

Limulus Amebocyte Lysate

CHROMO-LAL

Manufactured by:



Telephone: (508) 540-3444
Toll-Free: (888) 395-2221
Fax: (508) 540-8680
Technical Support: (800) 848-3248
Customer Service: (800) 525-8378

US License # 700

PN001087 Rev003 / December 2006

LIMULUS AMEBOCYTE LYSATE CHROMO-LAL

for the Detection and Quantitation of Gram Negative Bacterial Endotoxins (Lipopolysaccharides)

SUMMARY AND EXPLANATION OF TEST

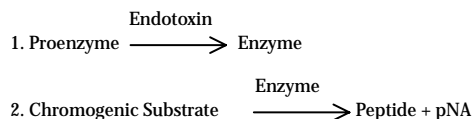
In the 1950's, Frederik Bang observed that infection from gram negative bacteria resulted in intravascular coagulation in *Limulus polyphemus*, the horseshoe crab (1). Levin and Bang demonstrated that the coagulation was caused by the activation of a number of enzymes located in the blood cells (amebocytes) of *Limulus polyphemus*, and that this activation was initiated by the endotoxin (lipopolysaccharide) in the gram negative bacterial cell walls (2, 3, 4).

Subsequently, the *Limulus* Amebocyte Lysate (LAL) test using LAL reagent prepared from horseshoe crab blood was shown to be the most sensitive and specific means of measuring bacterial endotoxins (5). The chromogenic test, introduced in 1977 (6, 7), is a modification that enables endotoxin concentration to be measured as a function of color intensity rather than by turbidity or gelation in the reaction mixture. Results obtained by this modified method are generally comparable to those obtained by the gel-clot or turbidimetric methods within the error of the tests.

In the Chromo-LAL test, co-lyophilized LAL and substrate reagent are mixed with test sample in a microplate and incubated in a reader at 37±1°C. Absorbance measurements are collected with time after addition of Chromo-LAL and analyzed by suitable software. The time (onset time) taken for a sample to reach a specified absorbance (onset OD) is calculated; and a standard curve, showing the linear correlation between the log onset time and the log concentration of standard endotoxin, is generated. The maximum range of endotoxin concentrations for the standard curve is 0.005 EU/mL - 50 EU/mL. The sensitivity (λ) of the assay is defined as the lowest concentration used in the standard curve. The maximum sensitivity of this test is 0.005 EU/mL.

BIOLOGICAL PRINCIPLE

LAL contains enzymes that are activated in a series of reactions in the presence of endotoxin. The last enzyme activated in the cascade splits the chromophore, paranitro aniline (pNA), from the chromogenic substrate, producing a yellow color.



The amount of pNA released and measured photometrically at 405 nm is proportional to the amount of the endotoxin in the system. The greater the endotoxin concentration, the faster the reaction.

REAGENTS

Reagents required to perform the Chromo-LAL Assay are listed below. Unopened reagents are stable at 2-8°C until the expiration date printed on the container label. Before reconstitution, bring the reagents to room temperature and tap the vials containing lyophilized material against a hard surface to cause loose material to fall to the bottom of the vial.

1. Chromo-LAL, Limulus Amebocyte Lysate co-lyophilized with chromogenic substrate

This reagent is an aqueous extract of amebocytes of *L. polyphemus*, buffered at pH 7, and co-lyophilized with the chromogenic substrate. Reconstitute Chromo-LAL immediately before use with 3.2 mL LAL Reagent Water (LRW). This solution is stable 24 hours at 2-8°C or for two weeks at -20°C or colder if frozen immediately after reconstitution and not contaminated. Chromo-LAL may be frozen and thawed once. Contamination may be indicated by a dark yellow color that develops rapidly after reconstitution. The reagent will turn yellow slowly under normal conditions of use.

2. Control Standard Endotoxin (CSE)

Control Standard Endotoxin (CSE) is not provided with Chromo-LAL and must be ordered separately. CSE obtained from Associates of Cape Cod, Inc., is used to construct standard curves, validate product, and prepare inhibition controls. Each vial contains a measured weight of endotoxin. USP Endotoxin Reference Standard may be obtained from the U.S. Pharmacopeial Convention, Inc. Follow manufacturer's directions for reconstitution and storage of standard endotoxins. CSE lots may show different potencies (EU/ng) when tested with different lots of Chromo-LAL. If using CSE, endotoxin concentrations can be expressed in EU/mL if the potency of a given lot of CSE has been determined with the Chromo-LAL lot in question. (11,12).

