⟨85⟩ BACTERIAL ENDOTOXINS TEST

Change to read:

✦Portions of this general chapter have been harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. Those portions that are not harmonized are marked with symbols (✦✦) to specify this fact.✦

The Bacterial Endotoxins Test (BET) is a test to detect or quantify endotoxins from Gram-negative bacteria using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*).

There are three techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex. Proceed by any of the three techniques for the test. In the event of doubt or dispute, the final decision is made based upon the gel-clot ■limit test■2S (USP35) unless otherwise indicated in the monograph for the product being tested. The test is carried out in a manner that avoids endotoxin contamination.

APPARATUS

Depyrogenate all glassware and other heat-stable materials in a hot air oven using a validated process.✦[1]✦ A commonly used minimum time and temperature is 30 min at 250°. If employing plastic apparatus, such as microplates and pipet tips for automatic pipetters, use apparatus that is shown to be free of detectable endotoxin and does not interfere in the test. [NOTE—In this chapter, the term "tube" includes any other receptacle such as a microtiter well.]

REAGENTS AND TEST SOLUTIONS

Amoebocyte Lysate—A lyophilized product obtained from the lysate of amoebocytes (white blood cells) from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This reagent refers only to a product manufactured in accordance with the regulations of the competent authority. [NOTE—*Amoebocyte Lysate* reacts to some β-glucans in addition to endotoxins. *Amoebocyte Lysate* preparations that do not react to glucans are available: they are prepared by removing the G factor reacting to glucans from *Amoebocyte Lysate* or by inhibiting the G factor reacting system of *Amoebocyte Lysate* and may be used for endotoxin testing in the presence of glucans.]

Water for Bacterial Endotoxins Test (BET)—Use Water for Injection or water produced by other procedures that shows no reaction with the lysate employed, at the detection limit of the reagent.

Lysate TS—Dissolve *Amoebocyte Lysate* in *Water for BET*, or in a buffer recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, according to the specifications of the manufacturer.

✦[1] For a validity test of the procedure for inactivating endotoxins, see *Dry-Heat Sterilization* under *Sterilization and Sterility Assurance of Compendial Articles* ⟨1211⟩. Use *Lysate TS* having a sensitivity of not less than 0.15 Endotoxin Unit per mL.✦

Change to read:

PREPARATION OF SOLUTIONS

Standard Endotoxin Stock Solution—A *Standard Endotoxin Stock Solution* is prepared from a USP Endotoxin Reference Standard that has been calibrated to the current WHO International Standard for Endotoxin. Follow the specifications in the package leaflet and on the label for preparation and storage of the *Standard Endotoxin Stock Solution*. Endotoxin is expressed in Endotoxin Units (EU). [NOTE—One USP Endotoxin Unit (EU) is equal to one International Unit (IU) of endotoxin.]

Standard Endotoxin Solutions—After mixing the *Standard Endotoxin Stock Solution* vigorously, prepare appropriate serial dilutions of *Standard Endotoxin Solution*, using *Water for BET*. Use dilutions as soon as possible to avoid loss of activity by adsorption.

Sample Solutions—Prepare the *Sample Solutions* by dissolving or diluting drugs ■■2S (USP35) using *Water for BET*. Some substances or preparations may be more appropriately dissolved, ■or diluted■2S (USP35) in other aqueous solutions. If necessary, adjust the pH of the solution to be examined (or dilution thereof) so that the pH of the mixture of the lysate and *Sample Solution* falls within the pH range specified by the lysate manufacturer, usually 6.0–8.0. The pH may be adjusted by use of an acid, base, or suitable buffer as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with *Water for BET* in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

Change to read:

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. Determine the MVD from the following equation:

$$\text{MVD} = (\text{endotoxin limit} \times \text{concentration of } \textit{Sample Solution})/(\lambda)$$

Endotoxin Limit—The endotoxin limit for parenteral drugs, defined on the basis of dose, equals K/M✦[2]✦, where K is a threshold pyrogenic dose of endotoxin per kg of body weight, and M is equal to the maximum recommended bolus dose of product per kg of body weight. When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period. The endotoxin limit for parenteral drugs is specified in the individual monograph in units such as EU/mL, EU/mg, EU/Unit of biological activity, etc.

Concentration of Sample Solution—

mg/mL: in the case of endotoxin limit specified by weight (EU/mg);

✦[2] ■K is 5 USP-EU/kg of body weight for any route of administration other than intrathecal (for which K is 0.2 USP-EU/kg of body weight). For radiopharmaceutical products not administered intrathecally, the endotoxin limit is calculated as 175 EU/V, where V is the maximum recommended dose in mL. For intrathecally administered radiopharmaceuticals, the endotoxin limit is obtained by the formula 14 EU/V. For formulations (usually anticancer products) administered on a per square meter of body surface, the formula is K/M, where K = 100 EU/m^2 and M is the maximum dose/m^2.✦■2S (USP35)

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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	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*	*Water for BET*	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D[d]	None/*Water for BET*	—	—	—	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 lysate sensitivity.
[d] *Solution D*: Negative control of *Water for BET*.

