Evoluzione delle biotecnologie: dalle sonde geniche alla proteomica

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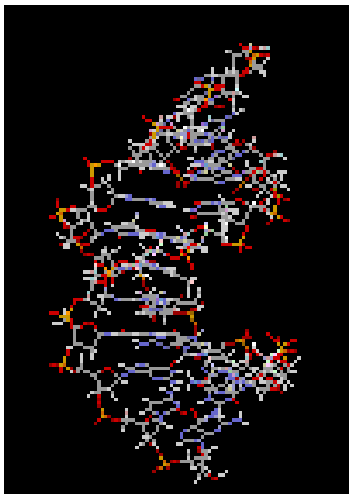
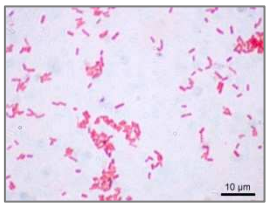
Nel 1953, la scoperta della struttura a doppia elica del DNA (James Watson e Francis Crick) ha segnato il passaggio alle biotecnologie innovative e la nascita dell' "*ingegneria genetica*"

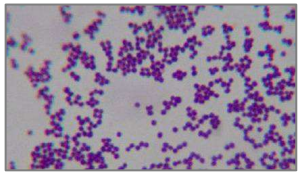
L'avvento della tecnologia del DNA ricombinante e l'evoluzione delle tecniche di sintesi chimica hanno permesso di realizzare **sonde genetiche** che, mediante reazioni di ibridazione degli acidi nucleici, sono state ampiamente impiegate in campo microbiologico **per la rilevazione ed identificazione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

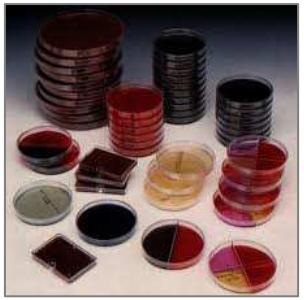


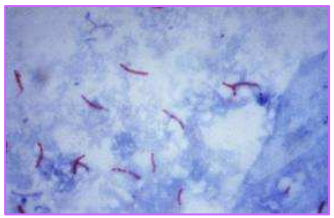
«Biotecnologia» : ogni applicazione tecnologica che si avvale di sistemi biologici, di organismi viventi o di loro derivati, per realizzare o modificare prodotti o procedimenti per un uso specifico. CONVENZIONE SULLA DIVERSITÀ BIOLOGICA Rio de Janeiro, 3-14 giugno 1992

Conventional methods in diagnostic microbiology





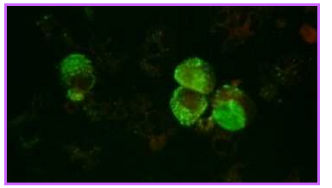


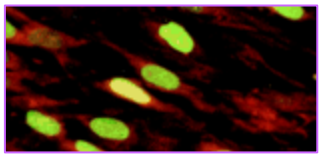


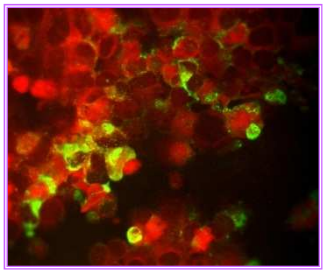
- Staining/microscope
- Culture
- Immunological methods

Problems

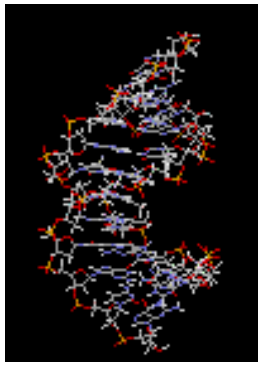
- Uncultivable/fastidious pathogens
- Some require cell culture lab./animal
- Discrimination between prognostic/ therapeutically important subgroups of pathogens





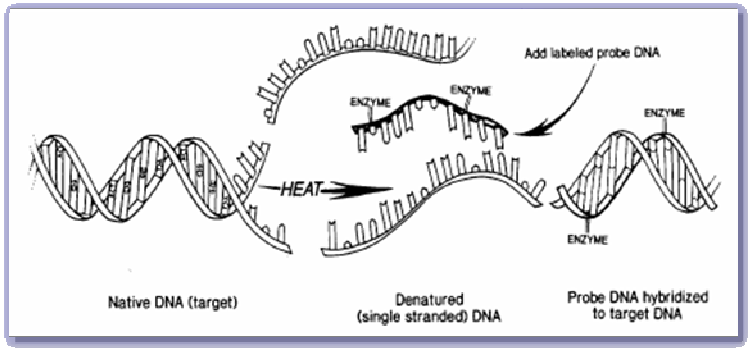


Nucleic Acid-based Techniques



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



Nucleic acid hybridization

Sensitivity and specificity

- Condition for hybridization (stringency)
- Selection of probe for detection
  - Genome sequences of the organisms should be known (gene or DNA fragment to detect)
- Sample preparation (nucleic acid extraction)

<u>Factors involved in hybridization reaction</u>

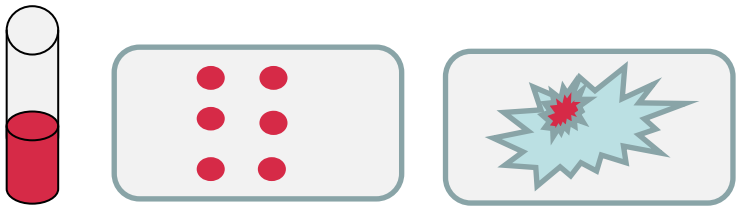
- Temperature
- Ionic strength
- Length of hybrid
- Percentage of complementary bases

$\Rightarrow\Rightarrow$ High/low stringent condition

Type of Nucleic Acid Hybridization

1. **Liquid phase hybridization** (detection)
2. **Solid phase(filter) hybridization** - format of choice for the simultaneous analysis of multiple samples . Target are bound to nylon or nitrocellulose and are hybridized with a probe in solution
    - dot blotting (detection)
    - Southern blot (detection, subtyping)
    - Northern blot (gene expression)

1. ***In situ* hybridization** (isotopica , cromogenica (CISH), fluorescente (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 **viral infections**
- Extent of infection
- Gene expression analysis
- Epidemiological studies/typing schemes to control and prevention of various infectious diseases

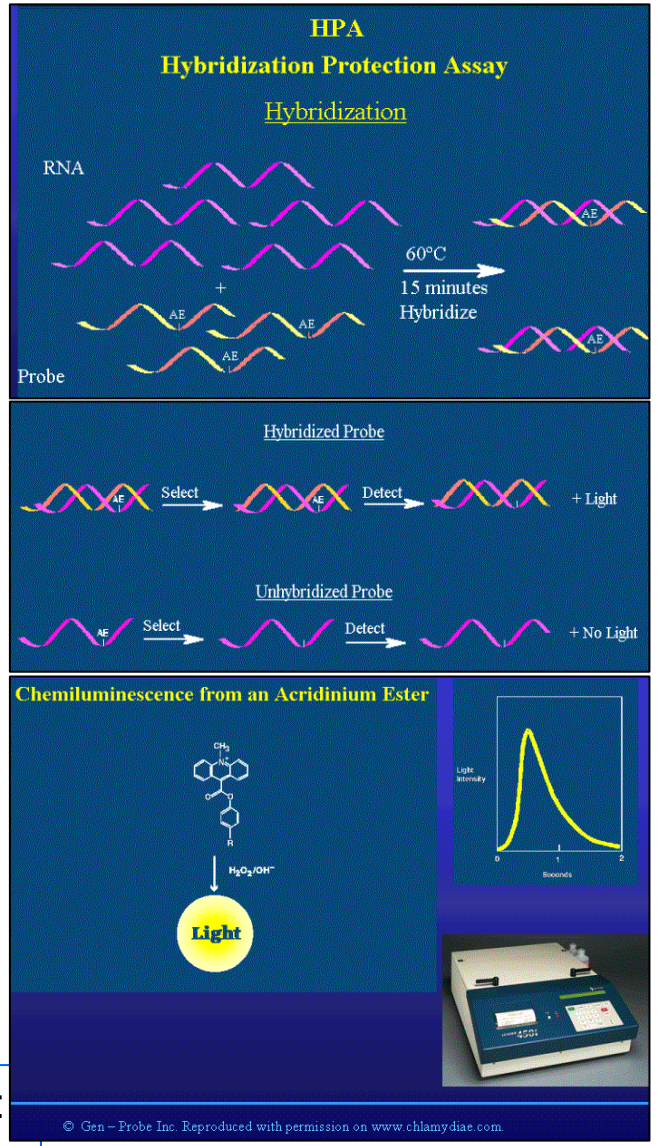
The first application of a DNA probe to detect bacteria

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.

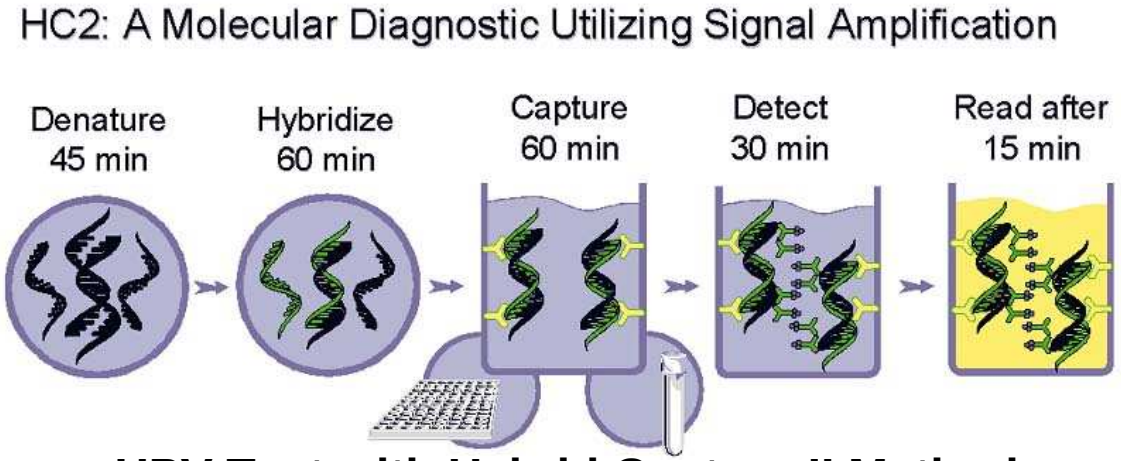
TABLE 2 Commercially available nucleic acid probes for detection of microbial pathogens[a]

Manufacturer	Primary application	Organism detected
Gen-Probe	Direct detection in clinical samples	*Chlamydia trachomatis* Group A streptococci *Mycobacterium tuberculosis* *Neisseria gonorrhoeae*
Gen-Probe	Culture confirmatory assays	*Blastomyces dermatitidis* *Campylobacter* spp. *Coccidioides immitis* Enterococci Group A streptococci Group B streptococci *Haemophilus influenzae* *Histoplasma capsulatum* *Listeria monocytogenes* *Mycobacterium avium* *Mycobacterium avium* complex *Mycobacterium gordonae* *Mycobacterium intracellulare* *Mycobacterium kansasii* *Mycobacterium tuberculosis* complex *Neisseria gonorrhoeae* *Staphylococcus aureus* *Streptococcus pneumoniae*
Digene	Direct detection in clinical samples	Human papillomavirus
Digene	Typing of isolates detected by the viral Pap test	Human papillomavirus

