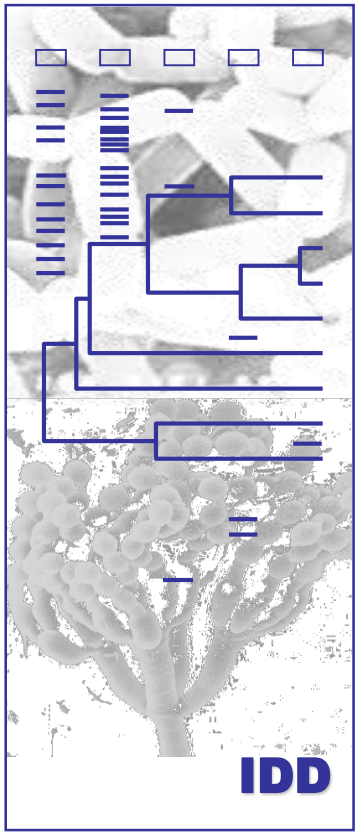
# Diagnosis



*Diagnostic […] will be particularly useful among experimental scientists in a variety of disciplines, including public health microbiologists, industrial research microbiologists, academic scientists, and all others who may need to establish the presence or absence of bacteria and/or their identities.*

*Howard & Whitcombe, 1995*

# Diagnosis

- **The concept of identification / diagnosis / detection**
- **Methods used in the diagnosis of microorganisms**
- **Validation of the molecular diagnosis methods**
- **The diagnostic methods applied to clinical, food and environmental samples**

# Methods Used for Microorganisms Diagnosis

| *Test* | *Ease of performance* | *Turnaround time* | *Result interpretation* | *Advantages* | *Disadvantages* |
|---|---|---|---|---|---|
| *Direct examination* | Could be performed in routine clinical lab and in nurse station | 1–3 h | Direct if correlated with symptoms | Rapid | Poor sensitivity and specificity; special skills are needed for interpretation |
| *Culture* | Could be performed in sophisticated clinical lab and in research lab | 2–14 days | Definite | For phenotypic drug susceptibility testing | Time-consuming; poor sensitivity; limited microorganisms are culturable |
| *Serology* | Could be performed in larger and sophisticated clinical lab | 4–6 h | Indirect | Automation | Results are generally retrospective; immunosuppressed host may be unable to mount a response |
| *Molecular diagnostics* | Could be performed in only a few very sophisticated research and clinical labs | 1–2 days | Direct without knowing microbial viability | High sensitivity and specificity | Facility requirement; false positive due to carryover contamination and false negative due to inhibitors in specimen |

*In* Tang & Persing, 2000

## Diagnostic Principles

- DNA probe technologies
- Amplification methods
- Nucleic acid sequencing strategies
- "New" approaches to detection of nucleic amplification products (*e.g.* biochips)

## Diagnostic Applications

- detect and characterize bacterial, viral, fungal, and parasitic pathogens
- identification of organisms isolated in pure culture
- rapid identification of organisms in "mixed" samples
- identification of slowly growing, fastidious or uncultivable organisms
- screening sterile samples for non-specific bacterial contamination

❖ laboratory standardization

❖ proficiency testing

❖ quality control

Bacterial sensing for the purpose of diagnosis can function in three ways:

- bacterial morphological visualization
- whole cell detection
- specific detection of bacterial component

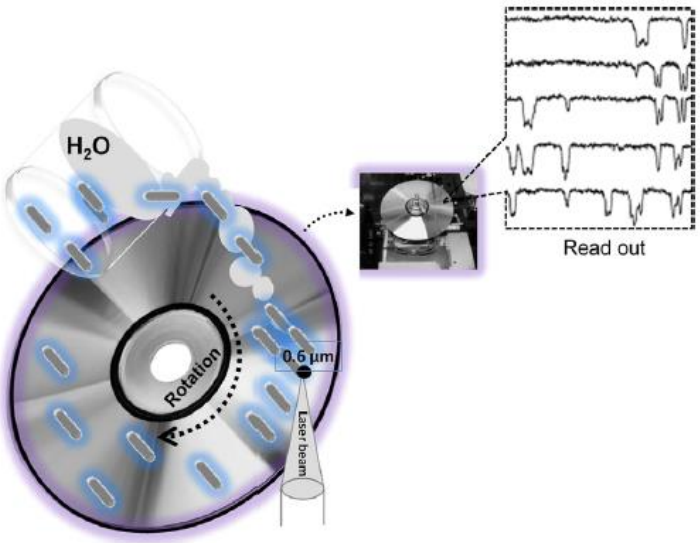



**Fig. 6.** Disc technology for detection of bacteria. Bacteria in water interact with the specific probe on the surface of the disc, resulting in a change in the signal read-out.

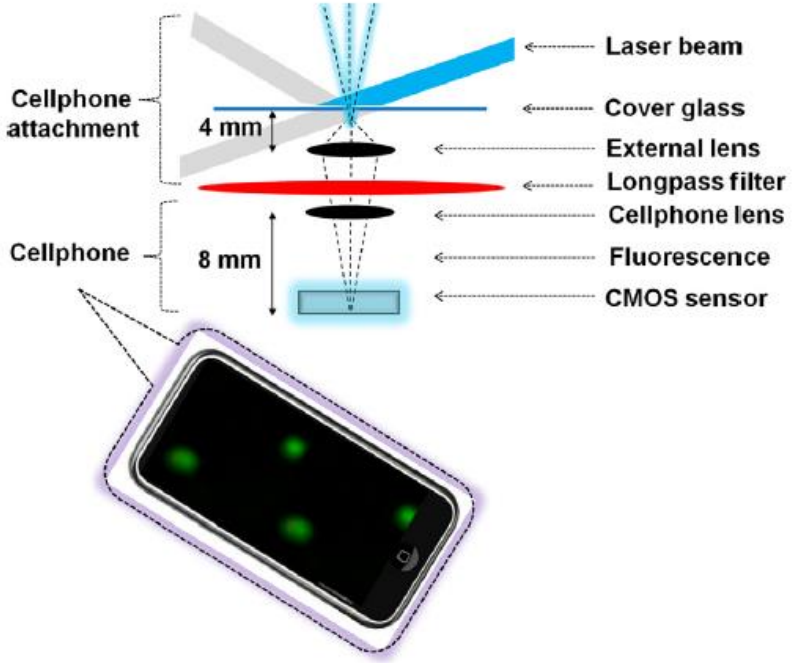
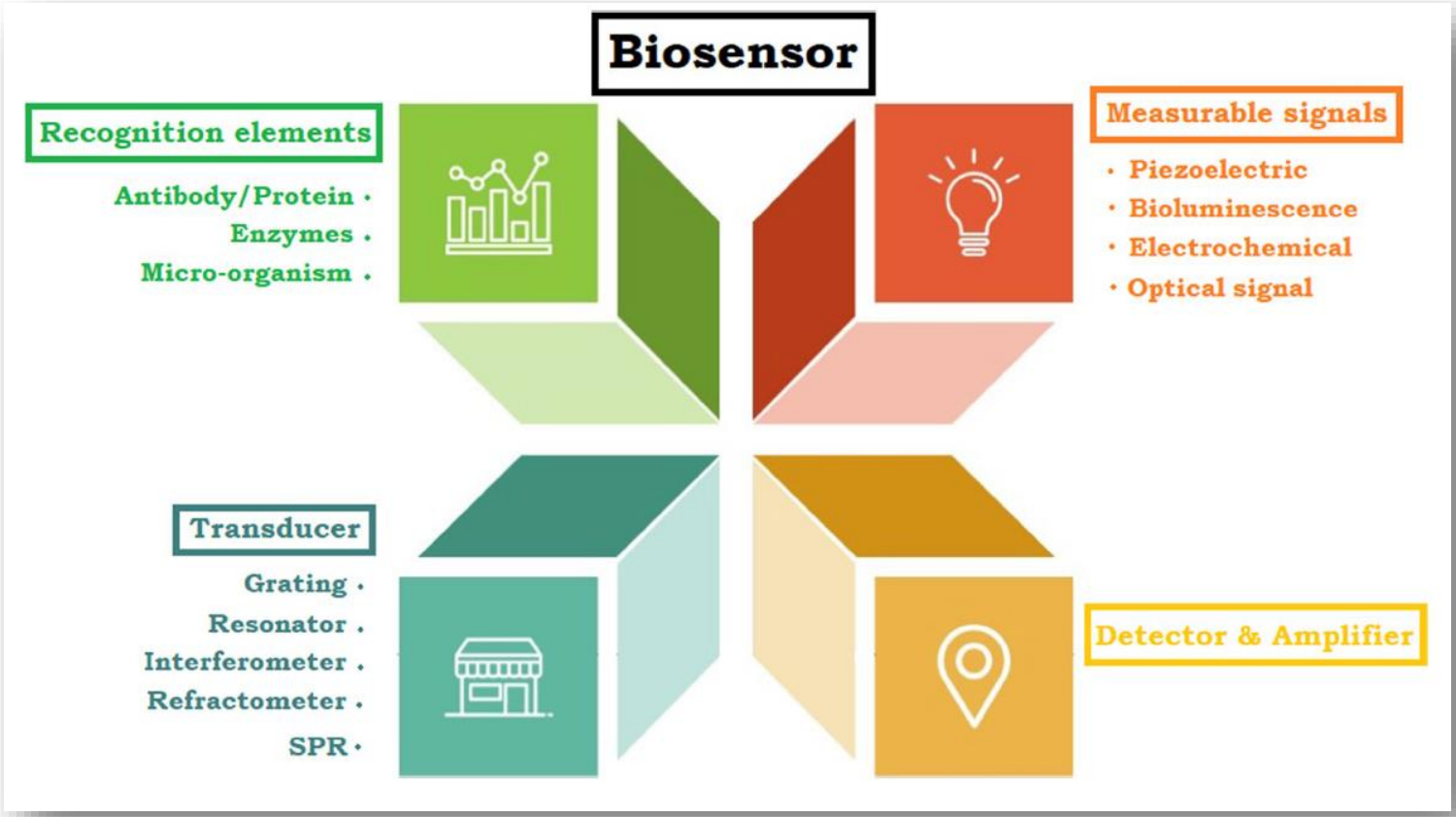



