á85ñ BACTERIAL ENDOTOXINS TEST

Change to read:

FPortions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopeia and/or the Japanese Pharmacopeia. Those portions that are not harmonized are marked with symbols ($^{F}_{F}$) to specify this fact.$_{F}$

This chapter provides a test to detect or quantify bacterial endotoxins that may be present in or on the sample of the article(s) to which the test is applied. It uses Limulus Amebocyte Lysate (LAL) obtained from the aqueous extracts of circulating amebocytes of horseshoe crab (*Limulus polyphemus*or *Tachypleus tridentatus*) which has been prepared and characterized for use as an LAL Reagent.[1]

There are two types of techniques for this test: the gel-clot techniques, which are based on gel formation, and the photometric techniques. The latter include a turbidimetric method, which is based on the development of turbidity after cleavage of an endogenous substrate, and a chromogenic method, which is based on the development of color after cleavage of a synthetic peptide-chromogen complex. Proceed by any one of these techniques, unless otherwise indicated in the monograph. In case of dispute, the final decision is based on the gel-clot techniques, unless otherwise indicated in the monograph.

In the gel-clot techniques, the reaction endpoint is determined from dilutions of the material under test in direct comparison with parallel dilutions of a reference endotoxin, and quantities of endotoxin are expressed in USP Endotoxin Units (USP-EU). [NOTE — One USP-EU is equal to one IU of endotoxin.]

Since LAL Reagents have been formulated to be used also for turbidimetric or colorimetric tests, such tests may be used to comply with the requirements. These tests require the establishment of a standard regression curve; the endotoxin content of the test material is determined by interpolation from the curve. The procedures include incubation for a preselected time of reacting endotoxin and control solutions with LAL Reagent and reading of the spectrophotometric light absorbance at suitable wavelengths. In the endpoint turbidimetric procedure the reading is made immediately at

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the end of the incubation period. In the endpoint colorimetric procedure the reaction is arrested at the end of the preselected time by the addition of an enzyme reaction-terminating agent prior to the readings. In the turbidimetric and colorimetric kinetic assays the absorbance is measured throughout the reaction period and rate values are determined from those readings.

Change to read:

APPARATUS AND GLASSWARE

Depyrogenate all glassware and other heat-stable materials in a hot-air oven using a validated process.[2] Commonly used minimum time and temperature settings are 30 minutes at 250°. If employing plastic apparatus, such as microplates and pipet tips for automatic pipetters, use only that which has been shown to be free of detectable endotoxin and not to interfere with the test. [NOTE — In this chapter, the term "tube " includes any other receptacle such as a micro-titer well.]

Change to read:

PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION AND STANDARD SOLUTIONS

The *USP Endotoxin RS* has a defined potency of 10,000 USP Endotoxin Units (EU) per vial. Constitute the entire contents of 1 vial of the RSE with 5 mL of LAL Reagent Water[3], mix intermittently for 30 minutes, using a vortex mixer, and use this concentrate for making appropriate serial dilutions. Preserve the concentrate in a refrigerator for making subsequent dilutions for not more than 14 days. Mix vigorously, using a vortex mixer, for not less than 3 minutes before use. Mix each dilution for not less than 30 seconds before proceeding to make the next dilution. Do not store dilutions, because of loss of activity by adsorption, in the absence of supporting data to the contrary.

Preparatory Testing

Use an LAL Reagent of confirmed label sensitivity.

The validity of test results for bacterial endotoxins requires an adequate demonstration that specimens of the article or of solutions, washings, or extracts thereof to which the test is to be applied do not of themselves inhibit or enhance the reaction or otherwise interfere with the test.

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Validation is accomplished by performing the inhibition or enhancement test described under each of the three techniques indicated. Appropriate negative controls are included. Validation must be repeated if the LAL Reagent source or the method of manufacture or formulation of the article is changed.

