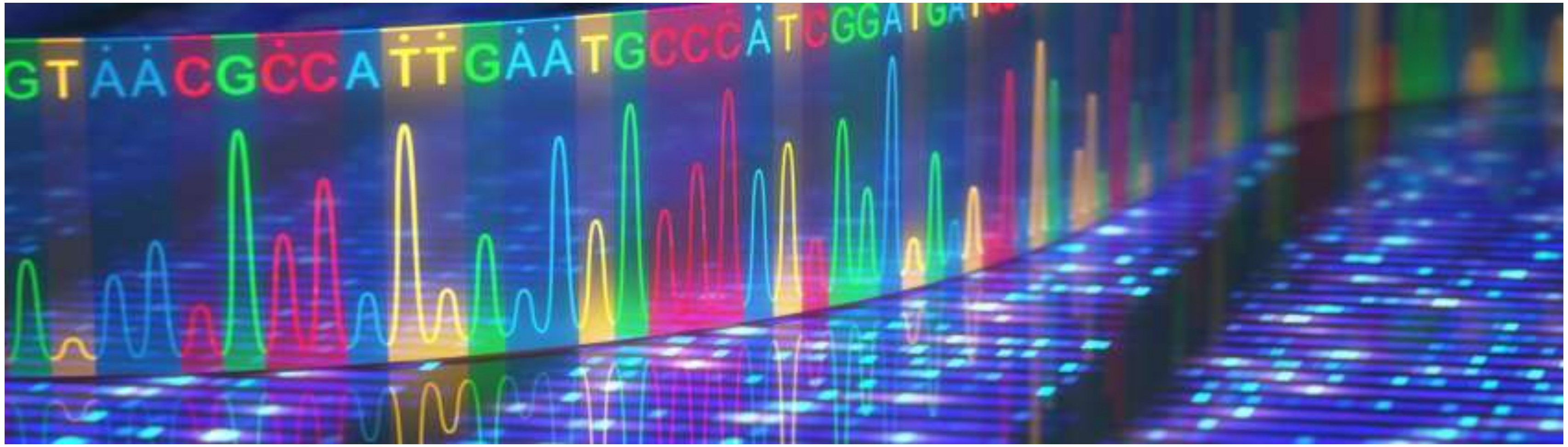
MOLECULAR MICROBIOLOGY



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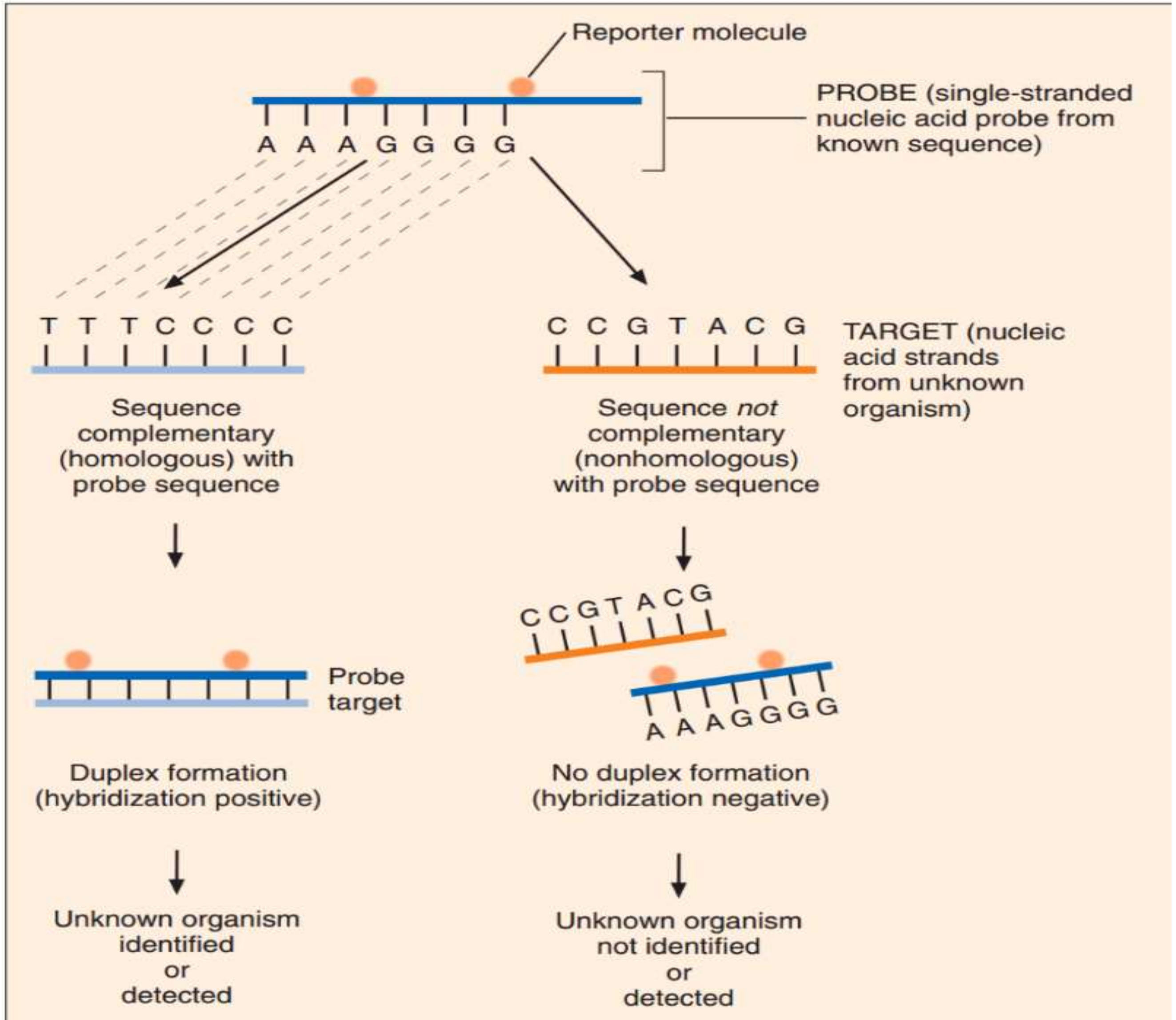
INTRODUCTION

- **Nucleic acid extraction**
- **Hybridization**
  - SOLID FORMAT
  - LIQUID FORMAT
- **Amplification techniques**
  - PCR BASED
  - NON PCR BASED
    - NASBA
    - SDA
- **Post amplification analysis**
  - ELECTROPHORESIS
  - DNA SEQUENCING
  - ENZYMETIC DIGESTION
- **MISCLLENEOUS**
  - LINE PROBE ASSAY
  - MALDI TOF
  - CB-NAAT

Nucleic acid hybridization

Hybridizations methods are based on the ability of two nucleic acid strands with complementary base sequence to bond specifically with each other and form a double stranded molecule, called duplex or hybrid.



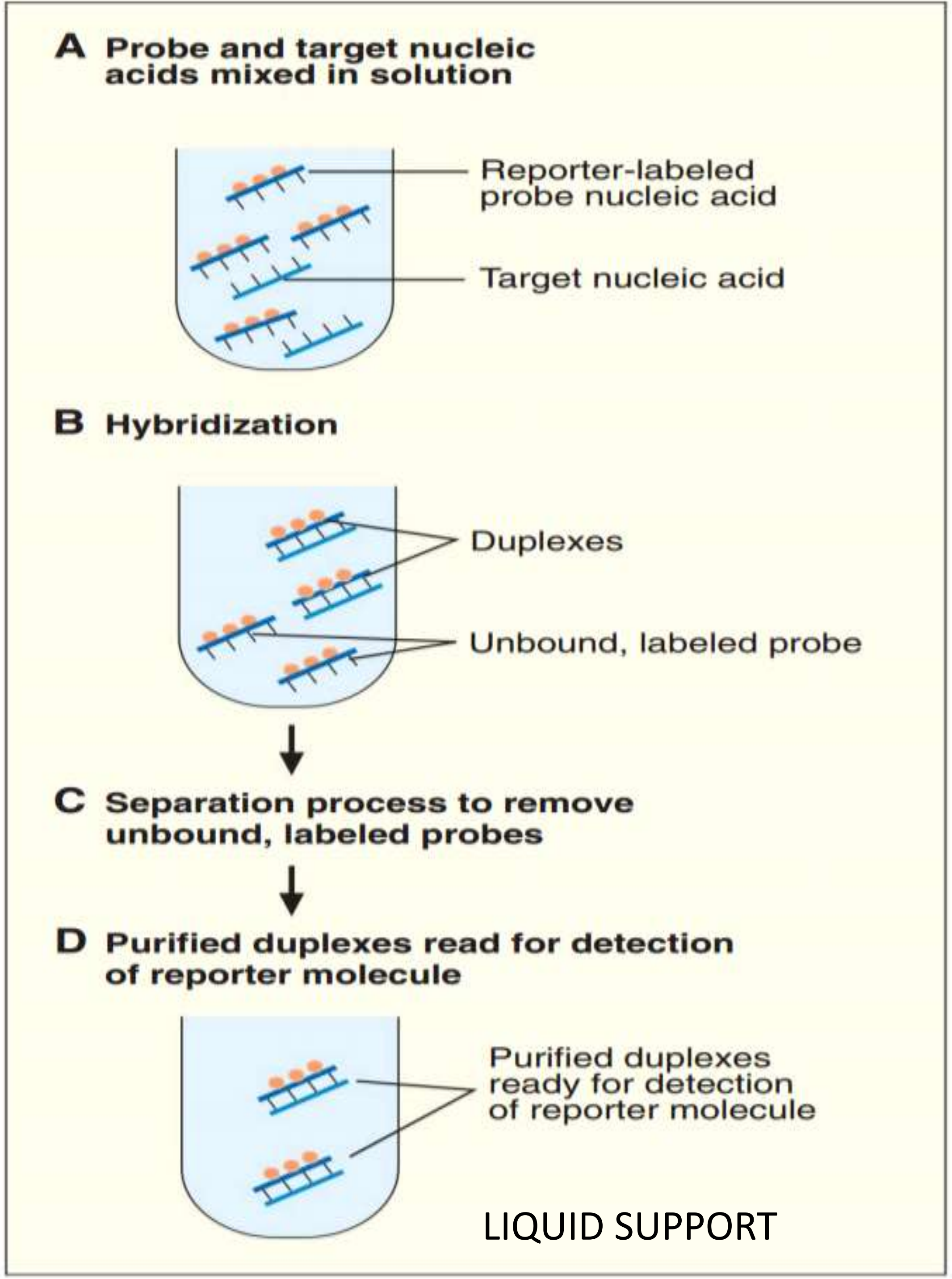
HYBRIDIZATION FORMATES

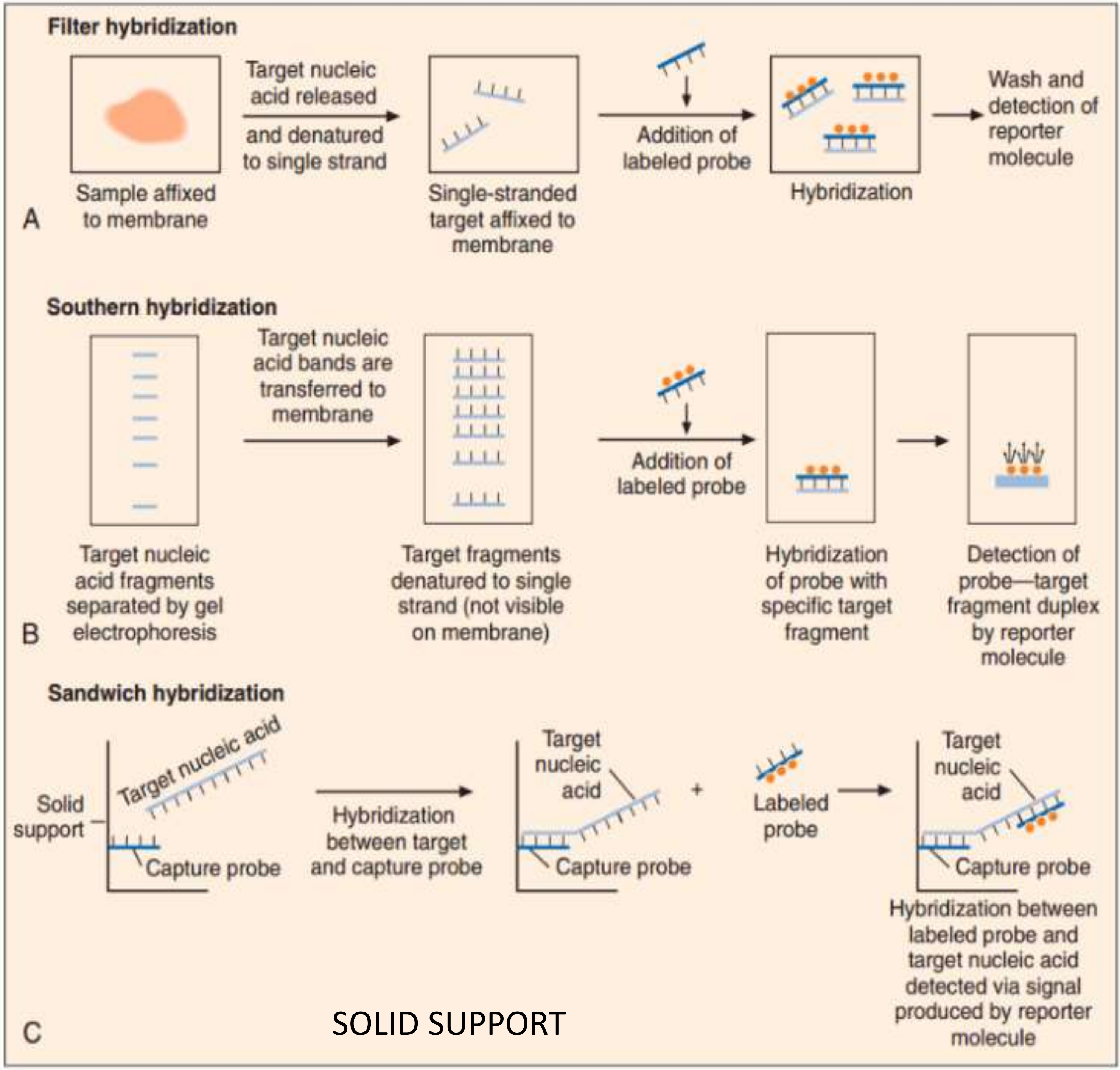
Liquid format

Probe and target are placed in a liquid reaction mixture, which facilitates duplex formation.

Solid support format:

- Filter hybridization
- Southern or northern hybridization
- Sandwich hybridization
- In Situ hybridization


A Probe and target nucleic acids mixed in solution
Reporter-labeled probe nucleic acid
Target nucleic acid
B Hybridization
Duplexes
Unbound, labeled probe
C Separation process to remove unbound, labeled probes
D Purified duplexes read for detection of reporter molecule
Purified duplexes ready for detection of reporter molecule
LIQUID SUPPORT


Filter hybridization
Sample affixed to membrane
Target nucleic acid released and denatured to single strand
Single-stranded target affixed to membrane
Addition of labeled probe
Hybridization
Wash and detection of reporter molecule
A
Southern hybridization
Target nucleic acid fragments separated by gel electrophoresis
Target nucleic acid bands are transferred to membrane
Target fragments denatured to single strand (not visible on membrane)
Addition of labeled probe
Hybridization of probe with specific target fragment
Detection of probe—target fragment duplex by reporter molecule
B
Sandwich hybridization
Solid support
Target nucleic acid
Capture probe
Hybridization between target and capture probe
Target nucleic acid
Capture probe
Labeled probe
Target nucleic acid
Capture probe
Hybridization between labeled probe and target nucleic acid detected via signal produced by reporter molecule
C
SOLID SUPPORT

In situ hybridization

- Allows the nucleic acid of the pathogen to be accessed in situ and denatured to a single strand with the base sequence intact for hybridization with the pathogen specific probe.
- When probe is attached to a fluorescent molecule, this hybridization is k/as fluorescent in situ hybridization (FISH)

Peptide Nucleic Acid Fluorescent In Situ Hybridization (PNA FISH):

- Standard DNA or RNA nucleic acid is replaced with a synthetic PNA probe.
- PNA probe- synthetic nucleic acid that have unique chemical characteristics in which the negatively charged sugar-phosphate backbone of DNAs is replaced by a neutral polyamide backbone.
- Individual nucleotide bases can be attached to this neutral backbone, which then allows the PNA probe to hybridize to complementary nucleic acid targets.
- It's a novel FISH that uses PNA probe to target species- specific rRNA sequence.
- Upon penetration of the microbial cell wall, the fluorescent-labeled PNA probes hybridize to multicopy rRNA sequences within the microorganism, resulting in fluorescent cells.

