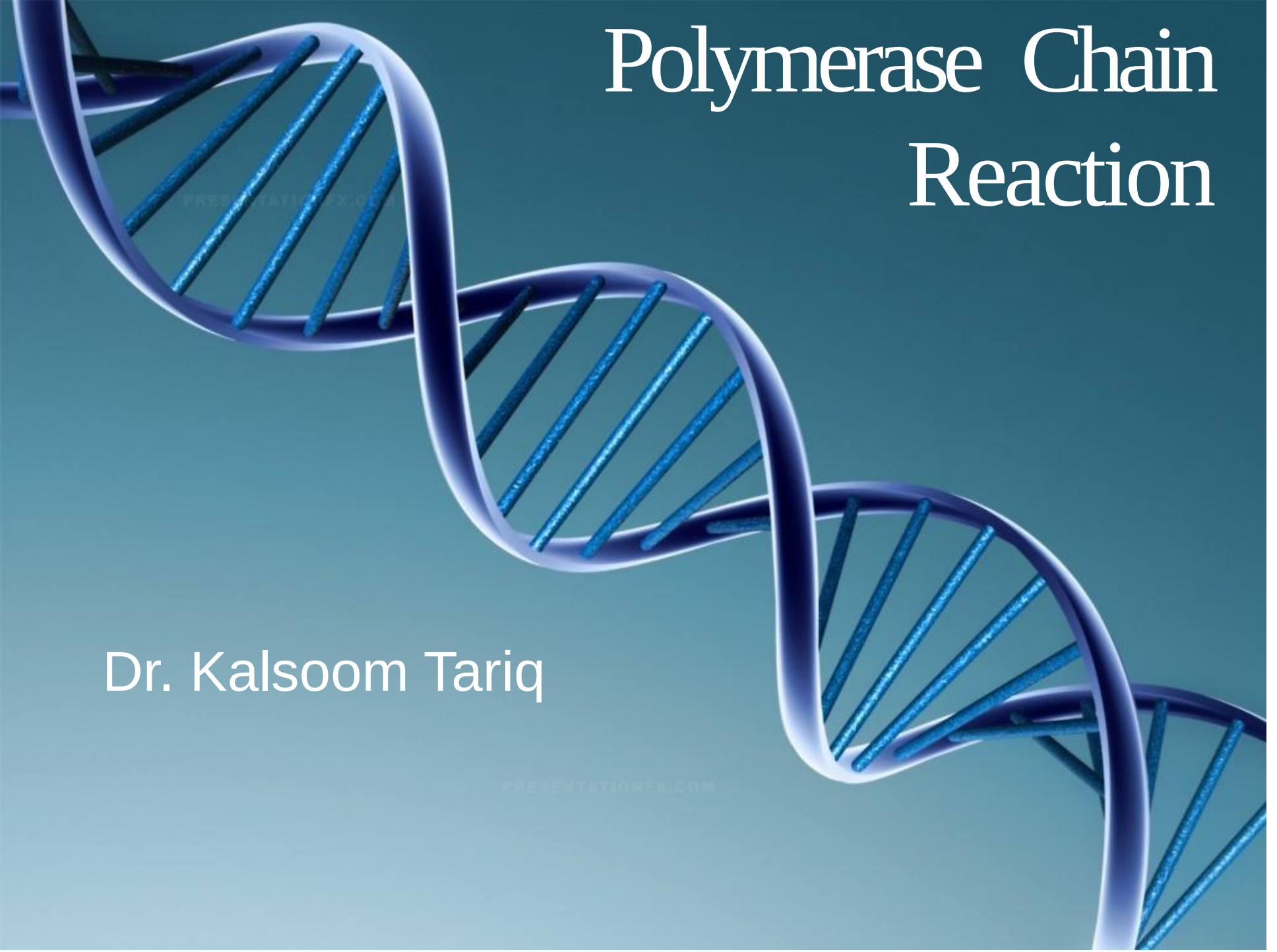
Polymerase Chain Reaction



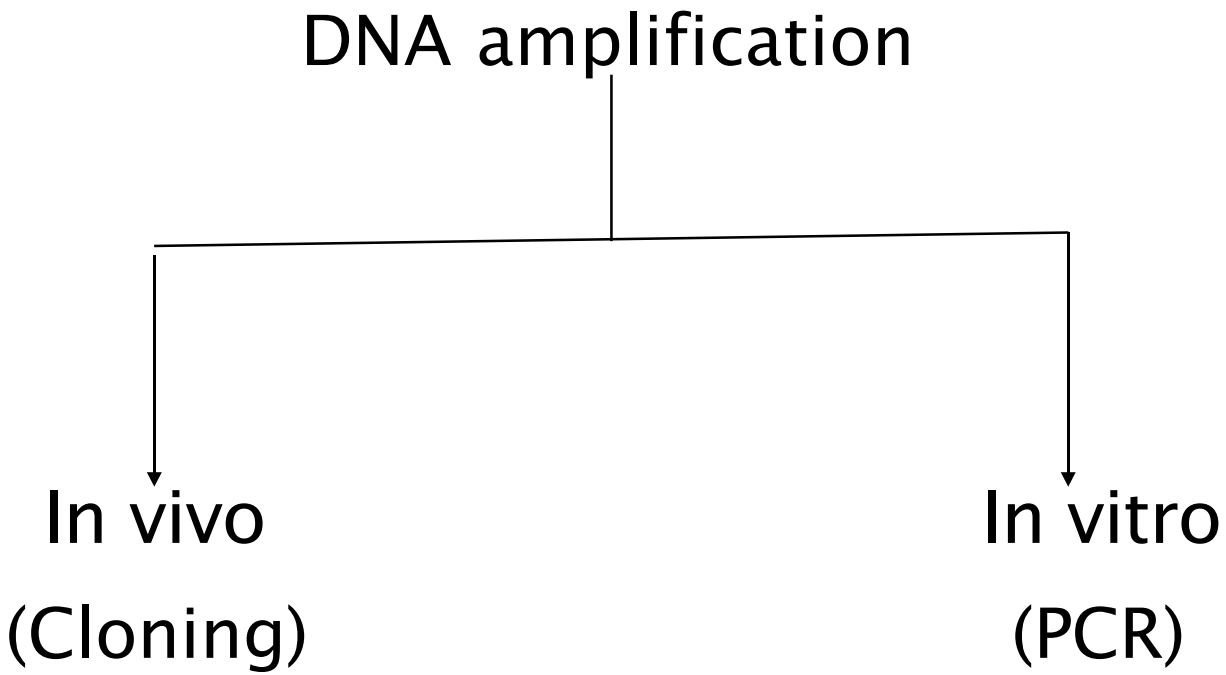
Dr. Kalsoom Tariq

Learning objectives:

- What is PCR?
- History of PCR
- Components of PCR
- Principle of PCR
- Basic Requirements
- Instrumentation
- Advantages of PCR
- Applications of PCR
- Problem in the use of PCR
- Types of PCR

DEFINITION

Amplification means making multiple identical copies (replicates) of a DNA sequence.



What is PCR?

- Polymerase Chain reaction is a technique that takes small amount of specific sequence of DNA & amplifies it to be used for further testing.

In vitro technique

Short History of PCR

- **1983**: Dr. Kary Mullis developed PCR, used the klenow fragment of DNA polymerase I from E.coli to describe the in vitro amplification of genes.

- **1988**: Saiki *et al.* used the DNA polymerase from Thermas aquaticus known as Taq polymerase & greatly increased the efficiency of PCR.

- **1989**: Science declares PCR as a major scientific development &Taq polymerase as the "molecule of the year".

Short History of PCR

- **1990**: amplification and detection of specific DNA sequences using a fluorescent DNA-binding dye, laying the foundation for future "real-time" or "kinetic" PCR.
- **1991**: RT-PCR is developed using a single thermostable polymerase, facilitating diagnostic tests for RNA viruses.
- **1993**:Dr. Kary Mullis shares Nobel Prize in Chemistry for conceiving PCR technology.

The Basic PCR reaction

Essential components required:

- Template DNA
- A thermostable DNA polymerase
- A pair of synthetic oligonucleotide primers.
- Divalent cations (Mg^{2+})
- dNTPs
- Buffer to maintain pH

Chemical Components

- Magnesium chloride: 0.5-2.5mM
- Buffer: pH 8.3-8.8
- dNTPs: 20-200µM
- Primers: 0.1-0.5µM
- DNA Polymerase: 1-2.5 units
- Target DNA: 1 µg

Basic requirements for PCR reaction

- 1) DNA sequence of target region must be known.
  Various types:
  Single or double stranded DNA,
  bacterial, viral, RNA etc.

