

**LIMULUS AMEBOCYTE LYSATE
ENDOCHROME™
U.S. LICENSE NO. 1197**

MULTI-TEST VIAL FOR ENDOTOXIN (PYROGEN) DETECTION

INTENDED USE

For the quantitative detection of bacterial endotoxin in aqueous solution by end-point chromogenic Limulus amoebocyte lysate (LAL) methods.

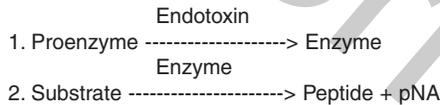
SUMMARY AND GENERAL INFORMATION

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 is easily recognized.^{1-4,6} 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.⁷ The USP Bacterial Endotoxins Test and USFDA Guideline for LAL testing provide standard methods for validating the LAL Test as a replacement for the rabbit pyrogen test.^{10,12}

The end-point chromogenic LAL test method is a simple, reproducible, quantitative test that is conducted by mixing ENDOCHROME™ and test specimen and monitoring the appearance of yellow color. With the aid of a photometer or a microplate reader, an end-point chromogenic assay may be done where the color intensity is directly related to endotoxin concentration in the sample. Endotoxin levels in unknown samples are determined by comparison to a standard curve. With quantitative measurements, lambda (λ) is the lowest point on the standard curve.

BIOLOGICAL PRINCIPLES

Bacterial endotoxin initiates activation of a proenzyme (a cascade of serine protease enzymes) in LAL that cleaves a peptide from coagulogen to produce opacity.⁶ However, in the presence of a colorless substrate (S-2423), it rapidly catalyzes the cleavage of the chromophore, p-nitroaniline (pNA). The pNA produces a yellow color that is measured spectrophotometrically at 405-410 nm.



Endotoxin measurement by an end-point chromogenic technique is dependent on a direct relationship between endotoxin concentration and color development.

REAGENTS

LAL Reagent: Lyophilized Endosafe® ENDOCHROME™ LAL Reagent contains buffered amoebocyte lysate stabilized by monovalent and divalent cations. The sensitivity (designated as λ) of a given kinetic assay is the lowest concentration used to generate an endotoxin (CSE or RSE) standard curve.

Reconstitution: 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 1.4 mL of LAL Reagent Water (LRW) immediately before use by pipetting it directly into the vial. Cover the vial with the inner side of Parafilm® when not in immediate use. LAL dissolves readily by gentle mixing into a colorless solution. Discard if the vacuum is absent or a marked turbidity is present.

Storage: Lyophilized LAL is relatively heat stable and should be stored at 2-8° C; avoid prolonged exposure to temperatures above 25° C. LAL reconstituted with LRW may be stored for 4 hours at 2-8° C or for two weeks at or below -20° C immediately after reconstitution. LAL may only be frozen and thawed once.

S-2423 Substrate: 10 mg lyophilized chromogenic substrate with mannitol filler. Rehydrate with 7.2 mL of LRW. Reconstituted substrate is stable for one month at 2-8° C.

Buffer: Endotoxin-free buffer solution of 0.05 M Tris, pH 9.0, for mixing with substrate.

E.coli Control Standard Endotoxin (CSE): Lyophilized CSE for positive controls. Refer to Certificate of Analysis for potency, rehydration, and storage information.

LRW: Non-LAL reactive LRW for preparing reagents and test specimen.

Substrate-Buffer Solution: Mix one volume of Substrate solution and one volume of buffer. The solution is stable for 8 hours at 20-25° C.

WARNINGS AND GENERAL PRECAUTIONS

Warnings: Endosafe® ENDOCHROME™ 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.

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 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.⁹

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 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.⁸ The Maximum Valid Dilution (MVD) is calculated by formulae presented in the previously mentioned documents and other pharmacopeia.^{10,12}

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

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

Note: For end-point chromogenic LAL 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 0.1 EU/mL of endotoxin is used to test this product, where the potency is 20 mg/mL, the MVD equals 1:34. Thus, cyclophosphamide may be diluted up to 1:34 to resolve potential inhibition.

