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Sterilization of health care products — Biological indicators — Guidance for the selection, use and interpretation of results

Stérilisation des produits de santé — Indicateurs biologiques — Directives générales pour la sélection, l'utilisation et l'interprétation des résultats

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 14161 was prepared by Technical Committee ISO/TC 198, *Sterilization of health care products*.

This second edition cancels and replaces the first edition (ISO 14161:2000), which has been technically revised.

Introduction

This International Standard provides guidance regarding the selection, use and interpretation of results of biological indicators when used to develop, validate and monitor sterilization processes. The procedures described in this International Standard are of a general nature and do not, of themselves, constitute a comprehensive development, validation or monitoring programme with regard to the sterilization of health care products. The intent of this International Standard is not to mandate the use of biological indicators in a process but, if they are used, to provide guidance for their proper selection and use in order to obviate misleading results.

In this International Standard, users will find guidance on selection of the correct biological indicator for their particular sterilization process and critical parameters as well as guidance on its appropriate use.

The user should select a biological indicator that is appropriate for the particular process to be used. There is a wide variety of sterilization processes in common use, and biological indicator manufacturers are not able to foresee all possible uses of their product. Manufacturers, therefore, label biological indicators according to their intended use. It is the responsibility of the users of biological indicators to select, use, recover and interpret the results as appropriate for the particular sterilization process used.

The certified performance of a biological indicator can be adversely affected by the conditions of storage and transport prior to its use, by the use of the biological indicator or by the sterilizer process parameters. In addition, the incubation procedure used after exposure to the process, including outgrowth temperature and culture medium type, supplier and specific lot, can affect measured resistance as a function of recovery and growth. For these reasons, the recommendations of the biological indicator manufacturer for storage and use should be followed. After exposure, biological indicators should be aseptically transferred (if applicable) and incubated as specified by the biological indicator manufacturer.

It should be noted that biological indicators are not intended to indicate that the products in the load being sterilized are sterile. Biological indicators are utilized to test the effectiveness of a given sterilization process and the equipment used, by assessing microbial lethality according to the concept of sterility assurance level. Suitably trained personnel should conduct these studies.

Sterilization of health care products — Biological indicators — Guidance for the selection, use and interpretation of results

1 Scope

This International Standard provides guidance for the selection, use and interpretation of results from application of biological indicators when used in the development, validation and routine monitoring of sterilization processes. This International Standard applies to biological indicators for which International Standards exist.

NOTE 1 See, for example, the ISO 11138 series.

NOTE 2 The general information provided in this International Standard can have useful application for processes and biological indicators not currently addressed by existing International Standards, e.g., new and developing sterilization processes.

This International Standard does not consider those processes that rely solely on physical removal of microorganisms, e.g., filtration.

This International Standard is not intended to apply to combination processes using, for example, washer disinfectors or flushing and steaming of pipelines.

This International Standard is not intended to apply to liquid sterilization processes.

2 Normative references

The following referenced documents are indispensable for the application 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 11135-1, *Sterilization of health care products — Ethylene oxide — Part 1: Requirements for development, validation and routine control of a sterilization process for medical devices*

ISO 11138-1:2006, *Sterilization of health care products — Biological indicators — Part 1: General requirements*

ISO 11138-2, *Sterilization of health care products — Biological indicators — Part 2: Biological indicators for ethylene oxide sterilization processes*

ISO 11138-3, *Sterilization of health care products — Biological indicators — Part 3: Biological indicators for moist heat sterilization processes*

ISO 11138-4, *Sterilization of health care products — Biological indicators — Part 4: Biological indicators for dry heat sterilization processes*

ISO 11138-5, *Sterilization of health care products — Biological indicators — Part 5: Biological indicators for low-temperature steam and formaldehyde sterilization processes*

ISO 11737-1, *Sterilization of medical devices — Microbiological methods — Part 1: Determination of a population of microorganisms on products*

ISO 14937, *Sterilization of health care products — General requirements for characterization of a sterilizing agent and the development, validation and routine control of a sterilization process for medical devices*

ISO 17665-1, *Sterilization of health care products — Moist heat — Part 1: Requirements for the development, validation and routine control of a sterilization process for medical devices*

ISO 18472:2006, *Sterilization of health care products — Biological and chemical indicators — Test equipment*

3 Terms and definitions

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

3.1
accreditation
procedure by which an authoritative body gives formal recognition that a body or person is competent to carry out specific tasks

NOTE 1 See ISO/IEC 17011[3].

NOTE 2 Accreditation does not itself qualify the laboratory to approve any particular product. However, accreditation can be relevant to approval and certification authorities when they decide whether or not to accept data produced by a given laboratory in connection with their own activities.

3.2
aseptic technique
conditions and procedures used to exclude the introduction of microbial contamination

3.3
bioburden
population of viable microorganisms on or in a product and/or sterile barrier system

[ISO/TS 11139, definition 2.2]

3.4
biological indicator
BI
test system containing viable microorganisms providing a defined resistance to a specified sterilization process

[ISO/TS 11139, definition 2.3]

3.5
D **value**
D_{10} **value**
time or dose required to achieve inactivation of 90 % of a population of the test microorganism under stated conditions

[ISO/TS 11139, definition 2.11]

3.6
holding time
period for which the sterilization variable within the sterilizer and at all points within the load are continuously within the limits specified for the sterilization stage

3.7
inoculated carrier
supporting material on or in which a defined number of viable test organisms have been deposited

NOTE 1 See ISO 11138-1.

NOTE 2 The test organism is a microorganism used for the manufacture of inoculated carriers.

3.8
installation qualification
IQ
process of obtaining and documenting evidence that equipment has been provided and installed in accordance with its specification

[ISO/TS 11139, definition 2.22]

3.9
inoculation
addition of a defined amount of a characterized microbial entity into or on to an item

3.10
log reduction
LR
reduction in number of viable microorganisms, expressed in log units

3.11
operational qualification
OQ
process of obtaining and documenting evidence that installed equipment operates within predetermined limits when used in accordance with its operational procedures

[ISO/TS 11139, definition 2.27]

3.12
performance qualification
PQ
process of obtaining and documenting evidence that the equipment, as installed and operated in accordance with operational procedures, consistently performs in accordance with predetermined criteria and thereby yields product meeting its specification

[ISO/TS 11139, definition 2.30]

3.13
process challenge device
PCD
item designed to constitute a defined resistance to a sterilization process and used to assess performance of the process

[ISO/TS 11139, definition 2.33]

3.14
process challenge location
PCL
site that simulates “worst case” conditions as they are given for sterilizing agent(s) in the goods to be sterilized

3.15
process parameter
specified value for a process variable

NOTE The specification for a sterilization process includes the process parameters and their tolerances.

[ISO/TS 11139, definition 2.34]

3.16
process variable
condition within a sterilization process, changes in which alter microbicidal effectiveness

EXAMPLE Time, temperature, pressure, concentration, humidity, wavelength.

[ISO/TS 11139, definition 2.35]

3.17
reference microorganism
microbial strain obtained from a recognized culture collection

[ISO/TS 11139, definition 2.39]

3.18
resistometer
test equipment designed to create defined reference combinations of the physical and/or chemical variables of a sterilization process

NOTE 1 Adapted from ISO 11138-1, definition 3.15 and ISO 18472:2006, definition 3.11.

NOTE 2 Also referred to as Biological Indicator Evaluator Resistometer (BIER).

3.19
spore-log-reduction
SLR
log of initial spore population, N_0, minus the log of the final population, N_F

3.20
sterile
free from viable microorganisms

[ISO/TS 11139, definition 2.43]

3.21
sterility assurance level
SAL
probability of a single viable microorganism occurring on an item after sterilization

NOTE The term SAL takes a quantitative value, generally 10^{-6} or 10^{-3}. When applying this quantitative value to assurance of sterility, an SAL of 10^{-6} has a lower value but provides a greater assurance of sterility than an SAL of 10^{-3}.

[ISO/TS 11139, definition 2.46]

3.22
sterilization
validated process used to render product free from viable microorganisms

NOTE In a sterilization process, the nature of microbial inactivation is exponential and thus the survival of a microorganism on an individual item can be expressed in terms of probability. While this probability can be reduced to a very low number, it can never be reduced to zero.

[ISO/TS 11139, definition 2.47]

3.23
sterilization cycle development
procedure for determination of the appropriate processing parameters that are consistent with attaining the desired specifications and label claims for a given product or group of products

3.24
sterilization cycle validation
documented procedure for obtaining, recording and interpreting the results required to establish that a process will consistently yield product complying with predetermined specifications

3.25
supplier
organization or person that provides a product

EXAMPLE Producer, distributer, retailer or vendor of a product, or provider of a service or information.

NOTE 1 A supplier can be internal or external to the organization.

NOTE 2 In a contractual situation, a supplier is sometimes called “contractor.”

[ISO 9000, definition 3.3.6]

3.26
survival-kill window
extent of exposure to a sterilization process under defined conditions where there is a transition from all biological indicators showing growth (survival time) to all biological indicators showing no growth (kill time)

[ISO 11138-1, definition 3.18]

3.27
third party
person or body that is recognised as being independent of the parties involved, as concerns the issue in question

NOTE 1 See ISO/IEC Guide 2[1].

NOTE 2 Parties involved are usually supplier (“first party”) and purchaser (“second party”) interests.

3.28
user
person or body employing biological indicators for a given purpose

NOTE 1 See ISO 9000[4].

NOTE 2 The user is the customer who is the recipient of a product provided by the supplier. In a contractual situation, the user is called “purchaser.” The user could be the customer, beneficiary or purchaser. The user can be either external or internal to the organization and represents the “second party.”

3.29
***z* value**
change in exposure temperature of a thermal sterilization process, which corresponds to a tenfold change in D value

NOTE See ISO 11138-3 and ISO 11138-4.

4 General

4.1 This International Standard provides guidance on biological indicators that can apply generally for any sterilization process, including new sterilization processes not yet covered by International Standards.

4.2 The use of biological indicators is normally documented in procedures and/or instructions.

NOTE Employing quality management systems such as ISO 13485[7] usually satisfies this provision.

4.3 Biological indicators should always be used in combination with physical and/or chemical measurements in demonstrating the efficacy of a sterilizing process. When a physical and/or chemical variable of a sterilization process is outside its specified limits, the reason for the sterilizer's inability to achieve its process parameters should be evaluated and the problem corrected. Systems and/or procedures should be established to evaluate any deviations from the cycle process limits, and reasons for accepting any deviation should be fully documented.

4.4 A suitable biological indicator consists of carrier material and packaging and has a microbiological component that is known to be suitable for handling without special containment facilities. The growth conditions should be well documented, and the use of the indicator should be as simple and well described as possible to avoid misinterpretation by the user.

4.5 No formal approval system exists, internationally, for biological indicators that are marketed and used for stated purposes or under stated conditions. Some national regulatory authorities, however, have particular requirements for biological indicators and for the choice and use of biological indicators for the validation and control of products marketed as sterile or sterilized.

4.6 A biological indicator represents a microbiological challenge to a sterilization process and is used to verify that a sterilization process has the ability to inactivate microorganisms that have a known resistance to a referenced sterilization process. Test organisms employed in biological indicators typically have resistance to sterilization which exceeds that of common bioburden microorganisms, although some organisms can exhibit a resistance to sterilization in excess of that of the test organisms. The appropriate biological indicator provides a challenge to the sterilization process which exceeds that of the bioburden through a combination of population and resistance. If there is reason to believe that the goods to be processed could be contaminated with particularly resistant organisms, extended sterilization processing, based on the bioburden, could be required.

4.7 Biological indicators are not intended for use in any process other than that specified by the manufacturer on the product labelling. Microbial species and strains are selected as biological indicator candidates based on their known resistance to the specific method of sterilization as certified by the manufacturer. The use of an inappropriate biological indicator can give misleading results.

The user should ensure that the biological indicator has been qualified for use with the particular range of sterilization conditions that are used. This could require information in addition to that given in the labelling. When biological indicators are used outside reference conditions, the user can require information on the reaction expected from the indicator, e.g., the effect of sub-optimal moisture conditions on the biological indicators used in an ethylene oxide process. Users who employ biological indicators outside the manufacturer's labelled recommendations should thoroughly characterize the resistance of the biological indicators to the particular sterilization process. The relationship of the response of the biological indicator to process parameters should be clearly demonstrated.

4.8 It is incumbent upon those responsible for the sterilization of product to ensure that the type of biological indicator employed to validate and/or routinely monitor a given sterilization process is appropriate for that use.

4.9 The manufacturer's recommendations for the use and storage of the biological indicators should always be followed. Failure to do so can compromise the integrity of the biological indicator. If the user removes the inoculated carrier from the biological indicator's primary packaging, changes in the resistance characteristics can occur. Guidance should be sought from the manufacturer on the extent of this change, or the user can evaluate changes in the resistance characteristics. The user should document that the performance characteristics of the inoculated carrier are appropriate for their use.

4.10 Biological indicators should not be used beyond the expiration date stated by the manufacturer.

4.11 Those who employ biological indicators for validation and/or routine monitoring of sterilization should be properly trained in their use. The time between completion of the sterilization process and the testing of the BI should be justified as described in 8.2.4. Transferral of microorganisms exposed to the sterilization process to the appropriate recovery medium should be done using aseptic technique.

4.12 The ISO 11138 series gives requirements for the information that the manufacturer should provide for biological indicators. The information may be provided on the label, as a packet insert or as a general specification accompanying the biological indicators. This series of International Standards also includes minimum requirements for resistance characteristics. Testing conditions and methods are given as reference methods.

4.13 Users of biological indicators come from a wide variety of industries, private enterprises and health care facilities. Users are not generally required to perform resistance assays on biological indicators but can have differing requirements for their quality assurance systems, which include audits of vendors and/or manufacturers (see 6.2.2).

4.14 The verification of resistance characteristics by the user is an alternative to and/or complementary to an audit, when necessary.

5 Characteristics of biological indicators

5.1 General

5.1.1 Biological indicators provide means to assess directly the microbial lethality of a sterilization process (see References [8] and [9]). When used in conjunction with physical and/or chemical process monitors, biological indicators can provide an indication of the effectiveness of a given sterilization process.

5.1.2 A sterilization process should be considered as satisfactory only when the desired physical and/or chemical parameters and microbiological results, as determined by an appropriate sterilization cycle development, validation and monitoring programme have been realized. Failure to achieve the desired physical and/or chemical parameters and/or microbiological challenge forms the basis for declaring the sterilization process as nonconforming (see ISO 13485[7] and ISO 9001[22]).

5.1.3 Biological indicators consist of a defined population of test organisms presented in such a manner as to allow their recovery following sterilization processing. For example, test organisms employed for ethylene oxide sterilization processes can be spores of a suitable strain of *Bacillus subtilis* or *Bacillus atrophaeus*, as noted in ISO 11138-2. For steam sterilization or moist heat sterilization, the test organisms employed can be spores of a suitable strain of *Geobacillus stearothermophilus*, as noted in ISO 11138-3. Test organisms other than bacterial spores can be used if they have been shown to provide appropriate resistance to the sterilization process.

5.1.4 The basis of all formulae used to determine biological indicator resistance characteristics such as D values is that the inactivation reaction follows first-order kinetics, with the requirement that the value for the coefficient of determination, r^2, for the linearity of the survivor curve be not less than 0,8 (see Annex E and Annex F). The strain, the production method, the suspension fluid, the carrier and packaging materials all affect the resistance characteristics of the finished product (see ISO 11138-1).

