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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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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.

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.

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]

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].

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

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

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)

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]

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.

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.

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

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:

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

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

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

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.

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

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 λ	4
		2-fold serial dilutions	λ	4
		of initial 2 λ prep	0,5 λ	4
			0,25 λ	4
Sample solution	Sample solution	None	NA	4
Standard control series	WBET	Prepare 2 λ solution, then	2 λ	2
		2-fold serial dilutions	λ	2
		of initial 2 λ prep	0,5 λ	2
			0,25 λ	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;

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.

7.3.3 Aqueous health care products

7.3.3.1 Aqueous health care products, biological components in aqueous form, or other aqueous samples may be tested without extraction. Samples may be diluted as determined in method suitability testing.

7.3.3.2 Powders, gels, and pastes shall be in suitable form for testing.

7.3.3.3 Detergents or chelating agents present on some products can cause interference. Dilution or other techniques (e.g. suitable buffers) can be necessary to overcome interference as long as the MVD is not exceeded.

7.3.4 Sample interference

If interference is observed in any BET, the sample extracts can be either diluted (not to exceed the MVD) or treated to overcome inhibition or enhancement, or both. Treatments to sample extracts to overcome interference, such as filtration, neutralization, dialysis or heat treatment, shall be validated or demonstrated to be suitable without loss of endotoxins. All sample manipulations shall be specified in the method suitability data/report and the same process shall be maintained during routine testing.

7.4 Reagent and analyst qualification

7.4.1 Gel-clot technique reagent qualification

The label claim sensitivity (λ) of each lot of lysate reagent shall be verified by testing. The test shall be performed in quadruplicate, with a series of twofold endotoxin dilutions that bracket λ (e.g. dilutions of 2 λ, λ, 0,5 λ, 0,25 λ). The geometric mean end point of the series shall confirm λ ± one twofold dilution. Once confirmed, the label claim sensitivity is used in all calculations. The geometric mean of the label claim sensitivity is calculated as shown in Formula (4):

$$\text{Geometric mean} = \text{antilog}\,(\Sigma e/f) \tag{4}$$

where

Σe is the sum of the log end point of each series;

f is the number of replicates.

NOTE Refer to Table A.7 for worked example.

7.4.2 Kinetic and end point method reagent qualification

Kinetic and end point methods (photometric quantitative techniques) require the demonstration of a linear standard curve across the range of endotoxin concentrations that will be routinely used in the analysis. At least three different endotoxin concentrations shall be used to generate the standard curve. Linearity requires that the absolute value of the correlation coefficient $|r|$ be $\geq 0{,}980$ for the range of endotoxin concentrations indicated by the lysate manufacturer's instructions.

If the standard curve range is greater than two logs, additional concentrations of the standard (e.g. 10-fold dilutions) should be included to bracket each log increase within the range of the standard curve. A standard curve should be no more than 4 logs, because a 5-log or greater curve can be interpreted incorrectly within the mid-range of the curve. If a standard curve is less than two logs, it is suggested that twofold dilutions be performed for the standard curve.

For cartridges pre-loaded with all the reagents needed for the test, acceptance information can be demonstrated using vendor supplied data or supplier certification.

The defined standard curve range that is used for reagent qualification shall be used during routine testing.

NOTE If the label claim sensitivity of a lysate lot against the endotoxin standard lot has already been performed, additional reagent qualification might not be necessary.

7.4.3 Analyst qualification

Each analyst performing the BET shall demonstrate competency by successful performance of the reagent qualification method.

NOTE Typically analyst qualification (including continued proficiency) is covered by the laboratory quality management system.

8 Routine testing, monitoring and interpretation of data

8.1 Routine testing

8.1.1 Gel-clot limit test

Prepare and test solutions as listed in Table 3 for routine testing. The sample solution and PPC shall be prepared using a dilution not greater than the MVD.

Table 3 — Preparation of solutions for gel-clot limit test

Solution	Diluent	Endotoxin spike	Number of replicates
Sample solution	Sample solution	None	2
Product positive control (PPC)	Sample solution	2 λ	2
Positive control	WBET	2 λ	2
Negative control	WBET	None	2

8.1.2 Gel-clot assay

Prepare solutions as listed in Table 4 for routine testing. The dilution series described for the sample solution is intended to dilute an endotoxin-containing sample to an end point to facilitate quantification. Testing the entire dilution series using twofold dilutions is not necessary unless a positive result is obtained on the initial sample solution. Additional dilutions may be used as needed to quantify the endotoxin in the sample. However, once a dilution with a positive result and a dilution with a negative result are found, twofold dilutions between the first positive and negative shall be made to obtain an endotoxin value.

Table 4 — Preparation of solutions for gel-clot assay

Test solution	Diluent	Endotoxin spike	Dilution factor	Endotoxin concentration	Minimum number of replicates
Sample solution	Sample solution	None	1	–	2
	WBET		2[a]	–	2
	WBET		4[a]	–	2
	WBET		8[a]	–	2
Positive product control (PPC)	Sample solution	2 λ	1	2 λ	2

[a] If the undiluted sample solution is positive, additional dilutions can be used to quantify endotoxins. Alternatively, in some circumstances, dilutions can be omitted.

Table 4 *(continued)*

Test solution	Diluent	Endotoxin spike	Dilution factor	Endotoxin concentration	Minimum number of replicates
Standard control series	WBET	Prepare 2 λ solution, then twofold dilutions of 2 λ prep	1	2 λ	2
	WBET		2	λ	2
	WBET		4	0,5 λ	2
	WBET		8	0,25 λ	2
Negative control	WBET	None	1	NA	2

[a] If the undiluted sample solution is positive, additional dilutions can be used to quantify endotoxins. Alternatively, in some circumstances, dilutions can be omitted.

8.1.3 Kinetic and end point methods (chromogenic and turbidimetric)

For routine testing, see Table 2.

8.2 Monitoring (test frequency)

BET shall be performed in accordance with a documented and justified sampling plan having a defined sampling frequency and sample size (see Clause 5). See A.5.2 and A.10 for additional guidance.

8.3 Interpretation of results

8.3.1 General

The test article is acceptable if the level of endotoxin is in conformance with the product endotoxin limit.

If the test article exceeds the product endotoxin limit, perform a failure investigation (see Annex C).

A valid routine test requires that the results given in Table 5 are observed.

Table 5 — Requirements for valid routine test

Criteria	Gel-clot assay	Kinetic and endpoint methods (chromogenic and turbidimetric)
standard control series criteria	standard control series confirms λ within a range of 0,5 λ to 2 λ	standard curve has a minimum value of correlation coefficient [r] of 0,980
negative control criteria	negative control is non-reactive	mean reaction time of the negative control is greater than mean reaction time of the lowest standard in the control series
PPC criteria	PPC is recovered (i.e. positive)	PPC is recovered in the range of 50 % to 200 % of the known added endotoxin concentration

An acceptable maximum CV% for replicates of standards, samples, spiked samples and negative controls should be established (see A.8.3.1.1).

If a sample is diluted, the total endotoxin per product unit may be calculated by applying the appropriate mathematical factors to the determined sample solution endotoxin concentration (i.e. sample/extract volume, product weight, sample-to-product ratio).

If the test is conducted with a diluted sample solution(s), calculate the concentration of endotoxin in the original sample solution by multiplying by the appropriate dilution factor.

8.3.2 Gel clot methods

8.3.2.1 For the gel-clot limit test, the article under test is acceptable when tested at or below the MVD, when the applicable parameters are met (see Table 3) and when negative results are found in both tubes containing the sample solution.

If positive results are found in any of the tubes containing the sample solution, when tested at the MVD, the test article exceeds the endotoxin specification. Refer to the guidance on OSL and failure investigation (see Annex C).

8.3.2.2 For the gel-clot assay, determine the endotoxin concentration in the sample solution by calculating the end point concentration for each replicate series and multiply each end point dilution factor by λ. The endotoxin concentration of the sample is the geometric mean end point concentration of the replicate dilution series. If the undiluted sample is negative, the endotoxin concentration is less than λ.

8.3.3 Kinetic and end point methods

For kinetic and end point methods (chromogenic and turbidimetric), the article under test is acceptable when the applicable parameters for validity are met and if the mean endotoxin concentration of the replicates of the sample solution, after correction for dilution and concentration, is in conformance with the product endotoxin limit. If the endotoxin level of the test article exceeds the endotoxin limit, refer to the guidance on OSL and failure investigation (see Annex C).

8.4 Data analysis

For the purpose of the BET and BET results, measurement uncertainty, precision and bias are typically incorporated in method validation and therefore this type of data analysis is not required.

Data derived from the endotoxin test can be used to identify trends.

8.5 Statistical methods

If used, the application of statistical methods shall be appropriate for the intended purpose.

9 Maintenance of the BET method

9.1 General

In order to detect inadvertent changes that can result in invalid test results, periodic demonstration of continued suitability shall be performed. This can be met by a valid PPC in the kinetic test method.

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

9.2 Changes to either the product or manufacturing process, or both

9.2.1 Changes to either the product or manufacturing process, or both, shall be reviewed to determine whether they are likely to alter bacterial endotoxin levels with consideration to the purpose for which BET data are to be used.

9.2.2 Re-assessment shall be performed for any changes that can impact the test, for example introduction of new materials, processing steps, product configuration changes, method of sterilization (for post-sterilization testing) or a different product manufacturing site. This assessment shall include

evaluation of the effect of the change on the outcome of determination. The results of this re-assessment shall be recorded, and suitability studies repeated as necessary.

9.2.3 For kinetic and end point methods, if a valid test is obtained, including a valid PPC, a one batch suitability study is considered sufficient for reassessment.

9.3 Changes to the BET method

9.3.1 Changes to a BET method shall be assessed. This assessment shall include evaluation of the effect of the change on the outcome of determination. The results of the assessment shall be recorded.

9.3.2 Affected elements of the suitability study shall be repeated for the following:

a) changes to the extraction method, including a change that involves alternate extraction methods or parameters that are outside of the defined extraction parameters (e.g. adding sonication, using alternate temperatures, use of solvents);

b) a change in BET technique (e.g. chromogenic to gel-clot, chromogenic to turbidimetric);

c) a change to the lysate manufacturer.

9.3.3 Re-assessment of suitability shall be performed for a change in BET testing laboratories or a change in materials/equipment that can affect the test.

9.3.4 For kinetic and end point methods, if a valid test is obtained, including a valid PPC, a one batch suitability study is considered sufficient.

10 Alternatives to batch testing

10.1 General

Non-pyrogenicity is typically confirmed through the use of end-product batch testing for product release. Alternatives to batch testing may be used if it has been demonstrated that the manufacturing processes, process inputs (e.g. components, utilities) and the manufacturing environment are suitably controlled and capable of producing products with endotoxin levels that consistently meet specified limits. Such demonstration generally includes sufficient data from end-product testing, manufacturing process controls, and/or process input testing showing acceptable endotoxin levels.

