POLYMERASE CHAIN REACTION

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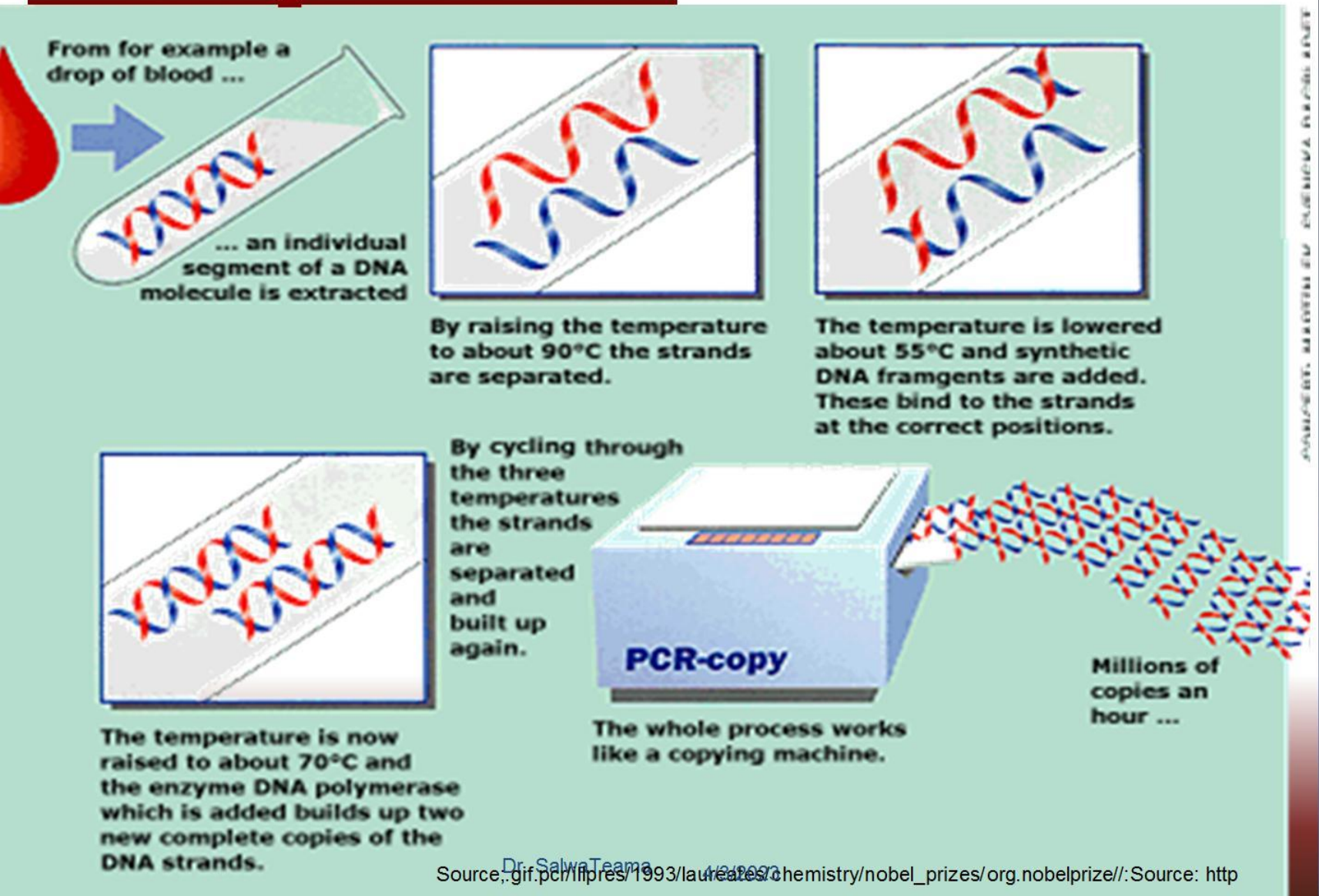
POLYMERASE CHAIN REACTION

Nobel prize 1993



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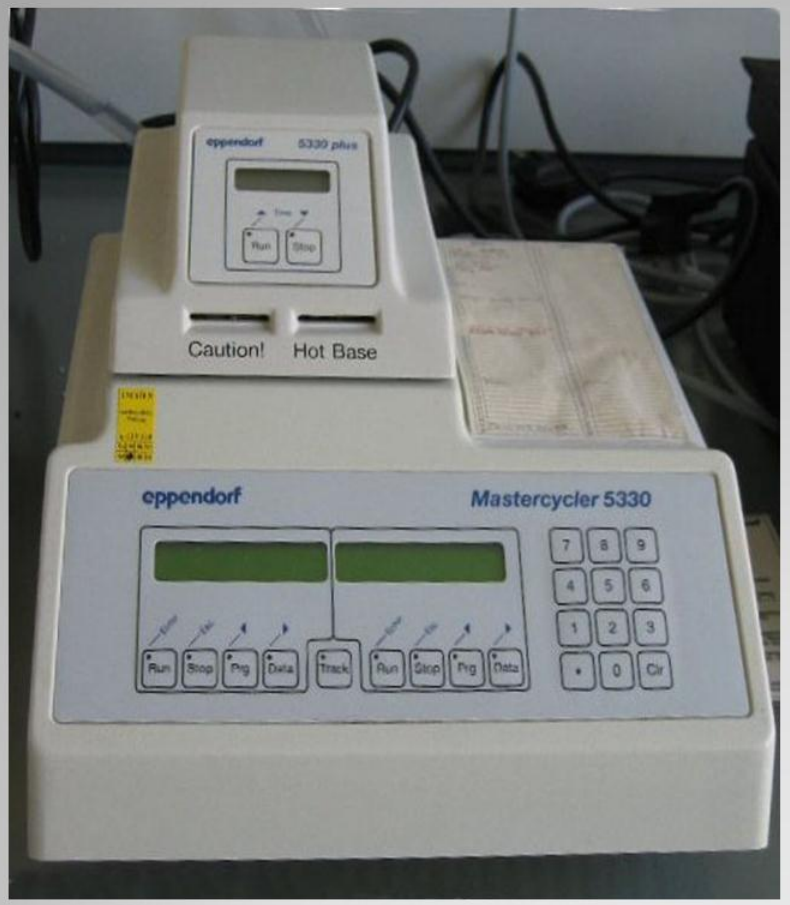

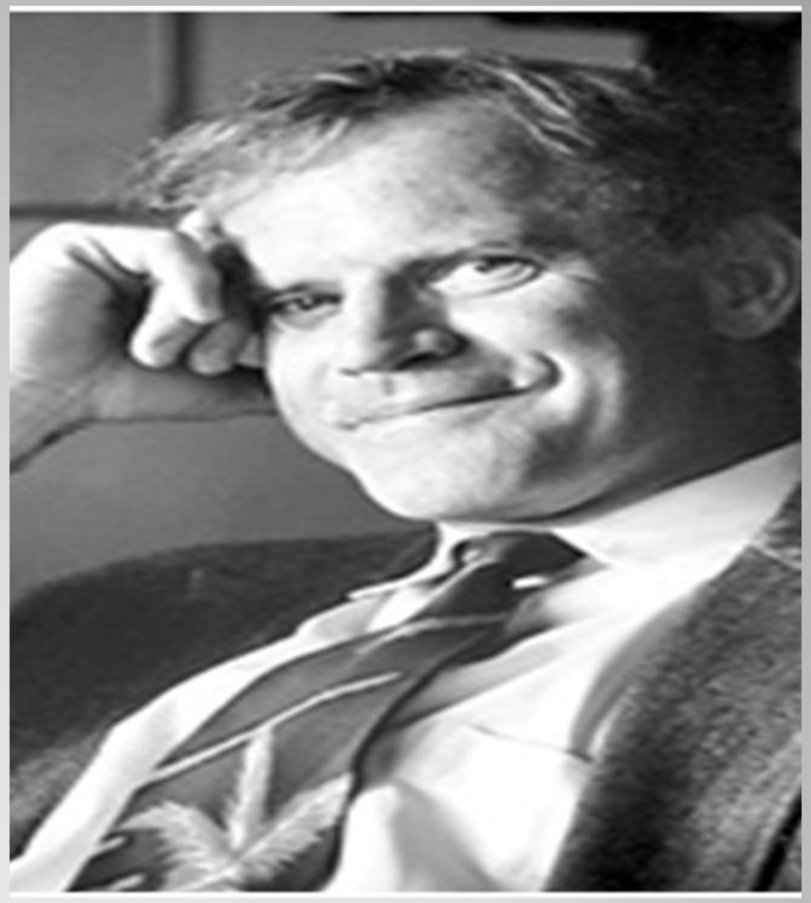

- Coping Machine for DNA Molecule
- Invented by Kary Mullis and his colleagues in the 1983

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

```
1   ACG GCC CTC ATC ACC TTC TTT CAC TTT TTG TTT TTG TGT AGG TGA GTC AAT TGC GTG TGG
61  CCT TCA AGT ATT TCG TAT TGT AAC AAT ATT CGA TCG GCA TCC AAA CAA AGG TGC ATG TAC
121 GGT TCC TAA GGG ATA CAA TTT TGT CTT AAA TCA TCG AGA AAG A TT AAG GTA AGT TGA TAG
181 GCG CGA TCT CGT ACC TAA CAC ATA CTC TCT AAA TAT TGA AGA A CT TGC ATG CGG CCT TCA
241 AGC CAC AAC GCG GTA TGA GTT CTT TGT TTG GGG GCT GCT TGC C CC TTC GCG TCG ACA AGG
301 AAA CTG AGG ACG ACA ATG G CA CC
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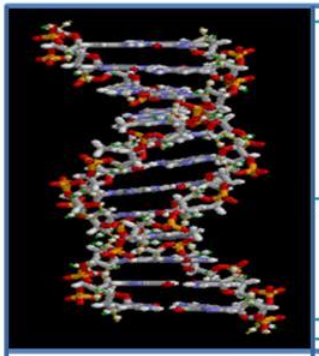
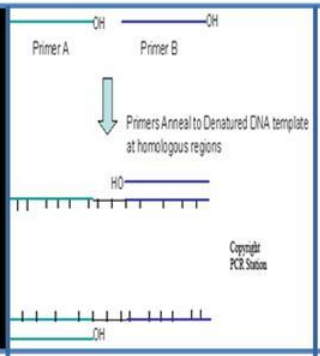
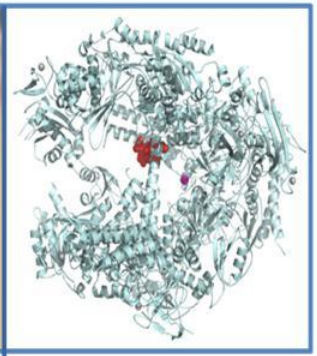
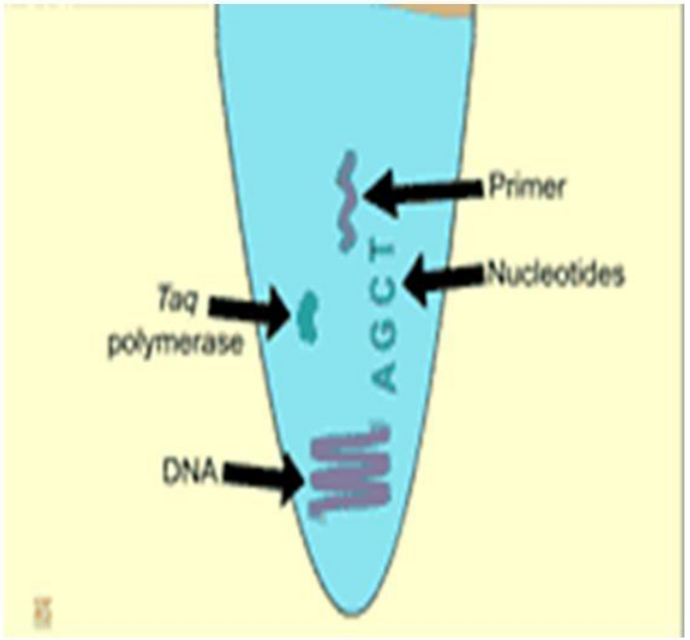
PCR REACTION COMPONENTS

				
Template	Two primers	Four (dNTPs)	Buffer system containing magnesium	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 50- 60% GC composition,
- Have a balanced distribution of G/C and A/T rich domains,
- The calculated Tm for a given primer pair should be balanced,
- Primer concentration between 0.1 and 0.6 μM are generally optimal,
- Contain no internal secondary structure,
- Are not complementary to each other at the 3' ends to avoid primer- dimer forming artifacts.

