
**Microbiology of the food chain —
Requirements and guidelines for
conducting challenge tests of food and
feed products —**

Part 2:

**Challenge tests to study inactivation
potential and kinetic parameters**

*Microbiologie de la chaîne alimentaire — Exigences et lignes
directrices pour la réalisation des tests d'épreuve microbiologiques —*

*Partie 2: Tests d'inactivation pour étudier le potentiel d'inactivation
et les paramètres de la cinétique d'inactivation*



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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).

A list of all parts in the ISO 20976 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Under the general principles of the Codex Alimentarius on food hygiene, it is the responsibility of the food business operators (FBOs) to control microbiological hazards in foods and to manage microbial risks. Therefore, FBOs implement validated control measures, within the hazard analysis and critical control point (HACCP) system, and conducts studies in order to investigate compliance with the food safety criteria throughout the food chain.

In the framework of microbial risk assessment (MRA), several complementary approaches are developed to estimate risks posed by pathogens or spoilage microorganisms in the food chain. MRA is adopted by regulators under the auspices of the international agency for setting food standards. Challenge testing is one of the recognized approaches used to validate control measures within the HACCP system, as well as to assess microbiological safety and quality of food, food production processes, food storage conditions, and food preparation recommendations dedicated to consumers.

Therefore, this document provides technical rules, calculations and approaches to investigate the ability of an inoculated microorganism of concern to grow, survive or be inactivated in the raw materials, intermediate or end products under reasonably foreseeable food processes, storage and use conditions. The objective and the scope of the study are to determine the experimental design and the selection of the study conditions, and to assess the extent of microbial inactivation. Regulatory authorities can have different recommendations, and these differences have been included as much as possible. It is, however, possible that specific requirements need to be incorporated to get a regulatory approval of the challenge test.

As the growth and inactivation studies are clearly different, the ISO 20976 series consists of two parts, under the general title *Microbiology of the food chain — Requirements and guidelines for conducting challenge tests of food and feed products*:

- *Part 1: Challenge tests to study the growth potential, lag time and the maximum growth rate;*
- *Part 2: Challenge tests to study inactivation potential and kinetic parameters.*

The use of the ISO 20976 series involves expertise in relevant areas such as food microbiology, food science, food processing and statistics. The statistical expertise encompasses an understanding of sampling theory and design of experiments, statistical analysis of microbiological data, and overview of scientifically recognized and available mathematical concepts used in predictive modelling.

For practical reasons, the term “food” includes feed.

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Microbiology of the food chain — Requirements and guidelines for conducting challenge tests of food and feed products —

Part 2:

Challenge tests to study inactivation potential and kinetic parameters

1 Scope

This document specifies the protocols for conducting microbiological challenge tests for inactivation studies on vegetative bacteria and bacterial spores in the raw materials and ingredients, intermediate or end products.

The use of this document can be extended to yeasts which do not form mycelium.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at <https://www.iso.org/obp>

— IEC Electropedia: available at <https://www.electropedia.org/>

3.1

bacterial spore

resistant form of bacteria which is dormant until the *germination* (3.9) step

[SOURCE: ISO 20976-1:2019, 3.1]

3.2

batch

group or set of identifiable food obtained through a given process under practically identical circumstances and produced in a given place within one defined production period

Note 1 to entry: The batch is determined by parameters established beforehand by the organization and may be described by other terms, e.g. lot.

[SOURCE: Commission Regulation (EC) No 2073/2005, Article 2 (e)^[10], modified — “food obtained through” has replaced “products obtained from” and Note 1 to entry has been added.]

3.3

bulk products

products that are not separated into individual items or units

[SOURCE: ISO/TS 17728:2015, 3.3.1]

3.4

challenge test

study of the growth or inactivation of microorganism(s) artificially inoculated in a food

[SOURCE: ISO 20976-1:2019, 3.5]

3.5

control unit

unit of food identical to the *test unit* (3.34) but not artificially inoculated (used as a blank)

[SOURCE: ISO 20976-1:2019, 3.4, modified — “inoculated” has replaced “contaminated”.]

3.6

D value

decimal reduction

time or dose required to achieve reduction of 90 % of the tested microorganism under stated conditions (e.g. temperature, pH or chemical composition) in case of log linear *inactivation kinetics* (3.10)

3.7

δ value

first decimal reduction

time or dose required to achieve the first reduction of 90 % of the tested microorganism under stated conditions (e.g.: temperature, pH or chemical composition) in case of non-log linear *inactivation kinetics* (3.10)

3.8

experimental datapoint

result of analysis of a *test unit* (3.34) per unit mass, per unit volume, or per unit area

Note 1 to entry: The enumeration results may be expressed in log₁₀ or most probable number (MPN).

[SOURCE: ISO 20976-1:2019, 3.6, modified — In the definition, “mass” has replaced “weight” and the units have been deleted. In Note 1 to entry, “for specific cases” has been deleted and “or most probable number (MPN)” has replaced “MPN”.]

3.9

germination

mechanism in which a *bacterial spore* (3.1) initiates its transformation into a *vegetative cell* (3.36)

[SOURCE: ISO 20976-1:2019, 3.9, modified — “initiates its transformation into” has replaced “starts becoming”.]

3.10

inactivation kinetics

change over time in the concentration of the target microorganism subjected to an inactivation process

3.11**inactivation parameter**

mathematical estimate that describes the resistance/sensitivity of the target organism to the *treatment* (3.35), obtained by fitting *primary models* (3.18) and *secondary models* (3.24)

Note 1 to entry: Examples of these parameters are D , δ and p for the primary models and z for the secondary models.

3.12**inactivation potential** **Δ value****log kill****log reduction**

difference in the log concentration (\log_{10} cfu/g or ml or cm^2) of the target microorganism between an earlier and a later time point expressed as \log_{10}

Note 1 to entry: In this document, the term “inactivation potential” refers to the type of inactivation study, and the terms “log kill” and “log reduction” refer to the result obtained.

