Near East University
DESAM Research Institute

Advanced Molecular Methods

Workshop

PCR technique and novel advances after COVID-19

Dr. Gökçe Akan

Polymerase Chain Reaction- DNA Photocopier

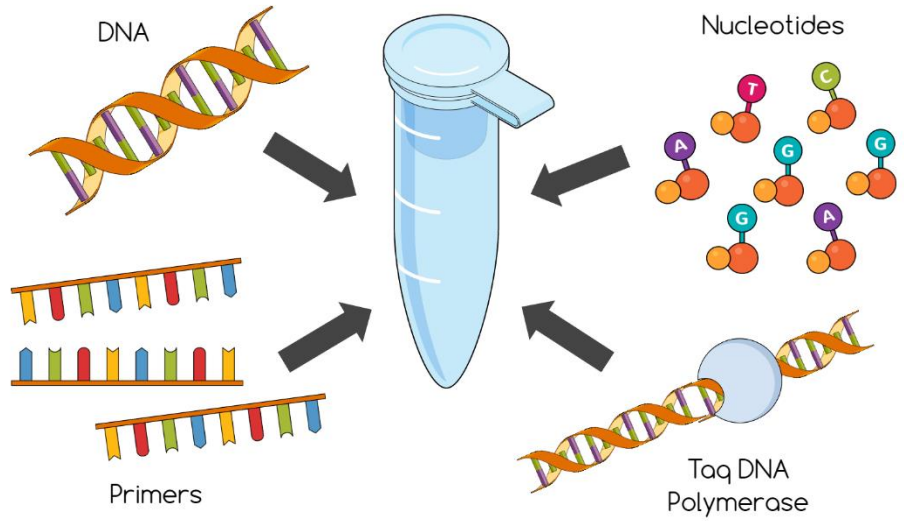
- PCR is an *in vitro* technique for the amplification of a region of DNA which lies between two regions of known sequence (primers).
  - ***in vitro* version of DNA Replication**
  - Multiple copies of specific DNA sequence
    - DNA photocopying

Types of PCR

- Conventional PCR
- Real-time PCR
- Quantitative real time PCR (Q-RT PCR)
- Reverse Transcriptase PCR (RT-PCR)
- Multiplex 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

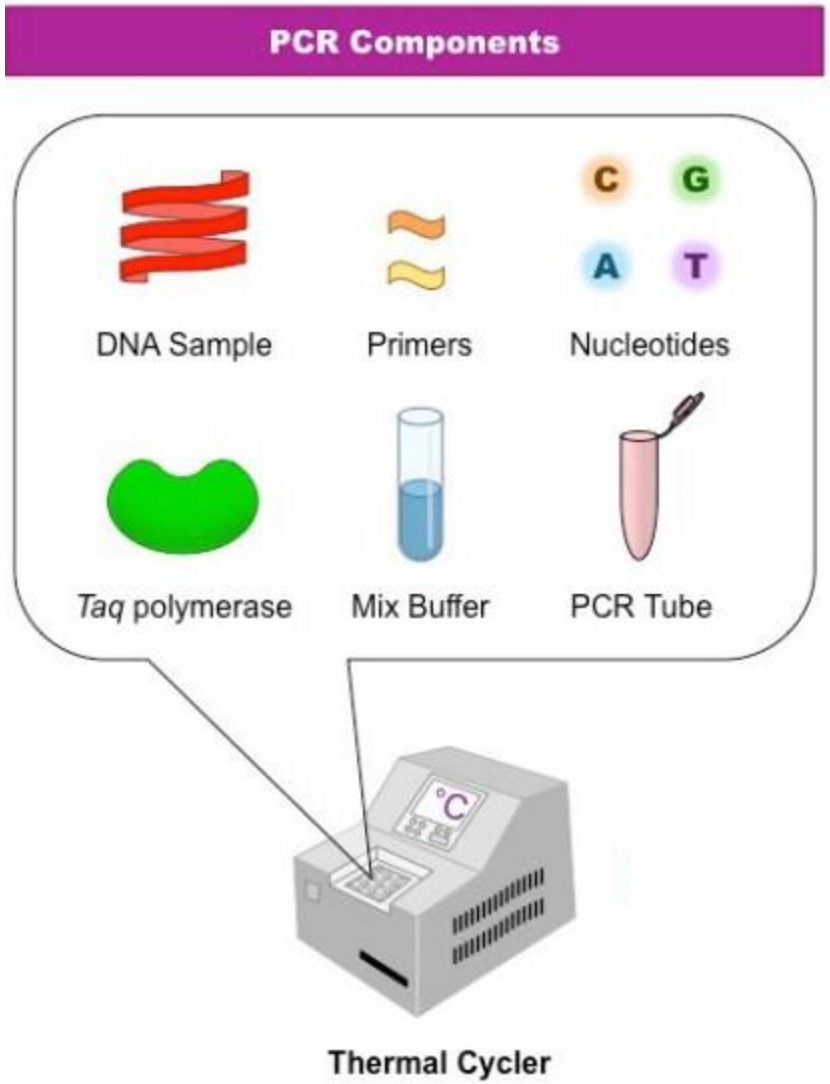
- Conventional PCR is a PCR technique that allows amplification of DNA sequences.
- It is used to detect qualitatively the amplification of the target genes.



Reagents for PCR

What we need in the laboratory:

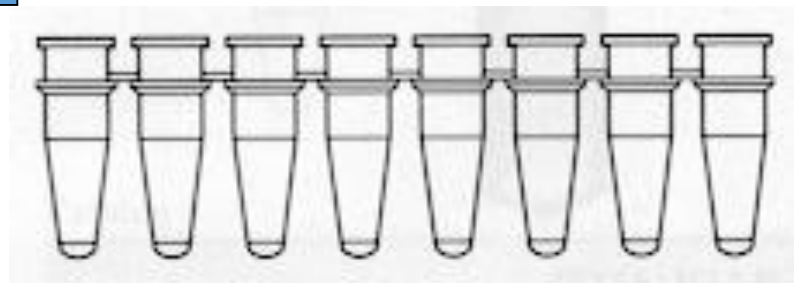
1. DNA template
2. Primers
3. DNA polymerase
4. Buffer
5. dNTPS (bases)
6. $MgCl_2$



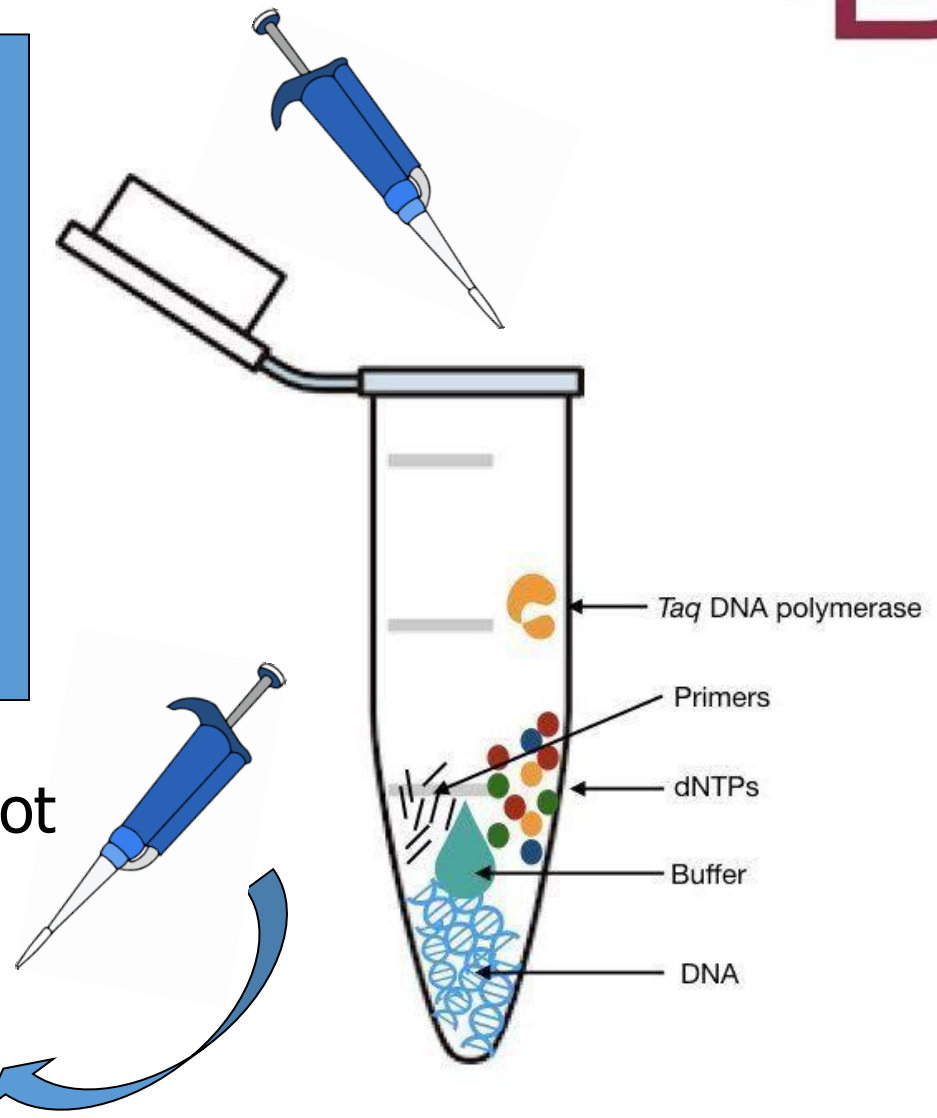
A Typical PCR Reaction

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
DNA Template	2.0 ul
Total Volume	20.0 ul

