National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Pilot validation study for confirmation of *Salmonella* following ISO/DIS 16140-6

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(Project leader ISO 16140-6)

ISO 16140 series

Microbiology of the food chain — Method validation —

Introduction

The ISO 16140 series has been elaborated in response to the need for various ways to validate or verify test methods. It is the successor of ISO 16140:2003, *Microbiology of food and animal feeding stuffs — Protocol for the validation of alternative methods.* ISO 16140 series consists of several parts with the general title, *Microbiology of the food chain — Method validation:*

— *Part 1: Vocabulary* → 2016

— *Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method* → 2016

— *Part 3: Protocol for the verification of reference and validated alternative methods implemented in a single laboratory*

— *Part 4: Protocol for single-laboratory (in-house) method validation*

— *Part 5: Protocol for factorial interlaboratory validation for non-proprietary methods*

— *Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures*

DIS stage

ISO 17468, *Microbiology of the food chain — Technical requirements and guidance on establishment or revision of a standardized reference method*[7], is a closely linked International Standard. This International Standard, which establishes technical rules for the development and validation of standardized methods, is intended for the development of standardized methods by ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology* and CEN/TC 275/WG 6, *Microbiology of the food chain.* → 2016

In general two stages are needed before a method can be used in a laboratory:

— The first stage is the validation of the method. This is either conducted in several laboratories (parts 2 and 5 of ISO 16140) or in one laboratory (part 4 of ISO 16140).

— The second stage is method verification, where a laboratory demonstrates that it can satisfactorily perform a validated method. This is described in part 3 of ISO 16140 (method verification). In

DRAFT INTERNATIONAL STANDARD

ISO/DIS 16140-6

ISO/TC 34/SC 9

Secretariat: **AFNOR**

Voting begins on:
2017-12-15

Voting terminates on:
2018-03-09

Microbiology of the food chain — Method validation —

Part 6:
Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures

Microbiologie de la chaîne alimentaire — Validation des méthodes —

Partie 6: Protocole pour la validation des méthodes alternatives (propriétaires) pour confirmation et identification microbiologique

DRAFT INTERNATIONAL STANDARD
ISO/DIS 16140-6

ISO/TC 34/SC 9

Secretariat: **AFNOR**

Voting begins on:
2017-12-15

Voting terminates on:
2018-03-09

Result of voting
P-Members voting: 21 in favour out of 21 = 100 % (requirement >= 66.66%) *(P-Members having abstained are not counted in this vote.)* **Member bodies voting: 0 negative votes out of 26 = 0 % (requirement <= 25%)** ***Approved***

Answers to Q.1: "Do you approve this draft as a European Standard"		
19 x	Yes	Austria (ASI), Bulgaria (BDS), Finland (SFS), France (AFNOR), Germany (DIN), Greece (NQIS ELOT), Hungary (MSZT), Ireland (NSAI), Lithuania (LST), Malta (MCCAA), Netherlands (NEN), Poland (PKN), Romania (ASRO), Serbia (ISS), Slovakia (SOSMT), Slovenia (SIST), Switzerland (SNV), Turkey (TSE), United Kingdom (BSI)
0 x	No	
14 x	Abstains	Belgium (NBN), Croatia (HZN), Cyprus (CYS), Czech Republic (UNMZ), Denmark (DS), Estonia (EVS), Iceland (IST), Italy (UNI), Latvia (LVS), Norway (SN), Portugal (IPQ), Spain (UNE), Sweden (SIS), The Former Yugoslav Republic of Macedonia (ISRM)

Result of voting

(National Members having abstained are not counted in this vote.)

