INSTITUTE OF APPLIED BIOSCIENCES
ΙΝΣΤΙΤΟΥΤΟ ΕΦΑΡΜΟΣΜΕΝΩΝ ΒΙΟΕΠΙΣΤΗΜΩΝ
CERTH

Detection of food pathogens

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Institute of Applied Biosciences - CERTH

SOUTH EAST EUROPE
Jointly for our common future

INNO-FOOD SEE

History

- Early 1900's
  - Contaminated food, milk and water caused many foodborne illnesses
- Sanitary revolution
  - Sewage and water treatment
  - Hand-washing, sanitation
  - Pasteurization of milk- 1908
  - Refrigeration in homes- 1913

History

- Animals identified as a source of foodborne pathogens
  - Improved animal care and feeding
  - Improved carcass processing
- Surveillance and research
- Outbreak investigations
- Laws and policies regarding food handling

Bacterial Diseases

- Airborne Bacterial Diseases
- Foodborne & Waterborne Bacterial Diseases
- Soilborne Bacterial Diseases
- Arthropodborne Bacterial Diseases
- Sexually Transmitted Bacterial Diseases
- Miscellaneous Bacterial Diseases

Foodborne Intoxications vs Infections

Foodborne intoxications: Caused by the exotoxin secreted by bacteria in contaminated food

Foodborne infections: Caused by the ingestion of live bacteria that colonize the digestive tract

Epidemiology

- Foodborne diseases each year in US
  - Affects 1 in 4 Americans
  - 76 million illnesses
  - 325,000 hospitalizations
  - 5,000 deaths
    - 1,500 of those deaths caused by *Salmonella*, *Listeria*, and *Toxoplasma*

Epidemiology

- Many unrecognized or unreported
  - Mild disease undetected
  - Same pathogens in water and person to person
  - Emerging pathogens unidentifiable
- Greatest risk
  - Elderly
  - Children
  - Immunocompromised

Foodborne & Waterborne Bacterial Diseases

- Botulism
- Staphylococcal Food Poisoning
- Clostridial Food Poisoning
- Typhoid Fever
- Salmonellosis
- Shigellosis
- Cholera
- Diseases associated with Escherichia coli
- Camphylobacteriosis and Helicobacteriosis

Foodborne diseases

- **Botulism**
  - *Clostridium botulinum*
  - *Clostridium perfringins*
  - *Clostridium tetani*
- **Staphylococcal Food Poisoning**
  - *Staph. aureus*
  - *Staph epidermidis*
- **Clostridial Food Poisoning**
  - *Clostridium perfringins*
- **Typhoid Fever**
  - *Salmonella typhi*
- **Salmonellosis**
  - *Salmonella enteriditis*
  - *Salmonella gallinarum*
  - *Salmonella typhimurum*

Foodborne diseases

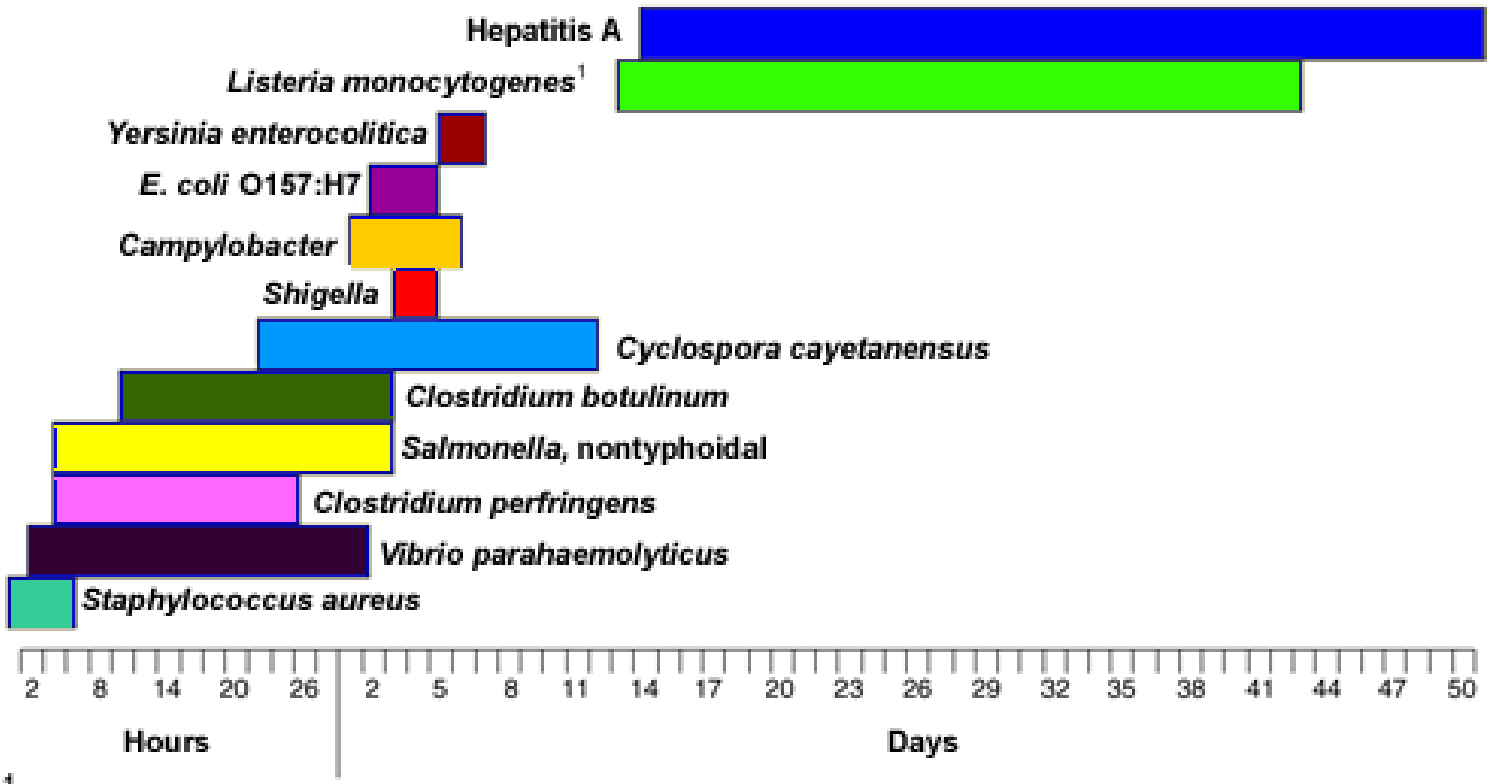
- **Shigellosis**
  - *Shigella sonnei*
  - *Shigella dysenteriae*
  - *Shigella flexneri*
  - *Shigella boydii*
- **Cholera**
  - *Vibrio cholerae*
  - *Vibrio parahaemolyticus*
- **Diseases associated with *Escherichia coli***
  - *E. coli strain O157:H7*
- **Camphylobacteriosis and Helicobacteriosis**
  - *Camphylobacter jejuni*
  - *Helicobacter pylori*

Soilborne Bacterial Diseases

- Anthrax
- Tetanus
- Gas Gangrene
- Leptospirosis
- Listeriosis (*Listeria monocytogenes)*

Incubation period of foodborne diseases

Figure 1
Usual incubation period ranges for select foodborne diseases



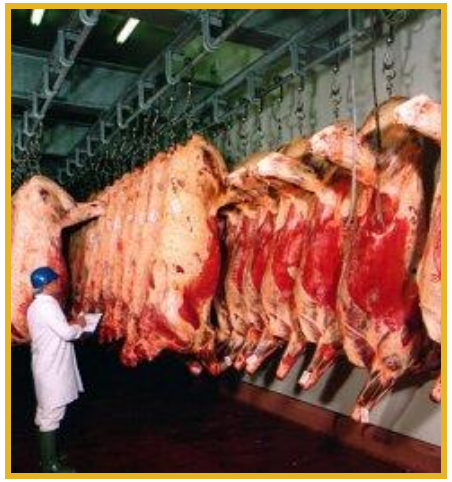
[1] Invasive form, incubation period for diarrheal disease unknown.
Source: Data on the "usual" incubation period obtained from the Centers for Disease Control and Prevention, "Surveliance for Foodborne Disease Outbreaks--United States, 1988-1992." MMWR 45, SS-6 (Oct. 25,1996):58-66

Table 1.1: Common Pathogenic Microorganisms

Microorganism	Associated Foods	Infective Dose (no. of organisms)	Incubaton Period
Campylobacter jejuni	Raw milk, and raw or under-cooked meat, poultry & shellfish	400-500	2 to 5 days
Salmonella spp.	Raw/undercooked eggs, poultry, and meat; raw milk and dairy products; seafood; chocolate; salad and spices	15–20	12 to 24 h
E. coli	Raw/undercooked eggs, poultry, and meat; raw milk and dairy products;	<10	2 to 4 days
L. monocytogenes	Soft cheese, raw milk, improperly processed ice cream, raw leafy vegetables; raw meat and poultry	< 1000	2 days to 3 weeks
Clostridium botulinum	Improperly canned foods and vacuum packaged and tightly wrapped food	< nano grams	12-36 h
Hepatitis A virus	Sandwiches, fruits and fruit juices, milk and milk products, vegetables, salads, shellfish, and iced drinks	10-100	Unknown
Norwalk virus/Norovirus	Raw oysters/shellfish, water and ice, salads, and frosting	Presumed to be low	1-2 days

At the Slaughter Plant

- FSIS target organisms
  - *Salmonella* and *E. coli*
- Control points
  - Removal of internal organs
  - Minimize contact between carcasses
  - Proper movement through facilities
  - Chilling
  - Cooking processes (time, temperature)



Irradiation

- Used since 1986 for *Trichina* control in pork
- Gamma rays
  - Poultry in 1990/1992
  - Meat in 1997/1999
  - Reduction of bacterial pathogens
- Kills living cells of organisms
  - Damaged and cannot survive

In the Home

- Drink pasteurized milk and juices
- Wash hands carefully and frequently
  - After using the bathroom
  - Changing infant's diapers
  - Cleaning up animal feces
- Wash hands before preparing food

In the Home

- Wash raw fruits and vegetables before eating
- After contact with raw meat or poultry
  - Wash hands, utensils and kitchen surfaces
  - Hot soapy water
- Defrost meats in the refrigerator

On Farm Strategies

- Testing and removal for *Salmonella*
  - Serologic, fecal culture, hide culture
- Vaccinating
  - Many serotypes
  - Varying effectiveness
- Minimize rodents, wild birds
- Isolation of new animals

Microbiological testing of foods

- an integral part of food production,
- most often applied for end-product control
  - ineffective because of:
    - logistical complexities in sampling and the heterogeneous distribution of contamination.
    - The implementation of the hazard analysis and critical control points (HACCP)
      - shifted the burden from testing to process control to ensure food safety (FAO⁄WHO, 2003).
- However
  - microbiological testing remains a critical tool in process control monitoring, quality control, surveillance, risk assessment.
  - Moreover, environmental sampling and analysis have become routine in food production as well as in outbreak tracing and tracking

Reasons for Testing

Primary purposes

- to establish the absence of pathogens or their toxins
- to ensure the safety of foods, and to enumerate total or indicator microbial load
- to monitor effectiveness of hygienic processing and to verify product quality and shelf-life stability

