DNA Extraction and PCR

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Objectives

- The principle of DNA extraction
- Explain the DNA extraction method
- Quantitation of DNA
- Types of PCR and how we select the best for our research
- Principle of Designing and Optimization of the Primers
- Determining the PCR cycle number
- preparation and calculation of PCR tube

DNA Extraction

- *Definition*
- DNA extraction is the technique used to isolate DNA from a biological sample.

- **<u>Initial development of DNA extraction techniques</u>**
- Extraction of nucleic acids is the starting point in any molecular biology study and hence is considered as a crucial process.
- The first crude extraction of DNA had been performed by the Swiss physician **Friedrich Miescher** in 1869.
- He had accidentally purified DNA from the nucleus while investigating proteins from leukocytes and found that the property of this substance was fundamentally different than proteins, hence coined the term “nuclein”

- However, it was only in 1958 that **Meselson and Stahl** developed a routine laboratory procedure for DNA extraction. They performed DNA extraction from bacterial samples of Escherichia coli using a salt density gradient centrifugation protocol.

Sources of DNA

DNA can be extracted from diverse clinical samples such as

- fine needle aspirates of body fluids and
- biopsy samples;
- forensic samples such as dried blood spots,
- buccal swabs
- plant and animal tissue, insects, protozoa, bacteria and yeast

The principle behind DNA extraction

disruption of cytoplasmic and nuclear membranes.

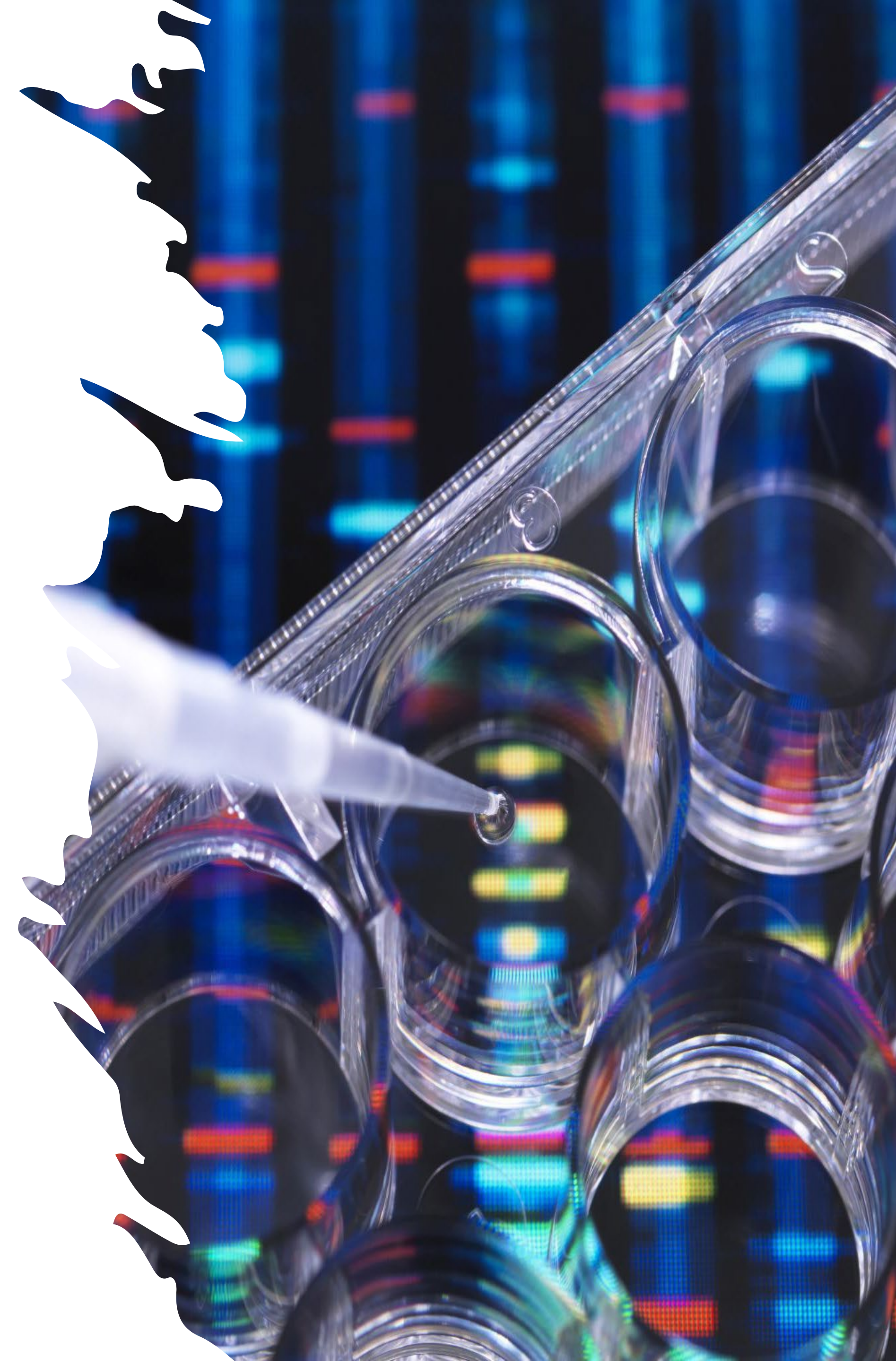
separation and purification of DNA from other components of the cell lysate such as lipids, proteins, and other nucleic acids.

concentration and purification of DNA

What are the Most Commonly used DNA Extraction Procedures ?

- Organic (Phenol-Chloroform) Extraction
- Non-Organic (Proteinase K and Salting out)
- Commercial kits (column technology)

- **When choosing a suitable method for DNA extraction, it is crucial to ensure**
  - ➢ the quality and quantity of the isolated DNA to carry out the intended downstream applications.
  - ➢ the time, cost, and potential toxicities.
  - ➢ laboratory equipment and expertise requirements
  - ➢ the required sample amount for the protocol



lysis of cells :

- For lysis, the cells (blood, tissue, etc.) of the sample must undergo a treatment to break the cell membrane and free the nucleic acid.

Lysis of cells :

- Depending on the target material, this can include the use of
- detergent or other buffers,
- proteinases or other enzymes,
- heating to various times/temperatures, or
- mechanical disruption such as cutting with a knife or homogenizer, using a mortar and pestle.

lysis of cells :

- **Sodium dodecyl sulfate** (SDS) and **Triton™ X-100** are examples of popular detergents used to solubilize cell membranes.
- Enzymes are also combined with detergents to target cell surface or cytosolic components.
- **Proteinase K** is a commonly used enzyme used in various protocols to cleave glycoproteins and inactivate RNases and DNases.

The Binding

- Buffer solution is added to the lysed sample along with ethanol or isopropanol.
- The sample in the binding solution is then transferred to a spin column, and the column is put either in a centrifuge or attached to a vacuum.
- The centrifuge/vacuum forces the solution through a silica membrane that is inside the spin column, where under the right ionic conditions, nucleic acids will bind to the silica membrane, as the rest of the solution passes through.

The wash

- This step is intended to maintain binding conditions, while removing the binding salts and other remaining contaminants.
- Generally, it takes several washes, often with increasing percentages of ethanol/isopropanol, until the nucleic acid on the silica membrane is free of contaminants.
- The last 'wash' is often a dry step to allow the alcohol to evaporate, leaving only purified nucleic acids bound to the column.

Elution

- Elution is the process of adding an aqueous solution to the column, allowing the hydrophilic nucleic acid to leave the column and return to the solution. This step may be improved with salt, pH, or heat.
- Finally, to capture the eluate/eluent, the column is transferred into a clean microtube prior to the last centrifugation step.

- **Methods and Workflow**
- DNA Extraction
- Genomic DNA was isolated from blood sample according to the protocol ReliaPrep™ Blood gDNA Miniprep System, Promega

- The ReliaPrep™ Blood gDNA Miniprep System provides a fast, simple technique to purify intact DNA from mammalian blood.
- Samples are processed using

a binding column in a microcentrifuge tube.

The ReliaPrep™ Blood gDNA Miniprep System uses a simple four-step method:

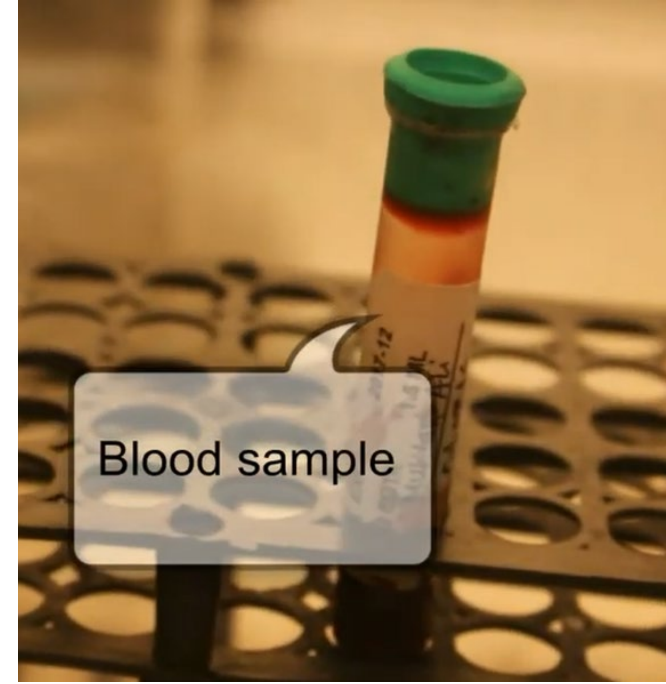
1. Effectively disrupting or homogenizing the starting material to release the DNA.
2. Binding DNA to the ReliaPrep™ Binding Column.
3. Removing impurities with wash solution.
4. Eluting purified DNA.

Each system contains

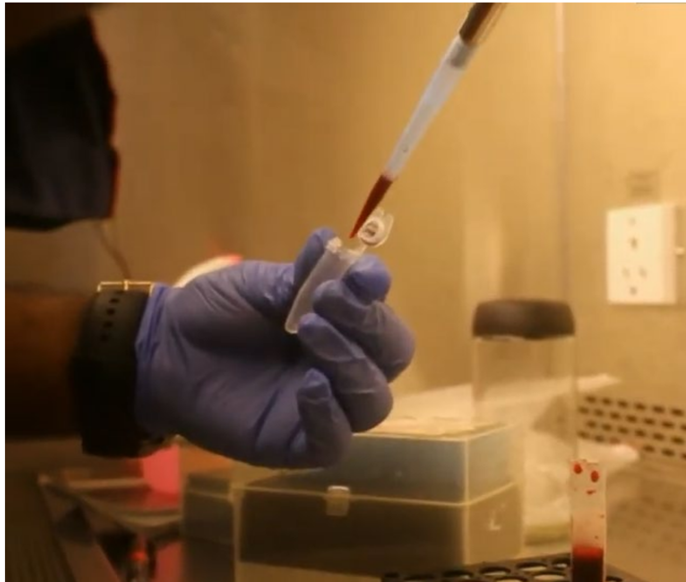
- ReliaPrep™ Binding Columns
- Collection Tubes
- Cell Lysis Buffer (CLD)
- Proteinase K (PK) Solution
- Binding Buffer (BBA)
- Column Wash Solution (CWD)
- Nuclease-Free Water

The steps:

• Blood sample was thoroughly mixed for 15 minutes in a roll mixer at room temperature.

