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Sterilization of health care products — Biological indicators —

Part 7: Guidance for the selection, use and interpretation of results

Stérilisation des produits de santé — Indicateurs biologiques —

Partie 7: Directives générales pour la sélection, l'utilisation et l'interprétation des résultats

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Foreword

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

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

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

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

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

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

This first edition cancels and replaces ISO 14161:2009, which has been technically revised.

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

Introduction

This document provides guidance regarding the selection, use and interpretation of results of biological indicators used to develop, validate and monitor sterilization processes. The procedures described in this document 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 document is not to stipulate 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 avoid misleading results.

In this document, users will find guidance on selection of the correct biological indicator for their particular sterilization process (see the ISO 11138 series) and critical parameters as well as guidance on its appropriate use.

The selection of an appropriate biological indicator for the particular process used is critical. 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 performance of a biological indicator can be adversely affected by the conditions of storage and transport prior to its use, by inappropriate/non-indicated use of the biological indicator or by the sterilizer process parameters. In addition, the incubation procedure used after exposure to the process, including incubation temperature and culture medium type, supplier and specific batch, can affect measured resistance as a function of recovery and growth. For these reasons, the recommendations of the biological indicator manufacturer for transportation, storage and use should be followed. After exposure, the aseptic transfer (if applicable) and incubation of biological indicators as specified by the biological indicator manufacturer is critical for obtaining correct results.

It is important to note 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. Suitable training is necessary for personnel conducting these studies.

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

Sterilization of health care products — Biological indicators —

Part 7: Guidance for the selection, use and interpretation of results

1 Scope

This document 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.

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

It is not applicable to combination processes using, for example, washer-disinfectors or flushing and steaming of pipelines.

It does not specify requirements for the selection and use of biological indicators intended to monitor vaporised hydrogen peroxide processes for isolator and room biodecontamination processes at atmospheric pressure.

It is not applicable to liquid immersion sterilization processes.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

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

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

— IEC Electropedia: available at http://www.electropedia.org/

3.1
aseptic technique
conditions and procedures used to minimize the risk of the introduction of microbial contamination

[SOURCE: ISO 11139:2018, 3.16]

3.2
bioburden
population of viable microorganisms on or in a product and/or sterile barrier system

[SOURCE: ISO 11139:2018, 3.23]

3.3
biological indicator
BI
test system containing viable microorganisms providing a specified resistance to a specified sterilization process

[SOURCE: ISO 11139:2018, 3.29, modified — "BI" has been added to term.]

3.4
***D* value**
D_{10} value
time or dose required under stated conditions to achieve inactivation of 90 % of a population of the test microorganisms

[SOURCE: ISO 11139:2018, 3.75]

3.5
holding time
period during which process parameters are maintained, within their specified tolerances

[SOURCE: ISO 11139:2018, 3.133]

3.6
inoculated carrier
supporting material on or in which a specified number of viable test microorganisms has been deposited

[SOURCE: ISO 11139:2018, 3.144]

3.7
inoculation
addition of a defined amount of a characterized microbial entity into or on to an item

3.8
log reduction
LR
reduction in number of viable microorganisms

Note 1 to entry: Expressed in log units.

3.9
operational qualification
OQ
process of obtaining and documenting evidence that installed equipment operates within predetermined limits when used in accordance with its operational procedures

[SOURCE: ISO 11139:2018, 3.220.3]

3.10
performance qualification
PQ
process of establishing by objective evidence that the process, under anticipated conditions, consistently produces a product which meets all predetermined requirements

[SOURCE: ISO 11139:2018, 3.220.4]

3.11
process challenge device
PCD
item providing a defined resistance to a cleaning, disinfection, or sterilization process and used to assess performance of the process

[SOURCE: ISO 11139:2018, 3.205]

3.12
process challenge location
PCL
site chosen within a load as the position at which the least microbiological inactivation is expected to be delivered

[SOURCE: ISO 11139:2018, 3.206]

3.13
process parameter
specified value for a process variable

Note 1 to entry: The specification for a process includes the process parameters and their tolerances.

[SOURCE: ISO 11139:2018, 3.211]

3.14
process variable
chemical or physical attribute within a cleaning, disinfection, packaging, or sterilization process, changes in which can alter its effectiveness

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

[SOURCE: ISO 11139:2018, 3.213]

3.15
reference microorganism
microbial strain obtained from a recognized culture collection

[SOURCE: ISO 11139:2018, 3.228]

3.16
resistometer
test equipment designed to create specified combinations of the physical and/or chemical parameters of a sterilization process

[SOURCE: ISO 11139:2018, 3.233]

3.17
spore log reduction
SLR
negative exponent to the base 10 describing the decrease in the number of spores

Note 1 to entry: It is expressed as a logarithm.

[SOURCE: ISO 11139:2018, 3.260]

3.18
sterile
free from viable microorganisms

[SOURCE: ISO 11139:2018, 3.271]

3.19
sterility assurance level
SAL
probability of a single viable microorganism occurring on an item after sterilization

[SOURCE: ISO 11139:2018, 3.275, modified — Note 1 to entry has been deleted.]

3.20
sterilization
validated process used to render product free from viable microorganisms

Note 1 to entry: 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.

[SOURCE: ISO 11139:2018, 3.277]

3.21
survival-kill window
extent of exposure to a sterilization process under specified conditions where there is a transition from all biological indicators showing growth to all biological indicators showing no growth

[SOURCE: ISO 11139:2018, 3.292]

3.22
third party
person or body that is recognized as being independent of the parties involved, as concerns the issue in question

Note 1 to entry: Parties involved are usually supplier ("first party") and purchaser ("second party") interests.

3.23
z **value**
change in temperature of a thermal sterilization or disinfection process that produces a tenfold change in *D* value

Note 1 to entry: It is expressed in degree Celsius (°C).

[SOURCE: ISO 11139:2018, 3.326]

4 General

4.1 This document provides guidance on biological indicators that can apply generally for any sterilization process, including new sterilization processes covered by ISO 14937.

4.2 The use of biological indicators is normally documented in the user's standard operating procedures (SOPs), procedures, or instructions.

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

4.3 Biological indicators should 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. Similarly, when an indicator failure takes place when the physical and/or chemical variables of the sterilization process are within the specified limits, the reason for the sterilizer's failure to inactivate the indicator 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 (e.g. Risk Group 1, WHO, 2004). 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 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 microorganisms employed in biological indicators typically have resistance to sterilization which exceeds that of common bioburden microorganisms, although some microorganisms can exhibit a resistance to sterilization in excess of that of the test microorganisms. 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 microorganisms, extended sterilization processing, based on the bioburden, could be required.

4.6 Biological indicators are not intended for use in any sterilization process other than that specified by the biological indicator 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. 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.

4.7 The user who is responsible for the sterilization of product should ensure that the type of biological indicator employed to validate and/or routinely monitor a given sterilization process is appropriate for that use.

4.8 The manufacturer's recommendations for the transportation, storage and use of the biological indicators should always be followed. Failure to do so can compromise the performance characteristics of the biological indicator. If the user removes the inoculated carrier from the biological indicator's primary packaging, or adds additional packaging over the 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.

