Use of this training material

- **Introduction** and overview (Clauses 1-3)
- **General principles** (Clause 4)
- **Qualitative** methods (Clause 5)
- **Quantitative** methods (Clause 6)
- **Confirmation** and **typing** methods (Clause 7)
- **Root cause analysis**
- **Non-validated** methods (Annex F)
- *Transition period for the implementation of ISO 16140-3*
Use of this training material

Use of this presentation material

- **Introduction** and overview (Clauses 1-3)
- **General principles** (Clause 4)
- **Qualitative** methods (Clause 5)
- **Quantitative** methods (Clause 6)
- **Confirmation** and **typing** methods (Clause 7)
- **Root cause analysis**
- **Non-validated** methods (Annex F)
- **Transition period for the implementation of ISO 16140-3**

The first two slides of this presentation are only included as background information for you as presenter or organizer of this training; you can use the complete presentation or choose certain modules. This presentation is separated into clauses within the standards as stand-alone modules, so presenters of this information can customize a presentation and pick and choose which modules they wish to deliver to their specific audience.

If you, for example, wanted to only focus on training for ‘quantitative methods’ you could chose to deliver only the content in these modules:

- (Click) Introduction and overview
- (Click) General principles
“Deep dive” into ISO 16140-3 ‘Method verification’
– an extended training for improving confidence in laboratory results
Introduction and overview
Why is this standard on verification needed?

ISO 17025 requirement

“The laboratory shall verify that it can properly perform methods before introducing them by ensuring that it can achieve the required performance. Records of the verification shall be retained.”

Not many protocols for verification available

• Differences – agency or country specific

Standard created with international input/consideration
Who participated in creation of this standard?

WG 3 ‘Method validation’:

• > 100 experts coming from 23 countries and liaison organizations, representation of:
  o government
  o industry
  o laboratories
  o academic and research bodies
  o method developers and validation bodies

• developed 7 standards and more standards will follow
Many opportunities for global review and input

Market driven

Consensus based

Ideas

Comments and/or votes

Preliminary Work Item (PWI)

New Work Item Proposal (NWIP)

Working Draft (WD)

Committee Draft (CD)

Draft International Standard (DIS)

Final Draft International Standard (FDIS)

International Standard (IS)
Additional survey: user laboratory participation

52 of 60 laboratories responded = 87% response rate!
Additional survey: user laboratory response

Lab Size
- Large (>15 FTEs): 21%
- Med (5-15 FTEs): 56%
- Small (≤ 5 FTEs): 23%

Lab Type
- Industry
- Contract
- Government
- Other
Additional user laboratory evaluation: *text comprehension*

**General**

- **Scope**: 95%
- **Terms**: 90%
- **General**: 90%
- **Implementation verification**: 90%
- **Type verification**: 55%
- **Performance characteristics**: 90%

**Acceptance criteria:**

75% ≥ 3 (neutral)
Additional user laboratory evaluation: *practice*

**Verification on site**

- **Implementation eLOD50**: 18 labs
- **Implementation eBias**: 11 labs
- **Implementation SIR**: 11 labs
- **Type verification eLOD50**: 14 labs
- **Type verification eBias**: 10 labs

**Acceptance criteria:**

75% ≥ 3 (neutral)
Objectives of this training

Familiarize you with ISO 16140-3

Help you understand:
• **Why** verification is done
• **What** methods can be verified
• **How** to verify methods
• **When** ISO 16140-3 will be implemented
Agenda

- **Introduction** and overview (+ Clauses 1-3)
- **General principles** (Clause 4)
- **Qualitative** methods (Clause 5)
- **Quantitative** methods (Clause 6)
- **Confirmation** and **typing** methods (Clause 7)

Additional information covered

- **Non-validated** methods (Annex F)
- *Transition period for the implementation of ISO 16140-3*
Why do we need to validate and verify methods?
Distinguishing **validation** and **verification** from ISO 16140-1:2016 and ISO 16140-3:2021

**2.81 validation**
establishment of the performance characteristics of a method and provision of objective *evidence that the performance requirements for a specified intended use are fulfilled*

**2.83 verification**
*demonstration that a validated method performs, in the user’s hands,*
according to the method’s specifications determined in the validation study and is fit for its intended purpose
ISO 17468:2016 ‘Microbiology of the food chain — Technical requirements and guidance on establishment or revision of a standardized reference method’

Editorial

European and International validation of 15 main reference methods in the microbiology of the food chain

2.59 reference method
internationally recognized and widely accepted method

2.4 alternative method (method submitted for validation)
method of analysis that detects or quantifies, for a given category of products, the same analyte as is detected or quantified using the corresponding reference method (2.59)

Note 1 to entry: The method can be proprietary. The term ‘alternative’ is used to refer to the entire ‘test procedure and reaction system’. This term includes all ingredients, whether material or otherwise, required for implementing the method.
Method validation – Alternative methods

‘Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method’

This document specifies the general principle and the technical protocol for the validation of alternative, mostly proprietary, methods for microbiology in the food chain.

ISO 16140-6:2019
‘Microbiology of the food chain — Method validation — Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures’

This document specifies the general principle and the technical protocol for the validation of alternative confirmation methods for microbiology in the food chain.
European Commission Regulation

COMMISSION REGULATION (EC) No 2073/2005

of 15 November 2005

on microbiological criteria for foodstuffs

<table>
<thead>
<tr>
<th>Food category</th>
<th>Micro-organisms/their toxins, metabolites</th>
<th>Sampling-plan</th>
<th>Limits</th>
<th>Analytical reference method</th>
<th>Stage where the criterion applies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes</td>
<td>Listeria monocytogenes</td>
<td>10 0</td>
<td>Absence in 25 g</td>
<td>EN/ISO 11290-1</td>
<td>Products placed on the market during their shelf-life</td>
</tr>
<tr>
<td>1.2. Ready-to-eat foods able to support the growth of L. monocytogenes, other than those intended for infants and for special medical purposes</td>
<td>Listeria monocytogenes</td>
<td>5 0</td>
<td>100 cfu/g</td>
<td>EN/ISO 11290-2</td>
<td>Products placed on the market during their shelf-life</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 0</td>
<td>Absence in 25 g</td>
<td>EN/ISO 11190-1</td>
<td>Before the food has left the immediate control of the food business operator, who as produced it</td>
</tr>
</tbody>
</table>

Article 5

Specific rules for testing and sampling

The use of alternative analytical methods is acceptable when the methods are validated against the reference method in Annex I and if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO standard 16140 or other internationally accepted similar protocols, is used.
COMMISSION REGULATION (EU) 2019/229 of 7 February 2019
amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs

Article 1

Amendments to Regulation (EC) No 2073/2005

(1) in Article 2 the following points are inserted after point (m):

‘(n) “a broad range of foods”, as referred to in EN ISO 16140-2, means food as defined by the first subparagraph of Article 2 of Regulation (EC) No 178/2002 of the European Parliament and of the Council(*);

(o) “independent certification body” means a body which is independent from the organisation that manufactures or distributes the alternative method, and which provides a written assurance, in the form of a certificate, testifying that the validated alternative method meets the requirements of EN ISO 16140-2;

(p) “production process assurance of the manufacturer” means a production process whose management system guarantees that the validated alternative method remains conform to the characteristics required by EN ISO 16140-2 and ensures that mistakes and defects in the alternative method are prevented;

Certification bodies: using ISO 16140-2 and ISO 16140-6

Method validation certificates and reports on their websites:

What about other validated methods?

“Fully validated” method:

- **Comparative study** – method compared to a reference method
- **Interlaboratory study** – method used with same (food) items in many laboratories

Interlaboratory study (ILS):

- ISO 16140-2
- ISO 17468
- AOAC INTERNATIONAL
  - AOAC® Performance Tested Methods℠ (PTM)
  - AOAC® Official Method of Analysis℠ (OMA)
2.19 Methods of analysis.
Where the method of analysis is not prescribed in a regulation, it is the policy of the Food and Drug Administration in its enforcement programs to utilize the methods of analysis of the AOAC INTERNATIONAL (AOAC) as published in the latest (21st) edition of their publication “Official Methods of Analysis of AOAC INTERNATIONAL”,

…use of an AOAC method does not relieve the practitioner of the responsibility to demonstrate that he can perform the method properly through the use of positive and negative controls and recovery and reproducibility studies.
So why do methods also need to be verified?

The airplane “works” (is validated for use)

But would you want to fly with someone who didn’t know how to properly use it? (proper use is verified)
Flow chart for application of the ISO 16140 series

START: Is the method validated (performance characteristics are given)?

NO

Are specific (e.g., legal) requirements given to use ISO 16140-2003 or ISO 16140-2?

NO

To validate alternative [proprietary] methods
- Choose ISO 16140-2

To validate non-proprietary methods
- Choose ISO 16140-5

To do a single-laboratory validation
- Choose ISO 16140-4

To validate reference methods
- Choose ISO 17468

YES

Choose ISO 16140-2

Is the method validated in accordance with ISO 16140-4?

YES

Apply method only in that particular laboratory (incl. scope extension)

NO

Is the (food) category to be tested in the scope of validation of the method?

NO

For extension of the scope of a reference method
- Choose ISO 17468

For extension of the scope of an alternative (proprietary) method validated in accordance with ISO 16140-2
- Choose ISO 16140-2

For extension of the scope of a non-proprietary method validated in accordance with ISO 16140-5
- Choose ISO 16140-5

YES

For use of the (food) type in a single laboratory, in the case of:
- a) an alternative (proprietary) method validated in accordance with ISO 16140-2; or
- b) a non-proprietary method validated in accordance with ISO 16140-5; or
- c) a reference method with performance characteristics; or
- d) a reference method without performance characteristics.
- Choose ISO 16140-4

Choose ISO 16140-3 (verification)
Verification: two stages

1. **Implementation** verification
   - Demonstrate the user laboratory can *run the method correctly*
   - Verify using ONE (food) item

2. **(Food) item** verification
   - Demonstrate the user laboratory can run the method with the *(food) items claimed by the user laboratory* (laboratory application)
   - Verify using categories tested in your laboratory
Scope of Method vs Validation vs Laboratory application

**Method**

It specifies the (group of) products (categories or types or items) for which the method is claimed to be applicable.

**Validation**

It specifies the (group of) products (categories or types or items) for which the method is claimed to be validated.

