BASICS OF PCR

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The Molecular Assays

<u>At the Beginning:</u> This type of the tests used for detection of only fastidious or uncultivated M.O.

<u>But Now:</u> It become one of the most important diagnostic tools for detection and characterization of M.Os.

Polymerase Chain Reaction (PCR)

- **It is a molecular technology aim to amplify a single or few copies of the DNA to thousands or millions of copies.**
- **Developed in 1983 by Kary Mullis, In 1993, Mullis was awarded the Nobel prize in Chemistry.**
- **PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.**

What is PCR?

- **The polymerase chain reaction (PCR) is a fast and inexpensive technique used to "amplify" - copy - small segments of DNA.**
- **Sometimes called "molecular photocopying,"**

3x3

- **DNA/RNA Extraction.**
- **Amplification →**
  - **Denaturation**
  - **Annulling**
  - **Extension**
- **Electrophoresis**

Polymerase Chain Reaction (PCR)

Laboratory requirements

Thermal cycler (PCR machine)

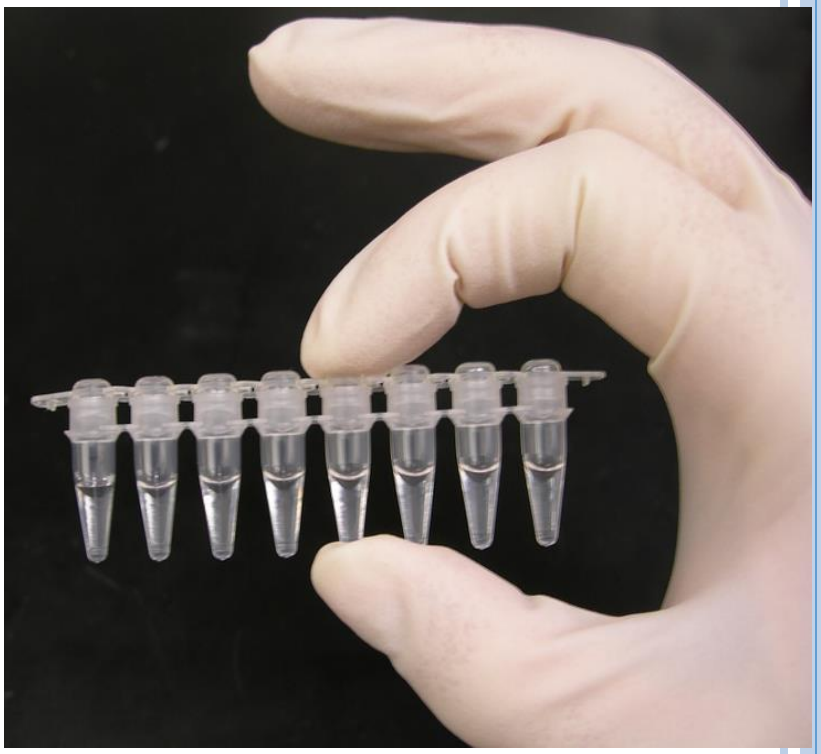
Denaturation at 94°C.

Annulling at 50:60°C.

Extension at72°C.

(PCR-Components)

1. Primers (Reverse and forward)
2. dNTPs
3. *Taq* polymerase (enzyme of Thermus aquaticus 1967)
4. Buffer



Template DNA:

An adequate amount of template DNA is between 0.1 and 1 µg of genomic DNA for a total reaction mixture of 100 µl.

Taq DNA polymerase:

- **The DNA polymerase (Taq polymerase) enzyme isolated from *Thermus aquaticus* bacterium.**
- **It withstand with the high temperatures which needed for DNA-strand separation.**

dNTPs:

The concentration of each dNTP (dATP, dCTP, dGTP, dTTP) in the reaction mixture is usually 200 µM.

These concentrations must be equal.