In cases where specific health care products require batch testing, it is possible that alternatives to batch testing will not be allowed.

NOTE See Annex E for specific guidance on alternatives to batch testing.

10.2 Criteria for establishing alternatives to batch testing

10.2.1 Alternatives to batch testing require identification of key process steps or control points, as well as additional risk assessment to demonstrate a process is appropriate for such an approach.

10.2.2 Alternatives to batch testing shall be supported via planning and validation (see 10.3.1), manufacturing process design (see 10.3.2), process control (see 10.3.3) and change control (see 10.4), as well as periodic review and adjustments made as necessary (see 10.5).

10.2.3 If alternatives to batch testing are used, the rationale for the alternative shall be documented and the sampling plan shall be defined. Additionally, if a failure occurs, an alternative sampling plan

and the requirements necessary to return to an alternative to batch testing plan or reduced sampling plan, should be considered and documented as appropriate.

10.2.4 Alternatives to batch testing can involve several options, including reducing the number of samples tested, reducing the frequency of testing, testing representatives from product families, or using alternatives to end product, e.g. surrogate product. See Annex E for examples of alternatives to batch testing and refer to B.10 for information on the Failure Mode and Effects Analysis (FMEA) approach to alternatives to batch testing.

10.3 Manufacturing process assessment

10.3.1 Quality planning of manufacturing processes

The quality planning of a manufacturing process shall be documented and records retained as specified in 4.1. Quality planning of a manufacturing process to ensure non-pyrogenic product shall include the following elements:

a) Process risk assessment: A documented analysis of the manufacturing process with key process elements identified by a risk assessment tool (e.g. FMEA, hazard analysis and critical control point, fault tree analysis).

b) Either process validation or applicable data, or both, to demonstrate sufficient manufacturing process controls.

NOTE A comprehensive review and assessment of the accumulated historical production, testing, control and other information for a product already in production and distribution can be used to satisfy the elements of process validation.

10.3.2 Process design

Manufacturing operations, including ancillary manufacturing operations (e.g. water system) shall be designed to minimize the presence of endotoxin on the product. Manufacturing operations shall be well characterized with established operating specifications. New and existing manufacturing processes shall operate in a state of control and shall be assessed for variables that could contribute to endotoxin contamination.

10.3.3 Process control

10.3.3.1 Manufacturing operation control shall be demonstrated through appropriate testing. A system of ongoing monitoring at key control points shall be in place to ensure the production of non-pyrogenic product is maintained.

The following shall be provided for each defined control point:

a) a sampling plan;

b) control levels (e.g. alert or action);

c) action to be taken when control levels are exceeded.

10.3.3.2 In the case of an unacceptable result for a sample that represents product employing an alternative to batch testing sampling plan approach (including surrogate product), consideration shall be given to the risk of all product represented by that sampling plan. The affected product shall be evaluated according to established non-conforming product procedures.

10.4 Change control

Process changes or deviations shall be assessed to determine the impact on the endotoxin level of the product or manufacturing operations or BET method suitability. Changes can include, e.g. product design, process deviations, changes in raw materials, water supply.

The extent of requalification or testing that is necessary shall be determined. The outcome of the assessment, including rationale for decisions reached, shall be documented.

10.5 Maintenance of risk assessment

A manufacturing process risk assessment shall be reviewed and evaluated on a periodic basis to assess the continued validity of the risk determination and risk controls established for alternatives to batch testing. This may be conducted based on trend reviews.

A risk assessment shall also be reviewed and evaluated whenever changes in the operation can adversely impact end-product endotoxin levels. In accordance with the overall risk management program defined by the quality system, the output of such review can include a recommendation for revalidation of the process controls or modification of the risk mitigation activities.

NOTE Guidance on risk management activities can be found in ISO 14971.

Annex A
(informative)

Guidance on bacterial endotoxin testing (following the subclauses in this document)

A.1 Scope

No guidance offered.

A.2 Normative references

The requirements given in documents that are included as normative references are requirements of this document only to the extent that they are cited in normative parts of this document; the citation can be to a whole standard or limited to specific clauses in which case the referenced standard is dated.

It should be noted in particular that it is not a requirement of this document to have a full quality management system. However, there are elements of a quality management system which are applicable to control the BET for medical devices. Attention is drawn to the standards for quality management systems for all stages of production of medical devices (see ISO 13485). National and/or regional regulations for the provision of medical devices can require the implementation of a complete quality management system and the assessment of that system by a third party.

A.3 Terms and definitions

No guidance offered.

A.4 General requirements

No guidance offered.

A.5 Selection of products

A.5.1 General

A.5.1.1 Products required to be non-pyrogenic are generally not required to be labelled as 'non-pyrogenic.' For products that are required to be non-pyrogenic, the decision on whether or not to label the product as 'non-pyrogenic' is typically based on national regulatory requirements or for marketing purposes.

Table A.1 illustrates the expectations for products that are labelled as non-pyrogenic and are expected to meet the same requirements for evaluation as those that are required to be non-pyrogenic based on their intended use and patient risk.

Table A.1 — Illustration of expectation for products labelled non-pyrogenic

Characteristic	Product required to be non-pyrogenic due to intended use	Product not required to be non-pyrogenic
Non-pyrogenic label claim	Testing required	Testing required
No non-pyrogenic label claim	Testing required	Testing not required

The claim 'non-pyrogenic' refers to bacterial endotoxins derived from Gram-negative bacteria and is typically supported by performing a BET. Gram-positive bacteria, while in some instances are capable of causing pyrogenicity, are not typically evaluated to support a non-pyrogenic claim. This is because extremely high levels of Gram-positive bacteria are required to elicit a pyrogenic response due to Gram-positive cell-wall constituents or intact bacteria having a potency several orders of magnitude less than endotoxin. [50][42][36]

For products labelled 'non-pyrogenic,' it can also be necessary to evaluate the product for material-mediated pyrogenicity (MMP) to fulfill regulatory requirements. This is in addition to any requirements for evaluating the product for bacterial endotoxins. See ISO 10993-11:2017, Annex G for clarification related to MMP associated with biological evaluation.[2] The MMP test is used to determine if pyrogenic chemicals are present and have leached from the device. The MMP test is typically conducted as part of the initial biocompatibility assessment and is not generally performed routinely or for batch-release. ISO 10993-11 is referenced here solely for information related to MMP. It is not intended that it be referenced or used as part of a BET plan. For example, it is not appropriate to reference ISO 10993-1,[1] ISO 10993-11,[2] or ISO 10993-12,[3] for determination of BET extraction parameters, extraction volumes, or sampling plans.

A.5.1.2 Studies in humans have supported a dose of 5 EU/kg as being a suitable tolerance limit for bacterial endotoxin contact with the circulatory system or lymphatic system. Using a body weight of 70 kg, the maximum amount of endotoxin that can be administered in one hour is 350 EU. The limit originally set by the United States Food and Drug Administration (US FDA) and currently used by US Pharmacopeia (USP) <161> reduced 350 EU to 200 EU to account for potential extraction inefficiencies and set the limit of 20 EU/device based on pooling extracts of 10 devices, assuming in a worst-case scenario that the 200 EU can originate from one device in the pooled extract of 10 devices.[2][20] If units are tested individually, this would mean the limit is 200 EU/device.

The limit of 2,15 EU/device was set for devices with intrathecal contact, because endotoxin in the intrathecal space has much more pyrogenic potency than endotoxin with intravascular contact.

Endotoxin limits higher than 20 EU/device can be justified and can require regulatory acceptance. For example, a limit of 35 EU/device or higher can be appropriate for medical devices without systemic exposure, given that the potency of the endotoxin to stimulate a pyrogenic response is lower and there is no need to add the additional reduction in the limit due to potential extraction inefficiencies.

Subcutaneous implantable health care products without systemic exposure are an example of a product where higher endotoxin limits can be justifiable. [26]

The current limits provided in this clause are conservative and appropriate for all patient populations including paediatric.

A.5.1.3 No guidance offered.

A.5.1.4 No guidance offered.

A.5.1.5 Examples of products with sealed portions that do not have patient contact can include the interior portions of balloon catheters or the interior of cardiac pacemaker implants.

A.5.1.6 A documented evaluation of the health care product will determine the portions of the product that are to not be considered non-pyrogenic and those that are excluded from testing.

A.5.1.7 Use of the term "non-pyrogenic" on a label implies that all the components in the kit that are in the sterile barrier system have been evaluated for endotoxin. Items within the kit typically not intended or labelled as non-pyrogenic (e.g. packaging, gauze, sterile drapes) can be excluded from the evaluation for endotoxin. However, a documented rationale for not testing these components, or a labelling exemption of these items from the non-pyrogenic claim should be considered.

A.5.2 Selection of product units

A.5.2.1 No guidance offered.

A.5.2.2 A sampling plan as shown in Table A.2 has been commonly used and is generally considered to be acceptable. Global regulatory requirements should also be considered.

Table A.2 — Selection of number of samples

Batch size	Number of samples
< 30	2
30 to 100	3
≥ 101	3 % of batch, up to a maximum of 10

Pharmacopeial requirements typically state not more than (NMT) 10 units should be tested. The number of product units chosen for routine testing is dependent on the size of the defined batch, level of control, statistical considerations, and historical performance. In most cases, each lot of product shall be tested using an appropriate number of samples, NMT 10, taken at random to represent the quality of the batch. Alternate sampling plans that use small sample sizes or that do not test each batch of product shall be clearly defined and supported/justified by a risk assessment (see Annex E).

Other statistically derived sampling plans, sometimes requiring a greater number of samples, can be necessary for validation or investigational purposes.

If sampling plans are used that require selecting more than 10 samples from a batch, no more than 10 samples should be pooled within a test.

A.5.2.3 No guidance offered.

A.5.2.3.1 Product families for the BET can be established based on an evaluation of products, processes, components, and materials. For routine testing for product families, the selection of a single product type from within a product family can be acceptable. It is possible that the designation of product families for endotoxin testing will not necessarily fit the criteria of product families as identified for other purposes, such as sterilization equivalence or bioburden assessment. However, each manufacturer should evaluate, analyse, and document the appropriate designation based on product components, manufacturing processes, and intended usage.

When defining a sampling group as a production batch for the purposes of endotoxin testing, the endotoxin batch can be defined as several similar product types or groupings with the same endotoxin risk. What is defined as a "batch" can differ from the definition of a "batch" for other purposes. For example, multiple production batches that each require separate endotoxin testing can be sterilized together in the same run or an endotoxin batch can be defined as several similar product types or groupings with the same endotoxin risk.

