List of Titles

American National Standard

ANSI/AAMI ST72:2002

Bacterial endotoxins—Test methodologies, routine monitoring, and alternatives to batch testing

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AAMI Association for the Advancement of Medical Instrumentation

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Particular care should be taken in applying a product standard to existing devices and equipment, and in applying a recommended practice to current procedures and practices. While observed or potential risks with existing equipment typically form the basis for the safety and performance criteria defined in a standard, professional judgment must be used in applying these criteria to existing equipment. No single source of information will serve to identify a particular product as "unsafe". A voluntary standard can be used as one resource, but the ultimate decision as to product safety and efficacy must take into account the specifics of its utilization and, of course, cost-benefit considerations. Similarly, a recommended practice should be analyzed in the context of the specific needs and resources of the individual institution or firm. Again, the rationale accompanying each AAMI standard and recommended practice is an excellent guide to the reasoning and data underlying its provision.

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American National Standard ANSI/AAMI ST72:2002

Bacterial endotoxins—Test methodologies, routine monitoring, and alternatives to batch testing

Developed by
Association for the Advancement of Medical Instrumentation

Approved 10 June 2002 by
American National Standards Institute, Inc.

Abstract: Specifies general criteria to be applied in the determination of bacterial endotoxins (pyrogens) on medical devices, components, or raw materials. Bacterial endotoxin methodologies covered include both qualitative (limit) methods and quantitative (end-point) methods using *Limulus* amebocyte lysate methodology. Determination of pyrogens other than bacterial endotoxins is not covered and acceptable levels for bacterial endotoxins are not covered.

Keywords: *Limulus* amebocyte lysate, LAL, pyrogenic labeling, maximum valid dilution, MVD, RSE:CSE standardization, analyst qualification, product qualification, gel-clot technique, chromogenic technique, turbidimetric technique, medical device, batch testing, laboratory quality system, product family, set, sample frequency, kinetic assay

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Association for the Advancement of Medical Instrumentation

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NOTE—Participation by federal agency representatives in the development of this Recommended Practice does not constitute endorsement by the federal government or any of its agencies.

Foreword

Pyrogens are any substance that can induce fever in a host. 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). The most significant pyrogens have been found to be endotoxins from Gram-negative bacteria. Although Gram-positive bacteria, fungi, and viruses can induce fever in the host, they do so through a different mechanism (systemic effects) and to a lesser degree than Gram-negative bacteria. Only Gram-negative bacterial endotoxin testing will be covered in this document.

Endotoxin is the high molecular weight lipopolysaccharide (LPS) component of the outer cell wall of Gram-negative bacteria that causes fever, meningitis, and a rapid fall in blood pressure if introduced into blood or tissues of the body. The outer cell wall components, which are composed primarily of proteins, phospholipids, and LPS, are constantly released into the environment when Gram-negative bacteria divide or lyse. Endotoxin contamination is difficult to prevent because it is ubiquitous in nature, stable, and small enough to pass through conventional sterilizing filters.

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

1) use of manufacturing techniques that produce non-pyrogenic products,

2) minimization of the source of pyrogens in the manufacturing process, and

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

This document will focus primarily on product manufactured under conditions that do not require a depyrogenation step as part of the manufacturing process.

The purpose of this document is to consolidate the requirements for testing for bacterial endotoxins. This includes the selection of product units for testing, selection and validation of testing technique, use of technique for routine testing, and interpretation of test results. This document also addresses the requirements for manufacturing operation validation that would support alternatives to batch testing.

The information included in the annexes provides:

— the background/history of endotoxin testing,

— guidance on endotoxin test methodologies, and

— guidance on alternatives to batch testing and validating manufacturing operations.

As used within the context of this document, “shall” indicates requirements strictly to be followed to conform to the recommended practice. “Should” indicates that among several possibilities, one is recommended as particularly suitable, without mentioning or excluding others, or that a certain course of action is preferred but not necessarily required, or that (in the negative form) a certain possibility or course of action should be avoided but is not prohibited. “May” is used to indicate that a course of action is permissible within the limits of the recommended practice. “Can” is used as a statement of possibility and capability. Finally, “must” is used only to describe “unavoidable” situations, including those mandated by government regulation.

The annexes to this Recommended Practice/American National Standard are for information only.

Suggestions for improving this Recommended Practice are invited. Comments and suggested revisions should be sent to Technical Programs, AAMI, 1110 N. Glebe Road, Suite 220, Arlington, VA 22201-5762.

NOTE—This foreword does not contain provisions of ANSI/AAMI ST72:2002, *Bacterial endotoxins—Test methodologies, routine monitoring, and alternatives to batch testing*, but it does provide important information about the development and intended use of the document.

List of Titles

American National Standard **ANSI/AAMI ST72:2002**

Bacterial endotoxins—Test methodologies, routine monitoring, and alternatives to batch testing

1 Scope

1.1 This document specifies general criteria to be applied in the determination of bacterial endotoxins on or in medical devices, components, or raw materials using bacterial endotoxin test methodology.

NOTE—Although the scope of this standard is limited to medical devices, it specifies requirements and provides guidance that may be applicable to other health care products.

1.2 The bacterial endotoxin test methodologies covered in this document include both qualitative methods and quantitative methods.

1.3 This document is not applicable to the determination of pyrogens other than bacterial endotoxins.

1.4 This document does not specify acceptable levels for bacterial endotoxins.

NOTE—For acceptable levels for bacterial endotoxins, reference the appropriate regulatory standards.

2 Normative references

The following documents contain provisions that, through reference in this text, constitute provisions of this guideline. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this guideline are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below.

ISO 9001:2000, *Quality Management Systems—Requirements.*

The United States Pharmacopoeia (USP). Easton: Mack, 2000, 24th ed., ⟨85⟩ & ⟨161⟩.

U.S. Food and Drug Administration:1998*, Quality System Regulation*, 21 CFR, Part 820.

U.S. Food and Drug Administration: *Guideline on validation of the* Limulus *amebocyte lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices*. DHHS, December (1987).

FDA Interim Guidance for Human and Veterinary Drug Products and Biologicals, *Kinetic LAL Techniques*, July 15, 1991.

3 Definitions

For the purpose of this document, the following definitions apply.

3.1 bacterial endotoxins test (BET): Assay for measuring active endotoxin by combining a liquid test sample with *Limulus* amebocyte lysate (LAL) reagent and measuring the resulting proportional reaction via visual, turbidimetric, chromogenic, or other validated means of detection.