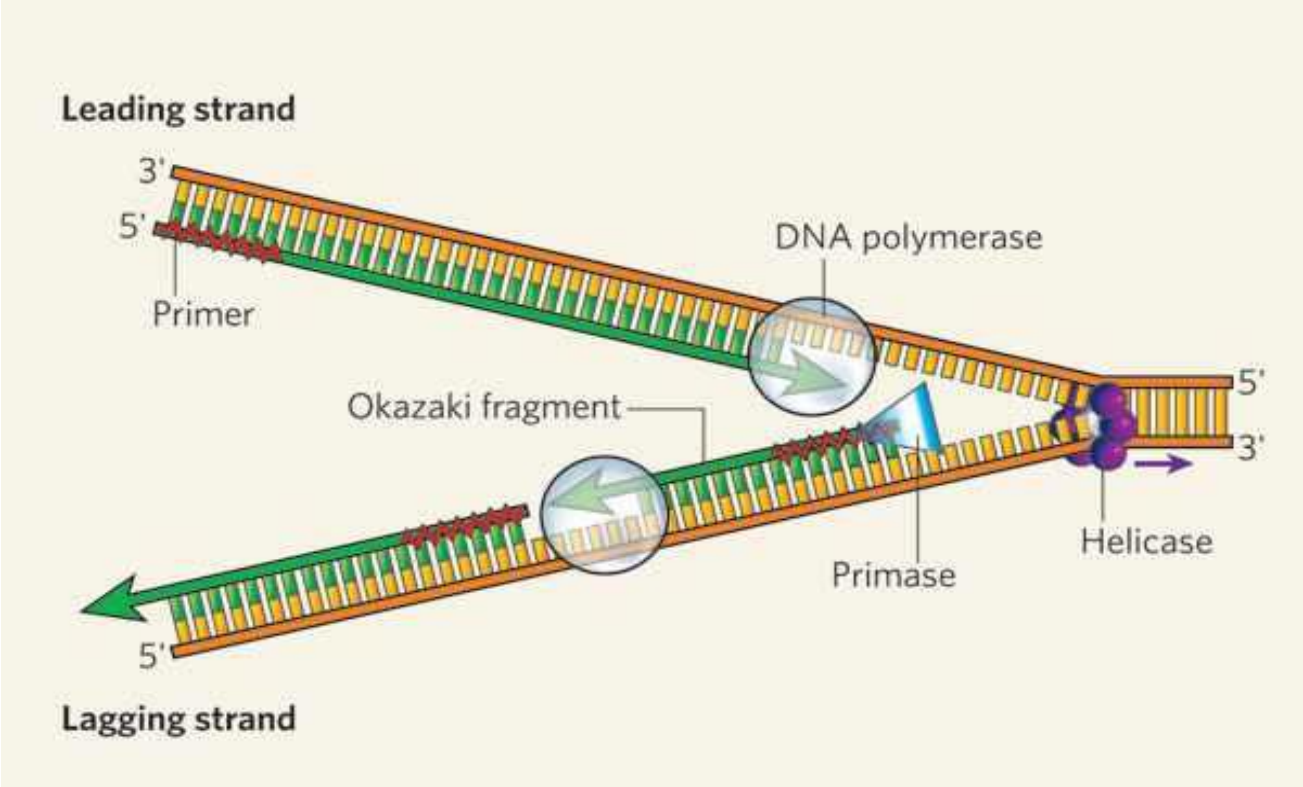
Primer: It is a small strand of nucleic acid that serves as a starting point for DNA replication.

**** DNA polymerases, can only add new nucleotides to an existing strand of DNA**

The polymerase starts replication at the 3'-end of the primer, and copies the opposite strand

5’-TCGAATATGCCGGATTC⟶

3’-AGCTTATACGGCCTAAGTTAGCTAGCTTGCA



Primers:

(1) Primers should be 10-24 nucleotides in length.

(2) The GC content should be 40%-60%.

(3)The primer should not be self-complementary or complementary to prevent primer-dimer and hairpin formation.

(4) Melting temperatures of primer pairs should not differ by more than 5°C, *so that the GC content and length must be chosen accordingly.*

(5) The melting and annealing temperatures of a primer are estimated as follows:

If the primer is shorter than 25 nucleotides, the approximate melting temperature is calculated with the formula: Tm = 4 (G + C) + 2 (A + T).

(6) The annealing temperature should be about 5°C lower than the melting temperature.

MgCl2 concentration: **Mg2+ ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of MgCl2 has to be selected for each experiment.**

- **Too few Mg2+ ions result in a low yield of PCR product, and too many will increase the yield of non-specific products.**
- **The recommended range of MgCl2 concentration is 1 to 3 mM,under the standard reaction conditions specified.**

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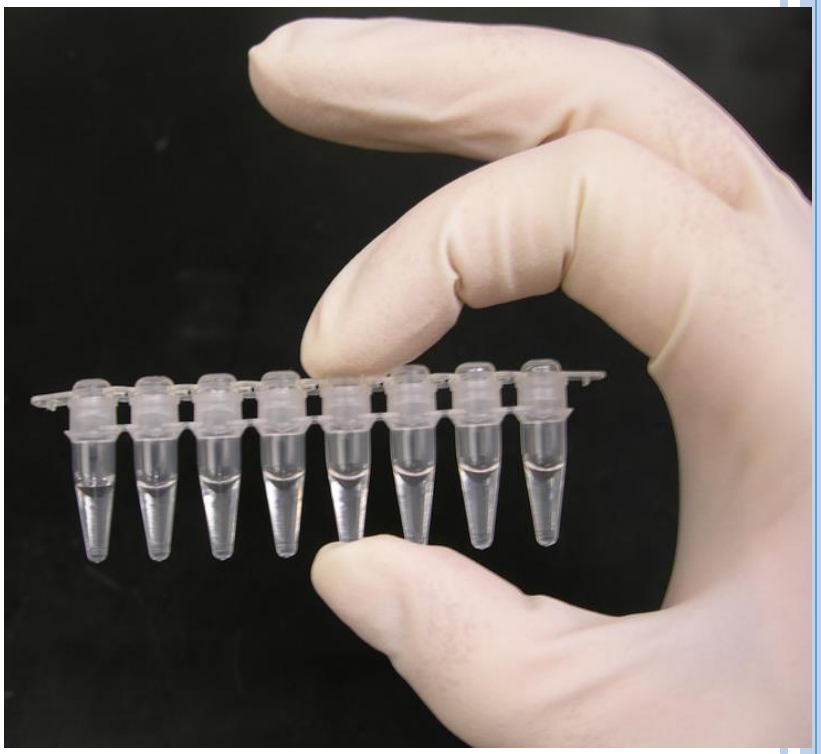

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PCR Three steps

1. **Separating the Target (DNA-Denaturation):**

During the first step of PCR, the DNA is heated to more than 90 degrees Celsius (194 degrees Fahrenheit) to separate the double-stranded DNA into two separate strands.

The high temperature breaks the relatively weak bonds between the nucleotides that form the DNA code.

