Guidance for Industry

Pyrogen and Endotoxins Testing: Questions and Answers (Edition 2)

U.S. Department of Health and Human Services
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Center for Biologics Evaluation and Research (CBER)
Center for Veterinary Medicine (CVM)
Center for Devices and Radiology Health (CDRH)
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Contains Nonbinding Recommendations

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Pyrogen and Endotoxins Testing: Questions and Answers (Edition 2)

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I. INTRODUCTION

This guidance provides recommendations for biological product, drug, and device firms on FDA's current thinking concerning the testing recommendations and acceptance criteria in the United States Pharmacopeia (USP) Chapter <85> "*Bacterial Endotoxins Test"*,[2] USP Chapter <161> "*Medical Devices – Bacterial Endotoxin and Pyrogen Tests"*,[3] and the Association for the Advancement of Medical Instrumentation (AAMI) ST72:2002/R2010, Bacterial Endotoxins—Test Methodologies, Routine Monitoring, and Alternatives to Batch Testing (AAMI ST72).[4, 5] These three documents describe the fundamental principles of the gel clot, photometric, and kinetic test methods, and recommend that appropriate components and finished products be tested for the presence of pyrogens and endotoxins.

This guidance does not cover the entire subject of pyrogen and endotoxins testing. Instead, it addresses those issues that may be subject to misinterpretation and are not covered in compendial procedures or in currently available guidance documents. You should already have a thorough understanding of these documents when using this guidance.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

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[1] This guidance has been prepared by the Division of Manufacturing and Product Quality, Office of Compliance, in the Center for Drug Evaluation and Research (CDER) in cooperation with the Center for Biologics Evaluation and Research (CBER), the Center for Veterinary Medicine (CVM), the Center for Devices and Radiological Health (CDRH), and the Office of Regulatory Affairs (ORA) at the Food and Drug Administration.

[2] United States Pharmacopeia (USP), 2018, Chapter <85> *Bacterial Endotoxins Test*.

[3] USP, 2017, Chapter <161>, *Medical Devices – Bacterial Endotoxin and Pyrogen Tests*.

[4] Association for the Advancement of Medical Instrumentation (AAMI), 2002/R2010, Bacterial Endotoxins — Test Methodologies, Routine Monitoring, and Alternative to Batch Testing.

[5] Veterinary drug requirements parallel the human drug requirements in safety evaluation for pyrogenicity (21 CFR 514.1(b)(5)(v), and 514.1(b)(5)(vii)(b)).

II. BACKGROUND

For more than 30 years, FDA has accepted the use of a Limulus Amoebocyte Lysate (LAL) test for endotoxins in lieu of the rabbit pyrogens test. In a November 4, 1977, *Federal Register* notice (42 FR 57749), FDA described conditions for using LAL as a finished product test.[6] By 1983, FDA indicated in guidance that an LAL test could be used as a finished product test for endotoxins. These tests were described in a series of draft and final guidance documents. *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*, was published in 1987 (the 1987 Guidance) and Version 1 of this Guidance was published in 2012.

FDA has found that the published USP and AAMI documents describing methods and calculation of pyrogen and endotoxins testing limits[7] provide industry with appropriate information. The Agency has withdrawn the 1987 Guidance because it no longer reflects the Agency's current thinking on the topic. However, because the compendial chapters and standards do not address certain regulatory perspectives, FDA is providing supplemental information in this guidance to explain our current thinking regarding the submission and maintenance of pyrogen and endotoxins testing for FDA-regulated products.

III. QUESTIONS AND ANSWERS

1. How do I establish sampling for in-process testing and finished product release?

The current good manufacturing practice (CGMP) regulations for finished pharmaceuticals require scientifically sound and appropriate sampling[8] and the medical device quality management system regulation requires validation using statistical techniques, as appropriate, with rationale for sample sizes.[9] Sampling information is addressed in AAMI ST72, but not USP Chapter <85>. Firms should include sampling as part of their application documentation. When sampling, firms should consider the potential for contamination in raw materials, in-process materials, and the finished product. Specifically, firms should take into account aspects of the manufacturing design, including consistency of a manufacturing process, impact of in-process hold times, endotoxins removal steps, and finished product endotoxins specifications. Sampling should be considered dynamic; firms should begin with maximum coverage and adjust their procedures as they gain confidence in the prevention of endotoxins in their manufacturing processes. Firms should update their regulatory filings when adjusting

[6] "Licensing of Limulus Amebocyte Lysate, Use as an Alternative for Rabbit Pyrogen Test" (42 FR 57749, November 4, 1977).

