



Limulus Amebocyte Lysate
PYROGENT™ Single Test Vials
US Licence No. 1775
Catalog No.: N189-06 Lot No.: 0000270627
Exp. Date: 25 OCT 2014 Store at 2 - 8° C
See Package Insert for Instructions and Warnings.

Lonza

Lonza
Walkersville, MD USA
301-898-7025, www.lonza.com

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Limulus Amebocyte Lysate (LAL) PYROGENT™ and PYROGENT™ Plus Single Test Vials

U.S. Licence No. 1775. Translated versions available at www.lonza.com

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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. LAL 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.

The Pharmacopeia outlines procedures that are considered necessary for:

1. Establishing endotoxin limits for pharmaceuticals and medical devices
2. Validating the use of LAL as an end-product endotoxin test
3. Developing a routine testing protocol¹⁰

The procedures described herein are based on the Pharmacopeial guidelines.

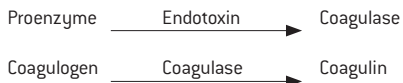
Warning

For *In Vitro* Diagnostic Use Only. The PYROGENT™ Assay is not intended to detect endotoxemia in man. The LAL Test may be substituted for the USP Rabbit Pyrogen Test when used according to the Pharmacopeial guidelines for end-product testing of human and animal parenteral drugs, biological products, and medical devices¹⁰.

Explanation of Test

The use of LAL for the detection of endotoxin evolved from the observation by Bang¹ 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 Bang⁴ prepared a lysate from washed amebocytes, which was an extremely sensitive indicator of the presence of endotoxin. Solum^{5,6} and Young, Levin, and Prendergast⁷ 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 LAL². 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 LAL. Once hydrolyzed, the resultant coagulin self-associates and forms a gelatinous clot.

Reagents Supplied and Storage Conditions

Limulus Amebocyte Lysate, Lyophilized, Single Test Vial (F211-06, F211-125, F211-25) Yellow-Labeled Vial

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

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

Lyophilized (unreconstituted) LAL must be stored 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.

E. coli Endotoxin 0111:B4, Lyophilized (C50-500) Red-Labeled Vial

One vial containing a lyophilized preparation of purified endotoxin from *E. coli*, strain 0111:B4. This vial, when prepared according to the instructions below, provides the user with a Control Standard Endotoxin (CSE). The potency of the endotoxin has been established using the current USP RSE according to the procedures described herein. The appropriate RSE/CSE ratio and resultant CSE potency is provided on the Certificate of Analysis (CoA). The CoA is available at www.lonza.com/coa.

This CSE preparation, with established potency, is an acceptable substitute for the USP RSE in all aspects of quality control provided the laboratory is using the designated lysate lot according to the procedures specified in this insert.

Store vial at 2–8°C prior to reconstitution. Reconstitute with 1.0 ml LAL Reagent Water. Potency (EU/ml) is stated on the CoA.

Store reconstituted vial at 2–8°C for up to 4 weeks. Prepare a 1.0 EU/ml dilution from this vial only in quantities as needed. See section on Reagent Preparation. Do not store or use diluted preparations for more than one day.

Warning: Contains human source material. Contents pyrogenic. Not to be administered to humans.

Note: Endotoxin is not included but required for lysate-only kits.

Materials and Equipment NOT Provided

1. LAL Reagent Water (#W50-640 or equivalent). LAL Reagent Water is equivalent to Water for Bacterial Endotoxins Test (BET).
2. Pipettes, 0.25 ml, 1 ml, and 5 ml, endotoxin-free.
3. 13 × 100 mm glass dilution tubes, endotoxin-free (#N207 or equivalent) (see Sample Collection and Preparation for sterilizing procedure).
4. Sodium hydroxide, 0.1N, or Hydrochloric acid, 0.1N dissolved in LAL Reagent Water, for pH adjustment of sample if necessary.
5. Endotoxin Standard (Control Standard Endotoxin that has been matched with the LAL).
6. Heating block or non-circulating hot water bath ($37^{\circ}\text{C} \pm 1^{\circ}\text{C}$).
7. Vial rack.
8. Timer.
9. Vortex mixer.

