

## REVISED GENERAL CHAPTER

### 2.2.3. Bacterial Endotoxins

The test for bacterial endotoxins (BET) measures the activity of bacterial endotoxins that may be present in the sample or on the article to which the test is applied using a lysate derived from the hemolymph cells or amoebocytes of the horseshoe crab, *Limulus polyphemus*. Other species of horseshoe crab namely *Tachypleus gigas*, *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* also yield amoebocyte lysate having similar activity.

The addition of a solution containing endotoxins to a solution of the lysate produces turbidity or gelation of the mixture. However, addition of a chromogenic substrate to a solution of the lysate results in development of colour due to release of chromophore from the substrate upon activation by the endotoxin present in the solution. The rate of reaction depends on the activity of endotoxin, the pH and the temperature. The reaction requires the presence of certain bivalent cations, a clotting cascade enzyme system and clottable protein, all of which are provided by the lysate.

The following methods can be used to monitor the endotoxin activity in a product official in the Pharmacopoeia and to determine whether the product complies with the limit specified in the monograph.

Method A. Gel-Clot Limit Test Method

Method B. Semi-quantitative Gel-Clot Method

Method C. Kinetic Turbidimetric Method

Method D. Kinetic Chromogenic Method

Method E. End-Point Chromogenic Method

When a monograph includes a test for bacterial endotoxins without mentioning a method, the test is carried out by Method A. Any one of the other four methods may be employed provided it yields results of equivalent reliability with the preparation under examination. **In the event of doubt or dispute, the final decision is made based upon the gel-clot limit test unless otherwise specified in the monograph for the product being tested.**

The quantities of endotoxins are expressed in defined Endotoxin Units (EU). With the adoption of the second International Standard for endotoxin by the Expert Committee on Biological Standards of the World Health Organization, 1 EU = 1 IU.

The endotoxin limit for a given test preparation is calculated from the expression  $K/M$ , where M is the maximum **recommended bolus** dose administered to an adult (taken as 70 kg for this purpose) per kg ~~per hour~~ **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.** K is the threshold pyrogenic dose of endotoxin per Kg of body mass. The value of K is 5.0 EU/kg for parenteral preparations except those administered intrathecally, and is 0.2 EU/kg for preparations intended to be administered intrathecally.

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For radiopharmaceutical products not administered intrathecally, the endotoxin limit is calculated as  $175/V$ , where  $V$  is the maximum recommended dose in ml. For intrathecally administered radio pharmaceuticals, the endotoxin limit is obtained by the formula  $14/V$ . For formulations (anticancer products) administered on a per square meter of body surface, the formula is  $K/M$ , where  $K= 2.5 \text{ EU/kg}$  and  $M$  is the  $(\text{maximum dose}/\text{m}^2/\text{hour} \times 1.80 \text{ m}^2)/70 \text{ kg}$ .

The test should be carried out in a manner that avoids endotoxin contamination. ~~If necessary, the containers should be treated to eliminate surface endotoxins that may be present by heating~~ **Depyrogenate all glassware and other heat-stable apparatus** in an oven at  $250^\circ$  for 30 minutes provides more than 3 log reductions or by using a validated oven cycle or by any other means.

**If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipettors, use apparatus shown to be free of detectable endotoxin and which does not interfere in the test.**

Before carrying out the test for endotoxins in the preparation under examination it is necessary to verify

- (a) in the case of gel-clot methods, the sensitivity of the lysate;
- (b) in the case of quantitative methods, the linearity of the standard curve;
- (c) the absence of interfering factors, which inhibit or enhance the reaction or otherwise interfere with the test on the preparation under examination;
- (d) the adequacy of the containers to resist adsorption of endotoxins.

### **Special Reagents**

**Endotoxin Reference Standard and Control Standard Endotoxin.** The Endotoxin Reference Standard (ERS) is the freeze-dried, purified endotoxin from the strain of *Escherichia coli* 0113:H10, and *Escherichia coli* 055:B5 is used as the Control Standard Endotoxin (CSE) which is calibrated in Endotoxin Units (EU) by comparison with the International Standard.

The freeze-dried endotoxin should be reconstituted with *water BET* by mixing intermittently for 30 minutes using a vortex mixer. The concentrate should be stored in a refrigerator ~~for not more than 28 days~~ **as recommended by the manufacturer**. Subsequent dilutions of the concentrate should be made by mixing vigorously for not less than 5 minutes before use. Each dilution should be mixed for not less than 1 minute before proceeding to make the next dilution.

