BS ISO 11737-3:2023

BSI Standards Publication

Sterilization of health care products — Microbiological methods

Part 3: Bacterial endotoxin testing

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National foreword

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Sterilization of health care products — Microbiological methods —

Part 3: Bacterial endotoxin testing

Stérilisation des produits de santé — Méthodes microbiologiques — Partie 3: Essai des endotoxines bactériennes

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Foreword

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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).

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

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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* (3.42) 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]

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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)

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].

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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

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, adj.
located or occurring within or administered through the eye

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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, adj.
located or occurring within or administered through the heart or blood vessels

3.23
intralymphatic, adj.
located or occurring within or administered through a lymph vessel

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

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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;

— 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 11139:2018, 3.166]

3.32
non-pyrogenic, adj.
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)

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3.35
pyrogen
substance that induces a fever

3.36
pyrogenic, adj.
inducing a fever

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

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 11139:2018, 3.313]

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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 11139:2018, 3.314]

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.

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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).

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.

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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.

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

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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:

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

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).

Product endotoxin limits can be reported in terms of EU/ml, provided an appropriate specified volume for rinsing/immersing has been determined to not exceed the MVD. Reporting the results in EU/device takes into account the initial extraction volume.

6.2.3 Maximum valid dilution (MVD)

6.2.3.1 Products can sometimes interfere with a BET, resulting in inhibition or enhancement due to the presence of interfering factors. The interfering factors shall be assessed. A common technique used to mitigate such interference is to dilute the product extract with water for bacterial endotoxins test (WBET) or another appropriate diluent. The interference can also be diluted by increasing the total extraction volume used to extract the product (e.g. rather than extracting with 40 ml, extract with 80 ml). Because these techniques will also dilute any endotoxin present, there is a limit to the extent of dilution that is allowed. This is referred to as the maximum valid dilution (MVD) and shall be calculated as shown in Formulae (2) and (3):

The MVD, in terms of extraction solution in EU/ml:

$$\frac{\text{EU/mL}}{\lambda} = \frac{\text{Endotoxin Limit of extract solution (EU / ml)}}{\lambda} \tag{2}$$

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or the MVD in terms of maximum extraction volume in EU/device:

$$\frac{\text{EU/device}}{\lambda} = \frac{\text{Endotoxin Limit (EU / device)}}{\lambda} \tag{3}$$

6.2.3.2 The value of the MVD indicates the greatest dilution or total extraction volume that may be used to overcome inhibition/enhancement, based on the sensitivity of the LAL. For example, an MVD of 10 means that a sample extract can be diluted no more than 1:10 without affecting the ability to detect the product endotoxin limit (see Tables A.5 and A.6 for examples of calculations).

6.2.3.3 If a product is tested at the MVD, the endotoxin test indicates a pass or fail result. Any endotoxin detected in the assay (a positive test result) that exceeds the defined endotoxin limit shall indicate a failing result. A negative result indicates a passing result.

NOTE 1 When a product is tested at a dilution less than the MVD, and endotoxin or interference is detected, it can be necessary to carry out further dilution to determine if a result meets or does not meet acceptance criteria.

NOTE 2 When a product cannot be tested at a dilution less than or equal to the MVD, a risk justified adjustment of the endotoxin limit (see 5.1.1) and recalculation of the MVD on this basis can be taken into account.

6.3 Critical test parameters

6.3.1 Temperature

The lysate manufacturer's instructions shall be referred to for appropriate temperatures. Incubation for BET methodology is typically carried out at 37 ± 1 °C.

6.3.2 Time

The lysate manufacturer's instructions shall be referred to for time intervals for reagent addition and incubation. Incubation time for the gel-clot BET methodology is typically 60 ± 2 min. For kinetic methods, the incubation time varies depending on the conditions of the test.

6.3.3 pH

The lysate manufacturer's instructions shall be referred to for the optimum pH range for the BET reaction. The pH range for BET methodology is typically 6 pH to 8 pH units. Performing the test outside of the optimum pH ranges can result in interference. When positive product controls are acceptable during suitability testing, it is possible that subsequent pH measurements will not be required. However, if adjustments in pH are made during suitability testing, then the pH shall be addressed during subsequent routine testing.

