RES056-EN.03

Recombinant Factor C Endotoxin Detection Kit

Pack Size: 48 Tests / 96 Tests

Catalog Number： RES-A056

IMPORTANT: Please thoroughly review this manual before conducting your experiment.

FOR RESEARCH USE ONLY. NOT For Use In Diagnostic Or Therapeutic Procedure

Contents List

INTENDED USE

The Recombinant Factor C Endotoxin Detection Kit is a non-shorecrab-derived pre-assemble kit for in vitro quality control during the manufacturing process, as well as final product endotoxin quantitative determination of parenteral drugs, biological products, infusion cells, medical devices and media for tissue cultures.

KIT FEATURES

- Comparable to Limulus Amoebocyte Lysate (LAL) based method - Endpoint fluorescent assay, and other chromogenic quantitative LAL methods.
- High specificity - Unlike LAL Assay, as Factor G is absent from the rFC test kit, false-positive results due to β-glucan activation are not expected to occur.
- Accuracy - Traceability of endotoxin standards in the kit against USP Standard (Catalog No: 1235503).
- Short time to obtain results - 1 hour.
- High sensitivity - 0.005-5 EU/mL.
- Extensive validation – Verify in accordance with the EUROPEAN PHARMACOPOEIA 11.0 and USP chapter<1225> pharmacopoeia, including specificity, sensitivity, precision, accuracy, applicability, and other aspects such as biological products, microplate readers and various buffer systems.
- Sustainable resource - This approach reduces dependence on horseshoe crab populations, alleviates fishing pressure, and ensures a consistent and long-term supply of materials, supporting both sustainability and ecological balance.
- Good inter batch consistency - Batch consistency of products is guaranteed due to the use of genetic recombination technology for kit manufacturing.

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PRECAUTIONS

1. This kit is for research use only and is NOT for use in diagnostic or therapeutic applications.
2. This kit must be utilized strictly in accordance with the provided instructions to ensure accurate and reliable results. Do NOT mix reagents from different lots.
3. Bring all reagents and samples to room temperature (20 - 25℃) before use.
4. Store the kit at 2-8℃ to maintain the performance and stability.
5. Please prepare the working solutions of each component in accordance with the protocol and experimental requirements. All prepared working solutions are intended for single use only and cannot be stored for future use.
6. All reagents and consumables used in the endotoxin testing process must be explicitly defined sterile and pyrogen free to prevent the contamination of testing reagents

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BACKGROUND

Endotoxins, also called lipopolysaccharides (LPS), are the component of the outer membrane of gram-negative bacteria. They are released into the surrounding environment when the intact bacteria are disrupted (either by death or cell lysis). It is known that endotoxin can trigger reactions in animals, with symptoms including high fever, vasodilation, and diarrhea. In extreme cases, it may lead to fatal shock. In vivo, it can result in the aforementioned complications. Endotoxin content is a critical quality control parameter for raw materials, biological products, and medical devices. It directly impacts product release and compliance. Therefore, developing sensitive, accurate, and rapid detection methods is essential to ensure product safety and quality.

The Limulus Amoebocyte Lysate test (LAL) has been widely used for decades for the quality assurance of injectable drugs and medical devices for endotoxin detection. The LAL test was developed based on the unique ability of Limulus polyphemus blood to form clots upon exposure to LPS. However, as an in vitro endotoxin detection tool, variations in the LAL's sensitivity and specificity towards to endotoxin, along with the decreasing supply of horseshoe crabs, are presenting the biotechnology industry with growing challenges. To address these issues, the recombinant factor C (rFC) assay was developed as a reliable alternative to the LAL test.

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PRINCIPLE OF THE ASSAY

The Recombinant Factor C Endotoxin Detection Kit represents a novel approach for endotoxin detection, leveraging recombinant technology. Recombinant Factor C, which is the key component in the horseshoe crab coagulation cascade reaction, gets activated upon exposure to endotoxin. Once activated, Factor C cleaves the fluorogenic substrate, thereby generating a fluorescent signal. There exists a positive correlation between the increment in the fluorescence signal and the amount of endotoxin. The experiment is conducted on a non-transparent 96-well plate, with measurements taken at the start (time zero) and after a one-hour incubation at 37℃. A fluorescence microplate reader is employed to measure at the excitation/emission wavelength of 380/440 nm to assess whether the sample is contaminated with endotoxin.

