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Appendix XIV C. Test for Bacterial Endotoxins[1]

(Bacterial Endotoxins, Ph. Eur. method 2.6.14)[1]

The test for bacterial endotoxins (BET) is used to detect or quantify endotoxins from gram-negative bacteria using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are 3 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 colour after cleavage of a synthetic peptide-chromogen complex.

The following 6 methods are described in the present chapter:

Method A.	Gel-clot method: limit test
Method B.	Gel-clot method: quantitative test
Method C.	Turbidimetric kinetic method
Method D.	Chromogenic kinetic method
Method E.	Chromogenic end-point method
Method F.	Turbidimetric end-point method

Proceed by any of the 6 methods for the test. In the event of doubt or dispute, the final decision is made based upon method A unless otherwise indicated in the monograph.

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

1. APPARATUS

Depyrogenate all glassware and other heat-stable apparatus in a hot-air oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. If employing plastic apparatus, such as microplates and pipette tips for automatic pipetters, use apparatus shown to be free of detectable endotoxin and which does not interfere in the test.

NOTE: in this chapter, the term 'tube' includes all types of receptacles, for example microplate wells.

2. REAGENTS, TEST SOLUTIONS

(1) Amoebocyte lysate

Amoebocyte lysate is a lyophilised product obtained from amoebocyte lysate 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 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.

(2) Lysate solution

Dissolve amoebocyte lysate in water for BET or in a buffer, as recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, as indicated by the manufacturer.

(3) Water for BET (water for bacterial endotoxins test)

Water for injections R or water produced by other procedures that shows no reaction with the lysate employed at the detection limit of the reagent.

3. PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION

The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*.

Endotoxin is expressed in International Units (IU). The equivalence in IU of the International Standard is stated by the World Health Organization.

NOTE: one International Unit (IU) of endotoxin is equal to one Endotoxin Unit (E.U.).

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for BET.

Use the solutions as soon as possible to avoid loss of activity by adsorption.

5. PREPARATION OF THE TEST SOLUTIONS

Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the lysate and test solution falls within the pH range specified by the lysate manufacturer, usually 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a 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.

6. DETERMINATION OF THE MAXIMUM VALID DILUTION

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:

$$\mathrm{MVD} = \frac{\mathrm{endotoxinlimit} \times \mathrm{concentrationo\ f\ testsolution}}{\lambda}$$

Endotoxin limit The endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass,

M = maximum recommended bolus dose of product per kilogram of body mass.

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 active substances administered parenterally is specified in units such as IU/mL, IU/mg, IU/Unit of biological activity, etc., in monographs.

Concentration of test solution:

— mg/mL if the endotoxin limit is specified by mass (IU/mg),

— Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit),

— mL/mL if the endotoxin limit is specified by volume (IU/mL).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/mL) or the lowest concentration used in the standard curve of the turbidimetric or chromogenic techniques.

7. GEL-CLOT TECHNIQUE (METHODS A AND B)

The gel-clot technique allows detection or quantification of endotoxins and is based on clotting of the lysate in the presence of endotoxins. The minimum concentration of endotoxins required to cause the lysate to clot under standard conditions is the labelled lysate sensitivity. To ensure both the precision and validity of the test, confirm the labelled lysate sensitivity and perform the test for interfering factors as described under 1. Preparatory testing.

1. PREPARATORY TESTING

(i) Confirmation of the labelled lysate sensitivity

Confirm in 4 replicates the labelled sensitivity λ, expressed in IU/mL, of the lysate solution prior to use in the test. Confirmation of the lysate sensitivity is carried out when a new lot of lysate is used or when there is any change in the test conditions which may affect the outcome of the test.

Prepare standard solutions of at least 4 concentrations equivalent to 2λ, λ, 0.5λ and 0.25λ by diluting the standard endotoxin stock solution with water for BET.

Mix a volume of the lysate solution with an equal volume of 1 of the standard solutions (such as 0.1 mL aliquots) in each tube. When single test vials or ampoules containing lyophilised lysate are employed, add solutions of standards directly to the vial or ampoule. Incubate the reaction mixture for a constant period according to the recommendations of the lysate manufacturer (usually at 37 ± 1°C for 60 ± 2 min), avoiding vibration. Test the integrity of the gel: for tubes, take each tube in turn directly from the incubator and invert it 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 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 end-point is the lowest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean end-point concentration by calculting the mean of the logarithms of the end-point concentrations of the 4 dilution series, take the antilogarithm of this value, as indicated by the following expression:

Geometric mean end-point concentration = $\text{antilog}\frac{\sum e}{f}$

$\sum e$ = sum of the $\log_{10}$ end-point concentrations of the dilution series used,

f = number of replicates.