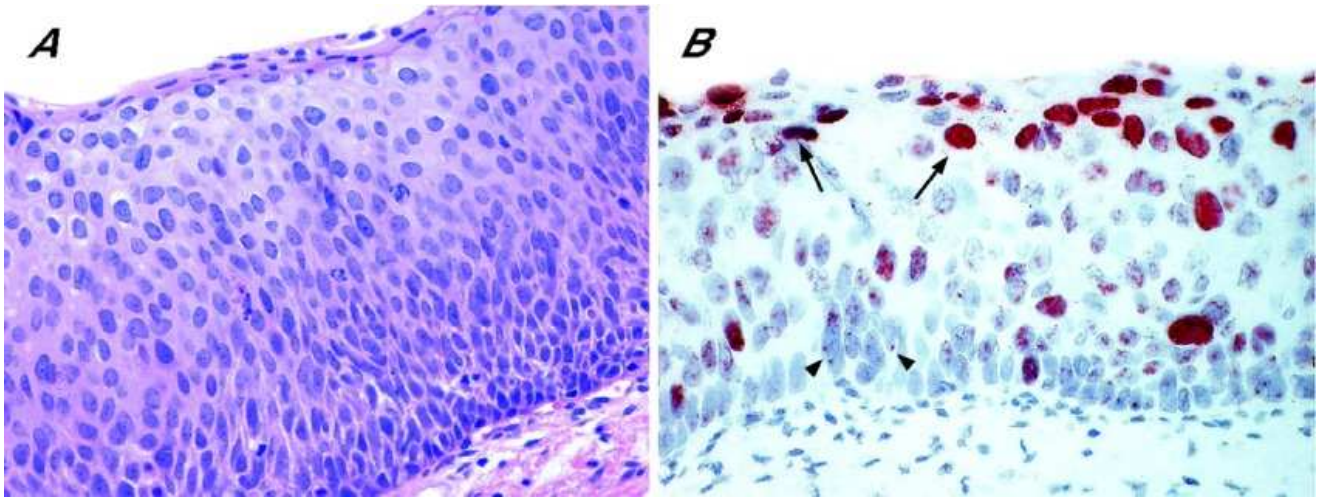
[a] All tests listed here have been cleared by FDA.



Liquid phase hybridization:
Chlamydia trachomatis

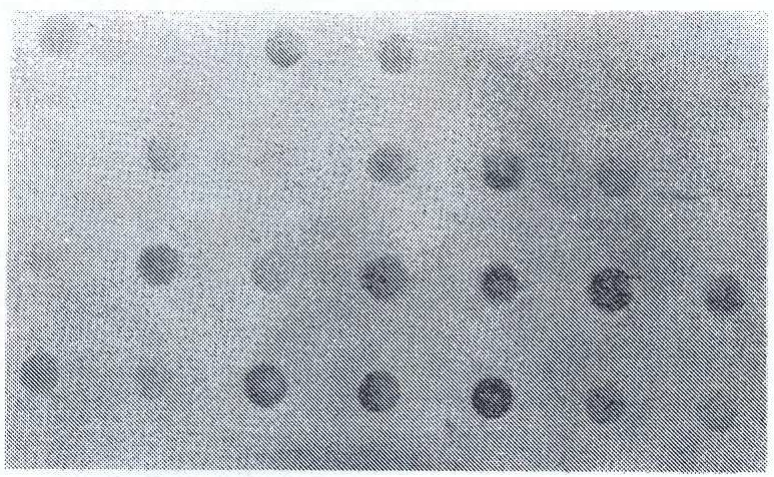


HPV Test with Hybrid Capture II Method



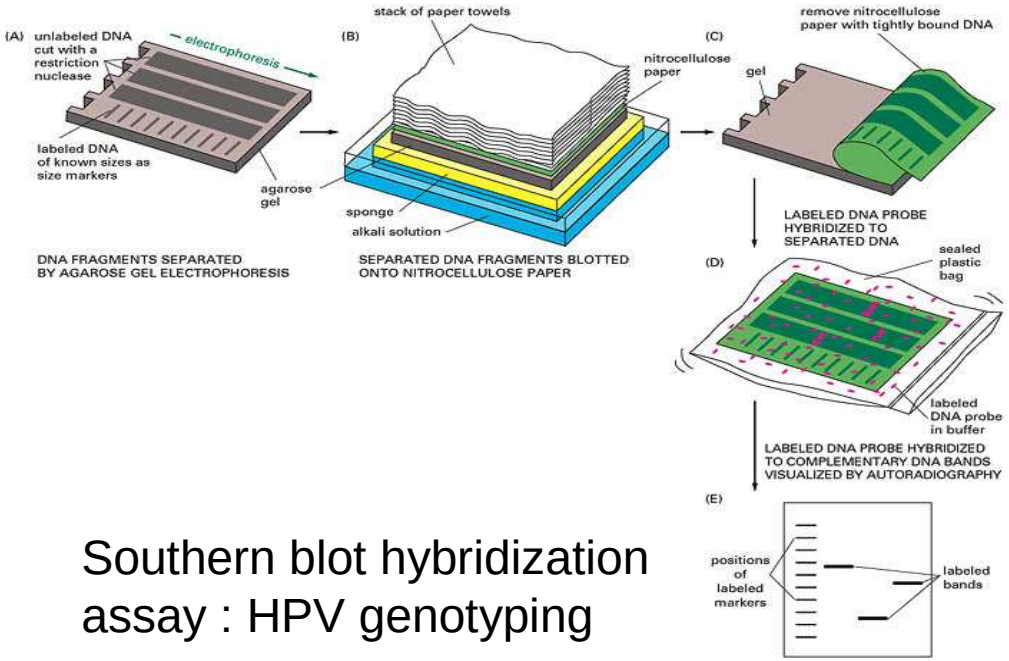
CISH : HPV

Our approach to squamous intraepithelial lesions of the uterine cervix.
Kalof AN, Cooper K.
J Clin Pathol. 2007 May;60(5):449-55. Epub 2006 Oct 17.



Dot blot hybridization assay of patient sera using biotin-labelled HBV DNA probe

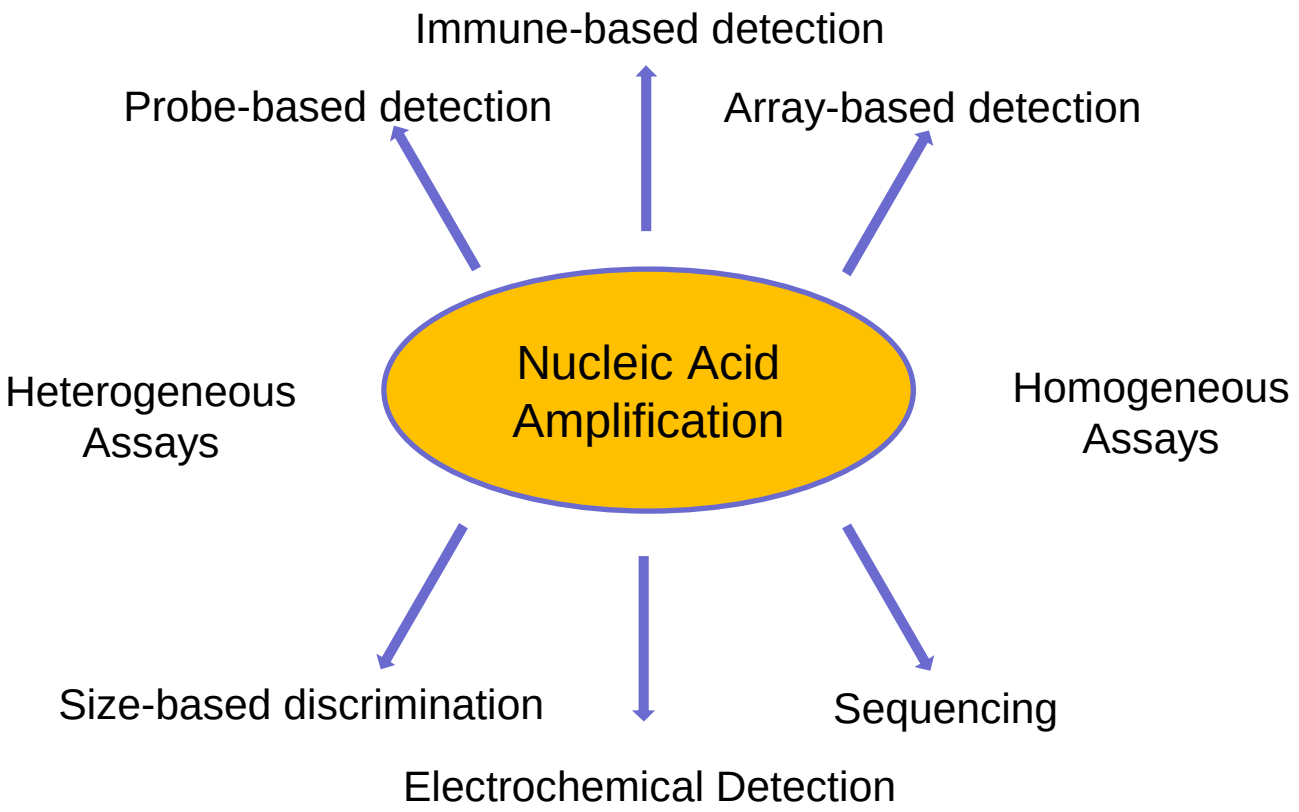
- Format of choice for the simultaneous analysis of multiple samples
- The length of time required and the complexity of the procedure limit its application in clinical pratice



Southern blot hybridization assay : HPV genotyping

Roe IH, Roe JH, Lee DH.
Detection of hepatitis B virus DNA in human serum by dot hybridization using a biotin-labelled probe.
Korean J Intern Med. 1988 Jan;3(1):9-14.

Nucleic acid amplification techniques



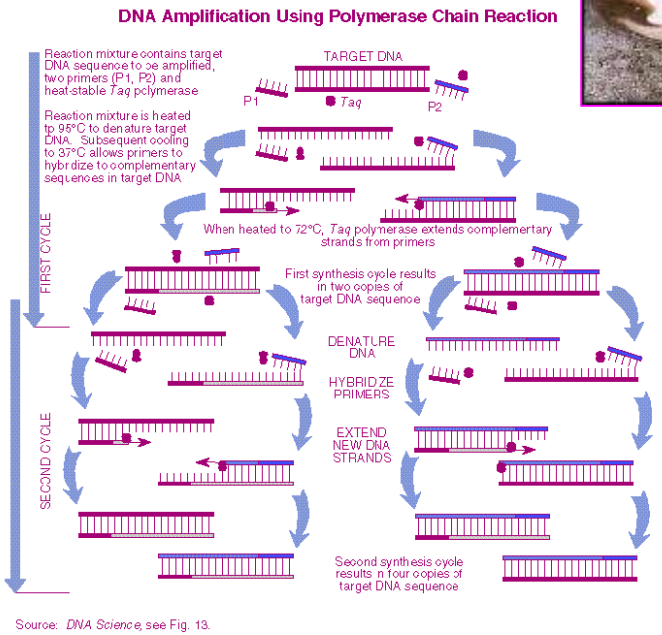
Use of nucleic acid amplification in clinical microbiology
Hayden R.T. in Molecular Microbiology: Diagnostic Principles and Pratice, 2004

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) 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.

