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Sterilization of health care products — Microbiological methods —

Part 3: Bacterial endotoxin testing

Stérilisation des dispositifs médicaux — Méthodes microbiologiques —

Partie 3: Essai des endotoxines bactériennes

FDIS stage

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: + 41 22 749 01 11
E-mail: copyright@iso.org
Website: www.iso.org

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Foreword

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

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*.

A list of all parts in the ISO 11737 series can be found on the ISO website.

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

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Introduction

A pyrogen is any substance that can induce fever. Testing for pyrogens is required for release of many health care products. Pyrogens can be classified into two groups: microbial (e.g. bacteria, fungi, viruses) and non-microbial (e.g. drugs, device materials, steroids, plasma fractions; see the ISO 10993 series). The predominant pyrogenic contaminants encountered in the manufacturing of health care products are bacterial endotoxins, which are components of the cell walls of Gram-negative bacteria. Although Gram-positive bacteria, fungi, and viruses can be pyrogenic, they do so through different mechanisms (systemic effects) and to a lesser degree than Gram-negative bacteria. Only the Gram-negative bacterial endotoxins test (BET) using amebocyte lysate reagents from *Limulus polyphemus* or *Tachypleus tridentatus* is covered in this document. Other endotoxin detection methodologies, such as monocyte activation and recombinant Factor C (rFc), are not included (see B.12) in this document.

Endotoxins are the molecular weight lipopolysaccharide (LPS) components of the outer cell wall of Gram-negative bacteria, that can cause fever, meningitis, and a rapid fall in blood pressure if introduced into the blood stream or certain other tissues of the body. The outer cell wall components, which are composed primarily of proteins, phospholipids, and LPS, are constantly released by the cell into the surrounding environment. Endotoxins are ubiquitous in nature, stable, and small enough to pass through conventional sterilizing filters. Sterilization processes will inactivate microorganisms on or in products, but usually do not inactivate endotoxin on products. With controlled processes, endotoxin contamination can be prevented.

The non-pyrogenicity of a health care product can be achieved through the following:

a) manufacturing techniques that prevent or control endotoxin contamination (e.g. contamination with Gram-negative bacteria);

b) depyrogenation by endotoxin inactivation (e.g. dry heat) or physical removal (e.g. rinsing, distillation, ultrafiltration).

The purpose of this document is to describe the requirements and guidance for testing for bacterial endotoxins. This includes product required to be non-pyrogenic based on either intended use or non-pyrogenic label claim, or both. Guidance is also provided on selection of product units, method suitability, use of techniques for routine testing, interpretation of test results, and alternatives to batch testing and risk assessment. Information on the following is provided in the annexes:

— guidance on bacterial endotoxin testing (Annex A);

— the history and background on the BET (Annex B);

— guidance on out of specified limits (OSL) and failure investigation (Annex C);

— guidance on in-process monitoring of manufacturing or component testing (Annex D);

— guidance on conducting a risk assessment to support alternatives to batch testing (Annex E);

— typical assignment of responsibilities (Annex F).

This document is based on ANSI/AAMI ST72. Several sections in this document have been restructured and extended or changed from ANSI/AAMI ST72.

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Sterilization of health care products — Microbiological methods —

Part 3:
Bacterial endotoxin testing

1 Scope

1.1 Inclusions

This document specifies general criteria to be applied in the determination of bacterial endotoxins on or in health care products, components or raw materials using bacterial endotoxins test (BET) methods, using amebocyte lysate reagents.

1.2 Exclusions

1.2.1 This document is not applicable to the evaluation of pyrogens other than bacterial endotoxins. Other endotoxin detection methodologies are not included (see B.12).

1.2.2 This document does not address setting specific endotoxin limit specifications.

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 terminology databases for use in standardization at the following addresses:

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

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

3.1
bacterial endotoxins test
BET
assay for measuring bacterial endotoxins by combining an aqueous test sample or test sample extract with *Tachypleus* amebocyte lysate *(TAL)* (3.41) or *Limulus* amebocyte lysate *(LAL)* (3.28) reagent and measuring the resulting proportional reaction via visual, turbidimetric or *chromogenic techniques* (3.3)

3.2
batch
defined quantity of a product intended or purported to be uniform in character and quality produced during a specified cycle of manufacture

[SOURCE: ISO 11139:2018, 3.21]

