APPLICATION NOTE

Detect endotoxin with the PyroGene Recombinant Factor C Assay

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Benefits

- **Assure sample safety with an endotoxin assay that uses a single enzymatic step and no animal-derived products**
- **Attain sensitivity beyond minimal requirements with SpectraMax readers**
- **Save time by automating a read-incubate-read sequence with a workflow in SoftMax Pro Software**

Introduction

Monitoring samples for contaminants is a critical step during the production process in the pharmaceutical and medical device industries. Endotoxin, found in the cell wall of gram-negative bacteria, is a frequent contaminant that can cause fever, inflammation, headache, nausea, and even death. Endotoxin is routinely detected by the Limulus amebocyte lysate (LAL) assay, whose central reagent is obtained from the blood of the horseshoe crab, *Limulus polyphemus*. In the presence of endotoxins, LAL coagulates via an enzyme-mediated cascade, which can be quantified using turbidimetric or chromogenic assays (Figure 1A). These methods involve multiple enzymatic steps and depend upon the availability of horseshoe crabs.

The PyroGene™ Recombinant Factor C (rFC) Assay from Lonza is a quantitative assay that works through a single enzymatic step, uses no animal-derived products, and offers the same sensitivity as the LAL method. In the presence of endotoxin, activated rFC will cleave a fluorogenic substrate, causing an increase in fluorescent signal (Figure 1B) that is proportional to the amount of endotoxin present.

Materials

- PyroGene Recombinant Factor C Endpoint Fluorescent Assay (Lonza cat. #50-658U)
- LAL Reagent Grade™ Multi-well Plates (Lonza cat. #25-340)
- Pyrogen-free Dilution Tubes (Lonza cat. #N207)
- LAL Reagent Water (Lonza cat. #W50-640)
- SpectraMax i3 or i3x Multi-Mode Microplate Reader (Molecular Devices; other SpectraMax microplate readers with fluorescence detection are also suitable)
- SoftMax Pro Software (Molecular Devices)

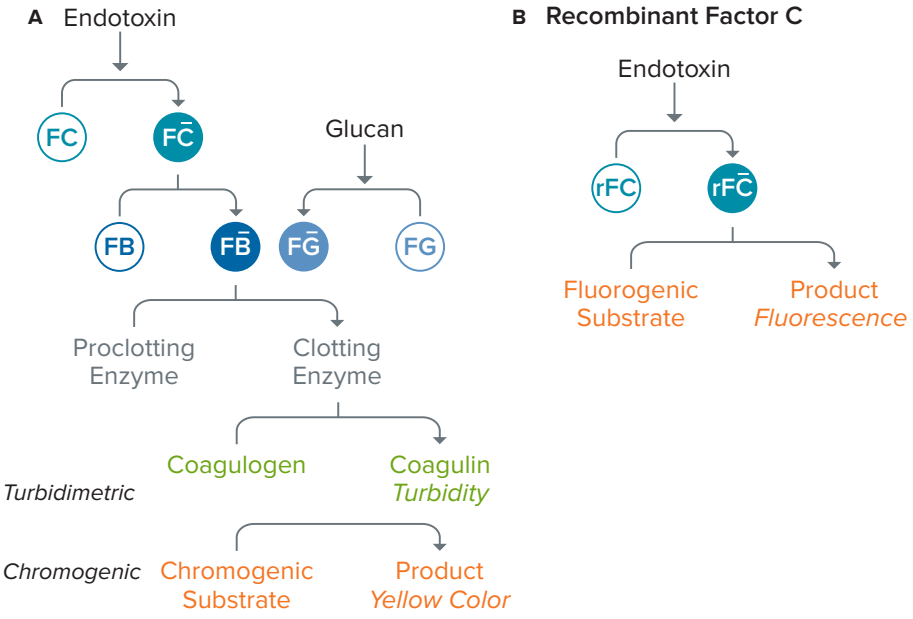

Figure 1. Enzymatic cascades for the LAL (A) and PyroGene rFC assay (B). The rFC assay involves a single enzymatic step and eliminates the need for LAL from the horseshoe crab.

Methods

The lyophilized *E. coli* O55:B5 endotoxin supplied with the kit was reconstituted with the volume of LAL Reagent Water indicated on the vial to yield a 20 EU/mL stock solution. The vial was shaken at high speed for 15 minutes using a vortex mixer to reconstitute completely. Standards of the following concentrations were prepared by diluting the stock solution with LAL Reagent Water and shaking vigorously at each dilution: 5 EU/mL, 0.5 EU/mL, 0.05 EU/mL, 0.005 EU/mL, and 0.001 EU/mL. The PyroGene assay has been optimized to be linear from 0.005 EU/mL to 5.0 EU/mL, but the additional standard concentration (0.001 EU/mL) was added in order to test the sensitivity of the SpectraMax i3 reader.

