3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

E-TOXATE™ Kits

Catalog Numbers **ET0100, ET0200, and ET0300**

TECHNICAL BULLETIN

Product Description

The E-TOXATE™ (*Limulus* Amebocyte Lysate) test kits are intended for the detection and semiquantitation 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] preclotting enzyme(s).[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]

Components

Sufficient reagents are provided to performed the indicated assays:

ET0200	20 Assays
ET0100	50 Assays
ET0300	100 Assays

- E-TOXATE Reagent, multiple test vial(s), dried concentrate from *Limulus polyphemus*. Limit of Sensitivity: 0.05–0.1 endotoxin units (EU) per ml.

  Catalog Number E8779 — 2 ml
  1 vial in ET0200
  5 vials in ET0300
  Catalog Number E 8904 — 5 ml
  1 vial in ET0100

- E-TOXATE Endotoxin Standard — 1 vial per kit
  Catalog Number E8029
  Endotoxin (*E. coli* 0.55:B5 lipopolysaccharide) containing 10,000–20,000 endotoxin units (EU) per vial. See lot-specific Certificate of Analysis for actual value. Standardized against USP Reference Standard Endotoxin (RSE).

- E-TOXATE Water, endotoxin-free, — 30 ml
  Catalog Number 2107
  1 vial in ET0100
  1 vial in ET0200
  5 vials in ET0300

Reagents and Equipment Required but Not Provided

- Sterile, pyrogen-free glassware or plasticware, including:
  Pipettes: 5 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 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 prescreened 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
Note: Endotoxin-free in this procedure is defined as producing a negative result when tested using the E-TOXATE assay.

- SIGMACOTE®, Catalog Number SL2 - Organic solvent based siliconizing solution for labware.
- E-TOXA-CLEAN® Concentrate, Catalog Number 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 liter of hot tap water.
- 0.1 N Hydrochloric acid, endotoxin-free, Catalog Number 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, endotoxin-free, Catalog Number 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, Catalog Number 2106 - 300 USP units/vial. Sufficient for 5 ml of blood.

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

It is recommended to read the entire technical bulletin before use.

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, e.g., E-TOXA-CLEAN, Catalog Number 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.
   a. Organic solvent-based siliconizing solution - If using SIGMACOTE, Catalog Number SL2, cover or immerse the glassware in the undiluted SIGMACOTE for 2–3 minutes. After treatment, remove the excess solution and allow the treated glassware to air dry in a hood. Rinse the siliconized glassware with water to remove the HCl by-products before use. For other commercially available organic solvent-based siliconizing solutions, follow the manufacturer's recommended procedure for application.
   b. 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 hot-air oven.
3. Dried pipettes are plugged with nonabsorbent 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 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 vial and add the required volume of E-TOXATE Water, Catalog Number 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.

Catalog Number	Volume of E-TOXATE Water for reconstitution
E 8779	2 ml
E 8904	5 ml

Notes: **Do Not shake vigorously.** This may be deleterious to the lysate. 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; 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, Catalog Number 2104, or endotoxin-free 0.1 N sodium hydroxide, Catalog Number 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 the removal of the LAL inhibitor. The choice of technique is determined by 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 Preparation Instructions, Endotoxin-Free Equipment, for suitable containers to use in preparing Endotoxin Standard Solutions.

Reconstitute the Endotoxin Standard, Catalog Number E8029, with an appropriate volume of E-TOXATE Water, Catalog Number 2107, to obtain an Endotoxin Standard Stock Solution with a concentration of 4,000 EU per 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 those wishing to use a preweighed endotoxin standard or other lipopolysaccharides as an endotoxin reference, the following steps for preparation of a stock endotoxin solution are recommended:

1. For preweighed endotoxin standards, reconstitute according to the manufacturer's instructions.
2. For bulk lipopolysaccharide powder:
   a. Using aseptic and endotoxin-free technique, accurately weigh a few milligrams of powder into an endotoxin-free capped polystyrene or siliconized glass culture tube.
   b. Add 1.0 ml of endotoxin-free water for each milligram of lipopolysaccharide, preparing a 1 mg/ml endotoxin solution. Recap tube.
   c. 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:

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

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.

Procedure

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

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 are 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 and 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 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 tubes containing the least (expected) endotoxin first, i.e, 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 mouths of tubes with foil or Parafilm and incubate for 1 hour undisturbed at 37 °C.
   Note: 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.

Table 3.