3.2 batch: Defined quantity of bulk, intermediate, or finished product, intended or purported to be uniform in character and quality, which has been produced during a defined cycle of manufacture.

3.3 chromogenic technique: BET methodology that quantifies or detects endotoxin on the basis of a measured color-producing reaction proportional to the interaction of LAL and endotoxin.

3.4 control standard endotoxin (CSE): Endotoxin preparation other than the reference standard endotoxin (RSE) that has been standardized against the RSE.

3.5 depyrogenation: Validated process designed to remove or inactivate endotoxin.

3.6 endotoxin: High molecular weight complex associated with the cell wall of Gram-negative bacteria that is pyrogenic in humans and specifically interacts with LAL.

3.7 endotoxin unit (EU): Standard unit of measure for endotoxin activity initially established relative to the activity contained in 0.2 ng of the U.S. Reference Standard Endotoxin Lot EC-2 (USP standard reference material).

NOTE—FDA's reference endotoxin 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 (Poole et al., 1997).

3.8 endpoint (gel clot): Last positive tube in a series of dilutions.

3.9 enhancement: BET 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.

3.10 gel-clot technique: BET methodology that quantifies or detects endotoxin on the basis of a clot-producing reaction proportional to the interaction of LAL and endotoxin.

3.11 inhibition: BET 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.

3.12 inhibition/enhancement test: Test used to determine whether a particular BET sample contains factors that diminish its accuracy by introducing enhancement or inhibition into the test system.

3.13 interfering factors test: See inhibition/enhancement test.

3.14 LAL reactive material (LAL-RM): Any non-endotoxin compound that will activate the LAL clotting cascade and therefore has the potential to cause enhancement.

3.15 LAL reagent water (LRW): Purified water or other qualified solutions employable as a solvent, diluent, and/or extractant in a BET, and as such must be non-reactive in the methodology in use.

3.16 lambda (λ): Labeled sensitivity of an LAL gel-clot reagent expressed in EU/mL or, for chromogenic or turbidimetric tests, the lowest point (endotoxin concentration) on the referenced standard curve.

3.17 *Limulus* amebocyte lysate (LAL): Reagent extracted from circulating amebocytes of the horseshoe crab, *Limulus polyphemus* or *Tachypleus tridentatus* (TAL), that interacts with endotoxin to form a gelatinous clot and is used to estimate endotoxin levels via the bacterial endotoxins test.

3.18 lipopolysaccharide: Gram-negative bacterial cell wall component typically composed of lipid A, a core polysaccharide, and an O-side chain.

3.19 lot: See **batch**.

3.20 maximum valid dilution (MVD): Maximum amount a sample can be diluted relative to the sensitivity of an LAL assay in which the specified test endotoxin limit can be detected.

3.21 non-pyrogenic: Term used to describe a health care product that does not induce a fever.

NOTE—May also be used to describe and label health care products that contain endotoxin levels less than specified limits.

3.22 product endotoxin limit: Maximum allowable level of endotoxin specified for a product, established below the threshold pyrogenic dose of endotoxin in humans.

3.23 pyrogen: Any substance that induces a fever.

3.24 pyrogenic: Term used to describe a health care product that induces a fever.

NOTE—May also be used to describe a health care product with an endotoxin level above specified limits.

3.25 reference standard endotoxin (RSE): USP Endotoxin Reference Standard that has a defined potency of 10,000 USP EUs per vial.

3.26 standard control series: Serial dilution series of RSE or CSE used to verify LAL sensitivity.

3.27 test endotoxin limit: Maximum endotoxin concentration allowable in a BET sample extract.

NOTE—This limit is determined by dividing the product endotoxin limit by the volume of the LRW used per unit for sample extraction.

3.28 turbidimetric technique: BET methodology that quantifies or detects endotoxin on the basis of a measured turbidity reaction proportional to the interaction of LAL and endotoxin.

3.29 validation: Documented procedure for obtaining, recording, and interpreting the results needed to demonstrate that a process will consistently yield a product complying with predetermined specifications.

4 General

4.1 Documentation

4.1.1 Approved procedures and instructions on the testing techniques to be employed and the use and operation of all relevant equipment shall be available and shall be controlled as specified in quality system requirements.

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

NOTE—If calculations are performed by electronic data processing techniques, the application of the software should be validated for the intended uses, and records of this validation should be retained.

4.1.3 Records of original observations, calculations, derived data, and final reports shall be retained as specified in quality system requirements. The records shall include the identity of personnel involved in the preparation and testing of samples.

4.2 Personnel

4.2.1 Responsibility for performing a BET shall be assigned to personnel as specified in quality system requirements.

4.2.2 Training shall be performed in accordance with documented procedures. Records of the relevant qualifications, training, and experience of technical personnel shall be maintained.

4.2.3 Analyst qualification shall be conducted prior to performance of a BET (see 8.3.3).

4.3 Equipment

4.3.1 All equipment required for performance of the specified tests and measurements shall be available, and planned maintenance and calibration shall be performed in accordance with documented procedures. Records of maintenance and calibration shall be retained.

4.3.2 All equipment shall be documented to perform according to specified criteria.

4.4 Reagents and materials

4.4.1 Methods shall be established and documented for the preparation of reagents, controls, and reference materials used in a BET, including appropriate quality tests.

NOTE—Appropriate quality tests should include confirmation of lambda.

4.4.2 Methods shall be established, validated, and documented for the depyrogenation of glassware and other heat-stable equipment used in a BET.

NOTE—Appropriate guidelines for depyrogenation of materials are referenced in USP, Weary, and PDA Technical Report No. 7.

4.4.3 Materials used in a BET shall be demonstrated to be free of detectable endotoxin if depyrogenation is not performed. This may be performed, for example, by testing a sample of purchased materials to demonstrate that they are non-pyrogenic or by accepting an appropriate vendor certificate.

NOTE—Containers used for sampling, storing, or diluting should be free of interference. For example, polypropylene has been shown to inhibit endotoxin detection (Rolansky, 1991).

5 Non-pyrogenic labeling

5.1 Products that directly or indirectly contact the cardiovascular system, lymphatic system, or cerebrospinal fluid, or present the potential for similar systemic exposures (e.g., solution administration sets, transfer sets, catheters, implants, and infusion assemblies), or ophthalmic products for intraocular use (e.g., silicone oil, viscoelastic products, intraocular lenses) shall be evaluated for endotoxin.