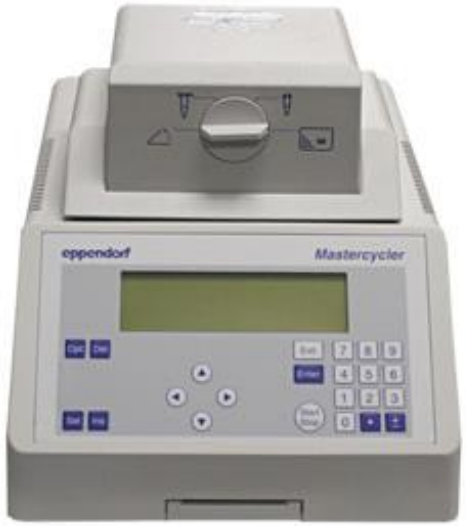
Add DNA as last step



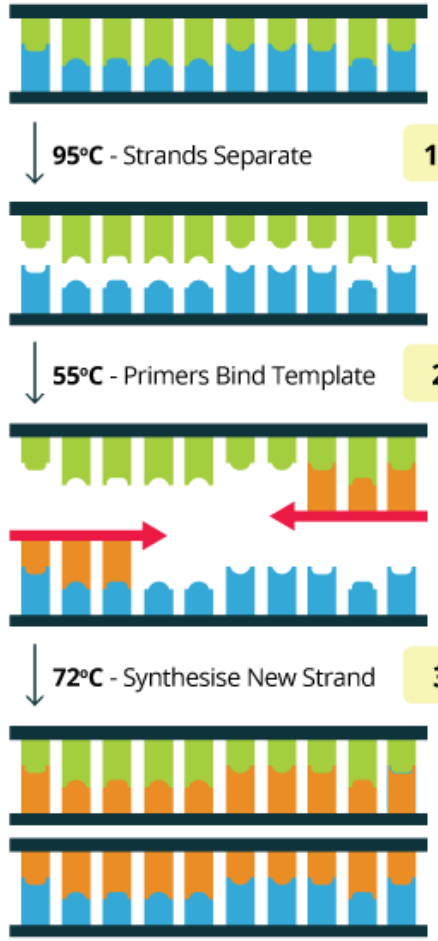
Aliquot



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

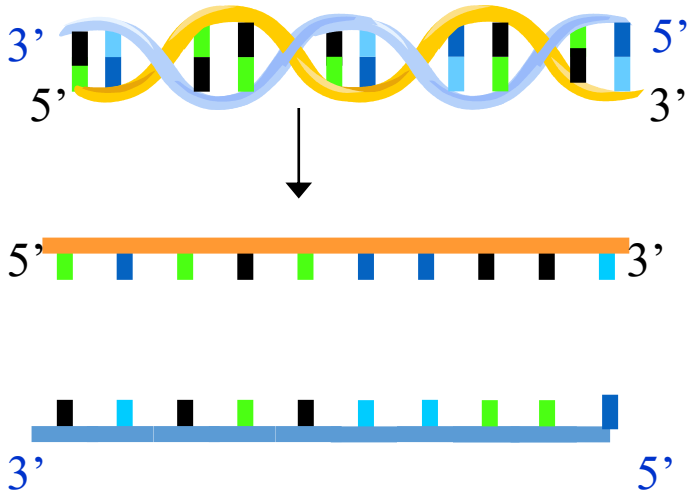
Elongation
72^{0}C
45-90secs

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

Steps in PCR

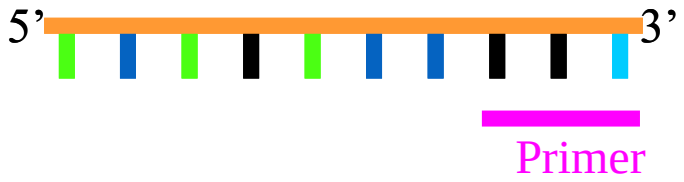
1. Denaturation

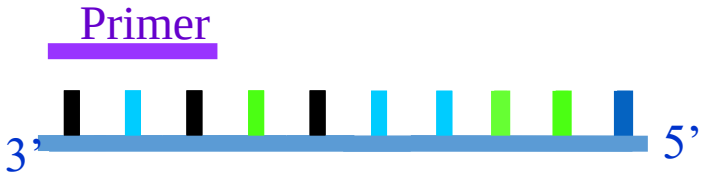
- Heat to separate double strands
- This occurs at 95 ºC



2. Annealing

- Primer **binds** to template sequence

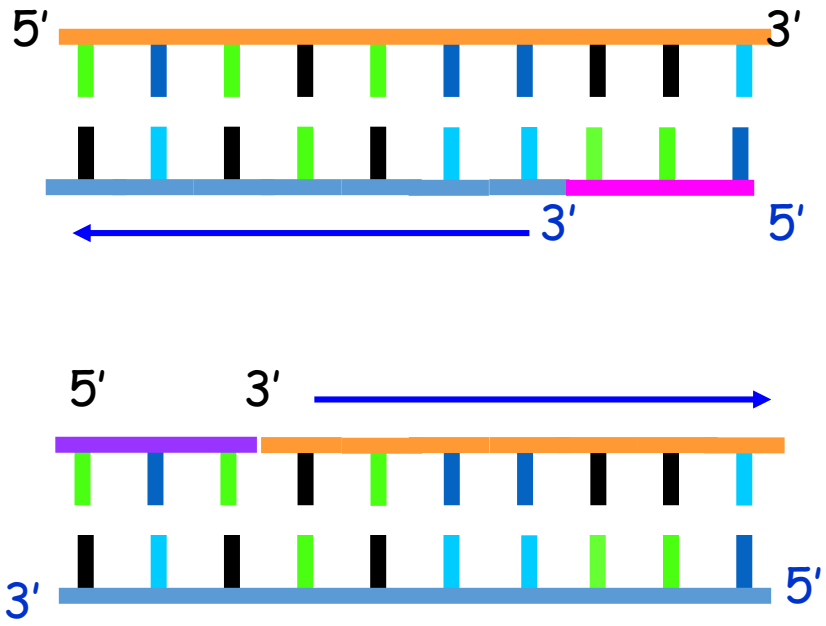




3. Elongation

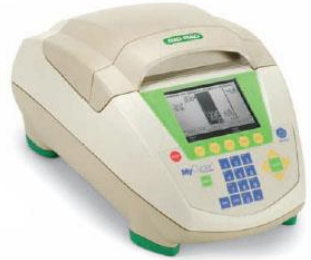
- Primer is extended with addition of **dNTPs** with ***Taq* polymerase**

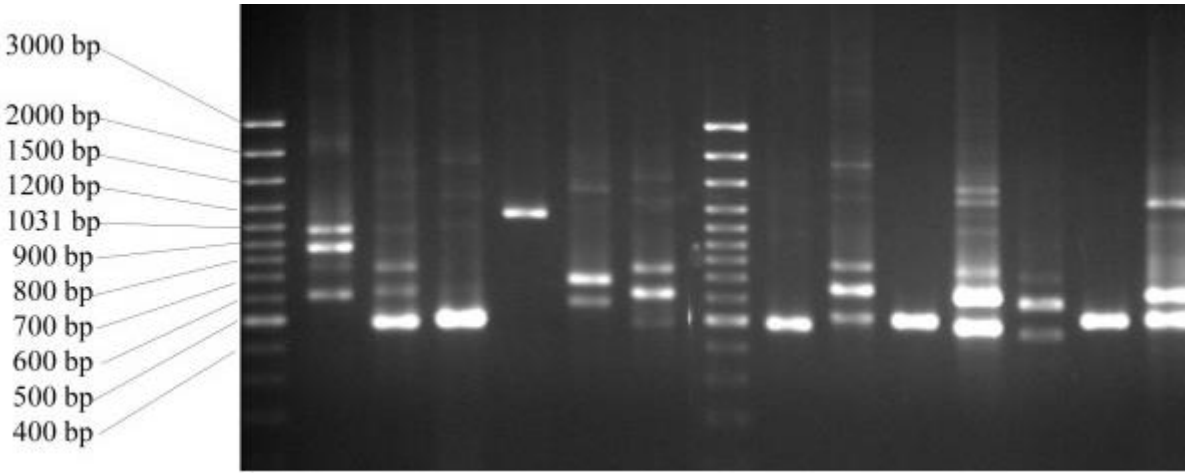
- the extension of the strand in the **5-3 direction** starting at the primers attaching the **appropriate nucleotide** (A-T, C-G)

