Bioendo™ rFC Endotoxin Test Kit

(Recombinant Factor C Fluorometric Assay) Instruction

CATALOG NUMBER

RFC96TS，RFC96T

Note: Bioendo™ rFC Endotoxin Test Kit (Recombinant Factor C Fluorometric Assay) is sufficient to do 96 tests per kit.

INTENDED USE

Bioendo™ rFC Endotoxin Test Kit (Recombinant Factor C Fluorometric Assay) is intended for use in the *In Vitro* quantitative detection of gram-negative bacterial endotoxins (Lipopolysaccharides) by recombinant Factor C.

WARNING

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

PRINCIPLE

Horseshoe Crab amebocyte Factor C is the first enzyme in amoebocyte of horseshoe crab serine protease catalytic cascade activated by endotoxin. This kit uses gene recombination technology to express Factor C of *Tachypleus* amebocyte in eukaryotic cells. After the recombinant factor C is activated by endotoxin, it is transformed from an inactive proteasome to a bioactive protease to recognize and catalyze the fluorescent substrate to generate fluorescent signals. The intensity of fluorescence signal is positively correlated with the concentration of endotoxin. The concentration of endotoxin in the sample is calculated by comparing with the standard curve.

STORAGE CONTIONS

Bioendo™ rFC Endotoxin Test Kit (Recombinant Factor C Fluorometric Assay) is stored at 2-8° C.

REAGENTS SUPPLIED

1. Recombinant Factor C

Catalog No. RFC48TS or RFC48T, Blue-Labeled Vial

Two vials of lyophilized Recombinant Factor C (rFC) , 2.5 ml each vial. The rFC is co-lyophilized with Fluorogenic Substrate. 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. For RFC96TS, the greatest sensitivity λ of this assay kit is 0.005EU/ml; for RFC96T, the greatest sensitivity λ of this assay kit is 0.01EU/ml. Each vial is sufficient to do 48 tests.

Store at temperature 2-8 °C is recommended. Avoid exposure to temperatures above 25 °C or to bright light for prolonged periods.
Reconstituted Recombinant Factor C could be stored at 2-8° C for 48 hours or -20 ° C for one month, only dissolve once.

2. Reconstitution Buffer

Catalog No. RFCRB48T, Green-Labeled Vial
Two bottles of Reconstitution Buffer, 3.0ml each bottle. The Reconstitution Buffer is used to rehydrate Lyophilized Recombinant Factor C. Reconstitution Buffer should be stored at 2-8 °C.

3. Water for BET

Catalog No. TRW50, Grey-Labeled Vial
One bottle of Water for BET, 50 ml. The endotoxin concentration of Water for BET is less than 0.003 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 °C.

4. Control Standard Endotoxin (CSE)

Catalog No. CSE10V, Red-Labeled Vial
Two vials of control standard endotoxin (CSE), bacterial strain *E.coli* O111:B4. Each vial contains 10-50 EU lyophilized endotoxin, the potency of CSE is labeled at the Certificate of Analysis. Store at 2-8 °C.
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 2-8°C, for endotoxin solution potency is equal or higher than 20 EU/ml, it is stable for at least one week at 2-8°C. Do not freeze endotoxin solution.

MATERIALS AND EQUIPMENT REQUIRED

1. Pyrogen-free pipettes, 1.0 milliliter and 100 microliters. Or automatic pipettes with pyrogen-free tips. (Supplied with the kit.)
2. Pyrogen-free black microplate strips for fluorogenic endotoxin assay (Catalog No. MPF96 or equivalent). (Supplied with the kit.)
3. Endotoxin-free glass dilution tubes for endotoxin dilution and sample dilution (Catalog No. T1310030，T1310005，T1310005C or equivalent). (Supplied with the kit.)
4. Test tube rack.
5. Timer.
6. Vortex Mixer.
7. Incubating Fluorescence Microplate reader, recommended model is H1F-SN from Agilent Technologies, Inc with excitation wavelength 380nm/ emission wavelength 440nm filters and Gen5 software.

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 °C.
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 °C for temporary storage (less than 24 hours), samples should be kept below -10 °C for long term storage.
The optimal pH range for rFC-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.

ENDOTOXIN STANDARD PREPARATION

Reconstitute Control Standard Endotoxin with 1ml Water for BET, mix vigorously for at least 5 minutes on a vortex mixer, obtaining Control Standard Endotoxin stock solution. For catalog number RFC96TS, the suggested standard curve range is 0.005-5EU/ml. To prepare endotoxin standards at concentrations of 0.005 EU/ml, 0.05 EU/ml, 0.5 EU/ml and 5 EU/ml. The following example is the Control Standard Endotoxin stock solution 20EU/ml:

1. Mix 0.5 ml of 20 EU/ml endotoxin with 1.5 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 5 EU/ml endotoxin.
2. Mix 0.1 ml of 5 EU/ml endotoxin with 0.9 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 0.5 EU/ml endotoxin.
3. Mix 0.1 ml of 0.5 EU/ml endotoxin with 0.9 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 0.05 EU/ml endotoxin.
4. Mix 0.1 ml of 0.05 EU/ml endotoxin with 0.9 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 0.005 EU/ml endotoxin.
5. Discard the remained endotoxin dilutions after 4 hours.