3.3
chromogenic technique
bacterial endotoxins test(BET) (3.1) methodology that quantifies endotoxins on the basis of a measured colour-producing reaction proportional to the interaction of *Limulus* amebocyte lysate *(LAL)* (3.28) and endotoxin

3.4
control standard endotoxin
CSE
endotoxin standard preparation whose potency has been standardized against the *Reference Standard Endotoxin (RSE)* (3.37) for a specific batch of *Limulus* amebocyte lysate *(LAL)* (3.28)

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3.5
depyrogenation
process used to remove or deactivate pyrogenic substances to a specified level

Note 1 to entry: Pyrogenic substances include bacterial endotoxins.

[SOURCE: ISO 11139:2018, 3.77]

3.6
direct contact
medical device or medical device component that comes into physical contact with body tissue

[SOURCE: ISO 10993-1:2018, 3.6]

3.7
end product
product samples that have completed the entire manufacturing process

Note 1 to entry: For the purposes of this document, end-product testing can be performed prior to sterilization (pre-sterilization samples) or after sterilization (post-sterilization samples). For limitations see 5.2.6.

3.8
endotoxin
bacterial endotoxin
lipopolysaccharide (LPS)(3.29) component of the cell wall of Gram-negative bacteria that is heat stable and elicits a variety of inflammatory responses in animals and humans

[SOURCE: ISO 11139:2018, 3.101]

3.9
endotoxin limit
maximum allowable amount of endotoxin present on the product or in a product extraction solution

3.10
endotoxin unit
EU
international unit
IU
standard unit of measure for endotoxin activity initially established relative to the activity contained in 0,2 ng of the *Reference Standard Endotoxin (RSE)* (3.37) Lot EC-2 [US Pharmacopeia (USP) standard reference material]

Note 1 to entry: Currently, the US RSE EC-6, USP Lot G, and the World Health Organization's primary international endotoxin standard (IS) are sub-lots of the same endotoxin preparation, making the EU and IU equal [45].

3.11
end point
most dilute concentration of a test or control solution for which a positive reaction for bacterial endotoxin is observed

Note 1 to entry: This definition is used for concentration dependent bacterial endotoxin testing, in contrast to dilution dependent end point methods described in A.6.1.1.

3.12
enhancement
bacterial endotoxins test (BET) (3.1) anomaly in which a non-endotoxin related factor, usually attributable to a characteristic of the test sample, elicits a test reaction greater than the amount of endotoxin present

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3.13
gel-clot technique
bacterial endotoxins test (BET) (3.1) methodology that quantifies or detects endotoxin on the basis of a clot-producing reaction proportional to the interaction of *Limulus* amebocyte lysate *(LAL)* (3.28) and endotoxin

3.14
geometric mean end point
antilog of the average of the logarithmic values with respect to the *end points* (3.11) from replicate dilution series converted back to a base 10 number used to establish the central tendency or typical value from a test solution

3.15
health care product
medical device, including in vitro diagnostic medical device, or medicinal product, including biopharmaceutical

[SOURCE: ISO 11139:2018, 3.132]

3.16
indirect contact
medical device or medical device component through which a fluid or gas passes, prior to the fluid or gas coming into physical contact with body tissue (in this case the medical device or medical device component itself does not physically contact body tissue)

[SOURCE: ISO 10993-1:2018, 3.11]

3.17
inhibition
bacterial endotoxins test (BET) (3.1) anomaly in which a non-endotoxin related factor, usually attributable to a characteristic of the test sample, elicits a test reaction less than the amount of endotoxin present

3.18
method suitability
inhibition/enhancement test
test used to determine whether a particular sample contains interfering factors that diminish its accuracy by introducing *enhancement* (3.12) or *inhibition* (3.17) into the test system

3.19
interference
interfering factor observed in the performance of the test that exceeds the acceptable threshold for a given *bacterial endotoxins test (BET)* (3.1) technique (e.g. positive product control that indicates a detected endotoxin level less than 50 % or greater than 200 % or ±2 lambda)

3.20
intraocular
located or occurring within or administered through the eye

3.21
interfering factors
non-endotoxin related factor, usually attributable to a characteristic of the test sample, that causes *inhibition* (3.17) or *enhancement* (3.12)

3.22
intravascular
located or occurring within or administered through the heart or blood vessels