5.2 Application of any non-pyrogenic product label statement or claim shall require explicit substantiation. Such substantiation may include:

— direct testing of the product employing a validated BET by qualified personnel,

— documented manufacturing operation validation for non-pyrogenicity, or

— other evidence of conformance to an appropriate standard and/or requirement.

5.3 All parts of products claimed to be non-pyrogenic shall be included in the testing process. A statement of "non-pyrogenic fluid path" shall be supported by appropriate evaluation of components and surfaces relevant to the fluid path in use.

5.4 For multi-component kit products, the labeling and/or claim shall be consistent with the documented evaluation of the contained sub-components for endotoxin conformance. Such labeling should be consistent with the intended clinical application of the kit and components. Use of the term "non-pyrogenic" without qualification for specific components shall indicate appropriate evaluation of all component parts.

5.5 Conformance limits associated with labeling shall be established by reference to appropriate regulatory standard(s) based upon clinical use of the product.

NOTE—Such requirements have been established by the FDA, and may be included in other relevant regulatory guidelines/ requirements.

6 Selection of product units

6.1 The sampling criteria for selection of product units for endotoxin testing is based on the premise that the manufacturing process is controlled and in compliance with quality system requirements.

6.2 The selection of product units for testing shall be based on criteria defined in a sampling plan. The sampling plan should specify the sample size (6.3), the group from which samples are to be drawn (6.4), and other pertinent sampling criteria (6.5–6.7).

6.3 Sample size shall be based on a rationale specified in the sampling plan. This rationale may be based on applicable regulatory requirements, drawn from published statistical plans or guidelines, or based on manufacturing operation validation.

6.4 The sampling group is generally defined as the production batch. Sample selection may be based on a sampling group other than the production batch if there is data to support a different selection basis. Products may be grouped into families for testing if alternatives to batch testing are used (see B.3.2). Requirements for using alternatives to batch testing can be found in clause 10.

NOTE—Specification of a sampling group other than production batch should include a risk assessment to evaluate the adequacy of the criteria used to establish the group.

6.5 Samples selected for testing should be produced and selected in the finished form. This includes all factors that might affect or contribute to the levels of endotoxin (e.g., packaging).

NOTE—Samples utilized for endotoxin testing may be selected from products that have been rejected for other production quality issues as long as the rejected samples are representative of non-rejected product.

6.6 Samples may be obtained prior to (pre-) sterilization or after (post-) sterilization. Post-sterilization samples encompass all of the factors that may affect the product or the endotoxin test. When pre-sterilization samples are selected for testing, the acceptability of this sampling shall be documented. The program for ongoing testing should consistently reflect either pre- or post-sterilization samples.

NOTE—For products that support microbial growth, the choice of pre-sterilization sampling may not be appropriate.

6.7 In the testing of multi-component kit products, either the individual components or the entire kit may be considered as a device entity. Standard test procedures should be applied in the case of individual component qualifications. Consideration of a kit as a single unit must address sample preparation in adherence to method requirements and the applicable product endotoxin limit. Additional guidance can be found in B.3.3 and B.3.4.

7 Selection of technique

7.1 The testing laboratory has three choices for BET techniques, all of which are described in various compendia, guidelines, and product inserts. The choice of technique should be made after careful thought and assessment of the laboratory's expertise, experience, sample throughput requirements, and data handling requirements, and the nature of the test sample. Current techniques and the methodologies for each are:

a) gel-clot technique: limit test and assay methods,

b) chromogenic technique: kinetic and endpoint methods, and

c) turbidimetric technique: kinetic and endpoint methods.

Information on each of these methodologies is presented in annex B.

7.2 The selected method shall be validated as specified in clause 8.

NOTE—If the test method or technique is changed, revalidation must be performed (see 8.6).

8 Validation of methodology

8.1 Test endotoxin limit qualification

Before conducting the BET, the test endotoxin limit for each product under test must be determined. The test endotoxin limit defines the maximum allowable concentration of endotoxin that can be present in a product extract solution, and is relative to the product endotoxin limit. The product endotoxin limit can be obtained from the applicable regulatory standard. The test endotoxin limit may be calculated as follows:

$$\text{Test endotoxin limit (EU/mL) for medical devices} = \frac{(K)\,(N)}{V} \qquad \text{(equation 1)}$$

where: K = Amount of endotoxin allowed per device

N = Number of devices tested

V = Total rinse/soaking solution in the combination of samples (mL)

8.2 Maximum valid dilution (MVD)

Recognizing that products can inhibit the BET, a provision has been made for the elimination of interfering substances through dilution in LAL reagent water (LRW) or other appropriate diluents. However, diluting the interference also means diluting any endotoxin that might be present, so there is a limit to the extent of dilution allowed. The MVD is calculated as follows:

$$\text{MVD} = \frac{\text{Test endotoxin limit}}{\lambda} \qquad \text{(equation 2)}$$

where: Test endotoxin limit is calculated (see equation 1)

λ = The confirmed label claim sensitivity of the lysate reagent (gel-clot) or the lowest endotoxin dilution used to construct the referenced standard curve (chromogenic and turbidimetric)

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 4 means that a 1:4 dilution can be used.

8.3 Reagent and analyst qualification

8.3.1 Preparatory testing (confirmation of label claim/demonstration of linearity)

For the gel-clot technique: The label claim sensitivity (λ) of each lot of lysate must be verified by testing, in quadruplicate, a series of endotoxin dilutions that bracket λ (e.g., dilutions of 2 λ, λ, 0.5 λ, 0.25 λ). The geometric mean endpoint of the series must 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 follows:

$$\text{Geometric mean} = \text{antilog}\,(\sum e/F) \qquad \text{(equation 3)}$$

where: $\sum e$ = Sum of the log endpoint of each series

F = Number of replicates

For chromogenic/turbidimetric techniques: Verification requires the demonstration of a linear standard curve across the range of endotoxin dilutions that will be routinely used in the laboratory. At least three endotoxin concentrations must be used to generate the standard curve. If the standard curve range is greater than two logs, additional standards should be included to bracket each log increase within the range. Each endotoxin concentration should be tested in triplicate. Individual points should not be averaged for the purpose of demonstrating linearity in an initial quality control test. Linearity requires that the absolute value of the correlation coefficient $|r|$ be ≥ 0.980 for the range of endotoxin concentrations indicated by the lysate reagent manufacturer.

8.3.2 RSE:CSE standardization

The activity of the CSE for each unique combination of LAL reagent lot number and CSE lot number in use in the laboratory must be determined and documented. Documentation in the form of a Certificate of Analysis may be obtained from the lysate manufacturer and should be kept on file. Alternatively, the standardization may be done in the laboratory.