**Fig. 7.** Detection of bacteria by smartphone. An opto-mechanical device containing a fluorescent imager is attached to the smartphone to capture images. (Wei et al., 2013).

Gopinath *et al.*, 2014

# Biosensors for the detection of foodborne pathogens

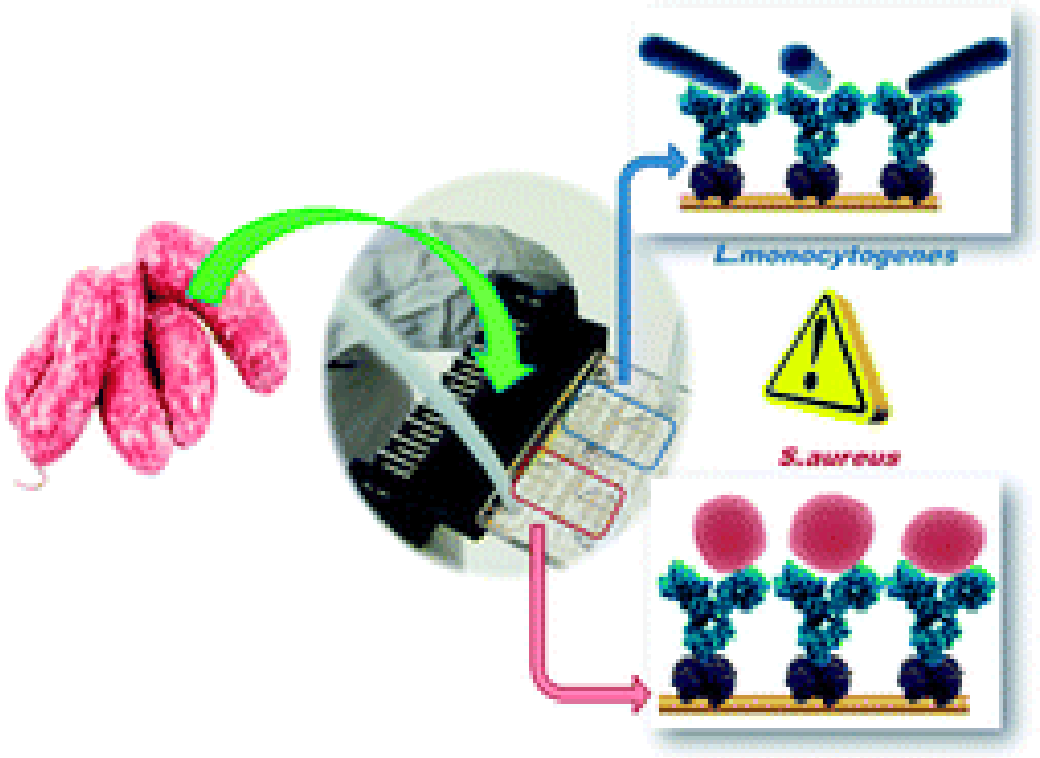



- **Biosensors** are analytical devices that **transform the biological response** into a **measurable signal** by incorporating **biorecognition element** with a **physical transducer**.
- The resulted signal is converted into measurable signals by **signal detection process**.

*SPR* surface plasma resonance.

