LIMULUS AMEBOCYTE LYSATE

ENDOSAFE®
U.S. License No. 1197

SINGLE-TEST Vial For Endotoxin (Pyrogen) Detection

INTENDED USE: Limulus amebocyte lysate (LAL), derived from *Limulus polyphemus* amebocytes, is intended for use in the qualitative detection of gram-negative bacterial endotoxins by the gel-clot method.

SUMMARY AND GENERAL INFORMATION: The LAL test is the most sensitive and specific means to detect and measure 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 readily recognized.[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 and U.S. Food and Drug Administration Guideline for LAL testing provide standard methods for validating the LAL Test as a replacement for the rabbit pyrogen test.[10,11]

The gel-clot LAL test method is a simple, reproducible, test that is conducted by mixing **ENDOSAFE®** LAL reagent and test specimen and promptly incubating the mixture undisturbed for 60 minutes at 37ºC. A positive response on the gel clot test indicates there is an amount of endotoxin in the sample which equals or exceeds the reagent's labeled sensitivity, represented by the symbol lambda, λ.

BIOLOGICAL PRINCIPLES: The development of a viable alternative to the rabbit pyrogen test began with the innovative work of John Hopkins Univ. investigators. Frederick Bang observed that bacteria caused intravascular coagulation in the American horseshoe crab, *Limulus polyphemus*. 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) produced a gelation reaction of amebocyte lysate by an enzymatic process. Serine protease zymogens found in amebocyte lysate are activated by endotoxin in the presence of divalent cations to initiate an enzymatic coagulation cascade that alters an abundant protein called coagulogen to produce a proteinaceous gel.[7]

The need for a suitable pyrogen test for radiopharmaceuticals led Cooper, Levin and Wagner to extend this new approach to drugs. A comparative study in 1970 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.[2,4,9]

The LAL reaction requires a neutral pH and is time and concentration dependent. The test is generally limited to aqueous solutions or extracts of test specimen. Most LAL test interferences are overcome by simple dilution.[8]

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 the Agency considers necessary to validate the use of LAL as an end-product endotoxin test.[10] Those who adhere to the guideline are considered in compliance with relevant cGMP provisions for drugs and devices and other applicable requirements. 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.[10]

GENERAL PRECAUTIONS: **ENDOSAFE®** LAL is intended for in vitro diagnostic purposes only. It is not to be used for detection of endotoxemia. Avoid direct contact with 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 200º C. It is our experience that plasticware labeled as sterile and disposable is endotoxin-free.

REAGENTS PROVIDED
Lyophilized LAL (**ENDOSAFE®**) is presented in single-test glass vials. The reagent contains buffered lysate and is stabilized by monovalent and divalent cations, and is sealed under approximately 1/2 atmosphere of dry nitrogen. Do not rehydrate until immediately prior to use.

Lyophilized LAL should be stored at 2-8ºC; avoid exposure to temperatures above 25ºC. The tube should be discarded if there is any yellow discoloration.

REAGENTS AND MATERIALS NOT PROVIDED
LAL Reagent Water (non LAL-reactive) must be used to prepare samples and positive controls. See Product No. W110.

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. High and low potency CSE are available from Endosafe.

A **water bath** or **heating block** is required to incubate the assay mixture at a temperature of 37º C, plus or minus 1º C. Sterile, endotoxin-free accessories are needed which include: 16 x 125 mm or larger reusable borosilicate tubes or equivalent, a calibrated mechanical pipetor with sterile, disposable plastic tips for accurate delivery of volumes less than 1 mL, and pipets for larger volumes. Test tube racks are needed for holding reaction tubes and standard endotoxin dilution tubes. Timers are useful in measuring incubation times and endotoxin mixing periods.

REAGENT PREPARATION: Caution: Single-test LAL must be incubated immediately after rehydration with test sample or control. The LAL is reconstituted by addition, directly into the test vial, of 0.2 mL of control solution or sample to be tested.

PREPARATION OF CONTROL STANDARD ENDOTOXIN (CSE)
Reconstitution: A CSE of **E. coli** is available from Charles River Endosafe which is suitable for confirmation of **LAL** labeled sensitivity and for preparation of positive controls. The CSE has a predetermined amount of endotoxin, as described in the Certificate of Analysis (COA), which was standardized with U.S. Reference Endotoxin. Note that the COA is specific to a lysate lot and CSE lot. The USP Reference Standard Endotoxin may be purchased from the U.S. Pharmacopoeial Convention, Inc., Rockville, MD 20852. The lyophilized endotoxin (CSE) must be prepared according to the package insert and the COA. Rehydrate the CSE with LAL Reagent Water and vortex vigorously for 5 minutes before further dilution. Dilution to 1 EU/mL should be made, then two-fold dilutions to bracket the labeled LAL Reagent sensitivity.

