FUJIFILM

Limulus Amebocyte Lysate

Wako

PYROSTAR™ ES-F/Plate

Multi Test Vial (2.0 mL and 5.2 mL)

Intended Use: *Limulus* amebocyte lysate (LAL) is intended for the detection of Gram-negative bacterial endotoxins. PYROSTAR™ ES-F/Plate is intended for the quantitative detection of endotoxins by kinetic turbidimetric methods. The quantitative range for the Kinetic Turbidimetric Assay (KTA) is:

KTA Quantitative Range (EU/mL)
0.01 to 10

SUMMARY AND GENERAL INFORMATION

Endotoxin (lipopolysaccharide or LPS) is a component of the outer membrane of Gram-negative bacteria. Since endotoxins, when injected or implanted, may cause fever and/or shock, the detection of bacterial endotoxins in pharmaceuticals and medical devices is critical.

The LAL assay is the most sensitive method for the detection of bacterial endotoxins currently approved by the U.S. Food and Drug Administration (FDA).[2] The first methodology used to determine the LAL test results was the formation of a gel-clot in the bottom of a glass reaction tube. It has also been observed that the test solution becomes turbid prior to gel-formation. The time required to produce a specified level of turbidity is inversely proportional to the amount of endotoxin in a sample.[1]

A photometric instrument such as the Microplate Reader (Tecan Sunrise™ Microplate Reader or equivalent) is used to measure the rate of turbidity change. This quantitative measurement procedure is often referred to as the Kinetic Turbidimetric Assay (KTA).

By utilizing these properties, FUJIFILM Wako Chemicals U.S.A. Corporation (Wako) has developed an LAL endotoxin test that can be used as a quantitative turbidimetric test.

The USP Bacterial Endotoxins Test <85>[6] provides standardized procedures for validation prior to routine use.

The Specificity of LAL; however, is not absolute.[7] It has been reported that LAL reacts not only with endotoxin but also with β-1,3-glucan. Although the cascade system activated by β-1,3-glucan has been shown to be different than the one activated by endotoxin,[8] the end result, gel-clot formation is indistinguishable.

The activation of LAL by glucan in a sample can be prevented by adding a large amount of carboxymethylated curdlan (CMC) to LAL. The presence of large amounts of glucan does not interfere with the quantitation of endotoxin. Wako first made use of these findings by developing an ES-buffer, which contains high concentrations of CMC. When the ES-buffer is used to reconstitute LAL, the LAL reagent becomes endotoxin-specific. PYROSTAR™ ES-F/Plate is a new preparation of LAL in which CM-curdlan is co-lyophilized with LAL. Reconstituting PYROSTAR™ ES-F/Plate test vials with LAL Reagent Water results in an endotoxin-specific LAL reagent.

HISTORY AND BIOLOGICAL PRINCIPLE

The *invitro* detection of bacterial endotoxin was pioneered by Levin and Bang.[3] Their findings showed that the blood of the horseshoe crab, *Limulus polyphemus,* clots in the presence of Gram-negative bacteria. They subsequently reported that all the components required for clot formation could be isolated from the circulating amebocytes found in *Limulus* blood.[3,4]

A guideline was released by the FDA in 1987[5] to inform manufacturers of human drugs and biologicals, animal drugs, and medical devices of procedures that FDA considers necessary to validate the use of LAL as an end-product endotoxin test. The FDA guideline was combined with the USP Bacterial Endotoxins Test in 2000 making the USP method the standard method for US manufacturers. The 1987 FDA Guideline was withdrawn in 2011.

WARNINGS AND GENERAL PRECAUTIONS

PYROSTAR™ ES-F/Plate is intended for the *invitro* detection of Gram-negative bacterial endotoxins. Exercise caution when handling LAL because its toxicity is unknown.

This test is not a diagnostic device and is not to be used to determine endotoxin levels in humans for diagnostic purposes.

REAGENTS PROVIDED

PYROSTAR™ ES-F/Plate Reagent: PYROSTAR™ ES-F/Plate is a lyophilized reagent containing *Limulus* amebocyte lysate, buffers, carboxy-methyl curdlan, monovalent and divalent cations.

Preparation: Tap the vial on a level surface to ensure that all powder is at the bottom of the vial. Gently remove the stopper and add 2.0 mL or 5.2 mL of LAL Reagent Water (LRW) to the vial. Replace the stopper and swirl gently to dissolve the contents without making contact to the stopper.