PCR Three steps

2. Binding Primers to the DNA (Annealing):

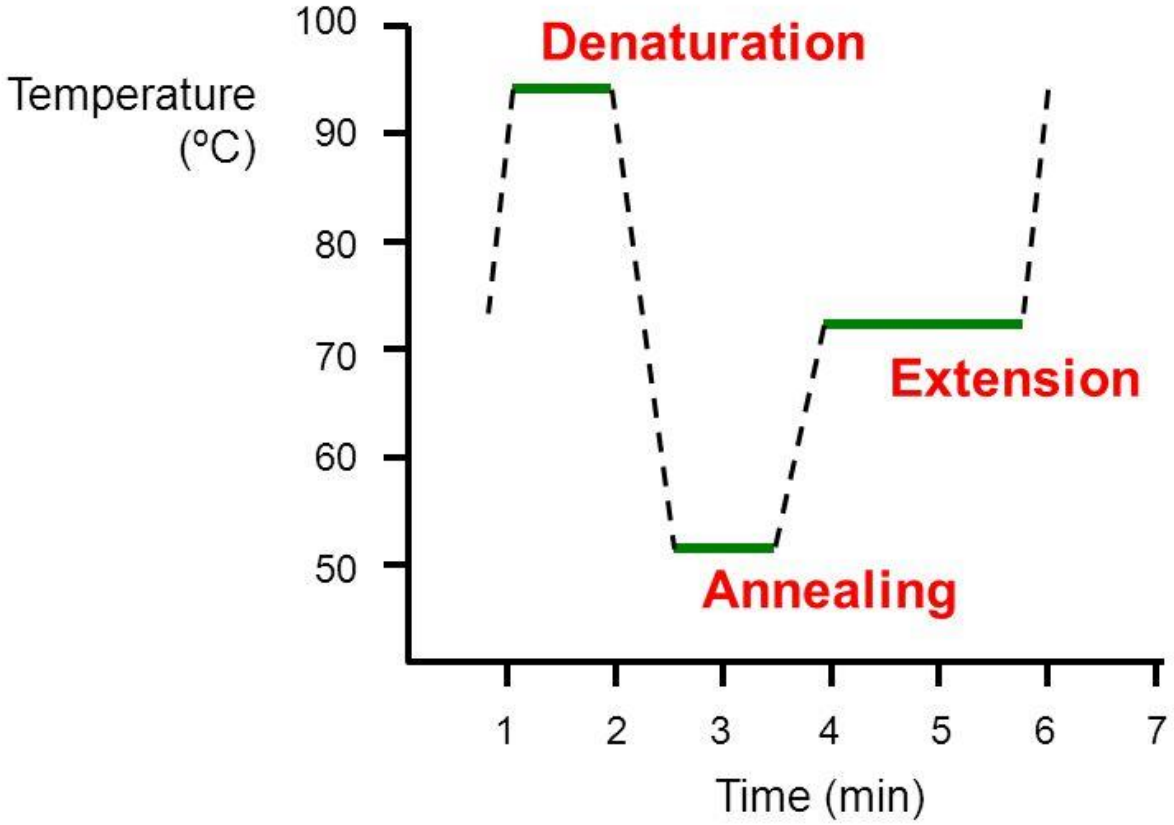
- **PCR does not copy the all of the DNA in the sample, It copies only a very specific sequence targeted by the primers.**
- **The primers bind to the beginning of the sequence to mark it for the extension.**
- **The tube cooled down untill the primer binding that occurs between 40 and 60 degrees Celsius.**

PCR Three steps

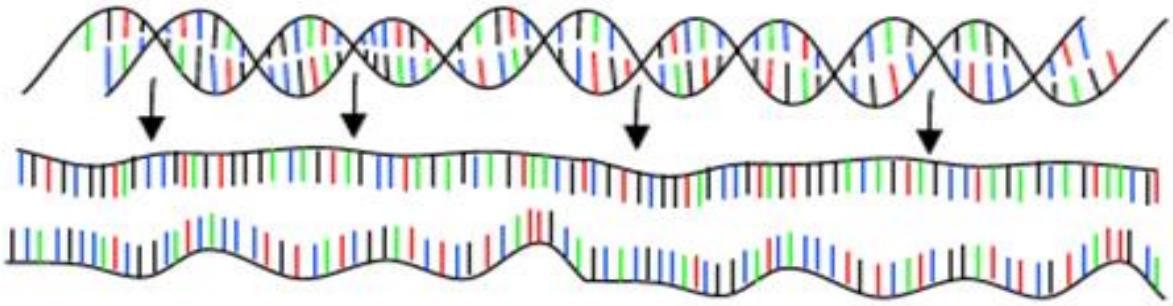
3. Making a Copy – Extension:

- **During the Extension, the temperature is increased to approximately 72 degrees Celsius.**
- **Nucleotides in the solution are added to the annealed primers by the DNA polymerase to create a new strand of DNA complementary to each of the single template strands.**