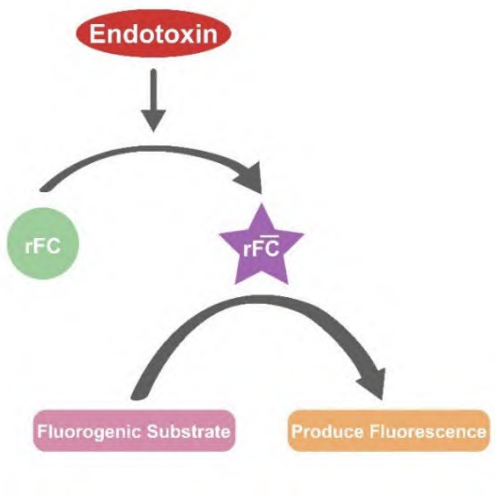

FIG.1 PRINCIPLE OF THE ASSAY

MATERIALS PROVIDED

TABLE 1. MATERIALS PROVIDED

Catalog	Components	Size (Tests)		Format	Storage
		48	96		
RES056-C01	Bacterial Endotoxin Standard	1vial		Powder	2-8℃
RES056-C02	Recombinant Factor C Protein	48	96	Powder	2-8℃
RES056-C03	Fluorogenic Substrate	48	96	Powder	2-8℃
RES056-C04	Water for Bacterial Endotoxins Test	50 mL		Liquid	2-8℃

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MATERIALS REQUIRED BUT NOT PROVIDED

Items	Specifications	Recommend Materials
Single channel and eight-channel pipettes	Must be calibrated pipettes, and sterile during the experiment	Different pipette has different precision, choose the appropriate precision pipettes
Tips	Endotoxin-free, low adsorption pipette tips	All tips need to fit the pipette. For tapered shank pipettes, a tapered nozzle is recommended, such as QSP tips (Cat. No. TF112-1000-Q or TF140-200-Q). For pipettes with cylindrical handles, dedicated tips are recommended.
96-well microplates	Endotoxin-free Black or white -walled 96 well microplates can be used.	Corning Black plates (Cat. No. 3603)
Glass tubes	Endotoxin-free.	t
Reagent reservoirs	Endotoxin-free.	Biofil Reagent Reservoirs (Cat. No. LTT-011-050) or equivalent
Timer	-	-
Vortex mixer		-
Constant-temperature Incubator	The incubator can be set at a temperature of 37 degrees Celsius.	-
96-well fluorescence microplate reader	Plate reader capable of measuring fluorescence with excitation/emission wavelengths of 380/440 nm.	BMG Labtech Clariostar Plus, BMG Omega, Agilent BioTek Synergy LX Multi-Mode Reader or equivalent.

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STORAGE AND VALIDITY INSTRUCTIONS

1. The kit is shipped with blue ice and must be stored at 2-8℃ upon receiving.
2. The expiration date is attached on the exterior packaging. Do not use expired reagents.

PREPARATION BEFORE EXPERIMENT

1. **Experimental environment preparation:** In order to ensure the accuracy of the detection of endotoxin, the operation process must be conducted in a manner to prevent the introduction of additional endotoxin. If the open lab environment cannot be confirmed to meet the requirements, the operation should be carried out in a biological safety cabin.
2. **Parameter settings of fluorescence microplate reader :**

Mode	Parameter settings
Excitation/emission	380/440nm
Gain	Fluorescence signals are usually recorded as Relative Fluorescence Units (RFU). As the actual fluorescence signal is converted into an electronic signal that can be adjusted via the gain or sensitivity setting, RFU is an arbitrary unit. Different microplate readers and gain values can yield different fluorescence signals. Adjust the parameters according to the equipment manual. Increase the gain if the signal of the lowest concentration point can't be measured. Reduce it when the background signal is too high. For instance, in the fixed Gain value mode, the fluorescence range of BMG CLARIOstar Plus and BMG Omega is 0 - 260,000. It's advisable that the RFU value of the highest concentration point not exceed 80% of the maximum 260,000 (i.e., 208,000) to prevent signal overflow. If there are no special requirements for the signal value and your instrument has an automatic calibration mode, you can also choose to automatically calibrate the Gain value based on your experiment to ensure no signal overflow.

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Note:

1) **the setting of instruments is not the same across different brands, please consult with the instrument vendor for details. Appropriate parameters setting is essential before the conducting experiment.**

3. **Materials Preparation:** Prepare materials and equipment corroding to "Materials required but not provided in page 5.
4. **Reagent Preparation:** Retrieve the kit and allow all buffer components to equilibrate to room temperature.

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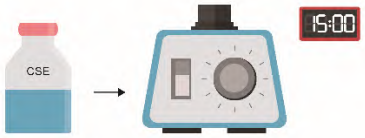
EXPERIMENTAL PROCEDURE FLOW CHART

Step 1

Reconstitute Control Standard Endotoxin (CSE) with LAL Reagent Water (LRW) to yield a solution containing 20 EU/mL.

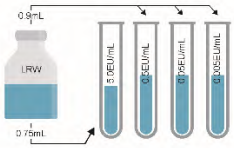
Step 2

Vortex for 15 minutes.



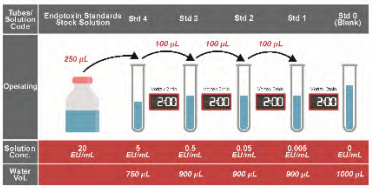
Step 3

Label the tubes with the appropriate endotoxin concentration and add 0.75 mL of LRW to the 5.0 EU/mL tube. Add 0.9mL of LRW to each of the remaining tubes.