**Laboratory**

It specifies the (group of) products (categories or types or items) for which the method is claimed to be used by the laboratory and are within the scope of validation.
Overlap of different scopes – Examples

**Method scope**  
– Broad range of foods + 3 others

**Validation scope**  
– Broad range of foods + 1 other

**Laboratory application**  
– Broad range of foods

**Method scope**  
– Broad range of foods + 3 other

**Validation scope**  
– Broad range of foods + 3 other

**Laboratory application**  
– Limited range of foods
Overlap of different scopes – Detailed example

Scope of the method

Method is applicable to:
- products intended for human consumption (15 categories);
- products intended for animal feeding (1 category);
- environmental samples in the area of food and feed production, handling (1 category);
- samples from the primary production stage (1 category).

Scope of validation

Method has been validated for a “broad range of foods” involving the following food categories and food items:
- heat-processed milk and dairy products [food item: pasteurized milk];
- raw meat and ready-to-cook meat products (except poultry) [food item: minced meat];
- eggs and egg products (derivates) [food item: whole liquid egg];
- chocolate, bakery products and confectionary [food item: bakery product with custard];
- multi-component foods or meal components [food item: refrigerated pasta salad].

Scope of laboratory application

The user laboratory wants to verify the method for a “broad range of foods”.

The user laboratory selects:
Implementation verification:
- food item: minced meat [category: raw meat and ready-to-cook meat products (except poultry)].

(Food) item verification, as these are challenging matrices and relevant to the food items tested by the user laboratory:
- blue cheese [category: raw milk and dairy products];
- smoked fish [category: ready-to-eat, ready-to-reheat fishery products];
- alfalfa [category: fresh produce and fruits];
- black pepper [category: dried cereals, fruits, nuts, seeds and vegetables];
- pizza [category: multi-component foods or meal components].
ISO 16140-3: Scope [Clause 1]

This standard provides the protocol for the verification of reference methods and validated alternative methods used for the analysis (detection and/or quantification), confirmation and typing of microorganisms in:

- products intended for **human consumption**;
- products intended for **animal feeding**;
- **environmental samples** in the area of food and feed production, handling;
- samples from the **primary production stage**.
## ISO 16140-3: Scope in relation to Annex A

Classification of (food) categories and suggested target combinations for *verification* studies

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw and ready-to-cook fish and seafoods (unprocessed)</td>
<td>Ready-to-eat, ready-to-reheat fishery products</td>
<td>Fresh produce and fruits</td>
<td>Processed fruits and vegetables</td>
<td>Dried cereals, fruits, nuts, seeds and vegetables</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chocolate, bakery products and confectionary</td>
<td>Multi-component foods or meal components</td>
<td>Pet food and animal feed</td>
<td>Environmental samples (food or feed production)</td>
<td>Primary production samples (PPS)</td>
<td></td>
</tr>
</tbody>
</table>

*Same categories are provided in ISO 16140-2:2016, Table A.1, for *validation* studies.*
An example: MicroVal certificate information

Scope of METHOD:
- Broad range of foods
- Environmental surfaces
- Animal feed

Tested in VALIDATION:
1. Meat products
2. Dairy and egg products
3. Fish and seafood products
4. Vegetable products
5. Ready-to-eat and ready-to-reheat
6. Animal feed
7. Production environment

Name and manufacturer of the method:

Certification of compliance
Lloyd’s Register Quality Assurance
hereby declares that the certification assessment has demonstrated that

has been validated and revealed to be at least equivalent to the reference method as demonstrated by the validation study report. The summary of the validation report is available on the MicroVal website: www.microval.org

Reference method:
1. ISO 4832:2006, Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coliforms – Colony-count technique
2. ISO 16649-2:2001, Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of β-gluconidase-positive Escherichia coli – Part 2: Colony count technique at 44 °C using 5-bromo-4-chloro-3-indolyl β-D-glucuronide

Scope: broad range of foods, select environmental surfaces and animal feed. Food categories tested: meat products, dairy and egg products, fish and seafood products, vegetable products, ready to eat and ready to reheat, animal food and samples from the industrial production environment.

The validation and certification have been performed in accordance with EN ISO 16140-2:2016 and the MicroVal Rules and Certification Scheme version 8.

Certificate no. 1000
First approval date: 13 December 2018
Surveillance date: 20 July 2020
Expiry date: 12 December 2022
5 food categories tested \( (= \text{broad range of foods}) \) + 2 other categories

Table A.1: Classification of categories and suggested target combinations for verification studies

<table>
<thead>
<tr>
<th>Category 1</th>
<th>Category 2</th>
<th>Category 3</th>
<th>Category 4</th>
<th>Category 5</th>
<th>Category 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs and egg products (derivatives)</td>
<td>Raw and ready-to-cook fish and seafoods (unprocessed)</td>
<td>Ready-to-eat, ready-to-reheat fishery products</td>
<td>Fresh produce and fruits</td>
<td>Processed fruits and vegetables</td>
<td>Dried cereals, fruits, nuts, seeds and vegetables</td>
</tr>
<tr>
<td>Infant formula and infant cereals</td>
<td>Chocolate, bakery products and confectionary</td>
<td>Multi-component foods or meal components</td>
<td>Pet food and animal feed</td>
<td>Environmental samples (food or feed production)</td>
<td>Primary production samples (PPS)</td>
</tr>
</tbody>
</table>

Scope of validation = Scope of method
Normative references [Clause 2]

ISO 6887 (all parts) ‘Preparation of test samples, initial suspension and decimal dilutions for microbiological examination’

ISO 7218 ‘General requirements and guidance for microbiological examinations’

ISO 16140-1 ‘Method validation - Part 1: Vocabulary’

Terms and definitions [Clause 3]

A total of 21 terms and definitions - 4 are unique to this standard:

- estimated bias
- estimated LOD_{50}
- scope of laboratory application
- user laboratory
General principles [Clause 4]
Implementation verification

Demonstrate competence of the user laboratory to perform the method

• **Qualitative** methods:
  o select 1 (food) item from the validation study also within the **scope of laboratory application**
  o use this **1 (food) item and the sample size** used in the validation study to perform implementation verification

• **Quantitative** methods:
  o select any (food) item within the scope of validation of the method
5 food categories tested (= broad range of foods) + 2 other categories

| Table A.1: Classification of categories and suggested target combinations for verification studies |
|-------------------------------------------------|------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Eggs and egg products (derivatives)             | Raw and ready-to-cook fish and seafoods (unprocessed) | Ready-to-eat, ready-to-reheat fishery products       | Fresh produce and fruits                         | Processed fruits and vegetables                  | Dried cereals, fruits, nuts, seeds and vegetables |
| Infant formula and infant cereals               | Chocolate, bakery products and confectionary      | Multi-component foods or meal components            | Pet food and animal feed                         | Environmental samples (food or feed production)  | Primary production samples (PPS)                |

**Implementation verification:**
- **Qualitative:** powdered egg
- **Quantitative:** pasteurized milk
(Food) item verification

Demonstrate the competence of the user laboratory to perform the validated method with (food) items that are tested in the user laboratory

The user laboratory shall:

1. select 1 challenging (food) item from each (food) category listed within the scope of validation, that is also a (food) category that is tested within the scope of laboratory application of the user laboratory, and

2. use this 1 (food) item to perform the (food) item verification.
Scope: limited range of foods

Scope of validation

- Validated reference method or alternative method validated in accordance with ISO 16140-2 or ISO 16140-5 "Limited range of foods" scope

- Food categories tested during the validation

Category 1 → Types → Items
Category 2 → Types → Items
Category 3 → Types → Items

Scope of laboratory application

- Implementation verification

- For qualitative methods: select one food item tested during the validation study belonging to the scope of laboratory application
- For quantitative methods: select any food item belonging to the scope of laboratory application

(Food) item verification

- If the scope of validation covers < 5 food categories, choose a minimum of one challenging food item from each of the food categories belonging to the scope of laboratory application

Figure 5 — Food items required when verifying a method for a "limited range of foods" scope
Scope: broad range of foods

Figure 4 — Food items required when verifying a method for a “broad range of foods” scope
Scope: broad range of foods and other categories

Figure 6 — Items required when verifying a method for a "broad range of foods and other categories" scope
## Number of (food) items to test

### Table 1 — Summary of the minimum number of (food) items required for verification

<table>
<thead>
<tr>
<th>Scope of validation</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Implementation verification</td>
</tr>
<tr>
<td>“Broad range of foods” scope ≥ 5 food categories</td>
<td>1</td>
</tr>
<tr>
<td>“Limited range of foods” scope N_{food} categories</td>
<td>1</td>
</tr>
<tr>
<td>“Broad range of foods” + other categories (N_{other}) scope</td>
<td>1</td>
</tr>
<tr>
<td>“Limited range of foods” N_{food} categories + other categories (N_{other}) scope</td>
<td>1</td>
</tr>
<tr>
<td>Other categories (N_{other}) scope only</td>
<td>1</td>
</tr>
</tbody>
</table>
Guidance on how to choose challenging (food) item(s) [Annex B]

B.2 Matrix effects to consider:

- **high background microbiota samples**, e. g. poultry minced meat, faecal samples, raw milk;
- **spoilage microorganisms**: the presence of this native microbiota can influence the recovery and growth of the target microorganism;
- **technological microbiota** such as microbial cultures and probiotics;
- **composition**, e. g. high fat content, lecithin, thickener, nutrient content;
- **pH**, e. g. pH < 4 to 5 (beverages, sauces, etc.);
- **oxidation reduction potential**;
- **water activity**, e. g. \( a_w < 0.85 \) (flour, low moisture foods);
- **antimicrobial constituents and growth inhibitors**, e. g. polyphenols, and others.
Consider the method principle: making (food) item choices

Table B.2 — Examples of characteristics of (food) items that can affect performance, categorized by method principles

<table>
<thead>
<tr>
<th>Method principle</th>
<th>High number of competitive (micro)organisms</th>
<th>Physical characteristics</th>
<th>Chemical compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Technological microbiota</td>
<td>High background microbiota, spoilage</td>
<td>pH</td>
</tr>
<tr>
<td>Cultural method</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Immuno-enzymatic</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Molecular test</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>ATP</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
# Classification of samples and their relevance for testing for various microorganisms

