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Specialized Modalities

Optimized rFC protocol for rapid endotoxin testing in high-throughput QC laboratories

Achieve reliable 0.1 EU/mL results in just 20 minutes

High-throughput environments, such as automated manufacturing lines and quality control laboratories, require fast, reliable endotoxin testing to match the pace of operations. In many cases, a detection threshold of 0.1 EU/mL is sufficient. However, while some assays offer greater sensitivity, they often require extended incubation times, creating delays in decision-making and workflow efficiency.

This application note demonstrates that the PyroGene® Recombinant Factor C (rFC) Endpoint Fluorescent Assay can be effectively adapted to a 20-minute incubation protocol while maintaining reliable detection at 0.1 EU/mL. Despite a reduced assay time, it continues to deliver accurate, precise, and reproducible results across multiple reagent lots and detection systems. This validated, sustainable approach to endotoxin testing is well-suited for laboratories that prioritize speed and efficiency without sacrificing performance.

Optimizing endotoxin detection for accelerated environments

The PyroGene® rFC Endpoint Fluorescent Assay offers a modern, recombinant approach to endotoxin detection that does not rely on blood harvested from horseshoe crabs. Unlike traditional limulus amebocyte lysate (LAL)-based assays, the PyroGene® Assay uses a single enzymatic step to achieve sensitive and specific results, thereby reducing assay complexity and enhancing consistency.

While the standard PyroGene® Assay protocol delivers high sensitivity, with a detection limit of 0.005 EU/mL using a 60-minute incubation, this duration can present a challenge in high-throughput workflows. In operations where hundreds of samples must be analyzed daily, a 60-minute fixed endpoint can become a bottleneck, particularly when a detection limit of 0.1 EU/mL is sufficient for the application.

In this study, we validate a modified, shortened PyroGene® Assay protocol (with a 0.1 EU/mL detection limit) that reduces incubation time to 20 minutes. We show that the assay reliably meets the performance criteria at this detection threshold, making it ideal for environments where speed is essential.

Experimental overview

Equipment and reagents

To evaluate the feasibility of a shortened 20-minute PyroGene® Assay, testing was conducted using three independent reagent lots and three Nebula® Multimode Readers. Each reagent lot included the recombinant Factor C (rFC) enzyme solution, assay buffer, fluorogenic substrate, and Control Standard Endotoxin (CSE). The CSE was reconstituted and diluted for use in standard curves and spike recovery assessments. Assays were performed using Lonza LAL Reagent Grade (Cat. No. 25-340) 96-well plates. All liquid handling was performed using endotoxin-free LAL Reagent Water (LRW) to ensure result integrity. Data was captured and analyzed using Lonza WinKQCL® Endotoxin Detection & Analysis Software.

Modified assay parameters

The PyroGene® rFC Assay was executed with a 20-minute endpoint instead of the standard 60-minute protocol. Each assay plate included standard concentrations of 0.1, 1, and 10 EU/mL, along with LRW blanks and 1 EU/mL positive product control (PPC) spikes. Assays were performed using three lots of reagents and three Nebula® Multimode Readers, totaling nine reader/lot combinations.

Each plate included 12 replicates per condition. Across the study, 99 duplicate data sets were collected, encompassing full replication across reagent and instrument variation.

	1	2	3	4	5	6	7	8	9	10	11	12
A	bl	bl	bl	bl	bl	bl	bl	bl	bl	bl	bl	bl
B	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C	1	1	1	1	1	1	1	1	1	1	1	1
D	10	10	10	10	10	10	10	10	10	10	10	10
E	LRW	LRW	LRW	LRW	LRW	LRW	LRW	LRW	LRW	LRW	LRW	LRW
F	PPC	PPC	PPC	PPC	PPC	PPC	PPC	PPC	PPC	PPC	PPC	PPC

Figure 1.
Plate configuration.

Acceptance criteria

To validate the modified protocol, the following acceptance criteria were applied:

- Coefficient of variation (CV) < 25% for all standards and PPC wells
- Standard curve R ≥ 0.980
- Standard curve slope between 0.760 and 1.10
- PPC recovery between 50% and 200%

Results and analysis

Assay sensitivity and reader consistency

All nine reader/lot combinations were successful and passed the acceptance criteria. The resulting sensitivity values are shown in Figure 2 below. While minor differences in sensitivity were observed between readers, performance was consistent across reagent lots. The variation across lots for a given reader did not exceed one, demonstrating reliable reproducibility.

