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Sterilization of medical devices — Microbiological methods —

Part 1: Determination of a population of microorganisms on products

Stérilisation des dispositifs médicaux — Méthodes microbiologiques —

Partie 1: Détermination d'une population de micro-organismes sur des produits

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Foreword

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

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

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

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 11737-1 was prepared by Technical Committee ISO/TC 198, *Sterilization of health care products*.

This second edition cancels and replaces the first edition (ISO 11737-1:1995) which has been technically revised and ISO 11737-3:2004 whose contents it now incorporates.

ISO 11737 consists of the following parts, under the general title *Sterilization of medical devices — Microbiological methods*:

— *Part 1: Determination of a population of microorganisms on products*

— *Part 2: Tests of sterility performed in the validation of a sterilization process*

Introduction

A sterile medical device is one that is free of viable microorganisms. International standards that specify requirements for validation and routine control of sterilization processes, require, when it is necessary to supply a sterile medical device, that adventitious microbiological contamination of a medical device prior to sterilization be minimized. Even so, medical devices produced under standard manufacturing conditions in accordance with the requirements for quality management systems (see, for example, ISO 13485) may, prior to sterilization, have microorganisms on them, albeit in low numbers. Such products are non-sterile. The purpose of sterilization is to inactivate the microbiological contaminants and thereby transform the non-sterile products into sterile ones.

The kinetics of inactivation of a pure culture of microorganisms by physical and/or chemical agents used to sterilize medical devices can generally best be described by an exponential relationship between the numbers of microorganisms surviving and the extent of treatment with the sterilizing agent; inevitably this means that there is always a finite probability that a microorganism may survive regardless of the extent of treatment applied. For a given treatment, the probability of survival is determined by the number and resistance of microorganisms and by the environment in which the organisms exist during treatment. It follows that the sterility of any one product in a population subjected to sterilization processing cannot be guaranteed and the sterility of a processed population is defined in terms of the probability of there being a viable microorganism present on a product item.

Generic requirements of the quality management system for design and development, production, installation and servicing are given in ISO 9001 and particular requirements for quality management systems for medical device production are given in ISO 13485. The standards for quality management systems recognize that, for certain processes used in manufacturing, the effectiveness of the process cannot be fully verified by subsequent inspection and testing of the product. Sterilization is an example of such a process. For this reason, sterilization processes are validated for use, the performance of the sterilization process is monitored routinely and the equipment is maintained.

International Standards specifying procedures for the validation and routine control of the processes used for the sterilization of medical devices have been prepared (see, for example, ISO 11135, ISO 11137 series and ISO 17665). However, it is important to be aware that exposure to a properly validated and accurately controlled sterilization process is not the only factor associated with the provision of assurance that the product is sterile and, in this respect, suitable for its intended use. Furthermore, for the effective validation and routine control of a sterilization process, it is important to be aware of the microbiological challenge that is presented in the process, in terms of number, characteristics and properties of microorganisms.

The term bioburden is used to describe the population of viable microorganisms present on or in product and/or a sterile barrier system. A knowledge of bioburden can be used in a number of situations as part of:

— validation and revalidation of sterilization processes;

— routine monitoring for control of manufacturing processes;

— monitoring of raw materials, components or packaging;

— assessment of the efficiency of cleaning processes;

— an overall environmental monitoring programme.

Bioburden is the sum of the microbial contributions from a number of sources, including raw materials, manufacturing of components, assembly processes, manufacturing environment, assembly/manufacturing aids (e.g., compressed gases, water, lubricants), cleaning processes and packaging of finished product. To control bioburden, attention must be given to the microbiological status of these sources.

It is not possible to enumerate the bioburden exactly and, in practice, a determination of bioburden is made using a defined method. Definition of a single method for use in the determination of bioburden in all situations is not practicable because of the wide variety of designs and materials of construction of medical devices. Nor is it possible to define a single technique to be used in all situations for the removal of microorganisms in preparation for enumeration. Furthermore, the selection of conditions for enumeration of microorganisms will be influenced by the types of microorganism likely to be present on or in medical devices.

This part of ISO 11737 specifies the requirements to be met in the determination of bioburden. The requirements are the normative parts of this part of ISO 11737 with which compliance is claimed. The guidance given in the informative annexes is not normative and is not provided as a checklist for auditors. The guidance provides explanations and methods that are regarded as being a suitable means for complying with the requirements. Methods other than those given in the guidance may be used, if they are effective in achieving compliance with the requirements of this part of ISO 11737.

Sterilization of medical devices — Microbiological methods —

Part 1: Determination of a population of microorganisms on products

1 Scope

This part of ISO 11737 specifies requirements and provides guidance for the enumeration and microbial characterization of the population of viable microorganisms on or in a medical device, component, raw material or package.

NOTE 1 The nature and extent of microbial characterization is dependent on the intended use of the bioburden data.

This part of ISO 11737 does not specify requirements for the enumeration or identification of viral or protozoan contaminants.

NOTE 2 Furthermore, the requirements specified in this part of ISO 11737 are not intended to address the removal and detection of the causative agents of spongiform encephalopathies such as scrapie, bovine spongiform encephalopathy and Creutzfeldt-Jakob disease.

This part of ISO 11737 does not specify requirements for the microbiological monitoring of the environment in which medical devices are manufactured.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10012, *Measurement management systems — Requirements for measurement processes and measuring equipment*

ISO 13485:2003, *Medical devices — Quality management systems — Requirements for regulatory purposes*

ISO/IEC 17025:2005, *General requirements for the competence of testing and calibration laboratories*

3 Terms and definitions

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

3.1
bioburden
population of viable microorganisms on or in product and/or sterile barrier system

[ISO/TS 11139:2006, definition 2.2]

3.2
correction
action to eliminate a detected nonconformity

NOTE A correction can be made in conjunction with a **corrective action** (3.4).

[ISO 9000:2005, definition 3.6.6]

3.3
correction factor
numerical value applied to compensate for incomplete removal from product and/or culture of microorganisms

3.4
corrective action
action to eliminate the cause of a detected nonconformity or other undesirable situation

NOTE 1 There can be more than one cause for a nonconformity.

NOTE 2 Corrective action is taken to prevent recurrence whereas **preventive action** (3.9) is taken to prevent occurrence.

NOTE 3 There is a distinction between **correction** (3.2) and corrective action.

[ISO/TS 11139:2006, definition 2.8]

3.5
culture conditions
combination of growth media and manner of incubation used to promote germination, growth and/or multiplication of microorganisms

NOTE The manner of incubation may include the temperature, time and any other conditions specified for incubation.

[ISO/TS 11139:2006, definition 2.10]

3.6
establish
determine by theoretical evaluation and confirm by experimentation

[ISO/TS 11139:2006, definition 2.17]

3.7
medical device
instrument, apparatus, implement, machine, appliance, implant, in vitro reagent or calibrator, software, material or other related article, intended by the manufacturer to be used, alone or in combination, for human beings for one or more of the specific purpose(s) of:

— diagnosis, prevention, monitoring, treatment or alleviation of disease;

— diagnosis, monitoring, treatment, alleviation of or compensation for an injury;

— investigation, replacement, modification or support of the anatomy or of a physiological process;

— supporting or sustaining life;

— control of conception;

— disinfection of medical devices;

— providing information for medical purposes by means of in vitro examination of specimens derived from the human body;

and which does not achieve its primary intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means

NOTE This definition from ISO 13485:2003 has been developed by the Global Harmonization Task Force (GHTF 2002).

[ISO 13485:2003]

3.8
microbial characterization
process by which microorganisms are grouped into categories

NOTE Categories may be broadly based, for example, on the use of selective media, colony or cellular morphology, staining properties or other characteristics.

[ISO/TS 11139:2006, definition 2.25]

3.9
preventive action
action to eliminate the cause of a potential nonconformity or other undesirable potential situation

NOTE 1 There can be more than one cause for a potential nonconformity.