2) Primers - typically 20-30 bases in size.
These can be readily produced by commercial companies.

2) Oligonucleotide primers

- A pair of synthetic primers is required to prime DNA synthesis. A forward and a reverse primer.
- Primers anneal to the flanking regions by complementary-base pairing (G≡C and A=T) using hydrogen bonding.
- The most crucial factor in PCR is the design of the oligonucleotide primers. Careful design of primers is required to,
  - Obtain desired products in high yields.
  - Suppress amplification of unwanted sequences.

3)

Thermostable DNA polymerase

- PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template
- A wide range of thermostable polymerases are available, which vary in their fidelity, efficiency and ability to synthesize large DNA products.
- Taq polymerase isolated from Thermus aquaticus is the first isolated and best known enzyme.

Taq polymerase

Source	Thermus aquaticus
Activity	5' - 3' polymerase activity, but lacks 3' - 5' exonuclease activity (no proofreading)
Stability	Half life of <5 min at 100 C, but retains activity up to 40 min at 95°C
Error rate	2×10^{-4} errors / base
Fidelity	low

When greater fidelity is required, other thermostable enzymes may have significant advantages.

Enzyme	Source	Optimum temp. C	Fidelity	Proofreading
rTth	*T. thermophilus*	75-80	Low	none
Pfu	*Pyrococcus furiosus*	72-78	High	Yes
Pwo	*P. woesei*	60-65	High	Yes
Deep Vent	*Pyrococcus* strain GB-D	70-80	High	Yes

Cocktails of different enzymes are also available that allow desired features like high efficiency, high fidelity, proofreading and generation of high yields of long targets. For e.g. a mixture of Pfu and Taq allows generation of products as long as 35 kb.

D

4) Divalent cations:

- All polymerases requires free divalent cations usually Mg^{2+} for activity. Some require Mn^{2+}

- Acts as cofactors in the catalytic addition of dNTPs to the growing DNA chain.

Basic requirements for PCR reaction

- 5) dNTPs: deoxy nucleotide tri phosphates

6)DNA thermal cycler - machine which programmed to carry out heating and cooling of samples over a number of cycles.

PCR Master Mix

- ✓ **PCR Master Mix** is a premixed, ready-to-use solution containing Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by **PCR**.

- ✓ **Ready to use PCR beads**

Principle of PCR

- Purpose:
- Condition/Procedure:
- Components:

Purpose

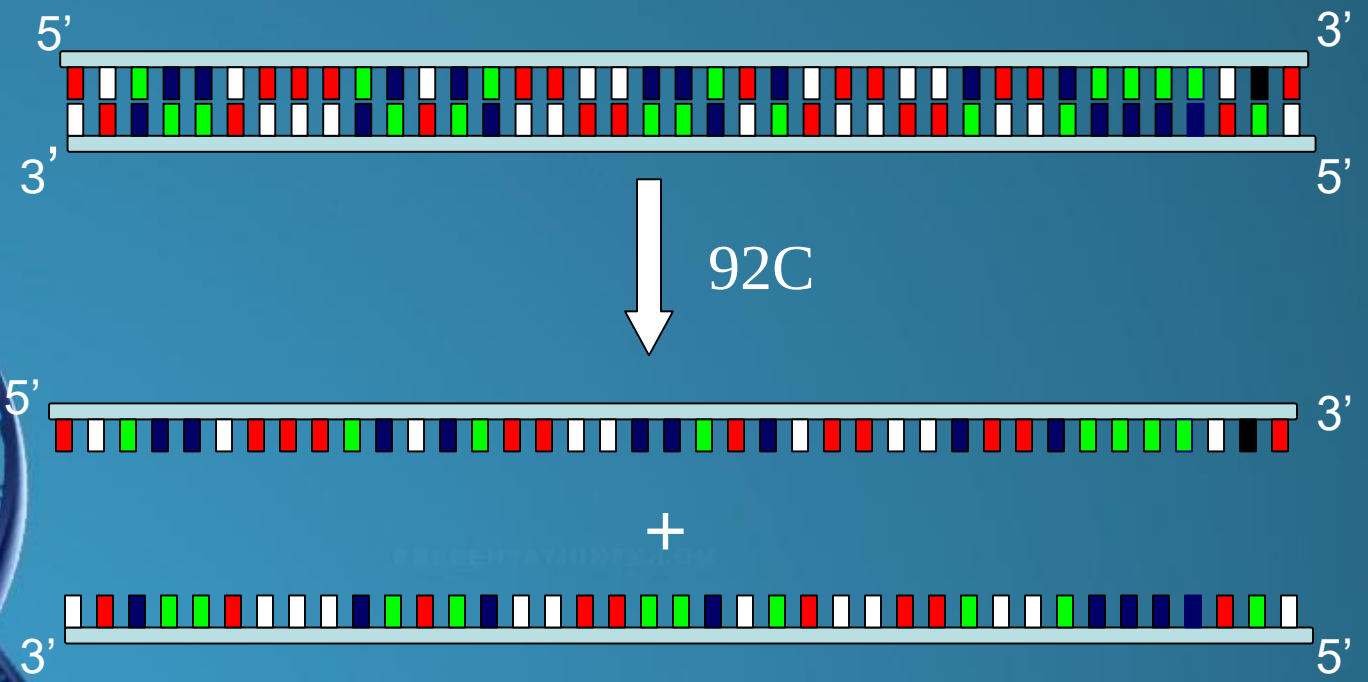
- To amplify a lot of double-stranded DNA molecules (fragments) with same (identical) size and sequence by enzymatic method and cycling condition.

Condition/Procedure:

- 1. Denaturation of ds DNA template
- 2. Annealing of primers
- 3. Extension of ds DNA molecules

