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AMENDMENT 1
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Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination —

Part 1: General rules for the preparation of the initial suspension and decimal dilutions

AMENDMENT 1: Requirements and guidance on the use of a larger test portion size for qualitative methods

Microbiologie de la chaîne alimentaire — Préparation des échantillons, de la suspension mère et des dilutions décimales en vue de l'examen microbiologique —

Partie 1: Règles générales pour la préparation de la suspension mère et des dilutions décimales

AMENDEMENT 1: Exigences et recommandations sur l'utilisation d'une taille de prise d'essai plus grande pour les méthodes qualitatives

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Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination —

Part 1:
General rules for the preparation of the initial suspension and decimal dilutions

AMENDMENT 1: Requirements and guidance on the use of a larger test portion size for qualitative methods

Clause 2

Add the following normative references:

ISO 16140-4:2020, *Microbiology of the food chain — Method validation — Part 4: Protocol for method validation in a single laboratory*

ISO 16140-4:2020/Amd 1:2024, *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*

3.2

Replace the text with the following:

3.2
composite sample
mixed sample of a number of the same *items* (3.13) of food, animal feed, animals or environment, prepared in or out of the laboratory from which a test portion is taken for examination

Note 1 to entry: See Figure A.1 for an illustration of a composite sample.

3.3

Replace the text with the following:

3.3
pooled sample
mixed sample of a number of the same *items* (3.13) of food, animal feed, animals or environment, prepared in or out of the laboratory where the complete mixture is the test portion and is taken as a whole for examination

Note 1 to entry: See Figure A.1 for an illustration of a pooled sample

Add the following terminological entries:

3.11
category
group of sample *types* (3.12) of the same origin

Note 1 to entry: Food categories are listed in ISO 16140-2:2016, Table A.1.

EXAMPLE Heat-processed milk and dairy products.

[SOURCE: ISO 16140-1:2016, 2.11, modified — Note 1 to entry added.]

3.12
type
for a given *category* (3.11), a group of *items* (3.13) processed in a similar way, with similar intrinsic characteristics and a similar microbial ecology

Note 1 to entry: Food types are listed in ISO 16140-2:2016, Table A.1.

EXAMPLE Food category: heat-processed milk and dairy products; food type: pasteurized dairy product.

[SOURCE: ISO 16140-1:2016, 2.78, modified — Note 1 to entry added.]

3.13
item
single specified food, feed, environmental or primary production matrix

Note 1 to entry: Examples for food items are listed in ISO 16140-2:2016, Table A.1.

EXAMPLE Food category: heat-processed milk and dairy products; food type: pasteurized dairy product; food item: milk-based desserts.

[SOURCE: ISO 16140-1:2016, 2.34, modified — Note 1 to entry added.]

3.14
larger test portion
measured (volume or mass) representative sample taken from the laboratory sample (3.1) or test sample (3.4) 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

9.3

Replace the text with the following:

9.3 Composite sample and larger test portion

9.3.1 Composite sample

A composite sample is where a number of the same items are mixed, and a test portion is taken for examination in the laboratory as illustrated in Figure A.1. The size of the test portion removed from the composite sample will remain the same as described in the original method and/or validation document.

Compositing shall be applicable to qualitative tests only.

A number of items may be composited at the sampling stage by the client (out of the laboratory) or by the laboratory (at client's request). Only items from the same origin or source (e.g. same batch or lot) shall be composited.

9.3.2 Larger test portion

9.3.2.1 General

A larger test portion is a sample that is larger than the test portion size that has been described in the original method and/or validation document.

Testing a larger test portion can be necessary:

a) to reflect the microbiological quality of a large batch of product;

b) in cases where a large number of environmental samples is taken;

c) as sometimes required by national or regional legislation.

A larger test portion can originate from a single larger test portion (see 9.3.2.2) or from the pooling of samples (see 9.3.2.3).

If a laboratory sample larger than the maximum sample size has been submitted, the sample can be split into multiple test portions based on the maximum sample size (e.g. if a laboratory sample of 750 g has been received for *Salmonella* testing it can be prepared as two 375 g test portions).