Procedure:

A drop from a positive blood culture bottle is added to a slide containing a drop of fixative solution

↓

A fluorescent- labelled PNA probe is added and allowed to hybridize

↓

Slides are washed and air dried

↓

After adding mounting medium and a coverslip, slides are examined under a fluorescent microscope

↓

Identification is based on the presence of bright green, fluorescent-staining organism
For negative results, only slightly red-stained background material is seen

Recent uses:

- To identify *Staphylococcus aureus*, *Candida albicans*
- To differentiate *Enterococcus fecalis* from other *Enterococci* in blood culture

Advantages:

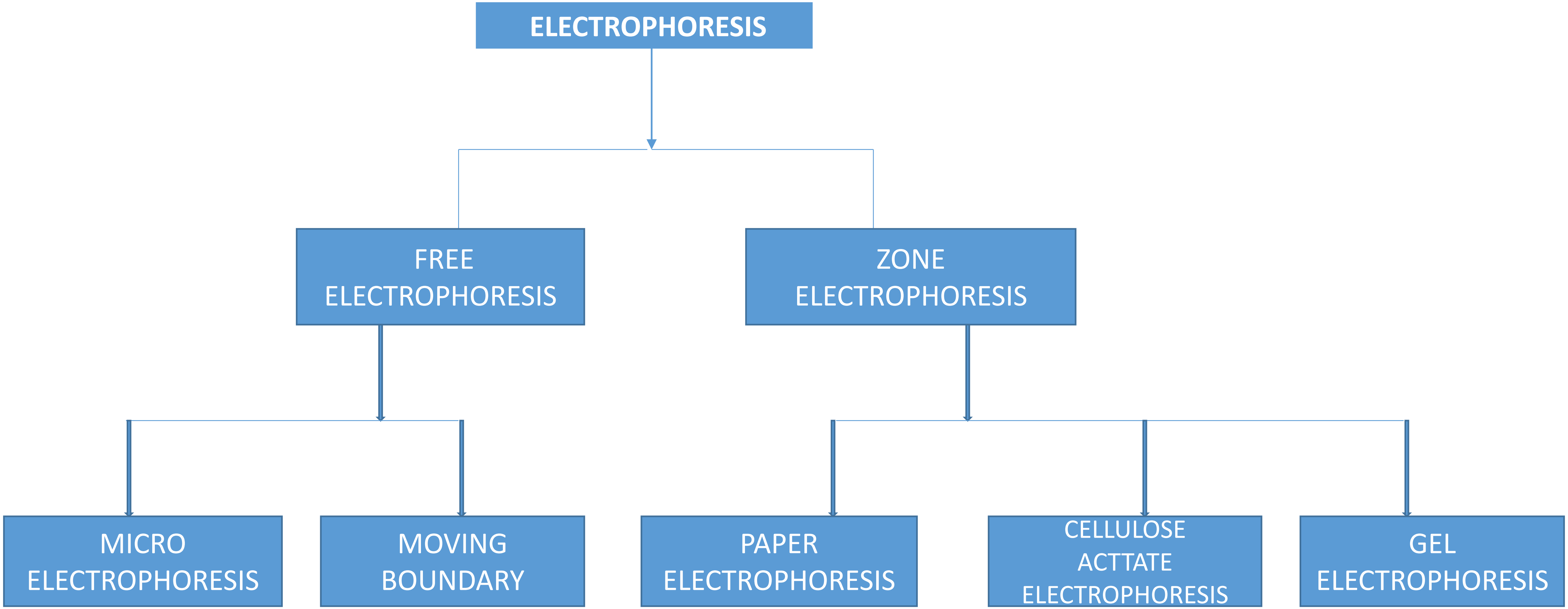
- Improved hybridization characteristics
- Providing faster and more specific results
- Probes are not degraded by nucleases and proteases,so they provide a longer shelf life

ELECTROPHORESIS (Arne Tiselius,1930)

It is a method whereby charged molecules in solution, chiefly proteins nucleic acids, migrate in response to an electrical field.

- Electrophoresis of positively charged particles is called cataphoresis, while negatively charged particles is called anaphoresis
- When they placed in electric field, charged biomolecules move towards the electrodes of opposite charge due to the phenomenon of electrostatic attraction
- Electrophoretic mobility of the molecules is directly proportional to charge density
- Higher the charge greater the electrophoretic mobility.
- An ampholytic become positively charged in acidic conditions & migrate to cathode, In alkaline conditions they become negatively charge and migrate to anode.

Types of Electrophoresis



Gel Electrophoresis

Prepare agarose gel

↓

Pour into casting tray with comb and allow to solidify

↓

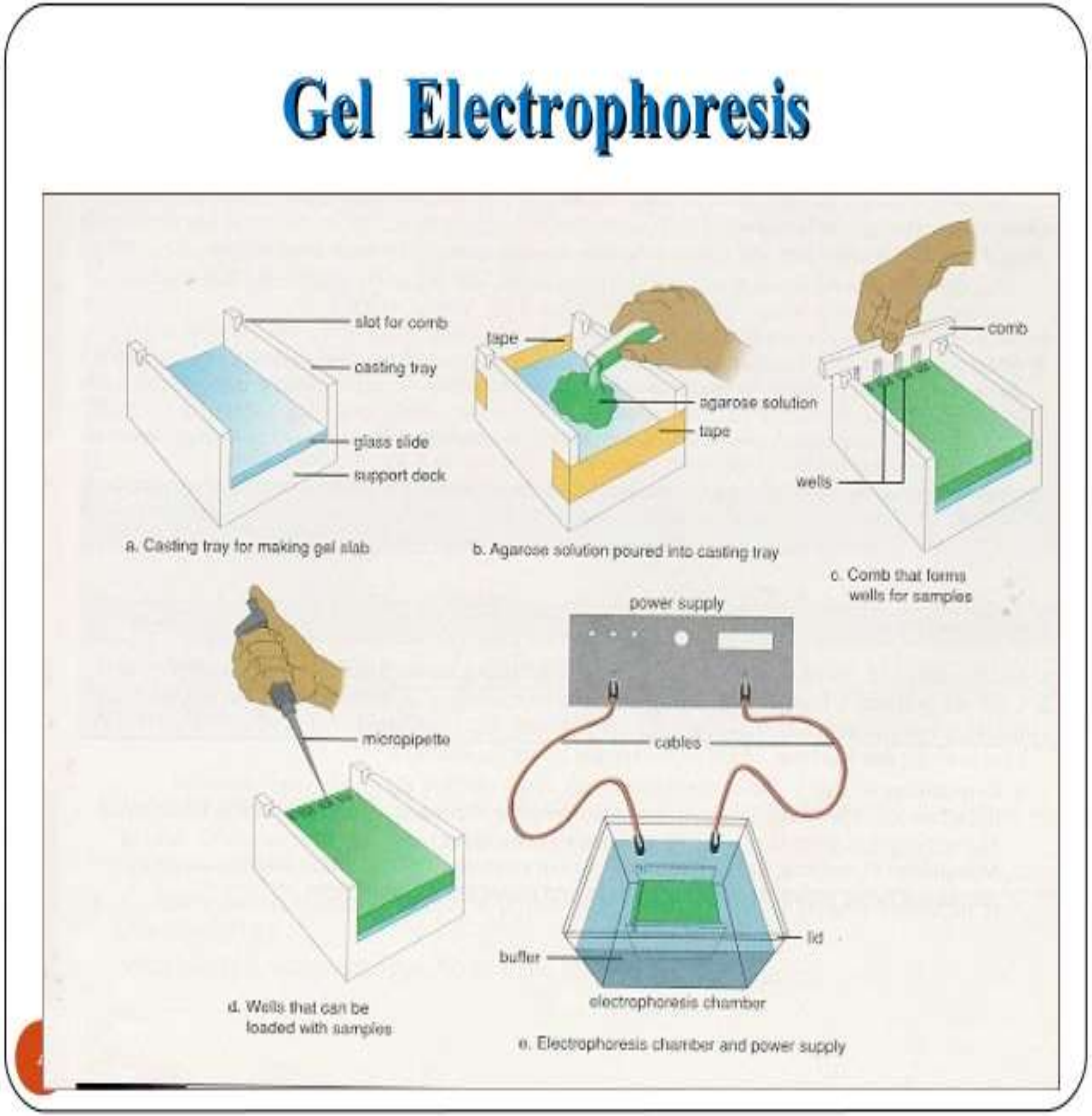
Add running buffer, load samples and marker

↓

Run gel at constant voltage until band separation occurs

↓

View DNA on UV light box and show results



Pulsed-Field Gel Electrophoresis (Schwarts& Cantor,1984)

- Specialized to separate larger fragments ranging from 10kb to 10Mb
- Current is not passed in one direction, instead alternatively at different angles and it is not continuous but in the form of pulses.

Bacterial cell suspensions

↓ **Mix with agarose**

Cast plugs

↓ **Chemical lysis & washing**

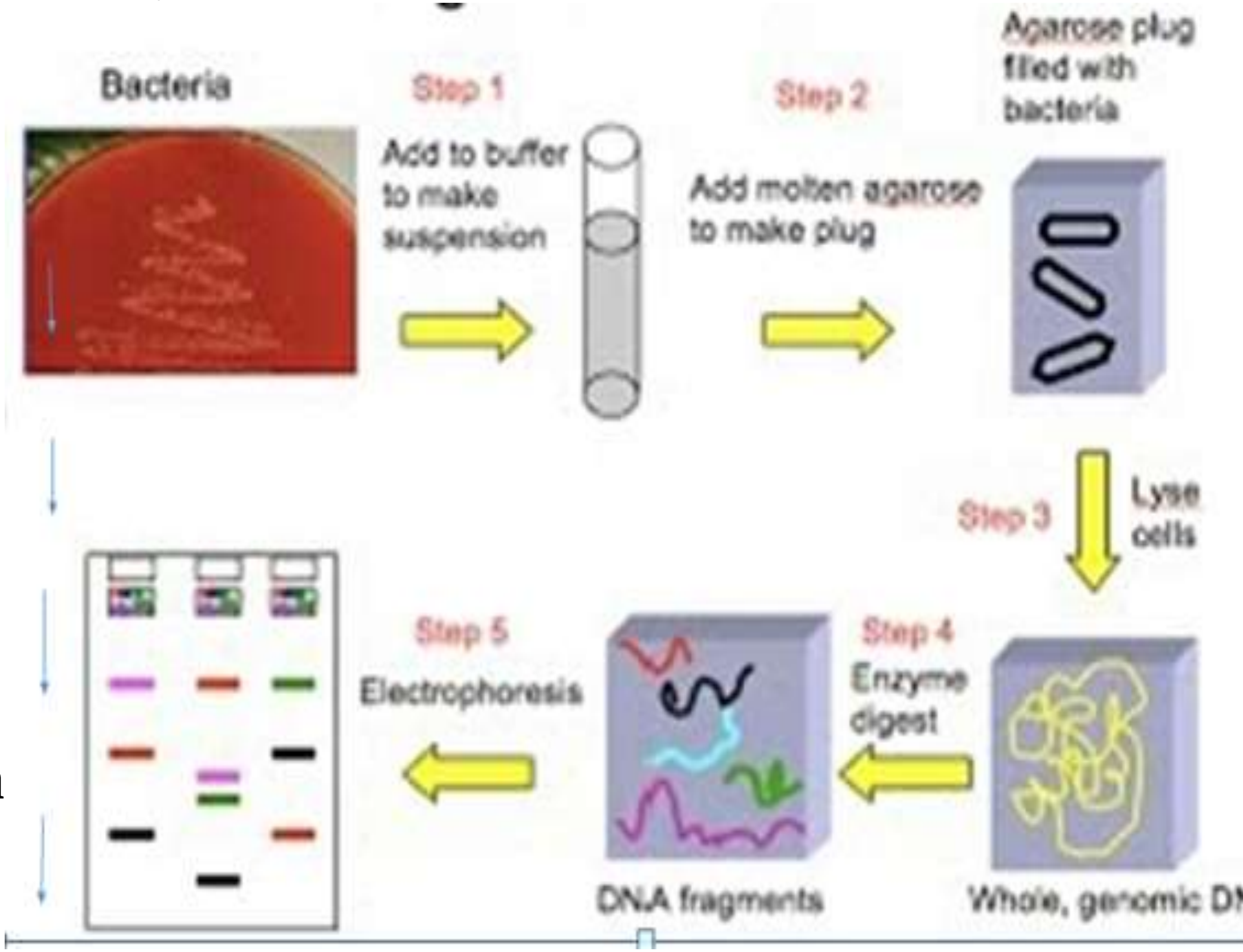
Genomic DNA in gel plug

↓ **Restriction enzyme**

PFGE electrophoresis

↓ **Gel image documentation**

Data analysis



Advantage	Disadvantage
• Gold standard for bacterial subtyping • Universal acceptability and high discriminatory power • Insertion or deletions of mobile genetic elements as well as large recombination events within genomic DNA will result in changes in the PFGE patterns	• Special electrophoresis equipment are required • Labor intensive and time consuming method • Lack the resolution power to distinguish bands of nearly identical size

Blotting Techniques

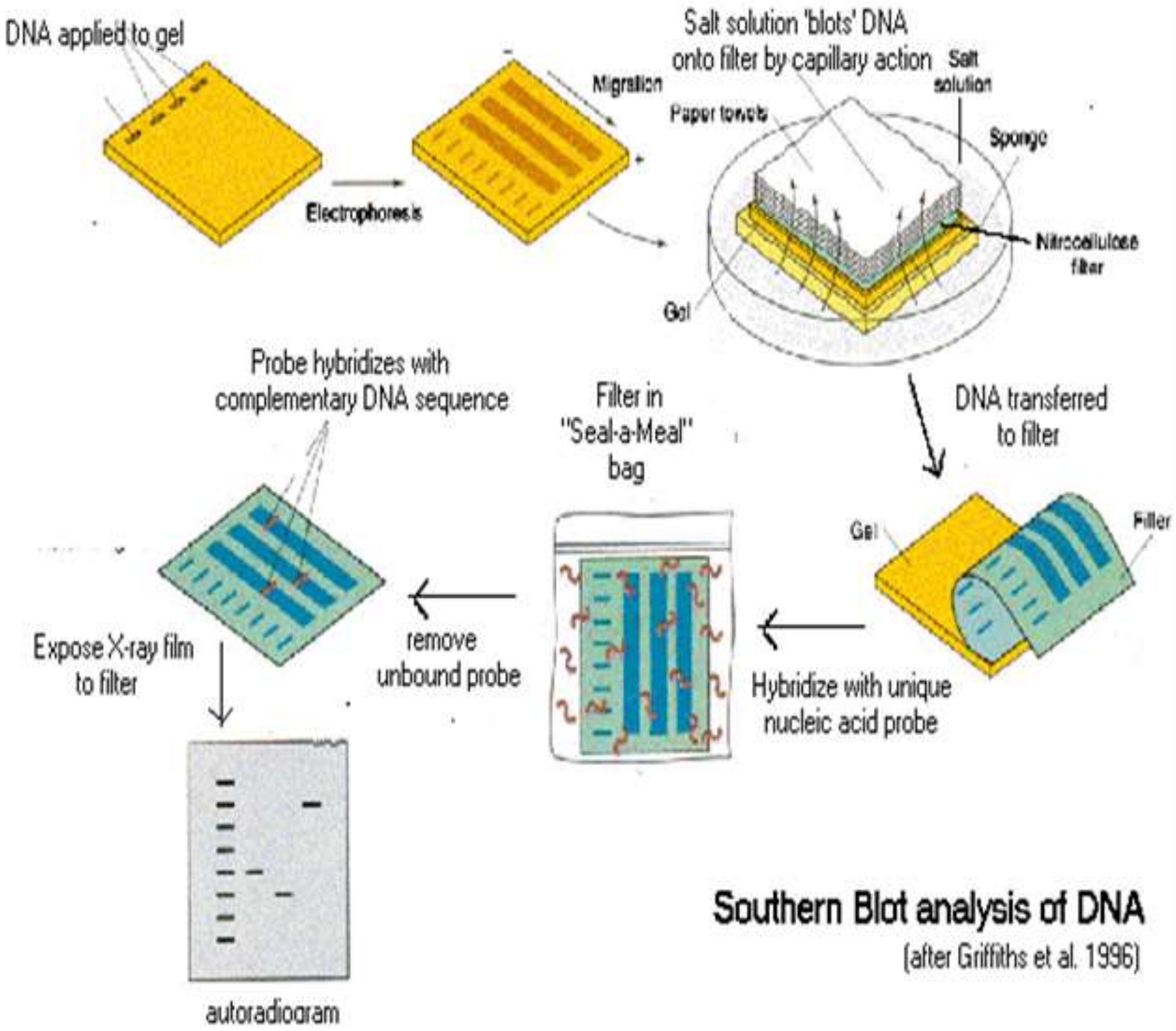
- Blot- method of transferring DNA, RNA and proteins from gel on to a carrier, followed by their detection by using specific nucleic acid probes or enzyme immunoassay.