The geometric mean end-point concentration is the measured sensitivity of the lysate solution (IU/mL). If this is not less than 0.5λ and not more than 2λ, the labelled sensitivity is confirmed and is used in the tests performed with this lysate.

(ii) Test for interfering factors

Table 2.6.14.-1

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

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 sensitivity.

Solution D = negative control (water for BET).

Prepare solutions A, B, C and D as shown in Table 2.6.14.-1, and use the test solutions at a dilution less than the MVD, not containing any detectable endotoxins, operating as described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The geometric mean end-point concentrations of solutions B and C are determined using the expression described in 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The test for interfering factors must be repeated when any changes are made to the experimental conditions that are likely to influence the result of the test.

The test is considered valid when all replicates of solutions A and D show no reaction and the result of solution C confirms the labelled lysate sensitivity.

If the sensitivity of the lysate determined with solution B is not less than 0.5λ and not greater than 2λ, the test solution does not contain interfering factors under the experimental conditions used. Otherwise, the test 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. The use of a more sensitive lysate permits a greater dilution of the preparation being examined and this may contribute to the elimination of interference.

Interference may be overcome by suitable validated treatment, such as filtration, neutralisation, 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.

2. LIMIT TEST (METHOD A)

(i) Procedure

Prepare solutions A, B, C and D as shown in Table 2.6.14.-2, and perform the test on these solutions following the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

Table 2.6.14.-2

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

Prepare solution A and solution B (positive product control) using a dilution not greater than the MVD and treatments as described in 1. Preparatory testing, (ii) Test for interfering factors. Solutions B and C (positive controls) contain the standard endotoxin at a concentration corresponding to twice the labelled lysate sensitivity. Solution D (negative control) consists of water for BET.

(ii) Interpretation

The test is considered valid when both replicates of solution 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 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. In the test, the preparation being examined complies with the test if a negative result is found for both replicates of solution A. The

Repeat

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.

3. QUANTITATIVE TEST (METHOD B)

(i) Procedure

The test quantifies bacterial endotoxins in the test solution by titration to an end-point. Prepare solutions A, B, C and D as shown in Table 2.6.14.-3, and test these solutions according to the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

(ii) Calculation and interpretation

The test is considered valid when the following 3 conditions are met:

(a) both replicates of solution D (negative control) are negative,

(b) both replicates of solution B (positive product control) are positive,

(c) the geometric mean end-point concentration of solution C is in the range of 0.5λ to 2λ.

To determine the endotoxin concentration of solution A, calculate the end-point concentration for each replicate, by multiplying each end-point dilution factor by λ.

The endotoxin concentration in the test solution is the end-point concentration of the replicates. If the test is conducted with a diluted test solution, calculate the concentration of endotoxin in the original solution by multiplying the result by the dilution factor.

If none of the dilutions of the test solution is positive in a valid test, report the endotoxin concentration as less than λ (or, if a diluted sample was tested, report as less than the lowest dilution factor of the sample × λ). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the largest dilution factor multiplied by λ (e.g. in Table 2.6.14.-3, the initial dilution factor × 8 × λ).

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

Table 2.6.14.-3

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

Solution	Endotoxin concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Endotoxin concentration	Number of replicates
			4	0.5λ	2
			8	0.25λ	2
D	None/Water for BET	-	-	-	2

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 a dilution series of 4 tubes containing the test solution at concentrations of 1, 1/2, 1/4 and 1/8, relative to the dilution used in the test for interfering factors. Other dilutions up to the MVD may be used as appropriate.

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

Solution C = a dilution series of 4 tubes of water for BET containing the standard endotoxin at concentrations of 2λ, λ, 0.5λ and 0.25λ.

Solution D = water for BET (negative control).

8. PHOTOMETRIC QUANTITATIVE TECHNIQUES (METHODS C, D, E AND F)

1. TURBIDIMETRIC TECHNIQUE (METHODS C AND F)

This technique is a photometric test to measure the increase in turbidity. Based on the test principle employed, this technique may be classified as being either the end-point-turbidimetric test or the kinetic-turbidimetric test.

The end-point-turbidimetric test (Method F) 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 (Method C) is a method to measure either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance or transmission, or the rate of turbidity development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually 37 ± 1 °C).

