Limulus Amebocyte Lysate

PYROTELL® STV

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Pyrotell® Single Test Vial
for the Detection and Quantitation of Gram-Negative Bacterial Endotoxins (Lipopolysaccharides)

The *Limulus* amebocyte lysate (LAL) test may be substituted for the U.S. Pharmacopeia (USP) Pyrogen Test (rabbit fever test) for the end-product testing of "human injectable drugs (including biological products), animal injectable drugs, and medical devices." The LAL test is recommended for the quantitation of endotoxin in raw materials used in production, including water, and for in-process monitoring of endotoxin levels. The USP Bacterial Endotoxins Test (1) is the official test referenced in specific USP monographs.

Summary of Test

Limulus amebocyte lysate is an aqueous extract of blood cells (amebocytes) from the horseshoe crab, *Limulus polyphemus*. The LAL test is performed by adding 0.2 mL of the test specimen to a single test vial (STV) of Pyrotell®. After the Pyrotell® dissolves (approximately a minute), the solution is mixed thoroughly and the STV is placed immediately in a dry block incubator or noncirculating water bath at 37 ± 1°C for 60 ± 2 min. After the incubation period, the STV is removed and inverted in one smooth motion. If a gel has formed and remains intact in the bottom of the tube after inversion of 180°, the test is positive; the concentration of endotoxin in the tube is greater than or equal to the sensitivity of the Pyrotell®. Any other state of the mixture constitutes a negative test indicating an endotoxin concentration less than the Pyrotell® sensitivity. Even if a gel has formed but breaks or collapses upon inversion, the test is negative. The LAL test is rapid, specific, easy to perform, and highly sensitive. Pyrotell® can detect as little as 0.03 Endotoxin Units (EU) per mL using the gel-clot technique.

History and Biologic Principle

Howell described the clotting of *Limulus* blood in 1885 (2). In the 1950's, Bang at the Marine Biological Laboratory, Woods Hole, MA, discovered that gram negative bacteria cause *Limulus* blood to clot (3). Levin and Bang later determined that the reaction is enzymatic and that the enzymes are located in granules in the amebocytes (4). They showed that clotting is initiated by a unique structural component of the bacterial cell wall called endotoxin or lipopolysaccharide (5). Current understanding is that the reaction leading to clot formation is a cascade of enzyme activation steps. While the complete reaction is not understood, the last step is well described. Clotting protein (coagulogen) is cleaved by activated clotting enzyme; the insoluble cleavage products coalesce by ionic interaction to form the gel matrix. More information about the LAL reaction and applications is available in the literature (6,7,8).

Reagent

Single test vials of Pyrotell® contain 0.2 mL lyophilized LAL.

Associates of Cape Cod, Inc. offers individual lots of Pyrotell® in sensitivities ranging from 0.03 to 0.25 EU/mL based on the USP Endotoxin Reference Standard (also referred to as the reference standard endotoxin or RSE). Sensitivity (λ) is the minimum concentration of RSE that produces a firm gel-clot under standard conditions. The lot sensitivity, EU/mL, is printed on the package labels. Specify the sensitivity desired when placing an order.

Use Pyrotell® for in-vitro testing purposes only. Do not use it for the detection of endotoxemia. The toxicity of this reagent has not been determined; thus, caution should be exercised when handling Pyrotell®.

Reconstitution of a Single Test Vial (STV)

1. The STV is rehydrated with 0.2 mL of the test sample during the test procedure (see "Performing the Test" under Test Procedure).

2. Gently tap the STV to cause loose Pyrotell® to fall to the bottom of the vial. Remove the crimp seal and break the vacuum by lifting the gray stopper. Do not contaminate the mouth of the vial. Remove and discard the stopper; do not inject through or reuse the stopper. A small amount of LAL left on the stopper will not affect the test.

3. The lyophilized LAL pellet will go into solution within a minute after the addition of the test specimen. After rehydration, thoroughly mix the contents of the vial to ensure homogeneity.

Storage Conditions

Freeze-dried Pyrotell® is relatively heat stable and, if kept refrigerated, will retain full activity through the expiration date on the vial label. Upon receipt, store the product at -20 to +8°C. Temperatures below -20°C shrink the stopper, leading to a loss of vacuum and possible contamination of Pyrotell®. Temperatures in excess of 37°C can cause rapid deterioration of lyophilized Pyrotell® as evidenced by loss of sensitivity and a distinct yellowing of the product. Pyrotell® is shipped with cold packs in insulated containers to protect against high temperatures.