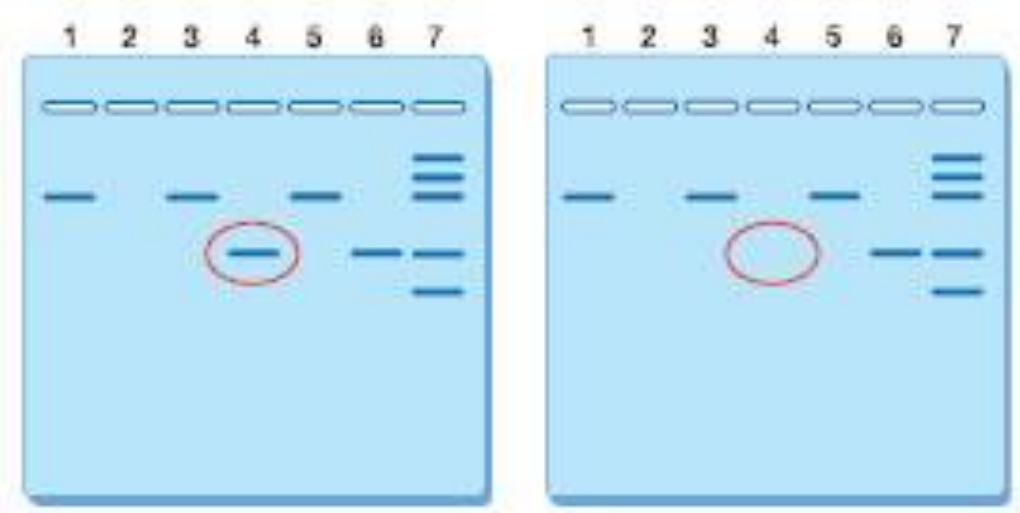


Gel Electrophoresis

- Contents of tubes are loaded onto an agarose gel.
- Fragmentation products of differing length are separated





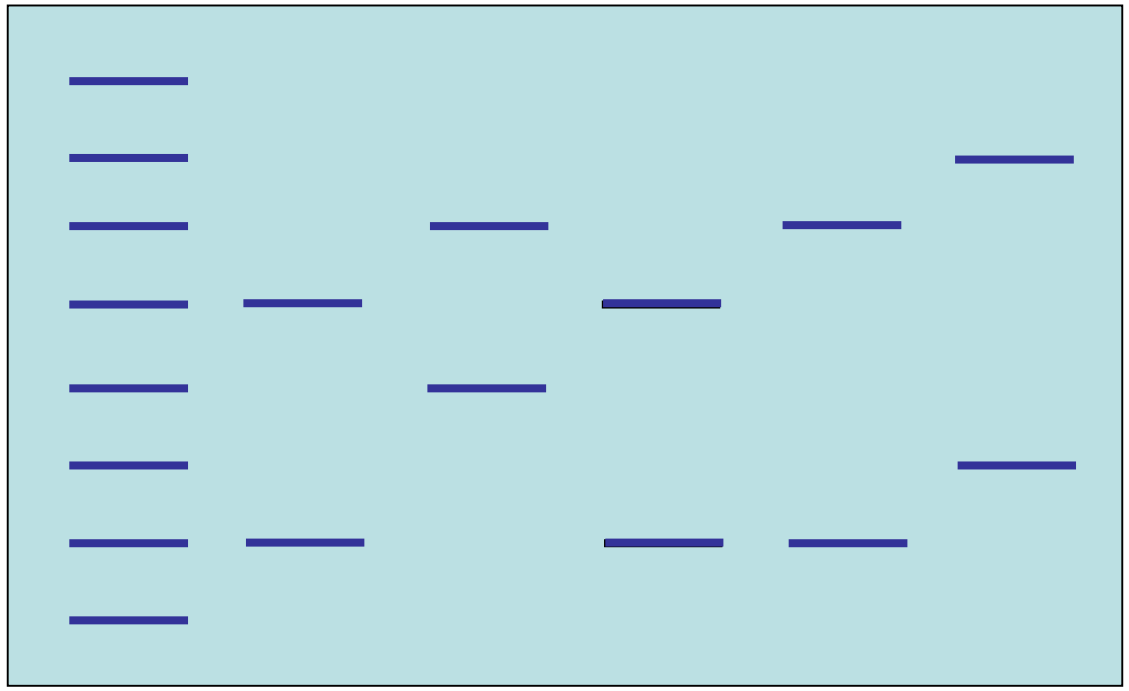


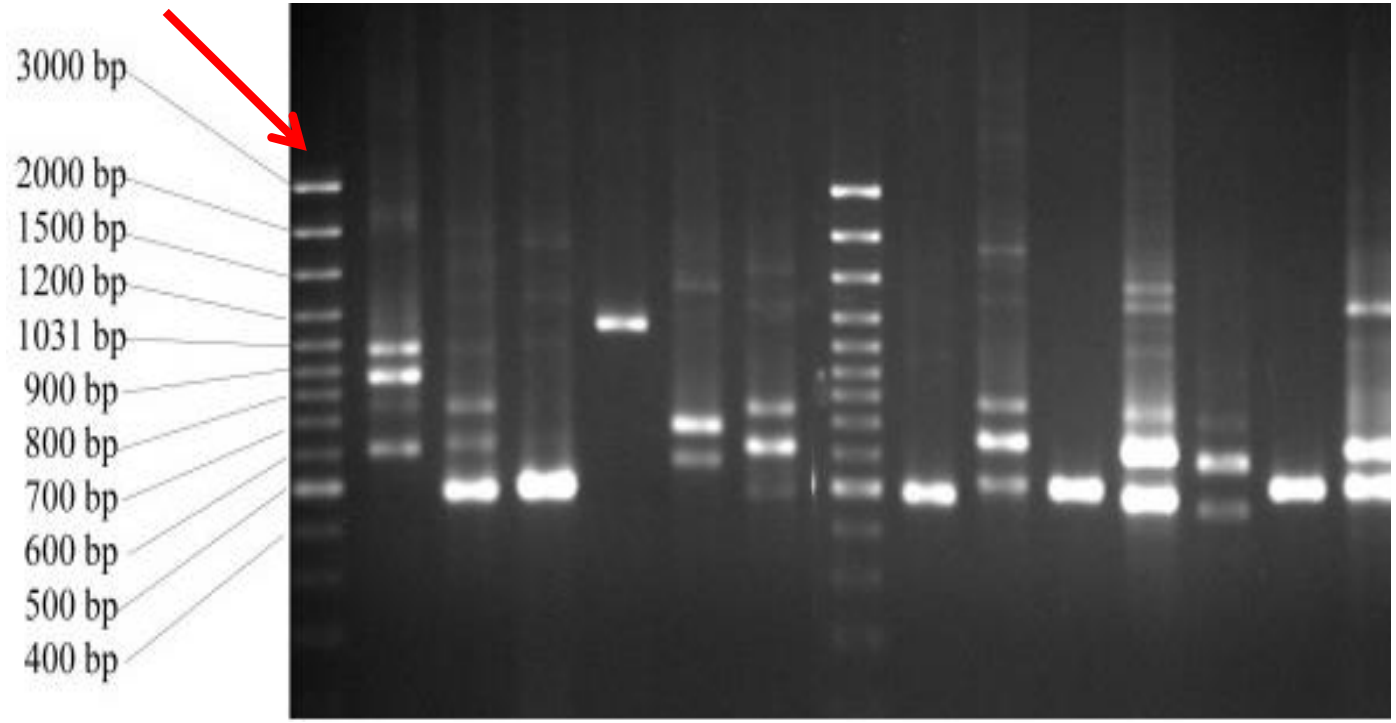
Separation of DNA fragments based on length

- 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:

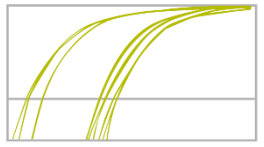


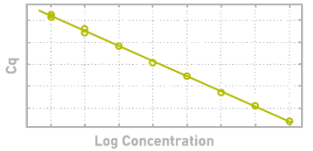


Bands can be compared against each other, and to known size-standards, to determine the presence or absence of a specific amplification product.

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.
- 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.