3.13**inactivation treatment**

process used to kill or inactivate the target microorganism

3.14**inoculum**

microbial suspension at the desired concentration used to contaminate *test units* (3.34)

[SOURCE: ISO 20976-1:2019, 3.12]

3.15**k value**

slope of the inactivation curve

3.16**p value**

parameter describing the shape of the inactivation curve

3.17**pH value**

measure of the concentration of acidity or alkalinity of a material in an aqueous solution

[SOURCE: ISO 5127:2017, 3.12.2.29, modified — The notes to entry have been deleted.]

3.18**primary model**

mathematical model describing the changes of microbial counts as a function of time

[SOURCE: ISO 20976-1:2019, 3.16]

3.19**organizing laboratory**

laboratory with responsibility for managing the *challenge tests* (3.4)

[SOURCE: ISO 20976-1:2019, 3.17]

3.20**pilot facility**

manufacturing location used to run an experiment or test before introducing more widely

3.21**processing facility**

location where products are made on a larger scale

3.22

sampling

selection of one or more units or portions of food such that the units or portions selected are representative of that food

[SOURCE: ISO 20976-1:2019, 3.18]

3.23

sampling point

time at which the *test units* (3.34) are taken for analyses

Note 1 to entry: When assessing *inactivation kinetics* (3.10), these are represented as *experimental datapoints* (3.8) on the inactivation graph.

[SOURCE: ISO 20976-1:2019, 3.19, modified — “taken for analyses” has replaced “analysed and which are represented as experimental datapoints on the kinetics graph” and Note 1 to entry has been added.]

3.24

secondary model

mathematical model describing the effects of the inactivation process factors (e.g. temperature, pH, a_w) on the parameters of the *primary model* (3.18) (e.g. D , δ)

[SOURCE: ISO 20976-1:2019, 3.20, modified — “inactivation process” has replaced “environmental” and “(e.g. D , δ)” has replaced “(e.g. growth rate)”.]

3.25

sporulation

mechanism by which *vegetative cell* (3.36) forms spore

[SOURCE: ISO 20976-1:2019, 3.21]

3.26

surrogate

non-pathogenic microorganism that has similar or more robust *survival* (3.27) capability compared to the pathogen of concern both in the matrix and under the processing conditions being studied

3.27

survival

state of continuing to live or exist without significant increase or decrease in viability

3.28

target reduction level

target inactivation level expressed in \log_{10}

3.29

t_0

time at which the treatment starts

3.30

t_{end}

time at which the treatment is finished

3.31

t_{inoc}

time at which the microorganism is inoculated in the food

3.32

t_{xD}

time of treatment needed for x log reduction of the target microorganism

3.33**test portion**

measured (volume or mass) representative sample taken from the *test unit* (3.34) for use in the analysis

[SOURCE: ISO 6887-1:2017, 3.5, modified — “test unit for use in the analysis” has replaced “laboratory sample for use in the preparation of the initial suspension” and Note 1 to entry has been deleted.]

3.34**test unit**

measured (volume or mass) amount of the food used for inoculation, subsequent *treatment* (3.35) and analysis

[SOURCE: ISO 20976-1:2019, 3.24, modified — “subsequent treatment and analysis” has been added.]

3.35**treatment**

any process, formulation or product characteristics, or a combination thereof, intended to inactivate the target microorganism

3.36**vegetative cell**

state of microbial form that is capable of growing under favourable environmental conditions

[SOURCE: ISO 20976-1:2019, 3.25]

3.37**water activity**

a_w

ratio of the water-vapour pressure in the foodstuff to the vapour pressure of pure water at the same temperature

[SOURCE: ISO 18787:2017, 3.1 modified — “water-vapour pressure in the foodstuff to the vapour pressure of pure water at” has replaced “partial water-vapour pressure in equilibrium with the product analysed to the water-vapour saturation pressure in equilibrium with”, and the formula and the notes to entry has been deleted]

3.38**z value**

change in *treatment* (3.35) (e.g. temperature, pH, a_w) that induces a 10-fold change in the *D value* (3.6)

Note 1 to entry: Temperature, pH and a_w can be indexed to the z value to denote the treatment being assessed.

