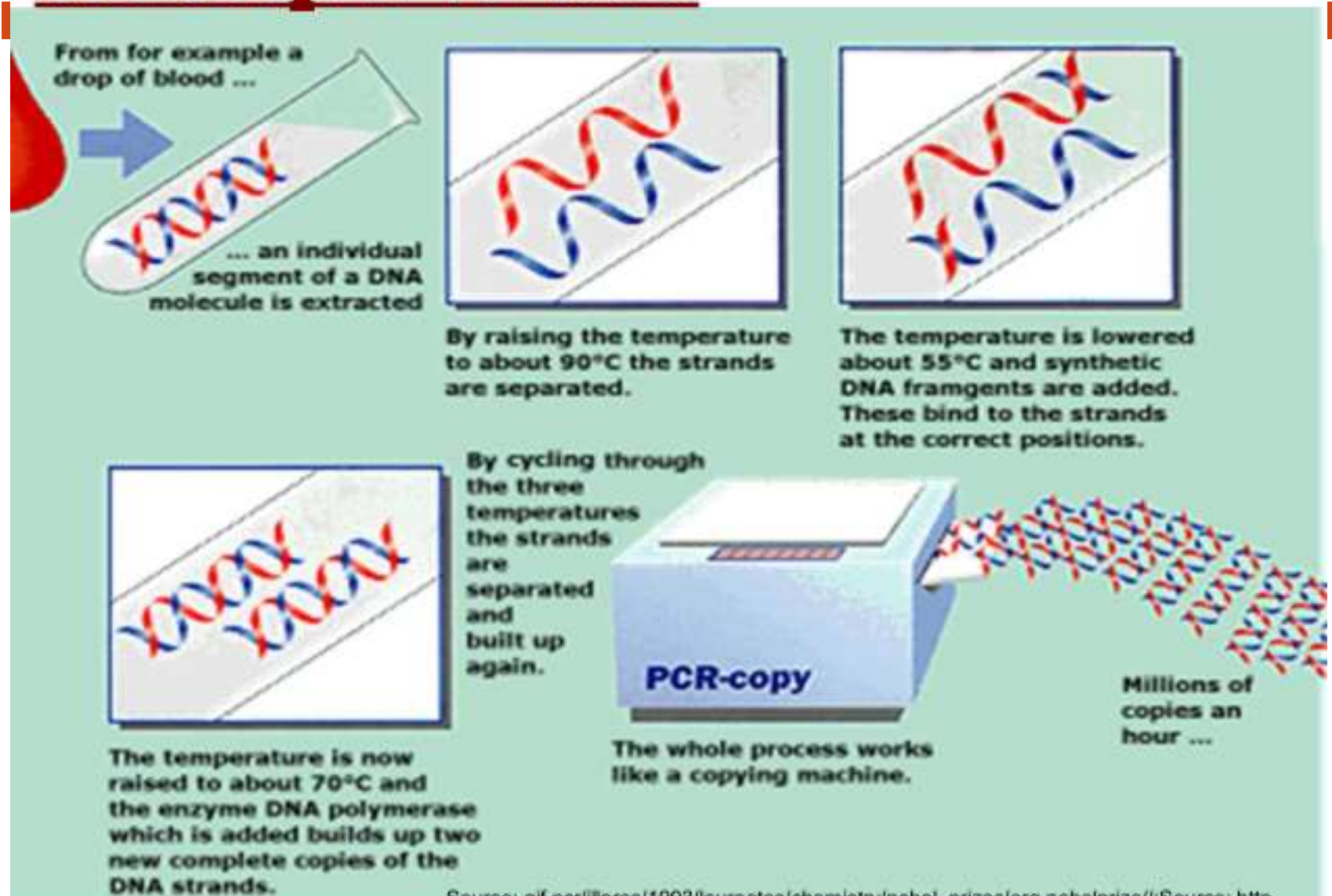


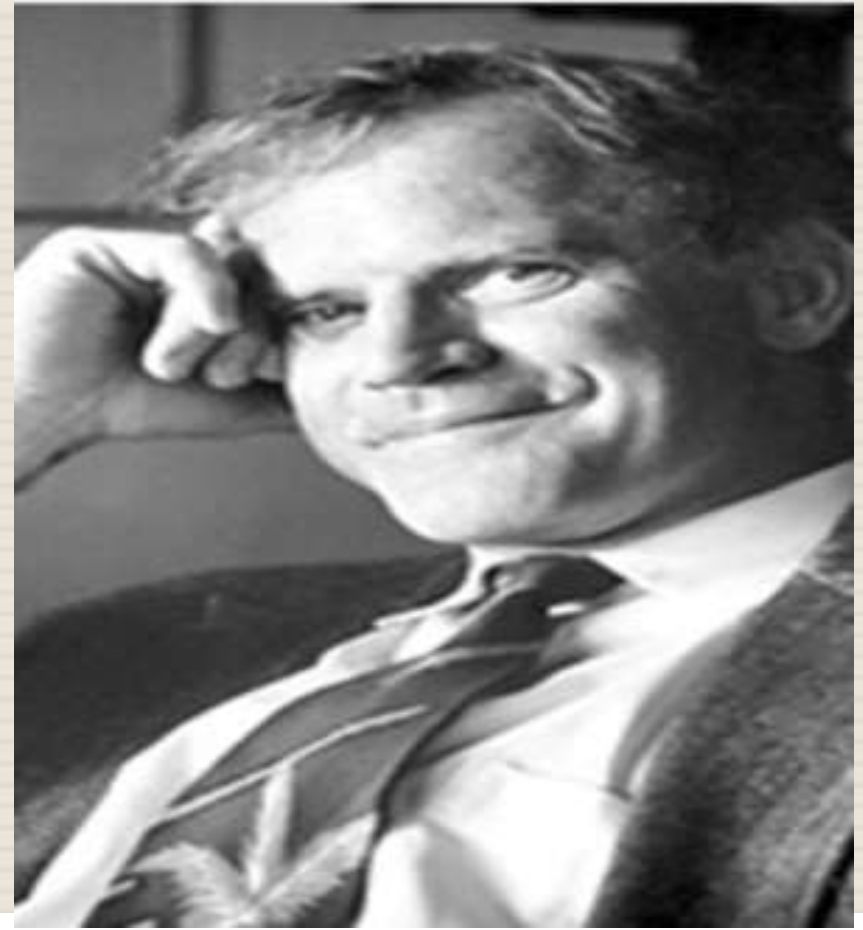
POLYMERASE CHAIN REACTION



POLYMERASE CHAIN REACTION

Nobel prize 1993



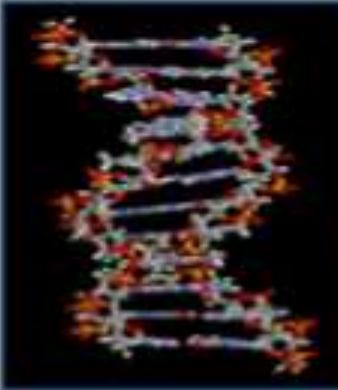
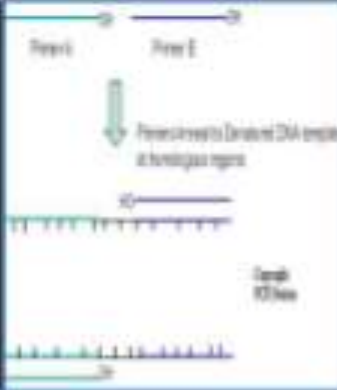


