POLYMERASE CHAIN REACTION

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1. INTRODUCTION

- Polymerase Chain Reaction
- Taq DNA polymerase
- Central Dogma of Gene Expression

1. INTRODUCTION

❑Polymerase Chain Reaction (PCR)

- PCR is an *in vitro* method in which a small **segment of DNA** or **gene** can be copied or amplified multiple times to yield millions of copies/ amplicons within short duration of time.
- Originally developed by the American biochemist **Kary Mullis** in 1983 and was published in 1986 [1]. He was awarded the **Nobel Prize in Chemistry in 1993** for his pioneering work.
- It is based on principle of **'DNA Replication'** and it exploits the ability of the **DNA polymerase** enzymes to create copies of the genetic material under laboratory conditions.
- In PCR, **Taq polymerase** (a thermostable DNA polymerase) isolated from *Thermus aquaticus* is used for gene amplification.

❑ Taq DNA polymerase

- This enzyme was isolated and purified from the hot springs bacterium ***Thermus aquaticus*** and was published in 1976.
- Enzyme's catalytic activity is expressed in Unit. 1 U **(µmol/min)** is defined as the **amount of the enzyme** that catalyzes the conversion of **one micromole of substrate per minute** under the specified conditions of the assay method.
- Optimum temperature for Taq polymerase activity is 72ºC.
- The half-life of Taq polymerase is <5 min at 100ºC but it retains its activity up to 40 minutes at 95°C.
- The error rate of *Taq* DNA Polymerase in PCR is **1.8 × 10^{-4} errors/base/doubling** [2].
- Pfu polymerase: *Pyrococcus furiosus.*

Hot Springs

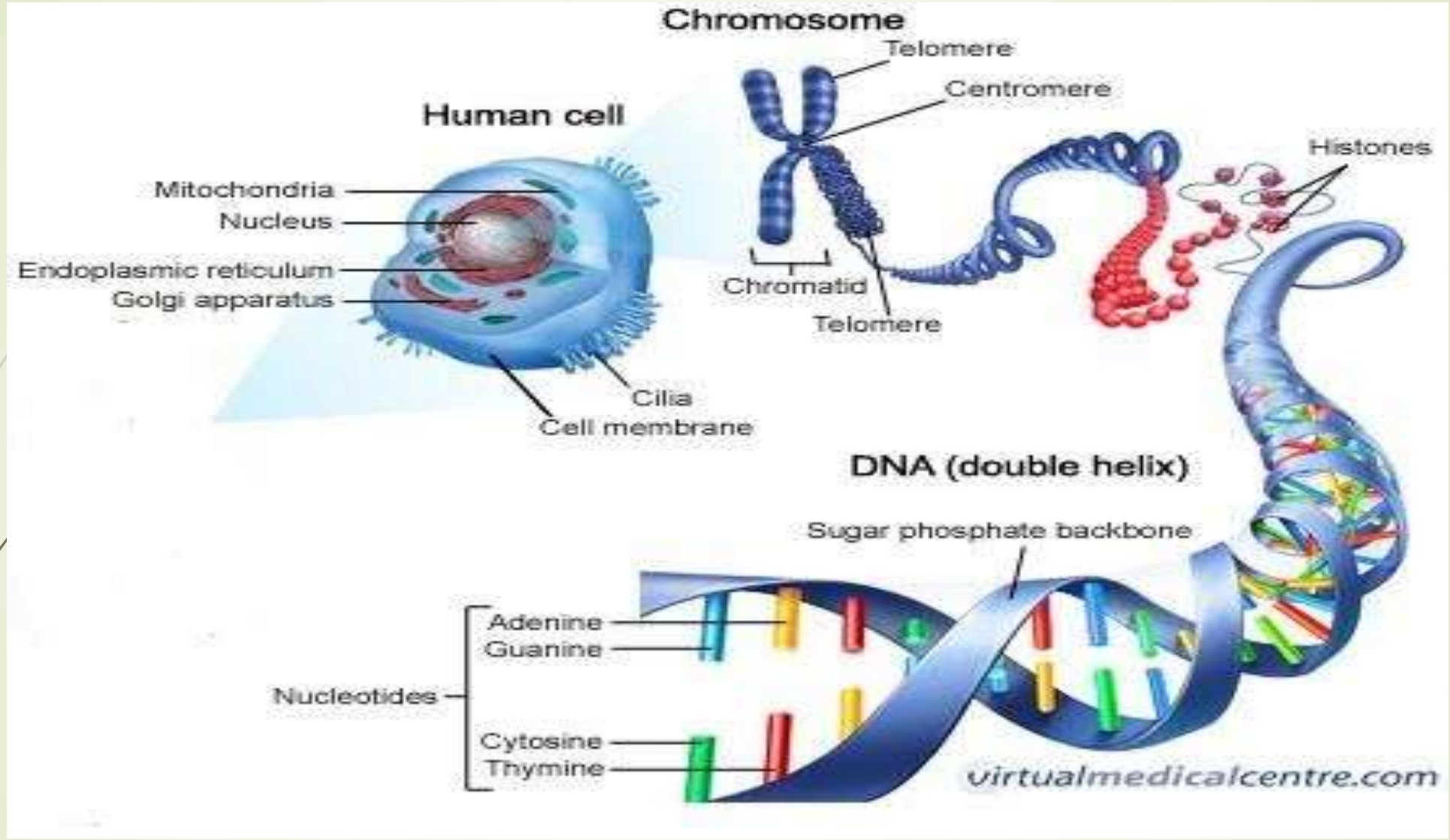
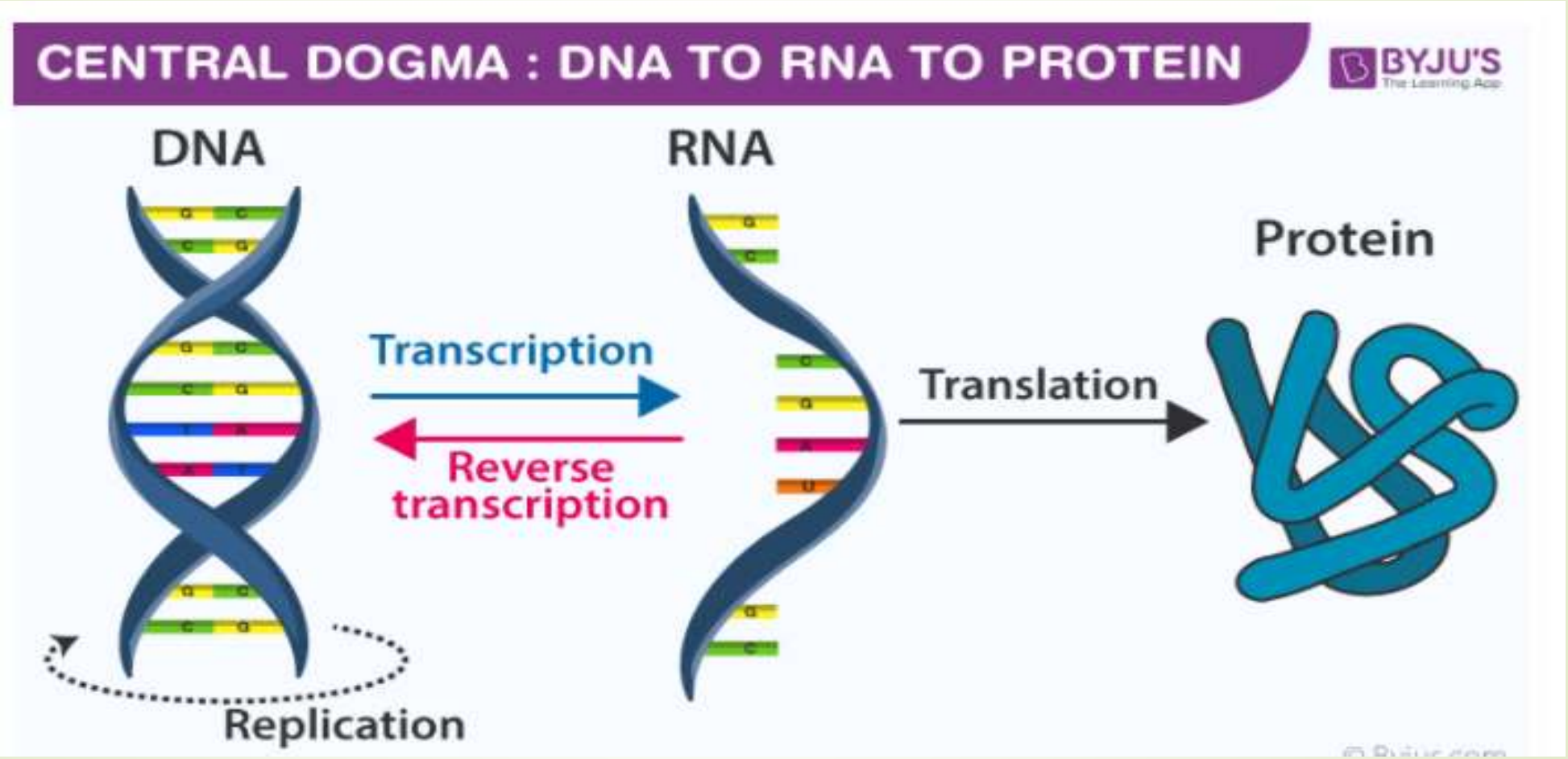
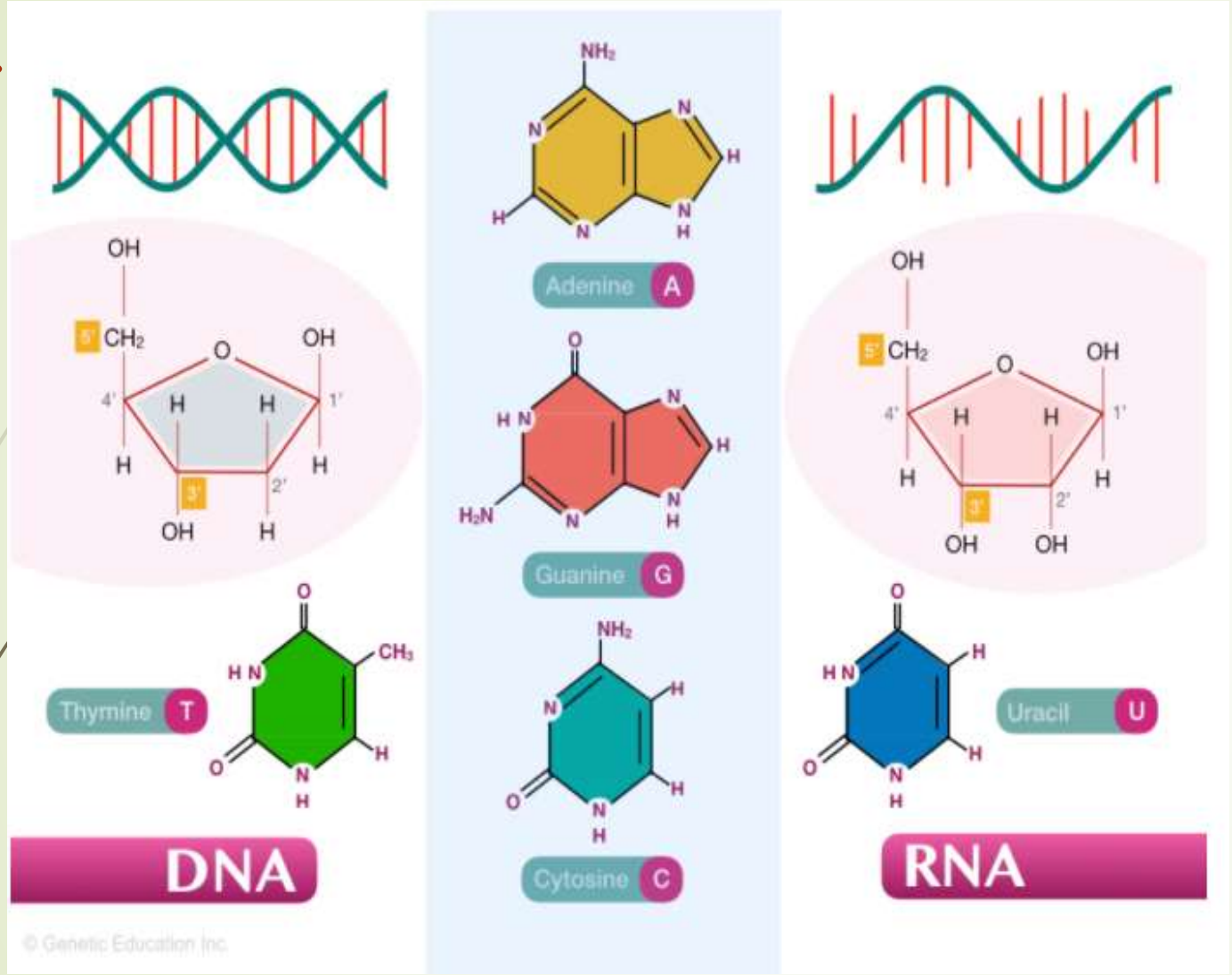