Types

1. Southern blotting
2. Northern blotting
3. Western blotting
4. Eastern blotting

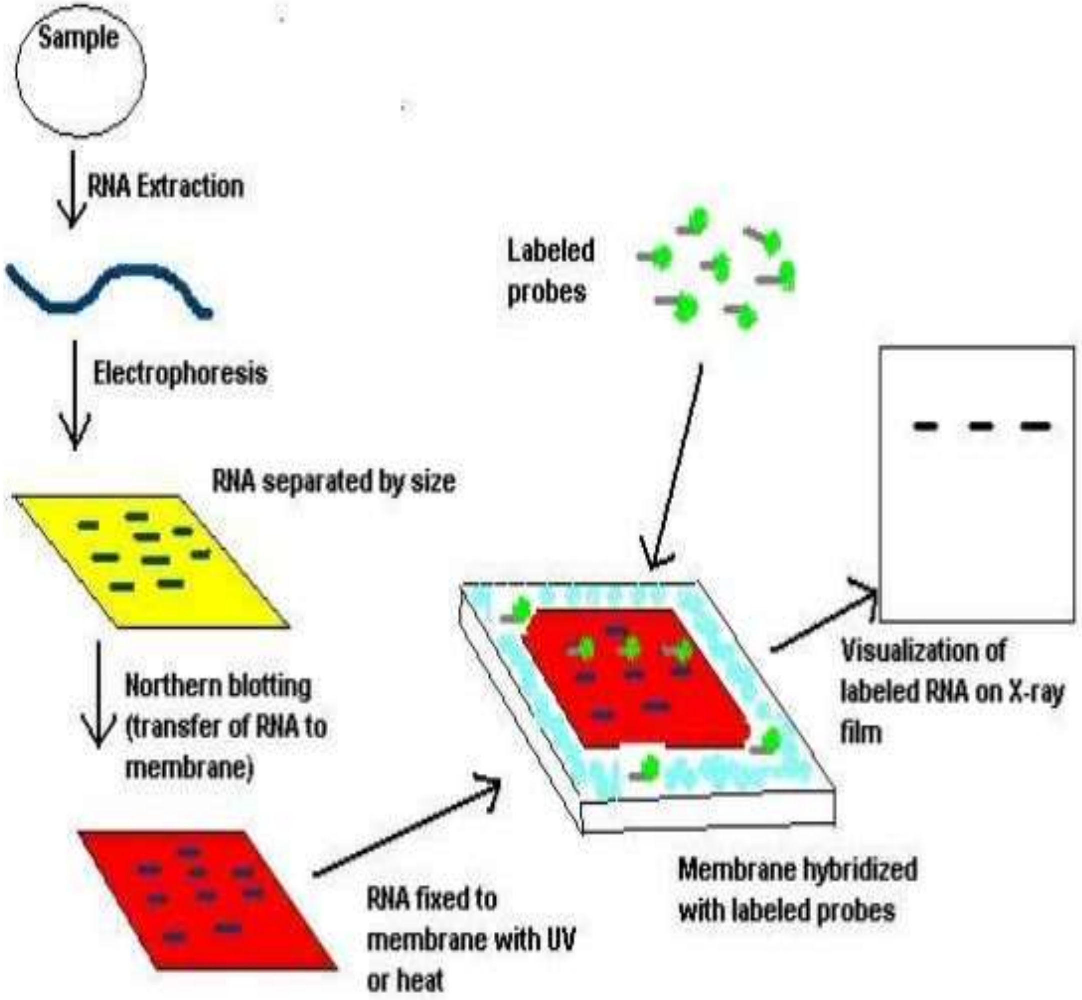
Southern blot

(Edwin Southern in 1975)



Northern Blot

James Alwine, David Kemp, George Stark (1977)



Southern blot	Northern blot
Advantage: • Effective way to detect a specific DNA sequence in a large, complex sample of DNA. • Can be used to quantify the amount of the present DNA • Cheaper than DNA sequencing	**Advantages:** • Simple method • Highly specific • Quality and quantity of gene can be measured
Disadvantages: • More expensive than most other tests • Complex and labor intensive • Time consuming and cumbersome	**Disadvantages:** • Relatively less sensitive than nuclease protection assay and RTPCR • Detection with multiple probes is a problem. • Time consuming procedure • RNA samples can be degraded by RNAse, so the quality of data and quantitation of expression is quite negatively affected.

Southern blot	Northern Blot
Applications: • To identify specific DNA in a DNA sample • To isolate desired DNA for construction of rDNA. • In gene discovery, mapping, evaluation and development studies • Identify mutations, deletions, and gene rearrangements • Used in prognosis of cancer and in prenatal diagnosis of genetic disease • Identification of the transferred genes in transgenic individual. • Diagnosis of HIV-1 and infectious diseases • In DNA fingerprinting- ➢ Paternity and maternity testing ➢ Criminal identification and forensics ➢ Personal identification	**Applications:** • A standard for the study of gene expression at the level of mRNA. • Detection of mRNA transcript size • Study RNA splicing • Study RNA half life • Often used to confirm and check transgenics

Western blot (Immunoblotting)	Eastern blot (Towbin,1979)
A technique for detecting specific proteins separated by electrophoresis by use of labelled antibodies. **<u>USE</u>** • It has excellent specificity, often used as a supplementary test to confirm the result of ELISA or other immunoassay having higher sensitivity. • To detect antibody in various diseases -HIV -Lyme’s disease -Herpes simplex virus infection -Cysticercosis -Toxoplasmosis	• Used to analyze Protein post translational modification , such as lipids ,phosphomoities and glyco-conjugates • Most often used to detect carbohydrate • Its considered an extension of the biochemical technique of western blotting **<u>USE</u>** • Detection of protein modification in bacterial species • PTM play an important role in translocation across biological membrane

Comparison between blottings

	Southern blot	Northern blot	Western blot
Molecule detected	DNA	RNA	Protein
Gel electrophoresis	Agarose gel	Formaldehyde agarose gel	Polyacrylamide gel
Gel pretreatment	Depurination , denaturation and neutralization	-	-
Blotting method	Capillary transfer	Capillary transfer	Electric transfer
Probe	DNA radioactive or nonradioactive	cDNA, cRNA radioactive or nonradioactive	Primary antibody

AMPLIFICATION TECHNIQUES

These are *in vitro* techniques used to replicate, or amplify, a specific molecule billions-fold in just a few hours

Signal Amplification

- Amplify the signal generated
- Minimum 10^3-10^5 nucleic acid targets can be detected
- Increase the sensitivity of the probe based assays
- b DNA
- Hybrid capture

Target Amplification

- Nucleic acid amplification procedures
- PCR based
- Non PCR based

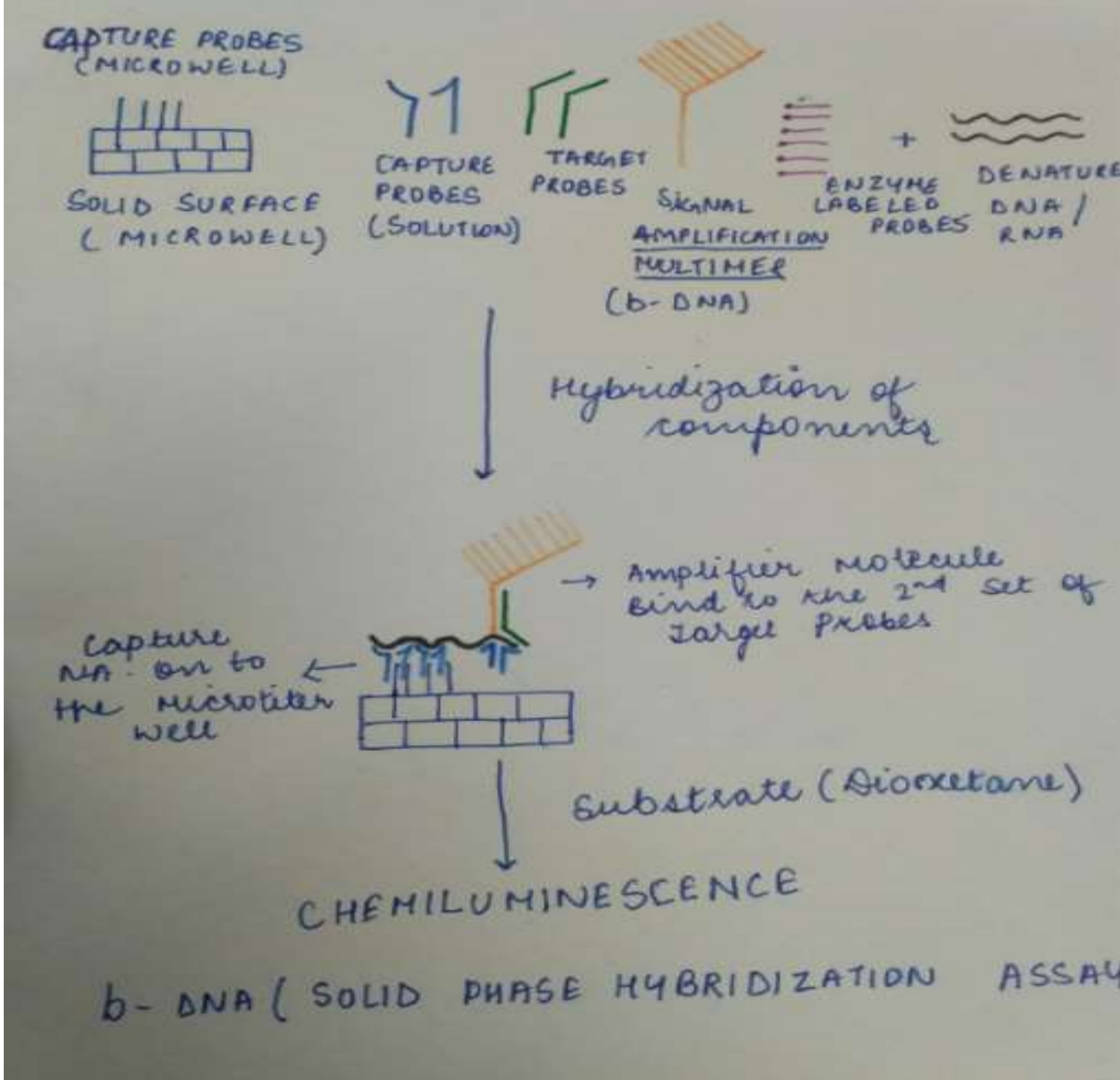
Probe Amplification

- Many copies of the probe that hybridizes the target nucleic acid are made ,
- Q beta replicase
- Ligase chain reaction

SIGNAL AMPLIFICATION TECHNIQUES

Branched DNA assay

Used in
b DNA-Detection and quantification of HIV,HBV and HCV



Hybrid capture assay

- Solution hybridization antibody capture method

Target DNA specimen is denatured

↓

Hybridized with a specific RNA probe

↓

Hybrids captured by antihybrid antibodies coated on surface of tube

↓

(alkaline phosphatase conjugated)

↓

chemiluminescent substrate

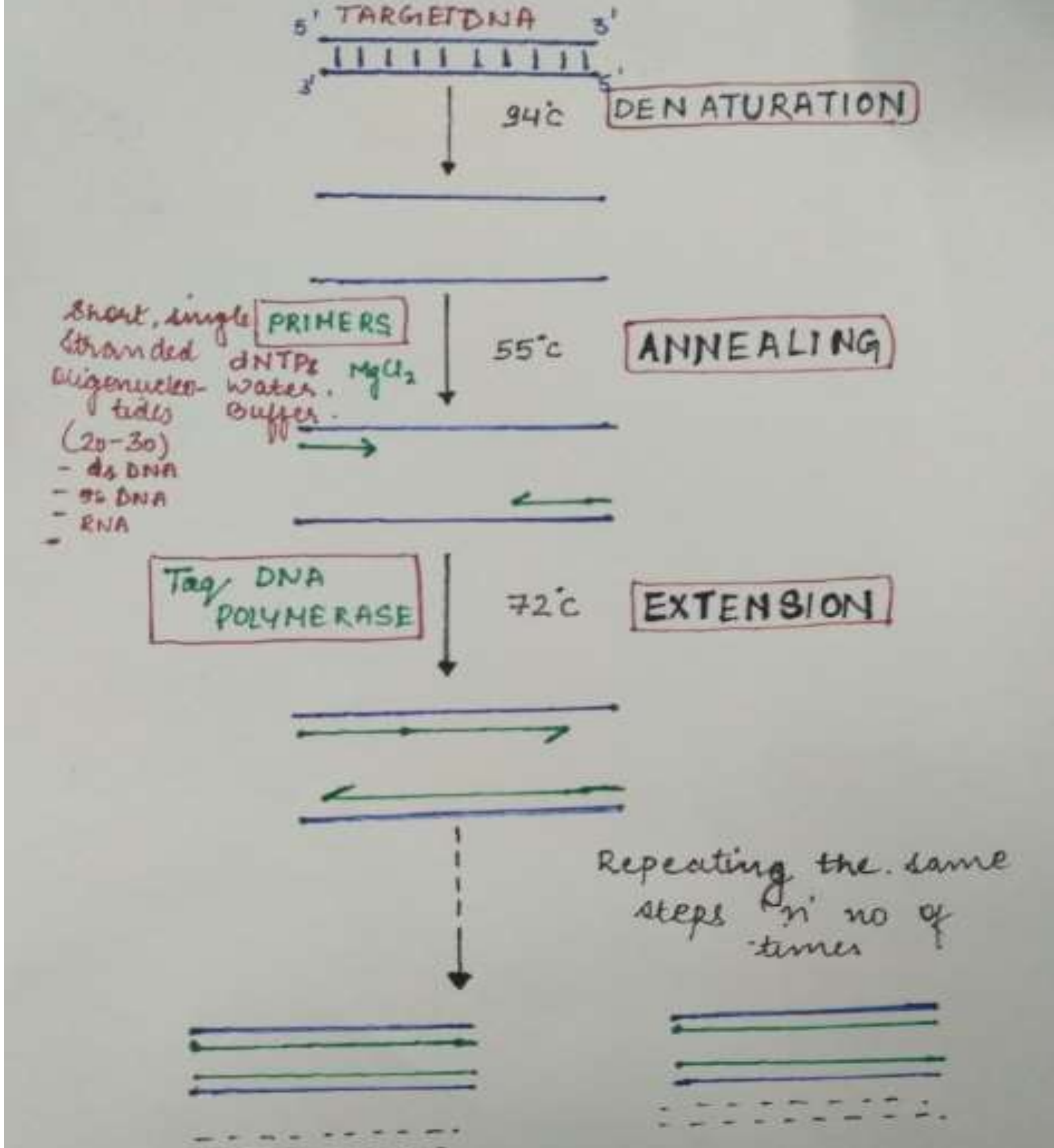
↓

Light emitted is measured in luminometer

AMPLIFICATION TECHNIQUES

POLYMERASE CHAIN REACTION
(1983- Dr Kary Mullis)

- It is an *in vitro* technique used to replicate, or amplify, a specific region of DNA billions-fold in just a few hours enzymatically



DENATURATION

- Initial Melt: 94°C for 2 minutes
- Melt: 94°C for 30 seconds
- At a temperature of 93◦C to 94◦C, the two strands of the DNA target are separated , or denatured
- Break the DNA ladder down the middle to create two strands, a 5’ to 3’ strand and a 3’ to 5’ strand
- At this temperature, all enzymatic reactions from a previous cycle, stop

ANNEALING

- temperature of the reaction is reduced to allow strands of DNA with complementary sequence to anneal
- 5’ primer to the 5’ to 3’ strand
- 3’ primer to the 3’ to 5’ strand
- Brownian motion of primers
- Ionic bonds are constantly formed and broken between the single-stranded primer and DNA target
- often between 50◦C and 60◦C (55°C for 30 seconds)

EXTENSION

- generally occurs at 72◦C.(72°C for 1 minute , Final Extension: 72°C for 6 minutes , Hold: 4°C)
- *Taq* DNA polymerase is most active at this temperature.
- Double-stranded section lengthens, the resulting ionic bond is greater than the forces that break these attractions.

