



International Standard

ISO 16140-4

Microbiology of the food chain — Method validation —

Part 4: Protocol for method validation in a single laboratory

AMENDMENT 1: Validation of a larger test portion size for qualitative methods

*Microbiologie de la chaîne alimentaire — Validation des
méthodes —*

*Partie 4: Protocole pour la validation de méthodes dans un seul
laboratoire*

*AMENDEMENT 1: Validation d'une taille de prise d'essai plus
grande pour des méthodes qualitatives*

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**AMENDMENT 1
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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

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Microbiology of the food chain — Method validation —

Part 4:

Protocol for method validation in a single laboratory

AMENDMENT 1: Validation of a larger test portion size for qualitative methods

Clause 3

Add the following terminological entry:

3.13

larger test portion size

measured (volume or mass) representative sample taken from the laboratory sample or test sample for use in the preparation of the initial suspension that is larger than the test portion that has been described in the original method and/or validation document

4.1

Add the following text after the second paragraph:

The protocol used to validate a larger test portion size for qualitative methods in a single laboratory shall be as given in Annex H.

5.1.1.4, note

Replace the text with the following:

NOTE An Excel®-based program¹ is available for performing the RLOD calculations from <https://standards.iso.org/iso/16140/-4/ed-1/en/amd/1/>.

5.1.2.4, Note 2

Replace the text with the following:

NOTE 2 An Excel®-based program² is available for performing the LOD₅₀ calculations from <https://standards.iso.org/iso/16140/-4/ed-1/en/amd/1/>.

6.1.2.3, Note 2

Replace the text with the following:

NOTE 2 An Excel®-based program⁴ is available for performing the LOD₅₀ calculations from <https://standards.iso.org/iso/16140/-4/ed-1/en/amd/1/>.

Annex H

Add the following annex after Annex G, before the Bibliography.

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Annex H (normative)

Validation of a larger test portion size for qualitative methods

H.1 General

This annex specifies a protocol for the validation of qualitative methods when using a larger test portion size. This protocol is intended to demonstrate the effect of analysing a test portion larger than the test portion initially used to validate the method. Validation of the larger test portion size applies only to the laboratory conducting the study and only to the specific (food) category used.

Qualitative reference methods which were validated using a larger test portion size in accordance with ISO 17468 and qualitative alternative (proprietary) methods which were validated using a larger test portion size in accordance with ISO 16140-2 only need to be verified by a laboratory following ISO 16140-3.

The larger test portion size can be used in other laboratories once this has been validated in a study in accordance with ISO 16140-2 or ISO 16140-5. See the flow diagram in Figure 1. Once validated in such a study, any laboratory can implement the larger test portion size after verification following ISO 16140-3. For the verification of a larger test portion size, the same (food) category shall be used. If not validated in accordance with ISO 16140-2 or ISO 16140-5, validation in accordance with the protocol in this annex is necessary for each laboratory wishing to use a larger test portion size.

Requirements with regards to pooling are specified in ISO 6887-1.

When testing a larger single test portion, pooled test portion or pooled (pre-)enrichment test portion, the dilution ratio (sample/diluent) used in the validated method shall remain the same as well as the other incubation conditions (e.g. time and temperature). This ratio may be increased to overcome the inhibitory effects coming from certain food materials as those mentioned in ISO 6887-4:2017, 9.1.4.4 (e.g. onion powder, garlic, oregano, peppers, certain teas and coffees, vitamin premixes, highly salted products).

