BIOENDO

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Gel Clot Lyophilized Amebocyte Lysate
Multi-test Vial

CATALOG NUMBER
G170030, G170060, G170125, G170250, G170500

INTENDED USE
Gel clot Lyophilized Amebocyte Lysate (AL, TAL or LAL) is intended for use in the *In Vitro* detection of gram-negative bacterial endotoxins (Lipopolysaccharides) with gel-clot method. The test procedures described herein are conformed to Chapter <85> Bacterial Endotoxins Test, United States Pharmacopeia.

WARNING
For *In Vitro* use only. Do not use it for the detection of endotoxemia in human.

EXPLANATION OF TEST
The gel clot AL assay is a qualitative assay for the detection of gram-negative bacterial endotoxins. The assay is conducted by mixing amebocyte lysate with test sample followed by incubating the mixture undisturbed for 60 min at 37°C. If a gel has formed and remains intact after inversion of 180°, the test is positive, indicating that the endotoxin concentration of the test sample is greater than or equal to the AL reagent's labeled sensitivity. If an intact gel is not formed, the test is negative, indicating that the endotoxin concentration of the test sample is less than the labeled sensitivity.

PRINCIPLES
Gel Clot LAL is an aqueous extract of circulating amebocytes of Chinese horseshoe crab (*Tachypleus tridentatus)*. The lysate contains a cascade of serine protease enzymes (proenzymes) which can be activated by bacterial endotoxins. Endotoxins activate the proenzymes to produce activated enzymes (termed coagulase), the latter further activates coagulogen to form coagulin, coagulin self-associates to form gelatinous clot.
When the endotoxin concentration is high enough, the formed gel is firm and remains intact after inversion of the test tubes, the test result is classified as positive. A positive result indicates that the endotoxin concentration of test sample is greater than or equal to the reagent's labeled sensitivity. If an intact gel is not formed, the test result is classified as negative, indicating the endotoxin concentration of test sample is less than the labeled sensitivity.

REAGENTS SUPPLIED
Gel Clot LAL:
Gel Clot LAL was produced from amebocyte lysate of *Tachypleus tridentatus*. The lysate was stabilized by monovalent and divalent cations. The sensitivity (λ) is the minimum concentration of Reference Standard Endotoxin to produce a firm gel-clot under standard conditions. The lot sensitivity, EU/ml, is printed on the package labels.
Storage Conditions:
Store at temperature 2~8° C. Avoid exposure to temperatures above 25°C or to bright light for

prolonged periods.

Reconstitution:

For multi test vials G17 series, reconstitute Gel Clot LAL by adding 1.7 ml Water for BET to the vial.

Reconstituted lysate should be used within 10 minutes.If freeze immediately at the temperature below -20°C, the lysate is stable for up to 28 days. The reconstituted lysate can only freeze-thaw once.

REAGENTS REQUIRED BUT NOT SUPPLIED

1. **Water for BET**: endotoxin concentration < 0.005 EU/ml.
2. **Control standard endotoxin (CSE)**: control standard endotoxin is used to confirm the sensitivity of Gel Clot LAL, validate product, and prepare inhibition controls. The potency of CSE is printed on the Certificate of Analysis.

MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

1. Pyrogen-free pipettes, 0.2 ml, 1.0 ml, 5.0 ml. Or automatic pipettors with pyrogen-free tips.
2. Endotoxin-free glass dilution tubes for endotoxin standards preparation.
3. Endotoxin-free 10 × 75 mm glass reaction tubes (For multi-test vials only).
4. Vortex Mixer.
5. Heating block or non-circulating hot water bath (37 ± 1°C).
6. Timer.
7. Test tube rack.

TEST SAMPLE AND REAGENT PREPARATION

All glassware, plasticware, and diluents to be in contact with test sample or test reagents should be endotoxin-free. Glassware and other heat-stable apparatus could be depyrogenated in oven using a validated process, a commonly used minimum time and temperature setting is 60 minutes at 250°C.

Use aseptic techniques all along.

Samples to be tested should be stored under conditions that bacteriological activities are stopped. Samples could be kept at 2-8°C for temporary storage (less than 24 hours), samples should be kept below -10°C for longer term storage.

The optimal pH range for AL-endotoxin reaction is from 6.0 to 8.0. Acidic and basic sample could be adjusted to desired pH range with endotoxin-free 0.1 N sodium hydroxide, 0.1 N hydrochloric acid, or endotoxin-free Tris buffer.

Potential of interference presence should be tested and eliminated following the description in **PRODUCT INHIBITION** section.

Preparation of Controls

1. Endotoxin standards
   Reconstitute control standard endotoxin with Water for BET, prepare endotoxin standard working solutions at concentrations of 2λ, λ, 0.5λ, and 0.25λ. Prepare the endotoxin solution immediately prior to use to avoid activity loss due to adsorption.
2. Positive control
   Positive control may be used in place of a series of endotoxin standards in certain circumstances. Positive control is the endotoxin solution at concentration of 2λ.

3. Positive product controls:
   Positive product controls are the endotoxin-spiked test sample. Spike concentration is usually at 2λ. See **PRODUCT INHIBITION** section.
4. Negative control:
   Use Water for BET as negative control.

Preparation of Gel Clot LAL

Caution: Reconstitute Gel Clot LAL immediately prior to use.

For multi test vials G17 series, reconstitute Gel Clot LAL by adding 1.7 ml Water for BET to the vial.

Swirl gently but thoroughly. Do not shake or vortex as contents will foam.

TEST PROCEDURE

For multi-test vial G17 series, add 0.1 ml Water for BET, endotoxin standards, or test sample into each reaction tube, then add 0.1 ml of Gel Clot LAL;

Mix gently but thoroughly. Failure to mix adequately is a common cause of unsatisfactory tests.