Approved by National Members

National Members approving: 19
National Members disapproving: 0
Number of Members approving: 100.000 % (requirement >= 55 %)
Weighted percentage of Population approving: 100.000 % (requirement >= 65 %)

Answers to Q.3: "Do you have any comments ?"		
6 x	Yes	France (AFNOR), Germany (DIN), Ireland (NSAI), Netherlands (NEN), Switzerland (SNV), United Kingdom (BSI)
27 x	No	Austria (ASI), Belgium (NBN), Bulgaria (BDS), Croatia (HZN), Cyprus (CYS), Czech Republic (UNMZ), Denmark (DS), Estonia (EVS), Finland (SFS), Greece (NQIS ELOT), Hungary (MSZT), Iceland (IST), Italy (UNI), Latvia (LVS), Lithuania (LST), Malta (MCCAA), Norway (SN), Poland (PKN), Portugal (IPQ), Romania (ASRO), Serbia (ISS), Slovakia (SOSMT), Slovenia (SIST), Spain (UNE), Sweden (SIS), The Former Yugoslav Republic of Macedonia (ISRM), Turkey (TSE)

- Plus comments at ISO level from: US, AU

- 203 comments (26 General, 103 Editorial, 74 Technical)

- Discussed at the 20th WG3 meeting in Helsinki, 23 -25 May 2018

* Updated document for pre-FDIS voting (final technical comments)

* Final document for FDIS voting (editorial comments only)

* Publishing of the Standard (part 6) by Spring 2018 (?!)

Discussed at the 20th WG3 meeting in Helsinki

ISO/DIS 16140 part 6, Scope

- Protocol for the validation of a part or complete **confirmation** procedure of a (qualitative or quantitative) reference method against a (proprietary) alternative confirmation method (e.g. API 20E or PCR test or Maldi-TOF).

- also applicable to validation of alternative **typing** methods (e.g. serotyping of *Salmonella*)

- Validated alternative confirmation methods can be used to replace (partly or completely) the confirmation procedure described in:
  - the reference method;
  - an alternative method validated according to part 2 of ISO 16140 as long as one of the isolation agars specified in the validation study of the alternative confirmation method is used.

ISO/DIS 16140 part 6, Example on application

- An alternative confirmation method based on PCR is validated to replace the biochemical confirmation for *Salmonella* as described in reference method ISO 6579-1. In the validation study XLD (mandatory agar according to ISO 6579-1) and BGA (optional agar according to ISO 6579-1) were used as the agars to start the confirmation.
- The validated PCR confirmation method can be used under the following conditions:
  - by laboratories using ISO 6579-1 to replace the biochemical confirmation;
  - by laboratories using an ISO 16140-2 validated alternative method that refers to ISO 6579-1 for (biochemical) confirmation;
  - by laboratories using an ISO 16140-2 validated alternative method that starts the confirmation from XLD and/or BGA agar.
- The validated confirmation method **cannot** be used under the following conditions:
  - by laboratories using an ISO 16140-2 validated alternative method that refers to a proprietary method for confirmation (e.g. a chromogenic agar);
  - by laboratories using an ISO 16140-2 validated alternative method that refers to other agars to start the confirmation (e.g. Hektoen agar);
  - by laboratories using an ISO 16140-2 validated alternative method that refers to a confirmation procedure not based on isolation on agar (e.g. an ELISA test).

ISO/preFDIS 16140 part 6, Example on application

- An alternative confirmation method based on ELISA is validated to replace the biochemical confirmation for *Salmonella* as described in reference method ISO 6579-1. In the validation study XLD (mandatory agar according to ISO 6579-1) and BGA plus a specified chromogenic agar (optional agars for second plating in accordance with ISO 6579-1) were used as the agars to start the confirmation.
- The validated confirmation method can be used to replace the biochemical confirmation under the following conditions:
  - by laboratories using ISO 6579-1; or
  - by laboratories using an ISO 16140-2 validated alternative method that refers to ISO 6579-1 for confirmation;
  - by laboratories using an ISO 16140-2 validated alternative method that starts the confirmation from XLD and/or BGA agar and/or the specified chromogenic agar.
- The validated confirmation method **cannot** be used under the following conditions:
  - by laboratories using an ISO 16140-2 validated alternative method that refers only to other agars to start the confirmation (e.g. Hektoen agar and SS agar only);
  - by laboratories using an ISO 16140-2 validated alternative method that refers only to a confirmation procedure that does not require isolation on agar (e.g. a PCR test).

