

American National Standard



ANSI/AAMI ST72:2019

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

Erratum

American National Standard
Bacterial endotoxin -Test methods, routine monitoring and alternatives to batch testing

TECHNICAL CORRIGENDUM 1

(ANSI/AAMI/ISO ST72:2019)

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Page 7, Equation 2 and Equation 3

On each equation replace the denominator, “1”, with a lambda, “ λ ”.

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

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Abstract: Specifies general criteria to be applied in the determination of bacterial endotoxins on or in medical devices, components, or raw materials employing bacterial endotoxins test (BET) methods using amebocyte lysate reagents *from Limulus polyphemus* or *Tachypleus tridentatus*. The document is not applicable to the evaluation of pyrogens other than bacterial endotoxins.

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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Committee representation

Association for the Advancement of Medical Instrumentation

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

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). The predominant pyrogenic contaminants 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* will be covered in this document. Other endotoxin detection methodologies, such as monocyte activation and recombinant Factor C (rFc), are not included (see A.12).

Endotoxin is the high molecular weight lipopolysaccharide (LPS) component of the outer cell wall of Gram-negative bacteria, which can cause 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. Because it is ubiquitous in nature, stable, and small enough to pass through conventional sterilizing filters, endotoxin contamination is difficult to prevent.

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

- a) manufacturing techniques that prevent or control endotoxin contamination,
- b) depyrogenation by endotoxin inactivation (e.g., dry heat) or physical removal (e.g., rinsing, distillation, ultrafiltration).

The purpose of this document is to consolidate the requirements and guidance for testing for bacterial endotoxins. This includes product required to be non-pyrogenic due to intended use and non-pyrogenic labelling. Details are 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:

- the background/history of endotoxin testing (Annex A),
- guidance on endotoxin test methods, routine monitoring, and alternatives to batch testing (Annex B),
- guidance on out of specification test results and investigation (Annex C),
- guidance on in-process monitoring of manufacturing processes and component testing (Annex D), and
- guidance on conducting a risk assessment to support alternatives to batch testing (Annex E).

The annexes to this document are for information only.

Bacterial endotoxins—Test methods, 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 employing bacterial endotoxins test (BET) methods using amebocyte lysate reagents from *Limulus polyphemus* or *Tachypleus tridentatus*.

NOTE Although the scope of this standard is limited to medical devices, it also includes requirements and provides testing guidance that might be applicable to other health care products, such as, biologics, tissue-based products and combination products.

1.2 This document is not applicable to the evaluation of pyrogens other than bacterial endotoxins.

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.

The United States Pharmacopoeia (USP) <85>, current edition, United States Pharmacopeial Convention (USP), Rockville MD.

The United States Pharmacopoeia (USP) <161>, current edition, United States Pharmacopeial Convention (USP), Rockville MD.

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

3 Definitions

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

3.1 bacterial endotoxins test (BET): Assay for measuring bacterial endotoxins by combining a liquid test sample or test sample extract with *Tachypleus* or *Limulus* amebocyte lysate (TAL/LAL) reagent and measuring the resulting proportional reaction via visual, turbidimetric, or chromogenic techniques.

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

[Source: ISO 11139:2018]

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

3.4 control standard endotoxin (CSE): Endotoxin standard preparation whose potency has been standardized against the Reference Standard Endotoxin (RSE) for a specific batch of LAL.

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

3.6 direct contact: Term used for a device or device component that comes into physical contact with body tissue

3.7 end product testing: Testing carried out on product samples that have completed the entire manufacturing process.

3.8 endotoxin or bacterial endotoxin: High molecular weight complex associated with the cell wall of Gram-negative bacteria that is pyrogenic in humans and specifically interacts with an endotoxin detection system.

3.9 endotoxin limit: Maximum allowable level of endotoxin specified for a product.

3.10 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).

3.11 endpoint (gel clot): The last point in a series of dilutions or concentrations of a test or control solution for which a positive reaction for bacterial endotoxin is observed.