Step 4

Prepare a series of endotoxin standards.

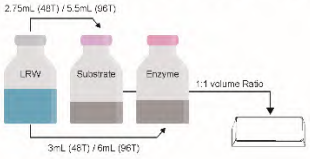


Step 5

Dispense the LRW Blank (100 µL), endotoxin standards (100 µL), product samples (100 µL), positive controls (100 µL), etc. into the appropriate wells of the microplate.

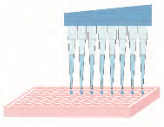
Step 6

Prepare the Mixture of Substrate working solution: Reconstitute the Recombinant Factor C Protein with 3mL (48T) / 6mL (96T) Endotoxin-free Water, and reconstitute the fluorescent substrate dry powder with 2.75mL (48T) / 5.5mL (96T) Endotoxin-free Water, Mix the two reconstructed solutions at 1:1 volume Ratio.



Step 7

Use an Muti-channel-pipettor to dispense 100 µL of the working reagent into the appropriate wells of the microplate.



Step 8

700 rpm/min, 20s ; Read 0 hour's Data.
Incubate at 37 ℃±1 ℃ for 1 hour; Read 1 hour's Data.

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RECOMMENDED PROTOCOL

1. Prepare the stock solution of Bacterial Endotoxin Standards

First, prepare the 20 EU/mL Endotoxin stock solution. Reconstitute the Bacterial Endotoxin Standard (RES056-C01) by adding the volume of Water for Bacterial Endotoxins Test (RES056-C04) as specified in the Certificate of Analysis (COA) to obtain a 20 EU/mL stock solution. Since prolonged storage may lead to endotoxin adsorption onto the glass tube wall, after adding water, vigorously shake on a vortex mixer at high speed (1000rpm) for approximately 10 minutes. Before subsequent use, the solution must be equilibrated to room temperature and vigorously vortexed again for around 10 minutes.

2. Preparation and Requirements for Bacterial Endotoxin Standard and Test Materials

According to the method, each well requires 100 µL of standard. Serially dilute the 20 EU/mL Bacterial Endotoxin Standard stock solution with the Water for Bacterial Endotoxins Test in endotoxin-free glass tubes to prepare the standards. Depyrogenate all glassware and other heat-stable materials in a hot air oven following a validated process. A commonly employed minimum time and temperature is 30 minutes at 250℃. If using plastic labware such as microplates and pipette tips, select labware that is proven to be free of detectable endotoxin and does not interfere with the test. All buffers should also be endotoxin-free.

Note:

1) **When establishing the endotoxin standard curve, prior to each experiment, the endotoxin stock solution should be serially diluted to obtain each concentration point required for the standard curve. Diluting the concentration points of the standard curve in advance and storing them for later use is not permitted.**

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2) **In order to counteract any standard sticking, we recommend changing tips between each dilution.**
3) **To prevent adsorption, it is recommended to prepare endotoxin standards in endotoxin-free glass tubes. Plastic tubes are not recommended to use.**

2.1 The endotoxin standard dilution procedure is listed and illustrated below:

2.1.1 If the 20 EU/mL endotoxin standards stock solution is left for a long time, remember to mix the stock solution at speed 1000rpm on the vortex mixer for more than 10 minutes.

2.1.2 Take 4 disposable endotoxin-free glass tubes and mark the concentration of the standard working solution of endotoxin on the tubes (St1: 0.005EU/mL, St2: 0.05EU/mL, St3: 0.5EU/mL and St4: 5EU/mL, respectively)

2.1.3 To obtain a concentration of 5 EU/mL (Std 4), dilute the 20 EU/mL standards stock solution four-fold with Water for Bacterial Endotoxins Test. Specifically, take 250 µL of the endotoxin standards stock solution and dilute it into 750 µL of Water for Bacterial Endotoxins Test. Then, mix the solution thoroughly on a vortex mixer at 1000 rpm for a minimum of 2 minutes.

2.1.4 To obtain a concentration of 0.5-0.005 EU/mL (Std 3-Std1) prepare 1:10 serial dilutions for the standard curve as follows (take 1 mL of each concentration of standards as example):

2.1.5 Dispense 900µL of Water for Bacterial Endotoxins Test into each glass tubes from Std 3 to Std 1;

2.1.6 Add 100 µL of 5 EU/mL endotoxin (Std 4) to 900µL of Water for Bacterial Endotoxins Test (Std 3) , mix well on the vortex mixer (1000rpm) for at least 2 min and repeat the

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serial dilution to make endotoxin standard solutions: Std 4, Std 3, Std 2, Std 1, this will create 4 standards for the quantitative analysis;

2.1.7 - Std 0 (Blank) is Water for Bacterial Endotoxins Test alone.