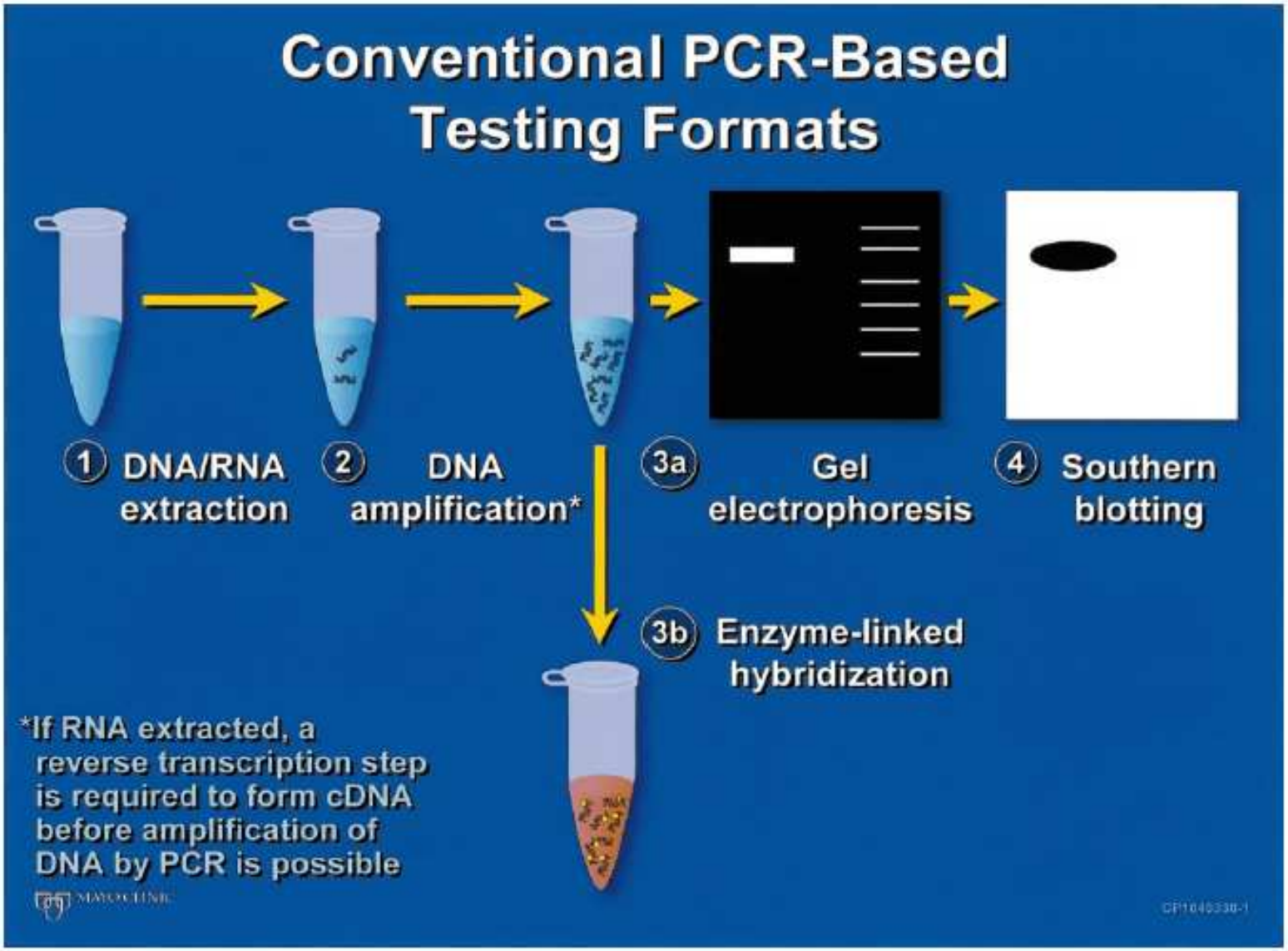
Nucleic acid amplification techniques

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

Nucleic acid amplification methods

Amplification	Enzyme used
Target amplification	**Enzyme used**
PCR	Thermophilic DNA polymerase
Transcription-Based Amplification System TAS, 3SR, NASBA, TMA	RT, Rnase H, RNA polymerase
SDA	Restriction endonucleases, DNA polymerase
LCR	Thermophilic DNA ligase
Signal amplification	
bDNA	None
Hybrid capture	None
Qβ replicase	Qβ replicase
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), branched-DNA (bDNA), cycling probe technologies (CPT), invader assays , rolling cycle amplification (RCA).

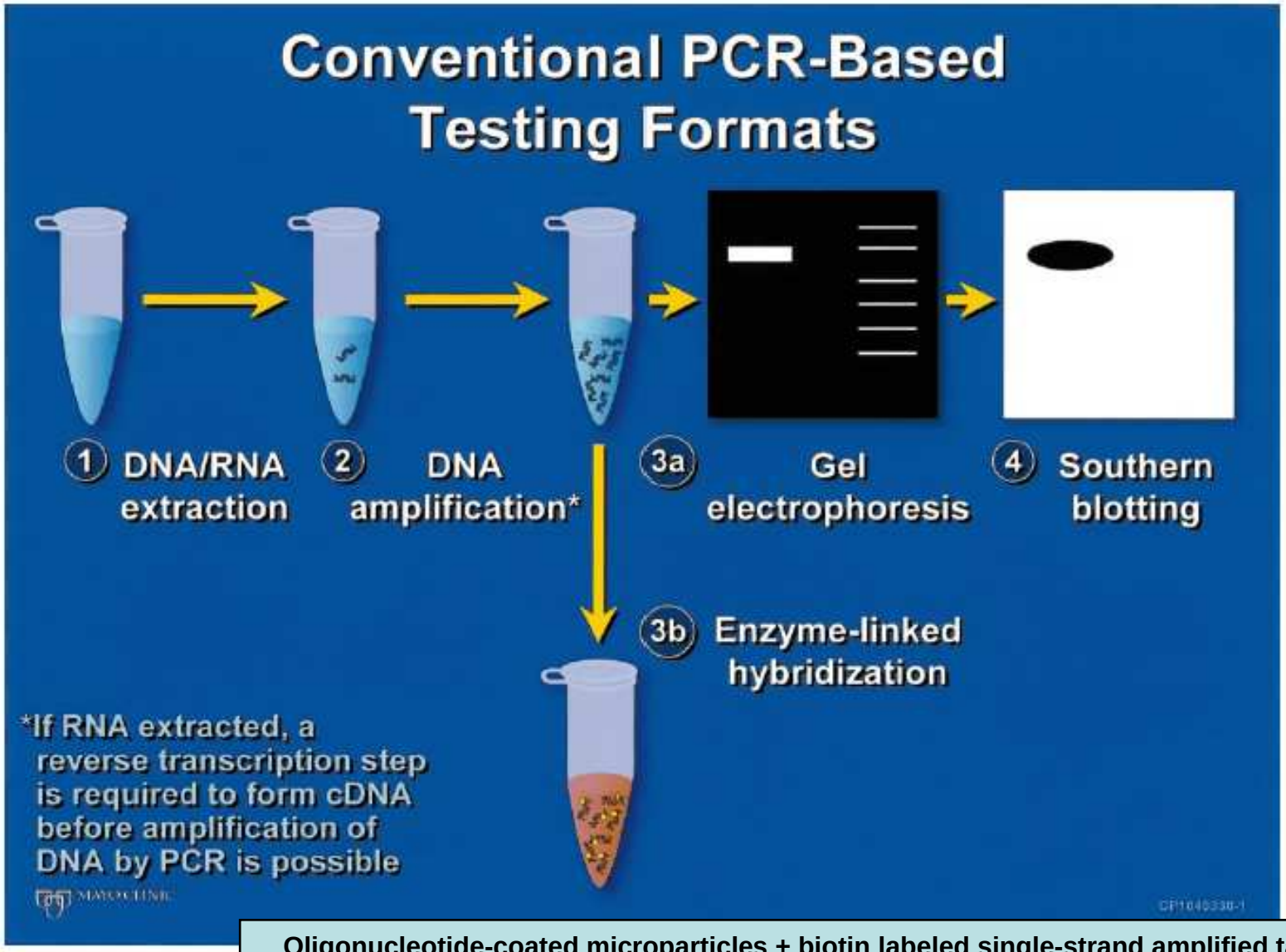

Conventional PCR-Based Testing Formats
1 DNA/RNA extraction
2 DNA amplification*
3a Gel electrophoresis
4 Southern blotting
3b Enzyme-linked hybridization
*If RNA extracted, a reverse transcription step is required to form cDNA before amplification of DNA by PCR is possible
MAYO CLINIC

Some of the problems with End-Point Detection

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

Agarose Gel

ABI: Real-Time PCR vs Traditional PCR (www)



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

Variations 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**
Arbitrarily primed PCR	Involves the use of a single short (10-15 base) arbitrarily chosen primer to amplify genomic DNA under low-stringency conditions. Useful in determining **whether 2 isolates of the same species are epidemiologically related**
Quantitative PCR	Normalization to internal or external standards. 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

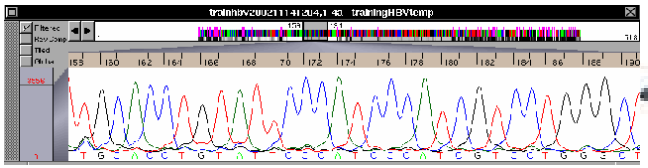
Automated PCR System

Assay for detection

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

Assay for quantification

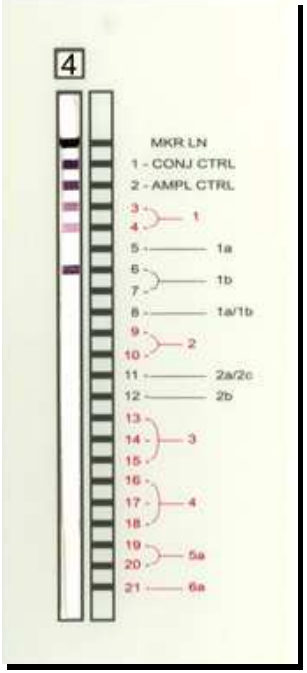
- HIV-1 viral load quantification
- CMV viral load quantification,
- Hepatitis B virus (HBV) viral load
- Hepatitis C virus (HCV viral load

