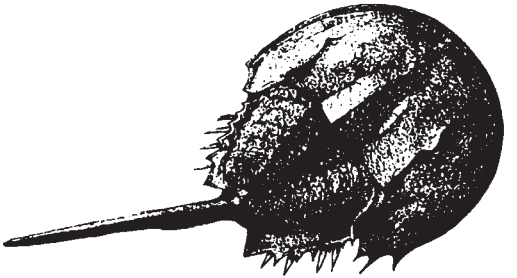
Lonza

Limulus Amebocyte Lysate (LAL)

PYROGENT™

Single Test Vials



Catalog Number: N189-06, N189-125, N189-25
F211U-125

Certificate of Analysis at www.lonza.com/coa

SINGLE TEST
LIMULUS AMEBOCYTE LYSATE

PYROGENT™
U.S. License No. 1775

Important: Read Entire Brochure
Before Performing Test

INTENDED USE

This product is intended as an *In Vitro* end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices. This product is not intended for the detection of endotoxin in clinical samples or as an aid in the diagnosis of human disease. The Limulus Amebocyte Lysate (LAL) test is a qualitative test for Gram-negative bacterial endotoxin. Limulus Amebocyte Lysate as supplied is to be reconstituted with the solution being tested. After incubation, and in the presence of endotoxin, gelation occurs; in the absence of endotoxin, gelation does not occur.

In December, 1987, the United States Food and Drug Administration (FDA) published the "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"[10]. This guideline outlines those procedures which the FDA considers necessary for: 1) establishing endotoxin limits for pharmaceuticals and medical devices, 2) validating the use of LAL as an end-product endotoxin test, and 3) developing a routine testing protocol.

The procedures described herein conform with those described in the FDA Guideline. Similar performance requirements for gel clot assays have been published and are updated regularly in the United States Pharmacopeia.[11]

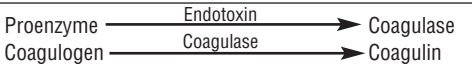
WARNING

For *In Vitro* Diagnostic Use Only. Not for the *In Vitro* Determination of Endotoxemia in Man. The LAL test may be substituted for the USP Rabbit Pyrogen Test when used according to the FDA Guideline for end-product testing of human and animal parenteral drugs, biological products, and medical devices[10].

EXPLANATION OF TEST

The use of LAL for the detection of endotoxin evolved from the observation by Bang[1] that Gram-negative infection of *Limulus polyphemus* resulted in fatal intravascular coagulation. Levin and Bang[2,3] later demonstrated that this clotting was a result of the action between endotoxin and a clottable protein in the circulating amebocytes of *Limulus* blood. Following the development of a suitable anticoagulant for *Limulus* blood, Levin and Bang[4] prepared a lysate from washed amebocytes which was an extremely sensitive indicator of the presence of endotoxin. Solum[5,6] and Young, Levin, and Prendergast[7] have purified and characterized the clottable protein from LAL and have shown the reaction with endotoxin to be enzymatic.

PRINCIPLE



Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the Limulus Amebocyte Lysate[7]. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme (coagulase) hydrolyzes specific bonds within a clotting protein (coagulogen) also present in Limulus Amebocyte Lysate. Once hydrolyzed, the resultant coagulin self-associates and forms a gelatinous clot.

REAGENTS SUPPLIED AND STORAGE CONDITIONS

Limulus Amebocyte Lysate (LAL), Lyophilized, Single Test Vial

Twenty-five vials containing lysate prepared from the circulating amebocytes of the horseshoe crab (*Limulus polyphemus*) standardized to detect the labeled concentration (EU/ml) of the FDA Reference Standard Endotoxin.

Contains buffered mono and divalent cations. Lysate is lyophilized and sealed under vacuum. Do not rehydrate until immediately prior to use.

Lyophilized Limulus Amebocyte Lysate must be stored under refrigeration at 2-8°C. Care should be taken to avoid exposing the lysate to temperatures in excess of 37°C. Lysate which has been exposed to prolonged periods of temperatures above 37°C or to bright light may turn yellow and/or become insoluble. Lysate which exhibits such characteristics should be discarded.

Note: The F211U kit contains 100 vials of lysate.

MATERIALS AND EQUIPMENT NOT PROVIDED

1. LAL Reagent Water which should not cause gelation of reconstituted lysate after 24 hours incubation at 37°C ± 1°C (#W50-640 or equivalent).
2. Pipettes, 0.25 ml, 1.0 ml, and 5.0 ml, endotoxin-free.

3. 13 x 100 mm glass dilution tubes, endotoxin-free (#N207 or equivalent) (see Specimen Collection and Preparation for procedure).
4. Endotoxin Standard.
5. Heating block or non-circulating hot water bath (37°C ± 1°C).
6. Vial rack.
7. Timer.
8. Vortex Mixer.

SPECIMEN COLLECTION AND PREPARATION

Careful technique must be used to avoid microbiological or endotoxin contamination. All materials coming in contact with the specimen or test reagents must be endotoxin-free. Clean glassware and materials may be rendered endotoxin-free by heating at 250°C for 30 minutes. Appropriate precautions should be taken to protect depyrogenated materials from subsequent environmental contamination.