8.3.3 Analyst qualification

Each analyst performing the BET must demonstrate competency. The procedure for analyst qualification is the same as for 8.3.1.

8.4 Product qualification/validation

8.4.1 General

Each product or product group (see B.3.2) must be qualified/validated to adequately demonstrate that the test articles do not of themselves cause inhibition, enhancement, or otherwise interfere with the accuracy and sensitivity of the BET system. For information on sample interference, see 8.5.

The methods for product qualification are given in 8.4.2 and 8.4.3.

8.4.2 Gel-clot technique

8.4.2.1 Three batches per product or product family shall be used for initial qualification/validation studies.

8.4.2.2 Prepare solutions as listed in Table 1. The sample solution must be at a dilution less than or equal to the MVD not containing any detectable endotoxin. Test each endotoxin-spiked dilution series and negative control. The geometric mean endpoint concentrations of each endotoxin-spiked dilution series should be determined using the equation listed in the preparatory testing section, 8.3.1.

Table 1—Preparation of solutions for interference test: Gel-clot technique

Solution	Diluent	Endotoxin spike	Endotoxin concentration	Number of replicates
Product positive control series	Sample solution	Prepare 2 λ solution, then 2-fold serial dilutions of initial 2 λ prep	2 λ λ 0.5 λ 0.25 λ	4 4 4 4
Product negative control	Sample solution	None	NA	4
Standard control series	LRW	Prepare 2 λ solution, then 2-fold serial dilutions of initial 2 λ prep	2 λ λ 0.5 λ 0.25 λ	2 2 2 2
Negative control	LRW	None	NA	2

8.4.2.3 The sample does not contain interfering factors if the LAL sensitivity in the product positive control series is between 0.5 λ and 2 λ, negative controls show no reaction, and the result of the standard control series confirms the labeled sensitivity of the LAL.

8.4.3 Chromogenic and turbidimetric techniques

8.4.3.1 Select an endotoxin concentration near the middle of the endotoxin standard curve. Prepare solutions as listed in Table 2. Test the solutions per the appropriate test method for the LAL reagent used.

Table 2—Preparation of solutions for interference test: Chromogenic and turbidimetric techniques

Solution	Diluent	Endotoxin spike	Minimum number of replicates
Positive product control	Sample solution	Middle concentration of the standard curve	2
Sample solution	Sample solution	None	2
Standard control series	LRW	Minimum of three different concentrations encompassing λ	2 per concentration
Negative control	LRW	None	2

8.4.3.2 Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the sample solution from the product positive control.

8.4.3.3 The sample or sample dilution is considered to be free of interfering factors if the measured concentration in the product positive control is within 50 to 200 percent of the known added endotoxin concentration (after subtraction of any endotoxin detected in the sample solution).

8.5 Sample interference

8.5.1 If interference is observed in any BET, the interference may be overcome by suitable treatment. The sample extracts may be diluted and/or treated to overcome inhibition or enhancement and determine the endotoxin concentration. Treatment of a sample may involve dilution, addition of reagents, heat denaturing, etc. If the MVD must be exceeded, it may be possible to overcome this by concentrating the sample extract using ultrafiltration and recovery, provided the methodology can be validated. All sample manipulations should be specified in the validation data and performed during routine testing.

8.5.2 The use of different lysate vendors, different lysate sensitivities, and different BET test methodologies are alternatives that may be used to eliminate interference.

8.5.3 Dilution of the sample in non-pyrogenic tris hydroxymethyl aminomethane (TRIS) buffer or the addition of non-pyrogenic NaOH or HCl may be used to neutralize the pH of the sample extract.

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

NOTE—An example of a substance that can cause ionic interference is heparin.

8.5.5 Samples exhibiting enhancement should be examined for LAL reactive material (LAL-RM). For example, samples that may have LAL-RM interference should be tested with and without an endotoxin-specific buffer solution to eliminate the possibility of enhancement.

NOTE—Some possible sources of LAL-RM are yeast and mold cell walls, and cellulosic materials.

8.5.6 The test for interfering factors should be repeated when any changes are made to the experimental conditions likely to influence the test results.

8.6 Maintenance of qualification/validation

8.6.1 A new validation consisting of three batches/lots shall be performed for the following:

a) a product change that may impact the test,

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

c) a change in test parameters (e.g., sample extraction parameters).

8.6.2 A verification consisting of one batch/lot shall be performed for the following:

a) a change in laboratories (where none of the changes in 8.6.1 have occurred),

b) a change in LAL reagent manufacturer,

c) a change in test materials/equipment that may impact the test, and

d) a change in LAL sensitivity.

NOTE—A positive product control (PPC) may be used for verification purposes in certain circumstances where the PPC would be sufficient to detect a change.

9 Use of technique

9.1 Critical test parameters

9.1.1 Temperature

Incubation for BET methodology is typically carried out at 37 ± 1 °C.

9.1.2 Time

Times for reagent addition and incubation are described in the lysate manufacturer's product instructions for use. Incubation time for the gel-clot BET methodology is typically 60 ± 2 minutes.

9.1.3 pH

The BET reaction occurs at an optimum pH of 6 to 8. Running the test outside of the optimum pH ranges may result in interference. A pH measurement should be taken on the extract reagent combination for each of three batches/lots during validation using an appropriate pH test system. If no pH adjustments are necessary during validation, subsequent pH measurements may not be required. However, if adjustments are made during validation, then the pH must be measured for each test.

9.2 Equipment and reagents

9.2.1 Due to the limited temperature range required for the BET, heat blocks or water baths used to incubate gel-clot tests should be mapped for heat distribution. Mechanical pipettors, including fixed, adjustable, and repeating units, must be calibrated periodically. If the laboratory is performing a chromogenic or turbidimetric technique, the hardware and software must be qualified according to the manufacturer's instructions and validation requirements. Materials that are not supplied non-pyrogenic (e.g., microtiter plates) should be carefully evaluated (see 4.4.3) prior to use to assure the testing lab that they will not interfere with the assay.

9.2.2 All LAL reagents used in testing must be licensed.

NOTE—In the United States, LAL products are licensed by the Center for Biologics Evaluation & Research.

9.2.3 The primary endotoxin standard in the United States is called the reference standard endotoxin (RSE). The activity of secondary standards or control standard endotoxins (CSEs) must be standardized against the RSE either by the testing laboratory or by the lysate manufacturer who will provide the testing lab with complete documentation describing the standardization.