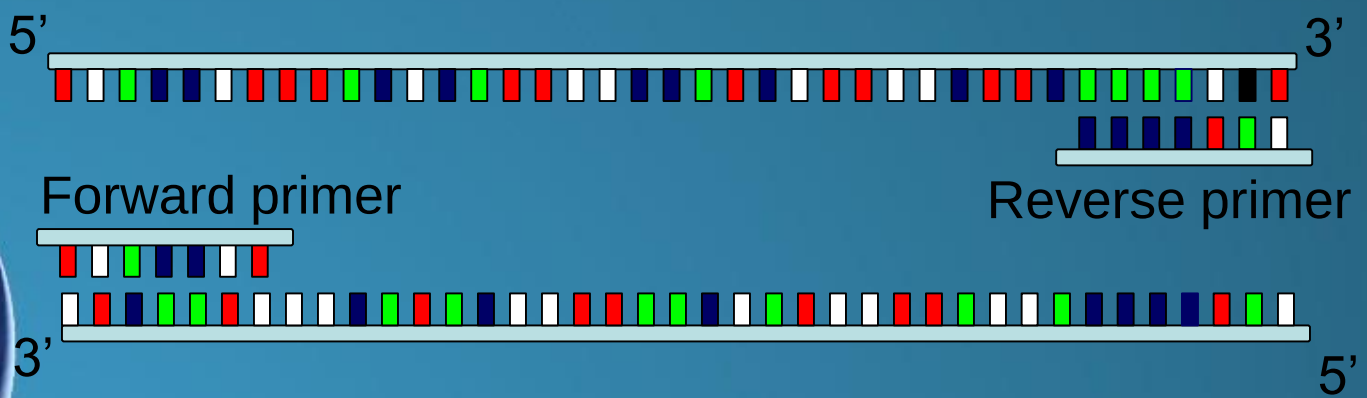
Denaturation

- Temperature: 92-94C
- Double stranded DNA melts ⟶ single stranded DNA



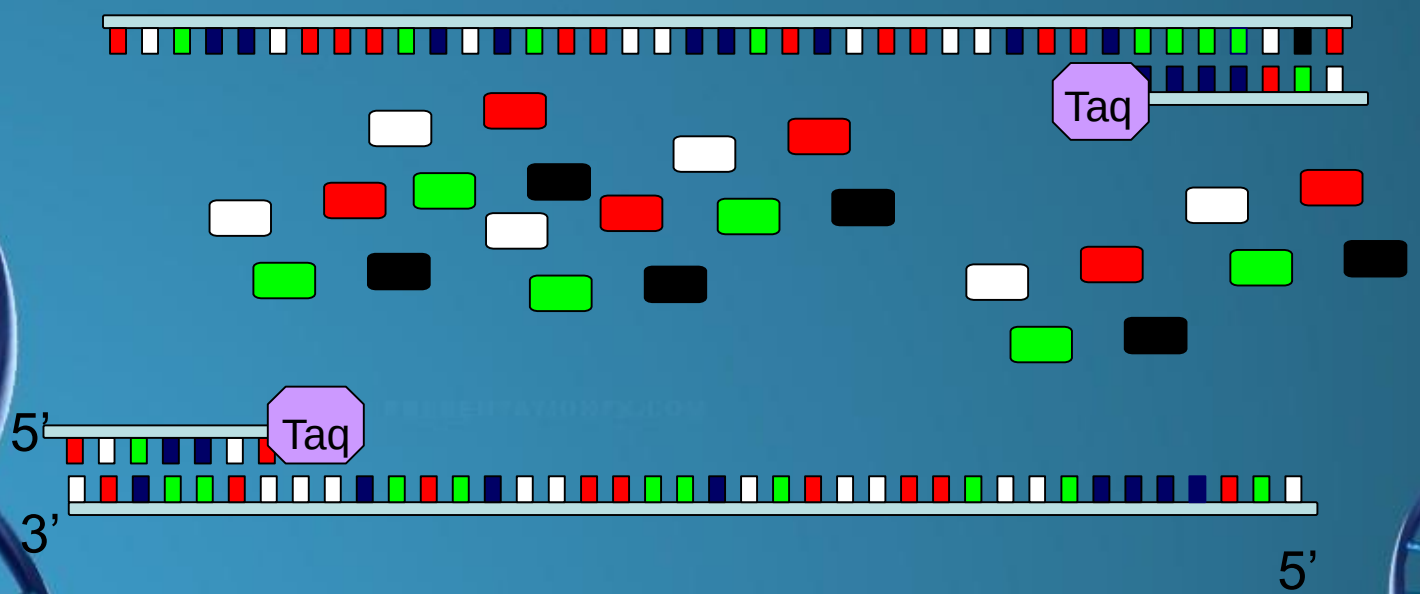
Annealing

- Temperature: ~50-70C (dependant on the melting temperature of the expected duplex)
- Primers bind to their complementary sequences



Extension

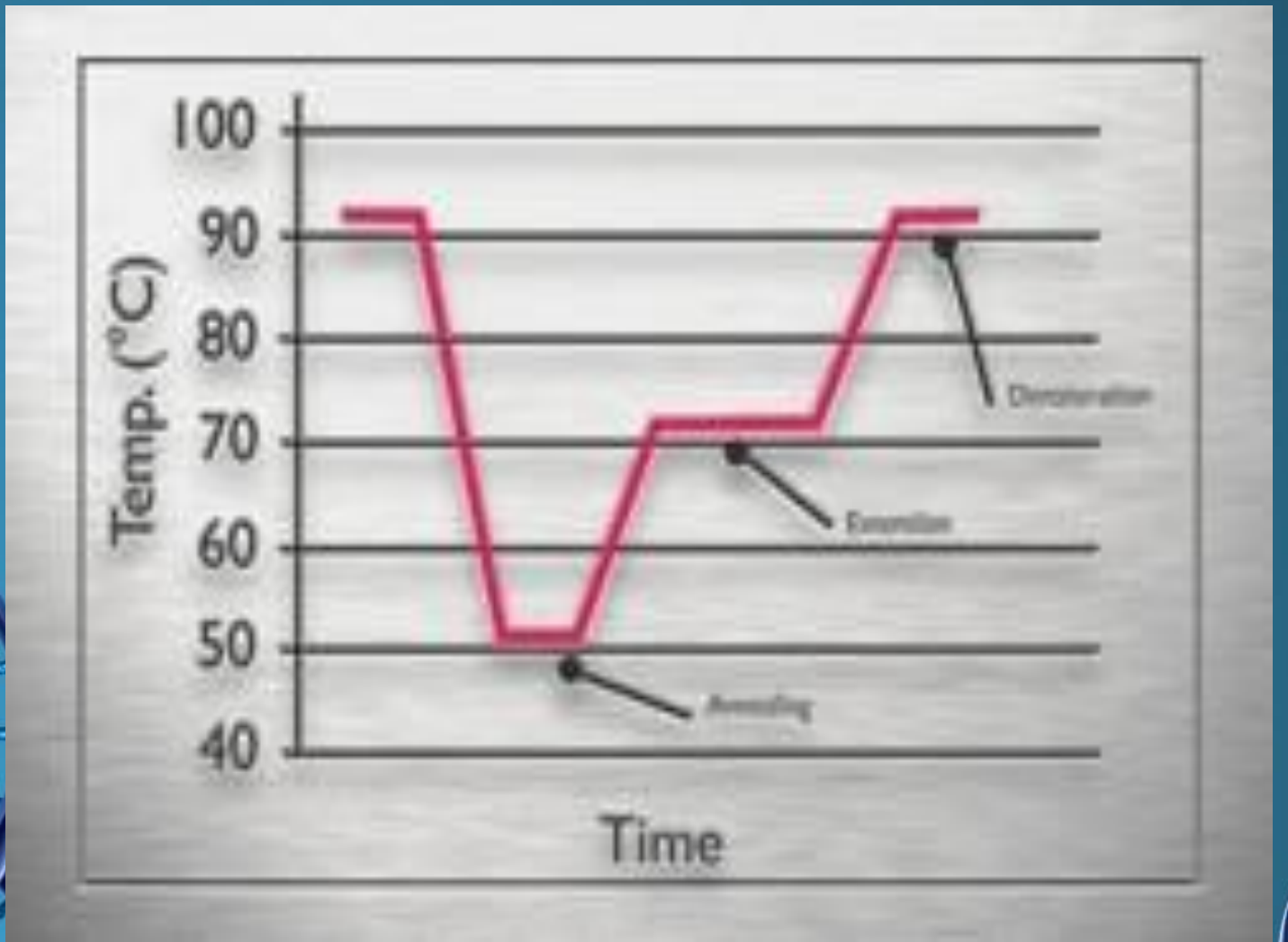
- Temperature: ~72C
- Time: 0.5-3min
- DNA polymerase binds to the annealed primers and extends DNA at the 3’ end of the chain



