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ProductInformation

E-TOXATE® Dry Concentrate
Product Codes: **E 9154**

TECHNICAL BULLETIN

Product Description

Sigma's E-TOXATE®(*Limulus* Amebocyte Lysate) Dry Concentrate is intended for use in 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.

E-TOXATE 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]

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

Components

E-TOXATE Dry Concentrate, Product No. E 9154
Dry concentrate from *Limulus polyphemus*.
Sensitive to 0.05 to 0.1 endotoxin units (EU) per ml.

Water, Endotoxin-free, Product No. W 1764
For your 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.

Reagents and Materials Required but not Provided

- Endotoxin Standard, Product No. E 8029
  Endotoxin (*E. coli* 0.55:B5 lipopolysaccharide).
  10,000 to 20,000 endotoxin units (EU) per vial.
  Actual value stated on label. Standardized against USP Reference Standard Endotoxin (RSE).
- Sterile, pyrogen-free glassware or plasticware, including:
  Pipets: 5 and 1 ml, serologic
  Syringes and needles
  Test tubes, glass (10 x 75 mm), (for endotoxin determination)
  Sterile, polystyrene culture tubes (for Endotoxin Standard Preparation)
- Sterile water for injection, USP or sterile water for irrigation, USP.
  Note: We do not recommend bacteriostatic water.
- Water bath or heating block, 37 °C. **Do not use air bath**
- pH meter or narrow-range (pH 6 to 8) pH indicator paper

Optional Reagents

SIGMACOTE®, Product No. SL-2
Organic solvent based siliconizing solution for labware.

E-TOXA-CLEAN Concentrate, Product No. E 9029
Alkaline detergent for preliminary cleaning of glassware prior to inactivation of endotoxins by steam sterilization and dry heating. Prepare a 1% solution by dissolving approximately 10 g E-TOXA-CLEAN in 1000 ml of hot tap water.

Hydrochloric acid, 0.1 N, Product No. H 4037
Endotoxin-free. For adjusting pH of samples when necessary. Suitable for use if introduction of contaminating organisms or endotoxin is avoided.

Sodium Hydroxide, 0.1 N, Product No. S 4067
Endotoxin-free. For adjusting pH of samples when necessary. Suitable for use if introduction of contaminating organisms or endotoxin is avoided.

Heparin, Product No. H 6162
Endotoxin-free, sodium salt, 300 USP units/vial. Sufficient for 5 ml of blood.

Note: Endotoxin-free in this procedure implies that a negative result was obtained when tested by the E-TOXATE assay.

Precautions and Disclaimer
Sigma E-TOXATE products are for research purposes only. E-TOXATE **may not** be used in the diagnosis of endotoxemia in man nor for final product testing of endotoxin in pharmaceuticals.

A material safety sheet (MSDS) for this product has been sent to the attention of the safety officer of your institution. Consult the MSDS for information regarding hazards and safe handling practices.

Storage/Stability
E-TOXATE Dry Concentrate: Store below 0 °C.
Water, Endotoxin-free: Store at room temperature.
Stable indefinitely if introduction of contaminating organisms or endotoxin is avoided.

