(1) Calibration curve method—Prepare standard solutions at more than 3 concentration levels, measure the specific absorption due to these standard solutions, and prepare the calibration curve of the atomic absorption against the concentration. Then measure the atomic absorption due to the sample specimen, in which the concentration of the element to be determined should be adjusted to be within the concentration range of the standard solutions, and determine the amount or the concentration of the element to be examined using the calibration curve.

(2) Standard addition method—To equal volumes of more than 3 sample solutions, prepared as directed in the monograph, add a measured quantity of the standard solutions to produce a series of solutions containing increasing amounts of the element to be examined, and further add a solvent to make up a constant volume. Measure the atomic absorption for the respective solutions, and plot the obtained values on a graph with the added amount or the concentration on the abscissa and the absorbance on the ordinate. Extrapolate the linear plot obtained by linking the data points, and determine the amount or the concentration of the element to be examined from the distance between the origin and the point where the plot intersects with the abscissa. This method is available only when the calibration curve obtained by Method (1) is confirmed to be linear and to pass through the origin.

(3) Internal standard method—Prepare a series of standard solutions of the element to be determined, each containing a definite amount of the internal standard element directed in the monograph. For these standard solutions, measure the atomic absorption due to the standard element and the internal standard element separately at the respective wavelengths under the same operating conditions, and obtain the ratio of absorbance by the standard element to that by the internal standard element. Prepare a calibration curve for the element to be determined, with the amount or the concentration of the standard element on the abscissa and the above-mentioned ratio of the absorbance on the ordinate. Then prepare sample solutions, adding the same amount of the internal standard element as contained in the standard solutions. Measure the ratio of the absorbance due to the element to be determined to that due to the internal standard element under the same conditions as employed for preparing the calibration curve, and determine the amount or the concentration of the element being examined by using the calibration curve.

Note: Reagents, test solutions, and gases used in this test should not interfere in any process of the measurement.

6. Bacterial Endotoxins Test

The Bacterial Endotoxins Test is a test to detect or quantify bacterial endotoxins of gram-negative bacterial origin using a lysate reagent prepared from blood corpuscle extracts of horseshoe crab (*Limulus polyphemus or Tachypleus tridentatus*). There are two types of techniques for this test: the gel-clot techniques, which are based on gel formation by the reaction of the lysate TS with endotoxins, and the photometric techniques, which are based on endotoxin-induced optical changes of the lysate TS. The latter include turbidimetric techniques, which are based on the change in lysate TS turbidity during gel formation, and chromogenic techniques, which are based on the development of color after cleavage of a synthetic peptide-chromogen complex.

Proceed by any one of these techniques for the test. In the event of doubt or dispute, the final decision is made based on the gel-clot techniques, unless otherwise indicated.

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. Commonly used minimum time and temperature settings are 30 minutes at 250°C. If employing plastic apparatus, such as multi-well plates and tips for micropipettes, use only that which has been shown to be free of detectable endotoxin and which does not interfere with the test.

Preparation of Standard Endotoxin Stock Solution

Prepare Standard Endotoxin Stock Solution by dissolving Endotoxin 10000 Reference Standard or Endotoxin 100 Reference Standard in water for bacterial endotoxins test (BET). Endotoxin is expressed in Endotoxin Units (EU). One EU is equal to one International Unit (IU) of endotoxin.

Preparation of Standard Endotoxin Solution

After mixing Standard Endotoxin Stock Solution thoroughly, 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.

Preparation of sample solutions

Unless otherwise specified, prepare sample solutions by dissolving or diluting drugs, using water for BET. Sample solutions for containers for medicines should be prepared according to other specified procedures. If necessary, adjust the pH of the solution to be examined so that the pH of the mixture of the lysate TS and sample solution falls within the specified pH range for the lysate reagent to be used. This usually applies to a sample solution with a pH in the range of 6.0 to 8.0. TSs or solutions used for adjustment of pH may be prepared using water for BET, and then stored in containers free of detectable endotoxin.

Determination of Maximum Valid Dilution

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a sample solution at which the endotoxin limit can be determined.

Determine the MVD from the following equation:

$$\mathrm{MVD} = \frac{\text{Endotoxin limit} \times \text{Concentration of sample solution}}{\lambda}$$

Endotoxin limit:

The endotoxin limit for injections, defined on the basis of dose, equals K/M, where K is a minimum pyrogenic dose of endotoxin per kg body mass (EU/kg), and M is equal to the maximum dose of product per kg of body mass in a single hour period.