*[Annex A, Table A.1]*

<table>
<thead>
<tr>
<th>Categories</th>
<th>Types</th>
<th>Items (some examples)</th>
<th>Total viable count</th>
<th>Lactic acid bacteria</th>
<th>Yeasts and moulds</th>
<th>Enterobacteriaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw meat and ready-to-cook meat products (except poultry)</td>
<td>Fresh meats (unprocessed)</td>
<td>Carcasses, meat cuts, carpaccio</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minced meat, meat preparations, carpaccio</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carcasses, swabs, rinsates</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ready-to-cook (processed)</td>
<td>Frozen burger patties, marinated beef shish-kabobs</td>
<td></td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cooked meat products</td>
<td>Cooked ham, pâté</td>
<td></td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Fermented or dried meat products</td>
<td>Salami</td>
<td></td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw cured (smoked) ($a_w &gt; 0.92$)</td>
<td>Filet de sax, lard</td>
<td></td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>Raw cured (smoked) ($a_w &lt; 0.92$)</td>
<td>Cobourg ham, dry cured ham</td>
<td></td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
</tr>
</tbody>
</table>
Challenging (food) item(s)

(Food) items out of scope
An example: MicroVal certificate information

Scope of METHOD:

- Broad range of foods
- Environmental surfaces
- Animal feed

Tested in VALIDATION:

1. Meat products
2. Dairy and egg products
3. Fish and seafood products
4. Vegetable products
5. Ready-to-eat and ready-to-reheat
6. Animal feed
7. Production environment
### Scope: broad range of foods and other categories

#### Table A.1: Classification of categories and suggested target combinations for verification studies

<table>
<thead>
<tr>
<th>Category</th>
<th>Item</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw poultry and ready-to-cook poultry products</td>
<td>Seasoned chicken breast</td>
<td>High background</td>
</tr>
<tr>
<td>Processed fruits and vegetables</td>
<td>Pickle</td>
<td>Low pH</td>
</tr>
<tr>
<td>Chocolate, bakery products and confectionary</td>
<td>Custard confectionary</td>
<td>High fat content</td>
</tr>
<tr>
<td>Heat-processed milk and dairy products</td>
<td>Ice cream</td>
<td>Lecithin</td>
</tr>
<tr>
<td>Eggs and egg products</td>
<td>Egg powder</td>
<td>Low $a_w$</td>
</tr>
<tr>
<td>Pet food and animal feed</td>
<td>Dry dog food pellets</td>
<td>Low $a_w$</td>
</tr>
<tr>
<td>Environmental samples (food or feed production)</td>
<td>Swabs</td>
<td>Low $a_w$</td>
</tr>
</tbody>
</table>
Table B.1 — Example of (food) items and its characteristics

<table>
<thead>
<tr>
<th>Category</th>
<th>Item</th>
<th>Challenging characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>pH</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Viscosity</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Fat content</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>High background microbiota and pH</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>Polyphenol</td>
</tr>
</tbody>
</table>
### Performance characteristics

**Table 2 — Required performance characteristics to be determined for verification**

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristic</th>
<th>Implementation verification</th>
<th>(Food) item verification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>Estimated LOD$<em>{50}$ (eLOD$</em>{50}$)</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Quantitative</td>
<td>Intralaboratory reproducibility standard deviation ($S_{IR}$)</td>
<td>✓</td>
<td>Not applicable</td>
</tr>
<tr>
<td></td>
<td>Estimated bias (eBias)</td>
<td>Not applicable</td>
<td>✓</td>
</tr>
</tbody>
</table>

**NOTE 1** The relationship between intralaboratory reproducibility standard deviation ($S_{IR}$) and ISO 19036 is explained in 6.1.

**NOTE 2** For the verification of qualitative method, three protocols are proposed to the user laboratory. The protocol 3 does not require a determination of an eLOD$_{50}$ but to target a concentration of 3 cfu to 5 cfu/test portion.

- **eLOD$_{50}$** – Three available protocols to determine the eLOD$_{50}$
- **$S_{IR}$** – Design is aligned with ISO 19036:2019
- **eBias** – Analyze in parallel the method to be verified with the (food) item versus inoculum for three levels of inoculation
Summary of introduction and overview

Methods need to have validation data before conducting verification

Distinction between scopes:
- Scope of method
- Scope of validation
- Scope of laboratory application

Two kinds of verification:
1. 4.2 Implementation verification: (food) item tested during method validation
2. 4.3 (Food) item verification: challenging (food) items tested in laboratory application that are included in the scope of validation

Defined verification performance characteristics
Qualitative method verification \textit{[Clause 5]}
Workflow: outline

Qualitative method verification

1. Choice of the method to be verified
2. Scope of validation of the method
3. Scope of the verification
4. Select (food) items
5. Protocol for verification
6. Analysis
7. Evaluation of results
Implementation and (food) item verification

Qualitative method verification

Estimated LOD$_{50}$ (eLOD$_{50}$) determination required for both:

1. Implementation verification: follow one of the technical protocols outlined
2. (Food) item verification: apply the same technical protocol

3.5 estimated LOD$_{50}$

determination of the LOD$_{50}$ (level of detection at 50 % probability of detection) based on the experimental design described in this document

Note 1 to entry: An accurate determination of the LOD$_{50}$ is not possible as the number of samples tested is small in comparison to the number of samples required in ISO 16140-2:2016. Therefore, the term “estimated LOD$_{50}$” (“eLOD$_{50}$”) is used in this document.

Annex C provides guidance and examples on preparation of samples and test portions
Method and scope to be verified

Qualitative method verification

**ISO 6579-1:2017** ‘Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 1: Detection of Salmonella spp.’

[Image of the cover of the journal 'International Journal of Food Microbiology']

International Journal of Food Microbiology 288 (2019) 1–2

Editorial

European and International validation of 15 main reference methods in the microbiology of the food chain

[Link to the article on ScienceDirect]

Select (food) items: ISO 6579-1 ‘Salmonella’

Qualitative method verification

Implementation verification:

<table>
<thead>
<tr>
<th>Category</th>
<th>Item</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk and dairy products</td>
<td>Fresh cheese curd</td>
<td>Validation study</td>
</tr>
</tbody>
</table>

(Food) item verification:

<table>
<thead>
<tr>
<th>Category (claimed, not tested)</th>
<th>Item</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw or heat processed milk and dairy products</td>
<td>Ice cream</td>
<td>Lecithin</td>
</tr>
<tr>
<td>Raw and ready to eat poultry products</td>
<td>Raw poultry</td>
<td>Background microbiota</td>
</tr>
<tr>
<td>Egg and egg products</td>
<td>Dried egg powder</td>
<td>Low $a_w$</td>
</tr>
<tr>
<td>Primary production samples (PPS)</td>
<td>Dust samples</td>
<td>Low $a_w$</td>
</tr>
</tbody>
</table>
Implementation verification
Choose a protocol from Table 3

Qualitative method verification

**Table 3 — Protocols to determine eLOD$_{50}$ and number of replicates needed per inoculation level**

<table>
<thead>
<tr>
<th>Protocol</th>
<th>High level 9 × LOD$_{50}$ / test portion</th>
<th>Intermediate level 3 × LOD$_{50}$ / test portion</th>
<th>Low level 1 × LOD$_{50}$ / test portion</th>
<th>3 cfu to 5 cfu / test portion</th>
<th>Blank</th>
<th>Total number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>–</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>3</td>
<td>5</td>
<td>–</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

NOTE The abbreviation of colony forming units is cfu.

- **Protocol 1**: Uncertain of achieving level of contamination (inoculation with *culture*)
- **Protocol 3**: Level of contamination is known (*inoculation with reference material*)
- **Protocol 2**: Use if 1$^{st}$ choice of protocol didn’t work, and need to repeat the experiment
Using Protocol 3 *(known inoculation of 3-5 cfu/test portion)*

*Qualitative method verification*

Reference Material (RM) certified 50 cfu/ml

1:10 dilution of RM
Expected 5 cfu/ml = dilution A

1:3 dilution of dilution A
Expected 1.7 cfu/ml = dilution B

Figure C.5 — Example of the inoculation of the test portions when using protocol 3
### Acceptability limits [Clause 8]

**Qualitative method verification**

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristics</th>
<th>Acceptability limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>eLOD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>For protocol 1 and 2: eLOD&lt;sub&gt;50&lt;/sub&gt; ≤ 4 × LOD&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For protocol 3: ≥ 6 out of 7 positive results</td>
</tr>
<tr>
<td>Quantitative</td>
<td>S&lt;sub&gt;IR&lt;/sub&gt;</td>
<td>S&lt;sub&gt;IR&lt;/sub&gt; ≤ 2 × lowest S&lt;sub&gt;R&lt;/sub&gt; mean value&lt;sup&gt;a&lt;/sup&gt; determined in the validation study</td>
</tr>
<tr>
<td></td>
<td>eBias</td>
<td>log&lt;sub&gt;10&lt;/sub&gt; cfu/g (inoculum) – mean log&lt;sub&gt;10&lt;/sub&gt; cfu/g (artificially contaminated [food] item) ≤ 0,5 log&lt;sub&gt;10&lt;/sub&gt; cfu/g for each of the inoculation levels&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Confirmation or typing</td>
<td>inclusivity and exclusivity</td>
<td>100 % agreement between methods</td>
</tr>
</tbody>
</table>

---

**Notes:**
- <sup>a</sup> S<sub>IR</sub> ≤ 2 × S<sub>R</sub> for validation studies with only one S<sub>R</sub> value.
- <sup>b</sup> For readability, only cfu/g is given but the results can also be expressed in cfu/ml.

**Blank = 0**

**Inoculation level = ≤ 5 cfu / ml**

**Positives ≥ 6 out of 7**
## Inoculation levels per protocol

*Qualitative method verification*

<table>
<thead>
<tr>
<th>Protocol</th>
<th>High level $9 \times \text{LOD}_{50}/\text{test portion}$</th>
<th>Intermediate level $3 \times \text{LOD}_{50}/\text{test portion}$</th>
<th>Low level $1 \times \text{LOD}_{50}/\text{test portion}$</th>
<th>3 cfu to 5 cfu/test portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>This should be at a maximum of nine times the expected LOD$_{50}$.</td>
<td>From the high inoculation level, perform a 1:3 dilution to achieve the intermediate level.</td>
<td>From the intermediate inoculation level, perform 1:3 dilution to achieve the low level.</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>This should be at a maximum of three times the expected LOD$_{50}$.</td>
<td>From the intermediate inoculation level, perform 1:3 dilution to achieve the low level.</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>The level of contamination of the inoculum is known, (e.g. reference material with known concentration).</td>
</tr>
</tbody>
</table>
ISO 6579-1:2017 ‘Salmonella’ validation study

Qualitative method verification

$\text{LOD}_{50}$ for fresh cheese curd sample = 5,7 cfu/test portion

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fresh cheese curd (blank)</th>
<th>Fresh cheese curd (low level contamination)$^a$</th>
<th>Fresh cheese curd (high level contamination)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participating collaborators</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Number of samples per collaborator</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Number of collaborators retained after evaluation of the data</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Number of samples retained after evaluation of the data</td>
<td>105</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td>Test portion size, in g</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Specificity, in %</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sensitivity, in %</td>
<td>—</td>
<td>74,3</td>
<td>83,8</td>
</tr>
<tr>
<td>$\text{LOD}_{50}$ (95 % confidence interval), in cfu/test portion</td>
<td>—</td>
<td>—</td>
<td>5,7 (4,0 to 8,1)</td>
</tr>
</tbody>
</table>

$^a$ Cheese samples were artificially contaminated with *Salmonella* Montevideo (lactose positive strain).