5.1.5 The design and construction of a biological indicator can result in unique resistance characteristics and can vary depending on whether the biological indicator is intended for use in the development and validation of a sterilization process or for use in routine monitoring. If the design of the biological indicator for use in routine monitoring differs from that employed to validate the sterilization processes, the challenge to the process during validation should be correlated with the challenge to the process during routine monitoring.

5.1.6 The resistance characteristics of biological indicators vary according to the manufacturing methods and the testing conditions. Depending upon placement within the load and the specific lethal conditions at those discrete locations, biological indicators from the same lot may show different survival capabilities (see 7.2.3). Users of biological indicators should note that ten indicators spread throughout the load are not considered replicates due to the differences in lethality that exist throughout the chamber and load (see Note to 11.3.1).

5.2 Test organism suspension for direct inoculation of products

5.2.1 Direct inoculation of test organisms on or in product can be necessary in cycle development and other studies when the use of a biological indicator is not feasible. Direct inoculation can be appropriate for assessing factors such as product sterilizability, identification of the more difficult to sterilize locations within the device, and localized microbiological effects, e.g., moist heat versus dry heat environments.

The rationale for the selection of the “most difficult to sterilize” site(s) on a medical device or within a sterilization load should be documented based on experimental data or derived from prior knowledge of the particular sterilization methodology. In practice, the “most difficult to sterilize” site represents those locations that are most likely to provide high resistance to the sterilization process. One should refer to specific sterilization standards (e.g., ISO 17665-1 and ISO 11135-1) for guidance in determining and selecting difficult-to-sterilize locations.

5.2.2 To assess the efficacy of sterilization at a particular site or location on the product, the desired species and population of test organisms can be inoculated at those sites. The use of suspensions of test organisms to prepare inoculated carriers or inoculated products requires caution. The materials on to which test organisms are inoculated can alter the test organisms' resistance characteristics. The resistance can be

higher or lower due to deposition as a monolayer or multilayer, coating effects, and/or bacteriostatic or bactericidal effects of the material. The methods employed to recover the test organisms following processing should be validated to ensure an adequate level of recovery from the product (see ISO 11737-1). Test organism recovery should be expressed in terms of percent recovery of the population of the original inoculum.

5.2.3 Direct inoculation of products or materials with a test organism suspension can cause prolonged or decreased survival of test organisms. This may affect the observed percent recovery of the original inoculum relative to what is expected under specified sterilization conditions. Inoculated products may be assayed with either survivor curve (enumeration/direct counting) or fraction-negative procedures (see Figure A.4). These assays require aseptic techniques.

5.2.4 The D value and, when appropriate, the z value, are constant values only under determined and defined conditions. The resistance characteristics of a spore suspension provided by a manufacturer or supplier of biological indicators might not correspond to the resistance characteristics for direct product inoculation studies. The resistance characteristics should be measured for the carrier employed (solid carrier material or fluid) as well as for the specific sterilization cycle employed.

5.3 Inoculated carriers

5.3.1 Inoculated carriers consist of a defined population of test organisms inoculated on or in a suitable carrier material (see ISO 11138-1:2006, Annex B). Caution should be exercised to ensure that the integrity of the carrier material selected is sufficient to withstand sterilization processing without degradation and to minimize the loss of the inoculated test organisms during transport and handling.

5.3.2 The resistance characteristics of a test organism in suspension can be considerably changed upon deposition on or in carriers. Several factors can influence the resistance characteristics, such as the surface on to which the suspension is inoculated (e.g., solid materials, viscous products or fluids), the way the spores are dispersed and otherwise treated, the methods of drying, etc.

5.3.3 If an inoculated carrier is removed from the biological indicator primary package for cycle development, cycle validation studies, or for process challenge devices used for routine process monitoring, then it is the responsibility of the user to provide a rationale for this application. It should be recognized that the resistance of the microorganism on the inoculated carrier could differ from the labelled resistance of the packaged biological indicator.

5.3.4 The resistance characteristics of an inoculated carrier provided by the manufacturer of biological indicators might not correspond to the resistance characteristics established in direct product inoculation studies.

5.3.5 The carrier material should be evaluated by the biological indicator manufacturer or the user to establish that the sterilizing agent for which the biological indicator is intended neither retains nor releases inhibitory substances (e.g. sterilizing agent residuals) to such an extent that the recovery of low numbers is inhibited (see ISO 11138-1:2006, 5.2).

5.4 Self-contained biological indicators

Self-contained biological indicators consist of either a) or b).

a) An ampoule containing growth medium and a carrier inoculated with test organisms contained within an outer vial so that the sterilizing agent obtains access to the inoculated carrier through a sterile barrier or a tortuous path.

   After exposure to the sterilization process, the growth medium is brought into contact with the inoculated carrier by breaking the ampoule of growth medium, thereby eliminating the need to aseptically transfer the inoculated carrier to a separate vial of growth medium. The biological indicator manufacturers' recommendations should be followed for incubation of self-contained biological indicators.

NOTE Due to the low volume and the possibility of evaporation of the growth medium, prolonged post-exposure incubation might not be possible.

Chemical residuals resulting from processes such as ethylene oxide or vapour hydrogen peroxide can inhibit growth of surviving organisms. The biological indicator manufacturer's recommendations should be followed for proper handling (including aeration) of biological indicators prior to incubation (see 8.2.4).

b) A hermetically sealed ampoule containing a suspension of test organisms in growth medium.

These are referred to as sealed-ampoule biological indicators. After exposure to the process, the sealed ampoule is incubated intact, and no aseptic transfer is required.

NOTE This type of indicator is sensitive only to exposure time and temperature and is primarily used to monitor moist heat sterilization of aqueous fluids.

Self-contained biological indicators are generally larger than biological indicators that consist only of an inoculated carrier in a primary packaging, and may not fit into locations within the device that represent the process challenge locations. Unless a biological indicator can be placed into a load without deforming it or otherwise potentially compromising its primary packaging, the biological indicator should not be used. Also, the user should be aware that the claimed resistance characteristics can be dependent on the air-removal method employed in the sterilization cycle.

5.5 Other biological indicators

Biological indicators in their simplest form consist of an inoculated carrier in primary packaging. The inoculated carrier can take a variety of forms, including paper strips, threads, metal coupons or other carriers suitable for inoculation. The primary packaging is chosen to permit the sterilizing agent to penetrate to the inoculated carrier while maintaining a sterile barrier after processing.

6 Selection of supplier

6.1 General

6.1.1 The user of biological indicators should, whenever possible, purchase to standard specifications, e.g., biological indicators manufactured according to specifications given in the ISO 11138 series, pharmacopoeial monographs or other applicable standards. The user should consider the particular sterilization process as the basis for the choice of biological indicator.

6.1.2 When the user has a process that requires performance characteristics that differ from the label claim for the biological indicator, it is the responsibility of the user to verify that the biological indicator has the performance characteristics needed.

6.1.3 The user of biological indicators should have a system in place to provide assurance that the biological indicators obtained consistently meet the specified characteristics. Such assurance may be provided by one or more of the following:

a) information from the manufacturer covering the performance characteristics of the lot of biological indicators prepared;

NOTE Requirements for information supplied by manufacturers of biological indicators are provided by the ISO 11138 series.

b) a statement of conformity from the manufacturer that the biological indicators meet the agreed specifications;

c) if needed, various degrees of testing of each lot of biological indicators received by the user, to verify that the performance characteristics meet the agreed specifications.

6.1.4 When the user has established a high level of confidence in the supplier (see 6.1.3), the testing performed by the user can be minimal. At a minimum, the user should have a mechanism to ensure that a shipment of biological indicators contains all agreed-upon documentation, such as appropriate label

information, packet inserts, storage and handling instructions, etc. There should be a mechanism to assure that the vendor continues to maintain the expected quality and manufacturing standards, such as a vendor's or manufacturer's declaration of conformity to standards. If the user has not established the vendor relationship required to be assured of consistent biological indicator performance, additional testing could be necessary until an appropriate assurance can be established that the biological indicators meet the vendors' and/or manufacturers' label claim and/or user requirements.

6.1.5 Testing by the user, if deemed necessary, can consist of population assays and survival-kill resistance tests on samples from each new lot of biological indicators received (see also 8.6 and Clause 11). Provided that the manufacturer of biological indicators manufactures to detailed standard specifications, i.e. the ISO 11138 series, and the user uses the biological indicator as intended by the manufacturer, testing of the resistance characteristics by the user is considered unnecessary.

The manufacturer's label claims for resistance, such as D values, z values (if any) and survival-kill results are determined using a resistometer (see ISO 18472).

6.2 Documentation

6.2.1 General

6.2.1.1 The labelling requirements for biological indicators are given in ISO 11138-1:2006, 4.3.

6.2.1.2 The labelling includes the information presented on the primary and secondary packaging of the biological indicator as well as any included package inserts providing additional information beyond that which can be printed on the packaging. The purchaser might need or want a certificate of conformity to product standards and/or quality system standards to include in appropriate documentation files.

6.2.1.3 When a statement of performance or compliance to a standard is provided by the manufacturer as a certificate, the user should have confirmation of the competence of the manufacturer as indicated by the second sentence of 6.2.2.1.

6.2.1.4 If an independent (third-party) test laboratory is used to confirm the performance characteristics of biological indicators, the test laboratory should be accredited for the specific test methods used (see ISO/IEC 17025[2] and ISO/IEC 17011[3]).

6.2.1.5 The status of the manufacturer of the biological indicator with regard to conformance to the appropriate quality standard, such as ISO 13485[7] or other quality assurance programmes should be verified. If compliance to the appropriate standards can be demonstrated, an audit may not be necessary.

6.2.2 Manufacturer audit

6.2.2.1 If necessary, the user should confirm that a qualified auditing body, e.g., a certification body, has performed an audit of the biological indicator manufacturer. Alternatively, the user could perform the audit.

NOTE The auditing standard ISO 19011[5] gives guidance on requirements for the process of auditing, the qualification criteria for quality system auditors, and the management of audit programmes.

6.2.2.2 A qualified auditor should perform the audit, as part of the purchaser's quality system. If an audit of the biological indicator manufacturer is performed, the following should be considered:

a) test organism:

   1) strain selection and maintenance;

   2) propagation of test organisms, including growth medium and components, growth temperature and incubation period;

   3) harvesting, purity and cleanliness of the test organism;

   4) viable test organism count and biochemical characteristics of the test organism;

b) biological indicator:

1) qualifying of components for use in the preparation of biological indicators, such as carrier material and primary packaging, and consideration of any potential toxic effects of these materials on the test organisms;

2) population of the test organisms during manufacture of the biological indicators;

3) consistency (e.g., growth promotion, pH, stability, etc.) and fill volume of any growth medium supplied with the biological indicator;

4) resistance of the biological indicator, including the type of test equipment and its calibration, the recovery medium employed and incubation conditions;

5) storage stability and continued resistance of the biological indicator until its expiration date;

c) quality control:

1) label claims for the finished product;

2) storage stability and continued conformance of the finished product to its label claims.

6.2.2.3 The manufacturer should be able to provide adequate documentation of the quality systems pertaining to the manufacturing of biological indicators and provide documentation of conformity of products to declared specifications.

7 Biological indicators in process development

7.1 General

7.1.1 For additional information on process development, refer to the sterilization standards for those processes (e.g., ISO 17665-1, ISO 11135-1 and ISO 14937).

7.1.2 If a biological indicator is used for process development, the appropriateness of the indicator should be determined.

7.1.3 Sterilization processes vary widely with regard to their operational characteristics and the type of products that are sterilized. While each application is unique, it can be acceptable to group similar products in the same category for the purposes of the development, validation and routine monitoring of a sterilization process. Careful consideration should be given to those aspects of product design or packaging which can impart an additional challenge to the sterilization process. Biological indicators can be used to determine those locations on the product which represent a rigorous challenge to the process, and likewise to establish the extent to which different types of product are related with regard to the challenge presented to the sterilization process. This could lead to the selection of a particular product configuration for further analysis.

7.1.4 Physical and/or chemical parameters and microbiological results should be reviewed and interpreted for acceptability prior to acceptance of the process.

7.1.5 Biological indicators should always be used in combination with appropriate physical and/or chemical measurements of the process parameters in order to demonstrate the efficacy of a sterilization process.

7.1.6 General advice on the number of biological indicators per sterilizer volume cannot be given, as this depends on the reproducibility of the cycles as well as the potential for differences in the process parameters throughout the load during sterilization. However, other sterilization standards could provide guidance on the recommended number of biological indicators to be used, e.g., ISO 11135-1. The correct number of biological indicators to use can be determined from data collected from the use of biological indicators and/or bioburden studies, as well as from documentation on the distribution of the sterilizing agent throughout the load.

7.2 Overkill approach

7.2.1 This method is often referred to as the “half-cycle method” or “overkill sterilization method” and is discussed in ISO 14937. This method is based on the following assumptions:

a) that the biological indicator (reference microorganism) provides a greater challenge than the bioburden;

b) that the full sterilization process achieves at least a twelve-log reduction of the biological indicator (see ISO 11135-1 and ISO 14937) or delivers an F_{bio} of 12 (see ISO 17665-1) with minimum resistance characteristics (see Figures A.1 and A.2 and ISO 11138-3);

c) that, at half cycle, the user can typically demonstrate at least a six-log reduction (see Figure A.1).

NOTE The ISO 11138 series permits resistance characteristics other than the minimum required for monitoring purposes.

7.2.2 These criteria can be addressed by placing biological indicators or inoculated carriers with, for example, a population of 10^6 test organisms meeting minimum resistance requirements at the process challenge location(s) within product throughout the load. These locations within the load should have been previously demonstrated to present a challenge to the sterilization process and correlate with the “most difficult to sterilize” locations, such that choosing these locations will secure the appropriate log reduction for the entire product load. At least a six-log reduction in the population of organisms should be demonstrated within one-half the normal holding time of the cycle to be validated when all of the biological indicators are statistically equivalent samples. The percentage of biological indicators showing growth can be correlated to the log reduction achieved [see Note to 11.3.1.c)]. If testing under half-cycle conditions demonstrates a reduction in the population of test organisms in excess of six logs, it is possible that no growth of the test organism could result, depending on the sample size. As illustrated in Figure A.1, there is a 1 % probability of positive growth with a spore-log-reduction of eight logs at the upper end of the half-cycle window.

7.2.3 Placement of the biological indicator either within the product or within the load is likely to alter its apparent resistance characteristics in comparison to the resistance noted on the labelling of the biological indicator. This can require adjusting the half-cycle exposure period to compensate for the additional resistance caused by the placement of the biological indicator in the product or load. Similar adjustments could be needed when test organism suspensions are used to prepare inoculated product (see 5.2).

7.2.4 Suitable physical and/or chemical probes should be used to establish temperature distributions, etc., that can aid in determining placement locations for the biological indicators. A sufficient number of probes should be prepared with biological indicators placed at the previously determined location within the product.

NOTE For moist heat sterilization, the z value of the biological indicator can be different from the z value of 10 °C, which is normally assumed for process lethality based on temperature measurements. This can lead to discrepancies between the integrated process lethality determined by use of biological indicators and the lethality determined by direct temperature measurements.

7.3 Combined biological indicator and bioburden method

7.3.1 The combined biological indicator and bioburden method requires the population and microorganism resistance of the product bioburden to be known. This method has the advantage of permitting a reduction in cycle exposure period and minimizing exposure of the product to the sterilizing agent and is discussed in ISO 14937.