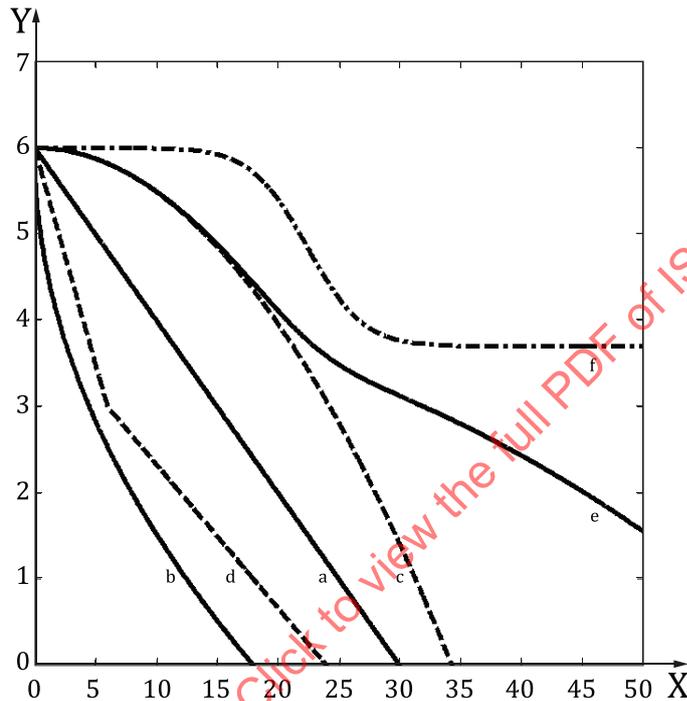
4 Principle

Inactivation studies are designed to determine the changes in the concentration of the target microorganism during the challenge test. These studies can be used to assess whether there is significant microbial inactivation in a foodstuff and to quantify the decrease in the target microorganism under a given set of processing conditions and/or formulation of the food product. The scope and the aim of the study shall be clearly specified (e.g. assessment/validation of the food process efficacy as a control measure, assessment of microbial stability and survival) including the target reduction level and the decision criteria. The experimental design shall be in accordance with that purpose and shall take into account the steps of the process and the food chain for which microbial inactivation is assessed. Knowledge from the FBO (e.g. on their products characteristics and production process) shall be combined with the expertise in food microbiology and analytical sciences to ensure the robustness of the study (see 14.3.2). Ideally, inactivation studies employ the target microorganism. In the challenge tests studies conducted within food production facilities, a validated surrogate shall be used in place of the target pathogen^[26].

The organizing laboratory shall have knowledge and skills in food microbiology, food science and technology, and statistics to design and conduct the studies, interpret the results and draw the conclusions. The analyses shall be conducted under a quality assurance system (e.g. ISO/IEC 17025).

To conduct an inactivation challenge study, the inoculum should be prepared such that the microbial cells or spores have been adapted to the environmental conditions that mimic the food processing environment, thereby encouraging natural microbial response once inoculated into the food.

The same microbial strain could exhibit various shapes as a function of the treatment (see [Figure 1](#))^[14]. The heterogeneous shapes of microbial inactivation curves are the results of the microbial resistance or adaptive response or cell heterogeneity^[16].



Key

- X inactivation treatment (time or dose)
- Y $\log_{10}(N)$
- a Log-linear.
- b Concave.
- c Convex or with a shoulder.
- d, e With a tail or biphasic.
- f Sigmoidal curve.

Figure 1 — Examples of microbial inactivation curve types

There are two types of inactivation study: the inactivation potential and the inactivation kinetics of a target microorganism.

The inactivation potential studies are most appropriate for process validation and/or product formulation. Inactivation potential results are specific to the conditions and matrix under study. To extrapolate to other conditions, inactivation kinetic parameters shall be used, or a new inactivation potential study conducted.

The inactivation kinetics studies are used to characterize the inactivation of the microorganism through the determination of inactivation parameters such as D, δ and z values. Those studies are more complex in terms of study design, execution, results interpretation and exploitation, particularly in the

case of nonlinearity (see [Figure 1](#)) but allow to extrapolate the results to non-tested conditions within the range of the study.

5 Apparatus

Routine microbiology labware specified in ISO 7218 shall be used. Specific labware, including the following, can also be needed to prepare the test portions, to store them under suitable conditions, or monitor how their characteristics change during the challenge test study.

5.1 Apparatus for packaging the samples under air, under vacuum or under a protective modified atmosphere.

5.2 Climate-control chamber, able to reach and hold setpoint temperature to $\pm 1^\circ\text{C}$ and to adjust relative humidity to $\pm 10\%$.

5.3 pH meter, able to perform measurements to a tolerance of $\pm 0,1$ pH units. pH meters shall give readings to a resolution of 0,01 pH units.

5.4 a_w meter, meeting the requirements of ISO 18787.

5.5 Headspace gas analyser, to measure gas composition (e.g. O_2 , CO_2).

5.6 Logger for measuring temperature conditions of the test unit.

5.7 Logger for measuring relative humidity conditions of the test unit.

5.8 Device for measuring the inactivation treatment (e.g. dosimeter for irradiation treatment, probe for heat treatment) **of the test unit.**

5.9 Apparatus (pilot facility or equipment in a laboratory) to run the inactivation treatment, which mimic foreseeable conditions of production leading to microbial inactivation.

5.10 Industrial piece of equipment to conduct the inactivation study at the factory level.

6 Culture media and reagents

Follow current laboratory practices as specified in ISO 7218.

For the preparation and performance testing of culture media and reagents, follow the procedures as specified in ISO 11133 and in the International Standard specific to the target microorganism. Use relevant internationally recognized and widely accepted methods for the detection or enumeration of injured target microorganisms (e.g. using a selective agar and non-selective agar with a dual layer method).

7 Study design and sampling

7.1 General

The study design and sampling will depend on the target level of reduction, the process, the formulation and the product characteristics. The factors impacting the inactivation shall be clearly identified and their range shall be specified.

There are two types of challenge tests to study inactivation: inactivation potential and inactivation kinetics. The aim of the study and the technologies used will determine the type (see [Annex A](#)).

For inactivation potential, test units shall be taken at a minimum at the beginning and the end of treatment.

For inactivation kinetics, test units shall be taken at each sampling point throughout the treatment duration. When sampling access during processing is not possible due to the equipment and/or technology assessed, independent treatments of varying lengths can be required.

The associated expected variability will determine the number of batches and test units to be considered (see [Annex B](#)).

The study design shall consider various sources of variability linked to:

- process characteristics (e.g. flow rate, residence time, temperature, moisture, equipment design);
- formulation and product characteristics (e.g. fat, sugar, salt, a_w , protein, acid, pH, particle size, chemical composition, background microorganisms, preservatives concentrations, gas atmosphere);
- where relevant (e.g. studies on preservatives), transportation and storage conditions (e.g. humidity, packaging) during the shelf-life.

The study design shall also consider the target reduction level.

7.2 Setting target reduction level for the inactivation study

Depending on the aim of the challenge test, the target reduction level shall be specified at the start of the study. It may be related to regulatory requirements or to internal requirements. Laboratories conducting inactivation studies in products that are subject to regulations should be aware of the most current requirements. The target reduction level influences the inoculum level to be used. It can vary depending on the food type (e.g. for *Salmonella* inactivation, a 4 log₁₀ reduction has been required for the treatment of almonds^[12] and a 7 log₁₀ reduction for poultry products^[13]). To be able to analyse the target reduction level, the limit of quantification of the analytical method used and the inoculum level shall be appropriate.