9.2.4 Storage requirements for freeze-dried and reconstituted reagents are described in individual lysate manufacturer's product inserts. If storage conditions utilized in the lab are different than those recommended by the manufacturer, the conditions of alternative storage shall be validated.

9.2.5 The BET requires the use of LRW, defined as water that is non-reactive with the test or assay in use in the lab. LRW is used to reconstitute reagents, dilute endotoxin standards, rinse/soak devices, and dilute samples.

9.3 Sample preparation

9.3.1 General

Product samples for testing should be collected and stored according to the manufacturer's instructions.

A routine BET must utilize the same method as used in the validation 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 label claim for the product.

NOTE—Historically, it is recognized that extractions performed for BET testing are not exhaustive (FDA, 1987). The endotoxin limits have been established with a safety factor to ensure patient safety (see A.8).

9.3.2 Medical devices

Devices are rinsed or soaked in LRW to obtain a sample extraction. Using depyrogenated instruments, the device(s) may be cut or disassembled for the extraction. The minimum extraction time should be 15 minutes at 37 °C, one hour at controlled room temperature (typically 18 to 25 °C), or other demonstrated equivalent conditions.

For devices labeled “nonpyrogenic fluid pathway,” fill the fluid pathway with LRW that has been heated to 37 °C, holding the extracting fluid in contact with the relevant pathway for not less than one hour at controlled room temperature (typically 18 to 25 °C).

The volume of LRW used to extract the devices may be adjusted depending on the size of the device. The MVD, or maximum amount of extraction fluid that may be used for the pooled extract, is calculated as described in 8.2. To overcome interference, the sample extract may be diluted to a level not to exceed the calculated MVD.

9.4 Routine testing and monitoring

9.4.1 Gel-clot limit test

Prepare and test solutions as listed in Table 3. The sample solution and product positive control should 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	LRW	2 λ	2
Negative control	LRW	None	2

9.4.2 Gel-clot assay

Prepare solutions as listed in Table 4. The sample solution must not be diluted more than the MVD. The dilution series described for the sample solution is intended to dilute an endotoxin-containing sample to an endpoint to facilitate quantification. Testing the entire dilution series is not necessary unless a positive result is obtained on the initial sample solution.

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
	LRW		2*	–	2
	LRW		4*	–	2
	LRW		8*	–	2
Product positive control (PPC)	Sample solution	2 λ	1	2 λ	2
Standard control series	LRW	Prepare 2 λ solution, then 2-fold dilutions of 2 prep	1	2 λ	2
	LRW		2	λ	2
	LRW		4	0.5 λ	2
	LRW		8	0.25 λ	2
Negative control	LRW	None	1	NA	2

* Testing necessary only if endotoxins are detected in the initial sample solution. In that case, prepare 2-fold dilutions of initial prep.

9.4.3 Chromogenic and turbidimetric techniques

For routine testing, see method and chart in 8.4.3.

9.4.4 Test frequency

A BET shall be performed in accordance with documented sampling plan(s) with defined sampling frequency and sample size (see clause 6). The frequency selected shall be adequate to ensure that endotoxin levels for all batches/lots produced will meet specified levels. Decisions regarding frequency of end-product testing also may be affected by the degree of control over the process and materials, and by procedures for testing of critical incoming raw materials and/or in-process monitoring. For a test frequency other than every batch/lot, see clause 10.

9.5 Interpretation of results

9.5.1 A valid routine test requires that the following results are observed:

a) For gel-clot techniques, the standard control series confirms λ within a range of 0.5 λ to 2 λ, and for chromogenic and turbidimetric techniques, the standard curve has a minimum |r| value of 0.980.

b) The negative control is non-reactive (all methods).

c) For gel-clot techniques, the PPC is recovered (i.e., positive), and for chromogenic and turbidimetric techniques, the PPC is recovered in the range of 50 to 200 percent of the "known added endotoxin concentration."

9.5.2 For the gel-clot limit test, the article under test is acceptable when the applicable parameters for validity are met (9.5.1) and negative results are found in both tubes containing the sample solution. If the validity parameters are not met, the following actions may be taken in conjunction with a laboratory investigation.

When a positive test is found in either or both of the tubes of the sample solution, the results may be confirmed (if desired) by repeating the test from the original extract using an appropriate number of test replicates.

If negative results are found in all of the tubes of the retest, the article under test is acceptable when the applicable parameters for validity are met (9.5.1).

If a positive result is found in any of the tubes of the retest, obtain and test additional samples from the same batch following the method in 9.4.1.

9.5.3 For the gel-clot assay, determine the endotoxin concentration in the sample solution by calculating the endpoint concentration for each replicate series and multiply each endpoint dilution factor by λ. The endotoxin concentration of the sample is the geometric mean endpoint concentration of the replicate dilution series. If necessary, 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, etc.).

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

The test article is acceptable if the level of endotoxin is determined to be less than the limit for the product.

9.5.4 For chromogenic and turbidimetric techniques, the preparation under test complies with the test if the mean endotoxin concentration of the replicates of the sample solution, after correction for dilution and concentration, is less than the product endotoxin limit.

9.5.5 If the endotoxin level exceeds the product specification, product shall be considered as out of specification and handled in accordance with documented procedures.

9.6 Change control

If the test technique is changed (e.g., gel-clot to chromogenic), a new validation consisting of three batches of product must be performed. See 8.6 for additional information on other changes and action to be taken for each.

10 Alternatives to batch testing

NOTE—See annex C for specific guidance on 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 and materials are well controlled and capable of producing products with endotoxin levels that consistently meet specified limits. Such demonstration should include sufficient data from batch testing showing acceptable endotoxin levels. In cases where

specific regulations/compendia require batch testing, alternatives to batch testing may not be allowed. (See C.1 for examples where specific regulations might not allow alternatives to batch testing.)

10.2 Criteria for establishing alternatives to batch testing

10.2.1 An alternative to batch testing shall be ensured via appropriate manufacturing operation design (see 10.3.1), validation (see 10.3.2), and control (see 10.3.3), as well as periodic review and requalification as necessary (see 10.5).

10.2.2 If an alternative to batch testing is used, the rationale for the alternative shall be documented and the sampling plan shall be defined.

10.2.3 Alternatives to batch testing may involve several options, including reducing the number of samples tested, reducing the frequency of testing, or using alternatives to finished product. See C.2 for examples of alternatives to batch testing.