*In* Saravanan *et al.*, 2021

# A multipurpose biochip for food pathogen detection




Biochips that are able to **quantitatively** detect

*Listeria monocytogenes* and *Staphylococcus aureus*

Primiceri *et al.*, 2016

# Culture-Independent Rapid Detection Methods for Bacterial Pathogens and Toxins in Food Matrices

Wang & Salazar, 2016

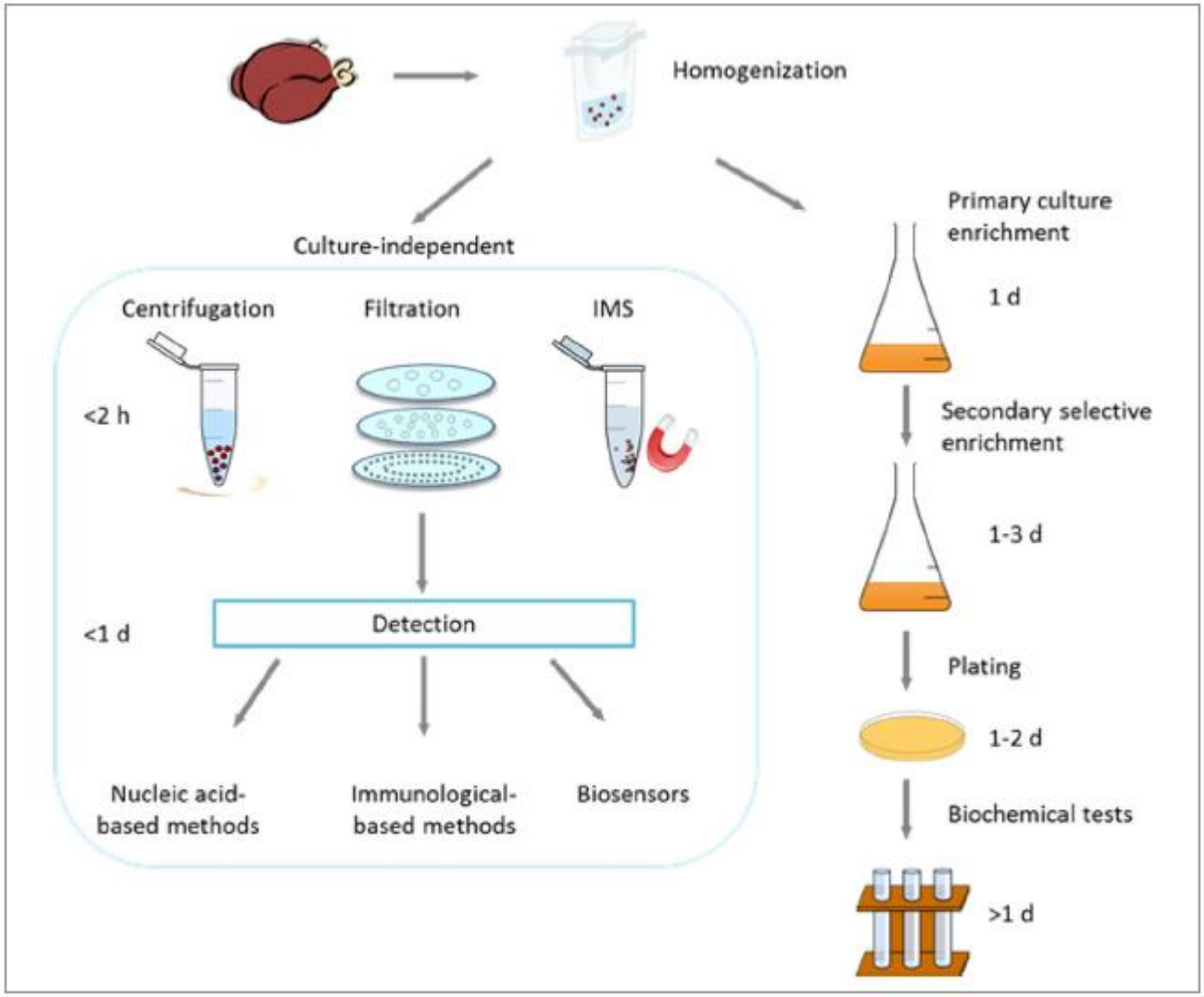
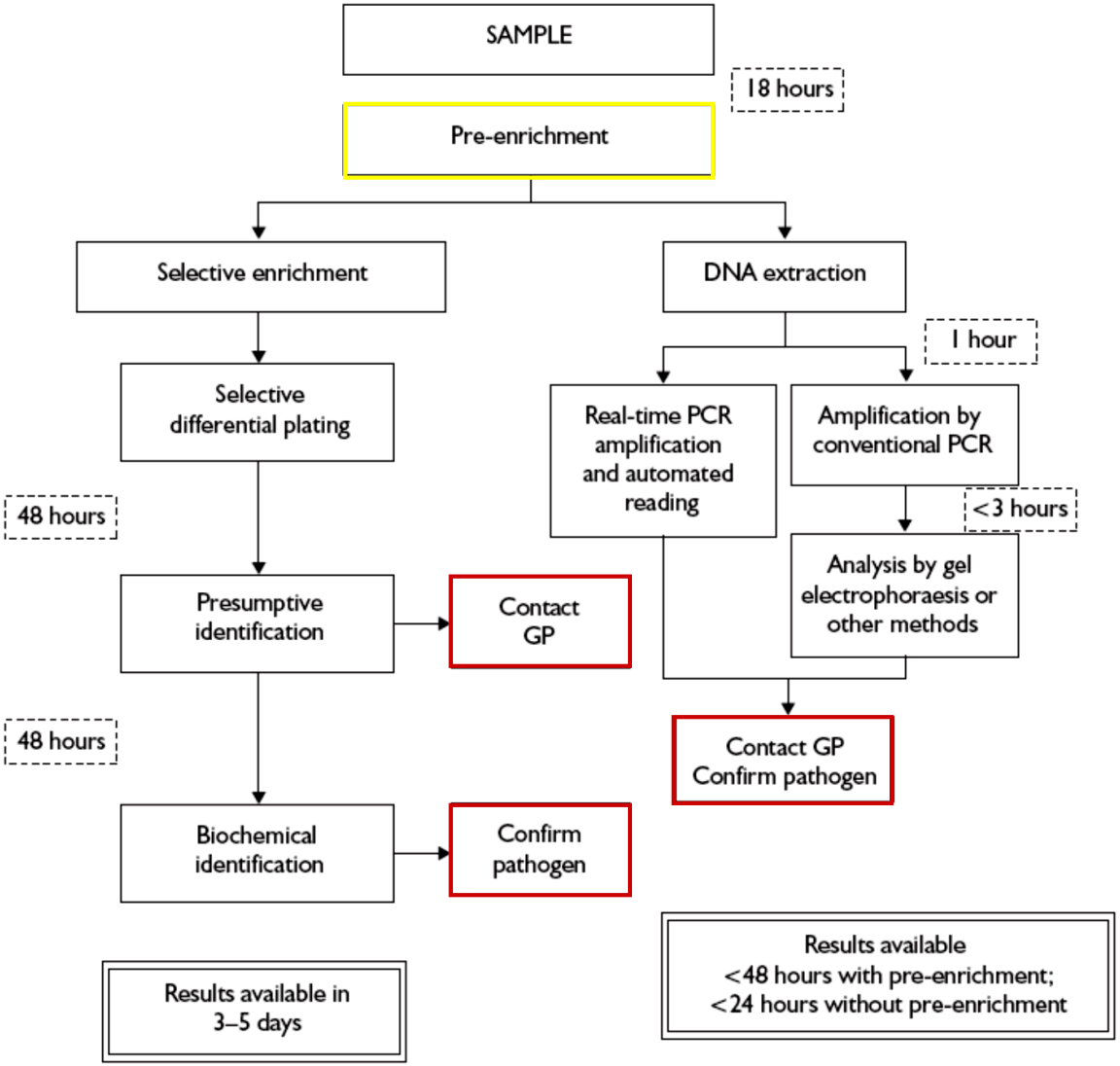



Figure 1–Comparison of culture-independent detection (left), including preprocessing techniques of centrifugation, filtration, and immunomagnetic separation (IMS), to conventional culture-based method (right). Culture-based detection may take place after enrichment or plating steps, which prolongs the total detection time.

# Detection and identification of bacterial intestinal pathogens in faeces and food




GP: microbiologists and consultants in communicable disease control

*In* Abubakar *et al.*, 2007

**Simplified flowchart of culture methods *vs.* PCR detection**

# Biosensors based on modularly designed synthetic peptides for recognition, detection and live/dead differentiation of pathogenic bacteria

- Engineered antimicrobial peptides (sAMP) are used as **synthetic receptors** for bacteria.
- sAMP enables detection and **live/dead differentiation** of bacterial pathogens.
- An impedimetric biosensor based on site-specifically attached sAMP is described.
- Detection limit of $10^2$ CFU/mL bacteria is demonstrated.

Liu *et al.*, 2016

TABLE 1. Detection of DT among isolates of pathogenic corynebacteria

| Species | No. of isolates examined | No. of toxigenic isolates determined by the following assay: | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | In vivo | Vero cell | Immunoblotting | Elek | | PCR | |
| | | | | | Conventional | Modified | Fragment A gene | Entire gene |
| *C. diphtheriae* var. *mitis* | 31 | 18 | 18 | 18 | 18 | 18 | 24 | 22 |
| *C. diphtheriae* var. *gravis* | 15 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| *C. diphtheriae* var. *belfanti* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. diphtheriae* var. *intermedius* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *C. ulcerans* | 5 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Total | 55 | 26 | 26 | 26 | 26 | 26 | 32 | 30 |

**Diphtheria toxin (DT), the main virulence factor**
**produced by organism *Corynebacterium diphtheriae*,**
**is a protein molecule with a molecular mass of 58,350 Da**

**Some isolates of *C. diphtheriae* possessed** **toxin genes**

**but failed to express a** **biologically active toxin**

**exclusion of toxigenicity**

*In* Efstratiou *et al.*, 1998

## PCR

***Investigative*** **mainly used for limited period**
**by a few technicians**
**for a specific research project**

***Diagnostic*** **has to perform reliably and consistently**
**day after day**
**in the hands of different staff**
**on different samples**

## Harmonization

**process of *synchronizing* various test *strategies* and *protocols***

## Standardization

**development of a single "gold standard" PCR protocol adopted in practice by all parties, and fulfilling all their requirements**

