IL TECNICO DI LABORATORIO E LA DIAGNOSTICA MOLECOLARE
INFETTIVOLOGICA: NUOVE COMPETENZE PER NUOVI SCENARI
**Pordenone, 5 maggio 2012**
Palazzo Montereale Mantica

# L'evoluzione della medicina molecolare nella diagnostica delle malattie infettive

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AO «S. Maria degli Angeli Pordenone»

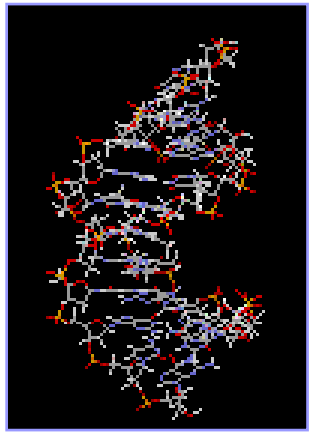
# The objectives of the **Molecular Medicine**

- provide an advanced understanding of the **molecular basis** of the pathogenesis, diagnosis and treatment of human diseases
- describe and discuss topics related to **infectious diseases**, chronic diseases, genetic diseases, endocrine disorders, malignancy and diseases arising from abnormal immune responses
- carry out **molecular-biological** and **bioinformatics techniques** for investigation of human diseases

# L’evoluzione della medicina molecolare nella diagnostica delle malattie infettive

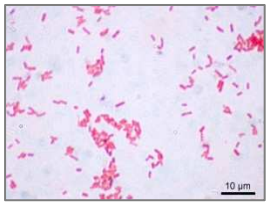
- Nel 1953, la scoperta della struttura a doppia elica del DNA (James Watson e Francis Crick ) ha segnato il passaggio alle biotecnologie innovative e la nascita dell’ “*ingegneria genetica*”

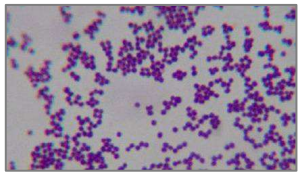
L’avvento della tecnologia del DNA ricombinante e l’evoluzione delle tecniche di sintesi chimica hanno permesso di realizzare **sonde genetiche** che, mediante reazioni di ibridazione degli acidi nucleici, sono state ampiamente impiegate in campo microbiologico

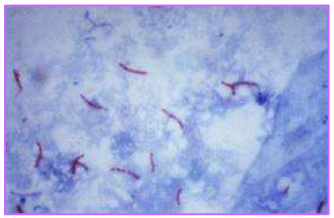


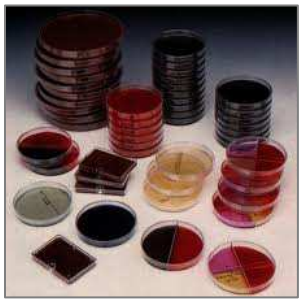
«Biotecnologia» : ogni applicazione tecnologica che si avvale di sistemi biologici, di organismi viventi o di loro derivati, per realizzare o modificare prodotti o procedimenti per un uso specifico. CONVENZIONE SULLA DIVERSITÀ BIOLOGICA Rio de Janeiro, 3-14 giugno 1992

# Conventional methods in diagnostic microbiology



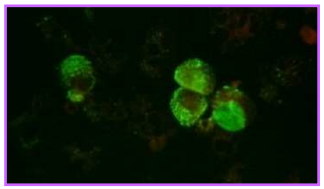


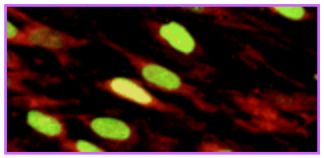


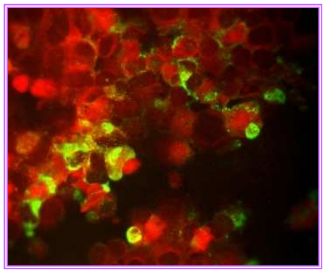
- Staining/microscope
- Culture
- Immunological methods

Problems

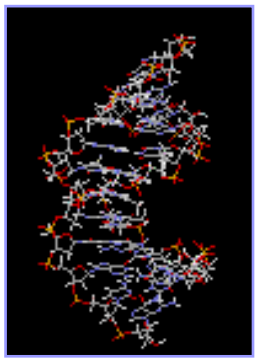
- Uncultivable/fastidious pathogens
- Some require cell culture lab./animal
- Discrimination between prognostic/ therapeutically important subgroups of pathogens





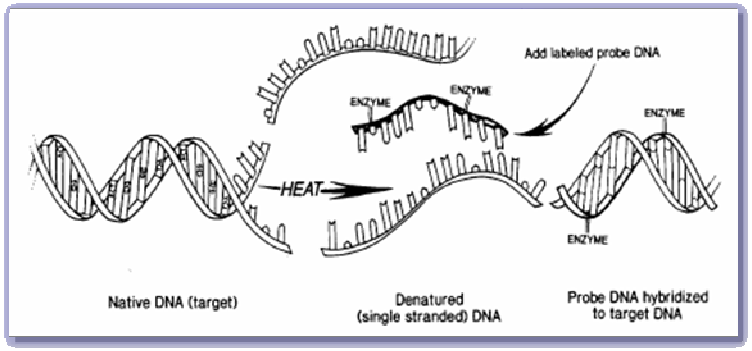


# Nucleic Acid-based Techniques



## Nucleic acid hybridization

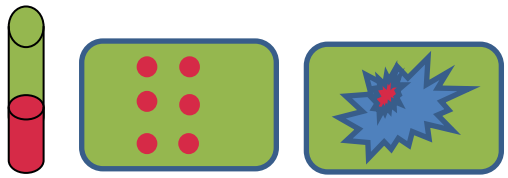
Target nucleic acids (DNA or RNA)
Probe = segments of nucleic acid labeled with radioisotopes, enzymes or chemiluminescent repoter molecules that can bind to complementary nucleic acid sequences with high degrees of specificity



# Type of Nucleic Acid Hybridization

1. **Liquid phase hybridization** (detection)
2. **Solid phase (filter) hybridization simultaneous analysis of multiple samples :** dot blotting (detection), Southern blot (detection, subtyping), Northern blot (gene expression)
3. **In situ hybridization** [isotopic tracer , chromogen (CISH), fluorescent (FISH)]

## Application of Nucleic Acid Hybridization in the early 1980s

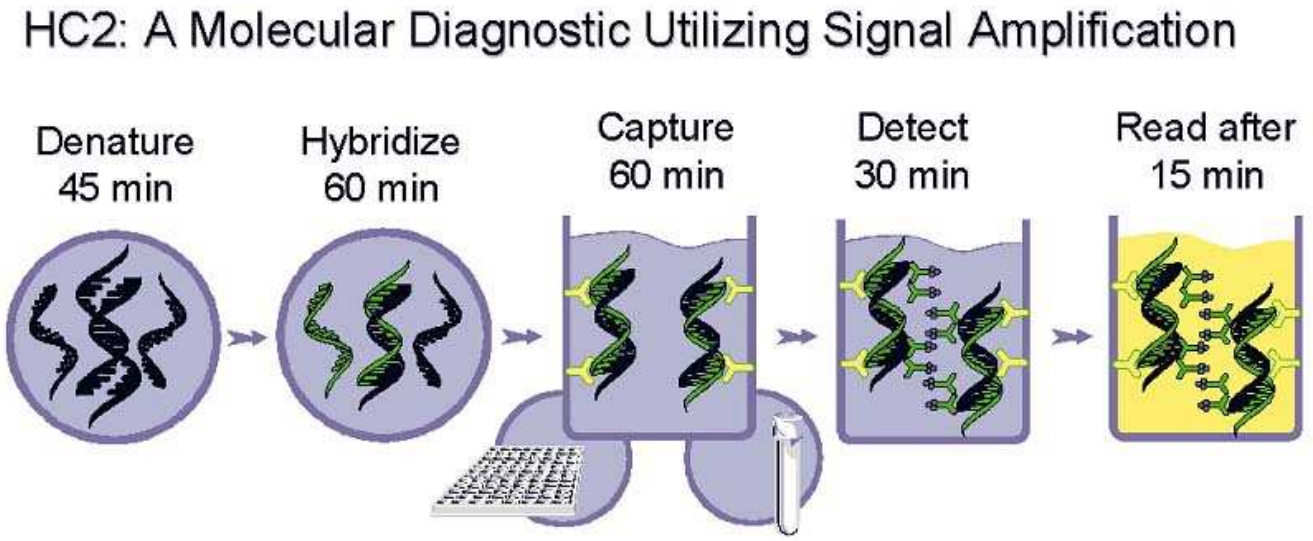


- **Direct rapid detection** in clinical specimens of fastidious or uncultivable organisms
- **Culture confirmation** and **rapid identification** (particularly of mycobacteria and systemic dimorphic fungi)
- **Differentiation** of pathogenic from avirulent strains
- Detection of **drug resistant** gene
- Detection of **latent viral infections**
- Extent of infection
- Gene expression analysis
- **Epidemiological studies**/typing schemes to control and prevention of various infectious diseases

**The first application of a DNA probe to detect bacteria**

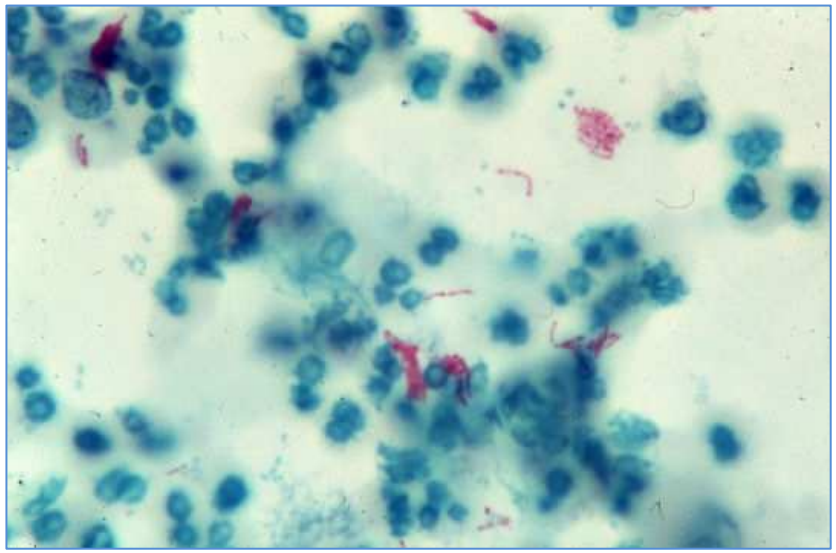
Moseley,S.L., Huq,I., Alim,A.R.M.A., So,M., Samadpour-Motalibi,M., and Falkow,S. Detection of enterotoxigenic Escherichia coli by DNA colony hybridization. J.Infect.Dis. 142:892-898, 1980.

# Hybrid Capture II HPV test

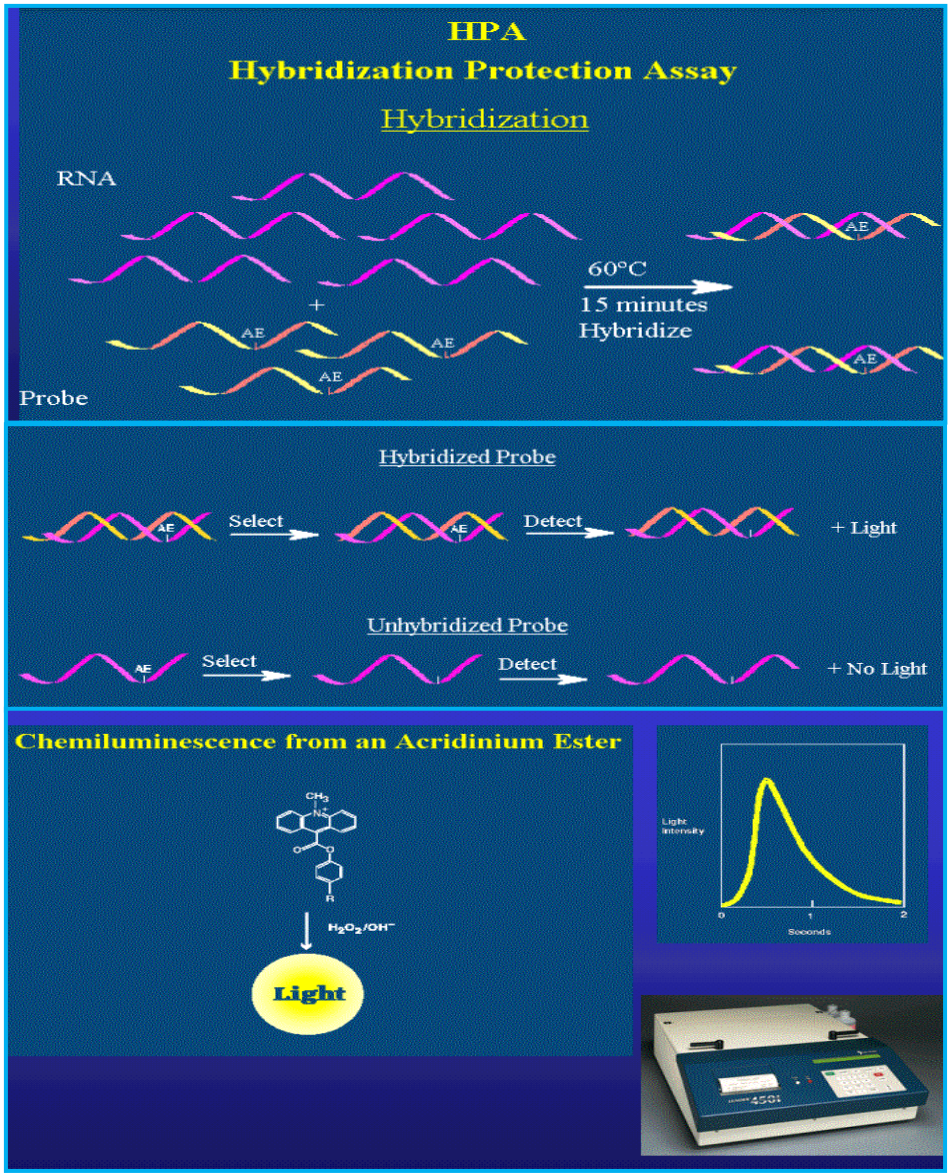



- Ibridazione in fase liquida
- sonda per 13 tipi di HPV HR (16,18,31,33,35,39,45,51,52,56,58,59,68)
- sonda per 5 tipi HPV LR (6,11,42,43,44)
- chemioluminescenza
- qualitativo e semiquantitativo: la quantità di luce emessa (Relative Light Units = RLU) è proporzionale alla quantità di HPV presente nel campione

