Limulus Amebocyte Lysate (LAL)

Rapid Endotoxin Detection

Pyrosate®

ASSOCIATES OF CAPE COD, INC.

Instructions for Use

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1. Intended Use

Pyrosate is intended for the *in vitro* detection and quantitation of endotoxin in end-product testing of human and animal injectable drugs (including biological products) and medical devices. It may also be used for testing raw materials and components, including water, and for in-process monitoring of endotoxin levels for such products. It can be used in other applications for detection of endotoxins.

Pyrosate is not intended for detection of endotoxin in clinical samples or for diagnosis of disease in humans or animals.

The United States Pharmacopeia (USP) Bacterial Endotoxins Test (BET) (1) is the official test referenced in specific USP monographs. This document describes four test methods for using Pyrosate. Two of these (Limit Test, method 1.1, and Quantitative Test, method 2) are fully consistent with the USP BET. **IMPORTANT:** Be sure to use the test method that is appropriate to meet regulatory requirements and your needs.

2. Summary and Explanation of the Test

History

In the 1950's, Bang discovered that gram negative bacteria cause *Limulus* blood to clot (2). Levin and Bang laid the foundation for endotoxin testing with LAL by demonstrating that the reaction is enzymatic, that the enzymes are located in granules in the amebocytes and that clotting is initiated by endotoxin (or lipopolysaccharide) (3, 4, 5).

Summary of the test

Limulus amebocyte lysate is an aqueous extract of blood cells (amebocytes) from the horseshoe crab, *Limulus polyphemus*. The test is performed by adding 0.5 mL of the test specimen to a single test vial (STV) of Pyrosate. After mixing to dissolve the reagent, 0.25 mL is removed from the vial and either discarded or, if PPC vials are being used, added to a PPC vial. The vials are then incubated at 37 ± 1°C for the time specified on the SPL and PPC package labels and the certificate of compliance. At the end of the incubation period, the vials are removed and inverted in one smooth motion. If a gel has formed and remains intact in the bottom of the vial after inversion, the test is positive; the concentration of endotoxin in the SPL vial is greater than or equal to the stated sensitivity of the Pyrosate (provided that the test is valid). Any other state of the mixture constitutes a negative test and indicates an endotoxin concentration less than the stated sensitivity.

Merits and limitations

A test with Pyrosate is rapid, easy to perform and specific for endotoxin. Pyrosate is available in sensitivities down to 0.03 Endotoxin Units (EU) per mL. Lower concentrations of endotoxin cannot be detected with Pyrosate. A limit test with Pyrosate gives a binary (positive or negative) result. The result depends on whether or not the specimen contains endotoxin at a concentration of at least the stated sensitivity of the reagent.

To perform a quantitative test for endotoxin concentrations equal to or greater than the stated sensitivity a series of dilutions of the specimen are tested and an endpoint (the greatest dilution to clot) is determined. The error of the test is plus or minus a twofold dilution (i.e. +/- a factor of two).

The gel that forms as a result of an LAL reaction is delicate and may be irreversibly broken if the vials are disturbed during incubation.

Certain substances interfere with the test and may reduce (or increase) the sensitivity of Pyrosate or prevent the detection of endotoxins. Appropriate interference controls are required. The test for interfering factors USP described in the BET chapter (1) should be performed to demonstrate that a particular type of specimen does not significantly interfere with the test.

3. Biological Principles of the Procedure

Endotoxin initiates an enzyme cascade culminating in activation of clotting enzyme. Clotting protein (coagulogen) is then cleaved by activated clotting enzyme and the

resulting insoluble protein (coagulin) coalesces by ionic interaction to form the gel-clot (6). In native LAL, clotting can also be initiated by (1→3)-β-D-glucan via an alternate (shorter) pathway. However, in Pyrosate this activation is inhibited by a glucan blocking component to render the reagent endotoxin specific for glucan concentrations up to 100 ng/mL.

4. Reagents

Pyrosate LAL Reagent (Blue cap, vial marked SPL XXX, where XXX is the component lot number): contains 0.5 mL lyophilized LAL reagent, which consists of only an aqueous extract of amebocytes of *L. polyphemus*, salts, glucan blocker and buffer. No preservatives or other ingredients have been added. The sensitivity of Pyrosate (which is designated λ) is determined using USP Endotoxin RS and is expressed in Endotoxin Units (EU)/mL.

Pyrosate Positive Product Control – PPC (Red cap, vial marked PPC YYY, where YYY is the component lot number): contains control standard endotoxin (extracted from *E. coli* O113:H10) to give a concentration at double the labeled sensitivity (2λ) of the accompanying LAL reagent (SPL vials – see above) in the kit when used as instructed. The endotoxin in the PPC is formulated with fillers.

Allergic reaction to LAL has been reported (7), but the toxicity of this reagent has not been determined. Exercise caution when handling Pyrosate to minimize hand contact with or inhalation of the reagents. The endotoxin in the PPC vial is a pyrogen and should not be injected into humans or animals. Pyrosate is a single use product. Do not attempt to reuse reagents.

Pyrosate may be disposed of in normal laboratory waste after use unless the specimen itself poses particular hazards, in which case dispose of Pyrosate vials as appropriate.

Reconstitution of Reagents

Gently tap the vial to cause loose reagent to fall to the bottom. Remove the crimp seal and aseptically remove and discard the gray stopper, taking care not to contaminate the mouth of the vial. Do not inject through or reuse the stopper. A small amount of reagent powder left on the stopper will not affect the test.