7.3.2 The combined biological indicator/bioburden methodology requires selecting process conditions that deliver a process lethality sufficient to inactivate the bioburden to the labelled product sterility assurance level. The number of replicate cycles required to demonstrate the appropriate efficacy will depend on the confidence in accuracy and degree of bioburden inactivation repeatability determined in bioburden evaluations. Figure A.1 shows the general relationship between the inactivation of a biological indicator and the inactivation of product bioburden. Since the bioburden is typically less resistant to the sterilizing agent than is the biological indicator, the desired sterility assurance level can normally be achieved with less treatment than would be recommended when using the overkill method. The extent of the required treatment is dependent upon the relationship between the biological indicator and bioburden relative to population and resistance to the sterilizing agent.

7.3.3 For this procedure, the appropriate biological indicator with a population of less than 10^6 can be employed, as long as the desired sterility assurance level can be demonstrated. The variation in bioburden should be considered when determining the minimum biological indicator population that should be used to validate a sterilization process. The population of the biological indicator should not be less than 10^3 test organisms per carrier (see Figure A.1).

7.3.4 Procedures for the estimation of the product bioburden are discussed in ISO 11737-1. Due to bioburden variation, it could be necessary to characterize bioburden and bioburden resistance on a routine basis.

Studies of the resistance of the total bioburden population could be required in order to assure that the challenge provided is less than that of the biological indicator. One possible method to determine this would be to run fractional sterilization cycles to determine that the bioburden population does not survive as great an exposure as does the biological indicator.

7.3.5 Implementation of the combined biological indicator/bioburden method requires consideration of many of the same factors noted in 5.4 and 7.2, with regard to the placement of the biological indicator in those locations within the product and load which present a rigorous challenge to the sterilization process. The method is only applicable when data are sufficient to be subjected to valid statistical analysis and there is a high level of confidence that the bioburden data is representative of "worst-case" conditions. There are many causes of variation, such as raw materials, process control, and seasonal variations. Consideration should be given to the presence and nature of the bioburden distribution in the product. As the distribution of bioburden within the product can vary significantly, it is important to determine how this distribution can affect the challenge presented by the product to the sterilization process and thereby affect the choice of biological indicator.

7.3.6 Determination of the product bioburden and its species and resistance distribution requires consideration of the factors discussed in the ISO 11737 series. The method selected shall be validated against the requirements of those standards and the statistical confidence of the bioburden estimate shall be established.

7.3.7 The fundamental criteria of the combined biological indicator/bioburden methodology requires selecting processing conditions that reduce the population of the bioburden to 10^0. An additional safety factor consistent with the labelling of the product should be applied to the holding period (see Figure A.1). The number of replicate cycles for which this should be demonstrated during sterilization cycle development depends on the accuracy and confidence limits of the bioburden estimates.

7.3.8 Microorganism strains whose resistance is greater than the overall resistance of the product bioburden may be employed as biological indicators if their sterilization kinetics satisfy the criteria for a biological indicator (kill curve log-linear within a coefficient of determination of 0,8). However, these indicator organisms may be less resistant to the process than those specified in the ISO 11138 series (see Figure A.2).

7.3.9 If the resistance of the bioburden is greater than that of commercially available biological indicators, resistant strains isolated from the bioburden should be considered for inclusion in process development studies (see ISO 11135-1 and ISO 17665-1). Alternatively, the holding period should be increased by a factor determined by the relative resistance of the bioburden and the biological indicator or a biological indicator with a higher population creating a challenge greater than or equal to the resistant strains may be used.

7.4 Bioburden method

7.4.1 The reader should refer to the ISO 11737 series for the appropriate microbiological methods to estimate the bioburden. Some bioburden microorganisms can have greater resistance than the biological indicators described in the ISO 11138 series. Bioburden microorganisms with higher resistance could be used as a model biological indicator (see 7.3); the method is discussed in ISO 14937.

7.4.2 For information on validation and routine control and the applicability of absolute bioburden methods, the reader is referred to the relevant International Standards for their particular sterilization process (see Clause 2). Detailed specifications for an absolute bioburden method, without any reference to biological indicators, are given in the ISO 11737 series.

8 Biological indicators in sterilization validation

8.1 General

If biological indicators are used in the validation process, one should also consider the type(s) of biological indicators that can be used in the routine monitoring. Different biological indicators can provide varying degrees of challenge to the sterilization process (see Figure A.2). If different biological indicators are used for validation and routine monitoring, both should be included in the validation studies so that the resistance relationship of the two can be established and documented.

8.2 Placement and handling of biological indicators

8.2.1 Validation of a sterilization process requires documentation that the process is capable of consistently producing product that meets its predetermined specifications (see ISO 17665-1, ISO 11135-1 and ISO 14937).

8.2.2 The number of biological indicators in products and/or product loads should be documented.

8.2.3 The user should document the placement of the chosen biological indicators in the sterilizer chamber, within the product load or a process challenge device (see Annex B). Other considerations to be addressed in biological indicator placement within the product load are loading patterns, load density and geometry, process challenge location(s), placement of physical and/or chemical sensors or probes, potential stratification of physical elements, the effect of packaging, etc.

8.2.4 The biological indicators should be removed from the sterilizer load as soon as possible after the process, without compromising the safety of personnel. They should be tested within a specified time interval that has been established for that product and process. The time intervals between preparation of the indicators and their use in the sterilization process, and between the end of the process and the culturing of the indicators should be justified in order to demonstrate that these intervals have no effect on the results of the biological testing of the sterilizer or sterilization process. These justified time intervals should not be exceeded. If the biological indicators are handled in a manner other than those stated by the manufacturer, the procedures should be validated to determine if they affect the performance of the biological indicator. Any established time intervals should be followed.

8.2.5 National or regional requirements for worker safety should be observed when removing the biological indicators from the sterilizer.

8.3 Sterilizer qualification

8.3.1 Initial qualification is performed to obtain and document evidence that the sterilizer, its services and ancillary equipment have been provided and installed in accordance with its specifications, and that the sterilizer functions within predetermined limits when operated in accordance with instructions (see ISO 17665-1 and ISO 11135-1).

Biological indicators can be used in OQ/PQ, for example, to establish evidence of uniformity of distribution of sterilizing agents.

8.3.2 Manufacturers of sterilizers might have performed tests in their factories prior to delivery using biological indicators for specific types of load (see Annex B).

8.4 Performance qualification

Following completion of sterilizer qualification (see 8.3), performance qualification (PQ) testing is conducted to document the reproducibility and the efficacy of the sterilization process, including its ability to produce product meeting its predetermined quality specifications. Validation establishes product compliance with predetermined specifications; relevant International Standards such as ISO 17665-1, ISO 11135-1 and ISO 14937 apply. Different biological indicators can provide varying degrees of challenge to the sterilization process (see Figure A.2). Correlation between the biological indicators used for cycle development and validation, performance qualification and routine monitoring should be established and documented.

8.5 Review and approval of validation

Upon successful completion of qualifications, a review of the validation documentation, including biological indicator performance, is necessary prior to beginning manufacture, in order to certify that the process conforms to requirements.

8.6 Requalification

8.6.1 When performing requalification, the same resistance characteristics, number of biological indicators, their placement in product load, etc., should be used. If a new biological indicator is being qualified for the process, it is important to establish and document a correlation between the new biological indicator and the previous biological indicator.

8.6.2 When using moist heat or ethylene oxide sterilization, ISO 17665-1 or ISO 11135-1 applies.

8.6.3 A minimum frequency for evaluating the resistance characteristics of a biological indicator lot should be established. Considerations leading to different intervals for requalification of the biological indicator system could include seasonal changes, product and material changes as well as equipment changes, etc. If the resistance characteristics of the biological indicator change outside the predetermined limits, requalification should be performed. If the recovery medium is changed, the new growth medium should be correlated to the previous one used and the choice of new growth medium should be validated (see also 12.4).

9 Biological indicators in routine monitoring

9.1 General

9.1.1 Biological indicators provide a method for demonstrating microbial lethality in a sterilization process; however, fractional (e.g. half cycles), may need to be conducted during validation in order to quantify the lethality. For well-developed processes where parametric release has been validated, biological indicators may not be necessary for routine monitoring of some sterilization processes (e.g. moist heat sterilization, see ISO 17655-1, ethylene oxide, see ISO 11135-1 or dry heat, see ISO 20857).

9.1.2 The type of biological indicator and its placement in the product or product load should be consistent with product load locations that have been determined during the sterilization development or the validation. If the microbiological challenge system used for routine monitoring of the sterilization process differs from that used in the validation of the process, the relationship between the system for validation and for routine monitoring should be documented.

9.1.3 The specified time intervals between preparation of the indicators and sterilization, and between the end of the process and the culturing of the indicators should be justified in order to demonstrate that these intervals have no effect on the outgrowth of the biological indicator (see 8.2.4).

9.1.4 Biological indicators for routine monitoring that are used in the combined biological indicator/bioburden method (7.3) will not comply with all parts of the 11138 series if the population and/or resistance is below the minimum requirements of the respective part (see ISO 11138-1:2006, 6.1.3).

9.2 Placement and handling of biological indicators

9.2.1 During cycle development and validation, biological indicators are placed in those sites within the product and load that present a rigorous challenge to the sterilization process. During routine monitoring, it could be desirable to place the biological indicators in more accessible locations using a process challenge device (see 9.3). In these situations the placement of the biological indicators should be correlated with the locations employed during cycle development or validation to ensure that the integrity of the sterilization process is not compromised. Consistent placement of the biological indicators employed for routine monitoring should also be ensured.

9.2.2 The directions of the supplier of the biological indicator should be followed with regard to the proper handling of the biological indicator subsequent to sterilization. In general, biological indicators should be removed from the load without compromising the safety of personnel and within a specified time period that is justified (see Clause 11). They should then be aseptically transferred to the appropriate growth medium within the defined time period and incubated at the proper temperatures (see Clause 12).

9.2.3 In addition to the qualification requirements on medium growth properties (see Clause 12) and viability of the biological indicator (see Clause 11), the user could also perform abbreviated versions of these checks during routine monitoring of the sterilization process. For example, using an unprocessed biological indicator incubated in the growth medium indicates both the viability of the indicator and the suitability of the growth conditions.

NOTE National guidance documents can require the use of unexposed controls.

9.2.4 The process should be considered acceptable only when the desired physical and/or chemical parameters have been reviewed and the microbiological results interpreted and both found to comply with the desired criteria.

9.3 Process challenge device (PCD)

9.3.1 A process challenge device in combination with biological indicators can be used both for validation and routine monitoring of sterilization cycles as well as for sterilizer testing by sterilizer manufacturers. Process challenge devices are designed so that the placement of the biological indicator within the process challenge device constitutes a location that is deemed to represent a suitably stringent challenge to the process. The design of the process challenge device can vary according to the nature of the product to be challenged (see Annex B for various examples of process challenge devices).

9.3.2 Process challenge devices should be designed with consideration given to the various process parameters that influence the sterilization process. Composition of a process challenge device depends on the type of cycle to be monitored, as well as the type of product to be sterilized.

9.3.3 Process challenge devices can be commercially available as prefabricated sets, often called “biological test packs.” Single-use biological test packs are manufactured by various companies and may be used instead of in-house process challenge devices. Process challenge devices and their placement in the product load should represent a challenge to the process that is equivalent to or greater than the challenge represented by the product load.

10 Results

10.1 General

10.1.1 The criteria for acceptance of a sterilization process as satisfactory should be decided upon during the sterilization cycle development, using relevant standards for the validation and control of the sterilization process.

10.1.2 In order to obtain reliable results, routine procedures should be established and maintained and should be carried out by trained technicians using appropriate equipment.

10.2 Interpretation of results

10.2.1 A validated sterilization process in which all the pre-set parameters have been met should show no growth of the biological indicator.

10.2.2 Based on the principles of the use of biological indicators in ISO 11135-1, a sterilization process where pre-set minimum parameters have not been met could show growth of the biological indicator.

10.2.3 Any biological indicator test results showing growth of the indicator when no growth would be expected can be an indication of an inadequate process, a defective biological indicator or a faulty test system

and should lead to an investigation. Action to be taken upon growth of a biological indicator subsequent to sterilization processing can vary with institutional and regulatory policies and could require that the lot of product be rejected as non-sterile. The identification of growth as that of the test organism should be confirmed, and an effort made to identify the cause of the growth. Consistent growth of biological indicators after sterilization processing can be indicative of a sterilization process that was not delivered in accordance with the validated parameters, an inadequate sterilization process or could possibly be due to the use of a biological indicator lot with an unusually high resistance to the sterilizing agent. If an investigation indicates that the sterilization process was delivered appropriately and there is no significant change in the biological indicator that would affect its performance in the sterilization process, then the sterilization process should be repeated. Gram staining in combination with colonial and cellular morphology may be useful in determining that the growth is not the indicator organism.

NOTE Some indicator organisms might be gram variable.

10.2.4 The particular sterilizer, type of product, and the loading of the product all affect the sterilization process. The resistance characteristics of the biological indicator system used in the process should be established for the overall system to be effective. Acceptable data from the biological indicator are only a part of the data necessary to show that the sterilization process has been successful.

10.2.5 Cultures showing growth that is not confirmed to be the indicator organism should be further investigated to determine the cause of the positive growth. Frequent test contaminants can indicate a faulty test system or inadequate training of personnel.

11 Application of biological indicator standards

11.1 General assessment of biological indicator performance by the user

11.1.1 The two main characteristics of a biological indicator are the nominal population of microorganisms and the resistance of the biological indicator to the sterilization process, expressed as the D value.

11.1.2 The biological indicator should be transported, stored and handled to ensure that the nominal population and resistance characteristics are maintained during the shelf life. Sterilization of culture medium, incubation conditions, equipment maintenance, and training of laboratory personnel are some areas that should be defined and controlled to ensure appropriate performance of the biological indicator. The user can periodically verify the biological indicator population. When the above-mentioned areas are controlled and validated, routine biological indicator testing by the user might not be necessary.

11.1.3 The user should note any deviation from the process that is employed and the reference set of the parameters that has been defined for the process. If the sterilizer cycle parameters or load are the reason for the deviations in the biological indicator's resistance characteristics, the user should investigate the possibility of eliminating these variations and requalify the process.

11.1.4 Variations in resistometer performance can in some instances give different resistance characteristics results for the indicator. In such cases, the manufacturer should, on request, give information on details of the relevant testing conditions.

11.1.5 If the user establishes data on the nominal population count or the D value and these are outside the limits required by the relevant standards or outside the label information, the user is encouraged to seek information from the manufacturer to ensure that the same techniques, methods, and conditions are used to obtain the data (see References [12], [14], [18], [19], [20], [21], [30], [31] and [32]).

11.2 Nominal population of test organism

11.2.1 The manufacturer shall give the nominal population of test organisms of each biological indicator as part of the labelling information. The requirements are given in the standards for the minimum number of microorganisms on a biological indicator or inoculated carrier to ensure a minimum resistance of the indicator. When tested, the population should be between 50 % and 300 % of the nominal population. The biological indicator manufacturer should be consulted to ensure that the same techniques and procedures are used,

because variations in testing procedures can affect the population determination results. ISO 11138-1 requires the manufacturer to provide this information on request.

