Rapid methods in food microbiology
Identification of microorganisms

Sabina Purkrtová
Microbiological analysis procedures

CONVENTIONAL CELL CULTIVATION
• relatively easy to use, but time (requires several days), labour (lots of procedural steps) and material consuming
• many of them are recognised as approved for ISO and they are gold standard procedures
  • Colony count method (CCM)
    • pour plate techniques
    • spread plate techniques
  • Membrane filtration
• Most Probable Number (MPN)

RAPID METHODS
• immunonological method (based on antigen/antibody-binding)
• based on molecular biological method (based on PCR)
• others (ATP Photometry, Direct Epifluorescent Filter Techniques (DEFT), Electrical impedance method, Flow cytometry, etc.)
IMMUNOLOGICAL METHODS
Immunonological method

A bacterial antigen – a molecule on the surface of bacterial cell

Immunoglobulins Ig (also antibodies) are glycoprotein molecules produced by B cells plasma cells (white blood cells) according to antigen to mark remaining bacteria for destruction. The antibody immune response is highly complex and exceedingly specific.
The various immunoglobulin isotypes differ in their biological features, structure, target specificity and distribution.
**Immunological method**

*Polyclonal antibodies*
The immune response to an antigen generally involves the activation of multiple B-cells all of which target a specific epitope on that antigen. As a result a large number of antibodies with different specificities and epitope affinities are produced.

*Monoclonal antibodies* are generated by a single B lymphocyte to one specific epitope. For production B cell is isolated from from the spleen and lymph nodes of immunised animals and fuse with immortal heteromyleoma. The produced hybridoma cells produce only one antibody within the supernatant.
Immunonological method

- immunonological method
  (based on antigen/antibody-binding)
Isolation on selective agar plates
MacConkey agar with sorbitol (instead of lactose) (SMAC)
E. coli O157 is sorbitol negative – no fermentation – colourless colonies
Fluorocult® E. coli O157 Agar
Chromogenic medium – chromogenic substrate for β-D-glucuronidase – E. coli O157 is negative -colourless colonies
37±°C for 24± 3h

Confirmation
4. Day: Isolation of a characteristic colony on Nutrient Agar and incubation at 37±°C for 24± 3h
5. Day: testing for positive indole reaction

Immunomagnetic beads separation
ISO 16654:2001 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of Escherichia coli O157

Selective enrichment
1. day: 25 g of sample + 225 ml of Modified Tryptone Soya Broth (mTSB) with novobiocine; homogenization
   Incubation at 41.5 °C

Immunomagnetic isolation
after 8 and 16 hours (2. day)

Isolation on selective agar plates
MacConkey agar with sorbitol (instead of lactose) (SMAC)
E. coli O157 is sorbitol negative – no fermentation – colourless colonies
Fluorocult® E. coli O157 Agar
Chromogenic medium – chromogenic substrate for β-D-glucuronidase – E. coli O157 is negative -colourless colonies
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Confirmation
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5. Day: testing for positive indole reaction

SmAC agar
Escherichia coli
O157:H7 (colourless)

• 1 ml of homogenate + 100 µl of paramagnetic beads covered with antibody against E. coli O157
• Present cells E. coli O157 are trapped and separated by applying a magnet
• The homogenate with other bacteria is taken away.
• The beads with trapped E. coli O157 are washed (to add a washing buffer – to release a magnet – to mix – to apply a magnet)
• Plating out by 50 µl of paramagnetic beads with trapped E. coli O157 on 2 selective agar plates.
Enzyme-linked immunosorbent assay (ELISA)

How to detect the formation of complex antigen-antibody?

• Different systems with antibodies linked to enzyme (e.g. horseradish peroxidase, alkaline phosphatase) – then to add substrate – its transformation is measured (e.g. changes in the absorbance)
Enzyme-linked immunosorbent assay (ELISA)

When the complex antibody-antigen is formed, the secondary antibody conjugated with enzyme is to be bound to the antigen and not to be washed off.

After adding the substrate, the substrate is changed and give the signal (e.g. Measured as absorbance).
**Enzyme-linked immunosorbent assay (ELISA)**

- monoclonal or polyclonal antibodies coated microtitre trays to capture target antigen. The captured antigens detected using a second antibody which is conjugated to an enzyme. The addition of enzyme substrate enables the presence of the target antigen to be visualised.

- considerable specificity

- can be automated; commercially available (*Salmonella, L. monocytogenes, Campylobacter, ...etc.*)
VIDAS or mini-VIDAS Analyzers

- the automated, multiparametric immunoanalyser
- based on an enzyme immunoassay which detect **target** antigens using the **ELFA** (Enzyme Linked Fluorescent Assay)

![Diagram of VIDAS analysis](image)

- 4-methyl-umbelliferone (fluorescent molecule) is released by alkaline phosphatase = fluorescence
Each test is composed of two parts:

1. The SPR® acts as a Solid Phase Receptacle for the reaction. The SPR is coated with anti-**target** antibodies adsorbed on its surface

2. The **Strip** contains all ready-to-use reagents necessary for the test: washing solution, alkaline phosphatase-labeled anti-**target** antibodies and substrate
VIDAS or mini-VIDAS Analyzers

Video: http://www.biomerieux-industry.com/food/vidas-listeria-monocytogenes-detection#VIDAS LMO2
https://www.youtube.com/watch?v=ZFRuJYynLwk

bioMérieux
http://www.biomerieux.com/
**VIDAS or mini-VIDAS Analyzers**

*Salmonella* spp.

**Detection using VIDAS Easy SLM**

The sample = the sample diluted and enriched in *Xpress 2 broth* (different from ISO 6579)

The used broth must be convenient for the method.

Validated by ISO 16140 against ISO 6579 (AFNOR, N°BIO 12/16-09/05)
Detection of *Salmonella* spp. according to ISO 6579:2002

ISO 6579:2002 Microbiology of food and animal feeding stuffs
-- Horizontal method for the detection of *Salmonella* spp.

**Pre-enrichment**
1. day

- 25 g of sample + 225 ml of buffered peptone water; homogenization
- Incubation at 37±1°C for 18±2 h

**Selective enrichment**
2. day

- 1 ml of homogenate to 10 ml **MKTTn**
  - Incubation at 37±1°C for 24±3 h
- 0.1 ml of homogenate to 10 ml **RVS**
  - Incubation at 41.5°C for 24±3 h

**Isolation on selective agar plates**
3. day

- Plating out by 10 µ on two selective media and incubation at 37±°C for 24± 3h:
  - Xylose Lysine – Desoxycholate Agar (**XLD**)
  - Any other selective medium (BGA, HE, BS, SS, DC, chromogenic media...)

**Confirmation**
4.-6. day

- Isolation of a characteristic colony on Nutrient Agar and incubation at 37±°C for 24± 3h
- Confirmation
VIDAS kits

- **E. coli O157** - VIDAS® UP *E. coli* O157 (including H7)
- **E. coli O157** - VIDAS® ECO
- **E. coli O157 (confirmation)** - VIDAS® ICE
- **Salmonella spp.** - VIDAS® SLM
- **Salmonella spp.** - VIDAS® Easy SLM
- **Listeria spp.** - VIDAS® LIS
- **Listeria spp.** - VIDAS® LSX
- **Listeria monocytogenes** VIDAS® LMO2
- **Staphylococcal enterotoxins** - VIDAS® SET2
- **Campylobacter spp.** - VIDAS® CAM
GLISA-Rapid Test

GLISA-Rapid Test (Gold Labelled ImmunoSorbent Assay) – Lateral Flow Test

Detection limit: 10^5 CFU/ml in a sample (homogenate of selective enrichment or suspension of colony) (sometimes heat-treatment required - 20 min at 80 °C = cell lysis = better motility of the cell walls with antigens)

Singlepath® for detection of Listeria monocytogenes (Merck)
### Merck's Rapid Test Product Portfolio
#### 8 Rapid tests for the most common pathogens

<table>
<thead>
<tr>
<th><strong>Listeria:</strong></th>
<th><strong>Salmonella:</strong></th>
<th><strong>E. coli O157 / VTEC:</strong></th>
<th><strong>Campylobacter:</strong></th>
<th><strong>Bacillus cereus:</strong></th>
<th><strong>Legionella:</strong></th>
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<tr>
<td>LEB Base</td>
<td>Tetrathionate</td>
<td>mEC + n</td>
<td>Bolton Broth</td>
<td>M.Y.P. Agar</td>
<td>CYE Agar</td>
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<td>Fraser Suppl.</td>
<td>Rappaport.-Vas.</td>
<td>mTSB + n</td>
<td>CCD Agar</td>
<td>CGY Broth</td>
<td>BCYE</td>
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<td>Salmosyst</td>
<td>SMAC</td>
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<td>OXFORD Plates</td>
<td>M Broth</td>
<td>BHI</td>
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<td>Chromocult®</td>
<td>XLT₄, XLD, Anaerocult® C</td>
<td>CAYE</td>
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<tr>
<td>Listeria Agar</td>
<td>Rambach®</td>
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</tbody>
</table>

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Rapid Detection of Pathogens
ISO Standard 6579 for Detection of Salmonella

**Day 1**
- 25 g/ml test sample in 225 ml BPW
- 16 - 20 h / 35 - 37 °C

**Day 2**
- 0.1 ml BPW to 10 ml RVS Broth
  - 24 h at 41.5°C

- 1 ml BPW to 10 ml MKTTn Broth
  - 24 h at 37°C

**Day 3**
- XLD Agar
  - 24 h / 35-37°C
  - 1 plate 14 cm / 2 plates 9 cm

- Any other Salmonella Agar
  - 1 plate 14 cm / 2 plates 9 cm

**Day 4**
- Interpreting of Growth on Plates
  - 11 media

**Day 5**
- For confirmation take 5 suspected colonies from each plate and streak onto Nutrient agar
  - 18 - 24 h / 35 - 37 °C

**Day 6**
- Biochemical / Serological Confirmation Tests
  - TSI / Urea / Lysin / β-Gal / VP / Indol
Rapid Testing
Singlepath® Salmonella

Singlepath® Salmonella

- For detection of *Salmonella* spp. in foods directly after 2-step enrichment

- No post-enrichment step needed:
  - sample directly taken from RVS
  - + 15 min heating,
  - transfer to sample port
  - read results within 20 min

- Advantage over competitor products:
  - Detection of multiple *Salmonella* serological groups
  - Lower detection limit: \(10^5\) cfu / ml
Rapid Testing
Singlepath® Salmonella - Screening

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
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<tbody>
<tr>
<td>25 g/ml TEST SAMPLE</td>
<td>0.1 ml BPW to 9.9 ml RVS Broth</td>
</tr>
<tr>
<td>In 225 ml Buff. Peptone Water</td>
<td>42°C for 24 h</td>
</tr>
<tr>
<td>35 - 37°C for 16 - 20 h</td>
<td></td>
</tr>
</tbody>
</table>

**Day 3**

**NO**
Salmonella not present

**Day 3**

**YES**
Salmonella present

**Day 4**

If positive, confirmation by culture and biochemical tests required

STREAK OUT ONTO RAMBACH 37°C FOR 24 h
Validation of procedure

Validation of an alternative method
• the validation of an alternative method is the procedure to demonstrate if the alternative method provides equivalent or better results compared to the reference methods.