Image credits: https://www.researchgate.net/figure/Cell chromosome-and-DNA-6

❑ Central Dogma of Gene Expression



e.g. Gene for Insulin → mRNA for Insulin → Insulin Protein



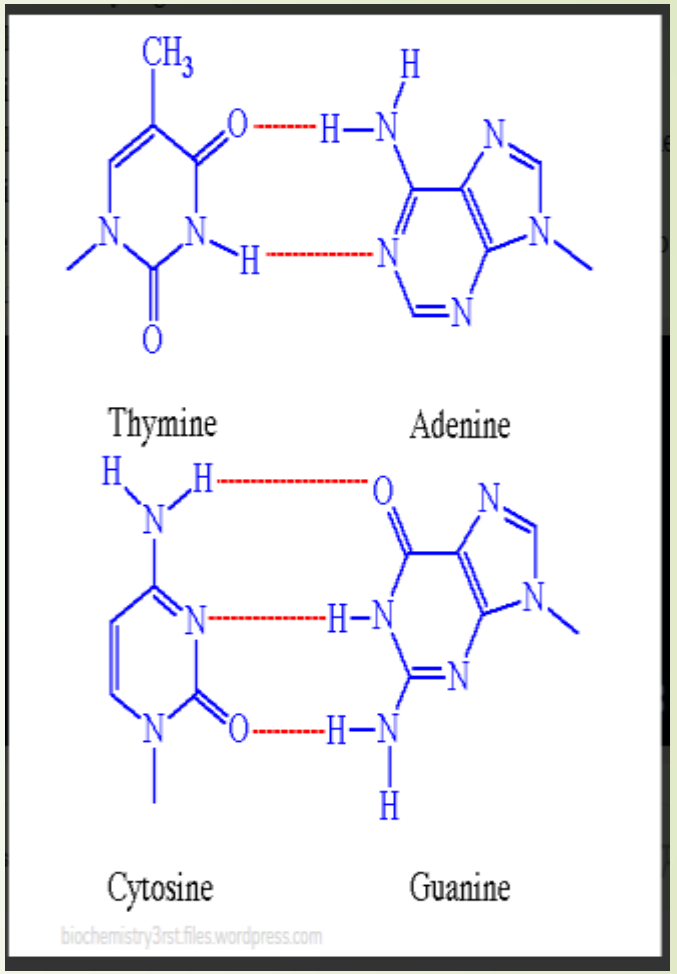


Image credit: https://geneticeducation.co.in/purines-vs-pyrimidines/

2. METHODOLOGY

- Primer Designing
- DNA Isolation
- Performing PCR
- Visualization of Amplicons Through Gel Electrophoresis.

Primer Designing

- Introduction
- Melting Temperature (Tm)
- GC Content, Repeats and Runs
- Primer Secondary Structures
- Resources for General Purpose PCR Primer Design
- Primer3web Home Page

➢ Primer Designing

❑ Introduction:

- ❖ A primer is a short synthetic oligonucleotide which is used in many molecular techniques from PCR to DNA sequencing.
- ❖ These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which we wish the primer to anneal.
- ❖ There are many more fields where primer design skills are required, such as real-time PCR, population polymorphism study microsatellite.

- **Primer length determines the specificity and significantly affect its annealing to the template**
  - **Too short** - base pairs low specificity, resulting in non-specific amplification
  - **Too long** - decrease the template-binding efficiency at normal annealing temperature due to the higher probability of forming secondary structures such as hairpins.
- **Optimal primer length**
  - 18-24 base pairs for general applications
  - 30-35 base pairs for multiplex PCR
- **Optimal amplicon size**
  - 300-1000 base pairs for general application, avoid > 3 kilo base (kb)
  - 50-150 base pairs for real-time PCR, avoid > 400 base pairs (bp)

❑ Melting Temperature (Tm)

- **T_m is the temperature at which 50% of the DNA duplex dissociates to become single stranded**
  - Determined by primer length, base composition and concentration.
  - Also affected by the salt concentration of the PCR reaction mix
  - Working approximation: T_m=2(A+T) + 4(G+C).
- **Optimal melting temperature**
  - 52°C-60°C
  - T_m above 65°C should be generally avoided because of the potential for secondary annealing.
  - Higher T_m (75°C-- 80°C) is recommended for amplifying high GC content targets.
- **Primer pair T_m mismatch**
  - Significant primer pair T_m mismatch can lead to poor amplification
  - Desirable T_m difference < 1°C between the primer pair

❑ GC Content, Repeats and Runs

- **Primer length:** 18 to 23 nucleotides
- **G/C content**
  - **Optimal G/C content:** 45-55%
  - **Common G/C content range:** 40-60%
  - 3′ end of primer should end with G or C
- **Runs (single base stretches)**
  - Long runs increases mis-priming (non-specific annealing) potential
  - Avoid runs of 4 or more bases or dinucleotide repeats (e.g. ACCCC or ATATATAT)
- **Repeats (consecutive di-nucleotide)**
  - Repeats increases mis-priming potential
  - The maximum acceptable number of repeats is 4 di-nucleotide

❑ Primer Secondary Structures

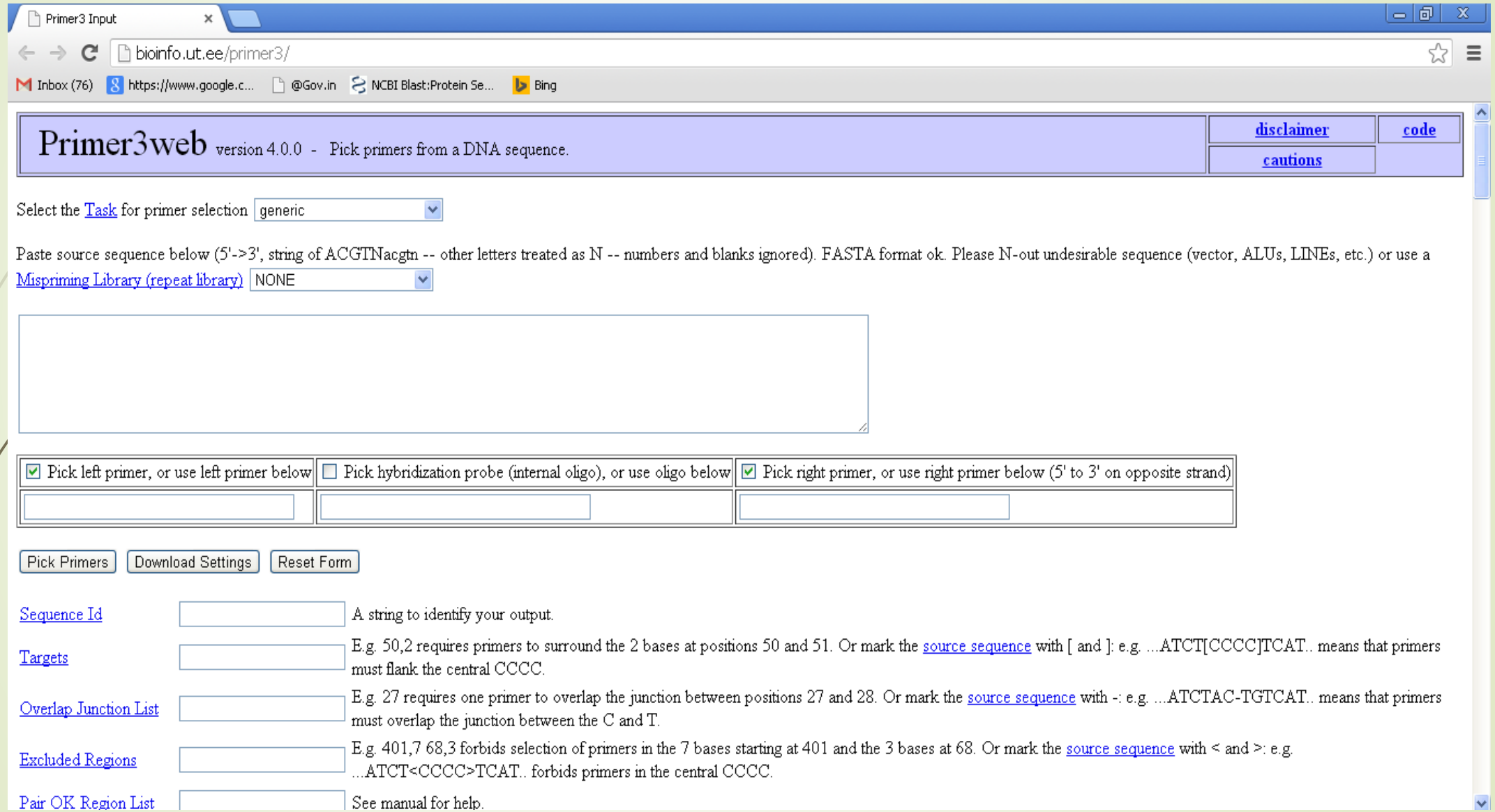
- **Hairpins**
  - Formed via intra-molecular interactions
  - Negatively affect primer-template binding, leading to poor or no amplification
- **Self-Dimer (homodimer)**
  - Formed by inter-molecular interactions between the two same primers
- **Cross-Dimer (heterodimer)**
  - Formed by inter-molecular interactions between the sense and antisense primers

Image Credit: https://geneticeducation.co.in/primer-dimer-zones-dna-amplification-by-pairing-with-foe/

❑ Resources for General Purpose PCR Primer Design

- **Primer3**
- **Primer3Plus**
- **Batch Primer3**
- **PrimerZ**
- **PerlPrimer**
- **Vector NTI Advantage 10**

- **Primer3Plus Home Page**



➢ DNA Isolation

- ❑ **Extraction/ Precipitation Method**
- ❑ **Quantification of DNA/RNA Through Spectrophotometry**

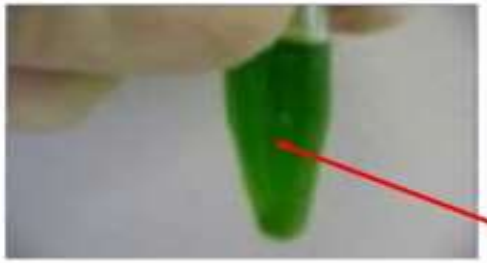
➢DNA Isolation

Extraction/Precipitation Method

Step 1: Disruption of cell walls by grinding

Grind sample into a fine powder to shear cell walls and membranes

Step 2: Lysis of cells in extraction buffer



Mix thoroughly with extraction buffer to dissolve cell membranes and inhibit nuclease activity