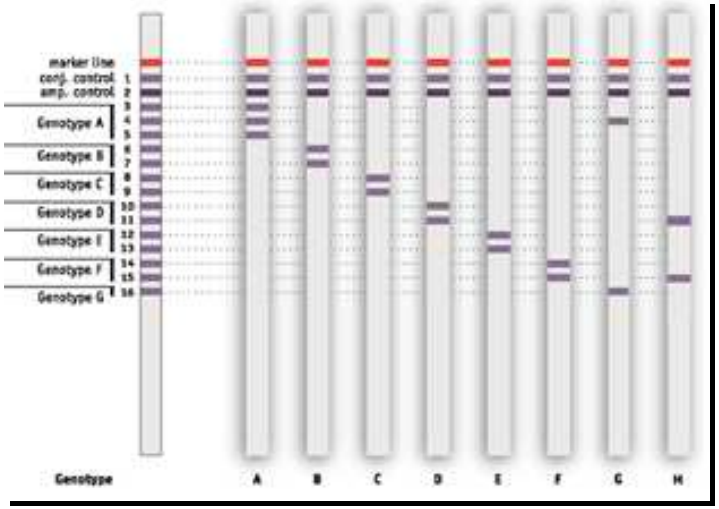


Automated Sequencing

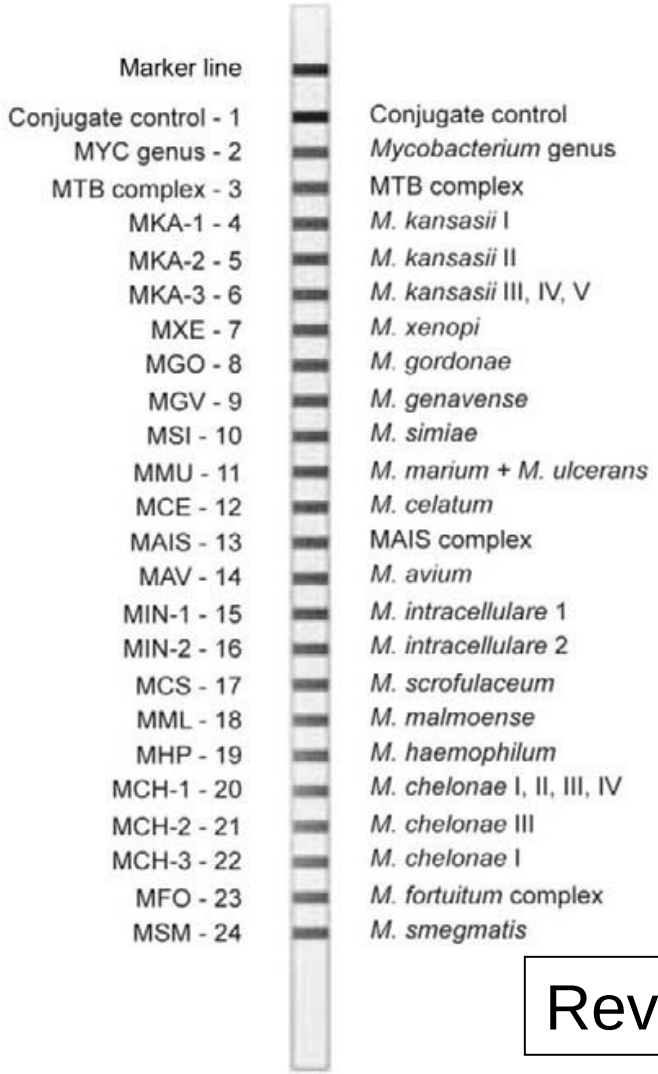
HIV, HCV, HBV (viral genotyping and resistance testing)

Versant HCV Genotype Assay

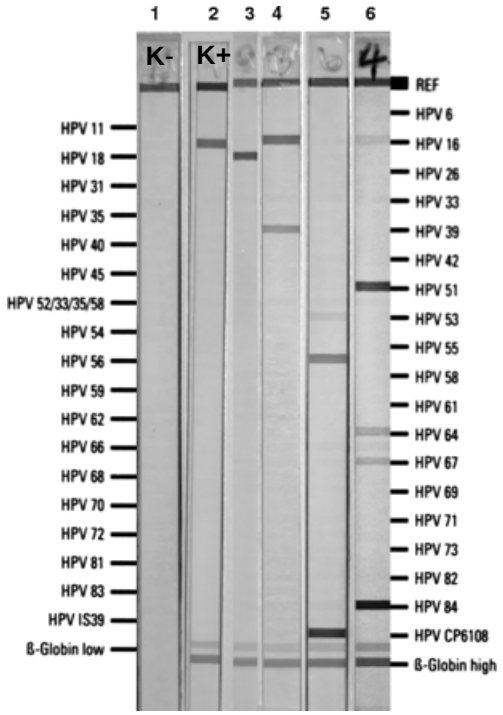




LiPA HBV



Linear Array HPV Genotyping Test



Reverse Hybridization

Mycobacterium tuberculosis

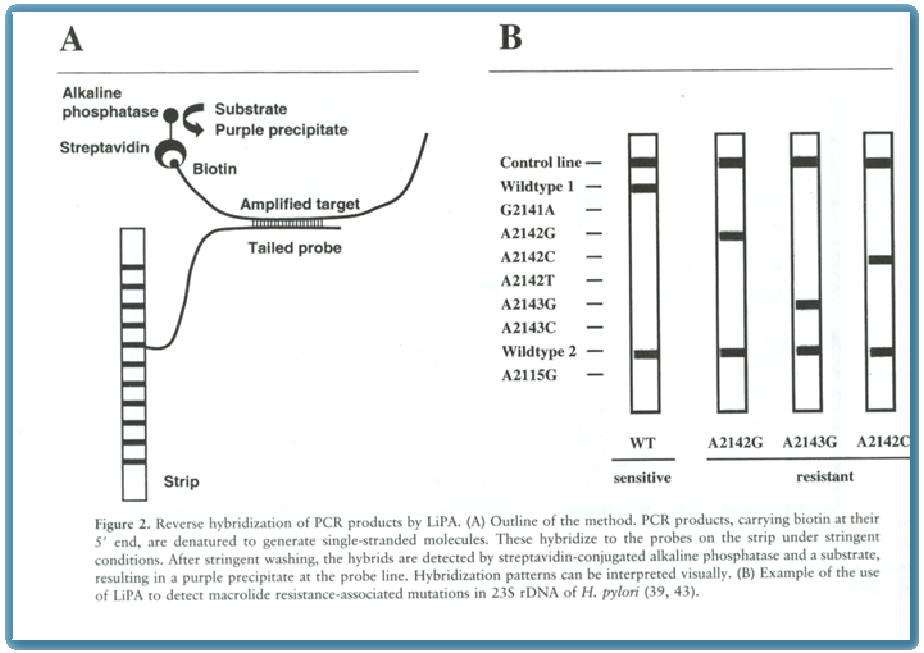
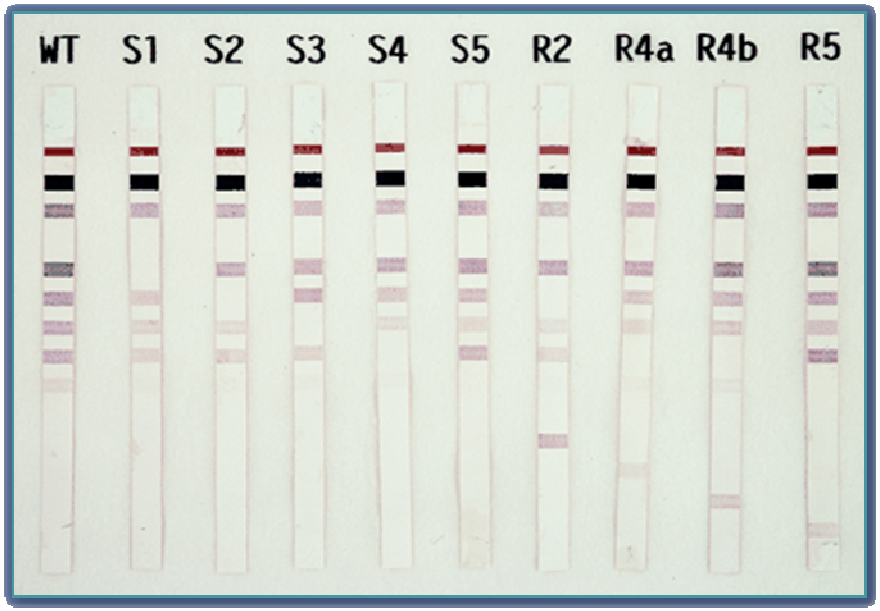


Figure 2. Reverse hybridization of PCR products by LiPA. (A) Outline of the method. PCR products, carrying biotin at their 5′ end, are denatured to generate single-stranded molecules. These hybridize to the probes on the strip under stringent conditions. After stringent washing, the hybrids are detected 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

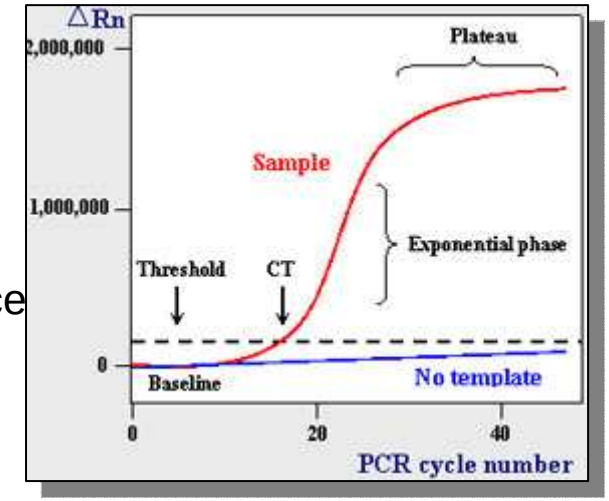
“Homogeneous, rapid cycle nucleic acid amplification”

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, Beacons)
  - Hybridization probes [Fluorescence resonance energy transfer (FRET) probes]

