Near East University
DESAM Research Institute

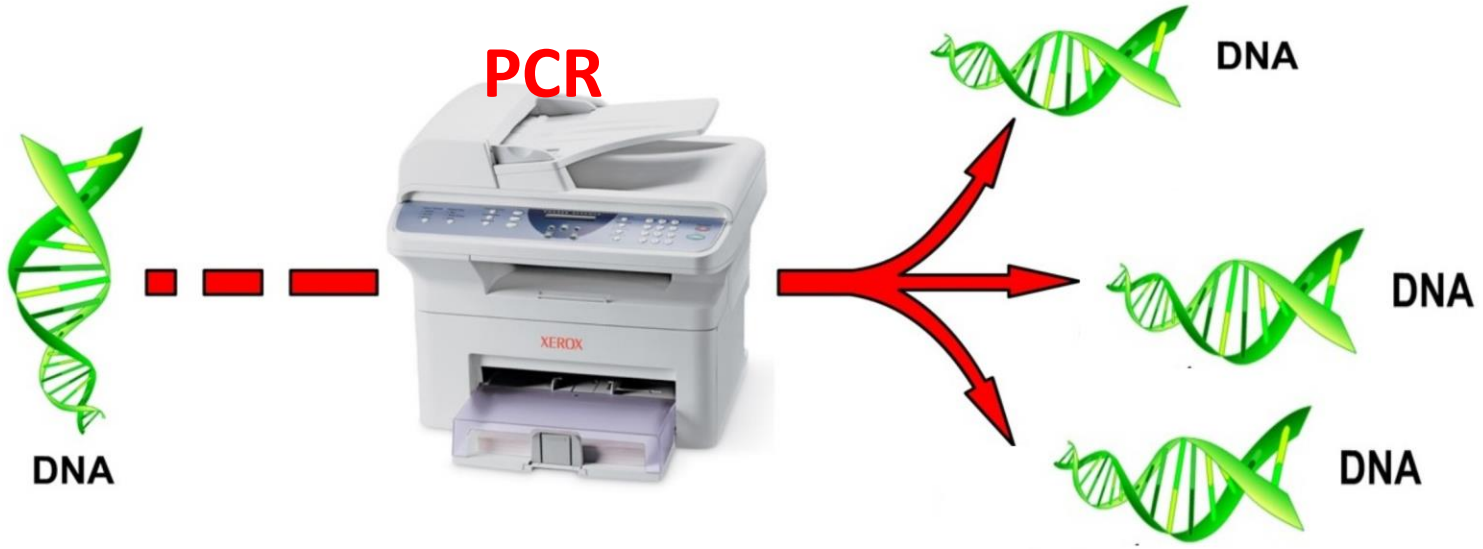
Third Generation Sequencing: from Wet-Lab to Informatics

Polymerase Chain Reaction (PCR)

Dr. Gökçe Akan

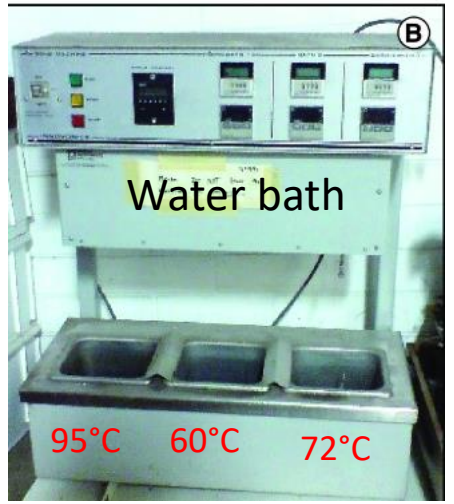
Polymerase Chain Reaction (PCR)- DNA Photocopier

- PCR is an *in-vitro* technique used in molecular biology for the amplification of a region of DNA which lies between two regions of known sequence (primers).
  - ***in vitro* version of DNA Replication**
  - Multiple copies of specific DNA sequence
    - DNA photocopying



History of PCR

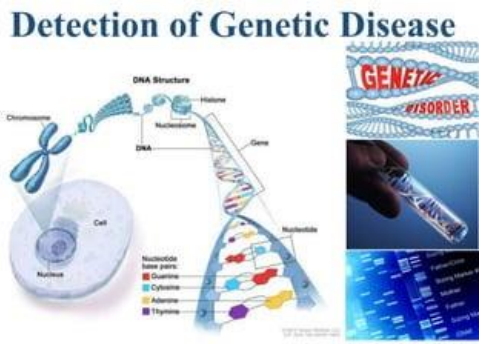
- PCR was developed in 1983 by **Kary Mullis**, an American biochemist who won the Nobel Prize for Chemistry in 1993 for his invention.
- Originally, PCR was developed for the detection of mutations in the HBB gene that causes sickle cell anemia.



- Before the development of the thermal cycler, water baths were used for PCR methods to amplify or generate copies of DNA fragments, which was time-consuming and labor-intensive.

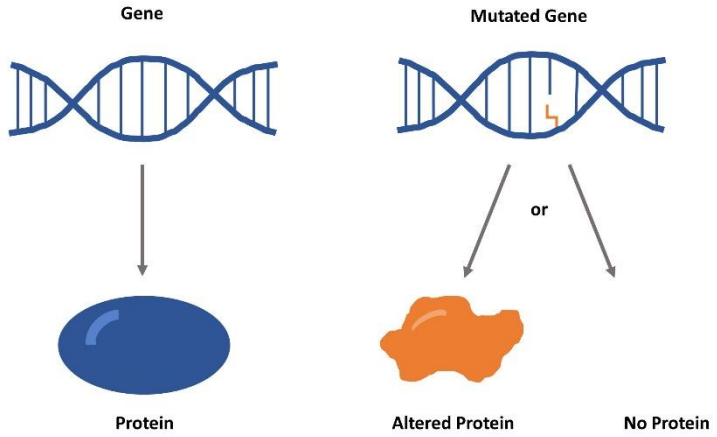
- The thermal cycler was designed to carry out PCR reactions, it can complete many rounds of replication, producing billions of copies of a DNA fragment, in only a few hours.

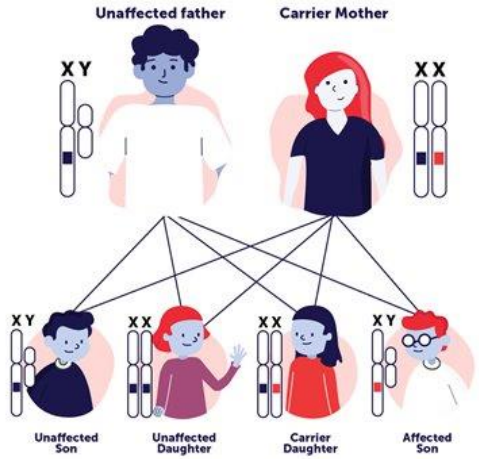
Applications of PCR



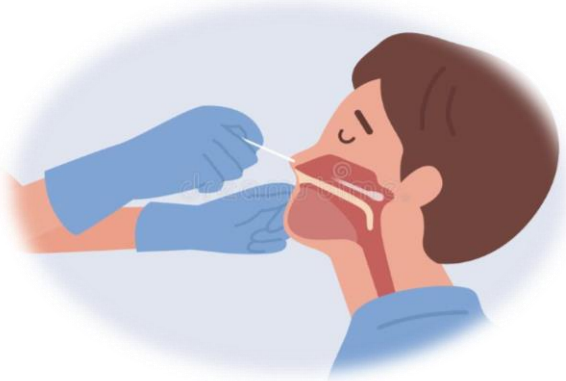
Medicine

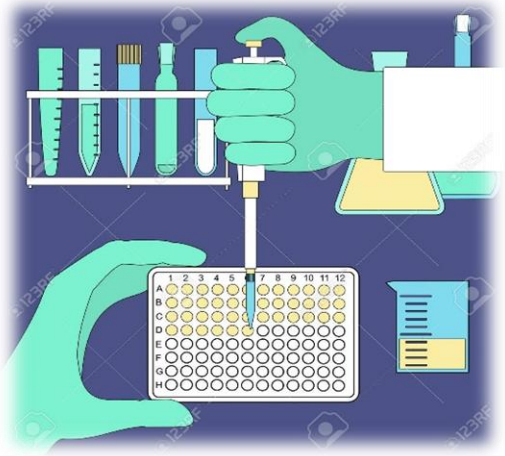
- Testing of genetic disease mutations.
- Detecting disease-causing genes in the parents
- Monitoring the gene in gene therapy.