3.12 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.

3.13 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.

3.14 geometric mean endpoint: The antilog of the average of the logarithmic values with respect to the endpoints 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 implantable medical device: A medical device inserted internally to replace a missing biological structure, support a damaged biological structure, or enhance an existing biological structure. It can be permanent or temporary.

3.16 indirect contact: A term used to describe the body contact for a device or 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 device or device component itself does not physically contact body tissue).

3.17 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.

3.18 inhibition/enhancement test: Test used to determine whether a particular sample contains factors that diminish its accuracy by introducing enhancement or inhibition into the test system. Also referred to as method suitability.

3.19 investigation test: Testing carried out to verify the validity of an original result that might include reanalysis of a sample extract/preparation or analysis of new samples

3.20 Interference. An interfering factor observed in the performance of the test that exceeds the acceptable threshold for a given BET technique (e.g. positive product control that indicates a known endotoxin recovery less than 50% or greater than 200% or ± 2 lambda)

3.21 intraocular: located or occurring within or administered through the eye

3.22 interfering factors: Non-endotoxin related factor, usually attributable to a characteristic of the test sample, that causes inhibition or enhancement.

3.23 intravascular: Used for a device within blood vessels or a blood vessel

[Source: The American Heritage Medical Dictionary, 2007]

3.24 intralymphatic: Within or introduced into a lymphatic vessel

[Source: Merriam-Webster Medical Dictionary, 2012]

3.25 intrathecal: Introduced into or occurring in the space under the arachnoid membrane of the brain or spinal cord

[Source: Merriam-Webster Medical Dictionary, 2012]

3.26 LAL reactive material (LAL-RM): Any non-endotoxin compound that will activate the LAL clotting cascade and cause enhancement.

3.27 LAL reagent water (LRW): Purified water employable as a solvent, diluent, and/or extractant in a BET that must be non-reactive and does not elicit interference in the methodology in use.

3.28 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.

3.29 *Limulus* amoebocyte lysate (LAL): Reagent extracted from amoebocytes taken from hemolymph of the horseshoe crab, *Limulus polyphemus*, which reacts with endotoxin, to form a gelatinous clot and is used to estimate endotoxin levels in BET methods.

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

3.31 maximum valid dilution (MVD): Maximum amount a sample can be diluted or the total extraction volume used relative to the sensitivity of a BET in which the specified endotoxin limit can be detected.

3.32 non-pyrogenic: Does not induce a fever. Term used to describe an item or product that contains endotoxin levels less than or equal to specified limits (see 5, 6).

3.33 out of specification (OOS): A sample with a valid BET result that exceeds a product endotoxin limit specification.

NOTE The term OOS applies only within the context of this document and does not imply compliance with any other regulatory guidance dealing with OOS results, such as the FDA's *Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production*.

3.34 product realization: All processes consistent with the quality system requirements that are needed to develop, manufacture, and deliver the finished product.

3.35 pyrogen: Any substance that induces a fever.

3.36 pyrogenic: Induces a fever. Term used to describe an item or product that contains endotoxin levels above specified limits.

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

3.38 retest: Test of additional product samples from a previously tested batch.

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

3.40 method suitability: see inhibition / enhancement test

3.41 *Tachypleus* amoebocyte lysate (TAL): Reagent extracted from amoebocytes 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 BET methods.

3.42 test endotoxin limit: Maximum endotoxin concentration allowable in a sample extract. The determined limit is used to calculate the Maximum Valid Dilution (MVD) of a sample extraction (pooled or single unit).

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

3.43 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.

3.44 validation: Confirmation process, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled.

[Source: ISO 11139:2018]

4 Quality

4.1 Documentation

4.1.1 Procedures for bacterial endotoxins tests shall be specified.

4.1.2 Documents and records required by this document shall be reviewed and approved by designated personnel (see 4.2.1). Documents and records shall be controlled in accordance with quality system requirements.