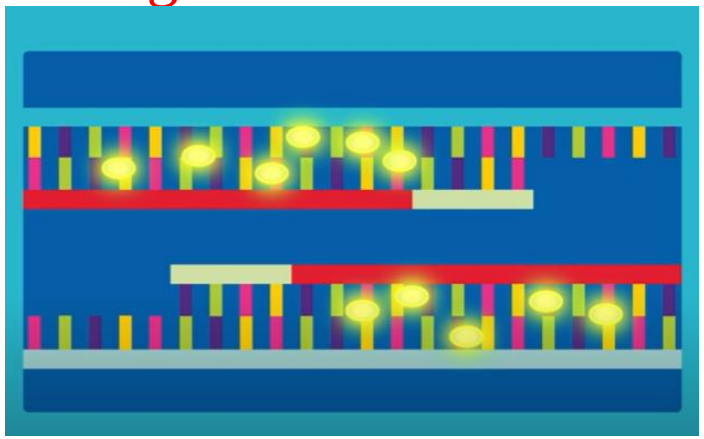




Which fluorescent dye using in Real-Time PCR

SybrGreen

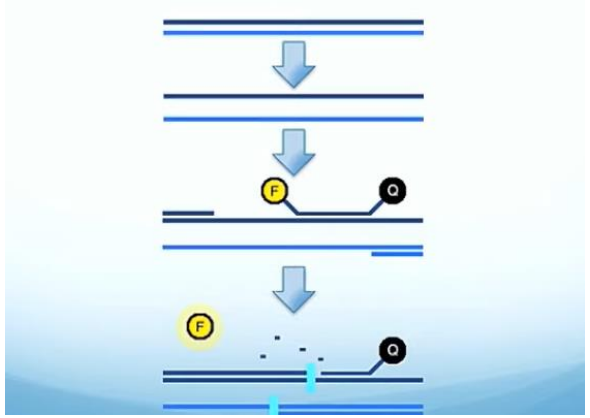
- DNA binding Dye
- Binds to dsDNA
- More binding → More fl.signal
- Unspesific
- Analysis with Melting Curve

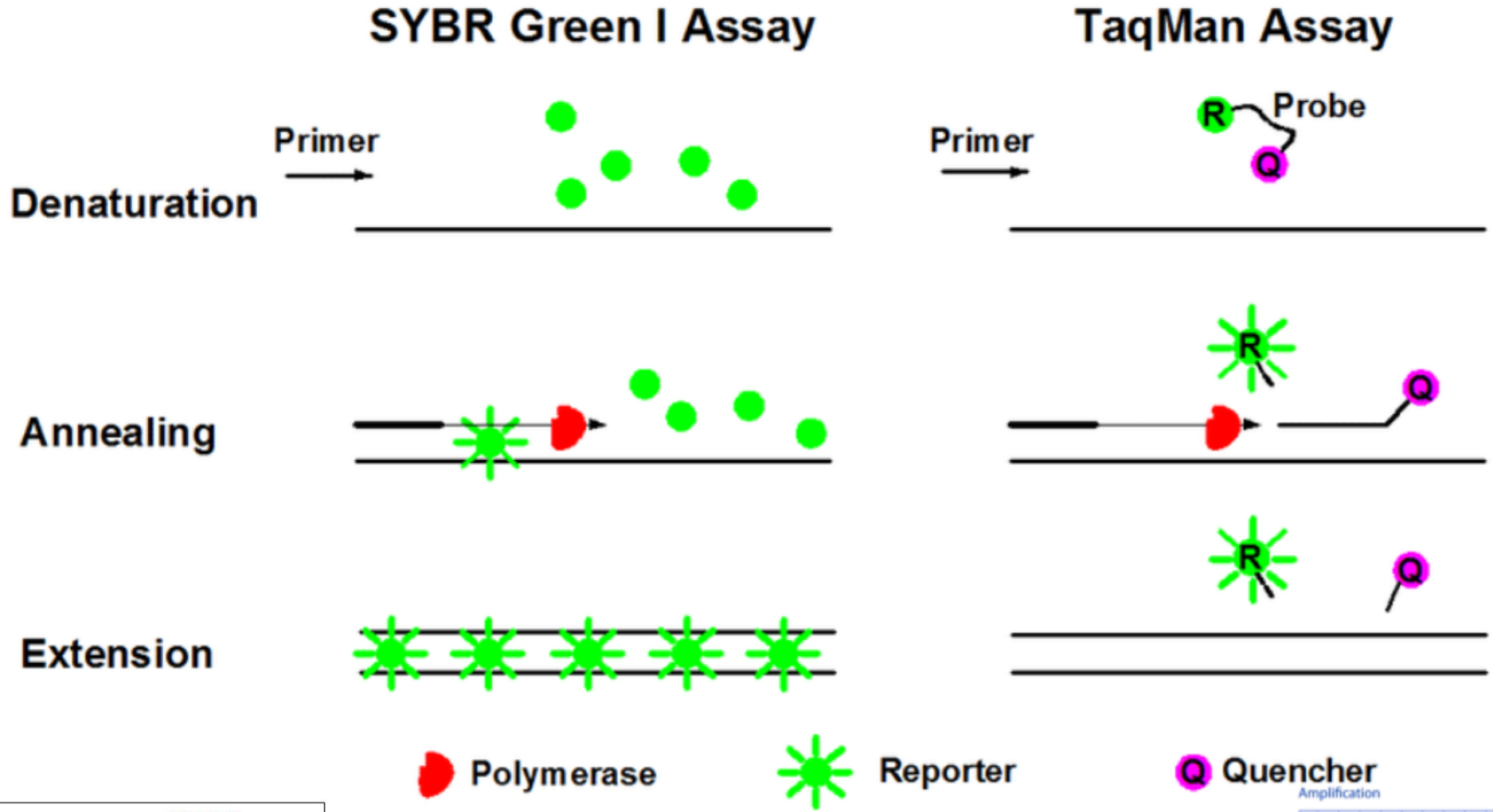


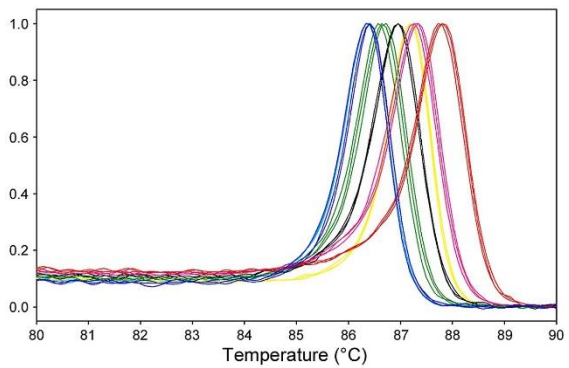
TaqMan Probe

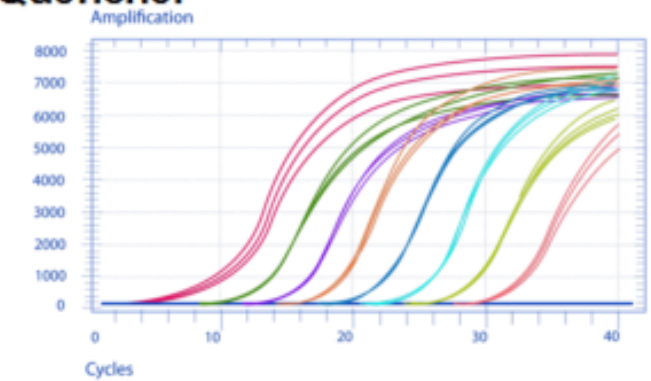
- A small segment of oligonucleotide
- Each TaqMan Probe has;
  - A Fluorescent molecule
  - A Quencher
- Binds to ssDNA
- Spesific for sequence
- Analysis with

Amplification Curve




SYBR Green I Assay
TaqMan Assay
Primer
Primer
R
Probe
Q
Denaturation
Annealing
Extension
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)

