BRIEFING

⟨**86**⟩ **Bacterial Endotoxins Test Using Recombinant Reagents.** This proposed new test chapter provides additional techniques using nonanimal derived reagents to the *Bacterial Endotoxins Test* ⟨85⟩.

This general chapter is not currently being introduced into a specific monograph or listed in *General Notices*. It is the responsibility of the user to review the supplier's primary validation package and to verify product suitability for use in testing specific products or materials. This verification must include specific experiments to confirm that the method is suitable for its intended purpose under the conditions of use for the material, drug substance, and/or drug product. The selected verification experiments should be based on an assessment of the complexity of the material to which the method is being applied. The user should refer to *Verification of Compendial Procedures* ⟨1226⟩. Regulatory authorities may require supplemental data prior to acceptance. An example of supplemental data may include a comparative study of the material tested by techniques described in this chapter and those in ⟨85⟩.

Under the *Rules and Procedures of the Council of Experts*, all comments submitted to USP in response to proposals published in the *Pharmacopeial Forum*, as well as the identities of the commenters, are considered public information unless clearly and specifically designated as confidential.

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Add the following:

▲⟨86⟩ BACTERIAL ENDOTOXINS TEST USING RECOMBINANT REAGENTS

The Bacterial Endotoxins Test (BET) described in this chapter contains additional techniques using nonanimal derived reagents to the *Bacterial Endotoxins Test* ⟨85⟩. Unless specified in an individual monograph, the tests in this chapter are considered alternative tests and must meet the requirements in *General Notices 6.30*. This test uses a reagent containing the recombinant Factor C (rFC) protein or a recombinant cascade reagent (rCR) containing recombinant Factor C, recombinant Factor B, and recombinant proclotting enzyme. These reagents are used to detect or quantify endotoxins from Gram-negative bacteria in test samples. The test is performed using reagent(s) based on the gene sequence(s) of the relevant factors of the horseshoe crab (*Limulus polyphemus*, *Tachypleus tridentatus*, or *Carcinoscorpius rotundicauda*).

There are two detection techniques that can be employed in this test: the endpoint fluorescence technique, based on the development of fluorescence after activation of a synthetic peptide-fluorophore complex; or the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromophore complex. To accurately detect endotoxins, the test is carried out using endotoxin-free materials with laboratory controls in place to prevent inadvertent endotoxin contamination.

It is the responsibility of the user to review the supplier's primary validation package and to verify that the recombinant reagent-based method is appropriate for use in testing specific products or materials. This verification must include performing specific experiments to confirm that the method is suitable for its intended purpose under the conditions of use for the material, drug substance, and/or drug product. These experiments should be commensurate with and based on an assessment of the complexity of the material to which the method is being applied. The user should refer to *Verification of Compendial Procedures* ⟨1226⟩. Regulatory authorities may require supplemental data prior to acceptance, and users are encouraged to consult each regulatory authority. An example of supplemental data may include a comparative study of the material tested by techniques described in this chapter and those in ⟨85⟩.

APPARATUS

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

REAGENTS AND TEST SOLUTIONS

Reagents

Recombinant reagents are based on the gene sequences of the relevant factors of the horseshoe crab (*L. polyphemus*, *T. tridentatus*, or *C. 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 as indicated by the manufacturer.

Water for Bacterial Endotoxins Test (BET)

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

PREPARATION OF SOLUTIONS

Standard Endotoxin Stock Solution

The *Standard Endotoxin Stock Solution* is prepared from an endotoxin reference standard that has been calibrated to the current WHO International Standard for Endotoxin, for example, USP Endotoxin RS.

Endotoxin is expressed in Endotoxin Units (EU). [NOTE—1 USP Endotoxin Unit (EU) is equal to 1 International Unit (IU) of endotoxin.]

Follow the specifications in the package leaflet and on the label for preparation and storage of the *Standard Endotoxin Stock Solution*.

Standard Endotoxin Solutions

After vigorously mixing the *Standard Endotoxin Stock Solution*, prepare appropriate serial dilutions of the *Standard Endotoxin Solutions* using *Water for BET*.

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

Sample Solutions

Prepare the *Sample Solutions* by dissolving or diluting the test article 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 solution (or dilution thereof) so the pH of the mixture of the reagent(s) and *Sample Solution* falls within the pH range specified by the test kit manufacturer, usually 6.0–8.0. The validity of the positive product control (*Solution B* in *Table 1*) is indicative of the potential necessity to adjust the sample pH. The pH may be adjusted with an acid, base, or 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 free of detectable endotoxin and interfering factors.

DETERMINATION OF MAXIMUM VALID DILUTION

The maximum valid dilution (MVD) is the maximum allowable dilution of a test article at which the endotoxin limit can be determined.