Concentration of sample solution:

mg/mL in the case of endotoxin limit specified by mass (EU/mg)

mEq/mL in the case of endotoxin limit specified by equivalent (EU/mEq)

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

mL/mL in the case of endotoxin limit specified by

volume (EU/mL)

λ : the labeled lysate reagent sensitivity in the gel-clot techniques (EU/mL) or the lowest point used (EU/mL) in the standard regression curve of the turbidimetric or chromogenic techniques

Gel-clot techniques

The gel-clot techniques detect or quantify endotoxins based on clotting of the lysate TS in the presence of endotoxin. To ensure both the precision and validity of the test, perform the tests for confirming the labeled lysate reagent sensitivity and for interfering factors as described under Preparatory testing.

(1) Preparatory testing

(i) Test for confirmation of labeled lysate reagent sensitivity

The labeled sensitivity of lysate reagent is defined as the lowest concentration of endotoxin that is needed to cause the lysate TS to clot under the conditions specified for the lysate reagent to be used.

The test for confirmation of the labeled lysate reagent sensitivity is to be carried out when each new lot of lysate reagent is used or when there is any change in the experimental conditions which may affect the outcome of the test. Perform the test by the following procedures.

Prepare standard solutions having four concentrations equivalent to 2 λ, λ, 0.5 λ and 0.25 λ by diluting the Standard Endotoxin Stock Solution with water for BET. Prepare the lysate TS by dissolving the lysate reagent with water for BET or a suitable buffer. Mix a volume of the lysate TS with an equal volume of one of the standard solutions (usually, 0.1 mL aliquots) in each test tube. When single test vials or ampoules containing lyophilized lysate reagent are used, add solutions directly to the vial or ampoule.

Keep the tubes (or containers such as vials or ampoules) containing the reaction mixture usually at 37 ± 1°C for 60 ± 2 minutes, avoiding vibration. To test the integrity of the gel after incubation, invert each tube or container through approximately 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 either a firm gel is not formed, or if a fragile gel has formed but flows out upon inversion.

Making the standard solutions of four concentrations one set, test four replicates of the set.

The test is not valid unless 0.25 λ of the standard solution shows a negative result in each set of tests. If the test is not valid, repeat the test after verifying the test conditions.

The endpoint is the last positive test in the series of decreasing concentrations of endotoxin. Calculate the geometric mean endpoint concentration using the following formula:

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

Σe = the sum of the log endpoint concentrations of the dilution series used

f = the number of replicates

If the geometric mean endpoint concentration is not less than 0.5 λ and not more than 2.0 λ, the labeled sensitivity is confirmed.

(ii) Test for interfering factors

This test is performed to check for the presence of enhancing or inhibiting factors for the reaction in sample solutions.

Following Table 1, prepare solutions A and B using a sample solution under test, and solutions C and D using water for BET. Test solutions A and B and solutions C and D in quadruplicate and in duplicate, respectively. Concerning the incubation temperature, incubation time, and procedure for the confirmation of gel formation, follow the procedure under (i) Test for confirmation of labeled lysate reagent sensitivity of (1) Preparatory testing.

The geometric mean endpoint concentrations of B and C solutions are determined by using the formula described in (i) Test for confirmation of labeled lysate reagent sensitivity of (1) Preparatory testing.

This test must be repeated when there is any change in the experimental conditions which may affect the outcome of the test.

Table 1

Solution	Concentration of added endotoxin in each solution/Solution to which endotoxin is added	Diluent	Dilution factor	Concentration of added endotoxin after dilution	Number of replicates
A	0/Sample solution	—	—	—	4
B	2λ/Sample solution	Sample solution	1 2 4 8	2λ 1λ 0.5λ 0.25λ	4
C	2λ/Water for BET	Water for BET	1 2 4 8	2λ 1λ 0.5λ 0.25λ	2
D	0/Water for BET	—	—	—	2

The test is valid if solutions A and D show no reaction and the result for solution C confirms the labeled sensitivity.

If the geometric mean endpoint concentration of solution B is not less than 0.5 λ and not greater than 2.0 λ, the sample solution being examined does not contain interfering factors and complies with the test for interfering factors. Otherwise the sample solution 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. Furthermore, interference of the sample solution or diluted sample solution may be eliminated by suitable treatment, such as filtration, neutralization, dialysis or heat treatment.

(2) Limit test

Based on the formation of a firm gel in the presence of endotoxin at above labeled lysate reagent sensitivity, this method tests whether a sample solution contains endotoxin not greater than the endotoxin limit.

