INTRODUCTION TO REVERSE TRANSCRIPTION PCR (RT-PCR)

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Objective of PCR

To provide a solution to one of the most pressing problems facing biology at the time: the replication of DNA.

To eliminate the use of radioisotopes or toxic chemicals.

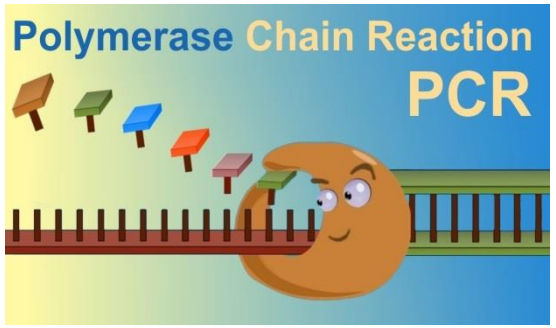
To amplify a given region of DNA (region of interest).

Polymerase Chain Reaction (PCR) conceptualized in 1983 by American biochemist Dr Kary Banks Mullis

Nobel Prize-winner in chemistry in 1993,
for the invention of the PCR.

Theory of PCR

- The Driver of PCR is the **Polymerase** enzyme
- A polymerase will synthesize a complementary sequence of bases to any single strand of DNA **providing it has a double stranded starting point**