3. LAL Reagent Water (LRW)

LRW is sterile water prepared by distillation or reverse osmosis that shows no detectable endotoxin when tested with Chromo-LAL. Additional vials of LRW may be obtained from Associates of Cape Cod, Inc.

Precautions and Warnings: Chromo-LAL is for *in vitro* diagnostic use only. Do not use these reagents for the detection of endotoxemia. Exercise caution when handling Chromo-LAL reagent because its toxicity has not been determined and allergies to LAL have been reported (8). Correct application of this test requires strict adherence to all items in the recommended procedure. Aseptic technique must be used. All materials coming in contact with specimens and reagents must be free of detectable endotoxin. Heat stable materials, including clean glassware, may be rendered free of detectable endotoxin by exposure to dry heat at a minimum temperature of 250°C for a minimum of 30 minutes (9).

SPECIMEN COLLECTION AND PREPARATION

Collect samples in a way that avoids microbial contamination. Use aseptic technique when handling specimens and reagents. Test any samples as soon as possible after collection; otherwise store them at 2-8°C. If bacterial growth is expected, samples may be frozen. Confirm that storage

does not reduce the level of endotoxin detectable in the sample (endotoxin may adsorb to the container surface). Many substances interfere with the Chromo-LAL test. Interference is indicated by inhibition, the recovery of less endotoxin than known to be present in a sample; or by enhancement, the recovery of more endotoxin than the amount known to be present (see "Procedure" for detection of interference). Interference is generally overcome by diluting the sample with LAL Reagent Water. Do not dilute beyond the Maximum Valid Dilution (MVD; 10, 11, 12, 13).

$$\text{MVD} = \frac{\text{endotoxin limit} \times \text{product concentration}}{\lambda}$$

Certain compounds may need special treatment in addition to dilution to remove interference. For example, blood products containing activated enzymes may cause false positive results. These types of samples may be diluted with LAL Reagent Water and heated at a minimum temperature of 75°C for a period of time shown to eliminate interference without loss of endotoxin activity. Samples that absorb strongly at 405 nm may interfere with the test and may require prior dilution.

TEST PROCEDURE

Reagents required to perform the Chromo-LAL Assay are listed under reagents.

Equipment and materials required but not provided:

1. Test tubes and/or microplates free of detectable endotoxin. Both available through Associates of Cape Cod, Inc.
2. Pipettes and pipette tips that are free of detectable endotoxin. Available through Associates of Cape Cod, Inc..
3. Repetitive pipettes with dispensing syringes free of detectable endotoxin.
4. Vortex mixer.
5. Microplate reader equipped with suitable software and capable of maintaining a uniform temperature across the microplate of 37±1°C. Available through Associates of Cape Cod, Inc.
6. Kinetic software. Software that collects and stores optical density (OD) readings at short intervals is necessary. The software must also calculate "onset time" for the sample in each well. An onset time is the time taken for the OD in a given well to reach a specified OD value (onset OD). The chosen value may be between 0.03 and 0.2 OD units; however the same value should be used for routine testing as was used for the validation of the assay for that product.

The software should generate the standard curve parameters (slope, intercept, and correlation coefficient) and calculate the endotoxin concentrations in the unknown samples. The software may perform additional calculations such as calculating the concentration of the endotoxin recovered in the positive product control after subtraction of any endogenous endotoxin in the sample.

Standard curve

Include a standard curve consisting of at least three concentrations of endotoxin, in duplicate, with each set of test samples. Additional concentrations should be added such that there is at least one standard per log increment of the range (11). Prepare the standard endotoxin concentrations by serial dilution starting with the highest or "stock" concentration. Mix the stock concentration approximately 30 seconds with a vortex mixer before making the first transfer.

Any dilution scheme may be used to prepare standard endotoxins and the concentrations used to construct the curve may encompass any range within the limits of 0.005 to 50 EU/mL. The lowest concentration retained in the curve is the sensitivity (λ) of the assay.