**Digene's Hybrid Capture® Technology**

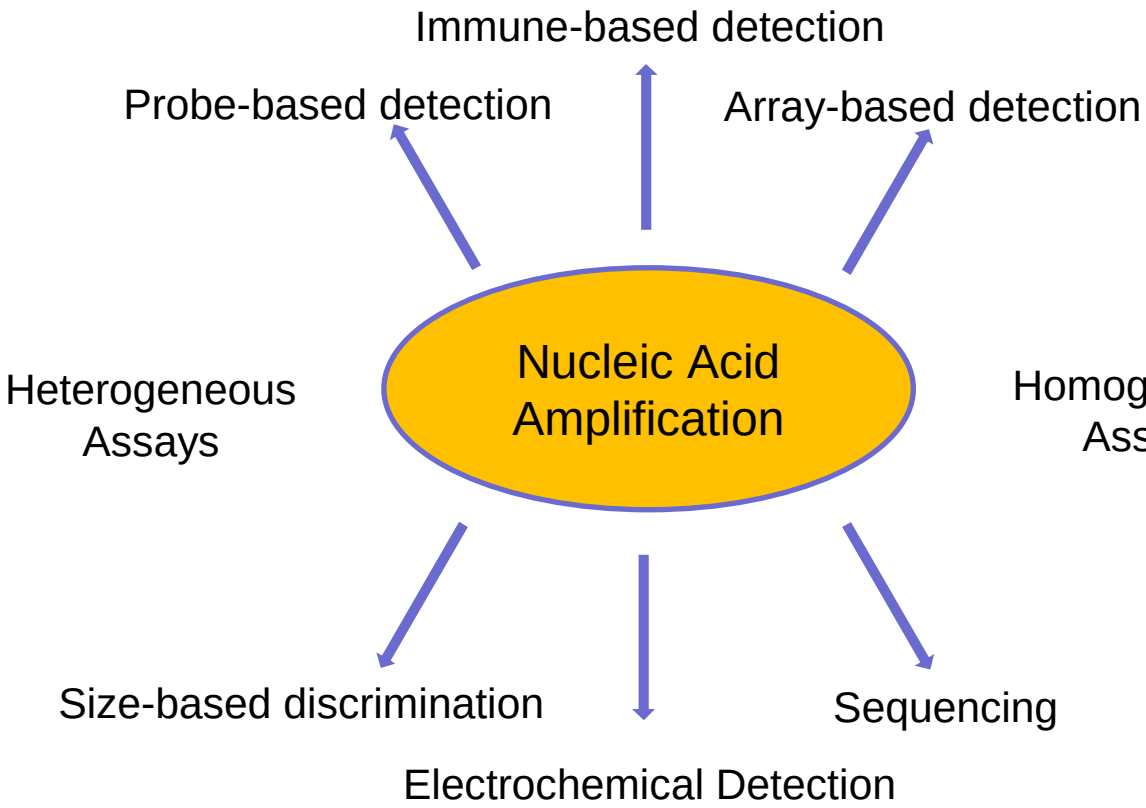


- Bersaglio: rRNA 16S
- Reazione: ibridizzazione in fase liquida, direttamente dalle colonie da terreno liquido o solido
- Rivelazione: in chemioluminescenza (*HPA*)
- Specificità:
  - *M. tuberculosis* complex
  - MAC o, in alternativa, *M. avium* e *M. intracellulare* separatamente
  - *M. kansasii*
  - *M. gordonae*




Liquid phase hybridization

# Nucleic acid amplification techniques




**Use of nucleic acid amplification in clinical microbiology**

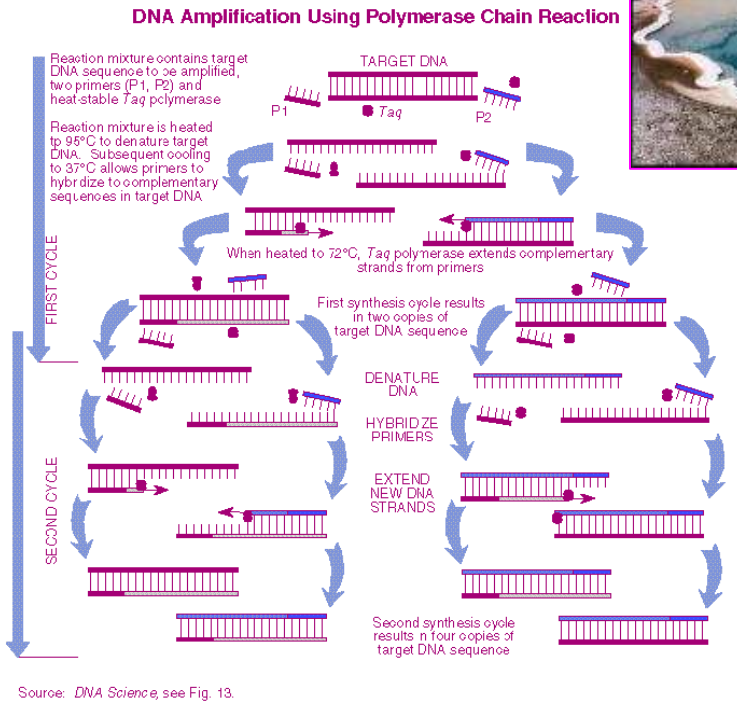
Hayden R.T. in Molecular Microbiology: Diagnostic Principles and Pratice, 2011

# Nucleic acid amplification techniques

K.B. Mullis received a Nobel Prize in chemistry in 1993, for his invention of the polymerase chain reaction (PCR). The process, which Kary Mullis conceptualized in 1983, is hailed as one of the monumental scientific techniques of the twentieth century.

PCR requires

1) A pre-defined DNA target sequence
2) two primers which hybridize to the ends of the target DNA
3) Thermostable DNA polymerase (*Thermus aquaticus*)
4) dNTPS

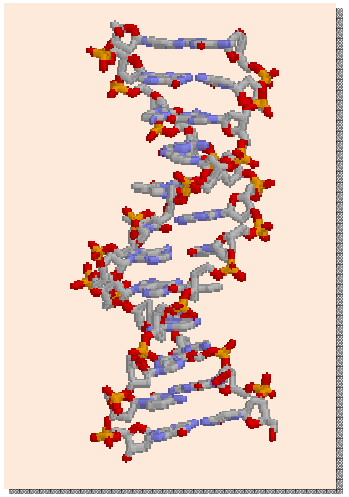




Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N.
**Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia.**
Science. 1985 Dec 20;230(4732):1350-4.

# Nucleic acid amplification techniques

- Less amount of samples
- Sensitivity
- Specificity
- Rapid TAT
- Simple
- Offer
  - Diagnosis (Qualitative)
  - Prognosis (Quantitative)
  - Therapeutic Monitoring (Quantitative)

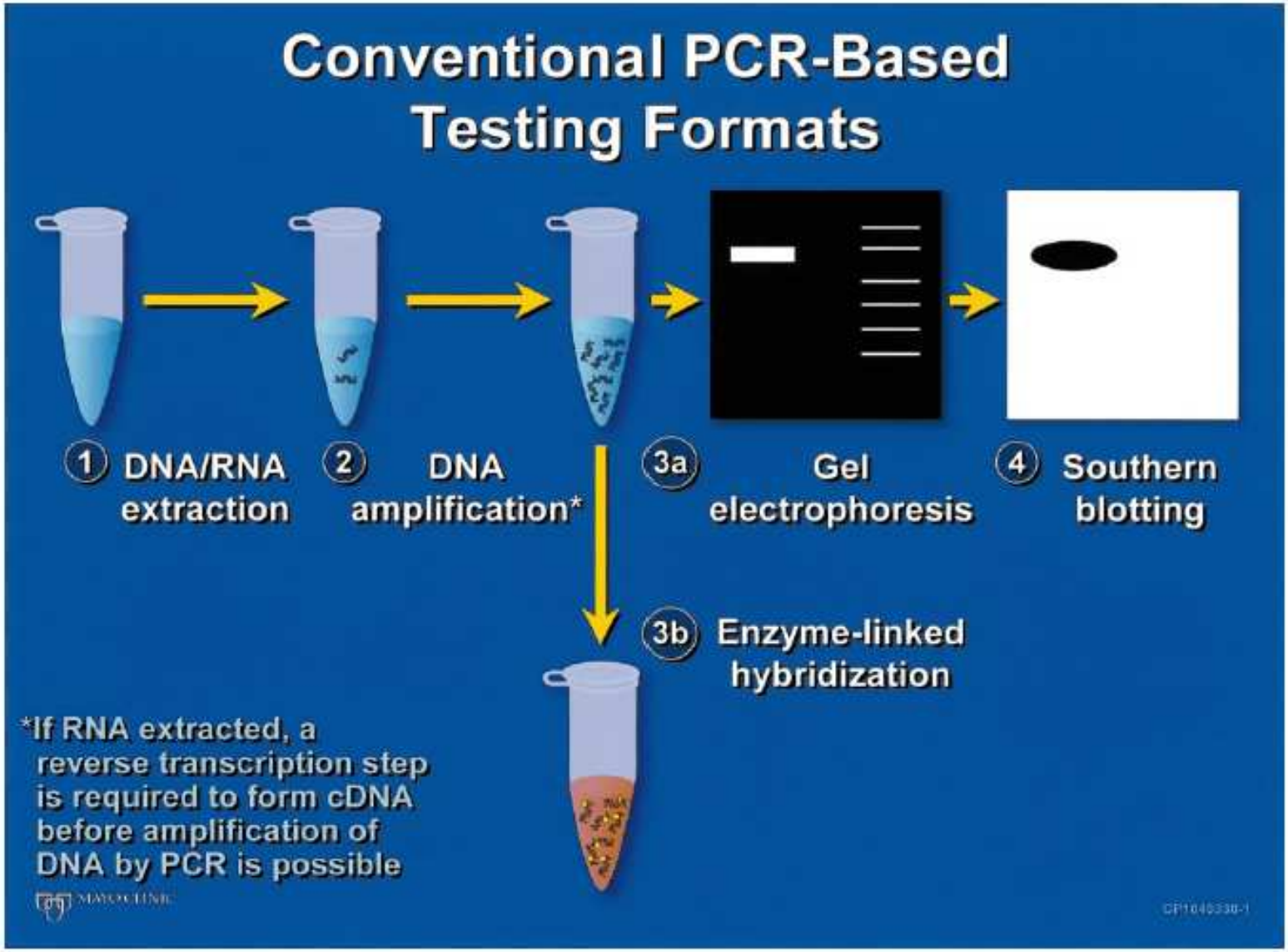


# Nucleic acid amplification methods

| Amplification method | Enzyme(s) used |
|---|---|
| **Target amplification** | **Enzyme used** |
| PCR | Thermophilic DNA polymerase |
| Transcription-Based Amplification System<br>TAS, 3SR, NASBA, TMA | RT, Rnase H, RNA polymerase |
| SDA | Restriction endonucleases, DNA polymerase |
| LCR | Thermophilic DNA ligase |
| LAMP | *Bst* DNA polymerase |
| **Signal amplification** | |
| bDNA | None |
| Direct Hybridization Assays | None |
| CPT | Rnase H |
| Invader | Cleavase |
| RCA | DNA polymerase |

**Polymerase chain reaction (PCR); transription-based amplification system (TAS); self-sustaining sequence amplification (3SR); Nucleic acid sequence-based amplification ( NASBA ); transcription-mediated amplification (TMA); strand displacement amplification (SDA); ligase chain reaction (LCR); loop -mediated isothermal amplification (LAMP); branched - DNA (bDNA); cycling probe technologies (CPT), invader assays ; rolling cycle amplification (RCA).**

**Application of Rapid-Cycle Real-Time Polymerase Chain Reaction for Diagnostic Testing in the Clinical Microbiology Laboratory** Franklin R. Cockerill III
Arch Pathol Lab Med—Vol 127, September 2003




**Oligonucleotide-coated microparticles + biotin labeled single-strand amplified target streptavidin-alkaline phosphatase - chromogen= color reaction**

# Technical Aspects and Modifications of PCR

| | |
|---|---|
| • Hot start PCR | Prevention of non-specific amplification |
| • Nested PCR | Designed mainly to increase sensitivity |
| • RT-PCR | Reverse transcriptase PCR (RT-PCR) was developed to amplify RNA targets |
| • Multiplex PCR | Two or more sets of primers specific for different targets are introduced in the same tube, allowing multiple target sequences to be amplified simultaneously |
| • Broad-range PCR | This application uses conserved sequences within phylogenetically informative genetic targets to diagnose infection : e.g. universal primers set to target herpesvirus infections; single pairs of primers targeting the bacterial 16S rRNA gene; panfungal primers |
| • Quantitative PCR | Competitive or noncompetitive<br>Normalization to internal or external standards<br>Value in determinig the clinical significance of a positive qualitative result for therapy, for clinical course and responsiveness to therapy |

# Increasing use of automation

## Sample extraction process

Instrument automates: manual steps are limited to loading and unloading, with reagents in sealed, bar-coded, ready-to-use cassettes for fast and accurate reagent data entry

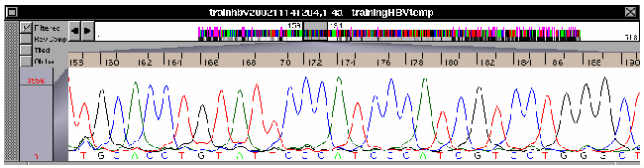
## Automated PCR System

### Assay for detection

- Chlamydia trachomatis
- Neisseria gonorrhea
- Mycobacterium avium
- Mycobacterium intracellulare

### Assay for quantification

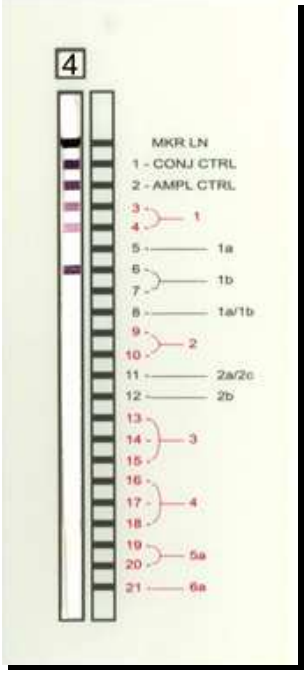
- HIV-1 viral load quantification
- CMV viral load quantification,
- Hepatitis B virus (HBV) viral load
- Hepatitis C virus (HCV viral load