11.2.2 Spores, such as *Geobacillus stearothermophilus*, can require a heat-shock procedure in order to obtain greater accuracy in counts. Several combinations of temperature and time have been used successfully. The results can be influenced by the mechanical treatment of the inoculated carrier and thus by the microorganisms, during preparation of the aliquots (see Reference [18]). Different laboratory practices and even variations in the performance of individual personnel can lead to different results.

11.2.3 The method for removing spores from the inoculated carrier should be validated and can include mechanical disintegration of the carrier or other methods, such as ultrasonication. If the user applies a different method from that recommended by the manufacturer, the method should be validated.

11.2.4 The fluid for disintegration should not influence the number of surviving microorganisms (e.g., it should not be a growth medium) and should not otherwise negatively influence the result by any inhibitory effect on the growth of the microorganism (see Clause 12).

11.2.5 The user should follow the manufacturer's recommended procedures for recovery to ensure comparable results.

11.2.6 The sterilized fluid and the processed, inoculated carrier should be treated aseptically to avoid any microbial contamination or cross-contamination that could bias the results.

11.2.7 Attention should be given to the accuracy of the plate counts. The accuracy of plate counts is dependent on a variety of factors including dilution and pipetting error, calibration of pipetting devices, technician training, and the number of colony-forming units (CFUs) per plate. It is generally accepted that plate counts should be between 30 CFUs and 300 CFUs per plate for greatest statistical accuracy.

11.2.8 The ISO 11138 series limits deviations from the labelled nominal number. The user should note that if the nominal population of an indicator from a given batch or lot of indicators is being tested by the user, the deviations can exceed the limits given in the relevant part(s) of ISO 11138. This could be caused, for example, by different culture media used or different enumeration and counting techniques (see Reference [17]).

11.3 Resistance determination

11.3.1 General

If the user chooses to verify the label claims or determine the D value of the biological indicator in or on the item to be sterilized, then they should employ the same conditions as the manufacturer. This includes employing the specific parameters for the relevant resistometer. The biological indicator resistance can be estimated or calculated using three approaches: the survivor curve method, the fraction-negative method and calculation of the survival-kill window. After exposure to incremental time intervals of the sterilization process (i.e. parts of the holding time), the indicators are tested using the methods outlined in a) to c). The ISO 11138 series provides requirements for each method, which can be used in combination to estimate resistance.

The main difference between the three approaches is given below.

a) Survivor curve method

This method requires counting of colonies. Depending upon the type of the inoculated carrier and the properties of the microorganism, this often implies the use of mechanical degradation of the inoculated carrier (performed using aseptic techniques) with subsequent retrieval and counting of the total retrievable count of colony forming units on solid medium (e.g., distinct colonies on agar plates).

b) Fraction-negative method

This method requires growth/no-growth determination and employs aseptic transfer of the intact inoculated carrier into the fluid culture medium. The transfer is performed without any mechanical, microbiological or thermal influence on the inoculated carrier.

c) The survival-kill window

This is based on a fraction-negative method, giving lower limits where all samples show growth and upper limits where none of the indicators shows growth after exposure to the whole sterilization process or after time intervals of the sterilization process (see ISO 11138-1:2006, Annex E).

NOTE The determination of D values from any series of exposures generally applies averaging in some fashion to the results from the sample set. Combining data in this fashion has a statistical restriction. The data can only be combined when the samples are statistical equivalents. For example, if ten samples are distributed throughout a sterilization chamber, they are not all true equivalents. However, multiple samples clustered in the same location within the sterilizer chamber can be considered true equivalents (see References [25] and [28]).

11.3.2 Survivor curve method

This method is also referred to as the "direct enumeration method" and the "enumeration number method." This method makes use of direct counting procedures (see ISO 11138-1) and should be performed on inoculated carriers (see Figure A.4).

For further details on procedures, see 11.2 and Annex F.

11.3.3 Fraction-negative method

There are several such methods in use, called fraction-negative or quantal methods. Growth or non-growth is observed relative to the number tested (see Figure A.4).

This International Standard gives the common reference method for the ISO 11138 series, which is the Limited Holcomb-Spearman-Karber Procedure (LHSKP). Two other commonly used statistical methods, the Holcomb-Spearman-Karber Procedure (HSKP) and the Stumbo-Murphy-Cochran Procedure (SMCP), may be used under particular conditions (see Annex C).

a) Limited Holcomb-Spearman-Karber Procedure (LHSKP):

This procedure can be used if the successive exposure periods, such as times or doses, differ by a constant time interval and if an identical number of replicates is exposed at each exposure period interval. For example, exposures could be at 3 min, 5 min, 7 min and 9 min, which represent a 2-min time interval. ISO 11138-1 specifies at least 20 replicates at each interval for the LHSKP (see Table 1 and Figure A.4).

NOTE The critical parameter "time" can be replaced by "dose" in some sterilization processes such as radiation and ozone.

The mean D value, $\overline{D}$, is calculated using Equation (1).

$$\overline{D} = \frac{U_{\mathrm{HSK}}}{\log_{10} N_0 + 0{,}250\,7} \tag{1}$$

where

N_0 is the initial inoculum of test organisms per test sample;

$$U_{\mathrm{HSK}} = U_k - \frac{d}{2} - \frac{d}{n}\sum_{i=1}^{k-1} r_i \tag{2}$$

Examples of calculations are presented in Annex C. For more information on LHSKP, see ISO 11138-1 and Block[9].

b) Holcomb-Spearman-Karber Procedure (HSKP):

This method is similar to the LHSKP but uses the generic formula which does not require use of the same number of replicates nor that constant time intervals be used.

The D value is calculated using Equation (3).

$$D = \frac{U_{\text{HSK}}}{\log_{10} N_0 + 0{,}250\,7} \tag{3}$$

where

$$U_{\text{HSK}} = \sum_{i=1}^{k-1} U_i \tag{4}$$

Examples of calculations are presented in Annex C.

c) Stumbo-Murphy-Cochran Procedure (SMCP):

The formula for the Stumbo-Murphy-Cochran Procedure requires one result in the fraction-negative range, consisting of time, t, the number of units negative for growth, r and the number of replicates, n, at one exposure time within the fraction-negative range, and the initial number of microorganisms per replicate, N_0.

The D value is calculated using Equation (5).

$$D = \frac{t}{\log_{10} N_0 - \log_{10}\left(\ln\frac{n}{r}\right)} \tag{5}$$

To obtain a higher level of confidence using the Stumbo-Murphy-Cochran Procedure, the D value should be calculated as the average of at least three runs in the fraction-negative range in order to confirm reproducibility.

For further details on procedures, see Annex C.

11.3.4 Survival-kill response characteristics

This method requires a total of 100 biological indicators with 50 replicates at the two conditions identified as the time at which all indicators will show growth and the time at which no indicators will show growth after exposure to the specified conditions (see Annex G).

11.4 z value determination

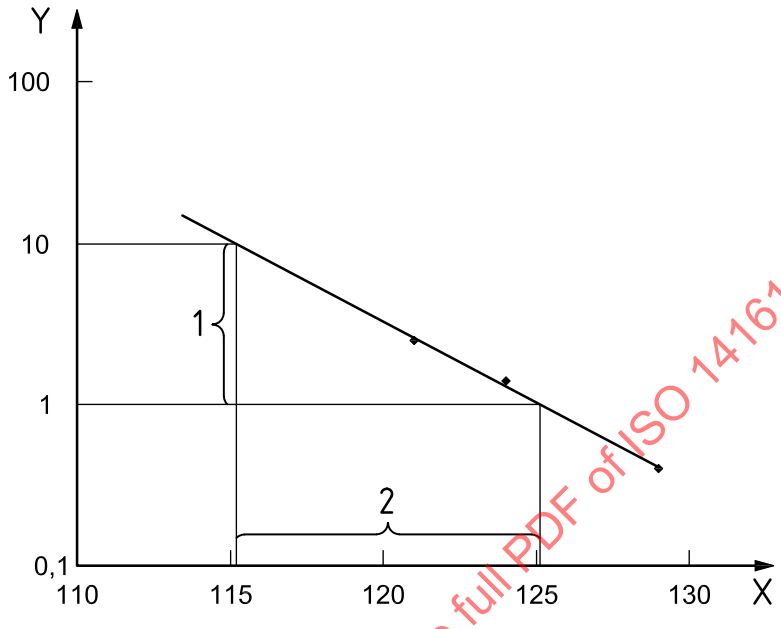
11.4.1 General

The z value is the change in exposure temperature of a thermal sterilization process, which corresponds to a tenfold change in D value. The z value allows the user to express equivalent lethality for thermal sterilization processes. The z value calculation is the only means of expressing the measured lethality under variable process conditions which can range from massive slow-to-heat loads to low density loads that achieve steady state conditions more quickly.

The z value can be determined with only two process D values. However, the resulting value will have wide confidence limits. For this reason it is preferred to calculate the z value from three or more process D values, as specified in ISO 11138-3. (See Figure A.3.)

11.4.2 Graphically plotting the z value

The z value can be determined by graphically plotting the log of the D value versus temperature on a semi-log scale (see Reference [33]). The line-of-best-fit will be linear (Figure 1) and the z value estimate is the negative reciprocal of the slope.



Key

Y D value (min) (plotted on logarithmic scale)
X temperature (°C)
1 one log reduction
2 z value (°C)

Figure 1 — Graphically plotting the z value

11.4.3 Mathematically calculating the z value

Mathematically calculating the line-of-best-fit is the statistical method to accurately represent the slope of the data. The line-of-best-fit can be found using log-linear regression analysis.

Equations (6) and (7) are used to derive the equation of a linear regression line.

$$\sum_{i=1}^{n} y_i = b + m \sum_{i=1}^{n} x_i \tag{6}$$

$$\sum_{i=1}^{n} x_i y_i = b \sum_{i=1}^{n} x_i + m \sum_{i=1}^{n} x_i^2 \tag{7}$$

where

b is the y intercept of the regression line;

m is the slope;

n is the number of x, y points.

The above equations are modified to include the x, log y pairs to reflect the log-linear scale, thus:

$$\sum_{i=1}^{n} \log y_i = bn + m\sum_{i=1}^{n} x_i \tag{8}$$

$$\sum_{i=1}^{n} x_i \log y_i = b\sum_{i-1}^{n} x_i + m\sum_{i=1}^{n} {x_i}^2 \tag{9}$$

Using the method of least squares, Equation (10) is established for the slope of the line:

$$m = \frac{n\sum_{i=1}^{n}(x_i \log y_i) - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} \log y_i}{n\sum_{i-1}^{n} {x_i}^2 - \left(\sum_{i=1}^{n} x_i\right)^2} \tag{10}$$

where

n is the number of D value/temperature pairs (data points);

x represents temperature;

y represents D value.

From this formula, the z value can be calculated by the absolute value of the reciprocal of this slope:

$$z = \left|\frac{1}{m}\right| \tag{11}$$

Examples of calculations are presented in Annex E (see also ISO 11138-3 and ISO 11138-4).

11.4.4 Correlation coefficient, r, for the z value

The correlation coefficient, r, is a mathematical value that indicates the precision with which the D values can be predicted by a linear regression of temperatures and respective D values. A correlation coefficient of + 1,000 0 or – 1,000 0 would indicate that all data points lie on the linear regression line. The square of the correlation coefficient, r^2, is referred to as the coefficient of determination. The coefficient of determination is a measure of how well the regression line represents the data. An r^2 value of 0,800 0 or greater is considered an acceptable model fit. The detailed calculations appear in Annex E.

NOTE The D values should be expressed to four decimal places to increase the accuracy of the above coefficients when used in the calculations in 11.4.3.

11.5 $F_{(T, z)}$ equivalent sterilization value determination

$F_{(T,\ z)}$ is defined as the process equivalent minutes at any reference temperature T_{ref}. Calculation of the $F_{(T_{\text{ref}},\ z)}$ requires the z value to be known.

$$F_{(T_{\text{ref}},\ z)} = 10^{\left(\frac{T - T_{\text{ref}}}{Z}\right)} \tag{12}$$

The most common F value is F_0, used in moist heat sterilization. F_0 is a specific F value for the equivalent minutes at 121,1 °C using a z value of 10,0 °C. This has become the worldwide standard for moist heat process design. F equivalent values have been applied in moist heat, dry heat and more recently in ethylene oxide sterilization processes (see Reference [29]). When validating and monitoring processes using biological indicators, the actual process time at any temperature is calculated to the specific reference temperature as a

function of the specific z value for that particular biological indicator. This allows the integration of lethality and is used to calculate the actual spore-log-reduction (SLR) value for the process.

11.6 Establishing spore-log-reduction (SLR)

The thermal-death-time curve is a method of comparing F values on a logarithmic scale versus temperature. This method allows the process to be expressed relative to its effectiveness in reducing the spore challenge. The F value represents equivalent time at a specified process temperature, which in conjunction with the D value, allows the calculation of the reduction in the number of spores of a homogenous population by a specific amount. This result is the lethality delivered in the process expressed as a spore-log-reduction (SLR) and can be expressed as the log of the initial spore population, N_0, minus the log of the final population, N_F. The formula for calculating F_T using this change in the microbial population at the specified process conditions is given by Equation (13).

$$F_{(T,\,z)} = D_T\,(\log N_0 - \log N_F) \tag{13}$$

where

T is the temperature;

z is the specific z value;

D_T is the D value at the specified temperature T;

N_0 is the initial spore population or bioburden;

N_F is the final spore population or bioburden.

This change in spore population is the spore-log-reduction (SLR) which is represented by Equation (14).

$$\text{SLR} = \log N_0 - \log N_F \tag{14}$$

The thermal-death-time can therefore be expressed using Equation (15).

$$F_T = D_T \times \text{SLR} \tag{15}$$

The process spore-log-reduction is then used to calculate the product sterility assurance level (SAL).

11.7 Sterility assurance level (SAL) calculation

The probability of a non-sterile unit from a microbial control process is expressed as sterility assurance level (SAL). The SAL calculation uses Equation (16).

$$\text{SAL} = 10^{-(\text{SLR}-\log N_0)} \tag{16}$$

where N_0 is the initial spore population or bioburden.

The commonly accepted minimum SAL is 10^{-6} or less than one chance in a million of having a non-sterile unit. Therefore, the total integrated lethality shall deliver an excess of six more spore-log-reduction equivalents than the microbial challenge that was sterilized.

NOTE This concept is graphically expressed in Figure A.1 and Figure A.2.

11.8 Test equipment

11.8.1 Particular attention should be given to the kind of resistometer used. ISO 18472 gives requirements for special resistometers to be used in order to comply with the requirements of the standards. Manufacturers of biological indicators shall comply with the relevant standard in the ISO 11138 series if they declare conformity to these standards.

11.8.2 A pilot plant sterilizer or a production sterilizer can provide useful information but should not be relied upon to verify manufacturers' label claims. Attention should be given to the set of parameters and the number of vacuum pulses as well as the depth of vacuum.

11.8.3 All technical equipment, including automatic or adjustable pipettes, should be periodically calibrated and/or controlled.

11.8.4 The control and maintenance of technical equipment should be documented.

12 Culture conditions

12.1 General

12.1.1 Manufacturers of biological indicators are required by ISO 11138-1 to provide information to the user as to the culturing conditions (i.e. incubation temperature, incubation period and choice of growth medium). If the user employs other culturing conditions, these should be validated by the user.