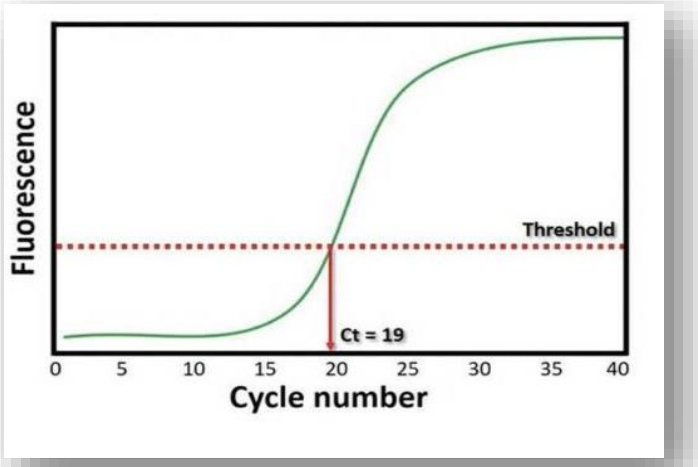


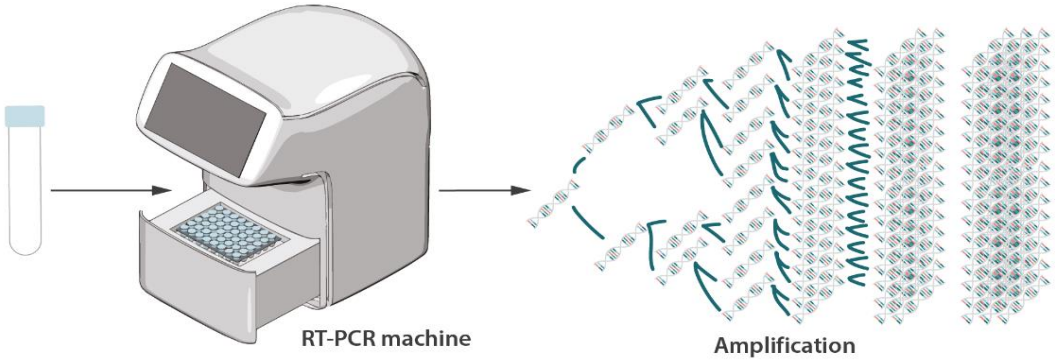


Detection of the pathogens





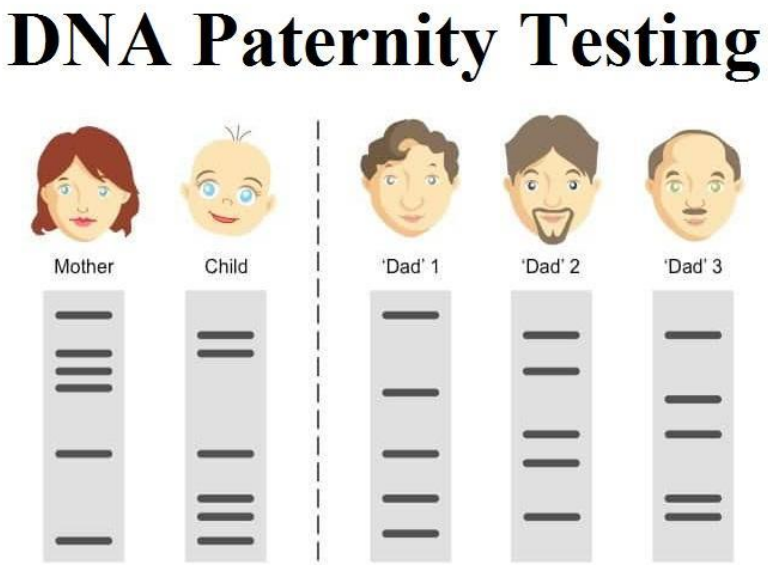




Applications of PCR

Forensic Science

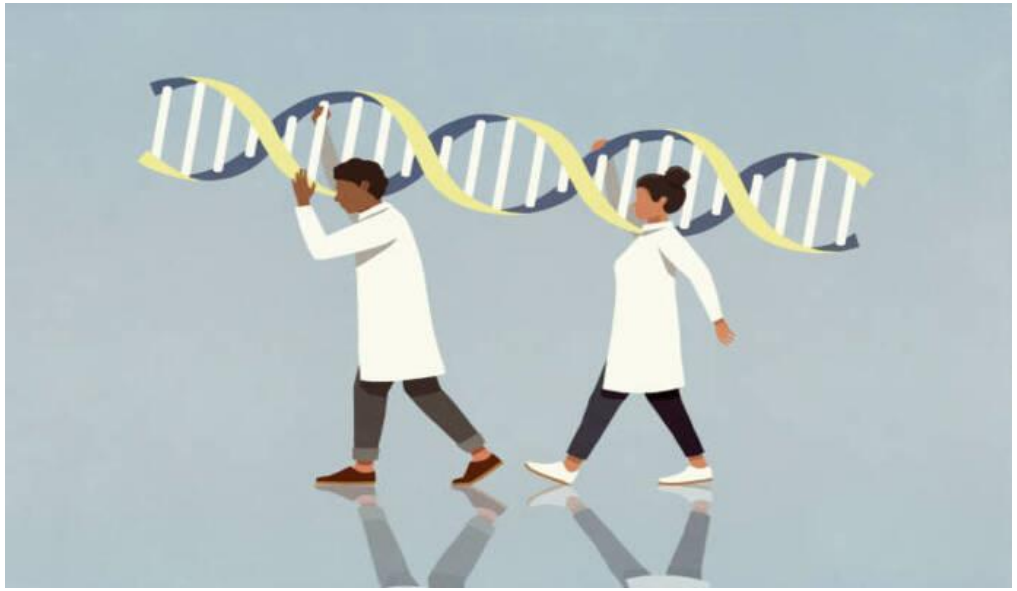
- Used as a tool in genetic fingerprinting.
- Identifying the criminal from millions of people.
- Paternity tests



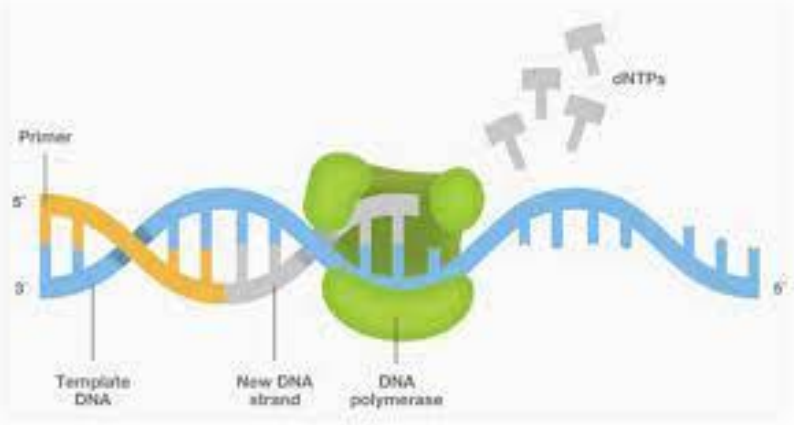
Applications of PCR

Research and Genetics

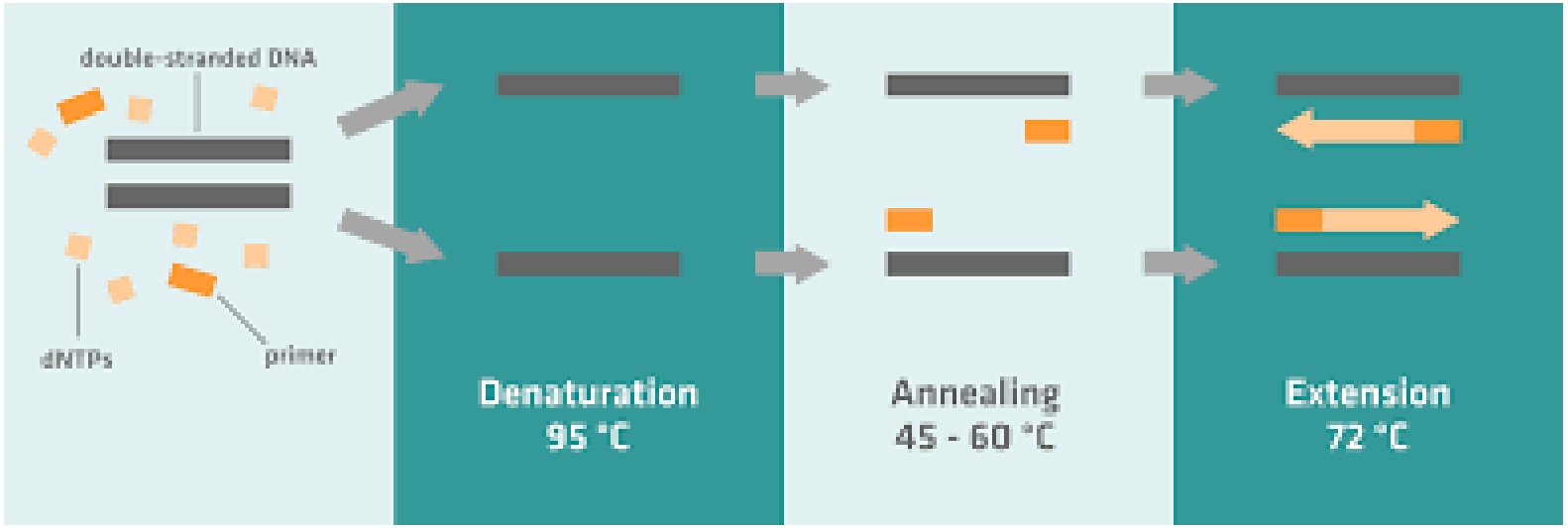
- Compare the genome of two organisms in genomic studies.
- In the phylogenetic analysis of DNA from any source such as fossils.
- Analysis of gene expression.
- Gene Mapping



Principle of PCR



- The PCR technique is based on the enzymatic replication of DNA.
- In PCR, a short segment of DNA is amplified using primer mediated enzymes.
- DNA Polymerase synthesizes new strands of DNA complementary to the template DNA.

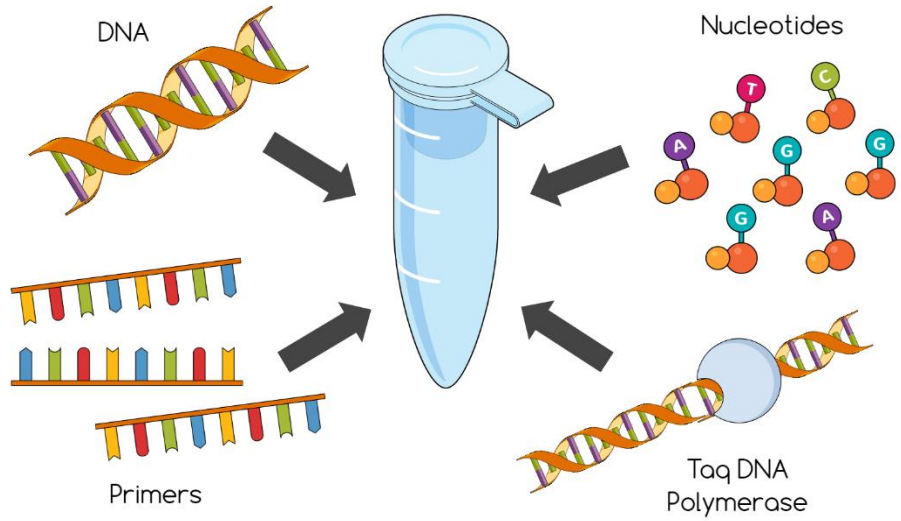


Types of PCR

- Conventional PCR
- Real-time PCR
- Quantitative real time PCR (Q-RT PCR)
- Reverse Transcriptase PCR (RT-PCR)
- Multiplex PCR
- Digital Droplet PCR
- Nested PCR
- Long-range PCR
- Single-cell PCR
- Fast-cycling PCR
- Methylation-specific PCR (MSP)
- Hot start PCR
- High-fidelity PCR
- In situ PCR
- Variable Number of Tandem Repeats (VNTR) PCR
- Asymmetric PCR
- Repetitive sequence-based PCR
- Overlap extension PCR
- Assemble PCR
- Intersequence-specific PCR(ISSR)
- Ligation-mediated PCR
- Methylation –specifin PCR
- Miniprimer PCR
- Solid phase PCR
- Touch down PCR

Conventional PCR

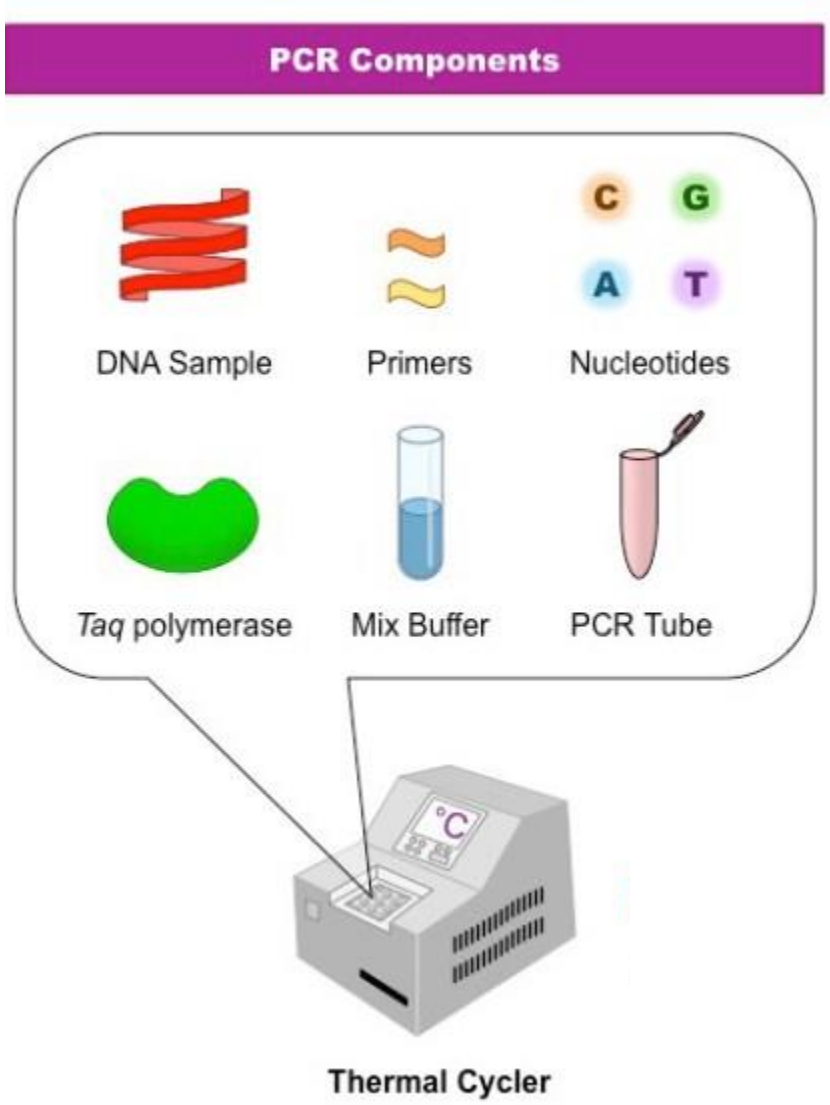
- Conventional PCR is a PCR technique that allows amplification of DNA sequences.
- It is used to detect qualitatively the amplification of the target genes.



