# LIMULUS AMEBOCYTE LYSATE
## ENDOSAFE® ENDOCHROME-K™
## U.S. License No. 1197

**MULTI-TEST VIAL FOR ENDOTOXIN (PYROGEN) DETECTION**

**INTENDED USE**
**Co-lyophilized Limulus amebocyte lysate (LAL) and a synthetic color producing substrate, which is intended for quantitative detection of endotoxins by kinetic-chromogenic 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.[1,5] 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 USFDA Guideline for LAL testing provide standard methods for validating the LAL Test as a replacement for the rabbit pyrogen test.[9,11]

With the aid of a microprocessor and microplate or tube reader, a kinetic colorimetric assay may be done where the early onset of color can be detected and precisely measured. The time for onset of color 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, lambda (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 color and gel-forming reaction enzymatically.

In the presence of a colorless substrate, the enzymatic reaction will cause a yellow color to develop upon cleavage of the chromophore, p-nitroaniline (pNA). pNA liberation can be measured spectrophotometrically.

## REAGENTS
**LAL Reagent:** Lyophilized Endosafe® Endochrome-K™ LAL Reagent contains a synthetic chromogenic substrate and buffered amebocyte lysate stabilized by monovalent and divalent cations.

**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 3.2 - 3.4 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 2-8° 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 for up to two weeks 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.

**LAL Reagent Water** (non-LAL active) must be used to rehydrate LAL reagent and prepare samples, controls and endotoxin standards.[11]

### WARNINGS AND GENERAL PRECAUTIONS
**Warnings:** Endosafe® Endochrome-K™ 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 on those materials that cannot be heat sterilized or those which are sold without an endotoxin-free label.

## SPECIMEN COLLECTION AND PREPARATION FOR ANALYSIS
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® Endochrome-K™ 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:** 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] Specific limits for compendial items have been adopted.[11] 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 the previously mentioned documents and other pharmacopeia.[9,11]

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 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. The curve must meet the criteria stated in “Performance Characteristics”.[10]

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.[10]

### β-GLUCAN

Endosafe® Endochrome-K™ reacts with some β-Glucans in addition to endotoxin. Endochrome-K™ 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.

Depyrogenated glass dilution tubes.

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

Depyrogenated 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 be less than the lowest endotoxin detection level of the test.

### KINETIC-COLORIMETRIC TEST PROCEDURE
Endochrome-K™ is approved for Kinetic-colorimetric analysis in a tube reader or microplate reader that has: 1) a detection system to make optical density measurements over time, and 2) a microprocessor and suitable software to analyze data by linear or polynomial regression. The Tecan Sunrise with Endoscan-V software[12] is an example of an incubating microplate reader with software designed to collect, process, and store data using protocols for LAL endotoxin measurement. Automated endotoxin measurement systems designed as tube readers are available for kinetic-colorimetric measurement. Endosafe® Endochrome-K™ 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.[8,9]

**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 four-log standard curve, from 50 to 0.005 EU/mL may be more suitable. Users should consider performing polynomial regression analysis when using standard curves with ranges greater than 3 logarithms.

The positive product control (PPC) should contain endotoxin equal to the concentration at or near the middle 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 Endochrome-K™ 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 and recovery of an endotoxin spike of 0.5 EU/mL in sample products is presented below.

**REPRESENTATIVE LINEAR ANALYSIS**

| SPECIMEN | CSE (EU/mL) | MEAN ONSET TIME (sec) | RECOVERY (EU/mL) |
|---|---|---|---|
| STD 1 | 50.0 | 337 | |
| STD 2 | 5.0 | 493 | |
| STD 3 | 0.5 | 801 | |
| STD 4 | 0.05 | 1400 | |
| STD 5 | 0.005 | 3025 | |
| AVG. SPL1 | ---- | **** | <0.005 |
| AVG. PPC | 0.5 | 822 | 0.706 (141%) |

Fitted regression equation: Log(y) = -0.236*Log(x) + 2.879
Correlation Coefficient: r = -0.991

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.

If the absolute value of the correlation coefficient is greater than 0.980, a polynomial regression model can be used to describe the standard curve. The polynomial regression is calculated for the user using Endoscan-V software[12], or equivalent software. For additional information, please refer to the related software manuals and the section describing polynomial regression below.

**POLYNOMIAL REGRESSION**
A polynomial regression model may be fitted to the standard curve, providing the absolute value of the linear correlation coefficient is greater than, or equal to 0.980. Software packages such as Endoscan-V[12] are capable of fitting the best polynomial function to the data.

NOTE: A polynomial standard curve can not be used for initial qualification assays. The FDA accepts linear regression models for such assays, per the regulatory guidelines.

The order of the polynomial regression is determined as follows:

Endoscan-V Software n-1

Where n = the number of standards utilized.

The example assay (above), analyzed using a fourth degree polynomial (five standards), would yield the following results:

**REPRESENTATIVE POLYNOMIAL ANALYSIS**

| SPECIMEN | CSE (EU/mL) | MEAN ONSET TIME (sec) | RECOVERY (EU/mL) |
|---|---|---|---|
| STD 1 | 50.0 | 337 | |
| STD 2 | 5.0 | 493 | |
| STD 3 | 0.5 | 801 | |
| STD 4 | 0.05 | 1400 | |
| STD 5 | 0.005 | 3025 | |
| AVG. SPL1 | ---- | **** | <0.005 |
| AVG. PPC | 0.5 | 822 | 0.445 (89%) |

Fitted regression equation:
$Log(y) = 0.0031*Log(x)^4 + 0.0001*Log(x)^3 + 0.011*Log(x)^2 - 0.216*Log(x) + 2.837$

Linear Correlation Coefficient: r = -0.991

## 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 Pyrogen Test.

## 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.[10] The absolute value of the coefficient of correlation, r, shall be greater than or equal to 0.980.[10]

**QUALITY CONTROL PROCEDURES FOR THE KINETIC-COLORIMETRIC METHOD**
Follow the U.S. Pharmacopeia, 25th revision for end-product testing using Kinetic Colorimetric methods, including the attainment of a Positive Product Control within 50 – 200% of theoretical concentration. Standard curves must have a correlation coefficient ≤ -0.980.

When consistency in standard curves is observed, an archived curve may be generated, using 3 assays, as described by the Interim Guidance.[10] If an archived curve is used, the positive water control must recover a mean endotoxin concentration within ± 25% of this point on the archived curve.[10]

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

## EXPECTED VALUES
Endochrome-K™ LAL Reagent is standardized against the U.S. Reference Standard Endotoxin (RSE). Endotoxin can be quantified if the concentration is within the range of 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.

Using the appropriate conditions, Endosafe® Endochrome-K™ has an effective range from 100 - 0.001 EU/mL. Factors influencing the selection of the standard curve range include 1) the parameters of the analytical instrument, 2) the choice of regression models, and 3) the quality of supporting analytical reagents and labware.

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