Sensitivity	Reader 1	Reader 2	Reader 3
Reagent Lot 1	77	83	85
Reagent Lot 2	78	83	85
Reagent Lot 3	78	83	84

Figure 2.
Sensitivity assay results.

Standard curve performance

Standard curves were generated using both linear and quadratic fits. An example curve from Reader 1 and Lot 1 is shown in Figure 3. The results show a small improvement when using a quadratic fit due to some non-linearity of the standard curve. The same slight curvature was present for all samples.

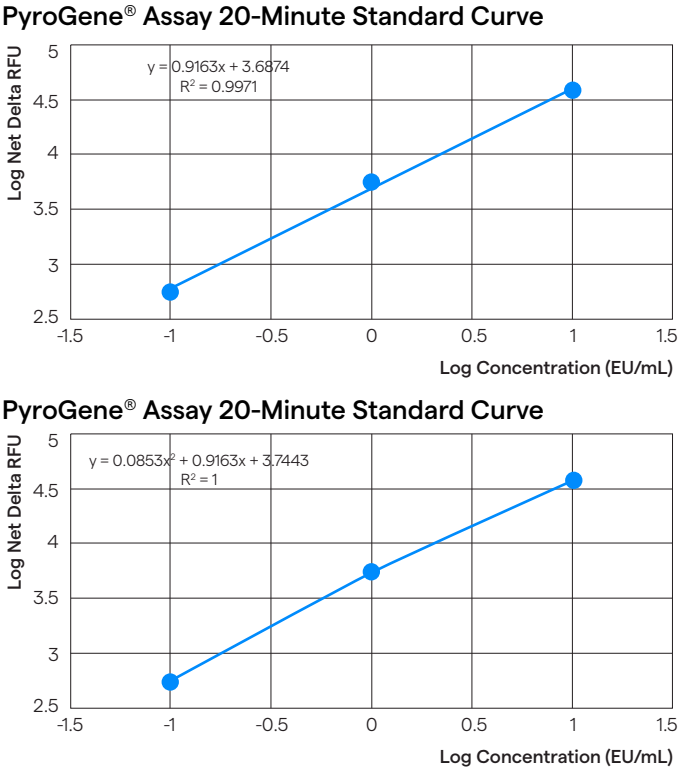

Figure 3.
Example standard curve with linear and quadratic fit.

A total of 99 standard curves were performed, and all met the current acceptance criteria (Figure 4). These results confirm that the 20-minute endpoint consistently supports accurate standard curve generation.

Curve parameter	Specification	Max observed	Min observed
Slope	0.760 to 1.10	0.945	0.887
Intercept	2.5 to 5.00	3.693	3.534
R	≥0.980	1.000	0.998

Figure 4.
Standard curve specifications and range of observed values.

Delta RFU and fluorescence signal separation

For each plate, fluorescence was measured at the beginning and end of a 20-minute incubation period to calculate the delta relative fluorescence units (RFU) for each well. Net delta RFU was calculated by subtracting the delta RFU of the blank from each sample or standard to remove background signal. Net delta RFU values were used to assess whether the assay could clearly differentiate low levels of endotoxin (0.1 EU/mL) from blanks.

Across all 99 duplicate data sets, the average net delta RFU at 0.1 EU/mL ranged from 401 to 556 RFU—well above the corresponding blank values. This strong signal separation was consistently observed across all reader and reagent lot combinations, demonstrating robust assay performance even at the lowest tested standard. Delta RFU distributions shown in Figure 5, visually confirm clear differentiation between blank and 0.1 EU/mL signals across all replicates. These results validate that the shortened 20-minute endpoint continues to deliver reliable fluorescence signal resolution at the 0.1 EU/mL detection level.