Storage: Store at 2-10°C. Refrigerate reconstituted reagent at 2-8°C, maximum 6 hours or at -15 ± 5°C for a maximum of 14 days. Reconstituted reagent can only be frozen and thawed one time.

MATERIALS AND EQUIPMENT NOT PROVIDED

Control Standard Endotoxin (CSE): A lyophilized reagent that contains refined endotoxin from *E. coli* and is used to confirm LAL reagent sensitivity, validate product test methods, and prepare inhibition controls. CSE can be obtained from FUJIFILM Wako Chemicals U.S.A Corporation.

Preparation: CSE should be reconstituted as specified by the manufacturer. After reconstitution, the vial should be rapidly shaken or vortexed as instructed by the manufacturer.

Storage: Reconstituted CSE should be stored as instructed by the manufacturer. CSE should not be stored at temperatures below freezing and should be rapidly shaken or vortexed prior to usage.

Reference Standard Endotoxin (RSE): USP Endotoxin Reference Standard that has a defined potency of 10,000 USP Endotoxin Units (EU).
LAL Reagent Water (LRW): Endotoxin-free water
Endotoxin free pipettes
Endotoxin free dilution tubes
Microplate Method
Microplate Reader (Tecan Sunrise™ Microplate Reader or equivalent)
Endotoxin free Microplates (Corning® 96 well clear flat bottom plate (Corning® catalog # 3370) or equivalent)

PREPARATION OF CONTROL STANDARD ENDOTOXIN DILUTIONS

Control Standard Endotoxin Preparation

Based on the information provided on the Certificate of Analysis, prepare a CSE standard to a concentration of 1,000 EU/mL. Vortex the 1,000 EU/mL CSE solution for 2 minutes at Room Temperature. Using the 1,000 EU/mL CSE solution, prepare the endotoxin dilution series as shown in Table 1.

Vortex each tube for 30 seconds between dilutions. Dilutions may be prepared in different volumes as long as the same ratio is maintained.

Table 1
Sample Endotoxin Dilution Scheme
(10-fold dilution series)

Initial endotoxin conc. (EU/mL)	Volume added to LRW (mL)	Final endotoxin conc. (EU/mL)
1000	0.4 + 3.6	100
100	0.4 + 3.6	10
10	0.4 + 3.6	1
1	0.4 + 3.6	0.1
0.1	0.4 + 3.6	0.01

SPECIMEN COLLECTION AND PREPARATION

LAL reactions are pH sensitive requiring that the LAL and sample mixture have a pH of 6.0 to 8.0. PYROSTAR™ ES-F/Plate contains buffering components that help bring the test mixture within the pH range in most cases. If a pH adjustment is necessary, the pH of the sample should be adjusted with endotoxin-free HCl or NaOH.

Product Interference

Prior to using the LAL test for the routine release of product, it is necessary to validate the absence of product interference by performing an inhibition/enhancement test for each product type. A product is determined to be non-interfering by assaying a sample of the product spiked with a known amount of endotoxin and detecting 50 – 200% of the spiked endotoxin.

Kinetic Turbidimetric Assay (KTA): Prepare a standard curve that covers the testing range. Spike the product to a level of endotoxin that is equal to or near the middle of the standard curve. The product is determined to be non-interfering if the level of endotoxin reported is 50 to 200% of the spiked endotoxin concentration. For example, if a standard curve is run between 0.1 and 10 EU/mL, the product should be spiked with endotoxin to yield a concentration of 1.0 EU/mL. The acceptable endotoxin recovery is between 0.5 and 2.0 EU/mL.

KINETIC TURBIDIMETRIC ASSAY PROCEDURE

A kinetic turbidimetric assay (KTA) can be performed on the Microplate Reader and accompanying software (Tecan Sunrise™ Microplate Reader or equivalent).

In addition to the product being tested, a valid assay will include endotoxin standards bracketing the analysis range, positive product controls, and negative controls. All test values need to be determined from at least duplicate samples.

Microplate Method:
All samples, standards and/or reagents should be at room temperature prior to beginning the following procedure:

Aseptically transfer 0.05 mL of each product sample or control to the respective microplate well beginning with the negative control and ending with the highest endotoxin concentration. Add 0.05 mL of PYROSTAR™ ES-F/Plate reagent into each microplate well containing standard or sample. Place the microplate into the Microplate Reader with the following specifications:

Wavelength:	405 nm
On set O.D.:	0.015
Measurement Interval:	40 sec
Temperature:	37°C

At the end of the incubation period, the Microplate software will perform regression analysis of the standard curve data and calculate endotoxin levels for each sample.