Reconstitute Pyrosate LAL Reagent (Blue cap, marked SPL) with 0.5 mL of the test specimen during the test procedure as described under "Performing the Test" below. The lyophilized LAL pellet will go into solution within about one minute of addition of the test specimen. Thoroughly mix (shaking the vials by hand) the contents of the vial to ensure homogeneity. Do not invert or tip vials past 45° while mixing. Multiple vials may be conveniently shaken together in a test tube rack. Use a pipette to remove 0.25 mL of the specimen/reagent mixture from the vial.

If PPC vials are used, add this 0.25 mL specimen/reagent mixture to a PPC vial to reconstitute it (see "Performing the Test" below). If standard endotoxin is used instead of PPC vials, discard the 0.25 mL volume of specimen/reagent mixture.

Storage Conditions

Freeze-dried Pyrosate reagents are relatively heat stable. Pyrosate is stored at 2 to 25 °C (see USP requirements for Controlled Room Temperature in chapter <659>, Packaging and Storage Requirements (8)). When stored under these conditions the product will retain full activity through the expiration date on the package labels.

Reconstituted Pyrosate LAL Reagent and PPC are used immediately after reconstitution and must not be stored for later use.

Indications of Instability or Deterioration

The pellet of lyophilized Pyrosate is white. A distinct yellow or a shriveled, wet looking pellet indicates deterioration. If the white pellet breaks into a powder during shipping, this will not affect the performance of the reagent. Reconstituted Pyrosate is usually colorless and slightly opalescent. The product lot may exhibit a slight, uniform turbidity; this is normal. Flocculent precipitation indicates deterioration or contamination.

5. Specimen Collection and Preparation for Analysis

Specimens (samples) should be collected aseptically in containers that are free of detectable endotoxin and of interfering substances. Containers should not adsorb endotoxin. Collect specimens in depyrogenated glassware or sterile, disposable, plastics that have been shown to be suitable. Containers (selected randomly from a batch) may be rinsed with a small volume of LAL Reagent Water (room temperature for one hour) and the LAL Reagent water tested as a specimen to determine if the batch is suitable for use. Test tubes and pipettes used for dilutions of specimens or standard endotoxin should meet the same requirements.

The pH of the reaction mixture (specimen added to Pyrosate) should be in the range of 6.9 – 7.9 ± 0.1 pH units at 25°C. Note that even though the pH of the reaction mixture may be out of range for the undiluted specimen, it may be in range at a dilution within the maximum valid dilution (MVD; see explanation of MVD under "Limitations of Procedure" below). If necessary, adjust the pH of the specimen with buffer (e.g. Pyrosol) or an appropriate concentration of hydrochloric acid or sodium hydroxide (free of detectable endotoxin). It should not be necessary to adjust the pH of unbuffered water or saline.

Substances that denature proteins, chelate cations, bind endotoxin or alter the aggregation state of endotoxin can interfere with the test. Interference can 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" below.

Specimens should be tested as soon as possible after collection. If specimens are to be stored, the container and storage procedure (time and temperature) should be validated to assure that endotoxin is not lost during storage.

6. Test Procedure and Interpretation of Results

Materials Provided

- Pyrosate LAL Reagent (Blue cap, SPL).

- Pyrosate Positive Product Control (Red cap, PPC). Note: PPC vials are specific to a particular kit and must not be used with Pyrosate LAL (SPL) vials for another kit lot number.

- Disposable pipettes (single use only) - only provided with Pyrosate 10 packs (kit catalog numbers with the suffix -10). These pipettes are available separately (catalog number PPT50).

Materials Not Provided

Reagents

- USP Endotoxin Reference Standard (RS) - if required. Available from U.S. Pharmacopeial Convention, Inc., 12601 Twinbrook Parkway, Rockville, Maryland 20852-1790, USA. Website: www.usp.org. Follow the manufacturers' directions for reconstitution and storage of standard endotoxins.

- LAL Reagent Water (LRW) (water for Bacterial Endotoxins Test) - if dilutions of specimens or standard endotoxin are required. Pyroclear® LRW is available from Associates of Cape Cod, Inc., (ACC) in a range of fill sizes and containers, catalog numbers: W0051 (5.5 mL), W020P (pack of 10 bottles each containing 20 mL), WP050C (pack of 30 plastic bottles, each containing 50 mL). See the ACC catalog for additional options.

Equipment and Other Materials

- Dry bath incubator or non-circulating water bath at 37 ± 1°C with a thermometer.
- Timer.
- Specimen containers (see "Specimen Collection and Preparation for Analysis" above).

- Pipettes or pipetters with pipette tips capable of pipetting at least 0.5 and 0.25 mL (if using Pyrosate bulk packs, catalog number suffix -30; disposable pipettes are provided with Pyrosate 10 pack kits, catalog number suffix -10). Pipettes and tips should be free of detectable endotoxin and of interfering substances. The disposable pipettes (catalog number PPT50) and Pyroclear® Pipette tips (catalog numbers: PPT25: up to 250 µL; PPT10: up to 1000 µL) are available from ACC. Do not attempt to reuse disposable pipettes and tips.

- Pipettes of appropriate volumes (typically 5 and/or 10 mL glass serological pipettes) for preparing dilutions of standard endotoxin and specimens (if required).

- Vortex-type mixer - if dilutions of endotoxin standard or test specimen are required.

- Dilution tubes – if required. Tubes should have adequate capacity for making dilutions of endotoxin standard or test specimen. See "Specimen Collection and Preparation for Analysis" for other containers suitable for dilutions. Pyrotubes® (e.g. catalog number: TB013) are available from ACC.

- Test tube rack.