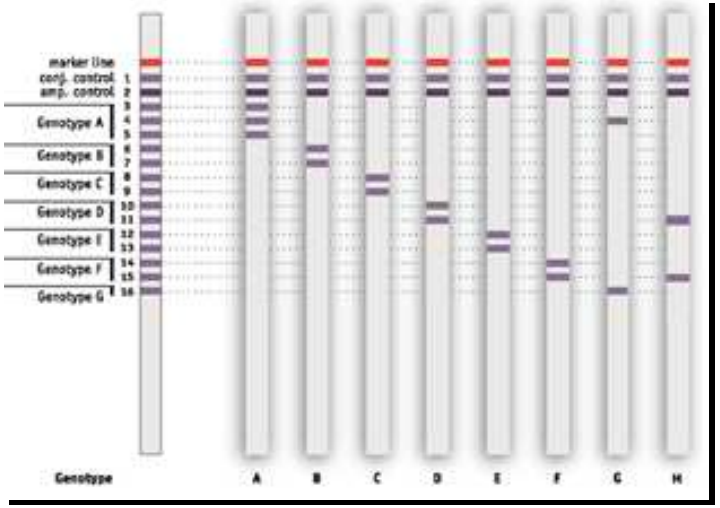


## Automated Sequencing

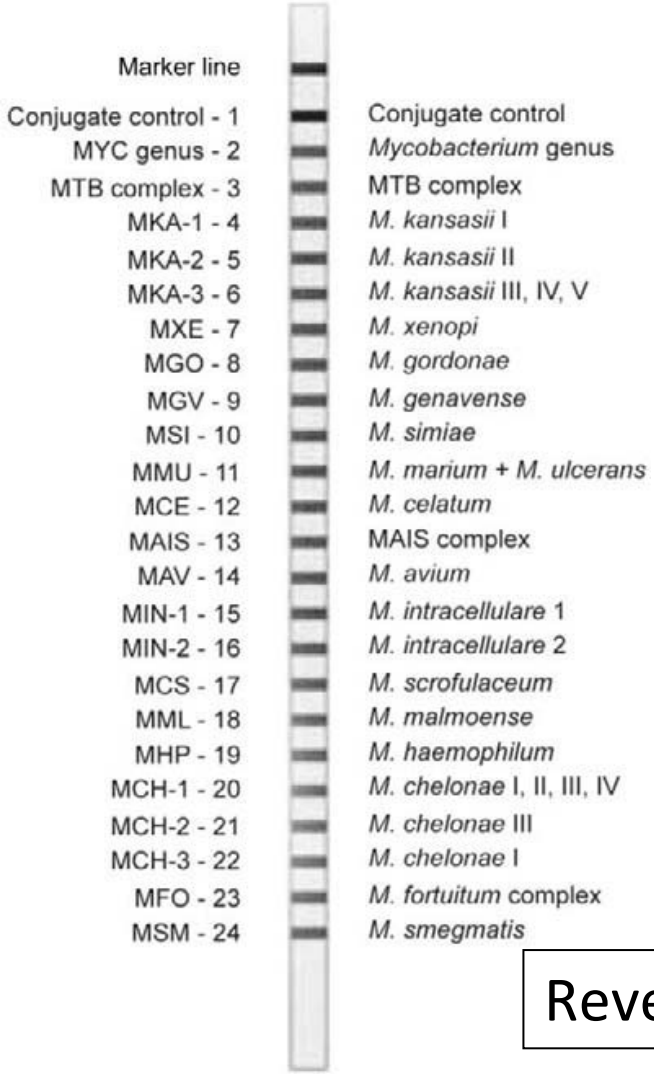
HIV, HCV, HBV (viral genotyping and resistance testing)

Versant HCV Genotype Assay



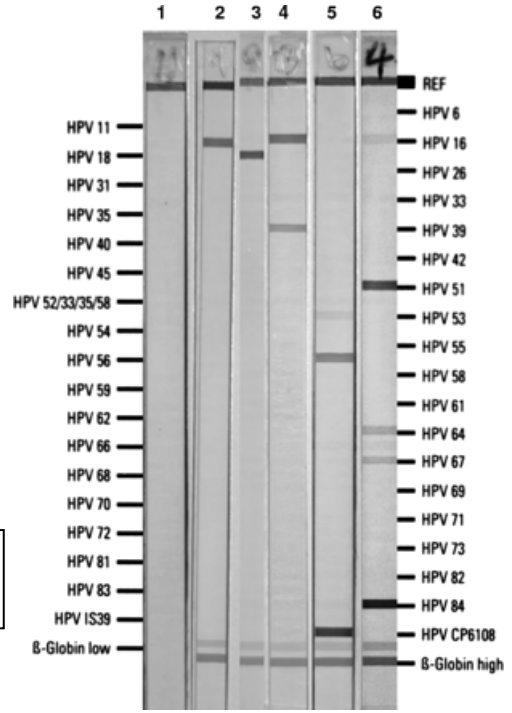




LIPA HBV




*Mycobacterium tuberculosis*

Linear Array HPV Genotyping Test




Reverse Hybridization

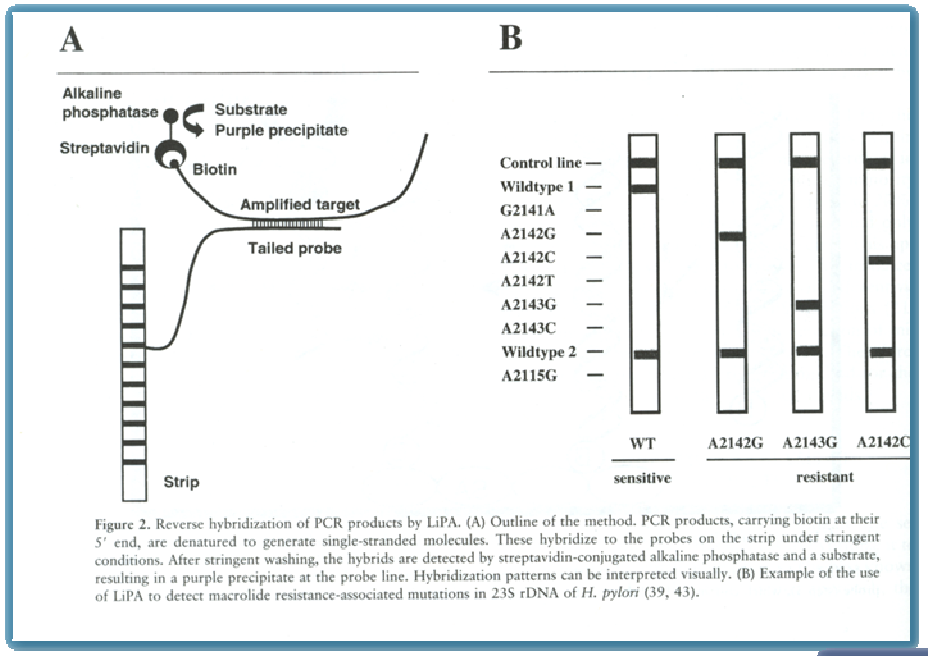
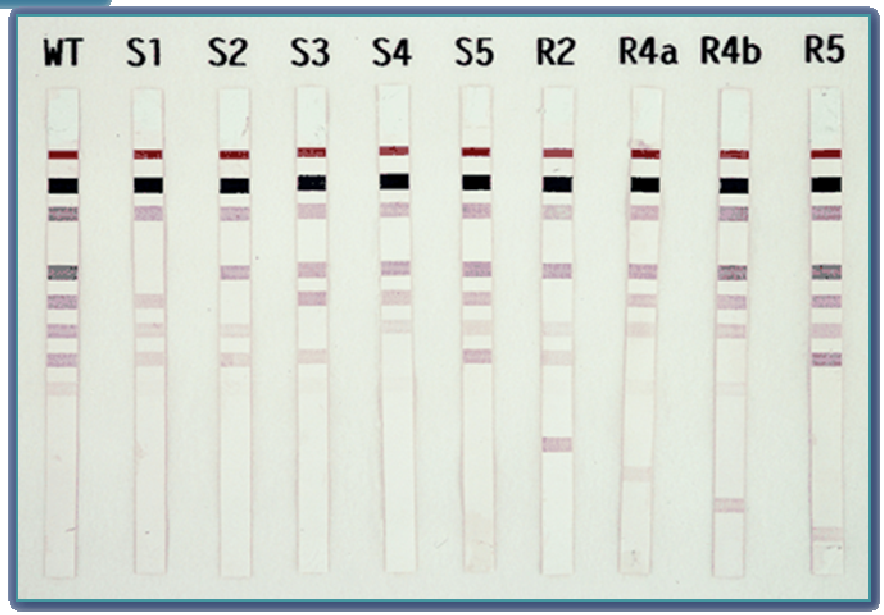



Figure 2. Reverse hybridization of PCR products by LiPA. (A) Outline of the method. PCR products, carrying biotin at their 5′ end, are denatured to generate single-stranded molecules. These hybridize to the probes on the strip under stringent conditions. After stringent washing, the hybrids are detected by streptavidin-conjugated alkaline phosphatase and a substrate, resulting in a purple precipitate at the probe line. Hybridization patterns can be interpreted visually. (B) Example of the use of LiPA to detect macrolide resistance-associated mutations in 23S rDNA of *H. pylori* (39, 43).

*H. pylori* mutazioni rDNA associate alla resistenza ai macrolidi

*Mycobacterium tuberculosis* mutazioni associate alla resistenza a rifampicina



# Real-time PCR

## “Homogeneous, rapid cycle nucleic acid amplification”

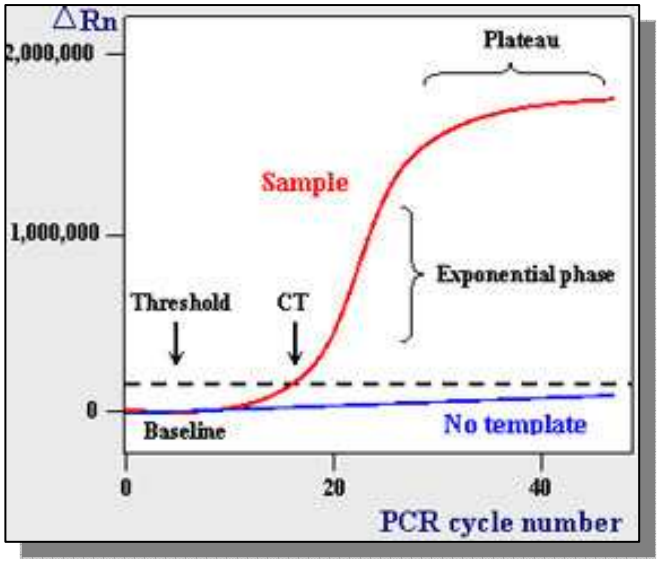
Rileva in tempo reale la fluorescenza associata all’amplificazione, durante la fase esponenziale della PCR, quando l’efficienza di amplificazione è minimamente influenzata dalle variabili di reazione, permettendo di ottenere risultati, analizzati tramite software, molto più accurati rispetto alla tradizionale PCR “end point”.

Real-Time PCR Principles

General methods for the quantitative assays

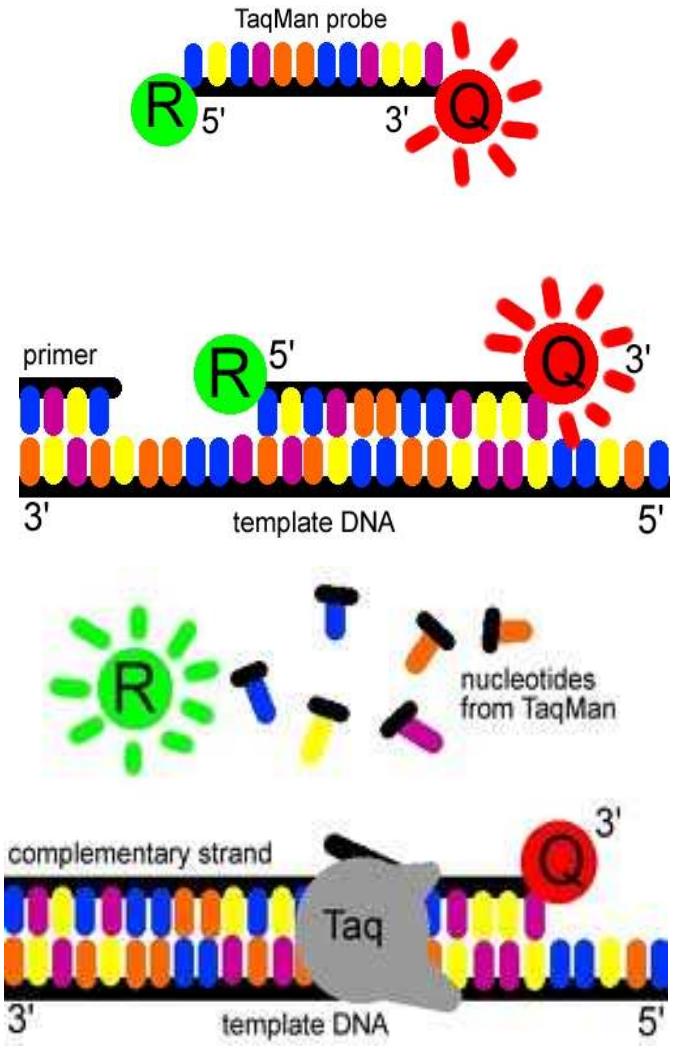
- DNA-binding agents (e.g. SYBR Green)
- Sequence specific fluorescent-labeled probes
    - Hydrolysis probes: TaqMan probes
    - Hybridization FRET(§) probes:
      LightCycler probes

(§) FRET : Fluorescence resonance energy transfer

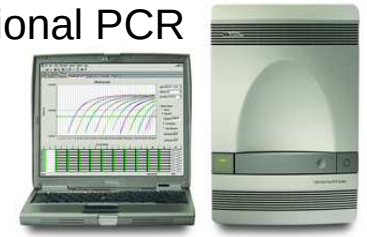



Higuchi, R., Dollinger, G., Walsh, P. S., and Griffith, R. (1992). "Simultaneous amplification and detection of specific DNA sequences." *Biotechnology* 10:413–417.