Tube		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

5. After 1 hour incubation, gently remove tubes or vials one at a time and 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 semi-quantitatively determine the endotoxin level of a sample yielding a positive result, make dilutions of the sample in E-TOXATE Water and 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 yielding 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 "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, i.e., 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 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.

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

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				Interpretation
A	**B**	**C**	**D–I**	
–	+	–	+	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

References

1. Bang, F.B., A bacterial disease of *Limulus polyphemus*. Bull. Johns Hopkins Hosp., **98**, 325 (1956).
2. Levin, J., and Bang, F.B., Clottable protein in *Limulus*: Its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemmorrh., **19**, 186 (1968).
3. Levin, J., and Bang, F.B., The role of endotoxin in the extracellular coagulation of *Limulus* blood. Bull. Johns Hopkins Hosp., **115**, 265 (1964).
4. U.S.P. Pyrogen Test, U.S. Pharmacopeia, 19th ed., 1975, p. 613. United States Pharmacopeial Convention, Inc., Rockville (MD). Technique for Rabbit Pyrogen Test.
5. Reinhold, R., and Fine, J., A technique for quantitative measurement of endotoxin in human plasma. Proc. Soc. Exp. Biol. Med., **137**, 334 (1971).
6. Tomasulo, P.A., et al., Biological activities of tritiated endotoxins: Correlation of the *Limulus* lysate assay with rabbit pyrogen and complement-activation assays for endotoxin. J. Lab. Clin. Med., **89**, 308 (1977).
7. Wachtel, R.F., and Tsuji, K., Comparison of *Limulus* amebocyte lysates and correlation with the United States Pharmacopeial pyrogen test. Appl. Environ. Microbiol., **33**, 1265 (1977).
8. Yin, E.T., et al., Picogram-sensitivity assay for endotoxin: Gelation of *Limulus* polyphemus blood cell lysate induced by purified lipopolysaccharides and lipid A from Gram-negative bacteria. Biochem. Biophys. Acta, **261**, 284 (1972).
9. Sullivan, J.D., Jr. and Watson, S.W., Purification and properties of the clotting enzyme from *Limulus* lysate. Biochem. Biophys. Res. Commun., **66**, 848 (1975).
10. Tai, J.Y., et al., Studies of *Limulus* amoebocyte lysate. II: Purification of the coagulogen and the mechanism of clotting. J. Biol. Chem., **252**, 4773 (1977).
11. Tai, J.Y., and Liu, T.Y., Studies on *Limulus* amoebocyte lysate. Isolation of preclotting enzyme. J. Biol. Chem., **252**, 2178 (1977).
12. Young, N.S., et al., An invertebrate coagulation system activated by endotoxin: Evidence for enzymatic mediation. J. Clin. Invest., **51**, 1790 (1972).
13. Solum, N.O., The coagulogen of *Limulus polyphemus* hemocytes. A comparison of the clotted and non-clotted forms of the molecule. Thromb. Res., **2**, 55 (1973).

14. Elin, R.J., and Wolff, S.M., Nonspecificity of the limulus amebocyte lysate test: positive reactions with polynucleotides and proteins. J. Infect. Dis., **128**, 349 (1973).
15. Sullivan, J.D., Jr. and Watson, S.W., Factors affecting the sensitivity of *Limulus* lysate. Appl. Microbiology, **28**, 1023 (1974).
16. Nandan, R., and Brown, D.R., An improved *in vitro* pyrogen test to detect picograms of endotoxin contamination in intravenous fluids using *Limulus* amebocyte lysate. J. Lab. Clin. Med., **89**, 910 (1977).
17. Rhodes, B.A., et al., The use of *Limulus* testing to reduce the incidence of adverse reactions to cisternographic agents. Neurology, **24**, 810 (1974).
18. Cooper, J.F., et al., The *Limulus* test for endotoxin (pyrogen) in radiopharmaceuticals and biologicals. Bull. Parenter. Drug Assoc., **26**, 153 (1972).
19. Levin, J., et al., Detection of endotoxin in human blood and demonstration of an inhibitor. J. Lab. Clin. Med., **75**, 903 (1970).
20. Levin, J., et al., Detection of endotoxin in the blood of patients with sepsis due to Gram-negative bacteria. N. Engl. J. Med., **283**, 1313 (1970).
21. Harris, R.I., et al., An improved chromogenic substrate endotoxin assay for clinical use. J. Clin. Pathol., **36**, 1145 (1983).
22. Greisman, S.E., and Hornick, R.B., Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. Proc. Soc. Exp. Biol. Med., **131**, 1154 (1969).

RBG,MAM 07/09-1