

Evaluation of the results If none of the 3 rabbits in the first test shows a temperature rise of 0.6°C or more, and the sum of the 3 temperature rises does not exceed 1.3°C, or if only one of the 5 rabbits in the second test shows a temperature rise of 0.6°C or more, and the sum of the 8 temperature rises does not exceed 3.5°C, the preparation being examined passes the test.

If more than one rabbit in either test show a temperature rise of 0.6°C or more; or the sum of the 8 temperature rises exceed 3.5°C, the preparation being examined is considered to be pyrogenic.

When the temperature rise is negative, the result is counted as a zero response.

1143 Test for Bacterial Endotoxin

The test for bacterial endotoxins is used to detect or quantify endotoxins of gram-negative bacterial origin using amoebocyte lysate from horseshoe crab (*Tachypleus tridentatus* or *Limulus polyphemus*).

Two methods are used for this test: the gel-clot method and the photometric method. The latter includes a turbidimetric method and a chromogenic method. Proceed by any one of these two methods for the test.

In the event of doubt or dispute, the final decision is made based on the gel-clot limit method, unless otherwise indicated in the monograph.

The test is carried out in a manner that avoids endotoxin contamination.

Endotoxin is expressed in Endotoxin Units (EU).

One Endotoxin Unit (EU) is equal to one International Unit (IU) of endotoxin.

The National Standard Endotoxin (NSE) is prepared and purified from *Escherichia coli*. That has been calibrated against the International Standard. It is used for calibration of the working standard endotoxin (WSE). It also is used for calibration, confirmation and arbitrating the sensitivity of lysate reagents. NSE can be used for bacterial endotoxin test as positive control (solution B and solution C), for interference test, confirmation of labelled lysate reagent sensitivity, and for the standard curve in photometric method.

The working standard endotoxin (WSE) has been calibrated against the NSE. It is used for bacterial endotoxins test as positive control (solution B and solution C), for interference test, confirmation of labelled lysate reagent sensitivity, and for the standard curve in photometric method.

Water for bacterial endotoxins test (water for BET) should meet the standard of sterile water for injection, and must be free of interfering factors. The concentration of bacterial endotoxins of water used in the gel-clot method is less than 0.015 EU/ml, while the concentration is less than 0.005 EU/ml in the photometric method.

Amoebocyte lysate is a lyophilised product obtained from the lysate of amoebocyte from the horseshoe crab. This reagent refers only to a product manufactured in accordance with the regulations of the competent authority. Amoebocyte lysate reacts with some β-glucans in addition to endotoxins. Amoebocyte lysate preparations which do not react with glucans are available; they are prepared by removing from amoebocyte lysate the G factor, which reacts with glucans, or by inhibiting the G factor reacting system of amoebocyte lysate. These preparations may be used for endotoxin testing in the presence of glucans.

Depyrogenate all glassware and other heat-stable apparatus

by heating in a hot-air oven at 250°C for equal or more than 30 minutes (or by any other validated suitable method) to eliminate extraneous endotoxin that may present. If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipettors, use apparatus shown to be free of detectable endotoxin and of interfering factors for the test.

Preparation of the test solutions Prepare the test solutions by dissolving or diluting active substances or extracting in aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the lysate reagents and test solution falls within the pH range of 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a suitable buffer. 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.

Establishment of endotoxin limits The endotoxin limit (*L*) for drugs or biological products is usually defined as follows;

$$L = K/M$$

Where: *L* is the endotoxin limit for active substances administered parenterally, which is specified in units such as EU/ml, EU/mg, or EU/Unit of biological activity;

K is the threshold human pyrogenic dose of endotoxin per kg of body mass in a single hour period, which is expressed as EU/(kg·h). For injections, *K* = 5 EU/(kg·h); for injections of radio-pharmaceuticals, *K* = 2.5 EU/(kg·h); and for intrathecal injections, *K* = 0.2 EU/(kg·h);

M is the maximum recommended bolus dose of product per kg of body mass in a single hour period, which is specified in units such as ml/(kg·h), mg/(kg·h), or U/(kg·h). Here the human average body weight is 60 kg; human body surface area is calculated according to 1.62 m², the injection period is calculated as 1 hour when the injection is completed within 1 hour. The dose of test product per square metre of body surface area multiplied by 0.027 can be converted to dose of product per kg of body mass (*M*).

The endotoxin limit calculated by human dose maybe adjusted according to the situation of manufacture and clinical use if necessary. In that case, appropriate reasons for adjustment have to be submitted.