Reagents for PCR

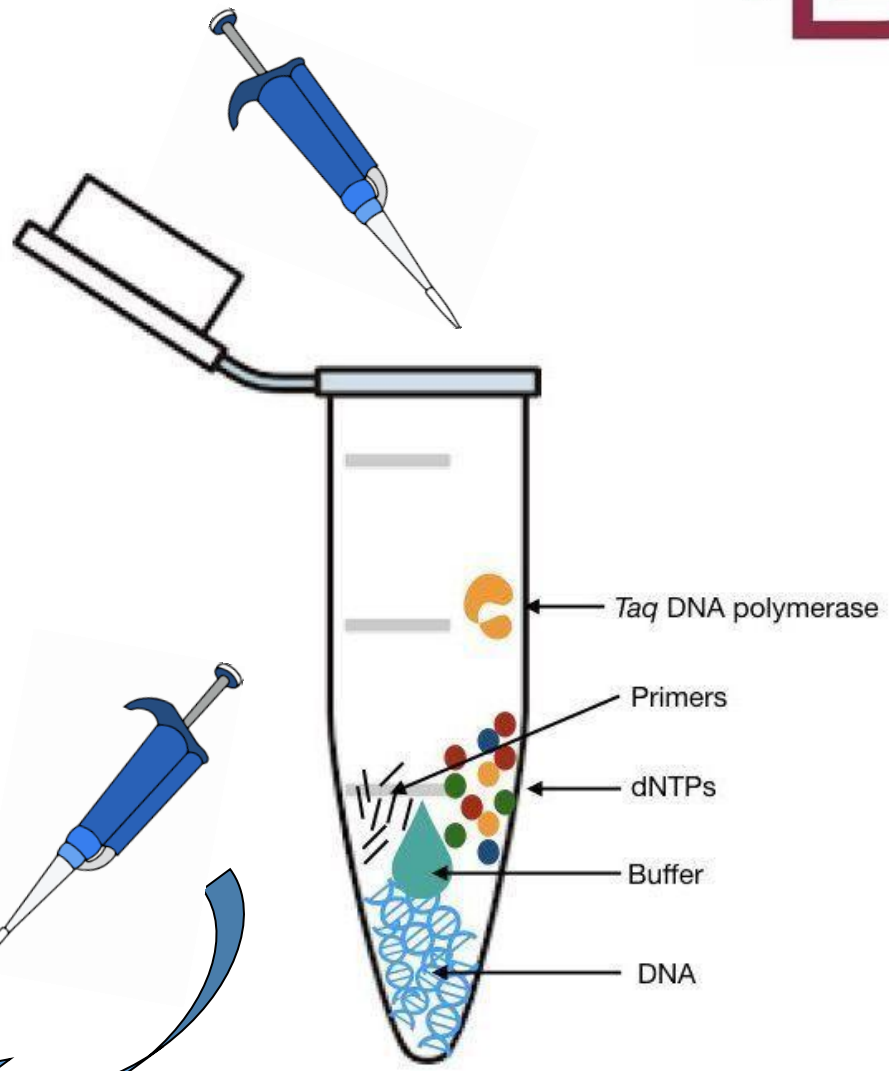
What we need in the laboratory:

1. DNA template
2. Primers
3. DNA polymerase
4. Buffer
5. dNTPS (bases)
6. $MgCl_2$



A Typical PCR Reaction

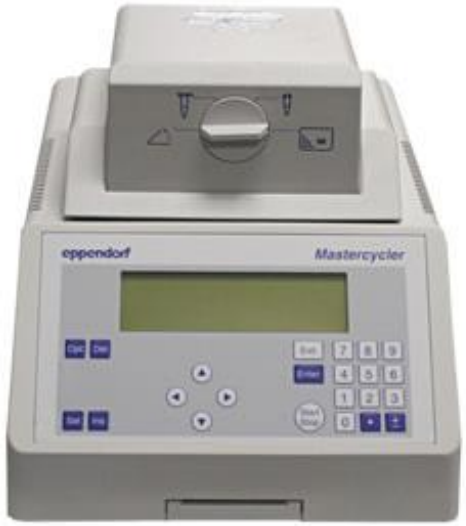
Mix Content	
Sterile Water	8.25 ul
10X PCR Buffer	5.0 ul
$MgCl_2$ (50mM)	1.5 ul
dNTP'mix(10mM)	1.0 ul
PrimerFWD (10 pmol/ul)	1.0 ul
PrimerREV	1.0 ul
DNA Polymerase	0.25 ul
Mix	18 ul
DNA Template	2.0 ul
Total Volume	20.0 ul



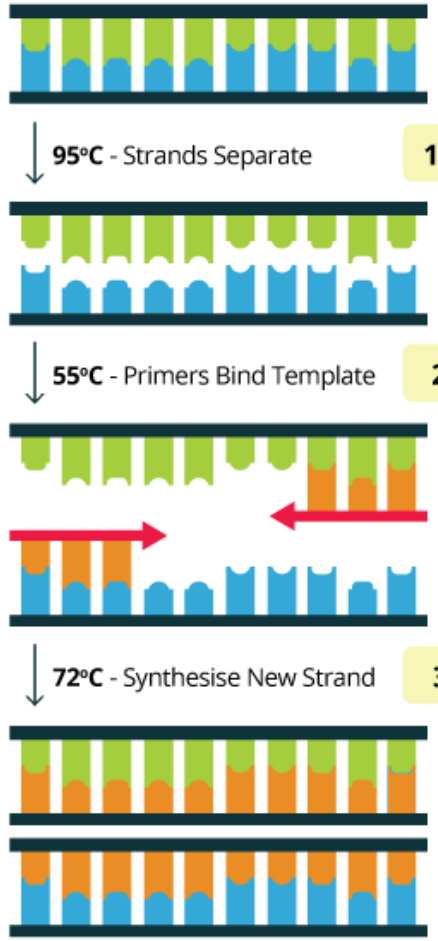
Aliquot

Add DNA as last step

PCR tube with all the reagents



Thermocycler



94-95^{0}C
2-5mins
dsDNA → ssDNA for primer binding

Primers bind on the template
54-63^{0}C
25-60secs

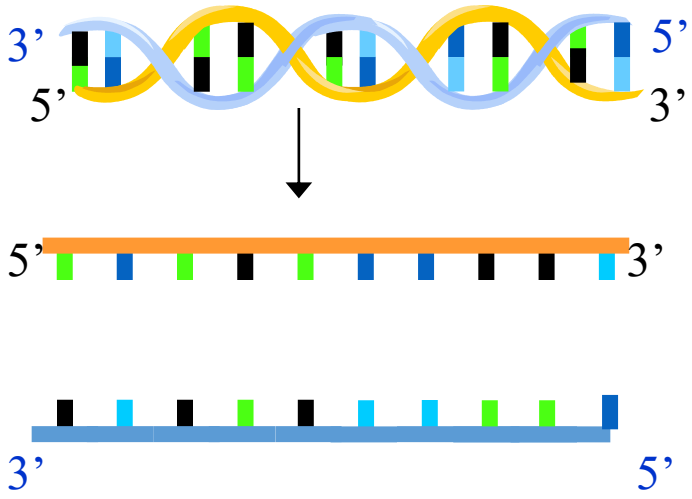
Elongation
72^{0}C
45-90secs

•The thermal cycler allows heating and cooling of the reaction tubes to control the temperature required at each reaction step.

Steps in PCR

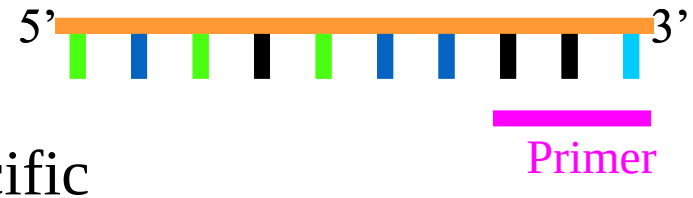
1. Denaturation

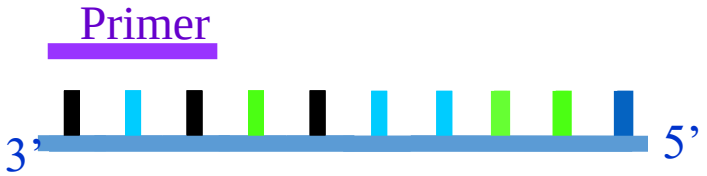
- Heat to separate double strands
- This occurs at 95 ºC



2. Annealing

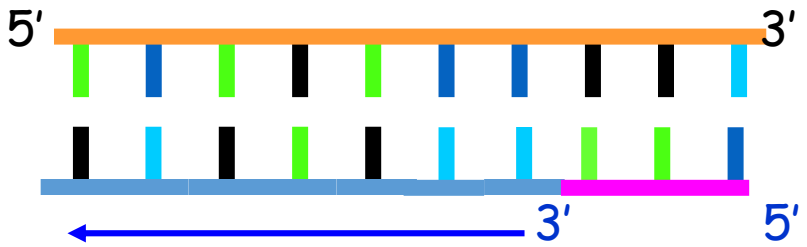
- Primer **binds** to template sequence at a specific temperature