Storage: Rehydrated endotoxin may be stored for 28 days at 2 to 8º C. Diluted endotoxin solutions should be made daily unless longer intervals have been validated.

SPECIMEN COLLECTION AND PREPARATION
Specimen for testing with Endosafe® LAL must be collected and prepared using depyrogenated materials and endotoxin-free reagents. If the positive product control fails and a pH related problem is suspected, the pH of the test specimen and LAL mixture should be measured to assure a pH within the range of pH 6.0 to 8.0. If pH adjustment is necessary, use endotoxin-free HCl or NaOH at a suitable concentration (generally 0.1N or less), or a Tris buffer from Charles River Endosafe. Do not arbitrarily adjust the pH of unbuffered solutions. **If the specimen contains interfering substances, dilute or modify the specimen to an extent that eliminates interference, as discussed in the PRODUCT INHIBITION Section.**

TEST PROCEDURE: The single-test vials containing LAL serve as the test container. Before use, collect the vial contents by gently tapping the bottom of the vial on a hard surface. Use aseptic technique when removing the rubber stopper. Test as follows:

Aseptically add 0.2 mL of each test specimen to assay tubes. Mix the contents gently until the contents are dissolved. Immediately place the reaction tubes in a 37ºC water or dry bath for 60 minutes (plus or minus 2 minutes). **Timing of the reaction of ENDOSAFE® LAL with endotoxin is critical. If large numbers of samples are to be tested in parallel, the reactions should be started at 2-4 minute intervals so as to permit reading of each test within the above time limit.**

Since the reaction of ENDOSAFE® LAL is temperature sensitive, the incubator must be monitored carefully. Also, the gel-forming reaction is delicate and may be irreversibly altered if the tubes are disturbed during the incubation period.

ENDOTOXIN CONTROL SERIES (Positive Water Controls)
An endotoxin standard series does not have to be run with each set of tests if consistency of standard endpoints has been demonstrated. It should be run at least once a day with the first set of tests and repeated if there is any change in LAL lot or test conditions.

A fresh CSE control dilution series should be prepared from a stock solution in a two-fold dilution series that brackets the labeled sensitivity (λ) of **ENDOSAFE®** LAL reagent. A 4-point series is usually made with two endotoxin dilutions above and below. Add 0.2 mL of each concentration of endotoxin directly to the assay tube. Mix and incubate as described above.
TEST CONTROLS: Prepare the Negative Control by adding 0.2 mL of the LAL Reagent Water to the assay tube. In the absence of an endotoxin series, add 0.2 mL of a 2 lambda concentration of the endotoxin standard. For a Positive Product Control, add 0.2 mL of a mixture containing a 2 lambda concentration of endotoxin in the test specimen, which may be modified or diluted consistent with validated conditions. This control assures the absence of interference. See section on **PRODUCT INHIBITION**.

INTERPRETATION OF RESULTS: Each tube in the gel-clot method is interpreted as either positive or negative. A **positive** result is defined as the formation of a firm gel capable of maintaining its integrity when the test tube is inverted 180º. 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.

EXPECTED VALUES: ENDOSAFE® LAL Reagent is standardized against the U.S. Reference Endotoxin, so that the sensitivity is expressed in Endotoxin Units per milliliter (EU/mL). Confirmation of label claim is an assay of the LAL by a 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 (λ) of 0.125 EU/mL is presented in Table I. A 4-point endotoxin dilution series was prepared to bracket the labeled sensitivity.

TABLE I: CONFIRMATION OF LABEL CLAIM ASSAY

	Endotoxin Dilution (EU/mL)				
Replicate	0.25	0.125	0.06	0.03	Endpoint
1	+	+	+	-	0.06
2	+	+	-	-	0.125
3	+	+	-	-	0.125
4	+	+	+	-	0.06

The LAL sensitivity is calculated by determining the geometric mean of the endpoint. Each endpoint of the quadruplicate assay is converted to $\log_{10}$. The individual $\log_{10}$ values are averaged and the LAL sensitivity is taken as the antilog of this average log value (see Table II).

