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Standard Guide for Determination of Endotoxin on Sterile Medical Gloves[1]

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INTRODUCTION

This guide is established and designed to determine the qualitative or quantitative presence of bacterial endotoxin on sterile medical gloves. Bacterial endotoxins are found in the outer membrane of Gram negative bacteria and may contaminate gloves during the manufacturing process. Consequences of endotoxin introduced into a patient during invasive procedures are dose dependent and may include inflammation, fever, nausea, pain, clot formation, hypoglycemia and reduced profusion of the heart, kidney, and liver as well as endotoxic shock. Endotoxins are not inactivated by routine methods utilized in the routine sterilization of medical gloves including irradiation (gamma or E-beam), ethylene oxide, or steam.

1. Scope

1.1 This guide covers a selection of methodologies for the determination of bacterial endotoxin on gloves when such a determination is appropriate.

1.2 As bacteria may continue to grow on non-sterile gloves, reportable endotoxin levels are only appropriate for gloves labeled as sterile. Because most environments contain endotoxin, once a box of gloves is opened and the gloves are manipulated, endotoxin levels will increase making it inappropriate to report endotoxin levels on boxed gloves (ex. examination gloves). This is true even if the box had undergone sterilization prior to distribution.

1.3 This guide may also be appropriate for internal quality control or alert purposes at different stages of manufacturing or during process change evaluations.

1.4 This guide is not applicable to the determination of pyrogens other than bacterial endotoxins.

1.5 The sample preparation method described must be used regardless of the test method selected. This method does not describe laboratory test method validation, analyst qualification, or reagent confirmation. Product-specific validation is addressed.

1.6 The safe and proper use of medical gloves is beyond the scope of this guide.

1.7 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory requirements prior to use.*

1.8 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *EN Standard:*[2]

EN 455-3:2015 Medical Gloves for Single Use—Part 3: Requirements and Testing for Biological Evaluation

2.2 *ANSI Standard:*[2]

ANSI/AAMI ST 72:2011 Bacterial Endotoxins—Test Methodologies, Routine Monitoring and Alternatives to Batch Testing

3. Terminology

3.1 *Definitions:*

3.1.1 *bacterial endotoxin test (BET)*—a method for determining the qualitative or quantitative presence of endotoxin in an aqueous test sample utilizing *Limulus* amebocyte lysate (*LAL*) reagent and measuring the resulting proportional reaction.

[1] This guide is under the jurisdiction of ASTM Committee D11 on Rubber and Rubber-like Materials and is the direct responsibility of Subcommittee D11.40 on Consumer Rubber Products.

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[2] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036.

3.1.2 *batch*—defined quantity of intermediate or finished product produced in a defined cycle of manufacture that is said to be of uniform quality.

3.1.3 *chromogenic (colorimetric) 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.

3.1.4 *control standard endotoxin (CSE)*—purified endotoxin product supplied at a known potency and utilized as a standard control in endotoxin testing.

3.1.5 *device*—with regard to medical gloves, a device is defined as a pair of gloves when they are packaged in pairs and a single glove when packaged singly.

3.1.6 *endotoxin*—high molecular weight, heat stable complex associated with the cell wall of gram-negative bacteria that is pyrogenic in humans and specifically interacts with *LAL*.

3.1.7 *endotoxin unit (EU)*—the standard unit of measure for endotoxin activity initially established relative to the activity in 0.2 ng of the U.S. Reference Standard Endotoxin (USP standard reference material).

3.1.7.1 *Discussion*—The FDA's endotoxin standard and that of the World Health Organization's International Endotoxin Standard (*IU*) are sub lots of the same endotoxin preparation, making *EU* and *IU* equal.

3.1.8 *endpoint (gel clot)*—last positive (coagulated or gel clot) tube in a series of dilutions.

3.1.9 *enhancement*—a type of interference that renders test results with higher values than the amount of endotoxin present.