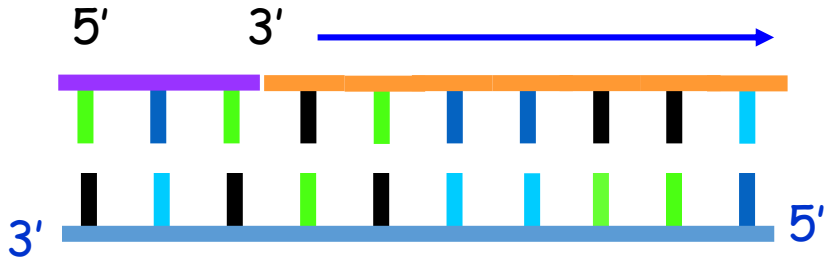


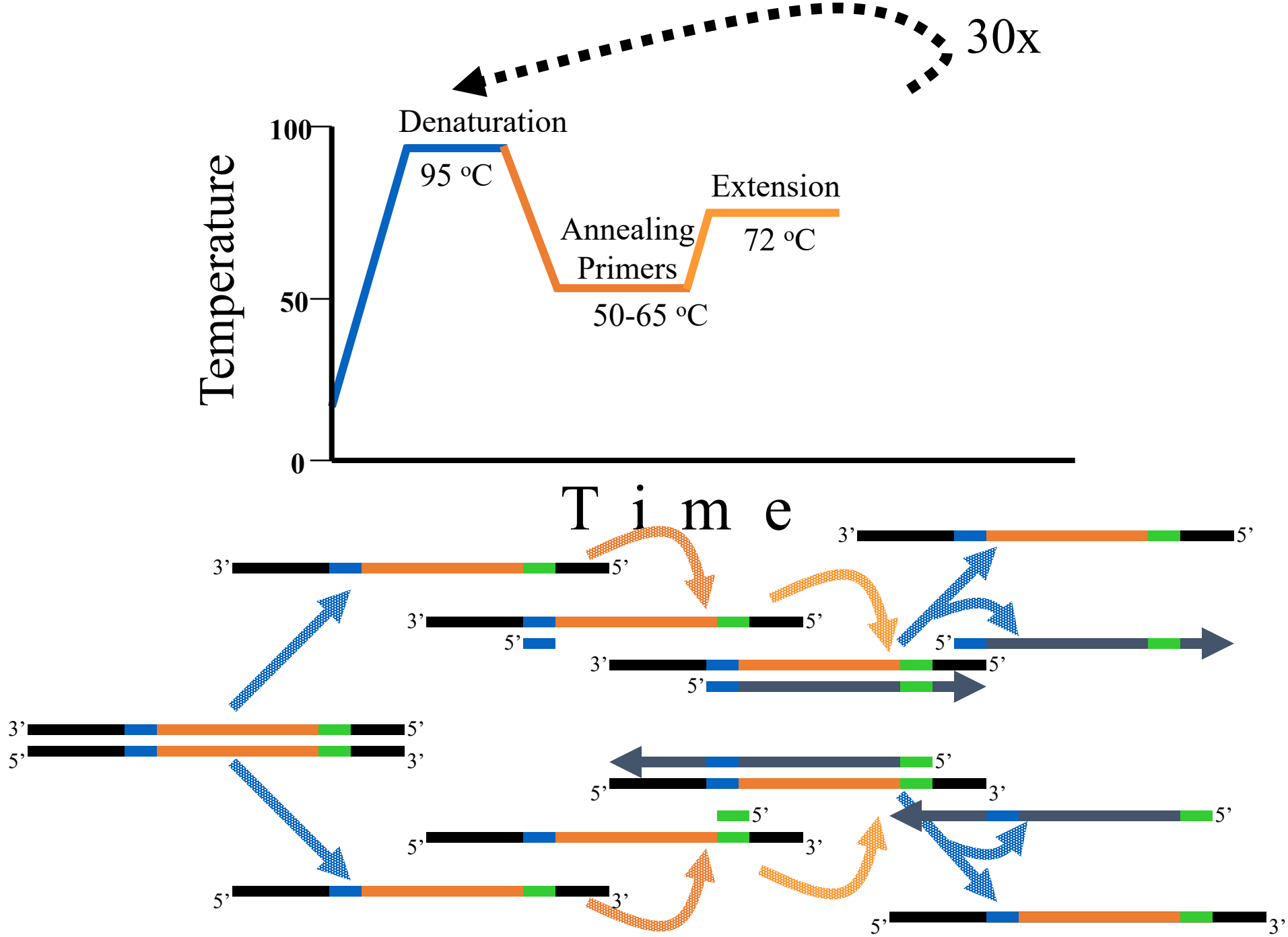
3. Extension

– Primer is extended with addition of **dNTPs** with ***Taq* polymerase**

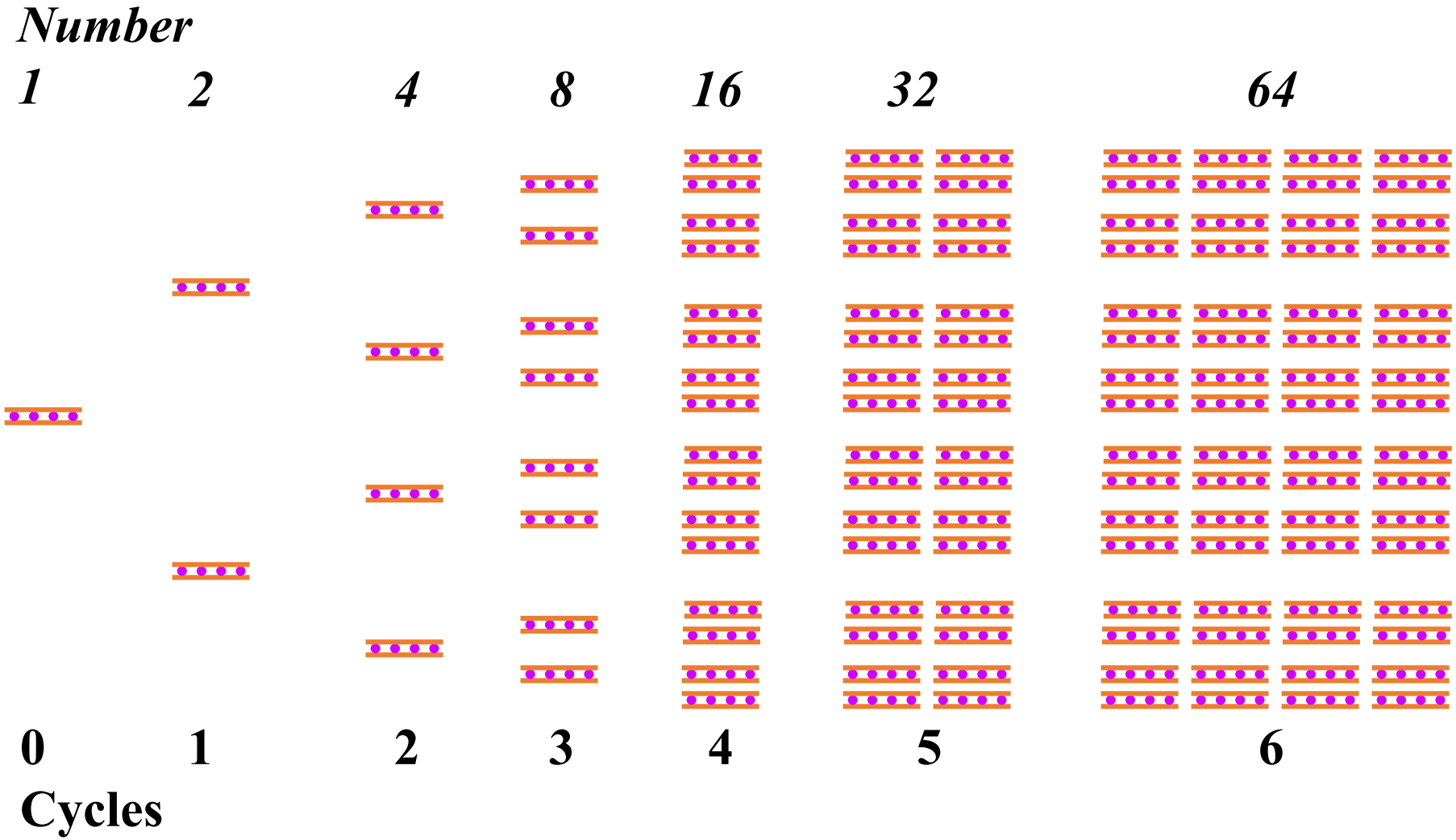


– the extension of the strand in the **5-3 direction** starting at the primers attaching the **appropriate nucleotide** (A-T, C-G)




30x
Denaturation
95 °C
Annealing
Primers
50-65 °C
Extension
72 °C
100
50
0
Temperature
T i m e
3’
5’

Doubles The Copy Number of the amplified gene generates in per cycle

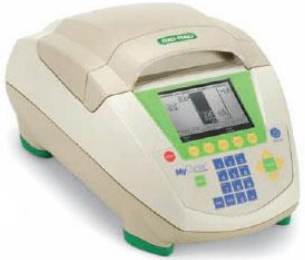


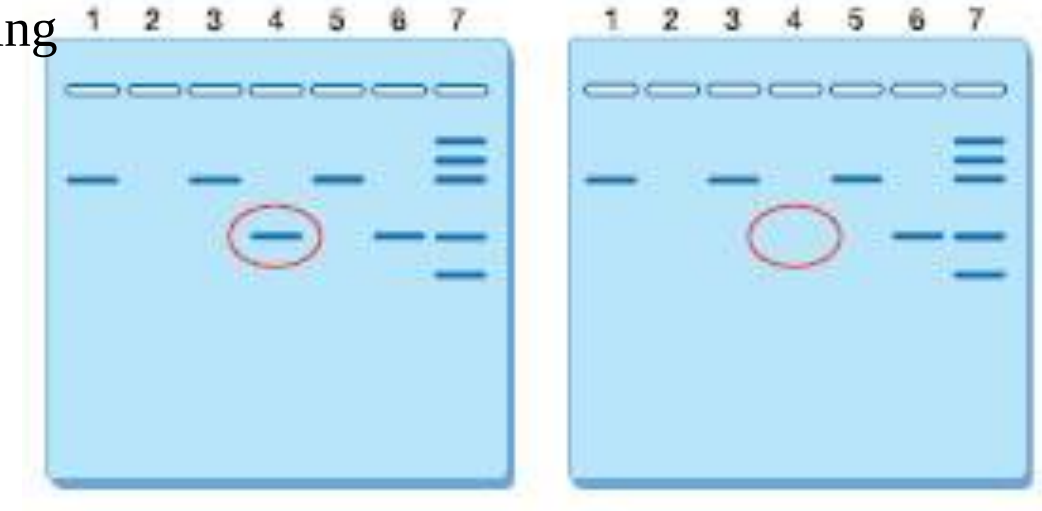
2^{n}(cycle)

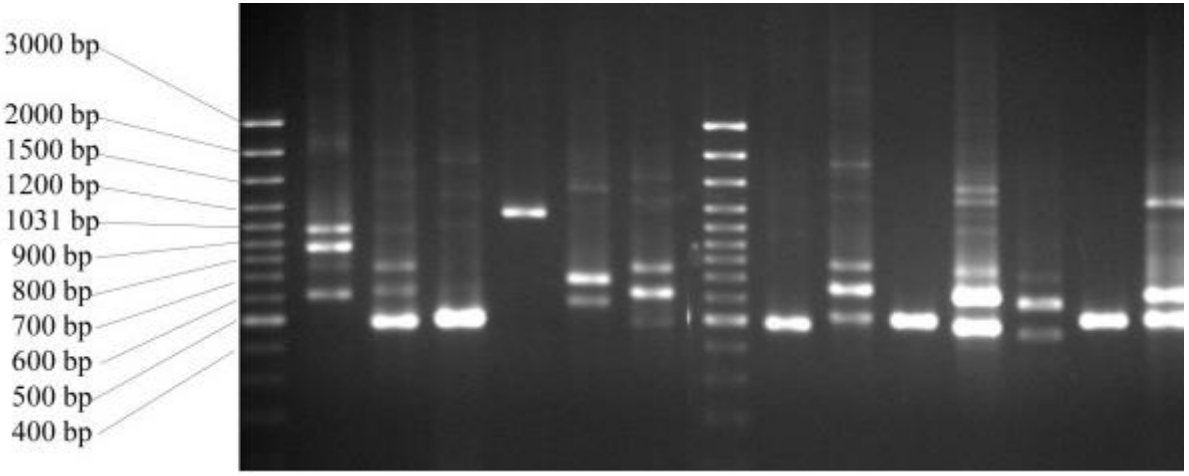
ten cycles ideally produces 2→4→ 8→ 16→ 32→ 64→ 128→ 256→ 512→1,024 (2^{10}) copies.