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

4.10 Users who employ biological indicators for sterilization process development, validation and/or routine monitoring of sterilization should be properly trained in their use.

4.11 The time between completion of sterilization process and incubation should be within the manufacturer's stated time or should be justified as described in 8.2.3.

4.12 Transfer of microorganisms exposed to the sterilization process to the appropriate recovery medium should be done using aseptic technique.

4.13 Self-contained biological indicators are specifically designed to eliminate the need for aseptic handling because all of the components required to effect post process incubation are enclosed within the primary packaging which need not be opened (see 5.4).

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

4.15 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 may include audits by regulators or Notified Bodies (see 6.2.2). If a user wishes to carry out a population and/or resistance determination it is essential that they use the method specified by the manufacturer.

4.16 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 ISO 13485 and Reference [16]). 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.

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.

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 process 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 and ISO 9001).

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 log-linear 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 Annexes E and F). The strain of the test organism, the production method, the suspension fluid, the carrier and packaging materials and the testing conditions all affect the resistance characteristics of the biological indicators (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 Depending upon placement within the load and the specific sterilization process conditions at those discrete locations, biological indicators from the same batch can show different survival capabilities (see 7.2.3). Users of biological indicators should note that 10 indicators spread throughout the load are

not considered replicates due to the differences in lethality that may 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 process 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 sterilisability, identification of the most 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 product 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) 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. This is because 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 (clumping), coating effects, and/or bacteriostatic or bactericidal effects of the material.

5.2.3 The methods used to recover test organisms 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. A change in survival characteristics of test organisms due to inoculation can affect the observed percent recovery of the original inoculum. 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 defined conditions. The resistance characteristics of a spore suspension provided by a biological indicator supplier 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 11138-1:2017, Annex B). Caution should be exercised to ensure that the carrier material selected is able to withstand sterilization processing without adversely affecting its performance characteristics 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 packaging or additional packaging is placed over the primary packaging for cycle development, cycle validation studies, or for process challenge devices (PCDs) 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 determine 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 11138-1:2017, 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 1 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 8.2.3).

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 2 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 might not fit into locations within the product that represent the process challenge locations (PCLs). If a biological indicator cannot be placed into a load without deforming it or otherwise potentially compromising its primary packaging, then a different biological indicator should 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.

6 Selection of supplier

6.1 General

6.1.1 The user of biological indicators should, whenever possible, make purchase decisions ensuring that the biological indicators chosen meet standard specifications. 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 can be provided by one or more of the following:

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

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

b) a statement from the manufacturer that the biological indicators meet the agreed specifications between the supplier and the user;

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

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 ensure that the BI supplier continues to maintain the expected quality standards, such as a BI supplier or BI manufacturer's declaration of conformity to standards. If the user has not established the supplier relationship required to be ensured of consistent biological indicator performance, additional testing could be necessary until an appropriate assurance can be established that the biological indicators meet the BI manufacturer's label claim and/or user requirements. (For information on documentation, see Annex D.)

6.1.5 Testing by the user, if deemed necessary, can consist of population assays and defined resistance tests such as *D* value or survival-kill time on samples from each new batch of biological indicators received (see also 8.6 and Clause 11). Testing should be conducted under exact conditions specified by the manufacturer. Provided that the biological indicator manufacturer produces the based upon detailed standard specifications, i.e. the ISO 11138 series, and the user uses the biological indicator as intended by the biological indicator 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 11138-1:2017, 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 user might need or want evidence 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 conformity 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 and ISO/IEC 17011).

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

6.2.2 Manufacturer audit

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

NOTE The auditing standard ISO 19011 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) conditions for culturing of test organisms, including growth medium and components, incubation temperature and 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) 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 biological indicator;

   2) storage stability and continued conformity of the biological indicator 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 11135, ISO 14937, ISO 17665-1 and ISO 20857).

7.1.2 When 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 product family 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 Biological indicators should 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.

7.1.5 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. 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.

7.1.6 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 are 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.

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 should be validated against the requirements of those standards and the statistical confidence of the bioburden estimate be established.

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 an SAL of at least 10^{-6} (see ISO 11135 and ISO 14937);

c) that, at half cycle, the user can typically demonstrate at least a 6-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 overkill 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 PCL(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 6-log reduction in the population of organisms should be demonstrated within one-half the normal holding time or dose of the sterilization process 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 (SLR) 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 measurements should be used to establish the process challenge locations throughout the load 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 (SAL). 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 SAL 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 test organism population of less than 10^6 CFU (colony-forming units) per carrier can be employed, as long as the desired SAL 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 ensure that the challenge provided is less than that of the biological indicator. One possible method to determine this would be to run sub-lethal sterilization cycles to determine that the bioburden population does not survive as great an exposure as does the biological indicator.

7.3.5 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.

7.3.6 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 might be less resistant to the process than those specified in the ISO 11138 series (see Figure A.2).

7.3.7 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 and ISO 17665-1). Alternatively, the holding period or dose 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 Reference should be made 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 Information on validation and routine monitoring and the applicability of absolute bioburden methods is given in the relevant International Standards for the particular sterilization process. 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

When 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.

Validation of a sterilization process requires documentation that the process is capable of consistently producing a product that meets its predetermined specifications (see ISO 11135, ISO 14937, ISO 17665-1 and ISO 20857).

8.2 Placement and handling of biological indicators

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

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

8.2.3 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.

8.2.4 Applicable requirements for worker safety should be observed when removing the biological indicators from the sterilizer.

8.3 Sterilizer qualification

8.3.1 IQ and OQ are 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 11135, ISO 17665-1 and ISO 20857).

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 type tests 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 process conformity with predetermined specifications; relevant International Standards such as ISO 11135, ISO 14937, ISO 17665-1 and ISO 20857 can 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 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 verify 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 processes, ISO 17665-1 or ISO 11135 should be used.

8.6.3 A minimum frequency for evaluating the resistance characteristics of a biological indicator batch 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).

NOTE For biological indicators purchased from a qualified supplier, resistance characteristics can be expected to remain within the expected tolerance (see ISO 11138-1) throughout the labelled shelf-life if stored as recommended on labelling.

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), might need to be conducted during validation in order to quantify the lethality. For well developed, validated processes parametric release may be possible (e.g. ethylene oxide, see ISO 11135; or dry heat, see ISO 20857), however local regulatory requirements can also require the use of biological indicators as part of process monitoring and batch release. For processes where the process variables cannot be reliably measured and therefore parametric release of batches is not possible, biological indicators provide the best available alternative for demonstrating microbial lethality in the sterilization process. See ISO 11135 for ethylene oxide and ISO 20857 for dry heat.

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 process 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 performance of the biological indicator (see 8.2.3).

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 ISO 11138 series if the population and/or resistance is below the minimum requirements of the respective part (see ISO 11138-1:2017, 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 PCD (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). When self-contained biological indicators are employed the need for aseptic handling is eliminated.