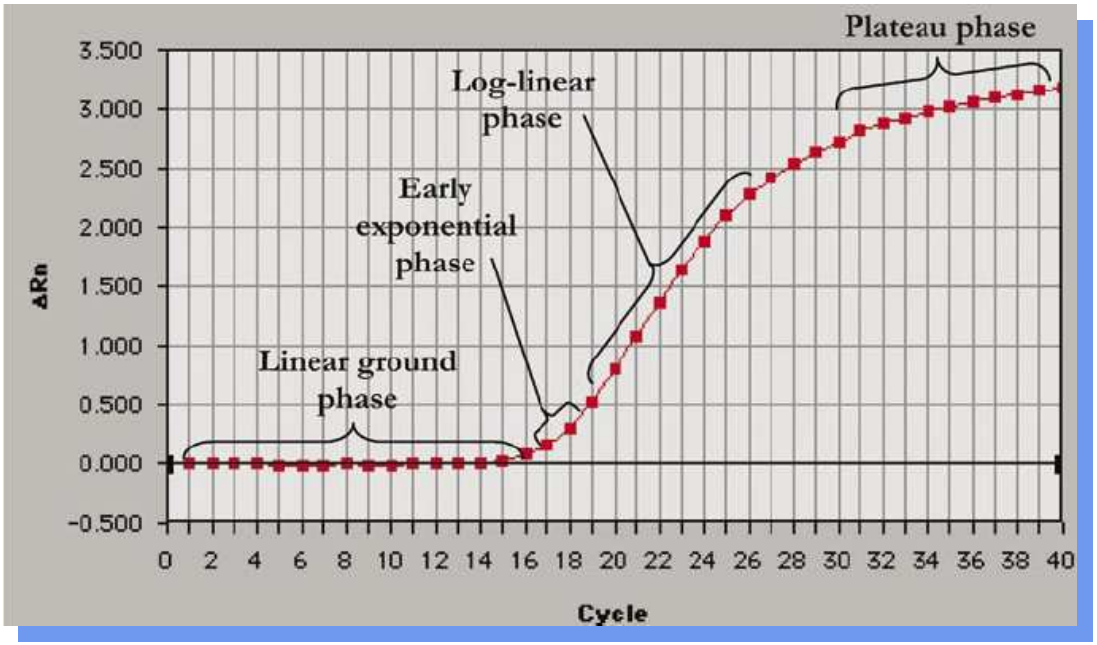


Higuchi, R., Dollinger, G., Walsh, P. S., and Griffith, R. (1992). "Simultaneous amplification and detection of specific DNA sequences." *Biotechnology* 10:413–417.

Real-time PCR is kinetic

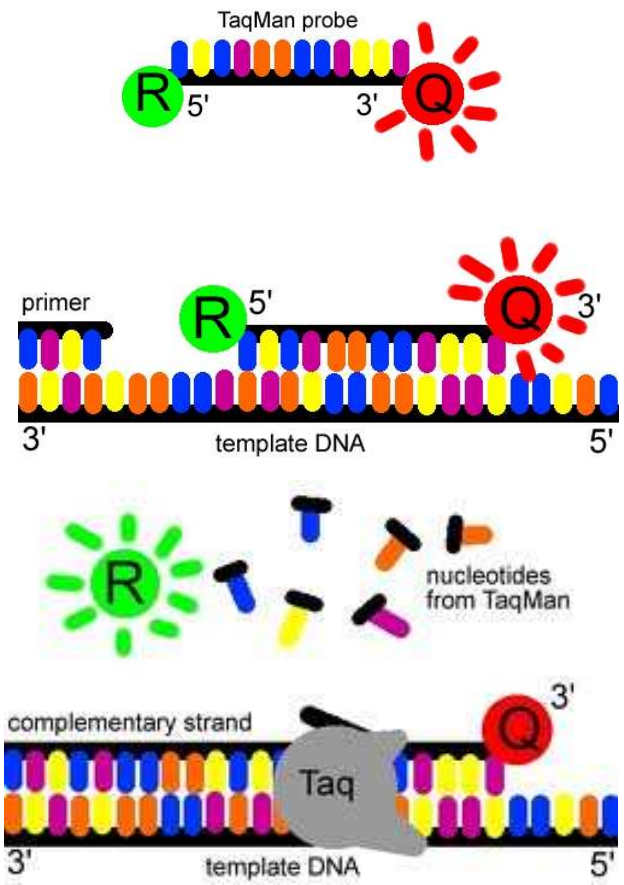
- Detection of " amplification-associated fluorescence" at each cycle during PCR
- No gel-based analysis at the end of a PCR reaction
- Computer based analysis of the cycle-fluorescence time course

Increasing fluorescence



Linear plot

PCR cycle



Real-time PCR advantages

- not influenced by non-specific amplification
- amplification can be monitored real-time
- 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
- confirmation of specific amplification by melting curve analysis
- most specific, sensitive (3 pg or 1 genome eq of DNA) and reproducible (CV <2.0%)
- allows for quantitation of results
- Software driven operation
- not much more expensive than conventional PCR (except equipment cost)

Multiplex Real-Time PCR
(fluorescein-labeled molecular beacon)

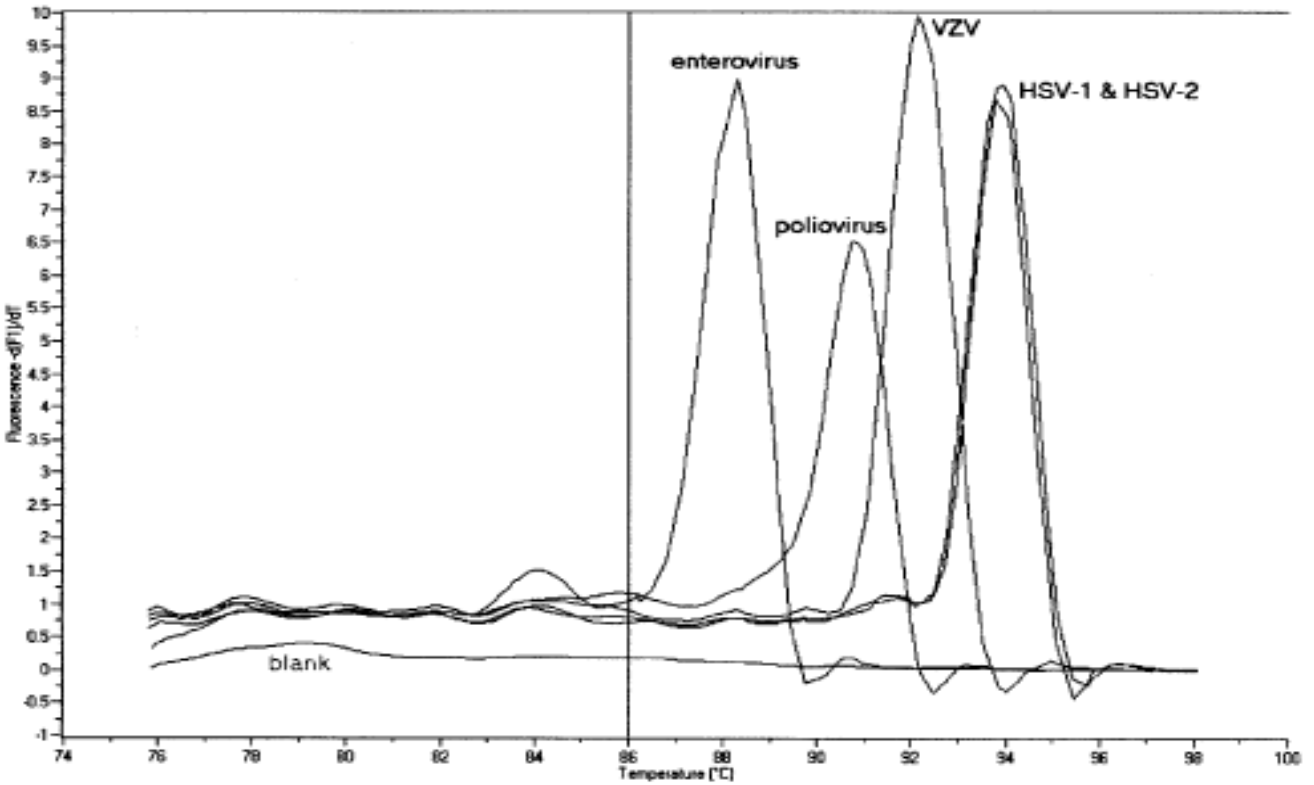
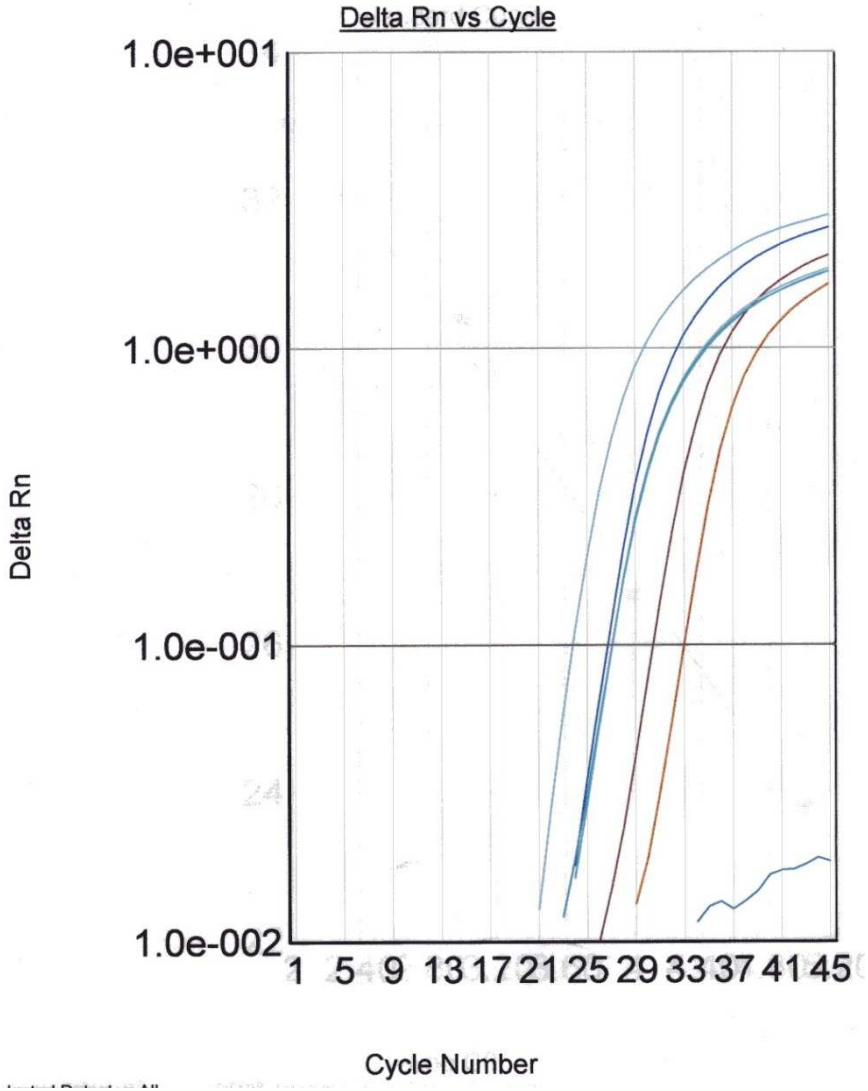


FIG. 1. LightCycler system melting curve analysis of ATCC isolates amplified by multiplex PCR. Enterovirus is echovirus type 1, ATCC VR-31, T_m of 88.20°C; poliovirus is poliovirus type 2 (attenuated), ATCC VR-301, T_m of 90.79°C; VZV is ATCC VR-586, T_m of 92.17°C; HSV-1 is ATCC VR-260, T_m of 93.83°C; and HSV-2 is ATCC VR-734, T_m of 93.91°C. The line designated "blank" represents the analysis of a PCR mixture without the addition of a target. The vertical line at 86°C represents the incubation temperature at which the levels of fluorescent signals were measured during each cycle of PCR. The *y* axis is the negative differential of fluorescence over temperature ($-dF/dT$).