Units/mL: in the case of endotoxin limit specified by unit of biological activity (EU/Unit);

mL/mL: when the endotoxin limit is specified by volume (EU/mL).

λ: the labeled sensitivity in the *Gel-Clot Technique* (EU/mL) or the lowest concentration used in the standard ■2S (*USP35*) curve for the *Turbidimetric Technique* or *Chromogenic Technique*.

Change to read:

GEL-CLOT TECHNIQUE

The gel-clot technique is used for detecting or quantifying endotoxins based on clotting of the lysate reagent in the presence of endotoxin. The minimum concentration of endotoxin required to cause the lysate to clot under standard conditions is the labeled sensitivity of the lysate reagent. To ensure both the precision and validity of the test, perform the tests for confirming the labeled lysate sensitivity and for interfering factors as described in *Preparatory Testing*, immediately below.

Preparatory Testing

Test for Confirmation of Labeled Lysate Sensitivity— Confirm in four replicates the labeled sensitivity, λ, expressed in EU/mL of the lysate prior to use in the test. The test for confirmation of lysate sensitivity is to be carried out when a new batch of lysate is used or when there is any change in the test conditions that may affect the outcome of the test. Prepare standard solutions having at least four concentrations equivalent to 2λ, λ, 0.5λ, and 0.25λ by diluting the USP Endotoxin RS with *Water for BET*.

Mix a volume of the *Lysate TS* with an equal volume (such as 0.1-mL aliquots) of one of the *Standard Endotoxin Solutions* in each test tube. When single test vials or ampuls containing lyophilized lysate are used, add solutions directly to the vial or ampul. Incubate the reaction mixture for a constant period according to the directions of the lysate manufacturer (usually at $37 \pm 1°$ for 60 ± 2 min), 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 considered valid when the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The endpoint is the smallest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean endpoint by calculating the mean of the logarithms of the endpoint concentrations of the four replicate series and then taking the antilogarithm of the mean value, as indicated in the following formula:

$$\text{geometric mean endpoint concentration} = \text{antilog}\ (\Sigma e/f)$$

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 lysate (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.

Test for Interfering Factors—Usually prepare solutions (A–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, operating as described for *Test for Confirmation of Labeled Lysate Sensitivity*. The geometric mean endpoint concentrations of *Solutions B* and *C* are determined using the formula described in the *Test for Confirmation of Labeled Lysate Sensitivity*. ●The test for interfering factors must be repeated when any condition changes that is likely to influence the result of the test.● (IRA 1-Apr-2011)

The test is considered valid when all replicates of *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 *Solution B* is not less than 0.5λ and not greater than 2λ, the *Sample Solution* does not contain factors that 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

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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	Endotoxin Concentration	Number of Replicates
A[a]	None/*Sample Solution*	*Water for BET*	1	—	2
			2	—	2
			4	—	2
			8	—	2
B[b]	2λ/*Sample Solution*	—	1	2λ	2
C[c]	2λ/*Water for BET*	*Water for BET*	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D[d]	None/*Water for BET*	—	—	—	2

[a] *Solution A*: *Sample Solution* under test at the dilution, not to exceed the MVD, with which the *Test for Interfering Factors* was completed. Subsequent dilution of the *Sample Solution* must not exceed the MVD. Use *Water for BET* to make a dilution series of four tubes containing the *Sample Solution* under test at concentrations of 1, 1/2, 1/4, and 1/8 relative to the concentration used in the *Test for Interfering Factors*. Other dilutions up to the MVD may be used as appropriate.
[b] *Solution B*: *Solution A* containing standard endotoxin at a concentration of 2λ (positive product control).
[c] *Solution C*: Two replicates of four tubes of *Water for BET* containing the standard endotoxin at concentrations of 2λ, λ, 0.5λ, and 0.25λ, respectively.
[d] *Solution D*: *Water for BET* (negative control).

that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described above using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

Limit Test

Procedure—Prepare *Solutions A, B, C,* and *D* as shown in *Table 2*, and perform the test on these solutions following the procedure above for *Preparatory Testing*, *Test for Confirmation of Labeled Lysate Sensitivity*.