For catalog number RFC96T, the suggested standard curve range is 0.01-10EU/ml. To prepare endotoxin standards at concentrations of 0.01 EU/ml, 0.1 EU/ml, 1 EU/ml and 10EU/ml. The following example is the Control Standard Endotoxin stock solution 20EU/ml:

1. Mix 0.5 ml of 20 EU/ml endotoxin with 0.5 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 1EU/ml endotoxin.
3. Mix 0.1 ml of 1 EU/ml endotoxin with 0.9 ml Water for BET in an endotoxin-free test tube, vortex for 1 minute, obtaining 0.1EU/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 0.01EU/ml endotoxin.

5. Discard the remained endotoxin dilutions after 4 hours.

SETTING FLUORESCENCE MICROPLATE READER

1. Set the incubator temperature at 37 °C.
2. Set the plate shaking speed as medium speed for 15 seconds before reading.
3. Set the reading wavelength: when the fluorescence microplate reader H1F-SN is used, the excitation wavelength 380nm / emission wavelength 440nm is recommended. Reading setting as the following example:

	HIF-SN
Time between readings (hh:mm:ss)	00:T:00 (see Certificate of Analysis)
Excitation filter (nm)	380:20
Emission filter (nm)	440:30
Scans per well	10
Delay before scanning (miliseconds)	200
Optics position	top
Sensitivity	25, 27, 29 (select by checking)

4. Sensitivity Setting for Fluorescent Reader
   Before testing, it is necessary to adjust the sensitivity of the fluorescence readout to make the fluorescence signal fall within a suitable range, so that the detection range of the standard curve can be optimized. If the sensitivity is set too low, the fluorescence signal of the point with low endotoxin concentration in the standard curve is too weak, resulting in hard to separate the lowest point of the standard curve and the negative control. If the sensitivity is set too high, the fluorescence signal of the point with high endotoxin concentration in the standard curve is too strong, which will exceed the detection range. Fluorescence signals are normally recorded as Relative Fluorescence Units (RFU). It is recommended to adjust the net △ RFU value (RFU at time T minus RFU at time zero corrected with the blank) at the point where the endotoxin concentration is 0.5EU/ml in the standard curve to about 3000 (range from 2000-4500). Users can adjust the sensitivity of the fluorescent reader by adjust the fluorescence gain value.
   Taking H1F-SN as an example, during the initial detection, multiple fluorescence gain values should be set for the test (the recommended fluorescence gain values for the test are 25, 27 and 29), and each concentration point of the standard curve should be detected. Select the fluorescence gain value that gives the net △ RFU value of about 3000 at the point where the endotoxin concentration is 0.5EU/ml, and the linear correlation coefficient of the standard curve is closest to 1, as the fluorescence gain setting value of this lot of reagents.

TEST PROCEDURE

1. Start to run assay after the fluorescent microplate reader incubator temperature reached 37 °C.

2. Run assay in duplicate or triplicate.
3. Vortex endotoxin standards and samples before loading.
4. Place the endotoxin-free black microplate strips into the holder.
5. Dispense 100 µl of Water for BET (as negative control), endotoxin standard solutions, and test sample into each well of the microplate. Avoid air bubbles.
6. At the end of step 5, the microplate should be preheated for 10 min on the microplate reader incubator.
7. After the microplate starts to preheat, reconstitute each vial of Recombinant Factor C with fluorogenic substrate with 2.5ml **Reconstitution Buffer.** Mix gently by tilting and swirling the vial until the contents are in solution. Do not use vortex mixer.
   Reconstituted Recombinant Factor C solution should be stored at 2-8° C for 48 hours or -20 ° C for one month, only dissolve once.
   If more than one vial is required, pool two or more vials before use.
8. After the preheated was done, take out the microplate, dispense 50 µl rFC solution into each well of the microplate rapidly. Avoid air bubbles. Leave the microplate uncover.
9. Place the microplate into the fluorescent microplate reader.
10. Start to run the reading program. The reading program should include a plate shaking step of medium speed for 15 seconds before the reading started to mix the rFC and test sample sufficiently.
11. After plate mix, read fluorescence at time zero.
12. Incubate the reaction for T minutes and read the plate at elapsed time T.

DATA COLLECTION AND ANALYSIS

1. Record the RFU values at time T and time Zero.
2. The difference of time T and time zero RFU readings are ΔRFU.
3. ΔRFU values are corrected with the blank to give net ΔRFU.
4. The log net ΔRFU is then plotted against log endotoxin concentration in a linear regression curve as following equation:
   The standard curve equation:
   $\log_{10}(Y) = B^* \log_{10}X + A$,
   where Y = net ΔRFU (RFU at time T minus RFU at time 0 corrected with the blank), X= endotoxin concentration,
   B= slope of the regression curve, A = the Y intercept.

INITIAL QUALIFICATION

As the requirements in the Pharmacopeia, validation of the 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 Recombinant Factor C, 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 Recombinant Factor C 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 of the value of ΔRFU (RFU at time T minutes minus RFU at time 0min) of endotoxin concentration at lowest concentration of the endotoxin standard curve

should be large than the ΔRFU of the negative controls.

4. The coefficient of variation (C.V.) equals the sample standard deviation of the netΔ RFU (RFU at time T minus RFU at time zero corrected with the blank) divided by the mean and is expressed as a percent. The %C.V. of the netΔRFU for the replicates should be less than or equal to 20%.

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