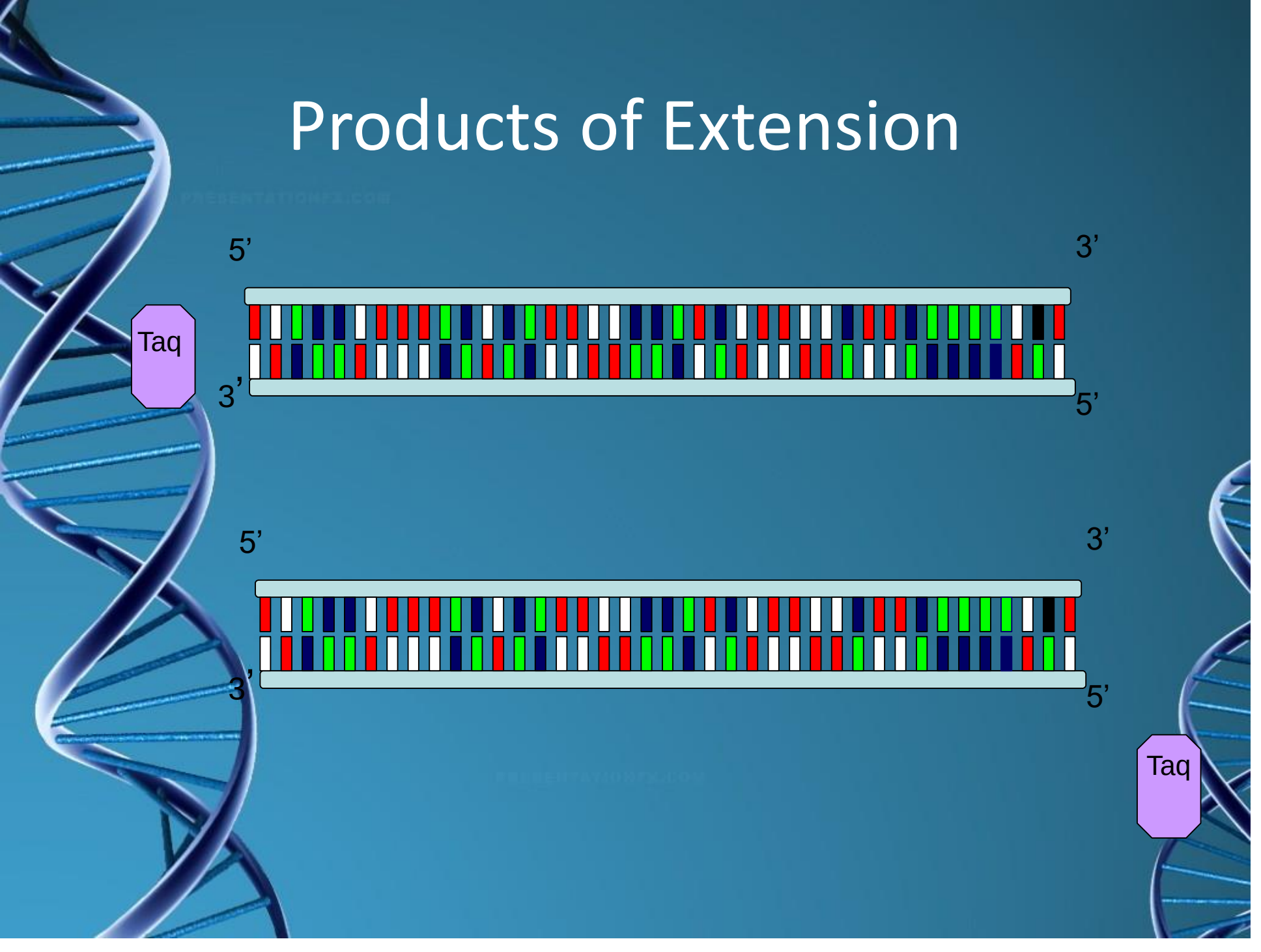
Example of PCR programme

- Initial denaturation 95C for 5 mins
- Thermo-cycle file - 30 cycles of
- Denaturation : 95C for 30 secs
- Annealing : 55C for 30 secs
- Extension : 72C for 45 secs
- Final extension 72C for 5 mins