From experience, most sterile, individually wrapped, plastic pipettes and pipette tips are endotoxin-free. However, these materials should be tested before regular use.

It may be necessary to adjust the pH of the sample to within the range 6.0-8.0 using endotoxin-free sodium hydroxide or hydrochloric acid.[8,9] Always measure the pH of an aliquot of the bulk sample to avoid contamination by the pH electrode. Do not adjust unbuffered solutions.

Samples to be tested must be stored in such a way that all bacteriological activity is stopped or the endotoxin level may increase with time. For example, store samples at 2-8°C for less than 24 hours and frozen for periods greater than 24 hours. It is the responsibility of the customer to validate the proper container and storage conditions for their samples.

REAGENT PREPARATION

Caution: Single-test lysate must be incubated immediately after rehydration with test sample. Allow reagents to equilibrate to room temperature prior to use.

1. Preparation of Positive Controls.

   Note: Plastic tubes are not recommended for making endotoxin dilutions.

   A. Reconstitute endotoxin control per label directions with LAL Reagent Water.

   B. Vortex the vial of endotoxin for at least 15 minutes or refer to product information sheet.

   C. Dilute the endotoxin with LAL Reagent Water to a concentration of 1 EU/ml. Each dilution should be vortexed for 60 seconds prior to proceeding to the next dilution.

   D. Using the 1 EU/ml endotoxin solution, prepare a serial two-fold dilution series that brackets the sensitivity of the lysate as shown in the following example:

Dilution Series for Use With Lysate of 0.125 EU/ml Sensitivity

Tube#	Water (ml)	Volume Added to Water	Endotoxin Concentration
1	1.0	1.0 ml from 1 EU/ml	0.5 EU/ml
2	1.0	1.0 ml from Tube 1	0.25 EU/ml
3	1.0	1.0 ml from Tube 2	0.125 EU/ml
4	1.0	1.0 ml from Tube 3	0.06 EU/ml
5	1.0	1.0 ml from Tube 4	0.03 EU/ml

2. LAL Reagent Water may be used as a negative control.
3. The lysate is reconstituted by addition of 0.25 ml of the control solution or sample to be tested to the lyophilized lysate in the test vial.

TEST PROCEDURE AND INTERPRETATION

The vials containing the lysate serve as the test containers. Before use, bring all the contents of the vial together by gently tapping the bottom of the vial on a hard surface. Use careful technique when removing the rubber stopper to avoid microbial and endotoxin contamination.

Prepare the test sample by adding 0.25 ml of the appropriate test solution to the lysate vial. Mix by tilting and gently swirling the vial until the contents are in solution.

Each Limulus Amebocyte Lysate vial is labeled with the minimum endotoxin concentration required for gelation. Prepare the positive control by adding 0.25 ml of an endotoxin standard that contains twice this labeled concentration to a vial of lysate. Mix as before.

Prepare a negative control by adding 0.25 ml of LAL Reagent Water to a vial of lysate. Mix as before.

Prepare a positive sample control, by adding 0.25 ml of the test sample containing twice the minimal concentration of endotoxin required for gelation of the lysate. Mix as before. See section PRODUCT INHIBITION.

Immediately after reconstitution, incubate each vial of lysate for 60 (±2) minutes at 37°C ±1°C. The 60 (±2) minute incubation time should be determined from the time each vial is placed in a 37°C ±1°C water or dry heat bath. Assay tubes should not be removed from incubation or disturbed prior to the time specified for reading the test.

At the end of the incubation period, carefully remove each vial and invert it 180°. Compare the sample vials to the control vials.

1 A positive reaction is characterized by the formation of a firm gel that remains intact momentarily when the tube is inverted. This should be observed in the positive control vial and in the positive sample control vial.

2. A negative test is characterized by the absence of solid clot after inversion. This should be observed in the negative control vial. The lysate may show an increase in turbidity or viscosity. This is considered a negative result.

3. Record positive and negative results for the test sample vials.

CONFIRMATION OF LABEL CLAIM

Each vial of LAL is labeled with the lysate sensitivity obtained using the FDA Reference Endotoxin, and is expressed in *Endotoxin Units*.

As part of an initial in-house validation, each user should reverify the labeled lysate sensitivity using an endotoxin standard whose potency is known. Prepare serial two-fold dilutions of the endotoxin standard which bracket the labeled lysate sensitivity. Each dilution, as well as a negative water control, should be assayed in quadruplicate. After the one hour incubation period, the positive and negative results are recorded. The endpoint dilution is determined as the last dilution of endotoxin which still yields a positive result.

Assay Results - Gel Clot Method

	Endotoxin Dilution (EU/ml)						
Replicate	0.50	0.25	0.125	0.06	0.03	H_2O	Endpoint
1	+	+	+	-	-	-	0.125
2	+	+	+	-	-	-	0.125
3	+	+	+	+	-	-	0.06
4	+	+	+	-	-	-	0.125

The lysate sensitivity is calculated by determining the geometric mean of the endpoint. Each endpoint value is converted to $\log_{10}$. The individual $\log_{10}$ values are averaged and the lysate sensitivity is taken as the antilog_{10} of this average log value.