7.3 Number of batches

The number of batches to be included in the study depends on the variability of the food production process and food characteristics (see [Annex B](#)). The characteristics of the studied batches shall be representative of the variability of the production process based on historical data.

A minimum number of three batches should be used for both inactivation potential and inactivation kinetics studies. Generally, the number of batches should be increased in situations of higher variability or uncertainty.

The use of a single batch shall be clearly justified, for example:

- a) evaluating the impact of a new formulation of the food;
- b) using a batch representing the most unfavourable inactivation conditions (worst case);
- c) when multiple levels of the treatment will be assessed in inactivation kinetics studies.

7.4 Preparation of the test units

Test units representative of the matrix (raw materials and ingredients, intermediate or end products) before the inactivation treatment can be either:

- aseptically-sampled portions of the bulk products from the production line;

- aseptically-sampled portions or complete content of the packaging unit(s).

The test units shall be maintained at appropriate conditions before inoculation, preferably inoculated as close as possible to the time of production unless otherwise specified by the aim of the study. Once inoculated, all test units, including t_0 (see [Figure 2](#)), shall be maintained under the same appropriate conditions, including transport and storage where necessary.

7.5 Number of control units and test units

The minimum number of units to be analysed will depend on the type of the inactivation study, the nature of the food and the processing conditions (see [Annex B](#)).

For inactivation potential studies, the number of sample points depends on the accessibility of the samples. In some cases, it is only possible to get samples at the start and the end of the process. In other cases, it is possible to get intermediate samples.

For inactivation kinetics studies, it is important to have enough experimental datapoints available to get an accurate estimation of the curve type (see [Figure 1](#)). For linear curve types, a minimum of six sampling points is required. For nonlinear curve types, usually more sampling points are needed.

The number of units should be increased in situations of high variability or uncertainty related to food and process characteristics. It is recommended to prepare additional units to cover unforeseen incidents.

8 Selection of strains

Each strain used shall be characterized biochemically and/or serologically and/or genetically in sufficient detail for its identity to be known. Strains previously isolated from the food matrix (raw materials, ingredients, end products) or from the production environment or from clinical/food/environmental samples in outbreaks involving the food are preferred.

The original source of all isolates should be known and they should be held in a local (e.g. laboratory which runs the challenge test), national or international culture collection to enable them to be used in future testing, if required. Use strains for which the inactivation parameter(s) (e.g. D, k or δ values) have been previously determined in a similar matrix (e.g. heat, pressure, acid resistance) so as to ensure that the selected strain(s) is/are fit for purpose. If the matrix is not similar, a preliminary study needs to be performed to measure the resistance^[15].

Cocktails of three to five fit for purpose strains are appropriate for inactivation potential studies. For the inactivation kinetics, strains shall only be tested individually.

Ideally, inactivation studies would employ the target microorganism. In case of pathogens, this is possible when the study is performed in a laboratory or in a pilot plant. However, this is not possible in factories or food processing facilities (see [Annex A](#)). In such cases, a surrogate shall be used. The suitability of the selected surrogate shall be justified and documented in relation to the pathogen, food matrix and treatment under assessment. Otherwise, a specific surrogate validation study shall be conducted and documented^[26].

9 Preparation of the inoculum

9.1 General

In order to take into account the dilution effect when inoculating the test units, the inoculum concentration shall be greater than the targeted log reduction. In some cases, preliminary studies need to be taken to achieve the target inoculum level such as concentrating by centrifugation or by growing on solid medium to minimize volume.

9.2 Preparation of the vegetative cells

Prepare the strains to obtain a standardised inoculum with physiological conditions and concentration which are fit for purpose.

The culture should be initially grown in non-selective media and under conditions suitable for optimal growth. This culture can be used under optimum conditions or be adapted in order to mimic conditions of potential contamination. Examples of adaptation are pH, acid type, temperature, a_w , salt content, sugar content, liquid or solid media and atmosphere.

The microbial concentration of the inoculum shall be estimated using internationally recognized and widely accepted methods or alternative methods validated according to internationally accepted protocols. This inoculum can be concentrated by centrifugation to meet the target concentration. When using a cocktail, strains should be individually prepared and mixed in equivalent concentrations.

Additionally, microbial count of the prepared inoculum shall be confirmed at t_{inoc} (see [Figure 2](#)) by enumeration on the same medium that will be used for the challenge study.

Vegetative cells are used for direct inoculation (wet inoculum) or to be prepared for further uses in dry applications.

Dry inoculum preparation techniques can be required for studies of low moisture foods. Inoculum can be prepared without a carrier (e.g. freeze drying, vacuum) or dried on a carrier (e.g. beads, sand, chalk, talcum powder, flour, powdered form of the product, or a product similar to the challenge food). For preparation of dehydrated inoculum, the microorganism can require several days to stabilize at a given concentration. Viable counts of the stabilized dried inoculum shall be obtained prior to use.

