APPLICATION OF MOLECULAR TECHNICS FOR DIAGNOSIS OF VIRAL INFECTIONS

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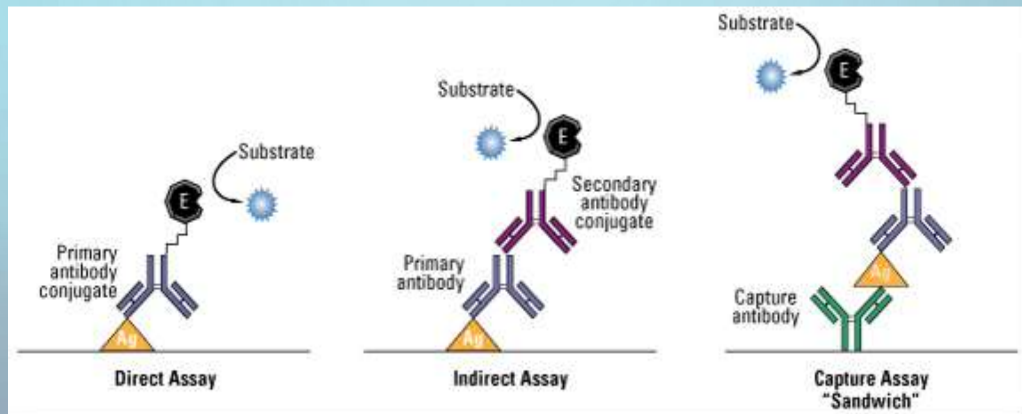
Basic Diagnostic Methods in Virology

Immunology and serology techniques

(Antigen-Antibody Reactions)

ELISA (Enzyme Link Immunosorbent Assay)



Advantages of Elisa

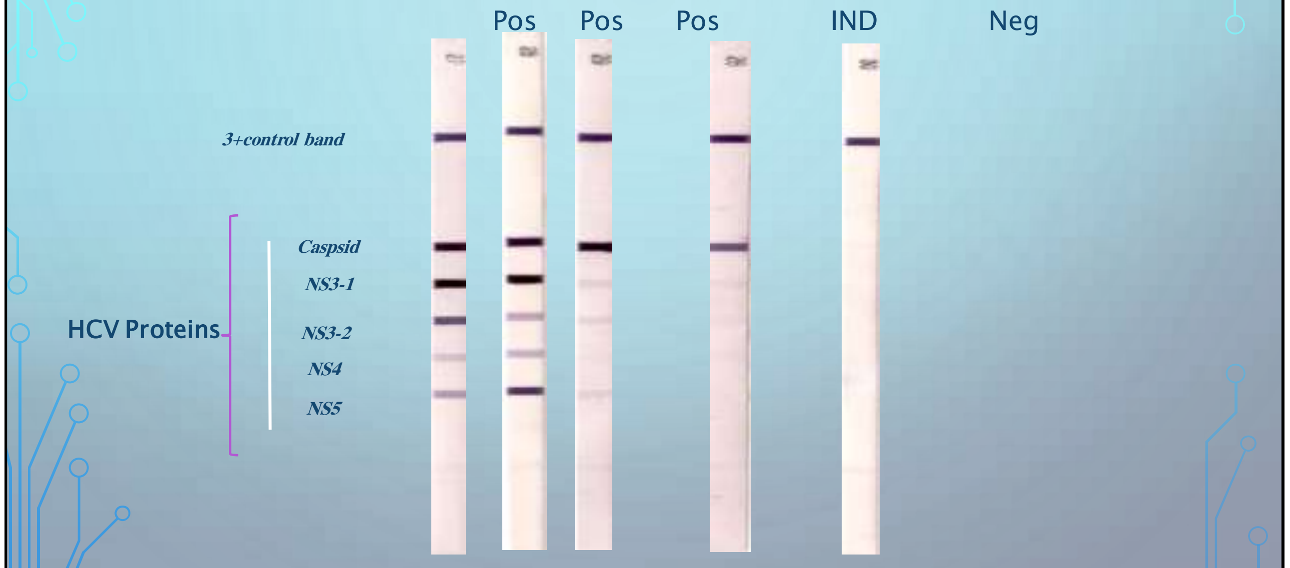
- Reagents are relatively cheap and long shelf life
- Highly specific and sensitive
- Equipment can be inexpensive and widely available
- Can be use to variety of infection
- Safe and No radiation hazards
- Easy to perform and quick procedure

Elisa Disadvantages

- Enzyme activity may be affected by plasma constituents(Natural inhibitor)
- False Positives and Negatives possible, especially with mutated/altered antigen
- Sensitivity of Enzyme to temperature
- Takes 3 to 8 weeks after exposure to the viral agent

WESTERN BLOT (IMMUNOBLOT) AND RIBA

- **RIBA (Recombinant ImmunoBlot Assays)**
- **RIBA is a " confirmatory method "**
- **RIBA is very useful in the confirmation of Antibody results**
- **RIBA in viral diagnosis is mostly used for the confirmation of positive ELISA of HCV and HIV antibodies**



Recombinant ImmunoBlot Assays (RIBA)

In Iran we recommend RIBA as a confirmatory test for a person who might be considered as HCV infected.

The reason for this recommendation are:

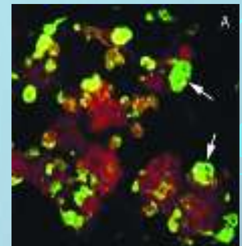
-Quality of ELISA kits

-High cost of NAT tests

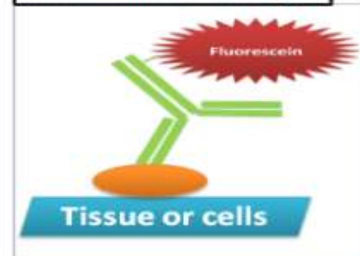
IFA

- **Most commonly used fluorescent dyes are**:
- Fluorescein: absorbs blue light and emits an intense yellow-green fluorescence
- Rhodamine: absorbs yellow-green range and emits a deep red fluorescence

Direct immunofluorescence

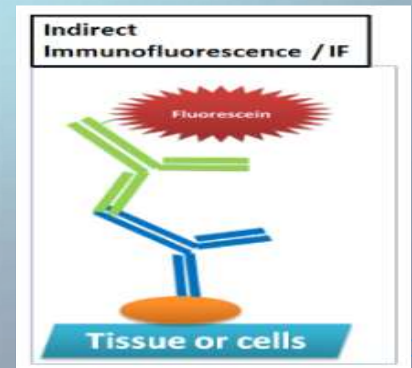


- Ag is fixed on the slide
- Fluorescein labeled Ab's are layered over it
- Slide is washed to remove unattached Ab's
- Examined under UV light in a fluorescent microscope
- The site where the Ab attaches to its specific Ag will show apple green fluorescence



Indirect immunofluorescence

- Indirect test is a double-layer technique
- The unlabelled antibody is applied directly to the tissue substrate
- Treated with a fluorochrome-conjugated
- anti-immunoglobulin serum