Specimen Preparation
For a limit test (a pass/fail test at a particular endotoxin concentration), dilute the specimen as required to test at a specific limit. The dilution factor is determined by dividing the desired pass/fail concentration (expressed in EU/mL) by the labeled lysate sensitivity (λ). The pass/fail concentration may be an alert limit, action limit or the endotoxin limit for the product being tested. For example, for a limit test at a concentration of 0.5 EU/mL using a reagent with a sensitivity of 0.125 EU/mL, the specimen must be diluted by a factor of 4. For a limit test at 0.125 EU/mL, the specimen is tested without dilution using a reagent of this sensitivity.

To perform a quantitative test, a series of dilutions of the specimen (twofold dilutions are commonly used) is tested. Prepare dilutions in test tubes (see "Equipment and Other Materials" above) to give sufficient volumes for the number of replicate tests required at each dilution. Examples of appropriate volumes are given in Quick Guide 3 for a quantitative test.

Performing the Test
Perform testing in a clean laboratory environment. It is generally not necessary to use a laminar flow hood but do not perform the test under an air vent or in a dusty or dirty location.

Note: Prior to performing the limit test or the quantitative test described below, it is recommended that qualification of the test method be performed as described under Preparatory Testing in the Gel-clot Technique section of the USP BET chapter(1) (or the equivalent chapter in the European or Japanese pharmacopoeia). This specifies use of the USP endotoxin reference standard. All testing should be performed by appropriately trained and qualified personnel.

1. Limit Test

1.1 Procedure for a Limit Test using USP Endotoxin RS per the USP BET (1)

For instructions with illustrations, see Quick Guide 1.

1. Dilute reconstituted USP Endotoxin RS with LRW to a concentration suitable for preparing a positive product control (PPC), e.g. forty times the labeled lysate sensitivity (40λ).

2. From this (e.g. 40λ) prepare a concentration of double the labeled lysate sensitivity (2λ) in a dilution tube for positive controls leaving a sufficient volume of the higher concentration (such as 40λ) to prepare PPCs for each of the specimens (step 3 below).

3. For each specimen to be tested prepare a PPC containing a small volume of added standard endotoxin giving a final concentration of 2λ. For a test performed per the USP BET, use the specimen concentration (or dilution of specimen) that was used in the Test for Interfering Factors (see the USP BET chapter (1)).

4. For each specimen to be tested, remove the blue caps and stoppers from eight SPL vials. Four of the vials will be used for controls (negative and positive, each in duplicate) and four vials for the specimen (sample and PPC, each in duplicate). Take care not to contaminate vials when removing stoppers. (Red capped Pyrosate PPC vials are not used in this test.)

   Place the vials in a test tube rack with the vials for PPCs next to the corresponding vials for that specimen.

   Set (but do not start) the timer to the incubation time specified on the SPL package labels (and on the certificate of compliance, which is available on the ACC website at www.acciusa.com).

   Prepare vials and start incubation (steps 5 – 8) rapidly (within about five minutes), taking care not to contaminate the vials. If a large number of specimens are to be tested, the procedure can be batched and steps 5-9 can be performed on a manageable number of specimens at each stage and timed separately. Provided that all testing is performed in one session without delays between batches, it is not necessary to repeat the negative and positive controls with subsequent batches. Batches may be overlapped if desired.

5. Transfer 0.5 mL LRW to each of two Pyrosate SPL vials for negative controls.

   Transfer 0.5 mL of 2λ in LRW to each of two Pyrosate SPL vials for the positive control (PC).

   Transfer 0.5 mL of specimen (or specimen dilution) to each of two Pyrosate SPL vials for the sample assay.

   Transfer 0.5 mL of 2λ in specimen (or specimen dilution) to each of two Pyrosate SPL vials for the positive product control (PPC).

   Use a new pipette (or tip) for negative controls, PCs, specimens, and PPCs.

6. Mix thoroughly and ensure complete reconstitution by shaking the test tube rack gently for 20 to 30 seconds in a horizontal plane. The contents of the vials should dissolve completely within about 60 seconds. Failure to mix adequately is a common cause of unsatisfactory tests.

7. From all vials, remove and discard 0.25 mL of the reconstituted lysate reagent using a new pipette (or tip) for each set of replicates.

   Note: If the long disposable pipettes (included in 10 test kits or available separately) are not used, vials must be tipped in order to make the transfers.

8. Transfer the vials to the heat block or water bath that is equilibrated at 37 ± 1°C and start the timer. Record the time and temperature at the start of incubation. If using a water bath, vials must be submerged above the level of the reaction mixture but not so deeply that they float.

   During the incubation period do not touch or agitate the vials or subject them to vibration. The gel is delicate and may be irreversibly broken if disturbed during incubation.

   Allow the vials to incubate for the time (within one minute) specified on the SPL package labels and the certificate of compliance.

   Record the time and temperature at the conclusion of the incubation period.

9. Remove and read the vials one at a time. Do not wipe the vials dry or bump them. To read the test, invert the vial by 180° in one smooth motion. Do not pause half way unless it is obvious that the gel has not formed. The test is positive if a gel has formed that does not collapse when the vial is inverted. Any other state of the reaction mixture constitutes a negative test, even if a gel formed but it breaks upon inversion.

1.2 Procedure for a Limit Test using Pyrosate PPC Vials

For instructions with illustrations, see Quick Guide 2.

1. For each specimen to be tested in duplicate, remove the blue caps and stoppers from four SPL vials and the red caps and stoppers from four PPC vials. Two of the SPL vials will be used for negative controls and two will be used for the specimen. Similarly, two of the PPC vials will serve as positive controls (without specimen) and two will be the positive product controls (PPCs) for the specimen.