PCR phases

Exponential :

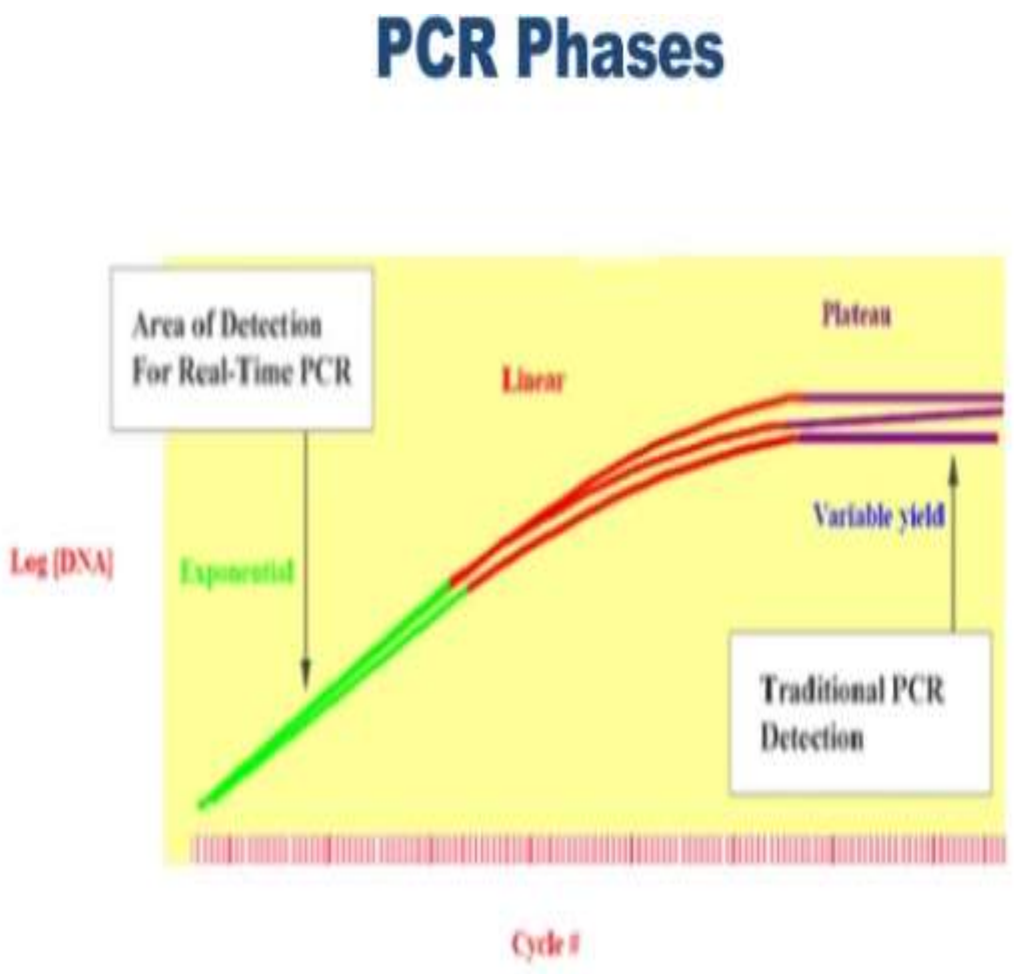
- Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise

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



CONSUMABLES

eppendorf

Pipette with tips

PCR tubes

Advantages

- Specific
- Quick
- Reliable
- Sensitive
- Relatively easy

Disadvantages

- Need for equipment
- Taq polymerase is expensive
- Contamination
- False reactions
- Internal control
- Cross-reaction
- Enrichment steps in (contaminated) samples
- Capacity building needed
- Unspecific amplification

Restrictions

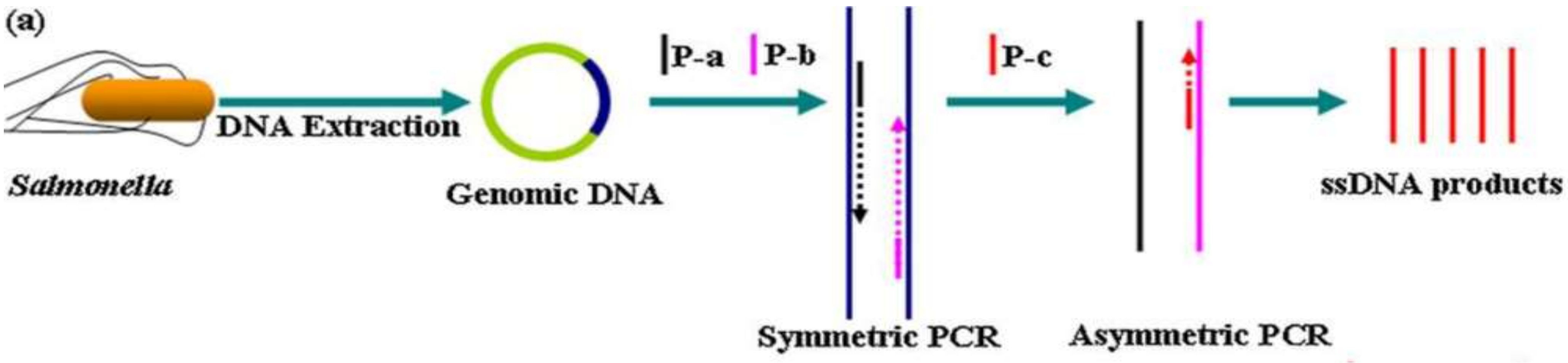
- Contamination of reagents or lab results in false positive results
- Failure due to a mistake in the protocol
- Different materials/parts of the sample can inhibit the PCR process

TYPES OF PCR

- Asymmetric PCR
- Nested PCR
- Multiplex PCR
- Competitive PCR
- RT-PCR (reverse transcriptase)
- Real-time PCR
- Hot start PCR
- Inverse PCR
- Long PCR
- Allele specific PCR
- Anchored PCR

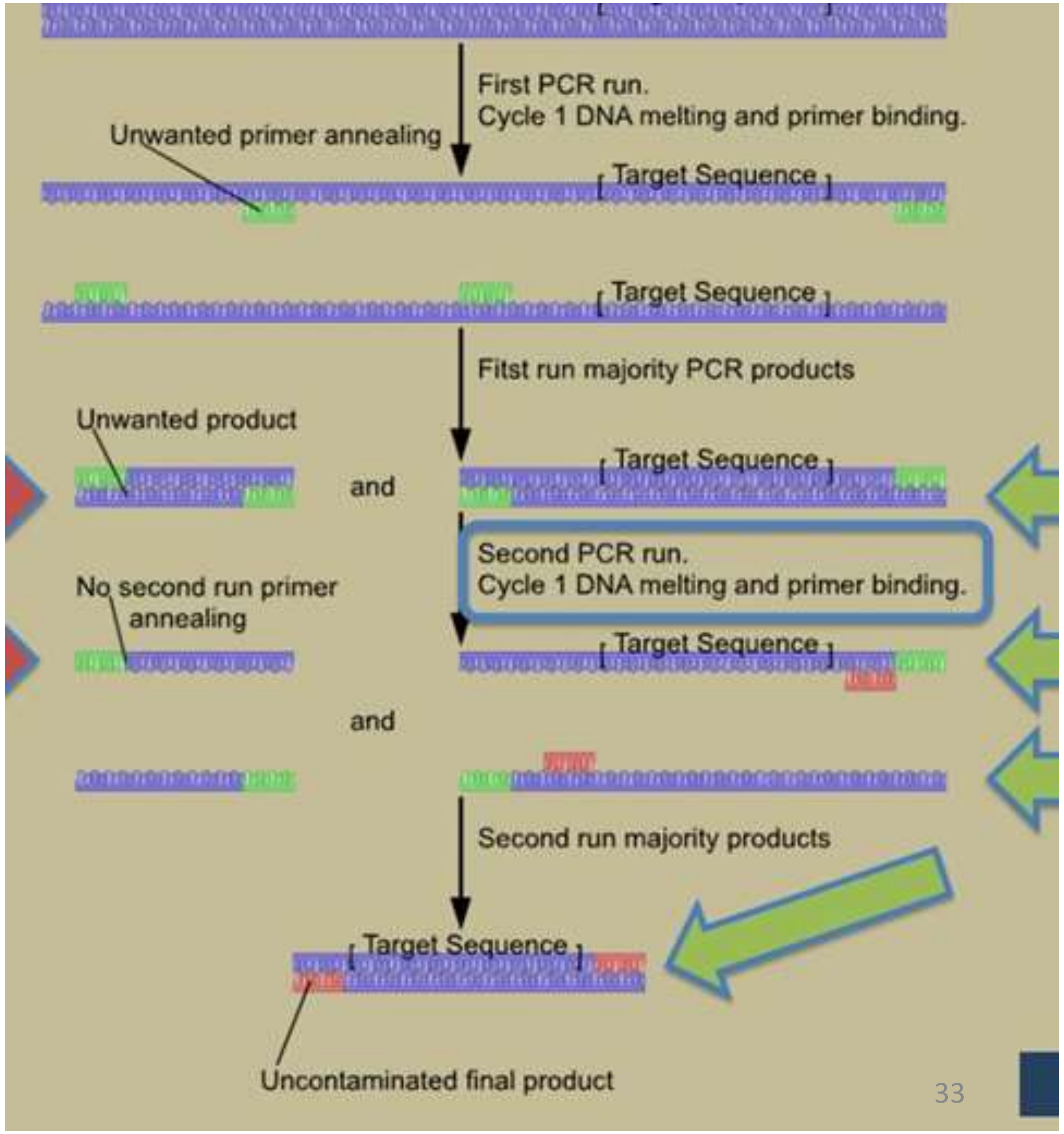
Asymmetric PCR

- For synthesis of Single stranded DNA molecules useful for DNA sequencing and hybridization probing where having only one of the two complementary strand is required
- PCR is carried out as usual , but with a great excess of the primers for the chosen strand
- The two primers are used in the 100:1 ratio so that after 20-25 cycles of amplifications one primer is exhausted thus single stranded DNA is produced in the next 5-10 cycles
- Recent modification - **L**inear **A**fter **T**he Exponential PCR (LATE–PCR) uses a limiting primer with a higher melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid reaction



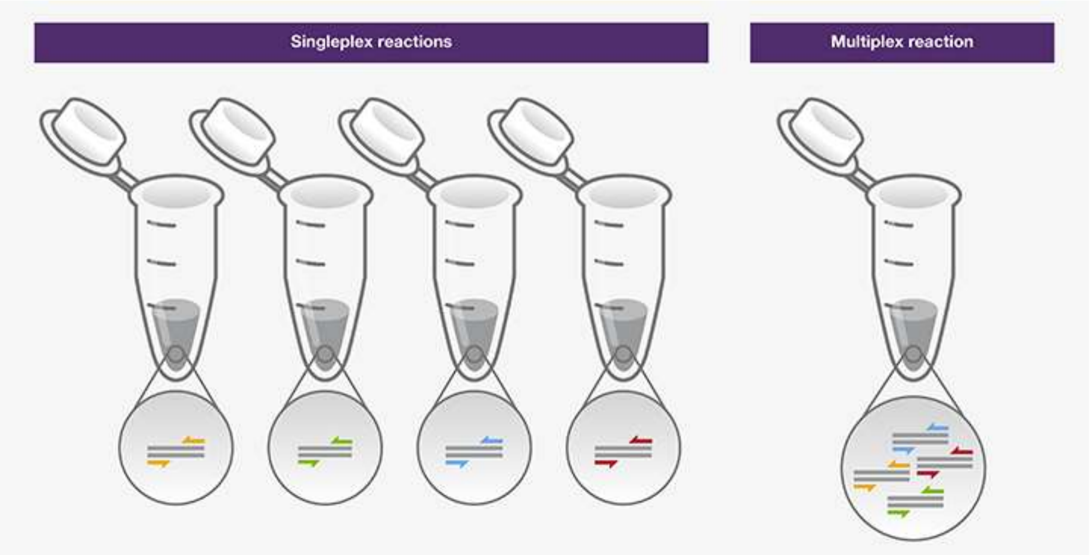
Nested PCR

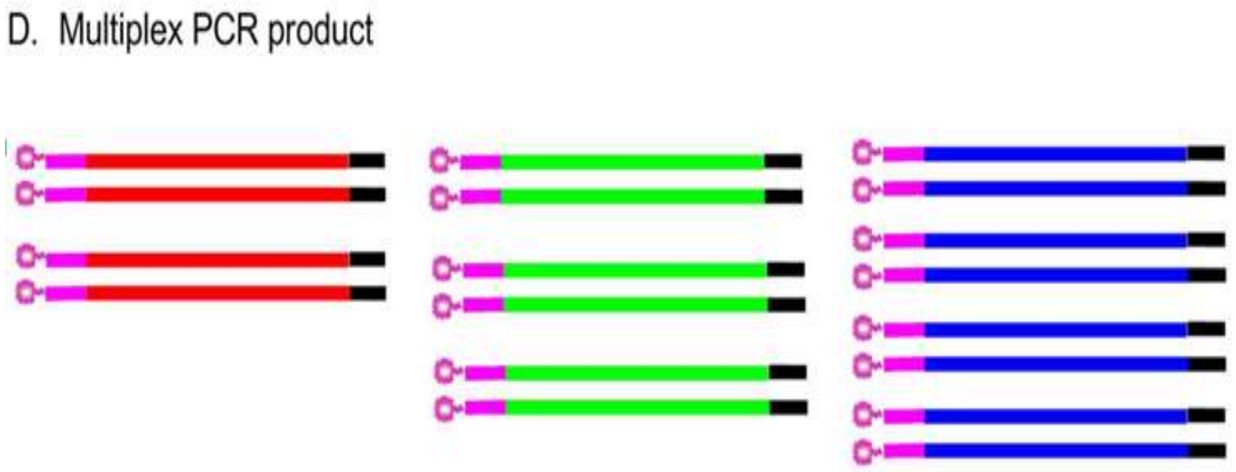
- Take product of PCR 1 use as target in reaction 2
- Increase specificity by having two sets of primers needed for amplification
- Increase sensitivity by amplifying target prior to second PCR
- Two sets of primers are used in two successive reactions(hidden or nested primers
- Valuable in detection of microorganisms that may be in low quantity in blood and tissues
- Advantage-if the wrong PCR fragment was amplified the probability is quite low that the region would be amplified a second time by the second set of primers



Multiplex PCR

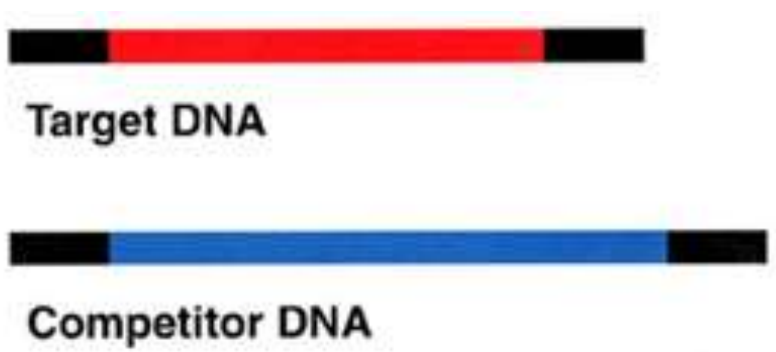
- In it more than one target sequence can be amplified by including more than one pair of primers in the reaction
- A modification of PCR in order to rapidly detect deletions or duplications in a large gene
- First described in 1988 as a method to detect deletions in the Dystrophin gene