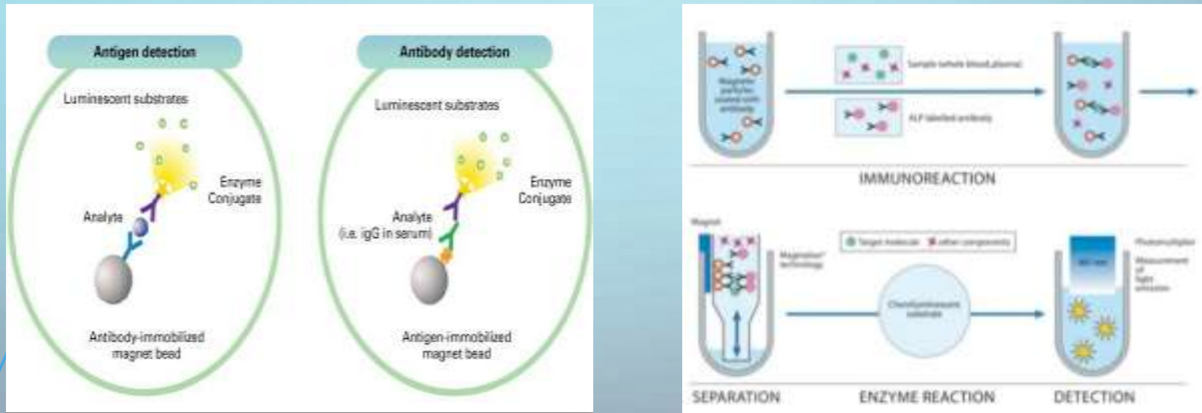
Advantages and Disadvantages

High Specificity	Subjective errors in reading
High Sensitivity	Expensive equipment
The expression of molecules can be observed directly	Cross reacting Ab
Fixed slide can persist for long time	Improper Immune system(False Negative)

Chemiluminescence assay

Enzymes for luminescent assay are AP(Alkaline phosphatase), HRP(HORSE RADISH PEROXIDASE)

SUBSTRATE → LUMINOL, ISOLUMINOL, LUCIFERIN



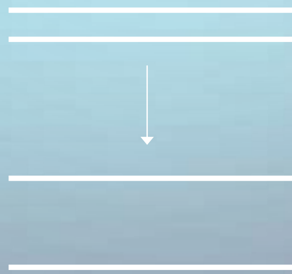
Advantages	Disadvantages
Sensitive	False Positive
Fast emission of light	Expensive Instrument
Short Incubation Period	
Absence of toxicity	

Molecular diagnostic

PCR (Polymerase Chang Reaction)

Step 1: **Denature** DNA

At 95°C, the DNA is denatured (i.e. the two strands are separated).

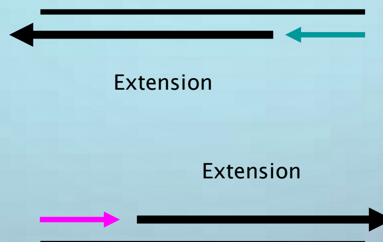


Step 2: Primers **Anneal**

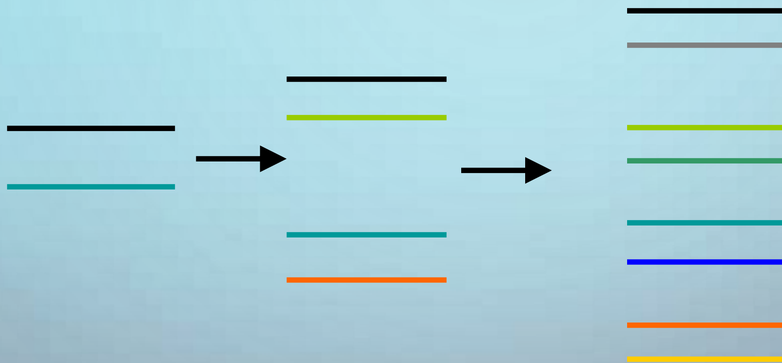
At 40°C– 65°C, the primers anneal (or bind to) their complementary sequences on the single strands of DNA

Step 3: DNA polymerase **Extends** the DNA chain

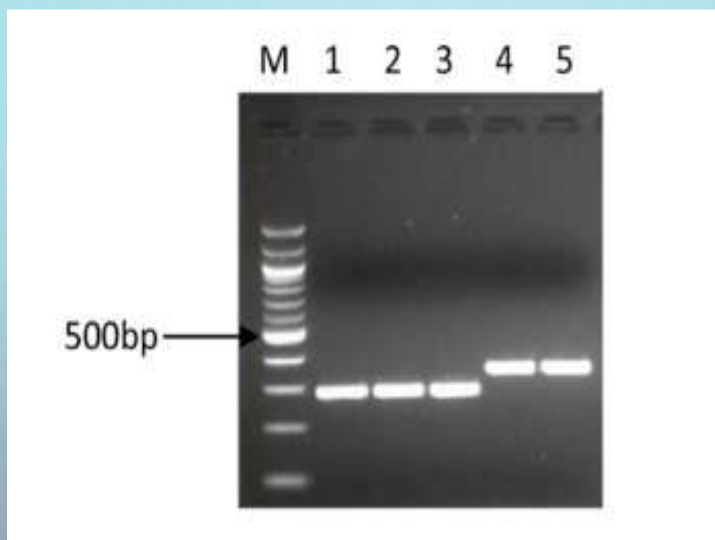
At 72°C, DNA Polymerase extends the DNA chain by adding nucleotides to the 3' ends of the primers



The **next cycle** will begin by denaturing the new DNA strands formed in the previous cycle



Gel Electrophoresis



Nested PCR

- ❖Nested PCR involves two sets of primers
- ❖ Nested reaction includes:

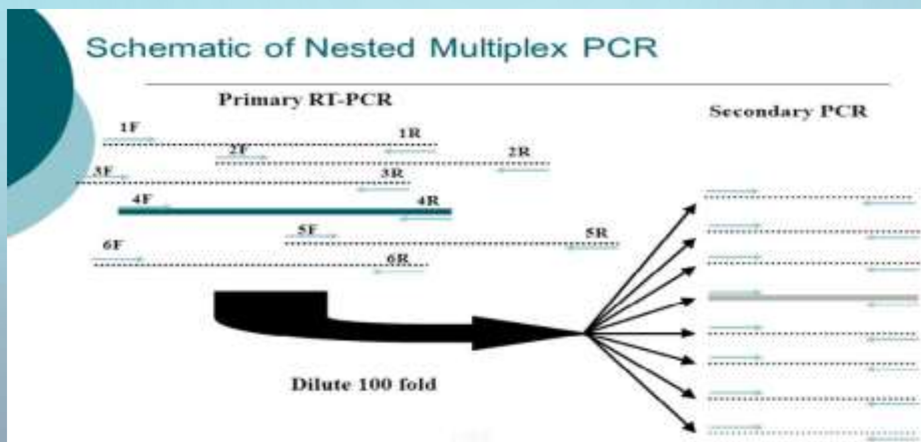
1. Outer PCR (PCR1)
2. Dilution
3. Inner PCR (PCR2)