- ☐ Coping Machine for DNA Molecule
- ☐ Invented by Kary Mullis and his colleagues in the **1983**

Polymerase Chain Reaction

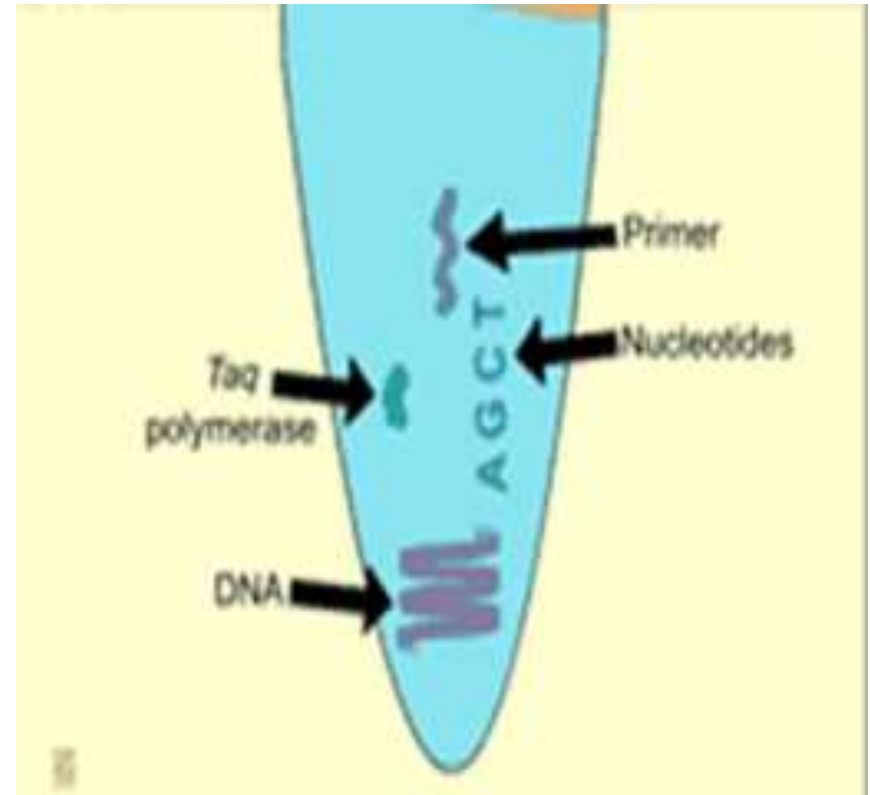
- **PCR:** Technique for in vitro (test tube) amplification of specific DNA sequences via the temperature mediated. DNA polymerase enzyme by simultaneous primer extension of complementary strands of DNA.
- **PCR:** This system for DNA replication that allows a "target" DNA sequence to be selectively amplified, several million-fold in just a few hours.

PCR reaction components

				
Template	Two primers (forward and reverse)	Four (dNTPs) A, G, C, T	Buffer system containing magnesium Mg^{2+}	DNA polymerase

PCR reaction components

- ❑ DNA template
- ❑ Two primers
- ❑ Four normal deoxynucleosides triphosphates
- ❑ Buffer system
- ❑ DNA polymerase I



DNA Template

Integrity

- ☐ **High molecular weight**

Purity

- ☐ **Pure**

Amount

- ☐ **Human genomic DNA should be up to 500ng**
- ☐ **Bacterial DNA 1-10ng**
- ☐ **Plasmid DNA 0.1-1ng**

Primers

- Typical primers are 18-28 bases in length,
- Having 40- 60% GC composition,
- Have a balanced distribution of G/C and A/T rich domains,
- The calculated T_m for a given primer pair should be balanced (difference no more than 5 °C),
- T_m =Temperature at which 2 strands of the duplex dissociate
- Primer concentration between 0.1 and 0.6 μM are generally optimal,
- Have a cytosine and guanine at the 3'-end because they form three hydrogen bonds with the matrix molecules, making a more stable hybridization

Calculation of Melting Temperature

$$T_m = 2\text{ }^{\circ}\text{C} \times (\text{number of A and T bases}) + 4\text{ }^{\circ}\text{C} \times (\text{number of G and C bases}).$$

Optimal annealing temperature are 5-10 $^{\circ}\text{C}$ lower than T_m values of the primers .

Four Normal Deoxynucleosides Triphosphate

- Final concentration of dNTPs should be 50-500 μM (each dNTP). Usually included at conc. of 200 μM for each nucleotide.
- Always use balanced solution of all four dNTPs to minimize polymerase error rate.

Buffer System Containing Magnesium

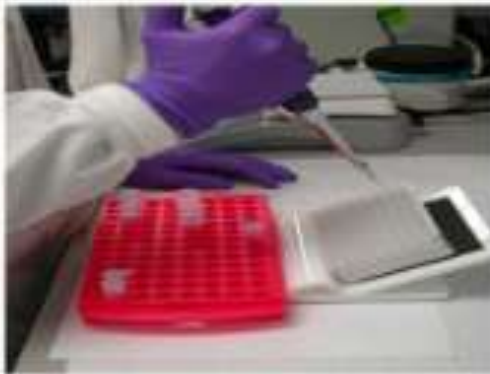
The standard PCR buffer contains:

- ❑ **Tris-HCl 10mM (10-50mM)** for dissolution of nucleic acids
pH 8.3 (pH 8.3-8.8 at 20C°)
- ❑ **KCl 50mM** promotes specificity of hybridization
- ❑ **MgCL2 1.5mM (0.5-10mM)** for stabilizing of complex between primers and matrix and for increasing of exit the special product of PCR
- ❑ **Gelatin or Bovine Serum Albumin 100 µg/ml**
frequent unfreezing-freezing at the temperature -20C

DNA Polymerase

- ❑ The most widely characterized polymerase is that from *Thermus aquaticus* (Taq), Thermophilic bacterium lives in hot springs and capable of growing at 70 -75 C°,
- ❑ Consist of a single polypeptide chain has a molecular weight of 95 Kd, and has an optimum polymerization temperature of 70 – 80 C° (72 C°).
- ❑ 0.5 – 2 units/50µl reaction. Too little will limit the amount of products, while too much can produce unwanted non specific products.

PCR



All one has to do is to mix the contents in the test tube and

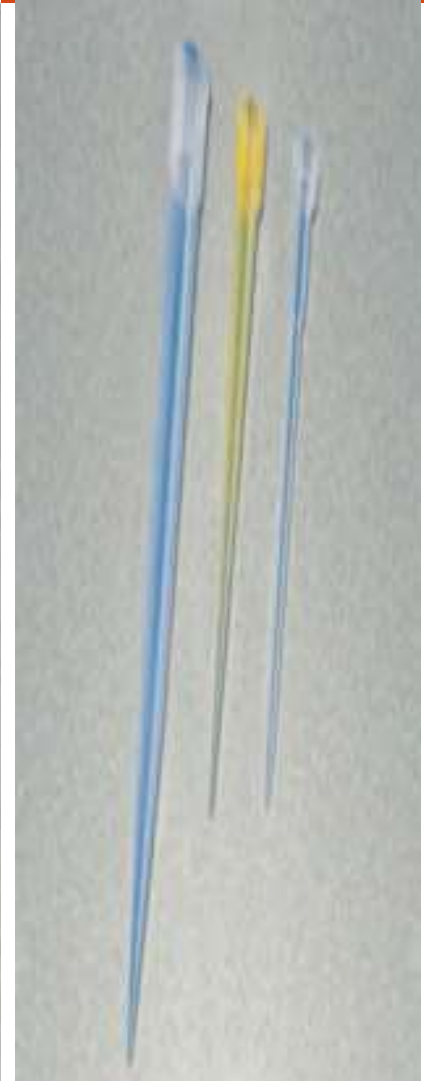


Seal the tube and then place it in a thermal cycler.