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)
  PH 8.3 (PH 8.3-8.8 at 20C °)
- KCl 50mM
- MgCL2 1.5mM (0.5-10mM)
- Gelatin or Bovine Serum Albumin 100 µg/ml

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.

Enhance The Specificity and or Efficiency of a PCR

Betadine
Bovine serum albumin
Dimethylysulfoxide
Gelatine
Glycerol
Pyrophosphate
Spermidine, Detergents, Gelatin,....

Calculation of Melting Temperature

Tm= 2 C ° X (number of A and T bases)+4 C ° X (number of G and C bases).

Optimal annealing temperature are 5-10 C ° lower than Tm values of the primers.

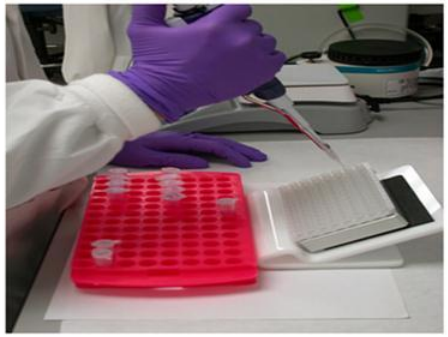
STANDARD PCR REACTION

		Vol./tube	final cons.
10 X	PCR buffer	5 ul	1 x
25 mM	$MgCl_2$	4 ul	2mM
10 mM	dATP	1 ul	0.2 mM
10 mM	dTTP	1 ul	0.2 mM
10 mM	dCTP	1 ul	0.2 mM
10 mM	dGTP	1 ul	0.2 mM
Taq	polymerase (5U/ul)	0.3 ul	1.5 units
Forward	primer (50 ng/ul)	2 ul	100 ng
Reverse	primer (50 ng/ul)	2 ul	100 ng
Template DNA (100 ng)		3 ul	100 ng

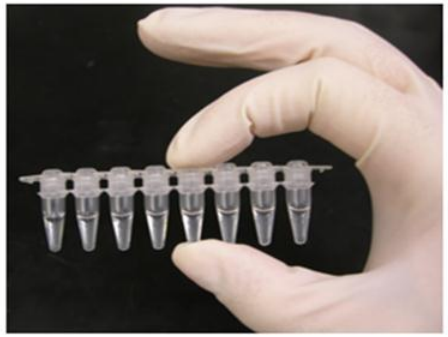
Add sterile H_2O to 50 ul and 30 ul mineral oil

Component	Amount	Your Reaction
10X PCR Buffer	5 µL	
25 mM MgCl2	4 µL	
10 µM forward primer	5 µL	
10 µM reverse primer	5 µL	
10 mM dNTP (mix)	1 µL	
100 ng template DNA (genomic DNA)	x µL	
Taq DNA polymerase (1U/µL) (add last)	1.0 µL	
diH_2O (use whatever volume y is correct to bring total reaction volume to 50 µL)	y µL	
TOTAL	50 µL	

PCR



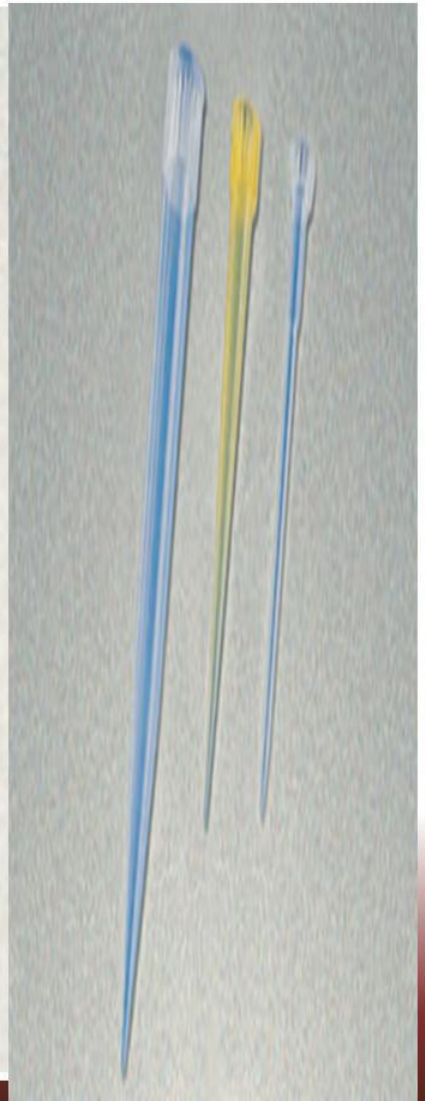
All one has to do is to mix the contents in the test tube and



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

Thermal cycler

AVOIDING CONTAMINATION



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.