# Real-time PCR advantages

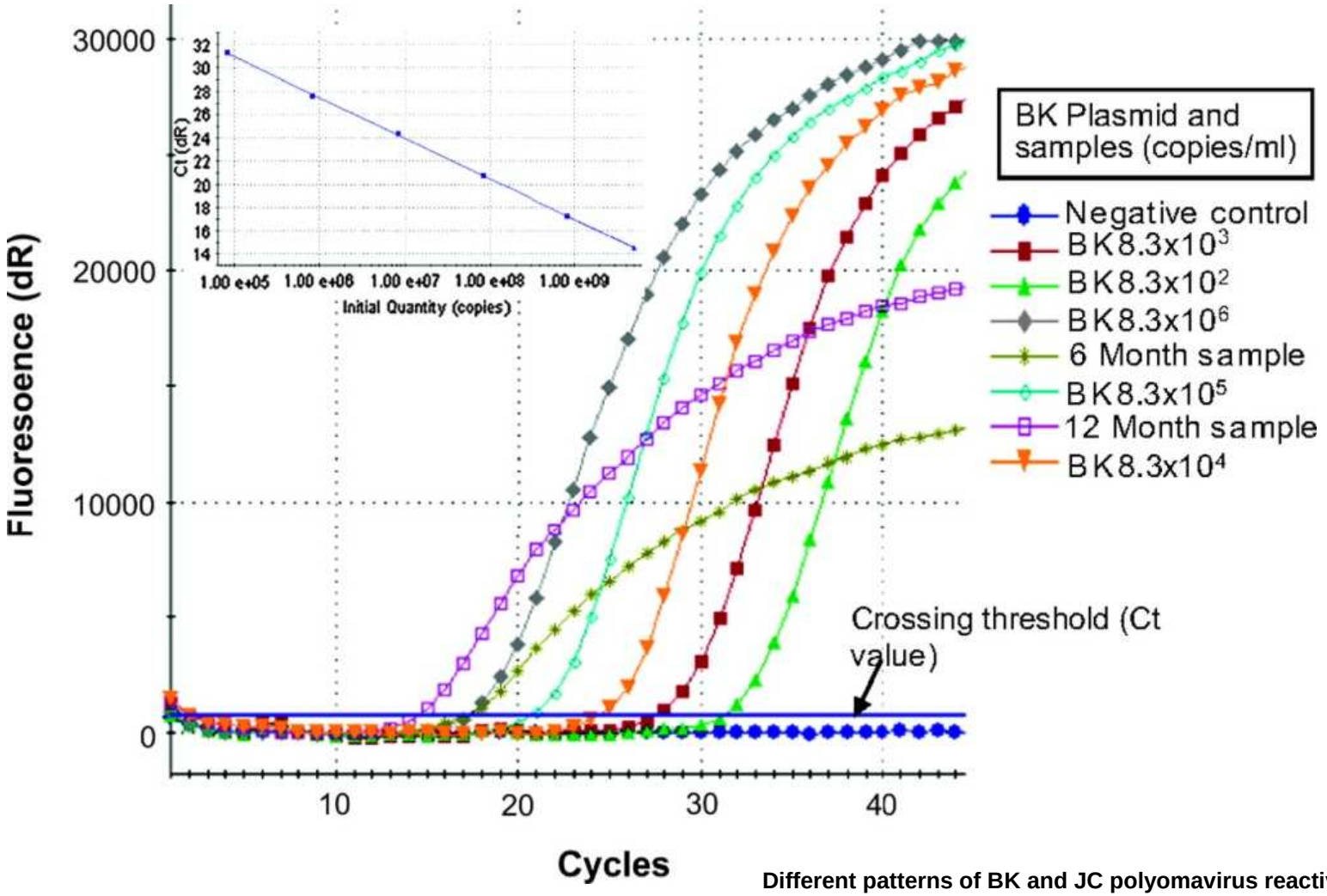



- amplification can be monitored real-time : detection of " amplification-associated fluorescence" at each cycle during PCR , real-time PCR is kinetic
- not influenced by non-specific amplification
- no post-PCR processing of products (high throughput, low contamination risk)
- ultra-rapid cycling (30 minutes to 2 hours) (TAT)
- wider dynamic range of up to $10^{10}$-fold (10-$10^{10}$ copies)
- requirement of 1000-fold less RNA than conventional assays (6 picogram = one diploid genome equivalent)
- detection is capable down to a two-fold change
- most specific, sensitive (3 pg or 1 genome eq of DNA) and reproducible (CV <2.0%)
- melting curve analysis: confirmation of specific amplification, detection sequence variants, detection and identification of clinically important pathogens
- allows for quantitation of results
- Software driven operation: computer based analysis of the cycle-fluorescence time course
- not much more expensive than conventional PCR (except equipment cost)



ABI 7300 Real-Time PCR System

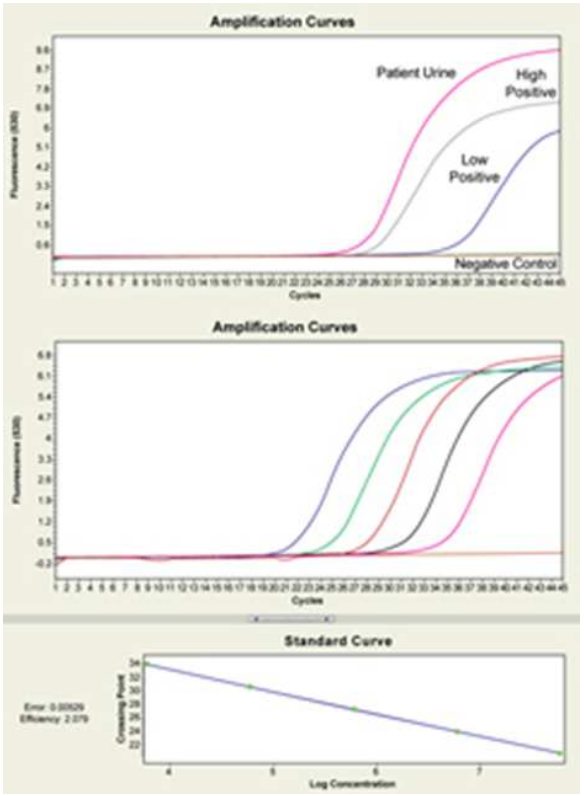
# Q-PCR




**Different patterns of BK and JC polyomavirus reactivation following renal transplantation**

***•Baljit K Saundh, et al.***

*J Clin Pathol* ***2010;63:8 714***




Test **validation** is the **ongoing process** of ensuring that the expected performance of an assay is consistently met in testing clinical specimens

Definitions used by CLIA, CLSI
Source: *Cumitech 31A*. 2009. ASM Press

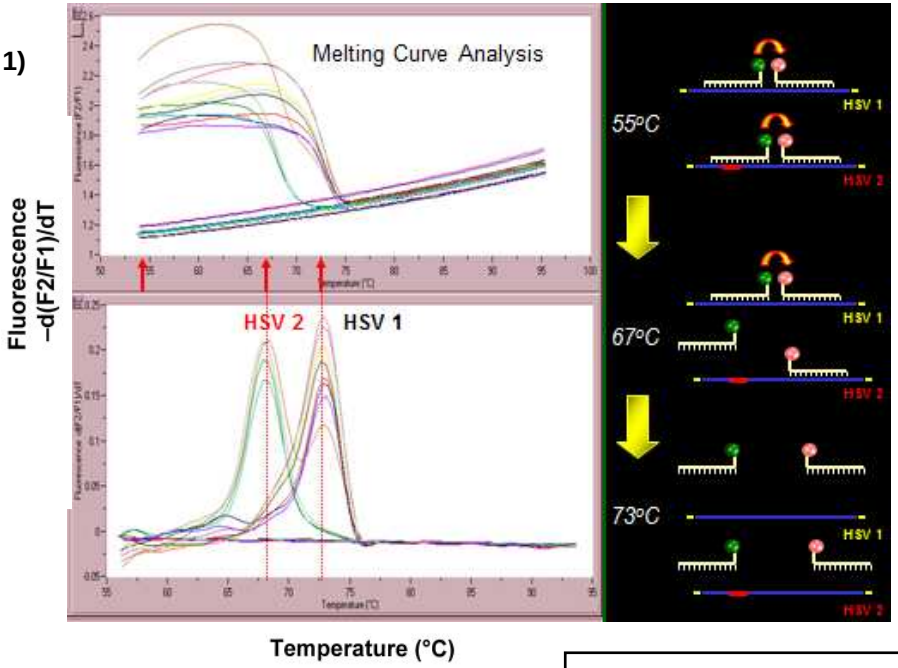
## Molecular infectious disease testing validation control

| Molecular test type | Control and calibrators | Comments |
|---|---|---|
| Qualitative | • IC<br>• Positive (low)<br>• Positive (high)<br>• Negative control<br>• No-nucleic-acid control | • IC in same tube as specimen or spliked into a second specimen tube<br>• Low positive close to the test cutoff value<br>• High positive in the test midrange<br>• Contain nonspecific nucleic acid<br>• Control has buffer in place of nucleic acid |
| Quantitative | • IC<br>• Positive (low)<br>• Positive (high)<br>• Negative control<br>• Calibrators | • IC, low-positive, high-positive, and negative controls similar to qualitative test<br>• Calibrators consist of at least three defined samples covering the dynamyc range of the test |
| Multiplex and microarray | • IC<br>• Multiple positive controls<br>• Negative control | • IC and negative control similar to qualitative test<br>• All positive controls should be included at a user-defined test frequency |

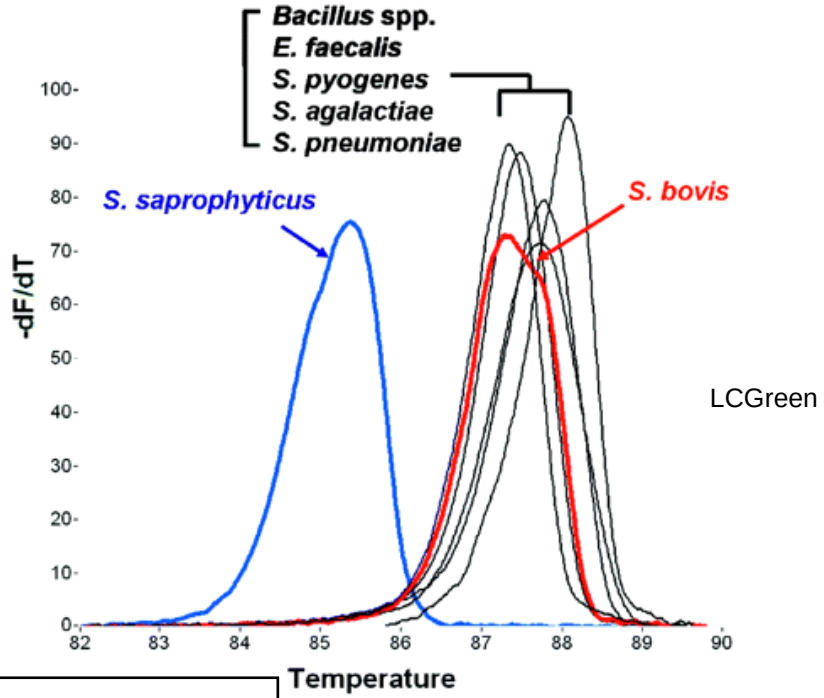
## Assay performance characteristics

***Analytical sensitivity : limit of detection*** **(LOD);** ***analytical specificity*** **: including interfering substances ; d*iagnostic sensitivity; diagnostic specificity; accuracy; repeatability*** **(short-term precision or intra-assay variance); r*eproducibility*** **(long-term precision or interassay variance); *Linear dynamic range.***

1)



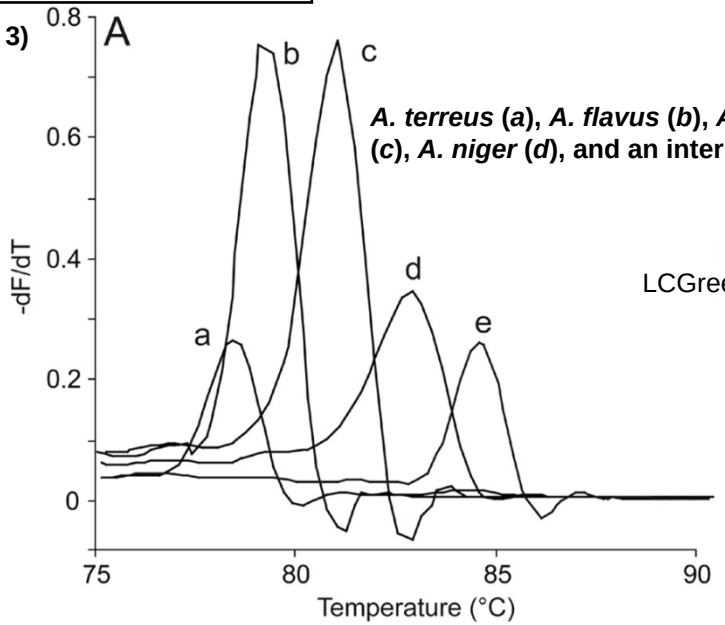

2)




LCGreen

# MELTING CURVE ANALYSIS

3)




***A. terreus* (*a*), *A. flavus* (*b*), *A. fumigatus* (*c*), *A. niger* (*d*), and an internal control (*e*).**

LCGreen


1) **Diagnosis of Herpes Simplex Virus Infections in the Clinical Laboratory by LightCycler PCR**
Mark J. Espy
Clin Microbiol. 2000 February; 38(2): 795–799

2) **Rapid detection and identification of clinically important bacteria by high-resolution melting analysis after broad-range ribosomal RNA real-time PCR.**
Cheng JC, et al.
Clin. Chem. 2006 Nov;52(11):1997-2004.