3.1.10 *gel-clot technique—BET* methodology that can be used to detect or quantify the presence of endotoxin based on clotting of the lysate reagent (gel formation) in the presence of endotoxin.

3.1.11 *inhibition—BET* anomaly wherein a non-endotoxin substance, usually contributed by the sample, elicits a test reaction less than the amount of endotoxin actually present.

3.1.12 *inhibition/enhancement (suitability) test*—test used to determine whether a particular *BET* sample contains factors that diminish its accuracy of the *BET* either by enhancement or inhibition of the results.

3.1.13 *interfering substances*—those substances that cause inhibition or enhancement.

3.1.14 *Limulus amebocyte lysate (LAL)*—the reagent extracted from amebocytes in the circulatory system of the horseshoe crab *Limulus polyphemus* or *Tachypleus tridentatus* (*TAL*), which forms a clot when brought into contact with substances containing endotoxin.

3.1.15 *lot*—see *batch*.

3.1.16 *lipopolysaccharide (LPS)*—the gram-negative cell wall component typically composed of lipid A, a core polysaccharide, and an O-side chain sugar moiety.

3.1.17 *LAL reagent water (LRW)—LAL* reagent water that has been validated to contain no detectable endotoxin, typically available from the lysate manufacturer for automated test systems.

3.1.18 *maximum valid dilution (MVD)*—the highest dilution a sample is permitted to attain in diluting out interfering substances while still being capable of detecting endotoxin in the sample extract.

3.1.19 *non-pyrogenic*—describes a product that does not induce a fever. Also used to label medical devices that contain endotoxin below a specified level.

3.1.20 *pyrogen*—any substance that can induce a fever. Endotoxins are one type of pyrogen.

3.1.21 *pyrogenic*—a term used to describe healthcare products with endotoxin levels above specified limits.

3.1.22 *reference standard endotoxin (RSE)*—the USP endotoxin reference standard defined to have a potency of 10 000 USP *EU*s per vial.

3.1.23 *turbidimetric technique—BET* methodology that detects or quantifies endotoxin based on the level of turbidity created proportional to the interaction of *LAL* and endotoxin.

3.1.24 *water for BET*—water that shows no reaction with the lysate employed, at the detection limit of the reagent. Examples – LRW and Water of Injection.

4. Summary of Guide

4.1 A standard method of sample preparation is specified in this guide.

4.2 Three variations of endotoxin determination test methods are identified and briefly described to facilitate selection of the appropriate method. The reader is referred to the referenced standards for complete instructions.

5. Significance and Use

5.1 This guide establishes a standard sample preparation method and provides a description of three established and recognized test methods for the determination of endotoxin on medical gloves. If interferences in a sample yield suspect results, a second method should be used.

5.2 This guide is appropriate for testing final product that has been subjected to all processes that could influence the final endotoxin level (either microbial contamination or processing agents/raw materials contaminated with endotoxin). As raw materials and processing conditions vary from lot to lot with regard to these parameters, it is appropriate to test for endotoxin on a routine basis if a product endotoxin claim is to be made (for example, non-pyrogenic). The user may find it beneficial to incorporate endotoxin testing for vulnerable areas of their manufacturing process as an alert mechanism.

6. Sampling, Sample Preparation, and Extraction

NOTE 1—All gloves must follow this sampling plan, sample preparation, and extraction method regardless of assay method chosen.

6.1 *Sampling*—The bacterial endotoxin test shall be carried out for each batch of gloves where a limit has been set. The sampling plan should be based on the batch size. Refer to Table 1.

6.1.1 Samples selected for testing should be produced and selected in the finished form. This includes all factors that

TABLE 1 Sample Size

Batch Size	# of Samples (Glove Pairs)
<30	2
30–100	3
≥101	3 % of lot, up to a maximum of 10

might contribute to the levels of endotoxin (for example, any manufacturing, physical handling, packaging, or delay in sterilization).