• **Validation** is a process, within which the method is demonstrated to be suitable for its purpose. *It documents methods validity!*

• During validation process, methods **performance characteristics** are estimated.

The validation of qualitative and quantitative methods comprises two phases:

1. a method comparison study of the alternative method against a reference method (performed by an expert laboratory).

2. a interlaboratory study of the alternative method (organised by an expert laboratory).

Expert laboratory (organising): laboratory having the qualified staff and skills to perform the method comparison study and organise the interlaboratory study.
Useful documents

ISO 16140:2003 – Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods

ISO 17025:2005 – General requirements for the competence of testing and calibration laboratories
Accreditation bodies that recognize the competence of testing and calibration laboratories should use this International Standard as the basis for their accreditation.

ISO/IEC 17011:2004 Conformity assessment - General requirements for accreditation bodies accrediting conformity assessment bodies
If a laboratory wishes accreditation for part of all of its testing and calibration activities, it should select an accreditation body that operates in accordance with ISO/IEC 17011.

It is expected that all laboratories involved in each step of a validation process will have a Quality System or quality assurance (QA) program in place to ensure standardization of laboratory operations, as well as adequate quality control (QC) activities.
Validation of procedure

Test characteristics
The test characteristics for alternative methods are shown:

Qualitative methods
1. Selectivity (inclusivity/ exclusivity)
2. Relative accuracy
3. Detection level
4. Relative sensitivity/Relative specificity
5. The agreement between the methods

Quantitative methods
1. Selectivity (inclusivity/ exclusivity)
2. Lowest validated level with satisfactory precision
3. Repeatability
4. Reproducibility
5. Uncertainty of the method
Rapid Testing
Singlepath® Salmonella

Evaluation of Singlepath® Salmonella:

• Evaluated with 105 *Salmonella* and 58 non-*Salmonella* species
  - skimmed milk powder
  - pet food
  - ground beef
  - black pepper
  - seafood
  - ground poultry

• Sensitivity and Specificity each >98%

• AOAC approval granted October 2004
Rapid Testing

Singlepath® Salmonella

Evaluation at University of Giessen: Comparison with CELSIS Path Stik Salmonella

- Tested with spiked + native ground meat, poultry, milk powder
- Both tests have 98% Sensitivity and Specificity (n = 50 food samples)
- Significant stronger bands than with PATHSTIK

Singlepath showed better Detection Limit (10^5 CFU / ml)

Evaluation at Agriquality (NZ): Comparison with TECRA ELISA Salmonella

- Tested with roasted chicken, grated cheddar cheese, whole milk powder, non-fat milk powder, environmental swabs (n = 279 food samples)
- Both assays give 100% identical results
- Both assays with >99% Sensitivity / Specificity
PCR METHODS
DNA, RNA, PROTEINS

Deoxyribonucleic acid: (DNA)
A linear polymer that consists of four nucleotides:
Adenine Cytosine Guanine Thymine Primer binding  A – T , C - G
PCR principle

PCR = Polymerase Chain Reaction (1983 by Kary Mullis)

_in vitro_ amplification of the part of DNA (usually 100 bp – 1500 bp) bordered by two primers (syntetically prepared oligonucleotides)

Typical number of cycles = 30 - 45

https://www.youtube.com/watch?v=iQsu3Kz9NYo
PCR principle

**DENATURATION** of double-stranded DNA by heat (app. 94-95°C, initially 5, then in each step 2-3 minutes)

**ANNEALING** of PRIMERS on the free single strand DNA
- forward primer on the 3′-5′ strand
- reverse primer sits on the 5′-3′ strand

\[ T_a = \text{annealing temperature} \]
- 50-65 °C – depends on the primers sequence
- Time: 30 s-60 s

**ELONGATION** by DNA polymerase
- *in vivo* starts to synthetize new DNA molecules from 3′-end of RNA primer
- *in vitro* PCR from 3′-end the primers
- 72 °C – optimal temperature
- 45 s-3 minutes (final elongation 5-15 minutes)

http://www.mun.ca/biology/scarr/PCR_sketch_3.gif
PCR principle

Mistake in the beginning – the most influence

**Exponential phase**
Duplication of PCR products amount in each cycle

**Linear phase**
The level of amplification is decreased (lower than duplication in each cycle)

**Plateau:**
Absence or degradation of some component
Reassociation can be preferred than primers anealing due to thermodynamical reasons
PCR principle

Primers (forward, reverse)

Mg$^{2+}$ (cofactor for DNA polymerase)

dNTP mix (dCTP, dATP, dTTP, dGTP) – building stones for newly synthetized DNA product

DNA (genomic, plasmid DNA)

Nuclease free water – without Dnase, Rnase presence (enzymes, which degrade DNA, RNA)

Thermostable DNA polymerase

MASTERMIX

Total volume – 25 μl – 50 μl
Polymerase

**Taq DNA polymerase**
- from thermophilic bacterium *Thermus aquaticus* (now prepared by recombination)
- opt. temp.: 75-80°C (150 nts/s)
- active at the room temperature –to work on ice,
- inactive at >90°C, but reactivation when cooled
- only 5´exonuklease activity, not 3´exonuklease activity (proofreading)
- 1 mistake at 10-20 000 nts

**Other polymerases:**
- Proofreading polymerases (with the 3´exonuclease activity),
- *Tth* polymerase - reverse transcription (Mn$^{2+}$) or polymerase activity (Mg$^{2+}$)

**Hot start polymerases**
- Activity at the room temperature is inhibited - binding on anti-polymerase antibodies on the wax balls
- activation after heating at 94 °C (antibodies destroyed)
1) Releasing DNA from the cell
- lysis of the cell wall by detergents or by heat, digesting cell surface proteins by proteinase K
- RNAsase treatment for RNA-free genomic DNA

2) Isolation DNA from the released cytoplasmatic contents
- Centrifugation the vaste in pellet (heat isolation)
- Ion-exchange chromatography (commercial kit) – binding DNA by ionic interactions to the column (DNA is negative charged) – proteins and RNA pass away – DNA is later released by using more concentrated salt solution (column is charged positively)
- Phase separation (aqueous/chlorophorm phase – CTAB method)
Detection of food-borne pathogens

DNA of microorganism = set of specific genes with specific sequences

*Primers are designed to be complementary to sequence of some specific gene*
  - if the complementary sequence is present, PCR product will be performed

**Main problems about primers designing and applying of PCR**
- Is the gene of interest specific enough (can be found only in the detected microorganism) ?
- Is the primers sequences complementary only to this gene ?
- Is the primer sequence designed well ? (requirements about easy and specific annealing – depends on the primers sequence)
- Primers can be designed commercially or by using software ([http://www.biocenter.helsinki.fi/bi/Programs/download.html](http://www.biocenter.helsinki.fi/bi/Programs/download.html))
- sequences and genomes needed - [www.pubmed.com](http://www.pubmed.com)
PCR products detection

The PCR product has got specific length and specific sequence.

**Visualiton by intercalating agents**

bind to double-stranded DNA - fluorescence signal

- *Ethidium bromide* – carcinogen/teratogen
  
If intercalated the fluorescence of EtBr is significantly higher than in aqueous solution
  
(UV absorbance maxima at 300 and 360nm/emitted as orange/yellow light at 590nm)

- *Sybr Green*
  
DNA-Sybr Green complex absorbs blue light at 488 nm and emits green light at 522 nm

**Fluorescent tags** at 5´-end of primers

**Other tag** at 5´-end of primers

- e.g. biotin - for immunodetection detected by anti-bodies linked with an enzyme

**Microarray**

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Fig. 3. (a) Ethidium bromide; (b) the process of intercalation, illustrating the lengthening and untwisting of the DNA helix.
Electrophoresis

Conventional electrophoresis
a single electrical field causes biomolecules to migrate through a matrix according to its mass-to-charge ratio effectively separates DNA fragments up to ~20-30 kb (according to the gel concentration), larger fragments will comigrate (a large band at the top of the gel)
Electrophoresis

Primers (OT1559 a 18–1) complementary to DNA coding 16S rRNA *Campylobacter jejuni, Campylobacter coli* a *Campylobacter lari.*
Product is 287 bp

60 V, 100 minutes, M – 100 bp marker, (1-5: positive controls) 1- *C. jejuni* CCM 6212, 2- *C. jejuni* CCM 6214, 3 – *C. coli* CCM 6211, 4 – *C. lari* CCM 4897, 5 – *C. upsaliensis* ATCC 43954, 6-15 : strains C. jejuni, Nt- no template control (negative control)
Electrophoresis

DUPLEX PCR = two pairs of primers

*C. jejuni*: C1 5' - CAA ATA AAG TTA GAGGTA GAA TGT-3'
C4 5' - GGA TAA GCA CTA GCTAGC TGA T-3'

Complementary to a part oxidoreductase, 159 bp

*C. coli*: 18F 5' - GGT ATG ATT TCT ACAAG ACAG-3'
519R 5'- ATA AAA GAC TAT CGT CGC GTG-3'

Complementary to aspartatekinase and flanking ORF, 500 bp

60 V, 100 minutes, M – 100 bp marker, (1-5: positive controls) 1- C. *jejuni* CCM 6212, 2- C. *jejuni* CCM 6214, 3 – C. *coli* CCM 6211, 4 – C. *lari* CCM 4897, 5 – C. *upsaliensis* ATCC 43954, 6- 15 : strains C. *jejuni*, Nt- no template control (negative control)
Melting curve

Intercalating dye binds to double-stranded DNA - producing fluorescent signal when excited by light.