Tubes/ Solution Code	Endotoxin Standards Stock Solution	Std 4	Std 3	Std 2	Std 1	Std 0 (Blank)
Operating	250 µL	100 µL, Vortex 2min	100 µL, Vortex 2min	100 µL, Vortex 2min	Vortex 2min	
Solution Conc.	20 EU/mL	5 EU/mL	0.5 EU/mL	0.05 EU/mL	0.005 EU/mL	0 EU/mL
Water Vol.		750 µL	900 µL	900 µL	900 µL	1000 µL

Note:

1) **Every time samples test requires simultaneous testing of the endotoxin working standard solution and the creation of a new standard curve.**

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3. Prepare the samples

3.1 Precautions and Requirements in Endotoxin Testing and Sample Handling

The test samples should be handled carefully to avoid microbiological or endotoxin contamination. **All materials in direct contact with the samples or test reagents MUST be endotoxin free**. Sample dilution should be carried out in endotoxin-free glass tubes with endotoxin-free water.

If the samples are not tested promptly, they must be stored under conditions where all bacteriological activity is halted or endotoxin levels will not escalate over time. To inhibit bacteriological activity, it is advisable to store the samples at a temperature range of 2 - 8℃ for a duration of less than 24 hours. End-user is responsible for ensuring that the containers and storage conditions are suitable for their specific samples.

3.2 Evaluation of Sample Interference in Endotoxin Detection

To determine whether there is interference or not, the recovery rate of endotoxin detection in each test sample after adding the standard substance should be examined. As the experimental data shows, when the detected value of the spiked sample minus the detected value of the sample without added endotoxin is divided by the known content of the added endotoxin, if the recovery rate of the added endotoxin standard substance falls within the range of 50% - 200%, it can be considered that there is no interference in the sample solution under these experimental conditions.

3.2.1 Design of Sample Spiking Experiments: This experiment can be performed by add a certain concentration of endotoxins in the linear range into the testing samples, for example, adding 1 part of the 0.5 EU/mL standard to 1 part of the test sample. This yields an added spike of 0.25 EU/mL, any endogenous endotoxins from the sample itself determined prior to spiking

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and corrected for by the 50% dilution of that sample should be subtracted from the value determined for the spiked sample, then calculated the concentration of endotoxins to give the recovery rate. If the endotoxins content of the sample itself exceeds the highest standard (5EU/mL), dilute the sample to a linear concentration and then add standards for recovery. Calculate Recovery based on the formula $\text{Recovery} = \frac{\text{CR-C}}{\text{Endotoxin concentration in spiking}} \times 100\%$. According to the design in the following table, the recovery rate is calculated as ((CR1 - C1) / 0.25) x 100%, or ((CR2 - C2) / 0.25) x 100%. If the calculated recovery rate falls within the range of 50% - 200%, the sample is considered to have no interference at this dilution factor.

Sample ID	Diluent Ratio	Sample and Standard Volume	Endotoxin concentration (EU/mL)
Sample 1-1	2	150 µL Sample 1-1+ 150 µL Water for Bacterial Endotoxins Test	C1
Sample 1-2	16	150 µL Sample 1-2 + 150 µL Water for Bacterial Endotoxins Test	C2
Sample 1-1	2	150 µL Sample 1-1 + 150 µL Std 3 (0.5EU/mL)	CR1
Sample 1-2	16	150 µL Sample 1-2 + 150 µL Std 3 (0.5EU/mL)	CR2

3.3 Elimination of Sample Interference in Endotoxin Detection

High concentrations of components in the sample, the presence of interfering substances, or improper pH values may all cause interference in endotoxin detection. For high-concentration components, appropriate dilution can be used to reduce the interference with endotoxin detection. The improper pH value can be adjusted according to the following method. If the interfering

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substances continue to cause interference after dilution, the buffer system of the sample must be replaced with an endotoxin-free buffer until the interference is eliminated.

3.3.1 If the sample recovery rate is not within the range of 50% - 200%, the sample can be diluted to reduce or lower the degree of interference. The dilution factor of the sample should be within the range of the Maximum Valid Dilution (MVD). MVD is the formula for calculating the maximum allowable dilution of the test sample for the endotoxin limit. The MVD formula is calculated as follows:

$$MVD = \frac{\text{endotoxin limit} \times \text{concentration of Sample Solution}}{\lambda}$$

In the formula, please consider the following example: If the endotoxin limit is 10 EU/mg and the sample concentration is 10 mg/mL, where λ represents the lowest concentration adopted in the standard curve of the kit, that is, 0.005 EU/mL. The calculation of MVD (Maximum Valid Dilution) = (10 EU/mg × 10 mg/mL) / 0.005 EU/mL, and the calculated maximum valid dilution factor is 20,000 times. When performing the calculation, attention should be paid to the units and their concentrations of the test samples. The conversion of the unit combinations used is shown in the following table:

Unit	Group1	Group2	Group3	Group4
Endotoxin limit the test sample	EU/mg	EU/U	EU/dose	EU/mL
The concentration of Sample Solution	mg/mL	U/mL	dose/mL	ml/mL

3.3.2 Given that the rFC endotoxin assay reagent employed for endotoxin determination involves an enzymatic reaction, it might be requisite to adjust the pH of the reaction system to fall within the range of 6.0 - 8.0. Should the pH of the reaction system deviate from this range, it can be adjusted using endotoxin-free hydrochloric acid, sodium hydroxide, or other buffer solutions.