6.2 *Sample Extraction:*

6.2.1 Handle everything with pyrogen-free instruments. Perform all extractions in non-pyrogenic containers.

6.2.1.1 *Glassware or Other Heat-Stable Materials*—Commonly used minimum time and temperature of 30 min at 250°C in hot air oven using validated process may be applied. Refer to U.S. Pharmacopoeia (**1**).[3]

6.2.1.2 *Plastics*—Use materials that have been shown to be free of detectable endotoxin and do not interfere in the test.

6.2.2 A sample extraction is prepared by immersing the outside surface of the gloves in *Water for BET*. The standard extraction method is to soak or immerse a sample or flush the fluid pathway with extracting fluid that has been heated to 37 ± 1.0°C, keeping the extracting fluid in contact with the relevant surface(s) for no less than 1 h. Alternate extraction or rinsing methods may be used, but must be demonstrated to be equivalent or better than the standard method.

6.2.3 The extraction should be performed in a way to ensure that all surfaces of the gloves that would have patient contact, come in contact with the extraction medium. For example, the glove may be lowered into a flask containing sufficient volume of *Water for BET* for the full exterior of the glove to be in complete contact with the water and the cuff (≤2 cm) folded over the opening at the neck of the flask and held in place by a non-pyrogenic stopper by the cuff.

6.2.4 Extracts or samples may be pooled for testing. For example, a glove may be extracted, removed and a second glove may be suspended in the same extraction liquid. Alternately, both gloves may be extracted simultaneously in the same flask.

6.2.5 Powder and other particulate matter can interfere with endotoxin determination assays. Interference should be overcome with sample dilution. Neither extract filtration nor centrifugation for clarification or the removal of particulates are acceptable treatment methods as endotoxin can be coincidentally removed from the test sample.

6.2.6 Test the sample by one of the methods identified in Section 7, Test Methods. If not tested immediately, the sample should be refrigerated to prevent microbial growth, which may increase endotoxin levels.

7. Test Methods

7.1 *Bacterial Endotoxin Test (BET) Methods*—The testing laboratory may choose bacterial endotoxin testing techniques, described in the various compendia, guidelines, and product inserts. The choice of the technique should be made after careful thought and assessment of the product and testing facility. Current techniques are: *(a)* Gel Clot, *(b)* Turbidimetric, and *(c)* Chromogenic (Colorimetric). These may be found in FDA Guidelines (**2**), U.S. Pharmacopoeia (**3-5**), ANSI/AAMI ST 72, and EN 455-3/European Pharrmacopeia (**6**). Thorough understanding of the listed standards is required. For subjects not covered in compendial procedures refer to FDA guidance (**2**).

7.1.1 *Gel Clot Techniques*—In the gel-clot test, equal volumes of test sample diluted to a validated concentration and *LAL* reagent are mixed in a 10 by 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. The disadvantage to this test is that it is a qualitative test method and has a lower test sensitivity. The test may be made semi-quantitative by diluting positive test samples and assaying each dilution until an end point (no clot) is obtained. The level of *EU* in the sample can then be determined by incorporating the dilution factor into the calculation.

7.1.2 *Turbidimetric Technique*—This technique measures the increases in reactant turbidity by either assaying the quantitative relationship between endotoxin concentration and turbidity (determined by optical density readings) of the reaction mixture at the end of a pre-determined incubation time (endpoint variant) or by measuring the time required, also refered to as onset time, for the reaction to produce a predetermined turbidity level (kinetic variant). The disadvantage to the kinetic turbidimetric technique is that it is not appropriate for turbid samples.

7.1.3 *Chromogenic Technique*—This assay measures the chromophore release from a suitable chromogenic peptide by the reaction of endotoxin with the lysate. This method also has kinetic and a chromogenic variants as described in 7.1.2, however, the detectable change is color intensity instead of turbidity. The disadvantage to the chromogenic test method is that it has often been found to be more subject to interference, in comparison with the kinetic turbidimetric method.

