LIMULUS AMEBOCYTE LYSATE

ENDOSAFE® KTA™

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MULTI-TEST VIAL FOR ENDOTOXIN (PYROGEN) DETECTION

INTENDED USE

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

WARNING

This product is intended as an in vitro end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices. This product is not intended for the detection of endotoxin in clinical samples or as an aid in the diagnosis of human disease.

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 is easily recognized.[1,5] 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.[6] The USP Bacterial Endotoxins Test <85> provides standard methods for validating the LAL Test as a replacement for the rabbit pyrogen test.[9]

The Gel-clot LAL test method is a simple, reproducible, semi-quantitative test that is conducted by mixing Endosafe® KTA™ and test specimen and observing for gelation after incubation for 60 minutes at 37° C. A positive response (gel) indicates that there is an amount of endotoxin in the sample which meets or exceeds the reagent's labeled sensitivity, represented by the symbol λ (lambda). 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, so endotoxin levels in unknown samples are determined by comparison to a standard curve. With kinetic measurements, λ is the lowest point on the standard curve.

BIOLOGICAL PRINCIPLES:

Frederick Bang observed that bacteria caused intravascular coagulation in the American horseshoe crab, *Limulus polyphemus*.[1] In collaboration, Levin and Bang[5] 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.

REAGENTS

LAL Reagent: Lyophilized Endosafe® KTA™ LAL Reagent, contains buffered amebocyte lysate stabilized by monovalent and divalent cations. Sensitivity, λ, is referenced to the U.S. Standard Endotoxin (RSE) and is stated in the labeling as EU/mL; it is defined as the lowest concentration of U.S. Standard Endotoxin (RSE) in Endotoxin Units per milliliter that produces a Gel-clot under standard conditions.

Reconstitution: Collect LAL powder into the bottom of the vial by tapping on a firm surface. Unseal and release the vacuum by slowly lifting the stopper, avoiding touch contamination. The small amount of LAL on the stopper is insignificant. Rehydrate with 5.2 mL of LAL Reagent Water or an Endosafe® reconstitution buffer by pipetting directly into the vial immediately before use. Remove and discard the stopper. Cover the vial with an endotoxin-free surface or the inner side of Parafilm® when not in immediate use. Gently swirl until LAL dissolves into a colorless solution. Discard if the seal integrity is breached or if color or opacity is present after rehydration.

Storage: Lyophilized LAL is relatively heat stable and should be stored at 2-25° 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 for up to twenty eight days after reconstitution and freezing, LAL may only be frozen and thawed once.

***E.coli* Control Standard Endotoxin (CSE)** is available from Charles River Endosafe to confirm LAL reagent sensitivity, validate product test methods, and prepare inhibition controls (positive water and positive product controls). Refer to the Certificate of Analysis for each CSE lot for potency, rehydration, and storage information. CSE must be ordered separately for this product.

LAL Reagent Water (non-LAL active) must be used to rehydrate LAL reagent and prepare samples, controls and endotoxin standards.[9] LAL Reagent Water must be ordered separately for this product.

WARNINGS AND GENERAL PRECAUTIONS

Warnings: Endosafe® KTA™ is intended for in-vitro diagnostic purposes only. Exercise caution when handling LAL because its toxicity is not known.

Correct application of this test requires strict adherence to all items in the recommended procedures. Positive controls should be included in LAL protocols to detect inhibitory conditions. All materials coming in contact with specimen or test material must be endotoxin-free. Glassware must be depyrogenated by validated conditions, such as three hours exposure at 200° C. It is prudent to test for endotoxin those materials that cannot be heat sterilized or those which are sold without an endotoxin-free label.

SPECIMEN COLLECTION AND PREPARATION

All materials or diluents 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. Use an endotoxin-free TRIS buffer (available from Endosafe) if pH adjustment is necessary. Do not arbitrarily adjust the pH of unbuffered solutions because Endosafe® KTA™ formulation is already buffered.

PRODUCT INTERFERENCE

A test method must be validated for each sample by demonstrating the absence of significant interference. Inhibition is usually concentration dependent, and is overcome by dilution with LAL Reagent Water (LRW). Common sources of inhibition include conditions that 1) interfere with the enzyme-mediated reaction, and 2) alter the dispersion of the endotoxin (Positive) control.[8]

Maximum Valid Dilution: USP <85> has listed endotoxin limits of 5 EU/kg for intravenous drugs and 0.2 EU/kg for intrathecal drugs.[9] Specific limits for compendial items have been adopted.[9] 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.[7] The Maximum Valid Dilution (MVD) is calculated by formulae presented in other pharmacopeia.[9]

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

$$MVD = \frac{\text{Endotoxin Limit} \times \text{Product Potency}}{\lambda}$$

Note: For Gel-clot testing λ is the labeled sensitivity of the LAL. For kinetic testing λ is the lowest point on the standard curve.

For example, the compendial limit for cyclophosphamide is 0.17 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 (one part to a total of 68 parts LRW).

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.

Select a point at or near the middle of the standard curve for interference testing. For example, the positive product control (spike) concentration would be 0.5 EU/mL for a standard curve with a range of 5 to 0.05 EU/mL. (See ROUTINE TESTING)

The calculated mean amount of endotoxin in the spiked drug product, when referenced to the standard curve, must be within 50–200% to be considered free of inhibition or enhancement. Failure to recover the spike within 50–200% indicates sample interference. Further dilute the sample in LRW, not to exceed the MVD, until the spike is recovered consistently by the assay.[9]

β-GLUCAN

Endosafe® KTA™ reacts with some β-Glucans in addition to endotoxin. Endosafe® KTA™ must be rendered unreactive to β-Glucans before testing samples that contain β-Glucan. This can be accomplished by using an endotoxin-specific (ES) buffer.

