

Limulus Amebocyte Lysate

For reference use only. Please refer to insert provided with the product when performing the assay.

PYROTELL-T®

for the Detection and Quantitation of Gram Negative Bacterial Endotoxins (Lipopolysaccharides)

The *Limulus* amebocyte lysate (LAL) test, when used according to U.S. Food and Drug Administration (FDA) guidelines (1, 2), 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 quantification of endotoxin in raw materials used in production, including water, and for in-process monitoring of endotoxin levels. The USP Bacterial Endotoxins Test (3) is the official test referenced in specific USP monographs.

SUMMARY OF TEST

Limulus amebocyte lysate (LAL) is an aqueous extract of blood cells (amebocytes) from the horseshoe crab, *Limulus polyphemus*. In the presence of endotoxin LAL becomes turbid and, under appropriate conditions, forms a solid gel-clot. The test is performed by adding a given volume of Pyrotell-T to a given volume of specimen and incubating the reaction mixture at 37° C. The higher the endotoxin concentration in the specimen, the faster turbidity will develop. Turbidity can be used to quantify endotoxin concentration in two ways.

In the kinetic turbidimetric LAL method, either the rate of increase in turbidity or the time taken to reach a particular level of turbidity (the onset time) is determined. Higher endotoxin concentrations give shorter onset times. The assay requires specialized instrumentation to incubate multiple samples at a controlled temperature (usually 37° C) and take optical density readings at regular intervals. Standard curves may be constructed by plotting the log onset time against the log concentration of standard endotoxin and are used to calculate endotoxin concentrations in specimens.

Alternatively, in the endpoint turbidimetric method, turbidity can be measured following a fixed incubation period. A standard curve, consisting of measured optical density plotted against known standard endotoxin concentration, is used to determine concentrations in specimens.

The turbidimetric LAL method is rapid, specific, easy to perform, and highly sensitive. The detection limit depends on the method and instrumentation used and may be as low as 0.001 Endotoxin Units (EU) per ml.

HISTORY AND BIOLOGIC PRINCIPLE

Howell described the clotting of *Limulus* blood in 1885 (4). In the 1950's, Bang at the Marine Biological Laboratory, Woods Hole, MA, discovered that gram negative bacteria cause *Limulus* blood to clot (5). Levin and Bang later determined that the reaction is enzymatic and that the enzymes are located in granules in the amebocytes (6). They showed that clotting is initiated by a unique structural component of the bacterial cell wall called endotoxin or lipopolysaccharide (7). Current understanding is that the reaction consists of 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 and the turbidity of the reaction mixture increases. The greater the amount of endotoxin present, the faster turbidity develops. More information about the LAL reaction and applications is available in the literature (8, 9, 10).

REAGENT

Pyrotell-T is packaged in lyophilized form in a 5 ml/vial fill size. Pyrotell-T contains an aqueous extract of amebocytes of *L. polyphemus*, human serum albumin (stabilizer), NaCl and other appropriate ions. No preservatives or buffers are added.

Pyrotell-T is not labelled with a specific sensitivity. Sensitivity in a given test (designated λ) is the lowest endotoxin concentration used to construct the standard curve. In the LAL-5000 Automatic Endotoxin Detection System (Associates of Cape Cod, Inc.) the detection limit, and thus the lowest possible value of λ , is 0.001 EU/ml.

Use Pyrotell-T 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-T.

Reconstitute Pyrotell-T as follows:

1. Gently tap the vial of Pyrotell-T to cause loose LAL to fall to the bottom. 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 on the stopper will not affect the test.
2. Reconstitute Pyrotell-T with LAL Reagent Water (LRW, see below) or Pyrosol® buffer (Associates of Cape Cod, Inc.). Do not rehydrate until immediately before use. Add 5 ml LRW as indicated on the vial label. The LAL pellet will go into solution within a few minutes. Before use, gently shake the vial to insure homogeneity. Mixing too vigorously may cause excessive foaming which can cause a loss of sensitivity. Cover the vial with Parafilm M® (American National Can™) when not in use.

STORAGE CONDITIONS

Freeze-dried Pyrotell-T is relatively heat stable and, if kept refrigerated, will retain full activity through the expiration date on the label. 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-T. Temperatures in excess of 37° C can cause rapid deterioration of lyophilized Pyrotell-T as evidenced by loss of sensitivity and a distinct yellowing of the product. Pyrotell-T is shipped with cold packs in insulated containers to protect against high temperatures.

