BIOENDO

Xiamen Bioendo Technology Co., Ltd.

Bioendo™ EC Endotoxin Test Kit (End-point Chromogenic Assay) 64 Tests/Kit

CATALOG NUMBER

EC64405S

INTENDED USE

Bioendo™ EC Endotoxin Test Kit (End-point Chromogenic Assay) is intended for use in the *In Vitro* quantitative detection of gram-negative bacterial endotoxins (Lipopolysaccharides).

WARNING

For *In Vitro* use only. Do not use it for the detection of endotoxemia in human.

EXPLANATION OF TEST

The end-point chromogenic endotoxin assay is conducted by adding a colorless artificial peptide substrate solution into amebocyte lysate and test sample mixture after a certain incubation period. If endotoxin is present in the sample, a yellow color will develop. Its absorbance (λmax = 405 nm) is related to the endotoxin concentration, therefore the endotoxin levels in unknown samples can be calculated against a standard curve.

PRINCIPLES

Lyophilized Amebocyte Lysate (AL, TAL or LAL) Reagent is an aqueous extract of circulating amebocytes of Chinese horseshoe crab (*Tachypleus tridentatus)*. The lysate contains a cascade of serine protease enzymes (proenzymes) which can be activated by bacterial endotoxins. Endotoxins activate the proenzymes to produce activated enzymes (termed coagulase), the latter catalyzes the split of the colorless substrate, releasing a yellow-colored product pNA. The released pNA can be measured photometrically at 405 nm.

The absorbance is positively correlated with the endotoxin concentration, based on which, the endotoxin concentration is quantified.

REAGENTS SUPPLIED

1. Lyophilized Amebocyte Lysate

Catalog No. EC17, Blue-Labeled Vial

Two vials of lyophilized AL, 1.7 ml each vial. Lyophilized AL was produced from amebocyte lysate of *Tachypleus tridentatus*, the lysate is stabilized by monovalent and divalent cations.

With different incubation time, the end-point chromogenic assay can detect different ranges of endotoxin concentration. Procedure for two ranges is provided in this assay kit, one is 0.1-1.0 EU/ml and the other is 0.01-0.1 EU/ml. The lysate is not labeled with a specific sensitivity. Sensitivity in a given test (designated λ) is the lowest

endotoxin concentration used to construct the standard curve. The greatest sensitivity λ of this assay kit is 0.01 EU/ml.
Store at temperature 2-8 ºC is recommended. Avoid exposure to temperatures above 25 ºC or to bright light for prolonged periods.
Reconstituted AL reagent should be used in 10 minutes. If freeze reconstituted AL reagent at the temperature below -20 ºC immediately after reconstitution, the reagent will be stable for up to 28 days. Dissolve only once.

2. Water for BET

Catalog No. TRW50, Grey-Labeled Vial
Three bottles of Water for BET, 50 ml each bottle. The endotoxin concentration of Water for BET is less than 0.005 EU/ml. Water for BET is used to rehydrate AL reagent, CSE and chromogenic substrate, to dilute endotoxin standards and test samples, and as a negative control (blank). Water for BET should be stored at 2-30 ºC.

3. Control Standard Endotoxin (CSE)

Catalog No. CSE10, Red-Labeled Vial
Two vials of control standard endotoxin (CSE), bacterial strain *E.coli* O111:B4. Each vial contains 10-60 EU lyophilized endotoxin, the potency of CSE is labeled at the Certificate of Analysis. Store at 2-8 ºC.
Reconstitute CSE with Water for BET. The reconstitution volume is indicated in the Certificate of Analysis. Mix vigorously for 5 minutes on a vortex mixer.
Reconstituted endotoxin solution of potency less than 20 EU/ml is stable for up to one day at 2-8ºC, for endotoxin solution potency is equal or higher than 20 EU/ml, it is stable for at least one week at 2-8ºC. Do not freeze endotoxin solution.

4. Chromogenic Substrate

Catalog No. ECCS17, Yellow-Labeled Vial
Four vials lyophilized chromogenic substrate, 1.7 ml each vial. Store at 2-8 ºC. Each vial of substrate is rehydrated with labeled amount of Water for BET. Reconstituted chromogenic substrate is stable for one week at 2-8 ºC.