(i) Procedure

Prepare solutions A, B, C and D according to Table 2. Making these four solutions one set, test two replicates of the set.

In preparing solutions A and B, use the sample solutions complying with (ii) Test for interfering factors of (1) Preparatory testing. Concerning the test conditions including the incubation temperature, incubation time, and procedure for the confirmation of gel formation, follow the procedure under (i) Test for confirmation of labeled lysate reagent sensitivity of (1) Preparatory testing.

Table 2

Solution	Concentration of added endotoxin in each solution/Solution to which endotoxin is added	Number of replicates
A	0/Sample solution	2
B	2λ/Sample solution	2
C	2λ/Water for BET	2
D	0/Water for BET	2

(ii) Interpretation

The test is valid when both replicates of solutions B and C are positive and those of solution D are negative.

The sample meets the endotoxin limit requirement of the test when a negative result is found for both replicates of solution A.

Repeat the test in duplicate when the test results are positive for one test but negative for the other one. The sample meets the endotoxin limit requirement of the test when a negative result is found for both replicates of solution A in the repeat test.

The sample does not meet the endotoxin limit requirement of the test when a positive result is found for both replicates of the solution A at a dilution equal to the MVD. If the test is positive for the sample at a dilution less than the MVD, the test should be performed at a dilution equal to the MVD.

(3) Assay

The test measures endotoxin concentrations of sample solutions by titration to an endpoint of gel formation.

(i) Procedure

Prepare solutions A, B, C and D according to Table 3. Making these four solutions one set, test two replicates of the set. When preparing solutions A and B, use sample solutions complying with (ii) Test for interfering factors of (1) Preparatory testing. Concerning the test conditions, follow the procedure under (i) Test for confirmation of labeled lysate reagent sensitivity of (1) Preparatory testing.

Table 3

Solution	Concentration of added endotoxin in each solution/Solution to which endotoxin is added	Diluent	Dilution factor*	Concentration of added endotoxin after dilution	Number of replicates
A	0/Sample solution	Water for BET	1 2 4 8	— — — —	2
B	2λ/Sample solution	—	1	2λ	2
C	2λ/Water for BET	Water for BET	1 2 4 8	2λ 1λ 0.5λ 0.25λ	2
D	0/Water for BET	—	—	—	2

* The dilution range of the dilution series of solution A may be changed as appropriate, but not exceeding the MVD.

(ii) Calculation and interpretation

The test is valid when the following three conditions are met: (a) both replicates of the negative control solution D are negative, (b) both replicates of the positive product control solution B are positive and (c) the geometric mean endpoint concentration of solution C is in the range of $0.5\ \lambda$ to $2\ \lambda$.

The endpoint is defined as the maximum dilution showing the last positive test in the dilution series of solution A, and the endotoxin concentration of solution A is calculated by multiplying the endpoint dilution factor by λ.

Calculate the geometric mean endotoxin concentration of the two replicates, using the formula given under (i) Test for confirmation of labeled lysate reagent sensitivity of (1) Preparatory testing.

If none of the dilutions of solution A is positive, report the endotoxin concentration solution A as less than $\lambda \times$ the lowest dilution factor of solution A.

If all dilutions are positive, the endotoxin concentration of solution A is reported as equal to or greater than the greatest dilution factor of solution A multiplied by λ.

Calculate the endotoxin concentration (in EU per mL, in EU per mg or mEq or in EU per Unit) of the sample, based on the mean endotoxin concentration of solution A. The sample complies with the Bacterial Endotoxins Test if the endotoxin concentration of the sample meets the requirement for the endotoxin limit (in EU per mL, in EU per mg or mEq or in EU per Unit) specified in the individual monograph.

Photometric techniques

(1) Turbidimetric technique

This technique measures the endotoxin concentrations of sample solutions based on the measurement of turbidity change accompanying gel formation of the lysate TS. 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 of the reaction mixture at a specified reaction time.

The kinetic-turbidimetric technique is based on the quantitative relationship between the concentration of endotoxins and either the time needed to reach a predetermined turbidity of the reaction mixture or the rate of turbidity development.

The test is usually carried out at 37 ± 1°C, and turbidity is expressed in terms of either absorbance or transmission.

(2) Chromogenic technique

This technique measures the endotoxin concentrations of sample solutions based on the measurement of chromophore released from a synthetic chromogenic substrate by the reaction of endotoxins with the lysate TS. 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 based on the quantitative relationship between the concentration of endotoxins and either the time needed to reach a predetermined absorbance (or transmittance) of the reaction mixture or the rate of color development.