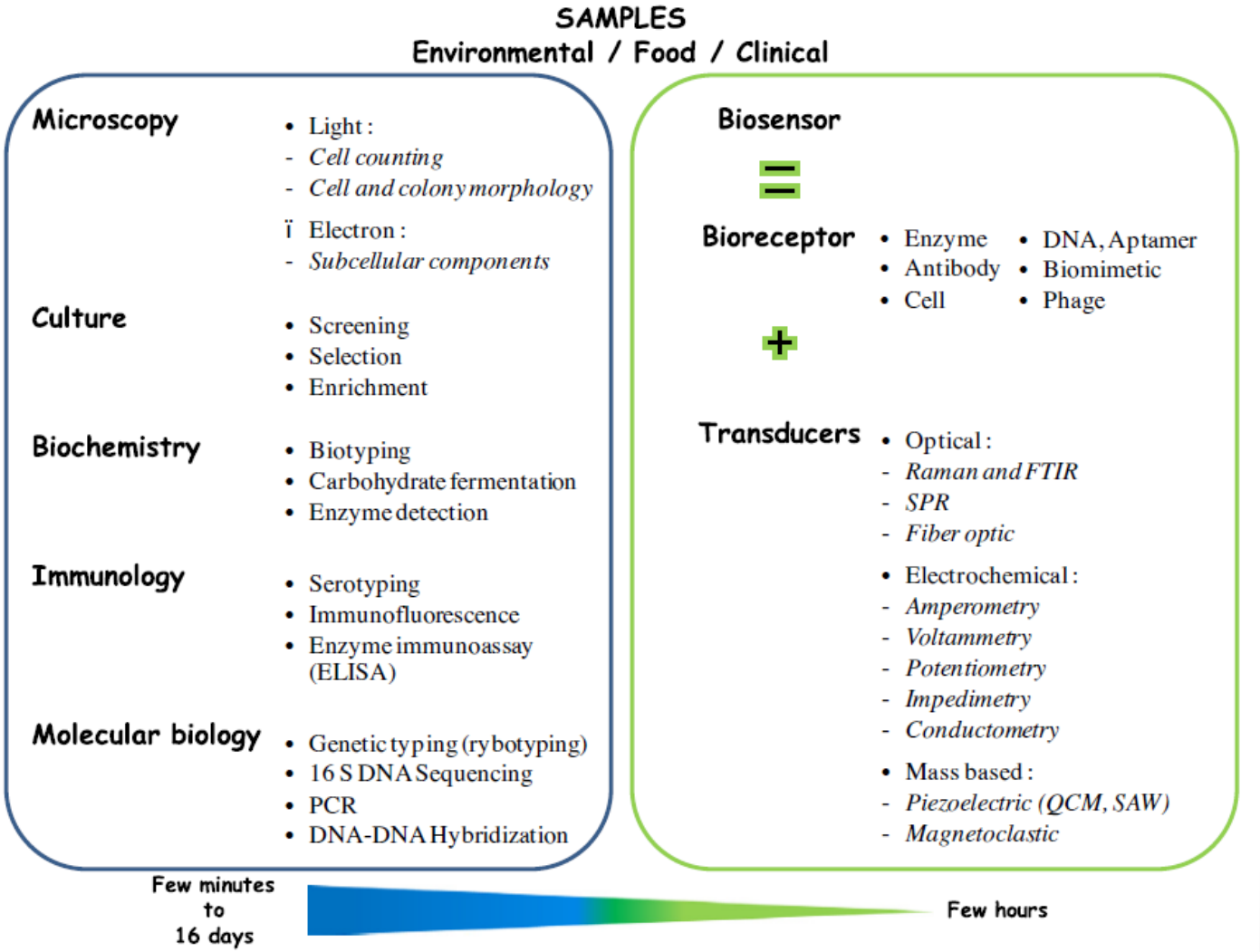
Secondary purposes

- Food safety emphasis on the farm-to-fork approach has increased field testing
- increased number of tests for large-scale data collection for risk assessment
- surveillance and monitoring at the primary production levels
- the increasing need of food producers to quantify the level of pathogens in food samples.

Methods

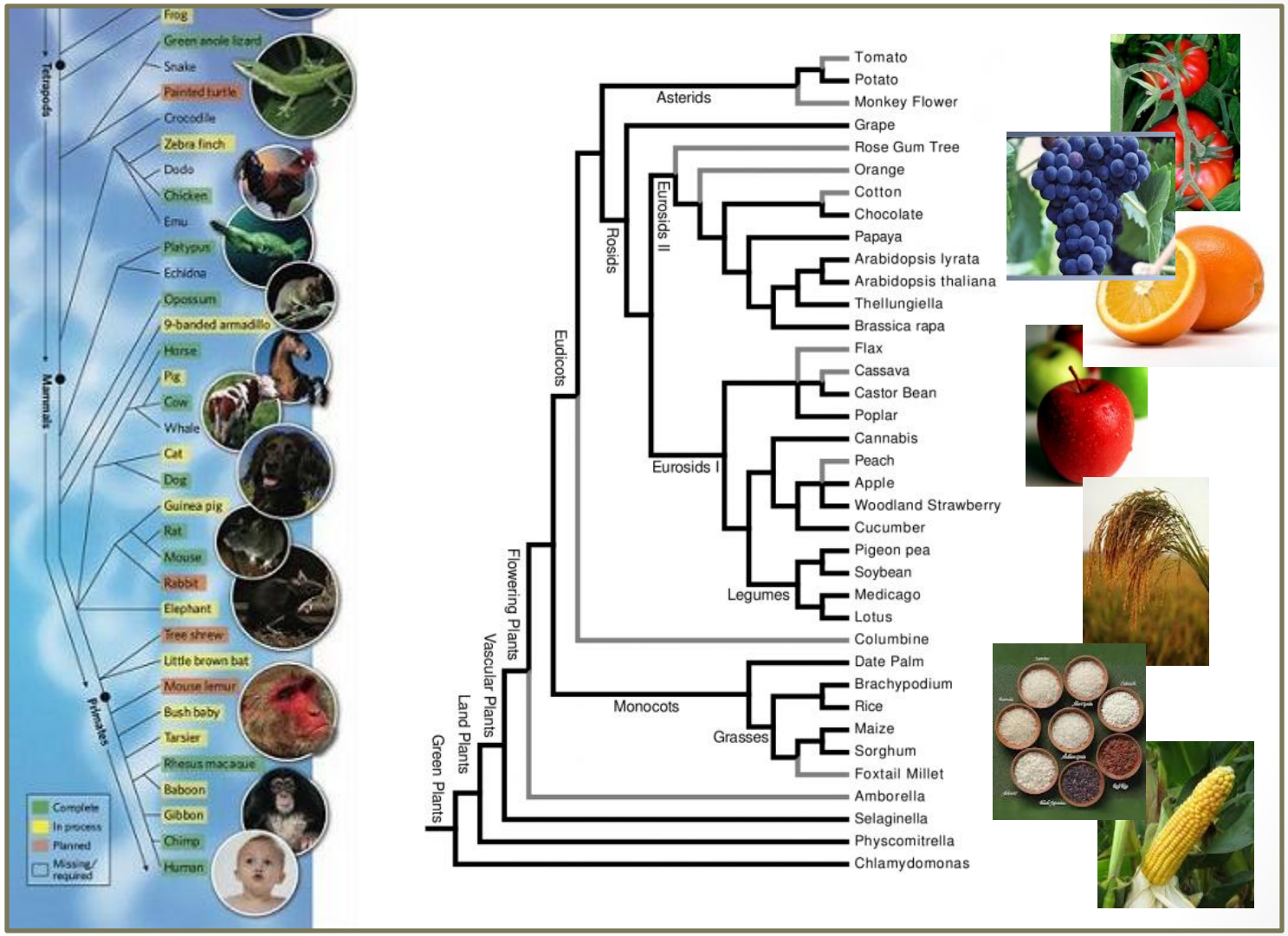
1. Detection method must be rapid
2. The method should detect the desired specific pathogen
3. Method must be sensitive to detect small numbers of pathogens
4. The detection method should produce a quantitative analysis to help determine the severity of the hazard
5. The method should be multiplex (i.e., capable of detecting more than one contaminant simultaneously)

Testing Methods

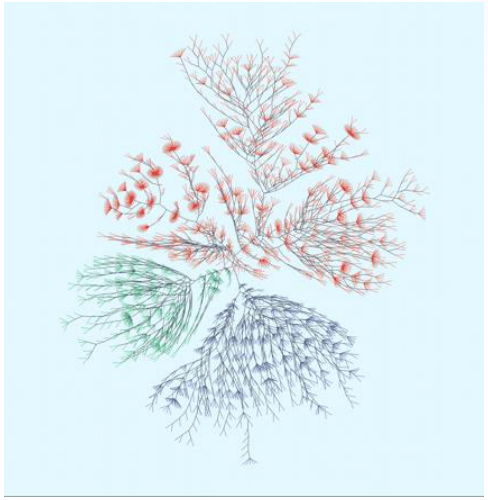


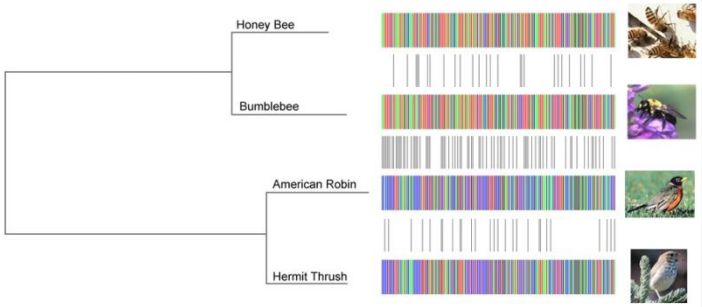
Paniel, N., et al.. Methods, 2013.

Sequenced Genomes



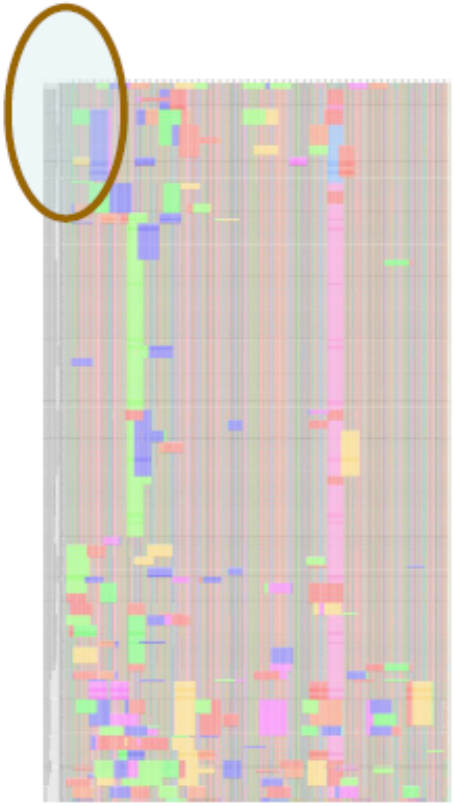
Bar Coding DNA





Barcode searching

Full length barcodes

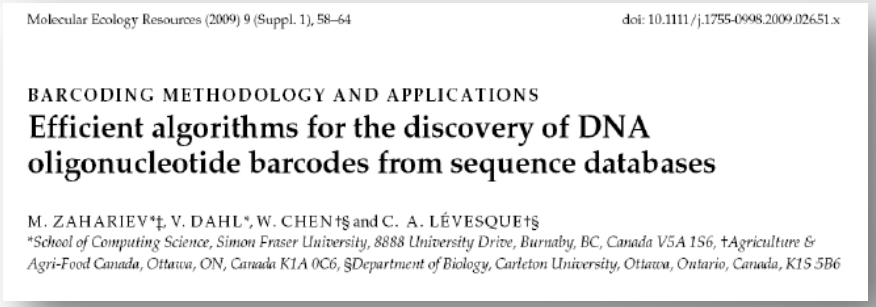


Microcodes

Summerbell, Seifert, Levesque, et al. (2005)
Phil. Trans. R. Soc. B (360), 1897-1903.