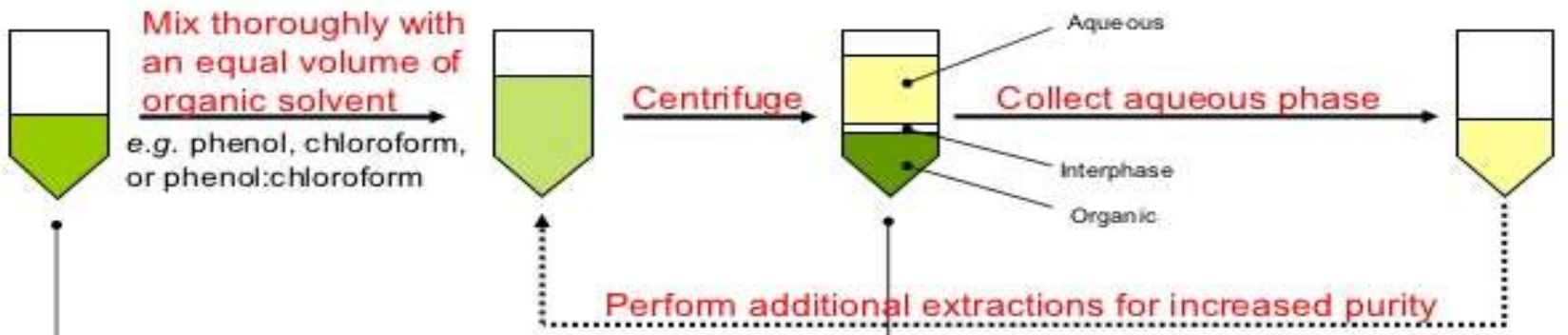
Step 1+2: mechanical disruption and homogenization in extraction buffer

A homogenizer allows cells to be mechanically disrupted within the extraction buffer

Crude lysate

Extraction/Precipitation Method

Step 3: Organic extraction



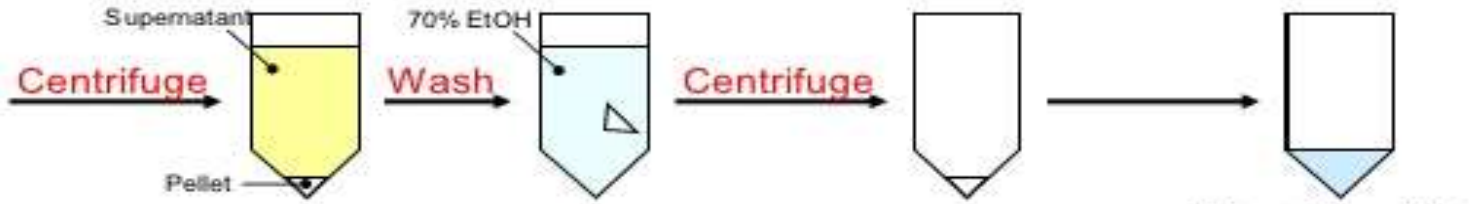
Crude lysate containing nucleic acids and other cell constituents

The **aqueous phase** contains water-soluble molecules, including nucleic acids. Proteins and lipids become trapped in the **organic phase**, and are thus separated away. Insoluble plant debris become trapped in the **interphase** between the two layers

Extraction/Precipitation Method

Step 4: Nucleic Acid Precipitation

Add alcohol and salt to precipitate nucleic acids from the aqueous fraction



- **Pellet down nucleic acids.**
- **Wash pellet with 70% ethanol to remove residual salts and other contaminants.**
- Discard ethanol and allow pellet to dry.

❑ Quantification of DNA/RNA Through Spectrophotometry

DNA concentration can be determined by measuring the intensity of absorbance with a spectrophotometers & comparing to a standard curve of known DNA concentration.

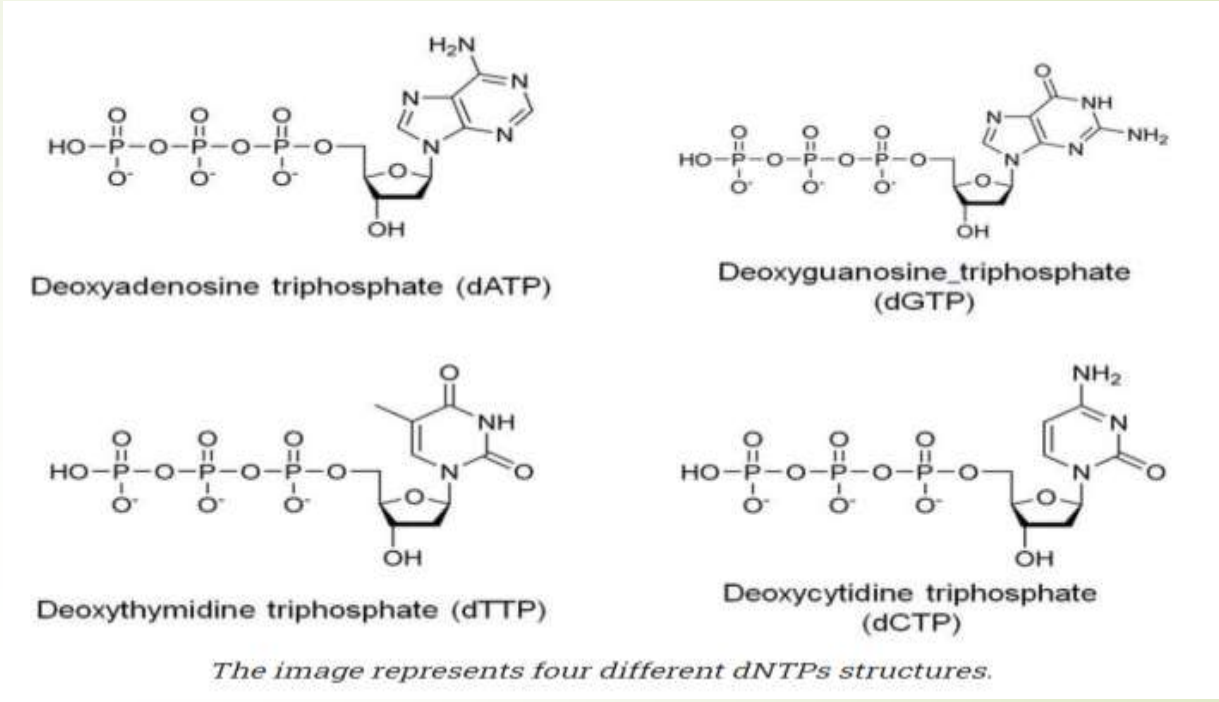
- Measuring the intensity of absorbance of the DNA solution at wavelength 260nm & 280nm is used as a measure of DNA purity
- **DNA purity:** A260/A280 ratio: 1.7 – 1.9
- Pure preparations of DNA and RNA have O.D 260/280 of 1.8 and 2.0 respectively
- **DNA concentration (µg/ml):** A260 X 50
- **DNA yield:** DNA conc. X Total volume of DNA solution
- **A260 x dilution x 40 =** [RNA] µg/ml
- DNA absorbs UV light at 260 &280 nm & aromatic proteins absorb UV light at 280 nm A pure sample of DNA has the 260/280 ratio at 1.8 & is relatively free from protein contamination.
- If the 260nm/280nm ratio is less than 1.6 for DNA and 2.0-2.3 for RNA, this indicates contamination, usually with protein.
- For DNA sample - If the OD ratio is higher than 2.0 it may be contaminated with RNA.

➢ Performing PCR

- ❑ Components of PCR
- ❑ Steps of PCR
- ❑ Schematic Representation of Different Stages of PCR

❑ Components of PCR

- Reaction Buffer
- $MgCl_2$
- dGTP
- dATP
- dTTP
- dCTP
- Taq DNA Polymerase
- Forward Primer
- Reverse Primer
- Template DNA
- Milli Q water



The image represents four different dNTPs structures.

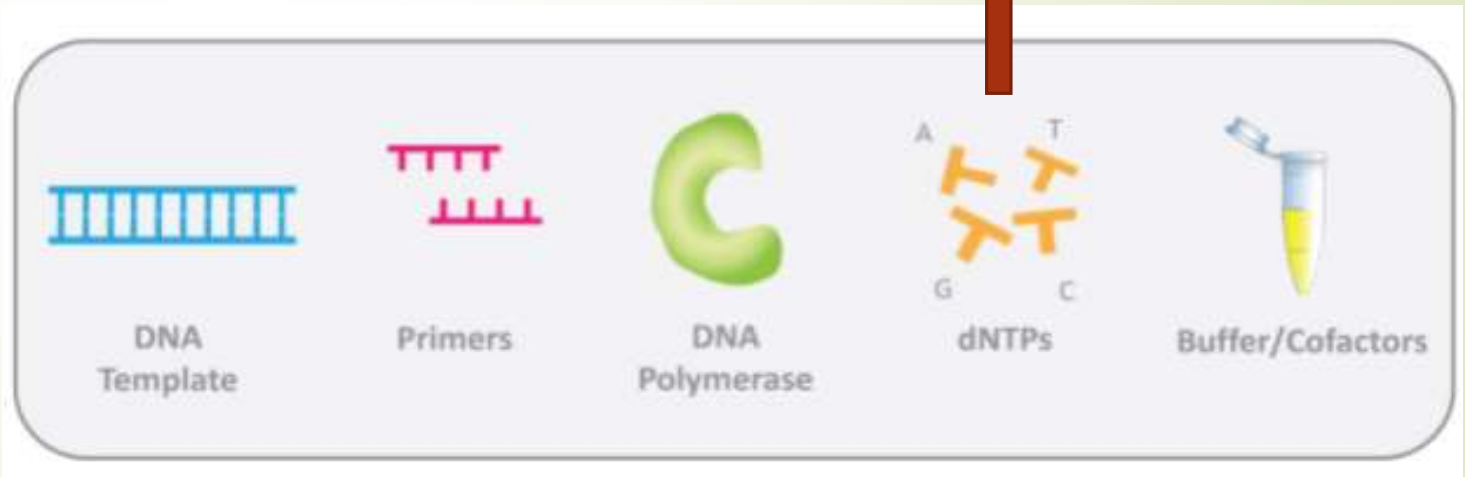


Image Credit: https://geneticeducation.co.in/the-function-of-dntps-in-pcr-reaction/