NOTE 2 Preventive action is taken to prevent occurrence whereas **corrective action** (3.4) is taken to prevent recurrence.

[ISO 9000:2005, definition 3.6.4]

3.10
product
result of a process

NOTE For the purposes of sterilization standards, the product is tangible and can be raw material(s), intermediate(s), sub-assembly(ies) and health care products.

[ISO 9000:2005, definition 3.4.2]

3.11
recognized culture collection
depository authority under the Budapest Treaty on “The International Recognition of the Deposit of Microorganisms for the Purposes of Patent and Procedure”

[ISO/TS 11139:2006, definition 2.38]

3.12
recovery efficiency
measure of the ability of a specified technique to remove and/or culture microorganisms from product

3.13
sample item portion
SIP
defined part of a medical device that is tested

3.14
specify
stipulate in detail within an approved document

[ISO/TS 11139:2006, definition 2.42]

3.15
validation
documented procedure for obtaining, recording and interpreting the results required to establish that a process will consistently yield product complying with predetermined specifications

NOTE In the context of determination of bioburden, the "process" is the test methodology and the "product" is the test result. The validation of a technique for the determination of bioburden consists of a series of investigations to assess the effectiveness and reproducibility of the test method.

[ISO/TS 11139:2006, definition 2.55]

4 Quality management system elements

4.1 Documentation

4.1.1 Procedures for determination of bioburden shall be specified.

4.1.2 Documents and records required by this part of ISO 11737 shall be reviewed and approved by designated personnel (see 4.2.1). Documents and records shall be controlled in accordance with ISO 13485 or ISO/IEC 17025.

4.1.3 Records retained shall include all original observations, calculations, derived data and final reports. The records shall include the identity of all personnel involved in sampling, preparation and testing.

4.1.4 Calculations and data transfers shall be subject to appropriate checks.

4.2 Management responsibility

4.2.1 The responsibility and authority for implementing and performing the procedures described in this part of ISO 11737 shall be specified. Responsibility shall be assigned to competent personnel in accordance with ISO 13485 or ISO/IEC 17025.

4.2.2 If the requirements of this part of ISO 11737 are undertaken by organizations with separate quality management systems, the responsibilities and authority of each party shall be specified.

4.2.3 All items of equipment required for correct performance of the specified tests and measurements shall be available.

4.3 Product realization

4.3.1 Procedures for purchasing shall be specified. These procedures shall comply with ISO 13485 or ISO/IEC 17025.

4.3.2 A documented system complying with ISO 13485, ISO/IEC 17025 or ISO 10012 shall be specified for the calibration of all equipment, including instrumentation for test purposes, used in meeting the requirements of this part of ISO 11737.

4.3.3 Methods shall be specified for the preparation and sterilization of materials used in the determination of bioburden, including appropriate quality tests.

4.4 Measurement, analysis and improvement — Control of nonconforming product

Procedures for investigation of out-of-specification results and for correction, corrective action and preventive action shall be specified. These procedures shall comply with ISO 13485 or ISO/IEC 17025.

5 Selection of product

5.1 General

5.1.1 The procedures for selection and handling of product for determination of bioburden shall ensure that selected product is representative of routine production including packaging materials and processes.

5.1.2 If product(s) are grouped for the purpose of determination of bioburden, the rationale for inclusion of a product within a group shall be recorded (see 4.1.2). The rationale shall include criteria to ensure that bioburden determined for a product selected from the group is representative of the whole group.

5.1.3 Consideration shall be given to the timing of the performance of determination of the bioburden relative to taking samples, because bioburden determination can be subject to change with the passage of time.

5.2 Sample item portion (SIP)

If the bioburden is demonstrated as being evenly distributed on and/or in the product item, the SIP may be selected from any portion of the item. Otherwise, the SIP shall consist of portion(s) of product, selected at random, which proportionally represent each of the materials from which product is made. If the bioburden distribution is known, the SIP may be selected from the portion of the product that is considered to be the most severe challenge to the sterilization process. The SIP can be calculated on the basis of length, mass, volume or surface area (see Table 1 for examples).

Table 1 — Examples of SIP calculation

Basis for SIP	Product
Surface area	Implants (non-absorbable)
Mass	Powders Gowns Implants (absorbable)
Length	Tubing (consistent diameter)
Volume	Fluid in water cup

NOTE If appropriate, the standard specifying requirements for validation and routine control of the sterilization process stipulates criteria for the adequacy of SIP.

6 Methods of determination and microbial characterization of bioburden

6.1 Determination of bioburden

6.1.1 Selection of an appropriate method

An appropriate method shall be selected for determination of bioburden. The method shall comprise techniques for:

a) removal of microorganisms, if appropriate;

b) culturing of microorganisms;

c) enumeration of microorganisms.

The precision shall be determined and shall be appropriate to the purpose for which the data are to be used.

6.1.2 Removal of microorganisms

6.1.2.1 For an identified product where removal of viable microorganisms is part of the method, the efficiency of removal shall be considered and the outcomes of this consideration recorded (see 4.1.3). Consideration shall, at least, be given to:

a) ability of the technique to remove microorganisms;

b) possible type(s) of microorganism and their location(s) on product;

c) effect(s) of the removal technique on the viability of microorganisms;

d) the physical or chemical nature of product under test.

6.1.2.2 For an identified product for which removal of viable microorganisms is not part of the method, the efficiency of enumeration of microorganisms shall be considered and the outcomes of this consideration recorded (see 4.1.3). Consideration shall, at least, be given to:

a) possible type(s) of microorganism and their location(s) on product;

b) the physical or chemical nature of the product to be tested;

c) aggregates of cells forming single colonies due to *in-situ* culturing.

6.1.2.3 If the physical or chemical nature of product is such that substances can be released that adversely affect either the number or the types of microorganism found, then a system shall be used to neutralize, remove or, if this is not possible, minimize the effect of any such released substance. The effectiveness of such a system shall be demonstrated.

NOTE Annex B describes techniques that may be used to assess the release of microbicidal or microbiostatic substances.

6.1.3 Culturing of microorganisms

Culture conditions shall be selected after consideration of the types of microorganism likely to be present. The results of this consideration and the rationale for the decisions reached shall be recorded (see 4.1.2).

6.1.4 Enumeration of microorganisms

The technique for enumeration shall be selected after consideration of the types of microorganism likely to be present. The results of this consideration and the rationale for the decisions reached shall be recorded (see 4.1.2).

6.2 Microbial characterization of bioburden

6.2.1 Appropriate techniques for microbial characterization of bioburden shall be selected.

NOTE Microbial characterization is necessary to detect a change to product microflora that might affect some aspect of the use of the bioburden data (e.g. establishing a sterilization process).

6.2.2 Microbial characterization shall be accomplished using one or more of the following:

a) staining properties;

b) cell morphology;

c) colony morphology;

d) use of selective culturing;

e) biochemical properties;

f) genetic sequence data for which there is an adequate data base.

7 Validation of method for determining bioburden

7.1 The method for determining of bioburden shall be validated and documented.

7.2 Validation shall consist of the following:

a) assessment of the adequacy of the technique for removal of microorganisms from product, if removal is part of the method;

b) determination of the recovery efficiency in order that a correction factor be derived;

c) assessment of the adequacy of the enumeration of microorganisms, including culture conditions and microbiological counting techniques;

d) assessment of the suitability of the technique(s) of microbial characterization.

8 Routine determination of bioburden and interpretation of data

8.1 Routine determination of bioburden shall be performed employing documented sampling plan(s) defining sample size and sampling frequency.

8.2 Determination of bioburden shall be performed using a method specified for a product or group of products (see 5.1.2).

8.3 Microbial characterization of bioburden shall be performed to a degree dependent on the purpose for which the data derived from the determination of bioburden are to be used (see 6.2).

If, on microbial characterization, isolates are recovered that are not part of the normal microflora, consideration should be given to assessing the properties of these isolates.