- ❖ With high sensitivity and specificity
- ❖Research and clinical applications

AUTOMATED NESTED MULTIPLEX PCR SYSTEM

BIOFIRE® FILMARRAY®

BIOMERIEUX COMPANY



Nested PCR

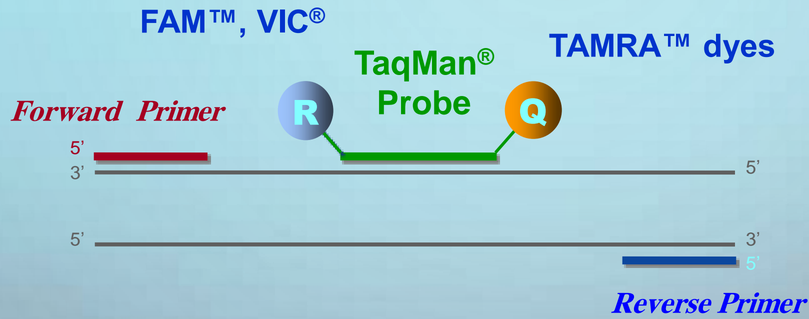
❖**Disadvantages**

❖**Extended time**
❖**Increases the risk of contamination**

Real time PCR

REAL TIME PCR

5' NUCLEASE ASSAY USING TAQMAN® PROBES

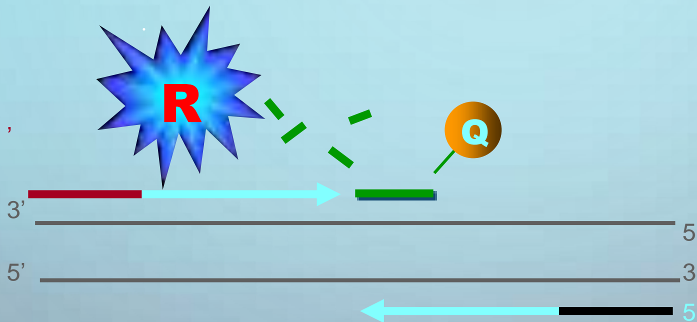


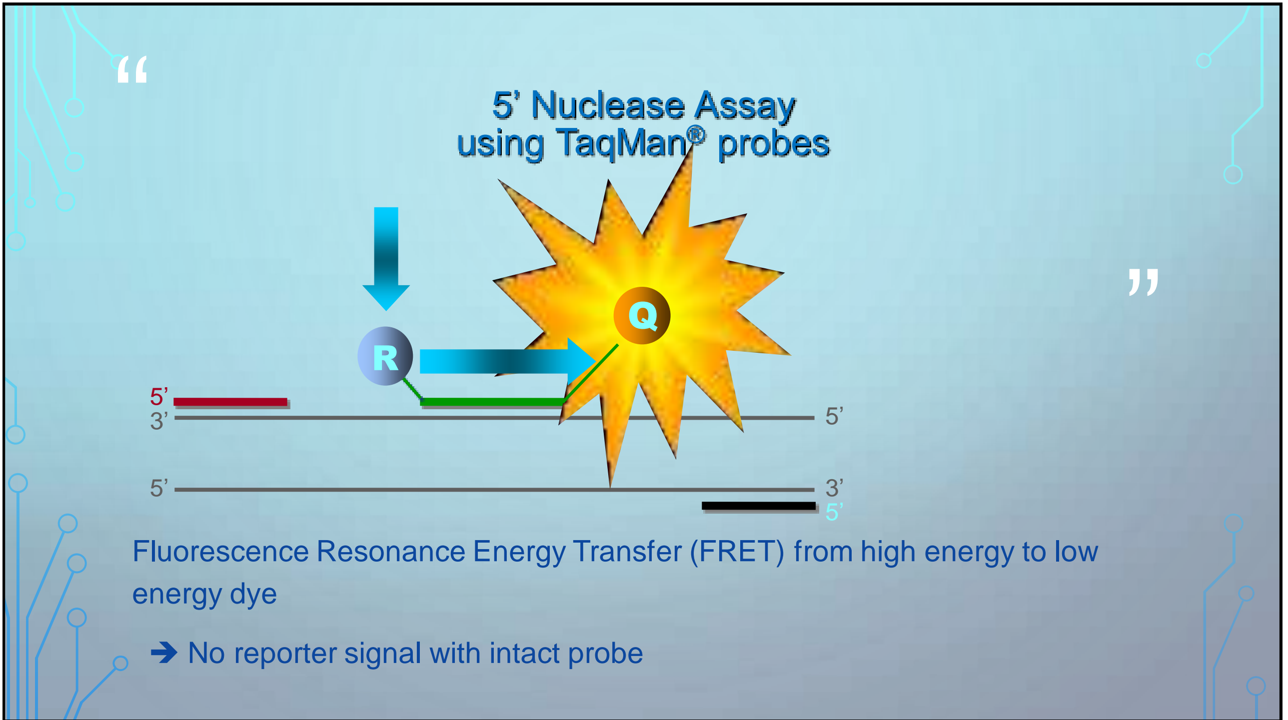
- **PCR specificity (primer)**
- **Hybridization specificity (probe)**