Comparative sequence based identification is only meaningful if:

- well-curated, robust and reliable databases are available
- that are populated with sequence data from:
  - type or reference strains (where possible)
  - a wide range of clinical strains
  - a wide variety of target species
- the strains have been rigorously validated in terms of their nomenclature



Molecular Ecology Resources (2009) 9 (Suppl. 1), 58–64 doi: 10.1111/j.1755-0998.2009.02651.x

BARCODING METHODOLOGY AND APPLICATIONS

Efficient algorithms for the discovery of DNA oligonucleotide barcodes from sequence databases

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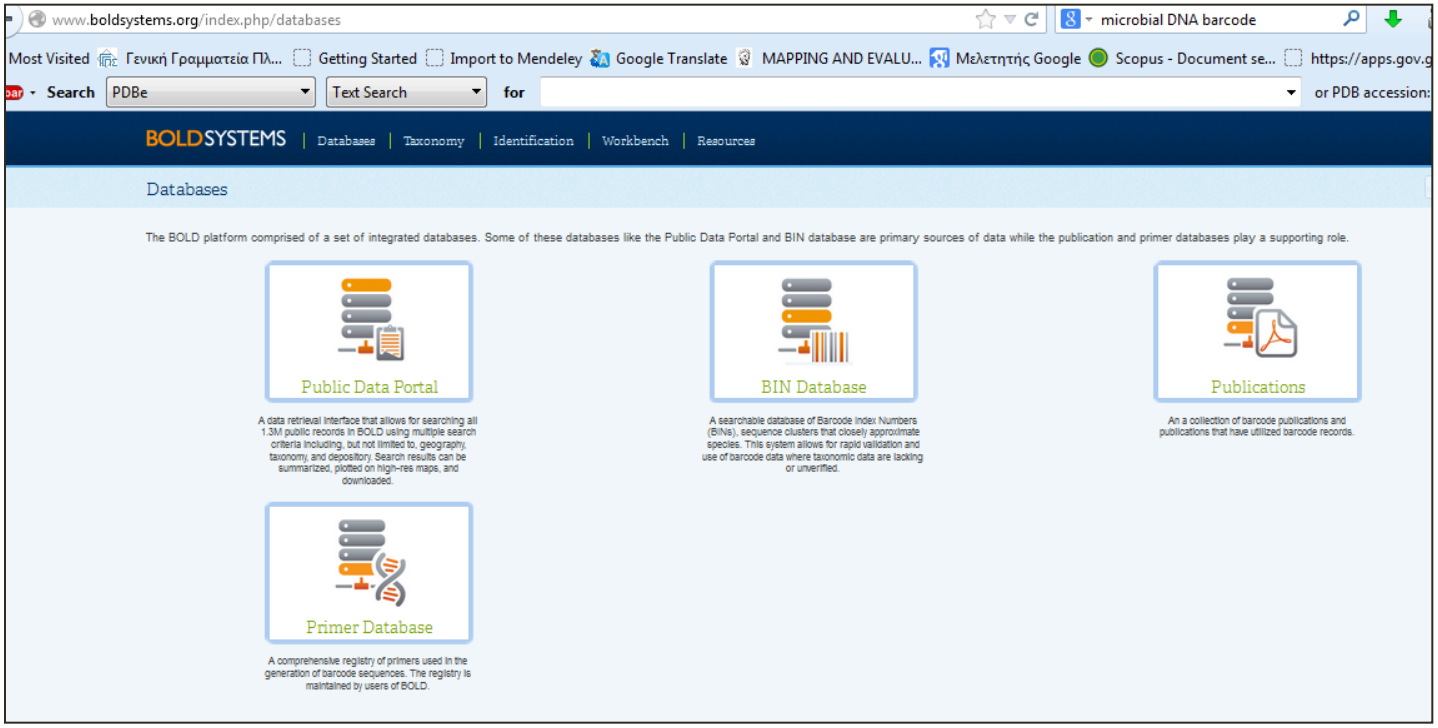
Which area of DNA?

Several loci have been suggested, a common set of standardized regions were selected by the respective communities:

- For **animals** and many other eukaryotes, the mitochondrial **COI** gene
- For **land plants**, the concatenation of the **rbcL** and **matK** chloroplast genes
- For **fungi**, the **internal transcribed spacer (ITS**) region
- For **bacteria**, the **16S** ribosomal genes

Barcode databases



www.boldsystems.org

BOLD is a web based workbench and database supporting the acquisition, storage, analysis, and publication of DNA barcode records. By assembling molecular, morphological, and distributional data, it bridges a traditional bioinformatics chasm. BOLD is the most prominently used barcoding software and is freely available to any researcher with interests in DNA barcoding.

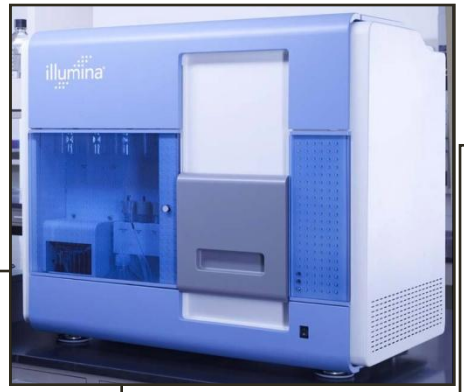
Molecular Biology Methods

Nucleic acid based

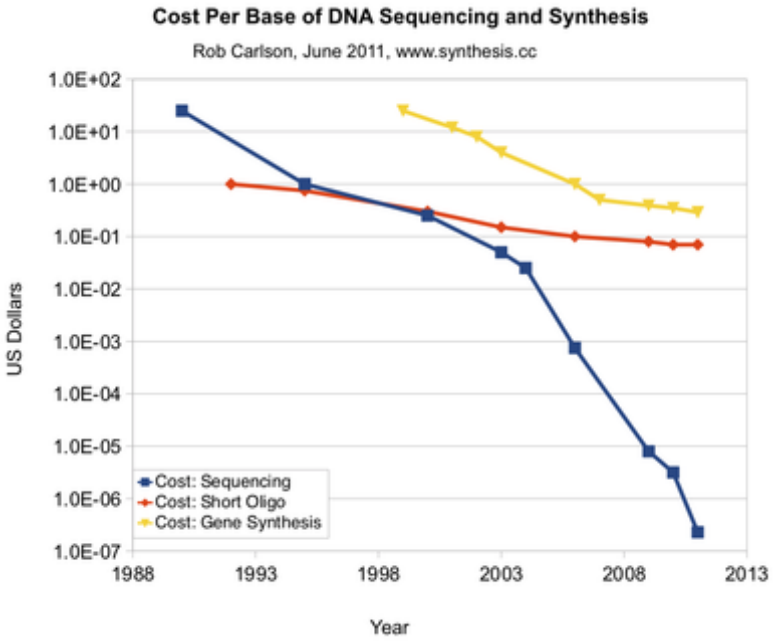
- Next Generation Sequencing
- Oligonucleotide DNA microarrays
- Nucleic acid sequence based amplification (NASBA)
- Ligation detection reaction-universal arrays (LDR-UA)
- Fragment analysis (PFGE, SSR, RAPD, iSSR etc)
- Polymerase chain reaction methods
- Real Time Quantitative PCR (TaqMan probes, HRM etc)
- loop-mediated isothermal amplification (LAMP)

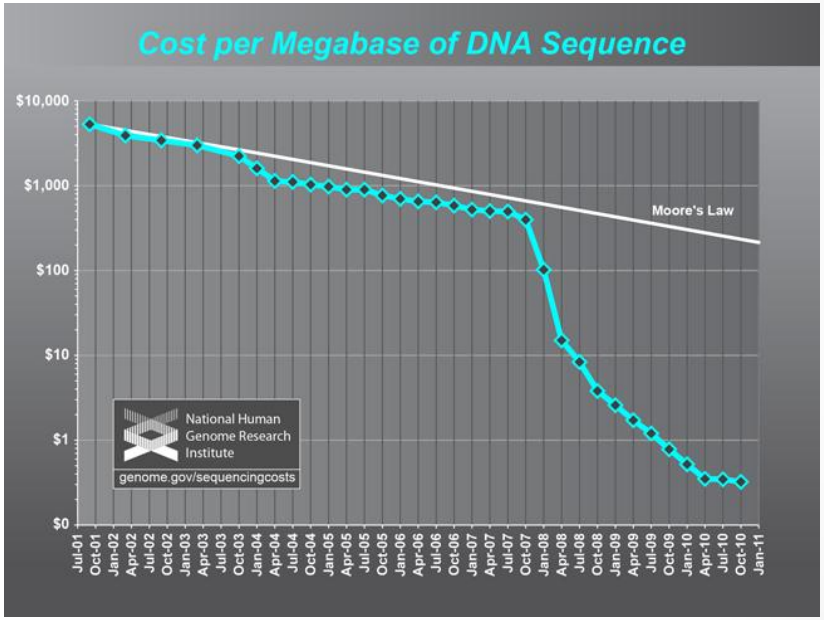
Sequencing Platforms

- Roche/454 FLX: 2004
- Illumina Solexa Genome Analyzer: 2006
- Applied Biosystems SOLiD™ System: 2007
- Helicos Heliscope™ : 2009
- Pacific Biosciences SMRT: 2010
- Ion Torrent: 2011
- Ion Proton 2012



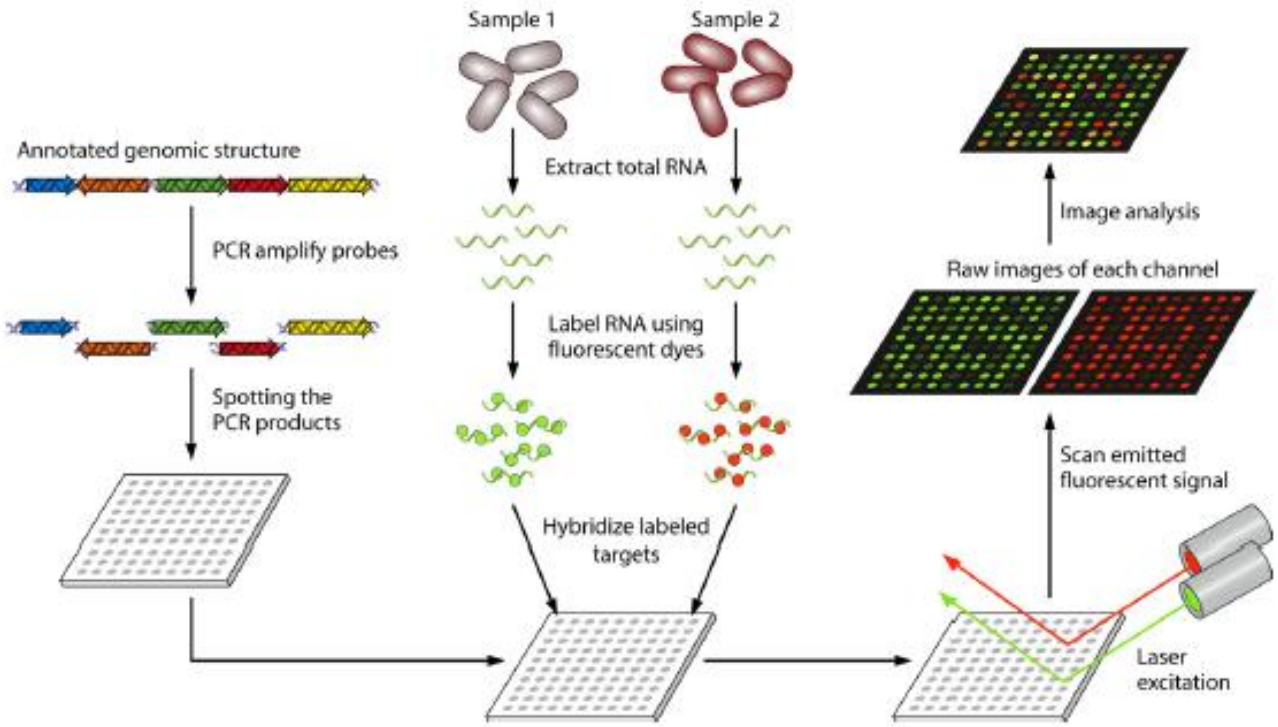
Sequencing Costs





High cost, time consuming, high level of expertise

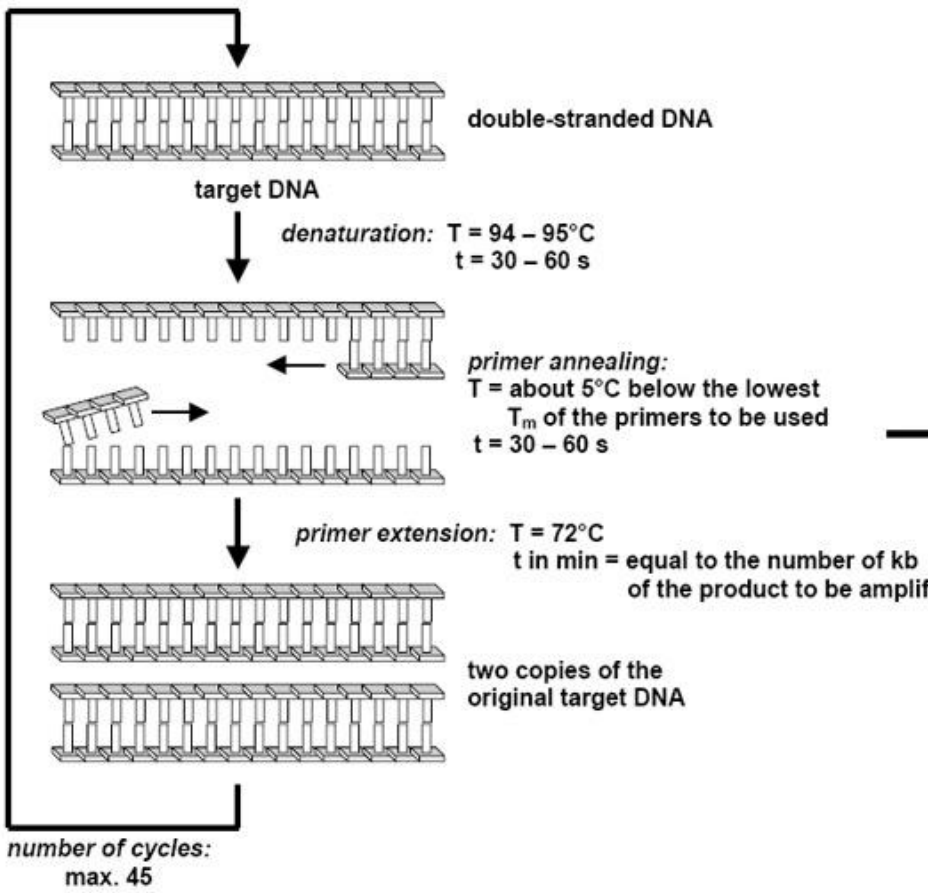
DNA microarrays



Miller, M.B. and Y.W. Tang Clin Microbiol Rev, 2009. **22**(4): p. 611-33.