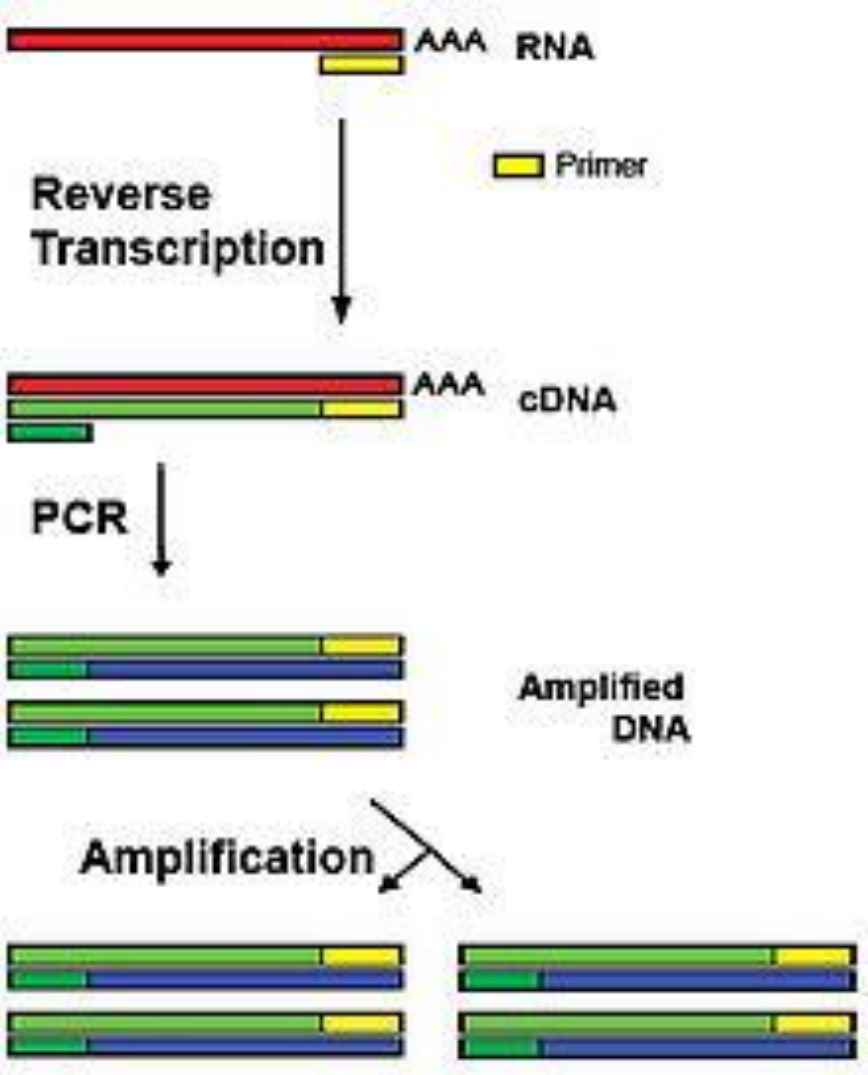


- Any gene can be specifically amplified by the polymerase in a mixed DNA sample by adding small pieces of complementary DNA
- These small pieces of DNA are known as **primers** because they prime the DNA sample ready for the polymerase to bind and begin copying the gene of interest.
- During a PCR, **changes in temperature** are used to control the activity of the polymerase and the binding of primers.

Reverse transcription polymerase chain reaction (RT-PCR)

1. RT-PCR refers to PCR that uses product of an Reverse Transcription (RT) reaction as template
2. A variant of polymerase chain reaction (**PCR**)
3. A technique commonly used in molecular biology to detect **RNA** expression
4. **RT-PCR** is often confused with real-time polymerase chain reaction (**qPCR**)
5. RT-PCR is used to **qualitatively** detect gene expression through creation of complementary DNA (**cDNA**) transcripts from RNA
6. **qPCR** is used to quantitatively measure the amplification of DNA using fluorescent dyes

RT-PCR Principle



Some priming methods for generating first strand cDNA:
Simplicity

1. Oligo(dT)-based priming: RNA with a poly(A) tail
2. Random hexamer priming: fragmented RNA (<500 b)
3. Gene-specific priming: low abundance RNA

RT-PCR is also used in gene cloning, transcriptomics;