9.3 Preparation of the spores

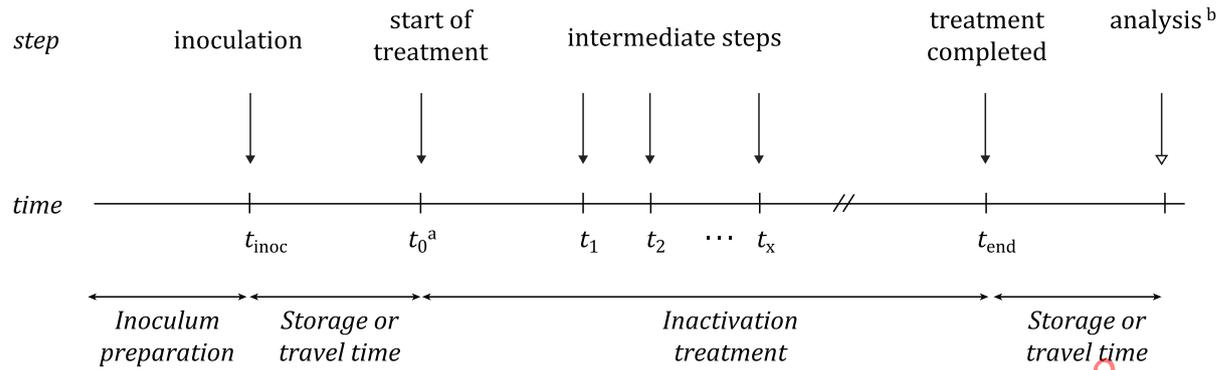
To prepare the spore crop, inoculate the target strain(s) into an appropriate culture medium and incubate under conditions that will promote a high sporulation rate (e.g. aerobic/anaerobic, medium, temperature). The length of this step will vary (from days to weeks) depending on the microbial species or strain(s) and the sporulation conditions. The extent of sporulation shall be checked with a microscope prior to harvesting the spores. Wash the spore crops to remove nutrients. Spores crops should be enumerated using internationally recognized and widely accepted methods or alternative methods validated according to internationally accepted protocols. Spore crops can then be stored at appropriate conditions to avoid germination until needed for the challenge study.

Prior to conducting the challenge study, enumerate the spore crop on the same medium that will be used for the challenge study after an appropriate heat treatment to inactivate the vegetative cells [\[19\]](#) [\[22\]](#). This concentration shall be adjusted to meet target criteria.

10 Inoculation of the test units

The inoculum procedure shall be described and justified. Its aim is to achieve the same target level of studied microorganism in all test units without significantly modifying their physico-chemical characteristics (pH, moisture and/or a_w included). The inoculum volume:sample mass (or sample volume) ratio should not exceed 1:100. The inoculation level should be at least one log higher than the targeted reduction level at the time of inoculation (t_{inoc}). The time between inoculation (t_{inoc}) and (t_0) immediately before the start of treatment should be minimized (see [Figure 2](#)).

NOTE For specific applications (e.g. low moisture food, acid food), an inoculated unit can need an equilibration period between t_{inoc} and t_0 .



- ^a If the treatment has an immediate inactivation effect to the inoculation, t_0 shall be sampled immediately before treatment starts. Otherwise sampling at t_0 is conducted when the treatment starts.
- ^b The time between sampling and the analysis should be minimized and documented.

Figure 2 — Possible steps for inactivation studies

If the goal is to investigate microbial behaviour in the whole product, the inoculum needs to be homogeneously distributed in the units.

If the goal is to characterize microbial behaviour in a specific part (e.g. core or surface) of the product, the inoculation should be done on this specific part.

Typically, when studying formulation and/or packaging, individual units are inoculated versus when studying process, bulk products inoculation can be used.

Depending on the product and process under study, use an appropriate inoculation procedure (see [Annex C](#)). For instance:

- in the core (e.g. injecting the centre of a whole ham);
- on the surface (e.g. by applying several deposits locally over the surface, by spraying of a sliced smoked salmon, by dipping of vegetables, by coating dry product);
- throughout the product (e.g. doughs, liquids, powders);
- in one or more components of a composite product (e.g. sauce in the sandwich).

Modified atmosphere packaged food (including vacuum) can be opened, inoculated and repacked according to 7.4 before applying the treatment. The units can also be inoculated directly by injecting the inoculum through adhesive septum/septa so as not to affect the packaging atmosphere. In this case, the laboratory shall ensure that the packaging remains hermetically sealed throughout the duration of the study.

The standard deviation of the target microorganism concentration (due to inoculation heterogeneity) at t_{inoc} , shall not be greater than the limit of $0,3 \log_{10}$ cfu/g, ml or cm^2 for individual test units and $0,5 \log_{10}$ cfu/g, ml or cm^2 for bulk test units. When analysing individually inoculated test units the results are more precise because the entire test unit is analysed. Thus, it is recommended to verify the concentration of inoculation at t_{inoc} to decide if the study is valid. If the standard deviation of the target microorganism concentration at t_{inoc} is higher than the proposed limits, the study shall be repeated.

If t_{inoc} is the same as t_0 , all the above shall apply to t_0 .

11 Controls

11.1 Uninoculated controls

The aim of the uninoculated controls is to verify the representativeness of the test units in comparison to the studied food. The food needs to be checked for relevant characteristics for the interpretation of the results (e.g. fat, sugar, salt, a_w , protein, acid, pH, particle size, chemical composition, background microorganisms preservatives concentrations, gas atmosphere).

The number of uninoculated controls to prepare for the inactivation challenge-test study is given in [Annex B](#).

11.2 Inoculated controls

The aim of the inoculated controls is to verify that the inoculation level is achieved at the time of the inoculation (t_{inoc}) and to act as a reference to prove that the treatment is causing the obtained inactivation. [Annex B](#) provides the minimum number of inoculated controls to be prepared.

The same test units may be used for measurement of physico-chemical characteristics, gas atmosphere and determination of the concentration of the background microorganisms or any relevant component likely to affect the inactivation of the target microorganism.

It is recommended that additional inoculated controls are prepared to cover unforeseen incidents, and in some cases, to measure possible changes in concentration when the time between inoculation and sample analysis is delayed due to transportation.

12 Treatment of the test units

All test units shall be treated under the selected conditions in order to mimic or reproduce the treatment under assessment.

Among product (e.g. pH/acidity, a_w , salt, moisture, core temperature) and process (e.g. ambient temperature, humidity, pressure, UV) characteristics, factors impacting the inactivation shall be identified, monitored and when possible controlled.

13 Analysis

Follow current laboratory practices as specified in ISO 7218.