5. REAGENTS REQUIRED BUT NOT SUPPLIED

25% v/v glacial acetic acid in water (Stop reagent), store at 2-8 ºC. Preparation method is as follows:

Water for BET (ml)	Glacial acetic acid (ml)	Stop reagent
15	5	20 ml/vial
75	25	100 ml/vial

MATERIALS AND EQUIPMENT REQUIRED

1. Pyrogen-free pipettes, 1.0 ml, 100 microliters. Or automatic pipettes with pyrogen-free tips.
2. Pyrogen-free microplate (Catalog No. MP96, MPC96 or equivalent).

3. Endotoxin-free glass dilution tubes for endotoxin dilution and sample dilution (Catalog No. T1310030，T1310005，T1310005C or equivalent).
4. Test tube rack.
5. Timer.
6. Vortex Mixer.
7. Microplate reader with 405 nm filter.
8. Dry Bath Heater at 37 ± 1 ºC with microplate adapter (if using a non-incubating microplate reader).
9. Pyrogen-free reagent reservoirs (optional).
10. Multi-channel pipettor(optional).

SAMPLE COLLECTION AND PREPARATION

All glassware, plasticware, and diluents to be in contact with sample or test reagents should be endotoxin-free. Glassware and other heat-stable apparatus could be depyrogenated in oven using a validated process, a commonly used minimum time and temperature setting is 60 minutes at 250 ºC.

Use aseptic techniques all along.

Samples to be tested should be stored under conditions that bacteriological activities are stopped. Samples could be kept at 2-8 ºC for temporary storage (less than 24 hours), samples should be kept below -10 ºC for long term storage.

The optimal pH range for AL-endotoxin reaction is from 6.0 to 8.0. Acidic and basic sample could be adjusted to desired pH range with endotoxin-free 0.1 N sodium hydroxide, 0.1 N hydrochloric acid, or endotoxin-free Tris buffer.

Always measure the pH of an aliquot of the bulk sample to avoid contamination by the pH electrode.

Potential of interference presence should be tested and eliminated following the description in **PRODUCT INHIBITION/ENHANCEMENT** section.

REAGENT PREPARATION

Caution: Allow reagents to warm to room temperature before use. All solution can be prepared at room temperature.

Preparation of Endotoxin Standards

Caution: Reconstitute CSE immediately prior to use.

1. Reconstitute CSE with Water for BET. The reconstitution volume is indicated in the Certificate of Analysis. Mix vigorously for 5 minutes on a vortex mixer, obtaining 10 EU/ml of endotoxin stock solution. Reconstituted endotoxin stock solution is stable for one day at 2-8 ºC, do not freeze endotoxin solution.
2. **For detection of endotoxin ranging from 0.1 to 1.0 EU/ml:**
   Prepare endotoxin standards at concentrations of 0.1 EU/ml, 0.25 EU/ml, 0.5 EU/ml and 1.0 EU/ml.
   Mix 0.2 ml 10 EU/ml of endotoxin with 1.8 ml Water for BET, vortex for 1 minute, obtaining 1.0 EU/ml endotoxin,
   Mix 1.0 ml 1.0 EU/ml of endotoxin with 1.0 ml Water for BET, vortex for 1 minute, obtaining 0.5 EU/ml endotoxin,

Mix 0.5 ml 1.0 EU/ml of endotoxin with 1.5 ml Water for BET, vortex for 1 minute, obtaining 0.25 EU/ml endotoxin,

Mix 0.2 ml 1.0 EU/ml of endotoxin with 1.8 ml Water for BET, vortex for 1 minute, obtaining 0.1 EU/ml endotoxin.

For detection of endotoxin ranging from 0.01 to 0.1 EU/ml:

Prepare endotoxin standards at concentrations of 0.01 EU/ml, 0.025 EU/ml, 0.05 EU/ml and 0.1 EU/ml.