Determination of the maximum valid dilution (MVD) The maximum valid dilution (MVD) is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. Determine the MVD using the following formulae;

$$MVD = cL/\lambda$$

Where: *L* is the endotoxin limit of the sample being examined;

c is the concentration of the test solution, when *L* is expressed as EU/mg or EU/U, the unit of *c* is mg/ml or U/ml; When *L* is expressed as EU/ml, *c* is 1.0 ml/ml. Minimum valid concentration (the concentration of substance in MVD), can be calculated using the formulae, *c* = λ/L ;

λ is the labelled lysate reagent sensitivity in the gel-clot method (EU/ml) or the lowest point used in the standard curve of the photometric method.

Gel-clot Method (Method 1)

The gel-clot method detects or qualifies endotoxins and is based on clotting of the lysate reagent in the presence of endotoxins.

Test for confirmation of labelled lysate reagent sensitivity

The minimum concentration of endotoxin required to cause the lysate to clot under standard conditions is the labelled sensitivity of the lysate reagent. The test for confirmation of the labelled lysate reagent sensitivity is carried out when each new batch of lysate reagent is used or when there is any change in the test conditions which may affect the outcome of the test.

Dissolve NSE or WSE using water for BET, mix continuously for 15 minutes or following the instruction of the standard using a vortex mixer. Prepare standard solutions of 4 concentrations equivalent to 2λ , 1.0λ , 0.5λ and 0.25λ by diluting the NSE or WSE with water for BET, mix each dilution for 30 seconds or following the instruction of the standard using a mixer. Mix a volume of the lysate solution with an equal volume (such as 0.1 ml/ aliquots) of one of the standard endotoxin solutions in each tube, every standard concentration in four replicates, and water for BET to each of 2 tubes as negative control. Mix gently after each addition, cover the tubes tightly and incubate the tubes vertically at $37^\circ\text{C} \pm 1^\circ\text{C}$ for 60 minutes ± 2 minutes avoid vibration. Take each tube in turn directly from the incubator and invert it

through approximately 180° in one smooth motion to test the integrity of the gel. 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. Handle the tubes with care to avoid vibration, or false negative may result. The test is not valid unless the highest concentration (2.0λ) of the standard solution shows positive results in all replicate tubes, the lowest concentration (0.25λ) shows negative results in all replicate tubes and the duplicated tubes of negative control show negative results. Calculate the geometric mean endpoint concentration, i. e. the measured sensitivity of the lysate reagent (λ_e), using the following expression:

$$\lambda_e = \text{antilog} (\sum X/n)$$

Where: X is the log endpoint concentration. The endpoint concentration is the last positive result in a series of decreasing concentrations of endotoxin; n is the number of replicates.

If the λ_e is not less than 0.5λ and not more than 2λ , the labelled sensitivity (λ) is confirmed and is used in tests performed with this lysate reagent.

Test for interfering factors Prepare solutions A, B, C and D as shown in Table 1, and use the test solutions at a dilution less than the MVD, not containing any detectable endotoxins, operating as described under the "Test for confirmation of labelled lysate reagent sensitivity".

Table 1 Preparation of solutions in the test for interfering factors by using gel-clot method

Solution	Endotoxin concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Initial endotoxin concentration	Number of replicates
A	None/Test solution	—	—	—	2
B	2 λ /Test solution	Test solution	1	2 λ	4
			2	1 λ	4
			4	0.5 λ	4
			8	0.25 λ	4
C	2 λ /Water for BET	Water for BET	1	2 λ	2
			2	1 λ	2
			4	0.5 λ	2
			8	0.25 λ	2
D	None/Water for BET	—	—	—	2

Note: Solution A: solution of the preparation being examined that is free of detectable endotoxins.

Solution B: test for interference.

Solution C: control of the labelled lysate reagent sensitivity.

Solution D: negative control (water for BET).

The test is not valid unless all replicates of solutions A and D show negative results and the result of solution C confirms the labelled lysate reagent sensitivity.

If the result of solution B confirms the labeled lysate reagent sensitivity, the test solution does not contain interfering factors under the experimental conditions used. Otherwise, the solution interferes with the test. If the preparation being examined interferes with the test at a dilution less than the MVD, repeat the test for interfering factors using a greater dilution, not exceeding the MVD.

Interference may be overcome by suitable treatment, such as filtration, neutralization, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

When establishing a method of bacterial endotoxin test for a new drug, the test for interfering factors should be carried out. When the source of the lysate reagent, or the formula or the manufacture process of the substance being examined are

changed, or there is any change in the experimental conditions that are likely to influence the result of the test, the test for interfering factors should be performed again.

Procedure**(1) Gel-clot limit test**

Prepare solutions A, B, C and D as shown in Table 2, and perform the test following the procedure in the "Test for confirmation of labelled lysate reagent sensitivity".