   Take care not to contaminate vials when removing stoppers. Place the vials in a test tube rack. It is recommended that each pair of SPL vials for a particular specimen be placed in the rack next to the corresponding pair of PPC vials.

   Set (but do not start) the timer to the incubation time specified on the time specified on the SPL and PPC package labels (and on the certificate of compliance, which is available on the ACC website at www.acciusa.com).

   Prepare vials and start incubation (steps 2 – 6) rapidly (within about five minutes), taking care not to contaminate the vials. If a large number of specimens are to be tested, the procedure can be batched and steps 2-7 can be performed for a manageable number of specimens at each stage and timed separately. Provided that all testing is performed in one session without delays between batches, it is not necessary to repeat the negative and positive controls. Batches may be overlapped if desired.

2. Add 0.5 mL LRW to each of the two SPL vials that will serve as negative controls.

   For each specimen (or specimen dilution), add 0.5 mL specimen to two SPL vials. Always use a new pipette (or tip) for each specimen.

3. Mix thoroughly and ensure complete reconstitution by shaking the test tube rack gently for 20 to 30 seconds in a horizontal plane. The contents of the vials should dissolve completely within about 60 seconds. Failure to mix adequately is a common cause of unsatisfactory tests.

4. Remove 0.25 mL from each of the SPL vials reconstituted with LRW and transfer it to the corresponding PPC vial using a new pipette (or tip) for each positive control (PC).

   Remove 0.25 mL from each of the SPL vials reconstituted with specimen and transfer it to the corresponding PPC vial using a new pipette (or tip) for each positive product control (PPC).

   Note: If the long disposable pipettes (included in 10 test kits or available separately) are not used, vials must be tipped in order to make the transfers.

5. Shake the rack again gently for 20 to 30 seconds in a horizontal plane to reconstitute and mix the PCs and PPCs.

6. Transfer the vials to the heat block or water bath that is equilibrated at 37 ± 1°C and start the timer. Record the time and temperature at the start of incubation. If using a water bath, vials must be submerged above the level of the reaction mixture but not so deeply that they float.

   During the incubation period do not touch or agitate the vials or subject them to vibration. The gel is delicate and may be irreversibly broken if the vials are disturbed during incubation.

   Allow the vials to incubate for the time (within one minute) specified on the SPL and PPC package labels and the certificate of compliance.

   Record the time and temperature at the conclusion of the incubation period.

7. Remove and read the vials one at a time. Do not wipe the vials dry or bump them. To read the test, invert the vial by 180° in one smooth motion. Do not pause half way unless it is obvious that the gel has not formed. The test is positive if a gel has formed that does not collapse when the vial is inverted. Any other state of the reaction mixture constitutes a negative test, even if a gel formed but breaks upon inversion.

   Note: A simplified limit test without positive and negative controls is described in Quick Guide 4. This simplified procedure does **not** include the controls specified in the USP BET chapter; it does meet the minimum requirement for tests of fluids for dialysis specified in ANSI/AAMI/ISO 23500:2011 (9).

1.3 Interpretation of Results of Limit Tests

1. For the test to be valid both replicates of the negative controls must test negative and both replicates of the positive controls must test positive. If these conditions are not met, the whole test is invalid.

2. For the test of an individual specimen to be valid, both replicates of the PPC for that specimen must test positive.

3. For each specimen in a valid test: if both replicates are negative, the specimen (or specimen dilution) contains an endotoxin concentration less than the labeled sensitivity. If both replicates are positive, the specimen (or dilution of specimen) contains an endotoxin concentration of at least the labeled sensitivity. If one replicate of a specimen tests positive and the other negative, the result is indeterminate and the test should be repeated. (If the negative replicate formed a gel, but not a firm clot, this indicates that the endotoxin concentration of the specimen is close to the labeled sensitivity of the reagent. If the negative replicate shows no sign of gelation, this suggests that either the positive replicate was contaminated or that the test was performed incorrectly.)

   Example of three specimen results using a lysate sensitivity of 0.125 EU/mL:

Controls		Specimen A		Specimen B		Specimen C	
Negative	**Positive**	**Test**	**PPC**	**Test**	**PPC**	**Test**	**PPC**
-	+	-	+	+	+	-	+
-	+	-	+	+	+	-	-

   Interpretation:

   - The test is valid for Specimens A and B because negative controls did not clot and the positive controls and PPCs did clot. Clots for PPCs indicate that there is no significant interference. The test for Specimen C is not valid because one of the PPCs did not clot, indicating possible interference (inhibition) or that the test was not properly executed.

   - The endotoxin concentration of Specimen A is less than 0.125 EU/mL (the labeled sensitivity of the lysate reagent), assuming no sample dilution.

   - The endotoxin concentration of Specimen B is equal to or greater than 0.125 EU/mL, again assuming no sample dilution.

   - The endotoxin concentration of Specimen C cannot be determined because the test is not valid due to the failure of one replicate of the PPC to clot.

4. If a dilution of the original specimen was tested, then multiply the result by the dilution factor. For example, if using a lysate sensitivity of 0.125 EU/mL to test specimens at a dilution of 1:4, a negative result is reported as < 4 x 0.125 EU/mL or < 0.5 EU/mL. A positive result is reported as ≥ 4 x 0.125 EU/mL or ≥ 0.5 EU/mL.

2. Quantitative Test

2.1 Procedure for a Quantitative Test per the USP BET (1)

Prepare a series of standard endotoxin concentrations of 2λ, λ, ½λ and ¼λ (where λ is the labeled lysate sensitivity) using USP Endotoxin RS. Also prepare a series of dilutions of specimen and prepare a PPC for the highest concentration to be tested (as described for specimens in section 1.1 for the USP limit test). Test the endotoxin standards, a negative control (prepared with LRW) and the specimen dilutions and PPC in duplicate.