[7] USP, 2018, Chapter <85> *Bacterial Endotoxins Test*; USP, 2017, Chapter <161> *Medical devices – Bacterial Endotoxin and Pyrogen Tests*; Association for the Advancement of Medical Instrumentation (AAMI), 2002/R2010, Bacterial Endotoxins—Test Methodologies, Routine Monitoring, and Alternatives to Batch Testing.

[8] See 21 CFR 211.160.

[9] See 21 CFR 820.7(b), which incorporates by reference ISO 13485:2016(E), Medical devices—Quality management systems—Requirements for regulatory purposes, Third edition, March 1, 2016. ISO 13485:2016(E) Clause 7.5, and Clause 8 and its subclauses require manufacturers to document verification plans, validation plans, and procedures for validation of processes that include, as appropriate, statistical techniques with rationale for sample sizes.

procedures. For drugs and biological products, these in-process changes should be submitted in a supplement or annual report, as appropriate.[10] For devices, a 30-day notice[11] may be appropriate.[12]

2. When is retesting appropriate?

When conflicting results occur within a test run, firms should consult USP Chapter <85>, Gel-Clot Limit Test, Interpretation, for guidance on repeat testing. As specified in Chapter <85>, if the test failure occurred at less than the maximum valid dilution (MVD), the test should be repeated using a greater dilution not exceeding the MVD. A record of this failure should be included in the laboratory results. If a test is performed at the MVD and an out-of-specification (OOS) test result occurs that cannot be attributed to testing error, the lot should be rejected.[13] All testing procedures, including those for retesting within the above limits, should be specified in advance in written standard operating procedures approved by the firm's quality control unit.

3. Is sample storage and handling important?

Yes. The ability to detect endotoxins can be affected by storage and handling. Firms should establish procedures for storing and handling (which includes product mixing) samples for bacterial endotoxins analysis using laboratory data that demonstrate the stability of assayable endotoxins content. Protocols should consider the source of endotoxins used in the study, bearing in mind that purified bacterial endotoxins might react differently from native sources of endotoxins.

4. Can finished product samples for analysis of bacterial endotoxins be pooled into a composite sample prior to analysis?

Yes. With some exceptions (see below), finished drug product units may be pooled into a composite sample and assayed for bacterial endotoxins. The composite sample may be represented by the entire unit or partial aliquots (equal volumes) of finished product containers from one manufactured lot of aqueous-based pharmaceuticals. Pooling would generally be accepted for small-volume parenterals (those with volumes of 100 mL or less) as long as the MVD is adjusted to a proportional, lower value because of the potential for diluting a unit containing harmful levels of endotoxins with other units containing lower, less harmful, levels of endotoxins. This "adjusted MVD" is obtained by dividing the MVD computed for an individual sample by the total number of samples to be pooled. FDA suggests pooling no more than three units per composite in keeping with the concept of testing representative

[10] See 21 CFR 314.81(b)(2)(iv), 314.98, and 601.12.
[11] See 21 CFR 814.39(f).
[12] For guidance regarding when and how to submit a 30-day notice, please refer to guidance for industry and FDA staff *30-Day Notices, 135-Day Premarket Approval (PMA) Supplements and 75-Day Humanitarian Device Exemption (HDE) Supplements for Manufacturing Method or Process Changes* (December 2019).
[13] For guidance regarding how to examine results that are above acceptable limits, please refer to guidance for industry *Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production* (May 2022). Although this guidance is not intended to address biological assays, many of the concepts in the guidance are applicable to bacterial endotoxins testing..

beginning, middle, and end finished product containers. If this reduction in MVD results in an inability to overcome product-related assay interference because of an insufficient dilution, then the samples should be tested individually.

Finished medical devices may also be pooled into a composite sample and assayed for bacterial endotoxins. Testing for medical devices should be conducted using rinsing/eluting and sampling techniques as described in ISO 10993-1[14] and ISO 10993- 12,[15] as also used for inhibition/enhancement. Sampling can be adjusted for special situations. After a suitable eluate/extract pool is obtained from a finished production lot, this pooled extract should be kept under conditions appropriate for stability until it is tested in duplicate.