Pyrotell® rehydrated with LAL Reagent Water (see "Test Reagents") is usually clear and slightly opalescent. Occasionally, a lot will exhibit a slight, uniform turbidity. The presence of small fibers or strands does not indicate contamination nor affect activity; however, flocculent precipitation or a distinct yellow color does indicate deterioration.

Specimen Collection and Preparation

Specimens should be collected aseptically in non-pyrogenic containers. Depyrogenated glassware or sterile, disposable, qualified plastics (per USP (9)) are recommended to minimize adsorption of endotoxin to container surfaces. Not all plastic containers are free of detectable endotoxin and an extractable substance from some types may interfere with the LAL test. Containers (selected randomly from a batch) may be rinsed with a small volume of LAL Reagent Water (room temperature for one hour) and the rinse tested as a specimen to determine if the batch is acceptable.

The pH of the reaction mixture (diluted sample added to Pyrotell®) should be 6 to 8. Adjust the pH of the specimen with HCl or NaOH (free of detectable endotoxin) or compatible buffer (e.g., Pyrosol®). Dilute concentrated HCl or NaOH with LRW to normalities that will not lead to significant dilution of the test specimen when adjusted. Do not adjust the pH of unbuffered saline or water.

Substances that denature proteins, chelate cations, bind endotoxin or alter endotoxin's hydrophobic state may interfere with the test. Interference may be detected as recovery of significantly more or less endotoxin than that expected when a known amount of standard endotoxin is added to the specimen (see "Limitations of Procedure"). In most cases, dilution of the specimen will reduce the concentration and activity of interfering substances and still yield valid test results. Appropriate controls and dilution schemes are discussed under "Test Procedure."

Specimens should be tested as soon as possible after collection. It may be advisable to freeze a nonsterile specimen that will be stored or shipped before testing. Specimens expected to contain low concentrations of endotoxin (less than 1 EU/mL) should be tested for loss of endotoxin during storage.

Test Procedure

Test Reagents

1. *Pyrotell® STV* (see description and method of reconstitution in section above).

2. *LAL Reagent Water* (LRW); not provided with Pyrotell®; order separately. Diluent water that shows no detectable endotoxin in the LAL test must be used. Recommended sources include Associates of Cape Cod, Inc., or USP Sterile Water for Injection or Irrigation (WFI, without bacteriostat). The endotoxin limit for USP WFI is 0.25 EU/mL; therefore, WFI may have detectable endotoxin with some lots of Pyrotell®. To certify a new lot of water as an LRW with a given lot of Pyrotell®, make dilutions of standard endotoxin with the new lot of water to confirm the sensitivity of the Pyrotell®. If the sensitivity of the lot is confirmed and the negative control shows no increase in viscosity and no flocculent precipitation, the water is suitable for use. Use LRW to reconstitute endotoxin standards and to dilute endotoxin standards and test specimens.

3. *Standard Endotoxin;* not provided with Pyrotell®; order separately. Control Standard Endotoxin (CSE) Cat# E0005, obtained from Associates of Cape Cod, Inc., is used to confirm the sensitivity of Pyrotell®, validate product, and prepare inhibition controls. Each vial contains a measured weight of endotoxin. USP Endotoxin Reference Standard may be obtained from the U.S. Pharmacopeial Convention, Inc. Follow manufacturers' directions for reconstitution and storage of standard endotoxins. CSE lots may show different potencies (EU/ng) when tested with various lots of Pyrotell®. Request a Certificate of Analysis for the potency of a CSE with a specified lot of Pyrotell®.

Materials and Equipment (not provided)

1. *Noncirculating water bath* or dry block incubator capable of maintaining 37 ± 1°C.

2. *Test tube racks.*

3. *Pipets*, automatic pipettors with pipet tips, or repeating pipettors with plastic syringe barrels. Sterile, disposables are recommended.

4. *Vortex-type mixer.*

5. *Nonpyrogenic dilution tubes* with adequate capacity for making dilutions of endotoxin standard or test specimen, e.g., Cat# TB013, TB240 or TB016C. See "Specimen Collection and Preparation" for other containers suitable for dilutions.

6. *Hot air oven* with 250°C capacity for depyrogenation of glassware. Commonly used minimum time and temperature settings are 30 minutes at 250°C (1, 10).

Controls

Controls are necessary to ensure a valid test. Required procedures are detailed by the USP (1).