A Control Standard Endotoxin (CSE) which is suitably standardized against the ERS may be used for routine bacterial endotoxin testing.

**Lysate.** A lysate of amoebocytes **is a lyophilized product obtained** from either of the species of the horseshoe crab, *Limulus polyphemus*, *Tachypleus gigas*, *Tachypleus tridentatus* or *Carcinoscorpius rotundicauda* reconstituted ~~as stated on the label~~ **in *water 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.** The species from which the lysate

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is obtained is stated on the label. **Limulus Amebocyte Lysate (LAL) or Tachypleus Amebocyte Lysate (TAL) or Carcinoscorpius Amebocyte Lysate (CAL)** refers only to a product manufactured in accordance with the regulations of the competent authority.

**Water BET.** Water BET is prepared by a suitable method that gives a negative result under the conditions prescribed in the test for bacterial endotoxins on the preparation under examination.

**0.1 M Hydrochloric acid BET.** Prepare from *hydrochloric acid* using *water BET*. After adjustment of the pH 6.0 to 8.0 with *0.1M sodium hydroxide BET* it gives a negative result under the conditions of the test.

**0.1 M Sodium hydroxide BET.** Prepare from *sodium hydroxide* using *water BET*. After adjustment of the pH to 6.0 to 8.0 with *0.1M hydrochloric acid BET* it gives a negative result under the conditions of the test.

**Tris-chloride buffer pH 7.4 BET.** Dissolve 0.6057 g of *tris (hydroxymethyl) methylamine* in 30 ml of *water BET*, add 0.33 ml of *hydrochloric acid*, dilute to 100 ml with *water BET* and mix. It gives a negative result under the conditions of the test.

**$\beta$ -D-Glucan blocking buffer.**  $\beta$ -D-Glucan blocking buffer is used to address the interference arising due to 1-3  $\beta$ -D-Glucans during endotoxin assay. The buffer inhibits the Factor G pathway and effectively blocks reactions with 1-3  $\beta$ -D-Glucans.

*NOTE* — Special reagents used in the test may use the suffix ‘LAL’, ‘TAL’ or ‘CAL’, as the case may be, to indicate the species of the horseshoe crab from which the amoebocyte lysate is derived. They have the same significance as the suffix ‘BET’.

### **Gel-Clot Methods**

Methods A and B depend on the formation of a firm gel when a solution containing bacterial endotoxins is incubated after mixing with the lysate. Method A is conducted as a limit test wherein both the replicate solutions of the preparation under examination must contain endotoxin in the concentration less than the endotoxin limit concentration specified in the individual monograph. Method B determines the endotoxin concentration semi-quantitatively in the preparation under examination.

**Confirmation of the labelled sensitivity of the lysate.** Confirm the labelled sensitivity of each new batch of lysate prior to use in the test using at least one vial of each batch of lysate. Prepare a series of dilutions of CSE to give concentrations of  $2\lambda$ ,  $\lambda$ ,  $0.5\lambda$  and  $0.25\lambda$ , where  $\lambda$  is the labelled sensitivity of the lysate in EU per ml. Perform the test as given under Method on these four standard concentrations in ~~duplicate~~ **quadruplicate** and include a negative control consisting of *water BET*. At least the final dilution in each series must give a negative result.

Dilution	Result
$2\lambda$	+
$\lambda$	+/-

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0.5λ	+/-
0.25λ	-

+ = positive (gel clot present); - = negative (gel clot absent)

Calculate the average of the logarithms of the lowest concentration of endotoxin in each series of dilutions for which a positive result is found. The geometric mean end-point concentration is the measured sensitivity of the lysate in EU/ml, which is calculated using the following expression:

Geometric mean end-point concentration =  $\text{antilog}(\Sigma e/f)$

Where,  $\Sigma e$  = sum of the  $\log_{10}$  end-point concentrations of the series of dilutions used;

$f$  = number of replicate test-tubes.

This average gives the estimated lysate sensitivity which must lie between 0.5λ and 2λ.

**Test for interfering factors.** The possibility of interference with the bacterial endotoxins test by certain factors should be borne in mind. For validation of the test results it must be demonstrated that the test preparation does not inhibit or enhance the reaction or otherwise interfere with the test. The validation must be repeated if the lysate vendor or the method of manufacture or the formulation of the sample is changed. Dilution of the test preparation with *water BET* is the easiest method for overcoming inhibition.