8.4 If bioburden data are to be used to establish the extent of treatment of a sterilization process, any requirements applicable to the use of bioburden data, specified in the appropriate standard for the development, validation and routine control of the sterilization process, shall be met.

8.5 Acceptable limits for bioburden on or in a medical device shall be specified. This specification shall be based on previously generated data. If these limits are exceeded, action shall be taken (see 4.4).

8.6 Data derived from determination of bioburden obtained over a period of time shall be used to identify trends. Acceptable limits shall be reviewed and revised as necessary.

8.7 The application of statistical methods to define sample size, sampling frequency and/or acceptable limits shall conform to ISO 13485.

9 Maintenance of the method of determination of bioburden

9.1 Changes to the product and/or manufacturing process

Changes to product and/or manufacturing processes shall be reviewed to determine whether they are likely to alter bioburden. The results of the review shall be recorded (see 4.1.2). If there is potential for alteration of bioburden, specific determinations of bioburden shall be performed to evaluate the extent and nature of any change.

9.2 Changes to the method of determination of bioburden

Any change to a routine method of bioburden determination shall be assessed. This assessment shall include:

a) evaluation of the effect of the change on the outcome of determination;

b) establishment of the recovery efficiency of the method following the change.

NOTE The assessment of the change could indicate that the previous validation and recovery efficiency are still applicable.

9.3 Revalidation of the method of determination of bioburden

The original validation data (see 7.2) and any subsequent revalidation data shall be reviewed at specified intervals in accordance with a documented procedure. The extent to which revalidation is to be undertaken shall be determined. The outcome of the review and any revalidation undertaken shall be recorded (see 4.1.3).

Annex A
(informative)

Guidance on determination of a population of microorganisms on product

NOTE For ease of reference, the numbering in this annex corresponds to that used in the normative part of this part of ISO 11737.

A.1 Scope

This annex contains guidance on the implementation of the requirements specified in this part of ISO 11737. The guidance given is not intended to be exhaustive, but to highlight important aspects to which attention should be given.

Methods other than those given in this annex may be used, but these alternative methods should be demonstrated as being effective in achieving compliance with the requirements of this part of ISO 11737.

This annex is not intended as a checklist for assessing compliance with the requirements of this part of ISO 11737.

A.2 Normative references

The requirements of documents included as normative references are requirements of this part of ISO 11737 only to the extent that they are cited in a normative part of this part of ISO 11737; the citation may be to an entire standard or limited to specific clauses.

A.3 Definitions

No guidance offered.

A.4 Quality management system elements

NOTE It is not a requirement of this part of ISO 11737 to have a full quality management system, but the elements of a quality management system that are the minimum necessary to control the determination of bioburden as used in the validation and monitoring of medical devices to be sterilized are normatively referenced at appropriate places in the text (see, in particular, Clause 4). Attention is drawn to the standards for quality management systems (see ISO 13485) that control all stages of production or reprocessing of medical devices. National and/or regional regulations for the provision of medical devices might require a complete implementation of a full quality management system and the assessment of that system by a third party.

A.4.1 Documentation

In ISO 13485, the requirements in the documentation section relate to the generation and control of documentation (including specifications and procedures) and records.

Computers may be used in laboratories for direct and indirect collection, processing and/or storage of data. Both the hardware and software used for such applications should be controlled.

The computer system in use should be identified, both in terms of hardware and software, and any changes in either of these aspects should be documented and subject to appropriate approval.

If calculations are performed by electronic data processing techniques, the software (e.g., spreadsheet calculations) should be validated prior to use and records of this validation should be retained.

For software, there should be documentation describing:

— applications software run on the computer system;

— operations software;

— data packages in use.

All software should be acceptance tested before being put into service.

If computer software is developed in-house, suitable procedures should be developed to ensure that:

— documentation on development, including the source code, is retained;

— records of acceptance testing are retained;

— modifications to programs are documented;

— changes in equipment are documented and formally tested before being put into use.

These controls should also be applied to any modification or customizing of commercial software packages.

There should be procedures to detect or prevent unauthorized changes to software programs.

Software programs that organize, tabulate and/or subject data to statistical or other mathematical procedures, or which otherwise manipulate or analyse the electronically stored data, should permit retrieval of original data entries. Special procedures for archiving computer data are likely to be required and these procedures should be documented.

Requirements for control of documents and records are specified in 4.2.3 and 4.2.4 of ISO 13485:2003, or 4.3 and 4.13 of ISO/IEC 17025:2005.

Requirements for technical records are specified in 4.13.2 and 5.4 of ISO/IEC 17025:2005.

See also ISO 90003 for guidance of the application of quality management systems to computer software.

A.4.2 Management responsibility

In ISO 13485, the requirements in the management responsibility section relate to management commitment, customer focus, quality policy, planning, responsibility, authority and communication, and management review.

In order that the data obtained from performing bioburden determinations are reliable and reproducible, it is important that the determinations be performed under controlled conditions. Therefore, the laboratory facilities used for the determinations, whether on the site of the manufacturer of the medical device or located at a remote location should be managed and operated in accordance with a documented quality system.

The determination of bioburden can involve separate parties, each of whom is responsible for certain elements of the method or procedure. This part of ISO 11737 requires that the party accepting particular responsibilities be defined and that this definition of responsibilities be documented. This definition of authority and responsibility is documented within the quality management system(s) of the identified parties. The party accepting responsibilities for defined elements is required to assign these elements to competent personnel, with competence demonstrated through appropriate training and qualification.

If bioburden determinations are performed in a laboratory under the direct management of the manufacturer of the medical device, the operation of the laboratory resides within the manufacturer's quality management system. If an external laboratory is used, the laboratory should be formally certified against an appropriate International Standard (e.g. ISO/IEC 17025).

Any laboratory should be committed to providing a quality service and this commitment should be documented as a quality policy. The lines of authority and responsibility within the laboratory organization should be formally established and documented. An individual should be nominated to be responsible for the establishment of the laboratory quality system and should have the authority to ensure that the system is implemented.

The operation of the laboratory should be subject to regular internal audits. The results of the audit should be documented and reviewed by the laboratory management. See 4.14 of ISO/IEC 17025:2005.

Requirements for responsibility and authority are specified in 5.5 of ISO 13485:2003 and requirements for human resources are specified in 6.2 of ISO 13485:2003.

Requirements for provision of resources are specified in ISO 13485 and requirements for equipment are specified in 5.5 of ISO/IEC 17025:2005.

A.4.3 Product realization

In ISO 13485, the requirements in the product realization section relate to the product lifecycle from the determination of customer requirements, design and development, purchasing, control of production, and calibration of monitoring and measuring devices.

There should be a system for identifying the maintenance requirements for each piece of laboratory equipment.

Equipment that does not require calibration should be clearly identified.

Any equipment, or parts thereof, that comes into contact with product, eluent, media, etc., during testing should be sterile. All microbiological media and eluents used to remove microorganisms from product should be prepared in a manner that ensures their sterility.

Appropriate quality tests should include growth promotion tests. Generally, growth promotion tests are performed on each batch of medium using an inoculum of low numbers [between 10 and 100 colony-forming units (CFUs)] of selected microorganisms. Growth promotion tests are described in pharmacopoeial monographs that detail suitable microorganisms. Other recognised quantitative and semi-quantitative methods for media quality control are also acceptable.

Requirements for purchasing are specified in 7.4 of ISO 13485:2003. In particular, it should be noted that the requirements in 7.4.3 of ISO 13485:2003 for verification of purchased product apply to all product and services received from outside the organization.

Requirements for calibration of monitoring and measuring devices are specified in 7.6 of ISO 13485:2003. Requirements for equipment and measurement traceability requirements are specified in 5.5 and 5.6 of ISO/IEC 17025:2005.

A.4.4 Measurement, analysis and improvement — Control of nonconforming product

In ISO 13485, the requirements in the measurement, analysis and improvement section relate to in-process monitoring, control of nonconforming product, analysis of data and improvement (including corrective and preventive actions).