Thermal cycler

AVOIDING CONTAMINATION



1-Sample Handling

- ❑ Use sterile techniques and always wear fresh **gloves**,
- ❑ Always use new or **sterilized** glassware, plasticware and pipettes to prepare the PCR reagents and template DNA,
- ❑ Autoclave and sterilize all **reagents** and solution,
- ❑ Have your own set of PCR reagent and Solution (store in small **aliquots**),
- ❑ Positive and negative control should be included.

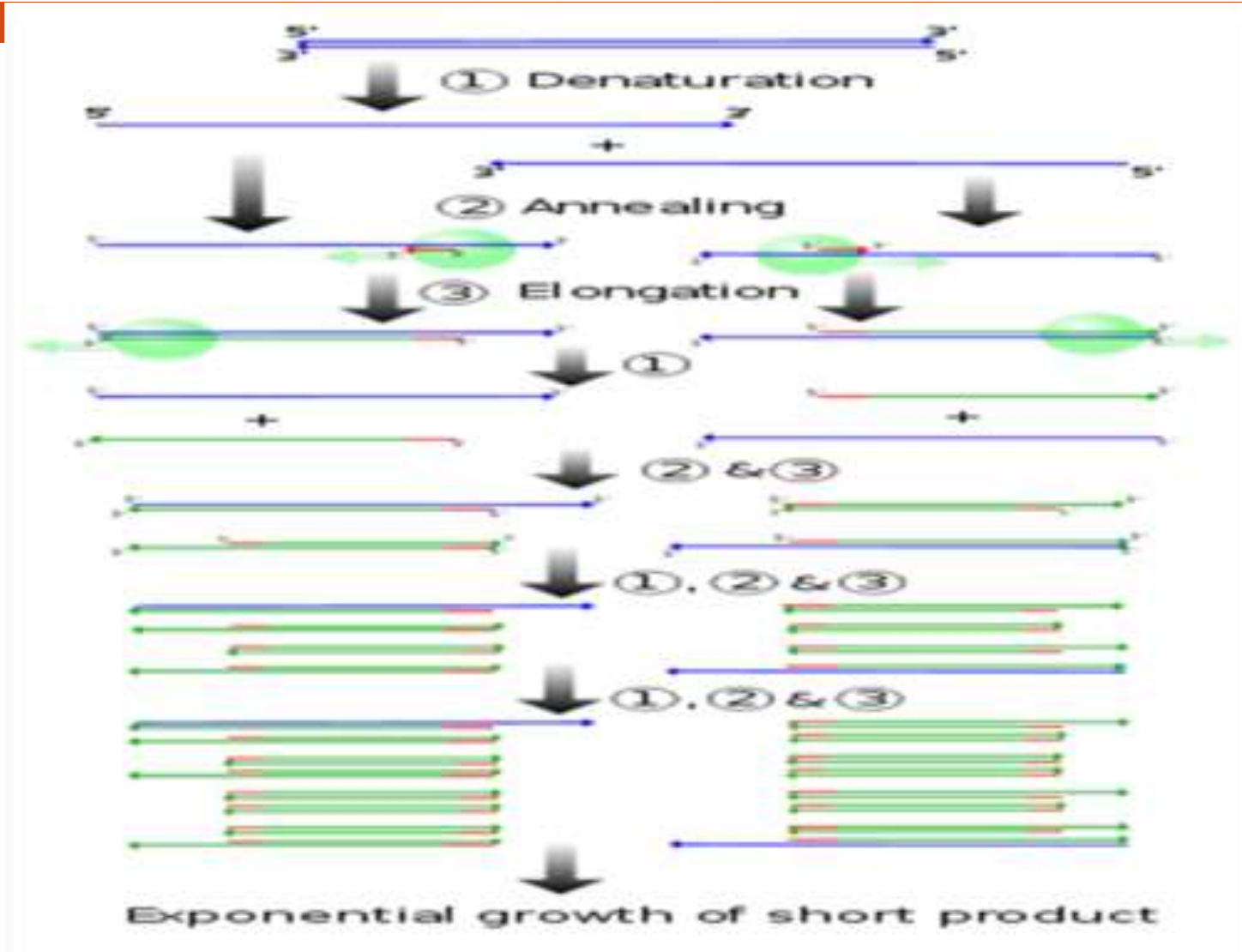
2-Laboratory Facilities

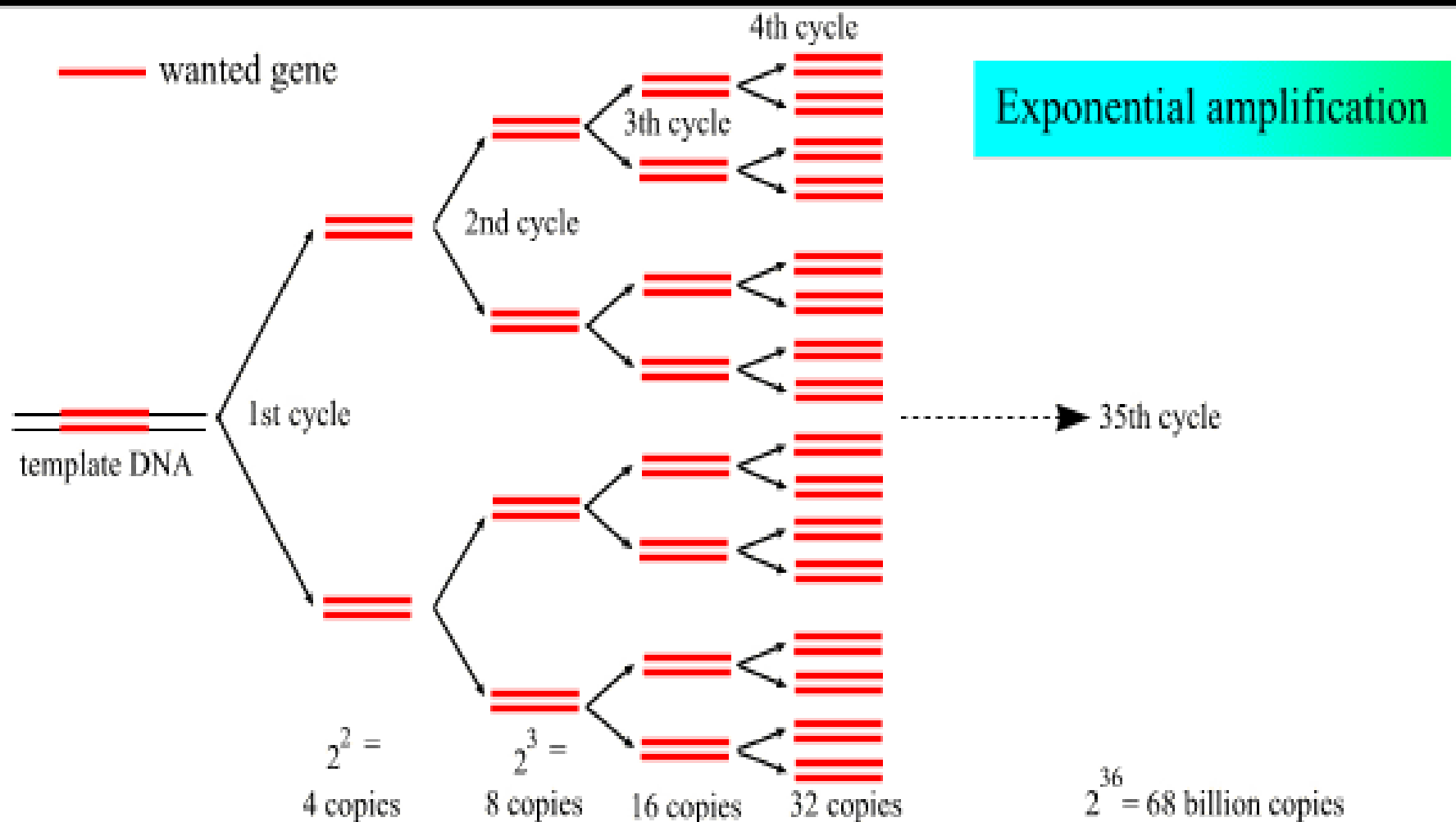
- ❑ Set up physically separated working places for:
 - Template preparation
 - Setting up PCR reactions
 - Post PCR analysis
- ❑ Use PCR only pipettes, micro-centrifuges and disposable gloves
- ❑ Use aerosol resistant pipette tips
- ❑ PCR reaction under a fume hood equipped with UV LIGHT.