PCR is a dance with 3 steps

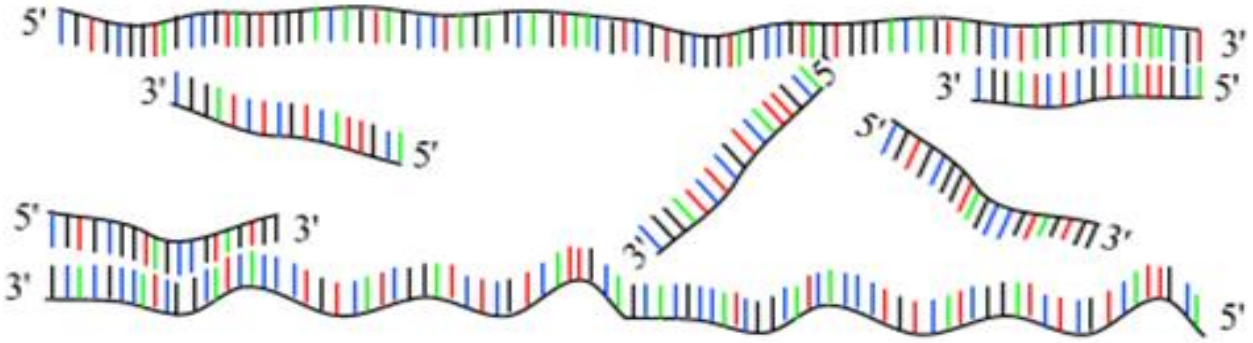


Adapted Brown 9.6



Step 1 : denaturation

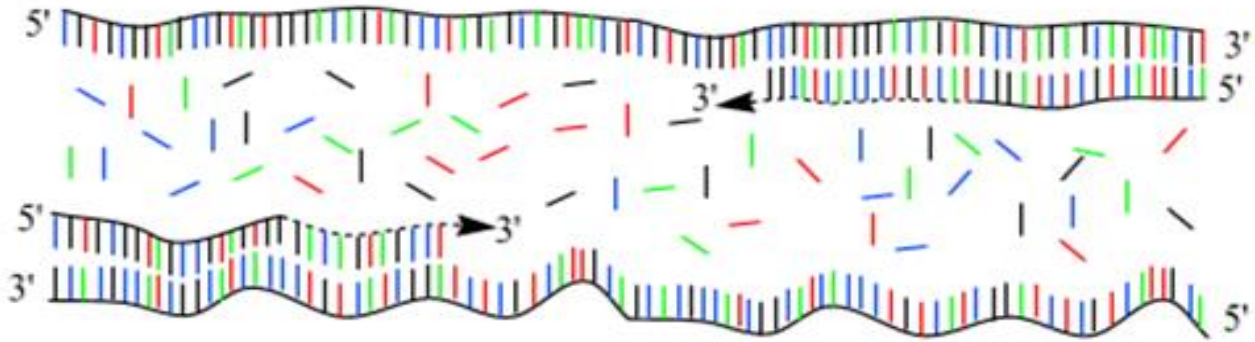
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!