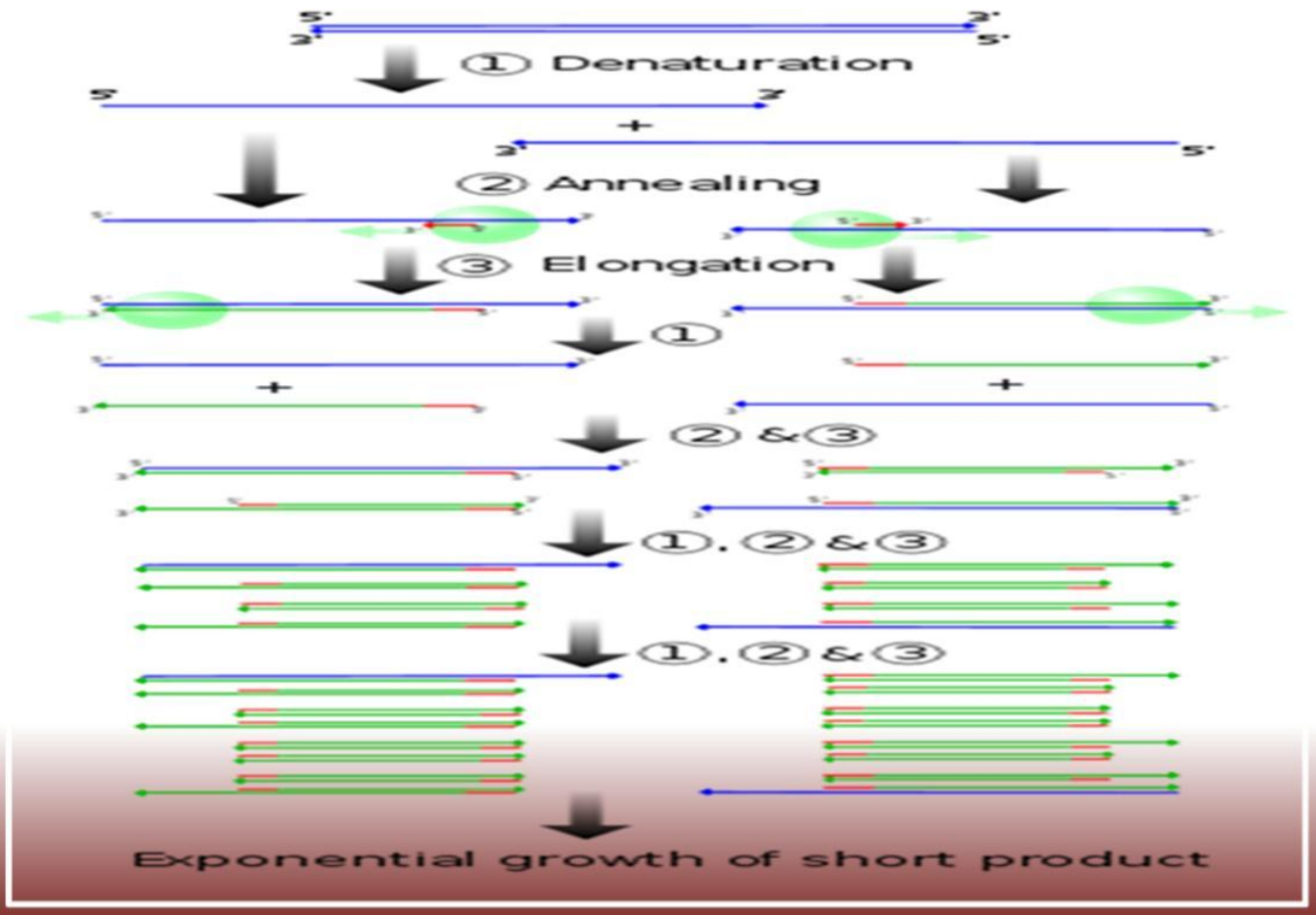
Laboratory Facilities

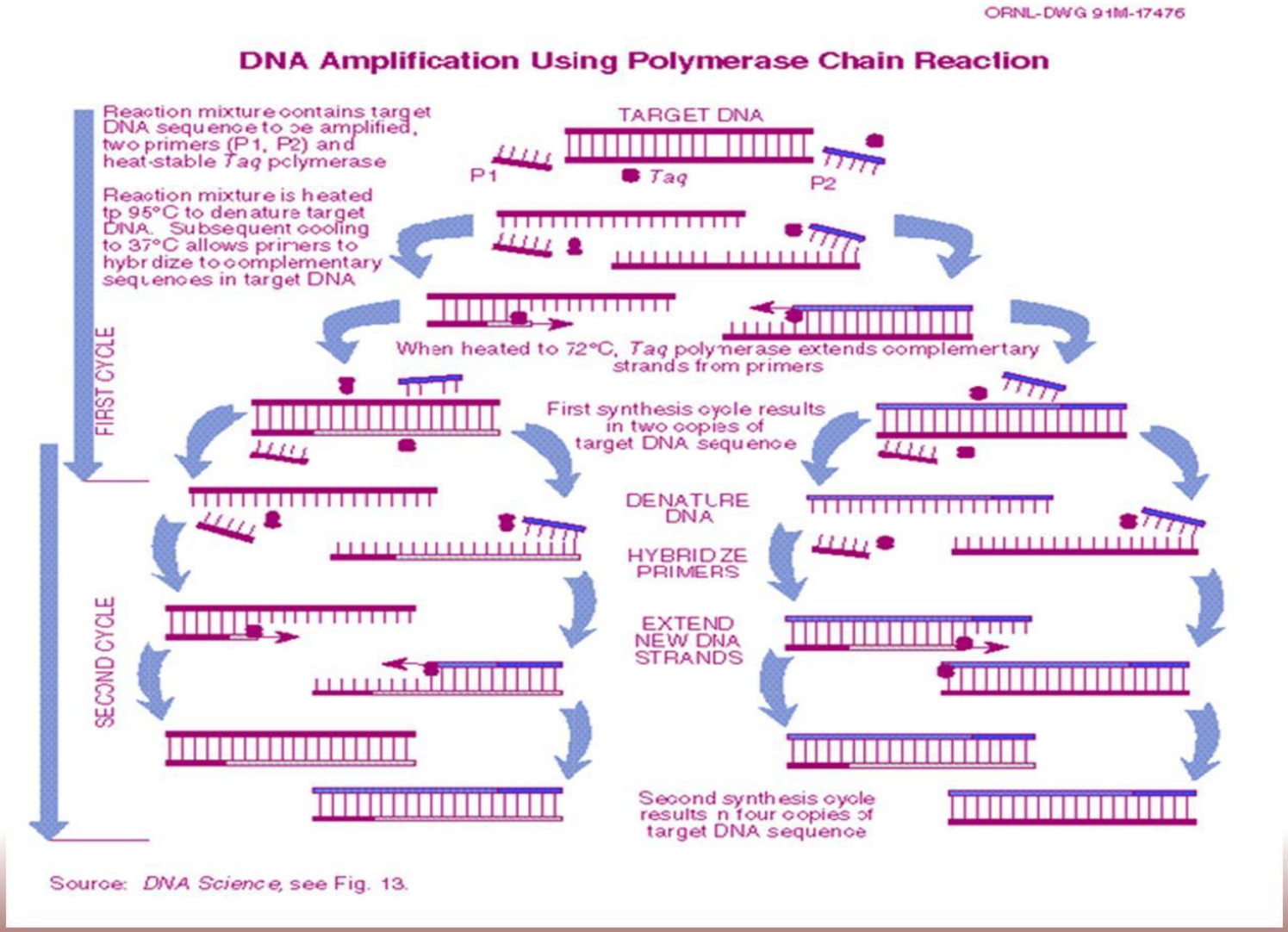
- Set up physically separated working places for:
  - Template preparation
  - Setting up PCR reactions
  - Post PCR analysis
- Use PCR only pipettes, micro-centrifuges and disposable gloves
- Use aerosol resistant pipette tips
- PCR reaction under a fume hood equipped with UV LIGHT.