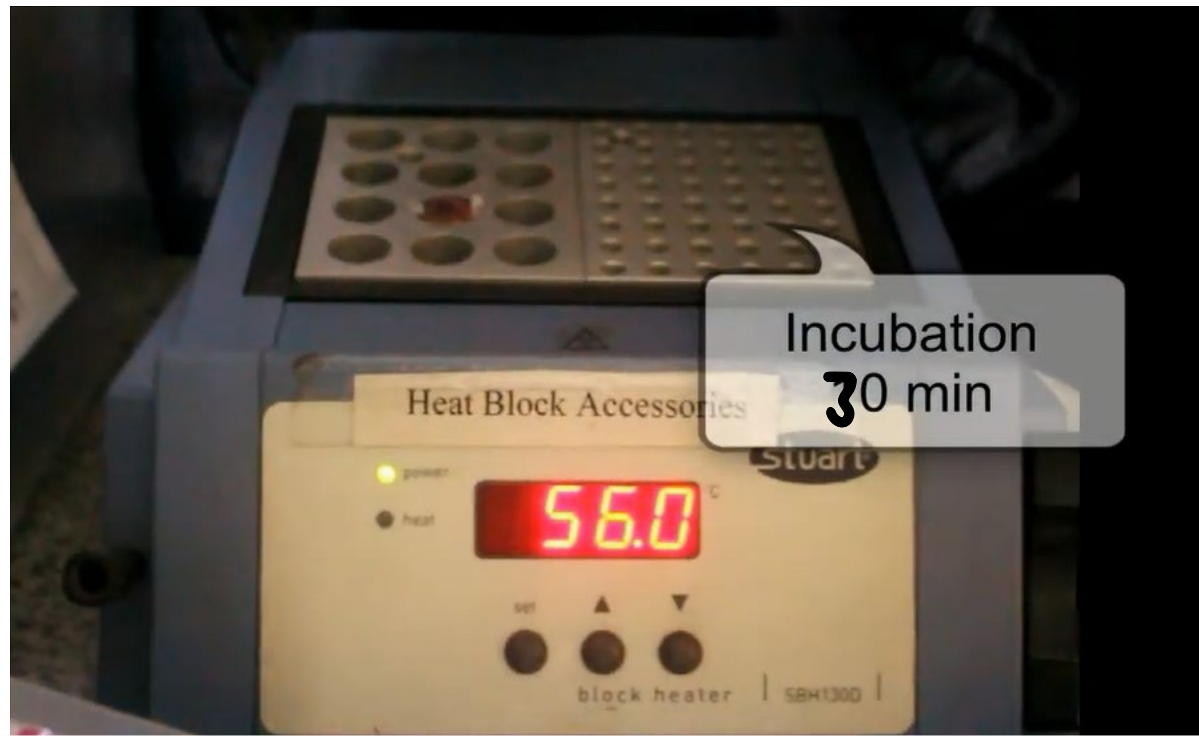


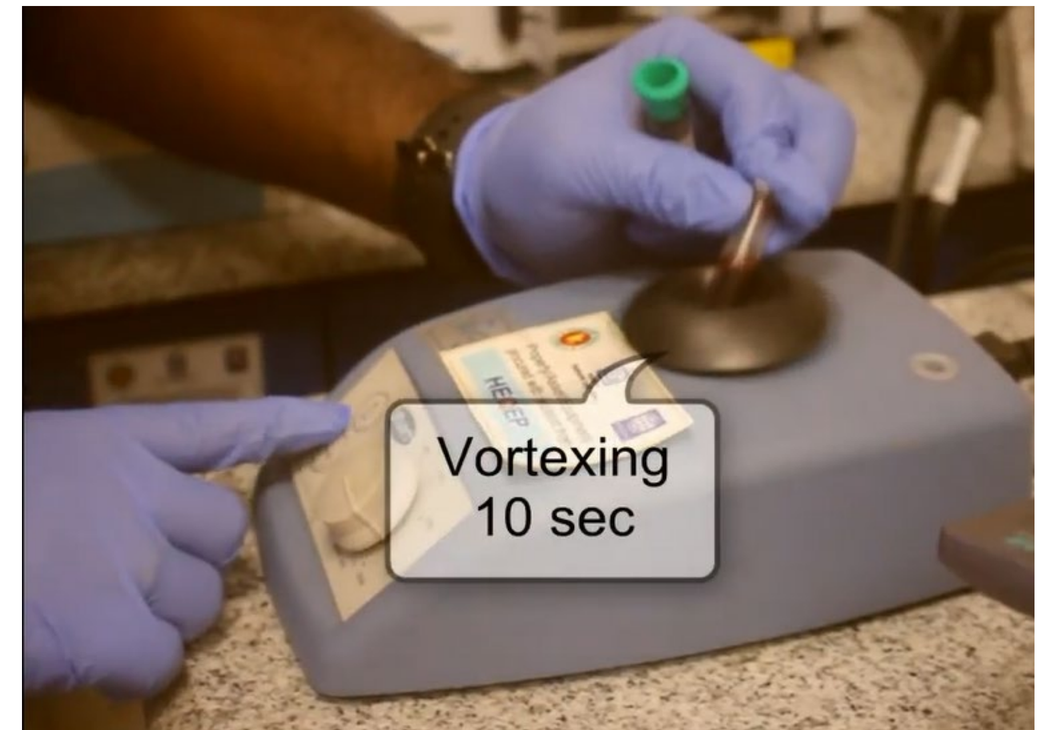
- The tube should be labeled
- For each 1.5ml micro centrifuge tube 20µl of Proteinase K (PK) Solution was dispensed then 200µl of blood was added and briefly mixed by vortex.
-



For Cells lysis, 200µl of Cell Lysis Buffer (CLD) was added to the tube and **mixed by vortex for 10 seconds.**

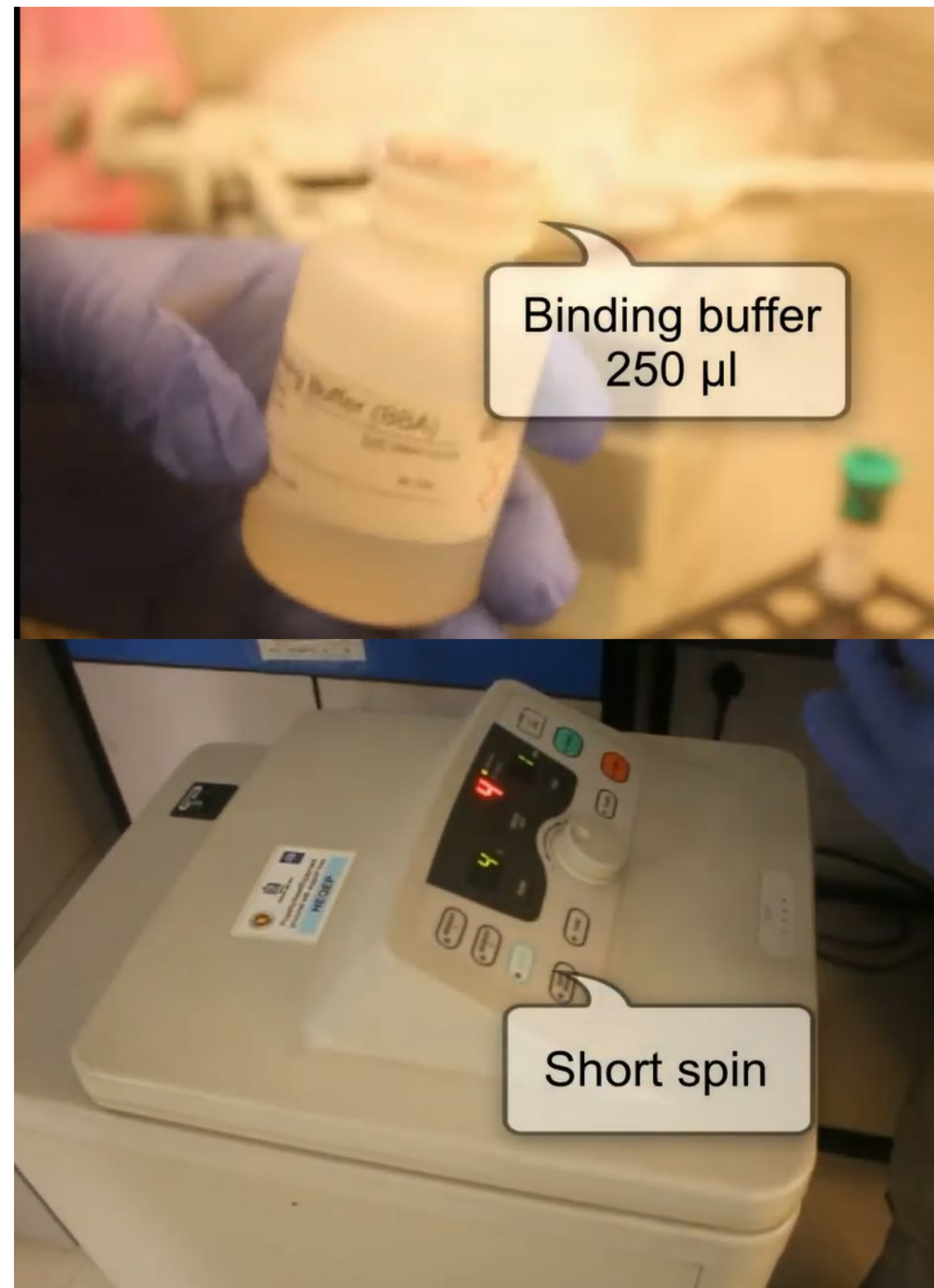
• All mixes were incubated in a water bath at 56°C for 30 minutes





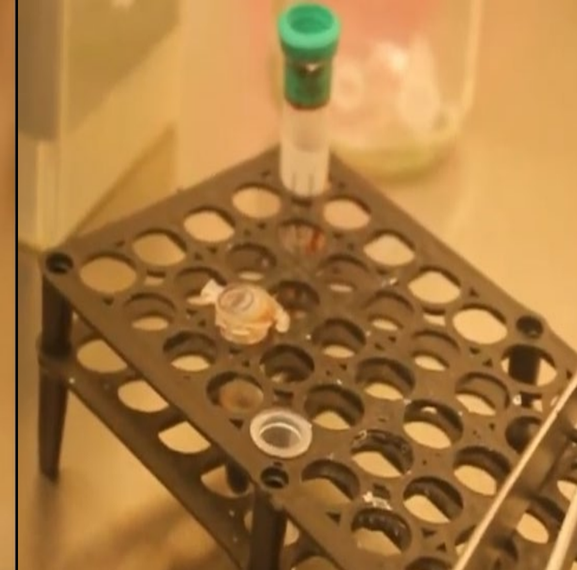
While the blood sample was incubated, a ReliaPrep™ Binding Column was placed into an empty collection tube.

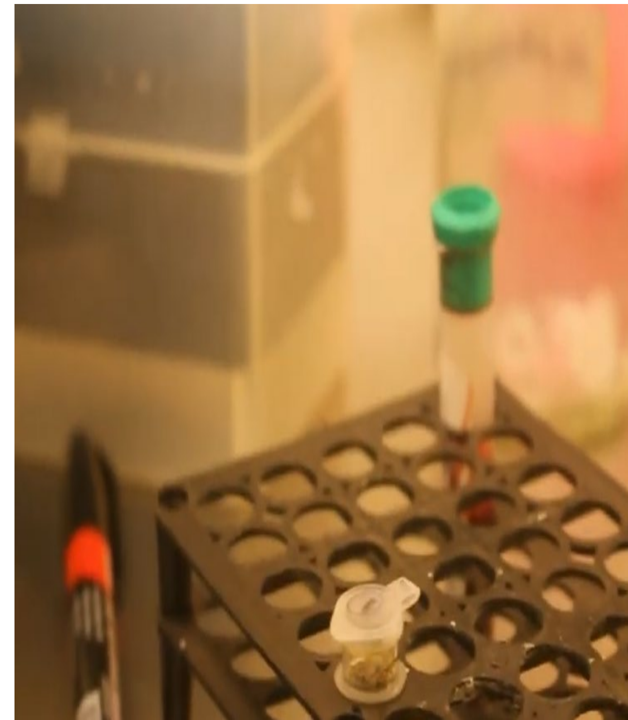
- After incubation, the tube removed from water bath and 250µl of Binding Buffer (BBA) was added and mixed by vortex for 10 seconds

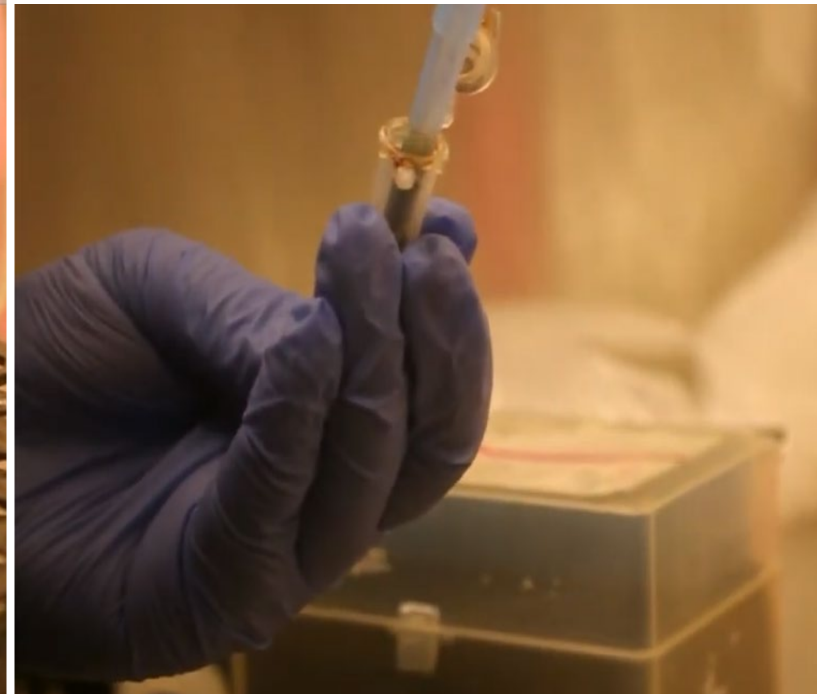


All the tube contents were transferred to the ReliaPrep™ Binding Column and centrifuged for 3 minutes at 12000rpm.