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

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

9.3.2 PCDs should be designed with consideration given to the various process parameters that influence the sterilization process. Composition of a PCD depends on the type of cycle to be monitored, as well as the type of product to be sterilized. Examples of process challenge devices can be found in local standards such as ANSI/AAMI ST79 (steam sterilization), EN 285 (steam sterilizers), EN 1422 (EO sterilizers).

9.3.3 PCDs can be commercially available as prefabricated sets, often called “biological test packs”. PCDs 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 Interpretation and acceptance criteria

10.1 General

10.1.1 The criteria for acceptance of a sterilization process as satisfactory should be decided upon during the sterilization process 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. A positive biological indicator could be the result of a sterilization process failure undetected by physical or chemical measurements resulting in inadequate lethality to the selected biological indicator.

10.2.2 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, a faulty test system or laboratory/operator error 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 batch 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 batch 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 or genetic identification methods can be useful in determining that the growth is not the indicator organism.

NOTE Some indicator organisms can be gram variable.

10.2.3 The particular sterilizer, type of product, and the loading of the product all affect the sterilization process. The resistance characteristics of the biological indicator 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.

10.2.4 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, test 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[17][18][19][20][21][22][23][24][25].

11.2 Nominal population of test organism

11.2.1 ISO 11138-1 requires the manufacturer to report 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 BI population as labelled by the manufacturer should be used in all calculations following verification. 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[19]. 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). The fluid should be sterilized prior to use and its sterility validated.

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.

11.3 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 differences between the three approaches are as described in a) to c).

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 CFUs 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) *Survival-kill response characteristics*: 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 or dose intervals of the sterilization process (see ISO 11138-1:2017, 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 10 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 EN 285 and Reference [26]).

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 exposed (see Figure A.4).

This document 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 conditions, such as times or doses, differ by a constant time interval and if an identical number of replicates is exposed at each exposure time 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 ozone.

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.

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

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.

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 2 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

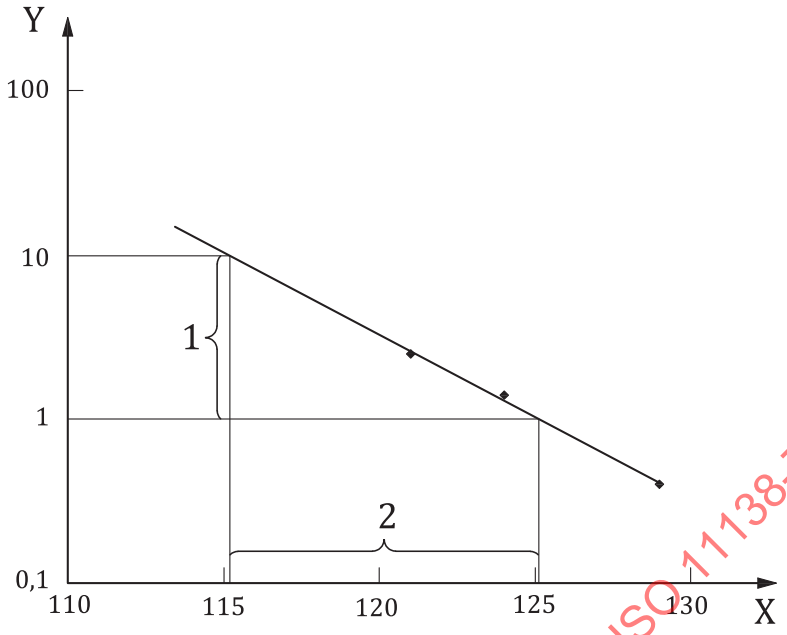
11.4.1 General

The z value is equal to the negative reciprocal of the slope of the best-fit rectilinear curve as determined by regression analysis. 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[27]. The line-of-best-fit will be linear (Figure 1) and the z value estimate is the negative reciprocal of the slope.



Key

X temperature, in °C

Y *D* value, in min, plotted on logarithmic scale

1 one log reduction

2 *z* value, in °C

Figure 1 — Graphically plotting the *z* value

11.4.3 Mathematically calculating the *z* value

The slope of the best-fit rectilinear curve is calculated using Formula (1):

$$m = \frac{(nG) - (AB)}{(nC) - (A^2)} \tag{1}$$

where

m is the slope of the best-fit rectilinear curve;

n is the number of *D* value/temperature pairs;

$G = \sum\left[t(\log_{10} y)\right]$;

$A = \sum\left[(t)\right]$;

$B = \sum\left[(\log_{10} y)\right]$;

$C = \sum\left[(t^2)\right]$;

t is exposure time;

y is minimum *D* value.

The z value is equal to the negative reciprocal of the slope obtained and is calculated using Formula (2):

$$z \text{ value} = -1\left(\frac{1}{m}\right) \tag{2}$$

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 above calculations are expressed to four decimal places to not reduce the accuracy of the above calculations. Rounding to one decimal place occurs at the end of the calculation.

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, as shown by Formula (3):

$$F_{\left(T_{\text{ref},z}\right)} = 10^{\left(\frac{T-T_{\text{ref}}}{Z}\right)} \tag{3}$$

where

T is the temperature;

T_{ref} is the reference temperature;

z is the specific z value.

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[28]. 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 SLR value for the process.

11.6 Establishing spore-log-reduction

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 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 Formula (4).

$$F_{(T,z)} = D_T\left(\log N_0 - \log N_F\right) \tag{4}$$

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 SLR which is represented by Formula (5):

$$\text{SLR} = \log N_0 - \log N_F \tag{5}$$

The thermal-death-time can therefore be expressed using Formula (6):

$$F_T = D_T \times \text{SLR} \tag{6}$$

The process SLR is then used to calculate the product SAL.

11.7 Sterility assurance level calculation

The probability of a non-sterile unit from a microbial control process is expressed as SAL. The SAL calculation uses Formula (7):

$$\text{SAL} = 10^{-(\text{SLR} - \log N_0)} \tag{7}$$

where

N_0 is the initial spore population or bioburden;

SLR is the spore-log-reduction.

The commonly accepted SAL is 10^{-6}, i.e. one in a million chance of having a non-sterile unit. Therefore, the total integrated lethality should deliver an excess of six more SLR equivalents than the microbial challenge that was sterilized.

NOTE This concept is graphically expressed in Figures A.1 and 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.

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 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. the time from the end of the sterilization process to the beginning of the incubation). 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 culture 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.

12.2 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* can be incubated at temperatures in the range of 30 °C to 37 °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 11138-1:2017, Annex B 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 incubation period information provided by the biological indicator manufacturer is validated using defined exposure and incubation conditions. The exposure conditions can vary from those in the user's facility. Therefore, the user may consider verification of the biological indicator manufacturer's recommendations for incubation time, based on the user's quality policies and procedures.

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 11138-1:2017, Annex B recommends an incubation period of 14 d.

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 sterilizing 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.

12.3.4 Self-contained biological indicators might not contain a sufficient volume of media to allow for recovery over prolonged incubation periods in some test settings, without media dry out. The manufacturer should be contacted for instructions on how to carry out extended incubations for their self-contained biological indicators.