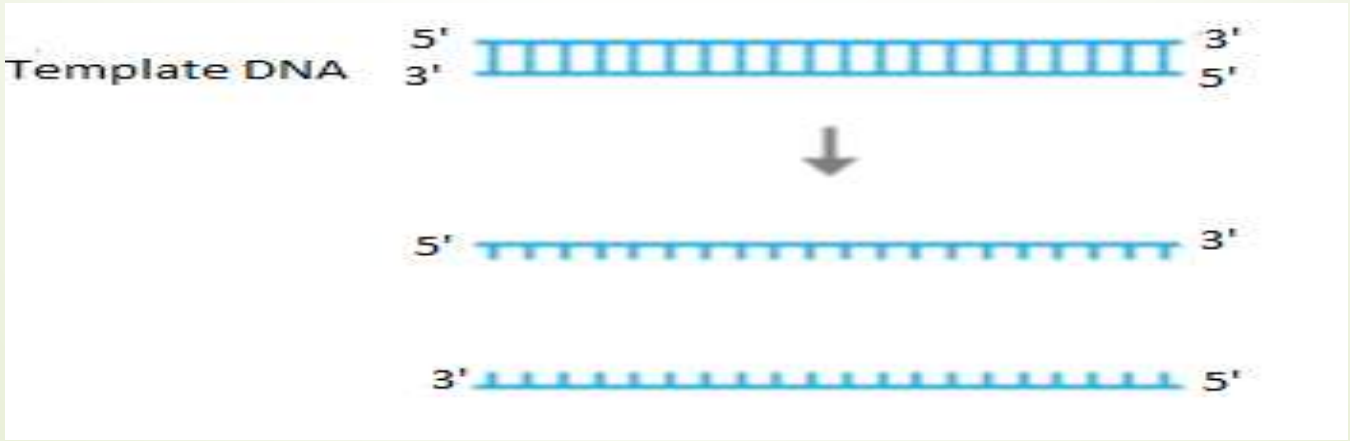
COMPONENTS	STOCK SOLUTION	WORKING SOLUTION	VOLUME (µl)
Reaction Buffer	10X	1X	2.5 µl
$MgCl_2$	25mM	2mM	2 µl
dGTP	1mM	1mM	1 µl
dATP	1mM	1mM	1 µl
dTTP	1mM	1mM	1 µl
dCTP	1mM	1mM	1 µl
Taq DNA Polymerase	5 Unit/µl	3 Unit/µl	1 µl
Forward Primer	100pM	10pM	1 µl
Reverse Primer	100pM	10pM	1 µl
Template DNA	100-150 ng/ µl	30-50 ng/ µl	3 µl
Milli Q water	-	-	10.5 µl
Total	-	-	**25µl**

Credit: https://geneticeducation.co.in/what-are-the-different-components-used-in-the-pcr-reaction-buffer/

❑ Steps of PCR

1. Denaturation

- The reaction mixture is heated to temperature between 90-98ºC so that the double stranded DNA is denatured into single strands.
- High temperature causes disruption of the hydrogen bonds between complementary nitrogenous bases.
- Duration of this step is 1-2 minutes.

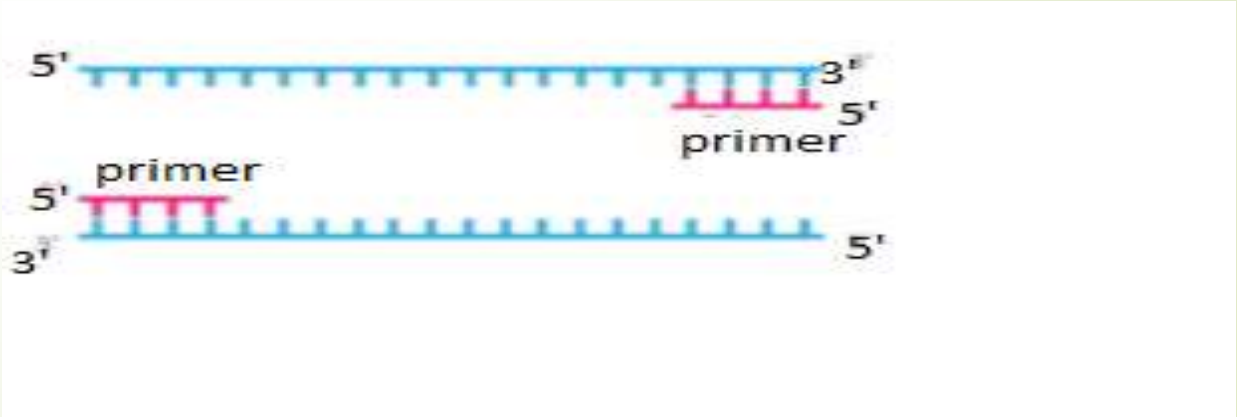


2. Annealing

- This step allows the hybridization of the two oligonucleotide primers, which are present in excess, to bind to their complementary sites the flank to target DNA.

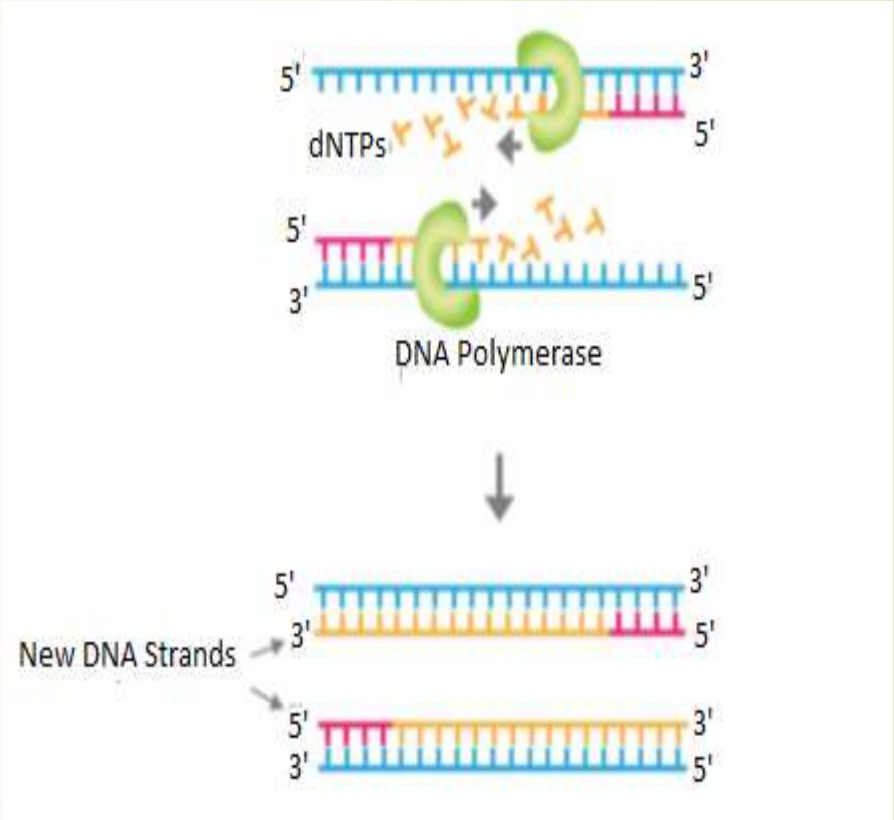
- The annealed oligonucleotide act as primers for DNA synthesis, since they provied a free 3′ hydroxyl group for Taq DNA polymerase.