9.3.2.2 Larger single test portion

A larger single test portion is a sample that is larger than the test portion size that has been described in the original method and/or validation document originating from a single sample. A larger single test portion can be applied to both qualitative and quantitative testing.

9.3.2.3 Pooled sample

A pooled sample is a sample where a number of items have been combined and the complete mixture is taken as a whole for examination in the laboratory. Items can be pooled:

a) out of the laboratory, where individual samples are combined into one larger pooled sample;

b) in the laboratory where individual test portions are combined into one larger (pre-)enrichment as illustrated in Figure A.1;

c) in the laboratory, where individual (pre-)enriched test portions items are combined into one and carried through as a single test as illustrated in Figure A.1.

A number of items may be pooled at the sampling stage by the client or laboratory (at the client's request). Pooling shall be applicable to qualitative tests only. Only items from the same origin or source (e.g. same batch or lot) shall be pooled.

9.3.3. Procedure for larger test portion size

Items can be composited or pooled out of 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].

To minimize the risk of false-negative results when testing a larger single test portion or pooled test portion, proceed as follows:

— For qualitative analysis, the primary pre-enrichment broth shall be pre-warmed to the intended incubation temperature with the same tolerance range.

— The temperature profile of the larger enrichment volume shall be checked to ensure that the time taken to reach the target incubation temperature and overall incubation time are as specified in the individual standard. For example, in ISO 6579-1:2017, 9.2, the pre-enrichment incubation time is between 16 h and 20 h. The temperature profile for the larger test portion size shall be between 34 °C and 38 °C with a minimum incubation time of 16 h. The time needed to reach the target incubation temperature should be minimized. Demonstrating compliance does not have to be for each individual sample but depends on

the temperature difference between the sample and the broth. Temperature profile shall be available for frozen, refrigerated and room-temperature samples.

— When testing a larger single test portion, pooled test portion and 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).

Additional pooling instructions may be described in individual standards (e.g. maximum sample size and food category).

9.3.4 Validation and verification of larger test portion size

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 ISO 16140-4:2020, Figure 1. Once validated in such a study, any laboratory can implement the larger test portion size after verification in accordance with 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 specified in ISO 16140-4:2020/Amd 1:2024, Annex H, is necessary for each laboratory wishing to use a larger test portion size.

Qualitative reference methods which were validated using 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.

Once the larger test portion size has been validated, all test portions smaller than the largest validated test portion size 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.

Laboratories applying sample or test portion pooling that exceeds the maximum sample size stated in the method shall carry out a validation using the protocol as specified in ISO 16140-4:2020/Amd 1:2024, Annex H .

The relative level of detection (RLOD) approach specified in ISO 16140-4:2020, 6.1.1.3, shall be used to demonstrate that a larger test portion size provides 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 corresponding protocol is specified in ISO 16140-4:2020/Amd 1:2024, Annex H.