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

4.1.4 Calculations and data transfers shall be subject to appropriate checks. If calculations are performed by electronic data processing techniques, the application of the software shall be validated for the intended uses, and records of this validation shall be retained.

4.2 Management responsibility

4.2.1 The responsibility and authority for implementing and performing the procedures described in this AAMI Standard shall be specified. Responsibility shall be assigned to competent personnel in accordance with quality system requirements.

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

4.3 Product realization

4.3.1 Procedures for purchasing shall be specified. These procedures shall comply with quality system requirements.

4.4 Personnel

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

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

4.4.3 Analyst qualification shall be conducted prior to performance of a BET (see 9.3.2). Continued competency shall be demonstrated.

4.5 Equipment

4.5.1 All equipment required to carry out the specified test shall be available, and planned maintenance and calibration shall be performed in accordance with documented procedures. Records of maintenance and calibration shall be retained.

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

4.6 Reagents and materials

4.6.1 Methods shall be specified for the preparation of materials used in the bacterial endotoxins tests, including appropriate quality tests (see section 9.3).

4.6.2 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.

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

NOTE Appropriate guidelines for depyrogenation of materials are referenced in USP [36], [37], ISO 20857, Weary [40].

4.6.4 Materials used for sampling, storing and diluting should be shown to not interfere with the validity of the test method. This may be accomplished, for example, by testing a sample of purchased materials to demonstrate that they are non-pyrogenic, by accepting an appropriate vendor certificate, or through test method suitability.

4.7 Measurement, analysis and improvement

Procedures shall specify requirements for handling unusual, unexpected or out of specification results including corrective and preventive action. These procedures shall comply with quality system requirements, such as ISO 13485 or ISO/IEC 17025. Additional guidance is provided in Annex C which addresses out of specification and failure investigations.

5 Determination of Product required to be non-pyrogenic due to intended use

5.1 Sterile implantable medical devices that contact non-intact tissue during use or medical devices that have direct or indirect intravascular, intralymphatic, intrathecal, and/or intraocular contact shall have an evaluation performed for bacterial endotoxins.

5.1.1 Some implantable medical devices are excluded and not required to be evaluated for bacterial endotoxins as the device only makes contact with intact tissue during use. Examples may include implantable medical devices such as, ureteral and biliary stents, gastric balloons, hearing aids, breathing strips, Foley catheters, etc.

5.2 All parts of products required 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 cord, etc.).

NOTE There are products that have portions of the device that are hermetically sealed and as such do not come into contact with the patient. Examples may include the interior portions of balloon catheters or cardiac pacemaker implants. Such portions of the product are not required or intended to be non-pyrogenic, and endotoxin testing is not required for such locations.

5.3 For multi-component kit products, only those components that have contact as outlined in 5.1 are required to have an evaluation performed for bacterial endotoxins.

5.4 Bacterial endotoxin limits shall be determined according to appropriate regulatory requirements and be consistent with intended clinical application.

NOTE 1 For endotoxin limits see current USP <161>, FDA guidance for devices labeled as sterile [51], FDA Q&A guidance [43], etc.

NOTE 2 For ophthalmic products, consideration should be given to the overall risk to patient endotoxin exposure when multiple healthcare products from the same manufacturer will be used together during the same surgical procedure or placement in the same surgical site[49].

NOTE 3 For subcutaneous implantable medical devices without systemic exposure higher endotoxin limits may be justifiable, with additional supporting data depending on the risk/benefit of the device. (See Cartmell, T., etc. al. [56] where the doses needed to elicit a subcutaneous response was 10 times that of the standard IV dose to produce a similar peak rise in temperature.)

6 Product with non-pyrogenic label claim

6.1 Products labelled as non-pyrogenic must be evaluated and meet the same applicable regulatory requirements for bacterial endotoxin content as products required to be non-pyrogenic due to intended use (see clause 5).