Labeling needed, high costs, time consuming, high level of expertise

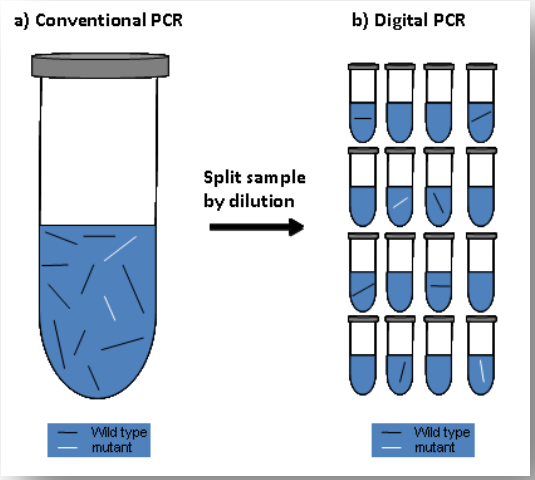
Polymerase Chain Reaction (PCR)



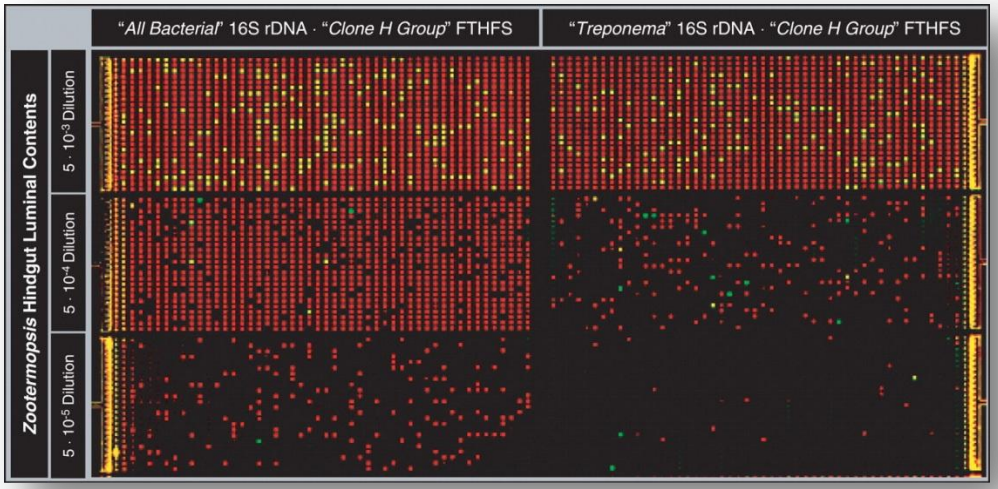
cycle number	target copies
1	2
2	4
3	8
4	16
5	32
6	64
20	1,048,576
30	1,073,741,824

Contamination prone

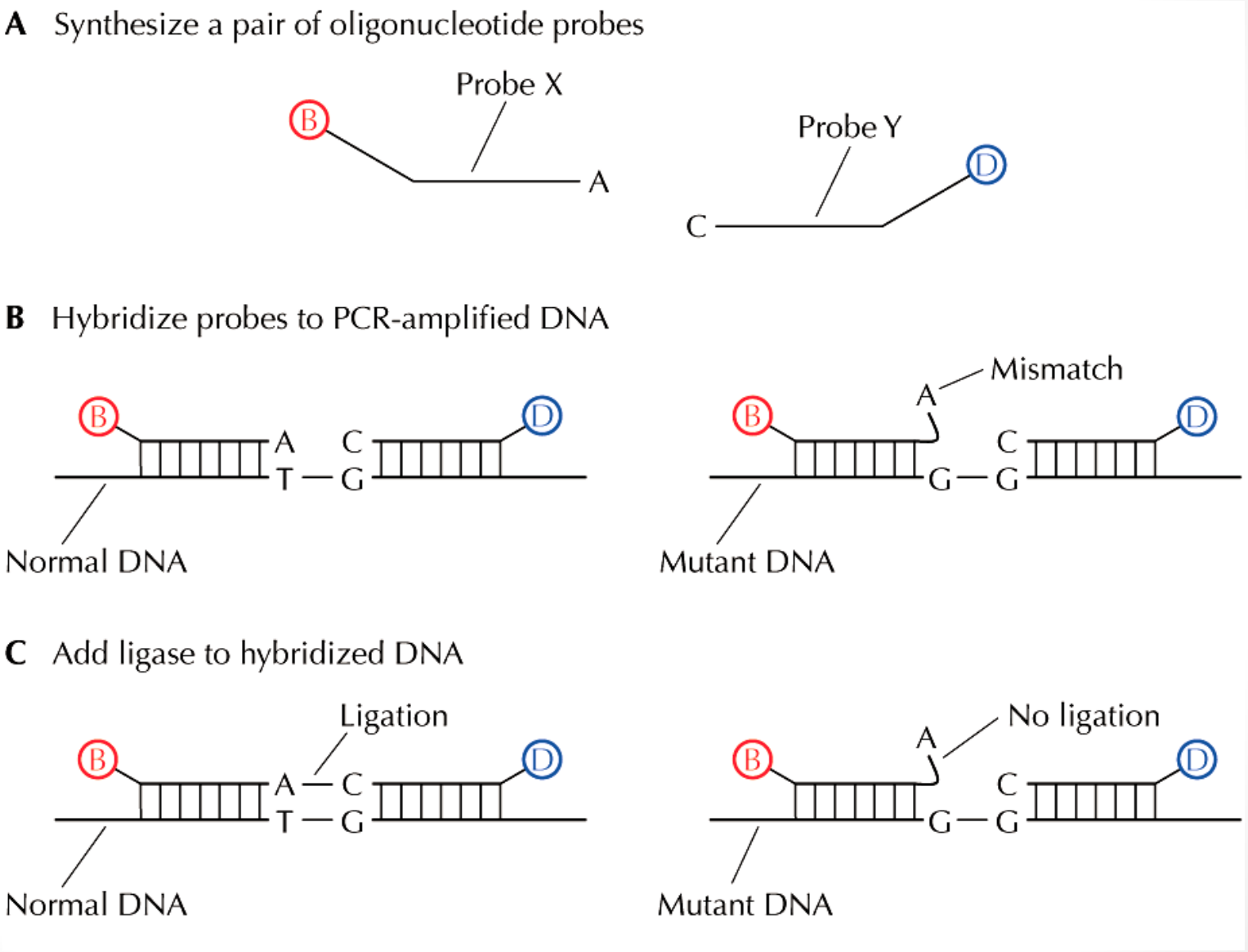
Digital PCR


a) Conventional PCR
b) Digital PCR
Split sample by dilution
Wild type
mutant
Wild type
mutant

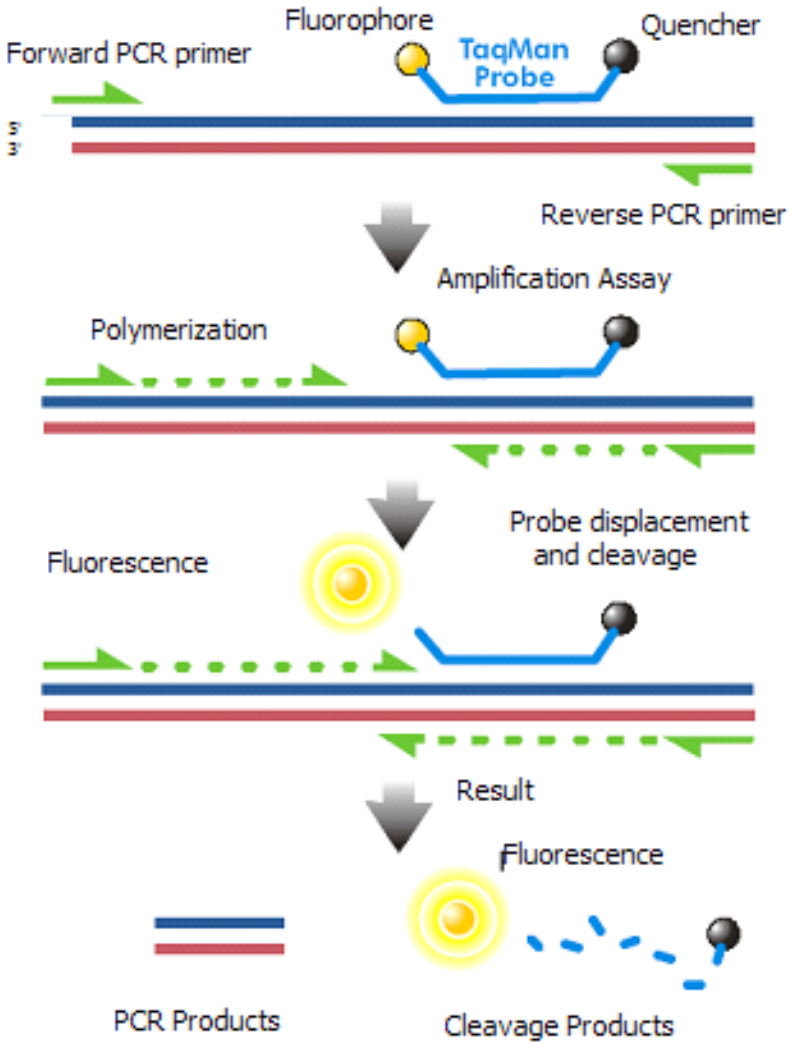

life


"All Bacterial" 16S rDNA · "Clone H Group" FTHFS
"Treponema" 16S rDNA · "Clone H Group" FTHFS
Zootermopsis Hindgut Luminal Contents
5 · 10-3 Dilution
5 · 10-4 Dilution
5 · 10-5 Dilution