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




ORNL-DWG 91M-17476
DNA Amplification Using Polymerase Chain Reaction
Reaction mixture contains target DNA sequence to be amplified, two primers (P1, P2) and heat-stable Taq polymerase
Reaction mixture is heated to 95°C to denature target DNA. Subsequent cooling to 37°C allows primers to hybridize to complementary sequences in target DNA
TARGET DNA
P1
Taq
P2
When heated to 72°C, Taq polymerase extends complementary strands from primers
FIRST CYCLE
First synthesis cycle results in two copies of target DNA sequence
DENATURE DNA
HYBRIDIZE PRIMERS
EXTEND NEW DNA STRANDS
SECOND CYCLE
Second synthesis cycle results in four copies of target DNA sequence
Source: DNA Science, see Fig. 13.

Thermal Cycling Profile for Standard PCR

- **Initial Denaturation:**
  Initial heating of the PCR mixture at 94- 95C ° 2 min. is enough to completely denature complex genomic DNA.

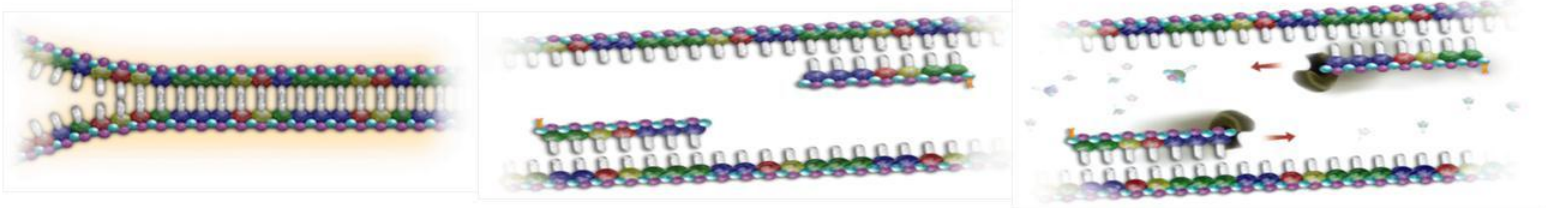
- **Each cycle includes three successive steps:** Denaturation, annealing and extension.

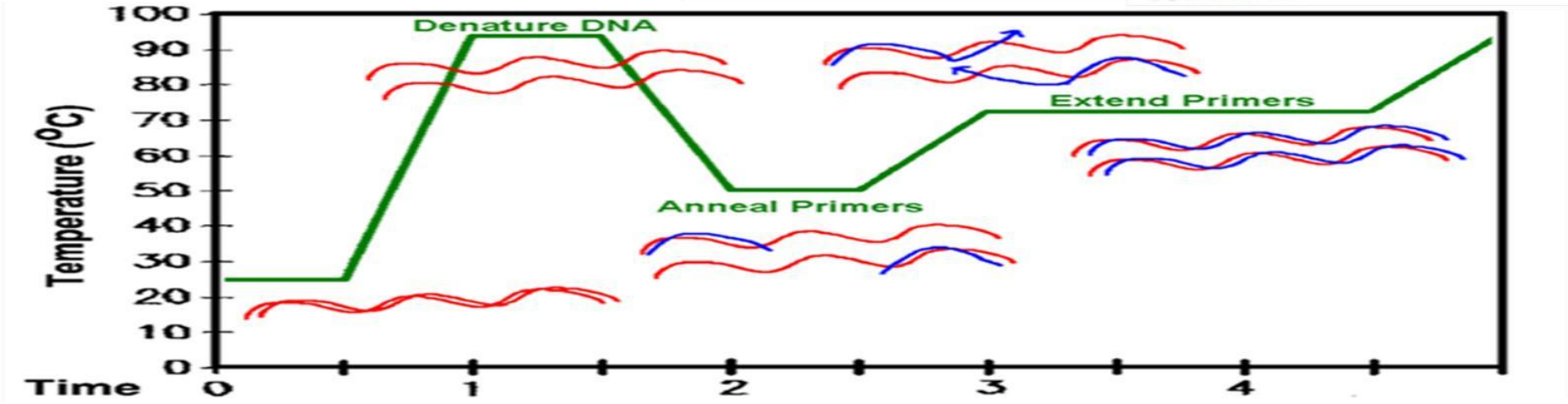
- **Post extension:**
  Cycling should conclude with a final extension at 72 C ° for 5 -15 minute to promote completion of partial extension products and then holding at 4 C ° .

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

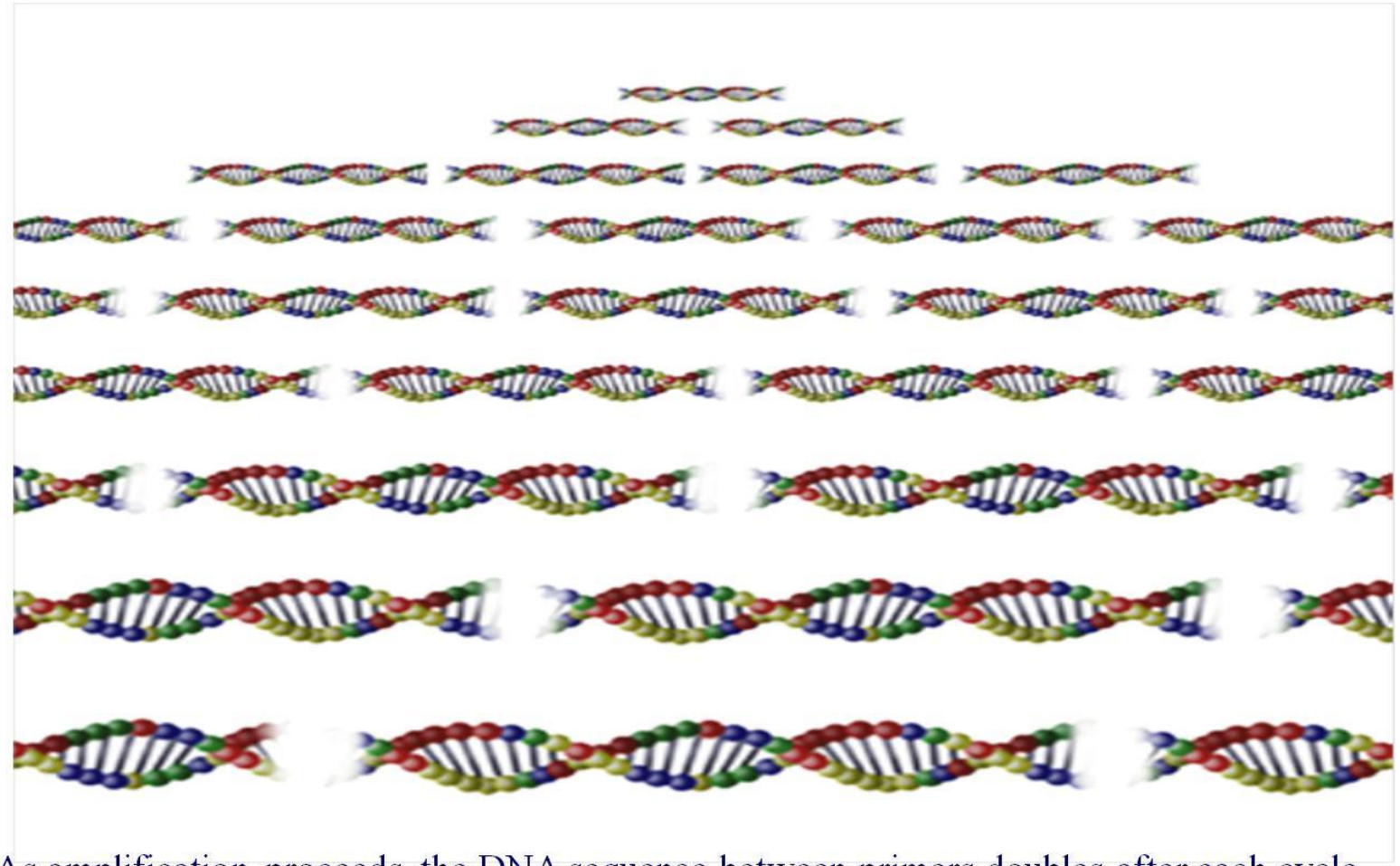
PCR





Source: Roche

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.