An example of the preparation of a broad range series with λ equal to 0.005 EU/mL is given in the table below.

Standard Concentrations EU/mL	LAL Reagent Water (μ L)	Endotoxin Standard Solution
50	950	50 μ L of the stock solution
5	900	100 μ L of the 50 EU/mL solution
0.5	900	100 μ L of the 5 EU/mL solution
0.05	900	100 μ L of the 0.5 EU/mL solution
0.005	900	100 μ L of the 0.05 EU/mL solution
Neg. Contr.	1000	

An archived standard curve (11,12) is not recommended. The parameters of the curve will change with the time after reconstitution of the reagent. Therefore, endotoxin standard concentrations should be included with each test.

Negative control

Negative controls, in duplicate, should be included with every set of samples. The negative control is the LRW used to dilute the samples for the test. The onset time of the negative control should be at least 10% greater than that of the least concentrated standard. Once the characteristic performance of the negative control is known, the operator may stop the assay before the preset run time is completed (see "Assay Performance Time" below). Stop the assay only if it can be concluded, from visual inspection of the reaction kinetics, that the lowest concentration of standard has reached the onset OD and that the OD of the negative control is characteristically low.

Detection of interference

A sample to which a known amount of endotoxin standard is added (spiked sample) is referred to as a positive product control; this control for detection of inhibition or enhancement is included in a routine test protocol. By comparing the recovery of the endotoxin concentration in the positive product control with the concentration known to have been added, it is possible to determine whether the sample inhibits (less endotoxin is detected than is present) or enhances (more endotoxin is detected than is present) the assay.

The calculated mean concentration of added endotoxin (concentration in the spiked sample minus the concentration in the sample) must be within 50% to 200% of the expected spike concentration to show that the product neither inhibits nor enhances the assay.

The concentration chosen for the spike will depend on the range of the standard curve and on the endotoxin limit for the dilution or concentration of sample under test (pass/fail cutoff, 11).

The spike concentration should be one of the concentrations used in the standard curve and should fall near the middle of the standard range. For the broad range curve illustrated above and with samples that have a pass/fail cutoff less than or equal to 1 EU/mL, an endotoxin concentration of 0.5 may be chosen. For the same range of standards and with samples that have a pass/fail cutoff greater than 1 EU/mL, as much as 5.0 EU/mL may be chosen. For narrower ranges of standard concentrations, for example from 0.005 to 1.6 EU/mL a 4 λ concentration or 0.02 EU/mL would be more appropriate, especially if the pass/fail cutoff is much lower than 1 EU/mL.

Assay

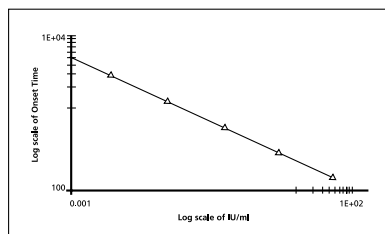
1. Bring samples and controls to room temperature. Mix each vigorously on a vortex mixer immediately before transfer to the microplate well(s).
2. Transfer 100 μ L of sample or control to the microplate.
3. Preincubate the microplate at 37 \pm 1 $^{\circ}$ C for at least 10 minutes.
4. Reconstitute the Chromo-LAL reagent with 3.2 mL LAL Reagent Water or GlucashieldTM Buffer.
5. Transfer 100 μ L of reconstituted Chromo-LAL reagent to each well as rapidly as possible by using a repetitive pipette.
6. Mix thoroughly without splashing reaction mixture; this is an option in some readers.
7. Place the microplate immediately in the microplate incubator/reader that has been set to read at 405 nm and incubate at 37 \pm 1 $^{\circ}$ C. Start the kinetic software.

Assay performance time

The time needed to complete the reaction depends on the range of endotoxin concentrations chosen for the standard curve and on lot specific characteristics. The "run time", using an onset OD of 0.05, is typically set for 60 minutes for an assay sensitivity of 0.05 EU/mL or for 100 minutes for a sensitivity of 0.005 EU/mL.