- The collection tube containing flow through was removed and discarded.
- The binding column was placed into a fresh collection tube.



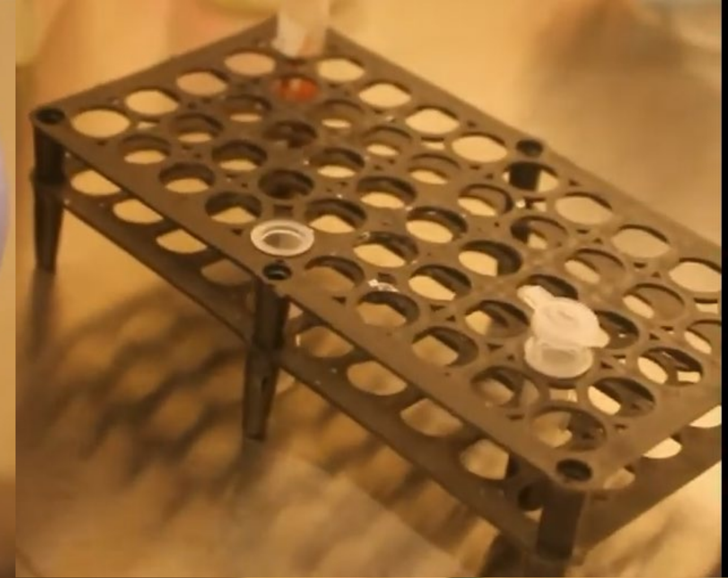


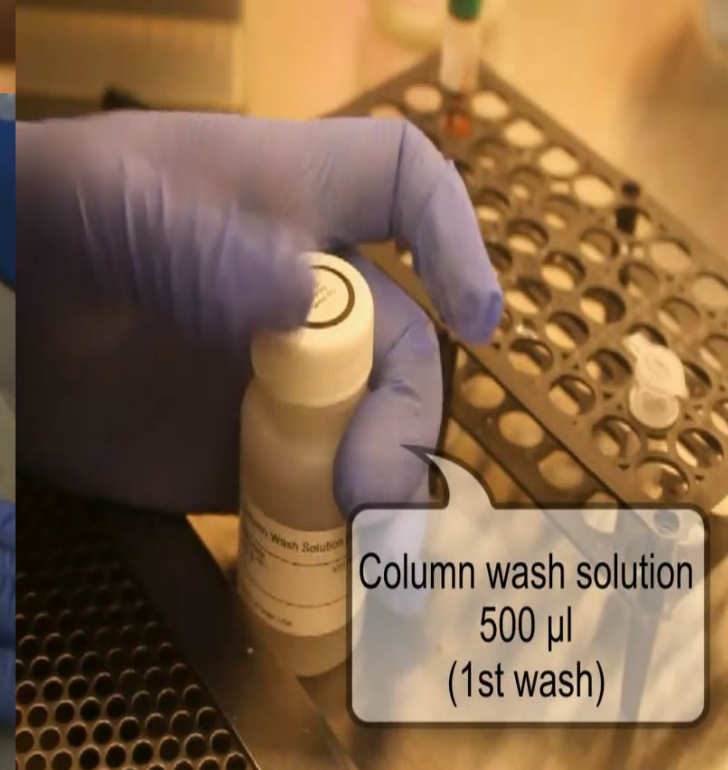


Centrifuged for 3 minutes at 12000rpm

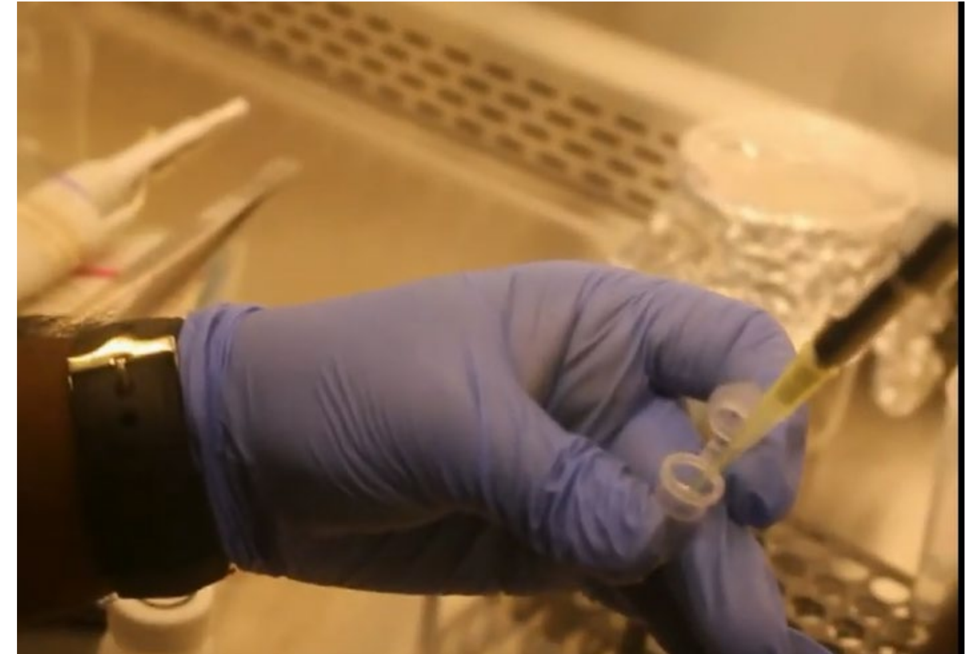
The binding column was placed into a fresh collection tube.

- For column washing 500µl of Column Wash Solution (CWD) was added to the column and centrifuged for 3 minutes at maximum speed, the flow through was discarded and this step was repeated three time.





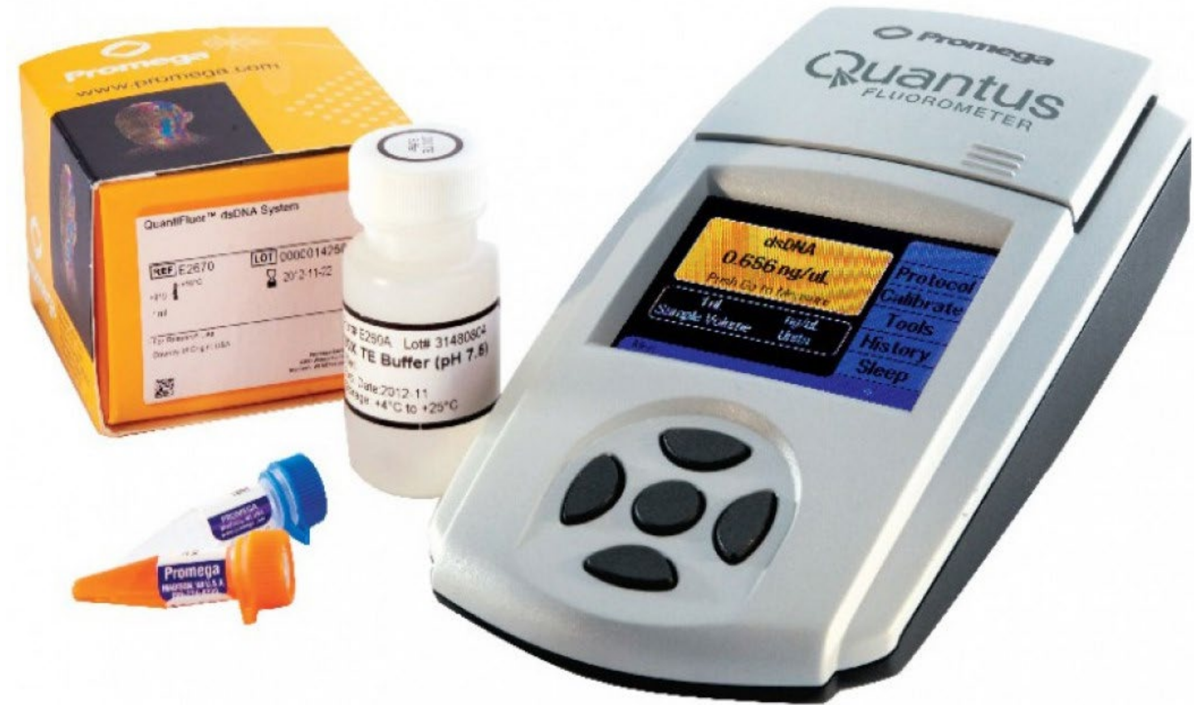
- After the washing step, the column was placed in a clean 1.5ml microcentrifuge tube, and 100µl of Nuclease-Free Water was added to the column.



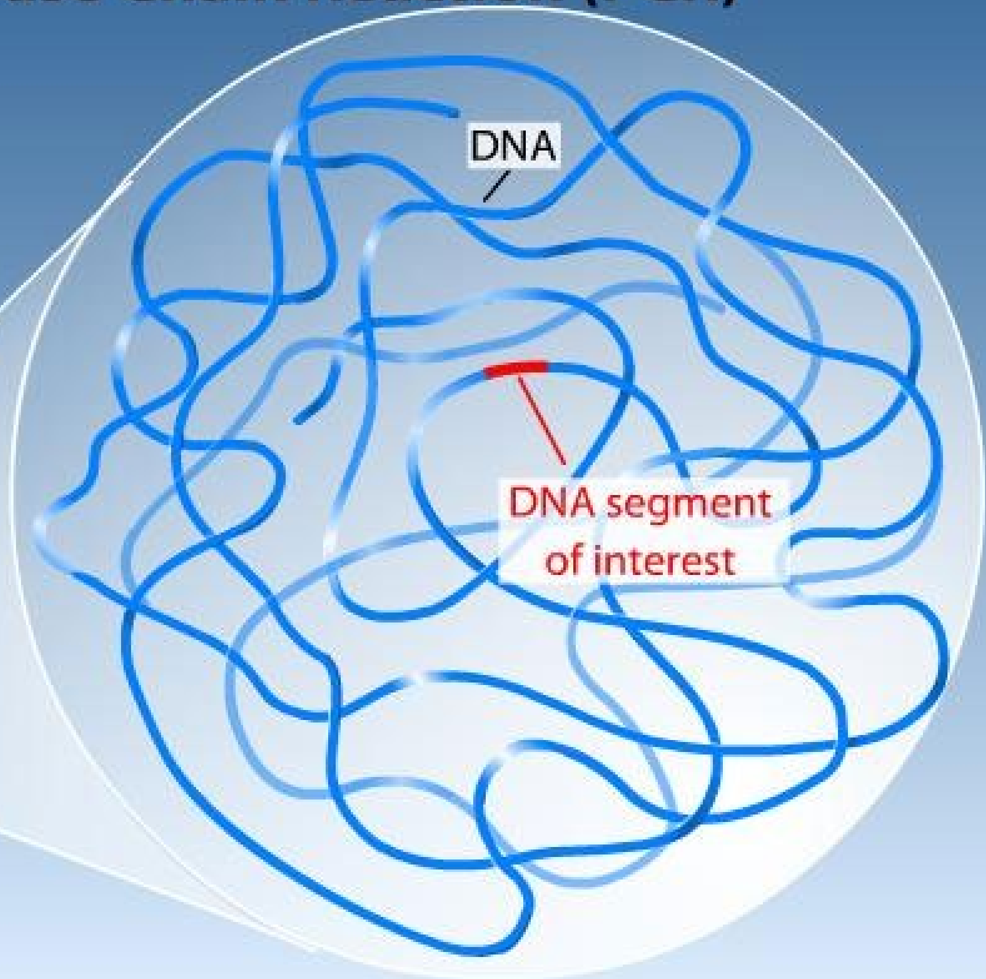
After 5 minutes, the 1.5ml tube with column centrifuge for 5 minutes at 5000rpm.