When samples are heated to melting point, double-stranded DNA denatures into single strands, freeing dye into solution:

- Melting temperature (Tm) is the point at which the DNA denatures.
- Dependent on the length of fragment and G-C content.
- More base pairs --> Increase in Tm.
- Higher G-C content --> Increase in Tm.

• Unbound dye has reduced fluorescent signal.
• Loss in signal generates melt curve.

- Intercalating dye

Signal: Increasing temperature.

No Signal
Inflex point
- 1. derivation = 0
- In this case 2.derivation is negative
C<sub>T</sub> (threshold cycle) – the lowest cycle, when fluorescence crosses the determinated value

Video: https://www.youtube.com/watch?v=kvQWKcMdyS4, https://www.youtube.com/watch?v=pRwoOBuk00c
Real-Time PCR

a  SYBR Green I
Annealing phase

Extension phase (I)

Extension phase (II)

End of PCR cycle

b  Hydrolysis probe

c  Hybridization probes
Food-borne pathogens detection by PCR

Identification of pure cultures - without problems

Detection/ enumeration from food matrix directly or after enrichment ?????

- How to isolate successfully DNA from few cells in 25 g/10 g????
  - Efficiency of DNA isolation (isolation by kits, heat-lysis, CTAB method)
- How many targets copies are needed for successfully PCR products detection ??
  - detection limits
  - presence of PCR inhibitors (inhibition of polymerase, primers annealing)
- How to distinguish DNA from dead and live cells ?? - Total DNA is detected (from dead and living cell)
- For distinguishing of dead cells DNA – e.g. application of EMA (ethidium monoazide) to the sample before DNA isolation – EMA irreversibly bound to DNA of dead cells – they are „invisible“ for PCR – no amplification

Direct enumeration/detection only in some specific case – simple liquid sample when the required limit is higher than the limit of detection

Standardly – for PCR is used enriched homogenate (sample diluted in enrichment broth and cultivated) – to obtain detectable level of microorganisms
BAX® System

Salmonella
Listeria monocytogenes
Genus Listeria
Enterobacter sakazakii
E. coli O157:H7
Staphylococcus aureus Real-time
Campylobacter jejuni/coli/lari Real-time
Vibrio cholerae/
parahaemolyticus/vulnificus Real-time
Yeast and molds

BAX – three major steps

On enriched/regrown samples

Prepare samples = perform heat-lysis in two stages

DNA present in the lysate

Hydrate PCR tablets

2. Process samples

Enrichment protocols vary depending on bacterial target and type of food you’re testing.

Salmonella, Enterobacter sakazakii, E. coli O157:H7, E. coli O157:H7 MP, Campylobacter jejuni/coli, Yeast and Mold

37° +/- 2°C for 20 minutes, then 95° +/- 3°C for 10 minutes

Listeria and L. monocytogenes

55° +/- 2°C for 60 minutes, then 95° +/- 3°C for 10 minutes

5 µl for all bacterial targets
20 µl for beef samples enriched in E. coli O157:H7 MP media
20 µl for yeast & mold

3. Review results
PCR tablets

- DNA-polymerase
- Primers
- Nucleotids
- Suplements
- SybrGreen or fluorescent probe
- Internal control + primer
- Mastermix in the tablet in PCR Eppendorf – minimal risk of contamination

For solving the problem of distinguishing dead and live cells the process of small volumes is used – only if live cells are present in the sample, they able to multiplied to the limit detection (10⁴ CFU/ml in the enrichment suspension used for lysis step). If the result of detection is positive, confirmation by plating out is done (live and dead cells).
Amplification of specific fragments

Target organism DNA + BAX® system primers in tablets → PCR → no detectable amplification

Background DNA + BAX® system primers in tablets → PCR

Detection of specific fragments

Fluorescent signal drops with heating to generate a melt curve
Melting curve

Melting curves display peaks within a set temperature range for the control and target (if present).

Strong positive result

Negative result

Positive control peaks

Target peak

Positive control peak
Detection

Melt curve: weak INPC in presence of strong *Salmonella* positive

Real time: weak INPC in presence of strong *Campylobacter* positives

Direct protocol does not display a positive or negative result

PCR was successful, Indeterminate, Signal error
Salmonella AFNOR (ISO 16140) protocol

Sample 1:10 BPW

18 h, 37°C

RVS

24 h, 41.5 / 37°C

Müller-Kauffmann

24 h, 37°C

5 suspicious colonies on TSA

24 h, 37°C

Biochemical and serological confirmation

22 h, 37°C

BHI

3 h, 37°C

1.5 d neg. and pos. result

Salmonella (ISO 6579:2003) versus BAX®-PCR

3 d neg., 5 d pos. results
International certificates

AOAC-RI Performance Tested Method
Salmonella #100201; L. monocytogenes #070202; L. spp #030502, S.aureus # 120701; Campylobacter coli/lari/jejuni

AFNOR Certification
Salmonella #QUA 18/3-11/02

Certificates from AFNOR and AOAC are equal

NordVal Certification
Salmonella #2006-30-5408
NordVal Certificate

<table>
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<tr>
<th>Issued for:</th>
<th>BAX Salmonella PCR (BAX Classic and BAX Q7) BAX System with Automated Detection PCR Assay for Screening Salmonella</th>
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<tr>
<td>NordVal No</td>
<td>030</td>
</tr>
<tr>
<td>First approval date</td>
<td>20 November 2003</td>
</tr>
<tr>
<td>Renewal date:</td>
<td>1 June 2007</td>
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<tr>
<td>Valid until:</td>
<td>1 June 2009</td>
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</tbody>
</table>

Oslo, 19 June 2007

CIG CXG AS Thermo Fisher Scientific Lundvæg 28, DK-2670 Greve, Denmark has applied for NordVal validation of BAX System with Automated Detection PCR Assay for Screening Salmonella in food, feed and environmental samples. The producer of the product is DuPont Qualicon, Bedford Bldg, 3531 Silverlake Road, Wilmington, DE 19810 USA.

The method is validated against ISO 6579:2002. BAX is also an AOAC method 2003.09. NordVal has studied the enclosures to the application and evaluated the results obtained in the validations by AFNOR and AOAC Research Institute. ISO 16140 was used for the AFNOR validation. NordVal has concluded that it has been satisfactorily demonstrated that the requirements of the NordVal validation protocol are fulfilled for foods, feeds and environmental samples. The limit of detection for the method is demonstrated to be 2-7 cfu/25 g or ml. The BAX Salmonella PCR System (BAX Classic and BAX Q7) can be used without further confirmation.

In the letter to the applicant it is stated that NordVal should be informed of any changes of the method, and that every two years an application for renewal of the certificate should be forwarded to NordVal. For the renewal of the NordVal certificate the method must be validated against the latest version of the reference method.

Yours sincerely

Sven Øverli
Chair of NordVal

Hide Shari Nori
NMKL Secretary General

NMKL / NordVal Tel. +47 23210249 / +47 46 986007 E-mail: nmkl@vetmed.no
OTHER
METHODS
ATP bioluminiscence

Principle: the detection of the present level of ATP (direct proportion to the presence of microorganisms or their products residues). The enzyme luciferase catalyzes the transformation of d-luciferin to oxyluciferin (indicated by emission of light at 560 nm)
ATP bioluminiscence

ATP Hygiene Monitoring System - SystemSURE II


http://www.bioxys.com/images2/figure-B11.gif
Cell-based sensors

Biosenzor = the whole cell/microorganism (CBS – cell-based sensors)

Patogenic microorganisms interact during the infection with mammalian cells – detection of cell or toxin by cell culture. The damage of cell culture is measured optically or electrically.

Bhunia, *Food Technol.* 65(2):38-43 (2011);
IDENTIFICATION OF MICROORGANISMS
**Definition of species in bacteria/archae**

**The bacterial species definition**
- eminent practical significance
- but a very difficult issue
  - very wide and often changes in DNA
  - mutations during replication of DNA
  - horizontal gene transfer through mobile elements, plasmids, also between species

The criterion for a bacterial or archaebal species

a collection of strains that have
- at least one diagnostic phenotypic trait
- ≥70% DNA–DNA hybridization of their purified molecules DNA
  - ~more than 97 % 16S rRNA sequence identity.

*Under the same conditions some eucaryotic order would be a single bacterial species.*
Identification based on genotype

Criterion for species in prokaryotic cells (bacteria and archaia):
Collection of strains characterised
- by at least one diagnostic feature
- their purified DNA molecules prove at least 70% hybridisation
which corresponds to at least 97% concordance in the sequence of **16S rRNA gene**

DNA isolation
DNA isolation
PCR amplification of 16S rRNA gene (usually)

Sequencing of PCR product

Comparison to sequences in databases (BLAST)

http://www.acgtinc.com/specialty_dna_sequencing.htm

Map of genome of B. cereus ATCC 14579
Identification of bacteria

PHENOTYPE METHODS
• Morphology of colony and cell (Gram staining, shape)
• Requirements for temperature, atmosphere, nutrition
• Biochemical tests - presence of specific enzymes
  • catalase, oxidase, fermentation of glucose
  • the way how they utilize different compounds
• Immunochemical tests - specific antigens in the cell wall

METHODS BASED ON PROTEIN ANALYSIS
• MALDI-TOF MS

METHODS BASED ON GENOTYPE
• looking for and comparing of specific DNA sequences
  • conserved universal genes (e.g. 16S rRNA)
  • genes specific for genus, species
• done by PCR methods, DNA chips, sequencing etc.
BIOCHEMICAL TESTING
Identification based on phenotype I

Physiological + biochemical features

appearance of colonies on selective or selective-diagnostic agars (for other testing to be reinoculated on non-selective agar)

• morphology of colonies and cells (Gram staining, shape)
• requirements for temperature, atmosphere, nutrient sources
• basic biochemical tests: oxidase, catalase, fermentation/oxidation of glucose, haemolysis etc.

the classification of an isolate into a certain group

(e.g. for bacteria: catalase positive/negative G+ cocci glucose fermenting/non-fermenting G- rods etc.)

biochemical tests specific for certain groups of microorganisms
Identification based on phenotype I