One-Step vs Two-Step RT-PCR

1. RNA
2. RNA and PCR Primers
3. Reverse Transcriptase
4. Buffer reagents
5. Taq polymerase

RT-PCR

One-Step
RT-PCR

- Simplicity
- Convenience
- Minimizes contamination
- Not flexible

1. RNA
2. RNA Primers
3. Reverse Transcriptase
4. Buffer reagents

Reverse Transcription

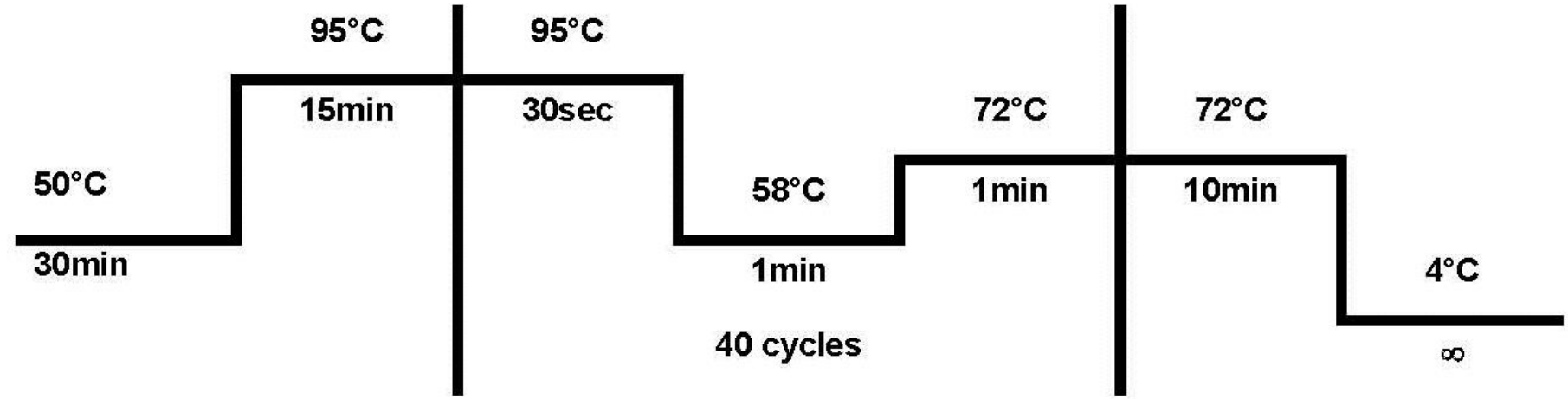
cDNA

[+] PCR Primers
[+] Taq

Two-Step
RT-PCR

cDNA can be re-used

One-Step Cycling Conditions



Reverse Transcription

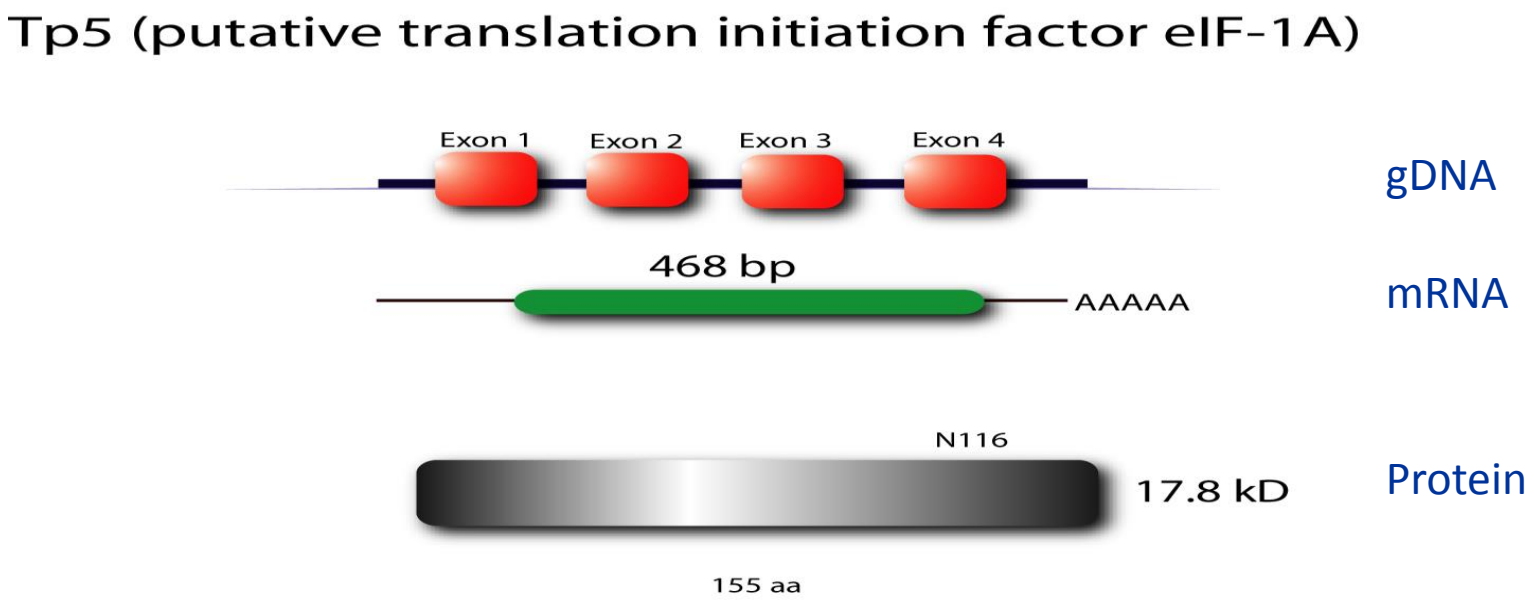
PCR

RT-PCR: Contamination gDNA

All RNA Isolation Methods Yield RNA Containing Residual Genomic DNA

- Should avoid detecting DNA contamination*;*
- Remove DNA:
  - DNase I treatment then phenol:CHCl3 extraction;
  - Lithium chloride (2.5M) precipitation then ethanol wash (but not very efficient for small RNA (<200 nt) and dNTPs; LiCl does not efficiently precipitate DNA, protein or carbohydrate;
  - Poly(A) RNA purification;
- Use primers that span an intro/exon boundary;

