



Limulus Amebocyte Lysate (LAL) PYROGENT™ and PYROGENT™ Plus

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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 LAL Reagent Water and then mixed in equal parts 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

Proenzyme $\xrightarrow{\text{Endotoxin}}$ Coagulase

Coagulogen $\xrightarrow{\text{Coagulase}}$ Coagulin

Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the LAL⁷. 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

(F245-06, F245-125, E209-06, E209-125, E209-25, E194-03, E194-06, E194-125) **Yellow-Labeled Vial**

A lysate prepared from the circulating amebocytes of the horseshoe crab (*Limulus polyphemus*) standardized to detect the labeled concentration (EU/ml) of the USP Reference Standard Endotoxin.

Contains buffered mono and divalent cations. Lysate is lyophilized and sealed under vacuum and is to be reconstituted with LAL Reagent Water. Do not rehydrate until immediately prior to use.

Lyophilized (unreconstituted) LAL should 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.

Reconstituted lysate may be stored at 2–8°C for 24 hours. For longer storage, reconstituted lysate can be stored at -10°C or colder. Freeze and thaw only once. The lysate should be protected from exposure to light during storage. Use within four weeks after reconstitution. Reconstitute before use with LAL Reagent Water per the following table:

PYROGENT™ LAL Reagent	# of Tests/Vial	LAL Reagent Water Required
F245-06	16	1.8 ml
F245-125	16	1.8 ml
E209-06	50	5.2 ml
E209-125	50	5.2 ml
E209-25	50	5.2 ml
E194-03	50	5.2 ml
E194-06	50	5.2 ml
E194-125	50	5.2 ml

***E. coli* Endotoxin 055:B5, Lyophilized (7360) Red-Labeled Vial**

A lyophilized preparation of purified endotoxin from *E. coli* strain 055:B5. Each vial when prepared according to the instructions below provides the user with a Control Standard Endotoxin (CSE) whose potency has been established using the current USP Reference Standard Endotoxin (RSE) and the enclosed lot of lysate 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 5.0 ml LAL Reagent Water. Potency (in EU/ml) is calculated from RSE/CSE ratio as in the following example: Potency (EU/ml) = RSE/CSE Ratio (in EU/ng) X ___ ng/vial ÷ 5.0 ml/vial.

Store reconstituted vial at 2–8°C for up to 4 weeks. Prepare 1.0 EU/ml dilution from this vial only in quantities as needed. See section on Reagent Preparation. Do not store or use diluted endotoxin preparations for more than 1 day.

Warning: 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, 1 ml, 5 ml, 10 ml and 100 microliter, endotoxin-free.
3. 10 × 75 mm glass reaction tubes, endotoxin-free (#N201, #N205 or equivalent) (see Sample Collection and Preparation for sterilizing procedure).
4. 13 × 100 mm glass dilution tubes, endotoxin-free (#N207 or equivalent) (see Sample Collection and Preparation for sterilizing procedure).
5. Sodium hydroxide, 0.1N, or Hydrochloric acid, 0.1N dissolved in LAL Reagent Water, for pH adjustment of sample if necessary.
6. Endotoxin Standard (Control Standard Endotoxin that has been matched with the LAL).
7. Heating block or non-circulating hot water bath ($37^{\circ}\text{C} \pm 1^{\circ}\text{C}$).
8. Test tube rack.
9. Timer.
10. 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°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.

If the container of diluent used to rehydrate the LAL has been opened previously or was not supplied by Lonza, the diluent alone must be tested for endotoxin contamination.

Reagent Preparation

Allow reagents to equilibrate to room temperature prior to use.

1. Preparation of LAL.

Caution: Do not rehydrate until immediately prior to use.

- A. Reconstitute lyophilized lysate by adding 1.8 ml LAL Reagent Water to the 16-test vial or 5.2 ml to the 50-test vial. Swirl gently but thoroughly for at least 30 seconds. Do not shake as contents will foam.
- B. Reconstituted lysate can be stored for up to 24 hours at 2–8°C without loss of sensitivity. Reconstituted lysate can be divided into more convenient volumes and stored below -10°C for up to four weeks. Frozen liquid lysate should be thawed immediately before use. Freeze and thaw only once.

2. Preparation of *E. coli* CSE.

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

- A. Reconstitute the vial of endotoxin with 5.0 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 to $1/X$, 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 $X = 21$ EU/ml:

Dilute 0.1 ml endotoxin with $0.1 (21-1) = 2.0$ 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 brackets the sensitivity of lysate as shown in the following example. Each dilution should be vortexed for 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

Test Procedure and Interpretation

Each assay should include serial two-fold dilutions of the CSE which bracket the labeled lysate sensitivity, dilutions of the test sample, and LAL Reagent Water to serve as a negative control. To avoid microbial or endotoxin contamination, carefully transfer 0.10 ml of standard, sample, or water into the appropriate 10 × 75 mm reaction tube.

Add 0.10 ml of the reconstituted lysate to each tube beginning with the blank then moving from lowest to highest concentration of endotoxin. Immediately following the addition of the lysate to each tube, the contents should be mixed thoroughly and the tube placed in a 37°C ± 1°C non-circulating hot water or dry heat bath. This procedure should be followed for each dilution of the endotoxin. The unknown test sample must be run in parallel with the CSE. The assay may be done either as a yes/no test at a single dilution or as a quantitative test via a dilution series. The incubation time should be determined from the time each tube is placed in the 37°C ± 1°C bath. Assay tubes should not be removed from incubation or disturbed prior to the time specified for reading the test. After 60 minutes (±2 minutes) of incubation, carefully remove each tube and invert 180°.

1. A positive reaction is characterized by the formation of a firm gel that remains intact momentarily when the tube is inverted.
2. A negative reaction is characterized by the absence of a solid clot after inversion. The lysate may show an increased turbidity or viscosity. This is considered a negative result.

- The reaction in each tube should be recorded in columns as either positive or negative.

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 an endotoxin standard whose potency is known.

Prepare serial two-fold dilutions of the CSE 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

Labeled Lysate Sensitivity = 0.125 EU/ml

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

Endpoint	Log ₁₀ Endpoint
1/8 (0.125)	-0.903
1/16 (0.0625)	-1.204

Mean = -1.054

Antilog₁₀ Mean 0.088 = 1/11.4

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

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 way to overcome product inhibition is through dilution. The 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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Notes

Notes

www.lonza.com/pharmabiotech

Certificate of Analysis: www.lonza.com/coa

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