3.23
intralymphatic
located or occurring within or administered through a lymph vessel

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3.24
intrathecal, adj,
located, or occurring within or administered through the space under the arachnoid membrane of the brain or spinal cord

3.25
kinetic method
photometric quantitative techniques (turbidimetric or chromogenic) for *bacterial endotoxins test (BET)* (3.1)

3.26
LAL reactive material
LAL-RM
Limulus **amebocyte lysate reactive material**
any non-endotoxin compound that will activate the *Limulus* amebocyte lysate *(LAL)* (3.28) clotting cascade and cause *enhancement* (3.12)

3.27
lambda
λ
labelled sensitivity of a *Limulus* amebocyte lysate *(LAL)* (3.28) gel-clot reagent, expressed in EU/mL or, for chromogenic or turbidimetric tests, the lowest point (endotoxin concentration) on the referenced standard curve

3.28
***Limulus* amebocyte lysate**
LAL
reagent extracted from amebocytes taken from hemolymph of the horseshoe crab, *Limulus polyphemus*, that reacts with endotoxin, to form a gelatinous clot and is used to estimate endotoxin levels in *bacterial endotoxins test (BET)* (3.1) methods

Note 1 to entry: The term LAL is sometimes used to describe *Tachypleus* amebocyte lysate *(TAL)* (3.41), as both are similar lysates that are used in the BET. They also are often generically referred to as "lysate".

3.29
lipopolysaccharide
LPS
Gram-negative bacterial cell wall component composed of lipid A, a core polysaccharide, and an O-side chain

3.30
maximum valid dilution
MVD
maximum amount a sample can be diluted, or the total extraction volume used relative to the sensitivity of a *bacterial endotoxins test (BET)* (3.1) in which the specified *endotoxin limit* (3.9) can be detected

3.31
medical device
instrument, apparatus, implement, machine, appliance, implant, reagent for in vitro use, or software material or other similar or related article, intended by the manufacturer to be used, alone or in combination, for human beings, for one or more of the specific medical 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;

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— control of conception;

— disinfection of medical devices;

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

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

Note 1 to entry: Products which can be considered to be medical devices in some jurisdictions, but not in others include:

— items specifically intended for cleaning or sterilization of medical devices;

— pouches, reel goods, sterilization wrap, and reusable containers for packaging of medical devices for sterilization;

— disinfection substances;

— aids for persons with disabilities;

— devices incorporating either animal or human tissues, or both;

— devices for in vitro fertilization or assisted reproduction technologies.

[SOURCE: ISO 13485:2016, 3.11, modified — The first two list items in the Note 1 to entry have been added. In Note 1 to entry, "may be considered" has been changed to "can be considered" to indicate possibility rather than permission.]

3.32
non-pyrogenic
not inducing a fever

Note 1 to entry: Describes an item or product that contains endotoxin levels that conform to specified limits.

3.33
out of specified limits
OSL
sample with a valid *bacterial endotoxins test (BET)* (3.1) result that exceeds a product *endotoxin limit* (3.9) specification

Note 1 to entry: The term OSL applies only within the context of this document and does not imply compliance with any other regulatory guidance dealing with out of specification (OOS) results.

3.34
product positive control
PPC
sample spiked with a known amount of endotoxin used for confirmation that the product being tested is not subject to *interfering factors* (3.21)

3.35
pyrogen
substance that induces a fever

3.36
pyrogenic
inducing a fever

Note 1 to entry: Describes an item or product that contains endotoxin levels above specified limits.

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3.37
Reference Standard Endotoxin
RSE
US Pharmacopeia (USP) endotoxin reference standard that has a defined potency of 10,000 USP EUs per vial

3.38
repeat test
analysis of additional product samples from a previously tested batch or another batch

3.39
retest
reanalysis of previously tested product samples or product sample preparation

3.40
standard control series
serial dilution series of *Reference Standard Endotoxin (RSE)* (3.37) or *control standard endotoxin (CSE)* (3.4) used to verify *Limulus* amebocyte lysate *(LAL)* (3.28) sensitivity

3.41
***Tachypleus* amebocyte lysate**
TAL
reagent extracted from amebocytes taken from hemolymph of the horseshoe crab, *Tachypleus tridentatus,* which reacts with endotoxin, to form a gelatinous clot and is used to estimate endotoxin levels in *bacterial endotoxins test (BET)* (3.1) methods

Note 1 to entry: The term TAL is sometimes used to describe *Limulus* amebocyte lysate *(LAL)* (3.28), as both are similar lysates that are used in the BET. They also are often generically referred to as "lysate".