- Temprature: 45-60ºC
- Time duration: 30 seconds

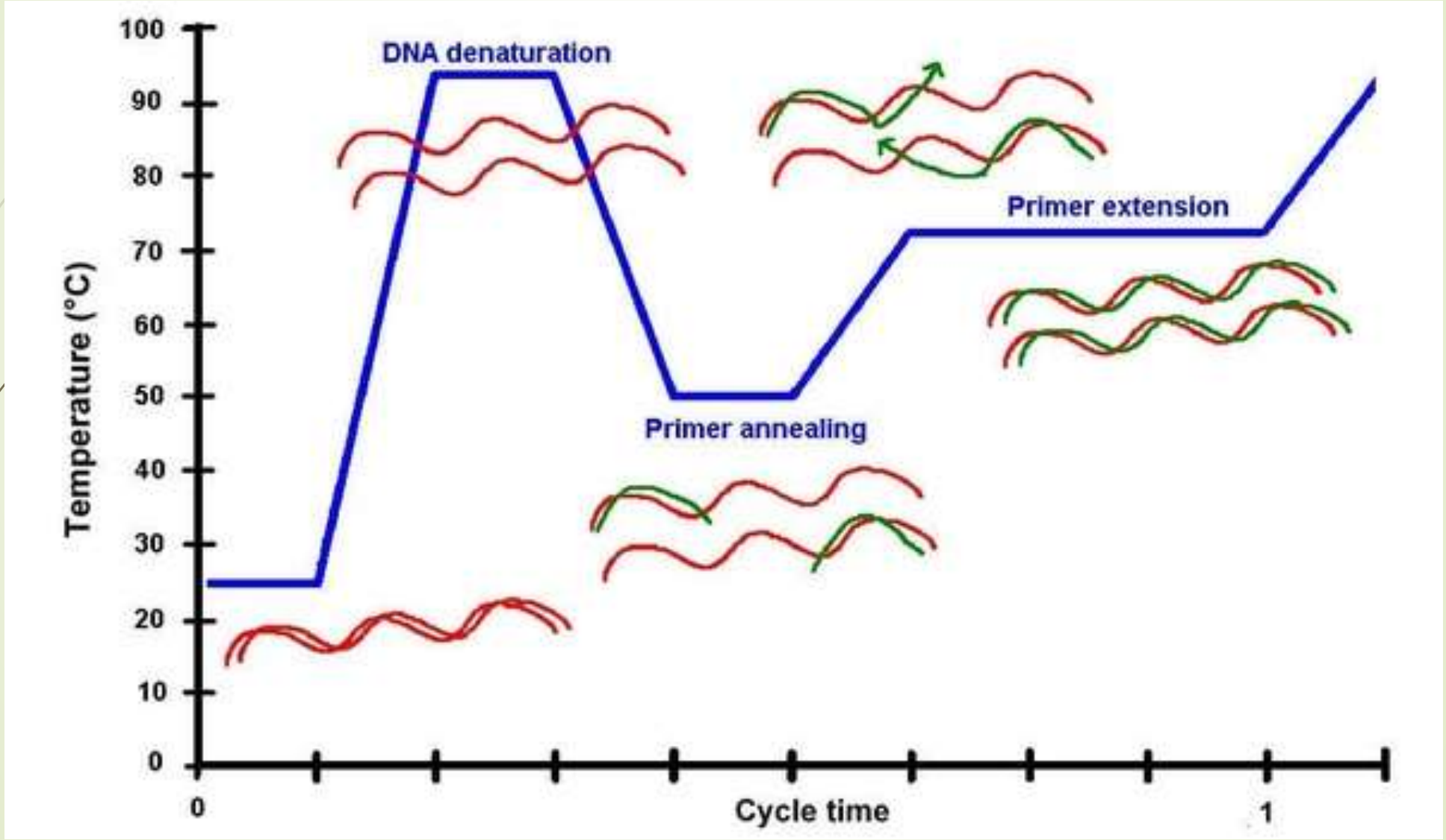


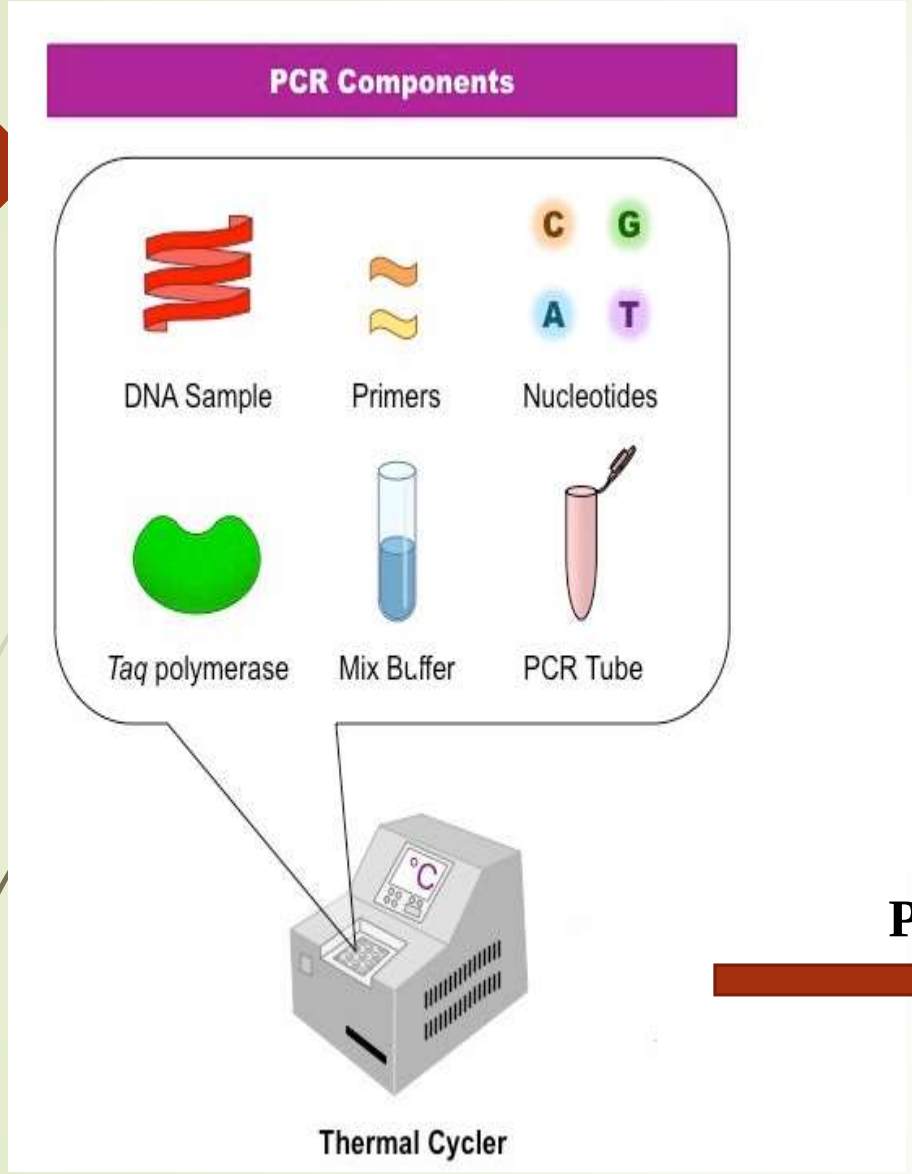
3. Extension

- The temperature is now raised to 72ºC which is optimum temperature for Taq polymerase.
- Time duration: 1min/kb
- Primers are extended by joining the bases complementary to DNA strands.
- Elongation step continues where the polymerase adds dNTPs from 5′ to 3′ direction while reading the template from 3′ to 5′ direction, the nitrogenous bases are added complementary to the template.
- With this the first cycle gets completed and next cycle is continued as PCR machine is automated the same cycle is repeated upto30-40 times.



❑ Schematic Representation of Different Stages of PCR





PCR

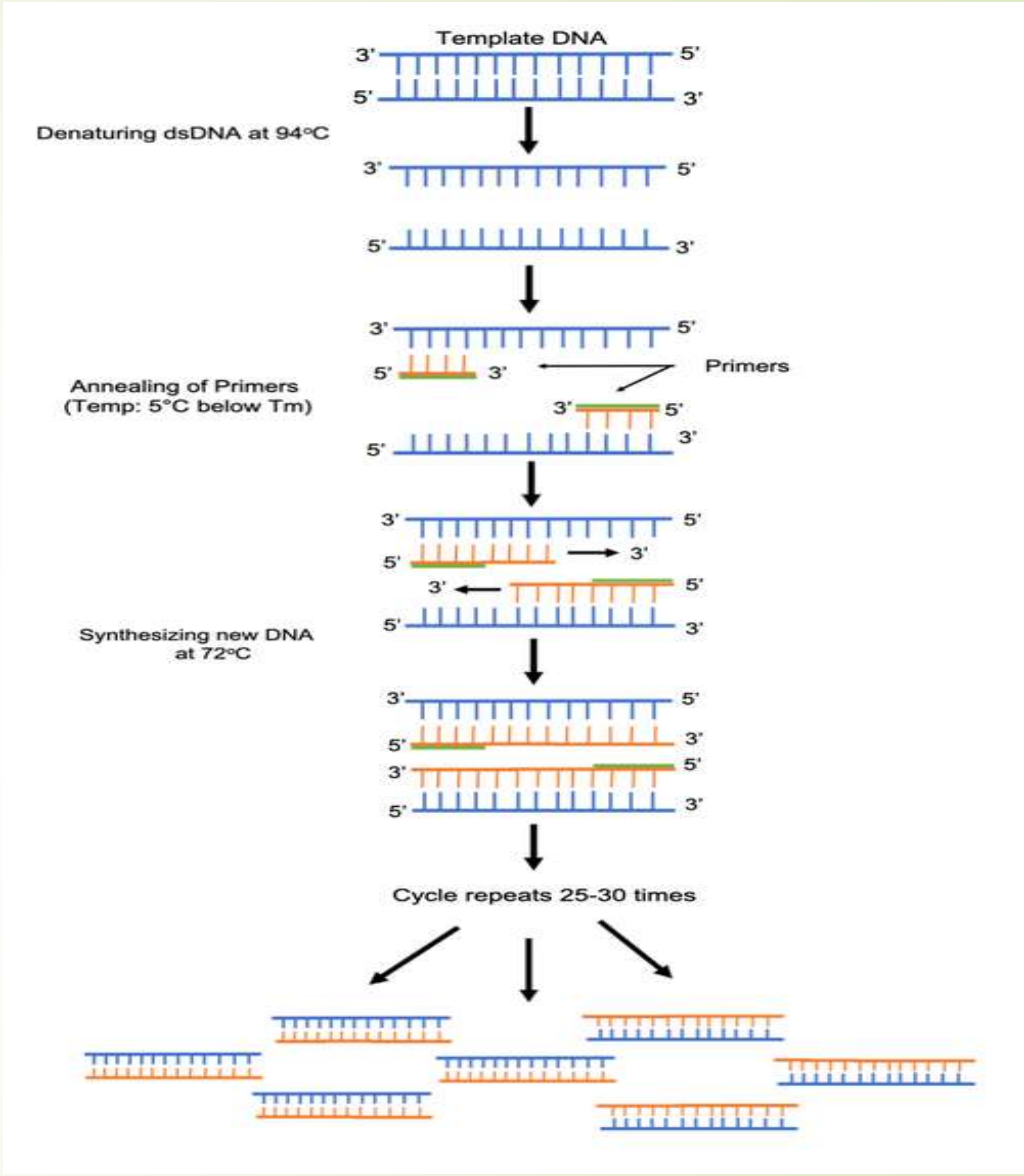


Image Credit: https://ib.bioninja.com.au/standard-level/topic-3-genetics/35-genetic-modification-and/pcr.html

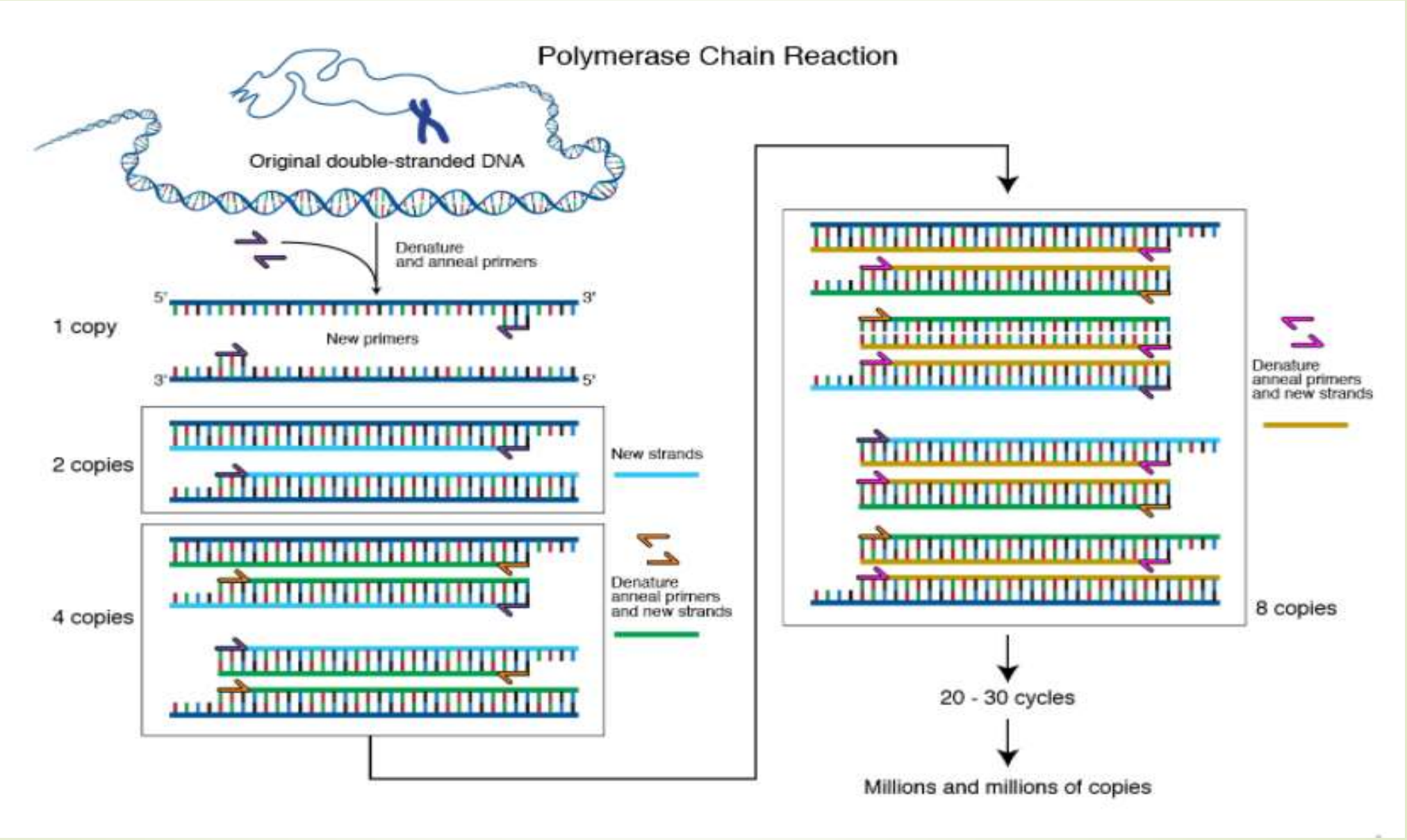


Image Credit: https://www.genome.gov/genetics-glossary/Polymerase-Chain-Reaction

➢ Visualization of Amplicons Through Gel Electrophoresis.

❑ Gel Electrophoresis

- Gel preparation
- Visualization in gel

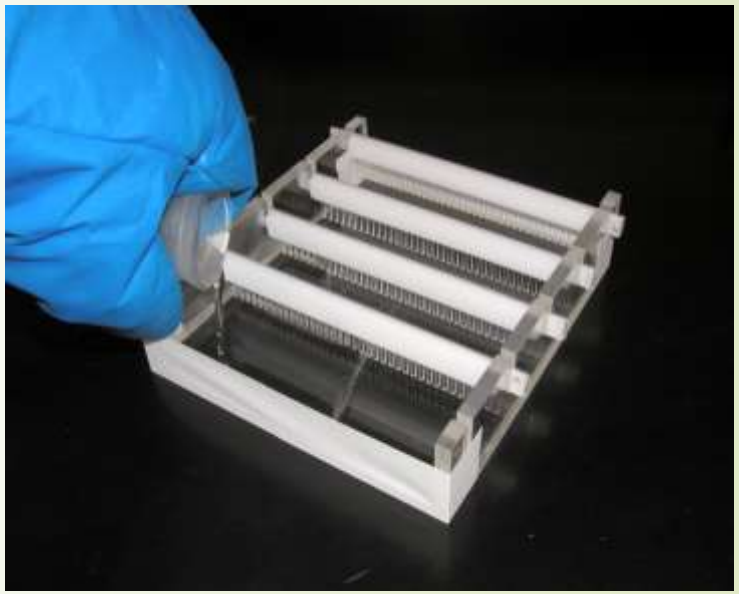
❑ Gel Electrophoresis: Gel electrophoresis is a procedure that separates molecules on the basis of their rate of movement through a gel under the influence of an electrical field..