- After centrifuge, the ReliaPrep™ Binding Column was discarded, and eluate saved.

- **Quantitation of DNA**
- Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the quality of samples.
- 1 µl of **DNA** and 199 µl of diluted **Quantifluor Dye** were mixed.
- After 5min incubation at room temperature, DNA concentration values were detected.



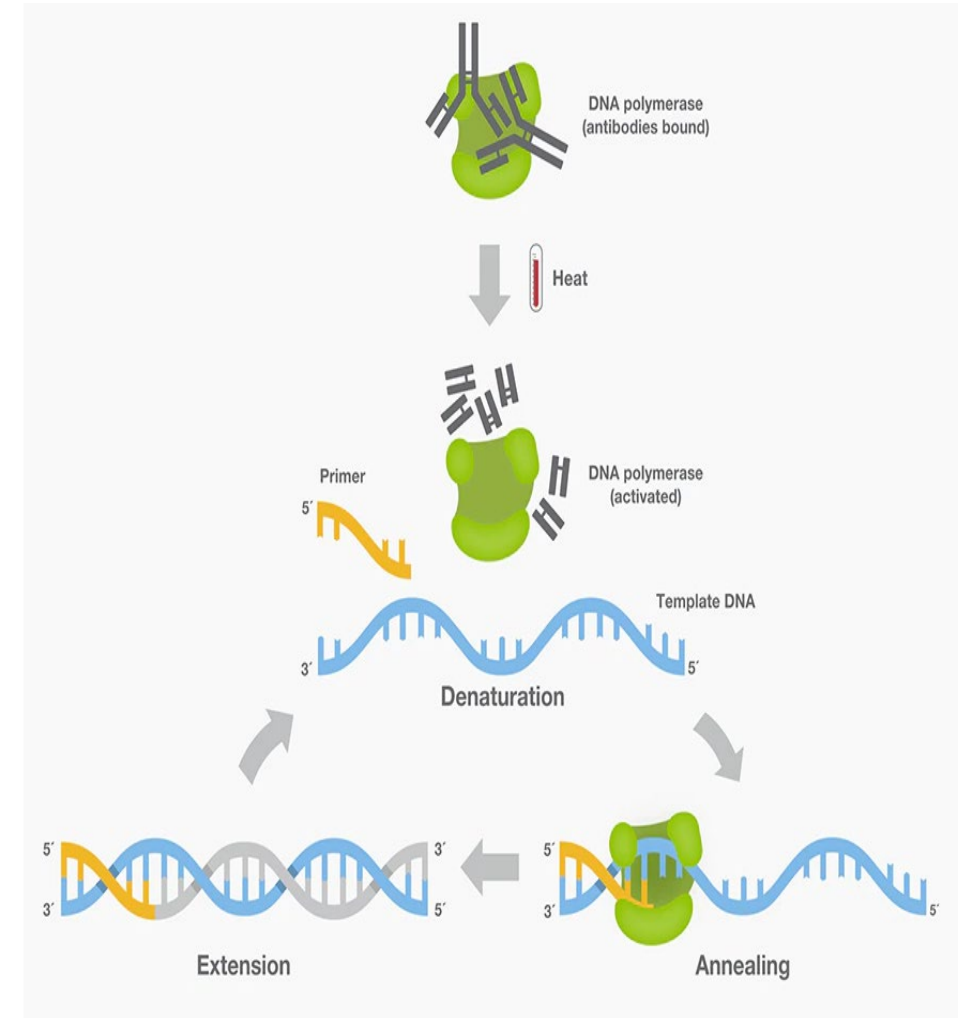
Polymerase Chain Reaction (PCR)



Polymerase chain reaction

Definition:

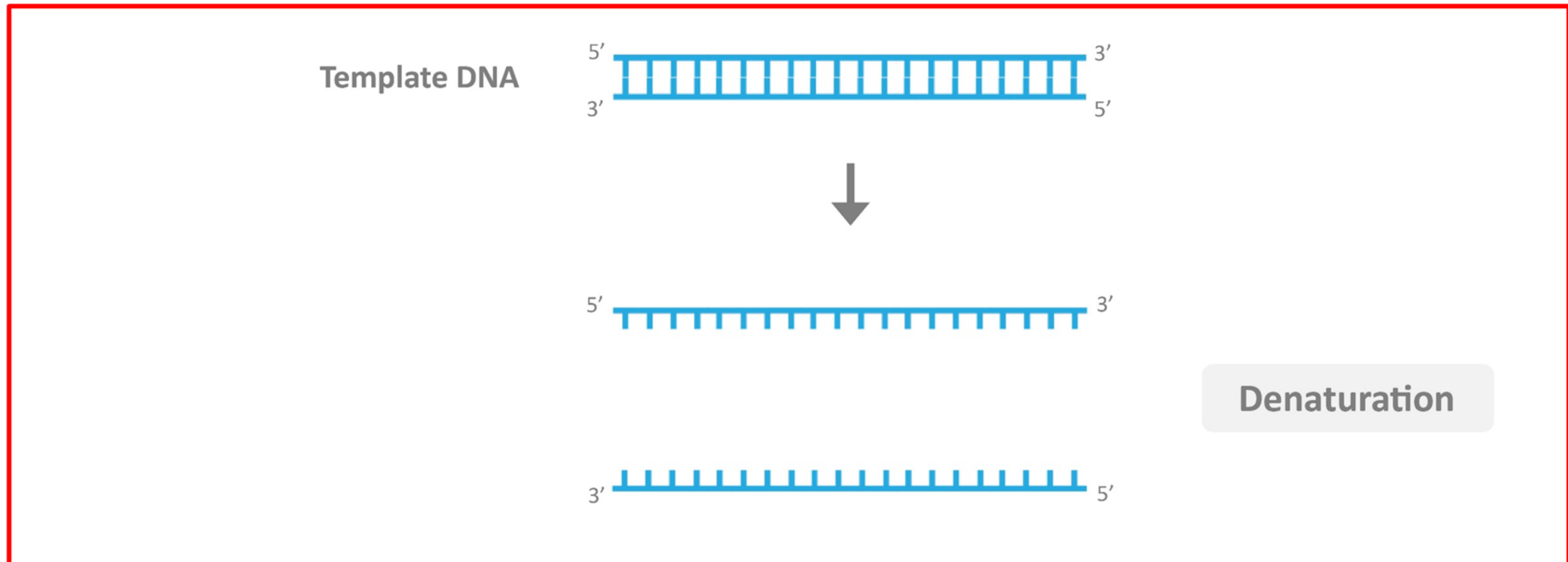
- Polymerase chain reaction (PCR) is a common laboratory technique used to make many copies (millions or billions!) of a particular region of DNA.
- This DNA region can be anything the experimenter is interested in. For example, it might be a gene whose function a researcher wants to understand, or a genetic marker used by forensic scientists to match crime scene DNA with suspects.



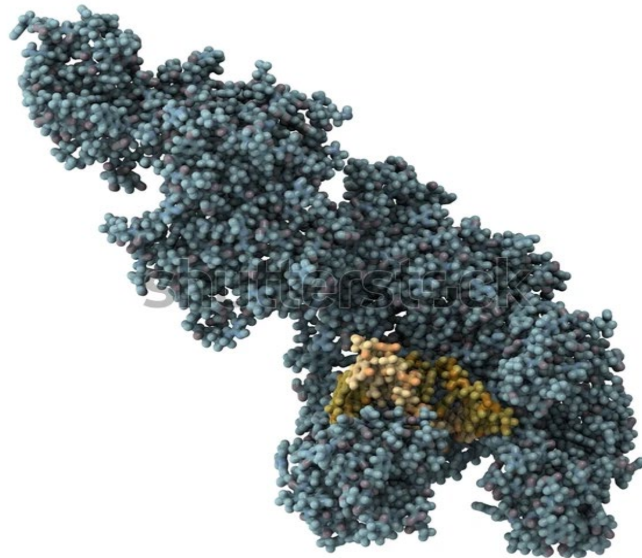
- Polymerase chain reaction (PCR) was invented by Dr. Kary Mullis in 1983.
- At that time, he was working at Cetus Corporation, one of the first biotechnology companies.
- In 1993, he received a Nobel Prize in Chemistry “for his invention of the polymerase chain reaction method.

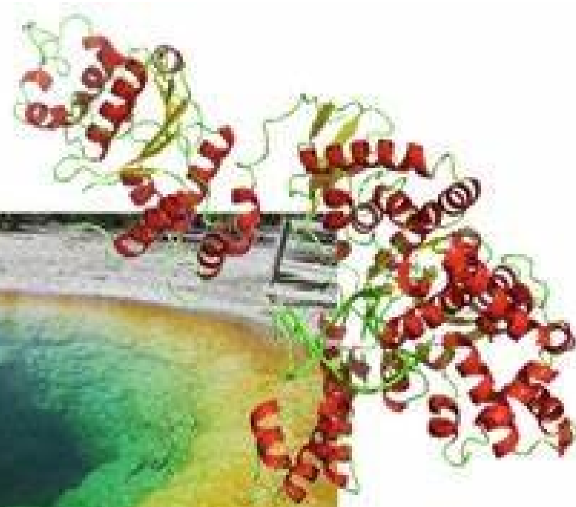
- Components of the PCR

1. DNA Template: The double stranded DNA (dsDNA) of interest, separated from the sample (genomic DNA or cDNA derived from RNA) that can be prepared from various sample



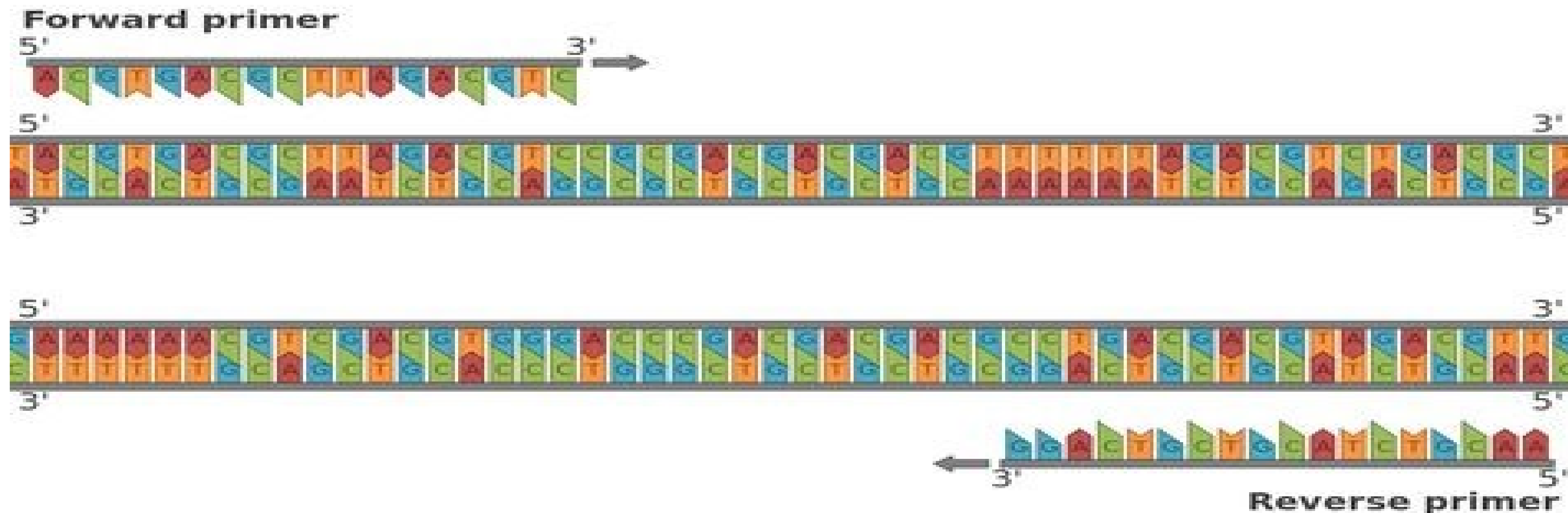
- DNA polymerase: Like DNA replication in an organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA, using existing strands as templates. The DNA polymerase typically used in PCR is called Taq polymerase, after the heat-tolerant bacterium from which it was isolated (*Thermus aquaticus*).