Most probable number (MPN) results of the artificially contaminated samples were the following:

- MPN/25 g
  - Low level: 0,7 (0,2 to 2.4)
  - High level: 37,2 (7,5 to 95,0)
Choose a protocol from Table 3

Qualitative method verification

Table 3 — Protocols to determine eLOD$_{50}$ and number of replicates needed per inoculation level

<table>
<thead>
<tr>
<th>Protocol</th>
<th>High level $9 \times$ LOD$_{50}$/test portion</th>
<th>Intermediate level $3 \times$ LOD$_{50}$/test portion</th>
<th>Low level $1 \times$ LOD$_{50}$/test portion</th>
<th>3 cfu to 5 cfu /test portion</th>
<th>Blank</th>
<th>Total number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>–</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>3</td>
<td>5</td>
<td>–</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

NOTE  The abbreviation of colony forming units is cfu.
Inoculation level per test portion

*Qualitative method verification*

**LOD\(_{50}\) from ISO 6579-1 validation study = 5,7 cfu/test portion**

**Inoculation levels:**

- High: \(9 \times 5,7 = 51\) cfu/test portion
- Mid: \(3 \times 5,7 = 17\) cfu/test portion
- Low: \(1 \times 5,7 = 6\) cfu/test portion

| Protocol | Inoculation levels for each protocol | 9 \(\times\) LOD\(_{50}\)  
\(51\) cfu | 3 \(\times\) LOD\(_{50}\)  
\(17\) cfu | 1 \(\times\) LOD\(_{50}\)  
\(6\) cfu | 3-5 cfu | Negative control | Number of samples |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>
Prepare culture for determination of inoculation level [Annex C]

**Qualitative method verification**

Figure C.1 — Example of preliminary determination of the inoculum level
Determination of inoculum level [*Annex C*]

**Qualitative method verification**

- **New** overnight culture
- **Serial dilute**
- **Plate inoculum**

- Previous culture = $6 \times 10^8$
- New culture = $5.4 \times 10^8$

**Figure C.6 — Example of the enumeration of the actual inoculum level**

- 10⁻⁶ dilution
  - Expected $6 \times 10^2$ cfu/ml
- 10⁻⁷ dilution
  - Expected 60 cfu/ml
- 10⁻⁶ dilution
  - Expected 6 cfu/ml

**Enumeration result:**
- $5.4 \times 10^9$ cfu/ml instead of $6 \times 10^8$ cfu/ml
### Inoculate test portions: protocol 1

**Qualitative method verification**

<table>
<thead>
<tr>
<th>Dilution A (10⁻⁷)</th>
<th>Dilution B (1:3 of A)</th>
<th>Dilution C (1:3 of B)</th>
<th>Dilution D (1:3 of C)</th>
<th>Dilution E (1:3 of D)</th>
<th>Dilution F (1:3 of E)</th>
<th>Dilution G (1:3 of F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure C.3 — Example of the inoculation of the test portions when using protocol 1
**Table 6 - Determination of eLOD$_{50}$ based on the number of positive results per level of contamination using protocol 1**

<table>
<thead>
<tr>
<th>High inoculation level</th>
<th>Intermediate inoculation level</th>
<th>Low inoculation level</th>
<th>Blank level</th>
<th>eLOD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>targeted $9 \times$ LOD$_{50}$/test portion</td>
<td>targeted $3 \times$ LOD$_{50}$/test portion</td>
<td>targeted $1 \times$ LOD$_{50}$/test portion</td>
<td>0/1</td>
<td>cfu/test portion</td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>4/4</td>
<td>0/1</td>
<td>$&lt; 1,0 \times $ LIL$^a$</td>
</tr>
<tr>
<td><strong>1/1</strong></td>
<td>4/4</td>
<td>3/4</td>
<td><strong>0/1</strong></td>
<td><strong>$= 0,5 \times $ LIL</strong></td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>2/4</td>
<td>0/1</td>
<td><strong>$= 0,7 \times $ LIL</strong></td>
</tr>
</tbody>
</table>

**Inoculum (cfu) at each level**

<table>
<thead>
<tr>
<th>High level</th>
<th>Intermediate level</th>
<th>Low level</th>
<th>eLOD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>18</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

**a LIL: Low inoculation level**

High level: $9 \times 5,7 = 54$ cfu/test portion
Intermediate level: $3 \times 5,7 = 18$ cfu/test portion
Low level: $1 \times 5,7 = 6$ cfu/test portion
Acceptability limits and results

**Qualitative method verification**

**Table 16 — Acceptability limits for the verification of validated methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristics</th>
<th>Acceptability limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>eLOD$_{50}$</td>
<td>For protocols 1 and 2: eLOD$<em>{50} \leq 4 \times$ LOD$</em>{50}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For protocol 3: $\geq 6$ out of 7 positive results</td>
</tr>
<tr>
<td>Quantitative</td>
<td>$S_{IR}$</td>
<td>$S_{IR} \leq 2 \times$ lowest $S_R$ mean value determined in the validation study</td>
</tr>
<tr>
<td></td>
<td>eBias</td>
<td>$</td>
</tr>
<tr>
<td>Confirmation or typing</td>
<td>inclusivity and exclusivity</td>
<td>100 % agreement between methods</td>
</tr>
</tbody>
</table>

$^a$ $S_{IR} \leq 2 \times S_R$ for validation studies with only one $S_R$ value.

**Acceptability limits:**
eLOD$_{50}$ should be $\leq 4 \times 5,7$ (LOD$_{50}$) = 22,8 cfu

**Implementation verification:**

- eLOD$_{50}$ = 3,0 cfu $\leq$ 22,8 cfu
- Meets acceptability limits
(Food) item verification
Determine results

*Qualitative* method verification

High inoculation level = 9 cfu/test portion
Intermediate inoculation level = 3 cfu/test portion
Low inoculation level = 1 cfu/test portion

---

**Table 6** – Determination of eLOD\(_{50}\) based on the number of positive results per level of contamination using protocol 1

<table>
<thead>
<tr>
<th>Inoculum (cfu) at each level</th>
<th>High inoculation level targeted 9 x LOD(_{50})/test portion</th>
<th>Intermediate inoculation level targeted 3 x LOD(_{50})/test portion</th>
<th>Low inoculation level targeted 1 x LOD(_{50})/test portion</th>
<th>Blank level</th>
<th>eLOD(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/1</td>
<td>4/4</td>
<td>4/4</td>
<td>0/1</td>
<td>&lt; 1,0 \times LIL(^a)</td>
</tr>
<tr>
<td></td>
<td>1/1</td>
<td>4/4</td>
<td>3/4</td>
<td>0/1</td>
<td>0,5 \times LIL</td>
</tr>
<tr>
<td></td>
<td>1/1</td>
<td>4/4</td>
<td>2/4</td>
<td>0/1</td>
<td>0,7 \times LIL</td>
</tr>
</tbody>
</table>

**Inoculum (cfu) at each level**

| 9 | 3 | 1 |

\(^a\) LIL: Low inoculation level

\[0.5 \times 1\] (LIL)

\[\text{eLOD}_{50} = 0.5\]
Acceptability limits and results

**Qualitative method verification**

### Table 16 — Acceptability limits for the verification of validated methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristics</th>
<th>Acceptability limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>eLOD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>For protocols 1 and 2: eLOD&lt;sub&gt;50&lt;/sub&gt; ≤ 4 × LOD&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For protocol 3: ≥ 6 out of 7 positive results</td>
</tr>
<tr>
<td>Quantitative</td>
<td>S&lt;sub&gt;IR&lt;/sub&gt;</td>
<td>S&lt;sub&gt;IR&lt;/sub&gt; ≤ 2 × lowest S&lt;sub&gt;R&lt;/sub&gt; mean value&lt;sup&gt;a&lt;/sup&gt; determined in the validation study</td>
</tr>
<tr>
<td></td>
<td>eBias</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confirmation or typing</td>
<td>inclusivity and exclusivity</td>
<td>100% agreement between methods</td>
</tr>
</tbody>
</table>

<sup>a</sup> S<sub>IR</sub> ≤ 2 × S<sub>R</sub> for validation studies with only one S<sub>R</sub> value.