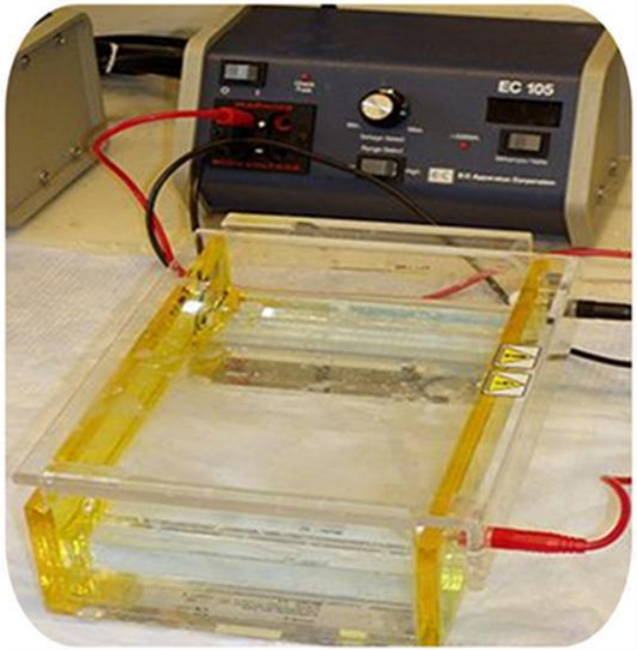
GEL ELECTROPHORESIS

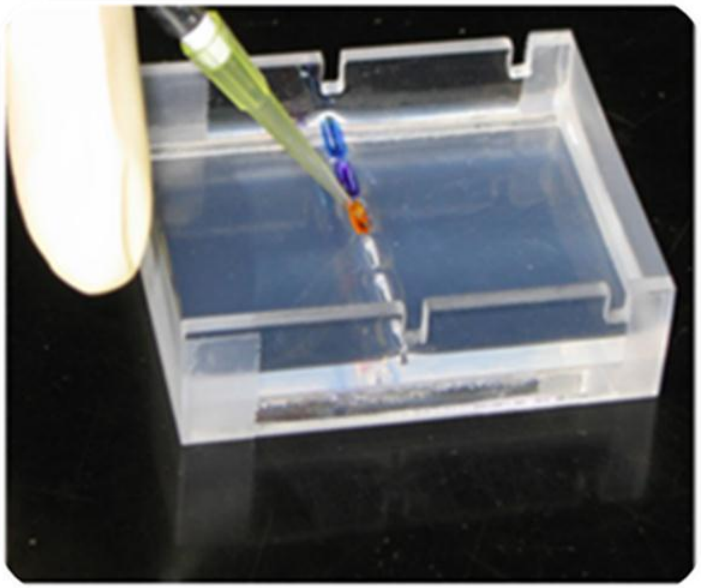
Gel Electrophoresis

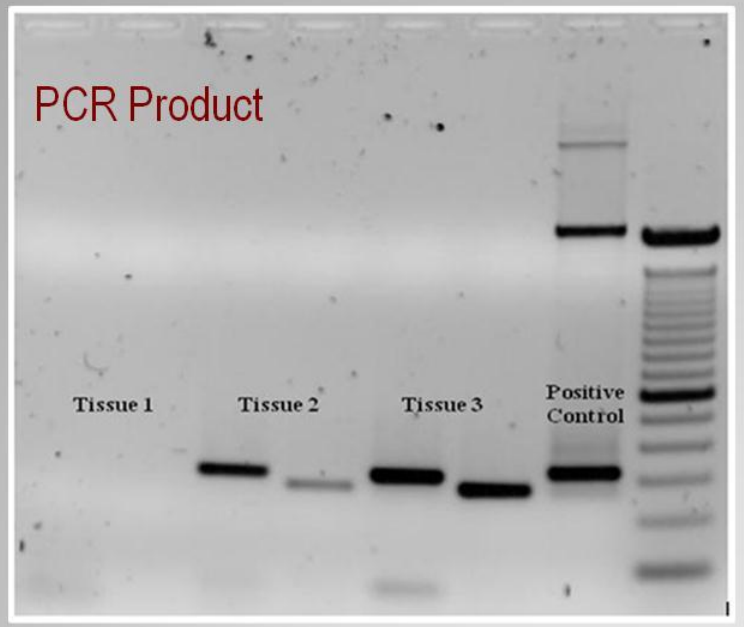
- **Agarose Gel Electrophoresis** is a method used in biochemistry and molecular biology to separate DNA, or RNA molecules based upon charge, size and shape.

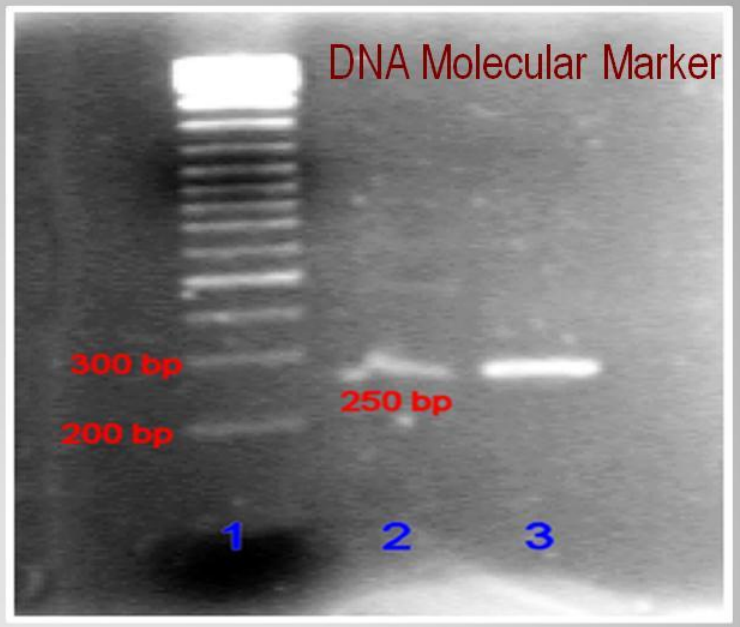
- Agarose is a polysaccharide derivative of agar.
- ……

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
- 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.