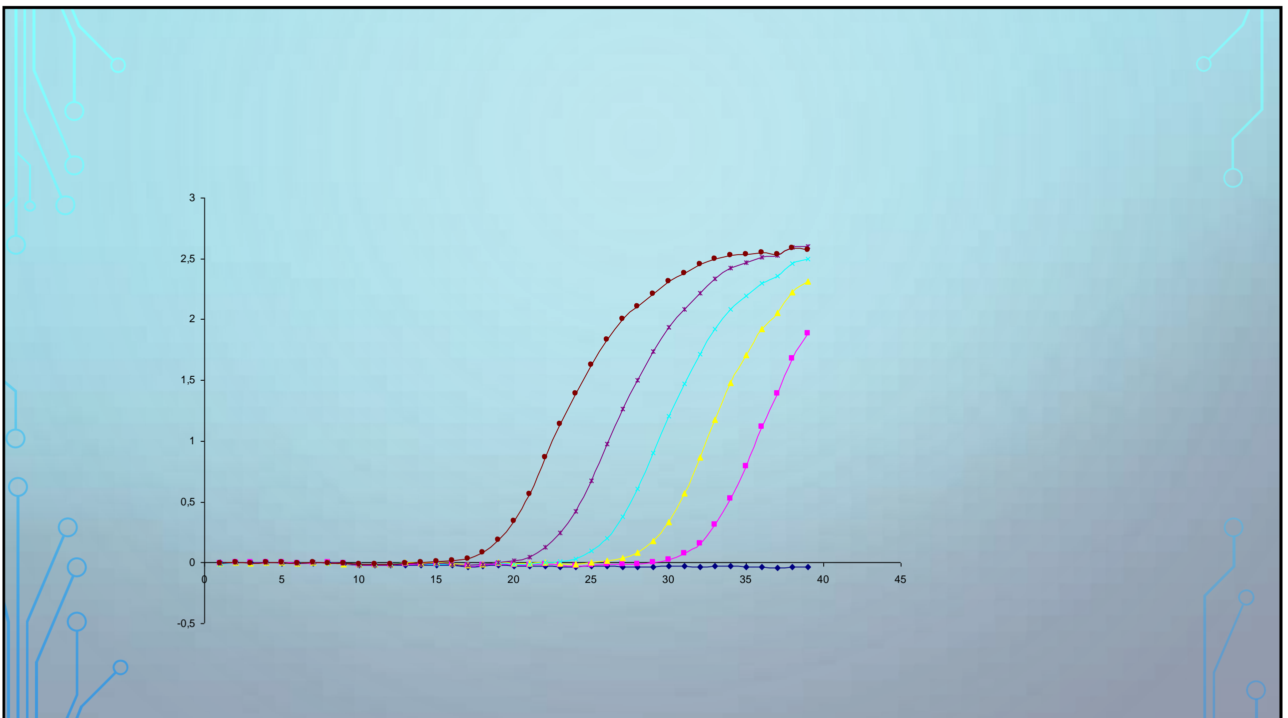
5' Nuclease Assay using TaqMan® probes

Cleavage of probe by 5' nuclease activity of Taq polymerase

➔ FRET disabled, generation of reporter signal




5' Nuclease Assay
using TaqMan® probes
R
Q
5'
3'
5'
5'
3'
5'
Fluorescence Resonance Energy Transfer (FRET) from high energy to low energy dye
➔ No reporter signal with intact probe


3
2,5
2
1,5
1
0,5
0
-0,5
0
5
10
15
20
25
30
35
40
45

“

COBAS TaqMan

”

- Lower Limit of Quantification: 6 IU/Ml
- Upper Limit of Quantification: 100,000,000IU/mL
- Conversion Factor: 5.82

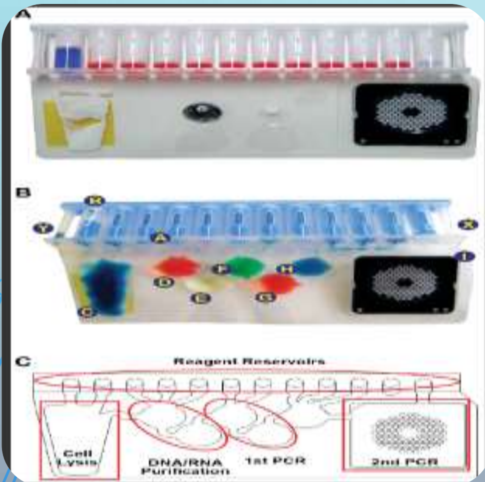
Advantages of Real Time PCR

- It does not required gel preparation for visualization
- It is not time consuming compare to conventional PCR
- Amplification can be monitored in Real Time
- Possibility of Confirmation of specific amplification by Melting point analysis

AUTOMATED NESTED MULTIPLEX PCR SYSTEM
BIOFIRE® FILMARRAY®

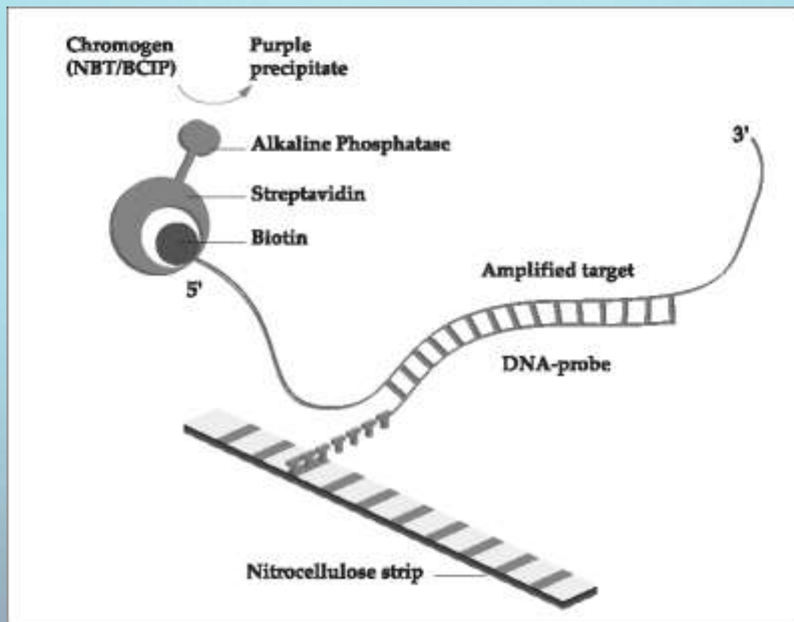
BIOMERIEUX COMPANY

- Integrates sample preparation, amplification, detection and analysis.

