

LIMULUS AMEBOCYTE LYSATE F AND HS-F

U.S. License No. 1197

SINGLE-TEST Vial For Endotoxin (Pyrogen) Detection

INTENDED USE

Limulus amoebocyte lysate (LAL), LAL F or HS-F, is an lysate derived from washed Limulus Polyphemus amoebocytes, which 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 equal parts of LAL F or HS-F 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 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 Johns Hopkins Univ. investigators. Frederick Bang observed that bacteria caused intravascular coagulation in the American horseshoe crab, Limulus polyphemus.¹ In collaboration, Levin and Bang⁵ found that the agent responsible for the clotting phenomena resided in the crab's amoebocytes, or circulating blood cells, and that pyrogen (bacterial endotoxin) produced a gelation reaction of amoebocyte lysate by an enzymatic process. Serine protease zymogens found in amoebocyte lysate are activated by endotoxin and 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.

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 biologics, 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

LAL F or HS-F 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.

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. Plasticware labeled as sterile and disposable is usually endotoxin-free. However, 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 LAL F or HS-F is presented in single-test glass vials. The reagent contains buffered amoebocyte 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 2-8°C; avoid exposure to temperatures above 25°C. 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 rehydrate LAL reagent and prepare samples and positive 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, plus or minus 1° C. Sterile, endotoxin-free accessories are needed which include: 16 x 125 mm or larger reusable borosilicate tubes, a calibrated mechanical pipettor 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 UKT-B, is available from WAKO 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 5 mL of 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 longer intervals have been validated.

SPECIMEN COLLECTION AND PREPARATION

Specimen for testing with LAL F or HS-F must be collected and prepared using depyrogenated materials and endotoxin-free reagents. Since the LAL-endotoxin reaction is pH dependent, an equal volume of the specimen to be tested and LAL should be mixed and demonstrated to have 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). Do not adjust the pH of unbuffered saline or water. 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

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:

Add 0.2 mL of each test specimen to an assay tubes. Mix the contents gently until the contents are dissolved. Immediately place the reaction tubes in a 37°C water or dry bath for 60 minutes (plus or minus 2 minutes). Timing of the reaction of LAL F or HS-F 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 LAL F or HS-F 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 set of dilutions should be prepared from a stock solution in a two-fold dilution series that brackets the labeled sensitivity (λ) of LAL F or HS-F reagent. A 5-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 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.

EXPECTED VALUES

LAL F or HS-F Reagent is standardized against the U.S. Reference Endotoxin, Lot EC-6, so that the sensitivity is expressed in Endotoxin Units per milliliter (EU/mL). Confirmation of label claim is an assay of the LAL by EC-6 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 (λ) of 0.125 EU/mL is presented in Table I. A 5-point endotoxin dilution series was prepared to bracket the labeled sensitivity.

TABLE I: RESULTS OF GEL-CLOT ASSAY

Replicate	Endotoxin Dilution (EU/mL)					Endpoint
	0.50	0.25	0.125	0.06	0.03	
1	+	+	+	-	-	0.06
2	+	+	+	-	-	0.12
3	+	+	+	-	-	0.12
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 log¹⁰. The individual log¹⁰ 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
0.12	-0.921
0.12	-0.921
0.06	-1.222
Mean =	-1.071
Antilog ₁₀ Mean =	0.085

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 (0.5λ) to two times (2λ) 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 level is calculated by dividing the LAL labeled sensitivity by the geometric mean endpoint.

TABLE III:
DETERMINATION OF ENDOTOXIN CONCENTRATION IN A SPECIMEN
LAL SENSITIVITY = 0.125 EU/mL

Replicate Tube	SPECIMEN DILUTION			
	1:2	1:4	1:8	1:16
1	+	+	+	+
2	+	+	+	-

Endpoint Specimen Dilution	Log ¹⁰ Endpoint
1:16 (0.0625)	-1.204
1:8 (0.125)	-0.903
Mean =	-1.054
Antilog ¹⁰ Mean =	0.088

$$\text{Endotoxin Concentration} = \text{LAL Sensitivity} \div \text{mean endpoint} \\ = 0.125 \text{ EU/mL} \div 0.088 = 1.42 \text{ EU/mL}$$

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)	= 0.12 EU/mL
Endpoint Positive Water Controls	= 0.12 EU/mL
Endpoint Positive Prod. A Controls	= 0.20 EU/mL
Endpoint Positive Prod. 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.⁸ 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 2λ Endotoxin	-	++	++	++
Product and LAL Reagent Water	-	-	-	-

Products which are acidic or basic may require pH adjustment to neutrality as well as dilution to resolve product inhibition.

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:

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

For example, the compendial limit for cyclophosphamide is 0.17 EU/mg. If a LAL Reagent with $\lambda = 0.125$ 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

Pharmaceuticals and medical devices should not be released by end-product LAL testing unless test conditions are validated. The LAL test is not valid for end-product testing if the specimen must be diluted greater than the MVD to overcome inhibition.

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