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 OF PCR

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

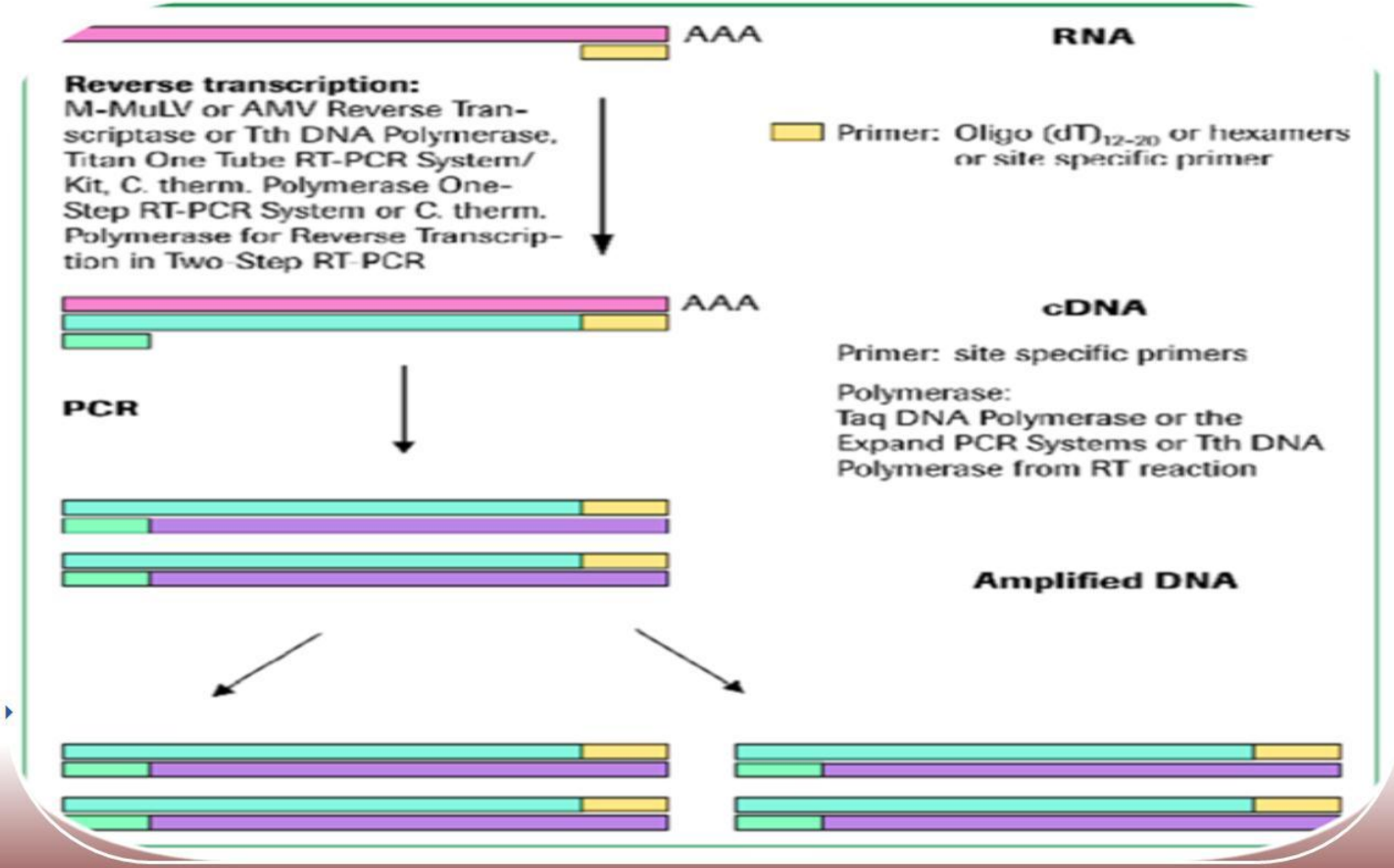
Reverse Transcriptase-PCR

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

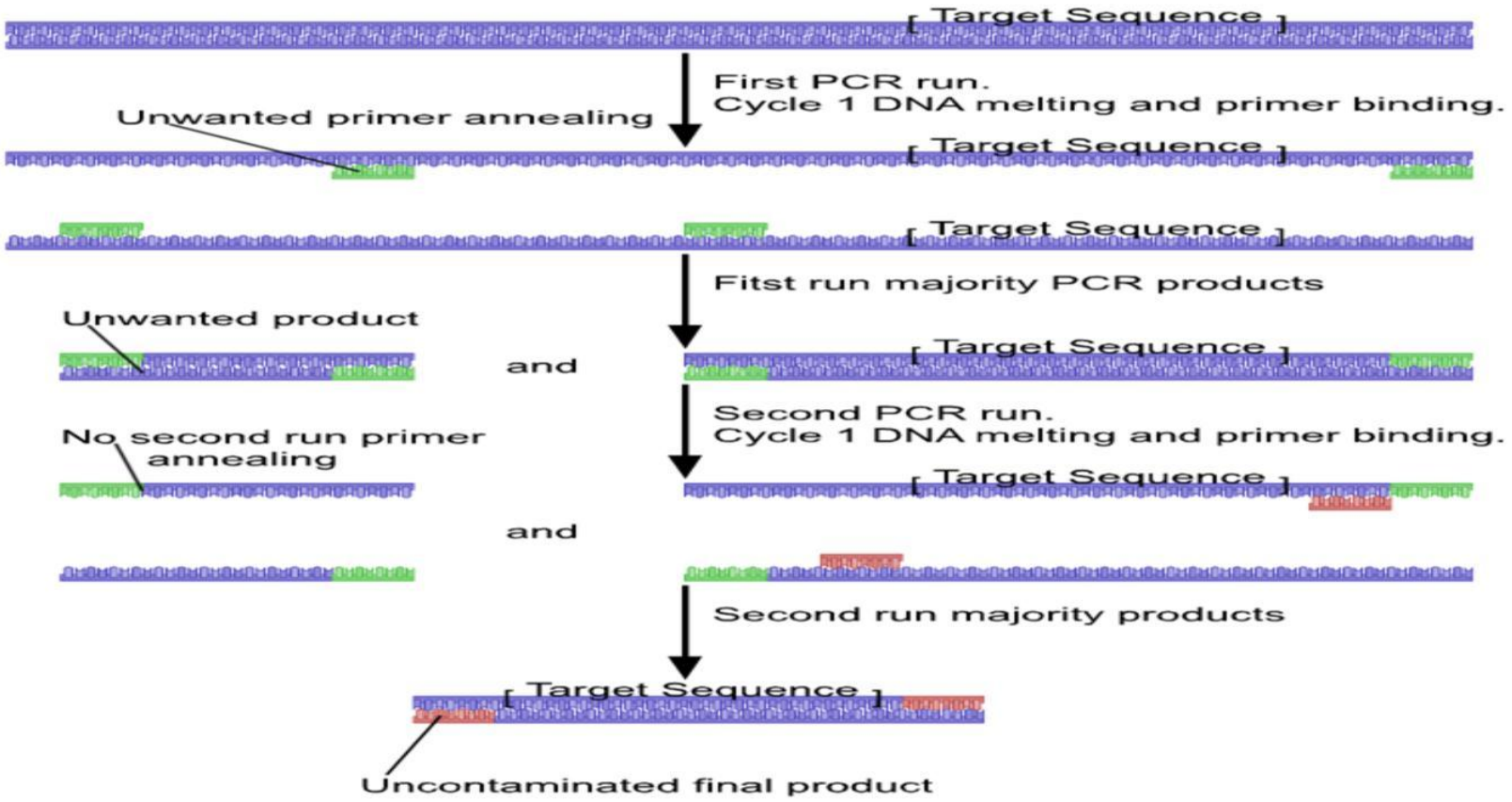

AAA
RNA
Reverse transcription:
M-MuLV or AMV Reverse Transcriptase or Tth DNA Polymerase, Titan One Tube RT-PCR System/ Kit, C. therm. Polymerase One-Step RT-PCR System or C. therm. Polymerase for Reverse Transcription in Two-Step RT-PCR
Primer: Oligo $(dT)_{12-20}$ or hexamers or site specific primer
AAA
cDNA
Primer: site specific primers
PCR
Polymerase:
Taq DNA Polymerase or the Expand PCR Systems or Tth DNA Polymerase from RT reaction
Amplified DNA

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

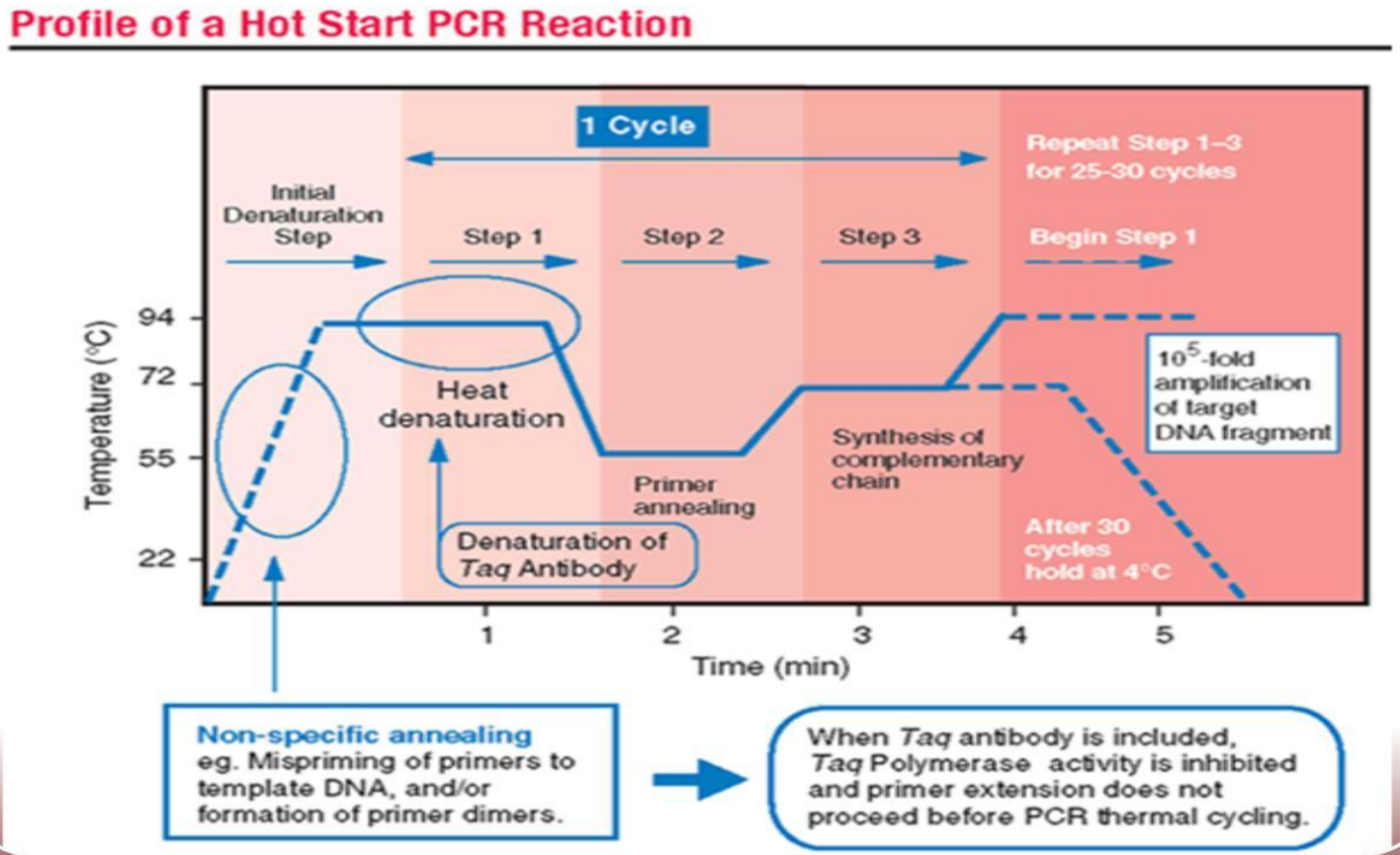

Target Sequence
First PCR run.
Cycle 1 DNA melting and primer binding.
Unwanted primer annealing
Target Sequence
Target Sequence
Fitst run majority PCR products
Unwanted product
and