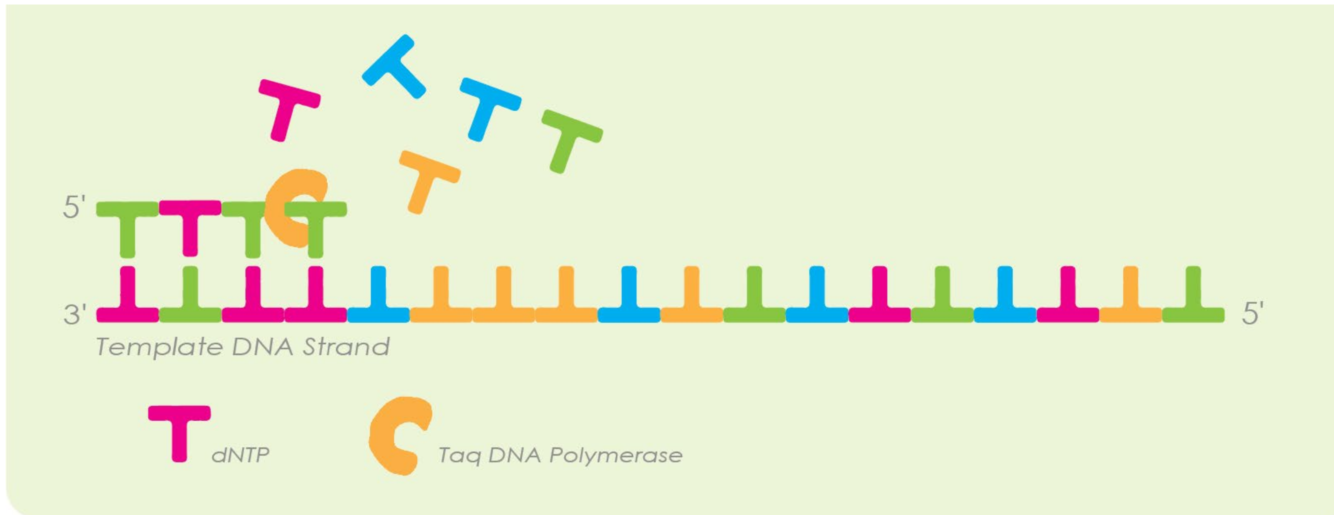


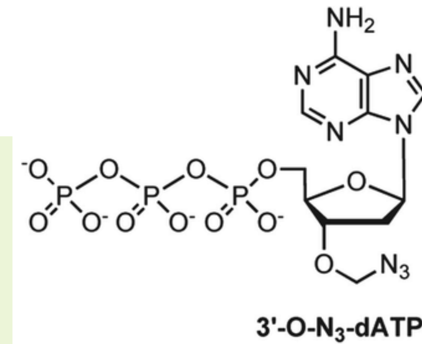
Thermus aquaticus

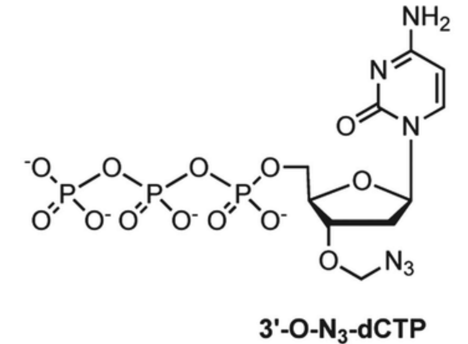
• Oligonucleotide primers: Like other DNA polymerases, Taq polymerase can only make DNA if it's given a primer, a Short sequence of single stranded DNA (often 20-30 base pairs) that provides a starting point for DNA synthesis. In a PCR reaction which are complementary to the 3’ ends of the sense and anti-sense strands of the target sequence.

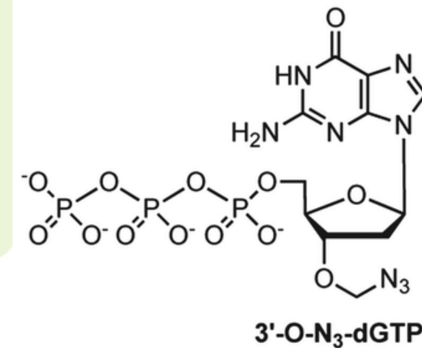


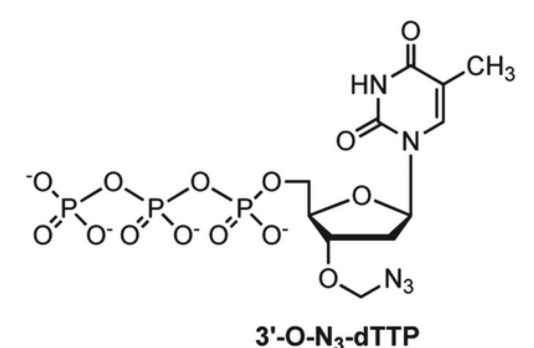
- Deoxynucleotide triphosphates: Single units of the bases A, T, G, and C (dATP, dTTP, dGTP, dCTP) provide the energy for polymerization and the building blocks for DNA synthesis.



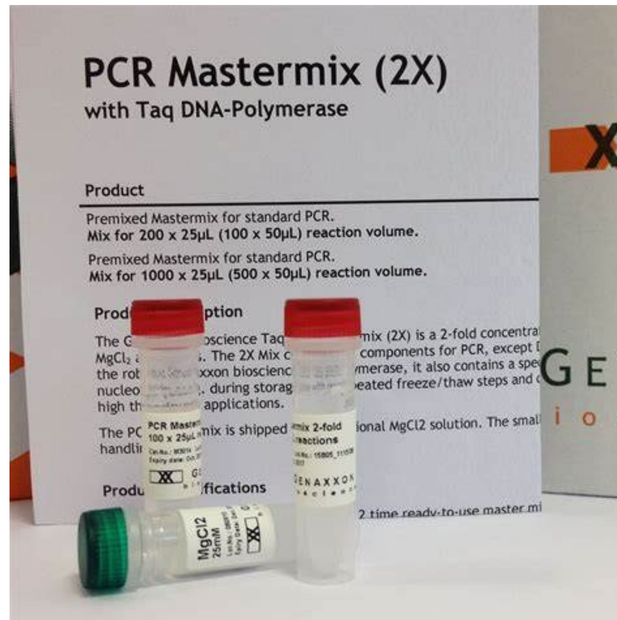


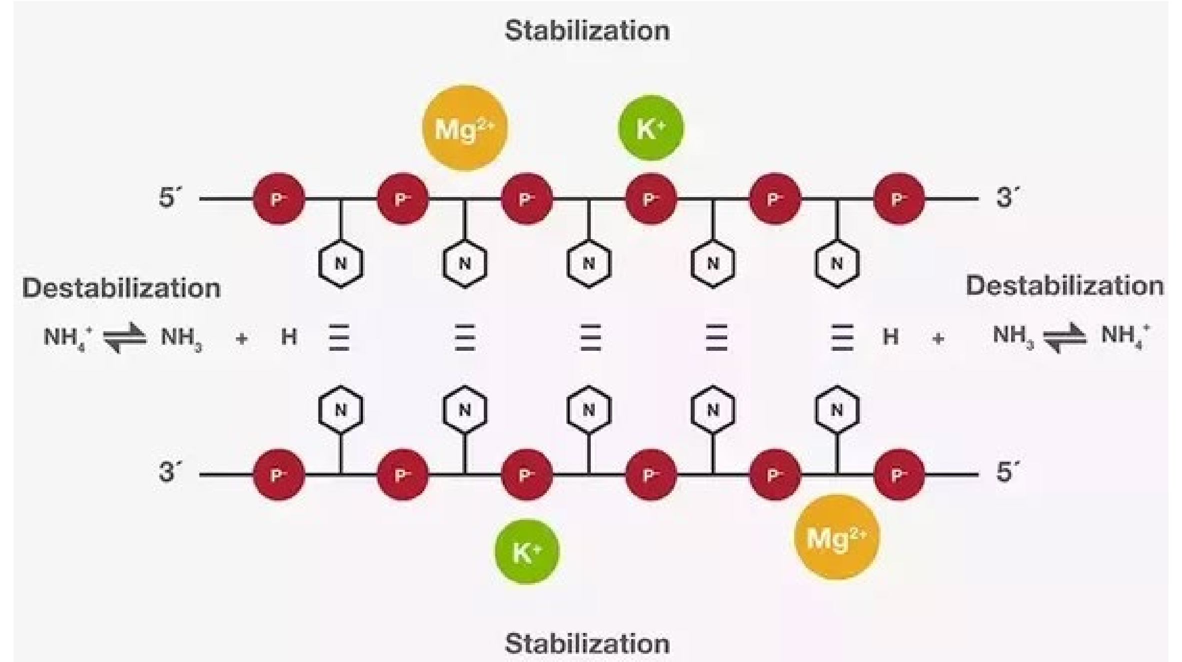






- Buffer system: Includes **magnesium and potassium** to provide the optimal conditions for DNA denaturation and renaturation; also important for polymerase activity, stability, and fidelity





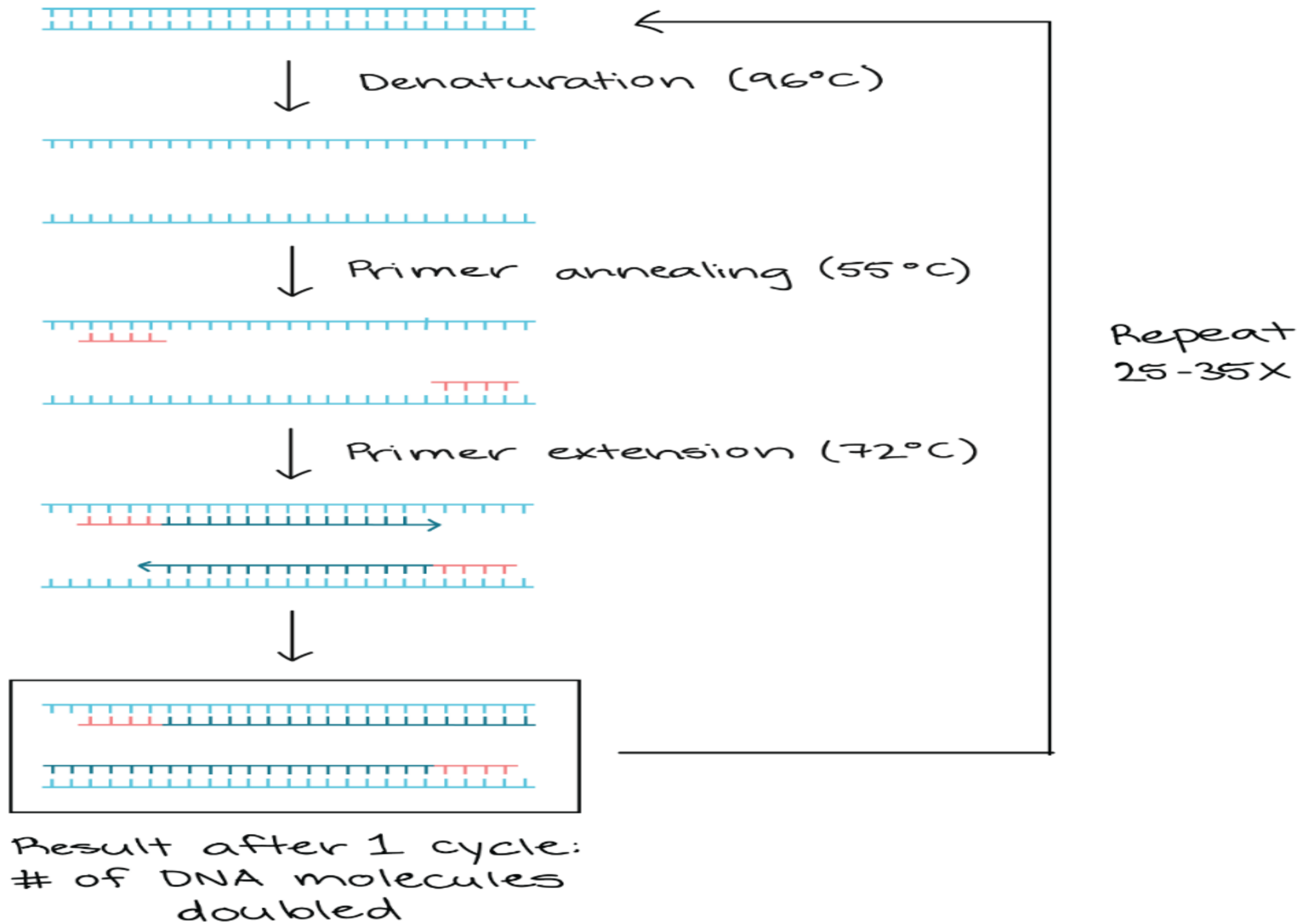
The steps of PCR

A. **Denaturation.** This step involves heating the reaction mixture to (94°C for 15-30) seconds. During this, the double stranded DNA is denatured to single strands due to breakage in weak hydrogen bonds.