Manufacturers are responsible for ensuring that the carrier and primary packaging of self-contained biological indicators do not retain or release enough residual sterilant to inhibit the growth of low numbers of surviving test organisms (ISO 11138-1). The manufacturer should be consulted for more information.

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 batches and to reserve suitable quantities of batches found to provide the desired growth performance. This would allow comparison with new batches.

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 User-supplied culture medium should not be overprocessed, 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[17][22][29][30].

12.4.5 Each batch of growth medium should be checked by a suitable growth promotion test and compared with a batch previously used, so as to determine batch-to-batch consistency. The growth promotion testing should include the specific test organism used in the biological indicator.

13 Third-party considerations

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 11138-1:2017, Annex B[24][25][26].

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 conformity with ISO/IEC 17025 or that has a recognized quality system for the service.

13.2 Minimum requirements from ISO 11138-1 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 conditions	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 11138-1:2017, Annex B)	50	2	100
Minimum total number depending on choice of combination of methods:			124 or 204

NOTE Common test conditions for specific sterilization methods have developed over time and are presented in subsequent parts of the ISO 11138 series.

[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 11138-1:2017, Annex B). The resistance characteristics are determined in accordance with 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[25].

ISO 11138-1:2017, Annex B 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 applies 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. Likewise, if a user purchases biological indicators and stores them at a central repository for subsequent further distribution, that user should ensure that conditions are adequate to retain the performance characteristics of the 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.

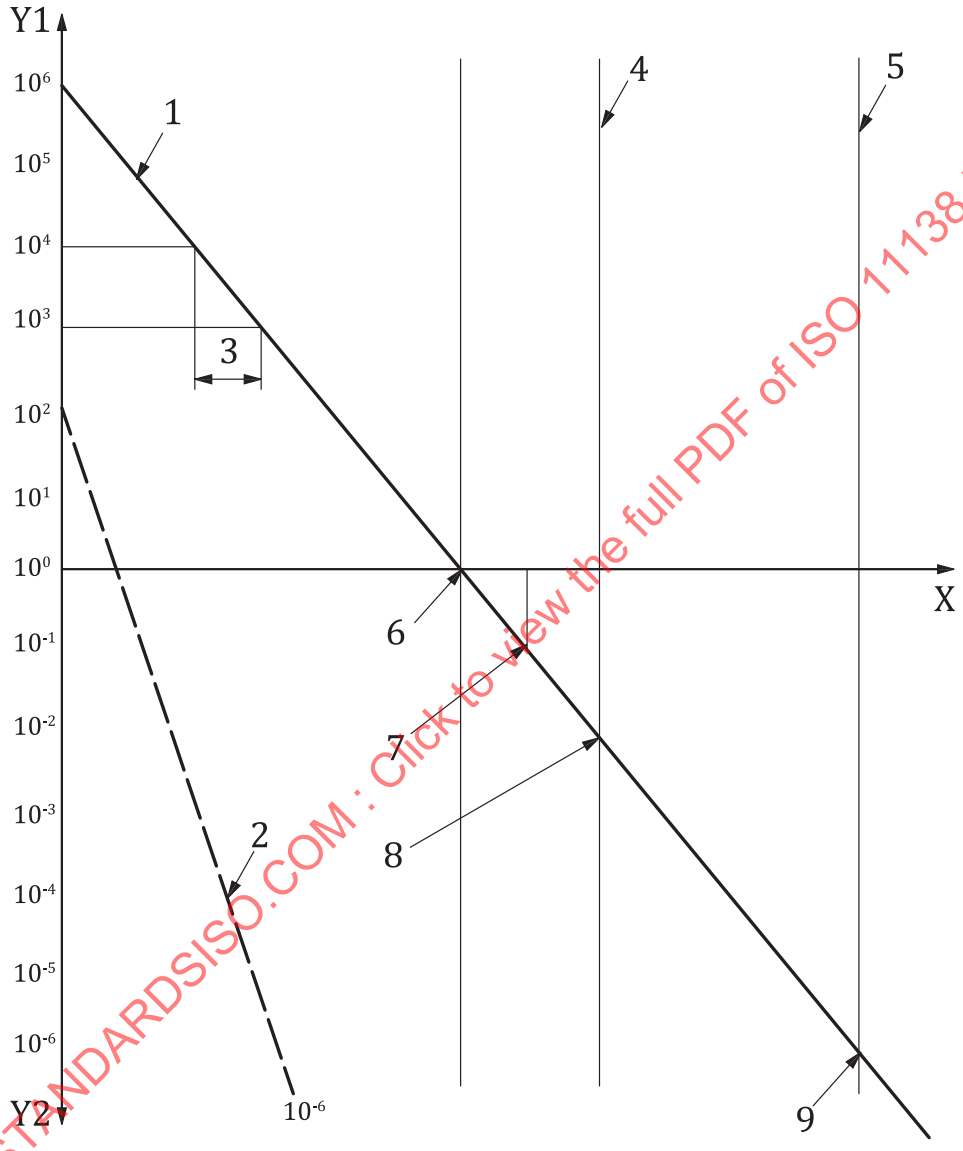
16 Disposal of biological indicators

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, non-sterile and 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.

Biological indicators can sometimes be defined as hospital waste.

Annex A
(informative)

