Product Information

E-TOXATE™ Kit

Sufficient for 50 assays

ET0100

Product Description

The E-TOXATE™ (*Limulus* Amebocyte Lysate) test kits are intended for the detection and semi-quantitation of endotoxins for research purposes.

The *Limulus* amebocyte lysate (LAL) test for endotoxins originated from the work of Bang and Levin.[1-3] When compared to the official USP rabbit test[4] which has historically been used for pyrogen testing, the LAL test was found to be not only more sensitive to endotoxins,[5-8] but also simpler, more rapid, and less expensive to perform.

The E-TOXATE™ Reagent is prepared from a lysate of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*. When exposed to minute quantities of endotoxin (lipopolysaccarides from the walls of Gram-negative bacteria), the lysate increases in opacity as well as viscosity and may gel, depending on the concentration of endotoxin. While the mechanism for this reaction is not completely understood, it appears to be analogous to the clotting of mammalian blood,[3] involving two steps:

- First, endotoxin in the presence of calcium ions activates a trypsin-like,[9,10] pre-clotting enzyme, or enzymes.[11,12]
- Then the activated enzyme(s) modify a "coagulogen" by limited proteolysis to produce a clottable protein.[10,13]

This endotoxin-mediated effect is closely tied to the biologically active or "pyrogenic" portion of the molecule, since it has been shown that "detoxified" endotoxin yields a negative Limulus lysate test.[6]

Precautions and Disclaimer

This product is for R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

The E-TOXATE™ kit may **not** be used in the diagnosis of endotoxemia in humans, nor for final product testing of endotoxins in pharmaceuticals.

The Endotoxin Standard is a **harmful pyrogen and may cause fever. Do not use if skin is cut or scratched.** Wash thoroughly after handling.

It is recommended to read the entire Product Information Sheet before use.

Components

This kit contains sufficient reagents for 50 assays.

Component Information

- E-TOXATE™ Reagent, dried concentrate from *Limulus polyphemus*, 5 mL, Cat. No. E8904: 1 vial
  - Limit of Sensitivity: 0.05-0.1 endotoxin units (EU) per mL
- E-TOXATE™ Endotoxin Standard, Cat. No. E8029: 1 vial per kit
  - Endotoxin (*E. coli* 0.55:B5 lipopolysaccharide) containing 10,000-20,000 endotoxin units (EU) per vial. See the lot-specific Certificate of Analysis for the actual value. Standardized against USP Reference Standard Endotoxin (RSE).
- E-TOXATE™ Water, endotoxin-free, 30 mL, Cat. No. 2107: 1 vial

Reagents and Equipment Required

(Not Provided)

Sterile, pyrogen-free glassware or plasticware, including:

- Pipettes: 5 mL and 1 mL, serologic
- Syringes and needles
- Test tubes, glass (10 × 75 mm), for endotoxin determination
- Sterile, polystyrene culture tubes for Endotoxin Standard preparation
- Water bath or heating block, 37 °C. **Do not use an air bath**.
- pH meter, or narrow-range (pH 6-8) pH indicator paper

- Pyrogen-free Water: for routine pyrogen-free water requirements, it is suggested that commercially available Sterile Water for Injection, USP, or Sterile Water for Irrigation, USP, preferably in small containers, be pre-screened for endotoxins with the E-TOXATE™ *Limulus* lysate test. Repeated sampling of large containers of pyrogen-free water over several days is not recommended.

  Note: The use of bacteriostatic water is not recommended.

Optional Reagents

Endotoxin-free in this procedure is defined as producing a negative result when tested using the E-TOXATE™ assay.