3-Working with RNA

- Do not **touch** a surface after putting the gloves to avoid reintroduction of RNase to decontaminated material.
- Designate a **special area** for RNA work only.
- Treat surface or benches and glassware with commercially available **RNase inactivating** agents.

Polymerase Chain Reaction





(Andy Vierstraete 1999)

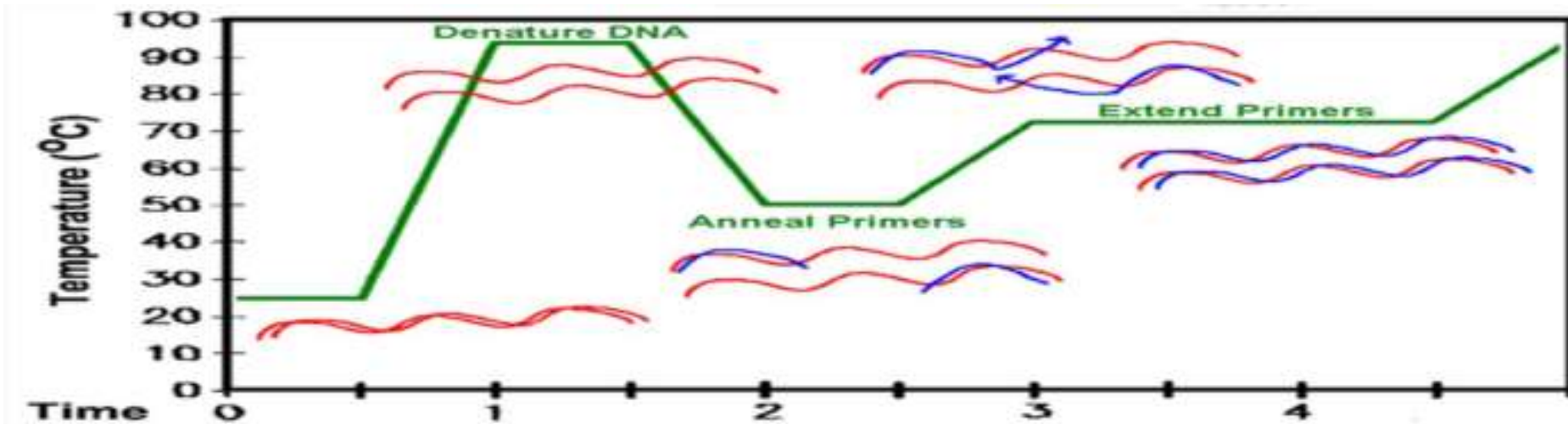
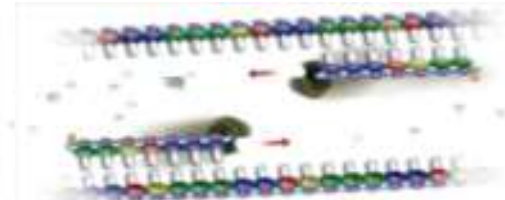
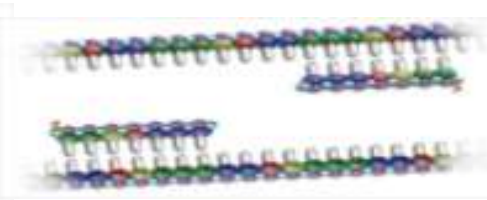
Thermal Cycling Profile for Standard PCR

- ❑ **Initial Denaturation:**
- ❑ Initial heating of the PCR mixture at 94- 95C within 2 min. is enough to completely denature complex genomic DNA.
- ❑ **Each cycle includes three successive steps:**
Denaturation, annealing and extension.
- ❑ **Post extension and holding:**
- ❑ Cycling should conclude with a final extension at 72 C° for 5 -15 minute to promote completion of partial extension products and then holding at 4 C°.

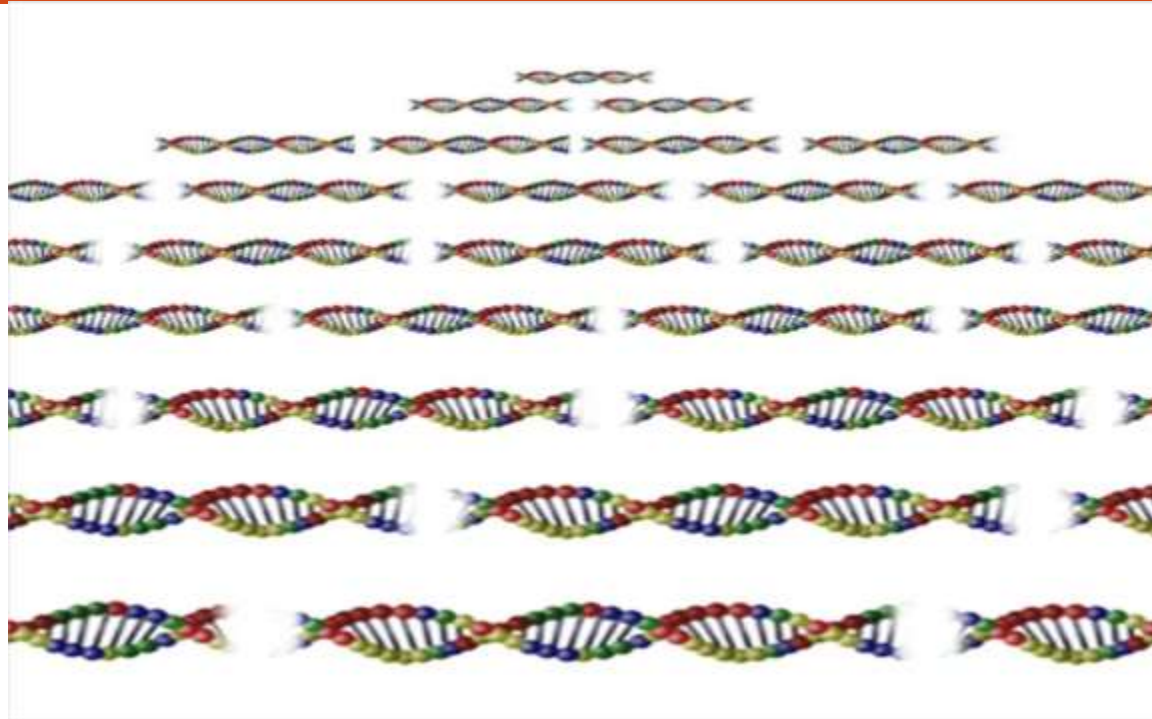
Each cycle includes three successive steps:

Denaturation	94C° 15sec-one minute	The DNA is denatured into single strands.
Annealing	34-72C° 30sec-two minute	The primers hybridize or "anneal" to their complementary sequences on either side of the target sequence.
Extension	72C° 1.5-3 minute	The polymerase binds and extends a complementary DNA strand from each primer

PCR



Exponential Amplification



As amplification proceeds, the DNA sequence between primers doubles after each cycle.

(The amplification of the target sequence proceeding in an exponential fashion (1 2 4 → 8 → 16... →) up to million of times the starting amount until enough is present to be seen by gel electrophoresis.

Number of Cycles

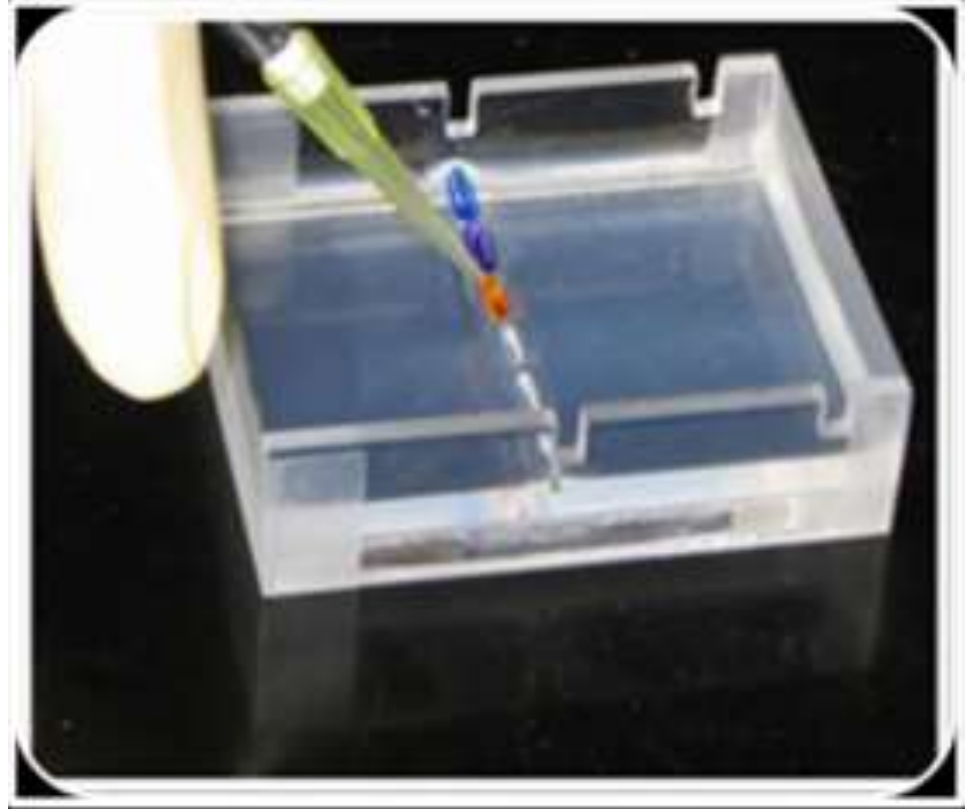
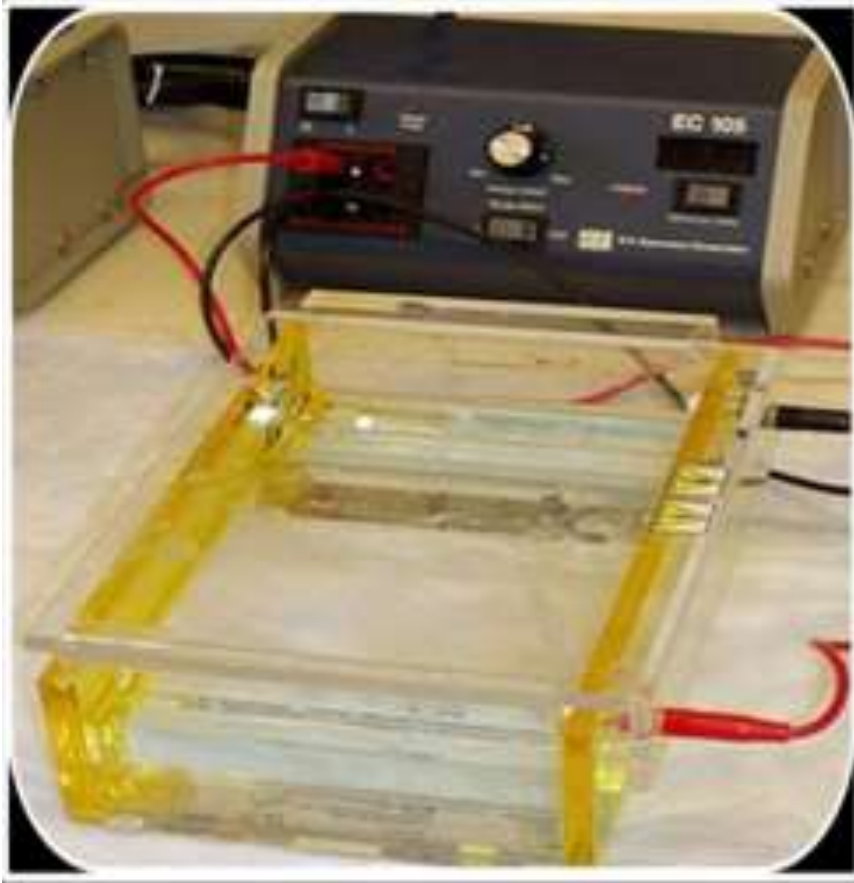
- The number of cycles required for optimum amplification varies depending on the **amount of the starting material**.
- Most PCR should, therefore, include only **25 – 35** cycles. As cycle increases, nonspecific products can accumulate.
- After **20- 40 cycles** of heating and cooling build up over a million copies of original DNA molecules.