Microbiological inactivation kinetics and enumeration techniques



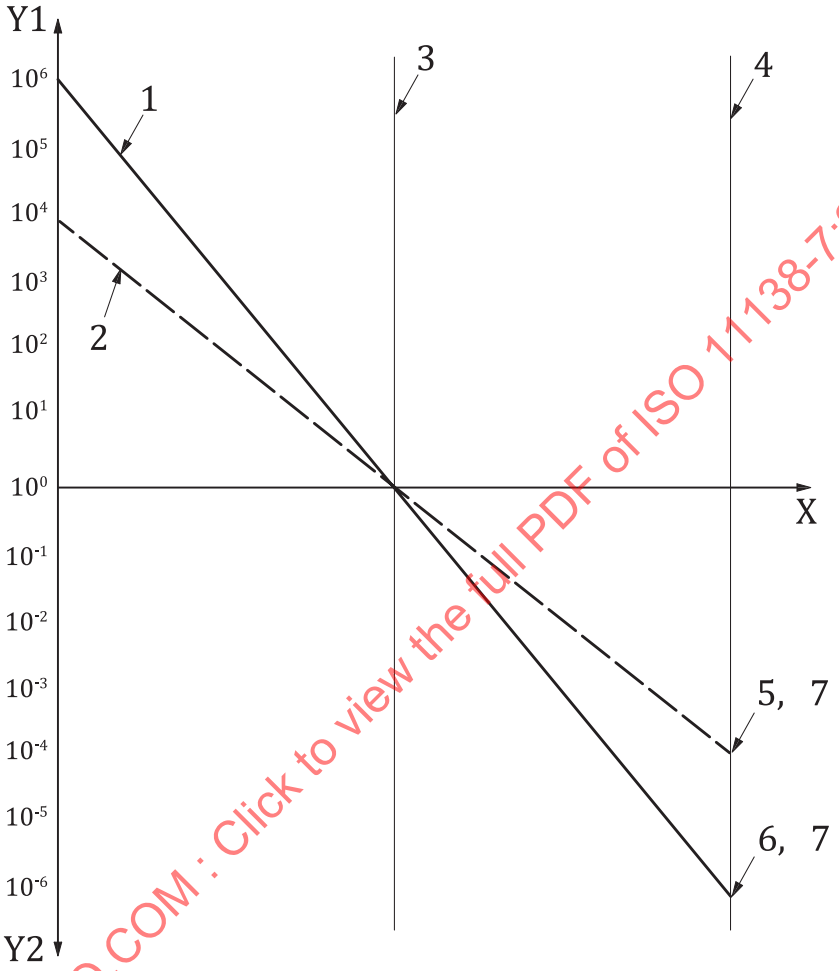
Key

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

NOTE 1 Log reduction for product achieved prior to the minimum sterilization process conditions.

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.

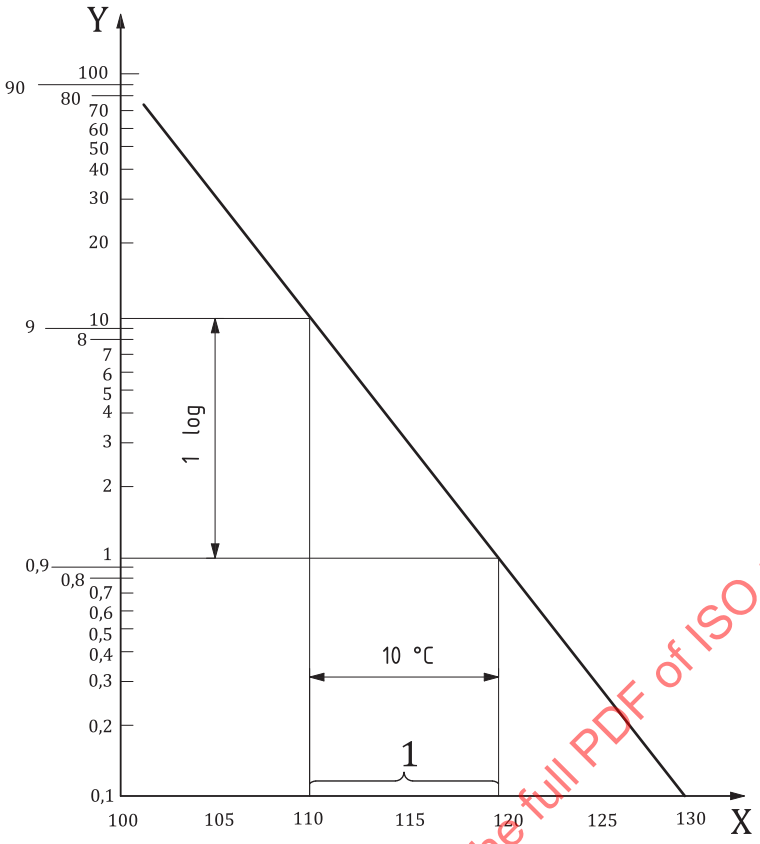
Figure A.1 — Examples of relationship between the biological indicator and the product bioburden in a reference microorganism method



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 conditions
5 theoretical eight log reduction (D_{BI} = 1,5 × 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



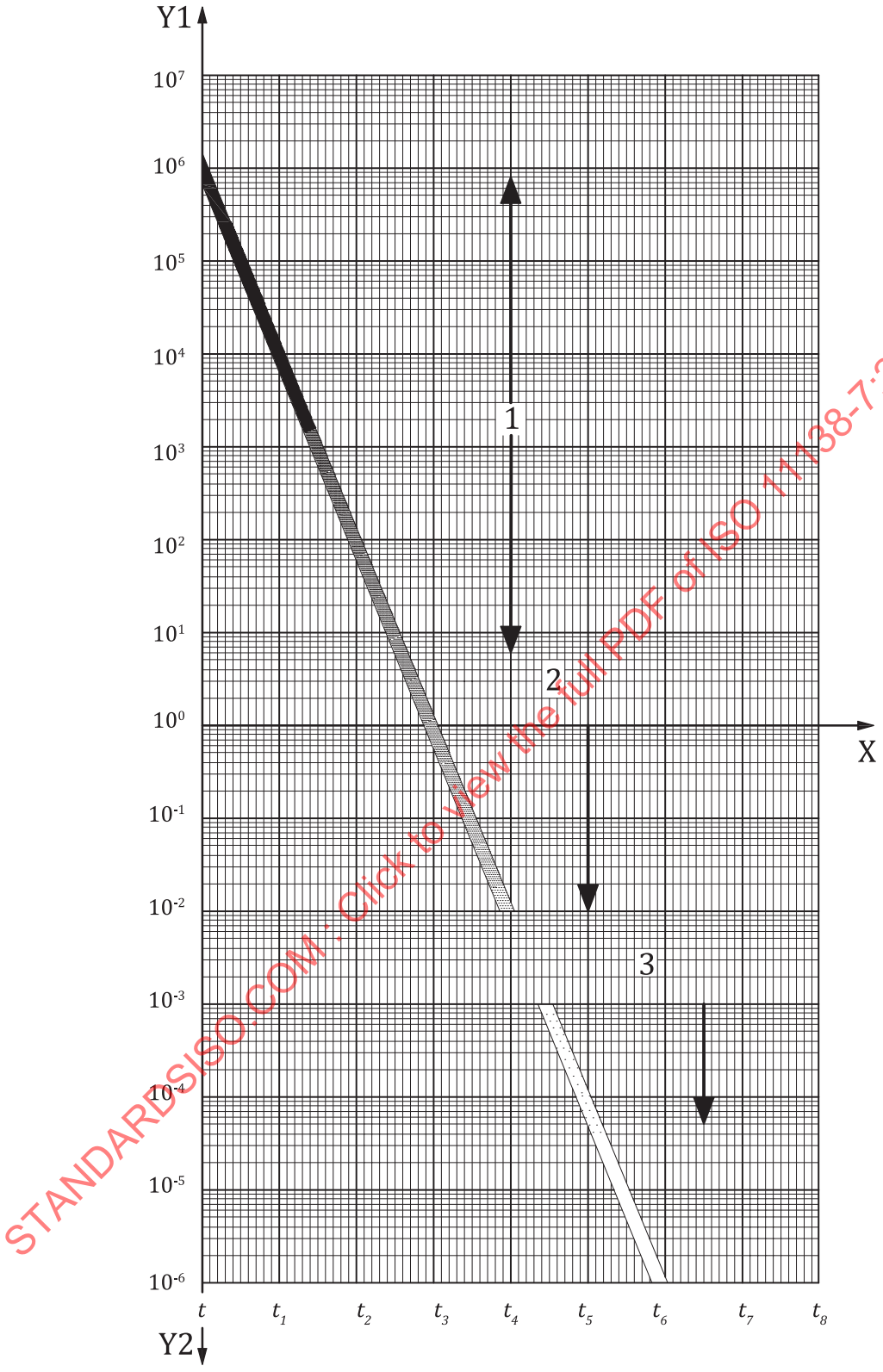
Key

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 PCD 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 PCD 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 PCD depends on the kind of goods to be sterilized and the operating cycle. The biological indicator should not interfere with the function of the PCD.