Interference (inhibition/enhancement) testing is done by spiking a sample or diluted sample with a known concentration of endotoxin (equal to 4λ) and testing for spike recovery in duplicate by the supplier's instructions. Non-interference is consistent with spike recovery within +/- 25% of the nominal endotoxin concentration. That is, the calculated mean amount of endotoxin in the spiked drug product, when referenced to the standard curve, must be within +/- 25% to be considered free of inhibition or enhancement. Further dilute the sample in LRW, not to exceed the MVD, until the spike is recovered consistently by the assay.

ADDITIONAL MATERIALS REQUIRED

Microplates.

Depyrogenated glass dilution tubes.

Repeating pipetter with individually wrapped, sterile dispensing syringes (Eppendorf Repeater™ with 2.5 mL Sterile Combipips®, or equivalent).

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

Vortex-type mixer.

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

TEST PROCEDURE

Timing of the test must begin when the LAL is added to the first microplate well. Then keep a consistent rate of pipetting (use a repetitive hand dispenser).

ADD IN A MICROPLATE:

Test Sample or standard (20-25° C)	50 µL
Incubate at 37° C for approximately 5 min.	
LAL solution	50 µL
Incubate at 37° C for approximately 7 min.	
Substrate-Buffer Solution (37° C)	100 µL
Mix and Incubate at 37° C (5 min.)	
Acetic Acid, 20%	100 µL
Mix Immediately	

• See the CSE Certificate of Analysis for exact times for high and low range assays.

Read the absorbance in a photometer reader at 405-410 nM.

END-POINT METHOD FOR THE DETERMINATION OF ENDOTOXIN IN WATER STANDARDIZATION

1. Concentration range 0.15-1.2 EU/mL (high range)

Run a complete standard curve in each run, or once daily if consistency of the standard curve has been established. In the latter case, include the standard representing the 4λ of the standard curve in each run. Check that this standard does not differ more than 25% from the corresponding standard of the curve. The standards should be run in duplicate.

The LAL / sample / standard incubation times may vary from 4-9 minutes and should be established during the assay validation. See the CSE Certificate of Analysis for the incubation interval recommended for each lot of Endosafe® ENDOCHROME™.

2. Concentration range 0.015-0.12 EU/mL (low range)

The low-range measurements require longer incubation periods than those for the high range of endotoxin detection. The LAL / sample / standard incubation times may vary from 6-20 minutes. This incubation period should also be established during the assay validation. See the CSE Certificate of Analysis for the low range incubation interval recommended for each lot of Endosafe® ENDOCHROME™.

CALCULATION OF ENDOTOXIN CONCENTRATION

The endotoxin concentration in unknowns may be determined by calculator or graphical methods. Ideally, a calculator or microprocessor with linear regression capability is used. First, enter the standard curve data. Then, determine the corresponding endotoxin concentration of the samples by substituting their absorbances into the standard linear regression.

For the graphical method, plot the values for the mean absorbance and corresponding endotoxin concentration in EU/mL. Construct a best fit straight line between these points and determine the endotoxin concentration of a sample graphically.

SAMPLES OTHER THAN WATER

For samples which contain elements other than water, the occurrence of interference such as inhibition or enhancement of the reaction must be examined. This is done by comparing the recovery after adding an equal amount (e.g., four times the lowest standard point) of the endotoxin to the sample and water respectively. Most interferences can be overcome by diluting the sample.

When the inhibition / enhancement has been reduced to an acceptable level, (<25%), the water procedure can be followed.

PERFORMANCE CHARACTERISTICS

Linearity: The linearity of the standard curve within the concentration range used to determine endotoxin levels must be verified. No less than 4 endotoxin standards, spanning the desired concentration range, and an endotoxin-free water blank should be assayed in quadruplicate.¹¹ The absolute value of the coefficient of correlation, r , shall be greater than or equal to 0.980.¹¹

Reproducibility: Replicate samples should be run to establish proficiency and low coefficient of variation. The coefficient of variation (CV) equals 100 times the standard deviation of a group of values, divided by the mean. The CV is expressed as a percentage, and should be less than 10%.

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Product **R160** is a 140-test (5-vial) kit with LRW, Substrate, Control Standard Endotoxin and Buffer.

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