INTENDED USE - Limulus amebocyte lysate (LAL), an aqueous extract derived from Limulus amebocytes, which is intended for quantitative detection of endotoxins by gel clot or kinetic-turbidimetric methods.

SUMMARY AND GENERAL INFORMATION - The LAL test is the most sensitive and specific means available to detect and measure bacterial endotoxin, a fever-producing byproduct of gram-negative bacteria commonly known as pyrogen. The basis of the test is that endotoxin produces an opacity and gelation in LAL that can be easily recognized. 3 The simplicity and economy of the LAL Test encourages the testing of in-process solutions and raw materials as well as end-product drugs, devices and biologics. 4 The USP Bacterial Endotoxin Test and USFDA Guideline for LAL testing provide standard methods for validating the LAL Test as a replacement for the rabbit pyrogen test. 11,12

The gel-clot LAL test method is a simple, reproducible, semi-quantitative test, conducted by mixing LAL HS-F and test specimen and observing for gelation after incubation for 60 minutes at 37°C. A positive response (gel) indicates that the amount of endotoxin in the sample meets or exceeds the reagent’s labeled sensitivity, represented by the symbol lambda, λ. With the aid of a microprocessor and microplate reader or tube reader, a kinetic turbidimetric assay may be done where the early onset of turbidity, which precedes gelation, can be detected and precisely measured. The time for onset of turbidity is inversely related to the amount of endotoxin in the sample; endotoxin levels in unknown samples are determined by comparison to a standard curve. With kinetic measurements, lambda (λ) is the lowest point on the standard curve.

BIOLICAL PRINCIPLES - Frederick Bang observed that bacteria caused intravascular coagulation in the American horseshoe crab, Limulus polyphemus. 5 Levin and Bang 6 found that the agent responsible for the clotting phenomena resided in the crab’s amebocytes, or circulating blood cells, and that pyrogen (bacterial endotoxin) triggered the turbidity and gel-forming reaction enzymatically. Serine protease zymogens found in amebocyte lysate are activated by endotoxin and divalent cations to initiate an enzymatic coagulation cascade that alters an abundant protein, coagulogen, to produce a proteinaceous gel. 7 A comparative study in 1976 demonstrated that the LAL test was more sensitive than the rabbit test and that LAL reactivity (gelation and increased opacity) correlated with endotoxin concentration. 3 Improvements in LAL reagents, the advent of standard methods and automated systems, and a better understanding of LAL reactivity make the LAL reagent readily adaptable to testing a variety of biologics, parenteral products and medical devices. 24,4

The endotoxin/LAL reaction requires a neutral pH and is time and endotoxin concentration dependent. The test is generally limited to aqueous solutions or extracts of test specimen. The reagent is susceptible to many interferences; fortunately, these effects are usually overcome by simple dilution or modification without significantly altering the sensitivity of the test.

USFDA GUIDELINE FOR END-PRODUCT TESTING - A guideline was released by the U.S. Food and Drug Administration in 1987 to inform manufacturers of human drugs and biologicals, animal drugs, and medical devices of procedures that FDA considers necessary to validate the use of LAL as an end-product endotoxin test. 11 Those who adhere to the LAL guideline would be considered in compliance with relevant provisions of CGMPs. The general endotoxin limit for parenteral drugs is 5 Endotoxin Units (EU) per Kg dose, except for a 0.2 EU/Kg limit for intrathecal drugs. Medical device eluates must not exceed 0.5 EU/ml; a 0.06 EU/ml limit applies to devices that contact cerebrospinal fluid. 11

WARNINGS AND GENERAL PRECAUTIONS - Warnings: LAL HS-F is intended for in vitro diagnostic purposes only. Exercise caution when handling LAL because its concentration must not exceed 0.5 EU/ml; a 0.06 EU/ml limit applies to devices that contact cerebrospinal fluid. Neurotoxicity effects are usually overcome by simple dilution or modification without significantly altering the sensitivity of the test.

LAL REAGENT - Lyophilized LAL HS-F LAL Reagent, contains buffered amebocyte lysate stabilized by monovalent and divalent cations. Sensitivity, λ, is referenced to the U.S. Standard Endotoxin and is stated in the labeling as EU/ml; it is defined as the lowest concentration of RSE in Endotoxin Units per milliliter that produces a gel clot under standard conditions.

Constitution: Collect LAL powder into the bottom of the vial by tapping on a firm surface. Release the vacuum by carefully removing the stopper. Rehydrate with 5.2 ml of LAL Reagent Water just before use by pipetting it directly into the vial. Cover the vial when not in immediate use. LAL dissolves by gentle mixing into a colorless solution. Discard if the vacuum is absent or if color or opacity is present.