Target Sequence
Second PCR run.
Cycle 1 DNA melting and primer binding.
No second run primer annealing
Target Sequence
and
Second run majority products
Target Sequence
Uncontaminated final product

Hot-Start PCR

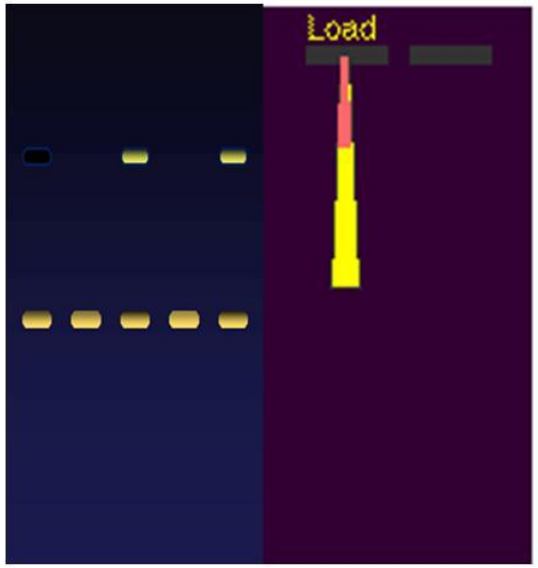
- Hot Start PCR significantly improves specificity, sensitivity and yield of PCR.

- 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.

Hot-Start PCR



WHAT IS WRONG WITH AGAROSE GELS?



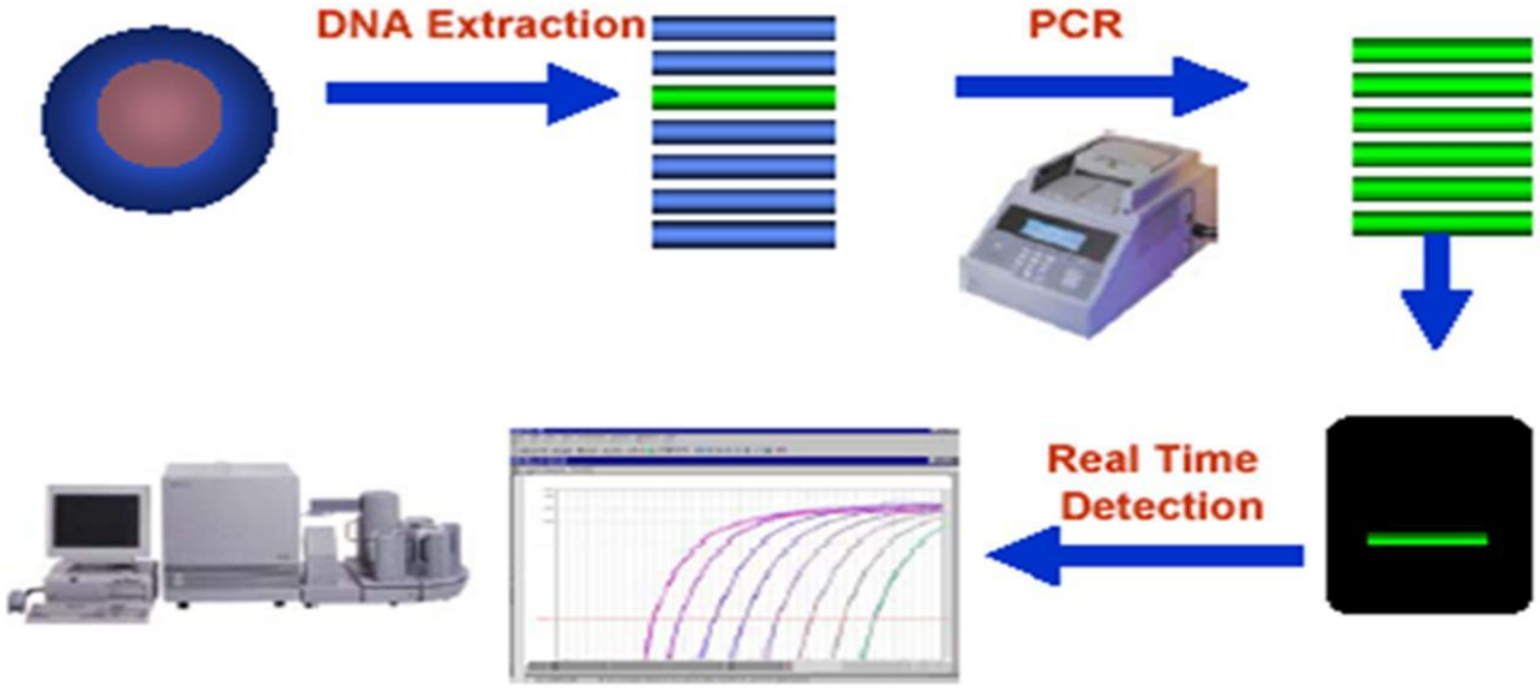
- Poor precision
- Low sensitivity
- Short dynamic range < 2 logs
- Low resolution
- Non-automated
- Size-based discrimination only
- Results are not expressed as numbers
- Ethidium bromide staining is not very quantitative

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



Source: www. AppliedBiosystem.com RealTime PCR

POLYMERASE CHAIN REACTION: USES

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

Infectious Diseases/ Cancer

Detection of infectious agents, such as Pathogenic bacteria, Viruses or Protozoa.