**Physiological + biochemical features:**  
**Example:** *Bacillus cereus*

**MYP agar** (Mannitol Egg Yolk Polymyxin Agar)  
pink colonies with a precipitation zone  
(no fermentation of manitol + enzyme lecininase)

β-haemolysis  
moisty and easy to grow colonies  
catalase positive

Set of biochemical tests:  
**Microgen® Bacillus ID Panel**  
for the identification of *Bacillus* spp. and related genera  
(time for performing: 48 hours)
Principle of biochemical testing

to rank the bacterium to some group having the same basic features
• e.g. catalase positive gram positive cocci
• Family *Enterobacteriaceae*

the identification on the genus or species level
• sets of biochemical tests specific for some group of bacteria
Gram staining procedure - principle

https://40.media.tumblr.com/25da2e5b25bbe336819bf1715849d171/tumblr_inline_ntrsgn9w1D1sv92o6_540.jpg
**Gram staining procedure - principle**

<table>
<thead>
<tr>
<th>Step</th>
<th>Microscopic Appearance of Cell (Gram (+))</th>
<th>Microscopic Appearance of Cell (Gram (-))</th>
<th>Chemical Reaction in Cell Wall (very magnified view) (Gram (+))</th>
<th>Chemical Reaction in Cell Wall (very magnified view) (Gram (-))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crystal violet</td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
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<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
</tr>
<tr>
<td>2. Gram’s iodine</td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
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</tr>
<tr>
<td>3. Alcohol</td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
</tr>
<tr>
<td>4. Safranin (red dye)</td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
<td><img src="https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg" alt="Image" /></td>
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</tr>
</tbody>
</table>

*First, crystal violet is added to the cells in a smear. It stains them all the same purple color.*

*Then, the mordant, Gram’s iodine, is added. This is a stabilizer that causes the dye to form large complexes in the peptidoglycan meshwork of the cell wall. The thicker gram-positive cell walls are able to more firmly trap the large complexes than those of the gram-negative cells.*

*Application of alcohol dissolves lipids in the outer membrane and removes the dye from the peptidoglycan layer—only in the gram-negative cells.*

*Because gram-negative bacteria are colorless after decolorization, their presence is demonstrated by applying the counterstain safranin in the final step.*

https://online.science.psu.edu/sites/default/files/micrb106/CellStructure/cow02354_03_15.jpg
Gram staining procedure

- 1-2 drops of sterile water on a slide
- Spread culture in thin filter in this drop
- Let dry in air/close to fire
- Pass slide through flame 2-3x to fix
- Add crystal violet – 15-20 s to act
- Add Lugol solution (KI/I₂) – 15-20 s to act
- Washing off with a mixture of ethanol:aceton (4:1) or acetone till the stain removes, but max. 15 s
- Rinse with water, decant excess liquid
- Add safranin: 1 minute
- Rinse with water, decant excess liquid
- Dry and put a drop of immersion oil, use an objective „OIL“
Gram staining procedure + Identification

**Enterobacter cloacae**

**Citrobacter freundii**

**Escherichia coli**

**Family Enterobacteriaceae**

**Gram-negative rods**

**Micrococcus luteus**

**Staphylococcus aureus**

**Bacillus cereus**

**Gram-positive cocci**

**Gram-positive rods (with endospores)**
Biochemical identification of G + bacteria

G+ bacteria
- cocci – in clusters, in pairs, diplococci
- bacilli
  - endospore
  - no endospore

For G+ cocci
- Catalase positive ? – *Staphylococcus* sp.
  - Coagulase + ?
    - *S. aureus* or some other few species
  - Coagulase - ?
    - Novobiocin S ?
      - *S. epidermidis* etc.
    - Novobiocin R ?
      - *S. saprophyticus* etc.
- Catalase negative ?
  *Streptococcus* sp.
  *Enterococcus* sp.
  - kind of haemolysis ?

Other important G + group – filamentous bacteria

Catalase test

Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it catalyzes the decomposition of hydrogen peroxide to water and oxygen.

\[ 2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2 \]

bubbling when culture is add in peroxide

catalase

Catalase testing: into a drop of 3% peroxide to transfer a loop of bacterial culture, catalase positive – releasing of air bubbles

Examples:

• G+ cocci:
catalase positive: Staphylococcus sp., Micrococcus sp., Kocuria sp., Dermacoccus sp.
Macrococcus sp., Rothia sp.
catalase negative: Streptococcus sp., Enterococcus sp.

• G+ rods (bacilli) endospores forming:
catalase positive: Bacillus sp.
catalase negative: Clostridium sp.
Resistance to ATB/haemolysis

Resistance e.g. to novobiocin

Hemolysis on blood agar

**alpha-hemolysis** - oxidizing the iron in the hemoglobin - green discoloration of the medium around

**beta-hemolysis** - rupture of erythrocytes - complete clearing medium around

**gamma-hemolysis** – no effect – no change

Measuring the diameter of inhibition zone (less than 17 mm resistant, more or equal to 17 mm sensitive) – differing in *Staphylococcus* sp.

(Clinical performance)
By cultivation on blood agar, bacteria can be differentiated based on their capacity to secrete hemolysins. The hemolysis will cause a clearing zone of the blood agar around the colonies. Bacteria can cause different types of hemolysis:

- **α-hemolysis**, which means an incomplete clearing (green haemolysis)
- **β-hemolysis**, which means a complete clearing
- Double hemolysis of some staphylococci consisting of an inner β-hemolysis zone and an outer α-hemolysis zone
- **No hemolysis**, which is sometimes referred to as γ-hemolysis, which may seem illogical.

Note that the α-hemolysin of staphylococci causes complete hemolysis, whereas their β-hemolysin causes incomplete hemolysis.

The capacity to produce hemolysins may vary between different strains of a particular bacterial species.
Bacterial hemolysins

Streptococcal hemolysins:
**α (alpha) hemolysis** - “zone of green discolouration” or “greening”, called partial or incomplete hemolysis produced by alpha-hemolytic streptococci such as *Streptococcus suis*. This type of hemolysis on BA is due to bacterial production of hydrogen peroxide which oxidises hemoglobin to methemoglobin.

**β (beta) hemolysis** – a clear zone of complete hemolysis produced by beta-hemolytic streptococci such as *S. equi* subsp. *equi*. This hemolysis is due to lysis of RBCs in the BA by the cytotoxin Streptolysin S.

Staphylococcal hemolysins:
Most coagulase-positive staphylococci (CPS) such as *Staphylococcus aureus*, *S. pseudintermedius* (but not *S. hyicus*) typically produce an distinctive pattern of “double zone” or “target” hemolysis on blood agar. Double-zone hemolysis indicates that the two hemolysins, one which produces “complete” and another one that produces “incomplete” hemolysis, are present, and this combined effect is visible due to different diffusion rates of these enzymes through the agar.

**The staphylococcal α (alpha) hemolysin causes a complete type of hemolysis.** This zone of complete hemolysis is narrow and immediately adjacent to the bacterial colony on blood agar.

**The staphylococcal β (beta) hemolysin causes an incomplete type of hemolysis at 37 °C.** This broader incomplete zone is outside of the inner complete zone and can be hard to see on early culture plates and also varies with the species of RBCs used to make the BA plate. Its action is enhanced by chilling at 4 °C and the hemolysis becomes complete (this effect is referred to as “hot-cold” hemolysis).

There are other staphylococcal cytotoxins that are also hemolysins, but their lytic actions are not seen on blood agar.

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The confusion arises due to the NAMES of the two STAPHYLOCOCCAL HEMOLYSINS and the TYPE of hemolysis they produce. These are the REVERSE of the two TYPES of STREPTOCOCCAL HEMOLYSIS. Strictly speaking, the descriptive terms “alpha” for “incomplete” and “beta” for “complete” hemolysis apply only to the hemolytic toxins of Streptococcus species. However, other organisms beside Streptococcus and Staphylococcus species have cytotoxins that cause hemolysis and this hemolysis can be either complete or incomplete. It is preferable to use the terms “complete” and “incomplete” rather than “beta” or “alpha” to describe the hemolysis caused by these organisms, but you will notice in bacteriology text books that the terms alpha and beta are commonly used.

Source: http://people.upei.ca/jlewis/html/hemolysins.html
Biochemical identification of G - bacteria

**G – bacteria**
- Cocci
- Bacilli (Rods)
- Oxidase positive/negative
- Glucose metabolism
  - fermentation
  - oxidation
  - Inactive
- Lactose metabolism for coliform
- Pigment formation for *Pseudomonas* sp.
Oxidase test

Oxidase
• abbreviated general name for some kind of cytochrome c oxidases (cytochrome oxidase or indophenol oxidase), enzymes involved in aerobic respiratory chain, when oxygen is used as the final receptor for hydrogen.
• Oxidases are usually present only in aerobic organisms.
• Presence or absence of oxidase(s) is an important feature of the bacterial metabolism, used mainly in the identification process of gram-negative bacteria.

<table>
<thead>
<tr>
<th>Oxidase positive (OXI +)</th>
<th>Aeromonas sp.</th>
<th>Pseudomonas sp.</th>
<th>Dark-blue to violet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase negative (OXI -)</td>
<td>Enterobacteriaceae</td>
<td>Bacillus sp.</td>
<td>Colourless</td>
</tr>
</tbody>
</table>

Detection:
Reaction of the culture with redox indicator as e.g N,N-dimethyl-p-phenylenediamine (DMPD): dark-blue when oxidized and colorless when reduced. Different test performances are possible.
Fermentation of carbohydrates

Type of utilisation of glucose or carbohydrates

- an important feature of the bacterial metabolism
- widely used in the identification process of bacteria
- **fermentation** strictly means to be under anaerobic conditions (but in bacterial metabolism, which can be but not have to be the same as the anaerobic conditions of cultivation), so very often is used in a general way like „utilisation“

- fermentation (like „utilisation“) of carbohydrates to
  - acid and gas (Durham tubes)
  - acid without gas
  - non-utilised

- Testing of fermentation or oxidation of carbohydrates
  - e.g. OF test for glucose
  - by oxidation under aerobic conditions
  - by fermentation under anaerobic conditions (an oil layer)
Fermentation of carbohydrates

Fermentation of sugars to acid

Peptone Water w/Phenol Red (HiMedia M028I)

Ingredients g/l
Peptic digest of animal tissue 10.000
Sodium chloride 5.000
Phenol red 0.020
Final pH (at 25°C) 6.8±0.2

+ after sterilisation at the room temperature to add aseptically sugar discs and inoculate:

- succrose, trehalose, rhamnose, xylose

**Incubation:** 30 °C, 24 h
**Enterobacteriaceae** – glucose fermentation

**Tryptone Yeast Extract Agar**

**Ingredients Gms / Litre**

- Casein enzymic hydrolysate 10.000
- Yeast extract 1.500
- Dextrose 10.000
- Sodium chloride 5.000
- Bromocresol purple 0.015
- Agar 15.000

**Final pH (at 25°C) 7.0±0.2**

Casein enzymic hydrolysate and yeast extract provide nitrogenous compounds, vitamin B complex and other growth nutrients. Dextrose is the fermentable carbohydrate and bromocresol purple acts as the pH indicator, with colour change from purple to yellow in acidic conditions. Sodium chloride maintains osmotic equilibrium.