B. **Annealing** The reaction temperature is rapidly lowered to (54-60°C for 20-40) seconds. This allows the primers to bind (anneal) to their complementary sequence in the single-stranded template DNA.

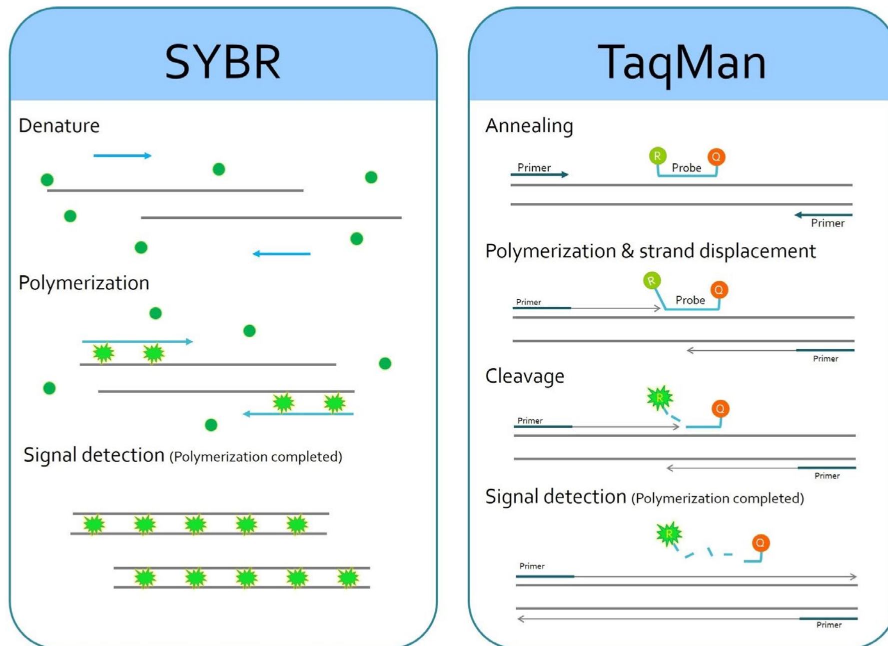
C. **Extension** Also known at elongation, this step usually occurs at 72-80°C (most commonly 72°C). In this step, the polymerase enzyme sequentially adds bases to the 3′ each primer, extending the DNA sequence in the 5′ to 3′ direction. Under optimal conditions, DNA polymerase will add about 1,000 bp/minute.

PCR steps

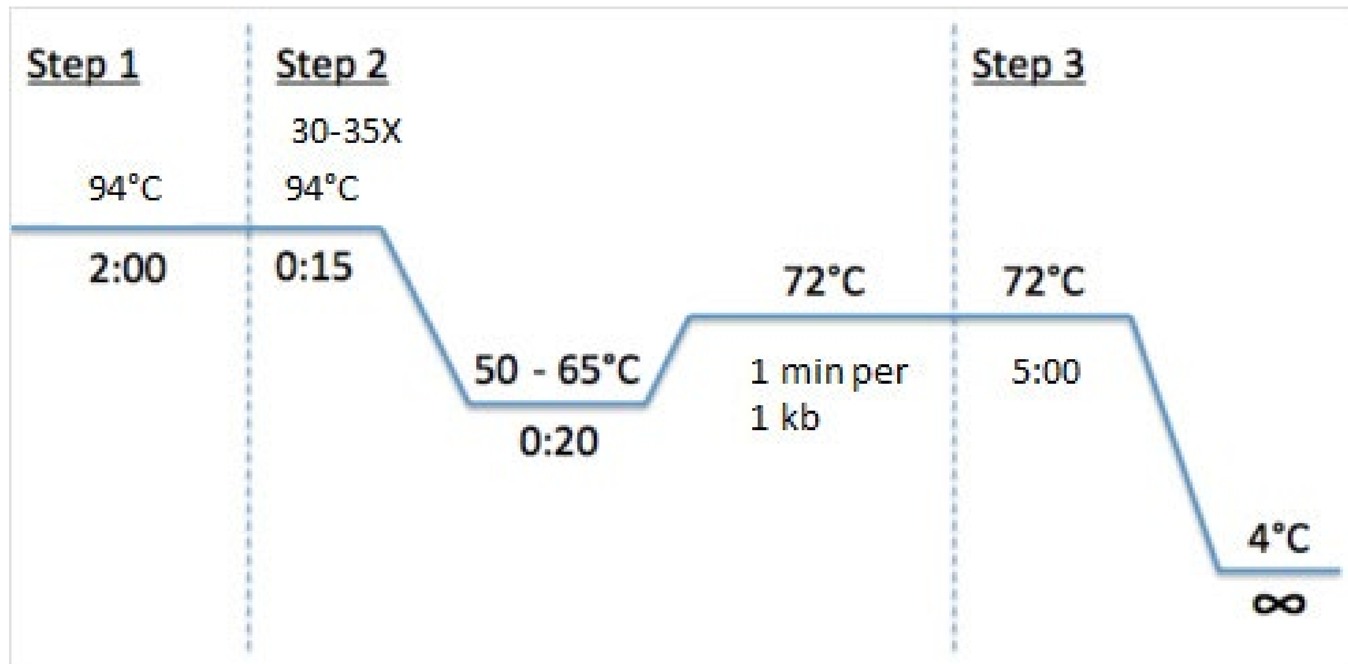


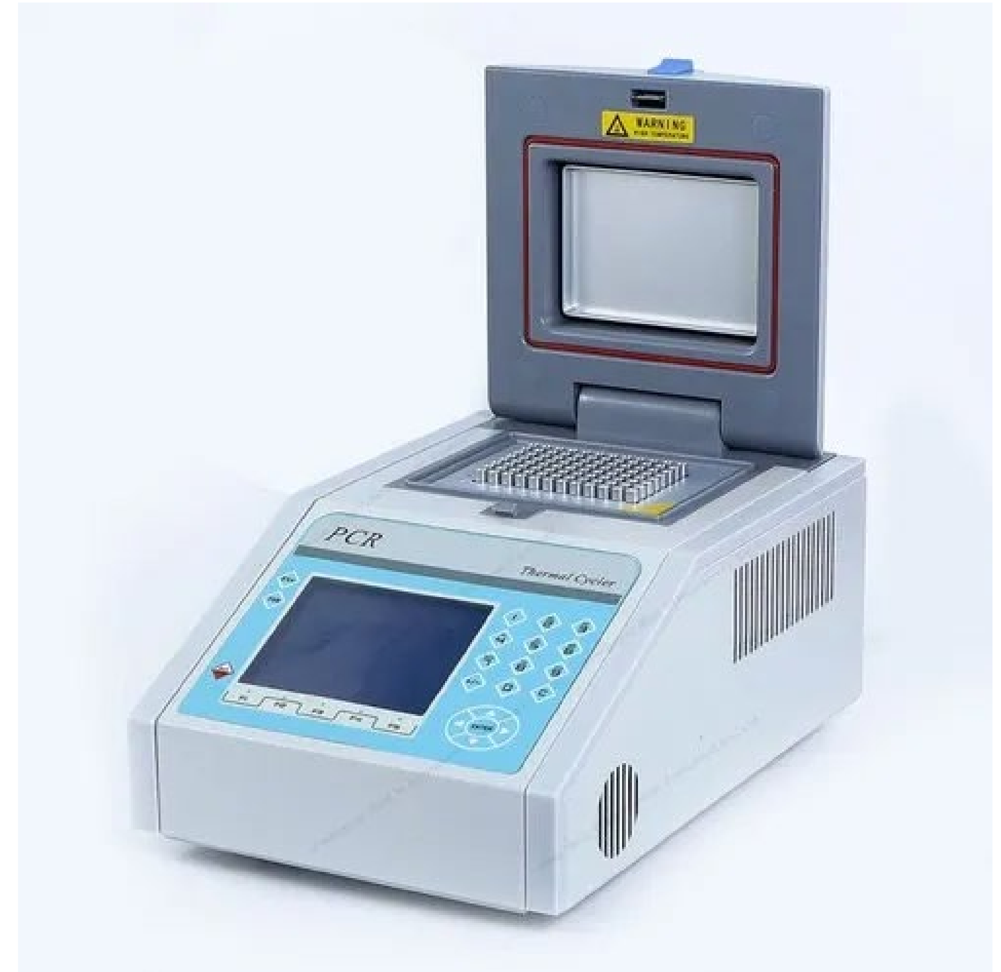
Types of PCR

1. **Quantitative PCR (Real-time PCR).** It is used to measure the specific amount of target DNA (or RNA) in a sample and used to detect the expression level of that fragment in the organism



2. **Conventional PCR:** gives qualitative expression of the presence of the gene and is used to detect the presence or absence of certain genomic fragments





What is the difference between real-time PCR and traditional PCR?

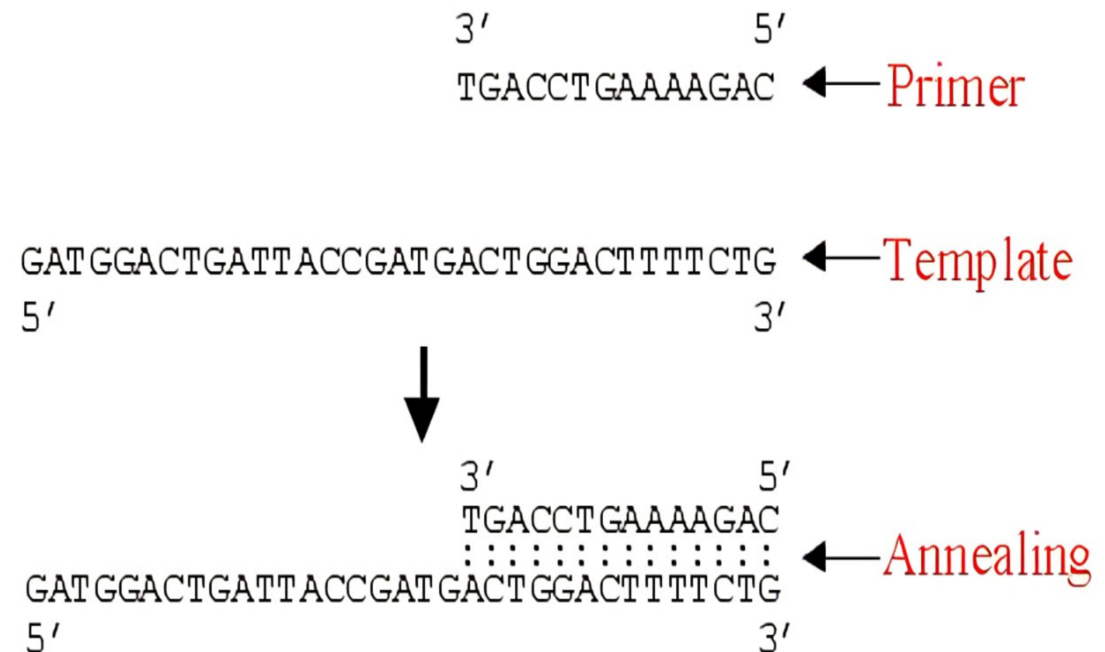
- Real-time PCR collects data at the exponential growth phase after each cycle of PCR, while traditional PCR collects data at the endpoint of the reaction.
- Conventional PCR is used to detect the presence or absence of certain genomic fragments, whereas Real-time PCR is used to detect the expression level of that fragment in the organism

Cycle number

- ❑The number of PCR cycles depends on
  - ➢ the amount of template DNA initially present in the reaction mix and
  - ➢ on the expected yield of the PCR product.
- ❑ For less than 10 copies of template DNA, 40 cycles should be performed.
- ❑If the initial quantity of template DNA is higher, 25-35 cycles are usually sufficient.

