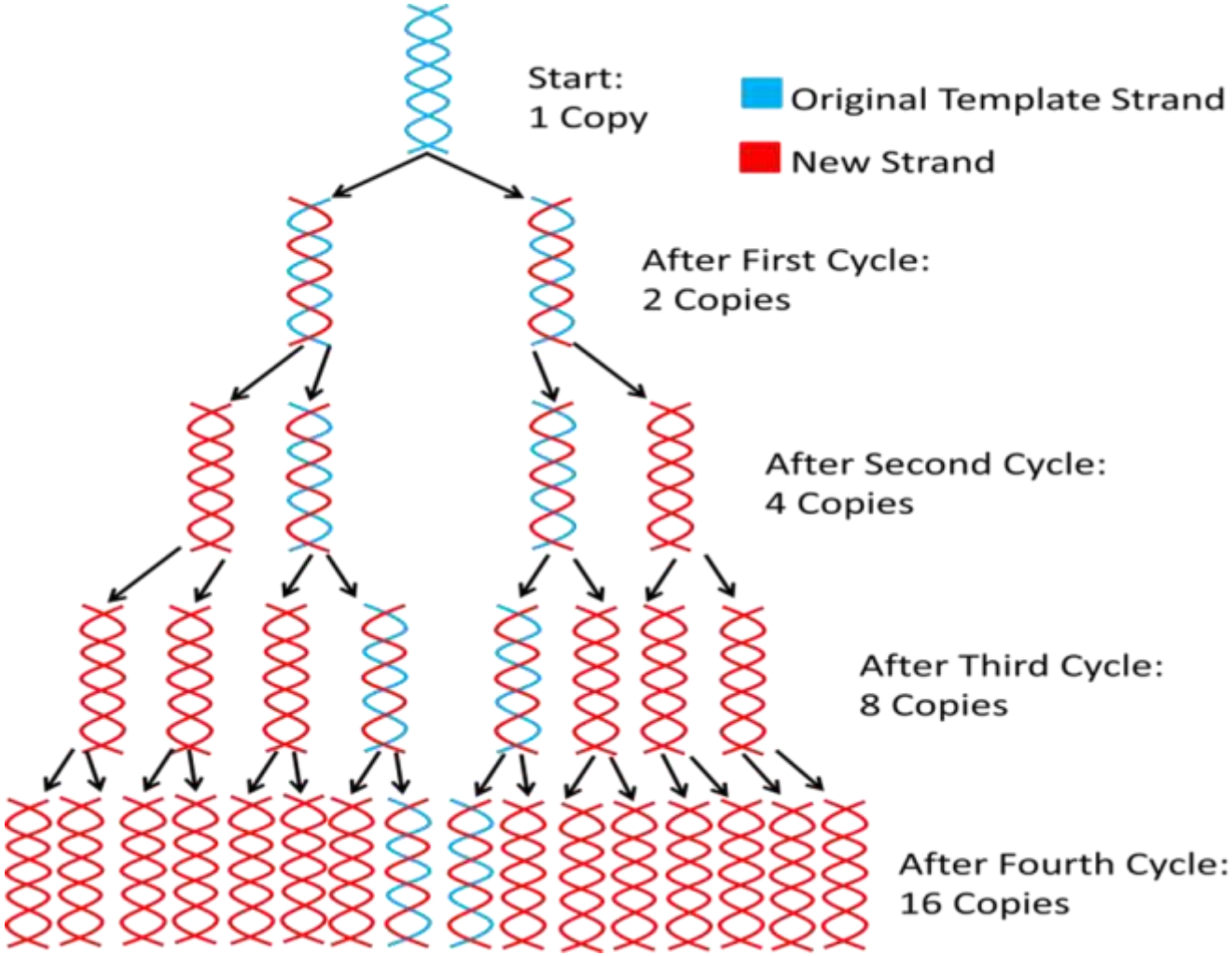
Anneal with primers

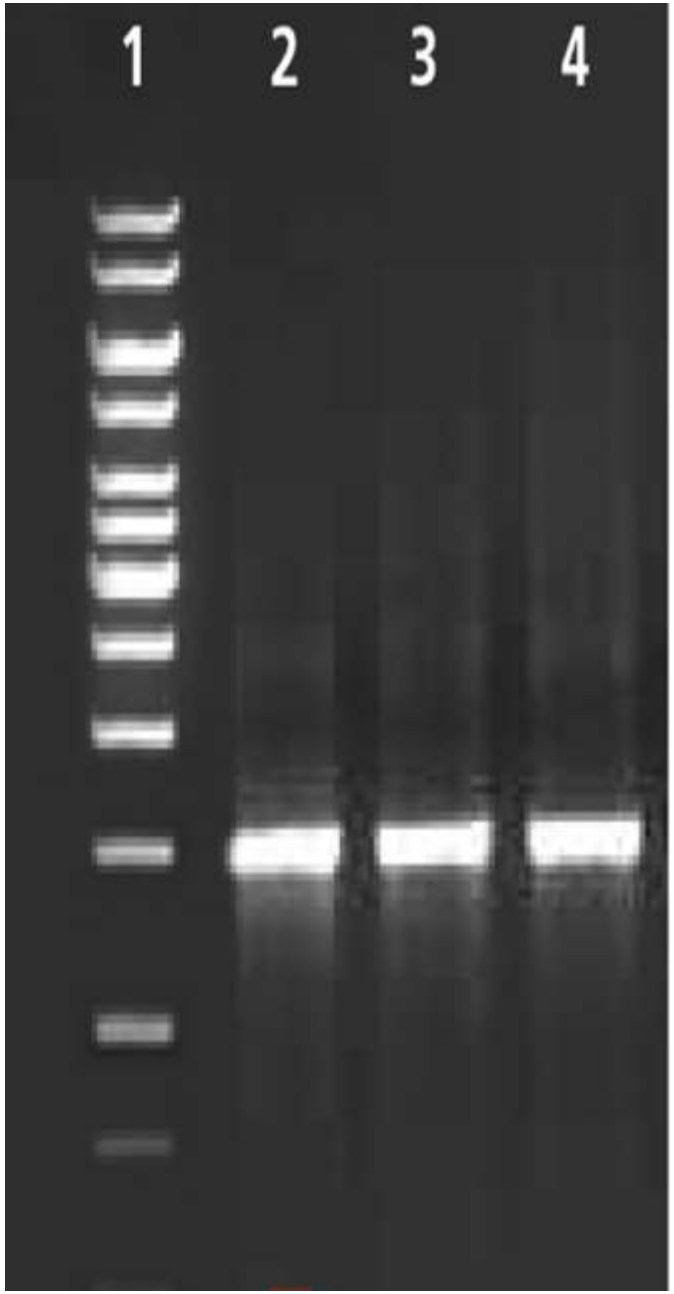
temperature from (55°C - 60°C)