The test is usually carried out at 37 ± 1°C.

(3) Preparatory testing

To assure the precision and validity of the turbidimetric or chromogenic techniques, perform both Test for assurance of criteria for the standard curve and Test for interfering factors, as indicated below.

(i) Test for 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 to generate the standard curve within the range of endotoxin concentrations indicated by the instructions for the lysate reagent used. Perform the test using at least three replicates of each standard endotoxin concentration according to the optimal conditions for the lysate reagent used (with regard to volume ratios, incubation time, temperature, pH, etc.). If the desired range is greater than two logs, 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.

If the test is not valid, repeat the test after verifying the test conditions.

The test for assurance of criteria for the standard curve must be repeated when any condition changes, which is likely to influence the result of the test.

(ii) Test for interfering factors

Prepare solutions A, B, C and D according to Table 4. Perform the test on these solutions following the optimal conditions for the lysate reagent used (with regard to volume of sample solution and lysate TS, volume ratio of sample solution to lysate TS, incubation time, etc.).

The test for interfering factors must be repeated when any condition changes, which is likely to influence the result of the test.

Table 4

Solution	Concentration of added endotoxin in each solution	Solution to which endotoxin is added	Number of test tubes or wells
A	0	Sample solution*1	Not less than 2
B	Middle concentration of the standard curve*2	Sample solution*1	Not less than 2
C	At least 3 concentrations*3	Water for BET	Each not less than 2
D	0	Water for BET	Not less than 2

*1 The sample solution may be diluted not to exceed the MVD.
*2 Add the Standard Endotoxin Solution to the sample solution to make an endotoxin concentration at or near the middle of the standard curve.
*3 The concentrations used in the test for the (i) Assurance of criteria for the standard curve of (3) Preparatory testing.

The test is not valid unless 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 recovery of the endotoxin added to solution B from the concentration found in solution B after subtracting the endotoxin concentration found in solution A.

When the recovery of the endotoxin added to solution B is within 50% to 200%, the sample solution under test is considered to be free of interfering factors.

When the endotoxin recovery is out of the specified range, the sample solution under test is considered to contain interfering factors. If the sample under test does not comply with the test, 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 treatment, such as filtration, neutralization, dialysis or heat treatment.

(4) Assay

(i) Procedure

Prepare solutions A, B, C and D according to Table 4, and follow the procedure described in (ii) Test for interfering factors of (3) Preparatory testing.

(ii) Calculation of endotoxin concentration

Calculate the endotoxin concentration of solution A using the standard curve generated with solution C. The test is not valid unless all the following requirements 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 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% to 200%.

3: 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.

(iii) Interpretation

The sample complies with the Bacterial Endotoxins Test if the endotoxin concentration of the sample calculated from the endotoxin concentration of solution A meets the requirement of the endotoxin limit (in EU per mL, in EU per mg or mEq or in EU per Unit) specified in the individual monograph.

7. Boiling Point and Distilling Range Test

The Boiling Point and Distilling Range are determined by Method 1 or Method 2 as described herein, unless otherwise specified. Boiling point is the temperature shown between when the first 5 drops of distillate leave the tip of the condenser and when the last liquid evaporates from the bottom of the flask. Distilling range test is done to determine the volume of the distillate which has been collected in the range of temperature directed in the monograph.

Method 1 This method is applied to a sample for which the permissible range of boiling temperature is smaller than 5°C.

(1) Apparatus

Use the apparatus illustrated in the figure.

(2) Procedure

Measure 25 mL of the sample, whose temperature is previously noted, using a volumetric cylinder G graduated in 0.1 mL, and transfer it to a distilling flask A of 50- to 60-mL capacity. Use this cylinder as the receiver for the distillate without rinsing out any of the adhering liquid. Put boiling chips into the distilling flask A, insert a thermometer B with an immersion line so that its immersion line C is on a level with the lower end of cork stopper D and the upper end of its mercury bulb is located in the center of the delivery tube, and connect condenser E with the distilling flask A and adapter F with the condenser E. Insert the open end of F into the mouth of cylinder G (receiver) so that air can pass through slightly. Use a hood with a height sufficient to shield A, and heat A with a suitable heat source. When direct flame is applied as the heat source, put A on a hole of an asbestos board (a wire gauge of 150 mL square bonded to asbestos 6 mm