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User Manual

Disclaimer: Products are intended for research use only

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EndoSynX™ Recombinant Cascade Reagent (rCR) (Kinetic-Chromogenic Assay) User Guide

PLEASE READ THE DOCUMENT CAREFULLY BEFORE PERFORMING THE TEST

Product No.: 1501112
Version: A/1
For Research Use Only

Biofargo, Inc.

■ Product Description

The EndoSynX™ **Recombinant Cascade Reagent (**rCR) is a recombinant endotoxin detection system that mimics the enzymatic cascade of natural *Limulus Polyphemus* Lysate and it contains Recombinant Factor C, Recombinant Factor B, and Recombinant Proclotting Enzyme. Upon exposure to Gram-negative bacterial endotoxin (lipopolysaccharide, LPS), recombinant Factor C is activated, which will then activate the cascade enzyme system of serine, generating a kinetic chromogenic signal that correlates with the concentration of endotoxin. The **EndoSynX™ rCR** does not contain Factor G, eliminating interference from β-D-glucan and Limulus Amebocyte Lysate-reactive materials in test samples. The kit is suitable for quantitative determination of endotoxin in pharmaceutical products.

The kit is for RESEARCH USE ONLY and not for clinical diagnostic use.

■ Kit Contents

Table 1. Kit Components

Reagent	Part No.	Quantity	Storage
Recombinant Cascade Reagent A	PNR018	4 vials	2-8℃
Recombinant Cascade Reagent B	PNR019	4 vials	
Recombinant Cascade Buffer	PNR016	2.5 mL × 2 vials	
96-well Microplate	/	2 Plates	
Water for BET	NND072	8 mL × 4 vials	

■ Package Size

96 tests/Kit

■ Sensitivity

0.005 - 5 EU/mL

■ Shelf Life

12 months at 2-8℃ (see product labels for expiry).

■ Additional Materials Required

➢ Sterile, pyrogen-free pipette tips: 10 μL, 100 μL, 200 μL and 1000 μL.

- Sterile, pyrogen-free glass tubes or penicillin bottles for serial dilutions of standard solutions or test samples.
- Sterile, pyrogen-free reservoirs: 50 mL

■ Additional Equipment Required

- An incubating kinetic microplate reader: 405 nm, kinetic reading and incubation at 37°C ± 1°C
- Calibrated automatic pipettes: 10 µL, 100 µL, 200 µL and 1000 µL
- Vortex-type mixer

Note: *Laboratory materials that need to be endotoxin-free should be validated or certified to be less than the lowest endotoxin detection level of the test.*

■ Maximum Valid Dilution (MVD)

The maximum valid dilution (MVD) is the maximum allowable dilution of a test sample at which the endotoxin limit can be determined.

$$MVD = (\text{endotoxin limit} \times \text{concentration of sample solution}) / (\lambda)$$

- λ= lowest concentration used in the standard curve, and it is 0.005 EU/mL of this kit.

For example, the endotoxin limit of antibody drugs is 0.1 EU/mg. If the lowest point of the standard curve is 0.005 EU/mL of assay, where the concentration of Sample Solution is 10 mg/mL, the MVD equals 1:200. Thus, the antibody drugs can be diluted up to 1:200 to settle potential interference (inhibition or enhancement).

Endotoxin Limit

Endotoxin limit (EU/mL, EU/mg, EU/Unit) is calculated as: USP <85> and Ph. Eur. <2.6.14> has listed endotoxin limits of 5 EU/kg for intravenous drugs and 0.2 EU/kg for intrathecal drugs.

$$\text{Endotoxin Limit} = K / M$$

- K = threshold pyrogenic dose of endotoxin per kilogram of body mass
- M = maximum recommended bolus dose of product per kilogram of body mass
- For infusions: maximum total dose administered in a single-hour period.

Concentration of Sample Solution

- mg/mL if the endotoxin limit is specified by mass (EU/mg);
- Units/mL if the endotoxin limit is specified by unit of biological activity (EU/Unit);
- mL/mL if the endotoxin limit is specified by volume (EU/mL).

■ Kinetic-colorimetric Test Procedure

1. Set the microplate reader before use:

Table 2. Instrument settings (e.g. MD SpectraMax M2)

Metric	Parameter
Temperature	37℃
Reading type	ABS, dynamic detection
Shaking (before detection)	Medium speed, 5-10 seconds
Wavelength	405 nm
Reading time	60 minutes
Reading interval	30-60 seconds
Onset OD	0.02-0.1

2. Test Samples Preparation

   Prepare the test sample and positive product controls (PPC). The concentration of spike endotoxin should follow the **Test for Interfering Factors** as below, equal to the concentration at or near the middle of the standard curve.

3. Preparation of Standard Endotoxin Stock Solution

   Use an reference standard endotoxin to the WHO International Standard, *e.g.*: USP Endotoxin RS, Catalog No. 1235503. Prepare and store according to the instructions of reference standard endotoxin.

4. Preparation of Standard Endotoxin Solutions

   Use sterile, pyrogen-free glass tubes or penicillin bottles to dilute the Standard Endotoxin Stock Solution at the concentrations of 5, 0.5, 0.05, and 0.005 EU/mL. **Vortex vigorously 2 minutes after each dilution**.