3) **Multiplex Single-Color PCR with Amplicon Melting Analysis for Identification of *Aspergillus* Species**
Maria Erali, et al.
*Clinical Chemistry* 52, No. 7, 2006

# Molecular microbiology instrument platforms

**QUIAGEN**

**Rotor-Gene Q**
Corbett Rotor-Gene

**QIAsymphony**

**NucliSENS easyMAG** **bioMérieux**

**7500 Real-Time PCR System**

**CFX96**

**Roche**

**LightCycler**
**Roche**

**LightCycler 480**

**BioRad**

**MyiQ5**

**TaqMan 48**

**Applied Biosystems**

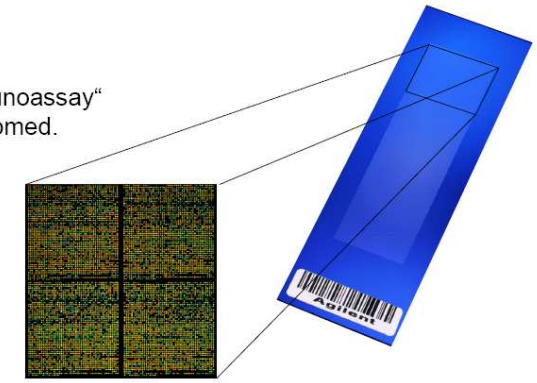
**7300 Real-Time PCR System**

# Hybridization Array Technology

As PCR has done in the last 25 years, and more recently real-time PCR, microarray technology will undoubtedly transform the diagnostic capabilities of clinical laboratories, ushering us into a new molecular revolution

Un microarray è un insieme ordinato e miniaturizzato di una moltitudine di reagenti immobilizzati su una superficie solida

First publication:
Roger Ekins et. al.,
„Multi-analyte immunoassay“
1989, J. Pharm. Biomed.
Anal. 7: 155 - 168



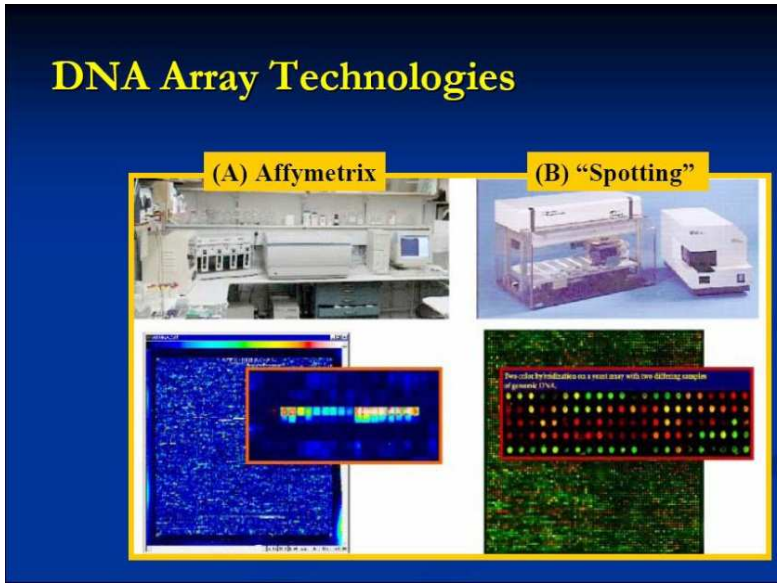
“A microarray is a collection of microscopic features (most commonly DNA) which can be probed with target molecules to produce either quantitative (gene expression) or qualitative (diagnostic) data”

**Mark Schena et al. 1995**
**Quantitative monitoring of gene expression patterns with a complementary DNA microarray**
**Science 270:467-470**

# Microarray

**A high throughput technology that allows detection of thousands of genes simultaneously**

- Principle: base-pairing hybridization
- Much rely on computer aids
- Central platform for functional genomics




**Features**

- Parallelism
  - Thousands of genes simultaneously
- Miniaturization
  - Small chip size
- Multiplexing
  - Multiple samples at the same time
- Automation
  - Chip manufacturing
  - Reagents

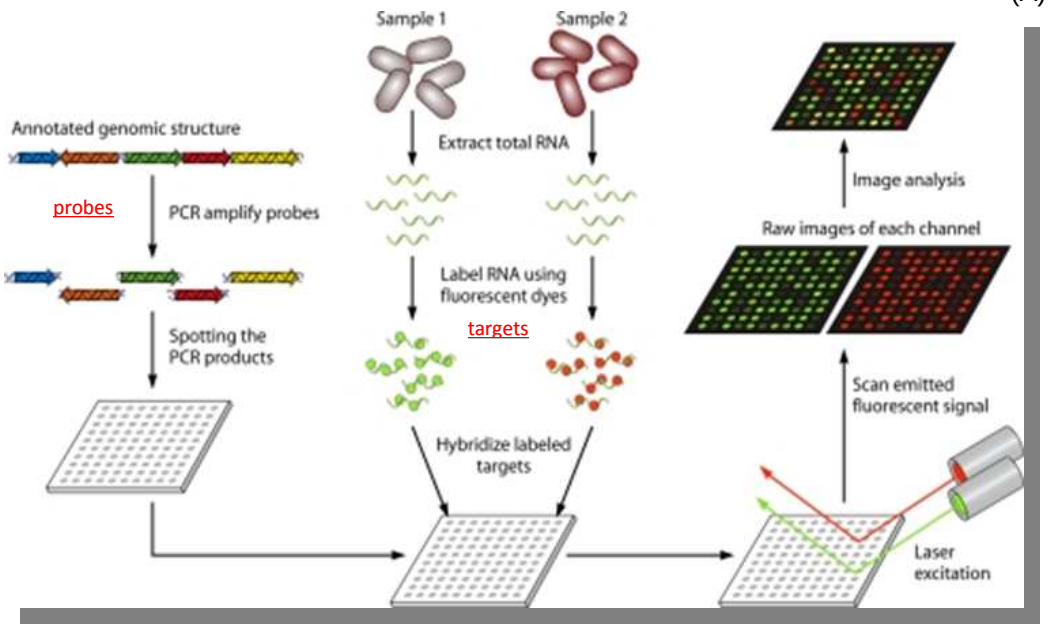
→ **Enormous analytical power**

# Microarrays characteristics

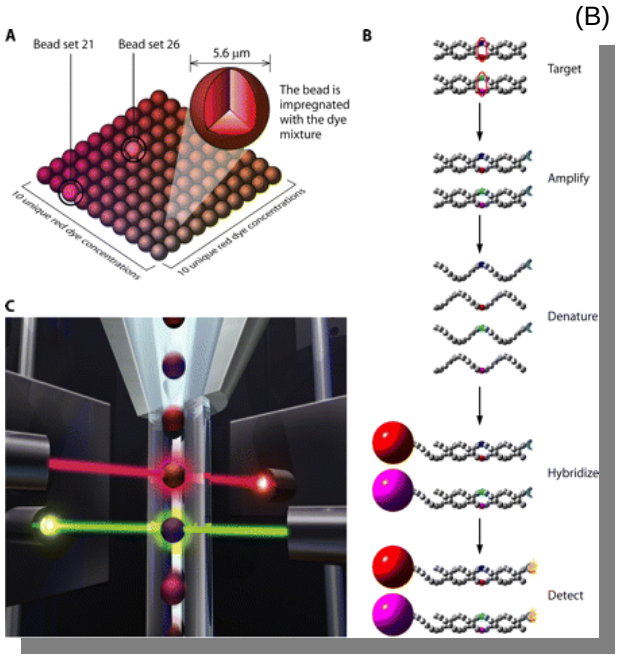
Microarrays can be distinguished based upon characteristics such as the nature of the probe, the solid-surface support used, and the specific method used for probe addressing and/or target detection

- Printed microarrays (A)
- In Situ-Synthesized Oligonucleotide Microarrays (Affymetrix )
- High-Density Bead Arrays (Illumina)
- Electronic Microarrays (Nanogen's RVA)
- Suspension Bead Arrays (Suspension bead array) (B)

(A)




(B)



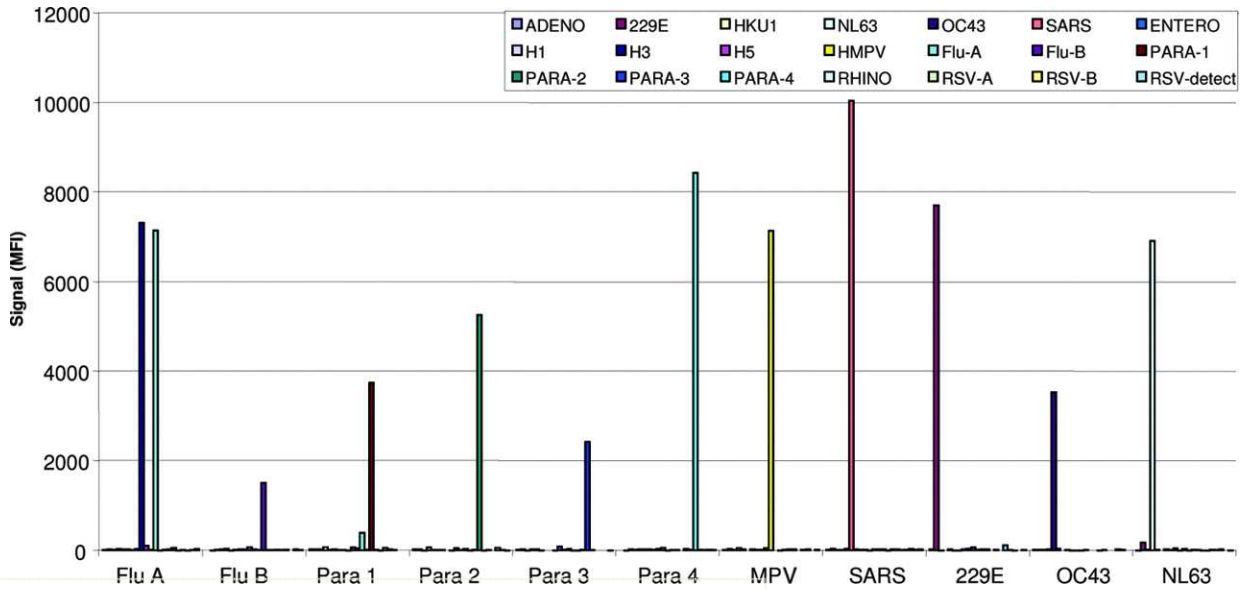
# Microarrays in Microbiology

| Array platform technology | Application |
|---|---|
| Microbial gene expression profiling | Drug discovery and drug development<br>Pathogenesis studies and microbial physiology<br>Vaccine development<br>Drug resistance detection |
| Host gene expression profiling during microbial infections | Selection of infection<br>Differentiation of infectious etiologies<br>Development of anti-inflammatory drugs |
| Applications in diagnostic microbiology | Microbial detection , identification, and typing<br>• sequencing (e.g. SARS-CoV, April 2003)<br>• broad-range PCR : bacterial, fungal, parasitic and viral pathogens<br>• multiplex PCR for enteropathogenic bacteria, for bacterial and viral pathogens – meningitis/ encephalitis, for respiratory viral pathogens, for sexually transmitted pathogens<br>• multiplex PCR for HPV testing<br>• broad-range PCR : to profile the development of infant intestinal microbiota<br>Antimicrobial resistance detection<br>• *S. aureus*, *M. tuberculosis*, HIV-1, *P. falciparum*<br>Detection of host polymorphisms associated with drug metabolism or differential immune response: e.g. IL28B polymorphisms on chromosome 19 |


Melissa B. et al.
Basic Concepts of Microarrays and Potential Applications in Clinical Microbiology.
Clinical Microbiology reviews,
Oct. 2009, p. 611–633 Vol. 22, No. 4

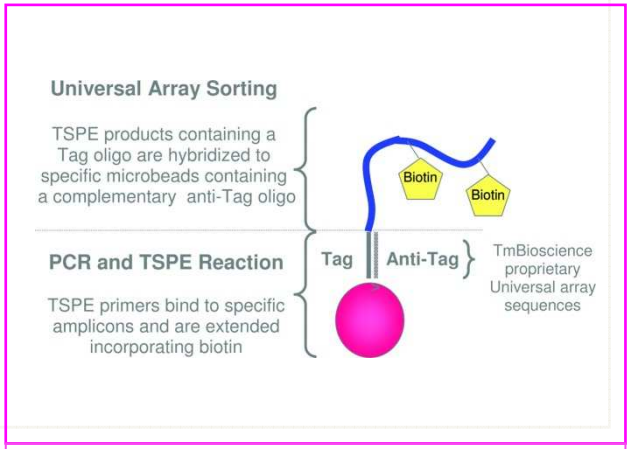
Melissa B.
Solid- and Liquid-Phase Array Technologies
*Molecular Microbiology: Diagnostic principles and Pratice*, 2nd Ed. 2011

**Detection and identification of TSPE reaction products captured onto microsphere beads containing anti-tag oligonucleotides (oligo) that hybridize to TSPE products containing a complementary tag oligonucleotide.**




Mahony J et al.
Development of a Respiratory Virus Panel Test for Detection of Twenty Human Respiratory Viruses by Use of Multiplex PCR and a Fluid Microbead-Based Assay

J. Clin. Microbiol. 2007;45:2965-2970



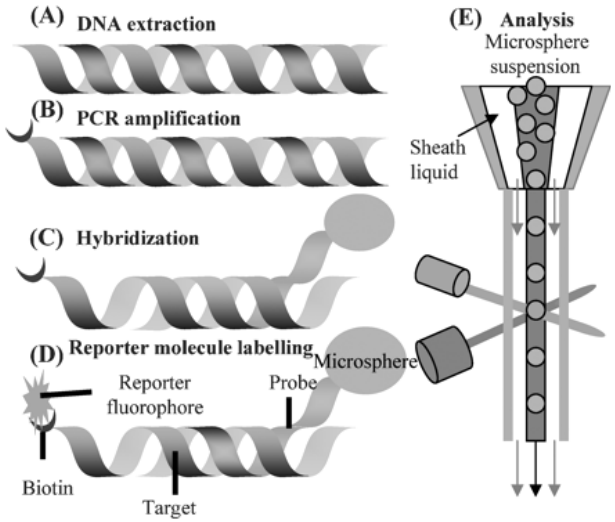

TSPE: a multiplex target-specific primer extension reaction

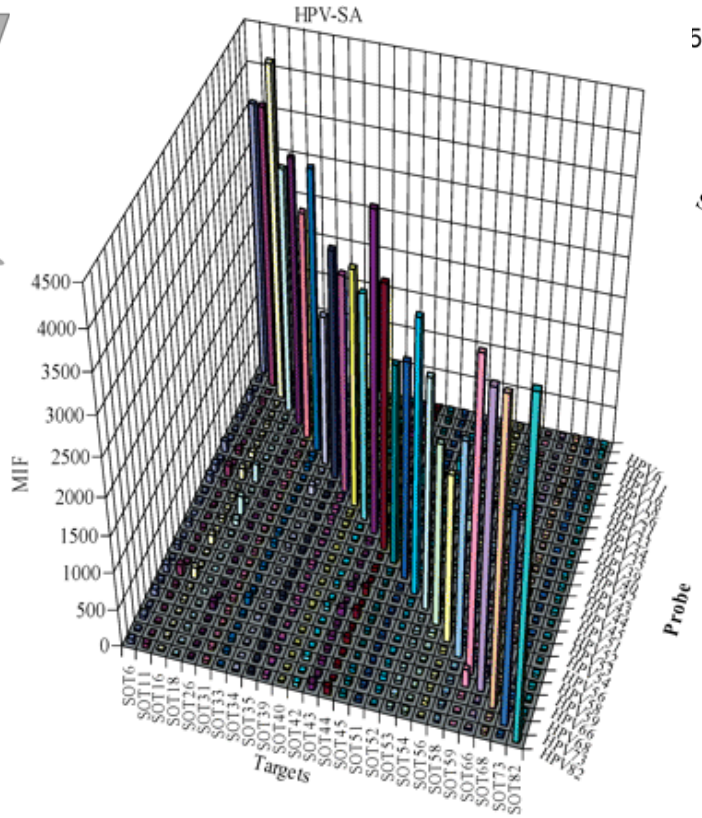

**Luminex x-MAP system**

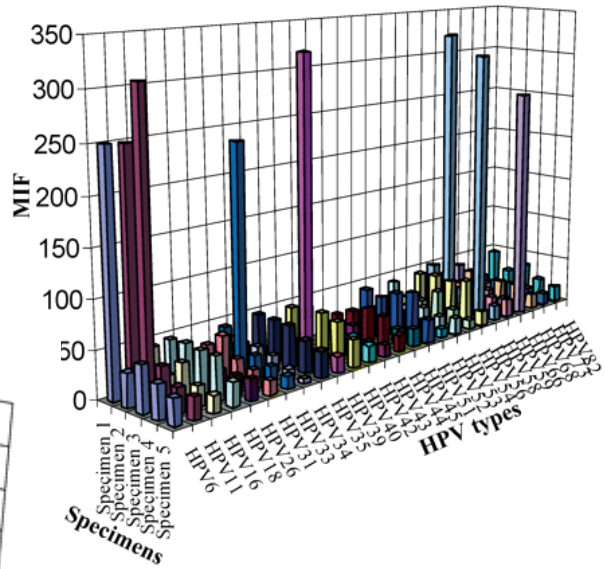
Jiang HL, Zhu HH, Zhou LF, Chen F, Chen Z