**standard guidelines**

# Implementation of a molecular assay in the clinical microbiology laboratory

**First phase is the assay development**

**Second phase is the validation**

**1 - once an appropriate target is selected**

**2 - primers and probes can be designed**

**3 - after appropriate target is established**

**4 - after detailed *in silico* analysis has been performed**

**to verify that the target is specific to the pathogen or gene of interest**

**5 - analytical sensitivity and specificity must be determined**

# Repeatability and reproducibility

**Repeatability**

repeat (*replicate*) 10% assays

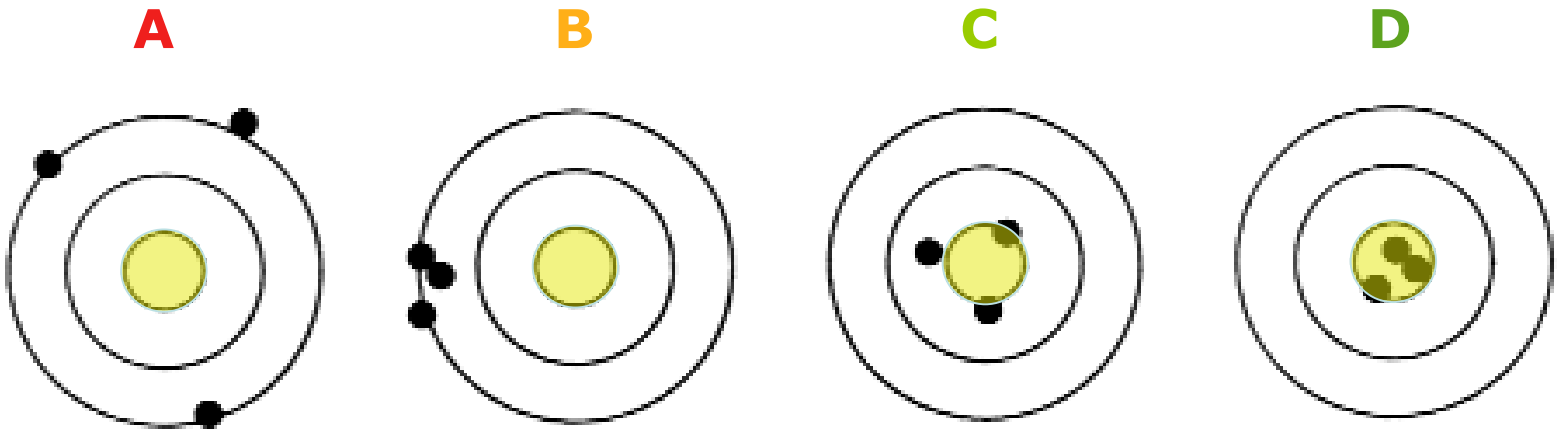
**Reproducibility** (*precision*)

agreement among results of samples tested in different laboratories

QT

- *Repeatability* ⇒ CVs for replicates ≤ 10%
- *Reproducibility* ⇒ regression analysis of normalized data among laboratories generally should not give significant differences at the 95% confidence level

# accuracy and precision




**A - imprecise and inaccurate**

**B - precise but inaccurate**

**C - accurate but imprecise**

**D - precise and accurate**

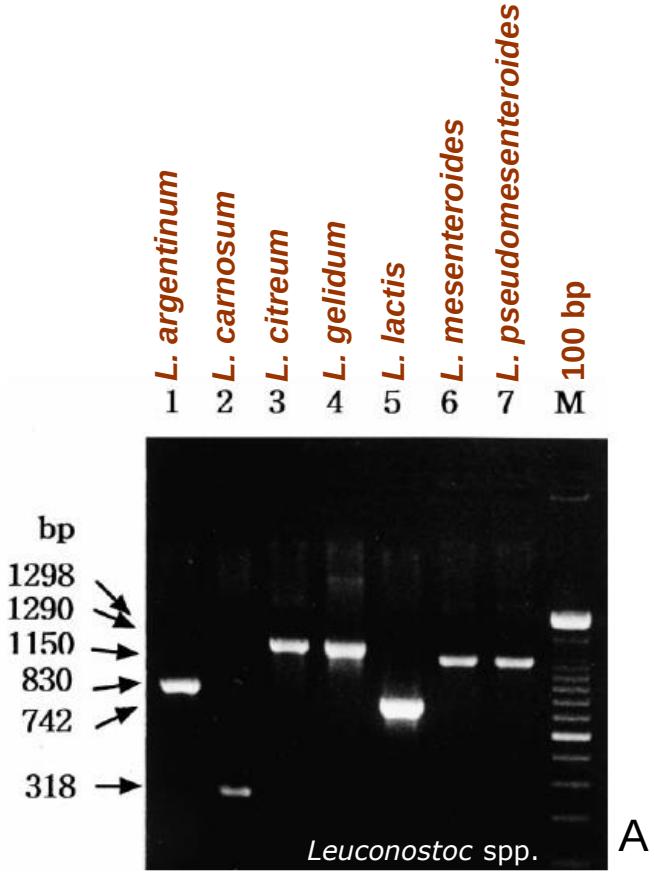
# Standardization of diagnostic PCR for detection of foodborne pathogens

| Criteria | Comment |
|---|---|
| Analytical and diagnostic accuracy | Low false-negative or false-positive results |
| Low detection limit | Less than one cell per 25 g |
| High robustness | Inter-lab reproducibility |
| Amplification controls | Reagent- and positive controls, internal amplification control |
| Low carry-over contamination risk | Separated working areas, UNG-treatment |
| High speed | At-line or on-line analysis |
| Acceptance | Validation and standardization, nonpatented primer sets |
| Low cost | Cost per analysis |
| Simplicity | User-friendliness and automation |
| Sample matrix flexibility | No PCR interference |
| Quantitative analysis | Food spoilage microorganisms |

*In* Malorny *et al.*, 2003

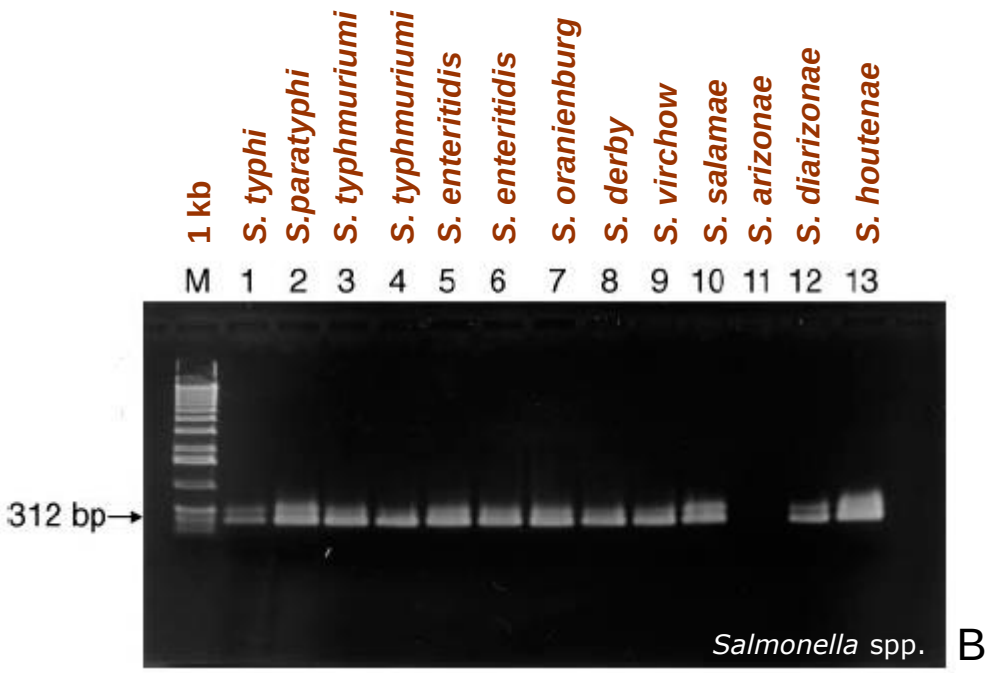
# Microorganisms isolation *vs.* direct detection in mixed samples