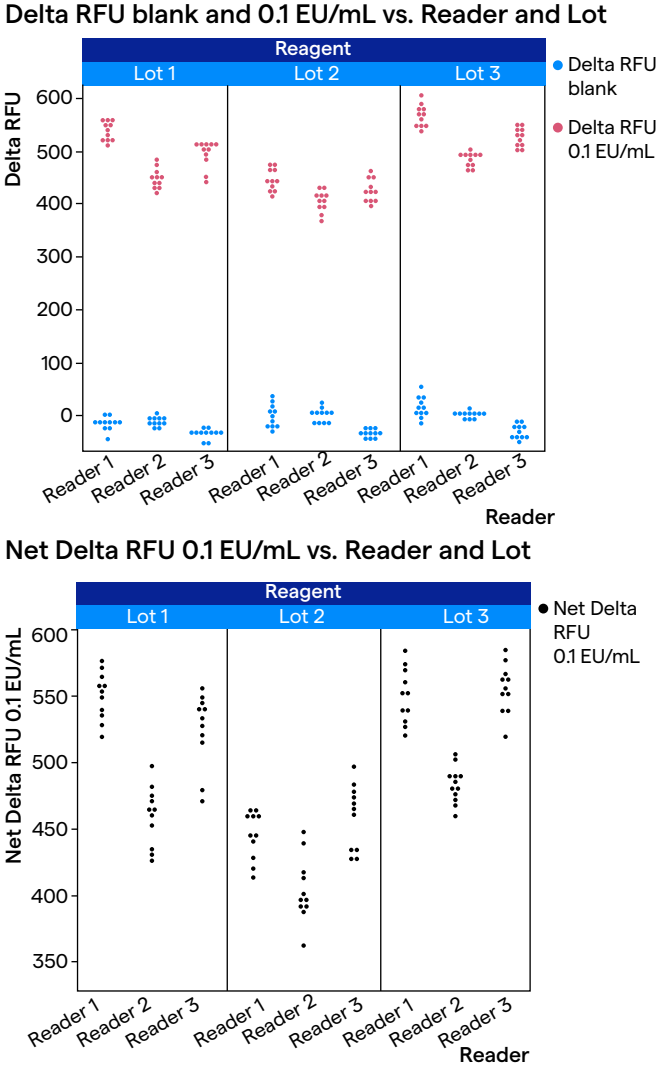

Figure 5.
Delta RFU values for the blank and 0.1 EU/mL standard (top) and the net delta RFU values for the 0.1 EU/mL standard (bottom) for each set of duplicate wells.

Precision (CV analysis)

Precision was assessed by calculating the CV for each standard concentration and PPC across all 99 duplicate data sets. The CV, which measures the relative variability between duplicate wells, serves as an indicator of assay repeatability. According to the current PyroGene® Assay specifications, CVs must be below 25% for all standards and sample wells. CVs were computed for all concentrations: 0.1 EU/mL, 1 EU/mL and 10 EU/mL, as well as the PPC, totaling 396 CV measurements. All CVs met the acceptance criteria, with the highest observed value at just 16.7% (Figure 6).

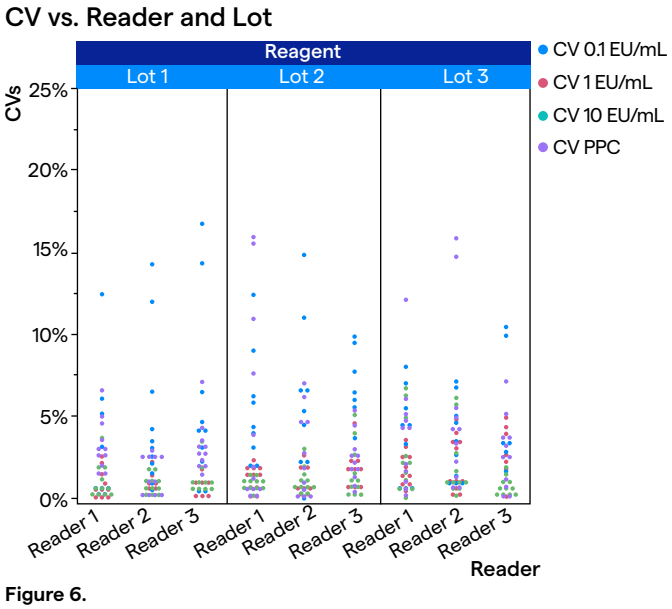

Figure 6.
Individual CV values for adjacent duplicates for all 3 standards and PPC wells. Points are grouped by reagent lot and reader.

As expected, the greatest variability occurred at the lowest concentration (0.1 EU/mL), due to a lower signal-to-noise ratio. To further evaluate reliability, statistical modeling (JMP) was applied, fitting an exponential model to the CV distribution (Figure 7). This model predicted a failure rate of only 0.3% for 0.1 EU/mL CVs, with a 95% confidence interval of 0% – 0.6%, indicating a very low risk of CV failures.