All bioburden results that exceed specification and that indicate an adverse trend require investigation. The initial phase of the investigation should involve assessing if the results are a true finding or are in error. The following can contribute to an error and should be addressed:

— inappropriate samples (e.g. non-representative, non-homogeneous rejected materials);

— inappropriate sampling materials (e.g. swabs, containers, packages);

— unsuitable conditions of transport/handling/storage;

— inappropriate test materials (e.g. storage, pipettes, filtration apparatus);

— incorrect handling or test method(s);

— inappropriate media or diluents;

— inappropriate laboratory environment;

— inappropriate incubation environment;

— errors of calculation or transcription.

If the results are due to an error, the bioburden result that exceeds the specified limit should be verified by the performance of a repeat determination employing samples from the same batch of product. If product supports microbial growth or if the same batch is no longer available, a new batch may be used.

If the original result is confirmed as a true finding, at least the following should be considered in the second phase of the investigation:

a) the implication of the result in relation to the effectiveness of the sterilization process;

b) the need to increase the sample size and/or frequency;

c) the outcome of an assessment of manufacturing processes; in such an assessment, the following should be addressed:

   1) raw materials/components (vendors, changes);

   2) cleaning/lubrication/manufacturing liquid;

   3) transport/holding containers;

   4) work surfaces;

   5) personnel attire/hygiene/practices;

   6) handling/assembly;

   7) environmental conditions and monitoring results (including seasonal factors, if any);

   8) packaging materials and procedures;

   9) storage conditions;

d) microbial characterization of organism(s) recovered, including

   1) potential sources;

   2) comparison with previous isolates.

Based on the results of the investigation, specific corrective action might be required. If corrective action is required, the effectiveness has to be demonstrated.

Procedures for corrective action are specified in 8.5.2 of ISO 13485:2003 and 4.11 of ISO/IEC 17025:2005.

A.5 Selection of product

A.5.1 General

A.5.1.1 Techniques of selecting and handling samples of product should be chosen and performed to avoid the introduction of inadvertent contamination and significant alterations to the numbers and types of microorganisms in the sample. Sampling techniques should be consistent to allow comparisons of bioburden to be made over a period of time.

In choosing samples of product for determination of bioburden, there are two possibilities:

a) take product at random

or

b) take product that is not suitable for sale, which may be scrapped or otherwise rejected.

The choice can depend on a number of factors but the first prerequisite is that product selected should possess bioburden representative of that of product. If the decision is made to utilize a rejected product, such product should have undergone all essential stages of production, including possible cleaning and packaging processes. Product taken as indicated in a) above constitutes the more desirable sample.

When sampling for determination of bioburden, product should be contained in its usual packaging.

A.5.1.2 In the grouping of product for bioburden determination, the following should be considered:

a) number of microorganisms;

b) types of microorganism;

c) size of product;

d) number of components;

e) complexity of product;

f) degree of automation used in manufacturing process;

g) manufacturing environment.

Product selected from the group for bioburden determination should be taken either 1) at random or 2) in accordance with a planned schedule.

A.5.1.3 If data from bioburden determinations are to be used to establish a sterilization process, the period of time that elapses between the selection of product samples and the determination of bioburden should reflect the time period between completion of the last manufacturing step and sterilization of product.

A.5.2 Sample item portion (SIP)

Whenever practicable, the determination of the bioburden should utilize the whole product, although this may not be feasible because the product cannot be accommodated in available laboratory glassware. In the latter instance, as large a portion of product as possible should be used, and the portion of the product used should

allow bioburden on the whole product to be determined. Therefore, careful selection of the portion of the product is necessary when large products like surgical gowns or external drainage kits are tested.

In preparing or assembling an SIP, care should be taken during manipulations of product. If portions are to be separated from product, this should be done under clean conditions in a controlled environment (e.g. inside a laminar flow cabinet) in order to avoid adding contamination.

A.6 Methods of determination and microbial characterization of bioburden

A.6.1 Determination of bioburden

A.6.1.1 Selection of an appropriate method

If appropriate, molecular typing methods, such as DNA sequencing, may be used to supplement culture methods. Figure A.1 is a decision tree having general application in the initial stages of selection of a method of bioburden determination.

A.6.1.2 Removal of microorganisms

See Annex B.

A.6.1.3 Culturing of microorganisms

The nature of raw materials, the method of manufacture and the conditions under which manufacture occurs, are factors that have to be considered when choosing the media and incubation conditions. Unless there are fastidious microorganisms that can be present, non-selective media and incubation conditions are appropriate.

When selecting media and incubation conditions, at least the following should be considered:

a) no single combination of medium and incubation conditions will enable the growth of all microorganisms;

b) validation exercises can require the use of a wider range of media and conditions of incubation than those used routinely;

c) direct plating on selective media might not permit growth of physiologically stressed or damaged microorganisms;

d) likely microbial contamination sources and the types of the microorganisms that could be encountered, bearing in mind that some contamination sources might vary seasonally.

Examples of media and incubation conditions are listed in Table A.1.

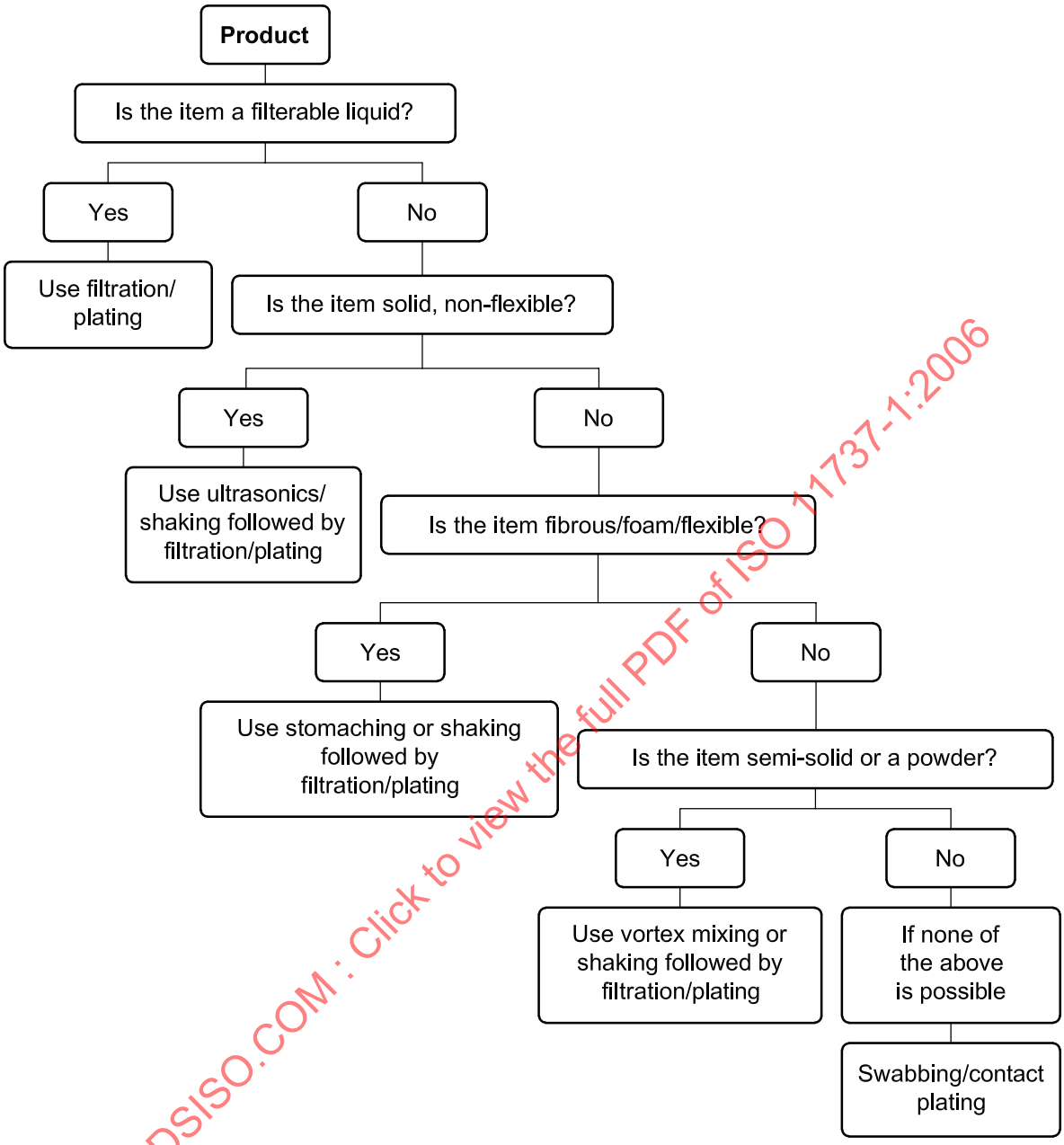

Figure A.1 — Bioburden method decision tree

Table A.1 — Examples of media and incubation conditions [a]