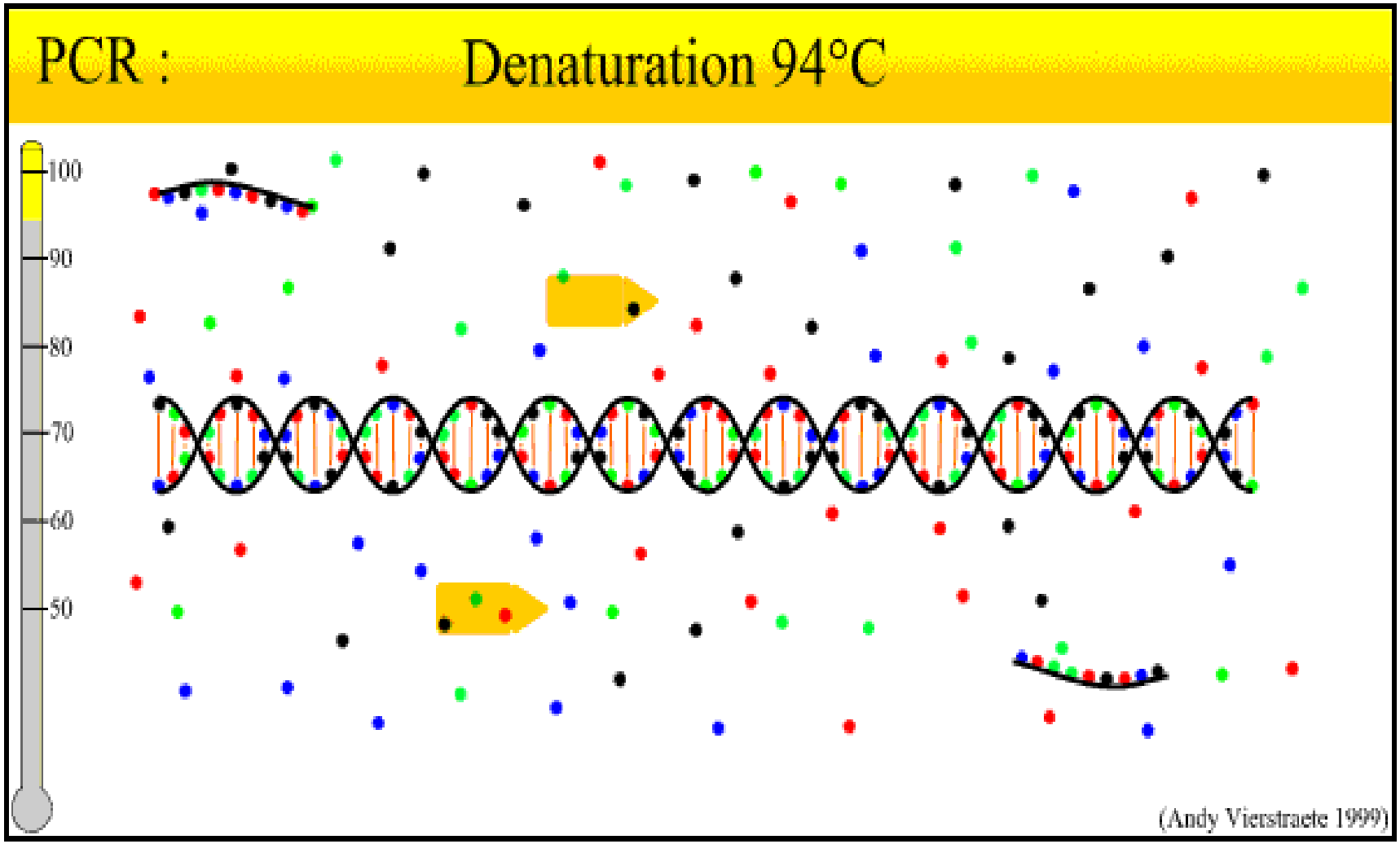


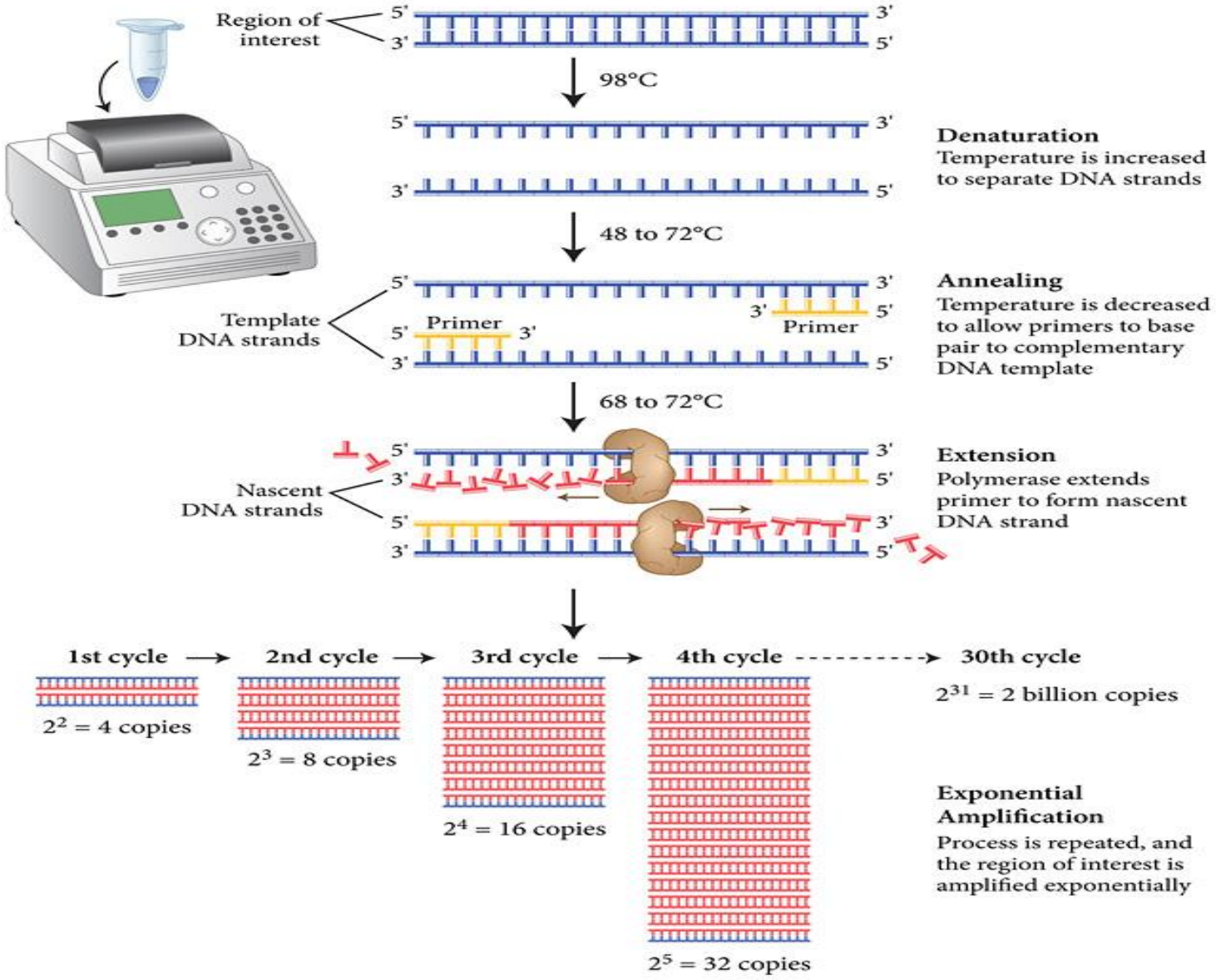
Step 3 : extension

2 minutes 72 °C

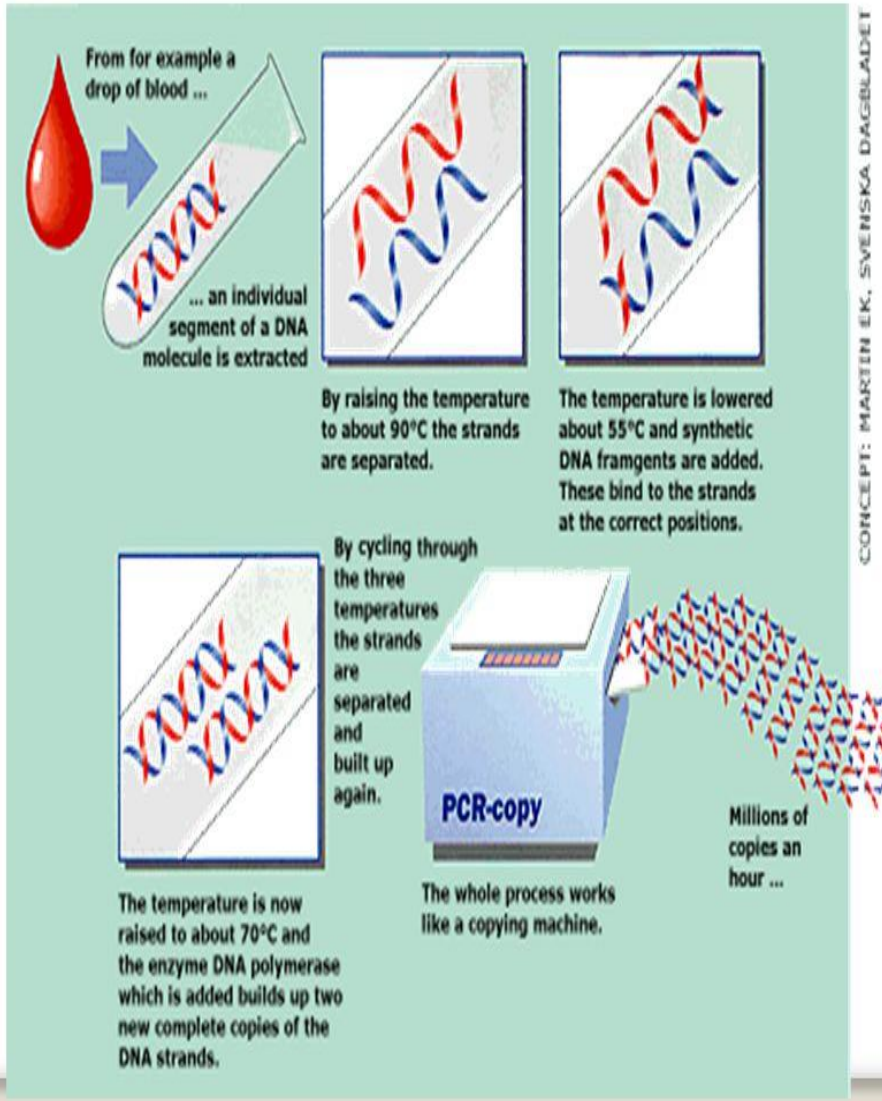
only dNTP's

(Andy Vierstraete 1999)


PCR : Denaturation 94°C
100
90
80
70
60
50
(Andy Vierstraete 1999)


Region of interest
5'
3'
3'
5'
98°C
Denaturation
Temperature is increased to separate DNA strands
48 to 72°C
Annealing
Temperature is decreased to allow primers to base pair to complementary DNA template
Template DNA strands
Primer
Primer
68 to 72°C
Extension
Polymerase extends primer to form nascent DNA strand
Nascent DNA strands
1st cycle