In some PCDs, 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 can 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 comprises 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 can include requirements for such standard test packs.

B.4 User's process challenge devices

This type of PCD is specially designed to meet the criteria for the PCL(s) in the load. The packaging and design should reflect the standardized load to be examined and varies with the load. The user's PCD 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 PCL(s).

B.5 Biological test packs

This is a common description of commercially available PCDs 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 *D* value determination by fraction-negative method

NOTE Adapted from ISO 11138-1:2017, Annex D.

C.1 Principles

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 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 can be applicable, particularly when the survival-kill window 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 conditions remaining within a defined window (steady-state), except the process variable used to calculate the *D* value. 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 may be used to calculate equivalent time "*U*". Both terms are found in the literature.

NOTE In this annex, the variable "*t*" can be replaced by any other process variable used to calculate the *D* value.

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 Procedure

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

C.3.1.1 Procedure

C.3.1.1.1 Test samples should be subjected to graded exposures to the defined exposure conditions 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 A minimum of five exposure conditions should be used including at least one set of samples in which all test samples show growth, two sets of samples in which a fraction of the test samples show growth, and two sets of test samples, from sequential exposures, in which no growth is observed.

NOTE Details of requirements for specific resistometer process parameters are provided in relevant parts of the ISO 11138 series.

C.3.1.1.3 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. 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 12.2 and 12.4). 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.

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 time or validated incubation time period (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 and broth medium. If the growth medium is an integral part of the biological indicator (e.g. a self-contained biological indicator), growth or no growth of the test organism might be indicated by a pH colour change and should be interpreted according to the manufacturer's instructions.

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, and

— 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 condition or constant time intervals between exposures.

C.3.1.2.2 The average D value is calculated using Formula (C.1). See 11.3.3.

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

where

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

N_0 is the average viable count per indicator determined by the total viable count method (see ISO 11138-1:2017, Annex A);

U_i is the ith treatment time;

k is the first exposure point with all units negative.

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

Table C.1 — Examples of data collected for HSKP

Time of exposure 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]

NOTE t_1 is defined as the longest exposure time to the sterilizing agent in the exposure set where all test samples show growth. Exposure times t_2 to t_5 are increasing exposure times in the fraction-negative area. Exposure times t_6 and t_7 are two sequential exposure periods at which all samples show no growth.

[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.

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 Formulae (C.2) and (C.3):

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

$$\gamma_i = \frac{r_{(i+1)}}{n_{(i+1)}} - \frac{ri}{ni} \tag{C.3}$$

where

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

n_i is the number of test samples exposed at exposure condition 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 Formula (C.4).

$$U_i = \chi_i \ \gamma_i \tag{C.4}$$

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, as shown by Formula (C.5):

$$U_{HSK} = \sum_{i=1}^{i=6} U_i \tag{C.5}$$

C.3.1.2.5 Where the interval between exposure times, d, is constant and the same number of test samples, n, is used at each exposure time, the mean to sterility, U_{HSK}, can be calculated using Formula (C.6).

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

where

U_k is the first exposure time when all units are negative;

d is the exposure interval.

C.3.1.2.6 The mean D value, $\overline{D}$, can be calculated using Formula (C.7). See 11.3.3.

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

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 11138-1:2017, Annex B).

C.3.1.2.7 The 95 % confidence interval for $\overline{D}$ (p = 0,05), D_{calc}, is calculated using Formula (C.8):

$$D_{calc} = \overline{D} \pm 2\sqrt{V} \tag{C.8}$$

C.3.1.2.8 The variance, V, is calculated using Formula (C.9).

$$V = a\left(\frac{2{,}302\,6}{\ln N_0 + 0{,}577\,2}\right)^2 \tag{C.9}$$

C.3.1.2.9 The "a" for the variance is calculated using Formula (C.10).

$$a = 0{,}25 \sum_{i=2}^{i=6} \left[\left(t_{(i+1)} - t_{(i-1)} \right)^2 \left(r_i \frac{(n_i - r_i)}{{n_i}^2 (n_{i-1})} \right) \right] \tag{C.10}$$

C.3.1.3 Example calculations using the HSKP

C.3.1.3.1 See Table C.2.

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

Exposure time 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.3.1.3.2 Calculate χ_i and γ_i for each exposure period, t_i:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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.3.1.3.3 Calculate U_i for each exposure period, t_i:

$$U_i = \chi_i \gamma_i$$

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

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

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

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

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

$U_6 = 65 \times 0 = 0$

C.3.1.3.4 The mean time to sterility, U_{HSK}, is calculated using Formula (C.11):

$$U_{HSK} = \sum_{i=1}^{i=6} \mu_i \qquad \text{(C.11)}$$

$U_{HSK} = \mu_1 + \mu_2 + \mu_3 + \mu_4 + \mu_5 + \mu_6$

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

C.3.1.3.5 The mean D value, $\overline{D}$, is calculated using Formula (C.12). See 11.3.3.

$$\overline{D} = \frac{U_{HSK}}{\log_{10} N_0 + 0{,}250\,7} \qquad \text{(C.12)}$$

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.3.1.3.6 The 95 % confidence interval for $\overline{D}$ (p = 0,05), D_{calc}, is calculated using Formula (C13). See C.3.1.2.7.

$$D_{calc} = \overline{D} \pm 2\sqrt{V} \tag{C.13}$$

C.3.1.3.7 The variance, V, is calculated using Formula (C.14). See C.3.1.2.8.

$$V = a\left(\frac{2{,}302\,6}{\ln N_0 + 0{,}577\,2}\right)^2 \tag{C.14}$$

C.3.1.3.8 The "a" in the variance formula for each t_i and summing all results is calculated using Formula (C.15). See C.3.1.2.9.