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Note:

1) The sample to be tested cannot be adjusted by pH electrode directly, to avoid the samples are contaminated by pH electrode resulting in false positive endotoxin. It is recommended to separate a part of the sample for pre-experiment to select an appropriate pH adjustment method, and the actual test sample should be adjusted by using endotoxin-free buffer as per the method.

3.4 Sample preparation

3.4.1 Samples that do not cause interference in endotoxin detection (with the spiked recovery rate ranging from 50% to 200%) during sample preparation can be directly tested without the need for dilution.

3.4.2 When the endotoxin concentration in all samples is higher than the highest standard (5 EU/mL, Std 4), they must be diluted with Bacterial Endotoxin Test Water before undergoing the bacterial endotoxin test.

3.4.3 When the total amount of added endotoxin and endogenous endotoxin in the sample itself is higher than the highest standard (5 EU/mL, Std 4), the sample also needs to be diluted to a concentration that can be detected within the standard curve.

3.4.4 If the sample contains interfering components, it is necessary to dilute the sample with Bacterial Endotoxin Test Water according to Sections 3.2 and 3.3 above to reduce the interference.

3.4.5 All samples should be diluted in endotoxin-free glass tubes. During each dilution step, the diluent should be mixed on a vortex mixer at a speed of 1000 rpm for at least 2 minutes.

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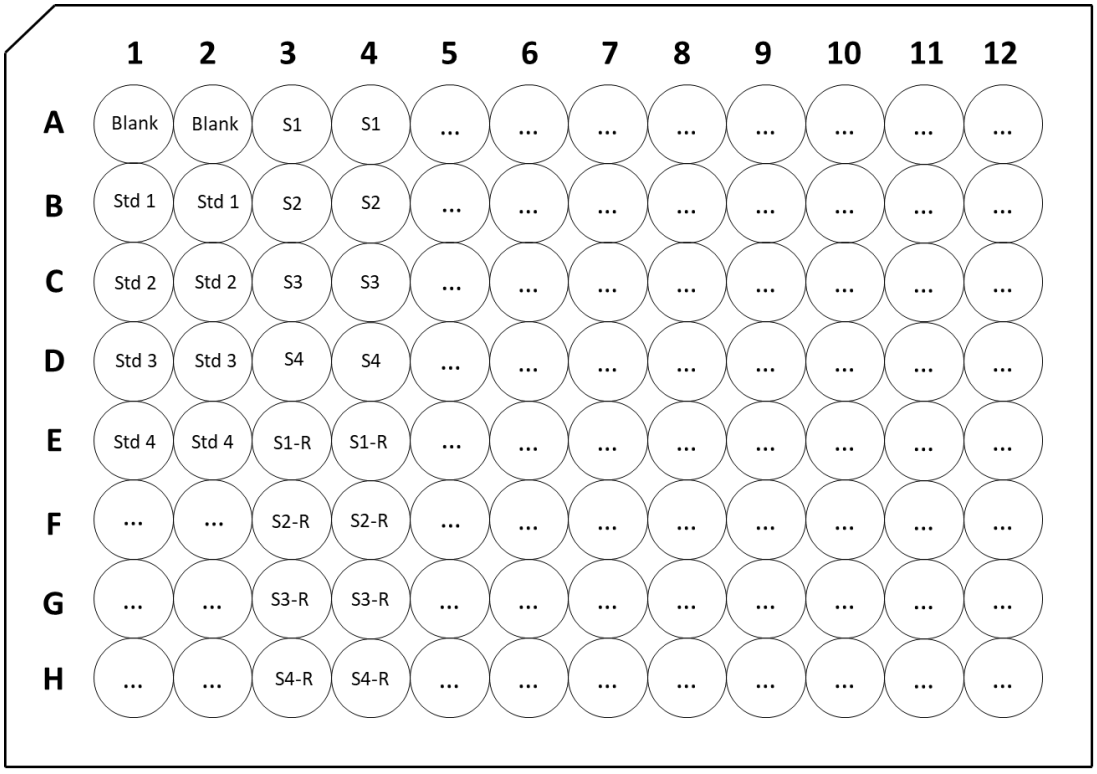
4. Add Standards and Samples to the plate

Add 100 μL of diluted endotoxin standards and samples to the endotoxin-free 96-well plates. It is recommended that each concentration of standards and your samples be reperforated. Place the plate after sample addition in a 37 ± 1℃incubator and preheat for 10 minutes.

Plate Layout



Note:

1) **The standards and all test samples should be measured at the same gain setting for the same plate.**

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5. Prepare the Mixture of Substrate working solution

First, reconstitute the Recombinant Factor C Protein (RES056-C02) and the fluorescent substrate dry powder (RES056-C03). The lyophilized substances should be dissolved into stock solutions using the Water for Bacterial Endotoxins Test (RES056-C04) per the instructions listed in the table below. Let the solutions sit for 15 minutes at room temperature with gentle mixing. Avoid vigorous shaking or vortexing.