Follow the ISO 6887 series for the preparation of the initial suspension and the decimal dilutions.

For the preparation and performance testing of culture media and reagents, follow the procedures as specified in ISO 11133 and in the International Standard specific to the microorganism target studied.

Control units and test units should be handled in the same way and as described below.

Microbiological enumeration and physico-chemical characteristics measurements should be performed at different sampling points according to the study's design.

In [Figure 2](#), the time between sampling and analysis should be minimized and documented to limit any changes in the test unit conditions. Only two sample points (t_0 and t_{end}) are considered in calculation for the inactivation potential, but, when possible, it is strongly advised that intermediate sampling points are included to follow the inactivation trend during the treatment.

If the treatment time is short (e.g. roasting, thermal treatment, high pressure processing), the samples should be analysed at the same time. When the treatment takes a long time, intermediate samples should be taken throughout and analysed as quickly as possible.

The impact of transport conditions on the cell viability, recovery or metabolic activity shall be minimized.

Whenever possible, the entire test unit should be used for analysis.

The physico-chemical analyses shall be performed using internationally recognized and widely accepted methods or alternative methods validated according to internationally accepted protocols.

The microbiological analysis shall be performed using relevant internationally recognized and widely accepted methods for the detection or enumeration of injured target microorganisms.

The sources of analytical uncertainty are essentially the sampling and the analytical method.

The number of analyses per sampling point should also be increased when the aim is to reduce the analytical uncertainty.

NOTE 1 In cases where the food has no background microorganisms, a non-selective agar can be used to enumerate the target microbial population, as this will favour the recovery ability of injured cells.

NOTE 2 The microbiology laboratory can adapt the volumes plated on the culture medium (in accordance with ISO 7218) in order to lower the limit of quantification of the method used.

NOTE 3 The microbiology laboratory can adapt the conditions of incubation of plates (e.g. extending the incubation time, using a selective agar and non-selective agar with a dual layer method, adapting the incubation temperature and/or atmosphere) in order to recover injured cells after the treatment, thus avoiding an overestimation of the inactivation effects of the treatment.

NOTE 4 When running challenge tests, it is not mandatory to run full confirmation tests on typical colonies as part of the enumeration technique procedure.

14 Expression of the results

14.1 General

Convert the enumeration results of the test portions to \log_{10} cfu/g, ml or cm^2 .

The homogeneity of the inoculation process in the test units shall be verified. The challenge study is rejected if the standard deviation of the target microorganism concentration (due to inoculation heterogeneity) at t_{inoc} (see [Figure 2](#)) is greater than the limit of $0,3 \log_{10}$ cfu/g, ml or cm^2 for individual test units and $0,5 \log_{10}$ cfu/g, ml or cm^2 for bulk test units.

When multiple time points are obtained, it is possible to calculate an inactivation potential using concentrations obtained at t_{inoc} (or when applicable t_0) and t_{end} or inactivation kinetics parameters using all the time points. In such case, requirements of [Annex B](#) on calculating the inactivation potential shall apply.

The obtained inactivation potential or inactivation kinetics parameters of the test units shall be compared to the results of the inoculated controls to evaluate the efficacy of the treatment.

14.2 Inactivation potential

The inactivation potential (Δ) or the log kill of the target microorganism is the difference between the concentration before and after the treatment under assessment (both expressed as \log_{10} cfu/g, ml or cm^2). For instance, as shown by [Formula \(1\)](#):

$$\Delta = \log_{10} N_0 - \log_{10} N_{\text{end}} = \log_{10} (N_0 / N_{\text{end}}) \quad (1)$$

where

N_0 is the concentration before treatment (t_0) expressed in cfu/g, ml or cm^2 ;

N_{end} is the concentration when the treatment is completed (t_{end} , see [Figure 2](#)) expressed in cfu/g, ml or cm^2 .

Therefore, the log kill is dimensionless as shown in the formula, thus expressed as \log_{10} .

For each batch, calculate the mean of $\log_{10} N_0$, if more than one test unit has been analysed. Then, individually subtract each $\log_{10} N_{\text{end}}$ value from the mean of $\log_{10} N_0$ to obtain individual log kill values for each treated test unit. [Annex B](#) provides the minimum number of inoculated units to be prepared per batch. Depending on the study objectives, these values can be used, for instance, to:

- identify the lowest log kill representing the worst case scenario;
- identify the log kill value(s) not complying with the pre-established target.

The final log kill is the lowest value obtained from the different batches. The log kill shall be higher or equal to the target reduction level.

14.3 Inactivation kinetics parameters

14.3.1 General

Curves may be fitted first by using recognized and commonly accepted primary models. Subsequently, when applicable, secondary models can be fitted to the primary kinetic parameters.

The standard errors shall be provided for the fit and the estimations of all parameters.

14.3.2 Primary inactivation kinetics parameters

Kinetics, representing the \log_{10} cfu/g, ml or cm^2 count throughout the treatment, may be fitted by using a recognized and commonly accepted primary model to estimate those parameters.

Different models are available to estimate the primary inactivation kinetics parameters (e.g. D , k , δ and p values) with appropriate tools.

The two following models are frequently used:

- In cases where the \log_{10} cfu/g, ml or cm^2 counts show a linear trend over time, apply a linear regression to obtain D or k values. The slope of the linear regression is k and the inverse of the slope is D , representing the time needed for one \log_{10} reduction, as shown by [Formula \(2\)](#):

$$\log_{10}N(t) = \log_{10}N_i - k \cdot t = \log_{10}N_i - (1/D) \cdot t \tag{2}$$

where

$N(t)$ is the number or the concentration of survivors after the treatment at time (t);

N_i is the fitted parameter representing the initial microbial population at time $t = 0$

- In cases where the \log_{10} cfu/g, ml or cm^2 counts show a nonlinear trend over time, apply the appropriate nonlinear model to estimate inactivation kinetics parameters. The δ and p values are estimated using the Weibull model^{[8][14][16][22][23][25]} as shown by [Formula \(3\)](#):

$$\log_{10}N(t) = \log_{10}N_i - (t/\delta)^p \tag{3}$$

where

- $N(t)$ is the number or the concentration of survivors for a treatment time (t);
- N_i is the fitted parameter representing the initial microbial population at time $t = 0$;
- δ is the treatment time needed for the first \log_{10} reduction;
- p is a parameter describing the shape of the curve:
 - if $p > 1$, the curve is concave;
 - if $p < 1$, the curve is convex;
 - if $p = 1$, the curve is log-linear.