## PCR amplification – "single" molecular marker



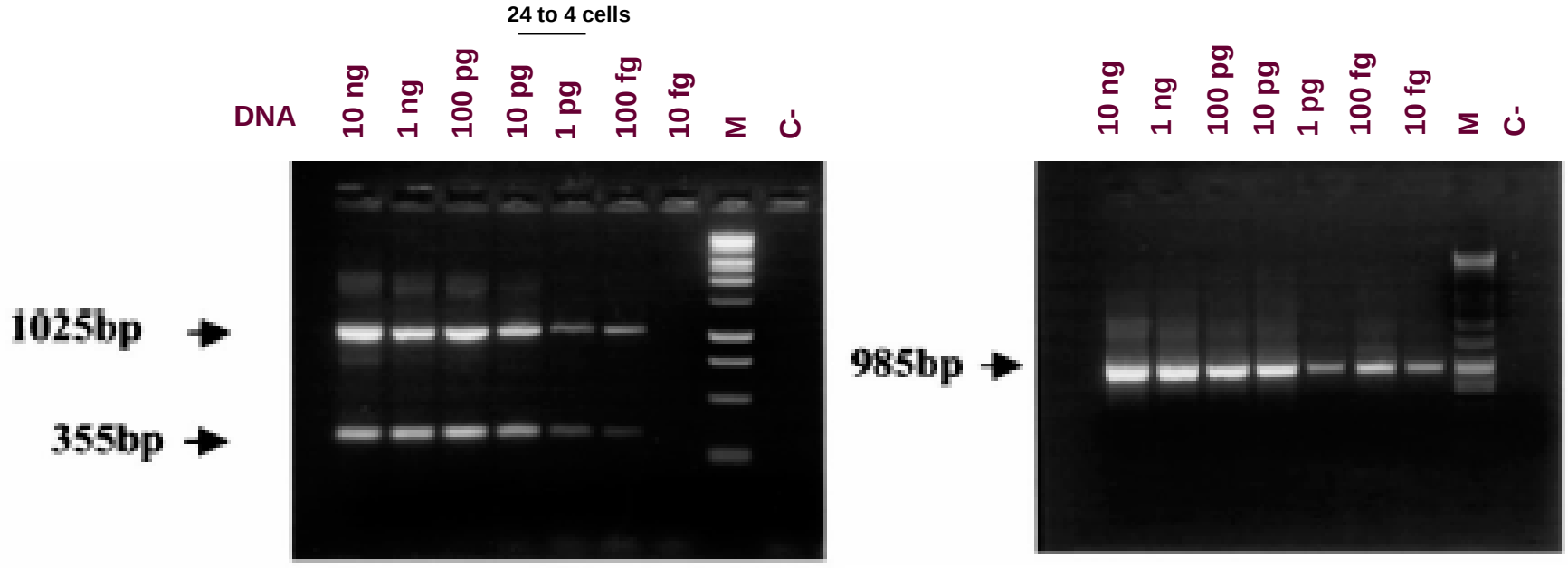

**PCR amplification products obtained from each species-specific primer set**

*In* Lee *et al.*, 2000




**Nested PCR amplification products obtained by lysed bacterial cultures**

*In* Waage *et al.*, 1999




## Detection threshold - Evaluating primers sensitivity

***S. aureus***

**NF-NR: universal primers for bacteria: 1025 bp**

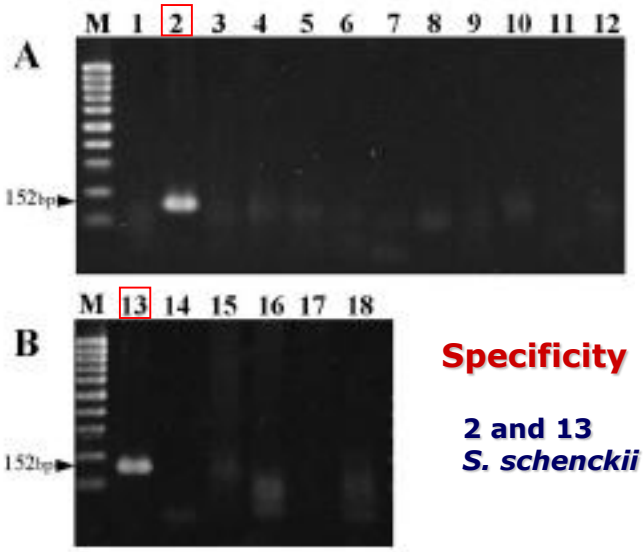
**P2F-NR: specific primers for bacteria G+: 355 bp**

***E. coli***

**NF-N6R: specific primers for bacteria G-: 985 bp**

*In* Carroll *et al.*, 2000

*In* Hu *et al.*, 2003




**Specificity**

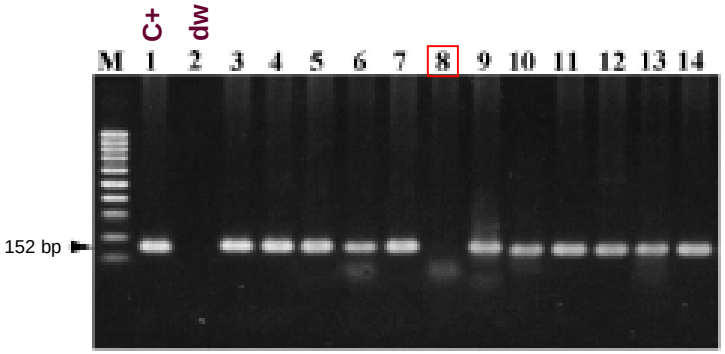
**2 and 13**
***S. schenckii***

**1 to 18: DNA from fungi, bacteria, mycobacterium and human skin**

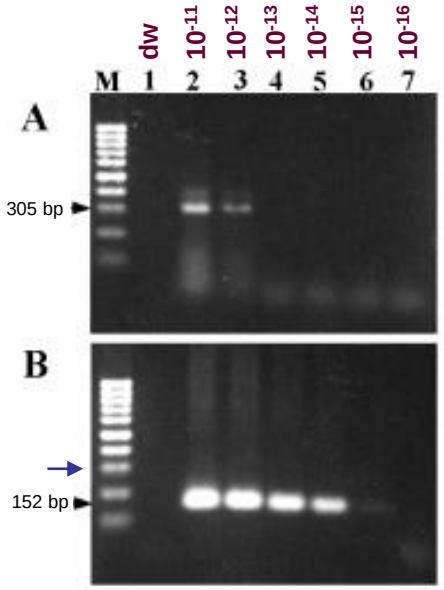
TABLE 1. Detection of *S. schenckii* in tail tissues of experimentally infected mice[a]

| Mouse no. | Result by: | | |
|---|---|---|---|
| | Histology (PAS stain) | First-round PCR | Nested PCR |
| 1 | + | + | + |
| 2 | − | − | + |
| 3 | − | − | + |
| 4 | − | − | + |
| 5 | + | + | + |
| C1 | ND | − | − |
| C2 | ND | − | − |

[a] C, control; +, positive; −, negative; ND, not done.




