**INTENDED USE**

Co-lipophilized Limulus amebocyte lysate (LAL) and a synthetic colorimetric 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 method available to detect and measure bacterial endotoxin, a fever-producing byproduct of gram-negative bacteria commonly known as pyrogen. The assay of the test is a 24 – 48 hour gelation and gelation in LAL that is easily recognized. The simplicity and economy of the LAL Test makes it the test of choice for the solutions and buffer used in drug products, devices, and biologicals. The USP Bacterial Endotoxins Test and USP/EP Guideline for LAL testing provide standard methods for validating the LAL Test as a replacement for the rabbit pyrogen test.

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 (\(\lambda\)) 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. In collaboration, Levin and Bang 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 clotting-gelation reaction.

In the presence of a colorless substrate, the enzymatic reaction will cause a yellow color to develop upon cleavage of the chromogen, p-nitroaniline (pNA). pNA is metabolically cleaved by the LAL enzyme and released as a yellow color, which is measured spectrophotometrically.

**REAGENTS**

**LAL Reagent:** Lyophilized Endosafe® Endochrome™ 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 touching the glass. The reconstituted LAL Reagent produces an opalescent yellow liquid. Store 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 be frozen and thawed once.

**Storage:** Lyophilized LAL is relatively heat stable and should be stored at 2-8°C; avoid prolonged exposure to temperatures above 25°C. Reconstituted LAL should be stored 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 be frozen and thawed once.

**E.coli Control Standard Endotoxin (CSE)** is available from Charles River Endosafe to detect and confirm the LAL reagent is functioning properly. The CSE provides inhibitory 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 (LRW):** (non-LAL active) must be used to rehydrate LAL reagent and prepare samples, controls and endotoxin standards.

**WARNINGS AND GENERAL PRECAUTIONS**

When using Endochrome™ formulation 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 all equipment 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 highly sensitive to both heat and contamination, it is prudent to test for endotoxin contamination at all steps of the assay procedure.

**PRODUCT INTERERENCE**

A method must be validated for each sample by demonstrating the absence of significant interference. Inhibition is usually concentration dependent, and is overcome by diluting with LAL Reagent Water (LRW). Concentrations of inhibition include conditions that 1) interfere with the enzyme-mediated reaction, and 2) alter the dispersion of the endotoxin (Positive) control.

**Maximum Valid Dilution:** The U.S. Food and Drug Administration has established endotoxin limits of 0.1 EU/mL for active ingredients of drug products. Specific limits for commercial items have been adopted. Maximum Valid Dilution (MVD) is calculated by formula presented in the previously mentioned documents and other pharmacopoeia.

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

\[
\text{MVD} = \frac{\text{Endotoxin Limit} \times \text{Product Potency}}{0.05 \text{ EU/mL}}
\]

Note: For kinetic testing, \(0.5\) is the lowest point on the standard curve.

For example, the compendial limit for cyclophosphamide is 0.17 EU/mL. If a standard curve with a lowest level of 0.05 EU/mL of endotoxin is used to test this product, where the MVD is 20 mg/mL, the MVD equals 1.68. Thus, cyclophosphamide must be diluted up to 1:68 to resolve potential interference (one part to 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 in LRW 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”.

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 prepared with a range of 0.5 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. The spike must be in LRW, not to exceed the MVD, until the spike is recovered consistently by the assay.

**β-GLUCAN**

Endosafe® Endochrome™ reacts with some β-glucans in addition to endotoxin. Endochrome™ 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 pipette with individually wrapped, sterile dispensing syringes. (Eppendorf® Repeater® or equivalent with 0.5 mL and 5.0 mL Sterile Combipots®, or equivalent).
- Depyrogenated glass pipettes (recommended), and calibrated automatic pipetters with sterile, endotoxin-free tips.
- Vertex-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**

Endosafe® Endochrome™ is approved for kinetic-colorimetric analysis in a tube reader or microplate reader that: 1) a detection system to make optical density measurements over time, and 2) a microprocessor and suitable software to analyze data. There are two linear or polynomial regression. The Tecan Sunrise with Endosafe® software is an example of an incubating microplate reader with software designed to collect, process, and store data using protocols for LAL endotoxin measurement. Additional information about the method as tube readers for kinetic-colorimetric measurement. Endosafe® Endochrome™ may be used in these systems under validated conditions.

Each assay should include samples or dilutions of samples or products, positive product controls, and negative controls. Assay at least in duplicate. Follow the specific directions supplied with the automated endotoxin measurement system for sample analysis.

**METHOD**

Asceptically 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 step heating may require pre- incubation of the assay plate at room temperature. Please follow the 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 with a micro-assay test dispenser, beginning with the negative control and ending with the highest endotoxin concentration. If the plate reader does not mix the solutions automatically, gently mix the samples and promptly initiate the time course 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 should be used. Endotoxin concentrations covering the desired standard curve range should be tested including positive water control, and a negative control. Follow the 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 with a micro-assay test dispenser, beginning with the negative control and ending with the highest endotoxin concentration. If the plate reader does not mix the solutions automatically, gently mix the samples and promptly initiate the time course observation. After the desired monitoring period is completed, initiate the programmed analysis of results.

**CALCULATION OF ENDOTOXIN CONCENTRATION**

The positive product control (PPC) should contain endotoxin equal to the concentration of the positive product control (PPC) or the 100% endotoxin standard (see “Performance Characteristics”). Failure to recover the spike within 50 – 200% indicates sample interference. The spike must be in LRW, not to exceed the MVD, until the spike is recovered consistently by the assay.

**RESULTS**

**Detection 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...
LAL Reagent is standardized against the U.S. Reference Standard Products and Pharmaceuticals. The linearity of the standard curve within the concentration range used to fit 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**

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

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>CSE (EU/mL)</th>
<th>MEAN ONSET TIME (sec)</th>
<th>RECOVERY (EU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD 1</td>
<td>50.0</td>
<td>357</td>
<td></td>
</tr>
<tr>
<td>STD 2</td>
<td>5.0</td>
<td>493</td>
<td></td>
</tr>
<tr>
<td>STD 3</td>
<td>0.5</td>
<td>801</td>
<td></td>
</tr>
<tr>
<td>STD 4</td>
<td>0.05</td>
<td>1400</td>
<td></td>
</tr>
<tr>
<td>STD 5</td>
<td>0.005</td>
<td>3025</td>
<td></td>
</tr>
<tr>
<td>AVG. SPL1</td>
<td>---</td>
<td>***</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>AVG. PPC</td>
<td>0.5</td>
<td>822</td>
<td>0.706 (141%)</td>
</tr>
</tbody>
</table>

**Fitted regression equation:**

\[ \text{Log}(y) = -0.236\times \text{Log}(x) + 2.879 \]

**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.

The absolute value of the coefficient of correlation, \( r \), should be greater than or equal to 0.980.

### 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 \( r \leq 0.980 \).

When consistency in standard curves is observed, an archived curve may be generated, using 3 assays, as described by the Interim Guidance. 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.

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 0.001 – 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.

### BIBLIOGRAPHY

12. Reference Guide for Endoscan-V, Charles River Laboratories, 1023 Wappoo Road, Suite 43B Charleston, SC, 29407 USA

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