Extension by DNA polymerase + dNTPs

temperature at72°C

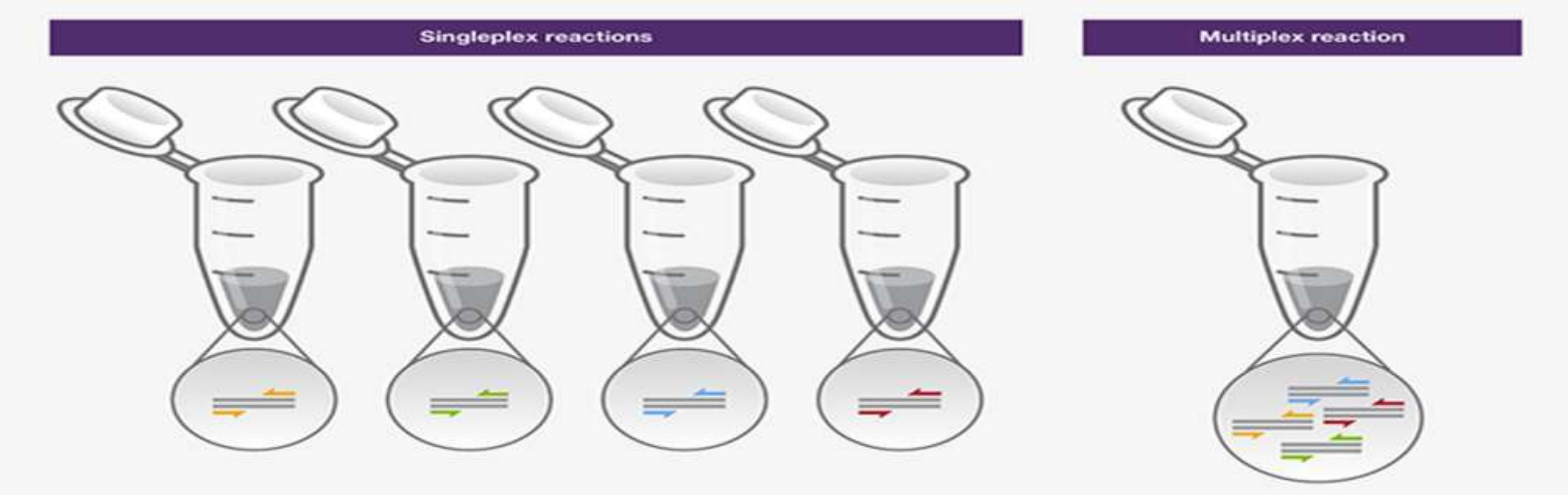


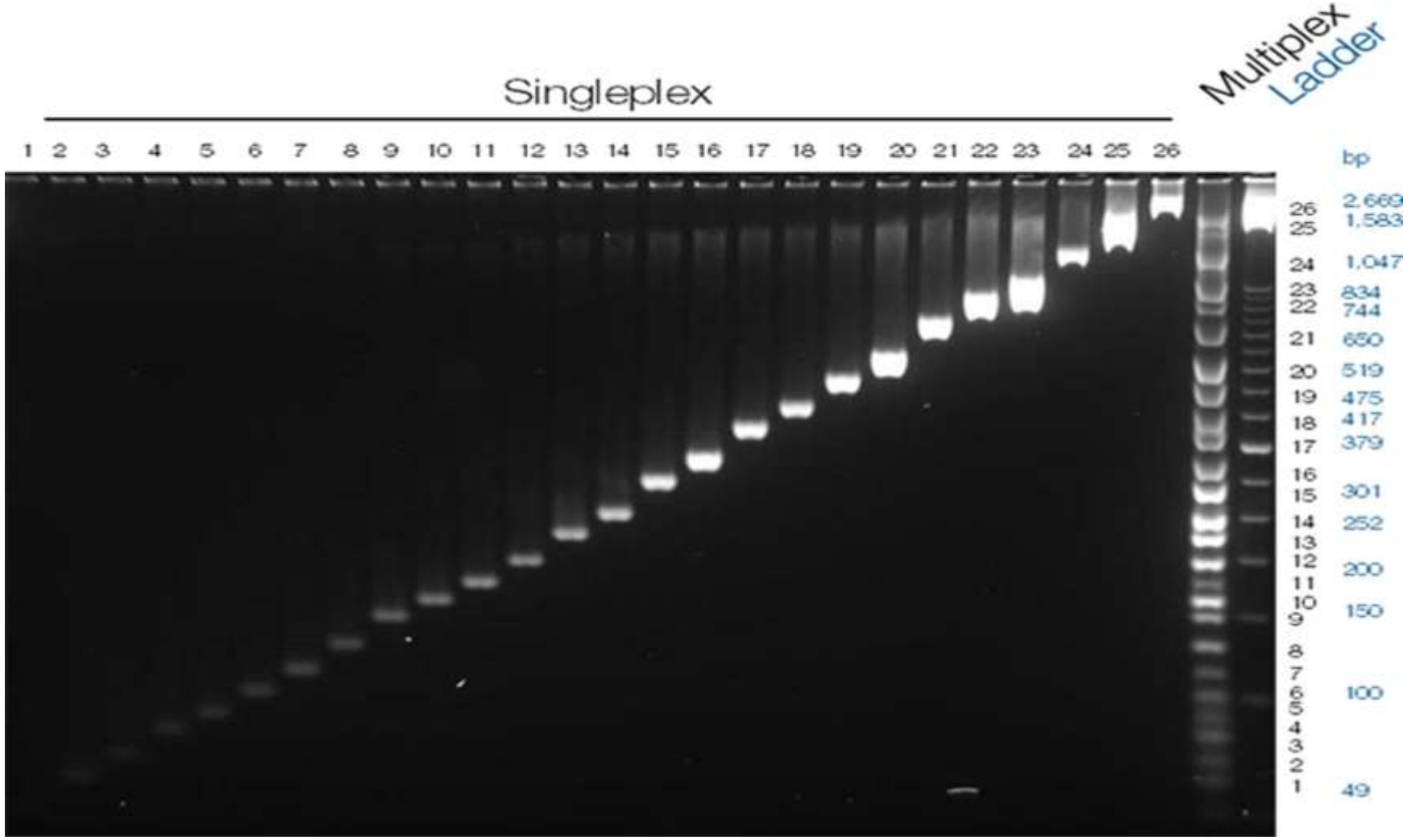

Start:
1 Copy
Original Template Strand
New Strand
After First Cycle:
2 Copies
After Second Cycle:
4 Copies
After Third Cycle:
8 Copies
After Fourth Cycle:
16 Copies


1
2
3
4

Multiplex PCR

It allows concurrent amplification of different targets in a single PCR tube. Multiplexing not only saves time, reagents, and samples but also makes simultaneous comparison of multiple amplicons possible.



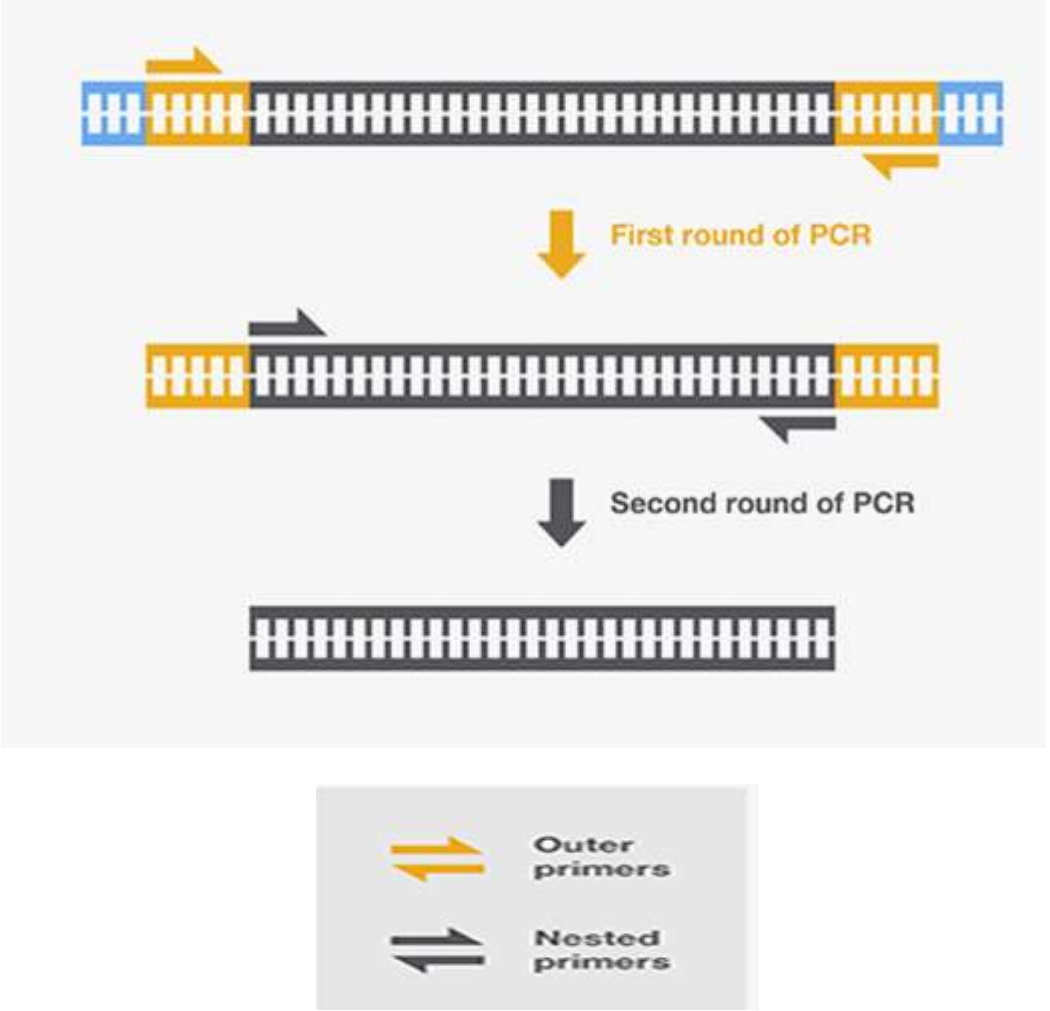

Singleplex
Multiplex Ladder
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
bp
26 2,669
25 1,583
24 1,047
23 834
22 744
21 650
20 519
19 475
18 417
17 379
16
15 301
14 252
13
12 200
11
10
9 150
8
7
6 100
5
4
3
2
1 49

Although multiplex PCR is routinely performed as endpoint reactions, the approach is more popular with real-time PCR because of capabilities in multi-labeling for detection of target amplicons.

Limitation: mixing different primers can cause some interferences in the amplification, so that optimizing conditions can be difficult.