Storage: Lysylated LAL is relatively heat stable and should be stored at 2-8°C; avoid prolonged exposure to temperatures above 25°C. Rehydrated LAL ideally should be stored on a cold surface or in a refrigerator at 2-8°C during intermittent use, for up to 24 hours; Otherwise, store LAL below minus 20°C up to 28 days after reconstitution and freezing; LAL may be frozen and thawed only once.

SPECIMEN COLLECTION AND PREPARATION - All materials coming in contact with specimen or test reagents must be endotoxin-free. Use aseptic technique at all times. Since the LAL-endotoxin reaction is pH dependent, the specimen-LAL mixture should yield a pH of 6.5 to 8.0. If pH adjustment is necessary, use endotoxin-free HCl or NaOH at an appropriate concentration, generally 0.1N or less. Do not arbitrarily adjust the pH of unpurified solutions.

PRODUCT INTERFERENCE - A test method for each sample must be validated by demonstrating the absence of significant interference. Inhibition is usually concentration dependent, and is overcome by dilution with LAL Reagent Water. Common sources of inhibition include conditions that 1) interfere with the enzyme-mediated gelation reaction, and 2) alter the dispersion of the endotoxin (Positive control). 10

Maximum Valid Dilution: The U.S. Food and Drug Administration has established endotoxin limits of 5 EU/kg for intravenous drugs and 0.2 EU/kg for intrathecal drugs. 11 Specific limits for compendial items have been adopted. 12 These limits may be used to determine the extent of dilution that may be used to overcome an interference problem without exceeding the limit endotoxin concentration. The Maximum Valid Dilution (MVD) is calculated by formulae presented in the previously mentioned documents. 11,12

For drug products that have a published limit, the MVD may be calculated by the following formula:

\[
MVD = \frac{\text{Endotoxin Limit} \times \text{Product Potency}}{\lambda}\n\]

For example, the compendial limit for cyclophosphamide is 0.017 EU/mg. If a standard curve with a lowest level of 0.05 EU/ml of endotoxin is used to test this product where the potency is 20 mg/ml, the MVD equals 1.68. Thus, cyclophosphamide may be diluted up to 1.68 to resolve potential inhibition.

Interference (inhibition/enhancement) testing by kinetic methods is done by spiking a sample or diluted sample with a known concentration of endotoxin and testing for spike recovery in duplicate by the supplier’s instructions. This testing requires a standard curve prepared using either RSE or CSE (refer to Certificate of Analysis for CSE). The standard curve shall consist of at least three RSE or CSE concentrations. An additional standard shall be included to bracket each 10-fold increase in the range of the standard curve. The curve must meet the criteria stated in “Performance Characteristics”.

Select the mid-point on the standard curve for interference testing. For example, the positive product control (spike) concentration would be 0.5 EU/mg for a standard curve with a range of 5 to 0.05 EU/mL.

The calculated mean amount of endotoxin in the spiked drug product, when referenced to the standard curve, must be within ±50% to be considered free of inhibition or enhancement. Failure to recover the spike within ±50% indicates sample interference. Further dilute the sample in LRW, not to exceed the MVD, until the spike is recovered consistently by the assay. Alternatively, test for interference by recovery of a 4 lambda endotoxin spike within ±50%. 11

REAGENTS/MATERIALS REQUIRED BUT NOT SUPPLIED - E coli ITBK-B Control Standard Endotoxin (CSE) is used to confirm LAL reagent sensitivity, validate product control and prepare inhibition controls (positive water and positive product controls). Refer to the CSE Certificate of Analysis for potency, rehydration and storage information.

LAL Reagent Water (non-LAL active) must be used to rehydrate LAL reagent and prepare samples, controls and endotoxin standards.

A heating block or water bath is required to incubate the assay mixture at a temperature of 37±1°C. Sterile, endotoxin-free accessories are needed which include: 10 x 75 mm tubes or microplates for the assay medium, borosilicate tubes of larger capacity for endotoxin standards, and a calibrated mechanical pipette with endotoxin-free plastic tips. Depyrogenated covers may be used to cover test tubes and rehydrated LAL Reagent. Test tube racks of suitable size are used to organize the assay. Timers are useful for measuring incubation times and endotoxin mixing periods.

KINETIC-TURBIDIMETRIC TEST PROCEDURE - Kinetic-turbidimetric analysis can be conducted in a tube or microplate reader that has a detection system to make optical density measurements over time and a microprocessor and suitable software to analyze data by linear (USA) regression. LAL HS-F may be used to assay endotoxin in these systems under validated conditions. The Toxinometer ET-201 (Wako Pure Chemical Industries, Ltd., Japan), an automated endotoxin measurement system designed as a tube reader, is available for kinetic-turbidimetric measurement.