$$a = 0{,}25 \sum_{i=2}^{i=6}\left[\left(t_{(i+1)} - t_{(i-1)}\right)^2 \left(r_i \frac{n_i - r_i}{{n_i}^2 (n_i - 1)}\right)\right] \tag{C.15}$$

$$a = 0{,}25\left[\left(t_{(1+1)} - t_{(1-1)}\right)^2 \left(r_1 \frac{(n_1 - r_1)}{{n_1}^2 (n_1 - 1)}\right) + \left(t_{(2+1)} - t_{(2-1)}\right)^2 \left(r_2 \frac{(n_2 - r_2)}{{n_2}^2 (n_2 - 1)}\right) + \left(t_{3+1} - t_{3-1}\right)^2 \left(r_3 \frac{(n_3 - r_3)}{{n_3}^2 (n_3 - 1)}\right) +$$
$$\left(t_{(4+1)} - t_{(4-1)}\right)^2 \left(r_4 \frac{(n_4 - r_4)}{{n_4}^2 (n_4 - 1)}\right) + \left(t_{(5+1)} - t_{(5-1)}\right)^2 \left(r_5 \frac{(n_5 - r_5)}{{n_5}^2 (n_5 - 1)}\right) +$$
$$\left(t_{(6+1)} - t_{(6-1)}\right)^2 \left(r_6 \frac{(n_6 - r_6)}{{n_6}^2 (n_6 - 1)}\right)\Bigg]$$

$$a = 0{,}25 \times (28-10)^2 \times 4\left(\frac{19-4}{361 \times 18}\right) = 2{,}991\,7 +$$
$$(40-18)^2 \times 8\left(\frac{21-8}{441 \times 20}\right) = 5{,}707\,0 +$$
$$(50-28)^2 \times 12\left(\frac{20-12}{400 \times 19}\right) = 6{,}113\,7 +$$
$$(60-40)^2 \times 16\left(\frac{20-16}{400 \times 19}\right) = 3{,}368\,4 +$$
$$(70-50)^2 \times 20\left(\frac{20-20}{400 \times 19}\right) = 0{,}000\,0$$

a = 0,25 (2,991 7 + 5,707 0 + 6,113 7 + 3,368 4 + 0,000 0) = 0,25 × 18,180 8

a = 0,25 × 18,180 8 = 4,545 2

C.3.1.3.9 The variance, V, is calculated using Formula (C.16) now that "a" is calculated. See C.3.1.2.8.

$$V = a\left(\frac{2{,}302\,6}{\ln N_0 + 0{,}577\,2}\right)^2 \tag{C.16}$$

where

N_0 $= 1 \times 10^5$;

$$V = 4{,}545\,2\left[\frac{2{,}302\,6}{\ln(1\times10^5)+0{,}577\,2}\right]^2 = 4{,}545\,2\left(\frac{2{,}302\,6}{11{,}513+0{,}577\,2}\right)^2 = 4{,}545\,2\times(0{,}190\,45)^2 =$$

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

C.3.1.3.10 The 95 % confidence interval for $\overline{D}$ (p = 0,05), D_{calc}, is calculated using Formula (C.17). See C.3.1.2.7.

$$D_{calc} = \overline{D} \pm 2\sqrt{V} \tag{C.17}$$

C.3.1.3.11 Lower confidence limit:

$$D_{calc} = \overline{D} - 2\sqrt{V}$$

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

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

C.3.1.3.12 Upper confidence limit:

$$D_{calc} = \overline{D} + 2\sqrt{V}$$

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

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

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 conditions 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, and

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

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

Exposure time to sterilizing agent (min) t	Number of test samples exposed n	Number of test samples showing negative results r_i
t_1 (U_1)	n_1	r_1 ($r = 0$)
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 replicates ($r = 0$), at the exposure preceding U_1, and all negative replicates, i.e. all replicates showing a positive result ($r = n$) at the exposure subsequent to U_k.

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 condition and constant time intervals between exposures.

C.3.2.1.3 The mean time to sterility, U_{HSK}, is calculated using Formula (C.18):

$$U_{HSK} = U_k - \frac{d}{2} - \frac{d}{n}\sum_{i=1}^{k-1} r_i \tag{C.18}$$

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 Formula (C.19). See 11.3.3.

$$\overline{D} = \frac{U_{HSK}}{\log_{10} N_0 + 0{,}250\,7} \tag{C.19}$$

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 Formula (C.20):

$$V = \frac{d^2}{n^2(n-1)} \times \sum_{i=1}^{k-1} r_i (n - r_i) \tag{C.20}$$

C.3.2.1.6 The standard deviation (SD) is calculated using Formula (C.21).

$$SD = \sqrt{V} \tag{C.21}$$

C.3.2.1.7 The 95 % confidence interval for $\overline{D}$ (p = 0,05), D_{calc}, are calculated using Formula (C.22).

$$D_{calc} = \overline{D} \pm 2SD \tag{C.22}$$

C.3.2.1.8 Lower confidence limit

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

C.3.2.1.9 Upper confidence limit

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

C.3.2.2 Example calculations using the LHSKP

C.3.2.2.1 See Table C.4.

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

Exposure time to sterilizing agent (min) t	Number of test samples exposed n	Number of test samples showing negative results r_i
t_1 = 20 (U_1)	n_1 = 20	r_1 = 0 (r = 0)
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 a positive result (r = n) at the exposure subsequent to U_k.

C.3.2.2.2 The mean D value, $\overline{D}$, is calculated using Formula (C.23). See 11.3.3.

$$\overline{D} = \frac{U_{HSK}}{\log_{10} N_0 + 0{,}250\,7} \tag{C.23}$$

where

$N_0 = 1 \times 10^6$.

C.3.2.2.3 The mean exposure time, (time to sterility), U_{HSK}, required to obtain no growth (sterility) is calculated using Formula (C.24). See C.3.2.1.3.

$$U_{HSK} = U_k - \frac{d}{2} - \frac{d}{n}\sum_{i=1}^{k-1} r_i \qquad \text{(C.24)}$$

where

U_k = 30;

D = 2;

N = 20.

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

$$\overline{D} = \frac{24{,}8}{6{,}000 + 0{,}2507} = 3{,}97 \text{ min}$$ (rounded to one decimal place D = 4,0 min).

C.3.2.2.4 The variance, V, is calculated using Formula (C.25). See C.3.2.1.5.

$$V = \frac{d^2}{n^2(n-1)} \times \sum_{i=1}^{k-1} r_i (n - r_i) \qquad \text{(C.25)}$$

$$= \frac{2^2}{(20)^2(20-1)} \times \left[(1\times19)+(7\times13)+(15\times5)+(19\times1)\right] = 0{,}107\,4$$

C.3.2.2.5 The standard deviation (SD) is calculated using Formula (C.26). See C.3.2.1.6.

$$\text{SD} = \sqrt{V} \qquad \text{(C.26)}$$

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

C.3.2.2.6 The 95 % confidence interval for $\overline{D}$ (p = 0,05), D_{calc}, are calculated using Formula (C.27). See C.3.2.1.7, C.3.2.1.8 and C.3.2.1.9.

$$D_{calc} = \overline{D} \pm 2\text{SD} \qquad \text{(C.27)}$$

C.3.2.2.7 Lower confidence limit

$$D_{calc} = \frac{U_{HSK} - 2\text{SD}}{\log_{10} N_0 + 0{,}2507}$$

$$= \frac{24{,}8 - (2\times0{,}322\,7)}{6{,}000 + 0{,}2507} = \frac{24{,}144}{6{,}250\,7} = 3{,}86 \text{ min}$$

where

$N_0 = 1 \times 10^6$.

C.3.2.2.8 Upper confidence limit

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

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

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

C.3.3.1 Procedure

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 time within the fraction-negative range and the initial number of microorganisms per replicate, N_0.