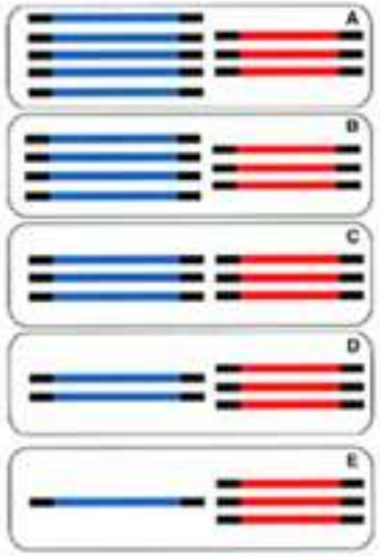


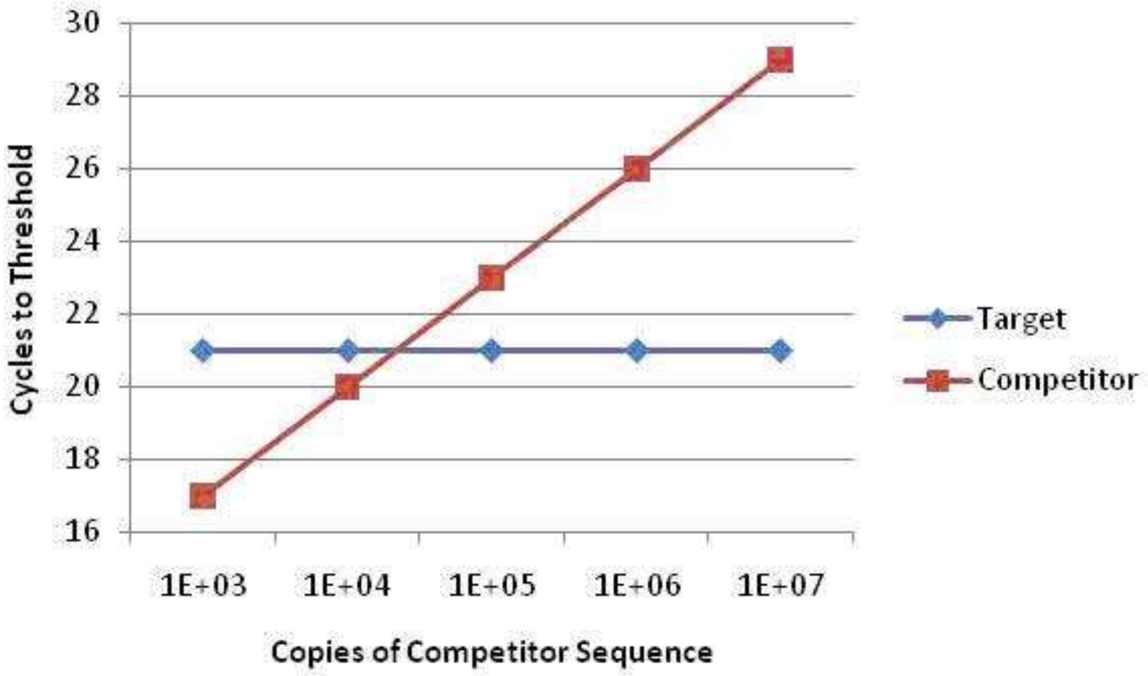


Competitive PCR

- Allows quantification of the amount of DNA
- Competitor DNA is a known sequence of DNA that has the same primer binding sites (black sequences) as the target sequences and is synthetically generated in laboratory
- A known amount of competitive DNA is added to a series of PCR reaction tubes in decreasing amounts
- A standard volume of target DNA is added to each PCR reaction tube
- During the annealing phase of the PCR cycle ,primers compete for binding
- PCR products are separated by gel electrophoresis

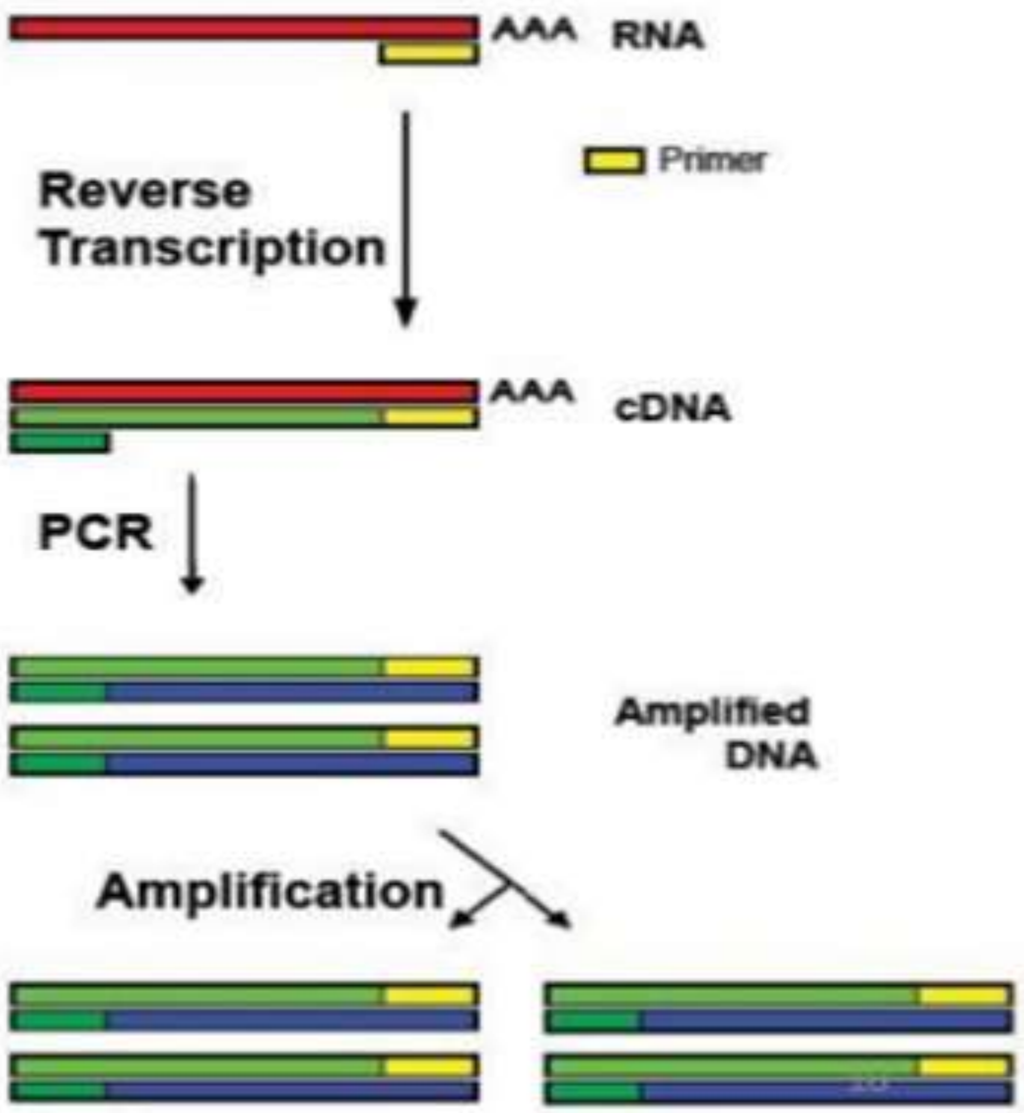






RT (Reverse Transcriptase) PCR

- For amplifying DNA from RNA
- Reverse transcriptase reverse transcribes RNA into c DNA , which is then amplified by PCR
- Some thermostable DNA polymerases used in the PCR such as Tth have reverse transcriptase activity under certain buffer conditions



One step RT PCR

- All reaction components are mixed in one tube prior to initiation of reaction
- Simple
- Convenient
- Minimizes the risk of contamination
- Resulting cDNA can not be used for detecting multiple messages from a single RNA

Two step RT PCR

- Involves 2 steps the RT reaction and PCR amplification
- RNA is first reverse transcribed into cDNA using enzyme reverse transcriptase
- Resulting cDNA is used s templates for subsequent PCR amplification using primers specific for one or more genes

Anchored PCR

- Small sequences of nucleotides can be attached or tagged to target DNA
- The anchor is frequently a poly G to which a poly C primer is used

Allele specific PCR

- Selective PCR amplification of the alleles to detect single nucleotide polymorphism(SNP)
- Selective amplification is achieved by designing a primer such that the primer will match or mismatch one of the alleles at the 3' end of the primer

Long PCR

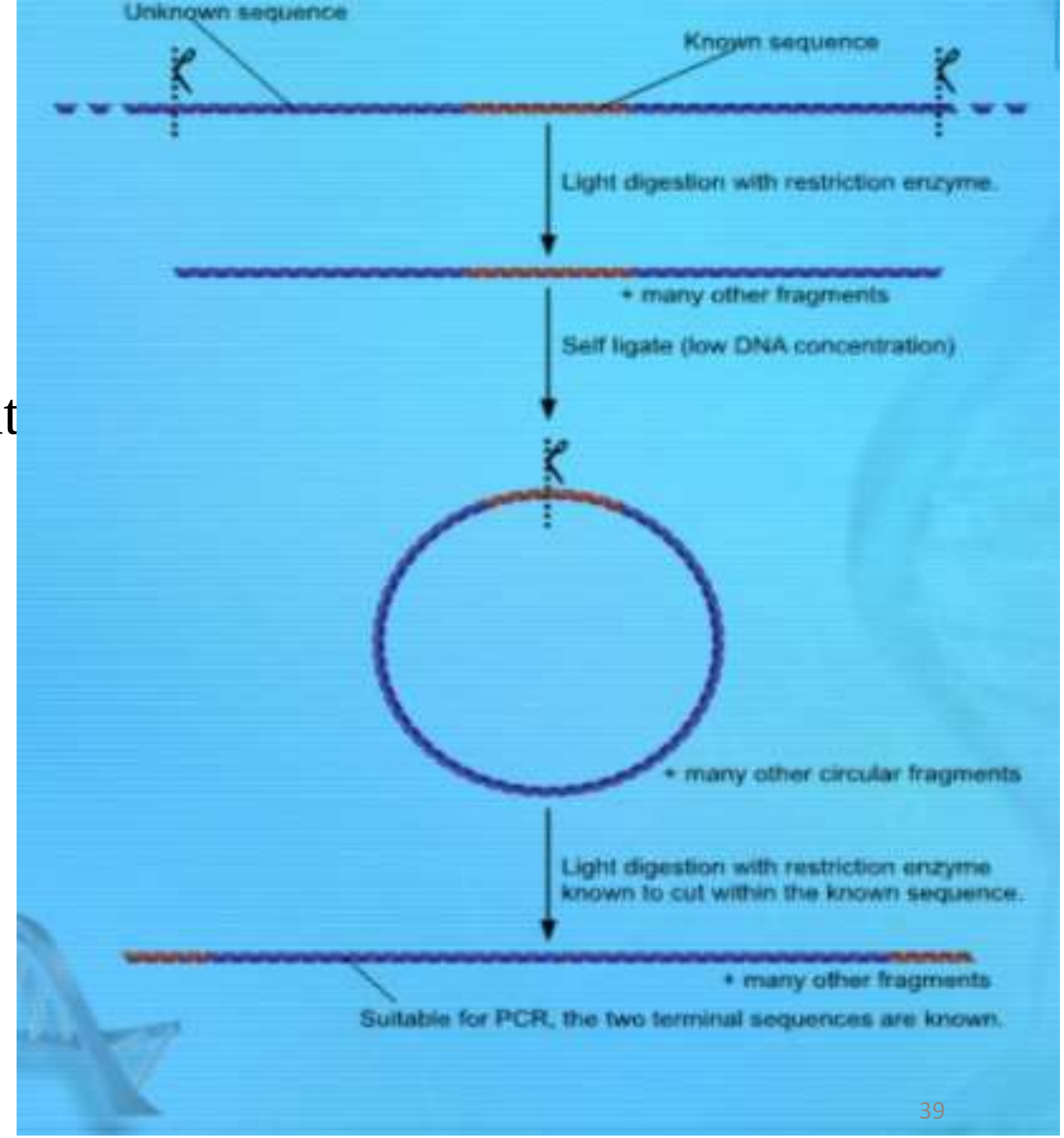
- Used if the DNA amplification up to 27kb fragments
- Method relies on – Thermostable DNA Polymerase DNA (*Taq* polymerase), Proofreading DNA (eg. *Pfu)*
- DNA is cut with two restriction enzymes to
- generate specific sequences , which are than amplified suitably

Random amplified polymorphic DNA

- DNA is cut and amplified using short single primers at low annealing temperature, resulting in amplification at multiple loci
- Lack specificity due to low annealing temperature and easier reaction conditions

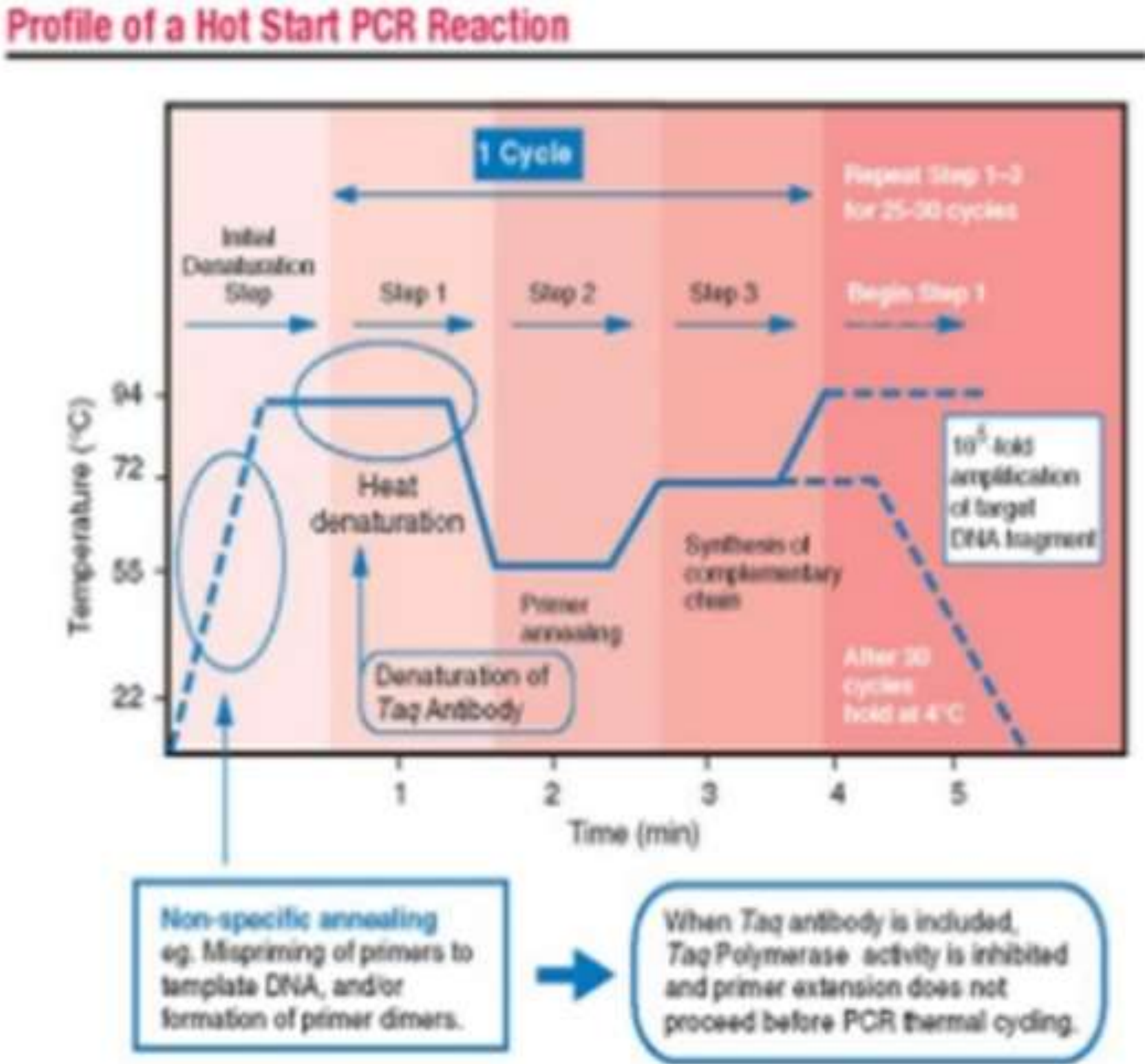
Inverse PCR

- Amplification of DNA of unknown sequence is carried out from known sequence
- Useful in identifying flanking sequences of various genomic inserts



Hot Start PCR

- Eliminates production of primer dimers caused by primer annealing at low temp (55-56 c)before the start of thermocycling
- Reduces non-specific amplification during the initial setup stages of the PCR
- Antibodies or covalently bound inhibitors are used to inhibit polymerase activity at ambient temperature
- Significantly improves specificity , sensitivity and yield of PCR



Thermal asymmetric interlaced PCR (TAIL – PCR)

- Makes use of three nested T-DNA specific primers in on end and a short arbitrary degenerate (AD) primer in the other end
- Three different PCR reactions are performed with these primer sets
- The primary PCR reaction involves different primer annealing temperatures and low and high stringent cycles to facilitate annealing of arbitrary and specific primers respectively
- This step results into both specific as well as nonspecific amplification of products
- In the next two steps of PCR reactions the non-specific products are eliminated amplifying predominantly the T-DNA flanking genomic DNA

Real time PCR

- Perform detection , analysis and quantification of the sample during PCR i.e. in real time
- Detection-find out the presence of targeted gene sequences which is assured by the presence of the amplification curve
- Quantification-done by using the cycleno. Needed to obtain the threshold value of detector and PCR efficiency
- Analysis-by studying or comparing the melting curve with the sequences of the database
- Uses a fluorescent reporter signal to measure the amount of amplicon asv it is generated
- quantify the amount of genome in sample
- Advantages over normal PCR-