Determine the MVD:

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

λ = lowest concentration used in the standard curve

Endotoxin Limit

The endotoxin limit for medicinal products administered parenterally, is defined on the basis of dose.

Calculate the endotoxin limit as follows:

$$\text{Result} = K/M$$

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 medicinal products can be specified in EU/mL, EU/mg, EU/Unit of biological activity, etc.

Concentration of Sample Solution

- mg/mL if the endotoxin limit is specified by mass (EU/mg)
- Units/mL if the endotoxin limit is specified by unit of biological activity (EU/Unit)
- mL/mL if the endotoxin limit is specified by volume (EU/mL)

QUANTITATIVE TECHNIQUES

Fluorometric 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 typically used as an endpoint fluorescence test.

The endpoint fluorescence 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. This is expressed, for example, as ΔRFU and blank-corrected:

$$\Delta RFU = RFU_{end} - RFU_{start}$$

RFU_{end} = fluorescence of the reagent mixture at the end of the incubation period

RFU_{start} = 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°).

Chromogenic Absorbance Technique

This technique is an assay to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with lysate. The kinetic-chromogenic assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture or the rate of color development. The test is carried out at the incubation temperature recommended by the test kit manufacturer (usually 37 ± 1°).

Preparatory Testing

Preparatory tests are conducted to ensure the fluorometric or chromogenic technique is valid. These tests demonstrate that the standard curve criteria and the test solution do not interfere with the test.

Supplemental validation for the test method is required when any changes are made to the experimental conditions that are likely to influence the test result.

STANDARD CURVE CRITERIA

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

Instrument sensitivity must be adjusted in accordance with the recommendations of the test kit manufacturer to ensure the placement of the standard curve within the dynamic range of the instrument.

Using the *Standard Endotoxin Solution*, prepare at least three endotoxin concentrations within the range indicated by the test kit manufacturer to generate the standard curve. If the desired range is greater than 2 log10, additional standards should be included to bracket each log change of the standard curve.

Perform the assay using at least two replicates of each concentration of the endotoxin standard. Volume ratios, incubation time, temperature, pH, and other conditions are as recommended by the manufacturer.

The absolute value of the correlation coefficient, $|r|$, must be greater than or equal to 0.980 for the range of the prepared *Standard Endotoxin Solutions*.

VERIFICATION—TEST FOR INTERFERING FACTORS

As recombinant reagents are not susceptible to the Factor G false-positive pathway in naturally derived reagents, false-positive results due to β-glucan activation are not expected to occur. This potential interference must be considered when the method is compared to other bacterial endotoxin quantitation methods.

Select an endotoxin concentration at or near the middle of the endotoxin standard curve.

Prepare *Solutions A–D* as shown in *Table 1*. Perform the test on at least two replicates of each of *Solutions A–D*, as recommended by the test kit manufacturer (volume of test solution and reagent test kit mixture, volume ratio of test solution to reagent mixture, incubation time, etc.).

Table 1. Preparation of Solutions for the Inhibition/Enhancement Test

Solution	Endotoxin Concentration	Solution to Which Endotoxin Is Added	Number of Replicates
A[a]	None	*Sample Solution*	Not less than 2
B[b]	Middle concentration of the standard curve	*Sample Solution*	Not less than 2

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

[a] *Solution A*: The *Sample Solution* may be diluted but not to exceed MVD.

[b] *Solution B* (positive product control): The 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.

[c] *Solution C*: The *Standard Endotoxin Solution* at the concentrations used in the method as described in *Standard Curve Criteria*.

[d] *Solution D*: *Water for BET* (negative control).

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

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

Calculate the mean recovery of the added endotoxin by subtracting the blank-corrected mean endotoxin concentration in the solution, if any (*Solution A*, *Table 1*), from the blank-corrected mean of the positive control (*Solution B*, *Table 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 50%–200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is outside 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 treatment such as filtration, neutralization, dialysis, or heat treatment. To establish that the chosen treatment effectively eliminates interference without loss of endotoxin, repeat the test for interfering factors using *Solution B* and applying the chosen treatment.

TEST PROCEDURE

Follow the procedure described for *Verification—Test for Interfering Factors*.

Calculation

Calculate the endotoxin concentration of each *Solution A* replicate using the standard curve generated by the endotoxin standard in *Solution C*.

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

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

Interpretation

The preparation being examined complies with the test if the mean endotoxin concentration of the replicates of *Solution A*, after correcting for dilution and concentration, is less than the endotoxin limit for the product.

USP Reference Standards ⟨11⟩

USP Endotoxin RS ▲ (USP 1-May-2025)

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