Preparation of Sample Solutions

Prepare sample solutions by dissolving or diluting drugs or extracting medical devices using LAL Reagent Water. Some substances or preparations may be more appropriately dissolved, diluted, or extracted in other aqueous solutions. If necessary, adjust the pH of the solution (or dilution thereof) to be examined so that the pH of the mixture of the LAL Reagent and sample falls within the pH range specified by the LAL Reagent manufacturer. This usually applies to a product with a pH in the range of 6.0 to 8.0. The pH may be adjusted using an acid, base, or suitable buffer as recommended by the LAL Reagent manufacturer. Acids and bases may be prepared from concentrates or solids with LAL Reagent Water in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

DETERMINATION OF MAXIMUM VALID DILUTION (MVD)

The Maximum Valid Dilution is the maximum allowable dilution of a specimen at which the endotoxin limit can be determined. It applies to injections or to solutions for parenteral administration in the form constituted or diluted for administration, or, where applicable, to the amount of drug by weight if the volume of the dosage form for administration could be varied. The general equation to determine MVD is:

$$MVD = (\text{Endotoxin limit} \times \text{Concentration of sample solution}) / (\lambda)$$

where the concentration of sample solution and λ are as defined below. Where the endotoxin limit concentration is specified in the individual monograph in terms of volume (in EU per mL), divide the limit by λ, which is the labeled sensitivity (in EU per mL) of the LAL Reagent, to obtain the MVD factor. Where the endotoxin limit concentration is specified in the individual monograph in terms of weight or Units of active drug (in EU per mg or in EU per Unit), multiply the limit by the concentration (in mg per mL or in Units per mL) of the drug in the solution tested or of the drug

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constituted according to the label instructions, whichever is applicable, and divide the product of the multiplication by λ, to obtain the MVD factor. The MVD factor so obtained is the limit dilution factor for the preparation for the test to be valid.

Change to read:

ESTABLISHMENT OF ENDOTOXIN LIMITS

The endotoxin limit for parenteral drugs, defined on the basis of dose, is equal to K/M, [4] where K is the threshold human pyrogenic dose of endotoxin per kg of body weight, and M is equal to the maximum recommended human dose of product per kg of body weight in a single hour period.

The endotoxin limit for parenteral drugs is specified in individual monographs in units such as EU/mL, EU/mg, or EU/Unit of biological activity.

GEL-CLOT TECHNIQUES

The gel-clot techniques detect or quantify endotoxins based on clotting of the LAL Reagent in the presence of endotoxin. The concentration of endotoxin required to cause the lysate to clot under standard conditions is the labeled sensitivity of the LAL Reagent. To ensure both the precision and validity of the test, tests for confirming the labeled LAL Reagent sensitivity and for interfering factors are described under *Preparatory Testing for the Gel-Clot Techniques.*

Preparatory Testing for the Gel-Clot Techniques

Test for Confirmation of Labeled LAL Reagent Sensitivity — Confirm the labeled sensitivity using at least 1 vial of the LAL Reagent lot. Prepare a series of two-fold dilutions of the *USP Endotoxin RS* in LAL Reagent Water to give concentrations of 2 λ, λ, 0.5 λ, and 0.25 λ, where λ is as defined above. Perform the test on the four standard concentrations in quadruplicate and include negative controls. The test for confirmation of lysate sensitivity is to be carried out when a new batch of LAL Reagent is used or when there is any change in the experimental conditions that may affect the outcome of the test.

Mix a volume of the LAL Reagent with an equal volume (such as 0.1-mL aliquots) of one of the standard solutions in each test tube. When single test vials or ampuls containing lyophilized LAL

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Reagent are used, add solutions directly to the vial or ampul. Incubate the reaction mixture for a constant period according to directions of the LAL Reagent manufacturer (usually at 37 ± 1° for 60 ± 2 minutes), avoiding vibration. To test the integrity of the gel, take each tube in turn directly from the incubator and invert it through about 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed. The test is not valid unless the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The endpoint is the last positive test in the series of decreasing concentrations of endotoxin. Calculate the mean value of the logarithms of the endpoint concentration and then the antilogarithm of the mean value using the following equation:

$$\text{Geometric Mean Endpoint Concentration} = \text{antilog}\,(\Sigma e / f),$$

where Σe is the sum of the log endpoint concentrations of the dilution series used, and f is the number of replicate test tubes. The geometric mean endpoint concentration is the measured sensitivity of the LAL Reagent (in EU/mL). If this is not less than 0.5λ and not more than 2λ, the labeled sensitivity is confirmed and is used in tests performed with this lysate.