➢ Does not require gel preparation

➢ Non time consuming

➢ Less complexity at the quantification of sample

Applications OF PCR

- Detection of specific genome
- Screening specific genes for unknown mutations
- Genotyping using short primers or primer pairs that are often repeated in the genome
- Detection of microbial pathogens
- Identification of clinical isolates
- Strain subtyping
- Detection of viral nucleic acid (of RNA viruses) in clinical specimen
- To prepare cDNA library of mRNA
- Viral load of HCV and HIV
- Qualitatively detect gene expression through creation of cDNA

CB NAAT/ GENEXPERT

Automated cartridge based nucleic acid amplification test (CBNAAT)

- Integrated sample processing & **real-time PCR**
- Uses 3 primers & 5 molecular probes
- Minimal hands on technical time
- Results in <2 hrs
- Identifies M.tb & detects rifampicin resistance simultaneously
- Samples – **Pulmonary (FDA approved)**
  - Extrapulmonary
- **POINT OF CARE TEST**
- **Endorsed by WHO in 2010**
- Sensitivity : **131 cfu/ml sputum**
  (comparable to culture)

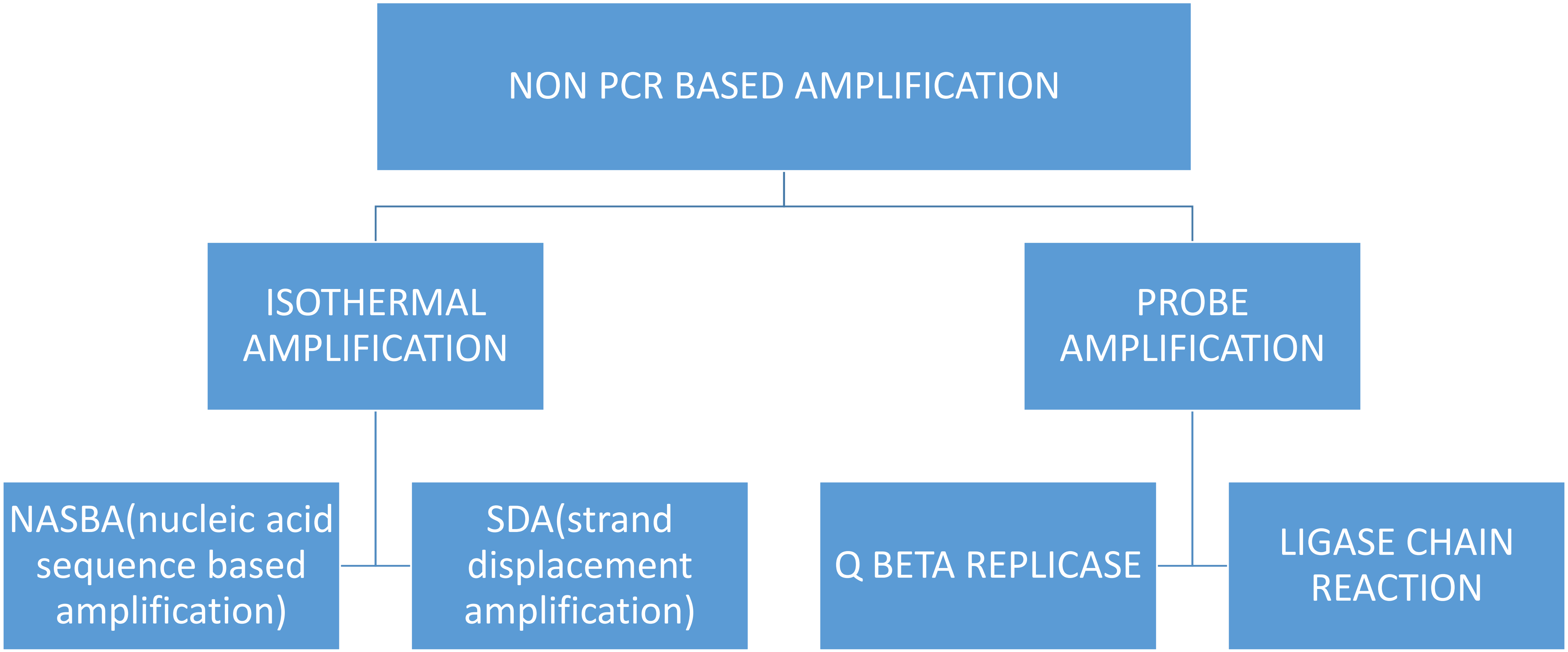
WHO Recommendations FOR Xpert MTB

Xpert MTB **should be used** :

- As the initial diagnostic test in adults & children presumed to have **MDR TB** or **HIV associated TB**

- As the initial diagnostic test in testing CSF specimens from patients presumed to have **TB meningitis**

NON-PCR BASED AMPLIFICATION METHODS


NON PCR BASED AMPLIFICATION
ISOTHERMAL AMPLIFICATION
PROBE AMPLIFICATION
NASBA(nucleic acid sequence based amplification)
SDA(strand displacement amplification)
Q BETA REPLICASE
LIGASE CHAIN REACTION

NASBA (Compton, 1991):

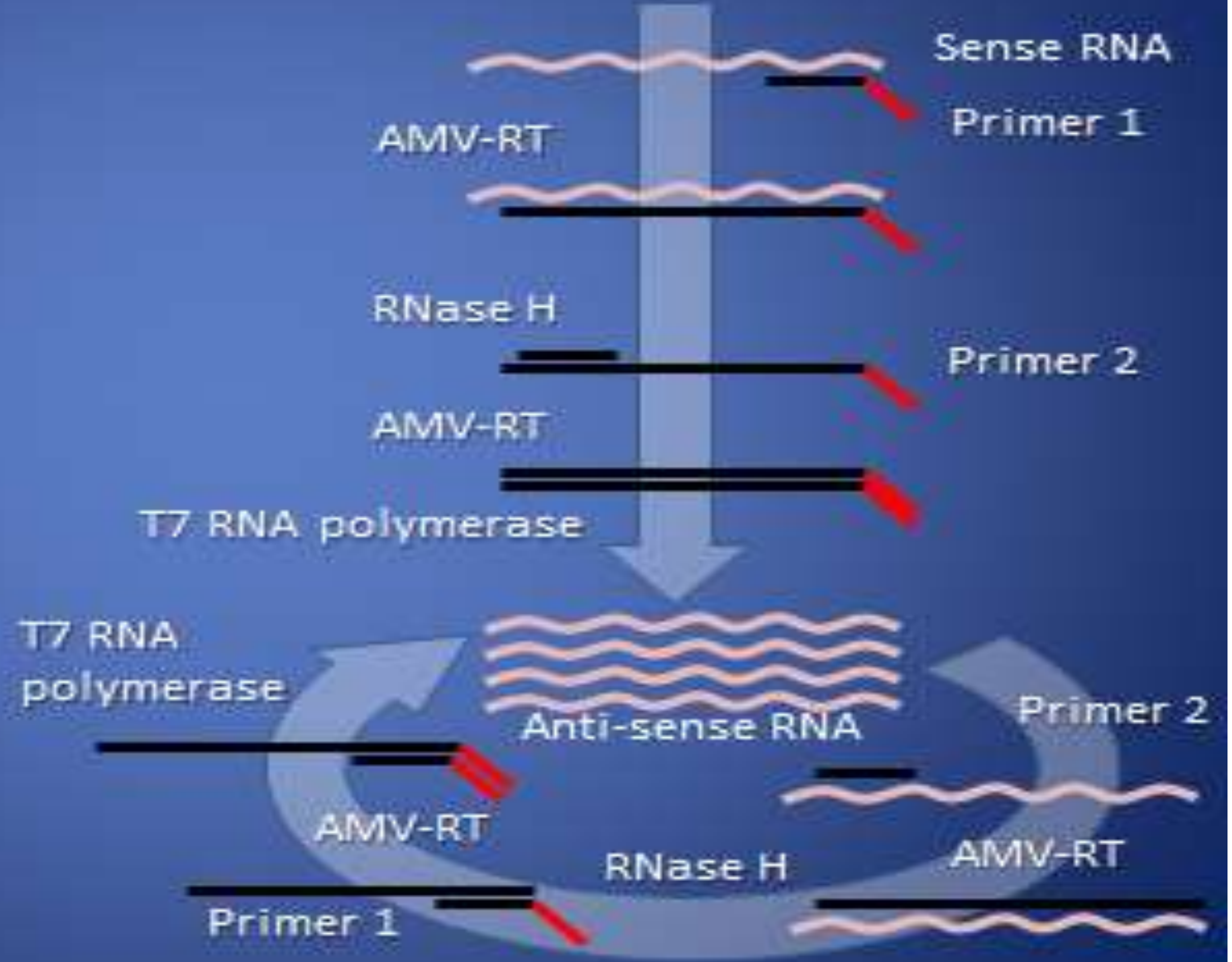
- Transcription based amplification system used in amplification of ssRNA rather than DNA.
- cDNA of the single stranded RNA is created using reverse transcriptase which is used as template for transcription.
- 3 enzymes are used in reaction mixture-

    1. Reverse transcriptase (from avian Myeloblastosis virus)
    2. E.coli RNase H
    3. Bacterophage T7 DNA dependent RNA polymerase

- **ADVANTAGE-** performance of the test in iso-thermic conditions and no requirement of a thermocycler
- **USE-** in detection of point mutations resulting in Zidovudine resistance in HIV-1.

Overview of NASBA

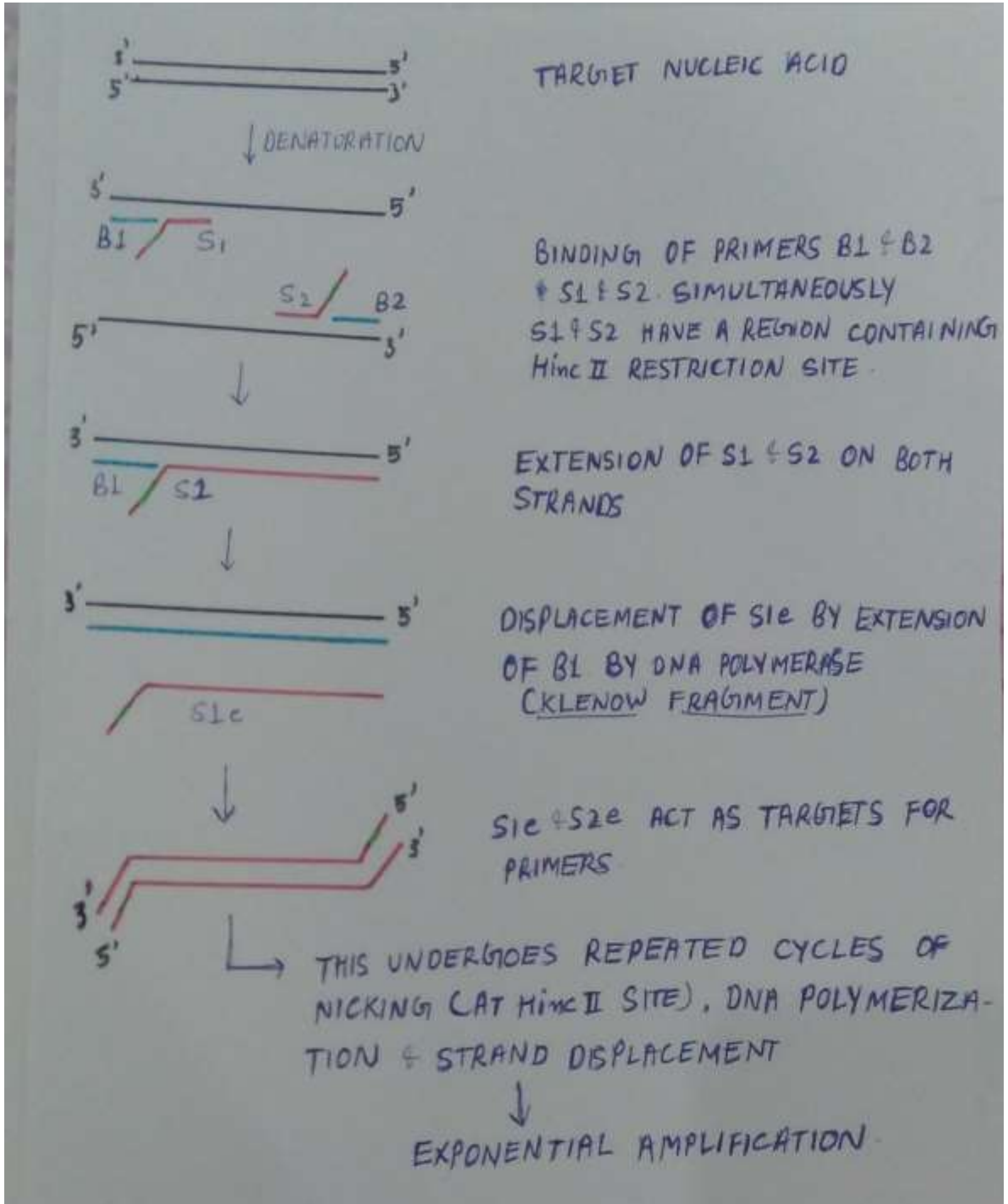
- **Exponential amplification of specific RNA targets**
- **Requires two oligonucleotide primers**
- **Catalyzed by three enzymes working in concert**
- **Carried out isothermally at 41°C**



SDA

- capable of detecting less than 10 target copies.

- Its efficiency decreases 10 folds for each 50 nucleotide increase in length. So, its more efficient for small targets (<200bp).



PROBE AMPLIFICATION

1. LIGASE CHAIN REACTION
2. Q BETA REPLICASE

LCR

- **Principle :**

  - A pair of oligonucleotide probes bind adjacently on one of the DNA target strands
  - A second oligonucleotide pair hybridize to same regions on the complementary DNA
  - DNA polymerase fills the gap & probes are ligated by ligase
  - The ligated probes now become templates for subsequent binding of more probes

Q BETA REPLICASE:

- Q beta replicase is a RNA dependent RNA polymerase derived from the bacteriophage Q-beta.
- This enzyme is capable of replicating a limited family of RNA molecules. Q beta replicase is remarkable in three respects:

(i) It effects a 10,000-fold amplification of the 4200-nucleotide single-stranded RNA of Q beta during the very short interval,

(ii) It specifically replicates viral genomic RNA in the presence of vast excess of host RNA.

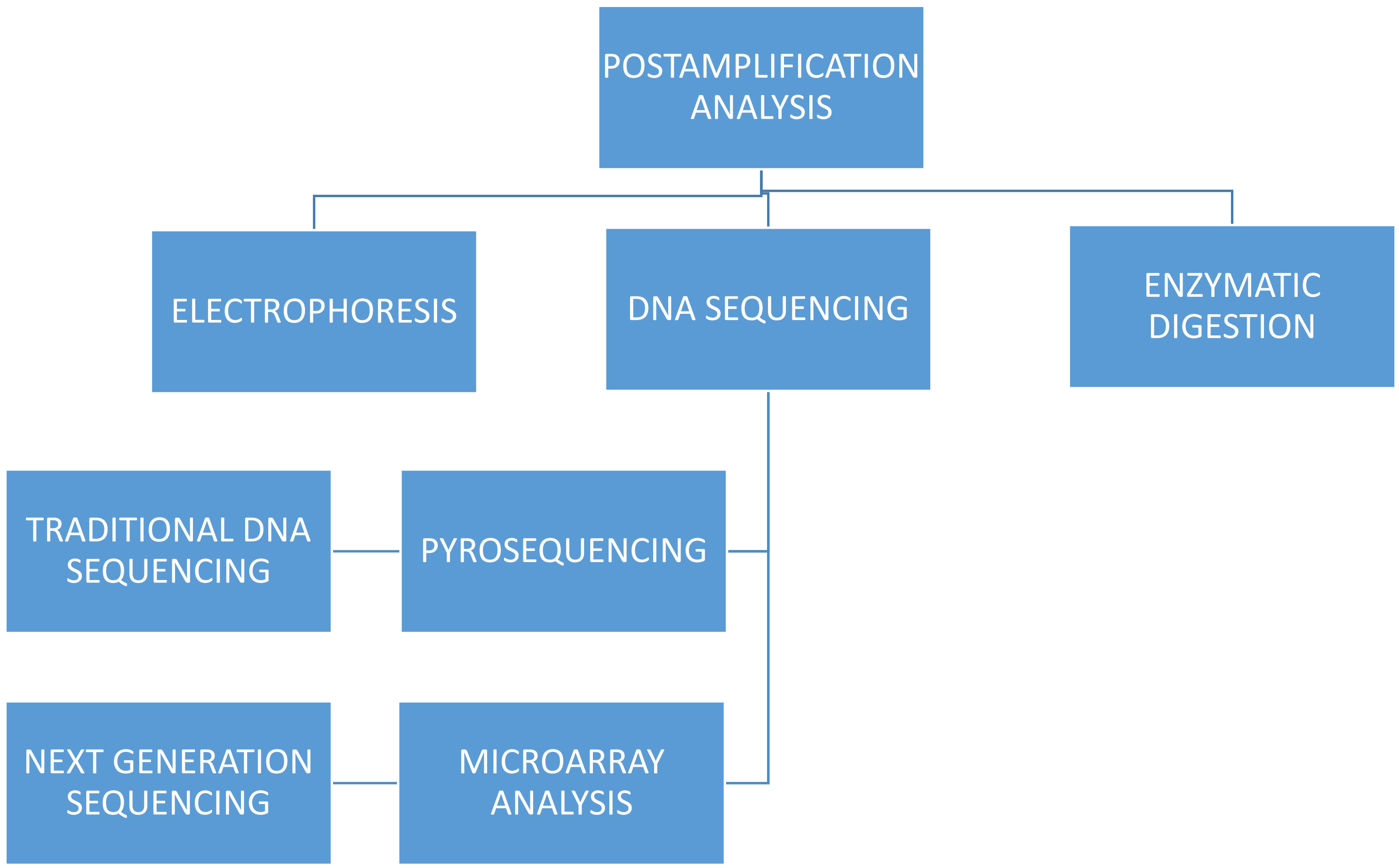
(iii) It copies entire template RNAs, from 3' terminus to 5' terminus, without utilizing endogenous primers.