### Acceptability limits:

eLOD<sub>50</sub> should be ≤ 4 × 1 (LOD<sub>50</sub>) = 4 cfu

### (Food) item verification:

- eLOD<sub>50</sub> = 0,5 cfu ≤ 4 cfu
- Meets acceptability limits
<table>
<thead>
<tr>
<th>Steps</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Scope of validation</td>
<td>‘Limited range of foods’ and one other category (primary production samples)</td>
</tr>
<tr>
<td>3. Scope of verification</td>
<td>‘Limited range of foods’ and one other category (primary production samples)</td>
</tr>
<tr>
<td>4. Select (food) items</td>
<td>Implementation verification: fresh cheese curd (Food) item verification: ice cream, raw poultry, dried egg powder, and dust samples</td>
</tr>
<tr>
<td>5. Protocol</td>
<td>Protocol 1 - inoculation with culture (3 inoculation levels + 1 blank)</td>
</tr>
<tr>
<td>6. Analysis</td>
<td>1 (food) item for implementation verification + 4 (food) items for (food) item verification = 5 items Analyse using the full method procedure</td>
</tr>
<tr>
<td>7. Evaluation</td>
<td>Determination of eLOD$<em>{50}$ Acceptability limit: eLOD$</em>{50} \leq 4 \times$ LOD$_{50}$</td>
</tr>
</tbody>
</table>
Quantitative method verification [Clause 6]
Workflow: outline

Quantitative method verification

1. Choice of the method to be verified
2. Scope of validation of the method
3. Scope of the verification
4. Select (food) items
5. Protocol for verification
6. Analysis
7. Evaluation of results
ISO 21528-2:2017 ‘Microbiology of the food chain — Horizontal method for the detection and enumeration of Enterobacteriaceae — Part 2: Colony-count technique’
Select (food) items: ISO 21528-2 ‘Enterobacteriaceae’

**Quantitative method verification**

**Implementation verification:**

<table>
<thead>
<tr>
<th>Category</th>
<th>Item</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chocolate, bakery and confectionary</td>
<td>Tiramisu</td>
<td>Validation study</td>
</tr>
</tbody>
</table>

**(Food) item verification:**

<table>
<thead>
<tr>
<th>Category (claimed, not tested)</th>
<th>Item</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat processed milk and dairy products</td>
<td>Ice cream</td>
<td>Lecithin</td>
</tr>
<tr>
<td>Eggs and egg products (derivatives)</td>
<td>Powdered egg</td>
<td>Low aw</td>
</tr>
<tr>
<td>Raw meat and ready-to-cook meat products</td>
<td>Seasoned meat</td>
<td>Antimicrobial (seasoning)</td>
</tr>
<tr>
<td>Chocolate bakery products and confectionary</td>
<td>Cocoa powder</td>
<td>Low aw</td>
</tr>
<tr>
<td>Pet food and animal feed</td>
<td>Dried pet food</td>
<td>Low aw</td>
</tr>
</tbody>
</table>
Implementation verification
Implementation verification

Quantitative method verification

Intralaboratory reproducibility standard deviation ($S_{IR}$):
- Any (food) item used in the method validation
- $S_{IR}$ determination is based on ISO 19036:2019
- Run the full procedure of the method as described, including the confirmation procedure for each test portion

Annex D provides guidance and examples on preparation of samples and test portions
Select (food) item: ISO 21528-2 ‘Enterobacteriaceae’

Quantitative method verification

Implementation verification:

<table>
<thead>
<tr>
<th>Category</th>
<th>Item</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chocolate, bakery and confectionary</td>
<td>Tiramisu</td>
<td>Validation study</td>
</tr>
</tbody>
</table>

10 samples:
- Different batches
- Manufacturers
- Other variations?
Prepare the inoculum

Quantitative method verification – implementation verification

1:10 dilution e.g. 1 ml in 9 ml diluent

New overnight culture
Expected $6 \times 10^8$ cfu/ml

$10^{-1}$ dilution

$10^{-2}$ dilution

$10^{-6}$ dilution
Expected $6 \times 10^2$ cfu/ml

$10^{-7}$ dilution
Expected 60 cfu/ml

$10^{-8}$ dilution
Expected 6 cfu/ml

Perform the enumeration in parallel with the test portions inoculation
Inoculate test portions to represent a range

Quantitative method verification – implementation verification

Table 10 — Test results

<table>
<thead>
<tr>
<th>Laboratory sample number</th>
<th>Expected contamination level (cfu/g)</th>
<th>Result A ($x_{IA}$) (cfu/g)</th>
<th>Result B ($x_{IB}$) (cfu/g)</th>
<th>$\log_{10}$ result A ($y_{IA} = \log_{10}(x_{IA})$)</th>
<th>$\log_{10}$ result B ($y_{IB} = \log_{10}(x_{IB})$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>&lt; 40 (10)</td>
<td>&lt; 40 (30)</td>
<td>≤ 1,60</td>
<td>≤ 1,60</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>110</td>
<td>182</td>
<td>2,04</td>
<td>2,26</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>410</td>
<td>620</td>
<td>2,61</td>
<td>2,79</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
<td>640</td>
<td>330</td>
<td>2,81</td>
<td>2,52</td>
</tr>
<tr>
<td>5</td>
<td>600</td>
<td>690</td>
<td>570</td>
<td>2,84</td>
<td>2,76</td>
</tr>
<tr>
<td>6</td>
<td>600</td>
<td>780</td>
<td>640</td>
<td>2,89</td>
<td>2,81</td>
</tr>
<tr>
<td>7</td>
<td>600</td>
<td>620</td>
<td>1300</td>
<td>2,79</td>
<td>3,11</td>
</tr>
<tr>
<td>8</td>
<td>600</td>
<td>870</td>
<td>1500</td>
<td>2,94</td>
<td>3,18</td>
</tr>
<tr>
<td>9</td>
<td>6000</td>
<td>8600</td>
<td>6400</td>
<td>3,93</td>
<td>3,81</td>
</tr>
<tr>
<td>10</td>
<td>6000</td>
<td>16000</td>
<td>5000</td>
<td>4,20</td>
<td>3,70</td>
</tr>
<tr>
<td>11</td>
<td>6000</td>
<td>&gt; 15000</td>
<td>13400</td>
<td>&gt; 4,18</td>
<td>4,13</td>
</tr>
<tr>
<td>12</td>
<td>30000</td>
<td>20000</td>
<td>32000</td>
<td>4,30</td>
<td>4,51</td>
</tr>
</tbody>
</table>
Implementation verification: $S_{IR}$

*Quantitative method verification*

![Diagram of method verification process]

*Test sample is infrequently used in microbiological examinations. In that case, the laboratory sample is directly used for homogenization.*

*Figure D.1 — Preparation of samples for intralaboratory reproducibility standard deviation determination*
Implementation verification: $S_{IR}$

*Quantitative method verification*
Implementation verification: $S_{IR}$

Quantitative method verification

Figure D.2 — Suggestions for variations for intralaboratory reproducibility standard deviation determination
### Implementation verification: $S_{IR}$

**Quantitative method verification**

#### Table 10 — Test results

<table>
<thead>
<tr>
<th>Laboratory sample number</th>
<th>Expected contamination level (cfu/g)</th>
<th>Result A ($x_{iA}$) (cfu/g)</th>
<th>Result B ($x_{iB}$) (cfu/g)</th>
<th>$\log_{10}$ result A $y_{iA} = \log_{10}(x_{iA})$</th>
<th>$\log_{10}$ result B $y_{iB} = \log_{10}(x_{iB})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>&lt; 40 (10)</td>
<td>&lt; 40 (30)</td>
<td>≤ 1,60</td>
<td>≤ 1,60</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>110</td>
<td>182</td>
<td>2,04</td>
<td>2,26</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>410</td>
<td>620</td>
<td>2,61</td>
<td>2,79</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
<td>640</td>
<td>330</td>
<td>2,81</td>
<td>2,52</td>
</tr>
<tr>
<td>5</td>
<td>600</td>
<td>690</td>
<td>570</td>
<td>2,84</td>
<td>2,76</td>
</tr>
<tr>
<td>6</td>
<td>600</td>
<td>780</td>
<td>640</td>
<td>2,89</td>
<td>2,81</td>
</tr>
<tr>
<td>7</td>
<td>600</td>
<td>620</td>
<td>1 300</td>
<td>2,79</td>
<td>3,11</td>
</tr>
<tr>
<td>8</td>
<td>600</td>
<td>870</td>
<td>1 500</td>
<td>2,94</td>
<td>3,18</td>
</tr>
<tr>
<td>9</td>
<td>6 000</td>
<td>8 600</td>
<td>6 400</td>
<td>3,93</td>
<td>3,81</td>
</tr>
<tr>
<td>10</td>
<td>6 000</td>
<td>16 000</td>
<td>5 000</td>
<td>4,20</td>
<td>3,70</td>
</tr>
<tr>
<td>11</td>
<td>6 000</td>
<td>&gt; 15 000</td>
<td>13 400</td>
<td>&gt; 4,18</td>
<td>4,13</td>
</tr>
<tr>
<td>12</td>
<td>30 000</td>
<td>20 000</td>
<td>32 000</td>
<td>4,30</td>
<td>4,51</td>
</tr>
</tbody>
</table>
Implementation verification: $S_{IR}$

Quantitative method verification

### Table 11 — Calculation of $S_{IR}$

| Laboratory sample number | Log$_{10}$ result A $y_{iA} = \log_{10}(x_{iA})$ | Log$_{10}$ result B $y_{iB} = \log_{10}(x_{iB})$ | Absolute difference $|y_{iA} - y_{iB}|$ | Squared difference $(y_{iA} - y_{iB})^2$ |
|--------------------------|-----------------------------------------------|-----------------------------------------------|--------------------------------------|-------------------------------------|
| 1                        | $\leq 1,602.1$                               | $\leq 1,602.1$                               | Not used                            | Not used                            |
| 2                        | 2,041.4                                      | 2,260.1                                      | 0,218.7                             | 0,047.8                             |
| 3                        | 2,612.8                                      | 2,792.4                                      | 0,179.6                             | 0,033.2                             |
| 4                        | 2,862.2                                      | 2,518.5                                      | 0,343.7                             | 0,118.0                             |
| 5                        | 2,838.8                                      | 2,755.9                                      | 0,083.0                             | 0,006.9                             |
| 6                        | 2,892.1                                      | 2,806.2                                      | 0,085.9                             | 0,007.4                             |
| 7                        | 2,792.4                                      | 3,113.9                                      | 0,321.6                             | 0,103.4                             |
| 8                        | 2,939.5                                      | 3,176.1                                      | 0,236.6                             | 0,056.0                             |
| 9                        | 3,934.5                                      | 3,868.2                                      | 0,128.3                             | 0,016.5                             |
| 10                       | 4,204.1                                      | 3,699.0                                      | 0,505.1                             | 0,255.2                             |
| 11                       | > 4,176.1                                    | 4,127.1                                      | Not used                            | Not used                            |
| 12                       | 4,301.0                                      | 4,505.1                                      | 0,204.1                             | 0,041.7                             |

| Sum                      |                                               |                                               | 0,650.0                             |                                    |
| Sum/(2×10)               |                                               |                                               | 0,032.5                             |                                    |
| $S_{IR} = \sqrt{\frac{1}{2n} \sum_{i=1}^{n} (y_{iA} - y_{iB})^2}$ |                                             |                                               | 0,032.5                             |                                    |

The calculated $S_{IR}$ value of 0.18 is compared to the results of the validation study (data taken over from ISO 21528-2). Table 12 lists the $S_R$ values obtained from that validation study.
# Implementation verification: $S_{IR}$