- SIGMACOTE®, Cat. No. SL2: Organic solvent-based siliconizing solution for labware.
- E-TOXA-CLEAN® Concentrate, Cat. No. E9029: Alkaline detergent for preliminary cleaning of glassware prior to inactivation of endotoxins by steam sterilization and dry heating. Prepare a 1% solution by dissolving ~10 g of E-TOXA-CLEAN® in 1 L of hot tap water.
- 0.1 N Hydrochloric acid (HCl), endotoxin-free, Cat. No. 2104: For adjusting pH of samples when necessary. Suitable for use if introduction of contaminating organisms or endotoxin is to be avoided.
- 0.1 N Sodium Hydroxide (NaOH), endotoxin-free, Cat. No. 2105: For adjusting pH of samples when necessary. Suitable for use if introduction of contaminating organisms or endotoxin is to be avoided.
- Heparin, sodium salt, endotoxin-free, Cat. No. 2106: 300 USP units/vial. Sufficient for 5 mL of blood.

Storage/Stability

Upon opening the kit, store the E-TOXATE™ Reagent at –20 °C, the Endotoxin Standard at 2-8 °C, and the E-TOXATE™ Water, endotoxin-free, at room temperature. The water is stable indefinitely if introduction of contaminating organisms or endotoxins is avoided.

Preparation Instructions

Endotoxin-Free Equipment

Since extremely minute quantities of endotoxin can cause the E-TOXATE™ Reagent to gel, all equipment coming in direct contact with it must be free of endotoxin contamination. Commercially available "pyrogen-free" disposable glassware and/or plasticware should be evaluated for suitability prior to routine use and substitution of materials from other manufacturers should not be made without pre-evaluation. If possible, start with new glassware and set it aside for endotoxin assays only. New glassware does not generally require presoaking and rinsing but should be subjected to heat treatment as described in Steps 3 and 4.

The following procedure is recommended for contaminated glassware:

1. Soak glassware, preferably overnight, in a 1% solution of an alkaline detergent, such as E-TOXA-CLEAN® (Cat. No. E9029). When possible, scrub with a clean brush.

   Note: Sterile, endotoxin-free siliconized glass or polystyrene tubes are recommended for making dilutions of samples and standards, since lipopolysaccharides absorb onto untreated glass and polypropylene surfaces.

   - Organic solvent-based siliconizing solution: If using SIGMACOTE® (Cat. No. SL2), cover or immerse the glassware in the undiluted SIGMACOTE® for 2-3 minutes. After treatment, remove the excess solution. Allow the treated glassware to air-dry in a hood. Rinse the siliconized glassware with water to remove HCl by-products before use. For other commercially available organic solvent-based siliconizing solutions, follow the manufacturer's recommended procedure for application.
   - Aqueous-based siliconizing solution: If using a commercially available aqueous-based siliconizing solution, follow the manufacturer's recommended procedure for application.
2. Rinse all glassware 8-10 times with warm, running tap water, 5 times with distilled or deionized water, and finally once with pyrogen-free water. Dry in a hot-air oven.

3. Dried pipettes are plugged with non-absorbent cotton and placed tip down in a stainless-steel pipette can or wrapped several to a package in aluminum foil. Other glassware may be placed in foil-covered beakers or other containers, or simply wrapped in foil. Cap the test tubes with Bakelite caps and rubber liners that will withstand heat treatment.
4. Autoclave covered material at 121 °C for 1 hour. Follow with heating in an oven at 175 °C for a minimum of 3 hours.

E-TOXATE™ Reagent Working Solution

- Before reconstituting the E-TOXATE™ Reagent, give the vial a sharp tap on a firm surface to dislodge any loose powder at the top of the vial.
- Carefully open the vial.
- Add the required volume of E-TOXATE™ Water (Cat. No. 2107), using an endotoxin-free pipetting device (see Table 1). Some laboratories may prefer to add water aseptically using a sterile syringe and needle.
- After adding water, swirl to dissolve.

Table 1.