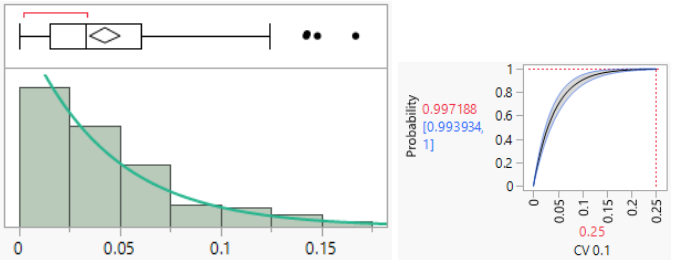

Figure 7.
The distribution of the 0.1 EU/mL CVs along with the distribution profiler.

PPC recovery

A PPC was included on every assay plate to test for inhibition or enhancement in the assay matrix. Inhibition occurs when a component in the sample interferes with the assay reaction, resulting in a lower-than-expected recovery of the spiked endotoxin and potentially leading to an underestimated endotoxin concentration or a false negative result.

PPC wells were prepared by spiking LAL Reagent Water (LRW) with 1 EU/mL of CSE, using 10 µL of a 10 EU/mL CSE stock solution. The recovered endotoxin concentration was calculated using both linear and quadratic standard curve fits, with acceptable recovery defined as 50% to 200% of the spiked value.

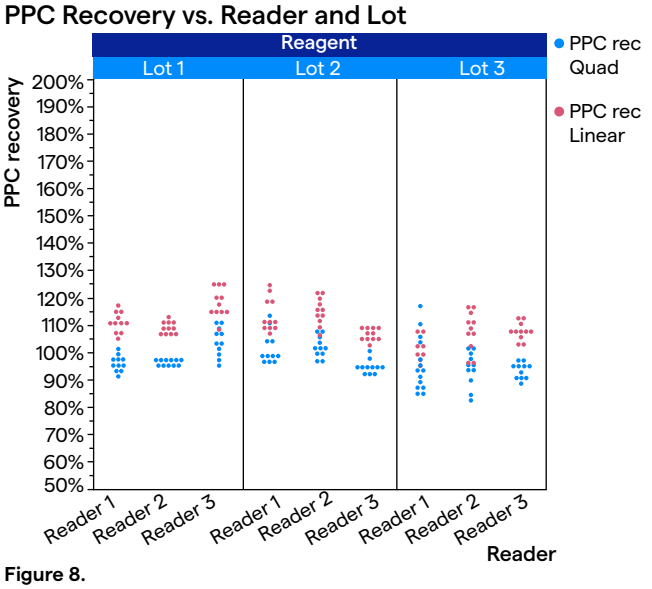

Figure 8.
PPC recovery for each set of duplicates.

All 99 PPC replicates met the recovery criteria using both fitting methods. Linear fits produced slightly higher recovery values, ranging from 95.1% to 125.2% with an average of 109.9%. Quadratic fits, which tend to offer more conservative estimates in the mid-range, yielded a narrower recovery range of 82.6% to 113.1%, with an average of 96.6%.

The consistency of PPC recovery across all reagent lots and instrument platforms confirms the accuracy of the shortened 20-minute assay and its resistance to interference from the sample matrix.

Discussion

This evaluation confirms that the 20-minute PyroGene® rFC Endpoint Fluorescent Assay is a reliable and efficient method for detecting endotoxin at the 0.1 EU/mL level. This detection threshold is sufficient for many quality control and manufacturing settings.

The shortened protocol is particularly well-suited to

high-throughput laboratories and automated testing environments, where assay time often limits productivity. Across 99 replicate data sets, the 20-minute assay consistently demonstrated acceptable precision, clear signal separation, and accurate recovery, even at the lowest tested concentration.

The use of positive product controls also ensures the assay can detect potential inhibition or enhancement in the test matrix, enhancing its robustness for real-world applications. While this modified protocol offers clear efficiency benefits, the trade-off in sensitivity should be considered. The standard 60-minute PyroGene® Assay protocol achieves a lower detection limit of 0.005 EU/mL, which is necessary for many applications. However, when 0.1 EU/mL is acceptable, the 20-minute format offers a validated, sustainable alternative that does not rely on reagents derived from horseshoe crab blood.

A faster, reliable option for 0.1 EU/mL endotoxin testing

The 20-minute PyroGene® rFC Assay delivers precise, reproducible detection at 0.1 EU/mL, ideal for high-throughput and time-sensitive workflows. It maintains full compliance with performance criteria across instruments and reagent lots, while offering a sustainable alternative to traditional LAL methods. For labs that prioritize speed without compromising confidence, this streamlined format is a reliable solution.

For more information on the PyroGene® Recombinant Factor C Assay, a sustainable method for endotoxin detection that works through a single enzymatic step, visit: lonza.com/PyroGene

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