***Sporothrix schenckii* detection**
**DNA from 12 clinical samples**




**1st PCR**

**Sensitivity**

**DNA of *S. schenckii***

**Nested PCR**

# Molecular diagnosis: method evaluation

**Methods Results**

| Microorganism Presence | + | - | |
|---|---|---|---|
| + | A | B | *Se* |
| - | C | D | *Sp* |
| | *PV+* | *PV-* | |

A : True (+)

B : False (-)

C : False (+)

D : True (-)

***Sensitivity*** = A/(A + B) x 100%

***Specificity*** = D/(C + D) x 100%

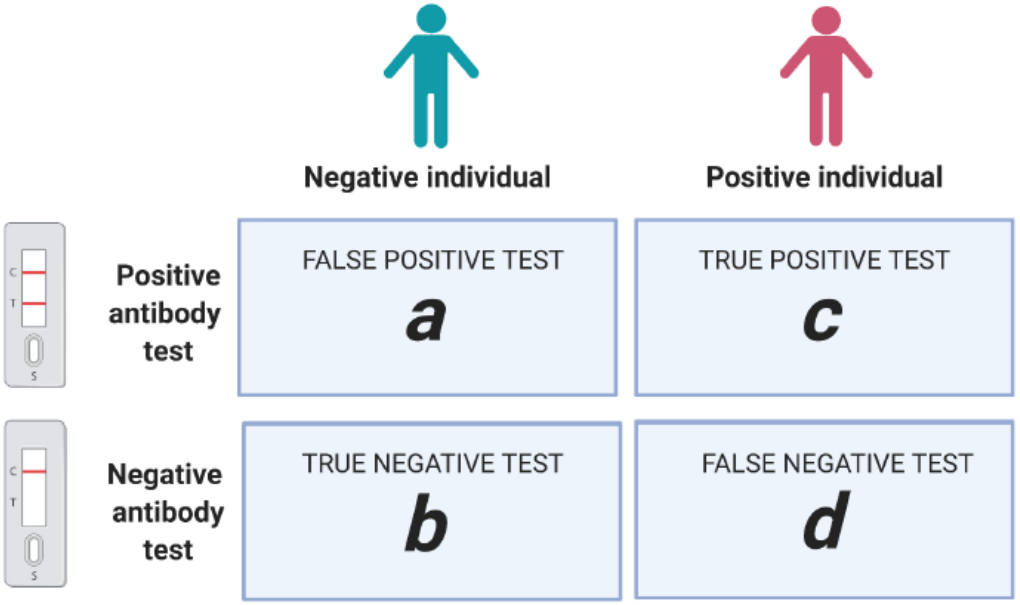
False (-) = 1 - Sensitivity

False (+) = 1 - Specificity

***PV (+)*** = A/(A + C) x 100%

***PV (-)*** = D/(B + D) x 100%

***PV*** = Predictive Value




***Sensitivity***

What proportion of individuals with prior exposure will test positive?

= c / (c+d) x100%

***Positive predictive value***

What proportion of all positive tests represent individuals who are truly positive?

= c / (a+c) x100%

***Specificity***

What proportion of individuals without prior exposure will test negative?

= b / (a+b) x100%

***Negative predictive value***

What proportion of all negative tests represent individuals who are truly negative?

= b / (b+d) x100%

*In* National COVID Testing Scientific Advisory Panel, 2020

# Reasons for Obtaining False-Positive and False-Negative Results in Molecular Diagnostic Assays

| Reasons for false-positive results | Reasons for false-negative results |
|---|---|
| Carry over contamination (amplicons) from previously amplified products | Inhibition of PCR reaction |
| Presence of exogenous target DNA in reagents, water, kits, sterile blood culture material | Inadvertent loss of template nucleic acid target owing to poor extraction, handling and storage protocols |
| Poor primer design (nonspecificity) | Digestion of nucleic acid template with endogenous DNAses and RNAses |
| Inadequate amplification conditions | Poor primer design (nonconserved regions at primer site[s] in variants) |
| Contamination from laboratory personnel | Poor intrinsic sensitivity of nucleic acid amplification/analysis detection system |
| | Poor sensitivity of nucleic acid amplification/analysis reaction |
| | Poor specificity |
| | Inadequate amplification conditions |

*In* Millar & Moore, 2004

**DNA amplification from dead organisms**

⇒ **diagnostically correct as positive**

⇒ **clinically as false-positive**

**Test controls necessary for performance of diagnostic PCR**

**Internal**

**Positive**

**Negative**

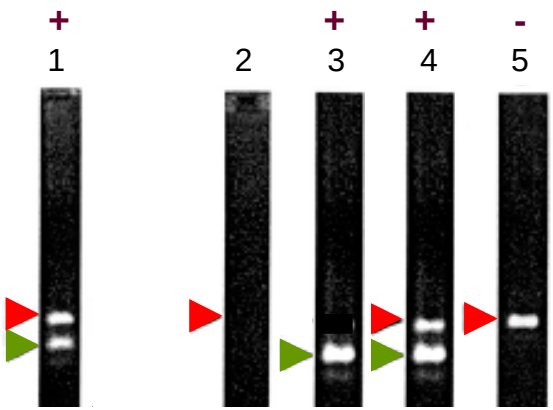
**No-template**

**Premise**

**Standard []**

| Type of control | Description |
|---|---|
| Internal amplification control (IAC) | Containing chimeric nonrelevant DNA added to master mixture and amplifiable by the same primer set as the target DNA, but resulting in an amplicon size visually distinguishable from that of the target DNA |
| Processing positive control (PPC) | A negative sample spiked with a sufficient amount of the pathogen and processed through the entire protocol |
| Processing negative control (PNC) | A negative sample spiked with a sufficient amount of a closely related, but nontarget strain processed through the entire protocol |
| Reagent (negative) control | Containing all reagents, but no nucleic acid apart from the primers |
| Premise control | A tube containing the master mixture left open in the PCR setup room to detect possible contaminating DNA in the environment (to be done at regular intervals as part of the quality assurance program) |
| Standard concentrations | Three to four samples containing serial 10-fold dilutions of a known number of target DNA copies in a range above the detection limit (necessary only for quantitative PCR) |