PCR/OLA

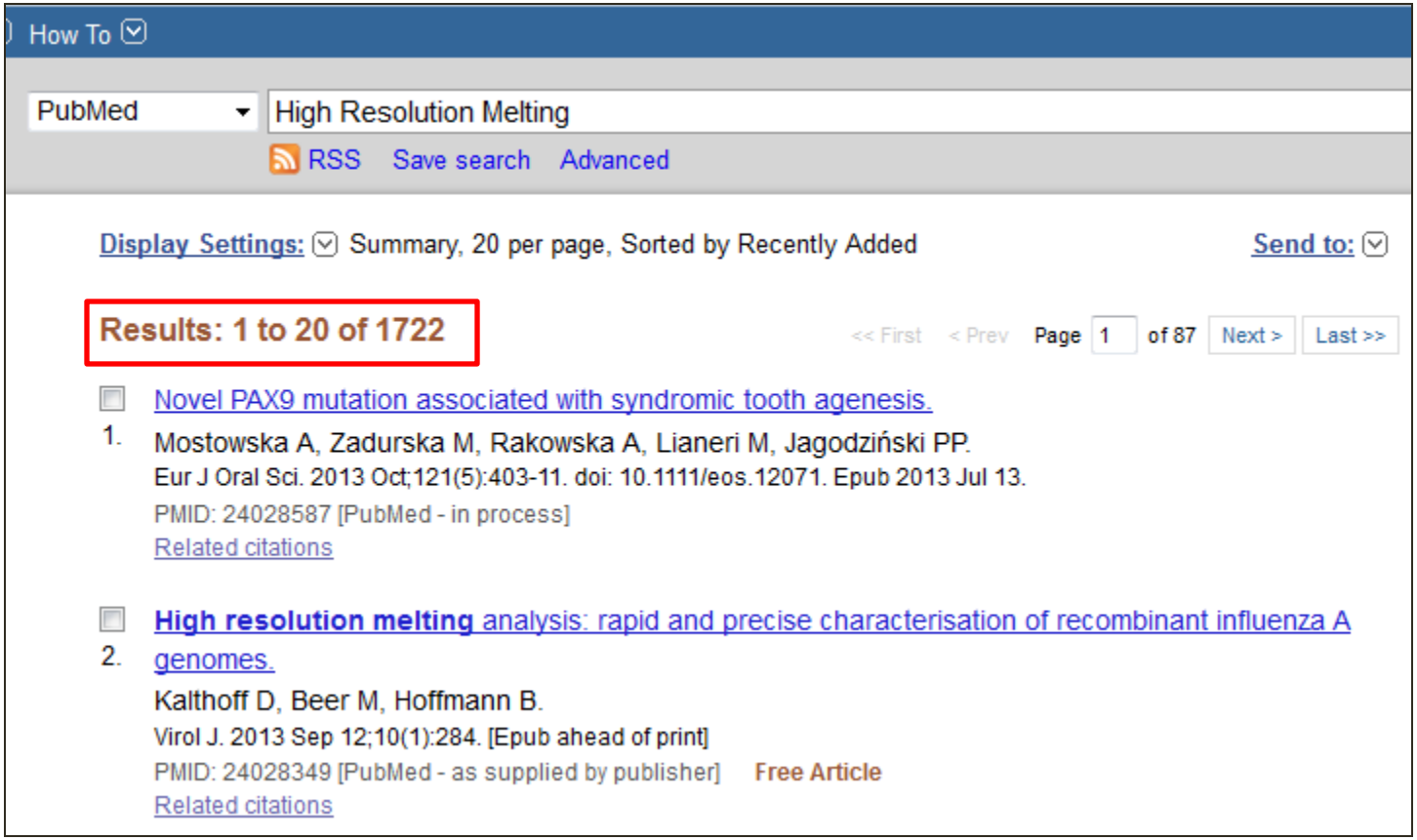

A Synthesize a pair of oligonucleotide probes
Probe X
B
A
Probe Y
D
C
B Hybridize probes to PCR-amplified DNA
B
A
T
C
G
D
Normal DNA
Mismatch
A
B
G
C
G
D
Mutant DNA
C Add ligase to hybridized DNA
Ligation
B
A
T
C
G
D
Normal DNA
No ligation
A
B
G
C
G
D
Mutant DNA

TaqMan Assay

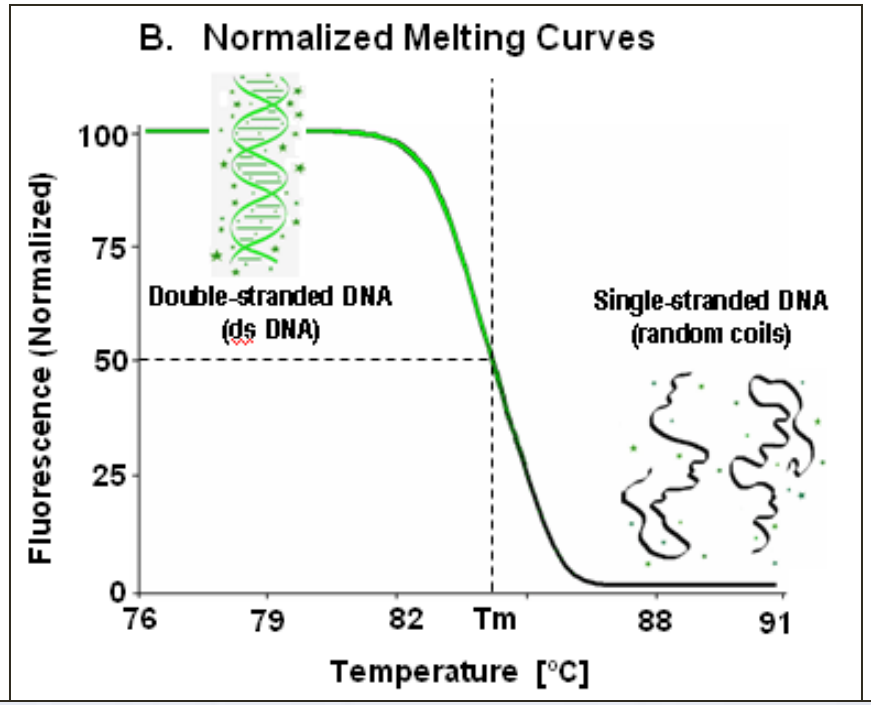


Labeling needed, close tube approach

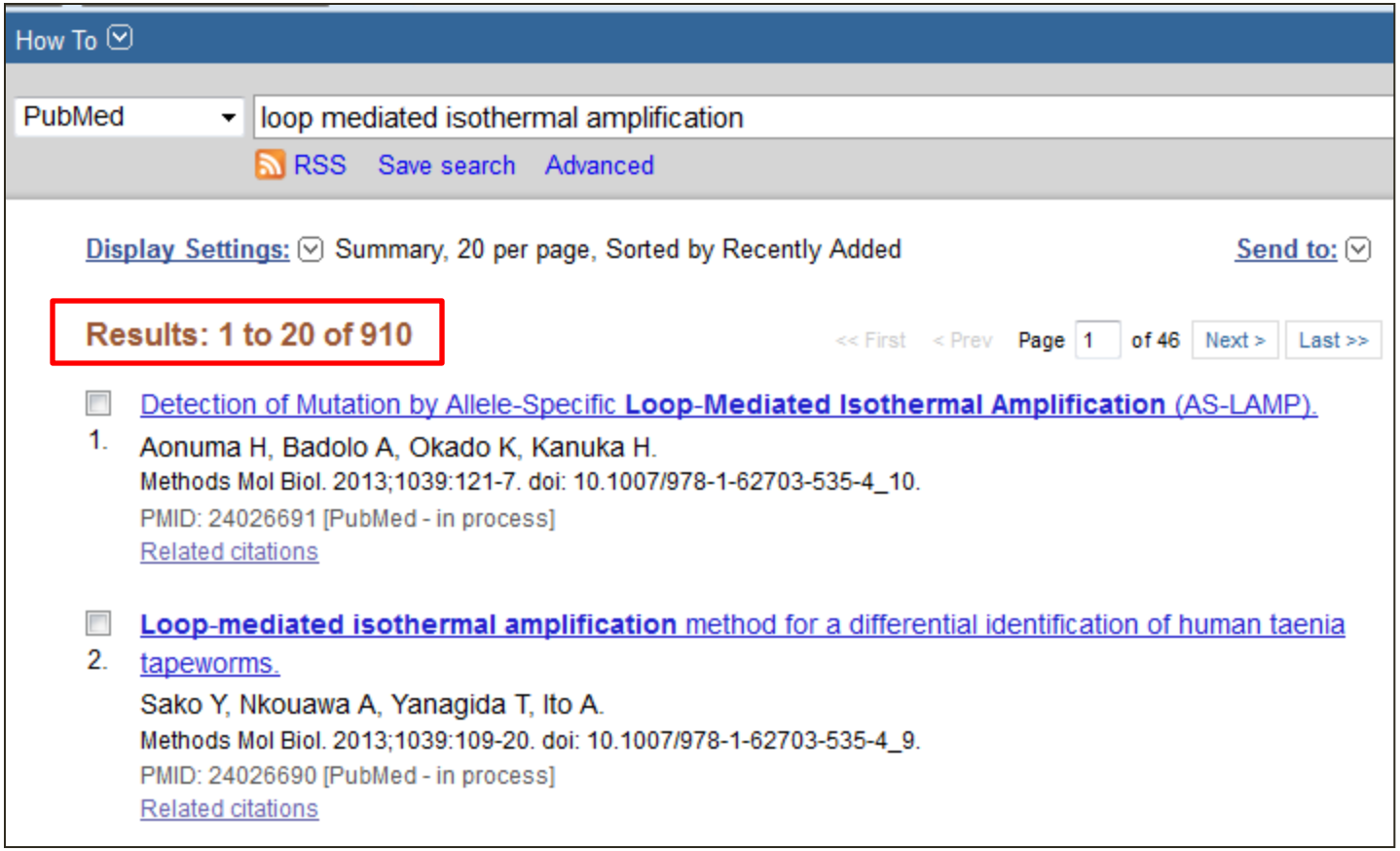
High Resolution Melting (HRM)