Catalog	Components	Amount	Reconstitution Buffer and Vol.
RES056-C02	Recombinant Factor C Protein	48tests	3mL Water for Bacterial Endotoxins Test
RES056-C03	Fluorogenic Substrate	48tests	2.75mL Water for Bacterial Endotoxins Test
RES056-C02	Recombinant Factor C Protein	96tests	6mL Water for Bacterial Endotoxins Test
RES056-C03	Fluorogenic Substrate	96tests	5.5mL Water for Bacterial Endotoxins Test

Each well requires 100 µL of the Mixture of Substrate working solution. Calculate the total volume of the Mixture of Substrate working solution based on the number of wells in the experiment. Mix equal volumes of the Recombinant Factor C Protein stock solution and the Fluorogenic Substrate stock solution. For example, if there are 96 experimental wells, 9.6 mL of the Mixture of Substrate working solution is needed. To be on the safe margin, prepare 10 mL： mix 5 mL of the Recombinant Factor C Protein stock solution and 5 mL of the Fluorogenic Substrate stock solution to obtain 10 mL of the Mixture of Substrate working solution for testing.

Note:

1) **Do not use vortex to mix the Mixture of Substrate working solution. Just gently shake and mix. It is required to prepare and use the mixture of Substrate working solution promptly.**

Please refer to the following methods to prepare the solution:

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Tests	Working solution	Recombinant Factor C Protein stock solution	Fluorogenic Substrate working solution
12 Tests	1.6 mL	0.8 mL	0.8 mL
24 Tests	2.8 mL	1.4 mL	1.4 mL
36 Tests	4 mL	2 mL	2 mL
42 Tests	4.6 mL	2.3 mL	2.3 mL
48 Tests	5.2 mL	2.6 mL	2.6 mL
54 Tests	5.8 mL	2.9 mL	2.9 mL
60 Tests	6.4 mL	3.2 mL	3.2 mL
66 Tests	7 mL	3.5 mL	3.5 mL
72 Tests	7.6 mL	3.8 mL	3.8 mL
78 Tests	8.2 mL	4.1 mL	4.1 mL
84 Tests	8.8 mL	4.4 mL	4.4 mL
90 Tests	9.4 mL	4.7 mL	4.7 mL
96 Tests	10 mL	5 mL	5 mL

6. Add the mixture of substrate working solution

Retrieve the preheated 96-well plate and dispense 100 µL of the Mixture of Substrate working solution into each well by means of an eight-channel pipettor.

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7. Detection of fluorescence signal at Zero Hour

Agitate the plate at a speed of 700 rpm/min on an orbital shaker for 20 seconds and promptly measure the fluorescence value at the zero hour time point. The Excitation/Emission wavelength is set at 380/440 nm.

8. Incubation

Seal the plate with microplate cover and incubate at 37±1℃ for 1 hour.

9. Data Recording

Read the fluorescence signal at 1 hour, with Excitation/Emission wavelength 380/440 nm.

10. Data analysis

10.1 Export the fluorescence values read by the fluorescence plate reader (at zero hour and one-hour) to a spreadsheet.

10.2 Subtract the fluorescence at time zero from the fluorescence at one-hour to obtain Δ RFU of all wells. Calculate the mean Δ RFU for each standard and sample.

10.3 Subtract the Δ RFU of blank control standard from Δ RFU of each standard and sample.

10.4 Linearity was analyzed based on the results obtained using the four endotoxin standard concentrations (5EU/mL, 0.5EU/mL, 0.05EU/mL, 0.005EU/mL). A standard curve was calculated using a regression model by fitting a linear model log(Y) = Alog(X) + B. The standard concentration as X and the calibrated Δ RFU value as Y. The absolute value of the correlation coefficient, |r|, must be greater than or equal to 0.980 for the range of the prepared

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Standard Endotoxin Solutions Calculate the endotoxin concentration of samples and spiked samples using the standard curve formula and calculate the recovery.

10.5 Calculate the endotoxin concentration of the undiluted samples by taking into account the dilution factor of those samples that have a qualified spiked recovery rate.

10.6 If the calibrated Δ RFU of the sample to be tested is higher than the highest standard (5 EU/mL), the sample shall be diluted with Water for Bacterial Endotoxins Test and assay should be repeated. If the calibrated Δ RFU of the sample to be tested is lower than 0.005EU/mL, the sample residual should be reported＜0.005EU/mL.