Nested PCR

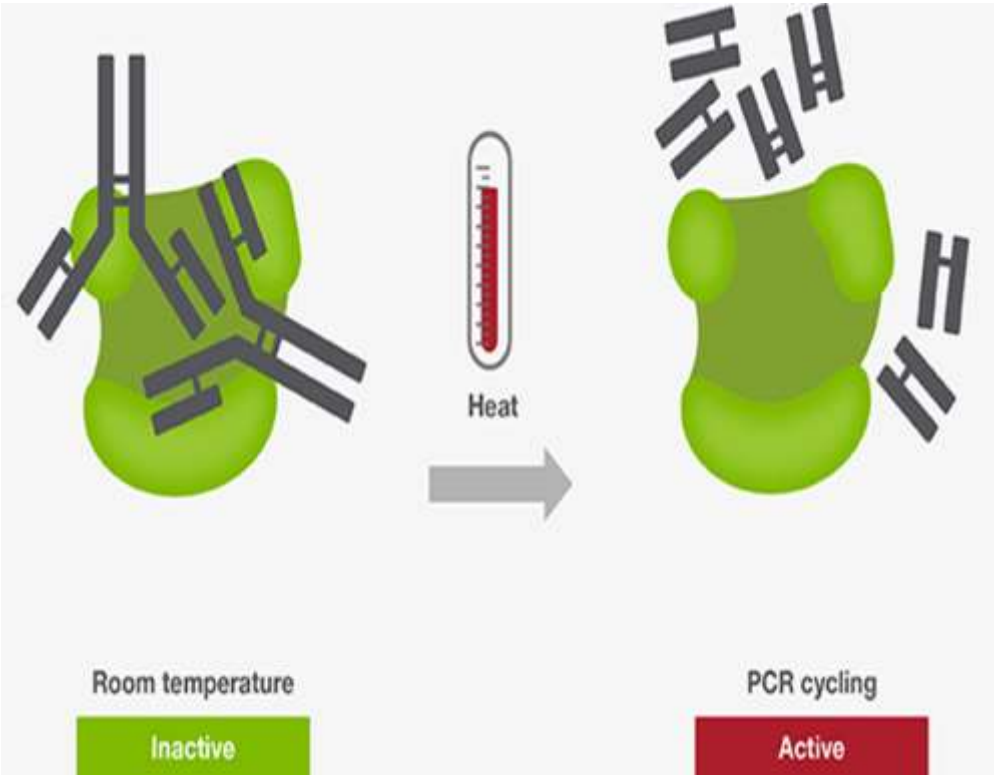
- one set of primers flanks a region of DNA containing the sequence of interest in a first PCR round.
- The product of the first round serves as a template in the second round of PCR
- a second primers set corresponds to the precise region of DNA to be amplified .



- Nested PCR is a variation of standard PCR that enhances the specificity and yield of the desired amplicons.
- this approach helps to obtain a sufficient yield of the desired target from a limited amount of input DNA.

Hot Start PCR

The DNA polymerase is activated in an initial heating step for 5-10 minutes that is added to PCR protocols during which the polymerase enzyme inhibitor is released at a high temperature (usually above 90°C).

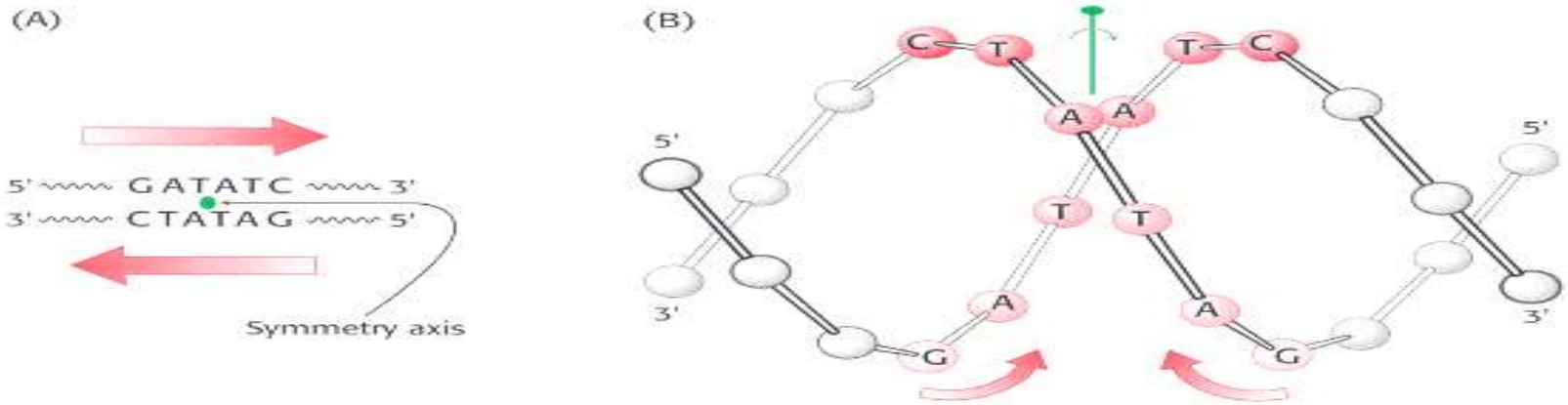


Hot start PCR decreases the risk of non specific amplification.

Restriction endonucleases (REs)

- Molecular scissors that cut double stranded DNA molecules at specific points (An important tool for manipulating DNA).
- Recognition sites of restriction enzymes

Restriction enzymes have corresponding symmetry to facilitate recognition and usually cleave the DNA on the axis of symmetry.



Biological Role

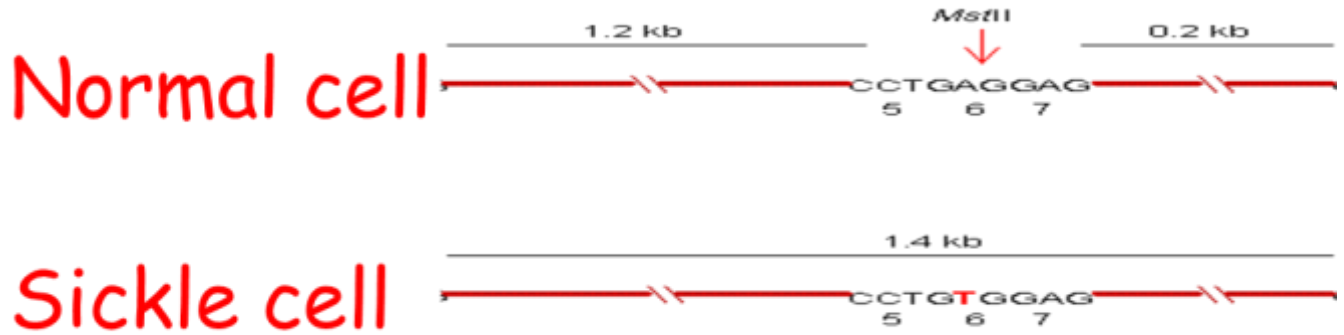
- Most bacteria use REs as a defense against bacteriophages.
- REs prevent the replication of the phage by cleaving its DNA at specific sites.
- Host DNA is protected by Methylases which add methyl groups to adenine or cytosine bases within the recognition site thereby modifying the site and protecting the DNA.

Mechanism of action

Restriction Endonuclease scan the length of the DNA , binds to the DNA molecule when it recognizes a specific sequence and makes one cut in each of the sugar phosphate backbones of the double helix. It can either cleave at the center of both strands to yield blunt end or at staggered position leaving overhang called sticky end. (examples of RE as EcoR1 and hind II)

Restriction fragment length polymorphism PCR (RFLP-PCR)

- It involves fragmenting a sample of DNA after PCR amplification by a restriction enzyme, which can recognize and cut DNA wherever specific short sequence occurs. Resulting DNA fragments are separated by length on agarose gel electrophoresis.



