

Limulus Amebocyte Lysate

PYROTELL® MTV

Manufactured by: **ASSOCIATES OF CAPE COD INCORPORATED**
Specialists in Endotoxin and Glucan Detection

Telephone: (508) 540-3444
Toll-Free: (888) 395-2221
Fax: (508) 540-8680
Technical Support: (800) 848-3248
Customer Service: (800) 525-8378

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Pyrotell® Multitest 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 0.1 mL reconstituted Pyrotell to 0.1 mL of the test specimen in a 10 x 75 mm depyrogenated, soda lime glass, reaction tube. The reaction solution is mixed thoroughly and placed immediately in a dry block incubator or noncirculating water bath at 37 ± 1°C for 60 ± 2 minutes. At the end of the incubation period, the tube is removed from the incubator and inverted. If a gel has formed and remains intact in the bottom of the reaction 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 reaction 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 (3). In the 1950's, Bang at the Marine Biological Laboratory, Woods Hole, MA, discovered that gram negative bacteria cause *Limulus* blood to clot (4). Levin and Bang later determined that the reaction is enzymatic and that the enzymes are located in granules in the amebocytes (5). They showed that clotting is initiated by a unique structural component of the bacterial cell wall called endotoxin or lipopolysaccharide (6). 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 its applications is available in the literature (7, 8, 9).

Reagent

Pyrotell *Limulus* Amebocyte Lysate (LAL) is packaged in lyophilized form in 2, and 5 mL/vial fill sizes.

Associates of Cape Cod, Inc. offers individual lots of Pyrotell in sensitivities ranging from 0.03 to 0.5 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 vial and package labels. Specify the sensitivity desired when placing an order.

Use Pyrotell for in vitro diagnostic 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.

Reconstitute Pyrotell as follows:

1. Gently tap the vial of Pyrotell to cause loose LAL 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. Do not inject through or reuse the stopper. A small amount of LAL left on the stopper will not affect the test. Cover the vial with Parafilm “M”® (American National Can™) when not in use.
2. Reconstitute Pyrotell with LAL Reagent Water (LRW, see “Test Reagents”) or compatible buffer (Associates of Cape Cod, Inc.). Add 2.0 or 5.0 mL as indicated on the vial label. The lyophilized LAL pellet will go into solution within a few minutes. Before use, gently mix the contents of the vial to ensure homogeneity. Mixing too vigorously may cause excessive foaming which can cause a loss of sensitivity.

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.

Reconstituted Pyrotell is usually clear and slightly opalescent. An occasional 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.

Reconstituted Pyrotell is less stable than the freeze-dried product; vials can be held for up to 24 hours at 2 to 8°C. Reconstituted Pyrotell can be frozen once. The product will retain activity for three months if frozen immediately after reconstitution and held at or below -20°C. After thawing, the same visual criteria for quality are applied as for initial reconstitution.

Specimen Collection and Preparation

Specimens should be collected aseptically in non-pyrogenic containers. Reused, depyrogenated glassware or sterile, disposable, polystyrene plastics 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 LRW (room temperature for one hour)

and the rinse tested as a specimen to determine whether or not the batch is acceptable.

The pH of the reaction mixture (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 (See #3 below). 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 multitest* vial (see description and method of reconstitution in section above).
2. *LRW*, not provided with Pyrotell; order separately. Lyophilized Pyrotell must be reconstituted with water that shows no detectable endotoxin in the LAL test. 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 when tested with a more sensitive Pyrotell. To certify a new lot of water as an LRW, reconstitute Pyrotell and make dilutions of standard endotoxin with the new lot of water to confirm the sensitivity of the Pyrotell. If the test 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 Pyrotell and endotoxin standards and to dilute endotoxin standards and test specimens.
3. *Buffer*, not provided with Pyrotell; order separately if required. Pyrosol buffer (Cat# BR051 or BC554) or Glucashield™ buffer (Cat# GB051) can be used instead of LRW to reconstitute Pyrotell to help overcome a sample pH problem or interference from glucans.
4. *Standard Endotoxin*, not provided with Pyrotell; order separately. Control Standard Endotoxin (CSE), 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. *Reaction tubes*, 10 x 75 mm, depyrogenated, soda lime glass (Cat# TS050). Some brands exhibit inhibitory

properties with certain lots of Pyrotell. Depyrogenated tubes are available from Associates of Cape Cod, Inc.