12.1.2 The procedures should be performed in a laboratory area defined for this purpose, giving proper attention to aseptic technique and good laboratory practice. It is good practice to include negative controls with each assay performed when using general laboratory areas for these assays. If a defined area cannot be found for this purpose, or if there is any risk of cross-contamination, the procedure should be performed in a defined critical zone (e.g., a sterile bench or biosafety cabinet with no air exchange between the critical zone and surrounding areas where biological indicators or microorganisms of the same or similar growth properties are being manufactured, packaged or otherwise handled).

12.1.3 Culture conditions recommended by the manufacturer of the biological indicator should be followed. If incubation conditions other than those recommended by the manufacturer are employed, they should be validated to determine their effect on the performance of the biological indicator.

12.1.4 For validation purposes, the incubation conditions should be carefully considered. The user is advised to seek information in available International Standards, such as the ISO 11737 series (see Clause 2).

12.2 Incubation temperature

12.2.1 The labelling of the biological indicator should always be consulted with regard to the recommended incubation temperature. Failure to incubate biological indicators at the appropriate incubation temperature can invalidate the test results.

12.2.2 Test organisms that have been exposed to a sterilization process can exhibit increased sensitivity to variations in incubation temperature. Some test organisms can exhibit increased recovery at incubation temperatures below the recommended incubation temperature and decreased recovery at incubation temperatures above the recommended incubation temperature. In general, biological indicators of *Geobacillus stearothermophilus* can be incubated at temperatures in the range of 55 °C to 60 °C, and biological indicators of *Bacillus atrophaeus* and *Bacillus subtilis* can be incubated at temperatures in the range of 30 °C to 39 °C or according to the specifications provided by the manufacturer.

12.3 Incubation period

12.3.1 The incubation period required could vary with the nature of the biological indicator and sterilization process; ISO 11138-1:2006, 7.3.2 recommends a period of 7 d for biological indicators for established processes. The labelling of the biological indicator and other information provided by the manufacturer should be consulted in this regard.

The information provided by the biological indicator manufacturer is established under specific conditions that might not apply in the user's facility. Therefore, the user should consider verification of the biological indicator manufacturer's recommendations for incubation time.

12.3.2 For non-standard or new sterilization processes not covered by current International Standards, the incubation period should be validated against current national requirements; ISO 11138-1:2006, 7.3.2 recommends an incubation period of 14 d.

Self-contained biological indicators might not have been designed with sufficient volume of media to allow for recovery over prolonged incubation periods.

Care is needed when considering the use of self-contained biological indicators in validating and monitoring processes where delayed growth is possible due to the potential of sterilizing agent residuals.

12.3.3 A biological indicator manufacturer may validate a design of a spore carrier/recovery medium combination (e.g. a spore strip sold with recovery medium as a kit, or a self-contained biological indicator). The reduced incubation time validation for this product does not have to be repeated by the end user, as long as the end user uses the product with the same sterilization agent as that used in the validation (e.g. design validated and used in ethylene oxide). If the end user intends to use a spore carrier/recovery medium/incubation temperature combination that has not been validated by a manufacturer (e.g. a spore strip used with a recovery medium not sold as part of a kit), the end user should validate a reduced incubation time using a defined statistical sampling plan and procedure with pre-established acceptance criteria.

12.4 Choice of growth medium

12.4.1 Most manufacturers of biological indicators either provide the culture medium directly or provide information regarding the preparation of a suitable culture medium. The culture medium employed by different manufacturers can vary significantly; thus, it is important to follow the biological indicator manufacturer's recommendations.

12.4.2 For validation of biological indicator incubation times, the inherent variability of the culture medium makes it advisable to screen the performance of several medium lots and to reserve suitable quantities of lots found to provide the desired growth performance. This would allow comparison with new lots.

12.4.3 Selection of a suitable culture medium requires consideration of many variables, such as the pH of the culture medium and the presence of inhibitory substances such as salts, pH indicators or antibiotics. Other substances in the culture medium can affect the recovery of sterilizing agent-stressed test organisms.

12.4.4 Users should not overprocess the culture medium, as extended sterilization can induce changes that can affect its growth-promoting properties. The ability of the culture medium to promote the growth of low numbers of microorganisms should be demonstrated (see References [10], [12], [13] and [21]).

12.4.5 Each lot of growth medium should be checked by a suitable growth promotion test and compared with a lot previously used, so as to determine lot-to-lot consistency.

13 Third-party requirements

13.1 General

13.1.1 Additional testing, whether in-house or by third-party laboratories, might not be able to duplicate a manufacturer's label claims due to inherent variation in test systems and personnel. For this reason, tolerances are stated in ISO 11138-1:2006, 6.3.2 and 6.4.3 (see References [31], [32], and [33]).

13.1.2 Third-party facilities should employ the test equipment and test methods, including all parallel and repeated tests, that are required by the relevant International Standards.

13.1.3 A third-party testing facility could be a testing laboratory that is in compliance with ISO/IEC 17025[2] or that has a recognised quality system for the service.

13.2 Minimum requirements for replicates and total number of biological indicators

Table 1 — Minimum samples according to method

Test method in accordance with ISO 11138-1	Minimum number of test samples	Minimum number of exposure periods	Minimum total number of test samples
Initial count of viable test organism [a]	4	–	4
Survivor curve method (see Annex F)	4	5	20
Fraction negative method (see Annex C)	20	5 [b]	100 [b]
Survival-kill window (ISO 11138-1:2006, Annex E)	50	2	100
Minimum total number depending on choice of combination of methods:			124 or 204

[a] The viable count of the unprocessed inoculated carrier or biological indicator.

[b] The extra set of testing conditions at the exposure subsequent to t_6 (see Table C.1) is not used in the calculations, but is a condition for accepting the test results as valid.

A total of at least 20 biological indicators is needed for the survivor curve method (see Annex F), with at least five exposure periods and four replicates for each period. Annex C covers the minimum requirements for the LHSKP, with a total of at least 100 biological indicators. A minimum of five graded exposure periods are used with 20 replicates each. One exposure period should result in all positive biological indicators. There should be at least two sequential exposures that result in no positive biological indicators. There should be a minimum of two intermediate exposures that result in fractional responses. The survival-kill window characterization requires a total of 100 biological indicators with 50 replicates at the two conditions (ISO 11138-1:2006, Annex E). The resistance characteristics are determined according to ISO 11138-1, which requires application of at least two methods out of the three mentioned. This implies the use of at least 124 or 204 biological indicators respectively, depending upon the methods chosen.

At least three D value determinations at three different temperatures are required to estimate the z value for moist heat sterilization processes in accordance with ISO 11138-3 and dry heat sterilization processes in accordance with ISO 11138-4. (See Reference [32].)

ISO 11138-1:2006, Annex A requires four replicates for the determination of viable count.

13.3 Test equipment

A testing laboratory that performs tests in accordance with the ISO 11138 series needs to apply the required test equipment, including the relevant resistometer (see ISO 18472).

For further information, see 11.8.

14 Personnel training

Personnel responsible for the placement, retrieval, testing, and all other handling of biological indicators need to be suitably trained. This training should be documented, and the adequacy of the training should be periodically assessed. There should be written procedures for the testing and handling of the biological indicators as well as for supporting activities such as preparing and sterilizing culture medium.

When aseptic techniques are used, particular attention should be given to training personnel in these techniques.

15 Storage and handling

15.1 The vendor or supplier is responsible for shipment or transport to the user and should ensure that temperature variations that occur during transport do not have an adverse effect on the labelled resistance characteristics. The vendor or supplier should make agreements with the user on means of transportation, to ensure that conditions are adequate to retain the performance characteristics of biological indicators during the transportation.

15.2 The recommendations of the manufacturer with regard to the storage and handling of biological indicators should always be followed. Failure to follow these recommendations could adversely affect the integrity and performance of the biological indicator and lead to incorrect assumptions regarding the efficacy of the sterilization process. In general, biological indicators should always be maintained in their protective packaging until ready for use. A biological indicator is delivered ready-to-use and in a packaging system that protects it from extraneous microbiological influences. Biological indicator storage should take into account temperature, relative humidity, chemical influences and light.

15.3 Biological indicators consisting of non-hazardous microorganisms can be handled without restriction, and shipment and transport should follow international rules for transport of non-hazardous microorganisms.

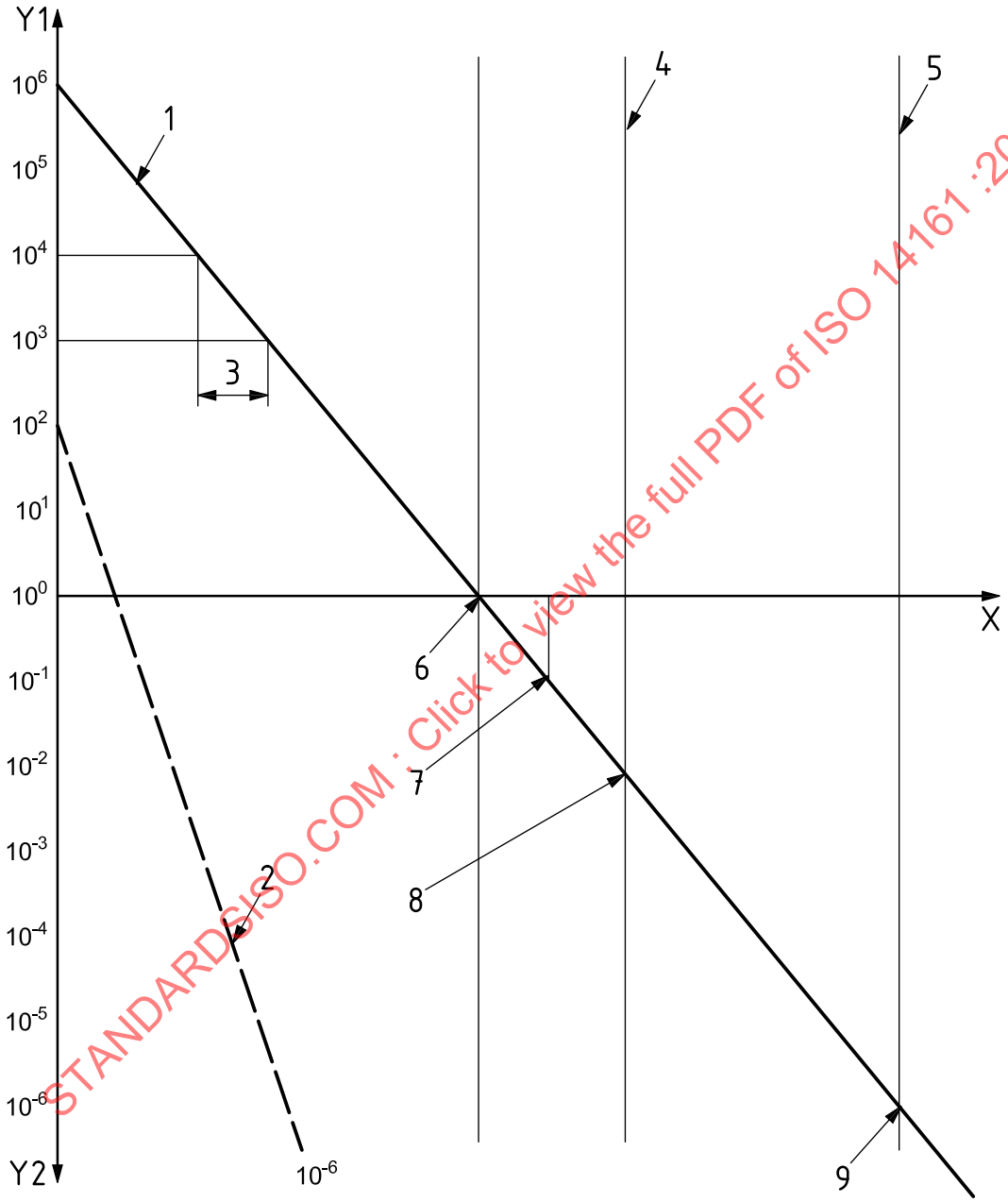
16 Disposal of biological indicators

According to ISO 11138-1, the biological indicator manufacturer is required to provide disposal instructions. Inactivated biological indicators can be disposed of as household waste. Expired or unused indicators can also be disposed of as household waste if the microorganism is of a non-hazardous nature. However, manufacturers' disposal instructions, which often require sterilization prior to disposal, should be followed.

NOTE National regulations can define biological indicators as hospital waste, and disposal of these biological indicators could be covered by regional or national regulations.

Annex A
(informative)

Microbiological inactivation kinetics and enumeration techniques



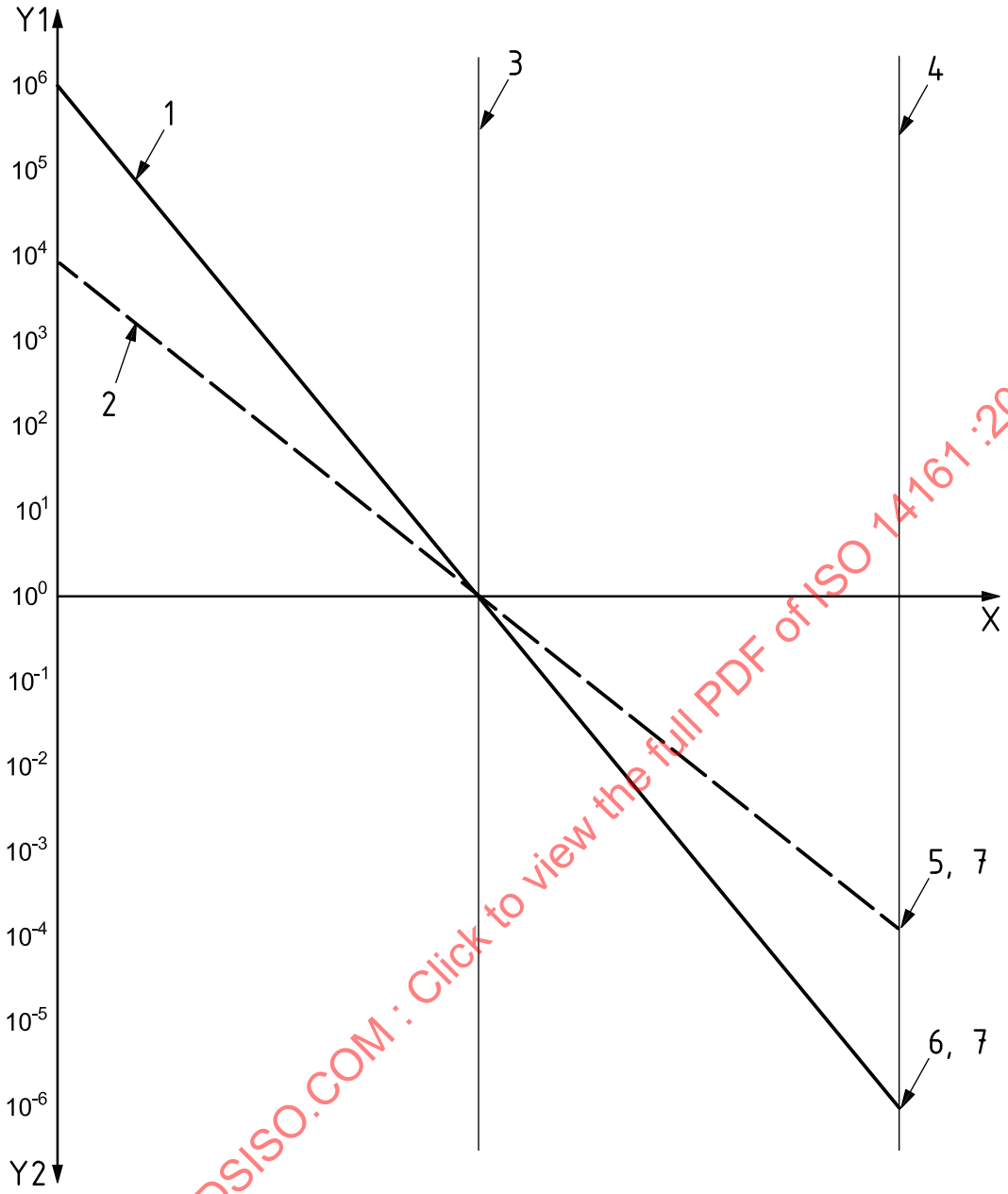
Key

X time or dose
Y1 number of surviving microorganisms (plotted on logarithmic scale)
Y2 probability of surviving microorganisms (plotted on logarithmic scale)
1 biological indicator
2 bioburden
3 D value
4 half-cycle window
5 minimum sterilization process time
6 six log reduction (63 % positives)
7 seven log reduction (10 % positives)
8 eight log reduction (1 % positives)
9 theoretical twelve log reduction (0,000 1 % positives)

NOTE 1 Log reduction for product achieved prior to the minimum sterilization process time (see 3.10).