Reconstituted Pyrotell-T 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-T is less stable than the freeze-dried product; vials can be held for up to 24 hours at 2 to 8° C. Reconstituted Pyrotell-T can be frozen once. It 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. Labware can be tested for acceptability by randomly selecting containers from a batch, rinsing them with a small volume of LRW at room temperature for one hour, and testing the rinse as a specimen. The rinse should contain significantly less endotoxin than the lowest standard concentration to be used. Also, the rinse should neither inhibit nor enhance the test as determined by recovery of a known amount of added endotoxin.

The pH of the reaction mixture (4 volumes of specimen or specimen dilution mixed with 1 volume of Pyrotell-T or other ratio used in the test protocol) should be 6 to 8. Adjust the pH of the specimen with HCl, NaOH, or buffer (free of detectable endotoxin). Dilute concentrated HCl or NaOH with LRW and use in normalities that will not lead to significant dilution of the test specimen. If a precipitate forms in the sample upon pH adjustment, dilute the sample (not to exceed the MVD — see “Limitations of Procedure”) before adjusting the pH. Alternatively, reconstitute the Pyrotell-T with Pyrosol buffer and check the pH of the reaction mixture. Do not adjust the pH of unbuffered saline or water. Note that dilution alone may overcome pH problems.

Substances that denature proteins, chelate ions, 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 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 non-sterile specimens 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-T (see description and method of reconstitution above).
2. LAL Reagent Water (LRW), not provided with Pyrotell-T; order separately. Lyophilized Pyrotell-T must be reconstituted with water (or buffer, see item 3 below on Pyrosol buffer) that shows no detectable endotoxin in the LAL test. Recommended sources for water include Associates of Cape Cod, Inc. or any commercially available USP Sterile Water for Injection (sterile WFI) without bacteriostat or USP Water for Irrigation. Any of these may be used provided they have been shown to be acceptable for use as an LRW. The endotoxin limit for USP sterile WFI is only 0.25 EU/ml; therefore, sterile WFI may contain detectable endotoxin and be unsuitable for use.

To certify that water is acceptable as an LRW, test it as a specimen with a positive product control (see item 1.c. in the section on “Controls”). Use certified LRW to reconstitute Pyrotell-T, to make dilutions of standards, and to prepare positive controls (see items 1.a. and 1.b. under “Controls”). Construct a standard curve from the onset times for the standards. The correlation coefficient should be at least 0.980 (absolute value). The endotoxin concentration of the water being tested can be estimated by extrapolation of the standard curve below the lowest endotoxin concentration and should be significantly less than that of the lowest standard. Also, the endotoxin concentration of the positive product control should be within 50% to 200% of the nominal concentration of the added endotoxin “spike.”

3. Pyrosol buffer, not provided with Pyrotell-T; order separately if required. Pyrosol buffer can be used instead of LRW to reconstitute Pyrotell-T to help overcome a sample pH problem or interference.
4. Standard Endotoxin, not provided with Pyrotell-T; order separately. Control Standard Endotoxin (CSE), obtained from Associates of Cape Cod, Inc., is used to construct standard curves, 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 manufacturer's directions for reconstitution and storage of standard endotoxins. CSE lots may show different potencies (EU/ng) when tested with different lots of Pyrotell-T. If using CSE, endotoxin concentrations can be expressed in EU/ml if the potency of a given lot of CSE has been determined with the Pyrotell-T lot in question (1, 2).

MATERIALS AND EQUIPMENT (NOT PROVIDED)

1. Reaction vessels. Choice of type depends on the instrumentation used to measure turbidity. Reaction tubes for the LAL-5000 system are 10 x 75 mm, depyrogenated, borosilicate glass culture tubes. These are available from Associates of Cape Cod, Inc. Some plastics interfere with the LAL reaction or adsorb endotoxin. Thus, microtiter plates should be checked for suitability before use.
2. Optical reader. For the kinetic turbidimetric method, use an incubating optical reader such as the LAL-5000 Automatic Endotoxin Detection System, Associates of Cape Cod, Inc. For the endpoint method use a spectrophotometer capable of reading at less than 450 nm with appropriate cuvettes or use a plate reader and microtiter plates.
3. Non-circulating water bath or dry block incubator capable of maintaining $37 \pm 1^\circ \text{C}$ is required for the end-point method only.
4. Test tube racks to hold and/or incubate reaction tubes.
5. Pipets, automatic pipettors with pipet tips, or repeating pipettors with plastic syringe barrels. Sterile, disposable pipets and tips are recommended.
6. Vortex-type mixer.
7. Parafilm M®. The side in contact with the paper backing is normally nonpyrogenic.
8. Nonpyrogenic test tubes with adequate capacity for making dilutions of endotoxin standard or test specimen (18 x 150 mm glass test tubes with stainless steel Morton closures are reusable and convenient). See “Specimen Collection and Preparation” for other containers suitable for dilutions.
9. Hot air oven with 180° or 250°C capacity for depyrogenation of glassware. Recommended depyrogenation cycles are a minimum of 180°C for 3 hours (11) or 250°C for 30 minutes (3, 12).