*In* Malorny *et al.*, 2003

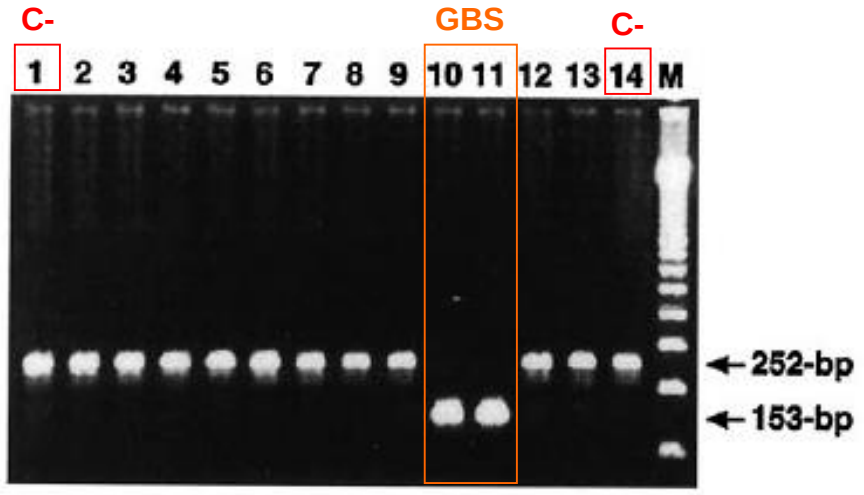



**2** - PCR inibition

**3** = 2 purified **4** = 2 diluted

**Coamplification of *Leishmania* DNA from clinical samples and one positive control (540 bp)**

Adapted from Marfurt *et al.*, 2003




GBS = "group B streptococci" or *Streptococcus agalactiae*

**Signal of PCR GBS-specific = 153 bp**

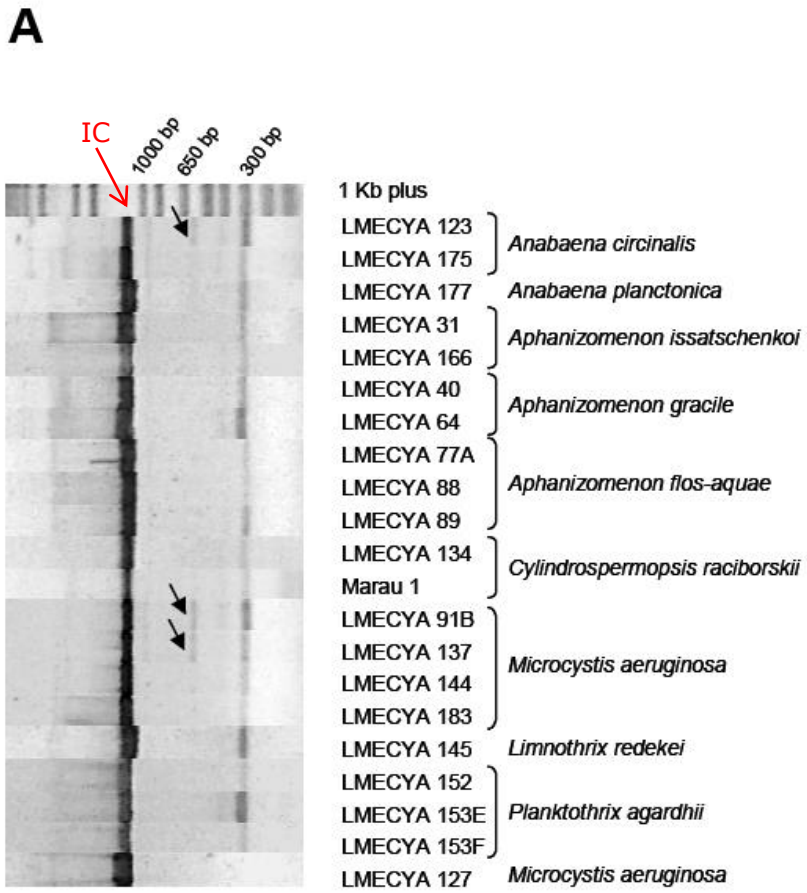
**Internal control = 252 bp**

**10 and 11 = positive samples**

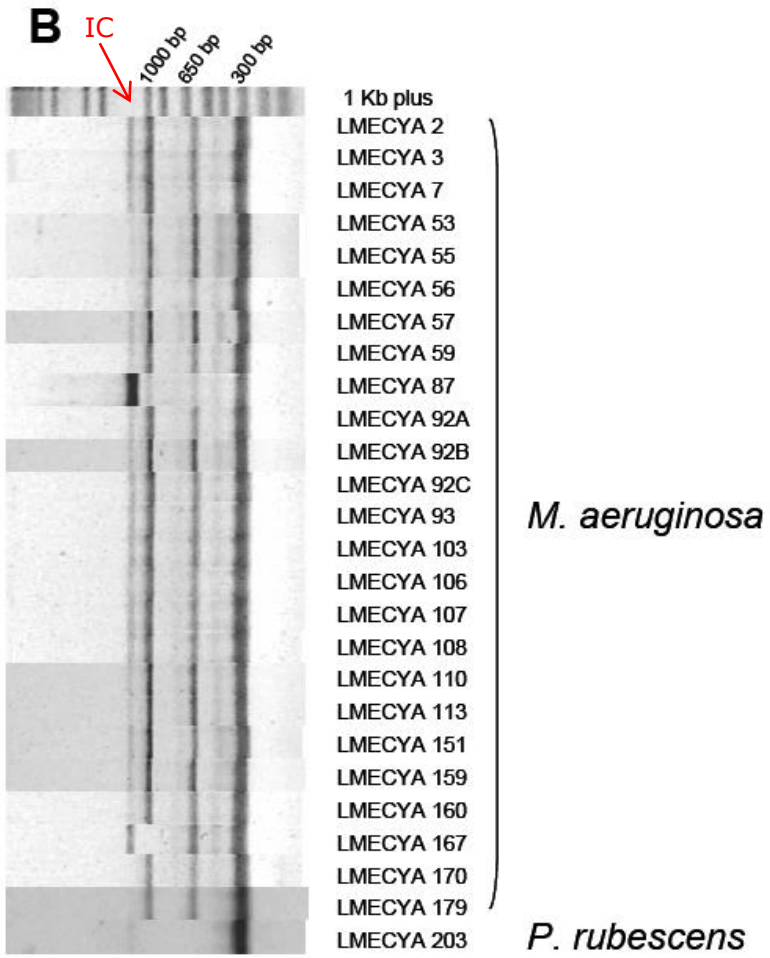
*In* Ke *et al.*, 2000

# Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples

*In* Valério, 2008

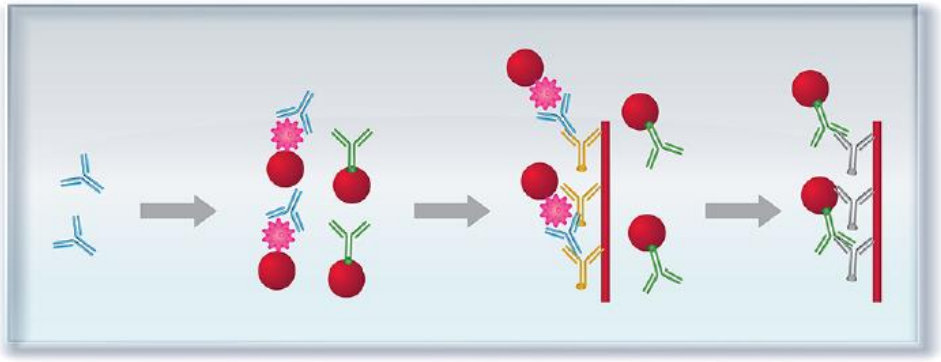



**Multiplex PCR assay for the simultaneous amplification of *mcy*A-cd, *mcy*AB and *mcy*B in 21 non-toxic isolates**




**Multiplex PCR assay results of the 26 toxic isolates**

**The amplification of a cyanobacterial 16S rDNA gene fragment was also included as a PCR internal control**



**Lateral Capillary Flow (Nitrocellulose Membrane)**

- Human anti-SARS-CoV-2 antibody
- SARS-CoV-2 antigen
- Tag
- Control antibody to validate assay
- Immobilized anti-human antibody
- Immobilized antibody against control antibody

**Lateral flow immunoassay for detection of anti-SARS-CoV-2 antibodies.**

- Samples move via capillary flow on the nitrocellulose membrane.
- When **anti-SARS-CoV-2 antibodies** are present, they bind to the **labeled antigen** and continue to move until they are captured by the **immobilized antihuman antibodies**.
- The presence of the captured antibody−antigen complex is visualized as a colored **test band**.
- The **labeled control antibodies** comigrate until they are **captured** at the control band.

Carter *et al.*, 2020

# Reliability of *mcy*-based PCR methods tested for diagnosis of microcystin-producing isolates

## HPLC analysis of microcystins was used as the "gold standard"

| Parameter | No. of isolates based on mcyA-cd PCR | No. of isolates based on mcyAB PCR | No. of isolates based on mcyB PCR | No. of isolates based on multiplex PCR |
|---|---|---|---|---|
| True positive | 10 (15.2)[c] | 10 (15.2) | 9 (13.6) | 24 (19.4) |
| False positive | 7 (10.6) | 5 (7.6) | 7 (10.6) | 0 (0) |
| True negative | 49 (74.2) | 51 (77.3) | 49 (74.2) | 98 (79.0) |
| False negative | 0 (0) | 0 (0) | 1 (1.5) | 2 (1.6) |
| Sensitivity | 100.0 | 100.0 | 90.0 | 92.3 |
| Specificity | 87.5 | 91.1 | 87.5 | 100.0 |
| PPV[a] | 58.8 | 66.7 | 56.3 | 100.0 |
| NPV[b] | 100.0 | 100.0 | 98.0 | 98.0 |
| Number of tested isolates | 66 | 66 | 66 | 124 |