ISO/DIS 16140 part 6, Principles

- Comparison between reference confirmation procedure and alternative confirmation method.

- Validation starts with a bacterial strain (colony on an agar plate) and not with a (food) sample.

- Based on the inclusivity/exclusivity study of ISO 16140-2, using well characterised strains.

- Organising laboratory:
  - involved in validation studies
  - Method Comparison Study
  - Interlaboratory Study, at least 10 participants

ISO/DIS 16140 part 6, Use

- Can also be used within the scope of ISO 16140 part 4
  - single laboratory (in-house) validation

- Can also be used within the scope of ISO 16140 part 5
  - interlaboratory validation of non-proprietary methods

- **Verification** will be described in ISO 16140 part 3
  - user laboratory to demonstrate that it can satisfactorily perform a validated method

ISO/DIS 16140 part 6, differentiation between:

- Confirmation to the Family level (non-*Salmonella*)
  - e.g. a confirmation test for *Enterobacteriaceae*
- Confirmation to the Genus level (non-*Salmonella*)
  - e.g. a confirmation test for *Campylobacter* spp.
- Confirmation to the Species level (non-*Salmonella*)
  - e.g. a confirmation test for *Listeria monocytogenes*
- Confirmation/typing to the Microbial (sub)type level (non-*Salmonella*)
  - e.g. typing of *E.coli* O157

ISO/DIS 16140 part 6, differentiation between:

- Confirmation to the *Salmonella* Genus or Species level
  - e.g. confirmation of *Salmonella enterica*

- Confirmation/typing to the *Salmonella* serovar level
  - e.g. serotyping of *Salmonella* Typhimurium

Method Comparison Method (non-*Salmonella*)

Level :	Inclusivity:	Exclusivity:
Family	**200** different target strains	**100** different non-target strains
Genus	**150** different target strains	**100** different non-target strains
Species	**100** different target strains per species	- **50** different strains from non-target genus - **50** different strains from non-target species within target genus
Microbial (sub) type	**25** different target strains per microbial (sub)type If more than 4 microbial (sub)types: at least **100** strains and a minimum of 5 strains per microbial (sub)type	- at least **25** different strains from non-target genus - at least **25** different strains from non-target microbial (sub)type within target species - add up to a minimum of **100** strains in total

Method Comparison Method (*Salmonella*)

Level :	Inclusivity:	Exclusivity:
Genus or species	**150** different target strains	**100** different non-target strains
Serovar	**25** different target strains per serovar If more than 10 serovars: at least **250** strains and a minimum of 5 strains per serovar	- **25** different strains from non-target genus - **75** different strains from non-target serovar within target subspecies

preFDIS:

— If the alternative confirmation method claims confirmation of *Salmonella* spp., target strains include at least 2strains each of *S. bongori*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, *S. enterica* subsp. *indica*. To be supplemented with strains of *S. enterica* subsp. *enterica* covering common serovars, and preferably including at least one representative of each (somatic) O-antigen described (ISO/TR 6579-3).

NOTE More information on 'common' serovars can be found on the following websites: www.cdc.gov, www.ecdc.europa.eu, www.efsa.europa.eu.

Interlaboratory Study

- Inclusivity: a total of 16 different target strains
- Exclusivity: a total of 8 different non-target strains
- To be selected and pre-tested by the organising laboratory
- At least 10 participating laboratories
  - 24 strains to be tested per participant
  - Alternative confirmation/typing method to be tested
  - ~~Optionally,~~ Reference confirmation procedure ~~may~~ to be tested

ISO/DIS 16140-6, Evaluation of results

- Presentation of tabulated results in study report

- First interpretation of results
  - Alternative method compared to reference method
- In case of discrepancies: Second interpretation of results
  - Alternative method compared to identity of the strain
- Final interpretation of results
  - Inclusivity/Exclusivity Agreements (IA, EA)
  - Inclusivity/Exclusivity Deviations (ID, ED)

- Evaluation of results (ID, ED) according to Acceptability Limits (AL)
  - Method Comparison Study
  - Interlaboratory Study