In establishing a sampling group and selecting samples for endotoxin testing, the following factors can be considered:

— raw materials or components (e.g. product containing extruded tubing from the same supplier exposed to the same manufacturing processes and only differing in dimensions);

— production quantities from a single shift or defined time period (e.g. 8 h or 24 h, products with similar endotoxin risks sterilized in the same sterilization load);

— product produced on specific equipment, or in the same manufacturing environment with equivalent endotoxin risk (e.g. all product cleaned in the same bath);

— product families (e.g. different sizes of the same product);

— product risk factors (e.g. products that undergo the same endotoxin reduction steps, products that have the same endotoxin limits);

— product produced using equivalent manufacturing processes (e.g. handling, automated versus manual processes, drying, storage);

— other logical divisions that contribute, control, or result in consistent end-product endotoxin levels.

In-process sampling (i.e. sampling of raw materials or components) can be useful for process monitoring and risk control. In-process sampling can be used towards the justification for an alternative to batch testing approach. Refer to Annex D for guidance on establishing endotoxin limits for in-process sampling.

For the test method suitability of a BET where the same testing process and parameters are used for multiple products (see Clause 7), a manufacturer can logically divide its products into groups according to common components (chemical formulations) and can then choose representative product from each such group. The product chosen from each group should ideally be:

— the one with the greatest surface area being extracted (e.g. fluid path), thus contributing to the largest source of extractables; and/or

— the chemical formula that can present the most inhibition (e.g. pH, divalent ions) or enhancement (e.g. glucans), if applicable.

A.5.2.3.2 No guidance offered.

A.5.2.4 While it would not be necessary for the samples selected for end-product testing to be packaged with all packaging materials and product literature that are used in the end product, at a minimum the packaging should include all materials that would normally come into direct contact with the end product.

Consideration should be given to packaging materials necessary to protect the samples and prevent contamination while in transit to the testing laboratory.

A.5.2.5 No guidance offered.

A.5.2.6 For products that support microbial growth (e.g. products that contain non-preserved aqueous liquids or gels), endotoxin levels can increase if there is a significant increase in bioburden levels of Gram-negative microorganisms on or in the product prior to sterilization. In such cases, post-sterilization testing can be necessary to ensure that test results are representative of the end product.

Some pharmacopeia state that pre-sterilization testing is inappropriate for products that support microbial growth.

For products that do not support microbial growth, a documented justification based on an assessment in consideration of factors such as the materials, manufacturing processes, historical data (e.g. shown by comparative endotoxin testing), should be sufficient to allow for pre-sterilization testing. LAL has been shown to respond to both living bacteria as well as endotoxins released from non-viable bacteria. Provided that a product does not support microbial growth, it should be expected that pre-sterilization and post-sterilization testing would be equivalent. [40][10][46][34][35]

If it is unknown whether a particular material supports microbial growth, bioburden determinations can be performed in accordance with ISO 11737-1 at multiple time points on an appropriate number of samples from the same batch to assess the ability of the product to support growth and hence the suitability of pre-sterilization testing. Other methods for assessing the capability of supporting growth

can include inoculation with selected microorganisms or a determination of water activity for the product. [13]

A.5.2.7 Multi-component kits or sets

A.5.2.7.1 Sets

For the purpose of this document, a set is defined as a collection of components presented in a sterile barrier system that is assembled at the point of use to form the health care product. In such cases, the specified endotoxin limit applies to the assembled product and not for each subcomponent (see Table A.3). When testing the subcomponents of a set, combining the extracts is acceptable if the necessary ratio of extraction fluid per assembled product is maintained. Refer to A.7.3.2.4 for further guidance on combining extracts and A.7.3.2.5 for further guidance on various extraction strategies.

In the case of a non-conforming result from a combined sample, additional individual testing would be recommended to investigate the source of contamination among the subcomponents.

A.5.2.7.2 Kits

A kit is defined as a collection of individual health care products in its sterile barrier system, or a variety of procedure-related health care products. Each individual type of health care product can have its own product endotoxin limits and should be tested and evaluated on an individual basis, if necessary, to support individual claims (see Table A.3). Alternately, all health care products can be tested together if the collective results support the claim for the product with the lowest endotoxin limit. Refer to A.7.3.2.4 for further guidance on combining extracts and A.7.3.2.5 for further guidance on various extraction strategies.

When considering if a single endotoxin limit should be applied to a kit, the decision should also be based on the risk to the patient. For example, per some regulatory requirements, it can be necessary to set a single endotoxin limit for all components in an ophthalmic kit that would have intraocular contact. This is because the interior of the eye is generally more sensitive to bacterial endotoxin compared with other parts of the body.

Table A.3 — Selection of product units for testing

Product	Packaging	Item for testing	Basis for non-pyrogenic claim	Rationale
a) One health care product	One sterile barrier system	Individual health care product or applicable patient contact portion	The health care product and its use	The health care product is used in clinical practice as a unit
b) Set: Components assembled into health care product at the point of use	One or more sterile barrier systems	All components or applicable patient contact portion	The assembled health care product and its use	Components combined to assemble the health care product before its use in clinical practice
c) Number of identical health care products[a]	One sterile barrier system	Individual health care product or applicable patient contact portion	One health care product within the sterile barrier system	Each health care product can be used independently in clinical practice

[a] Manufacturers can choose to test the procedure-related health care products together to provide a single collective non-pyrogenic claim, however this is not required. Manufacturers that combine procedure-related health care products can support non-pyrogenicity by using supplier certification or by performing testing.

Table A.3 *(continued)*

Product	Packaging	Item for testing	Basis for non-pyrogenic claim	Rationale
d) Kit: Procedure-related health care products[a]	Within a single sterile barrier system	Each type of health care product or applicable patient contact portion	The health care product and its use	Each type of health care product can be used independently in a procedure and can have different product endotoxin limits
e) Kit: Procedure-related health care products[a]	Health care products each in a sterile barrier that are combined in secondary packaging	Each type of health care product or applicable patient contact portion	The health care product and its use	Each type of health care product is used in clinical practice and might have different product endotoxin limits
[a] Manufacturers can choose to test the procedure-related health care products together to provide a single collective non-pyrogenic claim, however this is not required. Manufacturers that combine procedure-related health care products can support non-pyrogenicity by using supplier certification or by performing testing.				

A.6 Methods for BET

A.6.1 General

A.6.1.1 The three commonly accepted BET techniques are as follows:

a) Gel-clot techniques: limit test and assay methods

The gel clot methods are simple in terms of technical expertise required to perform the assay and data interpretation/analysis. Investment in the gel clot equipment is minimal, requiring only a properly qualified and maintained water bath or heating block and accessories. In the gel-clot test, equal volumes of test sample diluted to the concentration established during method suitability testing and LAL reagent are mixed in a glass reaction tube. After incubation, individual reaction tubes are carefully removed from the incubating device and slowly inverted 180°. A firm gel that maintains its integrity upon inversion is scored as a positive test. Anything other than a firm gel is scored as a negative test. The detection limit is normally between 0,03 EU/ml and 0,25 EU/ml of solution used in the test, depending on the LAL reagent used. A solid gel clot will not be formed if the endotoxin concentration is below the detection limit. These results are generally considered qualitative, i.e. pass (no gel clot) or fail (gel clot).

b) Chromogenic photometric technique: end point method

The end point methods for the chromogenic techniques are based on the linear relationship between endotoxin concentration and formation of a colour (chromogenic) measured by optical density (OD) at a given wavelength, which is assessed over a relatively short range of standard dilutions. A standard curve is constructed by plotting the optical densities of a series of endotoxin standards prepared in WBET as a function of the endotoxin concentration. Using linear regression analysis, the resulting "best fit" standard curve covers an endotoxin range of approximately 1 log, usually 1,0 EU/ml to 0,1 EU/ml or 0,1 EU/ml to 0,01 EU/ml. The correlation coefficient |r| is a statistical measure of the scatter of the observed points relative to the calculated regression line. Linearity is typically defined as a correlation coefficient of an absolute value of ≥ 0,980. The endotoxin level in an unknown is calculated by measuring the OD of the sample and interpolating the endotoxin concentration from the standard curve. Chromogenic end point methods are generally performed in multiwell plates and require a heating block, a qualified microplate reader, and software with a statistical package (linear regression analysis) for the construction of standard curves and analysis of samples. These methods are dependent on good analyst technique. Knowledge of basic statistics is helpful when analysing and interpreting data.

c) Chromogenic and turbidimetric photometric techniques: kinetic methods

The kinetic methods for the chromogenic and turbidimetric techniques measure the amount of time it takes for a series of standards to reach a pre-determined optical density, sometimes called the onset OD or reaction OD. A standard curve is constructed by plotting the log of the onset or reaction time (i.e. the time it takes for each standard or sample to reach the onset OD) as a function of the log of the endotoxin concentration. This log/log treatment of the data results in a linear standard curve. The range of the curve for a kinetic assay is up to four logs as compared to the one log curve generated in the end point method. Unless approved alternate regression analyses are used, the resulting standard curve is constructed using linear regression analysis across the observed points. A correlation coefficient of $|r| \geq 0{,}980$ is typically the minimum linearity requirement for a kinetic method. As with the end point methods, the endotoxin content of the unknown is calculated by interpolation from the standard curve using the logarithm of the onset time of the sample. The kinetic methods can be performed in multiwell plates, glass tubes, or other validated technology. Either method requires qualified equipment to incubate, a spectrophotometer to read the results, and software with a statistical package (regression analysis) for the construction of standard curves and analysis of samples. The use of a spreadsheet or database software package in conjunction with these methods greatly aids in the instant analysis and longitudinal trending of data. As with the end point method, the demonstration of good, consistent laboratory technique on the part of the analyst is important. Knowledge of basic statistics is helpful when analysing and interpreting data.

A.6.1.2 No guidance offered.

A.6.2 Consideration of an applicable endotoxin limit

A.6.2.1 Product endotoxin limits are subject to interpretation, depending on the configuration of the sample extraction: pooled or single unit testing. Regulatory requirements to endotoxin limits can apply. An example of a specific country endotoxin limit for medical devices is defined by USP <161> [20]:

> The endotoxin limit for the finished device is NMT 20 USP EU per device and NMT 2,15 USP EU per device for devices in contact with cerebrospinal fluid.

For devices that directly or indirectly contact the intraocular environment, a lower endotoxin limit can apply. [15]

Generally, BET guidelines allow for samples to be pooled for testing. This is accomplished by either extracting all of the sample units together in a common extract solution or combining all or part of the solutions from individual unit extractions. The endotoxin limit expressed per ml of extract (calculated using the formula in Table A.4) does not change when test articles are pooled.

Alternative endotoxin limits can be considered for in-process sampling. The endotoxin limits for in-process sampling should be established based on an assessment of risk, keeping in mind the risk that acceptable levels of endotoxins on individual types of components can collectively still result in unacceptable endotoxin levels on the end product.