Cancer

Detection of malignant diseases by PCR, Recurrence of hematological cancers has also been evaluated and Detection of micro-metastasis in blood, lymph nodes and bone marrow.

Genetic Disease

Single point mutations can be detected by modified PCR techniques such as the ligase chain reaction (LCR) and PCR-single-strand conformational polymorphisms (PCR-SSCP) analysis.

Detection of variation and mutation in genes using primers containing sequences that were not completely complementary to the template.

Inherited disorders diagnosed using PCR protocols

- Alpha1 antitrypsin
- B thalassaemia
- Cystic fibrosis
- Duchenne muscular dystrophy
- Myotonic dystrophy
- Haemophilia A and B
- Huntington`s chorea
- Phenylketonuria
- Sickle cell anaemia
- Familial adenomatous polyposis
- ……

Prenatal Diagnosis

Prenatal sexing: Often required in families with inherited sex-linked diseases.

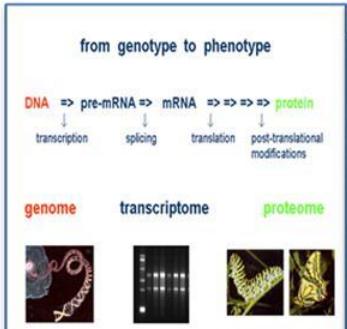
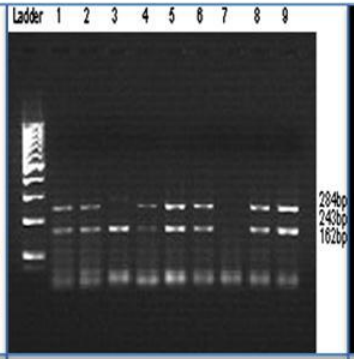
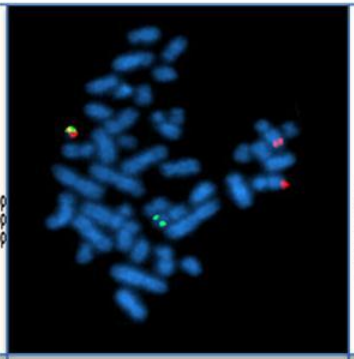
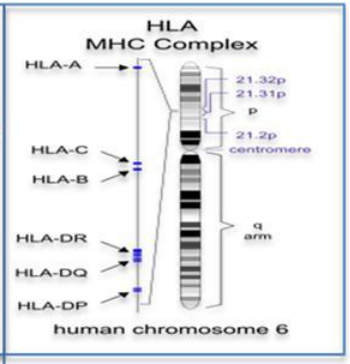
Prenatal Diagnosis of diseases: Prenatal diagnosis of many of the inborn errors of metabolism is possible by DNA markers.

Research

PCR is used in research laboratories in DNA cloning procedures, Southern blotting, DNA sequencing, recombinant DNA technology.

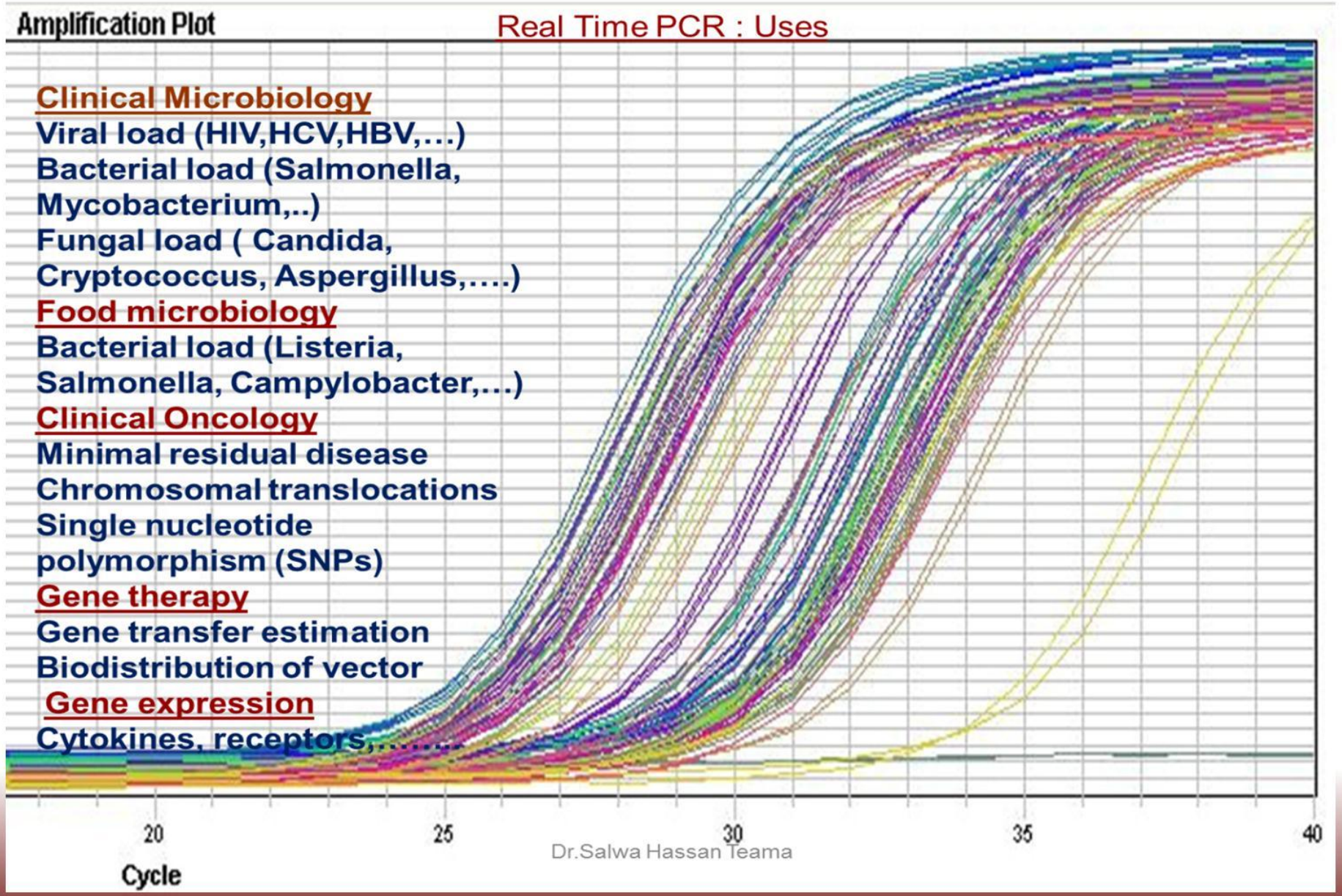
Major role in the human genome project.

Polymerase Chain Reaction

				
Gene expression analysis	Multiplex PCR , •made it possible to compare two or more complex genomes, for instance to detect chromosomal imbalances.	Combining in situ hybridization with PCR •made possible the localization of single nucleic acid sequences on one chromosome within an eukaryotic organism.	Amplification of archival and forensic material.	HLA Typing •Identify testing for transplantation,.

Source: Wikipedia

Real Time PCR : Uses



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,……

References & Further Reading

- PCR Applications Manual. www.Roche Molecular Biochemicals: PCR Applications Manual
- PCR Techniques. www.Roche Molecular Biochemicals: PCR Techniques
- Real Time PCR. www. AppliedBiosystem.COM
- Trends in molecular medicine Vol. 8 No.6 june 2002. Quantification using real time PCR technology, application and limitation.


THANK YOU