7.1.4 For details regarding the performance and test parameters for the gel clot, turbidimetric, and chromogenic test methods refer to U.S. Pharmacopoeia (**3**) and ANSI/AAMI ST 72.

7.1.5 A batch of gloves that fails one of the *BET* methods described above may be retested once by the same method used originally or by one of the other methods.

7.1.6 For samples that cannot be tested by any of the *BET* methods because of non-removable inhibition or enhancement, the Rabbit Pyrogen Test may be utilized. Refer to U.S. Pharmacopoeia (**4**).

7.2 *Product Validation:*

7.2.1 The validity of the test results requires demonstration that the test article does not inhibit or enhance the LAL reaction. This is demonstrated through suitability testing for each product or product family by performing inhibition/enhancement testing.

[3] The boldface numbers in parentheses refer to the list of references at the end of this standard.

7.2.2 The continued suitability of the assay should be re-assessed when significant changes are made that could adversely affect the accuracy of the BET result.

8. Test Parameters

8.1 *Time:*

8.1.1 The gel-clot methods are typically incubated for 60 ± 2 min.

8.1.2 The kinetic methods measure the time it takes for a series of standards and unknown to read a pre-determined optical density or color intensity.

8.2 *Temperature*—All *BET* methodologies are typically carried out at the incubation temperature recommended by the lysate manufacturer (typically 37 ± 1°C).

8.3 *pH*—The *LAL* test (a mixture of the sample with lysate) performs optimally at a pH between 6 and 8 pH units. Refer to the lysate manufacturer's package insert for further information and methods of adjustment.

8.4 *Product Validation:*

8.4.1 *Recovery:*

8.4.1.1 *Turbidimetric and Chromogenic Methods*—The positive product control (*PPC*) for each unknown must have a spike recovery of 50 to 200 % of the specified standard control level for the endotoxin test to be valid.

NOTE 2—If the control series is less than one log, the recovery should be within ±25 %.

8.4.1.2 *Gel-Clot Method*—The geometric mean of the endpoints for the sample solution with endotoxin added must be within 0.5 and 2 λ for the endotoxin test to be valid.

8.4.2 *Neutralization of Sample Interference*—For the kinetic methods, a spike recovery <50 % suggests inhibition, whereas spike recovery >200 % suggests enhancement. For the gel clot methods, inhibition can be determined by the sample solution not clotting within 2 λ. Enhancement is difficult to determine with the gel-clot assay. If interference is observed in any *BET*, the interference may be overcome by suitable treatment.

8.4.2.1 Treatment of a sample may involve dilution, addition of reagents, heat denaturing, and the like.

8.4.2.2 The sample extracts may be diluted or treated, or both, to overcome inhibition or enhancement and determine the endotoxin concentration.

(1) LAL reagent water is the optimal diluent for the *LAL* test and should be used as the first option for dilution of interfering substances.

(2) 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 (pH extremes can interfere with normal enzymatic activity).

(3) Dilution of the sample in a cationic buffer ($MgSO_4$ or $MgCl_2$) prepared with non-pyrogenic water can be used to adjust the ionic concentration, which may otherwise interfere with the BET.

(4) LAL Reactive Material Neutralization—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 identify and eliminate the possibility of enhancement.

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

8.4.2.4 All sample manipulations should be specified in the validation data and performed during routine testing.

9. Interpretation

9.1 A packaged pair of medical gloves is considered one medical device. Thus all *EU* determinations must represent the sum value of each of their levels. If the glove is packaged as a single medical glove, the definition of a device is the single glove.

9.2 No endotoxin limits have been established for medical gloves. The U.S Pharmacopoeia set a 20 *EU* per device limit for medical devices that contact circulating blood. Devices meeting this limit are referred to as "non-pyrogenic."

10. Keywords

10.1 endotoxin; gloves; lipopolysaccharide; LPS; non-pyrogenic; pyrogenic

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