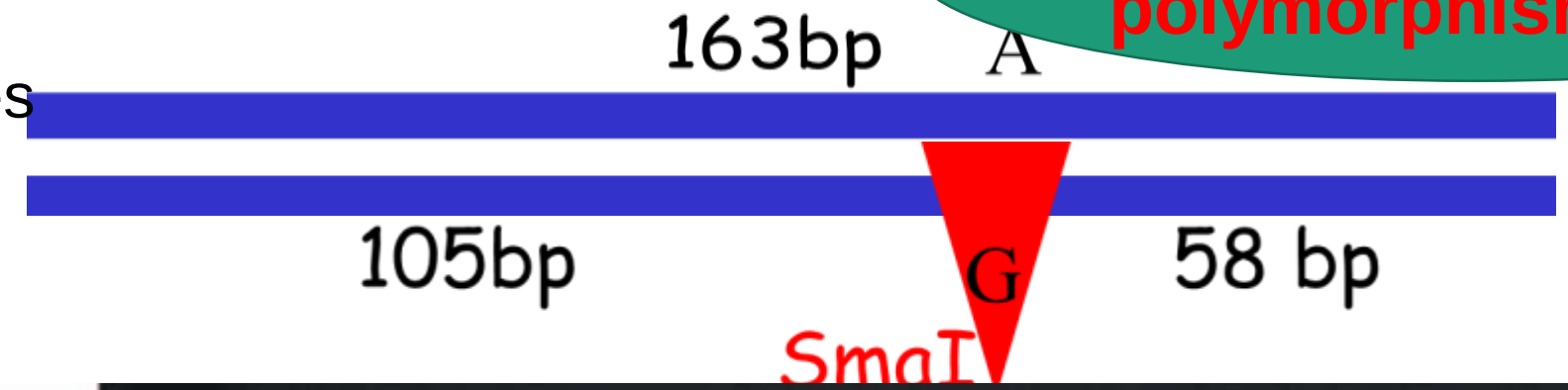
MMP-9 gene polymorphism (SNP)

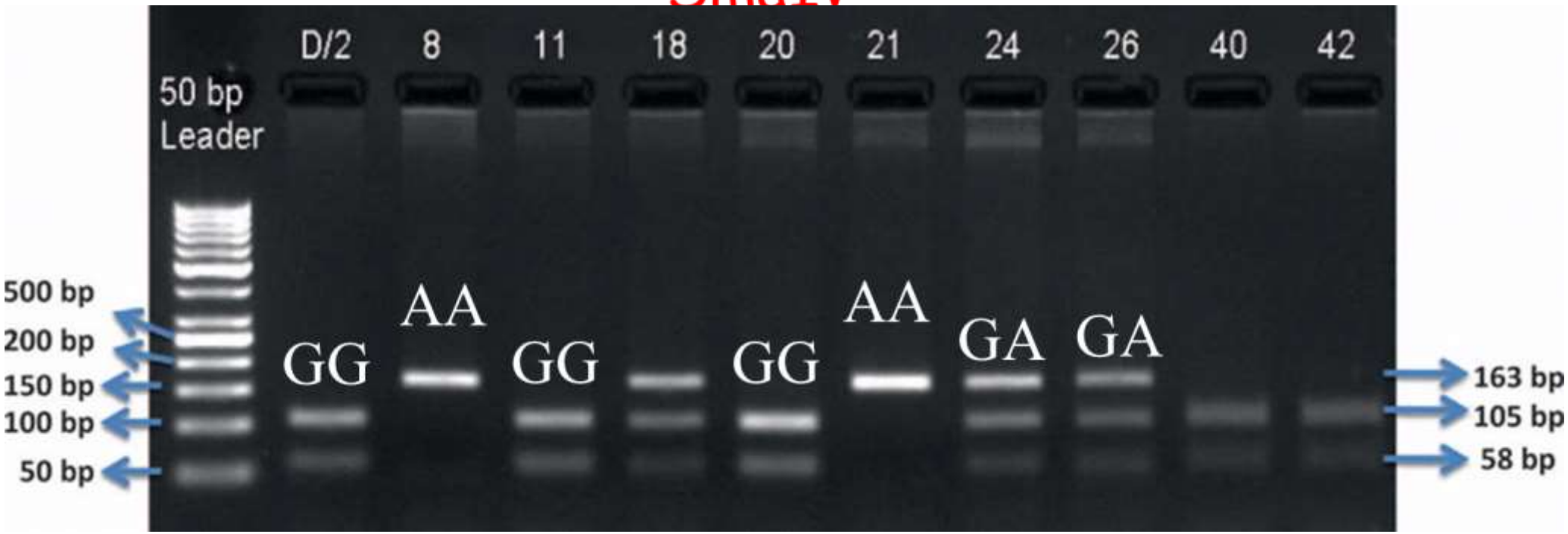
Genotypes

GG

GA

AA

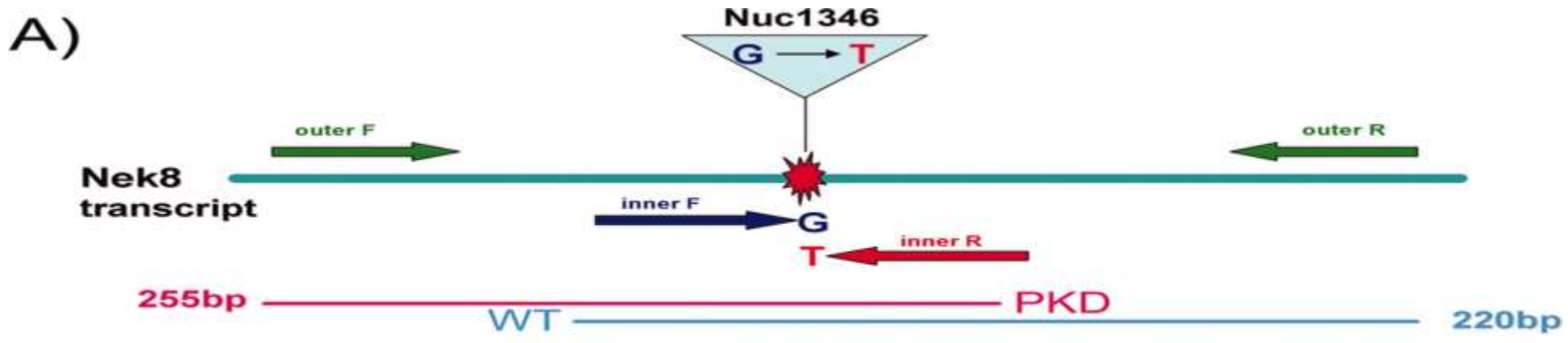


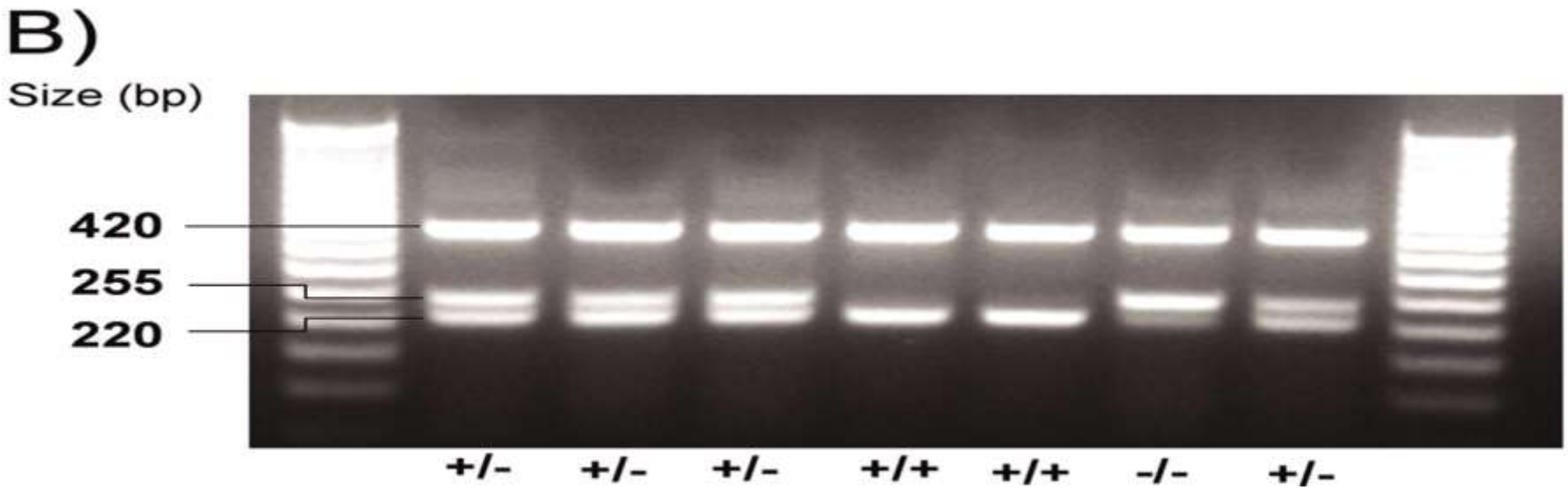


Amplification refractory mutation system PCR (ARMS-PCR)