Types of microorganism	Solid media	Liquid media	Incubation conditions [b]
Facultative, non-fastidious, aerobic bacteria	Soybean casein digest agar (Tryptone soya agar) Nutrient agar Blood agar base Glucose tryptone agar	Soybean casein digest broth (Tryptone soya broth) Nutrient broth	30 °C to 35 °C for 3 d to 7 d
Yeasts and molds	Sabouraud dextrose agar Malt extract agar Rose Bengal agar Chloramphenicol agar Soybean casein digest agar (Tryptone soya agar) Potato dextrose agar	Sabouraud dextrose broth Malt extract broth Soybean casein digest broth (Tryptone soya broth)	20 °C to 25 °C for 5 d to 7 d
Anaerobic bacteria	Reinforced clostridial agar [c] Schaedler agar [c] Pre-reduced blood agar [c] Fastidious anaerobe agar [c] Wilkens-Chalgren agar [c]	Robertson's cooked meat broth Fluid thioglycollate broth	30 °C to 35 °C for 3 d to 7 d

[a] This list is not exhaustive.

[b] The incubation conditions listed indicate conditions that are commonly used for the types of microorganism listed.

[c] Cultured under anaerobic conditions.

It should be noted that all non-selective anaerobic culture methods may also permit the growth of facultative anaerobic organisms.

A.6.1.4 Enumeration of microorganisms

See B.6.

A.6.2 Microbial characterization of bioburden

The degree of microbial characterization necessary for the bioburden of product is based on the purpose for which the data are used.

A wide range of methods can be used for the microbial characterization of microorganisms comprising the bioburden on or in a medical device. Identification of an isolate based on classical tests such as morphology, Gram and spore stain reactions and simple biochemical reactions (e.g. catalase, oxidase, indole) usually provides some indication of the family or genus to which a microorganism belongs. More complex biochemical, serological and molecular tests can identify isolates to genus or species level.

Table A.2 provides information on common categorization methods.

Table A.2 — Common bioburden categorization methods

Method	Example	Specificity
Staining properties	Gram stain, spore stain	Low to moderate
Cell morphology	Rods, cocci	Low to moderate
Colony morphology	Shape, colour, texture	Low to moderate
Use of selective culturing	Heat shock for spores, selective fungal agar	Moderate to high
Identification of isolates	Genus and species	High

A.7 Validation of method for determining bioburden

A.7.1 In general, standard classical microbiological methods present a challenge to the user in the validation of the determination of bioburden. It is not usually necessary to validate classical microbiological methods, other than to establish their applicability, to verify staff competence in their performance and, if applicable, to include appropriate positive and negative reference control microorganisms or chemicals. Such actions are usually sufficient to confirm the validity of the determination of bioburden.

Laboratories are generally encouraged to use methods described in national and International Standards, such as those published by pharmacopoeias, ISO, AOAC, ASTM, etc. Recognised reference methods, as defined in standards or reference tests, have undergone interlaboratory comparison. A laboratory using such recognised methods should need only to verify their accuracy and reliability under its unique conditions of use.

All strains of test microorganisms used as controls during validation should be obtained from a recognised culture collection.

A.7.2 There are essentially two approaches available for validation of the efficiency of removal of a test microorganism from medical devices. These approaches are:

— repetitive treatment of a sample product or

— product inoculation with known levels of microorganism,

followed by quantitative assessment of the extent of recovery.

The first of these approaches has the advantage of utilizing the naturally occurring microorganisms but usually needs a relatively high initial bioburden. The second approach creates a model system for testing purposes. The use of such a system raises questions as to its applicability to the natural situation. However, this approach cannot be used for products with low levels of bioburden.

The culture conditions (i.e. media and incubation conditions), selected for use in determination of bioburden cannot be expected to detect all potential microorganisms. In practice, therefore, it is inevitable that bioburden will be underestimated. Nevertheless, a decision on appropriate culture conditions must be made.

One approach to the assessment of culture conditions consists of selecting the culture conditions based on a knowledge of the manufacturing process, environment and materials, and then comparing the microorganisms enumerated under these culture conditions with those detected by alternative combinations of medium and incubation conditions. If this approach indicates that a low proportion of the bioburden is being enumerated, the proposed culture conditions should be reconsidered in order to optimize the determination. However, this approach can be used for products with low levels of bioburden.

For further guidance on enumeration, see B.6.

A determination of bioburden can only be an estimate of the bioburden on product. In improving the percentage recovery for a particular technique, the accuracy of the determination of bioburden can be increased. Since there are a variety of techniques that can be used to remove microorganisms, the percentage recovery obtained using a particular technique is a factor in deciding the technique to be used. If the percentage recovery is found to be less than 50 %, improvements to the technique or the use of alternative techniques should be considered. It is noted that there can be certain circumstances where it is not possible to have a recovery above 50 %.

When selecting techniques for use in the microbial characterization of environmental and industrial microorganisms, laboratory personnel should be mindful of:

— the appropriateness of tests and kits, which have been developed primarily for use in clinical settings;

— the ability of laboratory staff to interpret results for microorganisms displaying minimal metabolic activity in biochemical tests (e.g. non-fermentative gram-negative bacteria).

A.8 Routine determination of bioburden and interpretation of data

A.8.1 In order to demonstrate that effective control of microbiological quality has been implemented and maintained, a programme of monitoring product and/or components should be developed.

It is common practice to use a sample size of between 3 to 10 items for routine monitoring of bioburden levels.

A rational choice of sample size primarily depends upon two factors:

a) the change in bioburden to be detected;

NOTE This will depend upon the consequences associated with a change (either increase or decrease) in bioburden level and how the bioburden information is being applied. For early detection of a small change in the mean bioburden level, a large sample size could be needed.

b) the variation in estimates of the number of viable microorganisms present on individual items.

NOTE The degree of this variability will determine the sample size necessary to detect a given change. Small item-to-item variation in such estimates will require a smaller sample size to detect a change than that required for large item-to-item variation.

Table A.3 (provided as an illustration only) demonstrates how sample size and variability of bioburden affects the ability to detect a given change in the magnitude of bioburden. Clearly, large sample sizes provide increased confidence in detecting significant changes.

It should be recognized that the manner in which bioburden data are used could influence the desired level of confidence in detecting a change of a given magnitude. A rational choice of the magnitude of change to be detected and the probability of achieving that detection should be made.

A rational choice for the frequency of monitoring should be made, taking into account a variety of factors including:

— the availability of historical data;

— the purpose for generating the data;

— the nature of the manufacturing process;

— the production frequency for the product;

— the criticality of detecting bioburden changes in a timely fashion;

— seasonal and environmental variations.