NOTE—It should be recognized that using alternatives to batch testing may result in a reduction in the ability to detect an inadvertent change within the manufacturing process that may result in a non-conforming endotoxin level on a product. Consequently, the risk associated with a reduction in ability to detect inadvertent changes should be evaluated before proceeding with using alternatives to batch testing.

10.3 Manufacturing operation qualification

NOTE—See C.3 for specific guidance on manufacturing operation qualification.

10.3.1 Design

The manufacturing operation shall be designed to minimize the presence of endotoxin on the product. Manufacturing operations shall be well characterized with established operating specifications. Existing processes shall be operating in a state of control and shall be assessed for variables that could contribute to endotoxin contamination. The manufacturing operation shall be evaluated to identify key process steps or control points for potential endotoxin contamination. These key process steps should be included during the qualification of the manufacturing operation.

10.3.2 Validation

Procedures for validation of a manufacturing operation shall be documented and records of the validation retained as specified in 4.1. Validation of a manufacturing operation for endotoxin levels should include these elements:

a) Process qualification—An adequately designed installation qualification, operational qualification, and performance qualification as applicable.

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

b) Process risk assessment—A detailed schematic of the manufacturing process with key process elements identified by a risk assessment tool (e.g., failure mode effects analysis).

10.3.3 Control

Manufacturing operation control may require a system of monitoring at key control points to ensure that the operation is maintained in a controlled state that produces non-pyrogenic product. If key process steps or control points are applicable, they should be identified. For each defined control point:

a) a sampling plan shall be defined,

b) control limits shall be specified, and

c) the action to be taken when control limits are exceeded shall be specified.

10.4 Change control

Process changes or deviations shall be assessed to determine the impact on the endotoxin level of the product and/or manufacturing operation qualification. Changes may include product design, process deviations, changes in raw materials, water supply, etc. The extent of qualification that is necessary shall be determined. The outcome of the assessment, including rationale for decisions reached, shall be documented.

NOTE—The magnitude of the change is considered in determining the extent to which operational qualification is undertaken.

10.5 Maintenance of qualification

The manufacturing operation qualification shall be reviewed and evaluated on a periodic basis to assess the continued validity of the operation, as well as the cumulative effects of all minor and major changes on the product endotoxin levels. Requalification shall be performed where appropriate.

Annex A
(informative)

Background on the bacterial endotoxins test

A.1 The rabbit pyrogen test was introduced just prior to World War II to prevent pyrogenic materials from entering the health care system. (1) The *Limulus* amebocyte lysate (LAL) test was introduced in 1971 as a potential replacement for the rabbit bioassay. (2, 3) The parenteral drug industry and the U.S. Food and Drug Administration agreed on a guideline in 1987 to replace the pyrogen test with the LAL endotoxin test because the *in vitro* test had greater sensitivity, specificity, accuracy, and economy. (4) The LAL test became the official replacement for the majority of parenteral products in the United States in 1993, with a sweeping revision of the bacterial endotoxins test (BET) and adoption of product endotoxin limits; the current revision of the BET contains more than 650 limits for U.S. Pharmacopeia (USP) articles. (5) Although BET and LAL are widely used acronyms for these methods, BET is now the appropriate abbreviation for the test.

A.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. (6, 7) 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 ng/mL as the product endotoxin limit for device extracts. (8) A HIMA study further verified that the threshold pyrogenic dose of a Difco endotoxin (*E. coli 055:B5)* was approximately 1 ng/kg in rabbits.

A.3 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 reference standard endotoxin (RSE) from a purified LPS derived from *E. coli 0113*. (9) A collaborative study by LAL producers and the Bureau of Biologics established biological activity of the RSE in endotoxin units (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. (10)

A.4 Since 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 of endotoxin. Product endotoxin limits first appeared in appendix E of "FDA's Guideline on validation of the *Limulus amebocyte* test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices." (4) This guideline specified product endotoxin limits based on the formula K/M, where K is the tolerance limit for endotoxin 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. Studies in humans have supported a dose of 5 EU/kg as being a suitable tolerance limit. (11) The M in the K/M formula signifies that endotoxic response is related to dose. For large volume parenterals (LVP) 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 shall contain no more than 0.5 EU/mL of endotoxin (5 EU/kg/10mL = 0.5 EU/mL). Products designed for injection or instillation into intrathecal spaces have a more stringent product endotoxin limit because this exposure is more endotoxic than intravenous administration. (12)

A.5 The question of non-endotoxin pyrogens was a major concern for the pharmaceutical industry. In 1979, an LVP producer reported on its policy for LAL testing in place of pyrogen testing. (13) 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 that:

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; and

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

A.6 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. The LAL reagent has proven to be highly specific; only one non-specific activator has been recognized, 1→3-β-D-glucan, formerly known as LAL-reactive material. (14)

A.7 There are two documents in the United States that are primarily used for LAL testing: the USP BET (5) and the FDA LAL Test Guideline (4), as amended by the Interim Guidance for kinetic tests (1991). The FDA Guideline is referenced more than the USP BET because it describes all LAL methods. The USP BET is similar to the FDA Guideline, but the former is a referee method that only describes the gel-clot method. USP <161> contains a chapter titled, "Transfusion and infusion assemblies and similar medical devices" that specifies extraction procedures. (5) In addition to applying the previously mentioned guidelines, the FDA uses good manufacturing (quality systems) regulations in its enforcement program to require the BET for validating depyrogenation cycles and for screening water, raw materials, and in-process samples.

A.8 The BET for pharmaceutical solutions usually requires mixing equal parts of LAL reagent and a validated dilution of the test sample. The typical product endotoxin limit for a (whole body) dose of a drug compound is usually 350 EU. Validity of the assay is ensured by recovery of an endotoxin-spiked control. In contrast to pharmaceuticals, endotoxins must be extracted or flushed from medical devices and then the extract/effluent subsequently mixed with LAL reagent. Studies by FDA investigators have demonstrated that recovery extraction of endotoxin from spiked device materials does 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 must still be demonstrated by use of a spiked endotoxin control.

A.9 Pharmacopeia from developed nations contain bacterial endotoxins test chapters with similar requirements. The next regulatory advance will be a harmonized BET by the International Conference on Harmonization (ICH). The ICH document has reached a high level of approval, and is being proposed for adoption in the European Pharmacopeia (EP) (15) and the USP (16).