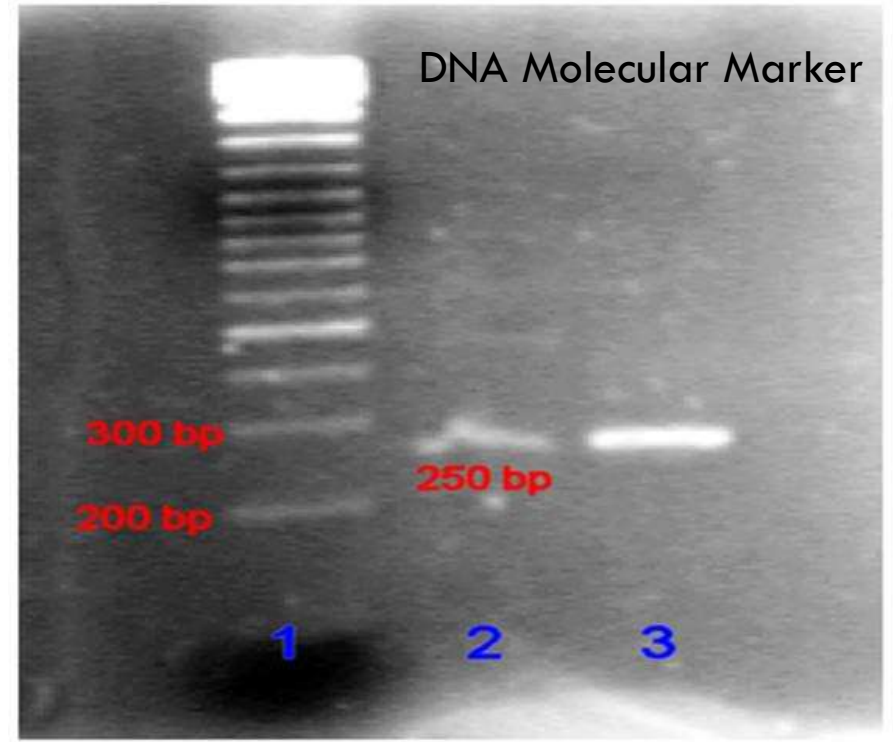
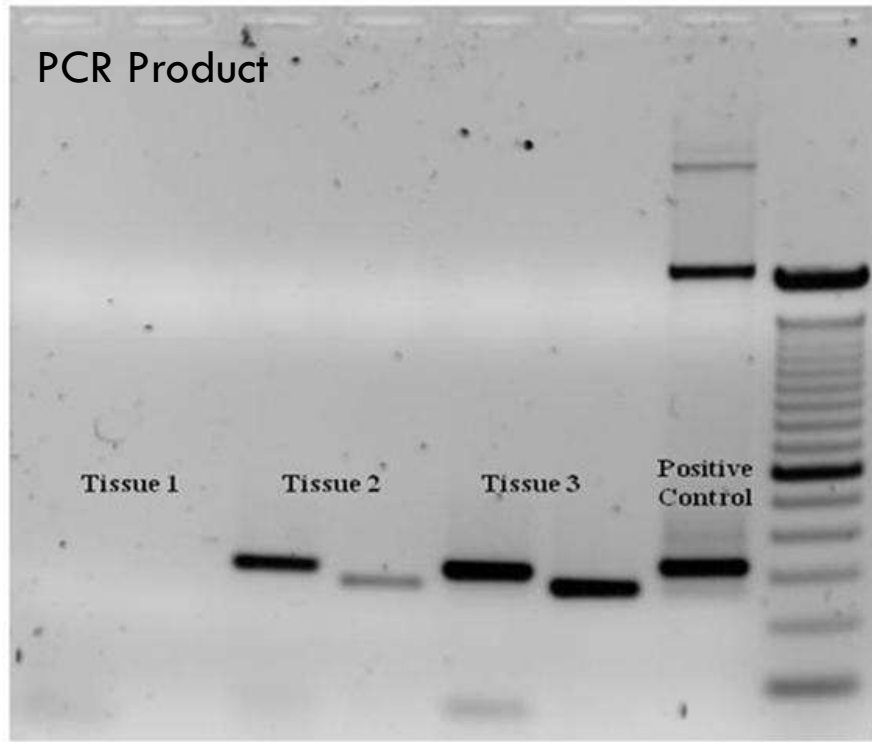
GEL ELECTROPHORESIS

Agarose Gel Electrophoresis

- ❑ It is a method used in **biochemistry** and molecular **biology** to **separate DNA**, or **RNA** molecules based upon charge, size and shape.
- ❑ Agarose is a polysaccharide derivative of agar.

Gel Tray/ Loading





❑ **Amplified fragments** can be visualized easily following staining with a chemical stain such as ethidium bromide.

❑ The DNA fragments are separated by charge and the relative sizes of fragments are determined by comparing to a standard DNA ladder.

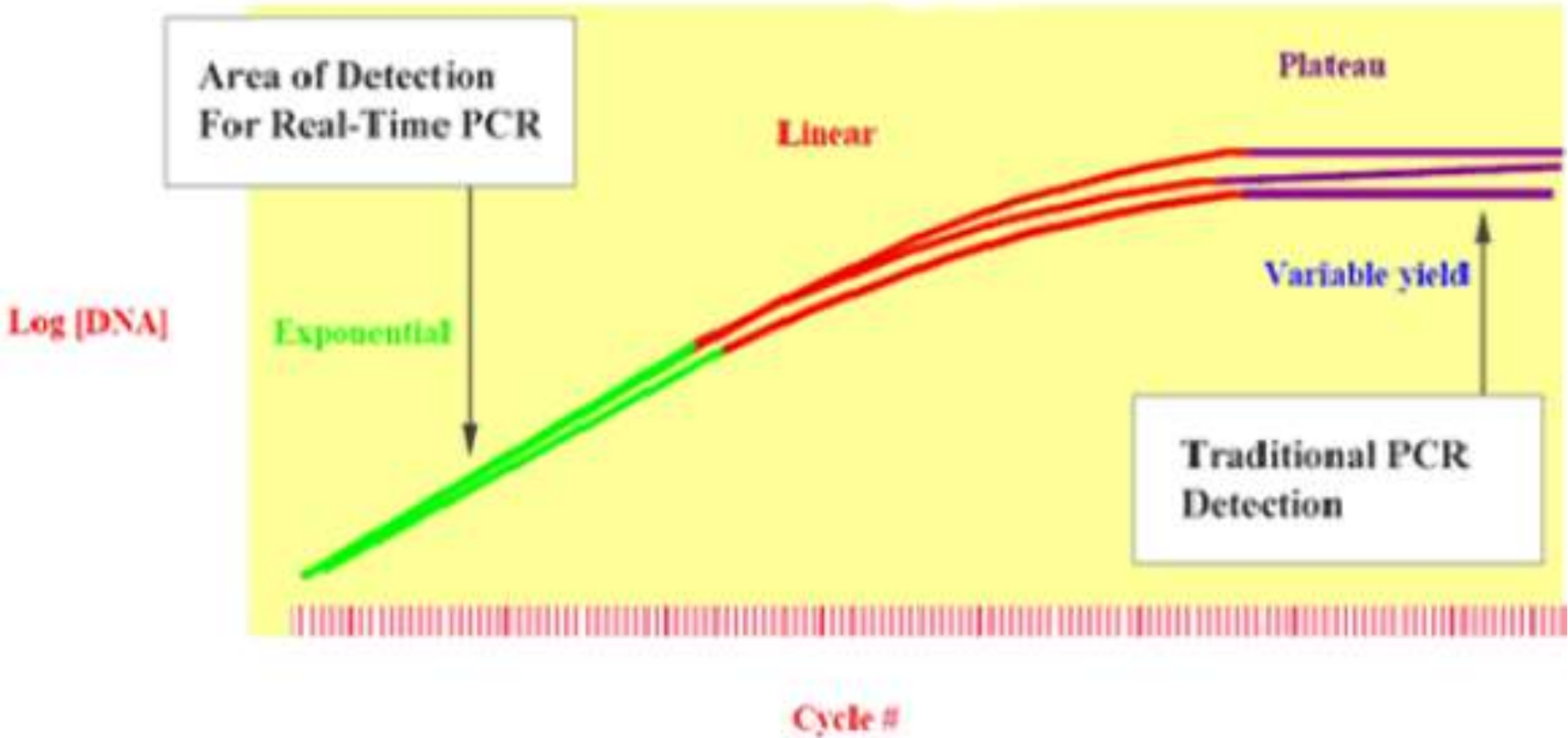
» Factors, affect the mobility of molecules in gel

- Charge
- Size
- Shape
- Buffer conditions
- Gel concentration and
- Voltage

PCR: Three Phases

- **Exponential**: Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise.
- **Linear**: The reaction components are being consumed; the reaction is **slowing**, and products are starting to degrade.
- **Plateau**: The reaction has **stopped**; no more products are being made and if left long enough; the PCR products will begin to degrade.