6.4 Equipment and materials

6.4.1 Due to the limited temperature range required for the BET, equipment such as heat blocks or water baths used to incubate gel-clot tests, shall be mapped for heat distribution during qualification and maintenance activities. Mechanical pipettors, including fixed, adjustable, and repeating units, shall be calibrated periodically and the calibration documented.

6.4.2 If the laboratory is performing a chromogenic or turbidimetric technique, the BET instrument hardware shall be qualified and software shall be validated.

6.4.3 Materials that are not supplied as certified to be non-pyrogenic, e.g. multi-well plates, shall be evaluated prior to use to ensure that they will not interfere with the assay. This can be accomplished, for example, by testing a sample of materials to demonstrate that they do not interfere with the assay.

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6.4.4 The equipment used for the depyrogenation of glassware and other equipment used in a BET shall be qualified and maintained. Methods used for depyrogenation shall be established, validated and documented.

6.4.5 Ensure all materials used to collect, store, or in other ways perform the BET, do not themselves introduce enhancement or inhibition into the test system, e.g. due to leachables or the adherence of endotoxins to the material.

6.5 Reagents

6.5.1 LAL reagents shall be obtained from licensed manufacturers as applicable.

6.5.2 The primary endotoxin standard is called the Reference Standard Endotoxin (RSE). The activity of secondary standards or control standard endotoxins (CSEs) shall be standardized against the reference standard and documented by a certificate of analysis or similar record.

6.5.3 Storage requirements for freeze-dried and reconstituted reagents are described in individual lysate or endotoxin manufacturer's product instructions. If storage conditions utilized in the laboratory are different from those recommended by the manufacturer's instructions, the conditions of alternative storage shall be validated.

7 Method suitability for BET (BET validation)

7.1 General

The BET method shall be determined to be suitable to adequately demonstrate that the test articles do not of themselves cause inhibition, enhancement, or otherwise interfere with the accuracy and sensitivity of the test. This is accomplished through method suitability. Method suitability consists of multiple aspects, including product and test method suitability, as well as reagent and analyst qualification.

The number of batches chosen for product and test method suitability shall reflect the level of control of the manufacturing process and shall be justified. A minimum of one batch per product or product family shall be used to demonstrate product and test method suitability. The number of replicates for reagent and analyst qualification are detailed in 7.4.

NOTE 1 Laboratory quality management systems can already include these items as part of either internal method development or qualification, or both. In this case, further method suitability testing might not be necessary.

NOTE 2 Development of a suitable test method can be required for products demonstrating enhancement or inhibition due to interfering factors in the sample extract. Additional information can be found in A.7.3.4.

NOTE 3 For health care products, an endotoxin recovery efficiency study is generally not required because of the conservative approach used in establishing endotoxin limits for devices (see B.5 and B.9).

7.2 Product and test method suitability

7.2.1 Gel-clot technique

7.2.1.1 Prepare solutions as listed in Table 1. The sample solution shall be at a dilution less than or equal to the MVD and shall not contain any detectable endotoxin in the test system used. Test each endotoxin-spiked dilution series and negative control. The geometric mean end point concentrations

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of each endotoxin-spiked dilution series can be determined using the formula listed in the preparatory testing in 7.4.1.

Table 1 — Preparation of solutions for test method suitability — Gel-clot technique

Solution	Diluent	Endotoxin spike	Endotoxin concentration	Number of replicates
Positive product control (PPC) series	Sample solution	Prepare 2 λ solution, then 2-fold serial dilutions of initial 2 λ prep	2 λ λ 0,5 λ 0,25 λ	4 4 4 4
Sample solution	Sample solution	None	NA	4
Standard control series	WBET	Prepare 2 λ solution, then 2-fold serial dilutions of initial 2 λ prep	2 λ λ 0,5 λ 0,25 λ	2 2 2 2
Negative control	WBET	None	NA	2

7.2.1.2 The sample meets the criteria for method suitability if:

a) the LAL sensitivity in the PPC series is within 0,5 λ and 2 λ;

b) negative controls show no reaction;

c) the result of the standard control series is within 0,5 λ and 2 λ.