Table 3. Standard Endotoxin Solutions (Starting with 10 EU/mL as an example)

Final Conc. (EU/mL)	Water for BET (mL)	Previous Conc. (EU/mL)	Endotoxin Added (mL)
5	0.5	10	0.5
0.5	0.9	5.0	0.1
0.05	0.9	0.5	0.1
0.005	0.9	0.05	0.1

5. Loading Samples into Microplate

   Add 100 μL of Standard Endotoxin Solutions, Water for BET (NND072, as negative control), test samples, and positive product controls (PPC) into the 96-well Microplate, respectively. Prepare at least 2 replicates for each sample.

Note: *Each assay should include samples or dilutions of samples or products, positive product controls (PPC), either a series of dilutions covering the desired standard curve or a positive water control, and a negative control.*

6. Preparation of Reaction Mixture Solution

1) Recombinant cascade reagent A solution (rCR-A): Dispense 0.75 mL of Water for BET into the vial of Recombinant Cascade Reagent A (PNR018) and gently dissolve the powder until the solution clears.

2) Recombinant cascade reagent B solution (rCR-B): Dispense 0.75 mL of Water for BET into the vial of Recombinant Cascade Reagent B (PNR019) and gently dissolve the powder until the solution clears.

3) Calculate the number of reaction wells to decide the volume of Reaction Solution (including the pippeting loss).

Table 4. Preparation of Reaction Mixture Solution

Wells	Buffer (μL)	rCR-A (μL)	rCR-B (μL)	Total (μL)
1	50	25	25	100
24	1300	650	650	2600
48	2500	1250	1250	5000

Note:

1) Each vial of reagent needs to be equilibrated to room temperature before use.

2) Addition order: Recombinant Cascade Buffer →Reagent A solution → Reagent B solution.

3) Mix by gentle inversion (no vigorous shaking).

7. Add Reaction Mixture Solution

Add 100 μL of Reaction Mixture Solution to each well and insert the plate into microplate reader immediately.

Note: *To ensure the accuracy of the test, the dispensing of Reaction Mixture Solution into each well of the 96-well Microplate must be completed within 5 minutes.*

8. Start the test

Run using preset kinetic parameters of microplate reader.

■ Data analysis

1. Standard Curve

The relationship between start-up time (T) and log concentration (C)

$$\lg T = b \lg C + a$$

- b = slope
- a = intercept.

2. Calculation of Sample Concentration

Determine the endotoxin concentration by interpolation from the standard curve.

■ Test for Interfering Factors

Select an endotoxin concentration at or near the intermediate endotoxin standard curve. Prepare Solutions A, B, C and D as shown in Table 5. It is recommended that the interference test for Solutions A, B, C and D be performed on at least two replicates.

Table 5. Preparation of Solutions for the Inhibition / Enhancement Test

Solution	Endotoxin Concentration	Solution to Which Endotoxin Is Added	Number of Replicates
A[a]	None	Sample Solution	≥ 2
B[b]	Middle concentration of the standard curve	Sample Solution	≥ 2
C[c]	At least three concentr-ations (lowest concentr-ation is designated λ)	Water for BET	≥ 2 each
D[d]	None	Water for BET	≥ 2

[a] *Solution A: The Sample Solution may be diluted not to exceed MVD*

[b] *Solution B (positive product control): The concentration at the same dilution as Solution A, containing given concentration of endotoxin equal to or near the intermediate concentration of standard curve.*

[c] *Solution C: Standard curve, we recommend to prepare Standard Endotoxin Solutions from 0.005-5 EU/mL.*

[d] *Solution D: Water for BET (negative control).*

The test is considered valid when the following conditions are met:

1. The absolute value of the correlation coefficient generated by the standard curve using Solution C is greater than or equal to 0.980.
2. The result with Solution D is less than the endotoxin detection limit of the recombinant reagent employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution, if any (Solution A), from that containing the added endotoxin (Solution B). In order to be considered free of factors that interfere with the assay under the conditions of the test, the measured concentration of the endotoxin added to the Sample Solution must be within 50%–200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added

endotoxin.

■ Warnings and Precautions

1. For Bacterial Endotoxins Tests only.
2. Use endotoxin-free materials and prevent contamination.
3. Discard the dissolved Recombinant cascade reagent A if the solution turns yellow or any insoluble substances appear.
4. Sample pH must be 6.0-8.0.

Caution: *As a novel assay, it is the responsibility of the user to review the supplier's primary validation package and to verify that the recombinant reagent-based. This verification must include performing specific experiments to method is appropriate for use in testing specific products or materials confirm that the method is suitable for its intended purpose under the conditions of use for the material, drug substance, and/or drug product. The user should refer to Verification of Compendial Procedures<1226> and the Verification--Test for interfering Factors section.*

■ References

- USP <85> Bacterial Endotoxins Test
- USP <86> Bacterial endotoxins test using recombinant reagents
- Ph. Eur. <2.6.14> Bacterial Endotoxins

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Support & Contact

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