2. *Noncirculating water bath* or dry block incubator (Cat# TH120) capable of maintaining 37±1°C.
3. *Test tube racks* to hold and/or incubate reaction tubes.
4. *Pipets*, automatic pipettors with pipet tips, or repeating pipettors with plastic syringe barrels. Sterile, disposables are recommended.
5. *Vortex-type mixer*.
6. *Parafilm “M”*®. The side in contact with the paper backing is normally nonpyrogenic.
7. *Nonpyrogenic test tubes* with adequate capacity for making dilutions of endotoxin standard or test specimen. See “Specimen Collection and Preparation” for other containers suitable for dilutions.
8. *Hot air oven* with 250°C capacity for depyrogenation of glassware. Commonly used minimum time and temperature settings are 30 minutes at 250°C (2, 11).

Controls

Controls are necessary to ensure a valid test. Recommended procedures are detailed by the FDA (1) and USP (2).

1. Endotoxin controls

- a. **Endotoxin standard series.** Prepare a fresh set of appropriate serial dilutions from the stock endotoxin solution each day and vortex each dilution for not less than 30 seconds. Make dilutions such that a final series of twofold dilutions will bracket the sensitivity (λ) of the Pyrotell. Concentrations of 2 λ , λ , 0.5 λ , and 0.25 λ 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 in the absence of a series of standard concentrations in certain circumstances. Refer to the FDA guideline (1) under “Routine Testing of Drugs by the LAL Test” for details. The positive control concentration should be 2 λ .
- 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 2 λ .

2. Negative controls

LRW negative control(s) should be included with each batch of specimens tested. During product validation or inhibition/enhancement testing (1, 2), the specimen used to dilute standard endotoxin is also treated as a negative control.

Specimen Preparation for Limits Test or Assay

Either dilute the specimen to the required concentration to perform a limits (pass/fail) test or perform an assay by testing a series of concentrations (examples of the two types of tests are given in “Results and Interpretation”). Dilutions may be made in test tubes and the test volume transferred to the reaction tubes, or dilutions may be made directly in the reaction tubes to leave the test volume, 0.1 mL, in each tube. The dilution tested for a limits test is determined from the sensitivity of the Pyrotell and the endotoxin limit for the specimen. Refer to “Limitations of Procedure” or to the FDA guideline (1) for explanation and calculation of Minimum Valid Concentration (MVC) and Maximum Valid Dilution (MVD).

Performing the Test

Consistent technique is necessary to obtain satisfactory results.

1. Add 0.1 mL of reconstituted Pyrotell to each reaction tube containing 0.1 mL test specimen or control. Use a graduated (0.1 mL increments) pipet, or an automatic or repeating pipetter. Add Pyrotell to the negative control(s) first and from the lowest to highest concentration in each test series where carryover may be a problem. A fresh pipet or pipet tip is recommended for each entry into the Pyrotell vial. 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 LAL is added to the test specimen 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 reaction tubes 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. Do not wipe the tubes dry or bump them against the side of the rack during removal. Invert the tube in one smooth motion; do not pause half way 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, 2) that bracket the labeled sensitivity (i.e., 2λ, λ, 0.5λ, and 0.25λ). 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 has deteriorated, the endotoxin lost potency (often because of adsorption to container surface), or the test has not been 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 achieve 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 Specimen 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 interfering with (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, 2) at a specimen concentration that is greater than the minimum valid concentration (MVC), then the LAL test cannot be substituted for the USP Pyrogen Test. The MVC is calculated as follows:

$$MVC = \frac{(\lambda) (\text{specimen dose})}{(\text{endotoxin tolerance limit})}$$

where λ is in EU/mL, specimen dose is in units per kg body weight, and the endotoxin tolerance limit is in EU/kg.

The maximum valid dilution (MVD) is the specimen dilution containing the MVC (1). It is the initial specimen concentration divided by the MVC.

The endotoxin tolerance limit (1) is 0.2 EU/kg for drugs with an intrathecal route of administration and 5 EU/kg for all other parenterals. The limit for medical devices is expressed per mL of an extraction or rinse volume obtained as described in the FDA guideline (1). For devices that contact cerebrospinal fluid, the limit is 0.06 EU/mL; for those that do not, it is 0.5 EU/mL. The limit for liquid devices is the same as that for drugs.

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 (12).

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.