Examples described in ISO 16140 part 6

- Informative Annex B: Example of the validation of an alternative confirmation method to the species level (*Listeria monocytogenes)*

- Informative Annex C: Example of the validation of an alternative typing method to the *Salmonella* serovar level (15 different serovars claimed)

Example: first confirmation-validation pilot study

Validation of a confirmation method according to ISO/DIS 16140-6:2017
A MicroVal pilot study using the MALDI Biotyper
as an alternative for *Salmonella* spp. confirmation

B. Bastin[1], P. Bird[1], E. Crowley[1], B. Diep[2], I. Ferro[3], T. Hammack[4], W. Jacobs[5], M. Kostrzewa[6], C. Le Doeuff[7], S. Peron[7], M. Rannou[7], D. Sohier[6], M. Timke[6], P. in 't Veld[8], J. Witsenburg[3]

The ISO 16140 standard provides technical and interpretation rules for method validation and verification, and consists of 6 different parts. Part 6 is currently at the DIS (Draft International Standard) stage and describes the protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures. The study design was set up during the past years, and acceptability limits for the data interpretation were defined based on expert opinion, i.e. maximum number of positive or negative deviations between the reference and alternative method.

Evaluation of the ISO/DIS 16140-6:2017: Do the technical rules give sufficient detail to conduct the method comparison and inter-laboratory studies? Are the proposed acceptability limits (AL) fit for purpose or too restrictive? A pilot study was coordinated by MicroVal as a proof of concept.

The MALDI Biotyper (Bruker) was tested as an alternative to confirm *Salmonella* spp. from non-selective and selective agars. A method comparison and an inter-laboratory studies were realized. 150 *Salmonella* spp. strains and 100 non-target strains were tested by two expert laboratories in the method comparison study. The collaborative study was run by involving a minimum of 10 organizations to produce 10 valid data sets with 16 target and 8 non-target strains.
See Tables 1 and 2, with the Tested strains (N), Deviation (D) and Acceptability Limit (AL).

TABLE 1: Summary of the Method Comparison Study

Tested Media	Tested Panel of Strains	N	D	AL
Nutrient Agar	Inclusivity	150	0	Accepted
	Exclusivity	100	0	Accepted
XLD	Inclusivity	150	0	Accepted
	Exclusivity	100	0	Accepted
BGA	Inclusivity	150	0	Accepted
	Exclusivity	100	0	Accepted
RAPID' *Salmonella*	Inclusivity	150	0	Accepted
	Exclusivity	100	0	Accepted
Brilliance *Salmonella*	Inclusivity	150	0	Accepted
	Exclusivity	100	0	Accepted
ASAP	Inclusivity	150	0	Accepted
	Exclusivity	100	0	Accepted

TABLE 2: Summary of the Inter-Laboratory Study

Tested Media & Number of Labs	Tested Panel of Strains	N	D	AL
Nutrient Agar - 14 Labs	Inclusivity	224	0	Accepted
	Exclusivity	112	0	Accepted
XLD 13 Labs	Inclusivity	208	0	Accepted
	Exclusivity	104	0	Accepted
RAPID' *Salmonella* 12 Labs	Inclusivity	192	0	Accepted
	Exclusivity	96	0	Accepted

The MicroVal reviewers and the expert laboratories encountered no specific difficulties in setting up the project, organizing the testing, and interpreting the generated data. The collaborating laboratories could easily understand the protocol of the ISO/DIS 16140-6:2017 and achieve the required number of tests. The defined AL were easily passed as all the *Salmonella* spp. strains were correctly confirmed with the MALDI Biotyper on all tested media in the method comparison and inter-laboratory studies.

The ISO/DIS 16140-6:2017 provides valuable technical rules and interpretation concept to validate confirmation methods. The observed results were excellent; therefore Microval issued a certificate of validation based on the ISO/DIS 16140-6:2017. The certificate is available on www.microval.org.