A.6.2.2 Worked example of calculating the endotoxin limit for the product extract solution is given in Table A.4.

Table A.4 — Calculation of endotoxin limit of extract solution (within a sterile barrier system)

Term	Value	Comment
Step 1		
K	20 EU	Amount of endotoxin allowed per device (product endotoxin limit)
N	10	Number of device samples tested
V	400	Total rinse/soaking solution in the pooling of samples (ml)
Step 2		

Table A.4 *(continued)*

Term	Value	Comment
Endotoxin limit of extract solution in terms of EU/ml	0,5 EU/ml	Calculate endotoxin limit using Formula (A.1) Endotoxin limit of extract solution = $\frac{(K)(N)}{V}$ Endotoxin limit of extract solution = $\frac{(20\ \text{EU})(10)}{400\ \text{ml}} = 0{,}5\ \text{EU/ml}$. where K is the amount of endotoxin allowed per device (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.

A.6.2.3 Maximum valid dilution (MVD)

A.6.2.3.1 With devices that require an extraction, the sample can be diluted by diluting the extract solution or by increasing the extraction volume used. Worked examples for calculating the MVD are given in Table A.5 and Table A.6.

A.6.2.3.2 From a practical perspective, since product sizes vary and some devices require large volumes of WBET to adequately fill or immerse during the extraction process, there is a maximum amount of extract volume allowed in order to not dilute potential endotoxins beyond the endotoxin limit. Determining the initial maximum extraction volume can be helpful, especially when device extracts are not expected to be inhibitory or require further dilution beyond the initial extraction process. The maximum extraction volume can easily be calculated by dividing the product endotoxin limit by the sensitivity of the BET assay. For example, devices with a 20 EU per device limit, tested with a lysate sensitivity (λ) of 0,01 EU/ml, cannot be filled/immersed with greater than 2 000 ml per device without exceeding the MVD.

Table A.5 — Working example of the maximum valid dilution (MVD) of extract solution

Term	Value	Comment
Step 1		
Endotoxin limit of product extract solution	0,5 EU/ml	Maximum allowable level of endotoxin specified for a product
Lambda (λ)	0,01 EU/ml	The confirmed label sensitivity of the LAL reagent or the lowest endotoxin concentration used to construct the referenced standard curve (chromogenic and turbidimetric)
Step 2		
MVD (Maximum valid dilution)	50	Calculate the MVD using Formula (2) $\text{MVD} = \frac{(\text{Endotoxin Limit of extract solution})}{\lambda}$ $\text{MVD} = \frac{(0{,}5\ \text{EU/ml})}{0{,}01\ \text{EU/ml}}$ where MVD is 50. The value of the MVD indicates the dilution that can be used to overcome inhibition, based on the sensitivity of the LAL. For example, an MVD of 50 means that a 1:50 dilution can be used.

Table A.6 — Working example of maximum valid dilution (MVD) using extraction volume

Term	Value	Comment
Step 1		
Product endotoxin limit	20,0 EU/device	Maximum allowable level of endotoxin specified for a product
Lambda (λ)	0,01 EU/ml	The confirmed label sensitivity of the LAL reagent or the lowest endotoxin concentration used to construct the referenced standard curve (chromogenic and turbidimetric)
Step 2		
MVD by determining the Maximum Extraction Volume	2 000 ml/device	Calculate the MVD in terms for total extraction volume using Formula (3) $MVD = \frac{(\text{Endotoxin Limit})}{\lambda}$ $MVD = \frac{(20\ \text{EU / device})}{0{,}01\ \text{EU / mL}}$ where MVD in terms of maximum extraction volume = 2 000 ml per device.

A.6.2.3.3 No guidance offered.

A.6.3 Critical test parameters

A.6.3.1 Temperature

No guidance offered.

A.6.3.2 Time

No guidance offered.

A.6.3.3 pH

While all LAL reagents efficiently detect endotoxin, individual formulations are proprietary and differ in buffering capacity and divalent cation levels. Because of the buffering provided in the reagent, pH measurements should be taken using an appropriate pH test system on a mixture of the same LAL reagent and test solution ratio as that used in the assay.

Measurements for pH can be taken on the sample/lysate mixture after completion of the assay, to conserve lysate.

A.6.4 Equipment and materials

A.6.4.1 No guidance offered.

A.6.4.2 No guidance offered.

A.6.4.3 Generally, multiwell plates used in the performance of quantitative assays are not manufactured solely for the purpose of endotoxin testing, and they can contain random endotoxin contamination among the wells (“hot wells”). Therefore, sufficient sampling should be performed to demonstrate that the plates are appropriate for use. In practice, “hot wells” tend to occur at very low

levels of endotoxin (i.e. blanks, samples wells where endotoxin contamination is at or close to the limit of detection).

Depending on the product being tested, there can be situations even when certified non-pyrogenic materials would need to be free of detectable endotoxins.

A.6.4.4 No guidance offered.

A.6.4.5 No guidance offered.

A.6.5 Reagents

A.6.5.1 No guidance offered.

A.6.5.2 Typically, suitable CSE should have a potency range between 2 EU/ng and 50 EU/ng.

A certificate of analysis of the standardization of CSE against RSE should be obtained from the CSE supplier.

A.6.5.3 Concentrated CSE preparations provided by LAL reagent manufacturers should be stored according to the product instructions. However, storing endotoxin dilutions prepared in WBET should have a validation supporting the length of time and temperature of storage, the container used for storage and the minimum volumes of the dilutions to be stored.

A.7 Method suitability for BET (BET validation)

A.7.1 General

Three batches of product have been historically (or commonly) used for method suitability testing. When it is determined that more than one batch should be tested, this number is generally considered to be acceptable when BET history is not available for a new product.

A.7.2 Product and test method suitability

A.7.2.1 Gel-clot technique

No guidance offered.

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

A.7.2.2.1 No guidance offered.

A.7.2.2.2 No guidance offered.

A.7.2.2.3 Caution should be given to performing initial suitability testing simultaneously with routine testing as efforts can be required to further mitigate inhibition or enhancement.

A.7.3 Sample preparation

A.7.3.1 General

A.7.3.1.1 If sample extracts are either not immediately tested or are retained for re-analysis, or both, the sample extracts should be refrigerated and tested as soon as possible or frozen to prevent microbial

growth over longer storage time. [27] Suitable storage conditions should be evaluated to ensure endotoxin stability.

Extracts have been shown to be stable when stored in inert containers at refrigerated temperatures for up to 24 h (refer to ISO 10993-12:2021, 10.3.7). Other time durations can be qualified.

A.7.3.1.2 No guidance offered.

A.7.3.2 Solid health care products

A.7.3.2.1 Pooling can be used to reduce test efforts in established situations. Individual product testing can be used to measure individual unit endotoxin levels or to evaluate unit-to-unit variability, which could be applicable after setup or modification of a production or OSL situation.

When samples are pooled there is less visibility to the individual endotoxin results.

A.7.3.2.2 Depending on the product being tested, there can be situations where the instruments for cutting or disassembly would need to be free of detectable endotoxins.

A.7.3.2.3 Vertical standards/guidance documents (e.g. recommendations for ophthalmic devices [15]) recommend extraction be performed at 37 °C to 40 °C with agitation for a minimum of 60 min (only if necessary) or that the extraction medium is pre-warmed to 37 ± 1 °C, then extraction is performed at 37 ± 1 °C for not less than 1 h.[16] Applicable international standards/guidance documents can be considered when determining the extraction conditions.

A.7.3.2.4 The aliquot should be mixed prior to analysis to ensure homogeneity.

If combining eluates from separate extractions into a single pooled sample, each aliquot should also be mixed prior to pooling. Once all eluates from separate extractions are combined the pooled aliquot should be mixed to ensure homogeneity of the prepared (pooled) sample.

A.7.3.2.5 When testing a large product or kit, it is particularly important to determine the most appropriate extraction and test method to ensure that the limit of detection of the test does not exceed the specified endotoxin limit for the product or kit. Examples of common strategies used for testing large products or kits can include:

— use of a more sensitive lysate and test method (e.g. the kinetic chromogenic method using a lysate with a sensitivity level of 0,001 EU/ml instead of the kinetic turbidimetric method or kinetic chromogenic method using a lysate with a sensitivity level of 0,005 EU/ml);

— use of a different configuration of extraction container to allow the product to be fully immersed using a lower volume of extraction fluid;

— multiple use of the same extraction fluid (e.g. extract half of the components for the specified time and temperature, then use the same extraction fluid to extract the other half of the components for the specified time and temperature);

— use of continuous agitation during the extraction process to provide continuous rinsing/extraction over the entire device (in lieu of complete immersion);

— testing and applying discrete endotoxin limits to each component within a kit;

NOTE 1 Whether or not discrete endotoxin limits can be applied to each component within a kit will depend on the results of a risk assessment. Refer to risk considerations in Annex E and Table A.3 to determine whether the risk assessment demonstrates an acceptable level of risk based on the intended use of the kit.

— extracting only the components or portions of a component falling under the patient contact categories that would require evaluation of bacterial endotoxins.

NOTE 2 Extracting less than the entire product or less than the entire contents of a kit can affect how non-pyrogenic labelling can be applied (A.5.1.5 and A.5.1.6).

When assessing options for testing a kit, the assessment should take into account the manufacturing processes for each kit component as well as related endotoxin controls. For example, an injection moulded component that has limited or no additional contact with water or other sources of endotoxin might present less risk to the process than a machined component that is subsequently cleaned in an ultrasonic water bath, where water is not controlled for endotoxin. In this scenario it can be beneficial to demonstrate method suitability for the extraction procedure based upon data from each component extracted in isolation as well as pooled.

In circumstances where the above measures are not successful and results in the MVD being exceeded, consider adjusting the endotoxin limit based on a risk analysis.

A.7.3.3 Aqueous health care products

A.7.3.3.1 No guidance offered.

A.7.3.3.2 Powders, pastes, and gels should be dissolved, reconstituted, thoroughly saturated or extracted as necessary. Healthcare products that are used to form a pliable mass (e.g. powders, pastes and gels) should not be extracted by rinsing only the exterior of the mass, because this might not access all endotoxin within the product. In development of the test method it should be determined if the product can be broken down or treated in a way to maximize surface area to the extraction liquid that is representative of intended product use.

A.7.3.3.3 No guidance offered.

A.7.3.4 Sample interference

Treatment of a sample to eliminate or reduce interference can involve, e.g. dilution with WBET or suitable buffer, addition of reagents, or heat denaturing. See examples in NOTE 1 and NOTE 2 of this subclause. Any sample manipulation, with the exception of dilution with a qualified diluent (such as WBET), should be evaluated to demonstrate that the method does not remove, denature, or inhibit the detection of endotoxin.