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/*Water for BET*	2
D	None/*Water for BET*	2

* Prepare *Solution A* and the positive product control *Solution B* using a dilution not greater than the MVD and treatments as described for the *Test for Interfering Factors* in *Preparatory Testing*. The positive control *Solutions B* and *C* contain the *Standard Endotoxin Solution* at a concentration corresponding to twice the labeled lysate sensitivity. The negative control *Solution D* consists of *Water for BET.*

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

When a positive result is found for one replicate of *Solution A* and a negative result is found for the other, repeat the test. In the repeat test, the preparation under test complies with the test if a negative result is found for both replicates of *Solution A*. The preparation does not comply with the test if a positive result is found for one or both replicates of *Solution A.* However, if the preparation does not comply with the test at a dilution less than the MVD, the test may be repeated using a greater dilution, not exceeding the MVD.

Quantitative Test

Procedure—The test quantifies bacterial endotoxins in *Sample Solutions* by titration to an endpoint. Prepare *Solutions A, B, C,* and *D* as shown in *Table 3*, and test these solutions by following the procedure in *Preparatory Testing*, *Test for Confirmation of Labeled Lysate Sensitivity.*

Calculation and Interpretation—The test is considered valid when the following three 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 0.5λ to 2λ.

To determine the endotoxin concentration of *Solution A*, calculate the endpoint concentration for each replicate by multiplying each endpoint dilution factor by λ. The endotoxin concentration in the *Sample Solution* is the endpoint concentration of the replicates. 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, report as less than λ times the lowest dilution factor of the sample). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the greatest dilution factor multiplied by λ (e.g., initial dilution factor times eight times λ in *Table 3*).

The preparation under test meets the requirements of the test if the concentration of endotoxin in both replicates is less than that specified in the individual monograph.

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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 three concentrations (lowest concentration is designated λ)	*Water for BET*	Each not less than 2
D[d]	None	*Water for BET*	Not less than 2

[a] *Solution 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 for *Assurance of Criteria for the Standard Curve* under *Preparatory Testing* (positive controls).
[d] *Solution D*: *Water for BET* (negative control).

PHOTOMETRIC QUANTITATIVE TECHNIQUES

Turbidimetric Technique

This technique is a photometric assay measuring increases in reactant turbidity. On the basis of the particular assay principle employed, this technique may be classified as either an endpoint-turbidimetric assay or a kinetic-turbidimetric assay. The endpoint-turbidimetric assay 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 assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance or transmission of the reaction mixture, or the rate of turbidity development. The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°).

Chromogenic Technique

This technique is an assay to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with lysate. On the basis of the particular assay principle employed, this technique may be classified as either an endpoint-chromogenic assay or a kinetic-chromogenic assay. The endpoint-chromogenic assay 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 assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture, or the rate of color development. The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°).

Preparatory Testing

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 interfere with the test. Validation for the test method is required when conditions that are likely to influence the test result change.

Assurance of Criteria for the Standard Curve—The test must be carried out for each lot of lysate reagent. Using the *Standard Endotoxin Solution,* prepare at least three endotoxin concentrations within the range indicated by the lysate manufacturer to generate the standard curve. Perform the assay using at least three replicates of each standard endotoxin concentration according to the manufacturer's instructions for the lysate (volume ratios, incubation time, temperature, pH, etc.). If the desired range is greater than two logs in the kinetic methods, additional standards should be included to bracket each log increase in 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 set up.

Test for Interfering Factors—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, according to the instructions for the lysate employed, for example, concerning volume of *Sample Solution* and *Lysate TS,* volume ratio of *Sample Solution* to *Lysate TS,* incubation time, etc.

The test is considered valid when the following conditions are met.

1. The absolute value of the correlation coefficient of the standard curve generated using *Solution C* is greater than or equal to 0.980.
2. The result with *Solution D* does not exceed the limit of the blank value required in the description of the lysate reagent employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution, if any (*Solution A, Table 4*), from that containing the added endotoxin (*Solution B, Table 4*). In order to be considered free of factors that interfere with the assay under the conditions of the test, the measured concentration of the endotoxin added to the *Sample Solution* must be within 50%–200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified range, the *Sample Solution* under test is considered to contain interfering factors. Then, repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the *Sample Solution* or diluted *Sample Solution* not to exceed the MVD may be eliminated by suitable validated treatment such as filtration, neutralization, dialysis, or heat treatment. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described above, using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

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Test Procedure

Follow the procedure described for *Test for Interfering Factors* under *Preparatory Testing*, immediately above.

Calculation

Calculate the endotoxin concentration of each of the replicates of *Solution A*, using the standard curve generated by the positive control *Solution C*. The test is considered valid when the following three requirements are met.

1. The results of the control *Solution C* comply with the requirements for validation defined for *Assurance of Criteria for the Standard Curve* under *Preparatory Testing*.
2. The endotoxin recovery, calculated from the concentration found in *Solution B* after subtracting the concentration of endotoxin found in *Solution A*, is within the range of 50%–200%.
3. The result of the negative control *Solution D* does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Interpretation

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.

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