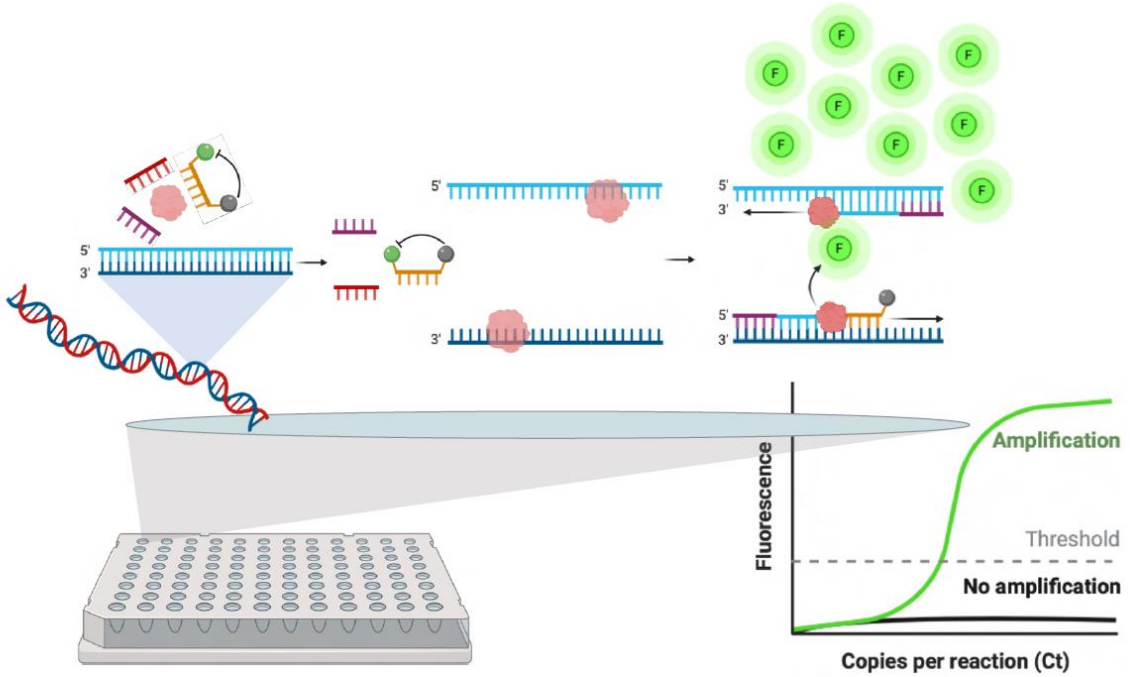

Amplification
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
Cycles

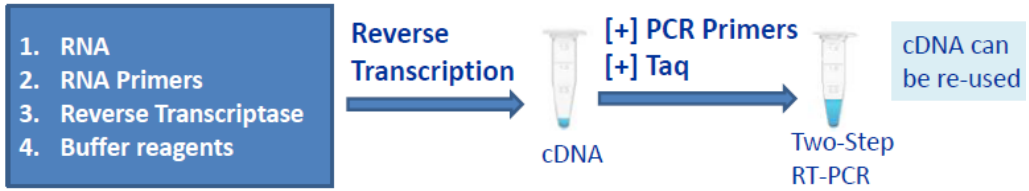
Real Time PCR

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

Quantitative real time PCR (Q-RT PCR)

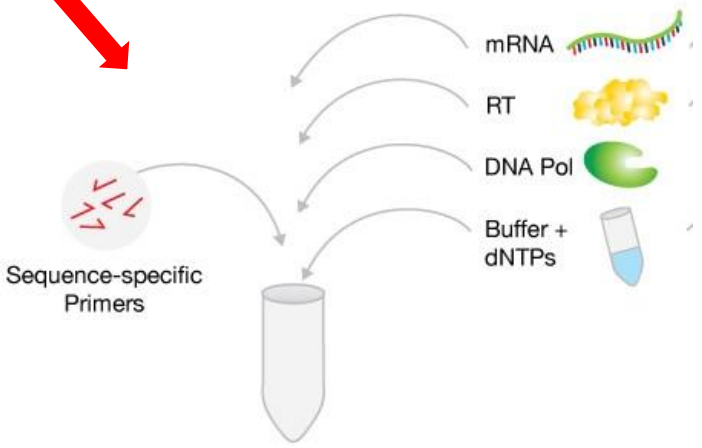
- Q-RT PCR is used to quantitatively measure the amplification of DNA using fluorescent dyes
- the starting material is double-stranded DNA (DNA or cDNA)





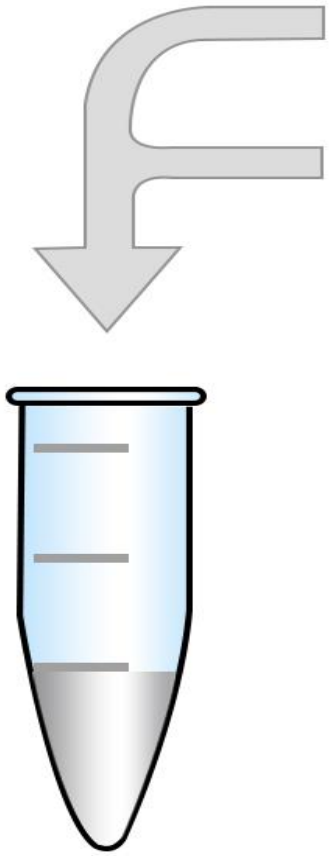
Q-RT PCR

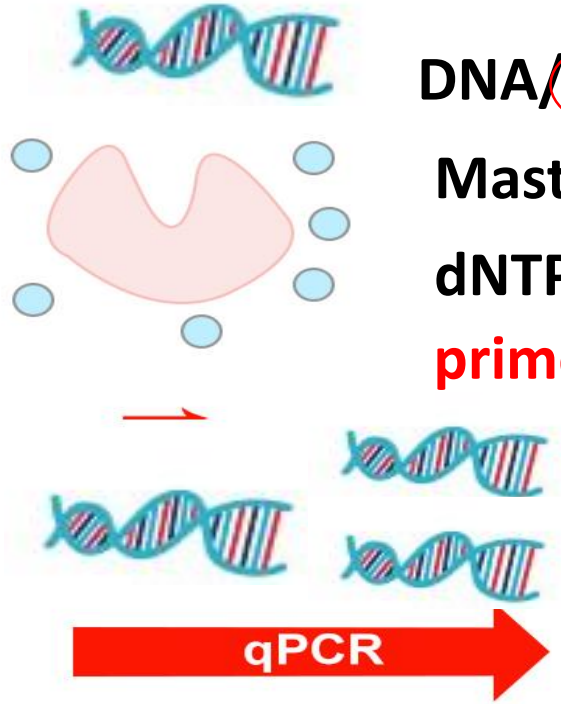
RNA

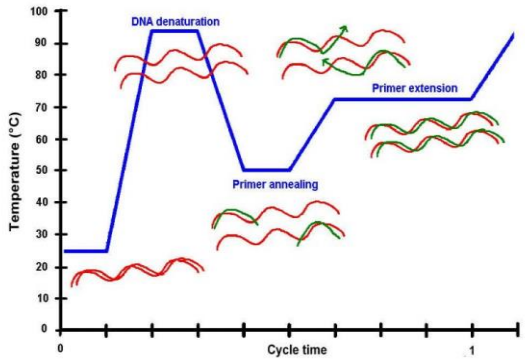


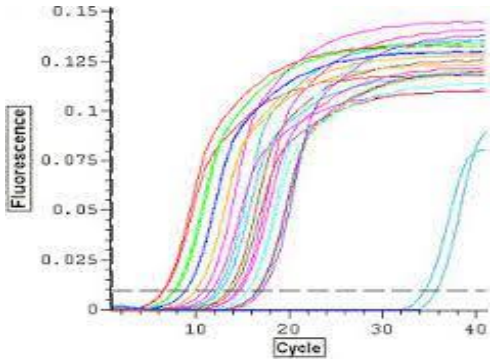
DNA/cDNA

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



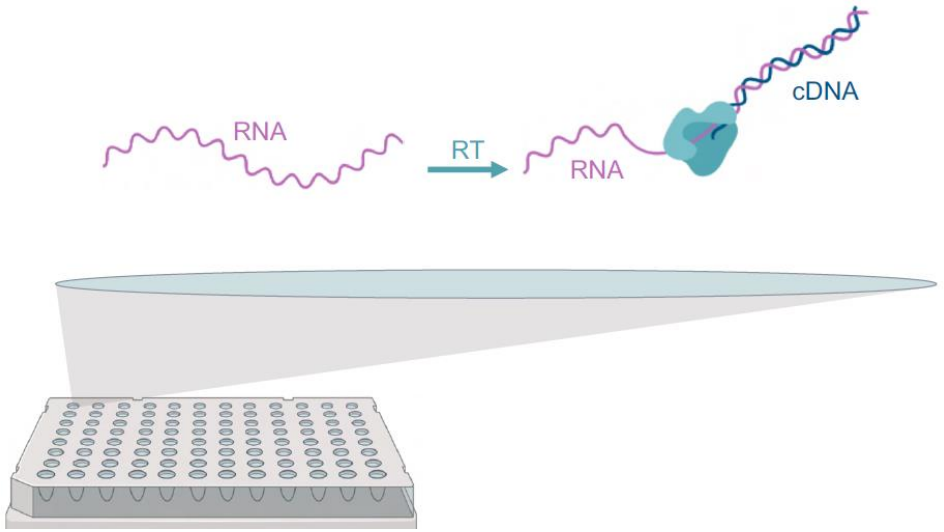






Reverse Transcriptase PCR (RT-PCR)

- RT-PCR is often confused with real-time PCR
- A technique commonly used in molecular biology to detect RNA expression
- RT-PCR is used to qualitatively detect gene expression through creation of cDNA transcripts from RNA