Cat. No.	Volume of E-TOXATE™ Water for reconstitution
E 8779	2 mL
E 8904	5 mL

Notes:

- Do not shake vigorously, as this may be deleterious to the lysate. The solution may appear hazy.
- Chill in ice bath immediately after reconstitution.
- It is preferable to use the entire solution the same day it is reconstituted, although the E-TOXATE™ Reagent Working Solution may be stored frozen with a minimal loss of sensitivity. However, sensitivity of the lysate will decrease with repeated freeze-thaw cycles.

Precautions to prevent contamination of the E-TOXATE™ Reagent Working Solution

- Do not return pipettes, needles, etc., or excess reagent back to the vial containing the bulk of the E-TOXATE™ Reagent Working Solution, as this may introduce contamination.
- When sampling the E-TOXATE™ Reagent, remove only the quantity required for assays.
- Discard the pipette or other glassware, and any removed reagent that was unused, rather than risking back contamination of the reagent remaining in the vial.

Fluid Samples other than Plasma (pH Adjustment)

For fluids other than plasma, the pH of the solution to be tested must be between 6 and 8 (optimal range 6.8–7.5).[18,19] The pH may be adjusted as needed with endotoxin-free 0.1 N hydrochloric acid (Cat. No. 2104), or endotoxin-free 0.1 N Sodium hydroxide (Cat. No. 2105).

Note: pH electrodes may contaminate the solution. The pH of sample can usually be determined by applying drops to pH indicator paper with pyrogen-free Pasteur pipettes. Alternatively, the pH of an aliquot of the sample may be checked and adjusted with a pH meter to determine the amount of acid or alkali needed to adjust the sample pH.

Plasma Samples

For plasma or other biological materials that may be contaminated with blood, refer to either the chloroform extraction technique[20] or the dilution-heating technique[21] for removal of the LAL inhibitor. The choice of technique is determined by the sensitivity of the E-TOXATE™ Reagent and by endotoxin levels deemed significant.[22] Unless grossly bloody, fluids other than plasma do not require inhibitor removal.

Endotoxin Standard Stock Solution

- See the Preparation Instructions, Endotoxin-Free Equipment section, for suitable containers to use in preparing Endotoxin Standard Solutions.
- Reconstitute the Endotoxin Standard (Cat. No. E8029) with an appropriate volume of E-TOXATE™ Water (Cat. No. 2107), to obtain an Endotoxin Standard Stock Solution with a concentration of 4,000 EU/mL. The actual volume of water required to reconstitute the vial is reported on the lot-specific Certificate of Analysis.
- Mix vigorously (vortex mixer) for at least 2 minutes.
- Then vortex ~30 seconds at 10-minute intervals over a 30-minute period. The Endotoxin Standard Stock Solution remains active when stored in a refrigerator for at least 2 weeks, if kept free of contamination.
- Before each use, mix as previously described.
- **Do not freeze**.

For researchers who wish to use a pre-weighed endotoxin standard or other lipopolysaccharides as an endotoxin reference, the following steps for preparation of a stock endotoxin solution are recommended:

1. For pre-weighed endotoxin standards, reconstitute according to the manufacturer's instructions.
2. For bulk lipopolysaccharide powder:
   2.1. Using aseptic and endotoxin-free technique, accurately weigh a few milligrams of powder into an endotoxin-free capped polystyrene or a siliconized glass culture tube.
   2.2. Add 1.0 mL of endotoxin-free water for each mg of lipopolysaccharide, for a 1 mg/mL endotoxin solution. Recap the tube.
   2.3. Vortex the endotoxin solution for ~20 minutes. Store overnight at 2-6 °C to improve solubility before making further dilutions.

The prepared endotoxin solution should be vortex-mixed for 20 minutes prior to use in preparing Endotoxin Standard Dilutions.

Endotoxin Standard Dilutions

Dilutions of the Endotoxin Standard Stock Solution, containing ≥400 EU/mL, generally remain active for at least one week stored in a refrigerator, if kept free from contamination. More dilute solutions should be prepared fresh daily.