Treatment of the sample with LAL reagent from different manufacturers, different LAL reagent sensitivities, and different BET techniques are options that can be used for eliminating interference.

Optimum LAL-Endotoxin reactivity requires neutral pH, availability of divalent cations and a dispersed endotoxin. For example, if invalid results are obtained, the pH of the sample should be checked to see if the pH of the mixture of the LAL reagent and sample solution falls within the pH range specified by the LAL reagent manufacturer, usually pH 6,0 to 8,0. If necessary, the pH can be neutralized by use of an acid, base, or suitable buffer (tris hydroxymethane [TRIS] buffer) as recommended by the LAL reagent manufacturer. Non-buffered LAL reagents can be rehydrated with a suitable buffer as prescribed by the LAL reagent manufacturer. Any buffers, acid, or base used should be shown to contain endotoxin levels below that which could affect the validity of the test and interfering factors.

Dilution of the sample in a cation buffer ($MgSO_4$ or $MgCl_2$) can be used to adjust the ionic concentration.

NOTE 1 Examples of substances that can cause ionic interference are metal ions such as iron (e.g. caused by corrosion), copper, nickel, or sodium or product coatings such as heparin.

If these options do not work and the MVD is exceeded, it can be possible to overcome this by concentrating the sample extract using ultrafiltration. Alternately, consider adjusting the endotoxin limit based on a risk analysis.

Samples exhibiting enhancement should be examined for LAL reactive material (LAL-RM). For example, samples that can have LAL-RM should be tested with and without an endotoxin-specific buffer solution to determine if the LAL-RM is affecting the test. In some cases when there is enhancement due to LAL-

RM, the PPC results can be in the acceptable range. Evidence of glucan interference can be obtained by comparing the results of an assay performed using separate aliquots of the same extract that are tested with and without an endotoxin-specific buffer solution.

NOTE 2 Some possible sources of LAL-RM are yeast and mould cell walls and cellulosic materials containing β-D-glucans. Serine protease can also mimic endotoxin, which can be overcome by denaturing the enzymes prior to the LAL assay with a heat treatment. Glucan-blocking reagents validated to be suitable can be used to overcome glucan interference.

Product or processing residuals can have an influence on interfering factors.

Diluents can also cause interference (e.g. salt solutions can elicit an inhibitory response). When using diluents other than WBET, method suitability with the product being tested should be demonstrated.

A.7.4 Reagent and analyst qualification

A.7.4.1 Gel-clot technique qualification

Table A.7 — Calculation of geometric mean – Worked example

Term	Value	Comment
Step 1		
∑e	−3	Sum of the log end point of each series Series 1 end point = 0,125 log 0,125 = −0,903 1 Series 2 end point = 0,25 log 0,25 = −0,602 1 Series 3 end point = 0,25 log 0,25 = −0,602 1 Series 4 end point = 0,125 log 0,125 = −0,903 1 (−0,903 1) + (−0,602 1) + (−0,602 1) + (−0,903 1) = −3,010
f	4	Number of replicate dilution series
Step 2		
Geometric mean	0,177 EU/ml	Calculate geometric mean using Formula (4) Geometric mean = Antilog $\frac{\sum e}{f}$ where e = the $\log_{10}$ of each end point; f = the total number of replicate end points. Geometric mean = Antilog $\frac{-3,010}{4} = 0,176\ 7$
Step 3		
Lambda (λ) (LAL reagent labelled sensitivity)	0,125 EU/ml	Confirmation of labelled lysate sensitivity (λ) Geometric mean endotoxin concentration = 0,177 EU/ml Labelled lysate sensitivity (λ) = 0,125 EU/ml The labelled sensitivity (λ) was confirmed because the geometric mean was within 0,5-2λ (0,06 EU/ml to 0,25 EU/ml)

A.7.4.2 Kinetic and end point method qualification

No guidance offered.

A.7.4.3 Analyst qualification

Continued competency of the analyst should be demonstrated to ensure that a qualified analyst maintains the appropriate skills and training to perform the assay, subsequent to the initial analyst qualification. Competency can be demonstrated by:

— demonstration of consistent acceptable performance of the assay (trending);

— proficiency testing;

— analyst requalification; and/or

— non-conformance trending/analysis.

A.8 Routine testing, monitoring and interpretation of data

A.8.1 Routine testing

The BET need not be performed in a controlled environment (e.g. certified cleanroom, HEPA-filtered hood or biological safety cabinet) unless required by product related safety concerns for the analyst, such as testing human blood products. However, it is important that analysts understand and follow basic aseptic techniques to prevent contamination of the sample. Documented training of all analysts in those tasks for which they have responsibility is essential.

NOTE For storage and re-test of the extract, aseptic technique and prevention of contamination of the sample are of high importance.

A.8.1.1 Gel-clot limit test

No guidance offered.

A.8.1.2 Gel-clot assay

No guidance offered.

A.8.1.3 Kinetic and end point methods (chromogenic and turbidimetric)

No guidance offered.

A.8.2 Monitoring (test frequency)

The frequency selected for testing should be adequate to ensure that endotoxin levels for all products produced meet specified levels. Decisions regarding frequency of end-product testing can also be affected by the degree of control over the process and materials, and by procedures for testing of either critical incoming raw materials or in-process monitoring, or both.

A.8.3 Interpretation of results

A.8.3.1 General

A.8.3.1.1 This subclause provides guidance on coefficient of variation (CV%).

An acceptable maximum CV% for replicates of standards, samples, and spiked samples should be established. If available, consideration should be given to CV% recommendations provided by the lysate manufacturer.

Consideration should be given to individual replicate values when the negative control indicates a reaction and the mean result is less than the mean of the lowest standard in the control series. An

acceptable maximum CV% between the replicates should be established or a rationale should be provided specifying when observed negative control variation is acceptable.

A.8.3.1.2 No guidance offered.

A.8.3.2 Gel clot methods

No guidance offered.

A.8.3.3 Kinetic and end point methods

Acceptance is typically based on the mean result of the replicates with quantitative chromogenic and turbidimetric methods. However, when items are tested individually to obtain the mean of the sample results, consideration should be given to individual sample values that exceed the endotoxin limit even though the mean of the sample results is below the product endotoxin limit. An acceptable maximum sample CV% between the samples should be established or a rationale should be provided when product is released on the mean result in these circumstances and variation is observed and deemed acceptable.

A.8.4 Data analysis

When the sample is tested at the MVD or by a limit test, trending is not applicable.

When results are consistently below the detection limit, it is possible that trending will not be applicable.

A.8.5 Statistical method

Statistical methods can be used to define sample size, sampling frequency and/or endotoxin levels and can be based on risk analysis.

A.9 Maintenance of the BET method

A.9.1 General

No guidance offered.

A.9.2 Changes to either the product or manufacturing process, or both

A.9.2.1 No guidance offered.

A.9.2.2 For product changes, evaluations should be performed to confirm that the manufacturing process is capable of producing product meeting the established limits following the change. The number of samples/batches tested should be justified.

A.9.2.3 No guidance offered.

A.9.3 Changes to the BET method

No guidance offered.

A.10 Alternatives to batch testing

A.10.1 General

Alternatives to batch testing require risk assessment (see Clause 10 and Annex E) and corresponding data demonstrating that the manufacturing process is capable of producing product that consistently meets specified endotoxin limits. Such data typically includes, e.g. testing of a specified number of

batches, testing over a specified period of time, testing representative master products, raw materials/component, in-process testing, or verification of manufacturing operational controls, particularly water processes. See Figure A.1 for key questions related to the risk and appropriateness of alternatives to batch testing.

A review of historical product test data is a key component to determining if a product is an appropriate candidate for alternatives to batch testing.

Cases where alternatives to batch testing can be considered include:

a) products with a successful history of end-product endotoxin control;

b) products with lower endotoxin contamination risk due to no exposure to water that has not been qualified to control endotoxin levels;

c) products that undergo a manufacturing process that has been validated to depyrogenate the device;

d) products that, based on their intended patient contact, have an expected minor or negligible risk of a pyrogenic reaction (see Annex E).

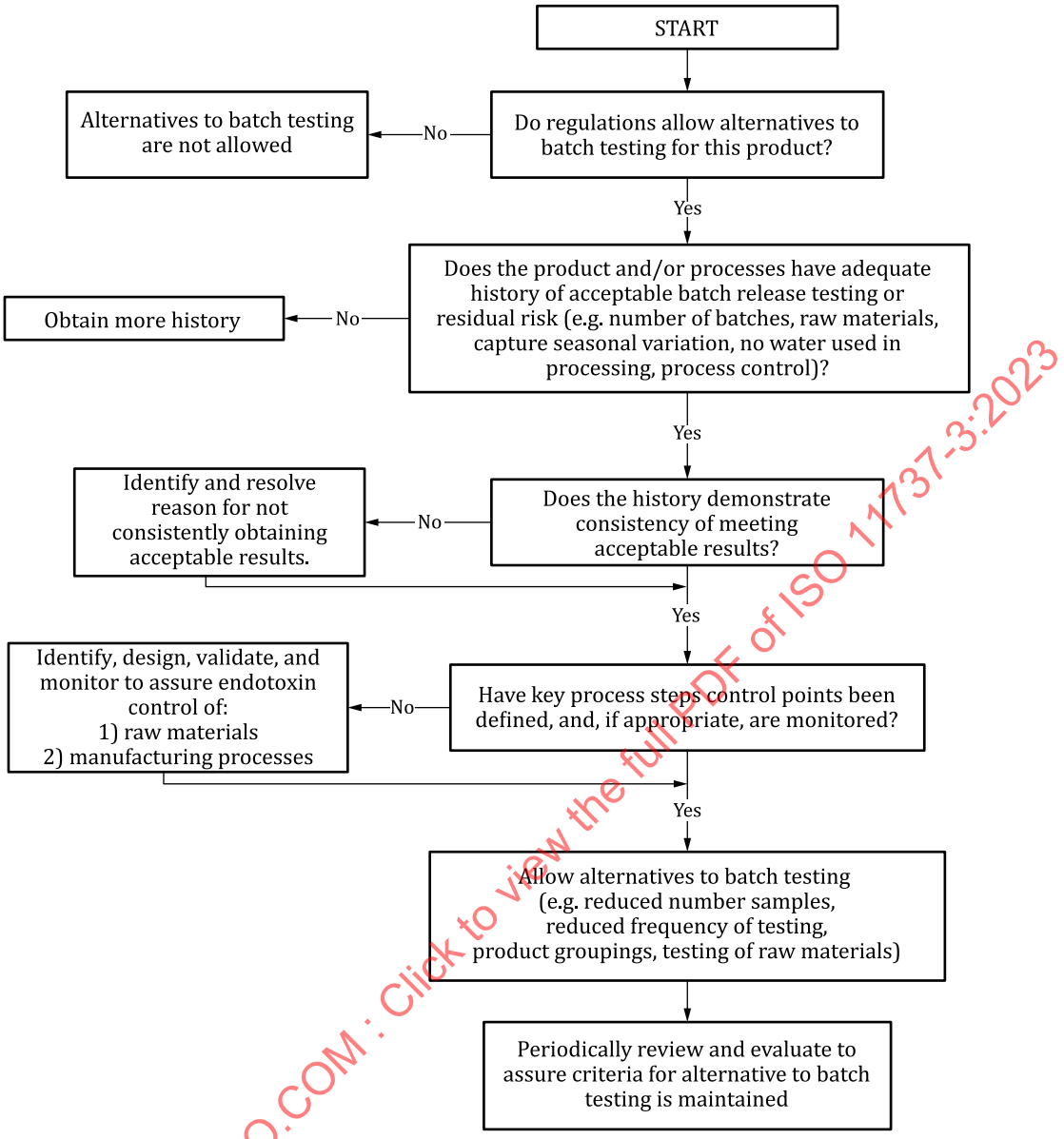

Figure A.1 — Key questions in evaluating the appropriateness and risk associated with alternatives to endotoxin batch testing