Interfering Factors Test for the Gel-Clot Techniques — Prepare solutions A, B, C, and D as shown in Table 1, and perform the inhibition/enhancement test on the sample solutions at a dilution less than the MVD, not containing any detectable endotoxins, following the procedure in the *Test for Confirmation of Labeled LAL Reagent Sensitivity* above. The geometric mean endpoint concentrations of solutions B and C are determined using the equation in that test.

Table 1. Preparation of Solutions for the Inhibition/Enhancement Test for Gel-Clot Techniques

Solution	Endotoxin Concentration/Solution to which Endotoxin is Added	Diluent	Dilution Factor	Initial Endotoxin Concentration	Number of Replicates
A[a]	none/sample solution	—	—	—	4
B[b]	2λ/sample solution	sample solution	1	2λ	4
			2	1λ	4
			4	0.5λ	4
			8	0.25λ	4
C[c]	2λ/water for BET	LAL Reagent Water	1	2λ	2

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Table 1. Preparation of Solutions for the Inhibition/Enhancement Test for Gel-Clot Techniques

Solution	Endotoxin Concentration/Solution to which Endotoxin is Added	Diluent	Dilution Factor	Initial Endotoxin Concentration	Number of Replicates
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D[d]	none/LAL Reagent Water	—	—	—	2

a Solution A: a sample solution of the preparation under test that is free of detectable endotoxins.

b Solution B: test for interference.

c Solution C: control for labeled LAL Reagent sensitivity.

d Solution D: negative control of LAL Reagent Water.

This test must be repeated when any condition that is likely to influence the test results changes. The test is not valid unless Solutions A and D show no reaction and the result of Solution C confirms the labeled sensitivity.

If the sensitivity of the lysate determined in the presence of the sample solution under test of Solution B is not less than 0.5λ and not greater than 2λ, the sample solution does not contain factors which interfere under the experimental conditions used. Otherwise, the sample solution to be examined interferes with the test.

If the sample under test does not comply with the test at a dilution less than the MVD, repeat the test using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the sample to be examined and this may contribute to the elimination of interference.

Interference may be overcome by suitable treatment, such as filtration, neutralization, dialysis, or heating. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described below using the preparation to be examined to which *USP Endotoxin RS* has been added and which has been subjected to the selected treatment.

Gel-Clot Limit Test

This test is used when a monograph contains a requirement for endotoxin limits.

Procedure — Prepare Solutions A, B, C, and D as shown in Table 2, and perform the test on these solutions following the procedure in the *Test for Confirmation of Labeled LAL Reagent*

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Sensitivity under *Preparatory Testing for the Gel-Clot Techniques.*

Table 2. Preparation of Solutions for the Gel-Clot Limit Test

Solution*	Endotoxin Concentration/Solution to which Endotoxin is Added	Number of Replicates
A	none/diluted sample solution	2
B	2λ/diluted sample solution	2
C	2λ/LAL Reagent Water	2
D	none/LAL Reagent Water	2

*Prepare Solution A and positive product control Solution B using a dilution not greater than the MVD and treatments as directed in the *Interfering Factors Test for the Gel-Clot Techniques* under *Preparatory Testing for the Gel-Clot Techniques.* Positive control Solutions B and C contain the standard endotoxin preparation at a concentration corresponding to twice the labeled LAL Reagent sensitivity. The negative control Solution D is LAL Reagent Water.

Interpretation — The test is not valid unless both replicates of positive control Solutions B and C are positive and those of negative control Solution D are negative. The preparation under test complies with the test when a negative result is found for both tubes containing Solution A. The preparation under test does not comply with the test when a positive result is found for both tubes containing Solution A.

Repeat the test when a positive result is found for 1 tube containing Solution A and a negative result for the other one. The preparation under test complies with the test when a negative result is found for both tubes containing Solution A in the repeat result. If the test is positive for the preparation under test at a dilution less than the MVD, the test may be repeated at a dilution not greater than the MVD.

Gel-Clot Assay

This assay quantifies bacterial endotoxins in sample solutions by titration to an endpoint.