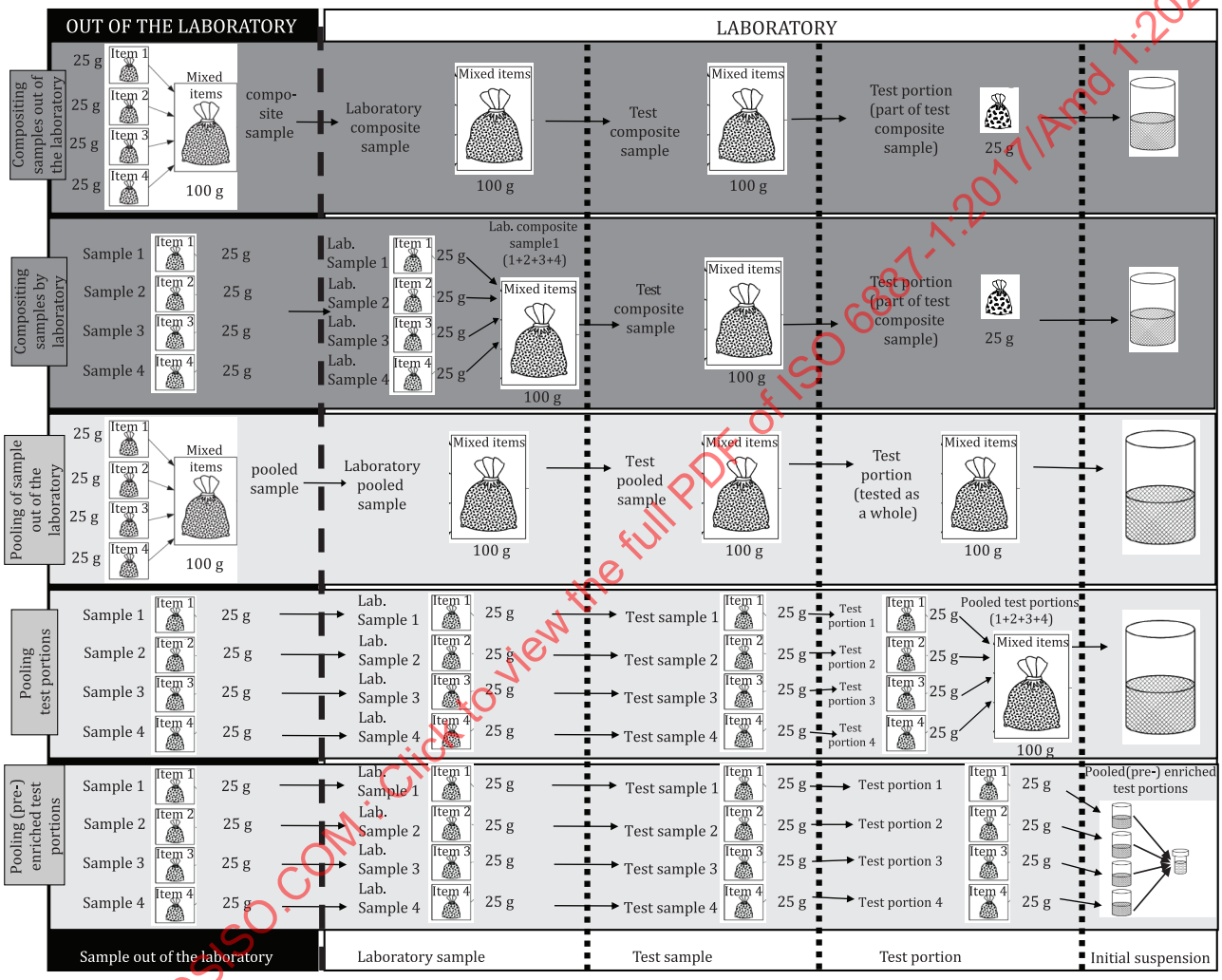
Annexes A, C and D

Replace each annex with the following text:

Annex A
(informative)

Compositing and pooling procedures

Figure A.1 illustrates the compositing and pooling procedures, which can be performed out or in the laboratory.



NOTE 1 Numbers of items, mass of 25 g and 100 g, are used as examples. This mass may be changed.

NOTE 2 Reported results for both rows of compositing are “Detected or not detected” in 25 g. Reported results for the three last rows of pooling are “Detected or not detected” in 100 g.

Figure A.1 — Illustration of compositing and pooling procedures

Annex C
(informative)

Data showing reliability of test results according to size of test portions

C.1 General

Data are available to show that the larger the test portion size used, the less variance occurs between replicate test results of the same samples.[6] The data presented in this annex are based on an experimental protocol developed and used in The Netherlands and France.

C.2 Dutch study comparing different sample sizes and effects of sample homogenization

Laboratory samples of 600 g from three sample types (pre-cut vegetables, Chinese rice dish and milk shake or soft ice cream) were used to compare differences in the homogeneity of the individual sample types, together with four different sample preparation procedures. The test portions for two samples were taken without prior homogenization, while 100 g from two of the laboratory samples were homogenized before taking the test portions (see Table C.1).

Table C.1 — Study design to show effects of sample preparation method and test portion size

Sample preparation method	Homogenization of laboratory sample	Test portion size	Test portion dilution
T1	No	10 g	1 in 10
T2	Yes (100 g)	10 g	1 in 10
T3	Yes (100 g, 1 to 1 with diluent)	20 g	1 in 5
T4	No	35 g	1 in 10

For the laboratory samples prepared without homogenization, two test portion sizes were used: the minimum of 10 g specified for enumeration tests in many specific standards (T1) and a larger test portion of 35 g (T4).