A.10.2 Criteria for establishing alternatives to batch testing

A.10.2.1 It should be recognized that using alternatives to batch testing can result in a reduced ability to detect an inadvertent change within the manufacturing process. This can result in an undetected out of specified endotoxin level on a product. Before proceeding with using an alternative to batch-testing sampling plan, the risk associated with a reduction in ability to detect inadvertent changes should be evaluated.

A.10.2.2 No guidance offered.

A.10.2.3 No guidance offered.

A.10.2.4 No guidance offered.

A.10.3 Manufacturing process assessment

A.10.3.1 Quality planning of manufacturing processes

A comprehensive review and assessment of the accumulated historical production, testing, control, and other information for a product already in production and distribution can be used to satisfy some of the elements of process validation, and/or evaluate the existing controls that would be included in the risk assessment of the alternative to batch testing.

A comprehensive process risk assessment of the manufacturing operation should be conducted to identify key process steps or control points (e.g. failure mode effects analysis, hazard analysis and critical control point, fault tree analysis). For non-pyrogenic products, this would include any process step for which a change would likely affect the endotoxin level on the product. These can include but are not limited to the following:

a) raw materials;

b) manufacturing process aids that do not become part of the final healthcare product (e.g. moulding aids);

c) extrusion operations;

d) aqueous steps in production;

e) drying or curing processes;

f) in-process aqueous leaching or soaking;

g) product/component handling;

h) manual versus automated assembly;

i) product or material storage.

NOTE Product or material storage is especially important for material that supports microbial growth prior to sterilization.

For validation of alternatives to batch testing, the product selected should be the worst-case product (i.e. largest amount of potential endotoxins, as well as potentially the greatest source of inhibition/enhancement). This can be a product with the largest surface area that contacts the patient (directly or indirectly), or a product with the largest amount of potential endotoxins based on raw materials, aqueous manufacturing steps, and/or handling operations that are most likely to contribute to the presence of endotoxins.

Validation of a manufacturing operation for the control of endotoxin should include the steps listed below.

— Establish that the manufacturing operation has the capability of producing non-pyrogenic product when operated within specified parameters.

— Demonstrate that the key process elements identified during the process risk assessment are in control.

— Demonstrate that the equipment and instrumentation are capable of controlling, monitoring, and/or measuring endotoxin within the parameters prescribed for the manufacturing operation equipment.

— Perform testing on replicated manufacturing batches representing the specified operational range of the equipment to demonstrate that the product consistently meets requirements for non-pyrogenicity.

— Document the revalidation or requalification criteria and frequency.

A.10.3.2 Process design

The manufacturing operation should be designed to minimize the level of endotoxin on the product. Considerations in the manufacturing operation design should include the following:

a) selection of appropriate materials and suppliers (including understanding the sources of endotoxin contamination and potential endotoxin contamination activities/risks);

b) minimizing and controlling materials and components that can contribute to the level of endotoxin on products (e.g. natural materials, or materials that support microbial growth);

c) control of aqueous processing solutions that directly contact product;

d) demonstration of the adequacy of drying, if aqueous processes are employed;

e) controlling processes that can contribute to the level of endotoxin on products (e.g. handling).

Critical factors for evaluating can include, but are not limited to:

— "wet" manufacturing steps, in which water or other aqueous material is utilized as part of the manufacturing process (e.g. rinsing, soaking);

— dry manufacturing (e.g. heat extruded plastics) or assembly of materials (i.e. kit assembly lines), in which the entire process is possible without exposure to water or other aqueous processing.

Health care products that contain pharmaceuticals (e.g. nebulizer bottles, blood collection bags containing anti-coagulants) should be tested and evaluated according to pharmaceutical/drug sampling requirements.

Dry products that are produced under high temperatures or in controlled environments do not normally present the same risk of endotoxin contamination as a "wet" process in which water is present in the process.

Water is the most significant and prevalent source of endotoxin contamination in manufacturing processes. Control of any water source used in manufacturing, and cleanliness of all surfaces, equipment, and storage are necessary. In addition, for components of microbial (e.g. fermentation), plant, or animal tissue origin consider controls to prevent and/or control the growth of microorganisms.

A.10.3.3 Process control

Depending on the risks associated with the various manufacturing operations, validation, verification, and on-going monitoring programs shall be defined as deemed appropriate. For example, for a process deemed as having a low endotoxin contamination risk, monitoring process inputs and/or end product with alternatives to batch testing can be acceptable. For a process deemed as having a high endotoxin risk, monitoring both process inputs and end product on a batch basis can be required.

A.10.3.3.1 Manufacturing operation control for endotoxins can include:

a) supplier quality assurance and/or endotoxin testing of incoming materials, components, or subassemblies (e.g. vendor endotoxin certification or specification requirements for no water contact);

b) monitoring and control of process water or other aqueous processing solutions (e.g. control of process water can be demonstrated by periodically monitoring chlorine residual levels, microbial counts, water endotoxin levels, or component endotoxin levels);

c) monitoring product in process at specified control points (e.g. time, temperature for drying process);

d) periodic maintenance and cleaning of equipment, especially those used to convey or contain aqueous product contacting processing materials (e.g. UV light, proper filtration);

e) microbial control of the environment and processing materials.

A.10.3.3.2 When an alternative to batch testing sampling plan is employed, the impact of an out of specified limit result on untested batches or the end product should be evaluated according to established non-conforming product procedures. The evaluation should consider risk to all product that is represented by the sampling plan. As part of the investigation, previously released batches associated with alternatives to batch testing should be considered and a risk assessment undertaken on their suitability for use. The investigation and subsequent actions should be recorded.

A.10.4 Change control

The magnitude of the change is considered in determining the extent to which verification, validation, or revalidation is undertaken.

A.10.5 Maintenance of risk assessment

No guidance offered.

Annex B
(informative)

History and background on the bacterial endotoxins test (BET)

B.1 The rabbit pyrogen test was introduced just prior to World War II to prevent pyrogenic materials from entering the health care system.[44] The *Limulus* amebocyte lysate (LAL) test was introduced in 1971 as a potential replacement for the rabbit pyrogen test.[29][41] The parenteral drug industry and the US Food and Drug Administration (FDA) agreed on a guideline in 1987 to replace the rabbit pyrogen test with the LAL endotoxin test, because the in vitro test had greater sensitivity, specificity, accuracy, and economy.[16] In 1993 the LAL test became the official pyrogen test for the majority of parenteral products in the United States. This action included a sweeping revision of the BET and adoption of more than 650 product endotoxin limits for USP articles.[21] Although BET and LAL are widely used abbreviations for the test, BET is considered the appropriate identifier

B.2 It was essential to clarify issues of equivalency, safety, endotoxin tolerance limits, and regulatory control before the new test could be accepted. The FDA's Bureau of Biologics (now the Center for Biologics Evaluation and Research) elected to regulate LAL reagents as an in vitro biologic, because of its potential as a human diagnostic test and a replacement for the rabbit pyrogen test.[22][51] LAL reagents were first marketed in 1977, but their use was restricted to in-process testing of parenterals. A collaborative study involving medical device manufacturers and an FDA laboratory, supported by the Health Industry Manufacturers Association (HIMA) and sanctioned by the FDA, established 0,1 nanograms (ng)/ml as the product endotoxin limit for device extracts.[33] This HIMA study further verified that the threshold pyrogenic dose of a Difco endotoxin (*E. coli* 055:B5) was approximately 1 ng/kg in rabbits when administered at a dose of 10 ml/kg. [35]

B.3 The question of non-endotoxin pyrogens was a major concern for the pharmaceutical industry. In 1979, a large volume parenteral (LVP) producer reported on its policy for LAL testing in place of pyrogen testing.[43] The study described the results of 143 196 LAL tests and 28 410 rabbit tests performed on intravenous fluids and health care products. The data confirmed the following:

1) all pyrogens in fluids and devices were endotoxins;

2) no unexplained, false-negative LAL results occurred;

3) most endotoxin pyrogens detected by LAL were undetected by rabbits, because of LAL's greater sensitivity;

4) the rabbit pyrogen test often gave equivocal results that were reproducible in the LAL test.

The study had an enormous impact. The FDA approved the LAL assay because of concern about the relative insensitivity and unreliability of the rabbit test.

B.4 Any inter-laboratory comparison of endotoxin data was hindered by the lack of a uniform standard. This deficiency was corrected when the USP and FDA collaborated to produce a RSE from a purified LPS derived from *E. coli* 0113.[47] The 1991 FDA Interim Guidance for Human and Veterinary Drug Products and Biologicals,[18] a collaborative study by LAL producers and the Bureau of Biologics established biological activity of the RSE in EU. Laboratories could now standardize methods and report endotoxin content in a measure of biological activity. An international reference standard became effective in 1996 to permit reference to a single global endotoxin standard. [45]

B.5 Because endotoxin is ubiquitous in nature, it was necessary to assign an allowable amount of endotoxin for a health care product that represented a safe level. Product endotoxin limits first appeared in Annex E of FDA's 1987 guideline for the LAL test.[16] This guideline specified product endotoxin

limits based on the formula K/M, where K is the tolerance limit for endotoxin, 5,0 EU/kg unless the drug is administered intrathecally then the limit is 0,2 EU/kg, and M is the adult dose per kilogram of body weight in a one-hour period, where an average weight of 70 kg was assigned to humans.