Notes
- 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. If glassware is to be siliconized using an organic solvent-based siliconizing solution, follow procedure (a), then continue with Step 2 under "Preparation of Endotoxin-Free Equipment" section. If glassware is to be siliconized using an aqueous-based siliconizing solution, follow procedure (b) then continue with Step 2 under "Preparation of Endotoxin-Free Equipment" section.
  a. If using Sigma's SIGMACOTE®, Product No. SL-2, cover or immerse the glassware in the undiluted SIGMACOTE for 2 to 3 minutes, excess solution removed and the treated glassware is allowed to air dry in a hood. Rinse the siliconized glassware with deionized or distilled water to remove the HCl by-products before use. For other commercially available organic solvent-based siliconizing solutions, follow their recommended procedure for application.
  b. If using a commercially available aqueous-based siliconizing solution, follow their recommended procedure for application
- Do not return pipets, needles, etc., or excess lysate back to stock lysate solution vial; this may introduce contamination. When sampling E-TOXATE, remove only the quantity required for assays, discarding pipet or other glassware and lysate residue, if any, rather than risking back contamination of remainder.
- The given protocol incorporates the use of Endotoxin Standard, Product No. E 8029. For those wishing to use the FDA Endotoxin Reference, Lot EC-5, or other lipopolysaccharides as endotoxin reference, we recommend the following steps for preparation of a "stock" endotoxin solution from:
  a. FDA (or other) preweighed Endotoxin Reference: Reconstitute according to accompanying instructions
  b. Other (bulk) lipopolysaccharide powder:
     1. Using aseptic and endotoxin-free technique, accurately weigh a few milligrams of powder into an endotoxin-free capped polystyrene or siliconized glass culture tube.
     2. Add 1.0 ml of endotoxin-free water for each milligram of lipopolysaccharide, preparing a 1 mg/ml endotoxin solution. Recap tube.
     3. Vortex mix the endotoxin solution for approximately 20 minutes. Store overnight at 2 to 6 °C to improve solubility before making further dilutions.
     4. The endotoxin solution should be vortex-mixed for 20 minutes prior to use in preparing further dilutions, as described under "Endotoxin Standard Dilutions" section.
- 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.

Notes (continued from previous page)

- Some test samples may exhibit "enhancement" of the lysate reaction by amplifying the expected endotoxin sensitivity, thereby yielding erroneously higher results. 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 that in place of Endotoxin-free Water, use the minimum test sample dilution in step 1 above as diluent to prepare the standard dilutions.
  3. Prepare a series of Endotoxin Standard Dilutions using Endotoxin-free Water as described under "Endotoxin Standard Dilutions" section.
  4. Perform side-by-side testing of each dilution from steps 2 and 3 above by mixing 0.2 ml with E-TOXATE Reagent 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 above 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 step 2 and step 3 above, yielded positive tests at 0.06 and 0.125 EU/ml, respectively.
  Since these results are within one dilution (see dilution scheme under "Endotoxin Standard Dilutions" section), it may be concluded that there is no enhancement of lysate sensitivity by the sample.

- False positives are reportedly caused by:
  Trypsin and trypsin-like enzymes[9-10]
  Thrombin, thromboplastin, polynucleotides and ribonuclease[14]
  Enhancement of lysate sensitivity (as described in note above) by various substances including calcium has been reported.[15]
- False negatives are reportedly caused by:
  Trypsin inhibitors, EDTA and other calcium binding reagents[2]
  High molar (>2 M) salt concentration[16]
  Semisynthetic penicillins[17]

Preparation of Endotoxin-free Equipment

Since extremely minute quantities of endotoxin can cause E-TOXATE 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 below. The following procedure is recommended for contaminated glassware:

1. Soak glassware, preferably overnight, in 1% solution of an alkaline detergent, e.g., E-TOXA-CLEAN® Product No. E 9029. When possible, scrub with a clean brush. If glassware is to be siliconized, see "Notes" section above.
2. Rinse all glassware 8 to 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 pipets are plugged with nonabsorbent cotton and placed tip down in a stainless steel pipet 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. Screw-capped test tubes with Bakelite caps and rubber liners 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.

Preparation of 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 (optimum range 6.8 to 7.5[18,19]). The pH may be adjusted as needed with Hydrochloric acid, endotoxin-free, Product No. H 4037, or Sodium hydroxide, endotoxin-free, Product No. S 4067

Caution: pH electrodes may contaminate the solution. The pH of sample can usually be checked by applying drops to pH indicator paper with pyrogen-free Pasteur pipets. Alternatively, 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.

Preparation of Plasma Samples

For plasma or other biological materials that may be contaminated with blood, researchers are referred to the following procedures for the removal of the LAL inhibitor:

The chloroform extraction technique of Levin, et al.[20]

The dilution-heating technique of Harris, et al.[21]

Note: The choice of technique is determined by sensitivity of the lysate and by endotoxin levels deemed significant.[22] Unless grossly bloody, fluids other than plasma do not require inhibitor removal.