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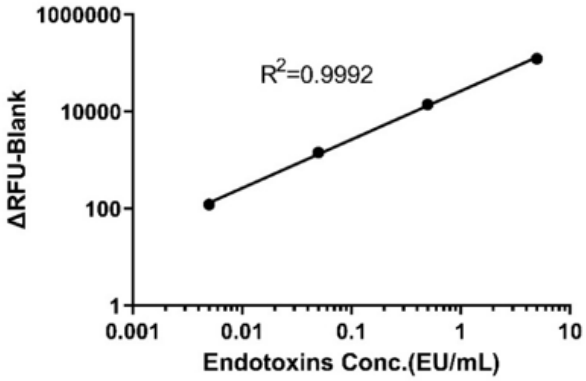
TYPICAL DATA

1. Standard curve of the Kit:

For each experiment, a standard curve needs to be set for each microplate, and the RFU value may vary depending on different laboratories, testers, or equipment. Different microplate reader and different gain value may give different fluorescence signal. Please adjust instrument parameters according to the instrument manual. Reduce the gain value when the signal is too high. The following data is from the BMG CLARIOstar Plus. This following data is for reference only.



Standard Num.	Concentration (EU/mL)	ΔRFU	ΔRFU-Blank
Standard 4	5	124229	123698
Standard 3	0.5	14582	14051
Standard 2	0.05	1974	1442
Standard 1	0.005	655	123
Standard 0	0	532	0

2. Sensitivity

Assay range (EU/mL)	Limit of quantification (LoQ*)
0.005-5 EU/mL	0.005EU/mL

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3. Precision and Accuracy（Intra-Assay）

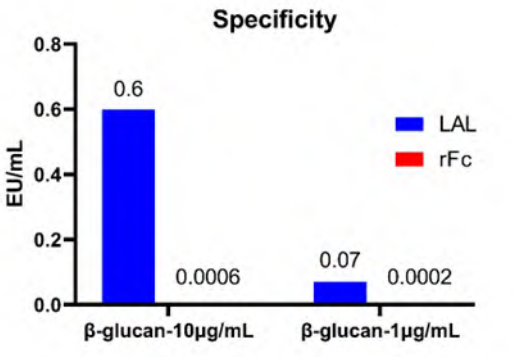
Sample Conc. (EU/mL)	5	3.75	0.2	0.01	0.005
Number of Replicate	10	10	10	10	10
Mean (EU/mL)	4.205	3.562	0.239	0.010	0.005
Standard Deviation	0.037	0.080	0.011	0.001	0.001
Coefficient of Variation (%)	0.9%	2.2%	4.5%	11.2%	18.2%
Recovery	84.1%	95.0%	119.6%	101.1%	94.9%

4. Precision and Accuracy（Inter-Assay）

Sample Conc.(EU/mL)	5	3.75	0.2	0.01	0.005
Number of Replicate	10	10	10	10	10
Mean (EU/mL)	4.338	3.712	0.239	0.009	0.005
Standard Deviation	0.168	0.148	0.028	0.001	0.001
Coefficient of Variation (%)	3.9%	4.0%	11.5%	14.9%	16.3%
Recovery	86.8%	99.0%	119.6%	89.9%	90.3%

5. Specificity

Unlike LAL Assay, Factor G is absent from the rFC test kit, false-positive results due to β-glucan activation are not expected to occur. 10 μg/mL β-glucan and 1 μg/mL β-glucan was test in LAL Assay and rFC Assay. In the LAL assay, non-specific signals were detected with values of 0.6 EU/mL and 0.07 EU/mL. There are no non-specific signals be detected in recombinant factor C method.



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6. Applicability

The kit is applicable in endotoxin detection of injectable drugs such as Recombinant Human Interferon α-1b and Human insulin injection.

Sample	Endotoxin limit	MVD	Dilution factor	Endotoxin Detection value	Recovery
Recombinant Human Interferon α-1b	10EU/mL	2000	32	< 0.16EU/mL	102%
Human insulin injection	32EU/mL	6400	4	< 0.02EU/mL	97%

7. Interfering Substances

The reagent exhibits excellent buffer compatibility. For a specific buffer, it is recommended that you verify the recovery rate to determine the minimum dilution factor. Please refer to the following reference table for buffer solution dilution.

Matrix	Recovery	Dilution Factor
1M NaCl	140%	16
20mM $CaCl_2$	82%	8
20mM $MgCl_2$	98%	4
1M Sodium acetate, pH5.0	73%	16
50mM Sodium acetate, pH3.5	74%	20
100mM Tris, pH10.9	113%	80
100mM Glycine, pH3.5	109%	16