For LVPs and device extracts, the rabbit pyrogen test dose of 10 ml/kg, which usually exceeds a human dose, was used to calculate the product endotoxin limits. An LVP or device extract contained no more than 0,5 EU/ml of endotoxin (5 EU/kg/10ml/kg = 0,5 EU/ml). Products designed for injection or exposure to the intrathecal space were given a lower product endotoxin limit of 0,06 EU/ml. When changing the intrathecal limit of 0,2 EU/kg to 0,06 EU/ml the endotoxin limit was arbitrarily set considering the most sensitive LAL reagents available at the time from any BET manufacturer.

Studies in humans have supported a dose of 5 EU/kg as being a suitable tolerance limit for parenteral drugs and devices in contact with the circulatory system or lymphatic system.[37] However, exposure to endotoxin in the intrathecal space of humans has not been evaluated. Endotoxin in the subarachnoid space of dogs, rabbits, and cats is at least 1 000 times more pyrogenic than intravascular contact.[28]

B.6 Given that K is equal to 5,0 EU/kg for parenteral drugs and M is equal to 70 kg, the maximum amount of endotoxin that could be administered in a one-hour time period is 350 EU. Historically, the endotoxin limit for health care products that do not contact cerebrospinal fluid was based upon a reduction of the typical 350 EU limit for pharmaceuticals. The limit was reduced to 200 EU to account for potential inefficiency in the extraction method. This limit was further reduced to 20 EU based upon a maximum amount of endotoxin of 200 EU for the combined extracts from 10 samples. The assumption being that in the worst-case, all of the endotoxin in the pooled extract could have come from a single device.

B.7 Conveniently, when extracted with 40 ml per device the historical FDA endotoxin limit of 0,5 EU/ml is equivalent to the 20 EU/device limit established by USP. The same is not true for the 2,15 EU/device USP limit established for devices that contact the central nervous system (intrathecal). First the initial endotoxin limit established of 0,2 EU/kg for drugs administered by an intrathecal route does not provide the same reduction to account for potential inefficiencies in the extraction method. With a dose of 0,2 EU/kg and a 70 kg weight the maximum dose would be 14 EU. In addition, the original limit for health care products of 0,06 EU/ml listed in both the FDA LAL guidance document from 1987 and USP <161> does not convert to 2,15 EU/device.[24] When a 40 ml per device extraction is used the value would be 2,4 EU/device. USP <161> later changed the requirement from 0,06 EU/ml to the more stringent limit of 2,15 EU/device in the first supplement of USP 23 (1995). There is no documentation in USP regarding the rationale for changing the limit from 2,4 EU/device to 2,15 EU/device. Although the 20 EU/device and the 2,15 EU/device limits have historically been thought of as having similar risks, the risks when moving to a reduced sampling plan or justifying an unconfirmed out of specification, should be considered. The tolerance limit is unknown and the rationale behind the assigned limit is not as well documented.

B.8 In contrast to pharmaceuticals, endotoxins are extracted or flushed from medical devices and then the extract/effluent subsequently mixed with LAL reagent. Studies by FDA investigators have demonstrated that extraction of endotoxin from spiked device materials might not achieve complete recovery; therefore, a more stringent product endotoxin limit of 20 EU per device was established to account for any potential inefficiency in the extraction method. If this assumption is applied, then there is no requirement for performing efficiency testing for each medical device. However, as with pharmaceutical solutions, the validity of the assay is demonstrated by use of a spiked endotoxin control.

B.9 In 2004, a task group within the Microbiological Methods working group of the American Association of Medical Instrumentation (AAMI) Sterilization Committee was tasked with evaluating the appropriateness of carrying out endotoxin recovery efficiency for the extraction of medical devices for endotoxin testing. [24]

The first of four steps was to obtain historical information or data that was used to establish the methodology. Step two was to review a body of knowledge for published recovery studies on medical devices. Step three included a search of documents on regulatory actions related to poor recovery of endotoxin that led to endotoxin detection problems with health care products. The fourth step was

to request unpublished data from AAMI members who could contribute recovery experimentation to further support this decision.

In summary, the Task Group found that endotoxin limits established by FDA and USP have an adequate safety factor based on less than a 100 % recovery. Studies showed that: 1) recovery experiments conducted with high levels of endotoxin do not represent the normal level of naturally occurring endotoxin present on devices, 2) there is no new data that indicates current methods of extraction are inadequate at the current limits for devices, 3) there is no historical evidence of patient injury related to poor extraction methods and 4) current methods outlined in USP and FDA will ensure non-pyrogenicity of health care products.

In conclusion, the Task Group decided that validation of extraction efficiency for endotoxin testing is not recommended. These same conclusions were agreed upon by AAMI Sterilization Working Group 8 (AAMI ST-WG08, Microbiological Methods) during the most recent revision processes, and no additional requirements were added related to the need for validation of extraction efficiency. [9]

B.10 Failure mode and effects analysis (FMEA) is an assessment tool for risk analysis when developing alternatives to batch testing. A practical example of the FMEA approach to risk analysis was published to support this concept.[39] This approach enables a medical device manufacturer to successfully conduct an analysis of bacterial endotoxin risk for a medical device manufacturing process that can use potentially contaminated process water. FMEA is not a zero-risk system but is designed to minimize the risk of potential hazards. An FMEA provides supporting documentation toward developing a rationale for alternative batch release testing.

B.11 The original FDA guideline for the LAL test published in 1987[16] created endotoxin limits for parenteral products and described procedures for validating test conditions for end-product testing. When the USP harmonized <85> BET, much of the information in the 1987 LAL Guideline, including endotoxin limits in Annex E, was no longer necessary. This historic guideline was retired in 2011. The FDA released a new guidance in 2012 in a question-and-answer format that addressed endotoxin issues of concern that are not discussed elsewhere.[18] The FDA guidance recognizes ANSI/AAMI ST72 and indicates that endotoxin limit and extraction procedures for medical devices are adequately described in ANSI/AAMI ST72 and USP chapters.

B.12 The FDA Q&A LAL Guidance[18] specifies that non-compendial tests for pyrogens should be validated as described in the USP General Chapter <1225>, Validation of Compendial Procedures, and shown to have an advantage over compendial tests. This requirement applies to the monocyte activation test (MAT) using whole blood or peripheral monocytes, where endotoxin stimulated cytokines are measured after overnight incubation. The release of cytokines is assessed by ELISA as a measure of pyrogenic activity. A monograph for the MAT was introduced into the European Pharmacopoeia (EP) in 2010 (EP chapter 2.6.30), which is not specific for health care products. A revised general chapter for the MAT went into effect July 2017, see Ph.Eur Supplement 9.2 (2.6.30). [11]

Recombinant Factor C (rFC) assay contains synthesized rFC, but no other enzyme in the clotting cascade. Activated rFC acts on synthetic substrate reagent that incorporates a fluorogen. [12]

Peptidoglycan (PG) is many orders of magnitude less pyrogenic than endotoxin. PG can be assayed with the Silk Worm Larva Test (SLP). The reagent responds to PG and β-glucan with the release of melanin. The SLP test was used in an investigation of PG levels in peritoneal dialysis solutions. [42]

B.13 Our knowledge of endotoxins, particularly outer membrane vesicles (OMV) has expanded in recent years. Endotoxins are fever causing agents that are also structural constituents of the Gram-negative outer cell membrane (OM). The biologically active component of endotoxin is LPS, an amphipathic molecule consisting of hydrophobic Lipid A, the toxic moiety of the molecule that is embedded in the membrane, and a hydrophilic polysaccharide chain that is in contact with the extracellular environment. In nature, LPS is not secreted from the cell as a purified molecule in the same manner as an exotoxin, but rather LPS is released from a Gram-negative cell during its normal growth cycle as an integral part OMV.[48][23] OMV are buds of the outer membrane that are pinched off and float freely in the extracellular environment. They are hearty, but unable to replicate, because they

do not contain DNA. It is OMVs that are contaminants in parenteral products and health care products. As bacteria adapt to stresses in their environments, the chemical composition of the LPS in the OM can change as well to maintain the integrity of the OM and ensure survival of the organism.[38][25]

Early in the history of the BET, the FDA recognized a need to calibrate the various LAL reagents that were being manufactured to ensure consistency in sensitivity. The RSE, which is a Westphal (hot phenol) extracted and formulated preparation of purified LPS, was created for the purposes of assigning and calibrating LAL reagent sensitivity. Secondary standards called Control Standard Endotoxins (CSE) that are provided in kits are currently purified and formulated LPS as well. Whereas these calibration standards are used for creating standard curves and for preparing PPCs, they are not reflective of actual endotoxin contamination because they are not OMV.

B.14 Knowledge has also improved related to the interferences with the BET that arise from two general sources. The first is a condition or agent that acts on the proteins in the LAL cascade to cause nonspecific interferences; examples include non-neutral pH, LAL-reactive glucans and serine proteases. The second group of interferences act on the LPS in the RSE or CSE to change the aggregation state of the purified material causing it to be less reactive.[49] Fortunately, most LAL reagents are sensitive enough to allow dilution of sample extracts to avoid most interference without exceeding the MVD.

B.15 The development and more widespread use of automated techniques and equipment allowing for a reduction of variation and errors has occurred since the publication of ANSI/AAMI ST72. For example, automated liquid handling system, the use of robots. Also, a cartridge-based kinetic chromogenic LAL test system contains a reagent-impregnated cartridge that eliminates the need for reagent preparation and creation of a standard curve. The platform measures absorbance and compares the observed value with an archived standard curve. Weber reported that the unit reduced sample handling, test time, deviations and BET-related investigations. [52]

B.16 The clotting enzymes in LAL reagent can be activated by solutions that contain (1,3)-β-D-glucan, a non-pyrogenic polysaccharide found in cellulose and yeast cell wall.[30] To avoid false-positive endotoxin tests, the BET allows the use of LAL reagents that contain a β-D-glucan blocking agent. These reagents enable a BET for products that have been exposed to cellulosic products, depth filters, yeast fermentation and other sources of LAL reactive materials. Glucans can be suspected if there is an unexpectedly high endotoxin level, nonlinear reactions are observed when multiple dilutions are tested, and an enhancement in the positive controls of a kinetic BET.

B.17 Several documents have been published that are primarily used for endotoxins testing. There are two USP chapters USP <85> BET,[21] USP <161> Medical Device – Bacterial Endotoxin and Pyrogen testing,[20] and several FDA documents: DHHS, Guidance for Industry: Pyrogen and Endotoxin Testing: Questions and answers (2012),[14] Endotoxin Testing Recommendations for Single Use Intraocular Ophthalmic Devices (2015),[15] and Submission and Review of Sterility Information in Premarket Notification (510(k)) Submissions for Devices Labelled as Sterile (2016).[17] FDA also uses good manufacturing (quality systems)[19] regulations in its enforcement program to require the BET for validating depyrogenation cycles and for monitoring water, raw materials, and in-process samples.