For more detailed instructions with illustrations, see Quick Guide 3.

2.2 Interpretation of Results of a Quantitative Test

1. For the test to be valid the following conditions must be met:

   1.1. Both replicates of the negative controls must test negative.

   1.2. The sensitivity of the lysate reagent (λ) must be confirmed within a factor of two in the series of endotoxin standards tested. This means that the lowest concentration of the endotoxins standards to clot (the endpoint) must be 2λ, λ, or ½λ. Any higher concentrations should also clot – e.g. if ½λ clots, λ and 2λ concentrations should also clot. The ¼λ concentration must not clot, λ or ½λ may clot, but 2λ should always clot. If both replicates have endpoints at the same endotoxin concentration, that is the result for the standards. If the endpoints are different for the two replicate series, the geometric mean endpoint endotoxin concentration is determined as follows:

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

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

2. For the test of an individual specimen to be valid, both replicates for the PPCs for that specimen must test positive.

3. For a specimen for which the test is valid (PPCs clot), if both replicates of all dilutions are negative, report the result as less than the concentration of λ of the Pyrosate reagent (multiplied by the dilution factor of any initial dilution, if applicable, as described in the Note in item 5 below).

4. For each of the replicates in which at least one of the concentrations of specimen tests positive, determine the "endpoint dilution factor". This is the dilution factor of the greatest dilution of the specimen to form a solid clot. The next dilution in the series must give a negative result (no clot) to demonstrate that the endpoint has been reached (see the example results in step 7 below).

   Note: the endpoint dilution may be different for the two replicates tested.

5. Calculate the endotoxin concentration by multiplying the endpoint dilution factor by λ. Thus, if using a lysate sensitivity (λ) of 0.125 EU/mL and the endpoint dilution factor is 4, the result for that replicate series is 4 x 0.125 EU/mL, or 0.5 EU/mL.

   Note: if the initial concentration tested was a dilution of the original specimen, that dilution factor must be taken into account when calculating the concentration of the original (undiluted) specimen. This is also applicable to negative results, as stated in item 3. In the example above, if the specimen had been initially diluted by a factor of 10 and the twofold dilutions were made from there, the final endotoxin concentration for the sample would be 4 x 0.125 EU/mL x 10 = 5 EU/mL.

6. If both replicate endotoxin concentrations determined in step 5 are the same, that concentration is the reportable result. If they are different, calculate the geometric mean concentration as described above for the endotoxin standards (step 1.2 in this section).

7. If all dilutions of the specimen test positive, additional dilutions may be tested to determine the endpoint. Alternatively, report the result as greater than or equal to λ multiplied by the greatest dilution factor tested (taking into account any initial dilution as described in item 5 above). Thus, using a reagent sensitivity (λ) of 0.125 EU/mL, if 8x was the greatest dilution of specimen and it tested positive, the result would be ≥ 8 x 0.125 EU/mL, or ≥ 1.0 EU/mL.

Example of a test for a single specimen using a lysate reagent sensitivity (λ) of 0.125 EU/mL:

Controls

Negatvie control	¼λ 0.03 EU/mL	½λ 0.06 EU/mL	λ 0.125 EU/mL	2λ 0.25 EU/mL
-	-	-	+	+
-	-	-	+	+

Specimen

8x dilution	4x dilution	2x dilution	1x (undiluted)	
			Specimen	PPC
-	-	+	+	+
-	-	+	+	+

Interpretation

The test is valid because:

(1) the negative controls did not clot

(2) the endpoint concentration of the standard series is at λ (0.125 EU/mL), confirming the labeled sensitivity of the lysate reagent within a factor of two

(3) the PPCs clotted.

For both replicates of the specimen the endpoint is at the twofold (2x) dilution (i.e. the endpoint dilution factor = 2). The endotoxin concentration of the specimen is therefore 2 x λ or 0.25 EU/mL.

3. Simple Test

A simplified procedure is given in Quick Guide 4.

7. Limitations of the Procedure

The capacity of the specimen to interfere with the LAL reagent or endotoxin may limit the sensitivity of the test or even preclude a valid test. Many materials interfere with the endotoxin test but the interference can often be overcome by dilution of the specimen. If a valid test of a specimen cannot be performed at a dilution that does not exceed the maximum valid dilution (MVD), then the test cannot be used. The MVD is the greatest dilution at which the endotoxin limit for the specimen can be detected. It is calculated as follows:

$$MVD = \frac{\text{Endotoxin limit x Concentration of the specimen}}{\lambda}$$

where λ is the sensitivity of the LAL reagent in EU/mL and the endotoxin limit is expressed in EU/unit of specimen (e.g. EU/mg, EU/mEq or EU/mL) and the concentration is expressed in units of specimen/mL. Endotoxin limits are given in pharmacopeial monographs or may be calculated (or verified) using the information in the USP BET chapter(1).

Trypsin and other serine proteases 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 interfering factors prior to testing (10, 11).

Inhibition has been observed for samples that have been taken using syringes, possibly due to silicone lubricant. If syringes are used to collect or hold samples, it is recommended that they be tested for interference.

8. Expected Values

Endotoxin can be quantified if the concentration is greater than or equal to the Pyrosate sensitivity. Dilution of the specimen will reduce the sensitivity of the test in proportion with the dilution factor. 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.