- Midivariant (MDV) RNA is a most extensively studied non-viral substrate for Q-beta replicase into which probe sequences are inserted. So, the probe gets amplified along with MDV RNA by beta replicase.

Advantage:

- It is a very sensitive probe amplification method- One billion or more progeny molecules can be produced from a single starting template MDV molecule in approximately 30 minutes.

POSTAMPLIFICATION ANALYSIS:



TRADITIONAL DNA SEQUENCING:

- **DNA sequencing** is the process of determining the sequence of nucleotide bases (As, Ts, Cs, and Gs) in a piece of DNA.

- **Sanger sequencing** also called **the chain termination method** is the traditional method of dna sequencing.

SANGER SEQUENCING : THE CHAIN TERMINATION METHOD (Fred Sanger, 1977)

- Regions of DNA up to about 900 base pairs in length are routinely sequenced using this method.
- It involves making copies of a target, so its ingredients are similar to those needed for DNA replication.
- Unique ingredient – di-deoxy or chain terminating versions of all four nucleotides.

USES AND LIMITATIONS:

- It's typically used to sequence individual pieces of DNA (upto 900bp), such as bacterial plasmids or DNA copied in PCR
- However, it is expensive and inefficient for larger-scale projects, such as the sequencing of an entire genome or Metagenome .

SAMPLE DNA + PRIMER + DNA POLYMERASE + NUCLEOTIDES + DYE-LABELLED CHAIN TERMINATING NUCLEOTIDES.

→

NEW DNA SYNTHESIS CONTINUES UNTIL A DIDEOXYNUCLEOTIDE IS ADDED, THUS TERMINATING THE PROCESS

↓

REPEATED IN NO. OF CYCLES

↓

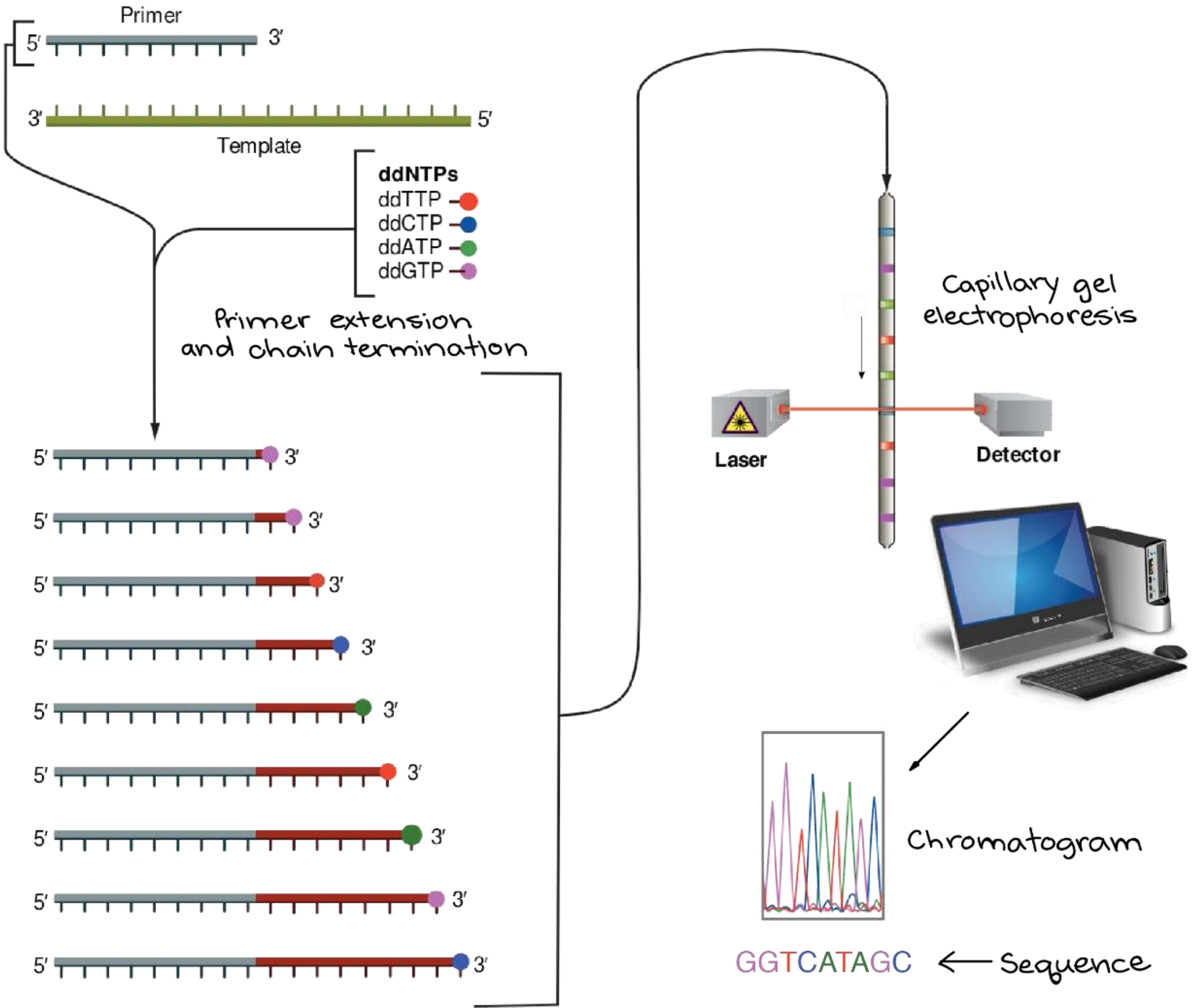
FRAGMENTS OF DIFFERENT LENGTHS ARE OBTAINED (IT IS VIRTUALLY GUARANTEED THAT A DIDEOXYNUCLEOTIDE WILL HAVE BEEN INCORPORATED AT EVERY SINGLE POSITION OF TARGET DNA.

↓

FRAGMENTS RUN THROUGH CAPILLARY GEL ELECTROPHORESIS

←

DATA RECORDED BY DETECTOR IN THE FORM OF PEAKS IN THE CHROMATOGRAM



Primer
5′
3′
3′
5′
Template
ddNTPs
ddTTP
ddCTP
ddATP
ddGTP
Primer extension and chain termination
Capillary gel electrophoresis
Laser
Detector
Chromatogram
GGTCATAGC
Sequence

NEXT GENERATION SEQUENCING:

The most recent set of DNA sequencing technologies are collectively referred to as **next-generation sequencing**.
next-generation sequencing is kind of like running a very large number of tiny Sanger sequencing reactions in parallel.

Most share a common set of features that distinguish them from Sanger sequencing:

- **Highly parallel:** many sequencing reactions take place at the same time
- **Micro scale:** reactions are tiny and many can be done at once on a chip
- **Fast:** because reactions are done in parallel, results are ready much faster
- **Low-cost:** sequencing a genome is cheaper than with Sanger sequencing
- **Shorter length:** reads nucleotides range from 50 -700 nucleotides in length

.

PYROSEQUENCING:

It is based on nucleotide incorporation into the newly synthesized strand of DNA i.e sequencing by synthesis.

Amplified DNA +other reagents

↓

automated addition of nucleotides

↓

addition of appropriate nucleotide in growing strand

↓

generation of pyrophosphate(natural by product of nucleotide incorporation)

↓

pyrophosphate converted to light by enzymatic reactions

↓

light generated recorded by instrument

↓

sequence is determined from pyrogram

USES:

- Extensively used in the analysis of SNPs associated with genetic diseases and Neoplasia.
- Used for identification and differentiation of microorganisms.

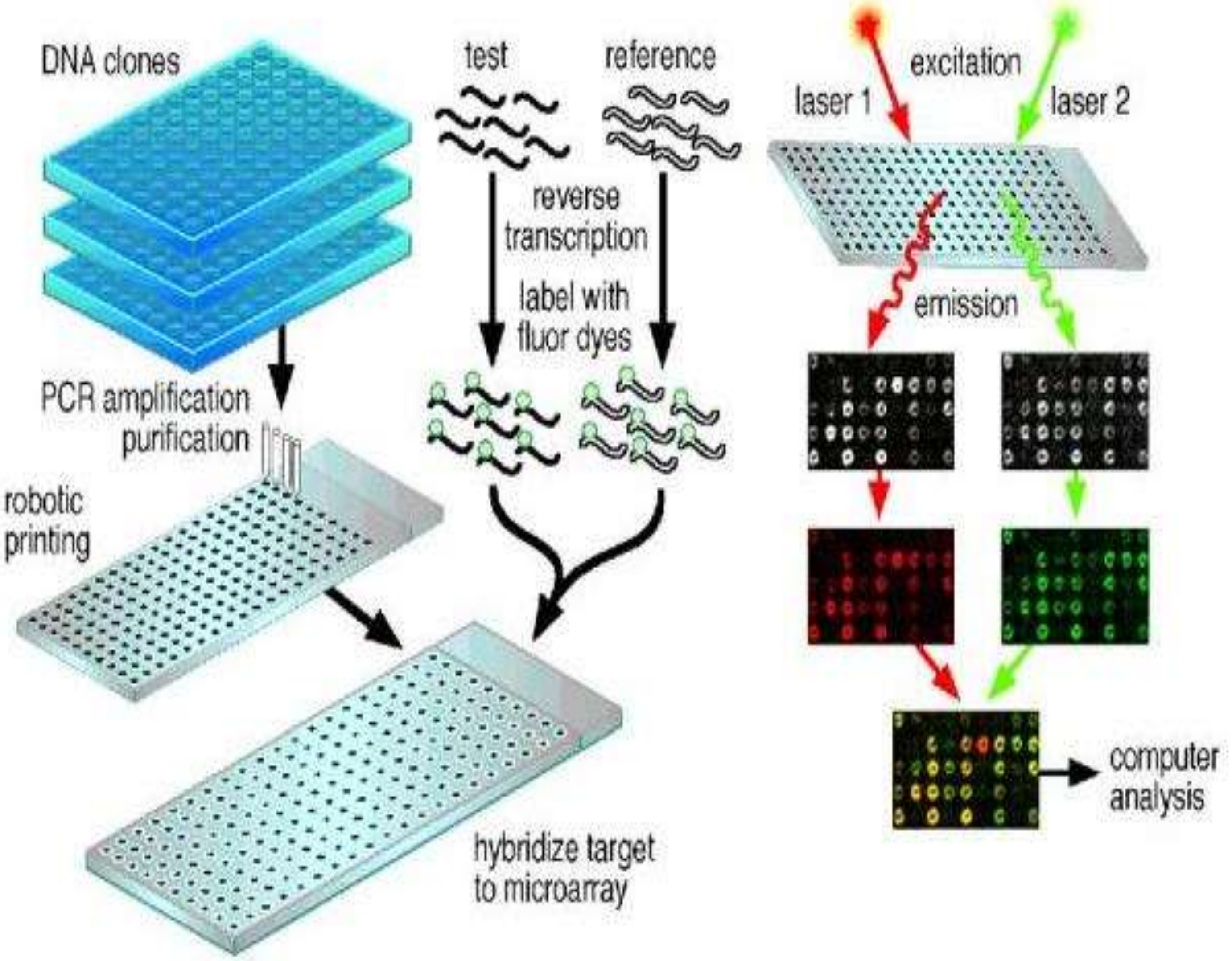
LIMITATIONS:

- Ability to generate relatively short sequences
- Problems generating a sequence when extensive secondary structure is present.

MICROARRAY:

- A DNA microarray is a molecular platform in which a few hundred to thousands of specific DNA oligonucleotides or short sequences (capture molecules) are bound to a matrix.

- The array detects specific DNA sequences(target sequences) in a test sample of DNA from the organism being investigated by hybridization followed by detection of the array bound test DNA.


DNA clones
PCR amplification
purification
robotic
printing
test
reference
reverse
transcription
label with
fluor dyes
hybridize target
to microarray
excitation
laser 1
laser 2
emission
computer
analysis

Uses:

- For viral genotyping of human rotavirus, hepatitis B virus, HIV, measles virus, human and avian influenza viruses.

Advantages:

- It allows rapid and accurate analysis of large numbers of different DNA molecules so it is useful in bacterial identification and subtyping.
- DNA array analysis unveils the genetic region responsible for diversity, often allowing phenotypic predictions at the same time.

Limitations:

- Initial high cost for the synthesis of target specific primers and fluorophores used to label the reaction.

Biofire filmArray

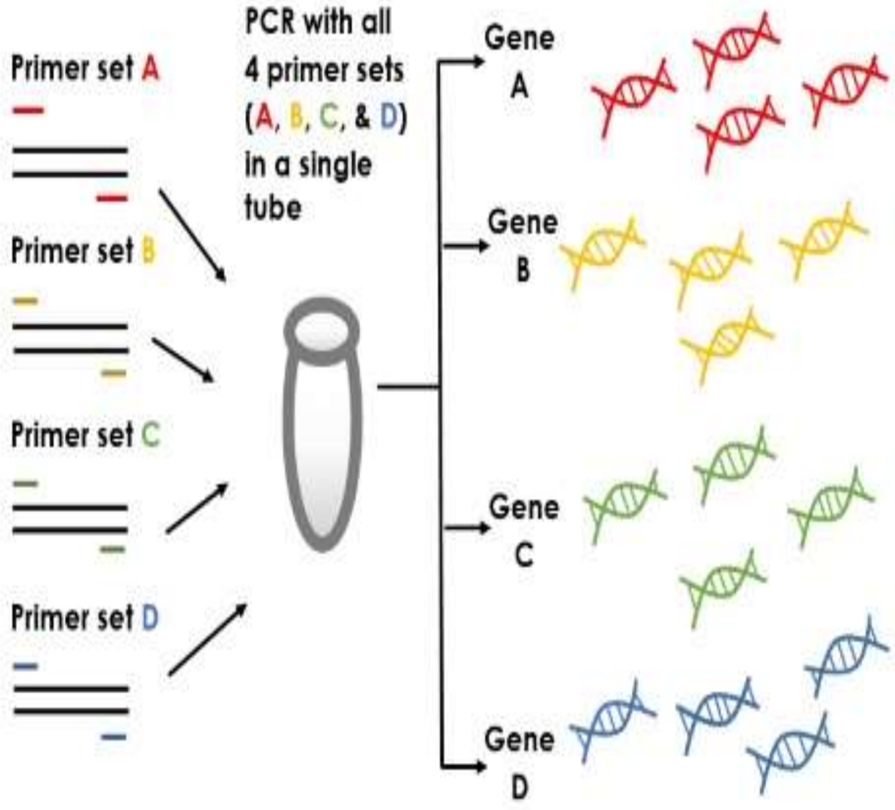
- A commercially available system that employs nested , multiplex , and single-plex PCR reactions for the detection of various pathogens
- Examples