Designing The Primers

- Designing appropriate primers is essential to the successful outcome of a PCR experiment.
- When designing a set of primers to a specific region of DNA desired for amplification, one primer should anneal to the plus strand, (also known as the sense strand), and the other primer should complement the minus strand, (antisense or template strand).

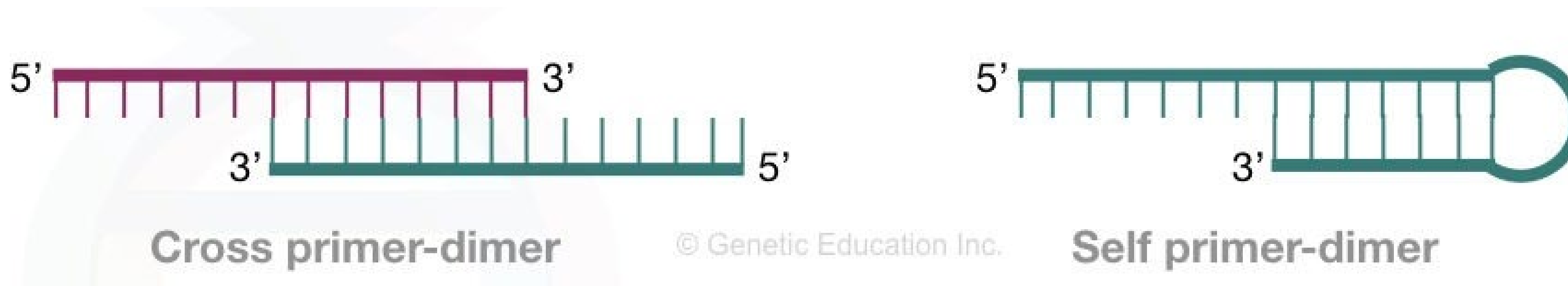


Characteristics that should be considered when designing primers

1. Primer length should be 15-30 nucleotide residues (bases). Longer primers provide higher specificity.
2. Optimal G-C content should range between 40-60%.
3. The 3' end of primers should contain a G or C in order to clamp the primer and prevent the "breathing" of ends, increasing priming efficiency.

DNA "breathing" occurs when ends do not stay annealed but fray or split apart. The three hydrogen bonds in GC pairs help prevent breathing but also increase the melting temperature of the primers.

4. The 3' ends of a primer set, which includes a plus-strand primer and a minus-strand primer, should not be complementary to each other, nor can the 3' end of a single primer be complementary to other sequences in the primer. These two scenarios result in the formation of primer dimers and hairpin loop structures, respectively.



5. Optimal melting temperatures (T_m) for primers range between 52-58 °C, although the range can be expanded to 45-65 °C. The final T_m for both primers should differ by no more than 5 °C.

6. Di-nucleotide repeats (e.g., GCGCGCGCGC or ATATATATAT) or single base runs (e.g., AAAAA or CCCCC) should be avoided as they can cause slipping along the primed segment of DNA and or hairpin loop structures to form. If unavoidable due to the nature of the DNA template, then only include repeats or single base runs with a maximum of 4 bases.

How to design a primer?

There are many computer programs designed to aid in designing primer pairs. *NCBI Primer design tool*

http://www.ncbi.nlm.nih.gov/tools/primer-blast/

and *Primer3* European research infrastructure for biological information.

http://frodo.wi.mit.edu/primer3/

are recommended websites for this purpose.

National Library of Medicine
National Center for Biotechnology Information

Log in

Primer-BLAST

A tool for finding specific primers

Finding primers specific to your PCR template (using Primer3 and BLAST).

Primers for target on one template | Primers common for a group of sequences

Retrieve recent results | Publication | Tips for finding specific primers | Save search parameters | Res

PCR Template

Enter accession, gi, or FASTA sequence (A refseq record is preferred) ? Clear

Range ? Clear

	From	To
Forward primer		
Reverse primer		

Or, upload FASTA file Choose File No file chosen

Primer Parameters

Use my own forward primer (5'->3' on plus strand) ? Clear

Use my own reverse primer (5'->3' on minus strand) ? Clear

	Min	Max
PCR product size	70	1000
# of primers to return	10	

Primer3web version 4.1.0 - Pick primers from a DNA sequence.	disclaimer	code
	cautions	

Select the Task for primer selection generic

Template masking before primer design (available species)			
Select species	Example: Mus musculus	Nucleotides to mask in 5' direction	1
Primer failure rate cutoff	< 0.1	Nucleotides to mask in 3' direction	0

Paste source sequence below (5'->3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a Mispriming Library (repeat library) NONE

☑ Pick left primer, or use left primer below	☐ Pick hybridization probe (internal oligo), or use oligo below	☑ Pick right primer, or use right primer below (5' to 3' on opposite strand)

Pick Primers | Download Settings | Reset Form

Sequence Id — A string to identify your output.

Targets — E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the source sequence with [and]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.

Estimation of the melting and annealing temperatures of primer:

If the primer is shorter than 25 nucleotides, the approx. melting temperature (Tm) is calculated using the following formula:

Tm= 4 (G + C) + 2 (A + T)

G, C, A, T - number of respective nucleotides in the primer.

- Annealing temperature should be approx. 5°C lower than the melting temperature.
- If the primer is longer than 25 nucleotides, the melting temperature should be calculated using specialized computer programs where the interactions of adjacent bases, the influence of salt concentration, etc. are evaluated.

Primer preparation

These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in nuclease-free water to give a final concentration of 100pmol/μl as a stock solution. A working solution of these primers was prepared by adding 10μl of primer stock solution (stored at freezer -20 C) to 90μl of nuclease-free water to obtain a working primer solution of 10pmol/μl.

Primer optimization

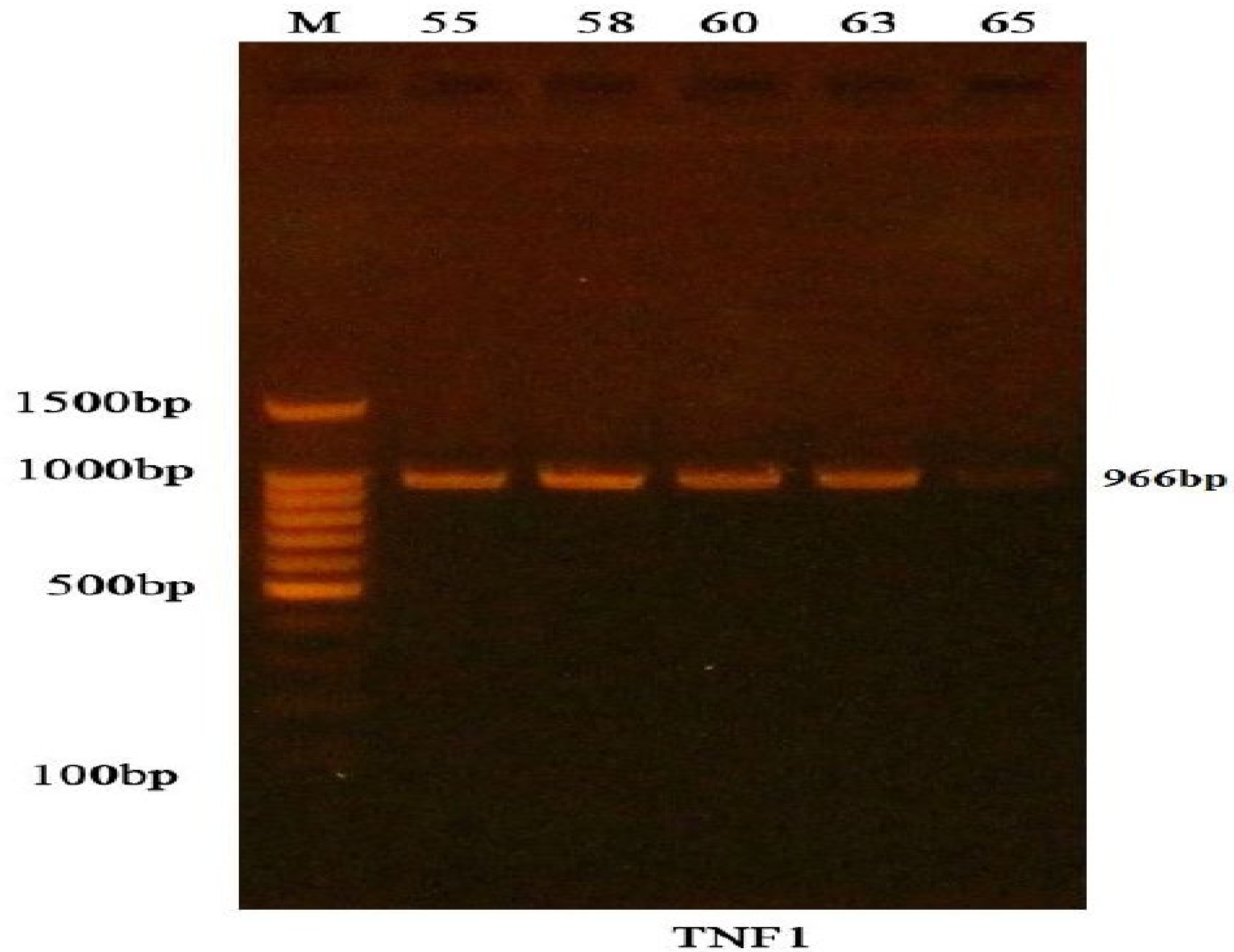
➢ Because of the differences in sequence, length, and composition of the primers, it is often difficult to have similar annealing temperatures

- Reaching the optimum Ta is critical for **reaction specificity**, as non-specific products may be formed as a result of **non-optimal Ta.**

- **HOW?**

➔Optimization done by applying temperature gradient PCR, where **PCR carried with different Ta starting at 5 °C** below the lowest calculated melting temperature (Tm) of the primer pair.

- Analyse the results using 2% agarose gel, and determine the optimum Ta.



Preparation

- When setting up a PCR experiment, it is important to be prepared. Wear gloves to avoid contaminating the reaction mixture or reagents.
- Arrange all reagents needed for the PCR experiment in a freshly filled ice bucket and let them thaw completely before setting up a reaction.
- Keep the reagents on ice throughout the experiment.

- Start by making a table of reagents that will be added to the reaction mixture.
- Next, label PCR tube(s) with the ethanol-resistant marker.