Sampling may be performed at a frequency based on time (e.g. monthly), or on production volume (e.g. alternate batches). However, in order to establish baseline levels, it is common practice to determine bioburden at a higher frequency during the initial production of a new product and for this frequency to be reduced as a knowledge of bioburden develops.

The frequency of determinations of bioburden should allow detection of changes in bioburden, for example, due to seasonal variations, manufacturing changes or changes in materials.

A.8.2 The selection of a method for determination of bioburden should consider the possible occurrence of biofilm on or in product. Medical devices incorporating tissue have a potential for biofilm occurrence. Biofilm could form on or in product in contact with liquids.

A.8.3 See A.6.2

A.8.4 No guidance offered.

A.8.5 A predetermined course of action must be taken when specified limits are exceeded. If corrective actions lead to changes to the process that affect the bioburden, new data should be obtained and new limits established for product.

The limits used for bioburden are based upon historical data for product. In the absence of such historical data, tentative limits can be set upon evaluating the first three batches of a given product. Based upon successive test results, these should be re-evaluated after a period of time to verify whether the original limits are appropriate.

Data derived from bioburden determinations for a given product might not precisely follow a well-recognized mathematical distribution. In particular, some data exhibit many zero counts with a few high counts and a histogram representing such bioburden data will exhibit a long tail to the right. If a mathematical distribution can be fitted to a given set of data, upper limits for the data may then be set accordingly. Thus, an upper probability limit can be chosen (perhaps the 95 % or 99 % probability limit) for the bioburden that should not be exceeded if the production process continues to operate in the same manner as that used to obtain the historical data.

In the absence of a fit to a mathematical distribution, it is possible to apply the principle of control charting to data derived from bioburden determinations and set limits accordingly. The preparation of control charts does not strictly depend upon any underlying assumed distribution. The quantities that are plotted on control charts are the mean and standard deviation (or range). Means will tend to have a more symmetrical distribution than individual values. Transformation of raw data may further improve the applicability of a standard Shewhart control chart. Experience gained in fitting empirical distributions to such data sets suggests that the following two transformations of individual counts might be suitable.

a) For product with an overall average bioburden of less than 10 CFU per unit, the suggested transformation to improve symmetry is:

$$Y = \sqrt{N}\ \log_{10}\left(\sqrt{x/N} + \sqrt{x/N+1}\right) \qquad \text{(A.1)}$$

where

Y is the transformed count;

x is the original untransformed count;

N is a scaling factor based upon variance and is equal to mean^2/(variance – mean).

For those cases where the historical mean exceeds the historical (within group) variance, ignore the transformation since the constant N will be undefined.

b) For product with an overall average bioburden exceeding 10 CFU per unit, the distribution could again tend to be skewed with a long tail to the right. In practice, such data can be best approximated by a lognormal distribution. In practice, taking logarithms of the individual counts will make the data approximate to a normal distribution. Since bioburden counts of zero may be observed, a positive constant of 0,1 should be added to all counts before taking logarithms. The suggested transformation then becomes:

$$Y = \log_{10}(x + 0{,}1) \qquad \text{(A.2)}$$

where

Y is the transformed count;

x is original untransformed count.

Control limits for a standard Shewhart control chart of the average bioburden can be established after a sufficient number of historical counts have been collected for a product. (See ISO 8258 and ISO 7871 for control charting procedures). If a sufficient number of historical data is not available for a given product, conditional control limits may be established with fewer values. The limits can be revised when more data become available. Where appropriate, seasonal variation should be taken into account when setting limits. Any data identified as outliers should be discarded in setting limits.

Data identified as unusually large or small should be investigated. If they are thought to be other than laboratory error or the occasional high values found in the manufacturing process, the data may be considered an outlier and can be omitted from calculations in setting the limits for bioburden monitoring. When bioburden data are analysed for use in a quality-related decision, individual test outcomes such as “no growth” or “too numerous to count (TNTC),” are included in the analysis.

A.8.6 Graphical representation of data collected over time can be useful in distinguishing actual trends from sampling variability. Graphical representation can also indicate that a significant change in the microbiological population has occurred even though the bioburden values reside within the preset limits.

Before statistical calculations can be performed on data derived from bioburden determinations, especially where many observations are recorded, it can be necessary to manipulate the data in such a way that the significant features are revealed. This can be done in a qualitative manner by grouping the measurements to form frequency tables and charts. Upon completion, the data can be examined for trends.

There is a number of techniques for trending which can be applied to bioburden. These trending techniques can be, but are not limited to, Shewhart control charts, control based on range (BOR), or cumulative sum charts (ISO 7871). Each of these different techniques can be used to establish a possible shift from the usual random spread of results and to highlight out-of-specification results.

In some instances, it can be appropriate to utilize more than one of these techniques to determine whether or not action is to be taken based upon the available data set or whether additional data are required.

A.8.7 Subclause 8.1 of ISO 13485:2003 requires the planning and implementation of appropriate methods of measurement and analysis, including selecting suitable statistical techniques. The examination of data derived from determinations of bioburden for a wide range of products illustrates the variability of such data. Determinations from a group will vary within the group of items, and, therefore, analyses of data generally use means. Clearly, these means can take high, intermediate or low values, and mean values will vary over time. Furthermore, the types of microorganism that comprise the bioburden can also vary.

A commonly observed characteristic of the frequency distributions of data derived from determinations of bioburden is that distributions are extremely skewed and frequently show extremely long tails. For low or intermediate data, the modal value is zero. In these circumstances, the bioburden is generally low but there may be occasional high values, even though the control measures are effectively applied.

The extreme asymmetry of these skewed frequency distributions means that the established techniques of quality control based on symmetrical distributions are not always appropriate. Special statistical techniques may have to be developed for individual cases, either:

a) using transformational techniques to make the distribution of the data symmetrical and applying standard techniques

or

b) developing a new technique specifically suited to a skewed distribution.

A.9 Maintenance of method of determination of bioburden

No guidance offered.

Table A.3 — Probability of a Shewhart control chart for bioburden data detecting a tenfold change in bioburden level with changing sample size and within sample variability

Sample size	Within-sample variability							
	Standard deviation							
	0,3	0,4	0,5	0,6	0,7	0,8	0,9	1,0
3	0,99723	0,908 26	0,678 71	0,454 92	0,299 57	0,201 88	0,141 08	0,102 41
4	0,99988	0,977 25	0,841 34	0,630 56	0,443 20	0,308 54	0,218 35	0,158 66
5	1,000 00	0,995 20	0,929 51	0,766 32	0,577 06	0,418 82	0,303 11	0,222 45
6	1,000 00	0,999 11	0,971 22	0,860 48	0,691 21	0,524 66	0,390 37	0,290 98
7	1,000 00	0,999 85	0,989 03	0,920 67	0,782 20	0,620 65	0,475 97	0,361 58
8	1,000 00	0,999 98	0,996 06	0,956 74	0,850 97	0,703 86	0,556 74	0,431 89
9	1,000 00	1,000 00	0,998 65	0,977 25	0,900 73	0,773 37	0,630 56	0,500 00
10	1,000 00	1,000 00	0,999 56	0,988 41	0,935 43	0,829 67	0,696 25	0,564 46
11					0,958 90	0,874 06	0,753 37	0,624 24
12					0,974 34	0,908 26	0,802 06	0,678 71
13					0,984 25	0,934 09	0,842 83	0,727 59
14					0,990 49	0,953 24	0,876 44	0,770 85
15					0,994 34	0,967 21	0,903 77	0,808 66

NOTE Table A.3 has been compiled as the basis of the following:

— logarithmic transformation of bioburden estimates;

— use of standard Shewhart control charts;

— transformed data follow a normal distribution;

— a tenfold increase in bioburden has occurred;

— the sample mean takes a value greater than the three sigma control limit.

Annex B
(informative)

Guidance on methods of determination of bioburden

B.1 General

B.1.1 Bioburden determinations can be employed in a variety of situations. The individual responsible for the conduct of such determinations should take account of the particular circumstances under which the determinations are made in deciding the sampling rate, the nature of culture media and the incubation conditions, together with the extent of method development and validation.