Organizations involved in the international technical committee of MicroVal and in the study: [1]Q-Laboratories (OH, USA), [2]Nestlé Research Center (CH), [3]MicroVal (NL), [4]FDA (MY, USA), [5]RIVM (NL) & project leader of the ISO 16140-part 6, [6]BRUKER, [7]ADRIA, (FR), [8]NVWA (NL) & convenor of the ISO 16140 working group

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CERTIFICATE OF COMPLIANCE

LLOYD'S REGISTER QUALITY ASSURANCE

hereby declares that the certification assessment has demonstrated that

MALDI Biotyper®

Complete Solution for the confirmation of *Salmonella* spp.

Manufactured and supplied by:
Bruker Daltonik GmbH
Fahrenheitstraße 4
D-28359 Bremen
GERMANY

has been validated and revised 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: ISO 6579-1 (2017) and ISO 6579-2 (2012) - Microbiology of food and animal feeding stuffs - Horizontal method for the detection, enumeration and serotyping of *Salmonella* spp. - Part 1: detection of *Salmonella* spp. - Part 2: Enumeration by a miniaturized most probable number technique

Scope: Confirmation of *Salmonella* spp. from colonies isolated on XLD, BGA, Chromogenics based on C8-esterase activity detection (RAPID' *Salmonella*, Brilliance *Salmonella*, ASAP®) and non-selective nutrient agars.

The validation and certification has been performed in accordance with ISO/DIS 16140-6:2017 and the MicroVal Rules and Certification Scheme version 8

Certificate no.: 2017U873 — First approval date: 13 December 2018
Expiry date: 11 February 2022

ISSUED BY: Lloyd's Register Nederland B.V.
Rotterdam, The Netherlands

Example: confirmation of *Salmonella* spp.

- Alternative confirmation method: Bruker MALDI Biotyper® (MTB)
  - MTB (smart) Microflex LT/SH (smart) instruments
  - MTB Compass and 4.0 software
  - MBT Compass Library (6903 version and higher)

Reference method: ISO 6579-1 (2017) and ISO 6579-2 (2012) - Microbiology of food and animal feeding stuffs - Horizontal method for the detection, enumeration and serotyping of *Salmonella* spp. - Part 1: detection of *Salmonella* spp. - Part 2: Enumeration by a miniaturized most probable number technique

Scope: Confirmation of *Salmonella* spp. from colonies isolated on XLD, BGA, Chromogenics based on C8-esterase activity detection (RAPID' *Salmonella*, Brilliance™ *Salmonella*, ASAP®) and non-selective nutrient agars.

The validation and certification has been performed in accordance with ISO/DIS 16140-6:2017 and the MicroVal Rules and Certification Scheme version 8.

Example: Confirmation of *Salmonella* spp.

- Method Comparison Study (MCS)

For inclusivity, at least 150 different target strains shall be tested.

— If the alternative confirmation method claims confirmation of *Salmonella* spp., target strains include at least 2strains each of *S. bongori*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, *S. enterica* subsp. *indica*. To be supplemented with strains of *S. enterica* subsp. *enterica* covering common serovars, and preferably including at least one representative of each (somatic) O-antigen described (ISO/TR 6579-3).

NOTE More information on 'common' serovars can be found on the following websites: www.cdc.gov, www.ecdc.europa.eu, www.efsa.europa.eu.

For exclusivity, at least 100 different non-target strains shall be tested.

— If the alternative confirmation method claims confirmation of *S. enterica*, include:

  — at least 2 strains of *S. bongori*;

  — at least 75 strains from the target family (*Enterobacteriaceae*).

Example: Confirmation of *Salmonella* spp. (MSC)

Table 5a — Summary of the results in the method comparison study, non-selective nutrient agar

	N	PA	IN	IP	NA			IA/EA	ID/ED
					NA *(growth)*	NA *(no growth)*	NA *(total)*		
Inclusivity	150	150	0	0	0	0	0	150	0
Exclusivity	100	0	0	0	100	0	100	100	0

Table 5b — Summary of the results in the method comparison study, selective agar(s)

	N	PA	IN	IP	NA			IA/EA	ID/ED
					NA *(growth)*	NA *(no growth)*	NA *(total)*		
Inclusivity	150	150	0	0	0	0	0	150	0
Exclusivity	100	0	0	0	67	33	100	100	0

PA: Positive Agreement, IN: Inconsistent Negative result, IP: Inconsistent Positive result, NA: Negative Agreement, IA: Inclusivity Agreement, EA: Exclusivity Agreement, ID: Inclusivity Disagreement, ED: Exclusivity Disagreement.