A.10 In 1995, a new whole blood pyrogen test was developed based on the human fever reaction. (17) The whole blood test is not restricted to endotoxins from Gram-negative bacteria, but detects all classes of pyrogens. Human whole blood is used to carry out the pyrogen test where monocytes react to pyrogens by the formation of fever-inducing mediators such as interleukin-1. After incubation at 37 °C, the release of interleukin-1 is assessed by ELISA as a measure of pyrogenic contamination. The EP has a monograph under development for the whole blood pyrogen test, and validation of this new application currently is underway.

Endnotes

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Annex B
(informative)

Guidance on test methodologies and routine monitoring

B.1 Introduction

B.1.1 This annex provides guidance on the implementation of the specified requirements. The guidance given is not intended to be exhaustive, but to highlight important aspects that should be given attention.

B.1.2 This annex is not intended as a checklist for assessing compliance with the requirements.

B.2 General

B.2.1 Laboratory quality systems

B.2.1.1 For the data obtained from performing the BET to be reliable and reproducible, it is important that the tests be performed under controlled conditions. The laboratory facilities used for the tests, whether on the site of the medical device manufacturer or at a remote location, should be managed and operated in accordance with a documented, quality system and according to approved laboratory methodology.

B.2.1.2 If a BET is performed in a laboratory under the direct management of the medical device manufacturer, the operation of the laboratory should be within the manufacturer's quality system. If an external laboratory is used, it is recommended that such a laboratory be formally certified to an appropriate International Standard (e.g., ISO/IEC 17025).

B.2.1.3 Any laboratory should be committed to providing a quality service, and this commitment should be documented as a quality policy. The lines of authority and responsibility within the laboratory organization should be formally established and documented. An individual who is knowledgeable of quality systems should be responsible for the establishment of the laboratory quality system and have sufficient authority to ensure that the system is implemented.

B.2.1.4 The operation of the laboratory should be subject to regular internal audits. The results of the audit should be documented and reviewed by the laboratory management.

B.2.1.5 Further information on quality management is available in ISO 9001. ISO/IEC 17025 outlines requirements for laboratory quality systems. Particular requirements for quality systems for the manufacture of medical devices are available in ISO 13485 and ISO 13488.

B.2.2 Personnel/training

Although the BET need not be performed in a cleanroom or a HEPA-filtered environment (unless there are concerns about protecting the analyst from the sample, as when testing human blood products), it is important that analysts understand and follow basic aseptic technique to prevent contamination of the sample. Documented training of all analysts in those tasks for which they have responsibility is essential.

B.2.3 Equipment

B.2.3.1 It is expected that all equipment for measuring/maintaining the temperature for the BET be properly calibrated, qualified, and documented for performance. Refrigerators and freezers used to store reagents should be qualified and on a regular maintenance schedule.

B.2.3.2 Time and temperature requirements for ovens used to depyrogenate glassware used in the performance of the BET should be validated according to conventional methods.

B.3 Selection of product units

B.3.1 Sample size

According to the FDA 1987 guidelines for end-product testing of medical devices, the recommended number of samples depends on the size of the batch under test as follows.

Table B.1—Selection of number of samples

Lot size	# of samples
<30	2
30–100	3
≥101	3 % of lot, up to a maximum of 10

USP 24, Section 161, *Transfusion and Infusion Assemblies & Similar Medical Devices*, states “select not less than 3 and not more than 10 devices.”

B.3.2 Product families/sampling groups

Product families for the BET may be established based on an evaluation of products, processes, components, and materials. For routine testing for product families, the selection of a single product from within a product family may be acceptable. The designation of product families for endotoxin testing may not necessarily fit the criteria of product families as identified for sterilization equivalence or bioburden families. Endotoxin-related families may or may not fit into these other family classifications. However, each manufacturer should evaluate, analyze, and document the appropriate designation based on product components, manufacturing processes, and intended usage.

Factors for the sampling group that might be considered with supporting data in selecting samples for endotoxin testing include:

— raw materials or component lots,

— production quantities from a single shift or defined time period (e.g., 24 hours),

— product produced on specific equipment,

— product family groupings,

— sterilization loads,

— other logical divisions.

For the qualification/validation of a BET (see 8.4), a manufacturer may logically divide its devices into groups of products according to common components (chemical formulations), and may then validate representative product from each such group. The product chosen from each group should ideally be:

— the one with the greatest surface area that contacts the patient directly or indirectly (e.g., fluid path), thus contributing to the largest source of extractables; and

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

B.3.3 Sets

B.3.3.1 A set is defined as a collection of components presented in a primary package that is assembled at the point of use to form the healthcare product. In such cases, the specified EU limit is for the assembled device and not for each subcomponent (see Table B.2). When testing the subcomponents of a set, combining the extracts may be utilized as long as the necessary ratio of extraction fluid per assembled device is maintained. For example, a device may have three subcomponents. The total volume of extraction fluid used for the subcomponents must not exceed the MVD (e.g., 40 mL). This may result in using the same extraction fluid for all three subcomponents, or using a portion of the fluid for each of the three subcomponents and then combining, or using a combination of these two approaches. The same principle of maintaining the ratio of extraction fluid to device applies when multiple sample units of devices are being tested.

B.3.3.2 In the case of a non-conforming result from a combined sample, additional individual testing would be required to investigate the source of contamination among the subcomponents.

B.3.4 Kits

A kit is defined as a collection of individual health care products in its primary package or a variety of procedure-related health care products. Each individual type of device may have its own product endotoxin limits and should be tested and evaluated on an individual basis (see Table B.2).

Table B.2—Selection of product units for testing

Product unit	Item for testing	Basis for non-pyrogenic claim	Rationale
a) Individual medical device in primary package	Individual medical device	Individual medical device	Each medical device is used independently in clinical practice
b) Set of components in primary package	Combination of components	Combination of components	Components are assembled as a product and used together in clinical practice
c) Number of identical medical devices in primary package	Single medical device taken from the primary package	Single medical device taken from the primary package	Each medical device is used independently in clinical practice
d) Kit of procedure-related medical devices	Each type of medical device that has a non-pyrogenic claim *OR* All items together*	Each type of medical device based on its non-pyrogenic claim *OR* All items collectively*	Each type of medical device is used independently in clinical practice and may have different product endotoxin limits

* For a kit of procedure-related medical devices, two options are allowed for flexibility. The first option typically will be used by companies that prepare a large number of kits (e.g., re-packer) that are assembled from a multitude of products, where the individual medical devices may be purchased with the vendor providing certification of non-pyrogenicity to the re-packer, or the re-packer may provide certification of non-pyrogenicity by subsequent testing. The second option for manufacturers that have a small number of components within a kit and/or produce a small number of kits, is that all items in the kit may be tested together to provide a single non-pyrogenic claim.