NOTE 1 Products are not 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 2 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 of 'non-pyrogenic fluid path' shall be supported by appropriate evaluation of components and surfaces relevant to the fluid path in use.

NOTE 3 The claim 'non-pyrogenic' refers to bacterial endotoxins derived from Gram-negative bacteria and is typically supported by performing 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, with a potency several orders of magnitude less than endotoxin. (Vanhaedeke [44]; The Lancet [45]; Pharmaceutical Technology (1987) [46]). Such levels are easily notable by an increase in bioburden counts.

NOTE 4 For products labelled 'non-pyrogenic,' it might also be necessary to evaluate the product for material-mediated pyrogenicity to fulfill regulatory requirements. This is in addition to any requirements for evaluating the product for bacterial endotoxins. See ISO 10993-11 (Annex G) for clarification related to material-mediated pyrogenicity (MMP) associated with biological evaluation. The material-mediated pyrogen 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 material-mediated pyrogenicity. It is not intended that it be referenced or used as part of a BET plan. For example, it is not appropriate to reference the ISO 10993-1 [52], ISO 10993-11 [53], or ISO 10993-12 [54] for determination of BET extraction parameters, extraction volume, or sampling plans.

6.2 Application of any non-pyrogenic product label statement or claim 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,
- Alternatives to batch testing (see B.11).

6.3 When non-specific non-pyrogenic labeling is used for a multi-component kit, all items in the kit are considered non-pyrogenic. If the non-pyrogenic label only applies to certain components this shall be identified on the label and/or other associated product literature.

7 Selection of product units

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

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

7.2 The selection of product units for testing shall be based on criteria defined in a sampling plan. This rationale may be based on:

- a) applicable regulatory requirements,
- b) published statistical considerations,
- c) assessment of risk,
- d) manufacturing process validation, or
- e) historical performance.

7.3 The sampling group is generally defined as the production batch. In most cases, each batch of product must be tested using an appropriate number of samples, not more than (NMT) 10, taken randomly to represent the quality of the batch. If there is data to support a different selection basis, or alternatives to batch testing, sample selection may be based on a sampling group other than the production batch. If alternatives to batch testing are used, see Clause 11, and B.7 through B.9 and B.11 for guidance.

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 (see Annex B and Annex E).

7.4 For end-product testing, samples selected for testing should be in the finished form. This includes all factors that might affect or contribute to the levels of endotoxin (see B.7.4 for guidance).

NOTE Samples utilized for endotoxin testing may be selected from routine production, products that have been rejected for other production quality issues, or surrogate samples representative of the full manufacturing process and representative endotoxin levels of product.

7.5 For end-product testing, samples may be obtained prior to (pre-) sterilization or after (post-) sterilization. Post-sterilization samples encompass all of the factors that could 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 the finished product shall be justified and documented. The program for ongoing testing should consistently reflect either pre- or post-sterilization samples. Guidance is provided in B.7.5 for assessing the acceptability of pre-sterilization testing.

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

7.6 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 must address sample preparation in adherence to method requirements and the applicable endotoxin limit. Additional guidance can be found in B.7.6.

8 Selection of technique

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

- a) gel-clot technique: limit test and assay methods,
- b) chromogenic technique: kinetic and endpoint methods, and
- c) turbidimetric technique: kinetic method.

Information on each of these methods is presented in Annex B.

8.2 The selected method shall be determined to be suitable as specified in Clause 9. Continued suitability shall be confirmed as specified in Clause 9.6.

9 Method suitability

9.1 Endotoxin Limit

9.1.1 The endotoxin limit defines the maximum allowable concentration of endotoxin that can be present on the product or in a product extract solution. Refer to Annex B for guidance on establishing endotoxin limits.

9.1.2 The endotoxin limit for the extract solution in EU/mL is calculated as:

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

where:

K = Amount of endotoxin allowed per device (endotoxin limit)

N = Number of devices tested

V = Total volume of the extract or rinse (mL) that can be adjusted for the size and configuration of the device

NOTE Endotoxin limits for devices can also be reported in terms of EU/mL, provided an appropriate specified volume for rinsing/immersing has been determined to not exceed the MVD.