„**Anaerobic**“ conditions – inoculation by stabbing to used fresh prepared media (oxygen expelled by heating medium in boiling water or running steam for 15 min, then to be cooled down quickly)
Fermentation of carbohydrates

Test for oxidation/fermentation of glucose (O/F glucose test) detection of acid production by acidobazic indicator change to yellow (acid present) in lower pH from green (no acid). Anaerobic atmosp. – covering by sterile mineral oil. If fermented anaerobically, then also runs aerobically.
Principle of biochemical tests

- The first step in identification is to rank the bacterium to some group of bacteria having the same basic features – e.g. catalase positive gram positive cocci.
- The identification on the genus or species level is finished by other biochemical tests specific for each group of bacteria.
- The tests can be done in tubes or miniaturized plate.

**ENTEROtest 24N (Erba Lachema)**
(24 means 24 biochemical tests, N= new)
- designed for routine identification of clinically important species of family Enterobacteriaceae without the use of reagents.
- A strip test OXITest or OFtest in microtitration wells can be used to confirm that the isolate belongs to Enterobacteriaceae family.
- The results are read visually or automatically using reader MIKROLA®.
- The identification can be supplemented by diagnostic strip tests: INDOLtest, COLItest and VPtest.

**API 20E (bioMérieux)**

**API® Gram negative Identification**
- API 20E – Species/subspecies identification of Enterobacteriaceae and group/species identification of non-fermenting Gram negative bacteria
- API Rapid 20E – 4-hour identification of Enterobacteriaceae
- API 20NE – 24 to 48-hour identification of Gram negative non-Enterobacteriaceae
- API Campy – 24-hour identification of Campylobacter species
### ENTEROtest 24 N

**Example:** *Escherichia coli*

| Identification        | IND | H  | URE | G  | ARG | F  | ORN | E  | LYS | D  | H₂S | C  | SCI | B  | MAL | A  | ONP | H  | SAL | G  | SOR | F  | MLB | E  | CEL | D  | LAC | C  | TRE | B  | MAN | A  | GLR | H  | DUL | G  | ADO | F  | ART | E  | SUC | D  | INO | C  | RAF | B  | ESL | A  | bXY |
|-----------------------|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|----|
| *Escherichia coli*     |     | (+)|    | -  | (-) | d  |    | (+)|    | d  |    |    | d  |    | (+)|    | d  |    | (+)|    | d  |    |    |    |    |    |    | (+)|    | d  |    |    |    |    |    |    |    |    |    |    |    |    |

**Legend:**
- + = positive reaction
- - = negative reaction
- d = variable reaction
- (+) = mostly positive reaction
- (-) = mostly negative reaction

<table>
<thead>
<tr>
<th>Test</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease</td>
<td>URE</td>
</tr>
<tr>
<td>Arginine</td>
<td>ARG</td>
</tr>
<tr>
<td>Ornithine</td>
<td>ORN</td>
</tr>
<tr>
<td>Lysine</td>
<td>LYS</td>
</tr>
<tr>
<td>Hydrogen sulphide</td>
<td>H₂S</td>
</tr>
<tr>
<td>Simmons citrate</td>
<td>SCI</td>
</tr>
<tr>
<td>Malonate</td>
<td>MAL</td>
</tr>
<tr>
<td>β - Galactosidase</td>
<td>ONP</td>
</tr>
<tr>
<td>Salicine</td>
<td>SAL</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>SOR</td>
</tr>
<tr>
<td>Melibiose</td>
<td>MLB</td>
</tr>
<tr>
<td>Celllobiose</td>
<td>CEL</td>
</tr>
<tr>
<td>Lactose</td>
<td>LAC</td>
</tr>
<tr>
<td>Trehalose</td>
<td>TRE</td>
</tr>
<tr>
<td>Mannitol</td>
<td>MAN</td>
</tr>
<tr>
<td>β - Glukuronidase</td>
<td>GLR</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>DUL</td>
</tr>
<tr>
<td>Adonitol</td>
<td>ADO</td>
</tr>
<tr>
<td>Arabitol</td>
<td>ART</td>
</tr>
<tr>
<td>Sucrose</td>
<td>SUC</td>
</tr>
<tr>
<td>Inositol</td>
<td>INO</td>
</tr>
<tr>
<td>Raffinose</td>
<td>RAF</td>
</tr>
<tr>
<td>Esculin</td>
<td>ESL</td>
</tr>
<tr>
<td>β - Xylosidase</td>
<td>bXY</td>
</tr>
</tbody>
</table>
Biochemical testing - *Enterobacteriaceae*

- **TSI** Triple Sugar Iron Agar (use a straight wire to stab the butt and streak the agar surface)
  - to determine whether organisms can ferment glucose, sucrose and/or lactose with or without production of gas and the ability of the organism to produce hydrogen sulphide from thiosulphate in an acid environment is also tested.

**Formula / Liter**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic Digest of Casein</td>
<td>5 g</td>
</tr>
<tr>
<td>Enzymatic Digest of Animal Tissue</td>
<td>5 g</td>
</tr>
<tr>
<td>Yeast Enriched Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>10 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10 g</td>
</tr>
<tr>
<td>Ferric Ammonium Citrate</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Sodium Thiosulfate</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.025 g</td>
</tr>
<tr>
<td>Agar</td>
<td>13.5 g</td>
</tr>
</tbody>
</table>

Final pH: 7.3 ± 0.2 at 25 °C

**Results**

An alkaline slant-acid butt (red/yellow) - fermentation of dextrose only.

An acid slant-acid butt (yellow/yellow) - fermentation of dextrose, lactose and/or sucrose.

An alkaline slant-alkaline butt (red/red) - dextrose or lactose not fermented (non-fermenter).

Phenol red – alkaline = red, acid = yellow

Inoculation in the butt by stab inoculation on the slant on the surface

- **Fermentation of dextrose (glucose) to acid**
  - anaerobically - yellow butt
  - aerobically leads to products, which are later oxidised by air and pH remains alkaline (red)

- **Fermentation of glucose to gas** (anaerobically - in the stab)
  - Cracks, splits, or bubbles in medium indicate gas production

- **Production of hydrogen sulfide** ($H_2S$) – reaction with ferrous sulphate and sodium thiosulphate - a black precipitate in butt

- **Lactose and/or sucrose utilisation to acid**
  - Acid production – yellow butt and slant

Bacterium fermenting lactose and/or succrose are able to ferment glucose
Biochemical testing - *Enterobacteriaceae*

- **TSI Triple Sugar Iron Agar** (use a straight wire to stab the butt and streak the agar surface)
  
  - to determine whether organisms can ferment glucose, sucrose and/or lactose with or without production of gas and the ability of the organism to produce hydrogen sulphide from thiosulphate in an acid environment is also tested.

---

**Expected Cultural Response:** Cultural response in Triple Sugar Iron Agar at 35 ± 2°C after 18 – 24 hours of incubation.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Approx. Inoculum (CFU)</th>
<th>Expected Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> ATCC® 25922</td>
<td>Heavy</td>
<td>Recovery</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> ATCC® 12453</td>
<td>Heavy</td>
<td>growth</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC® 27853</td>
<td>Heavy</td>
<td>growth</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> ATCC® 14028</td>
<td>Heavy</td>
<td>growth</td>
</tr>
<tr>
<td><em>Shigella flexneri</em> ATCC® 12022</td>
<td>Heavy</td>
<td>growth</td>
</tr>
</tbody>
</table>

The organisms listed are the minimum that should be used for quality control testing.

KEY: A, acid, K, alkaline, +, positive, -, negative, +/-, usually negative, positive, growth
Biochemical testing - Enterobacteriaceae

Urea Hydrolysis

Urea

C=O(NH₂)₂ + H⁺ + 2H₂O → HCO₃⁻ + 2(NH₄⁺)

Bicarbonate

Ammonium ions

And then...

HCO₃⁻ → CO₂⁻ + OH⁻

Phenol red:
Medium is acidic - yellow (pH 6.8±0.2)
Alkaline reaction by urea hydrolysis - pink colour

Nutrition:
Peptic digest of animal tissues, dextrose
Sodium chloride - osmotic equilibrium
Phosphates - buffering of the medium.

Composition Gms / Litre
Peptic digest of animal tissue 1.000
Dextrose 1.000
Sodium chloride 5.000
Disodium phosphate 1.200
Monopotassium phosphate 0.800
Phenol red 0.012
Agar 15.000

Final pH (at 25°C) 6.8±0.2

Biochemical testing - Enterobacteriaceae

Urease Test

a agar (Christensen) (use a 1 μl loop full)
determine whether an organism can split urea into ammonia and on dioxide by the action of the enzyme urease.

http://image.slidesharecdn.com/lab17-141201085153-conversion-gate01/95/-10-638.jpg?cb=1417424219

Biochemical testing - *Enterobacteriaceae*

**L-lysine decarboxylase** (use a 1 μl loop full)
- to determine whether an organism can decarboxylate an amino acid (lysine) leading to formation of an amine. After inoculation both tubes are overlayed with sterile paraffin oil.