9. Specific Performance Characteristics

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

10. Bibliography

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11. Roth, R.I., F.C. Levin, and J. Levin. 1990. Optimization of the detection of bacterial endotoxin in plasma with the limulus test. J. Lab. Clin. Med. 116:153-161.

Limulus Amebocyte Lysate (LAL)

Rapid Endotoxin Detection

Instructions for Use

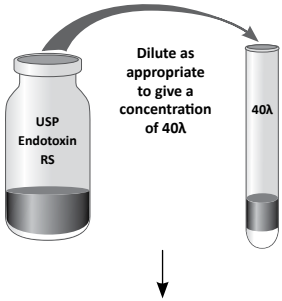
Fold Out Quick Guides

Quick Guide 1

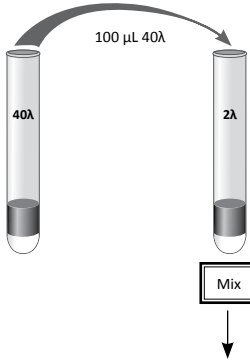
Limit Test Using Pyrosate and USP Endotoxin RS

IMPORTANT: Be sure to use the test method that is appropriate to meet regulatory requirements and your needs.

1. Dilute reconstituted USP Endotoxin RS with LRW to a concentration suitable for preparing a positive product control (PPC), such as forty times the labeled lysate sensitivity (40λ).

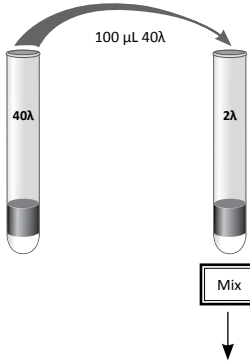


2. From this concentration (e.g. 40λ) prepare a concentration of double the labeled lysate sensitivity (2λ) in a dilution tube for positive controls. For example:



Leave enough of the higher concentration (e.g. 40λ) to prepare PPCs for each of the specimens (step 3).

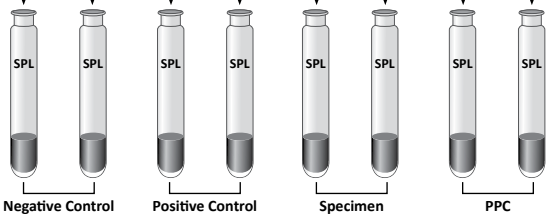
3. Prepare a PPC for each specimen (or specimen dilution) to be tested. For example (for a single specimen):



Always use a new pipette (or tip) for each specimen.

4. Set up eight SPL vials; remove the blue caps and stoppers.

5. Add 0.5 mL LRW | Add 0.5 mL 2λ in LRW | Add 0.5 mL Specimen | Add 0.5 mL 2λ in Specimen



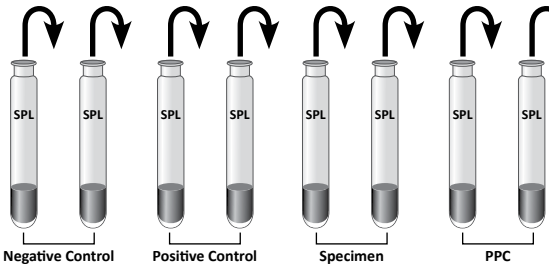
Prepare vials and start incubation (steps 5 – 8) rapidly (within about five minutes).

Use a new pipette (or tip) for each specimen, PPC, positive control and negative control.

The contents of the vials should dissolve completely within about 60 seconds.

6. Mix for 20 to 30 seconds by shaking the test tube rack gently in a horizontal plane.

7. Remove and discard 0.25 mL from each of the eight vials.

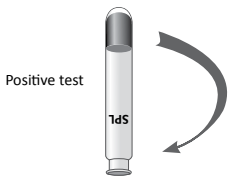


Use a new pipette (or tip) for each specimen, PPC, positive control and negative control.

8. Incubate at 37 ± 1°C for the time specified on the SPL package labels (± 1 minute).

Record the time and temperature at the start and end of incubation. Do not disturb the vials during incubation.

9. Read the test by inverting each vial one at a time.



If a firm gel forms that withstands inversion, the test is scored as positive (+). All other results are negative (-), even if a gel forms but the clot breaks.

10. Interpretation

10.1. Verify test validity: The negative controls should both test negative; all replicates of the positive controls and the PPCs should test positive. If these conditions are not met the test is invalid.

10.2. If the test is valid, determine the result for each specimen (or specimen dilution):

If one replicate of a specimen tests positive and the other negative, the result is indeterminate, so repeat the test.

If both replicates are negative, report the result for the specimen as less than the labeled reagent sensitivity (λ).

If both replicates are positive, report the result as greater than or equal to the concentration of λ.

If a dilution of the specimen was tested, then multiply the result (including a negative result) by that dilution factor.

See the main text (section 1.3 Interpretation of Results of Limit Tests) for further details and examples of test interpretation.

Quick Guide 2

Limit Test Using Pyrosate PPC Vials

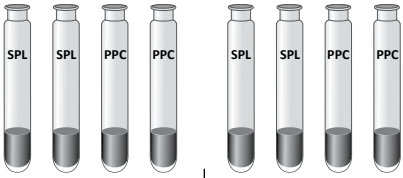
IMPORTANT: Be sure to use the test method that is appropriate to meet regulatory requirements and your needs.

1. Set up four blue capped SPL vials and four red capped PPC vials in a test tube rack. Remove the caps and stoppers. Perform steps to the initiation of incubation rapidly (within about five minutes).

2. Add 0.5 mL LRW to two of the SPL vials

   Add 0.5 mL specimen to two of the SPL vials

   Always use a new pipette (or tip) for each specimen.