Use primers that span an intro/exon boundary

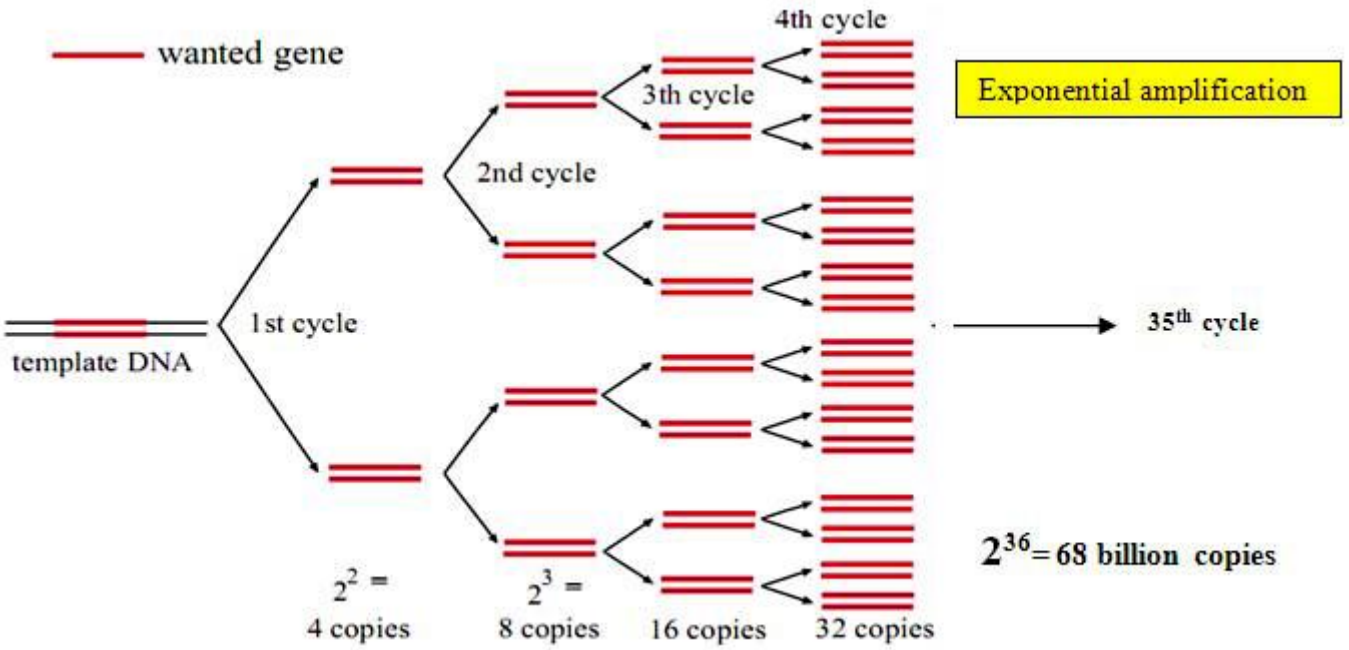


Principle of PCR 1

1. The reaction's temperature is raised to 95^{o}C to denature all double stranded DNA into single strands: **Denaturation**
2. The temperature is then lowered to 55-65^{o}C to allow the primers to bind to your gene of interest: **Annealing**
3. The optimal temperature for the Taq to operate is 72^{o}C. So the temperature is raised to 72^{o}C: **Extension**
   There are now twice as many copies of your gene of interest as when you started
4. To do its job the Taq requires a supply of DNA building blocks, i.e., the nucleotides: A, T, C and G