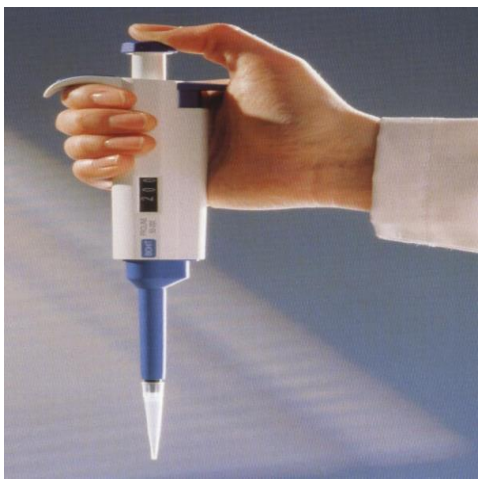
Cycling

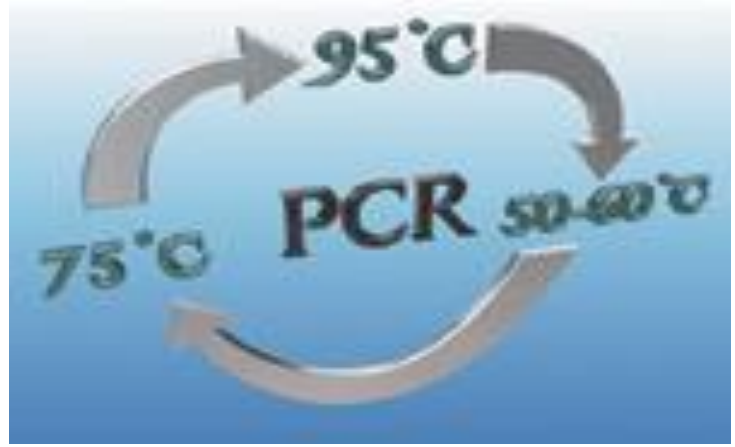


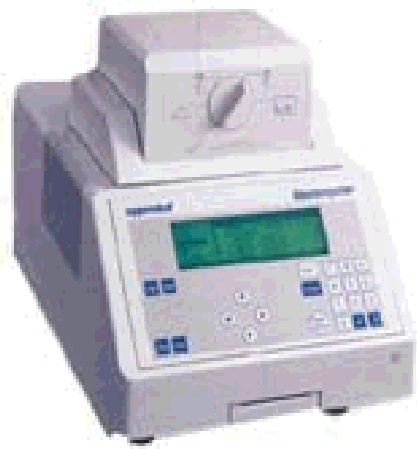
Products of Extension



The Reaction







PCR tube

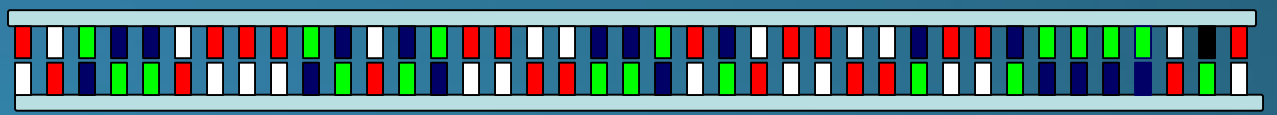
THERMOCYCLER

Variants of PCR

Instrumentation

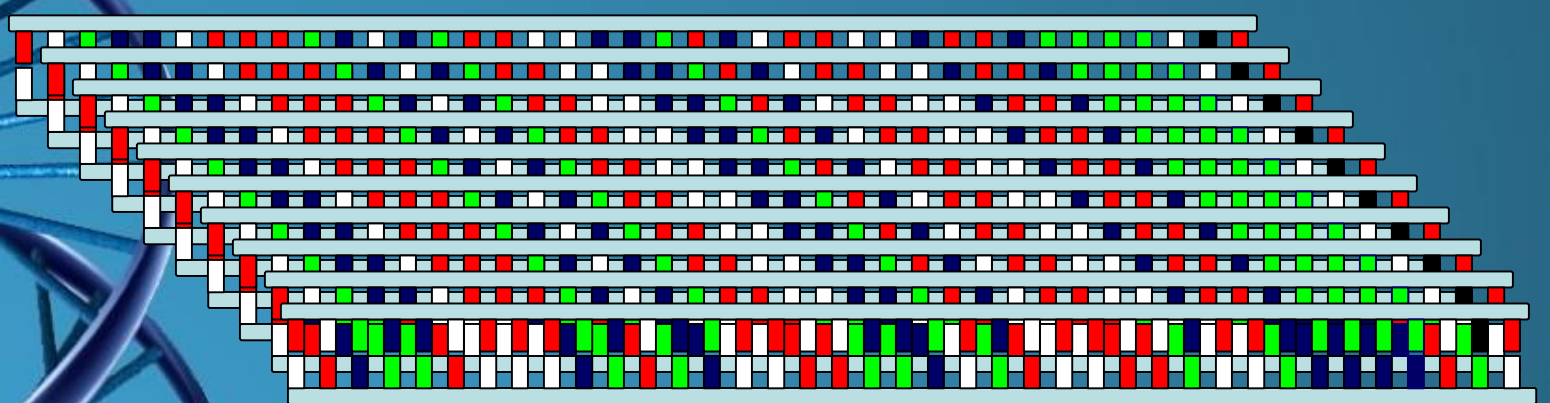
Overall Principle of PCR

- DNA – 1 copy



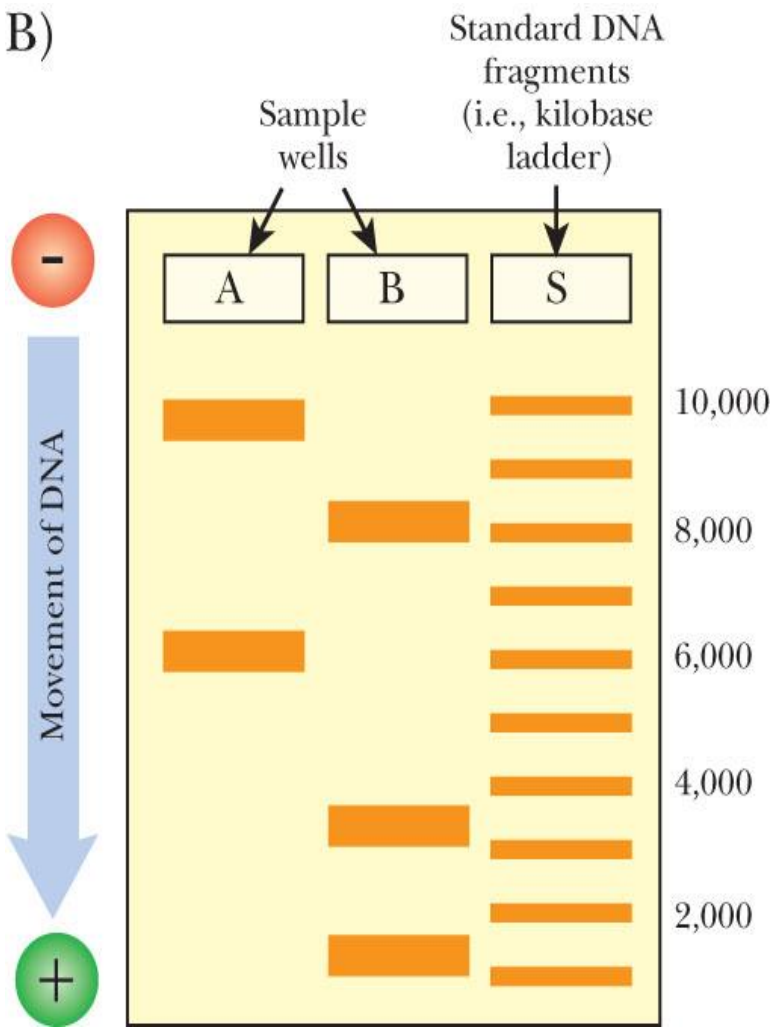
Known sequence Sequence of interest Known sequence

- PCR

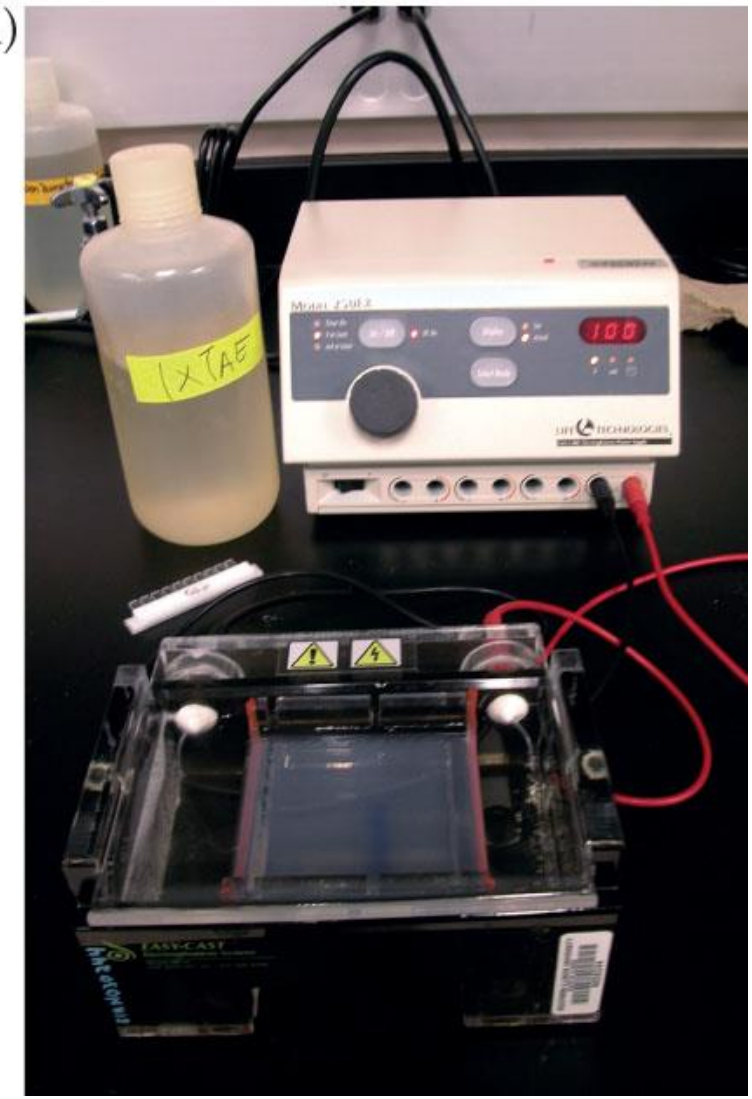


How Gel Electrophoresis of DNA Works

B)



A)



- **Staining of the Gel ;** one of the most important technique of gel electrophoresis is staining, with an agent which is appropriate for the sample.
  In case of DNA , the stain used is Ethidium bromide(10ng) .
- Silver chloride dye can also be used(1ng).
- **Visualization :** DNA molecules are easily visualized under UV lamp after electrophoresis by soaking the gel in a solution of Ethidium bromide.

Following PCR, the amplification product can be detected using gel electrophoresis where visualization of a band containing DNA fragments of a particular size indicates the presence of the target sequence in the original starter DNA sample.

Properties of PCR

- Specificity
- Efficiency
- Fidelity(accuracy)

Things to try if PCR does not work

- A) If no product (of correct size) produced:
  - 1 Check DNA quality
  - 2 Reduce annealing temperature
  - 3 Increase magnesium concentration
  - 4 Add dimethylsulphoxide (DMSO) to assay (at around 10%) to inhibit sec. structures formations.
  - 5 Use different thermostable enzyme
  - 6 Throw out primers - make new stocks

Things to try if PCR does not work

- B) If extra spurious product bands present
  - 1 Increase annealing temperature
  - 2 Reduce magnesium concentration
  - 3 Reduce number of cycles
  - 4 Try different enzyme

Advantages of PCR

- Small amount of DNA is required per test
- Result obtained more quickly .
  Inexpensive & easy procedure.
- Usually not necessary to use radioactive material (32P) for PCR.
- PCR is much more precise in determining the sizes of alleles - essential for some disorders.
- PCR can be used to detect point mutations.

Potential Pitfalls of PCR

- The target DNA template may not amplify due to the presence of PCR inhibitors in the extracted DNA
- Amplification may fail due to sequence changes in the primer binding region of the genomic DNA template
- Contamination from other human DNA sources besides the forensic evidence at hand or previously amplified DNA samples is possible without careful laboratory technique and validated protocols

PCR in Clinical Diagnosis:

1)In detection of bacterial infection : Tuberculosis

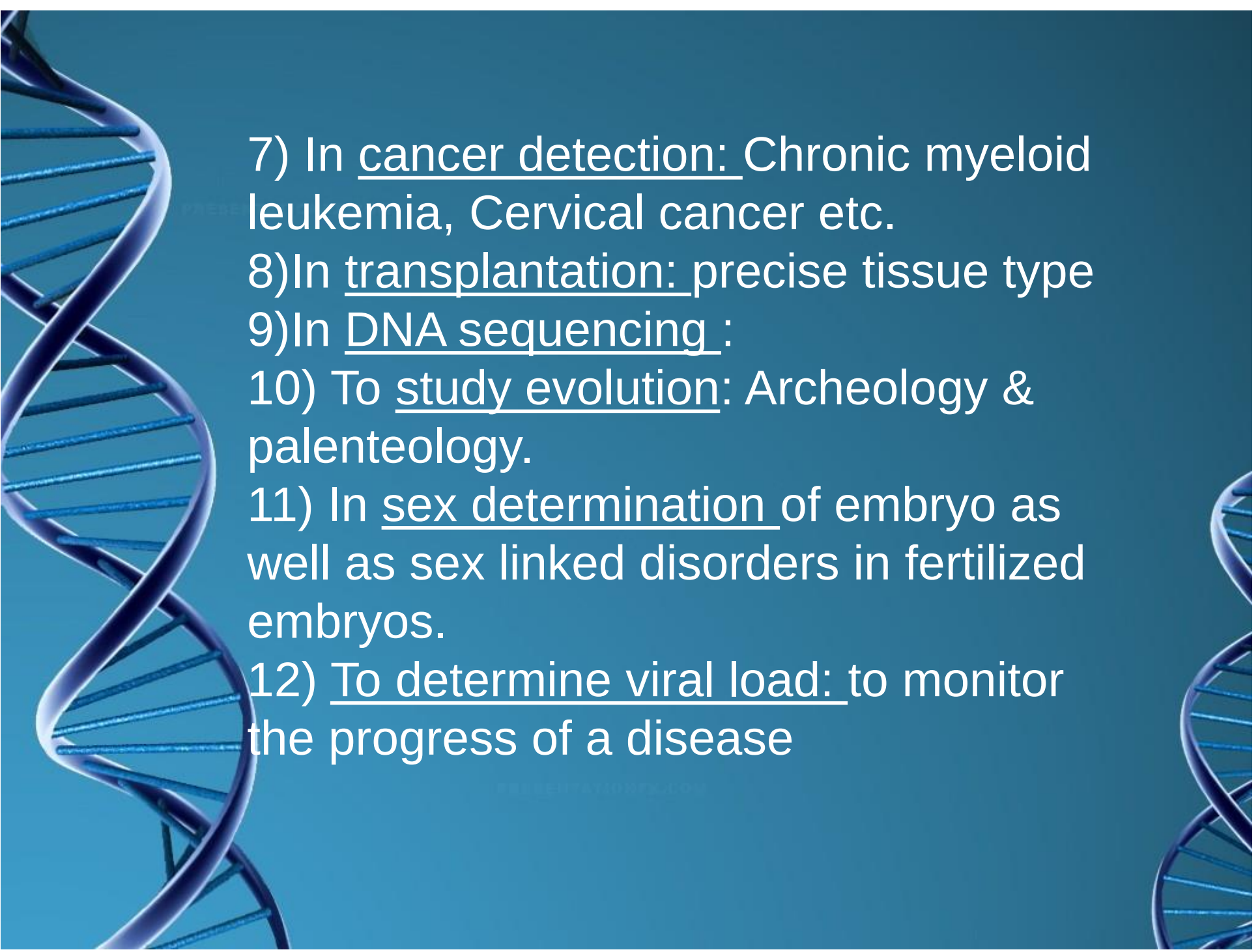
2) In diagnosis of retroviral infection: HIV infection