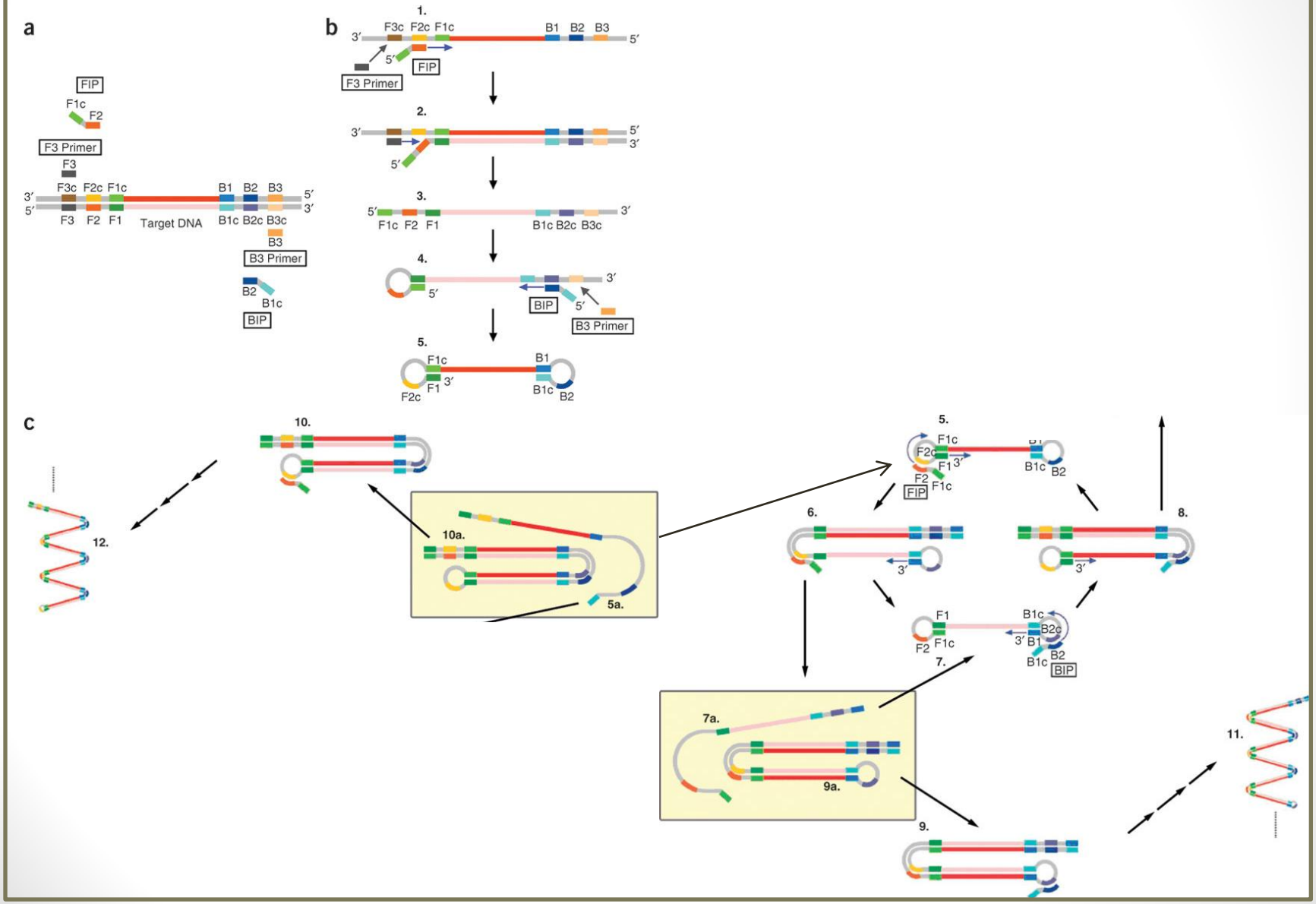
High Resolution Melting (HRM) principle



loop mediated isothermal amplification

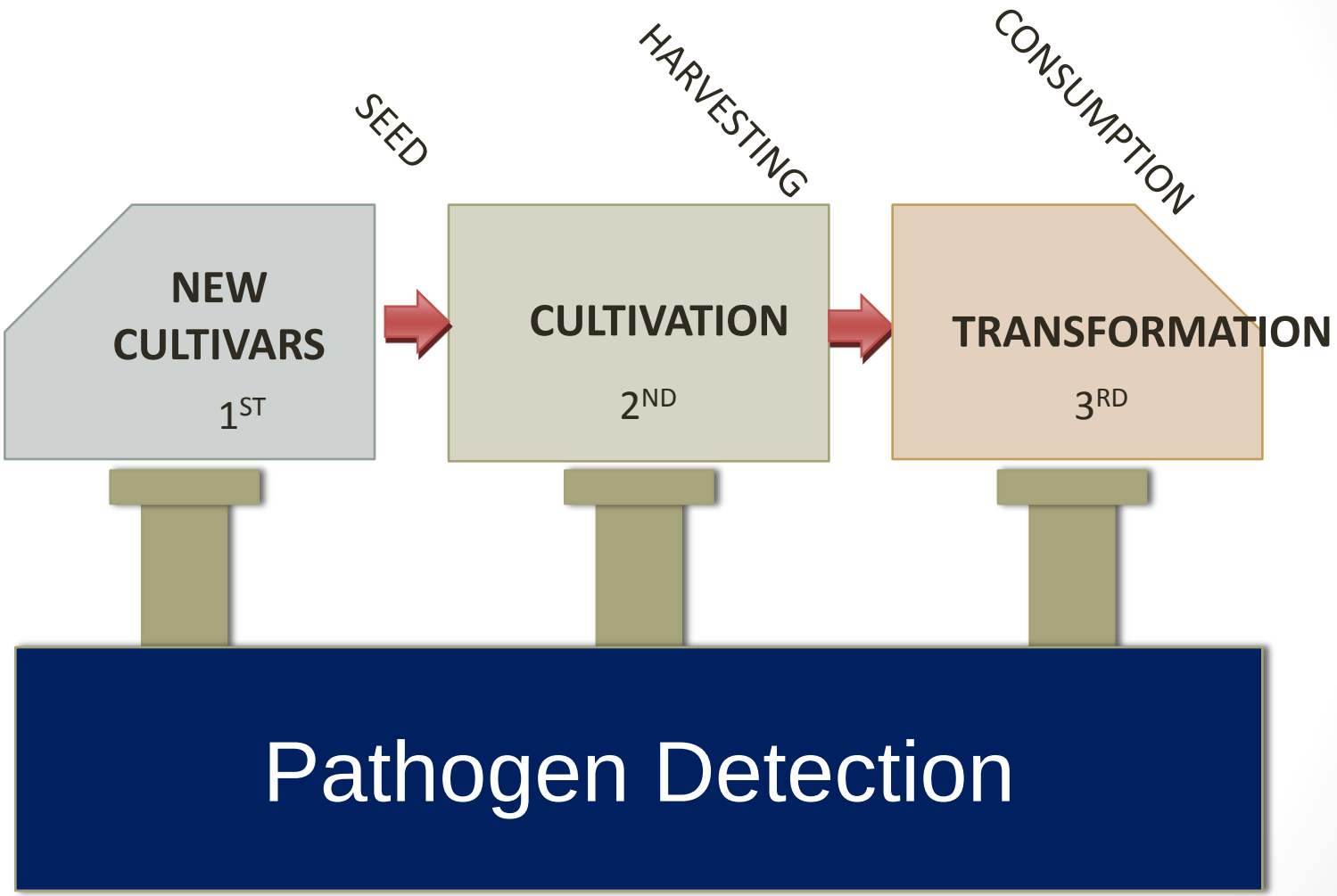


loop mediated isothermal amplification



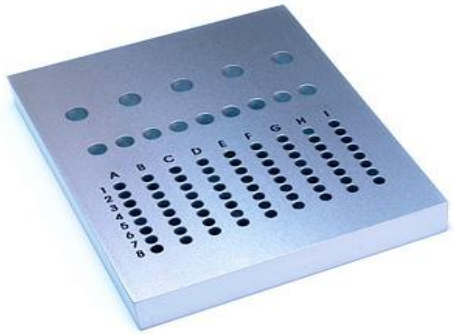
DNA Extraction

Methods	Basis & format	Starting material	Extraction buffer	Elution buffer	Reference
Epicentre	Solution-based; selective precipitation of DNA	5 – 9 mg	300 µL buffer[a]	50 µL TE buffer[b]	Master Pure Purification Kit.
Modified CTAB	Solution-based; selective precipitation of DNA	100 mg	1000 µL buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8.0)	150 µL TE buffer[b]	Tinker *et al.*, (1993)
NucleoSpin	Silica membrane binding; spin-column format	120 mg	550 µL buffer[a]	200 µL buffer[a]	Genomic DNA from food
Qiagen	Silica membrane binding; spin-column format	60 mg	400 µL buffer[a]	150 µL buffer[a]	DNeasy Plant Handbook
CTAB	Solution-based; selective precipitation of DNA	100 mg	1000 µL buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8.0)	150 µL TE buffer[b]	Gryson *et al.*, (2004)
Roche	Solution-based; magnetic glass particle technology	50 mg	800 µL buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 2 mM EDTA, 1% SDS)	100 µL buffer[a]	Sakai *et al.*, (2002); MagNA Pure LC DNA Kit 1
Wizard	Silica resin binding; vacuum manifold format	250 mg	3.0 mL buffer (150 mM NaCl, 2 mM EDTA, 1% SDS, 10 mM Tris base pH 8.0)	100 µL TE buffer[b]	Spoth, and Strauss, (1998)


SEED
HARVESTING
CONSUMPTION
NEW CULTIVARS
1ST
CULTIVATION
2ND
TRANSFORMATION
3RD
Pathogen Detection

Isolation and typing of Lactic acid bacteria

- Number of samples = 100 (300 carcasses)
- Initial characterization by the method of the double layer inhibition
- Selection of psychrotrophic lactic acid bacteria
- Detection for the production of biogenic amines (HPLC)
- Biochemical typing (API 50 CH)
- Molecular typing (sequencing and HRM)



High Resolution Melting analysis (HRM)

- Post PCR closed tube method
- DNA isolation
- PCR (16s rRNA)
- HRM analysis

❑ Controls:

(*Leuconostoc lactis, Lactobacillus salivarius, Lactobacillus fermentum, Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactobacillus brevis, Pediococcus acidilactici*)

Differences between strains

>gb|CP000233.1| **_Lactobacillus salivarius_ UCC118, complete genome**
Length=1827111
rRNA-23S ribosomal RNA