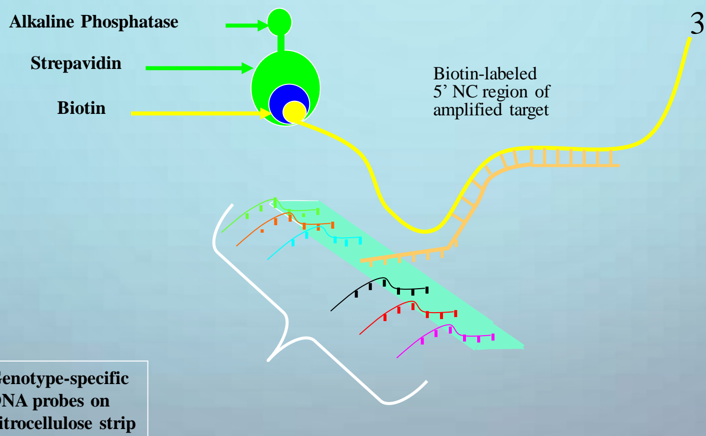


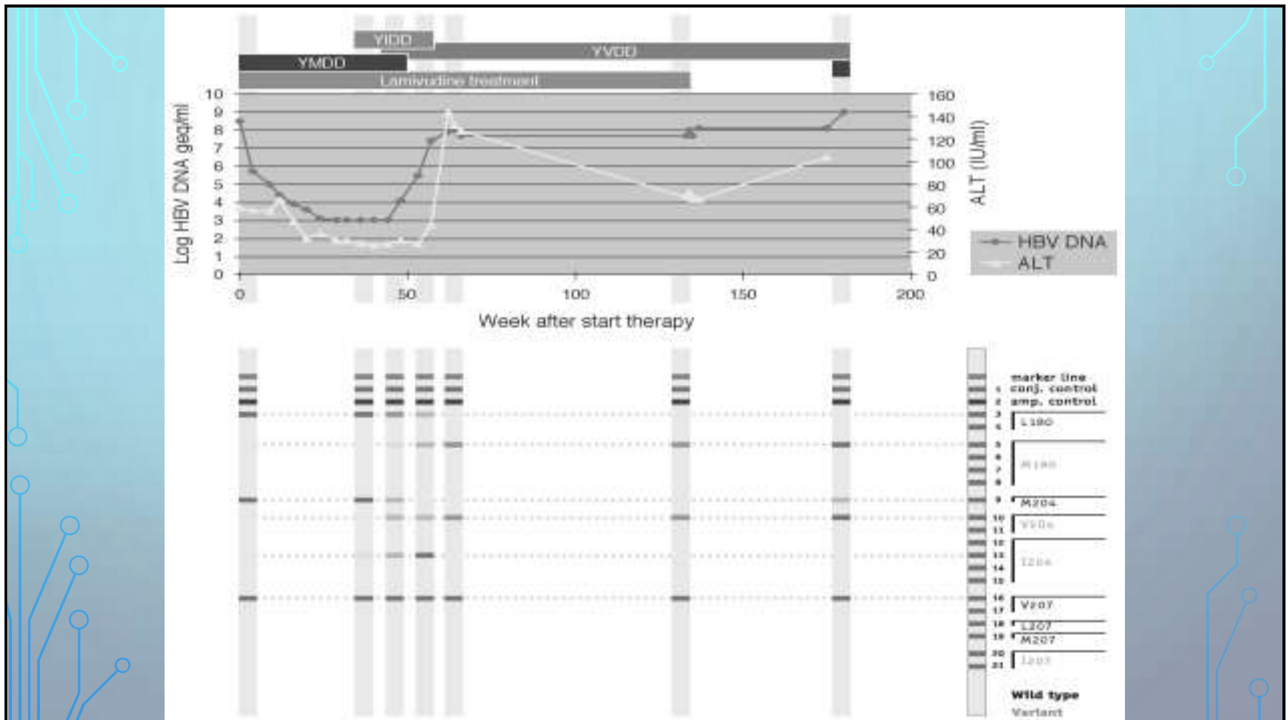
REVERS DOT BLOLT

- Able to detect different pathogens in one strip
- Able to differentiate different genotypes
- Able to detect mutants
- Mostly used for "genotyping" and "mutant detection"



REVERSE DOT BLOT METHOD





Lab-on-a-Chip

Microarray

- **Oligonucleotides microarray:**

hybridization of the targets(labeled nucleic acids of RNA or DNA with fluorescent dyes) and the probes(several individual nucleic acid species immobilized on a solid surface in the spots form

Oligonucleotides microarray

- **Sample preparation**

Extraction RNA or DNA

- **Amplification with dNTP labelled with fluorescent**

Cyanine dyes (i.e., Cy3 and Cy5)

- **Denatured PCR products**

Hybridized target products with specials probe

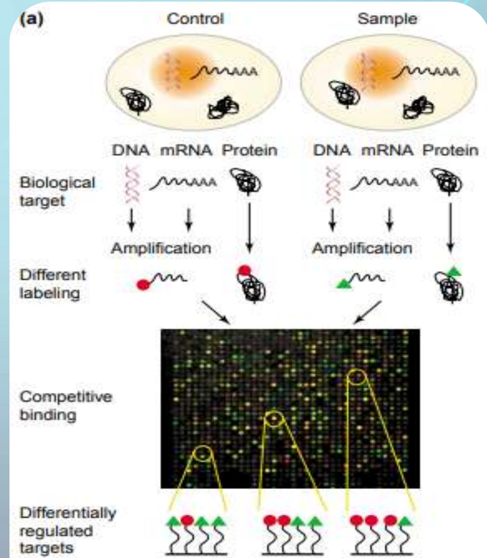
Spotted on a glass slide.

Microarray software

Green spots → Up regulated genes.

Red spots → Down regulated genes

Yellow spots → Equal regulated genes



Microarray-based Genotyping and drug-resistant HBV mutations

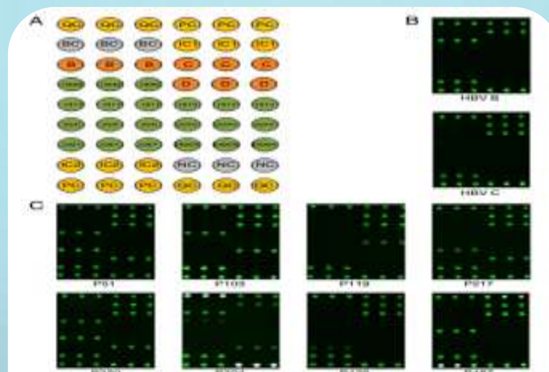


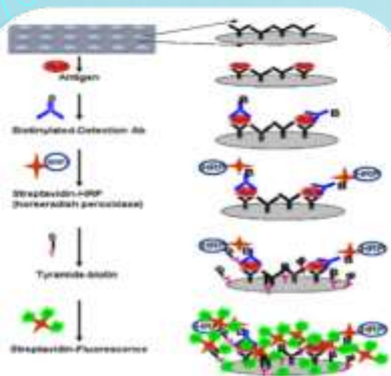
Fig. 1 – Microarray-based HBV genotyping and drug resistance-related mutations. (A) Layout of the microarray used in the current study. All probes, with the exception of QC and PC, were spotted in three replicates; QC and PC were spotted in six replicates. QC and BC were the positive and negative controls for array production, respectively. PC and NC were the positive and negative controls for hybridization, and IC1 and IC2 were the internal controls for HBV amplification, respectively. B, C, and D represent HBV genotypes B, C, and D, respectively. 180M, 181T/V, 204I/V, and 236T represent the rtL180M, rtA181T/V, rtM204I/V, and rtN236T, respectively, mutations in the HBV reverse transcriptase region. 1896A denotes the G1896A mutation in the HBV precore region. (B) Hybridization results of the classical HBV samples with genotypes B and C. (C) Hybridization results of eight classical NA-resistant samples.

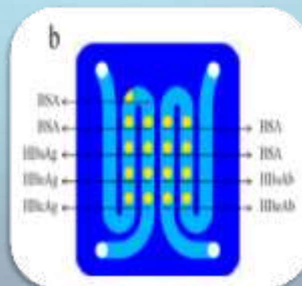
Protein microarray: Protein – Protein interaction

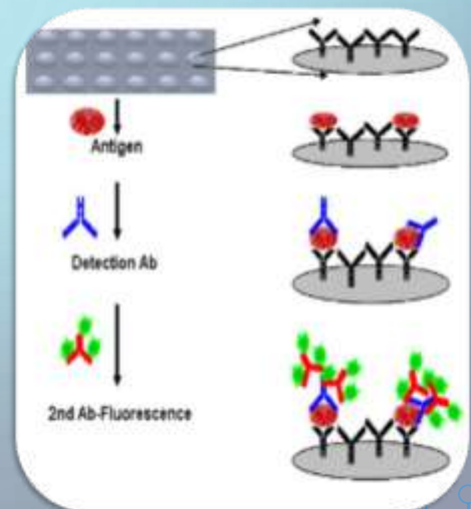
Prepared by immobilizing proteins(Antigen or Antibodies) onto a glass slide

Screened for protein ability to bind other protein in a complex ,antibody's ,enzymes ….

Protein microarray







Advantages

- Provide data from thousands of genes
- One experiment instead of many
- Fast and easy to obtained results
- Specific and Sensitive
- Need less volume sample and reagent.