**Two steps detection:**

1. **step**
   - Dextrose is fermented – H⁺ is released
   - Acidic conditions
     - bromocresol purple changes colour to yellow
     - in acidic conditions lysine decarboxylase is activated

2. **step**
   - L-lysine is decarboxylated and OH⁻ released
   - Alkaline conditions
     - bromocresol purple changes colour back to purple

**Lysine Decarboxylase Broth**

Typical Composition (g/litre)
- Peptic digest of animal tissue 5.000;
- Yeast extract 3.000; Dextrose 1.000 (*glucose prepared from starch, chemically equivalent to glucose*);
- L-Lysine hydrochloride 5.000; Bromocresol purple 0.020

**Final pH (at 25 °C) 6.8±0.2**

Inoculate below the surface, cover by sterile paraffin oil

\[
\text{L-lysine} \rightarrow \text{Cadaverine} + \text{CO}_2 + \text{OH}^-
\]

http://fce-study.netdna-ssl.com/images/upload-flashcards/1029530/2332621_m.jpg
• **β-galactosidase (ONPG)** (use a 1 μl loop full)
  - to determine whether an organism has the enzyme β-galactosidase by using the compound o-nitrophenyl-beta-D-galactopyranoside (ONPG).
  - this test is used to differentiate between lactose-negative and lactose-delayed organisms.
  - ONPG is hydrolysed by the enzyme to o-nitrophenol, which is yellow, and galactose.

**Lactose fermenting bacteria (ONPG positive):**
- **two enzymes**
- Permease – transferring of the lactose molecule into the bacterial cell
- **β-galactosidase** - cleaving the galactoside bond, producing glucose and galactose
  - inducible enzyme - made ONLY in the presence of the lactose substrate

**Coliform bacteria as E.coli, Klebsiella spp., Enterobacter spp.**

**Late lactose fermenting bacteria**
- Permease – not present
- delayed in the production of acid from lactose because of sluggish permease activity
- **β-galactosidase – present**
  - give a positive ONPG test
  - *Citrobacter* spp., *Arizona* spp,

**Non lactose fermenter (ONPG Negative):**
- Permease – not present
- **β-galactosidase – not present**
  - *Salmonella* spp; *Shigella* spp; *Proteus* spp; *Providencia* spp. and *Morganella* spp.

O-Nitrophenyl-β-D-galactopyranoside (ONPG) is structurally similar to lactose (i.e. ONPG is an analog of lactose), except that orthonitrophenyl has been substituted for glucose. ONPG is colorless compound: O-nitrophenol is yellow, visual evidence of hydrolysis.

---

**Biochemical testing - *Enterobacteriaceae***

**β-D-Galactose**

**O-Nitrophenol**

hydrolysis
Biochemical testing - *Enterobacteriaceae*

**β-galactosidase (ONPG)**

**Procedure**

**Culture**
Bacteria grown in medium containing lactose (to induce the production of the galactosidase enzyme), such as Kligler iron agar (KIA) or Triple sugar Iron (TSI) agar, (Note: β-galactosidase enzyme (inducible enzyme) is made ONLY in the presence of the lactose substrate).

**Inoculation**
1. A loopful of bacterial growth is emulsified in 0.05mL of physiologic saline to produce a heavy suspension
2. One drop of toluene is added to the suspension and vigorously mixed for a few seconds to release the enzyme for bacterial cells.
3. An equal quantity of buffered ONPG solution is added to the suspension.
4. The mixture is placed in a 37 °C water bath
5. When Using ONPG Tablets
6. A loopful of bacterial suspension is added directly to the ONPG substrate resulting from adding 1mL of distilled water to a tablet in a test tube.
7. This suspension is also placed in a 37°C water bath

**Results and Interpretations**
The rate of hydrolysis of ONPG to o-nitrophenol may be rapid for some organisms; producing a visible yellow color reaction within 5 to 10 minutes. Most tests are positive within 1 hour; however, reactions should not be interpreted as negative before 24 hours of incubation. The yellow color is usually distinct and indicates that the organism has produced o-nitrophenol from the ONPG substrate through the action of β-galactosidase.

![Proteus vulgaris (Left): ONPG Negative Escherichia coli (Right): ONPG Positive](image-url)
Biochemical testing - *Enterobacteriaceae*

**Voges–Proskauer (VP) test** (use a 1 μl loop full)
- To determine whether an organism can produce acetyl-methylcarbinol (acetoin) from fermentation of glucose. If present, acetyl-methyl carbinol is converted to diacetyl in the presence of α- naphthol (to be added first), strong alkali (40% KOH), and atmospheric oxygen. The **diacetyl** and **quanidine-containing compounds** found in the peptones of the broth then condense to form a **pinkish red polymer**.

**Procedure of Voges–Proskauer (VP) Test**
1. Prior to inoculation, allow medium to equilibrate to room temperature.
2. Using organisms taken from an 18-24 hour pure culture, lightly inoculate the medium.
3. Incubate aerobically at 37 °C, 24 hours.
4. Following 24 hours of incubation, aliquot 2 ml of the broth to a clean test tube.
5. Re-incubate the remaining broth for an additional 24 hours.
6. Add 6 drops of 5% alpha-naphthol, and mix well to aerate.
7. Add 2 drops of 40% potassium hydroxide, and mix well to aerate.
8. Observe for a pink-red color at the surface within 30 min. Shake the tube vigorously during the 30-min period.

**Positive Reaction:**
A pink-red color at the surface
Examples: Viridans group streptococci (except *Streptococcus vestibularis*), *Listeria*, *Enterobacter*, *Klebsiella*, *Serratia marcescens*, *Hafnia alvei*, *Vibrio* *ello*, *Vibrio alginolyticus*, etc.

**Negative Reaction:**
A lack of a pink-red color
Examples: *Streptococcus* *mitis*, *Citrobacter* sp., *Shigella*, *Yersinia*, *Edwardsiella*, *Salmonella*, *Vibrio furnissii*, *Vibrio* *fluvialis*, *Vibrio* *vulnificus*, and *Vibrio parahaemolyticus* etc.

**A copper color should be considered negative. A rust color is a weak positive reaction.**

**Quality Control of Voges–Proskauer (VP) Test**
- VP positive: *Enterobacter aerogenes* (ATCC13048)
- VP negative: *Escherichia coli* (ATCC25922)

**MRVP broth (pH 6.9)**
Ingredients per liter of deionized water:
- Buffered peptone= 7.0 gm
- Glucose= 5.0 gm
- Dipotassium phosphate= 5.0 gm

**Voges–Proskauer Reagent A: Barritt’s reagent A**

<table>
<thead>
<tr>
<th>Alpha-Naphthol, 5%</th>
<th>50 gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute Ethanol</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Voges–Proskauer Reagent B: Barritt’s reagent B**

<table>
<thead>
<tr>
<th>Potassium Hydroxide</th>
<th>400 gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Voges–Proskauer (VP) test

**Principle:**
The substrate for acetoin formation is sodium pyruvate in paper zone of strip. The strip of VP test is inserted into saline suspension of strain tested. After incubation for time recommended the reagents of VPT I (α-naphthol) and VPT II (potassium hydroxide) is added. The presence of acetoin is indicated by red colour.

**Procedure:**
- From a pure culture of strain tested prepare suspenion in saline
- The suspension must have a turbidity recommended in table "Turbidity and incubation"
- Insert the VP test strip into tube with suspension; both zones of the paper strip must be immersed into saline
- Incubate at 37°C for time recommended
- After incubation add 3 drops of reagent VPT I and VPT II and shake the tube thoroughly
- Incubate for another 30 min at 37°C to develop colour reaction
- Read the reaction in accordance with the table "Interpretation of VP test"

### Suspension turbidity and incubation time

<table>
<thead>
<tr>
<th></th>
<th>McFarland turbidity scale No.</th>
<th>Incubation (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>enterobacteria</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>streptococci and enterococci</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>staphylococci</td>
<td>2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

### Results of control strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>CCM</th>
<th>VPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>1484</td>
<td>+</td>
</tr>
<tr>
<td>Micrococcus sedentarius</td>
<td>2699</td>
<td>–</td>
</tr>
</tbody>
</table>
Rapid test for confirmation of *E. coli* – typical features:

- β-D-glucuronidase (97 % strains), production of indole from tryptophan (typical for *E. coli*)

Performance:

- Previous testing: Gram-negative (e.g. able to grow on VRBD agar), oxidase negative
- From pure culture to prepare suspension in saline solution (app. 5 ml) at the density 3 McFarland
- To transfer 0.5 – 1 ml of this suspension into a new tube – to add the strip of COLImtest (chemicals – fluorogenic substrate for β-D-glucuronidase, tryptophane are present in the pad at the end of the strip)
- Incubation: 37 °C, 4 hours
- Detection of β-D-glucuronidase (to be done at first): UV (360 nm) – blue fluorescence (GLR + ...β-D-glucuronidase positive) – GLR test can be read after 1 hour
- Detection of indole formation from tryptophane (to be done at second): to add Kovacs reagent (IND + ...formation of red ring)
Biochemical testing - *Enterobacteriaceae* 
**COLItest**

Fluorogenic culture medium

- One or more fluorogenic substrates
- For detection of specific enzyme, which are able to cleave out the fluorogenic substrate, that a designed fluorophore (responsible for the UV fluorescence) is released

**FLUOROGENIC MEDIUM (Colilert):** *E. coli* use β-glucuronidase to metabolize MUG and create fluorescence (UV 360 nm). Since most non-coliforms do not have these enzymes, they are unable to grow and interfere.

[E. coli image](http://ga.water.usgs.gov/projects/bacteria/pictures/summaryanalysecoli.jpg)
Biochemical testing - *Enterobacteriaceae*

**COLItest**

- **Indole test** (use a 1 µl loop full)
  - to determine whether an organism can split indole from tryptophan.