ADDITIONAL MATERIALS REQUIRED

Microplates.

Glass dilution tubes.

Repeating pipettor with individually wrapped, sterile dispensing syringes. (Eppendorf® Repeater™ with 0.5 mL and 5.0 mL Sterile Combitips®, or equivalent).

Glass pipettes (recommended) and calibrated automatic pipetters with sterile endotoxin-free tips.

Vortex-type Mixer.

An incubating kinetic microtiter plate reader or tube reader.

Note: Laboratory materials that need to be endotoxin-free should be validated or certified to less than the lowest endotoxin detection level of the test.

KINETIC-TURBIDIMETRIC TEST PROCEDURE

Kinetic-turbidimetric analysis may 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 regression analysis. The BioTek ELx808IU with Endoscan-V[10] software is an example of an incubating microplate reader with software designed to collect, process and store data using protocols for endotoxin measurement using LAL. Automated endotoxin measurement systems designed as tube readers are available for kinetic-turbidimetric measurement. Endosafe® KTA™ may be used in these systems under validated conditions.

Each assay should include samples or 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 selected for analysis.[8]

Method: Aseptically transfer 0.1 mL of each sample into the bottom of each well of a microplate or tube matrix, guided by the template which assigns each component. Microplate readers which are not equipped with stage heating may require pre-incubation of the plate to achieve temperature uniformity. Please follow the LAL manufacturer's guidelines to establish the appropriate operating parameters for the related equipment. Then, quickly add 0.1 mL of LAL (ambient temperature) to each well using a multi-test dispenser, beginning with the negative control and ending with the highest endotoxin concentration. If the plate reader does not mix the solutions automatically, tap the side of the plate several times and promptly initiate the timed observation. After the desired monitoring period is completed, initiate the programmed analysis of results.

ROUTINE TESTING

Routine testing is most efficiently done with a two-log standard curve; 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. For in-process or raw-materials testing, a three-log standard curve, from 50 to 0.05 EU/mL may be more suitable.

The positive product control (PPC) should contain endotoxin equal to the concentration at or near the mid-point of the standard curve. For example, when using a 10 – 0.01 EU/mL standard curve the spike value should be equal to or less than 1 EU/mL but greater than or equal to 0.1 EU/mL; when using a 50 – 0.005 EU/mL standard curve the spike value should be equal to or less than 5 EU/mL but greater than or equal to 0.05 EU/mL. The mean endotoxin concentration of the positive product control must be within 50–200% of the corresponding standard curve concentration. An endotoxin standard series should be run when confirming measured contamination. The efficient spiking technique recommended by Endosafe is direct addition of the endotoxin spike to the test specimen in the specified microplate well. With this technique the positive product controls are prepared by spiking 10 µl of the 5.0 EU/mL RSE or CSE into a microplate well containing 100 µl of test specimen before adding the KTA™ LAL Reagent.

RESULTS

CALCULATION OF ENDOTOXIN CONCENTRATION

Throughout the assay, the tube or microplate reader has monitored the increase in absorbance. The reader records the time required for the absorbance to increase significantly over background, usually 0.050 to 0.200 OD units. This time is termed the ONSET TIME. The reader's software automatically 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.

REPRESENTATIVE ANALYSIS

SPECIMEN	CSE (EU/mL)	MEAN ONSET TIME (sec)	RECOVERY (EU/mL)
STD 1	5.0	798	
STD 2	0.5	1344	
STD 3	0.05	2163	
SPL 1	----	<<<<	>>>>>
PPC	0.5	1334	0.508 (102%)
SPL 2	----	<<<<	>>>>>
PPC	0.5	1387	0.426 (85%)

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, should be assayed at least in triplicate.[9] The coefficient of correlation, r, shall be greater than or equal to the absolute value of 0.980.[9]

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. Aseptically transfer 0.1 mL of each component into their respective 10 x 75 reaction tube.

Add 0.1 mL of the reconstituted LAL to each 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 one hour, undisturbed. After one hour of 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 $\lambda = 0.25$ EU/mL. The endotoxin concentration was found to contain at least 2 EU/mL by the following calculation:

$$(E) = (\lambda)\ (8/1) = (0.25\ \text{EU/mL})(8) = 2\ \text{EU/mL}$$

QUALITY CONTROL PROCEDURES FOR KINETIC-TURBIDIMETRIC AND GEL-CLOT METHODS:

Follow USP <85> 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.[9] Refer to the Endosafe® KTA™ Package Insert for additional information on Gel-clot assays.

Charles River Endosafe has developed a guide for initial qualification of kinetic incubating microplate readers.

EXPECTED VALUES

LAL Reagent is standardized against the U.S. Reference Standard Endotoxin (RSE) 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 endotoxin 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 Bacterial Endotoxins Test.

The error of the Gel-clot method is plus or minus two-fold dilution at the endpoint of the assay.

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10. Reference Guide for Endoscan-V, Charles River Laboratories, Inc., 1023 Wappoo Road, Suite 43B, Charleston, SC, 29407 USA

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