9.2 Maximum valid dilution (MVD)

9.2.1 Products can sometimes interfere with a BET resulting in inhibition or enhancement due to the presence of interfering factors. A common technique used to mitigate such interference is to dilute the product extract in LAL reagent water (LRW) or other appropriate diluent, or increase the total extraction volume. 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 and is calculated as follows:

$$MVD \text{ (in terms of extract solution)} = \frac{\text{Endotoxin Limit of extract solution (EU/mL)}}{1} \quad \text{(equation 2)}$$

or

$$MVD \text{ (in terms of maximum extraction volume)} = \frac{\text{Endotoxin Limit (EU/Device)}}{1} \quad \text{(equation 3)}$$

where:

The endotoxin limit for the extract solution in EU per mL is calculated (see equation 1) or the endotoxin limit in EU per device is defined.

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

The value of the MVD indicates the greatest dilution or total extraction volume that can be used to overcome inhibition/enhancement, based on the sensitivity of the LAL. For example, an MVD (in terms of extract solution) 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 Annex B for Table B.5 for explanation of calculations).

The dilution used during method suitability should be less than the MVD, if possible.

9.2.2 If a product is tested at the MVD the endotoxin test should be considered a qualitative test, which indicates a pass or fail. Any endotoxin detected in the assay (a positive test result) indicates that an endotoxin level is at or exceeds the defined endotoxin limit, which should be interpreted as a failing test. A negative result indicates a passing test.

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

9.3 Reagent and analyst qualification

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

9.3.1.1 For the gel-clot technique

The label claim sensitivity (λ) of each lot of LAL reagent must be verified by testing. The test must be performed in quadruplicate, with a series of two-fold endotoxin dilutions that bracket λ (e.g., dilutions of 2λ , λ , 0.5λ , 0.25λ) and a

negative control. The geometric mean endpoint of the series must confirm $\lambda \pm$ one two-fold dilution. Once confirmed, the label claim sensitivity is used in all calculations. The geometric mean of the label claim sensitivity is calculated as follows:

$$\text{Geometric mean} = \text{antilog} (\Sigma e/F) \quad (\text{equation 4})$$

where:

Σe = Sum of the log endpoint of each series

F = Number of replicates

9.3.1.2 For photometric quantitative techniques (chromogenic and turbidimetric)

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 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. Linearity requires that the absolute value of the correlation coefficient $|r|$ be ≥ 0.980 for the range of endotoxin concentrations indicated by the LAL reagent manufacturer.

If a standard curve is less than two logs, it is suggested that 2-fold dilutions be performed for the standard curve. If the standard curve 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.

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

9.3.2 Analyst qualification

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

9.4 Product and test method suitability

9.4.1 General

The BET method must be determined to be suitable with each product or product group (see B.7.3) 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. For information on sample interference, see 9.5.

The number of batches of product chosen for the suitability study shall reflect the level of control of the process and must be justified. A minimum of one batch per product or product family shall be used to demonstrate test method suitability. For photometric techniques, a valid positive product control (PPC) fulfills the requirement for test method suitability (see Table 2).

NOTE 1 Development of a suitable test method might be required for products demonstrating inhibition/enhancement due to interfering factors in the sample extract. Additional information can be found in 9.5 and B.9.5.

NOTE 2 A recovery efficiency study is not required. (see A.9 for discussion).

9.4.2 Gel-clot technique

9.4.2.1 Prepare solutions as listed in Table 1. The sample solution must be at a dilution less than or equal to the MVD and must not contain any detectable endotoxin in the test system used. Test each endotoxin-spiked dilution series and negative control. The geometric mean endpoint concentrations of each endotoxin-spiked dilution series can be determined using the equation listed in the preparatory testing 9.3.1 (equation 4).

