FUJIFILM

Limulus Amebocyte Lysate

PYROSTAR™ ES-F

Multi Test Vial (2.0 mL and 5.2 mL) with Control Standard Endotoxin (CSE)

Intended Use: *Limulus* amebocyte lysate (LAL) is intended for the detection of Gram-negative bacterial endotoxins. PYROSTAR™ ES-F is intended for the qualitative detection of endotoxins by gel-clot or quantitative detection by kinetic turbidimetric methods. The quantitative range for the Kinetic Turbidimetric Assay (KTA) is based on the lysate gel-clot labeled sensitivity, refer to the table below:

Gel-clot Labeled Lysate Sensitivity (EU/mL)	KTA Quantitative Range (EU/mL)
0.015	0.001 to 10
0.03 to 0.25	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 Toxinometer® (FUJIFILM Wako Pure Chemical Corporation) 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 either as a quantitative turbidimetric test or as a qualitative gel-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 is a new preparation of LAL in which CM-curdlan is co-lyophilized with LAL. Reconstituting PYROSTAR™ ES-F multi test vials with LAL Reagent Water results in an endotoxin-specific LAL reagent.

HISTORY AND BIOLOGICAL PRINCIPLE

The *in vitro* 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 is intended for the *in vitro* 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 Reagent: PYROSTAR™ ES-F 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 with the stopper.

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

Note: For sensitivity 0.015 EU/mL reagent, guaranteed stability is for a maximum 4 hours after reconstitution.

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.

Preparation: Release the vacuum by gently pulling up and removing the rubber stopper from the vial. The vial contains 500 ng of endotoxin. Each Control Standard Endotoxin is tested in comparison to the FDA Reference Endotoxin Standard and the conversion factor (EU/ng) is determined. Determine the volume of LRW to be added to the vial to produce a 1,000 EU/mL solution. Add the determined volume of LRW to the vial. Replace the rubber stopper, invert the vial several times and vortex vigorously for 2 minutes.

Storage: Reconstituted CSE can be stored at 2-10°C for 1 month. CSE should not be stored at temperatures below freezing. When the stored solution is used, vigorously vortex the solution for 1 minute before use.

MATERIALS AND EQUIPMENT NOT PROVIDED

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
Toxinometer® Method
Toxinometer® (FUJIFILM Wako Pure Chemical Corporation)
Endotoxin-free test tubes and aluminum caps for Toxinometer®
Gel-clot Techniques
Water bath or heating block incubator capable of maintaining 37 ± 1°C
Endotoxin-free test tubes and aluminum caps

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, as described above (Please see the CSE vial label for CSE potency, EU/vial, for all 8-digit production code products). 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 or 2.

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
(2-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	2.0 + 2.0	0.5
0.5	2.0 + 2.0	0.25
0.25	2.0 + 2.0	0.125
0.125	2.0 + 2.0	0.06
0.06	2.0 + 2.0	0.03
0.03	2.0 + 2.0	0.015
0.015	2.0 + 2.0	0.008
0.008	2.0 + 2.0	0.0039

Table 2
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
0.01	0.4 + 3.6	0.001

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 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.

Gel-clot Techniques: Two independent CSE dilution series are prepared using the product and LRW as diluents, respectively. Assay the CSE dilution series prepared in LRW in duplicate (n=2), and the CSE dilution series prepared in product in quadruplicate (n=4). The geometric mean endpoints of both dilution series must fall within two-fold of the labeled endotoxin sensitivity.

KINETIC TURBIDIMETRIC ASSAY PROCEDURE

A Kinetic Turbidimetric Assay (KTA) can be performed on the Toxinometer® and accompanying software (FUJIFILM Wako Pure Chemical Corporation).

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.

> Toxinometer® Method: Aseptically transfer 0.1 mL of PYROSTAR™ ES-F reagent into endotoxin-free Toxinometer® tubes. Add 0.1 mL of each product sample or control to the respective test tubes beginning with the negative control and ending with the highest endotoxin concentration. After each addition of each product sample or control, quickly mix the contents of the tube without foaming. Place the tube into the Toxinometer® in the order of the assigned tube position. At the end of the incubation period, the Toximaster® 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 Toxinometer®. The time required for a sample to reach a determined absorbance level over background is measured. This time is referred to by the Toximaster® software as Tg (gelation time).