Microfluidics Technology

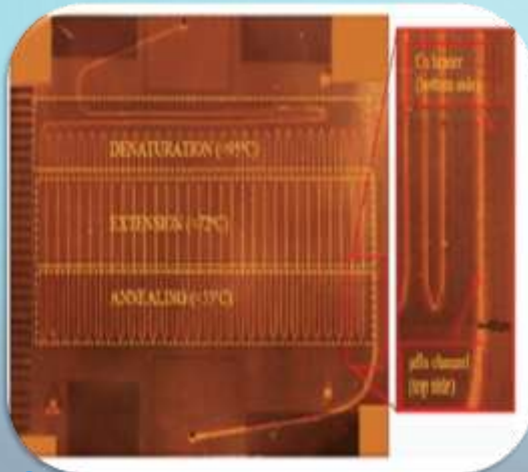
- **process small quantities of fluids by using tiny channels having dimensions at the microscale – typically tens to hundreds of micrometers.(10-9 to 10-18 liters)**

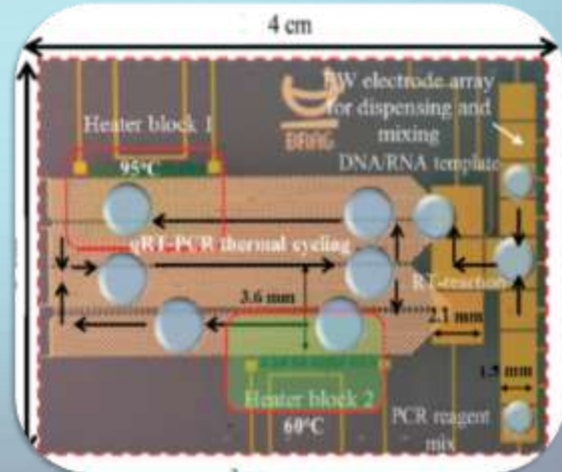
- Microducts
- Micronozzles
- Micropumps
- Microturbines
- Microvalves
- Microsensors
- Microfilters
- Microneedles
- Micromixers
- Microreactors
- Microdispensers
- Microseparators

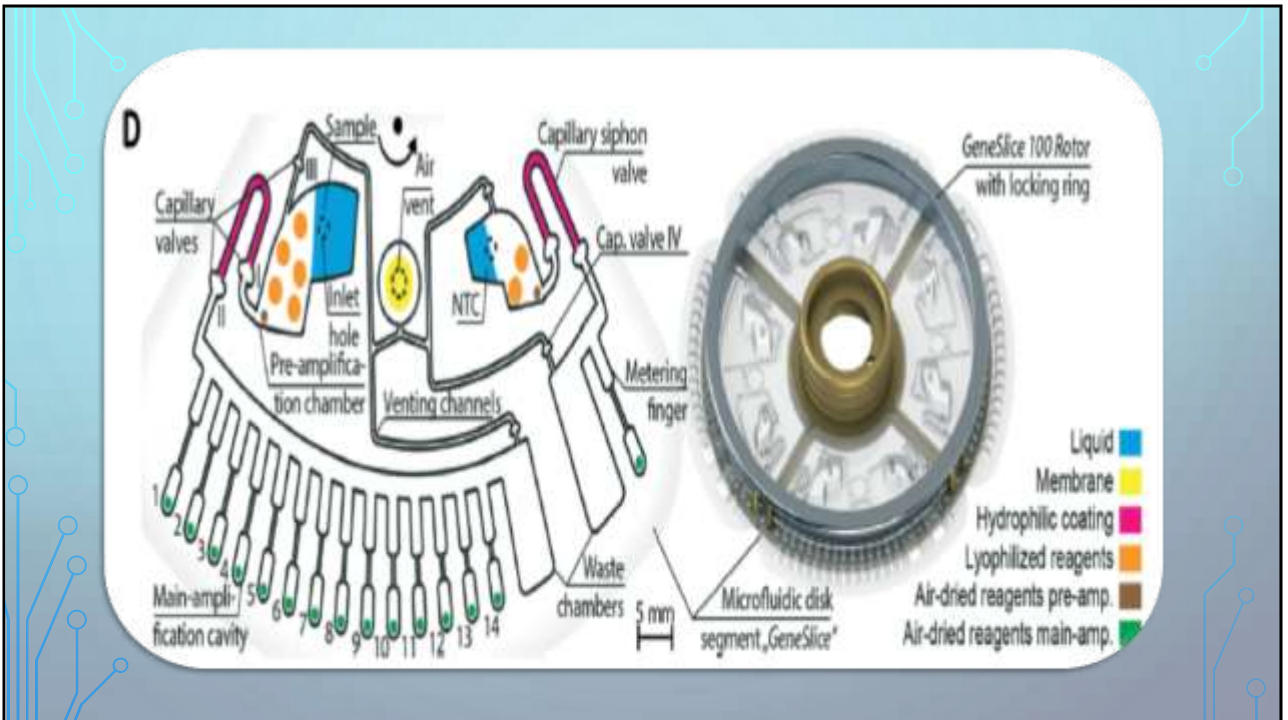
Microfluidics Technology

- **Reducing the size will increase the volume-to –surface ratio.**

1. **Surface tension.**
2. **Diffusion (allow faster transfer).**
3. **Fast thermal relaxation (the larger heat distribution, especially for PCR, which requires a different system and different stages of cold and heat).**