PCR Phases



Polymerase Chain Reaction

□ Advantages of PCR

- Useful non- invasive procedure.
- Simplicity of the procedure.
- Sensitivity of the PCR

□ Disadvantages of PCR

- False positive results (cross contamination).
- False negative results

Variant PCR

- ❑ Reverse transcriptase-PCR.
- ❑ Nested-PCR.
- ❑ Hot-start PCR.
- ❑ Quantitative PCR.
- ❑ Multiplex-PCR.
- ❑ Mutagenesis by PCR.
- ❑ Allele specific PCR.
- ❑

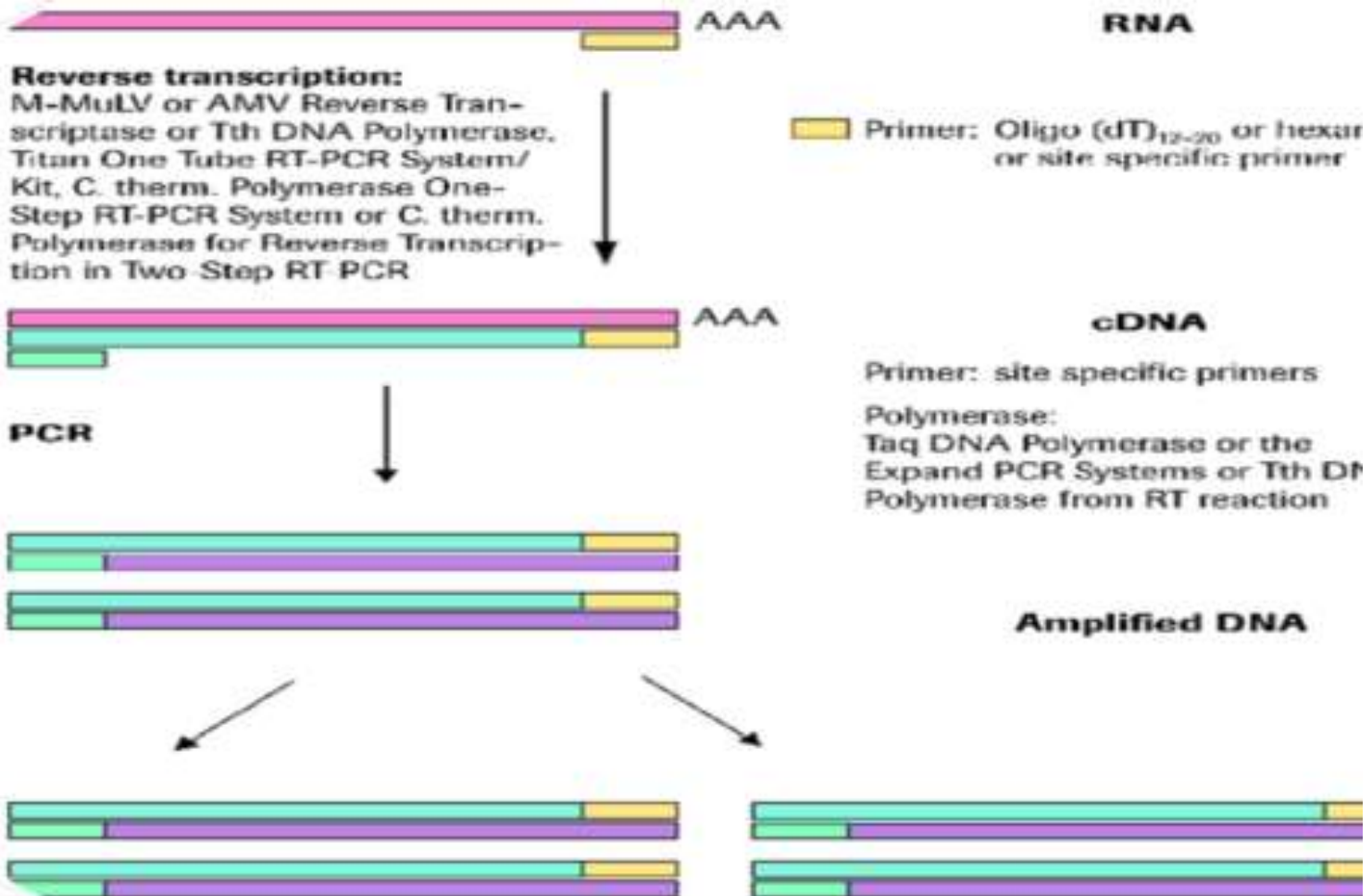
Reverse Transcriptase - PCR

RT-PCR, one of the most sensitive methods for the detection and analysis of rare mRNA transcripts or other RNA present in low abundance.

RNA cannot serve as a template for PCR.

RNA must be first transcribed into cDNA with reverse transcriptase from Moloney murine leukemia virus or Avian myeloblastosis virus, and the cDNA copy is then amplified.