Sample Collection and Preparation

Careful technique must be used to avoid microbial or endotoxin contamination. All materials coming in contact with the sample 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 $2-8^{\circ}\text{C}$ for less than 24 hours and frozen for periods greater than 24 hours. It is the responsibility of the end-user 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 *E. coli* CSE

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

- A. Reconstitute endotoxin vial with 1.0 ml LAL Reagent Water.
- B. Vortex the vial of endotoxin for at least 15 minutes.
- C. Dilute the endotoxin with LAL Reagent Water to a concentration of 1.0 EU/ml.

This is accomplished by diluting the reconstituted endotoxin $1/X$, where X is the CSE potency in EU/ml as specified on the CoA. Using X as defined above, the general formula is 0.1 ml reconstituted Endotoxin diluted with $0.1 (X-1)$ ml LAL Reagent Water.

Example for $X = 11$ EU/ml:

Dilute 0.1 ml endotoxin with $0.1 (11-1) = 1.0$ ml LAL Reagent Water.

Vortex 60 seconds before proceeding.

- D. Using the 1.0 EU/ml endotoxin solution, prepare a serial two-fold dilution series that bracket the sensitivity of the lysate as shown in the following example. Each dilution should be vortexed 60 seconds prior to proceeding to the next dilution.

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 1.0 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 negative control, positive sample control, control standard endotoxin dilution 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 LAL 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 minutes (± 2 minutes) at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The 60 minute incubation time should be determined from the time each vial is placed in a $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ 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 USP RSE, and is expressed in Endotoxin Units.

As part of an initial in-house validation, each user should reverify the labeled lysate sensitivity using the endotoxin standard provided in the kit. 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

Labeled Lysate Sensitivity = 0.125 EU/ml

Replicate	Endotoxin Dilution (EU/ml)					H ₂ O	Endpoint
	0.50	0.25	0.125	0.06	0.03		
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

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

Endpoint (EU/ml)	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

$$\begin{aligned} \text{Endotoxin Concentration} &= \text{lysate sensitivity} \times \text{endpoint dilution} \\ &= 0.125 \text{ EU/ml} \times 11.4 = 1.4 \text{ EU/ml} \end{aligned}$$

Product Inhibition

The LAL 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.

See the example on the following page.

Product Inhibition Testing

Labeled Lysate Sensitivity = 0.125 EU/ml

Endotoxin		Endotoxin Dilution (EU/ml)				
		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.

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11. European Directorate for the Quality of Medicines. Chapter 2.6.14 Bacterial Endotoxins Test. *European Pharmacopoeia* (EP).
12. Ministry of Health, Labour, and Welfare, General Chapter 4.0.1 Bacterial Endotoxins Test. *Japanese Pharmacopoeia* (JP).
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Notes

Notes

www.lonza.com/pharmabiotech

Certificate of Analysis: www.lonza.com/coa

Contact Information

North America

Customer Service: 800 638 8174 (toll free)

order.us@lonza.com

Scientific Support: 800 521 0390 (toll free)

scientific.support@lonza.com

Europe

Customer Service: +32 87 321 611

order.europe@lonza.com

Scientific Support: +32 87 321 611

scientific.support.eu@lonza.com

International

Contact your local Lonza distributor

Customer Service: +1 301 898 7025

Fax: +1 301 845 8291

scientific.support@lonza.com

International Offices

Australia	+61 3 9550 0883
Belgium	+32 87 321 611
Brazil	+55 11 2069 8800
France	0800 91 19 81 (toll free)
Germany	0800 182 52 87 (toll free)
India	+91 40 4123 4000
Japan	+81 3 6264 0660
Luxemburg	+32 87 321 611
Singapore	+65 6521 4379
The Netherlands	0800 022 4525 (toll free)
United Kingdom	0808 234 97 88 (toll free)

Lonza Walkersville, Inc. – Walkersville, MD 21793

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