Calculation of Geometric Mean Endpoint

Endpoint (EU/ml)	Log_{10} Endpoint
0.125	-0.903
0.125	-0.903
0.06	-1.222
0.125	-0.903
	Mean = -0.983
	Antilog_{10} Mean = 0.10 EU/ml

Acceptable variation is one half to two times the labeled lysate sensitivity.

DETERMINATION OF ENDOTOXIN IN AN UNKNOWN

To determine the endotoxin concentration of an unknown solution, test serial two-fold dilutions of sample until an endpoint is reached. Calculate the geometric mean dilution as before and multiply by the labeled lysate sensitivity.

Determination of Endotoxin Concentration in an Unknown

Lysate Sensitivity = 0. 125 EU/ml

	Sample Dilution					
Replicate	1/2	1/4	1/8	1/16	1/32	1/64
1	+	+	+	-	-	-
2	+	+	+	+	-	-

Endpoint Dilution	Log_{10} Endpoint
1/8 (0.125)	-0.903
1/16 (0.0625)	-1.204
Mean =	-1.054
$Antilog_{10}$ Mean =	0.088 = 1/11.4

Endotoxin Concentration = lysate sensitivity x endpoint dilution
= 0.125 EU/ml x 11.4 = 1.4 EU/ml

PRODUCT INHIBITION

The Limulus Amebocyte Lysate reaction is enzyme mediated and, as such, has an optimal pH range and specific salt and divalent cation requirements. Occasionally, test samples may alter these optimal conditions to an extent that the lysate is rendered insensitive to endotoxin. Negative results with samples which inhibit the LAL test do not necessarily indicate the absence of endotoxin.

Initially, each type of sample should be screened for product inhibition. Prepare a series of two-fold dilutions of endotoxin in LAL Reagent Water and a similar series of endotoxin dilutions using sample as diluent. Assay each series in parallel using standard procedures. At the end of the incubation period, record positive and negative results and calculate the geometric mean endpoint for both series of endotoxin dilutions. Products are said to be free of product inhibition if the geometric mean endpoint of endotoxin in product is within 1/2 to 2 times the labeled lysate sensitivity.

Product Inhibition Testing

		Endotoxin Dilution (EU/ml)				
Endotoxin		0.50	0.25	0.125	0.06	0.03
in Water	1)	+	+	+	-	-
	2)	+	+	+	-	-
	3)	+	+	+	+	-
	4)	+	+	+	-	-
		geometric mean endpoint = 0.10 EU/ml				
in Product A	1)	+	+	-	-	-
	2)	+	+	+	-	-
	3)	+	+	+	-	-
	4)	+	+	+	-	-
		geometric mean endpoint = 0.15 EU/ml				
		non-inhibitory				

in Product B	1)	+	-	-	-	-
	2)	+	-	-	-	-
	3)	+	-	-	-	-
	4)	+	-	-	-	-

geometric mean endpoint = 0.50 EU/ml inhibitory

The easiest means to overcome product inhibition is through dilution. This initial dilution factor must be taken into account when calculating the total endotoxin concentration in a test sample. As a quick screen to determine a non-inhibitory dilution of product, prepare a series of increasing dilutions of the product containing an endotoxin spike equal in concentration to twice the lysate sensitivity. Assay each spiked product dilution using standard procedures. Positive results indicate when product inhibition has been overcome. Products which are extremely acidic or basic may require pH adjustment as well as dilution in order to completely overcome product inhibition.

A NOTE FOR OUR INTERNATIONAL CUSTOMERS

Other regulatory agencies may adopt other performance standards which will need to be satisfied in order to be in compliance in their jurisdictions.

REFERENCES

1. Bang, F.B. (1956) Bull. Johns Hopkins Hosp. 98:325-351.
2. Levin, J. and F.B. Bang (1964) Bull. Johns Hopkins Hosp. 115:337.
3. Levin, J. and F.B. Bang (1964) Bull. Johns Hopkins Hosp. 115:265-274.
4. Levin, J. and F.B. Bang (1968) Thrombos. Diath. Haemorrh. 19:186-197.
5. Solum, N.O. (1970) Thrombos. Diath. Haemorrh. 23:170-181.
6. Solum, N.O. (1973) Throm. Res. 2:55-70.
7. Young, N.S., J. Levin and R.A. Prendergast (1972) J. Clin. Investig. 51:1790-1797.
8. Nachum, R., A. Lipsey and S.E. Siegel (1973) N. Eng. J. Med. 289:931-934.
9. Cooper, J.F., H.D. Hochstein and E.B. Seligman, Jr. (1972) Bull. Parent. Drug Assoc. 26:153-162.
10. U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration Guideline on the Validation of the Limulus Amebocyte Lysate Test As an End-product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices (1987).
11. Chapter <85> Bacterial Endotoxins. Rockville,MD: United States Pharmacopeia.

TRADEMARKS

NOTES

NOTES

Lonza

8830 Biggs Ford Road | Walkersville, MD 21793
301-898-7025 | www.lonza.com

08286
PN189-8
01/11