Gel Electrophoresis

- Contents of tubes are loaded onto an agarose gel.
- Agarose gel electrophoresis that is an electric field is used to drive charged molecules and it is the most effective way of separating DNA fragments on the basis of their molecular weight
- Fragmentation products of differing length are separated





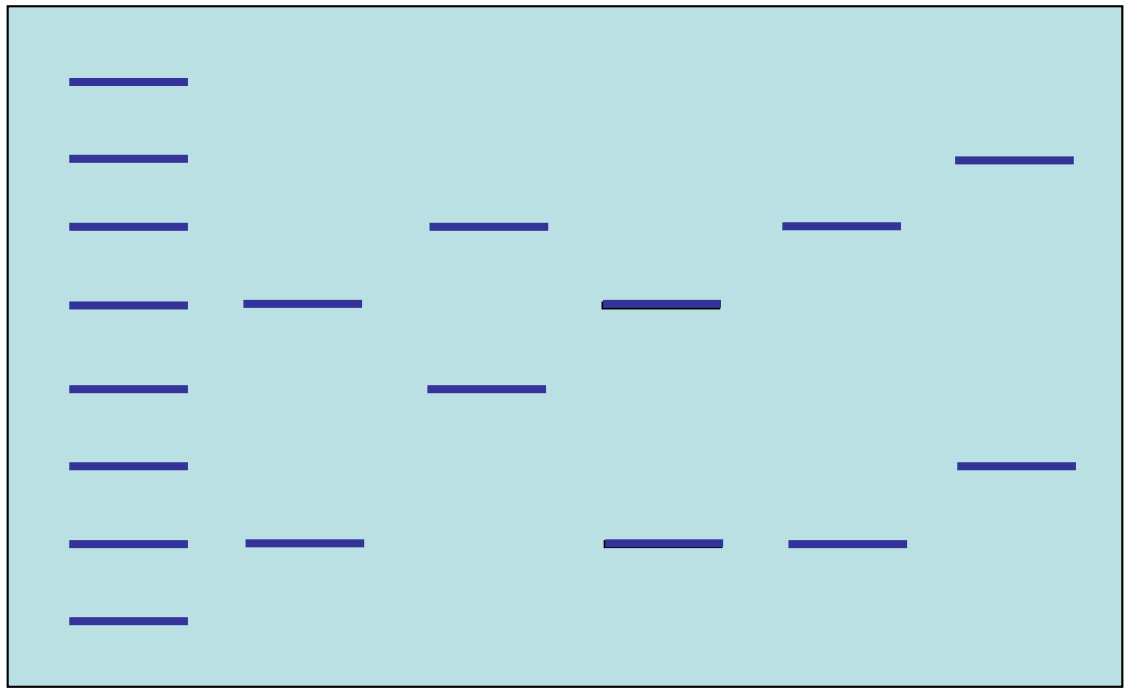


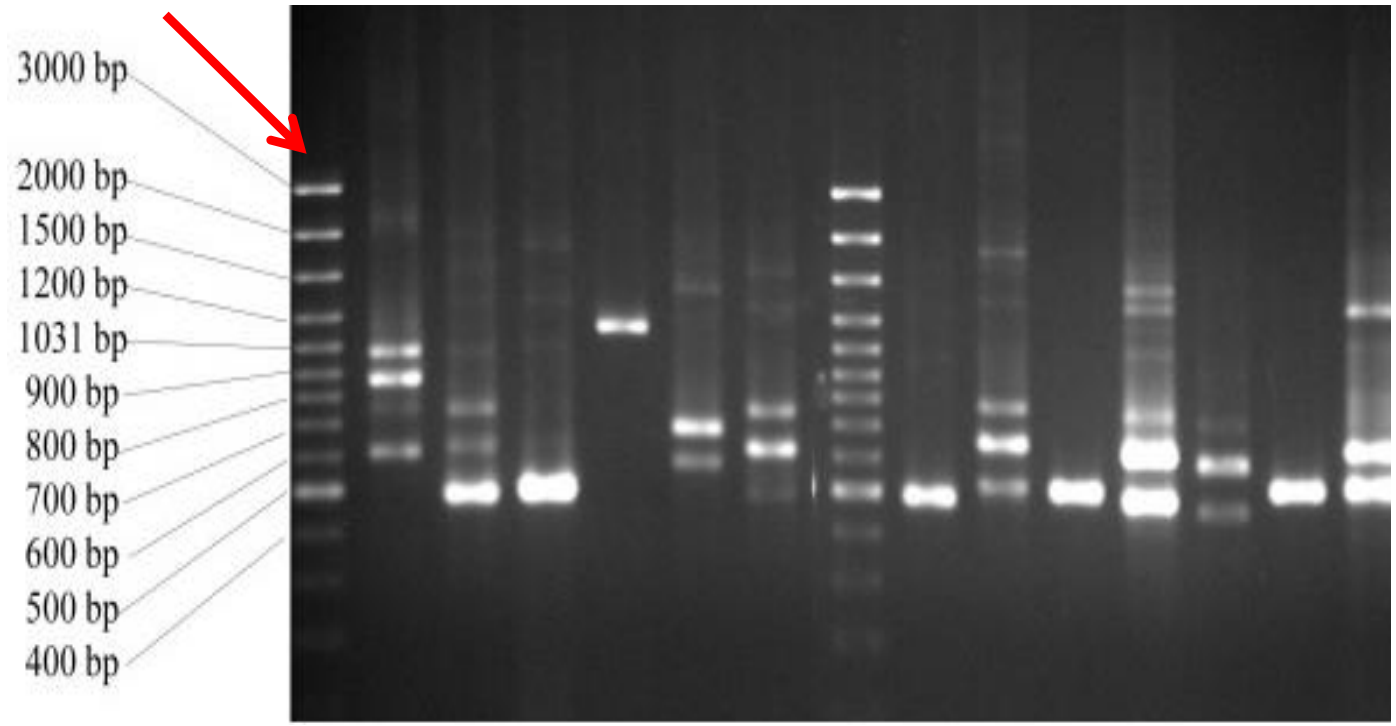
Separation of DNA fragments based on length

- Shorter fragments move farther along the gel
- Longer fragments move slower.

PCR Visualizing Results

The final result of the conventional PCR procedure is a gel with a series of bands:





Bands can be compared against each other, and to known size-standards, to determine the presence or absence of a specific amplification product.

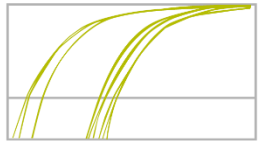
Real Time PCR

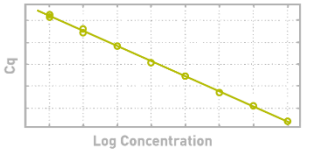
- It can be used for both qualitative and quantitative analysis for amplification of the target genes
- Specialized technique that allows a PCR reaction to be visualized "in real time" as the reaction progresses.

It allows us to <u>measure</u> minute amounts of DNA sequences in a sample!

- Real-time detection of PCR products is enabled by the inclusion of a fluorescent reporter molecule in each reaction well that yields increased fluorescence with an increasing amount of product DNA.

the change in fluorescence over time is used to calculate the amount of amplicon produced in each cycle.

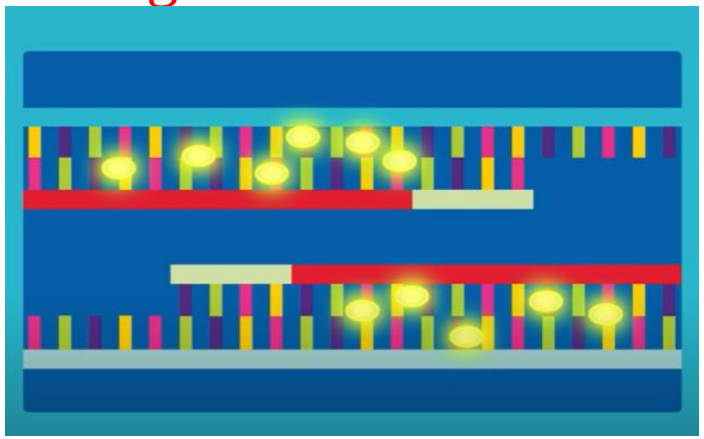




Which fluorescent dye using in Real-Time PCR

SybrGreen

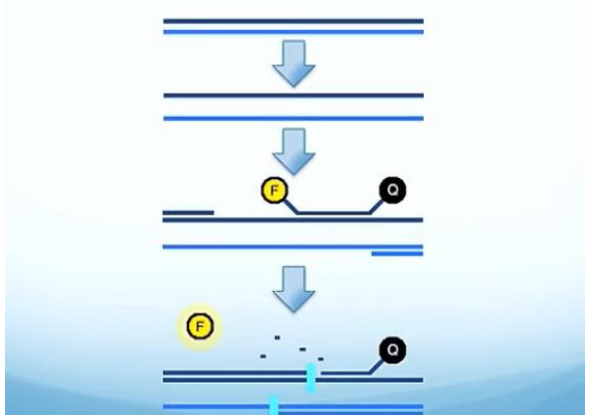
- DNA binding Dye
- Binds to dsDNA
- More binding → More fl.signal
- Unspesific
- Analysis with Melting Curve