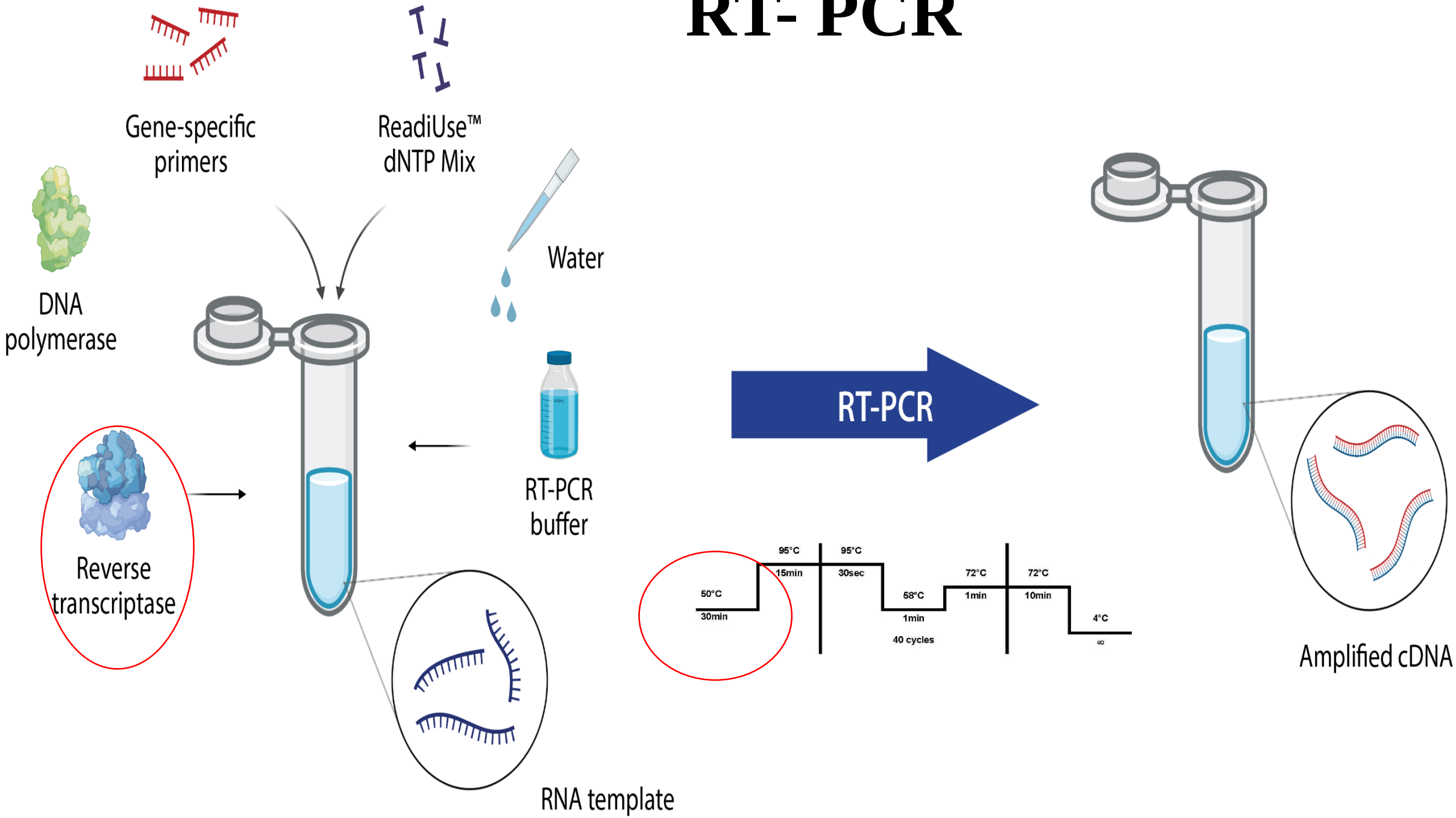
1. RNA
2. RNA and PCR Primers
3. Reverse Transcriptase
4. Buffer reagents
5. Taq polymerase

RT-PCR

One-Step RT-PCR

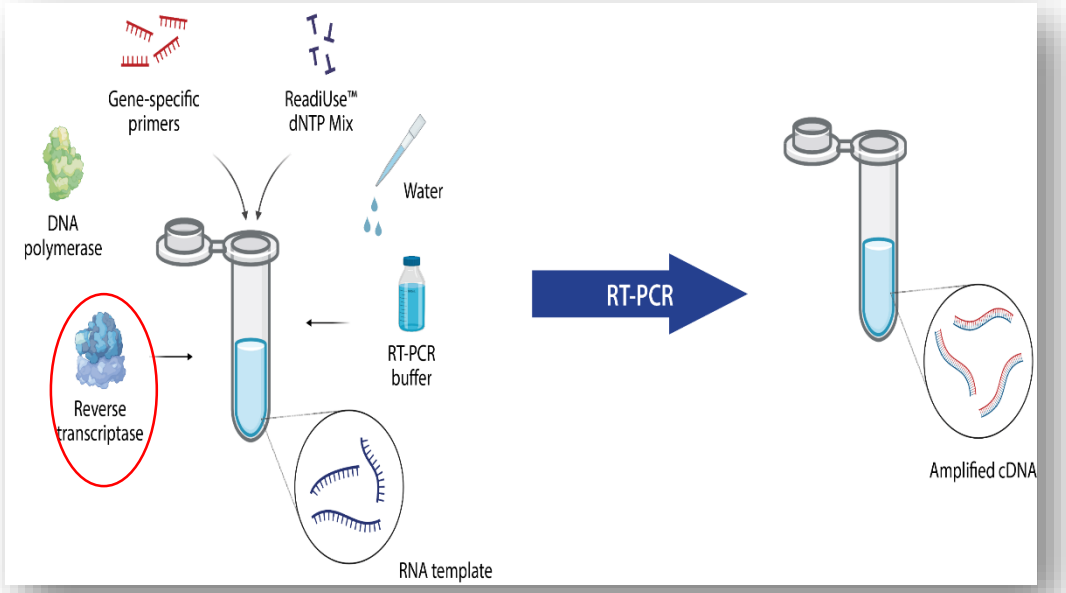
- Simplicity
- Convenience
- Minimizes contamination
- Not flexible

RT- PCR

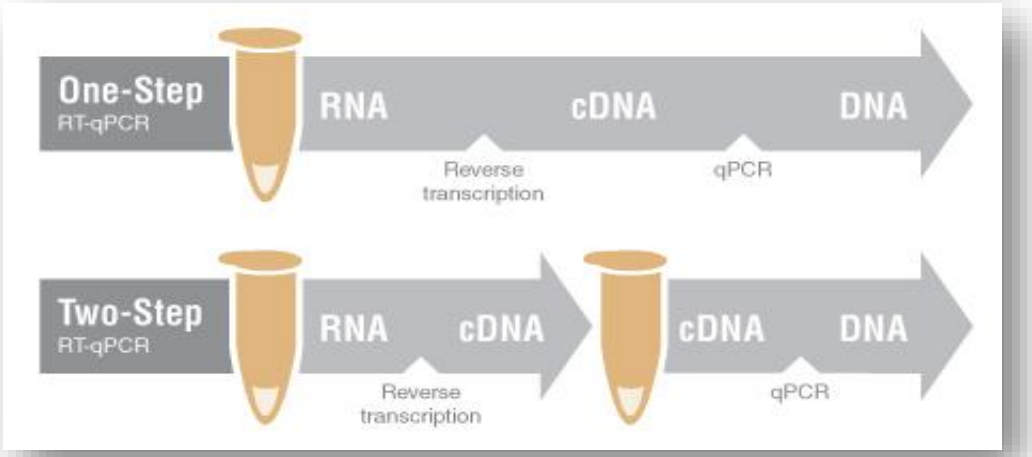

Gene-specific primers
ReadiUse™ dNTP Mix
DNA polymerase
Water
RT-PCR buffer
Reverse transcriptase
RNA template
RT-PCR
95°C
15min
95°C
30sec
50°C
30min
58°C
1min
72°C
1min
72°C
10min
4°C
∞
40 cycles
Amplified cDNA