NOTE 2 For the purposes of this illustration, time and dose are shown as highly controlled steady-state conditions and might not apply to process vessel conditions.

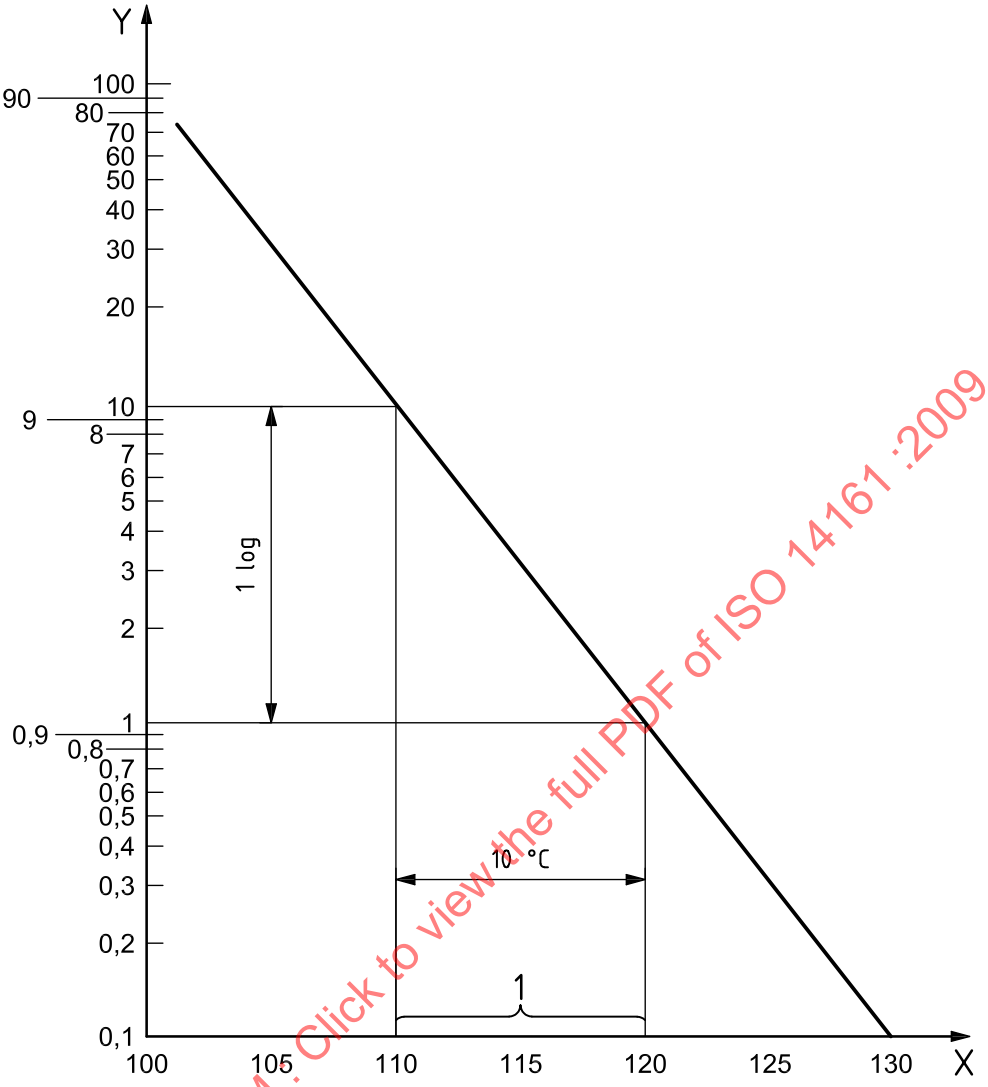
Figure A.1 — Examples of relationship between the biological indicator and the product bioburden in a reference microorganism method



Key

X time or dose
Y1 number of surviving microorganisms (plotted on logarithmic scale)
Y2 probability of a surviving microorganism (plotted on logarithmic scale)
1 curve representing a six log reduction of BI with minimum specified resistance
2 curve representing a four log reduction of BI with 1,5 × minimum specified resistance
3 minimum half-cycle window
4 minimum sterilization process time
5 theoretical eight log reduction ($D_{BI} = 1{,}5 \times D_{min}$)
6 theoretical twelve log reduction ($D_{BI} = D_{min}$)
7 equivalent challenges

Figure A.2 — Examples of equivalent biological challenges with preparations of different resistance



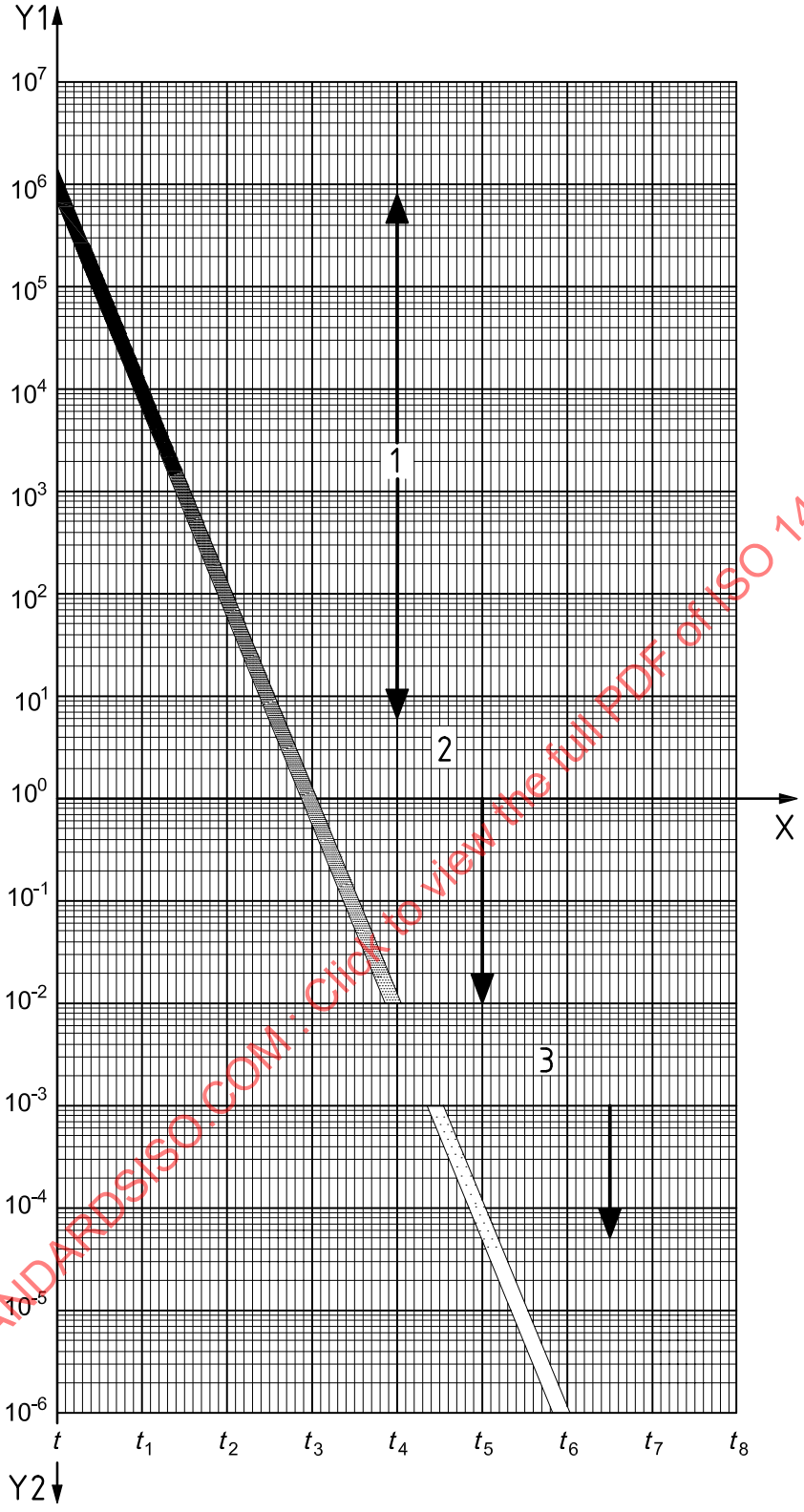
Key

X temperature (°C)

Y D value, minimum (plotted on logarithmic scale)

1 z value (°C)

Figure A.3 — Example of a z value determination (see 11.4)



Key

X exposure time or dose
Y1 number of surviving microorganisms (plotted on logarithmic scale)
Y2 probability of a surviving microorganism (plotted on logarithmic scale)
1 direct enumeration method: number of microorganisms determined by counting colonies formed by viable organisms
2 fraction-negative method: number of microorganisms estimated from fraction-negative methods
3 total inactivation (or “kill”) method: fraction-negative methods for showing no growth of the indicator

Figure A.4 — Areas for D value determination methods under uniform conditions

Annex B
(informative)

Process challenge devices

B.1 General

A process challenge device can have several configurations and uses. It is an item designed to constitute a defined resistance to a sterilization process and used to assess performance of the process. The commercially available process challenge device should represent a similar or greater challenge to the user's sterilization process than would be represented by the load.

The device is constituted so that a biological indicator can be arranged in the place most difficult for the sterilizing agent to reach. The design of the process challenge device depends on the kind of goods to be sterilized and the sterilization procedure. The biological indicator should not interfere with the function of the process challenge device.

In some process challenge devices, an inoculated carrier can be used in place of a biological indicator.

B.2 Helices

The helix consists of a coiled tube with a gas-tight capsule for the inoculated carrier at one end, intended for challenging sterilizing agent penetration into long hollow instruments.

NOTE National standards for specific sterilizers could include requirements for such helices.

B.3 Standard test packs

Standard test packs are used in large steam sterilizers for porous loads to check that rapid and uniform penetration of steam into the pack is attained at the levels at which the process variables are set.

The standard test pack is comprised of porous sheets wrapped in a particular configuration with biological indicators inside. They are designed to test the efficacy of the steam sterilization process for porous loads.

NOTE National standards for specific sterilizers could include requirements for such standard test packs.

B.4 User's process challenge devices

This type of process challenge device is specially designed to meet the criteria for the process challenge location(s) in the load. The packaging and design should reflect the standardized load to be examined and varies with the load. The user's process challenge device serves as a “dummy” to replace the actual goods for this location and to allow removal of biological indicators without destroying the goods to be sterilized. The user could need one or several devices to cover the process challenge location(s).

B.5 Biological test packs

This is a common description of commercially available process challenge devices that have a claimed level of resistance. Biological test packs can be re-usable or single-use items, depending on the materials used and the process parameters.

Annex C
(informative)

Formulae for fraction negative methods for D value calculations
(adapted from ISO 11138-1:2006, Annex D)

C.1 General

C.1.1 This method establishes the number of surviving test organisms by indirect calculation based on the recoverable number of microorganisms as determined by visual observation of growth in fluid growth medium. The method referred to as "fraction negative analysis" is a method in which a fraction of the test samples shows no growth (the fraction negative range) and the calculation is based on the results obtained with these data. A "total kill analysis" is also a fraction negative method in which all the test samples show no growth and the calculation is based on the results obtained with this requirement. Fraction negative method is used when the recoverable number of test organisms is less than 5×10^0 CFUs/unit of measure.

C.1.2 The Holcomb-Spearman-Karber Procedure (see C.3.1) and Limited-Holcomb-Spearman-Karber Procedure (see C.3.2) require successive exposures which span the fraction negative range.

NOTE Other methods could be applicable, particularly when the fraction negative range is known. One such alternative method is provided by the Stumbo-Murphy-Cochran Procedure (see C.3.3).

C.1.3 Test samples should be subjected to defined exposure periods with all process variables, except time, remaining within defined windows (steady-state). Where the process variables are considered to be acceptably narrow, time is expressed as "t". Where the control of process variables is too wide to be considered constant, methods of integration can be used to calculate equivalent time "U". Both terms are found in literature references.

C.1.4 The number of samples exposed, n, in each exposure and the intervals between sequential exposures, d, both affect the reliability of the test.

C.2 Materials

C.2.1 Test samples should be representative of spore suspensions, inoculated carriers or packaged biological indicators.

C.2.2 The relevant resistometer should be used.

NOTE Test methods are given in subsequent parts of the ISO 11138 series. Specifications for resistometers are given in ISO 18472.

C.2.3 The incubator should be set to provide, and monitored to confirm, the temperature specified in the culture conditions.

C.2.4 The growth medium should be as specified in the culture conditions.

C.3 Methods

C.3.1 Holcomb-Spearman-Karber Procedure (HSKP)

C.3.1.1 Introduction

C.3.1.1.1 Test samples should be subjected to graded exposures to the defined exposure period with all process variables, except time, remaining constant. The total number of test samples should be not less than 100. A minimum number of 20 replicates should be used for each exposure.

C.3.1.1.2 When the sterilizing agent leaves a residue in or on the test samples, this should be neutralized as rapidly as possible so as not to interfere with the test results. If a neutralization procedure is required, it should be validated.

C.3.1.1.3 To obtain a higher level of confidence using the HSKP, samples should be cultured after exposure according to the manufacturer's specified method.

C.3.1.1.4 Each inoculated carrier is transferred aseptically to a test tube containing an adequate volume of the specified growth medium. The volume of medium should be the same for each replicate. If the growth medium is included by the manufacturer as an integral part of the biological indicator, the manufacturer's culturing instructions should be followed. The manufacturer of the biological indicators should identify or make available a suitable recovery medium and/or the complete data for preparing one (see also 12.1 and 12.4).

C.3.1.1.5 The test samples should be incubated following the manufacturer's specified methods. The cultures should be examined after the manufacturer's recommended incubation period or validated incubation time (see also 12.2 and 12.3). Growth of the test organism can be indicated by turbidity of the broth medium, growth on the surface of the broth, or sediment at the bottom of the tube, depending upon the characteristics of the test organism. If the growth medium is an integral part of the biological indicator (e.g. a self-contained biological indicator), growth or the lack of growth of the test organism should be interpreted according to the manufacturer's instructions.