The software produces a log(x axis)/log(y axis) correlation of the Tg of each standard with its corresponding endotoxin concentration. 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)	Tg (min)	Log Concentration	Log Tg (min)
NC	0	NR	-	-
STD1	10.0	8.0	1.000	0.903
STD2	1.0	13.2	0.000	1.121
STD3	0.1	23.6	-1.000	1.373

slope	-0.2349
y-intercept	1.132
coefficient of correlation	-0.999

		Tg (min)	Log Tg (min)	Calculated EU/mL	% Recovery
Product 1	NPC1	NR	-	-	-
	PPC1	13.6	1.134	0.987	98.7%
Product 2	NPC2	NR	-	-	-
	PPC2	13.0	1.114	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 to the Verification of Criteria for the Standard Curve in USP. The absolute value of the coefficient of correlation, |r|, shall be greater than or equal to the value of 0.980.[6]

GEL-CLOT TECHNIQUES PROCEDURE:

To ensure both the precision and validation of the PYROSTAR™ ES-F test, the sensitivity indicated on the label must be verified as described in the USP Bacterial Endotoxins Test <85>.[6]

Each assay should include NC (negative control), 2λ PC (2λ CSE control), 2λ PPC (2λ positive product control) and the product to be tested, where λ is the PYROSTAR™ ES-F sensitivity.

1. Add 0.1 mL of the reconstituted PYROSTAR™ ES-F to each assay tube.
2. Aseptically transfer 0.1 mL of each product sample or control into each assay tube, beginning with the negative control and ending with the highest endotoxin concentration.
3. Quickly mix the contents of the tubes and incubate them undisturbed in a 37 ± 1°C heating block or heated water bath for 60 minutes ± 2 minutes.
4. After incubation, examine each tube for gelation. Each tube in the gel-clot method is interpreted as either positive or negative. A positive test is defined as the formation of a firm gel capable of maintaining its integrity when the assay tube is inverted 180°. A negative test is characterized by the absence of gel or by the formation of a viscous mass which does not hold when the assay tube is inverted.
5. Test results are only valid when the 2λ PC and 2λ PPC are positive, and the NC is negative.

ENDOTOXIN ASSAY BY DILUTION SERIES

The sensitivity indicated on the PYROSTAR™ ES-F label can be confirmed by preparing a two-fold dilution series of RSE or CSE (with a confirmed potency) that brackets the stated sensitivity. The dilution series needs to be assayed in quadruplicate and include negative controls. The sensitivity is then determined by calculating the geometric mean of the obtained endpoints. An example of PYROSTAR™ ES-F with a labeled sensitivity of 0.125 EU/mL (λ) is described below:

Results of Gel-Clot Assay: Endotoxin Dilution (EU/mL)

Replicate	0.25	0.125	0.06	0.03	NC	End point	Log_{10} End point
1	+	+	+	-	-	0.06	-1.222
2	+	+	-	-	-	0.125	-0.903
3	+	+	-	-	-	0.125	-0.903
4	+	+	+	-	-	0.06	-1.222

Convert each endpoint of the quadruplicate assay to its log_{10} value. The individual log_{10} values are averaged and the PYROSTAR™ ES-F sensitivity is taken as the $antilog_{10}$ of this mean log value. The mean of the log_{10} value in the above example is -1.063 and the geometric mean ($antilog_{10}$) is 0.087.

DETERMINATION OF ENDOTOXIN IN AN UNKNOWN BY GEL-CLOT

To determine the endotoxin concentration of an unknown solution, test two-fold dilutions of the product until an endpoint has been reached. Calculate the geometric mean dilution factor and multiply by the labeled endotoxin sensitivity.

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

Endpoint Dilution Factor	Log_{10} Endpoint
8	0.903
16	1.201
Mean	1.054
$Antilog_{10}$	11.3

Endotoxin Concentration = 0.125 EU/mL times 11.3 = 1.4 EU/mL

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