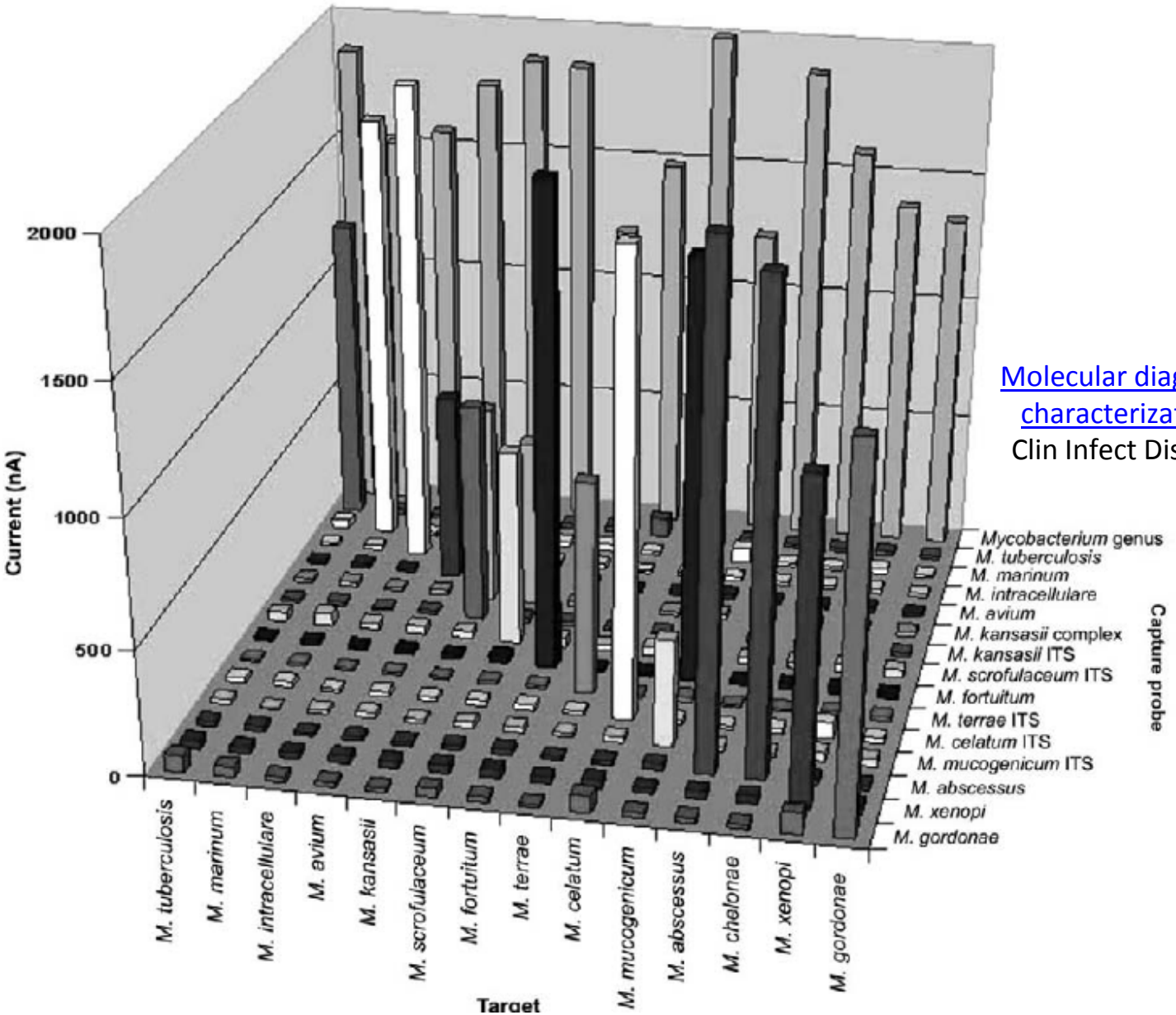
**Genotyping of human papillomavirus in cervical lesions by L1 consensus PCR and the Luminex xMAP system.**

J Med Microbiol. 2006 Jun;55(Pt 6):715-20.



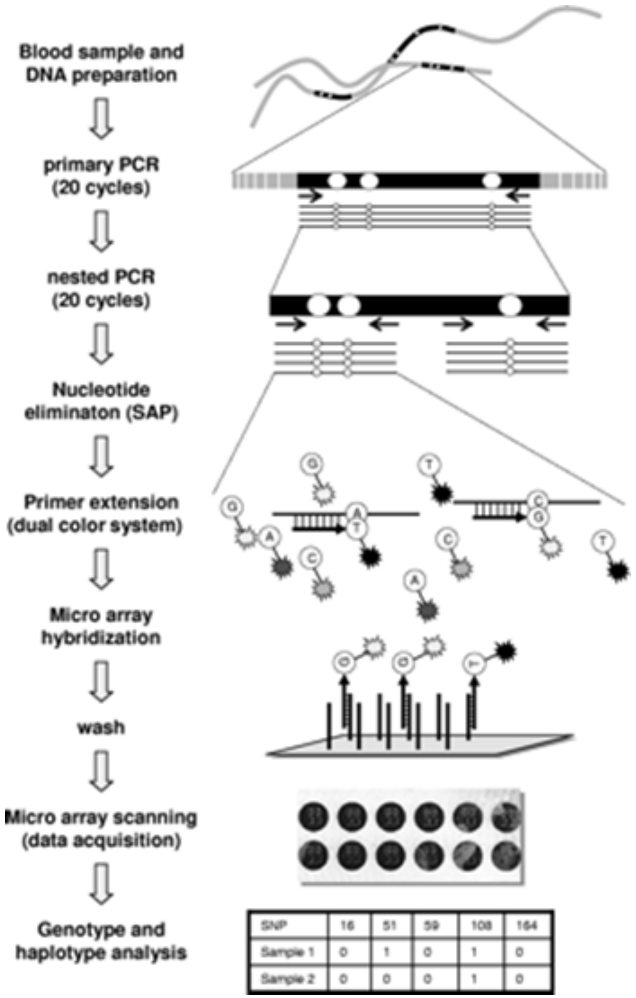






Procop GW.
[Molecular diagnostics for the detection and characterization of microbial pathogens.](#)
Clin Infect Dis. 2007 Sep 1;45 Suppl 2:S99-S111.

**Figure 5.** Limited or small-scale bioelectric microarray demonstrating the feasibility of this type of technology to differentiate most of the clinically important mycobacteria. A *Mycobacterium* genus site is located on the far side of the microarray, whereas the remainder is occupied by species-specific or complex-specific (e.g., *Mycobacterium tuberculosis* complex) hybridization sites. ITS, internal transcribed spacer region.



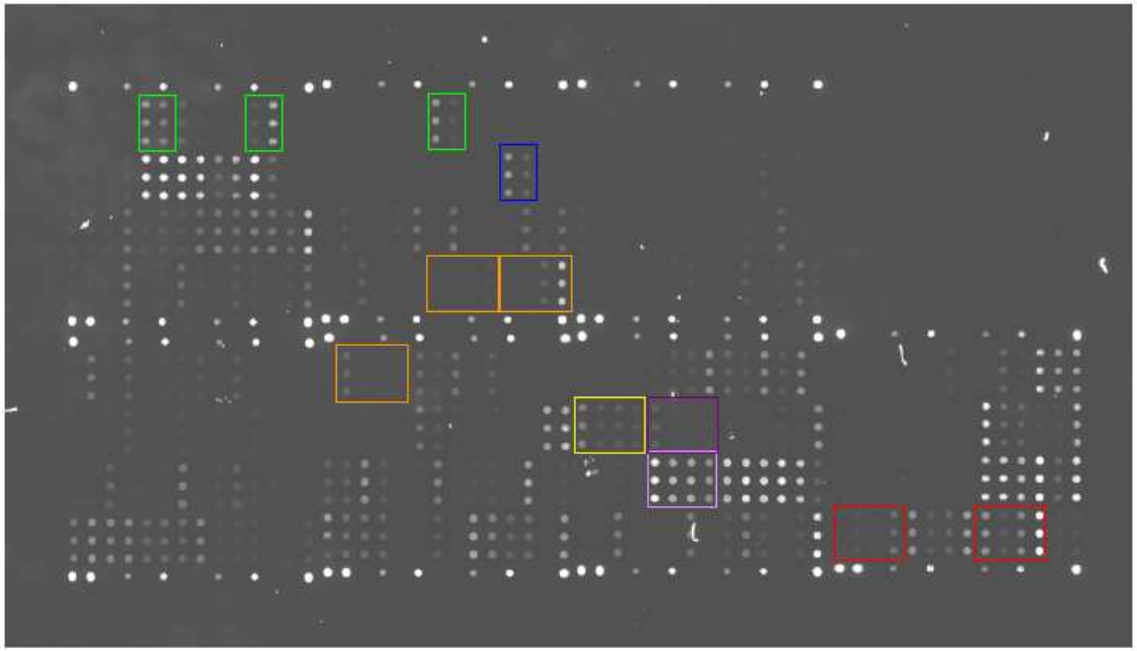

# A rapid and affordable microarray technique for application in epidemiological studies of malaria drug resistance

Molecular markers (29 SNP sites) of *P. falciparum* resistance are available for only a few drugs (chloroquine, pyrimethamine, cycloguanil, sulfadoxine, atovaquone), while for others they are not yet determined

**Rapid microarray-based method for monitoring of all currently known single-nucleotide polymorphisms associated with parasite resistance to antimalaria drugs.**

Crameri A, Marfurt J, Mugittu K, Maire N, Regös A, Coppee JY, Sismeiro O, Burki R, Huber E, Laubscher D, Puijalon O, Genton B, Felger I, Beck HP. J Clin Microbiol. 2007 Nov;45(11):3685-91.

DNA microarray hybridization pattern of a multidrug-resistant *P. aeruginosa* clinical isolate



Weile J, Knabbe C.
Current applications and future trends of molecular diagnostics in clinical bacteriology.
Anal Bioanal Chem. 2009 Jun;394(3):731-42.

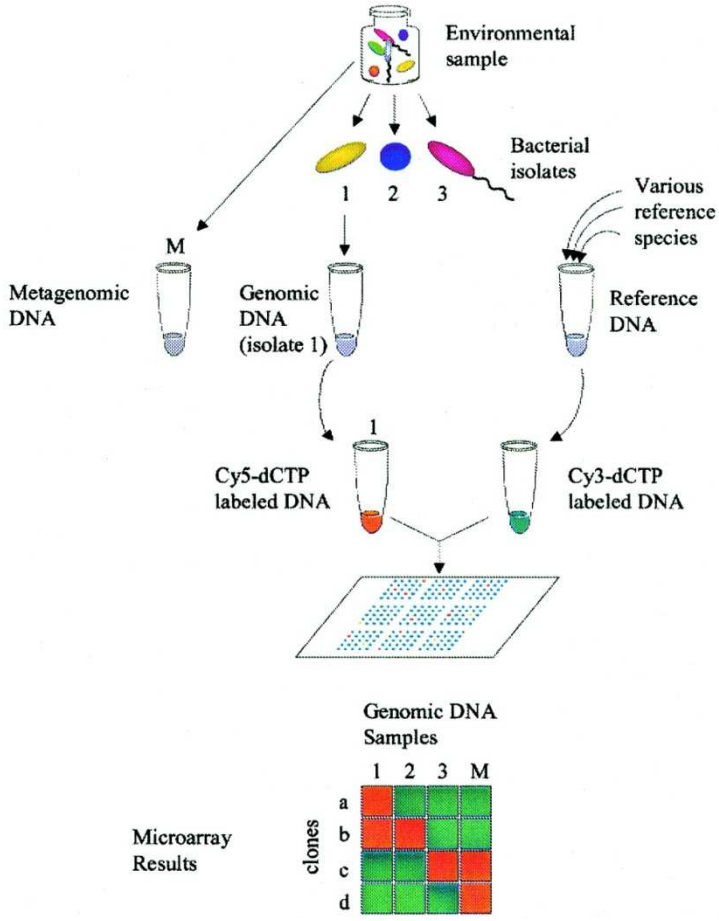
## Detection of antimicrobial resistance

