LIMULUS AMEBOCYTE LYSATE
ENDOSAFE®
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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 qualitative detection of 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.[9,10]

The gel-clot LAL test method is a simple, reproducible test that is conducted by mixing equal parts of **Endosafe® LAL** reagent and test specimen and promptly incubating the mixture undisturbed for 60 minutes at 37°C. A positive response on the gel clot test indicates that there is an amount of endotoxin in the sample which equals or exceeds the reagent's labelled 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 Johns Hopkins Univ. investigators. 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) produced a gelation reaction of amebocyte lysate by an enzymatic process.[1,4]

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.[2,4,7]

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.

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.[9] Those who adhere to the LAL 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.[9]

GENERAL PRECAUTIONS

Endosafe® LAL is intended for in vitro diagnostic purposes only. It is not to be used for detection of endotoxemia. Exercise caution when handling LAL because its toxicity is not known.

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.

REAGENTS PROVIDED

Lyophilized **Endosafe® LAL** is presented as a 5.2 mL vial for 50 tests or a 1.2 ml vial for 10 tests. The reagent contains buffered amebocyte lysate and is stabilized by monovalent and divalent cations.

REAGENTS AND MATERIALS NOT PROVIDED

LAL Reagent Water (non LAL-reactive) must be used to rehydrate LAL reagent and prepare endotoxin controls.

Control Standard Endotoxin (CSE) or Reference Endotoxin (RSE) is necessary for preparation of positive controls and endotoxin standard solutions. A **vortex mixer** is best suited for mixing of endotoxin solutions and subsequent dilutions.

A **water bath** or **heating block** is required to incubate the assay mixture at a temperature of 37° C, ±1° C. Sterile, endotoxin-free accessories are needed which include: 10 x 75 mm glass test tubes for the gel-clot assay, 16 x 125 mm or larger reuseable borosilicate tubes, 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 neeed for holding reaction tubes and standard endotoxin dilution tubes. Timers are useful in measuring incubation times and endotoxin mixing periods.

PREPARATION AND STORAGE OF Endosafe® LAL REAGENT

Reconstitution: Collect LAL powder into the bottom of the vial by tapping on a hard surface. Rehydrate with the indicated amount of LAL Reagent Water just before use by pipeting it directly into the vial after removing the stopper. Cover the vial with the stopper or Parafilm when not in immediate use. Mix the LAL gently until it dissolves. Avoid touch contamination of closures. Discard if seal integrity is breached or if color or opacity is present after rehydration.

Storage: Lyophilized **LAL** 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 -20°C up to 28 days after reconstitution and freezing; LAL can be frozen and thawed only once.

PREPARATION OF CONTROL STANDARD ENDOTOXIN (CSE)

Reconstitution: E. coli CSE 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. Serial dilutions 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 8° C. Diluted endotoxin solutions should be made daily unless storage conditions and periods 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. Use aseptic technique at all times. **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

A. PREPARATION OF ASSAY PROCEDURE AND INCUBATION.

1. Add 0.1 mL of each dilution of test specimen to an assay tube; test at least in duplicate.

2. Add 0.1 mL of reconstituted **Endosafe® LAL** to each tube, starting with the negative control and ending with the highest endotoxin concentration. Next, mix the assay tubes by hand or low-speed vortexing to avoid mixing artifacts.

3. Place the reaction tubes in a 37±1°C water or dry bath for 60 minutes (±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.

B. ENDOTOXIN CONTROL SERIES (Positive Water Controls)

1. An endotoxin standard series does not have to be run with each set of tests if endpoints are consistent. 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.

2. Prepare a CSE series according to the directions provided in the Certificate of Analysis for the specified LAL and CSE. The series should consist of four CSE concentrations prepared by serial two-fold dilutions to yield 2λ, λ, 1/2λ and 1/4λ and bracket the labeled sensitivity (λ) of **Endosafe® LAL** reagent. Add 0.1 ml of each endotoxin standard to a reaction tube; test at least in duplicate.

3. Starting with the negative control add 0.1 ml of LAL to each reaction tube and mix as directed (step A.2).

4. Incubate the reaction tubes undisturbed for 60 minutes at 37°C (step A.3).

C. TEST CONTROLS

1. **NEGATIVE CONTROLS.** Add 0.1 mL of the LAL Reagent Water used in the assay to duplicate the tubes.

2. **POSITIVE WATER (ENDOTOXIN) CONTROLS.** In the absence of an endotoxin series, add 0.1 mL of a 2λ concentration of endotoxin standard.

3. **POSITIVE SPECIMEN CONTROL.** Add 0.1 mL of a mixture containing a 2λ concentration of endotoxin in the test specimen, which may be modified or diluted in accordance with validated conditions. This interference control assures the absence of inhibition, and it is prepared by adding endotoxin to an aliquot of the test specimen so that there is a 2λ concentration in the specimen. Concentrated endotoxin solutions may be used to prepare product controls.

4. Finally, add 0.1 ml of LAL reagent to each tube, mix, and incubate as directed above (Steps A.2 and A.3).

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 valid only when the positive water controls perform as expected, the interference controls are positive at the 2λ endotoxin concentration, and the negative controls are negative as described above.

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 reference 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 (lambda) 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: RESULTS OF GEL-CLOT 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 to conform with the FDA guideline[9]. Each analyst, using a single lot of LAL and a single lot of endotoxin (CSE or RSE), should satisfactorily complete the test for confirmation of labeled LAL sensitivity. Acceptable variation is one half (0.5 λ) to two times (2 λ) the 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 not less than 4 replicate vials. 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 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 4 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:

$$(E) = (\lambda)(8) = (0.25\ EU/ml)(8) = 2\ EU/ml$$

Alternatively, the endotoxin level may be calculated by dividing the LAL labeled sensitivity by the geometric mean endpoint.

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.[7] 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 (lambda)	=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 2λ endotoxin concentration.[7,9] 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 lambda Endotoxin	--	-+	++	++
Product and LAL Reagent Water	--	--	--	--

Endosafe® LAL includes a buffer which neutralizes many unfavorable pH conditions. Measurement or adjustment of pH is unnecessary in the presence of valid positive product (interference) controls and validated test conditions. When interference controls fail, it is prudent to measure pH because the LAL-endotoxin reaction is optimum at neutrality. Measure the pH of equal-part mixtures of LAL and the test specimen when sub-optimum conditions are suspect. Ideally, neutralize with a suitable buffer or predetermined amounts of dilute acid or base.

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.[9] The U.S. Pharmacopeia has adopted specific limits for compendial items such as 175 EU per dose of radiopharmaceutical.[10] 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.[8,9]

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

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

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.

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

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 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.[9,10]

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

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