The allowable dilution level or Maximum Valid Dilution (MVD) is dependent on the concentration of the product, the endotoxin limit for the product and the lysate sensitivity. It is calculated by the following expression:

$$\text{MVD} = \frac{\text{Endotoxin limit} \times \text{Concentration of the test solution}}{\lambda}$$

where, λ is the labelled sensitivity of the lysate (EU/ml).

\* Concentration of the test solution is expressed as mg/ml in case the endotoxin limit is specified by weight (EU/mg), or as Units/ml in case the endotoxin limit is specified by Unit (EU/Unit), or as 1.0 ml/ml in case the endotoxin limit is specified by volume (EU/ml).

**Preparation of test solutions.** Prepare replicates of solutions A to D as indicated in the table.

Solution	Final concentration of added CSE in the solution	Number of replicates
A	–	4
B	2 λ	4
	λ	4
	0.5 λ	4
	0.25 λ	4
C	2 λ	2
	λ	2
	0.5 λ	2
	0.25 λ	2
D	–	2

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Solution A = Solution of the product at a dilution at or below MVD (test solution).

Solution B = Test solution spiked with indicated CSE concentrations (Positive Product Control; PPC).

Solution C = Standard solution with indicated CSE concentrations in *water BET*.

Solution D = *Water BET* (Negative Control; NC).

**Method.** Carry out the following procedure in receptacles such as tubes, vials or wells of micro-titre plates. Into each of the chosen receptacle, add an appropriate volume of negative control (NC), standard CSE solutions in *water BET*, test solution and positive product control (PPC). At intervals that will permit the reading of each result, add to each receptacle an equal volume of the appropriately constituted lysate unless single test vials are used. Mix the sample-lysate mixture gently and place in an incubating device such as a water-bath or a heating block, accurately recording the time at which the receptacles are so placed. Incubate each receptacle at  $37^{\circ} \pm 1^{\circ}$  undisturbed for  $60 \pm 2$  minutes. Remove the receptacles and examine the contents carefully. A positive reaction is characterised by the formation of a firm gel that retains its integrity when inverted through  $180^{\circ}$  in one smooth motion. Record this result as positive (+). A negative result is characterised by the absence of such a gel or by the formation of a viscous gel that does not maintain its integrity. Record such a result as negative (-). Handle the receptacles with care to avoid subjecting them to unwanted vibrations as false negative observations may result.

Calculate the geometric mean end-point concentration of solutions of series B and C by using the formula described under Sensitivity of the lysate.

**Calculation and interpretation of results.** The test for interfering factors is valid if

- (a) solutions of series A and D give negative results;
- (b) the results obtained with solutions of series C confirm the labelled sensitivity of the lysate;
- (c) the geometric mean of the end-point concentration of solutions of series B is not more than  $2\lambda$  or not less than  $0.5\lambda$ .

If the result obtained is outside the specified limit, the test preparation under examination is acting as an inhibitor or enhancer. The interfering factors may be eliminated by further dilution (not greater than MVD), filtration, neutralization, and inactivation or by removal of the interfering substances. The use of a more sensitive lysate permits the use of greater dilution of the preparation under examination.

Ultrafiltration with suitable consumables or affinity chromatography may be used, if necessary, when the interfering factor passes through a filter with a nominal separation limit corresponding to a molecular weight of 10,000 to 20,000. Such filters should be checked for the presence of any factors causing false positive results. The material retained on the filter, which contains the endotoxins, is rinsed with *water BET* or *tris-chloride buffer pH 7.4 BET*. The endotoxins are recovered in the *water BET* or the buffer. The endotoxin concentration in the test volume and the final volume are determined for each preparation under examination.

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Establish that the chosen treatment effectively eliminates interference without removing endotoxins by repeating the test for interfering factors using the preparation under examination to which the CSE has been added and which has been submitted to the chosen treatment.

### **Method A. Gel-Clot Limit Test Method**

**Preparation of test solutions.** Unless otherwise prescribed, prepare the solutions and dilutions with *water BET*. If necessary, adjust the pH of the solution under examination to 6.0 to 8.0 using sterile *0.1M hydrochloric acid BET*, *0.1M sodium hydroxide BET* or a suitable buffer prepared with *water BET*.

Prepare the sample solution at any dilution at or below MVD. Use *water BET* as negative control (NC) and two positive controls. One of the positive controls consists of the CSE at a concentration of  $2\lambda$  and the other consists of the test solution spiked with CSE to give a concentration of  $2\lambda$  (PPC).