B.1.2 The sequence of key steps of the process for the determination of bioburden is illustrated in Figure B.1. The individual responsible for the conduct of such determinations should use the knowledge of the raw materials, components, manufacturing environment, production processes and the nature of the product to select appropriate techniques for the various steps.

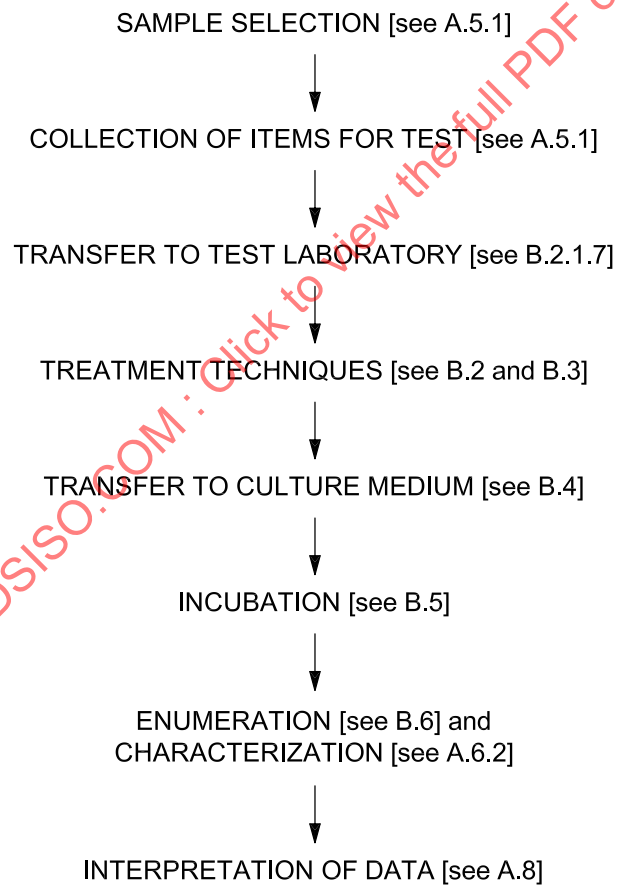

Figure B.1 — Sequence of key steps of the process for the determination of bioburden

B.2 Methods where removal of microorganisms is used

B.2.1 General

B.2.1.1 Several methods described in this annex may be combined to increase the number of organisms found and reduce variability.

B.2.1.2 The degree of adhesion of microorganisms to surfaces varies with the nature of the surface, the microorganisms involved and other materials present (e.g. lubricants). The origin of the contamination will also influence the degree of adhesion. To remove microorganisms, treatments used may consist of rinsing together with some form of physical force or direct surface sampling. A surfactant may be used to enhance recovery but it should be recognized that surfactants at high concentrations could be inhibitory to the growth of microorganisms.

B.2.1.3 For materials in contact with non-sterile fluids, microorganisms can occur as a biofilm. A biofilm is a structure in which microorganisms are encapsulated in a matrix that adheres strongly to surfaces. Microorganisms in biofilms can exhibit increased resistance to sterilization processes. Biofilms can be initiated within minutes and can develop to a much greater extent on medical devices incorporating tissue or on used devices. In such instances, consideration should be given to the potential for biofilm formation and it should not be assumed that the treatments outlined in B.2.2 would be appropriate for liberating microorganisms completely from a biofilm. An indication that a biofilm is present can be obtained during validation of the removal technique if repeated high microbial counts are recorded during repetitive recovery.

B.2.1.4 Any treatment used during bioburden determination should be reproducible and should avoid conditions that are likely to affect the viability of microorganisms, such as excessive cavitation, shear forces, temperature rise or osmotic shock.

B.2.1.5 Some treatments are easier to control than others. The variables and ways of controlling them should be considered when selecting a treatment and devising suitable conditions of treatment. For example, for a given treatment, the time may be extended or the nature of mechanical agitation modified to increase the removal of organisms.

B.2.1.6 Certain methods of treatment can disaggregate the product under test (e.g. disintegration, stomaching and vortexing). The presence of disaggregated material can render enumeration of microorganisms difficult. Additional treatment, for example to separate the disaggregated material from the eluent, can be necessary. Care should be taken to ensure that the counts obtained are representative.

B.2.1.7 Every effort should be made to transfer items for testing to the laboratory as quickly as possible. If delay in transfer is unavoidable, the conditions under which the items are stored should be selected to prevent changes in the microbial population. The maximum storage time should be specified. Desiccation can be the cause of significant decreases in numbers of microorganisms and should be considered in the selection of storage conditions and storage times.

B.2.2 Removal techniques

B.2.2.1 Stomaching

B.2.2.1.1 The test item and a known volume of eluent are enclosed in a sterile stomacher bag. Reciprocating paddles operate on the bag forcing the eluent through and around the item.

B.2.2.1.2 The time of treatment should be defined.

B.2.2.1.3 This method is particularly suitable for soft, fibrous and/or absorbent materials but it would be unsuitable for any materials that would puncture the bag (e.g. devices containing needles or rigid items).

B.2.2.1.4 This method may yield a suspension having a low concentration of microorganisms if a relatively large volume of eluent is used. If practicable, the eluent should be filtered.

B.2.2.2 Ultrasonication

B.2.2.2.1 The test item is immersed in a known volume of eluent within a suitable vessel. Either the vessel and contents are treated in an ultrasonic bath or an ultrasonic probe is immersed in the contained eluent. Microorganisms can also be inactivated by ultrasonication, especially with more energy transfer, and inactivation is more likely with probe use than in an ultrasonic bath; the sonication method should be validated in accordance with B.9.

B.2.2.2.2 The nominal frequency of sonication and duration of treatment should be defined. Furthermore, the position(s) in which items are placed in an ultrasonic bath should be defined. Consideration should be given to limiting the number of items to be processed concurrently as some of the sonication power can be reduced through shielding.

B.2.2.2.3 The technique is particularly suitable for solid impermeable items and for products with complex shapes. It can be destructive to some medical devices, particularly those containing electronic components, such as implantable pulse generators.

B.2.2.2.4 The sonication energy and duration of sonication should not cause disruption and death of microorganisms or cause the eluent to overheat.

B.2.2.3 Shaking (mechanical or manual)

B.2.2.3.1 The test item is immersed in a known volume of eluent within a suitable vessel and shaken on a mechanical shaker (e.g. reciprocating, orbital or wrist action) in order to assist the removal of microorganisms. Manual shaking might be used but its effectiveness can vary depending on the operator.

B.2.2.3.2 The time and frequency of shaking should be defined.

B.2.2.3.3 Glass beads of a defined size may be added to increase surface abrasion and thereby recovery efficiency. The size of added glass beads, together with the time and frequency of shaking, should not be such as to cause overheating and/or possible damage to the microorganisms.

NOTE The addition of glass beads will increase the surface area to which microorganisms can adhere.

B.2.2.4 Vortex mixing

B.2.2.4.1 The test item is immersed in a known volume of eluent in a closed container that is placed on the rotating pad of the vortex mixer so that a vortex is created. The vortex produced will depend upon the pressure applied manually. Variations in the vortex can cause variable removal.

B.2.2.4.2 The container to be used, the time of mixing and the speed at which the mixer is set should be defined.

B.2.2.4.3 The method is quick and simple to perform but is mainly suitable for small items.

B.2.2.5 Flushing

B.2.2.5.1 The eluent is passed through the internal lumen of the test item. Liquid flow can be induced by gravity or pumping. Alternatively, the product can be filled with the eluent, clamped and agitated.

B.2.2.5.2 The time of contact between the device and eluent, the rate of flushing and the volume of fluid should be defined.

B.2.2.5.3 Device configurations and lumen sizes can limit the physical forces necessary to remove organisms completely from internal surfaces.