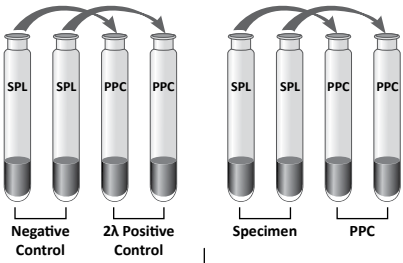
   The contents of the vials should dissolve completely within about 60 seconds.

3. Mix for 20 to 30 seconds by shaking the test tube rack gently in a horizontal plane.

4. Transfer 0.25 mL from the SPL vial containing LRW to a PPC vial

   Transfer 0.25 mL from the SPL vial containing specimen to a PPC vial

   Always use a new pipette (or tip) for each specimen.

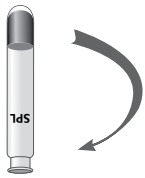


5. Mix again (shake rack 20-30 seconds).

6. Incubate at 37 ± 1°C for the time specified on the SPL and PPC package label (± 1 minute).

   Record the time and temperature at the start and end of incubation. Do not disturb the vials during incubation.

7. Read the test by inverting each vial one at a time.



   If a firm gel forms that withstands inversion, the test is scored as positive (+). All other results are negative (-), even if a gel forms but the clot breaks.

8. Interpretation

   8.1. Verify test validity: The negative controls should both test negative; all replicates of the positive controls and the PPCs should test positive. If these conditions are not met the test is invalid.

   8.2. If the test is valid, determine the result for each specimen (or specimen dilution):

   If one replicate of a specimen tests positive and the other negative, the result is indeterminate, so repeat the test.

   If both replicates are negative, report the result for the specimen as less than the labeled reagent sensitivity (λ).

   If both replicates are positive, report the result as greater than or equal to the concentration of λ.

   If a dilution of the specimen was tested, then multiply the result (including a negative result) by that dilution factor.

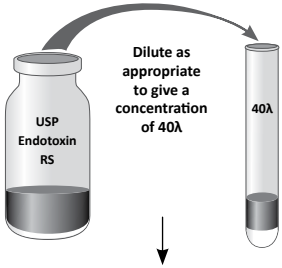
See the main text (section 1.3 Interpretation of Results of Limit Tests) for further details and examples of test interpretation.

Quick Guide 3

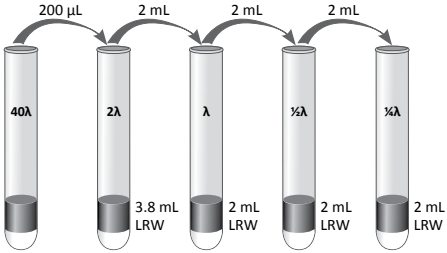
Quantitative Test Using Pyrosate

IMPORTANT: Be sure to use the test method that is appropriate to meet regulatory requirements and your needs.

1. Dilute reconstituted USP Endotoxin RS with LRW to a concentration suitable for preparing a positive product control (PPC), such as forty times the labeled lysate sensitivity (40λ, where λ is the labeled lysate sensitivity).

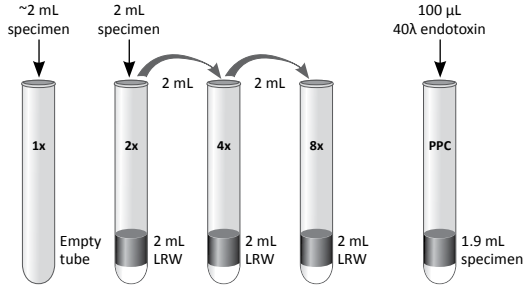


2. From this concentration (e.g. 40λ) prepare concentrations of 2λ, λ, ½λ and ¼λ in dilution tubes. For example:



Mix tubes thoroughly between dilutions.

3. Prepare a series of twofold dilutions of the specimen and a PPC (highest specimen concentration containing 2λ of endotoxin). For example:

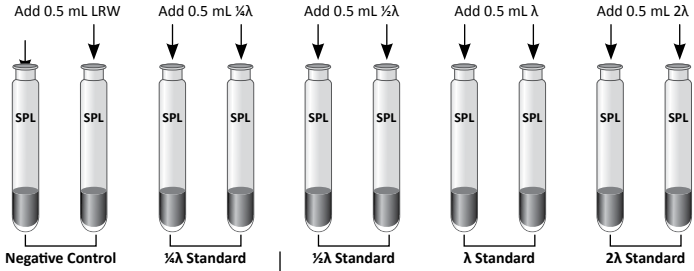


Mix tubes thoroughly between dilutions.

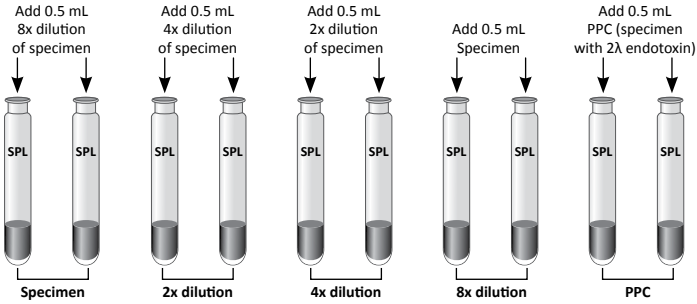
4. Set up twenty SPL vials in a test tube rack and remove the blue caps and stoppers, taking care not to contaminate the vials.

Prepare vials and start incubation (steps 5 – 9) rapidly (within about five minutes).