Mix 0.2 ml 10 EU/ml of endotoxin with 1.8 ml Water for BET, vortex for 1 minute, obtaining 1.0 EU/ml endotoxin,

Mix 0.2 ml 1.0 EU/ml of endotoxin with 1.8 ml Water for BET, vortex for 1 minute, obtaining 0.1 EU/ml endotoxin,

Mix 1.0 ml 0.1 EU/ml of endotoxin with 1.0 ml Water for BET, vortex for 1 minute, obtaining 0.05 EU/ml endotoxin,

Mix 0.5 ml 0.1 EU/ml of endotoxin with 1.5 ml Water for BET, vortex for 1 minute, obtaining 0.025 EU/ml endotoxin,

Mix 0.2 ml 0.1 EU/ml of endotoxin with 1.8 ml Water for BET, vortex for 1 minute, obtaining 0.01 EU/ml endotoxin.

3. Discard the remained endotoxin dilutions after 4 hours.

Preparation of AL reagent and chromogenic substrate

Caution: Reconstitute immediately prior to use.

1. Gently tap the vial allowing AL reagent powder to fall to the bottom of the vial, add 1.7 ml Water for BET to lyophilized AL. Mix gently by tilting and swirling the vial until the contents are in solution. A small amount of AL powder left on the stopper will not affect the test.
2. Rehydrate the chromogenic substrate with 1.7 ml Water for BET.

TEST PROCEDURE

Caution: The following experiments must be performed at 37±1.0° C.

The microplate should be preheated for 10 min on the microplate reader or dry bath heater before adding reagent.

For detection of endotoxin ranging from 0.01 to 0.1 EU/ml:

1. Pre-equilibrate the microplate at 37 ºC ± 1.0 ºC in the heating block adapter.
2. While leaving the microplate at 37 ºC ± 1.0 ºC, dispense 50 µl of Water for BET (as blanks), 0.01 EU/ml, 0.025 EU/ml, 0.05 EU/ml, 0.1 EU/ml endotoxin standard solution and test samples into the appropriate microplate well. Run all the reaction in duplicate.
3. Dispense 50 µl AL into each well. (If using multi-channel pipettor, pipette adequate amount of AL into a reagent reservoir, then dispense 50 µl AL into each well.) Mix well immediately. It is important to be consistent in the order of reagent addition from well to well or row to row, and in the rate of pipetting.
4. Place microplate cover, let the plate stay in the heating block for the time indicated in Certificate of Analysis.
5. Pipette adequate chromogenic substrate solution into a reagent reservoir (optional).

Dispense 100 µl chromogenic substrate solution into each well. Mix well immediately. The order of adding chromogenic substrate solution should be consistent with Step 3.
6. Place the microplate cover, let the plate stay in the heating block for the time indicated in Certificate of Analysis.
7. Pipette adequate amount of stop reagent into a reagent reservoir (optional). After Step 6's incubating time ended, dispense 50 µl stop reagent into each well. Maintain the same pipetting order as in Step 3 and Step 5. Mix well immediately.
8. Read the absorbance of each well at 405 nm.
The table below outlines the test procedure.

	Endotoxin Standard Wells	Sample Wells	Blank Wells
Pre-equilibrate the microplate at 37 ºC ± 1.0 ºC.			
Add Water for BET			50 µl
Add endotoxin standard	50 µl		
Add sample		50 µl	
Add AL reagent	50 µl	50 µl	50 µl
Mix well, incubating at 37 ºC ± 1.0 ºC for the time (T1) indicated in Certificate of Analysis.			
prewarm chromogenic substrate solution at 37 ºC ± 1.0 ºC for 5 minutes			
Add chromogenic substrate solution	100 µl	100 µl	100 µl
Mix well, incubating at 37 ºC ± 1.0 ºC for the time (T2) indicated in Certificate of Analysis.			
Add 50 µl stop reagent	50 µl	50 µl	50 µl
Mix well, read the absorbance at 405 nm			

For detection of endotoxin ranging from 0.1 to 1.0 EU/ml:
All of the test procedures are the same as the 0.01 to 0.1 EU/ml range, except in the step 2, the endotoxin standard solutions are 0.1 EU/ml, 0.25 EU/ml, 0.5 EU/ml and 1.0 EU/ml. And in step 4 and step 6 the incubation time should be change to what indicated in Certificate of Analysis.

DATA COLLECTION AND ANALYSIS
1. Obtain the absorbance values at 405 nm.
2. Construct the standard curve
   Y = b X + a, where
   Y = absorbance, X = endotoxin concentration,
   b = slope of the regression curve, a = the Y intercept.