Read SJ et al. J Clin Microbiol 2001



CSF : HSV-1 DNA
112.408 copie/mL

Methods used for amplification product detection and identification

Method	Signal molecules	Amplification and detection	Clinical applicatıcns	Elapsed time (including amplification)	Comments[a]
Gel electrophoresis	Ethidium bromide staining	Separate	Detection	1 day	When used with SSCP or RFLP, this method can be used for differentiation and subtyping
Southern blot hybridization	Radioactive or chemiluminescence	Separate	Detection	1–2 days	No longer a routine method in the clinical laboratory
Microtiter plate	Enzyme	Separate	Detection and differentiation	4–8 h	No expensive equipment needed; adaptable to automation
Molecular beacons	Fluorescence	Simultaneous	Detection and differentiation	4 h	Point mutation detection and differentiation; minimal carryover contamination due to the closed system
Direct sequencing	Fluorescence	Separate	Detection, typing, and resistance determination	1–2 days	Unique ability to identify unknown and highly mutated species
Real-time PCR	Fluorescence	Simultaneous	Detection, differentiation, and quantitation	0.5–2 h	Shortest test turnaround time; minimal carryover contamination due to the closed system
Matrix hybridization (DNA chip)	Fluorescence	Separate	Detection, differentiation, and resistance determination	6–12 h	Able to resolve complex mixtures of amplicons; high capital requirement

[a] SSCP, single strand conformation polymorphism; RFLP, restriction fragment length polymorphism.

* Solid- and liquid-phase microarray analysis

Tang Y.W. et al. in Molecular Microbiology: Diagnostic Principles and Pratice, 2004

DISADVANTAGES OF REAL-TIME PCR

- Current technology has limited capacity for multiplexing
- Development of protocols needs high level of technical skill and/or support
- High capital equipment costs ($ 50,000 -160,000)

Rotor-Gene
Corbett

iCycler
BioRad

LightCycler
Roche

TaqMan 48
Roche

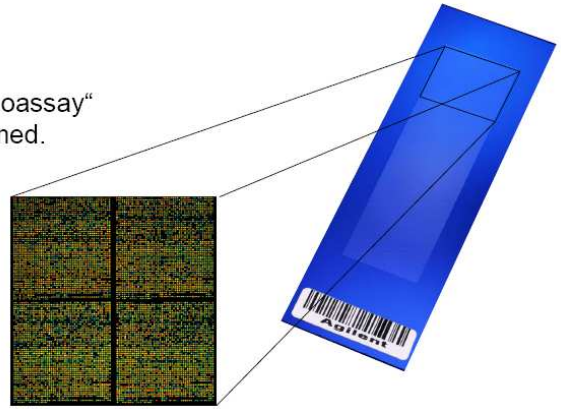
7300 Real-Time PCR System
Applied Biosystems

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

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

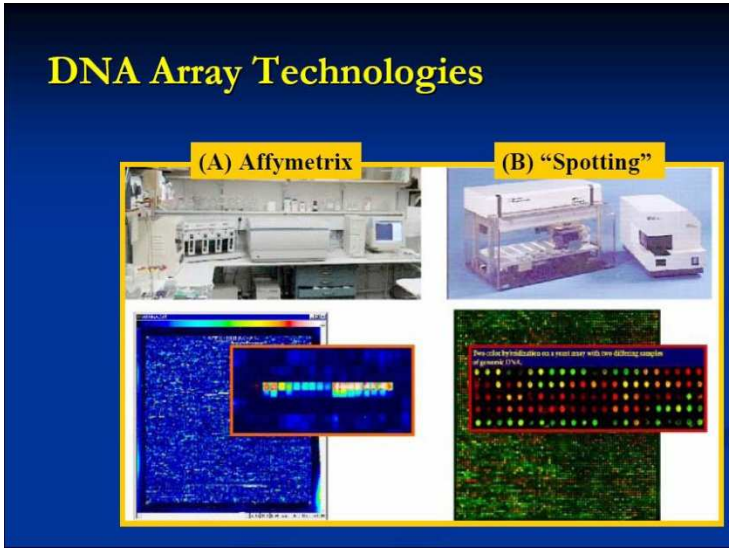
Microarray

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



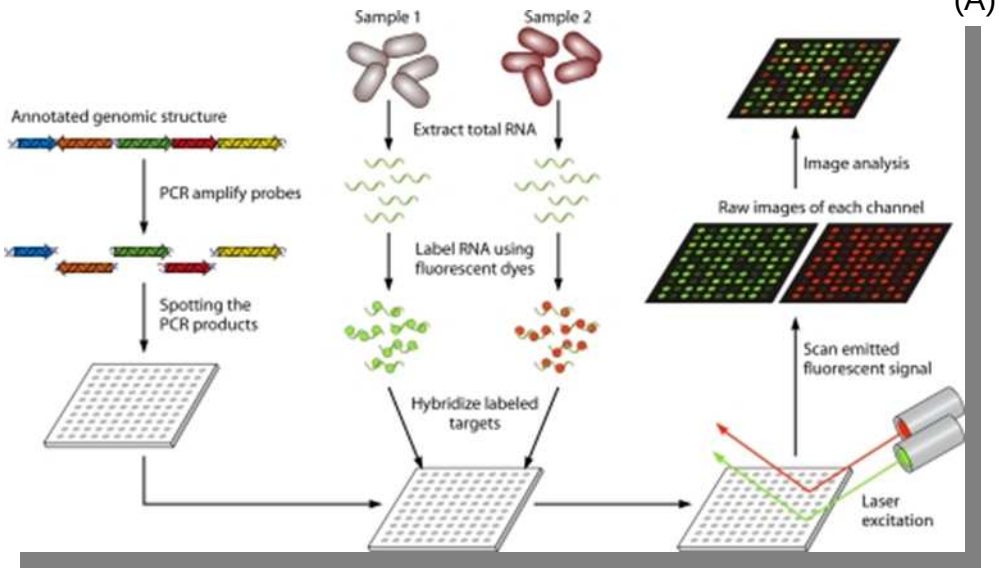
Enormous analytical power

Microarrays characteristics

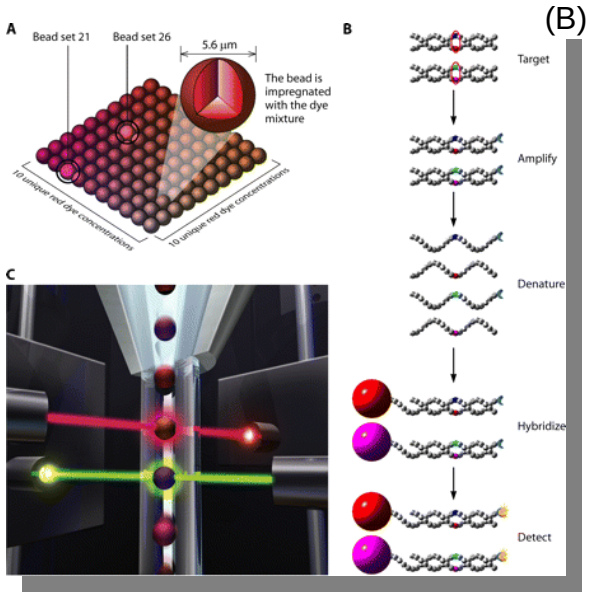
Microarrays can be distinguished based upon characteristics such as the nature of the probe, the solid-surface support used, and the specific method used for probe addressing and/or target detection

- 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	Drug discovery and drug development Pathogenesis studies and microbial physiology Vaccine development Drug resistance detection
Host gene expression profiling	Selection of infection Differentiation of infectious etiologies Development of anti-inflammatory drugs
Diagnostic sequencing	Microbial detection, identification, and typing • sequencing (e.g. SARS-CoV, April 2003) • broad-range PCR : bacterial, fungal, parasitic and viral pathogens • multiplex PCR for enteropathogenic bacteria, for bacterial and viral pathogens –meningitis, for respiratory viral pathogens • multiplex PCR for HPV testing Detection of drug resistance • *S. aureus*, *M. tuberculosis*, HIV-1 Detection of host polymorphisms associated with drug metabolism or differential immune response

Stover A.G. et al. in Molecular Microbiology: Diagnostic Principles and Pratice, 2004

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

TABLE 1. Comparison of microarray platforms[a]

Microarray	Principle(s)	Format(s)	Density	Relative cost	Diagnostic application(s)	References
Printed	Glass slides are used as the solid support for printing DNA probes	For dsDNA, PCR amplicons (200–800 bp) from known genomic sequence, shotgun library clones, or cDNA are used; for oligonucleotides, 25-80-bp probes are synthesized	Moderate (~10,000–30,000)	$$$	No commercially available applications; pathogen detection and identification, antimicrobial resistance detection, viral discovery, molecular surveillance	21, 32, 153, 167, 172, 195, 206–208
In situ synthesized	Oligonucleotides are synthesized directly on the surface of a quartz wafer using photochemistry; multiple probe sets (one perfect-match probe and one mismatch probe) are included per target	Affymetrix GeneChips, 20-25-bp probes; Roche NimbleGen, 60-100-bp probes; Agilent, 60-bp probes	High (Affymetrix, $>10^6$; NimbleGen and Agilent, 15,000–$>10^6$)	$$$$	No commercially available applications; pathogen detection and identification, antimicrobial resistance detection, viral discovery, molecular surveillance, strain typing	59, 117, 150, 151, 157, 200, 215
High-density bead arrays	Sequence-tagged beads are randomly assorted onto fiber-optic bundles or silicon slides	SAM, 96 samples; Sentrix BeadChip; 1-16 samples	High (~50,000-10^6)	$$$	No commercially available applications; potential use in microbiology but no studies published to date	52, 71, 146
Electronic	Electric fields are used to promote active hybridization of nucleic acids on a microelectronic device; streptavidin-biotin bonds immobilize the probes on the array surface	NanoChip 400; capture probe down; amplicon down; sandwich assays	Low (400 max)	$$	Commercially available products discontinued; pathogen detection and identification	10, 108, 185, 226
Liquid-bead suspension	Spectrally unique microspheres provide solid support for application of probes or universal sequence tags; bead hybridization with fluorescently labeled target DNA is measured using flow cytometry	Direct DNA hybridization; competitive DNA hybridization; solution-based chemistries (ASPE/TSPE, OLA, SBCE)	Low (100 max)	$$	FDA-cleared xTAG RVP assay; pathogen detection and identification, antimicrobial resistance detection, strain typing	18, 73, 79, 109, 112, 148, 191

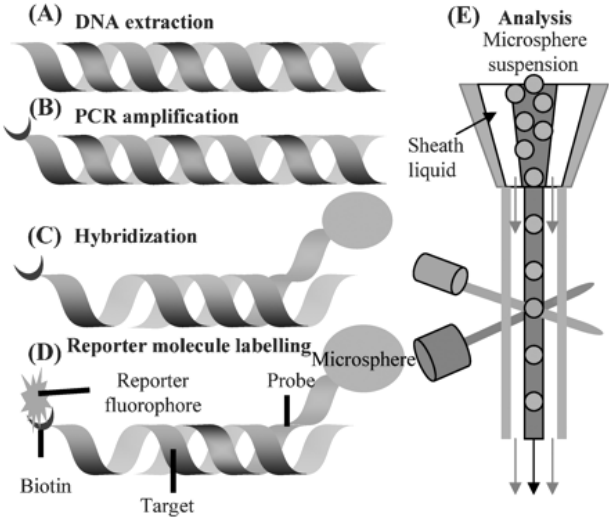
[a] Data from reference 135. $$, low-moderate cost; $$$, moderate cost; $$$$, high cost.