- Mechanisms of resistance are varied and, for any given antimicrobial, can be due to one or multiple genetic polymorphism in one or multiple genes
- Molecular genetic basis for many types of antimicrobial resistance is not currently known
- Resistance patterns and mechanisms are constantly evolving

| Antibiotic | Phenotype |
|---|---|
| Levofloxacin (LEV) | R |
| Ciprofloxacin (CIP) | R |
| Gentamicin (GM) | R |
| Tobramycin (TO) | R |
| Amikacin (AK) | R |
| Aztreonam (AZT) | R |
| Piperacillin /Tazobactam (PIP/TAZ) | R |
| Ceftazidim (CAZ) | R |
| Cefepim (FEP) | R |
| Meropenem (MER) | R |
| Imipenem (IMP) | R |
| Colistin (COL) | S |
| Fosfomycin (FOS) | S |

| Array detected genes/mutations | Function/Description | Expected antibiotic resistance due to genotype |
|---|---|---|
| SNP in gyrA (248 C-> T) | ***gyrA*** gene, gyrase, involved in transcription/replication, target for fluoroquinolones | LEV ,CIP |
| SNP in parC (260 C->T) | ***parC*** gene; topoisomerase; involved in transcription / replication, target for fluoroquinolones | LEV ,CIP |
| aadA1, aac(6')Ib, aph(3') | Aminoglycoside modifying enzymes; aac (=acetylation); aad (adenylation); aph (=phosphorylation) | GM, TO, AK |
| SNP in mexR (327 G->A; 377 T->A; 384 G->T) | ***mexR*** gene, regualtor of multidrug efflux transporter MexAB-OprM | LEV, CIP, PIP, CAZ, FEP, AZT |
| SNP in nalC (212 C->T) | ***nalC*** gene, regualtor of multidrug efflux transporter MexAB-OprM | LEV, CIP, PIP/TAZ, CAZ, FEP, AZT |
| vim-1 | ***vim*** gene; metallo-beta-lactamase; plasmid encoded | PIP/TAZ, CAZ, FEP, IMP, MER |
| ampD (443 C->G), ampR (341 T->G) | ***ampD*** and ***ampR*** genes; regulators of chromosomal AmpC beta-lactamase | PIP/TAZ, CAZ, FEP, AZT |

Advances in various techniques (PCR quantitative, microarrays, next-generation sequencing, bioinformatic…) have allowed the creation of a new area of research ( "metagenomics"), allowing an exhaustive study of microbial communities, including the non-cultivable members




**Metagenomic Profiling: Microarray Analysis of an Environmental Genomic Library**

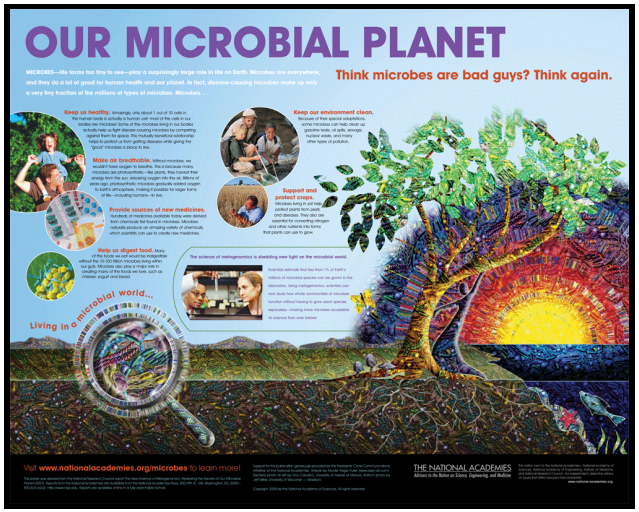
Sebat J L et al. Appl. Environ. Microbiol. 2003;69:4927-4934

**"Next-Generation Sequencing (NGS)"**

**Applied Biosystems (ABI) 3730 DNA Analyzer**

**The HiSeq 2000 Illumina**

**Roche 454 FLX pyrosequencing platforms**



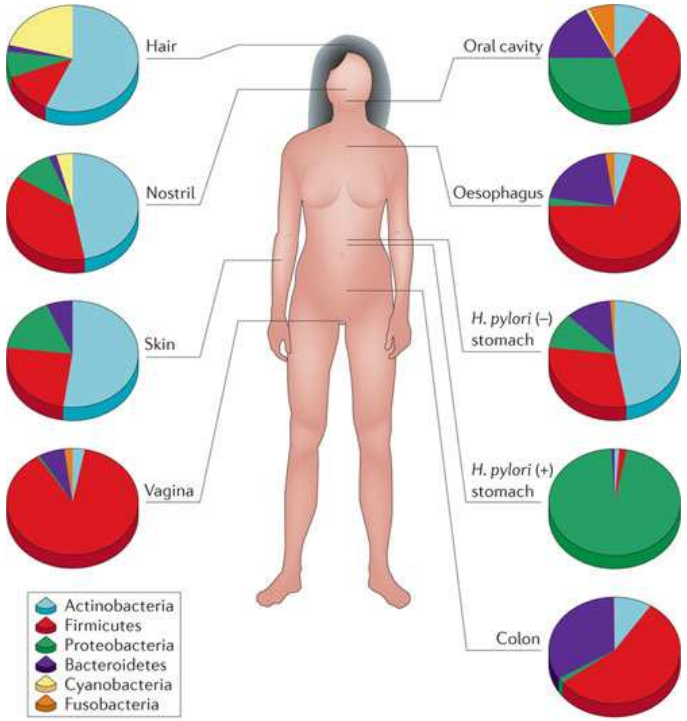

**Metagenomics** is the study of **metagenomes**, genetic material recovered directly from environmental samples

The metagenomic approach enables analysis of genetic material derived from complete microbial communities in their own natural environment

## The human Microbiome Project

A strategy to understand the microbial components of the human genetic and metabolic landscape and how they contribute to normal physiology and predisposition to disease

**The human Microbiome Project**
Turnbaugh, P.J. ***et al.***
***Nature* 449**, 804–810 (18 Oct 2007)

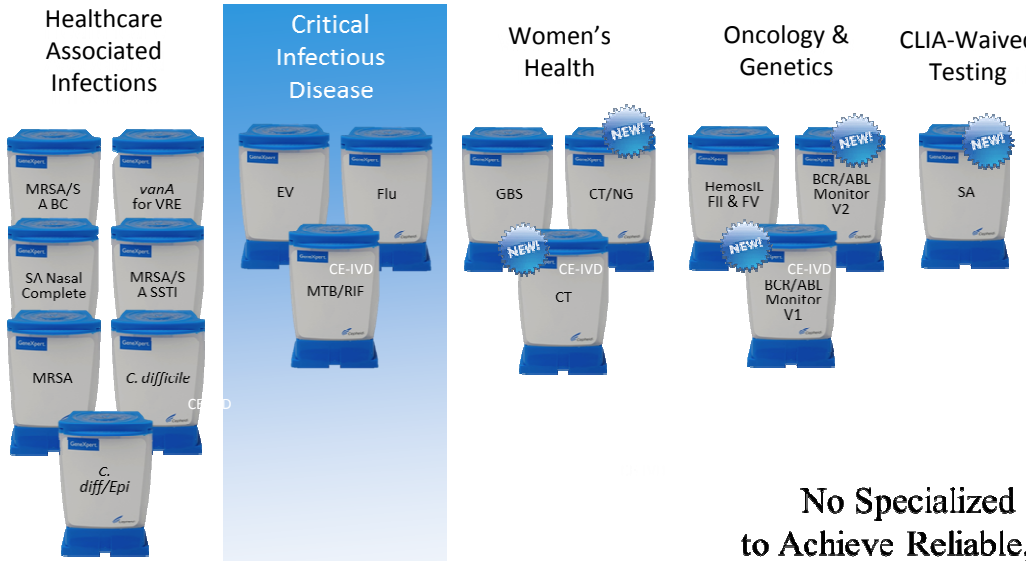



The human microbiome: at the interface of health and disease
Ilseung Cho & Martin J. Blaser
***Nature Reviews Genetics* 13, 260-270 (April 2012)**

Nature Reviews | Genetics

# Integrated fluidic lab-on-a-chip devices for molecular diagnostics

## WW Xpert Test Portfolio: End 2012




LAB on a Chip Platforms

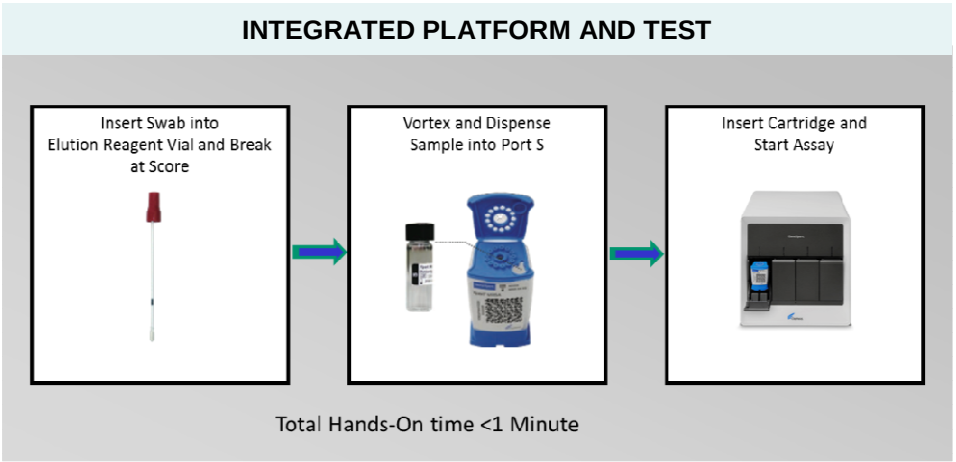
No Specialized Training Required
to Achieve Reliable, Reproducible Results

GeneXpert® System

A closed, self-contained, fully-integrated and automated platform that represents a paradigm shift in the automation of molecular analysis, producing accurate results in a timely manner with minimal risk of contamination

Is the only system to combine on-board sample preparation with real-time PCR amplification and detection functions for fully integrated and automated nucleic acid analysis

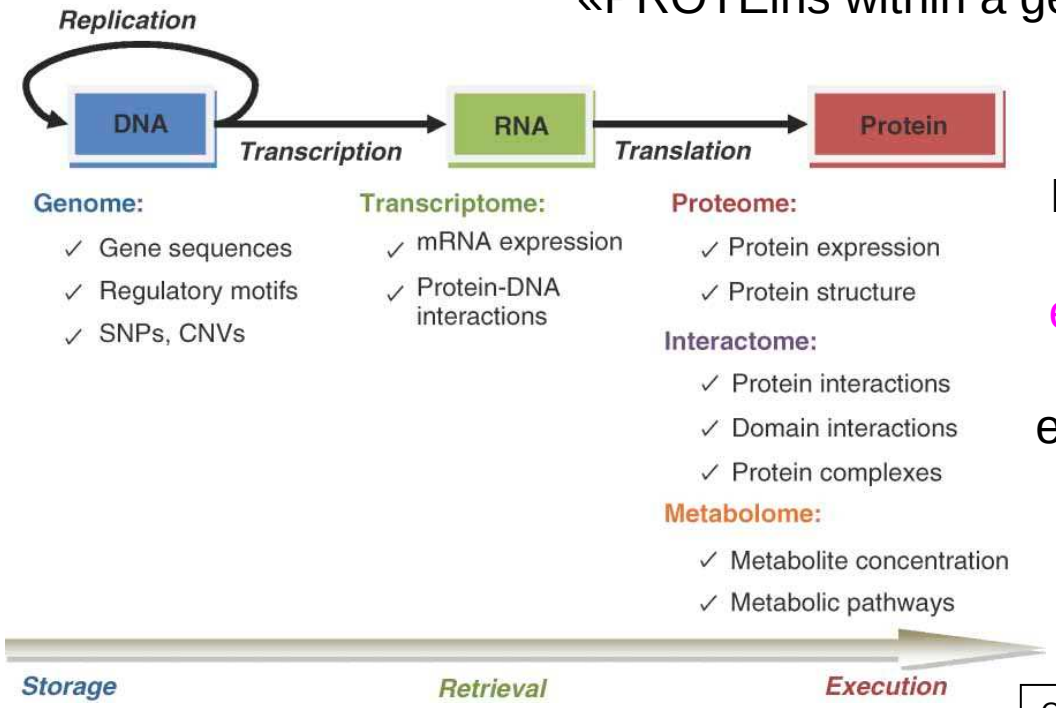
Modular in design, the GeneXpert System has a variety of configurations to meet the broad range of testing demands of any clinical environment



# Proteomics meets microbiology

The ever increasing number of completed sequences for important human pathogens will lead to a similar rise in demand for new methods to facilitate identification and functional analysis of the gene products

PROTEOMA
«PROTEins within a genOME»




Proteomics can be defined as the study of the full set of proteins expressed by an organism, tissue or cell, and the change in their expression patterns under different conditions

Carolyn I. Phillips et al. **Proteomics meets microbiology: technical advances in the global mapping of protein expression and function** Cellular Microbiology (2005)**7**(8),1061

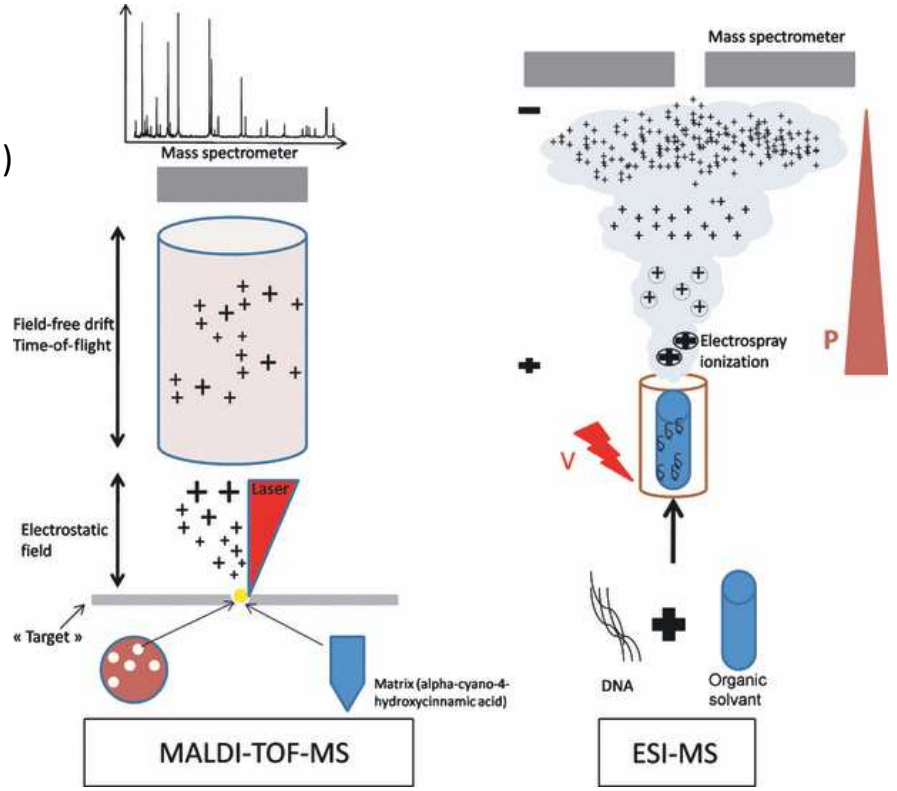
**MASS SPECTOMETRY**

# Proteomic methods

- High resolution two-dimensional electropresis (D-GE)
- High performance liquid chromatografy (HPLC)
- Mass spectrometry (MS)
- Protein microarray

The development of automated, high-throughput proteomic technologies such as MALDI-TOF MS has enabled large numbers of samples to be analyzed simultaneously in a short time

**J. B. Fenn and K.Tanaka**
Nobel Prizes in Chemistry 2002 *"for their development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules"*




Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)
Electrospray ionization mass spectrometry (ESI-MS)

S. Emonet, et al. Application and use of various mass spectrometry methods in clinical microbiology . Clin Microbiol Infect 2010; 16: 1604–1613

**The first description of the use of MS for bacterial identification**

Anhalt JP, Fenselau C. **Identification of bacteria using mass-spectrometry**. Anal Chem 1975; 47: 219–225.