3.42
turbidimetric technique
bacterial endotoxins test (BET) (3.1) methodology that quantifies or detects endotoxin on the basis of a measured turbidity reaction proportional to the interaction of *Limulus* amebocyte lysate *(LAL)* (3.28) and endotoxin

3.43
validation
confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled

Note 1 to entry: The objective evidence needed for a validation is the result of a test or other form of determination such as performing alternative calculations or reviewing documents.

Note 2 to entry: The word "validated" is used to designate the corresponding status.

Note 3 to entry: The use conditions for validation can be real or simulated.

[SOURCE: ISO 9000:2015, 3.813]

3.44
verification
confirmation, through the provision of objective evidence, that specified requirements have been fulfilled

Note 1 to entry: The objective evidence needed for a verification can be the result of an inspection or of other forms of determination such as performing alternative calculations or reviewing documents.

Note 2 to entry: The word "verified" is used to designate the corresponding status.

[SOURCE: ISO 9000:2015, 3.8.12, modified — The original Note 2 to entry has been deleted and Note 3 has been renumbered as Note 2 to entry.]

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3.45
water for bacterial endotoxins test
WBET
purified water employable as a solvent, diluent, and/or extractant that is non-reactive with the lysate employed at the detection limit of the reagent, and does not elicit *interference* (3.19) with methodology in use [typically *Limulus* amebocyte lysate *(LAL)* (3.28) reagent water, water for injection, or other appropriate solution meeting these requirements]

4 General requirements

4.1 The development, validation and routine control of products with acceptable endotoxin levels are critical elements in the realization of some types of health care products. To ensure the consistent implementation of the requirements specified in this document, the necessary processes shall be established, implemented and maintained. Processes of particular importance in relation to the development, validation and routine endotoxin control of a process include but are not limited to:

— control of documentation, including records,

— assignment of management responsibility,

— provision of adequate resources, including competent human resources and infrastructure,

— control of product provided by external parties,

— identification and traceability of product throughout the process, and

— control of non-conforming product.

NOTE ISO 13485 covers all stages of the life cycle of medical devices in the context of quality management systems for regulatory purposes. National and/or regional regulatory requirements for the provision of health care product can require the implementation of a full quality management system and the assessment of that system by a recognized conformity assessment body.

4.2 A process shall be specified for the calibration of all equipment, including instrumentation for test purposes, used in meeting the requirements of this document.

5 Selection of products

5.1 General

5.1.1 The types of products required or labelled to be non-pyrogenic and the associated bacterial endotoxin limits shall be determined and be consistent with the intended clinical application.

Products should not be labelled as 'pyrogen free' because complete freedom from bacterial endotoxins cannot be demonstrated by testing due to the detection limits inherent in current test methods. The term 'non-pyrogenic' should be used.

NOTE 1 See A.5.1.1 and Annex B for risks associated with endotoxins and for commonly used limits.

NOTE 2 National regulatory requirements can apply regarding non-pyrogenic labelling.

5.1.2 For some products, higher endotoxin limits can be justifiable, with additional supporting data depending on the risk/benefit of the device. Likewise, for other products, more stringent limits can be required (e.g. devices with intrathecal contact).

5.1.3 Product required or labelled to be non-pyrogenic shall require explicit substantiation employing a suitable BET method. Such substantiation shall include at least one of the following:

— end-product testing for each batch;

— alternative-to-batch testing (see Clause 10 and Annex E).

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5.1.4 All parts of products required or labelled to be non-pyrogenic shall be included in the testing process. The exclusion of any part of the product shall be justified and documented (e.g. a handle or a power cord).

5.1.5 There are health care products that have portions of the product that are sealed and as such do not come into contact with the patient. Such portions of the product that do not have patient contact are not required or intended to be non-pyrogenic, and may be excluded from endotoxin testing.

5.1.6 For products for which a claim of non-pyrogenicity applies only to a portion of the product (e.g. the fluid path in an administration set for intravenous infusion), endotoxin testing does not apply to the portions of product not intended to be non-pyrogenic. A statement about the portion of the product to which the claim applies (such as 'non-pyrogenic fluid path') shall be supported by appropriate evaluation of components and surfaces relevant to that portion of the product.