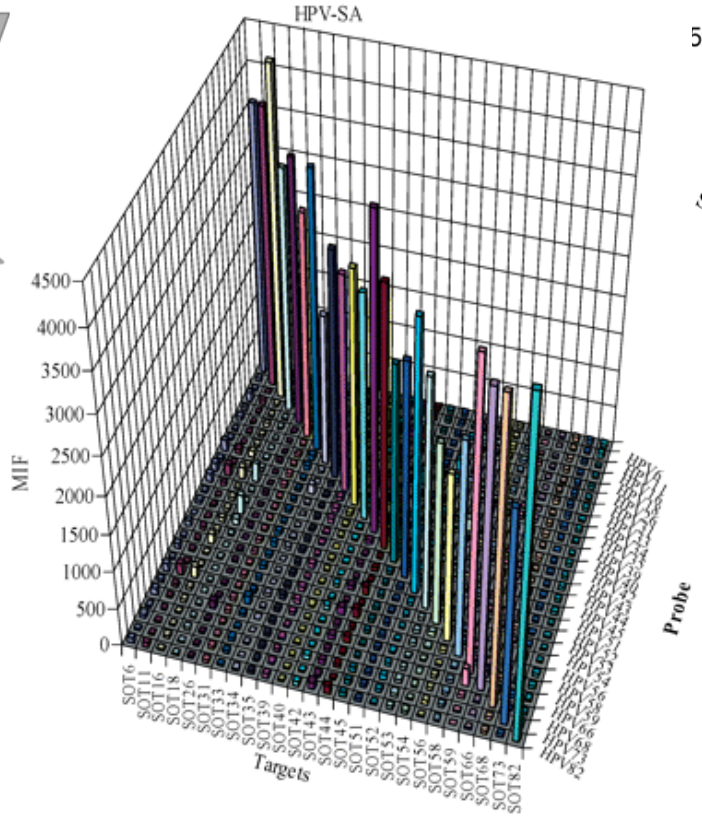
CLINICAL MICROBIOLOGY REVIEWS, Oct. 2009, p. 611–633 Vol. 22, No. 4
Basic Concepts of Microarrays and Potential Applications in Clinical Microbiology
Melissa B. Miller and Yi-Wei Tang

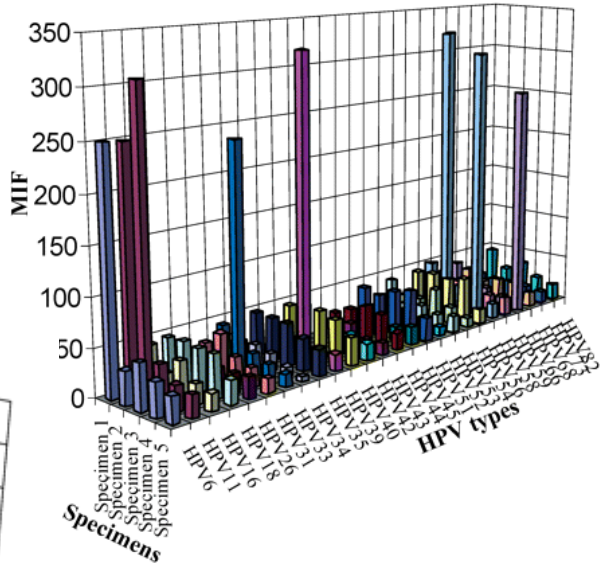
Jiang HL, Zhu HH, Zhou LF, Chen F, Chen Z

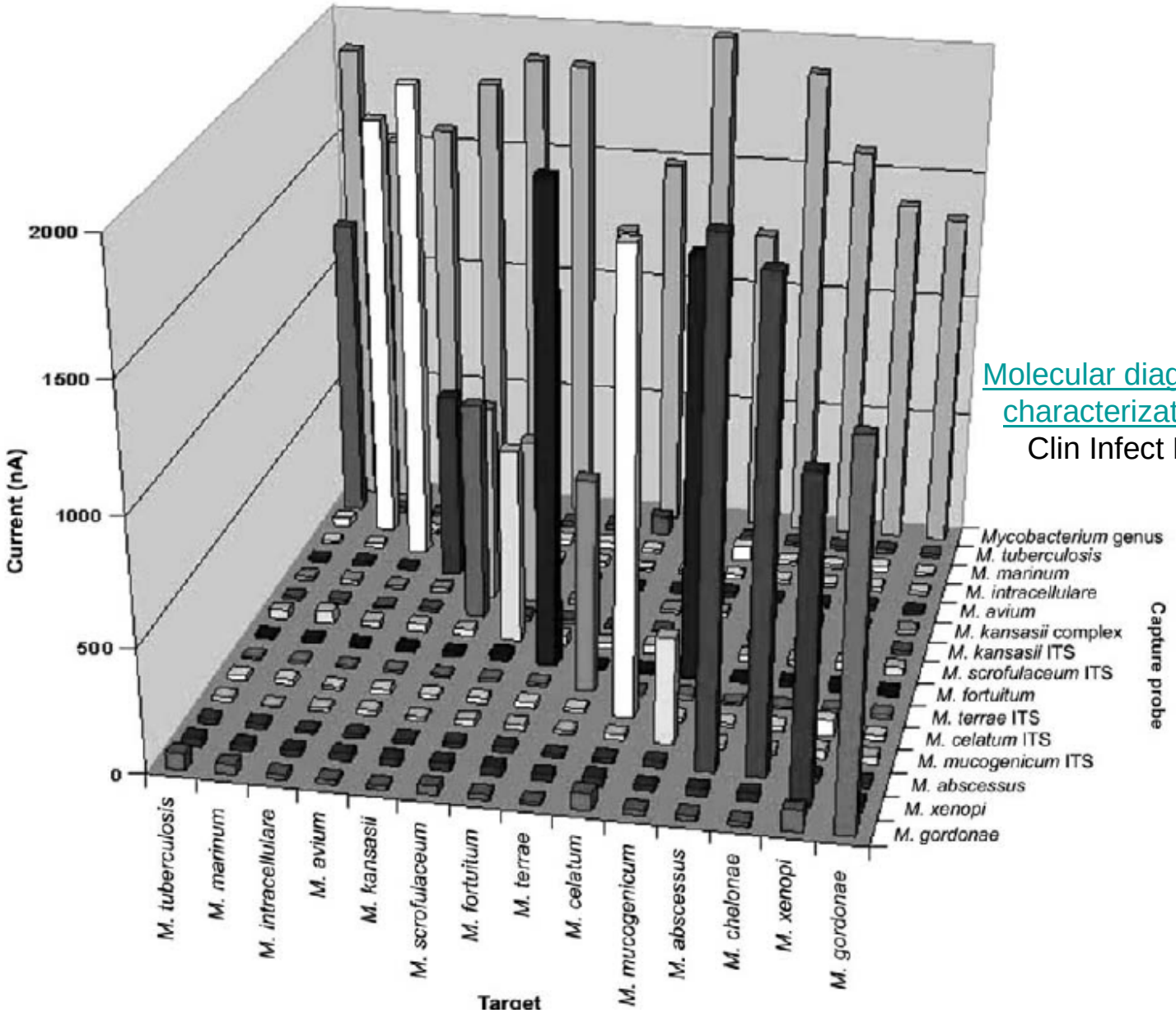
Genotyping of human papillomavirus in cervical lesions by L1 consensus PCR and the Luminex xMAP system.

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





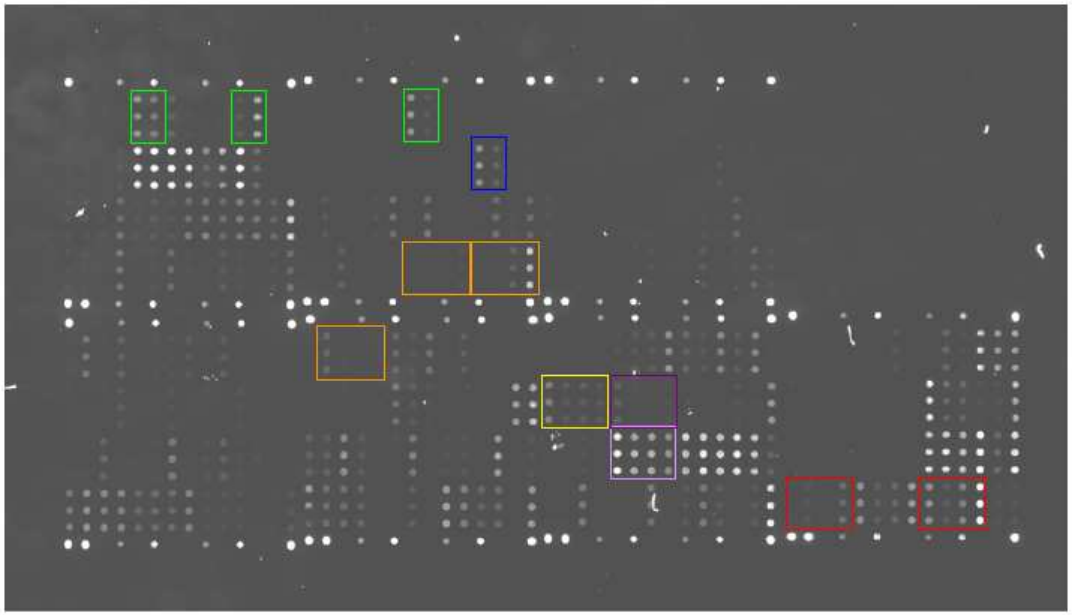




Procop GW.
Molecular diagnostics for the detection and characterization of microbial pathogens.
Clin Infect Dis. 2007 Sep 1;45 Suppl 2:S99-S111.

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.