The plate reader temperature was set to 37°C and allowed to reach 37°C before proceeding. 100 µL of blank or endotoxin standard (in triplicate) was added to wells of the microplate. The plate was preincubated in the reader at 37°C for a minimum of 10 minutes.

During preincubation, the working reagent was prepared by mixing fluorogenic substrate, assay buffer, and rFC enzyme solution in a 5:4:1 ratio, respectively. 100 µL of working reagent was added to each well after the 10-minute preincubation step.

The SpectraMax i3 reader temperature was kept at 37°C for the duration of the assay. The plate was read at time zero (after addition of working reagent) using the settings shown in Table 1. Setting the PMT Gain to Automatic in SoftMax Pro Software ensured that accurate results were obtained across the full range of samples without the need to determine a sensitivity setting. Plate height optimization was carried out initially, and the optimized 2-mm read height was used for subsequent measurements.

After the first (T0) read, the plate was incubated in the reader at 37°C for one hour and then read a second time using the same settings. The read-incubate-read sequence can be automated using the workflow feature in SoftMax Pro Software (Figure 2).

Parameter	Setting
Optical configuration	Monochromator
Read mode	FI (Fluorescence)
Read type	Endpoint
Wavelength	Ex: 380 nm, bandwidth 15 nm Em: 440 nm, bandwidth 25 nm
Plate type	96 Well Costar clear
Read area	(user selected)
PMT and optics	PMT Gain: Automatic Flashes per read: 6 Read from Top (default) Read Height: 2 mm

Table 1. Instrument settings for the SpectraMax i3 reader in SoftMax Pro Software. Setting the PMT Gain to Automatic negates the need to determine a sensitivity setting for the reader.

Figure 2. Workflow setup in SoftMax Pro Software. The initial read at time zero is followed by a one-hour delay (incubation), and then the plate is read again. The instrument is set to 37°C prior to start of the workflow.

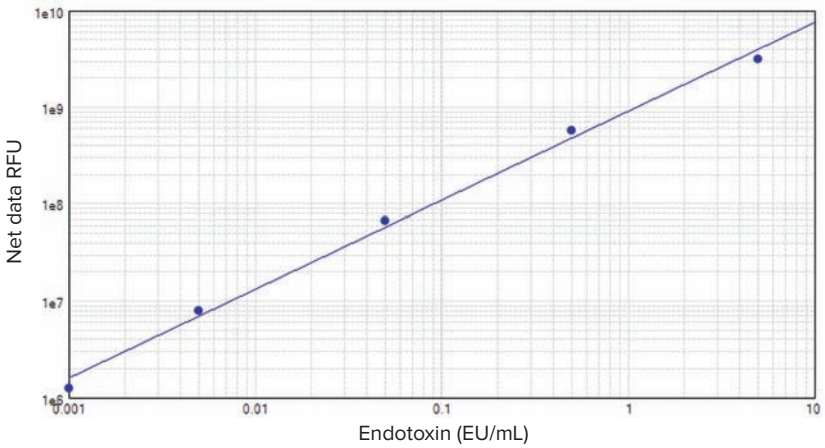

Figure 3. PyroGene rFC assay standard curve. The standard curve was generated in SoftMax Pro Software using the log-log curve fit and was composed of five dilutions of control standard endotoxin in duplicate from 0.001 EU/mL to 5 EU/mL (R^2 = 0.996).

After the second (T60) plate read, net ΔRFU was calculated for each standard using the following formula: $(STD_{T60}-STD_{T0}) – (BL_{T60}-BL_{T0})$, where...

- STD_{T60} is the standard RFU at the one-hour timepoint
- STD_{T0} is the standard RFU at time zero
- BL_{T60} is the blank RFU at one hour
- BL_{T0} is the blank RFU at time zero

Results

To demonstrate the suitability of the SpectraMax i3 reader to perform the PyroGene assay, a standard curve was produced by plotting the log of the net ΔRFU vs. the log of the concentration in SoftMax Pro Software (Figure 3).

The assay was carried out using five endotoxin dilutions ranging from 0.001 EU/mL to 5 EU/mL. The CV for duplicate standards was below 5%. With an R^2 value of 0.996, the standard curve shows excellent linearity throughout this extended concentration range. The SpectraMax i3 reader was able to detect a lower concentration of endotoxin than the range suggested in the product insert (0.005 EU/mL to 5.0 EU/mL), showing an increased sensitivity for the detection of endotoxin in a fluorescence assay.

Conclusion

The generation of linear standard curves with low variance between replicates, and sensitivity below the suggested lower limit of detection of the assay, demonstrates the excellent performance of the SpectraMax i3 Multi-Mode Microplate Reader with the PyroGene Recombinant Factor C Assay. Similar results can be obtained with the SpectraMax i3x reader and other SpectraMax readers with fluorescence detection.

SoftMax Pro Software was used to analyze data, and the net ΔRFU values calculated were used to plot a standard curve, generate the R^2 value, and show the sensitivity of this reader in detecting very low endotoxin levels.

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