➢ Gastrointestinal panel

➢ Respiratory panel

➢ Blood culture identification panel

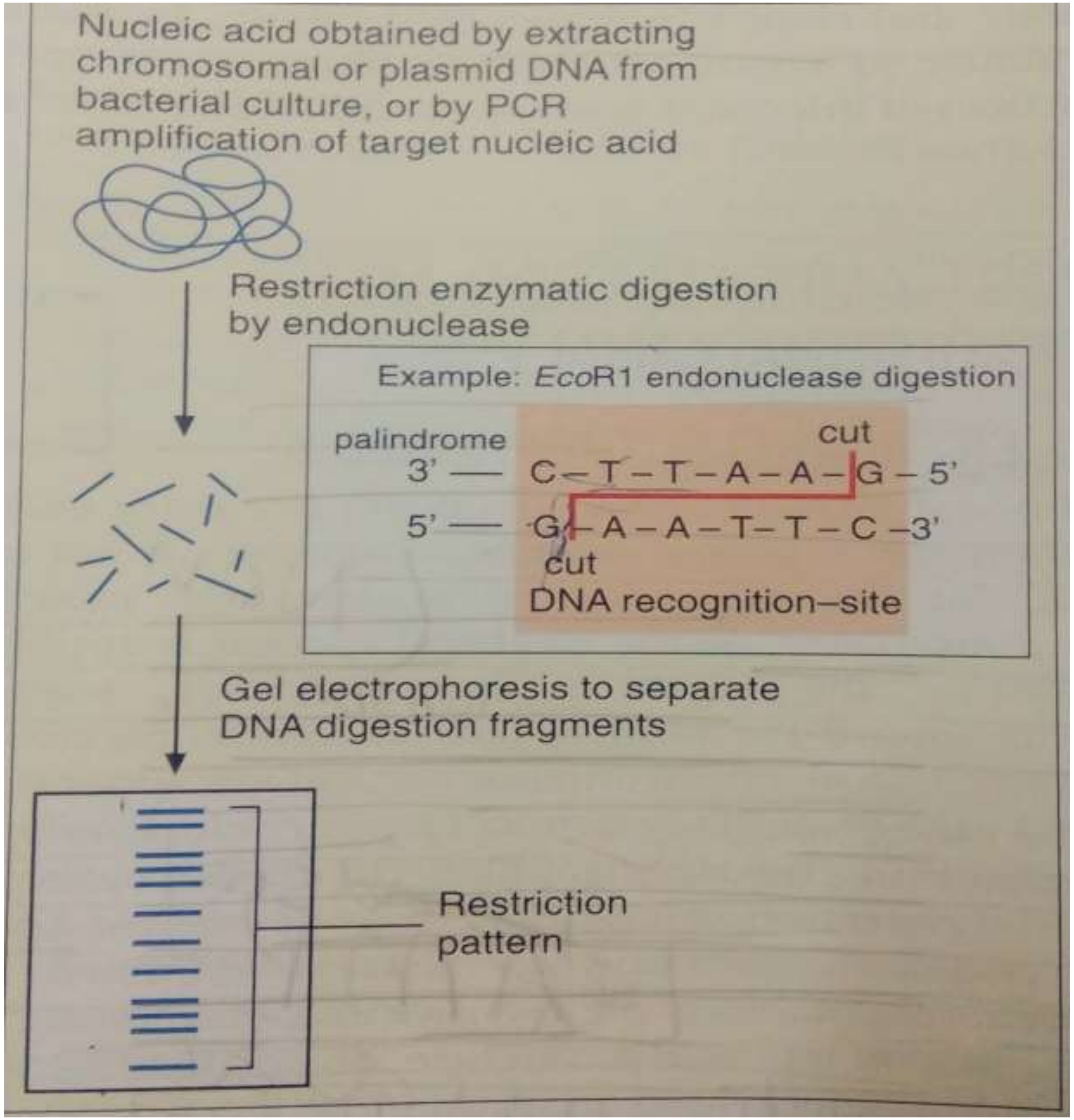
➢ Meningitis panel



ENZYMATIC DIGESTION:

- Digestion of DNA using restriction Endonucleases.

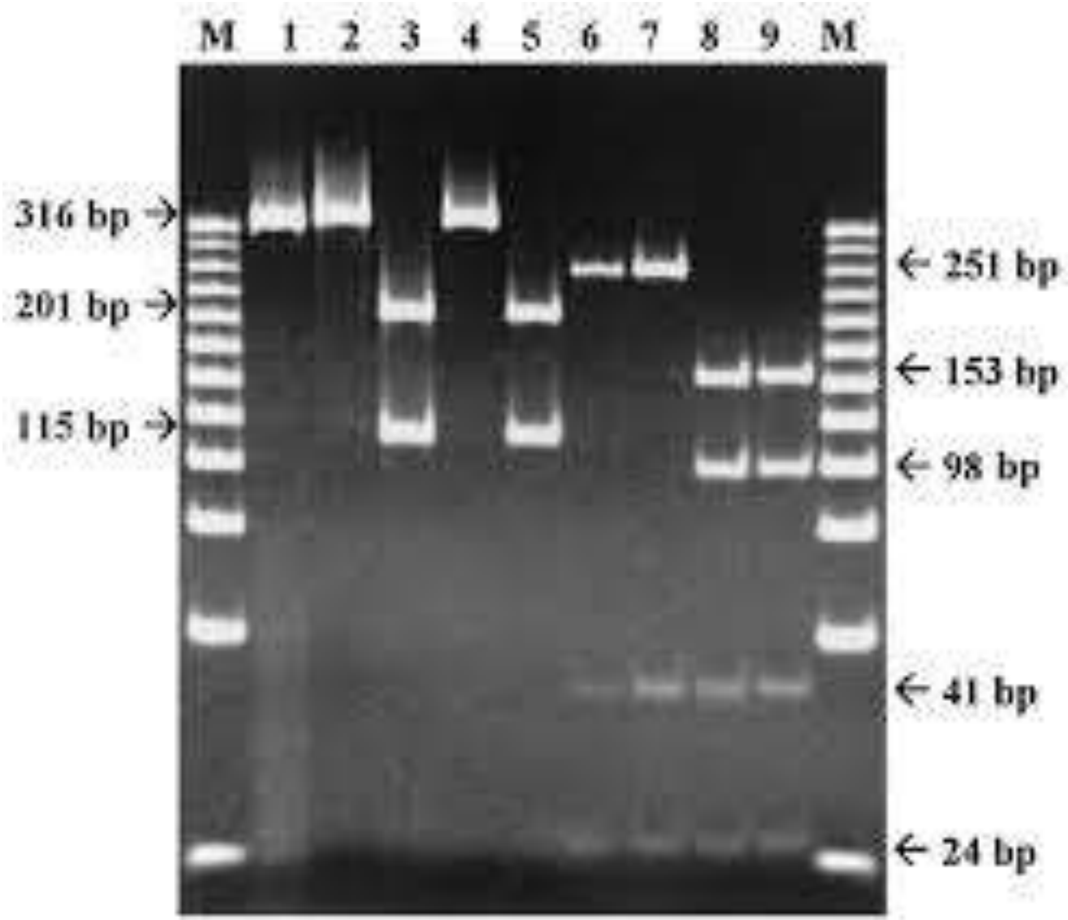
- Pattern obtained after electrophoresis are referred as **restriction Patterns.**



- Differences between the restriction patterns of various microorganism known as **Restriction Fragment Length Polymorphism**

(RFLPs).

- Use- for analyzing relatedness among organisms.
- Another variant of this is **Ribotyping**



RIBOTYPING

- Method that can identify and classify bacteria based upon differences in rRNA (16S and 23SrRNA).
- Technique is same as RFLP
- As all bacteria contain ribosomal genes, a hybridization pattern will be obtained with almost any isolate, only the pattern will vary depending on the arrangement of genes in particular strain.

MISCELLENEOUS

LPA (Line probe assays)

- Commercial product of **reverse hybridization** technology
- Principle-All the probes of interest are immobilized on a nitrocellulose strip and then apply the amplicon to the strip, and determine which probe hybridized with the amplicon
- USES-

➢HCV genotyping

➢Screening for the mutations resulting in resistance to antiretroviral agents in HIV

- ADVANTAGES

➢Less time consuming and less labour intensive then southern hybridization

➢Uses chromogenic reaction rather then radioactivity to detect hybridization

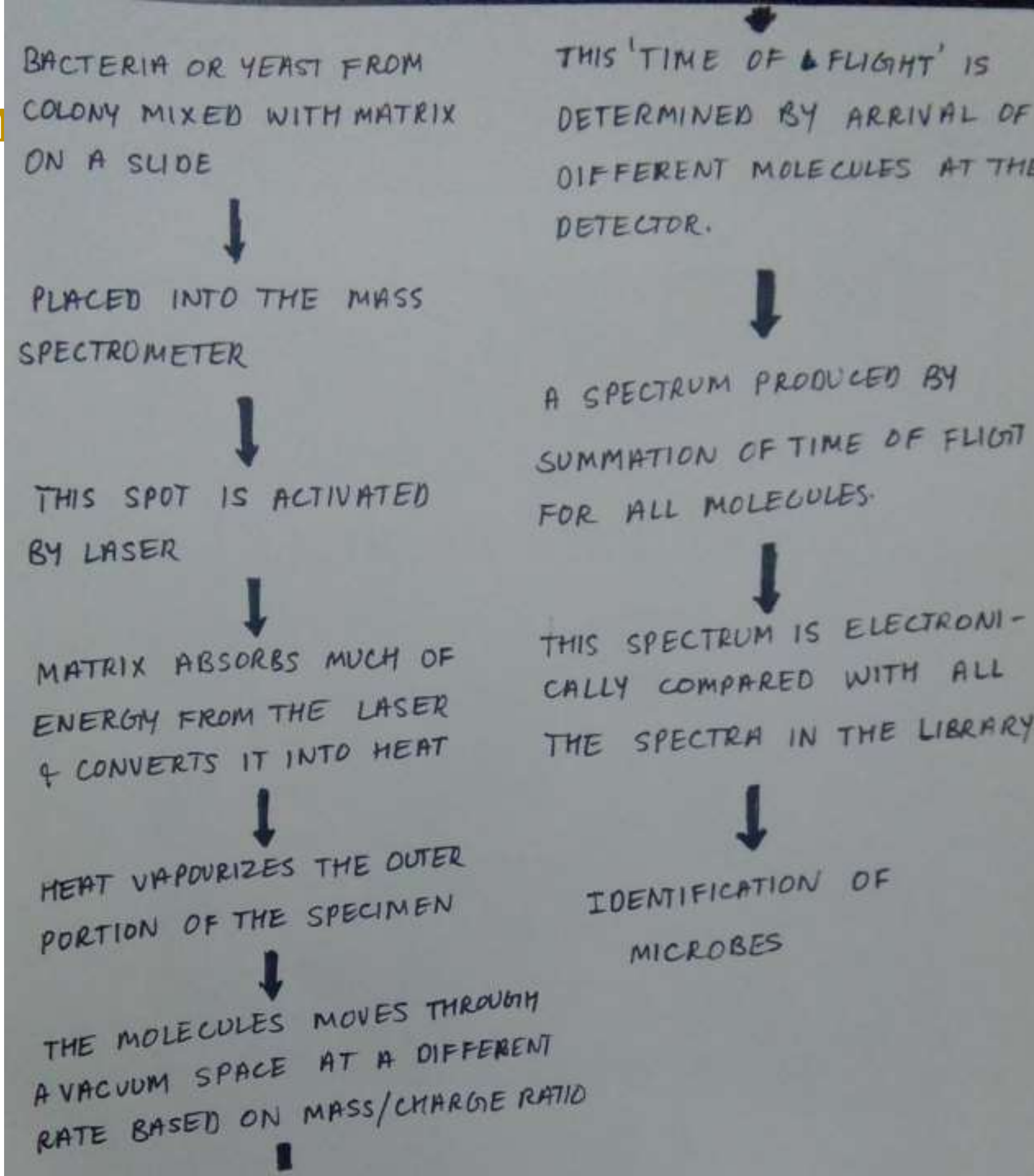
MALDI-TOF:
(Matrix Assisted Laser Desorption Ionization-Time Of Flight)

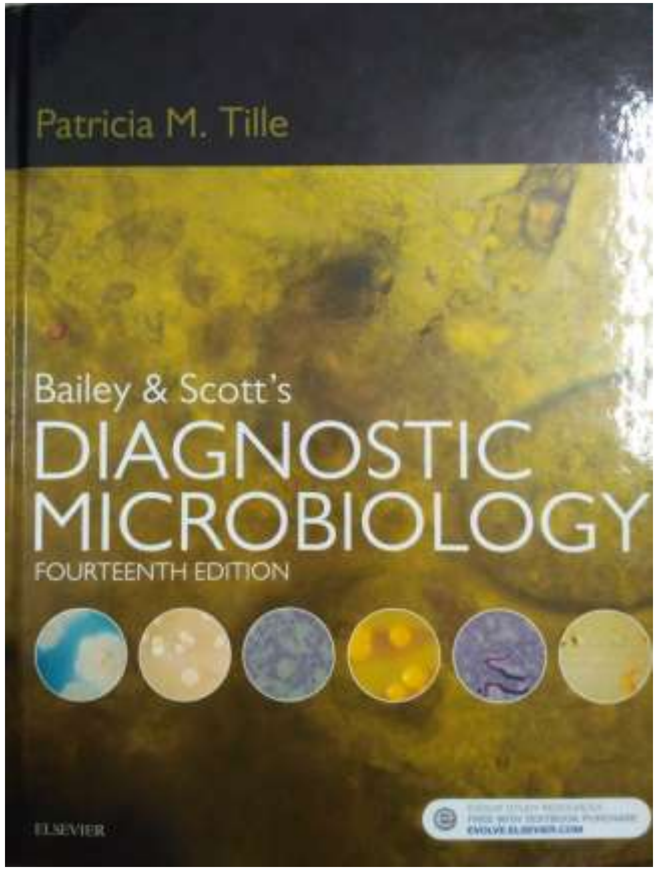
- It is a type of mass spectrometry used in clinical microbiology.
- FDA approved commercially available mass spectrometer are-

  1.MALDI Biotype

  2.Vitek MS

- Limitation- It cannot differentiate *Shigella* from *Escherechia* coli

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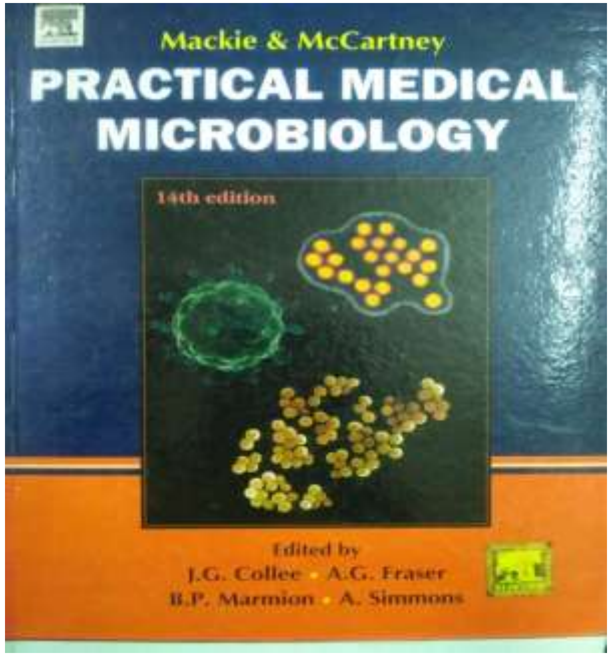
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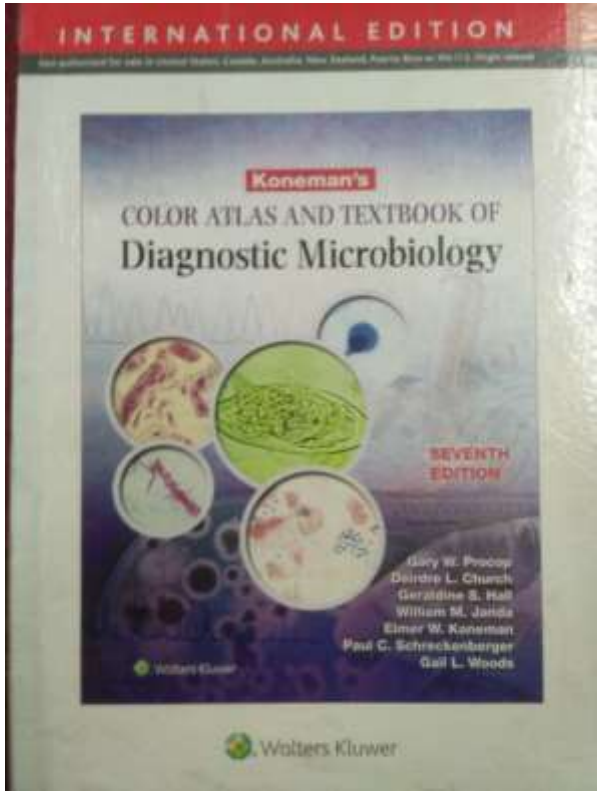
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