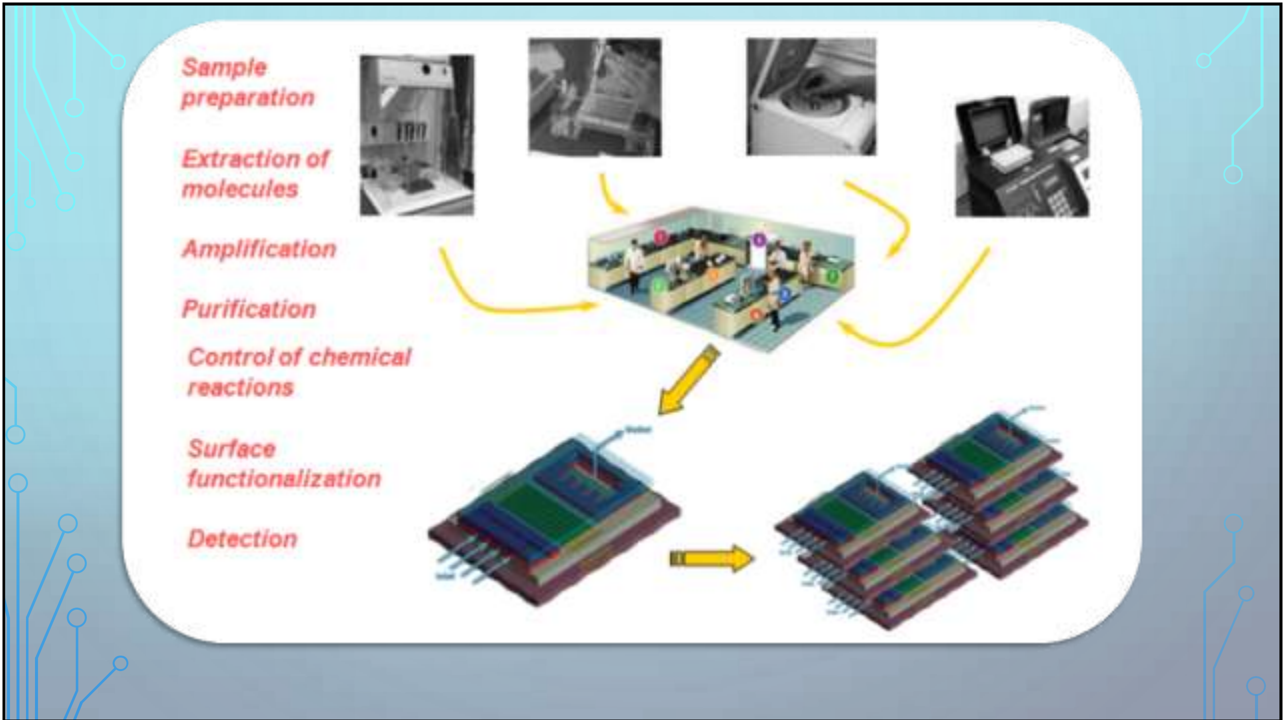




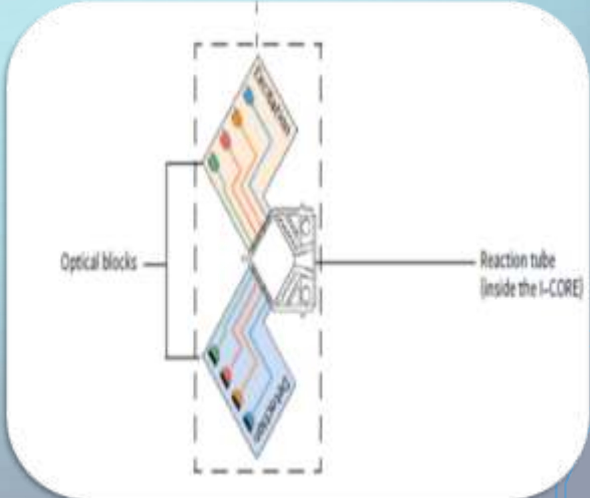


Advantages

- Low cost
- Physically small .
- Ease of use and compactness
- Reduction of human error
- Faster response time and diagnosis
- Low volume sample
- Real time process control and monitoring increase sensitivity.
- Expendable: automation and low energy consumption.



GeneXpert



DNA Sequencing

- Determining the precise order of nucleotides in a pieces of DNA

- DNA sequence is useful in studying fundamental biological processes and in applied fields such as diagnostic or forensic research

- DNA sequencing methods have been around for 40 years, and since the mid-1970s

Sequencing Methods

Sanger

- B- Enzymatic method (Sanger, 1981)
- Uses dideoxy nucleotides to terminate DNA synthesis.
- DNA synthesis reactions in four separate tubes
- Radioactive dATP is also included in all the tubes so the DNA products will be radioactive.
- With addition of enzyme (DNA polymerase), the primer is extended until a ddNTP is encountered.
- The chain will end with the incorporation of the ddNTP
- With the proper dNTP: ddNTP ratio (about 100:1), the chain will terminate throughout the length of the template.
- Yielding a series of DNA fragments whose sizes can be measured by electrophoresis.

Sequencing Methods

Gilbert

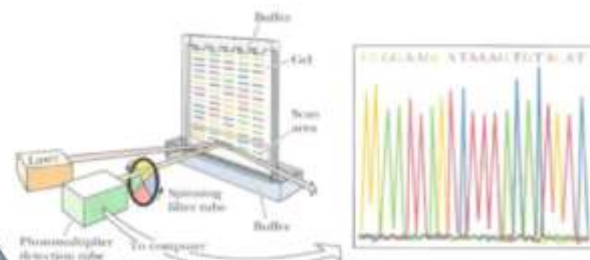
- Two basic methods for DNA sequencing :-
- A- Chemical cleavage method (Maxam and Gilbert, 1977)
- Base-specific cleavage of DNA by certain chemicals
- Four different chemicals, one for each base – A set of DNA fragments of different sizes
- DNA fragments contain up to 500 nucleotides

Dye Terminator Sequencing

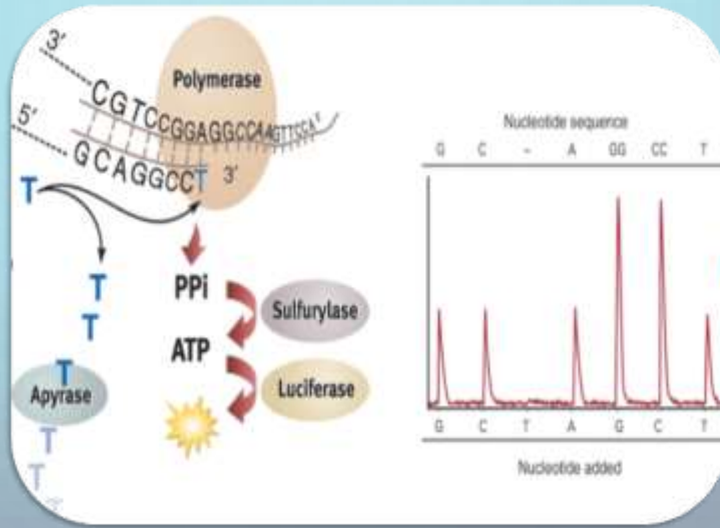
- A distinct dye or "color" is used for each of the four ddNTP.
- Since the terminating nucleotides can be distinguished by color, all four reactions can be performed in a single tube.

The fragments are distinguished by size and "color."

Automated Version of the Dideoxy Method



Pyrosequencing



pyrosequencing (Roche)

Polymerase

APS

PPi

ATP sulfurylase

ATP

Luciferase

Luciferin

Light and oxyluciferin

Pyrosequencing
As a base is incorporated, the release of an inorganic pyrophosphate triggers an enzyme cascade, resulting in light

Single nucleotide addition
Only one dNTP species is present during each cycle; multiple identical dNTPs can be incorporated during a cycle, increasing emitted light