INITIAL QUALIFICATION
As the requirements in the Pharmacopeia, validation of the Amebocyte Lysate endotoxin assay should be performed when conditions that are likely to influence the test result change.

1. Standard Curve Validation

Once received a new lot of AL reagent, the test to validate the standard curve must be performed. Prepare at least three endotoxin concentrations within the standard curve range indicated by the Certificate of Analysis. Perform the assay using at least three replicates of each standard endotoxin concentration. Unlike the **ROUTINE TESTING**, do not average the absorbances of all replicates. The absolute value of the correlation coefficient, r, must be greater than or equal to 0.980 for the range of endotoxin concentrations set up.

2. Test for Interfering Factors

The test for interfering factors must be repeated when any conditions that is likely to influence the result of the test change. This includes but not limit to the change of the formulation of test samples, or change a new AL reagent provider. Please see **PRODUCT INHIBITION/ENHANCEMENT** section.

PRODUCT INHIBITION/ENHANCEMENT

If there is potential that the sample contains interfering substances, recovery rate test should be run. Prepare a Positive Product Control (PPC), PPC is a sample of product to which a known amount of endotoxin spike has been added. The spike concentration of endotoxin (λm) should be in the middle of the standard curve range.

1. Analyze the spiked sample (PPC) along with the un-spiked sample.
2. Determine the endotoxin concentration in spiked test sample (Cs) and the endotoxin concentration in un-spiked test sample (Ct).
3. Calculate the recovery rate (R): R= (Cs－Ct)/ λm ×100%.
4. Recovery rates within the range of 50% to 200% suggest non-significant interference. Recovery rates out of the range of 50% to 200% suggest significant interference. Dilution and modification are commonly employed to reduce the interference to non-significant level.
5. If R is outside 50% to 200%, repeat the inhibition test in a series of dilution (the dilution should not exceed the Maximum Valid Dilution) of test sample.
6. It is better to select a dilution factor of test sample at which R is closest to100% for the routine endotoxin concentration assay.

ROUTINE TESTING

In a routine testing, prepare a series of endotoxin standards, assay the endotoxin standards and unknown samples at the same time under the same condition. Calculate the concentration of endotoxin in unknowns by comparison to the performance of the standards. Each standard concentration and unknown samples should be run at least duplicate. Average the absorbances of all replicates during the calculation. The user may include a Positive Product Control (PPC) as a monitor for product inhibition or enhancement.

PERFORMANCE CHARACTERISTICS

The test is valid only when all of the following requirements met,

1. Prepare at least three endotoxin concentrations within the concentration range

along with blanks to generate the standard curve. Perform the assay using at least three replicates of each standard endotoxin concentration.

2. The absolute value of the correlation coefficient (r) of the calculated standard curve should be ⩾0.980.
3. Both absorbances of the negative controls are lower than that of the lowest concentration of the endotoxin standard curve.
4. The coefficient of variation (C.V.) equals the standard deviation of the absorbances divided by the mean and is usually expressed as a percent. The %C.V. of the absorbances for the replicates should be less than 10%.

COLORED SAMPLES

Sample blank should be run for colored samples, and samples which produce yellow product in acid environments. The only difference between sample blank and sample is replacing AL reagent with equal volume of Water for BET for sample blank. If the absorbance of the sample blank is greater than 0.5, the sample should be diluted and reanalyzed.

REFERENCES

1. Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices. U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration, December 1987.
2. Bacterial Endotoxins Test, USP 26 NF 21. 2003.
3. Bang, F. B. 1956. A Bacterial Disease of Limulus Polyphemus. Bull. Johns Hopkins Hosp. 98:325-351.
4. Levin, J., and F. B. Bang. 1964. A description of cellular coagulation in the Limulus. Bull. Johns Hopkins Hosp. 115:337-345.
5. Levin, J., and F. B. Bang. 1964. The role of endotoxin in the extracellular coagulation of Limulus blood. Bull. Johns Hopkins Hosp. 115:265-274.
6. Levin, J., and F. B. Bang. 1968. Clottable protein in Limulus: its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. 19:186-197.