Example: Confirmation of *Salmonella* spp. (MCS)

Table 6 — Evaluation of the method comparison study results

	ID/ED	AL	ID/ED ≤ AL	Evaluation
Inclusivity	0	1	0 ≤ 1	Accepted
Exclusivity	0	2	0 ≤ 2	Accepted

NOTE The Acceptability Limit (AL) values are taken from Table D.1 (non-*Salmonella*) or Table D.2 (*Salmonella*).

Example: Confirmation of *Salmonella* spp.

- Interlaboratory Study (ILS)
- Inclusivity: a total of 16 different target strains
- Exclusivity: a total of 8 different non-target strains
- To be selected and pre-tested by the organising laboratory
- At least 10 participating laboratories
  - 24 strains to be tested per participant
  - Alternative confirmation method to be tested
  - ~~Optionally,~~ **Reference confirmation procedure** ~~may~~ to be **tested**
  - In the example study: Nutrient agar, XLD, RAPID' *Salmonella* plates

Example: Confirmation of *Salmonella* spp. (ILS)

Table 8a — Summary of the results in the interlaboratory study, non-selective nutrient agar

	N	PA	IN	IP	NA			IA/EA	ID/ED
					NA *(growth)*	NA *(no growth)*	NA *(total)*		
Inclusivity	224	224	0	0	0	0	0	224	0
Exclusivity	112	0	0	0	112	0	112	112	0

Table 8b — Summary of the results in the interlaboratory study, selective agar(s)

	N	PA	IN	IP	NA			IA/EA	ID/ED
					NA *(growth)*	NA *(no growth)*	NA *(total)*		
Inclusivity	208	208	0	0	0	0	0	208	0
Exclusivity	104	0	0	0	104	0	104	104	0

PA: Positive Agreement, IN: Inconsistent Negative result, IP: Inconsistent Positive result, NA: Negative Agreement, IA: Inclusivity Agreement, EA: Exclusivity Agreement, ID: Inclusivity Disagreement, ED: Exclusivity Disagreement.

Example: Confirmation of *Salmonella* spp. (ILS)

Table 9 — Evaluation of the interlaboratory study results

	ID/ED	AL	ID/ED ≤ AL	Evaluation
Inclusivity	0	3	0 ≤ 3	Accepted
Exclusivity	0	3	0 ≤ 3	Accepted

NOTE The Acceptability Limit (AL) values are taken from Table D.1 (non-*Salmonella*) or Table D.2 (*Salmonella*).

Example: Confirmation of *Salmonella* spp.

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The MicroVal reviewers and the expert laboratories encountered no specific difficulties in setting up the project, organizing the testing, and interpreting the generated data. The collaborating laboratories could easily understand the protocol of the ISO/DIS 16140-6:2017 and achieve the required number of tests. The defined AL were easily passed as all the *Salmonella* spp. strains were correctly confirmed with the MALDI Biotyper on all tested media in the method comparison and inter-laboratory studies.

The ISO/DIS 16140-6:2017 provides valuable technical rules and interpretation concept to validate confirmation methods. The observed results were excellent; therefore Microval issued a certificate of validation based on the ISO/DIS 16140-6:2017. The certificate is available on www.microval.org.

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Pre-FDIS commenting ISO 16140-6 in Autumn 2018…

Thank you for your attention !

Ačiü, Aitäh, Bedankt, Благодаря, Ďakujem, Danke, Děkuji, Dziękuję, ευχαριστώ, Go raibh maith agat, Gracias, Grazie, Grazzi, Hvala, Hvala, Kiitos, Köszönöm, Merci, Mulţumesc, Obrigado, Paldies, Tack, Tak , Thank you.

Any questions ?

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