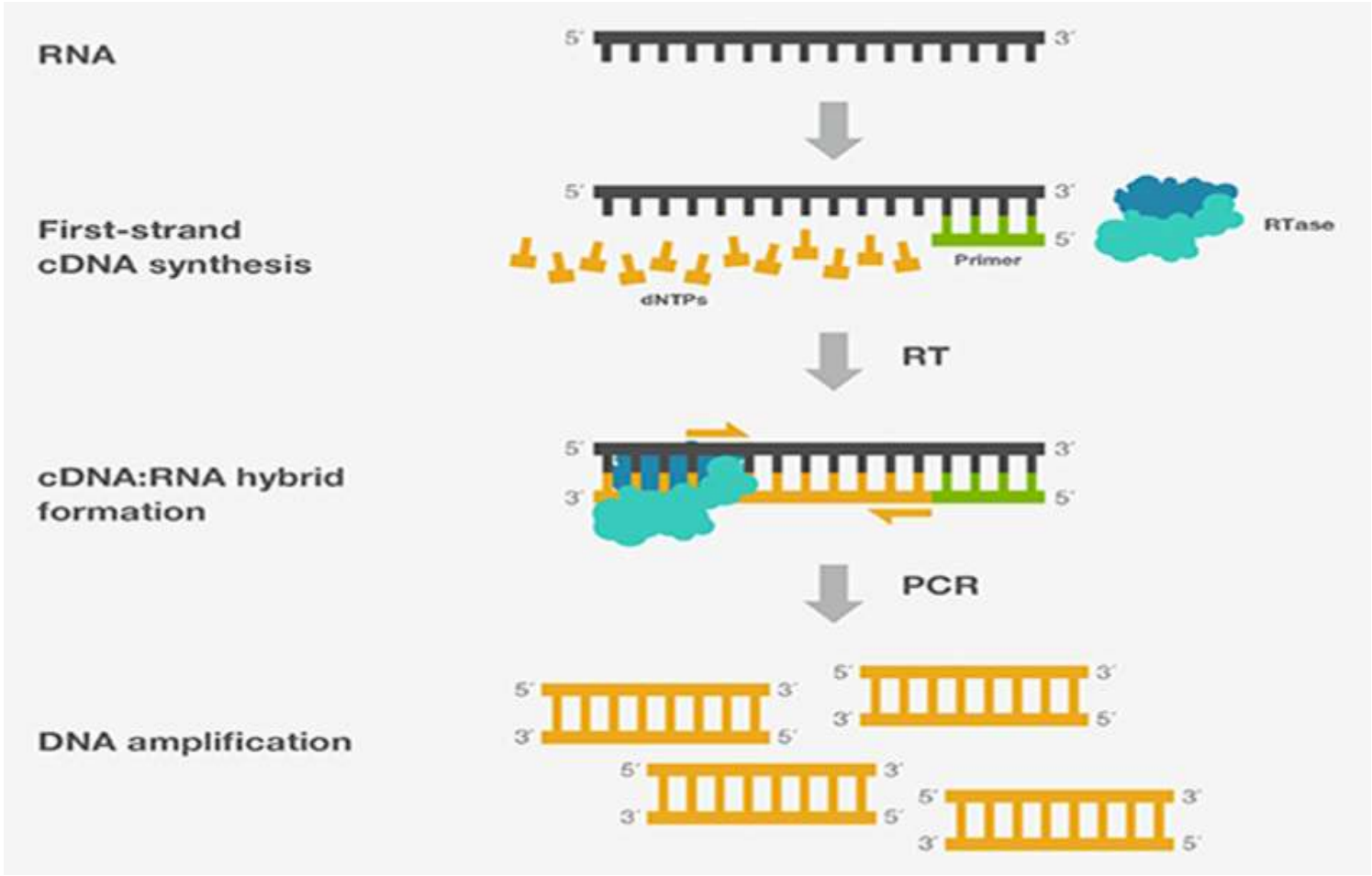
It employs two pairs of primers to amplify two alleles in one PCR reaction. The primers are designed such that the two primer pairs overlap at a SNP location. One primer for mutant allele with the SNPs and second primer for the normal allele. Both primers are combined in a single PCR reaction.

It’s the gold standard method for thalassemia and sickle cell anaemia diagnosis.


A)
Nuc1346
G → T
outer F
outer R
Nek8
transcript
inner F
G
T
inner R
255bp
PKD
WT
220bp


B)
Size (bp)
420
255
220
+/-
+/-
+/-
+/+
+/+
-/-
+/-

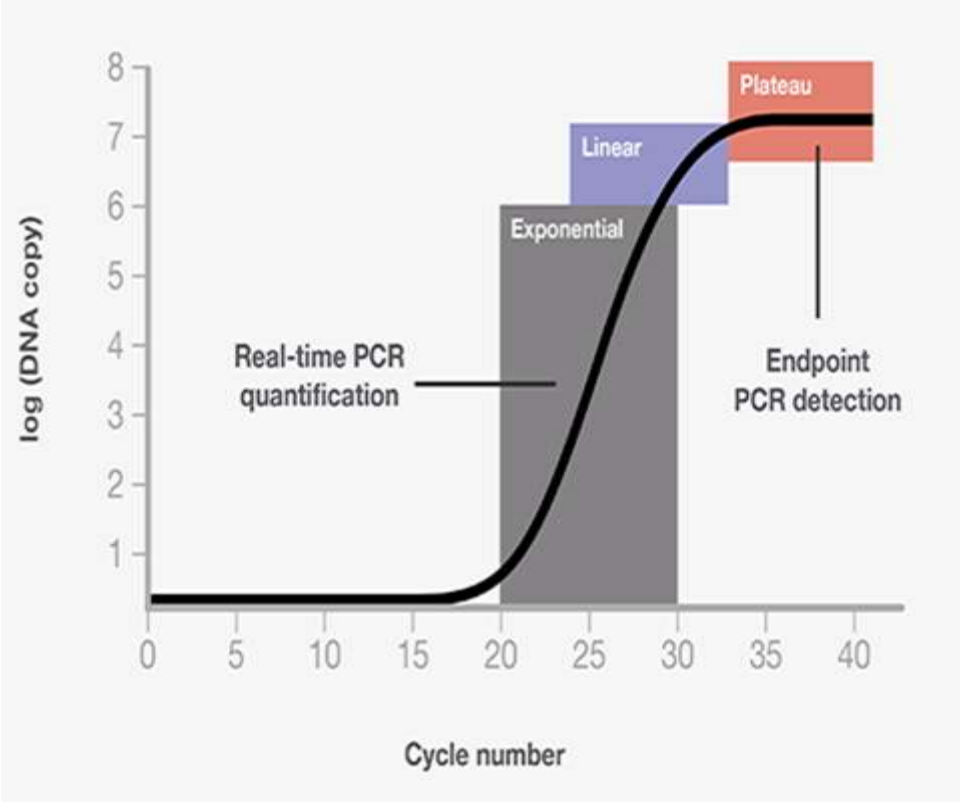
B. Reverse transcription PCR (RT-PCR)



- It is commonly used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA.

C. Real-Time PCR (RTQ-PCR)

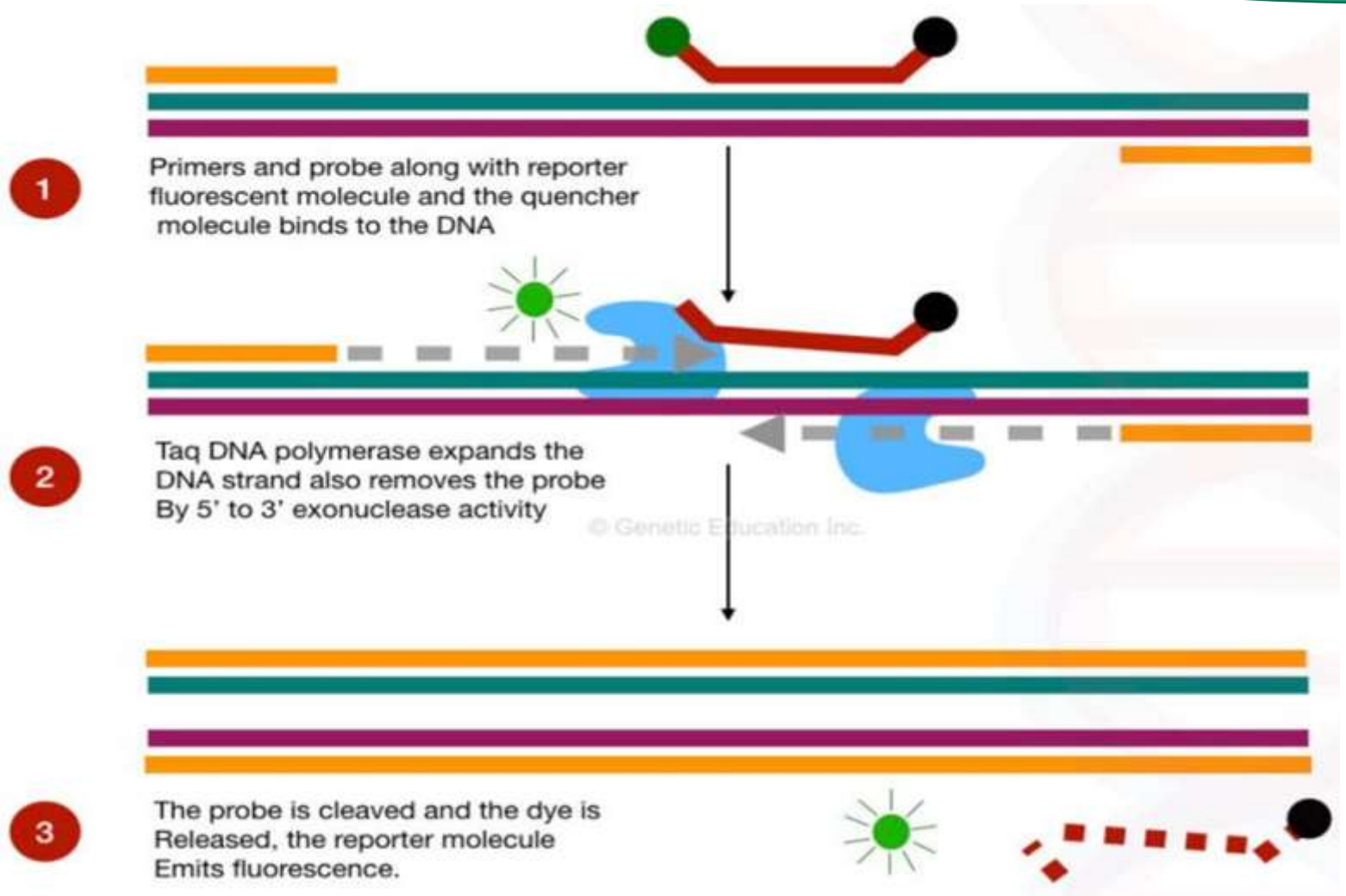
- **Real-Time PCR** is used to detect the expression level of the target nucleic acid in the organism.
- Quantitative PCR relies upon real-time monitoring of fluorescence intensity associated with target amplification during the exponential phase by using intercalating dye (e.g. SYBR Green) or a hydrolysis probe (e.g.TaqMan), based on detection solution