B.3.5 Sample frequency

To determine the appropriate sampling frequency, each manufacturer should assess the potential for pyrogenicity of each device. Critical factors for evaluating the potential for pyrogenicity may include, but are not limited to, some of the following issues:

a) "Wet" manufacturing steps, in which water or other aqueous material is utilized as part of the manufacturing process (e.g., rinsing, washing, etc.); and

b) 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.

NOTE 1—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.

NOTE 2—Dry health care products that contain wet ingredients (e.g., nebulizer bottles, blood collection bags containing anti-coagulants) or pharmaceuticals as part of the device should be tested and evaluated according to pharmaceutical/drug sampling requirements.

B.4 Selection of BET methodology

B.4.1 Gel-clot techniques

The gel clot methods are the simplest of the BET methods, both in terms of technical expertise required to perform a valid assay and data interpretation/analysis. Investment in 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 a validated concentration and LAL reagent are mixed in a 10 x 75 mm glass test tube. After incubation, individual test 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.

B.4.2 Chromogenic and turbidimetric techniques—Endpoint methods

The endpoint methods for the chromogenic and turbidimetric techniques are based on the linear relationship between endotoxin concentration and formation of a color (chromogenic) or turbidity measured based on optical density 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 LRW 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 to 0.1 EU/mL or 0.1 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 currently defined by the FDA as a correlation coefficient of ≥0.980 (FDA, 1987). The endotoxin level in an unknown is calculated by measuring the optical density of the sample and interpolating the endotoxin concentration from the standard curve. Endpoint methods are generally performed in microtiter 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 more dependent on good analyst technique than the gel-clot test. A knowledge of statistics is helpful when analyzing and interpreting data.

B.4.3 Chromogenic and turbidimetric 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 (kinetic turbidimetric) or color intensity (kinetic chromogenic), 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 endpoint method. Unless approved alternate regression analyses are utilized (currently authorized by the FDA only on an individual reagent manufacturer basis), the resulting standard curve is constructed using linear regression analysis across the observed points. A correlation coefficient of |r| ≥0.980 is the minimum linearity requirement for a kinetic method. As with the endpoint 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 may be performed in microtiter plates, glass tubes, or other validated technology. Either method requires qualified equipment 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 data base software package in conjunction with these methods greatly aids in the instant analysis and longitudinal trending of data. As with the endpoint method, the demonstration of good, consistent laboratory technique on the part of the analyst is important, and a knowledge of basic statistics is helpful when analyzing and interpreting data.

B.5 BET parameters

B.5.1 Time

B.5.1.1 The gel-clot methods are typically incubated for 60 ± 2 minutes.

B.5.1.2 The chromogenic and turbidimetric kinetic methods measure the time it takes for a series of standards and unknowns to reach a pre-determined optical density or color intensity. It is expected that timing devices be appropriately calibrated and qualified.

B.5.2 Temperature

All BET methodologies are typically carried out at 37 ± 1 °C.

B.5.3 pH

B.5.3.1 While all lysates 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 measurement should be taken using an appropriate pH test system on a mixture of equal parts lysate reagent and test solution.

B.5.3.2 Dilution in LRW is the easiest and most appropriate approach to neutralize any interfering factor, and in most cases will result in a test specimen with no interference. Dilution, however, may not exceed the calculated MVD. If dilution in LRW does not solve pH problems, one alternative is to use a buffer (e.g., tris) in the first dilution.

B.5.4 Materials and supplies

Microtiter plates used in the performance of quantitative assays are not manufactured solely for the purpose of endotoxin testing, and they could contain random endotoxin contamination in the wells (“hot wells”). Therefore, sufficient sampling should be performed to demonstrate that the plates are appropriate for use.

B.5.5 Storage

Concentrated CSE preparations provided by lysate manufacturers may be stored according to the product insert. However, storing endotoxin dilutions prepared in LRW will require a validation describing the length of time and temperature of storage, the container used for storage, the particular endotoxin dilutions to be stored, and the volumes of the dilutions to be stored.

Annex C
(informative)

Guidance on alternatives to batch testing

C.1 General

Alternatives to batch testing require data showing that the manufacturing process is capable of consistently meeting specified limits. Such data might include testing of a specified number of batches or testing over a specified period of time.

Cases where specific regulations/compendia might require batch testing are:

a) products to be used for infusion/transfusion,

b) products to be used for wound or tissue irrigation,

c) products that contain ingredients/components of biological origin, and

d) products that contain water other than water for injection or inhalation.

C.2 Criteria for alternatives to batch testing

Alternatives to batch testing may include one or more of the following:

a) reduced number of samples (e.g., from 10 samples per batch to three samples per batch),

b) reduced frequency of sampling (e.g., from every batch to every n^{th} batch OR from every batch to one batch per shift or per day),

c) specified combinations of products based upon product grouping (see B.3.2),

d) testing of raw materials (e.g., testing critical components or rinse water), and

e) other logical alternatives.

C.3 Manufacturing operation qualification

C.3.1 Manufacturing operation design

The operation should be designed to minimize the level of endotoxin on the product. Considerations in the operation design should include such things as:

a) selection of appropriate materials and suppliers,

b) minimizing and controlling materials and components that may 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, and

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

C.3.2 Manufacturing operation validation

C.3.2.1 Prior to qualification/validation activity, a process risk assessment of the manufacturing operation should be conducted to identify key process steps or control points (e.g., failure mode effects 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 may include but are not limited to:

a) raw materials,

b) extrusion operations,

c) aqueous washing,

d) drying or curing processes,

e) in process aqueous leaching or soaking,

f) product/component handling,

g) manual versus automated assembly, and

h) product or material storage.

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

C.3.2.2 For validation, 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 may 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.

C.3.2.3 Validation of a manufacturing operation for the control of endotoxin should address the following principles:

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

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

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

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

e) Maintain the qualification/validation through periodic monitoring of the manufacturing operation, including periodic testing of product, to ensure that it continues to produce non-pyrogenic product.

f) Document the requalification criteria and frequency.

C.3.3 Manufacturing operation control

Manufacturing operation control for endotoxins may include:

a) supplier quality assurance and/or endotoxin testing of incoming materials, components, or subassemblies;

b) monitoring and control of process water or other aqueous processing solutions;

c) monitoring product in process at specified control points;

d) periodic maintenance and cleaning of equipment, especially those used to convey or contain aqueous product contacting processing materials;

e) antimicrobial control of the environment and processing materials.

Annex D
(informative)

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