CONTROLS

Controls are necessary to insure a valid test. Recommended procedures are detailed by the FDA (1, 2) and USP (3).

1. Endotoxin controls

- a. Endotoxin standard series.** Prepare a fresh set of dilutions from the stock endotoxin solution for each test. Do not use previously prepared dilutions unless you have demonstrated the stability of that range of concentrations. Make dilutions in a geometric series (e.g. twofold, fourfold or tenfold dilutions) to give the range of endotoxin concentrations required. Concentrations of 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 EU/ml are recommended to verify Pyrotell-T performance. The lowest endotoxin concentration in any standard series is the detection limit of that particular test and is designated λ . To get to the range of standards required, use as few dilutions as possible with appropriate pipet volumes to maximize accuracy.
- b. Positive control** (a single standard endotoxin concentration) should be included if the standard series (see a. above) is not prepared in the same way as the positive product controls (see c.). The endotoxin concentration of the positive control should equal that of a standard from the middle of the standard curve. A 4λ concentration is appropriate for standard curves constructed from 4, 5 or 6 twofold dilutions of standard endotoxin (e.g., $4\lambda = 0.125$ EU/ml in the standard series given in section a. above). For standard concentrations of 0.001, 0.01, 0.1, 1.0 and 10 EU/ml, an appropriate concentration for positive controls is 0.1 EU/ml. If a standard series is not included in a test, a positive control must be included to verify that it is appropriate to use the parameters of a previous standard curve to calculate endotoxin concentrations. Refer to the FDA guideline (1) and Interim Guidance (2) under Routine Testing of Drugs (or Devices) by the LAL Test for details.
- c. Positive product controls** are inhibition/enhancement controls and consist of the specimen or dilution of specimen to which standard endotoxin is added. The concentration of added endotoxin in the test specimen should be the same as that of the positive control. See section b. above for selection of the appropriate endotoxin concentration for the positive product control. The added endotoxin is frequently referred to as a "spike."

2. Negative controls

LRW negative controls should be included with each test.

Specimen Preparation - Determination of Test Dilution

If a test protocol has been developed previously for the type of specimen under test, make the necessary dilution for the assay and proceed as directed under "Performing the Test" below. If a protocol has not been developed for the specimen type, make a series of tenfold dilutions of the specimen. Do not exceed the Maximum Valid Dilution (MVD) of the product by more than a factor of 10. [Refer to the "Limitations of Procedure" below or the FDA Guideline (1) for explanation and calculation of MVD and Minimum Valid Concentration (MVC)].

Prepare appropriate dilutions of all specimens with a positive product control for each one.

Performing the Test

Consistent technique is necessary to obtain satisfactory results. All controls and specimens should be tested in (at least) duplicate.

1. Prepare test instrumentation as necessary. In an automated system this usually involves entering sample descriptors and setting test parameters.
2. Add the appropriate volume (0.4 ml for the LAL-5000 system) of sample (negative control, endotoxin standard, specimen or positive product control) to the reaction vessel or microtiter plate.
3. Add Pyrotell-T as appropriate for procedure.
 - a. For methods using individual reaction tubes or cuvettes the timing of the reaction in each tube is critical. To each reaction vessel in turn, add Pyrotell-T, mix for approximately 2 seconds and place the vessel into the incubating optical reader (for the kinetic method) or the incubator bath (for an endpoint method). The volume of Pyrotell-T added is 0.1 ml for the LAL-5000 system and most other methods. Failure to mix adequately is a common cause of unsatisfactory tests. Pyrotell-T may be added most conveniently using a repeating pipetter. A fresh pipet or pipet tip is recommended for each entry into the Pyrotell-T vial. Different volumes of Pyrotell-T may be appropriate for other test protocols.