## Quantitative method verification

## Table 16 — Acceptability limits for the verification of validated methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristics</th>
<th>Acceptability limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>eLOD$_{50}$</td>
<td>For protocols 1 and 2: eLOD$<em>{50}$ ≤ 4 × LOD$</em>{50}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For protocol 3: ≥ 6 out of 7 positive results</td>
</tr>
<tr>
<td>Quantitative</td>
<td>$S_{IR}$</td>
<td>$S_{IR} \leq 2 \times$ lowest $S_{R}$ mean value determined in the validation study</td>
</tr>
<tr>
<td></td>
<td>eBias</td>
<td>$</td>
</tr>
<tr>
<td>Confirmation or typing</td>
<td>inclusivity and exclusivity</td>
<td>100 % agreement between methods</td>
</tr>
</tbody>
</table>

\(^{a} S_{IR} \leq 2 \times S_{R} \) for validation studies with only one $S_{R}$ value.
Implementation verification: $S_R$ values from validation study report

Quantitative method verification

**Table 12 — Summary of $S_R$ values from the validation study for ISO 21528-2**

<table>
<thead>
<tr>
<th>(Food) item</th>
<th>$S_R$ values from the validation study</th>
<th>Low inoculation level</th>
<th>Intermediate inoculation level</th>
<th>High inoculation level</th>
<th>Mean value of three inoculation levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg product</td>
<td></td>
<td>0,32</td>
<td>0,50</td>
<td>0,48</td>
<td>0,43</td>
</tr>
<tr>
<td>Raw meat</td>
<td></td>
<td>0,28</td>
<td>0,36</td>
<td>0,57</td>
<td>0,40</td>
</tr>
<tr>
<td>Animal feed</td>
<td></td>
<td>0,18</td>
<td>0,17</td>
<td>0,20</td>
<td>0,18</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td></td>
<td>0,24</td>
<td>0,18</td>
<td>0,19</td>
<td>0,20</td>
</tr>
<tr>
<td>Tiramisu</td>
<td></td>
<td>0,22</td>
<td>0,28</td>
<td>0,13</td>
<td>0,21</td>
</tr>
</tbody>
</table>

**Acceptability limits:** $S_{IR} \leq 2 \times \text{lowest } S_R \text{ mean value}

- Lowest $S_R$ mean value = $2 \times 0,18 = 0,36$
- $S_{IR}$ obtained in implementation verification study = 0,18
- $0,18 \leq 0,36$
- Meets acceptability limits
(Food) item verification
(Food) item verification: eBias

Quantitative method verification

Estimated bias (eBias):

1. Select (food) items

2. Artificially contaminate at 3 levels
   - Different laboratory sample or batch for each level
   - Each level performed in duplicate

3. Enumerate the contaminated (food) item and the inoculum

4. Test uninoculated test portion for each to determine background microbiota
(Food) item verification: prepare inoculum

Quantitative method verification

- Prepare inoculum:
  - 1:10 dilution e.g. 1 ml in 9 ml diluent
  - .....
  - 10⁴ dilution
  - 10⁷ dilution

Determine inoculation level:

- Initial concentration: 5x10⁷ cfu/ml

Figure D.3 — Example of the preliminary determination of the inoculum level

Repeat, and prepare to inoculate:

- New overnight culture: Expected 5x10⁷ cfu/ml
- 10⁻¹ dilution: Expected 5x10⁶ cfu/ml
- 10⁻³ dilution: Expected 5x10⁴ cfu/ml
- .....

Figure D.4 — Example of the preparation of the inoculum
(Food) item verification: inoculation of test portions

Quantitative method verification

Figure D.5 — Example of the inoculation of the test portions
(Food) item verification: inoculation of test portions

Quantitative method verification

Figure D.6 — Example of quantitative method verification (eBias) using artificial contamination
## (Food) item verification: eBias determination

**Quantitative method verification**

### Table 13 — Test results obtained using the method to be verified

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean result</th>
<th>For comparison</th>
<th>eBias: absolute difference in results between artificially contaminated (food) item per test portion and the inoculum suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Artificially contaminated (food) item (log10 cfu/g or ml)</td>
<td>Artificially contaminated (food) item (log10 cfu/test portion)</td>
<td>Inoculum suspension [without (food) item] (log10 cfu/ml)</td>
</tr>
<tr>
<td>$10^1$</td>
<td>Laboratory sample 1 (from batch 1), test portion 1</td>
<td>2.06 (average of 1.87 and 2.25)</td>
<td>3.06</td>
</tr>
<tr>
<td>$10^3$</td>
<td>Laboratory sample 2 (from batch 2), test portion 1</td>
<td>3.11 (average of 3.16 and 3.06)</td>
<td>4.11</td>
</tr>
<tr>
<td>$10^5$</td>
<td>Laboratory sample 3 (from batch 3), test portion 1</td>
<td>3.99 (average of 3.93 and 4.04)</td>
<td>4.99</td>
</tr>
</tbody>
</table>

---

*a This example is based on the use of a 10-gram test portion inoculated with 1 ml of inoculum.
## (Food) item verification: eBias determination

*Quantitative method verification*

### Table 16 — Acceptability limits for the verification of validated methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristics</th>
<th>Acceptability limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>eLOD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>For protocols 1 and 2: eLOD&lt;sub&gt;50&lt;/sub&gt; ≤ 4 × LOD&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For protocol 3: ≥ 6 out of 7 positive results</td>
</tr>
<tr>
<td>Quantitative</td>
<td>(S_{IR})</td>
<td>(S_{IR} \leq 2 \times ) lowest (S_R) mean value determined in the validation study</td>
</tr>
<tr>
<td></td>
<td>eBias</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confirmation or typing</td>
<td>inclusivity and exclusivity</td>
<td>100 % agreement between methods</td>
</tr>
</tbody>
</table>

\(S_{IR} \leq 2 \times S_R\) for validation studies with only one \(S_R\) value.
(Food) item verification: eBias determination

Quantitative method verification

| Laboratory sample 1 (from batch 1), test portion 1 | 2.06 (average of 1.87 and 2.25) | 3.06 | 3.17 | 0.11 | Meets |
| Laboratory sample 1 (from batch 1), test portion 2 | 3.11 (average of 3.16 and 3.06) | 4.11 | 4.05 | 0.06 | Meets |
| Laboratory sample 2 (from batch 2), test portion 1 | 3.99 (average of 3.93 and 4.04) | 4.99 | 5.29 | 0.30 | Meets |

Table 13 — Test results obtained using the method to be verified

<table>
<thead>
<tr>
<th>Mean result</th>
<th>For comparison</th>
<th>eBias: absolute difference in results between artificially contaminated (food) item per test portion and the inoculum suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artifically contaminated (food) item (log_{10} cfu/g or ml)</td>
<td>Result</td>
<td>Result</td>
</tr>
<tr>
<td>(log_{10} cfu/g or ml)</td>
<td>Artificially contaminated (food) item (log_{10} cfu/test portion)</td>
<td>Inoculum suspension [without (food) item] (log_{10} cfu/ml)</td>
</tr>
</tbody>
</table>

This example is based on the use of a 10-gram test portion inoculated with 1 ml of inoculum.
Workflow: summary

*Quantitative method verification*

<table>
<thead>
<tr>
<th>Steps</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Scope of validation</td>
<td>4 food categories and 1 other category (pet food)</td>
</tr>
<tr>
<td>3. Scope of verification</td>
<td>4 food categories and 1 other category (pet food)</td>
</tr>
<tr>
<td>4. Select (food) items</td>
<td>Implementation verification: <em>tiramisu</em></td>
</tr>
<tr>
<td></td>
<td>(Food) item verification: <em>ice cream, powdered egg, seasoned meat, cocoa powder, and dried pet food</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Implementation verification</th>
<th>(Food) item verification</th>
</tr>
</thead>
<tbody>
<tr>
<td>5. Protocol</td>
<td>Intralaboratory reproducibility standard deviation ($S_{IR}$)</td>
</tr>
<tr>
<td></td>
<td>Estimated bias ($eBias$)</td>
</tr>
<tr>
<td>6. Analysis</td>
<td>1 (food) item = 10 samples</td>
</tr>
<tr>
<td></td>
<td>5 (food) items × 9 tests each = 45</td>
</tr>
</tbody>
</table>

  *Analyze using the full method procedure*

<table>
<thead>
<tr>
<th>7. Evaluation</th>
<th>Acceptability limits: $S_{IR} \leq 2 \times$ lowest $S_R$ mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acceptability limits: $\leq 0.5 \log_{10}$ for each inoculation level</td>
</tr>
</tbody>
</table>

Prepared by ISO/TC 34/SC 9/WG 3 “Method validation” (version: 20240308)
Validated alternative confirmation and typing methods – Technical protocol for verification [Clause 7]
Workflow: outline

Confirmation and typing method verification

1. Choice of the method to be verified
2. Scope of validation of the method
3. Scope of the verification
4. Select (food) items target/non target strains
5. Protocol for verification
6. Analysis
7. Evaluation of results
Confirmation and typing method verification require only implementation verification

• Review method validation data

• Choose 1 selective agar plate used in the validation study

• Use this agar to perform implementation verification
  • If no selective agar plate was tested, select and use one non-selective agar plate tested during the validation study

• Test selected inclusivity and exclusivity strains – according to the method being verified

Annex E provides guidance and examples for confirmation and typing method verification
### Selection of strains

*Confirmation and typing method verification*

#### Table 14 — Number of strains for implementation verification of validated alternative confirmation or typing methods

<table>
<thead>
<tr>
<th>Level of the confirmation</th>
<th>Inclusivity study</th>
<th>Exclusivity study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Genus</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Species</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microbial (sub)type (e.g. serotyping of <em>Salmonella</em>)</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
# Test and tabulate results

*Confirmation and typing method verification*

<table>
<thead>
<tr>
<th>Tested strains</th>
<th>Inclusivity/exclusivity</th>
<th>Characteristics of the strain</th>
<th>Expected confirmation/typing result</th>
<th>Result of the confirmation/typing method being verified</th>
<th>Interpretation&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</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>
</tr>
<tr>
<td>...</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Agreement or deviation between the expected result and the result of the tested confirmation or typing method.