1. **Endotoxin controls**
   a. **Endotoxin standard series**. Prepare a fresh set of dilutions from the stock endotoxin solution. Make dilutions such that a final series of twofold dilutions will bracket the sensitivity (λ) of the Pyrotell®. Concentrations of 2λ, λ, 0.5λ, and 0.25λ are recommended to confirm Pyrotell® sensitivity. Use as few dilutions as possible with appropriate pipet volumes to maximize accuracy.

   b. **Positive controls** may be used instead of a series of standard concentrations for the Limit Test per USP (1). The positive control concentration should be 2λ.

   c. **Positive product controls** are inhibition controls and consist of the specimen or dilution of specimen to which standard endotoxin is added. The final concentration of the added endotoxin in the test specimen should be 2λ.

2. **Negative controls**
   LRW negative control(s) should be included with each batch of specimens tested. When performing the Test for Interfering Factors (1), the specimen used to dilute standard endotoxin is also treated as a negative control.

Specimen Preparation for Limits Test or Quantitative Assay

Either dilute the specimen to the required concentration to perform the Limits (pass/fail) test (1) or perform the Quantitative assay (1) by testing a series of concentrations (examples of the two types of tests are given in "Results and Interpretation"). Dilutions should be made in dilution tubes and the test volume of 0.2 mL transferred to the STVs. The dilution tested for the Limits Test is determined from the sensitivity of the Pyrotell® and the endotoxin limit for the specimen. Refer to "Limitations of Procedure".

Performing the Test

Consistent technique is necessary to obtain satisfactory results.

1. Add 0.2 mL of the test specimen or control directly to the STV using a graduated (0.1 mL increments) pipet or an automatic pipettor. Prepare the negative control(s) first. Add the standard endotoxin concentrations to each STV from the lowest to highest concentration in each series. Shake the rack of tubes vigorously for 20 to 30 seconds to ensure thorough mixing. If there are only a few tubes, each may be vortex mixed for 1 to 2 seconds. Failure to mix adequately is a common cause of unsatisfactory tests.

2. Place the reaction tubes in a 37 ± 1°C water or dry bath for 60 ± 2 minutes. The reaction begins when the test specimen is added to the LAL but does not proceed at an optimum rate until the mixture reaches 37°C. If large numbers of specimens are tested in parallel, the tests should be batched and started at intervals that permit the reading of each within the time limit.

   Do not disturb the STVs during the incubation period. The gel-forming reaction is delicate and may be irreversibly terminated if the tubes are handled, agitated, or vibrated. Do not use a water bath with a stirrer or other source of vibration. Submerge tubes above the level of the reaction mixture but not so deeply that they float or move about in the racks.

3. Remove and read reaction tubes one at a time directly from within the water or dry bath. Do not wipe the tubes dry or bump them against the side of the rack. Invert the tube in one smooth motion; do not pause halfway in the inversion unless it is obvious that the gel has not formed. A positive test is indicated by the formation of a gel which does not collapse when the tube is inverted.

Results and Interpretation

Example of Standard Endotoxin Series

Confirm the sensitivity of the Pyrotell® and qualify the laboratory or technician by performing the LAL test on a series of known standard endotoxin concentrations (1) that bracket the labeled sensitivity (i.e., 2λ, λ, 0.5 λ, and 0.25 λ). For this example, the Pyrotell® sensitivity (λ) is 0.25 EU/mL:

Endotoxin Concentration	Test Result
0.5 EU/mL(2λ)	+
0.25 EU/ml (λ)	+
0.125 EU/mL (0.5λ)	-
0.06 EU/mL (0.25λ)	-
LRW (negative control)	-

The endpoint of this assay is defined as the least concentration of endotoxin to give a positive test. The labeled sensitivity of the Pyrotell® is confirmed if the endpoint is λ plus or minus a twofold dilution. In this example, the concentration of endotoxin in the last positive tube in the series is 0.25 EU/mL or λ; therefore, the sensitivity is confirmed. The test would be valid (sensitivity confirmed) if the endpoint were 0.125 to 0.5 EU/mL (the error of the method). To show an endpoint of 0.125 EU/mL, the 0.06 EU/mL level must be present in the series and be negative.

When the endotoxin assay is replicated, sensitivity is expressed as the geometric mean (GM) of the individual sensitivities:

$$GM = \text{antilog}((\Sigma e)/f)$$

where Σe = sum of log endpoints, and f = number of replicate endpoints.

The LRW **negative control** should give a negative test. If the negative control clots, the LRW, glassware, or Pyrotell® is contaminated. The mixture should be clear with no increase in viscosity. "Snowflake" or flocculent precipitation indicates an endotoxin concentration less than the Pyrotell® sensitivity.