One step vs Two step

One Step



Two Step

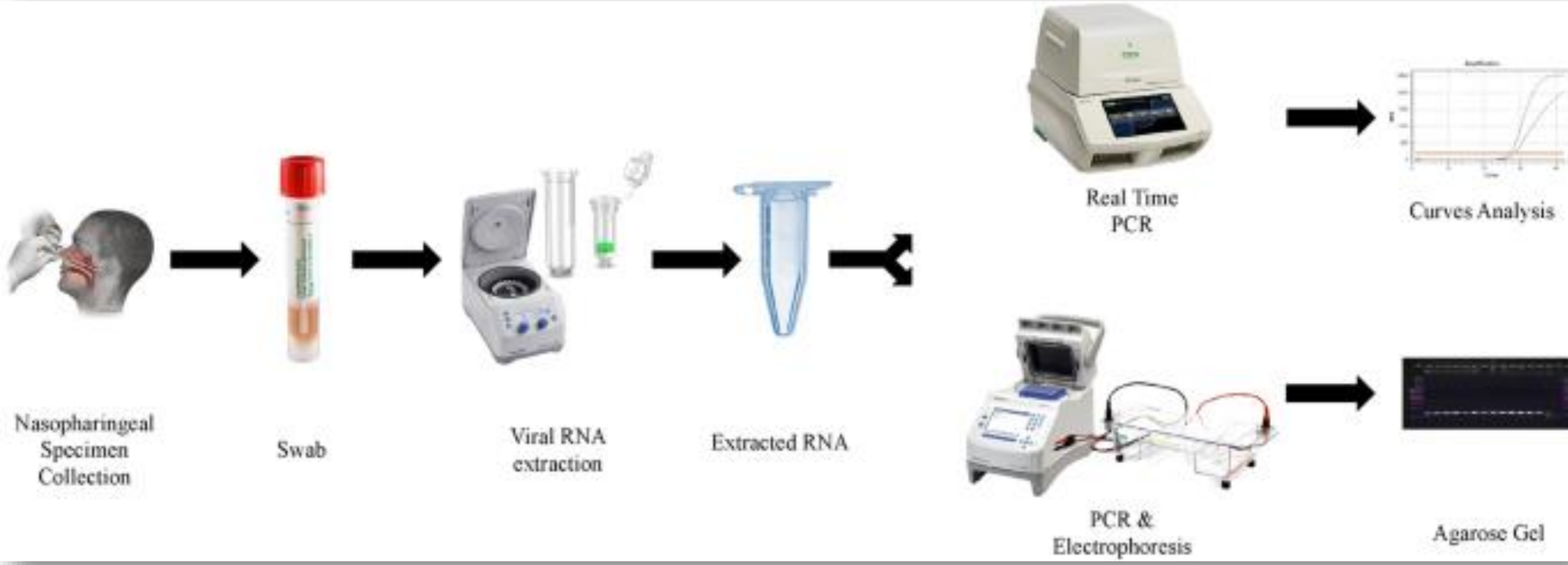


- More pipetting step
- Time consuming
- Contamination risks
- Reduce errors
- Simple

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

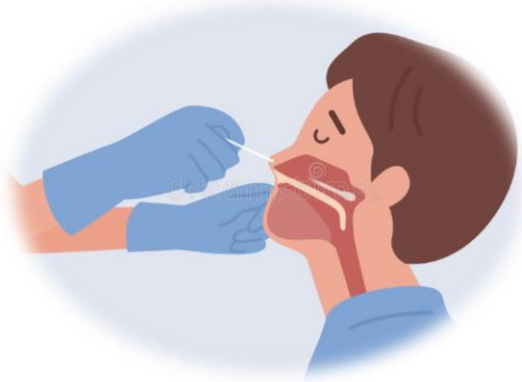
COVID-19 & PCR

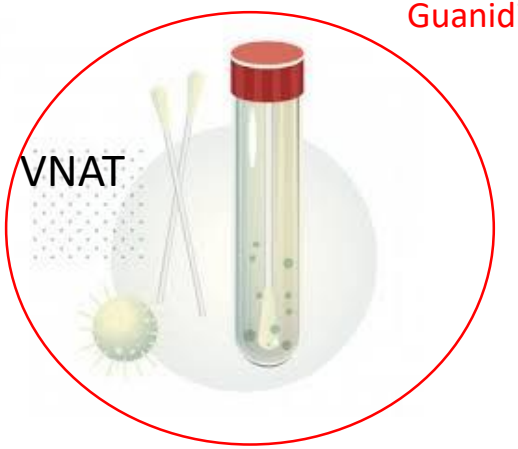

Nasopharingeal Specimen Collection
Swab
Viral RNA extraction
Extracted RNA
Real Time PCR
Curves Analysis
PCR & Electrophoresis
Agarose Gel

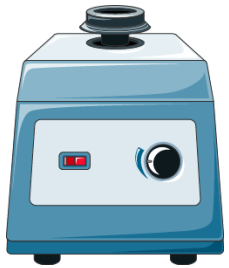
COVID-19 & PCR

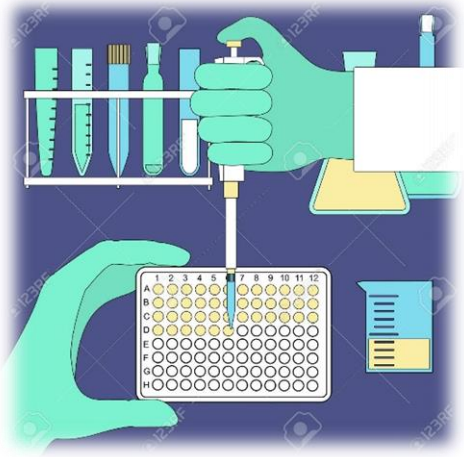
Polyethyleneimine coated tetradecyl dimethyl benzyl
Ammonium chloride
Tween-20

Guanidinium thiocyanate

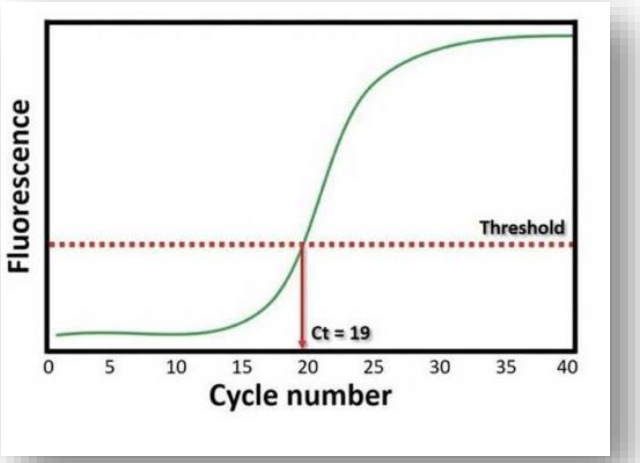


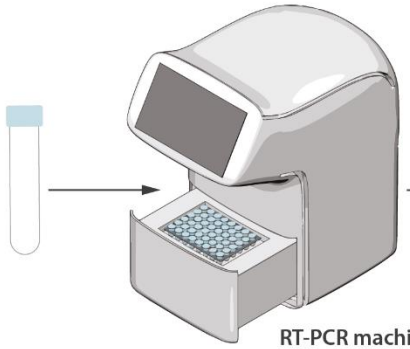


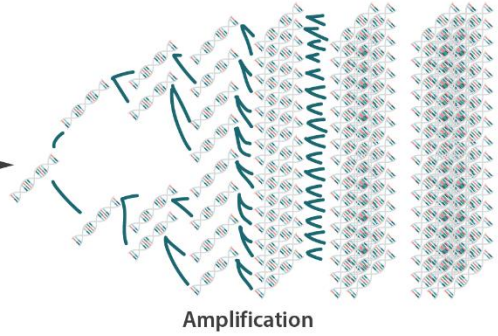




Bovine Serum Albumin







PCR-Future

S/N	Manufacturer	Viral gene targe
1	China, CDC	ORF1b, N
2	India, TruPCR	E, RdRP, N
3	Thailand, NIH	N
4	Japan, NIID	SP, Pancorona &
5	Hong Kong SAR, HKU	ORF1b-nsp14, N
6	Germany, Charité	RdRP, E, N
7	France, IIP	RdRP (2 targets)
8	USA, CDC	N gene (3 target

CDC; centre for disease control and prevention, E; envelop university of Hong Kong, IIP; institut pasteur paris, N; nucleoc national institute of health, NIID; national institute of infectious structural protein, ORF; open reading frame, RdRP; RNA polymerase, SP; spike protein, USA; United States of America.

test approved for COVID-19 is based on everse transcriptase polymerase chain ime RT-PCR). Real–time RT-PCR for nosis is a quantitative test that amplifies enes for RNA virus detection. Numerous CR kits developed by different countries, companies are available (FIND, 2020). A

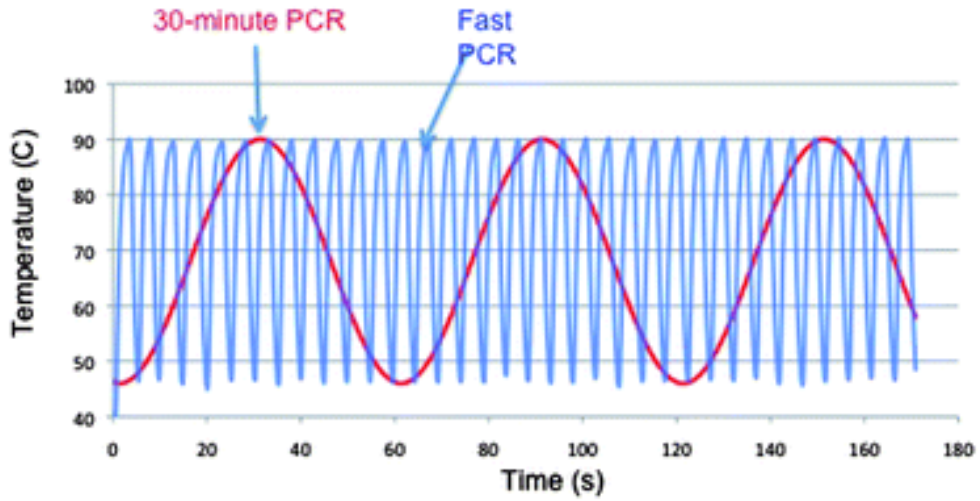
artificially create is a means by v compared bet genomics, uniq are identified. T determine the establish wheth