TABLE II: CALCULATION OF GEOMETRIC MEAN ENDPOINT

Endpoint (EU/mL)	Log_{10} Endpoint
0.06	-1.222
0.125	-0.903
0.125	-0.903
0.06	-1.222
Mean=	-1.0625
Antilog_{10} Mean=	0.0865

INITIAL QUALITY CONTROL PROCEDURE FOR A TESTING LABORATORY: The variability of a test laboratory and its analysts should be assessed before any official tests are done. Each analyst, using a single lot of LAL and a single lot of endotoxin (CSE or RSE), should correctly and satisfactorily complete the test for confirmation of labeled LAL sensitivity. Acceptable variation is one half (0.5 λ) to two times (2 λ) labeled sensitivity (λ).

TEST FOR CONFIRMATION OF LABELED LAL REAGENT SENSITIVITY: The labeled sensitivity must be confirmed before a new LAL lot is introduced into a test laboratory. A single lot of LAL should be assayed by a single lot of endotoxin (CSE or RSE) by testing in quadruplicate vials (see Table I). The geometric mean of the endpoints must be within the limits of labeled claim, as defined and illustrated above.

DETERMINATION OF ENDOTOXIN IN AN UNKNOWN:
To determine the endotoxin concentration in an unknown, 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 aliquot was diluted by preparing a series of two-fold dilutions with LAL Reagent Water. A test of each product dilution yielded an endpoint at the 1:8 dilution when tested with LAL Reagent having a labeled sensitivity (λ) equal to 0.25 EU/mL. The endotoxin titer was determined to contain at least 2 EU/mL by the following calculation:

(E) = (λ)(8/1) = (0.25 EU/ml)(8) = 2 EU/ml

PRODUCT INHIBITION: Before routine LAL testing is started, the potential for product inhibition must be excluded. Inhibition is usually concentration dependent, and is easily 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 control. Inhibition exists if the endpoint of an assay of a two-fold endotoxin dilution series made with the specimen (Positive Product Controls) differs more than one two-fold dilution from the endpoint of a similar endotoxin series in water (Positive Water Control). Product inhibition may be recognized as follows:

Labeled LAL Sensitivity (λ)	= 0.125 EU/mL
Endpoint Positive Water Controls	= 0.125 EU/mL
Endpoint Positive Product A Controls	= 0.20 EU/mL
Endpoint Positive Product B Controls	= 0.50 EU/mL

Product A is considered within limits whereas Product B exhibits inhibition. The easiest method to determine the non-inhibitory product concentration is to prepare a series of increasing dilutions of the product containing a 2 lambda endotoxin concentration.[8,10] Assay this series as well as a series of the product diluted with water. The following results are consistent with a product that is non-inhibitory at a 1:20 dilution or greater, and is endotoxin-free.

Specimen Dilution	1:4	1:10	1:20	1:40
Product and 2 λ Endotoxin	-	-+	++	++
Product and LAL Reagent Water	-	-	-	-

Raw materials may be acidic or basic and require pH adjustment to neutrality as well as dilution to resolve product inhibition. Endosafe LAL reagent is particularly resistant to interference because of its high buffer capacity and balanced divalent and monovalent cation formulation.

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.[10] The U.S. Pharmacopeia has adopted specific limits for compendial items such as 175 EU per dose of radiopharmaceutical.[11] These limits may be used to determine the extent of dilution that may be applied to overcome an interference problem without exceeding the limit endotoxin concentration. The Maximum Valid Dilution (MVD) may be calculated by formulae presented in the previously mentioned documents.

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

$$\text{MVD} = \frac{\text{Endotoxin Limit x Potency of Product}}{\text{Labeled Sensitivity, } \lambda}$$

For example, the compendial limit for cyclophosphamide is 0.17 EU/mg. If a LAL Reagent with λ = 0.125 is used to test this product where the potency is 20 mg/mL, the MVD equals 1:27.

$$\text{MVD} = \frac{0.17 \text{ EU/mg} \times 20 \text{ mg/mL}}{0.125 \text{ EU/mL}} = 27.2$$

Under these conditions, cyclophosphamide may be diluted up to 1:27 in order to resolve an inhibition that might be present.

LIMITATIONS: Samples may be tested by LAL methods provided that no inhibition or enhancement conditions are present that cannot 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.[10]

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

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