RESULTS

The endotoxin concentration for the corresponding onset time of the unknown sample is read from the standard curve which is a log-log plot of the onset times vs. the standard concentrations, or an arithmetic plot of the logs of onset times vs. the logs of the standard concentrations. A typical standard curve is illustrated below.



The log-log line equation generated for the illustrated standard curve is $Y = -0.2X + 3.14$, where $Y = \log$ onset time and $X = \log$ endotoxin concentration. The concentration of endotoxin in an unknown sample with a mean onset time of 1630 seconds would be calculated by converting the onset time to its log value, 3.212, solving the equation for X , and taking the antilog of X to obtain concentration:

$$X = (Y - 3.14) / -0.2$$
$$X = (3.212 - 3.14) / -0.2$$
$$X = -0.36$$
$$\text{Antilog } (-0.36) = 0.44 \text{ EU/mL}$$

LIMITATION OF THE PROCEDURE

The procedure is limited by the extent of the inhibition or enhancement demonstrated by the test sample. If the interference can not be overcome by dilution or other means at the MVD, then the Chromo-LAL assay cannot be used to measure endotoxin in that sample.

EXPECTED VALUES

Endotoxin in the test sample can be quantified between the range of the endotoxin concentrations used to construct the standard curve. In order to report results in endotoxin units (EU) or international units (IU) of endotoxin, it is necessary to use either the standard reference endotoxin (USP 2nd International Standard) or a control standard with potency calibrated against the reference.

If it is necessary to dilute the test sample to overcome inhibition or enhancement, the least amount of endotoxin that can be detected will be increased accordingly.

PERFORMANCE CHARACTERISTICS

The linearity of the standard curve, within the concentration range used to determine endotoxin levels, must be verified by performing the test on the appropriate number of standard concentrations (see curve above) in triplicate (11). Calculate the standard curve parameters without averaging the onset times of replicates. The absolute value of the coefficient of correlation, r , should be greater than or equal to 0.980. The same criterion for linearity applies to the standard curves that are included with routine tests (see standard curve above).

REFERENCES

1. Bang, F.B. The toxic effect of a marine bacterium on Limulus and the formation of blood clots. Biol. Bull. (Woods Hole, MA) 105:361-362 (1953).
2. Levin, J., and F.B. Bang. A description of cellular coagulation in the Limulus. Bull. Johns Hopkins Hosp. 115:337-345 (1964).
3. Levin, J., and F.B. Bang. The role of endotoxin in the extracellular coagulation of Limulus blood. Bull. Johns Hopkins Hosp. 115:265-274 (1964).
4. Levin, J., and F.B. Bang. 1968. Clottable protein in Limulus: its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. 19: 186-197.
5. Hochstetl, H.D. 1987. The LAL test versus the rabbit pyrogen test for endotoxin detection: Update >87. Pharm. Technol. 11(6):124-129.
6. Nakamura, S., T. Morita, S. Iwanaga, M. Niwa, and K. Takahashi. 1977. A sensitive substrate for the clotting enzyme in horseshoe crab hemocytes. J. Biochem. 81:1567-1569.
7. Iwanaga, Morita, Harada, Nakamura, Niwa, Takada,

Kimura, and Sakakibara. 1978. Chromogenic Substrates for Horseshoe Crab Clotting Enzymes Its application for the assay of Bacterial Endotoxins. Haemostasis 7: 183-188.

8. Ebner, C., D. Kraft, F. Prasch, R. Steiner, and H. Ebner. Type I allergy induced by Limulus Amebocyte Lysate (LAL). Clinical and Experimental Allergy 22:417-419 (1992).
9. Tsuji, K. and S.J. Harrison. Dry-heat destruction of lipopolysaccharide: Dry-heat destruction kinetics. Appl. Env. Microbiol. 36:710-714 (1978).
10. Bacterial endotoxins test. Current edition.
11. Interim Guidance for Human and Veterinary Drug Products and Biologicals. U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration. July 15 (1991).
12. Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs. Biological Products, and Medical Devices. U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration, December (1987).
13. European Pharmacopoeia, third edition publ. Section 2.6.14. Bacterial Endotoxins. European Pharmacopoeia Secretariat, Strasbourg, June (1996).