3)In detection of latent infectious agents

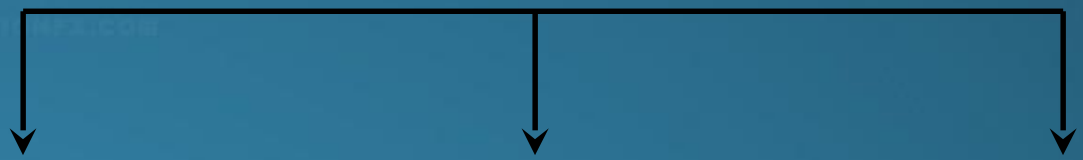
4)Application in forensic medicine: DNA in a single cell, hair follicle or sperm etc.

5)In diagnosis of inherited disorders: sickle cell anemia, thalassemia & cystic fibrosis.

6) In prenatal diagnosis : by using chorionic villus samples or cells from amniotic fluid


7) In cancer detection: Chronic myeloid leukemia, Cervical cancer etc.
8)In transplantation: precise tissue type
9)In DNA sequencing :
10) To study evolution: Archeology & palenteology.
11) In sex determination of embryo as well as sex linked disorders in fertilized embryos.
12) To determine viral load: to monitor the progress of a disease

Applications of PCR



Molecular Identification
Molecular Archaeology
Molecular Epidemiology
Molecular Ecology DNA fingerprinting
Classification of organisms
Genotyping
Pre-natal diagnosis
Mutation screening
Drug discovery
Genetic matching
Detection of pathogens

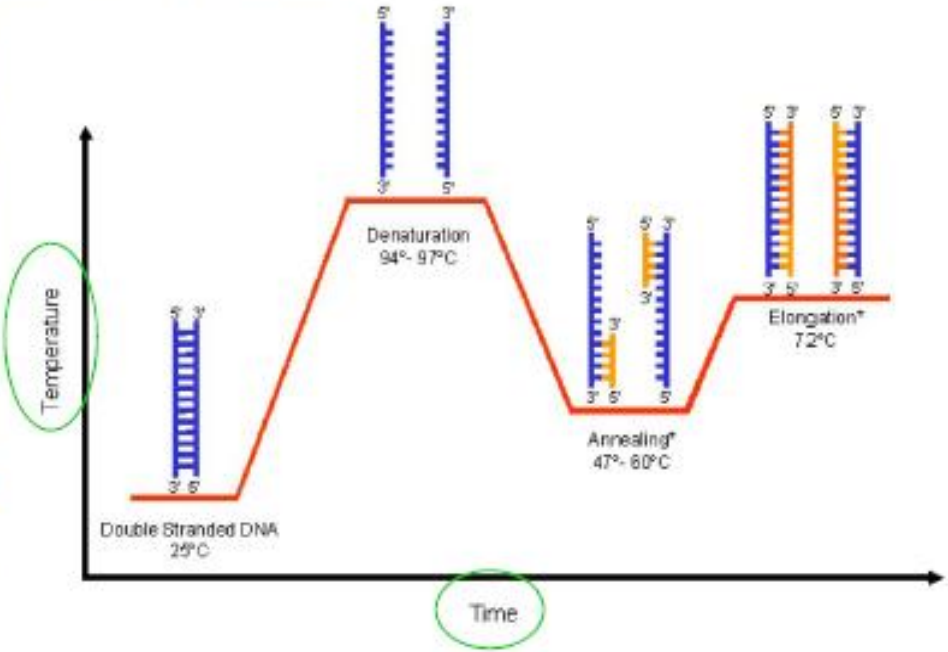
Sequencing
Bioinformatics
Genomic Cloning
Human Genome Project

Genetic Engineering
Site-directed mutagenesis
Gene Expression Studies

TYPES OF PCR:

1)Conventional PCR
2)Nested PCR
3)Multiplex PCR
4)Reverse transcriptase PCR
5)Real-time PCR
6)Asymmetric PCR
7)Quantitative competitive PCR

Conventional PCR



Conv. PCR uses thermostable DNA polymerase to amplify DNA sequence of interest

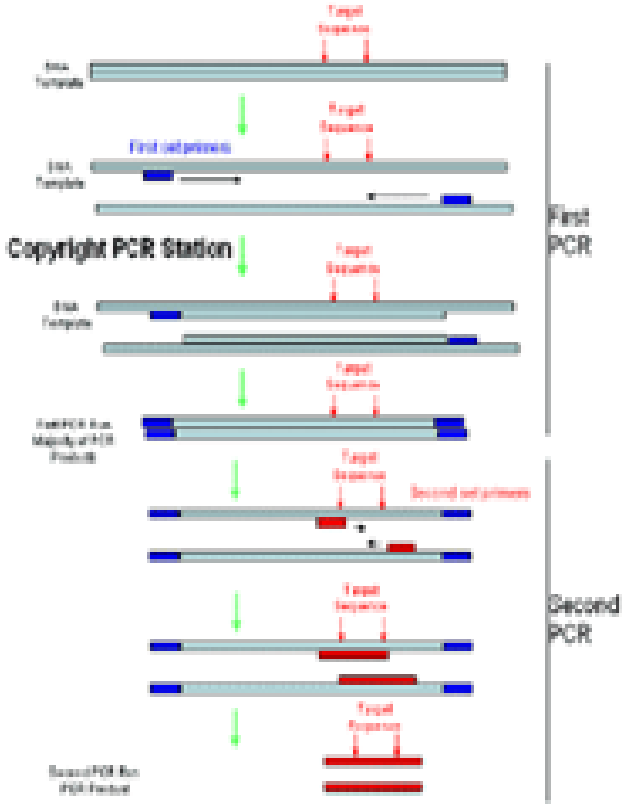
First step - **denaturation**, the sample is heated to separate the two strand (15-60s)

Followed by **annealing** step, in which the reaction temp. lowered allowing the primers to bind to the separated single strands of the template DNA (30-60s)

The last step is **elongation**, during which the temperature is raised, allowing specific enzymes to make the complementary copy of the template nucleic acid

Nested PCR

- Conventional PCR with a second round of amplification using a different set of primers
- The second set of primers is specific to a sequence found within the initial conv. PCR amplicon.
- Second amplifications step with nested primer set results in reduced background
- Nested PCR increases sensitivity and specificity of PCR.