When more than one food batch has been evaluated, inactivation parameters shall be estimated for each batch.

It is recommended to estimate the primary inactivation kinetics parameters at constant treatment conditions. The estimated inactivation kinetics parameters qualifying the microbial resistance can be used for further simulations in both static (e.g. a lower pH than the pH of the tested food matrix) and dynamic conditions using predictive modelling. These estimates should be paired with a confidence interval.

Calculation sheets or tools are freely available online to estimate the inactivation kinetics parameters obtained under constant treatment conditions (e.g. Curve Fitting from Sym'Previus^[33], GInaFit^[30], iPMP^[31]).

14.3.3 Secondary inactivation kinetics parameters

When several constant levels of treatment (e.g. different temperatures, pressures, concentrations of preservatives higher than the minimum inhibitory concentration, irradiation doses) have been assessed, a secondary model can be applied. For example, in case of isothermal heat treatment when $\log_{10} D$ or $\log_{10} \delta$ values show a linear trend with temperature, the inverse of the slope corresponds to the z value, which can be used for further simulations, as shown by [Formula \(4\)](#):

$$\log_{10} D_T = \log_{10} D_0 - (1/z) * T \quad (4)$$

where

- D_T is the D value at the temperature of interest;
- D_0 is the D value at the reference temperature;
- z is the change in temperature that induces a 10-fold change in the D value;
- T is the temperature of interest.

14.4 Simulation of inactivation

The inactivation kinetics parameters are used to predict the effects of inactivation treatment (e.g. temperature, pressure, antimicrobial preservative) at non-tested levels, in the same product, within the range of tested levels. Extrapolation outside this range is not advisable without caution^{[17][19][21][28]}.

When z is known, it is possible to calculate a D value for a new, non-experimentally tested temperature. Based on this D value, it is possible to calculate the time of treatment to reach the target decimal reduction level ($t_{x,D}$) or to calculate the concentration of the target microorganism after the treatment time. The equation used for the simulation shall be the same used for the fitting. Finally, the log kill can be calculated by subtracting the obtained value from the initial concentration at t_{inoc} (when appropriate

t_0). This simulated log kill shall be equal or higher than the target inactivation level. Calculation sheets or tools are freely available online for this purpose (e.g. MicroHibro^[32], FDA-iRISK^[29], Sym'Previus^[33]).

15 Test report

15.1 General

The test report shall include:

- the aim, type and design of the inactivation study, the target reduction level and the method(s) used;
- all the data needed for interpreting the challenge test results;
- the results obtained with their standard deviations and/or standard errors mentioned in [Clause 14](#);
- any deviations from the procedure;
- any unusual features observed;
- a reference to this document, i.e. ISO 20976-2;
- the date of the test.

15.2 Aim of the study, type of challenge test and target reduction level

The aim of the study shall be clearly specified (e.g. assessment/validation of the food process efficacy as a control measure, assessment of microbial stability and survival). Information describing the characteristics of the food, inactivation treatment and storage under test shall be provided, such as:

- the type of challenge test shall be clearly identified (inactivation potential or inactivation kinetics);
- the target level of reduction and the acceptance criteria shall be clearly specified.
- type of inactivation treatment;
- test batch identification and date of manufacture;
- process parameters (e.g. time, dose, temperature);
- product parameters:
 - description of composition and structure of the food relevant to the test (e.g. including all ingredients, food additives);
 - physico-chemical, microbiological and packaging characteristics of the food before, during and after the inactivation treatment (e.g. pH, a_w , moisture, microorganisms, preservative concentrations, gas composition);
- storage conditions recommended on the packaging and expected shelf-life of the food and the foreseeable storage conditions throughout the food chain.

NOTE Photographs are very useful to illustrate the study focus and design.

Based on this information, the rationale for the selection and design of the type of challenge test and the target microorganisms shall be justified. In cases using a surrogate, the justification of its suitability shall be incorporated into the final report.

15.3 Experimental protocol

The experimental protocol shall include the following:

- information on the microbial strain(s) used:
 - identification and original source of the strain(s) used and justification of selection (see [Clause 8](#));
 - inoculum concentration;
 - conditions under which the inoculum was prepared, including the preparation of the different cultures and the dilution medium/media used, concentration method(s), any adaptation/stress procedures used as relevant to the food and process under test (e.g. adaptation to low temperature, pH or a_w stress);
- number of batches studied and the justification;
- process treatment or product formulation description and justification that they are representative of the production process and/or a worst case scenario;
- information on the sample unit preparation (individual or bulk test units):
 - mass or volume or surface of the inoculated test units;
 - ratio of inoculum to the test and the contamination mode (e.g. at-core or at-surface);
 - packaging characteristics (e.g. material, permeability properties, gas composition);
 - storage conditions (e.g. time, temperature and, if relevant, relative humidity);
- sampling plan justification including additional samples in case of delay between the inoculation (t_{inoc}) and the start of treatment (t_0), see [Figure 2](#), and in case of unforeseen incidents.