Table 2 Preparation of solutions in gel-clot limit test

Solution	Endotoxin concentration/Solution to which endotoxin is added	Number of replicates
A	None/Diluted test solution	2
B	2 λ /Diluted test solution	2
C	2 λ /Water for BET	2
D	None/Water for BET	2

Note: Solution A: solution of the preparation being examined.

Solution B: positive product control.

Solution C: positive control.

Solution D: negative control (water for BET).

Prepare solution A and solution B using a dilution not greater than the MVD and treatments as described in Test for interfering factors.

Interpretation The test is not valid unless both replicates of the two positive control solutions B and C are positive and those of negative control solution D are negative.

When a negative result is found for both replicates of solution A, the preparation being examined complies with the test. When a positive result is found for both replicates of solution A, the preparation being examined 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 by 4 replicates of solution A. The preparation being examined complies with the test when a negative result is found for all replicates of solution A in the repeat test. Otherwise, the preparation being examined does not comply with the test.

However, if the preparation does not comply with the test at a dilution less than the MVD, the test may be repeated at a dilution of MVD.

(2) Gel-clot semi-quantitative test

The test quantifies bacterial endotoxins in the test solution by titration to an endpoint. Prepare solutions A, B, C and D as shown in Table 3, and perform the test following the procedure in the "Test for confirmation of labelled lysate reagent sensitivity".

Calculation and interpretation The test is not valid unless the following 3 conditions are met: ① both replicates of solution D (negative control) are negative; ② both replicates of solution B (positive product control) 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 series of dilutions by multiplying each endpoint dilution factor by λ . If the test is conducted with a diluted test solution, calculate the concentration of endotoxin in the original solution (c) by multiplying the result by the dilution factor.

The preparation being examined meets the requirements of the test if the endotoxin concentration is less than that specified in the individual monograph. The endotoxin concentration in the test solution is the geometric mean endpoint concentration of the replicates [$c_E = \text{antilog}(\sum \lg c/2)$]. If none of the dilutions of the test solution is positive in a valid test, record the endotoxin concentration as less than λ (or, if a diluted sample was tested, as less than $\lambda \times$ the lowest dilution factor of the sample).

The preparation being examined does not meet the requirements of the test, if the endotoxin concentration is not less than that specified in the individual monograph. If all dilutions are positive, the endotoxin concentration is recorded as equal to or greater than the greatest dilution factor multiplied by λ .

Table 3 Preparation of solution in the gel-clot semiquantitative test

Solution	Endotoxin concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Initial endotoxin concentration	Number of replicates
A	None/Test solution	Water for BET	1	not more than 1λ	2
			2	—	2
			4	—	2
			8	not less than 0.25λ	2
B	2λ /Test solution		1	2λ	2
C	2λ /Water for BET	Water for BET	1 2 4 8	2λ 1λ 0.5λ 0.25λ	2 2 2 2
D	None/Water for BET		—	—	2

Note: Solution A: test solution at the dilution, not exceeding the MVD, with which the test for interfering factors was carried out. Subsequent dilution of the test solution must not exceed the MVD. Use water for BET to make two dilution series of 1, 1/2, 1/4, and 1/8, relative to the dilution with which the test for interfering factors was carried out.

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

Solution C: series of water for BET containing the standard endotoxin at concentrations of 2λ , λ , 0.5λ , and 0.25λ , respectively.

Solution D: negative control (water for BET).

Photometric Method (Method 2)

The photometric method includes a turbidimetric method and a chromogenic method. The turbidimetric method measures the endotoxin concentrations of test solutions based on the measurement of the increase in turbidity during the gel formation of the lysate reagent. Depending on the test principle used, this method is classified as being either the endpoint-turbidimetric test or the kinetic-turbidimetric test. The endpoint-turbidimetric test is based on the quantitative relationship between the endotoxin concentration and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period. The kinetic-turbidimetric test is a method to measure either the onset time needed for the reaction mixture to reach a predetermined absorbance, or light transmittance, 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 lysate reagent. Depending on the test principle employed, this method is classified as being either the endpoint-chromogenic test or the kinetic-chromogenic test. The endpoint-chromogenic test is based on the quantitative relationship between the endotoxin concentration and the quantity of chromophore released at the end of an incubation period. The kinetic-chromogenic test is a method to measure either the onset time needed for the reaction mixture to reach a predetermined absorbance or light transmittance, or the rate of colour development.

All photometric tests are usually carried out by special instrumentation at the incubation temperature of $37^\circ\text{C} \pm 1^\circ\text{C}$. The volume ratios of the substance being examined and the lysate reagent, incubation time, etc. employed in the test are decided according to the related instructions of instruments and the lysate reagents.