VanBogelen RA, Abshire KZ, Moldover B, Olson ER, Neidhardt FC. **Escherichia coli proteome analysis using the gene-protein database.** Electrophoresis. 1997 Aug;18(8):1243-51.

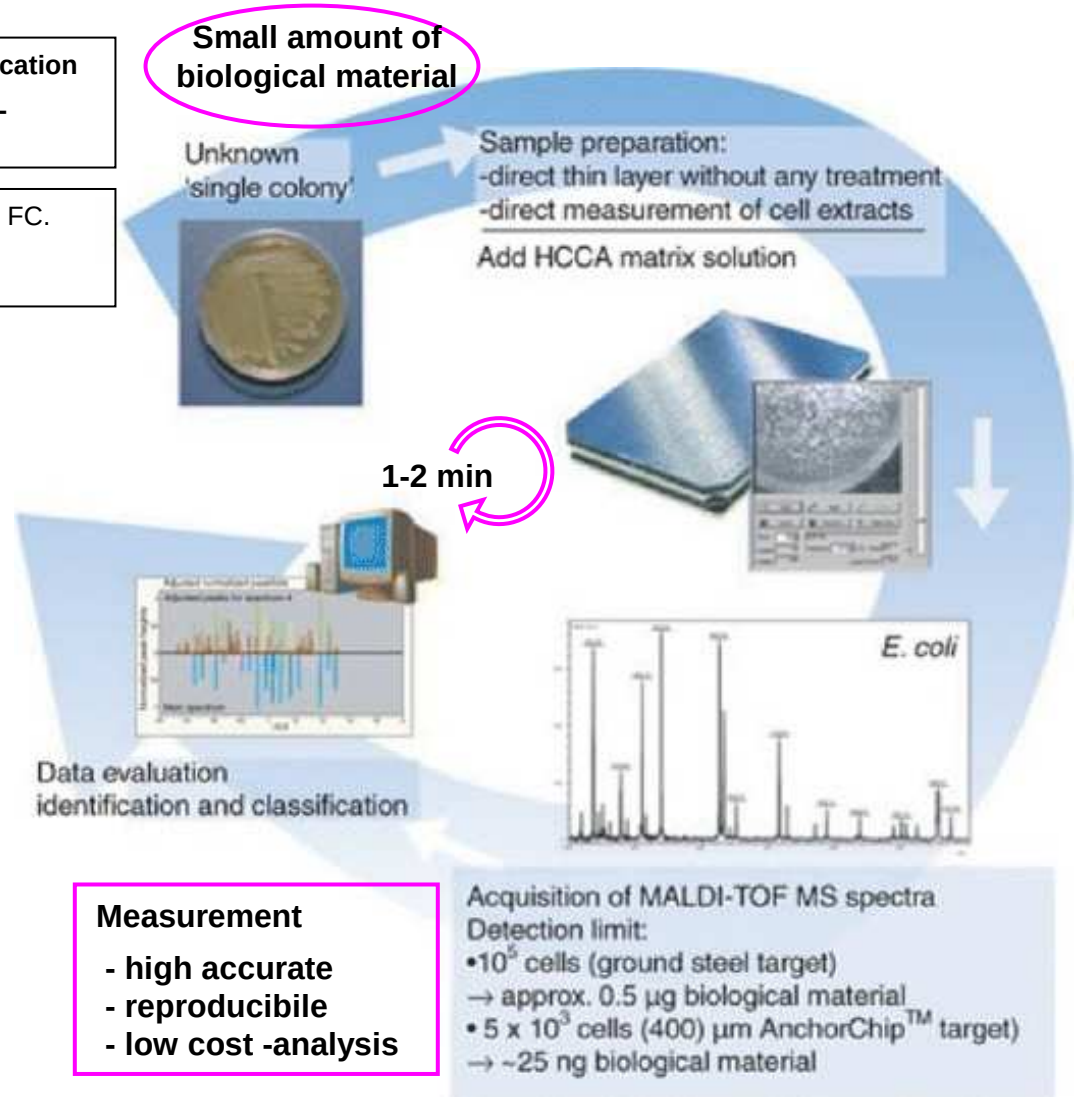
## MALDI-TOF MS

- Automated and rapid molecular identification of microorganisms
    - Enterobacteriaceae
    - Non-fermenting bacteria
    - Staphylococci
    - Enterococci
    - β-haemolytic streptococci
    - Anaerobes
    - Yeast
    - Mycobacteria

-Virulence/resistance factors

## Challenges

- Sample type , quality, specific storage
- Hardware/software/database




Maier, T. , Klepel, S. , Renner, U. , & Kostrzewa, M. . (2006). Fast and reliable MALDI-TOF MS-based microorganism identification. *Nature Methods Application Notes*, *25*(2), 68-71

# Proteomic application in Microbiology

- detection of infectious agents , characterization and their host interaction involved in infection and pathogenesis
  - direct identification on clinical specimens (§)
  - diseases diagnosis of emerging infectious diseases
  - detection of specific virulence or resistance protein markers
  - identification of new potential drug and vaccine target
- epidemiology and taxonomy of human microbial pathogens
- bioterrorism defence
- biomarkers for the diagnosis of diseases and for the monitoring of their progress
- characterisation of the proteomes of bacterial pathogens growing in their natural hosts remains a future challenge

(§) QCMD Quality Control for Molecular Diagnostics **MALDIBAC12 Maldi-TOF Bacterial EQA**

# MALDI-TOF Bacterial

MALDIBAC12 Catalogue Number QAB124155

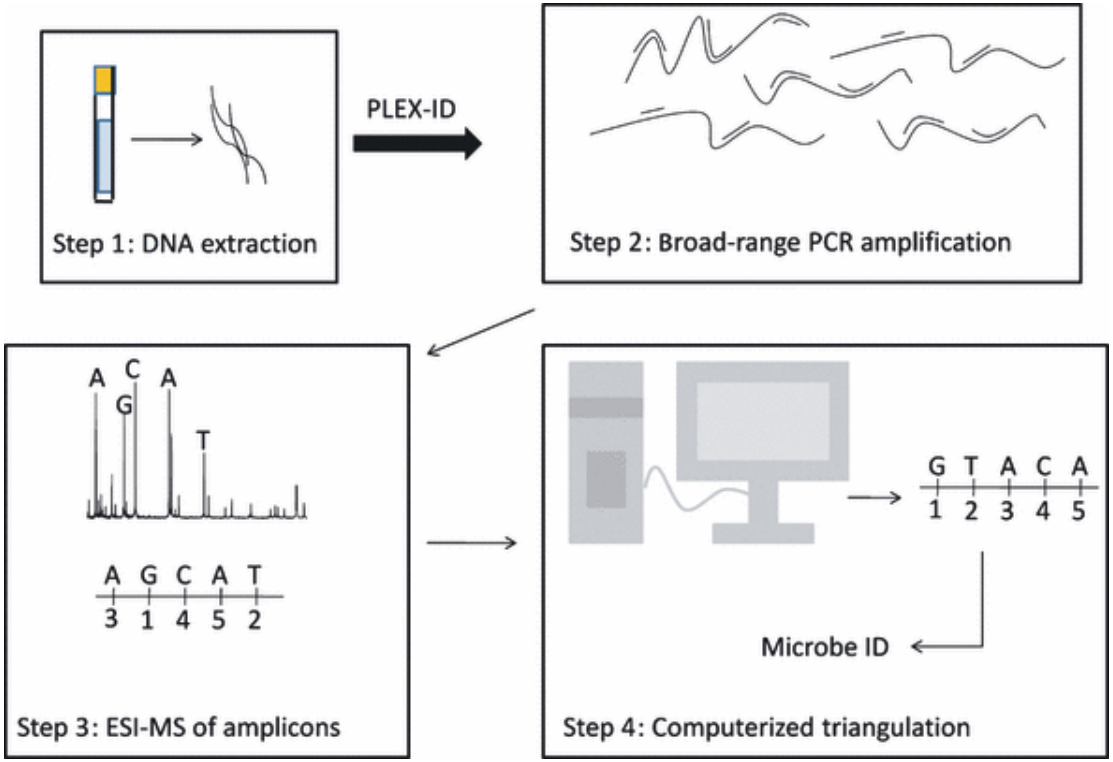
Matrix-Assisted Laser Desorption Ionisation – Time of Flight (MALDI-TOF) is becoming an important diagnostic tool in the microbiological laboratory for the routine identification of bacterial species based on protein and in some cases nucleic acid composition.

MALDI-TOF and similar technologies have been shown to be fast, reliable and cost-effective. The technology has potential to reduce the risk of misidentifying unusual organisms and is reportedly capable of correctly identifying the most common bacterial isolates at the species level in 84.1 to 93.6% instances . MALDI-TOF therefore has the potential to complement or possibly replace conventional bacterial phenotypic identification methods.

MALDI-TOF does still have some current limitations and these include the identification of some microbial species including; Shigella, pneumococci, and streptococci. These current limitations are often due to the lack of suitable reference strains, standards and in some cases clinical isolates. This means that it can be difficult to obtain sufficient quality data with which to define appropriate reference spectra to update the reference databases.

The primary goal of this EQA pilot study is to evaluate the ability of laboratories in the detection and determination of different clinically relevant bacterial strains using MALDI-TOF and other similar mass spectrometry based technologies in the routine microbiology laboratory.

| Feature | Specifications |
|---|---|
| Number of Panel Members | 8 to 12 |
| Sample NA Target Source | Clinical material |
| Matrix panel format | Physiological |
| Panel Member Target Range | Clinically relevant range of bacteria for detection & determination |
| Panel Analysis type | Qualitative |
| Storage / Shipment Conditions | <-20°C / Dry-ice |




## PCR/ESI-MS

**The PLEX-ID technology**.

- Step 1: extraction of microbial DNA from the clinical sample. All of the following steps take place in the PLEX-ID instrument and are fully automated
- Step 2: PCR amplification with primers targeting ribosomal and housekeeping protein genes
- Step 3: determination of the base composition of the amplicons (no linking order) by electrospray ionization mass spectrometry (ESI-MS)
- Step 4: computerized triangulation of the base composition of several genomic regions is used to identify the microorganism.to the species level

New technology for rapid molecular diagnosis of bloodstream infections.
Ecker DJ et al.
Expert Rev Mol Diagn. 2010 May;10(4):399-415.

### PLEX-ID BAC Spectrum Assay

Status: in clinical studies
Sample volume: 1,5 mL
Sample preparation: magnetic beads
Amplification: broad-range PCR
Detection: mass spectrometry
Bacteria: > 300 species, 3 antibiotic determinants *mecA*, *vanA* and *vanB*, $bla_{KPC}$; Candida speciation

An amplification reaction can be analyzed in the mass spectrometer in 30 s.


**PCR-ESI-MS (PLEX-ID, Abbott Molecular)**

# S.C. Microbiologia e Virologia

| Microrganismi | Hybridization probe | Real-time PCR sonde FRET | Real-time PCR sonde TaqMan | Multiplex PCR | PCR nested | Sequenza | LAB on Chip |
|---|---|---|---|---|---|---|---|
| *M. tuberculosis* complex; *M. intracellulare*, *M. avium*; M. avium complex; *M. gordonae* | X | | | | | | |
| *M. tuberculosis* /rpoB | | | | | | | X |
| *S. aureus* MRSA | | | | | | | X |
| *Clostridium difficile* | | | | | | | X |
| Sepsi: n.6 Gram+, n.8 Gram –, n.6 funghi | | X | | | | | |
| Meningiti batteriche (§) | | | X | | | | |
| HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, HHV-8; Enterovirus; Adenovirus; *parvovirus B19;* BKV, JCV | | | Xq | | | | |
| HHV-7; TOSV | | | | | X | | |
| Virus respiratori n.15 | | | | X | | | |
| *C. pneumoniae*, *M. pneumoniae*, *L. pneumophila* | | | X | | | | |
| *C. trachomatis* | | | X | | | | |
| HPV n. 37 genotipi ad alto e basso rischio | | | | X (Linear Array HPV genotyping) | | | |
| *L. interrogans* | | X | | | | | |
| *P. jiroveci* | | | | | x | | |
| *B. anthracis* | | X | | | | | |
| HIV, HCV, HBV | | | X | | | X | |

(§) *K. pneumoniae*, *E. coli*, *S. agalactiae*, *L. monocytogenes*, *N. meningitis*, *S. pneumoniae*, *H. influenzae*

La dotazione tecnologica di cui disponiamo deve essere inserita in una organizzazione del lavoro tale da garantire una qualità analitica di eccellenza in tempo utile per la cura del paziente

Solo così i nostri risultati diventano clinicamente significativi

Labor Berlin

Kompetenz von Charité und Vivantes

# Alerts via Smartphones and Tablet-Computers


Positive Blood Cultures

AST


Multidrug Resistant Organisms

Prof. Dr. med. Dr. rer. nat. Ulf B. Göbel

«Still, much progress remains to be made. To press the Star Trek analogy further, what the universe needs now is the **diagnostic equivalent** of the Tricorder : a device or approach that can ascertain a patient's condition comprehensively so that well-informed treatment and management decisions can be made in real time»

David H. Persing

MOLECULAR MICROBIOLOGY Diagnostic Principles and Pratice
second edition 2011 ASM Press

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