Procedure — Prepare Solutions A, B, C, and D as shown in Table 3, and test these solutions by following the procedure in the *Test for Confirmation of Labeled LAL Reagent Sensitivity* under *Preparatory Testing for the Gel-Clot Techniques.*

Table 3. Preparation of Solutions for the Gel-Clot Assay

Solution	Endotoxin Concentration/Solution to which Endotoxin is Added	Diluent	Dilution Factor	Initial Endotoxin Concentration	Number of Replicates

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Table 3. Preparation of Solutions for the Gel-Clot Assay

Solution	Endotoxin Concentration/Solution to which Endotoxin is Added	Diluent	Dilution Factor	Initial Endotoxin Concentration	Number of Replicates
A[a]	none/sample solution	LAL Reagent Water	1	—	2
			2	—	2
			4	—	2
			8	—	2
B[b]	2λ/sample solution	—	1	2λ	2
C[c]	2λ/LAL Reagent Water	LAL Reagent Water	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D[d]	none/LAL Reagent Water	—	—	—	2

aSolution A: a sample solution under test at the dilution, not to exceed the MVD, with which the *Interfering Factors Test for the Gel-Clot Techniques* was completed. Subsequent dilution of the sample solution must not exceed the MVD. Use LAL Reagent Water to make dilution series of four tubes containing the sample solution under test at concentrations of 1, ½, ¼, and 1/8 relative to the dilution with which the *Interfering Factors Test for the Gel-Clot Techniques* was completed. Other dilutions may be used as appropriate.

b Solution B: Solution A containing standard endotoxin at a concentration of 2 λ (positive product control).

c Solution C: two series of 4 tubes of LAL Reagent Water containing the standard endotoxin at a concentration of 2 λ, λ, 0.5 λ, and 0.25 λ, respectively.

d Solution D: LAL Reagent Water (negative control).

Calculation and Interpretation — The test is not valid unless the following conditions are met: (1) both replicates of negative control Solution D are negative; (2) both replicates of positive product control Solution B are positive; and (3) the geometric mean endpoint concentration of Solution C is in the range of 0.5 λ to 2 λ.

To determine the endotoxin concentration of Solution A, calculate the endpoint concentration for each replicate series of dilutions by multiplying each endpoint dilution factor by λ. The endotoxin concentration in the sample is the geometric mean endpoint concentration of the replicates (see the formula given in the *Test for Confirmation of Labeled LAL Reagent Sensitivity* under *Preparatory Testing for the Gel-Clot Techniques*). If the test is conducted with a diluted sample solution, calculate the concentration of endotoxin in the original sample solution by multiplying by the dilution factor. If none of the dilutions of the sample solution is positive in a valid assay, report the endotoxin concentration as less than λ (if the diluted sample was tested, less than λ times the lowest dilution factor of the sample.) If all dilutions are positive, the endotoxin concentration is reported as equal to

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or greater than the greatest dilution factor multiplied by λ (e.g., initial dilution factor times 8 times λ in Table 3).

The article meets the requirements of the test if the concentration of endotoxin is less than that specified in the individual monograph.

PHOTOMETRIC TECHNIQUES

The turbidimetric method measures increases in turbidity. Depending on the test principle used, this technique is classified as either endpoint-turbidimetric or kinetic-turbidimetric. The endpoint-turbidimetric technique is based on the quantitative relationship between the concentration of endotoxins and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period. The kinetic-turbidimetric technique is a method to measure either the onset time needed to reach a predetermined absorbance of the reaction mixture or the rate of turbidity development.

The chromogenic method measures the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the LAL Reagent. Depending on the test principle employed, this technique is classified as either endpoint-chromogenic or kinetic-chromogenic. The endpoint-chromogenic technique is based on the quantitative relationship between the concentration of endotoxins and the release of chromophore at the end of an incubation period. The kinetic-chromogenic technique is a method to measure either the onset time needed to reach a predetermined absorbance of the reaction mixture or the rate of color development.

All photometric tests are carried out at the incubation temperature recommended by the LAL Reagent manufacturer, which is usually 37 ± 1°.

Preparatory Testing for the Photometric Techniques

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to verify that the criteria for the standard curve are valid and that the sample solution does not inhibit or enhance the reaction. Revalidation for the test method is required when conditions that are likely to influence the test result change.