Advantages:

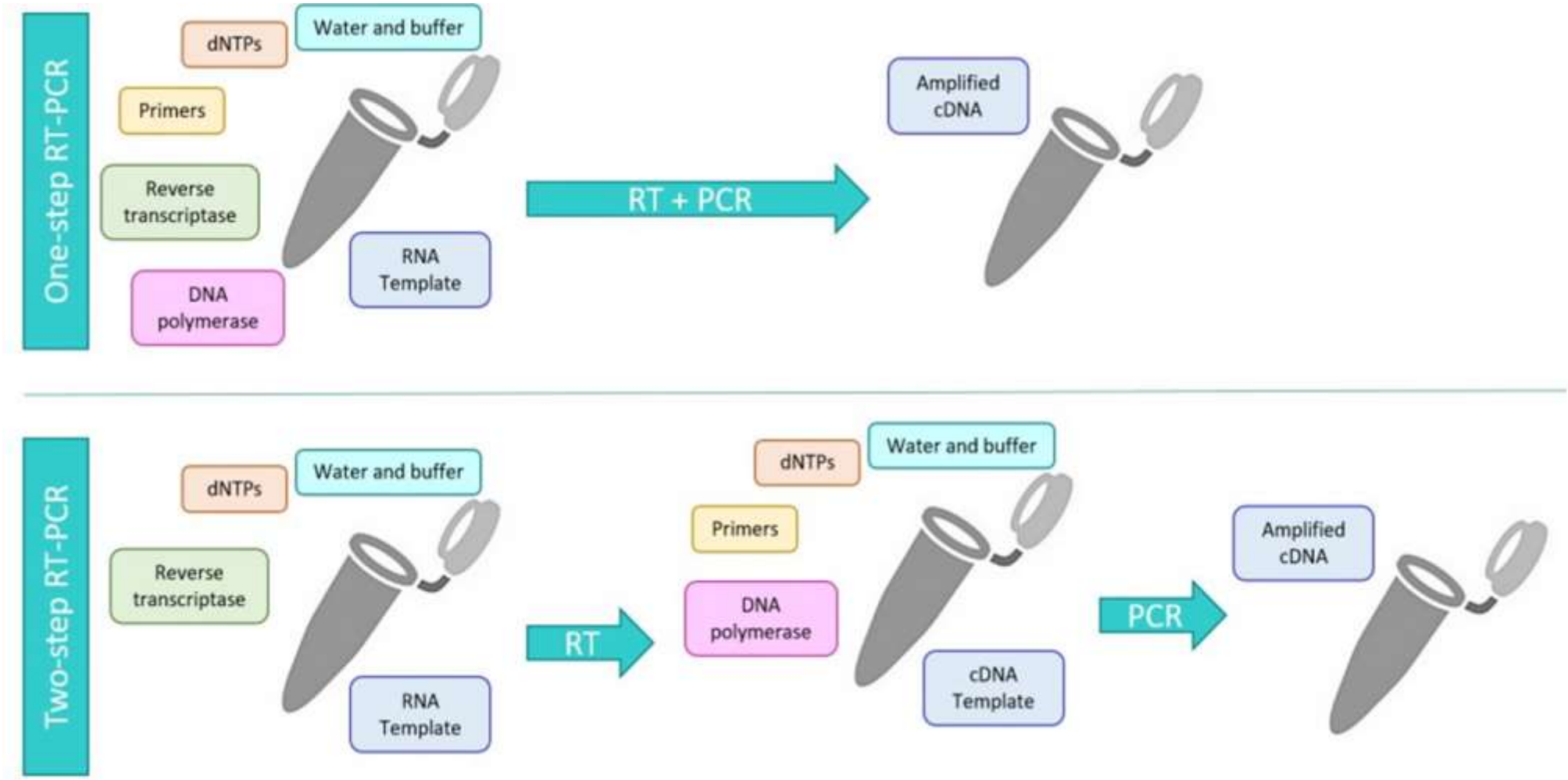
- Quantitative
- Both amplification & detection can be accomplished in one vessel without opening → no cross contamination.
- Less time is needed→ no post-PCR detection as in conventional PCR , as electrophoresis is used after amplification → 20 – 30 min.
- Multiplex real time PCR : using different probes for target sequence.
- Avoiding the pitfalls of endpoint PCR quantitation.

The principle of Real Time PCR



Quantitative reverse transcription PCR (RT-**qPCR**) is used when the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA or messenger RNA (mRNA).

Then cDNA is used as the template fort he **qPCR** reaction

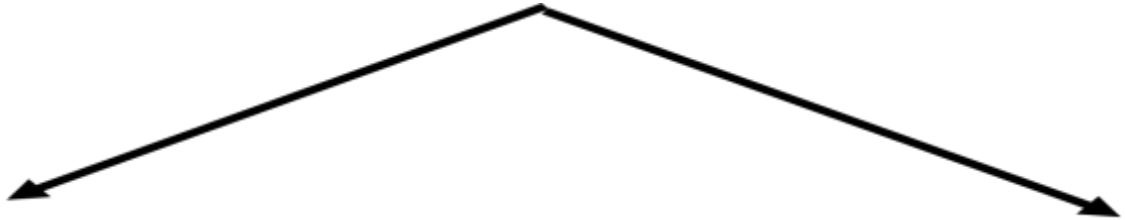

One-step RT-PCR
dNTPs
Water and buffer
Primers
Reverse transcriptase
DNA polymerase
RNA Template
RT + PCR
Amplified cDNA
Two-step RT-PCR
dNTPs
Water and buffer
Reverse transcriptase
RNA Template
RT
dNTPs
Water and buffer
Primers
DNA polymerase
cDNA Template
PCR
Amplified cDNA

PCR

The speed and ease of use, sensitivity, specificity and robustness of PCR has made it the most widely used and powerful technique with great spectrum of research and diagnostic applications.