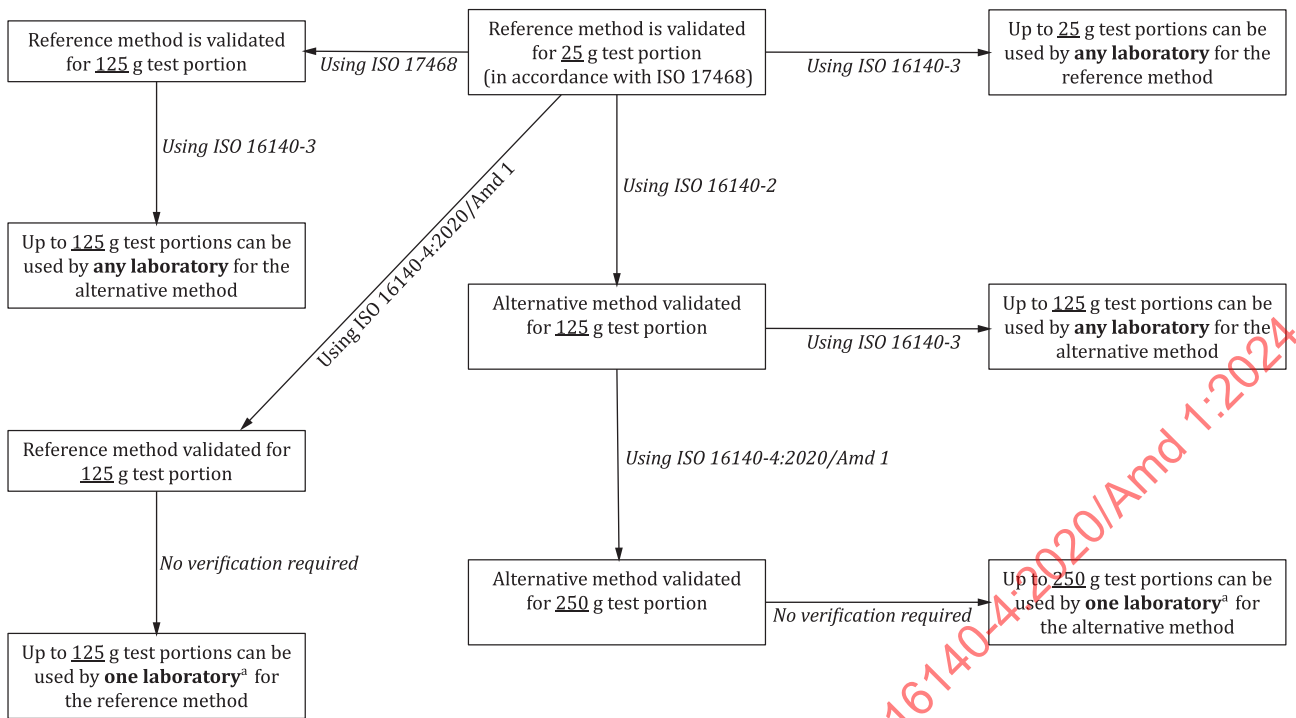
A reduction in, for example, the dilution ratio (sample/diluent) requires a validation study (details are given in Figure H.1).

Items can either be composited, pooled out of the laboratory or in the laboratory as a test portion or as a (pre-)enriched test portion but not as two or more combinations [i.e. pooling of (pre-)enriched as well as test portion is not allowed].

When a laboratory, for example, wants to pool test portions as well as (pre-)enriched test portions, two separate validation studies shall be conducted in accordance with this protocol.

Once the larger test portion size has been validated, all test portions smaller than the largest validated test portion can be used for routine testing for this particular (food) category at the same sample/diluent ratio. For example, a method that has been validated for 375 g test portions can be used for 25 g, 100 g, etc., up to 375 g test portions.

The protocol is intended to demonstrate that a larger test portion size provides a similar or lower level of detection (LOD_{50}) compared to the LOD_{50} of the (validated) test portion size as specified in the method. The relative level of detection (RLOD) approach specified in 6.1.1.3 shall be used. For calculation of the data, the larger test portion size corresponds to the alternative method and the original test portion size corresponds to the reference method.



^a The “one laboratory” is the laboratory that performed the validation in accordance with this document.

Figure H.1 — Steps to validate a larger test portion size and the implementation in a laboratory

H.2 Selection of item, number of samples and replicates tested

The (food) item selected for the validation of a larger test portion size shall be a relevant (food) item which is routinely analysed in the laboratory. When various (food) items belonging to the same (food) category are of interest for testing with a larger (but the same) test portion size, the most challenging (food) item shall be selected for the validation study. The rationale for the selection of the challenging (food) item shall be documented. Each (food) category shall be validated by using at least one (challenging) (food) item.