**Indole production from tryptophan**

Production of indole is detected by Kovac's reagent

![Reaction diagram](image)

**Interpretation of the reactions**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>GLR (fluorescence)</th>
<th>IND (colour reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive</td>
<td>blue fluorescence</td>
<td>red to pink colour</td>
</tr>
<tr>
<td>negative</td>
<td>no fluorescence</td>
<td>yellow to yellowish colour</td>
</tr>
</tbody>
</table>

**Explanations:**

- GLR .................. β-glucuronidase test
- IND .................. indole production test

**Indole Kovacs Reagent:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Dimethylaminobenzaldehyde</td>
<td>50.0 gm</td>
</tr>
<tr>
<td>Hydrochloric Acid, 37%</td>
<td>250.0 ml</td>
</tr>
<tr>
<td>Amyl Alcohol</td>
<td>750.0 ml</td>
</tr>
</tbody>
</table>

**Control strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>CCM</th>
<th>ATCC</th>
<th>GLR</th>
<th>IND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>3988</td>
<td>10536</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>303</td>
<td>13880</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Biochemical testing - *Enterobacteriaceae*

**INDOLe test – quick variant**

**Procedure:**
- on the filtration paper to add a drop of INDOL suspension
- to spread into by a loop some culture
- To incubate – at the room temperature for 5 minutes
- To read results

<table>
<thead>
<tr>
<th>INDOL Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)</td>
</tr>
<tr>
<td>(-)</td>
</tr>
</tbody>
</table>
routine identification of clinically important species of family *Enterobacteriaceae*. The test is focused only on some species often isolated in clinical practices. Strains from other sources can be slightly different in the dominant biochemical profiles.

Intended exclusively for the identification of family *Enterobacteriaceae*, the preliminary ranking into family *Enterobacteriaceae* must be done (Gram staining, oxidase, glucose fermentation).

The results of reactions obtained in this modified micromethod may differ from the results obtained using conventional tests and results available in literature sources. In case of problem to control positive/negative reactions by control strains or to use for this tests tube variant to check the functionality of the kit not to check the accuracy or success of the identification!
Preparation of suspension from **over-night culture**

**growing on non-selective agar**

(for staphylococci Columbia agar blood with 5 % of sheep blood is recommended)

Inoculation of microtitre plates, anaerobic reaction covered by mineral oil

**Incubation**

Reading results by both visual and instrumental mode, respectively

**Standard off-line tests**

INDOLtest or COLItest

**Other off-line tests**

OXItest, OFtest, VPtest, Acetoin test
ENTEROtest 24N

- Tube with 5 ml of sterile physiol.sol.
- Suspension (1 McFarland)
- Application: 100 µl to each hole
- 2 drops of parafin oil into holes D-H of the first line (anaerobic conditions)
- Incubation 37°C, 24h
- For check the purity of suspension to streak suspension on TSA (incubation: 37 °C – 24 h)
**ENTEROtest 24 N - evaluation**

<table>
<thead>
<tr>
<th></th>
<th>H</th>
<th>G</th>
<th>F</th>
<th>E</th>
<th>D</th>
<th>C</th>
<th>B</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>➕</td>
<td>➕</td>
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<td>➕</td>
<td>➕</td>
<td>➕</td>
<td>➕</td>
<td>➕</td>
</tr>
</tbody>
</table>

**Identification code:** 023426

**Escherichia coli**

**H:** 0+0+0=0  
**G:** 0+2+0=2  
**F:** 1+2+0=3 ....

**CCM 4225 Escherichia coli (ATCC 35218)**

<table>
<thead>
<tr>
<th></th>
<th>URE</th>
<th>ARG</th>
<th>ORN</th>
<th>LYS</th>
<th>H₂S</th>
<th>SCI</th>
<th>MAL</th>
<th>ONP</th>
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<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>d</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td></td>
<td></td>
<td>—</td>
</tr>
</tbody>
</table>

---

**Notes:**
- The evaluation results are based on the color reactions in the test cards, indicating the presence or absence of specific enzymes or metabolic reactions.
- The identification code 023426 is specific to the test results for *Escherichia coli*.
- The **H**, **G**, and **F** values are calculated by summing the positive (+) and negative (−) reactions.
## ENTEROtest 24 – example of biochemical testing

| Identification                  | IND | HURe | G ARG | FORN | E LYS | D H₂S | C SCI | B MAL | A ONP | H SAL | G SOR | F MLB | E CEL | D LAC | C TRE | B MAN | A GLR | H DUL | G ADO | F ART | E SUC | D INO | C RAF | B ESL | A bXY |
|--------------------------------|-----|-------|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Yersinia aldovae               | -   | d     | -     | d    | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     |
| Yersinia bercovieri            | -   | d     | -     | (+)  | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     |
| Yersinia enterocolitica ssp. enterocolitica | d  | d     | -     | +    | -     | -     | -     | +     | d     | +     | -     | d     | -     | +     | +     | +     | -     | -     | -     | -     | -     | -     | -     | -     |
| Yersinia frederiksenii         | +   | d     | -     | +    | -     | -     | (-)   | -     | +     | d     | +     | +     | d     | +     | -     | -     | -     | +     | d     | (+)   | d     | d     | (+)   | d     |
| Yersinia intermedia            | +   | (+)   | -     | +    | -     | -     | (+)   | +     | (+)   | +     | d     | +     | +     | +     | -     | -     | d     | (+)   | d     | +     | -     | -     | -     | -     |
| Yersinia kristensenii          | d   | d     | -     | (+)  | -     | -     | -     | d     | (-)   | -     | +     | -     | (-)   | +     | +     | +     | d     | -     | -     | (-)   | d     | +     | -     | -     |
| Yersinia mollaretii            | -   | d     | -     | (+)  | -     | -     | -     | d     | (-)   | +     | -     | +     | d     | +     | -     | -     | -     | -     | +     | +     | -     | -     | -     | -     |
| Yersinia pestis                | -   | -     | -     | -    | -     | -     | d     | d     | d     | d     | d     | -     | +     | +     | +     | -     | -     | d     | -     | -     | -     | -     | -     | -     |
| Yersinia pseudotuberculosis    | -   | +     | -     | -    | -     | -     | d     | d     | -     | d     | d     | -     | +     | +     | +     | -     | -     | d     | -     | -     | -     | -     | -     | -     |
| Yersinia rohdei                | -   | d     | -     | d    | -     | -     | d     | +     | d     | d     | d     | -     | +     | +     | +     | -     | -     | +     | -     | +     | -     | -     | -     | -     |
| Yersinia ruckeri               | -   | -     | +     | d    | -     | -     | d     | -     | d     | -     | -     | +     | +     | +     | +     | -     | -     | +     | -     | +     | -     | -     | -     | -     |

### Explanations:
- **+** = 90–100% of positive reactions
- **(+)** = 75–89% of positive reactions
- **d** = 26–74% of positive reactions
- **−** = negativní reakce
- **(−)** = 11–25% of positive reactions
- *** = orientation identification**

### Performance of the kit:
The kit was tested on a set of 97 strains, including INDOLOtest a VPtest:
The identification of 93% of the strains was correct.
The identification of 7% of the strains was to the genus level.
The result = a set of biochemical properties (+/-), which is compared to the sets of biochemical properties of different chosen genera and species for them the test is designed.

Identification score:
the measure of similarity to proposed taxon

T-index: how is the likelihood of the different properties?
The combination of identification and T-index = the reliability of the result

Excelent, very good, good (this case), acceptable, intermediary strain, needs some additional tests, genus level, non-existing taxon.

---

<table>
<thead>
<tr>
<th>% id.</th>
<th>Tin.</th>
<th>T.proti</th>
<th>Rozlišující testy</th>
<th>T.again</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>INTERMEDIÁRNÍ KMen / LOW DISCRIMINATION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>78.17</td>
<td>0.44</td>
<td>LYS 90</td>
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<td>95</td>
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<tr>
<td>Escherichia coli inactive</td>
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<td>0.25</td>
<td>TRE 90</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>Yersinia rohdei</td>
<td>6.05</td>
<td>0.11</td>
<td>IND GLR</td>
<td>1</td>
<td>1</td>
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<tr>
<td><strong>INTERMEDIÁRNÍ KMen / LOW DISCRIMINATION</strong></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Escherichia coli</td>
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<td>0.78</td>
<td>LYS 90</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>Yersinia rohdei</td>
<td>12.51</td>
<td>0.51</td>
<td>IND GLR</td>
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<td>1</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>3.87</td>
<td>0.30</td>
<td></td>
<td>99</td>
<td>1</td>
</tr>
</tbody>
</table>

**Evaluation by using the identification code**
- By using a code book
- By using software TNW

---

Citrobacter freundii | 6.59 | 0.35 | LYS 1 | 99 | 1 | 022713 |
| Citrobacter gillenii | 63.43 | 0.48 | MAL ONF | 99 | 67 |
| Salmonella enterica ss. houtenae | 3.04 | 0.10 | | 1 | 1 |

**RODOVA IDENTIFIKACE / IDENT.TO THE GENUS LEVEL**

**INTERMEDIÁRNÍ KMen / LOW DISCRIMINATION**

Citrobacter freundii | 71.75 | 0.33 | LYS 1 | MAL | 022716 |
| Yersinia rohdei | 21.12 | 0.11 | 1 |
| Citrobacter gillenii | 7.14 | 0.08 | 99 |

**DOBA IDENTIFIKACE / GOOD IDENTIFICATION**

Citrobacter freundii | 97.57 | 0.45 | LYS 1 | 022717 |
| Citrobacter gillenii | 2.43 | 0.08 | |
**ENTEROtest 24 – example of biochemical testing**

**The way of identification:**
the obtained profile is compared with profiles in database
- The most similar profiles are found
  - **Identification score** = % of similarity
- **But what about the different reactions?**
  - T-index is calculated
    - how is the probability of the different reactions?
      - It depends on their importance – typical reactions should be the same
- **Reliability of the result**
  - the combination of identification score and T-index =
  - Excelent, very good, good, acceptable, intermediary strain, needs some additional tests, genus level, non-existing taxon

| Identification  | IND | HRE | ARG | FNE | ERE | DRE | SRE | BNE | ANP | HNE | GNE | SOR | MLB | FN  | CEL | DLC | CNE | BNE | ANE | HNE | GNE | GNE | ANE | BNE | ME | ERE | DRE | SRE | (+) | (+) |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cronobacter sakazakii | (-) | -   | +   | (+) | -   | +   | -   | +   | -   | +   | +   | +   | -   | +   | +   | +   | -   | +   | +   | +   | +   | +   | +   | +   | d   | +   | +   | +   | d   | +   | +   | +   | +   | +   |

**Explanations:**

- + = 90–100% of positive reactions
- (+) = 75–89% of positive reactions
- d = 26–74% of positive reactions
- (-) = 11–25% of positive reactions
- = neonatni reakce
- * = orientation identification
Biochemical tests - divisions

STAPHYtest 24
Genus *Staphylococcus* isolated from clinical material and for their distinguishing from related genera of other gram positive, catalase positive cocci

STREPTOtest 24
*Streptococcus, Enterococcus* and related gram positive, catalase negative cocci isolated from clinical material

ENTEROtest 24
Species of family *Enterobacteriaceae*

NEFERMtest 24
Gramnegative non-fermenting bacteria and families *Vibrionaceae, Aeromonadaceae* and *Pleiomonas shigelloides* present mainly in clinical material.