FDA recommends that pooled samples be a composite of aseptically removed aliquots (after at least 30 seconds of vigorous mixing) from each of the product containers.[16] In this way, the original, individual containers will be available for possible retesting in the event the pooled sample displays an OOS result.

Some product types should not be pooled. Two examples are drug products that have an initial low MVD (see discussion above of "adjusted MVD") and products that are manufactured as a suspension, because sample aliquot homogeneity may present significant interference issues. FDA also does not recommend pooling in-process samples from different in-process stages of the manufacturing process because it may be difficult to ensure the homogeneity of these materials.

5. May a firm use alternative assays to those in the USP for a compendial article?

Yes, firms may use alternative methods and/or procedures if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, and in other special circumstances. Such alternative procedures and methods should be validated as described in the USP General Chapter <1225>, "*Validation of Compendial Procedures*",[17, 18] and should be shown to achieve equivalent or better results.[19] When a difference appears or in the event of a dispute, the final decision is made based upon the USP compendial gel-clot method unless otherwise indicated in the monograph for the product being tested.[20]

Below is an example of an alternative assay.

Monocyte Activation Type Pyrogen Test

Product-specific validation is necessary to establish whether a particular test substance or material is appropriate for evaluation of the monocyte activation method. The validation should include, but is not limited to, interference testing, accurate detection of pyrogen in

[14] ISO 10993-1:2009 Biological evaluation of medical devices Part 1: Evaluation and testing in the risk management process.
[15] ISO 10993-12:2007 Biological evaluation of medical devices Part 12: Sample preparation and reference materials.
[16] See 21 CFR 211.84(c)(3).
[17] USP General Notices and Requirements 6.30 *Alternative and Harmonized Methods and Procedures*.
[18] USP, 2017, Chapter <1225> *Validation of Compendial Procedures*.
[19] USP General Notices and Requirements 6.30 *Alternative and Harmonized Methods and Procedures*.
[20] USP, 2018, Chapter <85> *Bacterial Endotoxins Test.*

individual test samples, and, for devices, ability of test system to provide direct contact to the monocytes.

6. What is the best process for transitioning from one alternate bacterial endotoxins test (BET) method to another?

The transition between tests that measure the same entity can be made by comparing the two tests to verify the equivalence of the new method.[21] The comparison of the limit of detection and inhibition/enhancement is fundamental. The sensitivity of the new method can be evaluated on spiked product samples.[22] In addition to using spiked samples, a battery of field samples of product found to be positive may be a good source for comparing results from the methods. The method validation should also attempt to address the variability found in the normal use of the method and the manufacturing environment (e.g., source materials or seasonal changes).[23]

For drug, animal drug, and biological products, the transition to a new method should be submitted in a prior approval supplement (PAS). Alternatively, once a firm has established a general method for making the transition between tests, it may submit the method for review in a PAS—comparability protocol (CP). The CP should describe, in detail, the methods used to transition between assays and the acceptance criteria used to establish the equivalence of the new method. After approval of the CP, results of implementation of the CP may be reported in a reduced reporting category (21 CFR 314.81, 314.98, and 601.12). The firm should maintain the study protocol, final report, and all data at the facility for FDA review. The firm should confirm the filing process with the appropriate review division before submitting a CP. For Class III devices, the transition to a new assay may be appropriate for a 30-day notice filed under 21 CFR 814.39(f).[24] Manufacturing changes for Class I and II devices must be in accordance with the quality management system regulation, 21 CFR part 820, which incorporates ISO 13485:2016 by reference. Design control, production and process control requirements can be found at ISO 13485:2016 Clause 7, including subclauses 7.3 and 7.5.

For devices, a 30-day notice may be appropriate for changes to quality control testing used on incoming components, raw materials, the in-process device, or the finished device, including performing end-product pyrogen testing on nonsterile samples prior to sterilization.[25] Manufactures of medical devices should demonstrate a sensitivity that is consistent with the route of administration for the device and the type of body contact. Manufacturers may be able to use another endotoxin test after demonstrating a reproducible correlation between methods and the USP reference standard.