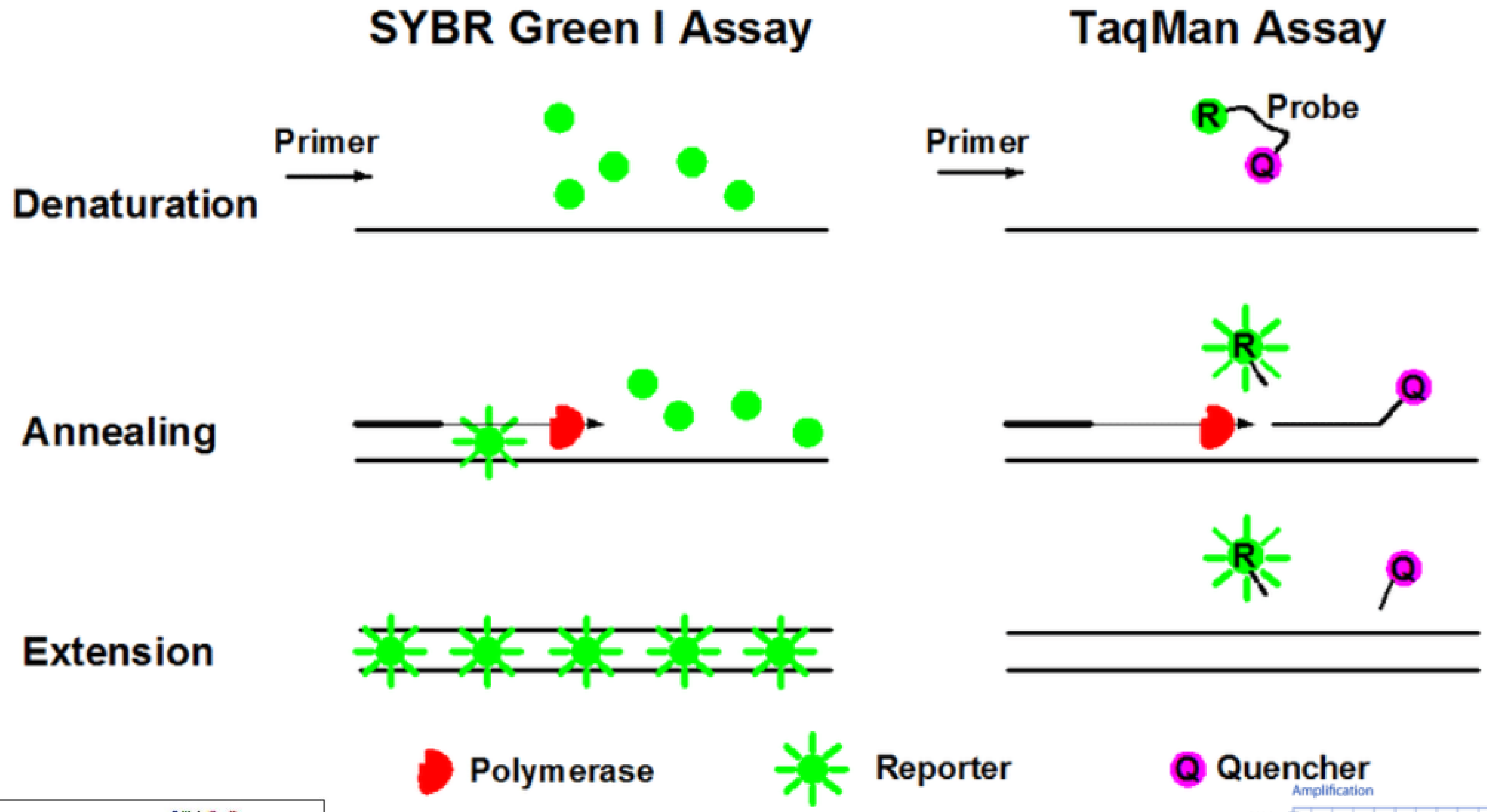


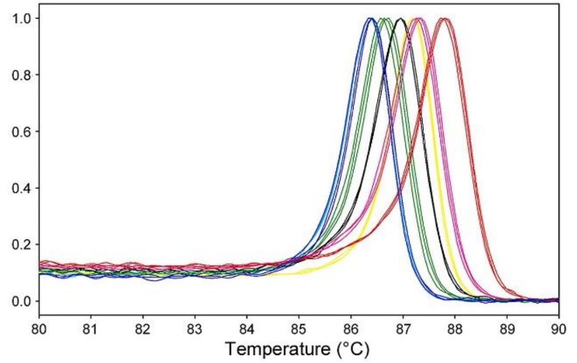
TaqMan Probe

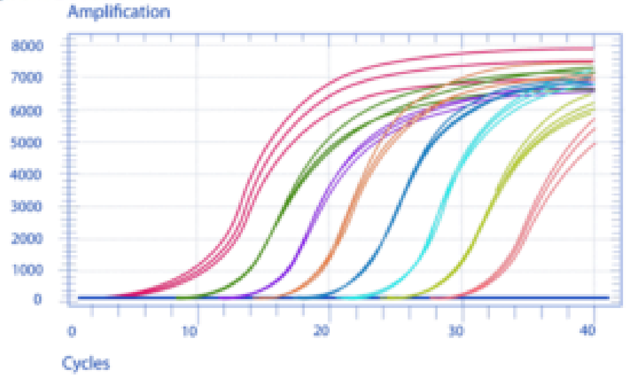
- A small segment of oligonucleotide
- Each TaqMan Probe has;
  - A Fluorescent molecule
  - A Quencher
- Binds to ssDNA
- Spesific for sequence
- Analysis with

Amplification Curve




SYBR Green I Assay
TaqMan Assay
Denaturation
Annealing
Extension
Primer
Primer
Probe
R
Q
R
Q
R
Q
Polymerase
Reporter
Q
Quencher


1.0
0.8
0.6
0.4
0.2
0.0
80
81
82
83
84
85
86
87
88
89
90
Temperature (°C)

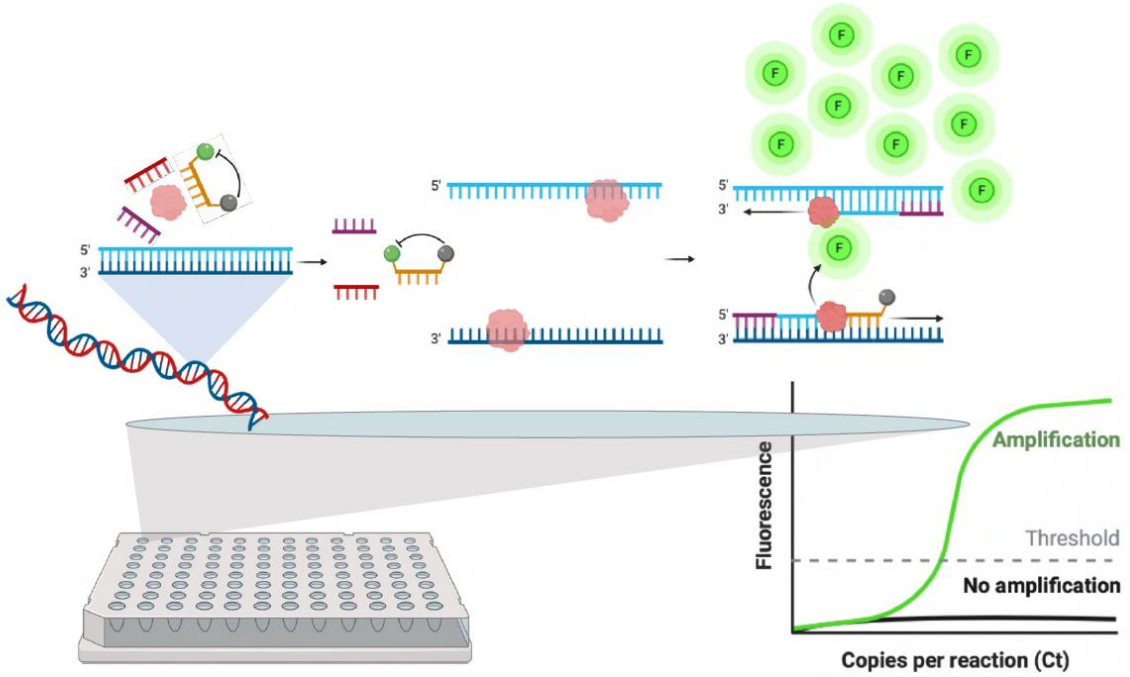

Amplification
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
Cycles

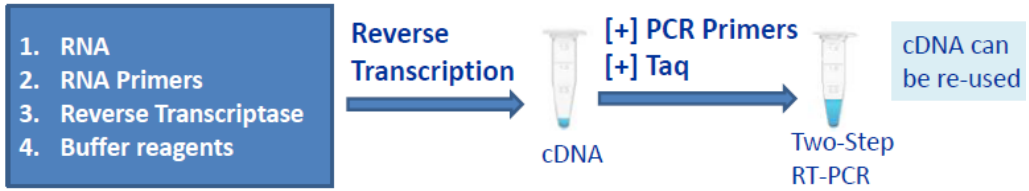
Real Time PCR

- Quantitative real time PCR (Q-RT PCR)
- Reverse Transcriptase PCR (RT-PCR)

Quantitative real time PCR (Q-RT PCR)

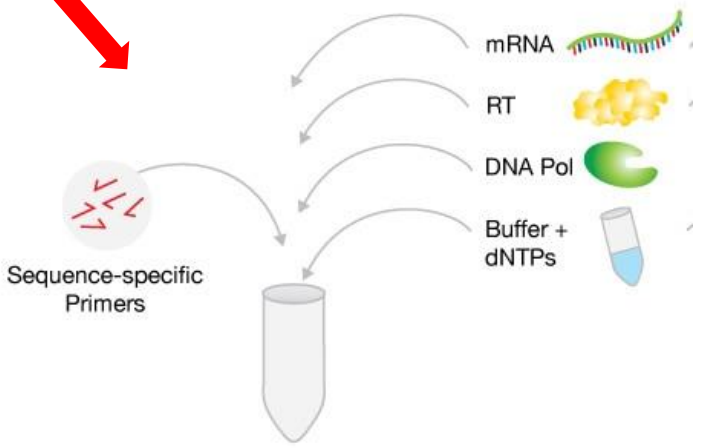
- Q-RT PCR is used to quantitatively measure the amplification of DNA using fluorescent dyes
- the starting material is double-stranded DNA (DNA or cDNA)





Q-RT PCR

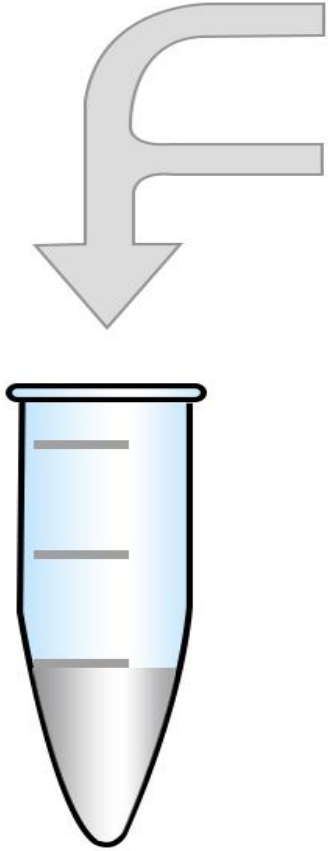
RNA

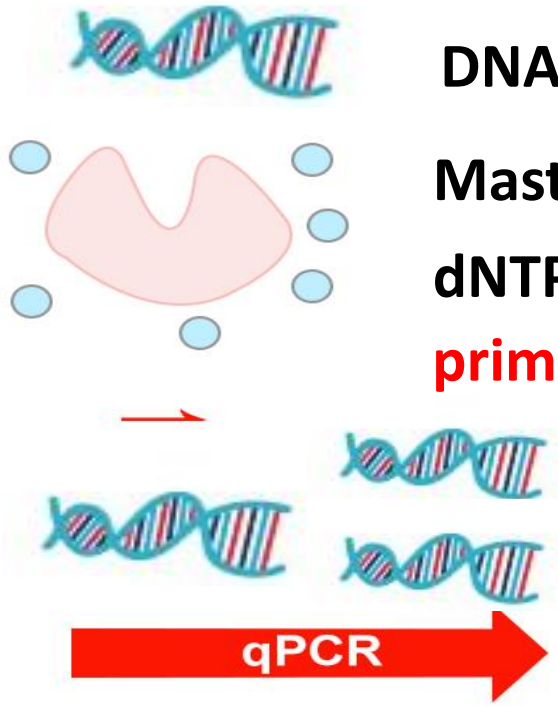


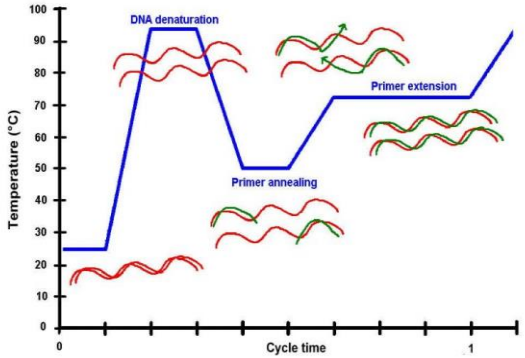
DNA/cDNA

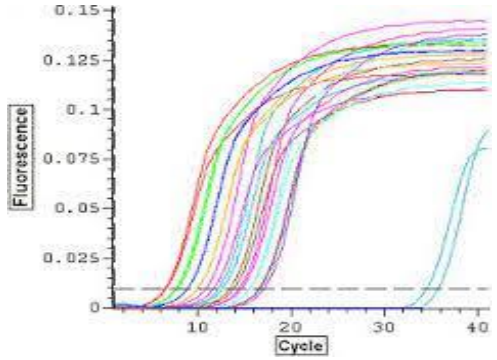
Master mix with DNA polymerase, dNTPs, Mg_2Cl, Buffer and Dye

primers



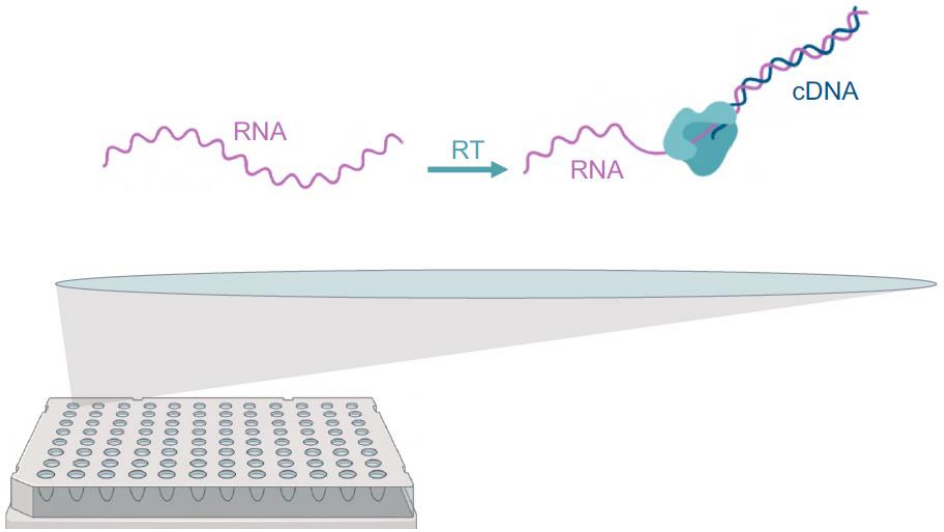






Reverse Transcriptase PCR (RT-PCR)