ANAEROtest 23
**ANAEROBIC BACTERIA (G + AND G-)***
Routine identification of anaerobic bacteria (G + AND G-) in clinical material and in food
Biochemical tests

API® Gram positive Identification
API Staph – Overnight identification of clinical staphylococci and micrococci
API 20 Strep – 4 or 24-hour identification of streptococci and enterococci
API Coryne – 24-hour identification of Corynebacteria and coryne-like organisms
API Listeria – 24-hour identification of all Listeria species

API® Gram negative Identification
API 20E – Species/subspecies identification of Enterobacteriaceae and group/species identification of non-fermenting Gram negative bacteria
API Rapid 20E – 4-hour identification of Enterobacteriaceae
API 20NE – 24 to 48-hour identification of Gram negative non-Enterobacteriaceae
API Campy – 24-hour identification of Campylobacter species

API® Anaerobe Identification
API 20A – 24-hour identification of anaerobes
Rapid ID 32 A – 4-hour identification of anaerobes
Biochemical tests

**MicrogenTM GN-ID**
Convenient 12 substrate identification system for commonly encountered *Enterobacteriaceae* (GNA).
Extended 24 substrate identification system for the complete range of *Enterobacteriacea* and oxidase positive non-fastidious Gram negative bacilli (GNA+B).

**MicrogenTM Listeria-ID**
Enhanced built-in haemolysis test.

**MicrogenTM Bacillus-ID**
24 substrate system for the identification of mesophilic *Bacillus* spp. isolated from foods and related samples.

**MicrogenTM Staph-ID**
12 substrate system minimizes handling and manipulation.
Substrates selected specifically for Staphylococcus and related organisms.

**MicrogenTM Strep-ID**
Substrates selected specifically for *Streptococcus, Enterococcus* and related species. Provides excellent differentiation of Enterococci spp. especially *E. casselilavus, E. mundtii* and *E. gallinarum.*
Identification of microorganisms by MALDI-TOF MS

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1) Sample Preparation
- Microbial culture or its proteins extract is smearing onto a steel plate and covered over by matrix.
- Matrix enables the sample (A) to be desorbed and ionised as pseudomolecule ionts \([A+H]^+\).

2) MALDI-TOF MS Analysis
Unique mass spectral fingerprint of desorbed microbial cell components (mainly intracellular proteins), different among genera, species or also some strains.

3) Identification:
Comparison of mass spectrum to those of reference strains in database.
MALDI-TOF MS: PRINCIPLE

MALDI (Matrix Assisted Laser Desorption Ionization)

1. Irradiation
2. Desorption
3. Desolvation & ionization

Matrix
(Poly)peptide

Time of flight is a function of the specific ion mass (m/z)

\[ \frac{m}{z} = 2eU \frac{t^2}{L^2} \]

- \( m \) mass, \( z \) charge,
- \( L \) length of drifting zone, \( e \) elementary charge, \( U \) speeding voltage

Soft ionisation method:
- low level of sample fragmentation

Animation: http://cmgm.stanford.edu/pan/section_html/MS/

MALDI-TOF MS: SAMPLE AND MATRIX

Sample preparation
Direct transfer
- intact cells spread directly onto a steel plate
- lysis of cells occurs during the contact with acid matrix and by laser desorption
- most bacteria

Proteins extraction
- previous extraction of proteins by organic acids and/or alcohol (e.g. ethanol and 70% formic acid)
- yeasts, moulds, some species of bacteria (depending on the cell wall composition)

Matrix:
- able to absorb the energy of the laser (usually 337 nm)
- to crystallise with samples - usually acid character (proton ionisation of sample), dissolved in organic solvent

CHC: α-Cyano-4-hydroxycinnamic acid
(organic solvent: 50% acetonitrile with 2.5% trifluoracetic acid)

SA: 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid)
DHB: 2,5-Dihydroxybenzoic acid

MALDI-TOF MS: MICROORGANISMS

Mass spectrum protein profile

$z$ equals usually to $1+$ (so m/z usually corresponds to molecule mass)
the range usually used for identification: 2000 -20 000 m/z
the intensity of single peaks corresponds to the abundance of the protein

Which proteins dominates in the protein profile?
abundant, basic and mediumly hydrophobic
mainly ribosomal proteins
further cold-shock and heat-shock proteins, chaperons etc.

Analysis is recomended (and validated) to be performed from colonies grown onto non-selective agar

conserved house-keeping gene = conserved proteins = the accordance with identification based on DNA

Visualisation of mass spectrum protein profile – (software mMass 5, Strohalm et al., 2010)
MALDI-TOF MS: ANALYSIS

Comparison of mass spectrum protein profile of unknown sample with these of reference strains present in database by software

BioTyper:
The statistical analysis for correlation includes peak positions, intensities and frequencies across the complete range of microorganisms.

Score value:
0 (none similarity) - 1000 (absolute similarity)
But it is expressed in decadic logarithm
log(score value): 0-3

Commercial databases from different MALDI-TOF MS producers

Bruker Daltonics – MALDI BIOTYPER
Shimadzu - Shimadzu Launchpad software + SARAMIS database
Biomérieux - VITEK® MS
Other databases compatible with different hardware systems (e.g. Andromas)

<table>
<thead>
<tr>
<th>Range</th>
<th>Description</th>
<th>Symbols</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.300 ...</td>
<td>highly probable species identification</td>
<td>( +++  )</td>
<td>green</td>
</tr>
<tr>
<td>3.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.000 ...</td>
<td>secure genus identification, probable species identification</td>
<td>( ++  )</td>
<td>green</td>
</tr>
<tr>
<td>2.299</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.700 ...</td>
<td>probable genus identification</td>
<td>( +   )</td>
<td>yellow</td>
</tr>
<tr>
<td>1.999</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.000 ...</td>
<td>not reliable identification</td>
<td>( -    )</td>
<td>red</td>
</tr>
<tr>
<td>1.699</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### bacillus cereus

**Rank (Quality)** Matched Pattern                  Score Value   NCBI Identifier
1 (+++)
Bacillus cereus DSM 31T DSM                  2.554        1396
2 (++)
Bacillus cereus 994000168 LBK                  2.203        1396
3 (++)
Bacillus weihenstephanensis DSM 11821T DSM          2.158        86662
4 (++)
Bacillus mycoides DSM 2048T DSM                  2.155        1405
5 (++)
Bacillus cereus 4080 LBK                        2.147        1396
6 (++)
Bacillus thuringiensis DSM 2046T DSM              1.975        1428
7 (+)  
Bacillus pseudomycoide DSM 12442T DSM            1.787        64104
8 (-)  
Bacillus bataviensis DSM 15601T DSM              1.369        220685
9 (-)  
Brevibacterium linens IMET 11075T HKJ            1.347        1703
10 (-)  
Acinetobacter towneri DSM 14962T HAM            1.345        202956

**Range** Description                      Symbols Color
2.300 ... 3.000 highly probable species identification (+++ green
2.000 ... 2.299 secure genus identification, probable species identification (++) green
1.700 ... 1.999 probable genus identification (+) yellow
0.000 ... 1.699 not reliable identification (-) red
**MALDI-TOF MS - PROCEDURE**

**Direct method:** smearing sample in four parallels in lower and higher cells concentrations – after drying to cover over by matrix (1-2 µl) and let to crystallise at room temperature

**Matrix:** solution of α-Cyano-4-hydroxycinnamic acid (10 mg/ml) in 50% acetonitrile with 2,5% trifluoroacetic acid (prepared with 10% TFA solution)

**Protein standard** (1 µl): Bruker Bacterial Test Standard (Bruker Daltonics, SRN) – proteins extracted from *E. coli* DH5alpha BRL + some others

**Equipment**
- Bruker Autoflex Speed
- Database MALDI Biotyper 3.1
MALDI-TOF MS: PROTEIN STANDARD

Bruker Bacterial Test Standard (referred to as ‘Bruker BTS’) contains a carefully manufactured extract of Escherichia coli DH5 alpha that shows a characteristic peptide and protein profile in MALDI-TOF mass spectra. The extract is spiked with two additional proteins that extend the upper boundary of the mass range covered by Bruker BTS. The overall mass range covered by Bruker BTS is 3.6 to 17 kDa.

Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reference mass (average mass)</th>
<th>± 300 ppm range</th>
</tr>
</thead>
<tbody>
<tr>
<td>RL29 [M+2H]^2+</td>
<td>3637.8 Da</td>
<td>3636.7 Da – 3638.6 Da</td>
</tr>
<tr>
<td>RS32 [M+H]^+</td>
<td>5096.8 Da</td>
<td>5095.3 Da – 5098.3 Da</td>
</tr>
<tr>
<td>RS34 [M+H]^+</td>
<td>5381.4 Da</td>
<td>5379.8 Da – 5383.0 Da</td>
</tr>
<tr>
<td>RS33meth [M+H]^+</td>
<td>6255.4 Da</td>
<td>6253.5 Da – 6257.3 Da</td>
</tr>
<tr>
<td>RL29 [M+H]^+</td>
<td>7274.5 Da</td>
<td>7272.3 Da – 7276.7 Da</td>
</tr>
<tr>
<td>RS19 [M+H]^+</td>
<td>10300.1 Da</td>
<td>10297.0 Da – 10303.2 Da</td>
</tr>
<tr>
<td>RNAseA [M+H]^+</td>
<td>13683.2 Da</td>
<td>13679.1 Da – 13687.3 Da</td>
</tr>
<tr>
<td>Myoglobin [M+H]^+</td>
<td>16952.3 Da</td>
<td>16947.2 Da – 16957.4 Da</td>
</tr>
</tbody>
</table>

Analyte name | Rank (Quality) | Matched Pattern | Score Value
---|---|---|---
F4 | 1 ( +++ ) | *Escherichia coli DH5alpha BRL* | 2.439
Thank you for your attention

Questions to be sent to
Sabina.Purkrtova@vscht.cz