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[21] USP, 2017, Chapter <1225> *Validation of Compendial Procedures*.
[22] International Conference on Harmonisation (ICH) *Q2(R1) Validation of Analytical Procedures: Text and Methodology* (1994); USP, 2017, Chapter <1225> *Validation of Compendial Procedures*.
[23] Thompson, M. et al., 2002, "Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis: IUPAC Technical Report," *Pure Appl. Chem*., Vol. 74, No. 5, pp. 835-855.
[24] See guidance for industry and FDA staff, *Modifications to Devices Subject to Premarket Approval (PMA) - The PMA Supplement Decision-Making Process* (December 2008).
[25] See 21 CFR 814.39(f).

7. What happened to the endotoxins limit table in Appendix E of the 1987 Guidance?

The endotoxins limit table is out of date due to the increase in numbers of dosage (regimes) and drug strengths since the publication of the 1987 Guidance. The appropriate way to establish the endotoxins limit is to use the calculation methods provided in the USP or AAMI standards. Monograph limits may also not account for current product strengths or dosage regimes; these should also be checked using the calculations recommended in the standards.

If there are several components in a finished product, then the overall endotoxins limit for parenterally-administered products should not exceed the overall threshold limit specified in the USP <85> "*Bacterial Endotoxins Test*", regardless of an individual component endotoxins limit. Intrathecally-administered products, ophthalmics, or devices (see question 11 for devices) may have endotoxins limits that are not based on the calculation for parenterally administered products. FDA encourages firms to check with the appropriate office or review division about these products.

8. How can Quality by Design concepts support endotoxins limits?[26]

When implementing Quality by Design concepts, the strategy for endotoxins testing should be based upon product and process understanding in combination with risk management to ensure consistent final product quality. The appropriate in-process testing should be used to evaluate the production process areas at risk of endotoxins formation or incursion. Many firms already have programs for monitoring incoming ingredients and components, including the processing water, for endotoxins contamination. The finished product release specification should be considered when determining in-process limits for each phase of manufacturing tested. For purposes of evaluating the relative risk of product contamination, quantitative testing may be preferable to limit testing to facilitate product quality trending and to identify and correct excursions before they exceed the specification and cause product failure. An endotoxins limit should be justified on a case-by-case basis, and will be evaluated as a part of each relevant marketing application or supplement.

9. When is the USP Chapter <151> "*Pyrogen Test*" (the rabbit pyrogen test) appropriate?

For certain biological products, 21 CFR 610.13(b) requires a rabbit pyrogen test. The requirement in 21 CFR 610.13(b) may be waived if a method equivalent to the rabbit pyrogen test is demonstrated in accordance with 21 CFR 610.9.

For human and animal drugs, some USP monographs still require a rabbit pyrogen test. Even with such monographs, a firm may substitute an endotoxins test or alternative cell- based test if the firm can demonstrate equivalent pyrogen detection. The appropriate FDA review division will consider alternative methods, such as monocyte activation, on a case-by-case basis.

[26] ICH Harmonised Tripartite Guideline, *Q8(R2) Pharmaceutical Development*, Step 4 Version (Nov 2009).

For devices and drug materials, firms should assess the risk of the presence of non- endotoxin pyrogens. If the risk assessment indicates that non-endotoxin pyrogens may be present, it may be more appropriate to use the rabbit pyrogen test.

Bacterial endotoxins assays are subject to a variety of interferences related to the physical and chemical properties of the test article. Where such interferences cannot be mitigated through sample dilution (up to the MVD) or other validated means of sample preparation, firms should use the rabbit pyrogen test.

10. How would an appropriate endotoxins limit be determined for a veterinary product that targets multiple species?

For a veterinary product labeled for use in multiple species, the limit should be based on the maximum product dose used on the smallest species. If the label indicates that the product may be used on juvenile and adult animals, the juvenile is considered the worst case. If the weight of the animal is required to calculate the dose, firms should use an average weight for that species. For a listing of some average animal weights, see the FDA guidance for industry *Demonstrating Bioequivalence for Soluble Powder Oral Dosage Form Products and Type A Medicated Articles Containing Active Pharmaceutical Ingredients Considered to Be Soluble in Aqueous Media* (June 2023). For animal weights not listed in that guidance, please contact FDA's Center for Veterinary Medicine.