Applications of PCR

According to research type



Basic Research

Gene expression studies
Mutation screening
Genomic cloning
Genotyping

Applied Research

Detection of pathogens
Prenatal diagnosis
DNA fingerprinting
Gene therapy

Molecular Identification

DNA is unique for single type of organism ⟹ DNA can be used to identify the organism

PCR allows easy manipulation of DNA

Organisms can be identified by using PCR

Molecular markers are the foundation for molecular identification, and both mitochondrial DNA and ribosomal DNA are species-specific and can be used as molecular markers for species identification

Detection of Pathogens

A sample that may contain the pathogen

PCR for pathogen gene

Yes PCR → YES Pathogen

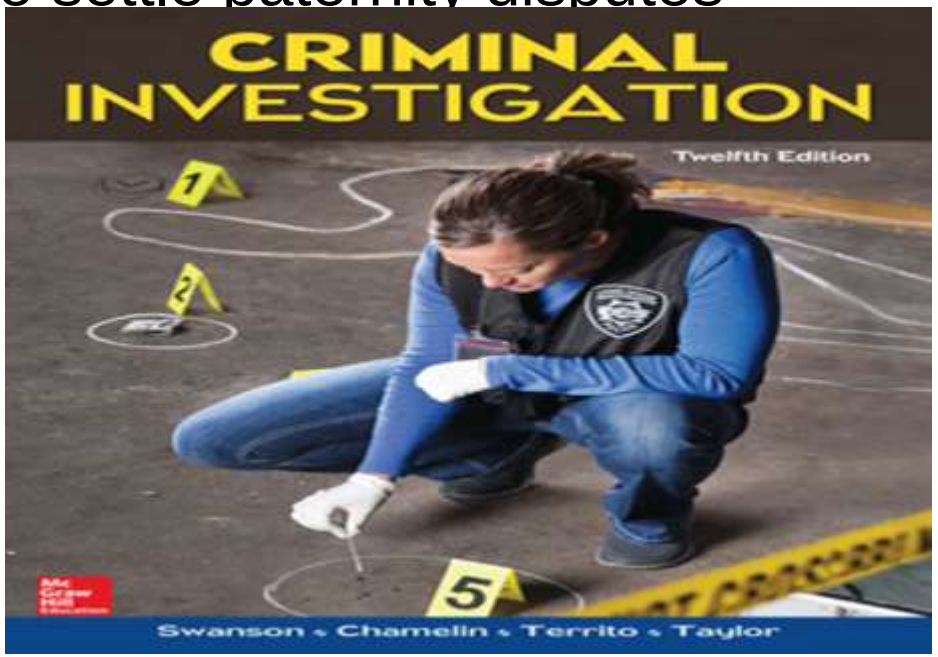
No PCR → NO Pathogen

PCR assays are available for a variety of pathogens , including HIV, HCV, hepatitis B , cytomegalovirus, M.tuberculosis and nisseria gonorrhea.

Generally the principal of detection is based on detection of the pathogen specific DNA/RNA region, amplification of that sequence and analyzing the presence or absence of detection amplicon on agarose gel electrophoresis

criminal and paternity investigations

- DNA profiling (DNA typing, genetic fingerprinting, DNA testing) is a technique by which individuals can be identified and compared via their respective DNA profiles
- DNA profiling is commonly used in criminal investigations (forensics) and to settle paternity disputes

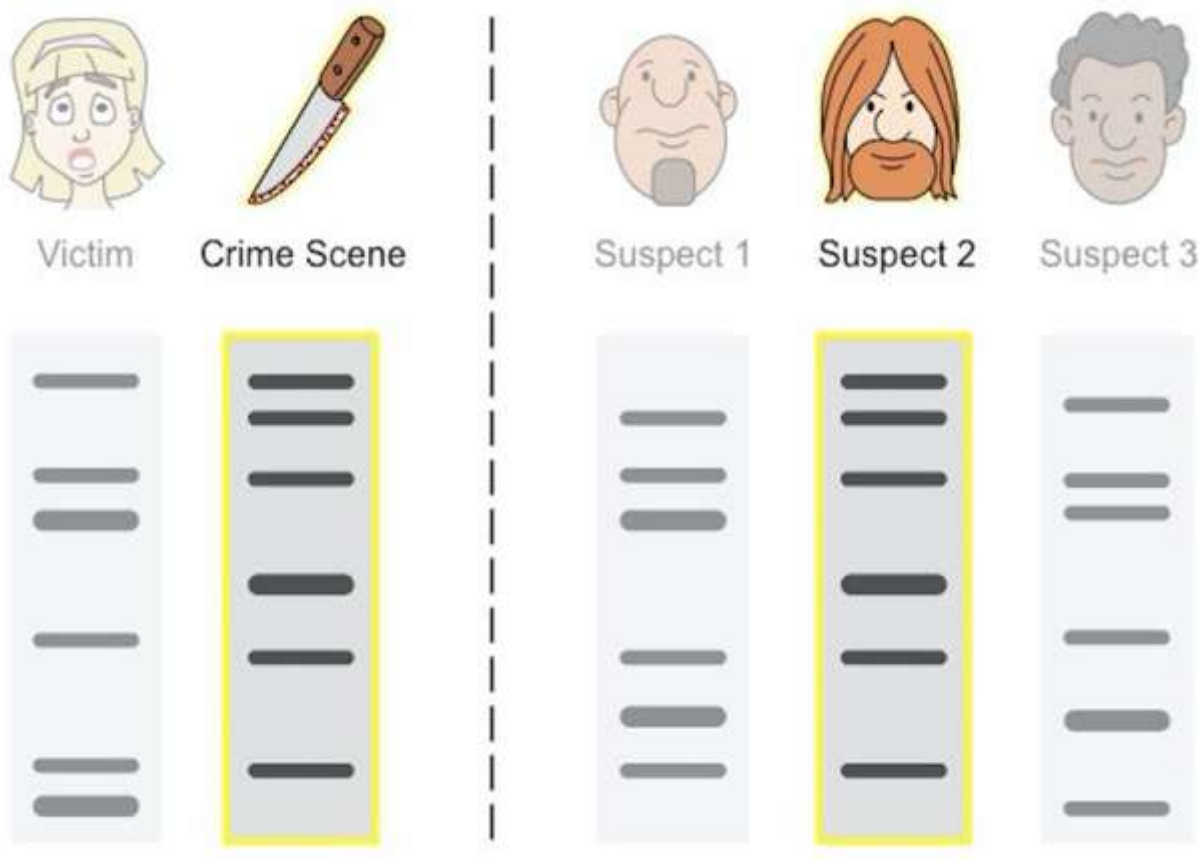


The procedure involved is common for both:

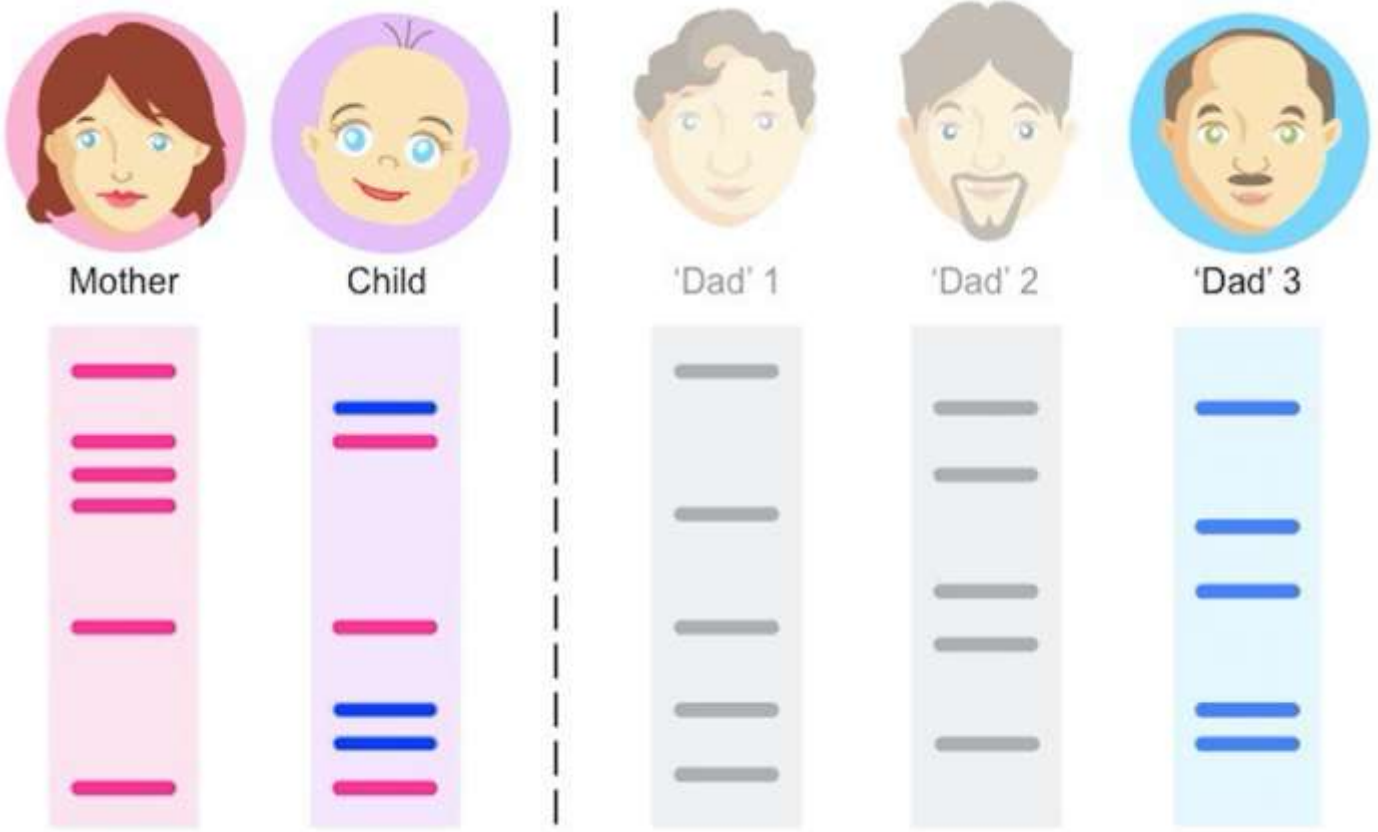
- A DNA sample is collected (e.g. from blood, semen, saliva, etc.) and then amplified using PCR
- Satellite DNA (with STR sequences) are cut with specific restriction enzymes to generate fragments
- Fragment length will differ between individuals due to the variable length of their short tandem repeats
- The fragments are separated using gel electrophoresis and the resulting profiles are compared