5. Add 0.5 mL Standard Endotoxin Concentrations and LRW (for the Negative Controls) to SPL vials as follows:



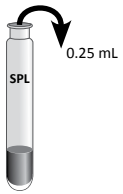
6. Add 0.5 mL Specimen, Specimen Dilutions and PPC to SPL vials as follows:



7. Mix for 20 to 30 seconds by shaking the test tube rack gently.

The contents of the vials should dissolve completely within about 60 seconds.

8. Remove and discard 0.25 mL from each of the 20 vials.



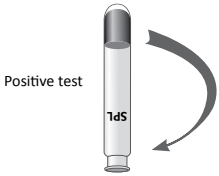
For standards and specimens the same pipette may be used for each provided that you start from the greatest dilution and work towards the highest concentration in each case. Take care to avoid contamination. If in doubt, use a fresh pipette (or tip).

Always change pipettes between endotoxin and specimen or between different specimens.

9. Incubate the vials at 37 ± 1°C for the time specified on the SPL package label (± 1 minute).

Record the time and temperature at the start and end of incubation. Do not disturb the vials during incubation.

10. Read the test by inverting each vial one at a time.



If a firm gel forms that withstands inversion, the test is scored as positive (+). All other results are negative (-), even if a gel forms but the clot breaks.

11. Interpretation

11.1. Verify test validity: The negative controls should both test negative; the sensitivity of the lysate reagent (λ) should be confirmed (i.e. the geometric mean endpoint of the standards must be between ½λ and 2λ – see main text for details); and the PPCs should test positive. If these conditions are not met the test is invalid.

11.2. For a valid test of a specimen, if both replicates of all dilutions are negative, report the result as less than the concentration of λ (multiplied by the dilution factor of any initial dilution, if applicable).

11.3. For each of the replicates of the specimen dilutions in which at least one of the concentrations of specimen test positive, determine the endpoint dilution factor. (See main text, section 2.2, Interpretation of Results of a Quantitative Test, for explanation of "endpoint dilution factor.")

11.4. Calculate the endotoxin concentration by multiplying the endpoint dilution factor by λ. Thus, if λ = 0.125 EU/mL and the endpoint dilution factor is 4, the result is 4 x 0.125 EU/mL, or 0.5 EU/mL.

If both replicate endotoxin concentrations (calculated as described above) are the same, that concentration is the reportable result. If not, calculate and report the geometric mean of the two.

If all dilutions of the specimen test positive, either test additional dilutions to determine the endpoint or report the result as greater than or equal to λ multiplied by the highest dilution factor tested.

Note: If a dilution of the original specimen was tested, then the result is multiplied by that dilution factor.

See the main text (section 2.2, Interpretation of Results of a Quantitative Test) for further details and examples of test interpretation.

Quick Guide 4

Simplified Limit Test Using Pyrosate PPC Vials

IMPORTANT: Be sure to use the test method that is appropriate to meet regulatory requirements and your needs.

1. Set up a blue capped SPL vial and a red capped PPC vial in a test tube rack. Remove the caps and stoppers. Perform steps to the initiation of incubation rapidly (within about five minutes).

2. Add 0.5 mL specimen to the SPL vial

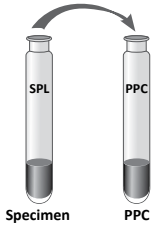
Always use a new pipette (or tip) for each specimen.

3. Mix for 20 to 30 seconds by shaking the test tube rack gently in a horizontal plane.

4. Transfer 0.25 mL from the SPL vial containing specimen to a PPC vial:

The contents of the vials should dissolve completely within about 60 seconds.

Always use a new pipette (or tip) for each specimen.

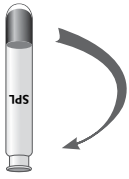


5. Mix again (shake rack 20-30 seconds).

6. Incubate at 37 ± 1°C for the time specified on the SPL and PPC package label (± 1 minute).

Record the time and temperature at the start and end of incubation. Do not disturb the vials during incubation.

7. Read the test by inverting each vial one at a time.



If a firm gel forms that withstands inversion, the test is scored as positive (+). All other results are negative (-), even if a gel forms but the clot breaks.

8. Interpretation

8.1. PPC (Positive Product Control) vial: This SHOULD clot. If it does not, the test is invalid and should be repeated. A failure of the PPC tube to clot indicates either inhibition of the reaction by the sample or improper performance of the assay.

8.2. SPL (Sample) vial: This vial may or may not clot, depending on the endotoxin concentration in the specimen. Absence of a clot means the specimen contains endotoxin at a concentration less than the labeled sensitivity of the reagent. If the SPL vial clots, the specimen contains an endotoxin concentration of at least the labeled reagent sensitivity.

Summary of the possible results of a two vial test (one PPC vial and one SPL vial):

RESULT SCENARIO	CONTROL (PPC)	SAMPLE (SPL)	RESULT
1.	Clot (+)	Clot (+)	Valid assay. Sample contains ≥ labeled reagent sensitivity
2.	Clot (+)	No clot (-)	Valid assay. Sample contains < labeled reagent sensitivity
3.	No clot (-)	Clot (+)	Assay is NOT valid. Repeat the test.
4.	No clot (-)	No clot (-)	Assay is NOT valid. Repeat the test.

Note: If a dilution of the original specimen was tested (as may be necessary for testing dialysate), then the result is multiplied by that dilution factor.

For example, if a fourfold (4x) specimen dilution is tested with a lysate sensitivity of 0.125 EU/mL, a negative result is reported as < 4 x 0.125 EU/mL or < 0.5 EU/mL. A positive result is reported as ≥ 4 x 0.125 EU/mL or ≥ 0.5 EU/mL.

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