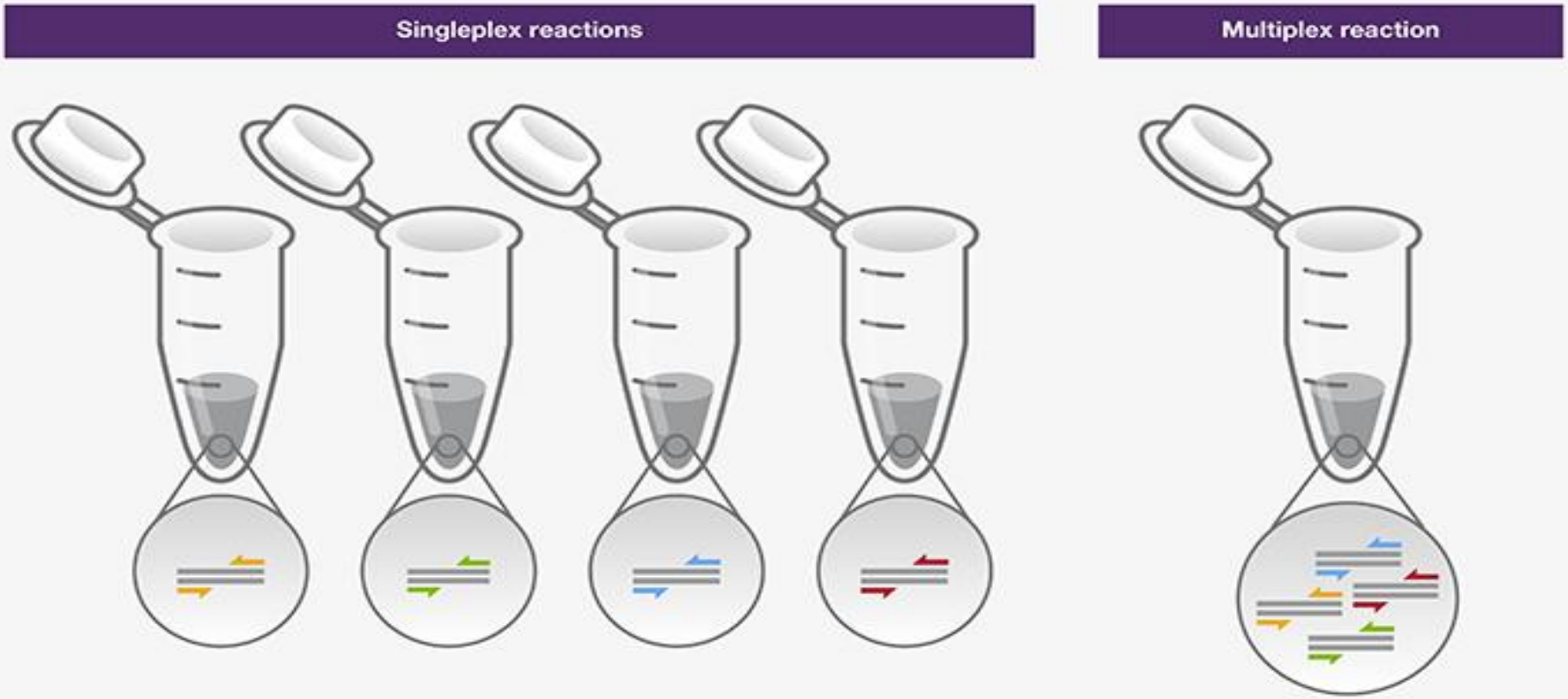
http://www.pcrstation.com

Multiplex polymerase chain reaction

- refers to the use of PCR to amplify several different DNA targets (genes) simultaneously
- amplifies genomic DNA samples using multiple primers and temperature-mediated DNA polymerase in a thermal cycler
- primer design for all primers pairs has to be optimized
- so that all primer pairs can work at the same annealing temperature during PCR.

Multiplex PCR

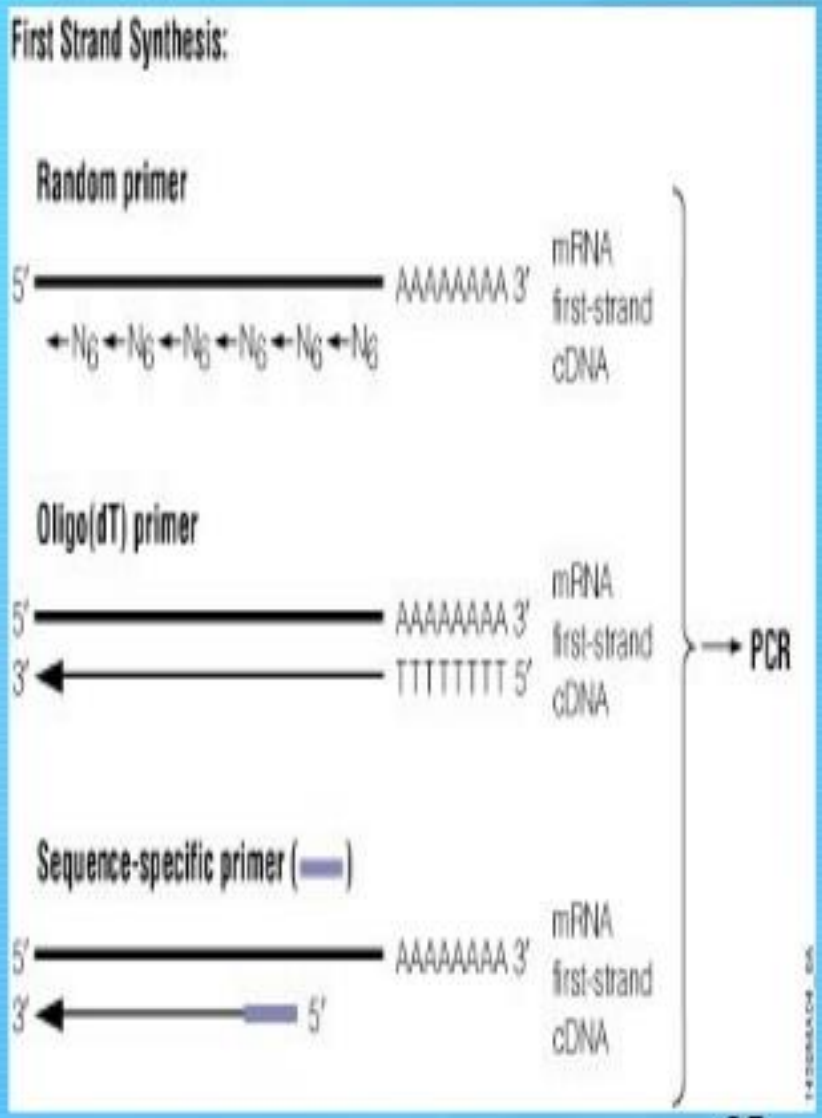
- Modification of conventional PCR in which two or more different PCR products are amplified
- While multiplex PCR provides a potential time saving by allowing simultaneous detection of multiple targets, significant optimization is required.



❑ Reverse transcriptase PCR

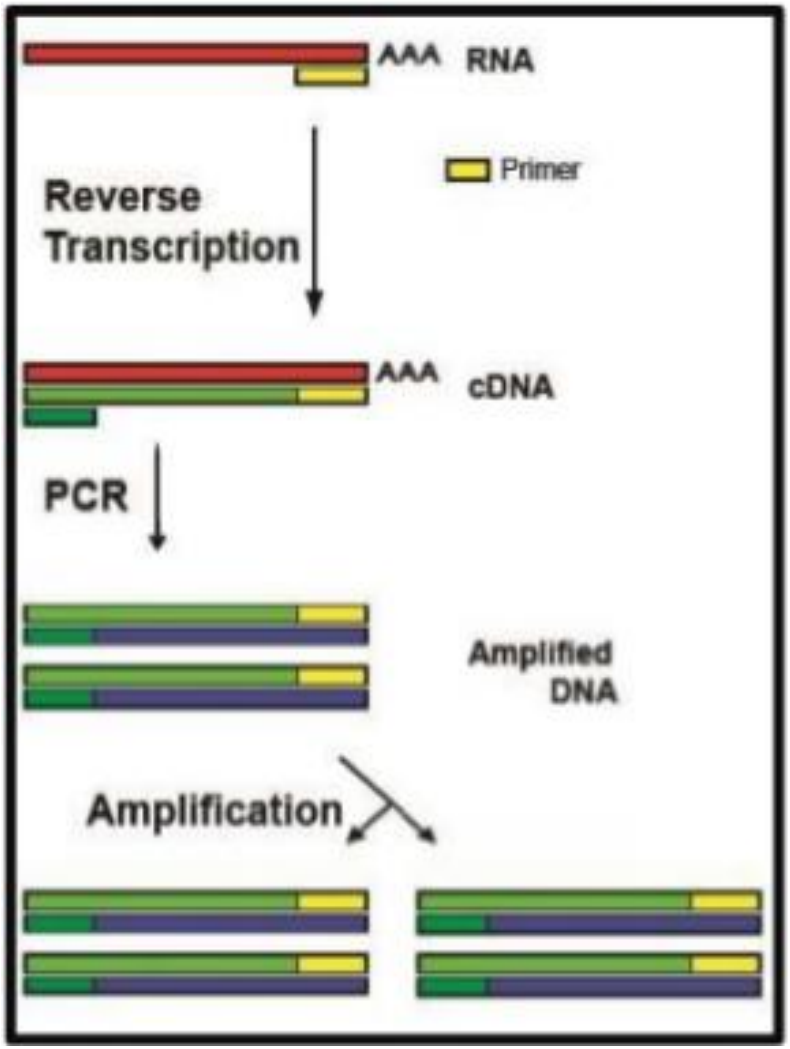
-It is employed for amplification of RNA molecules .