Growth of the test organism in self-contained biological indicators can be shown by a colour change caused by a change in pH.

C.3.1.1.6 The results are recorded as the ratio of inoculated carriers with non-recoverable test organisms to the total number of inoculated carriers tested at each sub-lethal exposure.

C.3.1.2 Calculations using the HSKP

C.3.1.2.1 The calculations are based on a minimum of five exposure periods and should include at least:

— one set of samples in which all tested samples show growth;

— two sets of samples in which a fraction of the samples shows growth;

— two sets of samples, from sequential exposures, in which no growth is observed (see Table C.1).

NOTE HSKP is similar to LHSKP (see C.3.2), except that it uses a generic formula which is not limited to the same number of replicates at each exposure period or constant time intervals between exposures.

C.3.1.2.2 The mean D value, $\overline{D}$, is calculated using Equations (1) and (4). See 11.3.3.

$$\overline{D} = \frac{U_{\mathrm{HSK}}}{\log_{10} N_0 + 0{,}250\ 7} \tag{1}$$

where

$$U_{HSK} = \sum_{i=1}^{k-1} U_i \tag{4}$$

N_0 is the average viable count per indicator determined by the total viable count method (see ISO 11138-1:2006, Annex A).

The data required for the calculation are given in Table C.1.

Table C.1 — Examples of data collected for HSKP

Exposure period to sterilizing agent (min) t	Number of test samples exposed n	Number of test samples showing no growth r_i
$t_1(U_1)$	n_1	$r_1(r=0)$[a]
t_2	n_2	r_2
t_3	n_3	r_3
t_4	n_4	r_4
$t_5(U_{k-1})$	n_5	r_5
$t_6(U_k)$	n_6	$r_6(r=n_6)$
t_7	n_7	$r_7(r=n_7)$[a]
[a] The test is valid if there are no negative units, i.e. no negative test samples ($r=0$), with all units showing growth at the exposure preceding t_1, and all negative test samples ($r=n_7$), i.e. none showing growth at the exposure subsequent to t_6.		
NOTE t_1 is defined as the longest exposure period to the sterilizing agent in the exposure set where all test samples show growth. Exposure periods t_2 to t_5 are increasing exposure periods in the fraction negative area. Exposure periods t_6 and t_7 are two sequential exposure periods at which all samples show no growth.		

C.3.1.2.3 For times of exposure to sterilizing agent, t_1 to t_6, the factors χ and γ are calculated using Equations (C.1) and (C.2).

$$\chi_i = \frac{t_i + t_{(i+1)}}{2} \tag{C.1}$$

$$\gamma_i = \frac{r_i + 1}{n_i + 1} \frac{ri}{ni} \tag{C.2}$$

where

r_i is the number of test samples showing no growth at exposure period t_i;

n_i is the number of test samples exposed at exposure period t_i.

At t_1, all test samples show growth and so γ_i is the number of test samples $\frac{r_i + 1}{n_i + 1}$.

From the calculated values of χ_i and γ_i above, the value U_i can be calculated for each exposure period, t_i, using Equation (C.3).

$$U_i = \chi_i \ \gamma_i \tag{C.3}$$

C.3.1.2.4 The mean time to sterility, U_{HSK}, from any of the test samples can then be calculated as the sum of U_i for each exposure period t_1 to t_6:

$$U_{HSK} = \sum_{i=1}^{i=6} U_i \tag{C.4}$$

C.3.1.2.5 Where the interval between exposure period, d, is constant and the same number of test samples, n, is used at each exposure period, the mean time to sterility, U_{HSK}, can be calculated using Equation (C.5).

$$U_{HSK} = U_k - \frac{d}{2} - \frac{d}{n}\sum_{i=1}^{i=6} r_i \tag{C.5}$$

C.3.1.2.6 The mean D value, $\overline{D}$, can be calculated using Equation (1). See 11.3.3.

$$\overline{D} = \frac{U_{HSK}}{\log_{10} N_0 \ + \ 0{,}250\ 7} \tag{1}$$

NOTE 1 The Euler constant = 0,577 2.

NOTE 2 0,577 2/ln10 = 0,250 7.

where N_0 is the initial viable count of test organisms per test sample (see ISO 11138-1:2006, Annex A).

C.3.1.2.7 The 95 % confidence interval for $\overline{D}$ ($p = 0{,}05$), D_{calc}, is calculated using Equation (C.6).

$$D_{calc} = \overline{D} \pm 2\sqrt{V} \tag{C.6}$$

C.3.1.2.8 The variance, V, is calculated using Equation (C.7).

$$V = a\left(\frac{2{,}302\ 6}{\ln N_0 + 0{,}577\ 2}\right)^2 \tag{C.7}$$

C.3.1.2.9 The "a" for the variance is calculated using Equation (C.8).

$$a = 0{,}25\sum_{i=1}^{i=6}\left[\left(t_{(i+1)} - t_{(i-1)}\right)^2\left(\frac{r_i\left(n_i - r_i\right)}{{n_i}^2\left(n_i - 1\right)}\right)\right] \tag{C.8}$$

C.3.1.3 Example calculation of the Holcomb-Spearman-Karber Procedure (HSKP)

Table C.2 — Examples of data with non-constant time intervals and non-constant number of samples

Exposure period to sterilizing agent (min) t	Number of test samples exposed n	Number of test samples showing no growth r_i
$t_1 = 10$	$n_1 = 20$	$r_1 = 0$
$t_2 = 18$	$n_2 = 19$	$r_2 = 4$
$t_3 = 28$	$n_3 = 21$	$r_3 = 8$
$t_4 = 40$	$n_4 = 20$	$r_4 = 12$
$t_5 = 50$	$n_5 = 20$	$r_5 = 16$
$t_6 = 60$	$n_6 = 20$	$r_6 = 20$
$t_7 = 70$	$n_7 = 20$	$r_7 = 20$

C.3.1.3.1 Calculate χ_i and γ_i for each exposure period, t_i:

$$\chi_i = \frac{t_i + t_{(i+1)}}{2} \tag{C.9}$$

$$\chi_1 = \frac{t_1 + t_{(1+1)}}{2}$$

$$\chi_1 = \frac{10 + 18}{2} = 14$$

$$\chi_2 = \frac{18 + 28}{2} = 23$$

$$\chi_3 = \frac{28 + 40}{2} = 34$$

$$\chi_4 = \frac{40 + 50}{2} = 45$$

$$\chi_5 = \frac{50 + 60}{2} = 55$$

$$\chi_6 = \frac{60 + 70}{2} = 65$$

$$\gamma_i = \frac{r_i + 1}{n_i + 1} - \frac{r_i}{n_i}$$

$$\gamma_1 = \frac{r_1 + 1}{n_1 + 1} - \frac{r_1}{n_1}$$

$$\gamma_1 = \frac{4}{19} - \frac{0}{20} = 0{,}21$$

$$\gamma_2 = \frac{8}{21} - \frac{4}{19} = 0{,}17$$

$$\gamma_3 = \frac{12}{20} - \frac{8}{21} = 0{,}22$$

$$\gamma_4 = \frac{16}{20} - \frac{12}{20} = 0{,}2$$

$$\gamma_5 = \frac{20}{20} - \frac{16}{20} = 0{,}2$$

$$\gamma_6 = \frac{20}{20} - \frac{20}{20} = 0$$

NOTE For the calculations of γ_4 and γ_5, both γs = 0,2. This happens because the number of test samples showing no growth increase at a constant rate in this example.

C.3.1.3.2 Calculate U_i for each exposure period, t_i:

$$U_i = \chi_i \times \gamma_i \tag{C.10}$$

$U_1 = \chi_1 \times \gamma_1 = 14 \times 0{,}21 = 2{,}94$

$U_2 = 23 \times 0{,}17 = 3{,}91$

$U_3 = 34 \times 0{,}22 = 7{,}48$

$U_4 = 45 \times 0{,}2 = 9{,}0$

$U_5 = 55 \times 0{,}2 = 11{,}0$

$U_6 = 65 \times 0 = 0$

C.3.1.3.3 The mean time to sterility, U_{HSK}, is calculated using Equation (C.11).

$$U_{\text{HSK}} = \sum_{i=1}^{i=6} U_i \tag{C.11}$$

$U_{\text{HSK}} = U_1 + U_2 + U_3 + U_4 + U_5 + U_6$

$U_{\text{HSK}} = 2{,}94 + 3{,}91 + 7{,}48 + 9{,}0 + 11{,}0 + 0 = 34{,}33$

C.3.1.3.4 The mean D value, $\overline{D}$, is calculated using Equation (1). See 11.3.3.

$$\overline{D} = \frac{U_{\text{HSK}}}{\log_{10} N_0 + 0{,}250\,7} \tag{1}$$

where

N_0 is the initial population of 1×10^5;

$$\overline{D} = \frac{34{,}33}{5{,}000 + 0{,}250\,7} = 6{,}54 \text{ min.}$$

C.3.1.3.5 The 95 % confidence interval for $\overline{D}$ ($p = 0{,}05$), D_{calc}, is calculated using Equation (C.6). See C.3.1.2.7.

$$D_{\text{calc}} = \overline{D} \pm 2\sqrt{V} \tag{C.6}$$

C.3.1.3.6 The variance, V, is calculated using Equation (C.7). See C.3.1.2.8.

$$V = a\left(\frac{2{,}302\,6}{\ln N_0 + 0{,}577\,2}\right)^2 \tag{C.7}$$

C.3.1.3.7 The "a" in the variance formula for each t_i and summing all results is calculated using Equation (C.8). See C.3.1.2.9.

$$a = 0{,}25 \sum_{i=1}^{i=6} \left[\left(t_{(i+1)} - t_{(i-1)}\right)^2 \left(r_i \frac{(n_i - r_i)}{n_i^{\,2}(n_i - 1)} \right) \right] \tag{C.8}$$

$$a = 0{,}25\left[\left(t_{(1+1)} - t_{(1-1)}\right)^2\left(r_1\frac{(n_1 - r_1)}{n_1{}^2(n_1 - 1)}\right) + \left(t_{(2+1)} - t_{(2-1)}\right)^2\left(r_2\frac{(n_2 - r_2)}{n_2{}^2(n_2 - 1)}\right) + \left(t_{3+1} - t_{3-1}\right)^2\left(r_3\frac{(n_3 - r_3)}{n_3{}^2(n_3 - 1)}\right) +\right.$$

$$\left(t_{(4+1)} - t_{(4-1)}\right)^2\left(r_4\frac{(n_4 - r_4)}{n_4{}^2(n_4 - 1)}\right) + \left(t_{(5+1)} - t_{(5-1)}\right)^2\left(r_5\frac{(n_5 - r_5)}{n_5{}^2(n_5 - 1)}\right) +$$

$$\left.\left(t_{(6+1)} - t_{(6-1)}\right)^2\left(r_6\frac{(n_6 - r_6)}{n_6{}^2(n_6 - 1)}\right)\right]$$

$$a = 0{,}25 \times (28 - 10)^2 \times 4\left(\frac{19 - 4}{361 \times 18}\right) = 2{,}991\ 7 +$$

$$(40 - 18)^2 \times 8\left(\frac{21 - 8}{441 \times 20}\right) = 5{,}707\ 0 +$$

$$(50 - 28)^2 \times 12\left(\frac{20 - 12}{400 \times 19}\right) = 6{,}113\ 7 +$$

$$(60 - 40)^2 \times 16\left(\frac{20 - 16}{400 \times 19}\right) = 3{,}368\ 4 +$$

$$(70 - 50)^2 \times 20\left(\frac{20 - 20}{400 \times 19}\right) = 0{,}000\ 0$$

$$a = 0{,}25\ (2{,}991\ 7 + 5{,}707\ 0 + 6{,}113\ 7 + 3{,}368\ 4 + 0{,}000\ 0) = 0{,}25 \times 18{,}180\ 8$$

$$a = 0{,}25 \times 18{,}180\ 8 = 4{,}545\ 2$$

C.3.1.3.8 The variance, V, is calculated using Equation (C.7) now that "a" is calculated. See C.3.1.2.8.

$$V = a\left(\frac{2{,}302\ 6}{\ln N_0 + 0{,}577\ 2}\right)^2 \qquad \text{(C.7)}$$

where

$$N_0 = 1 \times 10^5;$$

$$V = 4{,}545\ 2\left[\frac{2{,}302\ 6}{\ln(1 \times 10^5) + 0{,}577\ 2}\right]^2 = 4{,}545\ 2\left(\frac{2{,}302\ 6}{11{,}513 + 0{,}577\ 2}\right)^2 = 4{,}545\ 2 \times (0{,}190\ 45)^2 =$$

$$= 4{,}545\ 2 \times 0{,}036\ 27 = 0{,}164\ 9.$$

C.3.1.3.9 The 95 % confidence interval for $\overline{D}$ ($p = 0{,}05$), D_{calc}, is calculated using Equation (C.6). See C.3.1.2.7.

$$D_{calc} = \overline{D} \pm 2\sqrt{V} \qquad \text{(C.6)}$$

C.3.1.3.10 Lower confidence limit is calculated using Equation (C.12):

$$D_{calc} = \overline{D} - 2\sqrt{V} \quad \text{(C.12)}$$

$$= 6{,}54 - 2\ \sqrt{0{,}164\ 9}$$

$$= 6{,}54 - (2 \times 0{,}406\ 1) = 5{,}73$$

C.3.1.3.11 Upper confidence limit is calculated using Equation (C.13):

$$D_{calc} = \overline{D} + 2\sqrt{V} \quad \text{(C.13)}$$

$$= 6{,}54 + \ 2\sqrt{0{,}164\ 9}$$

$$= 6{,}54 + (2 \times 0{,}406\ 1) = 7{,}35$$

C.3.2 Limited Holcomb-Spearman-Karber Procedure (LHSKP)

C.3.2.1 Calculations using the LHSKP

C.3.2.1.1 The calculations for the LHSKP are based on a minimum of five exposure periods and should include at least:

— one set of samples in which all tested samples show growth;

— two sets of samples in which a fraction of the samples shows growth;

— two sets of samples in which no growth is observed (see Table C.3).

C.3.2.1.2 LHSK procedure is similar to HSKP (see C.3.1), except that it uses a formula which requires the same number of replicates at each exposure period and constant time intervals between exposures.

Table C.3 — Examples of data collected for LHSKP with constant time intervals and constant number of samples

Exposure period to sterilizing agent (min) t	Number of test samples exposed n	Number of test samples showing no growth r_i
$t_1\ (U_1)$	n_1	$r_1\ (r = 0)$ [a]
t_2	n_2	r_2
t_3	n_3	r_3
t_4	n_4	r_4
$t_5\ (U_{k-1})$	n_5	r_5
$t_6\ (U_k)$	n_6	$r_6\ (r = n)$
t_7	n_7	$r_7\ (r = n)$ [a]

[a] The test is valid if there are no negative units, i.e. no negative test samples ($r = 0$), with all units showing growth at the exposure preceding U_1, and all negative replicates ($r = n$), i.e. no replicate showing growth at the exposure subsequent to U_k.

C.3.2.1.3 The mean time to sterility, U_{HSK}, is calculated using Equation (C.14).

$$U_{HSK} = U_k - \frac{d}{2} - \frac{d}{n}\sum_{i=1}^{k-1} r_i \qquad \text{(C.14)}$$

where

U_{HSK} is the mean time to sterility;

U_k is the first exposure to show no growth of the replicates;

d is the time or dose interval between exposures (being identical);

n is the number of replicates at each exposure (identical number at each exposure, e.g. 20);

$\sum_{i-1}^{k-1} r_i$ is the sum of the negatives between U_2 and U_{k-1} inclusive.