- A technique commonly used in molecular biology to detect RNA expression
- RT-PCR is used to qualitatively detect gene expression through synthesis of cDNA transcripts from RNA



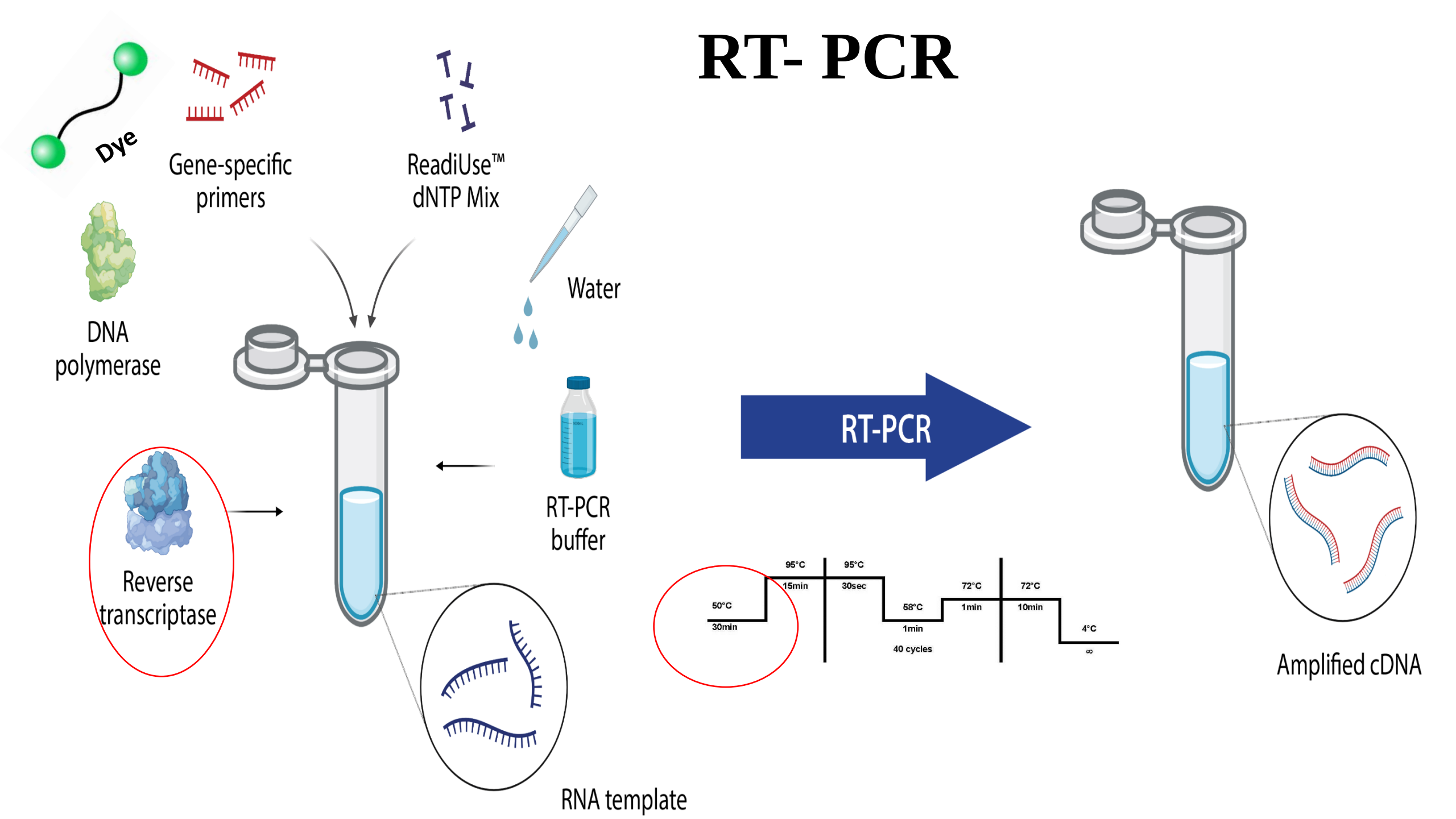
1. RNA
2. RNA and PCR Primers
3. Reverse Transcriptase
4. Buffer reagents
5. Taq polymerase

RT-PCR

One-Step RT-PCR

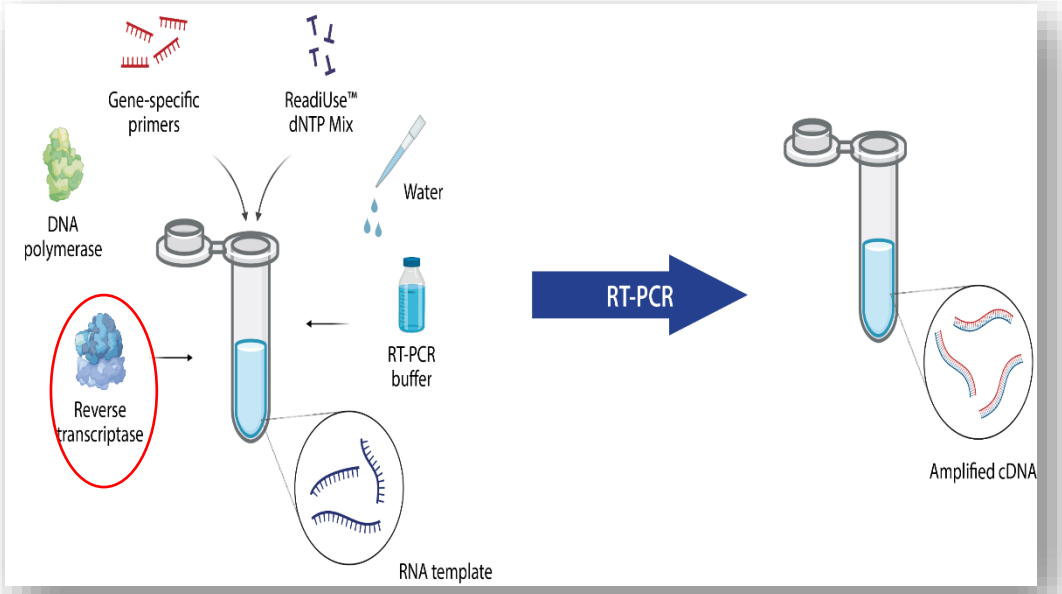
- Simplicity
- Convenience
- Minimizes contamination
- Not flexible

RT- PCR

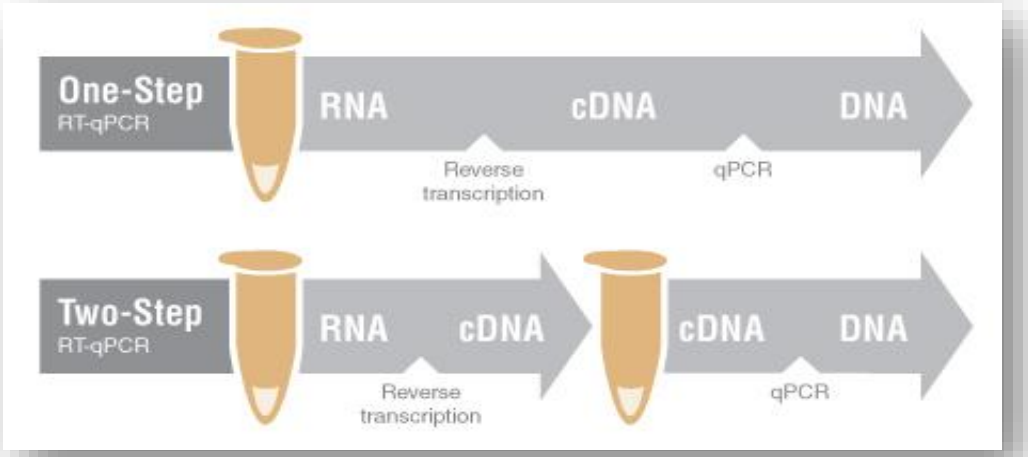

Dye
Gene-specific primers
ReadiUse™ dNTP Mix
DNA polymerase
Water
RT-PCR buffer
Reverse transcriptase
RNA template
RT-PCR
95°C
15min
95°C
30sec
50°C
30min
58°C
1min
72°C
1min
72°C
10min
4°C
∞
40 cycles
Amplified cDNA

One step vs Two step

One Step



Two Step

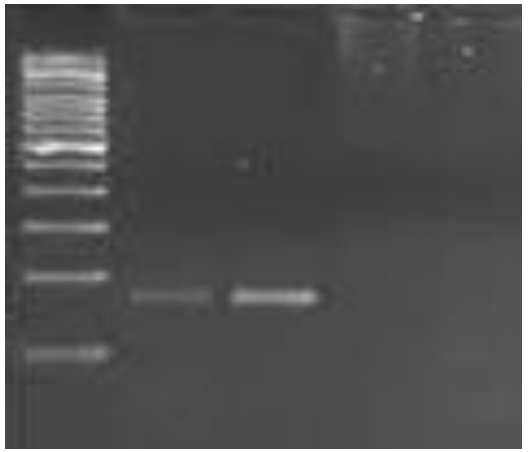


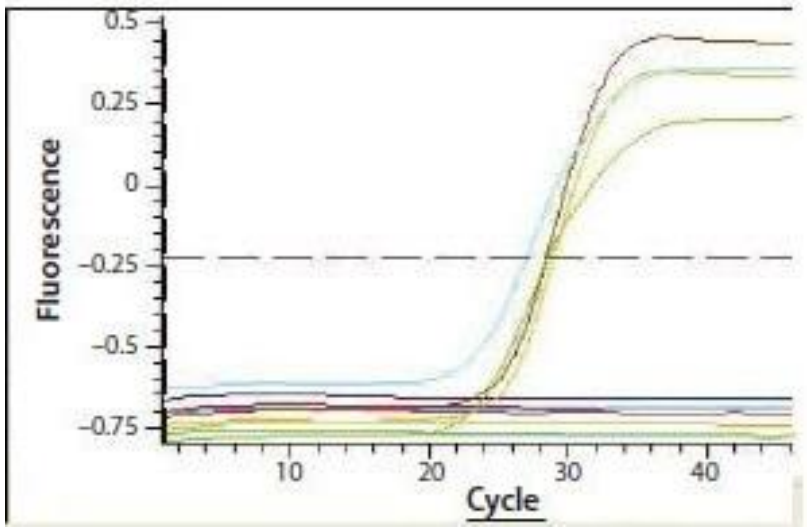
- More pipetting step
- Time consuming
- Contamination risks
- Reduce errors
- Simple