15.4 Sample analysis

The information regarding sample analysis shall include the following:

- sample storage conditions (e.g. time, temperature and, if relevant, relative humidity);
- number of test portions analysed at each sampling point;
- mass or volume or area of the test units;
- reference to each method used for microbial analysis (target microorganism and other microorganisms, if performed) and their limit of quantification and, if needed, limit of detection;
- reference to each method used for physico-chemical analyses (e.g. pH, a_w , moisture, preservative concentrations and/or gas composition in the packaging on control samples when performed) and apparatus used;
- where relevant, any delay(s) shall be documented and justified.

15.5 Results

The results shall include the following:

- the monitoring results of the treatment conditions during the study;
- the physico-chemical analyses at each relevant sampling point (e.g. pH, a_w , moisture, preservative concentrations and/or gas composition);
- the microbiological analyses performed for the tested microorganisms;

- when relevant, the concentration of microorganisms other than the target microorganism (e.g. mesophilic microorganisms, psychrotrophic microorganisms, lactic acid bacteria, yeasts and moulds);
- if the purpose of the challenge test is to estimate inactivation potential, the lowest log kill value representing the worst case scenario and/or the log kill value not complying with the pre-established target;
- if the purpose of the challenge test is to estimate and use inactivation kinetics parameters,
 - the primary model and, if relevant, the secondary model and the tools used for the fitting;
 - the graph(s) showing the experimental datapoints and the fitted curve(s);
 - the estimated inactivation kinetics parameters, and their associated standard errors;
 - the simulation results and the tools used according to [14.2](#), when performed.

All raw data shall be made available.

The results shall be compared to the pre-defined target reduction level.

Any deviations from the procedure and any unusual features observed shall be mentioned.

15.6 Conclusions

Based on the results, conclusion(s) shall be provided according to the aim of the study and shall be limited to the process and product under study.

When inactivation potential studies are used, if the log kill is higher or equal to the target reduction level the process is validated. Otherwise, the process is not validated and should be revised.

When inactivation kinetics are used, if the simulated log kill is higher or equal to the target reduction level, the process is validated. Otherwise, the process is not validated and should be revised.

15.7 Reference documents

List the bibliographic references used to write the report for the study, if relevant.

Annex A (informative)

Selection of the type and the location of inactivation study

There are two types of challenge tests to study inactivation: inactivation potential and inactivation kinetics. The selection depends on the aim of the study and the technologies used. [Figure A.1](#) proposes a decision tree for the selection of the type of inactivation study for the process validation or the formulation assessment.

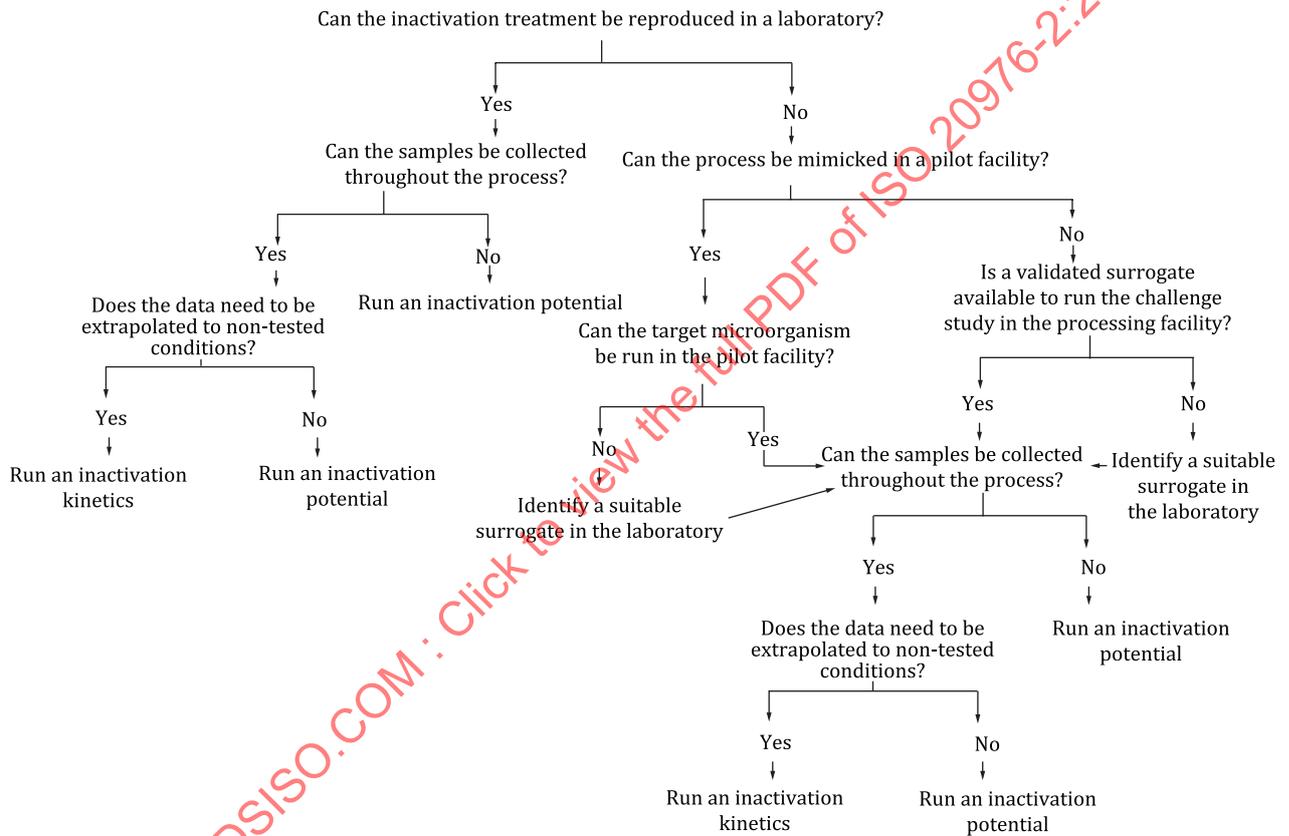


Figure A.1 — Decision tree to select the type of inactivation study