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

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

9.4.2.2 The sample does not contain interfering factors if:

- the LAL sensitivity in the product positive control series is within 0.5 λ and 2 λ ;
- negative controls show no reaction; and
- the result of the standard control series conforms with the labeled sensitivity of the LAL.

9.4.3 Photometric quantitative techniques (chromogenic and turbidimetric)

9.4.3.1 Select an endotoxin concentration near the middle of the endotoxin standard curve. Prepare solutions as listed in Table 2. The sample solution must be at a dilution less than or equal to the MVD.

Table 2—Preparation of solutions for method suitability test: chromogenic and turbidimetric techniques

Solution	Diluent	Endotoxin spike	Minimum number of replicates
Product positive control (PPC)	Sample solution	Middle concentration of the standard curve	2
Sample solution	Sample solution	None	2
Standard control series ^{a)}	LRW	Minimum of three different concentrations	2 per concentration
Negative control	LRW	None	2
^{a)} Can be achieved with an archived standard curve.			

9.4.3.2 Calculate the recovery of the added endotoxin (PPC) by subtracting the mean endotoxin concentration in the sample solution from the mean endotoxin concentration in the product positive control, and dividing by the known endotoxin concentration.

9.4.3.3 The sample or sample dilution is considered to be valid if the measured concentration in the PPC is within 50 % to 200 % of the known added endotoxin concentration (after subtraction of any endotoxin detected in the sample solution).

9.5 Sample interference

If interference is observed in any BET, the sample extracts can be diluted (not to exceed the MVD) and/or treated to overcome inhibition or enhancement. Treatments to sample extracts, such as filtration, neutralization, dialysis or heat treatment shall be demonstrated to be suitable without loss of endotoxins.

9.6 Demonstration of continued suitability

9.6.1 The demonstration of continued suitability is intended to detect changes in interfering factors that cause inhibition and enhancement that could result in invalid test results. The same BET technique and extraction methods documented in the successful suitability study shall be used for routine testing.

9.6.1.1 A suitability study (inhibition/enhancement test) shall be performed for the following:

- a) a change in BET test technique (e.g., chromogenic to gel-clot), changes to the extraction method, including a change that involves alternate methods or parameters that are outside of the defined extraction parameters (e.g. adding sonication, using alternate temperatures, use of solvents);

9.6.1.2 Re-assessment and a suitability study (if determined to be necessary) shall be re-executed for the following:

- a) a product change that can impact the test, including introduction of new materials, processing steps, or product configuration, or new product manufacturing site;
- b) a change in BET testing laboratories,
- c) a change in BET reagent source,
- d) a change in materials/equipment that could affect the test, or
- e) any other change that could adversely affect the accuracy of the BET result.

10 Use of technique

10.1 Critical test parameters

10.1.1 Temperature

Incubation for BET methodology is typically carried out at 37 ± 1 °C. Refer to the lysate manufacturer's product instructions for appropriate parameters.

10.1.2 Time

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

10.1.3 pH

The optimum pH range for the BET reaction will be specified by the manufacturer of the LAL reagent in use. Running the test outside of the optimum pH ranges can result in interference. When positive product controls are acceptable during suitability testing, subsequent pH measurements may not be required. However, if adjustments in pH must be made during suitability testing, then the pH must be addressed during subsequent routine testing.

10.2 Equipment and reagents

10.2.1 Due to the limited temperature range required for the BET, 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, 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 as certified to be non-pyrogenic, e.g., multiwell plates, etc. shall be carefully evaluated (see 4.6.4) prior to use to ensure that they will not interfere with the assay.

10.2.2 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 LAL reagent manufacturer who should provide the testing lab with a Certificate of Analysis or other documentation describing the standardization.

10.2.3 Storage requirements for freeze-dried and reconstituted reagents are described in individual LAL reagent or endotoxin standard manufacturer's product inserts. If storage conditions utilized in the lab are different from those recommended by the manufacturer, the conditions of alternate storage shall be validated.