2. CHROMOGENIC TECHNIQUE (METHODS D AND E)

This technique is used to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the lysate. Depending on the test principle employed, this technique may be classified as being either the end-point-chromogenic test or the kinetic-chromogenic test.

The end-point-chromogenic test (Method E) 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 (Method D) measures either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance, or the rate of colour development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually 37 ± 1 °C).

3. PREPARATORY TESTING

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to show that the criteria for the standard curve are satisfied and that the test solution does not interfere with the test.

Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

(i) 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 3 endotoxin concentrations within the range indicated by the lysate manufacturer to generate the standard curve. Perform the test using at least 3 replicates of each standard endotoxin solution as recommended by the lysate manufacturer (volume ratios, incubation time, temperature, pH, etc.).

If the desired range is greater than 2 $\log_{10}$ in the kinetic methods, additional standards must be included to bracket each $\log_{10}$ 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.

(ii) 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 2.6.14.-4. Perform the test on at least 2 replicates of these solutions as recommended by the lysate manufacturer (volume of test solution and lysate solution, volume ratio of test solution to lysate solution, incubation time, etc.).

Table 2.6.14.-4

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2
B	Middle concentration 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

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 as described under 3. Preparatory testing, (i) Assurance of criteria for the standard curve (positive controls).

Solution D = water for BET (negative control).

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

— the absolute value of the correlation coefficient of the standard curve generated using solution C is greater than or equal to 0.980;

— 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 2.6.14.-4) from that in the solution containing the added endotoxin (solution B, Table 2.6.14.-4).

The test solution is considered free of interfering factors if under the conditions of the test, the measured concentration of the endotoxin added to the test solution is within 50-200 per cent 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 test solution is considered to contain interfering factors. Repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the test solution or diluted test solution not to exceed the MVD may be eliminated by suitable validated treatment, such as filtration, neutralisation, 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.

4. TEST

(i) Procedure

Follow the procedure described in 3. Preparatory testing, (ii) Test for interfering factors.

(ii) Calculation

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the positive control solution C.

The test is considered valid when the following 3 requirements are met:

(1) the results obtained with solution C comply with the requirements for validation defined under 3. Preparatory testing, (i) Assurance of criteria for the standard curve,

(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 50-200 per cent,

(3) the result obtained with solution D (negative control) 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.

(iii) 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.

Guidelines on the test for bacterial endotoxins are given in general chapter 5.1.10.

Test for Bacterial Endotoxins using Recombinant Factor C

(Ph. Eur. general text 2.6.32)

The test for bacterial endotoxins using recombinant factor C (rFC) is carried out to quantify endotoxins from gram-negative bacteria. It is performed using rFC based on the gene sequence of the horseshoe crab (*Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas* or *Carcinoscorpius rotundicauda*), using a fluorimetric method.

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

1. EQUIPMENT

Depyrogenate all glassware and other heat-stable equipment in a dry-heat oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. Where plastic equipment (such as microplates and pipette tips for automatic pipettes) is employed, it must be shown to be free of detectable endotoxin and not to interfere with the test.

2. REAGENTS

Reagents

Recombinant factor C is based on the gene sequence of the horseshoe crab (*Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas* or *Carcinoscorpius rotundicauda*). All reagents, including the fluorogenic substrate and assay buffer, must be free of detectable endotoxin.

Reagent solutions

If necessary, prepare the reagents according to the test kit manufacturer's instructions. Store the reagents, refrigerated or frozen, as indicated by the manufacturer.

Water for BET (water for bacterial endotoxins test)

Water for injections R or water produced by other procedures that shows no reaction with the reagent employed at the detection limit of the reagent.

3. PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION

The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*.

Endotoxin is expressed in International Units (IU). The equivalence in IU of the International Standard is stated by the World Health Organization.

NOTE: 1 International Unit (IU) of endotoxin is equal to 1 Endotoxin Unit (EU).

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for BET.

Use the solutions as soon as possible to avoid loss of activity by adsorption.

5. PREPARATION OF THE TEST SOLUTIONS

Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the reagent(s) and test solution falls within the pH range specified by the test kit manufacturer, usually 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a suitable buffer, as recommended by the test kit 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.

6. DETERMINATION OF THE MAXIMUM VALID DILUTION

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 formula:

$$\frac{endotoxinlimit \times concentrationo\ \ f\ testsolution}{\lambda}$$

Endotoxin limit The endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass;

M = maximum recommended bolus dose of product per kilogram of body mass.

