BIOENDO

Xiamen Bioendo Technology Co., Ltd.

# Bioendo™ KC Endotoxin Test Kit (Kinetic Chromogenic Assay)

## CATALOG NUMBER
KC0828S, KC5028S

## INTENDED USE
Bioendo™ KC Endotoxin Test Kit (Kinetic Chromogenic Assay) is intended for use in the *In Vitro* quantitative detection of gram-negative bacterial endotoxins (Lipopolysaccharides) with kinetic chromogenic method.
The test procedures described herein are conformed to Chapter <85> Bacterial Endotoxins Test,United States Pharmacopeia.

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

## EXPLANATION OF TEST
The kinetic chromogenic Amebocyte Lysate endotoxin assay is a quantitative assay for the detection of gram-negative bacterial endotoxins. The assay is conducted by mixing Chromogenic Amebocyte Lysate with test sample followed by recording the mixture's absorbance at 405 nm for 120 min at 37 ºC. The velocity of the absorbance increase is related to the endotoxin concentration of the test sample. A fast absorbance increase indicates high endotoxin concentration, whereas a slow one indicates low endotoxin concentration. Endotoxin concentration is determined based on the fastness of the absorbance increase.

## PRINCIPLES
Lyophilized Chromogenic Amebocyte Lysate (AL, TAL or LAL) 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. With the formation of pNA, the absorbance at 405 nm is increased. The velocity of the absorbance increase is positively correlated with the endotoxin concentration. That is, the time needed to reach a certain absorbance increase (onset OD), termed onset time, is negatively correlated with the endotoxin concentration. Based on which, the endotoxin concentration of test sample can be quantified.

## REAGENTS SUPPLIED
### 1. Chromogenic Amebocyte Lysate
Catalog No. KC28S, Blue-Labeled Vial
Lyophilized Chromogenic AL reagent was a mixture of Amebocyte Lysate and chromogenic substrate.Chromogenic AL reagent was produced from amebocyte lysate of *Tachypleus*

*tridentatus,* the lysate was stabilized by monovalent and divalent cations. The detection limit of provided assay kit for endotoxin test is 0.001 EU/ml. Reconstitute by Reconstitution Buffer, reconstitution volume is stated on the label.
Store at 2-8 ºC. Avoid exposure to bright light. Reconstituted Chromogenic AL reagent should be used in 10 minutes. If freeze reconstituted Chromogenic AL reagent at the temperature below -20 ºC immediately after reconstitution, the reagent will be stable for up to 14 days. Dissolve only once.

**2. Reconstitution Buffer**
Catalog No. KCRB35, KCRB60 or KCRB 30, Green-Labeled Vial
Reconstitution Buffer is used to rehydrate Chromogenic AL Reagent.
Allow buffer to warm to room temperature before use.
Store Reconstitution Buffer at 2-8 ºC.

**3. Control Standard Endotoxin (CSE)**
Catalog No. CSE10, Red-Labeled Vial
Control Standard Endotoxin (CSE), each vial contains 20-199 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 is stable for 7 days at 2-8 ºC. Do not freeze endotoxin solution.

**4. Water for BET**
Catalog No. TRW50, Grey-Labeled Vial
Water for BET, 50 ml each bottle. The endotoxin concentration of Water for BET is less than 0.001 EU/ml. Water for BET is used to rehydrate CSE, to dilute endotoxin standards and test samples, and as a negative control (blank). Water for BET should be stored at 2-30 ºC.
**Note: Water for BET is included in KC0828S, not in KC5028S.**

**MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED**
1. Single-channel pipettors with pyrogen-free tips.
   Multiple-channel pipettor with pyrogen-free reagent reservoirs, or repeating pipettor with pyrogen-free syringe reservoirs.
2. Endotoxin-free glass dilution tubes for preparation of endotoxin standard and test sample (Catalog No. T1310030，T1310005，T1310005C or equivalent).
3. Vortex Mixer.
4. Pyrogen-free 96-well microplates (Catalog No. MP96, MPC96 or equivalent).
5. Incubating microplate reader (BioTekTM ELx808IULALXH，BioTek™ ELx808IU) with kinetic assay software.

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

**ENDOTOXIN STANDARD PREPARATION**

Reconstitute Control Standard Endotoxin with Water for BET, mix vigorously for at least 5 minutes on a vortex mixer, obtaining 50 EU/ml standard stock solution. Reconstituted endotoxin stock solution is stable for up to 7 days at 2-8 ºC. Do not freeze endotoxin solution. Refrigerated endotoxin solution should be mixed vigorously for 5 minutes on a vortex mixer prior to use.
To prepare endotoxin standards at concentrations of 0.001 EU/ml, 0.01 EU/ml, 0.1 EU/ml and 1 EU/ml，**If necessary, the standard curve can reach 10 EU/ml.**

1. Mix 0.1 ml of 50 EU/ml endotoxin with 0.4 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 10 EU/ml endotoxin.
2. Mix 0.1 ml of 10 EU/ml endotoxin with 0.9 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 1.0 EU/ml endotoxin.
3. Mix 0.1 ml of 1.0 EU/ml endotoxin with 0.9 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 0.1 EU/ml endotoxin.
4. Mix 0.1 ml of 0.1 EU/ml endotoxin with 0.9 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 0.01 EU/ml endotoxin.
5. Mix 0.1 ml of 0.01 EU/ml endotoxin with 0.9 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 0.001 EU/ml endotoxin.
6. Discard the remained endotoxin dilutions after 4 hours.