Incubate the mixture at 37°C for 60 minutes. Avoiding vibration during incubation.

If large numbers of samples are tested in parallel, the tests should be batched and started at intervals that permit the reading of each within the time limit.

Result Reading

Remove and read reaction tubes one by one. Invert the tube in one smooth motion. A positive result is indicated by the formation of a gel which does not collapse when the tube is inverted. A negative result is characterized by the absence of solid clot after inversion. The increase in turbidity or viscosity is considered negative result.

RESULTS AND INTERPRETATION

The results for all negative control replicates should be negative, positive results suggest the contamination of Gel Clot LAL, Water for BET, or glassware.

The results for all positive control replicates should be positive, negative results suggest the possibilities of enzyme activity loss of Gel Clot LAL, potency loss of CSE, and incorrectness of endotoxin dilution.

The results for positive product control should be positive. If the result for positive product control is negative whereas that for positive control is positive, the presence of interference in the test sample is hinted. See **PRODUCT INHIBITION** section.

Determination of the Maximum Valid Dilution

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a substance to be examined at which the endotoxin limit can be determined.

Determine the MVD using the following formula:

$$MVD = \frac{L \times C}{\lambda}$$

L refers to the endotoxin limit of the substance to be examined, L is specified in units such as

EU/ml, EU/mg, EU/Unit.

L = K/M, where

K refers to the maximum allowable dose of endotoxin per kilogram body weight per hour,

M refers to the maximum allowable dose of substance per kilogram body weight per hour.

C refers to the concentration of the substance to be examined, C is specified in mg/ml if the endotoxin limit is specified by mass (EU/mg), and in Units/ml if the endotoxin limit is specified by unit of biological activity (EU/Unit).

λ = the labeled Gel Clot LAL sensitivity.

Confirmation of Labeled Gel Clot LAL Sensitivity (λ)

Run gel clot assay in 4 replicates with Gel Clot LAL to be tested and endotoxin standard solutions at concentrations of 2λ, λ, 0.5λ, and 0.25λ.

The test is valid only when results for all replicates of 2λ are positive, results for all replicates of 0.25λ and negative controls are negative. The labeled sensitivity is confirmed if the observed sensitivity is between 0.5λ and 2λ.

The observed sensitivity is equal to the Geometric Mean (***G***) of the endpoint (e).

$$\boldsymbol{G} = (e_1 \times e_2 \times \ldots \times e_f)^{1/f} = \lg^{-1}(\sum(\lg e)/f)$$

The endpoint (e) is the lowest endotoxin concentration with positive result in series, *f* is the number of replicate test tubes.

An example of the confirmation test for a sample with labeled Gel Clot LAL sensitivity (λ) of 0.25 EU/ml.

Replicates *f*	Endotoxin Concentration (EU/ml)				Negative control	Endpoint (EU/ml) e	Log_{10} e	Mean of Log_{10} e	G (EU/ml)
	2λ	λ	0.5λ	0.25λ					
	0.5	0.25	0.125	**0.0625**					
1	+	+	−	−	−	0.25	-0.602	-0.677	0.21
2	+	+	+	−	−	0.125	-0.903		
3	+	+	−	−		0.25	-0.602		
4	+	+	−	−		0.25	-0.602		

The observed sensitivity 0.21 EU/ml is between 0.125 and 0.5 EU/ml, therefore the labeled sensitivity 0.25 EU/ml is confirmed.

Gel Clot Semi-Quantitative Assay

Quantify the endotoxin concentration by finding the endpoint in a series of sample dilutions. In the following example, the sample is diluted with Water for BET, Gel Clot LAL sensitivity is 0.25 EU/ml.

Replicates *f*	Sample dilution						Negative control	Endpoint dilution	Log_{10} Endpoint	Mean	Log_{10}^{-1}
	1:2	1:4	1:8	1:16	1:32	1:64					
1	+	+	+	−	−	−	−	1:8 (0.125)	-0.903	-1.054	0.088 (1:11.3)
2	+	+	+	+	−	−	−	1:16 (0.0625)	-1.204		

Endotoxin Concentration = Gel Clot LAL sensitivity × endpoint dilution

= 0.25 EU/ml×11.3

= 2.83 EU/ml

Gel Clot Limit Test

The test is applied when a monograph contains a requirement for endotoxin limits.

Run the diluted sample along with positive product controls, positive controls and negative controls, dilution factor is MVD. If the test is valid (see **RESULTS AND INTERPRETATION** section), read the results.

Negative results for all test samples replicates indicate that the endotoxin concentration in this sample is less than the endotoxin limit, which means the sample pass the gel clot limit test.

Positive results for all sample replicates indicate that the endotoxin concentration in this sample exceeds the endotoxin limit, which means the sample fail the gel clot limit test. If one replicate is positive while the other is negative, repeat the test with four replicates of the sample, the sample will pass the test only if all the four replicates test negative in the repeated test.

PRODUCT INHIBITION

Product inhibition test is frequently employed to evaluate the existence of interference in the test sample. Prepare a series of two-fold dilutions of endotoxin in both Water for BET and test sample matrix, run assay with two series in parallel, calculate the geometric mean endpoint for each series.

If the geometric mean endpoint of endotoxin in test sample matrix is within the range of 0.5λ to 2λ, the sample is considered free of product inhibition. Otherwise, the existence of interference in the sample is suggested.

Product inhibition is usually concentration dependent, and could be reduced by dilution with Water for BET. The dilution should not exceed the MVD. The application of more sensitive Gel Clot LAL allows greater dilution of the sample, which may better the elimination of interference.

REFERENCES

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