Verification of Criteria for the Standard Curve — Using the Standard Endotoxin Solution,

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prepare at least three endotoxin concentrations to generate the standard curve. Perform the test using at least three replicates of each standard endotoxin concentration according to the manufacturer's instructions for the LAL Reagent (with regard to volume ratios, incubation time, temperature, pH, etc.). If the desired range in the kinetic methods is greater than two logs, additional standards should be included to bracket each log increase within the range of the standard curve. The absolute value of the correlation coefficient, $|r|$, must be greater than or equal to 0.980 for the range of endotoxin concentrations indicated by the manufacturer of the LAL Reagent.

Interfering Factors Test for the Photometric Techniques — Select an endotoxin concentration at or near the middle of the endotoxin standard curve. Prepare Solutions A, B, C, and D as shown in Table 4. Perform the test on Solutions A, B, C, and D at least in duplicate following the instructions for the LAL Reagent used (with regard to volume of sample and LAL Reagent, volume ratio of sample to LAL Reagent, incubation time, etc.).

Table 4. Preparation of Solutions for the Inhibition/Enhancement Test for Photometric Techniques

Solution	Endotoxin Concentration	Solution to which Endotoxin is Added	Number of Replicates
A[a]	none	sample solution	not less than 2
B[b]	middle concentration of the standard curve	sample solution	not less than 2
C[c]	at least 3 concentrations (lowest concentration is designated λ)	LAL Reagent Water	each not less than 2
D[d]	none	LAL Reagent Water	not less than 2

aSolution A: the sample solution may be diluted not to exceed MVD.

b Solution B: the preparation under test at the same dilution as Solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.

c Solution C: the standard endotoxin at the concentrations used in the validation of the method described in *Verification of Criteria for the Standard Curve* under *Preparatory Testing for the Photometric Techniques* (positive control series).

d Solution D: LAL Reagent Water (negative control).

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) from that containing the added endotoxin. In order to be considered free of interfering factors under the conditions of the test, the measured concentration of the endotoxin added to the sample solution must be within 50% to 200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin.

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When the endotoxin recovery is out of the specified ranges, the interfering factors must be removed as described in the *Interfering Factors Test for the Gel-Clot Techniques* under *Preparatory Testing for the Gel-Clot Techniques.* Repeating the *Interfering Factors Test for the Gel-Clot Techniques* validates the treatment.

Procedure for the Photometric Techniques

Follow the procedure described in the *Interfering Factors Test for the Photometric Techniques* under *Preparatory Testing for the Photometric Techniques.*

Calculation for the Photometric Techniques

Calculate the endotoxin concentration of each of the replicates of test Solution A using the standard curve generated by positive control series C. The test is not valid unless the following conditions are met: (1) the results of control series C comply with the requirements for validation defined under *Verification of Criteria for the Standard Curve* under *Preparatory Testing for the Photometric Techniques;* (2) the endotoxin recovery, calculated from the concentration found in Solution B after subtracting the endotoxin concentration found in Solution A is within 50 to 200%; and (3) the result of negative control series D does not exceed the limit of the blank value required in the description of the LAL Reagent used.

Interpretation of Results from the Photometric Techniques

In photometric assays, the preparation under test complies with the test if the mean endotoxin concentration of the replicates of Solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

F 1 LAL Reagent reacts with some β-glucans in addition to endotoxins. Some preparations that are treated will not react with β-glucans and must be used for samples that contain glucans. [F]

F 2 For a validity test of the procedure for inactivating endotoxins, see *Dry-Heat Sterilization* under Sterilization and Sterility Assurance of Compendial Articles 〈 1211〉. Use an LAL Reagent having a sensitivity of not less than 0.15

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Endotoxin Unit per mL. F

F 3 *Sterile Water for Injection* or other water that shows no reaction with the specific LAL Reagent with which it is to be used, at the limit of sensitivity of such reagent. F

F 4 *K* is 5 USP-EU/kg for any route of administration other than intrathecal (for which *K* is 0.2 USP-EU/kg body weight). For radiopharmaceutical products not administered intrathecally the endotoxin limit is calculated as 175/ *V*, where *V* is the maximum recommended dose in mL. For intrathecally administered radiopharmaceuticals, the endotoxin limit is obtained by the formula 14/ *V*. For formulations (usually anticancer products) administered on a per square meter of body surface, ! the formula is *K*/ *M*, where *K* = 5 EU/kg and *M* is the (maximum dose/m^2/hour × 1.80 m^2)/70 Kg. ! 1S (*USP26*) F

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