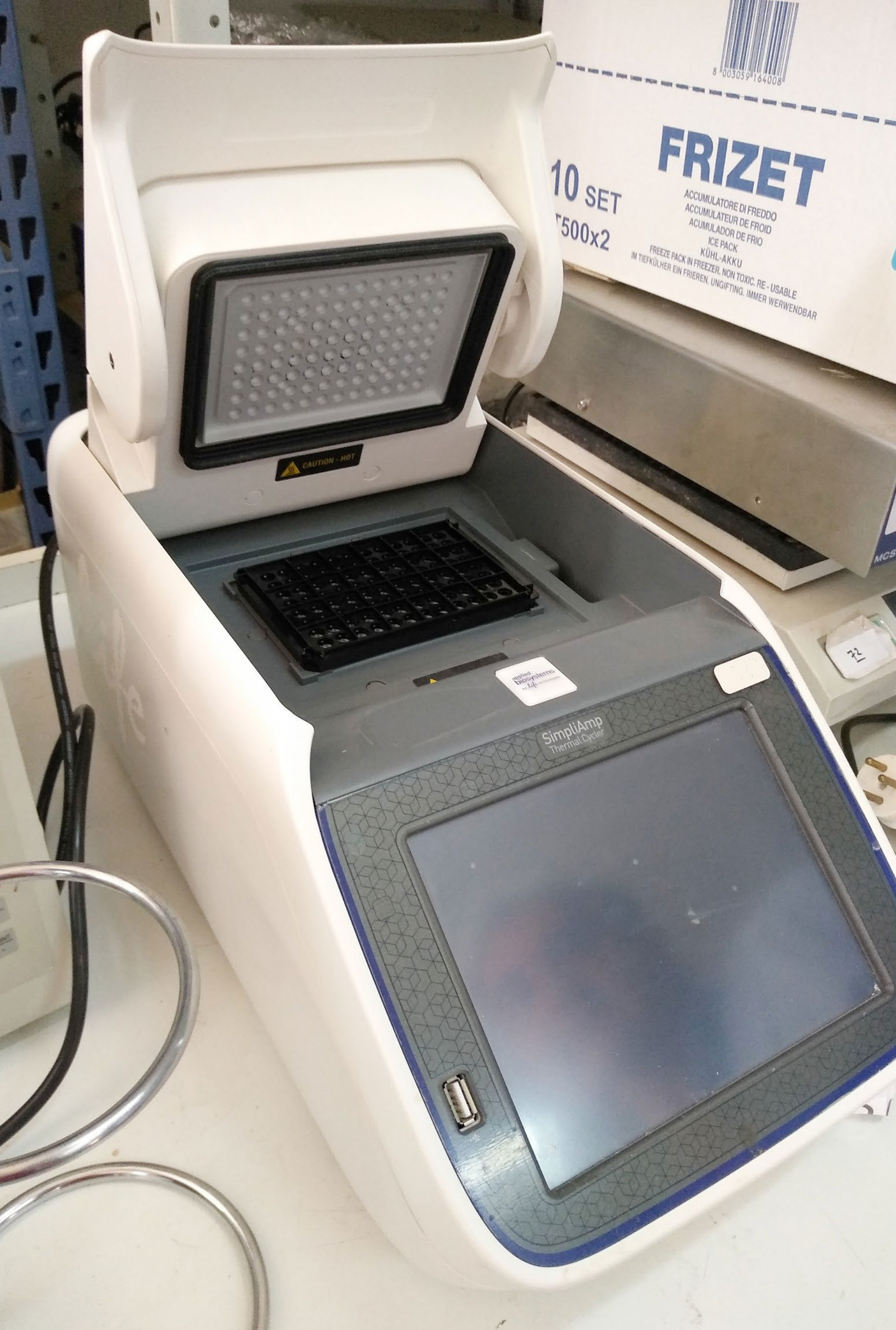
Standard concentrations of PCR components

Component	Final concentration
Taq polymerase	0.5–2.0 units, ideally 1.25 units.
Deoxy-nucleotides (dNTPs)	Typical concentration is 200 μM of each dNTP.
Magnesium Concentration	1.5-2.0 mM is optimal for Taq DNA Polymerase.*
Forward Primers	Typically 0.1-0.5 μM .
Reverse Primer	Typically 0.1-0.5 μM.
DNA Template	1ng–1μg of genomic templates.

PCR tube content

PCR amplifications were performed with 25µl volumes containing 12.5µl GoTaq Green Master Mix (2X); 1µl for each primer (10pmol); 7.5µl nuclease free water and 3µl of template DNA.

Master mix components	**Stock**	**Unit**	**Final**	**Unit**	**Volume**	
					1 Sample	80.05 Samples
Master Mix	2	X	1	X	12.5	1000.6
Forward primer	10	µM	1	µM	1	80.1
Reverse primer	10	µM	1	µM	1	80.1
Nuclease Free Water					7.5	600.4
DNA		ng/µl		ng/µl	3	
Total volume					25	
Aliquot per single rxn	22µl of Master mix per tube and add 3µl of Template					

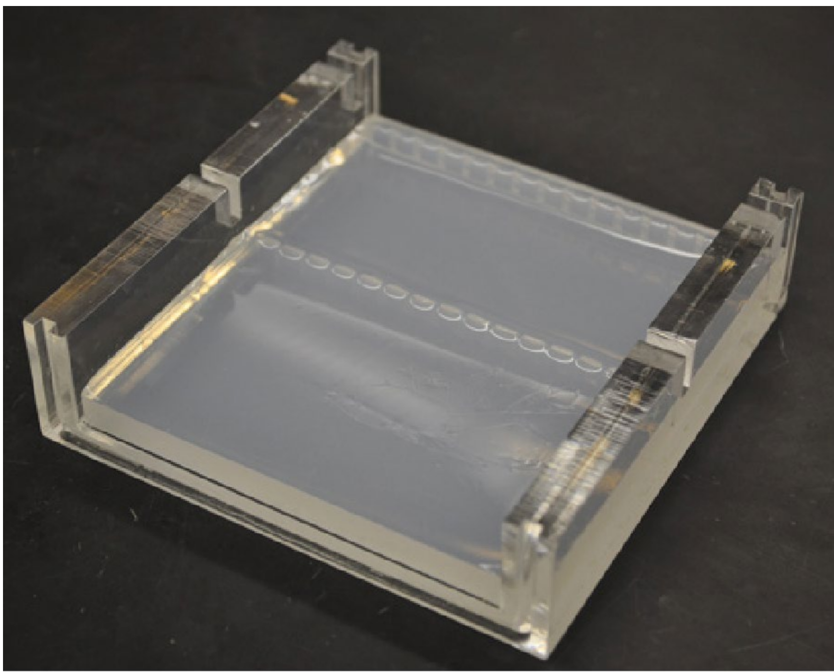


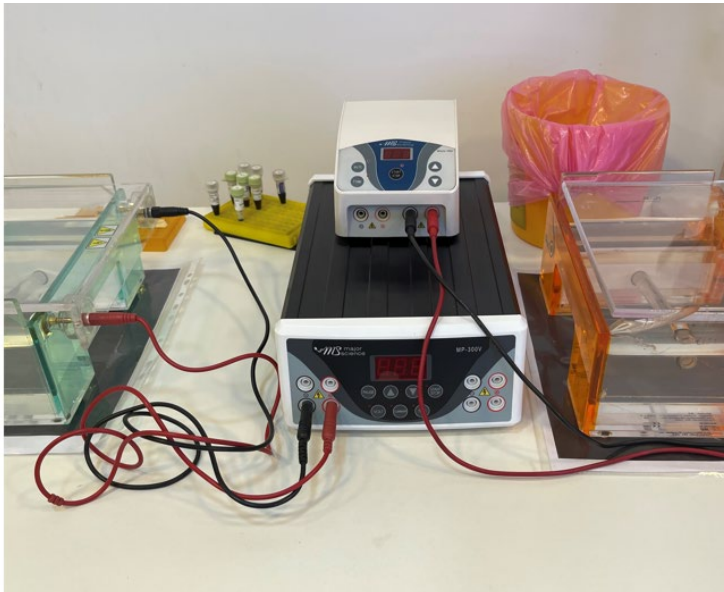
PCR Cycling

Stage	Step	Temperature	Duration	Cycle
Stage 1	Initial denaturation	94–97 °C	3 min	x1
Stage 2	Denaturation	94–97 °C	30 sec	x (25-35)
	Annealing	50-65 °C	30 sec	
	Elongation	72-80 °C	30-60 sec	
Stage 3	Final elongation	75-80 °C	5-7 min	x1

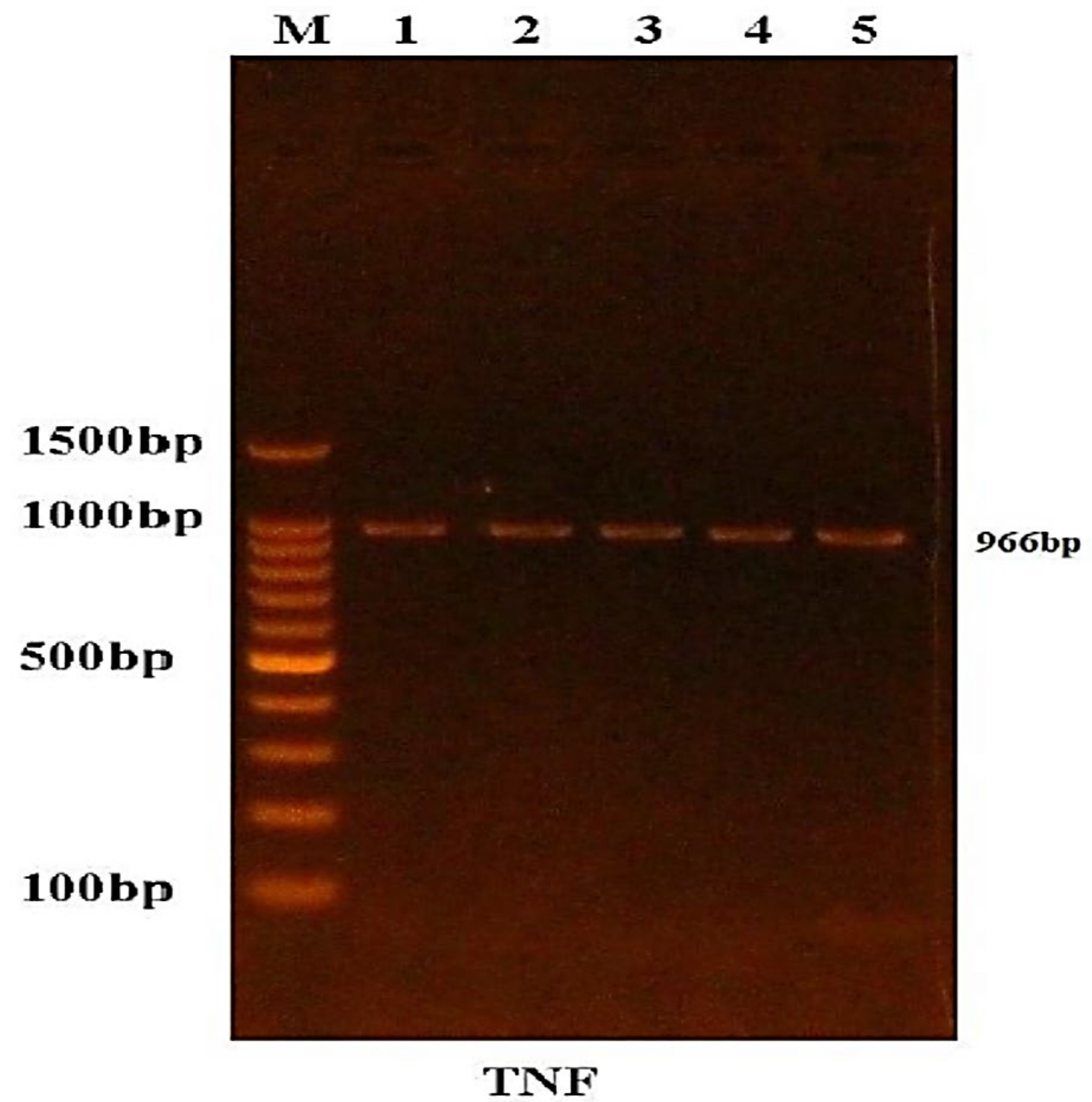
PCR Program

<table>
<tr><th>Steps</th><th>°C</th><th>m: s</th><th>Cycle</th></tr>
<tr><td>Initial Denaturation</td><td>95</td><td>05:00</td><td>1</td></tr>
<tr><td>Denaturation</td><td>95</td><td>00:30</td><td rowspan="3">30</td></tr>
<tr><td>Annealing</td><td>60</td><td>00:30</td></tr>
<tr><td>Extension</td><td>72</td><td>01:00</td></tr>
<tr><td>Final extension</td><td>72</td><td>07:00</td><td rowspan="2">1</td></tr>
<tr><td>Hold</td><td>10</td><td>10:00</td></tr>
</table>





- **Agarose Gel Electrophoresis**
- After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.


M
1
2
3
4
5
1500bp
1000bp
500bp
100bp
966bp
TNF

The End