NOTE 1 Guidance for choosing a challenging (food) item is given in ISO 16140-3:2021, Annex B.

The samples shall be artificially inoculated. Procedures for the preparation of artificially inoculated samples are specified in ISO 16140-2:2016, Annex C.

A minimum of three levels per (food) item shall be prepared consisting of at least a negative control level, a low level, and a higher level. Ideally, the low level shall be the theoretical detection level (i.e. 0,7 cfu per test portion) and the higher level just above the theoretical detection level (e.g. 1 cfu to 1,5 cfu per test portion).

At least 1 replicate shall be prepared for the negative control level (L_0), 20 replicates for the low level (L_1) and 5 replicates for the higher level (L_2). An estimate for the level of contamination (except for the negative control) shall be made.

It is important to select a batch of the (food) item from which the test portions are prepared that does not contain the target microorganism, e.g. by using a batch that has been tested beforehand and shown negative for the target microorganism.

The replicates shall be analysed with both test portion sizes [specified (validated) test portion size in accordance with the reference method and the alternative larger test portion size].

Negative control samples shall not produce positive results. When a positive result is obtained, repeat the experiment for all levels.

The low level should have fractional recovery using the either the validated or the larger test portion size (fractional recovery at the low level should be between 25 % and 75 % of the number of samples tested).

NOTE 2 To give better assurance that fractional recovery will be obtained, additional levels of contamination can be prepared and tested.

H.3 Calculation and interpretation of the RLOD

The RLOD is defined as the ratio of the LODs of the larger test portion size (larger) and the original (org) test portion size, see Formula (H.1):

$$\text{RLOD} = \frac{\text{LOD}_{\text{larger}}}{\text{LOD}_{\text{org}}} \quad (\text{H.1})$$

where

$\text{LOD}_{\text{larger}}$ is the LOD obtained with the larger test portion size;

LOD_{org} is the LOD obtained with the specified (validated) test portion size in accordance with the method.

The RLOD shall be estimated by fitting a complementary-log-log (CLL) model to the combined detected/not detected data as a function of test portion size. The contamination levels are not required for the calculations of the RLOD since they are included in the model; the resultant curves are plotted in a graph of probability of detection versus log dose (contamination level). The statistical model and the calculations are given in ISO 16140-2:2016, Annex D.

Calculations can be performed using the Excel®-based program¹⁾ for this document. The Excel®-based program for calculating RLOD values is freely available for download at <https://standards.iso.org/iso/16140/-4/ed-1/en/amd/1>. Select the file “RLOD”. For calculations using this Excel®-based program, the option of “unknown concentration” shall be used.

NOTE In this Excel®-based program, data obtained with a reference and alternative method are used. To use it for this protocol, the term “reference method” is replaced by “the specified (validated) test portion size according to the method” and the term “alternative method” is replaced by “larger test portion size”.

An Acceptability Limit (AL) for the RLOD specifies the maximum increase in LOD of the larger test portion size versus the test portion size in accordance with the method to decide if the larger test portion size used is fit for purpose.

The AL for the larger test portion size (unpaired study design) is set at 2,5; meaning that the LOD for the larger test portion size shall not be higher than 2,5 times the LOD of the (validated) test portion size of the method. The AL is not met when the observed value is higher than 2,5. In this case, carry out investigations (e.g. root cause analysis) to provide an explanation for the observed results. Based on the AL and the additional information, decide whether the larger test portion size is fit for purpose for the (food) item or (food) category involved. Reasons for acceptance of the larger test portion size if the AL is not met shall be justified.

Two examples, using artificial data, to calculate RLOD for validation of larger test portion sizes are given below.

EXAMPLE 1 The ISO method for the detection of *Salmonella* (see ISO 6579-1) was evaluated for the use of 375 g test portions for cocoa powder (category: Chocolate, bakery products and confectionary). The enrichment for both test portion sizes was performed in accordance with ISO 6887-4 using a 1 in 10 dilution in non-fat dried milk supplemented with brilliant green. The results are presented in Table H.1.

1) Excel® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.