C.3.2.1.4 The mean D value, $\overline{D}$, can be calculated using Equation (1). See 11.3.3.

$$\overline{D} = \frac{U_{HSK}}{\log_{10} N_0 + 0{,}250\ 7} \qquad \text{(1)}$$

NOTE When following the method above, the LHSK procedure makes it possible to calculate the variance, V, the standard deviation (SD) and the 95 % confidence interval (the upper and lower confidence limits).

C.3.2.1.5 The variance, V, is calculated using Equation (C.15).

$$V = \frac{d^2}{n^2(n-1)} \times \sum_{i=1}^{k-1} r_i (n - r_i) \qquad \text{(C.15)}$$

C.3.2.1.6 The standard deviation (SD) is calculated using Equation (C.16).

$$SD = \sqrt{V} \qquad \text{(C.16)}$$

C.3.2.1.7 The 95 % confidence limits for $\overline{D}$ ($p = 0{,}05$), D_{calc}, are calculated using Equations (C.17), (C.18) and (C.19).

$$D_{calc} = \overline{D} \pm 2SD \qquad \text{(C.17)}$$

C.3.2.1.8 Lower confidence limit

$$D_{calc} = \frac{U_{HSK} - 2SD}{\log_{10} N_0 + 0{,}250\ 7} \qquad \text{(C.18)}$$

C.3.2.1.9 Upper confidence limit

$$D_{calc} = \frac{U_{HSK} + 2SD}{\log_{10} N_0 + 0{,}250\ 7} \qquad \text{(C.19)}$$

C.3.2.2 Example calculations of the Limited Holcomb-Spearman-Karber Procedure (LHSKP)

Table C.4 — Examples of data with constant time intervals and constant number of samples

Exposure period to sterilizing agent (min) t	Number of test samples exposed n	Number of test samples showing no growth r_i
$t_1 = 20$ (U_1)	$n_1 = 20$	$r_1 = 0$ ($r = 0$) [a]
$t_2 = 22$	$n_2 = 20$	$r_2 = 1$
$t_3 = 24$	$n_3 = 20$	$r_3 = 7$
$t_4 = 26$	$n_4 = 20$	$r_4 = 15$
$t_5 = 28$ (U_{k-1})	$n_5 = 20$	$r_5 = 19$
$t_6 = 30$ (U_k)	$n_6 = 20$	$r_6 = 20$ ($r = n$) [a]
$t_7 = 32$	$n_7 = 20$	$r_7 = 20$ ($r = n$)

[a] The test is valid if there are no negative units, i.e. no negative replicates ($r = 0$), at the exposure preceding U_1, and all negative replicates, i.e. all replicates showing growth, ($r = n$) at the exposure subsequent to U_k.

C.3.2.2.1 The mean D value, $\overline{D}$, is calculated using Equation (1). See 11.3.3.

$$\overline{D} = \frac{U_{\text{HSK}}}{\log_{10} N_0 + 0{,}250\,7} \tag{1}$$

where $N_0 = 1 \times 10^6$.

C.3.2.2.2 The mean exposure period, (time to sterility), U_{HSK}, required to obtain no growth (sterility) is calculated using Equation (C.14). See C.3.2.1.3.

$$U_{\text{HSK}} = U_k - \frac{d}{2} - \frac{d}{n}\sum_{i=1}^{k-1} r_i \tag{C.14}$$

where

$U_k = 30$;

$d = 2$;

$n = 20$.

$$U_{\text{HSK}} = 30 - \frac{2}{2} - \frac{2}{20} \times (0 + 0 + 1 + 7 + 15 + 19) = 24{,}8$$

$$\overline{D} = \frac{24{,}8}{6{,}000 + 0{,}250\,7} = 3{,}97 \text{ min (rounded to one decimal place } D = 4{,}0 \text{ min)}$$

C.3.2.2.3 The variance, V, is calculated using Equation (C.15). See C.3.2.1.5.

$$V = \frac{d^2}{n^2\,(n-1)} \times \sum_{i=1}^{k-1} r_i\,(n - r_i) \tag{C.15}$$

$$= \frac{2^2}{(20)^2\,(20-1)} \times \left[(1 \times 19) + (7 \times 13) + (15 \times 5) + (19 \times 1)\right] = 0{,}107\,4$$

C.3.2.2.4 The standard deviation (SD) is calculated using Equation (C.16). See C.3.2.1.6.

$$\text{SD} = \sqrt{V} \tag{C.16}$$

$$\text{SD} = \sqrt{0{,}107\,4} = 0{,}327\,7$$

C.3.2.2.5 The 95 % confidence limits for $\overline{D}$ ($p = 0{,}05$), D_{calc}, are calculated using Equations (C.17), (C.18) and (C.19). See C.3.2.1.7, C.3.2.1.8 and C.3.2.1.9.

$$D_{\text{calc}} = \overline{D} \pm 2\text{SD} \tag{C.17}$$

C.3.2.2.6 Lower confidence limit

$$D_{\text{calc}} = \frac{U_{\text{HSK}} - 2\text{SD}}{\log_{10} N_0 + 0{,}250\,7} \tag{C.18}$$

$$= \frac{24{,}8 - (2 \times 0{,}322\,7)}{6{,}000 + 0{,}250\,7} = \frac{24{,}144}{6{,}250\,7} = 3{,}86 \text{ min}$$

where $N_0 = 1 \times 10^6$.

C.3.2.2.7 Upper confidence limit

$$D_{\text{calc}} = \frac{U_{\text{HSK}} + 2\text{SD}}{\log_{10} N_0 + 0{,}250\,7} \tag{C.19}$$

$$= \frac{24{,}8 + (2 \times 0{,}322\,7)}{6{,}000 + 0{,}250\,7} = \frac{25{,}455}{6{,}250\,7} = 4{,}07 \text{ min}$$

C.3.3 Stumbo-Murphy-Cochran Procedure (SMCP)

C.3.3.1 Introduction

C.3.3.1.1 Other methods of analysing fraction negative data may be used when equivalence with the methods of C.3.1 and C.3.2 is demonstrated.

C.3.3.1.2 The formula for the SMCP requires one result in the fraction negative range consisting of time, t, the number of units negative for growth, r, the number of replicates, n, at one exposure period within the fraction negative range and the initial number of microorganisms per replicate, N_0.

C.3.3.1.3 To obtain a higher level of confidence using the SMCP, the D value should be calculated as the average of at least three runs in the fraction negative range in order to confirm reproducibility.

C.3.3.1.4 The same materials apply as those in C.2.

C.3.3.1.5 For a confidence interval of 95 %, not less than 50 replicates at each exposure period should be used and the condition $r/n < 0{,}9$ should be met in order to establish test criteria equivalent to C.3.1 and C.3.2. (See Reference [35].) Test samples should be subjected to a defined exposure period within the fraction negative range of the batch/lot.

C.3.3.2 Calculations using the SMCP

C.3.3.2.1 The D value is calculated using Equation (C.20).

$$D = \frac{t}{\log_{10} A - \log_{10} B} \tag{C.20}$$

where

t is the exposure period;

$\log_{10} A$ is $\log_{10}$ of initial population, N_0, per replicate;

$\log_{10} B$ is $\log_{10}$ of population after exposure period, t.

C.3.3.2.2 This equation can be restated for fraction negative data sets. See 11.3.3.

$$D = \frac{t}{\log_{10} N_0 - \log_{10}\left(\ln\frac{n}{r}\right)} \tag{5}$$

or

$$D = \frac{t}{\log_{10} N_0 - \log_{10} N_{\mu_i}} \tag{C.21}$$

where

N_{μ_i} is the natural log of the quotient of the number of replicates per test divided by the number of negative samples;

n is the number of replicates per exposure period;

r is the number of units sterile or showing no growth.

C.3.3.2.3 The 95 % confidence interval for $\overline{D}$ ($p = 0{,}05$), D_{calc}, is calculated using Equation (C.22).

$$D_{\text{calc}} = \frac{t}{\log_{10} N_0 - \log_{10}\left[\ln\left(\frac{1}{a}\right)\right]} \tag{C.22}$$

where $a = \frac{r}{n} \pm 1{,}96 \times \sqrt{\frac{r}{n} \times \frac{1 - r/n}{n}}$

C.3.3.2.4 The formula above can only be used if $n \times \frac{r}{n} \times \frac{n - r}{n} \geqslant 0{,}9$.

C.3.3.3 Example calculations of the Stumbo-Murphy-Cochran Procedure (SMCP)

Table C.5 — Calculations of D value using only one data set in the fraction negative zone

Exposure period to sterilizing agent (min) t	Number of test samples exposed n	Number of test samples showing no growth r_i
24	100	37

C.3.3.3.1 The D value is calculated using Equation (5). See 11.3.3.

$$D = \frac{t}{\log_{10} N_0 - \log_{10}\left[\ln\left(\frac{n}{r}\right)\right]} \tag{5}$$

where

t is the exposure period;

N_0 is the initial viable count per test organism per sample $= 1 \times 10^6$;

n is the number of replicates per exposure period;

r is the number of units sterile or showing no growth.

$$D = \frac{24}{6{,}000 - \log_{10}(\ln 2{,}702\,7)}$$

$$D = \frac{24}{6{,}000 - \log_{10}(0{,}994\,3)}$$

$$D = \frac{24}{6{,}000 - (-0{,}002\,5)}$$

$$D = \frac{24}{6{,}002\,5} = 4{,}00 \text{ min (rounded to one decimal place } D = 4{,}0 \text{ min)}$$

C.3.3.3.2 The 95 % confidence interval for $\overline{D}$ ($p = 0{,}05$), D_{calc}, is calculated as follows.

If $n \times \frac{r}{n} \times \frac{n-r}{n} \geqslant$ to 0,9, then the 95 % confidence interval can be calculated using Equation (C.22). See 3.3.2.3.

Lower confidence limit:

$$D_{calc} = \frac{t}{\log N_0 - \log_{10}\left[\ln\left(\frac{1}{a}\right)\right]} \tag{C.22}$$

where $a = \frac{r}{n} + 1{,}96 \times \sqrt{\frac{r}{n} \times \frac{1 - r/n}{n}}$

$$D_{\text{calc}} = \frac{24}{6{,}000 - \log_{10}\left[\ln\left(\frac{1}{a}\right)\right]}$$

where

$$a = \frac{37}{100} + 1{,}96 \times \sqrt{\frac{37}{100} \times \frac{1 - 37/100}{100}}$$

$$= 0{,}37 + 1{,}96 \times \sqrt{0{,}37 \times \frac{0{,}63}{100}}$$

$$= 0{,}37 + 1{,}96 \times \sqrt{0{,}37 \times 0{,}006\,3}$$

$$= 0{,}37 + 1{,}96 \times \sqrt{0{,}002\,331}$$

$$= 0{,}37 + 1{,}96 \times 0{,}048\,28$$

$$a = 0{,}465$$

$$D_{\text{calc}} = \frac{24}{6{,}000 - \log_{10}\left[\ln\left(\frac{1}{0{,}465}\right)\right]}$$

$$= \frac{24}{6{,}000 - \log_{10}(0{,}765\,7)}$$

$$= \frac{24}{6{,}000 - (-0{,}115\,9)}$$

$$= \frac{24}{6{,}000 + 0{,}115\,9}$$

$$D_{\text{calc}} = \frac{24}{6{,}115\,9} = 3{,}92$$

Upper confidence limit:

$$D_{\text{calc}} = \frac{t}{\log_{10} N_0 - \log_{10}\left[\ln\left(\frac{1}{a}\right)\right]} \qquad \text{(C.22)}$$

where $a = \frac{r}{n} - 1{,}96 \times \sqrt{\frac{r}{n} \times \frac{1 - r/n}{n}}$

$$D_{\text{calc}} = \frac{24}{6{,}000 - \log_{10}\left[\ln\left(\frac{1}{a}\right)\right]}$$

where

$$a = \frac{37}{100} - 1{,}96\sqrt{\frac{37}{100} \times \frac{1 - 37/100}{100}}$$

$$= 0{,}37 - 1{,}96\sqrt{0{,}37 \times \frac{0{,}63}{100}}$$

$$= 0{,}37 - 1{,}96\sqrt{0{,}37 \times 0{,}006\ 3}$$

$$= 0{,}37 - 1{,}96\sqrt{0{,}002\ 331}$$

$$= 0{,}37 - 1{,}96 \times 0{,}048\ 28$$

$$a = 0{,}37 - 0{,}095 = 0{,}275$$

$$D_{\text{calc}} = \frac{24}{6{,}000 - \log_{10}\left[\ln\left(\frac{1}{0{,}275}\right)\right]}$$

$$= \frac{24}{6{,}000 - \log_{10}(1{,}291)}$$

$$= \frac{24}{6{,}000 - 0{,}111}$$

$$D_{\text{calc}} = \frac{24}{5{,}889} = 4{,}08$$

Annex D
(informative)

Examples of documentation for biological indicators prepared by the user

D.1 General

D.1.1 Sources of microorganisms

There are different sources of microorganisms for biological indicators. Biological indicators could be delivered from a manufacturer as a ready-to-use system with resistance characteristics in accordance with the ISO 11138 series. Consult the main document for commercially available biological indicators. Commercially available suspensions of microorganisms delivered from a manufacturer of biological indicators can be used to inoculate products, thereby using the product as the carrier. Alternatively, an in-house-made suspension could be produced from a commercially available strain. In particular circumstances, microorganisms isolated from the production plant (in-house isolates) can represent the most resistant microorganisms likely to be found in or on the products to be sterilized. In such cases, biological indicators can be produced using the relevant microorganism (see 7.3 and 7.4).

Biological indicators can be prepared from microorganisms from one of three sources:

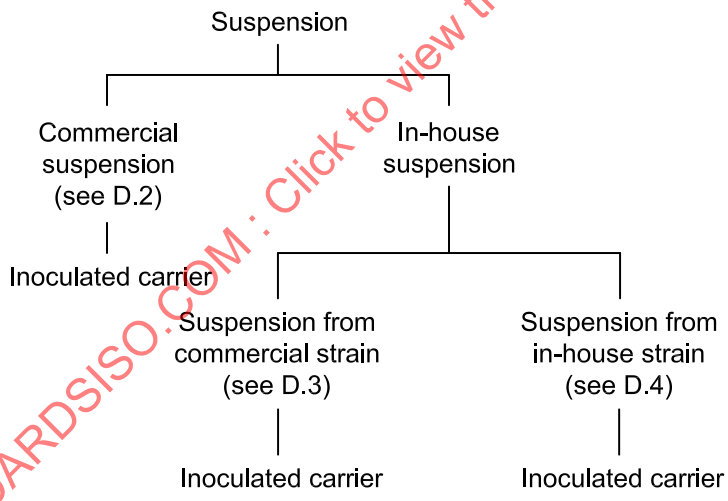


Figure D.1 — Sources of microorganisms

D.1.2 Documentation

A list of relevant documents can include:

a) work instructions for production and handling of microbiological strains, preparation of in-house suspensions, inoculation of carriers, and handling of inoculated carriers and in-house biological indicators;

b) work instructions for handling of the microbiological systems after sterilization-cycle processing;

c) protocols for validation studies and results.