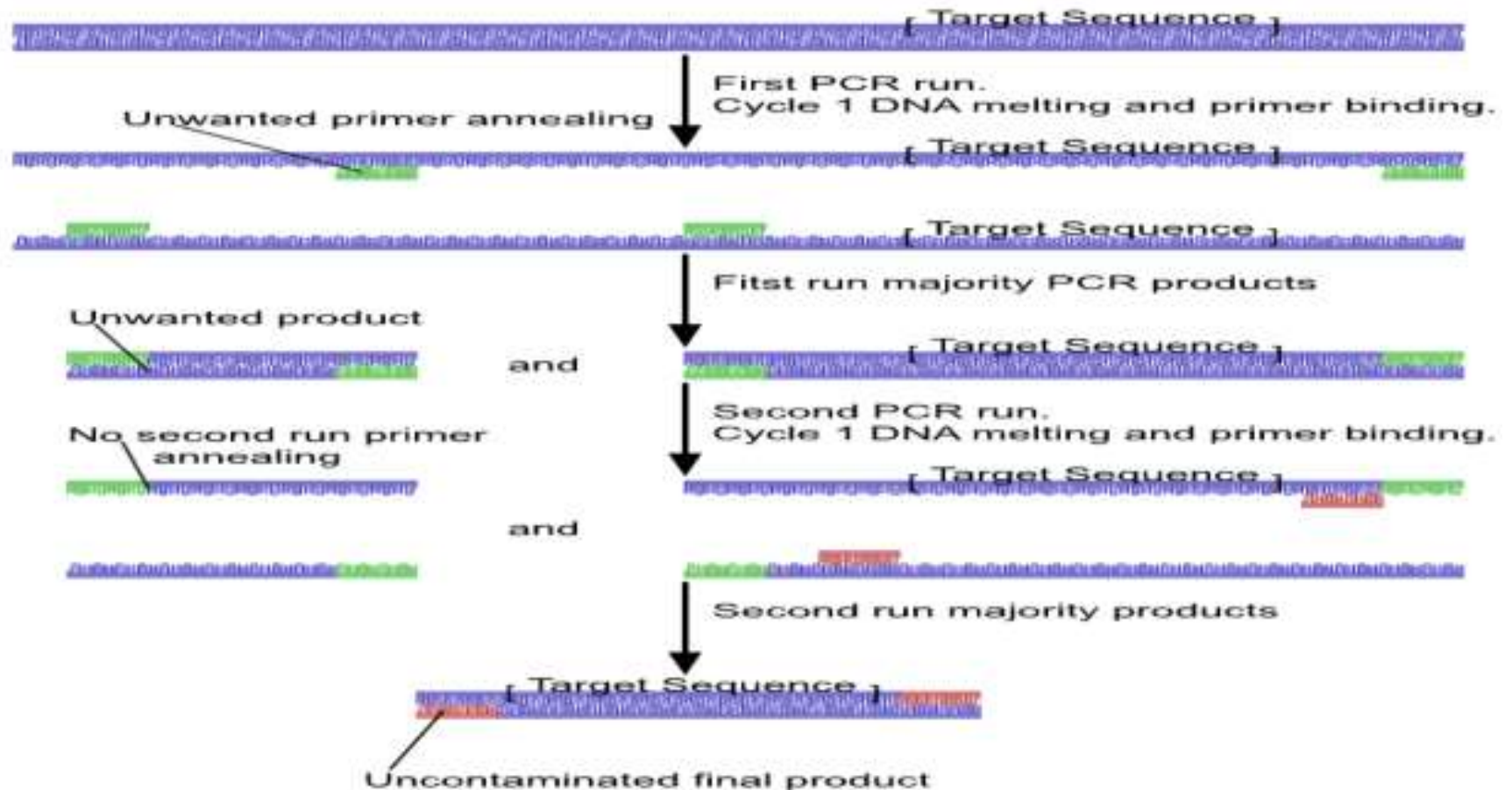
RT- PCR



Nested PCR

- Nested PCR is a very specific PCR amplification.
- Nested PCR use two pairs (instead of one pair) of PCR primers are used to amplify a fragment.

Nested - PCR



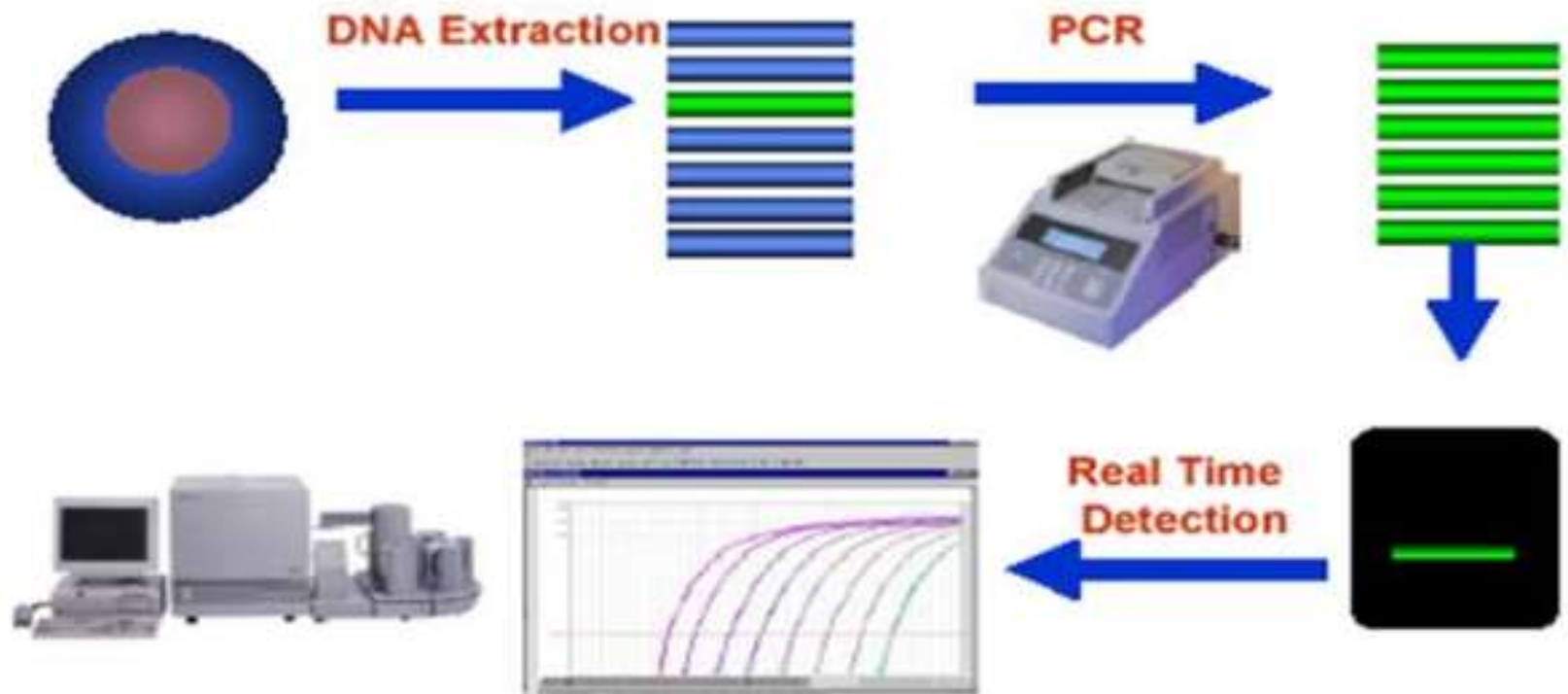
Hot - Start PCR

- Hot Start PCR significantly improves specificity, sensitivity and yield of PCR.
- The technique may be performed **manually** by **heating** the reaction components to the melting temperature (e.g., 95°C) before adding the polymerase. Specialized enzyme systems can be used.

Real Time PCR

- Traditional PCR has advanced from detection at the end-point of the reaction to detection while the reaction is occurring (Real-Time).
- Real-time PCR uses a fluorescent reporter signal to measure the amount of amplicon as it is generated . This kinetic PCR allows for **data collection after each cycle** of PCR instead of only at the end of the 20 to 40 cycles.

Real Time PCR



POLYMERASE CHAIN REACTION: USES

- Molecular biology,
- Microbiology,
- Genetics,
- Diagnostics clinical laboratories,
- Forensic science,
- Environmental science,
-

Clinical Microbiology

Viral load (HIV,HCV,HBV,...)

Bacterial load (Salmonella,
Mycobacterium,...)

Fungal load (Candida,
Cryptococcus, Aspergillus,...)

Food microbiology

Bacterial load (Listeria,
Salmonella, Campylobacter,...)

Clinical Oncology

Minimal residual disease

Chromosomal translocations

Single nucleotide
polymorphism (SNPs)

Gene therapy

Gene transfer estimation

Biodistribution of vector

Gene expression

Cytokines, receptors,...