Each assay should include dilutions of samples or products, positive product controls, either a series of dilutions covering the desired standard curve or a positive water control, and a negative control. Assay at least in duplicate. Follow the specific directions supplied with the automated endotoxin measurement system.

1. Microplate Method: Aseptically transfer 0.1 ml of each sample into the bottom of each well of a microplate, guided by the template which assigns each component, Pre-
incubate the plate for at least 5 minutes while preparing for the addition of LAL. Then, quickly add 0.1 ml of LAL (Wako-LAL HS-F) into reaction tubes. Add 0.1 ml of each sample or control to the respective reaction tube, beginning with the negative control and ending with the highest endotoxin concentration. After adding the LAL, quickly mix the contents of the tube without foaming. Place the tube into the kinetic tube reader in the order of the assigned tube position, and initiate the timed observation of each tube. After the monitored incubation period is completed, begin analysis of results; use the designated software to conduct regression analysis on the standard curve’s Onset Time (Gelation Time for the Toximeter) data and to calculate endotoxin levels for each sample.

2. Tube Method: Aseptically transfer 0.1 mL of LAL (Wako-LAL HS-F) into reaction tubes. Add 0.1 mL of each sample or control to the respective reaction tube, beginning with the negative control and ending with the highest endotoxin concentration. After adding the LAL, quickly mix the contents of the tube without foaming. Place the tube into the kinetic tube reader in the order of the assigned tube position, and initiate the timed observation of each tube. After the monitored incubation period is completed, begin analysis of results; use the designated software to conduct regression analysis on the standard curve’s Onset Time (Gelation Time for the Toximeter) data and to calculate endotoxin levels for each sample.

ROUTINE TESTING - Each assay should contain a standard curve with at least 3 points, tested in duplicate, such as 5, 0.5 and 0.05 EU/mL. When consistency in standard curves is observed, an archived curve may be generated, using 3 replicates, for a 5-point standard curve over a two-log range. If an archived curve is used, the positive water control should be the endotoxin concentration at the mid-point of the standard curve, on a log basis (0.5 EU/mL in the above example). This standard control must yield a mean endotoxin concentration within ± 25% of the mid-point of the archived curve.12

The positive product control (PPC) should contain endotoxin equal to the concentration at the mid-point of the standard curve. The mean endotoxin concentration of the positive product control must be within ± 50% of the standard curve concentration. An endotoxin standard series must be run when confirming end-point contamination.

CALCULATION OF ENDOTOXIN CONCENTRATION - Throughout the assay, the tube or microplate reader has monitored the increase in turbidity. The reader records the time required for the absorbance to increase significantly over background. This time is termed the ONSET TIME. The reader’s software produces a log/log correlation of the Onset Time of each standard with its corresponding endotoxin concentration. Standard curve features are then displayed and evaluated to determine if the analysis is valid. An example of a standard series (r = 0.999) and recovery of an endotoxin spike of 0.5 EU/mL in sample products is presented below

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>CSE (EU/mL)</th>
<th>MEAN ONSET TIME (sec)</th>
<th>RECOVERY (EU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD 1</td>
<td>5.0</td>
<td>798</td>
<td></td>
</tr>
<tr>
<td>STD 2</td>
<td>2.0</td>
<td>1030</td>
<td></td>
</tr>
<tr>
<td>STD 3</td>
<td>0.5</td>
<td>1344</td>
<td></td>
</tr>
<tr>
<td>STD 4</td>
<td>0.11</td>
<td>1812</td>
<td></td>
</tr>
<tr>
<td>STD 5</td>
<td>0.05</td>
<td>2163</td>
<td></td>
</tr>
<tr>
<td>SPL 1</td>
<td>---</td>
<td>&lt;&lt;&lt;&lt;</td>
<td>&gt;&gt;&gt;&gt;</td>
</tr>
<tr>
<td>PPC</td>
<td>0.5</td>
<td>1334</td>
<td>0.500 (102%)</td>
</tr>
<tr>
<td>SPL 1</td>
<td>0.1</td>
<td>1334</td>
<td></td>
</tr>
<tr>
<td>SPL 1</td>
<td>0.5</td>
<td>1387</td>
<td>0.420 (85%)</td>
</tr>
</tbody>
</table>

In this example: the positive product control (PPC) for each sample (SPL) yielded endotoxin recovery consistent with absence of inhibition. The negative control should be significantly lower than the lowest standard endotoxin concentration.