NOTE Characteristics of the individual strains are as a minimum: the name of the strain, (culture) collection number and origin of the strain. Other available characteristics can be added as well.
### Example: Overview of verification results [see Table E.1]

**Alternative confirmation method verification**

<table>
<thead>
<tr>
<th>Tested strains</th>
<th>I/E*</th>
<th>Characteristics of the strain</th>
<th>Expected result</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td><em>L. monocytogenes</em> (serotype 4b) WDCM 00021 Human isolate</td>
<td>Positive</td>
<td>Positive</td>
<td>Agreement</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td><em>L. monocytogenes</em> (serotype 1/2a) WDCM 00109 Guinea-pig isolate</td>
<td>Positive</td>
<td>Positive</td>
<td>Agreement</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td><em>L. monocytogenes</em> (genotype IV) 12MOB112LM Meat isolate</td>
<td>Positive</td>
<td>Positive</td>
<td>Agreement</td>
</tr>
<tr>
<td>4</td>
<td>I</td>
<td><em>L. monocytogenes</em> (genotype II) 12MOB118LM Dairy isolate</td>
<td>Positive</td>
<td>Positive</td>
<td>Agreement</td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td><em>L. monocytogenes</em>, Field strain LM01 Smoked salmon isolate</td>
<td>Positive</td>
<td>Positive</td>
<td>Agreement</td>
</tr>
<tr>
<td>6</td>
<td>E</td>
<td><em>L. innocua</em> WDCM 00017</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
<tr>
<td>7</td>
<td>E</td>
<td><em>L. ivanovii</em> WDCM 00018</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
<tr>
<td>8</td>
<td>E</td>
<td><em>Bacillus cereus</em> WDCM 00001</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
<tr>
<td>9</td>
<td>E</td>
<td><em>Enterococcus faecalis</em> WDCM 00009</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
<tr>
<td>10</td>
<td>E</td>
<td><em>Staphylococcus aureus</em> WDCM 00034</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
</tbody>
</table>

*I/E = inclusivity / exclusivity*
**Example: Overview of verification results**  [see Table E.2]

Alternative **typing** method verification

<table>
<thead>
<tr>
<th>Tested</th>
<th>I/E*</th>
<th>Characteristics of the strain</th>
<th>Expected result</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>S. Anatum  (3,{10}{15},{15,34}:e,h:1,6) Field strain Salm01 Dairy product</td>
<td>S. Anatum</td>
<td>S. Anatum</td>
<td>Agreement</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>S. Enteritidis  (1,9,12:g,m:-) WDCM00030</td>
<td>S. Enteritidis</td>
<td>S. Enteritidis</td>
<td>Agreement</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td>S. Hadar  (6,8:z10:e,n,x) Field strain Salm02 Poultry meat</td>
<td>S. Hadar</td>
<td>S. Hadar</td>
<td>Agreement</td>
</tr>
<tr>
<td>4</td>
<td>I</td>
<td>S. Infantis  (6,7,14:r:1,5) Field strain Salm03 Egg product</td>
<td>S. Infantis</td>
<td>S. Infantis</td>
<td>Agreement</td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td>S. Typhimurium  (1,4,[5],12:i:1,2) WDCM00031 Chicken tissue isolate</td>
<td>S. Typhimurium</td>
<td>S. Typhimurium</td>
<td>Agreement</td>
</tr>
<tr>
<td>6</td>
<td>E</td>
<td>S. Panama  (1,9,12:l,v:1,5) Field strain Salm04 Human isolate</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
<tr>
<td>7</td>
<td>E</td>
<td>S. Saintpaul  (1,4,[5],12:e,h:1,2) Field strain Salm05 Turkey isolate</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
<tr>
<td>8</td>
<td>E</td>
<td><em>Citrobacter freundii</em> WDCM 00006</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
<tr>
<td>9</td>
<td>E</td>
<td><em>Escherichia coli</em> WDCM 00012</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
<tr>
<td>10</td>
<td>E</td>
<td><em>Hafnia alvei</em> WDCM 00095</td>
<td>Negative</td>
<td>Negative</td>
<td>Agreement</td>
</tr>
</tbody>
</table>

*I/E = inclusivity / exclusivity*
# Acceptability limits

*Confirmation and typing method verification*

## Table 16 — Acceptability limits for the verification of validated methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristics</th>
<th>Acceptability limits</th>
</tr>
</thead>
</table>
| Qualitative           | $eLOD_{50}$                 | For protocols 1 and 2: $eLOD_{50} \leq 4 \times LOD_{50}$  
                        |                             | For protocol 3: $\geq 6$ out of $7$ positive results |
| Quantitative          | $S_{IR}$                    | $S_{IR} \leq 2 \times \text{lowest } S_{R} \text{ mean value}^a$  
                        |                             | determined in the validation study |
|                        | $eBias$                     | $| \log_{10} \text{ cfu/ml (inoculum)} - \text{mean } \log_{10} \text{ cfu/test portion}$  
                        |                             | (artificially contaminated [food] item) |  
                        |                             | $\leq 0,5 \log_{10}$ for each of the inoculation levels |
| Confirmation or typing| **inclusivity and exclusivity** | **100 % agreement between methods** |

---

^a $S_{IR} \leq 2 \times S_{R}$ for validation studies with only one $S_{R}$ value.
**Workflow: summary**

**Confirmation and typing method verification**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Example</th>
</tr>
</thead>
</table>
| 1. Methods                      | Confirmation: *Listeria monocytogenes* - commercially available PCR-test  
Typing: *Salmonella* - commercially available PCR-test                                                                                                                                                  |
| 2. Scope of validation          | Confirmation: *L. mono* isolated on several selective & non-selective nutrient agars  
Typing: 15 *Salmonella enterica* subsp. *enterica* serovars                                                                                                                                           |
| 3. Scope of verification        | Confirmation: *L. mono* isolated on several selective & non-selective nutrient agars  
Typing: 15 *Salmonella enterica* subsp. *enterica* serovars                                                                                                                                           |
| 4. Select strains               | Confirmation and Typing: 5 target strains and 5 non-target strains                                                                                                                                       |
| 5. Protocol                     | Inclusivity and exclusivity study                                                                                                                                                                        |
| 6. Analysis                     | Testing selected target/non-target strains using the full procedures of the methods                                                                                                                                               |
| 7. Evaluation                   | Acceptability limits: 100 % agreement in inclusivity and exclusivity studies                                                                                                                                  |
Root cause analysis
It didn’t work – now what?!

Conduct a root cause analysis to determine error(s):

• analytical error due to lack of good laboratory practice
• analytical error in protocol application
Example: fishbone diagram

Root cause analysis

Cause

- Samples
  - (Food) item choice
  - Homogenization
- Manpower
  - Technician
- Verification interpretation
  - Validation data
  - Averaging, rounding
- Equipment and material
  - Appropriate dilution
  - Inoculation level
  - Verification protocol

Effect

- Method to be verified
  - Correct application
- did not meet

Acceptability Limits

Prepared by ISO/TC 34/SC 9/WG 3 “Method validation” (version: 20240308)
Conclusions

Root cause analysis

When the issue is identified:

• implement corrective actions
• repeat the test

If an issue cannot be identified after repeating the test, contact:

• method supplier for support to resolve (proprietary methods)
• standardization body (reference methods)
• certification body (if method is certified and no resolution can be found)
Protocol for the verification of non-validated reference methods in a single laboratory [Annex F]
Scope of Method vs Validation vs Laboratory application

Non-validated reference methods

Method

It specifies the (group of) products (categories or types or items) for which the method is claimed to be applicable

Laboratory

It specifies the (group of) products (categories or types or items) for which the method is claimed to be used by the laboratory and are within the scope of validation
(Food) item verification

Non-validated reference methods

Demonstrate the competence of the user laboratory to perform the non-validated reference method with (food) items that are tested in the user laboratory

[no implementation verification – because there is no validation study]

The user laboratory shall:

• select 1 non-challenging (food) item from a (food) category claimed in the scope of the reference method

• select 1 challenging (food) item from each (food) category, based on the scope of the reference method, that is also under the scope of the laboratory application

Annex F: Protocol for the verification of non-validated reference methods in a single laboratory
Scope: broad range of foods

*Non-validat*ed reference method verification

**Figure F.1 — Food items required when verifying a non-validated reference method for a “broad range of foods” scope**

- Implementation verification
  - Do not perform
- (Food) item verification
  - Choose first one non-challenging food item and then a minimum of 5 challenging food items, each one from a different food category, belonging to the scope of laboratory application
Scope: limited range of foods

*Non-validated* reference method verification

**Scope of the method**

Reference method with no published validation data

*“Limited range of foods” scope*

**Category 1 → Types → Items**
**Category 2 → Types → Items**
**Category 3 → Types → Items**

**Scope of laboratory application**

Implementation verification

- Do not perform

- If the scope of the method covers < 5 food categories, choose first one non-challenging food item and then a minimum of one challenging food item from each of the food categories, belonging to the scope of laboratory application

**Figure F.2 — Food item required when verifying a non-validated reference method for a “limited range of foods” scope**
Scope: ‘broad range of foods’ and other categories

Non-validated reference method verification

Scope of the method

Reference method with no published validation data
“Broad range of foods and other categories” scope

- Category 1 → Types → Items
- Category 2 → Types → Items
- Category 3 → Types → Items
- Category 4 → Types → Items
- Category 5 → Types → Items
- Category 6 ...
- ... Category 15

Category Pet food and animal feed
→ Types → Items
Category Environmental samples
→ Types → Items
Category Primary production samples → Types → Items

Scope of laboratory application

“Broad range of foods and other categories” scope

- Implementation verification
  - Do not perform
- (Food) item verification
  - Choose first one non-challenging food item and then a minimum of 5 challenging food items, each one from a different food category, belonging to the scope of laboratory application
  - If other categories are included, choose one challenging item from each of these other categories, belonging to the scope of laboratory application

Figure F.3 — Items required when verifying a method for a “broad range of foods and other categories” scope
### Table F.1 — Summary of the minimum number of (food) items required for verification of a non-validated reference method