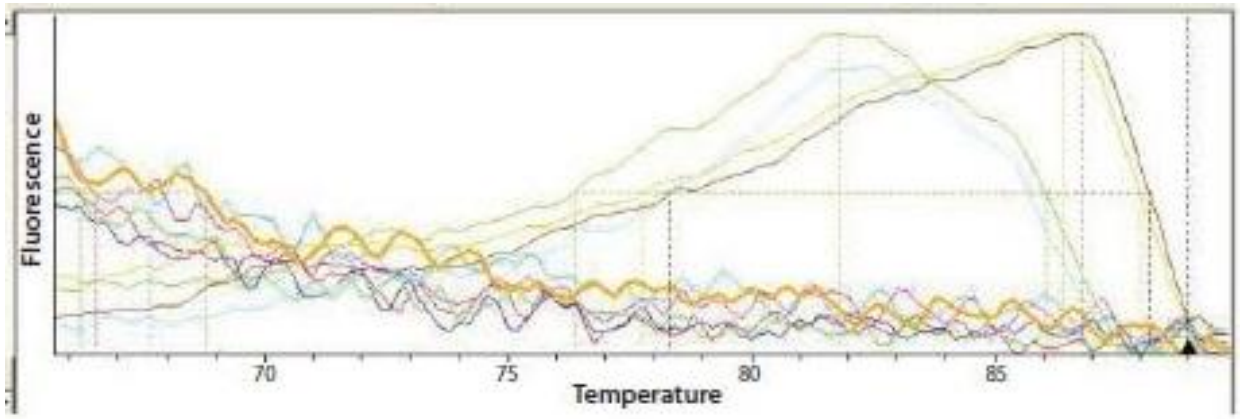
Conventinal PCR vs Real time PCR

	Conventional PCR	Real time PCR
Sensitivity	Low	High
Specifity	Low- only size discrimination	High-use spesific probes
Quantitave results	No- EtBr staining	Yes-spesific flourescence
Detection method	Agarose gel electrophoresis	Probe-spesific flourescence
Detection range	Short-range	Wide range
Reaction time	3-5 hours	1 hour
Post-PCR step	Agarose gel electrophoresis	No
Cross-contamination	Yes Open system and Multiple steps	No Closed system and Single step

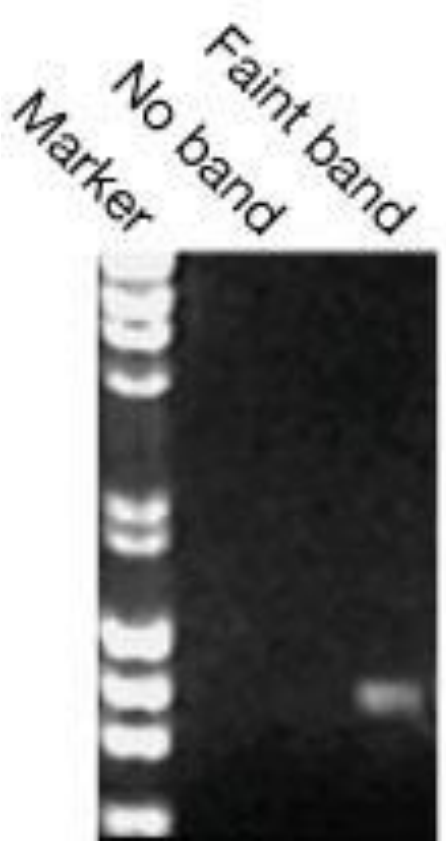
Problems and Solutions during PCR







Problems and Solutions during PCR



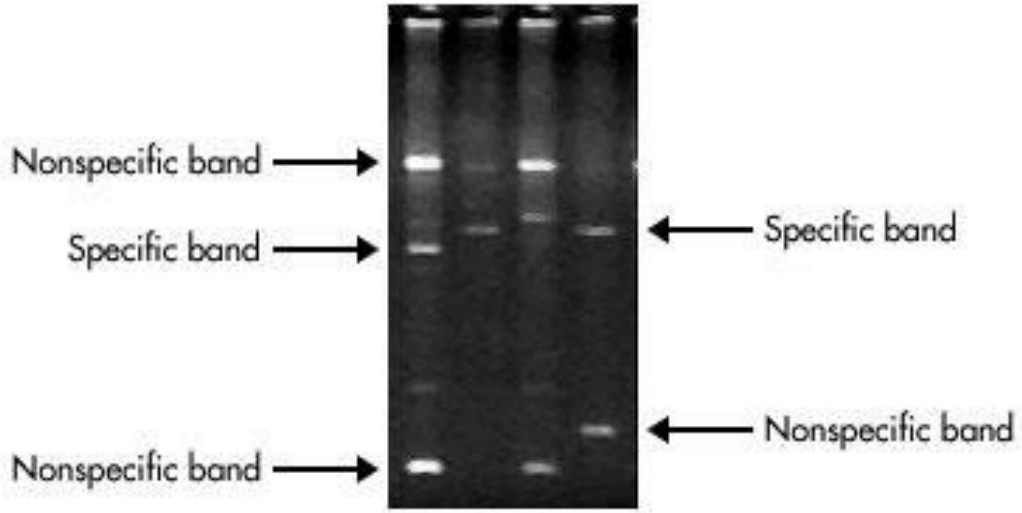
No band or faint band

- Too few cycles
- Short extension time.
- Short annealing time.

Extend the time and cycle

Problems and Solutions during PCR

Nonspesific band or primer dimers



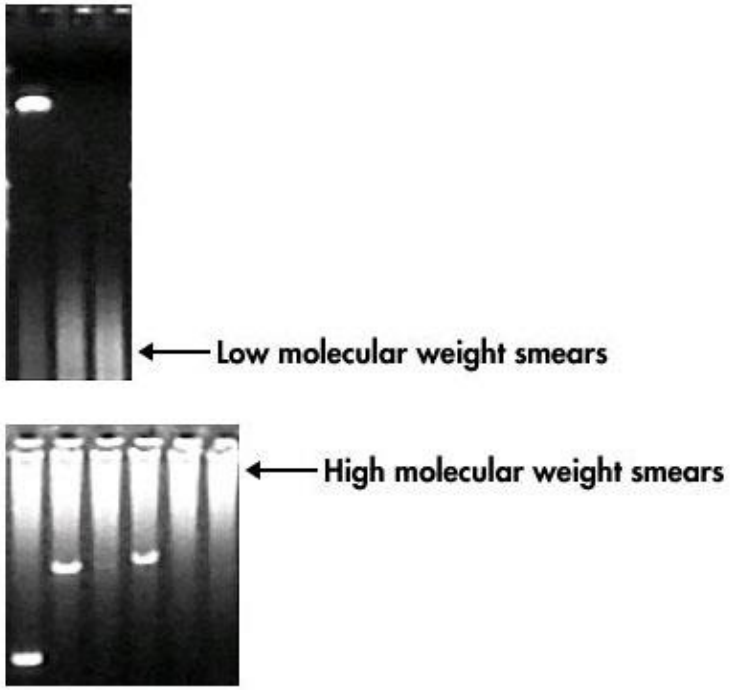
Reduce

- Long extension time.
- Annealing temperature too low. **Increase**
- Low $MgCl_2$ concentration.

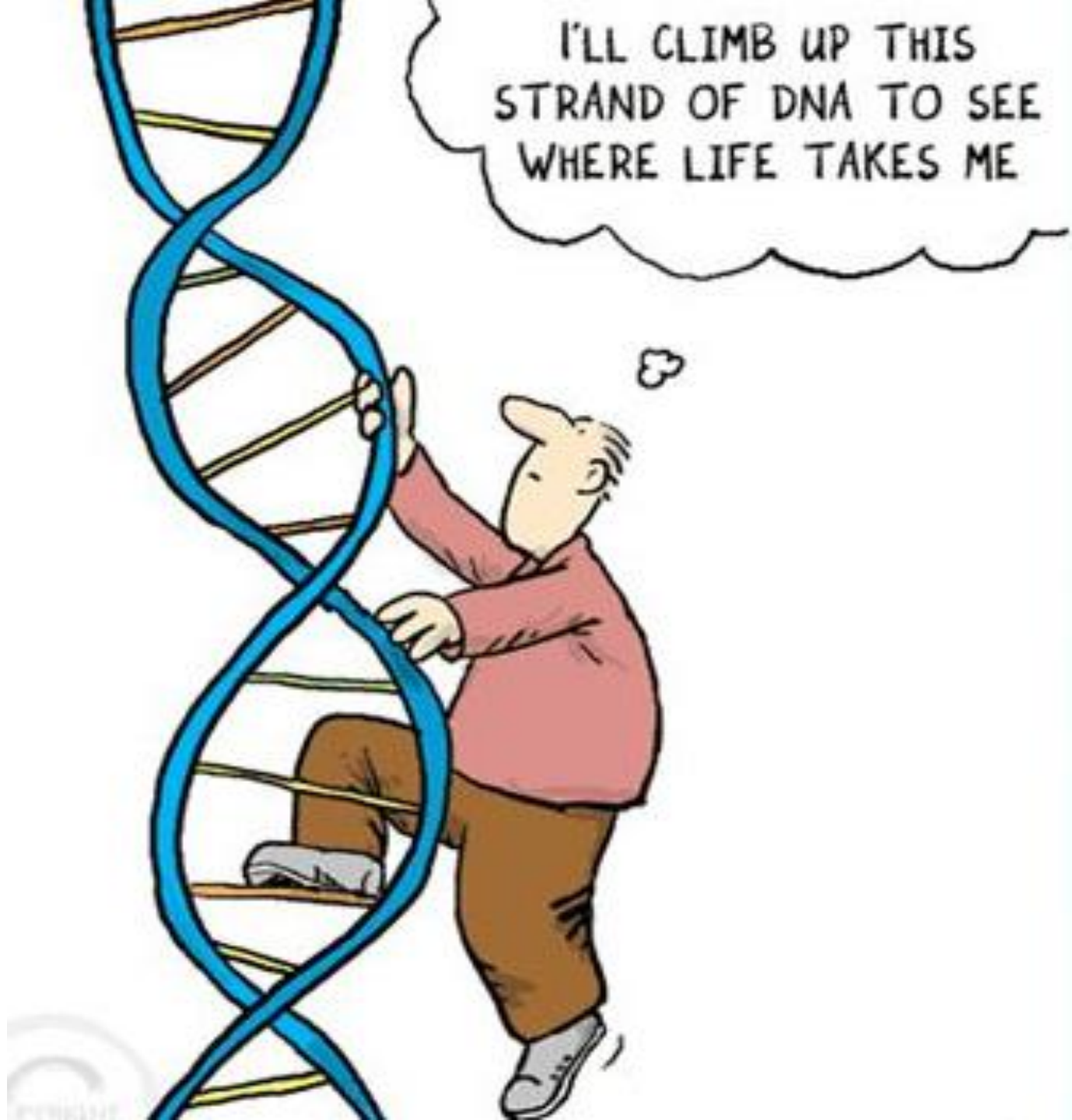
Increase

Problems and Solutions during PCR

Smeared bands



- Too much template
- Degraded template
- Contamination


I'LL CLIMB UP THIS STRAND OF DNA TO SEE WHERE LIFE TAKES ME