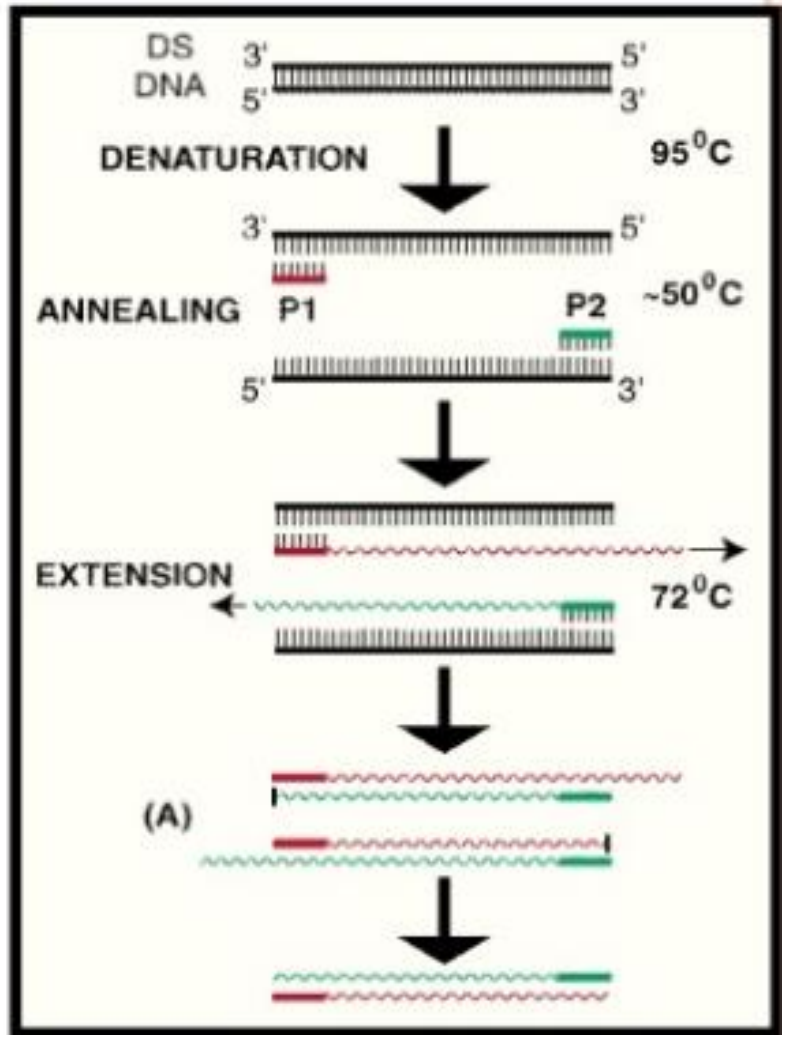
-RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript.



RT-PCR V/S PCR

Clip slide





Real-Time PCR:

- Detects & measures the amplification target DNA as they are produced.
- Unlike, conventional PCR, real time PCR uses an oligonucleotide probe labeled with fluorescent dyes (SYBR green & taqman) & a thermo cycler equipped with the ability to measure fluorescence.
- As the number of gene copies increases during the reaction , the fluorescence increases.
- This is advantageous because the efficiency and rate of the reaction can be seen.
- There is also no need to run the PCR product out on a gel after the reaction.

There are many different markers used as the marker of Real Time PCR.
There are mainly two types of marker are used for this purpose.

1.Taqman probe.

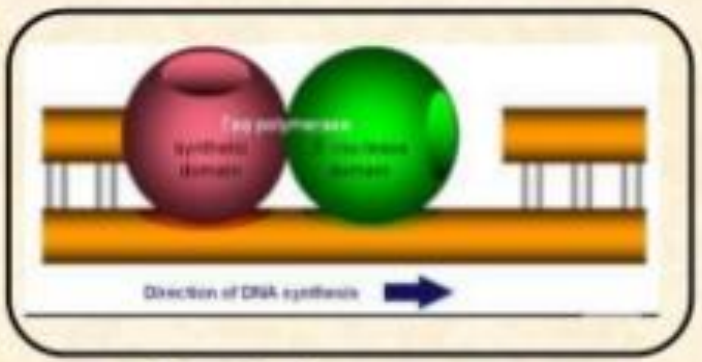
2.SYBR Green.

Taqman Probe:

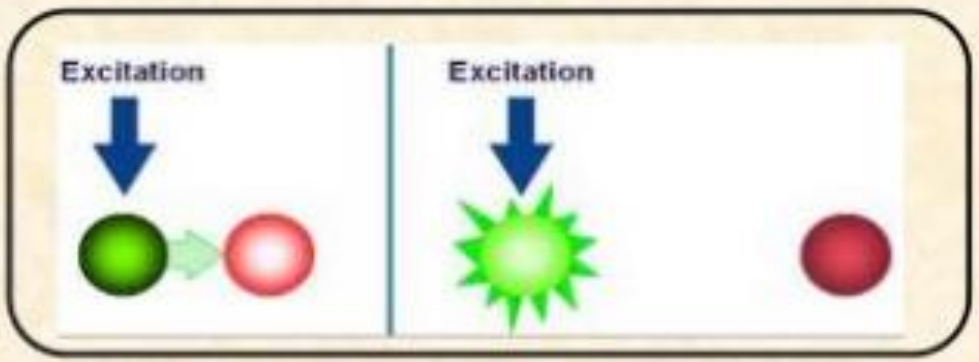
This is a hydrolysis probe, it bear a reporter dye , often fluorescein(FAM) at its 5' end and a quencher tetramethylrhodamine (TAMRA), attached to the 3' end of a oligonucleotide .In normal condition the probe remain coiled on itself bringing the fluorescence dye near the quencher causing quenching of fluorescent signal of the dye.

TaqPolymerase

The oligonucleotide of the Taqpolymerase has a homologous s region with the target gene, when the target sequence is present in mixture it bind with the sample DNA. As the taqpolymerase start to replicate new DNA strand at the extension stage it Causes degradation of the probe by 5' end nuclease activity and the fluorescein is get Separated from the quencher and fluorescence signal is generated. As this procedure Continue at each cycle the no. of signal molecule increase causing the increase of Signal which is positively related with the amplification of DNA.



TaqPolymerase



Excitation with quenching(left)
Excitation without quenching(right)

ASYMMETRIC PCR:

- Used for generating single strand for sequencing experiments
- Amplifies just one strand of target DNA
- First produce double strand DNA
- Then produce single strand DNA by adjusting primer concentration to favor one strand.
- Unequal primer concentrations.

Quantitative competitive (QC) PCR:

- Is used to measure or quantify specific amount of target DNA or RNA in a sample
- Co amplification of the sequence of interest with diluted synthetic DNA fragment of known concentration which is called ‘Competitor’
- The quantity of target molecule in the sample is calculated from the ratio of competitor & amplicons generated during PCR using single set of primers.

Conclusion

PCR is not only vital in the clinical laboratory by amplifying small amounts of DNA for STD detection, but it is also important for genetic predisposing for defects such as Factor V Leiden.

The PCR technology can also be employed in law enforcement, genetic testing of animal stocks and vegetable hybrids, and drug screening along with many more areas.


THANK YOU