Additionally, CHMPICH guideline Q4B Annex 14 note for evaluation and recommendation of pharmacopoeial texts for use in the ICH regions on BET – general chapter (CHMP). The ICH Harmonized Tripartite Guideline[53] became effective in May 2013.

Several Pharmacopeia contain BET test chapters with similar requirements. For example, portions of the USP <85> have been harmonized with the European Pharmacopeia and Japanese Pharmacopeia.

Annex C
(informative)

Guidance on out of specified limits (OSL) and failure investigation

C.1 General considerations

A failure investigation into an out of specified limit (OSL) result can include the laboratory, raw materials, and/or the manufacturing process. See Figure C.1.

An OSL result assumes that a valid BET has been performed.

It is recommended that all test samples/extracts be retained until data have been reviewed and assessed.

When alternatives to batch testing are used and a batch exceeds the product endotoxin limit, the continued use of alternatives to batch testing should be reassessed (refer to 10.2.3).

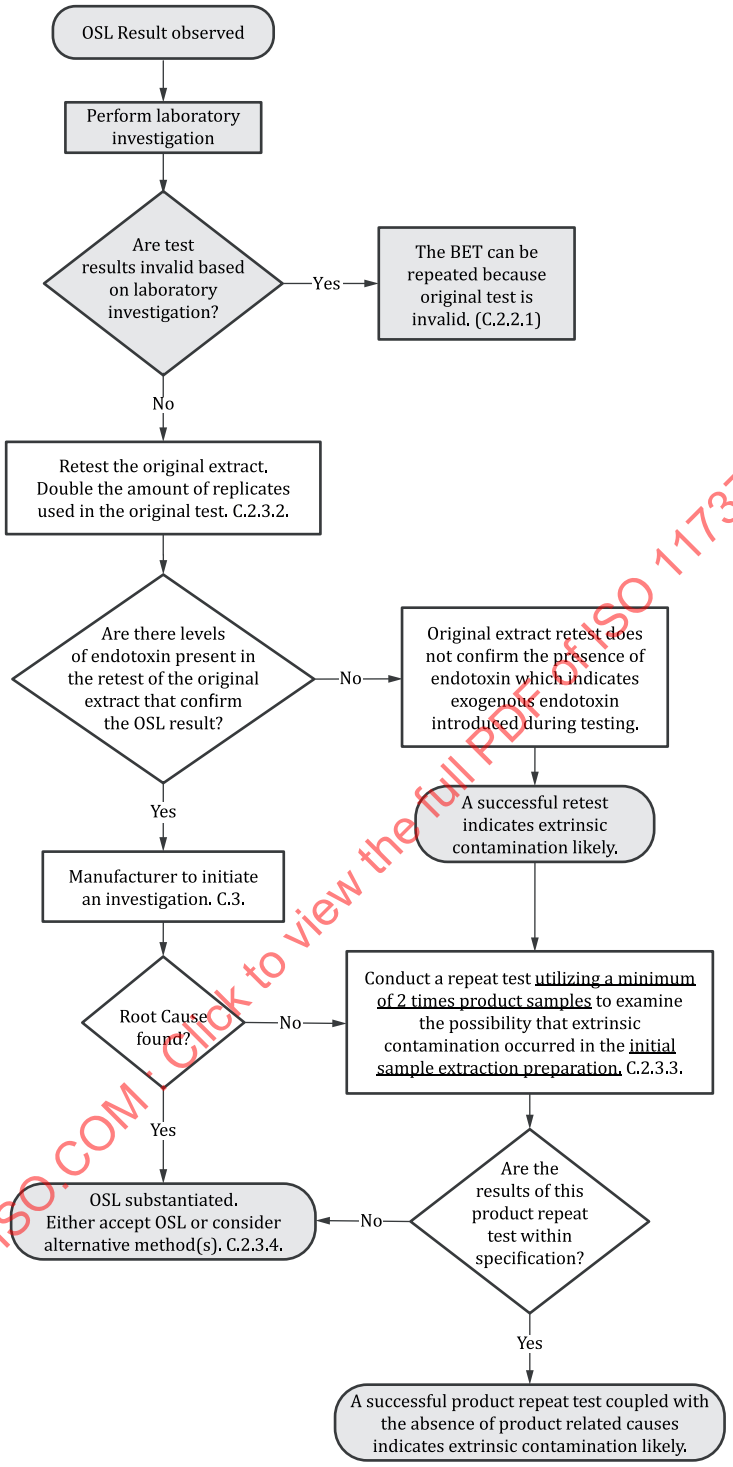

Figure C.1 — Flow chart

C.2 Laboratory investigation

C.2.1 Items to consider

Items to consider in the laboratory investigation include the following:

a) raw data review and verification of calculations;

b) supplies and reagents used during testing (e.g. pipette tips, dilution tubes, multi-well plates, water, lysate);

c) standard curve parameters (e.g. onset times of the control standards and samples, correlation coefficient, y-intercept and slope criteria);

d) equipment performance (e.g. microplate readers, heat block, micro pipettors);

e) input of laboratory analyst to identify anomalies during testing process (e.g. evaluate contacted packaging materials, tape);

f) proper identification and storage of the samples, reagents, controls and standards;

g) initial sample method suitability testing review;

h) calibration records of applicable equipment/tools (e.g. pipettors);

i) qualification of the test system;

j) training records of analyst(s);

k) testing history of the product, including changes to the test procedure.

Laboratory quality management systems can already include these items as part of standard processes prior to release of the laboratory result. In this case, it is possible that further laboratory investigation will not be necessary.

C.2.2 Results of laboratory investigation

C.2.2.1 If the laboratory investigation indicates that the test result is invalid for causes unrelated to the product endotoxin content, the initial test can be considered invalid (i.e. a "no-test") and the BET can be repeated (repeat test) using new product samples and the original sample size.

C.2.2.2 If the laboratory investigation fails to identify a root cause for the OSL, then additional investigational tests (e.g. extract retest) to verify the validity of the original result should be conducted to examine the possibility that extrinsic contamination occurred during the initial BET (refer to C.2.3). Extrinsic contamination is contamination arising from sources other than the product raw materials, processing aids, processing of the product, or product packaging.

NOTE A successful product or extract retest by itself does not necessarily prove extrinsic contamination.

C.2.2.3 The retest or repeat test results and conclusion of the laboratory OSL investigation should be documented (see A.7.3.1.1 for storage conditions).

C.2.3 Additional investigational tests

C.2.3.1 Additional optional investigational tests should be conducted to examine the possibility that extrinsic contamination occurred during the initial BET. Investigational testing can include testing at the MVD, testing using glucan blocker or other appropriate techniques.

C.2.3.2 Extract retest: using the original extract preparations to examine the possibility that extrinsic contamination occurred in the initial BET, at least twice (2x) the number of replicates of the original sample extraction preparation used during the initial BET should be tested. For example, in a test that was initially performed as a single test in duplicate, the investigation retest would consist of two tests, each performed in duplicate. If sample extracts are to be retained, the storage conditions should be assessed regarding the recoverability of endotoxin over time (refer to A.7.3.1.1). If the initial OSL result occurred at less than the MVD, the test can be repeated using a greater dilution not exceeding the MVD.

C.2.3.3 Product repeat test: in the event that the extract confirms the presence of endotoxin, then a product repeat test using new additional product samples should be conducted to examine the possibility that extrinsic contamination occurred during the initial sample extraction preparation. In a product repeat test, it is recommended that the number of product samples be increased from the initial number tested. For example, at least twice (2x) the initial test article(s) should be tested during the product repeat test.

C.2.3.4 Alternatively, the sample can be analysed with another BET as allowed by regulatory guidance. Alternate formulations can aid in identifying factors such as test interference.

C.2.3.5 If the investigational test(s) indicate that the sample does not conform to the product endotoxin limit, the product does not pass the test. The manufacturer can coordinate an investigation to identify the source of contamination in the raw material and/or manufacturing process (refer to C.3).

C.2.3.6 If the additional investigational testing indicates a laboratory error, in that contamination can have occurred during extraction or during the initial BET, the initial test can be considered invalid (i.e. a "no-test") and the BET can be repeated using new product samples and the original sample size.

C.3 Raw material and manufacturing processes investigation

C.3.1 Items to consider in the raw material and manufacturing process investigation include key process steps or control points that can lead to contamination, such as those listed below:

a) incoming raw materials or components;

b) extrusion operations;

c) aqueous steps in production;

d) manufacturing cleaning process;

e) drying or curing processes;

f) in-process aqueous leaching or soaking;

g) water in compressed air system;

h) product/component handling;

i) manual versus automated assembly;

j) product or material storage.

C.3.2 Individual unit testing can be conducted to determine endotoxin distribution among units within the batch.

Annex D
(informative)

Guidance on in-process monitoring of manufacturing processes or component testing

D.1 General

D.1.1 In-process monitoring of either manufacturing process or component sampling, or both, is commonly performed for a variety of reasons, some of which include, but are not limited to:

a) risk control (i.e. mitigation) for critical processes;

b) monitoring of critical points in a process;

c) obtaining data to support alternatives to batch testing.

D.1.2 Performing in-process monitoring and component testing is a key aspect for an alternative to batch testing sampling plan rationale except where routine batch testing over a significant period of time has demonstrated that the product as a whole does not have pyrogens associated with manufacture, and water or other natural materials are not part of the process. If the product has demonstrated no recovery of endotoxins, these steps could be eliminated.

In-process monitoring and actions to be taken can improve the capability of the manufacturing process to produce product meeting endotoxin limit requirements when operated within specified parameters.

D.2 Selection of product units

D.2.1 The sampling plan used in the selection of product units for component testing depends on the purpose for which the data are used, such as:

a) It is common to select a sampling plan of 3 % of a given batch, with a minimum of 2 samples for a batch size less than 30 and a minimum of 3 samples for a batch size between 30 and 100 and a maximum of 10 samples taken at random to represent the quality of the batch (see A.5.2.2). A larger sampling plan can be used. For example, with a subassembly that has risk controls in the process, a smaller sampling can be appropriate for verification of other process controls, based on the risk assessment.

b) Where water is part of the process, a water sample or representative sampling from a homogenously exposed group from the same water can be appropriate.

c) The sampling plan can be based on a statistically derived sampling plan (e.g. a sampling plan that might require greater than 10 samples).

d) Components might be pooled or tested individually. The endotoxin specification of the component should consider the risk of endotoxin on individual units.

D.2.2 The component(s) and/or time point(s) selected for sampling should be based on an assessment of the risk of how either the components or processes, or both can impact end-product endotoxin levels. Some examples of criteria to consider can include, but are not limited to:

a) manufacturing processes, including the potential for whether individual processes can introduce bacterial endotoxins;