- Gel Preparation

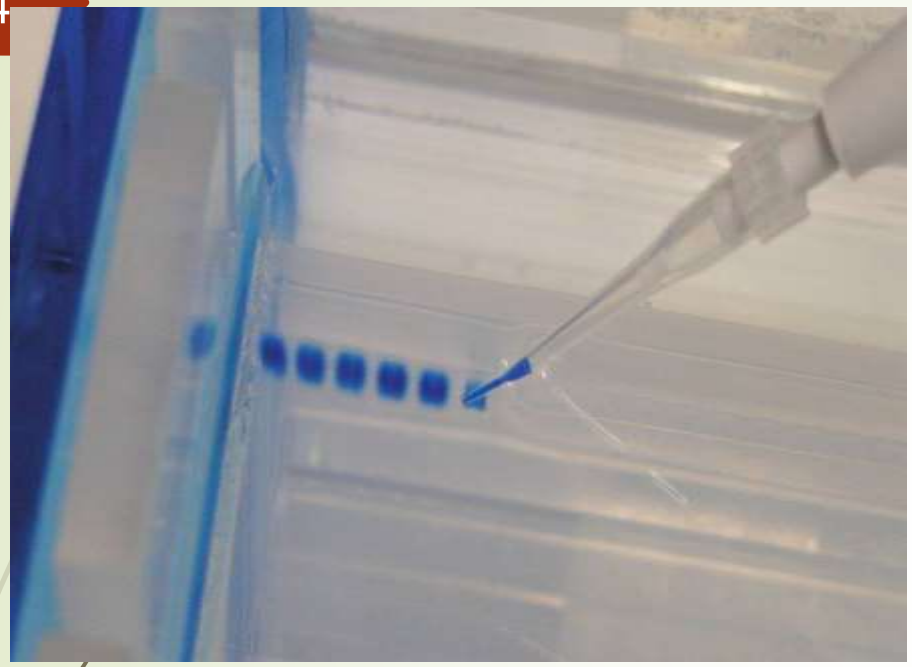
1 gm of Agarose is weighed

100 ml of double distilled water is added

The agarose solution is boiled until clear as agarose is insoluble at room temperature.

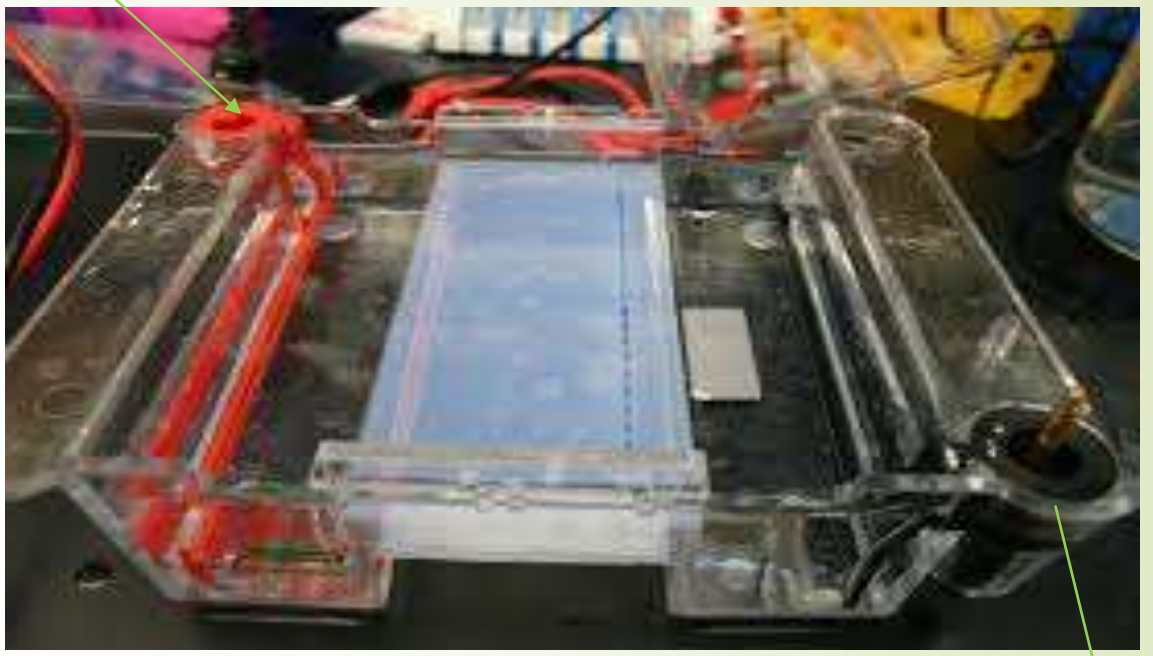


After cooling till 40˚C gel is poured into casting tray avoiding air bubbles.



2 µl of sample is mixed with 2 µl loading dye (EtBr/Bromophenol blue/Glycerol) and is loaded into well setting of the gel.

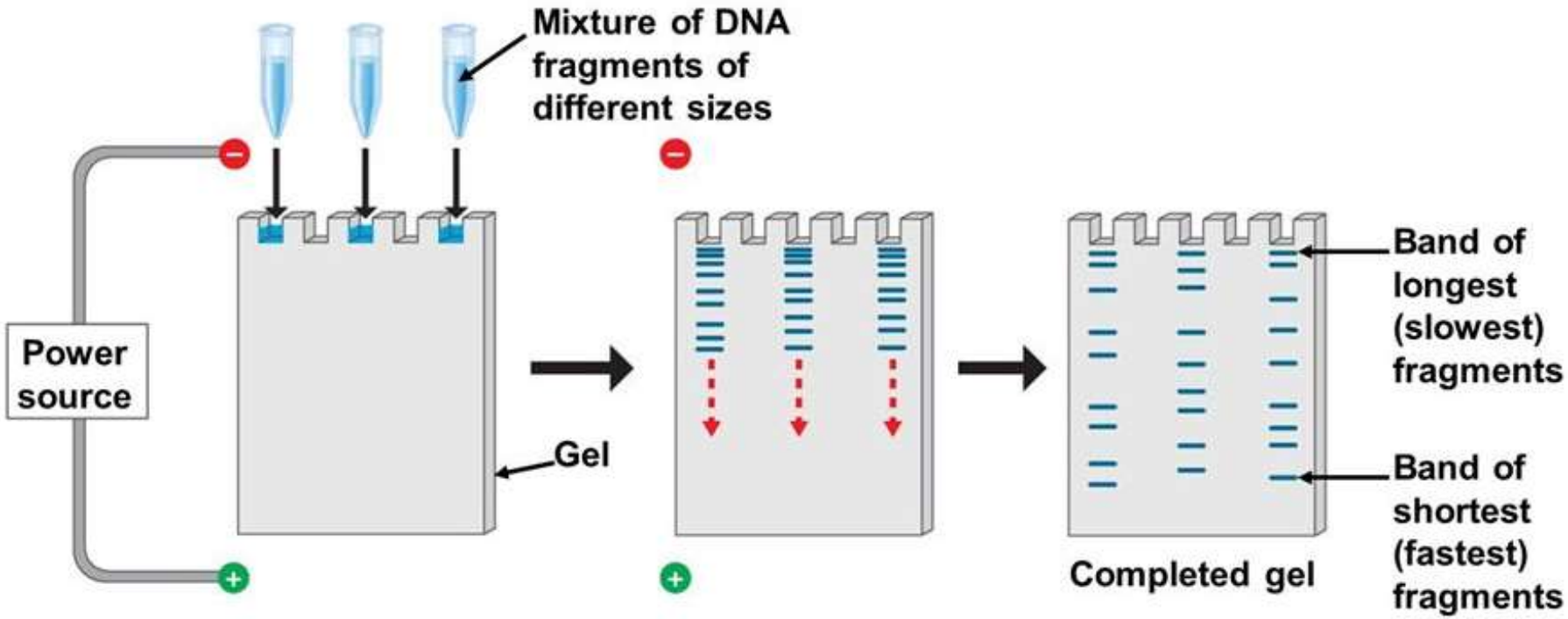
ANODE



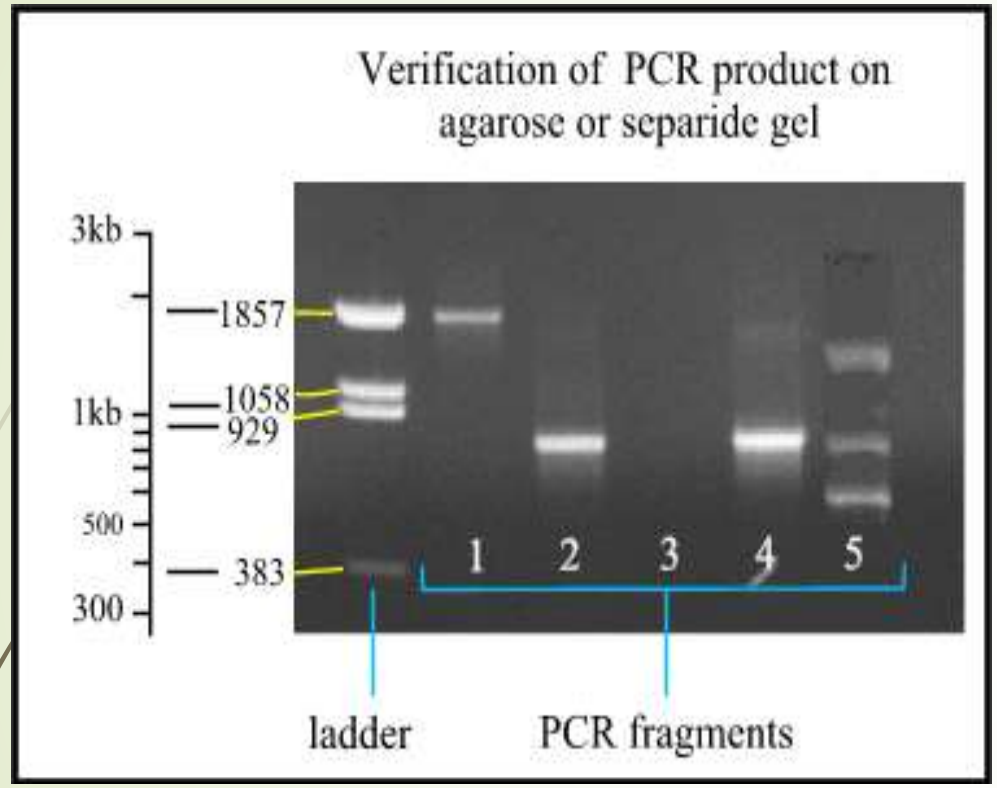
CATHODE

Filling tank with **Tris/Acetate/EDTA (TAE)** Buufer solution and allowing movement of DNA under influence of electric field.