Cycle 1

Cycle 2

Cycle 3

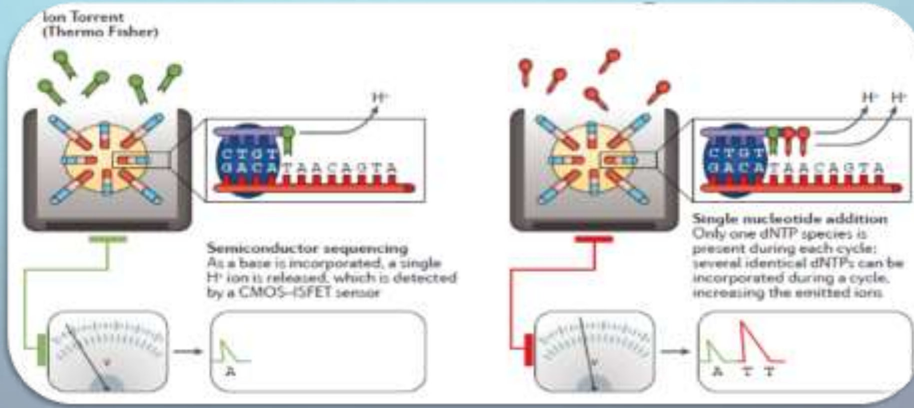
Cycle 4

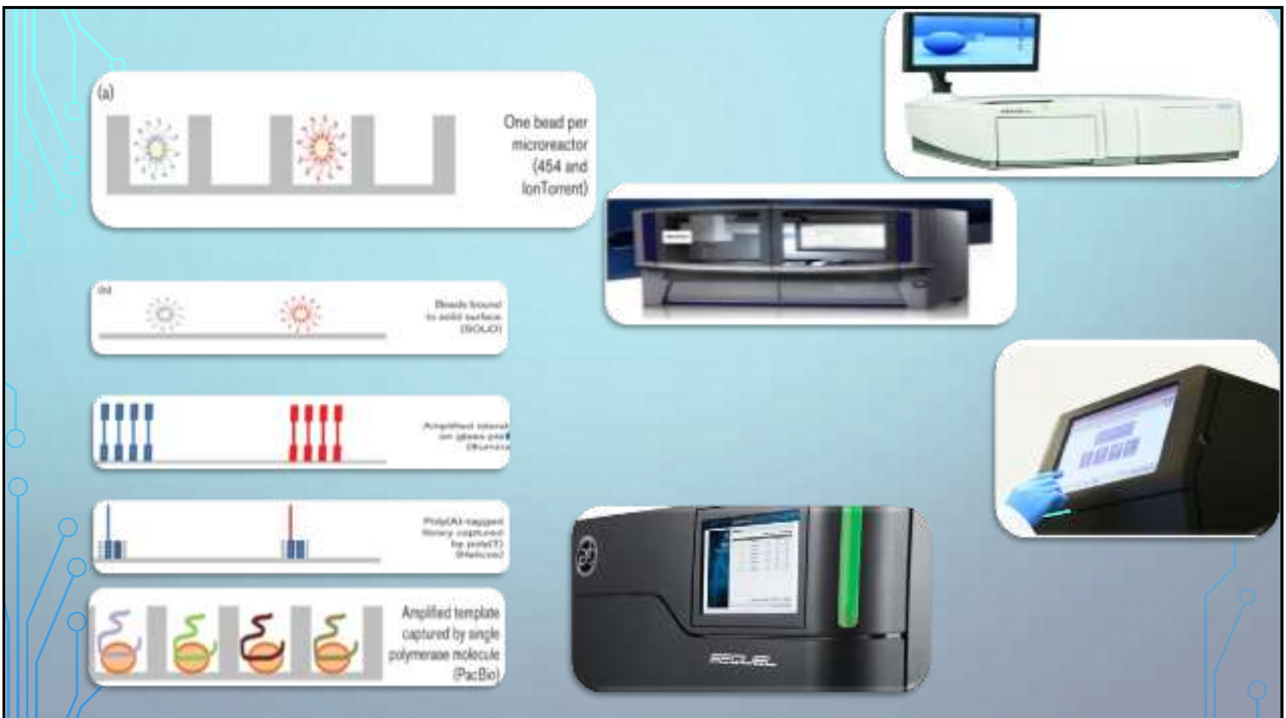
Ion Torrent

After bead-based template enrichment, beads are carefully arrayed into a microtitre plate.

Nucleotide species are added to the wells one at a time and a standard elongation reaction is performed.

As each base is incorporated, a single H+ ion is generated as a by-product. The H+ release results in a 0.02 unit change in pH, detected by acomplementary metal-oxide semiconductor (CMOS) and an ion-sensitive field-effect transistor (ISFET) device.





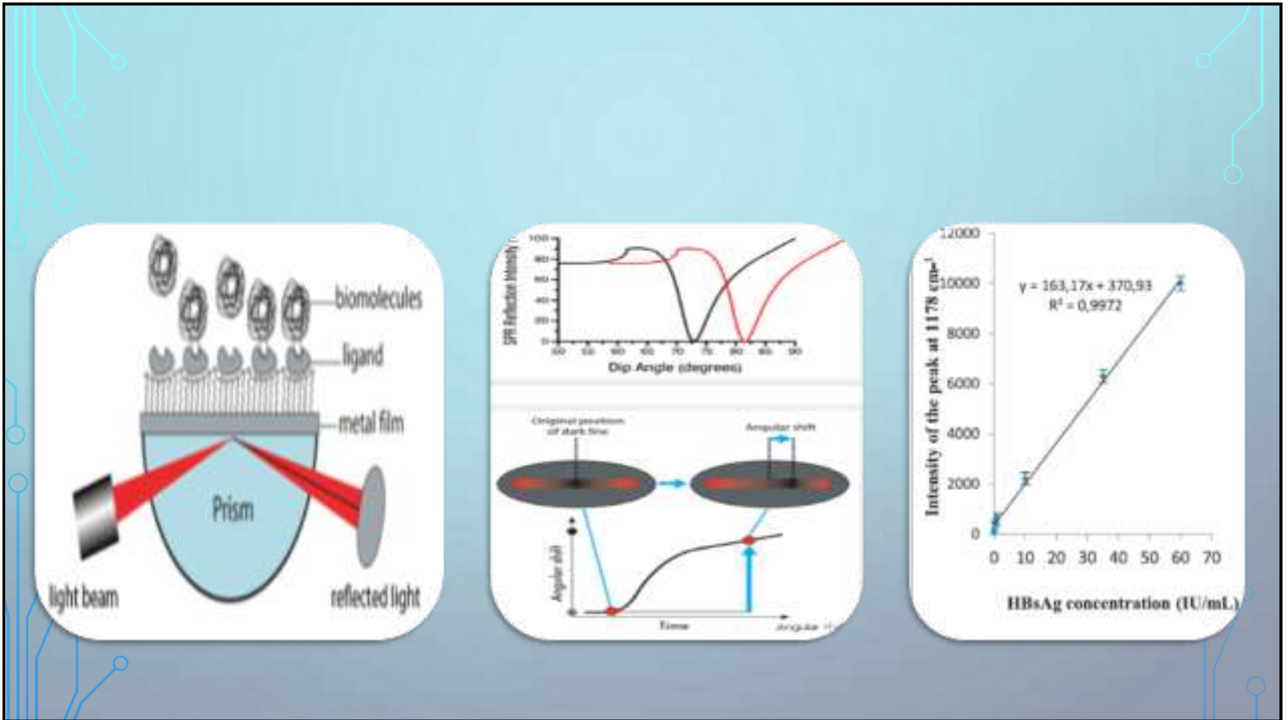
Advantages

NGS technologies

Cost-effective
Fast
Ultra throughput
Cloning-free
Short reads

Molecular Diagnostic in Pipeline

Microarray integrated with SPR (Surface Plasma Resonance)


biomolecules
ligand
metal film
Prism
light beam
reflected light
Dip Angle (degrees)
y = 163,17x + 370,93
R² = 0,9972
Intensity of the peak at 1178 cm-1
HBsAg concentration (IU/mL)