PERFORMANCE CHARACTERISTICS - Linearity: The linearity of the standard curve within the concentration range used to determine endotoxin levels must be verified. No less than 3 endotoxin standards, spanning the desired concentration range, and an endotoxin-free water blank should be assayed at least in triplicate, consistent with an INITIAL QUALIFICATION assay.12 The coefficient of correlation, r, shall be greater than or equal to the absolute value of 0.990.12

TEST PROCEDURE FOR GEL CLOT ASSAY - Each assay should include dilutions of a sample or product, a negative water control, a positive product control, and either a 2 lambda water control or a series of two-fold dilutions from an endotoxin standard which bracket the labeled LAL sensitivity. Add 0.1 ml of the reconstituted LAL to each assay tube. Aseptically transfer 0.1 ml of each sample/control into each assay tube, beginning with the negative control and ending with the highest endotoxin concentration. Then, quickly mix the contents of the tubes and incubate them in a 37°C dry or water heat bath for 60 minutes ± 2 minutes, undisturbed. After incubation, examine each tube for gelation.

INTERPRETATION OF RESULTS - Each tube in the gel-clot method is interpreted as either positive or negative. A positive test is defined as the formation of a firm gel capable of maintaining its integrity when the test tube is inverted 180°. A negative test is characterized by the absence of gel or by the formation of a viscous mass which does not hold when the tube is inverted. Test results are only valid when the positive water and specimen controls are positive at the 2 lambda endotoxin concentration, and the negative-controls are without gelation.

DETERMINATION OF ENDOTOXIN IN AN UNKNOWN BY GEL CLOT - To determine the endotoxin concentration in a specimen, test serial two-fold dilutions of the specimen until an endpoint is reached. The endotoxin concentration (E) in a sample is calculated by multiplying the LAL labeled sensitivity by the reciprocal of the dilution representing the endpoint. For example, a product sample was diluted for 5 two-fold dilutions, yielding an endpoint at the 1:8 dilution when tested with LAL Reagent where λ = 0.25 EU/mL. The endotoxin concentration was found to contain at least 2 EU/mL by the following calculation:

\[ E = \left( \frac{A}{B} \right) \times (0.25 \text{ EU/mL}) = 2 \text{ EU/mL} \]

ENDOTOXIN ASSAY BY DILUTION SERIES - Confirmation Of Label Claim is an assay of the LAL by U.S. Standard Endotoxin or standardized control endotoxin which yields an endpoint that is equal to or within a two-fold dilution of the labeled sensitivity. The results of an endotoxin assay of a LAL Reagent labeled with a sensitivity (A) of 0.12 EU/mL is presented below. A 4-point endotoxin dilution series was prepared to bracket the sensitivity.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Endpt. Log</th>
<th>Endpt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.06</td>
<td>-1.222</td>
</tr>
<tr>
<td>2</td>
<td>0.12</td>
<td>-0.921</td>
</tr>
<tr>
<td>3</td>
<td>0.12</td>
<td>-0.921</td>
</tr>
<tr>
<td>4</td>
<td>0.06</td>
<td>-1.222</td>
</tr>
</tbody>
</table>

The LAL sensitivity is calculated by determining the geometric mean of the endpoint. First, each endpoint of the quadruplicate assay is converted to log10. The individual log10 values are averaged and the LAL sensitivity is taken as the antilog of this mean log10 value. The geometric mean of the above example is 1.071 and the antilog value is 0.655 EU/mL.

QUALITY CONTROL PROCEDURES FOR KINETIC-TURBIDIMETRIC AND GEL-CLOT METHODS: Follow the FDA’s LAL Test Guideline for end-product testing for gel-clot quality control procedures which include 1) Initial quality control procedure for a testing laboratory; and 2) Test for confirmation of labeled LAL Reagent sensitivity.11

EXPECTED VALUES - LAL Reagent is standardized against the U.S. Standard Endotoxin so that the sensitivity is expressed in Endotoxin Units per milliliter (EU/mL). Endotoxin can be quantified if the concentration is greater than the labeled sensitivity or within the standard curve. Water and materials derived from biological sources may contain measurable levels of endotoxin if purification efforts are incomplete. Determined endotoxin content should be compared to the endpoint limit to assess its significance.

LIMITATIONS OF PROCEDURE - Samples may be tested by LAL methods provided that no inhibition or enhancement conditions are present that can not be eliminated by an acceptable dilution (refer to MVD calculation) or sample-pretreatment, such as buffering. If the LAL method cannot be validated at a concentration within the maximum valid dilution, the LAL test cannot be substituted for the USP Pyrogen Test.

SPECIFIC PERFORMANCE CHARACTERISTICS - The error of the gel-clot method is plus or minus two-fold dilution at the endpoint of the assay.

BIBLIOGRAPHY