**Method.** Carry out the procedure on the test solutions in duplicate as described under Test for interfering factors.

**Interpretation of results.** The product under examination complies with the bacterial endotoxin test if the positive product control is positive and the negative controls as well as the test solutions are negative. The test is not valid if the positive product control is negative or if the negative control is positive.

The product under examination meets the requirements of the test if the endotoxin content is less than the endotoxin limit stated in the individual monograph.

**Retests.** If a positive result is found for one of the test solution duplicates and a negative result for the other, the test may be repeated as described above. The results of the retest should be interpreted as for the initial test. If the results are still inconclusive, the sample fails the test.

### **Method B. Semi-Quantitative Gel-Clot Method**

**Preparation of test solutions.** Prepare test solutions at concentrations of MVD, 0.5 MVD, 0.25 MVD or any other appropriate dilutions relative to the dilution at which the test for interfering factors was completed. Additionally, prepare a similar series of test solutions spiked with  $2\lambda$  of CSE each (PPC).

**Method.** Carry out the procedure on the test solutions in duplicate as described under Test for interfering factors.

**Calculation and interpretation of results.** To calculate the endotoxin concentration in the product, determine for the series of test solutions the lowest concentration or the highest dilution giving a positive (+) reaction. Multiply this dilution factor with  $\lambda$  to obtain the endotoxin concentration of the product.

For instance, if MVD is equal to 8 and the positive reaction was obtained at 0.25 MVD and  $\lambda$  was equal to 0.125 EU/ml, the endotoxin concentration in the test solution will be  $8 \times 0.25 \times 0.125 = 0.25$  EU/ml.

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If none of the dilutions of the series gives a positive reaction, the endotoxin concentration will be less than the value obtained by multiplying the lowest dilution factor with  $\lambda$ . If all the dilutions of the series give a positive reaction, the endotoxin concentration will be more than the value obtained by multiplying the highest dilution factor with  $\lambda$ .

Calculate the endotoxin content of the product under examination from the endotoxin concentration. The product under examination meets the requirements of the test if the endotoxin content is less than the endotoxin limit stated in the individual monograph.

### **Photometric Quantitative Methods**

The photometric quantitative methods include

- Kinetic Turbidimetric Method (Method C),
- Kinetic Chromogenic Method (Method D) and
- End-Point Chromogenic Method (Method E).

These methods make use of an appropriate regression analysis of the log response with the log endotoxin concentration.

To ascertain the precision or validity of the turbidimetric and chromogenic methods, preparatory tests are conducted to see that the criteria for the standard curve are valid and that the test solution does not interfere with (inhibit or enhance) the reaction.

#### **Method C. Kinetic Turbidimetric Method**

The kinetic turbidimetric method is a photometric assay measuring the increase in turbidity caused by the reaction of the endotoxin with the lysate. The kinetic turbidimetric assay is a method measuring either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture or the rate of turbidity development.

#### **Method D. Kinetic Chromogenic Method**

The kinetic chromogenic method is a photometric assay measuring the colour developed by the chromophore released from a chromogenic substrate by the reaction of the endotoxin with the lysate. The kinetic chromogenic assay is a method measuring either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture or the rate of colour development.

**Test for interfering factors.** For validation of the test results, it must be demonstrated that the test preparation does not inhibit or enhance the test or otherwise interfere with the test. The validation must be repeated if the lysate vendor or the method of manufacture or formulation of the sample is changed. The initial dilution may be prepared using the following expression:

$$\text{Initial dilution} = \frac{\text{Endotoxin limit of the test solution}}{C^*}$$

\*C is the lowest CSE concentration of the standard curve expressed in EU/ml.

**Preparation of test solutions.** Prepare solutions A to D as given below.

Solution A = Solution of the product under examination at the initial dilution (test solution).

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Solution B = Test solution spiked with CSE at a concentration at or near the middle of the standard curve (PPC).

Solution C = Standard solutions of CSE in *water BET* covering the linear part of the standard curve.

Solution D = *Water BET* (NC).

The pH of the solutions must be in the range specified by the manufacturer of the lysate, usually between 6.0 and 8.0. Adjust the pH, if necessary, by addition of sterile *0.1 M hydrochloric acid BET*, *0.1 M sodium hydroxide BET* or a suitable buffer prepared with *water BET*.

**Method.** Carry out the test in duplicate receptacles such as wells of a micro-titre plate. Into each chosen receptacle, add an appropriate volume of solution D (NC), standard CSE solutions in *water BET* (solution C), test solution (solution A) and solution B (PPC). Add the lysate and carry out the assay in accordance with the instructions given by the lysate manufacturer.