1. Vortex the Endotoxin Standard Stock Solution (4,000 EU/mL). All endotoxin dilutions should be prepared in sterile, capped polystyrene tubes.
2. Prepare dilutions of Endotoxin Standard Stock using E-TOXATE™ Water (see Table 2):
3. Vortex dilutions for 30-60 seconds prior to further dilution or assay. Any endotoxin solution standing for more than 30 minutes should be vortexed prior to use.

Table 2.

Tube No.	Endotoxin	E-TOXATE™ Water (mL)	Final Concentration (EU/mL)
1	0.2 mL Endotoxin Std. Stock Soln.	1.8	400
2	0.2 mL from Tube No. 1	1.8	40
3	0.2 mL from Tube No. 2	1.8	4
4	0.3 mL from Tube No. 3	2.1	0.5
5	1 mL from Tube No. 4	1.0	0.25
6	1 mL from Tube No. 5	1.0	0.125
7	1 mL from Tube No. 6	1.0	0.06
8	1 mL from Tube No. 7	1.0	0.03
9	1 mL from Tube No. 8	1.0	0.015

Procedure

All assays using multiple test vials are performed in 10 × 75 mm glass culture tubes (not siliconized). The mouths of the tubes may be covered with small squares of foil or Parafilm® during incubation. Unless the incubation environment is extremely contaminated, covering the mouths of the tubes may be unnecessary.

Table 3.

Tube ID	Tube sample	Sample	E-TOXATE™ Water	Endotoxin Std. Dilution	E-TOXATE™ Reagent working solution
A	test for endotoxin in sample	0.1 mL	–	–	0.1 mL
B	test for inhibitor in sample	0.1 mL	–	0.01 mL of 4 EU/mL	0.1 mL
C	negative control	–	0.1 mL	–	0.1 mL
D	standard	–	–	0.1 mL of 0.5 EU/mL	0.1 mL
E	standard	–	–	0.1 mL of 0.25 EU/mL	0.1 mL
F	standard	–	–	0.1 mL of 0.125 EU/mL	0.1 mL
G	standard	–	–	0.1 mL of 0.06 EU/mL	0.1 mL
H	standard	–	–	0.1 mL of 0.03 EU/mL	0.1 mL
I	standard	–	–	0.1 mL of 0.015 EU/mL	0.1 mL

Notes:

- False positives are reportedly caused by trypsin and trypsin-like enzymes,[9,10] thrombin, thromboplastin, polynucleotides, and ribonuclease.[14]
- False negatives are reportedly caused by trypsin inhibitors, EDTA and other calcium-binding reagents,[2] high molar (>2 M) salt concentration,[16] and semisynthetic penicillins.[17]

1. Label 9 tubes as shown in Table 3:
   - One set, of Tubes A and B, is needed for each sample to be tested.
   - Tubes D, E, F, G, H, and I are used to determine the sensitivity of the E-TOXATE™ Reagent Working Solution. These also serve as positive controls.
   - Tubes E, F, G, H, and I may be omitted if sensitivity information is unnecessary.
   - Tube B may be omitted if sample has been previously shown to be free of lysate inhibitor.
2. Add sample, water, and Endotoxin Standard Dilutions directly to the bottom of tubes (volumes as indicated in Table 3).
3. Add E-TOXATE™ Reagent Working Solution to each tube by inserting the pipette to just above the contents and allowing lysate to flow down the side of tube, thereby avoiding contact and possible cross-contamination. Adding the reagent to the tubes that contain the least (expected) endotoxin first (for example, Tube C followed by Tube A, then lowest through highest positive standard(s) and finally Tube B) will reduce possible cross-contamination.
4. Mix tube contents gently. Cover the mouths of the tubes with foil or Parafilm®. Incubate for 1 hour undisturbed at 37 °C.

   Notes:

   - Once the incubation begins, tubes must remain stationary. Do not disturb the tubes, as this may disrupt gel structure and cause an irreversible liquefaction.
   - In addition, a mixture in the process of gelation may never gel if shaken, but only increase in viscosity.
   - When examining tubes, handle as gently as possible.