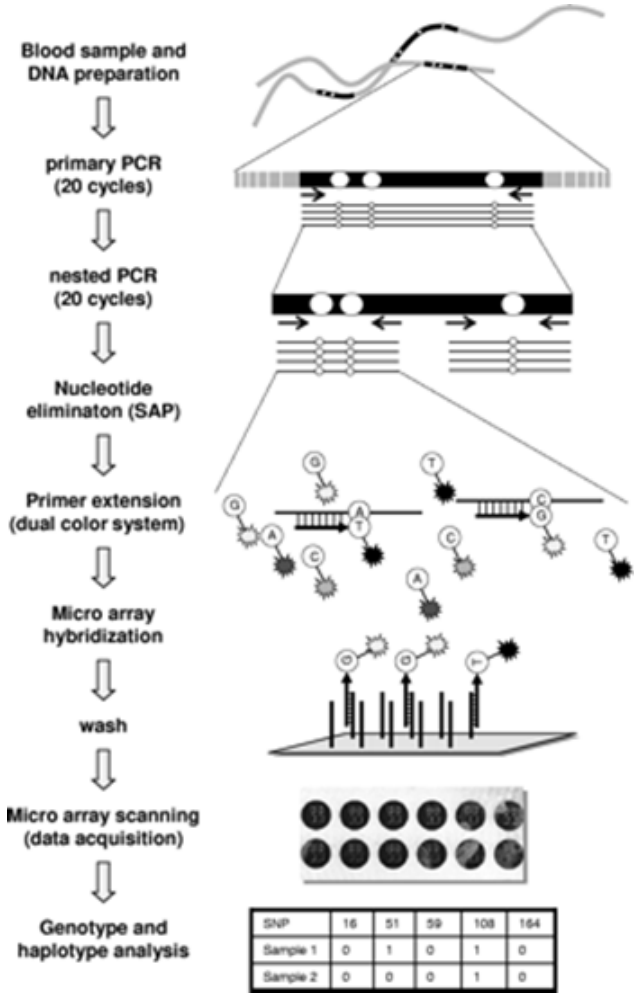
DNA microarray hybridization pattern of a multidrug-resistant *P. aeruginosa* clinical isolate



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

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



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.

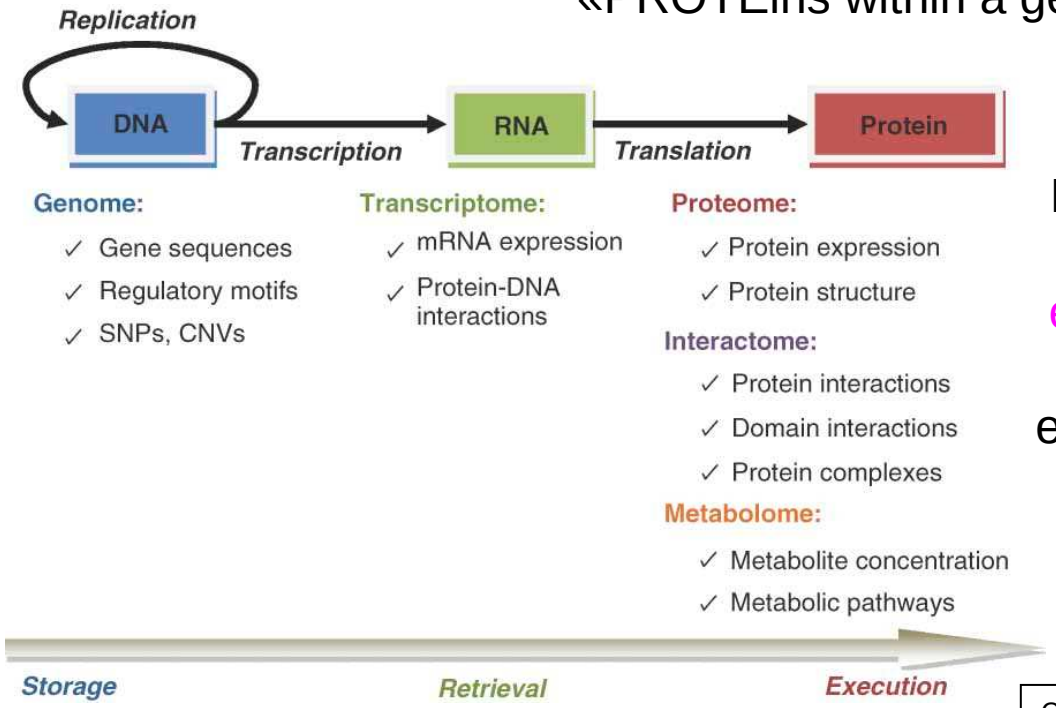
Crameri A, Marfurt J, Mugittu K, Maire N, Regös A, Coppee JY, Sismeiro O, Burki R, Huber E, Laubscher D, Puijalon O, Genton B, Felger I, Beck HP.

J Clin Microbiol. 2007 Nov;45(11):3685-91.

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

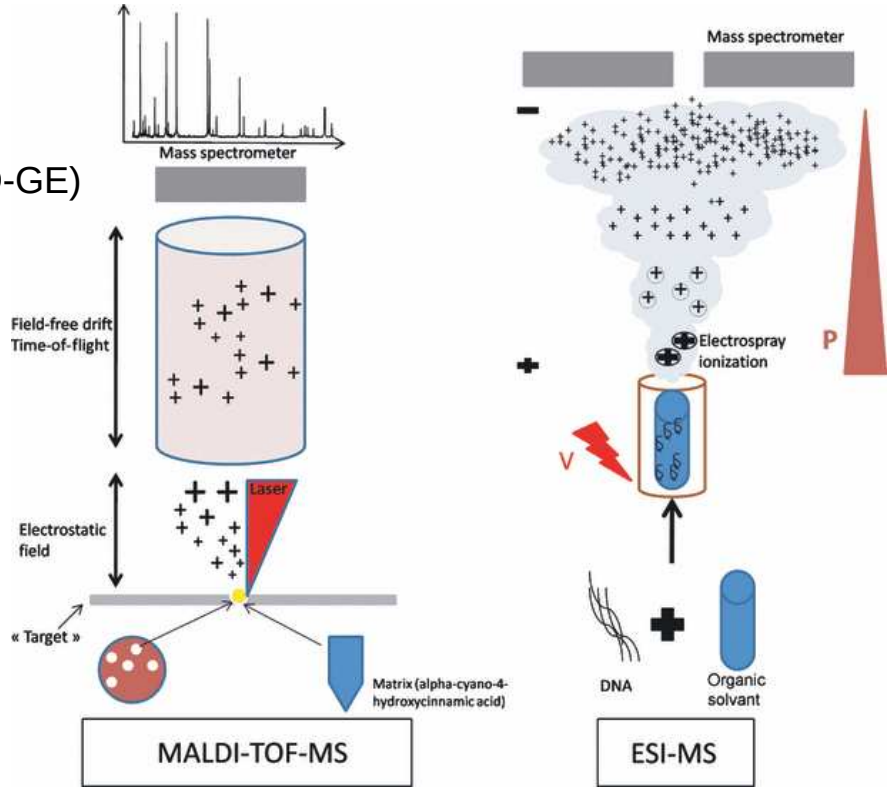
Carolyn I. Phillips et al. **Proteomics meets microbiology: technical advances in the global mapping of protein expression and function** Cellular Microbiology (2005)**7**(8),1061

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.

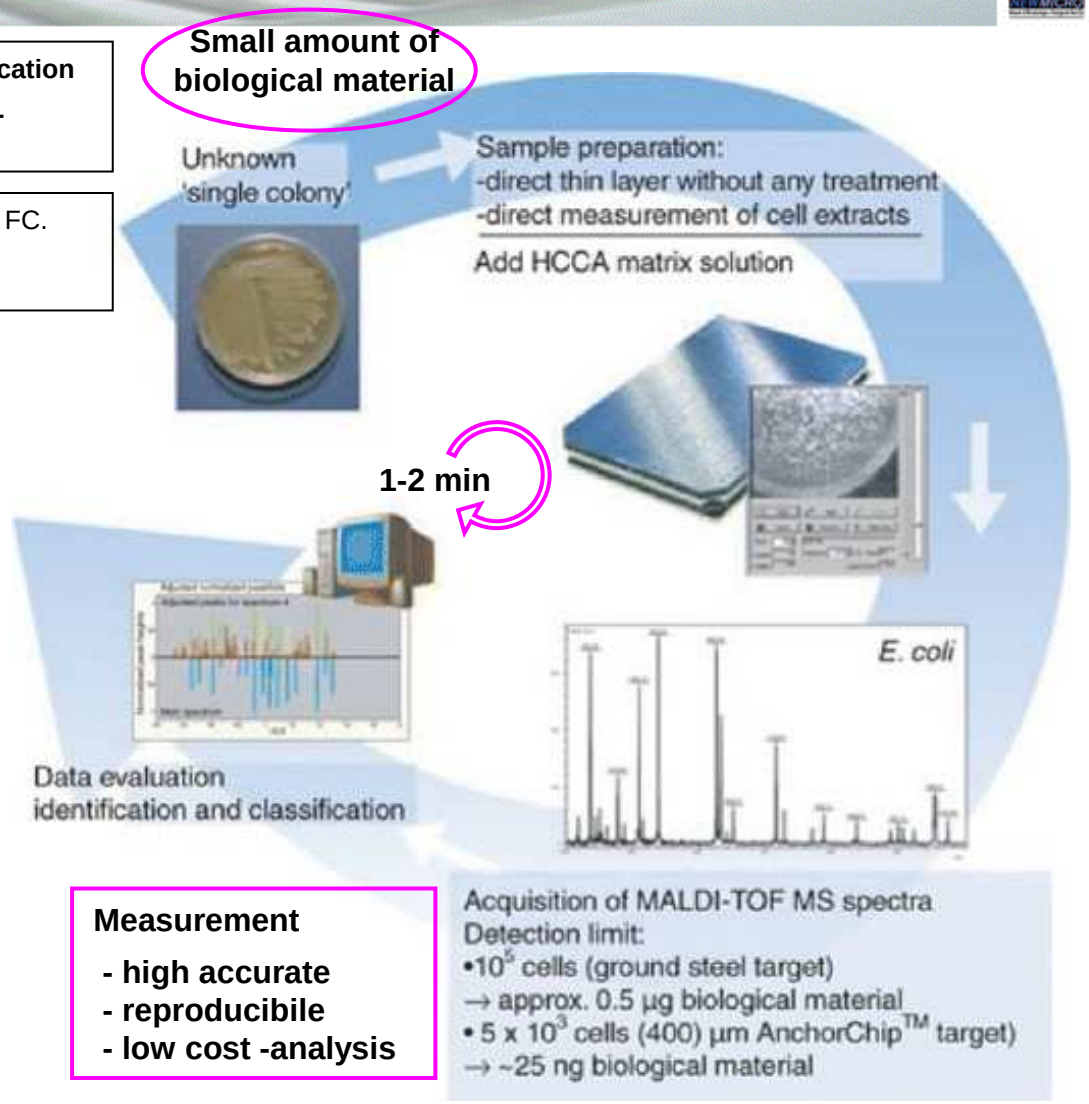
MALDI-TOF MS

- 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

Dipartimento di Medicina di Laboratorio

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
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			
L. interrogans		x					
P. jiroveci					x		
B. anthracis		x					
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 dei risultati di qualità analitica di eccellenza in tempo utile per la cura del paziente.

Solo così i nostri risultati diventano clinicamente significativi.

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