2nd cycle
3rd cycle
4th cycle
30th cycle
2^2 = 4 copies
2^3 = 8 copies
2^4 = 16 copies
2^5 = 32 copies
2^{31} = 2 billion copies
Exponential Amplification
Process is repeated, and the region of interest is amplified exponentially

PCR Steps



PCR Cycles	Target Copies
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024
15	32,768
20	1,048,576
25	33,554,432
30	1,073,741,842

GEL ELECTROPHORESIS

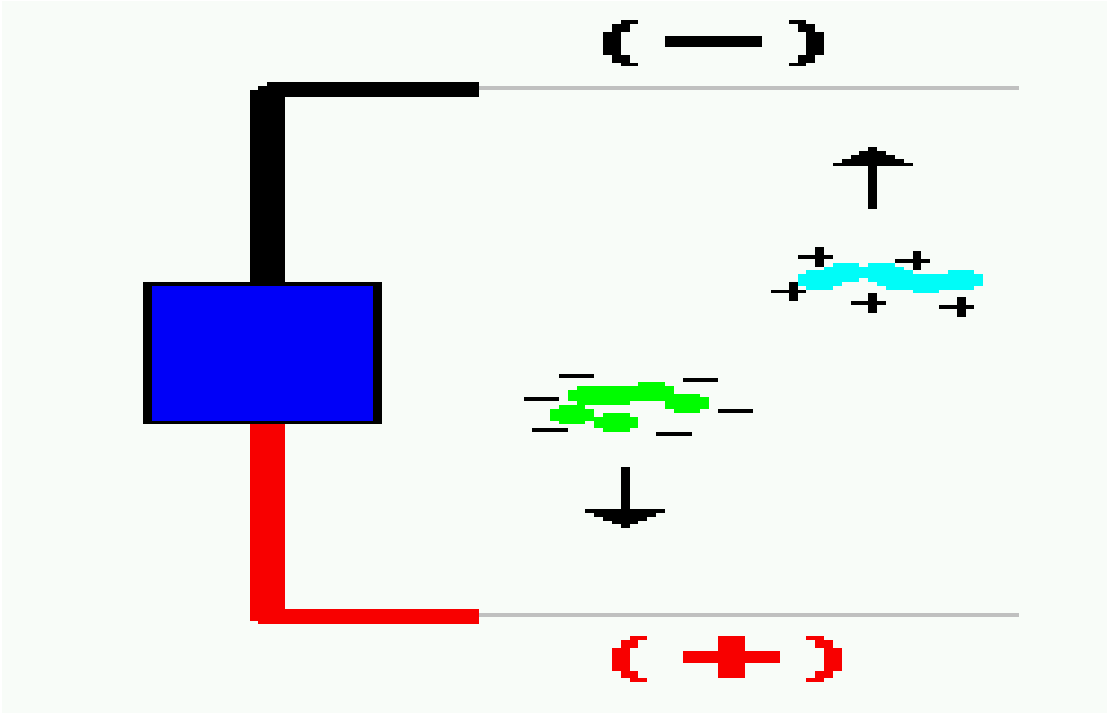
This phenomenon is called Sieving

Nucleic acid and Proteins' molecules are separated by applying an electric field to move molecules through a matrix of agarose.

Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel.

"Electrophoresis“

Refers to the *Electromotive force* (EMF) that is used to move the molecules through the gel matrix.



Electrophoresis is a technique used for sorting of macromolecules molecules based on size and charge.

- The gel is placed in an electrophoresis chamber, which is then connected to a power source.
- The electric field consists of a negative charge at one end which pushes the molecules through the gel and a positive charge at the other end that pulls the molecules through the gel.

Method For Electrophoresis

Prepare agarose gel
Melt, cool and add Ethidium Bromide. Mix thoroughly.

⇩

Pour into casting tray with comb and allow to solidify

⇩

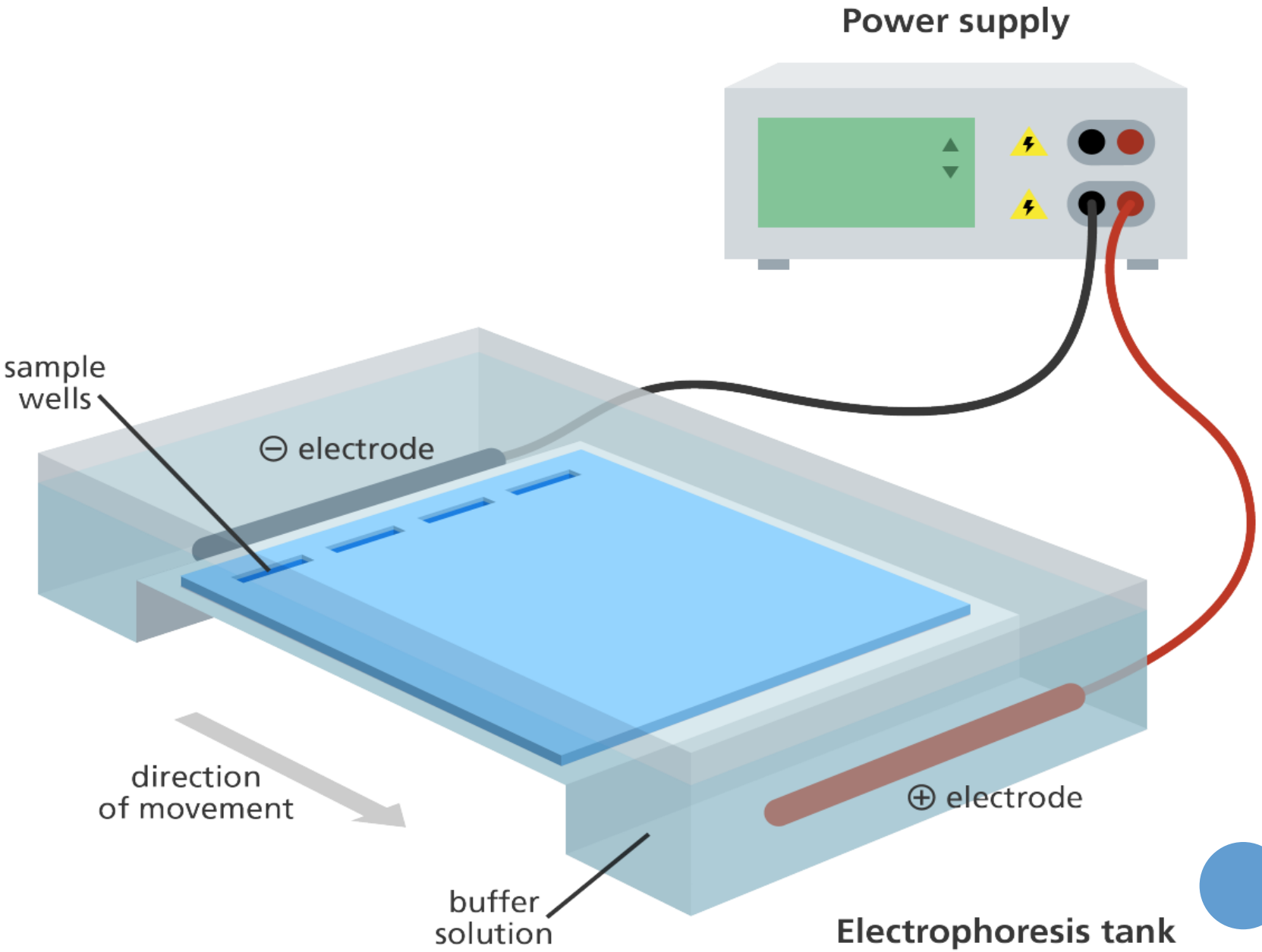
Add running buffer, load samples and marker