Preparation of Endotoxin Standard Stock Solution

See "Notes" section regarding suitable containers for use in preparing Endotoxin Standard Working Solutions.

Reconstitute Endotoxin Standard, Catalog No. E 8029, with appropriate amount of Endotoxin-free Water stated on label to obtain Endotoxin Standard Stock Solution, 4000 EU per ml. Mix vigorously (vortex mixer) for at least 2 minutes. Then vortex approximately 30 seconds at 10 minute intervals over a 30 minute period. Solution is stable stored in refrigerator for at least 2 weeks if kept free of contamination. Before each use, mix as previously described. **Do not freeze.**

Endotoxin Standard Dilutions

Endotoxin standard dilutions containing at least 400 EU/ml are generally stable for at least one week stored in refrigerator if kept free from contamination. All other dilutions should be prepared fresh daily. See "Notes" section for use of endotoxin standards other than Endotoxin Standard, Product No. E 8029.

1. Mix Endotoxin Standard Stock Solution (4000 EU/ml) using vortex mixer. All endotoxin dilutions should be prepared in sterile, capped polystyrene tubes
2. Prepare dilutions of Endotoxin Standard Stock using Endotoxin-free Water as indicated below:

Tube No.	Endotoxin	Endotoxin-free Water (ml)	Final Conc. (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 to 60 seconds prior to further dilution or assay. Any endotoxin solution standing for more than 30 minutes should be vortexed prior to use.

E-TOXATE Assay Procedure

All assays using single test vials are performed directly in the vial of E-TOXATE, Product No. E 9154. Remove aluminum seal and loosen rubber stopper after labeling.

1. Label 8 vials (Product No. E 9154) as in chart below. One set of Vials A and B are needed for each sample to be tested. Vials D, E, F, G, and H are used to determine the sensitivity of the E-TOXATE lot being used and also serve as positive controls. Vials E, F, G and H may be omitted if sensitivity information is unnecessary. Vial B may be omitted if sample has been previously shown to be free of E-TOXATE inhibitor.
2. Add test sample, water and Endotoxin Standard Dilutions to vials as indicated below. Make additions by carefully lifting rubber stopper from each vial, adding solutions directly to bottom and replacing stopper.

Tube		Sample	Endotoxin-free water	Endotoxin Std. Dilution
A	test for endotoxin in sample	0.2 ml	–	–
B	test for E-TOXATE inhibitor in sample	0.2 ml	–	0.01 ml of 4 EU/ml
C	negative control	–	0.2 ml	–
D	standard	–	–	0.2 ml of 0.25 EU/ml
E	standard	–	–	0.2 ml of 0.125 EU/ml
F	standard	–	–	0.2 ml of 0.06 EU/ml
G	standard	–	–	0.2ml of 0.03 EU/ml
H	standard	–	–	0.2 ml of 0.015 EU/ml

3. Very briefly vortex-mix each vial to ensure homogeneity. Avoid excessive mixing and foaming. Incubate tubes undisturbed for 1 hour at 37 °C. See "Notes" section regarding incubation precautions.
4. To evaluate test results see "Reading and Interpretation of E-TOXATE Assay" section below.

Reading and Interpretation of E-TOXATE Assay

Reading:

After 1-hour incubation, gently remove tubes or vials one at a time and slowly invert 180 degrees 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, 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 endotoxin-free water and test each dilution as under "Tube A" until a negative test result is obtained. Note the greatest dilution of sample and lowest concentration of Endotoxin Standard yielding positive test results. The endotoxin level, EU/ml is then derived 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:

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

(+) Hard gel

(–) Absence of hard gel

Tube				Interpretation
A	**B**	**C**	**D**	
–	+	–	+	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, glassware or E-TOXATE 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 E-TOXATE. Test is not valid.
–/+	–/+	–	–	E-TOXATE or Endotoxin Standard has deteriorated. (Sample results are not valid unless Tubes B and D show hard gels.)

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