7.2.2 Kinetic and end point methods (chromogenic and turbidimetric techniques)

7.2.2.1 Select an endotoxin concentration at or near the middle of the endotoxin standard curve. Prepare solutions as listed in Table 2. The sample solution shall be at a dilution less than or equal to the MVD. A valid PPC fulfils the requirement for test method suitability (see Table 2).

Table 2 — Preparation of solutions for test method suitability — Kinetic and end point methods

Solution	Diluent	Endotoxin spike	Minimum number of replicates
Product positive control (PPC)	Sample solution	At or near the middle concentration of the standard curve	2
Sample solution	Sample solution	None	2
Standard control series[a]	WBET	Minimum of three different concentrations	2 per concentration
Negative control	WBET	None	2

[a] Can be achieved with an archived standard curve.

7.2.2.2 Calculate the recovery of the added endotoxin by subtracting the mean endotoxin concentration in the sample solution from the mean endotoxin concentration in the PPC and dividing by the known endotoxin concentration.

7.2.2.3 The sample meets the criteria for method suitability and is valid if:

a) the measured concentration in the PPC is within 50 % to 200 % of the known added endotoxin concentration;

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b) the mean reaction time of the negative control is greater than the mean reaction time of the lowest standard in the control series;

c) the result of the standard control series conforms to the absolute value of the correlation coefficient, $|r| \geq 0{,}980$.

The data to support the criteria for method suitability may be gathered simultaneously with performing a routine test on product.

7.3 Sample preparation

7.3.1 General

7.3.1.1 Product samples and sample extracts for testing shall be collected and stored in a manner to maintain stability of the endotoxin content.

NOTE Sterile products in their final packaging configuration or products that do not support microbial growth (see 5.2.6) in protective packaging will not typically exhibit an increase or decrease in endotoxin levels over time.

7.3.1.2 A routine BET shall use the same sample preparation/extraction method used in the method suitability study of the BET. Products may be flushed or immersed for preparation of the eluate/extract for testing. The extraction method used depends on the specific non-pyrogenic claim (e.g. entire device, fluid pathway) or intended use for the product.

7.3.2 Solid health care products

7.3.2.1 Solid health care products are tested either individually or pooled for extraction when performing a routine BET.

NOTE Typically, the endotoxin limits for a batch take into consideration the pooling of a maximum of 10 health care products, maintaining the safety of the patient pyrogenic threshold. See A.5.1.1 and Annex B for additional information.

7.3.2.2 Using depyrogenated instruments or instruments demonstrated not to interfere with the assay, the device(s) may be cut or disassembled for the extraction.

7.3.2.3 Extract the samples using one of the following or other demonstrated acceptable conditions:

— Fill with WBET or immerse in WBET and hold for a minimum of 1 h at ambient temperature.

— Fill with WBET or immerse in WBET and hold at 37 °C to 40 °C for a minimum of 15 min.

NOTE Regulatory requirements can prescribe alternate acceptable BET extraction conditions.

7.3.2.4 Agitation (intermittent or continuous) is recommended to aid in the extraction process. If product units are extracted individually but are intended to be tested as a pooled sample, combine the eluates from each extraction to obtain a pooled sample, as appropriate.

NOTE Where the product configuration precludes total immersion, extraction can be performed by agitation that provides intermittent or continuous rinsing/extraction over the entire product. For guidance on how to address large devices or kits refer to A.7.3.2.5.

7.3.2.5 The MVD, or maximum amount of extraction fluid that may be used for the pooled extract for a given endotoxin limit, is calculated as described in 6.2.3. The volume of WBET used to extract the product units may be adjusted to facilitate extraction, depending on the size of the device. To overcome interference, the sample extract may be further diluted to a level not to exceed the calculated MVD, as described in 6.2.3.

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