<table>
<thead>
<tr>
<th>Scope of the reference method</th>
<th>Implementation verification</th>
<th>Number of samples</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Broad range of foods” scope</td>
<td>Not applicable</td>
<td>1 non-challenging +</td>
<td>≥ 6</td>
</tr>
<tr>
<td>5 food categories</td>
<td></td>
<td>(N_{\text{food}} \geq 5) challenging food items</td>
<td></td>
</tr>
<tr>
<td>“Limited range of foods” scope</td>
<td>Not applicable</td>
<td>1 non-challenging +</td>
<td>((N_{\text{food}} + 1) \leq 5)</td>
</tr>
<tr>
<td>(N_{\text{food}}) categories</td>
<td></td>
<td>(N_{\text{food}} \leq 4) challenging food items</td>
<td></td>
</tr>
<tr>
<td>“Broad range of foods” +</td>
<td>Not applicable</td>
<td>1 non-challenging +</td>
<td>≥ 6 + (N_{\text{other}})</td>
</tr>
<tr>
<td>other categories ((N_{\text{other}})) scope</td>
<td></td>
<td>(N_{\text{food}} \geq 5) challenging food items</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 challenging item from each of the (N_{\text{other}}) other categories</td>
<td></td>
</tr>
<tr>
<td>“Limited range of foods”</td>
<td>Not applicable</td>
<td>1 non-challenging +</td>
<td>((N_{\text{food}} + N_{\text{other}} + 1) \leq 8)</td>
</tr>
<tr>
<td>(N_{\text{food}}) categories + other categories ((N_{\text{other}})) scope</td>
<td></td>
<td>(N_{\text{food}} \leq 4) challenging food items</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 challenging item from each of the (N_{\text{other}}) other categories</td>
<td></td>
</tr>
<tr>
<td>Other categories ((N_{\text{other}})) scope only</td>
<td>Not applicable</td>
<td>1 non-challenging (food or other) + (N_{\text{other}}) ≤ 3 challenging items</td>
<td>((N_{\text{other}} + 1) \leq 4)</td>
</tr>
</tbody>
</table>
## Performance characteristics

**Non-validated reference method verification**

### Table F.2 — Required performance characteristics to be determined for verification of a non-validated reference method

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristic</th>
<th>Implementation verification</th>
<th>(Food) item verification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>Estimated LOD$<em>{50}$ (eLOD$</em>{50}$)</td>
<td>Not applicable</td>
<td>✓</td>
</tr>
<tr>
<td>Quantitative</td>
<td>Intralaboratory reproducibility standard deviation ($S_{LR}$)</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td></td>
<td>Estimated bias (eBias)</td>
<td>Not applicable</td>
<td>✓</td>
</tr>
</tbody>
</table>

**NOTE** For the verification of a qualitative method, three protocols are proposed to the user laboratory. The protocol 3 does not require a determination of an eLOD$_{50}$ but to target a concentration of 3 cfu to 5 cfu/test portion.
Determination of eLOD\textsubscript{50}

Qualitative non-validated reference method verification

### Table 3 — Protocols to determine eLOD\textsubscript{50} and number of replicates needed per inoculation level

<table>
<thead>
<tr>
<th>Protocol</th>
<th>High level 9 $\times$ LOD\textsubscript{50} / test portion</th>
<th>Intermediate level 3 $\times$ LOD\textsubscript{50} / test portion</th>
<th>Low level 1 $\times$ LOD\textsubscript{50} / test portion</th>
<th>3 cfu to 5 cfu / test portion</th>
<th>Blank</th>
<th>Total number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>--</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>--</td>
<td>3</td>
<td>5</td>
<td>--</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>7</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

NOTE: The abbreviation of colony forming units is cfu.

### Table F.3 — Protocols to determine eLOD\textsubscript{50} and number of replicates needed per inoculation level for a non-validated reference method

<table>
<thead>
<tr>
<th>Protocol</th>
<th>High level 9 cfu/test portion</th>
<th>Intermediate level 3 cfu/test portion</th>
<th>Low level 1 cfu/test portion</th>
<th>3 cfu to 5 cfu/test portion</th>
<th>Blank</th>
<th>Total number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>--</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>--</td>
<td>3</td>
<td>5</td>
<td>--</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>7</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

NOTE: The abbreviation of colony forming units is cfu.
## Inoculation levels

**Qualitative non-validated reference method verification**

<table>
<thead>
<tr>
<th>Protocol</th>
<th><strong>High level 9 cfu/test portion</strong></th>
<th><strong>Intermediate level 3 cfu/test portion</strong></th>
<th><strong>Low level 1 cfu/test portion</strong></th>
<th>3 cfu to 5 cfu/test portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>This should be at a maximum of nine times the assumed LOD$_{50}$.</td>
<td>From the high inoculation level, perform a 1:3 dilution to achieve the intermediate level.</td>
<td>From the intermediate inoculation level, perform a 1:3 dilution to achieve the low level.</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>This should be at a maximum of three times the assumed LOD$_{50}$.</td>
<td>From the intermediate inoculation level, perform a 1:3 dilution to achieve the low level.</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>The level of contamination of the inoculum is known, (e.g. reference material with known concentration).</td>
</tr>
</tbody>
</table>
**Summary of acceptability limits**

*Non-validated reference method verification*

Table F.5 — Acceptability limits for the verification of non-validated reference methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristics</th>
<th>Acceptability limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>eLOD$_{50}$</td>
<td>For protocols 1 and 2: eLOD$_{50}$ ≤ 4 cfu/test portion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For protocol 3: ≥ 6 out of 7 positive results</td>
</tr>
<tr>
<td>Quantitative</td>
<td>eBias</td>
<td>$</td>
</tr>
</tbody>
</table>
# Determination of eLOD$_{50}$

**Qualitative** non-validated reference method verification

## Table 6 — Determination of eLOD$_{50}$ based on the number of positive results per level of contamination using protocol 1

<table>
<thead>
<tr>
<th>High inoculation level</th>
<th>Intermediate inoculation level</th>
<th>Low inoculation level</th>
<th>Blank level</th>
<th>eLOD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>targeted 9 cfu/test portion</td>
<td>targeted 3 cfu/test portion</td>
<td>targeted 1 cfu/test portion</td>
<td>0/1</td>
<td>cfu/test portion</td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>4/4</td>
<td></td>
<td>&lt; 1,0 × LIL$^a$</td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>3/4</td>
<td>0/1</td>
<td>= 0,5 × LIL</td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>2/4</td>
<td>0/1</td>
<td>= 0,7 × LIL</td>
</tr>
</tbody>
</table>

**Inoculum (cfu) at each level**

| 8 | 3 | 1 |

**eLOD$_{50}$ shall be ≤ 4 cfu/test portion**

0,5 eLOD$_{50}$ is ≤ 4 cfu/test portion = **Meets acceptability limits**
## Determination of eBias

**Quantitative non-validated reference method verification**

### Table 13 — Test results obtained using the method to be verified

<table>
<thead>
<tr>
<th>Mean result Artificially contaminated (food) item (log$_{10}$ cfu/g or ml)$^a$</th>
<th>For comparison</th>
<th>Result Artificially contaminated (food) item (log$_{10}$ cfu/test portion)$^a$</th>
<th>Result Inoculum suspension [without (food) item] (log$_{10}$ cfu/ml)</th>
<th>eBias: absolute difference in results between artificially contaminated (food) item per test portion and the inoculum suspension</th>
<th>≤ 0,5 log</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory sample 1 (from batch 1), test portion 1</td>
<td>2,06 (average of 1,87 and 2,25)</td>
<td>3,06</td>
<td>3,17</td>
<td>0,11</td>
<td>Meets</td>
</tr>
<tr>
<td>Laboratory sample 1 (from batch 1), test portion 2</td>
<td>3,11 (average of 3,16 and 3,06)</td>
<td>4,11</td>
<td>4,05</td>
<td>0,06</td>
<td>Meets</td>
</tr>
<tr>
<td>Laboratory sample 2 (from batch 2), test portion 1</td>
<td>3,99 (average of 3,93 and 4,04)</td>
<td>4,99</td>
<td>5,29</td>
<td>0,30</td>
<td>Meets</td>
</tr>
</tbody>
</table>

$^a$ This example is based on the use of a 10-gram test portion inoculated with 1 ml of inoculum.
Transition period for implementation of ISO 16140-3:2021
Introduction

Transition period for implementation

This document and ISO 16140-3 are intended to be used by:

• user laboratories (accredited and non-accredited)
• (technical) assessors involved in the evaluation of verification data generated for reference methods and validated alternative methods
• accreditation bodies

It can also be useful for regulatory authorities, risk managers and customers
The transition arrangement is as follows:

- **until 2027-12-31**, user laboratories may perform method verification of non-validated reference methods and in accordance with ISO 16140-3, Annex F
- **from 2028-01-01**, only validated reference methods are applicable for method verification

After this date, reference methods (including ISO or CEN standards) shall be validated before a verification can be performed in accordance with ISO 16140-3

**Reminder:**
- ISO standards are *voluntary* documents
- ISO develops standards but has *no authority* over their implementation
Differentiation is made between three situations

Transition period for implementation

1. Methods already accredited under the scope of laboratory application:
   • do not need to re-verify, unless changes made to the method

2. Methods or (food) categories new to the scope of laboratory application:
   • verify methods introduced to the laboratory after publication of ISO 16140-3
   • verify new (food) category additions to accredited methods under scope of laboratory application
Differentiation is made between three situations

Transition period for implementation

3. Methods revised after they have been accredited under the scope of laboratory application:

REFERENCE methods:

a) **Validated reference methods:**
   
   No re-verification:
   
   • minor (technical) change
   • major (technical) change with no/minor impact
   
   Re-verification:
   
   • a major (technical) change with major impact

b) **Non-validated reference methods:**
   
   • can only be used for verification during the transition period
   • from 1 January 2028, only validated reference methods can be used
Differentiation is made between three situations

Transition period for implementation

3. Methods revised after they have been accredited under the scope of laboratory application:

Validated ALTERNATIVE methods:
Re-validation or additional validation, may also require re-verification

- a major (technical) change in the reference method(s)
- a major (technical) change or revision of ISO 16140-2 or ISO 16140-6
- a change to the scope of the method (extensions or exclusions)
- technical changes to parameters or application
Public website of ISO/TC 34/SC 9 ‘Microbiology’

- Information: method validation and verification
- Background: six parts of ISO 16140 series

Supporting materials*
- Transition document: implementation of ISO 16140-3
- Excel®-based program for assistance on statistics
- Recording of the webinar from 2 March 2021

Presentations:
- Overview of the entire ISO 16140 series
- Overview of ISO 16140-3 ‘Method verification’
- “Deep-dive training” on ISO 16140-3 ‘Method verification’

*All these materials are available on the SC 9-website: https://committee.iso.org/home/tc34sc9

Method validation and method verification

The ISO 16140 series is dedicated to the validation and verification of microbiological methods. These International Standards are designed to help food and feed testing laboratories, test kit manufacturers, competent authorities, and food and feed business operators to implement microbiological methods.

Learn more about ISO 16140 series, and the necessary stages of validation and verification of methods before use.
Thank you
Questions?