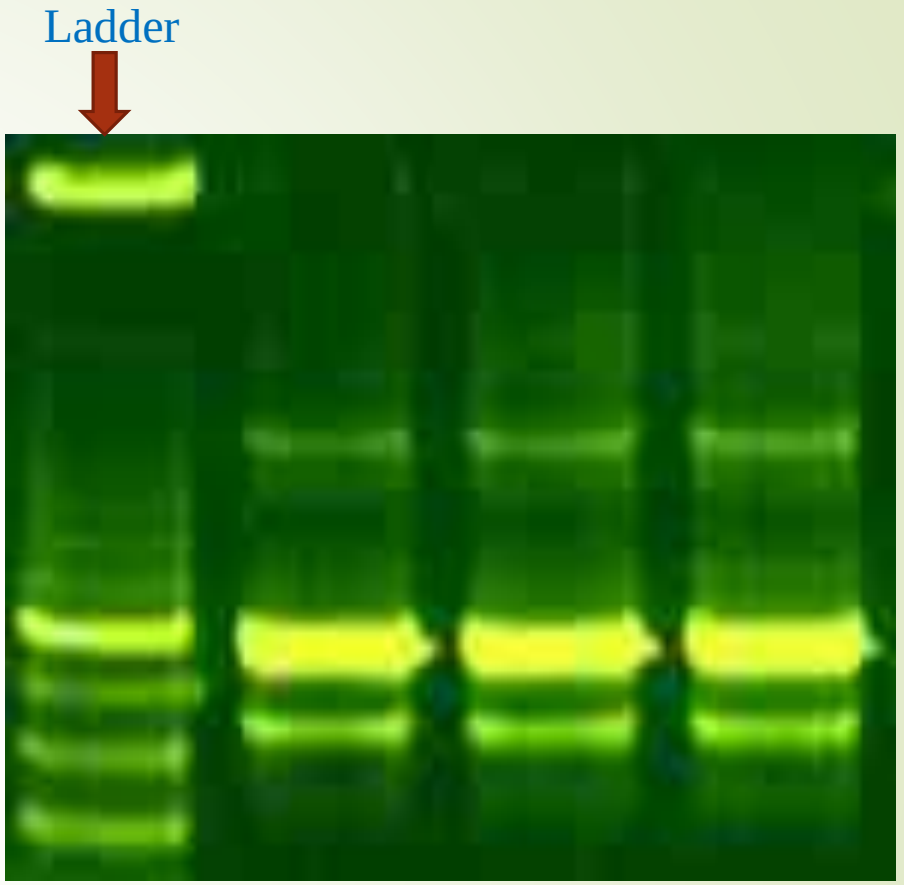
Gel Electrophoresis of DNA



- Visualization in gel



Ethidium Bromide stained gel



Sybr Green stained gel

The lane in the left side of the picture on the left is a “ladder” which has fragments of **known** base pair (bp) sizes. This allows determination of the size of isolated fragments.

3. TYPES OF PCR

- End point PCR
- Real Time PCR or Quantitative PCR (qPCR)
- Reverse Transcription PCR (RT-PCR)
- Multiplex PCR
- High Fidelity PCR
- Hot Start PCR
- Touch Down PCR
- Colony PCR
- Inverse PCR

•Reverse Transcription PCR (RT-PCR)

- For amplifying DNA from RNA
- Reverse Transcriptase enzyme reverse transcribes RNA into cDNA, which is then amplified by PCR
- Some thermostable DNA polymerase used in the PCR such as Tth (*Thermus thermophilus*) DNA Polymerase have a reverse transcriptase activity under certain buffer conditions.

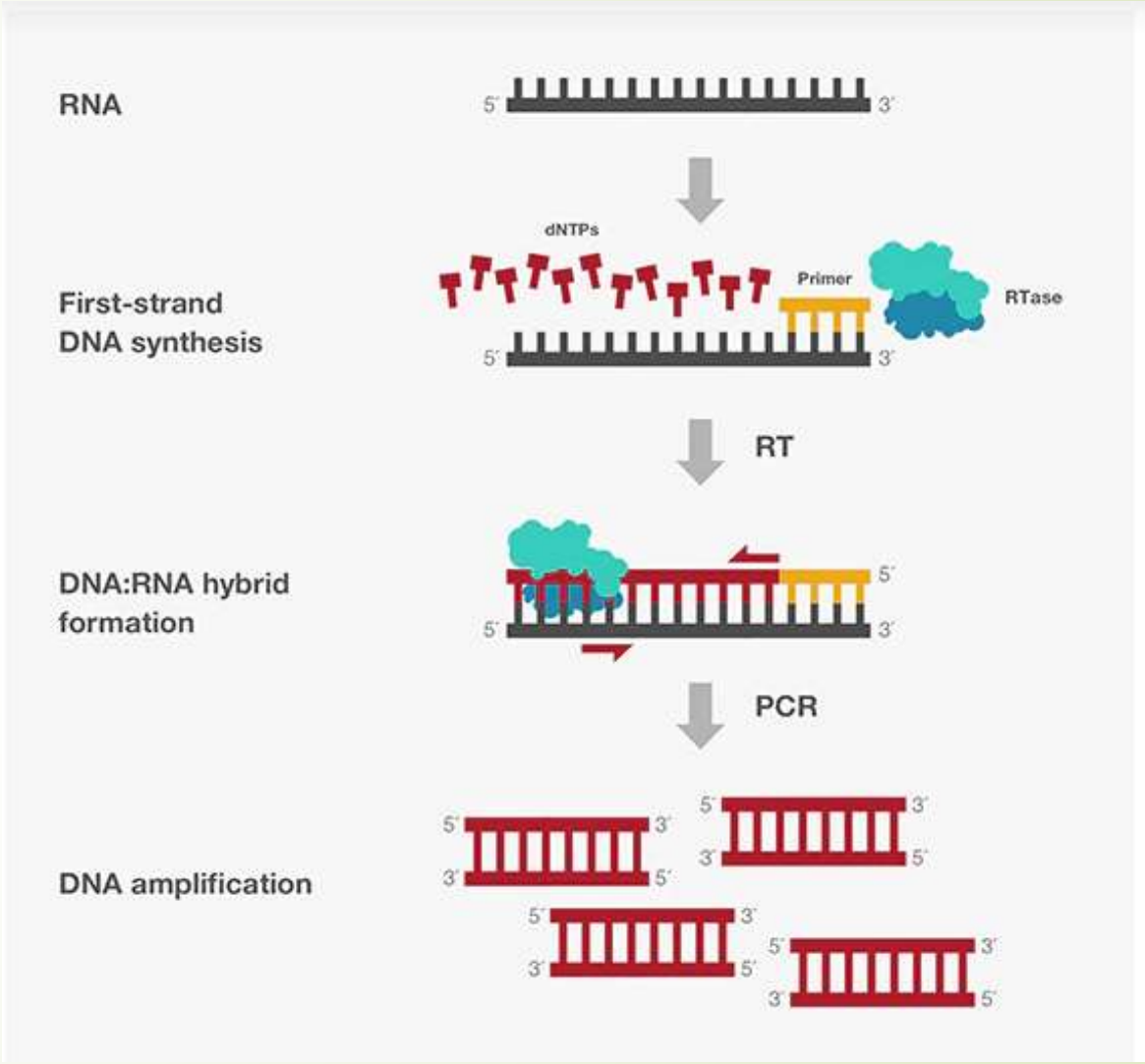
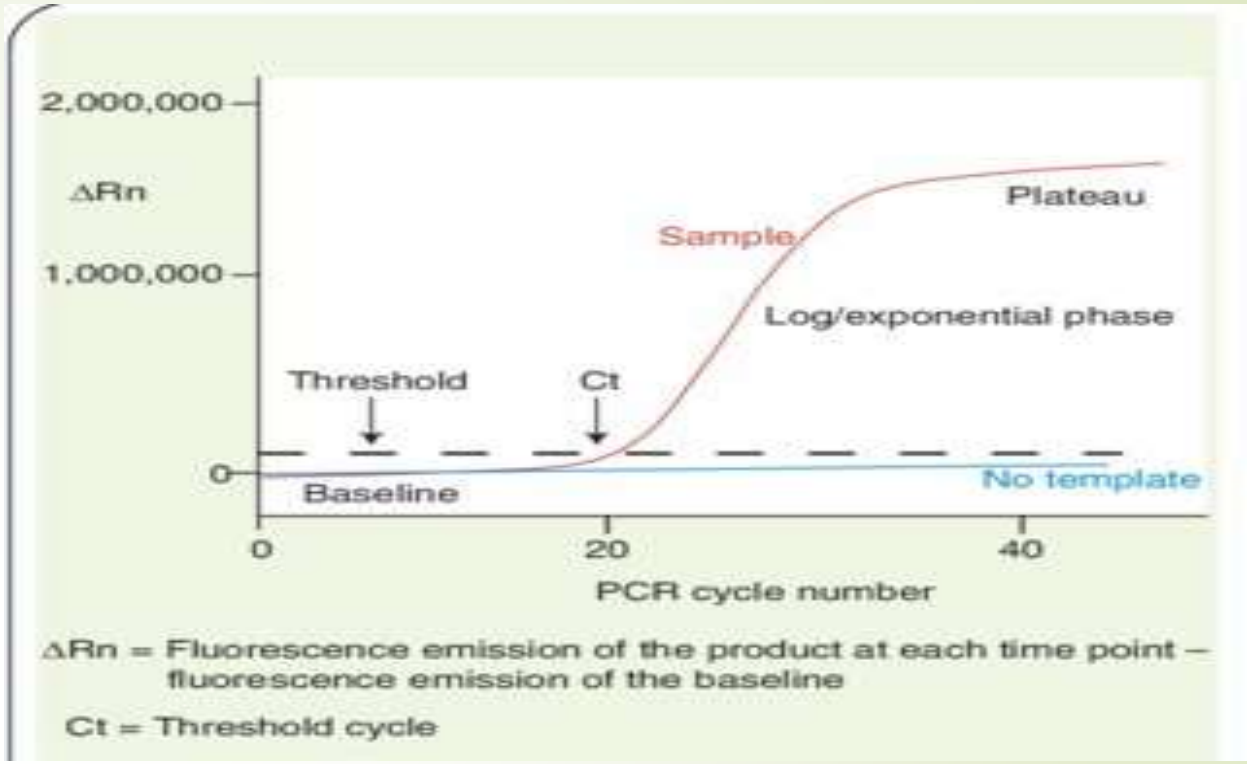
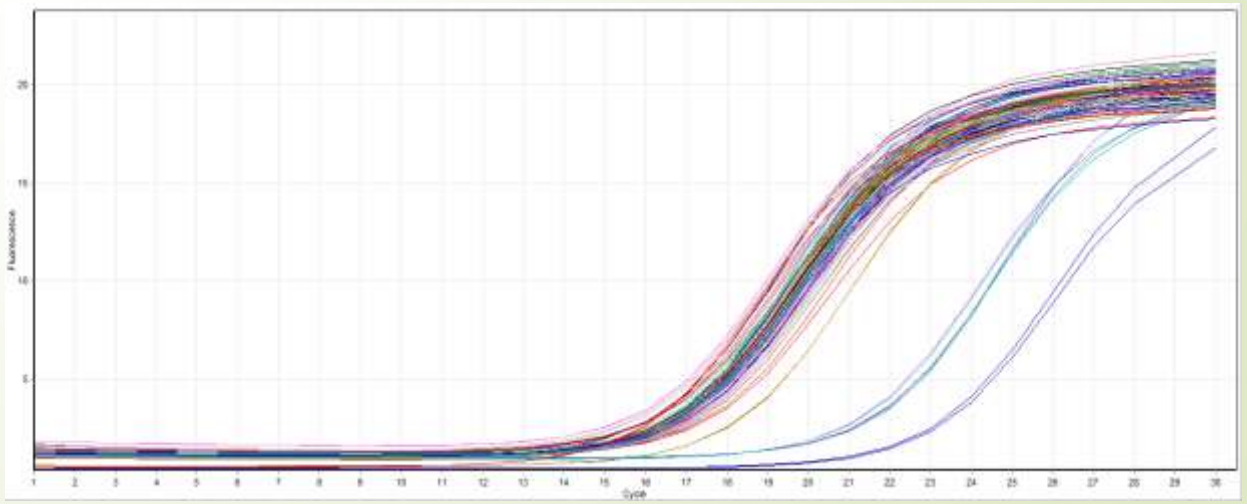