- b. For a microtiter plate, add Pyrotell-T as rapidly as possible to all samples and mix on a plate shaker for 30 seconds. Either put the plate on an incubator block (for an endpoint test) or into an incubating plate reader (for a kinetic test).
4. Once the incubation has started, do not disturb the reaction vessel(s). The laboratory bench carrying the incubator/optical reader should be free from excessive vibration.
5. Read the test.
 - a. For the kinetic method, allow the test to run until all samples have incubated for significantly longer than the time required for the lowest standard endotoxin concentration to become turbid. Automated test systems will usually terminate the test after a preset period.
 - b. For the endpoint method, after a precisely timed incubation, remove the reaction tubes or plate from the incubator, mix if necessary, and read the optical density (OD) in a spectrophotometer or plate reader (as appropriate) at 360 nm (405 nm is also satisfactory). Note: The test should be set up and read so that the incubation time for each sample is the same (within ± 30 seconds). The incubation period depends on the endotoxin concentration range desired and is likely to vary with different lots of Pyrotell-T. Preliminary tests may be necessary to determine the correct incubation period. Instrument manufacturers may recommend appropriate incubation periods. Contact Associates of Cape Cod, Inc. for a copy of a suggested protocol.

RESULTS

1. Preliminary calculations.

- a. For the kinetic method, determine the time taken for specimens to reach a particular optical density threshold (usually 0.02 OD units) after any data corrections have been made. Optical density readings must be relative to an initial reading taken to be 0 OD units. The system software will do this in the LAL-5000 system. The time taken to reach the OD value is called the onset time.
- b. For the endpoint method, subtract the (mean) optical density of the negative control from that of all other readings.

2. Construct a standard curve.

- a. For the kinetic method, construct a standard curve by regression of the log onset time on the log endotoxin concentration for the standards. (This is performed by the software in the LAL-5000 system). The equation for the regression line describes the standard curve.
- b. For the endpoint method, construct a standard curve by plotting optical density readings against standard endotoxin concentrations.

3. Calculate the endotoxin concentrations.

Calculate endotoxin concentrations of all specimens (including standard controls) using the line equation for a straight line:

$$Y = aX + b$$

where:

- Y = log onset time (kinetic method), or optical density (endpoint method);
- X = log endotoxin concentration (kinetic method), or endotoxin concentration (endpoint method);
- a = slope of the line
- b = the Y intercept.

This calculation is performed by the software in the LAL-5000 system.

INTERPRETATION

1. In order for a test to be valid, the endotoxin concentration of negative controls (estimated by extrapolation of the standard curve) should be significantly less than that of the lowest standard concentration.
2. When a standard curve is included with the test, the absolute value of the correlation coefficient for the standard curve should be greater than 0.980.
3. The mean measured endotoxin concentration of positive controls must be within 25% of the nominal

concentration. Thus, if the positive control is 0.125 EU/ml, the measured concentration must be between 0.09375 and 0.15625 EU/ml.

4. For the kinetic method, determine the mean range of onset times for the standard curve. For example, if the onset times of two replicates of the highest standard endotoxin concentration are 1079 and 1087 seconds and those for the lowest concentration are 1954 and 1968, the mean range of onset times is 1083 to 1961. Valid endotoxin concentrations can only be calculated for specimens with a mean onset time that falls within the mean range of onset times of the standard curve.

For example, a valid result can be given for an unknown that gives onset times of 1949 and 1965 (mean = 1957), despite the fact that one of the replicates lies outside the standard range.

For the endpoint method, valid endotoxin concentrations can only be calculated from OD values that lie on the linear portion of the standard curve.

5. In order to demonstrate that the specimen does not significantly interfere with the LAL/endotoxin reaction, the measured endotoxin concentration of the positive product control must be within 50 to 200% of the nominal concentration of the added endotoxin 'spike.' Before determining whether the spike is recovered within these limits, subtract the endotoxin concentration measured in the (unspiked) specimen. For example, in order to be considered free of significant interference, the measured endotoxin concentration in a 0.125 EU/mL positive product control (after subtraction of any endotoxin in the unspiked specimen) must be within the range 0.0625 - 0.25 EU/mL (50 to 200% of 0.125 EU/mL). If the measured endotoxin concentration in the unspiked specimen is 0.028 EU/mL and that in the positive product control is 0.163 EU/mL, the endotoxin attributable to the spike is $0.163 - 0.028 = 0.135$ EU/mL. This is within the range and subject to other requirements being met; the test of the sample is valid.

LIMITATIONS OF PROCEDURE

The procedure is limited by the extent of the inhibition or enhancement capacity of the specimen under test. If the procedure cannot be validated (1, 2, 3) 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:

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

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, albumin, and plasma may interfere with turbidimetric assays.

EXPECTED VALUES

Endotoxin in specimens can be quantified between the range of standard endotoxin concentrations used to construct the standard curve. If it is necessary to dilute the specimen to overcome any inhibition or enhancement, the least amount of endotoxin that can be detected will be increased accordingly. Materials derived from biological sources, even after biochemical purification, may still 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.

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