See "Bacterial Endotoxins Test" in The U.S. Pharmacopeia and refer to sections describing standard curve and preparation of solutions.[6]

CALCULATION OF ENDOTOXIN CONCENTRATION

During a KTA reaction the turbidity of the test solutions is continually monitored by the Microplate Reader. The time required for a sample to reach a determined absorbance level over background is measured. This time is referred to by the Microplate Reader software as Ta (activation time).

The software produces a log (x-axis) / log(y-axis) linear correlation of the Ta (Activation Time) of each standard with its corresponding endotoxin concentration. The Ta is the time required for the delta optical density (O.D.) of the standard/sample to reach 0.015. Endotoxin concentrations in unknown samples are calculated from their corresponding Ta using the calculated standard curve. An example of a standard series and recovery of an endotoxin spike of 1.0 EU/mL in product is presented below:

Representative Analysis

Standards	CSE (EU/mL)	Ta (sec)	Log Concentration	Log Ta (sec)
NC	0	NR	------	-------
STD1	10.0	480	1.000	2.681
STD2	1.0	792	0.000	2.899
STD3	0.1	1416	-1.000	3.151
slope			-0.2349	
y-intercept			2.910	
coefficient of correlation			-0.999	

		Ta (sec)	Log Ta (sec)	Calculated EU/mL	% Recovery
Product 1	NPC1	NR	------		------
	PPC1	816	2.912	0.987	98.7%
Product 2	NPC2	NR	------	------	------
	PPC2	780	2.892	1.196	119.6%

NR= Nonreactive

In this example, the positive product control (PPC) for each product sample yielded endotoxin recovery consistent with the spiked level indicating there is no detectable product enhancement or inhibition. The negative product control (NPC) and negative control (NC) showed significantly lower endotoxin levels than the lowest standard endotoxin concentration.

PERFORMANCE CHARACTERISTICS:

The linearity of the standard curve within the concentration range used to determine endotoxin levels must be verified. No less than 3 endotoxin standards, spanning the desired concentration range, and LRW water blank should be assayed in at least triplicate.Refer the Verification of Criteria for the Standard Curve in USP. The absolute value of the coefficient of correlation, lrl, shall be greater than or equal to the value of 0.980.[6]

BIBLIOGRAPHY

1. Cooper, J.F., Levin, J., and Wagner, H.N. "Quantitative Comparison of in Vitro and In Vivo Methods for Detection of Endotoxin" J. Lab, Clin, Med., 78, p.128 (1971).
2. Hochstein, H.D. "The LAL Test versus the Rabbit Pyrogen Test for Endotoxin Detection; Update '87." Pharm. Technol., 11(6), p. 124 (1987).
3. Levin, J. and Bang, F.B. "Clottable protein in *Limulus*: Its Localization and Kinetics of its Coagulation by Endotoxin." Thromb. Dieth. Haemonti., 19, p. 186 (1966).
4. Tai, J.Y. and Llu. T.Y. "Studies on *Limulus* Lysate, Isolation of Pro-clotting Enzyme." J. Biol, Chem., 252, p.2176 (1977).
5. Guideline on Validation of the *Limulus* Amebocyte Lysate Test as an End-Product Endotoxin Test of Human and Animal Parenteral Drugs, Biological Products and Medical Devices. U.S. Dept. of Health & Human Services, FDA, December 1987.
6. "Bacterial Endotoxins Test." The United States Pharmacopeia, most current version. U.S. Pharmacopeial Convention, Rockville, MD.
7. Cooper, J.F., Weary, M.E., and Jordon, J,T. "The Impact of Nonendotoxin LAL-Reactive Materials on *Limulus* Amebocyte Lysate Analysis." L. Parent, Sci $ Tech, 51(1), (1966).
8. Tsuchiya, M., Oishi, H., Takaoka, A., Fusamoto, M. and Matsuura, S. "Discrimination between endotoxin and (1 – 3) beta-D-glucan using turbidimetric kinetic assay with *Limulus* amebocyte lysate." Chem Pahrm Bull (Tokyo), 38(9). p. 2523 (1990).

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