SCIENCE

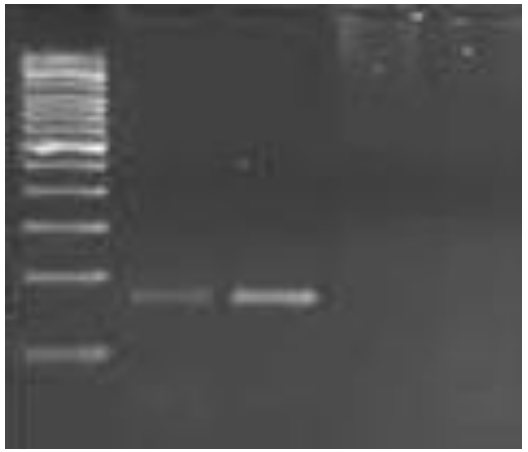
COVID: Chinese researchers develop 4-minute PCR test

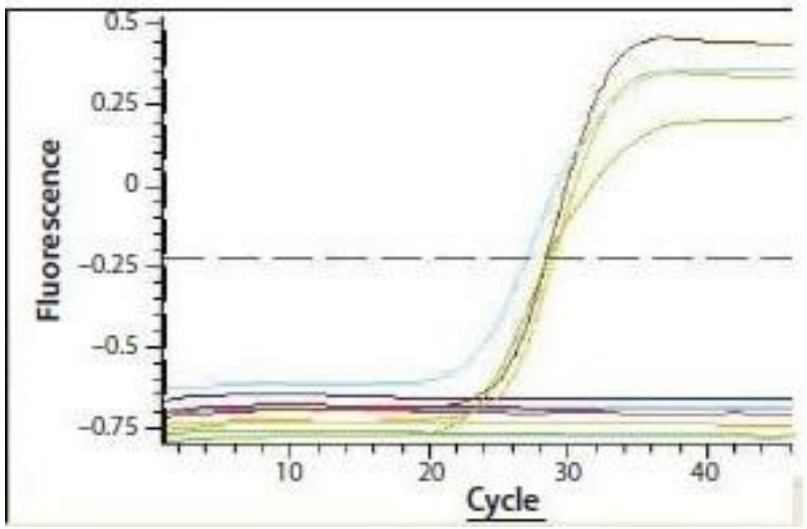
Researchers at Fudan University in Shanghai say they have developed a technology combining the speed of the rapid antigen test with the accuracy of PCR testing.

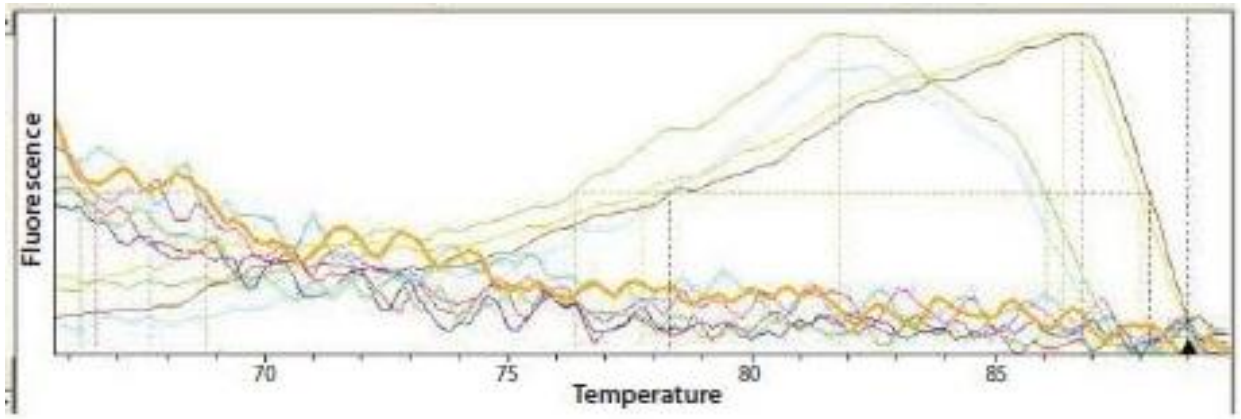
Feb.2022



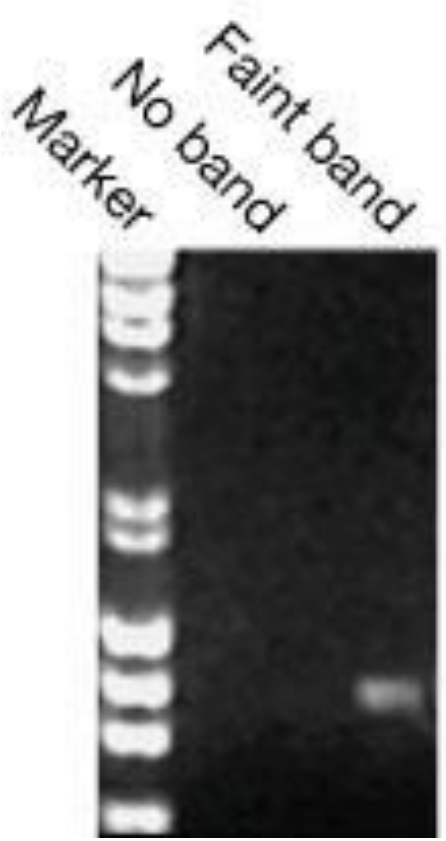
Problems and Solutions during PCR







Problems and Solutions during PCR

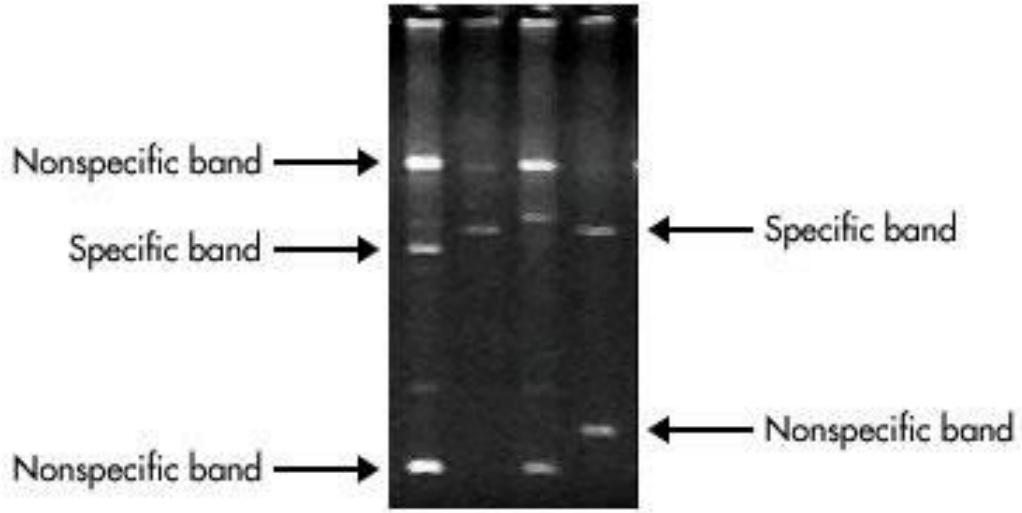


No band or faint band

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

Problems and Solutions during PCR

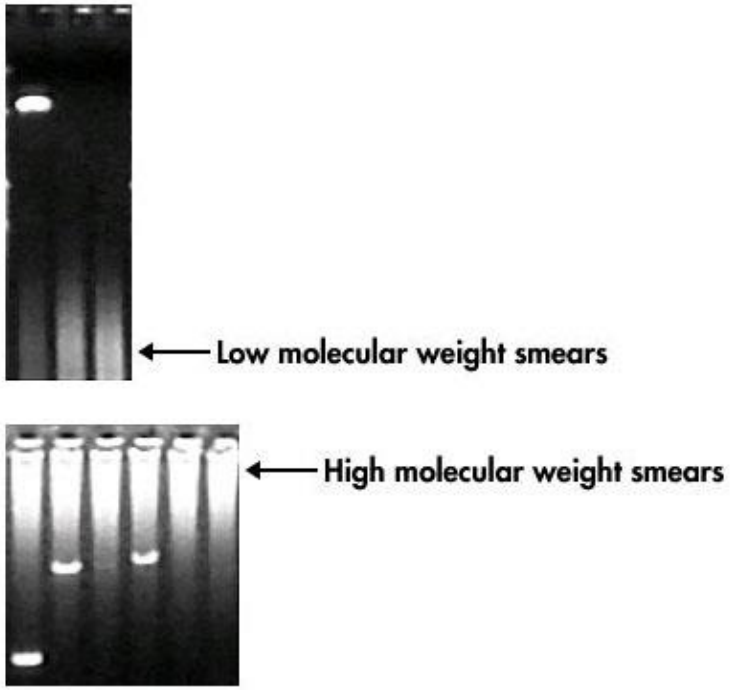
Nonspesific band or primer dimers



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

Problems and Solutions during PCR

Smeared bands



- Too much template
- Degraded template
- Contamination

Thank you for your attention!