Identities = 741/743 (99%), Gaps = 0/743 (0%)

```
Query  1      CTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAAC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  75921  CTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAAC  75980

Query  61     CGCAAGGAGCCAGCCGTCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAG  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  75981  CGCAAGGAGCCAGCCGTCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAG  76040

Query  121    CCGTAGGAGAACCTGCGGCTGGATCACCTCCTTTCTAAGGAATAATTACGGAACCTGTAC  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76041  CCGTAGGAGAACCTGCGGCTGGATCACCTCCTTTCTAAGGAATAATTACGGAACCTGTAC  76100

Query  181    ATTTATCGAATACTTTGTTTAGTTTTGAGAGGTCATATCTCTCAAGATTTTGTTCTTTGA  240
              |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76101  ATTTATCGGATACTTTGTTTAGTTTT GAGAGGTCATATCTCTCAAGATTTTGTTCTTTGA  76160

Query  241    AAACTAGATATTGATTTATTTCTTAAAAATAAACCGAGAACACCGCGTTTTAAAGAGTTT  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  301    AAAACAAGAATTATAGTTCTTAATCGCTAAACTCATAACCTATTATCGTTAGATAATATT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  361    AGGTTAAGTTATTAAGGGCGTATGGTGGATGCCTTGGCACTAGGAGCCGATGAAGGACGT  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76281  AGGTTAAGTTATTAAGGGCGTATGGTGGATGCCTTGGCACTAGGAGCCGATGAAGGACGT  76340

Query  421    GACTAACTGCGATATGCTTCGGGGAGTTGTAAGTAAACTATGATCCGGAGATTTCCGAAT  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76341  GACTAACTGCGATATGCTTCGGGGAGTTGTAAGTAAACTATGATCCGGAGATTTCCGAAT  76400

Query  481    GGGGAAACCTAACAG GTTTTACCGCCTGTTATCACTAAGTGAATTCATAGCTTAGTTGAA  540
              ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76401  GGGGAAACCTAACAGGTTTTACCGCCTGTTATCACTAAGTGAATTCATAGCTTAGTTGAA  76460

Query  541    GGTAGACGTGGGGAACTGAAACATCTAAGTACC CACAGGAAGAGAAAGAAATTCGATTCC  600
              ||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  76461  GGTAGACGTGGGGAACTGAAACATCTAAGTACCCACAGGAAGAGAAAGAAATTCGATTCC  76520

Query  601    CTCAGTAGCGGCGAGCGAACCGGGAAGAGCCCAAACTAAGAAGCTTGCTTC TTAGGGTTG  660
              |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  76521  CTCAGTAGCGGCGAGCGAACCGGGAAGAGCCCAAACTAAGAAGCTCGCTTCTTAGGGTTG  76580

Query  661    TAGGACTGAACATTTGAGTTACCAAGAAATGAAGTAGTTGAATAATCTGGGAAGATTAGC  720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76581  TAGGACTGAACATTTGAGTTACCAAGAAATGAAGTAGTTGAATAATCTGGGAAGATTAGC  76640

Query  721    CAAAGAGAGTGATAGCCTCGTAA  743
              |||||||||||||||||||||||
Sbjct  76641  CAAAGA GAGTGATAGCCTCGTAA  76663
```
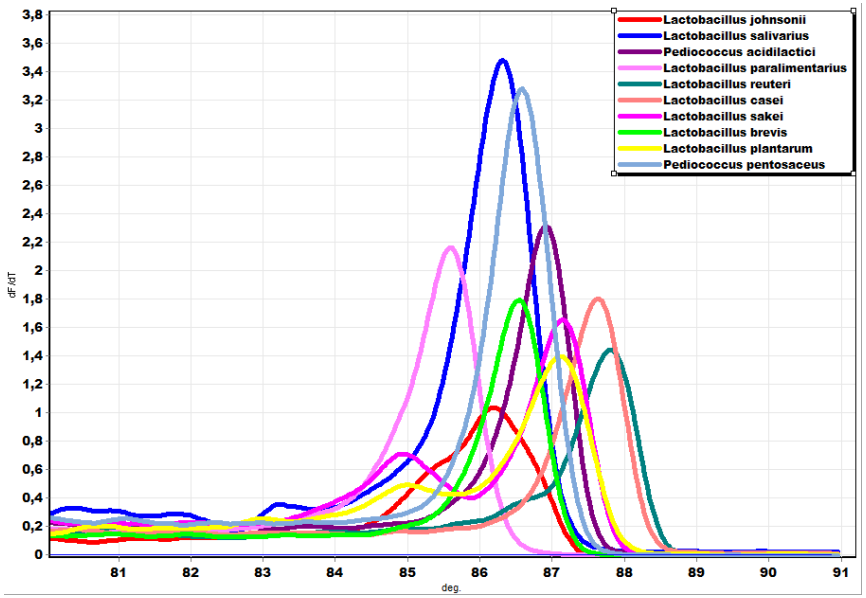
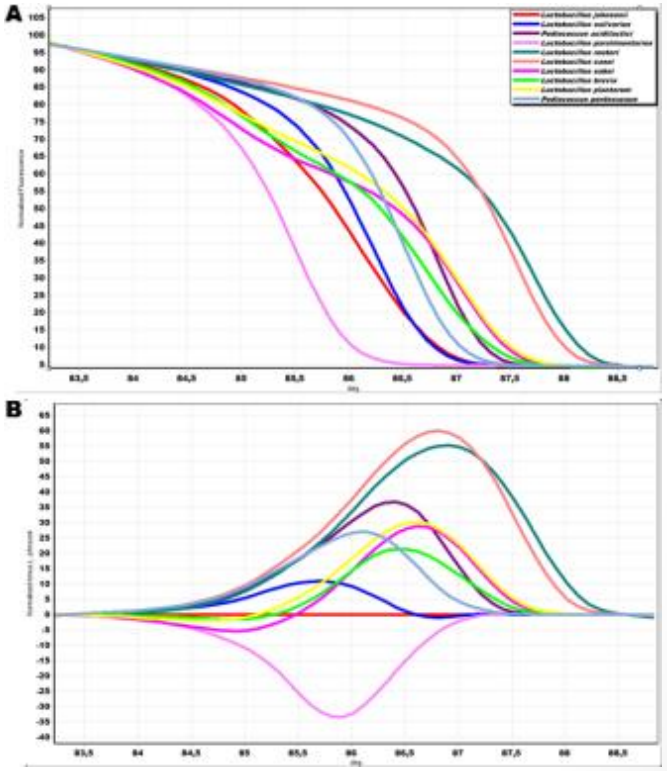
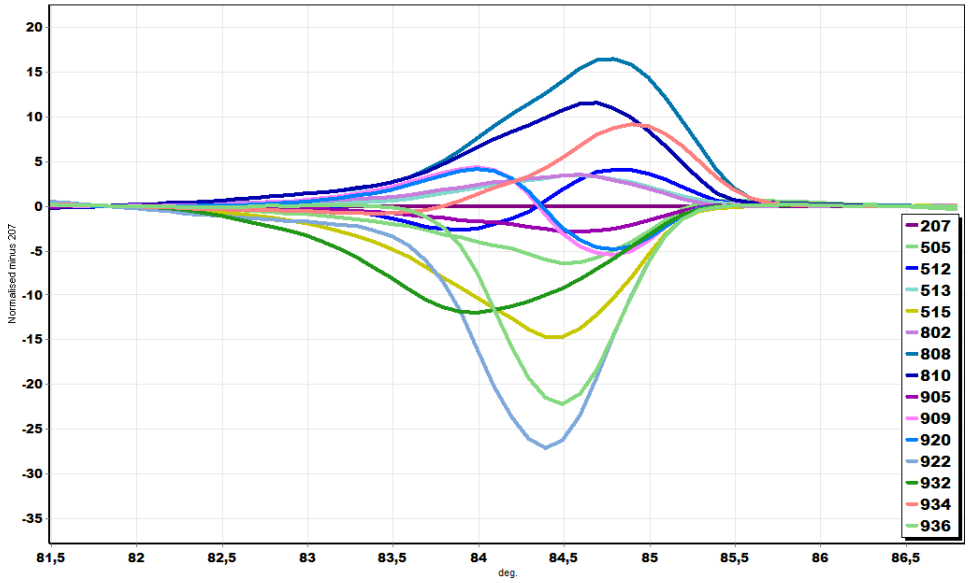
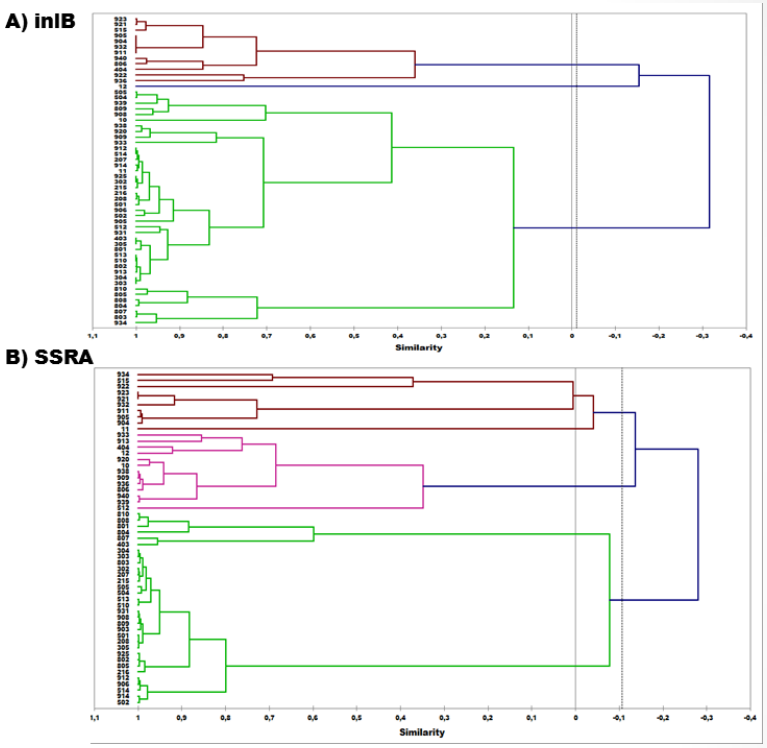

HRM on Lactic Acid Bacteria





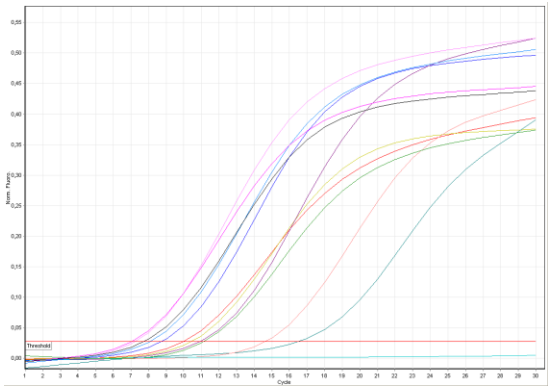
Genotyping Listeria strains using HRM

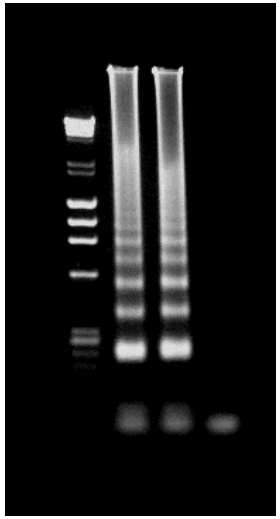


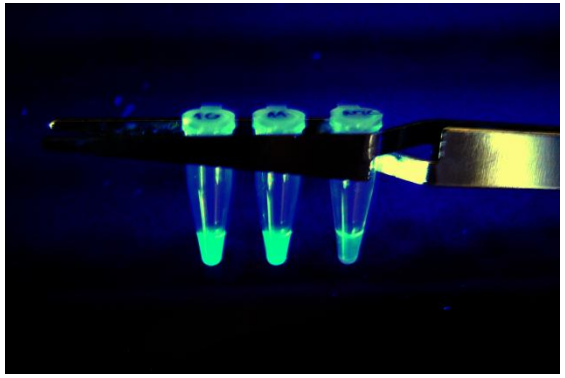


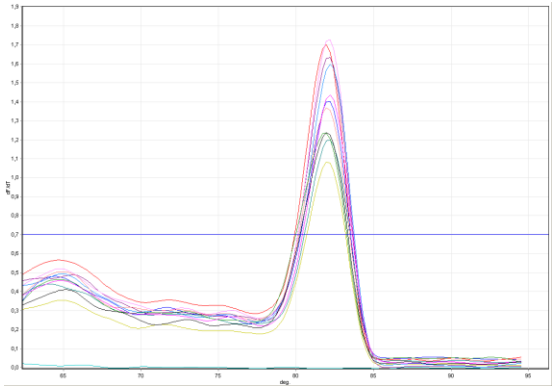
Genotyping of *Listeria monocytogenes* isolates from poultry carcasses using High Resolution Melting (HRM) analysis
Sakaridis, Ganopoulos, Madesis, Tsaftaris and Argiriou
Biotechnology and Biotechnological Equipment, 2013

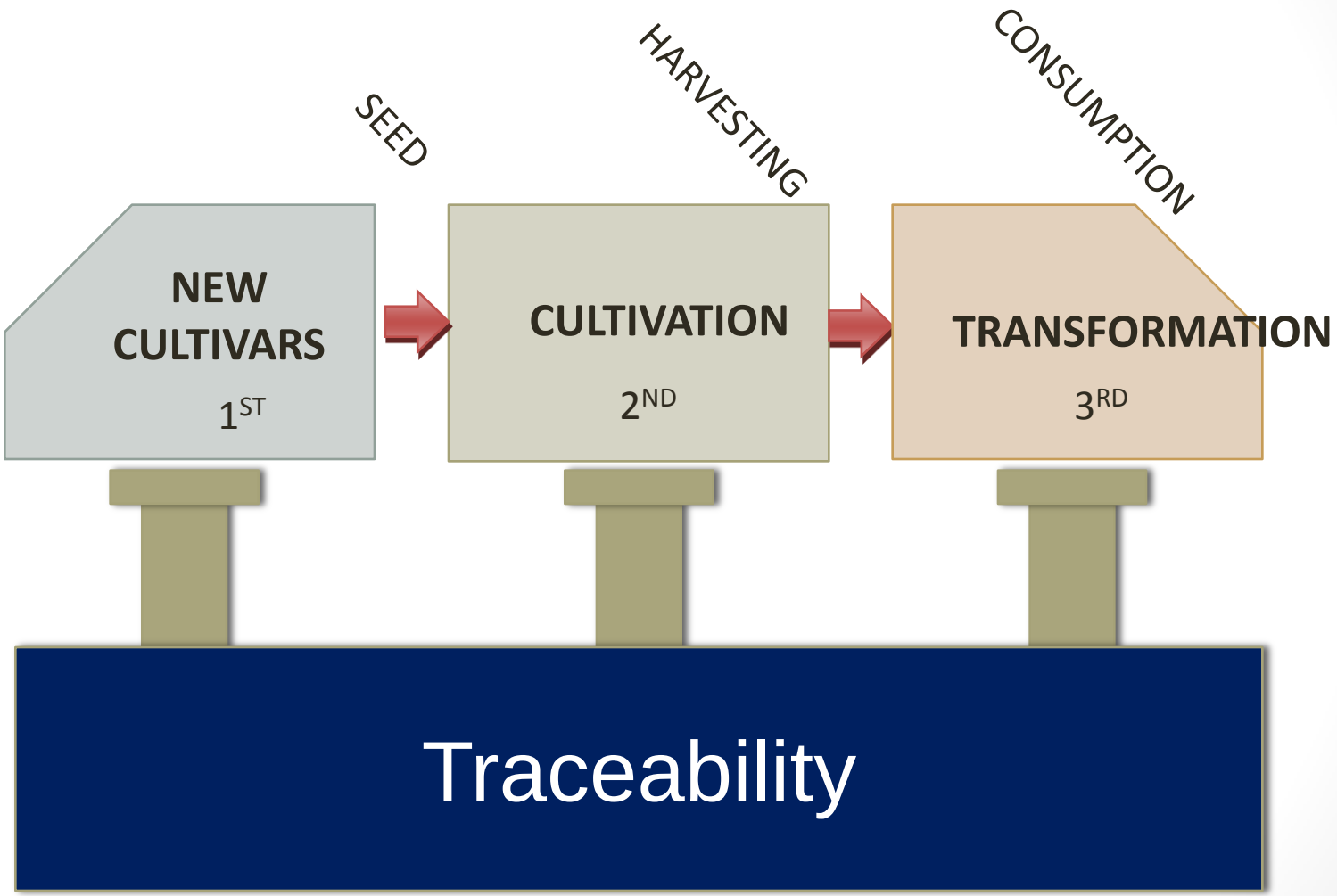
Detection of *Listeria monocytogenes* using LAMP