Principle of PCR 2

1. The cycle of changing temperatures (95°C, 55°C and 72°C) is then repeated and two copies become four. Another cycle and four become eight, up to 30-35 cycles. After 'n' cycles = $2^{(n+1)}$ copies



Thermal Cycler

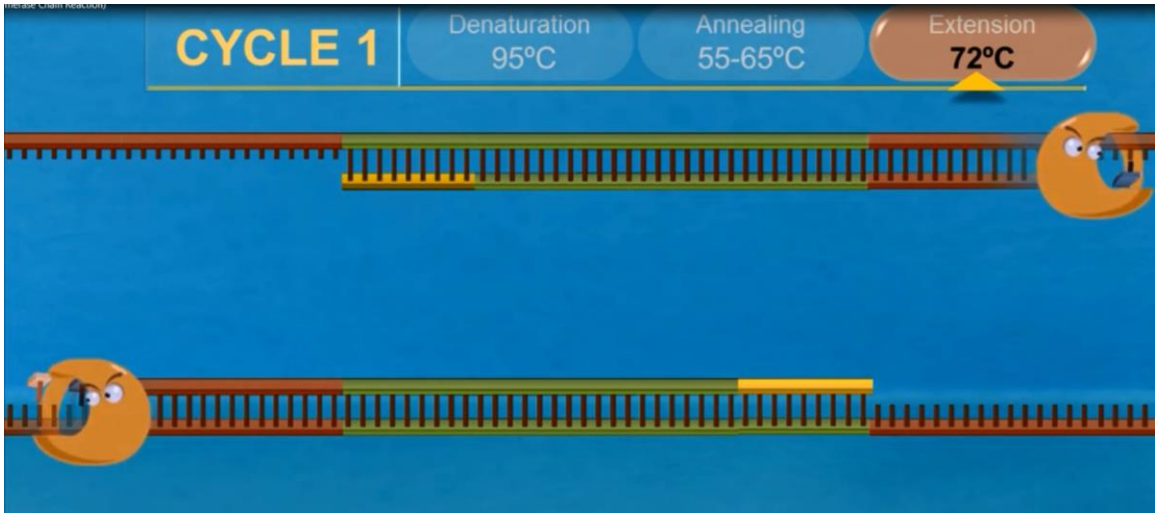
Also known as:

- Thermocycler
- PCR machine
- DNA amplifier

A very early PCR machine
Samples moved mechanically

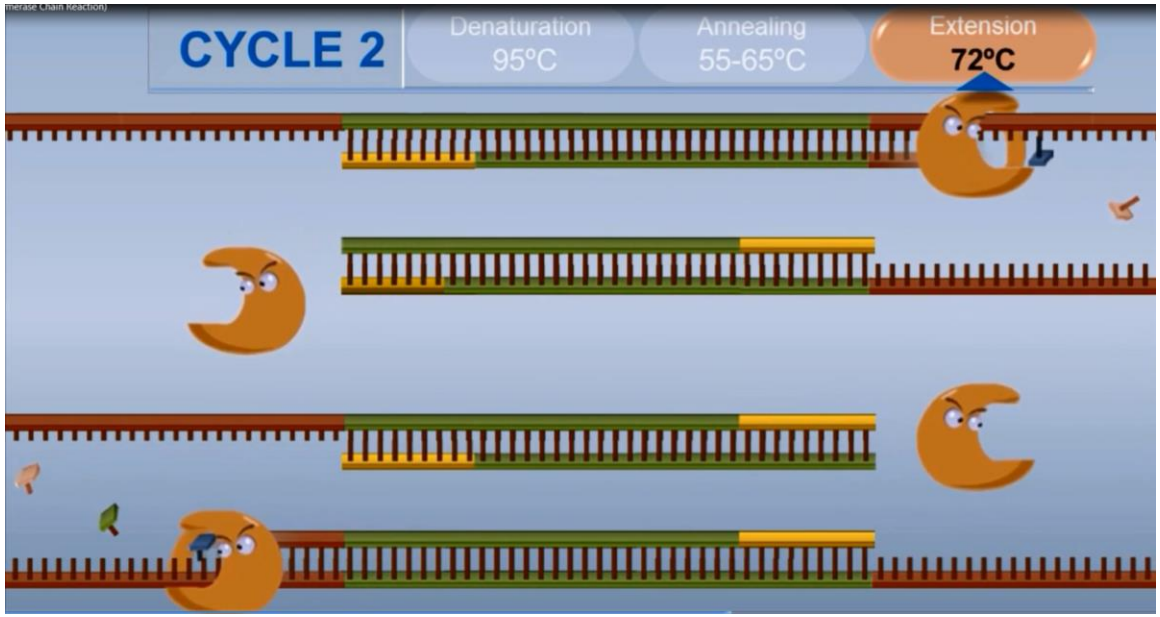
Modern Machines
Using the Peltier effect
1834 by Jean-Charles Peltier