⇩

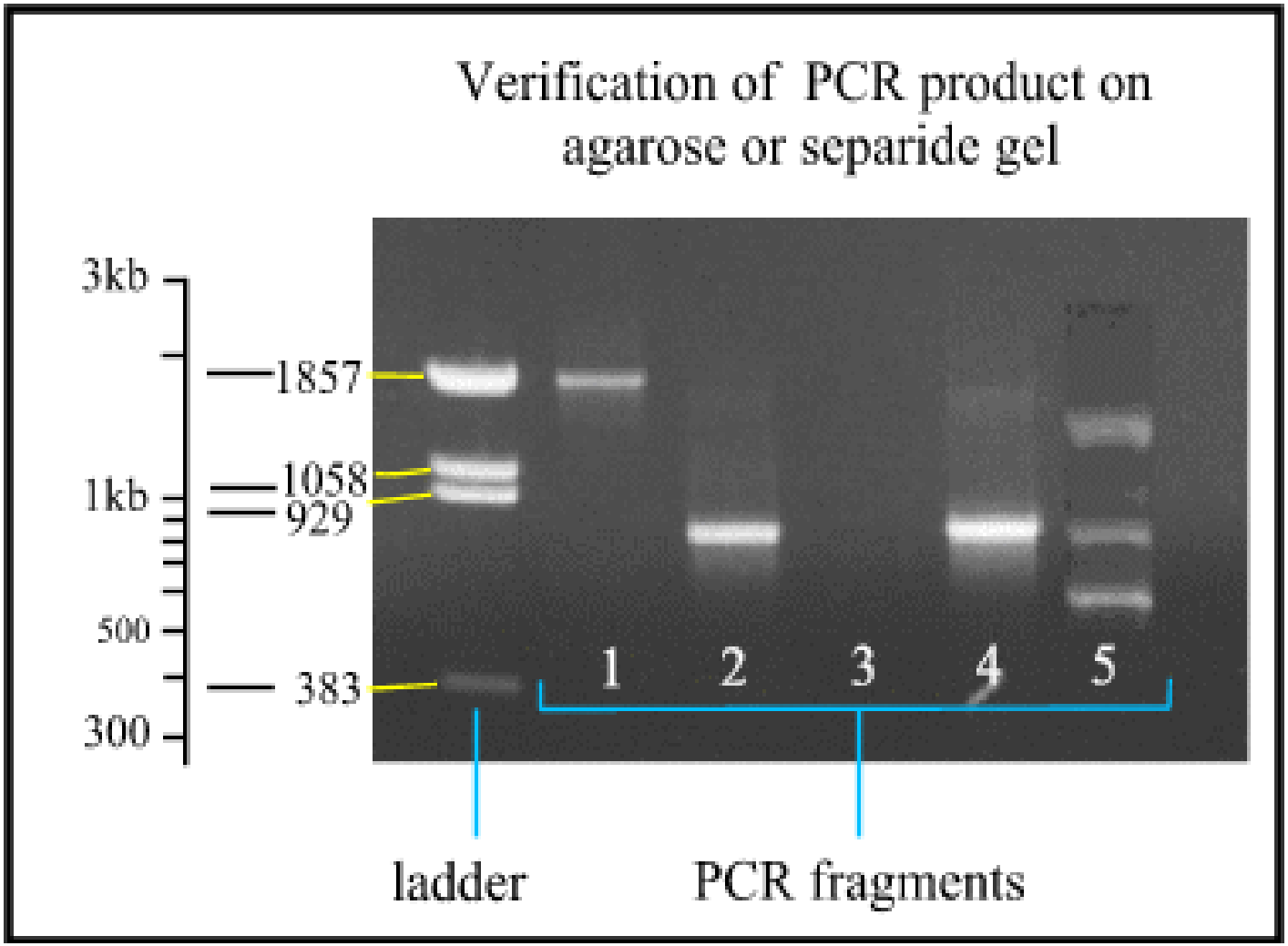
Run gel at constant voltage until band separation occurs

⇩

View DNA on UV light box and show results


Power supply
sample wells
⊖ electrode
direction of movement
⊕ electrode
buffer solution
Electrophoresis tank

Agarose gel electrophoeresis



Application of PCR

1. **Diagnosis of infectious diseases (Medical uses):**

- **PCR is a highly sensitive tool for diagnosis of various diseases in human, animals and plants.**
- **It provides a rapid and highly specific diagnosis.**
- **PCR also permits identification of non-cultivatable or slow-growing M.O. such as *Mycobacteria spp*, anaerobic bacteria.**
- **Discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.**

2. **Detection of new virulent sub-types.** **The sub-types of an organism that were responsible for earlier epidemics can also be determined by PCR analysis.**

3. **Detection of genetic diseases:**

The occurrence of genetic diseases can be identified by the length of Restriction fragment length polymorphism (RFLP).

4. **The amount of virus ("viral load"): can also be quantified by PCR-based DNA quantitation techniques.**
5. **Detection of Mutation: mutation resulting due to some change in the DNA.**
6. **Diagnosis of retroviral infection and cancers. (oncogenes)**
7. **sex determination of embryos.**
8. **Prenatal testing:**

9. **Forensic science: PCR is very important for the identification of criminal through the DNA fingerprinting technique is used in forensic science.**
10. **Gene Therapy: PCR helps to monitor the gene in gene therapy**
11. **Genomic studies: PCR helps to compare the genomes of two organisms and identify the difference between them.**

12. **Evolutionary studies: It plays an important role in phylogenetic analysis. Minute quantities of DNA from any source such a fossilized material, hair, bones, mummified tissues can be amplified using PCR technique.**
13. **Tissue typing: vital to organ transplantation.**

2008, the traditional antibody-based tests was replaced by molecular techniques.

14. **Research applications:**

DNA sequencing, DNA cloning, Sequence-tagged sites, gene expression and genetic mapping.

15. **PCR in Comparative Studies of Genomes:**

PCR has revolutionized the studies in palaentology and archaelogy. The movie 'Jurassic Park' has created public awareness of the potential applications of PCR!

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