5.1.7 For multi-component kit products for which a claim of either non-pyrogenicity or label claim, or both, applies to only a portion of the kit, endotoxin testing does not apply to the portions of the kit not intended to be non-pyrogenic. The non-pyrogenic portions of the kit shall be supported by appropriate documented rationale.

5.2 Selection of product units

5.2.1 The sampling criteria for selection of product units for endotoxin testing are based on the premise that the manufacturing process, as well as the processes identified in 4.1, are controlled (refer to A.2).

NOTE See Annex D for guidance on in-process monitoring of manufacturing processes or component testing.

5.2.2 The selection of product units for testing shall be based on criteria defined in a sampling plan that includes an assessment of components and processing. This rationale should consider the following:

a) applicable regulatory requirements;

b) assessment of risk;

c) historical performance;

d) manufacturing process validation;

e) statistical considerations.

5.2.3 There are two types of sampling plans: batch testing and alternatives to batch testing.

5.2.3.1 For batch testing, non-pyrogenicity is confirmed through the use of end-product testing. The batch may be defined as each production lot or a product intended or purported to be uniform in character and quality produced during a specified cycle of manufacture. This should be supported with documented rationale or risk assessment (refer to A.5.2 for guidance on the number of samples).

5.2.3.2 Alternatives to batch testing may be used if it has been demonstrated that the manufacturing process and materials are suitably controlled. If alternatives to batch testing are performed, a risk assessment to evaluate the criteria used to establish the sampling plan shall be performed (see Clause 10 and Annex E).

5.2.4 Samples selected for testing shall include all factors that can affect or contribute to the levels of endotoxin.

5.2.5 Samples used for endotoxin testing can be selected from routine production, products that have been rejected for other production quality issues that have no effect on endotoxin content, or surrogate samples that are representative of the full manufacturing process and representative of product endotoxin levels.

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5.2.6 Samples may be obtained prior to sterilization (pre-sterilization) or after sterilization (post-sterilization). Post-sterilization samples encompass all the factors that can affect the product or the endotoxin test. When pre-sterilization samples are selected for testing, the acceptability of the samples in representing the endotoxin level on sterilized product shall be justified and documented. The program for ongoing testing should consistently reflect either pre- or post-sterilization samples. Guidance is provided in A.5.2.6 for assessing the acceptability of pre-sterilization testing.

NOTE For products that support microbial growth, see A.5.2.6.

5.2.7 In the testing of multi-component kits (procedure packs) or sets of individual products within the same sterile barrier system, depending upon how the product is used, there are instances where each component may be evaluated individually and other instances where the entire contents may be considered as a single entity. Consideration of a set or a kit as a single unit shall address sample preparation in adherence to method requirements and the applicable endotoxin limit. The total volume of extraction fluid used for the subcomponents should not exceed the maximum extraction volume determined by the MVD.

6 Methods for BET

6.1 General

6.1.1 There are currently three commonly accepted BET techniques. The choice of technique should be based upon an assessment of the laboratory's capability, experience, sample throughput requirements, data handling requirements, and the nature of the test sample. The current techniques and associated methods are:

a) gel-clot techniques: limit test and assay methods;

b) chromogenic photometric technique: end point method;

NOTE A turbidimetric end point is available but is not commonly used.

c) chromogenic and turbidimetric photometric techniques: kinetic methods.

Information on each of these methods is presented in A.6.

6.1.2 The selected method shall be determined to be suitable as specified in Clause 7. Continued suitability shall be confirmed as specified in Clause 9.

6.2 Consideration of an applicable endotoxin limit

6.2.1 Endotoxin limit

The endotoxin limit defines the maximum allowable amount of endotoxin present on the product or in a product extract solution.

6.2.2 Calculation of endotoxin limit for the extract solution

The endotoxin limit for the extract solution in endotoxin units per ml (EU/ml) shall be calculated as shown in Formula (1) as:

$$\frac{(K)(N)}{V} \quad (1)$$

where

K is the product endotoxin limit;

N is the number of devices tested;

V is the total volume of the extract or rinse (ml) that can be adjusted for the size and configuration of the device(s).

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