**Calculation and interpretation of results.** Calculate the endotoxin concentration of solutions A and B from the regression equation obtained with solutions of series C. Calculate the mean percentage recovery of the added endotoxin by subtracting the mean endotoxin concentration in solution A from the mean endotoxin concentration in solution B.

The test for interfering factors is valid only if

- (a) the negative control (solution D) does not yield a value higher than the limit for the value required in the description of the lysate employed;
- (b) the CSE solutions of series C comply with the requirements given under Preparation of the standard curve;
- (c) the mean percentage recovery of added endotoxin in solution B is between 50 per cent and 200 per cent.

If the mean percentage recovery is beyond the specified range, the interfering factors must be removed by the procedure described under the Gel-Clot Method.

**Preparation of the standard curve.** Using CSE, prepare solutions of 3 point, 2 log curve for endotoxin concentrations to obtain a linear standard curve. Carry out the procedure using at least two replicates of each standard endotoxin solution in accordance with the instructions of the lysate manufacturer (volume ratios, incubation times, temperature, pH, etc.).

The regression line must have linearity with the coefficient of correlation,  $r$ , greater than or equal to 0.980 or  $\geq 0.980$  for the range of endotoxin concentrations.

**Preparation of test solutions.** Unless otherwise prescribed, prepare the solutions to be employed in the test using *water BET*. If necessary, adjust the pH of the solution under examination to 6.0 to 8.0 using sterile *0.1 M hydrochloric acid BET*, *0.1 M sodium hydroxide BET* or a suitable buffer prepared with *water BET*.

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Prepare the test solution at a suitable dilution. Use not less than three CSE concentrations to prepare a linear standard curve. Use *water BET* as negative control and one positive control. The positive control consists of the test solution spiked with CSE to give an endotoxin concentration at the middle or below the middle point of the standard curve (PPC).

**Method.** Carry out the procedure described under Test for interfering factors.

**Interpretation of results.** The assay is valid only if

- (a) the standard curve is linear for the range of CSE concentrations used;
- (b) the co-efficient of correlation,  $r$ , should be greater than or equal to 0.980.
- (c) the mean percentage recovery of the added endotoxin in the positive product control is between 50 per cent and 200 per cent.

The product under examination meets the requirements of the test if the mean endotoxin content of the replicates, after correction for dilution and concentration, is less than the endotoxin limit stated in the individual monograph.

### **Method E. End-Point Chromogenic Method**

The end-point chromogenic method is a photometric assay measuring the colour developed by the chromophore released from a chromogenic substrate by the reaction of the endotoxin with the lysate. The end-point assay is a method measuring the colour intensity at the end of an incubation period after the reaction is stopped by the addition of a suitable acid.

**Preparation of test solutions.** Unless otherwise prescribed, prepare the solutions to be employed in the test using *water BET*. If necessary, adjust the pH of the solution under examination to 6.0 to 8.0 using sterile *0.1M hydrochloric acid BET*, *0.1M sodium hydroxide BET* or a suitable buffer prepared with *water BET*.

Prepare the test solution at a suitable dilution. Prepare a reagent blank and not less than three dilutions of CSE in *water BET* to prepare a linear standard curve. Use *water BET* as negative control and one positive control. The positive control consists of the test solution spiked with CSE to give an endotoxin concentration at the middle or below the middle point of the standard curve (PPC).

**Method.** Carry out the procedure described under Test for interfering factors. The chromogenic substrate and lysate are added to the solution and incubated for the recommended time. Stop the reaction and measure the absorbance at the wavelength specified by the lysate manufacturer.

Perform the linear regression analysis of the absorbance on the endotoxin concentration using standard statistical methods (method of least squares is usually suitable). Do not average the absorbance values of the replicates of each standard before performing the linear correlation regression analysis. Determine the endotoxin concentration of the test solution from the standard curve.

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**Interpretation of results.** The assay is valid only if

- (a) the standard curve is linear for the range of CSE concentrations used;
- (b) the co-efficient of correlation,  $r$ , greater than or equal to 0.980;
- (c) The mean percentage recovery of the added endotoxin in positive product control is between 50 per cent and 200 per cent.

The product under examination meets the requirements of the test if the mean endotoxin content of the replicates, after correction for dilution and concentration, is less than the endotoxin limit stated in the individual monograph.