Fluorescence-based bacterial endotoxin testing: High sensitivity detection with a flexible and streamlined workflow

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Key takeaways

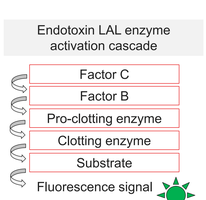
- **Qubit and Quant-iT Endotoxin Detection Assays utilize a streamlined single-incubation step workflow for endotoxin quantification and offer a broad detection range of 0.01 – 1.0 EU/mL when using 50 µL of sample.**
- **The Quant-iT Endotoxin Detection Assay is compatible with fluorescence microplate readers and is tailored for high-throughput runs. When processing data from microplate readers, first log transform the data and perform a linear fit to confirm data viability ($r \geq 0.98$). For optimal results, calculate concentrations using the log transformed data with a background-subtracted nonlinear curve fit.**

Introduction

Endotoxins are frequent contaminants of recombinant proteins and nucleic acids purified from gram-negative bacteria such as E. coli. Contamination in samples can trigger shock, inflammation, or sepsis in animals and cell culture. To minimize the risk of endotoxin contamination, bacterial endotoxin testing is a mainstay of plasmid preparation, cell culture, and transfection workflows. Amebocyte lysates are widely used as a simple and sensitive assay for the detection of endotoxin.

When endotoxin encounters the amebocyte lysate, a series of enzymatic reactions results in the activation of Factor C, Factor B and pro-clotting enzyme. The activated enzyme catalyzes a cleavage event in the substrate to produce a strong fluorescent signal. After stopping the reaction, the resulting signal can be measured on the Qubit Flex Fluorometer or fluorescence microplate reader. The correlation between fluorescent signal is proportional to the endotoxin concentration in the sample.

Here we review best practices for bacterial endotoxin testing and demonstrate the performance of the Invitrogen Qubit and Quant-iT Endotoxin Detection Assays.



Assay kit components:
- Amebocyte lysate reagent
- Endotoxin standards
- Endotoxin free water
- Fluorescence substrate
- DMSO

Invitrogen™ Qubit™ Flex Fluorometer

Materials and Methods

Qubit and Quant-iT Endotoxin Detection Assays (Cat. Nos. Q32891 and Q32892) were tested for performance across the quantification ranges. Using pyrogen-free consumables, dilutions of the endotoxin control were prepared in accordance with the user guide (MAN0029395 and MAN0029394) and assayed in replicates. The Qubit and Quant-iT assays were both performed with a 50 µL sample input. Additionally, the Qubit Endotoxin Detection Assay was performed with a 5 µL sample input (Table 1). At the lot-specific incubation time, the reactions were quenched in accordance with the protocol.

Sample Input Volume	Endotoxin Free Water Dilution	Dynamic Range
5 µL	45 µL	1.0 - 10 EU/mL
25 µL	25 µL	0.02 - 2 EU/mL
50 µL	0 µL	0.01 - 1 EU/mL

Table 1. Qubit and Quant-iT Endotoxin Detection Assay Dynamic Range

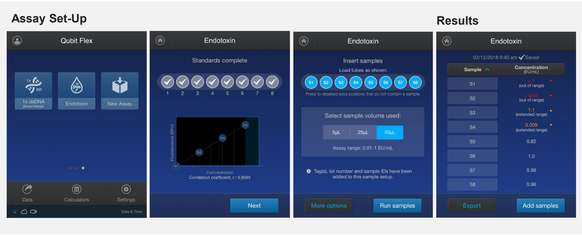


Figure 1. Qubit Flex Instrument Workflow. The endotoxin assay is selected from the home page of the Qubit Flex Fluorometer. Standards are used to generate a 4-point standard calibration curve that is used to measure endotoxin concentration. Up to 8 samples can be measured at a given time using the Qubit Flex Pyrogen Free Assay Tube Strips. Endotoxin levels are reported on the instrument with alerts if values fall out of the assay detection range.

Quant-iT assay analysis was performed at room temperature using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader (Cat. No. VLB000GD0) with an excitation/emission of 485/520 nm and a 12 nm bandwidth. Qubit assay results were obtained using a Qubit Flex Fluorometer (Cat. No. Q33327) (Figure 1).