B.2.2.6 Blending (disintegration)

B.2.2.6.1 The test item is immersed in a known volume of eluent within a suitable vessel. The item is blended or chopped for a specified time.

B.2.2.6.2 The specified time depends on the item and the blender, but should not be extended such as to cause overheating of the eluent and possible damage to the microorganisms.

B.2.2.6.3 This technique provides a way of dividing an item into small enough parts so that the microorganisms can be enumerated by a plating technique.

B.2.2.7 Swabbing

B.2.2.7.1 Swabs consist of absorbent material usually mounted on some form of stick or handle. The sampling material may be soluble or insoluble.

B.2.2.7.2 The normal method of use is to moisten the swab with eluent and wipe a pre-determined surface area of the item. The recovery efficiency can be improved in some circumstances by first moistening the surface and then swabbing with a dry swab. The swab is transferred to diluent and agitated to remove microorganisms from the swab. Alternatively, in the case of soluble swabs, the swab is dissolved in diluent.

B.2.2.7.3 Swabs are a useful method of sampling irregularly shaped or relatively inaccessible areas. They are also useful when a large area is to be sampled.

B.2.2.7.4 This technique is particularly prone to errors due to variation in the way the swab is manipulated. Furthermore, it is unlikely that all microorganisms on the surface will be collected by the swab. Some of the microorganisms that are collected can become trapped in the matrix of the swab itself and therefore not be detected.

B.2.2.7.5 There should be no microbicidal or microbiostatic agents present in the swab.

B.2.3 Eluents, diluents and transport media

B.2.3.1 During bioburden determination, eluents can be used to remove microorganisms from the product. Transport media can be used to transfer removed microorganisms for enumeration and diluents used to obtain suspensions containing microorganisms in countable numbers.

B.2.3.2 The nature of the eluents and diluents can have a marked influence on the overall efficiency of the method used. In selecting a diluent or eluent, consideration should be given to its composition (e.g. constituents and their concentrations, osmolarity and pH). The composition should be such that proliferation or inactivation of microorganisms does not occur.

B.2.3.3 When a liquid is used for removal of microorganisms from solid surfaces, the incorporation of a surfactant may be considered.

B.2.3.4 Eluents and diluents commonly used include those listed in Table B.1.

Table B.1 — Examples of eluents and diluents

Solution	Concentration in water	Applications
Buffered peptone water	0,067 M phosphate 0,43 % sodium chloride 0,1 % peptone	General
Calgon Ringer	1/4 strength	Dissolution of calcium alginate swabs
Peptone water	0,1 % to 1,0 %	General
Phosphate buffered saline	0,02 M phosphate 0,9 % sodium chloride	General
Ringer	1/4 strength	General
Sodium chloride	0,25 % to 0,9 %	General
Thiosulphate Ringer	1/4 strength	Neutralization of residual chlorine
Water	NA	Dilution of aqueous samples. Preparation of isotonic solutions of soluble materials prior to counting
NOTE This list is not exhaustive. A surfactant such as polysorbate (Tween) 80 may be added to eluents and diluents. A concentration of between 0,01 % and 0,1 % is generally used, depending upon the specific application. The appropriate concentration to be used with any particular treatment needs careful selection because foaming can occur.		

B.3 Methods where removal of microorganisms by elution is not used

B.3.1 Contact plating

B.3.1.1 Contact plates or slides are means by which solidified culture medium can be applied to a surface with the intention that viable microorganisms will adhere to the surface of the medium. The plate or slide can then be incubated to produce colonies that are enumerated.

B.3.1.2 Such systems have the advantage of being easy to use. Results are directly related to the area in contact with the solidified culture medium.

B.3.1.3 The natural clumping of cells on surfaces, spreading of colonies at the agar interfaces, drying out of the agar and possibility of anaerobic locations are potential disadvantages.

B.3.1.4 This method should be used only when other methods are not applicable because the efficiency is generally low. Contact plates and slides are generally only useful on flat or at least regular surfaces.

B.3.2 Agar overlaying

B.3.2.1 Agar overlaying involves coating the surfaces of a product with a molten agar medium (at a temperature of approximately 45 °C) and allowing it to solidify, followed by incubation to produce visible colonies. This method may be applicable when the bioburden is low and the product configuration suitable.

B.3.2.2 The natural clumping of cells on surfaces, spreading of colonies at the agar interfaces, drying out of the agar and possibility of anaerobic locations are potential disadvantages.

B.3.3 Most Probable Number (MPN)

B.3.3.1 The MPN method is a well-established and fully documented method of estimating the number of viable microorganisms in product in which the microorganisms are randomly distributed. Its principal uses are in the food and water industries where it is used with liquid, powdered and semi-solid products or raw materials. The method is particularly appropriate for product having bioburden of low mean number.

B.3.3.2 The method consists of taking replicate samples of product (by volume or weight) which contain, on average, the same number of viable microorganisms in each of them (hence the requirement for randomness of distribution) and scoring each sample individually for the presence of viable microorganisms by means of transferring to liquid growth media and incubation. When a sufficient volume of eluent is available, a range of dilutions can be inoculated into nutrient medium such that a fraction of the inoculated media does not produce visible growth on subsequent incubation. From the frequency of the occurrence of positive tests within a set of replicates, an estimate is made of the number of viable microorganisms present in the sample or the bulk product from which the sample has been taken; the 95 % confidence limits about the estimate are relatively wide. The estimate and its confidence limits are derived from published MPN Tables (DeMan [18]), which have been developed on the assumption that numbers of viable microorganisms present in replicate samples are distributed around a mean number in accordance with Poisson distribution.

The key requirement for the application of the MPN method is the random distribution of microbial population throughout the product under investigation. Accordingly, the MPN method may have value for the determination of bioburden for liquid medical devices, viscous fluids, powders or in situations where the bioburden is being estimated in a liquid used as an eluent for a single product. However, it is difficult to see where the method has general application for bioburden determinations performed on populations of solid medical devices. Typically, the distribution of numbers of microorganisms comprising low average bioburden on such devices is not generally random; a population of such items invariably has a high proportion possessing no detectable organisms and a small number of items with “spikes” that contribute substantially to the average bioburden. In an MPN exercise, the “spike” would simply be recorded as a positive and, as such, would contribute to the average in a formularistic manner only and not numerically. This could lead to an underestimate of the bioburden, an undesirable outcome.

B.3.3.3 MPN methods are simple to perform, and the statistical basis for the method makes it more appropriate for general assessment rather than accurate determinations.

B.3.3.4 If microbicidal or microbiostatic substances are present, the considerations outlined in this regard in B.8 will apply.

B.4 Transfer to culture medium

B.4.1 General

B.4.1.1 Treatment will usually produce a suspension of microorganisms. Enumeration of the viable microorganisms in the suspension can be undertaken using one of the techniques described below.

B.4.1.2 Prior to transfer to culture medium, additional treatment might be necessary in order to disrupt aggregates of microorganisms and thereby reduce under-estimation. In some cases, the technique used to remove the microorganisms from the item under test might also disrupt such aggregates.

B.4.1.3 The presence of microbicidal or microbiostatic substances may influence the choice of culture method. If microbicidal or microbiostatic substances are present in the eluent, these may be reduced to an ineffective concentration by dilution, removed by filtration or chemically inactivated.

B.4.2 Membrane filtration

B.4.2.1 Filtration of an eluent, followed by incubation of the filter on an appropriate growth medium to give visible colonies, is an effective means of enumerating viable microorganisms. A filter of appropriate nominal pore size not greater than 0,45µ is generally adequate to capture microorganisms.

B.4.2.2 A vacuum, or in some instances pressure, source is usually required. Care should be exercised in order to avoid excessive backpressures, which can cause distortion of or damage to the membrane filter.

B.4.2.3 Membrane filtration of eluents containing particulates, such as remnants of fibrous products, could be difficult, as the particulates may block the filter.