11. What are the endotoxins limits for medical devices?

The Center for Devices and Radiological Health (CDRH) has adopted the USP Endotoxin Reference Standard and endotoxin limits for medical device extracts expressed in EU/mL as stated in USP Chapter <161> "*Medical devices – Bacterial Endotoxin and Pyrogen Tests*". This USP chapter provides the endotoxin limits for medical devices within its scope. These endotoxin limits for a medical device are dependent on the intended use of the device and what the device contacts (e.g., blood, the cardiovascular system, cerebrospinal fluid, intrathecal routes of administration, permanently implanted devices, and devices implanted subcutaneously).[27]

For medical devices, using the extraction volume recommendations described below, the endotoxin limit is 0.5 EU/mL or 20 EU/device for products that directly or indirectly contact the cardiovascular system and lymphatic system. For devices in contact with cerebrospinal fluid, the endotoxin limit is 0.06 EU/mL or 2.15 EU/device. For devices that are in direct or indirect contact with the intraocular environment, a lower endotoxins limit may apply. Please contact the appropriate review division for specific recommendations.

The process of preparing an eluate/extract for testing may vary from device to device. Some medical devices can be flushed, some may have to be immersed, while others may need disassembly. Unless otherwise directed by another compendial standard, our recommended rinse volumes include the following: (1) each of the 10 test units should be rinsed with 40 mL of non-pyrogenic water; (2) for unusually small or large devices, the surface area of the device that contacts the patient may be used as an adjustment factor in selecting the rinse or extract volume. The endotoxins limit can be adjusted accordingly. In any case, the rinse/extract

[27] USP, 2017, Chapter <161> *Medical devices – Bacterial Endotoxin and Pyrogen Tests*.

procedure should not result in a greater dilution of endotoxin than recommended in USP <85>. For inhibition/enhancement testing, both the rinse/extract solution and the device eluate/extract should be tested.

Examples of medical devices with testing or interference challenges include devices that are coated with anticoagulant, contain heavy metals, or that have particulates. In these situations, treatments for interferences can include digestion, dilution, and addition of buffers, centrifugation, or filtration.

During the same surgical procedure or placement in the same surgical site, multiple units of the same device from one manufacturer should generally meet the same endotoxins limit as a single device administered during the procedure. In instances where multiple units of the same device are known or intended for use in a single procedure, manufacturers should justify any deviation from the overall endotoxins limit identified in this guidance.

When a manufacturer of medical devices plans to use testing that deviates significantly from this guidance or recognized standards, a premarket notification (510(k)) under section 510(k) of the Federal Food, Drug, and Cosmetic Act (the Act) or a premarket approval application (PMA) supplement under section 515 of the Act may be required. Significant deviations include, but are not necessarily limited to: higher endotoxin concentration release criteria, sampling from fewer than three (3) lots for inhibition/enhancement testing, lesser sensitivity to endotoxins, and a device rinsing protocol resulting in greater dilution of endotoxins than that recommended in this guidance.

12. What is the FDA's expectation for regular screening of therapeutic drug products?

Ideally, the undiluted product should be screened as long as there is no interfering/enhancing property within the test. However, in some product formulations, the ingredients interfere with the test. For such formulations, the USP recommends that the product be diluted to overcome interference or enhancement properties. The calculated MVD is the dilution of a sample at which the endotoxins limit would be detected, but it should not be the regular testing dilution. When product interference is encountered during development, FDA recommends that the firm determine the lowest product dilution that would neutralize the interfering condition.

FDA recommends that firms begin subsequent product screening at a product dilution just above the level that neutralized the interference. For example, if the product has an MVD of 1:100, and the product displays inhibition at the 1:10, but not at the 1:20, it may be best to screen product at 1:30. If bacterial endotoxins are detected at this level, then the firm should conduct full enumeration with the product to titrate the true amount of endotoxins.

13. Are control standard endotoxins still acceptable for use in running bacterial endotoxins tests?

Control standard endotoxins (CSEs) are endotoxin preparations other than the international or national reference standards that are traceable in their calibration to the international reference endotoxins standard. CSEs may be secondary or tertiary standards and are usually manufactured and certified by a reagent manufacturer for use with a specific lot of reagent

under defined assay conditions. CSEs have become an accepted source for preparation of standard curve calibrators and as assay controls, and have provided a cost saving to end users and helped to preserve the inventory of primary standards. FDA encourages the continued use of CSEs that are suitably calibrated to the international reference endotoxins standard.