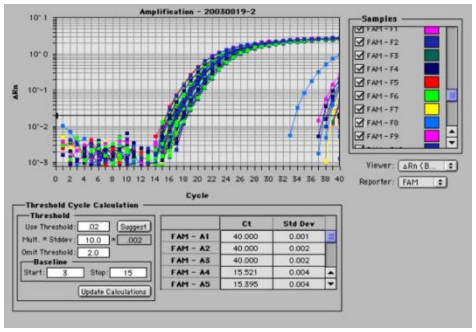




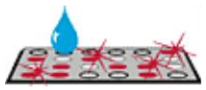


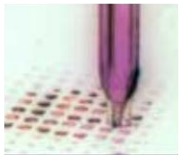

SEED
HARVESTING
CONSUMPTION
NEW CULTIVARS
1ST
CULTIVATION
2ND
TRANSFORMATION
3RD
Traceability

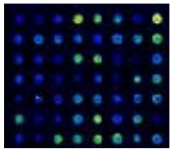
GMO DETECTION

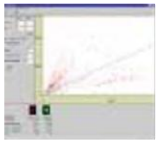


Substrate Selection → Microarray Production → Hybridization → Scanning → Data Analysis









BarCode chips

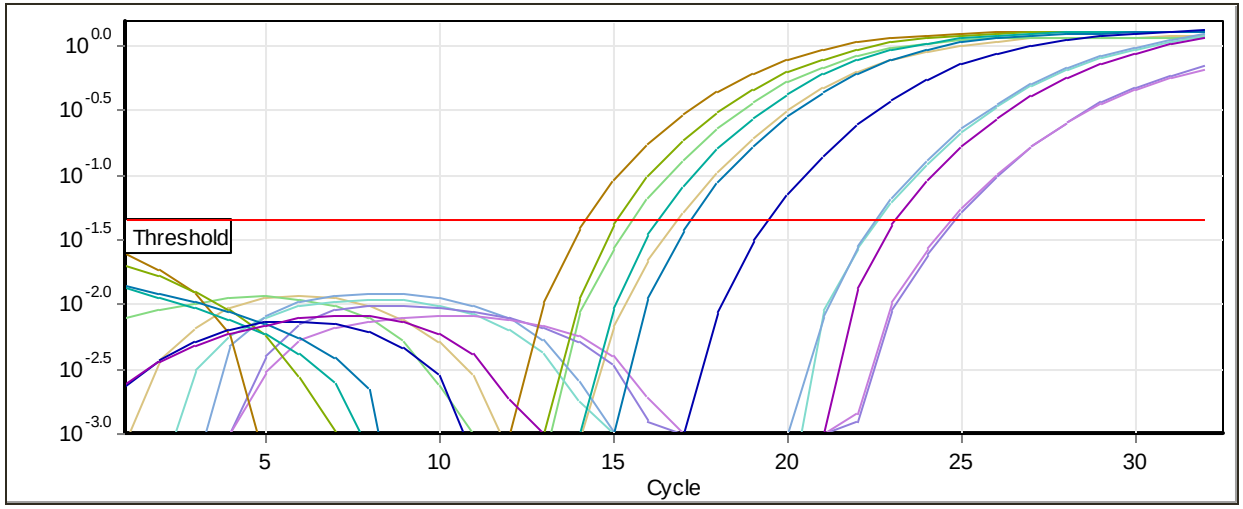
High-Density Microarray of Small-Subunit Ribosomal DNA Probes

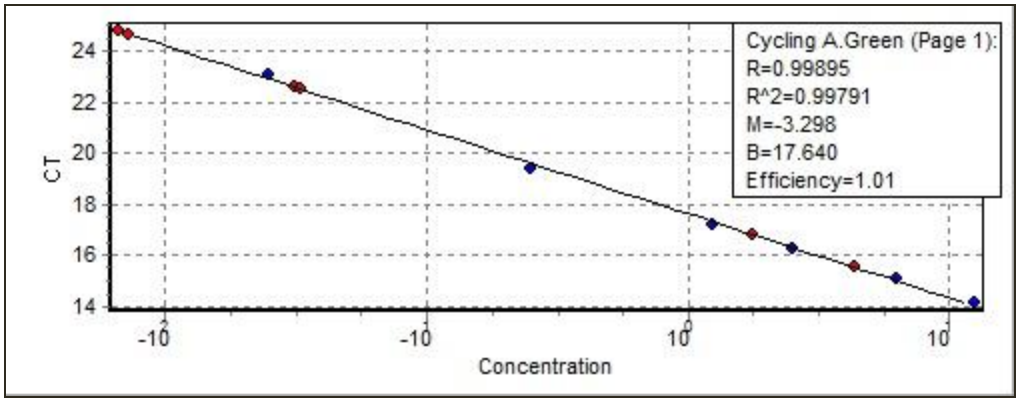
*Kenneth H. Wilson,[1] Wendy J. Wilson,[2] Jennifer L. Radosevich,[2] Todd Z. DeSantis,[2] Vijay S. Viswanathan,[2] Thomas A. Kuczmarski,[2] and Gary L. Andersen[2]**

Some Examples

- Certifying products made from specific plant species or cultivars and animal species or races using DNA Barcoding techniques
  - Unique signature to be used in all the production chain – traceability
  - Protect from fraudulent products the consumers
  - Maintain the added value of traditional products
  - Protect investments made to develop new food products
- **Obtained Results:** Certification of Mozzarela di buffala, Feta cheese, Hammon from Greek black pig, PDO legumes etc.

Detection of Horse DNA in Meat products

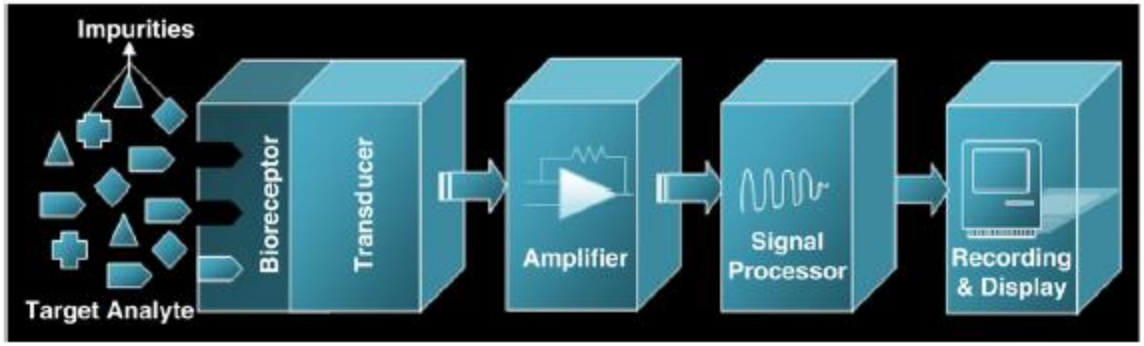


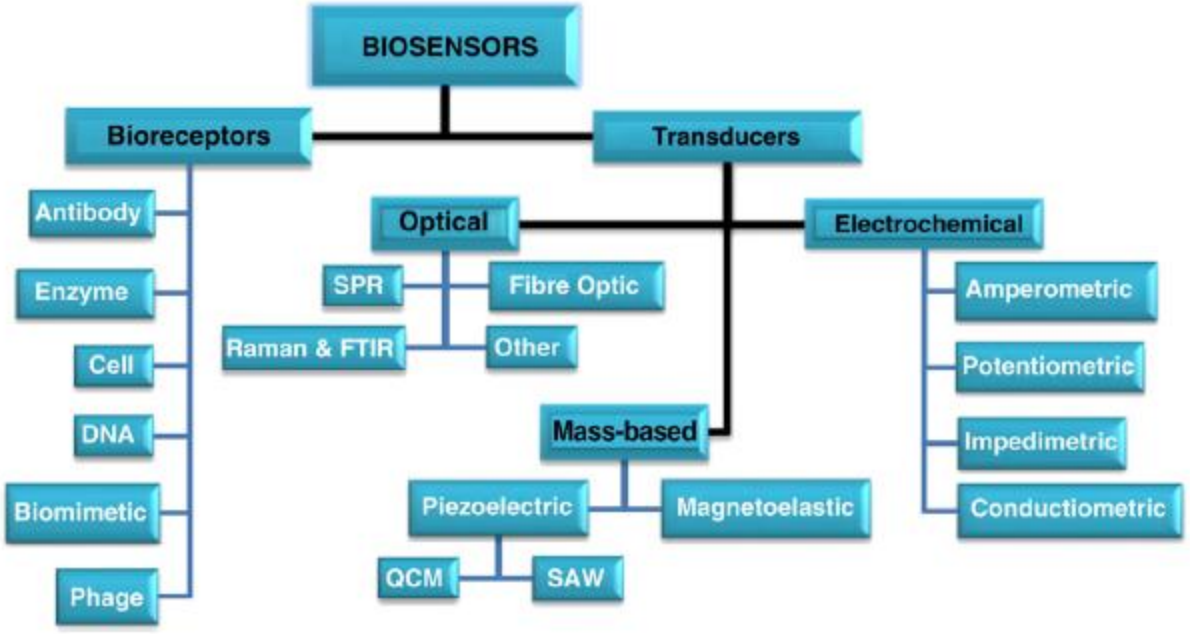


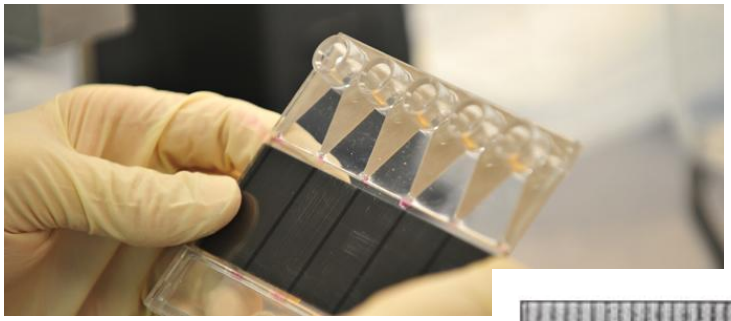
Available also for:

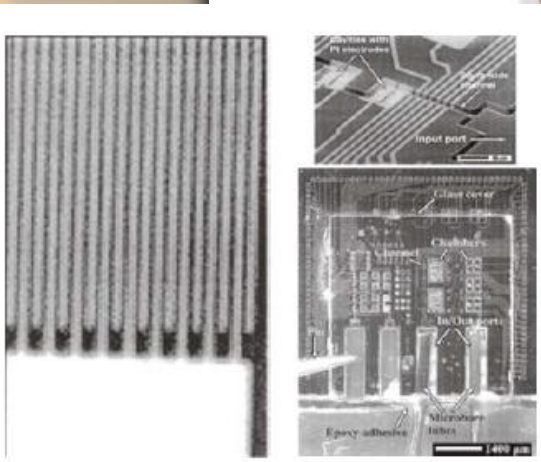
- Donkey
- Mule
- Chicken
- Turkey
- Pig (Halal and Kosher certification)

Biosensors


Impurities
Target Analyte
Bioreceptor
Transducer
Amplifier
Signal Processor
Recording & Display


BIOSENSORS
Bioreceptors
Transducers
Antibody
Enzyme
Cell
DNA
Biomimetic
Phage
Optical
SPR
Fibre Optic
Raman & FTIR
Other
Electrochemical
Amperometric
Potentiometric
Impedimetric
Conductiometric
Mass-based
Piezoelectric
Magnetoelastic
QCM
SAW





A B

Figure 6. Electron microscopy zoom of an interdigitated microelectrode (A) and of the channels of a biochip belonging to a micro fluid device (B).

Conclusions

- Many DNA methods available for Food pathogen detection and Food Traceability
- Most of them require high cost equipment, high expertise and are time consuming
- Most promising (in our lab):
  - HRM (requires RT-PCR system with HRM capabilities) and LAMP (in principle is enough a water bath)
- **Future trends:**
  - Low cost NGS
  - Biosensors

INSTITUTE OF APPLIED BIOSCIENCES
ΙΝΣΤΙΤΟΥΤΟ ΕΦΑΡΜΟΣΜΕΝΩΝ ΒΙΟΕΠΙΣΤΗΜΩΝ
CERTH

Thank you for your attention

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SOUTH EAST EUROPE
Jointly for our common future

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