Image Credit: www.thermofisher.com

•Real Time PCR or Quantitative PCR (qPCR)

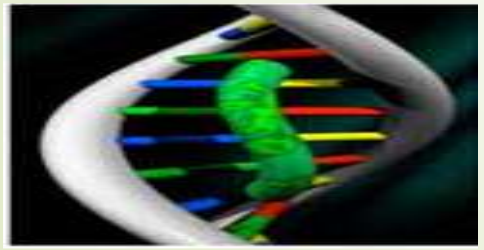
- It monitors the amplification of the targeted DNA molecules during the PCR.
- Ability to monitor PCR reaction in real time, because of use of fluorescent dye (e.g. Syber green) or labeled primers.
- Accurate quantification of the amount of starting material (template) added to a reaction.





qPCR Using SYBER Green dye

- Dye binds to the minor grooves of DNA
- Fluorescence is much stronger when bound to DNA
- More product, more dye bound, more fluorescence.
- Cheap
- Not specific
- At early stage check product on gel and perform dissociation analysis (melt curve)



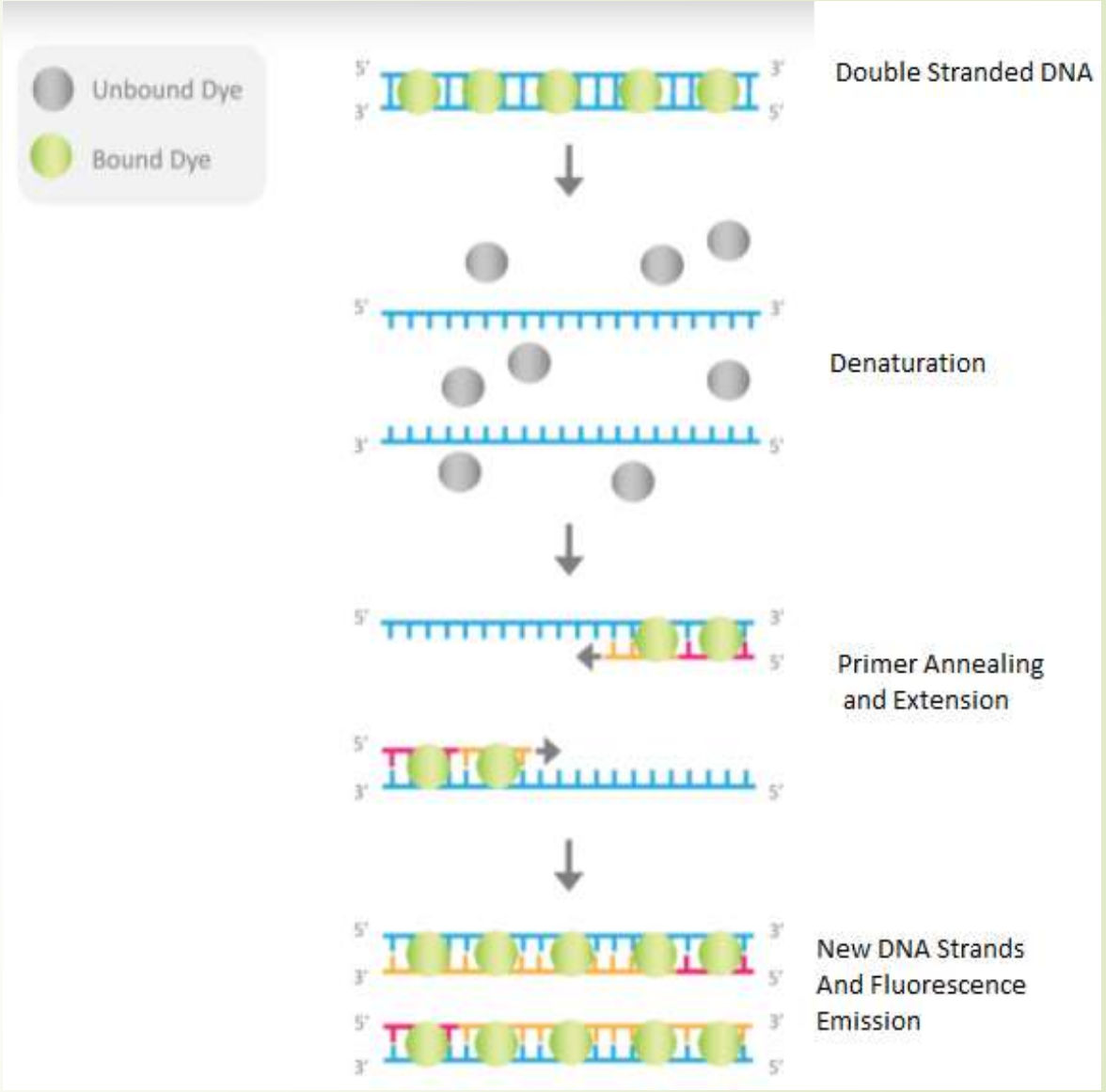
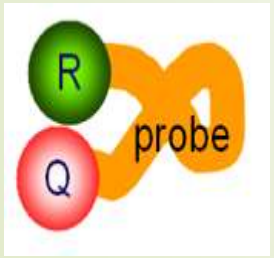
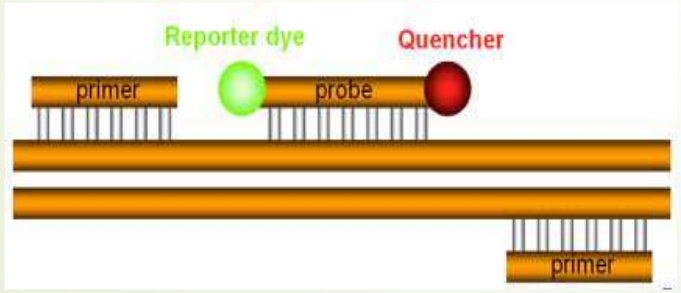


Image Credit: www.thermofisher.com

qPCR Using Probe

- Reporter dye and quencher on either ends of the probe
- Nuclease activity of polymerase removes the Reporter dye and quencher, during PCR
- More amplicons, more fluorescence
- No requirement of melt curve
- Allows multiplexing





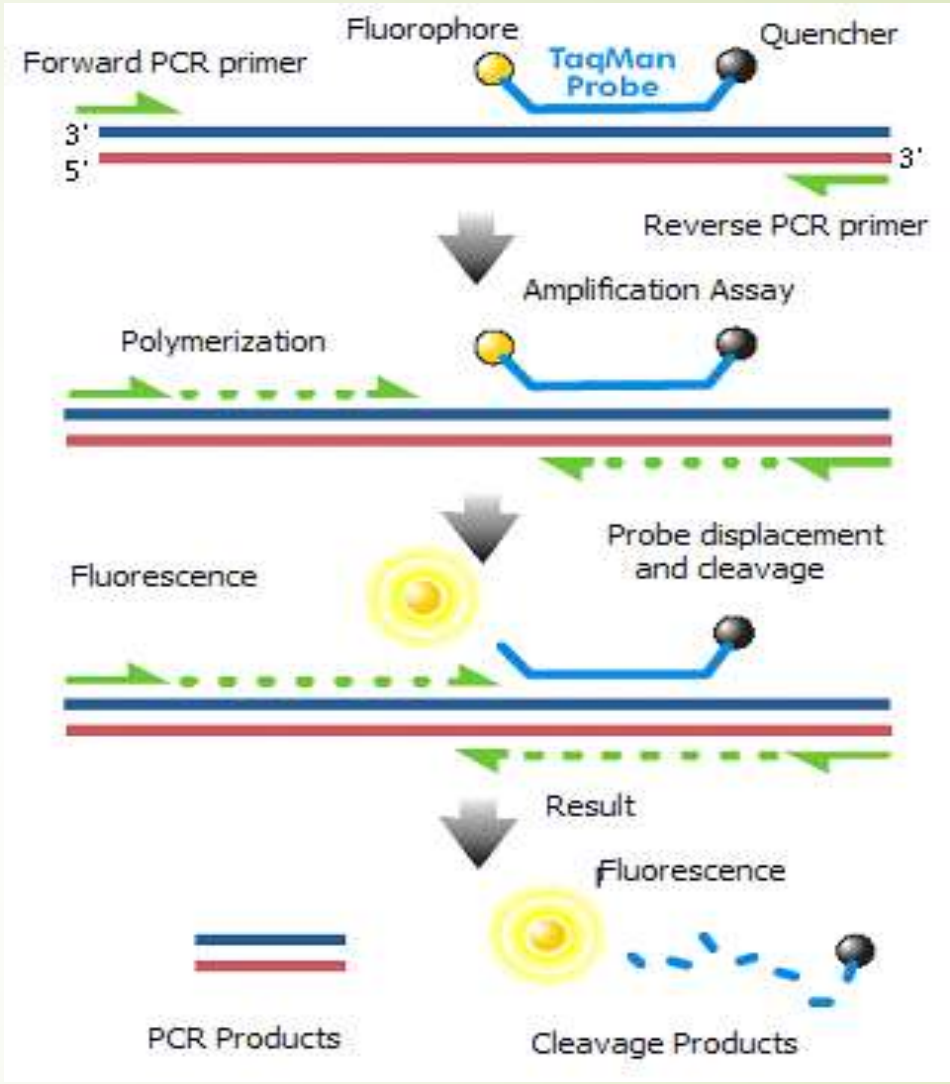


Image Credit: TaqMan probe chemistry mechanism

4. APPLICATIONS OF PCR

- The amplification of gene fragments.
- The mutagenesis or modification of DNA fragments.
- The sensitive detection of pathogenic microorganisms.
- DNA analysis of archaeological specimens.
- The detection of mutations relevant for inherited diseases, malignant transformation or tissue typing.
- The analysis of genetic markers for forensic applications, for paternity testing and for the mapping of hereditary traits.
- The species-specific amplification of DNA segments between interspersed-repeat elements.
- The study of gene expression.

Applications in Ayurveda

Preparation of Anti- Diabetic or Anti-Cancerous Herbal or Polyherbal Formulation

↓

Administered to animal model e.g. Rat

↓

Isolation of Tissues and Isolation of whole cell RNA

↓

Perform RT-PCR and qPCR for Upregulation and Downregulation of ‘Genes of Interest’

It will reveal the impact of the herbal formulation at the level of genes

5. LIMITATIONS OF PCR

1. **Prior information** about the **target sequence** is necessary in order to generate the primers that will allow its selective amplification.[
2. Even the **smallest amount of contaminating DNA** can be amplified, resulting in misleading or ambiguous results.
3. Like all enzymes, **DNA polymerases** are also **prone to error**, which in turn causes mutations in the PCR fragments that are generated.

6. DISCUSSION AND CONCLUSION

- PCR is powerful biotechnique which founds its applications from basic scientific research to clinical diagnostics to forensic sciences/ investigation.
- It can be utilized to study the impact of polyherbal formulation for treatment of any disease at the level of gene expression in animal model.
- At the level of gene expression, RT-PCR and qPCR could be used to study the up-regulation or down-regulation of any gene upon treatment with the herbal drug.
- Such molecular level studies shall provide legitimacy of the use of herbal drugs for treatment of the diseases.

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THANK YOU