For the two laboratory samples prepared with homogenization, test samples of 100 g were taken and homogenized: one (T2) was homogenized directly, while the other (T3) was diluted 1 to 1 with diluent before homogenization.

All sample preparations were tested for aerobic colony count, using a final dilution factor of 1 in 10.

The results of the study were tested for variance using the F-test and these are shown in Table C.2.

Table C.2 — Effect of four sample preparation techniques on the variance of test results from three sample types

Matrix (no. of samples)	Results	Sample preparation T1 (10 g)	Sample preparation T2 (100 g)	Sample preparation T3 (100 g)	Sample preparation T4 (35 g)
Pre-cut vegetables (18)	Mean ($\log_{10}$ cfu / g)	0,150	0,164	0,111	0,172
	Standard deviation	0,128	0,179	0,071	0,183
	Variance	0,016	0,032	0,005	0,003
Chinese rice dish (22)	Mean ($\log_{10}$ cfu / g)	0,285	0,218	0,104	0,216
	Standard deviation	0,261	0,239	0,072	0,237
	Variance	0,068	0,057	0,005	0,056
Milk shake or soft ice cream (8)	Mean ($\log_{10}$ cfu / g)	0,094	0,064	0,069	0,115
	Standard deviation	0,142	0,035	0,042	0,092
	Variance	0,020	0,001	0,002	0,008

These data are in agreement with other published works[6] showing that the least variances for all three different food samples of differing homogeneity was mainly obtained when the largest samples (100 g) were homogenized, with prior 1- to 1-dilution.

C.3 French study comparing seven different sample preparation techniques

Samples of three different sample types (pâté, cheese and mixed salad) were prepared using seven different techniques (T1 to T7) involving variations of sample size and homogenization as shown in Table C.3.

Table C.3 — Study design to show effects of sample preparation method and test portion size

Sample preparation method	Homogenization of laboratory sample	Test portion size	Test portion dilution
T1	No	10 g from 1 area	1 in 10
T2	No	10 g taken from 5 areas	1 in 10
T3	Yes (whole sample)	10 g	1 in 10
T4	Yes (whole sample, 1 to 1 with diluent)	20 g	1 in 5
T5	No	35 g from 1 area	1 in 10
T6	No	35 g taken from 5 areas	1 in 10
T7	Yes (100 g, 1 to 1 with diluent)	20 g	1 in 5

The study was similar to that described in Clause C.2, with a further variation of taking the test portions from a single area of the sample (T1 and T5) or from five different areas (T2 and T6) across the sample. Technique T7 was similar to technique T4 except that a sample of 100 g was used rather than the whole sample.

All sample preparations were tested for aerobic colony count, using a final dilution factor of 1 in 10.

The results of the study were tested for variance and these are shown in Table C.4.

Table C.4 — Effect of seven sample preparation techniques on the variance of test results from three sample types

Technique (test portion size)		**T1** (10 g)	**T2** (10 g)	**T3** (10 g)	**T4** (20 g)	**T5** (35 g)	**T6** (35 g)	**T7** (20 g)
Sample size		Whole	Whole	Whole	Whole or ≈100 g	Whole	Whole	100 g
Homogenized (Yes/No)		No	No	Yes	Yes (1 to 1 with diluent)	No	No	Yes (1 to 1 with diluent)
No. of samples		129	124	130	135	6	10	16
Food sample		Pâté, cheese	Pâté, cheese	Pâté, cheese	Pâté, cheese	Pâté	Pâté, mixed salad	Pâté, mixed salad
RESULTS (550 samples in duplicate)	sd	0,81	0,59	0,36	**0,17**	0,33	0,30	**0,15**
	Variance	0,66	0,35	0,13	**0,03**	0,11	0,09	**0,02**

These data also illustrate that the least variance for all three different food samples of differing homogeneity was obtained when the largest samples (100 g) were homogenized, with prior 1 to 1 dilution.