PCR Cycling



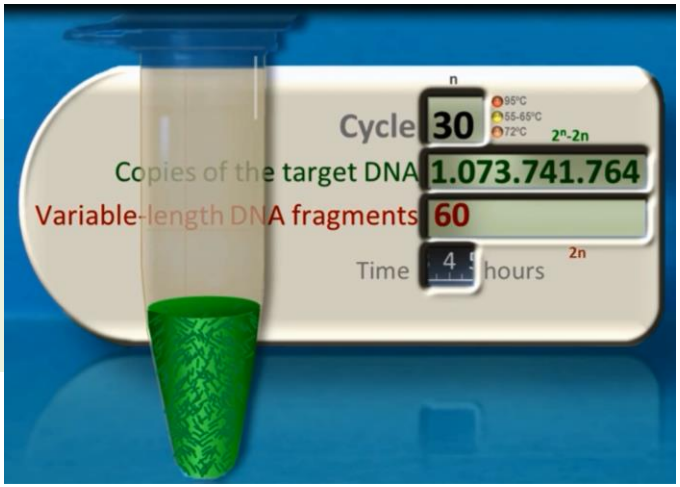
DNA Copy Types:

1. Target DNA
2. Long-length DNA

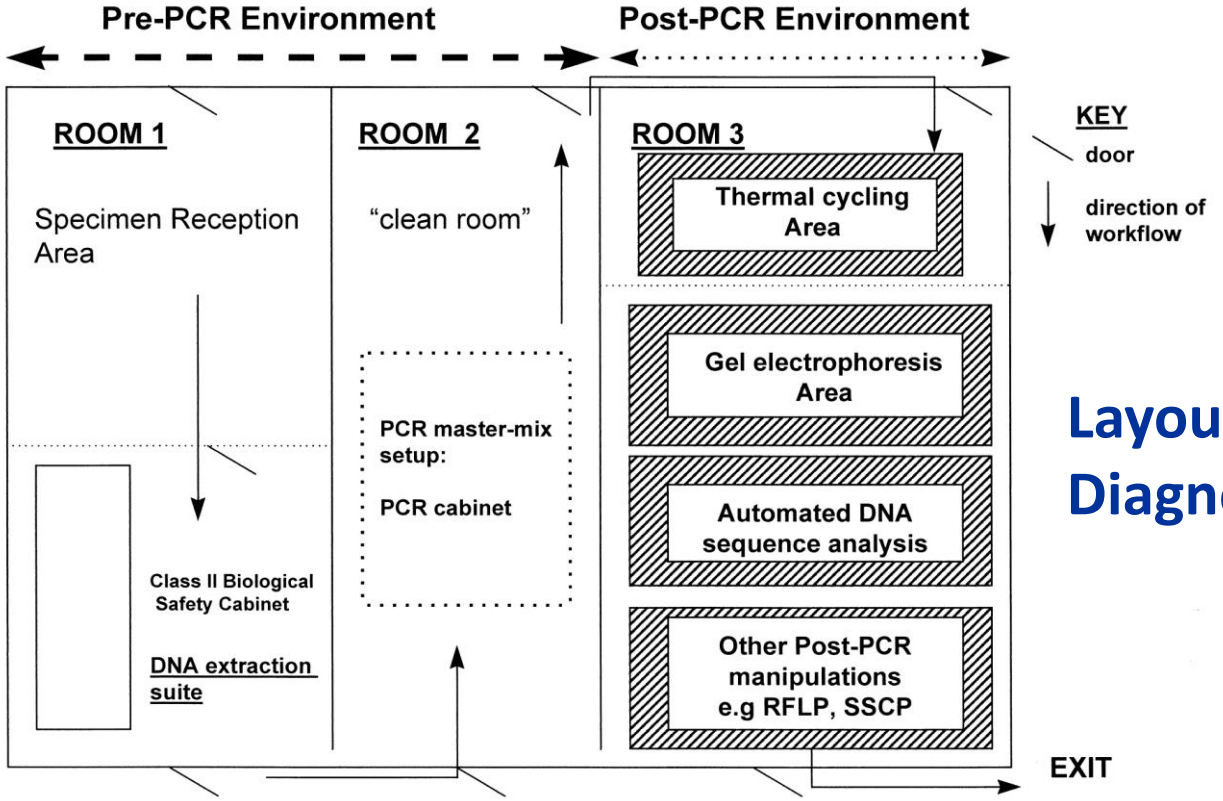


Complete PCR

After 30 cycles, from only one molecule:
1,073,741,764 copies of target DNA
60 longer molecules



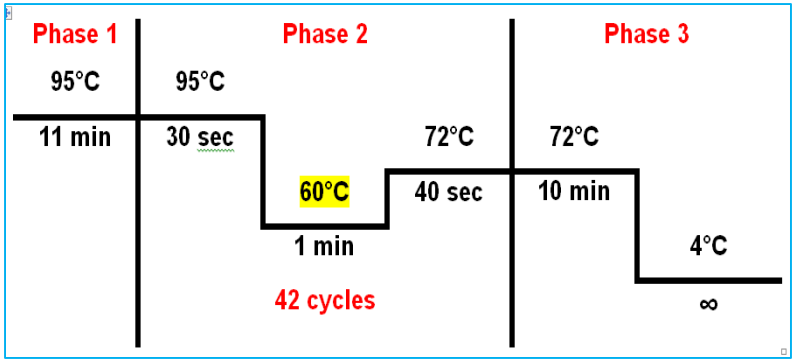
---Flow of samples for PCR analysis------>

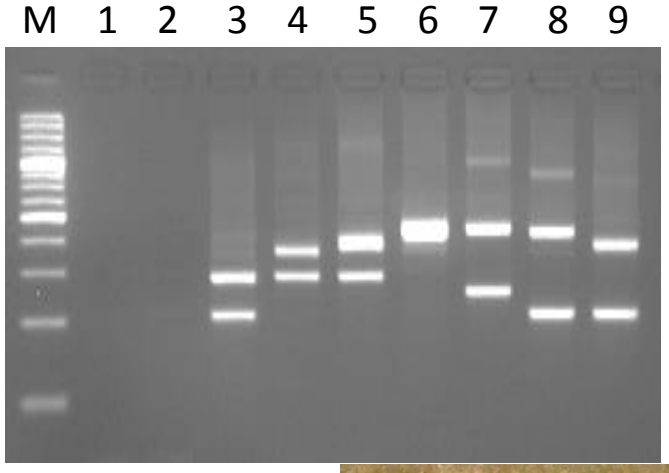


Layout of PCR Diagnostic Lab

Analysis of PCR products

After amplifying your gene into many millions of copies, it is possible to run the amplicons on an agarose gel and stain it with a dye to visualize it.





Play Principle of PCR Movie

Thank you

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