To assure the precision or validity of the turbidimetric and chromogenic tests, preparatory tests are conducted to assure

that the criteria for the standard curve are satisfied and that the test solution does not interfere with the test.

Assurance of criteria for the standard curve The test for Assurance of criteria for the standard curve must be carried out when each new batch of lysate reagent is used or any changes are made to the experimental conditions that are likely to influence the result of the test. Using the standard endotoxin solution, prepare at least three endotoxin concentrations (the dilution factor of adjacent concentrations is not greater than 10) to generate the standard curve within the range of endotoxin concentrations indicated by the lysate reagent manufacturer. The mixing time for every dilution is the same as that of gel-clot method. Perform the test using at least three replicates of each standard endotoxin solution, and duplicate of negative control solution at the same time. When absorbances of the duplicate of negative control solution are less than that of the lowest concentration, or light transmittances, or both reaction times of the duplicate of negative control solution are greater than that of the lowest concentration, perform statistical analysis of linear regression for all the data.

The test is not valid unless the absolute value of the correlation coefficient, $|r|$, is greater than or equal to 0.980, otherwise repeat the test.

Test for interfering factors Select an endotoxin concentration (λ_m) at or near the middle of the endotoxin standard curve. Prepare solutions A, B, C and D as shown in Table 4.

Table 4 Preparation of solutions in the test for interfering factors by using photometric method

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2
B	Middle concentration (λ_m) of the standard curve	Test solution	Not less than 2
C	At least 3 concentrations (lowest concentration is designated λ)	Water for BET	Each concentration not less than 2
D	None	Water for BET	Not less than 2

Note: Solution A: test solution that may be diluted not to exceed the MVD.

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

Solution C: standard endotoxin solution at the concentrations used in the validation of the method described in assurance of criteria for the standard curve.

Solution D: negative control (water for BET).

Calculate the content of endotoxin contained in the solution A (c_T) and solution B (c_S), respectively, and calculate the recovery of the endotoxin added to solution B as follows;

$$R = [(c_S - c_T)/\lambda_m] \times 100\%$$

If under the conditions of the test, the recovery of the endotoxin added to solution B is within 50% to 200%, the test solution is considered free of interfering factors. When the endotoxin recovery is out of the specified ranges, the interfering factors must be removed as described in the Test for interfering factors under *Gel-clot Method*. The

efficiency of the treatment is verified by repeating the test for interfering factors.

When the source of the lysate reagent, or the origin, formula and the manufacture process of the substance being examined are changed, or there is any change in the experimental conditions that are likely to influence the result of the test, the Test for interfering factors should be performed again.

Procedure Follow the procedure described in the "Test for interfering factors" under *Photometric Method*. Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the series of positive controls, solution C.

The test is not valid unless the following 3 requirements are met;

- (1) the results obtained with the series of positive controls, solution C, comply with the requirements for validation defined in the "Assurance of criteria for the standard curve" under *Photometric Method*;
- (2) the endotoxin recovery, calculated from the endotoxin concentration found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50% to 200%;
- (3) the result obtained with solution D (negative control) is less than the lowest concentration of the standard curve, or the reaction time of solution D (negative control) are greater than that of the lowest concentration of the endotoxin standard curve.

Calculation and interpretation The preparation being examined 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. Otherwise, the preparation being examined does not meet the requirements of the test.

Note: In this chapter, the term "tube" includes all types of receptacles, for example, microtiter plate wells.

1144 Test for Vasopressor Substance

The vasopressor substance of the preparation being examined (T) is determined by comparing its vasopressor activity on anaesthetized rat with that of standard preparation of lysine vasopressin (S) under the condition of the following method.

Preparation of standard solution Dissolve the lysine vasopressin standard in sodium chloride injection to produce a standard solution of 0.1 Unit per ml immediately before the test.

Preparation of test solution Dissolve the preparation being examined in water for injection (or sodium chloride injection) to produce a solution of suitable concentration specified in the monograph. Adjust the concentration of the solution so that of the volume of the solution being injected will be equal to that of the standard dilution.

Procedure Carry out the test on a healthy male rat weighing over 300 g which anaesthetized with suitable anaesthetics (such as i.p. 1 g urethane per kg body weight). Tie the rat on its back to a operating table and maintain the body temperature of the rat during the test. Dissect the trachea for cannulation when necessary. Insert the femoral (or carotid) vein a cannula filled with sodium chloride injection for intravenous injection. Inject through the venous cannula 50-100 Units of heparin per 100 g body weight. Insert another cannula filled with a heparinised sodium chloride injection into the common carotid artery and connect it to a device capable of giving a continuous record of the blood pressure.