In the absence of the endotoxin series (1), a **positive control** may be included with the tests. The positive control at 2λ is the 0.5 EU/mL level in the example above. If the positive control is negative, the Pyrotell® sensitivity is less than twofold of the labeled sensitivity and the specimen test is invalid. Loss of sensitivity may mean the Pyrotell® deteriorated, the endotoxin lost potency (often because of adsorption to container surface), or the test was not conducted properly.

Example of a Limits (Pass/Fail) Test

It is possible to test one specimen concentration with a given sensitivity of Pyrotell® and have the result indicate whether or not the test specimen has more or less endotoxin than its limit. In this example, the specimen concentration is 1 mg/mL and the desired or predetermined endotoxin limit for the specimen is 3 EU/mg (see "Limitations of Procedure"). The limit expressed in EU/mL,

$$(3 \text{ EU/mg})\ (1 \text{ mg/mL}) = 3 \text{ EU/mL},$$

is greater than the sensitivity of the Pyrotell®, 0.25 EU/mL, so the specimen must be diluted to perform a pass/fail test. Determine the specimen dilution that will indicate a pass, < 3 EU/mL, or a fail, ≥ 3 EU/mL, by dividing the endotoxin limit in EU/mL by the sensitivity of the LAL:

$$3 \text{ EU/mL} / 0.25 \text{ EU/mL} = 12.$$

Combine one part specimen with 11 parts LRW to prepare the 1:12 dilution and test. The result will indicate whether the specimen passes the test at the 3 EU/mL limit. Positive product controls are included at the specimen dilution to rule out false negative results.

Example of a Quantitation Assay

Endotoxin is quantified in an assay by finding the endpoint in a series of specimen dilutions. In the example below, the specimen is diluted with LRW and the dilutions in the table are tested; λ is 0.25 EU/mL. The results are scored as positive or negative.

Specimen Dilution	Test Result
undiluted	+
1:2	+
1:4	+
1:8	-
1:16	-
1:32	-
negative control	-

To calculate the concentration of endotoxin in the specimen, multiply the Pyrotell® sensitivity (λ) by the reciprocal of the dilution at the endpoint:

$$\text{Conc.} = (\lambda)\ (4/1) = (0.25 \text{ EU/mL})\ (4) = 1 \text{ EU/mL}.$$

The concentration for replicate assays is expressed as the geometric mean.

A **positive product control** (specimen spiked with 2λ standard endotoxin) must be present and test positive to rule out false negative results. If the positive product control is negative and the positive control is positive, the specimen is inhibiting the LAL test. The specimen should be retested at a greater dilution (not to exceed the MVD; see "Limitations of Procedure").

Limitations of Procedure

The procedure is limited by the capacity of the specimen to inhibit or enhance the LAL test. If the procedure cannot be validated (1) at a specimen dilution not exceeding the Maximum Valid Dilution (MVD), then the LAL test cannot be substituted for the USP Pyrogen Test. The MVD is calculated as follows:

$$MVD = (\text{Endotoxin Limit})\ (\text{Concentration of Sample Solution}) / (\lambda)$$

where λ is the labeled sensitivity in EU/mL

Per USP (1), the endotoxin limit for parenteral drugs is defined on basis of dose as K/M, where K is the threshold pyrogenic dose of endotoxin per kg of body weight (0.2 EU/kg for drugs with an intrathecal route of administration and 5 EU/kg for all other parenterals) and M is the maximum human dose.

Per USP (12), the endotoxin limit for finished medical devices is not more than 20 EU/device and not more than 2.15 EU/device for devices in contact with cerebrospinal fluid. The endotoxin limit for the device extract is calculated as: (K x N) / V, where K is the limit for each device, N is the number of extracted devices and V is the total volume of the extract.

Trypsin will cause a false positive result unless denatured by heat treatment before testing. Materials such as blood, serum, and plasma should be treated to inactivate inhibitors prior to testing (11).

Expected Values

Endotoxin can be quantified if the concentration is greater than or equal to the Pyrotell® sensitivity. Materials derived from biological sources, even after biochemical purification, may contain measurable levels of endotoxin. Water obtained by distillation, reverse osmosis, or ultrafiltration may contain less endotoxin than detectable as long as the purification process is operating correctly, and the water is not contaminated after production.

Specific Performance Characteristics

The error of the gel-clot method is plus or minus a twofold dilution at the endpoint of the assay.

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Our experienced staff will be pleased to discuss the practical and theoretical aspects of the LAL test. Please call if you have problems using Pyrotell®. We will replace any of our products that do not perform to product specifications; you must notify us before returning product.