Results

For both assays, the data was evaluated for viability by confirming that the correlation coefficient, *r*, was greater than or equal to 0.980. The Qubit Flex Fluorometer automatically calculates the correlation coefficient using log-transformed linear regression as described by the U.S. Pharmacopeia. Then the Qubit Flex data is analyzed using log transformed data and a background corrected quadratic fit. Using the Qubit Flex, 5 µL and 50 µL samples generated accurate and reproducible results (Table 2).

Expected Concentration (EU/mL)	0.010	0.050	0.100	1.00	1.00	5.00	10.00
Average Concentration (EU/mL)	0.0098	0.045	0.099	0.98	0.97	4.96	10.0
CV	5%	2%	8%	5%	4%	6%	10%
Relative Error	2%	11%	1%	2%	3%	1%	0%

Table 2. Qubit Endotoxin Detection Assay Performance. The assay generated accurate and precise results across the dynamic range. Five microliter samples (gray) had an average CV < 7% and average relative error < 5%. Fifty microliter samples had an average CV of 5% and average relative error < 5%.

Data from the Varioskan LUX was analyzed using the Skan-iT software (v7.0.2). Data was plotted using background subtraction, then both the x and y variables were log transformed, and then concentration calculations were performed using a 4PL extrapolated fit (Figure 2). Where possible, allow for the data to be extrapolated beyond the standards for best data sampling. If the data is analyzed in Microsoft Excel, a background corrected, log transformed quadradic is recommended as detailed in the Quant-iT user guide (MAN0029394). Using the Varioskan LUX, 50 µL samples generated accurate and reproducible results (Table 3).

Expected Concentration (EU/mL)	0.010	0.050	0.100	0.50	1.00
Average Concentration (EU/mL)	0.010	0.051	0.10	0.50	1.00
CV	14%	1%	1%	3%	6%
Relative Error	0%	2%	0%	1%	0%

Table 3. Quant-iT Endotoxin Detection Assay Performance. The assay generated accurate and precise results across the dynamic range. Fifty microliter samples had an average CV of 5% and average relative error < 2%.

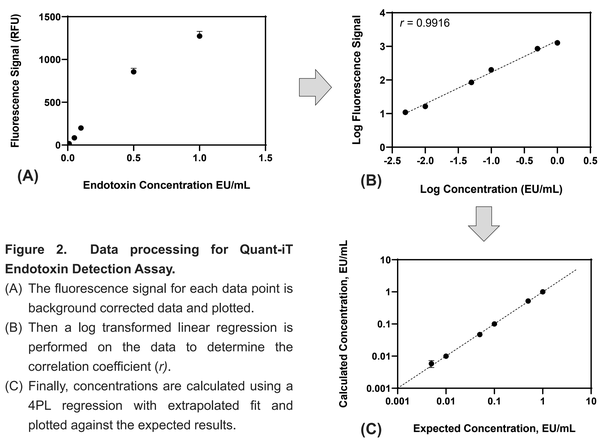


Figure 2. Data processing for Quant-iT Endotoxin Detection Assay.
(A) The fluorescence signal for each data point is background corrected data and plotted.
(B) Then a log transformed linear regression is performed on the data to determine the correlation coefficient (*r*).
(C) Finally, concentrations are calculated using a 4PL regression with extrapolated fit and plotted against the expected results.

Conclusion

Here we demonstrate the performance of the Qubit and Quant-iT Endotoxin Detection Assays. Both assays offer an easy-to-use workflow with a single incubation step while providing sensitive measurements across a broad dynamic range. Using pyrogen free consumables and the data processing techniques described here, the assays can generate accurate and precise measurements for scientists performing cell culture, vector prep, and protein expression studies.

Key benefits of the Qubit Endotoxin Detection Assay Kit:

- **High sensitivity with a broad range** – detect as little as 0.01 EU/mL to 10.0 EU/mL
- **Flexible** – suitable for wide range of samples, including protein, peptide, antibody, or nucleic acid samples
- **Easy-to-use** – when paired with the Qubit Flex Fluorometer, calculations are performed automatically reducing the potential for error

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