5. After 1 hour incubation, gently remove tubes or vials one at a time. Slowly invert 180° while observing for evidence of gelation.
    - A positive test is the formation of a hard gel that permits complete inversion of the tube or vial without disruption of the gel.
    - All other results (soft gels, turbidity, increase in viscosity, or clear liquid) are considered negative.
    - To determine semi-quantitatively the endotoxin level of a sample yielding a positive result, make dilutions of the sample in E-TOXATE™ Water. Test each dilution as under "Tube A" until a negative test result is obtained. Determine the greatest dilution of sample and lowest concentration of Endotoxin Standard that yields positive test results.

Note: Some test samples may exhibit enhancement of the lysate reaction by amplifying the expected endotoxin sensitivity, thereby yielding erroneously higher results. Enhancement of lysate sensitivity by various substances including calcium has been reported.[15] This potential enhancement may be identified by the following steps:

1. Determine the minimum dilution of test sample required to obtain a negative result.
2. Prepare a series of Endotoxin Standard Dilutions as described under the "Endotoxin Standard Dilutions" section, except in place of E-TOXATE™ Water, use the minimum test sample dilution in Step 1 as diluent to prepare the standard dilutions.
3. Prepare a series of Endotoxin Standard Dilutions using E-TOXATE™ Water as described under "Endotoxin Standard Dilutions" section.
4. Perform side-by-side testing of each dilution from Steps 2 and 3, by mixing 0.1 mL with E-TOXATE™ Working Solution as required.

Positive test endpoints of the two dilution series should be within one dilution. A difference of greater than one dilution may suggest sample enhancement of the lysate sensitivity.

Example: The minimum dilution under Step 1 of a test sample required to obtain a negative result was found to be 1/256, or in other words:

- 1/64 and 1/128 dilutions positive,
- but 1/256 and 1/512 dilutions negative.

Side-by-side testing of Endotoxin Standard Dilutions prepared as described under Steps 2 and 3, yielded positive tests at 0.06 EU/mL and 0.125 EU/mL, respectively. Since these results are within one dilution (see Table 2), it may be concluded that there is no enhancement of lysate sensitivity by the sample.

Results

Calculate the endotoxin level (EU/mL) by multiplying the inverse of the highest dilution of sample found positive by the lowest concentration of Endotoxin Standard found positive.

Example: Sample is positive at 1/64 dilution, and negative at 1/128. Endotoxin Standard is positive at 0.06 EU/mL and negative at 0.03 EU/mL.

$$\text{Endotoxin} = \frac{1}{(1/64)} \times 0.06 \text{ EU/mL} = 3.8 \text{ EU/mL}$$

Interpretation of Results: Table 4 explains the interpretation of results of the sample (Tube A), test for E-TOXATE™ inhibitor (Tube B), negative control (Tube C), and standards (Tubes D–I).

Note: A hard gel in Tube B shows that the sample is free of E-TOXATE™ Inhibitor.

Table 4.

Tube ID				
A	**B**	**C**	**D-I**	**Interpretation**
–	+	–	+	Sample does not contain endotoxin or else contains endotoxin at a level below the detection limits of assay.
+	+	–	+	Sample contains endotoxin equal to, or greater than, the amount present in the most dilute Endotoxin Standard giving a positive result.
+	+	+	+	Since negative control shows a hard gel, contamination of water, E-TOXATE™ Reagent, or glassware by endotoxin is present. Sample result may not be valid.
–	–	–	+	Absence of hard gel in Tube B and presence of hard gel in Tube D show that sample contains an inhibitor of the E TOXATE™ Reagent. Test is not valid.
±	±	–	–	E-TOXATE™ Reagent or Endotoxin Standard has deteriorated. Sample results are not valid unless Tubes B and D show hard gels.

(+) Hard gel

(–) Absence of hard gel

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