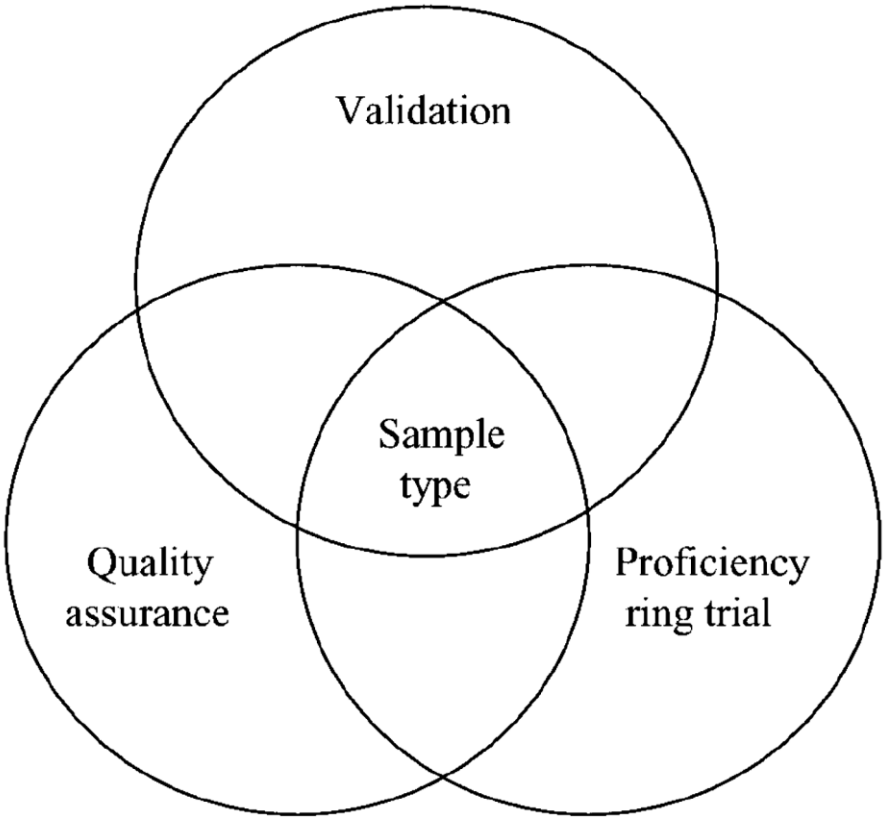
[a]PPV, predictive value of positive test; [b]NPV, predictive value of negative test. [c]Values in parentheses refer to percentages of the total of tested isolates.

*In* Valério, 2008

# An integrated approach to establishment of diagnostic PCR

## Validation

**demonstrating that the new method can generate results that are comparable - if not better - to those obtained by the current reference method**




*In* Hoorfar *et al.*, 2004

# PCR Workflow

The workflow needs to be well defined and procedures to prevent contamination must be strictly followed

| | |
|---|---|
| **1** | Nucleic acid extraction and specimen processing |
| **2** | PCR mastermix reaction setup only |
| **3** | The specimen nucleic acid is added to the PCR mastermix |
| **4** | Positive control material is added |
| **5** | PCR instruments |

# Are Molecular Tools Solving the Challenges Posed by Detection of Plant Pathogenic Bacteria and Viruses?

*In* López *et al.*, 2009

## Comparison of sensitivity, specificity, feasibility, rapidness and cost of different techniques in detection of plant pathogenic bacteria and viruses

| Technique | Sensitivity[a] | Specificity[b] | Feasibility[c] | Rapidness | Cost |
|---|---|---|---|---|---|
| Molecular hybridisation | +[d] | ++++ | ++ | + | +++ |
| FISH | ++ | ++ | +++ | + | ++ |
| Conventional PCR | +++ | ++++ | +++ | +++ | +++ |
| Nested PCR in a single tube | ++++ | ++++ | +++ | ++ | +++ |
| Cooperational-PCR[e] | ++++ | ++++ | +++ | +++ | +++ |
| Multiplex PCR | +++ | ++++ | +++ | +++ | +++++ |
| Multiplex nested PCR | ++++ | ++++ | ++ | +++ | ++++ |
| Real-time PCR[f] | +++++ | +++++ | ++++ | +++++ | +++ |
| NASBA[g] | +++++ | ++++ | ++++ | ++++ | ++ |
| LAMP | ++++ | ++++ | +++ | ++++ | ++ |
| Microarrays | + | +++++ | + | ++ | + |

[a] Sensitivity: probability of detecting true positives.

[b] Specificity: probability of detecting true negatives.

[c] Feasibility: practicability in routine analysis, execution and interpretation.

[d] The number of + symbols indicates how methods rate regarding each considered criterion, from acceptable (+) to optimum (+++++).

[e] Coupled with hybridisation and colorimetric detection.

[f] Using TaqMan probes.

[g] Using Molecular Beacons probes.

# Advantages and disadvantages of detection methods

| S. no | Detection method | Advantages | Disadvantages |
|---|---|---|---|
| 1 | Culture-dependent method | Ability to detect single bacterial strain<br>Recognition of viable cells<br>Appropriate toward suitable media | Risk for contamination<br>Intense requirement of time and resource |
| 2 | Culture-independent method | Accurate and multiplex detection | High-level skill is required in obtaining results |
| 3 | Piezoelectric biosensor | High amplitude and frequency range | Depends on temperature<br>Less energy efficiency |
| 4 | Bioluminescence biosensor | Simplicity, inexpensiveness and high intensity | Depends on the process of emission of light |
| 5 | Electrochemical biosensor | Portable system, faster response and high stability | Performance is influenced by temperature and ph |
| 6 | Optical biosensor | Faster optimization and easy design | Only optimization and no quantification |
| 7 | Spectroscopic techniques | Sensitive toward the surface of the molecule | Interference of fluorescence and time-consuming |
| 8 | Gas chromatography–mass spectrometry (GC–MS) | Volatile and nonvolatile compounds can be analyzed<br>Accuracy and low running cost | Sample preparation is difficult |
| 9 | Enzyme-linked immunosorbent assay (ELISA) | Quick and easy to carry out<br>Highly specific | Difficulty is faced in measuring the enzyme activity |
| 10 | Enzyme-linked fluorescent assay (ELFA) | Much sensitive toward biochemical tests | Depends on the emission of fluorescence |
| 11 | Immunomagnetic separation (IMS) | Effective isolation of cells from fluids | Preparation of beads is required in the case of magnetic beads separation |
| 12 | DNA microarray | High throughput<br>Analysis of multiple genes | Confusing to first time users<br>Results are not reproducible |
| 13 | DNA hybridization | Reduced cost, rapid detection | Based on DNA assay |
| 14 | Multiplex PCR | Less time and effort consumption | Optimization is difficult and increased cost |
| 15 | Real-time PCR | Simple and rapid data analysis | Emission spectra overlapping and nonspecific binding |

*GC–MS* gas chromatography–mass spectrometry, *ELISA* enzyme-linked immunosorbent assay, *ELFA* enzyme-linked fluorescent assay, *IMS* immunomagnetic separation, *DNA* deoxyribonucleic acid, *PCR* polymerase chain reaction

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# Definition of terms used in validation of PCR testing

| | |
|---|---|
| Validation | Results obtained by PCR should be comparable to those obtained by the reference method. |
| Qualitative PCR | The test response is either the presence or absence of PCR product (amplicon), detected either by observation or with equipment. |
| Quantitative PCR | The test response can be correlated with the DNA copy number of amplicon, related to the number of target microorganisms. |
| Detection limit (DL) | The smallest number of culturable target microorganisms necessary to create a PCR-positive response. |
| Selectivity | Measure of inclusivity of target strains (from a wide range of strains), and exclusivity (the lack of amplicon from a relevant range of closely related non-target strains). |
| Positive deviation (PD) | PCR-positive case when the reference method gives a negative result (false positive). |
| Negative deviation (ND) | PCR-negative case when the reference method gives a positive result (false negative). |
| Positive agreement (PA) | Sample positive by both PCR and the reference method. |
| Negative agreement (NA) | Sample negative by both PCR and the reference method. |
| Diagnostic accuracy (AC) | Degree of correspondence between the response obtained by PCR and the response obtained by the reference method on identical samples (AC=(PA+NA)/ total number of samples). |
| Diagnostic sensitivity (SE) | Ability of PCR to detect the microorganism when it is detected by the reference method ((PA/N+) ×100). |
| Diagnostic specificity (SP) | Ability of PCR to not detect the microorganism when it is not detected by the reference method ((NA/N−) ×100). |
| Robustness | Reproducibility by other laboratories using different batches and brands of reagents and validated thermal cyclers and equipment. |

N− is the total number of negative results with the reference method.
N+ is the total number of positive results with the reference method.

*In* Hoorfar *et al.*, 2004

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*In this article some sentences were highlight, they are related to the subject presented in the slides.*