**SETTING MICROPLATE READER**

Set the incubator temperature at 37 ºC.
Set up the template and procedure.
Set wavelength at 405 nm and onset OD at 0.2.
Set the reading parameters as kinetic reading for 120 minutes with interval of 60 seconds.
Set the plate shaking speed as medium speed for 5 seconds before the kinetic reading started.

**TEST PROCEDURE**

1. Start to run assay after the kinetic incubator temperature reached 37 ºC.
2. Run assay in duplicate.
3. Vortex endotoxin standards and samples before loading.
4. Transfer 100 µl of Water for BET (as negative control), endotoxin standard solutions, and test sample into each well of the microplate. Avoid air bubbles.
5. At the end of step 4, the miroplate should be preheated for 10 min on the kinetic incubator.

6. After the microplate starts to preheat, reconstitute each vial of Chromogenic AL reagent with labeled amount of **Reconstitution Buffer.** Mix gently by tilting and swirling the vial until the contents are in solution. Do not use vortex mixer. Reconstituted Chromogenic AL reagent solution should be used within 10 minutes at room temperature. If more than one vial is required, pool two or more vials before use.
7. Transfer Chromogenic AL reagents into a reagent reservoir, mix by rocking the reservoir from side to side. Add 100 µl Chromogenic AL reagent into each well of the microplate rapidly. Application of multiple-channel pipettor or repeating pipettor is recommended. Avoid air bubbles. Leave the microplate uncover.
8. Place the microplate into the kinetic incubating reader.
9. Start to run the kinetic program. The kinetic program should include a plate shaking step of medium speed for 5 seconds before the kinetic reading started to mix the Chromogenic AL reagent and test sample sufficiently. Record the kinetic readings every 60 seconds for 120 minutes at wavelength of 405 nm.

## DATA COLLECTION AND ANALYSIS

### Linear Regression

1. Obtain the onset time for onset OD 0.2 at 405 nm.
2. Construct the standard curve
   $\log_{10} y = b (\log_{10} x) + a$, where
   y = reaction time (onset time), x= endotoxin concentration,
   b = slope of the regression curve, a = the Y intercept.

### Polynomial Regression

If the absolute value of the correlation coefficient (r) is $\geqslant$0.980, a polynomial regression can be used to construct a standard curve. The polynomial regression improves the accuracy of predicting endotoxin concentration over the entire endotoxin range.

When using polynomial regression, a standard curve is generated using the $\log_{10}$ reaction time values and their corresponding $\log_{10}$ endotoxin concentration to define a polynomial equation. The order of the polynomial equation used to generate the regression curve is determined by the number of endotoxin standards in the assay. The order of the polynomial will always be one less than the number of endotoxin standards, with a maximum of a fourth-order polynomial for assays with five or more endotoxin standards and a minimum of a second order polynomial for assays with three standards. A fourth-order polynomial equation as following example:

$$\log_{10} y = A + B(\log_{10} x) + C(\log_{10} x)^2 + D(\log_{10} x)^3 + E(\log_{10} x)^4$$

It is important to note that the polynomial regression **CANNOT** be used for **INITIAL QUALIFICATION** assays.

## INITIAL QUALIFICATION

As the requirements in the Pharmacopeia, validation of the Chromogenic 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 Chromogenic 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. The endotoxin standards should be included to bracket each log increase in the assay. For example, if the standard range is 0.001-1 EU/ml, the endotoxin standards could be 0.001, 0.01, 0.1, 1 EU/ml. Perform the assay using at least three replicates of each standard endotoxin concentration. Unlike the **ROUTINE TESTING**, do not average the onset time 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 are 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 Amebocyte Lysate 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 onset time of all replicates during the calculation.

The user may include a Positive Product Control (PPC) as a monitor for product inhibition or enhancement. For example, in a 0.001-1 EU/ml standard curve range, PPC wells could be prepared by transfer 10 µl of the 1.0 EU/ml endotoxin solution into each 0.1 ml of test sample (or dilution). The well will have a 0.1 EU/ml endotoxin concentration. Mix gently by tapping the side of the plate.

**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. The onset time of both of the negative controls are longer than that of the lowest concentration of standard.
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**

Colored sample if the absorbance is larger than 1.5 absorbance units, should be diluted until absorbance is less than 1.5 absorbance units.

**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.
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