N.B Within the non-coding regions of an individual's genome there exists satellite DNA – long stretches of DNA made up of repeating elements called *short tandem repeats* (STRs)

Forensic Investigations:

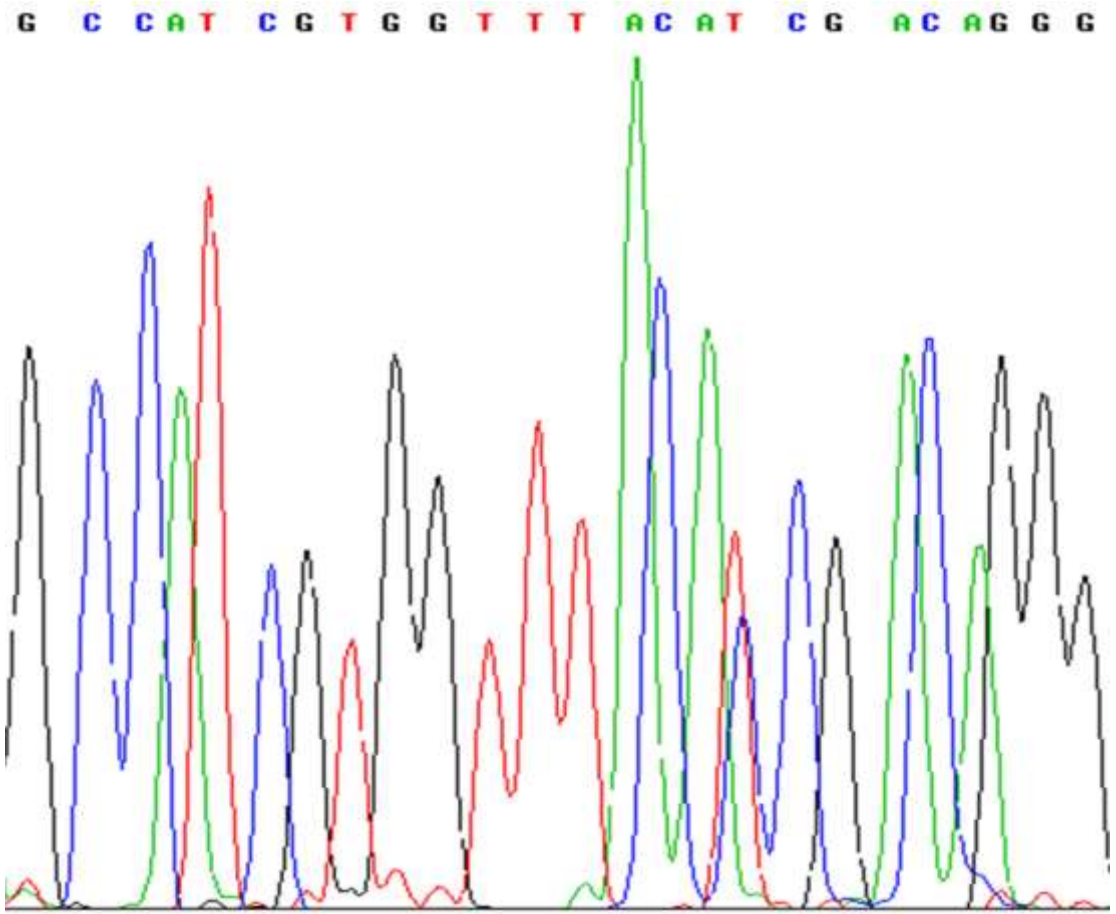


Paternity Testing:

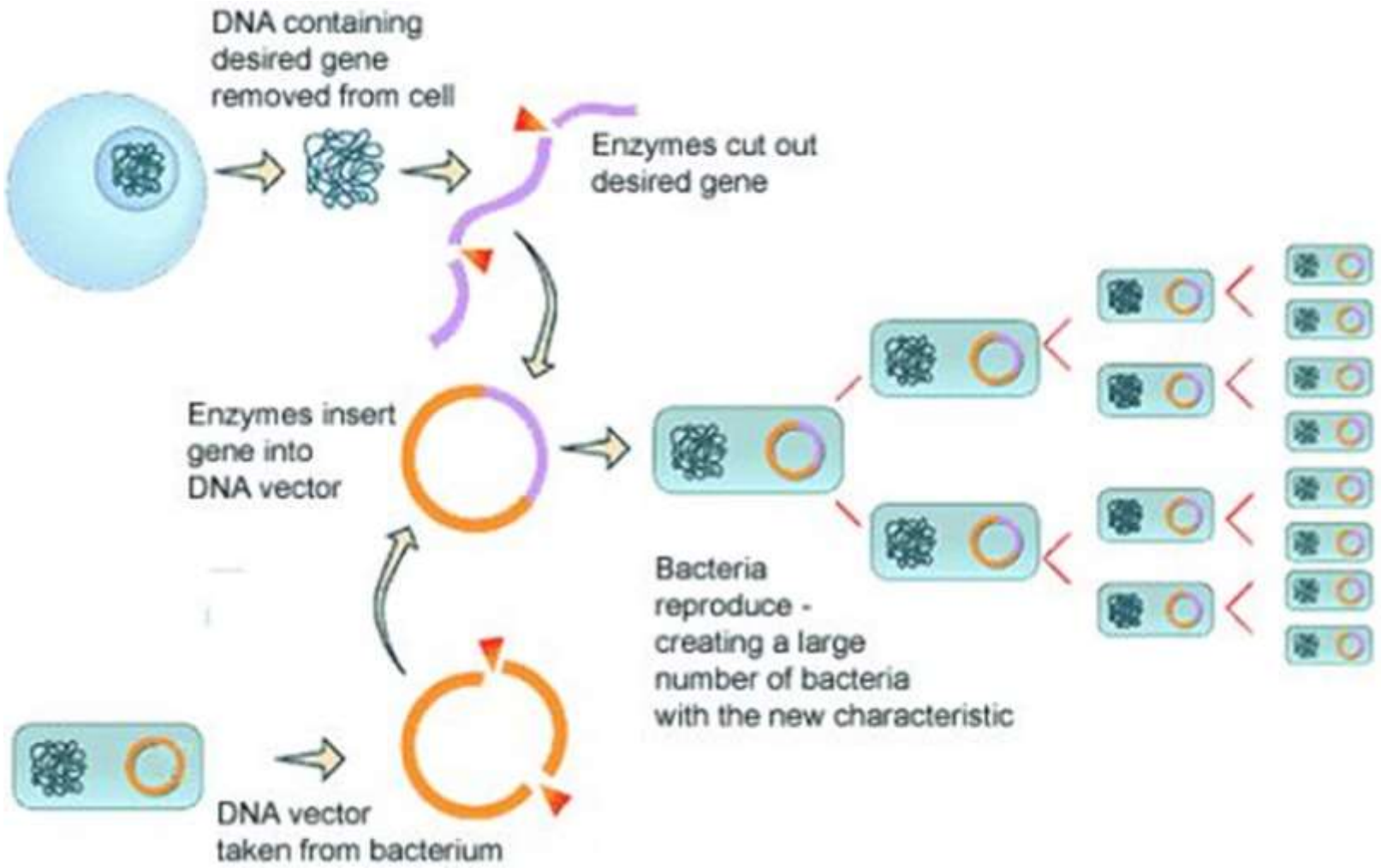


Sequencing

- DNA sequencing is a laboratory technique used to determine the exact sequence of bases (A, C, G, and T) in a DNA molecule. The DNA base sequence carries the information a cell needs to assemble protein and RNA molecules. DNA sequence information is important to scientists investigating the functions of genes.
- Sequencing employs a technique known as electrophoresis to separate pieces of DNA that differ in length by only one base.



Genetic engineering



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- https://www.ncbi.nlm.nih.gov/tools/primer-blast/
- https://blast.ncbi.nlm.nih.gov/Blast.cgi

Thank you