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 active substances administered parenterally is specified in units such as IU/mL, IU/mg, IU/Unit of biological activity, etc., in monographs.

Concentration of test solution:

— mg/mL if the endotoxin limit is specified by mass (IU/mg);

— Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit);

— mL/mL if the endotoxin limit is specified by volume (IU/mL).

λ = the lowest concentration used in the standard curve.

7. FLUORIMETRIC QUANTITATIVE TECHNIQUE

This technique is used to measure the fluorescence (relative fluorescence units; RFU) emitted by a fluorescent substrate (reagent) after cleavage by endotoxin-activated factor C. It is used as an end-point-fluorescent test.

The end-point-fluorescent test is based on the quantitative relationship between the endotoxin concentration and the fluorescence of the reagent mixture at the end of the incubation period, expressed for example as ΔRFU:

$$\Delta RFU = RFU_{t_{end\text{-}point}} RFU_{t_0}$$

$RFUt_{end\text{-}point}$ = fluorescence of the reagent mixture at the end of the incubation period;

$RFUt_0$ = fluorescence of the reagent mixture at the start of the incubation period.

The test is carried out at the incubation temperature recommended by the test kit manufacturer (usually 37 ± 1 °C).

8. PREPARATORY TESTING

Preparatory tests are conducted to ensure that the fluorometric technique is valid. These tests demonstrate that the criteria for the standard curve are satisfied (8-1) and that the test solution does not interfere with the test (8-2).

Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

8-1 ASSURANCE OF CRITERIA FOR THE STANDARD CURVE

The test must be carried out for each lot of recombinant factor C reagent.

Instrument sensitivity must be adjusted in accordance with the recommendations of the test kit manufacturer.

Using the standard endotoxin solution, prepare at least 3 endotoxin concentrations within the range indicated by the test kit manufacturer to generate the standard curve. If the desired range exceeds the range indicated by the manufacturer by more than 2 $\log_{10}$, additional standards must be included to bracket each log increase in the range. Perform the test using at least 3 replicates of each standard endotoxin solution as recommended by the manufacturer (volume ratios, incubation time, temperature, pH, etc.).

The absolute value of the correlation coefficient, | r |, must be greater than or equal to 0.980, for the range of endotoxin concentrations prepared.

8-2 INTERFERING FACTORS

As factor G is absent from the test kit, false-positive results due to β-glucan activation are not expected to occur. This must be taken into account when the method is compared to other bacterial endotoxin quantification methods.

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 2.6.32.-1. Perform the test on at least 2 replicates of these solutions as recommended by the test kit manufacturer (volume of test solution and reagent test kit mixture, volume ratio of test solution to reagent test kit mixture, incubation time, etc.).

Table 2.6.32.-1

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
B	Middle concentration 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

Solution A = test solution, which may be diluted but not exceeding the MVD.

Solution B (positive product control) = 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 as described in section 8-1.

Solution D (negative control) = water for BET.

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

— the absolute value of the correlation coefficient of the standard curve generated using solution C is greater than or equal to 0.980;

— the result with solution D does not exceed the limit of the blank value required in the description of the reagent mixture employed, or it is less than the endotoxin detection limit of the rFC employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) (solution A, Table 2.6.32.-1) from that in the solution containing the added endotoxin (solution B, Table 2.6.32.-1).

The test solution is considered free of interfering factors if, under the conditions of the test, the measured concentration of the endotoxin added to the test solution is within 50-200 per cent of the known added endotoxin concentration, after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is outside the specified range, the test solution is considered to contain interfering factors. Repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the test solution or diluted test solution (not exceeding the MVD) may be eliminated by suitable validated treatment, such as filtration, neutralisation, dialysis, heat treatment or endotoxin-specific binding steps (enrichment of endotoxin from the test solution prior to detection in the absence of the interfering matrix). 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.

9. TEST

9-1 PROCEDURE

Follow the procedure described in section 8-2.

9-2 CALCULATION

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the standard endotoxin solution C.

The test is considered valid when the following 3 requirements are met:

(1) the results obtained with solution C comply with the requirements for validation defined in section 8-1;

(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 50-200 per cent;

(3) the result obtained with solution D (negative control) does not exceed the limit of the blank value required in the description of the reagent mixture employed, or it is less than the endotoxin detection limit of the rFC employed.

9-3 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.

Guidelines on the test for bacterial endotoxins are given in general chapter 5.1.10.

[1] This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8 Pharmacopoeial harmonisation*.