C.3.3.1.3 To obtain valid data 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 condition should be used and the condition r/n less than 0,9 should be met in order to establish test criteria equivalent to C.3.1 and C.3.2 (see Reference [31]). Test samples should be subjected to a defined exposure condition within the fraction-negative range of the batch.

C.3.3.2 Calculations using the SMCP

C.3.3.2.1 The D value is calculated using Formula (C.28).

$$D = \frac{t}{\log_{10} A - \log_{10} B} \tag{C.28}$$

where

t is the exposure time;

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

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

C.3.3.2.2 Formula (C.28) can be restated for fraction-negative data sets. See 11.3.3.

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

or

$$D = \frac{t}{\log_{10} N_0 - \log_{10} N_{\mu_i}}$$

where

$\log_{10} N_{\mu i}$ = $\log_{10}$ (ln n/r) or $\log_{10}$ [2,303 $\log_{10}$ (n/r)]

$N_{\mu i}$ 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 Formula (C.29).

$$D_{calc} = \frac{t}{\log_{10} N_0 - \log_{10}\left[\ln\frac{(1)}{a}\right]} \quad \text{(C.29)}$$

where

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

C.3.3.2.4 Formula (C.29) can only be used if $n \cdot \frac{r}{n} \cdot \frac{n-r}{n}$ is greater than or equal to 0,9.

C.3.3.3 Example calculations using the SMCP

C.3.3.3.1 See Table C.5.

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

Exposure time (min) t	Number of test samples exposed n	Number of test samples showing no growth r
24	100	37

C.3.3.3.2 The D value is calculated using Formula (C.30). See 11.3.3.

$$D = \frac{t}{\log_{10} \mathrm{A} - \log_{10} \mathrm{B}} \quad \text{(C.30)}$$

where

t is the exposure time;

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

$\log_{10}$A = $\log_{10}$ of initial population, N_0, per sample;

$\log_{10}$B = $\log_{10}$ of population after exposure time, t, or

$= \log_{10}(\ln n/r)$ or $\log_{10}[2{,}303 \log_{10}(n/r)]$;

n is the number of replicates per exposure time;

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

$$D = \frac{24}{6{,}000 - \log_{10}(\ln 2{,}7027)}$$

$$D = \frac{24}{6{,}000 - \log_{10}(0{,}9943)}$$

$$D = \frac{24}{6{,}000 - (-0{,}0025)}$$

$$D = \frac{24}{6{,}0025} = 4{,}00 \text{ min}$$ (rounded to one decimal place D = 4,0 min)

C.3.3.3.3 The 95 % confidence interval for $\overline{D}$ (p = 0,05), D_{calc}, is calculated as follows.

If $n \cdot \frac{r}{n} \cdot \frac{n-r}{n}$ is greater than or equal to 0,9, then the 95 % confidence interval can be calculated using Formulae (C.31) and (C.32). See C.3.3.2.3.

Lower confidence limit:

$$D_{calc} = \frac{t}{\log N_0 - \log_{10}(\ln 1/a)} \quad \text{(C.31)}$$

where

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

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

where

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

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

$$= 0{,}37 + 1{,}96 \times \sqrt{0{,}37 \times 0{,}0063}$$

$$= 0{,}37 + 1{,}96 \times \sqrt{0{,}002331}$$

$$= 0{,}37 + 1{,}96 \times 0{,}04828$$

a = 0,465

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

$$= \frac{24}{6{,}000 - \log_{10}(0{,}7657)}$$

$$= \frac{24}{6{,}000 - (-0{,}1159)}$$

$$= \frac{24}{6{,}000 + 0{,}1159}$$

$$D_{calc} = \frac{24}{6{,}1159} = 3{,}92$$

Upper confidence limit:

$$D_{calc} = \frac{t}{\log_{10} N_0 - \log_{10}\left[\ln\left(\frac{1}{a}\right)\right]} \quad \text{(C.32)}$$

where

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

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

where

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

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

$$= 0{,}37 - 1{,}96\sqrt{0{,}37 \times 0{,}0063}$$

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

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

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

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

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

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

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

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. The main document should be consulted 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 as shown by Figure D.1: Commercial suspension; suspension from commercial strain; and suspension from in-house strain.

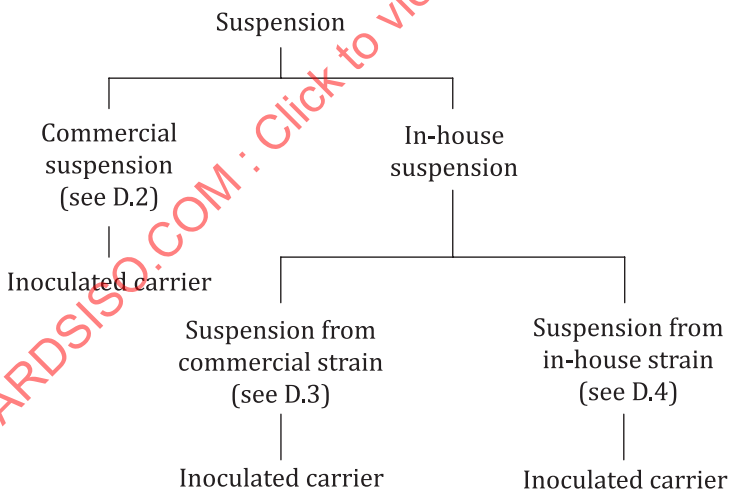


Figure D.1 — Sources of microorganisms

D.1.2 Documentation

D.1.2.1 A list of relevant documents can include the following:

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 processing;

c) protocols for validation studies and results.

D.1.2.2 The user is advised to define basic elements such as the following:

a) the method of sterilization to be used in the routine production of products, e.g. moist heat, dry heat, gaseous or other sterilization method;

b) which of the critical variables of the sterilization process is measured directly during the routine production of products, e.g. time, temperature, pressure.

NOTE This list of elements to document is not necessarily exhaustive.

D.2 Commercially available suspension

When using commercially available suspensions, the following elements should be documented:

a) name of manufacturer of suspension;

b) name and identification of the microorganism;

c) origin of the strain/reference to a recognized culture collection number;

d) number of microorganisms to be detected per defined volume of suspension;

e) suspending medium;

f) batch number or other means of identification;

g) resistance characteristics as given by the manufacturer of the suspension (see 5.2 and 5.3);

h) recommended culture conditions for retrieval of viable microorganisms from the suspension, e.g. time, temperature and growth medium;

i) storage conditions;

j) stability information (expiry date or equivalent).

NOTE This list of elements to document is not necessarily exhaustive.

D.3 Suspension from a commercially available strain

When using suspensions prepared from a commercially available strain, the following elements should be documented:

a) name of manufacturer or supplier of the strain;

b) name of the microorganism;

c) reference to a recognized culture collection number;

d) the manufacturer's instructions for handling of the strain;

e) relevant information on processing elements for preparing the suspension from the strain;

f) storage conditions;

g) means of identification of the stored and processed strain/batch number;

h) stability information.

NOTE This list of elements to document is not necessarily exhaustive.