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Matrix	Recovery	Dilution Factor
1×PBS, pH7.5	102%	40
1×PBS, pH6.0	91%	20
50 mM Tris,100 mM Glycine,225 mM Arginine,150 mM NaCl,0.005% Tween 80, pH7.5 with 11% Trehalose	81%	4
50 mM Tris,100 mM Glycine,25 mM Arginine,150 mM NaCl,pH7.5 with 0.01% Tween80 with 11%Trehalose	88%	16
Essential 8™ Flex Basal Medium (Thermofisher, Cat.No. A2858501)	74%	4
mTeSR™ Plus (Stemcell, Cat.No. 100-0276)	82%	32
CelThera™ GMP T Cell Expansion Medium (Acrobiosystems, Cat.No. GMP-CM3101)	93%	4
RPMI 1640 Medium (Hyclone, Cat.No. SH30809.01)	109%	8
DMEM Medium (Basalmedia, Cat.No. L120KJ)	104%	8
CTS™ OpTmizer™ T-Cell Expansion SFM, no phenol red, bottle format(Gibco, Cat.No. A3705001)	91%	16
12.5 mM Histidine Buffer,pH6.5	88%	4
Keytruda Formulation(1.55 mg/mL L-histidine, 0.2mg/mL polysorbate 80, 70mg/mL sucrose in water, pH5.2-5.8)	76%	64
Hemlibra Formulation (26.1 mg/mL L-arginine, 3.1 mg/mL L-histidine, 0.5mg/mL poloxamer 188, adjusted to pH 6.0 with L-aspartic acid)	100%	4
30%DMSO	99%	16

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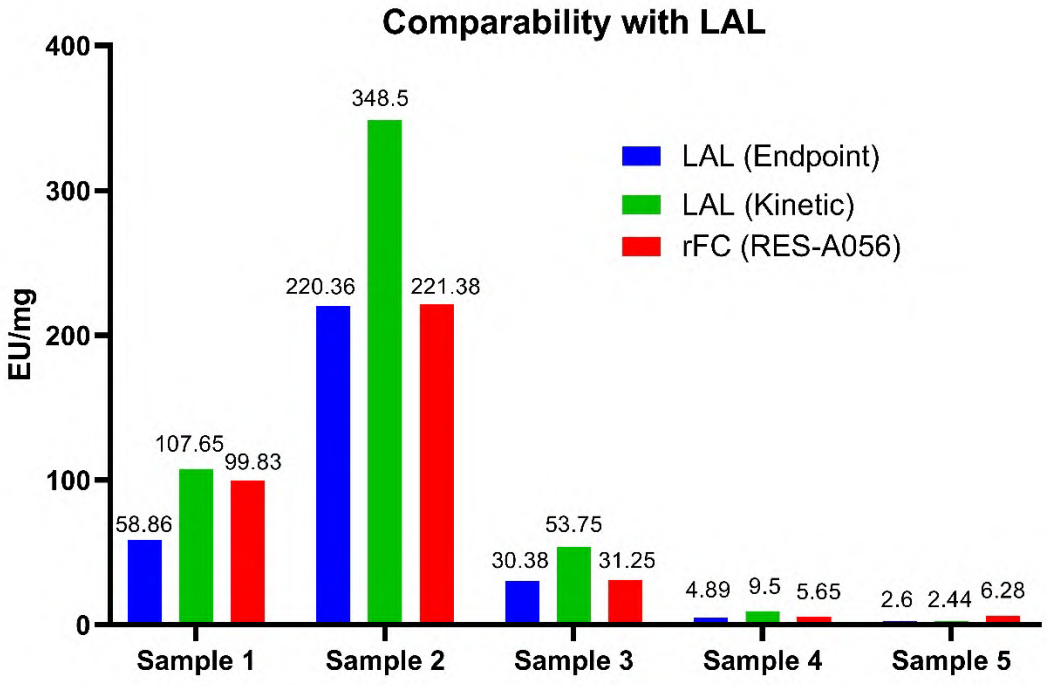
Matrix	Recovery	Dilution Factor
HSA(25mg/mL)	73%	64
Toripalimab(40mg/mL)	83%	40
Multiple Electrolytes Injection (Baxter)	96%	4
100%FBS	109%	10

8. Comparability with LAL

Different methods were used to detect endotoxin residues in five samples, and the deviation between the detection results of rFC method and LAL method is within 2 times.



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FREQUENTLY ASKED QUESTIONS

Problem	Cause	Solution
Poor standard curve	* Inaccurate pipetting * Inaccurate dilution * Inappropriate parameters of fluorescence plate reader	* Check pipettes and repeat experiments * Repeated experiments * Check if the the fluorescence of 5EU/mL exceeds the detection range of the reader and reread after adjusting appropriate parameters
Large CV	* Inaccurate pipetting * Materials pollution	* Check pipettes and repeat experiments * Use the pyrogen free materials to repeat experiments
High background	* LPS pollution Substrate working solution (Recombinant Factor C Protein stock solution, Fluorogenic Substrate)	* Use newly opened reagents and be sure to use endotoxin-free experimental materials
Very low readings across the plate	* Incorrect wavelengths * The gain value of the reader is too low	* Check filters/reader * Increase the gain value of the reader
Samples are reading too high, but standard curve looks fine	* Samples contain endotoxin levels above assay range	* Dilute samples and run again
Samples and spiked samples are reading too low, but standard curve looks fine	* The sample has interference effects * The pH value of the sample is too high or too low	* Increase the sample dilution factor as much as possible within the MVD to reduce interference * Adjust the pH of the test solution using endotoxin-free hydrochloric acid or sodium hydroxid to range of 6.0 to 8.0.

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